454 Sequencing System Software Manual, v 2.5p1

Part D – GS Amplicon Variant Analyzer

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454 SEQUENCING

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Part D

GS Amplicon Variant Analyzer

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1. GS AMPLICON VARIANT ANALYZER APPLICATION

- This section describes the GS Amplicon Variant Analyzer (AVA) application through its Graphical User Interface (GUI). The AVA software also features a Command Line Interface (CLI) that may be more appropriate for large Projects, especially when large amounts of data need to be imported into, exported from, or automated within a Project. See section 3 for a full description of the CLI, the language that was developed for it, and all the commands it includes.
- Projects are compatible with each other regardless of whether they are set up or computed using the Graphical User Interface (GUI) or the Command Line Interface (CLI). For certain projects, some may find it useful to set up portions of the project definition using the CLI and then enter the GUI for all subsequent tasks.

The sequencing results of Amplicon libraries are designed primarily to identify and quantitate both known and novel DNA variants (*e.g.* rare alleles) by the Ultra Deep Sequencing coverage of one or more region(s) of interest. This is supported by the "GS Amplicon Variant Analyzer" (AVA) software described in this section.

Briefly, the AVA application computes the alignment of reads from Amplicon libraries obtained on the GS Junior or Genome Sequencer FLX Instrument, and identifies differences between the reads and a reference sequence. Variations are displayed both graphically with a histogram indicating positions of variation, and textually with a color-coded multiple alignment that emphasizes regions and bases of difference from the reference sequence.

The software specifically reports the frequency of user-defined and software-identified variants in a summary Table, allowing for the high-throughput detection and quantitation of known and putative variants in the samples sequenced. In addition, various tools and views allow the user to examine the read alignments in detail to assess whether the software-identified variants appear to be legitimate, and possibly identify new ones. The user can then define these new variants into the system, and decide which variants to include in the analysis for quantitative reports.

1.1 Introduction to the GS Amplicon Variant Analyzer Application

1.1.1 Definitions

A few important terms have special meanings or characteristics in the context of the AVA software. These are defined and described below.

1.1.1.1 Project

An Amplicon **Project** is the main container of an Amplicon Sequencing experiment. In it, you specify the Reference Sequence(s) to which the sequencing reads will be compared, in search for Variants; the Amplicon(s) that constitute the library(ies) you sequenced [and hence, the reads in the Read Data Set(s)]; the Variant(s) that you specifically want the software to search and report on; and the Sample(s) that constitute the organizational basis for the analysis. If the Amplicon library(ies) contain Multiplex Identifiers (MIDs), the Project should further specify the

MIDs used and Multiplexers to define the relationship between MIDs and Samples. All these terms correspond to “elements” that constitute the Amplicon Project, and are further defined in the following sub-sections. The Project format allows the user to incrementally add new information (Read Data Sets, of course, but also Sample, Amplicon, Variant and even new Reference Sequence or MID/Multiplexer definitions) to a Project, *e.g.* as the sequencing results from new Runs/regions become available.

1.1.1.2 Reference Sequence

The basic definition of a **Reference Sequence** is quite straightforward: it is simply a string of A, T, G, C (or N) characters representing a DNA sequence against which the sequencing reads will be aligned and compared so variations can be identified and reported. The Reference Sequence(s) also provide the coordinates used to localize other elements defined in the Project (Amplicons and Variants; each Reference Sequence starts at coordinate “1”). You can define any number of Reference Sequences in a Project.

It is important to note that only “nucleotide” characters (A, T, G, C, or N) are accepted when you enter a Reference Sequence into the AVA software (by typing or pasting). For convenience, when pasting sequences, characters that are not nucleotide characters and are also not IUPAC ambiguity characters (such as R for purine, Y for pyrimidine, *etc*.) are removed from the pasted entry. This is useful when pasting sequences from sources that may include non-sequence information (such as white space or numerical position information in the margin of each line). During such pastes, any IUPAC ambiguity characters are converted to “N” characters, as the other ambiguity characters are not supported by the software (typing individual “ambiguity” characters, however, does not result in their conversion to “N”; these are simply ignored and the text “Only ATGC and N” at the top of the Edit Sequence window turns bold and red to alert you that an invalid character was used). The restriction that no ambiguity characters other than N be present in a sequence is a requirement of many alignment algorithms and is not unique to the 454 Sequencing System software.

It is also important to be aware that shorter Reference Sequences are more efficient for computation in the AVA software, whereas a long Reference Sequence could result in unnecessarily long computation times and slow navigation and scrolling in the application’s windows. Since in Amplicon sequencing, the interest is in one or a few small regions of DNA, the user should specify such region(s) when defining the Reference Sequence(s) for a Project rather than entering, for example, the entire genome of the organism. If you want to monitor together multiple targets that are distant from one another in the reference genome (for example exons of a given gene), you can create an “artificial” Reference Sequence by concatenating the segments of interest; it is useful to insert a few “N” characters between the concatenated targets if you create artificial Reference Sequences.

1.1.1.3 Amplicon and Target

The term **Amplicon** is used in the AVA software to represent essentially the same entity (sequence) as in the preparation of an Amplicon library, except that it does not include the 19 bp “Primer A” and “Primer B” parts of the Fusion Primers. As such, therefore, they match the sequencing reads from the Read Data Set(s).

In the AVA software, however, an Amplicon is a virtual entity defined *relative to a Reference Sequence* by specifying two primers (the “template-specific” parts of the Fusion Primers). This

relative definition is also *directional*: the AVA software names the two template-specific primers "Primer 1" and "Primer 2" in the 5'-Primer 1 --> Primer 2-3' orientation of the Reference Sequence. Therefore, Amplicon orientation is internal to the AVA software, and is NOT dependent upon the "Primer A" and "Primer B" parts of the Fusion Primers used in library construction.

You can define any number of Amplicons in a Project, each associated with a specific Reference Sequence; you can also associate multiple Amplicons, even overlapping ones, with a given Reference Sequence. Thus, a Reference Sequence may be associated with multiple Amplicons, but an Amplicon may only be associated with one Reference Sequence. Amplicons are also associated with Read Data Sets and with Samples (see below).

The term **Target** specifies the part of an Amplicon that is between the two primers (*i.e.*, the non-primer portion of the Amplicon). This is the sequence that is actually aligned to the Reference Sequence during the computations. It is important to trim the primers before alignment because any variant found therein would be a reflection of primer design (or errors in primer synthesis) rather than representing variations in the DNA sample used to prepare the Amplicon library, and therefore would not have any biological significance.

1.1.1.4 Read Data Set and Read Group

A **Read Data Set** is a group of sequencing reads derived from an Amplicon library. In a Project, Read Data Sets exist within a Read Group (this helps to organize the data) and are associated with pairings of Amplicons and Samples:

- the Amplicon association specifies which Amplicon(s) were included in the sequencing Run that produced this Read Data Set; the reads are identified as belonging to an Amplicon by virtue of their template-specific primers (see section 1.1.1.3, above)
- the Sample association specifies in which Sample(s) to report the results of the computations (see section 1.1.1.6, below, for a more detailed explanation of "Samples").

You can include any number of Read Data Sets in a Project; and associate them with any number of Amplicon-Sample pairs. However, an Amplicon cannot be associated with more than one Sample within a given Read Data Set unless MIDs are used to further associate the reads with specific Samples; see section 1.1.1.7 for more details on this. Note also that the AVA software can only process reads from Amplicon libraries.

In the current release of the AVA software, a Read Data Set is equivalent to an SFF file, *e.g.* as output by the data processing pipeline of the 454 Sequencing System, each file corresponding to a region of the PTP Device. On the GS Junior there is only one region per run and on the Genome Sequencer FLX there can be two or more regions per run depending on the gasket format employed. Using the SFFTools (see Part C, Section 3) from the command line, a user may reorganize the SFF files into multiple separate files prior to importing them as Read Data Sets into a Project. More typically, the SFF files are taken as-is from the data processing pipeline and so for the GS Junior, there will typically be one Read Data Set for each Amplicon sequencing Run you import into the Project, and for Amplicon Sequencing performed on a Genome Sequencer FLX, there will usually be one Read Data set for each region of the PTP Device of the Run you import

1.1.1.5 Variant

Simply put, a **Variant** is a sequence difference relative to a Reference Sequence. Like Amplicons, Variants are thus defined *relative to a Reference Sequence*. Four kinds of variations can be defined in the AVA software: substitutions, deletions, insertions, and required matches; and a defined Variant can include any number of these, in any combination (haplotypic variations). You can define any number of Variants in a Project, each associated with a specific Reference Sequence; you can also associate any number of Variants to a given Reference Sequence.

Though the multiple alignment views of the AVA software show all variations between the reads displayed and their Reference Sequence, a Variant must be defined in the Project to be reported in the application's Variants tab. Known Variants (*e.g.* from the scientific literature) can be defined directly in a Project, and putative substitution and deletion Variants will be automatically identified and defined by the AVA software if they are detected at a preset minimum abundance during computation of the Project; alignments of these putative Variants can be examined in detail, to allow you to formally "accept" them as legitimate Variants or "reject" them as noise. You can also define new Variants from the variations observed between the Reference Sequence(s) and the reads included in your Project.

The Variants tab thus reports statistics on the observed incidence (in all the reads included in the last computation of the Project) of each defined Variant, broken out by Sample. A Variant's definition specifies one or more Reference Sequence positions whose nucleotide identity must be matched or mutated in some way. Only reads that, in their multiple alignment to the Reference Sequence, span the entire set of Variant positions are eligible to contribute to the statistics computed for that Variant.

1.1.1.6 Sample

The term **Sample**, in the context of the AVA software, can be defined very generically as a virtual "container" specified by the user only as a name (and an optional annotation), and used to group reads for analysis and reporting. The Samples thus represent the organizational foundation for the analysis, whose primary output is the Variants Tab, such that the frequency of any or all defined Variants can be compared between the different "Samples" defined in the Project. You can define any number of Samples in a Project, each associated with one or more Read Data Sets and with one or more Amplicons. For example, Samples could correspond to sequencing data from an Amplicon library prepared from a "control" DNA sample; and those associated with a second Sample, to a library prepared from the DNA of an "experimental" tissue or individual. Or, different Samples could correspond to multiple replicate libraries of a biological sample, *e.g.* to allow for statistical comparison between them.

Within a Read Data Set, reads may correspond to one or more Samples. In order to demultiplex the reads, *i.e.* assign them each to the proper Sample, the reads must contain reliably identifiable Sample-specific features. The AVA software can use either of two mechanisms to assign reads to Samples:

1. It can use the known template-specific part of the Adaptor used to prepare the Amplicon library. This works well when the Amplicon identity alone is sufficient to assign the reads to Samples; this restricts one to the case where any given Amplicon within a Read Data Set provides reads for only one Sample (though it allows reads from different Amplicons to belong to the same Sample.)

2. It can use Multiplex Identifiers (MIDs) in conjunction with the template-specific part of the Adaptor. This is required if reads from a given Amplicon need to be assigned to more than one Sample in the same Read Data Set; the MIDs then provide the additional context necessary to resolve the reads to the appropriate Samples.

To perform the read to Sample assignments, the AVA software relies on user-specified, three-way associations between Read Data Sets – Samples – Amplicons (first mechanism), or Read Data Sets – Multiplexers – Amplicons (second mechanism). In the second case, the Multiplexers (see sections 1.1.1.7 and 1.1.1.8) provide the MID to Sample assignment information. Within one Read Data Set, a given Amplicon cannot belong to more than one such three-way association because the software would then be unable to unambiguously determine which association mechanism to use in order to assign reads from that Amplicon to their proper Samples.

Once the read to Sample assignment is made, the AVA software can compute the prevalence of Variants found in the reads, broken out by Sample. These statistics are reported in the Variants tab (section 1.5). Be aware, however, that while you can examine Variant frequency statistics for all the Samples of the Project in the Variants tab, you can view read alignments of only one Sample at a time (*e.g.* in the Global Align tab).

1.1.1.7 MID and MID Group

An **MID** (or Multiplex Identifier) is a short, recognizable sequence tag that can be added to the design of the Adaptors used for library preparation, between the sequencing key and the template-specific primer, to help determine the provenance of the read (see section 4.6). Multiple Amplicon libraries (the Project's Samples) can be prepared that include the same Amplicon target sequences (with the same template-specific primers), each labeled with different MID tags. The MID sequences provide extra context that, in concert with the template-specific primers, allow flexible demultiplexing options, and specifically enable the sequencing of the same Amplicon across multiple Samples within the same Read Data Set: when using MIDs, the Sample-Amplicon associations are indirectly specified in the software by associating Amplicons with Multiplexers (see section 1.1.1.8), which themselves specify the relationship between MIDs and Samples and then apply that information to the associated Amplicons. Note that both non-MID and MID-tagged Amplicons may be used in a Project, but within a given Read Data Set, all the reads for any individual Amplicon must be of one type or the other.

Contrary to the situation with Shotgun (sstDNA) libraries, where an MID sequence can only be on Adaptor A, Amplicon libraries can be constructed with MIDs at either or both ends of the reads. This provides for considerable flexibility in the design of the MID Amplicon libraries. In particular, if the Amplicons are designed such that the read length of the sequencing Run allows full read-through of the Amplicon reads, then placing MIDs at both ends of the reads makes it possible to use them combinatorially, such that a small number of MID tags can encode a much larger number of Samples (per the "Both" encoding; see section 1.1.1.8).

If multiple sets of MIDs are used in a laboratory, it may be useful to define **MID Groups** for each set, allowing them to be referred to as a group. A common grouping may be by length of the MID tags, because there is a restriction that all MIDs used at one end of any given Amplicon be the same length (see section 1.3.2.6). The AVA software is delivered with an MID Group named "454Standard", containing 14 MIDs carefully chosen to be resilient to sequencing and primer synthesis errors.

1.1.1.8 Multiplexer

A **Multiplexer** specifies the association between MIDs and Samples, *i.e.* how the MIDs should be used to assign reads to Samples. Depending on the design of the Amplicon libraries, Multiplexers allow four types of encoding (see section 4.6 for a description of Amplicon library design, in the context of MIDs):

- **Primer 1 MID:** This encoding provides an MID signature only on the end of the read that contains the template-specific primer defined as "Primer 1" in the Project. This will be at the beginning of the "forward" reads, or at the end of "reverse" (complemented) reads. These MIDs are then used to assign the reads to the proper Sample, as defined by the Multiplexer.
- **Primer 2 MID:** This encoding is the same as Primer 1 MID encoding, except that the MID appears at the "Primer 2" end of the Amplicons.

- **Both:** This encoding provides MIDs at both ends of the Amplicons and requires that read length be sufficient to read through to the distal MID, in both orientations. The *paired combination* of MIDs located on the Primer 1 and Primer 2 sides is used to assign reads to their proper Sample, as defined by the Multiplexer.

- **Either:** This encoding also provides MIDs at both ends of the Amplicons, but assigns the reads to their proper Sample on the basis of only the proximal MID on the read, in either orientation. This allows for proper assignment of both forward and reverse reads even if the Amplicon is longer than the read length provided by the sequencing Run script. Note that even if full read-through to the distal end of the read is possible, only the proximal MID will be used for Sample assignment (and any contradiction between the MIDs seen at the two ends will be assumed to be the effect of sequencing artifacts at the distal end of the read).

Selecting the proper encoding: It is crucially important to select the encoding method that truly corresponds to the way the libraries were prepared. For example, if a library was prepared with the 'Either' chemistry in mind, it may be tempting to use a 'Primer 1 MID' or 'Primer2 MID' encoded Multiplexer since the distal MID gets discounted in favor of the proximal MID, in 'Either' encoding. However, the AVA software needs to know that MIDs are expected to be found at both ends: without that knowledge, the trimmer might get a suboptimal alignment of the distal primer, which in certain cases could drop valid reads out of the analysis.

Multiplexers specify the assignment of reads that contain each defined MID (or MID pair) to each specific Sample, *within a Read Data Set*. Different Amplicons within a Read Data Set may simultaneously be sequenced even if they use different Multiplexer encoding methods, or no encoding at all (*i.e.* are sequenced without the use of MIDs), but any given Amplicon can only be sequenced in a single manner within a given Read Data Set. In the software, Multiplexers are associated with Read Data Sets and then one or more Amplicons are associated with those Multiplexers, in the context of the Read Data Sets (creating Read Data Sets – Multiplexers – Amplicons triads). The software then assigns the reads from those Amplicons to Samples according to the rules of the Multiplexer encoding. Operationally, the

same restriction exists regarding the association of Amplicons to Multiplexers as exists regarding the association of Amplicons to non-MID Samples (see section 1.1.1.6): a given Amplicon cannot belong to more than one Multiplexer within one Read Data Set, because the software would then be unable to unambiguously resolve which Multiplexer to use to determine the proper Sample assignment for the Amplicon reads.

Multiplexers conveniently encapsulate the correspondence between MIDs and Samples. Without Multiplexers, each instance of an Amplicon in a Project, distinguished from one another only by a choice of different MIDs in their library preparation, would require that a separate Amplicon be defined in the Project. Multiplexers also allow the correspondence between MIDs and Samples to be specified only once and shared across multiple Amplicons that may be sequenced simultaneously, a common experimental design. These and other benefits of using Multiplexers, including the more accurate decoding of MIDs and the reduction of errors in Sample assignment during the demultiplexing phase of computation, are further described in section 2.6.5.

1.1.2 Launching the GS Amplicon Variant Analyzer GUI Application

The GS Amplicon Variant Analyzer GUI application is launched by double clicking on its desktop icon.

From a Linux terminal window on an attendant PC or a DataRig, where the data analysis software package is installed, the following command can be used to launch the GS Amplicon Variant Analyzer GUI application:

```
gsAmplicon [<advanced options>]
```

The `gsAmplicon` command supports 3 advanced, optional, command line parameters. The typically most useful of these is the "`--cpu`" option that can accelerate computation through the use of multiple processors. These advanced options are the same as those available to the command line interface and are documented in detail in section 3.3.2.1 (Note: the gsAmplicon command shares only the "advanced options" of the command line interface, not the other "basic options").

gsAmplicon command interruption: If you interrupt the gsAmplicon command, accidentally or otherwise, by typing control-C, the application will immediately shutdown, not giving you a chance to save any of your recent Project changes. For additional safety, you may prefer to start the command in the background, ending the command line with a '&', as in: "`gsAmplicon &`". Other modes of exiting the application, such as clicking the "Exit" button, would elicit warning dialogs if the Project contains any unsaved changes.

This will open the AVA GUI application main window in its Overview tab (Figure 1-1). [A splash screen identifying the application and its version number will be displayed briefly (not shown); you can also view this splash screen at any time, after launching the application, by clicking on the "About" button.] Until a Project is open, the Overview tab provides a brief textual description

of the AVA application's usage and capabilities. There are two ways to open a Project at this point: you can create a new Project by clicking on the "New" button, or you can open an existing Project by clicking on the "Open" button (see section 1.1.3.1). Note that if you do not have write-permission to the Project folder, or if another user already has it open, you can open the Project as "Read-Only", but you will be unable to save any changes or carry out any computations (which requires writing to disk). See section 4.1 for more details on this kind of situation.

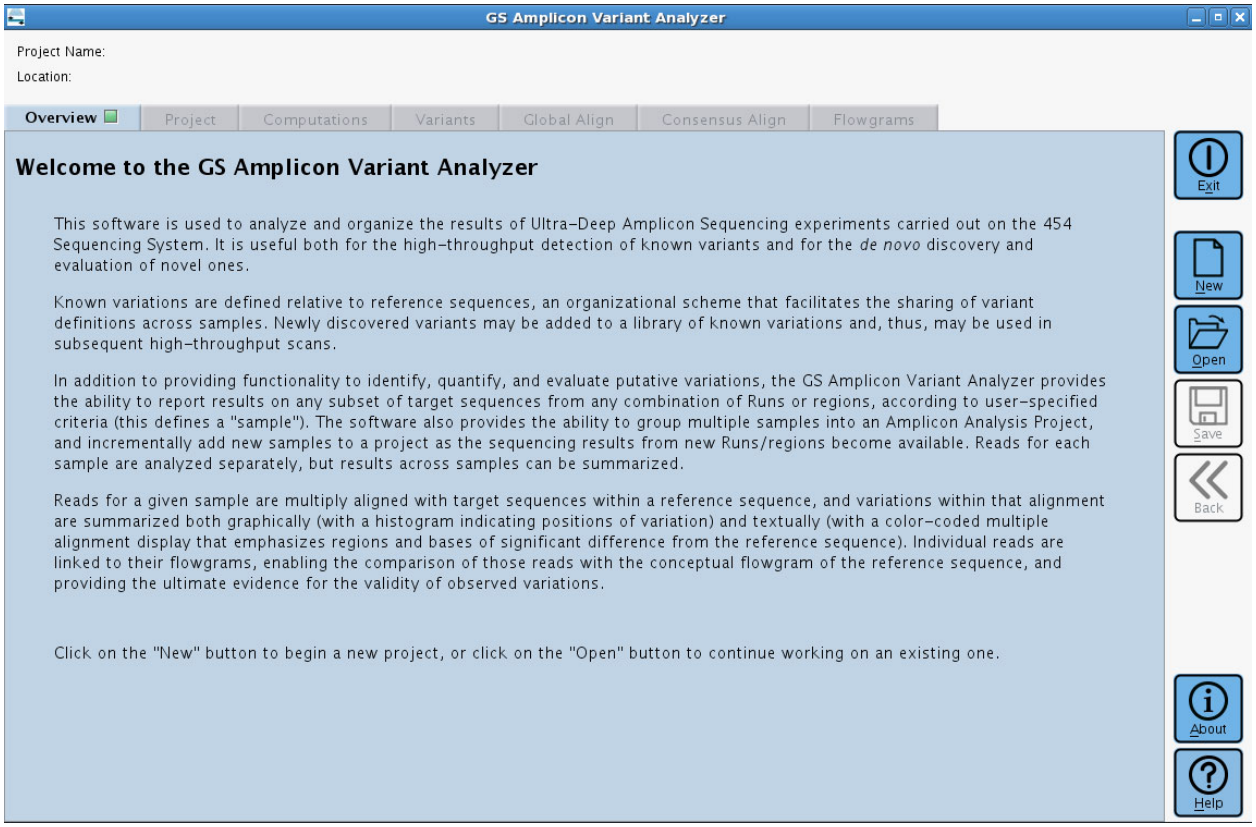


Figure 1-1: The Overview tab showing the textual description of the application, before a Project is open

1.1.3 GS Amplicon Variant Analyzer Application Interface Overview

The top of the main AVA window shows the name and path of the Amplicon Project being displayed; and a set of seven main buttons are located along the right-hand side of the window. The rest of the AVA window is occupied by a selection of tabs, described in the sections below.

- Make sure to set the resolution of your computer screen to at least 1024x768 pixels (1280x1024 recommended), or the AVA application may not be able to display all the features described below. If the resolution is too low (or if the application window or one of its constituent tabbed panels are resized too small), the software attempts to prioritize which feature to omit. For example, the Global Align tab may not show the color code legend on its lower-left corner; or the button column to the left of the Variation Frequency Plot on the top panel of the Global Align or the Consensus Align tabs may not show the "Save plot data to spreadsheet file" and/or the "Save plot snapshot to image file" buttons. While program features may be omitted in this way, scrollbars are used as needed to ensure that all data is viewable regardless of the screen resolution or window size.
- The AVA software also features a Command Line Interface (CLI) that may be more appropriate for large Projects, especially when large amounts of data need to be imported into, exported from, or automated within a Project. See section 3 for a full description of the CLI, the language that was developed for it, and all the commands it includes.

1.1.3.1 Main Buttons

Seven main buttons are always visible, along the right-hand side of the GS Amplicon Variant Analyzer window (Save and Back are grayed-out when their function is not applicable, *e.g.* no Project changes to save):

Icon	Description
	The **Exit** button closes the AVA application.
	The **New** button opens a "New Amplicon Project" window in which you can provide a name for a new Project, as well as a file-system location where to save it and a free-text description (Figure 1-2). Clicking "OK" in the "New Amplicon Project" window initializes the Project and takes you to the Project Tab of your new Project. For more details on Project initialization, see section 4.4. (See section 2.2.2 for more details on ways to create a new Project, especially synchronizing the Project and Location names, and navigating your file-system using the "folder" icon to the right of the 'Location' field.)
	The **Open** button allows you to browse your file-system to find an existing Amplicon Project and open it in the AVA application.
	The **Save** button saves the current state of the Project to the disk, *i.e.* all the primary elements and their associations.
	The **Back** button takes you back to the previous AVA view. (Note that this button does not carry an "undo" function; see Note, below.)
	The **About** button opens a splash screen providing some information about the AVA application. (Click the "Close" button to close it).
	The **Help** button opens an electronic version of the user manual.

The AVA software does not offer the possibility to "undo" an action such as a computation, an element definition entry, a display selection in a multi-alignment view, *etc*. In some cases, the opposite action or a "clear" action may be available. You can also revert to the last saved state of the Project by re-opening the Project without first clicking the "Save" button.

Saving *vs.* computing a project: Saving a Project does NOT update the results displayed in the Variants, Global Align, Consensus Align or Flowgrams tabs. To update these displays after making changes in a Project, you must re-compute it. Conversely, these results do not require that you save the Project to persist in the Project.

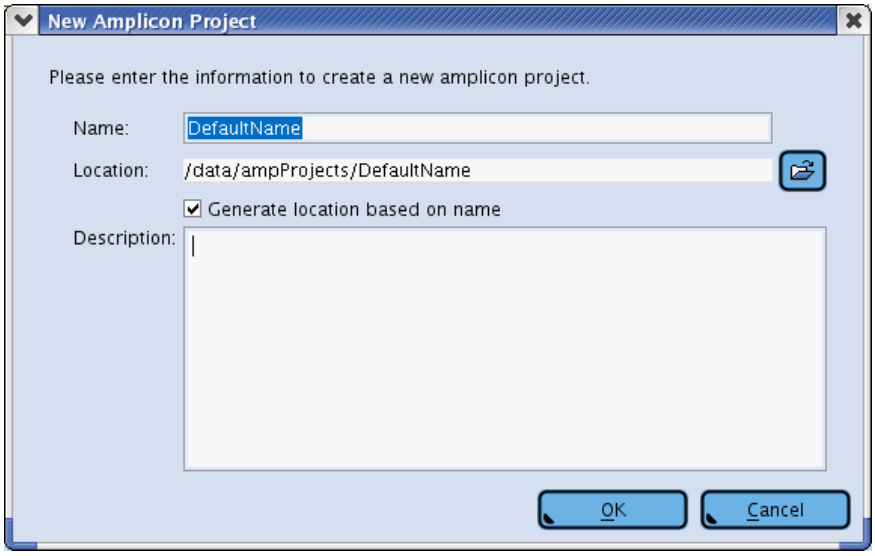


Figure 1-2: The New Amplicon Project window, with fields to enter a Project's name, file-system location, and textual description. If the "Generate location based on name" box is checked, typing a Project's name in the Name field also enters it at the end of the path in the Location field. Be aware that later changing the name of the Project from within the application will NOT change the name of the folder that contains it, causing a mismatch between the two; a mismatch would also occur if the "Generate location based on name" box is not checked, and you type different names for the Project (in the Name field) and the folder (at the end of the path). If there is a problem with the selected location, the OK button will be disabled, the location field will be highlighted in red, and, if you position the mouse over the location field, a screen tip will appear indicating the nature of the problem. The button to the right of the Location field allows you to browse your file-system to set a location path. See section 2.2.2 for an example and additional details on ways to create a new Project in the AVA software.

1.1.3.2 The Tabs and Sub-Tabs

The AVA application displays the various aspects of the Amplicon Project in a series of 7 tabs, with the Project tab separated into two panels (themselves comprising 4 and 7 sub-tabs, respectively). When a tab has no content, its name is grayed out and the tab is unavailable. When a tab does have content, a green square icon appears next to its name; clicking on an available tab or sub-tab name brings the information it contains to the front for viewing, as listed below. If the size of your screen does not allow you to view all the tabs, a pair of arrow buttons in each panel allows you to scroll the set of tabs to bring hidden ones into view. The contents and usage of the information included in the tabs are described in full detail in sections 1.2 through 1.8.

- The **Overview** tab provides a basic summary of the Amplicon Project.
- The **Project** tab is used to set up the Project (define all the elements that compose it and their associations) and to navigate it and select particular Sample-Amplicon pairs to view in the Global Align tab. The Project tab contains two panels:
    - The left panel comprises 4 sub-tabs that show various representations of the Project in "tree" form, thus displaying the associations between the various elements that compose it.
    - The right panel comprises 7 sub-tabs that list and show all the characteristics of the various "elements" defined in the Project, in tabular form.

- The **Computations** tab allows the user to compute (or re-compute) the Project, and lists the progress and state of each computation step.

- The **Variants** tab provides summary results of the GS Amplicon Variant Analyzer, listing the observed frequency of each defined Variant in each relevant Sample. A Sample is relevant to a Variant when the Read Data Set(s) with which it is associated contain reads that cover all the Reference Sequence positions specified in the Variant's definition.

- The **Global Align** tab is populated with the multiple alignment (against the appropriate Reference Sequence) of the reads corresponding to one or more selected Sample-Amplicon pair(s). Such a selection is done by right-clicking on an appropriate object in any of the Project Trees or in the Variants Tab (only one Sample-Amplicon pair can be selected this way), or by using a navigation dialog found within the Global Align tab itself (multiple pairs selection possible). The reads displayed may be Individual reads, corresponding to sequence reads directly extracted from the Read Data Set(s), or Consensus reads, corresponding to sets of Individual reads that were collapsed into a single representative read (in order to simplify the display and eliminate noise from the data). In either case, the tab comprises two data panels:

    - The top panel is a stacked histogram indicating the frequency of all the variations observed between the selected reads and the Reference Sequence associated with the Amplicon(s) from which the reads were derived. The histogram is based on a gapped multiple alignment and, thus, may contain data for positions of the alignment that occur between positions of the Reference Sequence itself. This graph also shows depth of coverage for each position of the alignment.
    - The bottom panel displays the (gapped) sequence multi-alignment of the reads, aligned below the Reference Sequence. Color codes help to highlight variations in the reads in comparison with the Reference Sequence; the reads (Individual or Consensus) can be filtered for display to help identify specific variations and potentially discover haplotypes.
- The **Consensus Align** tab displays the same information as the Global Align tab, but for the set of individual reads that were collapsed into a consensus on the Global Align tab. A line highlighting the differences between that consensus sequence and the Reference Sequence is displayed directly below the Reference Sequence in the alignment.

- The **Flowgrams** tab, finally, displays a tri-flowgram view of an individual read, selected from either the Global Align or the Consensus Align tab. This display is designed to help evaluate the significance of differences between an individual read and a Reference Sequence. As such, the read flowgram displayed is not the raw flowgram of the read, but is a computationally processed version designed to facilitate the comparison of the read flowgram with an idealized flowgram for the Reference Sequence. In particular, flow cycle-shifts may be introduced into one or both flowgrams in order to optimize their alignment, and the flowgram of the read may be computationally reverse-complemented in order that the display always be in the 5'-->3' orientation of the Reference Sequence. Finally, the flowgram only displays the subset of flows relevant to the read's sequence alignment as displayed in the Global Align or Consensus Align tabs. The display is divided into three panels:

    - The top panel shows an aligned, idealized flowgram for the Reference Sequence.

- The middle panel shows the aligned, (possibly reverse-complemented) flowgram of the read.
- The bottom panel shows a difference flowgram (read minus reference), where any variation from the Reference Sequence is seen as a non-zero value. Specifically, extra signals in the read, relative to the Reference Sequence, are displayed as positive differences in this panel; and missing signals in the read, relative to the Reference Sequence, are displayed as negative differences.

When a tab is divided into panels, the panels can be resized by dragging the separator. Also, when the panels are stacked vertically (as in the case of the Global Align, the Consensus Align and the Flowgrams tabs), two small buttons are present at the left edge of the separator(s); these buttons allow you to collapse one of the panels, leaving the entire height of the window to the other one(s). This action is reversible (use the other button to re-expand the panel).

1.1.3.3 Buttons and Plots

The plots, multi-alignment views and data tables displayed in the various tabs of the AVA application are scrollable and/or zoomable graphical elements. They share certain common buttons and functions, *e.g.* to perform the scrolling and zooming. When they do appear, these graphic elements have some or all of the following features (see in Figure 1-3, an example for a Global Align window which has many of these elements):

- Scroll bars for horizontal and/or vertical scrolling (appearing below and to the right of the element, if necessary).
- A column of buttons along the upper left edge of the graphic elements, used for navigation (including various zooming functions) and/or to save snapshot images or text files of the displayed data.

- Additional functional buttons, also in the column at the left of graphic elements, to carry out actions such as applying a selection filter on the reads currently displayed, defining novel Variants, assembling consistent reads (which might span overlapping Amplicons) into consensi, running a computation of the Project, adding or removing Project elements or associations, *etc*.

- “Mousing” functions (pointing, clicking or dragging the mouse, touchpad, pen, *etc*. over the graphical element) to view data values and adjust the zoom level.

- When a plot with DNA sequence information is present (Variation Frequency Plot and Flowgrams), a legend is shown, giving the color code for the nucleotides [as well as gaps (“-”) and depth of coverage (“#”), if appropriate].

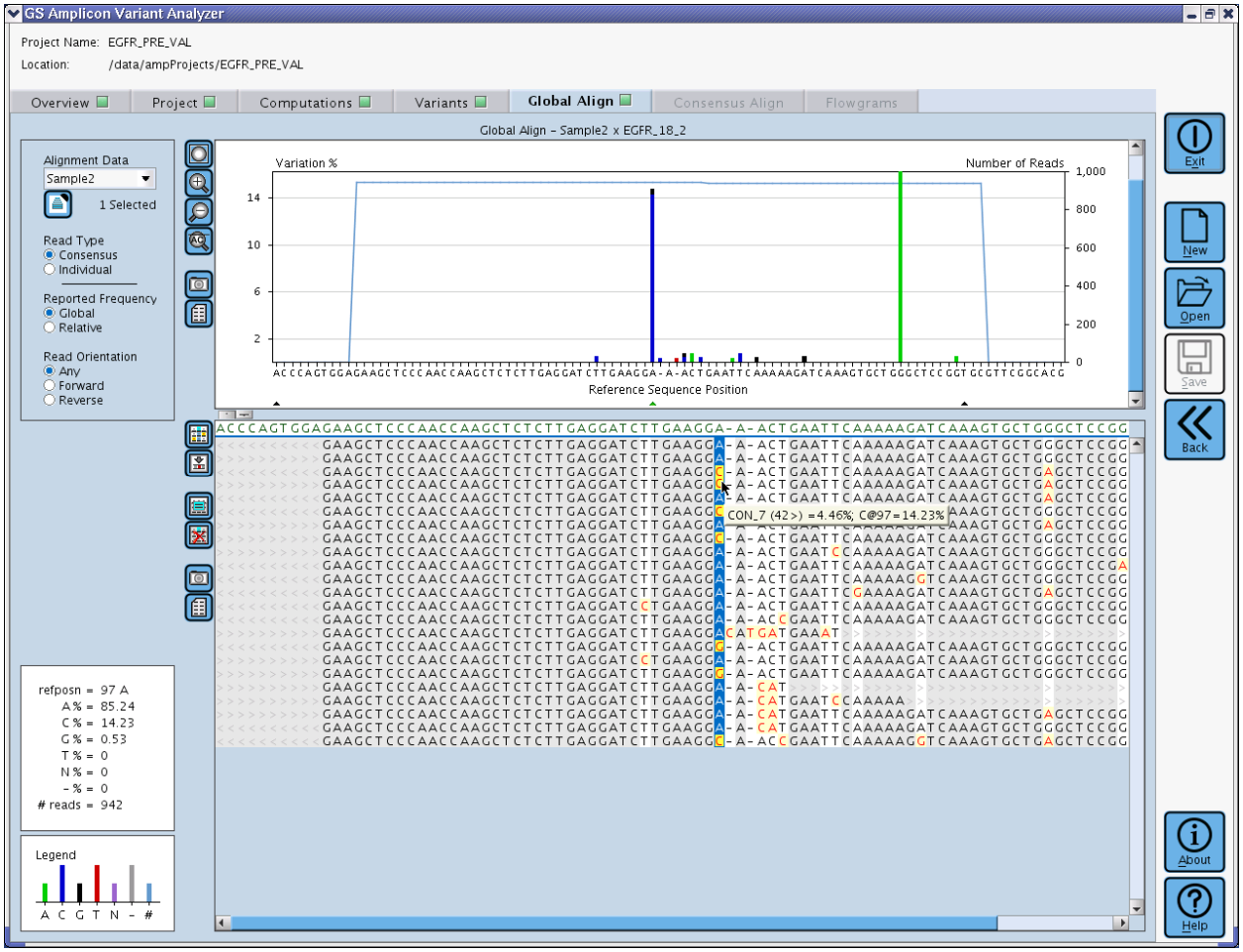


Figure 1-3: An example Global Align tab showing many of the common graphical element functions

1.1.3.3.1 Scroll Bars

The scroll bars have the standard functions, and appear when the data in either direction is too large to be shown in the viewable area. In Figure 1-3, for example, the horizontal scrollbar appears in the multi-alignment pane, and the vertical one appears in the plot pane; others are not displayed because axes scales are set such that the full breadth of the data can be seen.

1.1.3.3.2 Navigation Buttons

Many of the buttons appearing to the left of an element are used for navigation on the element, and have the following general functions:

Button	Name – Description
	**Fit** – Fit means to scale out to the limits of the data. The y-axis is rounded to an attractive-looking number rather than stopping at the exact data limit.
	**Zoom in** – Zoom in by a factor of 1.5. This button zooms only the primary (left) y-axis scale; use the **Zoom to labels** and **Freehand zooming** functions described below to zoom the x-axis.
	**Zoom out** – Zoom out by a factor of 1.5. This button will zoom only the primary y-axis scale and, unlike other zoom operations, this will zoom out past the data limits (to allow you to get a better perspective of the data, especially when attempting to visually separate data on the primary and secondary y-axes).
	**Zoom to labels** – This button zooms the x-axis of the flowgram so that the nucleotide/flow characters can fit below the axis.
	**Snapshot** – Save a snapshot image of the current view to disk. This will open a dialog asking for the location and filename to save a PNG format snapshot image. The saved image contains only the currently visible region of the element: in particular, if the element is displayed in the context of a scrollbar, only the current, scrolled view is saved.
	**Text file** – Save a text-formatted version of the element. This will open a dialog asking for the location and filename to save the text file. It then saves the data, along with summary information describing the data source. In most cases the user has the option of exporting a tab or comma-separated text file of the underlying data element. For plots, the text file includes data that may be outside of the current view (due to scrollbars). For a flowgram view (see section 1.8), the data for all three plots are saved to one file, with some white space between the three sections of plot data. For summary data tables, the file contains the tabular data, also including data that may be beyond the current scroll region. For multiple-alignment displays, four output formats are supported: FASTA, Clustal, Ace and Table. For the Variants Table, finally, the text file includes an extra column (Variant Status), as compared to the Table visible in the GUI, placed between the Variant Name and Max Value columns (*i.e.* at column 3 in the text output); note that although the GUI lacks an explicit column for this, the Variant Status data is accessible via the tooltips as you pause the mouse over Variants).

1.1.3.3.3 Mousing Functions

When the mouse cursor is located over a graphical element, additional functions can be performed by moving, clicking or dragging the mouse:

- **Mouse Tracker** – Whenever the cursor is located over a position in a plot or a multi-alignment display, detailed data values for that position are shown in a related Mouse Tracker area, at the bottom left of the window. This allows you to see the specific numerical value for any data point as well as other detailed data associated with the display position.

- **Freehand Zoom In** – The mouse can be used to zoom in on specific regions of a plot. To zoom in, hold the left mouse button down and drag a box around the area of interest (see Figure 1-4). Releasing the button zooms to the area circumscribed by the box. If the plot has both primary and secondary Y-axes, only the primary axis data is zoomed.

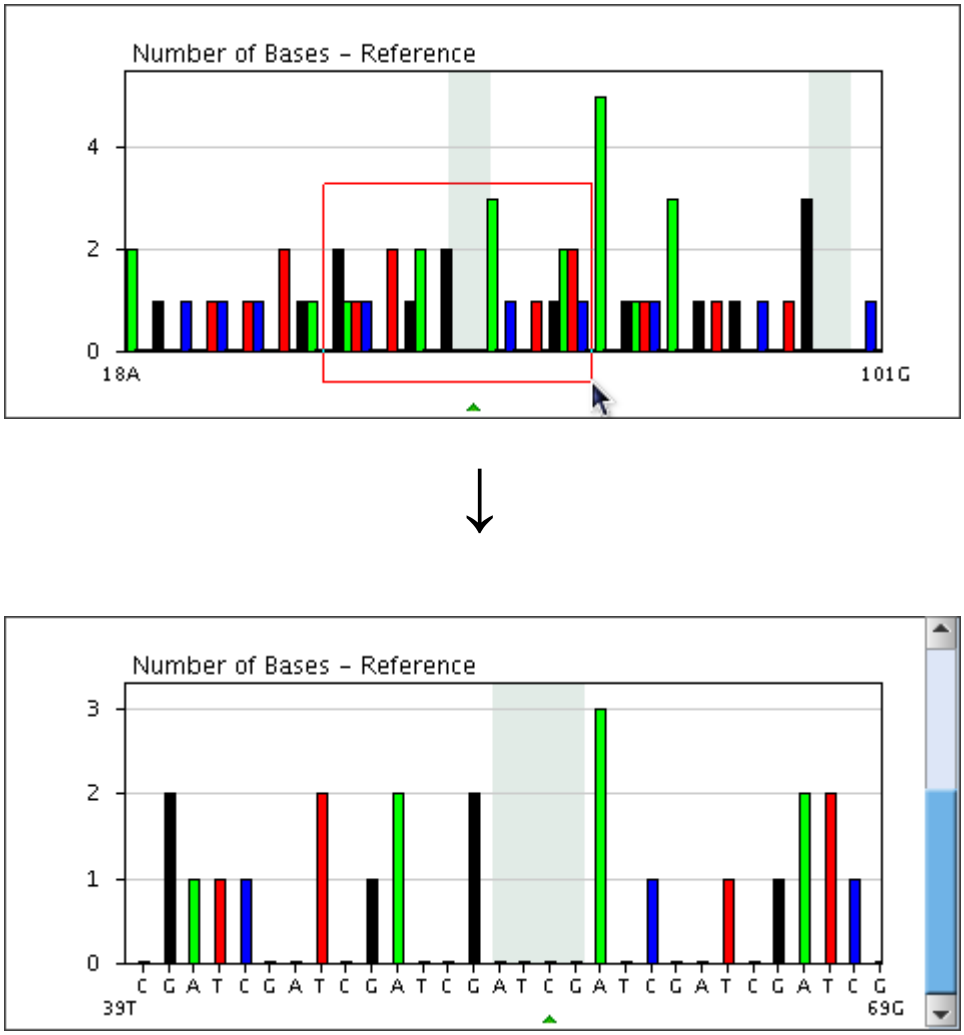


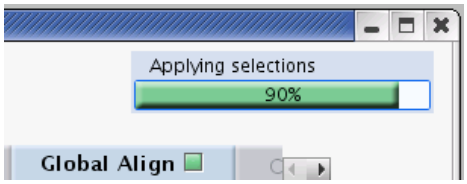
Figure 1-4: Freehand zoom in to a flowgram region

- **Freehand zoom out** – For plots only, right-clicking the plot will cause the plot to zoom out by a factor of 1.5 in both the X and (primary) Y directions, centered on the middle of the current view. This zoom will not zoom farther than the limits of the data.
- **Screen tips** – When you hover the mouse over a button or other display control, over an element definition data, or over most of the Variants or multi-alignment results, a screen tip appears providing some information about the object under the pointer. Some of the most useful examples of this are specified in the tab sections, below.

1.1.3.3.4 Progress bars

When an operation takes more than a few seconds, a Progress bar appears temporarily in the upper-right corner of the application window, to display the progress of the operation (Figure 1-5**A**). Double-clicking on this progress bar opens the Progress window, which contains individual progress bars for the operation or any of its sub-processes (Figure 1-5**B**). In certain contexts, you can cancel an operation by clicking the “Cancel” button that appears to the right of a progress bar in the Progress Window (when cancelling is not possible, such as when new data is being loaded, the Cancel button is present, but grayed out). The Progress window stays open, even after the entire operation completes, until you close it manually.

A



B

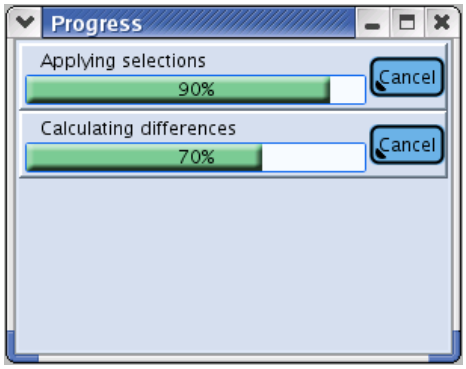


Figure 1-5: (A) A Progress bar (B) The Progress window

1.1.3.3.5 Special Action Buttons

Another functional category of buttons appears to the left of some graphic elements or in the display options area of certain tabs. Since these are tab-specific, these buttons are described in the corresponding tab sections, below.

1.1.3.4 File Browsing in Linux

The Linux File Brower used for opening Projects, finding Read Data Sets, and creating New Projects lacks some of the usually expected controls. For example, there are no buttons for going up a directory, creating a new folder, *etc*. However, these functions can be found by right-clicking in the browser window and choosing from the options presented in the contextual menu that will open (Go up, Go Home, View Details/List, Refresh, and New Folder).

1.2 The Overview Tab

This simple tab provides a basic summary of the Amplicon Project (Figure 1-6): the name, location and description of the Project entered in the New Application Project window when the Project was created (or as edited thereafter, *e.g.* from the Project Tab); and the number of Reference Sequences, Amplicons, Read Data Sets, Samples, Variants, MIDs and Multiplexers defined in the Project. These numbers also appear on the seven Definition Table sub-tabs of the Project tab.

- When the application is launched, but before a Project is open, the Overview tab displays a brief, general description of the GS Amplicon Variant Analyzer application's usage and capabilities (see Figure 1-1).
- Most of the screenshots in this section are derived from the Project shown in Figure 1-6. Since this Project does not use MIDs, the 454Standard MID set that is automatically loaded when a new Project is created (see section 4.4), has been manually removed to simplify the display of the Project.

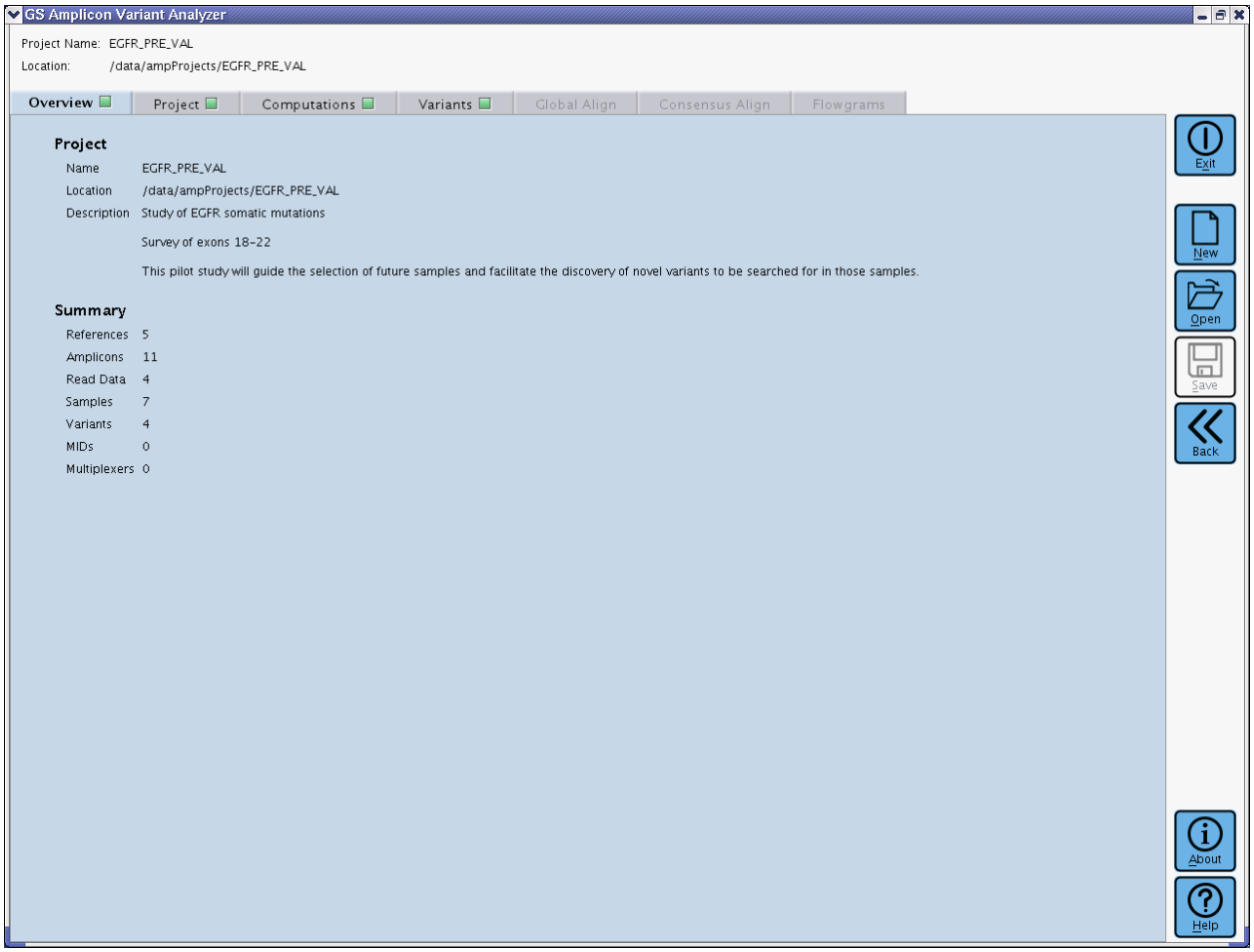


Figure 1-6: The Overview tab

1.3 The Project Tab

This is one of the most complex tabs of the AVA application. It is used to set up and navigate an Amplicon Project. Setting up an Amplicon Project means to define all the elements that constitute it, and all their associations. There are seven types of Project elements: Reference Sequences, Amplicons, Read Data Sets / Read Groups, Samples, Variants, and optionally, MIDs / MID Groups, and Multiplexers.

The Project tab is divided into two panels (Figure 1-7): the left-hand panel comprises four sub-tabs, each with one "Tree" representation of the Project that show the diverse interrelations between the Project's elements; and the right-hand panel comprises seven sub-tabs, each with the "Definition Table" for one type of element. Clicking on an element in any tree view that is represented in a Definition Table (Reference, Amplicon, Read Data, Sample, Variant, MID, or Multiplexer) causes the right-hand panel to load the appropriate sub-tab Definition Table with the element selected. Expanding or collapsing a node in the tree or clicking on an item in the tree that is not represented in a Definition Table, such as a Read Group or an MID Group, does not impact the right-hand panel. You can click and drag the divider between the two panels to adjust the space assigned to each panel. If the size of your screen or the position of the panel divider does not allow you to view all the sub-tabs of a panel, a pair of arrow buttons appears in the upper-right corner of that panel, allowing you to scroll the set of sub-tabs to bring hidden ones into view.

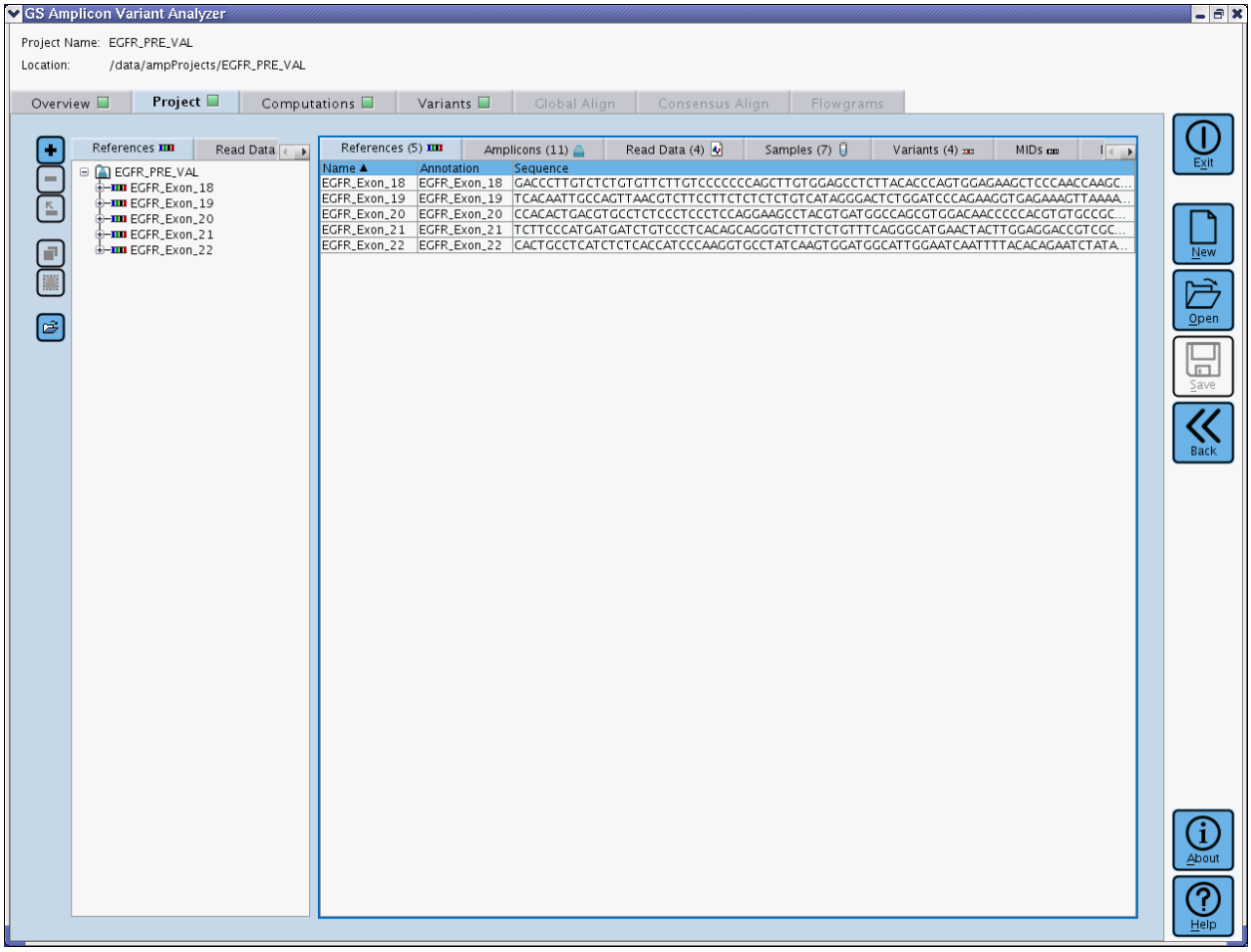


Figure 1-7: The Project tab

A column of six buttons runs down the left edge of the Project Tab (some of which are inactive and grayed-out in Figure 1-7), whose individual functions are discussed in detail in sections 1.3.1 and 1.3.2. These buttons are context sensitive in that the target of their actions can be either an object in the Tree or in the Definition Table panel, whichever was clicked last: the application provides a visual reminder of which panel is "active" (and will be subjected to the action of the left margin buttons) by surrounding the active panel with a thin blue, rectangular border. For example, Figure 1-8 shows a Reference Sequence selected in the left panel and a Sample selected in the right panel, and some of the left margin buttons are active; the blue border is around the right panel, so the Sample from the right panel is the current target of the available buttons.

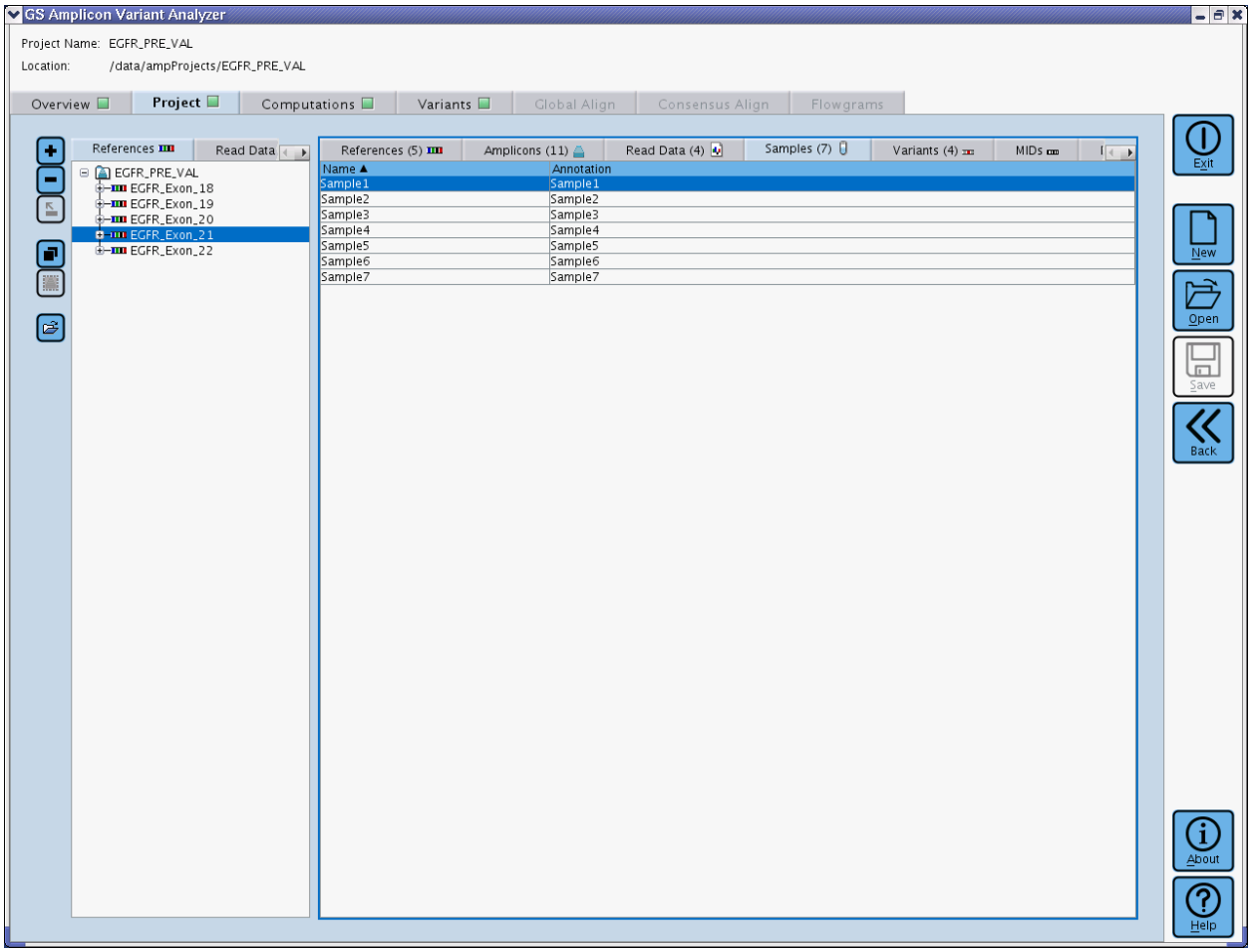


Figure 1-8: The Project Tab with the right-hand Definition Table panel highlighted with a rectangular blue border indicating that the panel is "active". The left margin buttons that are active (*i.e.* not grayed-out) will operate on the "Sample1" that is selected on the right, and not the "EGFR_Exon_21" that is selected in the tree on the left.

Re-computing a changed Project: Project results in the Variants, Global Align, Consensus Align, or the Flowgrams tabs are representative of the state of the Project as defined in the Project Tab, at the time of the last computation (see section 1.4). If a change is made to a Project element that is germane to these results, the results will remain but will be out of date until you re-compute. This includes changes in the definition of Reference Sequences, Amplicons, Variants and Samples, and the addition or removal (or inactivation) of Read Data Sets; as well as changes in their associations, including changes in the definition of MIDs or Multiplexers. If you find that the data in these tabs does not reflect the current state of the Project, try re-computing it.

1.3.1 The "Project Tree" Sub-Tabs

In a new Project, each "Project Tree" tab contains a single folder representing the Project itself. Right-clicking on this folder opens a contextual menu that includes a "Properties" option; this opens a "Project Properties" window in which you can enter or edit the name and description of the Project. You cannot modify the location of the Project from within the Project. (Note that changing the name of the Project this way will NOT also change the name of the folder that contains it, in your file-system, so be aware of the possibility of mismatch between the Project name and its file-system location.)

The "tree" Project views provide a convenient way to add, remove and organize ("associate") the various elements that compose a Project, and to navigate it afterwards. There are 5 ways to construct or otherwise manipulate a tree.

- You can use the buttons located to the left of the window's left panel
- You can right-click on an existing element in a Project Tree, which opens a contextual menu that includes the same actions of the buttons

- You can drag elements from the Definition Tables on the right panel of the tab, to the appropriate element in a tree view (see section 1.3.2)

- As a special case, the associations between Amplicons or Variants and their Reference Sequences can also be specified in the Definition Tables of these elements, and will then appear in the References Tree.

- As another special case, when MIDs and Multiplexers are used, editing the associations between MIDs and Samples for a given Multiplexer (see section 1.3.2.7.3) may cause any Amplicons previously associated with Samples using that Multiplexer to shift to new positions in the tree, to reflect associations to the Multiplexer's new Samples.

The functions of the five action buttons to the left of the Tree panel that are applicable to trees are described below. Right-clicking on an element in a tree opens a contextual menu that contains the same actions [plus, if you right-click on a Sample-Amplicon pair, the "Global Align" action (described in section 1.6.1)].

Button	Name – Description
	**Add** – allows you to create a new element in the tree (except for Read Data Sets; see the "Import Data" button, below), as a branch under the element selected at the time you clicked, automatically creating an association between the two. This action is contextual, *i.e.* the type of element you can create with this button depends on which "tree" you are in and which type of element is selected at the time you click the button. When the context allows for the creation of more than one type of element, a contextual menu opens to let you choose; if there is only one possibility, the new element and association are created directly.
	**Remove from Project** – deletes the element selected from the Project altogether. Of course, this severs all the associations it may have had with other elements. If the association is based on a definitional relationship then those other elements are deleted as well (specifically, deleting a Reference Sequence will delete any Amplicons or Variants that are defined based on their association with the Reference Sequence). If there are related elements in purely associational relationships, then it does NOT delete those other elements, even if they were in a lower branch of the tree in the particular tree view from which the operation is carried out. For example, if you remove a Sample from the Sample Tree, all Amplicons associated with that Sample remain in the Project (even if they are no longer associated with any Sample at all) and keep their full definition and all other associations they may have. In all cases, a warning message is displayed to indicate exactly what elements or associations will be removed from the project as a result of the removal action.
	**Remove association and remain in project** – severs the association between the element selected at the time you click the button and the element above it in the tree, but leaves all elements otherwise fully defined in the Project. This button is contextual as well, as not all links can be severed. For example, you cannot sever the link between a Sample and an Amplicon in the References Tree (though you can in the Read Data and Samples Trees).
	**Select Amplicons associated with item** – provides the ability to select, within the Definition Table, all the Amplicons associated with an item in the tree, based on the relationships of that item that exist in the tree. For example, selecting a Reference, a Sample, or a Multiplexer in one of the trees, and clicking this button causes the Definitions Table panel to switch to the Amplicons Definition Table sub-tab. Within that Amplicon table, the subset of the Amplicons that are associated with the Reference, Sample, or Multiplexer used to trigger the operation will be multi-selected. This multi-selection in the Table can be dragged to another valid tree location by holding down the control key before left-clicking on one of the members of the multi-selection with the mouse. (Note: The Amplicon on which you clicked will initially become deselected, but it will be added back to the selection at the moment the selection gets dragged.) The primary utility of this button is that it allows complex Sample-Amplicon or Multiplexer-Amplicon relationships to be cloned to another Sample or Multiplexer with a single drag-and-drop operation, rather than making many individual manual selections, drags, and drops to re-create the whole set. See also section 1.3.2 for information on how dragged Amplicons can "trickle" down a Tree.

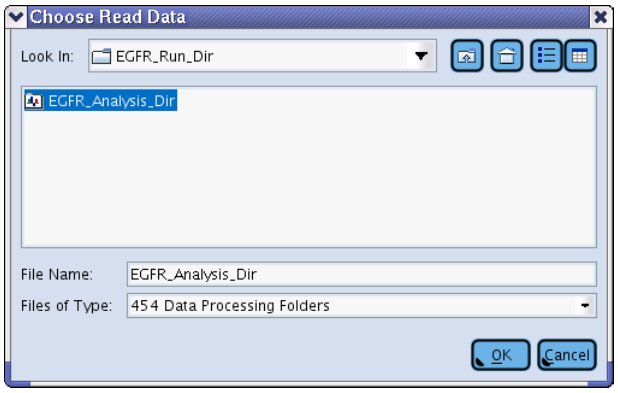
	**Import data** – allows you to either add Read Data Set(s) to the Project, or to import a file containing specifications to create any of the other project entities that have a dedicated tab in the Tree or Definition Table panel of the GUI (References, Amplicons, Samples, Variants, MIDs, Multiplexers). The "Import data" button operates on the item type which has focus when the button is clicked, as indicated by the rectangular blue outline (Figure 1-8). More details on this function are provided below.

Clicking the "Import data" button when the ReadData Tree or Table has focus opens a "Choose Read Data" window from which you can browse your file-system to select the Read Data Set(s) of interest. This can be:

- The Data Processing ('D_') folder of a sequencing Run: select '454 Data Processing Folders' in the 'Files of Type' drop down menu, at the bottom of the window; (Figure 1-9**A**; Data Processing directories are marked by a special icon:).
- Individual SFF file(s): select '454 SFF Files' in the 'Files of Type' drop down menu (Figure 1-9**B**).

Note that the data set(s) must comprise reads from an Amplicon library(ies) to be useable in the AVA software.

A



B

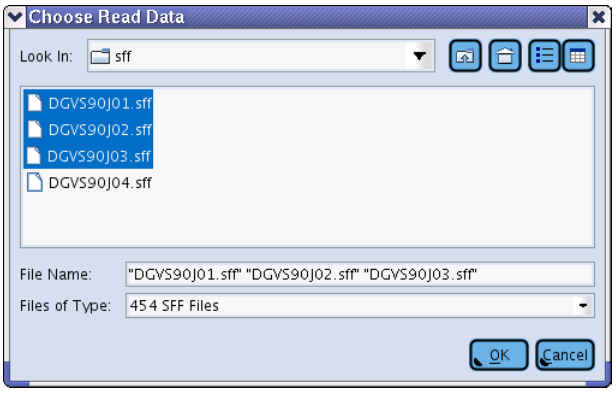


Figure 1-9: The Choose Read Data window showing (A) an example with a Data Processing Directory selected, and (B) an example with some SFF files selected.

When the selection is made and you click the "OK" button to accept it, an "Import Read Data" window opens (Figure 1-10). In this window, you can:

- select the exact file(s) to import (*e.g.* from the list of SFF files corresponding to the sequencing Run, if a Data Processing ('D_') folder was selected in the previous step) by clicking the "Import all" or the appropriate check box(es) to the left of the Read Data Set name(s);
- determine whether to import full copies of the Read Data Set(s) into the Project, or only symbolic links to the Set(s) selected, by clicking the "Link all" or the appropriate check box(es) to the right of the Read Data Set name(s) (see first Note below);

- incorporate the Read Data Set(s) you are importing into an existing Read Group or into a new one (which you can name in the appropriate field, in this window) (see second Note below).

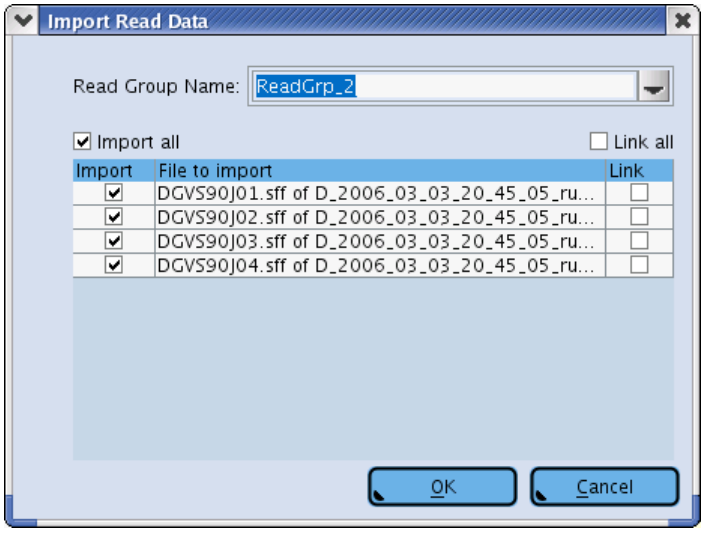


Figure 1-10: The Import Read Data window

- All the files related to an Amplicon Project are collected in a single folder, at a location determined by the user at the time the Project was created (or as modified thereafter). To save file transfer time and disk space, you can choose to import only a symbolic link to the data file(s) rather than the files themselves. However, be aware that if you do this and then the file that is the target of the link is moved, the AVA software will not be able to compute (or re-compute) the Project.
- Read Groups are distinct entities (which can be created at the root node of the Read Data Tree, using the "Add" button), and although you can rename an existing Read Group to match the name of another pre-existing Read Group, this will not cause the Read Data Sets to be merged into the same group.

Clicking the "Import data" button, when a non-Read Data Tree or Table has focus, opens a "Choose File to Import" window from which you can browse your file-system to select a tab- or comma-delimited file containing definition information for the selected entity type (Figure 1-11). The content of the file being imported should be of the same format as one that would be provided as the "-file" option for the AVA Command Line Interface (CLI) "create" command for that entity ("`create` *`entity`* `-file` *`providedFile`*", see section 3.4.4).

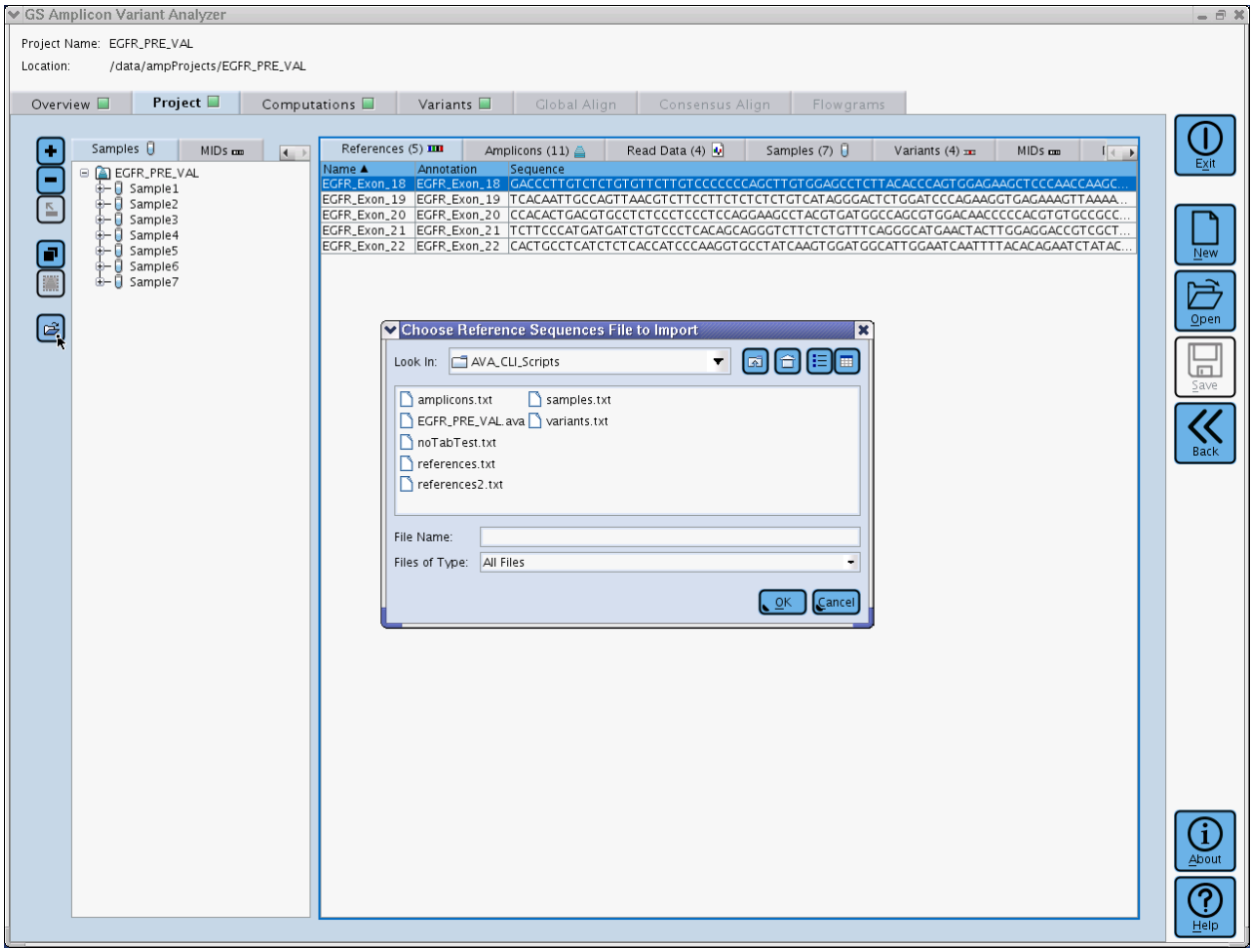


Figure 1-11: A "Choose Reference Sequences File to Import" window has been opened by clicking on the "Import data" button. The Tree view is displaying Samples and the Table view is displaying references. The import window label mentions "Reference Sequences" rather than Samples because the References Table has focus, as indicated by the blue outline.

When a file is imported, it is subjected to the same error checking as if it was being provided to the CLI. The header line should contain field names that are appropriate for the entity being created and the file should only be used to create new entities. If the file uses unknown fields in the header, or attempts to create an entity that already exists, an error window will be generated. If no errors are encountered, the newly created entities will appear in the appropriate Tree and Table. The newly created entities are not permanent until the project gets saved.

- **Importing the incorrect file type:** Because the "Import data" button is context-sensitive, care should be taken to make sure that the window that pops up is for the expected object by checking the title of the window. Some entities, such as the Sample, have header fields that are not unique compared to other entities, so it is possible to select a file for that entity by accident without triggering any error. In Figure 1-11, for example, suppose the intent was to import a Sample file, and the blue focus outline and window title indicating Reference import were ignored, and a Sample file was chosen. In such a case, the Samples would end up being created as incomplete References without triggering any warning or error.
- **Partial file imports:** If an import operation is terminated due to an attempt to create an entity that already exists or some other error condition (e.g. attempting to create a Variant while referring to a non-existent Reference), the import halts at the point where the error is encountered. Any entities created prior to the error will have been successfully added to the project, and any entities after the error are never encountered, resulting in a partial upload. The simplest way to back out of such a situation is to reopen the project without saving the results of the partial import. This provides an opportunity for a corrected file to be re-imported without running into conflicts with partially imported files.

- Files can only be imported for project entities that have a dedicated tab in the Tree or Definition Table panel of the GUI (References, Amplicons, Samples, Variants, MIDs, Multiplexers). Entities such as Read Data Groups and MID Groups can not be imported in this manner; they must be created using the GUI or the CLI.
- Since the files to be loaded must be compatible with the CLI "create" command, they can only be used to create basic versions of more complex entities such as Multiplexers. The only associations that can be created are the ones inherent to the creation command for the entity, so an Amplicon can be created that is associated with a Reference because "-reference" is one of the keywords available to the "create amplicon" command. A Multiplexer can be created and assigned an annotation and an encoding, but the association of MIDs to the multiplexer and the associations of Samples to MID combinations are not accessible via the create command and those associations must be established via the GUI or via the "associate" command using the CLI (3.4.1).

The "Add" action above will only create empty Project elements with generic names. You can re-name an element from either a Project Tree or a Definition Table. In a Project Tree, click twice with a slight pause between the clicks to activate an editor directly on the name of the element in the tree. (Note that you can also change the name of the Project this way but that this will NOT also change the name of the folder that contains it, in your file-system, so be aware of the possibility of mismatch between the Project name and its file-system location.) Once elements are created (and possibly named), they can be fully defined in the corresponding "Definition Table" tab for the element type (see section 1.3.2). The approach of creating Project elements in a Project Tree view before fully defining them is convenient because it allows the user to set the structure of the Project up-front, possibly even before any sequencing reads are imported; see the documentation on "Samples" (sections 1.1.1.6, 1.3.1.3, 1.3.2.4, and 2.6.1) for an explanation of the usefulness (and complexity) of this.

For large Projects, especially when large amounts of data need to be imported into, exported from, or automated within a Project, the Command Line Interface (CLI) of the AVA software may be more appropriate than the Graphical User's Interface (GUI) described in the present section. See section 3 for a full description of the CLI, the language that was developed for it, and all the commands it includes.

1.3.1.1 The References Tree

The **References** Tree sub-tab shows the Reference Sequences as the main limbs of the Project Tree, with the Amplicons and Variants associated with each Reference Sequence as the next branching level, and the Samples associated with each Amplicon in the third level (Figure 1-12). This tree is useful to easily visualize all the Amplicons and defined Variants that are associated with each Reference Sequence, and all the Samples that will report on each Amplicon. You can also use this tree to populate the Global Align tab with the multi-alignment of the reads of any Sample-Amplicon pair you have created in your Project that has had computations run for it (see section 1.6.1).

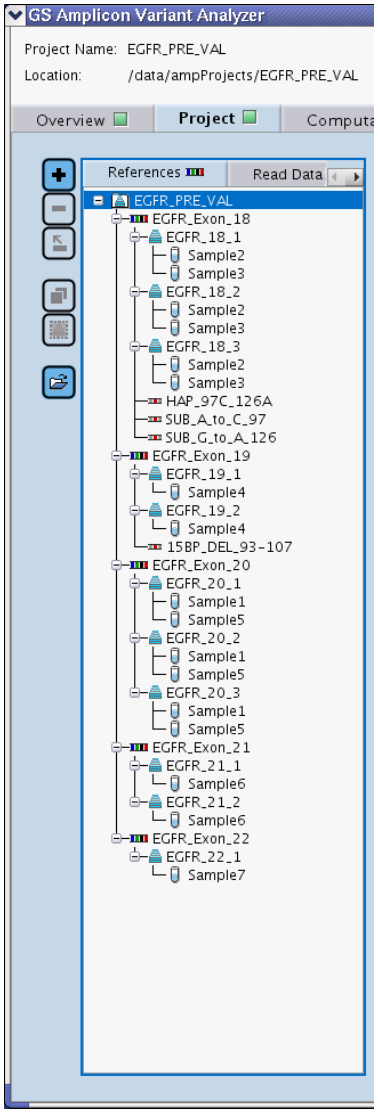


Figure 1-12: The References Tree sub-tab of the Project Tab's left-hand panel

1.3.1.2 The Read Data Tree

The **Read Data** Tree sub-tab shows the Read Groups / Read Data Sets as the main limbs of the Project Tree, with the Samples associated to each Read Data Set as the next branching level, and the Amplicons associated to each Sample in the last level (Figure 1-13). If the libraries were prepared with MIDs and Multiplexers are defined in the Project, the Multiplexers are displayed in this Tree, between the Read Data Sets and the Samples. (Read Groups are only a means to associate several Read Data Sets together, *e.g.* the various PicoTiterPlate Device regions of a Genome Sequencer FLX sequencing Run, for better ease of handling.)

You can use this tree to populate the Global Align tab with the multi-alignment of the reads of any Sample-Amplicon pair you have created in your Project that has had computations run for it (see section 1.6.1). The principal use of the Read Data Tree, however, is to establish which Read Data sets supply the Amplicon reads to particular Samples. Thus, rather than merely establishing that reads from some Amplicon generally exist in the Project for a given Sample (as

on the Sample Tree; see section 1.3.1.3), the Read Data Tree represents specifically which Read Data Set supplies those reads (and which Multiplexer defines the read assignments to the Samples, if applicable).

The AVA software will not allow you to associate a given Amplicon with more than one Sample or Multiplexer within the branch of a Read Data Set. This is important because the demultiplexing phase of computation (see section 1.4) depends on the uniqueness of such associations.

False Amplicon associations in the Read Data Tree:

Be careful to limit the Amplicons "lower" branches of this tree to those to which the specific Read Data Set truly contributes. False Sample-Amplicon associations could easily creep into a Read Data Set branch of your Project set up when you use the dragging method (section 1.3.2) to associate Samples with Read Data Sets; while convenient, this method brings the Sample *with all its associated Amplicons* into the Read Data Tree (unless any of these Amplicons are already associated with another Sample in this branch of the tree; see Note above). Similarly, if you drag one or more Amplicons to the root node or to a Read Group node in the Read Data Tree, they will get associated with every eligible Sample under the receiving node (see section 1.3.2). After you create such associations, therefore, make sure to "prune" the tree of any Amplicons that don't belong to any given Read Data Set branch or to any given Sample, by using the "Remove association and remain in project" button or its equivalent right-click contextual menu option. (Note that deleting an association between a Sample and an Amplicon within the Read Data Tree has no effect on the association between those entities in the Samples Tree; see section 1.3.1.3.)

This is important because the Read Data Tree provides the specific Run information for each of the Sample-Amplicon pairs used by the AVA software to determine which read sequences (Amplicons) to look for in each Read Data Set at the time of computation (and to which Sample each read belongs). The presence of false Amplicon associations in this tree would not only needlessly lengthen the Trimming step computing time (as the software would search all the reads for primer pairs that are, in fact, not present), but it could even result in the assignment of a read to a non-existent Amplicon should a spurious match occur.

Note that the Samples Tree, by comparison, represents all the Sample-Amplicon associations relevant to the Project *design*, whether or not any Read Data Set(s) containing such reads have (yet) been imported into the Project (see section 1.3.1.3): all Sample-Amplicon associations seen in any branch of the Read Data Tree are also seen in the Samples Tree; but Sample-Amplicon associations present in the Samples Tree do not (or should not) necessarily be present in any given branch of the Read Data Tree.

- Dragging a Sample from its Definition Table onto a Read Data Tree node that already contains this Sample will not create a duplicate “Sample” sub-branch on the tree. However if, at the time of this dragging action, the Sample has Amplicon associations that are not displayed in the tree node, these associations will be added to the branch (unless any of these Amplicons are already associated with another Sample in this branch of the tree; see the first Note above). As explained above, you should then “prune” the Read Data Tree of any false Read Data-Amplicon associations that may have been created.
- A Sample must have at least one Amplicon associated with it to be associated with a Read Data. This is because, as seen above, the association of Amplicons to Read Data is intrinsic to this tree, just as the Sample-Read Data is. One can view the information contained in this tree as Read Data-Sample-Amplicon association triads.
- For Amplicon libraries created with MIDs, the method of dragging a Sample with its associated Amplicons to a Read Data Tree node is NOT used. Rather, a Multiplexer is first associated with the Read Data Tree node, and then one or more Amplicons are dragged to the Multiplexer node. All Sample associations (to Read Data Sets and to Amplicons) are made indirectly, as defined by the Multiplexer *i.e.*, using the MIDs.

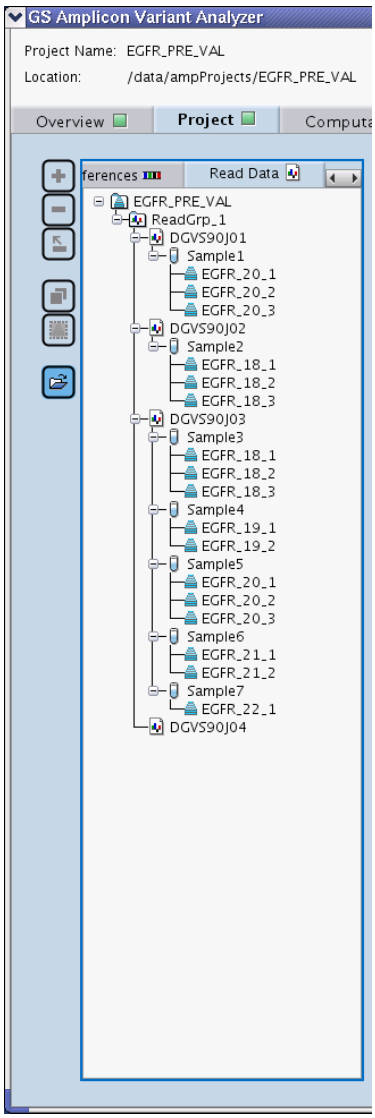


Figure 1-13: The Read Data Tree sub-tab of the Project Tab's left-hand panel. In this example we see that Sample1 has reads for Amplicon EGFR_20_3 which can be found in Read Data Set DGVS90J01. Sample5 also has reads for EGFR_20_3, but those are found in a different Read Data Set (DGVS90J03), which is allowed. Read Data Set DGVS90J04 has been imported into the Project, but no Sample-Amplicon pairs have yet been associated with it. Hence, DGVS90J04 would be excluded from Computations if carried out at this point, since the AVA software would not know to which Samples and Amplicons its reads belong (see section 1.4).

1.3.1.3 The Samples Tree

The **Samples** Tree sub-tab shows the Samples as the main limbs of the Project Tree, with the Amplicons associated to each Sample as the next branching level, and the Read Group / Read Data Sets associated to each Amplicon in the third/fourth level (Figure 1-14). Since this tree representation lists together all the Amplicons associated with each Sample, it is useful to navigate the results for a given Sample, irrespective of which Read Data Set supplied the reads for each Amplicon. You can use it to design your project, showing not only Sample-Amplicon pairs for which Read Data Set(s) already exists in your Project (shown in the Read Data Tree), but also any other Sample-Amplicon pairs that you expect to see over the course of the entire Project, irrespective of whether the Read Data has yet been imported into the Project (or even

yet exists). As such, this tree does not have the functional constraints of the Read Data Tree which provides the specific Run information for each of the Sample-Amplicon pairs, to be used for computation by the AVA software (see section 1.3.1.2). You can also use this tree to populate the Global Align tab with the multi-alignment of the reads of any Sample-Amplicon pair you have created in your Project that has had computations run for it (see section 1.6.1).

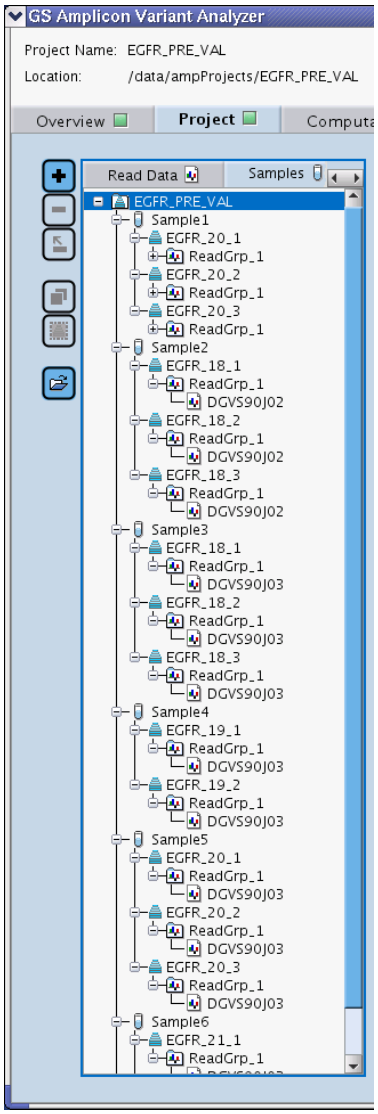


Figure 1-14: The Samples Tree sub-tab of the Project Tab's left-hand panel

1.3.1.4 The MIDs Tree

This is the simplest of the Tree sub-tabs, as it simply lists the MIDs that are defined in the Project, with the optional MID Groups if applicable (Figure 1-15).

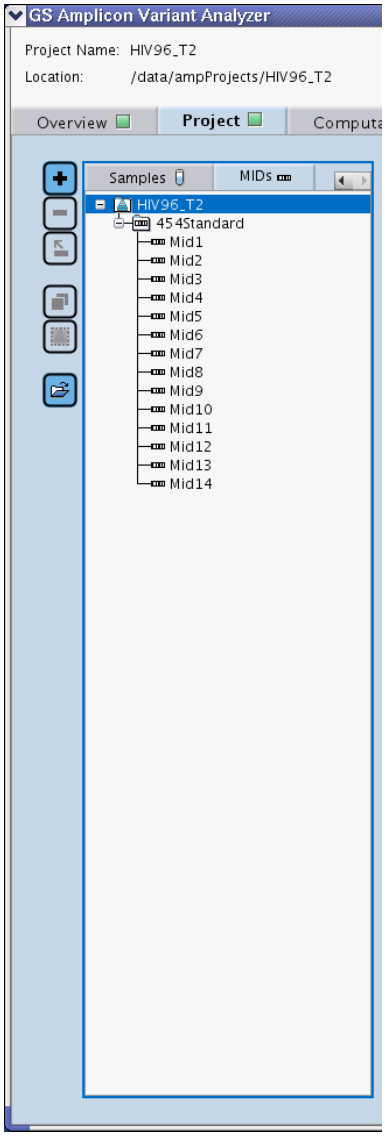


Figure 1-15: The MIDs Tree sub-tab of the Project Tab's left-hand panel

1.3.2 The "Definition Table" Sub-Tabs

The right-hand panel of the Project tab contains seven sub-tabs, one for each type of element that makes up an Amplicon Project (except "Group" element types). The number of elements listed in a Definition Table appears next to the tab name, in parenthesis. The row for a particular element in a Definition Table can be selected by clicking on any of its cells and you can select multiple rows by holding down the 'shift' or 'control' keys while making your selections. The Tables on these sub-tabs can be used to create or delete elements of the corresponding type for your Project, using most of the same buttons or right-click functions as for the "Tree" sub-tabs of the left panel (see section 1.3.1).

Button	Name – Description
	To add a new element in the Project (except for Read Data Sets and Groups), either click in the appropriate sub-tab to make it the focus of the application and click the "Add" button to the left of the Project Tab; or right-click on an existing element in the appropriate Definition Table and select the "Add" option from the contextual menu that appears. The "Add" action is used to create new elements that can be completely defined using tools of the Project tab. However, certain data, specifically the Read Data Sets, are data which are defined outside the application and must be imported into the Project. For this data there is the "Import" action.
	To import Read Data Sets in the Project, either click in the Read Data sub-tab to make it the focus of the application and click the "Import data" button to the left of the Project Tab; or right-click on an existing element in the Read Data Definition Table and select the "Import" option from the contextual menu that appears. To import en-masse definitions for other project elements for which there is a sub-tab (References, Amplicons, Samples, Variants, MIDs, Multiplexers) the same mechanism may be used (see section 1.3.1 for a more detailed discussion of this feature).
	To remove an existing element from the Project (including a Read Data Set), either select it in its Definition Table and click the "Remove from project" button to the left of the Project Tab; or right-click on the element to be deleted in its Definition Table and select the "Remove" option from the contextual menu that appears. A confirmation window will appear; click "Yes" to delete the element. If you make multiple selections and choose the "Remove" action, the confirmation window will prompt you to remove each one individually, or you can click the "Yes to all" button to confirm the removal of all selected elements at once (Figure 1-16).
	The "Duplicate item" button is used to create copies of items in the Definition Tables. This is another contextual button that operates on an item that is selected in one of the Definition Table sub-tabs. Clicking on this button while a single item is selected in the Definition Table will add an extra row to the table that is identical to the selected item except that its name will have a suffix of the form "_copy_NUM" (where "NUM" increments from 1 when more than one copy is made of an original item). A copy of a copy adds another copy suffix (*e.g.*, "ItemName_copy_2_copy_1" would result from a duplication of "ItemName_copy_2"). The duplication operation only duplicates data that is explicitly associated with the item in the Table row: it does not duplicate any associations the item might have as implied by the tree structures (such as Sample-Amplicon associations) unless they are specified in the Table (such as the Reference association in the Amplicon and Variant Definition Tables, and the content of Multiplexers). The duplication of Read Data is not currently supported.

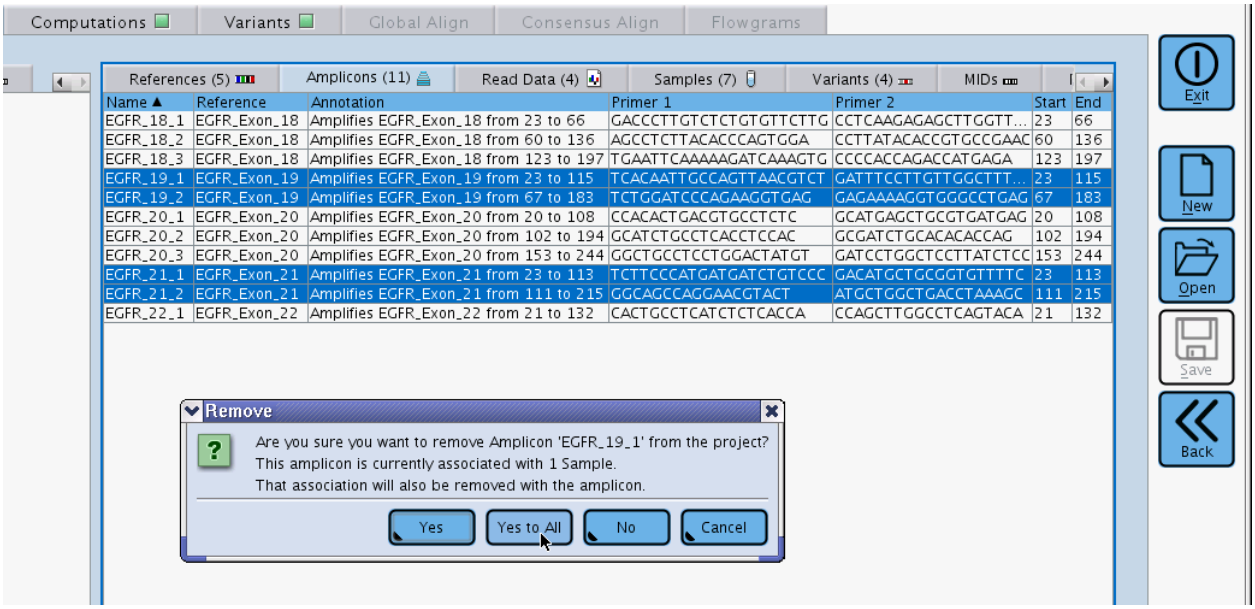


Figure 1-16: The Amplicons Definition Table with a multiple selection applied to rows. The user is prompted to remove the selected rows one at a time, which can be done by clicking "Yes" to each; or you can remove all the selected rows at once using the "Yes to All" button.

Contrary to the case with the tree tabs, the only associations you can create or modify within the element Definition Tables are the ones between:

- Amplicons or Variants and their Reference Sequence,
- Read Data Sets and their Read Data Groups, or MIDs and MID Groups, which are done via a drop down menu that appears when you double-click in the corresponding cell, in these Tables

- The triads Amplicons / Read Data Sets / Samples (indirectly), when MIDs and Multiplexers are used. Specifically, modifying the MID and Sample associations for a given Multiplexer, using the functionality found in the Multiplexer Definition Table, will dynamically update the associated Samples for any Amplicons of a Read Data Set that are associated with the changed Multiplexer.

The "Remove association and remain in project" button or its right-click equivalent are never available when the focus of the application (the last place you clicked) is on a sub-tab of the right-hand panel because not all associations are explicit in the Definition Tables (especially the multiple associations an element may have); indeed, the ability to view the network of element associations in the Project is one of the main benefits of the Project Tree views.

However, associations can also be established by dragging an element from its Definition Table on a right-hand sub-tab, to an appropriate element on a tree view in a left panel sub-tab: hold the mouse button until the name of the "destination" element on the tree view turns green, and then release the mouse button (Figure 1-17).

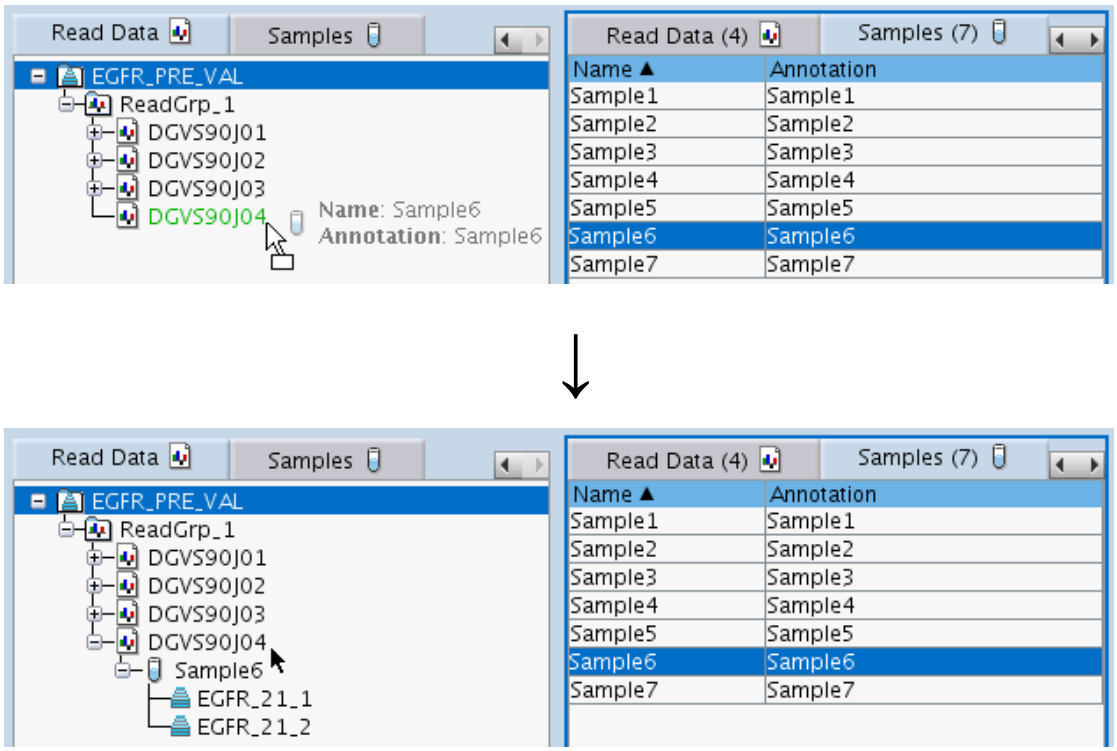


Figure 1-17: Creating an association by dragging an element from its Definition Table on a right-hand sub-tab to an appropriate element on a tree view in a left panel sub-tab. In this case, Sample6 (with its two previously-associated Amplicons EGFR_21_1 and EGFR_21_2) are being associated to Read Data Set DGVS90J04. (See the "Caution" in section 1.3.1.2 for special information about dragging Samples into the Read Data Tree.)

- The directionality of the dragging capability is always from a Definition Table on the right to an appropriate tree node on the left you cannot establish an association by dragging a tree node object to an object in its Definition Table.
- The software will not allow you to establish invalid associations, such as linking an Amplicon to a Variant: only elements that are valid destinations for the element you are dragging will turn green and allow the creation of the new association.
- If you drag one (or more) valid element(s) to the root node (the "Project" folder) in the Samples Tree, or to the root node, a Read Group node or a Read Data node in the Read Data Tree, the element will become associated with all the relevant elements in the nodes below and will be added to all the corresponding branches of that tree (unless this would cause an Amplicon to become associated with more than one Sample or Multiplexer within a Read Data Set branch of the Read Data Tree; in such a case, the existing association remains and only new, non-conflicting associations are created).
    - This is particularly useful when the experimental design requires the association of one or more Amplicons to a large number of Samples, in one or more Read Data Sets. Such a design may be especially common for experiments using MIDs, where the same Amplicon(s) are to be monitored in multiple MID-tagged libraries (Samples). In this case, Amplicons dragged to an upper node will "trickle down" and become associated with all the relevant and eligible objects below that node. However, the restriction that an Amplicon be associated with only one Sample (or Multiplexer) within a given Read Data Set imposes the following behavior for this function:
        - The "trickle" will only be accepted if there is at least one Read Data at or below the level of the drag that has at most one Sample or Multiplexer currently associated with it [so it is unambiguous which Sample or Multiplexer the dragged Amplicon(s) should be associated with] and if the Amplicons are not already associated with those Samples or Multiplexers.
        - If multiple Amplicons are dragged together and some of them are already associated with some of the Samples or Multiplexers under the recipient node, but others aren't, then the non-associated Amplicons will become associated, and the others will be ignored.

In general, to add or edit the information for an element in its Definition Table, double-click in the corresponding cell in the Table. In some cases, the content of the cell will be highlighted and you can type in the new content; in other cases, the double-click opens a separate window for data entry. Two characteristics, Name and Annotation, are common to all element types; the method to enter or edit them is provided below. The editing methods applicable to the other characteristics of each element type are specified in the sub-sections below.

To edit a Project element's Name

1. Double-click in the "Name" cell for the element you want to re-name, in its Definition Table.
2. Overtype the new name.
3. Press "Enter" or click elsewhere.

To enter or edit a Project element's Annotation

1. Double-click in the "Annotation" cell for the element you want to re-name, in its Definition Table. An "Edit Annotation" window will open (Figure 1-18).
2. Type or overtype the information desired.
3. Click "OK".

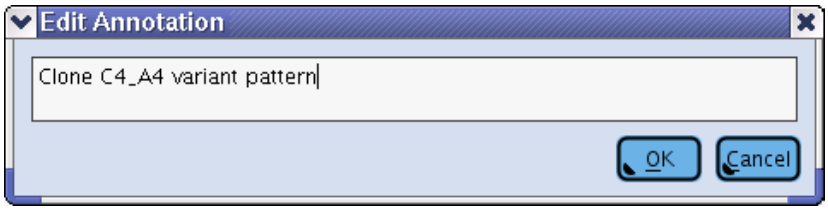
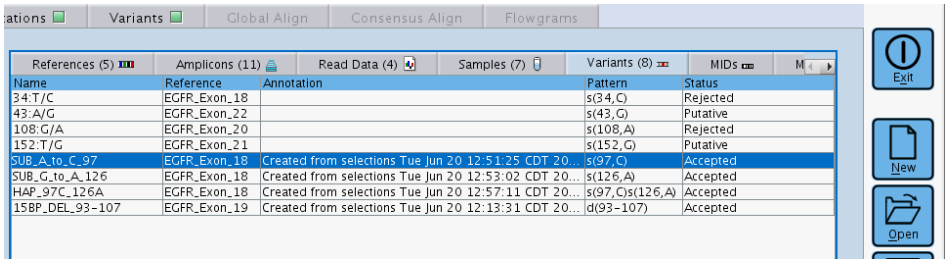


Figure 1-18: The Edit Annotation window, used to enter or edit the Annotation from any type of Project element

Each Definition Table sub-tab has a header row that labels the content type of each column. The data in the table can be sorted by column content. A black triangle indicator appears to the right of the header label of a column if a sort has been applied to it: an upward-pointing triangle indicates that an ascending sort has been applied to the table data, while a downward-pointing triangle indicates a descending sort. Keep in mind that not all sorts are purely alphabetical or numerical; Project element Names, in particular, are broken down into numerical and non-numerical sections and the sections are sorted either numerically or alphabetically as appropriate.

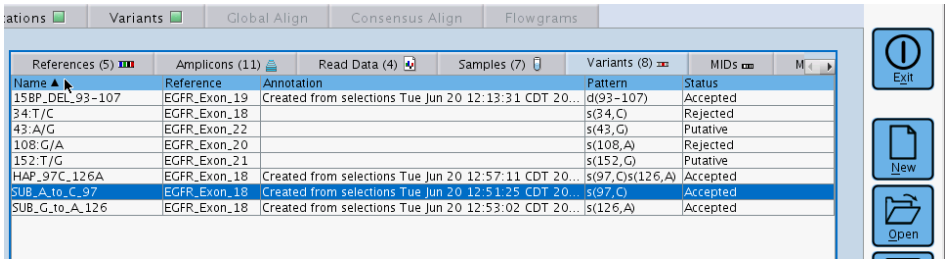
Clicking on a column header that does not currently have a sort applied to it will cause an ascending sort of the table based on the data in that column. Clicking on a column header that already has an existing sort applied to it will toggle the sort type (ascending or descending). Only one column may have an active sort applied to it at a time, but the sort operations are stable and maintain the prior table order in cases where ties are encountered during the sort operation. This allows you to apply more than one sort at a time. For instance, if you wanted to sort the Variants Definition Table (Figure 1-19A) by status with the entries subsorted by variant name, you would first click on the 'Name' column header to sort by variant name (Figure 1-19B) and then you would click on the 'Status' column to sort the entries by status (Figure 1-19C).

A



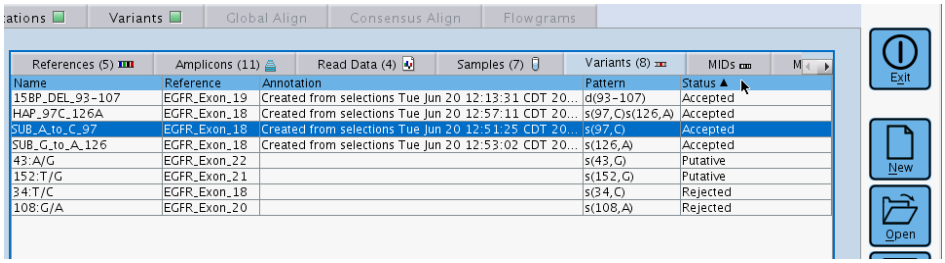
Name	Reference	Annotation	Pattern	Status
34:T/C	EGFR_Exon_18		s(34,C)	Rejected
43:A/G	EGFR_Exon_22		s(43,G)	Putative
108:G/A	EGFR_Exon_20		s(108,A)	Rejected
152:T/G	EGFR_Exon_21		s(152,G)	Putative
SUB_A_to_C_97	EGFR_Exon_18	Created from selections Tue Jun 20 12:51:25 CDT 20...	s(97,C)	Accepted
SUB_G_to_A_126	EGFR_Exon_18	Created from selections Tue Jun 20 12:53:02 CDT 20...	s(126,A)	Accepted
HAP_97C_126A	EGFR_Exon_18	Created from selections Tue Jun 20 12:57:11 CDT 20...	s(97,C)s(126,A)	Accepted
15BP_DEL_93-107	EGFR_Exon_19	Created from selections Tue Jun 20 12:13:31 CDT 20...	d(93-107)	Accepted

B



Name ▲	Reference	Annotation	Pattern	Status
15BP_DEL_93-107	EGFR_Exon_19	Created from selections Tue Jun 20 12:13:31 CDT 20...	d(93-107)	Accepted
34:T/C	EGFR_Exon_18		s(34,C)	Rejected
43:A/G	EGFR_Exon_22		s(43,G)	Putative
108:G/A	EGFR_Exon_20		s(108,A)	Rejected
152:T/G	EGFR_Exon_21		s(152,G)	Putative
HAP_97C_126A	EGFR_Exon_18	Created from selections Tue Jun 20 12:57:11 CDT 20...	s(97,C)s(126,A)	Accepted
SUB_A_to_C_97	EGFR_Exon_18	Created from selections Tue Jun 20 12:51:25 CDT 20...	s(97,C)	Accepted
SUB_G_to_A_126	EGFR_Exon_18	Created from selections Tue Jun 20 12:53:02 CDT 20...	s(126,A)	Accepted

C



Name	Reference	Annotation	Pattern	Status ▲
15BP_DEL_93-107	EGFR_Exon_19	Created from selections Tue Jun 20 12:13:31 CDT 20...	d(93-107)	Accepted
HAP_97C_126A	EGFR_Exon_18	Created from selections Tue Jun 20 12:57:11 CDT 20...	s(97,C)s(126,A)	Accepted
SUB_A_to_C_97	EGFR_Exon_18	Created from selections Tue Jun 20 12:51:25 CDT 20...	s(97,C)	Accepted
SUB_G_to_A_126	EGFR_Exon_18	Created from selections Tue Jun 20 12:53:02 CDT 20...	s(126,A)	Accepted
43:A/G	EGFR_Exon_22		s(43,G)	Putative
152:T/G	EGFR_Exon_21		s(152,G)	Putative
34:T/C	EGFR_Exon_18		s(34,C)	Rejected
108:G/A	EGFR_Exon_20		s(108,A)	Rejected

Figure 1-19: (A) A Variants Definition Table with unsorted entries. (B) A Variants Definition Table with entries sorted by name. Clicking on the 'Name' header performs an ascending sort of the rows as indicated by the upward-pointing black triangle. (C) A Variants Definition Table with entries sorted by status. Clicking on the 'Status' header performs an ascending sort of the rows as indicated by the upward-pointing black triangle. Since an ascending name-sort was performed first (panel B), the rows within each 'Status' category have an ascending name-sort.

If you add a new entity to a Definition Table and there is a sort applied to a column for which the new row has a default value (such as the 'Name' field), the new row will be inserted into the table in its proper place according to the default value and the sort order. If you edit a cell in a sorted column and you change the value to something that would cause the row to change its position according to the sort order, however, sorting is automatically turned off for that column. This behavior prevents the row from moving out from under you as you enter an edit in a cell in a sorted column.

When the sorting is turned off, none of the header labels for the table will have the black triangle indicator. You can re-enable sorting by clicking on any column header you want to sort by. If the first column header you click on for sorting is the same one that was used for sorting just prior to sorting deactivation, you will restore the same sort order (ascending or descending) that was last used for the column. Clicking on any other column header will result in the default ascending sort for that column.

1.3.2.1 The References Definition Table

The **References** Definition Table lists all the Reference Sequences defined in the Project, with the following three characteristics (Table columns; see Figure 1-20):

- Name
- Annotation (free user-entered text)
- Sequence

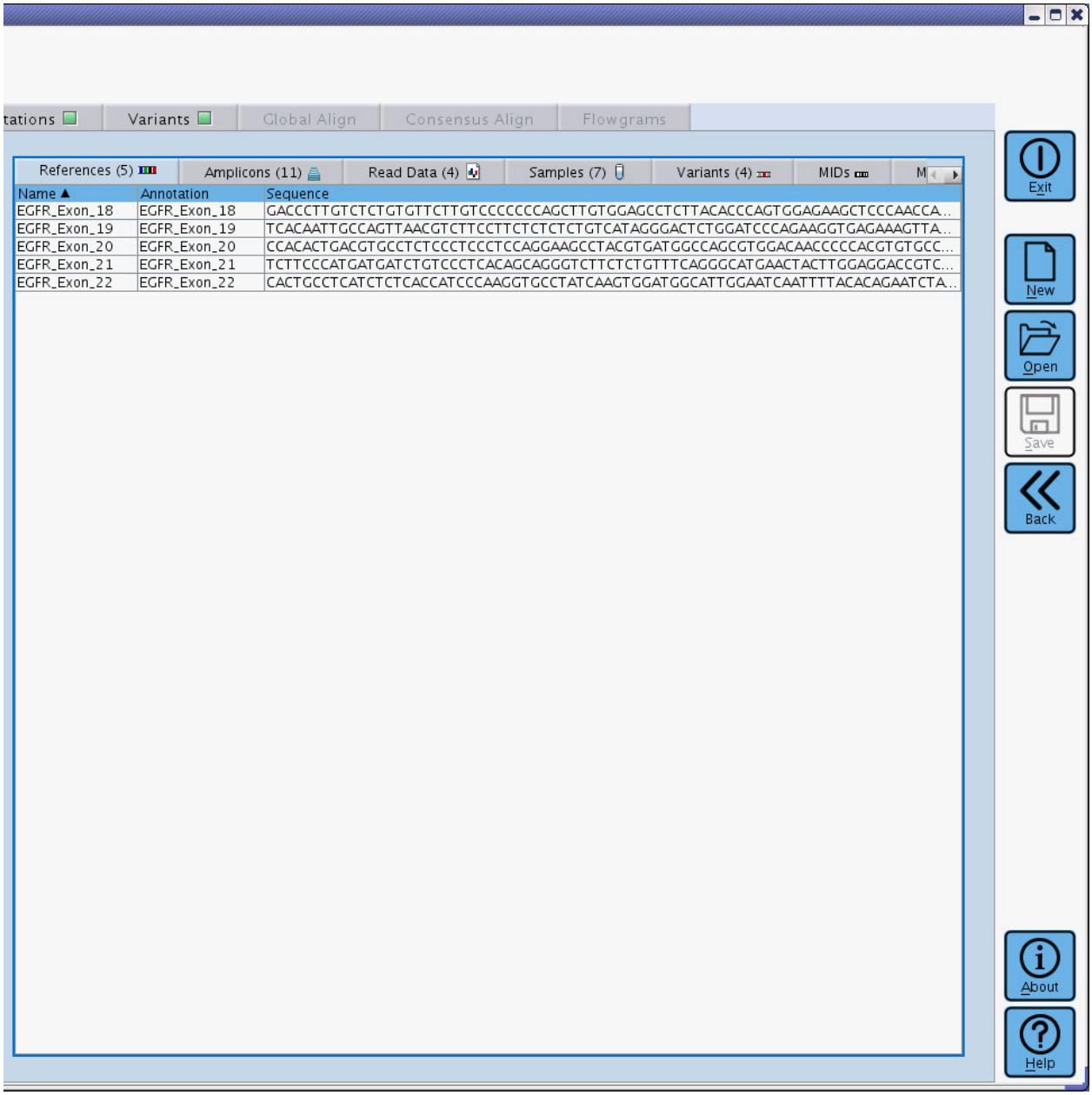


Figure 1-20: The References Definition Table sub-tab of the Project Tab's right-hand panel

For the procedures to add or remove Reference Sequences in a Project, see section 1.3.2 (or 1.3.1, to accomplish this in a "Project Tree" view). For the procedures to enter/edit the Name or Annotation information for a Reference Sequence, see section 1.3.2. The sub-section below provide the procedure to enter/edit the other characteristic of Reference Sequences, the DNA sequence itself.

1.3.2.1.1 To Enter or Edit the DNA Sequence of a Reference Sequence

1. Double-click in the "Sequence" cell of the Reference Sequence you are defining, in its Definition Table. An "Edit Sequence" window will open (Figure 1-21).
2. Paste or type the sequence (only A, T, G, C, or N characters; see Caution, below).
3. Click "OK".

Characters restriction: Be aware that only "nucleotide" characters (A, T, G, C, or N) are accepted when you enter a Reference Sequence into the AVA software (by typing or pasting). For convenience, when pasting sequences, characters that are not nucleotide characters and are also not IUPAC ambiguity characters (such as R for purine, Y for pyrimidine, *etc.*) are removed from the pasted entry. This is useful when pasting sequences from sources that may include non-sequence information (such as white space or numerical position information in the margin of each line). During such pastes, any IUPAC ambiguity characters are converted to "N" characters, as the other ambiguity characters are not supported by the software (typing individual "ambiguous" characters, however, does not result in their conversion to "N"; these are simply ignored and the text "Only ATGC and N" at the top of the Edit Sequence window turns bold and red to alert you that an invalid character was used). The restriction that no ambiguity characters other than N be present in a sequence is a requirement of many alignment algorithms and is not unique to the 454 Sequencing System software.

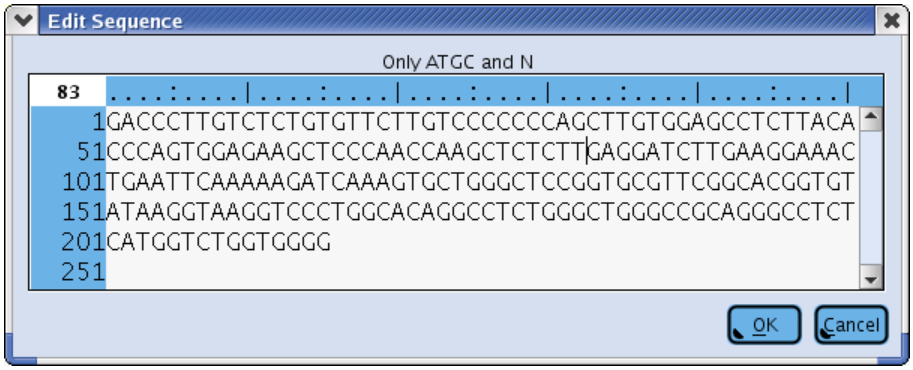


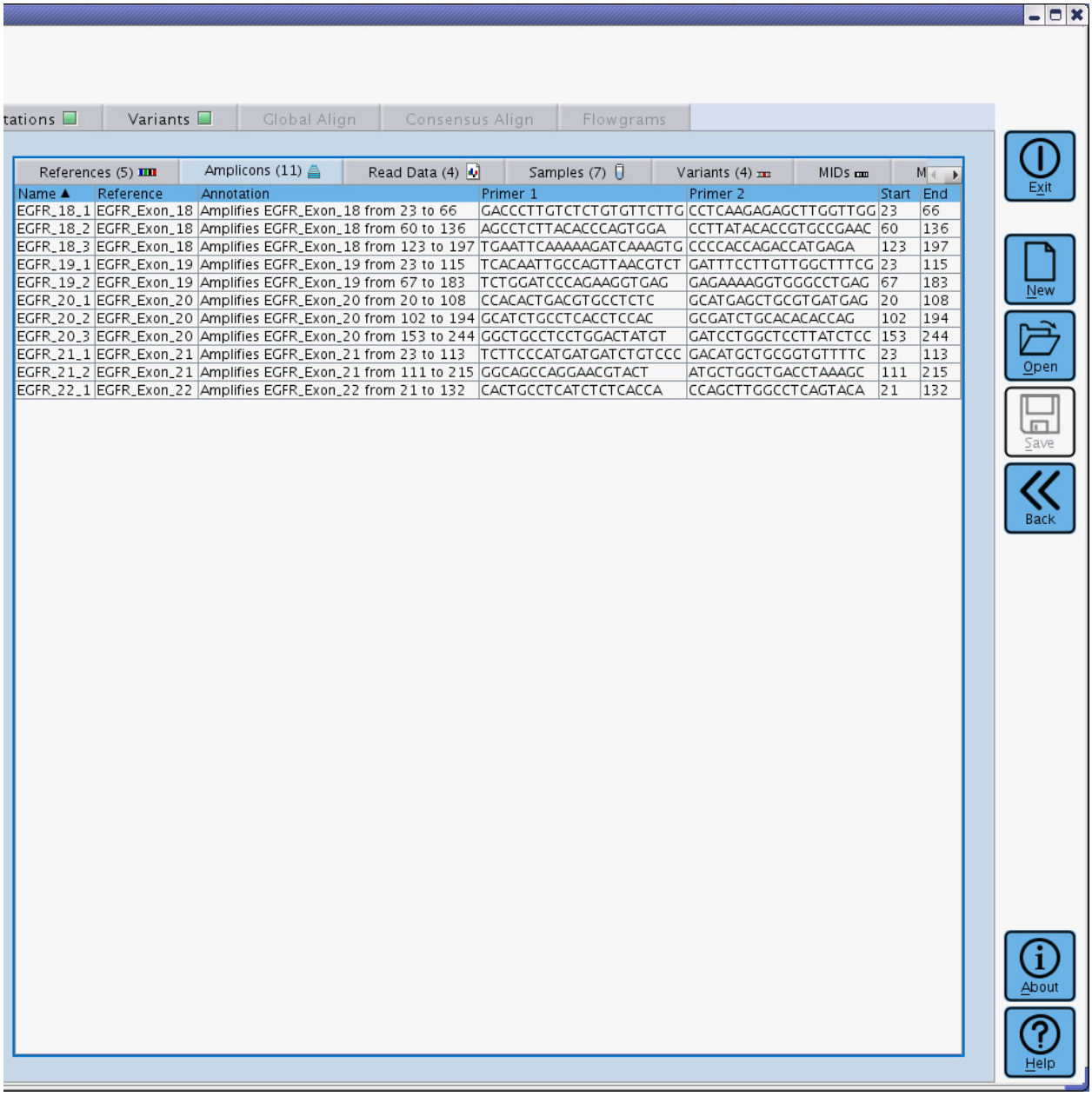
Figure 1-21: The Edit Sequence window, used to enter or edit the DNA sequence of a Reference Sequence element

1.3.2.2 The Amplicons Definition Table

The **Amplicons** Definition Table lists all the Amplicons defined in the Project, with the following seven characteristics (Table columns; see Figure 1-22):

- Name

- Reference Sequence (to which the Amplicon is associated)
- Annotation (free user-entered text)
- Primer 1 (5'-->3' sequence of the forward primer of the Amplicon)
- Primer 2 (5'-->3' sequence of the reverse primer of the Amplicon)
- Start (first nucleotide of the Target, on the Reference Sequence)
- End (last nucleotide of the Target, on the Reference Sequence



Name	Reference	Annotation	Primer 1	Primer 2	Start	End
EGFR_18_1	EGFR_Exon_18	Amplifies EGFR_Exon_18 from 23 to 66	GACCCTTGTCTCTGTGTTCTTG	CCTCAAGAGAGCTTGGTTGG	23	66
EGFR_18_2	EGFR_Exon_18	Amplifies EGFR_Exon_18 from 60 to 136	AGCCTCTTACACCCAGTGGA	CCTTATACACCGTGCCGAAC	60	136
EGFR_18_3	EGFR_Exon_18	Amplifies EGFR_Exon_18 from 123 to 197	TGAATTCAAAAAGATCAAAGTG	CCCCACCAGACCATGAGA	123	197
EGFR_19_1	EGFR_Exon_19	Amplifies EGFR_Exon_19 from 23 to 115	TCACAATTGCCAGTTAACGTCT	GATTTCCTTGTTGGCTTTCG	23	115
EGFR_19_2	EGFR_Exon_19	Amplifies EGFR_Exon_19 from 67 to 183	TCTGGATCCCAGAAGGTGAG	GAGAAAAGGTGGGCCTGAG	67	183
EGFR_20_1	EGFR_Exon_20	Amplifies EGFR_Exon_20 from 20 to 108	CCACACTGACGTGCCTCTC	GCATGAGCTGCGTGATGAG	20	108
EGFR_20_2	EGFR_Exon_20	Amplifies EGFR_Exon_20 from 102 to 194	GCATCTGCCTCACCTCCAC	GCGATCTGCACACACCAG	102	194
EGFR_20_3	EGFR_Exon_20	Amplifies EGFR_Exon_20 from 153 to 244	GGCTGCCTCCTGGACTATGT	GATCCTGGCTCCTTATCTCC	153	244
EGFR_21_1	EGFR_Exon_21	Amplifies EGFR_Exon_21 from 23 to 113	TCTTCCCATGATGATCTGTCCC	GACATGCTGCGGTGTTTTC	23	113
EGFR_21_2	EGFR_Exon_21	Amplifies EGFR_Exon_21 from 111 to 215	GGCAGCCAGGAACGTACT	ATGCTGGCTGACCTAAAGC	111	215
EGFR_22_1	EGFR_Exon_22	Amplifies EGFR_Exon_22 from 21 to 132	CACTGCCTCATCTCTCACCA	CCAGCTTGGCCTCAGTACA	21	132

Figure 1-22: The Amplicons Definition Table sub-tab of the Project Tab's right-hand panel

For the procedures to add or remove Amplicons in a Project, see section 1.3.2 (or 1.3.1, to accomplish this in a "Project Tree" view, and concurrently create associations). For the

procedures to enter/edit the Name or Annotation information for an Amplicon, see section 1.3.2. The sub-sections below provide the procedures to enter/edit the other characteristics of Amplicons.

1.3.2.2.1 To Enter or Edit the Reference Sequence to which an Amplicon is associated

1. Ensure that the column labeled “Reference” in the table is wide enough to allow you to distinguish among the Reference Sequences from which you want to select. The column may be widened by clicking on the separator line, in the table header, between the Reference and Annotation columns, and dragging the separator to the right.
2. Double-click in the “Reference Sequence” cell for the Amplicon you are defining, in its Definition Table. A drop down menu will expand, showing all the Reference Sequences currently listed in the Project.
3. Select the proper Reference Sequence from the drop down menu. The new association will automatically appear on the References Tree, on the left panel.

If the Reference Sequence does not yet contain a DNA sequence (see section 1.3.2.1.1), you will still be able to associate Amplicons to it, but you will not be able to fully define them. In particular, you will not be able to specify the Target Start and End for the Amplicons (see section 1.3.2.2.3, below) because these are set using the position numbering from the Reference Sequence.

1.3.2.2.2 To Enter or Edit the Primer Sequences for the Amplicon

As mentioned earlier (section 1.1.1.3), Primer 1 and Primer 2 correspond to the “sequence-specific” part of the two Fusion Primers used to construct the Amplicon library, excluding the 19 bp “Primer A” and “Primer B” parts of the Fusion Primers.

- Both Primer 1 and Primer 2 should be entered as their true 5’-->3’ sequence. To find the End of the Target (section 1.3.2.2.3, below), the software automatically determines the reverse-complement of Primer 2 (Primer 2’) and aligns this to the Reference Sequence.
- The AVA software does not require any knowledge of A vs. B beads from the emPCR Amplification kits; reads that align in the same orientation as the given Reference Sequence are considered ‘forward’ reads, and those that must be reverse complemented to align are considered ‘reverse’ reads.

1. Double-click in the “Primer 1” (or “Primer 2”) cell for the Amplicon you are defining, in its Definition Table. An “Edit Primer 1” (or “Edit Primer 2”) window will open (Figure 1-23).
2. Paste or type the sequence (only A, T, G, C, or N characters; see Caution, below).
3. Click “OK”.

Characters restriction: Be aware that only “nucleotide” characters (A, T, G, C, or N) are accepted when you enter a Primer Sequence into the AVA software (by typing or pasting). For convenience, when pasting sequences, characters that are not nucleotide characters and are also not IUPAC ambiguity characters (such as R for purine, Y for pyrimidine, *etc*.) are removed from the pasted entry. This is useful when pasting sequences from sources that may include non-sequence information (such as white space or numerical position information in the margin of each line). During such pastes, any IUPAC ambiguity characters are converted to “N” characters, as the other ambiguity characters are not supported by the software (typing individual “ambiguous” characters, however, does not result in their conversion to “N”; these are simply ignored and the text “Only ATGC and N” at the top of the Edit Sequence window turns bold and red to alert you that an invalid character was used). The restriction that no ambiguity characters other than N be present in a sequence is a requirement of many alignment algorithms and is not unique to the 454 Sequencing System software.

Although the AVA software allows “N” characters in the primer sequences, the primer trimming and demultiplexing computational steps perform better if only A, T, G, or C characters are used. If your primer design involves “wobble" positions in which more than one base may appear, it is preferable to define the primer sequence with one of the alternative bases rather than an N at those positions. For record keeping purposes, you may document the choice of data entry in the corresponding Amplicon’s Annotation field.

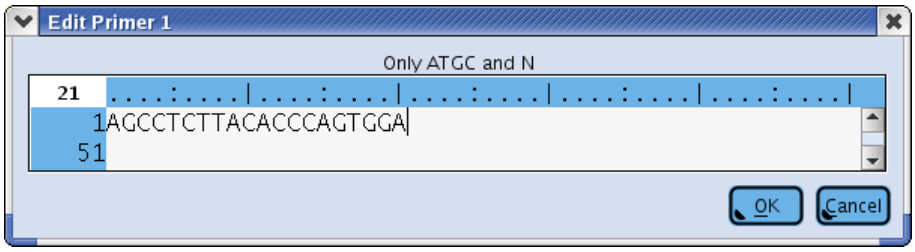


Figure 1-23: The Edit Primer 1 window, used to enter or edit the DNA sequence of Primer 1 for an Amplicon element

1.3.2.2.3 To Enter or Edit the Target Start and End Positions

There is no requirement that the Reference Sequence contain either of the two Primer sequences, although they typically will be contained therein. The Target, however, is required to be present in the Reference Sequence, and the Target Start and End positions indicate the first and last bases of the Target, inclusive, relative to the Reference Sequence.

To simplify the setting of the Start and End values, and reduce the risk of data entry errors, the AVA software searches for the Primers within the Reference Sequence, on the assumption that they will most likely be present and, if found, uses the primer positions to establish default values for the Target Start and End (see below). As such, prior to entering or editing the Target Start and End positions, the sequence of the Reference Sequence with which the Amplicon is associated and the two Amplicon Primers must already be defined (sections 1.3.2.1.1 and 1.3.2.2.2).

1. Double-click in either the “Start” or the “End” cell for the Amplicon you are defining, in its Definition Table. Clicking in either the “Start” or the “End” cell opens the same “Edit Start/End” window (Figure 1-24). This window includes:
    a. a brief set of instructions
    b. a pair of data entry boxes for the Start and End nucleotides
    c. the Reference Sequence to which the Amplicon is associated, with a color-coded overlay for the Primer sequences (matched and mismatched), the Target sequence (the part of the Reference Sequence between the two Primers), and the “unused” sequence (the part of the Reference Sequence outside the two Primers)
    d. the sequence of the two Primers, plus the reverse-complement of Primer 2 (Primer 2’)

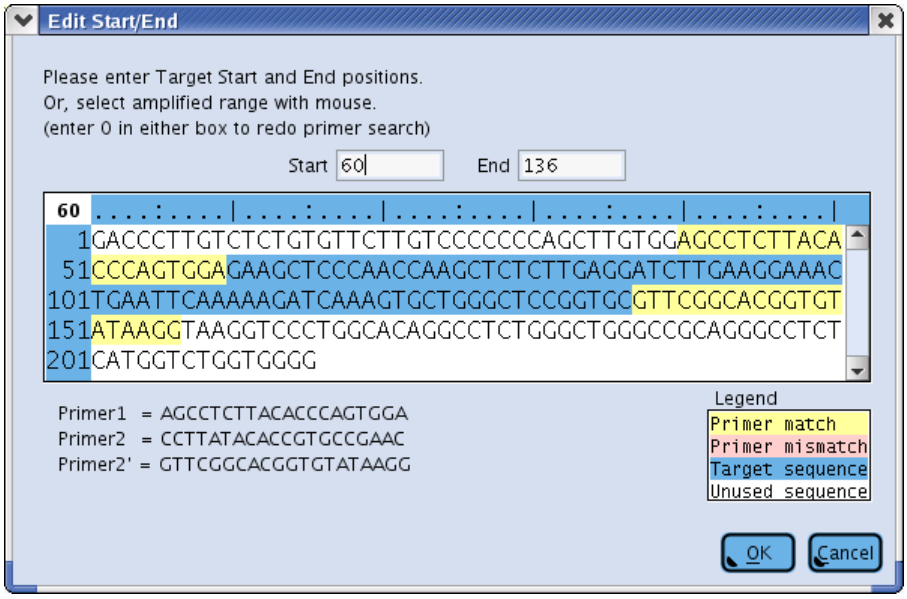


Figure 1-24: The Edit Start/End window, used to define the start and end of the Target for an Amplicon element (the part excluding the Primers), by locating the Primers 1 and 2 on the Reference Sequence with which the Amplicon is associated

2. There are 3 ways to set (or reset) the Start and End nucleotides of the Target:
    a. If the Target’s Start and End have not been specified for this Amplicon before (*i.e.* the Start and End cells were empty when you double-clicked them), the software automatically searches for the Primers (Primer 1 and Primer 2’) in the Reference Sequence; if it finds them (exact matches only), the software marks the Primers in yellow and the Target sequence (between the two Primers) in blue, and specifies default values for the Target’s Start and End positions in the boxes at the top of the window. The user should verify that the default positions are correct since, in some rare circumstances, there may be multiple Primer1-Primer2’ pairs of matches within the same Reference Sequence and the software simply gives the first such pair it finds. This Primer search function can also be elicited by typing a “0” (or a negative number) in either the Start or the End entry box. It is possible that exact matches for the Primers are not found in the Reference Sequence, as either or both Primers may actually not be represented by the Reference Sequence or, due to design

considerations (or primer synthesis or sequencing errors), the Primers may slightly differ from the Reference Sequence so that they have a close, but inexact match. Whatever the reason, if no exact match can be found for Primer1, the AVA software will default the Target Start to the first base of the Reference Sequence; if no exact match can be found for Primer2', the default for Target End will be the last base of the Reference Sequence. If this happens, verify that you have correctly defined the Primer and the Reference Sequence to which this Amplicon is associated; if the sequences are correct, but the default values supplied are incorrect, use one of the following methods to specify the Target Start and End positions.

b. If you know the exact positions of the Target's Start and End relative to the Reference Sequence, you can type them in the entry boxes at the top of the window. The AVA software will automatically update the color-coded display to indicate how well Primer1 and Primer2' match to the Reference Sequence in the regions abutting the supplied Start and End positions. If you know that the Reference Sequence doesn't actually contain the Primers themselves, then you can safely ignore this feedback. However, if the Reference Sequence is supposed to contain the primers and there are one or more bases of mismatch indicated by pink highlighting in the display, you should check that you entered the Start and End positions correctly, that you entered the correct Primers (and that both are in the 5'-->3' orientation), that you have the correct Amplicon-Reference sequence association, and that the Reference Sequence itself has the correct sequence.
c. You can also use the mouse drag method: the software interprets click-and-dragging the mouse in the sequence as an attempt at selecting the amplified range (the Target), so it aligns the sequence beyond the drag point with the Primers (Primer 2' if dragging to the right and Primer 1 if dragging to the left). Matching nucleotides are overlaid in yellow and non-matching nucleotides, in pink. This method may be especially useful if the Primers you used to generate the Amplicon library did not exactly match the Reference Sequence you are using for analysis. To use this method, do the following:
    i. Click near the beginning of the Reference Sequence and drag the mouse to the right. The stretch of sequence (the length of Primer 2') beyond the drag point will be displayed in color to indicate matches with the Primer. Stop and release the mouse button when the whole stretch is yellow, indicating a perfect match with Primer 2' (or allow for mismatches, shown in pink, if appropriate). The software enters the start and stop points of the dragging action in the Start and End entry boxes, respectively; this sets the Target's End nucleotide.
    ii. Click again on the last nucleotide of the Target, as just defined, and drag the mouse to the left. Stop and release the mouse as before, when you have located Primer 1. Again, the software enters the start and stop points of the dragging action, but in the End and Start entry boxes, respectively; this sets both the Target's Start and End nucleotides.

3. Click "OK".

1.3.2.3 The Read Data Definition Table

The **Read Data** Definition Table lists all the Read Data Sets defined in the Project, with the following four characteristics (Table columns; see Figure 1-25):

- Name

- Annotation (free user-entered text)
- Group (the Read Data Set to which the Read Data Set belongs)
- Active (if checked, the Read Data Set will be included in the next computation of the Project)

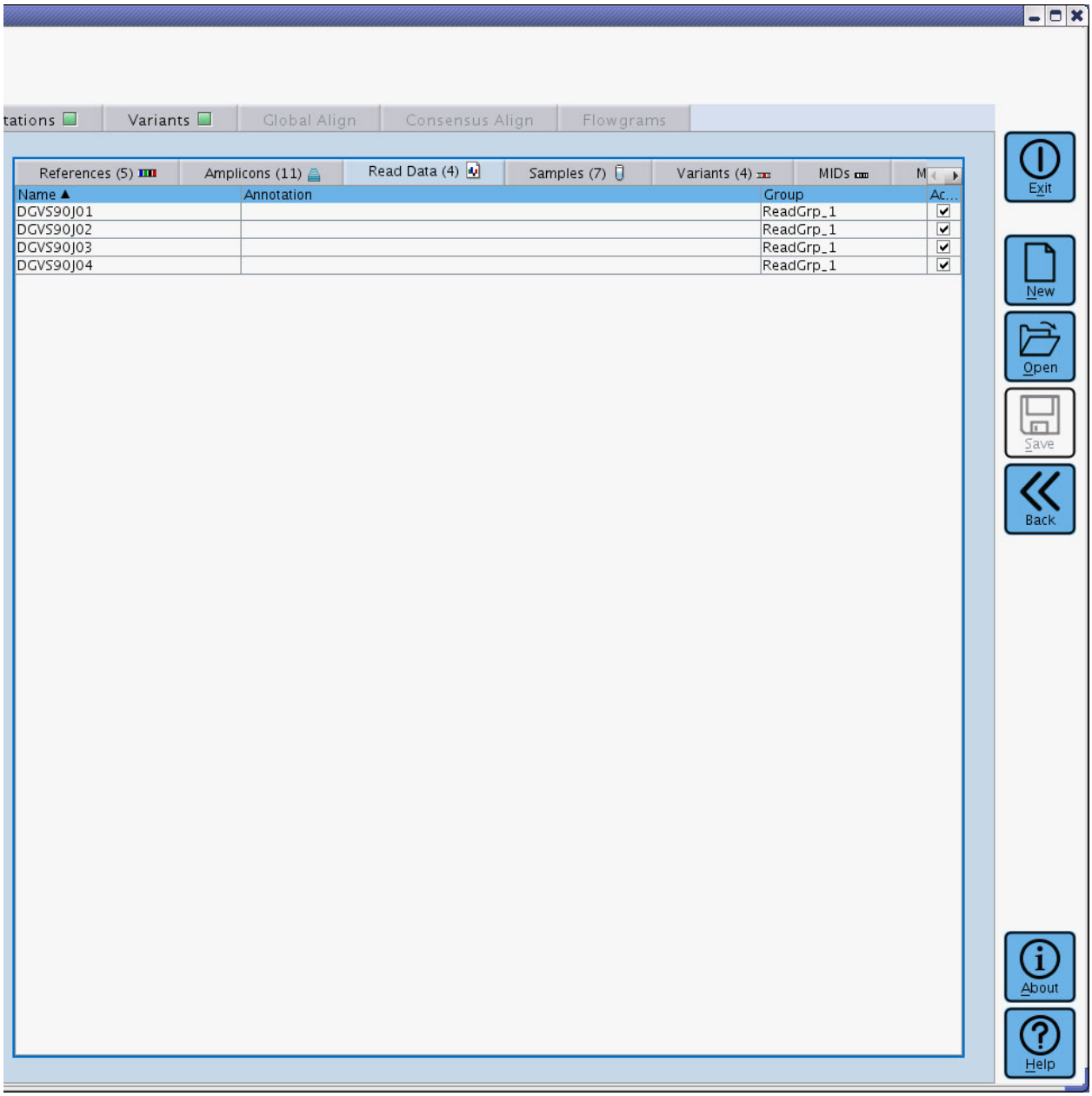


Figure 1-25: The Read Data Definition Table sub-tab of the Project Tab's right-hand panel

For the procedures to add or remove Read Data Sets in a Project, see section 1.3.2 (or 1.3.1, to accomplish this in a "Project Tree" view, and concurrently create associations). For the procedures to enter/edit the Name or Annotation information for a Read Data Set, see section

1.3.2. The sub-sections below provide the procedures to enter/edit the other characteristics of Read Data Sets.

The Read Data files generated by the standard data processing pipelines are named uniquely, and within the files, the read accnos (which are based on the file name) are also unique. Read Data files with duplicate names are not allowed in AVA. So, if unmanipulated Read Data files are imported into an AVA project, all reads and their accnos in the project will be unique.

However, manipulation of Read Data files by either renaming them or using SFF Tools to merge or filter them, can result in situations where reads are present in a project in duplicate (and depending on the project, the duplicates may appear in the same alignment). If the Read Data files are manipulated, care should be taken to ensure that reads are not unintentionally duplicated within a project.

1.3.2.3.1 To Edit the Read Group of a Read Data Set

If you want to transfer a Read Data Set to another pre-existing Read Data Group, double-click the drop down menu in the Group cell for the Read Data Set and select the Read Group you want from the available choices. You can also reassign a Read Data Set to a different Read Group by dragging the Read Data Set to a Read Group node of the Read Data Tree (a multiple-selection of Read Data Sets will assign them all to the Read Group on which you drop them). While you cannot change the name of a Read Group from within the Read Data Definition Table, you can do so in the Read Data Tree (as with any other rename operation in the tree, click once on the Group name, pause and click a second time to activate the name editor). Note that all Read Groups are distinct entities, and although you can rename an existing Read Group to match the name of another pre-existing Read Group, this will not cause the Read Data Sets to be merged into the same group.

Note also that you cannot import the same Read Data Set more than once in a Project, even if you intend to assign them to different Read Groups. If you attempt to do so, an error message will appear on screen.

1.3.2.3.2 To Edit the “Active” status of a Read Data Set

You may elect to include or exclude from future computations, any Read Data Set defined in the Project. Excluding a Read Data Set from future computations does not remove it from the Project. If you discover that a Read Data Set is unsuitable for the Project in some manner, it can be useful to keep it in the project but mark it as inactive (and possibly update its Annotation) to serve as a reminder not to reintroduce the data at some future point.

1. To include a Read Data Set in future computations of the Project, check its box in the “Active” column of the Read Data Definition Table.
2. To exclude a Read Data Set in future computations of the Project, uncheck its box in the “Active” column of the Read Data Definition Table.

1.3.2.4 The Samples Definition Table

The **Samples** Definition Table lists all the Samples defined in the Project, with only the following two characteristics (Table columns; see Figure 1-26):

- Name
- Annotation (free user-entered text)

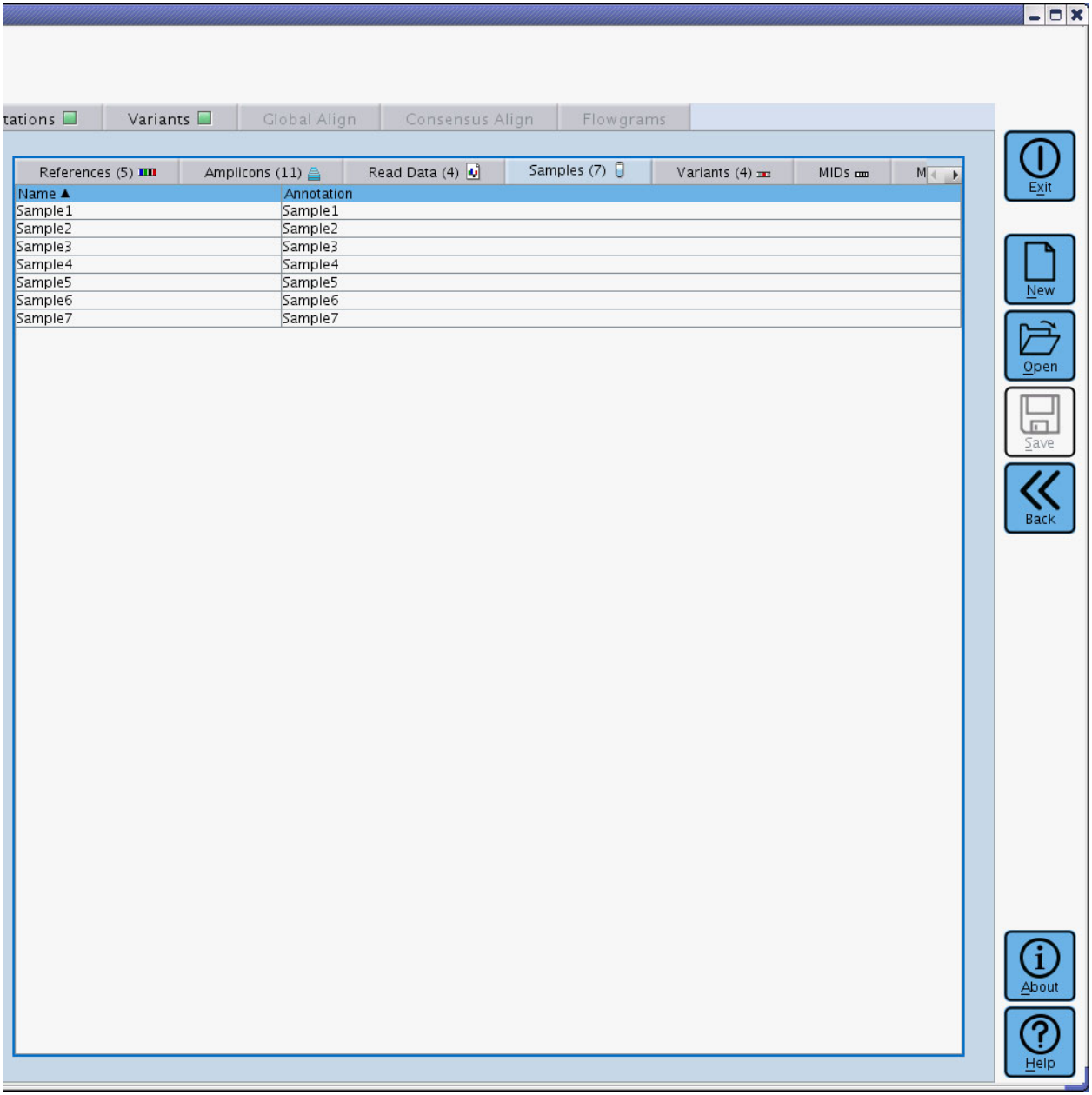


Figure 1-26: The Samples Definition Table sub-tab of the Project Tab's right-hand panel

For the procedures to add or remove Samples in a Project, see section 1.3.2 (or 1.3.1, to accomplish this in a “Project Tree” view, and concurrently create associations). For the procedures to enter/edit the Name or Annotation information for a Sample, see section 1.3.2.

1.3.2.5 The Variants Definition Table

The **Variants** Definition Table lists all the Variants defined in the Project, with the following five characteristics (Table columns; see Figure 1-27):

- Name
- Reference Sequence (with which the Variant is associated)
- Annotation (free user-entered text)
- Pattern (definition of the nature of the Variant)
- Status (workflow category)

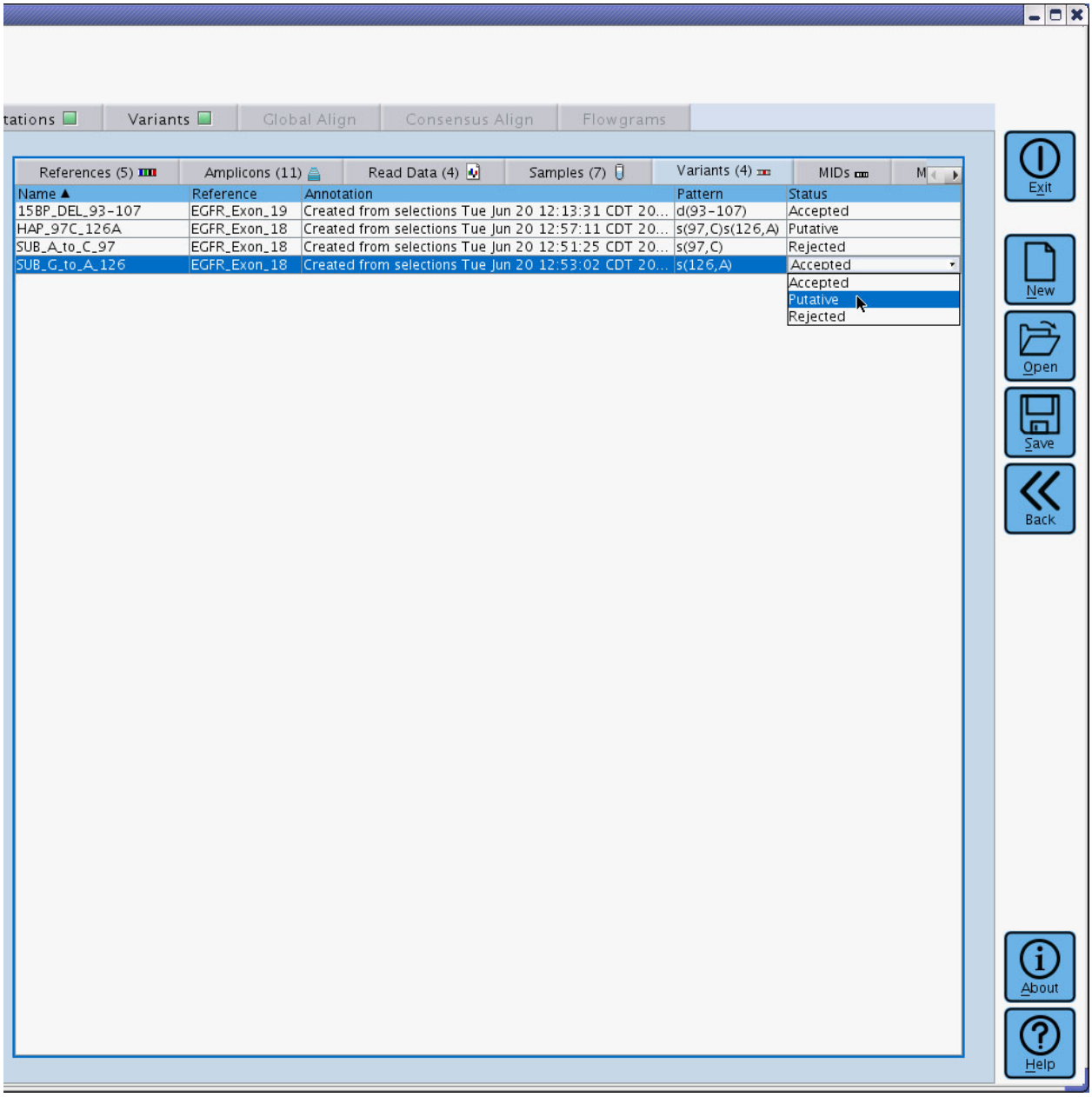


Figure 1-27: The Variants Definition Table sub-tab of the Project Tab's right-hand panel

For the procedures to add or remove Variants in a Project, see section 1.3.2 (or 1.3.1, to accomplish this in a "Project Tree" view, and concurrently create associations). For the procedures to enter/edit the Name or Annotation information for a Variant, see section 1.3.2. The sub-sections below provide the procedures to enter/edit the other characteristics of Variants.

1.3.2.5.1 To Enter or Edit the Reference Sequence to which a Variant is associated

1. Ensure that the column labeled “Reference” in the table is wide enough to allow you to distinguish among the Reference Sequences from which you want to select. The column may be widened by clicking on the separator line, in the table header, between the Reference and Annotation columns, and dragging the separator to the right.
2. Double-click in the “Reference Sequence” cell for the Variant you are defining, in its Definition Table. A drop down menu will expand, showing all the Reference Sequences currently listed in the Project.
3. Select the proper Reference Sequence from the drop down menu. The new association will automatically appear on the References Tree, on the left panel.

If the Reference Sequence does not yet contain a DNA sequence (see section 1.3.2.1.1), you will still be able to associate Variants to it, but you will not be able to fully define them. In particular, you will not be able to specify the Pattern for the Variant (see section 1.3.2.5.2, below) because this is set using the position numbering from the Reference Sequence.

1.3.2.5.2 To Enter or Edit the Pattern of a (Known) Variant

If you already know one or more Variants (*e.g.* from the scientific literature or from previous experiments), you can define them in the Project and have the AVA software report on the frequency at which they occur in the Read Data Sets included in the Project. Note that novel Variants observed in the reads of the Project itself can also be defined as described below, but the best way to specify novel Variants is to examine the multiple alignments of the putative Variants found by the AVA software during computation and to “Accept” them if you determine that they are legitimate (see section 1.3.2.5.3, below); also, you can “declare” novel Variants not identified by the software after you identify and evaluate them in the Global Align or Consensus Align tabs (see sections 1.6 and 1.7).

The AVA software uses 4 types of constraints to define Variants, and writes them following a strict Variant Definition Syntax, summarized in Table 1-1. A Variant can be specified by one or more constraints which, collectively comprise the “Pattern” that defines the Variant.

Constraint Type	Syntax	Description
Must match	m(p) or m(p_1-p_2)	A read satisfies this constraint when the nucleotide(s) at position "p" or in the range "p_1-p_2" (inclusive) of the Reference Sequence are identical to those of the Reference Sequence.
Substitute base	s(p,n)	A read satisfies this constraint when the nucleotide at position "p" is "n" (where "n" is different from the nucleotide at that position in the Reference Sequence).
Insert bases	i(p.5, n…)	A read satisfies this constraint when the (one or more) nucleotide(s) "n…" are present between positions "p" and "p+1" of the Reference Sequence. Note that a deletion may not also exist at positions "p" or "p+1", as this combination of insertion and deletion would rather define a substitution.
Delete bases	d(p) or d(p_1-p_2)	A read satisfies this constraint when the nucleotide(s) at position "p" or in the range "p_1-p_2" (inclusive) of the Reference Sequence are absent. Note that directly neighboring insertions may not also exist, as this combination would rather define a substitution.

Table 1-1: The language of variations in the AVA software: the Variant Definition Syntax. A Variant may comprise multiple constraints (though any given nucleotide may only have a single constraint) enabling the encoding of haplotypes or other complicated variation Patterns. In these more complicated cases, the Variant is encoded by specifying multiple, concatenated constraints, optionally separated by whitespace.

To enter or edit the Pattern defining a Variant in the Variants sub-tab of the Project Tab, do the following:

1. Double-click in the "Pattern" cell for the Variant you are defining, in its Definition Table. An "Edit Pattern" window will open (Figure 1-28). This window includes:
    a. a "Pattern" data entry box to define and view the nature of the Variant, using the Variant Definition Syntax (see above)
    b. a box containing a DNA sequence based on the Reference Sequence to which the Variant is associated, with a color-coded overlay for the graphical definition and visualization of the Variant

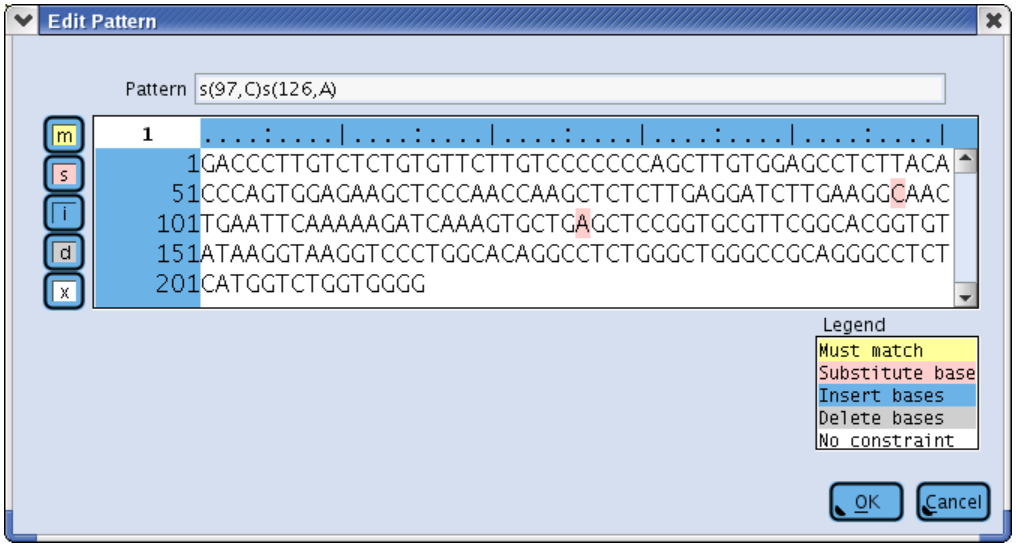


Figure 1-28: The Edit Pattern window, used to specify the difference(s) compared to the Reference Sequence that define the Variant

2. There are 2 ways to set (or reset) the definition of a Variant, using this window:
    a. You can enter it directly in the Pattern box at the top of the window, using the Variant Definition Syntax (see above). The software automatically adds variations entered in the Pattern box to the graphic sequence box, below it. If the syntax used is incorrect, the AVA software parses the entry and suggests a correction or provides a tip, in the area below the sequence box
    b. You can enter it graphically by selecting nucleotide(s) in the sequence field and assigning the appropriate type of constraint using the buttons to the left of the sequence box. The software automatically adds constraints entered graphically, in the Pattern box. There are 4 buttons, matching the four types of constraints, and a fifth button for clearing a previously specified constraint, that can be used to graphically define a Variant. The 5 buttons, and their use, are as follows:
        i.  Must match (shown in yellow overlay)
            01. Select one nucleotide (click) or a nucleotide range (click-and-drag) in the sequence.
            02. Click the Must match button.
        ii.  Substitute base (shown in pink overlay)
            01. Select one nucleotide (click) in the sequence.
            02. Click the Substitute base button; the “One base” window will open (not shown).
            03. Type the substituting nucleotide.
            04. Click OK; the sequence changes to that of the Variant.
        iii.  Insert bases (shown in blue overlay)
            01. Select the one nucleotide (click) in the sequence before which the insertion is located.
            02. Click the Insert bases button; the “Enter insert sequence” window will open (not shown).
            03. Type the nucleotide(s) to be inserted (only A, T, G, or C characters).

04. Click OK; the insertion appears in the sequence. The position of the inserted nucleotides use decimals so that the original Reference Sequence positions are maintained (*e.g.* position 66.5 means that the insertion is between the nucleotides at positions 66 and 67 of the Reference Sequence).

iv. d Delete bases (shown in gray overlay)

01. Select one nucleotide (click) or a nucleotide range (click-and-drag) in the sequence.

02. Click the Delete bases button; the nucleotides in the sequence are replaced with dashes.

v. x No constraint (shown with white background; the Reference Sequence)

01. Select one nucleotide (click) or a nucleotide range (click-and-drag) in the sequence that already have one of the above changes assigned

02. Click the No constraint button; the nucleotide(s) in the sequence revert to the Reference Sequence. (This function is useful if you incorrectly entered a constraint in the definition of a Variant.)

3. Click OK.

If an erroneous pattern is directly entered into the Pattern field of the "Edit Pattern" window, the AVA software will remove portions of the pattern until what remains is valid both syntactically and semantically, as seen in Figure 1-29. In this case, one or more parsing error messages will appear describing the nature of the problem, in the context of the full, erroneous pattern entered.

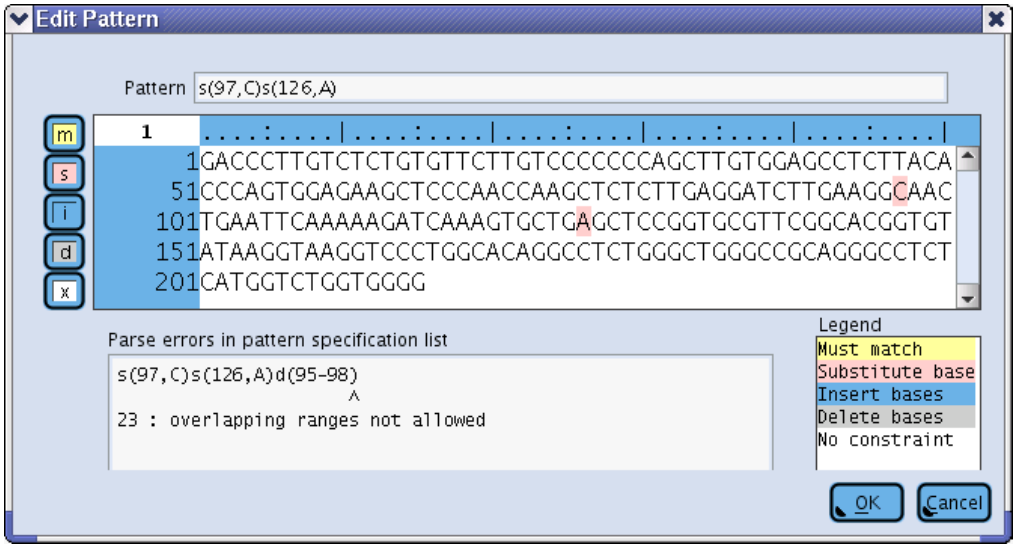


Figure 1-29: The Edit Pattern window, with an error in the pattern specification. The user attempted to specify both a substitution at positions 97 and 126 as well as a deletion spanning positions 95-98. Since position 97 cannot both be deleted and involved in a substitution, the software automatically removed the deletion from the Pattern specification at the top of the window, but shows the full erroneous pattern with an appropriate error message in an error window. The compatible substitution at position 126 is left as part of the pattern.

1.3.2.5.3 To Edit the Status of a Variant

Variants exist within a Project, with one of three possible Status values: “Accepted”, “Putative”, or “Rejected”. Variants defined manually by the user (see section 1.3.2.5.2) receive the “Accepted” status by default. By contrast, variations between the Read Data Sets and the References that are identified by the AVA software (during computation; see section 1.4), are initially proposed as “Putative” Variants. After you have examined the data underlying a Variant and determined whether you believe it to be legitimate or not, you can change its assigned Status as described below.

Note, however, that the main use of the Variant Status feature is as part of a “Discovery Workflow”, on the Variants tab. The purpose of this process is precisely to determine whether the Variants included in the Project appear to be legitimate or not and to mark them as such (“Accepted” or “Rejected”); The Variants tab is also where the control is located to “load” the putative Variants discovered by the software, into the Project. For these reasons, Variant Status assignment might more often be done on the Variants tab than on the Variants sub-tab of the Project tab, as described here. See section 1.5 for a description of the Variants tab and for more details on this Discovery Workflow.

To edit the Status of a Variant in the Variants sub-tab of the Project Tab, do the following:

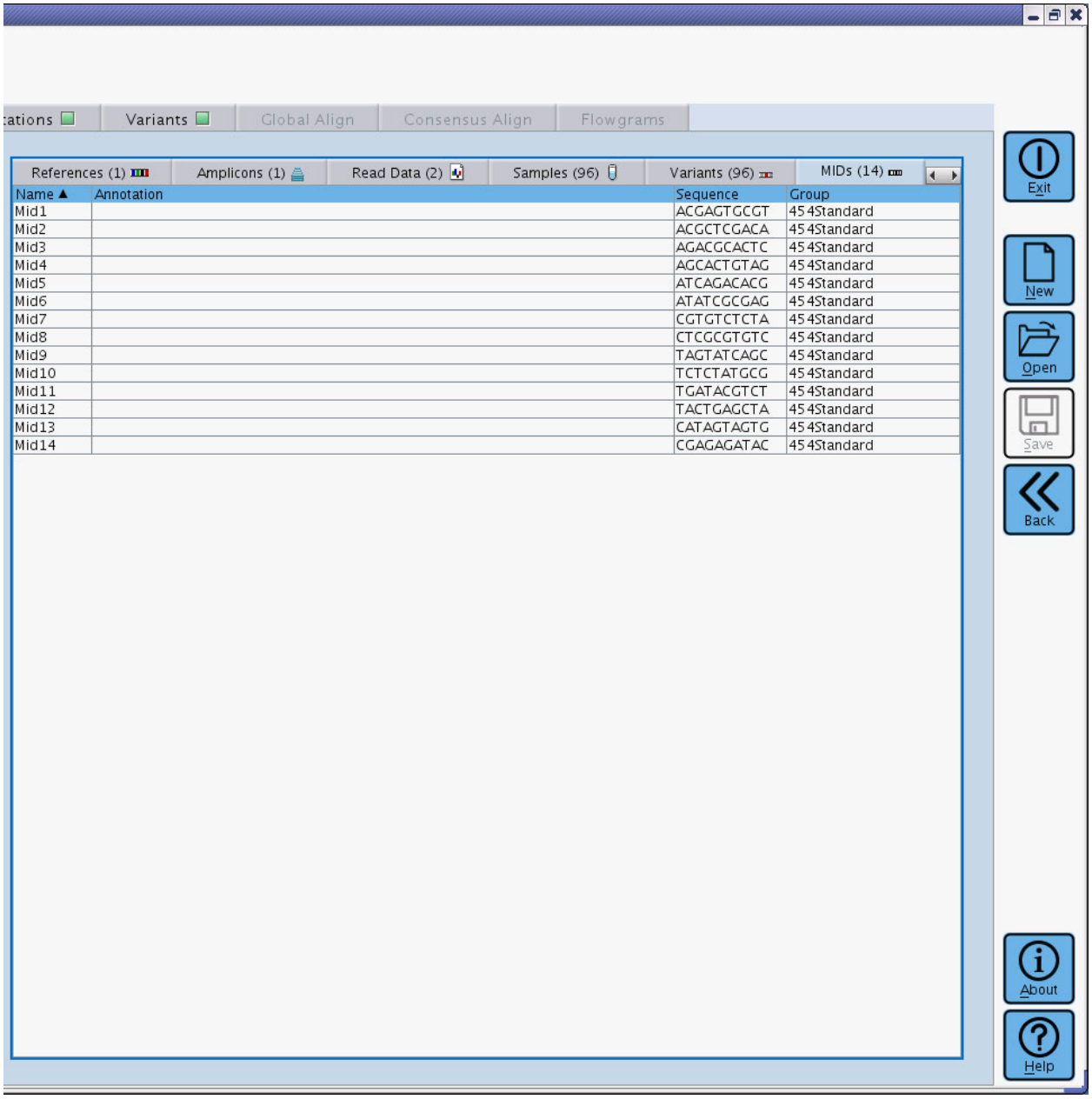
1. Ensure that the “Status” column in the Variants Definition Table is wide enough to allow you to distinguish among the Status choices. The column may be widened by clicking on the separator line, in the table header, between the Pattern and Status columns, and dragging the separator to the left.
2. Double-click in the “Status” cell for the Variant you are defining. A drop down menu will expand, showing the available Status options.
3. Select the proper Status from the drop down menu.

- If you open a Project from a prior version of the AVA software which did not have the Status field, the status value for any pre-existing Variants in the Project will be set to “Accepted”.
- It is often more useful to use the “Rejected” status than to actually remove a Variant from the Project. When you mark a Variant as rejected, you prevent it from being rediscovered by the auto-variant detection process, during further computations of the Project. Variants can safely be removed after you are done adding new data to the Project.

1.3.2.6 The MIDs Definition Table

The **MIDs** Definition Table lists all the MIDs defined in the Project, with the following four characteristics (Table columns; see Figure 1-30):

- Name
- Annotation (free user-entered text)
- Sequence
- Group (the MID Group to which the MID belongs)



Name ▲	Annotation	Sequence	Group
Mid1		ACGAGTGCGT	454Standard
Mid2		ACGCTCGACA	454Standard
Mid3		AGACGCACTC	454Standard
Mid4		AGCACTGTAG	454Standard
Mid5		ATCAGACACG	454Standard
Mid6		ATATCGCGAG	454Standard
Mid7		CGTGTCTCTA	454Standard
Mid8		CTCGCGTGTC	454Standard
Mid9		TAGTATCAGC	454Standard
Mid10		TCTCTATGCG	454Standard
Mid11		TGATACGTCT	454Standard
Mid12		TACTGAGCTA	454Standard
Mid13		CATAGTAGTG	454Standard
Mid14		CGAGAGATAC	454Standard

Figure 1-30: The MIDs Definition Table sub-tab of the Project Tab's right-hand panel

MIDs may be created (and assigned to MID Groups) in the MIDs Definition Table even before the sequence of the MID has been filled in by the user. Such MIDs, without defined sequences, may even be used in the definitions of the Samples encoded by Multiplexers (section 1.3.2.7.1). This flexibility allows users to define of the logical structure of an experiment in advance of knowing the specifics of the MID sequences themselves.

For the procedures to add or remove MIDs in a Project, see section 1.3.2 (or 1.3.1, to accomplish this in a "Project Tree" view). For the procedures to enter/edit the Name or Annotation information for an MID, see section 1.3.2. The sub-sections below provide the procedure to enter/edit the other characteristics of MIDs.

Criteria for good MID Sets; standard and custom MID Groups:

An MID Group ("454Standard") containing 14 ten-base MIDs is provided and recommended for use when designing multiplexing libraries for the AVA software. This group includes the set of 12 tags known as MID1 through MID12 that are available in kit form to make Shotgun (sstDNA) MID libraries, and includes two additional MIDs not included in these kits (MID13 and MID14). The MIDs of the 454Standard MID Group have been especially selected for the following qualities:

- Their sequences are as divergent as possible and include only single nucleotide homopolymers, minimizing the risk that eventual sequencing errors would make any of these MIDs look like one of the other MIDs of the Group (which would cause the assignment of the read to a wrong Sample). Indeed, no fewer than six changes (insertions, deletions, and/or substitutions) separate any two MIDs of the 454Standard MID Group.)
- All MIDs of the Group are of the same length (10-mers, in this case). The AVA software requires that all the MIDs used for a given end of the Amplicons (Primer A/Primer 1 or Primer B/Primer 2) have the same length, so they are on equal footing for error distance calculation purposes. It is permitted, however, to use a different MID length on each side of the Amplicons being handled by a Multiplexer. For instance, custom 5 bp MIDs might be used on the Primer 1 side and 10 bp MIDs might be used on the Primer 2 side.
- None start with a "G" nucleotide, the last base of the sequencing key, to ensure clear reading of the MID tag.
- All MIDs are read within 4 or 5 nucleotide flow cycles, to minimize usage of Run flows to read the MID tags and leave as much as possible for the sample sequence.

The software does not constrain the user to only these 14 MIDs, however: any other set of sequences can be designed for use as multiplexing tags, incorporated between the sequencing key and the template-specific primer of the Adaptors used to prepare the Amplicon libraries, and defined as MIDs (with optional custom MID Groups) in the Amplicon Project. This flexibility can be useful, for example, if the user prefers to use shorter MIDs; or if Amplicon libraries already exist that have intrinsic sequences that can be used for demultiplexing; or if the experiment requires the multiplexing of more Samples than can be differentiated with the 14 MIDs of the 454Standard MID Group. If you design your own MIDs, it is recommended that you keep in mind the 4 criteria listed above, for best results.

Amplicon library chemistry requires the inclusion of the MID sequence as part of the Fusion Primers used in library preparation. These must be obtained separately by the user because they contain sequences that are specific to each Amplicon, so MID Adaptors used for Amplicon libraries are not available as kit reagents. A naming convention has been adopted to distinguish between Shotgun (sstDNA) and Amplicon library MIDs; fully uppercase MID names are Shotgun (sstDNA) library MIDs, and initial uppercase Mids are MIDs used in Amplicon libraries. Thus, MID1 and Mid1 refer to the same sequence content for an MID, but are each used in the context of their respective library types; MID1 is an Adaptor available in a kit, while Mid1 is not.

Contrary to the situation with the GS *De Novo* Assembler and the GS Reference Mapper applications, the number of acceptable "reading errors" in the MIDs is not set by the user in the AVA software. Rather, the software dynamically calculates how many errors can be accepted by analyzing the set of MIDs used and determining how close they are to each other in terms of the minimum number of insertions, deletions, or substitutions that would be required to transform one MID into another.

1.3.2.6.1 To Enter or Edit the DNA Sequence of an MID

1. Double-click in the "Sequence" cell of the MID you are defining, in its Definition Table. An "Edit Sequence" window will open (Figure 1-31).
2. Paste or type the sequence (only A, T, G, or C characters; see Caution, below).
3. Click "OK".

Characters restriction: Be aware that only "nucleotide" characters (A, T, G, C) are accepted when you enter an MID Sequence into the AVA software (by typing or pasting). For convenience, when pasting sequences, characters that are not nucleotide characters and are also not IUPAC ambiguity characters (such as R for purine, Y for pyrimidine, *etc.*) are removed from the pasted entry. This is useful when pasting sequences from sources that may include non-sequence information (such as white space or numerical position information in the margin of each line). If any IUPAC ambiguity characters are included, the paste will be cancelled entirely, and an error message will be displayed explaining the problem. If you directly type individual "ambiguous" characters, however, or any character other than A, T, G, or C, these characters are simply ignored.

The restriction that no ambiguity characters be present in an MID sequence is crucial because MIDs are intended to designate specific Samples. If you have a degenerate MID design in which multiple MID sequences specify the same Sample, enter all the specific MID sequences into the system and use the Multiplexer Sample editor to specify all the MIDs that encode each Sample (see section 1.3.2.7.3)

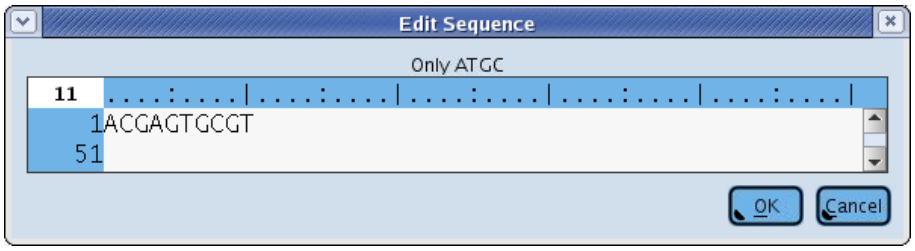


Figure 1-31: The Edit Sequence window, used to enter or edit the DNA sequence of an MID Sequence element

1.3.2.6.2 To Edit the MID Group of an MID

To transfer an MID to another pre-existing MID Group, double-click the drop down menu in the Group cell for the MID and select the MID Group you want from the available choices. You can also reassign an MID to a different MID Group by dragging the MID to an MID Group node of the MIDs Tree (a multiple selection of MIDs will assign them all to the MID Group on which you drop them). While you cannot change the name of an MID Group from within the MIDs

Definition Table, you can do so in the MIDs Tree (as with any other rename operation in the tree, click once on the Group name, pause and click a second time to activate the name editor). Note that all MID Groups are distinct entities, and although you can rename an existing MID Group to match the name of another pre-existing MID Group, this will not cause the MIDs to be merged into the same group.

A valid MID Group should contain MIDs with sequences of the same length, and each MID sequence should be distinct. When assigning MIDs with defined sequences to an MID Group, the software will prevent you from making an inconsistent assignment, such as adding an MID with an a defined sequence to an MID Group that already has at least one defined MID sequence of a different length: if dragging to the MIDs Tree, the MID Group node will not activate to allow you to release the dragged MID; if using the drop down menu from the MIDs Definition Table, the drop down menu will only display valid MID Group choices.

Although the AVA application is designed to minimize the possibility of creating inconsistent MID Groups, to allow flexibility in editing, it allows the addition of MIDs with undefined sequences to MID Groups, and the editing of MID sequences even after they have already been assigned to an MID Group. During such editing you are allowed to bring an MID Group into a temporarily inconsistent state, with the assumption that you will eventually fix it prior to computation. If you use inconsistent MIDs when defining Multiplexers, the Multiplexer setup dialogs will provide you with error messages (section 1.3.2.7.2), and you will also be provided with error warnings prior to computation (section 1.4). Ignoring the warnings will prevent the portions of the computation that depend on the faulty Multiplexers from executing.

1.3.2.7 The Multiplexers Definition Table

The **Multiplexers** Definition Table lists all the Multiplexers defined in the Project, with the following six characteristics (Table columns; see Figure 1-32):

- Name
- Annotation (free user-entered text)
- Encoding
- Primer 1 MIDs
- Primer 2 MIDs
- Samples

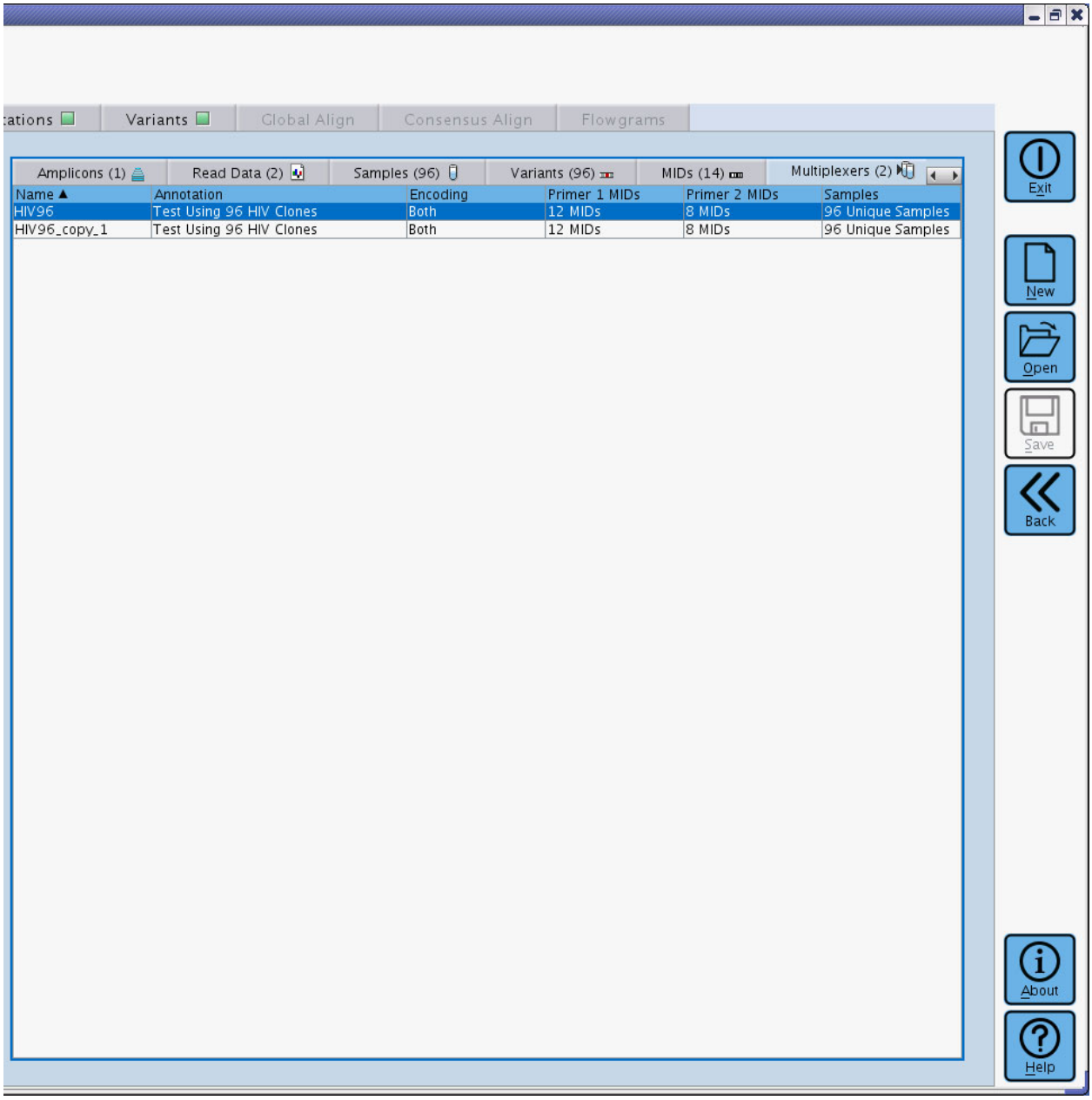


Figure 1-32: The Multiplexers Definition Table sub-tab of the Project Tab's right-hand panel

For the procedures to add or remove Multiplexers in a Project, see section 1.3.2 (or 1.3.1, to accomplish this in a "Project Tree" view). For the procedures to enter/edit the Name or Annotation information for a Multiplexer, see section 1.3.2. The sub-sections below provide the procedure to enter/edit the other characteristics of Multiplexers.

Note on Sample encoding using MIDs and Multiplexers:

In the standard non-MID demultiplexing scheme, the AVA software looks for the template-specific primer sequences (Primer 1 and Primer 2) of the defined Amplicons at the beginning of each read. Once the Amplicon to which a read belongs is identified, the Sample-Amplicon associations defined for the Read Data Set that the read comes from are used to assign the read to its appropriate Sample. In other words, when MIDs are not used, the assignment of a read to an Amplicon, using the template-specific primers, is sufficient to further assign the read to the proper Sample. As explained before (see section 1.1.1.6, and Note and Caution sidebars in section 1.3.1.2), this scheme imposes the restriction that an Amplicon may only belong to a single Sample within a Read Data Set, to allow for unambiguous Sample assignment of the reads.

MIDs and Multiplexers allow this restriction to be lifted and experiments to be designed in which reads from a given Amplicon are monitored in multiple Samples, in the same Read Data Set. In this scheme, the MID sequence detected within a read is used to assign reads to Samples. This MIDs to Samples assignment is the function of Multiplexers, as described in this section.

The Primer 1 and Primer 2 sequences of a read are still used, however, to determine to which Amplicon a read belongs. Moreover, different Amplicons, within a Read Data Set, may be associated with different Multiplexers. Thus, when MIDs and Multiplexers are used, the demultiplexing process involves:

1. decoding the Primer1 and Primer2 regions of the read to determine which Amplicon it represents;

2. using the user-specified association between Amplicons and Multiplexers for the Read Data Set to determine the appropriate Multiplexer to apply; and

3. using the MID sequence detected in the reads, in conjunction with that Multiplexer, to assign the read to the proper Sample.

1.3.2.7.1 To Enter or Edit the Sample Encoding using Multiplexers

The AVA software provides 4 ways to encode the Sample to which a read belongs in the Multiplexer, based on the construction of the libraries (see section 4.6 for details on Amplicon library design with MIDs). The proper option must be selected from a drop down menu in the Multiplexers Definition Table (Figure 1-33). The options, further described in the sub-sections below, are:

- Both
- Either
- Primer 1 MID
- Primer 2 MID

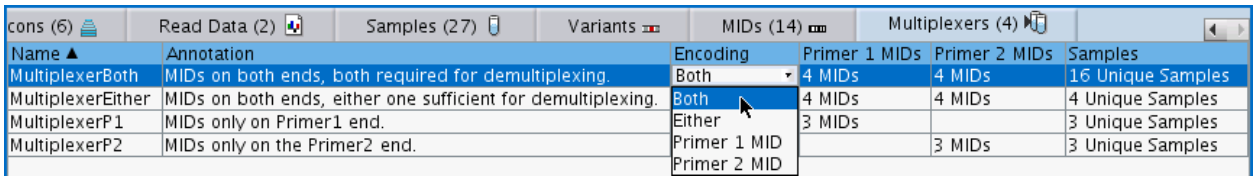


Figure 1-33: The Encoding drop down menu, on the Multiplexers tab

Selecting the proper encoding: It is crucially important to select the encoding method that truly corresponds to the way the libraries were prepared. For example, if libraries were prepared with 'Either' chemistry in mind, it may be tempting to use a 'Primer 1 MID' or 'Primer2 MID' encoded Multiplexer since the distal MID gets discounted in favor of the proximal MID in 'Either' encoding. However, the AVA software needs to know that MIDs are expected to be found at both ends: without that knowledge, the trimmer might get a suboptimal alignment of the distal primer, which in certain cases could drop valid reads out of the analysis.

1.3.2.7.1.1 "Primer 1 MID" and "Primer 2 MID" Encoding

The "Primer 1 MID" and "Primer 2 MID" encoding options assume that the libraries were prepared with MID sequences incorporated in the design of only one of the Adaptors used in the preparation of the Amplicon libraries. The MID is placed between the sequencing key and the template-specific primer that will be identified either as Primer 1 or as Primer 2 (as entered in the Amplicon definition table).

When either of these encoding options is selected for a Multiplexer, only the corresponding Primer MID field, Primer 1 MIDs or Primer 2 MIDs, needs to be filled in the Multiplexer's Definition Table, to identify the MIDs used in the scheme (see section 1.3.2.7.2). For example, a Multiplexer encoded as "Primer 1 MID" will have an empty column in the Definition Table for the "Primer 2 MIDs" field. The maximum number of Samples that can be encoded with this scheme is equal to the number of MIDs defined in the Primer 1 MIDs or Primer 2 MIDs field.

The AVA software uses the encoding type to automatically determine where to search for MIDs within reads, taking read orientation into account. For example, if both forward and reverse reads are sequenced for an experiment where the "Primer 1 MID" encoding is being used, forward reads will have the MID at the beginning of the read, just before of the template-specific Primer 1 sequence; and reverse reads will have the reverse complement of the MID near the end of the read, just after the reverse complement of the template-specific Primer 1 sequence. For this reason, if reads will be obtained in both orientations (as is recommended) and Primer 1 MID or Primer 2 MID encoding is used, it is important to design Amplicons of a length shorter than the read length provided by the sequencing Run. If the ability to read through the Amplicons is in doubt, the "Either" encoding (section 1.3.2.7.1.3) should be used instead, to guarantee that both forward and reverse reads have an MID at their beginning.

For details on Sample assignment using the Primer 1 MID or Primer 2 MID encoding options, see section 1.3.2.7.3.1.

1.3.2.7.1.2 "Both" Encoding

"Both" encoding involves the incorporation of MID sequences in both Adaptors used in the preparation of the Amplicon libraries, such that each read contains both a Primer 1 MID and a Primer 2 MID. Therefore, both the "Primer 1 MIDs" and the "Primer 2 MIDs" fields of the Multiplexer Definition Table must have at least one MID selection (see section 1.3.2.7.2).

With this encoding scheme, both a Primer 1 MID and a Primer 2 MID must be observed in a read to assign it to the proper Sample. Note that there is no requirement that the same set of MIDs be used at both ends of the Amplicons: the two MIDs used to determine Sample assignment are completely independent from one another. In addition, with the "Both" encoding, the order of the appearance of MIDs is significant: for example, "Primer 1 Mid1 – Primer 2 Mid6" is a different encoding than "Primer 1 Mid6 – Primer 2 Mid1". Given this combinatorial feature, the maximum number of Samples that can be encoded with this scheme is equal to the product of the number of MIDs defined in the Primer 1 MIDs and the Primer 2 MIDs fields.

It is also important to remember that in order to be able to read the distal MID, the length of the Amplicon library product must be within the read length provided of the sequencing Run script. For details on Sample assignment using the Both encoding option, see section 1.3.2.7.3.2.

1.3.2.7.1.3 "Either" Encoding

The "Either" encoding method is a hybrid between the single primer MID and "Both" methods. The libraries are tagged with MIDs on both the Primer 1 and Primer 2 ends, but only one MID needs to be observed on a read to assign it to the proper Sample. This can be useful if the Amplicon library products are longer than the read length of the sequencing Run, and you are sequencing them from both ends. One limitation of this encoding scheme is that a given MID can be used for only one Sample, for each of the Primer 1 and Primer 2 ends.

As with the Both encoding scheme, there is no requirement that the same set of MIDs be used at both ends of the Amplicons. Although the number of MIDs used at the Primer 1 and Primer 2 ends will typically be the same, the software even allows degenerate designs in which the "Either" encoding is used and the number of Primer 1 MIDs and Primer 2 MIDs differ. Regardless, a read must be able to be uniquely assigned to the same Sample whether its Primer 1 MID or the Primer 2 MID are used for demultiplexing. Therefore, the maximum number of Samples that can be encoded with a Multiplexer that uses this scheme is equal to the smaller of the number of MIDs defined in the Primer 1 MIDs and Primer 2 MIDs field.

For details on Sample assignment using the Either encoding option, see section 1.3.2.7.3.3.

1.3.2.7.2 To Enter or Edit the Primer 1 MIDs and Primer 2 MIDs

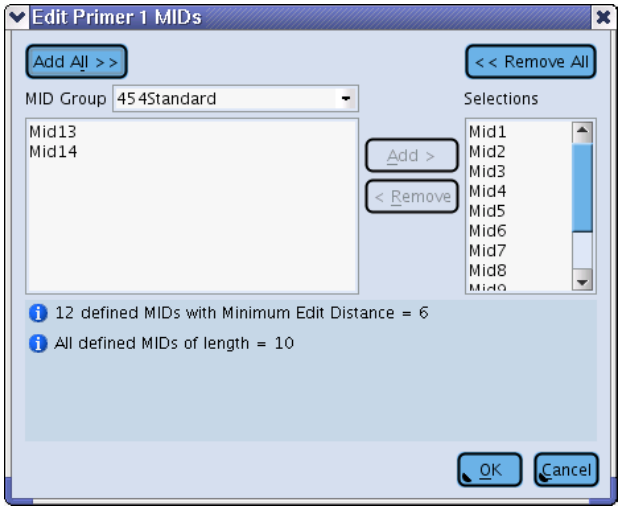
The user must specify the list of MIDs that the AVA software must search for to demultiplex reads using a Multiplexer. This information is set in the Primer 1 MIDs and the Primer 2 MIDs columns of the Multiplexer Definition Table. If Primer 1 MID or Primer 2 MID encoding is chosen, only the corresponding 'Primer MIDs' cell is available for that Multiplexer; if Either or Both encoding is chosen, both cells are available and must be filled.

To specify the MIDs for one end of a Multiplexer, double-click on the appropriate 'Primer MIDs' cell for that Multiplexer. The Edit Primer 1 MIDs (or Edit Primer 2 MIDs) window opens (Figure

1-34). The window will not open unless at least one MID entry has already been specified into the MID Definition Table (though the MIDs do not have to have sequences defined at this stage). Select the MIDs of interest on the list on the left, and click Add > . To remove MIDs that have been previously selected, highlight them in the list on the right, and click < Remove .

Certain shortcuts are available to carry out these tasks, such as an Add All >> and a << Remove All button; and an MID Group drop down menu allows the user to restrict the list on the left to the MIDs contained in any of the MID Groups available in the Project. Also, if the Primer 1 and Primer 2 MIDs are the same, you can first define the Primer 1 MIDs and then use the Clone Primer 1 MIDs button in the Edit Primer 2 MIDs window (Figure 1-34**B**), to create the same list for that set.

A



B

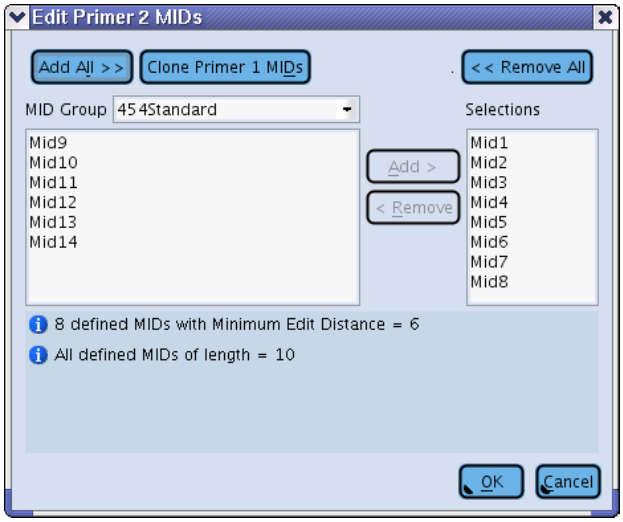


Figure 1-34: (A) The Edit Primer 1 MIDs window and (B) the Edit Primer 1 MIDs window. Note the Clone Primer 1 MIDs button in the Edit Primer 2 MIDs window.

The MID Group drop down menu also contains some “virtual” groups that are automatically generated by the AVA software based on the MIDs currently defined in the Project. Figure 1-35 shows an example where all 14 of the 454Standard MIDs (10-mers) have already been loaded into the Project, and four new MIDs have been added without groups: two 6-base MIDs (Mid15 and Mid16) and two MIDs for which no sequence has yet been defined (Mid17 and Mid18).

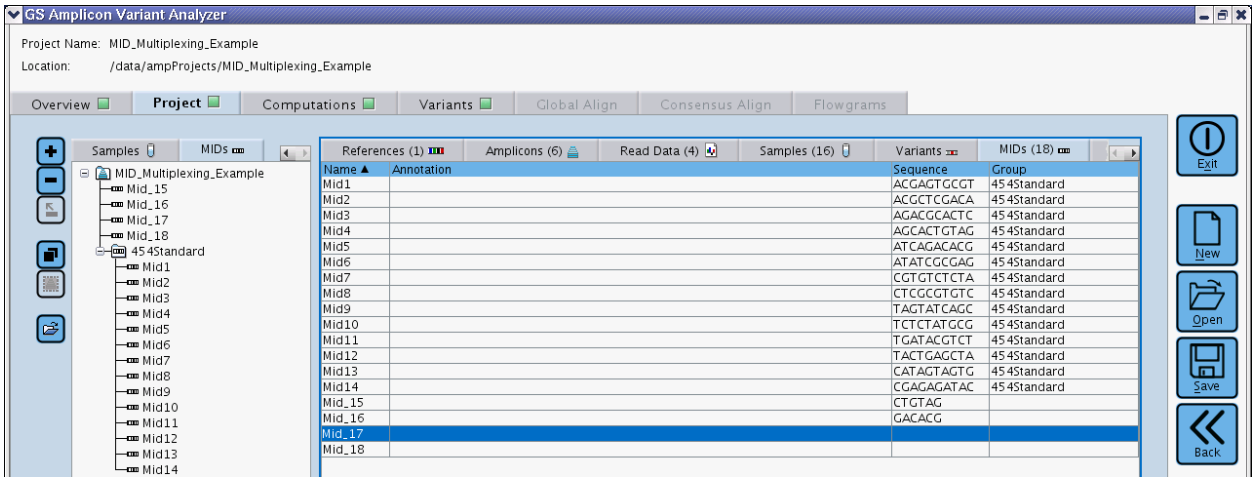


Figure 1-35: MID Tree and Definition Table view showing the 454Standard group MIDs, plus four newly defined MIDs (Mid15 - Mid18), two 6-mers and two that have no defined sequence.

Figure 1-36**A**, then shows the MID Group drop down menu for the MIDs in Figure 1-35:

- The AVA software always provides an "[All MIDs]" option on this menu to allow all the MIDs defined in the Project to be viewed in the left list, irrespective of their group status (and even if an MID in the Project has not yet been assigned a sequence).
- In addition, the software creates virtual MID Groups based on the length of the MIDs defined in the Project. This is useful because, as mentioned above (see Note in section 1.3.2.6), all the MIDs used on a given end of an Amplicon must be of the same length.
    - Note that MIDs without a defined sequence will appear in all length-restricted lists (*e.g.* see Figure 1-36**B**). This allows undefined MIDs to be selected in a Multiplexer scheme and defined later. Once an MID has a sequence defined, it will lose its wild card status and will only appear in the list appropriate to its length.

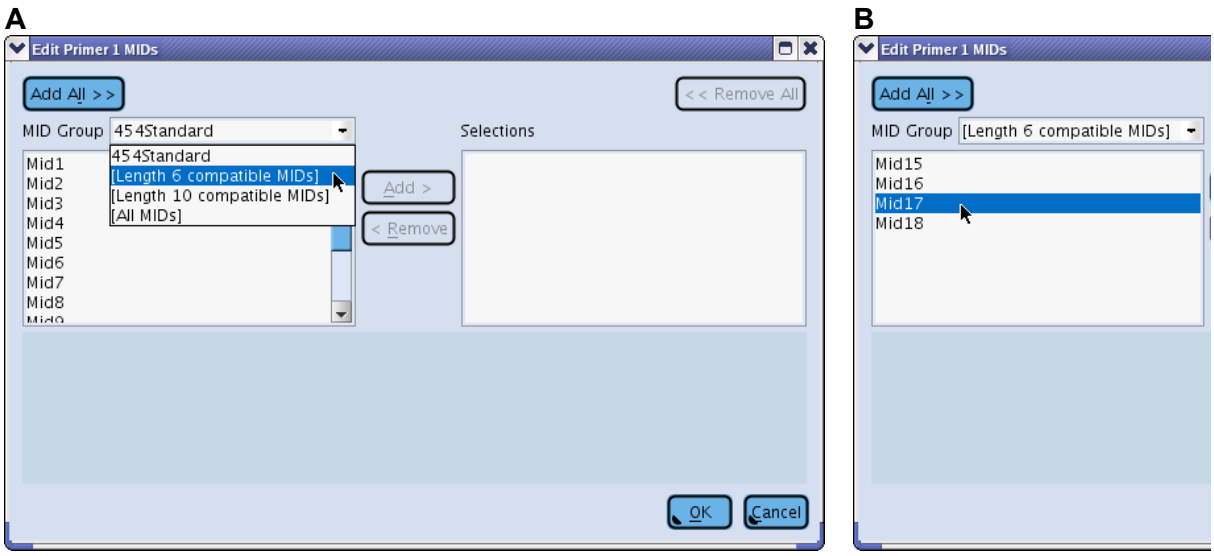


Figure 1-36: (A) The Edit MIDs window showing the MID Group drop down menu. A defined group "454Standard" is listed along with three custom automatically generated groups, the "[All MIDs]" group and two groups based on MID length. (B) The "[Length 6 compatible MIDs]" group restricts the left list to those MIDs that are exactly 6 bases long, along with those that have no sequence yet defined.

The lower part of the Edit Primer 1 MIDs (or Edit Primer 2 MIDs) window provides information (and errors or warnings, as appropriate) concerning the MIDs selected. For example, this includes a summary of the number of MIDs selected, their length, and the minimum edit distance (the minimum number of insertion, deletion, or substitution sequencing errors that could turn one of the selected MID sequences into one of the others) (see Figure 1-34, above).

The types of errors and warnings provided may include MIDs not all the same length, or undefined MIDs (Figure 1-37). Note that the software gives the benefit of the doubt to undefined MIDs, and calls the attention of the user with a warning but does not assume an error. This provides the advantage that the structure of a Multiplexer can be defined independently, and possibly in advance, of the knowledge of the MID sequences themselves. However, prior to computation, all the MIDs used in defining Multiplexers that are associated with active Read Data Set must, naturally, be defined. The software also calculates the minimum edit distance even for defined MIDs of different lengths, assuming that corrections will be made prior to Project computation (*i.e.* that MIDs of unequal length will be corrected or eliminated).

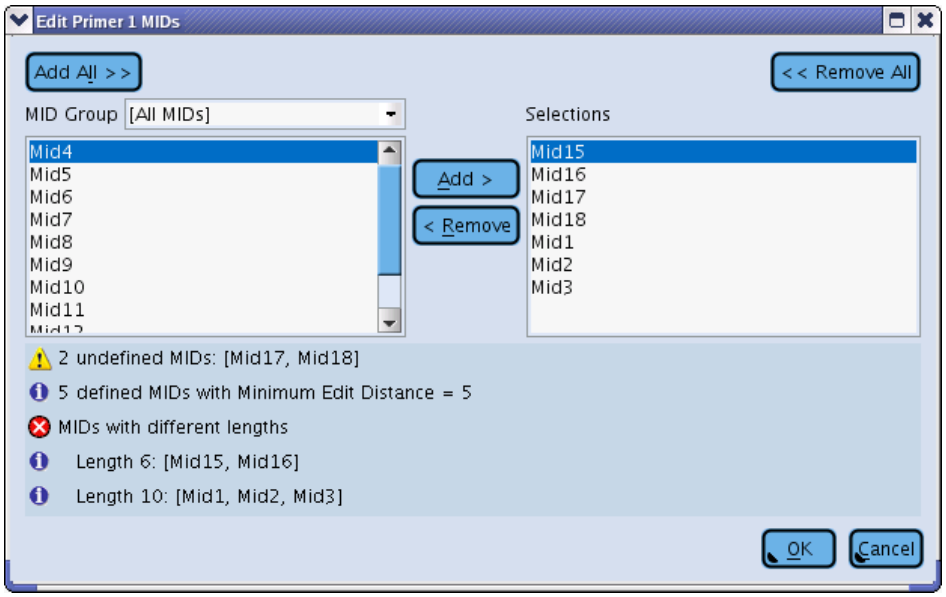


Figure 1-37: Examples of errors and warnings flagged on the Edit Primer 1 MIDs window.

1.3.2.7.3 To Enter or Edit the Samples Assignment

The most complex part of setting up a Multiplexer is to specify the assignment of the MIDs to the Samples. This is done in the Edit Samples window, which is accessed by double-clicking in the cell of the Samples column, for the Multiplexer of interest (in the Multiplexer Definition Table). This window can take 3 different forms, depending on the type of encoding selected, as described in the sections below. Note that Primer 1 MIDs and/or Primer 2 MIDs, as appropriate, must have been selected for that Multiplexer for the Edit Samples window to be available; if any errors or warnings exist regarding the selected MIDs (see section 1.3.2.7.2), these will be displayed in the Edit Samples window as well.

1.3.2.7.3.1 Sample Assignment with "Primer 1 MID" or "Primer 2 MID" Encoding

With these single-end MID encoding schemes, the Edit Samples window simply lists all the MIDs selected for the Multiplexer (see section 1.3.2.7.2), and the Sample is selected from the drop down menu (or can be typed) in the cell to the right of each MID name (Figure 1-38**A**). The user can also type into the cells the names of Samples that have not yet been defined in the project: new samples with those names will automatically be created and appear in the Project's Samples Definition Table when the user clicks 

. These samples will not be created, however, if the user clicks 

. A Sample assignment may be removed from a given MID by choosing the "--remove--" option from the drop down menu.

If the drop down menus are too narrow to display the full Sample names, you can widen the Edit Samples window, making more room for the 'Sample' column.

Certain shortcuts are available on this window as well: clicking the 

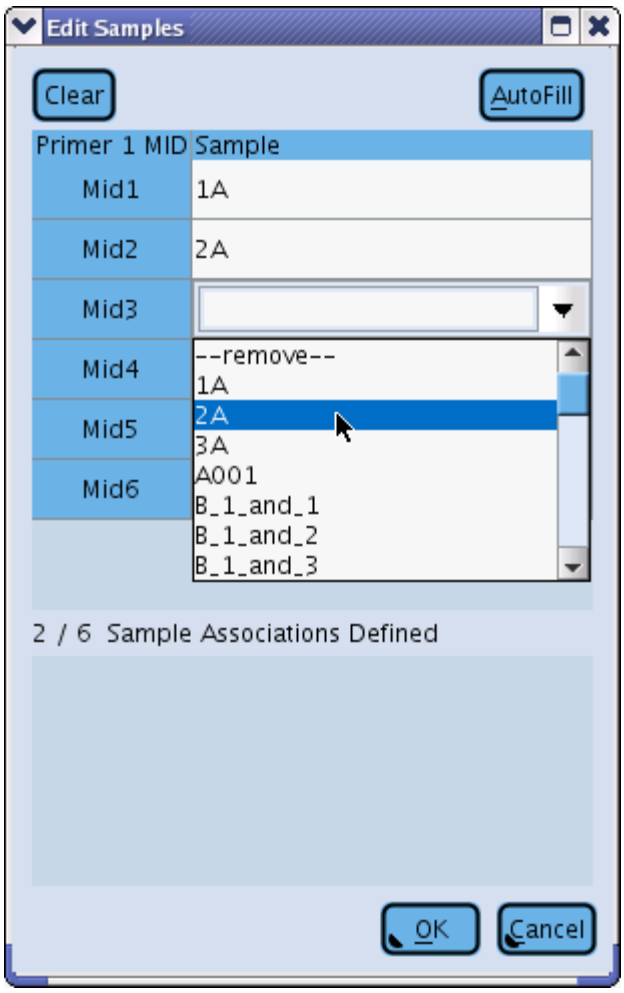
button assigns default-named Samples to any MID that does not yet have an assigned Sample (Figure 1-38**B**); and a

Clear button empties all the Sample cells. The default Sample names contain three parts, in the following format:

Sample_<Multiplexer name>_<MID name>

As with typed-in novel Sample names, the Autofill Samples will only be added to the Project if the user clicks the OK button.

A



B

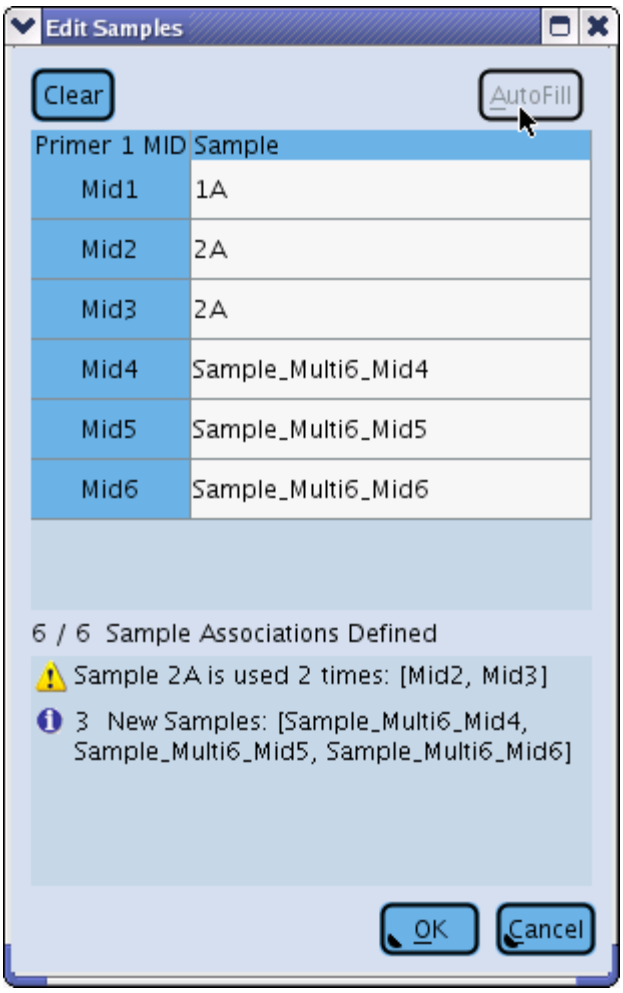


Figure 1-38: The Edit Samples window, for Primer 1 MIDs encoding.

A functional Multiplexer must specify at least one Sample assignment, but it is not formally necessary to fill all cells of the Table. This can be useful if a subset of the selected MIDs have not been used in the experiment, but were known to have been used in a previous experiment: specifying them allows the system to search for these MIDs as potential contaminants and prevent them from being misinterpreted as an erroneous version of one of the MIDs actually used in the Project to demultiplex Samples. On the other hand, it is possible to assign the same Sample to multiple MIDs (but not the opposite). Since this could be a legitimate experimental set

up, it does not elicit an error message; however, a warning is displayed to draw the user's attention to this unusual assignment (Figure 1-38**B**).

In a manner analogous to the Edit Primer 1 MIDs or Edit Primer 2 MIDs windows (section 1.3.2.7.2), a summary of the assignment scheme is provided at the bottom of the Edit Samples window, including information on the number of MID-Sample associations defined and the total number that can be defined with the MIDs selected (Figure 1-38). Any errors and warnings associated with the MIDs are also shown here, to alert the user that action must be taken to complete or correct the MID definitions or the Sample assignments (Figure 1-39).

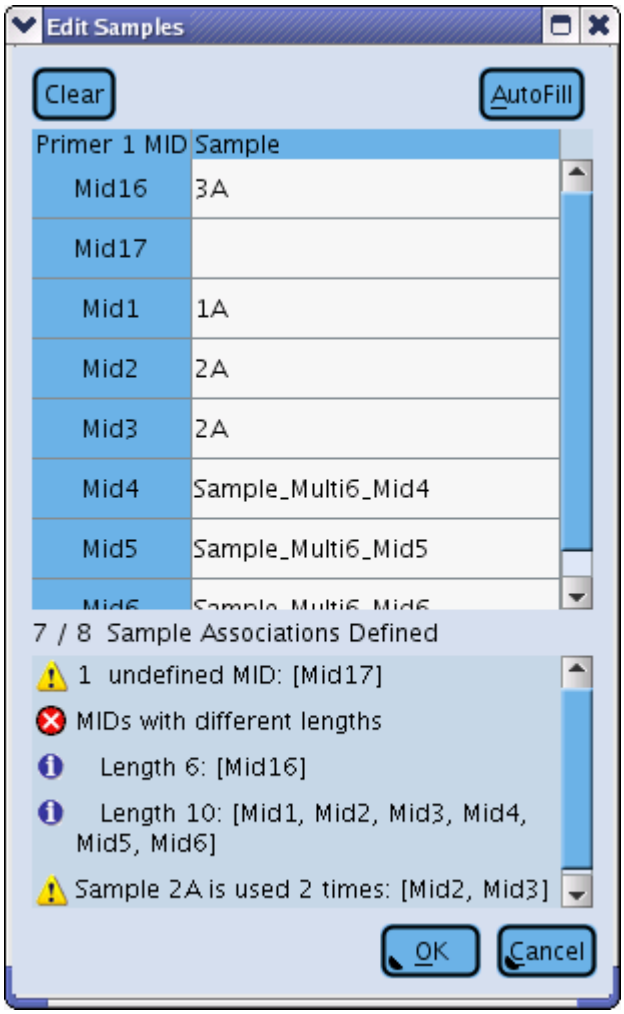


Figure 1-39: The Edit Samples window for Primer 1 MID encoding, showing one error and two warnings regarding either the MIDs currently selected for this Multiplexer, or the Sample assignments. The MIDs are listed in sorted order based on their MID Group (not displayed, but visible in the tooltip that will appear if the user hovers the mouse over the MID name) and then their MID name. MIDs without an associated MID Group appear before those with an MID Group. In this example, Mid16 and Mid17 were not assigned to an MID Group and so appear before Mid1. Had they been part of the same MID group as Mid1 they would have appeared later in the list, as expected.

1.3.2.7.3.2 Sample Assignment with "Both" Encoding

With the "Both" encoding scheme, two MIDs must be specified for each Sample, one attached to Primer 1 and one to Primer 2. In this case, therefore, the Edit Samples window displays a two-dimensional Table where the MIDs selected for the Primer 1 side occupy one dimension (rows or columns) and those for Primer 2 occupy the other (Figure 1-40). In a manner analogous to the single-MID Sample encoding seen above, Sample assignment is done by selecting the Sample name from the drop down menu (or by typing it) in the cell at the intersection of the two encoding MID names.

If the drop down menus are too narrow to display the full Sample names, you can widen the Edit Samples window, making more room for the 'Sample' columns. You can also resize individual columns by dragging on the column header separators to the left or right, until the column of interest has the proper width to allow the display of the full Sample names.

The other features of this window (can have empty cells, shortcut buttons, summary and error/warning reporting, *etc.*) are the same as for the Primer 1 MID or Primer 2 MID encoding, described above. For "Both" encoding, the names of the Autofill Samples are of the form:

Sample_<Multiplexer name>_<Primer 1 MID name>_<Primer 2 MID name>

It is important to be aware of the directionality of the Amplicons when assigning the Samples to MID pairs: MIDs are selected separately for the Primer 1 and Primer 2 sides to support this directionality. In the Edit Samples window, the side corresponding to the two selected MID sets are identified by a "1" and a "2" with arrowheads, on the top-left corner of the Table. This is illustrated on Figure 1-40: in this example, the table of the Edit Samples window has been populated using the AutoFill button; the Primer 1 – MID1: Primer 2 – MID2 pair encodes Sample Sample_Multi7_Mid1_Mid2, while the Primer 1 – MID2: Primer 2 – MID1 pair encodes Sample Sample_Multi7_Mid2_Mid1. For convenience, a Flip Table button can transpose the table.

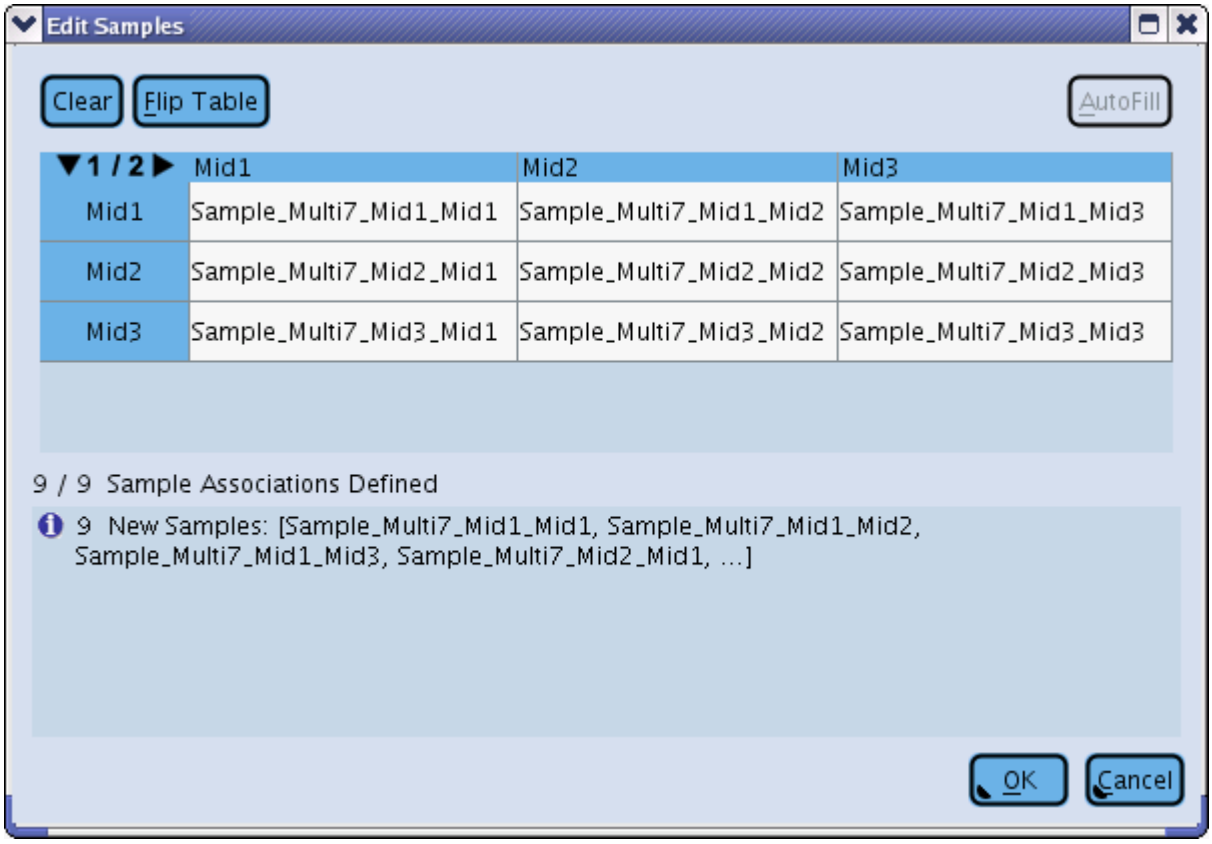


Figure 1-40: The Edit Samples window for the "Both" encoding scheme, showing Samples that were assigned using the AutoFill button.

1.3.2.7.3.3 Sample Assignment with "Either" Encoding

With the "Either" encoding, Amplicons also have two MIDs so the Edit Samples Table is two-dimensional as well. However, contrary to the situation with Both encoding, the MID from only one end is used to assign any given read to the proper Sample. This situation is akin to the Primer 1 MID and Primer 2 MID encoding cases, and likewise, each MID (at a given end) can encode only one Sample.

To help in this, the software grays out cells that become ineligible as Samples are assigned to Primer 1 MID – Primer 2 MID pairs. In the simplest case, the libraries are designed such that the same MIDs are placed at both ends of each Amplicon (Figure 1-41**A**). For an Either encoded Multiplexer, the AutoFill function is only enabled for this type of symmetric design (AutoFill expects to make sample assignments along the diagonal where the same MID is used on each end of the read: Mid1-Mid1, Mid2-Mid2, *etc*.).

However, asymmetric designs are also legitimate. The software flags this with a warning in case the asymmetry was unintended (Figure 1-41**B**). Even if the same set of MIDs are selected for both the Primer 1 MIDs and the Primer 2 MIDs series (a symmetrical design), the Sample assignment does not have to be along the diagonal in the grid (Mid1-Mid1, Mid2-Mid2, *etc*.) as it would be with an AutoFill. As long as no MID at either end is assigned to more than one Sample, and every MID on one side that has a Sample assignment has some corresponding

MID on the other side with the same Sample assignment, the design is still valid. Again, mis-assignment is prevented by graying out the ineligible cells (Figure 1-41**C**).

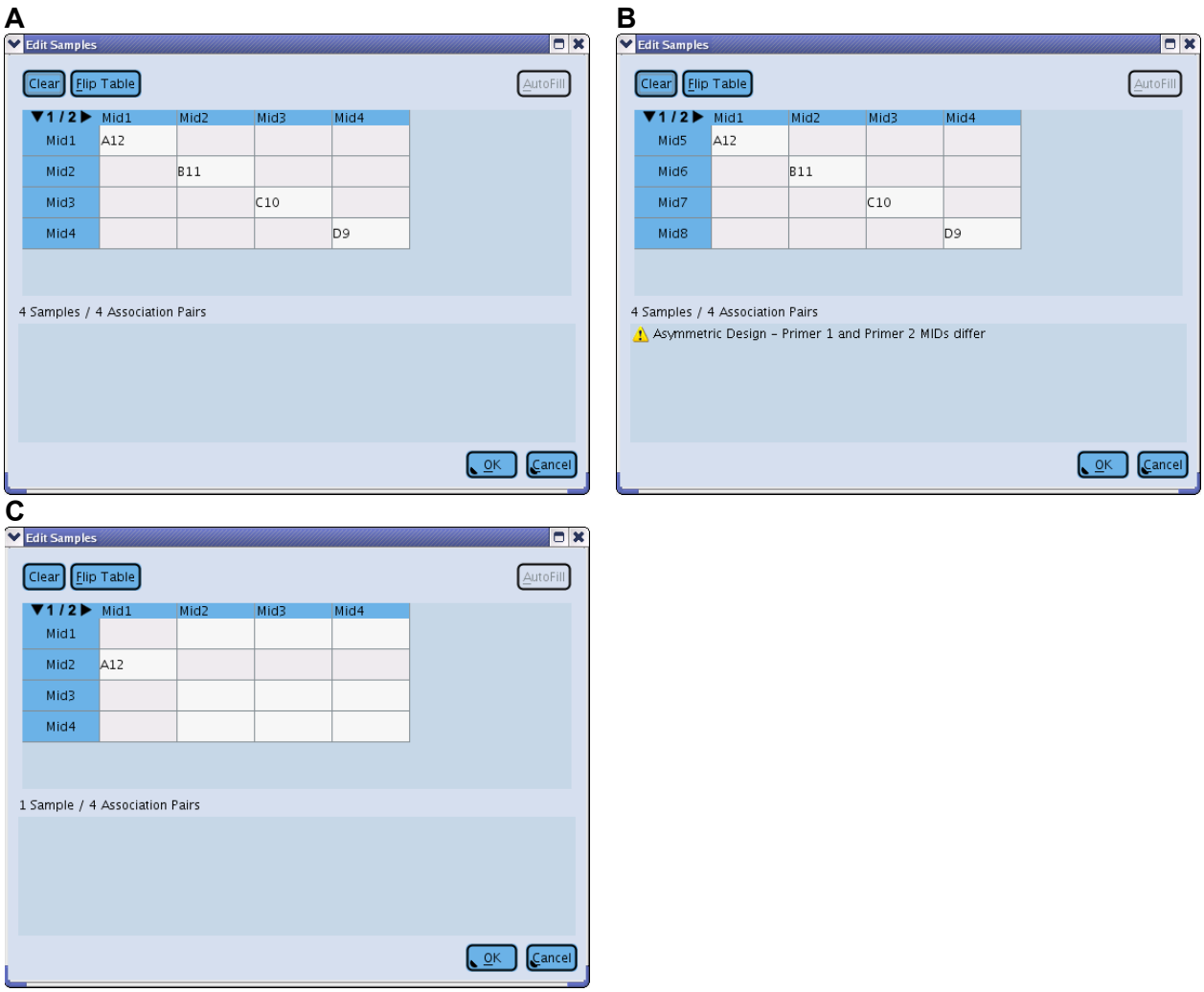


Figure 1-41: The Edit Samples window, for Either encoding. (A) Symmetrical design with Sample assignment down the diagonal. (B) Asymmetrical design with different sets of Primer1 MIDs and Primer 2 MIDs and a warning about the asymmetry. (C) Symmetrical design but with a Sample assignment deviating from the diagonal.

In fact, it is even possible to have a different number of MIDs selected on the Primer 1 and Primer 2 sides. When this kind of design is used, the software displays a warning that there are unequal numbers of Primer 1 and Primer 2 MIDs, and specifies the number of unbalanced associations (Figure 1-42). In this special case, one or more MIDs will have to be used more than once, yet the constraint that a given MID at a given end of the Amplicons must specify a single Sample (to allow for unambiguous assignment of the reads) must be respected. To accomplish this, the AVA software restricts the Sample choices in cells that may receive such secondary assignments (highlighted with a thicker gray border), to Samples already specified for a Primer 1 MID or a Primer 2 MID. Some of the specific circumstances one might encounter are illustrated in Figure 1-42.

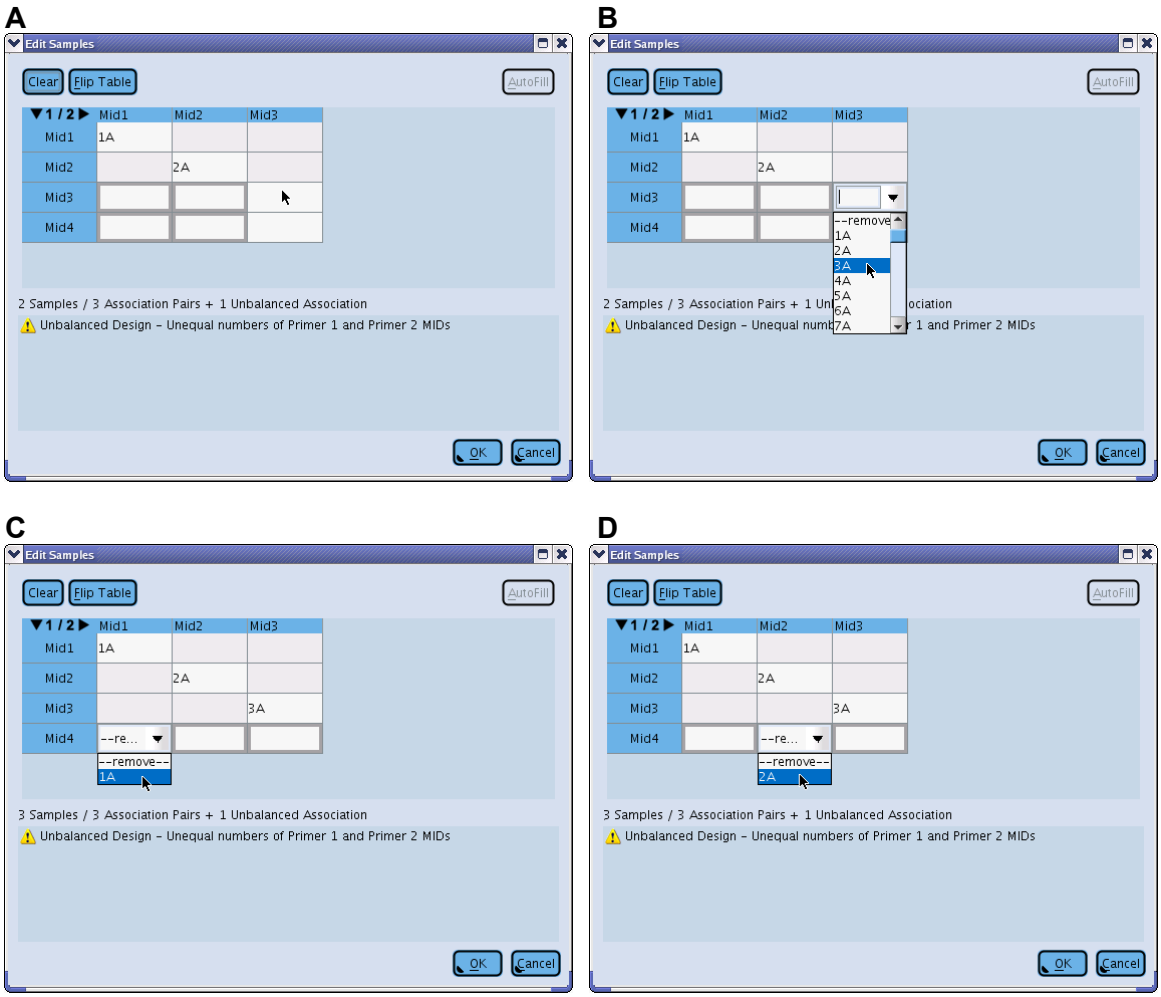


Figure 1-42: Edit Samples window for an Either-encoded Multiplexer with an unbalanced design. There are 4 Primer 1 MIDs and 3 Primer 2 MIDs. (A) With two sample assignments already made, the only cells that are not constrained at all (Mid3-Mid3 and Mid4-Mid3) are shown as totally white. Cells that have a thicker gray border are available for selection but are constrained to a single choice by previous Sample assignments. (B) The Mid3-Mid3 cell has an unconstrained Sample list and Sample 3A is being selected. (C) After the Sample assignment, the Mid4 row consists of three constrained cells with thick gray borders. The Mid4-Mid1 cell only allows Sample 1A to be selected because the Primer 2 MID Mid1 is already being used to encode that Sample. (D) Similarly, The Mid4-Mid2 cell only allows Sample 2A to be selected because the Primer2 MID Mid2 is already being used to encode Sample 2A.

Again the other features of the Edit Samples window for “Either” encoding (can have empty cells, shortcut buttons, summary and error/warning reporting, *etc.*), are the same as for the Primer 1 MID, Primer 2 MID, or Both encoding, described above. Autofill Samples are handled the same way as for Both encoding.

As above, if the drop down menus are too narrow to display the full Sample names, you can widen the Edit Samples window, making more room for the ‘Sample’ columns. You can also resize individual columns by dragging on the column header separators to the left or right, until the column of interest has the proper width to allow the display of the full Sample names.

1.3.2.7.4 Using Multiplexers for more than one Read Data

Once a Multiplexer has been created and defined, it can be used on any number of Read Data Sets in the Project, as long as these Read Data Sets share the same MIDs, encoding, and MID-to-Sample associations. Furthermore, each Multiplexer-Read Data Set pairing can even be used to demultiplex distinct sets of Amplicons, as defined by the Sample-Amplicon-Read Data Set triad associations (Figure 1-43).

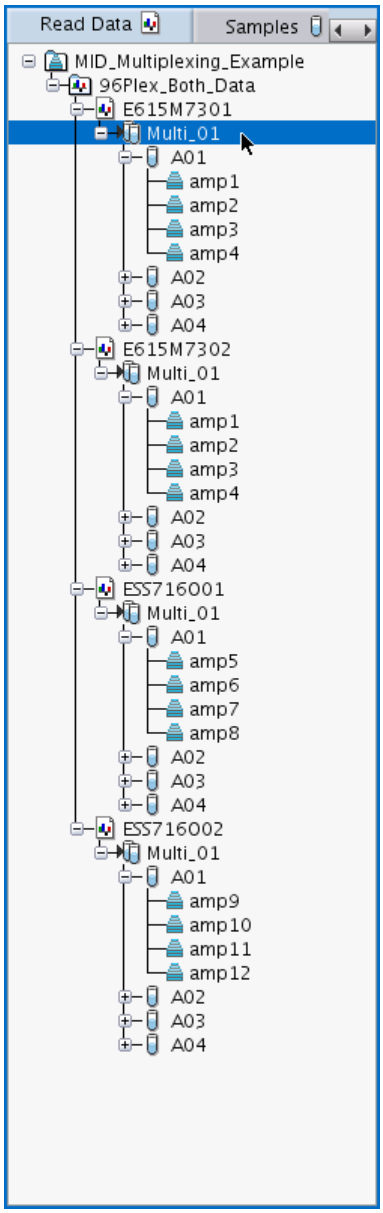


Figure 1-43: A single Multiplexer (Multi_01) is associated with 4 different Read Data Sets in the Read Data Tree. In the context of the first two Read Data Sets, the same set of Amplicons is being measured (amp1-amp4), but different Amplicons are being measured for each of the remaining Read Data Sets (amp5-amp8 for the third Read Data Set and amp9-amp12 for the fourth Read Data Set).

The AVA software also allows for Multiplexers to be duplicated. Although it is not necessary to define multiple exact copies of Multiplexers within a Project, as just discussed, duplication may be useful if multiple Multiplexers need to be defined, that share common baseline features such as the encoding scheme and specific MIDs on each side of their Amplicons. This is done using the “Duplicate item” button on the left margin of the Project Tab (see section 1.3.2): a copy of a Multiplexer created this way retains the encoding and MID settings of the original.

The “Select Amplicons associated with item” button can also provide a very useful shortcut when a given set of Amplicons is to be measured by multiple Read Data Set – Multiplexer pairs. This button is also located on the left margin of the Project Tab, and its functionality is described in section 1.3.1. Selecting a large number of disparate Amplicons from the Amplicons Definition Table, to associate them to a Multiplexer, can be laborious and painstaking; if many Multiplexers require the same (or similar) Amplicon associations, you need to create only the first of these Multiplexers manually, then select it in the Read Data Tree and click the “Select Amplicons associated with item” button; the software will switch to the Amplicons Definition Table sub-tab, and the subset of the Amplicons that are associated with the original Multiplexer will be selected, ready to be dragged to another Multiplexer in the Tree.

1.4 The Computations Tab

The Computations tab has only one function: carry out the computations on the Amplicon Project, with the elements currently defined and the “active” Read Data Set(s). This requires that the Project has been “set up”, including the definition of the various elements that constitute it (Reference Sequences, Amplicons, Read Data Sets / Read Groups, Samples, Variants, and, optionally, MIDs / MID Groups and Multiplexers), and their associations as appropriate. (See sections 1.3 on the Project Tab, and the example in section 2.2, for details on how to set up a Project before computation.)

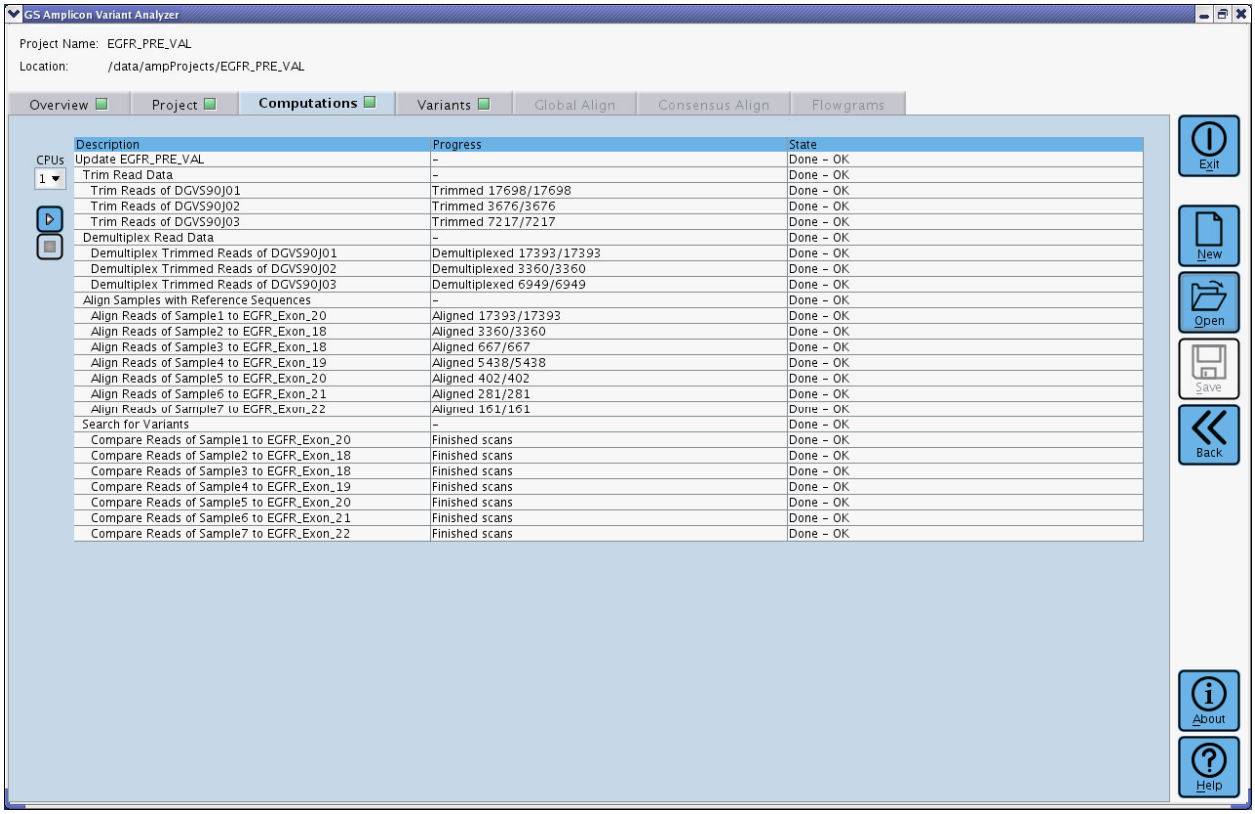


Figure 1-44: The Computations tab

Before starting the computation, you have the option of selecting the number of CPUs that will be utilized for the computation. The default value is 1. Increasing the number of CPUs allows certain parts of the computation, mainly trimming and alignment, to be split into concurrent parallel processes, potentially reducing the computation time.

The number of CPUs is selected from the dropdown list above the “Start Computation” button (Figure 1-44). The dropdown list contains values from 0 through the number of processors available on the Attendant PC or Datarig. The value 0 represents “All available processors” and is equivalent to selecting the highest value in the list. The number of CPUs option is the GUI equivalent to the command line option –cpu, which is described in section 3.3.2.1.

Computations with large references require large amounts of memory. If high numbers of CPUs are used on systems with insufficient memory, the system could freeze due to memory swapping issues.

To carry out the computations, simply click the “Start Computation” button. The Computations Status Table will be populated as the computations progress. The Table comprises 3 columns: Description (of each computational step); Progress; and Status. You cannot enter any information into this Table.

The main steps of a Project’s computations are as follows:

1. Trim Read Data: for each Read Data Set, the Primer sequences and MIDs, if used, of all the reads are identified for demultiplexing purposes and the trim points are noted for the "Target" sequences. (As mentioned in section 1.1.1.3, trimming the Primers is important because any variations found therein would have no biological significance, and therefore should not be reported by the AVA software.)
2. Demultiplex Read Data: for each Read Data Set, each read is identified as belonging to one defined Amplicon and assigned to the appropriate Sample, taking into account any relevant MID information. If MIDs are not used, the Amplicon must be associated with one specific Sample, and the read's Sample is so established. If MIDs are used, the Amplicon is used to determine the relevant Multiplexer associated with the Read Data Set, and then the MIDs found within the read, in conjunction with the MID encoding of Samples defined by the Multiplexer, are used to determine the read's Sample. Demultiplexing may involve:
    a. splitting the reads of a Read Data Set over multiple Samples, *e.g.* if the experiment was set up such that one or more Amplicons (which are associated with different Samples either directly, or through the use of MIDs) were present in a PicoTiterPlate Device (GS Junior Instrument) or PicoTiterPlate region (Genome Sequencer FLX) of a sequencing Run; and/or
    b. joining of the reads from multiple Read Data Sets into any given Sample, *e.g.* if the experiment was set up such that multiple regions of a PicoTiterPlate Device (Genome Sequencer FLX) and/or sequencing Runs contributed reads to the Amplicons that are associated with the Sample.
3. Align Samples with Reference Sequences: the reads of each Sample are multiply aligned to the Reference Sequences corresponding to the Amplicons with which the Samples are associated. Initially, the reads are aligned with their primers included, but after the reads find their place in the alignment, the primer regions get trimmed off. Using the primers in this way can provide more alignment context and produce more sensitive and accurate alignments at the edges of amplicons (particularly amplicons where there is a sizeable deletion near the target sequence boundaries). Similar Individual reads are grouped into Consensus reads, and the multiple alignments of Individual and Consensus reads are constructed for later viewing in the Global Align and the Consensus Align tabs.
4. Search for Variants: For each defined Variant, the multiple alignments of the previous step are scanned to determine which Individual and Consensus reads span all the positions of the Variant's Pattern and, of those that do span all these positions, which reads satisfy all the constraints specified by the Pattern. Statistics are calculated for forward and reverse reads separately, and then pooled together, in order to provide estimates of Variant frequencies. The results of this search are reported in the Variants tab.

5. In addition, the AVA software automatically searches for potential Variants not explicitly entered into the system. These Auto-Detected "Putative" Variants receive "Intelligent Names" that are unique and compact, yet descriptive (see section 4.2); these Auto-Detected Variants are not automatically loaded into the Project, but can be manually loaded based on particular selection criteria on the main Variants Tab (see section 1.5.2.6). The AVA software automatically searches for substitution (SNP) and block deletion (>=3 bp) Variants. (Insertion Variants and block deletion Variants <3 bp are not currently automatically detected, so manual browsing of the alignments may be needed if these types of Variants are anticipated.) Combined with the ability to selectively load and subsequently sort and filter these Variants based on their frequencies and read-orientation support, the vast majority of interesting Variants can be easily discovered and evaluated from the view of the data provided in the Variant tab's Sample – Variants Table. By providing the ability to both edit the Variant Status, and filter by that Status, the AVA software provides a simple

Discovery Workflow to determine which Variants have been evaluated and what the outcome of that evaluation was. See section 1.5.2.7 for more details on the proposed Variant Discovery Workflow process.

If you modified some information in the Project after computing it (*e.g.* imported new Read Data Sets, defined new Variants, *etc*.), you must re-compute it to update the results reported in all the tabs. When you re-compute a Project, the AVA software uses cached results, if possible, for any step that has not changed (except for demultiplexing, which is brief and is always carried out). This can save a lot of time in what would otherwise be needless repetition of calculations.

The Computations tab also has a "Stop computation" button that you can use to abort calculations, *e.g.* if you decide to make further changes to your Project set up while calculations are on the way. This can be useful for very large Projects, where the calculations can take some time. When you do this, the AVA software accepts the results that have already been re-computed, but it also keeps the results from the previous computation that have not yet been altered by the re-computation. The reason for this is that since Amplicon Projects are incremental, re-calculations are often done after adding Read Data Set(s) or Sample(s) to the Project in a manner such that much of the previously computed results are still valid. On the other hand, the results that were in the process of being re-computed at the time of the interruption could truly be corrupted; the results for these computations, caught in an intermediate state, are removed from the computation's output (such as in the Variants Tab) and the navigation elements that would be used to load these results are disabled (such as those used to load multiple alignments in the Global Align tab).

Interrupted computations: If a computation (or re-computation) is interrupted, there is a risk that part of the output may not match the state of the saved Project. While the AVA software withholds the potentially corrupted results from the data that was being processed at the time of the interruption, it also maintains the results from previous computations that had not yet been altered at the time of the interruption. Be aware that those older results may not be consistent with more recent updates to the Project. The outcome of this is similar to the case described in the "Caution" at the end of section 1.3 (editing a Project in a manner that is germane to previously computed results). If you find that the data in these tabs does not reflect the current state of the Project, try re-computing it. The only way to be completely sure that the Project is consistent is to allow the computation to run to completion, without interruption.

Before starting the actual computations, the AVA software validates the computationally relevant aspects of the Project setup. This includes most Project elements' definitions and their associations, such as a Variant pattern and its relationship to the Reference Sequence, or the particular pairings of Samples and Amplicons on particular Read Data Sets. If the software finds any problems, warning messages are displayed, giving the user a chance to address them prior to running the computation. The user may elect to ignore the warnings and proceed; the computation will still run, and shouldn't throw any errors, but the results may be incomplete because the computation will skip the problematic elements.

Figure 1-45 shows the different kinds of warning messages that can occur:

- The warning that the Project has been modified, but not saved, so the computation might produce results that are out of sync with the Project's current state.
- The message indicating that further messages in the Computation Warning window are based on the Project in its current state, *i.e.* as computation would see it if it were saved; this gives you warning of problems in a Project even before you save it to disk. If your Project is up to date on the disk, the other messages below may still occur but they would then concern the Project as saved.

- A warning that a Read Data Set is active in the Project, but has no associated Amplicons (because no Sample-Amplicon pairs have been assigned to it). This may be an oversight on the part of the user, in which case a large part of the expected output might be missing if the computation were carried out. This warning also appears if a valid Multiplexer is associated with the Read Data Set but no Amplicons are associated with the Multiplexer (and there are no other Amplicons associated with the Read Data Set directly via Samples or indirectly via another Multiplexer).

- A warning that one or more Amplicons that are associated with some Read Data Set have problems. Such problems may include:

    - the Amplicon is incompletely defined (no primers, or target start/end not specified)
    - the Amplicon appears to be inconsistent with its Reference Sequence (the Start/End of the Target are outside the range of the Reference Sequence; this may occur if the user edited the Reference Sequence subsequent to having assigned the Start/End of the associated Amplicon)
    - the Reference Sequence itself is incompletely defined (*e.g.* it was given a name, but no actual sequence; in this case the Amplicon wouldn't likely have any Target Start/End set either).
- A warning that one or more Variants that are potentially associated with a Read Data Set (the Variant location on its Reference Sequences is spanned by an Amplicon that is associated with the Read Data Set) have problems. Possible Variant definition problems include:

    - the Variant pattern is inconsistent with the Reference Sequence [*e.g.* a substitute constraint specifies the substituted nucleotide as the same as the one already in the Reference Sequence (should be a match constraint instead)]
    - some or all the positions of the Variant Pattern are not in the Reference Sequence (as above, this may occur if the user edited the Reference Sequence subsequent to having defined the Pattern of the associated Variant)
- A warning that the file for an active Read Data Set is missing. This warning would be triggered if a Read Data Set was imported as a symbolic link, and the file that the link points to has been moved, deleted, or become corrupted. The warning message tells where the link was expecting to find the file so that it can be restored to the proper location.

- A warning that an inconsistent Multiplexer is associated with a Read Data Set. Problems with a Multiplexer may include:

    - The Multiplexer has not been completely defined (the Encoding, MIDs, and/or Samples have not been specified).

- There is a problem with the set of MIDs on either the Primer 1 or Primer 2 sides being used by the Multiplexer to encode Samples. Examples of MID problems are:
  - An MID is undefined.
  - At least 2 MIDs are defined and they are not of uniform length (at least one of them has a different sequence length than another).
  - An MID in the set has an identical sequence to another MID in the set.

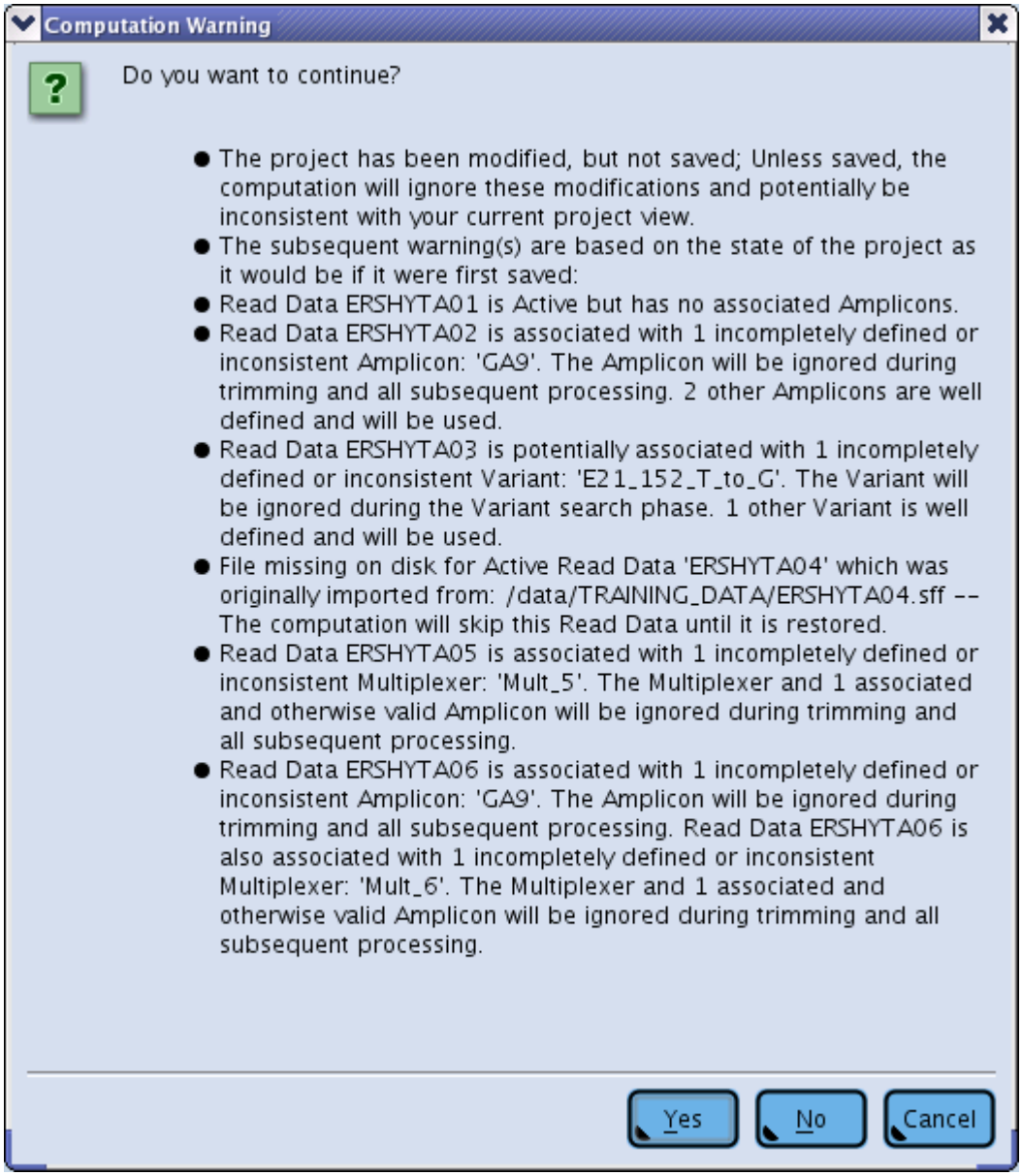


Figure 1-45: A Computation Warning message showing several of the types of message that can occur. The final warning message illustrates that more than one warning for a single Read Data Set can get merged together into a single message. Some of the additional warning messages that can occur, related to Multiplexing, include: Multiplexers that are associated with a ReadData that contain invalid combinations of MIDs, possibly including MIDs that are undefined; or Multiplexers associated with ReadData that have no MID -->Sample associations defined but do have Amplicons associated with the Multiplexer and the ReadData; or the similar case where MID --> Sample associations are defined for the Multiplexer but no Amplicons have been associated with the ReadData-Multiplexer combination.

1.5 The Variants Tab

The Variants tab shows the frequency at which all the Variants defined in the Project were observed, for each Sample defined in the Project, at the time of the last computation (Figure 1-46). This is presented in a convenient tabular form that allows the user to compare the

frequencies of the Variants across Samples. This tab is not to be confused with the Variants sub-tab of the Project Tab, which is used to store and edit the definitions of Variants. In addition to the Variants Frequency Table, the Variants tab also contains controls to modify the content and format in which the Variant frequency data is displayed. The customary Mouse Tracker, on the left, is also present to provide additional details as you mouse over the cells and headers of the Variants Frequency Table.

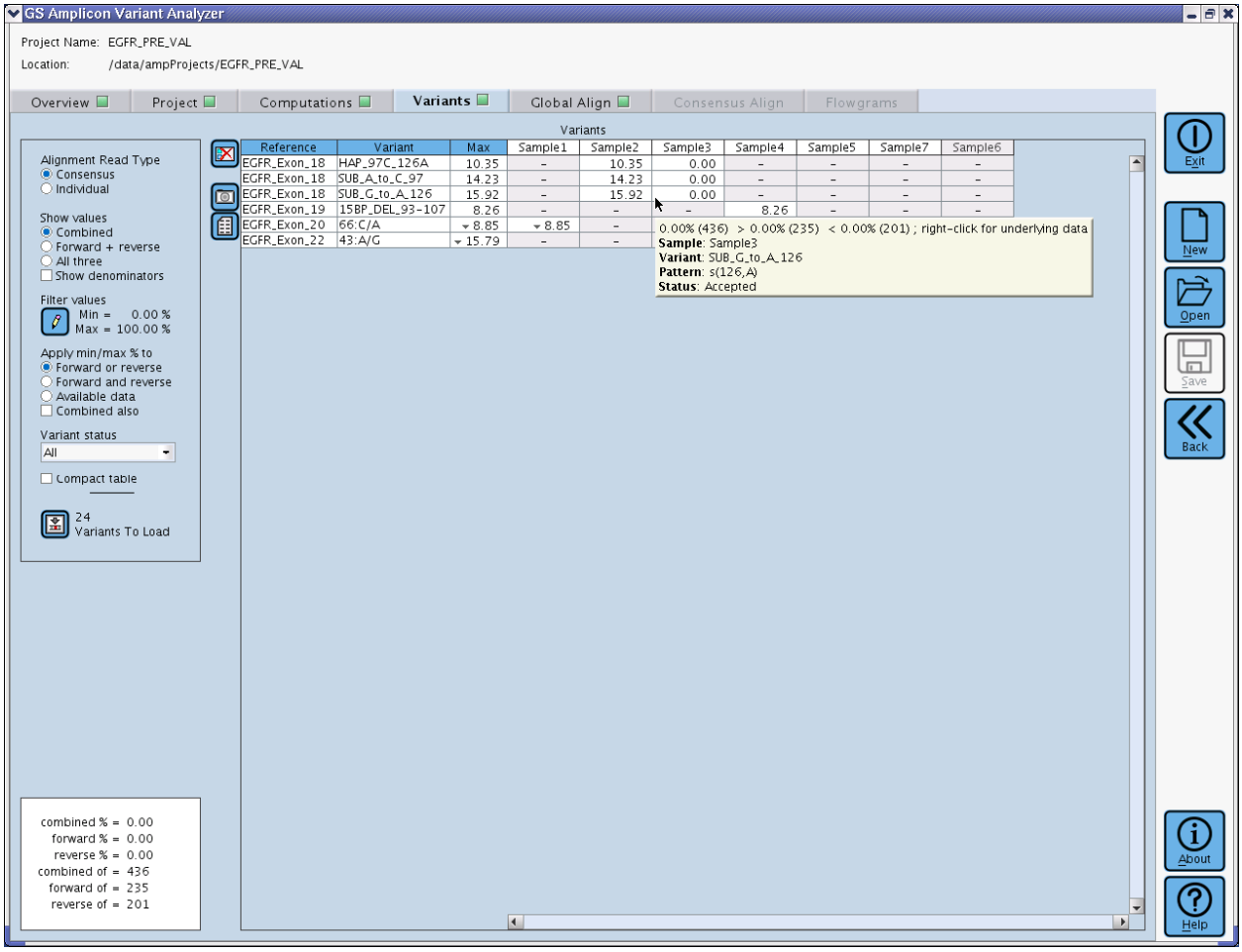


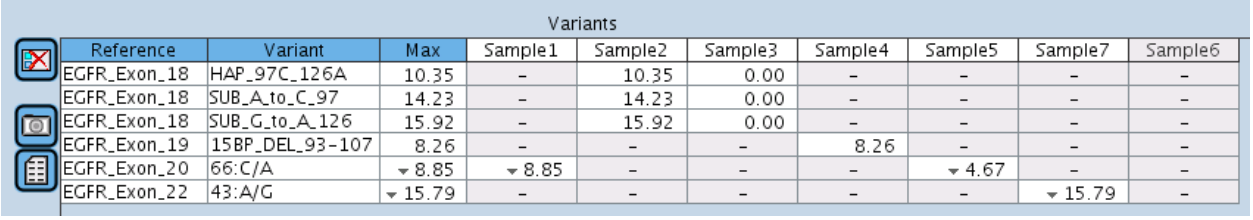
Figure 1-46: The Variants tab

1.5.1 The Variants Frequency Table

1.5.1.1 General Organization

The Variants Frequency Table shows results one Variant per row and one Sample per column (Figure 1-47). Initially, cells that contain data are white and cells that contain no data are grayed-out (this can happen, for example, if a Sample has no associated Amplicons whose sequence covers the Variant; see below). When an entire row or column is grayed-out, it is moved to the bottom or right of the Table, respectively. This grayed-out scheme is also used by various display features whereby you can filter out the data according to certain criteria, leaving the data of most interest in white cells, in the upper-left area of the Table. (The 'Compact table'

option from the Variant data display controls can then remove from view all completely grayed-out rows and columns; see section 1.5.2.4)



Variants

Reference	Variant	Max	Sample1	Sample2	Sample3	Sample4	Sample5	Sample7	Sample6
EGFR_Exon_18	HAP_97C_126A	10.35	-	10.35	0.00	-	-	-	-
EGFR_Exon_18	SUB_A_to_C_97	14.23	-	14.23	0.00	-	-	-	-
EGFR_Exon_18	SUB_G_to_A_126	15.92	-	15.92	0.00	-	-	-	-
EGFR_Exon_19	15BP_DEL_93-107	8.26	-	-	-	8.26	-	-	-
EGFR_Exon_20	66:C/A	8.85	8.85	-	-	-	4.67	-	-
EGFR_Exon_22	43:A/G	15.79	-	-	-	-	-	15.79	-

Figure 1-47: The Variants Frequency Table

With respect to columns, the Table is divided into two main parts:

- The first three columns (with blue Header cells) act as “Headers” to the Variant rows:
  - The ‘Reference’ column gives the names of the Reference Sequences to which the Variants (in the second column) are associated. The rows are initially sorted from top to bottom in alphabetical order of the Reference Sequence names (this applies separately to rows that contain at least one white cell and to the grayed-out rows that appear at the bottom of the Table).
  - The ‘Variants’ column gives the names of the Variants whose occurrence frequency for each Sample are given in each row. If two or more Variants are associated with any given Reference Sequence, the Variant names are used for second level sorting in the initial display.
  - The ‘Max’ column displays the maximum frequency observed for each Variant across all the Samples displayed in the Table. This can be a convenient indicator of whether the software detected a given Variant at sufficient frequency in the computed data to warrant further examination in the individual ‘Sample’ columns, to the right.
- All other columns give the occurrence frequency observed for each Variant for one Sample (identified in the column Header), one column for each Sample. These columns (excluding the first three) are initially sorted from left to right in alphabetical order of the Sample names (this applies separately to columns that contain at least one white cell and to the grayed-out columns that appear at the right end of the Table).

While the first three columns are always visible, you can scroll through the Sample columns using the scroll bar located at the bottom-right of the Table [see Figure 1-48, which displays the same data as in Figure 1-47 but in a more expanded display (see section 1.5.2), showing the scroll bar]. As you scroll to the right, the leftmost Sample columns appear to slide behind the first three rows, so you may end up in situations where you display a partial column just after the ‘Max’ column.

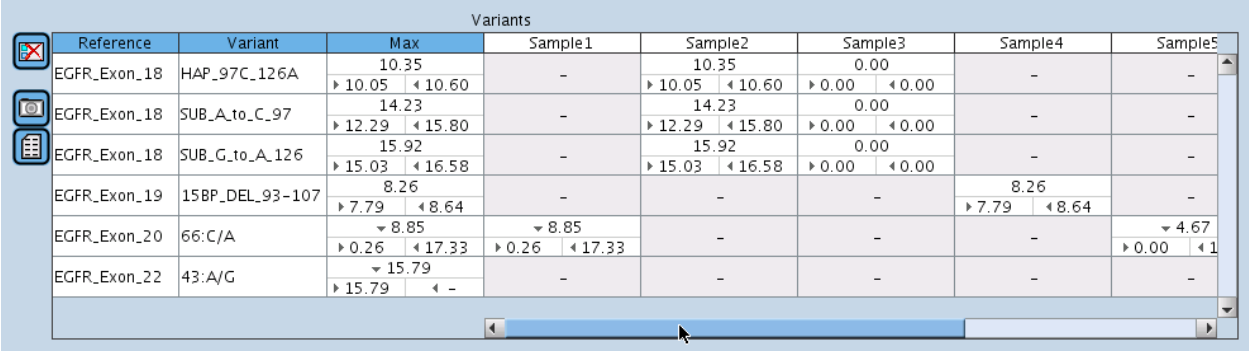


Figure 1-48: The Variants Frequency Table showing the same data as in Figure 1-47, but in a more expanded form, showing the scrolling feature that applies to the 'Sample' columns.

Since Variants are defined in the context of a Reference Sequence, and Samples are associated with Amplicons (which are in turn defined in the context of a Reference Sequence), there may be Samples in your Project that are not valid candidates for a particular Variant scan. This happens if all the Amplicons associated with the Sample are defined relative to a different Reference Sequence than the Variant; or even if they are from the same Reference Sequence, if they do not cover regions of the Reference Sequence where the Variant is defined. It may even be that the Sample was associated with an Amplicon that should have covered the region of variation, but for some reason no actual reads were sequenced that covered the variant (as distinguished from the case where some reads cover the region of variation, but none of them satisfy the constraints given by the Variant's Pattern). In these cases, the corresponding Sample-Variant cell in the Table will be grayed-out and contain a single dash ('-') character.

The Variants tab also has various common features such as a Mouse Tracker and the "Save table snapshot to image file" and "Save Table to Text file" buttons. Note that since each cell can contain up to 6 values (frequency in forward, reverse, and combined reads, and the three associated denominators of those frequencies; see section 1.5.2.2), the spreadsheet may be constructed with multiple columns for each sample to accommodate all these values.

Another useful feature is that if you pause the mouse over any cell of the Table, a screen tip will open providing relevant information about the content of that cell, as follows:

- Column header cell
  - Instructions on the right-click options (see section 1.5.1.2)
- Reference cell
  - Name of the Reference Sequence
  - Beginning of its DNA sequence
  - Reference Sequence Annotation
- Variant cell
  - Name of the Variant
  - "Pattern" of the Variant, in the Variant Definition Syntax (see section 1.3.2.5.2)
  - Variant Annotation
  - "Status" of the Variant (see section 1.3.2.5.3)
- All "Frequency" cells ('Max' or 'Sample' columns; shown in Figure 1-46):
  - Frequency (and number of reads) for the combined orientations and for each orientation; and instructions on the right-click options (see section 1.5.1.2)
  - Name of the Sample

- Name of the Variant
- "Pattern" of the Variant, in the Variant Definition Syntax (see section 1.3.2.5.2)
- "Status" of the Variant (see section 1.3.2.5.3)

1.5.1.2 Organizing Data in the Variants Frequency Table

The Variants Frequency Table contains the summary results of your Amplicon Project provided by the AVA software: the frequency at which each defined Variant was observed in each Sample, in the Read Data Set(s) analyzed, expressed as a percentage of number of reads included in the calculation. As you examine these results, however, it may be convenient to sort the data or to bring the focus on only certain Variants or Samples at a time. This would be especially true in a large Project with many defined Variants and/or many Samples.

To help with this, right-clicking on any column or row "header" cell opens a contextual menu that offers several sorting or filtering options (remember that the cells in the first three columns are all "row headers"; see section 1.5.1.1):

- If you right-click on the Reference or Variant column header, the contextual menu will include show/ignore options that apply to rows along with reversion options but no column sort options are available (Figure 1-49 **A-B**).
- If you right-click on the 'Max', column header, the contextual menu will contain hybrid options: the sort options will apply to the given column, but the show and ignore options will apply to rows (Figure 1-49 **C)**.

- If you right-click on a Sample column header, the options in the contextual menu will apply to that column, with additional options that apply to all the other Sample columns collectively; the sort options sort the values found in the column, effectively reordering the rows of Variant data (Figure 1-49 **D**).

- If you right-click on a cell in the column underneath the Reference label, there will be options that apply to all rows collectively and also to that subset of rows that are associated with the specific Reference Sequence: *i.e.*, all those rows that have data for Variants associated with that Reference Sequence (Figure 1-49 **E**).

- If you right-click on a cell in the columns underneath the Variant or Max headers, the options in the contextual menu will apply to that row or to all rows of Variants collectively; the sort options sort the values found in the row, effectively reordering the columns of Sample data. The menu also has a Variant Status option that pops up a set of radio buttons for Status selection (Figure 1-49 **F**).

- If you right-click on a Sample/Variant intersection cell, the contextual menu contains options to view the Global Alignment from which the frequency data for that cell was derived, and to edit the Variant Status or remove the Variant from the project (Figure 1-49 **G**).

- If you right-click on a cell in the columns underneath the Variant or Max headers or if you right-click on a Sample/Variant intersection cell, there is also a "Define Haplotype" option (Figure 1-49 **F** and **G**). This option is inactive unless rows for two or more Variants from

the same Reference are selected. The “Define Haplotype” option is described in section 1.5.1.4.

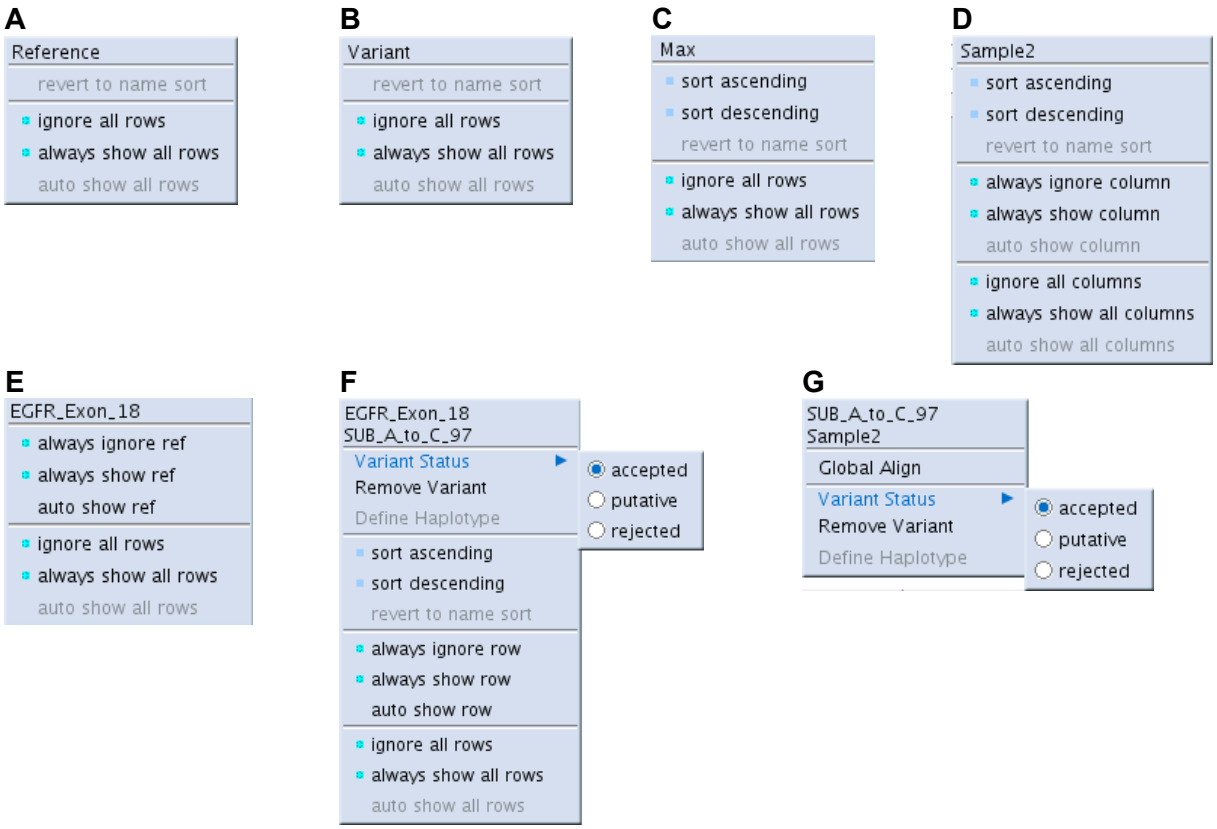


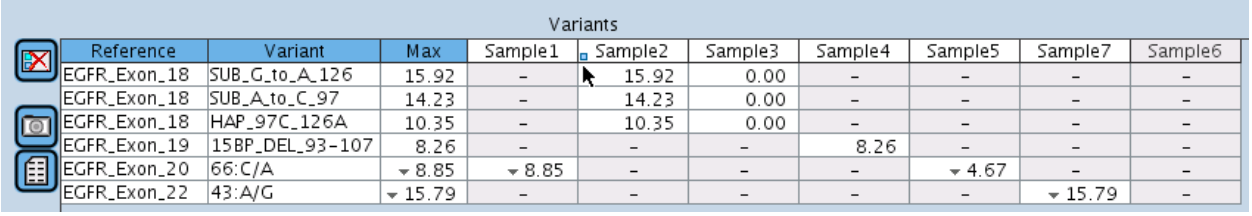
Figure 1-49: The contextual menus available in the Variants tab (see description above)

The data organization tools offered in these contextual menus include sorting, ignore filters, show filters, and option reversions. These are described below.

1.5.1.2.1 Sort options

- sort ascending
- sort descending

These options sort the columns/rows according to the ‘Combined’ Variant frequency in the column/row on which you right-clicked. A blue marker appears in the lower-left corner of the header cell (the ‘Max’ header for rows) in the column/row according to which the sorting was done. The ‘Max’ column header can be sorted and marked just like the Sample columns. You may apply a sort to only one row and only one column at a time, but you can sort the Table according to both one row and one column simultaneously.



Variants

Reference	Variant	Max	Sample1	Sample2	Sample3	Sample4	Sample5	Sample7	Sample6
EGFR_Exon_18	SUB_G_to_A_126	15.92	-	15.92	0.00	-	-	-	-
EGFR_Exon_18	SUB_A_to_C_97	14.23	-	14.23	0.00	-	-	-	-
EGFR_Exon_18	HAP_97C_126A	10.35	-	10.35	0.00	-	-	-	-
EGFR_Exon_19	15BP_DEL_93-107	8.26	-	-	-	8.26	-	-	-
EGFR_Exon_20	66:C/A	8.85	8.85	-	-	-	4.67	-	-
EGFR_Exon_22	43:A/G	15.79	-	-	-	-	-	15.79	-

Figure 1-50: The Variants Frequency Table, sorted (descending) according to the Sample2 column. Note the blue marker in the lower-left corner of the Sample2 column header; compare with Figure 1-47 .

1.5.1.2.2 Ignore filters

- always ignore column/row
- ignore all columns/rows

As indicated above (section 1.5.1.1), the software grays-out cells that contain no data and shifts rows and columns that contain only gray cells to the bottom or right ends of the Table. This focuses the cells of interest (white) to the upper-left area of the Table. With the 'always ignore column/row' options, you can gray-out any columns/rows (which moves them to the right/bottom of the Table) to help focus on data of current interest. If you want to gray-out most columns/rows, you can use the 'ignore all' option to gray them all first, and then apply the 'show' filter (see below) to re-focus on only the ones for which you have a current interest. (Cells will also be grayed-out if they fail the Min/Max filter, from the Variant data display controls; see section 1.5.2.3.)

A blue marker appears in the upper-left corner of the header cell (the 'Max' header for rows) in the column/row that you chose to ignore, to remind you that this filter was applied manually. You can ignore any number of columns or rows. You can also ignore a column or a row that was used for sorting; in this case, the corresponding header cell will have both blue markers, in its upper- and lower-left corners.

1.5.1.2.3 Show filters

- always show column/row
- always show all columns/rows

These filters have the opposite effect of the 'ignore' filters (including moving the columns/rows to the upper-left area of the Table). They use the same upper-left blue marker in the header cell as the 'ignore' filters, to indicate that the show filter was applied manually to the column/row. They will override the application of an 'ignore' filter, as described above, and can be used to force a Sample or Variant into the display even if it has failed the Min/Max filters (see section 1.5.2.3). This can be particularly useful for forcing the inclusion of negative controls in the display (which would typically fail a minimum filter).

1.5.1.2.4 Option reversions

The right-click 'revert' options below can be used to selectively undo any of the option choices you have made.

- 'revert to name sort' removes the numerical sort on the values of column or row (reverts to the default alphabetical order of the column or row header labels), and causes the lower-left blue marker in the header cell of the column/row that had been used to sort to disappear. You don't have to click on the specific column/row that was the object of the sorting to get this option; the 'revert to name sort' from another column/row would have the same effect.
- 'auto show column/row' reverts any show or ignore filters that may have been applied to the column or row on whose header cell you right-clicked. If that column/row was also used for sorting, sorting is not effected.

- 'auto show all columns/rows' reverts all show or ignore filters that may have been applied to any column or row (without removing any sorting that may have been applied to the table).

If you want to undo all the sorts and filters you applied (*i.e.* restore all defaults), use the 'Reset table' button.

Button	Name – Description
	The '**Reset table**' button removes all sorting and ignore/show filters that have been applied to the table and restores the table to its state prior to the filters. Note that this does not affect any of the table formatting options from the Variant data display controls (see section 1.5.2), including the Min/Max filter settings (section 1.5.2.3).

1.5.1.3 Populating the Global Align Tab from the Variants Tab

As described above, right-clicking on a column or row header cell in the Variants Frequency Table opens a contextual menu with the sorting and filter options. By contrast, right-clicking on a cell in the 'body' of the Table opens a contextual menu that allows you to populate the Global Align tab with the multi-alignment of the reads belonging to the Sample-Variant combination whose Variant frequency information the cell contains. This is the best way to initiate your exploration of the underlying reads if you are specifically interested in results for a given Variant, as it will selectively include reads from all Amplicons that cover that Variant (and that are associated with that Sample). Such a selection could not be done directly from any of the tree views (see sections 1.3.1 and 1.6.1) because these only allow you to choose a single Sample-Amplicon pair. When you navigate to the Global Align tab from a specific Variant on the Variants Tab, the position of the alignment containing the leftmost position of the Variant Pattern is highlighted to help you visually key in on the variation.

1.5.1.4 Defining a Haplotype from the Variants Tab

Right-clicking on a cell in the columns underneath the Variant or Max headers, or right-clicking on a Sample/Variant intersection cell, reveals a "Define Haplotype" option (Figure 1-49 **F** and **G**) in the contextual menu. This option uses a multi-row selection of individual Variants in the Variant Frequencies Table to propose a new Variant that requires that all the selected Variants be found together as a haplotype. This option is grayed-out and inactive unless rows for two or more Variants from the same Reference are selected (a multi-selection of Variant rows from mixed References will inactivate the option even if any of the selected Variants share a

Reference). After selecting two or more such Variant rows, right-clicking over any cell in the selection except for a Reference cell provides access to an active “Define Haplotype” option (Figure 1-51).

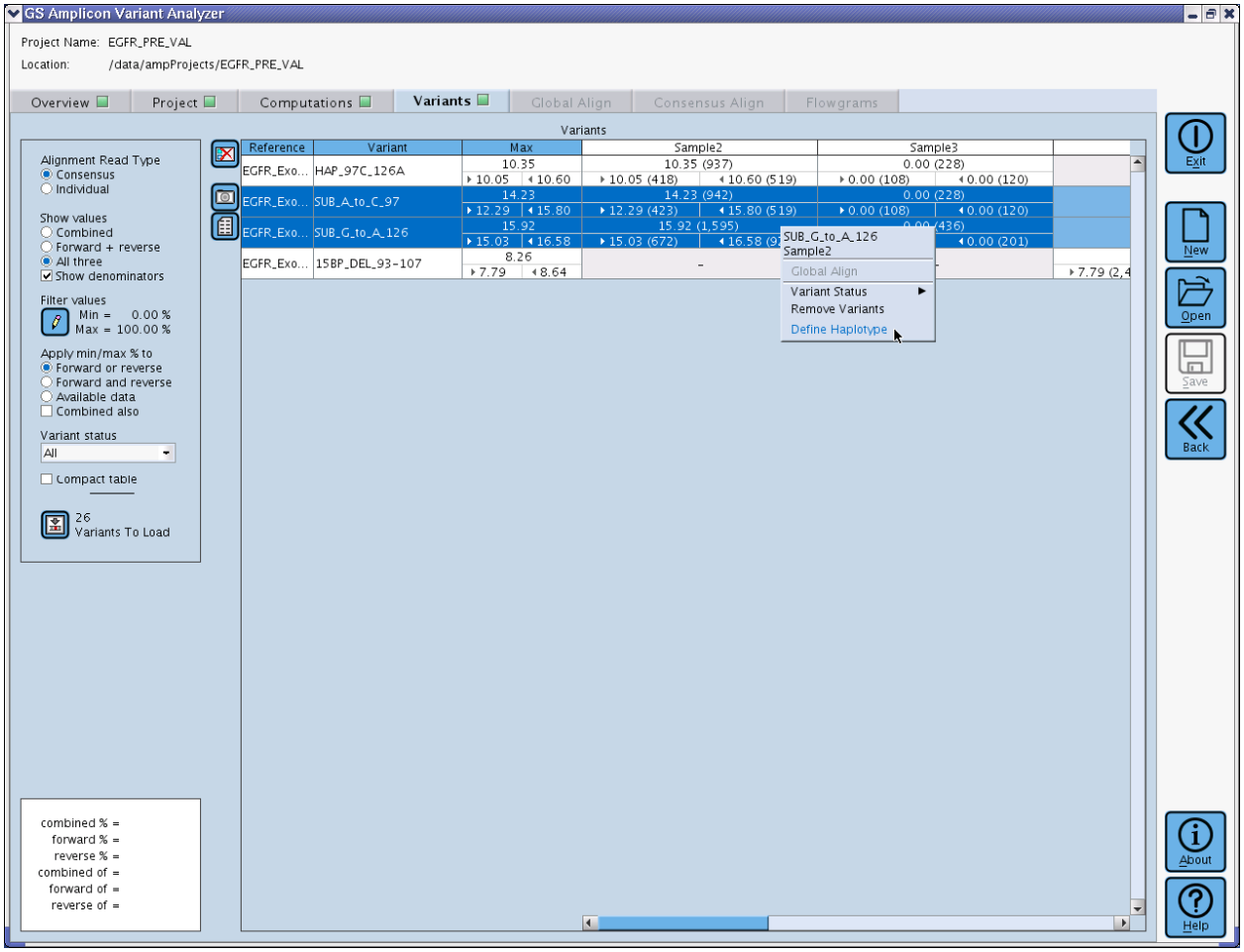


Figure 1-51: The Variants Frequency Table with two individual Variant rows selected. Right-clicking over a non-Reference cell in the selection provides a contextual menu with an active “Define Haplotype” option that can be used to propose a new Variant that requires the selected Variants to be found together as a haplotype.

Choosing the option will bring up the “Define Haplotype” window (Figure 1-52). This window functions similarly to the “Approve new variant” window used by the “Declare project variant” function of the Global and Consensus Alignment tabs (see section 1.6.3.3, Figure 1-62, and Figure 2-36, below). The main difference between the use of “Define Haplotype” and “Approve new variant” is a matter of context. The “Approve new variant” function is triggered in the context of a multiple alignment where the linkage of Variants in a haplotype can be visually verified. The “Define Haplotype” function is triggered from the main Variant Tab wherein similar Variant frequencies may be suggestive of a haplotype, but which may also simply be coincidental. Accordingly, the “Define Haplotype” window defaults to creating the haplotype with a “Putative” Status.

If the Variants selected as the haplotype constituents have any contradictory elements (*e.g.*, specifying two different SNPs for the same position or specifying both a SNP and a deletion for the same position), then a window similar to that encountered when the user enters erroneous variant patterns (Figure 1-29) will be displayed to explain the problem. The user then has the ability to edit the resulting pattern and create a semantically correct haplotype definition; more likely, however, one would select the Cancel button since any identified errors would actually disprove the coincidental existence of the Variants as a valid haplotype.

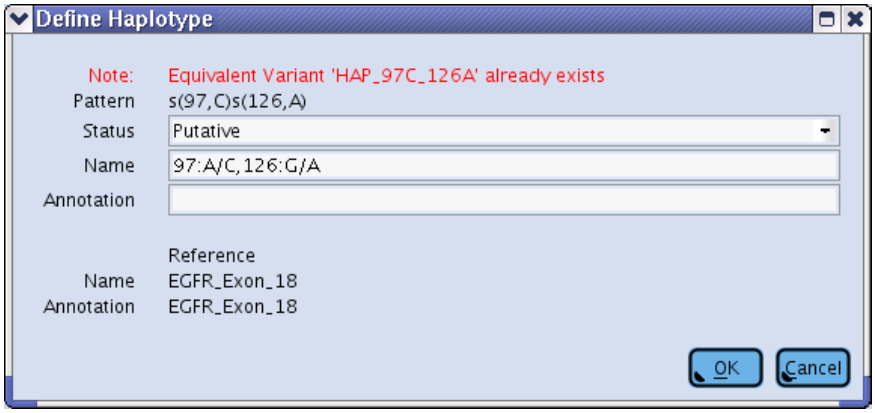


Figure 1-52: The "Define Haplotype" window with a Pattern built from the Variant selections made in Figure 1-51 . The Status defaults to Putative but can be edited, and a red warning is given to indicate that a variant with the same pattern already exists.

The Auto-Detected Variant system does not propose haplotypes (beyond the special case of multiple base pair deletions), so haplotypes must be entered into the system by the "Define Haplotype" or "Approve new variant" methods, or they must be defined from scratch in the Variant Definition Table. In large projects it may become difficult to keep track of all the proposed haplotypes that have already been entered into the system, so if an attempt is made to use the "Define Haplotype" or "Approve new variant" functions to propose a haplotype that already exists, a red warning message stating the redundancy will appear in the window (Figure 1-52).

1.5.1.5 Editing/Removing Variants from the Variants Tab

Right-clicking on a cell in the "body" of the Variants Frequency Table, at a Sample/Variant intersection (as above in section 1.5.1.3 and as shown in Figure 1-49 **G**), provides a contextual menu that includes options for editing the Variant Status and for removing Variants from the project. The same functions can be carried out using the Variants Definition Table in the Variants sub-tab of the Project Tab (see section 1.3.2.5). The Variants Frequency Table is a convenient place to decide on the Status of a Variant because the incidence frequency across Samples is available for examination. You can use the shift or control keys to help select multiple rows and you can delete them or adjust their Status as a bulk operation. In the case of removing Variants you are provided with a "Yes to All" confirmation window (similar to the case described in section 1.3.2 and Figure 1-16). A bulk Status edit occurs without any confirmation step.

- The Status is applied to the Variant and not a Sample. You can't mark the Status for a Variant to "Accepted" for one Sample and "Putative" for another; the Status is applied globally to the Variant regardless of the Sample(s) in which it is found.
- If you remove a Variant from the Project it gets removed from every Sample column in the Variants Frequency Table, not just from the Sample corresponding to the cell on which you clicked. If the Variants you remove happen to be Auto-Detected Variants, they could be re-imported the next time you load the Auto-Detected Variants (depending on your filter settings for the load). For this reason, it is usually better to mark the Variants' Status to "Rejected" rather than remove them from the Project, until you are sure that you will not be loading any more Auto-Detected Variants into the Project.

1.5.1.6 The Mouse Tracker

The Mouse Tracker on the Variants Tab is slightly more complex than on other tabs because the information it displays is highly context-sensitive:

- If the mouse is over a header cell in the Variants Frequency Table, the Mouse Tracker gives general information about how many different items (Reference Sequences for the 'Reference' column; Variants for all other columns) are present in the column, and how many of these comprise data that meet the current Min/Max filter settings.
- If the mouse is over a specific 'Reference' cell, the Mouse Tracker shows the number of Variants and Samples associated with that Reference Sequence, as well as a count of how many of those Samples comprise data that meet the current Min/Max filter settings.

- If the mouse is over a specific 'Variant' cell, the Mouse Tracker shows how many Samples are in the table for that Variant, as well as a count of how many of those Samples comprise data that meet the current Min/Max filter settings.

- If the mouse is over a specific Sample-Variant cell, the Mouse Tracker shows a set of frequency statistics for that Sample-Variant combination including the frequencies at which that Variant occurred in this Sample among reads in the forward, reverse, and combined orientations; and corresponding denominators (number of reads covering the Variant position) used in these frequency calculations. All these values are shown in the Mouse Tracker even if the setting of the 'Show values' option (see section 1.5.2.2) is more restrictive; this way, you have access to all the information even if you choose a more compact Table view.

- If the mouse is over a specific 'Max' cell, finally, the Mouse Tracker behaves as if you were hovering the mouse over the actual Sample-Variant cell that contains those maximum values. Note that 'Max' cells in the Table do not display denominators even when the 'Show denominators' option is chosen (section 1.5.2.2), but the Mouse Tracker does.

1.5.2 Variant Data Display Controls

A box located in the top-left corner of the Variants Tab contains various display option tools that allow you to control the display of the Variant data in the Variants Frequency Table (Figure 1-53).

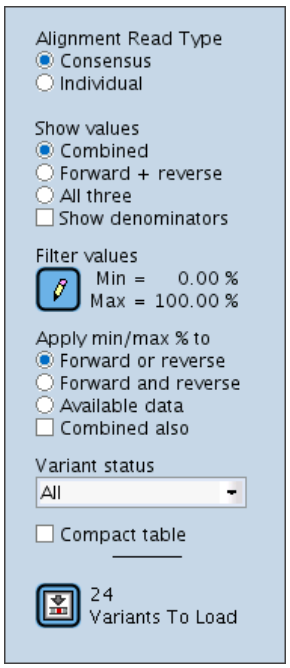


Figure 1-53: The Variant data display control tools

1.5.2.1 The Alignment Read Type Controls

The 'Alignment Read Type' radio buttons allow you to select 'Consensus' or 'Individual'. Consensi are a collapsed representation of multiple similar reads (see section 1.6.4.2) and have a single coverage value over their entire length. The intention of creating consensus reads is to simplify the data analysis and eliminate noise. However, there are sometimes discrepancies in read length within the consensus, making the coverage non-uniform. If a Variant is located in one of the regions of the consensus with lower actual coverage, the Variant frequencies reported with the 'Consensus' option can be misleading. Similarly, if a true variation is misinterpreted as noise, it might be eliminated from all the constructed consensi and a Variant of interest might go unnoticed. Looking at Variant frequencies based on individual reads rather than consensi gives more literal values. It is good to look at and compare Variant frequencies from both types of reads. If the numbers are in close agreement, they bolster one another, but if they are significantly different from each other, you may need to dig into the consensi to get a better understanding of the situation.

1.5.2.2 The Show Values Controls

The 'Show values' set of radio buttons control how to include orientation-specific Variant frequency data in the Table. Variants can potentially be found in reads or consensi of both orientations, but there may be some situations in which the design of your Amplicon libraries or

the combination of Amplicon length and read length is such that certain regions of the Reference Sequence are only covered by reads of a single orientation, and Variants defined in those regions are likewise limited to single orientation coverage. Even when both orientations are available to scan for Variants, there can sometimes be discrepancies between Variant frequencies in one orientation versus the other.

- Choosing the 'Combined' option merges the forward and reverse results together; the Sample-Variant cells in the Table each contain a single Variant frequency value. The Variant frequencies for the reads in each orientation are still calculated, however, and if the AVA software detects a significant difference between the combined value and the individual forward and reverse values, a small down-pointing triangle appears to the left of the value to alert you.
- Choosing 'Forward + reverse' alters the format of the table so that the Sample-Variant cells are divided into two side-by-side sub-cells. The sub-cells show the Variant frequency values broken out by orientation (identified by arrowheads) rather than showing a single combined value.

- Choosing the 'All three' option results in a more complex table format arrangement where each Sample-Variant cell gets subdivided into three sub-cells: the two side-by-side orientation-specific sub-cells are surmounted by the third, 'Combined' sub-cell (see Figure 1-48, above). Again, a small down-pointing triangle appears to the left of the combined value if the AVA software detects a significant difference between the combined Variant frequency value and that of either orientation.

The 'Show denominators' checkbox adds read counts to the Sample-Variant cells as a number in parentheses following the Variant frequency values, in any displayed cell or sub-cells. This allows you to judge the reliability of Variant frequencies based on sample size and can also be of assistance when comparing Variant frequencies by orientation. If one orientation is much more highly represented than the other, you may choose to ignore the value from the underrepresented orientation. All these values (Variant frequencies and number of reads) can also be seen in the Mouse Tracker when the mouse is over a Sample-Variant cell of the Table.

1.5.2.3 The Min / Max Filters

The 'Filter values' controls allow you to set a minimum and maximum Variant frequency on which you want to focus in the Variant Frequency Table: Sample-Variant cells that do not meet the min and max filters are grayed-out, and if any rows or columns are entirely grayed-out, they get moved to bottom or right of the Table; the 'Compact table' option can remove grayed-out rows and columns from view in the Table (see section 1.5.2.4).

- Clicking the 'Change min/max %' button opens the 'Set min / max filter %' window where you can set the minimum and maximum Variant frequency values (percentage) to use as filters, to 2 decimal places (Figure 1-54). Values must be within the range '0.00' to '100.00', and the maximum value must be greater than or equal to the minimum value. Click 'OK' to accept and apply your min/max filter selections. The 'All' button resets the values to a minimum of 0.00 and a maximum of 100.00 (thus All Variant frequency values pass filter).

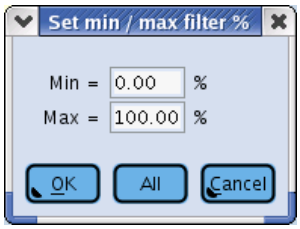


Figure 1-54: The 'Set min / max filter %' window

- The behavior of the minimum and maximum filters is modified by the 'Apply min/max % to' set of radio buttons, located beneath the 'Change min/max %' button. These controls are used to determine what set of Variant frequencies (forward, reverse, and/or combined) for each Sample-Variant cell is used when deciding if the cell survives the min/max filters.
    - 'Forward or reverse' causes the min/max settings to be applied only to the orientation-specific Variant frequency values. *Either* the forward *or* the reverse Variant frequency (or both) must meet both the minimum and the maximum filters for the Sample-Variant cell to survive the filters and remain in the table as a cell with a white background (rather than being grayed-out for failing the filter).
    - 'Forward and reverse' requires that the forward and reverse Variant frequency values *both* meet the minimum and maximum filters independently. If one orientation fails, the cell does not survive the filter and is grayed-out.
    - 'Available data' is a more sophisticated version of the 'Forward and reverse' option. In some cases, you may have intentionally sequenced only a single orientation, or the length of your Amplicon may be such that at the read length provided by the sequencing Run, the forward and reverse reads cannot provide double-orientation coverage in the region where your Variant is located. In those cases, you may not want to penalize a Variant for being represented by a single orientation when it was impossible for it to be represented in both. The 'Available data' option checks to see if there is read coverage from both orientations at the Variant position. If the coverage is all of one orientation, the min and max filters are applied to the Variant frequency value for that orientation. If coverage of the variant position comes from both orientations, both the forward and the reverse frequencies must independently survive the min and max filters as with the 'Forward and reverse' option.
- The 'Combined also' checkbox can be used to also take the 'Combined' Variant frequency value into consideration when applying the min/max filters. If you have chosen the 'Forward or reverse' option, the 'Combined' frequency is treated like another value to be added to the 'or' logic, so if any of the three values meets the min/max criteria, the cell survives. When using 'Combined' with the 'Forward and reverse' or the 'Available data' options, the 'Combined' value becomes another value to be added to the 'and' logic, and all must pass the min/max filter or the cell fails and gets grayed-out.

1.5.2.4 The Variant Status Filter

The "Variant Status" filter is presented as a drop down menu, and its action is cumulative with that of the other filters, described above. The choices available are "All", "Accepted or Putative", "Accepted", "Putative", and "Rejected" (Figure 1-55). Rows that do not meet the Variant Status

criteria get grayed-out and moved to bottom of the Table. This filter can be useful as part of a workflow process for user verification of the data (section 1.5.2.7).

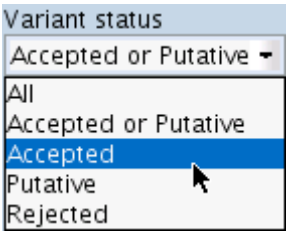


Figure 1-55: The Variant Status filter drop down menu

1.5.2.5 The Compact Table Checkbox

The 'Compact table' checkbox is used to temporarily hide columns or rows that are entirely grayed-out due to a combination of lack of data, the min/max and Variant Status filter criteria, and individual row and column 'ignore' filter settings. This gives a less cluttered view of the data that is a better candidate for the 'Save table snapshot' or Save Table to Text' options. You can uncheck the 'Compact table' option to reveal the rows/columns that have been hidden. In a large project that has accumulated many Samples and Variants, the judicious use of filters combined with the Compact table checkbox allow you to focus the table contents onto a meaningful domain, *e.g.* for preparing a report.

1.5.2.6 The Auto-Detected Variant Load Button

The Auto-Detected Variant Load button is the last control at the bottom of the Variant Display Control box. When you run a computation on the Project, the AVA software attempts to automatically detect potential substitution (SNP) and block deletion variations in the Samples that are processed (see section 1.4), and it creates a list of Variant definitions (without duplicating any Variants already saved in the Project). This queued list of potential Variants can be loaded into the Project by pressing the Load button.

The Load button obeys all the other filters in the Variant Display Control box except the Variant Status filter. The Min/Max filter values are inclusive, so if the Min is set to zero and the Max is set to 100, pressing the Load button would accept all the Auto-Detected Variants surviving the other filters.

The number of unloaded Variants that meet the collective filter criteria is indicated on the right-hand side of the Load button (Figure 1-56**A**). If there are no Auto-Detected Variants that meet the current filter criteria in the queue (*i.e.* that have not yet been loaded), the button is grayed out and the text to the right of the button states this (Figure 1-56**B**). If the Project was last computed with a version of the AVA software that did not support the Auto-Detected Variants feature (versions 1.1.01 and prior), the button is also grayed out and the text to the right states that the Project must be recomputed Figure 1-56**C**).

A B C

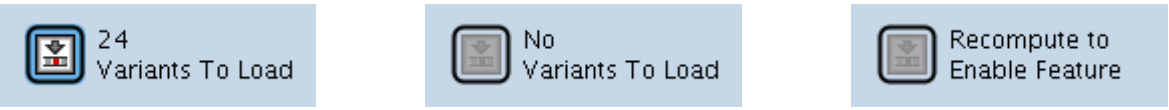


Figure 1-56: The "Variants Load" button in its 3 states: (A) Twenty-four detected Variants currently meet the Variants Tab filter settings, but are not yet loaded into the Project; (B) all Variants that currently meet the Variants tab filter settings (if any) have already been loaded into the Project; and (C) the current Project was computed using an earlier version of the AVA software that did not support Auto-Detected Variants (the Project must be recomputed with the current software in order to enable the Load button).

If the number of Variants to load is high, you can do a partial load by making the filter criteria more restrictive and then deal with the remainder later. For instance, you might set the filters to include Variants with a minimum frequency of 5% with support in both the Forward and Reverse Reads and press the Load button. This would give you the subset of the Variants most likely to hold up to scrutiny. A default Status of "Putative" is assigned to all the Auto-Detected Variants that get loaded into the Project.

Note that the filters in the Variant Display Control box select for Variants that meet the chosen criteria in *some* Sample, not ones that meet the criteria in *all* Samples. Thus, even with a minimum frequency setting of 5%, a Variant will be loaded if it appears at 5% or greater in one Sample, even if it is not observed at all in any of the other Samples.

No progress indicator is provided when loading Auto-Detected Variants. If the filters are set to liberal values such that a very large number of Variants are being loaded, the interface may appear to pause for few seconds during the loading process.

The loading of Auto-Detected Variants into the system is not permanent until you click the "Save" button for the Project. If you close your Project after a Variant load without clicking "Save", the Auto-Detected Variants won't be lost, but they will move back into the queue of the Load button and be available for import again when you reopen the Project. The full set of Auto-Detected Variants that don't have the same pattern as any existing Project Variant is updated every time the Project is computed. In addition, the Load queue is maintained when the Project or the AVA application are closed, so it is not necessary to immediately load Variants after a computation completes.

1.5.2.7 Variant Discovery Workflow

The AVA software provides features that, when combined, provide the ability to manage a Discovery Workflow for identifying and evaluating meaningful variations. The key components of this process are the ability to load automatically detected Variants; the ability to easily set the Status of one or more Variants at a time via a right-click menu item with rows selected in the Variants Frequency Table of the main Variants Tab; and the ability to filter the content of the Variants Frequency Table based on Variant Status.

This constellation of features allows the main Variants tab to be the hub of operations for Discovery Workflow. One can choose to load Variants that have been automatically detected into the Project with the click of a single "Load" button on the Variants tab. If the volume of Variants that are available to load, as displayed to the right of the "Load" button, is large, Project

clutter can be prevented by applying a selection on the Variants to load, via the filters associated with the Variants Frequency Table. For example, you could choose settings such as "Consensus" for "Alignment Read Type", with a Min/Max of 5.00%/100.00% applied to "Forward and reverse" reads. This would allow you to load the subset of Auto-Detected Variants most likely to withstand scrutiny.

For Discovery Workflow purposes the status options have the following intended meanings:

- Accepted – a Variant that is expected to be found in at least one Sample; or a Variant that has previously been found and verified by a user
- Putative – a Variant that is known from the literature but may or may not be found in a Project Sample; or a Variant that has been automatically computed but not verified by a user

- Rejected – a Variant that has been flagged as invalid because a user has determined that it was detected due to some type of artifact, such as from Sample processing or an alignment problem.

Despite these definitions, the AVA software simply treats the Variant Status as a tag that can be used for data filtering. Thus, you can choose to interpret the status values differently, if necessary, to better meet your needs.

Loaded Auto-Detected Variants are automatically assigned an initial Status of "Putative". Any other Variants that are manually defined (section 1.3.2.5.2) or declared via filter selections on Global and Consensus alignments (see sections 1.6.3.3 and 1.7.3) will have defaulted to a Status of "Accepted". Presuming that "Accepted" Variants have already been validated, one can set the "Variant Status" filter of the Variants Frequency Table to "Putative" and click on the "Compact Table" box. This causes any "Accepted" or "Rejected" Variant rows to be grayed-out and demoted to the bottom of the table. The "Compact Table" option then hides those rows so that the only visible rows are those that have a Status of "Putative".

With a Project set up as above, one can begin validating the "Putative" Variants. Right-clicking on individual Sample-Variant intersection cells in the Table allows the use of the "Global Align" link to load the Global Align tab with the alignment of Read Consensi that cover the region of the corresponding Variant of interest. After exploring the underlying alignments and flowgrams to determine if the Variant appears to be legitimate, one can return to the Variants Tab to change the Status of the "Putative" Variant to either "Accepted" or "Rejected". This is done via the "Variant Status" submenu that appears when right-clicking over a Sample-Variant intersection cell. Once the Status has been changed from "Putative", the Variant row will no longer meet the "Variant Status" filter of the table and the row will be automatically hidden because of the active "Compact Table" option. Variants judged as invalid should be marked as "Rejected" rather than deleted entirely. This will both prevent the Variant from being added back to the Load queue, and prevent the automatic Variant detection mechanism from potentially re-proposing the same Variant after the completion of the next computation cycle (which would force you to re-evaluate this Variant each time Variants are loaded).

This method provides a shrinking pool of "Putative" Variants to work with. Eventually, after all Variants have been evaluated, the Table will be empty. If one starts the process with a partial Variant Load, looser filter settings for the Variants Frequency Table can be tried to see if the "Load" button indicates any additional Variants to load. For instance, one might keep the current

filters but just change the “Forward and reverse” option to “Available data”. This would pick up Variants in regions of Amplicons that have coverage from reads of only a single orientation. Or one might try switching the “Alignment Read Type” from “Consensus” to “Individual” to catch any cases where variations are hidden because they were distributed over several Consensi at low enough levels that their changes were not incorporated into the Consensus sequences. By manipulating the filters in such a way, one can fine-tune a new set of “Putative” Variants to load and then load and validate them as before.

Eventually, the filters will be set to as restrictive values as one is willing to go, or to where there are no Variants left to load. To determine if there are any available Variants at all, one can choose the loosest settings with “Alignment Read Type” set to “Individual”, Min/Max to 0.00%/100.00%, with the Min/Max applied to “Forward or reverse” reads. At any point, one can reset the “Variant Status” filter to see “All” the Variants, just those “Accepted”, just those “Accepted or Putative”, or even those “Rejected”, and later switch back to the “Putative” only view to continue working through the list of Putative Variants.

Keep in mind that the automatic Variant detection does not currently report insertions or monomer deletions shorter than 3 bases (one must manually define those types of Variants if their statistics are to appear in the Variants Frequency Table). Also, remember that a Variant load and any changing of Variant Status are not permanent until the Project is saved. This can provide a useful form of “undo” if a Project is accidentally cluttered with an errant load. In such a case, simply re-open the Project without a “Save” in order to restore the Project’s previous list of Variants.

1.6 The Global Align Tab

The Global Align tab allows you to view the underlying alignment information that is used in the calculation of Variant frequencies. It is divided into two results panels (Figure 1-57): the top panel contains a stacked histogram / depth of coverage plot of all the variations observed in the reads included in the last computation, relative to the Reference Sequence while the bottom panel contains the multiple alignment of those reads to the Reference Sequence. In addition, a set of display option tools (for data navigation and filtering) as well as a “Mouse Tracker” display (section 1.1.3.3.3) and color legend for the Variation Frequency Plot are available on the left-hand side of the tab. The Global Align tab can only display data for one Sample-Reference Sequence combination at a time, but it can display data for multiple Amplicons together, provided that they are all associated with both this Sample and this Reference Sequence.

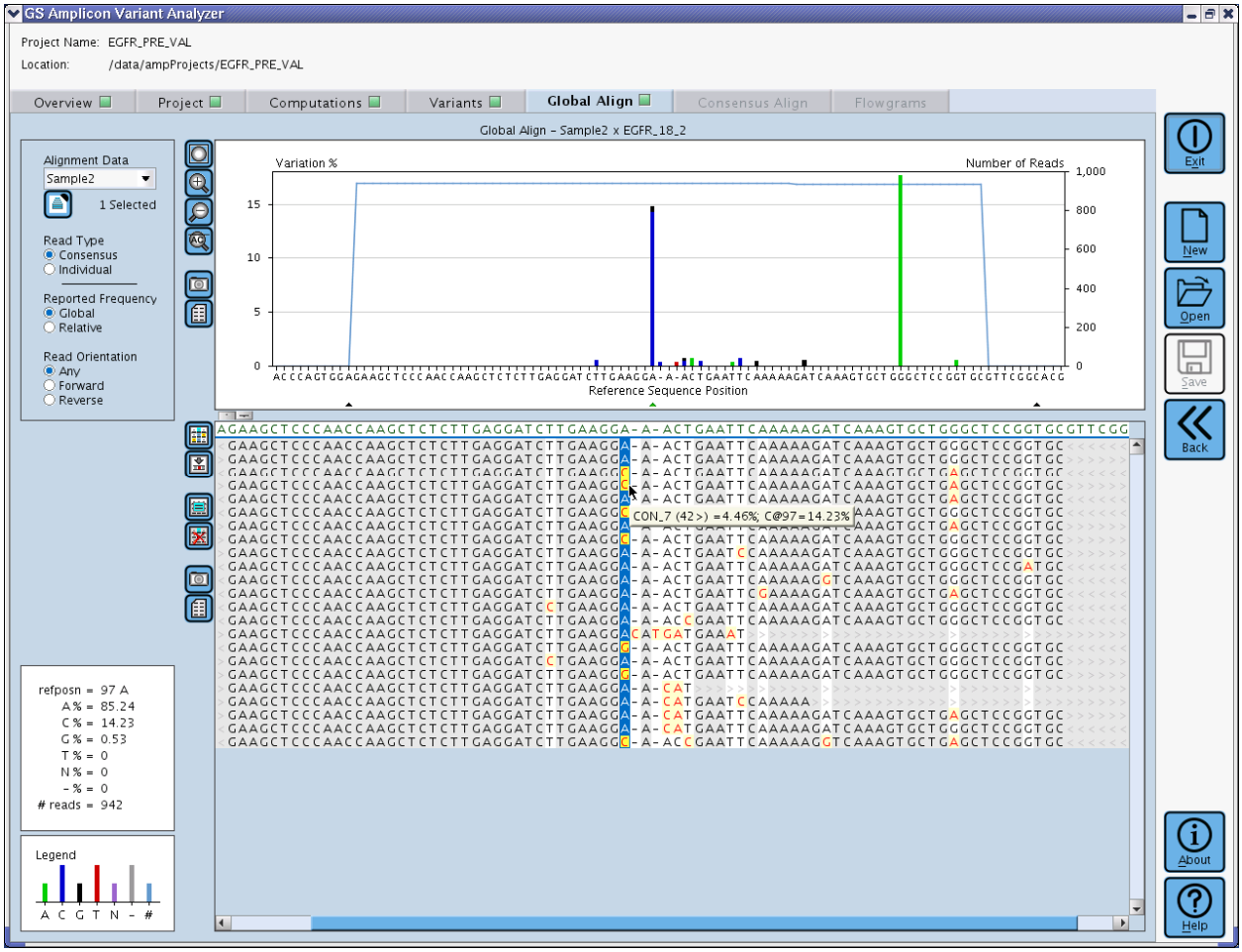


Figure 1-57: The Global Align tab

1.6.1 Populating the Global Align Tab

When you open an Amplicon Project in the AVA software, the Global Align tab has no content and is grayed-out. To populate it from this state, you must use a right-click “Global Align” action from one of the following two sources:

- A Sample-Amplicon pair, from any of the 3 Project Tree views, on the Project Tab [see sections 1.3.1.1, 1.3.1.2, and 1.3.1.3; note that the Amplicon must be fully defined (section 1.3.2.2) and the computation must have been carried out (section 1.4)]. Right-clicking on a Sample (in the References Tree) or an Amplicon (in the Read Data Tree or the Samples Tree) opens a contextual menu that includes a “Global Align” option; choosing this will populate the Global Align tab with the multi-alignment of the reads for the Sample-Amplicon pair on that branch of the tree.
- A Sample-Variant pair, from the Variants Table, on the Variants tab [see section 1.5.1.3; note that the Variant must be fully defined (section 1.3.2.5) and the computation must have been carried out (section 1.4)]. Right-clicking on an appropriate cell of the Variants Table opens a contextual menu that includes a “Global Align” option; choosing this will populate the Global Align tab with the multi-alignment of the reads of all the Amplicons

that cover this Variant on the Reference Sequence and that are associated with this Sample.

Once the Global Align tab is populated, you can still use the right-click method above, and replace the multi-alignment displayed with another one. But from inside the Global Align tab, you have another, more powerful option: this tab has two “Alignment data” controls in its upper-left corner that allow you to browse through and navigate all the alignments of your Project without leaving this tab, and even to view the data for multiple Amplicons together (for a given Sample-Reference Sequence pair). These controls are described in detail in section 1.6.4.1.

1.6.2 The Variation Frequency Plot

The Variation Frequency Plot (Figure 1-58) located in the top panel of the Global Align tab shows graphically all the variations (relative to the Reference Sequence) observed in the reads included in the last computation and associated with the Sample-Reference Sequence combination and the Amplicon(s) selected, and the depth of coverage at each position of the multi-alignment.

- The horizontal axis represents the Reference Sequence, gapped as needed to accommodate any insertions in the reads.
- The left vertical axis shows the percentage frequency of the variations, whereby individual variants are represented as colored bars keyed to the legend at the bottom-left of the tab for each position of the Reference Sequence; if more than one variation occurs at any given position, the bars are stacked vertically.

- The right vertical axis shows the depth of coverage for each Reference Sequence position, in number of reads, shown on the plot by a light blue line.

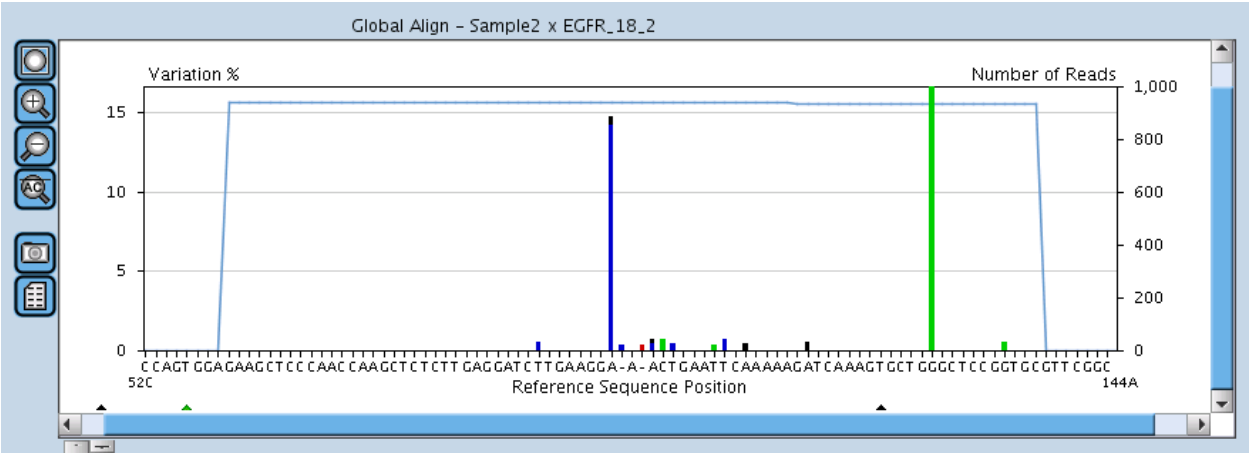


Figure 1-58: The Variation Frequency Plot. In this example, the plot was zoomed in to show the Reference Sequence along the horizontal axis.

The plot has all the standard navigation features (scrollbars, zoom buttons, mouse tracker, *etc*.) described in section 1.1.3.3. In addition, the Variation Frequency Plot and the multiple alignment of the bottom panel are reciprocally linked, in the following ways:

- Three small triangles (two black and one green) located at the bottom of the plot panel have the following meanings:
    - The black triangles indicate the boundaries of the subset of the plot that is visible in the multi-alignment panel.
    - The green triangle shows the position in the plot that corresponds to the highlighted nucleotide in the alignment.
- Clicking in either the plot or the multi-alignment panel centers the other panel on the position clicked.

    - If you clicked on the plot, the nucleotide column in the multi-alignment is also highlighted.
    - If you clicked in the multi-alignment, the position of the tracking triangles on the Variation Frequency Plot is also adjusted accordingly.

1.6.3 The Multiple Alignment Display

The multiple alignment display (Figure 1-59) located in the bottom panel of the Global Align tab shows the alignment of all the reads selected, to the Reference Sequence. These reads may be grouped into consensi and/or selected for certain observed variations (see below). Scrollbars appear when necessary, and the Mouse Tracker and Screen Tip features (1.1.3.3.3) are also active in the multiple alignment display.

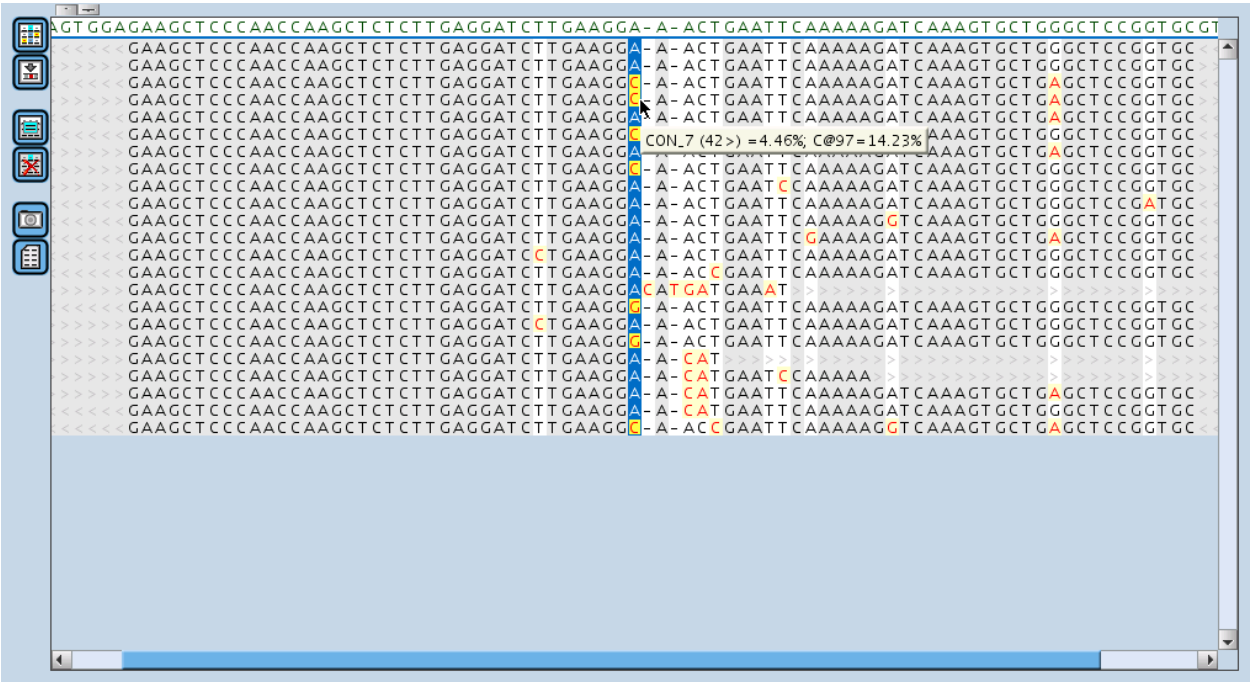


Figure 1-59: The multiple alignment display of the Global Align tab

1.6.3.1 The Reference Sequence

The Reference Sequence runs along the multiple alignment display's top strip and is shown in green characters. Pausing the mouse over the Reference Sequence displays a screen tip showing the position of the nucleotide under the pointer.

Gaps ('-') in the Reference Sequence indicate virtual positions where one or more aligned reads have insertions. These insertion positions are numbered with decimals based on the Reference Sequence position to the left of the insertion (*e.g.* a two nucleotide insertion between Reference Sequence positions 362 and 363 will be labeled positions 362.1 and 362.2). Do not confuse the decimal positions used in the multiple alignments and those used in specifying insertions in Variant Patterns (section 1.3.2.5.2). In Variant Patterns, the location of an insertion of any length between two specific Reference Sequence positions *p* and *p+1* is always noted as "*p.5*". In a multiple alignment, by contrast, each inserted nucleotide is given its own "virtual" decimal position identifier.

Note that only gaps that correspond to inserted nucleotides *in the reads or consensi displayed in the multi-alignment below* are shown in the Reference Sequence. If you apply selection(s) to the reads or consensi (using a right-click 'Select' option or the 'Assemble consistent reads' button; see section 1.6.3.2, below), some of the inserted nucleotides may not be represented in the remaining reads; those gaps will not be shown in the Reference Sequence. However, their decimal coordinate number will be maintained in the alignment, such that the decimal number of the gaps displayed may not always be consecutive. (This also applies to the display of the reads from a single consensus on the Consensus Align tab, which is another form of read selection; see section 1.7.)

1.6.3.2 The Multi-Alignment

Below the Reference Sequence are the aligned reads (or consensi). Initially, the 'Read Type' display control (from the display option tools in the upper-left corner of the tab) is set to 'Consensus', whereby the aligned reads are grouped into consensus representations of reads which are substantially similar to each other. If 'Individual' is chosen instead, all the individual underlying reads are displayed. The Read Type setting is maintained within the session as you navigate from alignment to alignment. See section 1.6.4.2 for a more complete description of these display options.

The multiple alignment display has the following functions and features:

- The beginning and ending of reads or consensi that don't start at the first or end at the last alignment position are filled with light gray '>' or '<' characters. These characters are indicators that the sequencing reads are 'forward' or 'reverse', respectively, relative to the Reference Sequence.
- The background color of the alignment columns provide an at-a-glance way to focus on the positions that may be of most interest:
    - Gray columns are tagged as uninteresting because all the reads or consensi match the Reference Sequence at that position.
    - White columns, by contrast, contain at least one read or consensus that differs from the Reference Sequence and are thus worthy of attention.

- In the white alignment columns, the specific nucleotides that do not match the Reference Sequence are shown in an eye-catching red on yellow background, while the matching ones are black on white.

- Pausing the mouse over a nucleotide in the multi-alignment displays a screen tip (Figure 1-60**A,D**) that provides:

    - the name of the read or consensus
    - the number of reads represented in the consensus (always 1 if 'Read Type' is set to 'Individual'; see section 1.6.4.2) and its orientation

    - frequency information (subject to the 'Global' or 'Relative' selection made in the 'Reported Frequency' tool; see section 1.6.4.3), as follows:

        - the proportion (as %) of the reads represented by this read or consensus

- if the 'Read Type' control is set to 'Consensus', the consensi are sorted in decreasing order of the number of reads they comprise

- if the 'Read Type' control is set to 'Individual', obviously, all the reads will display the same frequency

        - the proportion (as %) of the reads that have this nucleotide at this position

- Left-clicking on a nucleotide in the Reference Sequence or any of the aligned sequences highlights the column. The highlighting switches reference-matching nucleotides to white lettering with a dark blue background while mismatches remain as red letters, but the background switches to yellow with blue left and right edges. As seen above (section 1.6.2), this also centers the Variation Frequency Plot on the coordinate clicked, and places the green tracking triangle underneath it.

- Right-clicking on a nucleotide in the multi-alignment display opens a contextual menu like the one shown in Figure 1-60**B,E**.

    - The first option will depend on the setting of the 'Read Type' control:

        - If set to 'Consensus', the first item will be 'Open Consensus Alignment' (Figure 1-60**B**); this action will take you to the Consensus Align tab (see section 1.7) and populate it with the multi-alignment of the reads that are included in the consensus on which you clicked.
        - If set to 'Individual', the first item will be 'Open Flowgrams' (Figure 1-60**E**); this action will take you to the Flowgrams tab (see section 1.8) and populate it with the tri-flowgram corresponding to the read on which you clicked and focused on the flow corresponding to the base on which you clicked.

    - The option at the bottom of the menu, called "Properties" pops up a new window displaying specific sequence information of the consensus or read on which you right-clicked. The data is presented in a format that allows you to copy information to the clipboard so you can export it to external programs for further analysis. For further details about the data available in the properties menus and for suggestions on how the data can be useful, see section 4.3.

- The rest of the options allow you to restrict the display to the reads or consensi that contain a specific selected nucleotide (or a gap) at the position on which you clicked. When you make a selection, the corresponding nucleotide in the Reference Sequence is highlighted with a cyan background color, and all reads that do not contain the selection are hidden from view. You can make multiple successive selections in a multiple alignment, at one or more positions, further restricting the number of reads or consensi displayed at each selection. This can be useful to explore linkage between variations in the read data.

If following your selections a given position consists only of gaps (including in the Reference Sequence), this gap position will be removed from display; this results in a more compact and more readily understandable alignment. Because of this collapsing of gapped columns, the decimal "virtual" positions in the Reference Sequence, while always increasing, may not always be consecutive in a "selected" multi-alignment display (see section 1.6.3.1 for more details on decimal position numbering in a gapped alignment). This also applies to the display of the reads from a single consensus on the Consensus Align tab, which is another form of read selection, and whose view features these same selection tools (see section 1.7).

- Right-clicking on a nucleotide in the multi-alignment display at a position that is already the object of a selection opens a contextual menu like the one shown in Figure 1-60**C,F**.
  - The first option is the same as what is seen when no selection is active
  - The Properties option also is the same as what is seen when no selection is active
  - The second (middle) option indicates the currently active selection(s). If deactivated, all reads currently hidden by the selection will be reintroduced into the visible multi-alignment and the cyan highlight at the top of the alignment column will be removed. Selections may be removed from an alignment in any order, regardless of the order in which they were added to the alignment.

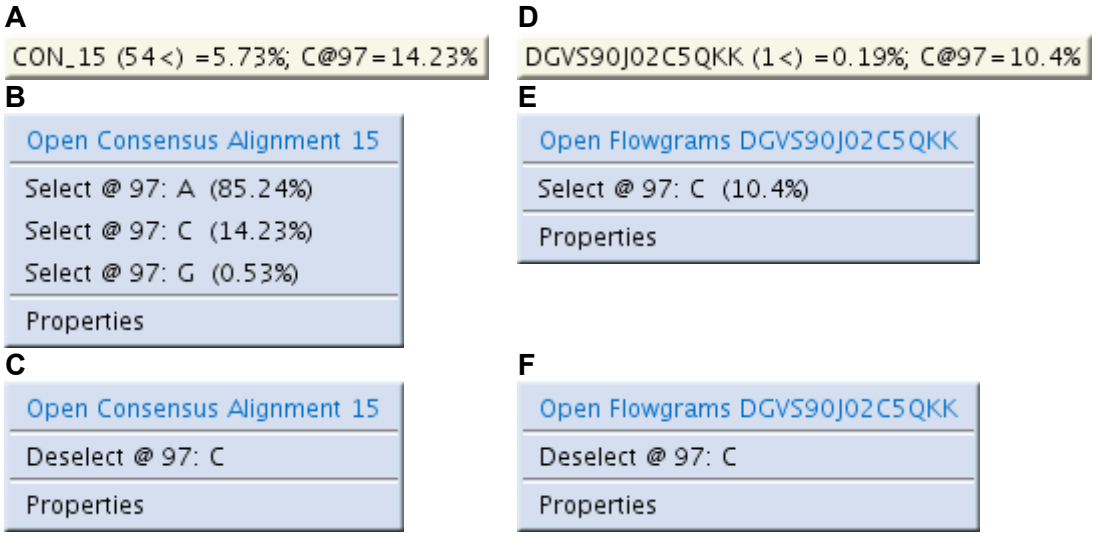


Figure 1-60: Screen tips and contextual menus that can appear in Global Align and Consensus Align tabs. A-C may only be seen in the Global Align Tab when "Read Type" is set to "Consensus". D-F may be seen in the Consensus Align tab or in the Global Align Tab when "Read Type" is set to "Individual". (A, D) The screen tip displayed when you pause the mouse over a nucleotide in the multi-alignment. (B, E) The contextual menu that opens when you right-click on a nucleotide in the multi-alignment. (C, F) The contextual menu that opens when you right-click on a nucleotide in a multi-alignment that is already the object of a selection.

1.6.3.3 Special Function Buttons

Various advanced functions can be carried out using the special function buttons located to the left of the multiple alignment. These can help you explore and exploit the reads or consensi displayed in the multi-alignment, to identify (or "declare") variations you believe to be valid Variants (see section 2.4 for guidelines and factors to consider when trying to determine whether a Variant is genuine).

Button	Name – Description
	**Deselect menu** – Every time you use a right-click 'Select' option on a nucleotide in the multiple alignment (and also when you use the 'Assemble consistent reads' function, below), your selections are added to a list. Clicking this button opens the "Remove Selections" window showing the list of selections, sorted by reference position, and allows you to remove any or all of the selections (Figure 1-61). As the selections are removed, the sequences hidden by those selections will be added back to the multiple alignment.

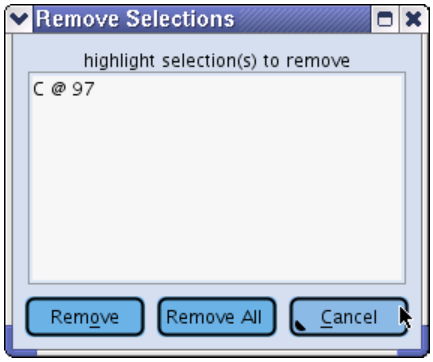


Figure 1-61: The Remove Selections window

This function is critical to removing selections that correspond to gaps in the Reference Sequence. If some reads contained an insertion relative to the Reference Sequence, and a '-' was selected by the user at that position, then the reads with the insertion would be eliminated from the display. At that time, the multiple alignment of the remaining reads would all have a gap character at that position. Since the AVA software automatically collapses from the display any columns that consist entirely of gaps, that gap position of the alignment would be removed and there would be no cyan indicator at the top of the alignment that the '-' selection was performed. The only way to remove the '-' selection at that point would be through the Deselect menu.

Button	Name – Description
	**Declare project variant** – Clicking this button takes the current set of 'Select' choices you made on the multi-alignment, and converts it into a new Variant on the Variants sub-tab of the Project Tab; this Variant will then be searched for during the next computation, with reporting of the search results in the Variants Tab. An "Approve new variant" window (Figure 1-62) will first open and show how your 'Select' choices have been converted into a valid 'Pattern' compatible with the Variant scanning function; the 'Reference' Sequence will have been determined based on the alignment you were viewing. The window also has fields in which you can select a Status for the Variant from a drop down (defaults to Accepted) and in which you can enter a Name and an Annotation for the new Variant, before accepting it. If the Pattern is equivalent to that of a Variant already defined in the Project, the window will display a warning of this fact to help prevent the incorporation of a redundant Variant. Keep in mind that during Variant scanning, a read must overlap all the positions involved in the Variant to qualify as containing the Variant, so the 'Select' choices should be as compact and succinct as possible before declaring a Variant. Note also that there must be at least one 'Select' choice made prior to clicking the 'Declare project variant' button or the 'Approve new variant' window will not open. In some cases, the current selections may be close to, but not exactly, the Variant Pattern you want to use to define the new Variant. The "Approve new variant" window does not, however, let you edit the synthesized Variant Pattern. In this case, you should approve the addition of the Variant to the Project and subsequently edit it in the Variants sub-tab of the Project Tab (section 1.3.2.5). Since possible Variants are automatically proposed by the AVA software, potentially in quite large numbers, the software attempts to provide meaningful but unique default names (see section 4.2). This also applies to Variants declared manually, via the Approve New Variant window.

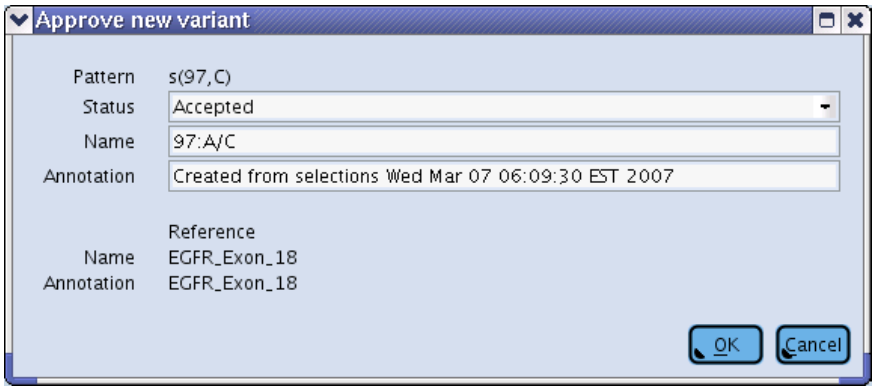


Figure 1-62: The Approve new variant window.

Button	Name – Description
	**Assemble consistent reads** – This button provides a means of mining for consistent patterns out of the sequences in the multiple alignment. “Consistent reads” means reads that are identical in the portion over which they overlap (*i.e.* overhanging nucleotides due to reads of different lengths do not penalize the “consistency”). This is more useful when the ‘Read Type’ is set to ‘Individual’ as opposed to ‘Consensus’ (in which case the consensus process has already gathered up similar reads). Using the reads on display in the multiple alignment as input (but not those already hidden away by ‘Select’ choices), the assembly process makes a set of automated ‘Select’ choices to identify sets of consistent reads for display. This is typically used in conjunction with the ‘Remove reads’ button discussed next, as a means to recursively mine for patterns in the alignment. As you identify patterns in the sequence variations, you can discard those sequences temporarily and search through the remaining sequences for additional variation patterns. There isn’t a direct undo operation after a round of assembly, but you can use the ‘Remove all’ option of the ‘Deselect menu’ button (see above) to wipe out the selections made by the assembly process (along with any other selections you may have made prior to the assembly). When you normally add selections to alignment positions, only those reads that explicitly have the selected nucleotide (or gap) remain visible; in particular, reads that do not extend all the way to the selected column are not given the benefit of the doubt that they might have matched that position, and are therefore hidden from view. Since the “Assemble consistent reads” action only requires agreement in the areas of overlap, the automatically generated ‘Select’ choices behave differently and allow reads to remain so long as they are consistent with the selections in the columns for which the read has coverage. Following an Assembly operation, the selection mechanism stays in this more “inclusive” mode even for additional selections or de-selections made via a right-click by the user. The selection mode is made clear to the user by having an ‘Inclusive Select’ rather than simply a ‘Select’ menu item appear in the right-click contextual menu. The AVA software reverts to the original non-inclusive behavior as soon as all the selections have been removed (either via the “Deselect menu” or by using the “Remove reads, reselect selections” button described next).
	**Remove reads, reset selections** – Clicking this button takes all the displayed sequences in the multiple alignment to the right and discards them from memory. The full set of alignment position filters is cleared, and any remaining sequences that were hidden by prior selections are revealed in the alignment. This button is typically used in conjunction with the ‘Assemble consistent reads’ button described above, as a means to recursively mine for patterns in the alignment. Once you click this button you cannot undo the sequence discard, but the sequences are only discarded from memory and not from the underlying multiple alignment. Reopening the global alignment via the Project Trees or the Variants Tab, or using the “Alignment data” display control tools (section 1.6.4.1), will reload the original alignment and restore the full complement of reads or consensi. (Similarly, if you are in the Consensus Align tab, you can restore the full complement of reads by returning to the Global Align tab and re-loading the same consensus; see section 1.7.)

	**Save table snapshot to image file** – This button saves the visible portion of the multiple sequence alignment at the right as a PNG image file. A file browser window will open, allowing you to assign a name and a destination for the file.
	**Save the alignment as ...** – This button takes the multiple sequence alignment at the right and writes it out as a FASTA, CLUSTAL, ACE or Table formatted file, so you can import it into a suitable third-party application. A file browser window will open, allowing you to choose the file type. A filename with the appropriate extension is automatically generated, but you are free to rename it. You should maintain the standard file suffixes since some applications will expect them when importing the file.

1.6.4 Display Option Tools

The upper-left corner of the Global Align tab contains various navigation and filtering tools that modify the information displayed on the Variation Frequency Plot and the multiple alignment panels of the tab (Figure 1-63).

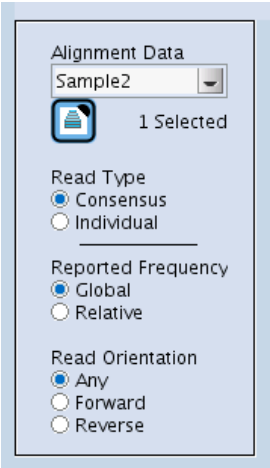


Figure 1-63: The display option tools of the Global Align tab

1.6.4.1 Alignment Data

There are two navigation controls located at the top of the display option tools box, that allow you to select new sets of data to display in the Global Align tab. The first is a drop down menu that contains a list of all the Samples defined in the Project that are associated with at least one of the Amplicons you are viewing in the multiple alignment currently displayed. Selecting a new Sample from the drop down menu will update the 'Global Align' tab with the alignment data for the new Sample, replacing the current data. This allows you to quickly compare various Samples over a single or a given set of Amplicon(s).

The second Alignment data control is the Amplicon selection button, located just below the 'Alignment Data' drop down menu (Figure 1-63). Clicking this button opens the "Choose Alignment Data" window (Figure 1-64).

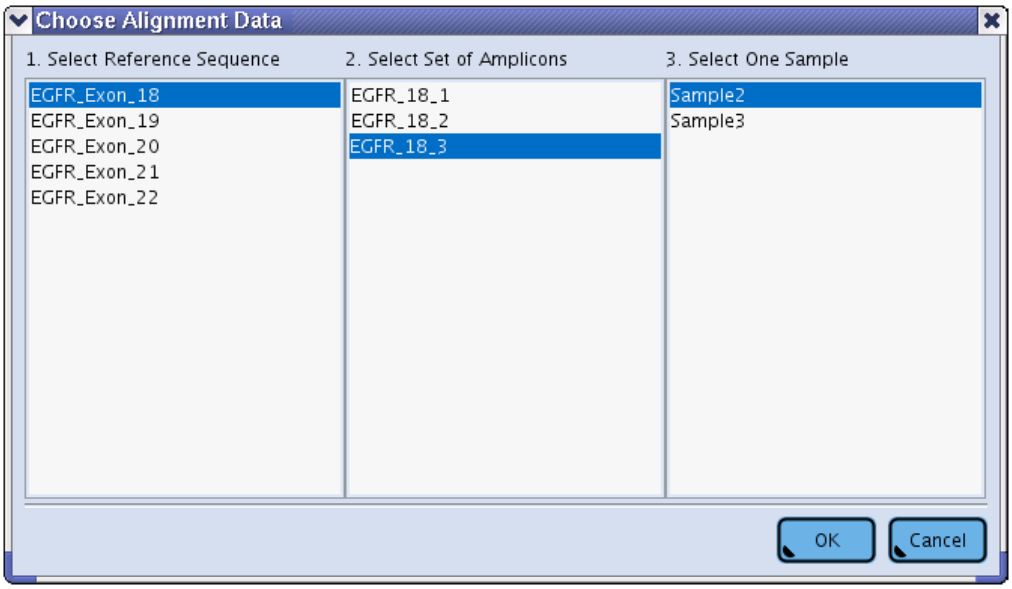


Figure 1-64: The Choose Alignment Data Window

This window allows you to browse over the entire Project and select data for display in the Global Align tab. It is used in three steps:

- Step 1: choose a Reference Sequence for which you want to display the data. This will update the list of available Amplicons, in the second column, to those that are associated with that Reference Sequence and for which there is an alignment computed for at least one Sample (excluding, however, Amplicons associated with the Reference Sequence selected but for which no Read Data sets have supplied any reads).
- Step 2: select one or more of the available Amplicon(s) of interest. The Global Align tab can display the reads from multiple Amplicons, merged into a single multi-alignment, as long as they all belong to the same Reference Sequence. This selection will update the list of eligible Samples, in the third column, to those that are associated with at least one of the Amplicons selected and for which there are currently computed results.

- Step 3: select one of the eligible Samples, and click “OK” to load the selected alignment in the Global Align tab. The number of Amplicons included in the displayed multi-alignment is indicated to the right of the Amplicon selection button.

If you activate the “Choose Alignment Data” window and click “OK” without changing any of the currently selected choices, the AVA software will freshly reload the currently selected alignment. This can be useful to reset the view of the alignment after using the “Remove reads, reset selections” action (see section 1.6.3.3)

1.6.4.2 Read Type

The ‘Read Type’ radio buttons (see Figure 1-63) give you the choice to display the reads in one of two ways:

- Consensus: This is the default read type. When this option is selected, reads that are substantially similar are grouped together into consensi, which results in fewer sequence entries in the multi-alignment table. The consensi are listed in the multi-alignment in decreasing order of the number of reads they represent, so the ones near the top have the more "weight". This option is intended to reduce noise and speed up navigation.
- Individual: This option displays every read as a separate sequence line in the multi-alignment, even if they are identical to other reads. This can greatly increase the volume of alignment lines and slow navigation, but hides no noise.

It is usually easiest to perform an initial analysis with the default Consensus view, with its lower volume and decreased noise. Delving into the individual reads can be useful if you need to search for a particular variation that may have been erroneously spread amongst several consensi, treated as noise in basecalling, rather than being exposed as a separate variation.

1.6.4.3 Reported Frequency

The next set of radio buttons controls the type of 'Reported Frequency' (see Figure 1-63). This applies to:

- the Variation Frequency Plot left axis (which relates to the histogram bars)
- the information reported in the mouse tracker panel
- the frequency information for a nucleotide, in the screen tips that appear when you pause the mouse over a nucleotide in the multi-alignment panel

- the frequency information for the nucleotide selection options, in the contextual menu that appears when you right-click on a nucleotide in the multi-alignment panel

- the reported read depth in all of the above

The two options are as follows:

- The default 'Global' frequency option uses the coverage from the full data set as the read depth denominator when calculating the frequency of occurrence of a given nucleotide at a given alignment position, regardless of any positional 'Select' filters that may have been applied to hide reads (or consensi) from the multi-alignment display. (This also applies to the display of the reads from a single consensus on the Consensus Align tab, which is another form of read selection; see section 1.7.)
- The 'Relative' option recalculates frequencies using only the visible data, *i.e.* ignoring reads (or consensi) hidden from the multi-alignment display after you applied any selection(s). This can be useful when you have selected reads (or consensi) for a variation at a given coordinate and you want to examine other variations relative to the first selection(s) (now set at 100%); for example, variations linked as a haplotype should show near 100% relative frequency in this situation. (This is also useful when examining the reads from a single consensus on the Consensus Align tab, which is another form of read selection; see section 1.7.)

If you did not make any 'Select' filter choices on alignment positions, the 'Global' and 'Relative' frequencies will be the same (but not so on the Consensus Align tab, where all results displayed

are inherently selected for a single consensus; see section 1.7). Once you make selections to focus on a subset of the data, you will notice the difference between the reported frequency types. Note that the reported read depth is that used in the frequency calculations. So, if you want to know how many reads are present amongst the selected reads of the multiple alignment, you must switch to 'Relative' frequencies.

1.6.4.4 Read Orientation

The final set of radio buttons controls the display of the reads or consensi by 'Read Orientation' (see Figure 1-63).

- The default is 'Any' which means both forward and reverse reads are presented in the multi-alignment.
- Choosing 'Forward' or 'Reverse' will restrict the multi-alignment view to display only the reads of the selected orientation. This can be useful when you have coverage of a variation in reads from both orientations; the presence of the variation at a similar frequency in both orientations would be a strong argument that it is "real", whereas its presence in only one orientation (or a large discrepancy in its frequency between the two orientations) would be an indication that the variation might be due to an artifact. (See section 2.5 for guidelines and factors to consider when trying to determine whether a Variant is genuine).

In a restricted orientation view, the behavior of the 'Global' reported frequency option is slightly modified so that the coverage in the denominator comes from the full data set, but restricted by orientation. This prevents the global frequencies from deceptively dropping by about 50% when an orientation is chosen. As mentioned in the description of the Variants Tab (section 1.5.2.2), failure of a variation to show in one orientation for which a good number of reads are available is an indication of a possible artifact; however, you may want to not "penalize" a variation on this account if it is covered in only one orientation, or if too few reads cover it in one orientation to provide for statistically valid data.

1.7 The Consensus Align Tab

The Consensus Align tab (Figure 1-65) is useful when you need to see the individual reads comprised in a consensus (from the Global Align tab), to evaluate the variations that the software has considered "noise". This removal of "noise" is what allows the Global Align tab to simplify the display of the Project's data by collapsing groups of similar reads into consensi, when its Read Type control is set to "Consensus" (see section 1.6.4.2). The Consensus Align tab, thus, is very similar to the Global Align tab, except that it displays the multi-alignment of the individual reads that comprised a Global Align tab consensus.

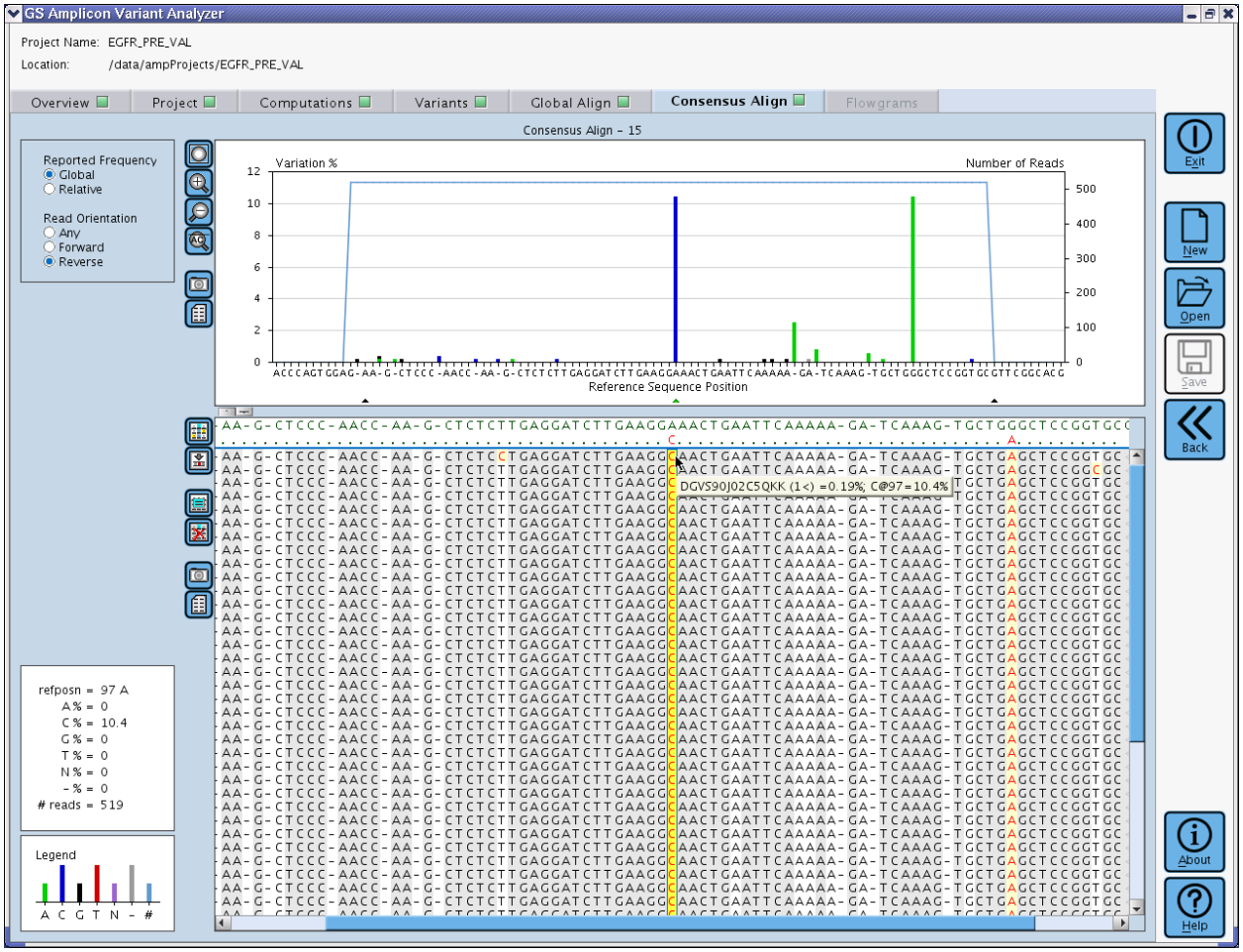


Figure 1-65: The Consensus Align tab

1.7.1 Populating the Consensus Align tab

When you open an Amplicon Project in the AVA software, the Consensus Align tab has no content and is grayed-out. To populate it, go to the Global Align tab, make sure that its Read Type control is set to “Consensus”, and right-click on the consensus whose reads you want to explore in detail. A contextual menu will appear which will include an “Open Consensus” option. Selecting this option will populate the Consensus Align tab with the multi-alignment of the reads that are grouped in the consensus on which you right-clicked.

Since the purpose of the Consensus Align tab is to “drill down” on the reads of a given consensus, as opposed to viewing data from the whole Project, it lacks the “Alignment data” controls that allow you to browse through all the alignments, on the Global Align tab (see section 1.6.4.1). Also, since it always displays individual reads, it lacks the “Read Type” controls as well, that allow you to choose to display consensi or individual reads, on the Global Align tab (see section 1.6.4.2).

1.7.2 The Variation Frequency Plot

The appearance and all features of the Variation Frequency Plot in the Consensus Align tab are identical to those of the corresponding panel in the Global Align tab, except that this one shows only data from the reads of the consensus selected for display in this tab. See section 1.6.2 for a full description of this plot's features.

1.7.3 The Multiple Alignment Display

The appearance and all features of the multiple alignment display in the Consensus Align tab are very similar to those of the corresponding panel in the Global Align tab (see section 1.6.3 for a full description of this display's features). They do, however, have the following differences:

- In the Consensus Align tab, only data from the individual reads of the consensus selected for display in this tab are shown. Therefore, the reads are never grouped into consensi as is possible in the Global Align tab, and there are no 'Read Type' display options.
- The consensus sequence of the aligned reads is shown just below the Reference Sequence, at the top of the panel. Matching positions are displayed as dots ".", whereas the consensus nucleotides are shown explicitly for positions that differ from the Reference Sequence.

1.7.4 Display Option Tools

This aspect of the Consensus Align tab differs from the Global Align tab in that it lacks the 'Alignment Data' and Read Type' controls, as these would not apply in this context. The other display controls, for 'Reported Frequency' and 'Read Orientation', are the same as in the Global Align tab (see section 1.6.4 for a full description of these display options features).

Note that consensi (in the Global Align tab) are always constructed from reads of the same orientation and from the same Amplicon; forward and reverse reads, and reads from two Amplicons, even if they overlap, will not be commingled in a single Consensus read. The system can thus automatically select the appropriate 'Read Orientation' option for the Individual reads of the Consensus at the time the Consensus Align tab is populated. This is important so that the estimated frequency of variation be correctly calculated when the Global Reported Frequency is selected, *i.e.* without including the depth of both read orientations in the denominator of the calculated frequency (see section 1.6.4.4 for more details on this).

1.8 The Flowgrams Tab

The Flowgrams tab allows you to view the flow-by-flow signals of any individual read included in the Project, highlighting any departure from the signal intensities that would be expected of the Reference Sequence to which that read (Amplicon) is associated. The display is designed to help you evaluate the significance of differences between an individual read and a Reference Sequence. To this end, the tab does not simply display the raw flowgram of the read, but rather a computationally processed version of it. In particular, flow cycle-shifts may be introduced into one or both flowgrams in order to optimize their alignment; and the flowgram of the read may be

computationally reverse-complemented in order that it is always shown in the 5'-->3' orientation of the Reference Sequence. Finally, the flowgram only displays the subset of flows relevant to the read's sequence alignment as displayed in the Global Align or Consensus Align tabs.

The Flowgrams tab's main feature is a "tri-flowgram" plot showing (Figure 1-66):

- an aligned, idealized flowgram for the Reference Sequence.
- an aligned, (possibly reverse complemented) flowgram of the read.
- a difference flowgram (read minus reference), where any variation from the Reference Sequence is seen as a non-zero value (whereby extra signals in the read, relative to the Reference Sequence, show up as positive differences in this panel).

In addition to the tri-flowgram plot, the Flowgrams tab contains a small set of display option and navigation tools in the upper-left corner, and the usual "Mouse Tracker" and color-code legend, in the lower-left corner.

Examining flowgrams can be useful when trying to assess whether a variation may be genuine or due to an artifact. For example, the flowgram of a mononucleotide from the Reference Sequence called as a dinucleotide repeat in a given read may show that the signal was barely over the threshold for calling a two-nucleotide incorporation, casting doubt on the second base of the call. Conversely, variations that induce a cycle shift in the flow alignment are particularly compelling since such shifts would not be expected as a result of simple overcalling or undercalling during signal processing, nor would they result from sequencing artifacts such as incomplete extension or carry forward. If the flowgram indicates that a variation appears genuine, the user should still consider whether it also occurs in other, overlapping reads, especially in the opposite orientation, or in a replicate experimental Sample; or could the variation simply be due to a PCR artifact introduced early in the sample preparation process? Anticipating these types of questions should play a large role in the experimental design of an Amplicon sequencing experiment.

A brief explanation of the information contained in a flowgram is provided along with a full description of the tri-flowgram display, in section 1.8.2. For more details on flowgrams and on the processing of data that generates them, see in the description of the Wells tab of the GS Run Browser in Part B, Section 3 of this manual.

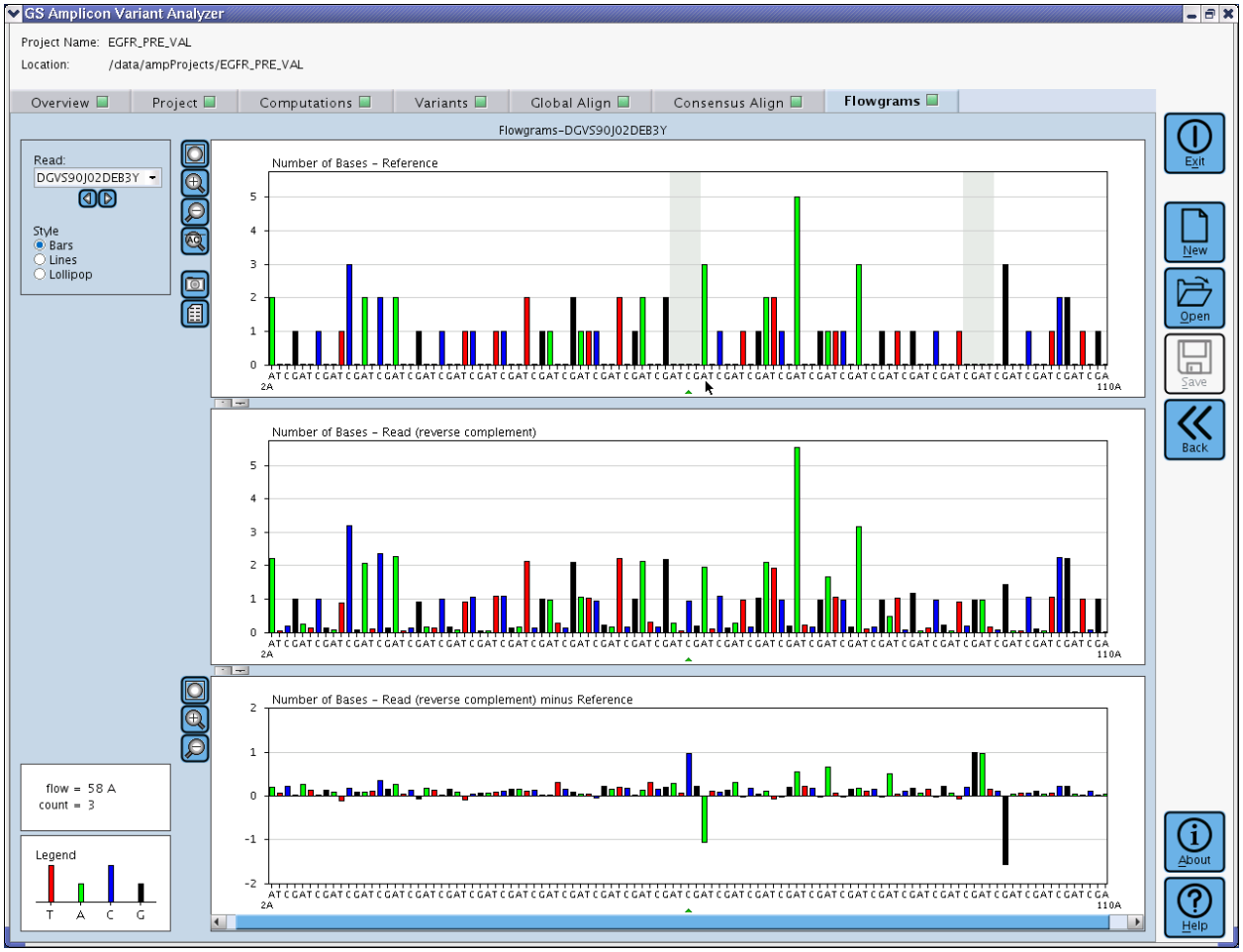


Figure 1-66: The Flowgrams tab

The flowgram alignment algorithm works only by introducing cycle shifts, with the goal of minimizing the sum of the absolute value of the signals in the difference plot while simultaneously attempting to minimize the number of cycle shifts introduced. The alignment algorithm does not attempt to split larger individual flow values into multiple flows of lesser magnitude, which could allow it to produce results that more closely mimic the alignments one would obtain by working in nucleotide-space (for an example where this situation arises, see section 2.3.2, below Figure 2-30).

1.8.1 Populating the Flowgrams Tab

When you open an Amplicon Project in the AVA software, the Flowgrams tab has no content and is grayed-out. To populate it, you must use the “Open Flowgrams <Name of the Read>” action from the contextual menu that appears when you right-click one of the following two sources:

- A single read on the Global Align tab (make sure that its Read Type control is set to “Individual”)
- A single read on the Consensus Align tab (which always displays individual reads)

Once the Flowgrams tab is populated, a small green arrow points to the flow corresponding to the nucleotide upon which you right-clicked. To load the Flowgrams tab with another read, you can use again the right-click method above and replace the displayed tri-flowgram by another one. But when in the Flowgrams tab, you have another, more powerful option: this tab has two "Read" controls in its upper-left corner that allow you to browse through and navigate all the reads that are present in the source tab that generated the one currently displayed (the Global Align or the Consensus Align tab). These controls are described in detail in section 1.8.3. As you navigate to other reads with these controls, the AVA software attempts to maintain the focus of the green arrow on the same corresponding nucleotide in the source tab. By observing the distribution of signals for a particular nucleotide over many reads, one may obtain increased (or diminished) confidence for a given variation.

1.8.2 The Triflowgram Plot

A flowgram is a graphic representation of the number of nucleotides added to the nascent DNA strands present in a given well of a PicoTiterPlate Device, during each nucleotide flow of a sequencing Run. Simply put, it shows the succession of nucleotide flows of the sequencing Run on the horizontal axis, and the number of nucleotides incorporated during each flow on the vertical axis (as histogram bars, with the nucleotides color-coded per the legend at the lower-left of the tab).

In Amplicon sequencing, because the software not only knows the data from the reads but also has a Reference Sequence to which each read is to be compared, the AVA application can calculate an "ideal flowgram" of the Reference Sequence corresponding to the read (*i.e.* the Amplicon, in AVA software language), and display the difference. The AVA software presents this to the user in the form of a "tri-flowgram". The three plots of a tri-flowgram show the following:

- The top plot shows the calculated flowgram of the segment of the Reference Sequence corresponding to the Amplicon that produced the read being displayed. This is simply the even number of bases that would result from the perfect incorporation of all nucleotides along a sequencing template of that sequence, during a sequencing Run on the GS Junior or Genome Sequencer FLX Instrument.
- The middle plot shows the exact signals recorded at each flow of the sequencing Run for the read being displayed, converted into "nucleotide" units. If the read was from the DNA strand opposite the Reference Sequence, the plot will display the signals after calculating the reverse complement of the read sequence, so the read flowgram will align with that of its Reference Sequence.

- The bottom plot is not truly a flowgram; it shows the difference between the two plots above (read minus Reference), such that if the read matches the Reference Sequence at a given flow, the histogram bar height is zero; if the read has a stronger signal than expected from the known Reference, the bar height is greater than zero; and if the read has a weaker signal than expected from the known Reference, the bar height is less than zero. This "difference flowgram" thus conveniently shows all flow-by-flow variations between the read and its Reference Sequence as departures from zero.

The tri-flowgram plot has all the standard navigation features (scrollbars, zoom buttons, mouse tracker, *etc*.) described in section 1.1.3.3. A separate set of zoom buttons is available for the

difference flowgram because it is sometimes convenient to examine it at a different scale than the Reference and read flowgrams. Also, the “Save plot snapshot to image file” and “Save plot data to spreadsheet file” buttons will save all three plots together, in a single file. The histogram bars can be displayed in your choice of three styles (Bars, Lines, and Lollipop), by selecting the corresponding radio button near the upper-left corner of the tab. The three plots of the Flowgrams tab can also be resized and collapsed, as described in section 1.1.3.2.

The tri-flowgram plots have another feature that is important to maintain the alignment between the two upper plots: due to the interplay between the nature of a variation and the flow order of the nucleotide cycle during the sequencing Run, the situation sometimes occurs whereby the Reference and read flowgrams fall out of phase by one or more cycles of four flows. If this were allowed to happen, all the flows beyond the cycle shift would be misaligned, and all the values in the difference flowgram would be wrong. Cycle shifts are therefore inserted in either the Reference or the read flowgram, as appropriate to maintain their synchronicity; these “empty” inserted cycles are marked in gray on the flowgrams (Figure 1-67).

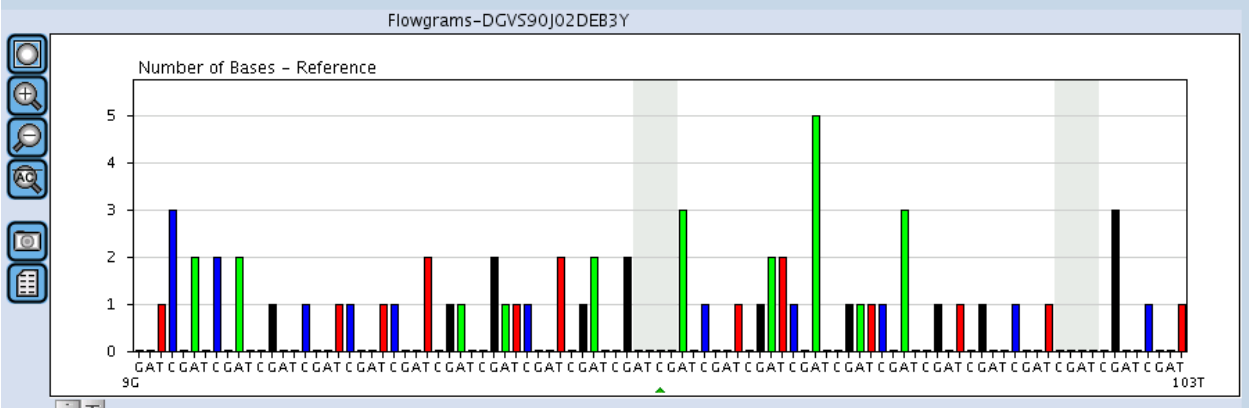


Figure 1-67: Example of a flowgram with inserted nucleotide cycles

1.8.3 Navigation on the Flowgrams Tab

There are two navigation controls located at the upper-left corner of the tab that allow you to select new flowgrams to display in the Flowgrams tab. The first is a drop down menu (see Figure 1-66, above) that contains a list of all the reads that are present in the source tab that generated the one currently displayed (the Global Align or the Consensus Align tab). Selecting a new read from the drop down menu will update the Flowgrams tab with the tri-flowgram for the new read, replacing the current data. This allows you to quickly compare the flowgrams of various reads over the data group available (*e.g.* all the reads of a Sample, for a single or a given set of Amplicon(s), as displayed in the Global Align tab; or a sub-list of the reads of a given consensus, as restricted by one or more selection(s) applied on the Consensus Align tab).

The second Read display control allows you for even faster scanning of the set of reads. It consists in a pair of arrow buttons located just below the ‘Read’ drop down menu. Clicking one of these buttons directly replaces the tri-flowgram currently displayed by the one of the read next to (or preceding) it in the list. This update is very fast and allows you to quickly scan the flowgrams for a large number of reads to see if a variation (most readily seen on the difference flowgram) is rare or, on the contrary, present in many (or most) of them.

2. EXAMPLE AMPLICON PROJECT DESIGN AND ANALYSIS

To help better guide the reader through the process of an Amplicon Sequencing experiment, this section provides a fictitious example of the whole procedure, starting with setting the objectives and including the design of the Amplicon libraries, sequencing, and the full analysis of the results through the determination of the frequency of previously known as well as novel Variants observed in the Sample. Emphasis, however, will be on software.

This example describes a Project that does not make use of MIDs or Multiplexers. For an example of how these features are used in an Amplicon Project (using the CLI), see section 3.6.

2.1 Experimental Design

In this example, we will look for Variants in five exons from the human Epidermal Growth Factor Receptor gene, EGFR exons 18 through 22, in a single DNA source. The sequences of the 5 exons are known (*e.g.* from public databases), and are shown in **Error! Reference source not found.**. We further posit that there is a known Variant in exon 19 that we want to track: a 15 bp deletion at positions 93-107 (inclusive) of the exon.

In order to be able to gather sequencing data from both orientations over the full length of the exons (as much as possible), we define a set of overlapping Amplicons whereby every nucleotide is within about 100 bp from each of two facing primers, providing nearly full coverage of each exon in both orientations (see **Error! Reference source not found.**). A Primer Design software can be used to assist in this task.

Note that this example was created for the Genome Sequencer 20 System but that the GS Junior and Genome Sequencer FLX Systems feature read lengths of over 400 bp for the GS FLX Titanium chemistry: this would allow a GS Junior or Genome Sequencer FLX System user to design longer Amplicons than those used in this example. See the other Manuals and Guides for the relevant chemistry for more details.

Overall, we define 11 Amplicons, as listed in Table 2-1. To ensure proper representation of all Amplicons in our experiment, we will generate 11 single-peak Amplicon libraries (as opposed to attempting a multiplex amplification). Amplicon libraries are made using Fusion Primers; for our experiment, all forward primers (“Primer1” from Table 2-1) are fused to Primer A, in the configuration 5’-PrimerA-Primer1-3’; and all reverse primers (“Primer2” from Table 2-1) are fused to Primer B, in the configuration 5’-PrimerB-Primer2-3’. Since the experiment comprises only one Sample, we do not need to use MIDs; the software will recognize the Amplicon to which each read belongs by looking at the Primer 1 or Primer 2 sequence that it will see at the beginning of the read.

Amplicon Name	Primer1 (Forward; 5'-->3')	Primer2 (Reverse; 5'-->3')
EGFR_18_1	GACCCTTGTCTCTGTGTTCTTG	CCTCAAGAGAGCTTGGTTGG
EGFR_18_2	AGCCTCTTACACCCAGTGGA	CCTTATACACCGTGCCGAAC
EGFR_18_3	TGAATTCAAAAAGATCAAAGTG	CCCCACCAGACCATGAGA
EGFR_19_1	TCACAATTGCCAGTTAACGTCT	GATTTCCTTGTTGGCTTTCG
EGFR_19_2	TCTGGATCCCAGAAGGTGAG	GAGAAAAGGTGGGCCTGAG
EGFR_20_1	CCACACTGACGTGCCTCTC	GCATGAGCTGCGTGATGAG
EGFR_20_2	GCATCTGCCTCACCTCCAC	GCGATCTGCACACACCAG
EGFR_20_3	GGCTGCCTCCTGGACTATGT	GATCCTGGCTCCTTATCTCC
EGFR_21_1	TCTTCCCATGATGATCTGTCCC	GACATGCTGCGGTGTTTTC
EGFR_21_2	GGCAGCCAGGAACGTACT	ATGCTGGCTGACCTAAAGC
EGFR_22_1	CACTGCCTCATCTCTCACCA	CCAGCTTGGCCTCAGTACA

Table 2-1: Names of the Amplicons defined for the EGFR experiment, and the Primers used to create them

Figure 2-1: DNA sequence of the five human EGFR exons in which we will be searching for Variants, including the location of the regions (colored underlines) with Primers (colored arrows) used to generate Amplicon libraries for sequencing. The known deletion Variant in exon 19 is boxed.

With these Fusion Primers on hand (and the initial DNA sample), we can proceed with the preparation of the 11 Amplicon libraries. Proper amounts are subjected to the emPCR Amplification process using both emPCR Amplification kits II and III (GS FLX standard chemistry), so that we will have reads that will start from both the Primer A and Primer B ends of each Amplicon.

For the GS FLX Titanium chemistry, one would use the GS FLX Titanium emPCR Kit Lib-A (of the appropriate size for the number of reads desired for each Amplicon) to prepare Amplicons in both the A and the B orientations.

Preparing for the sequencing proper, we calculate that with an expected minimum of 33,000 high quality reads per medium region of a PicoTiterPlate Device, we can pool all 11 Amplicon libraries and load them together in a single region and carry out a single sequencing Run: we can then expect approximately 3000 reads for each Amplicon (about 1500 in each orientation), not counting for overlaps, a depth of coverage sufficient to detect and reasonably quantitate Variants down to a frequency of approximately 3-5% (this depends on the nature of the variation, the sequence environment, and other factors), which is sufficient for our purpose. (As above, this example was created for the Genome Sequencer 20 System; for the GS Junior or Genome Sequencer FLX Systems, with the GS FLX Titanium chemistry, one would typically expect 70,000 reads per PicoTiterPlate Device, or 130,000 to 200,000 reads per medium region of a PicoTiterPlate Device, respectively, for a good quality library.)

2.2 Project Setup in the AVA Software

For our EGFR experiment example, let's posit that a 4-region sequencing Run generated four SFF files, one of which, named "DGVS90J03.sff", contains all the reads of our combined 11 Amplicon libraries and is now available on the local file-system.

Also, while each exon could be used individually as Reference Sequences in the Variant analysis, we decide that it would be more convenient to report on all the Variants found in all five exons together. To simplify the analysis, therefore, we create an "artificial" Reference Sequence by concatenating the sequences of the 5 exons, with strings of 20 'N' characters to separate them. The resulting single Reference Sequence is shown in Table 2-2.

EGFR Exons 18-22

GACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACCCAGTGGAGAAGCTCCCAACCAAGCT
CTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGATCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAA
GGTAAGGTCCCTGGCACAGGCCTCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGNNNNNNNNNNNNNNNN
NNNNTCACAATTGCCAGTTAACGTCTTCCTTCTCTCTCTGTCATAGGGACTCTGGATCCCAGAAGGTGAGAAAGTTA
AAATTCCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTCGATGTGAGTTTC
TGCTTTGCTGTGTGGGGGTCCATGGCTCTGAACCTCAGGCCCACCTTTTCTCNNNNNNNNNNNNNNNNNNNNCCACA
CTGACGTGCCTCTCCCTCCCTCCAGGAAGCCTACGTGATGGCCAGCGTGGACAACCCCCACGTGTGCCGCCTGCTGG
GCATCTGCCTCACCTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCGGCTGCCTCCTGGACTATGTCCGGGAA
CACAAAGACAATATTGGCTCCCAGTACCTGCTCAACTGGTGTGTGCAGATCGCAAAGGTAATCAGGGAAGGGAGATA
CGGGGAGGGGAGATAAGGAGCCAGGATCNNNNNNNNNNNNNNNNNNNNTCTTCCCATGATGATCTGTCCCTCACAGC
AGGGTCTTCTCTGTTTCAGGGCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACG
TACTGGTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGAAGAGAAAGAA
TACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCATNNNNNNNNNNNNNNNNNNNNCACTGC
CTCATCTCTCACCATCCCAAGGTGCCTATCAAGTGGATGGCATTGGAATCAATTTTACACAGAATCTATACCCACCA
GAGTGATGTCTGGAGCTACGGTGAGTCATAATCCTGATGCTAATGAGTTTGTACTGAGGCCAAGCTGG

Table 2-2: "Artificial" Reference Sequence comprising exons 18 through 22 of EGFR, concatenated and with separating strings of 20 N characters

Many of the operations described through the end of section 2.4 can be done in more than one way. For example, most actions that can be performed by clicking a button can also be accessed via a contextual menu and/or by double-clicking in a field in a Table. Only one way is given in the example below; for more information, see the detailed description of each tab.

2.2.1 Launching the AVA Application

The next step is to launch the GS Amplicon Variant Analyzer (AVA) application, which is done from the command line, using the "`gsAmplicon`" command (see section 1.1.2); the AVA software splash screen appears briefly, while the application is launching, and then the AVA main window is displayed with its Overview tab showing the AVA introduction text and the 7 main buttons, to the right of the window; the 'Project Name' and 'Location' fields at the top left are initially blank and all the other tabs are grayed-out, since no project is open (see Figure 2-2).

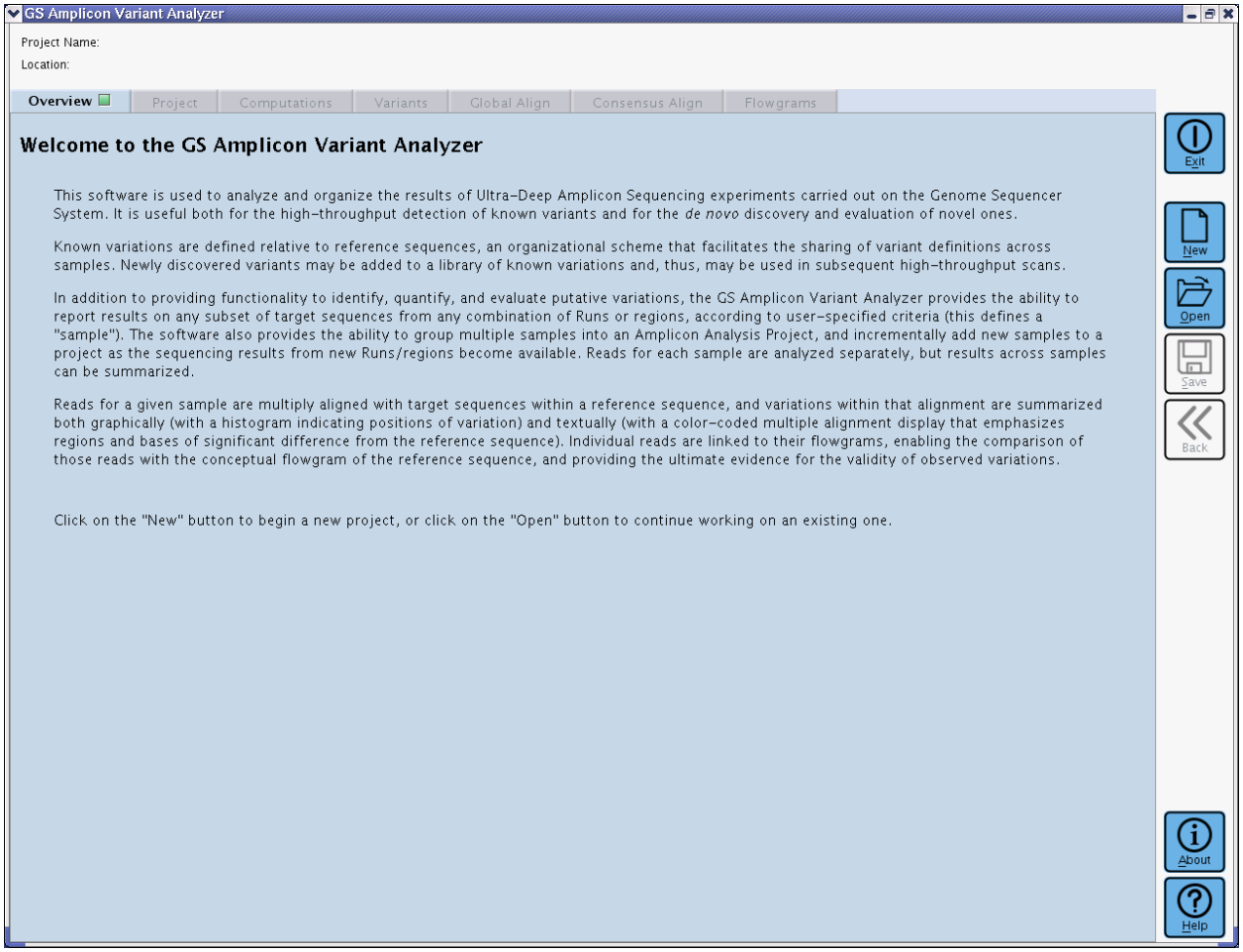


Figure 2-2: The AVA main window at start up

2.2.2 Creating a New Project

Since we want to create a new Project, we click the “New” button. (The “Open” button can be used to load a pre-existing Project.) The “New Amplicon Project” window opens, where we can specify the ‘Name’, ‘Location’, and ‘Description’ for the new Project (Figure 2-3). Note that since the ‘Generate location based on name’ box is checked, the ‘Name’ and ‘Location’ fields are linked such that as we type a name to replace the ‘DefaultName’ in the ‘Name’ field, the ‘DefaultName’ portion of the ‘Location’ will dynamically update to match the content of the ‘Name’ box; this is an easy way to ensure that the same name is used for the Project and for the folder that contains it (the “Location”), which is usually what one would want to do.

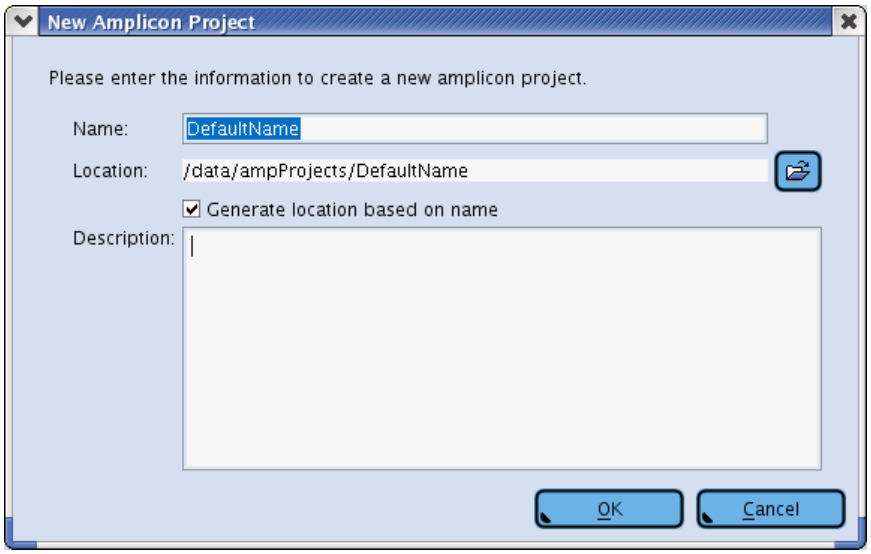


Figure 2-3: The New Amplicon Project window

To keep things simple, we initially leave the 'Name' field alone and first select the 'Location' we want. The folder icon to the right of the 'Location' field opens the "New Project Parent Location" window which allows us to navigate the file-system easily (Figure 2-4). The object is to identify a parent location where we want to store Project directories (as opposed to the full path to this particular new Project directory), providing a standard base of operations. It is important to choose a directory where we have both read and write permissions.

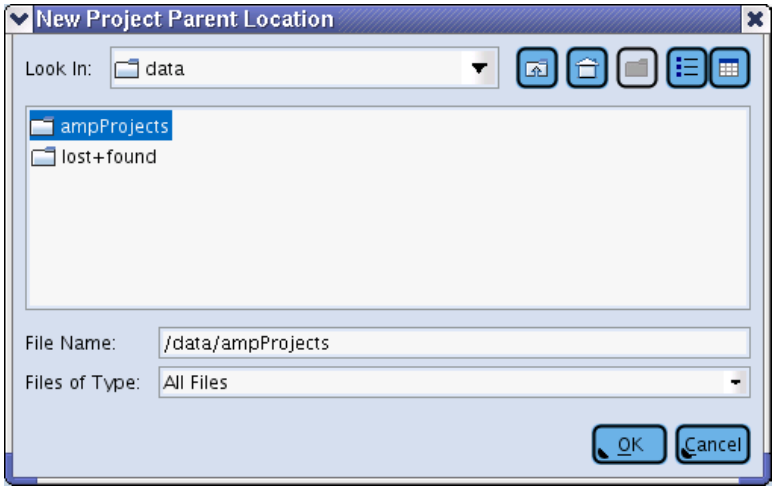


Figure 2-4: The New Project Parent Location window

To pursue our example, let's assume that we have read and write permissions in the '/data/ampProjects' directory on our local system, and that we chose 'ampProjects' as the parent location directory (Figure 2-4). The path to this directory is used to form a 'File Name'. Clicking 'OK' returns us to the "New Amplicon Project" window, where the path we just chose is reflected in the 'Location' field: '/data/ampProjects/DefaultName'. Editing the contents of the 'Name' field to 'myFirstTestProject' (with the 'Generate location based on name' box checked),

provides a full path for the Location of the new Project (Figure 2-5). This Figure also shows a short annotation entered in the 'Description' field.

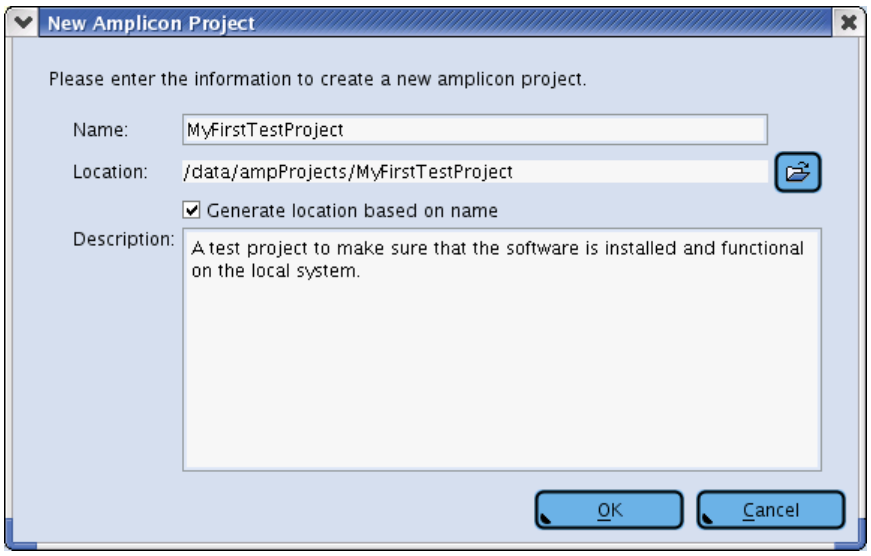


Figure 2-5: The New Amplicon Project window, with the Name, Location and Description of the new project

Clicking 'OK' at this point closes the "New Amplicon Project" window, creates the Project and the Location (including a proper subdirectory structure for the functioning of the Project computation and result storage), and opens the new Project in the AVA main window, in its "Project" tab (see next section).

Although this Project does not use MIDs, the 454Standard MID set is automatically loaded when a new Project is created (see section 4.4), which is why 14 MIDs will be present in the newly created project.

2.2.3 Defining the Reference Sequence

Figure 2-6 shows the AVA main window with its Project window in the front, showing the new (empty) Project. The 'Project Name' and 'Location' fields at the top left of the window match the new Project information we just entered. The 'Project' tab is in bold black lettering on blue background with a green square icon, indicating it is ready to be used to setup the Project. The other two accessible tabs are 'Overview' and 'Computations' (black type on gray background and green square icons), while the remaining tabs don't yet have any content and remain grayed-out. The References Tree (left panel of the Project Tab) contains a folder representing the Project, and nothing else. The 6 context-sensitive buttons in the upper left hand margin of the "tree" panel ('Add', 'Remove from project', 'Remove association and remain in project', 'Duplicate item', 'Select Amplicons associated with item', and 'Import data') start out inactive and grayed-out until some selection is made in the application that can give them context as to what needs to be added or removed (*e.g.* selecting an object type tab in the right table-view area).

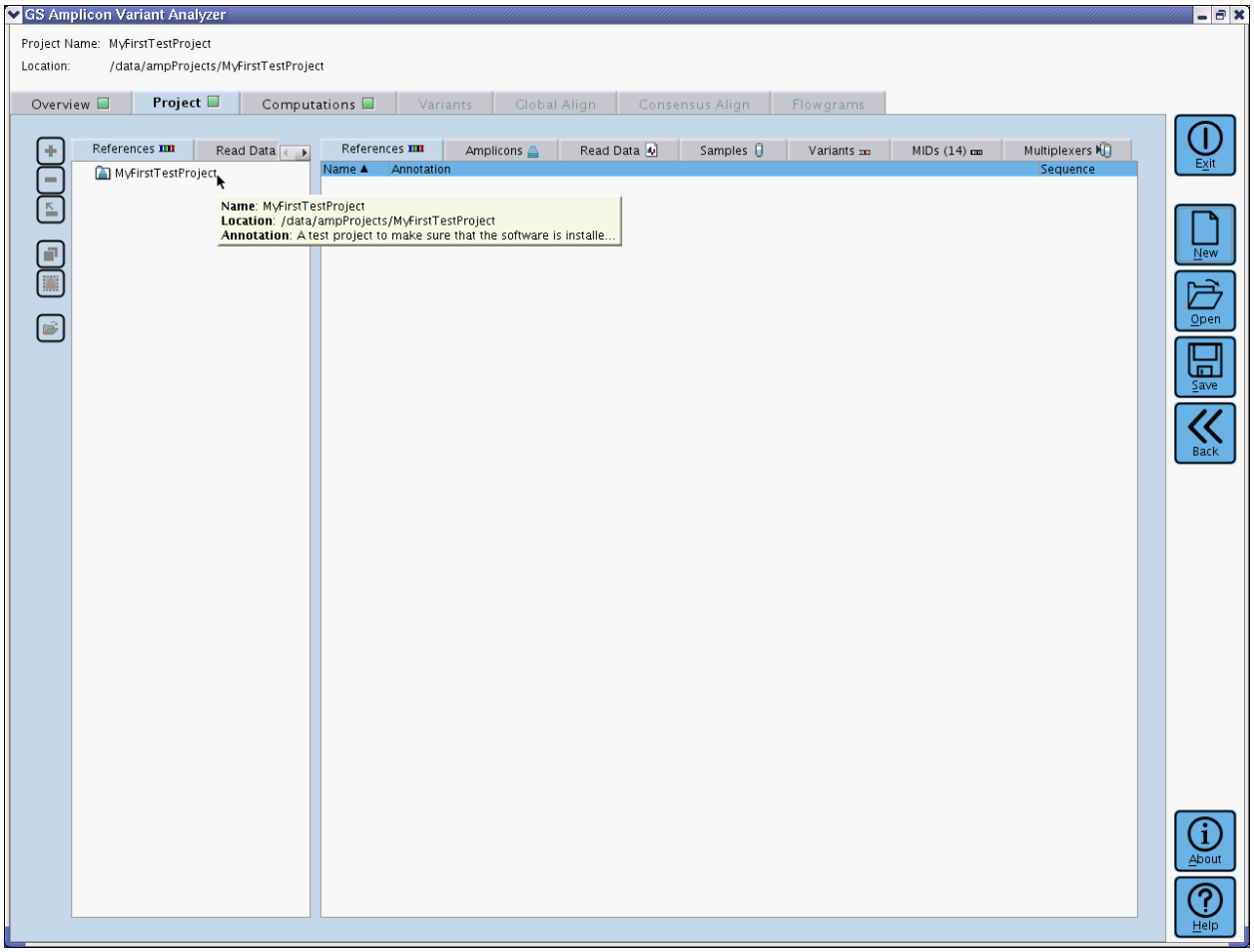


Figure 2-6: The AVA main window ('Project' tab) of a newly created Project

For the example Project, we want to enter a Reference Sequence first, so we click on the right panel's 'References' sub-tab, or References Definition Table. This enables the 'Add' button on the left margin (the button turns blue rather than gray) and a blue outline appears around the Definition Table Panel (indicating that the active buttons on the left can operate on the selected item in the Definition Table). Clicking the 'Add' button creates a new Reference Sequence entry in the References Definition Table and creates a corresponding reference node in the References Tree (Figure 2-7).

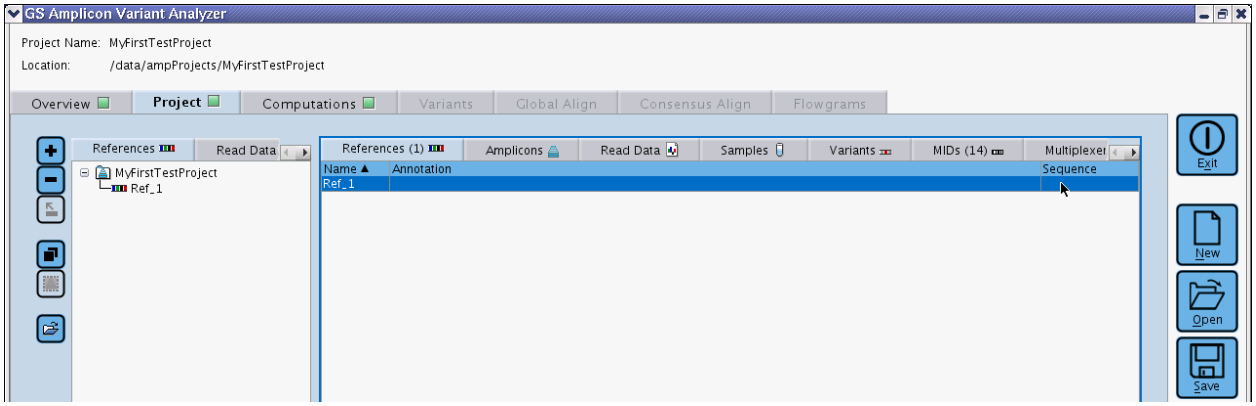


Figure 2-7: The Project Tab, with the References Tree and References Definition Table displayed in the left and right panels, respectively. Note that the 'Add' button is enabled; it was used to create a new Reference Sequence entry.

To define the Reference Sequence, we double-click on the fields in its row, in the References Definition Table. In our example, we edit the default Name "Ref_1" to "EGFR_Exons_18-22". Note that the tree and table views are linked and editing the Reference Sequence name in the table also changes its name in the tree. Annotations are optional; they are also entered by double-clicking in the corresponding field which, in this case, opens a text entry window (not shown). Finally, we double-click in the 'Sequence' field of our Reference Sequence; an 'Edit Sequence' window appears in which we paste the "artificial" Reference Sequence covering all five exons, prepared before (Figure 2-8).

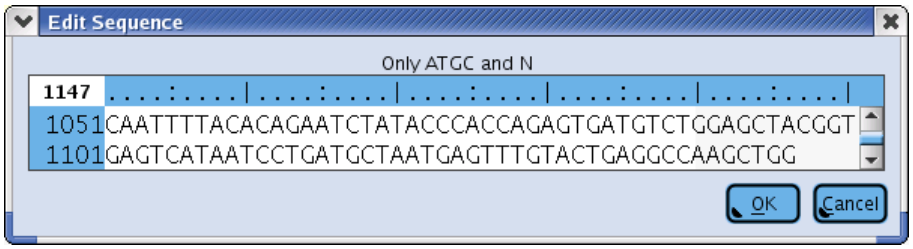


Figure 2-8: The Edit Sequence window, in which we pasted the 1146 nt "artificial" Reference Sequence covering exons 18-22 of EGFR

2.2.4 Defining the Amplicons

Now that we have a Reference Sequence, we can enter our Amplicons. To do so, we click on the 'Amplicons' sub-tab of the table-view. The 'Add' button on the left margin is now capable of creating new Amplicons, as this is its new context. For our EGFR Project, we need to add 11 Amplicons, so we click the 'Add' button 11 times, which adds 11 rows in the Amplicons Definition Table, with generic Amplicon Names. As before, the information is entered by double-clicking into the various fields for each Amplicon; the fields are: 'Name', 'Reference', 'Annotation' (optional), 'Primer 1', 'Primer 2', 'Start', and 'End').

- Since we have only one Reference Sequence, we can associate all our Amplicons with it at once by multi-selecting all the rows from the Amplicons Definition Table and dragging the selection to the proper (in our case, only) node in the References Tree, on the left.

This adds a branch of Amplicon nodes hanging off the Reference node; and the 'Reference' fields on the Amplicons Definition Table are updated so that each Amplicon now has this field populated with this Reference Sequence.

- Primer 1 and Primer 2 are those used to prepare the Amplicon library (or libraries); for our example, these are listed in Table 2-1 (above), along with the Amplicon Names. We enter the sequence of all the Primers by double-clicking in each of the 'Primer' fields; this opens a sequence editor window into which the sequence can be typed or pasted (always 5'-->3'; the software will compute the reverse complement of Primer 2 to align it to the Reference Sequence). For the Amplicon Names, we double-click in the 'Names' fields and type or paste the Amplicon Names in the Table cells.

- We choose not to enter any Annotations for our Amplicons.

After adjusting the width of the column (by dragging of the separation lines between the headers) so that all the fields are completely readable, the application view looks as shown in Figure 2-9.

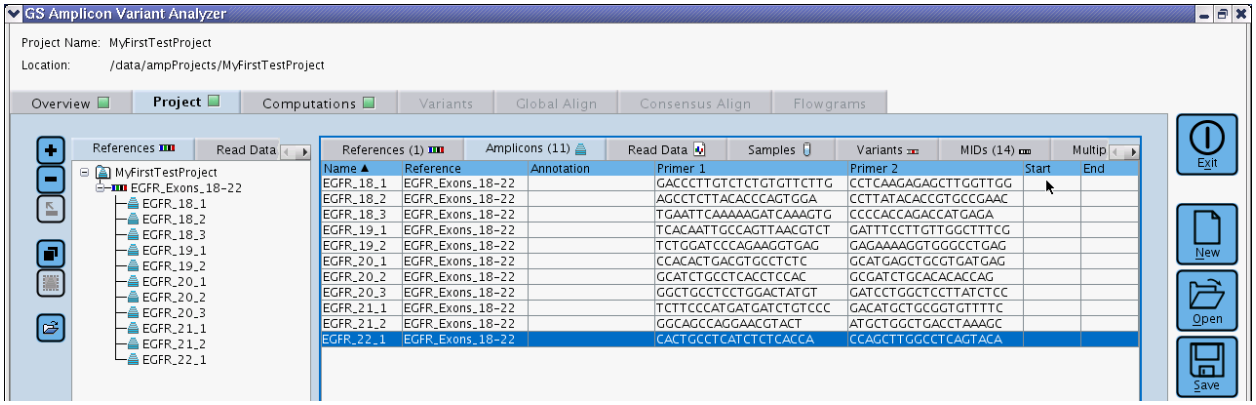


Figure 2-9: The AVA window after associating all 11 Amplicons to the single 'EGFR_Exons_18-22' Reference Sequence, and entering the Amplicons' names and Primer sequences

Next, we define the 'Targets' by specifying their Start and End (*i.e.* by positioning the Primers along the Reference Sequence), for each Amplicon. To do this, we double-click in either the 'Start' or the "End' field of each Amplicon. This opens the Edit Start/End window for this Amplicon and carries out an automatic search of its Primer 1 and the reverse-complement of its Primer 2 along its Reference Sequence (Figure 2-10). As long as no errors were made when entering the Primer sequences, each Primer in our EGFR example will find an exact unique match and be displayed with a yellow background, and the Start and End of the Target will appear in the corresponding fields in the Amplicons Definition Table.

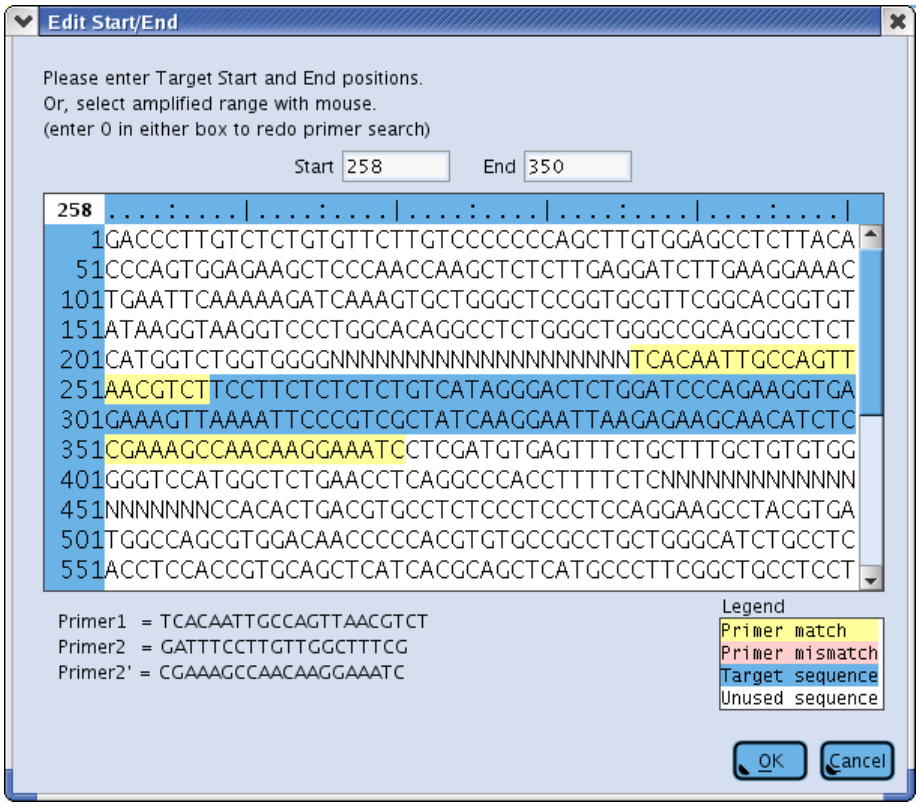
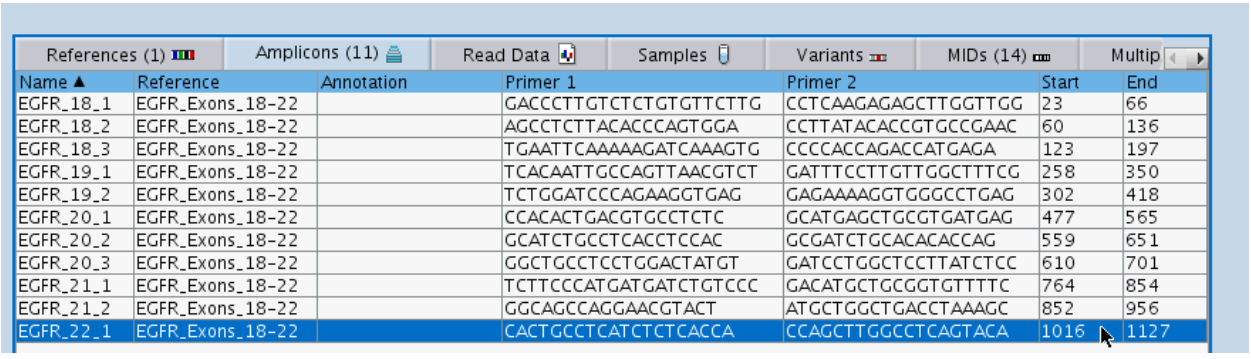


Figure 2-10: The Edit Start/End window for Amplicon EGFR_19_1, showing the two Primers with yellow background and the Target with blue background

After setting the Start and End points for the Targets within all Amplicons, the Amplicons Definition Table of out Project looks as shown in Figure 2-11.



Name ▲	Reference	Annotation	Primer 1	Primer 2	Start	End
EGFR_18_1	EGFR_Exons_18-22		GACCCTTGTCTCTGTGTTCTTG	CCTCAAGAGAGCTTGGTTGG	23	66
EGFR_18_2	EGFR_Exons_18-22		AGCCTCTTACACCCAGTGGA	CCTTATACACCGTGCCGAAC	60	136
EGFR_18_3	EGFR_Exons_18-22		TGAATTCAAAAAGATCAAAGTG	CCCCACCAGACCATGAGA	123	197
EGFR_19_1	EGFR_Exons_18-22		TCACAATTGCCAGTTAACGTCT	GATTTCCTTGTTGGCTTTCG	258	350
EGFR_19_2	EGFR_Exons_18-22		TCTGGATCCCAGAAGGTGAG	GAGAAAAGGTGGGCCTGAG	302	418
EGFR_20_1	EGFR_Exons_18-22		CCACACTGACGTGCCTCTC	GCATGAGCTGCGTGATGAG	477	565
EGFR_20_2	EGFR_Exons_18-22		GCATCTGCCTCACCTCCAC	GCGATCTGCACACACCAG	559	651
EGFR_20_3	EGFR_Exons_18-22		GGCTGCCTCCTGGACTATGT	GATCCTGGCTCCTTATCTCC	610	701
EGFR_21_1	EGFR_Exons_18-22		TCTTCCCATGATGATCTGTCCC	GACATGCTGCGGTGTTTTC	764	854
EGFR_21_2	EGFR_Exons_18-22		GGCAGCCAGGAACGTACT	ATGCTGGCTGACCTAAAGC	852	956
EGFR_22_1	EGFR_Exons_18-22		CACTGCCTCATCTCTCACCA	CCAGCTTGGCCTCAGTACA	1016	1127

Figure 2-11: The completed Amplicons Definitions Table for the EGFR example experiment

2.2.5 Defining the Sample

Our example experiment is the simplest case where we have only one Sample: the single DNA source used to prepare the 11 Amplicon libraries covering the five exons from the EGFR gene in which we are looking for sequence Variants (see section 2.1). (For more complicated situations, see section 2.6.1). To set up our single Sample in our Project, we click on the

'Sample' sub-tab (Sample Definition Table) and then click on the 'Add' button at the left of the tree view. This adds a Sample called 'Sample_1' to the Sample Definition Table. We will keep this Default Sample Name, and leave the 'Annotation' field empty. The AVA application window is now in the state shown in Figure 2-12.

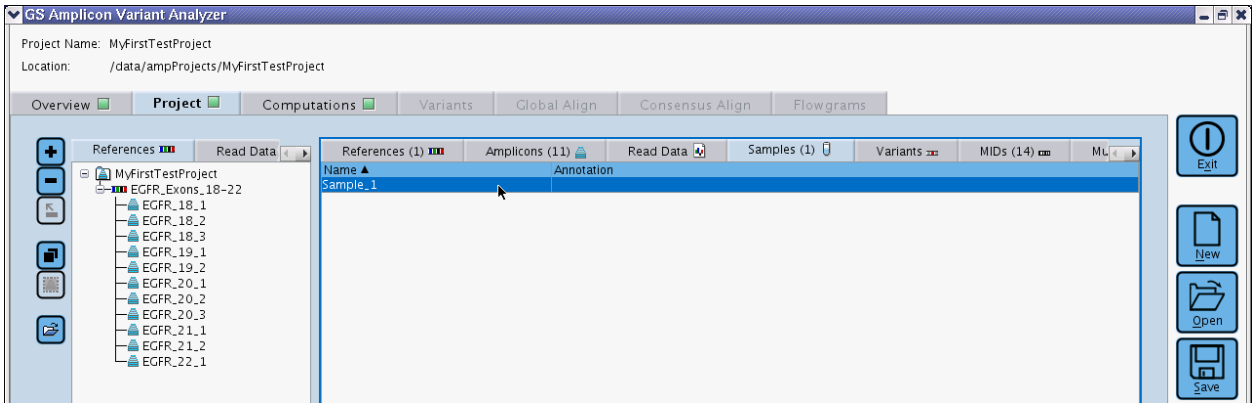


Figure 2-12: The AVA software window after creating a single Sample into the Project

We will next use the Tree sub-tabs to create the associations between the Sample and all 11 Amplicons defined in the Project. To do this, we select the Samples Tree sub-tab on the left panel (showing our single Sample hanging off the main project node), and the Amplicons Definition Table on the right panel. We then multi-select the full set of Amplicons from the Table (using the shift key) and drag them to Sample_1 in the Tree (Figure 2-13). This will associate all our Amplicons with our Sample (Figure 2-14).

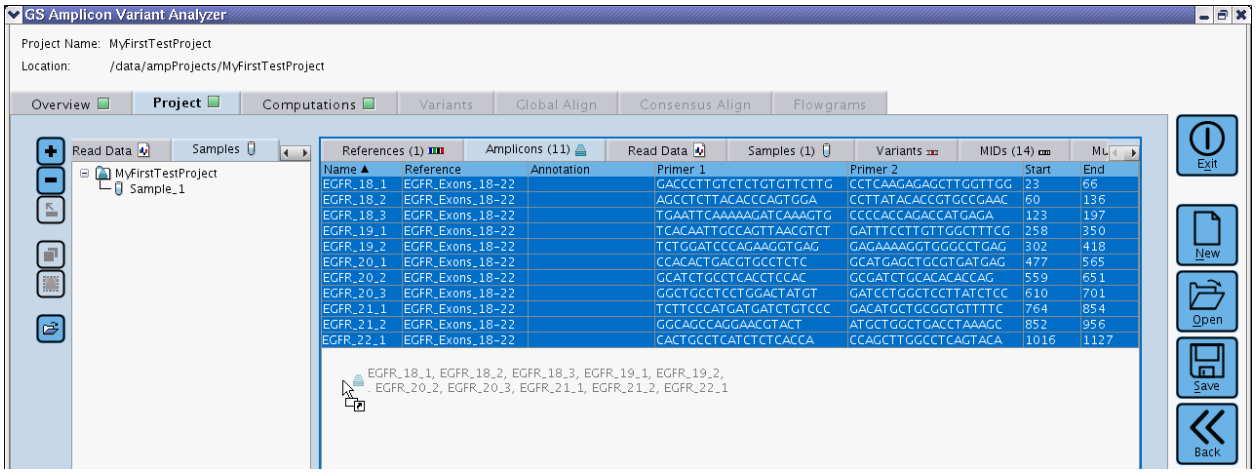


Figure 2-13: The AVA window, in the middle of a multi-select and drag of the Amplicons from their Definition Table to the Sample_1 node in the Samples Tree, to create the associations

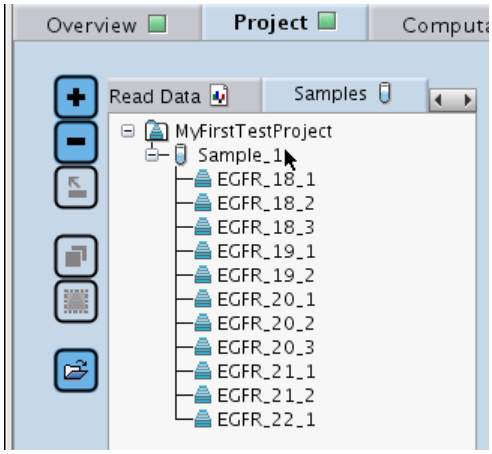


Figure 2-14: The Samples Tree after the 11 Amplicons have been associated with Sample_1

2.2.6 Defining the Known Variant

As mentioned at the beginning of this example (see section 2.1), there is a known 15 bp deletion Variant in exon 19 whose frequency we want to evaluate in our Sample. The corresponding sequence is in the "artificial" 'EGFR_Exons_18-22' Reference Sequence we already defined in our Project, so we can proceed with the definition of the Variant.

First, we click on the 'Variants' sub-tab on the right-hand panel of the 'Project' tab (the Variants Definition Table) and then choose the 'Add' button at the left margin. This creates a new entry in the Variant Definition Table. We decide to use the generic name, Var_1, for our Variant and to not enter any 'Annotation'. We are thus ready to associate the Variant to its Reference Sequence by click-and-dragging it to the 'EGFR_Exons_18-22' node on the References Tree. This fills in the 'Reference' field for this Variant and sets the Status to "Accepted" in the Definition Table, and also adds the Variant as a sub-node of the References Tree (Figure 2-15).

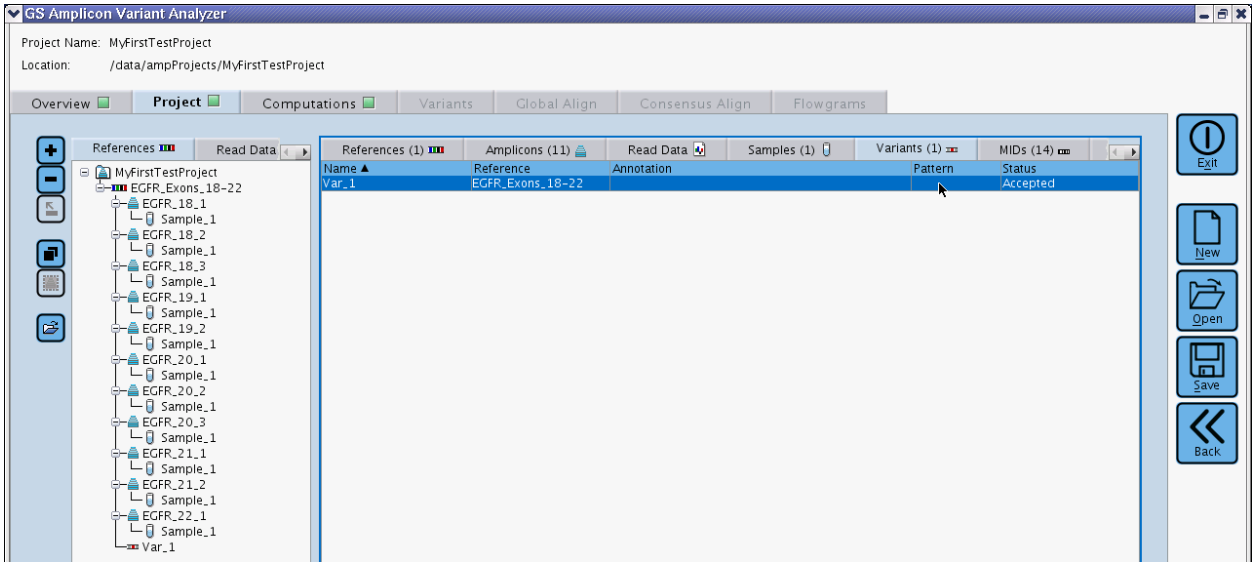


Figure 2-15: The AVA window after creating a Variant and associating it to the 'EGFR_Exons_18-22' Reference Sequence

To complete the definition of our Variant, we must enter a 'Pattern' of variation for the Variant with respect to the Reference Sequence. To do this, we double-click on the 'Pattern' cell for the Variant in the Variants Definition Table. This opens the 'Edit Pattern' window, pre-loaded with the Reference Sequence to which the Variant is associated (Figure 2-16).

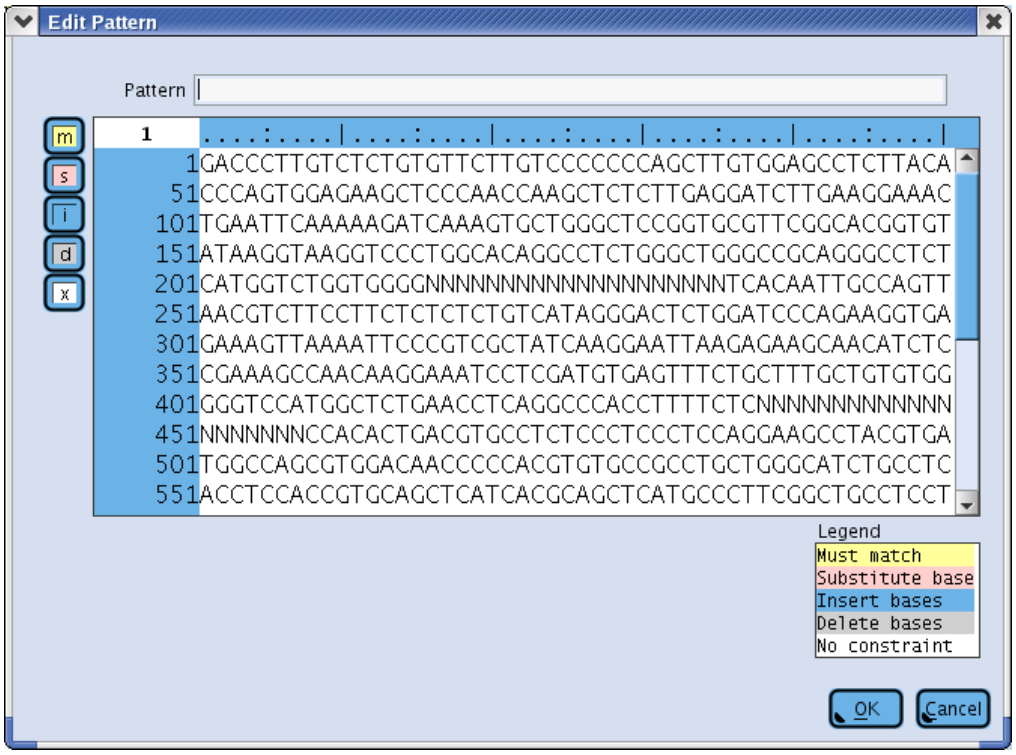


Figure 2-16: The Edit Pattern window, pre-loaded with the 'EGFR_Exons_18-22' Reference Sequence and ready to receive the 'Pattern' for the Var_1 Variant

We decide to type the Pattern directly into the 'Pattern' text area of the window, using the AVA software's Variant Definition Syntax. [Since the location of the deletion was initially defined relative to exon 19 (positions 93-107), we must first calculate its position in the artificial Reference Sequence we are using: 328-342.] So, we type: 'd(328-342)' and press 'Enter'. This highlights the Variant pattern in the Reference sequence according to the 'Legend' at the lower right corner (in this case highlighting a string of gaps in gray to represent the deletion; Figure 2-17).

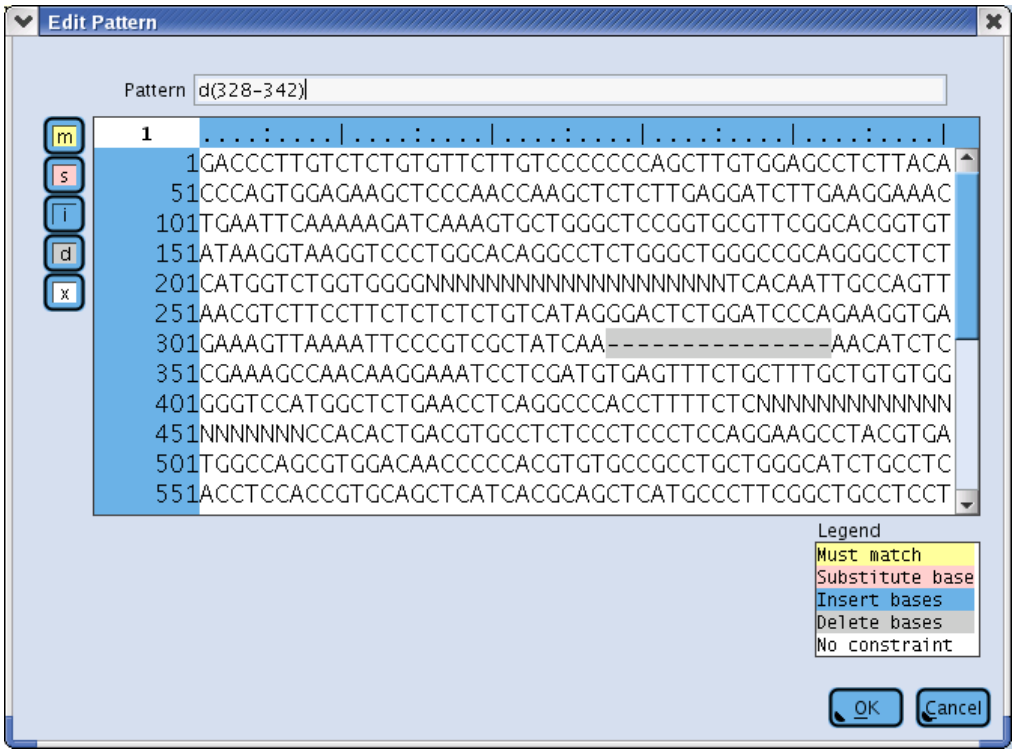


Figure 2-17: The Edit Pattern window after entering the 15 bp deletion at positions 328-342 of the Reference Sequence . The nucleotides in question are replaced by dashes and are highlighted in gray, per the legend.

Clicking OK accepts the Pattern specification into the 'Pattern' field of the Variant in the Variant Definition Table (Figure 2-18).

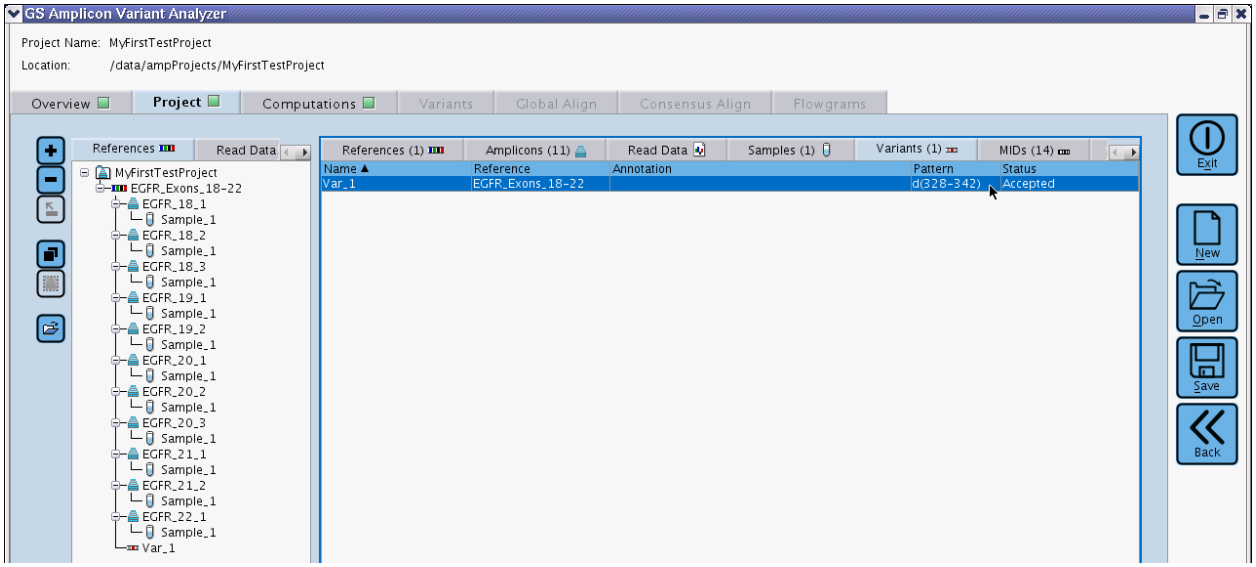


Figure 2-18: The AVA window after fully defining the Variant "Var_1"

2.2.7 Importing the Read Data Set

The next and final step in the set up of the Project is to add actual read data. This is done using the “Import” button at the left edge of the Project Tab. This button is enabled by selecting either the ‘Read Data’ Tree sub-tab (left panel) or the ‘Read Data’ Definition Table sub-tab (right panel) (Figure 2-19).

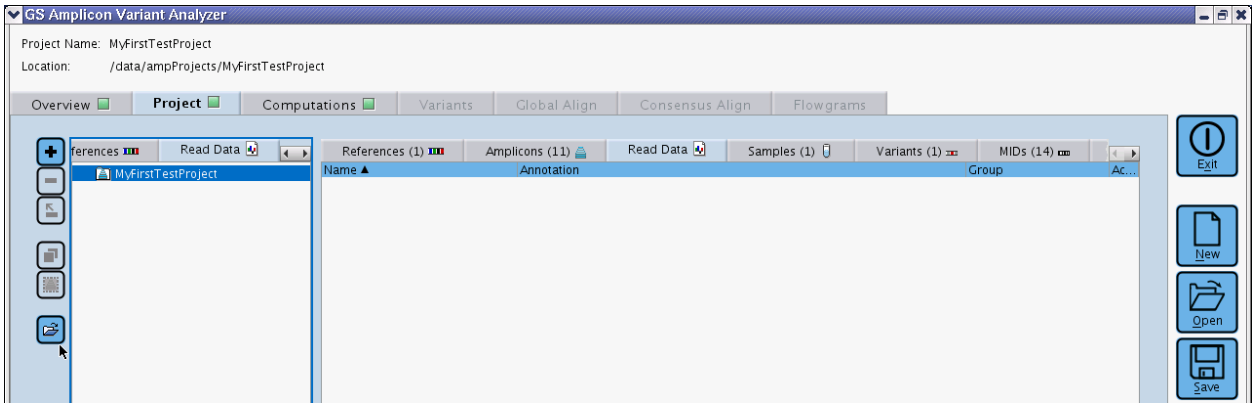


Figure 2-19: The AVA window with both the Read Data Tree and Read Data Definition Table visible. The tree panel was last clicked on, making it the currently “active panel” as indicated by the blue border that surrounds it. The Import button to the left is active and is associated with the type of data in the visible tab of the active panel. In this case, the Import button would allow the import of new Read Data into the project since the tree panel is the “active panel” and the Read Data Tree is selected and visible within it.

Clicking the “Import” button opens the “Choose Read Data” file browser window, which allows us to search for Read Data files to add to the Project. Since the data we want to import resides in a single region of a 4-region sequencing Run (and each region has its own SFF file), we select ‘454 SFF Files’ from the ‘Files of Type:’ drop down menu. We then navigate to the folder that contains the SFF files of the EGFR Run, and select the file that contains the read data we want to import (DGVS90J03.sff). This selection populates the ‘File Name:’ field, in the Choose Read Data window (Figure 2-20).

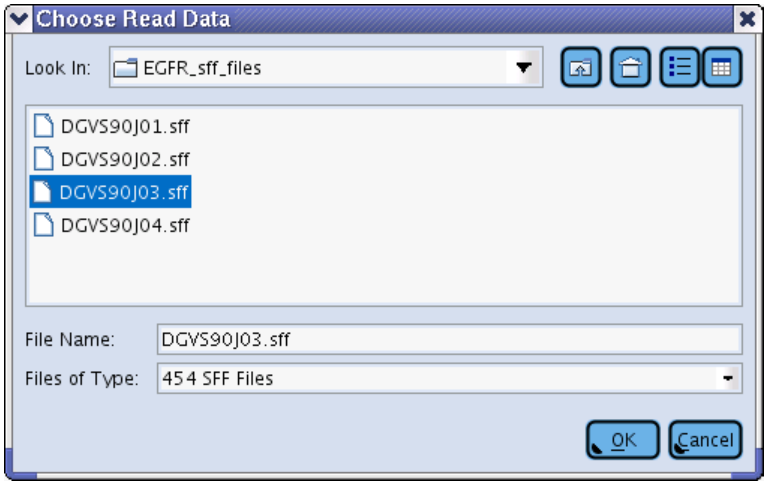


Figure 2-20: The Choose Read Data window, with the DGVS90J03.sff file selected

Clicking “OK” opens the Import Read Data window. We choose to use the default ‘Read Group Name’, and to import the data file itself (as opposed to simply creating a symbolic link) (Figure 2-21).

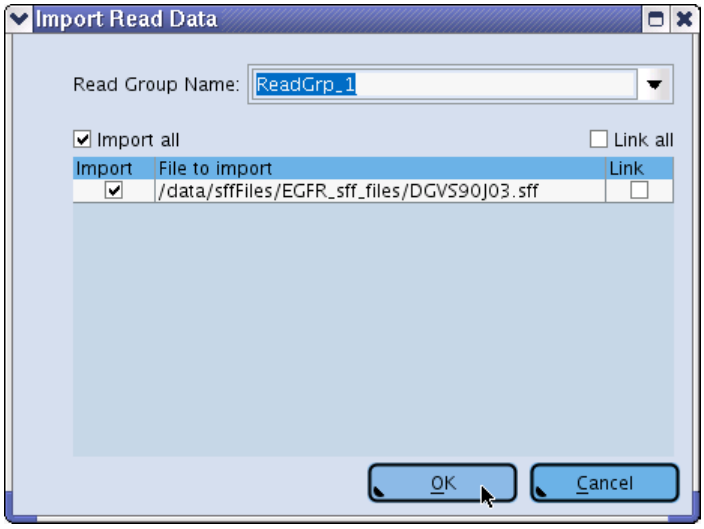


Figure 2-21: The Import Read Data window, ready to import the file selected

Clicking OK returns us to the AVA window and adds the new Read Data Set to both the Read Data Tree and the Read Data Table (Figure 2-22).

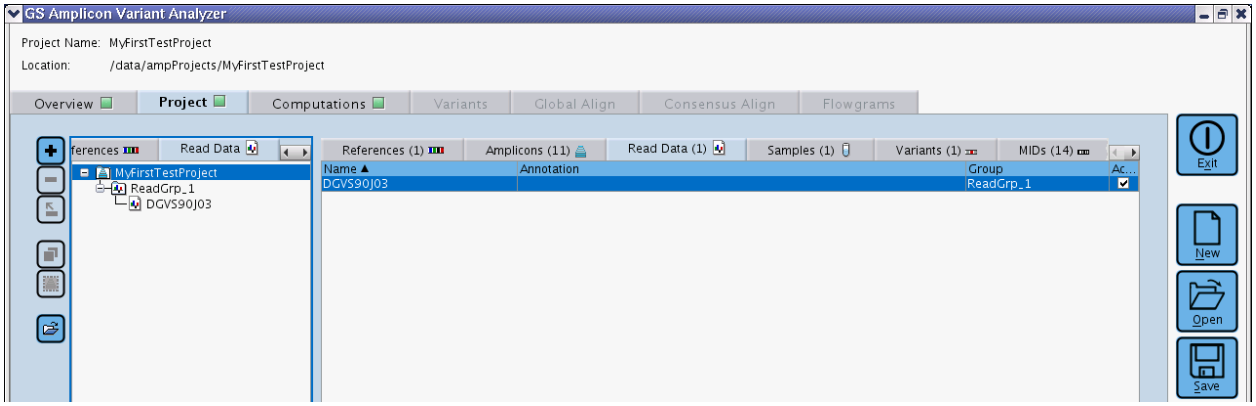


Figure 2-22: The AVA window, after importing the DGVS90J03.sff Read Data file

Finally, we must associate the Sample-Amplicon groups with the Read Data, so the AVA software can properly demultiplex the reads in the Read Data and assign them to their respective Amplicons. To do this, we select the Read Data Tree and the Samples Definition Table, and drag the Sample_1 to the DGVS90J03 Read Data node. This creates the association between the Sample and the Read Data, with the prior Sample-Amplicon associations also maintained (Figure 2-23). We then click the ‘Save’ button to save all the information we entered, in the Project folder.

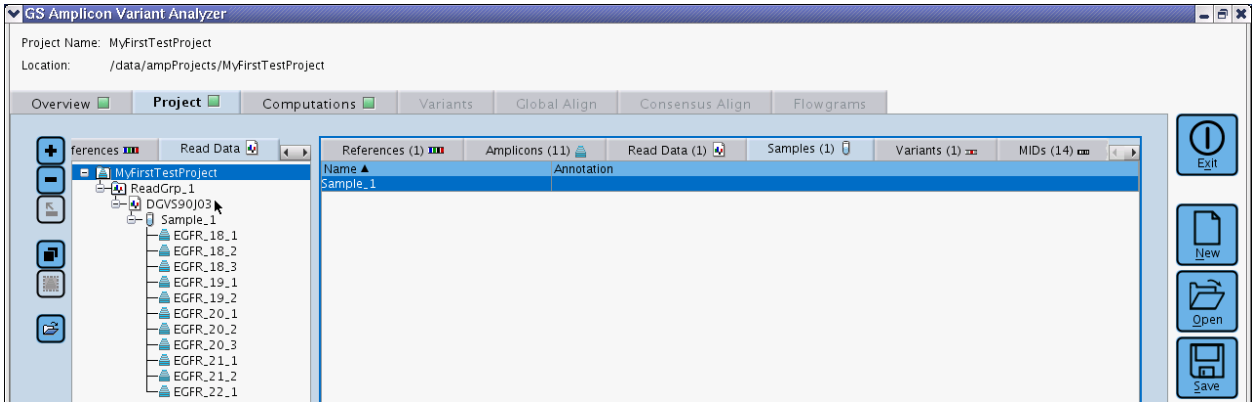


Figure 2-23: The AVA window after creating the association between the Sample_1 and the DGVS90J03 Read Data Set

2.3 Analysis of Known Variants

With the Project fully defined, we can now process ("compute") the Read Data and search for our known Variant, the 15 bp deletion in EGFR exon 19.

2.3.1 Compute the Project

To carry out the computation, we select the Computations tab and click on the "Start Computation" button. Status messages allow us to track progress. Computation is complete when all the 'State' messages say "Done - OK" and the "Start Computations" button is no longer grayed-out (Figure 2-24).

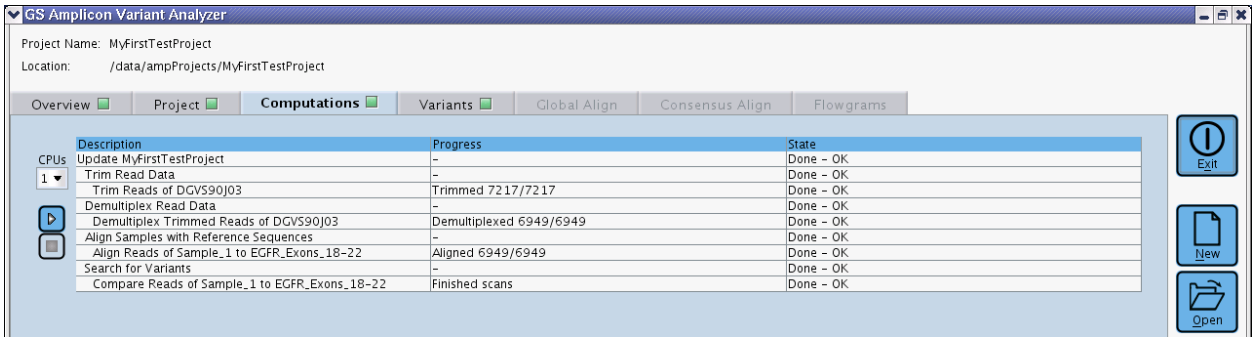


Figure 2-24: The Computation tab, with the computation complete

2.3.2 Frequency of Known Variants

A green square appears on the Variants Tab after completion of a computation that included at least one known (or auto-detected) Variant, which is our case. We click on the Variants tab to observe the results of the analysis (Figure 2-25). We choose to display the frequency and the number of reads of the Variant in the forward, reverse and combined orientations ('All three' and 'Show denominators' settings under "Show values"), to ascertain that the occurrence of the Variant isn't orientation-dependent; the fact that it isn't makes the observation more credible

(verification of support in both orientations is helpful to eliminate false-positives that may occur due to artifacts in alignment or sequencing).

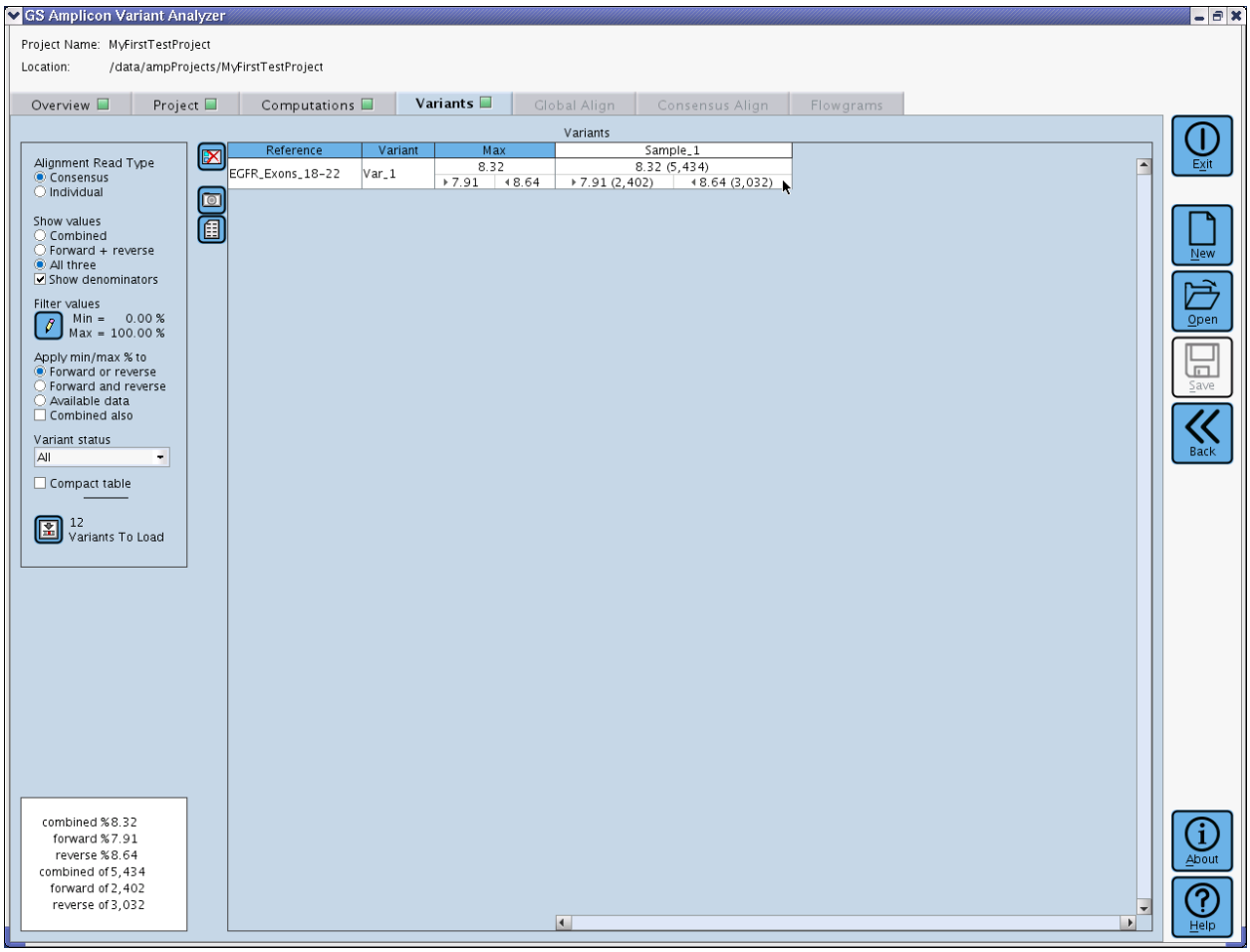


Figure 2-25: The Variants tab after completion of the computation, showing the frequency of the Var_1 Variant in the reads included in the Sample_1 Sample

To further explore our known Variant, we right-click on the cell that intersects with Variant 'Var_1' and 'Sample_1', in the Variants Tab, and choose 'Global Align' from the contextual menu. This loads all the reads corresponding to all the Amplicons that cover this Variant, in Sample_1 (Amplicons EGFR_19-1 and EGFR_19-2; see **Error! Reference source not found.**, above), and displays them in the Global Align tab (Figure 2-26).

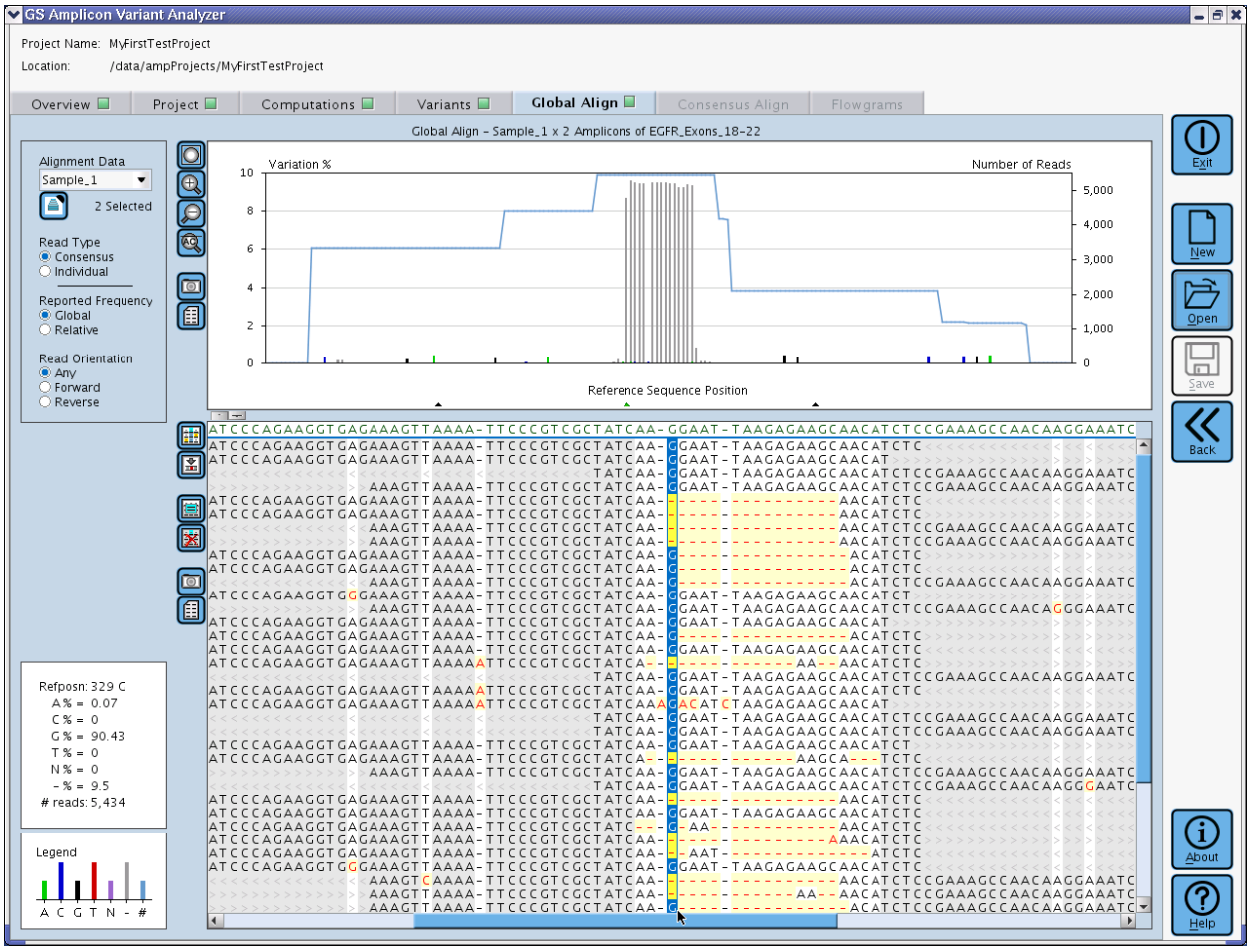


Figure 2-26: The Global Align tab, loaded with the reads relevant to Variant Var_1 in Sample_1

The area where the Variant is located is where a cluster of gray bars (indicating “gaps”, the deletion) can be seen, near the middle of the Variation Frequency Plot (the top panel). To get a better look, we draw a rectangle with the mouse, around the zone of interest in the Plot (this is the “Freehand Zoom In” tool). Then, by clicking on one of the gray bars in the plot, we can re-center the multi-alignment Table to the same area (Figure 2-27).

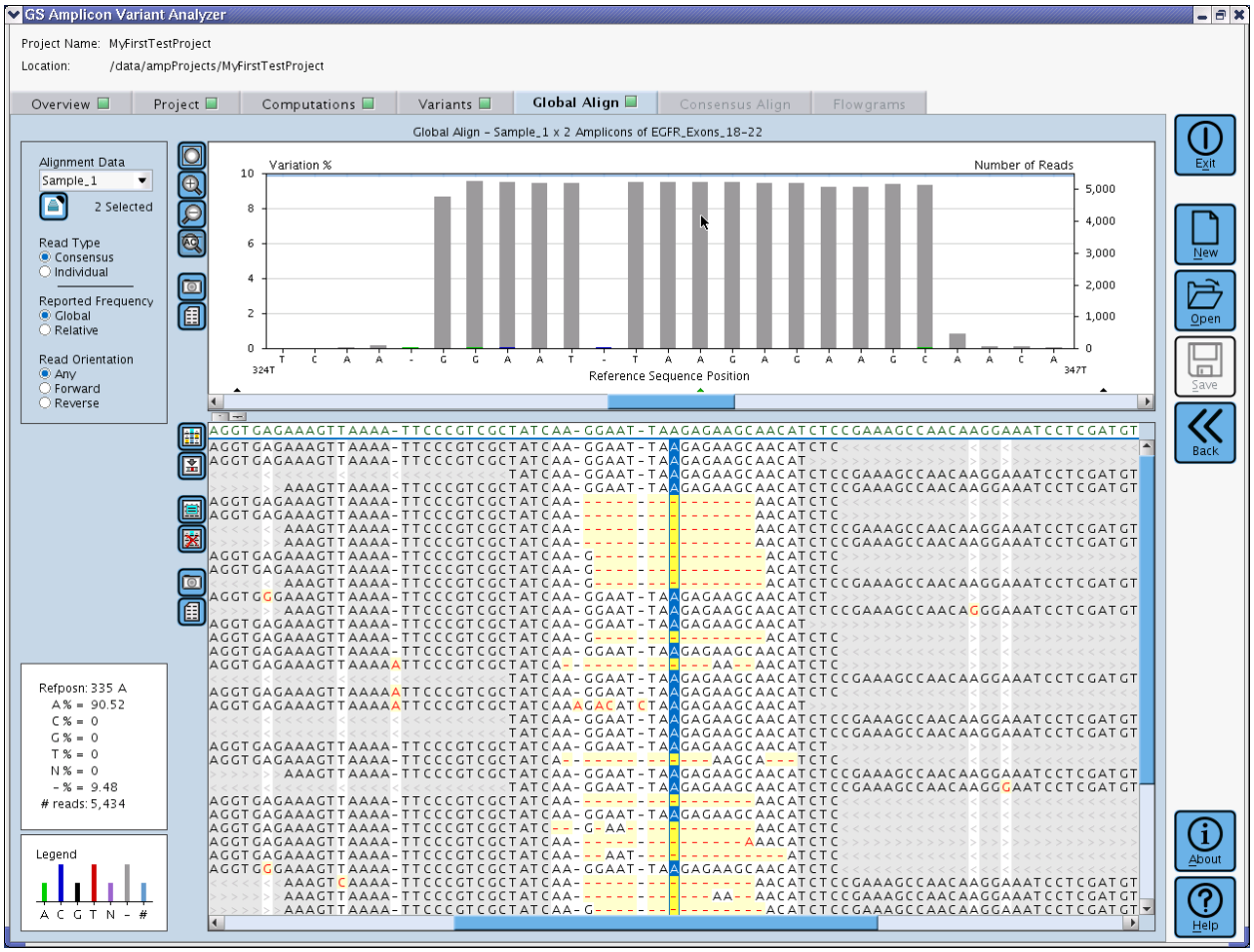


Figure 2-27: The Global Align tab for Var_1 in Sample_1, with the Variation Frequency Plot zoomed in around the deletion and the multi-alignment showing several consensi with a stretch of gaps

As can be seen, several of the consensi visible in the multi-alignment have many gaps in this region. To explore these in particular, we can select for viewing only the consensi with these gaps. This is done by right-clicking on a base (of any consensus) in a column within the stretch of gaps, and selecting for the gap character '-' in the contextual menu. The result, shown in Figure 2-28, is that only the consensi that have a gap at the position on which the selection was made (position 335 of the Reference Sequence in this case) are now displayed in the multi-alignment, and the Variation Frequency Plot is adjusted accordingly. Note in particular that the frequency axis (Variation %) is automatically re-scaled to best fit the data displayed, allowing us to clearly see that all the nucleotide positions in the stretch have the gap at a fairly consistent frequency, an observation consistent with a valid Variant. Note also that the frequency of 9.48% is close but a little on the high side compared to the value seen in the Variants Frequency Table for Var_1 (8.32%). The difference is caused by the fact that we made only one selection to focus the plot on the deletion area; not all the reads being displayed perfectly match our defined Variant. In part this is because there are some consensus reads representing basecalling/alignment problems that keep them from being counted as part of the Variant for the Variants Table frequency calculation. But more significantly, in this case, there is another deletion variant present that, as compared with Var_1, is shifted by a single base and is present at 0.82% (see the full list of automatically detected variants in Figure 2-44, below).

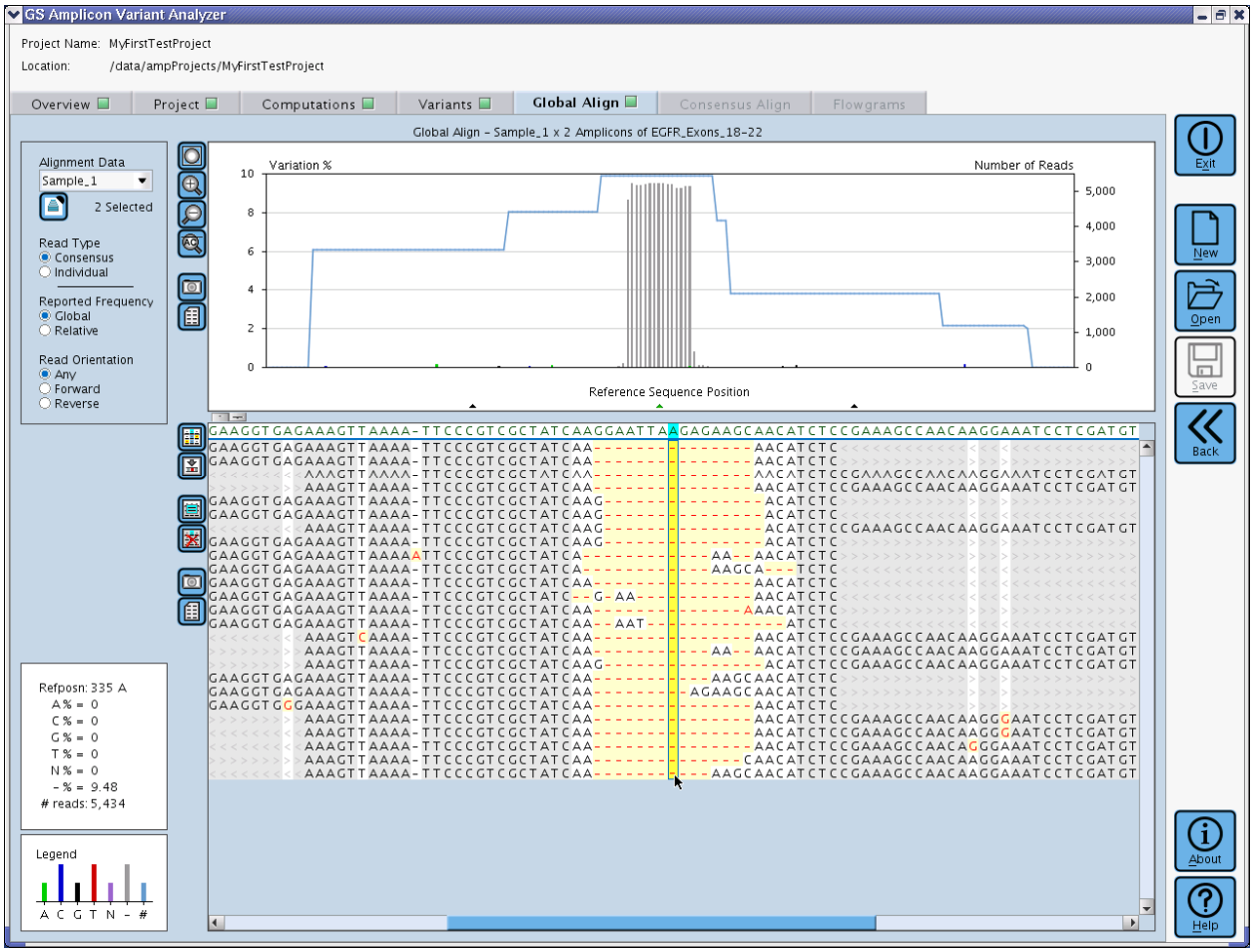


Figure 2-28: The Global Align tab for Var_1 in Sample_1, with a selection for gaps applied to position 335 of the Reference Sequence (in the stretch of gaps)

We will now dig further by examining the individual reads that comprise the third of these consensi (CON_46). To do this, we right-click on the nucleotide at position 335 of this consensus (to keep the focus at the same location) and select the 'Open Consensus Alignment' option from the contextual menu. This loads the Consensus Align tab with a multi-alignment of all the reads that contributed to the consensus on which we clicked (Figure 2-29). This view shows that certain reads lack an extra "A" nucleotide, compared to the rest of them. Looking at the sequence carefully we notice that the deletion has created a homopolymer of "A", suggesting that the minority "gap extension" may actually be due to an undercall of this homopolymer in the reads that show it; this is supported by the fact that this is especially observed in reads in the reverse orientation (as shown in Figure 2-29), which places an environment very rich in "A" nucleotides just before the gap.

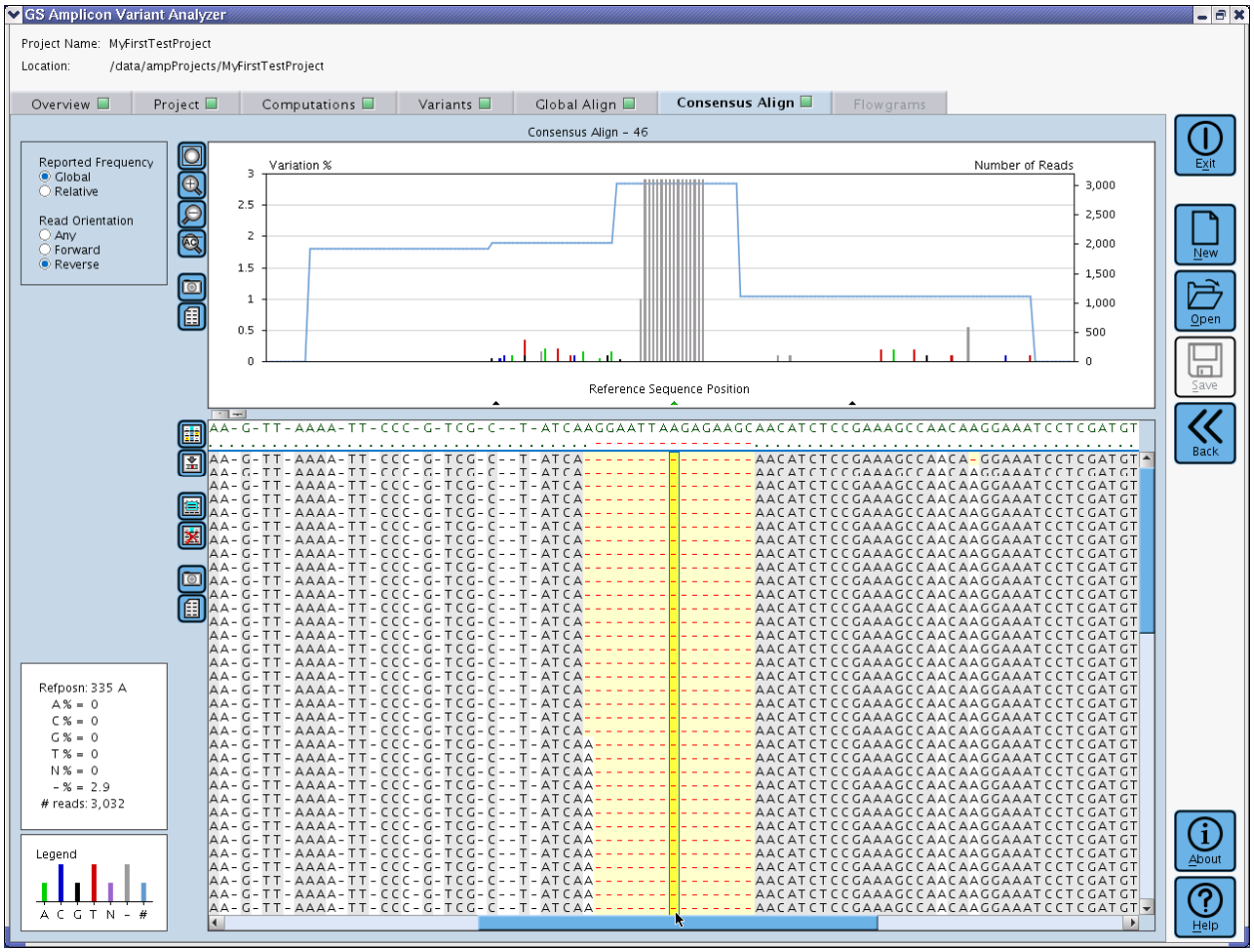


Figure 2-29: The Consensus Align tab for the reads included in the third consensus of the Var_1 Variant in the Sample_1 global alignment shown in Figure 2-28

Finally, we go to the finest level of detail by examining the flowgram of the first read in this multi-alignment. Again, we right-click on the nucleotide position corresponding to position 335 of the Reference Sequence for the convenience of keeping the focus at the same location, and we select the 'Open Flowgrams' option from the contextual menu. This loads the Flowgrams tab with the flowgram data for the read on which we clicked (Figure 2-30).

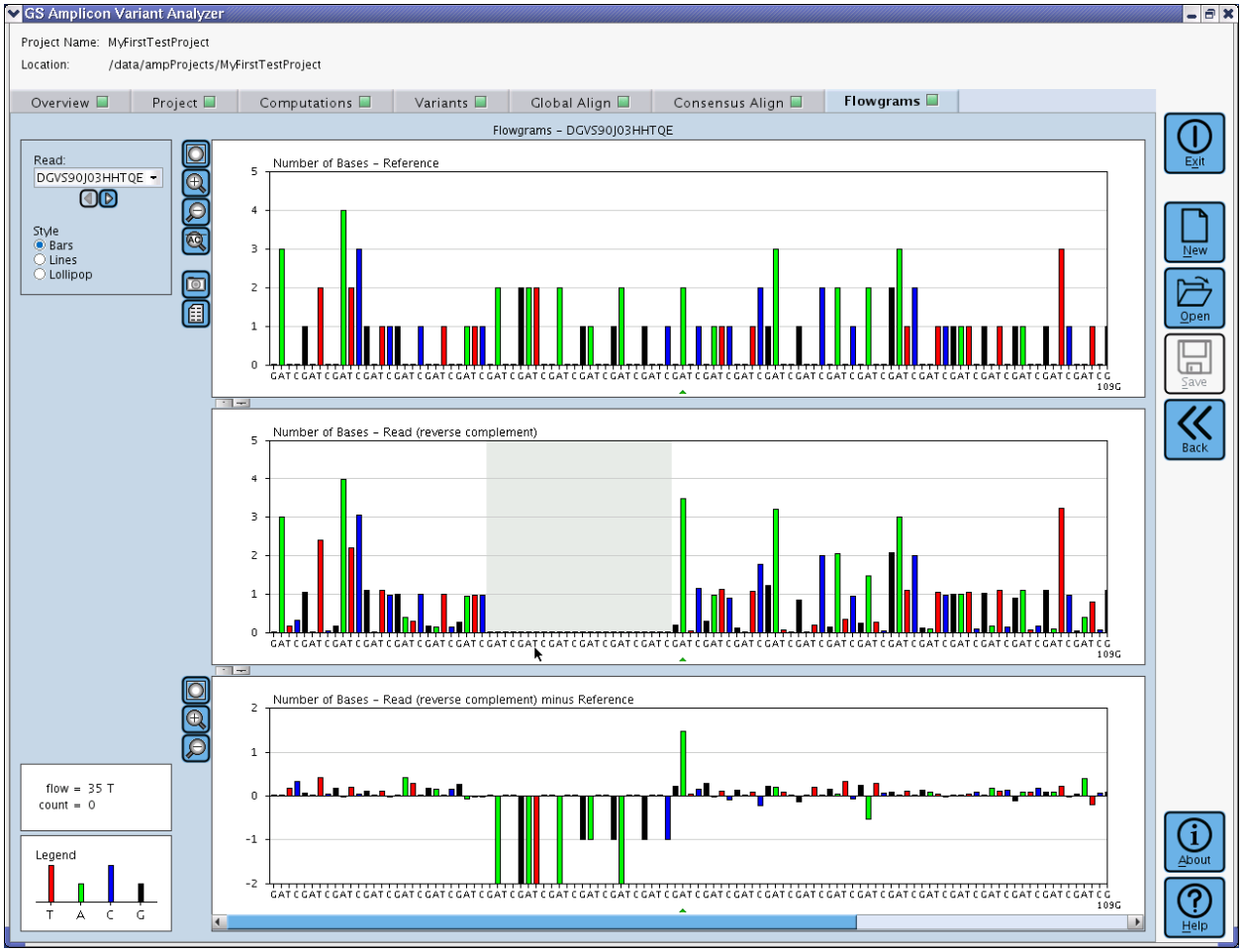


Figure 2-30: The Flowgram tab for the first read of the third consensus of Var_1 in Sample 1 Consensus Align view of CON_46 (in Figure 2-28), showing that a gap of several nucleotide flow cycles in the read allows it to maintain alignment with the Reference Sequence on both sides of the gap

We clearly see that in order to maintain the alignment with the Reference Sequence, the software introduced a gap of several nucleotide flow cycles at the position of the deletion (marked in gray in the read flowgram); this is very strong evidence for the presence of a true deletion. The elevated 'A' flow after the gap is caused by the splicing together of the two 'A' pairs on either side of the deletion into a single 'A' 4-mer.

We can use the "arrow" buttons at the top left of the tab to "scroll" over the flowgrams of the reads present in the Consensus Align tab and see how "stable" any particular flow or set of flows in the window seems to be. We can do this by focusing on a feature of the read on the difference flowgram as we scroll through the available flowgrams to see how the magnitude of the feature changes from read to read (the green triangle below each flowgram can serve as a useful focus point when scrolling through the reads).

The initial deletion peaks in the graph on the Global Align tab plot were seen at a moderate percentage range (8.65 - 9.48%). The underlying alignments show that the deletions were linked together as a 15-bp deletion haplotype. The underlying flowgrams of reads exhibiting the haplotype further show that the deletions were not due to marginal calls, and demonstrate that the flows needed to be shifted to align properly. Taken together, the evidence is compelling that

this 15 bp deletion is a true Variant in the sample. The 8.32% combined frequency for the Variant on the Variants Tab is a conservative estimate that seeks to measure perfect instances of the defined Variant in the context of consensus reads that, by their combination of individual reads, can distort the frequency statistics. So the actual frequency of the variation in the Sample is likely higher than 8.32%. As seen in Figure 2-44, below, the combined Var_1 percentage, based on individual reads is 8.79%, closer to the lower range of observed deletion peak values. Further inspection of the alignment suggests an overlapping deletion (see the 5^{th}, 6^{th} and other consensus lines of Figure 2-28 that end with a G just inside the deletion, rather than an A, and which end one base later inside the deletion, with a single A, rather than the double AA as occurs with Var_1). This additional deletion is reported as an automatically detected Variant (Figure 2-44) with a combined frequency of 0.82%, and helps explain the range of deletion peaks observed.

2.4 Mining a Project for New Variants

We started the project with only one predefined Variant. As part of the computation done to measure our defined Variant, the AVA software also examined the alignments in the Project to propose potential Variants. We can access them via the main Variants tab. If we look again at the view of the Variants tab in Figure 2-25, we can see that the “Load” button at the bottom left of the Variants Frequency Table filter control box states that there are 12 Variants to load. The automated Variant detector is sensitive and likely to include false positives, so it is wise to use some of the filters to narrow down the potential set of variants rather than just importing them all.

By setting the “Min” value to 5.00% and choosing the “Forward and reverse” filter on the Variants Tab, the status of the “Load” button changes to show that there is only one Variant to load that meets the criteria. Pressing the “Load” button adds the new Variant to the Project, and the “Load” button becomes grayed-out with the “No Variants To Load” status message (Figure 2-31).

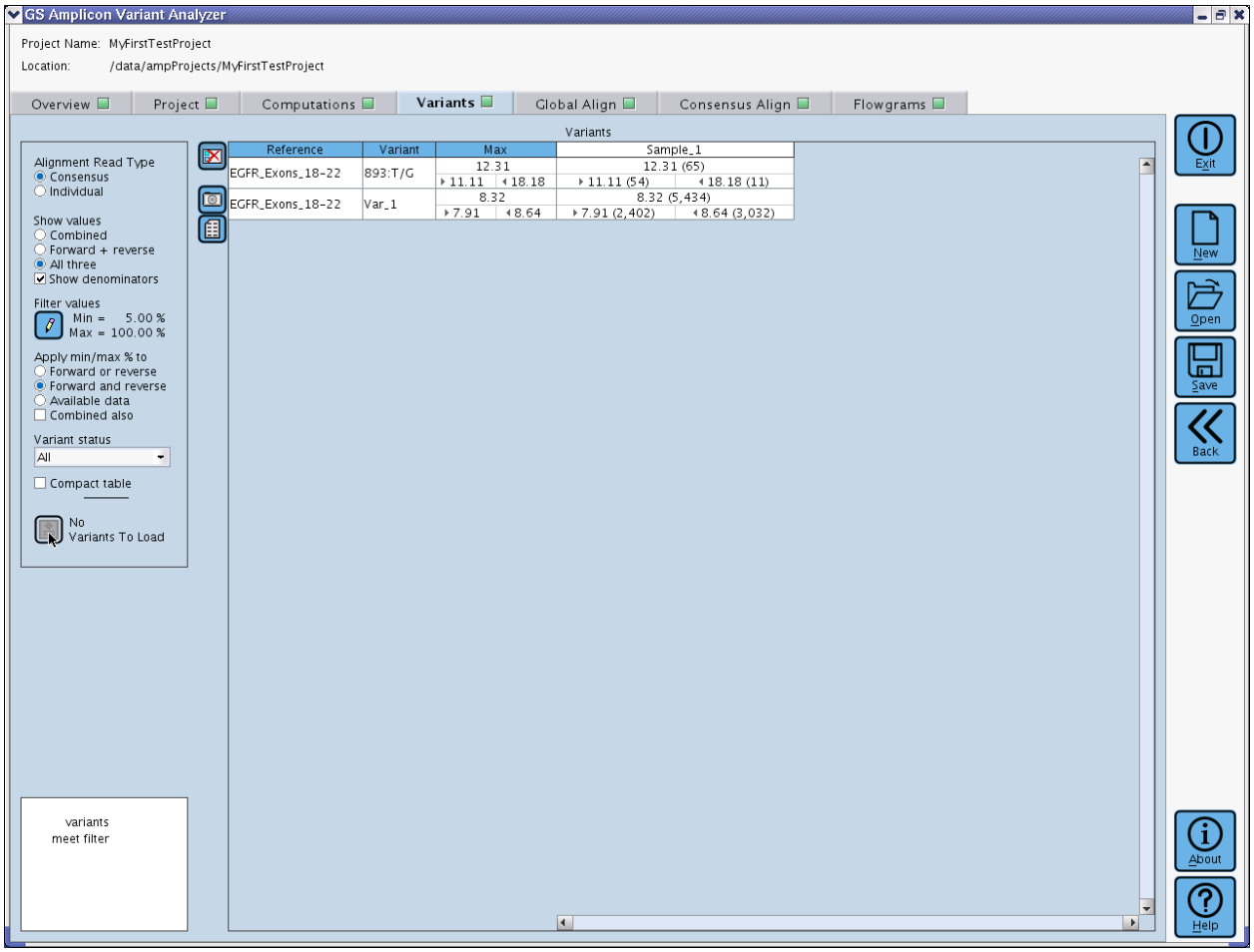


Figure 2-31: The Variants Tab after setting filters and loading the lone surviving Variant

After right-clicking on one of the frequency cells for the new Variant (893:T/G) in the Sample_1 column, we can use the “Global Align” in the menu to load the Global Align tab with the reads covering the Variant position for Sample_1. The global alignment (Figure 2-32) reveals that the Variant is covered by the EGFR_21_2 Amplicon and that there is an imbalance between forward and reverse read representation for this Amplicon. However, the Variant is present in both forward and reverse reads and has a combined frequency of over 12%, so it could well be a legitimate Variant. By right-clicking on the forward consensus containing the Variant, we can navigate to the consensus alignment.

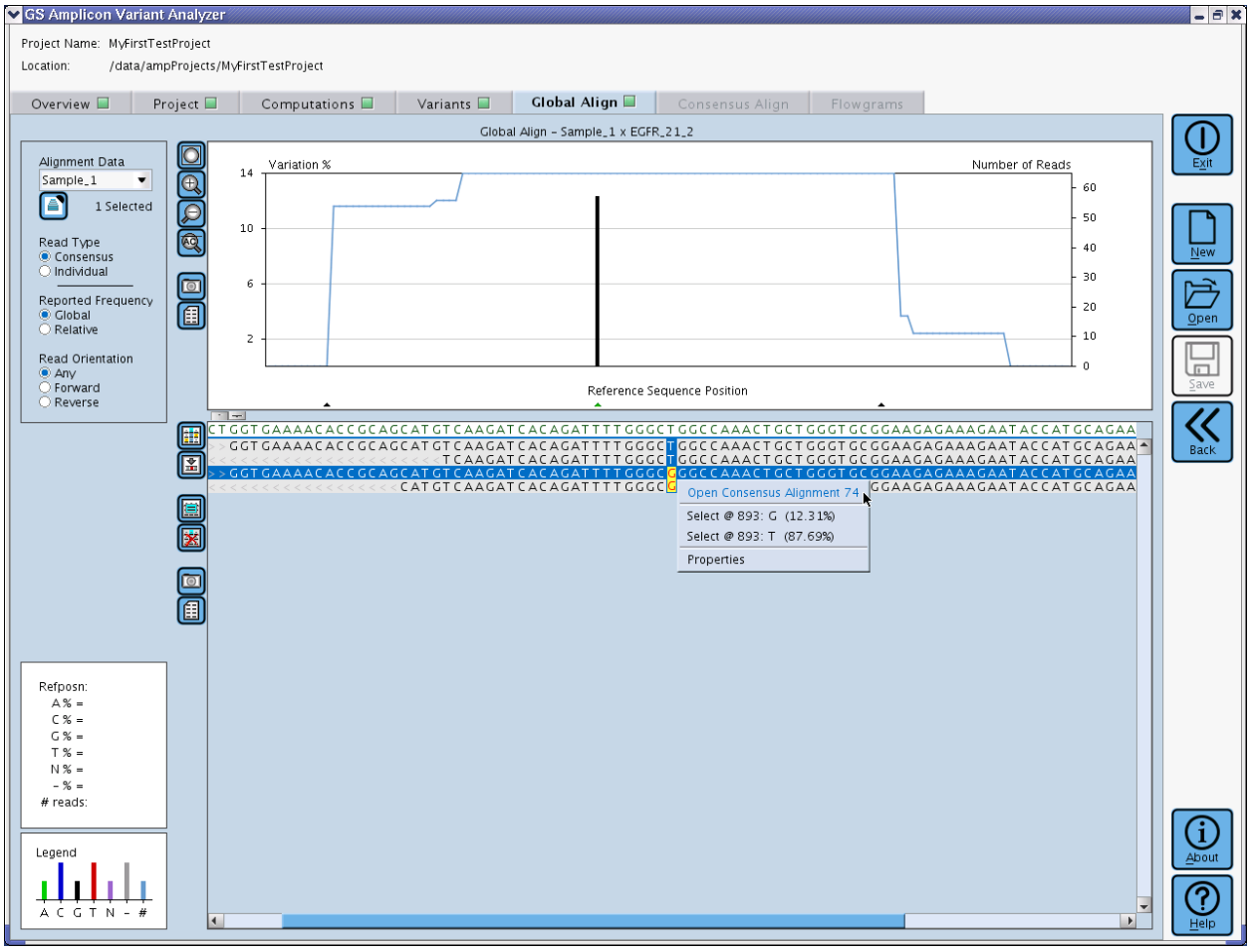


Figure 2-32: The Global Align tab displaying the Consensi for Sample_1 covering the region of the "893:T/G" Variant. The right-click context-sensitive menu of the forward consensus is shown in preparation for navigating to the Consensus Align tab.

The Consensus Align tab (see Figure 2-33) shows the 6 (forward) reads that comprise this Consensus, all of which contain the Variant of interest. However, one of those reads has an additional variation (an "A" to "G" substitution at position 915). The automated Variant detection does not scan for haplotypic variations (except for contiguous deletions), so even if this haplotype is real, we would never see it in the Variants Frequency Table unless we introduce the haplotypic variation to the Project manually (although we might encounter the parts of a haplotype in the table, individually).

To define this haplotype, we use the alignment filter selections to narrow down the view to meet the new haplotype: we right-click over the columns of interest in the alignment (893 and 915) and select the Variant base for those columns ("G" for both) (Figure 2-33).

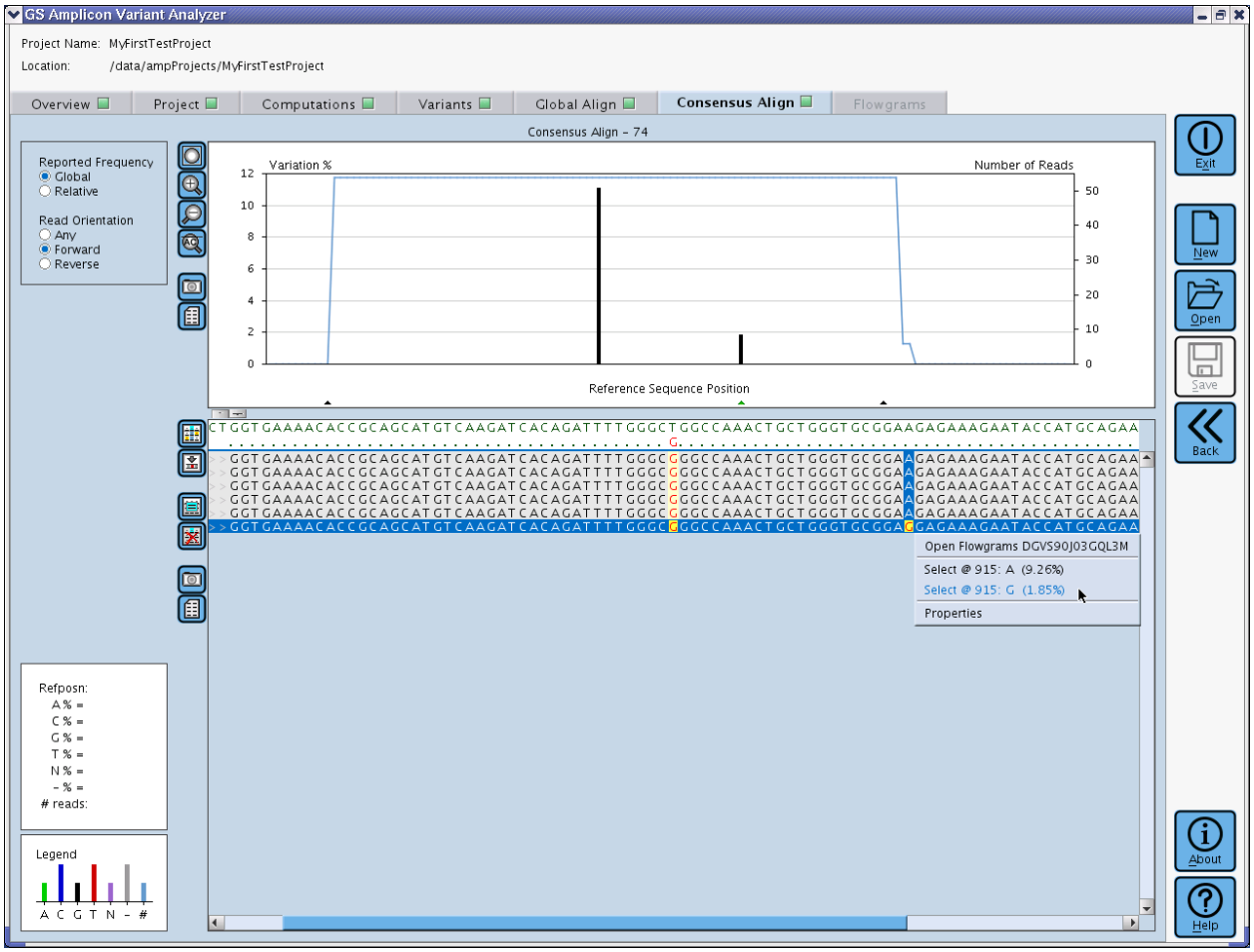


Figure 2-33: The Consensus Align tab displaying the forward reads for Sample_1 with the "893:T/G" Variant. The right-click context-sensitive menu is shown in preparation for making a second filter selection on the alignment (a "G" at position 915).

After both selections are made, we have narrowed down the view to a single read. Although a single read isn't very good evidence of a true Variant, we are going through this exercise just to show how a haplotype might be defined. We can right click over this read (Figure 2-34) to load the Flowgrams tab so we can judge whether the variations look real or not.

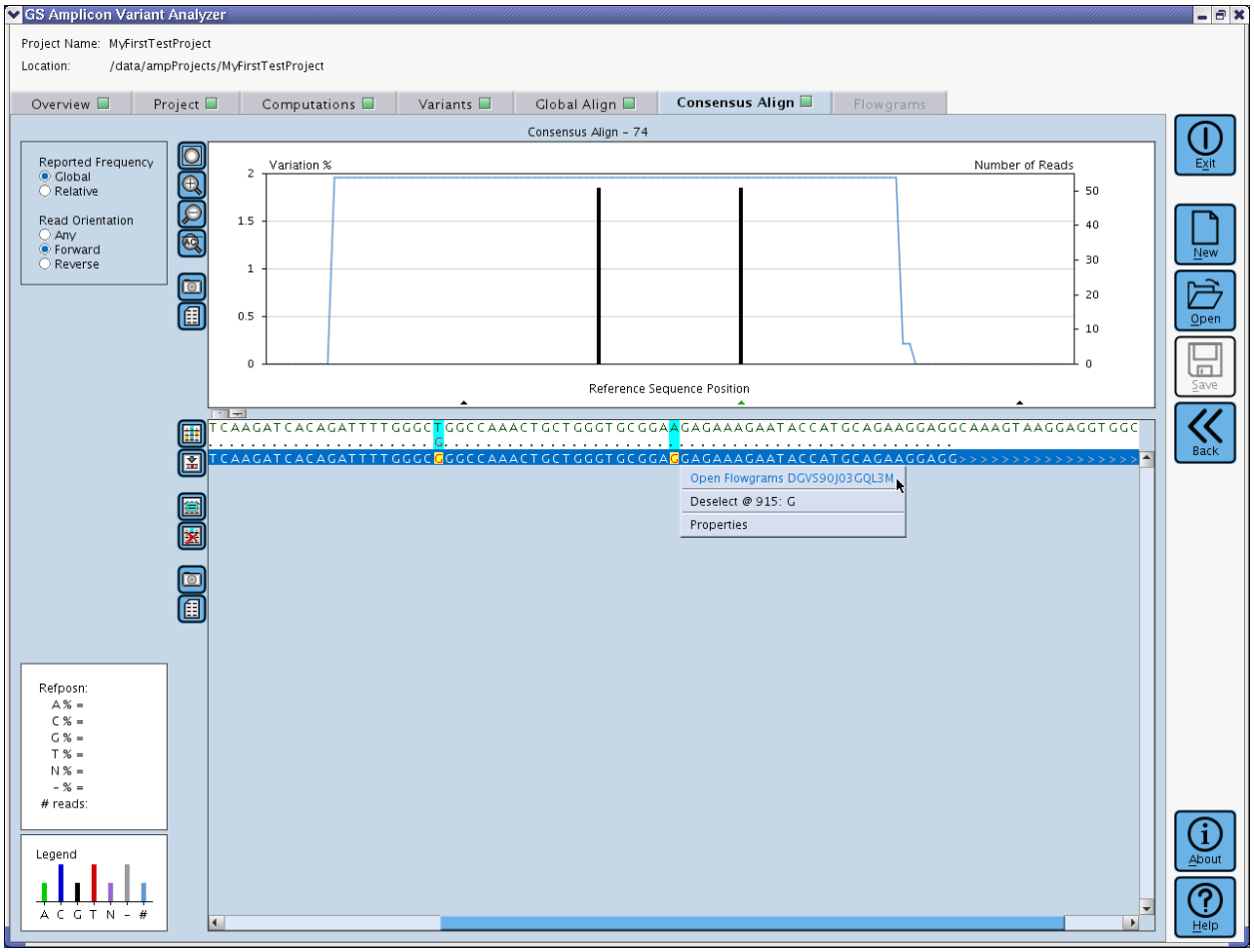


Figure 2-34: The Consensus Align tab displaying the only read with a haplotype including the "893:T/G" Variant and the "915:A/G" Variant. The right-click context-sensitive menu is shown in preparation for navigating to the Flowgrams tab.

The Flowgrams tab view (Figure 2-35) shows that based on the actual flows of the read, the haplotype appears convincing (or would be if it were supported by multiple reads): the original "893:T/G" substitution Variant exhibits a flow cycle shift (the gray column in the middle flowgram for the read). Furthermore, the flow values for the original Variant and the new Variant ("915:A/G") are not marginal: the difference flowgram at the bottom shows the reference bases for both Variants decreasing by a solid value of '1' each, and likewise, the replacement bases are each also increased by '1'. Although we have seen only one instance of this haplotype we will go ahead and set up the haplotype as a Project Variant so we can see if any other instances are to be found (it is conceivable that the haplotype could be hidden in other consensi).

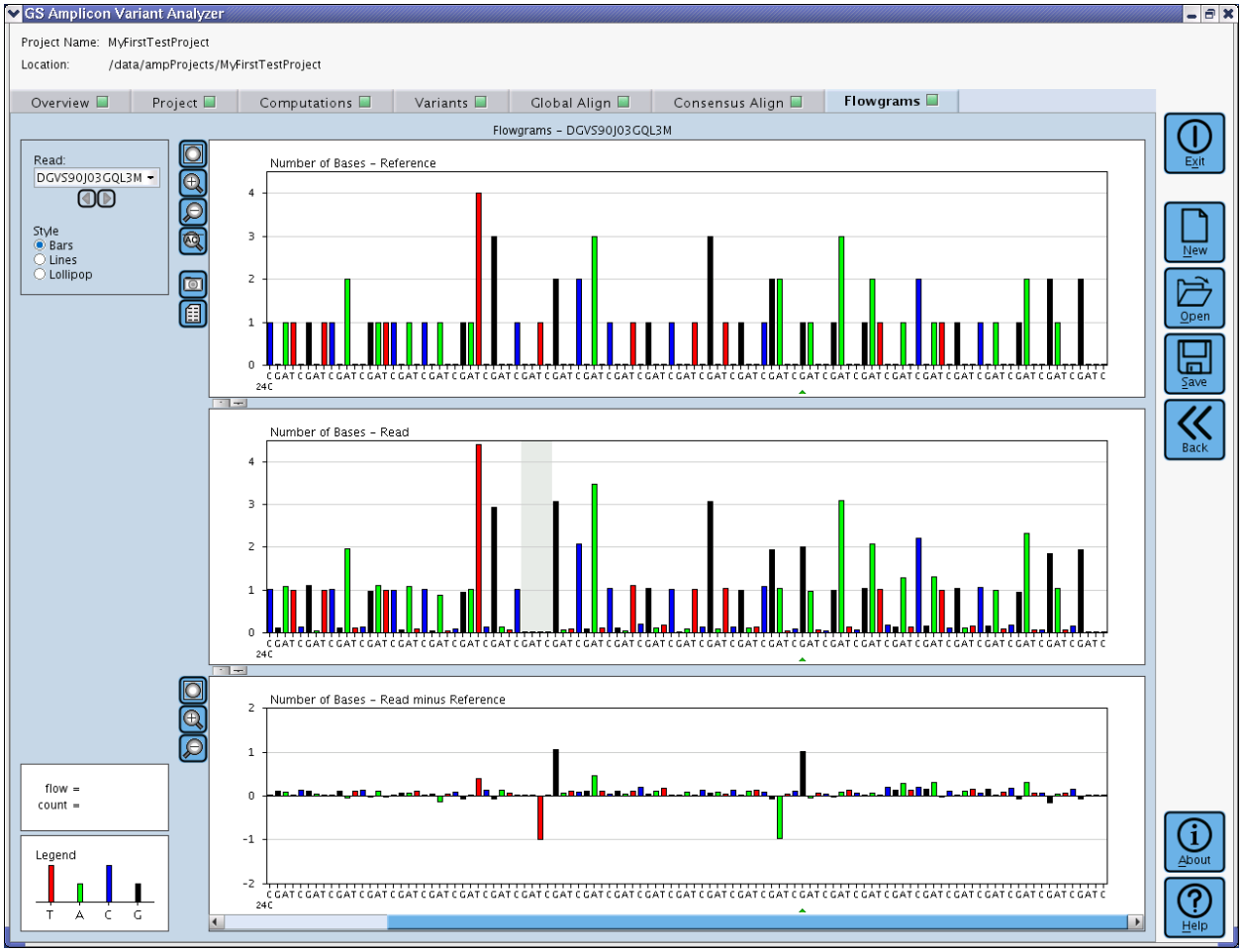


Figure 2-35: The Flowgrams tab for the read displaying the haplotype including the "893:T/G" Variant and the "915:A/G" Variant. The gray shaded column in the middle flowgram denotes a flow cycle shift caused by the "T" to "G" substitution of the first Variant, while the other Variant is evident in the loss of an "A" and the gain of a "G" in the bottom difference flowgram.

To add our haplotype to the Project as a Variant, we can return to the Consensus Align tab where we have already made the appropriate filter selections (Figure 2-34). We click on the "Declare project variant" button to the left of the alignment, and the "Approve new variant" window opens (Figure 2-36). The automatically created default name for the variant is a sensible concatenation of the two individual Variant names, sorted by position ("893:T/G,915:A/G"), and the rest of the defaults are reasonable so we can click "OK" to define the haplotype as a Variant to be searched for in subsequent rounds of computation.

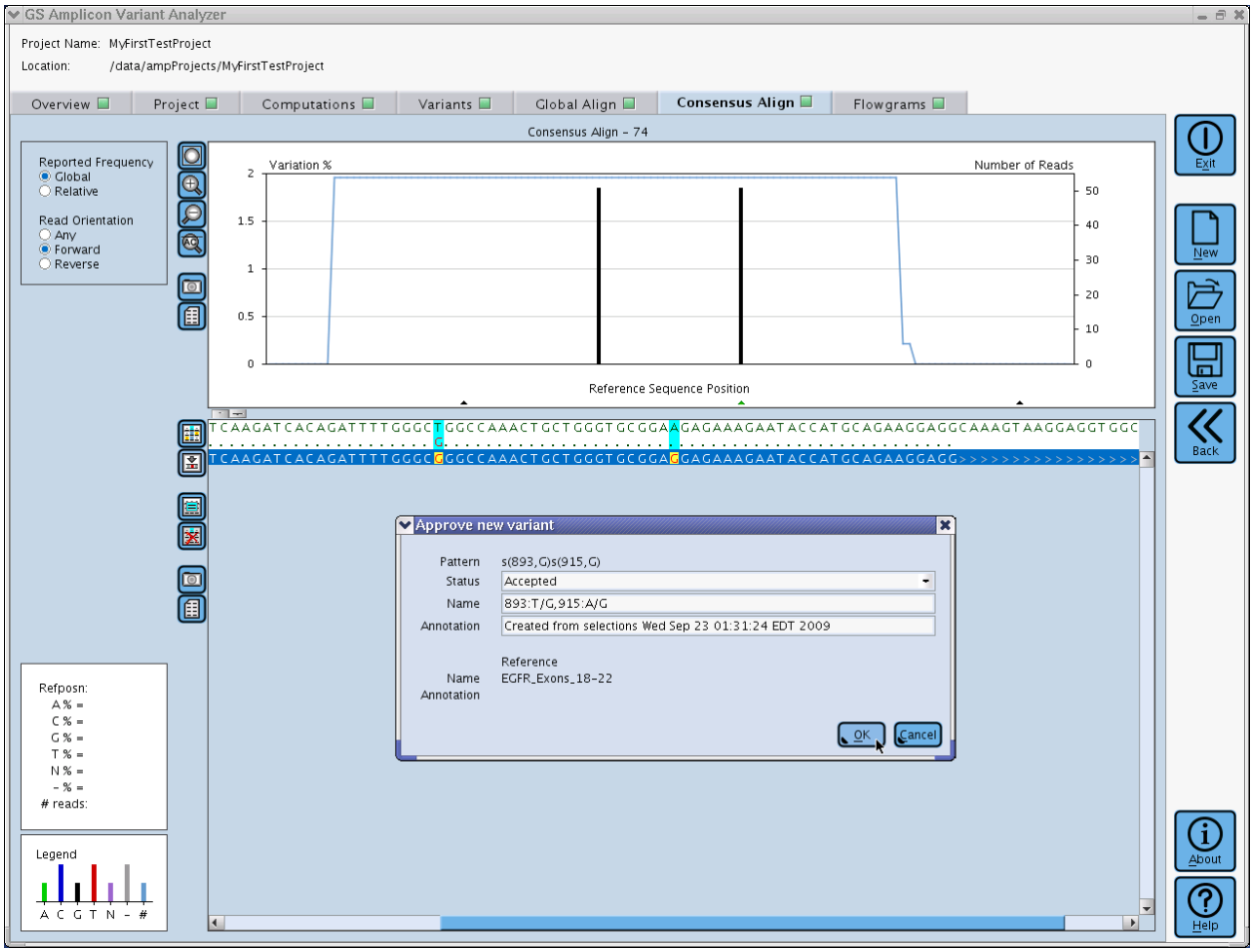


Figure 2-36: Creating a Variant from selection filters in the Consensus Align tab

Clicking on the “OK” button to define the haplotype Variant for the Project is a little anti-climactic because the creation takes place behind the scenes and you remain on the same tab we were on when we submitted the Variant. To view the Variant we just created, we can select the Variants sub-tab of the Project Tab to see the Variant definition table. That view also enables us to edit the Status of individual Variants in the Project. The Var_1 Variant that we entered manually (section 2.2.6) was automatically marked as “Accepted”. The Auto-Detected Variant that we loaded into the Project (“893:T/G”) defaulted to “Putative”. The haplotype Variant that we manually created from filter selections defaulted to “Accepted”. Now that we have had a chance to look at the data, we can make some reasonable Status changes by double-clicking on the Status field of Variants in the table (Figure 2-37). We should set the Auto-Detected Variant to “Accepted” rather than “Putative” since we saw ample evidence that it is real. The haplotype, however, is very questionable because it is supported by a single read, so we will demote it to “Putative”. Alternatively, we could have initially created the haplotype with the “Putative” status, changing the default “Accepted” status in the “Status” drop down menu (as seen in Figure 2-36) to “Putative”, prior to clicking the “OK” button in the “Approve new variant” popup.

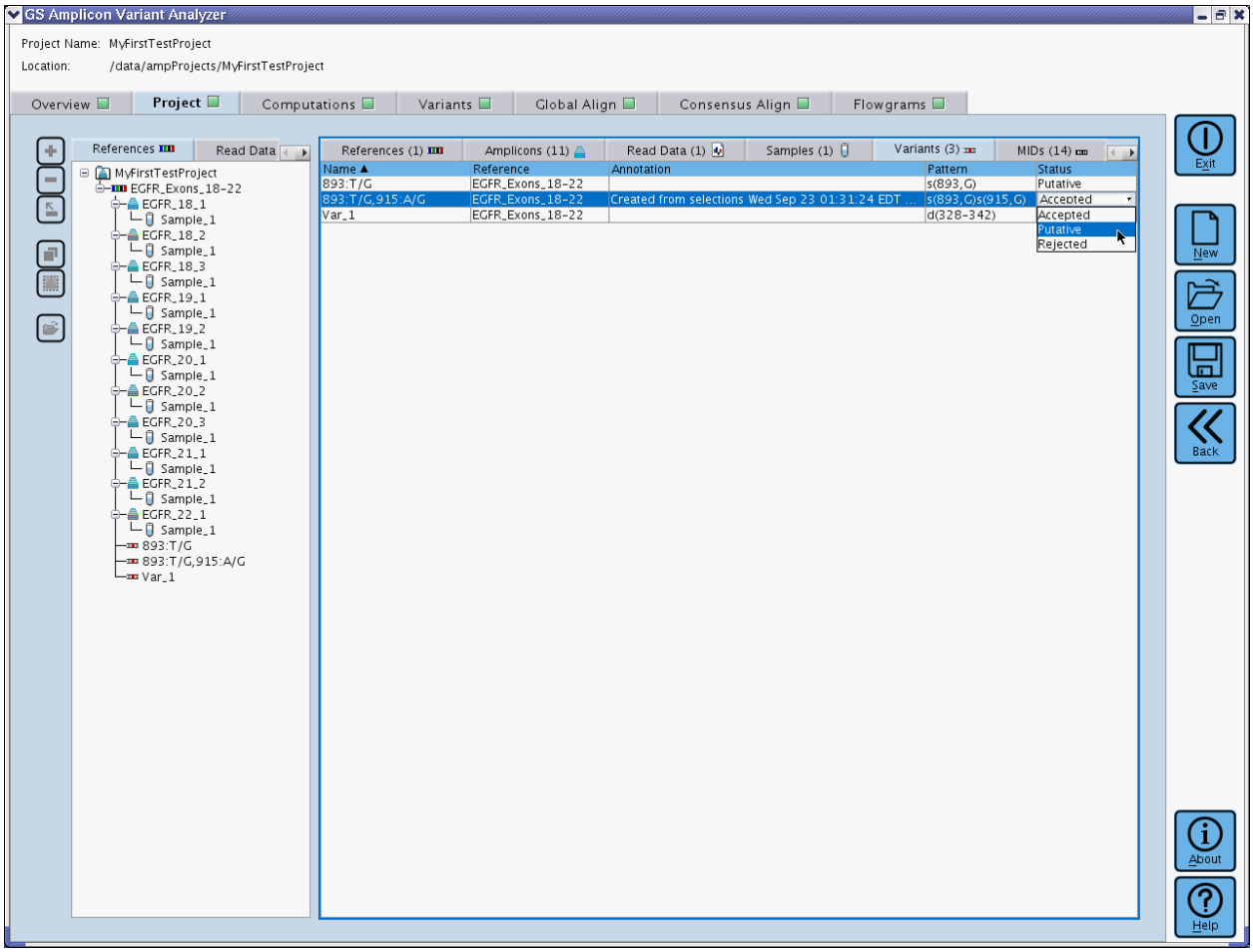


Figure 2-37: Changing the Status of Variants in the Variants sub-tab of the Project Tab

With our new Variants defined, we are ready to compute the Project again to get frequencies for the new Variants. If we rush off to the Computation tab and press the start button, we will get a warning (Figure 2-38): we forgot to save the Project first. After hitting “No” or “Cancel” and pressing the “Save” button for the Project, we should be able to start the computation successfully.

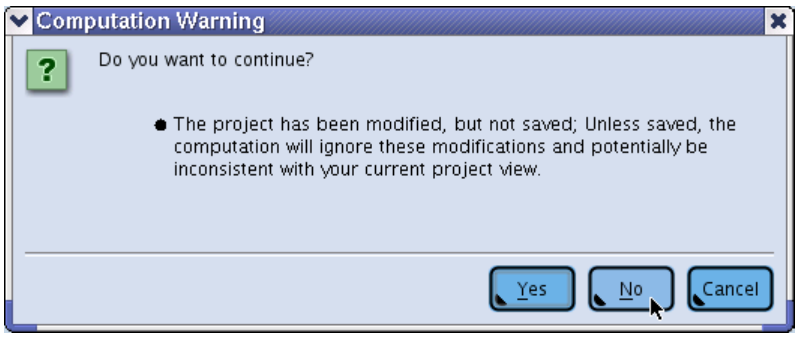


Figure 2-38: Warning message alerting the user that a re-computation is being set up but that some details of the Project have not been saved to disk

The computation should finish very quickly (Figure 2-39). Note that the computation made use of cached results from the previous computation (section 2.3.1). Except for the demultiplexing step, which is rerun with every computation, the only novel work the computation had to do was the "Search for Variants" step.

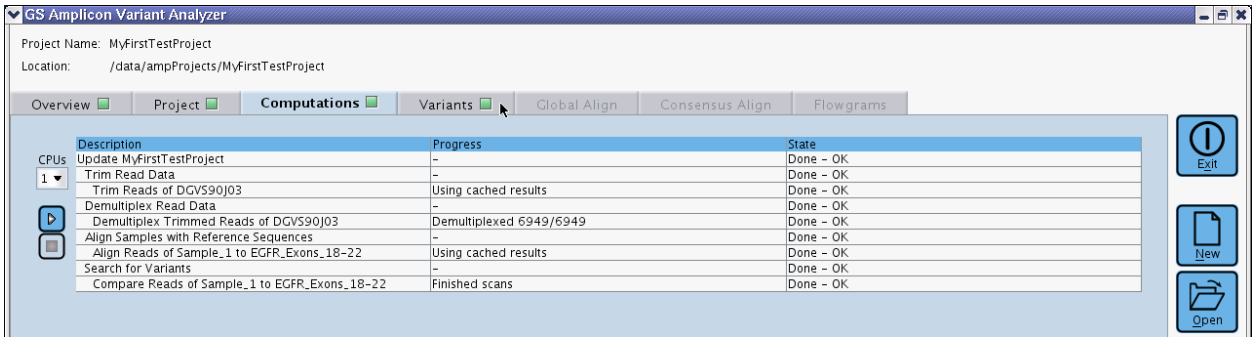


Figure 2-39: The Computations tab showing the results of a second round of computation on the Project, including the use of cached results but a new Variant search

After the computation is complete, we can click on the main Variants Tab to see the frequencies of our Variants in our Sample. The haplotype Variant we defined appears to not have been detected at all in the initial view of the Variants Frequency Table (Figure 2-40; frequency of 0.00% with a total of 65 reads, and grayed out row); this is because the haplotype Variant was defined from an individual read that was buried inside a consensus sequence, but the "Alignment Read Type" filter happens to be set to "Consensus" in the current view.

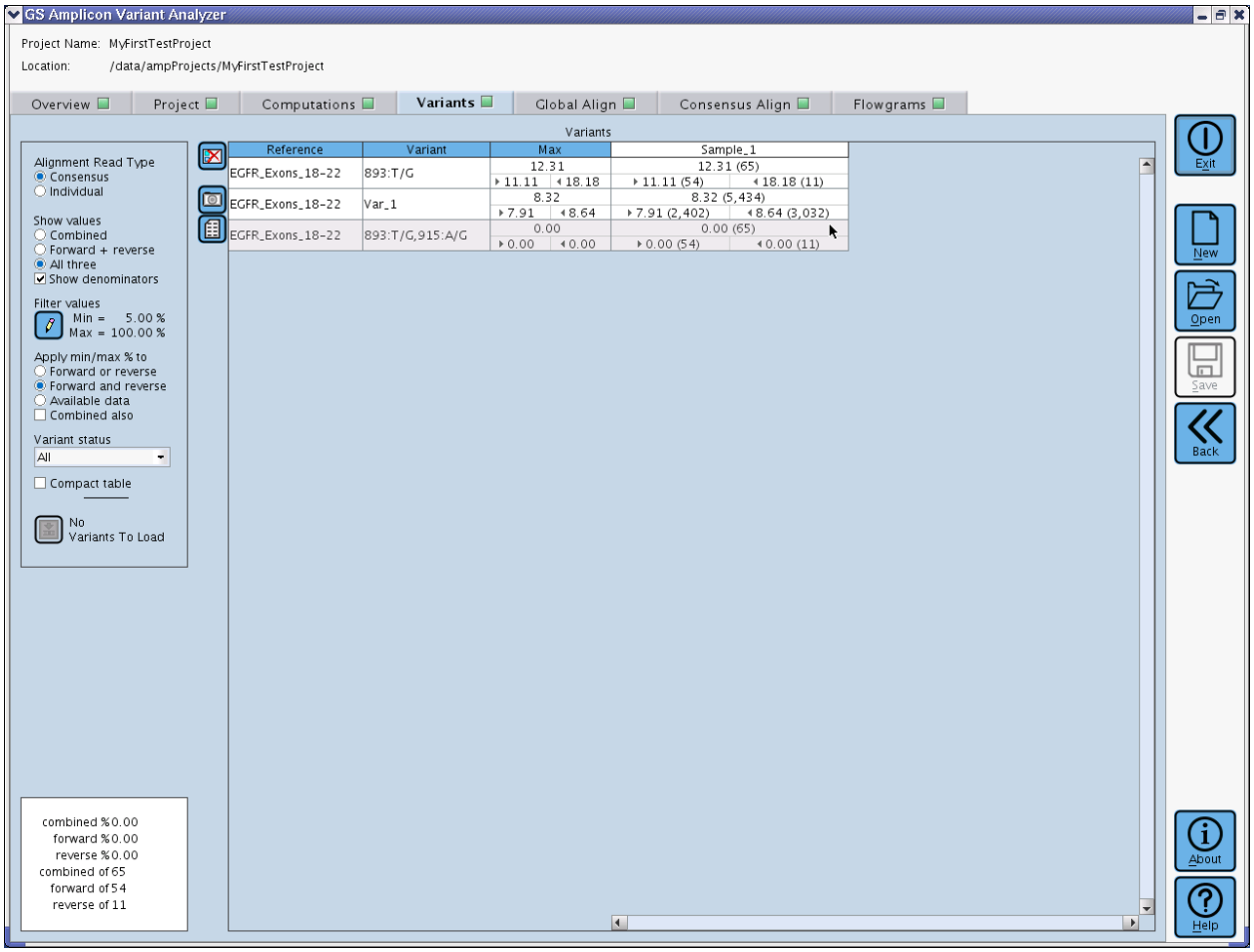


Figure 2-40: The Variants Tab showing the results of a second round of computation on the Project. The haplotype Variant was not detected in this view because the "Alignment Read Type" is set to "Consensus".

If we toggle the "Alignment Read Type" to "Individual", we can see that the haplotype Variant was not missing entirely (Figure 2-41). The frequency of 1.54% out of 65 reads for this Variant reveals that only one read was found with the haplotype (the very one we used to define it). Without further supporting evidence, this haplotype Variant should probably not be considered legitimate despite the fact that the flowgram evidence was good: it is most likely a read that had a PCR error at position 915.

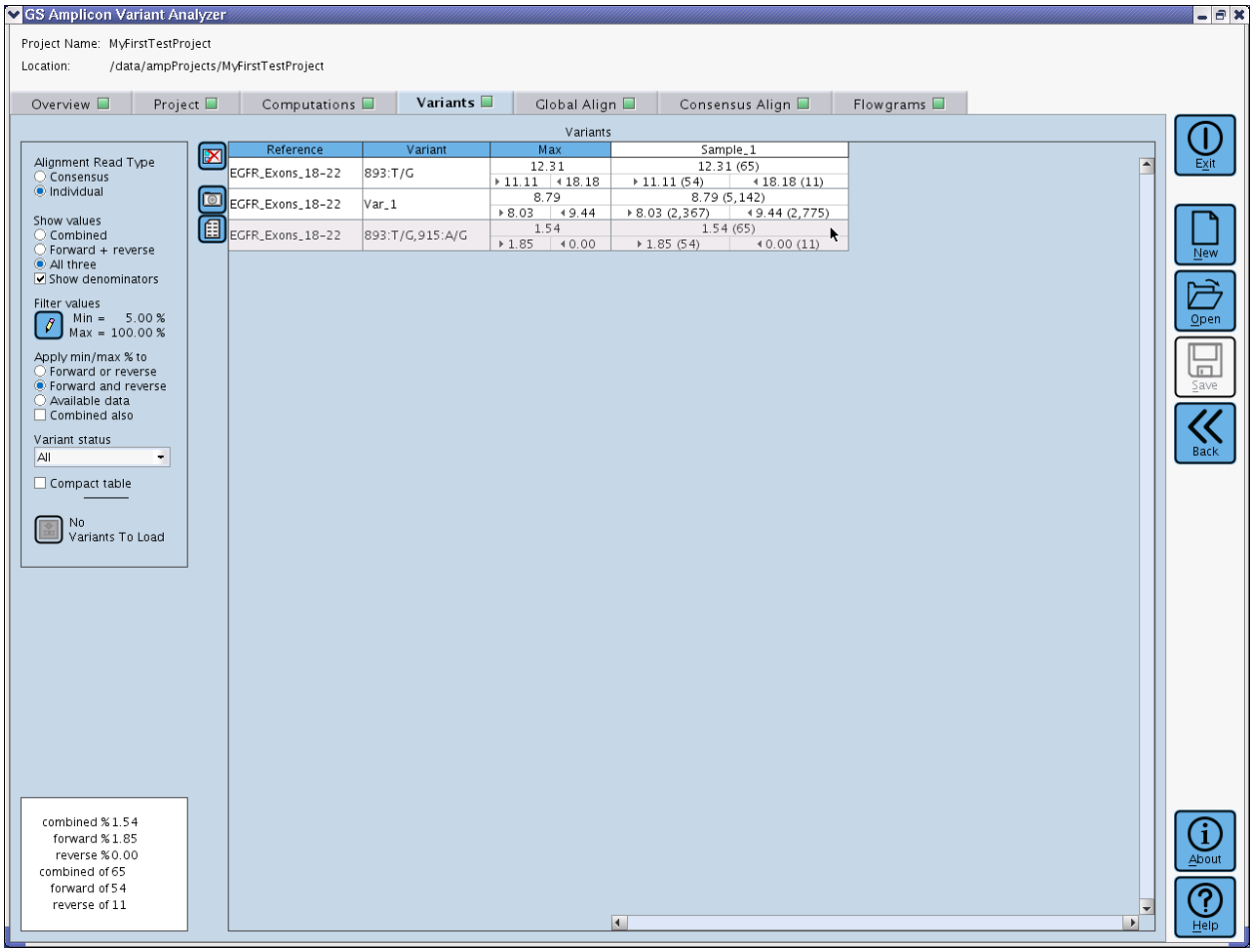


Figure 2-41: The Variants Tab with "Alignment Read Type" toggled to "Individual", showing that the haplotype Variant was detected after all, but only at 1.54% of 65 reads (a single read)

The first time we loaded Auto-Detected Variants, we used fairly stringent filters, to start with the most likely candidate Variants (there turned out to be a single Variant that met these criteria: the "893:T/G" Variant). However, there might be some Variants in the data that are real but in suboptimal contexts. Perhaps the Variant is in a position of the Amplicon that is only covered by reads from one direction or maybe the Variant is present at a frequency lower than the 5% cut-off we used for our first filter. We will now reset the filters to their most permissive values to allow all the remaining Auto-Detected Variants to be loaded into the Project. We do this by resetting the Min to 0.00% and selecting the "Forward or reverse" option. Under those selections, the "Load" button reveals that there are 11 variants to load (Figure 2-42). This also causes all the rows in the table to be displayed with a white background (the haplotype Variant row was previously grayed-out because of its low frequency).

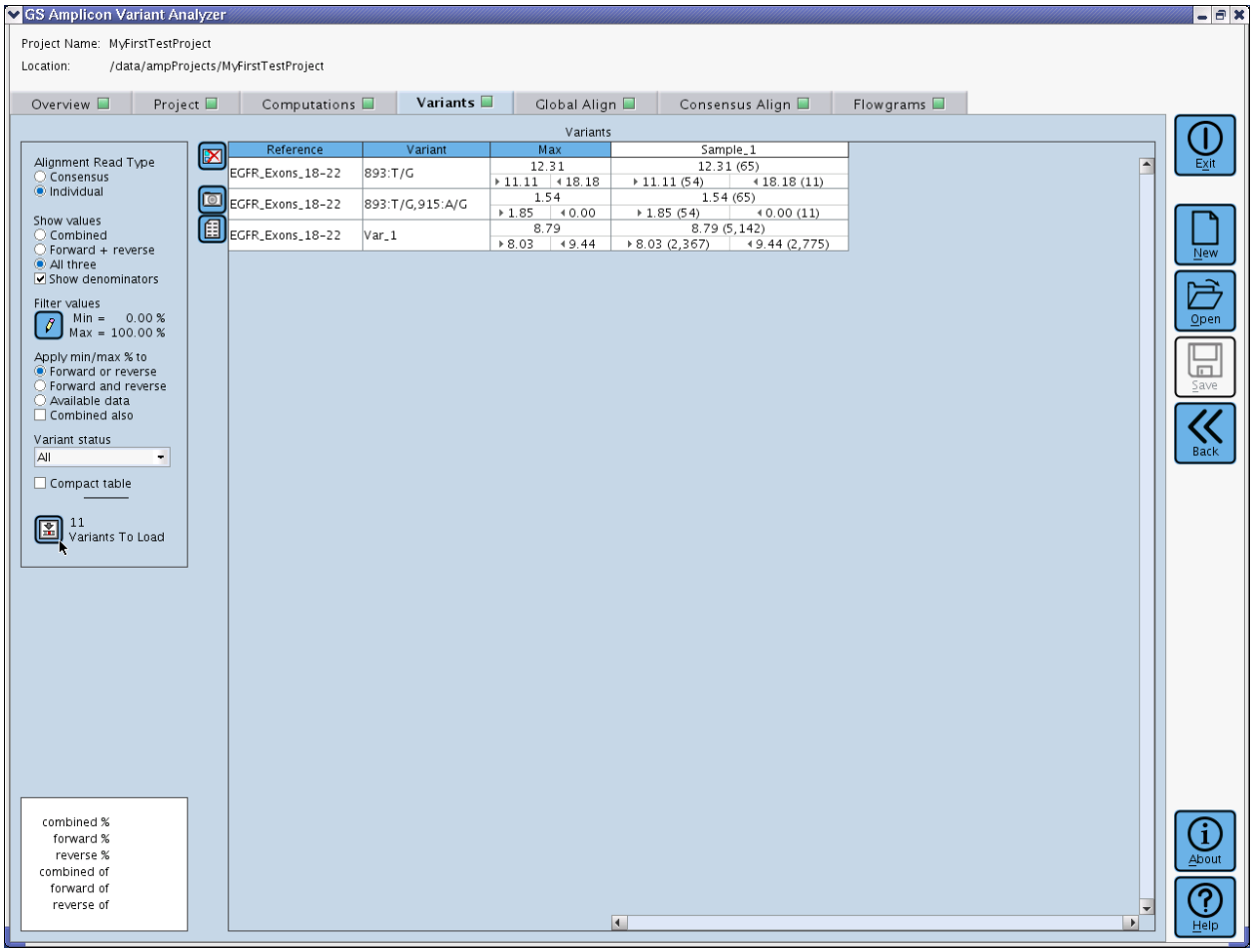


Figure 2-42: The Variants Tab with filters relaxed to allow loading of the rest of the Auto-Detected Variants

Prior to loading the Auto-Detected Variants, we can use the “Variant status” filter to prepare the Variants Frequency Table to assist us with workflow for examining the Variants. This filter influences the display by graying-out the rows of Variants in the table that do not meet the filter selection. If we set the filter to “Putative”, the rows for the two “Accepted” Variants that are already in the Project are grayed-out (Figure 2-43), but the haplotype Variant remains white because its Status was previously marked as “Putative”. Note that this filter has no influence on the “Load” button. Even though the “Load” button imports the Auto-Detected Variants as “Putative”, if you happened to set this filter to “Accepted” it would not change the fact that there are 11 Variants to load in this case, based solely on the other filter settings.

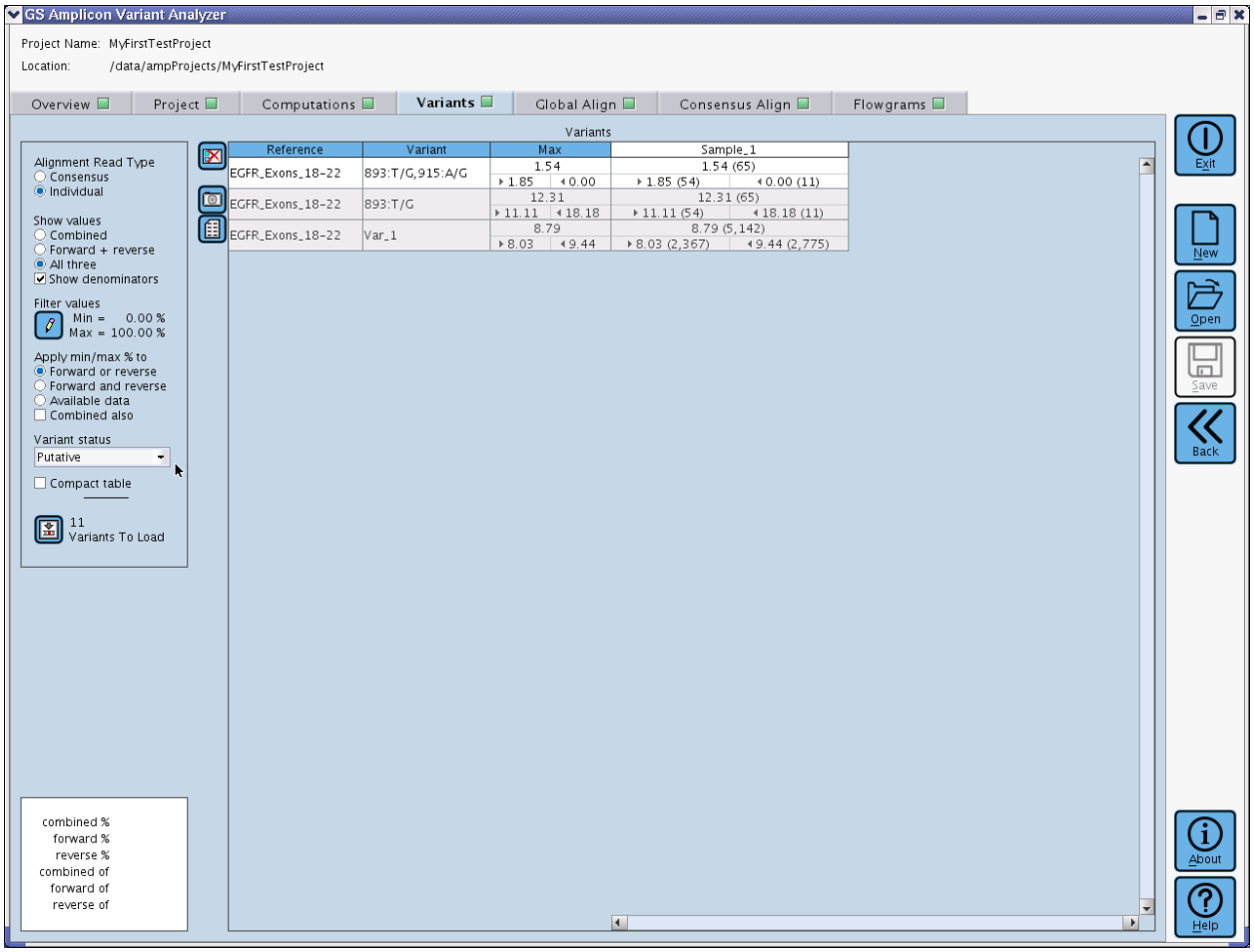
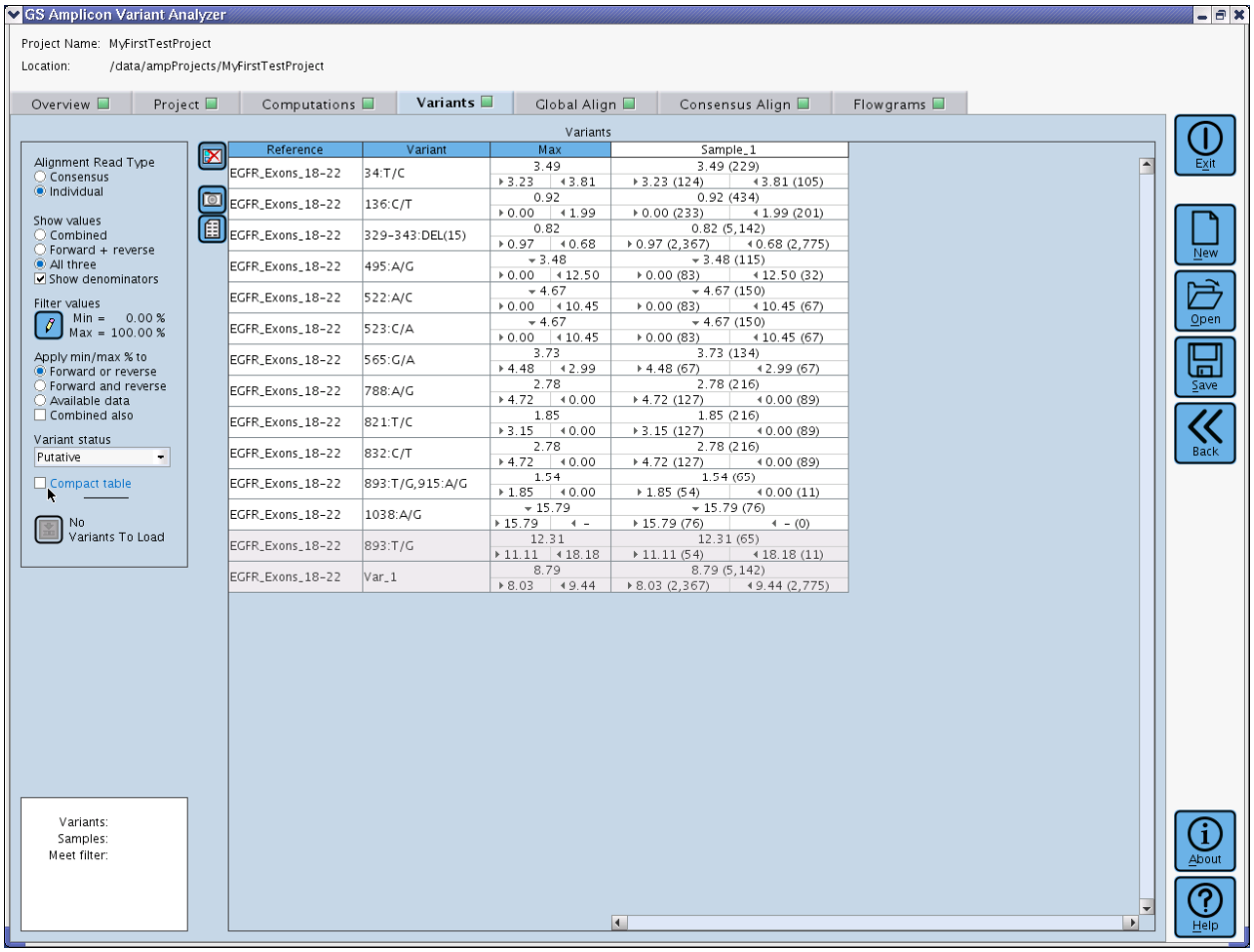


Figure 2-43: The Variants Tab with the "Variant status" filter set to "Putative". This causes the two "Accepted" variant rows to be grayed-out

Eleven Variants is a manageable number that we can reasonably load and examine at one time, so we click the "Load" button to import them. Once we do, the new Variants are all visible as white rows in the Variant Frequencies Table because the "Variant Status" filter is set to "Putative", the default for Auto-Detected Variants (Figure 2-44). The frequencies for these Variants are automatically filled out and are valid as of the completion of the last computation: we don't need to run another round of computation to update the frequencies until we make changes to the Project that would impact the calculation of the frequencies (such as new Samples or Read Data, or any change in the Reference Sequence). This is different from manually defined Variants, which require a round of computation after their definition in order to appear in the Table.



Reference	Variant	Max	Sample_1
EGFR_Exons_18-22	34:T/C	3.49	3.49 (229)
		3.23 | 3.81	3.23 (124) | 3.81 (105)
EGFR_Exons_18-22	136:C/T	0.92	0.92 (434)
		0.00 | 1.99	0.00 (233) | 1.99 (201)
EGFR_Exons_18-22	329-343:DEL(15)	0.82	0.82 (5,142)
		0.97 | 0.68	0.97 (2,367) | 0.68 (2,775)
EGFR_Exons_18-22	495:A/G	3.48	3.48 (115)
		0.00 | 12.50	0.00 (83) | 12.50 (32)
EGFR_Exons_18-22	522:A/C	4.67	4.67 (150)
		0.00 | 10.45	0.00 (83) | 10.45 (67)
EGFR_Exons_18-22	523:C/A	4.67	4.67 (150)
		0.00 | 10.45	0.00 (83) | 10.45 (67)
EGFR_Exons_18-22	565:G/A	3.73	3.73 (134)
		4.48 | 2.99	4.48 (67) | 2.99 (67)
EGFR_Exons_18-22	788:A/G	2.78	2.78 (216)
		4.72 | 0.00	4.72 (127) | 0.00 (89)
EGFR_Exons_18-22	821:T/C	1.85	1.85 (216)
		3.15 | 0.00	3.15 (127) | 0.00 (89)
EGFR_Exons_18-22	832:C/T	2.78	2.78 (216)
		4.72 | 0.00	4.72 (127) | 0.00 (89)
EGFR_Exons_18-22	893:T/G,915:A/G	1.54	1.54 (65)
		1.85 | 0.00	1.85 (54) | 0.00 (11)
EGFR_Exons_18-22	1038:A/G	15.79	15.79 (76)
		15.79 | -	15.79 (76) | - (0)
EGFR_Exons_18-22	893:T/G	12.31	12.31 (65)
		11.11 | 18.18	11.11 (54) | 18.18 (11)
EGFR_Exons_18-22	Var_1	8.79	8.79 (5,142)
		8.03 | 9.44	8.03 (2,367) | 9.44 (2,775)

Figure 2-44: The Variants Tab after the Auto-Detected Variants are loaded

The next phase of workflow control for the newly added Variants is to select the “Compact table” option while we leave the “Variant status” filter set to “Putative”. This hides any Variant rows where the Status is either “Accepted” or “Rejected”. In this case the immediate effect is to hide the rows of the two Variants that we have already validated and set to “Accepted” (Figure 2-45). Under this configuration of the Variants Frequency Table, we can right-click any Sample-Variant frequency cell to expose the “Global Align” navigation link as we did before for the first Auto-Detected Variant we loaded. After investigating the “Putative” Variants visible in the table and editing their status to either “Accepted” or “Rejected”, they will drop out of view. In this case, we have already decided that the haplotype Variant probably isn’t real, so we can go ahead and mark it as “Rejected”. The Status of a Variant can be changed via a sub-menu available when you right-click on a Variant cell in the Variants Frequency Table (this is shown for the haplotype Variant in Figure 2-45), or by editing the Status field of the Variant in the definition table, in the Variants sub-tab of the Project Tab.

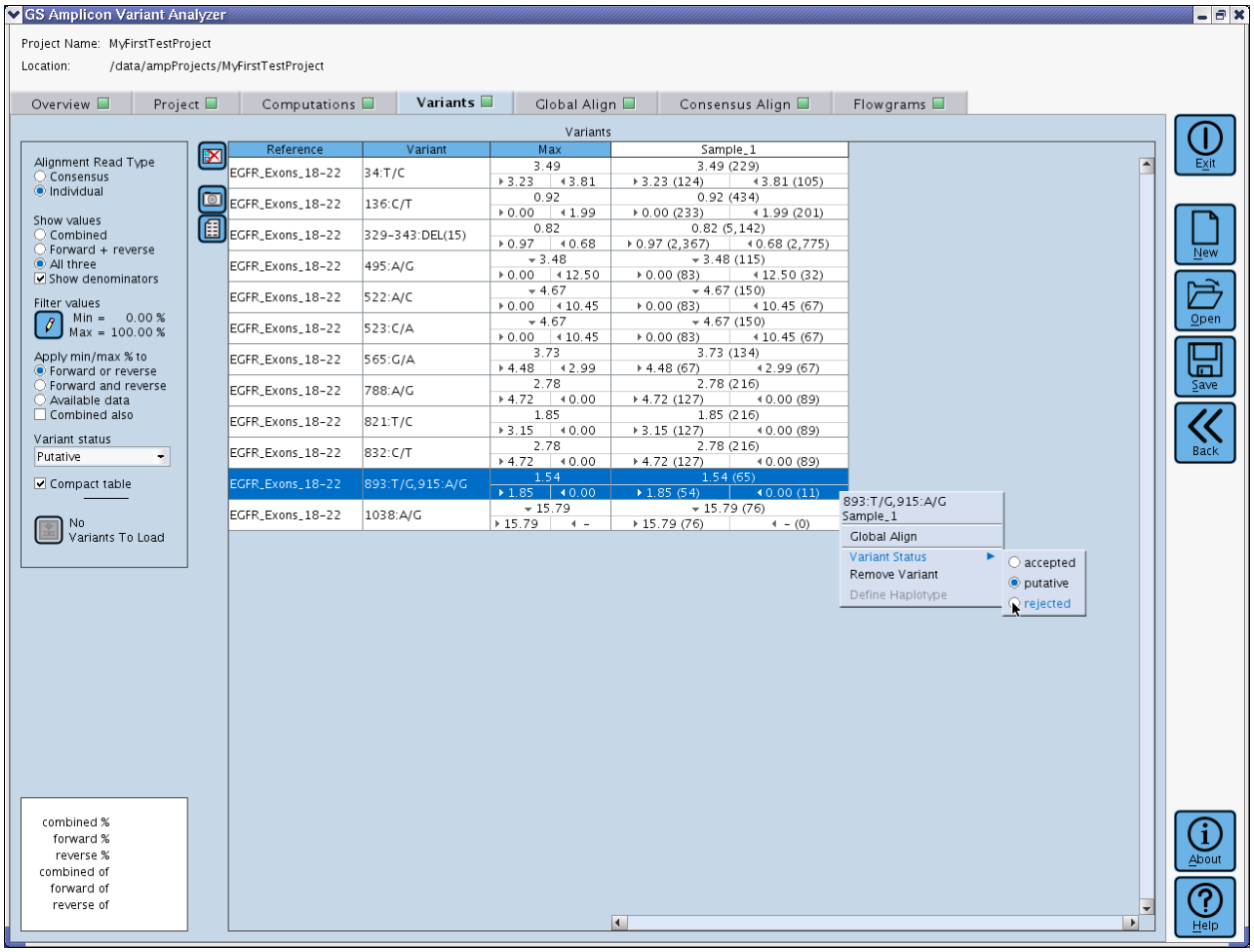
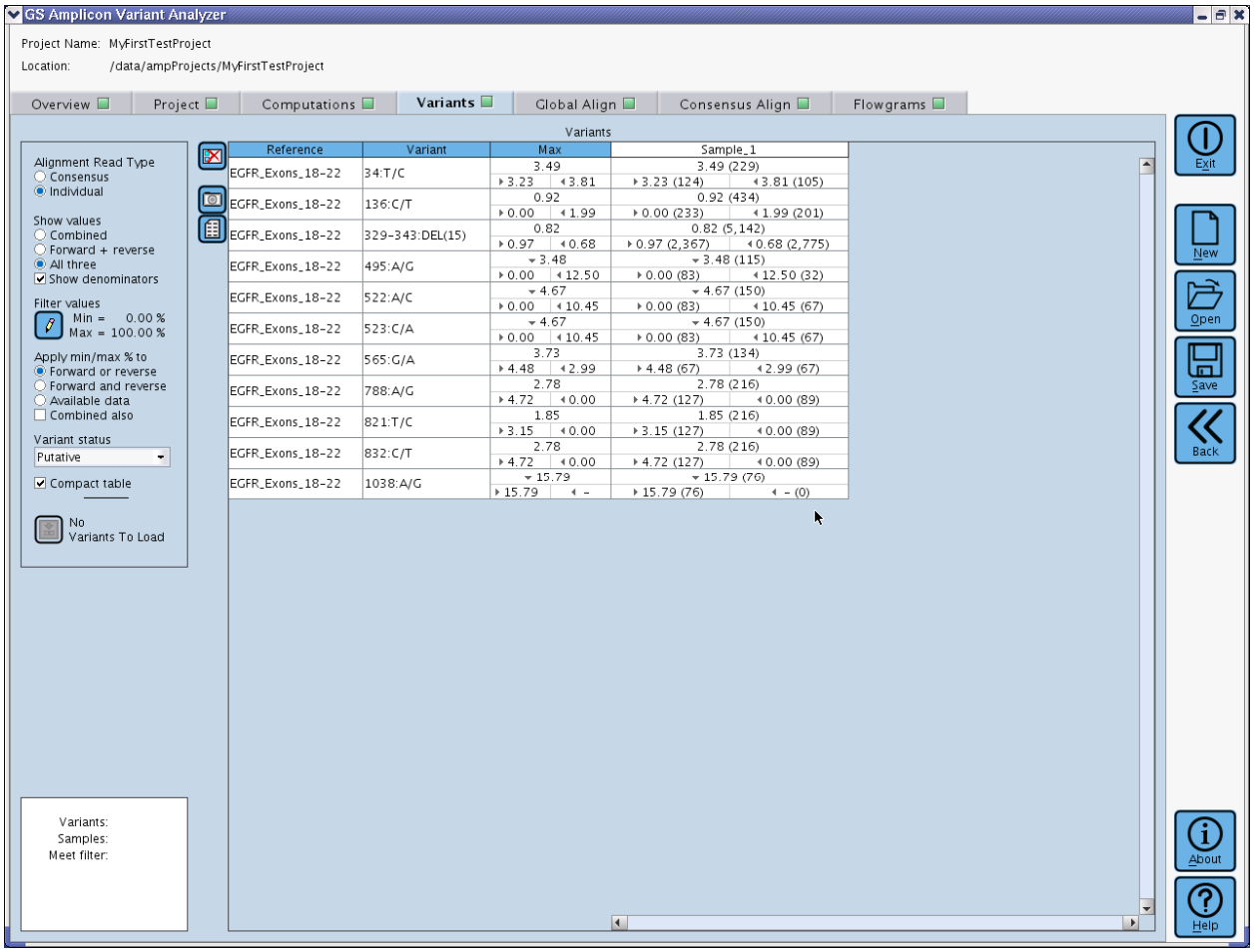


Figure 2-45: The Variants Tab after "Compact table" has been selected. This has hidden the two "Accepted" Variants that were previously grayed-out because of the "Putative" setting of the "Variant status" filter. The expanded right-click menus are poised to mark the haplotype Variant as "rejected".

After marking the haplotype Variant as "Rejected" it immediately disappears from view (Figure 2-46). Note that marking a Variant as "Rejected" rather than deleting it outright from the Project can be useful because this keeps the system from subsequently re-proposing it and forcing you to validate it more than once. Similarly, if we investigate one of the Auto-Detected Variants and determine that it is valid, we can change its Status to "Accepted" and it will also drop from view. In this way we can continue to work through Variants until all have been evaluated and the table is empty. At that point we could set the "Variant Status" filter to "Accepted" to display only the Variants in which we are confident, generating a convenient report table that we can export.



Reference	Variant	Max	Sample_1
EGFR_Exons_18-22	34:T/C	3.49	3.49 (229)
		3.23 | 3.81	3.23 (124) | 3.81 (105)
EGFR_Exons_18-22	136:C/T	0.92	0.92 (434)
		0.00 | 1.99	0.00 (233) | 1.99 (201)
EGFR_Exons_18-22	329-343:DEL(15)	0.82	0.82 (5,142)
		0.97 | 0.68	0.97 (2,367) | 0.68 (2,775)
EGFR_Exons_18-22	495:A/G	3.48	3.48 (115)
		0.00 | 12.50	0.00 (83) | 12.50 (32)
EGFR_Exons_18-22	522:A/C	4.67	4.67 (150)
		0.00 | 10.45	0.00 (83) | 10.45 (67)
EGFR_Exons_18-22	523:C/A	4.67	4.67 (150)
		0.00 | 10.45	0.00 (83) | 10.45 (67)
EGFR_Exons_18-22	565:G/A	3.73	3.73 (134)
		4.48 | 2.99	4.48 (67) | 2.99 (67)
EGFR_Exons_18-22	788:A/G	2.78	2.78 (216)
		4.72 | 0.00	4.72 (127) | 0.00 (89)
EGFR_Exons_18-22	821:T/C	1.85	1.85 (216)
		3.15 | 0.00	3.15 (127) | 0.00 (89)
EGFR_Exons_18-22	832:C/T	2.78	2.78 (216)
		4.72 | 0.00	4.72 (127) | 0.00 (89)
EGFR_Exons_18-22	1038:A/G	15.79	15.79 (76)
		15.79 | -	15.79 (76) | - (0)

Figure 2-46: The Variant tab after the haplotype Variant has been rejected. The haplotype is immediately hidden because the "Variant status" filter is set to "Putative", in combination with the "Compact table" option being activated.

2.5 Important Factors in the Assessment of New Variants

The examples above clearly show that variations observed in the reads of a sequencing experiment should be given careful scrutiny before they can be considered to be true Variants, existing physically in the DNA sample that was sequenced. This section enumerates and briefly describes some of the main features of the data to examine when making this kind of assessment.

2.5.1 Above the Noise

One major factor is the noise level in the Variation Frequency Plot. If you observe a lot of low-level frequency variation in the plot, a potential Variant would have to be convincingly above that noise level to be believed.

2.5.2 Coverage

If the depth of coverage at the potential Variant position is very low, a low frequency variant becomes less believable. At higher coverage, low frequency events become more believable provided they are convincingly above the noise. In general, one would want sufficient coverage to see several concrete instances of the variation. However, at extremely high coverage, you should be aware that identical noise type events can occur more than once, and seeing a small number of variant instances in such a case would not necessarily provide convincing evidence.

2.5.3 Bidirectional Support

If your experiment was designed so that the Target has been sequenced from both directions, you can use that information to probe the validity of a potential Variant. This is only useful if the position of your Variant is in a region of the alignment that is covered by both forward and reverse reads; if the alignment position is only covered by reads of one direction you shouldn't penalize the validity of the Variant for lack of bidirectional evidence.

If the specific Variant in question can be found in both forward and reverse reads, it is more believable as a true Variant. If the frequency of the Variant is similar in both directions, it is even more believable. If the frequency of the Variant is drastically different between the two directions, or if the Variant can only be found in one direction, you should be less likely to believe the Variant.

2.5.4 Homopolymers

If your potential Variant results from the overcall or undercall of a homopolymer, the length of the homopolymer and the sequence context will impact your assessment of the Variant. As homopolymer length increases, it becomes more likely that an erroneous overcall or undercall will occur. If there is a homopolymer of the same nucleotide in close proximity upstream or downstream of the one impacted by the Variant, known sequencing artifacts called carry-forward and incomplete extension could have caused the undercall or overcall.

2.5.5 Flowgram Evidence

If you filter the alignment to show only those reads containing your variant of interest, you can dig down into the flowgrams of the individual reads to see how convincing the Variant is.

The flowgram lowest on the page represents the subtraction of the reference flowgram from the read flowgram and shows which bases have been overcalled or undercalled in the read according to the reference. Does your potential Variant cause an appropriate shift in the heights of flowgram bars across the series of flowgrams? Is the intensity value of the shift always on the high end or low end of the expected value, or does it more appropriately form a narrow distribution around the middle of the expected value?

If your variant is an insertion or deletion that impacts a single flowgram bar, you only have the intensity of the bar to guide you. If your variant is a substitution, it will simultaneously impact the heights of at least two bars, making the Variant more believable. The most convincing flowgram evidence will be if your Variant happens to cause a nucleotide flow cycle shift in the flowgram.

This will be detectable as some inserted flows in the reference flowgram at the top plot or in the read flowgram in the middle plot, that are highlighted in grey.

2.5.6 Read Length

Sequencing quality can begin to drop off at the trailing edge of a read. You should closely examine potential Variants that are at the edges of reads. On the alignment tabs, the orientation of the reads with respect to the reference is indicated, so the end of a forward read is to the right and the end of a reverse read is to the left. If your Variant is at the end of a forward or reverse read, you should examine other types of corroborating factors like bidirectional support and flowgram evidence. If your candidate Variant only has support in a single direction, you should look at multiple reads in the same direction that share the Variant of interest. If the reads have multiple additional errors in close proximity to the Variant, it is likely an indication that the Variant isn't real but is the result of read quality drop off.

2.6 Other Issues of Special Interest

When you are familiar with the basics of the 'Amplicon Variant Analysis' software and you are ready to setup projects with your own data, you should take some time to consider the optimum setup for your project given the specifics of your experimental design and the way in which you intend to analyze the results. Primarily, you need to decide what the term 'Sample' means to you; you need to decide what type of project organization you need; and you need to decide on the relationships between your Amplicons and Reference Sequences.

2.6.1 What Does 'Sample' Mean?

One of the major decisions to make is what the term 'Sample' means to you within the context of your Project. The software recognizes a 'Sample' as a generic grouping unit of data. It is at its essence, merely a label with some optional annotation. It is up to you to decide how to best group your experimental data into Samples.

A typical Project might use a Sample to represent DNA from a distinct source, such as a tube of genomic DNA from a particular subject. Another Project might have a more specific definition of Sample and might split out different classes of DNA from a single individual and call them separate Samples such as control and experimental Samples or pre-treatment and post-treatment research Samples. Yet another project might define Samples as distinct replicates of a DNA source to allow for statistical comparison between them.

You are free to get more granular with Sample definitions (such as assigning each amplicon for an individual to its own Sample), but you should keep in mind that you can only view a single Sample-Reference Sequence alignment/difference plot at a time. You can examine cross-Sample Variant frequency statistics in the main 'Variant' tab, but reads from different Samples cannot be viewed in the same alignment. You can, however, navigate from Sample to Sample for a particular Reference Sequence.

There is a limit on the granularity of your Sample definitions. The fundamental unit of computation is the individual Read Data Set. If you intend to divide an individual Read Data Set into multiple Samples, it must be feasible for the software to assign the individual reads from

that Set to Samples. If MIDs are not used, this Sample assignment will be performed based solely on the primer content of the Amplicons being measured for the Samples. If MIDs are used, then a read's primer content will first be used to determine the Amplicon it represents, and then that Amplicon's association with a Multiplexer, within the read's Read Data Set of origin, will be used to assign the read to the appropriate Sample. It is important to remember that for a given Read Data Set, each Amplicon may be associated with at most one Sample, unless MIDs are used. With MIDs, a Multiplexer can associate an Amplicon with multiple Samples within a Read Data Set, but each Amplicon may still be associated with at most one Multiplexer.

2.6.2 How Should Your Project Be Organized?

Once you have decided on your preferred definition of 'Sample', you need to decide how your Samples and Amplicons should be organized within one or more Projects. Projects should be used to group together analyses for either ease of comparison or ease of navigation.

Much of the effort in setting up a Project has to do with defining Reference Sequences and Amplicons. Therefore, it is advisable to set up your Projects so that they contain Amplicons that will be measured across a variety of Samples. That way you can continue to add new Samples to the existing Project rather than starting a new Project for each batch of Samples.

In the case where you are scanning for a large number of different Amplicons from a single data source, collecting them within the same Project would also make sense because it would make navigation easier. It would eliminate unnecessary additional navigation clicks to open individual Projects when trying to jump from Amplicon to Amplicon during a review of your results.

You are free to organize your Sample-Amplicon analyses into one or more Projects as you find convenient. If you are a low volume user, you may be tempted to keep all of your analyses in the same Project even if they are unrelated to each other. However, you should keep in mind that pooling too many unrelated Samples together in a Project may unnecessarily clutter navigation menus and Variant summary pages.

2.6.3 Should Amplicons Share a Reference Sequence or Have Individual Ones?

When you are setting up your Amplicons for a Project, you will need to consider two opposing issues. The first issue is that smaller Reference Sequences are more efficient for computation. Excessively large Reference Sequences can lead to long computation times and slow scrolling and navigation, so shorter ones are preferable on that count. On the other hand, alignment views are restricted by Sample and Reference Sequence combination. This means that if you want to look at alignments or difference plots for two or more different Amplicons at the same time, those Amplicons must be defined from within the same Reference Sequence.

It makes sense to use a common Reference Sequence when your Amplicons actually overlap with one another and to use separate ones for Amplicons that don't overlap. However, you do have the capability to construct artificial Reference Sequences that allow you to view multiple unrelated Amplicons in a view at the same time. These artificial Reference Sequences can be constructed by concatenating Amplicon sequences together with a string of N's as separators. Such a Reference Sequence would be convenient if you have a small to moderate set of Amplicons that you are measuring in Samples with unknown variation content. You would then be able to look at the difference plot and get an overview of all of the Amplicons at the same time to identify obvious variations.

However, if you use an artificial Reference Sequence with too many Amplicons in it, you will get diminishing returns; the longer Reference Sequence will slow down computation, and the alignments will get more inconvenient to navigate. In general it is best to keep your Reference Sequences as compact as possible, thus if you wanted to measure a large number of exons from a particular gene, it would be better to use a Reference Sequence constructed by concatenating together the exons with N-separators than to use the full genomic sequence of the gene. As long as the exons don't overlap with each other, it would be even better to use separate Reference Sequences for each exon (provided viewing the exons within the same alignment or difference plot is not a priority).

2.6.4 When should MIDs be used?

The GS Amplicon Variant Analyzer (AVA) software provides a number of mechanisms for demultiplexing reads, allowing multiple Amplicons from the same or different Samples to be sequenced simultaneously within a PTP region. The simplest demultiplexing method, which has been available since the first release of the AVA software, exploits the template-specific primer regions of the Adaptors, used to prepare the library, to identify the Amplicons. The Amplicon library preparation method places these sequences at the beginning of the reads, just after the sequencing key (which is part of Primers A and B). If an experiment calls for measuring multiple distinct Amplicons from the same Sample, those Amplicons may be mixed together in a PTP region, and the Project can be set up such that reads of the various Amplicons are associated with the appropriate Sample by virtue of their known template-specific primer sequences.

But with the large number of sequencing reads that can be obtained in a single PicoTiterPlate Device region in the Genome Sequencer FLX System, the situation may be common whereby a single region would produce a vast excess of reads compared to what is necessary for any given Amplicon library (Sample). If the experiment includes multiple Samples, the obvious economical solution would be to load multiple Samples in each region such that each Sample will be covered at the appropriate depth, in a single sequencing Run. If different Amplicons were to be sequenced in each of the Samples, the standard demultiplexing method using the template-specific Primer 1 and Primer 2 sequences would be sufficient to assign each read to the proper Sample.

However, experiments where the same Amplicon, or set of Amplicons, are to be sequenced in several Samples are probably much more common. In such cases, one would face the restriction that an Amplicon can be associated with no more than one Sample, within a Read Data Set (equivalent to a PicoTiterPlate Device region, unless the data has been manipulated using the SFF Tools).

MIDs are short, recognizable sequence tags that can be added to the design of the Adaptors used for library preparation. Multiple Amplicon libraries (the Project's Samples) can be prepared that include the same Amplicon target sequences (with the same template-specific primers), each labeled with different MID tags. The MID sequences provide extra context that is specific to each library and that, in concert with the template-specific primers, allows flexible demultiplexing options and, specifically enables the sequencing of the same Amplicon across multiple Samples within the same PTP region.

2.6.5 What is the purpose of Multiplexers?

Multiplexers are used as a means to help the user avoid unnecessary duplication of effort when entering Project setup information and to help make the computation of Project results more efficient by allowing a consolidation of processing steps. To illustrate this, this section describes an example case whereby the Project is specified with and without the use of Multiplexers.

As the starting point, assume an experiment involving 16 different Samples where the same gene (single Amplicon) is being measured in each Sample, but each Sample has a specific pairing of MIDs at each end so that they can be multiplexed together for sequencing. The MID sequences being used are Mid1, Mid2, Mid3, and Mid4 and a 'both' encoding is being specified so those 4 sequences can be used combinatorially in pairs to indicate all 16 Samples. All the Samples are multiplexed into a pool and sequenced in two regions of the PicoTiterPlate Device (resulting in two Read Data Sets).

2.6.5.1 Non-Multiplexer Example

First, let's define the Amplicon to be measured in the 16 different Samples (Figure 2-47). Since the Samples will be pooled together on the same Read Data Set, MIDs are necessary.

References (1) | Amplicons (1) | Read Data (2) | Samples (16) | Variants (40) | MIDs (4)

Name ▲	Reference	Annotation	Primer 1	Primer 2	Start	End
Amp_1	HIV_Ref		TAGATGCATGCTCGAGCGGCC	CTAGGTATGGTAAATGCAGTA	22	175

Figure 2-47: An Amplicon to be measured in 16 different Samples

Using MIDs on both sides of the Amplicon, and assuming the final product is short enough to be sequenced in full within a read, only 4 MID sequences (Figure 2-48) are needed (combinatorially, using the "Both" encoding) to distinguish the Amplicons from all 16 Samples.

References (1) | Amplicons (1) | Read Data (2) | Samples (16) | Variants (40) | MIDs (4)

Name ▲	Annotation	Sequence	Group
Mid1		ACGAGTGCGT	454Standard
Mid2		ACGCTCGACA	454Standard
Mid3		AGACGCACTC	454Standard
Mid4		AGCACTGTAG	454Standard

Figure 2-48: The sequences of 4 MIDs being used to identify 16 Samples by employing a "Both" encoding

If Multiplexers were unavailable it would be necessary to define 16 different Amplicons. This is because in actuality there are 16 different Amplicon library products involved where the MID sequences would need to be considered as part of the template-specific portion of the Amplicon primers. Figure 2-49 shows the 16 different Amplicons that would need to be created for the experiment. The Amplicons are named according to their MID layout; "Amp_4_3" has Mid4 upstream of Primer1 and Mid3 upstream of Primer 2.

References (1) | Amplicons (16) | Read Data (2) | Samples (16) | Variants | MIDs | Mul

Name ▲	Reference	Annotation	Primer 1	Primer 2	Start	End
Amp_1_1	HIV_Ref		ACGAGTGCGTTAGATGCATGCTCGAGCGGCC	ACGAGTGCGTCTAGGTATGGTAAATGCAGTA	22	175
Amp_1_2	HIV_Ref		ACGAGTGCGTTAGATGCATGCTCGAGCGGCC	ACGCTCGACACTAGGTATGGTAAATGCAGTA	22	175
Amp_1_3	HIV_Ref		ACGAGTGCGTTAGATGCATGCTCGAGCGGCC	AGACGCACTCCTAGGTATGGTAAATGCAGTA	22	175
Amp_1_4	HIV_Ref		ACGAGTGCGTTAGATGCATGCTCGAGCGGCC	AGCACTGTAGCTAGGTATGGTAAATGCAGTA	22	175
Amp_2_1	HIV_Ref		ACGCTCGACATAGATGCATGCTCGAGCGGCC	ACGAGTGCGTCTAGGTATGGTAAATGCAGTA	22	175
Amp_2_2	HIV_Ref		ACGCTCGACATAGATGCATGCTCGAGCGGCC	ACGCTCGACACTAGGTATGGTAAATGCAGTA	22	175
Amp_2_3	HIV_Ref		ACGCTCGACATAGATGCATGCTCGAGCGGCC	AGACGCACTCCTAGGTATGGTAAATGCAGTA	22	175
Amp_2_4	HIV_Ref		ACGCTCGACATAGATGCATGCTCGAGCGGCC	AGCACTGTAGCTAGGTATGGTAAATGCAGTA	22	175
Amp_3_1	HIV_Ref		AGACGCACTCTAGATGCATGCTCGAGCGGCC	ACGAGTGCGTCTAGGTATGGTAAATGCAGTA	22	175
Amp_3_2	HIV_Ref		AGACGCACTCTAGATGCATGCTCGAGCGGCC	ACGCTCGACACTAGGTATGGTAAATGCAGTA	22	175
Amp_3_3	HIV_Ref		AGACGCACTCTAGATGCATGCTCGAGCGGCC	AGACGCACTCCTAGGTATGGTAAATGCAGTA	22	175
Amp_3_4	HIV_Ref		AGACGCACTCTAGATGCATGCTCGAGCGGCC	AGCACTGTAGCTAGGTATGGTAAATGCAGTA	22	175
Amp_4_1	HIV_Ref		AGCACTGTAGTAGATGCATGCTCGAGCGGCC	ACGAGTGCGTCTAGGTATGGTAAATGCAGTA	22	175
Amp_4_2	HIV_Ref		AGCACTGTAGTAGATGCATGCTCGAGCGGCC	ACGCTCGACACTAGGTATGGTAAATGCAGTA	22	175
Amp_4_3	HIV_Ref		AGCACTGTAGTAGATGCATGCTCGAGCGGCC	AGACGCACTCCTAGGTATGGTAAATGCAGTA	22	175
Amp_4_4	HIV_Ref		AGCACTGTAGTAGATGCATGCTCGAGCGGCC	AGCACTGTAGCTAGGTATGGTAAATGCAGTA	22	175

Figure 2-49: A table of all 16 Amplicons where the MID sequences have been incorporated into the template-specific Primer 1 and Primer 2 sequences. In the highlighted "Amp_1_1" sequence, both primers begin with "ACGAGTGCGT" which is the MID1 sequence from Figure 2-48 . Since the MID sequences are not actually present in the Reference, the Reference is constrained to the Amplicon being measured so that a single Reference could be used for all of the Amplicons. Otherwise 16 different MID-containing References would have to have been defined.

To finish the setup for a non-Multiplexer experiment, each of the 16 different Amplicons would have to be individually associated with its proper Sample, and those 16 Sample-Amplicon pairs would have to be associated with the Read Data Sets (Figure 2-50).

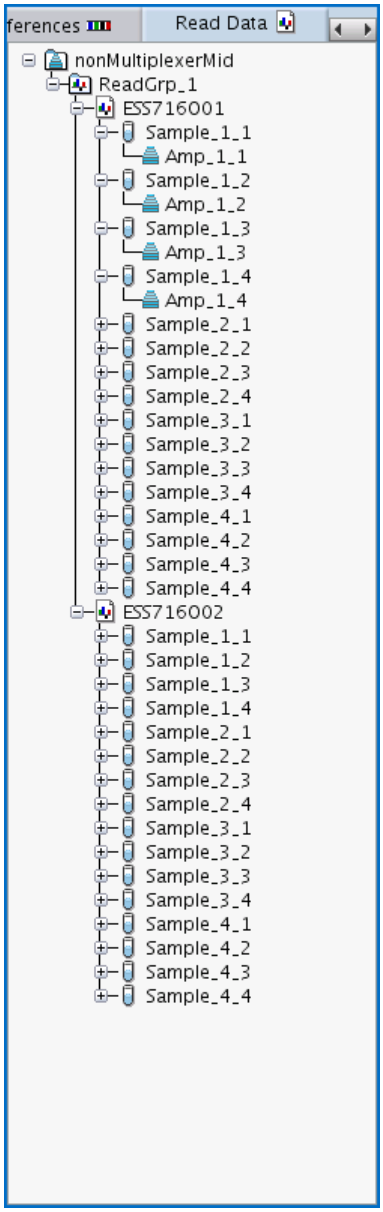


Figure 2-50: Read Data Tree without Multiplexers. Each of the 16 Amplicons had to be individually assigned to a separate Sample and the Sample-Amplicons had to be assigned to the Read Data Sets.

2.6.5.2 Multiplexer Example

With the introduction of Multiplexers, there is no need to define 16 different Amplicons. Only the basic Amplicon in Figure 2-47 needs to be defined and the Multiplexer contains the information necessary to assign the reads to their proper Sample based on their MID content. This experiment only requires a single Multiplexer that can be used on both Read Data sets. The Multiplexer needs to have the “Both” encoding with 4 MID choices (Mid1, Mid2, Mid3, and Mid4) for Primer 1 MIDs and the same four choices for Primer 2 MIDs. The Multiplexer definition table is shown in Figure 2-51.

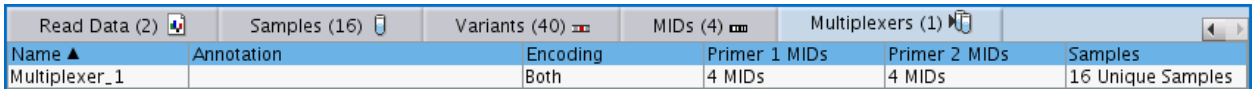


Figure 2-51: Multiplexer definition table entry

This Multiplexer setup provides a 16 cell grid of Primer 1 – Primer 2 MID pairs that can be assigned to the appropriate Samples (Figure 2-52).

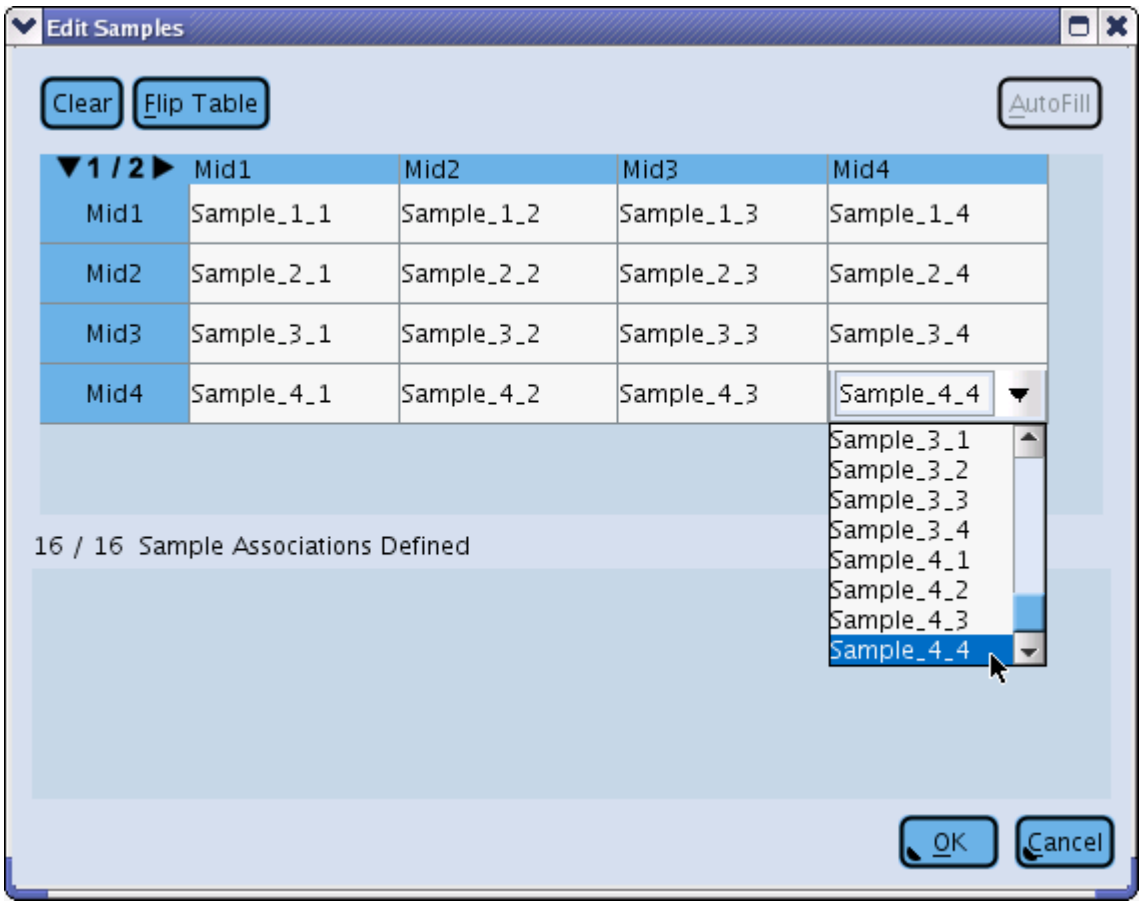


Figure 2-52: The Edit Samples window for the Multiplexer. The "Both" encoding being used allows all 16 cells in the MID grid to be assigned to distinct Samples.

To finish off the setup using Multiplexers, the Multiplexer has to be associated with the Read Data Sets. The single Amplicon being measured here can be associated with the Read Data Set –Multiplexer pair and the Amplicon automatically gets associated with each of the Samples encoded by the Multiplexer (see Figure 2-53).

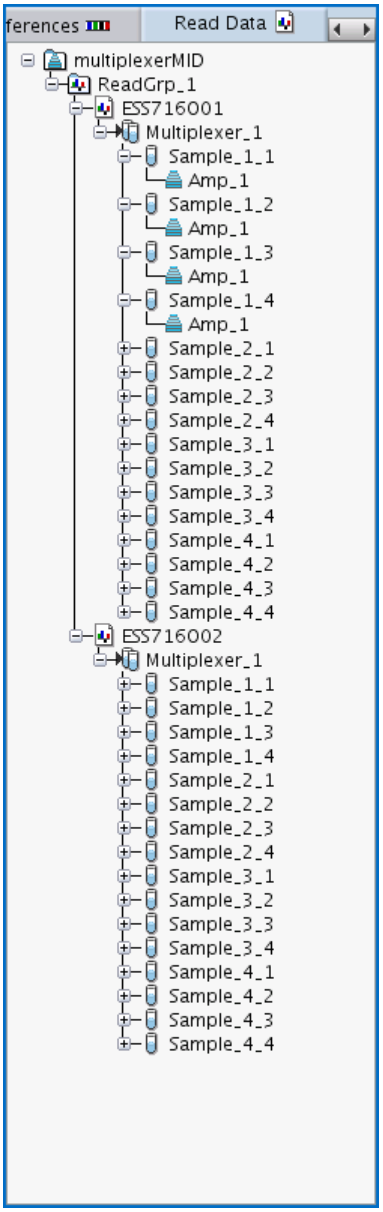


Figure 2-53: Read Data Tree with Multiplexers. The Multiplexer is associated with each Read Data Set and the single Amplicon gets associated with each Read Data Set – Multiplexer. The Read Data Set – Multiplexer automatically associates the Amplicon with each of the underlying Samples encoded by the Multiplexer.

2.6.5.3 Multiplexer Benefits Summary

Multiplexers help prevent redundant data entry during project setup when using MIDs. Without Multiplexers, every sample would have to have its own Sample-specific Amplicon defined, and all of those Amplicons would contain some level of duplication of MID sequences contained within them. With Multiplexers, the MID sequences only need to entered once, and one can define the common portion of the Amplicon library product as a single Amplicon rather than needing to define as many Amplicons as there are Samples. Since the Multiplexers also contain the rules for associating an Amplicon with its proper Sample, there is no need to manually make individual Sample – Amplicon associations prior to associating the Sample with the Read Data

Set. Instead, the Multiplexer gets associated with the Read Data set and associating the Amplicon with the Read Data Set – Multiplexer pair automatically generates the Sample – Amplicon relationships.

Beyond streamlined data entry, Multiplexers are also important for computational efficiency behind the scenes. The non-Multiplexer example provided in section 2.6.5.1 was included as an illustrative point, but it would run into trouble from a computational point of view. The 16 Amplicons only differ by at most 10 bases in each of their primers. When analyzing an individual read without any foreknowledge of MID specifics, the read needs to be compared against 16 very similar Amplicons. Allowing for distributed error in the read matches to the primer regions, it might be difficult to reliably assign a read to its proper Amplicon. With shorter MID sequences, this would be even more of a problem; the common portions of the primers from all of the Amplicons ends up making the differences in the MID regions seem less significant.

Multiplexers allow a read to be compared with expected template-specific primer sequences to first identify the Amplicon of the read. The knowledge of MID content and layout encoded by the Multiplexer allows the MID regions to be considered in a focused manner after the Amplicon assignment has already been established. This is more efficient and more likely to yield unambiguous results.

3. GS AMPLICON VARIANT ANALYZER COMMAND LINE INTERFACE

The GS Amplicon Variant Analyzer (AVA) software includes a Command Line Interface (CLI) that allows the user to carry out various functions batch-wise, *e.g.* on various objects simultaneously and/or with multiple tasks queued through a script (described in section 3.5.15). This can afford the user substantial time savings compared with entering all data and carrying out all actions one at a time via the Graphical User Interface (GUI).

The AVA-CLI is accessed via a command interpreter called "`doAmplicon`". The CLI has a flexible interface and depending on how it is invoked, you can either execute individual commands directly on the command line, read in a list of commands via a script file or a pipe, or type in commands manually in an interactive shell (see section 3.3.2.1 for the full usage statement for the `doAmplicon` command interpreter). The command language for the interpreter allows you to set up, manage, and compute Projects and trigger result reports (see section 3.4 for the full command language documentation).

3.1 Purpose of the CLI

The CLI in general allows many aspects of Project setup and management to be accomplished in a higher throughput manner than manipulating Projects via the GUI, where you must usually deal with elements on an individual basis. This can be especially useful in environments where large Amplicon Projects are carried out; or where Projects are carried out in large numbers, yet need to be kept strictly controlled and separate (*e.g.* if you are a sequencing service provider). More specifically, the CLI was primarily developed to meet four major needs: Data Import, Data Export, Automating the Triggering of Computations, and Result Reporting.

3.1.1 Data Import

In the GUI, you add Project objects one at a time; fully specifying objects like Reference Sequences and Amplicons can involve a lot of cutting and pasting. This is manageable for Projects where the number of Amplicons to be measured is small, but for more complex Projects, Project setup via the GUI can be taxing. The CLI remedies this situation by providing the ability to create Project objects via properly formatted tabular input information (see section 3.3.2.3 for more details on tabular input options). This means that files of Reference Sequences and Amplicons, *e.g.* provided by a client or perhaps generated from an in-house database, could be imported in bulk into the Project using "create" commands with the files as input (see section 3.4.4 for the create usage statement).

3.1.2 Data Export

It is often convenient to use an existing Project as the starting point for the creation of a new Project. For example, you may be measuring the same set of Amplicons for different Projects that have different owners and therefore need to be kept separate. Or, you may find that some set of Reference Sequences or Amplicons get reused across several different Projects. In such situations, the ability to export the information from an existing Project, and import it into a new Project, eliminates the duplication of labor and accumulated error risk of data re-entry. The CLI addresses this problem through a Project cloning command (the "utility clone" command usage

statement is given in section 3.4.17.4) and a set of more focused “list” commands (see section 3.4.7 for the usage statement).

The “utility clone” command allows you to safely make a copy of any existing Project (with or without its accompanying Read Data) to a new location. The cloned Project can be renamed and edited to be appropriately used as a new Project.

The “list” command can be used in a more focused manner to export data for particular Project objects rather than exporting the setup of an entire Project. For example, you could use the command “list reference -outputFile referenceExport.txt” to export a table of Reference Sequences from a Project that could then be imported into another Project with the command “create reference -file referenceExport.txt”.

3.1.3 Automating the Triggering of Computations

In the GUI you trigger the computation of a Project via a manual click on the Start button, on the Computation tab. In order to be able to truly automate the bulk of Amplicon Variant Analysis, you need a way to trigger computation independently of manual GUI interaction. The CLI has a set of “computation” commands (see section 3.4.3 for the usage statement) that allow you to control Project computation from the `doAmplicon` command interpreter, freeing you from GUI interaction through the computation stage.

3.1.4 Result Reporting

In the GUI, after you have run a computation, the Sample-Variant Table on the main Variants Tab gets updated with Variant frequencies, and you can export the data from that table to a file manually. The CLI allows you to trigger the generation of the report using the “report variantHits” command (see section 3.4.12.2 for the usage statement). You can then choose to process this report on your own to prioritize which Variants are the most promising for user verification in the GUI. The CLI also allows you to export the computed alignments, if you wish to analyze them by other means, using the “report alignment” command (see section 3.4.12.1 for the usage statement).

3.2 AVA-CLI Command Language Overview

The AVA-CLI command language consists mainly of a set of commands to create, modify, and associate Project entities and to perform, and report the results of, Project computations. Additional commands exist for Project validation, data export, and setting the behavior of the `doAmplicon` command interpreter itself, to assist in debugging problems in CLI scripts. The commands and their command option specifier are all case-insensitive. The Project entities that can be created or manipulated, and their associated commands, are listed below. The usage statement for the `doAmplicon` command interpreter itself is in section 3.3.2.1. Help information that applies generally to the command language is presented in section 3.3. Detailed usage statements for individual commands are listed in section 3.4, providing a Reference Guide to the command language. Section 3.5 provides a more high-level overview of the language, including a full example script to create a new Project in section 3.5.15. Finally, another (more limited) example script is provided in section 3.6, to display some of the particular features of MID-based Projects.

3.2.1 Entities

There are 10 Project object or "entity" types supported by the AVA-CLI. Many of the CLI language commands can act on more than one of these.

- **amplicon** – This entity type may be abbreviated to "amp". See section 1.1.1.3 for more information on Amplicons.
- **project** – This entity type may be abbreviated to "proj". See section 1.1.1.1 for more information on Projects.

- **readData** – This entity type may not be abbreviated, but it is case insensitive so you don't have to capitalize the "D" of "Data"; this is done here only to improve readability. See section 1.1.1.4 for more information on Read Data Sets.

- **readGroup** – This entity type may not be abbreviated, but it is case insensitive so you don't have to capitalize the "G" of "Group"; this is done here only to improve readability. See section 1.1.1.4 for more details on Read Groups.

- **reference** – This entity type may be abbreviated to "ref". See section 1.1.1.2 for more information on Reference Sequences.

- **sample** – This entity type may be abbreviated to "samp". See section 1.1.1.6 for more information on Samples.

- **variant** – This entity type may be abbreviated to "var". See section 1.1.1.5 for more information on Variants.

- **mid** – This entity type does not need to be abbreviated and is used as "mid". See section 1.1.1.7 for more information on Mids.

- **midGroup** – This entity type may not be abbreviated, but it is case insensitive so you don't have to capitalize the "G" of "Group"; this is done here only to improve readability. See section 1.1.1.7 for more details on Mid Groups.

- **multiplexer** – This entity type may be abbreviated to "mul". See section 1.1.1.8 for more information on Multiplexers.

3.2.2 Available Commands

The top level commands recognized by the AVA-CLI are introduced briefly below. Some of the commands like "set" don't act directly on any entity type, while many others can be used to act on more than one entity type. The full set of online help showing the usage statements for the many ways these commands can be used may be found in section 3.4.

In general, you can create the Project and the objects within it using the "create" command (except the Read Data Sets, which must be added to the Project via a specialized "load" command). Many of the commands accept input in a tabular format that enables bulk import and

allows you to process many objects at a time rather than forcing you to fully specify a command for each individual object. Once you have objects in the Project, they can be edited using the "update" and "rename" commands or they can be deleted from the Project entirely with the "remove" command. Associations between Read Data Sets, Samples, and Amplicons can be managed via the "associate" and "dissociate" commands. Computation can be automatically triggered and managed via the "computation" family of commands. Certain "utility" commands can be used to validate the Project and to export data. There are also several other commands (such as "set", "save", "close", and "exit") used to control interaction with the CLI and for other miscellaneous functions.

- **associate** – This command makes associations between appropriate records. It may be abbreviated to "assoc". It accepts tabular input. A full usage statement is available in section 3.4.1.
- **close** – This command closes the Project that is currently open. A full usage statement is available in section 3.4.2.
- **computation** – The command controls computations on the Project. It may be abbreviated to "comp". A full usage statement is available in section 3.4.3.
- **create** – This command creates entities including Projects, Reference Sequences, Amplicons, Samples, Variants, MIDs / MID Groups, Multiplexers, and Read Groups. It accepts tabular input. A full usage statement is available in section 3.4.4.
- **dissociate** – This command removes associations between records. It may be abbreviated to "dissoc". It accepts tabular input. A full usage statement is available in section 3.4.5.
- **exit** – This command exits the interpreter. A full usage statement is available in section 3.4.6.
- **list** – This command lists information about entities. It can optionally send output to a file. A full usage statement is available in section 3.4.7.
- **load** – This command loads Read Data Sets into the Project that is currently open. It accepts tabular input. A full usage statement is available in section 3.4.8.
- **open** – This command loads a pre-existing Project, making it the current open Project in the CLI. A full usage statement is available in section 3.4.9.
- **remove** – This command removes records from a Project. It accepts tabular input. A full usage statement is available in section 3.4.10.
- **rename** – This command renames entities. It accepts tabular input. A full usage statement is available in section 3.4.11.
- **report** – This command produces reports about computations including Variant frequencies and alignments, in multiple formats. A full usage statement is available in section 3.4.12.

- **save** – This command saves any modifications to the Project that is currently open. A full usage statement is available in section 3.4.13.

- **set** – This command sets environment variables. A full usage statement is available in section 3.4.14.

- **show** – This command is used to show various information about the interpreter. A full usage statement is available in section 3.4.15.

- **update** – This command updates entities' properties. It accepts tabular input. A full usage statement is available in section 3.4.16.

- **utility** - This command performs utility functions such as Project cloning. A full usage statement is available in section 3.4.17.

3.3 AVA-CLI General Online Help

This section provides the verbatim content of the general online help files for the AVA-CLI. To enter the upper level of the help files, run the CLI “help” command. Information to access help on more detailed topics is as indicated below. The online help file content for each individual command, providing the full command usage statement, is provided in section 3.4. A more gentle introduction to the commands, with a full example script for setting up and performing computations on a Project, is given in section 3.5, and a smaller Project script displaying MID features is given in section 3.6.

3.3.1 Help

```
This provides an overview of available commands.  For more specific
information, use 'help <command>' where '<command>' can be one of the
following.  For example, for help on the 'update' command, run 'help
update'.  For general help with the command interpreter, run
'help general'.


  create       Creates entities such as projects and project records.
  open         Opens projects.
  save         Saves the currently open project.
  close        Closes the currently open project.
  update       Updates entities' properties.
  rename       Renames entities.
  remove       Removes records from a project.
  load         Loads read data into the currently open project.
  associate    Makes associations between appropriate records.
  dissociate   Removes associations between records.
  computation  Controls computations on the project definition.
  report       Produces reports about computations.
  list         Lists information about entities.
  utility      Performs utility functions such as project cloning.
  set          Sets environment variables.
  show         Shows information about the interpreter settings.
  exit         Exits the interpreter.
```

3.3.2 General Help

```
Find help on general use of the command interpreter in the sections below.
Run 'help general <subsection>'.

  commandLine      Information about the command line arguments to start the
                   command interpreter itself.
  parsing          Information about how commands are parsed.
  filePaths        Information about how file paths are interpreted.
  tabularCommands  Information about using tables to succinctly construct
                   commands.
  recordNames      Information about record naming for the command line
                   interpreter.
  abbreviations    Information about abbreviations for options and commands that
                   can be used throughout the command line interpreter.
  multiplexing     Information about how the GS Amplicon Variant Analyzer software
                   supports multiplexing of amplicons and/or samples within a
                   single read data set.
```

3.3.2.1 CommandLine Help

```
doAmplicon [<advanced options>]
           [<files>] [-onErrors <"stop" or "continue">]
                     [-i[nteractive]]
                     [-v[erbose]]
                     [-c[ommand] <command>]
                     [-p[roject] <project path>]
                     [-h[elp]]
                     [-a[bout]]

Runs the command interpreter.  If no '<files>' are given, the interpreter
reads from standard input for its commands.  If one or more files is
given, each file is executed in order.  If "-" is encountered as one of
the file arguments, standard input is read for commands at that position.
For example:

     doAmplicon preamble.ava -

will execute the preamble and start reading commands from standard input.

The '-onErrors' option sets the value of the 'onErrors' parameter.  If
'onErrors' is set to "stop", the command interpreter will exit if an error
is encountered.  If 'onErrors' is set to "continue", the command
interpreter will abort the command that caused the error but will continue
running and executing subsequent commands.

The '-interactive' option indicates that the interpreter is being used
interactively.  A prompt is written to standard output and some commands
attempt to interact with the user for further input when necessary.

If neither '<files>' nor the '-command' option are given, the interpreter
implicitly enters interactive mode, even if '-interactive' is not
specified.  If "-" is supplied as one of the '<files>' option, then the
interpreter will read from standard input, but will not implicitly enter
interactive mode.  Thus, one syntax allows the interpreter to be used
as an interactive command line interface while the other facilitates the
creation of automated pipelined scripts, as in:

    generateScript | doAmplicon - > resultFile

Unless explicitly given an '-onErrors' option value, the interpreter, in
```

```
interactive mode, behaves as if 'onErrors' were set to "continue" and, in
non-interactive mode, behaves as if 'onErrors' were set to "stop."

The '-verbose' option will cause the interpreter to output information
about the commands that it is executing as it executes them.

The '-command' option can be used to execute a single command in the
interpreter.  For example, if you want to create an empty project, you
would execute:

    doAmplicon -command "create project /data/new/project/path"

The '-project' option can be used to open a project before executing the
rest of the specified commands.  For example, you may have a script that
removes all of the variants in a project.  You could choose on what project
to run this script by using the '-project' option.  For example:

    doAmplicon -project "/some/project" myRemoveVariants.ava

Combined with the "-command" option, this can be used to execute single
commands on a project.  For example:

    doAmplicon -project "/some/project" -command "list amplicon"

This command will list all of the amplicons of the project at
"/some/project".

The '-project' option attempts to open the project with exclusive control
and will fail if another instance of the program has control of the
project.  To attempt to preempt control of the project or open it in
a read-only fashion, requires the use of the 'open' command from within
the interpreter itself.

The '-help' option displays this help.  Online help for the interpreter
commands is available by entering the "help" command to interpreter
itself.

The '-about' option displays version information about the interpreter.

The <advanced options>, if provided, must all precede any of the files
or other basic options on the command line and may be one or more of:

  --maxPerm     A number indicating, in megabytes, the maximum amount of "PermGen"
                memory that doAmplicon's Java environment may use. (Default = 128)
  --maxHeap     A number indicating, in megabytes, the maximum amount of "Heap"
                memory that doAmplicon's Java environment may use. (Default = 500)
  --cpu         A number indicating the number of processes that doAmplicon may use
                to parallelize computations.
  --configDir   The location of configuration files used by doAmplicon. (Default -
                /opt/454/apps/amplicons/config/)

Note that all the advanced options are preceded by two dashes, unlike
the basic options that are preceded by only one.  Normally the default
values of --maxPerm or --maxHeap, which are 128 and 500 megabytes,
respectively, are sufficient.  If doAmplicon's underlying Java environment
runs out of memory, a message will be displayed indicating which
parameter needs adjusting.  Doubling the default values will typically
resolve any memory issues.  The --cpu option, which defaults to 1,
defines the number of parallel processes that may be used during the
Trimming and Alignment steps performed when computing a project via
the command 'computation start'.  Due to the memory and cpu resource
requirements of the Trimming and Alignment steps, the --cpu option
generally should not exceed the number of actual processors on the local
```

```
machine, as all the processes will be run on the local machine (i.e.,
not spread across a cluster).  If the amount of memory on the local machine
is limited, then it is advisable to limit the --cpu value, because the
parallelized steps will compete for memory resources and may lead to
excessive swapping of memory and degrade the responsiveness of the local
machine.  The memory used by the Trimming and Alignment steps is in
addition to that used by the Java environment as set by the --maxPerm and
--maxHeap parameters.  If --cpu value 0 (zero) is supplied, then all the
processors on the local machine will be used.
The –configDir option forces doAmplicon to use a configuration directory
other than the default.

Example usage:
Usage: gsAmplicon [--maxHeap <number>] [--maxPerm <number>] [--configDir
<directory>] [--cpu <number>]
```

3.3.2.2 Parsing Help

```
The interpreter is case insensitive with respect to its commands and
options.  For example, consider the two commands below:

create amplicon Amp1
CREATE AMPLICON Amp1

These commands are equivalent.  Note, however, that all strings that are
part of the project itself are case sensitive.  For example, consider the
two commands below:

create amplicon Amp1
create amplicon AMP1

These commands are not equivalent, since record names are case sensitive.

The character '#' may be used to document your scripts.  The '#' may
appear anywhere on the line, and everything from the '#' until the end
of the line is ignored.  For example:

# The next command line lists the Variants of the project
list variant
list amplicon # and this command lists the Amplicons

To use an argument that contain spaces or the comment character, surround
the argument with double quotes. For example, you can set an annotation
of an amplicon to an unusual string by running the following:

update amplicon Amp1 -annotation "My unusual string with a
  comment # character and even line breaks
"

If you need a double quote in your argument, "escape" it with a preceding
backslash.  For example:

update amplicon Amp1 -annotation "The \"Best\" Amplicon"


Inside of double quotes, the backslash (except when preceding a double
quote) and new line characters are treated literally.  Thus, in the
example:

update amplicon Amp1 -annotation "Testing 1 \
2 3"
```

```
both the backslash and new line will become part of the annotation.

Outside of double quotes, the backslash character can be used to make
any single character ordinary, avoiding the need to use double quotes.
Thus, the following two commands are equivalent:

create amplicon Amp\#1
create amplicon "Amp#1"

Note that without the '\' or surrounding quotes, the #1 in the both
of the commands above would have been treated as a comment, and an
amplicon simply named "Amp" would have been created.

Outside of double quotes, the backslash character can also be used
for line continuation, allowing you to split a command over multiple
lines.  A backslash immediately followed by a new line will join
the following line to the current line.  This allows you to format
commands nicely.  For example:

update amplicon Amp1 \
    -annotation "The best amplicon" \
    -reference "ref1"

is equivalent to the single line command:

update amplicon Amp1 -annotation "The best amplicon" -reference "ref1"
```

3.3.2.3 Tabular Commands Help

```
To facilitate high-throughput project setup and modification, it is
possible to run commands with tables of data as input.  The table column
headers are simply the options of the command that is to be run, but with
the leading "-" removed.  As with the command options themselves, the
column headers are case insensitive.  The tabular data may be supplied
from an external file, or from a table embedded in the command script
itself, using tab or comma separated value formats.

For example, suppose you need to add 100 amplicons to a project.  Instead
of adding them one by one with 'create amplicon' commands, you can issue
a single 'create amplicon' with a table as input.  For example:

create amplicon -file - << end_marker
Name     Reference
Amp1     Ref1
Amp2     Ref2
Amp3     Ref3
Amp4     Ref4
Amp5     Ref5
Amp6     Ref6
Amp7     Ref7
Amp8     Ref8
end_marker

This command will create 8 amplicons when run.  Let us examine each
element of this invocation.  First, the 'create amplicon' indicates that
we are creating amplicons.  The '-file - <<' option indicates that we are
going to be supplying a table in the form of a "Here" document.  A "Here"
document is essentially a document supplied to the command that can be
specified in place.  The 'end_marker' indicates that we are creating a
here document that terminates when 'end_marker' is seen by itself on a
```

```
line.

The document itself must be a tab-separated table whose first row indicates
what option each column represents.  Thus, when the second line of our
table is executed, it is precisely the same as if we were to have written:

create amplicon -name "Amp1" -reference "Ref1"

In fact, our table command is the same as executing the following:

create amplicon -name "Amp1" -reference "Ref1"
create amplicon -name "Amp2" -reference "Ref2"
create amplicon -name "Amp3" -reference "Ref3"
create amplicon -name "Amp4" -reference "Ref4"
create amplicon -name "Amp5" -reference "Ref5"
create amplicon -name "Amp6" -reference "Ref6"
create amplicon -name "Amp7" -reference "Ref7"
create amplicon -name "Amp8" -reference "Ref8"

However, it is much more succinct in table form.

This works for any command that takes a '-file' option.  For example:

update reference -annotation "Updated 2/12/07" -file - << end
Name     Sequence
Ref1     ATAGCAGATAGATAATATATAAAAAAGACGAT
Ref2     ATAGCAGATATAGATAGTGATGCAGTATAGACAGTAAGATAGACAG
Ref3     ATGAATAAAAAATCCCCCCCTAGTAGTACTTTTTTAAAAATA
Ref4     TGACGAAACATAGTGTAAACGTGTGCAGACAGCCCAC
Ref5     GCAGACGATAAAAAAAATGATGACGACGTAATACAATAT
Ref6     GACGCATTTTTTTTAGATATACTATATATT
Ref7     TATAATAAAAATTATATCGGGATAGTAGTGCAGAGAGAGAGTAGTAGCAC
Ref8     TACGACATATAGATGATAGACAAATAACAGATAGTAGTAGTAGAAGT
end

This time we are updating references rather than creating amplicons.  You
will also note that we specified an annotation in the main command and not
in the here document.  Options specified in this manner are applied to
each row of the command.  Our table command is the same as executing the
following:

update reference -annotation "Updated 2/12/07" -reference Ref1 -sequence ..
update reference -annotation "Updated 2/12/07" -reference Ref2 -sequence ..
update reference -annotation "Updated 2/12/07" -reference Ref3 -sequence ..
update reference -annotation "Updated 2/12/07" -reference Ref4 -sequence ..
update reference -annotation "Updated 2/12/07" -reference Ref5 -sequence ..
update reference -annotation "Updated 2/12/07" -reference Ref6 -sequence ..
update reference -annotation "Updated 2/12/07" -reference Ref7 -sequence ..
update reference -annotation "Updated 2/12/07" -reference Ref8 -sequence ..

Instead of using here documents, external files can be supplied using the
'-file' option.  For example:

create variant -file /data/variants.txt

In the previous examples, we specified the table in place using a here
document.  Here, we refer to the external file, "/data/variants.txt".  The
format of the external file is expected to be exactly the same as that of
the here document (without the need for an end marker, however).

So "/data/variants.txt" could look like this:

<begin /data/variants.txt>
```

```
Name     Reference     Status
Var1     Ref1     Accepted
Var2     Ref1     Accepted
Var3     Ref1     Accepted
Var4     Ref1     Rejected
Var5     Ref1     Rejected
Var6     Ref1     Accepted
<end /data/variants.txt>
```

You will also note in this example that the rows do not line up exactly. This is because we always expect one tab character to separate each column, regardless of the size of the data in the column.

If you prefer comma-separated columns, use the '-format' option. For example:

```
update readData -file - -format csv << end
Name,Active
Data1,true
Data2,true
Data3,false
Data4,true
Data5,false
end
```

Note the '-format csv' option. Valid values are "csv" and "tsv" to indicate comma-separated and tab-separated table formats, respectively. The default is "tsv", except when a file is provided with a ".csv" extension (such as those exported from Excel).

It is also important to note that empty cells are not omitted from the arguments. For example:

```
update variant -file - << end
Name     Reference
Var1     Ref1
Var2
Var3
Var4     Ref4
Var5
Var6     Ref6
Var7     Ref7
Var8     Ref8
end
```

Executing this command will make variants "Var2", "Var3", and "Var5" refer to no reference sequence.

Finally, note that the parsed table values are what are used to supply values to the command arguments, as opposed to the literal table text itself. This means that the table contents must follow the syntactic conventions of tab and comma separated values tables, not that of the command interpreter. In particular, this means that neither the interpreter's comment character '#' nor the special '\' constructs have any special meaning inside of tables. Similarly the conventions for quoting double quotes in tables should be followed.

Rather than, as one would embed a '"' in a command line argument:

```
"This is how \"double quotes\" are embedded for the interface"
```

in a table, one must use the double-double quote convention:

```
"Tables use ""double-double quotes"" to embed a quote character"

For more information on the interpreter's parsing of commands and special
characters, run 'help general parsing'.
```

3.3.2.4 Record Names Help

```
The command line interpreter primarily uses record names to identify and
distinguish records.  Duplicate record names lead to ambiguity that the
interpreter cannot resolve in most cases.  For example, it is technically
allowed for two reference sequences to have the same name, "Ref1".  If we
want to update one of these reference, we issue the command:

update reference "Ref1" -annotation "New annotation"

This will report an error, since the interpreter cannot discern which
record to update.  It is therefore recommended that unique names be used
for records.

There are exceptions to this rule.  Amplicons and variants can be
disambiguated by their reference sequences if duplicate names are found.
For example, if you have two amplicons named "Amp1", but one of them refers
to reference "Ref1" and the other to "Ref2", the '-ofRef' option of
commands dealing with amplicons can be used to disambiguate them.  For
example:

update amplicon "Amp1" -annotation "New annotation"

This will result in an error, since there are two amplicons named "Amp1".
However, consider this command:

update amplicon "Amp1" -ofRef "Ref1" -annotation "New annotation"

This is allowed because the '-ofRef' option has been used to determine
which amplicon to update.  This can be used in other commands as well:

associate -sample "Sam1" -amplicon "Amp1" -ofRef "Ref1"

Again, we are distinguishing between the duplicately named amplicons by
using the '-ofRef' option.

The 'utility validateNames' command is provided to help determine if your
project has any such ambiguity and if so, help correct.  Type
'help utility validateNames' for more information.
```

3.3.2.5 Abbreviations Help

```
Many commands and options can be abbreviated.  For example:

create amp Amp1

This command is the same as:

create amplicon Amp1

Such abbreviations are noted in the help documentation.  For example,
the documentation for 'create amplicon' specifies:

create amp[licon]
```

```
to indicate that it can be abbreviated as such.  This also goes for
some options.  For example:

assoc -sam "Sam1" -amp "Amp1"

This is the same as:

associate -sample "Sam1" -amplicon "Amp1"

The option abbreviations are also similarly noted in the help
documentation.
```

3.3.2.6 File Paths Help

```
File paths are used in commands to specify projects, script files, tabular
data, and, more generally, the location of input and output files.  For
example, records can be listed to a file:

    list amplicon -outputFile someFile.txt

or other scripts can be executed:

    utility execute someOtherScript.ava

In these examples, relative paths (i.e., paths that don't start with a '/')
specify the files to use.  These paths are considered relative to the
interpreter's current directory (currDir), which may be set with the
'set currDir' command.

When the interpreter starts, the currDir is initially set to the directory
in which the interpreter was invoked.  For example, if the current working
directory is /home/me/projects when doAmplicon is invoked, the initial
currDir will be /home/me/projects.  In this situation, for the example
above, the relative path someFile.txt would be resolved to the absolute
path /home/me/projects/someFile.txt.

If 'set currDir' is used, the file resolution will change.  For example:

    set currDir /some/other/directory
    list amplicon -outputFile someFile.txt

Now the relative path someFile.txt will be resolved to the absolute path
/some/other/directory/someFile.txt.

A few special path prefix shortcuts, denoted with a leading '%', are also
available to make specifying files easier.  The first of these, currDir,
has already been described.  This may be used to explicitly specify the
currDir in a path, but is entirely equivalent to the default interpretation
of relative paths.  For example, "%currDir/someFile.txt" and "someFile.txt"
will refer to the same file.

There is also a special path prefix shortcut to access the user's home
directory.  For example, if the user's home directory is /home/me, the path
"%homeDir/someFile.txt" will be resolved to the absolute path
/home/me/someFile.txt.

Finally, there is a special path prefix shortcut, libDir, to access a
system library path that is set up as part of installation of the software.
This provides access to a standard library that may be modified by the site
administrator.
```

```
Path prefixes are only recognized when they prefix the path and match a
known shortcut.  For example, suppose the values of the shortcuts are as
follows:

    currDir=/some/dir
    homeDir=/home/me
    libDir=/opt/454/apps/amplicons/config/lib

Only paths starting with "%currDir", "%homeDir", or "%libDir",
respectively, will be affected by shortcuts.  Here are some example
path specifications with their shortcut-expanded versions:

    %currDir/someFile.txt           => /some/dir/someFile.txt
    %homeDir/someFile.txt           => /home/me/someFile.txt
    %libDir/someFile.txt            =>
                          /opt/454/apps/amplicons/config/lib/someFile.txt
    someFile.txt                    => /some/dir/someFile.txt
    %otherDir/someFile.txt          => /some/dir/%otherDir/someFile.txt
    data/%currDir/someFile.txt      => /some/dir/data/%currDir/someFile.txt

The last example does not expand the %currDir shortcut because it
does not appear at the beginning of the path specification.  The second to
last example interprets '%otherDir' literally, and resolves the given path
relative to the currDir value of /some/dir, because %otherDir is not one of
the defined shortcuts.

Absolute paths (i.e., paths that begin with '/') may also be used.  Such
paths are entirely unaffected by the currDir and by shortcuts.

To see the values of the shortcut prefixes, use the 'show environment'
command.
```

3.3.2.7 Multiplexing Help

```
The GS Amplicon Variant Analyzer (AVA) software provides a number of
mechanisms for multiplexing reads, allowing multiple amplicons from
the same or different samples to be simultaneously sequenced within
a PTP region.

The simplest demultiplexing method relies on the sequence specific primer
regions of the amplicons.  If an experiment calls for measuring multiple
distinct amplicons from the same sample, those amplicons may be mixed
together in a PTP region.  The project setup allows different amplicons to
be associated with different samples, so it is also possible to multiplex
reads from different samples, providing the samples are constructed such
that each sample is comprised of reads from different amplicons.

However, if a user wants to sequence reads from different samples but the
same amplicons, the sequence specific primer information for the amplicons
is no longer sufficient for demultiplexing the reads to their appropriate
samples.  To allow multiplexing of samples with the same amplicon in a PTP
region, the Multiplex Identifier (MID) approach is supported, in which
bases are added adjacent to the sequence specific primer in order to label
an amplicon's sample.

MIDs are technically part of the amplicon primer, but if they were encoded
as such in a project, the user would have to enter as many versions of an
amplicon as there are samples to be demultiplexed in a given region.  For
simplicity, the AVA software allows the specification of amplicons in a
manner that is independent of whether MIDs are employed, and provides a
```

```
separate 'multiplexer' formalism that describes the MID to sample
relationships.  The AVA software automatically combines, for the user, the
MIDs of a multiplexer with the primers of an amplicon and applies the
multiplexer's MID-sample relationships to determine the sample to which a
given read belongs.  This facilitates project setup since multiple
amplicons can share the same MID to sample relationship information,
with that information being defined just once in a single multiplexer.

This also allows the MID specification (encapsulated in the multiplexer) to
be shared across multiple read data, in the event that the MID-sample
relationships are replicated in more than one read data of the experiment.

The use of multiplexers provides the following benefits:
    1) Separation of amplicon specification from the complexities of MIDs
    2) The sharing of MID to sample relationships across multiple amplicons
    3) The sharing of such information across multiple read data

The 'associate' command provides the ability to define both MID-based and
non-MID-based multiplexing relationships.  Run 'help associate' for more
details on how to create these multiplexing relationships.  For more
information on creating multiplexers, and their associated constituents,
run 'help create multiplexer', 'help create mid', and
'help create midGroup'.
```

3.4 AVA-CLI Command Usage Statements

This section provides the verbatim content of the online help files for each individual command, providing the full command usage statement. Each is accessed by typing “help <command>” in the CLI (as described in section 3.3.1).

3.4.1 associate

```
assoc[iate] -sam[ple] <sample name>
            -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
            [-file <file> [-format <format>]]

assoc[iate] -sam[ple] <sample name>
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

assoc[iate] -sam[ple] <sample name>
            -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

assoc[iate] -mul[tiplexer] <multiplexer name>
            [-primer1Mid <primer1Mid name>
                [-ofPrimer1MidGroup <primer1MidGroup name>]]
            [-primer2Mid <primer2Mid name>
                [-ofPrimer2MidGroup <primer2MidGroup name>]]
            -checkMid <boolean>
            [-file <file> [-format <format>]]

assoc[iate] -mul[tiplexer] <multiplexer name>
            [-primer1Mid <primer1Mid name>
                [-ofPrimer1MidGroup <primer1MidGroup name>]]
            [-primer2Mid <primer2Mid name>
                [-ofPrimer2MidGroup <primer2MidGroup name>]]
```

```
            -checkMid <boolean>
            -sam[ple] <sample name>
            [-file <file> [-format <format>]]

assoc[iate] -mul[tiplexer] <multiplexer name>
            -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

assoc[iate] -mul[tiplexer] <multiplexer name>
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

assoc[iate] -mul[tiplexer] <multiplexer name>
            [-primer1Mid <primer1Mid name>
                [-ofPrimer1MidGroup <primer1MidGroup name>]]
            [-primer2Mid <primer2Mid name>
                [-ofPrimer2MidGroup <primer2MidGroup name>]]
            -checkMid <boolean>
            -sam[ple] <sample name>
            -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

The 'associate' command is used to associate records in many-to-many
relationships.  Such relationships can exist between samples, amplicons,
read data, multiplexers, and MIDs.  When a particular association is made,
any more general associations that would be logically implied by the
original association will automatically be created (e.g., associating the
triplet of a sample, amplicon, and read data will implicitly create the
pairwise sample-amplicon association as well).

In any of the command forms above where '-amplicon' is being specified,
the '-ofRef' option can be used to disambiguate amplicons with the same
name but which are from different reference sequences.

The '-amplicon' option may be specified as a "*" to allow multiple
amplicons to be associated with a single command.  If a "*" is passed as
the '-amplicon' option value, with no '-ofRef' option, the "*" is
interpreted to indicate that all of the amplicons known in the project at
the time of the invocation will be involved in the association.  If both
the "*" value and '-ofRef' option are used, then all amplicons in the
project at the time of invocation that are of the given reference sequence
will be involved in the association.

In a similar manner to the '-ofRef' option for amplicons, the
'-ofPrimer1MidGroup' and '-ofPrimer2MidGroup' options can be used to
disambiguate '-primer1Mid' and '-primer2Mid' specifications, respectively.

The '-primer1Mid' and '-primer2Mid' options may also be specified as a
"*".  If no '-ofPrimer1MidGroup' or '-ofPrimer2MidGroup' option is
supplied, the "*" refers to all the MIDs of the project.  If a MID group
is specified, the "*" refers to only the MIDs of that MID group.

The '-checkMid' option is used to verify that the MIDs associated with the
primer1 side of the multiplexer are all mutually compatible as are the
primer2 side MIDs.  The definition of compatibility is the same as that
used by the '-checkMidGroup' option of the 'create mid' command (defined
sequences are compatible if they are of the same length and are
non-identical, with undefined zero-length sequences allowed).  This option
must be 'true' or 'false', and defaults to 'true' if not provided.

Read data can be specified in a command as either a specific read data
```

```
using the '-readData' option or as a collection of read data using the
'-readGroup' option.  When '-readGroup' is used, it is as if the command is
being run multiple times (once for each particular read data in the read
group at the time of invocation).  For example, if readGroup1 has 3
read data in it (readData1, readData2, and readData3), running the command:
    associate -sample sample1 -readGroup readGroup1
and running the commands:
    associate -sample sample1 -readData readData1
    associate -sample sample1 -readData readData2
    associate -sample sample1 -readData readData3
would have the same net effect.  In those situations where identical
associations can correctly be made with each of the read data in a read
group, the '-readGroup' allows the command to be more concise.

Explanations of the various command forms are as follows:

Run 'help general tabularCommands' for information about the '-file'
option.

assoc[iate] -sam[ple] <sample name>
            -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
            [-file <file> [-format <format>]]

When only a sample and amplicon are specified, an association is created
between them.  The '-ofRef' option can be used to disambiguate amplicons
with the same name that refer to different reference sequences.  If a "*"
is passed as the '-amplicon' option value, with no '-ofRef' option, all
amplicons known in the project are associated with the sample.  If both the
"*" value and '-ofRef' option are used, then all amplicons of the given
reference sequence are associated with the sample.

assoc[iate] -sam[ple] <sample name>
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

When a sample and read data are specified, associations are created between
the sample itself, all of the current amplicons associated with the sample,
and the read data.  For example, running
    associate -sample sample1 -readData read1
will associate "sample1" and all of its amplicons at the time of invocation
with "read1".

Similarly, when a sample and read group are specified, associations are
created between the sample itself, all of the current amplicons associated
with the sample, and all of the read data in the read group.  For example,
running
    associate -sample sample1 -readGroup group1
will associate "sample1" and all of its amplicons at the time of invocation
with all of the read data in "group1".

assoc[iate] -sam[ple] <sample name>
            -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

If a sample, an amplicon, and read data are specified, an association is
created between the sample itself, the amplicon, and the read data.  The
'-ofRef' option can be used to disambiguate amplicons with the same name
that refer to different reference sequences.  If a "*" is passed as the
'-amplicon' option value,  with no '-ofRef' option, all amplicons known
in the project are associated with the sample and read data.  If both the
"*" value and '-ofRef' option are used, then all amplicons of the given
reference sequence are associated with the sample and read data.  If a read
```

```
group is specified instead of a single read data, the sample and
amplicon(s) are associated with all of the read data in the read group.

In general, creating a triplet association between an amplicon, sample
and some read data implicitly creates a simultaneous paired association
between the amplicon and sample.  If an amplicon is already associated
with some sample on a particular read data set, any attempt to associate
the amplicon with a different sample on the same read data set will be
ignored (but with a warning) since the demultiplexing constraints only
allow amplicons to be associated with individual samples in the context
of any individual read data set (an amplicon may be associated with
different samples on different read data sets, and different amplicons
may be associated with different samples on the same read data, however).

assoc[iate] -mul[tiplexer] <multiplexer name>
            [-primer1Mid <primer1Mid name>
                [-ofPrimer1MidGroup <primer1MidGroup name>]]
            [-primer2Mid <primer2Mid name>
                [-ofPrimer2MidGroup <primer2MidGroup name>]]
            -checkMid <boolean>
            [-file <file> [-format <format>]]

When some combination of MIDs and a multiplexer are specified, the MIDs
will be associated with the multiplexer.  For either of the MID options
('-primer1Mid' and '-primer2Mid'), all of the MIDs in the project may be
specified at the same time by using a "*" for the value.  The
'-ofPrimer1MidGroup' and '-ofPrimer2MidGroup' options can be used to
restrict the "*" set of MIDs to those of a particular MID group (or to
disambiguate MIDs with the same name from different MID groups).  If it
becomes necessary to temporarily associate inconsistent MIDs on a primer
MID side, '-checkMid' can be set to 'false'.

Although it might be a more common case to use the next command below to
simultaneously associate a multiplexer, MIDs, and a sample in a single
operation (letting the implied multiplexer-MID associations be made
automatically), the above command can be useful to specify MIDs that are
not explicitly tied to samples.  In particular, if compatible MIDs have
been used in recent experiments, but are not present in this experiment,
associating them to the multiplexer without samples can help prevent
potential contaminants from being assigned to samples of this experiment.

assoc[iate] -mul[tiplexer] <multiplexer name>
            [-primer1Mid <primer1Mid name>
                [-ofPrimer1MidGroup <primer1MidGroup name>]]
            [-primer2Mid <primer2Mid name>
                [-ofPrimer2MidGroup <primer2MidGroup name>]]
            -checkMid <boolean>
            -sam[ple] <sample name>
            [-file <file> [-format <format>]]

When some combination of MIDs, a multiplexer, and a sample are specified,
the sample is associated with a particular MID configuration of the
multiplexer.  There are restrictions on how the MID options ('-primer1Mid'
and '-primer2Mid') can be used that depend on the '-encoding' type of the
multiplexer.  If the encoding type is 'both', it is required that both MID
options be provided in order to associate the sample with a pair of MIDs.
If the encoding type is 'either', 'primer1', or 'primer2', it is only
necessary to supply one of the MID options at a time.  In the 'either'
case, specifying both options at the same time is allowed.  In the
'primer1' and 'primer2' cases, the MID option of the proper type must be
used (e.g., the 'primer1' encoding type requires that the '-primer1Mid'
option be used).  Any implied multiplexer-MID associations that were not
explicitly set up previously will automatically be created as a consequence
```

```
of this command.  A sample may be associated with more than one MID
configuration, but each MID configuration may only map to a single sample
(e.g., in an 'primer1' configuration, sample1 may be associated with both
MID1 and MID2 on the primer1 side, but those MIDs could not simultaneously
be associated with a different sample2).

assoc[iate] -mul[tiplexer] <multiplexer name>
            -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

When a multiplexer, amplicon and read data are specified, the amplicon will
be associated with the read data-multiplexer context.  As a result, the
amplicon automatically becomes associated with each of the samples
associated with the multiplexer (creating any missing sample-amplicon
relationships along the way).  A "*" may be provided with the '-amplicon'
option to indicate that all amplicons in the project should be associated.
The '-ofRef' option can be used, if necessary, to disambiguate amplicons
with the same name or to restrict the "*" set of amplicons to those of the
specified reference sequence.  The association may be made with a single
read data using the '-readData' option, or the associations can be made
at once for all of the read data within a read group using the '-readGroup'
option.  Multiplexers may be associated with several different read data
sets.  Each of those read data-multiplexer contexts are allowed to have
different amplicon associations, but the internal MID-sample associations
remain the same in each of those contexts.  If the  multiplexer is not
already associated with the specified read data when this command is
invoked, those read data-multiplexer associations are created
automatically.

assoc[iate] -mul[tiplexer] <multiplexer name>
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

When a multiplexer and a read data are specified, the association between
the pair is created.  This provides a read data-multiplexer context that is
ready to accept amplicon associations (which get processed through the
multiplexer's MID configuration to get distributed to all the associated
samples).  This command is performed automatically as a consequence of the
more specific command above that also specifies an amplicon.

assoc[iate] -mul[tiplexer] <multiplexer name>
            [-primer1Mid <primer1Mid name>
                [-ofPrimer1MidGroup <primer1MidGroup name>]]
            [-primer2Mid <primer2Mid name>
                [-ofPrimer2MidGroup <primer2MidGroup name>]]
            -checkMid <boolean>
            -sam[ple] <sample name>
            -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
            -readData <read data name> | -readGroup <read group name>
            [-file <file> [-format <format>]]

It is also possible to specify all of the entities in a relationship at
once.  Provided that all of the individual entities have already been
created, this command creates a specific read data-multiplexer-amplicon
context where the multiplexer has an MID configuration that allows valid
mapping to samples.  This allows the AVA software to analyze reads at the
amplicon level and properly demultiplex them to their appropriate samples.
All of the necessary implied relationships in the command would be created
automatically.  A string of commands of this type might be used to define
the associations for an entire project.  This would not be a good strategy
when typing in commands by hand, but it could be convenient for setup
scripts that are programmatically generated using nested loops and tabular
```

```
commands.
```

3.4.2 close

```
close

Closes the current project.  This releases the lock held on the project
(unless it was opened "readOnly") and discards any unsaved changes.  A
project must be created or opened before executing commands that require an
open project.
```

3.4.3 computation

```
comp[utation] <computation command>

comp[utation] start
comp[utation] stop
comp[utation] status
comp[utation] loadDetectedVariants

The 'computation' command is used to control and query for information
about computations on the currently open project.

The following computation commands are available.  Run
'help computation <computation command>' for more detailed information.

start                   Starts a computation on the currently open project.
stop                    Stops a running computation on the currently
                            open project.
status                  Prints the status of computation on the currently
                            open project.
loadDetectedVariants    Loads variants into the currently open project that
                            were automatically detected during computation.
```

3.4.3.1 computation start

```
comp[utation] start

Starts a computation on the currently open project.
```

3.4.3.2 computation stop

```
comp[utation] stop

Stops a running computation on the currently open project.
```

3.4.3.3 computation status

```
comp[utation] status

Prints the status of computation on the currently open project.  If a
computation is currently running, "running" will be printed.  If no
computation is currently running, "stopped" will be printed.
```

3.4.3.4 computation loadDetectedVariants

```
comp[utation] loadDetectedVariants

Loads variants into the currently open project that were automatically
detected (i.e., not in list of predefined project Variants, but
automatically discovered by the software) during computation.
```

3.4.4 create

```
create <entity type> <other arguments>

The 'create' command is used to create new entities.  The type of entity to
create is determined by the '<entity type>' argument.  The
'<other arguments>' are determined by the entity type.  For example, to
create a project, you can run 'create project /path/to/new/project'.  This
will create a new project at '/path/to/new/project'.  To create a new
amplicon, you can run 'create amplicon "MyAmplicon"'.

The following entities are available for creation.  Run
'help create <entity type>' for more detailed information.

amplicon        Creates an amplicon in the currently open project.
mid             Creates an MID in the currently open project.
midGroup        Creates an MID group in the currently open project.
multiplexer     Creates a multiplexer in the currently open project.
project         Creates a new project.
readGroup       Creates a read group in the currently open project.
reference       Creates a reference sequence in the currently open project.
sample          Creates a sample in the currently open project.
variant         Creates a variant in the currently open project.
```

3.4.4.1 create amplicon

```
create amp[licon] <new amplicon name> [-orUpdate]
                                      [-ofRef <reference name>]
                                      [-annot[ation] <annotation>]
                                      [-ref[erence] <reference name>]
                                      [-primer1 <primer 1 sequence>]
                                      [-primer2 <primer 2 sequence>]
                                      [-start <target start index>]
                                      [-end <target end index>]
                                      [-checkPri[merMatch] <boolean>]
                                      [-file <file> [-format <format>]]

create amp[licon] -name <new amplicon name>
                  [-orUpdate]
                  [-ofRef <reference name>]
                  [-annot[ation] <annotation>]
                  [-ref[erence] <reference name>]
                  [-primer1 <primer 1 sequence>]
                  [-primer2 <primer 2 sequence>]
                  [-start <target start index>]
                  [-end <target end index>]
                  [-checkPri[merMatch] <boolean>]
                  [-file <file> [-format <format>]]
```

```
Creates a new amplicon in the currently open project.  In the first form,
the non-option argument is used as the name of the new amplicon.  In the
second, a name must be explicitly specified in option form.  If the
'-orUpdate' flag is given, an amplicon is only created if it does not
already exist.  If it already exists, the amplicon is merely updated.  The
'-ofRef' option can be used to disambiguate amplicons with the same name in
this case.  The remainder of the options are not required but can be used
to set properties of the new amplicon.

    -annotation         The annotation.
    -reference          The name of the reference sequence with which to
                            associate the amplicon.
    -primer1            The primer 1 sequence.  This must be a nucleotide
                            sequence string conforming to IUPAC
                            nomenclature.  Any ambiguous symbols are
                            considered 'N's.
    -primer2            The primer 2 sequence.  This must be a nucleotide
                            sequence string conforming to IUPAC
                            nomenclature.  Any ambiguous symbols are
                            considered 'N's.
    -start              The index of the target start position, or a '*'
                            to indicate the position should be
                            automatically assigned.
    -end                The index of the target end position, or a '*'
                            to indicate the position should be
                            automatically assigned.
    -checkPrimerMatch   Whether the system should check for a match between
                            the reference sequence and the primers in the
                            bases flanking the target region.  This must be
                            'true' or 'false', and defaults to 'true'.

The start and end options indicate the positional range of the amplified
target as measured from the first base of the associated reference
sequence.  In the case that the primer sequences are included in the
reference sequence, the system can automatically assign these positions by
finding matches of primer1 and the reverse complement of primer2 and
assigning the start and end positions to be just inside these matches.
Either, or both, of the start and end positions may be specified as a '*'
to request this search.  If one position is provided and the other is a
'*', then one position will be constrained as given and the search will
proceed on the other position.  If no such matching pair, or more than one
matching pair can be found, then an error is generated.  N's in either the
reference or primer sequences count as matches, but any match that involves
greater than 50% N's will be rejected.  Any other substitutions,
insertions, or deletions are not permitted.  Using a '*' for either the
start or end implies the checkPrimerMatch option and requires exact matches
of both primers in the reference sequence.  If the primers are not included
in the reference or if the primers contain bases that don't exactly match
the reference, the checkPrimerMatch option should be specified as 'false'
to prevent an error from being generated, and both start and end positions
should be explicitly provided.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.4.2 create mid

```
create mid <new mid name> [-orUpdate]
                          [-seq[uence] <sequence>]
```

```
                            [-annot[ation] <annotation>]
                            [-midGroup <midGroup>]
                            [-checkMidGroup <boolean>]
                            [-file <file> [-format <format>]]

    create mid -name <new mid name>
               [-orUpdate]
               [-seq[uence] <sequence>]
               [-annot[ation] <annotation>]
               [-midGroup <midGroup>]
               [-checkMidGroup <boolean>]
               [-file <file> [-format <format>]]

    Creates a new MID in the currently open project.  In the first form,
    the non-option argument is used as the name of the new MID.  In the
    second, a name must be explicitly specified in option form.  If the
    '-orUpdate' flag is given, an MID is only created if it does not
    already exist.  If it already exists, the MID is merely updated.  The
    remainder of the options are not required but can be used to set
    properties of the new MID.

        -annotation         The annotation.
        -sequence           The MID sequence.  This must be a non-zero length
                                nucleotide sequence string containing only the
                                bases A, C, T and G.
        -midGroup           The MID group of the MID, if it belongs to a
                                group.  This must be a pre-existing group,
                                created using the 'create midGroup' command.
        -checkMidGroup      Whether the system should check for compatibility
                                between the new MID sequence and other pre-
                                existing MID sequences belonging to the same
                                MID group.  This must be 'true' or 'false',
                                and defaults to 'true'.

    The name of the MID must be unique within the MID group it belongs to
    (or unique within the project if the MID is not assigned to an MID group).

    The rules for '-checkMidGroup' compatibility are as follows:

    - An MID with an undefined sequence is considered compatible with any MID
      group, under the assumption that its compatibility will eventually be
      assessed when a defined sequence gets assigned to the MID.
    - An MID with a defined sequence must have the same length as other
      defined MID sequences within an MID group to be compatible with the
      group. If the new MID sequence is the first defined sequence added to
      the MID group, the required sequence length for subsequent MIDs of the
      group with be the length of that first defined MID sequence.
    - An MID with a defined sequence must not be identical (ignoring case)
      with any other defined, pre-existing MID sequence of the same MID group.

    If it becomes necessary to edit existing MIDs in a way that temporarily
    leaves the MIDs in a group in an inconsistent state (such as changing the
    lengths of sequences in an MID group), '-checkMidGroup' should be set to
    'false'.

    Run 'help general tabularCommands' for information about the '-file'
    option.
```

3.4.4.3 create midGroup

```
create midGroup <new midGroup name> [-orUpdate]
```

```
                                    [-annot[ation] <annotation>]
                                    [-file <file> [-format <format>]]

create midGroup -name <new midGroup name>
                [-orUpdate]
                [-annot[ation] <annotation>]
                [-file <file> [-format <format>]]

Creates a new MID group in the currently open project.  In the first form,
the non-option argument is used as the name of the new MID group.  In the
second, a name must be explicitly specified in option form.  If the
'-orUpdate' flag is given, an MID group is only created if it does not
already exist.  If it already exists, the MID group is merely updated.
The remainder of the options are not required but can be used to set
properties of the new MID group.

    -annotation     The annotation.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.4.4 create multiplexer

```
create mul[tiplexer] <new multiplexer name>
                     [-orUpdate]
                     [-enc[oding] <encoding>]
                     [-annot[ation] <annotation>]
                     [-file <file> [-format <format>]]

create mul[tiplexer] -name <new multiplexer name>
                     [-orUpdate]
                     [-enc[oding] <encoding>]
                     [-annot[ation] <annotation>]
                     [-file <file> [-format <format>]]

Creates a new multiplexer in the currently open project.  In the first
form, the non-option argument is used as the name of the new multiplexer.
In the second, a name must be explicitly specified in option form.  If the
'-orUpdate' flag is given, a multiplexer is only created if it does not
already exist.  If it already exists, the multiplexer is merely updated.
The remainder of the options are not required but can be used to set
properties of the new multiplexer.

    -annotation         The annotation.
    -encoding           The MID layout type for the multiplexer, where
                            the choices are both, either, primer1, and
                            primer2.

The four '-encoding' types have the following definitions:

    both                 Both primer 1 and primer 2 MIDs are present and
                             necessary to determine the sample for each
                             read.
    either               Both primer 1 and primer 2 MIDs are present, but
                             either one is sufficient to determine the
                             sample.  For a given read, the MID at the
                             5' end, in the read's orientation, is used
                             to determine the sample.
    primer1              MIDs are only present adjacent to primer 1.
    primer2              MIDs are only present adjacent to primer 2.
```

```
Although a multiplexer can be initially created without specifying the
'-encoding' type, the '-encoding' type must be set before MIDs and
samples can be associated with the multiplexer.

If '-orUpdate' is used to change the '-encoding' type of a multiplexer,
then all pre-existing sample associations for the multiplexer will be
removed and certain pre-existing associations with MIDs may also be
removed. Specifically, if the '-encoding' type is changed to 'either'
and the numbers of already associated primer 1 and primer 2 MIDs are
not equal, then both sets of MID associations will be removed.  If the
'-encoding' type is changed to 'primer1', then any associated primer 2
MIDs will be dissociated and if the type is changed to 'primer2', any
associated primer 1 MIDs will be dissociated.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.4.5 *create project*

```
create proj[ect] <path for new project> [-name <new project name>]
                                        [-annot[ation] <annotation>]
                                        [-file <file> [-format <format>]]

create proj[ect] -path <path for new project>
                 [-name <new project name>]
                 [-annot[ation] <annotation>]
                 [-file <file> [-format <format>]]

Creates a new project.  In the first form, the non-option argument is
used as the path at which the new project will be created.  In the
second, a path must be explicitly specified in option form. The remainder
of the options are not required but can be used to set properties of the
new project.

When a new project is created, the previously open project is closed if
necessary, and the new project becomes the open project.

    -name           The name of the project.
    -annotation     The annotation describing the project.


Unlike with the creation of new projects from the gsAmplicon graphical user
interface (GUI), the 'create project' command does not initialize new
projects with any default contents.  To initialize a project with the same
default contents as it would have if created by the GUI, the following
command should be run subsequent the 'create project' command:

     utility execute %libDir/newProjectInit.ava

Run 'help general tabularCommands' for information about the '-file'
option.

Run 'help general filePaths' for more information about the interpretation
of relative paths when using the '-file' option or specifying the path for
the new project.
```

3.4.4.6 *create readGroup*

```
create readGroup <new read group name> [-orUpdate]
```

```
                                        [-annot[ation] <annotation>]
                                        [-file <file> [-format <format>]]

create readGroup -name <new read group name>
                 [-orUpdate]
                 [-annot[ation] <annotation>]
                 [-file <file> [-format <format>]]

Creates a new read group in the currently open project.  In the first form,
the non-option argument is used as the name of the new read group.  In the
second, a name must be explicitly specified in option form.  If the
'-orUpdate' flag is given, a read group is only created if it does not
already exist.  If it already exists, the read group is merely updated.
The remainder of the options are not required but can be used to set
properties of the new read group.

    -annotation     The annotation.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.4.7 create reference

```
create ref[erence] <new reference name> [-orUpdate]
                                        [-annot[ation] <annotation>]
                                        [-seq[uence] <sequence>]
                                        [-file <file> [-format <format>]]

create ref[erence] -name <new reference name>
                   [-orUpdate]
                   [-annot[ation] <annotation>]
                   [-seq[uence] <sequence>]
                   [-file <file> [-format <format>]]

Creates a new reference sequence in the currently open project.  In the
first form, the non-option argument is used as the name of the new
reference sequence.  In the second, a name must be explicitly specified in
option form.  If the '-orUpdate' flag is given, a reference sequence is
only created if it does not already exist.  If it already exists, the
reference sequence is merely updated.  The remainder of the options are not
required but can be used to set properties of the new reference sequence.

    -annotation     The annotation.
    -sequence       The nucleotide sequence string.  This sequence must use
                        IUPAC nomenclature.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.4.8 create sample

```
create sam[ple] <new sample name> [-orUpdate]
                                  [-annot[ation] <annotation>]
                                  [-file <file> [-format <format>]]

create sam[ple] -name <new sample name>
                [-orUpdate]
                [-annot[ation] <annotation>]
                [-file <file> [-format <format>]]
```

```
Creates a new sample in the currently open project.  In the first form, the
non-option argument is used as the name of the new sample.  In the second,
a name must be explicitly specified in option form.  If the '-orUpdate'
flag is given, a sample is only created if it does not already exist.  If
it already exists, the sample is merely updated.  The remainder of the
options are not required but can be used to set properties of the new
sample.

    -annotation     The annotation.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.4.9 *create variant*

```
create var[iant] <new variant name> [-orUpdate]
                                    [-ofRef <reference name>]
                                    [-annot[ation] <annotation>]
                                    [-ref[erence] <reference name>]
                                    [-pat[tern] <pattern>]
                                    [-stat[us] <status>]
                                    [-checkPat[tern] <boolean>]
                                    [-file <file> [-format <format>]]

create var[iant] -name <new variant name>
                 [-orUpdate]
                 [-ofRef <reference name>]
                 [-ref[erence] <reference name>]
                 [-annot[ation] <annotation>]
                 [-pat[tern] <pattern>]
                 [-stat[us] <status>]
                 [-checkPat[tern] <boolean>]
                 [-file <file> [-format <format>]]

Creates a new variant in the currently open project.  In the first form,
the non-option argument is used as the name of the new variant.  In the
second, a name must be explicitly specified in option form.  If the
'-orUpdate' flag is given, a variant is only created if it does not
already exist.  If it already exists, the variant is merely updated.  The
'-ofRef' option can be used to disambiguate variants with the same name in
this case.  The remainder of the options are not required but can be used
to set properties of the new variant.

    -annotation     The annotation.
    -reference      The name of the reference sequence to which the
                        variant refers.
    -pattern        The pattern that defines the nature of this variation.
    -status         The putative status.  This can be one of "accepted",
                        "rejected", or "putative"
    -checkPattern   Whether the system should check if the variant's
                        pattern is syntactically correct and consistent
                        with the variant's reference sequence. The
                        reference sequence must itself be set and have
                        a non-empty nucleotide sequence for this option
                        to take effect.  This value given must be 'true'
                        or 'false', and defaults to 'true'.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.5 dissociate

```
dissoc[iate] -sam[ple] <sample name>
             -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
             [-file <file> [-format <format>]]

dissoc[iate] -sam[ple] <sample name>
             -readData <read data name>
             [-file <file> [-format <format>]]

dissoc[iate] -sam[ple] <sample name>
             -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
             -readData <read data name>
             [-file <file> [-format <format>]]

dissoc[iate] -mul[tiplexer] <multiplexer name>
             [-primer1Mid <primer1Mid name>
                 [-ofPrimer1MidGroup <primer1MidGroup name>]]
             [-primer2Mid <primer2Mid name>
                 [-ofPrimer2MidGroup <primer2MidGroup name>]]
             [-file <file> [-format <format>]]

dissoc[iate] -mul[tiplexer] <multiplexer name>
             [-primer1Mid <primer1Mid name>
                 [-ofPrimer1MidGroup <primer1MidGroup name>]]
             [-primer2Mid <primer2Mid name>
                 [-ofPrimer2MidGroup <primer2MidGroup name>]]
             -sam[ple] <sample name>
             [-file <file> [-format <format>]]

dissoc[iate] -mul[tiplexer] <multiplexer name>
             -sam[ple] <sample name>
             [-file <file> [-format <format>]]

dissoc[iate] -mul[tiplexer] <multiplexer name>
             -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
             -readData <readData name>
             [-file <file> [-format <format>]]

dissoc[iate] -mul[tiplexer] <multiplexer name>
             -readData <readData name>
             [-file <file> [-format <format>]]

The 'dissociate' command is used to dissociate records in many-to-many
relationships.  If a general relationship is dissociated, the more
specific associations that depend on it automatically will be dissociated
as well (e.g., dissociating a sample from a read data will also result in
the dissociation of the sample's read data-sample-amplicon associations).
General relationships that are included as part of specific relationships
are not, however, automatically dissociated (e.g., when dissociating a
read data-sample-amplicon relationship, the more general sample-amplicon
relationship that it includes will not be dissociated).

In any of the command forms above where '-amplicon' is being specified,
the '-ofRef' option can be used to disambiguate amplicons with the same
name but which are from different reference sequences.

The '-amplicon' option may be specified as a "*" to allow multiple
amplicons to be dissociated with a single command.  In the context of a
command where '-amplicon' and '-sample' are both specified, the "*" is
interpreted to indicate that all of the amplicons of the sample should be
dissociated.  In the context of a command where '-amplicon',
'-multiplexer', and '-readData' are all specified, the "*" is interpreted
```

```
to indicate that all of the amplicons associated with the multiplexer in
the context of that read data should be dissociated.  In either case, the
'-ofRef' option can still be used to restrict the "*" selection of
amplicons to be that subset of amplicons belonging to the indicated
reference sequence.

In a similar manner to the '-ofRef' option for amplicons, the
'-ofPrimer1MidGroup' and '-ofPrimer2MidGroup' options can be used to
disambiguate '-primer1Mid' and '-primer2Mid' specifications, respectively.

The '-primer1Mid' and '-primer2Mid' options may also be specified as a
"*".  If no '-ofPrimer1MidGroup' or '-ofPrimer2MidGroup' option is
supplied, the "*" refers to all the MIDs of the project.  If a MID group
is specified, the "*" refers to only the MIDs of that MID group.

Explanations of the various command forms are as follows:

Run 'help general tabularCommands' for information about the '-file'
option.

dissoc[iate] -sam[ple] <sample name>
             -amp[licon] <amplicon name>
             [-ofRef <reference sequence name>]
             [-file <file> [-format <format>]]

If a sample and an amplicon are specified, they are dissociated.  The
'-ofRef' option can be used to disambiguate amplicons with the same name
that refer to different reference sequences.  If a "*" is passed as the
'-amplicon' option value, with no '-ofRef' option, all amplicons of the
sample are dissociated.  If both a "*" and the '-ofRef' option are used,
then all amplicons of the sample belonging to the indicated reference
sequence will be dissociated.

dissoc[iate] -sam[ple] <sample name>
             -readData <read data name>
             [-file <file> [-format <format>]]

If a sample and a read data are specified, the sample itself, all
amplicons of the sample currently associated with the read data, and the
read data are dissociated.

dissoc[iate] -sam[ple] <sample name>
             -amp[licon] <amplicon name>
             [-ofRef <reference sequence name>]
             -readData <read data name>
             [-file <file> [-format <format>]]

If a sample, amplicon, and read data are specified, the sample itself, the
amplicon, and the read data are dissociated.  The '-ofRef' option can be
used to disambiguate amplicons with the same name that refer to different
reference sequences.  If a "*" is passed as the '-amplicon' option value,
all amplicons of the sample are dissociated.  This is identical to using
the invocation form with only the sample and read data specified.

dissoc[iate] -mul[tiplexer] <multiplexer name>
             [-primer1Mid <primer1Mid name>
                 [-ofPrimer1MidGroup <primer1MidGroup name>]]
             [-primer2Mid <primer2Mid name>
                 [-ofPrimer2MidGroup <primer2MidGroup name>]]
             [-file <file> [-format <format>]]

If some combination of MIDs and a multiplexer are specified, the MIDs will
be dissociated from the multiplexer.  Any samples associated with those
```

```
MIDs via the multiplexer will be dissociated as well.  Note that a
multiplexer may be used on more than one read data, and MID dissociations
will impact sample associations on all of those read data at once.  Also
note, that depending on the pre-existing sample associations and
encoding type of the multiplexer (both, either, primer1, or primer2),
dissociating an MID might impact more than one sample (e.g., if the
multiplexer encoding is 'both' and there is a sample1 associated with
primer1Mid mid1 and primer2Mid mid2 and there is a sample2 associated with
primer1Mid mid1 and primer2Mid mid3, dissociating primer1 mid1 from the
multiplexer will cause both samples to be dissociated).

dissoc[iate] -mul[tiplexer] <multiplexer name>
             [-primer1Mid <primer1Mid name>
                 [-ofPrimer1MidGroup <primer1MidGroup name>]]
             [-primer2Mid <primer2Mid name>
                 [-ofPrimer2MidGroup <primer2MidGroup name>]]
             -sam[ple] <sample name>
             [-file <file> [-format <format>]]

If some combination of MIDs, a multiplexer, and a sample are specified,
the sample will be dissociated from the specific MID association, but the
MID associations with the multiplexer will be left intact.  The
'-primer1Mid' and '-primer2Mid' options are constrained by the encoding
type of the multiplexer.  Since this form of the command is expecting to
dissociate specific sample-MID associations, it must be given an
appropriate combination of MID options that are compatible with the
encoding type.  If the encoding type is 'both', '-primer1Mid' and
'-primer2Mid' options must both be specified along with the sample.
If the encoding type is 'either', it is permissible to provide both MIDs
or just a single MID along with the specified sample.

dissoc[iate] -mul[tiplexer] <multiplexer name>
             -sam[ple] <sample name>
             [-file <file> [-format <format>]]

If a multiplexer and a sample are specified, the sample gets dissociated
from all MID combinations that have been used to associate the sample
with the multiplexer.  The pre-existing multiplexer-MID associations are
left intact.

dissoc[iate] -mul[tiplexer] <multiplexer name>
             -amp[licon] <amplicon name> [-ofRef <reference sequence name>]
             -readData <readData name>
             [-file <file> [-format <format>]]

If a multiplexer, amplicon and readData are specified, the amplicon is
dissociated from the specific read data-multiplexer context.  If the
amplicon is simultaneously associated with the same multiplexer on a
different read data, that relationship will be left intact.  A "*" may be
provided with the '-amplicon' option to indicate that all amplicons
associated with the read data-multiplexer should be dissociated.  The
'-ofRef' option can be used, if necessary, to disambiguate among amplicons
with the same name or to restrict the "*" set of amplicons to those of
the specified reference sequence.  Severing the relationship of an
amplicon with a read data-multiplexer simultaneously dissociates the
amplicon from all of the samples associated with the multiplexer in that
read data context.  The general sample-amplicon relationships, however,
remain intact.

dissoc[iate] -mul[tiplexer] <multiplexer name>
             -readData <readData name>
             [-file <file> [-format <format>]]
```

```
If a multiplexer and a read data are specified, the multiplexer will be
dissociated from that specific read data.  The internal relationships of
the multiplexer such as MID and sample associations remain intact, but any
amplicons that were associated with the specific read data-multiplexer
will be dissociated.  If the multiplexer is simultaneously associated with
other specific read data, those associations remain unchanged.
```

3.4.6 exit

```
exit [<return code>]

Exits the command interpreter.  By default, 0 is used as the return code
for the command interpreter process.  If a return code is provided as an
argument, it is used instead.
```

3.4.7 list

```
list <entity type> <other arguments>

The 'list' command is used to list information about project entities or
the project itself.  The type of entity to list is determined by the
'<entity type>' argument.  The '<other arguments>' are determined by the
entity type.  For example, to list all amplicons in the currently open
project, you can run 'list amplicon'.

The following entities are available for listing.  Run
'help list <entity type>' for more detailed information.

amplicon        Lists amplicons in the currently open project.
mid             Lists MIDs in the currently open project.
midGroup        Lists MID groups in the currently open project.
multiplexer     Lists multiplexers in the currently open project.
project         Lists information about the currently open project.
readData        Lists read data in the currently open project.
readGroup       Lists read groups in the currently open project.
reference       Lists reference sequences in the currently open project.
sample          Lists samples in the currently open project.
variant         Lists variants in the currently open project.
```

3.4.7.1 list amplicon

```
list amp[licon] [-outputFile <file>] [-format <table format>]

Lists all of the amplicons in the currently open project.  The listing is
printed in the form of a table.  The table has columns for the following.

    Name            The name of the amplicon.
    Annotation      The annotation for the amplicon.
    Reference       The reference sequence to which the amplicon refers.
    Primer1         The first primer.
    Primer2         The second primer.
    Start           The index of the start of the target in the reference
                        sequence.
    End             The index of the end of the target in the reference
                        sequence.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
```

```
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.2 list mid

```
list mid [-outputFile <file>] [-format <table format>]

Lists all of the MIDs in the currently open project.  The listing
is printed in the form of a table.  The table has columns for the
following.

    Name            The name of the MID.
    Annotation      The annotation for the MID.
    Sequence        The nucleotide sequence of the MID.
    MidGroup        The MID group to which the MID belongs.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.3 list midGroup

```
list midGroup [-outputFile <file>] [-format <table format>]

Lists all of the MID groups in the currently open project.  The listing is
printed in the form of a table.  The table has columns for the following.

    Name            The name of the MID group.
    Annotation      The annotation for the MID group.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.4 list multiplexer

```
list mul[tiplexer] [-outputFile <file>] [-format <table format>]
```

```
Lists all of the multiplexers in the currently open project.  The listing
is printed in the form of a table.  The table has columns for the
following.

    Name            The name of the multiplexer.
    Annotation      The annotation for the multiplexer.
    Encoding        The encoding type of the multiplexer (both, either,
                        primer1, or primer2).

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.5 *list project*

```
list proj[ect] [-outputFile <file>] [-format <table format>]

Lists data about the currently open project.  The listing is
printed in the form of a table.  The table has columns for the following.

    Path            The directory path to the project.
    Name            The name for the project.
    Annotation      The annotation for the project.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.6 *list readData*

```
list readData [-outputFile <file>] [-format <table format>]

Lists all of the read data in the currently open project.  The listing is
printed in the form of a table.  The table has columns for the following.

    Name            The name of the read data.
    Annotation      The annotation for the read data.
    ReadGroup       The read group to which the read data belongs.
    SymLink         Whether the read data is symbolically linked into the
                        project.
    Active          Whether the read data is active in the project.
    SffDir          The SFF directory from which the read data was
                        imported.
    SffName         The name of the SFF file of the read data.

If no '-outputFile' option is given, the table is printed in a
```

```
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.7 list readGroup

```
list readGroup [-outputFile <file>] [-format <table format>]

Lists all of the read groups in the currently open project.  The listing is
printed in the form of a table.  The table has columns for the following.

    Name            The name of the read group.
    Annotation      The annotation for the read group.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.8 list reference

```
list ref[erence] [-outputFile <file>] [-format <table format>]

Lists all of the reference sequences in the currently open project.  The
listing is printed in the form of a table.  The table has columns for the
following.

    Name            The name of the reference.
    Annotation      The annotation for the reference.
    Sequence        The nucleotide sequence of the reference.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.9 list sample

```
list sam[ple] [-outputFile <file>] [-format <table format>]
```

```
Lists all of the samples in the currently open project.  The listing is
printed in the form of a table.  The table has columns for the following.

    Name            The name of the sample.
    Annotation      The annotation for the sample.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.7.10 list variant

```
list var[iant] [-outputFile <file>] [-format <table format>]

Lists all of the variants in the currently open project.  The listing is
printed in the form of a table.  The table has columns for the following.

    Name            The name of the variant.
    Annotation      The annotation for the variant.
    Reference       The reference sequence to which the variant refers.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.
```

3.4.8 load

```
load -readGroup <read group name>
     -sffDir <SFF directory>
     -sffName <SFF file name>
     [-symLink <boolean>]
     [-alias <alias prefix for command interpreter>]
     [-file <file> [-format <format>]]

load -readGroup <read group name>
     -analysisDir <analysis directory>
     -sffName <SFF file name>
     [-symLink <boolean>]
     [-alias <alias prefix for command interpreter>]
     [-file <file> [-format <format>]]

load -readGroup <read group name>
     -sffDir <SFF directory>
     -regions <comma-separated region list>
     [-symLink <boolean>]
     [-filePrefix <SFF file prefix>]
```

```
     [-alias <alias prefix for command interpreter>]
     [-file <file> [-format <format>]]

load -readGroup <read group name>
     -analysisDir <analysis directory>
     -regions <comma-separated region list>
     [-symLink <boolean>]
     [-filePrefix <SFF file prefix>]
     [-alias <alias prefix for command interpreter>]
     [-file <file> [-format <format>]]

The 'load' command is used load read data into the currently open project.
The different options combinations for running the 'load' command provide
different ways of specifying what read data to load.

For all forms of invocation, the read group into which the read data will
be loaded must be provided using the '-readGroup' option.

The '-symLink' option defaults to false, but may be specified as true.
When specified as true, the read data files are not actually copied into
the area of the disk that stores the Amplicon Project: a symbolic link
to the data is created instead.

The location of the read data files can be specified with either the
'-sffDir' or '-analysisDir' options.  Use the '-sffDir' option to specify a
directory that directly contains read data files (.sff files).  Use the
'-analysisDir' option to specify an analysis directory.

In addition to the location of the read data files, the specific read data
to load must also be specified with the '-sffName' or '-regions' options.
Use the '-sffName' option to specify the name of the SFF file to load.  Use
the '-regions' option to specify the regions to load.  Regions must be
specified in a comma separated list with no intervening spaces.  For
example, '-regions 1,2,4', specifies that regions 1, 2, and 4 should be
loaded.

If the '-regions' option is used to specify the read data files to load,
a '-filePrefix' option may be provided to restrict the loading to only
those regions whose SFF file names begin with a certain prefix.  For
example, in a given SFF directory there may be two region 1 files,
TEST01.sff and REAL01.sff.  If you specify '-regions 1', both of these
files will be loaded.  However, if you specify '-regions 1 -filePrefix
REAL', only the later file will be loaded.

An alias may be provided that allows loaded read data to be referenced by
subsequent commands.  For example, if we run
'load -readGroup MyGroup -sffDir some/path/sff -regions 1,2 -alias read',
we can subsequently refer to the imported region read data as "read01" and
"read02".  For example, we can run:
     'assoc -readData read01 -sample sample1 -amplicon amplicon1'
(assuming sample1 and amplicon1 exist).  The alias is constructed by taking
the value passed to the '-alias' option and appending two digits specifying
the region.  This option facilitates the creation of scripts that load from
analysis directories, wherein the regions of interest are known in
advance, but the actual SFF file names are not known (since they are
automatically given names by the pipeline software).

Here are some examples of valid 'load' invocations.

load -readGroup Group1 -sffDir /data/sff -sffName TEST01.sff

    This will load the read data in /data/sff/TEST01.sff into the read
    group named "Group1" of the currently open project.
```

```
load -readGroup Group1 -analysisDir /data/analysis1 \
                       -regions 1,2,4 -alias Read

    This will load the read data of regions 1, 2, and 4 inside the analysis
    directory /data/analysis1 into the read group named "Group1" of the
    currently open project.  Subsequent commands will be able to refer to
    the read data as "Read01", "Read02", and "Read04".

load -readGroup Group1 -analysisDir /data/analysis1 \
                       -regions 2 -filePrefix TEST -alias Read

    This will load the read data of region 2 inside the analysis directory
    /data/analysis1 into the read group named "Group1" of the currently
    open project.  Only SFF files with prefix "TEST" in the analysis will
    be considered.

Run 'help general tabularCommands' for information about the '-file'
option.

Run 'help general filePaths' for more information about the interpretation
of relative paths when using the '-file', '-analysisDir' or '-sffDir'
options.
```

3.4.9 open

```
open <project path> [-control <control mode>]

Opens a project at a given path.  When a project is opened, the previously
open project is closed if necessary.

    -control        The control mode.  This can be one of "preempt" or
                        "readOnly".  By default, this command attempts to
                        acquire control of the project, which is required
                        to modify and run computations on the project.  If
                        another application is already in control, the
                        attempt fails, and an error is reported.  If the
                        control mode is set to "preempt", this command
                        will preempt the control of the other application
                        and take control for itself.  If the control
                        mode is set to "readOnly", then control is not
                        taken, and attempts to save modifications to this
                        project will fail.

 Note: If a previous instance of the command line or graphical user
 interface had the project open and was prematurely terminated, it may
 erroneously appear to the system that the project is currently under the
 control of another program instance.   In this case, in order to obtain
 control over the project, the "-control preempt" option will need to
 be used.

Run 'help general filePaths' for more information about the interpretation
of relative paths if used when specifying the project path.
```

3.4.10 remove

```
remove <entity type> <other arguments>

The 'remove' command is used to remove entities.  The type of entity to
remove is determined by the '<entity type>' argument.  The '<other
```

```
arguments>' are determined by the entity type.  For project records, the
'<other arguments>' is generally the name of the record to remove.

The following entities are available for removing.  Run
'help remove <entity type>' for more detailed information.

amplicon        Removes an amplicon from the currently open project.
mid             Removes an MID from the currently open project.
midGroup        Removes an MID group from the currently open project.
multiplexer     Removes a multiplexer from the currently open project.
readData        Removes a read data from the currently open project.
readGroup       Removes a read group from the currently open project.
reference       Removes a reference sequence from the currently open
                    project.
sample          Removes a sample from the currently open project.
variant         Removes a variant from the currently open project.
```

3.4.10.1 remove amplicon

```
remove amp[licon] <amplicon name> [-ofRef <reference sequence name>]
                                  [-file <file> [-format <format>]]

remove amp[licon] -name <amplicon name>
                  [-ofRef <reference sequence name>]
                  [-file <file> [-format <format>]]

Removes an amplicon.  In the first form, the non-option argument is used as
the name of the amplicon to remove.  In the second, a name must be
explicitly specified in option form.

Amplicons are allowed to have duplicate names as long as the reference
sequences to which they refer are distinct.  The '-ofRef' argument can be
used to refer to such amplicons.  For example, if we have two amplicons
named "MyAmp", but one of them refers to "ReferenceSequence1" and the other
to "ReferenceSequence2", we can use the '-ofRef' option to distinguish
them.  We can run 'remove amplicon "MyAmp" -ofRef "ReferenceSequence1"' to
remove the former amplicon.

If the amplicon name is given as the character '*' then all amplicons will
be removed.  If the '-ofRef' option is also supplied, then all the
amplicons of just that reference sequence will be removed.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.10.2 remove mid

```
remove mid <mid name> [-ofMidGroup <midGroup>]
                      [-file <file> [-format <format>]]

remove mid -name <mid name>
           [-ofMidGroup <midGroup>]
           [-file <file> [-format <format>]]

Removes an MID.  In the first form, the non-option argument is used as
the name of the MID to remove.  In the second, a name must be
explicitly specified in option form.

MIDs are allowed to have duplicate names as long as they belong to
distinct MID groups.  The '-ofMidGroup' argument can be used to refer to
```

```
such MIDs.  For example, if we have two MIDs named "MyMID", but one of
them is a member of MID group "MID_Group1" and the other is a member of
MID group "MID_Group2", we can use the '-ofMidGroup' option to distinguish
them.  We can run 'remove mid "MyMID" -ofMidGroup "MID_Group1"' to remove
the former MID.

If the MID name is given as the character '*' then all MIDs will
be removed.  If the '-ofMidGroup' option is also supplied, then all the
MIDs of just that MID group will be removed.

Removing MIDs also results in the removal of any associations in which
they are participants.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.10.3 remove midGroup

```
remove midGroup <read group name> [-file <file> [-format <format>]]
remove midGroup -name <read group name> [-file <file> [-format <format>]]

Removes an MID group.  In the first form, the non-option argument is used
as the name of the read group to remove.  In the second, a name must be
explicitly specified in option form.

If an MID group is removed, then all the MIDs of that group are
also removed.

If the MID group name is given as the character '*' then all MID groups
will be removed.  This would remove all the MIDs that belong to MID groups
from the project at the same time, but it would leave behind any MIDs that
do not have an MID group assignment.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.10.4 remove multiplexer

```
remove mul[tiplexer] <multiplexer name> [-file <file> [-format <format>]]
remove mul[tiplexer] -name <multiplexer name>
                     [-file <file> [-format <format>]]

Removes a multiplexer.  In the first form, the non-option argument
is used as the name of the multiplexer to remove.  In the second, a
name must be explicitly specified in option form.

If a multiplexer is removed, then all the associations that include that
multiplexer (such as multiplexer-readData and multiplexer-MID associations)
are removed at the same time.

If the multiplexer name is given as the character '*' then all the
multiplexers will be removed along with the associations in which they
participate.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.10.5 remove readData

```
remove readData <read data name> [-file <file> [-format <format>]]
remove readData -name <read data name> [-file <file> [-format <format>]]

Removes a read data.  In the first form, the non-option argument is used as
the name of the read data to remove.  In the second, a name must be
explicitly specified in option form.

If the read data name is given as the character '*' then all read
data will be removed.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.10.6 remove readGroup

```
remove readGroup <read group name> [-file <file> [-format <format>]]
remove readGroup -name <read group name> [-file <file> [-format <format>]]

Removes a read group.  In the first form, the non-option argument is used
as the name of the read group to remove.  In the second, a name must be
explicitly specified in option form.

If a read group is removed, then all the read data of that group are
also removed.

If the read group name is given as the character '*' then all read groups
will be removed.  This would effectively remove all the read data from
the project at the same time.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.10.7 remove reference

```
remove ref[erence] <reference name> [-file <file> [-format <format>]]
remove ref[erence] -name <reference name> [-file <file> [-format <format>]]

Removes a reference sequence.  In the first form, the non-option argument
is used as the name of the reference sequence to remove.  In the second, a
name must be explicitly specified in option form.

If a reference sequence is removed, then all the amplicons and variants
associated with that reference sequence are removed at the same time.

If the reference sequence name is given as the character '*' then all
the reference sequences will be removed.  This would effectively remove
all the amplicons and variants at the same time.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.10.8 remove sample

```
remove sam[ple] <sample name> [-file <file> [-format <format>]]
remove sam[ple] -name <sample name> [-file <file> [-format <format>]]
```

```
Removes a sample.  In the first form, the non-option argument is used as
the name of the sample to remove.  In the second, a name must be explicitly
specified in option form.

If the sample name is given as the character '*' then all samples will be
removed.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.10.9 remove variant

```
remove var[iant] <variant name> [-ofRef <reference sequence name>]
                                [-file <file> [-format <format>]]

remove var[iant] -name <variant name>
                 [-ofRef <reference sequence name>]
                 [-file <file> [-format <format>]]

Removes a variant.  In the first form, the non-option argument is used as
the name of the variant to remove.  In the second, a name must be
explicitly specified in option form.

Variants are allowed to have duplicate names as long as the reference
sequences to which they refer are distinct.  The '-ofRef' argument can be
used to refer to such variants.  For example, if we have two variants named
"MyVar", but one of them refers to "ReferenceSequence1" and the other to
"ReferenceSequence2", we can use the '-ofRef' option to distinguish them.
We can run 'remove amplicon "MyVar" -ofRef "ReferenceSequence1"' to remove
the former variant.

If the variant name is given as the character '*' then all variants will
be removed.  If the '-ofRef' option is also supplied, then all the variants
of just that reference sequence will be removed.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.11 rename

```
rename <entity type> <other arguments>

The 'rename' command is used to rename entities.  The type of entity to
rename is determined by the '<entity type>' argument.  The '<other
arguments>' are determined by the entity type.  For project records, the
'<other arguments>' are generally the name of the record to rename followed
by the new name for the record.  For example, running 'rename amplicon
"Amp1" "Amp2"' will rename the amplicon named "Amp1" to "Amp2".

The following entities are available for renaming.  Run
'help rename <entity type>' for more detailed information.

amplicon        Renames an amplicon in the currently open project.
mid             Renames an MID in the currently open project.
midGroup        Renames an MID group in the currently open project.
multiplexer     Renames a multiplexer  in the currently open project.
project         Renames the currently open project.
readData        Renames a read data in the currently open project.
readGroup       Renames a read group in the currently open project.
reference       Renames a reference sequence in the currently open project.
```

```
sample          Renames a sample in the currently open project.
variant         Renames a variant in the currently open project.
```

3.4.11.1 rename amplicon

```
rename amp[licon] <name> <new name> [-ofRef <reference sequence name>]
                                   [-file <file> [-format <format>]]

rename amp[licon] -name <name>
                  -newName <new name>
                  [-ofRef <reference sequence name>]
                  [-file <file> [-format <format>]]

Renames an amplicon.  Amplicons are allowed to have duplicate names as long
as the reference sequences to which they refer are distinct.  The '-ofRef'
argument can be used to refer to such amplicons.  For example, if we have
two amplicons named "MyAmp", but one of them refers to "ReferenceSequence1"
and the other to "ReferenceSequence2", we can use the '-ofRef' option to
distinguish them.  We can run 'rename amplicon "MyAmp" "MyAmp2" -ofRef
"ReferenceSequence1"' to rename the former amplicon.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.11.2 rename mid

```
rename mid <name> <new name> [-ofMidGroup <midGroup>]
                             [-file <file> [-format <format>]]

rename mid -name <name>
           -newName <new name>
           [-ofMidGroup <midGroup>]
           [-file <file> [-format <format>]]

Renames an MID. MIDs are allowed to have duplicate names as long as they
belong to distinct MID groups.  The '-ofMidGroup' argument can be used to
refer to such MIDs.  For example, if we have two MIDs named "MyMID", but
one of them is a member of MID group "MID_Group1" and the other is a
member of MID group "MID_Group2", we can use the '-ofMidGroup' option to
distinguish them.  We can run:
       rename mid "MyMID" "MyMid2" -ofMidGroup "MID_Group1"
to update the former MID.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.11.3 rename midGroup

```
rename midGroup <name> <new name> [-file <file> [-format <format>]]

rename midGroup -name <name>
                -newName <new name>
                [-file <file> [-format <format>]]
```

```
Renames an MID group.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.11.4 rename multiplexer

```
rename multiplexer <name> <new name> [-file <file> [-format <format>]]

rename multiplexer -name <name>
                   -newName <new name>
                   [-file <file> [-format <format>]]

Renames an multiplexer.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.11.5 rename project

```
rename project <new name>

Renames the currently open project.
```

3.4.11.6 rename readData

```
rename readData <name> <new name> [-file <file> [-format <format>]]

rename readData -name <name>
                -newName <new name>
                [-file <file> [-format <format>]]

Renames a read data.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.11.7 rename readGroup

```
rename readGroup <name> <new name> [-file <file> [-format <format>]]

rename readGroup -name <name>
                 -newName <new name>
                 [-file <file> [-format <format>]]

Renames a read group.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.11.8 rename reference

```
rename ref[erence] <name> <new name> [-file <file> [-format <format>]]

rename ref[erence] -name <name>
                   -newName <new name>
                   [-file <file> [-format <format>]]

Renames a reference sequence.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.11.9 rename sample

```
rename sam[ple] <name> <new name> [-file <file> [-format <format>]]

rename sam[ple] -name <name>
                -newName <new name>
                [-file <file> [-format <format>]]

Renames a sample.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.11.10 rename variant

```
rename var[iant] <name> <new name> [-ofRef <reference sequence name>]
                                   [-file <file> [-format <format>]]

rename var[iant] -name <name>
                 -newName <new name>
                 [-ofRef <reference sequence name>]
                 [-file <file> [-format <format>]]

Renames an variant.  Variants are allowed to have duplicate names as long
as the reference sequences to which they refer are distinct.  The '-ofRef'
argument can be used to refer to such variants.  For example, if we have
two variants named "MyVar", but one of them refers to "ReferenceSequence1"
and the other to "ReferenceSequence2", we can use the '-ofRef' option to
distinguish them.  We can run 'update variant "MyVar" -ofRef
"ReferenceSequence1"' to update the former variant.

Instead of using arguments to specify the name and new name, the '-name'
and '-newName' options can be used.  This is useful when running this as a
tabular command.  Run 'help general tabularCommands' for information about
tabular commands and the '-file' option.
```

3.4.12 report

```
report <report type> <other arguments>

The 'report' command is used to generate reports about the currently open
project.  The type of report is determined by the '<report type>' argument.
The '<other arguments>' are determined by the report type.

The following report types are available.  Run
'help report <report type>' for more detailed information.

alignment       The alignments in the currently open project.
variantHits     The variant hits in the currently open project.
```

3.4.12.1 report alignment

```
report align[ment]
       -sam[ple] <sample name>
       -ref[erence] <reference sequence name>
       [-readT[ype] <"con[sensus]" or "ind[ividual]">]
       [-start <reference start position>] [-end <reference end position>]
       [-mar[gin] <size>]
       [-wrap[pingWidth] <width>]
       [-makeDir[ectory] <"all", "last" or "none">]
       [-outputFor[mat] <"fasta", "clustal", "ace",
                         "table" [-tableOutputFormat <tsv|csv>]> ]
       [-outputDir[ectory] <directory path>]
       [ [-outputFile <file>] |
         [ [-outputPre[fix] <prefix>]
           [-outputSuf[fix] <suffix>]
           [-mappingFile <file>]   ] ]
       [-annot[ationFileSuffix] <suffix>]
       [-fileFilter <"all", "linux", "mac", or "windows">]
       [-file <file> [-format <format>]]
       [<amplicon name 1> <amplicon name 2> ...]


The 'report alignment' command outputs sequence alignments in one of
several formats.  FASTA format is the default, but Clustal, Ace, and Table
may also be specified using the -outputFormat parameter.

Values for the '-sample' and '-reference' parameters are required, and if
specified as the names of a sample and reference sequence for which an
alignment has been computed in the project, then the corresponding
alignment will be output.  If no '-outputFile' option is given, the
alignment is printed to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the alignment
is written to that file.  Run 'help general filePaths' for more information
about specifying files.

Alternatively, either or both of the '-sample' or '-reference' parameters
may be specified as the (wildcard) character '*', in which case all
alignments that have been computed in the project for the indicated
combination of samples and reference sequences will be output.  When using
this form of the command, multiple alignments will typically be produced,
and so the output cannot be sent to standard output and the '-outputFile'
parameter cannot be used.  As explained below, the alignments are written
to files in a directory structure according to a file naming convention
that can be customized using the '-outputPrefix' and '-outputSuffix'
parameters.
```

```
Using the '-file' parameter, one or more of the parameter values may be
supplied from tabular input.  Run 'help general tabularCommands' for
information about the '-file' option.

The remaining parameters are described below, grouped by their use in
specifying the alignment region to output, formatting the alignment, and
determining where the output is to be written.

ALIGNMENT TYPE AND REGION PARAMETERS:

The '-readType' parameter specifies the type of read to include in the
alignment, and may be either "consensus" (the default if '-readType' is not
used) or "individual".

By default, the alignment output includes the target sequence regions of
all the amplicons for which there are computed alignment data for the given
'-sample' and '-reference' values.  An optional, space separated, list of
amplicon names may be provided to restrict the alignment output to the
target sequence neighborhoods of those specific amplicons.  The amplicon
names are interpreted relative to the given '-reference' value, and thus
this amplicon filtering ability is typically only useful if a non-wildcard
'-reference' value is supplied.

The '-start' and '-end' parameters may be used to precisely define
(in 1-based reference sequence positions) the bounds for the reads in the
alignment.

If '-start' and/or '-end' positions are specified along with a list of
specific amplicons (or all amplicons for the reference sequence, if a
specific list is not supplied), the alignment output will be restricted to
that region of reference base positions that constitute the (smallest)
intersection of all the specifications.

Bases of reads that extend outside the specified alignment region will be
trimmed from the output, and reads that align within these positions will
be padded on either side, as applicable, with gap characters ('-').  Reads
whose alignments have no overlap with the specified alignment region will
not be included in the output at all.

FORMATTING PARAMETERS:

The '-margin' parameter specifies a number of additional reference bases
to include on either side of the alignment region (as determined by the
amplicons, '-start' and '-end' parameters described above).  The bases
of the reads in the alignment will still be trimmed to the specified
alignment region, but the reference sequence, which is output as the first
sequence of the alignment output, will include the additional contextual
bases. Under these reference positions, the read alignments will be padded
with the gap character ('-').  If not specified, the default margin is
0 (zero).

The '-wrappingWidth' parameter defines the maximum number of alignment
characters to allow per line in the formatted alignment output. In FASTA
output only, the special value 0 (zero) may be given to indicate no
wrapping.  If no value is supplied, then the default value of 50 will be
used.  ACE formatted output currently ignores this option.

WRITING ALIGNMENT TO STANDARD OUTPUT:

If no wildcard ('*') specifiers are used for either the '-sample' or
'-reference' and no '-outputFile' parameter value is supplied (or one is
supplied, but it is the special value '-'), then the alignment
```

```
will be written to the standard output of the interpreter.

WRITING ALIGNMENT(S) TO FILE(S):

Alignment output may be written to files using a combination of the
'-outputDirectory' parameter and other parameters that depend on whether
or not a wildcard ('*') specification was provided for either of the
'-sample' or '-reference' parameters.

The '-outputDirectory' is optional, but can be used as a convenience to
factor out the specification of a containing directory from the remainder
of the output file path specification.   The value given for
'-outputDirectory' follows all the rules as explained for specifying
paths in 'help general filePaths' and, in particular, allows the use of
path shortcuts like %homeDir at the beginning of the path specification.

When wildcard ('*') specifications for '-sample' and '-reference' are not
used, the '-outputFile' parameter may be used to specify a single file for
the alignment output. The file is placed under the path specified
by the '-outputDirectory' parameter, if given.  If '-outputDirectory' is
not specified, then the file specified by '-outputFile' will be written
under the current directory unless the '-outputFile' itself contains some
additional, prefixed relative or absolute path specification as explained
in 'help general filePaths'.

When a wildcard ('*') specification for either '-sample' or '-reference' is
used,  the output file for a given sample / reference combination is a file
in the directory:

    outputDirectory/filteredSampleName/filteredReferenceName

where the outputDirectory is the current directory if '-outputDirectory'
is not specified.  The filteredSampleName and filteredReferenceName are the
original sample and reference names from the project, possibly changed
according to the value of the '-fileFilter' parameter, which is explained
below.

Within that directory structure, that alignment file is written to a file
of the automatically generated name:

outputPrefix +
    filteredSampleName + "_vs_" + filteredReferenceName + outputSuffix

where "+" indicates concatenation of the values.  The outputPrefix value
can be specified with the '-outputPrefix' parameter and defaults to the
empty string if not supplied.  The outputSuffix may be specified with the
'-outputSuffix' parameter to provide a filename extension; when unspecified
it defaults to the filename-extension associated with the type given in
-outputFormat, i.e., 'fasta'=".fna", 'clustal'=".aln", 'ace'=".ace".
Note that the "." that separates the file extension from the rest of the
file name is explicitly supplied as part of the outputSuffix itself, and
so the extension can be effectively eliminated by supplying an empty string
("") for the '-outputSuffix' parameter value.

When wildcards are used, the automatically generated filenames and the
directory structure that contains the alignment output, are based on the
names of the samples and reference sequences.  It is possible that these
names contain characters that are not allowed in filenames according to
the operating system where the files are initially created or may
eventually be viewed (if the files were copied to another machine).
Consequently, these names must be filtered to be compatible with file
naming conventions of the intended operating systems.
```

```
Filename filtering is controlled by the '-fileFilter' parameter that
ensures that the automatically generated output filenames and paths use
legal file system characters. If this parameter is not supplied, then its
value defaults to "all" which provides the most strict filtering and should
produce filenames that are compatible across all major operating systems.
Illegal characters are replaced with a hyphen and a unique index (for the
one invocation of the report alignment command) that uniquely encodes the
characters.  Less general, OS-specific filename filtering may be elected by
setting this parameter to "linux", "windows" or "mac".  Note, that this
setting does not filter the file-path value set by '-outputFile' when
wildcards are not used, where the user is in complete control of the
filename.

When wildcards are used, the '-mappingFile' parameter may optionally
designate the name of the file that should be created by the report
alignment command in the outputDirectory.  This file will contain a row of
data for each sample/reference name pair and specify the relative path to
the corresponding alignment output file for that pair.  Using this file,
a user, or automated process, can determine the alignment output file based
on the original sample and reference names, prior to any
filesystem-specific filename filtering.  The mapping file will be in comma
separated format if specified with a ".csv" extension, and will be
tab-separated otherwise.

When using wildcards, it is possible that the directory specified by
'-outputDirectory' does not already exist.  The '-makeDirectory' parameter
may be given to specify what to do in this case.  Providing the value "all"
will allow all sub-directories in the -outputDirectory path to be created
(i.e., if they don't already exist on the disk).  The value "last" will
allow the last directory on the path to be created, but if any of the
intermediate parent directories do not exist, the command will fail with
an error.  When not supplied, the default value is "none", in which case
the entire '-outputDirectory' path must already exist.  Regardless of this
value, the subdirectories based on the filtered sample and reference names
will automatically be created below the '-outputDirectory' location, and
do not have to pre-exist.

When not using wildcards, the '-makeDirectory' parameter is also available,
but is applied to the full directory path derived from the combination of
the values of the '-outputDirectory' and '-outputFile' parameters, rather
than just to the '-outputDirectory' value itself.

When writing to files, pre-existing files may be overwritten.  Run
'help set outputFileOverwritePolicy' to learn how to be alerted to, or
prevent, such file overwrites.

SUPPLEMENTAL ANNOTATION FILES

The -annotationFileSuffix may only be used in conjunction with
'-outputFormat clustal' or '-outputFormat ace' to generate two files: the
primary (i.e., clustal or ace) and the secondary, an ‘annotation file’ in
‘table’ format. The secondary file has the same name as the primary output
file plus the given annotation suffix.  If the suffix ends with ‘.csv’ the
annotation file format will be a table in comma separated value format, tab
separated value otherwise.  NOTE: annotation files can not be sent to
standard output, only to files.


BASIC EXAMPLES:

report alignment -sample Sample1 -reference EGFR_Exon_19

    Reports the consensus read alignment (default) for all amplicons in the
```

```
    EGFR_Exon_19 reference to the standard output of the command
    interpreter in FASTA format.  Default wrapping width of 50 characters
    is used.

report align -sam Sample1 -ref EGFR_Exon_19 -readType individual \
       -wrapping 0 -outputFile rpts/out.fna

    Reports the alignment of individual reads with no line wrapping and
    output going to the file:
       %currDir/rpts/out.fna

report align -sam Sample2 -ref HLA_Long_Amps -readType consensus \
       -wrappingWidth 60 -margin 15

    Reports, to standard output, the alignment of the consensus reads with
    a margin of 15 bases from the reference sequence added to both ends and
    then line wrapped on every 60th character.  Note: it is not necessary
    to use '-readType consensus' as this is the default report output.


AMPLICON FILTERING EXAMPLES:

report align -sam Sample1 -ref HLA_Long_Amps GA9 DE15

    Reports the consensus alignment for the amplicons GA9 and DE15
    in the reference to the standard output of the command interpreter in
    FASTA format.

report align -sam Sample1 -ref HLA_Long_Amps DD14 DE15 \
       -start 50 -end 350

    Reports the consensus alignment for the amplicons DD14 and DE15,
    clipping output to the given reference sequence positions
    [50, 350], inclusive.


WILDCARD SAMPLE AND REFERENCE EXAMPLES:

report align -sam * -ref *

    Reports the consensus alignment for all valid sample and reference
    pairs to a collection of files located in the current directory.

report align -sam Sample1 -ref * -outputDir dirA -makeDir last \
       -fileFilter linux -mappingFile map.tsv

    Reports the consensus alignment for all valid Sample1 and reference
    pairs to files (whose auto-generated names are linux OS compliant) in
    the %currdir/dirA directory, creating the 'dirA' directory if
    necessary, and creating a mapping file called "map.tsv" in the dirA
    directory as well.


FASTA ALIGNMENT OUTPUT FORMAT

The FASTA alignment output first begins with an entry for the reference
sequence as trimmed according to the '-start', '-end', amplicon list, and
'-margin' parameter values.  Subsequent entries are either the individual
or consensus reads (depending on the '-readType' parameter) that comprise
the alignment, padded as necessary with '-' gap characters.  Each entry
consists of a definition line prefixed with a '>' followed by the aligned
sequence data, wrapped according to the '-wrappingWidth' parameter.  The
definition line specifies the name of the reference sequence or read, as
```

applicable, followed by a set of keyword/value pairs that annotate the sequence. The general form of the definition line is:

```
>name keyword1=value1 keyword2=value2 ...
```

The particular keyword value pairs that appear on the definition line depend on whether or not the entry corresponds to the reference sequence or an individual or consensus read. The keywords are as follows, depending on the sequence type.

KEYWORD	R	C	I	DESCRIPTION OF CORRESPONDING VALUE
sample	x			name of the sample that is the read source
amplicon		x	x	name of the amplicon that is the read source
consensusLabel			x	consensus read containing the individual read
strand	x	x	x	+ = forward, - = reverse
forwardCount		!		# of + strand reads in consensus
reverseCount		!		# of - strand reads in consensus
refStart	x	x	x	start alignment position relative to reference
refEnd	x	x	x	end alignment position relative to reference
readStart		~	x	position of base within read at alignment start
readEnd		~	x	position of base within read at alignment end
alignedReadBases		x	x	number of aligned read bases

NOTE: R(x) = key is shown for the Reference Sequence (first output line).
I(x) = key is shown for Individual alignment reads.
C(x) = key is shown for Consensus alignment reads.
C(!) = key is shown for Consensus alignment reads only if value is non-zero.
C(~) = key is shown for Consensus alignment reads but positions are synthesized as [1..alignedReadBases].

For a given alignment output, all the reads will be derived from the same sample and so, for brevity, the sample keyword is only present on the definition line of the reference sequence that appears at the start of the output. All reported positions are given using a 1-based positioning system (i.e., the first base is base #1). For reads with a strand of '-', the readStart and readEnd are given relative to the original read orientation, and so in this case readStart will be greater than the readEnd.

TABLE OUTPUT FORMAT

The Table format is a tab or comma separated value table whose column headers are identical to FASTA's keywords, but with the first letter of each keyword in upper case (e.g., the "readEnd" values of the FASTA output would appear in a column labeled "ReadEnd"). Two additional columns of data are also included, 'Accno' and 'Alignment', specifying the identifier of a sequence and its (gapped) sequence alignment, respectively. The first row after the column labels contains data for the reference sequence and subsequent rows contain the data for the consensus or individual reads (depending on the value of the -readType parameter).

The '-tableOutputFormat' option controls the format of the table. If 'tsv' is specified, a tab-delimited format is used. Alternatively if 'csv' is given, then a comma-delimited format is used. If not specified, table will be tab-delimited, unless an output file is given (or is wildcard generated) with a ".csv" extension.

Example:

```
report alignment -sample Sample1 -reference EGFR_Exon_19 \
```

```
            -outputFormat table -outputFile S1_E19.dat \
            -tableOutputFormat csv
```

Reports the consensus read alignment (default) for all amplicons in the EGFR_Exon_19 reference to the file S1_E19.dat in a Table format, with data separated by commas.

The Table format can also, optionally, be used to supplement Clustal and Ace outputs formats to compensate for sequence annotations that are not fully supported by those formats. When used in this manner, the Alignment column of data is not included in the output (see Clustal Output Format documentation for an example).

CLUSTAL OUTPUT FORMAT

The Clustal output format is provided as another way to export AVA nucleotide sequence alignments. Output produced in this format is from the AVA alignments, and should not be misconstrued as being output from an actual Clustal-based alignment implementation.

For more information on specifics of the Clustal output format, and the basis of the AVA implementation of that format, see:

http://mcast.sdsc.edu/doc/clustalw-format.html

All 'report align' options used with CLUSTAL have similar effects as described for FASTA. One exception is -wrappingWidth, which for CLUSTAL is limited to a range of [1..60] and defaults to 50 if left unspecified.

Clustal format does not include space for key information, such as the forwardCount or reverseCount of reads contained within consensus reads or the true refStart and refEnd position of the Reference sequence and the readStart and readEnd positions of the reads in the type of local alignments performed by AVA (post primer trimming). A Table format output containing this additional information to annotate the Clustal formatted output can be generated along with the Clustal output by specifying a value for the '-annotationFileSuffix' option.

Example:

```
report align -sam * -ref * -outputFormat clustal \
        -annotationFileSuffix _annot.csv
```

In the above example, the wildcard expansion will generate file names based on the Sample and Reference names in the usual manner, and each file will contain alignments in Clustal format. For each such output file named X, an additional file named X_annot.csv will be generated in the Table format (see Table Output Format above) and contains the supplemental annotations.

NOTE: if -annotationFileSuffix is used, the report output can not be directed to the console's standard output.

ACE OUTPUT FORMAT

Using the option '-outputFormat ace', alignments are output in Ace format. Alignments in this format are still those of the AVA alignment algorithm and shouldn't be misconstrued as being output based on the Phrap assembly/alignment algorithm.

For more information on specifics of the Ace output format, see:

```
      http://www.phrap.org/consed/distributions/README.16.0.txt

In the current implementation, the "BQ" tagged quality score values are not
truly output (the constant value 30 is output for each base).

All 'report align' options used with ACE have similar effects as described
for FASTA. One exception is -wrappingWidth, which is ignored for ACE
because the width is fixed at 50.

The -annotationFileSuffix option may be used with the Ace format
(see Clustal Output Format for an example) to generate separate file(s)
containing supplemental annotation information for each aligned sequence
in tabular form.


READ ORDER IN ALIGNMENT:

Every alignment begins with an entry for the reference sequence.  Depending
on the specified '-readType', the consensus or individual reads that follow
are ordered as follows:

For the "consensus" reads:
    1.  Reads are grouped by amplicon, and the amplicon-based groups are
        ordered so that amplicons with smaller target start values appear
        first, and shorter (nested) amplicons with the same target start
        appear before the longer (containing) amplicons: i.e., reads from
        amplicons closest to the 5' end of the reference sequence appear
        before reads from amplicons that are closer to the 3' end.
    2.  Within an amplicon-based group, the consensus reads are ordered by:
        1.  Constituent read count: consensi with the largest forwardCount
            and reverseCount values appear first.
        2.  And if tied, then ordered by refStart: reads with fewer leading
            gaps appear first.
        3.  And if tied, then ordered by the aligned nucleotide sequence:
            these are sorted by their natural ASCII lexicographic order
            (i.e., - < A < C < G < N < T).
        4.  And if tied, then ordered by the strand: forward reads appear
            before reverse reads.
        5.  And finally, if necessary, ordered by the consensus read name.

For the "individual" reads:
    1.  Reads are first ordered by the refStart: reads with fewer leading
        gaps appear first.
    2.  And if tied, then ordered by the aligned nucleotide sequence:
        these are sorted by their natural ASCII lexicographic order
        (i.e., - < A < C < G < N < T).
    3.  And if tied, then ordered by the strand: forward reads appear
        before reverse reads.
    4.  And if tied, then ordered by the read identifier (i.e., as taken
        from the SFF file).
```

3.4.12.2 report variantHits

```
report variantHits [-outputFile <file>] [-format <table format>]

Reports variant hits.  Variant hits are reported in the form of a table.
The table has columns for the following.

    Reference Name
```

```
    Variant Name
    Variant Status
    Variant Pattern
    Sample Name
    Forward Hits
    Forward Denom
    Reverse Hits
    Reverse Denom
    Read Type

Data are provided for a Variant of a given Reference Sequence if there
are reads of a Sample that span the region of variation as described
by the Variant Pattern.  The number of forward and reverse reads that
span the region are reported in the Forward Denom and Reverse Denom
columns, respectively.  The number of these reads that have the variation
are given in the Forward Hits and Reverse Hits columns.  The Hit / Denom
ratio provides an estimate of the Variant frequency in the Sample.
Two rows of data are given for each Variant based on the Read Type,
which is either Consensus or Individual.

If no '-outputFile' option is given, the table is printed in a
tab-delimited format to the standard output of the interpreter.  An output
file of "-" has the same effect.  If an output file is given, the table is
written to that file.  Run 'help general filePaths' for more information
about specifying files.

The '-format' option controls the format of the printed table.  If "tsv", a
tab-delimited format is used.  If "csv", a comma-delimited format is used.
By default, the tab-delimited format is used, unless an output file is
given with a ".csv" extension.

Here are some examples.

report variantHits

    Reports the variant hits table to the standard output of the command
    interpreter in a tab-delimited format.

report variantHits -outputFile /reports/hits.csv

    Reports the variant hits table to the /reports/hits.csv file in a
    comma-delimited format.

report variantHits -outputFile -

    Reports the variant hits table to the standard output of the command
    interpreter in a tab-delimited format.
```

3.4.13 save

```
save

This command takes no arguments.  It saves the currently open project,
committing any modifications made since opening the project or since the
last save.  If no project is currently open, an error is reported.
```

3.4.14 set

```
set <parameter name> <value>
```

```
Sets the value of a parameter to a given value.  The following parameter
are available.  Run 'help set <parameter name>' for more detailed
information.

verbose     Sets the verbose mode.
onErrors    Sets the behavior of the interpreter when errors are
                encountered.
currDir     Sets the current directory.

outputFileOverwritePolicy
            Sets the file overwrite policy.
```

3.4.14.1 set verbose

```
set verbose <"true" or "false">

Sets the value of the 'verbose' parameter.  If the 'verbose' parameter is
set to 'true', extra information is provided about the commands that are
executed.  This may be useful to help debug scripts.
```

3.4.14.2 set onErrors

```
set onErrors <"stop" or "continue">

Sets the value of the 'onErrors' parameter.  If 'onErrors' is set to
'stop', the command interpreter will stop the current running script
if an error is encountered.  If 'onErrors' is set to 'continue', the
command interpreter will abort the command that caused the error but
will continue running and executing subsequent commands.

In the case that the interpreter stops due to an error, if it is running
a "top level" script (i.e., one that was not started from another script
with 'utility execute'), then the command interpreter will exit.  If the
running script was started from another script using the 'utility execute'
command, then control will be returned to the calling script and the
'utility execute' command in the calling script will be treated as if it
encountered an error.  The behavior in the calling script then depends on
how the 'onErrors' parameter is set in its environment.  If set to
'continue', the calling script will continue running the commands
subsequent to the 'utility execute'; otherwise, it will stop the calling
script:  this same rule is applied again in the case that the calling
script was itself invoked via a 'utility execute' from another script.

Run 'help utility execute' for information about how one script can execute
another script.
```

3.4.14.3 set currDir

```
set currDir <path>

Sets the current directory used to resolve relative file paths.  If the
indicated path does not exist or is not a directory, a warning is shown.

Run 'help general filePaths' for more information about file paths.
```

3.4.14.4 set outputFileOverwritePolicy

```
set outputFileOverwritePolicy <"allow", "warn", or "error">

Sets the value of the 'outputFileOverwritePolicy' parameter, which
determines what should happen when a command attempts to overwrite
a preexisting file.  When set to "allow" (the default), preexisting files
are silently overwritten.  When set to "warn", such files are also
overwritten, but a warning message is issued.  When set to "error",
an error message will be displayed and the command attempting to perform
the file overwrite will immediately be stopped.

This policy affects all commands that produce output, such as would be
generated using the '-outputFile' option of the various 'list' and 'report'
commands, as well as might be output using automatically generated file
names by the 'report alignment' command.  The policy additionally affects
the '-outputFile' and '-scriptOnly' options of
'utility makeSetupScript' and 'utility clone' commands, respectively.

This policy is not relevant to the internal files that are used to store a
project.  Thus, regardless of the 'outputFileOverwritePolicy', neither the
'create project' nor the 'utility clone' commands will let you overwrite a
preexisting project directory.  Similarly, there are no errors or warnings
involved when using the 'save' command to update an existing project or
when updating the internal files of a project to store results when the
'computation start' command is given.

Run 'help set onErrors' for information about how errors are handled within
an executed script.
```

3.4.15 show

```
show <show command> <other arguments>

The 'show' command is used to show various information about the
interpreter.

The following show commands are available.  Run 'help show <show command>'
for more detailed information.

environment             Shows the environment that defines the behavior of
                            the interpreter.
```

3.4.15.1 show environment

```
show env[ironment]

Shows the current environment in which commands are being run.  Here is
some example output:

libDir=/opt/454/apps/amplicons/config/lib
currDir=/home/me/data
homeDir=/home/me
verbose=false
onErrors=stop
outputFileOverwritePolicy=allow
project=MyProject (/home/me/data/MyProject)
```

```
The first three lines show the values of the path variables that may be
used in resolving relative file paths.  Run 'help general filePaths' for
more information about file paths.

The next line shows whether verbose mode is turned on.
Run 'help set verbose' for more information about this value.

The next line shows the behavior when errors are encountered.
Run 'help set onErrors' for more information about this value.

The next line shows the policy to use when a command attempts to overwrite
a preexisting file. Run 'help set outputFileOverwritePolicy' for more
information.

The next line shows the currently open project, indicating the project
name and location.  This is the project that will be affected by any
project-related commands.
```

3.4.16 update

```
update <entity type> <other arguments>

The 'update' command is used to update properties of entities.  For
example, you can update the annotation of an amplicon by running,
'update amplicon "My Amplicon" -annotation "New annotation"'.

The following entities are available for updating.  Run
'help update <entity type>' for more detailed information.

amplicon        Updates an amplicon in the currently open project.
mid             Updates an MID in the currently open project.
midGroup        Updates an MID group in the currently open project.
multiplexer     Updates a multiplexer in the currently open project.
project         Updates the currently open project.
readData        Updates a read data in the currently open project.
readGroup       Updates a read group in the currently open project.
reference       Updates a reference sequence in the currently open project.
sample          Updates a sample in the currently open project.
variant         Updates a variant in the currently open project.
```

3.4.16.1 update amplicon

```
update amp[licon] <new amplicon name> [-ofRef <reference sequence name>]
                                      [-annot[ation] <annotation>]
                                      [-ref[erence] <reference name>]
                                      [-primer1 <primer 1 sequence>]
                                      [-primer2 <primer 2 sequence>]
                                      [-start <target start index>]
                                      [-end <target end index>]
                                      [-checkPri[merMatch] <boolean>]
                                      [-file <file> [-format <format>]]

update amp[licon] -name <new amplicon name>
                  [-ofRef <reference sequence name>]
                  [-annot[ation] <annotation>]
                  [-ref[erence] <reference name>]
                  [-primer1 <primer 1 sequence>]
                  [-primer2 <primer 2 sequence>]
                  [-start <target start index>]
```

```
                    [-end <target end index>]
                    [-checkPri[merMatch] <boolean>]
                    [-file <file> [-format <format>]]

Updates an amplicon in the currently open project.  In the first form, the
non-option argument is used as the name of the amplicon to update.  In the
second, a name must be explicitly specified in option form.  Amplicons are
allowed to have duplicate names as long as the reference sequences to which
they refer are distinct.  The '-ofRef' argument can be used to refer to
such amplicons.  For example, if we have two amplicons named "MyAmp", but
one of them refers to "ReferenceSequence1" and the other to
"ReferenceSequence2", we can use the '-ofRef' option to distinguish them.
We can run 'update amplicon "MyAmp" -ofRef "ReferenceSequence1"' to update
the former amplicon.

The remainder of the options are not required but are used to set
properties of the amplicon.

    -ofRef              The name of the reference sequence to which the
                            amplicon currently refers to help disambiguate
                            amplicons with the same name.
    -annotation         The annotation.
    -reference          The name of the reference sequence with which to
                            associate the amplicon.
    -primer1            The primer 1 sequence.  This must be a nucleotide
                            sequence string conforming to IUPAC
                            nomenclature.  Any ambiguous symbols are
                            considered 'N's.
    -primer2            The primer 2 sequence.  This must be a nucleotide
                            sequence string conforming to IUPAC
                            nomenclature.  Any ambiguous symbols are
                            considered 'N's.
    -start              The index of the target start position, or a '*'
                            to indicate the position should be
                            automatically assigned.
    -end                The index of the target end position, or a '*'
                            to indicate the position should be
                            automatically assigned.
    -checkPrimerMatch   Whether the system should check for a match between
                            the reference sequence and the primers in the
                            bases flanking the target region.  This must be
                            'true' or 'false', and defaults to 'true'.

The start and end options indicate the positional range of the amplified
target as measured from the first base of the associated reference
sequence.  In the case that the primer sequences are included in the
reference sequence, the system can automatically assign these positions by
finding matches of primer1 and the reverse complement of primer2 and
assigning the start and end positions to be just inside these matches.
Either, or both, of the start and end positions may be specified as a '*'
to request this search.  If one position is provided and the other is a
'*', then one position will be constrained as given and the search will
proceed on the other position.  If no such matching pair, or more than one
matching pair can be found, then an error is generated.  N's in either the
reference or primer sequences count as matches, but any match that involves
greater than 50% N's will be rejected.  Any other substitutions,
insertions, or deletions are not permitted.  Using a '*' for either the
start or end implies the checkPrimerMatch option and requires exact matches
of both primers in the reference sequence.  If the primers are not included
in the reference or if the primers contain bases that don't exactly match
the reference, the checkPrimerMatch option should be specified as 'false'
to prevent an error from being generated, and both start and end positions
should be explicitly provided.
```

```
Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.16.2 update mid

```
update mid <mid name> [-ofMidGroup <midGroup>]
                      [-seq[uence] <sequence>]
                      [-annot[ation] <annotation>]
                      [-midGroup <midGroup>]
                      [-checkMidGroup <boolean>]
                      [-file <file> [-format <format>]]

update mid -name <mid name>
           [-ofMidGroup <midGroup>]
           [-seq[uence] <sequence>]
           [-annot[ation] <annotation>]
           [-midGroup <midGroup>]
           [-checkMidGroup <boolean>]
           [-file <file> [-format <format>]]

Updates an MID in the currently open project.  In the first form,
the non-option argument is used as the name of the MID to update.  In the
second, a name must be explicitly specified in option form.  MIDs are
allowed to have duplicate names as long as they belong to distinct MID
groups.  The '-ofMidGroup' argument can be used to refer to such MIDs.
For example, if we have two MIDs named "MyMID", but one of them is a member
of MID group "MID_Group1" and the other is a member of MID group
"MID_Group2", we can use the '-ofMidGroup' option to distinguish them.  We
can run 'update mid "MyMID" -ofMidGroup "MID_Group1"' to update the former
MID.

The remainder of the options are not required but can be used to set
properties of the MID.

    -annotation         The annotation.
    -sequence           The MID sequence.  This must be a non-zero length
                            nucleotide sequence string containing only the
                            bases A, C, T and G.
    -midGroup           The MID group of the MID if it belongs to a group.
                            This must be a pre-existing group such as one
                            created using the 'create midGroup' command.
    -checkMidGroup      Whether the system should check for compatibility
                            between the new MID sequence and other pre-
                            existing MID sequences belonging to the same
                            MID group.  This must be 'true' or 'false',
                            and defaults to 'true'.

The name of the MID must be unique within the MID group it belongs to
(or unique within the project if the MID is not assigned to an MID group).

The rules for '-checkMidGroup' compatibility are as follows:

- An MID with an undefined sequence is considered compatible with any MID
  group, under the assumption that its compatibility will eventually be
  assessed when a defined sequence gets assigned to the MID.
- An MID with a defined sequence must have the same length as other
  defined MID sequences within an MID group to be compatible with the
  group. If the new MID sequence is the first defined sequence added to
  the MID group, the required sequence length for subsequent MIDs of the
  group with be the length of that first defined MID sequence.
```

```
- An MID with a defined sequence must not be identical (ignoring case)
  with any other defined, pre-existing MID sequence of the same MID group.

If it becomes necessary to edit existing MIDs in a way that temporarily
leaves the MIDs in a group in an inconsistent state (such as changing the
lengths of sequences in an MID group), '-checkMidGroup' should be set to
'false'.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.16.3 update midGroup

```
update midGroup <midGroup name> [-annot[ation] <annotation>]
                                [-file <file> [-format <format>]]

update midGroup -name <midGroup name>
                [-annot[ation] <annotation>]
                [-file <file> [-format <format>]]

Updates an MID group in the currently open project.  In the first form,
the non-option argument is used as the name of the MID group to update.
In the second, a name must be explicitly specified in option form.  The
remainder of the options are not required but can be used to set
properties of the MID group.

    -annotation     The annotation.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.16.4 update multiplexer

```
update mul[tiplexer] <multiplexer name> [-enc[oding] <encoding>]
                                        [-annot[ation] <annotation>]
                                        [-file <file> [-format <format>]]

update mul[tiplexer] -name <multiplexer name>
                     [-enc[oding] <encoding>]
                     [-annot[ation] <annotation>]
                     [-file <file> [-format <format>]]

Updates a multiplexer in the currently open project.  In the first form,
the non-option argument is used as the name of the multiplexer to update.
In the second, a name must be explicitly specified in option form.

The remainder of the options are not required but can be used to set
properties of the new multiplexer.

    -annotation         The annotation.
    -encoding           The MID layout type for the multiplexer, where
                            the choices are both, either, primer1, and
                            primer2.

The four '-encoding' types have the following definitions:

    both                Both primer 1 and primer 2 MIDs are present and
                            necessary to determine the sample for each
                            read.
    either              Both primer 1 and primer 2 MIDs are present, but
```

```
                            either one is sufficient to determine the
                            sample.  For a given read, the MID at the
                            5' end, in the read's orientation, is used
                            to determine the sample.
    primer1             MIDs are only present adjacent to primer 1.
    primer2             MIDs are only present adjacent to primer 2.

If the multiplexer was initially created without specifying the
'-encoding' type, the '-encoding' type must be set using the 'update
multiplexer' command before MIDs or MID<->Sample associations can be
created using the multiplexer.

If the multiplexer already has a defined '-encoding' type and that type is
changed, then all pre-existing sample associations for the multiplexer
will be removed and certain pre-existing associations with MIDs may also
be removed. Specifically, if the '-encoding' type is changed to 'either'
and the numbers of already associated primer 1 and primer 2 MIDs are
not equal, then both sets of MID associations will be removed.  If the
'-encoding' type is changed to 'primer1', then any associated primer 2
MIDs will be dissociated and if the type is changed to 'primer2', then any
associated primer 1 MIDs will be dissociated.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.16.5 update project

```
update proj[ect] [-annotation <annotation>]

Updates the currently open project.  The options specify what properties of
the project to update.

    -annotation     The annotation describing the project.
```

3.4.16.6 update readData

```
update readData <read data name> [-annot[ation] <annotation>]
                                 [-readGroup <read group name>]
                                 [-active <boolean>]
                                 [-originalPath <original path>]
                                 [-file <file> [-format <format>]]

update readData -name <read data name>
                [-annot[ation] <annotation>]
                [-readGroup <read group name>]
                [-active <boolean>]
                [-originalPath <original path>]
                [-file <file> [-format <format>]]

Updates a read data in the currently open project.  In the first form,
the non-option argument is used as the name of the read group to update.
In the second, a name must be explicitly specified in option form.  The
remainder of the options are not required but are used to set properties of
the read data.

    -annotation     The annotation.
    -readGroup      The name of the read group to which this read data
                        belongs.
    -active         The active status of the read data.  This can be one of
                        "true" or "false".
```

```
    -originalPath   The original path of the read data.  The project
                        remembers the original path from which the read
                        data was imported.  This is used to update that
                        path.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.16.7 update readGroup

```
update readGroup <read group name> [-annot[ation] <annotation>]
                                   [-file <file> [-format <format>]]

update readGroup -name <read group name>
                [-annot[ation] <annotation>]
                [-file <file> [-format <format>]]

Updates a read group in the currently open project.  In the first form, the
non-option argument is used as the name of the read group to update.  In
the second, a name must be explicitly specified in option form.  The
remainder of the options are not required but are used to set properties of
the read group.

    -annotation     The annotation.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.16.8 update reference

```
update ref[erence] <reference name> [-annot[ation] <annotation>]
                                    [-seq[uence] <sequence>]
                                    [-file <file> [-format <format>]]

update ref[erence] -name <reference name>
                   [-annot[ation] <annotation>]
                   [-seq[uence] <sequence>]
                   [-file <file> [-format <format>]]

Updates a reference sequence in the currently open project.  In the
first form, the non-option argument is used as the name of the reference
sequence to update.  In the second, a name must be explicitly specified in
option form.  The remainder of the options are not required but are used to
set properties of the reference sequence.

    -annotation     The annotation.
    -sequence       The nucleotide sequence string.  This sequence must use
                        IUPAC nomenclature.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.16.9 update sample

```
update sam[ple] <sample name> [-annot[ation] <annotation>]
                              [-file <file> [-format <format>]]

update sam[ple] -name <sample name>
```

```
                [-annot[ation] <annotation>]
                [-file <file> [-format <format>]]

Updates a sample in the currently open project.  In the first form, the
non-option argument is used as the name of the sample to update.  In the
second, a name must be explicitly specified in option form.  The remainder
of the options are not required but are used to set properties of the
sample.

    -annotation     The annotation.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.16.10 update variant

```
update var[iant] <variant name> [-annot[ation] <annotation>]
                                [-ref[erence] <reference sequence name>]
                                [-pat[tern] <pattern>]
                                [-stat[us] <status>]
                                [-checkPat[tern] <boolean>]
                                [-file <file> [-format <format>]]

update var[iant] -name <new variant name>
                 [-ref[erence] <reference sequence name>]
                 [-annot[ation] <annotation>]
                 [-pat[tern] <pattern>]
                 [-stat[us] <status>]
                 [-checkPat[tern] <boolean>]
                 [-file <file> [-format <format>]]

Updates a variant in the currently open project.  In the first form, the
non-option argument is used as the name of the variant to update.  In the
second, a name must be explicitly specified in option form.  Variants are
allowed to have duplicate names as long as the reference sequences to
which they refer are distinct.  The '-ofRef' argument can be used to refer
to such variants.  For example, if we have two variants named "MyVar", but
one of them refers to "ReferenceSequence1" and the other to
"ReferenceSequence2", we can use the '-ofRef' option to distinguish them.
We can run 'update variant "MyVar" -ofRef "ReferenceSequence1"' to update
the former variant.

The remainder of the options are not required but are used to set
properties of the variant.

    -annotation     The annotation.
    -reference      The name of the reference sequence to which the
                        variant refers.
    -pattern        The pattern that defines the nature of this variation.
    -status         The putative status.  This can be one of "accepted",
                        "rejected", or "putative"
    -checkPattern   Whether the system should check if the variant's
                        pattern is syntactically correct and consistent
                        with the variant's reference sequence. The
                        reference sequence must itself be set and have
                        a non-empty nucleotide sequence for this option
                        to take effect.  This value given must be 'true'
                        or 'false', and defaults to 'true'.

Run 'help general tabularCommands' for information about the '-file'
option.
```

3.4.17 utility

```
util[ity] <utility command> <other arguments>

The 'utility' command is used to execute general utility commands.  For
example, running 'utility clone /data/clone1' will clone the currently open
project to /data/clone1.

The following utility commands are available.  Run
'help utility <utility command>' for more detailed information.

validateNames           Validates the record names in the currently open
                            project.
validateForComputation  Validates that the currently open project is ready
                            for computation.
makeSetupScript         Makes a setup script that, if run, would attempt to
                            recreate the currently open project.
clone                   Clones the currently open project.
execute                 Executes a script file.
```

3.4.17.1 utility validateNames

```
util[ity] validateNames [-fix] [-fixPrefix <prefix>] [-fixSuffix <suffix>]

Validates that the names of records in the currently open project conform
to the requirements of the command interpreter.  Since commands use record
names to identify records, duplicate record names can cause ambiguity.
Additionally, names that are empty or consist entirely of whitespace
can cause syntactic difficulties in certain situations.

This command exists to support the manipulation of projects that
were created using the Graphical User Interface, where the same naming
constraints are not currently applied.  Any project created or edited
with the Command Line Interface will automatically be compatible with
the Graphical User Interface.  This command provides a mechanism to
ensure that the reverse is true as well.

In its default form, this command will report an error if there are
records that will cause ambiguity or syntactic problems if they are
encountered by other commands.  If there are no problematic names, this
command does nothing.

If the '-fix' option is supplied, this command will construct unique,
non-empty names for the records that would have caused problems.  The
constructed names will have the word "FIX_" prepended to them and have
an underscore followed by a number appended to them for uniqueness.  The
prefix is added to provide a marker that this command has modified the
name of the record.

The '-fixPrefix' option specifies a custom string that will be prepended to
modified records instead of the default, "FIX_".  Setting this option
implies '-fix'.

The '-fixSuffix' option specifies a custom string that will appended to
modified records before the number is appended for uniqueness instead of
the default, "-".  Setting this option implies '-fix' as well.

For example, suppose you have a project with 3 samples all named "MyAmp".
Running 'utility validateNames' will report an error.  Running
```

```
'utility validateNames -fix' will rename the amplicons to be "FIX_MyAmp_1",
"FIX_MyAmp_2", and "FIX_MyAmp_3".  Running
'utility validateNames -fix -fixPrefix "FLAG" -fixSuffix "#" will rename
the amplicons to be "FLAG_MyAmp#1", "FLAG_MyAmp#2", and "FLAG_MyAmp#3".

Note that since amplicons and variants can be distinguished by the
reference sequence to which they refer, it is possible to have multiple
amplicons or variants with the same name but different reference sequences.
Such records will not be modified by this command, unless they are empty.
However, amplicons or variants with the same name and reference are
ambiguous and will be modified.
```

3.4.17.2 utility validateForComputation

```
util[ity] validateForComputation [-silent <boolean>]

Validates that the currently open project is ready for computation.  The
project is valid for computation if the following criteria are met.

- Reference sequences have a sequence that is at least 1 base.
- Amplicons refer to valid reference sequences and have target start and
  end coordinates that are contained within said reference sequence.
- Read data files are available that are associated with at least
  one Sample and one or more valid Amplicons.
- Optionally, Variants that refer to valid reference sequences and
  have non-empty patterns that are valid with respect to said reference
  sequence.

If some criteria are not met, warnings are reported describing the
problems, and an error is reported for the operation.  If '-silent' is set
to be true, no warnings are reported, but an error is still reported for
the operation.  If all criteria are met, this command has no effect.
```

3.4.17.3 utility makeSetupScript

```
util[ity] makeSetupScript [-outputFile <file>]

Makes a setup script that, if run with the command interpreter, would
attempt to recreate the currently open project.  Note that it will usually
not be possible to run this script after creating it, since the project
already exists in the same location.

If no '-outputFile' is given, the script is printed to the standard output
of the interpreter.  An output file of "-" has the same effect.  If an
output file is given, the script is written to that file.

Run 'help general filePaths' for more information about specifying files.
```

3.4.17.4 utility clone

```
util[ity] clone <clone project path>
                    [-projectName <clone project name>]
                    [-projectAnnotation <clone project annotation>]
                    [-copyReadData <boolean>]
                    [-scriptOnly <file>]

Clones the currently open project.  The project will be cloned to the path
given as the argument to this command.  By default, the read data and all
```

```
project records that depend on the read data will be excluded from the
clone.

By providing the '-projectName' option, the name of the clone project can
be set.  By default, the project name is set to the base name of the clone
project path.

By providing the '-projectAnnotation' option, the annotation of the clone
project can be set.  By default, the annotation of the clone project will
be set to be the same as the currently open project.

If '-copyReadData' is set to "true", the read data and all project records
that depend on the read data will be included in the clone.  If set to
"false" (the default), they will not be included.

If the '-scriptOnly' option is provided, no project will actually be
created.  Instead, a script will be created that, if run by the command
interpreter, will perform the clone.  This allows you to adapt and
customize the clone before actually performing it.  If "-" is provided as
the file, the script will be written to the standard output of the command
interpreter.

Note that the currently cloned project will be cloned as it currently
exists in the command interpreter.  This means that unsaved changes are
propagated to the clone.

Run 'help general filePaths' for more information about the interpretation
of relative paths when using the '-scriptOnly' option or specifying the
clone project path.
```

3.4.17.5 utility execute

```
util[ity] exec[ute] <script path>
                    [-withCurrDir <path>]
                    [-onMissingScript <"ignore", "warn", or "error">]

Executes a script file.  This allows useful sequences of commands to be
grouped and reused easily.  For example, it may be helpful to create a
script file that creates standard amplicons.  Executing this script will
create the amplicons in the currently open project.

The execution will inherit the environment of the caller.  For example, if
the verbose option is set in the caller, it will also be set in the script.
Modification of the environment by the called script will not be propagated
back to the caller.  For example, suppose the verbose option is set to
"true" in script A at the time it executes script B.  Script B then sets
the verbose option to "false".  Commands in script B will execute with a
"false" verbose option, but once the execution of script B is complete,
subsequent commands in script A will run with a "true" verbose option.

There are two important exceptions to this policy: the currently open
project and the current directory (currDir).

The currently open project is global.  For example, if script A executes
script B and script B opens a project, that project will continue to be
open in script A.

The current directory for the execution of a script is set by default to
the directory that contains the script itself.  For example, execution of
the script at "someDir/someScript.ava" will run with a current directory of
"someDir".  This default behavior allows a set of related scripts to
```

```
refer to each other using relative paths, independent of where the scripts
are actually installed.  It also allows the commands of a script to easily
refer to a tabular file with the '-file' option in a relative manner when
that tabular file is installed in a location relative to the script.

However, this current directory for the script execution can be modified by
using the '-withCurrDir' option.  If the '-withCurrDir' option is given,
the path passed to it will be used instead. In particular, to invoke a
script that will run with the same current directory as the calling script,
simply do:

     utility execute someDir/someScript.ava -withCurrDir %currDir

In this example, the shortcut path %currDir expands to the current
directory of the calling script, thereby setting the current directory
of the called script to be the same as that of the calling script.  Run
'help general filePaths' for more information on the use of relative paths
and other available shortcut paths.

The use of '-withCurrDir' has no effect on the current directory of the
calling script itself.

By providing the '-onMissingScript' option, the behavior of the command, if
the file specified by the script path cannot be found, is customized.  If
set to "ignore", a missing script will be ignored completely.  If set to
"warn", a warning will be shown.  If set to "error" (the default), an error
will be reported.

Run 'help set onErrors' for information about how errors are handled within
an executed script.
```

3.5 Creating and Computing a Project with the AVA-CLI

There are many different ways to use the AVA-CLI to create or perform computations on a project. These include running `doAmplicon` in interactive mode, typing commands individually, using a program to automatically generate a script that can be piped into the `doAmplicon` command, or manually authoring a script file and invoking `doAmplicon` to execute the commands in that file. The command line syntax for these different options are specified in section 3.3.2.1.

This section provides examples of ways one can piece together a script of commands for setting up a Project. This example Project uses the same underlying data as the example discussed in section 2. The differences for this particular example are that a separate Reference Sequence is being entered for each EGFR exon (rather than stitching them together as an artificial reference); and that all four Read Data files are being utilized (rather than just the region 3 file). Since the sections below often digress to illustrate the variety of commands available to accomplish a specific task, they are not meant to be followed as literal step-by-step instructions for Project creation. However, section 3.5.15 presents an integrated script that can be supplied to the `doAmplicon` command to perform the entire example Project setup, computation and processing (provided you have access to the same sff files and you edit the paths appropriately so the script can find them).

3.5.1 Setting CLI Parameters

You can use the “set” command to change some of the CLI environment parameters within a script (see section 3.4.14 for the usage statement). The “set” command allows you to change the value of three parameters (verbose, onErrors, and currDir).

```
set verbose false
set verbose true
set onErrors stop
set onErrors continue
set currDir <path>
```

Setting verbose to true enables additional logging to enhance troubleshooting capabilities. Each command is logged as it is executed. This is particularly useful for commands that are dynamically synthesized as a side effect of reading tabular input (see section 3.3.2.3). However, whether or not verbose mode is set to true, the CLI will report detailed locations (including, as appropriate, the file name of the script, the line of the script, and the line of any external file or table being read) when it encounters errors.

The onErrors parameter controls how the command interpreter handles any errors it encounters. If onErrors is set to stop (the default, unless running the interpreter in “interactive mode”), the command interpreter will halt and exit with a non-zero exit code when an error is encountered. If onErrors is set to continue, the command that encountered an error will be aborted, but the command interpreter will continue running and execute the rest of the commands in the script.

Because changes to a Project’s definition are not permanent until a “save” command is executed, setting the onErrors parameter set to “stop” allows the creation of “transactional” scripts that leave the Project in a consistent state and do not modify the Project definition unless all commands complete without error. This is simply achieved by placing the save command after all the commands that modify the project and with onErrors set to stop: if any of the commands fail, the script will halt, the save command will not be executed, and the Project definition will remain in its original state.

Setting the currDir parameter controls how relative file paths are interpreted by the commands to the CLI. For more information on file paths, see section 3.3.2.6.

3.5.2 Creating a New Project

The first step in creating a Project is setting up the Project directory structure that will store the Project configuration, data, and results. This is done using the “create project” command (see section 3.4.4.2 for the usage statement). Here is an example Project creation command:

```
create project /data/ampProjects/EGFR_CLI \
    -name EGFR_CLI \
    -annotation "CLI Example Project Creation Test"
```

Note the backslash character (“\”) used to indicate line continuation. This allows you to control the format of the command over multiple lines, to improve the readability of long commands. This command could also have been presented as one continuous line without the backslashes. Note also the multi-word annotation included within double-quotes. Double-quotes allow spaces and unusual characters to be included in argument values. See section 3.3.2.2 for other

specifics on how commands are formatted and parsed. Finally, note that creation of the directory structure for the Project occurs at the time that the “create project” command is executed and is the one aspect of Project definition that will not be reverted if you choose not to save before exiting the CLI.

The “create project” command can be used only once for any given Project. To continue the set up or other work on a Project that has been previously created, use the “open” command (see section 3.4.9 for the usage statement). To open an existing Project, you simply type the Project path after the open command, *e.g.*:

```
open /data/ampProjects/EGFR_CLI
```

Note that this is the actual path to the Project and not the name of the Project. The last part of the Project path and the Project name often coincide because the default name for a Project can be based on the Project directory (see section 2.2.2 for an example of this), but the Project path and the Project name can also diverge (such as if the Project is moved to a new location, perhaps for reasons related to disk space, in which case the Project name would stay the same but the Project path would change). If you try to open a Project that is already open by someone else and you are in interactive mode, a warning will appear and will give you the option to preempt control or to continue with read-only access. If you are using an open command in a script in non-interactive mode, the open command will fail and throw an error that will halt your script (unless onErrors is set to continue).

If you want to intentionally open a Project in read-only mode, you can use the “-control readonly” parameter as part of your open statement. You can also explicitly set the control to “-control preempt” to seize control of the Project you are opening even if someone else is working with it.

3.5.3 Creating References

The next step is to add Reference Sequences since they are necessary for the full specification of Amplicons. This is done using the “create reference” command (see section 3.4.4.7 for the usage statement). Multiple Reference Sequences can be created in a single invocation of the “create reference” command by saving to a file a table containing all the specific Reference Sequence features, and calling the “create reference” command on that file using the “-file” option. The file containing the reference features may be in either tab-separated value (tsv) or comma-separated value (csv) formats, but the file is assumed to be in the tsv format unless the “-format” option is set to “csv” or the file name ends in the suffix “.csv”. See section 3.3.2.3 for more details on using tabular files as input.

Generally speaking, any of the commands that support tabular input work by combining the given command line option values with the parameters specified in the table headers and associated values found in the table, to synthesize a set of command options that are the union of both sets of values. This allows all the parameters of the table to be nested within a constant set of options specified on the command line, reducing the chance of error that might occur when manually creating a table with the repeated constant values within the table itself. The example below is the simplest example of using a file as input, in which all the parameters to the “create reference” command are, in fact, coming from the supplied file. See section 3.5.7 for an example of a command that combines options on the command lines with values specified in a file.

Below is an example tsv-formatted table that could be used to create the 5 Reference Sequences for the EGFR Project. Note that the double quotes around the field names and values aren't strictly necessary for this particular example, but quotes would be necessary if the values contained the value delimiter character of the format being used. As explained in section 3.3.2.3, the syntax of the tabular input files follows the standard tsv and csv formatting conventions, not the syntax rules of the CLI itself.

Due to the limitations of this printed document, the "Sequence" entry for each Reference Sequence appears on a separate group of lines, when they are in fact on a single line and are separated from the "Annotation" entry only by a tab character. A similar situation occurs in various other file listings, throughout this Manual section.

```
"Name"     "Annotation"     "Sequence"
"EGFR_Exon_18"     "EGFR_Exon_18"
"GACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACCCAGTGGAGAAGCTCCCAA
CCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGATCAAAGTGCTGGGCTCCGGTGCGTTCG
GCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCCTCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTG
GGG"
"EGFR_Exon_19"     "EGFR_Exon_19"
"TCACAATTGCCAGTTAACGTCTTCCTTCTCTCTCTGTCATAGGGACTCTGGATCCCAGAAGGTGAGAAAG
TTAAAATTCCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTCGAT
GTGAGTTTCTGCTTTGCTGTGTGGGGGTCCATGGCTCTGAACCTCAGGCCCACCTTTTCTC"
"EGFR_Exon_20"     "EGFR_Exon_20"
"CCACACTGACGTGCCTCTCCCTCCCTCCAGGAAGCCTACGTGATGGCCAGCGTGGACAACCCCCACGTGT
GCCGCCTGCTGGGCATCTGCCTCACCTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCGGCTGCCTC
CTGGACTATGTCCGGGAACACAAAGACAATATTGGCTCCCAGTACCTGCTCAACTGGTGTGTGCAGATCGC
AAAGGTAATCAGGGAAGGGAGATACGGGGAGGGGAGATAAGGAGCCAGGATC"
"EGFR_Exon_21"     "EGFR_Exon_21"
"TCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGGGCATGAACTACTTGGAGGACC
GTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTGGTGAAAACACCGCAGCATGTCAAGATCACA
GATTTTGGGCTGGCCAAACTGCTGGGTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAG
GTGGCTTTAGGTCAGCCAGCAT"
"EGFR_Exon_22"     "EGFR_Exon_22"
"CACTGCCTCATCTCTCACCATCCCAAGGTGCCTATCAAGTGGATGGCATTGGAATCAATTTTACACAGAA
TCTATACCCACCAGAGTGATGTCTGGAGCTACGGTGAGTCATAATCCTGATGCTAATGAGTTTGTACTGAG
GCCAAGCTGG"
```

The header of the table shows the names of the parameters you want to supply to the "create reference command". In order to use the table to create the Reference Sequences in the Project, it can be saved as a file (*e.g.* "EGFR_CLI_references.txt") in the directory from which you plan to run the script, and cited as argument under the -file option of the "create reference" command:

```
create reference –file EGFR_CLI_references.txt
```

When you use a file as input to a command, the AVA-CLI uses the command and any of its other arguments (besides the "-file" argument) as a prefix command and applies them to each non-header line in the file, such that the contents of each row are converted into their command

line option form. Thus, the first Reference Sequence line in our example file gets converted into the following command:

```
create reference EGFR_Exon_18 –annotation EGFR_Exon_18 –sequence
GACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACCCAGTGGAGAAGCTCCCAAC
CAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGATCAAAGTGCTGGGCTCCGGTGCGTTCGG
CACGGTGTATAAGGTAAGGTCCCTGGCACAGGCCTCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGG
GG
```

Tabular input to commands can also be used without saving the table contents to a separate file: you can include the tables directly in your script using the “here” format (which is discussed in section 3.3.2.3). The symbols “- <<” after “-file” indicate that a table in “here” format follows, starting and ending with its “terminator” (in this case, “HERE_TERMINATOR”). The terminator text used to indicate the end of the table should obviously not be found in the table contents. The AVA-CLI will treat the lines following the command as if they were read from a separate file, until it encounters the “HERE_TERMINATOR” text at the beginning of a line.

```
create reference -file - << HERE_TERMINATOR
"Name"     "Annotation"     "Sequence"
"EGFR_Exon_18"     "EGFR_Exon_18"
"GACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACCCAGTGGAGAAGCTCCCAA
CCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGATCAAAGTGCTGGGCTCCGGTGCGTTCG
GCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCCTCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTG
GGG"
"EGFR_Exon_19"     "EGFR_Exon_19"
"TCACAATTGCCAGTTAACGTCTTCCTTCTCTCTCTGTCATAGGGACTCTGGATCCCAGAAGGTGAGAAAG
TTAAAATTCCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTCGAT
GTGAGTTTCTGCTTTGCTGTGTGGGGGTCCATGGCTCTGAACCTCAGGCCCACCTTTTCTC"
"EGFR_Exon_20"     "EGFR_Exon_20"
"CCACACTGACGTGCCTCTCCCTCCCTCCAGGAAGCCTACGTGATGGCCAGCGTGGACAACCCCCACGTGT
GCCGCCTGCTGGGCATCTGCCTCACCTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCGGCTGCCTC
CTGGACTATGTCCGGGAACACAAAGACAATATTGGCTCCCAGTACCTGCTCAACTGGTGTGTGCAGATCGC
AAAGGTAATCAGGGAAGGGAGATACGGGGAGGGGAGATAAGGAGCCAGGATC"
"EGFR_Exon_21"     "EGFR_Exon_21"
"TCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGGGCATGAACTACTTGGAGGACC
GTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTGGTGAAAACACCGCAGCATGTCAAGATCACA
GATTTTGGGCTGGCCAAACTGCTGGGTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAG
GTGGCTTTAGGTCAGCCAGCAT"
"EGFR_Exon_22"     "EGFR_Exon_22"
"CACTGCCTCATCTCTCACCATCCCAAGGTGCCTATCAAGTGGATGGCATTGGAATCAATTTTACACAGAA
TCTATACCCACCAGAGTGATGTCTGGAGCTACGGTGAGTCATAATCCTGATGCTAATGAGTTTGTACTGAG
GCCAAGCTGG"
HERE_TERMINATOR
```

The use of regular files or “here” files to input data is equivalent, and the choice is up to the user. Regular files can prove especially useful if the elements of the Project definition are supplied to you from a third-party (such as from the client of a sequencing center). On the other hand, “here” files allow you to encapsulate all the data except for the actual Read Data files into a single, relatively portable script. High-throughput environments with a workflow system might generate such scripts, thereby automating the project creation process.

The “create reference” command will normally fail if you try to create a Reference Sequence with a name that already exists. However, there are situations where it may be legitimate to attempt to do so. For example, a script may have correctly created Reference Sequences and then reached a save point before terminating early due to some error in the script; in such a case, it would be useful to be able to fix the problem in the script and just run it again without it throwing errors due to the Reference Sequences previously saved in the system. The optional flag “-orUpdate” allows this: if you try to create a Reference Sequence under a name that already exists in the Project, the “-orUpdate” flag converts the operation into an update, and the annotation and/or sequence provided in the “create” command are used to update the existing Reference Sequence. Without the flag, the name collision would throw an error.

This “-orUpdate” flag is available for most of the create commands, including those for Amplicons, Samples, and Variants. The flag is discussed in more detail for the Amplicon creation example (section 3.5.4).

3.5.4 Creating Amplicons

Now that there are Reference Sequences in the system, Amplicons can be completely specified for the Project. This is done using the “create amplicon” command (see section 3.4.4.1 for the usage statement). To add multiple Amplicons, it is best to use tabular input, as shown for the Reference Sequences, in section 3.5.3. (To best illustrate each command and parameters, the rest of this Project setup tutorial will show all tabular inputs using inline “here” files.)

```
create amplicon -file - << HERE_TERMINATOR
"Name"    "Annotation"    "Reference"    "Primer1"    "Primer2"    "Start"
"End"
"EGFR_18_1"   "Amplifies EGFR_Exon_18 from 23 to 66"   "EGFR_Exon_18"
"GACCCTTGTCTCTGTGTTCTTG"   "CCTCAAGAGAGCTTGGTTGG"   "23"   "66"
"EGFR_18_2" "Amplifies EGFR_Exon_18 from 60 to 136"    "EGFR_Exon_18"
"AGCCTCTTACACCCAGTGGA"   "CCTTATACACCGTGCCGAAC"   "60"   "136"
"EGFR_18_3"   "Amplifies EGFR_Exon_18 from 123 to 197"   "EGFR_Exon_18"
"TGAATTCAAAAAGATCAAAGTG"  "CCCCACCAGACCATGAGA"  "123"  "197"
"EGFR_19_1"   "Amplifies EGFR_Exon_19 from 23 to 115"   "EGFR_Exon_19"
"TCACAATTGCCAGTTAACGTCT"  "GATTTCCTTGTTGGCTTTCG"  "23"  "115"
"EGFR_19_2"   "Amplifies EGFR_Exon_19 from 67 to 183"   "EGFR_Exon_19"
"TCTGGATCCCAGAAGGTGAG"  "GAGAAAAGGTGGGCCTGAG"  "67"  "183"
"EGFR_20_1"   "Amplifies EGFR_Exon_20 from 20 to 108"   "EGFR_Exon_20"
"CCACACTGACGTGCCTCTC"  "GCATGAGCTGCGTGATGAG"  "20"  "108"
"EGFR_20_2"   "Amplifies EGFR_Exon_20 from 102 to 194"   "EGFR_Exon_20"
"GCATCTGCCTCACCTCCAC"  "GCGATCTGCACACACCAG"  "102"  "194"
"EGFR_20_3"   "Amplifies EGFR_Exon_20 from 153 to 244"   "EGFR_Exon_20"
"GGCTGCCTCCTGGACTATGT"  "GATCCTGGCTCCTTATCTCC"  "153"  "244"
"EGFR_21_1"   "Amplifies EGFR_Exon_21 from 23 to 113"   "EGFR_Exon_21"
"TCTTCCCATGATGATCTGTCCC"  "GACATGCTGCGGTGTTTTC"  "23"  "113"
"EGFR_21_2"   "Amplifies EGFR_Exon_21 from 111 to 215"   "EGFR_Exon_21"
"GGCAGCCAGGAACGTACT"  "ATGCTGGCTGACCTAAAGC"  "111"  "215"
"EGFR_22_1"   "Amplifies EGFR_Exon_22 from 21 to 132"   "EGFR_Exon_22"
"CACTGCCTCATCTCTCACCA"  "CCAGCTTGGCCTCAGTACA"  "21"  "132"
HERE_TERMINATOR
```

The Amplicons in this example are fully specified: the Reference Sequences used happen to have enough context to include both the Primer1 and Primer2 matches, and the exact “Start”

and “End” target coordinates are known and specified. If the exact coordinates of the targets were not known, asterisks (“*”) could be used as wild cards for the Start and End fields. This would force the application to try and determine the target coordinates by finding perfect matches for the primers, just as the GUI does (see section 2.2.4). “N” characters are counted as matches as long as they don’t make up more than 50% of the match. The results of an automatic primer match must yield one and only one pair of primer matches. If no pair match is found or if more than one match is found for a primer, an error is generated.

The software carries out the primer search, even if the asterisk notation is not used, to validate the target coordinates you supplied manually. This could lead to an error in the cases where your primers have an intentional mismatch with the reference, or where your primers are not included as part of the Reference Sequences. In such cases you would need to supply the correct target coordinates manually, and disable the automatic verification of the target coordinates using the “-checkPrimerMatch false” option to the “create amplicon” command.

The “create amplicons” command also has an “-orUpdate” flag like the one discussed for the create reference example (section 3.5.3), which can be used if a script terminates prematurely but after creating and saving one or more Amplicons: this flag will prevent errors due to “pre-existing” Amplicon names if you re-run the script (after fixing it), and will update the existing Amplicons with the new data instead.

Unlike with the creation of Reference Sequences, however, Amplicons cannot always rely solely on the use of the “-orUpdate” flag because the uniqueness requirement for the naming of Amplicons is only at the level of each Reference Sequence, not for the whole Project. In the case where Amplicons with the same name have been defined relative to different Reference Sequences, the “-orUpdate” flag would not know which Amplicon it is intended for. Such a situation can be resolved by using an “-ofRef” parameter to specify the Reference Sequence of the intended Amplicon. Without an “-ofRef” to properly disambiguate identically named Amplicons when using an “-orUpdate”, the “create amplicon” command will fail and throw an error.

3.5.5 Creating Variants

If known Variants exist, they can be added to the Project using the “create variant” command (see section 3.4.4.9 for the usage statement). As was the case for Amplicons (section 3.5.4), the Reference Sequence relative to which a Variant is defined must pre-exist in the project. An example using a “here” style table is below.

```
create variant -file - << HERE_TERMINATOR
"Name"   "Annotation"   "Reference"   "Pattern"   "Status"
"15BP_DEL_93-107"      "Pattern  entered  manually"      "EGFR_Exon_19"
"d(93-107)"   "accepted"
"HAP_97C_126A"        "Created   from   selections"        "EGFR_Exon_18"
"s(97,C)s(126,A)"   "accepted"
"SUB_A_to_C_97"       "Created   from   selections"       "EGFR_Exon_18"
"s(97,C)"   "accepted"
"SUB_G_to_A_126"       "Created   from   selections"       "EGFR_Exon_18"
"s(126,A)"   "accepted"
HERE_TERMINATOR
```

Another similarity with the “create amplicon” command is that a Project can also have multiple Variants of the same name, as long as they are defined relative to different Reference Sequences. So, the “create variant” command also has both the “-orUpdate” flag and the “-ofRef” parameter, which functions the same way as when used with the “create amplicon” command (section 3.5.4).

The “create variant” command has an additional option used to verify the “pattern” given for the Variants being created: “-checkPattern”. When this option is set to “true” (the default value), the “pattern” you set for each Variant is validated in three different ways.

1. The pattern is first checked to make sure that it is syntactically correct.
2. Secondly, the coordinates of the pattern are checked to make sure that they actually exist within the Reference Sequence specified for the Variant.
3. Thirdly, any substitution code must actually create a difference at the specified position (thus, specifying s(10,C) when position 10 is already a C in the Reference Sequence would be an error).

If a check is conducted and any of the three validation criteria are not met, an error will be thrown. However, the check does not occur if the Variant does not have a Reference Sequence assigned to it, or if the Reference Sequence’s nucleotide sequence is empty; this allows you to add incomplete Variant definitions or to add Variant place holders before you have specified your Reference Sequences, if you so desire, without causing the “create variant” command to fail. You can also disable the “-checkPattern” option by setting it to “false”.

3.5.6 Creating Samples

Samples are easily created as they consist only of a name and an optional annotation. Samples are added using the “create sample” command (see section 3.4.4.8 for the usage statement). Below is an example creating 7 Samples, using a “here” table.

```
create sample -file - << HERE_TERMINATOR
"Name"        "Annotation"
"Sample1"     "Sample1"
"Sample2"     "Sample2"
"Sample3"     "Sample3"
"Sample4"     "Sample4"
"Sample5"     "Sample5"
"Sample6"     "Sample6"
"Sample7"     "Sample7"
HERE_TERMINATOR
```

The “create sample” command has an “-orUpdate” flag like the one discussed for the “create reference” example, above (section 3.5.3).

3.5.7 Associating Samples with Amplicons

With the Amplicon and Samples defined, we can now associate them according to the requirements of the experiment. This is done using the “associate” command (see section 3.4.1 for the usage statement). For this example, the Samples are being used to pool Amplicons from

shared Reference Sequences. You can create commands to process a single Sample at a time using tabular file input (shown as a “here” block below):

```
assoc -sample Sample1 -file - << HERE_TERMINATOR
"amplicon"      "ofRef"
"EGFR_20_1"     "EGFR_Exon_20"
"EGFR_20_2"     "EGFR_Exon_20"
"EGFR_20_3"     "EGFR_Exon_20"
HERE_TERMINATOR
```

This example also illustrates how command line options are combined with tabular contents. In this case, the single given command is actually translated into the three separate commands:

```
assoc -sample Sample1 -amplicon EGFR_20_1 -ofRef EGFR_Exon_20
assoc -sample Sample1 -amplicon EGFR_20_2 -ofRef EGFR_Exon_20
assoc -sample Sample1 -amplicon EGFR_20_3 -ofRef EGFR_Exon_20
```

Alternatively, the sample name can be used as a field in the file rather than as an argument on the command line, allowing multiple Sample – Amplicon associations to be established from a single command:

```
assoc -file - << HERE_TERMINATOR
"sample"      "amplicon"      "ofRef"
"Sample1"     "EGFR_20_1"     "EGFR_Exon_20"
"Sample1"     "EGFR_20_2"     "EGFR_Exon_20"
"Sample1"     "EGFR_20_3"     "EGFR_Exon_20"
"Sample2"     "EGFR_18_1"     "EGFR_Exon_18"
"Sample2"     "EGFR_18_2"     "EGFR_Exon_18"
"Sample2"     "EGFR_18_3"     "EGFR_Exon_18"
"Sample3"     "EGFR_18_1"     "EGFR_Exon_18"
"Sample3"     "EGFR_18_2"     "EGFR_Exon_18"
"Sample3"     "EGFR_18_3"     "EGFR_Exon_18"
"Sample4"     "EGFR_19_2"     "EGFR_Exon_19"
"Sample4"     "EGFR_19_1"     "EGFR_Exon_19"
"Sample5"     "EGFR_20_2"     "EGFR_Exon_20"
"Sample5"     "EGFR_20_1"     "EGFR_Exon_20"
"Sample5"     "EGFR_20_3"     "EGFR_Exon_20"
"Sample6"     "EGFR_21_2"     "EGFR_Exon_21"
"Sample6"     "EGFR_21_1"     "EGFR_Exon_21"
"Sample7"     "EGFR_22_1"     "EGFR_Exon_22"
HERE_TERMINATOR
```

Note the “ofRef” field, used as a safety measure to make sure that the correct Amplicon is being specified, as described above (section 3.5.4). In this particular example, however, the “ofRef” field is not actually necessary since all the Amplicon names are unique within the whole Project, and is shown only for illustrative purposes.

For more uniform Projects, where the same Amplicons are measured in each Sample, an asterisk (“*”) can be used as a shortcut for the Amplicon names. This associates all the Amplicons defined in the Project at that time point with the specified Sample. You can also

combine the asterisk for Amplicons with an “ofRef” to selectively associate all and only the Amplicons derived from the “ofRef” Reference Sequence with the Sample specified.

Rather than directly associating Amplicons with Samples in advance, one might consider directly associating Samples with Amplicons and particular Read Data Sets. At such time, the corresponding Sample-Amplicon associations will be implicitly made. However, this requires that the Read Data Sets be first loaded into the Project as described in the section below (3.5.8).

3.5.8 Loading Read Data Sets

Read Data Sets are imported into the Project using the “load” command (see section 3.4.8 for the usage statement). The exact way to load the Read Data Sets into the Project depends on how the data is organized on the disk. In the case where individual SFF files are stored together in a given repository (*e.g.* /data/sffFiles/EGFR_sff_files) and you know the specific file names you need, you might load the files as shown below (shown in the “here” file format):

```
load –sffDir /data/sffFiles/EGFR_sff_files -file - << HERE_TERMINATOR
"SffName"           "ReadGroup"     "SymLink"     "Name"
"DGVS90J01.sff"     "ReadGrp_1"     "false"     "DGVS90J01"
"DGVS90J02.sff"     "ReadGrp_1"     "false"     "DGVS90J02"
"DGVS90J03.sff"     "ReadGrp_1"     "false"     "DGVS90J03"
"DGVS90J04.sff"     "ReadGrp_1"     "false"     "DGVS90J04"
HERE_TERMINATOR
```

Note that a Read Group can be specified as above even if it was not explicitly added to the Project beforehand: the load command will automatically create Read Groups of given names, as needed. To explicitly add a Read Group to the Project, use the “create readGroup” command (see section 3.4.4.6 for the usage statement). In the example above, if you had wanted to create the read group in advance you could have typed “`create readGroup ReadGrp_1`”.

In our example, the 4 SFF files we want to load into the Project happen to be from the same sequencing Run. This allows us to use a more compact form of the command, with the “-filePrefix” option:

```
load –sffDir /data/sffFiles/EGFR_sff_files \
–readGroup ReadGrp_1 -filePrefix DGVS90J \
–regions 1,2,3,4 –symLink “false”
```

Specifying the file prefix and choosing which regions to load gives the command enough context so each file does not need to be specified individually. Using the file prefix also provides sufficient specificity to prevent sff files from another Run from being erroneously picked up during the load. If there were a firstRun01.sff and a secondRun01.sff in the directory and you specified region 1 without a file prefix, both files would be imported.

If the SFF files you want to import are not gathered together in a repository but are instead still located in their respective Run analysis directories, the situation may be a bit different: while you might know what Runs and regions you want to import, you may not know the specific file prefix to use. This is because a Run’s SFF file names are assigned during analysis by the pipeline software. To get around this, one may set up an “alias” for the loaded files which you can use to

refer to the files by region without knowing their actual names. In this next example, we use the alias mechanism, and further use the “analysisDir” option to specify a Run analysis directory:

```
 load –analysisDir /data/sequencingRuns/EGFR_Run_Dir/EGFR_Analysis_Dir/ \
–readGroup ReadGrp_1 –regions 1,2,3,4 \
–symLink false –alias EGFR_reads
```

In the command above, the alias has been set to “EGFR_reads”. With this alias established, you would refer to the region 1 file as “EGFR_reads01” and to the region 4 file as “EGFR_reads04”. The files are still loaded into the Project with their actual names, but the alias enables you to refer to them without knowing those names. With this facility, you could actually create the script for processing an Amplicon Project in advance of the completion of Pipeline analysis.

In most cases, when Read Data Sets are loaded into a Project, actual copies of the read data files are stored within the directory structure of the Project. This makes the Project directory portable so it can be moved to another location and maintain its integrity as a functional Project. However, you can save disk space and transfer time by setting the “symlink” parameter to “true” (it defaults to “false”). Enabling symlinking causes the Read Data Sets loaded to be stored as symbolic links pointing to the original read data files instead of creating physical copies of the data in the Project folder. If you use this option, your Projects may become nonfunctional if you either move the Project to a location where the symlinks can no longer reach the original data (such as to a different computational host) or if you move or delete the original data. Without access to the Read Data, you will be unable to rerun computations for the Project, and you will also be unable to view Flowgrams.

3.5.9 Associating Read Data Sets with Samples

With the Sample-Amplicon associations already made and the Read Data Sets loaded into the Project, the Samples with their associated Amplicons can now be associated to the proper Read Data Sets. This uses the “associate” command, described in section 3.5.7 where it was used to associate Samples with Amplicons (the usage statement is in section 3.4.1). In this case, the command must supply a Sample and a Read Data Set (or a Read Group).

```
assoc -file - << HERE_TERMINATOR
"readData"      "sample"
"DGVS90J01"     "Sample1"
"DGVS90J02"     "Sample2"
"DGVS90J03"     "Sample6"
"DGVS90J03"     "Sample7"
"DGVS90J03"     "Sample3"
"DGVS90J03"     "Sample5"
"DGVS90J03"     "Sample4"
HERE_TERMINATOR
```

When you make the association between a Sample and a Read Data Set, all the Amplicons associated with that Sample at that time are used to form three-way Read Data Set - Sample - Amplicon associations. This is equivalent to dragging a Sample, in the GUI, onto a Read Data Set in the Read Data Tree. These associations are used during Demultiplexing to determine which reads of a given Read Data Set belong to which Samples. As with dragging a Sample

onto a Read Data Set in the GUI, when a Sample is associated with a Read Data Set, the Sample itself must already have an association with at least one Amplicon. An error is generated in the CLI if such a Sample-Amplicon association does not exist at the time you attempt to associate the Sample with the Read Data Set.

Rather than first creating Sample-Amplicon associations and using the Samples to then indirectly create Read Data Set – Sample – Amplicon associations, you can directly create these three-way associations by specifying all three entities in a single command. For example:

```
assoc -readData DGVS90J04 -sample Sample8 -amplicon EGFR_20_1
```

As this command only forms the association for the triad, the three individual entities must have been previously defined. It must be remembered that for a given Read Data Set, an Amplicon can only be assigned to one Sample; an “associate” command that would violate this constraint would return a warning message and the association will not be made. If the Sample and Amplicon specified in the “three-way association” form of the command, above, were not already associated with each other, that two-way association would simultaneously be made when the command executes.

In a Project where the same Sample (or list of Samples) is being measured against many Read Data Sets, the “associate” command can be simplified by the judicious use of Read Groups: associating a Sample (with its already associated Amplicons) to a Read Group forms the Read Data Set - Sample - Amplicon association triads with each Read Data Set present in the Read Group. For example:

```
assoc -readGroup ReadGrp_1 -sample Sample1
```

would associate Sample1 and all its currently associated Amplicons with each of the Read Data Sets of Read Group ReadGrp_1.

In a different context, where you may be measuring all the Amplicons defined in a Project for a Sample (or the same set of Amplicons for every Sample of the Project), you can more succinctly specify the triad association using the asterisk (“*”) notation for the Amplicons. For example:

```
assoc -readGroup ReadGrp_1 -sample Sample8 -amplicon *
```

The command above associates every Amplicon currently defined in the Project with the Sample specified and then associates the Sample and these Amplicons with every Read Data Set in the Read Group. You can include an “ofRef” parameter in the association command to restrict the Amplicons being associated to those derived from a single particular Reference Sequence.

Note that the command usage above is appropriate for establishing the Read Data Set – Sample – Amplicon associations in projects in which MIDs are not used. For an example of a CLI script that creates such associations for an MID-based experiment, see section 3.6

3.5.10 Editing Object Properties

After you have finished entering various objects into a Project, you may find it necessary to edit properties and correct mistakes. There are several commands that let you alter the Project data after they have already been entered into the system.

3.5.10.1 Updating an Object

One way to edit an object is to use the “update” command (see section 3.4.16 for the usage statement). In the example Project, we find that the region 4 Read Data Set is actually empty. Since the “load” command used to import Read Data Sets defaults the “active” flag to true, we need to change that flag to false, for this Read Data Set:

```
update readData EGFR_reads04 -active false
```

The update command is of the form “update <entity type> <name of entity> [other options]” where the “other options” are used to set the values for properties appropriate for the entity type. The only object property that cannot be updated via the update command is the object’s name. To change the name of an object, use the “rename” command (section 3.5.10.2, below).

When updating an Amplicon or a Variant, you can use an “ofRef” parameter to fully specify the entity of interest in cases where the multiple entities may share the same name (but are uniquely named in the context of their particular Reference Sequences). If an update command encounters an ambiguous Amplicon or Variant name, the command will fail and an error will be generated.

3.5.10.2 Renaming an Object

Although you can change most of the properties of an object using the update command (section 3.5.10.1), an object name change requires the “rename” command (see section 3.4.11 for the usage statement). For example:

```
rename sample –name Sample8 –newName Vial_XYZ
```

The general syntax of the rename command is: “rename <entity type> -name <existing name of entity> -newName <new name for entity>, whereby you provide the original name as the “name” parameter, and the name you want to change it to as the “newName” parameter. An alternative, more succinct, form of the command allows the “-name” and “-newName” option keywords to be left out with the old and new names being given positionally, as in:

```
rename sample Sample8 Vial_XYZ
```

As with the update command (section 3.5.10.1), if the Project contains Amplicons or Variants with duplicate names (but that are defined relative to different Reference Sequences), you must supply an “ofRef” parameter to specify which particular Amplicon or Variant you want to rename. If a rename command encounters an ambiguous Amplicon or Variant name, the command will fail and an error will be generated.

3.5.10.3 Removing an Object

If you find that you no longer have a need for an object, or if you find that an object you imported would require more work to edit than to create from scratch, you can use the "remove" command to eliminate it from the Project altogether (see section 3.4.10 for the usage statement). For example:

```
remove sample Sample8
```

Most of the remove commands have the syntax shown above, with the entity type and entity name as the only arguments to the command. Removing Amplicons and Variants, however, may also involve adding an extra "ofRef" parameter to resolve ambiguities where the Project contains Amplicons or Variants with duplicate names (but that are uniquely named relative to their particular Reference Sequences). If a remove command encounters an ambiguous Amplicon or Variant name, the command will fail and an error will be generated.

Be aware that removing an object can have a cascade of downstream consequences. If you delete a Read Data Set, the associations it has with any Sample-Amplicon sets will be severed (but the Samples will remain associated with the same Amplicons they had before the removal). Removing a Read Group will also remove all the Read Data Sets it contains (with the repercussions above). If you remove a Reference Sequence, you will also remove all the Amplicons and Variants associated with that Reference Sequence. Removing a Sample or an Amplicon severs any Read Data Set – Sample or Sample – Amplicon associations that it used to participate in. Removing a Variant does not impact other objects.

As for other commands, above, an asterisk ("*") can be used in place of an entity name to mean all instances of an entity type. This allows you to easily remove all the entities of the specified type. You can combine the "ofRef" parameter with an asterisk to remove only those entities (Amplicons or Variants) from the specified Reference Sequence.

3.5.10.4 Dissociating Relationships

Sometimes, the objects you have entered into the Project are all correct, but you may have made the wrong associations between some of them; or, you may have cloned a previous Project with similar objects, but the new Project structure may be slightly different. In cases such as these, you would want to dissolve the incorrect associations without removing the objects from the Project. The "dissociate" command serves that purpose (see section 3.4.5 for the usage statement).

This command is used to influence the three-way Read Data Set – Sample – Amplicon relationships (as displayed in the Project Tab's Read Data tree of the GUI) and the Sample-Amplicon relationships (displayed in the Project tab's Samples tree of the GUI). As such, it has two general flavors: one primarily affects the Samples tree and the other, primarily the Read Data tree; but there can be changes in both trees as a result of either command type.

If you are primarily trying to influence the Sample-Amplicon relationships (as seen in the Samples tree), you should use the form of the command where you supply a Sample and an Amplicon as arguments. For example:

```
dissoc -sample Sample6 -amp EGFR_21_1
```

The command above removes the association between Sample6 and Amplicon EGFR_21_1 but does not remove either object from the Project. Additionally, while removing this association, any three-way Read Data Set – Sample – Amplicon associations for Sample 6 and EGR_21_1 would also be removed. In the GUI this would be reflected as elements being removed from the corresponding Read Data Sets of the Read Data tree.

If more than one Amplicons of the same name are associated with a Sample (but are uniquely named relative to their particular Reference Sequences), you must use the "-ofRef" parameter to specify the Amplicon to which you want to apply the dissociation. If you don't use the "-ofRef" parameter in this situation, an error will be generated.

You can use an asterisk ("*") as the Amplicon specifier in the command to dissociate all Amplicons from a Sample, with the concomitant dissociation of the related Read Data – Sample – Amplicon associations that may exist. The asterisk notation can be combined with the "-ofRef" parameter to dissociate from the Sample all the Amplicons that are defined relative to a specified Reference Sequence (but maintain the associations of Amplicons from other Reference Sequences).

If you are primarily trying to influence the associations of a particular Read Data Set with its Samples and Amplicons, you should use the form of the command where you specify a Read Data, a Sample, and optionally an Amplicon as arguments. For example:

```
dissoc -readdata DGVS90J02 -samp Sample2 -amp EGFR_18_1
```

The command above dissociates the Read Data Set - Sample – Amplicon association triplet among DGVS90J02, Sample2, and EGFR_18_1, but all three objects still individually remain in the project. Dissociation of a Read Data Set - Sample – Amplicon triplet does NOT influence the corresponding Sample – Amplicon paired association, which is maintained.

If more than one Amplicon of the same name are associated with a Sample (but are uniquely named relative to their particular Reference Sequences), you must use the "-ofRef" parameter to specify the Amplicon to which you want to apply the dissociation. If you don't use the "-ofRef" parameter in this situation, an error will be generated.

You can use an asterisk ("*") as the Amplicon specifier in the command to dissociate all the Read Data Set - Sample – Amplicon triplets based on a given Read Data Set and Sample. As before, the dissociation of these triplet associations has no effect on the corresponding pairwise Sample-Amplicon relationships (as viewed in the Samples tree of the GUI). The asterisk notation can be combined with the "-ofRef" parameter to dissociate only those Read Data - Sample – Amplicon triplets where the Amplicons are defined relative to a specified Reference Sequence (but maintain the associations for Amplicons from other Reference Sequences).

A shortened form of the Read Data-centric command allows you to omit the Amplicon specification and only supply a Read Data and a Sample. This is interpreted as if you had specified the Amplicon as an asterisk.

```
dissoc -readdata DGVS90J01 -samp Sample1
```

3.5.11 Computation

The CLI can also be used to trigger the computation of a Project once it has been set up properly.

3.5.11.1 Validating the Project Before Computation

There are two utility commands that can be used to validate a Project before computation: “utility validateNames” and “utility validateForComputation”.

The GUI does not currently enforce unique names for individual objects because there is an internal accounting process other than naming that keeps entities distinct from each other. In the CLI, however, all objects are created and manipulated using their names, so non-unique names can be a problem. Some degree of name duplication can be tolerated for Amplicon and Variant objects if the Reference Sequences for those objects can be combined with the object names to unambiguously identify individual objects (using the “-ofRef” parameter available in various commands). Any name duplication that cannot be disambiguated in this manner must be fixed. Additionally, the GUI can allow objects to have empty names, which also would cause problems for the CLI.

The “utility validateNames” command can be used to detect and correct naming problems in Projects (see section 3.4.17.1 for the usage statement). Running the command without any arguments will report an error if any problem names are encountered (irresolvable duplicates or empty names). The command does nothing if there are no errors to report.

You can also use the “-fix” flag with the “utility validateNames” command to enable it to correct the naming problems it finds (rather than using it just as a problem detection tool as above). The default fix is to put a “FIX_” prefix and an underscore (“_”) suffix followed by a unique number. For example, two Reference Sequences with the same name “MyRef” would be converted to FIX_MyRef_1 and FIX_MyRef_2. You can specify alternate prefixes and suffix separators using the “-fixPrefix” and “-fixSuffix” options. The common prefix makes it easy to find these “fixed” names in the sorted Project Tab tables of the GUI and manually adjust them, if desired, to different unique names.

Even if the names are perfectly fine, there can be other problems with a Project that might impact its computation. The command “utility validateForComputation” checks for these problems (see section 3.4.17.2 for the usage statement). Specifically, the command verifies the following:

1. all Reference Sequences contain a sequence that is at least 1 base in length;
2. all Amplicons are associated to valid Reference Sequences and have target start and end coordinates that are contained within that Reference Sequence;
3. all Read Data files that are associated with at least one Sample and one or more valid Amplicons are available; and
4. if Variants are defined in the Project that are associated to valid Reference Sequences, they have non-empty patterns that are valid with respect to that Reference Sequence.

If any of these criteria aren’t met, warnings are reported and the command throws an error; otherwise it does nothing. The warnings can be silenced by setting the “-silent” option to “true”.

3.5.11.2 Managing the Computation

The “computation” command allows you to start or stop a computation, or check its status (see section 3.4.3 for the usage statement). You are only allowed to use the “computation start” and “computation stop” commands on Projects on which you have full control. If you were able to successfully do a simple “open” on a Project, or if you were able to open it with the “-control preempt” option, you have the appropriate level of control to start or stop a computation.

If you have read-only access to the Project, you cannot influence the course of the computation, but you can run the “computation status” command which will report “running” or “stopped”, as appropriate.

Unlike with the GUI, a computation started by the CLI is not run as a separate background process, but rather as part of the CLI process itself. This means that if the CLI instance that started a computation is terminated (via a control-C, for instance) the Project computation will abruptly stop as well. Additionally, it means that the next step of the current CLI script (or the display of the next command prompt, if in interactive mode) will not be executed until the computation finishes. Thus, the “computation status” command for checking the status of a computation is mostly useful for checking on the status of computations that were initiated either from the GUI or from other CLI instances, but not for computations that a particular CLI instance started itself. Unlike the GUI, therefore, the CLI does not currently provide the means to obtain status reporting to determine the current stage of computation or the dispositions of the processed and queued computation steps. However, if a computation were started from a CLI instance, you could open the project in the GUI (perhaps in read only mode, so as not to seize control from the CLI instance that may have additional steps to perform after the Project computation is finished) and track its detailed progress there.

Likewise, because computations run as part of the CLI process, you can’t execute a clean “computation stop” within the same instance of the CLI that you used to initiate the “computation start.” The safest way to halt a computation is to start another instance of the CLI (or open the GUI), open the Project in preempt mode, and type “computation stop” (in the CLI) or press the stop button on the main Computation tab (in the GUI).

3.5.11.3 Loading Automatically Detected Variants

Once a computation is complete, any Variants that were part of the Project prior to the computation will have their frequency statistics updated. As part of the computation, the application also attempts to automatically detect potential variations in the data. In the GUI, you have the option of importing the automatically detected variants using a “Load” button on the main Variants Tab, with the option to narrow down the set to import using a variety of filters. In the current version of the CLI, by contrast, no filters are provided and the import is an all or nothing proposition.

You can import the pool of automatically detected variants in the CLI by using the “computation loadDetectedVariants” command (see section 3.4.3.4 for the usage statement). Note that this command loads the automatically detected Variants into memory, but just like in the GUI, you must save the Project if you want the load to be permanent. Since the load is currently all or nothing, a good strategy may be to load the Variants but not save them, so you can run a report command (see section 3.5.12) on all the potential Variants for a Project without cluttering the GUI view of the Project with too many marginal Variants.

If you choose not to load the detected Variants after a computation triggered from the CLI (or you choose to load them but not save the Project), the Auto-Detected Variants will not be lost to you even if you exit the CLI: they will remain in the Project, in a queue to be loaded in a subsequent session. For instance, you could start a new instance of the CLI, reopen the Project and run the "computation loadDetectedVariants" command, and thus recover the Auto-Detected Variants you chose not to load or save the first time. Similarly, you could finish the computation in the CLI without loading (or with loading and not saving) the Auto-Detected Variants. Later, you can open the Project in the GUI, and you would be able to access the Auto-Detected Variants via the Load button on the main Variants Tab.

3.5.12 Reporting

After your Project has finished computing you can open it with the GUI to explore the results and alignments. As usual, the Variants Frequency Table on the main Variants Tab of the GUI can be manually exported by clicking on the "text file" button located next to it. The structure of this exported file is in the same two-dimensional geometry (Variants as the rows and Samples as the columns) as the table displayed in the GUI itself. Because of the two-dimensional structure, this format is not particularly amenable to high-throughput processing as one might want to do following a project Computation. The same information can also be generated in an automated fashion, and in a more useful linear form, using the "report variantHits" command from the CLI (see section 3.4.12.2 for the usage statement and output format). Although the GUI allows you to apply various filters to the data before exporting Sample-Variant data, the CLI currently only supports a bulk report of all the Variant statistics. You can generate the report in either tab-separated value (tsv) or comma-separated value (csv) formats. The report only includes Variants that are officially part of the Project, *i.e.* specifically defined Variants, or auto-detected Variants that were "loaded" using the "computation loadDetectedVariants" command (see section 3.5.11.3). If you have any unloaded automatically detected Variants, they will not be included in the output unless you use the "computation loadDetectedVariants" command prior to using "report variantHits."

With the CLI-generated report in hand you can do your own customized processing of the reported Variant frequencies. One suggestion would be to apply filter criteria on the reported data to highlight Variants with the most believable support; users can then focus on these "best candidates" and verify them by examination of the alignments in the GUI.

3.5.13 Finishing Touches

These few additional "Project management" commands can be useful when you have finished working with a Project and are ready to either move on to another one, or you want to shut down the CLI.

3.5.13.1 save

When you have gained control of a Project via a Project creation, a standard open, or an open in preempt mode, you have the freedom to save the modifications you make to that Project at any point you deem appropriate. This is done with the "save" command (see section 3.4.13 for the usage statement). If you close a Project (see section 3.5.13.2) that contains changes without first saving it, the unsaved changes will be discarded. Note that if you have made any modifications to the Project, you MUST run the "save" command to commit those changes to

the Project prior to triggering a computation with the “computation start” command. If you trigger a computation on a Project that contains unsaved modifications, an error will be generated.

3.5.13.2 close

When you have finished making changes to the Project, you can close it using the “close” command (see section 3.4.2 for the usage statement). The close statement discards any unsaved changes to the Project and frees the Project lock so someone else can access it without needing to preempt control. If there are unsaved changes to the Project, the CLI will show a warning to let you know that some changes are being discarded. If you are in interactive mode, a “yes”, “no”, “cancel” prompt will be shown to allow you to avoid discarding the changes if you didn’t really mean to close without saving. The CLI is still running after you use the close command, so you can open another Project and do more work.

3.5.13.3 exit

When you are ready to shut down the `doAmplicon` instance you are running, you can use the “exit” command (see section 3.4.6 for the usage statement). As with the close command (section 3.5.13.2, above), the exit command will warn you if you try to exit an Project that contains unsaved changes and, in interactive mode, will prompt you to decide if you want to save before exiting. Since the exit command actually shuts down the `doAmplicon` instance, it will terminate any chain of commands wherever it is introduced (*e.g.*, if you supply three scripts to be executed in succession by a `doAmplicon` instance and the first script has an exit in it, the other two scripts will not be executed).

3.5.14 Exporting from the Project

It can be convenient to use aspects, or the entire definition, of an existing Project as the basis for defining a new Project. Two utility commands are available (“utility makeSetupScript” and “utility clone”) that provide the means to export most or all aspects of an existing Project definition at once; and a set of “list” commands are provided to facilitate more focused exports of Project setup data.

3.5.14.1 utility makeSetupScript

The “utility makeSetupScript” command is basically a means of making a backup of the setup of your currently open Project in the CLI (see section 3.4.17.3 for the usage statement). If you provide the command with an “-outputFile” option, the CLI writes a script to that file that contains all the commands that you would have to input to `doAmplicon` to regenerate your Project setup.

The script will contain commands to create the Project directory structure and to create all the Project objects and the required associations between them. Load commands are included to handle the import of the Read Data Sets. However, the script does not back up any computed results for the Project. Generally, you will not be able to run the script immediately after you generate it, because your Project will still be in place and the “create Project” command in the script will fail because you can’t create a Project that already exists. The script is intended as a safety measure that you could use to reconstitute your Project should the Project directory

become corrupted. Note that the "utility makeSetupScript" command exports the Project setup based on the state of the open Project that you have in memory when you run the command (including any unsaved changes). The load commands generated for the Read Data Sets will try to find the data in the original locations where the data was located when originally imported into the Project (whether via the CLI or the GUI). If that data has been moved to a new location, you will need to manually edit the setup script to reflect the new location. If the data is no longer available, the load commands will fail. Note that the script only backs up the Project setup; it does not backup of the actual Read Data Sets.

3.5.14.2 utility clone

The "utility clone" command is used to create an exact copy of a Project setup to another location (see section 3.4.17.4 for the usage statement). The command is used in the context of an open Project in the CLI, and you provide the location where you want the clone to be created (*e.g.*, "utility clone /data/clonedProjectDirectory"). The command copies the Project directory structure and objects with their associations to the new location, but does not copy the Read Data Sets (unless specifically called for by setting the "-copyReadData" parameter to "true").

A "-scriptOnly" option can be used to prevent the actual execution of the clone operation and instead write out the commands necessary to carry out the cloning process to a script that you can edit and use later as input to a `doAmplicon` command (this is similar to running the "utility makeSetupScript" command, except that the Read Data Sets are not copied by default). The clone operation uses the state of the open Project at the time the command is run, so any unsaved changes involving the Project setup will be included in the clone operation. The cloning operation does not include any computed results for the Project.

If you choose to copy the Read Data Sets as part of the clone command, the read data will be obtained from the open Project and then the "OriginalPath" of the Read Data Sets will be updated to reflect the true original source of the data. This allows the clone to occur even if the original source of the Read Data Sets has been moved or deleted. The read data import should only fail if there are disk space issues or if the Project being cloned uses symbolic links and the links are invalid because the data was moved or deleted.

3.5.14.3 list

Sometimes you may want to export only a specific subset of the Project setup data rather than backing up or cloning an entire Project. Examples may be that you want to reuse some Reference Sequences from one Project in a new Project; or that you want to recycle some Amplicons and/or Variants associated with those Reference Sequences, but you don't want to import any Samples or Sample – Amplicon relationships (*e.g.* because your new Project will have its own Samples, different from the ones of the existing Project). In these cases you can use the "list" command to output tabular data that is suitable to import into a new Project (see section 3.4.7 for the usage statement). The tables returned by the list command can have their format set to tab-separated values (tsv) or comma-separated values (csv). Using the "-outputFile" option, you can specify what file the table should be written to or you can allow the table to be written to standard output.

To import a list table into a new Project, use the corresponding create command for the data type in the file, and either provide the table file using the "-file" parameter or the contents of the

file as part of a “here” format file (*e.g.*, “create ref -file listRefTable.tsv”, where listRefTable.tsv was previously created from another project via “list ref -outputFile listRefTable.tsv”).

The list commands can also be useful in interactive mode as a way to verify the content of the Project as you enter and edit Project objects. Even if you aren’t in interactive mode, the list commands can be used in scripts to enhance logging in scripts when troubleshooting.

List commands are available to review and export the basic entities of the Project, but not the associations between Samples, Amplicons, and Read Data Sets managed by the associate command. To view or export those associations one must inspect or run the scripts generated by the “utility makeSetupScript” and “utility clone” commands.

3.5.15 Integrated Project Script

Below is the commented text of an example script that could be used to set up and compute a Project (provided you have access to the Read Data files). You would execute this script by saving it to a file such as “projectSetupScript.ava” and executing it using the CLI (“doAmplicon projectSetupScript.ava”). Alternatively, you could start the CLI in interactive mode and type or copy and paste the commands into the interface individually. Note that the ‘#’ symbol at the beginning of a line is used to indicate a comment line that is ignored by the command interpreter. The data for the object types is supplied in tab-delimited “here” files. If you copy and paste the data, make sure that the space between fields in the “here” files remain tabs when you paste the data in the new location (in some combinations of applications the cut (or copy) and paste operation converts the tabs into spaces).

Due to the limitations of this printed document, certain lines of the script below appear on multiple lines. This occurs for certain tab-separated entries in the tables given as arguments to certain commands. Be aware that these should actually be single lines in the script.

```
# Script to create a project, compute it, and generate a report.
# Edit paths as necessary to conform to your system.

# Create the project architecture. Edit the path if you want to create the
# project in an alternate location.

create project /data/ampProjects/EGFR_CLI \
    -name EGFR_CLI \
    -annotation "CLI Example Project Creation Test"

# This command creates all the reference objects.

create reference -file - << HERE_TERMINATOR
"Name"     "Annotation"     "Sequence"
"EGFR_Exon_18"     "EGFR_Exon_18"
"GACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACCCAGTGGAGAAGCTCCCAACCAAGCTC
TCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGATCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGT
AAGGTCCCTGGCACAGGCCTCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGG"
"EGFR_Exon_19"     "EGFR_Exon_19"
"TCACAATTGCCAGTTAACGTCTTCCTTCTCTCTCTGTCATAGGGACTCTGGATCCCAGAAGGTGAGAAAGTTAAAATT
CCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTCGATGTGAGTTTCTGCTTTG
CTGTGTGGGGGTCCATGGCTCTGAACCTCAGGCCCACCTTTTCTC"
"EGFR_Exon_20"     "EGFR_Exon_20"
"CCACACTGACGTGCCTCTCCCTCCCTCCAGGAAGCCTACGTGATGGCCAGCGTGGACAACCCCCACGTGTGCCGCCTG
```

```
CTGGGCATCTGCCTCACCTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCGGCTGCCTCCTGGACTATGTCCGGG
AACACAAAGACAATATTGGCTCCCAGTACCTGCTCAACTGGTGTGTGCAGATCGCAAAGGTAATCAGGGAAGGGAGATA
CGGGGAGGGGAGATAAGGAGCCAGGATC"
"EGFR_Exon_21"     "EGFR_Exon_21"
"TCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGGGCATGAACTACTTGGAGGACCGTCGCTTG
GTGCACCGCGACCTGGCAGCCAGGAACGTACTGGTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCA
AACTGCTGGGTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCAT"
"EGFR_Exon_22"     "EGFR_Exon_22"
"CACTGCCTCATCTCTCACCATCCCAAGGTGCCTATCAAGTGGATGGCATTGGAATCAATTTTACACAGAATCTATACC
CACCAGAGTGATGTCTGGAGCTACGGTGAGTCATAATCCTGATGCTAATGAGTTTGTACTGAGGCCAAGCTGG"
HERE_TERMINATOR

# This command creates the amplicon objects.

create amplicon -file - << HERE_TERMINATOR
"Name"  "Annotation"  "Reference"  "Primer1"  "Primer2"  "Start"  "End"
"EGFR_18_1"  "Amplifies EGFR_Exon_18 from 23 to 66"  "EGFR_Exon_18"
"GACCCTTGTCTCTGTGTTCTTG"  "CCTCAAGAGAGCTTGGTTGG"  "23"  "66"
"EGFR_18_2"  "Amplifies EGFR_Exon_18 from 60 to 136"  "EGFR_Exon_18"
"AGCCTCTTACACCCAGTGGA"  "CCTTATACACCGTGCCGAAC"  "60"  "136"
"EGFR_18_3"  "Amplifies EGFR_Exon_18 from 123 to 197"  "EGFR_Exon_18"
"TGAATTCAAAAAGATCAAAGTG"  "CCCCACCAGACCATGAGA"  "123"  "197"
"EGFR_19_1"  "Amplifies EGFR_Exon_19 from 23 to 115"  "EGFR_Exon_19"
"TCACAATTGCCAGTTAACGTCT"  "GATTTCCTTGTTGGCTTTCG"  "23"  "115"
"EGFR_19_2"  "Amplifies EGFR_Exon_19 from 67 to 183"  "EGFR_Exon_19"
"TCTGGATCCCAGAAGGTGAG"  "GAGAAAAGGTGGGCCTGAG"  "67"  "183"
"EGFR_20_1"  "Amplifies EGFR_Exon_20 from 20 to 108"  "EGFR_Exon_20"
"CCACACTGACGTGCCTCTC"  "GCATGAGCTGCGTGATGAG"  "20"  "108"
"EGFR_20_2"  "Amplifies EGFR_Exon_20 from 102 to 194"  "EGFR_Exon_20"
"GCATCTGCCTCACCTCCAC"  "GCGATCTGCACACACCAG"  "102"  "194"
"EGFR_20_3"  "Amplifies EGFR_Exon_20 from 153 to 244"  "EGFR_Exon_20"
"GGCTGCCTCCTGGACTATGT"  "GATCCTGGCTCCTTATCTCC"  "153"  "244"
"EGFR_21_1"  "Amplifies EGFR_Exon_21 from 23 to 113"  "EGFR_Exon_21"
"TCTTCCCATGATGATCTGTCCC"  "GACATGCTGCGGTGTTTTC"  "23"  "113"
"EGFR_21_2"  "Amplifies EGFR_Exon_21 from 111 to 215"  "EGFR_Exon_21"
"GGCAGCCAGGAACGTACT"  "ATGCTGGCTGACCTAAAGC"  "111"  "215"
"EGFR_22_1"  "Amplifies EGFR_Exon_22 from 21 to 132"  "EGFR_Exon_22"
"CACTGCCTCATCTCTCACCA"  "CCAGCTTGGCCTCAGTACA"  "21"  "132"
HERE_TERMINATOR

# This command seeds the project with a few known variants.

create variant -file - << HERE_TERMINATOR
"Name"  "Annotation"  "Reference"  "Pattern"  "Status"
"15BP_DEL_93-107"  "Pattern entered manually"  "EGFR_Exon_19"  "d(93-107)"
"accepted"
"HAP_97C_126A"  "Created from selections"  "EGFR_Exon_18"  "s(97,C)s(126,A)"
"accepted"
"SUB_A_to_C_97"  "Created from selections"  "EGFR_Exon_18"  "s(97,C)"
"accepted"
"SUB_G_to_A_126"  "Created from selections"  "EGFR_Exon_18"  "s(126,A)"
"accepted"
HERE_TERMINATOR

# This command creates all the sample objects.

create sample -file - << HERE_TERMINATOR
"Name"        "Annotation"
"Sample1"     "Sample1"
"Sample2"     "Sample2"
"Sample3"     "Sample3"
"Sample4"     "Sample4"
"Sample5"     "Sample5"
```

```
"Sample6"     "Sample6"
"Sample7"     "Sample7"
HERE_TERMINATOR

# This command sets up the Sample-Amplicon associations.

assoc -file - << HERE_TERMINATOR
"sample"      "amplicon"      "ofRef"
"Sample1"     "EGFR_20_1"     "EGFR_Exon_20"
"Sample1"     "EGFR_20_2"     "EGFR_Exon_20"
"Sample1"     "EGFR_20_3"     "EGFR_Exon_20"
"Sample2"     "EGFR_18_1"     "EGFR_Exon_18"
"Sample2"     "EGFR_18_2"     "EGFR_Exon_18"
"Sample2"     "EGFR_18_3"     "EGFR_Exon_18"
"Sample3"     "EGFR_18_1"     "EGFR_Exon_18"
"Sample3"     "EGFR_18_2"     "EGFR_Exon_18"
"Sample3"     "EGFR_18_3"     "EGFR_Exon_18"
"Sample4"     "EGFR_19_2"     "EGFR_Exon_19"
"Sample4"     "EGFR_19_1"     "EGFR_Exon_19"
"Sample5"     "EGFR_20_2"     "EGFR_Exon_20"
"Sample5"     "EGFR_20_1"     "EGFR_Exon_20"
"Sample5"     "EGFR_20_3"     "EGFR_Exon_20"
"Sample6"     "EGFR_21_2"     "EGFR_Exon_21"
"Sample6"     "EGFR_21_1"     "EGFR_Exon_21"
"Sample7"     "EGFR_22_1"     "EGFR_Exon_22"
HERE_TERMINATOR

# This load command assumes that the data is sitting in an official analysis
# directory where the data is actually sitting in an sff subdirectory of the
# analysisDir. If you have data sitting in an alternate analysis directory,
# you can specify the analysis path

load   -analysisDir /data/sequencingRuns/EGFR_Run_Dir/EGFR_Analysis_Dir/ \
          -readGroup ReadGrp_1 -regions 1,2,3,4 \
          -symLink false -alias EGFR_reads

# If your read data is in a generic repository, rather than an official
# analysis directory, you can comment out the load above and replace it with
# one like the following where you have edited the sffDir path to point to the
# sff files on your system.

#load –sffDir /data/sffFiles/EGFR_sff_files \
#       –readGroup ReadGrp_1 -filePrefix DGVS90J \
#       –regions 1,2,3,4 –symLink “false”

# If you don’t have access to the specific Read Data for this project
# you have already set up as much of the project as you can and
# you won’t be able to run a computation on it. You should save the
# project setup here and exit. You can open the project in the
# GUI to see how the commands above have been translated into a
# project setup.

# save
# exit

# This command creates the associations between the Read Data and
# the Sample-Amplicon pairs. This command is only valid if you have
# imported the Read Data from an analysis directory using EGFR_reads
# as an alias. If you instead imported the Read Data from a
# repository using actual file names, you would need to change the aliases
# to actual file names (i.e. EGFR_reads01 to DGVS90J01).

assoc -file - << HERE_TERMINATOR
```

```
"readData"         "sample"
"EGFR_reads01"     "Sample1"
"EGFR_reads02"     "Sample2"
"EGFR_reads03"     "Sample6"
"EGFR_reads03"     "Sample7"
"EGFR_reads03"     "Sample3"
"EGFR_reads03"     "Sample5"
"EGFR_reads03"     "Sample4"
HERE_TERMINATOR

# The region 4 Read Data file is actually empty, so we should mark it as
# not active so it gets excluded from the analysis.

update readData EGFR_reads04 -active false

# You should save the project setup now.

save

# Run the validateNames utility to be on the safe side.

utility validateNames

# Check to see if there are any other problems with the project.

utility validateForComputation

# Trigger the start of the computation.

computation start

# Load the automatically detected variants discovered as part of the
# computation.

computation loadDetectedVariants

# Report the measured variant frequencies to a tab-delimited output file.

report variantHits -outputFile EGFR_variant_hits.txt

# Close the project without saving. This will prevent the automatically
# detected variants from being permanently added to the project.
# You will receive a warning about unsaved changes to the project.

close

# Exit the CLI. Your project setup and your computation results should now be
# able to be viewed if you open the project in the GUI.

exit
```

3.6 Creating and Computing an MID Project with the AVA-CLI

The EGFR example Project shown in detail in sections 2 (GUI version) and 3.5 (CLI version) does not display the usage of MIDs and Multiplexers. The example below briefly shows many of the special features of the AVA software that come into play when MIDs are used, and how they would be set up in a Project using the CLI.

A few things to remember are that in a Project that contains multiple Read Data sets, a given Multiplexer can be used for more than one Read Data set, or distinct Read Data sets can each have specific Multiplexer(s). It is also possible to associate more than one Multiplexer with the same Read Data set and to mix regular Samples with Multiplexers on a Read Data set. However, for a given Read Data set, an Amplicon can only be assigned to one entity (a regular Sample or a Multiplexer). Figure 3-1 shows the Read Data Tree and Multiplexers Definition Table for an example Project that is atypically complex for tutorial purposes, as it was intentionally constructed to illustrate a wide variety of Multiplexer features, whereby:

- 4 Multiplexers are used, showing all 4 encoding types (Both, Either, Primer 1 MID, and Primer2 MID)
- "MultiPlexerBoth" and "MultiplexerP1" are associated with Read Data ESS716O01 (as seen in the Tree), and "MultiplexerEither" and "MultiplexerP2" are associated with Read Data set ESS716O02

- the Tree nodes are partially expanded to reveal which Amplicons are being demultiplexed by which Multiplexer:

    - "MultiplexerBoth" is being used to assign a single amplicon "amp1" to 16 Samples
    - "MultiplexerP1" is being used to assign "amp4" to 3 different Samples
    - "MultiplexerEither" is being used to assign two different Amplicons ("amp2" and "amp3") to 4 different Samples
    - "MultiplexerP2" is being used to assign "amp5" to 3 different Samples
    - MIDs are not being used for "amp6" and it is being assigned to Sample "A001" on the basis of its template-specific primers without the benefit of a Multiplexer

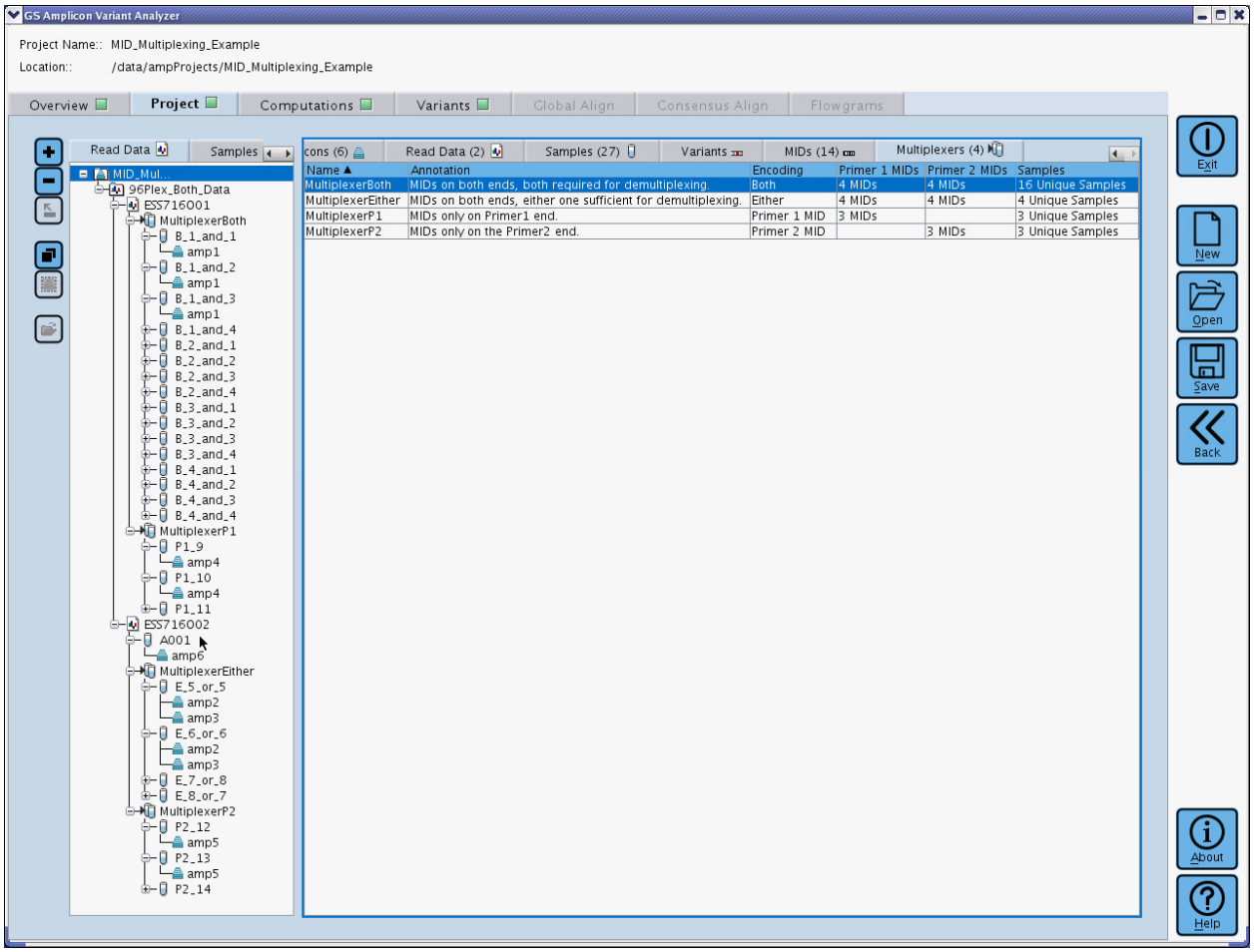


Figure 3-1: The Multiplexer Definition Table and Read Data Tree for the MID example Project described in this section. Note that this Project is atypically complex, as it serves to illustrate a wide variety of MID / Multiplexer features.

The usual CLI commands can be used to set up an MID Project, using the appropriate options. For example, the 'associate' command supports the definition of both MID-based and non-MID-based multiplexing relationships. Read section 3.4.1 (or run 'help associate') for more details on how to create these multiplexing relationships. For more information on creating Multiplexers and their associated constituents using the CLI, run 'help create multiplexer' (section 3.4.4.4), 'help create mid' (section 3.4.4.2), and 'help create midGroup' (section 3.4.4.3).

To display some of these commands in context, an example CLI script is provided below. This script would produce a Project setup that would match the one shown in Figure 3-1. Just as in non-MID project setup CLI scripts (as shown in the example in section 3.5), the standard objects such as References, Amplicons, and Samples must be created, and Read Data must be imported. The script shows these entities being loaded via the '-file' option to keep the script more succinct.

The "here" file contents that are shown are tab-delimited. The double-quoting of the "here" file contents isn't a requirement, but it is used here to help make it clear visually when particular fields have been deleted. These empty fields are generally interpreted as an intent to set an appropriate empty value for a field (such as an empty string for an annotation). However, the

"associate" command is unique in this regard, as empty fields are interpreted as "ignore this field for this line". This allows different types of associations to be specified within a single table (*e.g.* in the script below, the associations between Multiplexers, MIDs, and Samples for all four different types of Multiplexers are shown in the same association "here" file).

In the example script, the associations of the Multiplexers, MIDs, and Samples are split logically from the association of the Multiplexers, Read Data, and Amplicons, but they could have been combined into a single association table. This would have resulted in a larger, more complicated table with more repetition of fields across lines, which would be more difficult to create error-free by manual typing. However, the large association table would be convenient when creating the setup script via programmatic means (such as using Perl scripts to construct the commands by consulting a database or spreadsheet of the experimental design).

This script also illustrates the use of the new "utility execute" command to load the "454Standard" MID Group via an existing default script called "create454StandardMIDs.ava", which is also used as part of the automatic project initialization functionality. Documentation of the "utility execute" command is available in section 3.4.17.5 and more information on automatic project initialization can be found in sections 4.4 and 4.5.

Due to the limitations of this printed document, certain lines of the script below appear on multiple lines. This occurs for certain tab-separated entries in the tables given as arguments to certain commands. Be aware that these should actually be single lines in the script.

3.6.1 Example MID Project Script

```
# Create the project
create project /data/ampProjects/MID_CLI_Example \
    -name MID_CLI_Example \
    -annotation ""

# Load the references from a tab-delimited file
# containing data lines for each reference
# following  the format of the header below
# (which should be included at the top of the file):
#
# "Name"     "Annotation"     "Sequence"
#
# For this example, ref1-ref6 should be defined.

create reference -file referencesFile.txt

# Load the amplicons from a tab-delimited file
# containing data lines for each amplicon
# following  the format of the header below
# (which should be included at the top of the file):
#
#  "Name"        "Annotation"        "Reference"        "Primer1"        "Primer2"
"Start"     "End"
#
# For this example, amp1-amp6 should be defined
# (where amp1 is from ref1, amp2 is from ref2, etc.).

create amplicon -file ampliconsFile.txt

# For this example, the following samples need to be created.
```

```
create sample -file - << HERE_TERMINATOR
"Name"
"A001"
"B_1_and_1"
"B_1_and_2"
"B_1_and_3"
"B_1_and_4"
"B_2_and_1"
"B_2_and_2"
"B_2_and_3"
"B_2_and_4"
"B_3_and_1"
"B_3_and_2"
"B_3_and_3"
"B_3_and_4"
"B_4_and_1"
"B_4_and_2"
"B_4_and_3"
"B_4_and_4"
"E_5_or_5"
"E_6_or_6"
"E_7_or_8"
"E_8_or_7"
"P1_9"
"P1_10"
"P1_11"
"P2_12"
"P2_13"
"P2_14"
HERE_TERMINATOR

# Create a readGroup for the readData.
create readGroup -name "96Plex_Both_Data"


# Load the readData from a tab-delimited file
# containing data lines for each readData set
# following  the format of the header below
# (which should be included at the top of the file):
#
# "SffDir"     "SffName"     "ReadGroup"     "SymLink"     "Name"
#
# For this example, two sff files named
# ESS716O01 and ESS716O02 are being loaded.

load -file readDataFile.txt

# Use the utility execute command to run the
# default script that loads a project with
# the 454Standard group of 14 MIDs.

utility execute %libDir/create454StandardMIDs.ava

# For the sake of demonstrating functionality,
# this example assumes that you want to replace
# Mid9-Mid14 from the 454Standard group with
# your own custom set of 6 new MIDs.

# To simplify project, optionally remove the
# the 454Standard MIDs that are being replaced.
# Note: specifying the OfMidGroup option isn't
# technically necessary as the MID Names are unique
```

```
# in the project.

remove mid -file - << HERE_TERMINATOR
"Name"      "OfMidGroup"
"Mid9"      "454Standard"
"Mid10"     "454Standard"
"Mid11"     "454Standard"
"Mid12"     "454Standard"
"Mid13"     "454Standard"
"Mid14"     "454Standard"
HERE_TERMINATOR

# Create a midGroup for the new MIDs.

create midGroup -name "CustomMids"

# Load the custom MID sequences from a tab-delimited file
# containing data lines for each MID
# following  the format of the header below
# (which should be included at the top of the file):
#
# "Name"     "Annotation"     "Sequence"     "MidGroup"
#
# For this example, 6 MIDs are being defined
# with the names CMid9-CMid14.

create mid -file customMidFile.txt

# Create the four different multiplexers
# being used in the project.

create multiplexer -file - << HERE_TERMINATOR
"Name"     "Annotation"     "Encoding"
"MultiplexerBoth"     ""     "both"
"MultiplexerEither"   ""     "either"
"MultiplexerP1"       ""     "primer1"
"MultiplexerP2"       ""     "primer2"
HERE_TERMINATOR

# Set up the association of MIDs to samples
# in each of the multiplexers.
# Note: specifying the OfPrimer1MidGroup and OfPrimer2MidGroup
# options isn't technically necessary as the MID Names are unique
# in the project.

assoc -file - << HERE_TERMINATOR
"Multiplexer"        "Primer1Mid"        "OfPrimer1MidGroup"        "Primer2Mid"
"OfPrimer2MidGroup"  "Sample"
"MultiplexerBoth"  "Mid1"  "454Standard"  "Mid1"  "454Standard"  "B_1_and_1"
"MultiplexerBoth"  "Mid1"  "454Standard"  "Mid2"  "454Standard"  "B_1_and_2"
"MultiplexerBoth"  "Mid1"  "454Standard"  "Mid3"  "454Standard"  "B_1_and_3"
"MultiplexerBoth"  "Mid1"  "454Standard"  "Mid4"  "454Standard"  "B_1_and_4"
"MultiplexerBoth"  "Mid2"  "454Standard"  "Mid1"  "454Standard"  "B_2_and_1"
"MultiplexerBoth"  "Mid2"  "454Standard"  "Mid2"  "454Standard"  "B_2_and_2"
"MultiplexerBoth"  "Mid2"  "454Standard"  "Mid3"  "454Standard"  "B_2_and_3"
"MultiplexerBoth"  "Mid2"  "454Standard"  "Mid4"  "454Standard"  "B_2_and_4"
"MultiplexerBoth"  "Mid3"  "454Standard"  "Mid1"  "454Standard"  "B_3_and_1"
"MultiplexerBoth"  "Mid3"  "454Standard"  "Mid2"  "454Standard"  "B_3_and_2"
"MultiplexerBoth"  "Mid3"  "454Standard"  "Mid3"  "454Standard"  "B_3_and_3"
"MultiplexerBoth"  "Mid3"  "454Standard"  "Mid4"  "454Standard"  "B_3_and_4"
"MultiplexerBoth"  "Mid4"  "454Standard"  "Mid1"  "454Standard"  "B_4_and_1"
"MultiplexerBoth"  "Mid4"  "454Standard"  "Mid2"  "454Standard"  "B_4_and_2"
"MultiplexerBoth"  "Mid4"  "454Standard"  "Mid3"  "454Standard"  "B_4_and_3"
```

```
"MultiplexerBoth"    "Mid4"  "454Standard"  "Mid4"  "454Standard"  "B_4_and_4"
"MultiplexerEither"  "Mid5"  "454Standard"  "Mid5"  "454Standard"  "E_5_or_5"
"MultiplexerEither"  "Mid6"  "454Standard"  "Mid6"  "454Standard"  "E_6_or_6"
"MultiplexerEither"  "Mid7"  "454Standard"  "Mid8"  "454Standard"  "E_7_or_8"
"MultiplexerEither"  "Mid8"  "454Standard"  "Mid7"  "454Standard"  "E_8_or_7"
"MultiplexerP1"      "CMid9"  "CustomMids"  ""  ""  "P1_9"
"MultiplexerP1"      "CMid10"  "CustomMids"  ""  ""  "P1_10"
"MultiplexerP1"      "CMid11"  "CustomMids"  ""  ""  "P1_11"
"MultiplexerP2"      ""  ""  "CMid12"  "CustomMids"  "P2_12"
"MultiplexerP2"      ""  ""  "CMid13"  "CustomMids"  "P2_13"
"MultiplexerP2"      ""  ""  "CMid14"  "CustomMids"  "P2_14"
HERE_TERMINATOR

# Associate the non-MID sample directly
# with its amplicon and read data.

assoc -readData ESS716O02 -sample "A001" -amplicon "amp6" -ofRef "ref6"

# Associate the multiplexers with
# their read data and amplicons.

assoc -file - << HERE_TERMINATOR
"Multiplexer"        "readData"  "amplicon"  "ofRef"
"MultiplexerBoth"    "ESS716O01"  "amp1"  "ref1"
"MultiplexerP1"      "ESS716O01"  "amp4"  "ref4"
"MultiplexerEither"  "ESS716O02"  "amp2"  "ref2"
"MultiplexerEither"  "ESS716O02"  "amp3"  "ref3"
"MultiplexerP2"      "ESS716O02"  "amp5"  "ref5"
HERE_TERMINATOR

save
```

4. GS AMPLICON VARIANT ANALYZER – SPECIAL TOPICS

4.1 Addressing Simultaneous Multiple Users' Access to an Amplicon Project

Only one instance of the GS Amplicon Variant Analyzer can be "in control" of a given Amplicon Project at any given time, *i.e.* be able to save changes or carry out / stop computations in the Project. This is important because if multiple users or instances of the software had the same project open simultaneously and each were used to edit the project, saving from either instance would overwrite the changes of the other. To help minimize this risk, the AVA software presents a message window at the time a project is opened, if it appears to be in use by another user or another instance of the software (Figure 4-1).

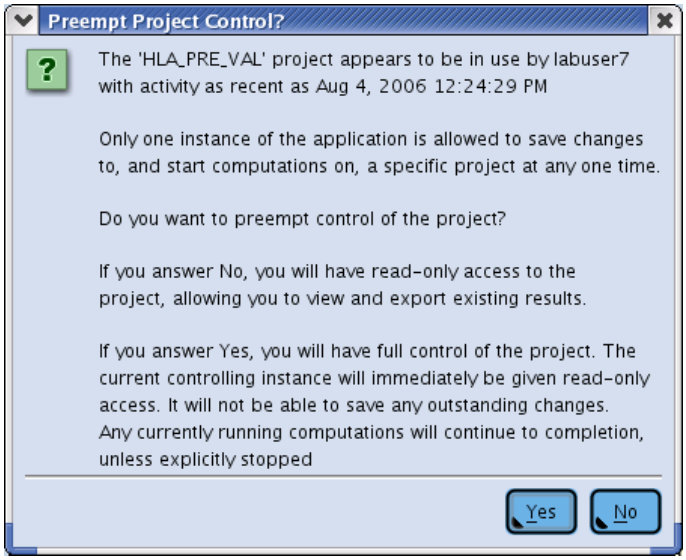


Figure 4-1: Message window alerting you that the Amplicon Project you are trying to open is already open in another instance of the AVA software. This message gives you the choice of opening the Project as Read-Only or to preempt control of the Project from the other instance.

This message provides you with:

- the logon name of the other user
- the approximate time at which that user last interacted with the Project (such as by saving an update or running a computation)

- the choice to load the Project
  - but to operate in a "read-only" mode, or
  - to preempt control of the Project from the other user.

The goal of this message is to inform users of a potential conflict, not to enforce any policy with respect to these conflicts. Thus, although it is possible to preempt control from the other AVA instance, it would likely be better to first contact the indicated user to see if they are still working with the Project, or if they might have simply neglected to shutdown the application. Another possibility is that the other instance may have been terminated with a control-C or was otherwise unable to carry out a clean shutdown. In such a case it would merely appear that

another instance is actively using the Project, when none actually is. If the other user cannot readily be contacted, the last activity indicator of the message may help you determine if it is safe to preempt control of the Project without unduly affecting the other user.

If you open the Project in Read-Only mode, you will be able to make changes internally to the application (define new Reference Sequences, *etc*.) and use all the features of the Variants, Global Align, Consensus Align, and Flowgrams Tabs, including the export of pre-existing results (.png snapshots, FASTA, Clustal, .ace or table files). However, you will not be able to save any changes to disk, or to use them in new calculations (which also involves writing the results to disk): the Save button will be grayed-out, which constitutes the only visual clue to the Read-Only status of the Project. Features like defining new Variants from selections on the Global and Consensus Align Tabs, though available, would be of little use since you would not be able to save the newly defined Variants to the disk or obtain frequency calculations for them in the Variants Tab. On the other hand, you would be able to observe (but not stop) a new computation of the Project if carried out by the currently controlling instance. If you have made changes to the Project and another user preempts control, the Save button may temporarily remain active (not grayed-out). If you then click on the Save button, you will be alerted to the transition to Read-Only mode, but you will not be able to save your changes. Clicking on either the Save, the Start computation or the Stop computation button, when in Read-Only mode, produces the following warning messages (Figure 4-2).

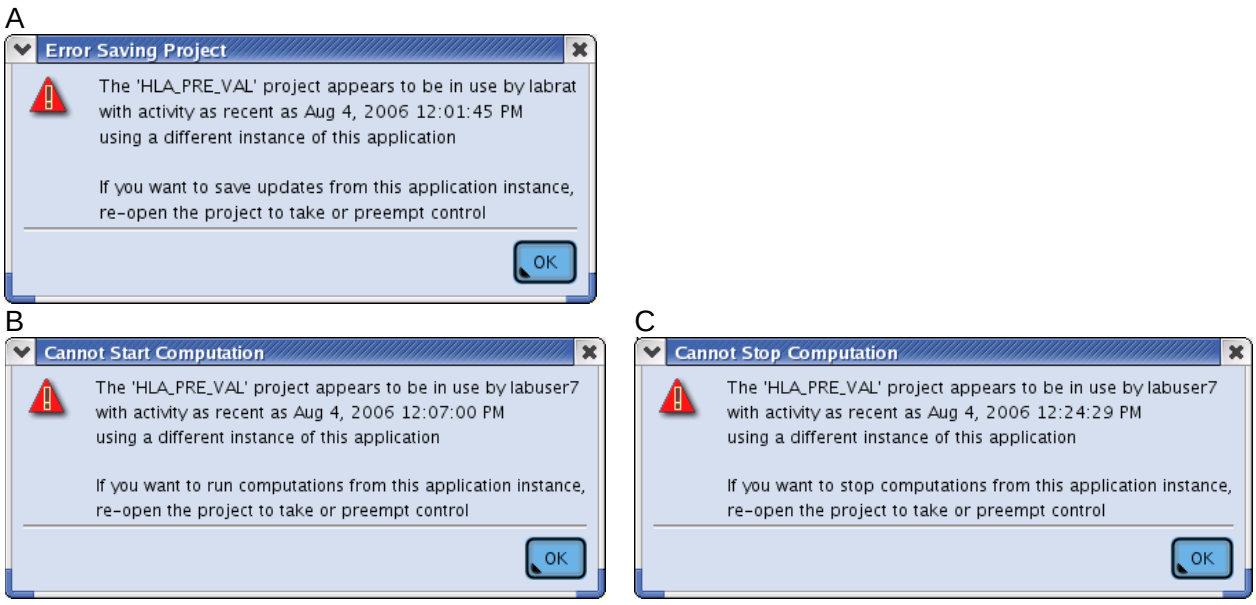


Figure 4-2: Messages indicating that you do not have "control" of the Amplicon Project (*i.e.* you are operating in Read-Only mode) and that you cannot (A) Save the changes to a Project, or (B) Start or (C) Stop computations in the Project.

If you preempt control of the Project from another user, either at this point or when you first opened the Project (see Figure 4-1), the other user will be automatically and *transparently* transitioned to Read-Only mode. Not only will any of their unsaved changes become irremediably unsaveable (even trying to preempt control back from you would involve exiting the Project and thus the loss of unsaved changes), but no message will be sent to inform the other user that this transition to Read-Only occurred. The only visual clue to this state is that the Save button will remain grayed-out even after changes are made. More obvious functional clues are

the reception of one of the messages of Figure 4-2, if the other ("preempted") user attempts to save Project changes or to start new computations (or stop your computation). Re-opening the Project would elicit the message of Figure 4-1, which identifies the person who currently has control over the Project.

In a related feature, if you attempt to open an Amplicon Project in a file-system on which you do not have writing permissions, the message shown in Figure 4-3 will be displayed, alerting you that the Project will open as Read-Only (assuming that you can actually read the files in that area of the file-system). Although the message specifies only the "Save" restriction, the computation restrictions apply as well.

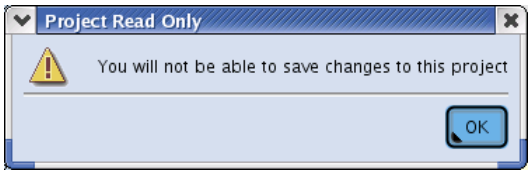


Figure 4-3: Alert message indication that the Amplicon Project you are trying to open will open in Read-Only mode

4.2 Intelligent Variant Naming

As part of its computation, the AVA software identifies certain differences between the Consensi or Reads corresponding to each Amplicon and their cognate Reference Sequences, and proposes them as possible Variants (see section 1.4). Depending on the size and complexity of the Project (and on whether or not the sequences you are working with are hypervariable), the Project could end up containing hundreds of thousands of potential Variants. Because the number of Variants can be so high, the AVA software features an automatic "intelligent" process for assigning meaningful names to the Variants, with the goal of generating Variant names that are unique for a given Reference Sequence, suitable for sensible sorting, and as informative as possible without being so long as to become unwieldy (above 25 characters).

The 4-tier naming convention described below is applied to the Auto-Detected Variants as well as to the Variants proposed manually by users via the "Declare project variant from current selections" functionality on the Global Align and Consensus Align tabs (see sections 1.6.3.3 and 1.7.3).

4.2.1 Tier 1 Naming

Tier 1 names are the preferred, and come in two forms. The first form has the prototype "Position:From-Sequence/To-Sequence" and the second has the prototype "PositionA-PositionB:From-Sequence/To-Sequence". Complicated Variants may be described using multiple names of either form, separated by commas. This is the most precise naming scheme as it explicitly specifies each base requirement that defines the Variant. Also, starting the name with the base position (relative to the Reference Sequence), is convenient for sorting a Table of Variants.

Table 4-1, below, shows some example Tier 1 names and what they mean.

Tier 1 Variant Name	Interpretation of the Variant Name
10:A/C	position 10 has a change from an A to a C
10-12:ACT/ATG	position 10 must match the Reference Sequence (and remain an A), while positions 11 and 12 change from a CT to a TG
10-12:ACT/ANG	position 10 must remain an A, position 11 can be any base, and position 12 has a change from a T to a G
10-12:ACT/---	the bases ACT at positions 10-12 are deleted
10.5:-/A	an A is inserted between positions 10 and 11
10-11:A-T/ACT	a C is inserted between A and T, which must be maintained, at positions 10 and 11
10:A/C,45:T/G	haplotype change including an A changed to a C at position 10 and a T changed to a G at position 45

Table 4-1: Examples of Tier 1 "intelligent" Variant names

4.2.2 Tier 2 Naming

If the attempt to explicitly specify out all the base changes in a Tier 1 name results in an identifier that is longer than 25 characters, Tier 2 naming takes over. Tier 2 names also come in two forms. The first form has the prototype "Position:Modifier[(count/value)]" and the second has the prototype "PositionA-PositionB:Modifier(count/value)". The modifiers are REF (must match the Reference Sequence), DEL (Deletion), INS (Insertion), and SUB (Substitution). For the first prototype, the "count/value" is optional for single base matches (REF) or single base deletions (DEL). This naming scheme is often more compact than Tier 1 names, especially when stretches of bases can be collapsed into a base count (second prototype), and it maintains the sorting advantage of starting names with the base position on the Reference Sequence. However, exact base changes are not always stated explicitly.

Table 4-2 shows examples that demonstrate the basics of Tier 2 naming.

Tier 2 Variant Name	Interpretation of the Variant Name
10:REF	base at position 10 must match the reference sequence
10-49:REF(40)	the 40 bases from 10-49 must match the reference sequence
10:DEL	base at position 10 is deleted
10-49:DEL(40)	the 40 bases from 10-49 are deleted
10.5:INS(ACG)	the bases ACG are inserted between positions 10 and 11
10:SUB(G)	base at position 10 has been changed to a G
10:SUB(C),45:SUB(G)	haplotype change at positions 10 (changed to a C) and 45 (changed to a G)

Table 4-2: Examples of Tier 2 "intelligent" Variant names. Note that some of these (like "10:REF") would not be used because their Tier 1 equivalent would be preferred. These are shown for illustrative purposes.

4.2.3 Tier 3 Naming

If the first and second tier attempts both produce names longer than 25 characters, the naming scheme resorts to Tier 3 naming, using the literal "pattern" used when defining a Variant manually (*i.e.* using the Variant Definition Syntax described in section 1.3.2.5.2). These are patterns such as d(10-50), s(10,C), or m(10-50)s(51,C)m(52-80). The Variant Definition Syntax can be more compact than the Tier 2 naming scheme because it uses single letter abbreviations

for the change types (m, d, i, s), as opposed to the 3-letter abbreviations seen above (REF, DEL, INS, SUB). Also, Tier 3 naming does not spell out the lengths of matches and deletions, and it concatenates haplotype codes without any separating characters like the comma used in Tier 2. However, these names are less convenient to sort through because they start with an abbreviated change type rather than a Reference position.

4.2.4 Tier 4 Naming

If a Variant can not be assigned a name that is 25 characters or less using any of the first three tier naming schemes, the software resorts to a generic but unique name following the prototype "Var_number". These are the same types of default Variant names used for Variants that are created from scratch in the Variant Definition Table of the Variants sub-tab of the Project Tab (see section 1.3.2.5.2): when a new Variant is created in that table using the "Add" function, it initially has no Reference and no pattern assigned to it, so there is no useful information with which to construct a meaningful default name. The generic name is constructed by obtaining a unique number from a counter and appending it to the prefix "Var_".

4.2.5 Naming Example

Table 4-3 shows how 4 different but related Variant Patterns end up being named by the naming scheme, showing an example of each Tier.

Final Naming Tier	Variant Pattern	Final Name
Tier 1	d(327)m(339-342)	327:A/-,339-342:AAGC/AAGC
Tier 2	d(327)m(339-343)	327:DEL,339-343:REF(5)
Tier 3	d(327-328)m(339-343)	d(327-328)m(339-343)
Tier 4	d(327-328)m(339-343)m(347)	Var_16

Table 4-3: Example final Variant names that could be used, for each of the 4 tiers schemes, using a set of Variant patterns of increasing complexity. See text below for more details.

The Tier 1 example shows that the Variant pattern can be expressed as a name exactly 25 characters in length. Since this meets the length constraint, the Tier 1 name is used as this Variant's final name.

In the Tier 2 example, the Variant pattern from the Tier 1 example has been altered to extend the match range by an extra base. If this pattern were converted into a Tier 1 name, it would read: "327:A/-,339-343:AAGCA/AAGCA" (assuming base 343 of the Reference Sequence were an A). This name exceeds the 25 character limit by two characters, so the software rejects it and constructs a Tier 2 name. The Tier 2 final name, "327:DEL,339-343:REF(5)", has 22 characters, so it is adopted as the final name for this Variant.

The Tier 3 example Variant pattern is the same as the Tier 2 pattern except that it has an extra base in its deletion. If this pattern were expressed as a Tier 2 name, it would read: "327-328:DEL,339-343:REF(5)". This name has 26 characters so the software rejects it and constructs a Tier 3 name, using the Variant Definition Syntax: "d(327-328)m(339-343)". Since the Tier 3 name is only 20 characters it is adopted as the final name for the Variant.

In the Tier 4 example, the Variant pattern from the Tier 3 example is altered by the addition of an extra match constraint. Since Tier 3 names are the same as the Variant Pattern, and the pattern here already exceeds 25 characters (see Table 4-3), the software resorts to the final tier, and the generic "Var_16" is used as the final name.

4.3 Properties Windows for Global and Consensus Alignments

As described in sections 1.6.3.2 and 1.7.3, above, right-clicking on a nucleotide in the alignment on the Global Align or the Consensus Align tab opens a context-sensitive menu that includes a "properties" option. Selecting this option opens a "properties" window containing sequence-specific information for the Consensus or read on which you right-clicked. The specific information that is provided in this window depends on whether the sequence is a Consensus, a forward read or a reverse read.

4.3.1 When is the Properties Information Useful?

The multi-alignment of the Global and Consensus Align tabs display only the non-Primer portions of the aligned reads. In certain situations, however, it may be useful to be able to examine the sequences flanking the aligned sequences (or indeed, the whole original sequencing read). For example, if a read appears to be aligned so poorly that you think it might be a contaminant, it might be useful to examine the part of the read that was considered as a Primer match by the alignment software, to determine if some mispriming might have occurred. Conversely, if a read appears to terminate too early in the alignment, one might want to check if the sequence is really that short, or if it was truncated by some unforeseen problem with the aligner, or if it was due to sequence quality issues. Or, if a read exhibits a large deletion at the edge of an alignment, it would be useful to see the sequence from the read beyond the edge of the alignment, to determine if there is a significant match that supports the deletion or if the aligner arbitrarily placed some trailing bases far from the adjacent bases of the read when they actually could have been aligned closer.

The "properties" window of a Consensus or a read provides the necessary information for such verifications, *e.g.* the full original sequence read, as well as the aligned and flanking sub-regions of the read (see details below, section 4.3.2). These are all provided in FASTA format, which can be copied to the clipboard and used in external search or analysis programs. In particular, one could BLAST a sequence to determine its identity if it is either aligned so poorly that it looks like a contaminant; or if it has such specific variation compared to the Reference Sequence that it looks like it might be a homolog or a paralog of the intended Amplicon rather than a regular Variant of the Amplicon. One can also compare a sequence to dbSNP to see if a particular Variant has already been identified in the literature.

4.3.2 Content of the Three "Properties" Window Types

The "properties" windows for each of the sequence types (Consensus, forward Read, and reverse Read) each have their own specific content, all displayed is one or more FASTA sequences.

4.3.2.1 Properties Window for a Consensus

The Consensus properties window (Figure 4-4) simply displays a FASTA version of the Consensus sequence displayed in the alignment, minus any gaps. Since the Consensus is formed from the alignment of many trimmed reads, there is no flanking sequence to report. The definition line of the sequence is annotated to provide the number and orientation of the reads that went into constructing the Consensus, and the length of the sequence.

Although the lack of flanking information precludes this sequence from being used to troubleshoot certain issues, it is perfectly suited for use in a dbSNP search. If you choose one of the Consensi with the most member reads in your alignment (which is likely to be less noisy than a Consensus with fewer members), you can copy it to the clipboard and use it in a dbSNP search to see if your Variant is novel or not.

The Consensus properties window is only accessible on the Global Align tab when “Read Type” is set to “Consensus” (see section 1.6.3.2).

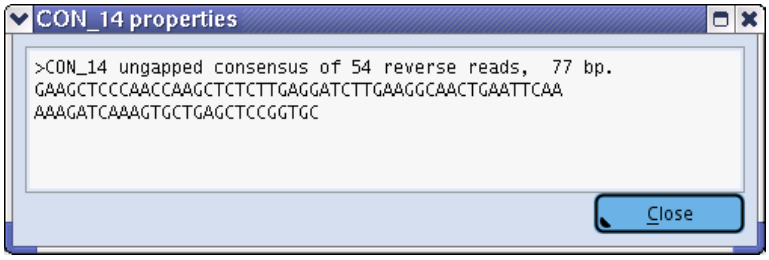


Figure 4-4: The Consensus properties window with the FASTA sequence of a Consensus and its annotated definition line

4.3.2.2 Properties Window for a Forward Read

The properties window of a forward read (Figure 4-5) contains up to 4 FASTA sequences. The window first displays a block of sequences based on the alignment data: The aligned portion of the Read, the unused 5’-flanking sequence, and the unused 3’-flanking sequence are provided as three separate FASTA sequences. Following this, the FASTA sequence of the entire read is shown, as obtained from the Read Data sff file. Note that the sequences can have mixed case characters: the lower case characters are used to represent the sequencing key and low quality read regions.

The flanking sequence information along with the knowledge of where the sequence quality might be trailing off can be used to troubleshoot alignment issues. The sequences are also available for copying so they can be used as queries to search external databases.

The properties window for individual reads is accessible from the Consensus Align tab (section 1.7.3), as well as from the Global Align tab when “Read Type” is set to “Individual” (see section 1.6.3.2).

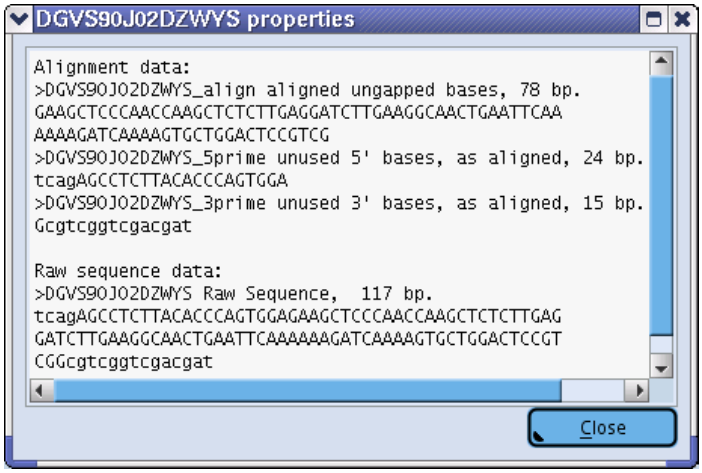


Figure 4-5: The forward read properties window with FASTA sequences showing the aligned portion of the read, the unused flanking sequences, and the full raw sequence from the Read Data file. Low quality stretches of bases and the sequencing key are denoted in lowercase letters in the sequences.

4.3.2.3 Properties Window for a Reverse Read

The properties window for a reverse read (Figure 4-6) has the same types of information and utility outlined for the forward read properties window, above (section 4.3.2.2) except that, as a convenience, the alignment data is presented in two blocks: in the first block, the sequences are presented as the reverse complements of the actual read data (thus the "_rc_" portion of the FASTA identifiers), so they can be easily related to the sequences you will see in the alignments, on the Global Align or Consensus Align tabs. The second alignment data block shows the same data (the aligned read and the flanking unused sequences) in the orientation of the read as it was sequenced (in the same orientation as the raw sequence from the Read Data file).

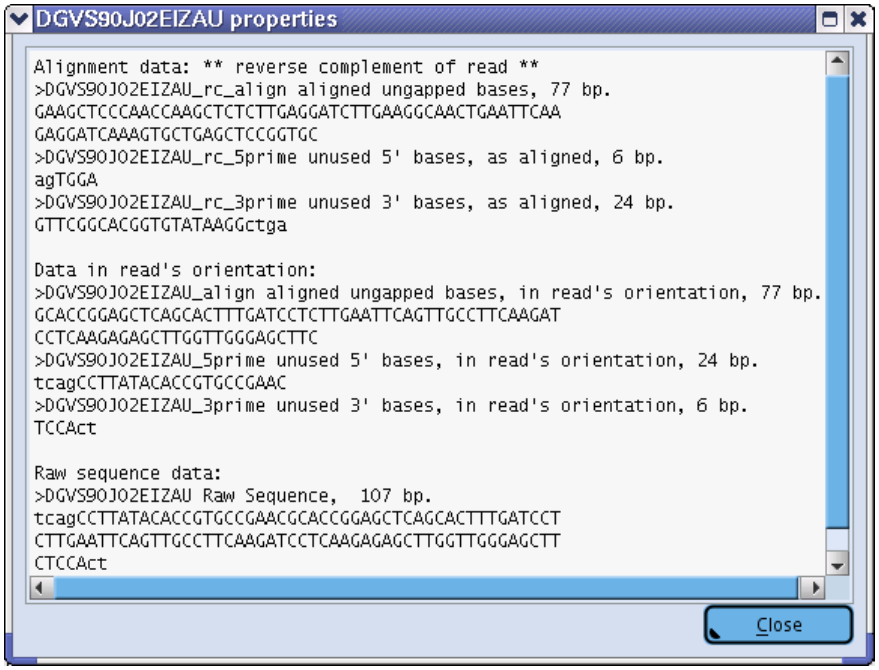


Figure 4-6: The reverse read properties window with FASTA sequences showing the aligned portion of the read, the unused flanking sequences, and the full raw sequence from the Read Data file. The alignment data is presented in two blocks, the first is reverse-complemented and the second is in the actual orientation of the read as it was sequenced. Low quality stretches of bases and the sequencing key are denoted in lowercase letters in the sequences.

4.4 Automatic Project Initialization in the GUI

When the “New” button in the AVA GUI is used to create a Project, an initialization script containing CLI-script commands is automatically carried out. This script is created as part of the software installation and is only automatically used when creating a new Project in the GUI; it is not used when opening pre-existing Projects and it is not automatically used when creating Projects via the CLI (section 4.5).

The default initialization script that is provided with the AVA software serves two main purposes: it automatically loads the 454Standard MID Group, and it provides the ability to automatically call through to optional customized initialization scripts in the users’ home directories. The functionality of the initialization script is optional, and can be disabled by an administrator by commenting out actions in the script (by placing a pound character (‘#’) in front of commands). However, the script should not be deleted entirely, or a ‘missing’ file warning will be encountered each time a new Project is created via the GUI.

4.4.1 Default Initialization Script Location

The default initialization script is located relative to the main software installation. If the software was installed to the standard location (‘/opt/454’), then the initialization script will be found as the following: /opt/454/apps/amplicons/config/lib/newProjectInit.ava. More generally, the file will be located at *installDir*/apps/amplicons/config/lib/newProjectInit.ava (where installDir is replaced by the main software installation path).

4.4.2 Default Initialization Script Contents

The contents of the default initialization script are provided below.

```
# GS Amplicon Variant Analyzer CLI script used to populate new projects
#
# This script adds the "Standard" 454 MIDs to the project and
# then runs the user-specific initialization script found in the user's
# home directory, if any.
#
# Sites may want to edit this file to further customize the initialization
# of new AVA projects.  To completely disable the actions of this file,
# comment out, or delete, all its lines:  do not delete the file itself,
# however, or else a warning message about a missing file will be displayed
# each time a new project is created from the gsAmplicon application GUI.



# Step 1: Populate with the "Standard" 454 MIDs
#         (To prevent this, comment out or delete the following line:)
utility execute create454StandardMIDs.ava

# Step 2: Run whatever "new project" scripts are in the
#         user's home directory.
#         (Note, due to the use of -onMissingScript with the value 'ignore',
#          it is not an error if the user has no such script)
utility execute -onMissingScript ignore %homeDir/.gsAmplicon_newProjectInit.ava
```

The script mainly contains comments (lines beginning with '#') and blank lines and only contains 2 actual commands. Both of the commands are 'utility execute' commands which run other scripts.

4.4.2.1 Step 1: Loading the "Standard" 454 MIDs

Fourteen MIDs were carefully selected four use with Amplicon libraries, in the 454 Sequencing System (section 1.3.2.6). As part of the default initialization process, these MIDs are automatically loaded into the Project as a convenience and as a means of reducing data entry error. The command '`utility execute create454StandardMIDs.ava`' runs a default script called '`create454StandardMIDs.ava`' that is located in the same directory as the default initialization script ('*installDir*/apps/amplicons/config/lib'). The script creates Amplicon MIDs Mid1-Mid14 in the Project.

4.4.2.2 Step 2: Running User-Customized Initialization Functions

Since the default initialization script is part of the main software installation, the average user probably will not have permissions to edit the script. To enable users to customize the automated project initialization, the default script calls through to a script in the user's home directory called '.gsAmplicon_newProjectInit.ava' by using the command '`utility execute -onMissingScript ignore %homeDir/.gsAmplicon_newProjectInit.ava`'. Thus, users can create a script called '.gsAmplicon_newProjectInit.ava' in their home directory, and fill it with CLI-commands to add extra functions to the initialization process. (Note that the file name

intentionally begins with a 'dot', which makes the file invisible to standard listings of the user's home directory.) The customized user script is entirely optional, however; no errors or warnings will be issued if the user does not create one.

The name of the user-customized script is dictated by the command issued in the default initialization script. If the administrator changes the name of the called script by editing the default initialization script *e.g.* so that the Step 2 command is '`utility execute -onMissingScript ignore %homeDir/.gsAmplicon_user_customization.ava`', then the corresponding user-customized script would have to be named '`.gsAmplicon_user_customization.ava`'.

4.4.3 Initialization Script Restrictions

Although the default initialization script can be edited and custom scripts can be nested within it (as illustrated by the call to the user's home directory script), the default initialization script and any of the subordinate scripts are precluded from utilizing certain specific CLI commands. The excluded commands are:

- open
- close
- exit
- create project
- any of the computation commands, such as computation start and computation stop

The reason for these exclusions is that automatic initialization is intended to work in the context of creating a new Project and leaving it in a state where more CLI commands can be issued. The banned commands would not make any sense in this context because they cause a switch to other Projects, shut down the Project, or attempt to prematurely compute the Project.

4.4.4 Initialization Script Error Handling

The automatic initialization script and any other scripts called within it are controlled by an error handler that reports problems encountered when running the scripts. Those errors, however, do not prevent the successful creation of a Project via the 'New' button in the GUI. So, errors might cause portions of an initialization to fail, but the new Project will be accessible. This is intended to prevent mistakes in editing of the default initialization file from locking users out of creating new Projects via the GUI. As mentioned earlier, a missing default initialization script will cause a warning to be issued, but a missing user-customized initialization file will be ignored.

4.5 Project Initialization and the CLI

When using the CLI, the 'create project' command is used to set up new Projects. This process is entirely under user control, and there is no attempt to automatically initialize the Project as is done in the GUI when using the 'New' button (section 4.4). Avoiding automatic initialization in the CLI maintains backward compatibility with pre-existing CLI scripts created for use with prior software versions.

However, this doesn't prevent a user from taking advantage of the default initialization script if desired. The script can be incorporated into CLI Project setup by using the 'utility execute' command. To take advantage of the default initialization script, use the command:

```
utility execute %libDir/newProjectInit.ava
```

If the only functionality that the user wants to borrow from the initialization is loading the '454Standard' MIDs (perhaps to add MIDs to a pre-existing Project), the user can directly call the script that the initialization script uses. To load the fourteen '454Standard' Amplicon MIDs to a Project, as shown in the example CLI script in section. 3.6, use the command:

```
utility execute %libDir/create454StandardMIDs.ava
```

4.6 Multiplex Amplicon Libraries

Multiplex Amplicon libraries are prepared the same way as standard Amplicon libraries with the exception that short Multiplex Identifier sequences, the MIDs, are added to the design of the Adaptors. Since the AVA software expects these sequences at the very beginning of the reads, the MID sequences must be positioned at the end of the Primer A and/or Primer B segments of the Adaptors, just past the sequencing key, and before the template-specific primers (see Figure 4-7).

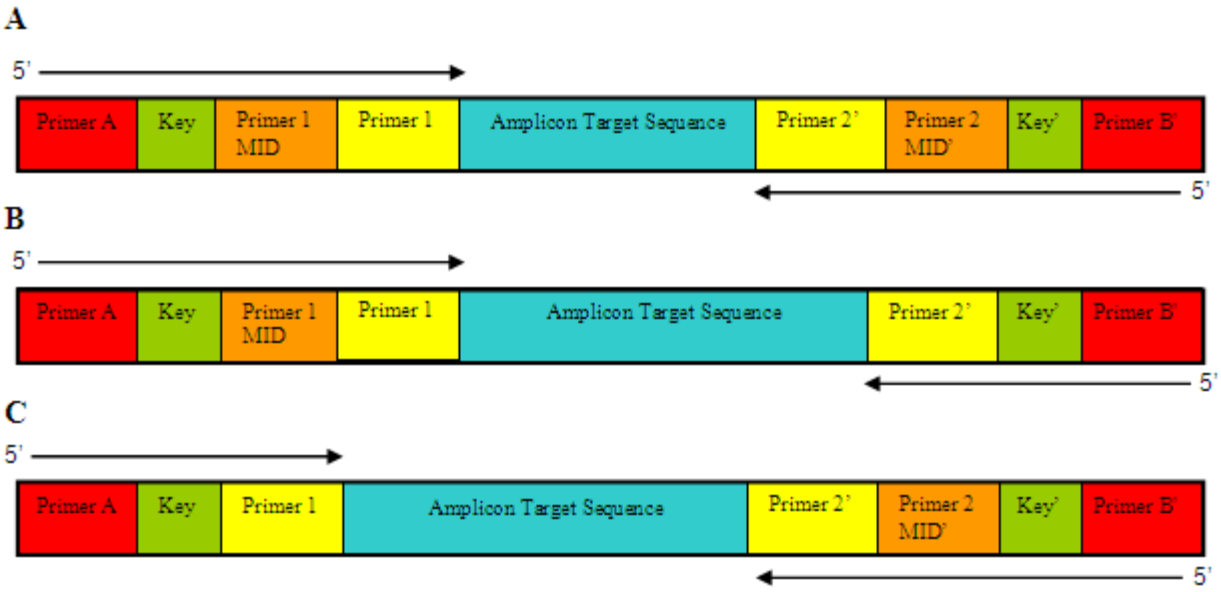


Figure 4-7: Diagrams of potential Amplicon structures using MIDs

(A) MIDs are inserted between the sequencing key and the sequence-specific primers (Primer 1 and Primer 2) on both ends of the Amplicon. A Multiplexer object describing Amplicons with this structure could have an encoding type of "both" (if unambiguous Sample assignment requires both MIDs be found), or "either" (if unambiguous Sample assignment can be made with the MID from either end independently).

(B) An MID is inserted between the sequencing key and the Primer 1 sequence-specific primer only, with no MID used with Primer 2. A Multiplexer object describing Amplicons with this structure must have an encoding type of "Primer 1 MID".

(C) An MID is inserted between the sequencing key and the Primer 2 sequence-specific primer only, with no MID used with Primer 1. A Multiplexer object describing Amplicons with this structure must have an encoding type of "Primer 2 MID".

An important difference between the way MIDs are used in Amplicon libraries, compared to Shotgun (sstDNA) libraries, is that Amplicon reads can carry an MID tag at each end, *i.e.* as part of both Adaptor A and Adaptor B. By contrast, "MID Adaptors" used to prepare Shotgun (sstDNA) libraries, such as the ones provided in the MID Adaptors Kits, carry an MID sequence only on Adaptor A, where the Sequencing Primer of the emPCR Amplification Kit II binds. (Note that the MID kits are not used to prepare Amplicon libraries, since the Adaptors for Amplicon libraries contain template-specific information and must be designed and obtained separately by the user.) The presence of MIDs at both ends of the reads in Amplicon libraries allows their use in a manner analogous to the use of the defined Primer 1 and Primer 2 in the standard (non-MID) AVA demultiplexing scheme; since Amplicons have fully defined sequences (unlike Shotgun library reads), the software knows from the experimental design exactly where the distal MID tag should be, and can thus look for it.

In addition, the possibility to use MIDs at both ends on Amplicons allows for combinatorial demultiplexing, which greatly increases the number of libraries that can be multiplexed with a given set of MIDs. For example, the 454Standard MID Group, which comprises 14 MIDs (see section 1.3.2.6), allows the multiplexing of up to 196 (14 x 14) separate Samples in a single PTP region (Read Data Set), when MIDs are placed at both ends.

However, as different experiments may require different amounts of multiplexing and read length considerations may make it impossible to exploit a demultiplexing scheme in which MIDs

are at both ends of the read, the software allows for a number of flexible “encoding” schemes: the user can choose to tag Amplicon libraries at only one end of the reads like in Shotgun libraries (which is simpler), at both ends (to take advantage of combinatorial demultiplexing or to guarantee the ability to demultiplex forward and reverse reads from amplicons that are too large to read all the way through), or at neither end, (*i.e.* not use MIDs at all and rely on the template-specific Primer sequences to carry out the demultiplexing); encoding schemes are described in more detail in section 1.3.2.7.1.

5. GLOSSARY

A

Amplicon Library - the output of the GS Junior or Genome Sequencer FLX Instrument. This output provides the input data to the GS Amplicon Variant Analyzer, which identifies both known and novel DNA variants.

AVA - Acronym for Amplicon Variant Analyzer application

B

Command Line Interface (CLI) - a means of running the software from the system command prompt.

E

Encoding types - (See Multiplexer)

F

Filters - When viewing Variant Frequency, filters allow you to focus the Variant Frequency Table display to specified minimum and maximum values.

M

MID - a unique identifier that is attached a DNA library to identify the library to which an individual read belongs. Allows multiple libraries tagged with different MIDs to be sequenced together, within an individual PTP Device..

Multiplexer - specifies the association between MIDs and Samples, i.e. how the MIDs should be used to assign reads to Samples. Depending on the design of the Amplicon libraries, Multiplexers allow four types of encoding: Primer 1 MID, Primer 2 MID, Both, Either.

P

Primer sequences - when defining Amplicons, you can define primers using a series of nucleotide characters (A, T, G, C, or N).

Project - the main container for an Amplicon Sequencing experiment. In it, you specify the Reference Sequence (s) to which the sequencing reads will be compared, in search for Variants - the Amplicon(s) that constitute the library(ies) you sequenced [and hence, the reads in the Read Data Set(s)] - the Variant(s) that you specifically want the software to search and report on - and the Sample(s) that constitute the organizational basis for the analysis.

R

Raw image - the data captured during a sequencing Run from the Genome Sequencer FLX or GS Junior Instrument fiber optic bundle camera. Consists of images of the PTP Device taken during each nucleotide flow, capturing the light released by the sequencing reaction in each well of the PTP Device.

Read Data Set - a group of sequencing reads derived from an Amplicon library. In a Project, Read Data Sets exist within a Read Group to help organize the data and are associated with pairings of Amplicons and Samples.

Read Length - the length of the sequence (number of bases) used for analysis.

Reference Sequence - the DNA sequence against which the sequencing reads are aligned by the alignment software. Reference sequences may only contain nucleotide characters (A, T, G, C or N) where N is ???. The software processes shorter Reference Sequences more quickly, however, users may create longer Reference Sequences by concatenating short sequences with “N” characters inserted between the Reference Sequence(s).

S

Sample - a virtual container that groups reads for analysis and reporting. A sample provides the inputs to the analysis and reporting software. You can define any number of Samples in a Project, each associated with one or more Read Data Sets and with one or more Amplicons.

Sample-Amplicon pair

T

Target - the part of the amplicon to be aligned to the Reference Sequence during processing. Primers are not part of the target, and must be trimmed before processing.

U

Ultra-deep sequencing: Sequencing the same target of DNA, using amplicons, many times to find rare mutations.

V

Variant - a variation in nucleotides relative to the Reference Sequence. The software identifies four kinds of variants: substitutions, deletions, insertions and required matches, and a defined variant that may include any of these types in any combination. You can define multiple Variants in a Project, each associated with a specific Reference Sequence. Any number of Variants may be associated with a given Reference Sequence.

Variant frequency - the frequency of Variants as reported by the xxx phase.
Variant Pattern - the constraints that define a Variant. The AVA software uses four types of constraints to define Variants: “Must match”, “Substitute base”, “Insert bases,” and “Delete bases.”

6. INDEX

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