SEQUENCER™

Tutorial for Windows and Macintosh

SNP Hunting

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SNP Hunting

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Sequencher's Variance Table gives you a summary analysis of your data that focuses on differences. The differences between two similar sequences may represent SNPs, polymorphisms, mutations, or just bases that require editing to be resolved. A Variance Table can compare two selected sequences or summarize all of the differences between each consensus sequence in your project and a common Reference Sequence.

You can use the Variance Table to validate your data. Each cell in the Variance Table is linked to the data used to generate the base call. The sorting tools in the Variance Table make it easy to find novel SNPs or to identify regions prone to base calling errors. You can create and export a variety of reports based on the Variance Table.

Getting Started

In this tutorial, you will use the Variance Table to identify and report on candidate SNPs. You will first need to open a project and trim the data within it.

- Launch Sequencher.
- Go to the **File** menu and select **Import > Sequencher Project…**
- Navigate to the Sample Data folder inside the Sequencher application folder.
- Select the **SNP Hunting** project and select **Open**.

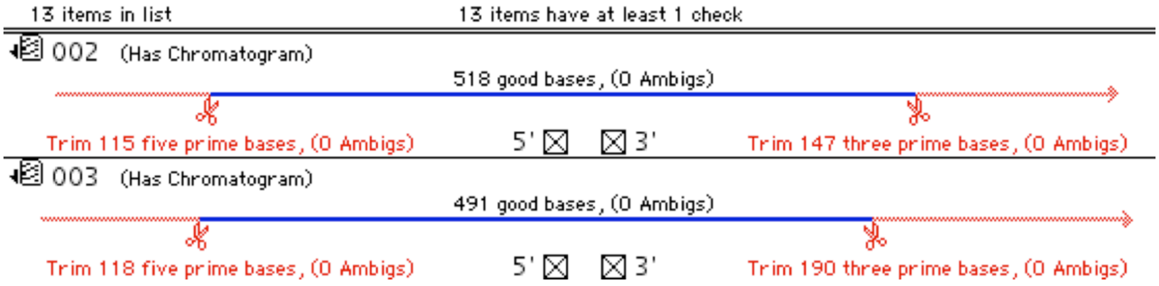
The SNP project contains 14 sequences. In addition to a Reference Sequence, the sequences include auto-sequencing data for one exon from different individuals.

Trimming Low Quality Data

The first step in analyzing data generated by an automated sequencer is trimming the data. Files from automated sequencers frequently have low quality data at the ends of the sequence. Low quality data may include miscalled bases. This can affect the outcome of the proposed assembly and so should be removed.

- On import, all of your sequences should be selected. If they are not, choose **Select** > **Select All**.
- Use **Apple + Click** on a Mac or **Ctrl + Click** on a PC to deselect the Reference Sequence.
- From the **Sequence** menu, select **Trim Ends…**

Sequencher will open the **Ends Trimming** window and display a graphic representation of the proposed trim for each of the sequences. You will see that each line has a red region at either end. The proposed trim site is indicated by a scissors icon.



13 items in list 13 items have at least 1 check

002 (Has Chromatogram)

518 good bases, (0 Ambigs)

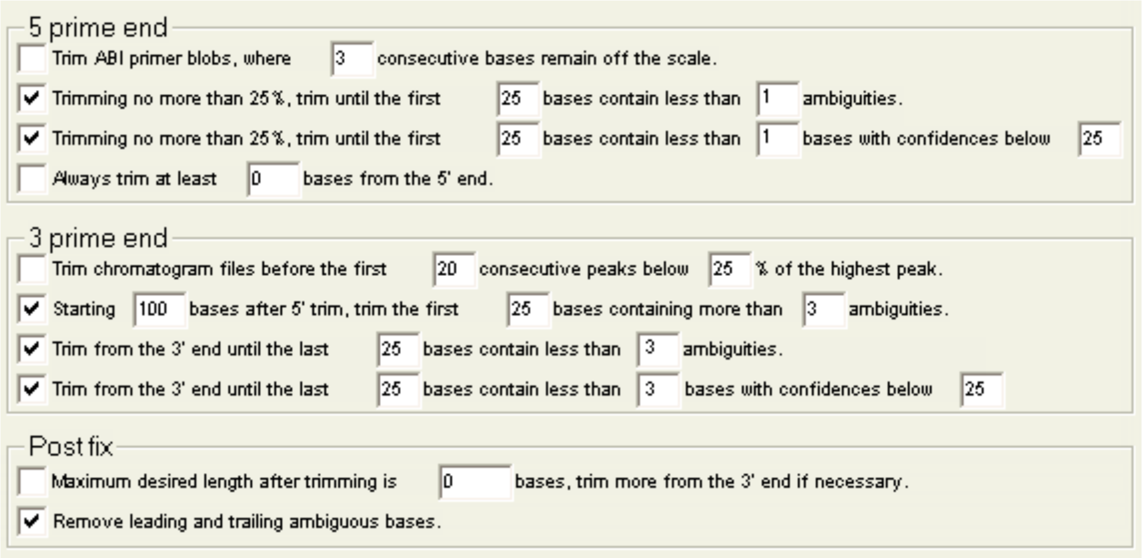
Trim 115 five prime bases, (0 Ambigs) 5' ☒ ☒ 3' Trim 147 three prime bases, (0 Ambigs)

003 (Has Chromatogram)

491 good bases, (0 Ambigs)

Trim 118 five prime bases, (0 Ambigs) 5' ☒ ☒ 3' Trim 190 three prime bases, (0 Ambigs)

- Go to the **Ends Trimming** button bar.
- Click on the **Change Trim Criteria** button.
- The **Ends Trimming Criteria** window will appear. Change the settings to match the following three selections:



5 prime end

□ Trim ABI primer blobs, where 3 consecutive bases remain off the scale.

☑ Trimming no more than 25%, trim until the first 25 bases contain less than 1 ambiguities.

☑ Trimming no more than 25%, trim until the first 25 bases contain less than 1 bases with confidences below 25

□ Always trim at least 0 bases from the 5' end.

3 prime end

□ Trim chromatogram files before the first 20 consecutive peaks below 25 % of the highest peak.

☑ Starting 100 bases after 5' trim, trim the first 25 bases containing more than 3 ambiguities.

☑ Trim from the 3' end until the last 25 bases contain less than 3 ambiguities.

☑ Trim from the 3' end until the last 25 bases contain less than 3 bases with confidences below 25

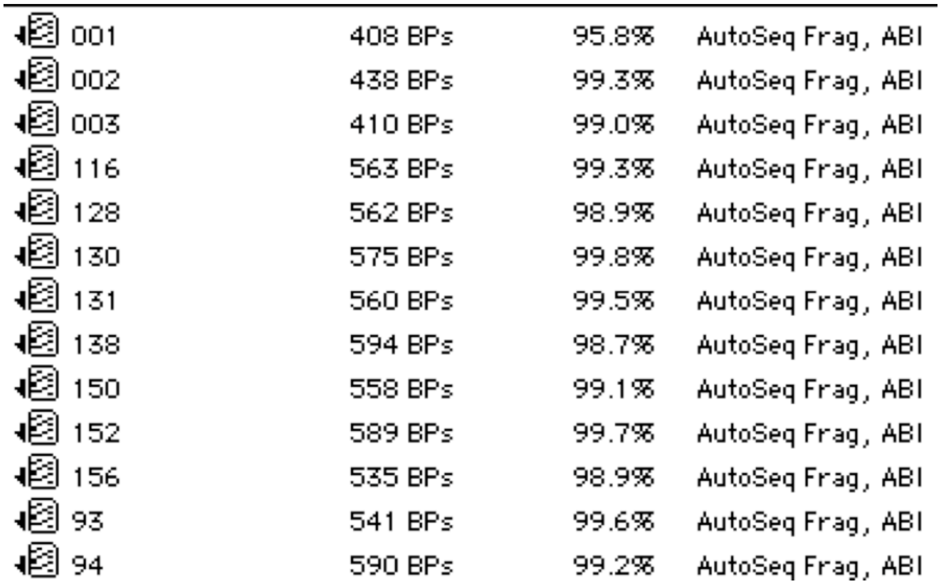
Post fix

□ Maximum desired length after trimming is 0 bases, trim more from the 3' end if necessary.

☑ Remove leading and trailing ambiguous bases.

- Click **OK** to close the window.
- Go to the **Ends Trimming** button bar and click the **Trim Checked Items** button.
- A caution window appears. Click the **Trim** button.
- Now only blue lines remain.
- Close the **Ends Trimming** window.

Notice the improvement of the values in the **Quality** column in the Project Window.



001	408 BPs	95.8%	AutoSeq Frag, ABI
002	438 BPs	99.3%	AutoSeq Frag, ABI
003	410 BPs	99.0%	AutoSeq Frag, ABI
116	563 BPs	99.3%	AutoSeq Frag, ABI
128	562 BPs	98.9%	AutoSeq Frag, ABI
130	575 BPs	99.8%	AutoSeq Frag, ABI
131	560 BPs	99.5%	AutoSeq Frag, ABI
138	594 BPs	98.7%	AutoSeq Frag, ABI
150	558 BPs	99.1%	AutoSeq Frag, ABI
152	589 BPs	99.7%	AutoSeq Frag, ABI
156	535 BPs	98.9%	AutoSeq Frag, ABI
93	541 BPs	99.6%	AutoSeq Frag, ABI
94	590 BPs	99.2%	AutoSeq Frag, ABI

The Reference Sequence

You are now ready to mark a sequence to serve as the Reference Sequence. The Reference Sequence has certain properties that are useful for characterizing SNPs. The Reference Sequence will set the base numbering and the orientation of the contig you are about to assemble. This allows you to reference a SNP in relation to a standardized position. In addition the Reference Sequence does *not* contribute to the consensus sequence. Having a Reference Sequence does not skew the consensus sequence calculation–an especially important fact in situations where you only have a few sequences in the contig.

- Click the sequence called NC_000022 to select it.
- Choose **Sequence > Reference Sequence.**

You will notice that the icon of the Reference Sequence now contains an R.
The sequence is now protected from editing.

NC_000022

Assembling your data

Sequencher provides several algorithms for data assembly. Each algorithm has been devised for a specific purpose and contains parameters you can control. In this tutorial you will use the default Assembly Parameters to create your contig.

- Choose **Select > Select All** and click on the **Assemble to Reference** button.
- Click the **Close** button to dismiss the Assembly Completed dialog.
- Choose **Contig > Consensus Inclusively.** Note the caution window.

Creating a Variance Table to locate SNPs

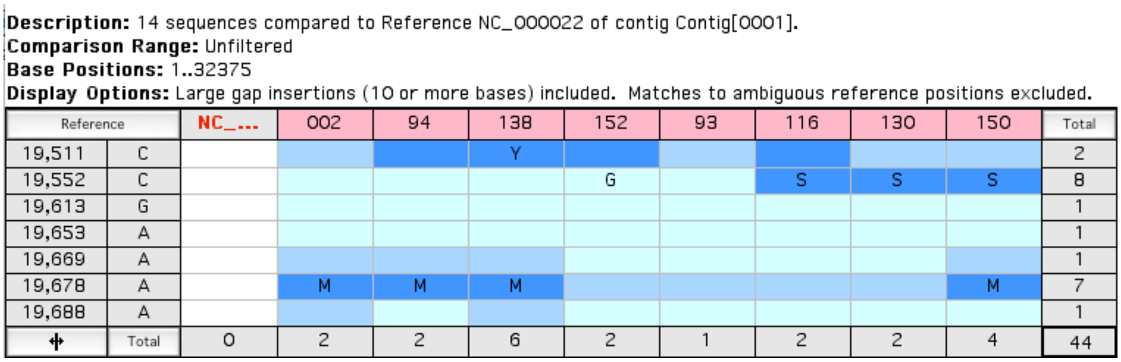
You are now ready to create a Variance Table with your trimmed data and identify candidate SNPs.

The Variance Table provides an overview of the differences within the same contig relative to a selected primary sequence or exemplar. These differences will be your candidate SNPs. You can choose which sequence you want to use for your exemplar. Your choices are the Reference Sequence, the Consensus Sequence, or the top sequence in your contig sequence list.

In this tutorial, you will use the Reference Sequence as the primary sequence.

- From the Project Window ensure that the contig is selected.
- From the **Sequence** menu, select the command **Compare Bases To > Reference Sequence**.

Sequencher generates the Variance Table. Your initial view of the Variance Table displays the candidate SNPs for the 14 sequences in the contig. Columns are in the order that sequences appear in the contig, and rows are ordered according to the positions of the differences relative to the Reference Sequence.



Description: 14 sequences compared to Reference NC_000022 of contig Contig[0001].
Comparison Range: Unfiltered
Base Positions: 1..32375
Display Options: Large gap insertions (10 or more bases) included. Matches to ambiguous reference positions excluded.

Reference		NC_...	002	94	138	152	93	116	130	150	Total
19,511	C				Y						2
19,552	C					G		S	S	S	8
19,613	G										1
19,653	A										1
19,669	A										1
19,678	A		M	M	M					M	7
19,688	A										1
✣	Total	0	2	2	6	2	1	2	2	4	44

The names of the sequences are quite long and are truncated at the default column width.

- Click once on the resize ✛ icon next to the bottom left Total button.

The table expands all of the columns to display the full name for each sequence.

- Click once on the resize ✛ icon button.

The column widths in the table change to display only a single base's width. This enables you to see more data in your viewing window.

- Click once on the resize ✛ icon button to return to the original display.

One sequence has a gray header. This is the Reference Sequence. Notice that the text in the header of the Reference Sequence is red. This matches the red icon label of the Reference Sequence. You will notice that each of the remaining column headings is shaded in pink. This indicates these sequences do not cover the full comparison range of the Reference Sequence. Notice that several of the cells contain a pink X. This indicates that this sequence does not have a base for the equivalent position in the Reference Sequence. The blue shading in some cells corresponds to one of the three base call Confidence Ranges (Low, Medium or High). Darker shading indicates the low Confidence Range. You alter the values that set the boundaries for the Confidence Ranges in the User Preferences.

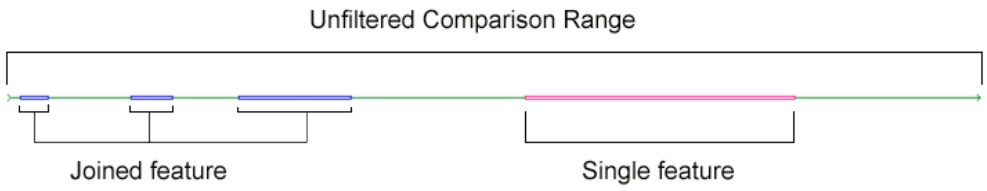
The **Total** cell in the bottom right corner shows that there are a total of 44 variants listed in the table.

- Move your cursor to the line that divides the columns between each sequence name until the arrow icon becomes the resize column icon.
- Drag the column divider for any individual cell and note that the column resizes to your chosen size.

FOCUSING ON REGIONS OF INTEREST ON THE REFERENCE SEQUENCE

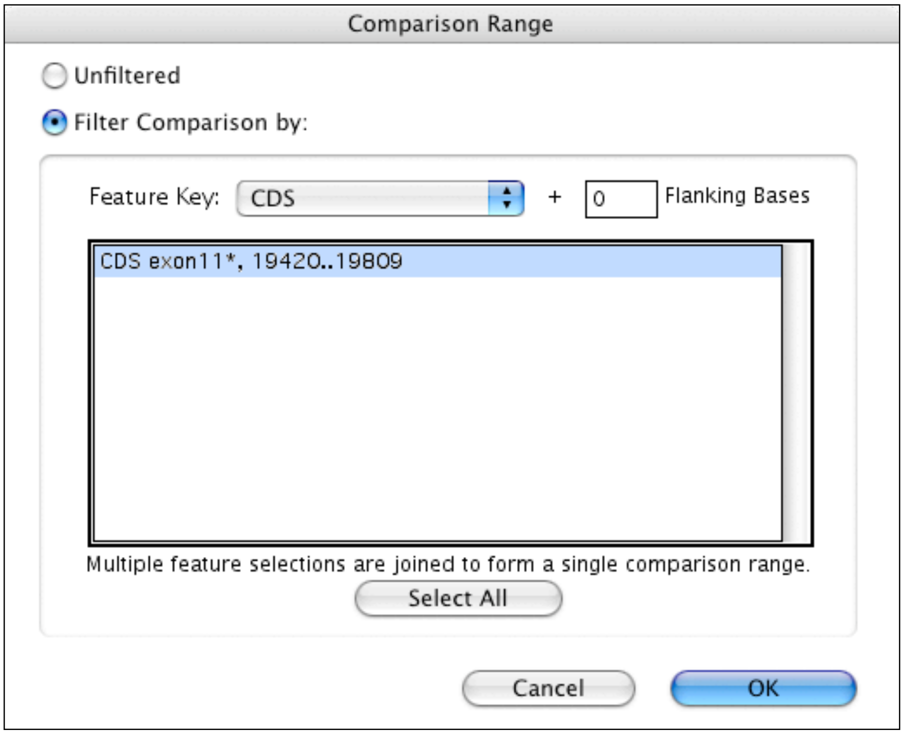
In some cases you want to explore the entire sequence. However, if your Reference Sequence contains features such as an exon or a CDS you can direct the Variance Table to focus on these features. This will reduce the amount of data you have to review. Sequences from GenBank are annotated with features using Feature Keys (a standardized method of referring to biologically important regions). The annotations are listed in a Feature Table that is read by Sequencher when the sequence is imported into a project.

The **Comparison Range** is defined by the bases numbered in the primary or exemplar sequence. In the example in this tutorial the **Comparison Range** is defined by the Reference Sequence from bases 1 to 32,375. This is the unfiltered **Comparison Range**.



You can restrict the Comparison Range by choosing the Feature used to annotate the Reference Sequence. You will use the CDS Exon 11* Feature in this tutorial.

- Go to the Variance Table button bar and click on the **Comparison Range** button.
- Check the **Filter Comparison by** radio button.
- Click on the **Feature Key** drop down menu and choose **CDS**.
- Select the **CDS Exon 11*, 19420..19809** feature from the list box.
- Click on the **OK** button to dismiss the **Comparison Range** dialog window.



Comparison Range

○ Unfiltered

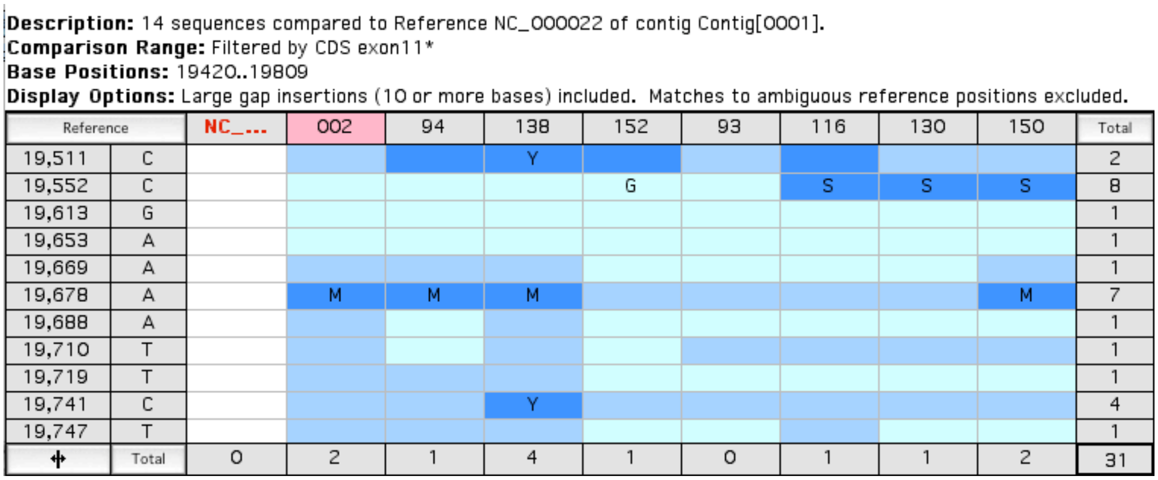
● Filter Comparison by:

Feature Key: CDS + 0 Flanking Bases

CDS exon11*, 19420..19809

Multiple feature selections are joined to form a single comparison range.

The Variance Table is redrawn and the only variants in the report are now within the selected feature. The **Total** cell at the bottom right of the table shows that there are now only 31 variants listed in the table.



Description: 14 sequences compared to Reference NC_000022 of contig Contig[0001].
Comparison Range: Filtered by CDS exon11*
Base Positions: 19420..19809
Display Options: Large gap insertions (10 or more bases) included. Matches to ambiguous reference positions excluded.

Reference		NC_...	002	94	138	152	93	116	130	150	Total
19,511	C				Y						2
19,552	C					G		S	S	S	8
19,613	G										1
19,653	A										1
19,669	A										1
19,678	A		M	M	M					M	7
19,688	A										1
19,710	T										1
19,719	T										1
19,741	C				Y						4
19,747	T										1
✢	Total	0	2	1	4	1	0	1	1	2	31

Reviewing the Data

The **Review** mode of the Variance Table lets you use the table of differences to navigate to areas of interest and explore the underlying data. When you click on the **Review** button in the button bar, or when you double-click on a cell in the table, Sequencher opens the Contig Editor and Contig Chromatogram windows. The data displayed in each of the windows updates to reflect your selection in the Variance Table.

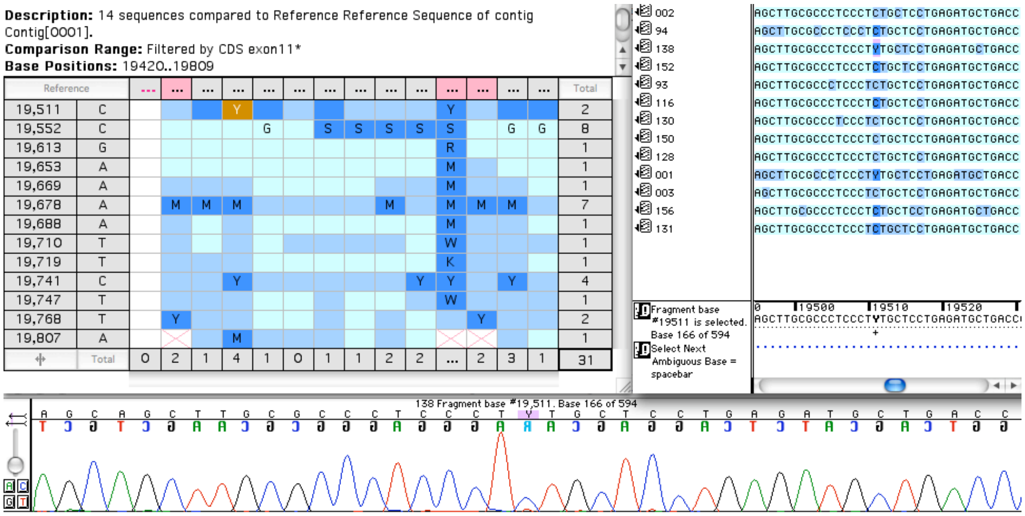
You can move quickly from one difference in the table to the next using a keyboard shortcut.

- Locate base position 19,511 and place your cursor in the first cell of the Variance Table at this position.
- While holding down the Option/Alt key, press the right arrow key.

The **Option/Alt** key plus arrow key combination moves your cursor to the next difference in the Variance Table.

- Go to the button bar and click on the **Review** button.
- Arrange the windows to suit your viewing preference.

The base call in this position is a "Y". There is a black + below the consensus sequence line indicating that there is an ambiguity here. You will notice that the background color of the cell containing the "Y" is dark blue indicating that the base call is low quality, which is typical for ambiguous base calls. The next step is to check the chromatogram underlying this base call.



Description: 14 sequences compared to Reference Reference Sequence of contig Contig[0001].
Comparison Range: Filtered by CDS exon11*
Base Positions: 19420..19809

Reference		...	...	...	...	...	...	...	...	...	...	...	...	...	...	Total
19,511	C				Y							Y				2
19,552	C					G		S	S	S	S	S		G	G	8
19,613	G											R				1
19,653	A											M				1
19,669	A											M				1
19,678	A		M	M	M					M		M	M	M		7
19,688	A											M				1
19,710	T											W				1
19,719	T											K				1
19,741	C				Y						Y	Y		Y		4
19,747	T											W				1
19,768	T		Y										Y			2
19,807	A				M											1
	Total	0	2	1	4	1	0	1	1	2	2	...	2	3	1	31

```
002  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
94   AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
138  AGCTTGCGCCCTCCCTYTGCTCCTGAGATGCTGACC
152  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
93   AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
116  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
130  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
150  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
128  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
001  AGCTTGCGCCCTCCCTYTGCTCCTGAGATGCTGACC
003  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
156  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC
131  AGCTTGCGCCCTCCCTCTGCTCCTGAGATGCTGACC

0      19500     19510     19520
AGCTTGCGCCCTCCCTYTGCTCCTGAGATGCTGACC
                +
```

Fragment base #19511 is selected. Base 166 of 594
Select Next Ambiguous Base = spacebar

138 Fragment base #19,511. Base 166 of 594

A G C A G C T T G C G C C C T C C C T Y T G C T C C T G A G A T G C T G A C C

You will now see the contig bases and chromatogram data for the sample. The base call is a "Y". This base call is not well supported by the underlying chromatogram.

- Use the right arrow key in your keyboard to view other samples at this position.

All of the samples at this position have a weak "C" peak. Underneath the "C" is a small "T" peak. This "T" is probably noise. It is not likely that there is a SNP at this position.

Editing data from the Variance Table

Although the Variance Table is used primarily for display and review of your data you can make use of its links to the underlying data in another way.

- Double click in Variance Table in sample **138** at base position **19,511**.

You will now see the contig bases and chromatogram data for the selected sample cell. The base call is a "Y", and you can see that there are two small peaks (relative to the surrounding data) at this position.

You decide to edit the "Y" base call, because the trace data in this sample matches "C" calls in the majority of the other samples at this position.

- Type a **C** into the cell.

The base now matches the Reference Sequence. Since you have removed the conflicting base, the cell is empty. Any edits you make are reflected immediately due to the dynamic link between the windows. They will also be updated in the Totals at the left and bottom of the Table.

- In the Variance Table, find the second "Y" call at position 19,511 in sample 001, and edit the call to a "C".
- Click on the **Refresh** button in the Variance Table button bar.

Note that the row for position 19,511 is no longer included in the table, because there are no longer samples with differences at this position.

- Close the Contig Editor and Contig Chromatogram windows.

Removing unwanted data from the table

There may be instances when you wish to remove data from your table before proceeding further with your analysis. In this example you will remove the samples that do not have any variants.

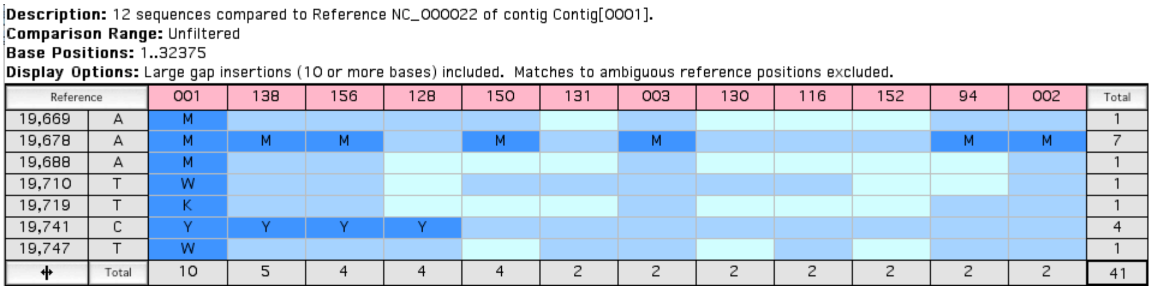
First sort the table so that all the sample sequences containing variants are grouped together.

- Click on the **Total** button at the bottom left of the table.

The samples with candidate SNPs are now grouped together at the left hand end of the table.

- Shift click in the column headers of the two columns that contain no variants.
- Choose **Edit > Remove from Table**.

This step only removes data from the table not from the underlying contig.



Description: 12 sequences compared to Reference NC_000022 of contig Contig[0001].
Comparison Range: Unfiltered
Base Positions: 1..32375
Display Options: Large gap insertions (10 or more bases) included. Matches to ambiguous reference positions excluded.

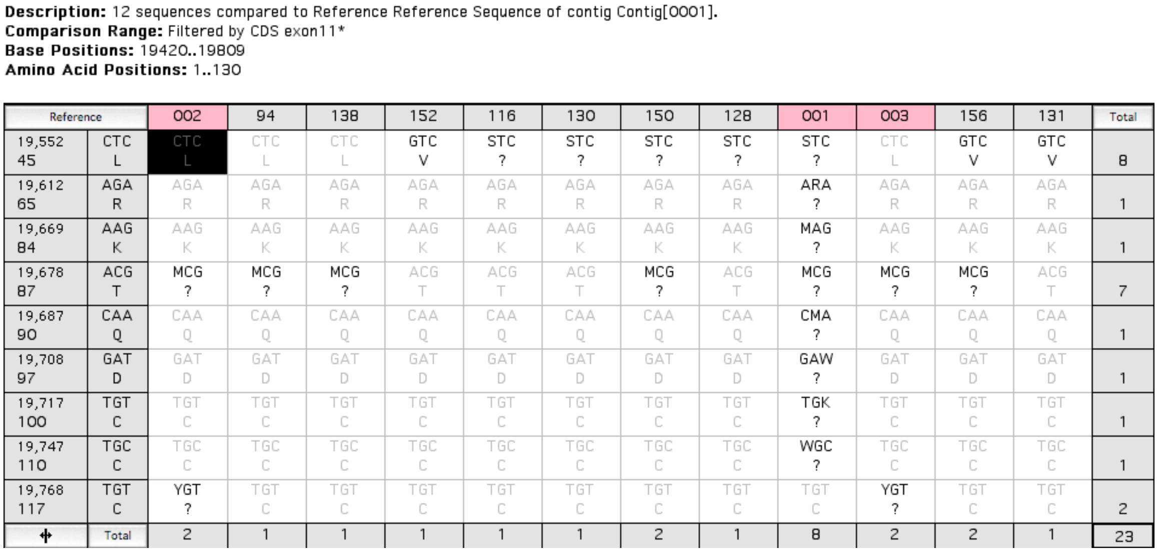
Reference		001	138	156	128	150	131	003	130	116	152	94	002	Total
19,669	A	M												1
19,678	A	M	M	M		M		M				M	M	7
19,688	A	M												1
19,710	T	W												1
19,719	T	K												1
19,741	C	Y	Y	Y	Y									4
19,747	T	W												1
✣	Total	10	5	4	4	4	2	2	2	2	2	2	2	41

Viewing the impact of candidate SNPs on translated regions

It is possible to view the coding impact of any difference in the Variance Table. The Translated Variance Table is the sister table to the Variance Table. This directs your attention to the putative effects a base difference has on a codon and its associated amino acid. This is most informative when your Reference Sequence has a feature which can be translated.

- Click on the **Translation** button on the Variance Table button bar.
- Click on the **Translation Range** button on the Translated Variance Table button bar.
- Check the **Filter Translation by:** radio button.
- Click on the **Feature Key:** drop down menu and choose **CDS.**
- Select the feature **CDS Exon 11*, 19420..19809**
- Click on the **OK** button to dismiss the **Translation Range** dialog window.

The Translated Variance Table shares many features with the Variance Table. You can move around from cell to cell using arrow keys. You can move across gaps using the **Option/Alt** + arrow key combination. The pink headers and Xs have the same function. You can also view the underlying base calls and trace data by using the **Review** mode.



Description: 12 sequences compared to Reference Reference Sequence of contig Contig[0001].
Comparison Range: Filtered by CDS exon11*
Base Positions: 19420..19809
Amino Acid Positions: 1..130

Reference		002	94	138	152	116	130	150	128	001	003	156	131	Total
19,552 45	CTC L	CTC L	CTC L	CTC L	GTC V	STC ?	STC ?	STC ?	STC ?	STC ?	CTC L	GTC V	GTC V	8
19,612 65	AGA R	AGA R	AGA R	AGA R	AGA R	AGA R	AGA R	AGA R	AGA R	ARA ?	AGA R	AGA R	AGA R	1
19,669 84	AAG K	AAG K	AAG K	AAG K	AAG K	AAG K	AAG K	AAG K	AAG K	MAG ?	AAG K	AAG K	AAG K	1
19,678 87	ACG T	MCG ?	MCG ?	MCG ?	ACG T	ACG T	ACG T	MCG ?	ACG T	MCG ?	MCG ?	MCG ?	ACG T	7
19,687 90	CAA Q	CAA Q	CAA Q	CAA Q	CAA Q	CAA Q	CAA Q	CAA Q	CAA Q	CMA ?	CAA Q	CAA Q	CAA Q	1
19,708 97	GAT D	GAT D	GAT D	GAT D	GAT D	GAT D	GAT D	GAT D	GAT D	GAW ?	GAT D	GAT D	GAT D	1
19,717 100	TGT C	TGT C	TGT C	TGT C	TGT C	TGT C	TGT C	TGT C	TGT C	TGK ?	TGT C	TGT C	TGT C	1
19,747 110	TGC C	TGC C	TGC C	TGC C	TGC C	TGC C	TGC C	TGC C	TGC C	WGC ?	TGC C	TGC C	TGC C	1
19,768 117	TGT C	YGT ?	TGT C	TGT C	TGT C	TGT C	TGT C	TGT C	TGT C	TGT C	YGT ?	TGT C	TGT C	2
✥	Total	2	1	1	1	1	1	2	1	8	2	2	1	23

Notice the difference in numbering between the two tables. The Variance Table reports the actual base position of the candidate SNP. For example the Variance Table reports a SNP at position 19,613. The Translated Variance Table reports the position of the first base in the codon containing the SNP, which is in this case 19,612. The numbering for both tables is relative to the Reference Sequence.

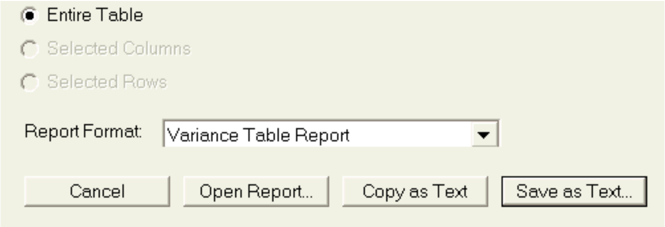
If you want to learn more about the Translated Variance Table refer to the manual and the associated tutorial.

Making a Report

Now that you have reviewed some of your results in the Variance Table, you can create a report and print or export it. Sequencher provides a number of report formats. The entire table can be exported as a single entity. It can be exported as individual column reports that reflect the original comparison sequences or you can export selected rows or selected columns. You will now create a Report as if you required it for printing.

- Click on the **Reports** button on the Variance Table button bar.

Sequencher will bring up the following Report dialog.



● Entire Table

○ Selected Columns

○ Selected Rows

Report Format: Variance Table Report

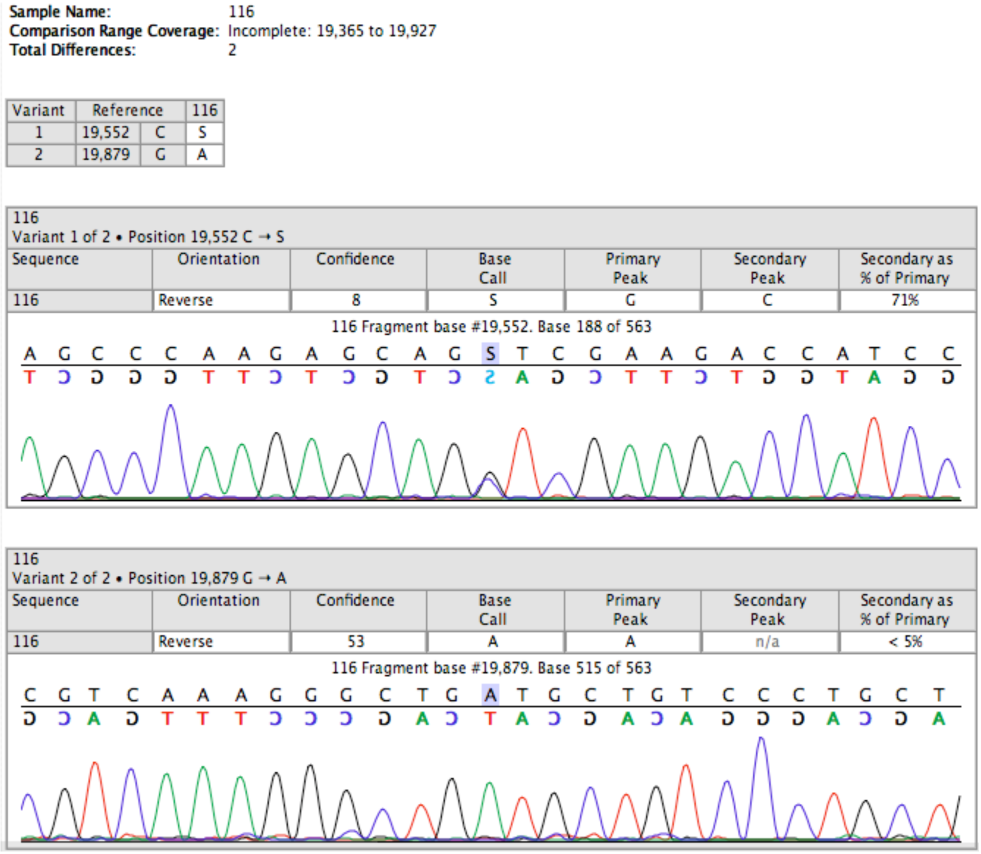
The **Report Format:** drop down menu provides four different report options: the Variance Table Report, Individual Variance Reports, the Variance Detail Report, and the Population Report. The **Open Report…** command displays a view of each report, which you can either print or save as a PDF (Portable Document Format). The Variance Table and the Individual Variance Reports are also available to either **Copy as Text** or **Save as Text** if you want to export your data.

The Variance Detail Report contains a wealth of information pertaining to each variant. It will contain the exact Confidence value, the base call, an extract of the trace (this is called a tracelet) around the variant, as well as information on any potential secondary peak.

- Choose **Variance Detail Report** from the Report Format drop down menu.
- Click on the **Open Report…** button.

Note: The Reporting functions are not accessible when you are running a copy of Sequencher in demo mode or using the special demonstration version of Sequencher.

- Scroll down the Variance Detail Report to view the data for sample 116.



Sample Name: 116
Comparison Range Coverage: Incomplete: 19,365 to 19,927
Total Differences: 2

Variant	Reference		116
1	19,552	C	S
2	19,879	G	A

116
Variant 1 of 2 • Position 19,552 C → S

Sequence	Orientation	Confidence	Base Call	Primary Peak	Secondary Peak	Secondary as % of Primary
116	Reverse	8	S	G	C	71%

116 Fragment base #19,552. Base 188 of 563

A G C C C A A G A G C A G S T C G A A G A C C A T C C
T Ɔ ⅁ ⅁ ⅁ T T Ɔ T Ɔ ⅁ T Ɔ ƨ A ⅁ Ɔ T T Ɔ T ⅁ ⅁ T A ⅁ ⅁

116
Variant 2 of 2 • Position 19,879 G → A

Sequence	Orientation	Confidence	Base Call	Primary Peak	Secondary Peak	Secondary as % of Primary
116	Reverse	53	A	A	n/a	< 5%

116 Fragment base #19,879. Base 515 of 563

C G T C A A A G G G C T G A T G C T G T C C C T G C T
⅁ Ɔ A ⅁ T T T Ɔ Ɔ Ɔ ⅁ A Ɔ T A Ɔ ⅁ A Ɔ A ⅁ ⅁ ⅁ A Ɔ ⅁ A

Sequencher reports the orientation, confidence score, and base call for the variants in this sample. The table also provides information on the exact ratio of the primary and secondary peaks in such a way that you can determine whether these are true heterozygotes or artifacts of the sequencing process. Snapshots of the supporting traces are included with the report.

You will notice that for position 19,879 the Confidence Value is 53. You will also notice that there is no base call for the secondary peak. The secondary peak is less than five percent of the primary peak height. This provides strong evidence in support of the pure base call.

You can save this report as a PDF if you want to archive your results.

- Click on the **Save as PDF…** button.
- Select a location and file name from the **Save as PDF File** dialog window.
- Click on the **Save** button to dismiss the window.
- Close the project without saving.
- **Quit** Sequencher.

Conclusion

In the example you've used in this tutorial, Sequencher's **Variance Table** immediately identified the candidate SNPS from sample sequences assembled to a Reference Sequence of 16,936 bases in length. With a few keystrokes you were able to define the Comparison Range so that you could further narrow your results to just the differences you wanted to see. The Review Mode and Variance Detail Report supported your validation of the candidate SNPs. You were able to make edits from the Variance Table. You were able to review the underlying trace data and obtain the Confidence Values for your candidates and confirm them efficiently.