FOOD SAFETY LABORATORY/MQIP

CORNELL UNIVERSITY

Automated Editing of DNA Sequences using SeqTrace

EFFECTIVE DATE: March 23, 2016

APPROVED BY: Martin Wiedmann

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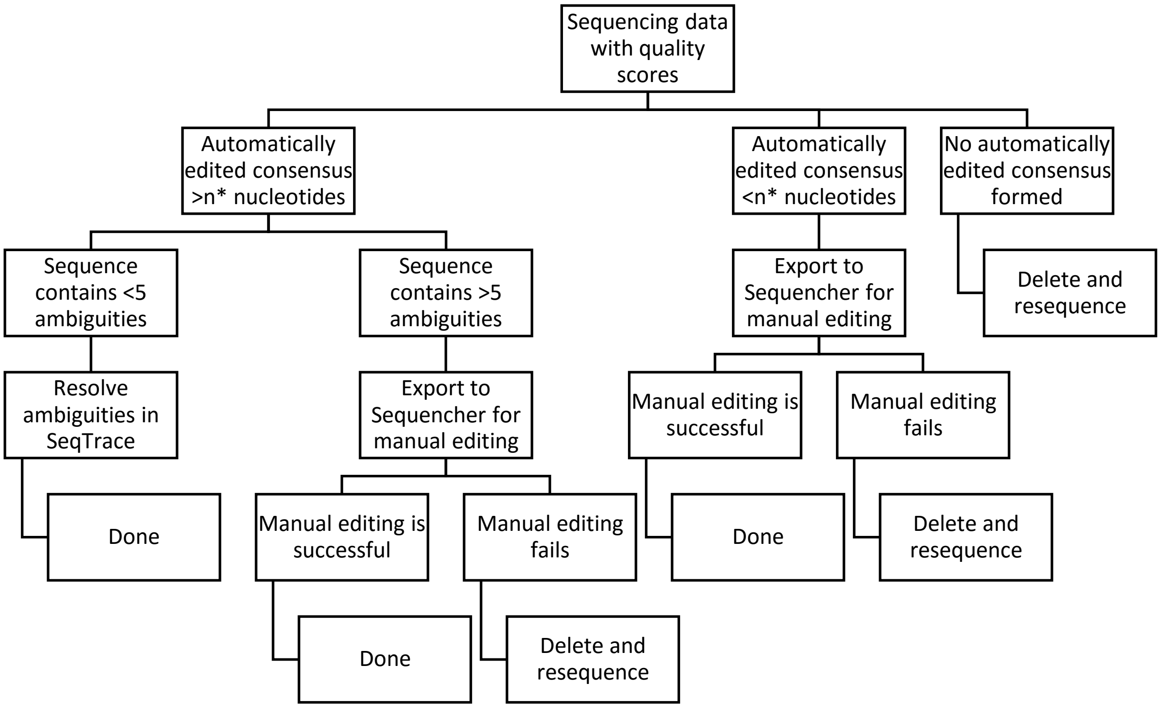
Dr. Martin Wiedmann Date

AUTHORED BY:

Samuel Reichler 2016-01-08

Automated Sequence Editing Decision Tree


Sequencing data with quality scores
Automatically edited consensus >n* nucleotides
Automatically edited consensus <n* nucleotides
No automatically edited consensus formed
Sequence contains <5 ambiguities
Sequence contains >5 ambiguities
Export to Sequencher for manual editing
Delete and resequence
Resolve ambiguities in SeqTrace
Export to Sequencher for manual editing
Manual editing is successful
Manual editing fails
Done
Manual editing is successful
Manual editing fails
Done
Delete and resequence
Done
Delete and resequence

***Recommended consensus length cutoff values:**

Gene	**Length (n)**
ITS	500
NLE	500
16s	600
rpoB	632
sigB	630

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SECTION 1 - INTRODUCTION

1.1 Purpose

To provide a functional automated platform for the alignment, editing, and resolution of sequence ambiguity necessary to efficiently process and BLAST large numbers of short DNA sequences, such as those produced by high-throughput Sanger sequencing.

1.2 Scope

This SOP may be used by all lab members at the discretion of the PI.

1.3 Definitions

1. **KB Basecaller**: A computer algorithm used to interpret the electropherograms produced by the Sanger sequencing instrument into a DNA sequence. It is more advanced than the standard ABI basecaller, and is capable of accurately extracting a greater number of bases from both the 3' and 5' ends of the sequence. It assigns a quality score to every base it calls, and is also capable of calling mixed base positions through the use of standard IUB ambiguity codes. More information can be found here: http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_041491.pdf
2. **Quality (Confidence) Score**: The quality score of an individual base call provides an indication of the confidence that the call is correct. The quality score Q is defined as a property which is logarithmically related to the base-calling error probabilities P: $Q = -\log_{10} P$, or $P = 10^{\frac{-Q}{10}}$. The table below demonstrates how quality scores are interpreted:

Quality Score	**Probability of incorrect base call**	**Base call accuracy**
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

3. **SeqTrace**: A free, open-source program that can be used for the automated alignment, editing, and ambiguity resolution of Sanger-sequenced DNA. It performs these tasks based on the quality scores of the individual base calls in the sequences.

1.4 Safety

There are no health or safety risks associated with this SOP.

SECTION 2 - MATERIALS

Materials

- A PC running Windows or Linux with network drive access and internet access
- PCR product or Ready-to-Load sequencing reactions ready to submit to the BRC, or DNA sequences already received from the BRC

SECTION 3 - PROCEDURES

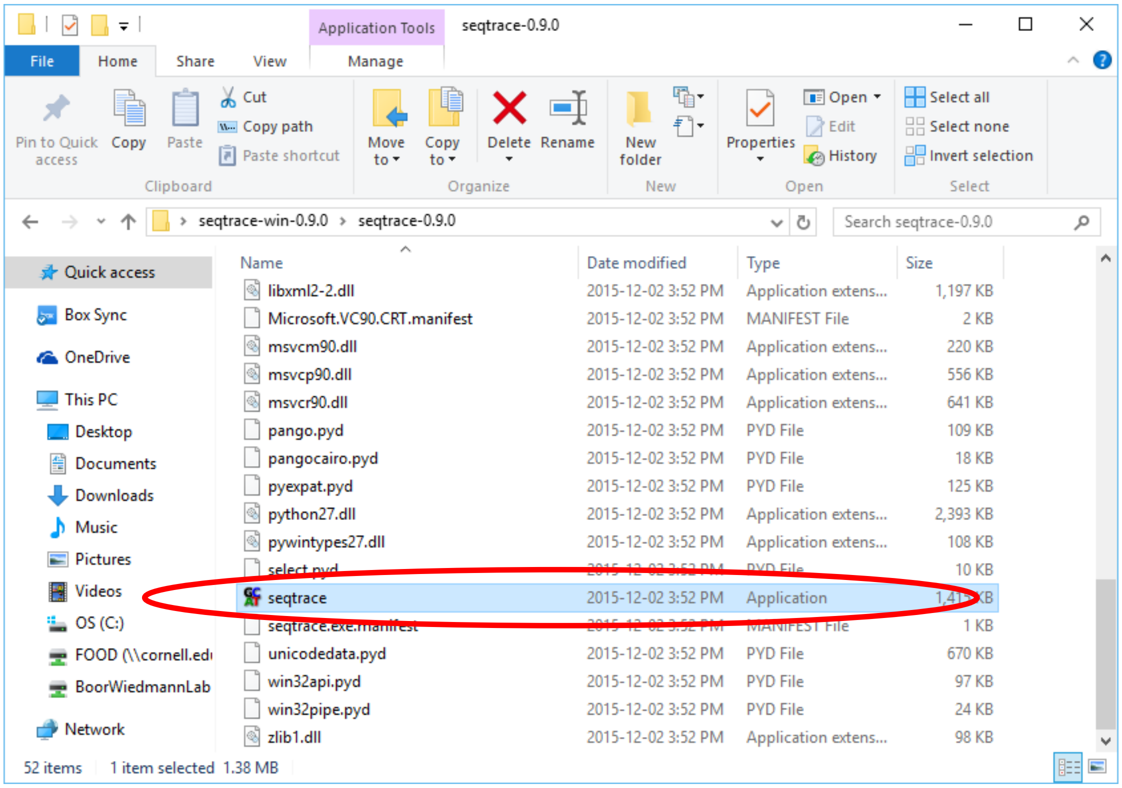
1. Software installation and running the software

1.1. Go to the following website: https://code.google.com/p/seqtrace/downloads/list

1.2. Download the version of SeqTrace 0.9.0 appropriate for your operating system.

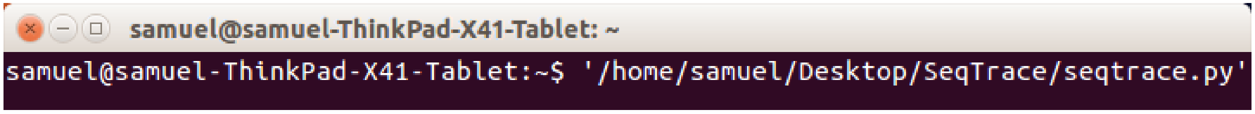
1.3. Extract the compressed file to an easily-accessible location on your computer. No installation is required.

1.4. **On Windows:** Open Seqtrace by navigating to the folder you extracted the downloaded file to and double-clicking on the SeqTrace application file, as shown in the image below:



Name	Date modified	Type	Size
libxml2-2.dll	2015-12-02 3:52 PM	Application extens...	1,197 KB
Microsoft.VC90.CRT.manifest	2015-12-02 3:52 PM	MANIFEST File	2 KB
msvcm90.dll	2015-12-02 3:52 PM	Application extens...	220 KB
msvcp90.dll	2015-12-02 3:52 PM	Application extens...	556 KB
msvcr90.dll	2015-12-02 3:52 PM	Application extens...	641 KB
pango.pyd	2015-12-02 3:52 PM	PYD File	109 KB
pangocairo.pyd	2015-12-02 3:52 PM	PYD File	18 KB
pyexpat.pyd	2015-12-02 3:52 PM	PYD File	125 KB
python27.dll	2015-12-02 3:52 PM	Application extens...	2,393 KB
pywintypes27.dll	2015-12-02 3:52 PM	Application extens...	108 KB
select.pyd	2015-12-02 3:52 PM	PYD File	10 KB
seqtrace	2015-12-02 3:52 PM	Application	1,41[illegible] KB
seqtrace.exe.manifest	2015-12-02 3:52 PM	MANIFEST File	1 KB
unicodedata.pyd	2015-12-02 3:52 PM	PYD File	670 KB
win32api.pyd	2015-12-02 3:52 PM	PYD File	97 KB
win32pipe.pyd	2015-12-02 3:52 PM	PYD File	24 KB
zlib1.dll	2015-12-02 3:52 PM	Application extens...	98 KB

1.5. **On Linux**: Open a terminal window by pressing *CTRL+ALT+T*, and provide the path to the file *seqtrace.py*. This can be done easily by navigating to the file in the graphical interface and dragging the file into the terminal window. Press enter to execute the file and open SeqTrace, as shown below:



```
samuel@samuel-ThinkPad-X41-Tablet:~$ '/home/samuel/Desktop/SeqTrace/seqtrace.py'
```

2. Obtaining Correctly Formatted Input Sequences

2.1. When filling out the online order form to submit samples to the BRC for Sanger sequencing, include the following comment in the "General Comments" section: "*Please use KB Basecaller software for trace processing, with mixed base identification set at a 70% detection level.*" It is also helpful to the BRC staff if you note the presence of this comment to them when dropping off your samples.

2.2. If you have recently received sequences from the BRC that were not processed using KB Basecaller, as evidenced by their lack of quality scores, you may contact the BRC and ask them to reprocess your sequence traces post-hoc using KB Basecaller. The BRC staff may be contacted either by using the contact form on their website (http://www.biotech.cornell.edu/contact-us, selecting the Genomics Facility as the message recipient), or directly at genomics@cornell.edu.

2.2.1. The following message may be used: "*Please reprocess the traces from order [order number] using the KB Basecaller with mixed base identification set at a 70% detection level.. Thank you!*"

2.3. If you have raw sequence data from the BRC more than a month or two old, they will most likely no longer be able to reprocess the data for you. If you wish to move forward with automated editing, there is software installed on Zeus, the Linux server, which is capable of assigning quality scores to any trace file. Upload your *.ab1 trace files to Zeus, use the terminal to navigate to the directory containing the files, and execute the following command:

2.3.1.1.1.

```
for f in *.ab1; do ttuner -het -c -cv3 $f && convert_trace < $(basename $f .ab1).scf > $(basename $f .ab1).ztr && rm $(basename $f .ab1).scf; done
```

2.3.1.1.2. This creates a new set of sequence trace files that include quality scores in the *.ztr file format. Sam Reichler or Dave Kent can help you perform this conversion.

3. Using SeqTrace for Automated Editing

3.1. Full software documentation for SeqTrace is available at https://code.google.com/p/seqtrace/wiki/DocumentationIntro

3.2. Open the SeqTrace program as described in §1.4 or §1.5.

3.3. SeqTrace Basic Features

3.3.1. The buttons at the top of the SeqTrace window have the following functions, from left to right:

3.3.1.1. Open a project file

3.3.1.2. Create a new project file

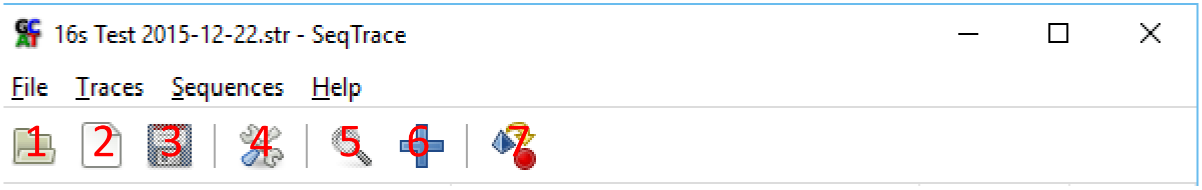
3.3.1.3. Save the current project

3.3.1.4. View and change the settings for the current project

3.3.1.5. View the selected trace file(s)

3.3.1.6. Add trace files to the project

3.3.1.7. Export all in-use sequences



3.4. Creating a Project and Setting Parameters

3.4.1. Create a new project file by clicking the 🗋 icon.

3.4.2. In the Trace settings tab of the Project Settings window, navigate to and select the folder containing the sequences you wish to process.

3.4.3. Assign search strings for identifying the forward and reverse trace files.

3.4.3.1. For instance, if the names of your forward and reverse trace files are r10222216sf.ab1 and r10222216sr.ab1, respectively, then your forward search string would be "16sf" and your reverse search string would be "16sr."

3.4.4. It is not necessary to enter the forward and reverse primer sequences if you do not want to. If you enter these sequences, they will be mapped onto your consensus sequence but it will not affect the resulting consensus sequence.

3.4.5. In the Sequence processing tab of the Project Settings window, set the minimum confidence score to 55.

3.4.6. Ensure that the Bayesian consensus algorithm is selected, that "Automatically Trim Sequence Ends" is selected, and that "Trim alignment end gap regions" is selected.

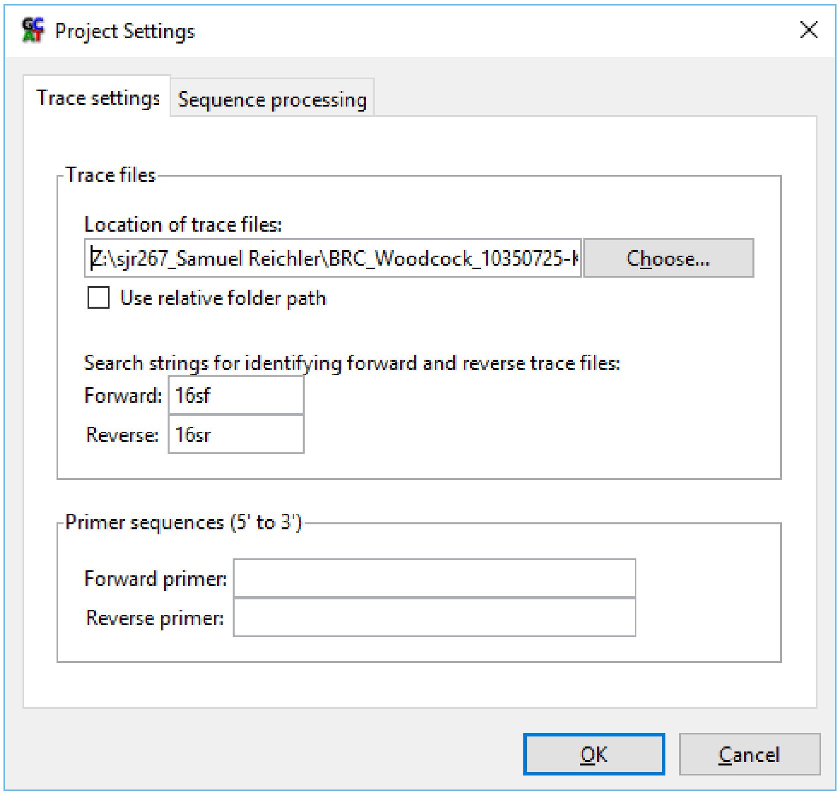
3.4.7. Ensure that "Trim primers if __% of the primer alignment matches" is NOT selected.

3.4.8. Ensure that "Trim until at least __ out of __ bases are correctly called" is selected, and change both values to the number 20.

3.4.8.1. Note: You must enter the second number before you will be allowed to enter the first number.

3.4.9. Click "OK" to finalize the settings.

3.4.10. Click the 💾 icon to save your project settings to a file.



Project Settings

Trace settings | Sequence processing

Trace files

Location of trace files: Z:\sjr267_Samuel Reichler\BRC_Woodcock_10350725-k

Choose...

□ Use relative folder path

Search strings for identifying forward and reverse trace files:

Forward: 16sf

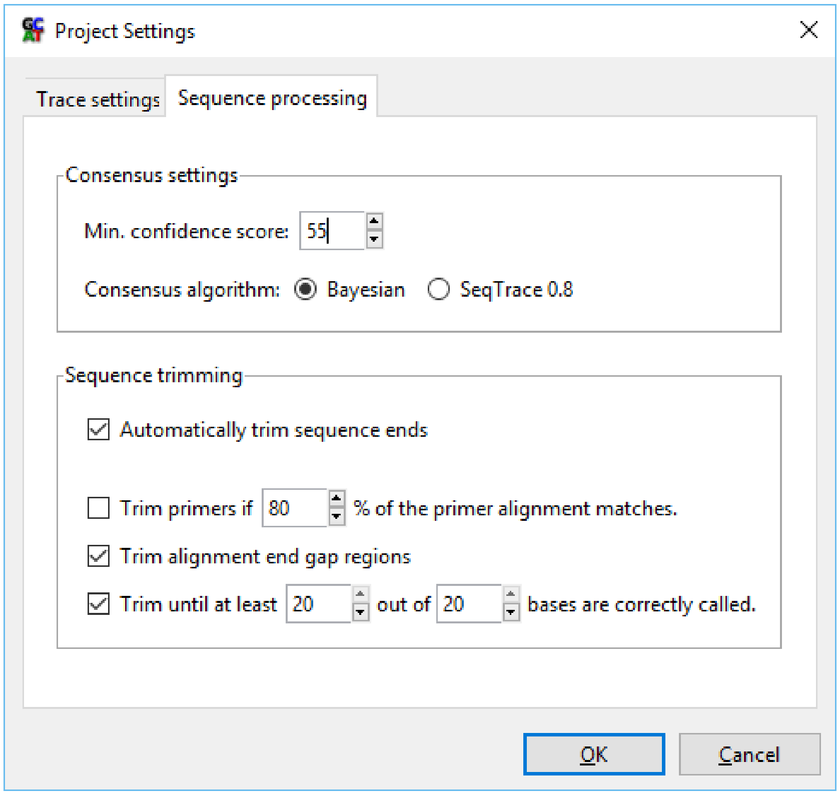
Reverse: 16sr

Primer sequences (5' to 3')

Forward primer:

Reverse primer:

OK | Cancel



Consensus settings

Min. confidence score: 55

Consensus algorithm: ● Bayesian ○ SeqTrace 0.8

Sequence trimming

☑ Automatically trim sequence ends

□ Trim primers if 80 % of the primer alignment matches.

☑ Trim alignment end gap regions

☑ Trim until at least 20 out of 20 bases are correctly called.

3.5. **Importing and Processing Sequences**

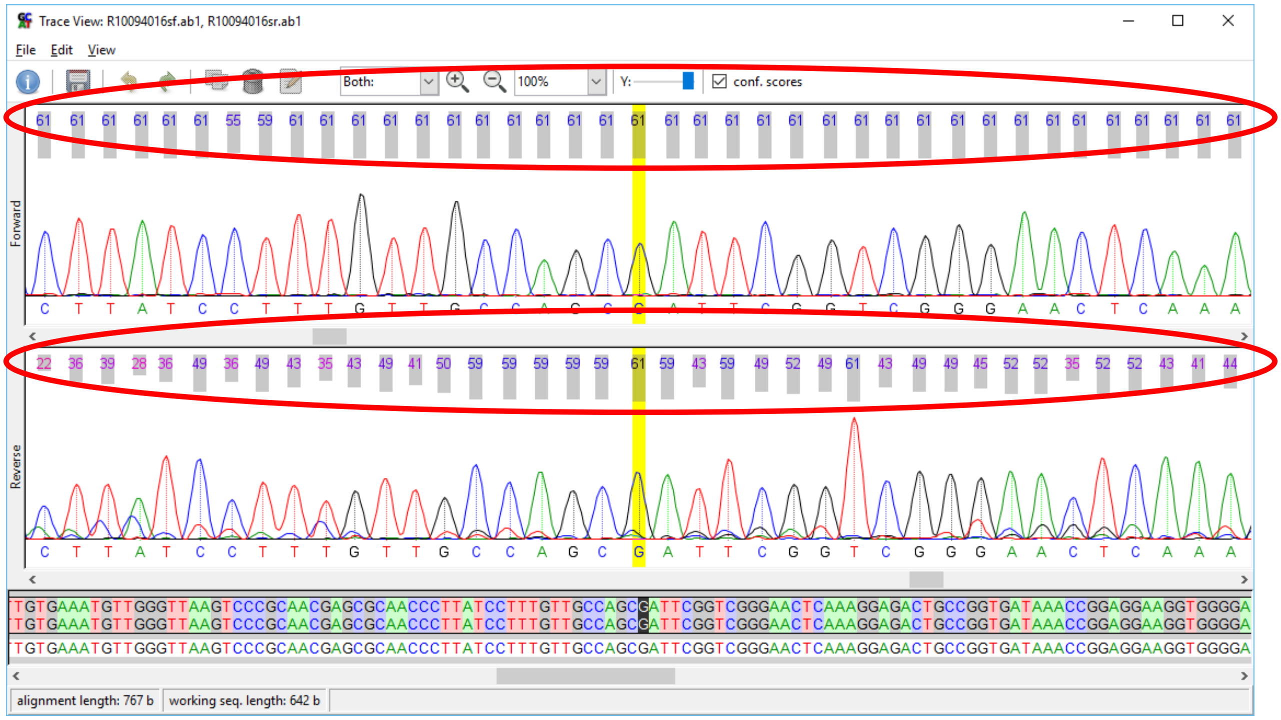
3.5.1. Click the ✚ icon at the top of the window to add sequences to the project. Sequences should be in the *.ab1 file format or the *.ztr file format. Multiple sequences can be selected by clicking on the first sequence desired, holding down the Shift key, then clicking on the last sequence desired. Click the Add button to add the sequences to the project.

3.5.2. At this point, it is a good idea to check a sequence trace file to verify that the KB Basecaller has been used and quality scores have been assigned to each base call.

3.5.2.1. Click on a sequence trace file in the window and then click on the 🔍 icon. This will open a separate Trace View window.

3.5.2.2. The quality scores, if present, should be clearly visible above the electropherogram. If all quality scores are zero, this means that the sequences were not processed with KB Basecaller and must be reprocessed as described above.

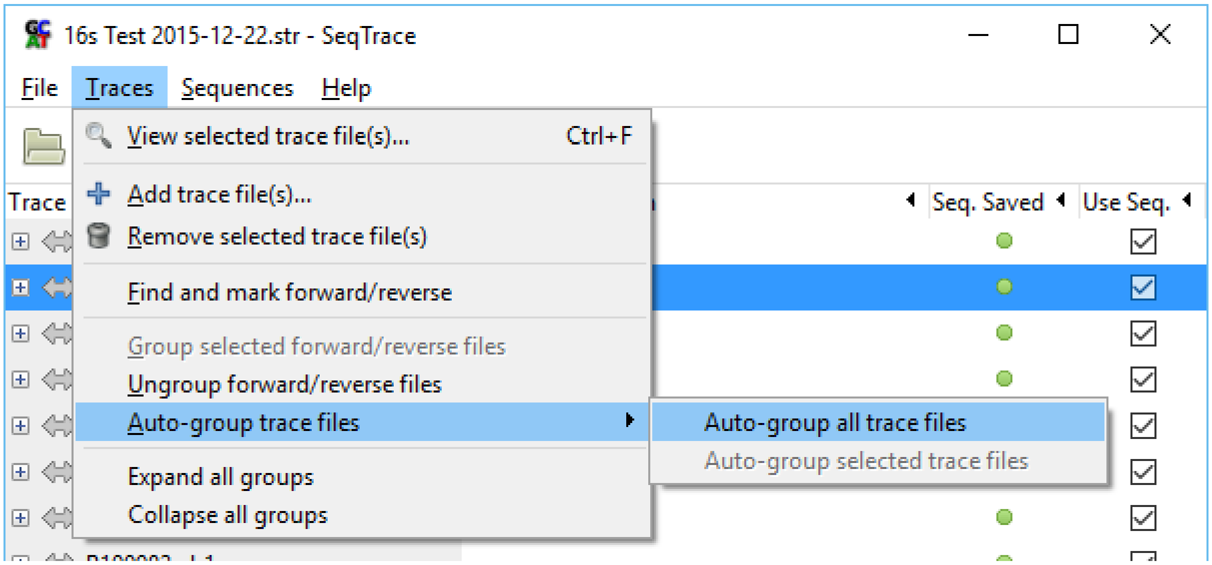
3.5.2.3. Close the Trace View window.



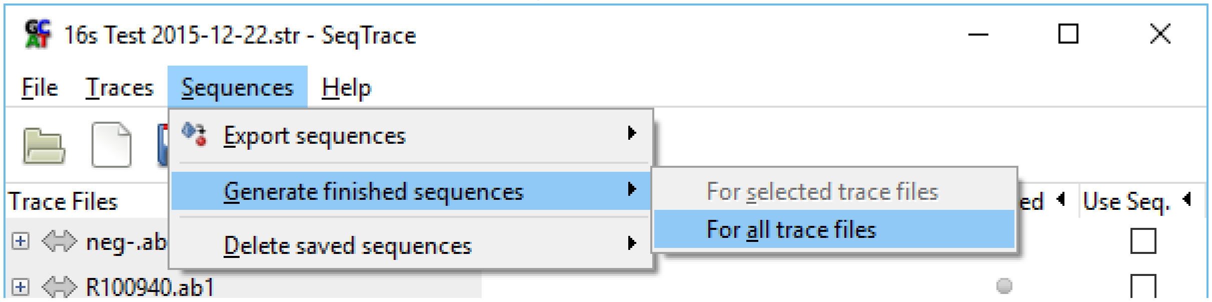
```
TTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGA
TTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGA
TTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGA
```

alignment length: 767 b | working seq. length: 642 b

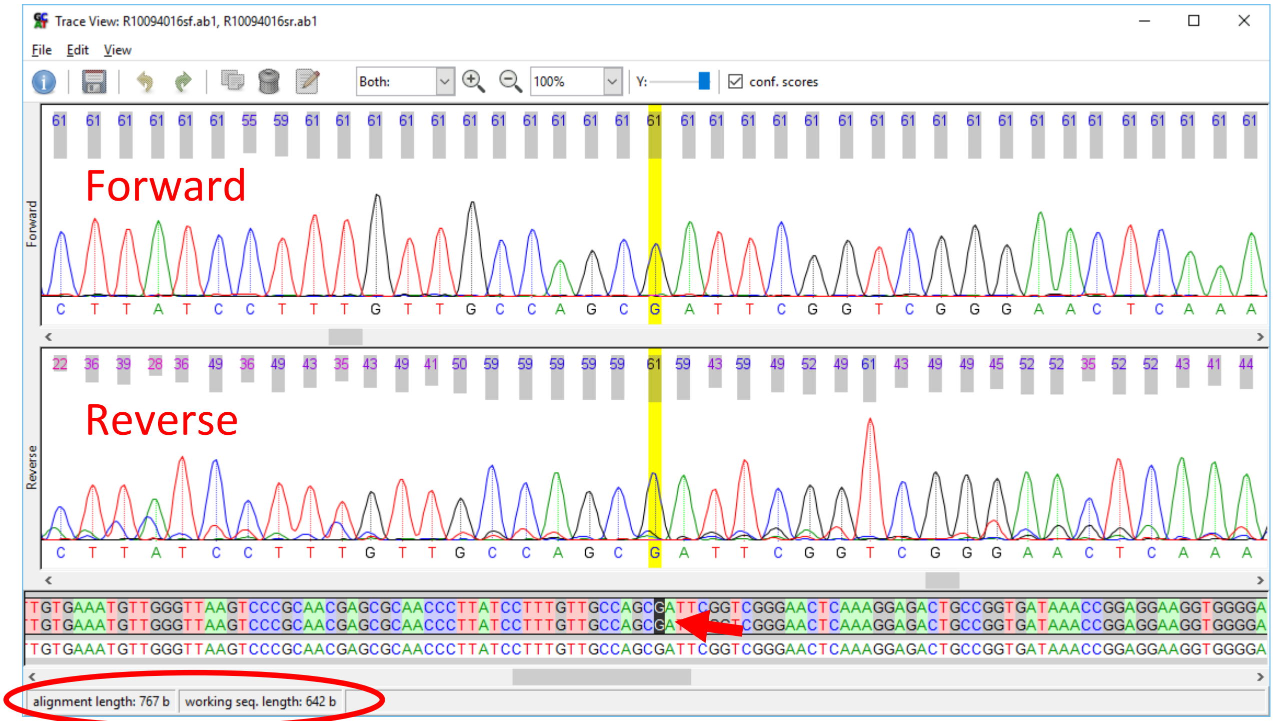
3.5.3. From the “Traces” menu at the top of the window, select “Auto-group trace files, and then “Auto-group all trace files.” A dialog box will appear that contains the first forward-reverse grouping done by SeqTrace. If the forward and reverse pairing is correct, you can click “Yes to All” for all the pairings to be done automatically. If the pairing is incorrect, confirm that the Project Settings match those listed above.


16s Test 2015-12-22.str - SeqTrace
File
Traces
Sequences
Help
View selected trace file(s)... Ctrl+F
Add trace file(s)...
Remove selected trace file(s)
Find and mark forward/reverse
Group selected forward/reverse files
Ungroup forward/reverse files
Auto-group trace files
Expand all groups
Collapse all groups
Auto-group all trace files
Auto-group selected trace files
Trace
Seq. Saved
Use Seq.

3.5.4. Generate the consensus sequences for all paired traces by selecting “Sequences” from the top of the screen, then “Generate finished sequences,” then “For all trace files.” If a conformational dialog box appears, select “Yes.”



3.5.5. At this point, you may view the consensus sequences by selecting a trace file in the main window and then clicking the 🔍 icon. The alignment length and the consensus sequence length are both displayed at the bottom of the Trace View window. The forward and reverse electropherograms are displayed at the top of the window, and below them is the consensus sequence. Clicking on a pair of bases in the consensus sequence causes those bases to be highlighted in the electropherograms.


Trace View: R10094016sf.ab1, R10094016sr.ab1
File
Edit
View
Both:
100%
Y:
☑ conf. scores
Forward
C T T A T C C T T T G T T G C C A G C G A T T C G G T C G G G A A C T C A A A
Reverse
C T T A T C C T T T G T T G C C A G C G A T T C G G T C G G G A A C T C A A A

```
TTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGA
TTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGA
TTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGA
```

alignment length: 767 b | working seq. length: 642 b

3.6. **Exporting and Quality-Checking Consensus Sequences**

3.6.1. For a large number of sequences, it would be inconsistent, unreliable, and extremely time-consuming to rely on manual inspection of every consensus sequence for markers of quality, such as the sequence length and lack of ambiguities. For this reason, we have a script called *ambiguityfinder* that examines the consensus sequences and output their length along with a list of the ambiguous bases present in the consensus.

3.6.2. To export all of your consensus sequences as a multi-FASTA file, click the icon at the top of the main SeqTrace window. Save this file into the folder …FOOD\FOOD-MQIP\Zeus\ambiguityfinder on the Food Science network drive. You may also save the file elsewhere and copy it into this folder.

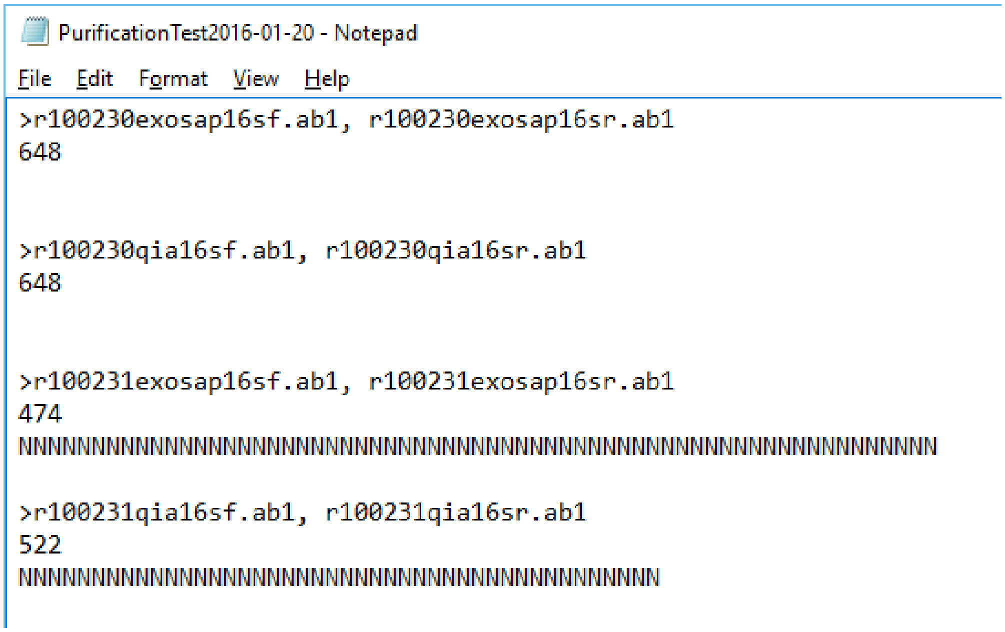
3.6.2.1. Do not include spaces in your file name. *Ambiguityfinder* cannot handle these. Dashes and underscores are fine.

3.6.3. Within 10 seconds of the FASTA file being in the *ambiguityfinder* folder, a *.txt file should appear with the same filename as your original FASTA file.

3.6.4. *Ambiguityfinder* will return one of two outputs for each consensus sequence in the multi-FASTA file:

3.6.4.1. If only the consensus sequence length is displayed, there are no ambiguities in the consensus sequence.

3.6.4.2. If the consensus sequence length is displayed and the next line contains a string of letters consisting of any of the ambiguity codes listed in §3.7.4.2, the consensus contains the listed ambiguities.


PurificationTest2016-01-20 - Notepad
File Edit Format View Help

```
>r100230exosap16sf.ab1, r100230exosap16sr.ab1
648


>r100230qia16sf.ab1, r100230qia16sr.ab1
648


>r100231exosap16sf.ab1, r100231exosap16sr.ab1
474
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

>r100231qia16sf.ab1, r100231qia16sr.ab1
522
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

3.7. **Editing Consensus Sequences in SeqTrace**

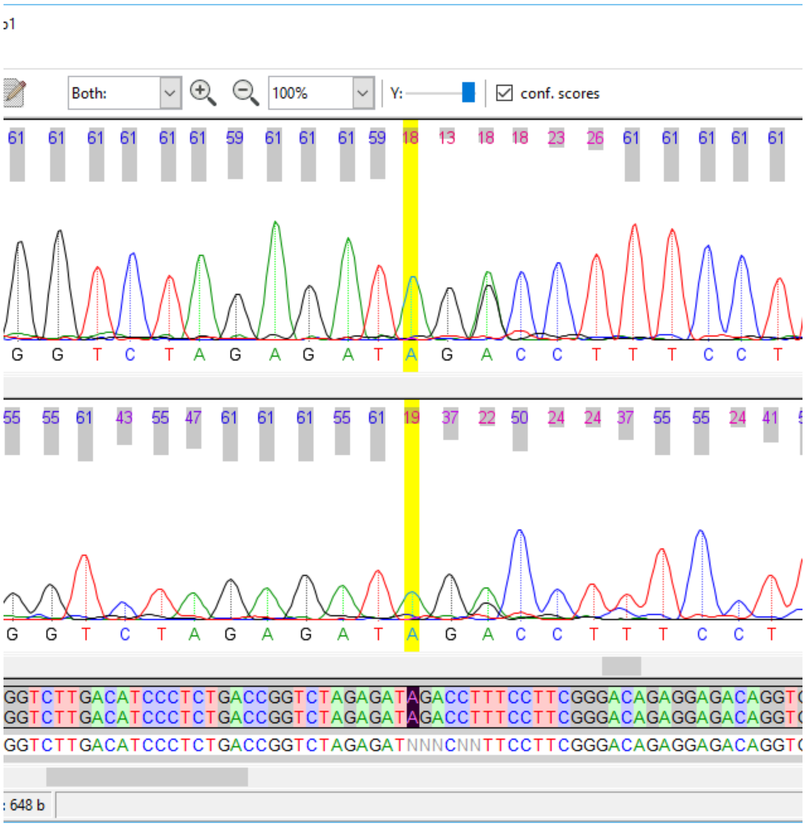
3.7.1. With the information obtained from *ambiguityfinder*, return to SeqTrace.

3.7.2. You may choose to delete the consensus sequences below a certain length from the project file by clicking the small plus sign to the left of the consensus sequence in the main window, selecting the forward and reverse sequences, and then selecting "*Remove selected trace file(s)*" from the Traces menu.

3.7.3. Consensus sequences that contain ambiguities should be manually inspected to see if it is possible to correct them. Select the consensus sequence you wish to view and then click the 🔍 icon in the menu bar to open the Trace View window.

3.7.4. Scroll horizontally through the consensus sequence, looking for the N ambiguity code(s) detected by *ambiguityfinder*. When one or more is located, you may proceed in one of three ways:

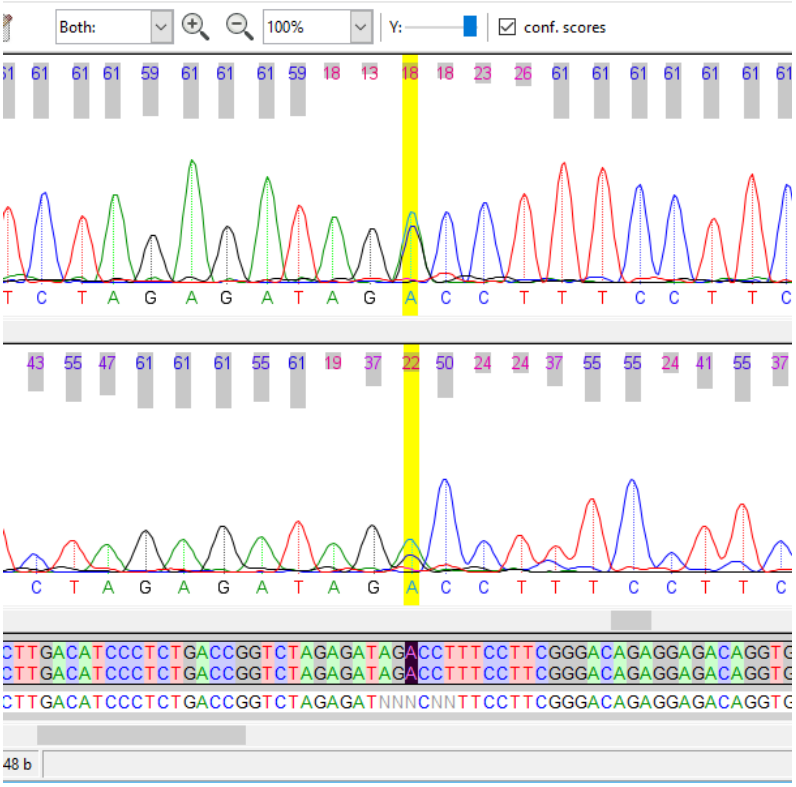
3.7.4.1. If, upon visual inspection of the electropherograms, there is no doubt that there is consensus between the forward and reverse base calls, as highlighted below, you may edit the sequence to contain this base call.



GGTCTTGACATCCCTCTGACCGGTCTAGAGATAGACCTTTCCTTCGGGACAGAGGAGACAGGT
GGTCTTGACATCCCTCTGACCGGTCTAGAGATAGACCTTTCCTTCGGGACAGAGGAGACAGGT
GGTCTTGACATCCCTCTGACCGGTCTAGAGATNNNCNNTTCCTTCGGGACAGAGGAGACAGGT

3.7.4.2. If, upon visual inspection of the electropherograms, there appears to be a disagreement between the two or if they contain double peaks, as is highlighted in the example below, the base call may be edited to contain an IUB ambiguity code:

IUPAC nucleotide code	Base
A	Adenine
C	Cytosine
G	Guanine
T (or U)	Thymine (or Uracil)
R	A or G
Y	C or T
S	G or C
W	A or T
K	G or T
M	A or C
B	C or G or T
D	A or G or T
H	A or C or T
V	A or C or G
N	A,C,T,G



```
CTTGACATCCCTCTGACCGGTCTAGAGATAGACCTTTCCTTCGGGACAGAGGAGACAGGTG
CTTGACATCCCTCTGACCGGTCTAGAGATAGACCTTTCCTTCGGGACAGAGGAGACAGGTG
CTTGACATCCCTCTGACCGGTCTAGAGATNNNCNNTTCCTTCGGGACAGAGGAGACAGGTG
```

3.7.4.3. If, upon visual inspection of the electropherograms, you can make no distinction between A, T, C, and G for a base call, you may leave it in the consensus as an N.

3.7.5. To edit the consensus sequence, click and drag to highlight the desired bases in the consensus sequence. Then click the 📝 icon at the top of the window. Type the desired bases into the dialog box and then click OK.

3.7.6. If you wish to delete bases from the consensus sequence, highlight them as described above, then click the 🗑 icon at the top of the window.

3.7.7. Once you have finished editing the sequence, click the 💾 icon at the top of the window before closing the Trace View window.

3.7.8. Repeat the above steps 3.7.3-3.7.7 to edit all consensus sequences containing ambiguity.

3.7.9. Once you believe you have addressed all instances of ambiguity, export the consensus sequences and analyze them once more using *ambiguityfinder*, as described above in §3.6. If *ambiguityfinder* finds any ambiguities that have been overlooked, repeat the above editing and rechecking process.

4. **Exporting and BLASTing Consensus Sequences**

4.1. Export all edited consensus sequences as a multi-FASTA file as described above in step 3.6.2, saving the file wherever you want to.

4.2. **BLASTing Consensus Sequences**

4.2.1. Instructions for conducting a BLAST using SequenceServer can be found on the Cornell Food Safety wiki at https://confluence.cornell.edu/pages/viewpage.action?spaceKey=MQIP&postingDay=2015%2F9%2F9&title=SequenceServer.

4.2.2. It is not necessary to follow the instructions for concatenating all sequences files into a multi-FASTA file as described in this tutorial, as SeqTrace does this automatically.

SECTION 4 – TROUBLESHOOTING

- Certain sequences that have had quality scores added post-hoc as described in §2.3 have been observed to crash SeqTrace. If SeqTrace freezes while processing your consensus sequences, look at the checkmarks in the “Use Seq.” column of the main window to see which sequence was last successfully processed. The next unchecked sequence in the list is the one causing the issue. Restart SeqTrace, delete the forward and reverse traces for this sequence, and rerun.
- Double peaks present an issue when editing using SeqTrace. KB Basecaller calls double peaks with ambiguity codes, and the quality scores for these calls typically falls below the set cutoff in SeqTrace. SeqTrace will therefore call double peaks as N in most cases, even when the ambiguity codes called in the trace files match and are legitimate. This is a particular issue for genes with multiple copies, such as 16s. Sequencher will call ambiguity codes in the consensus sequence at a lower quality score cutoff, so using Sequencher to manually edit sequences known to possess numerous double peaks may be recommended.
- If consensus sequences are too short or contain a large number of ambiguities, it is very likely that the sequences were noisy or of low relative quality. If it is desired to use them with automated editing regardless, the pros and cons of modifying the project parameters described above to obtain better results must be carefully weighed.

SECTION 5 - REFERENCES

https://code.google.com/p/seqtrace/

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3413935/

http://www.chick.manchester.ac.uk/SiteSeer/IUPAC_codes.html