

# Sanger Sequencing Best and Worst Practices

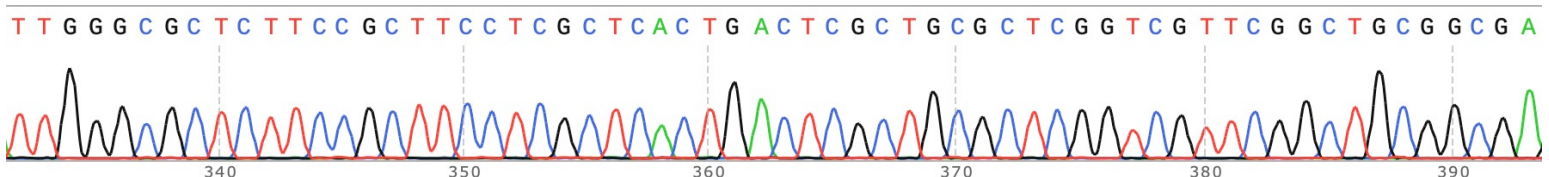
A resource for troubleshooting your Sanger Sequencing data

Sanger sequencing was first described by Fred Sanger *et al.* in 1977. In 1986, the Applied Biosystem's ABI 370A became the first automatic fluorescence-based Sanger sequencing instrument. That first instrument used a polyacrylamide gel for electrophoresis, could run 16 samples at a time, and produced about 450 bases of sequence per sample. In 1996, Applied Biosystems introduced the ABI 310, which replaced gel electrophoresis with electrophoresis in a single capillary. Improvements in chemistry increased read lengths to 600 bases. The ABI 3730 was released in 2002, and it offered the ability to sequence 48 samples at a time. The MSU Genomics Core has an ABI 3730XL, which sequences 96 samples at a time and can process many 96-well plates per day. Read lengths of 800 to 900 bases are possible with the 3730XL.

More than 45 years after the first description of Sanger sequencing and over 35 years since the first automated Sanger sequencing instrument was first introduced, Sanger sequencing is still an important tool for molecular genetics. While automated Sanger sequencing is extremely convenient, it has been around for so long that many researchers take it for granted. To keep costs low, most Sanger sequencing facilities prepare 1/16<sup>th</sup> reactions, which use very small amounts of template, primer and fluorescent reaction mix. The nature of these small volume reactions means that when templates and primers are not present at prescribed levels, the quality of the sequence data may suffer.

In the RTSF Genomics Core, we have seen many examples of how improper template or primer preparation can affect Sanger sequencing quality. This Sanger Sequencing guide has been developed to show best and worst practices when performing Sanger Sequencing. This guide shows examples of good- and poor-quality sequence. Explanations are provided about how results were obtained. In particular, examples of poor-quality sequences are provided to help researchers to troubleshoot their own Sanger sequencing results.

The pGEM-3Zf(+) control (supplied with BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, part number 4337456 or Promega, part number P2271) was used as the template for the examples in this guide (except where noted). Sequence Scanner 2 software (free from ThermoFisher) was used to view the results (.ab1 files) and capture images for this guide. There are many options available to view Sanger Sequencing (.ab1) files. The Genomics Core does not recommend a particular software. You will have to determine which software works best for your situation. A non-exhaustive list of available software is available on page 27.



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# Best Practices

The RTSF Genomics Core's Sanger Sequencing sample requirements are briefly described below. For detailed requirements, please see the following webpage:

<https://rtsf.natsci.msu.edu/genomics/sample-requirements/sanger-sequencing-sample-requirements.aspx>

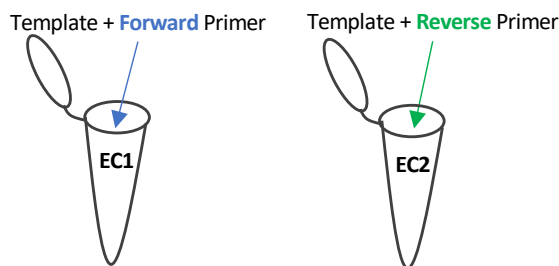
The RTSF Genomics Core accepts samples in which the researcher has already added their own primer, or the researcher can submit the sample and request that the Core add one of six available in-house primers (M13 forward, M13 reverse, SP6, T7, T7 terminator, or T3).

Fluorometric quantification, such as Qubit, is recommended. NanoDrop concentrations are inaccurate and often overestimate sample concentration by 10-fold or more. The most common culprit of poor sequencing results is insufficient template. The recommended DNA and primer concentrations are given in the table below.

Sample Type & Size	DNA Mass (ng)*	When primer is added by RESEARCHER		When primer is added by GENOMICS CORE	
		Volume of 10 $\mu$ M primer to add ( $\mu$ l)	Total Volume ( $\mu$ l)	Volume of 10 $\mu$ M primer to add ( $\mu$ l)	Total Volume ( $\mu$ l)
Plasmid					
Single-stranded DNA	200	3	12	0	8
Double-stranded DNA (up to 10 kb)	1000	3	12	0	8
Purified PCR Product					
<100 – 200 bp	2 - 6	3	12	0	8
200 – 500 bp	6 - 15	3	12	0	8
500 – 1000 bp	10 - 40	3	12	0	8
1000 – 2000 bp	20 - 80	3	12	0	8
>2000 bp	80 - 200	3	12	0	8

\*Please note that these are general recommendations and optimization may be required by the researcher.

To sequence in both the forward and reverse direction, two reactions are required. One primer is added per reaction.

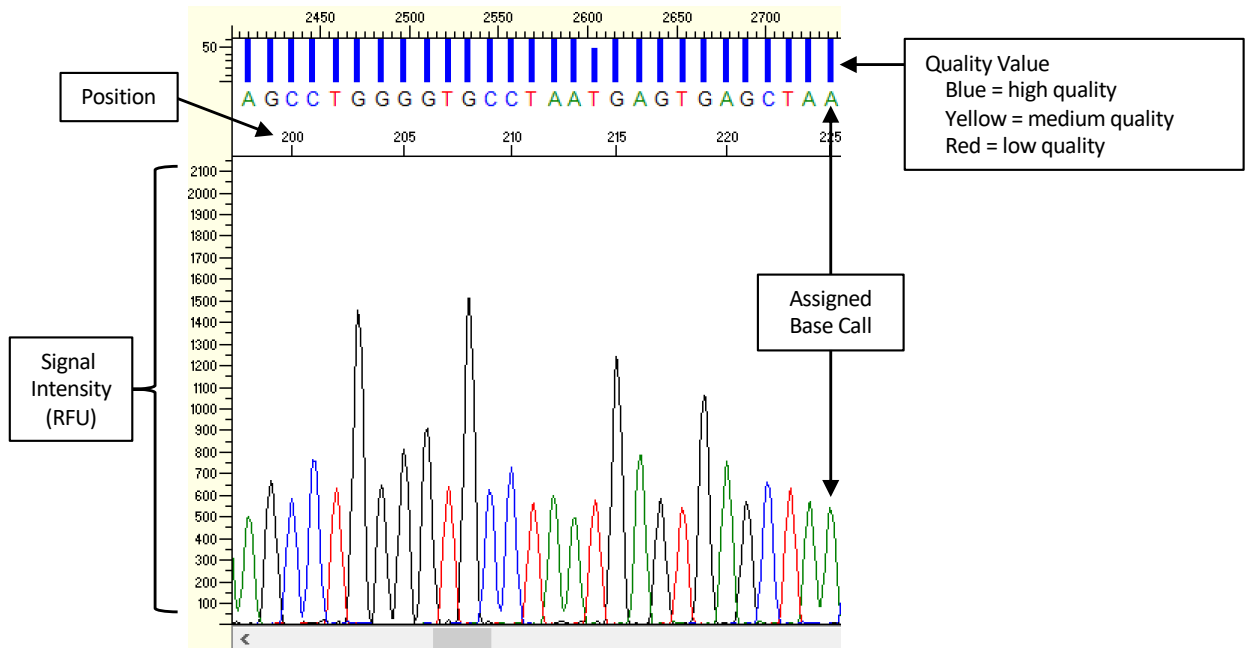


For PCR products it is crucial to purify the products to remove remaining primers, unincorporated nucleotides, enzyme, buffer, and salts. Recommended purification methods include bead or column-based purification, enzymatic cleanup (i.e. ExoSAP-IT), or gel extraction. Additionally, it is important to visualize PCR products on an agarose gel to confirm that only one product has been generated. Reactions containing multiple PCR products (i.e. desired product and an off-target product) will result in unusable data.

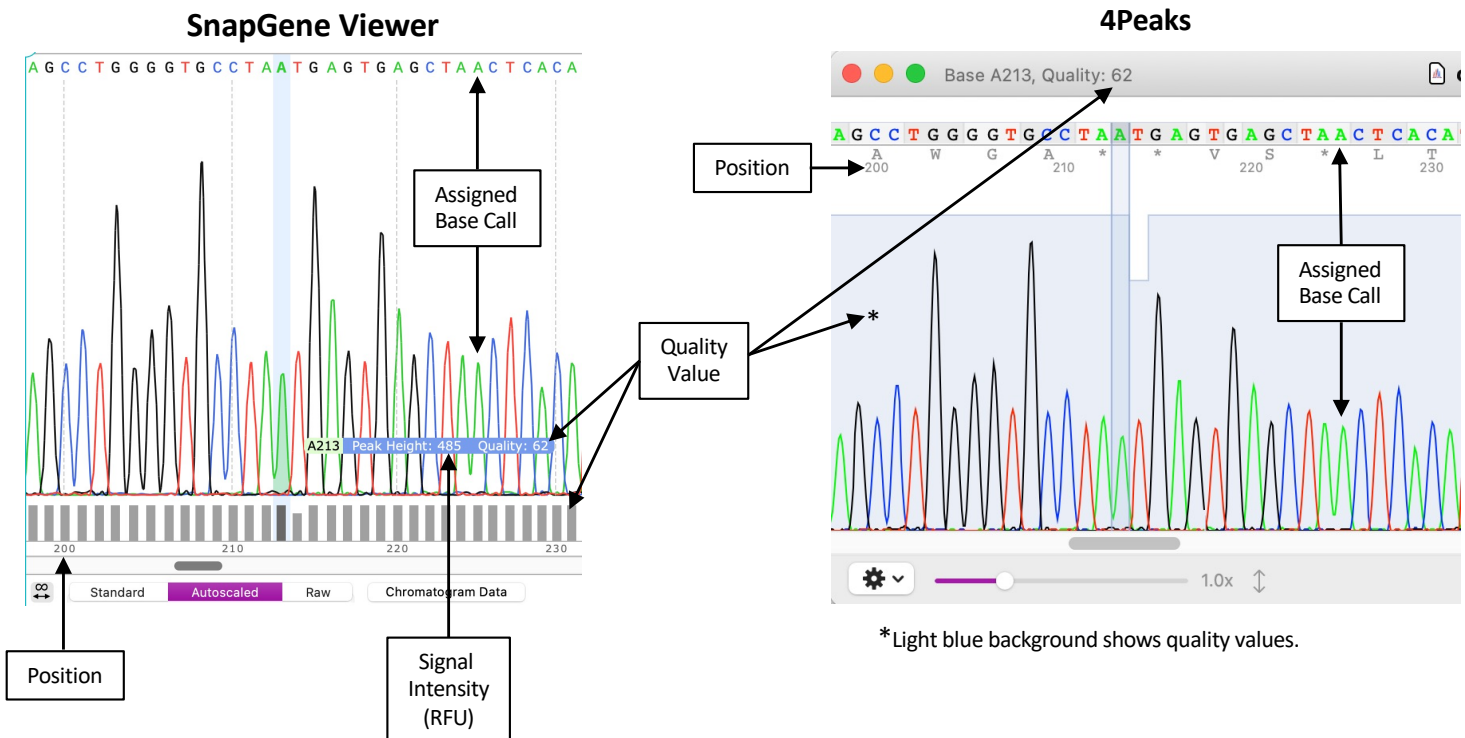
The Genomics Core will return the Sanger sequencing result in the form of an .ab1 file. One .ab1 file is returned per reaction. Results are uploaded to Genomics Depot for researchers to download. It is the responsibility of the researcher to download and save all data files. The Genomics Core will retain data for only 6 months from time of collection

# Anatomy of the Electropherogram

The electropherogram below is labeled to show the various aspects that should be reviewed when assessing the quality of the sequence. The sequence immediately below was viewed with Sequence Scanner 2 software. Sequence Scanner 2 was used to collect the images for this guide, except where noted.



Other software (free or paid) is available for viewing Sanger Sequencing data. The same sequence from the image above was viewed with two other (free) software programs, SnapGene Viewer (left image) and 4Peaks (right image), to show how they compare to Sequence Scanner 2.



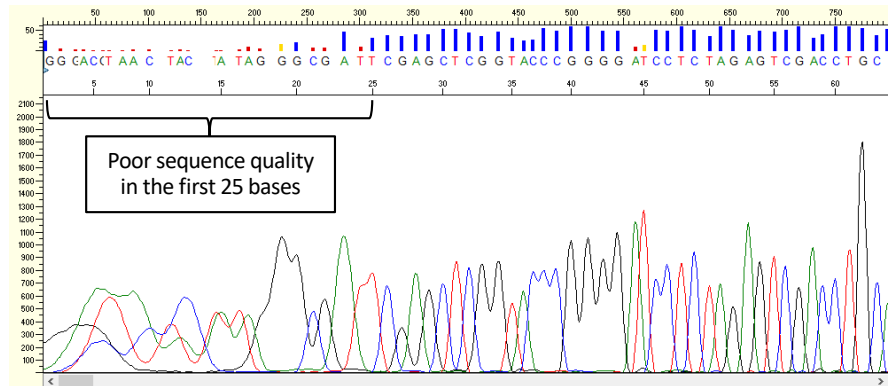
# Assessing your Sanger Sequencing results

When reviewing your Sanger sequencing results, it is important to **visually** review the electropherogram, sequence quality, and signal intensity to determine if your results are high quality. Start by viewing the electropherogram.

## Basic Expectations of the Electropherogram

### The beginning of the sequence

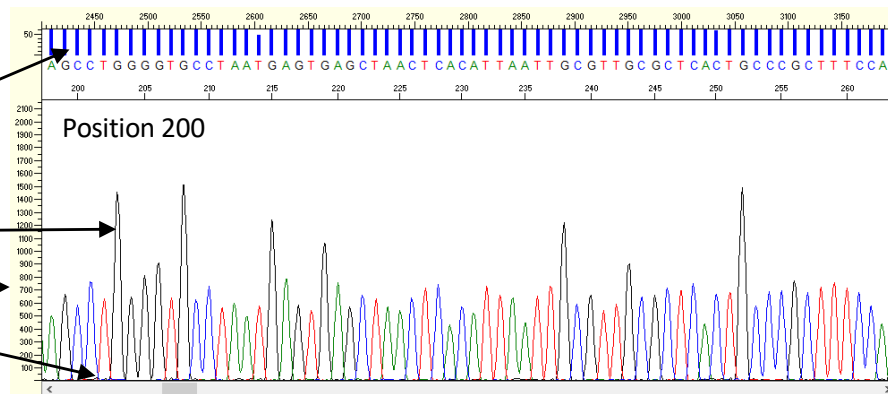
The quality of the first 25 to 35 bases of the sequence is expected to be poor. In this electropherogram, the quality is poor through the first 25 bases. After the initial poor-quality bases, the quality improves, the peaks are sharp and there is no background noise.



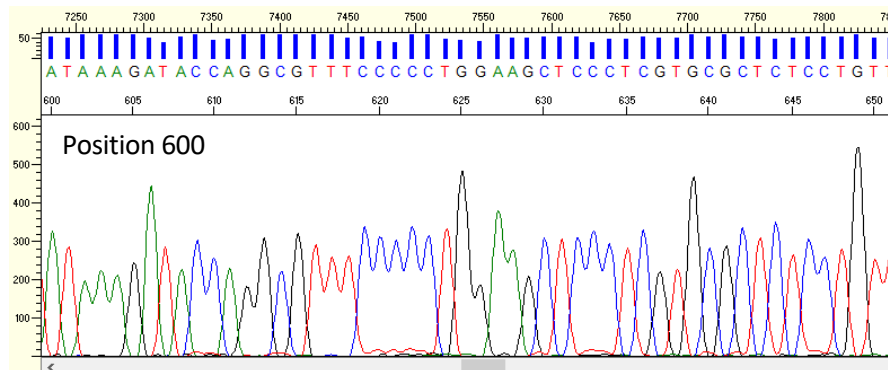
### The middle of the sequence

After the first 25-35 bases of a high-quality sample, you should expect:

- high quality values
- sharp peaks
- good signal intensity
- very little to no background noise

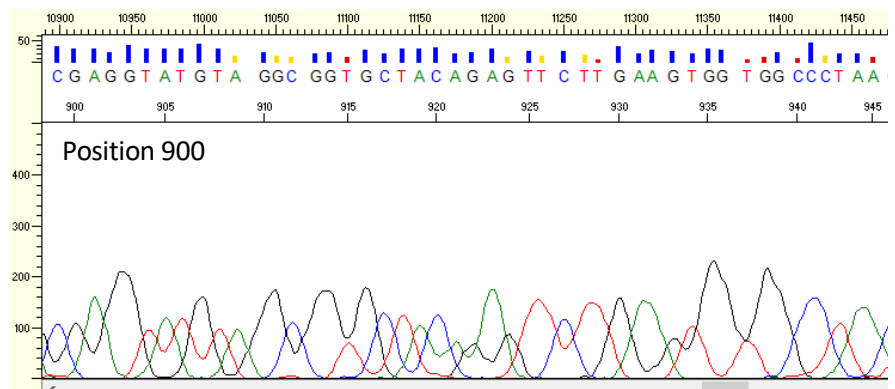


As the sequence continues, the signal intensity decreases. In the figure to the right, the signal intensity has dropped noticeably by position 600. The quality is still high – the peaks are well defined, the signal intensity is >175 RFU, and there is little to no background noise.



### The end of the sequence

Sequence lengths of 800 to 900 bases are possible for high-quality templates. It is common for the the signal intensity and quality to decrease as the read gets longer. In the electropherogram to the right, the signal intensity is lower than it was at the beginning and middle of the read, the quality is beginning to drop, and the peaks are broader. While sequence beyond 900 bases may be useful, those bases should be interpreted with caution.

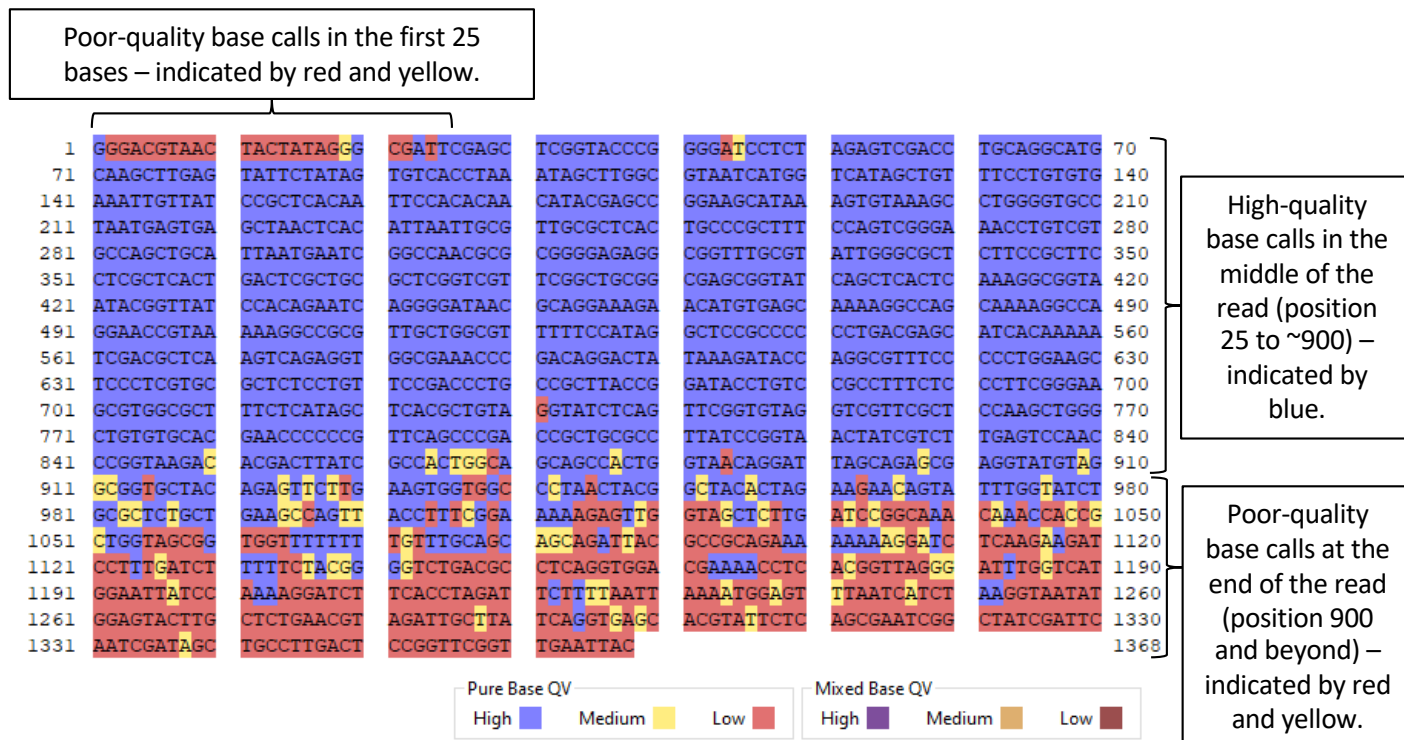




## Sequence and Base Call Quality

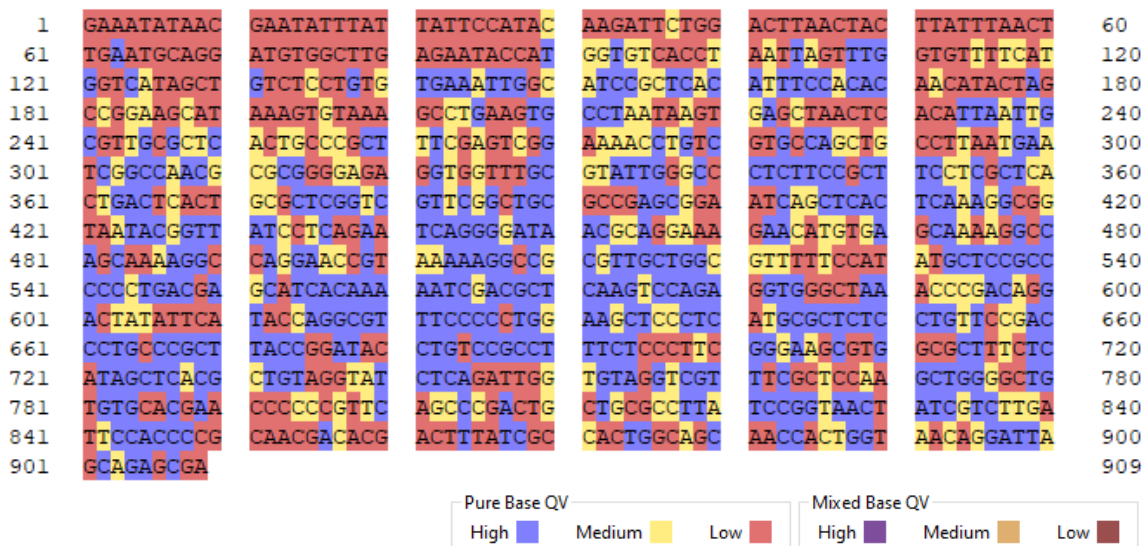
The base call quality can be assessed by looking at the quality bars on electropherogram itself, or if your program allows, you can review the base call quality in conjunction with the sequence as seen below. The sequence immediately below is the same sequence that was viewed in the electropherograms on the previous page.

### Example of a high-quality sequence



### Example of a poor-quality sequence

The base calls are low to medium quality throughout the entire sequence – indicated by red and yellow. There are very few consecutive high-quality base calls (blue). **This sequence is unusable.**



## Signal Intensity

The average raw signal intensities should be between 175 to 10,000 RFU (relative fluorescence units). Data with average raw signal intensities below 175 RFU or above 10,000 RFU often result in poor-quality data and should not be used. The example on the left has signal intensities in the acceptable range. The example on the right has very low signal intensities – this sequence data was unusable.

### Good Signal Intensities

▼ Data Analysis	
Basecaller	KB.bcp
Basecaller Version	KB 1.4.1.8
Mobility File	KB_3730_POP7_BDTv3.mob
Basecall Date/Time	2023-09-05 07:28:20 -04:00
Total # of Scans Collected	29961
Basecall Start Scan#	2183
Basecall Stop Scan#	25953
Peak 1 Scan#	2181
Base Spacing	20.1
Average Raw Signal Intensity	A(890), C(1324), G(664), T(1411)
Average Noise	A(7), C(7), G(6), T(8)
Average Raw Signal to Noise Ratio	A(121), C(193), G(117), T(167)
Trace Score	39
Contiguous Read Length	1001
QV20+ (# Bases w QV >=20)	1002

### Low/Poor Signal Intensities

▼ Data Analysis	
Basecaller	KB.bcp
Basecaller Version	KB 1.4.2.5
Mobility File	KB_3730_POP7_BDTv3.mob
Basecall Date/Time	2023-10-09 07:25:24 -05:00
Total # of Scans Collected	29961
Basecall Start Scan#	2260
Basecall Stop Scan#	25789
Peak 1 Scan#	2258
Base Spacing	20.18
Average Raw Signal Intensity	A(56), C(68), G(39), T(83)
Average Noise	A(9), C(10), G(7), T(13)
Average Raw Signal to Noise Ratio	A(5), C(6), G(5), T(6)
Trace Score	13
Contiguous Read Length	5
QV20+ (# Bases w QV >=20)	106

## Quality Values

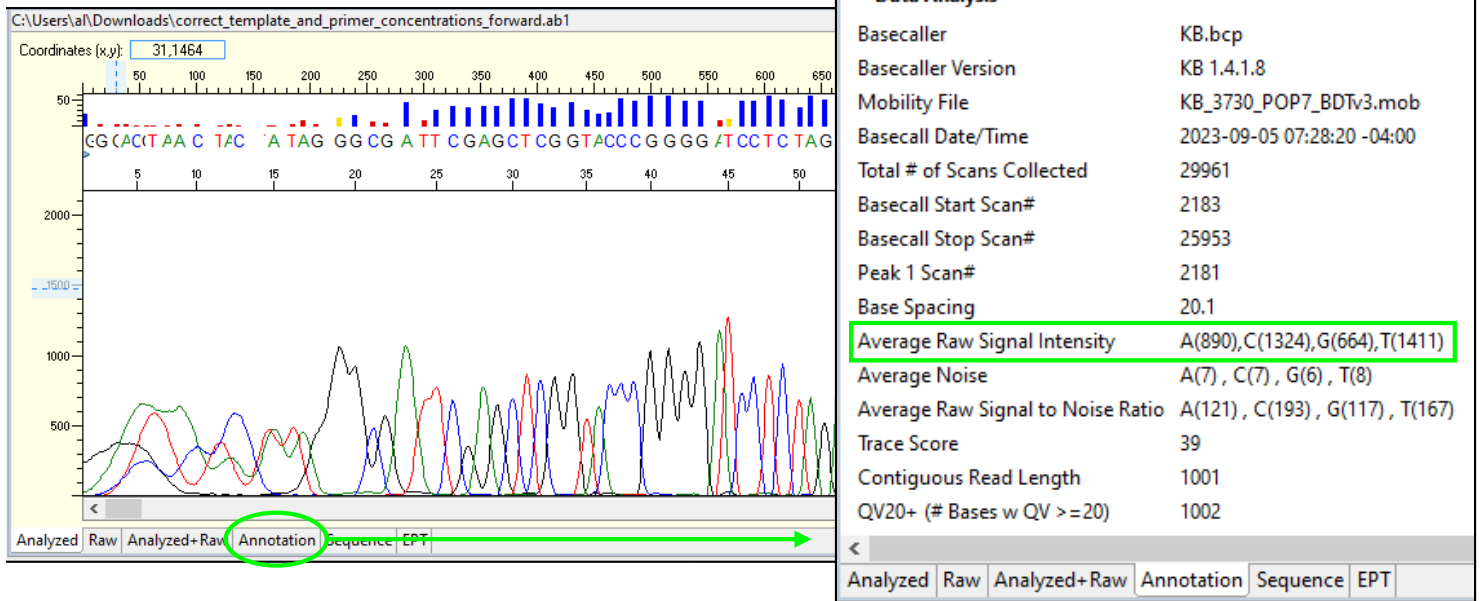
A quality value (QV) is given for each base. The QV is the probability of a basecall error. For example, a QV of 20 means that the error rate is 1% for that basecall.

<b>Quality Value = <math>-10\log_{10}(P_e)</math></b>					
Pe is probability of error					
KB basecaller generates QVs from 1 to 99					
Typical high quality pure bases will have QV 20- 50					
Typical high quality mixed bases will have QV 10-50					
Size and color of QVs bars are identical for QVs 50-99					
QV	Pe	QV	Pe	QV	Pe
1	79%	21	0.790%	41	0.0079%
2	63%	22	0.630%	42	0.0063%
3	50%	23	0.500%	43	0.0050%
4	39%	24	0.390%	44	0.0039%
5	31%	25	0.310%	45	0.0031%
6	25%	26	0.250%	46	0.0025%
7	20%	27	0.200%	47	0.0020%
8	15%	28	0.150%	48	0.0015%
9	12%	29	0.120%	49	0.0012%
10	10%	30	0.100%	50	0.0010%
11	7.9%	31	0.079%	60	0.0001%
12	6.3%	32	0.063%	70	0.00001%
13	5.0%	33	0.050%	80	0.000001%
14	4.0%	34	0.040%	90	0.0000001%
15	3.2%	35	0.032%	99	0.00000012%
16	2.5%	36	0.025%		
17	2.0%	37	0.020%		
18	1.6%	38	0.016%		
19	1.3%	39	0.013%		
20	1.0%	40	0.010%		

# Where do I find the signal intensity?

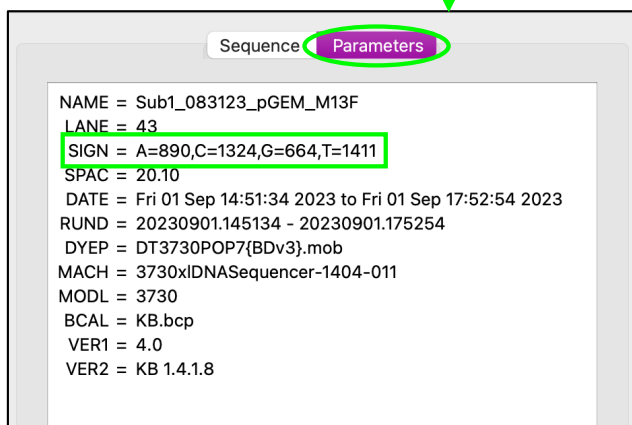
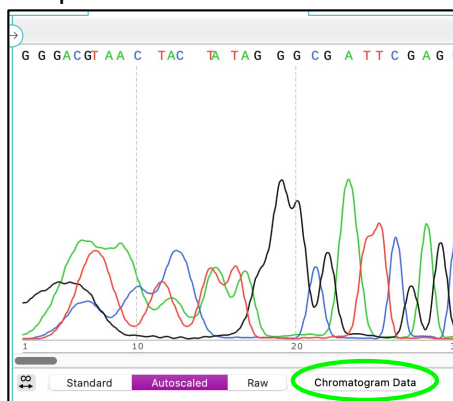
## Sequence Scanner 2

Open the file in Sequence Scanner 2; click “Annotation” at the bottom of the screen; and the window will switch to the annotation view.



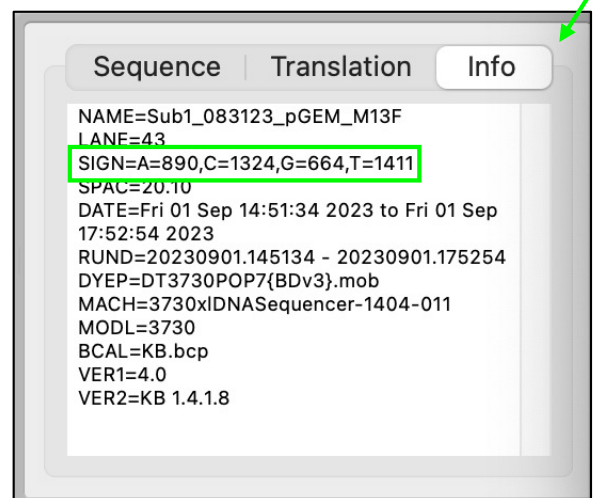
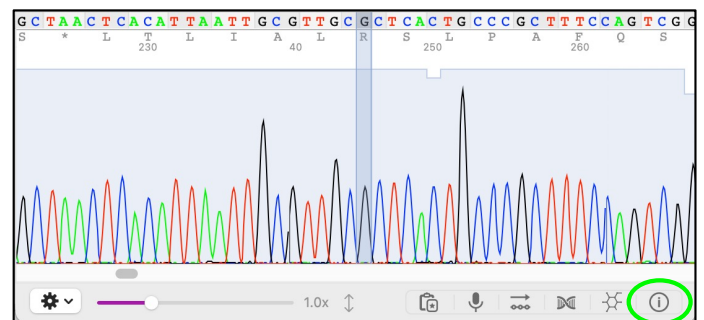
## SnapGene Viewer

Open the file in SnapGene Viewer; click “Chromatogram Data” at the bottom of the window. Click “Parameters” when the new window opens.



## 4Peaks

Open the file in 4Peaks; click the “i” icon in the bottom right corner and a new window will open.





# Example 1: Correct template and primer concentration (plasmid)

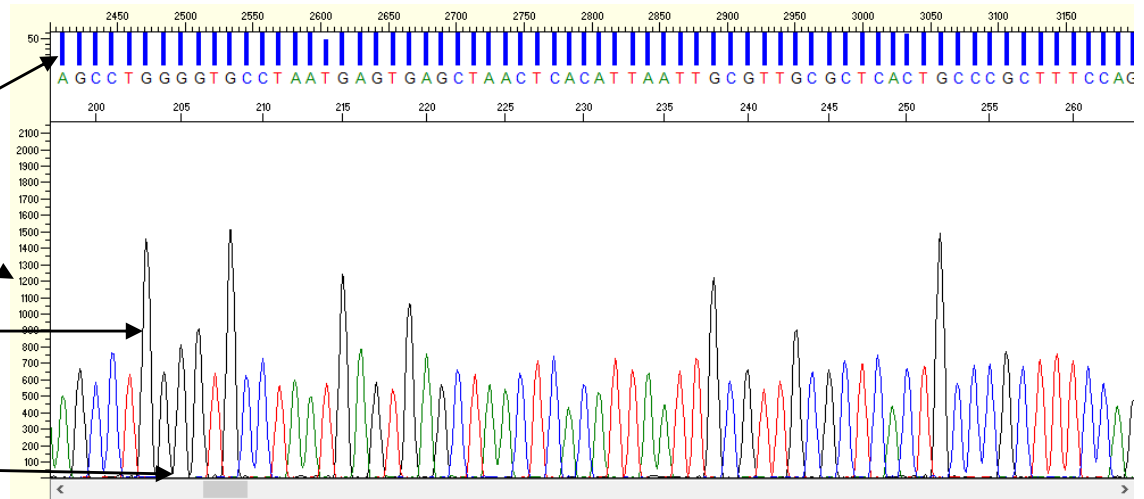
This is an example of a good sequencing reaction that resulted in high quality data. The template source is the pGEM-3Zf(+) control; a double stranded DNA plasmid that is 3,197 bp.

Template Mass	Number of Primers Added	Volume of 10µM Primer	Total Volume	File Name
1000 ng	1	3 µl	12 µl	1_correct_plasmid.ab1

## Position 200

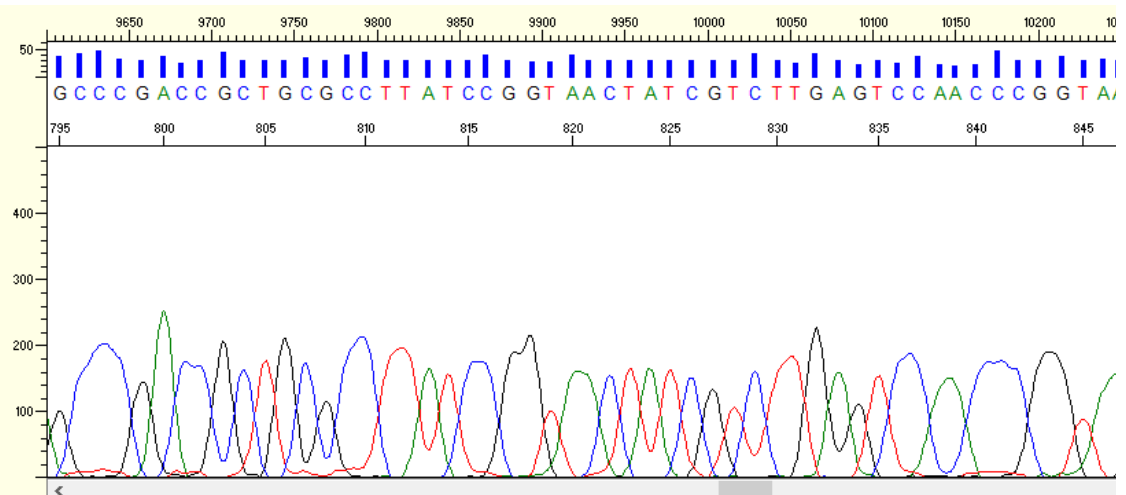
The criteria that make this a high-quality sequence are:

- Quality values are high throughout the read
- Signal intensity is > 175 RFU
- Each position only has one peak (i.e. one assigned base call)
- There is no background noise (i.e. peaks along the bottom of the electropherogram)



## Position 795

At position 795, the signal intensity has decreased, but continues to be ~200 RFU. The quality values continue to be high, each position has only one peak (i.e. assigned base call) and there is no background noise.



## Sequence Annotation and Quality

The base calls in blue are high-quality bases with only one base being called. The sequence quality begins to drop at base 909 (yellow and red bases).

1	GGGACGTAAC	TACTATAGGG	CGATTGAGC	TCGGTACCGG	GGGATCCTCT	AGAGTCGACC	TGCAGGCATG	70
71	CAAGCTTGAG	TATCTATAG	TGTCACCTAA	ATAGCTTGGC	GTAATCATGG	TCATAGCTGT	TTCTGTGTGT	140
141	AAATTGTTAT	CCGCTCACAA	TTCCACACAA	CATACGAGCC	GGAAAGCATAA	AGTGTAAGC	CTGGGGTGCC	210
211	TAATGAGTGA	GCTAACTCAC	ATTAATTCGG	TTGCGCTCAC	TGCCCGCTTT	CCAGTCGGGA	AACCTGTCTG	280
281	GCCAGCTGCA	TTAATGAATC	GGCCAAACGG	CGGGGAGAGG	CGGTTTCCGT	ATTGGGCGCT	CTTCGCGCTT	350
351	CTCGCTCACT	GACTCGCTGC	GCTCGCTGCT	TCCGCTGCGG	CGAGCGGTAT	CAGCTCACTC	AAAGGCGGTA	420
421	ATACGGTTAT	CCACAGAATC	AGGGGATAAC	CGAGGAAAGA	ACATGTGAGC	AAAAGGCCAG	CAAAAGGCCA	490
491	GGAAACGTA	AAAGCCCGCG	TTGCTGGCGT	TTTTCCATAG	GCTCCGCCCC	CCTGACGAGC	ATCACAAAA	560
561	TCGACGCTCA	AGTCAGAGGT	GGCGAAACCC	GACAGGACTA	TAAAGTACC	AGGCGTTTCC	CCCTGGAAAG	630
631	TCCTCTGTC	GCTCTCTGT	TCCGACCTGT	CCGCTTACCG	GATACCTGTC	CGCCTTCTC	CCTTCGGGAA	700
701	CGGTGGCGCT	TTCTCATAGC	TCACGCTGTA	GGTATCTCAG	TTCCGTTGAG	GTCGTTCCGT	CCAAGCTGGG	770
771	CTGTGTGAC	GAAACCCCGC	TTGAGCCCGA	CCGCTGCGCC	TTATCCGGTA	ACTATCGTCT	TGAGTCCAC	840
841	CCGGTAAGAC	ACGACTTATC	GCCACTGGGA	GCGAGCACTG	GTAACAGGAT	TAGCAGAGCG	AGGTATGTAG	910
911	CCGGTCTAC	AGAGTTCTG	AAGTGGTGGC	CCTAACTACG	GCTACACTAG	AAAGACAGTA	TTTGTATCT	980
981	CGGCTCTGCT	GAAAGCCAGT	ACCTTTCGGA	AAAAGAGTTG	GTAGCTCTTG	ATCCGGCAAA	CAAAACCCG	1050
1051	CTGGTAGCCG	TGGTTTTTTT	TGTTTCAGC	AGCAGATTAC	GCCGCAGAAA	AAAAGGATC	TCAAGAGAT	1120
1121	CTTTGATCT	TTTTCTACCG	GCTCTGAGCC	CTCAGTTGGA	CGAAAACCTC	ACGGTTAGGG	ATTTGGTCA	1190
1191	GGAAATATCC	AAAAGGATCT	TCACCTAGAT	TCTTTTAAAT	AAAATGAGT	TTAATCATCT	AAAGTATAT	1260
1261	GGAGTACTTG	CTCTGAACGT	AGATTGCTTA	TCAGGTTGAG	ACGTATCTC	AGCGAATCGG	GTATGATTG	1330
1331	AATCGATAGC	TGCCCTGACT	CCGTTTCGTT	TGAATTAC				1368

## Signal Intensity

Acceptable range: 664 to 1411 RFU.

### Data Analysis

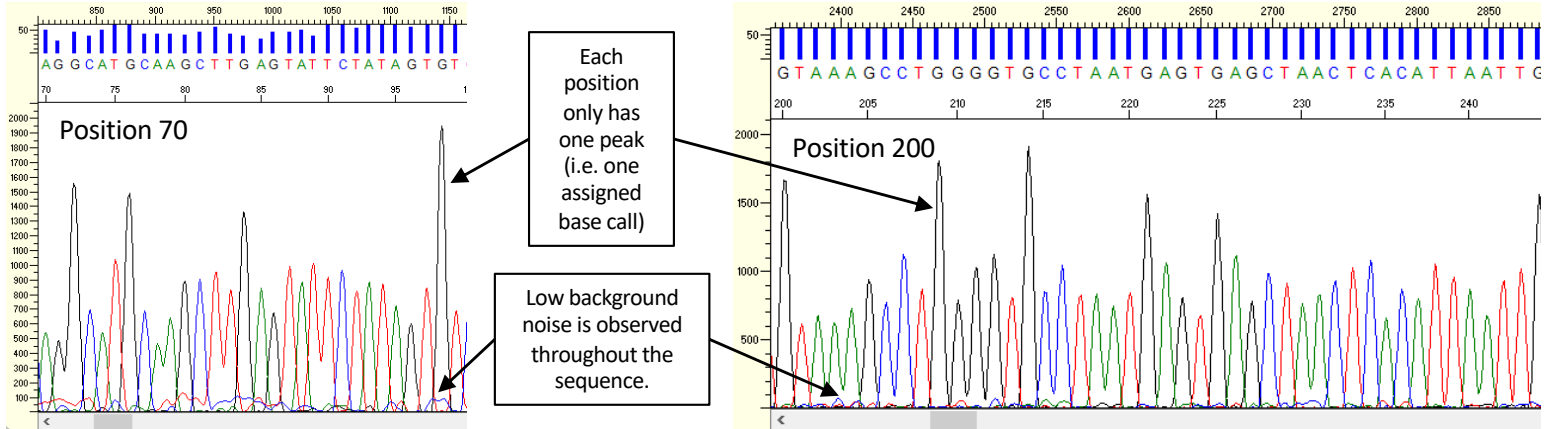
Basecaller	KB.bcp
Basecaller Version	KB 1.4.1.8
Mobility File	KB_3730_POP7_BDTv3.mob
Basecall Date/Time	2023-09-05 07:28:20 -04:00
Total # of Scans Collected	29961
Basecall Start Scan#	2183
Basecall Stop Scan#	25953
Peak 1 Scan#	2181
Base Spacing	20.1
Average Raw Signal Intensity	A(890), C(1324), G(664), T(1411)
Average Noise	A(7), C(7), G(6), T(8)
Average Raw Signal to Noise Ratio	A(121), C(193), G(117), T(167)
Trace Score	39
Contiguous Read Length	1001
QV20+ (# Bases w QV >=20)	1002

# Example 2a: 1/10<sup>th</sup> recommended template (plasmid)

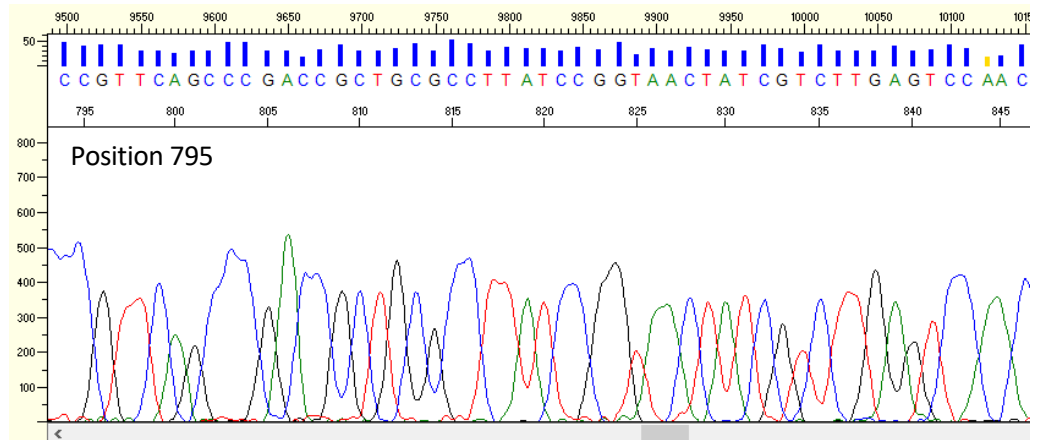
The template was diluted to 1/10<sup>th</sup> the recommendation. The primer was added at the correct concentration. The template source is the pGEM-3Zf(+) control; a double stranded DNA plasmid that is 3,197 bp.

Template Mass	Number of Primers Added	Volume of 10µM Primer	Total Volume	File Name
100 ng	1	3 µl	12 µl	2a_0.1_template_plasmid.ab1

The quality of this sequence is still good despite having a lower than recommended template mass. There are two characteristics that indicate that the template mass was lower than recommended. 1.) There is noise; these are the peaks along the bottom of the electropherogram. This is most pronounced around position 70 of this example, but it can be seen throughout the read. 2.) The signal intensities are lower than was observed in Example 1 (correct template), however, the signal intensities are still above the threshold of 175 RFU.



At position 795, the signal intensity has decreased, but continues to be >175 RFU. The quality continues to be high, and each position has only one peak (i.e. one assigned base call).



## Sequence Annotation and Quality

The base calls in blue are high quality with only one base being called. The sequence quality begins to drop around base 904 (yellow and red bases). The quality of this sequence is high, like Example 1 (correct template).

1	CAAAGATTGG	TCATCCCTAT	AGGGGCGTAT	TCGAGCTCGG	TACCGGGGA	TCCCTAGAG	TCGACCTGCA	70
71	GGCATGCAAG	CTTGAGTATT	CTATAGTGTC	ACCTAAATAG	CTTGGCGTAA	TCATGGTCAI	AGCTGTTTCC	140
141	TGTGTGAAAT	TGTTATCCGC	TCACAATTCC	ACACAACATA	CGAGCCGGAA	GCATAAAGTG	TAAAGCCTGG	210
211	GSTGCTAAAT	SAGTGAGCTA	ACTCACRTTA	ATTGCGITGC	GCTCACTGCG	CGCTTTCAG	TCGGGAACCC	280
281	TGTCGTGCCA	SGTCGATTA	TGAATCGGCC	AACGCGCGGG	SAGAGCCGGT	TTGCGTATG	GGCGCTCTC	350
351	CGCTTCCTCG	CTCACTGACT	CGTGCCTC	GGTGTTCGG	CTGCGCGGAG	CGGTATCAGC	TCACTCAAAG	420
421	CCGGTAATAC	GGTTATCCAC	AGAATCAGGG	GATAACGCG	GAAAGAACAT	GTGACAAA	GGCCAGCAA	490
491	AGGCCAGGAA	CCGTA AAAAG	CCCGCTTGC	TGGGTTTTT	CCATAGGCTC	CGCCCCCTG	ACGAGCATCA	560
561	CAAAAATCGA	CGCTCAAGTC	AGAGGTGGCG	AAACCCGACA	GGACTATAAA	GATACAGGG	GTTTCCCTCT	630
631	GGAAAGTCCC	TCGTGCGCTC	TCCTGTTCGG	ACCTTGCCGC	TTACCGGATA	CCTGTCCGCC	TTTCTCCCTT	700
701	CGGAAGCGT	GGCGCTTTCI	CATAGCTCAC	GCTGTAGGTA	TCTCAGITCG	GTGTAGTTCG	TTGCTCCAA	770
771	SCTGGCTGT	GTGCACGAAC	CCCGCGTTCA	CGCCGACCG	TGCGCTTAT	CCGTAACATA	TCGCTTTGAG	840
841	TCCAAACCGG	TAAGACACGA	CTTATCGCCA	CTGGGAGCAG	CCACTGGTAA	CAGGATTAGC	AGAGCGAGGT	910
911	ATGTAGCCGG	TCGTACAGAG	TTCTTGAAGT	GGTGGCCCTA	ACTAGGCTA	CACIAGARA	ACAGTATTG	980
981	GTATCTGGCC	TCTGCTGAAG	CCAGTTACCG	TTCCGGAAAA	AGAGTTGGT	AGCTTTGAT	CCGGGCAAC	1050
1051	AAACCACCGC	TGCTAGCCGG	TGTTTTTTTT	TTGTTTCCAA	GCACAGATT	ACGCGCAAGA	AAAAAAGGA	1120
1121	TCCTCAGAAA	GAATCCCTTT	GGATTCCTTT	TCTACGGGTC	TGACGCTCAG	GTGGCACGGA	CACCTACGTT	1190
1191	TAGGATTTGG	TCATGSGAAA	TTATCAAAG	GGATTCTTTG	CACCTAGATT	TTTTCAATA	AAATGGAGTT	1260
1261	GAATTCATCG	AGTAAATATG	AATACTGCCC	TGGACGTAGC	ATGCTTATCA	GTGAGGCACT	ATTCTAGCSA	1330
1331	ATCGGCTCTCA	AT						1342

## Signal Intensity

The Average Raw Signal Intensities are in the acceptable range.

A(194), C(232), G(230), T(241)

Sequence is good.  
No changes needed.

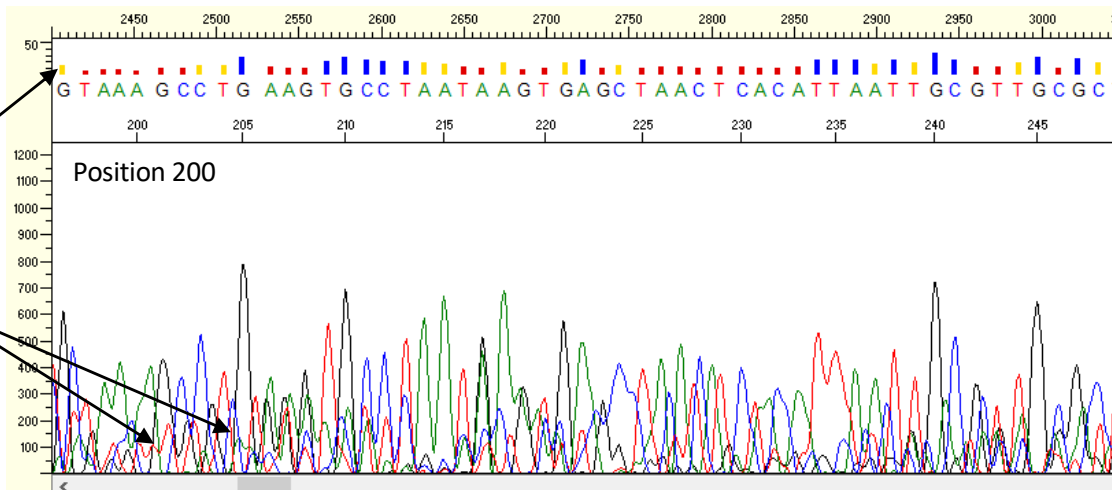
# Example 2b: 1/100<sup>th</sup> recommended template (plasmid)

The template was diluted to 1/100<sup>th</sup> the recommendation. The primer was added at the correct concentration. The template source is the pGEM-3Zf(+) control; a double stranded DNA plasmid that is 3,197 bp.

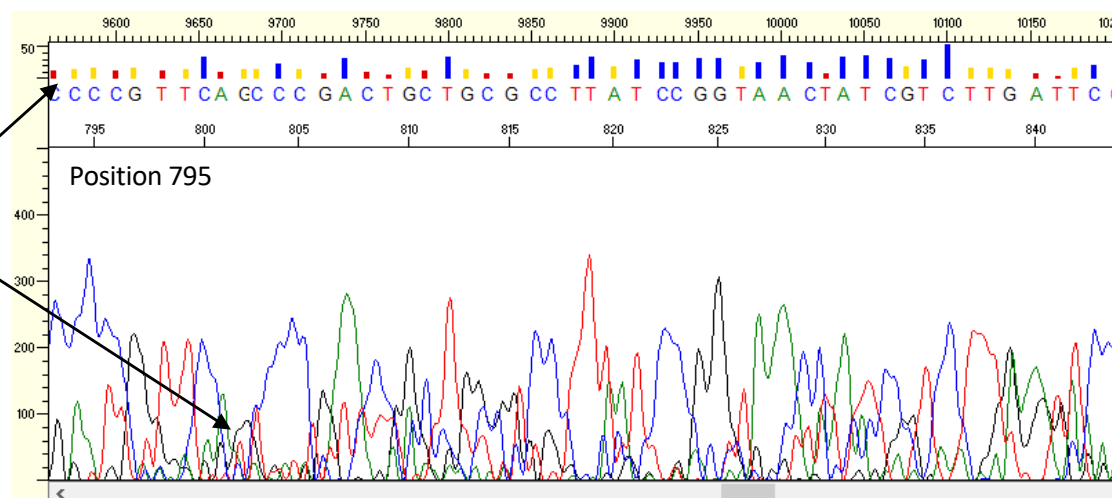
Template Mass	Number of Primers Added	Volume of 10µM Primer	Total Volume	File Name
10 ng	1	3 µl	12 µl	2b_0.01_template_plasmid.ab1

This is a poor-quality sequence that did not generate usable data.

- Quality values are low throughout the sequence.
- The background signal is high relative to the primary signal, which contributes to low quality base calls.



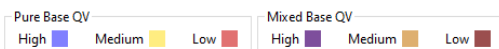
At position 795, the quality continues to be poor with multiple peaks per position as was seen at position 200 above.



## Sequence Annotation and Quality

The base calls in yellow and red are medium and low quality, respectively. Most of the sequence has medium to low quality base calls with a few bases that are high quality, but noncontinuous. **This sequence is poor quality and should not be used.**

1	GAAATATAAC	GAATATTAT	TATTCCATAC	AAGATTCTGG	ACTTAACTAC	TTATTAACT	60
61	TGAATGCAGG	ATGTGGCTTG	AGAATACCAT	GGTGTCACT	AAATTAGITG	GTGTTTTCAT	120
121	GGTCAATAGCT	GTCTCCTGTG	TGAAATTGGC	ATCCGCTCAG	ATTTCCACAC	AACATACTAG	180
181	CCGGAAGCAT	AAAGTGTAAA	GCCTGAAAGT	CCTAATAAGT	GAGCTAACTC	ACATTAAITG	240
241	CGTTGCGCTC	ACTGCCCGCT	TTCGAGTCGG	AAAACGTGTC	GTGCCAGCTG	CCTTAATGAA	300
301	TCGGCCAAAG	CGCGGGGAGA	GSTGTTTTCG	GTATTGGGCC	CTCTTCGGCT	TCCTCGCTCA	360
361	CTGACTCACT	GCCCTCGGTC	GTTCCGGCTGC	GCCGAGCGGA	ATCAGCTCAC	TCAAAGGCGG	420
421	TAATACGTTT	ATCCTCAGAA	TCAGGGGATA	ACGCAAGAAA	GAAATGTGTA	GCAAAAGGCC	480
481	AGCAAAGGC	CAGGAACCGT	AAAAAGGCCG	CGTTGCTGGC	GTTTTTCCAT	ATGCTCCGCC	540
541	CCCTGACGA	GCAATCAAAA	AATCGACGCT	CAAGTCCAGA	GSTGGGCTAA	ACCCGACAGG	600
601	ACTATATTCA	TACCAGGCGT	TTCGCCCTGG	AAGCTCCCTC	ATCCGCTCTC	CTGTTCCGAC	660
661	CCGCGCGCT	TACCGGATAC	CTGTCCGCTT	TTCCTCCCTC	GGGAAGCGTG	GCGCTTTCCT	720
721	ATAGCTCAG	CTGTAGGTAT	CTCAGATTGG	TGTAGTTCGT	TTCGCTCCAA	GCTGGGGCTG	780
781	TGTGCACGA	CCCGCGGTTT	AGCCCGACTG	CTGCGCTTAA	TCCGGTAACT	ATCGTCTTGA	840
841	TTCCACCCCG	CAACGACACG	ACTTATTCGC	CACTGGCAGC	AAACCCTSGT	AACAGGATTA	900
901	GCAGAGCGA						909



## Signal Intensity

The Average Raw Signal Intensities are below the cut off (175 RFU).

A(44),C(47),G(39),T(54)

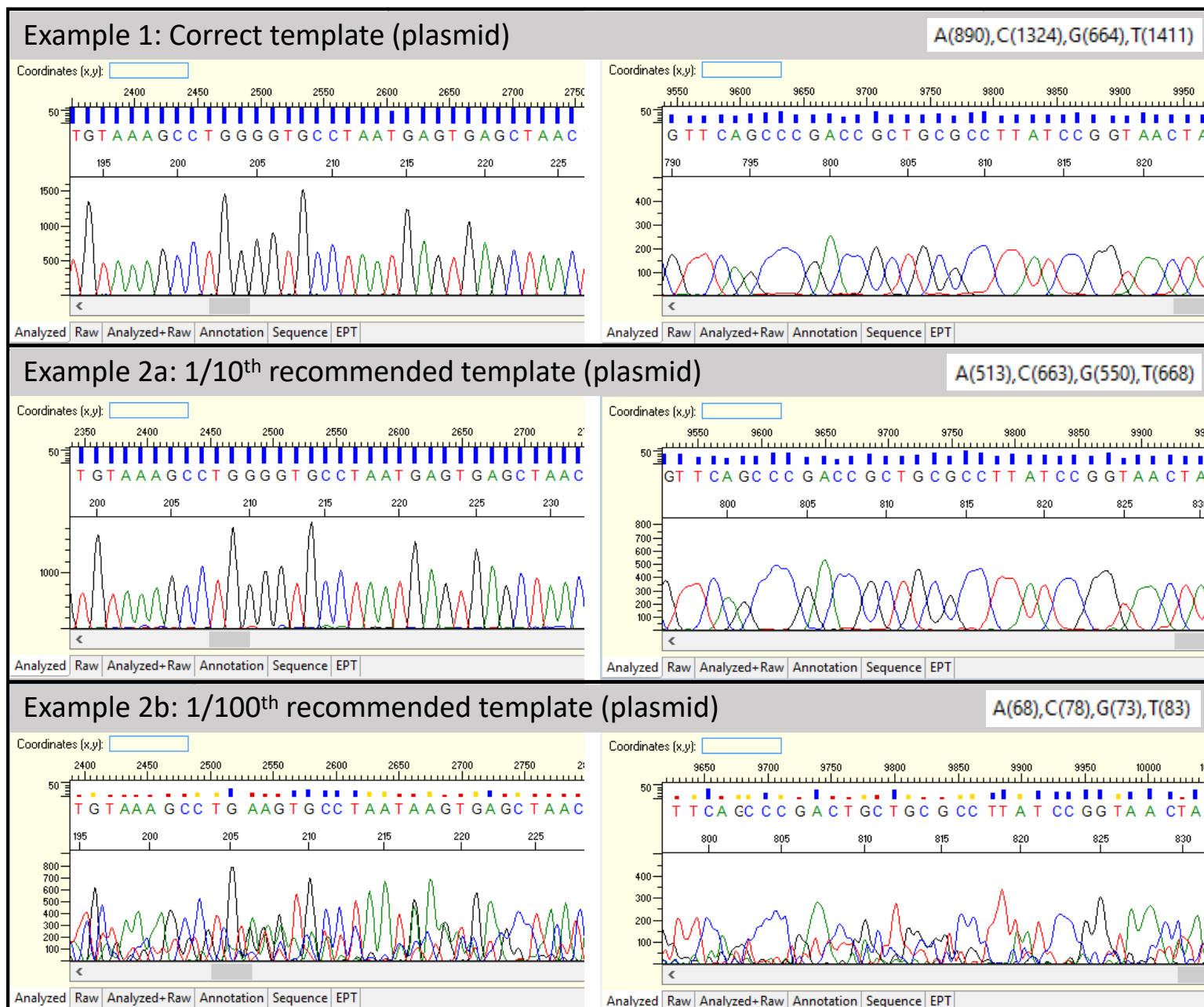
## Summary

Sequence is poor quality and should not be used.

To improve results, quantify sample with fluorometric method, such as Qubit. Submit fresh sample using recommended mass. Note that the NanoDrop is not an accurate method for quantifying nucleic acids.

# Comparison & Summary: Samples with too little template (plasmid)

The panel below shows how the quality of the sequence decreases as the amount of template decreases. The correct template mass (example 1) is on top, 1/10<sup>th</sup> the recommended template mass (example 2a) is in the middle, and 1/100<sup>th</sup> the recommended template mass (example 2b) is on the bottom. Two regions of the electropherogram are displayed: position 200 on the left and position 795 on the right. The average raw signal intensities are also displayed for each example.



Examples 1 and 2a are good quality sequences. While example 2a used 1/10<sup>th</sup> the recommended template and still achieved a good quality result, it should be noted that this is not necessarily expected or recommended. However, it does demonstrate that high quality templates may generate acceptable quality data even when deviating from recommended inputs. Example 2b is a poor-quality sequence and should not be used. Refer to pages 9 – 11 for a more detailed summary of each example.

# Example 3: Correct template and primer concentration (PCR product)

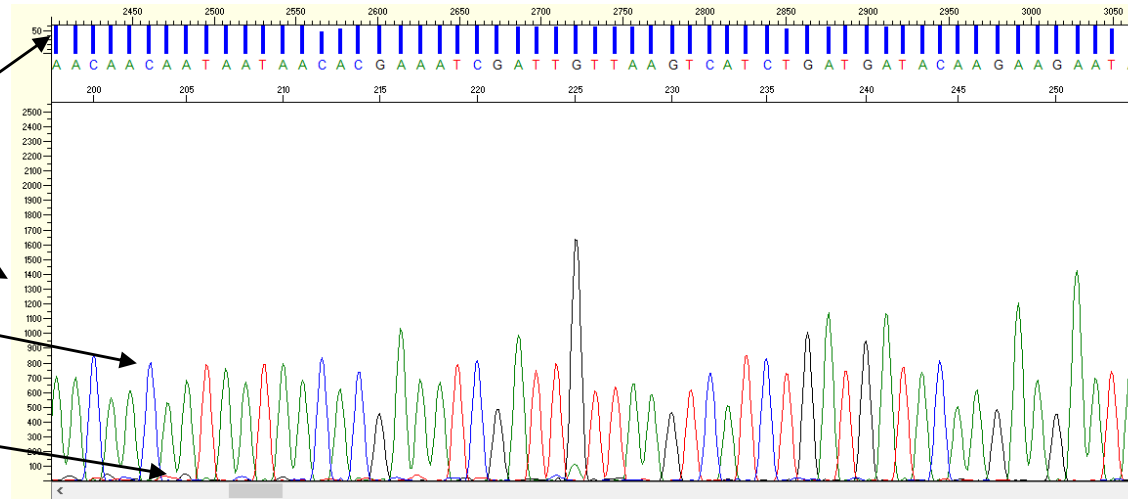
This is an example of a good sequencing reaction that resulted in high quality data. The template source is a 408 bp double stranded PCR product that was purified with ExoSAP-it.

Template Mass	Number of Primers Added	Volume of 10µM Primer	Total Volume	File Name
15 ng	1	3 µl	12 µl	3_correct_PCR_product.ab1

## Position 200

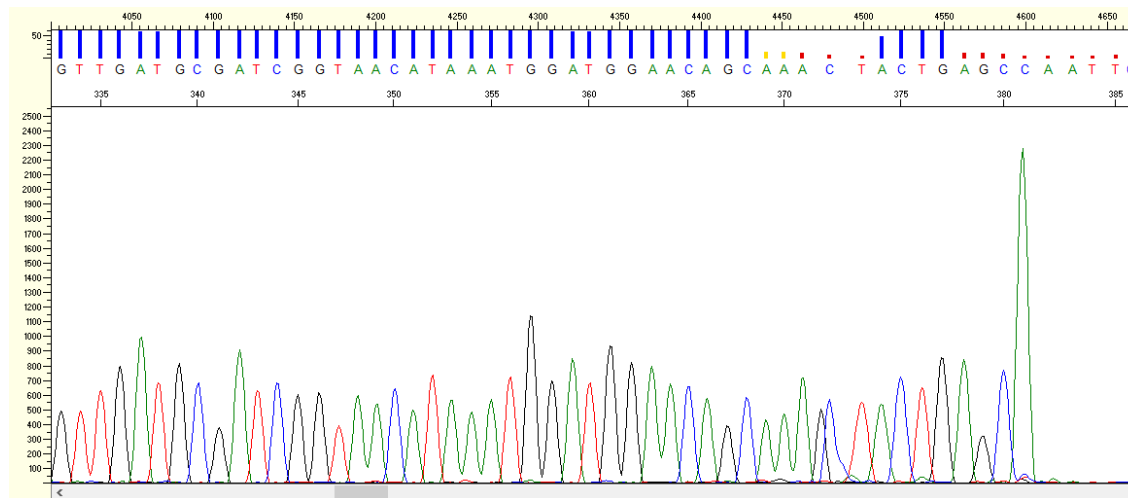
The criteria that make this a high-quality sequence are:

- Quality values are high throughout the read
- Signal intensity is > 175 RFU
- Each position only has one peak (i.e. one assigned base call)
- There is very little to no background noise (i.e. peaks along the bottom of the electropherogram)



## End of sequence

At position 381 the sequence abruptly ends. This is expected because the template is a PCR product.



## Sequence Annotation and Quality

1	GGGGGATTGG	GGGTGGGGCA	GATCGACCAG	GCGACATAAA	TGATGACAGG	AGAAGAATGC	ACCCGAGATC	70
71	TTCCTTTAAG	GCTTTTCTGG	AGGTCGTCAA	GTGGAGGAGT	GTCCCTTGGG	AAGATGTTGA	GATGGATGCA	140
141	ATCACTCGT	TGCAACTCAT	ATTGCGGGGT	TCCTTGCAAG	ACGAGATGCC	AATTGATAAC	AACAATAATA	210
211	ACACGAAATC	GATTGTTAAG	TCATCTGATG	ATACAAGAAG	AATACAATA	TATGATGAGT	TGAGAACAGT	280
281	TACTAATGAG	ATGGTTCGGC	TAATTGAGAC	AGCAACTGTC	CCTATATTGG	CAGTTGATGC	GATCGGTAAC	350
351	ATAAATGGAT	GGAACAGCAA	ACTACTGAGC	CAATTCATCT	AACATCATCT	TCTTCTTTTT	TTTTTTTTCAC	420
421	CGCCCCGGT	TGGGAGGGAA	TGAGAAACGA	ATATATAGTT	ACGGACTGTT	TTCTTCC		477

Pure Base QV: High (blue), Medium (yellow), Low (red)  
 Mixed Base QV: High (purple), Medium (orange), Low (brown)

The basecalls end at 381 in the electropherogram. Basecalls beyond this point should be ignored.

Signal Intensity is in the acceptable range

A(1522),C(863),G(950),T(1458)



# Example 4a: 1/10<sup>th</sup> recommended template (PCR product)

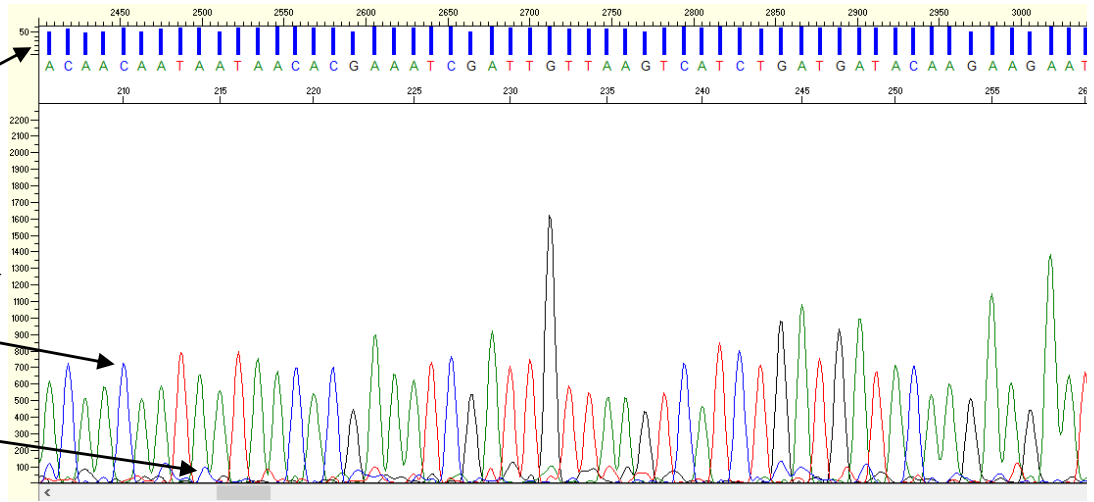
The template was diluted to 1/10<sup>th</sup> the recommendation. The primer was added at the correct concentration. The template source is a 408 bp double stranded PCR product that was purified with ExoSAP-it.

Template Mass	Number of Primers Added	Volume of 10µM Primer	Total Volume	File Name
1.5 ng	1	3 µl	12 µl	4a_0.1_template_PCR_product.ab1

## Position 210

The criteria that make this an acceptable quality sequence are:

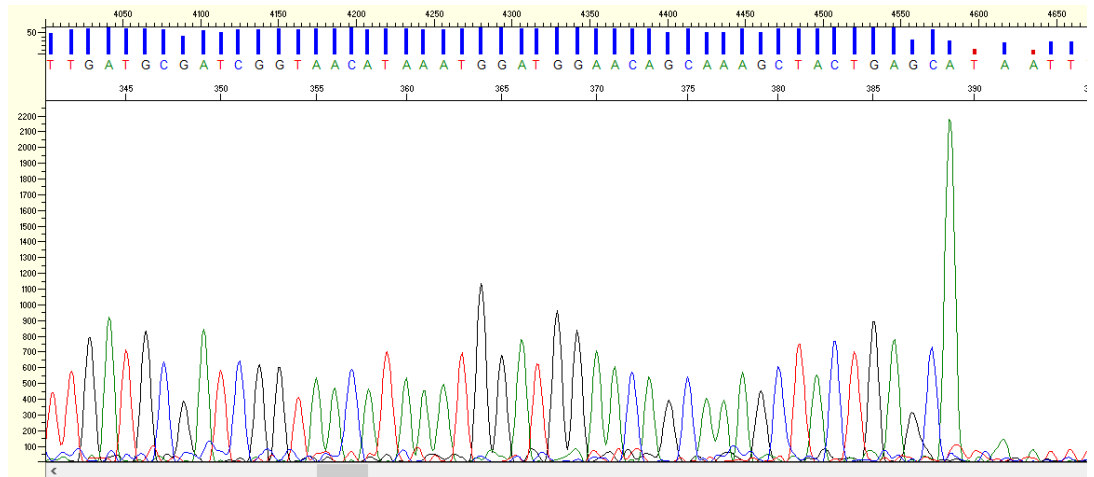
- Quality values are high throughout the read
- Signal intensity is > 175 RFU
- Each position only has one peak (i.e. one assigned base call)
- There is some background noise (i.e. peaks along the bottom of the electropherogram), but the true base call can be determined.



## End of sequence

Quality and signal intensity continue to be high until the end of the read. The background noise continues as well, but the base calls can still be determined.

At position 389 the sequence abruptly ends. This is expected because the template is a PCR product.



## Sequence Annotation and Quality

1	CGGGGGGTAA	GTGAGGTGGT	GTGGGCAGAT	CGACCAGGCG	ACATAAATGA	TGACAGGAGA	AGAATGCACC	70
71	CGAGATCTTC	CTTTAAGGCT	TTTCTGGAGG	TGTCGAAGTG	GAGGAGTGTC	CCTTGGGAAG	ATGTTGAGAT	140
141	GGATGCAATT	CACTCGTTGC	AACTCATATT	GCGGGGTTCC	TTGCAAGACG	AGATGCCAAT	TGATAACAAC	210
211	AATAATAACA	CGAAATCGAT	TGTTAAGTCA	TCTGATGATA	CAAGAAGAAT	ACAACATATAT	GATGAGTTGA	280
281	GAACAGTTAC	TAATGAGATG	GTTCCGGCTAA	TTGAGACAGC	AACTGTCCCT	ATATTGGCAG	TTGATGCGAT	350
351	CGGTAACATA	AATGGATGGA	ACAGCAAAGC	TACTGAGCAT	AATTTAATCT	ACCAGCTTCT	TTTTCTATTT	420
421	CTTTTTTATC	TCCGCCGCTT	GCTTGGAATA	AAAAACTAAC	CTATTGTTAA	CGACTGTTTT	TACTCTTCAA	490
491	GATGACAAA	ATACCAAATT	ACTCCTGCTG	TATT				524

The basecalls end at 389 in the electropherogram. Basecalls beyond this point should be ignored.

Pure Base QV: High (blue), Medium (yellow), Low (red)  
Mixed Base QV: High (purple), Medium (orange), Low (brown)

Signal Intensity is in the acceptable range

A(204), C(129), G(171), T(185)

Sequence is good.  
No changes needed.

# Example 4b: 1/100<sup>th</sup> recommended template (PCR product)

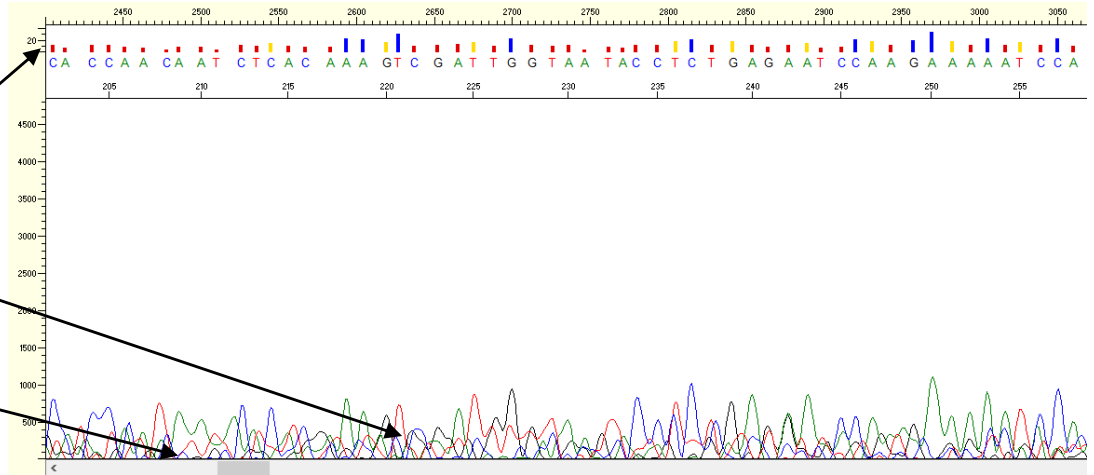
The template was diluted to 1/100<sup>th</sup> the recommendation. The primer was added at the correct concentration. The template source is a 408 bp double stranded PCR product that was purified with ExoSAP-it.

Template Mass	Number of Primers Added	Volume of 10µM Primer	Total Volume	File Name
0.15 ng	1	3 µl	12 µl	4b_0.01_template_PCR_product.ab1

## Position 205

The criteria that make this a poor-quality sequence are:

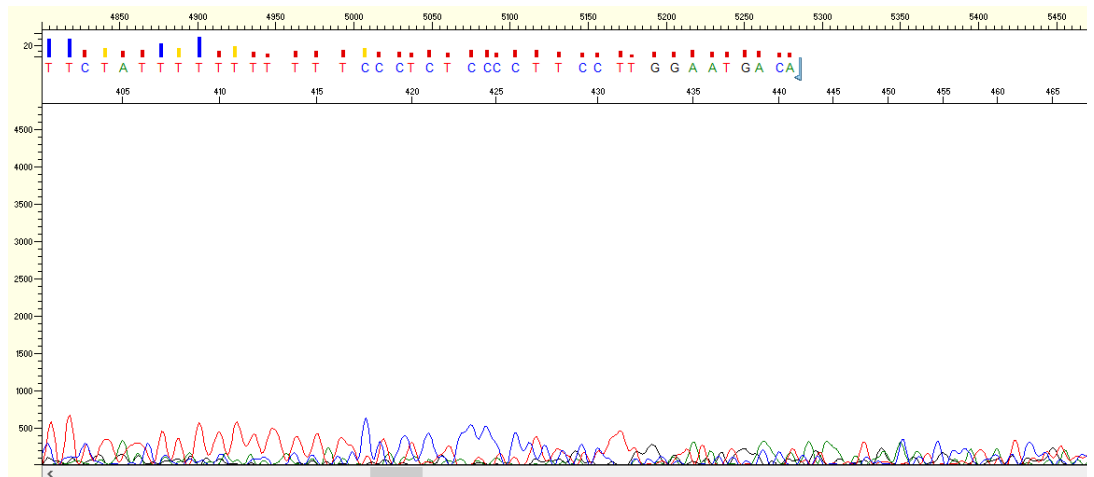
- Quality values are low throughout the read.
- Each position has multiple peaks and/or there is a lot of background noise.



## End of sequence

Bases are no longer called after position 440. This is beyond the expected length of the PCR product.

Quality values continue to be poor through the entire read and multiple peaks are called at each position.



## Sequence Annotation and Quality

This sequence is made up almost exclusively of low-quality base calls (red). This is a failed sequence and cannot be used.

1	CGCTCAATGG	TCITTTGGGG	GGITTAACCTC	TCCCGGGCGA	CTCTTTTGAT	TATGGAAAAG	ATGCCCGGGA	70
71	TTTTTTCITT	TCGGTITTTTC	TGGGGGCATT	AAGGGGCCGA	GTGCCCCCTTG	GGCTGATGTT	ATGATGCATG	140
141	AAATTCTGCC	CTTGCCTCCTA	ATATTGGGGG	GTCCCTGGIT	GAGCACTCTG	CTACTTGATG	ACACCAACAA	210
211	TCTCACAAAG	TCGATTGGTA	ATACCTCTGA	GAATCCAAGA	AAAATCCACC	CATTTTCTGA	TTTGAGATCA	280
281	CCTACTAATG	AAATGCTTCG	GCTAATTTGAA	AAACCCCTGT	CCCTATTTTG	CCTTGCCAGC	CTTCCGGTAC	350
351	AAAAAAGGGA	TGGAACACTG	TTATCTTCIG	ATTACCTCCG	CACCATCTTT	TTCTATTTTI	TTTTTTCCCT	420
421	CTCCCTTCC	TTGGAATGAC	A					441



Signal Intensity is below the acceptable range.

A(61),C(59),G(49),T(74)

## Summary

Sequence is poor quality and should not be used.

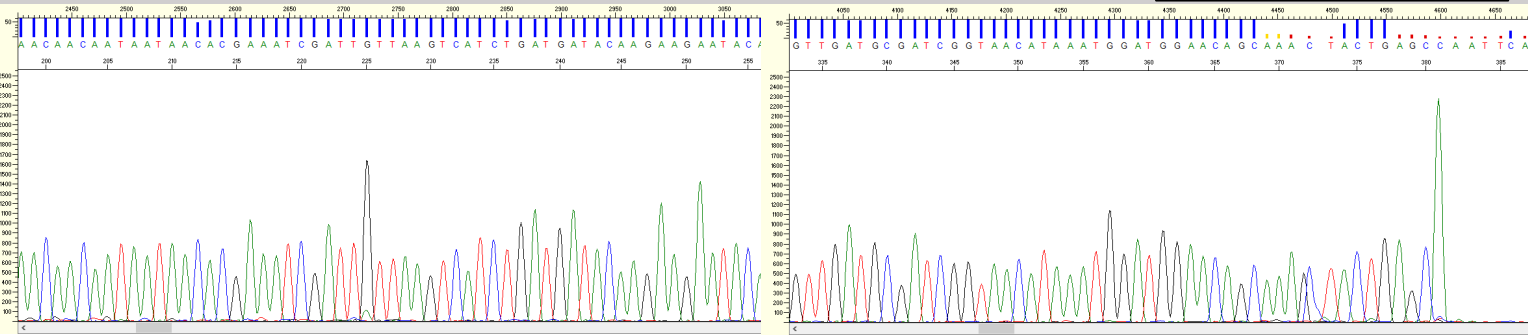
To improve results, quantify sample with fluorometric method, such as Qubit. Submit fresh sample using recommended mass. Note that the NanoDrop is not an accurate method for quantifying nucleic acids.

# Comparison & Summary: Too little template (PCR product)

The panel below shows how the quality of the sequence decreases as the amount of template decreases. The correct template mass is on top (example 3), 1/10<sup>th</sup> the recommended template mass (example 4a) is in the middle, and 1/100<sup>th</sup> the recommended template mass (example 4b) is on the bottom. Two regions of the electropherogram are displayed: position ~210 on the left and the end of the sequence on the right. The average raw signal intensities are also displayed for each example.

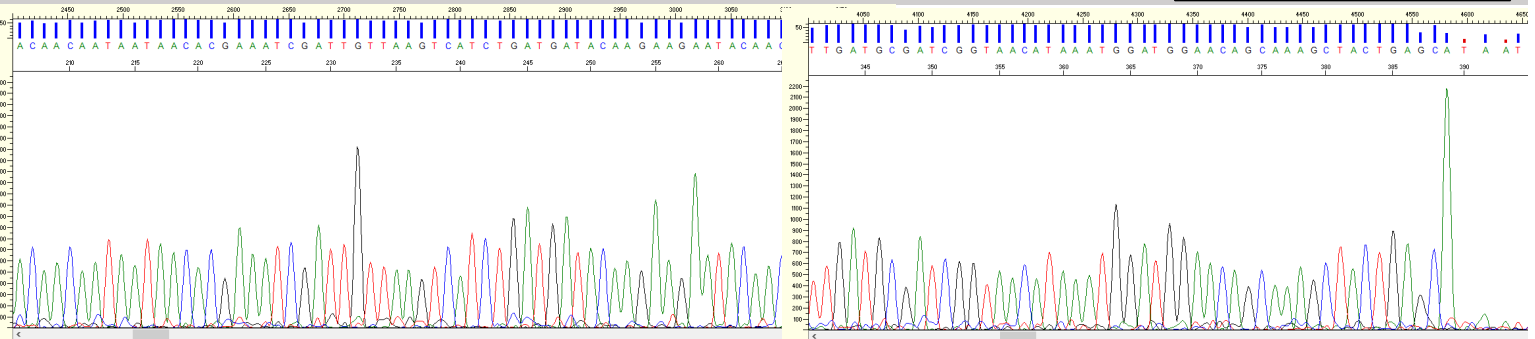
Example 3: Correct template (PCR product)

A(1522),C(863),G(950),T(1458)



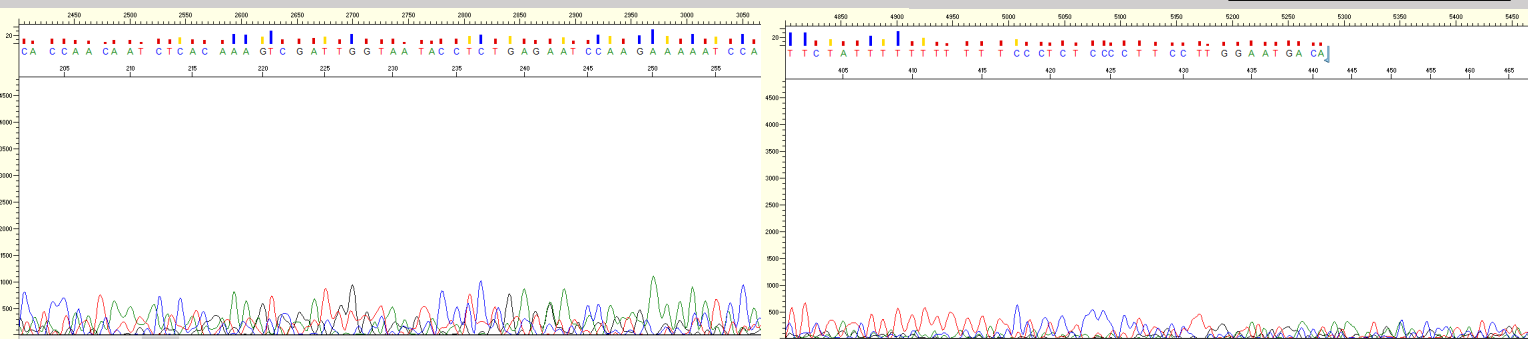
Example 4a: 1.5 ng template (PCR product)

A(204),C(129),G(171),T(185)



Example 4b: 0.15 ng template (PCR product)

A(61),C(59),G(49),T(74)



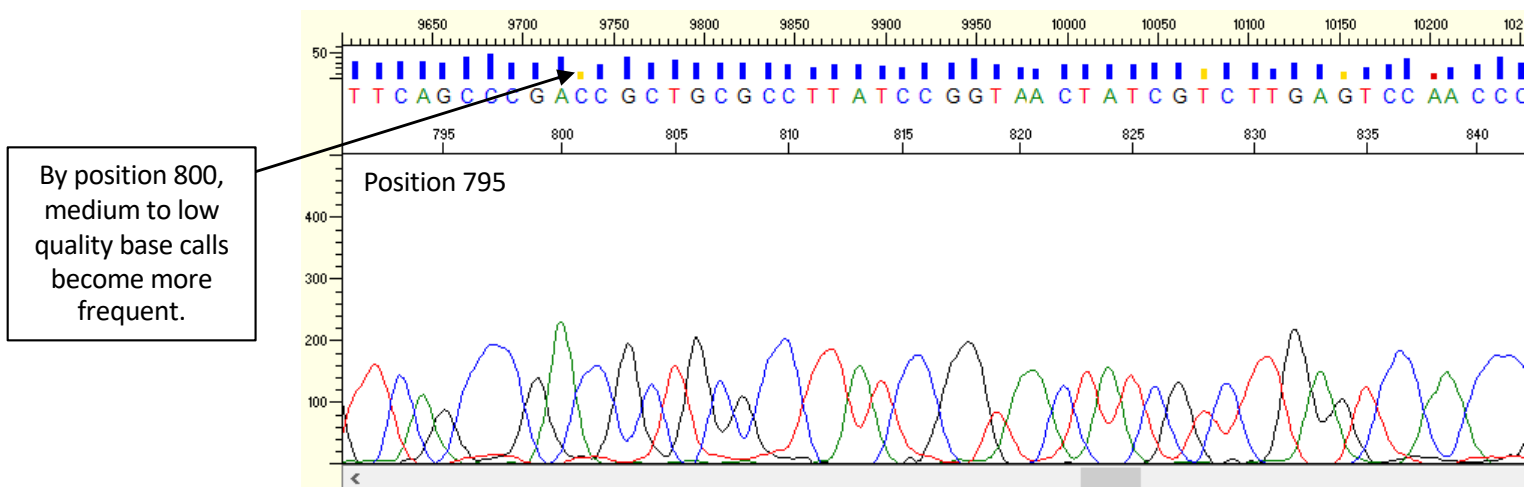
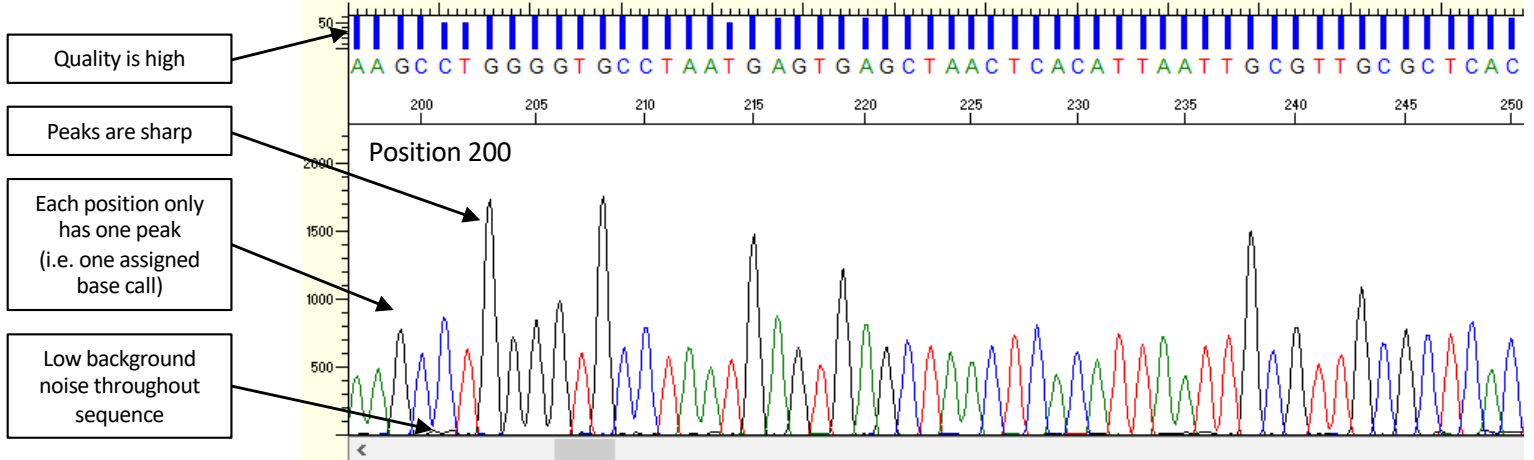
In the examples above you can see that a moderate deviation from the recommended template mass for PCR products generates acceptable results (example 4a). However, providing a very small amount of PCR product results in sequencing failure (example 4b). See pages 13-15 for more details on each of these examples.

# Example 5: 5X recommended template (plasmid)

The template was added at 5X the recommendation. The primer was added at the correct concentration. The template source is the pGEM-3Zf(+) control; a double stranded DNA plasmid that is 3,197 bp.

Template Mass	Number of Primers Added	Volume of 10µM Primer	Total Volume	File Name
5000 ng	1	3 µl	12 µl	5_5X_template_plasmid.ab1

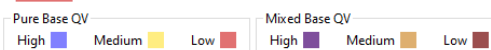
The quality of this sequence is still good despite the template being at a greater mass than is recommended.



## Sequence Annotation and Quality

The base calls in blue are high quality with only one base being called. Medium and low-quality values become more frequent around base 850 (yellow and red bases). The quality of this sequence is high, like example 1 (correct template). But, the usable sequence may be slightly shorter.

1	GGGGACATAC	TCIATAGGGG	CGATTCCGAGC	TCGGTACCCG	GGGATCCTCT	AGAGTCGACC	TCGAGGCATG	70
71	CAAGCTTGAG	TATTCTATAG	TGTCACCTAA	ATAGCTTGGC	GTAATCATGG	TCATAGCTGT	TTCTGTGTG	140
141	AAATTGTTAT	CCGCTCACAA	TTCCACACAA	CATACGAGCC	GGAAGCATAA	AGTGAAAGC	CTGGGGTGCC	210
211	TAATGAGTGA	GCTAACTCAC	ATTAATTGCG	TTGCGCTCAC	TGCCCGCTTT	CCAGTCGGGA	AACCTGTCTG	280
281	GCCAGCTGCA	TTAATGAATC	GGCCAAACGG	CGGGGAGAGG	CGGTTTGGCT	ATGGGGCGCT	CTTCCGCTTC	350
351	CTCGCTCACT	GACTCGCTGC	GCTCGGTGCT	TCGGCTGCGG	CGAGCGGTAT	CAGCTCACTC	AAAGGCGGTA	420
421	ATACGGTTAT	CCACAGAATC	AGGGGATAAC	GCAGGAAAGA	ACATGTGAGC	AAAAGGCGAG	CAAAAGGCCA	490
491	GGAAACCGTAA	AAAGGCGCGG	TTGCTGGGCT	TTTTCCATAG	GCTCCGCCCC	CCTGACGAGC	ATCCAAAAAA	560
561	TCGACGCTCA	AGTCAGAGGT	GGCGAAACCC	GACAGGACTA	TAAAGATAAC	AGGGGTTTCC	CCCTGGAAGC	630
631	TCCTCTGTCG	GCTCTCCTGT	TCGACCCCTG	CCGCTTACCG	GATACCTGTG	CGCCTTTCTC	CCTTCGGGAA	700
701	GCGTGGCGCT	TTCTCATAGC	TCACGCTGTA	GGTATCTCAG	TTCCGGTGTG	GTCCTTCGCT	CCAAGCTGGG	770
771	CTGTGTGCAC	GAACCCCGCC	TTCCAGCCCGA	CCGCTGGCGC	TTATCCGGTA	ACTATCGTCT	TGAGTCCAC	840
841	CCGGTAAGAC	ACGACTTATC	GCCACTGGCA	GCAGCCACTG	GTAACAGGAT	TAGCAGAGCG	AGGTATGTAG	910
911	GCGGTGCTAC	AGAGTCTTTC	AACTGGTGGC	CTAACTACGG	CTACACTAGG	AGACAGTATT	TGGTATCTGC	980
981	GCTCTGCTGA	AGCCAGTTAC	GTCGGAAAAA	AGAGTTGGGT	AGCTCTGATC	CGGCAACACG	CAGCCTGGTA	1050
1051	GCGGGTGGTT	TTTTTTTGTI	TGCASCAGTA	GATACGGGCG	AGAAAAAGG	ATCCTCAAGA	AGATCCTTGA	1120
1121	TCITTTTCTA	CGGGGTCTGA	CGCCTCAGTG	GACGAAAACT	CCACGTTAAG	GGAAITTTGC	ATGGAAATAT	1190
1191	CAAAACGAA	TCTCACTAGA	TCITTTAATA	AAATGAAGTT	CATCATCTAG	TATTATGAGT	AACTTGACTG	1260
1261	ACGCTACCAT	GCTATCATTG	AGACGTATCA	CAGCGA				1296



## Signal Intensity

The Average Raw Signal Intensities are in the acceptable range.

A(1818), C(2384), G(890), T(2914)

Sequence is good.  
No changes needed.

# Example 6: 5X recommended template (PCR product)

The template was added at 5X the recommendation. The primer was added at the correct concentration. The template source is a 408 bp double stranded PCR product that was purified with ExoSAP-it.

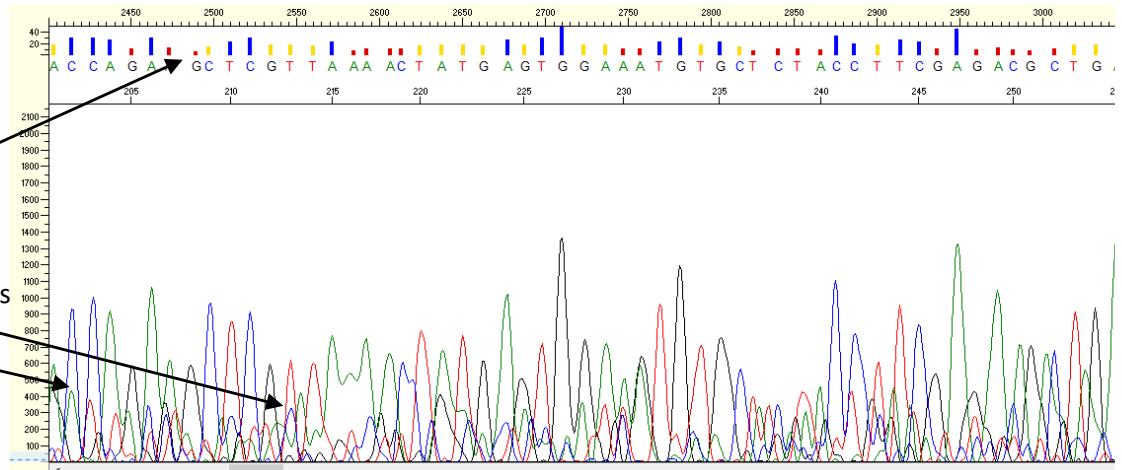
Template Mass	Number of Primers Added	Volume of 10µM Primer	Total Volume	File Name
75 ng	1	3 µl	12 µl	6_5X_template_PCR_product.ab1

The quality of this sequence is poor.

## Position 205

The criteria that make this a poor-quality sequence are:

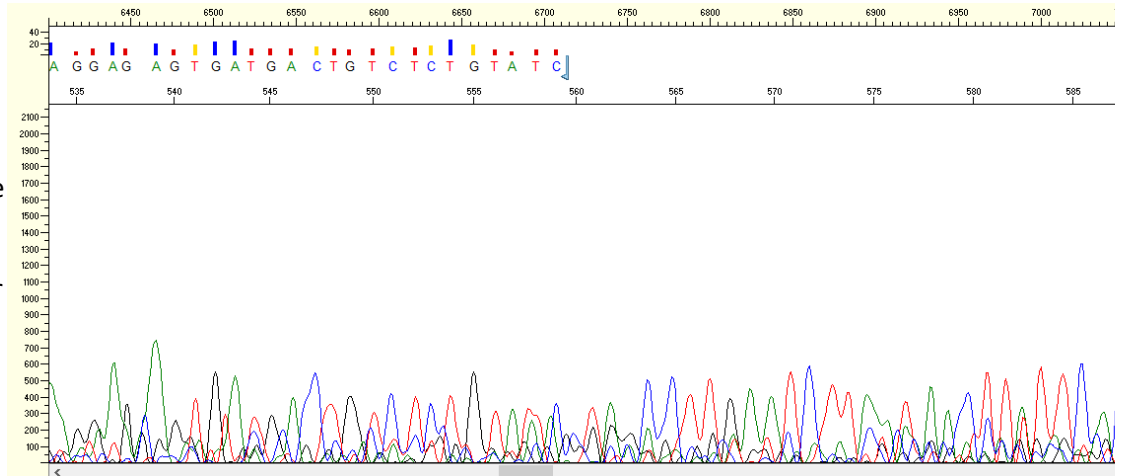
- Quality values are low throughout the read.
- Each position has multiple peaks and/or there is a lot of background noise.



## End of sequence

Bases are no longer called after position 559, however, this is beyond the expected length of the PCR product.

Quality values continue to be poor through the entire read and multiple peaks/noise are present at most, if not all, positions.



## Sequence Annotation and Quality

This sequence has mostly medium- to low-quality base calls. This is a failed sequence and cannot be used.

1	AGCAAATTTT	TTTTTGTITG	GGGTTAACCC	TCAAAGTCCT	TCGTCAAAT	ATATAACAAC	TGTCATCGAG	70
71	TTCTTATATT	TCCTACAATG	CGAAATAGGC	AGACTTATAC	ATCCAGCTCC	TGGTGTSCCT	GAAGCATTGA	140
141	TACAAGAGAT	GTTCCCCCAT	AACCGACACG	CATCGAGGGA	AGGTCCTTGGC	CTCTTCATCC	ACCAGAAGCT	210
211	CGTTAAAAC	ATGAGTGGAA	ATGTGCTCTA	CCTTCGAGAC	GGTGAAAAAT	CATCCTTTAT	TATCCTAATT	280
281	GAGTTACCCT	TGGTTCATAA	CTGGGGAAAGC	GTTTGAATAC	CAGGACATCA	CGATTCTCAC	TTGGCCGCCG	350
351	TCCTTTGGTA	AGTATTTGGG	ACCAATGTTTC	TGATTAGATT	TAATCTACCA	GCATCITTTT	CTATTTTTTT	420
421	TTTATCTCCG	CCACTTGCCT	GGAAATGAAAA	ATGAAGCTAT	TGTTAAAGAC	TGTTTTGACT	GTTCAAGATG	490
491	ACAAAAATAC	CAAATTAATC	CTGCTGTACT	GCGGAAGGAT	CCTAGGAGAG	TGATGACTGT	CTCTGTATC	559



Signal Intensity is below the acceptable range.

A(57),C(60),G(42),T(77)

## Summary

Sequence is poor quality and should not be used.

To improve results, quantify sample with fluorometric method, such as Qubit. Submit fresh sample using recommended mass. Note that the NanoDrop is not an accurate method for quantifying nucleic acids.



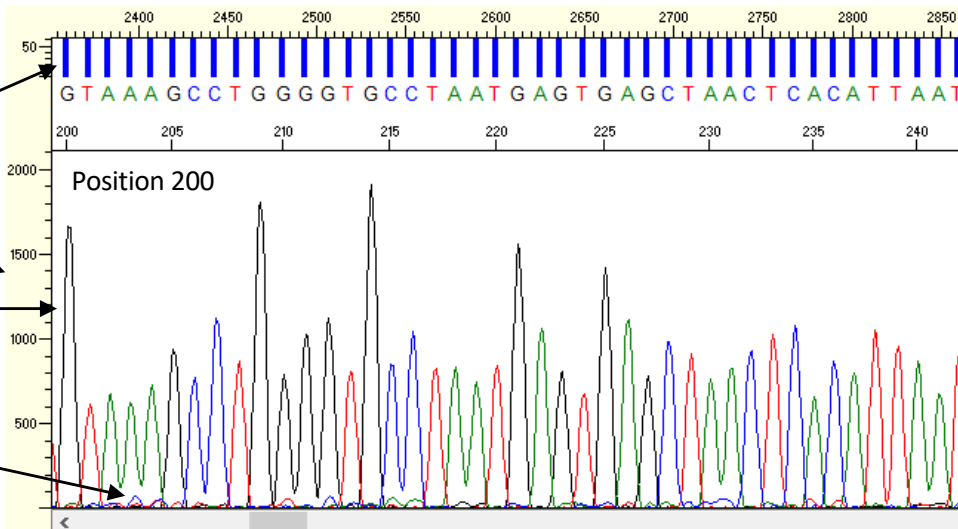
# Example 7a: 1/10<sup>th</sup> primer concentration but correct template

The primer was diluted to 1/10<sup>th</sup> the recommended concentration. The template was added at the correct mass. The template source is the pGEM-3Zf(+) control; a double stranded DNA plasmid that is 3,197 bp.

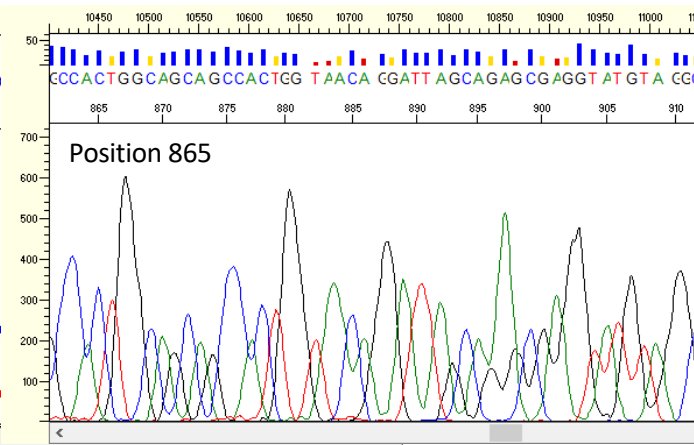
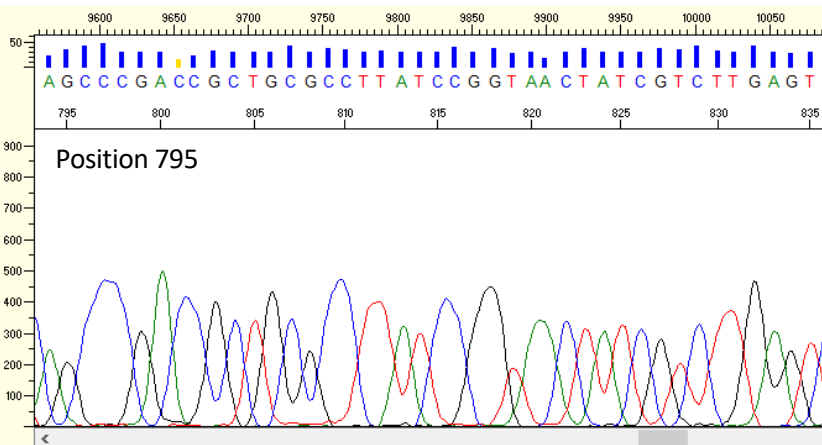
Template Mass	Number of Primers Added	Volume of 1µM Primer	Total Volume	File Name
1000 ng	1	3 µl	12 µl	7a_0.1_primer_plasmid.ab1

The quality of this sequence is still acceptable at the beginning and middle of the read:

- Quality is high throughout the read
- Signal intensity is > 175 RFU
- Each position only has one peak (i.e. base call) and peaks are sharp
- There is a very small amount of background noise (i.e. peaks along the bottom of the electropherogram)



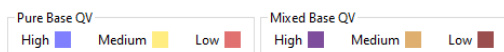
The quality of the sequence decreases earlier in the read than in example 1. At position 795, the signal intensity has decreased, but the quality is OK. The quality really begins to drop off by position 850.



## Sequence Annotation and Quality

The base calls in blue are high quality bases with only one base being called. The sequence quality begins to drop slightly earlier than in example 1.

1	GGGGGGCTAC	TACTATAGG	CGATTGCGAG	TCGGTACCG	GGGATCCTCT	AGAGTCGACC	TGCAGGCATG	70
71	CAAGCTTGAG	TATTTCTATAG	TGTCACCTAA	ATAGCTTGGC	GTAATCATGG	TCATAGCTGT	TTCCTGTGTG	140
141	AAATTTGTAT	CCGCTCACAA	TCCACACAA	CATACGAGCC	GGAAGCATAA	AGTGTAAAGC	CTGGGGTGCC	210
211	TAAITGAGTGA	GCTAACTCAC	ATTAATTCGG	TTGCGCTCAC	TGCCCGCTTT	CCAGTCGGGA	AACCTGCTGT	280
281	GCCAGCTGCA	TAAATGAATC	GGCCAAACCG	CGGGAGAGG	CGGTTTGGCT	ATTGGGCGCT	CTTCCGGTTC	350
351	CTCGCTCACT	GACTCGCTGC	GCTCGGCTGT	TGGGCTGCGG	CGAGCGGTAT	CAGCTCACTC	AAAGGGCGTA	420
421	ATACGGTTAT	CCACAGAAATC	AGGGGATAAC	GCAGGAAAGA	ACATGTGAGC	AAAAGGCCAG	CAAAAGGCCA	490
491	GGAAACCGTAA	AAAGGCCCGG	TTGCTGGCGT	TTTTCCATAG	GCTCCGCCCC	CCTGACGAGC	ATCACAAAAA	560
561	TCGACGCTCA	AGTCAGAGGT	GGCGAAACCC	GACAGGACTA	TAAAGATAAC	AGGCGTTTCC	CCCTGGAAGC	630
631	TCCCTCGTGC	GCTCTCCTGT	TCCGACCTGT	CCGCTTACCG	GATACCTGTC	CGCCTTTTCT	CCCTCGGGAA	700
701	CGCTGGCGCT	TTCTCATAGC	TCCAGCTGTA	GGTATCTCAG	TTCCGTTAG	GTCGTTGCTG	CCAAAGCTGGG	770
771	CTGTGTGAC	GAACCCCGCC	TTCAAGCCGA	CCGCTGCGCC	TTATCCGGTA	ACTATCGTCT	TGAGTCCAAC	840
841	CCGTAAGAC	ACGACTTATC	GCCACTGGCA	GCAGCCACTG	GTAACAGGAT	TAGCAGAGCG	AGTATGTAG	910
911	GCGGTGCTAC	AGAGTTCTTG	AAGTGGTGGC	CTAACTACGG	CTACACTAGA	AGACAGTAT	TTGGGTATCT	980
981	GCGCTCTGCT	GAAAGCCAGTT	ACCTTTCGGG	AAAAAGGAGT	TGGTAGCTC	TTGATCCGGG	CAAAACAAAC	1050
1051	ACCGCTGGGT	AGCGSTGGTT	TTTTTTTTGTT	TGCAGCAGCA	GATTACGCCG	CAGGAAAAAA	AGGATCTCAG	1120
1121	GAAGATCCTT	TGGATCTTTT	TCTACGGTTC	TGACCCGCCC	CAGSTGGAAAC	GAAACCTCCA	CGGTTAGGAT	1190
1191	TTGGTCAATG	AGATAATCAA	AAAAGGAATC	CACATAGACT	TTTAAATAAA	TGAGTTTAAI	CAATCTAAG	1260
1261	TATATATGGA	GTTABACCTG	CCTGAACGTT	AACATGCCTA	ATCAATGAGG	CCACCTATCT	CAACGGGAA	1330
1331	TTGGTTCATG	TCGGTCATAC	AGTCTAAGAT	TTGGCT				1366



## Signal Intensity

The Average Raw Signal Intensities are in the acceptable range.

A(513), C(663), G(550), T(668)

## Summary

Sequence is acceptable, but it may be necessary to trim lower quality bases from the end of the read.

To improve results, double check primer concentration and amount that was added to sample. Then submit a fresh sample with the appropriate amount of primer.

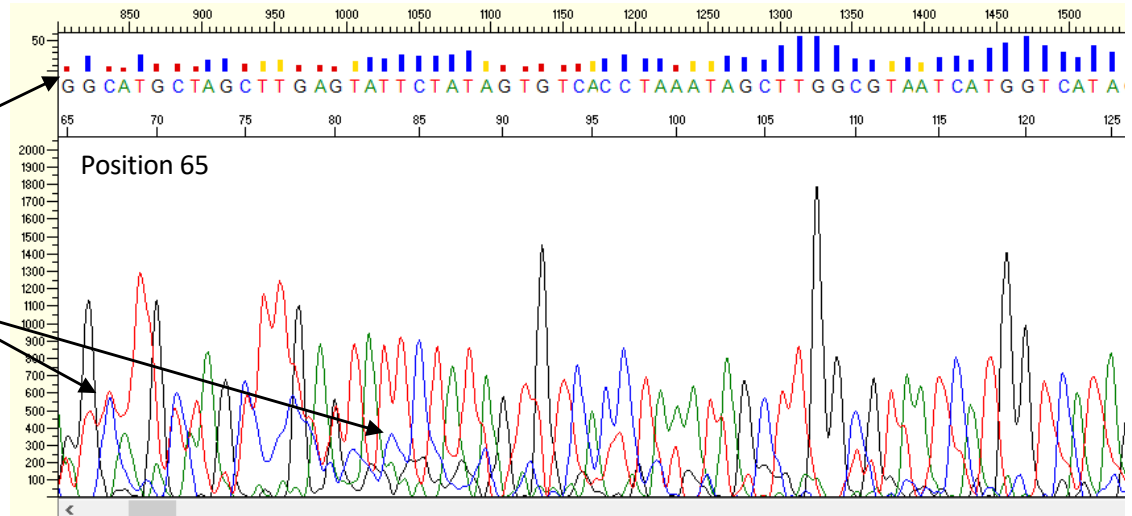
# Example 7b: 1/100<sup>th</sup> primer concentration but correct template

The primer was diluted to 1/100<sup>th</sup> the recommended concentration. The template was added at the correct mass. The template source is the pGEM-3Zf(+) control; a double stranded DNA plasmid that is 3,197 bp.

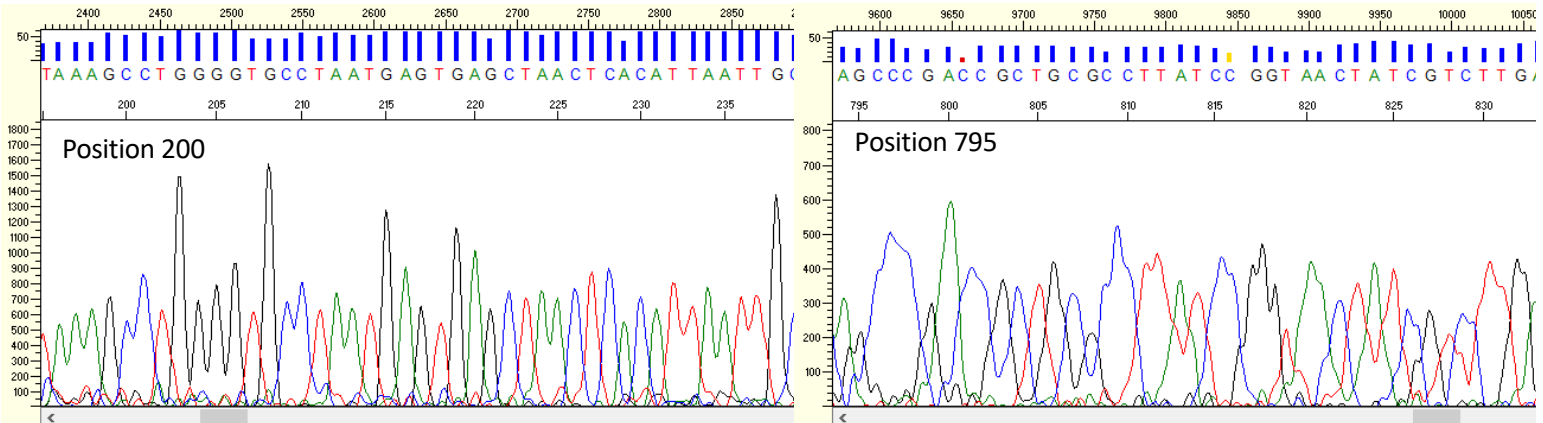
Template Mass	Number of Primers Added	Volume of 0.1µM Primer	Total Volume	File Name
1000 ng	1	3 µl	12 µl	7b_0.01_primer_plasmid.ab1

The base call quality is very poor in the first hundred bases. The quality improves after that.

There is background noise and multiple peaks per position.



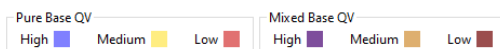
Some background noise continues throughout the sequence and the quality drops off earlier than in example 4a (1/10th primer concentration).



## Sequence Annotation and Quality

Sequence quality is poor in the first 100 bases and drops off around base 845.

1	GAAAAATCAC	GTATAGTCT	TACTACGAGC	TCGGTACC	GGGTTCTCC	ATAGTCGACC	TGCAGGCATG	70
71	CTAGCTTGAG	TATTCATAG	TGTCACCTAA	ATAGCTTGGC	GTATCATGG	TCATAGCTGT	TTCCGTGTGTG	140
141	AAATTGTTAT	CCGCTCACAA	TTCCACACAA	CATACGAGCC	GGAAGCATAA	AGTGTAAGC	CTGGGTGCCC	210
211	TAATGAGTGA	GCTAACTCAC	ATTAATTGGC	TTGCGCTCAC	TGCCCCGTTT	CCAGTCGGGA	AACTGTGCTG	280
281	GCCAGCTGCA	TAAATGAATC	GGCCAACGGC	CGGGAGAGGG	CGTITTCGCT	ATTGGGCGCT	CTTCCGCTTC	350
351	CTCGCTCACT	GACTCGCTGC	GCTCGGTCGT	TCGGCTCGGG	CGAGCGGTAT	CAGCTCACT	AAAGGCGGTA	420
421	ATACGGTTAT	CCACAGAATC	AGGGGATAAC	GCAGGAARAG	ACATGTGAGC	AAAAGGCCAG	CAAAAGGCCA	490
491	GGAACCGTAA	AAAGGCGCGC	TTGCTGGCGT	TTTTCCATAG	GCTCCGCCCC	CCTGACCGCC	ATCACAARAA	560
561	TCGACGCTCA	AGTCAGAGGT	GGCGAARACC	GACAGGACTA	TAAAGATACC	AGGCGTTTCC	CCCTGGGAGC	630
631	TCCTCGTGC	GCTCTCTGT	TCGACCCCTG	CCGCTTACCG	GATACCTGTC	CGCCTTCTC	CCTTCGGGAA	700
701	GCSTGGCGCT	TTCTCATAGC	TCACGCTGTA	GSTATCTCAG	TTCCGTTAG	STCGTTCGCT	CCAAGCTGGG	770
771	CTGTGTGCAC	GAAACCCCGC	TCAGGCCCGA	CCGCTGCGCC	TTATCCGGTA	ACTATCGTCT	TGAGTCCAAC	840
841	CCGGTARGAC	ACGACTTATC	SCCACTGGCA	GCAAGCCACTG	GTAACAGGAT	TAGCAGAAGC	GAGGTATGTA	910
911	GGCGGTGCTA	CAGAGTTCIT	GAGTGGTGGC	CTAACTACGG	CTAACACTAG	AAGAACAGTA	TTTGGTTATC	980
981	TGCGCTCTGC	CTGAAGCCCA	GTTACCTTCG	GGAAAAGAA	GTTGGTTAGC	CTCTTGATCC	GGGCAACCA	1050
1051	CCCACCGCTG	GGTAGCCGGG	TGGTTTTTTT	TGTTTGCAG	CCAGCAGATT	ACGGCCAAAG	AAAAAAGGAT	1120
1121	CCTCAAGAG	ATCCCTGATC	TGCTACGGGG	TCTGACCCGC	TCGTGAAAC	GAAACTCAA	CGGTAGGGG	1190
1191	ATTTTGTGT	TCAT						1204



## Signal Intensity

The average raw signal intensities are very low. Data quality may be compromised.

A(68),C(78),G(73),T(83)

## Summary

Sequence can be used, but manual review of electropherogram and quality scores must be done to determine what is usable.

To improve results, double check primer concentration and amount that was added to sample. Then submit a fresh sample with the appropriate amount of primer.

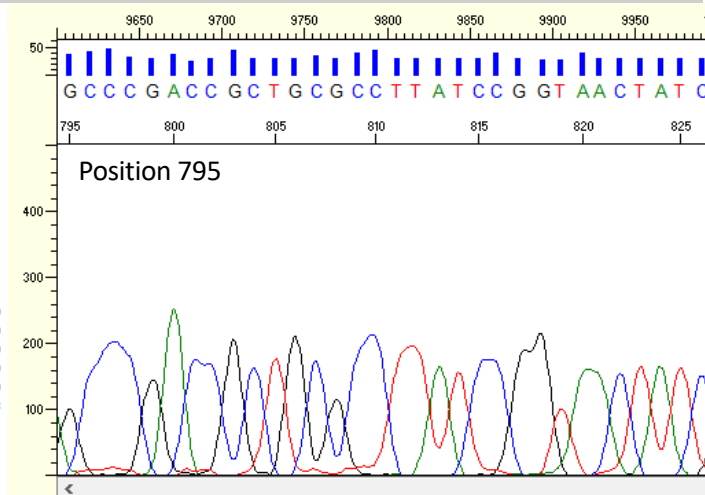
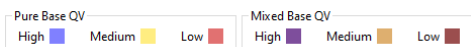
# Comparison & Summary: Samples with too little primer

The images below show the sequence and base call quality, the electropherogram at position 795, and the average signal intensities for comparison of a sample (plasmid) with the correct amount of primer and template (example 1), 1/10<sup>th</sup> recommended primer concentration (example 7a), and 1/100<sup>th</sup> recommended primer concentration (example 7b). The examples with too little primer still yielded acceptable results. However, manual review of the electropherogram and trimming to remove poor-quality bases at the beginning and end of the read is necessary with 1/100<sup>th</sup> the recommended primer concentration. Refer to pages 9 and 19-20 for a more detailed summary of each example.

## Example 1: Correct template (plasmid)

A(890),C(1324),G(664),T(1411)

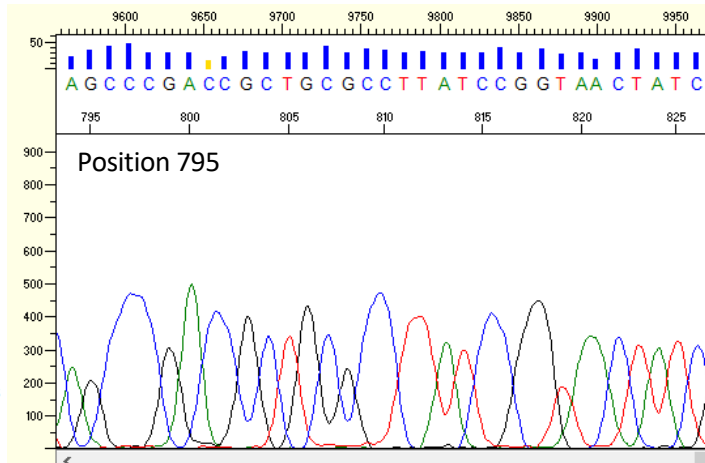
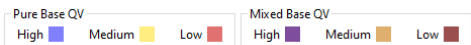
1	GGGAGTAA	TACTATAGG	CGATTCGAGC	TCGGTACCCG	GGGATCCTCT	AGAGTCGACC	TCGAGGCATG	70
71	CAAGCTTGAG	TATTCATATG	TGTCACCTAA	ATAGCTTGGC	GTAATCATGG	TCATAGCTGT	TTCCGTGTGG	140
141	AAATTTGTTAT	CCGCTCACAA	TTCCACACAA	CATACGAGCC	GGAAACATAA	AGTGTAAAGC	CTGGGGTGCC	210
211	TAATGAGTGA	GCTAACTCAC	ATTAATTTGG	TTGCCCTCAC	TCGCCCGTTT	CCAGTCGGGA	AACTCTGCTG	280
281	GCCAGCTGCA	TTAATGAATC	GGCCAACCGG	CGGGGAGAGG	CGGTTTGCCT	ATTGGGCGCT	CTTCCGCTTC	350
351	CTCGCTCACT	GACTCGCTCG	GCTCGCTCGT	TCGGCTCGGG	CGAGCGGTAT	CAGCTCACTC	AAAGGCGGTA	420
421	ATACGGTTAT	CCACAGAATC	AGGGGATAAC	SCAGGAARA	ACATGTGAGC	AAAAGGCCAG	CAAAAGGCCA	490
491	GGAAACGGTAA	AAAGGCCGGG	TTGCTGGCGT	TTTTCCATAG	GCTCCGCCCC	CCTGACGAGC	ATCACAAAAA	560
561	TCGACGCTCA	AGTCAGAGGT	GGCGAAACCC	GACAGGACTA	TAAAGATAAC	AGGCGTTTTCC	CCCTGGAAGC	630
631	TCCTCTGTCG	GCTCTCCTGT	TCGACCCCTG	CCGCTTACCG	GATACCTGTC	CGCCTTTCTC	CCTTCGGGAA	700
701	GGGTGGCGCT	TTCTCATAGC	TCACGCTGTA	GSTATCTCAG	TTGGGTGTAG	GTCGTTCCGT	CCAAGCTGGG	770
771	CTGTGTGCGAC	GAACCCCGCG	TTACGCCCGA	CCGCTGCGCC	TTATCCGGTA	ACTATCGTCT	TGAGTCCAA	840
841	CCGGTAAGAC	ACGACTTATC	GCCACTGGCA	GCAGCCACTG	GTAACAGGAT	TAGCAGAGCG	AGGTATGTAG	910
911	GCGGTGCTCA	AGAGTTCTTG	AAGTGGTGGC	CCTAACTACG	GCTACACTAG	AAGAACAGTA	TTTGGTATCT	980
981	TCGGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1050
1051	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1120
1121	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1190
1191	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1260
1261	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1330
1331	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1368



## Example 7a: 1/10<sup>th</sup> recommended primer concentration (plasmid)

A(513),C(663),G(550),T(668)

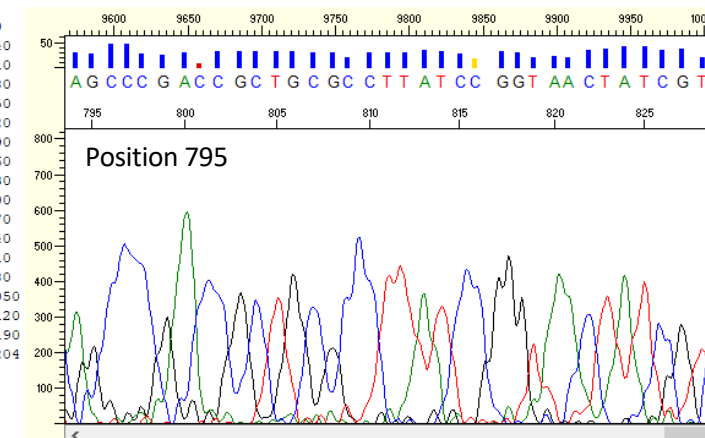
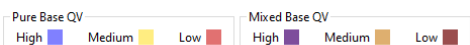
1	GGGGGGCTAC	TACTATAGG	CGATTCGAGC	TCGGTACCCG	GGGATCCTCT	AGAGTCGACC	TCGAGGCATG	70
71	CAAGCTTGAG	TATTCATATG	TGTCACCTAA	ATAGCTTGGC	GTAATCATGG	TCATAGCTGT	TTCCGTGTGG	140
141	AAATTTGTTAT	CCGCTCACAA	TTCCACACAA	CATACGAGCC	GGAAACATAA	AGTGTAAAGC	CTGGGGTGCC	210
211	TAATGAGTGA	GCTAACTCAC	ATTAATTTGG	TTGCCCTCAC	TCGCCCGTTT	CCAGTCGGGA	AACTCTGCTG	280
281	GCCAGCTGCA	TTAATGAATC	GGCCAACCGG	CGGGGAGAGG	CGGTTTGCCT	ATTGGGCGCT	CTTCCGCTTC	350
351	CTCGCTCACT	GACTCGCTCG	GCTCGCTCGT	TCGGCTCGGG	CGAGCGGTAT	CAGCTCACTC	AAAGGCGGTA	420
421	ATACGGTTAT	CCACAGAATC	AGGGGATAAC	SCAGGAARA	ACATGTGAGC	AAAAGGCCAG	CAAAAGGCCA	490
491	GGAAACGGTAA	AAAGGCCGGG	TTGCTGGCGT	TTTTCCATAG	GCTCCGCCCC	CCTGACGAGC	ATCACAAAAA	560
561	TCGACGCTCA	AGTCAGAGGT	GGCGAAACCC	GACAGGACTA	TAAAGATAAC	AGGCGTTTTCC	CCCTGGAAGC	630
631	TCCTCTGTCG	GCTCTCCTGT	TCGACCCCTG	CCGCTTACCG	GATACCTGTC	CGCCTTTCTC	CCTTCGGGAA	700
701	GGGTGGCGCT	TTCTCATAGC	TCACGCTGTA	GSTATCTCAG	TTGGGTGTAG	GTCGTTCCGT	CCAAGCTGGG	770
771	CTGTGTGCGAC	GAACCCCGCG	TTACGCCCGA	CCGCTGCGCC	TTATCCGGTA	ACTATCGTCT	TGAGTCCAA	840
841	CCGGTAAGAC	ACGACTTATC	GCCACTGGCA	GCAGCCACTG	GTAACAGGAT	TAGCAGAGCG	AGGTATGTAG	910
911	GCGGTGCTCA	AGAGTTCTTG	AAGTGGTGGC	CCTAACTACG	GCTACACTAG	AAGAACAGTA	TTTGGTATCT	980
981	TCGGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1050
1051	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1120
1121	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1190
1191	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1260
1261	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1330
1331	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1366



## Example 7b: 1/100<sup>th</sup> recommended primer concentration (plasmid)

A(68),C(78),G(73),T(83)

1	GAANAATCAC	GSTATAGGCT	TACTACGAGC	TCGGTACCCG	GGGATCCTCT	AGAGTCGACC	TCGAGGCATG	70
71	CAAGCTTGAG	TATTCATATG	TGTCACCTAA	ATAGCTTGGC	GTAATCATGG	TCATAGCTGT	TTCCGTGTGG	140
141	AAATTTGTTAT	CCGCTCACAA	TTCCACACAA	CATACGAGCC	GGAAACATAA	AGTGTAAAGC	CTGGGGTGCC	210
211	TAATGAGTGA	GCTAACTCAC	ATTAATTTGG	TTGCCCTCAC	TCGCCCGTTT	CCAGTCGGGA	AACTCTGCTG	280
281	GCCAGCTGCA	TTAATGAATC	GGCCAACCGG	CGGGGAGAGG	CGGTTTGCCT	ATTGGGCGCT	CTTCCGCTTC	350
351	CTCGCTCACT	GACTCGCTCG	GCTCGCTCGT	TCGGCTCGGG	CGAGCGGTAT	CAGCTCACTC	AAAGGCGGTA	420
421	ATACGGTTAT	CCACAGAATC	AGGGGATAAC	SCAGGAARA	ACATGTGAGC	AAAAGGCCAG	CAAAAGGCCA	490
491	GGAAACGGTAA	AAAGGCCGGG	TTGCTGGCGT	TTTTCCATAG	GCTCCGCCCC	CCTGACGAGC	ATCACAAAAA	560
561	TCGACGCTCA	AGTCAGAGGT	GGCGAAACCC	GACAGGACTA	TAAAGATAAC	AGGCGTTTTCC	CCCTGGAAGC	630
631	TCCTCTGTCG	GCTCTCCTGT	TCGACCCCTG	CCGCTTACCG	GATACCTGTC	CGCCTTTCTC	CCTTCGGGAA	700
701	GGGTGGCGCT	TTCTCATAGC	TCACGCTGTA	GSTATCTCAG	TTGGGTGTAG	GTCGTTCCGT	CCAAGCTGGG	770
771	CTGTGTGCGAC	GAACCCCGCG	TTACGCCCGA	CCGCTGCGCC	TTATCCGGTA	ACTATCGTCT	TGAGTCCAA	840
841	CCGGTAAGAC	ACGACTTATC	GCCACTGGCA	GCAGCCACTG	GTAACAGGAT	TAGCAGAGCG	GAGGTATGTAG	910
911	GCGGTGCTCA	AGAGTTCTTG	AAGTGGTGGC	CCTAACTACG	GCTACACTAG	AAGAACAGTA	TTTGGTATCT	980
981	TCGGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1050
1051	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1120
1121	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1190
1191	CCGCTCTGCT	GAGCCAGTTT	ACCTTTCCGG	AAAAGAGTGG	GTAGCTCTTG	ATCCCGCAAA	CAAAACCCCG	1204



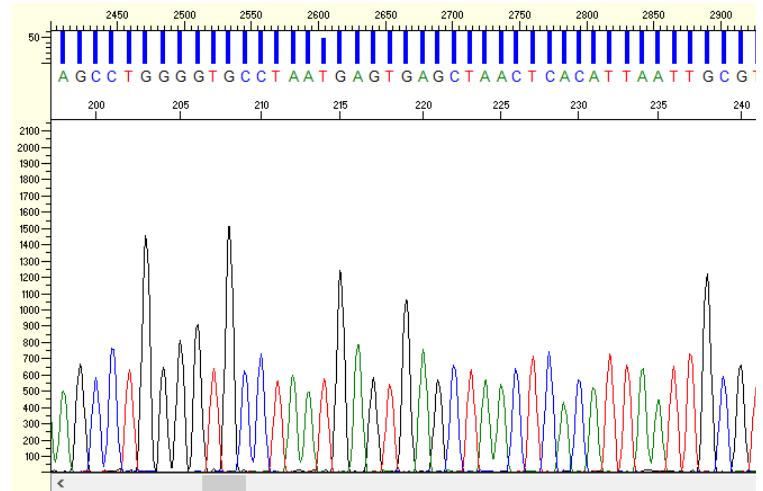
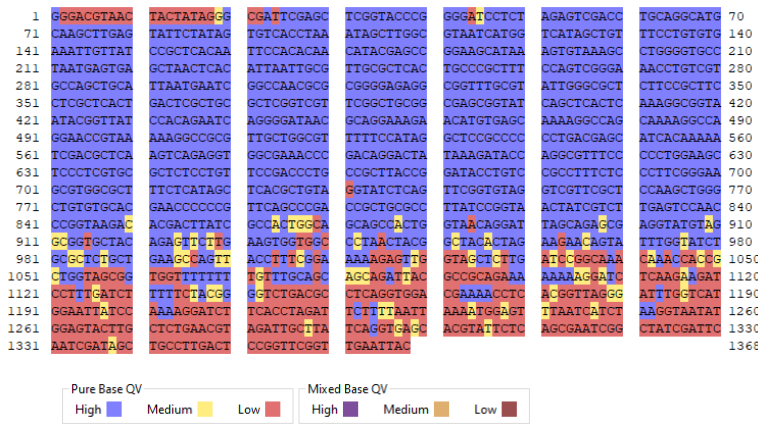


# Comparison & Summary: Samples with too much primer

We tested the effects of too much primer (10X and 40X recommended primer concentrations) with the correct template mass (plasmid). In both cases the quality of the sequence data was relatively unaffected, and the data was acceptable. The sequence and base call quality are presented below for comparison. The correct primer concentration is on top (example 1), 10X the recommended primer concentration is in the middle (example 8a), and 40X the recommended primer concentration is on the bottom (example 8b).

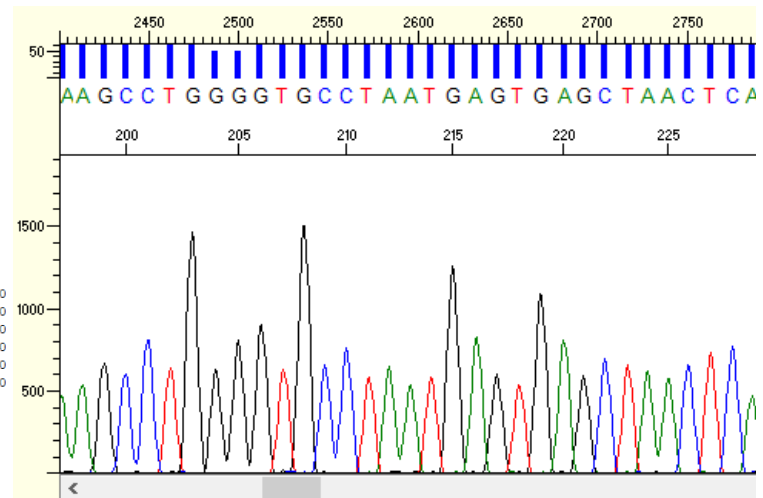
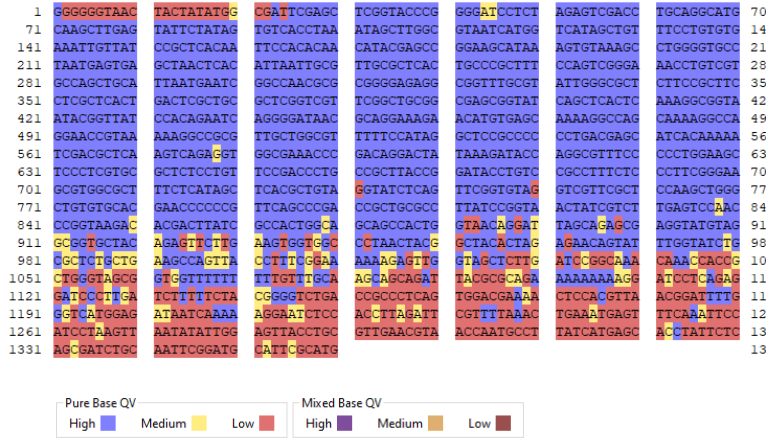
Example 1: Correct template (plasmid)

A(890),C(1324),G(664),T(1411)



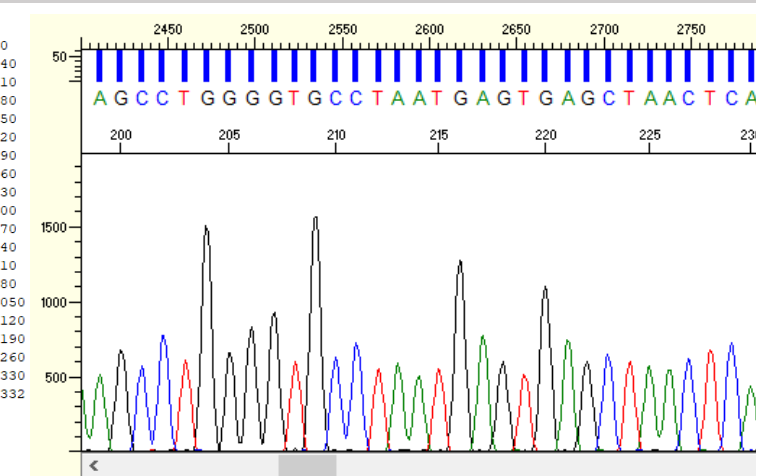
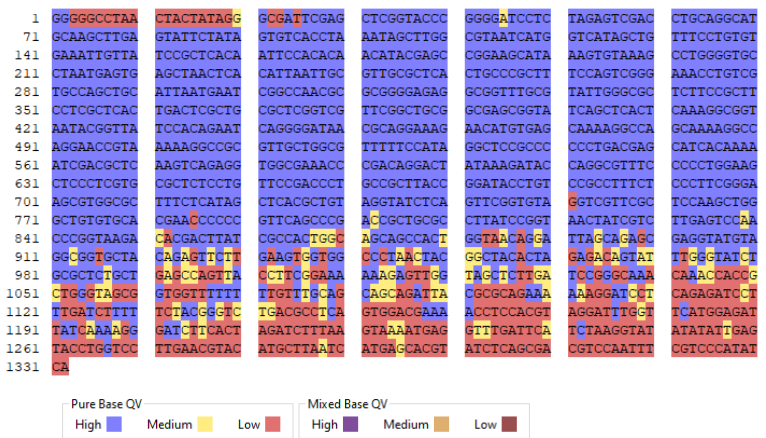
Example 8a: 10X recommended primer concentration (plasmid)

A(815),C(1210),G(597),T(1288)



Example 8b: 40X recommended primer concentration (plasmid)

A(851),C(1207),G(625),T(1323)

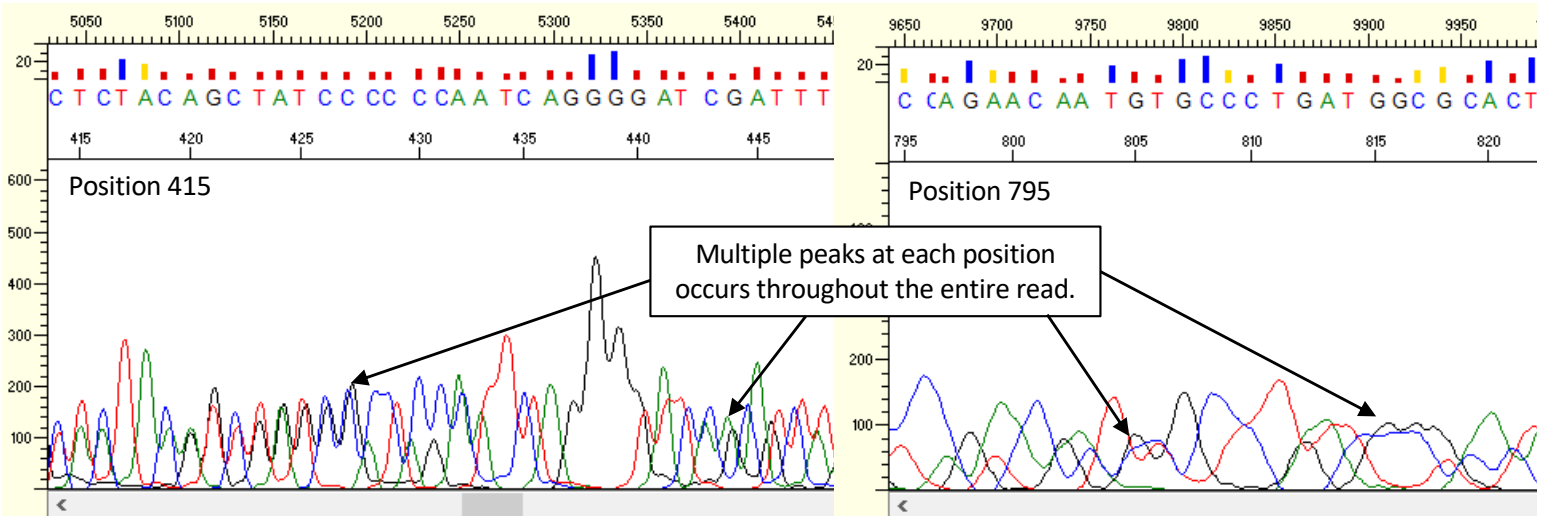


# Example 9a: Two primers and correct template

Two primers were added, both at the recommended concentration. The template was added at the correct mass. The template source is the pGEM-3Zf(+) control; a double stranded DNA plasmid that is 3,197 bp.

Template Mass	Number of Primers Added	Concentration and volume of primers	Total Volume	File Name
1000 ng	2	3 µl forward primer (10µM) 3 µl reverse primer (10µM)	12 µl	9a_two_primers_1X_plasmid.ab1

The quality of this sequence is poor throughout the entire read. This sequence cannot be used. The phenotype shown here is a mixed template, which means there are multiple peaks at each position.



## Sequence Annotation and Quality

The base calls are almost exclusively red, which are low-quality calls. **This sequence is cannot not be used.**

1	GGGCATTAAT	CTATGTGAGC	TATACGAGCT	CGGCCTTGG	TGATCTGCTA	GACTACACCT	GAGGATCTGC	70
71	GGCTTGAAGC	TCTATATCGT	CTATAGTGAG	CTTGATTACA	ATCATGGGGC	CGTTGTTTCC	TGTGTGAAAT	140
141	TGGGGAGCGCT	CTGATTCAC	CCAACTTACT	AGCCGTGGCG	ATAATCCGCC	TTTTCCGGGG	TGCCTAATGA	210
211	GTGAGCAAGC	CCGCATTAAT	TGCCCTTCCC	TCACTGCCCC	CTTTCTGATC	GGGAAACGGG	CGCGGCCTGC	280
281	TCCGTTACTG	AATCCGCGGC	GGCGGGGGGG	GAGGCGGTTT	CGGTATTGGG	CGCTTTCGG	CTTCCCTCGCT	350
351	CACTGACTCC	TTGGCGTTTC	TTCCCTTGGT	TGCTCGCAGG	GGTTCGGGT	TTTTCCGAGG	CGGCTCIACA	420
421	GCTATCCCCC	CAATCAGGGG	ATCGATTTAG	AGCTAAACGG	TGACCCAAAC	GCCAAAAACT	TGATTAGAAC	490
491	CGATGTTTCG	CCGCTGGGCT	GGCGTTTTTG	CTTAAGCGCC	GCCCCCCCGA	CGAGCATGGA	AAAATCGAGC	560
561	CTCAAGTCAG	GAGGTGGTGT	TCCCGGAGGG	GACTATACTC	ATACCTATCG	TTTTCCCGTG	TAATCACTCT	630
631	CGAGGGCTCT	CTGTTCGGGA	CCCTGCGGCT	TACCAGATAC	CTGTCCGGCT	TTCTCCCTTC	GAGCGGCGGT	700
701	GTGCTTTTAT	CATAGCTCAC	GCTGTATTGT	ATCTATGCTC	GGTGTTCGCT	GTACGCATCC	AAGCCGSTAT	770
771	GTGTACACCG	AACATGCGCG	TCTCCAGAA	CAATGTGCC	TGATGGCGCA	CTAGTTAACC	TGAGTCCGAC	840
841	CCCCGCGAC	ACCACGCTAT	CGCCGCTGT	GCAGCAGCCT	GCTGTGCACC	ACGGATATAC	CGCATAAGAG	910
911	ACTATGCTAG	TGCAGCTGTC	TACAGAGATT	CTTGCAAGTG	TGGACCGGTA	ACTGACCCGC	TCACTCTACG	980
981	AAGAACCAGC	AATACGTTATC	TGCGCCTCTG	ATGCAGCCTA	GTTTATCATT	ACGGTAAATA	GTACAGTGGG	1050
1051	TATCATCGGT	GATTCCTGGC	AACATCAAGC	TAGCCGCTT	GTCAGCGGG	AGAGTGTTCG	TCTGTAAGC	1120
1121	CACCTCAGTC	GAATAACGGG	CTAGRATAAC	AATGCAATCT	CATGTATGCA	CTCCCATGGA	TCCTATTCAA	1190
1191	CCGTGATCAG	AATGCCTTCA	TGAAACTGAA	AACATTGCGA	GTATATGATT	ATTGCTACAT	GTCACTTAGT	1260
1261	TCCAACACGG	TAATCTCCCG	TCITATAATG	CCGATTAGTC	ATAAACGTAT	TTTTCAATCA	GACTAACGGC	1330
1331	TGAGTTAAGG	TAAAGTTGGC	TGAACCGTTG	CCGAGGCTCA	AAGCTTGGGT			1380



## Signal Intensity

Average raw signal intensities can still be in the acceptable range even if the data is poor quality.

A(830),C(1449),G(464),T(1620)

## Summary

This example demonstrates the results if two primers are added to a Sanger reaction. Similar results can be seen when primers are not cleaned from a PCR product. The quality of this sequence is very poor and cannot be used.

To achieve usable results, prepare a fresh sample and add only one primer. If sequencing is needed in both the forward and reverse directions, two reactions are necessary.

If mixed template is observed in PCR products, visualize the products on an agarose gel to confirm that only one product is present. If there are multiple products, the sample will need to be purified so that only one product remains before Sanger sequencing.



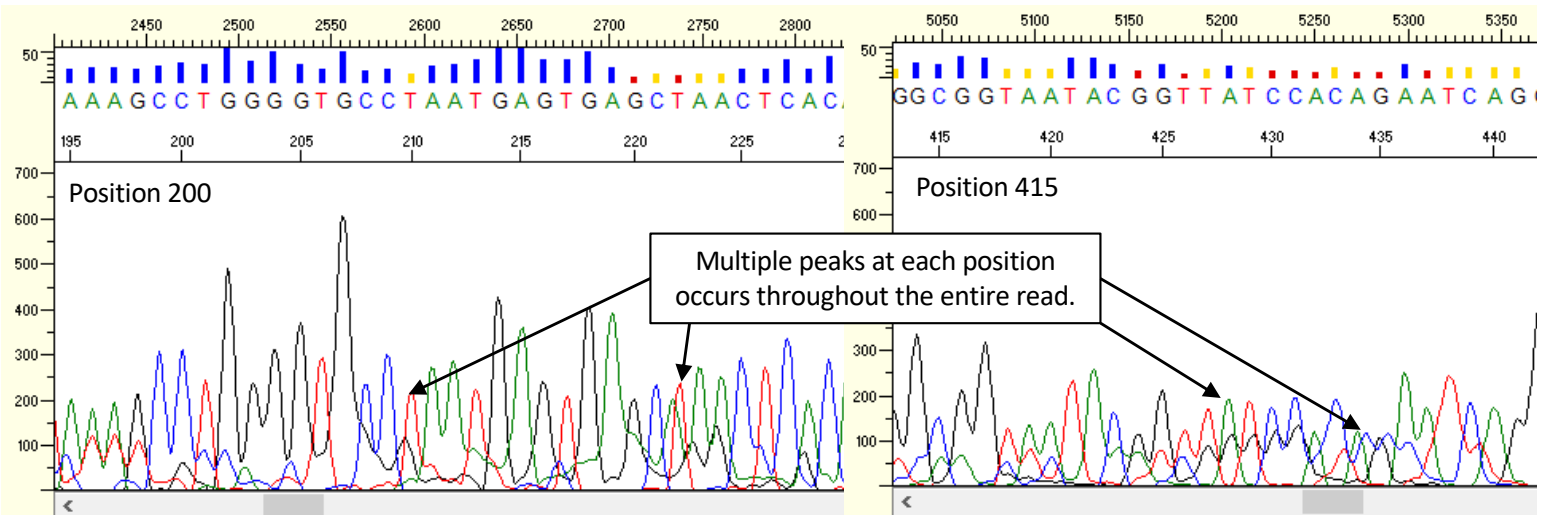
## Example 9b: Two primers and correct template

Two primers were added. One at the recommended concentration and the other at 1/10<sup>th</sup> the recommended concentration. The template was added at the correct mass.

The template source is the pGEM-3Zf(+) control; a double stranded DNA plasmid that is 3,197 bp.

Template Mass	Number of Primers Added	Concentration and volume of primers	Total Volume	File Name
1000 ng	2	3 µl forward primer (10µM) 3 µl reverse primer (1µM)	12 µl	9b_two_primers_1X_0.1_plasmid.ab1

The quality of this sequence is poor, showing the phenotype of mixed template at most positions throughout the read. Mixed template means that there are multiple peaks (base calls) at a single position.



### Sequence Annotation and Quality

Most of the base calls are red and yellow, which are low- and medium quality calls. **This sequence is poor quality and should not be used.**

1	TGGGGAATA	TCTATAGGG	GATTTCGAGCT	CGGTACC	GGATCCTCTA	GAGTCGACCT	GCAGGCATGC	70
71	GAGCTTGAGT	ATTCATAGT	GTCCACTAAA	TAGCTTGGCG	TAATCATGGG	CATAGCTGTI	TCCTGTGTGA	140
141	AATTGTTATC	CGCTCACAAT	TCCACACAA	ATACGAGCCG	GAGCATAAA	GTGTAAGCC	TGGGGTGCCT	210
211	AATGAGTGA	CTAACTCACA	TTAATTGCGT	TGCGCTCACT	GCCCCCTTTC	CAGTCGGGAA	ACCTGTCTGT	280
281	CCAGCTGCAT	TAATGAATCG	GCCAAACGCGC	GGGGAGAGCC	GGITTTGCGTA	TGGGGCGCTC	TTCGGCTTCC	350
351	TGGCTCACTG	ACTGCGTGGC	CTCGGTCTGT	CGGCTGCGGC	GAGCGGTATC	AGCTCACTCC	AGCGGGTAA	420
421	TACGGTATTC	CACAGATCA	GGGGATACG	CAGGAAAGAA	CATGTGAGCA	AAAGGCCAGC	AAAAGGCCAG	490
491	GACCCGTA	AAGGCCGCGT	TGCTGGCGTT	TTCCATAAG	CGCCGCCCC	CTGACGAGCA	TCACAAAAAT	560
561	CGAGGCTCAA	GTCCAGAGTG	GCGAACC	ACAGGACTAT	RAAGTACCA	GCGGTTTCC	CCTGGAGCT	630
631	CCCTCGTGG	CTCCCTGTI	CCGACCCTGC	CGCTTACCG	ATACCTGTCC	GCCTTTCTCC	CTTCGGGAAG	700
701	CGTGGCGCTT	TCTCATAGT	CACCGTGTAT	GTATCTCAGC	TCCGTTGAGC	TCCGTTGAGC	CAAGGTGGCC	770
771	TGTTGTCACG	AACCCCGCGT	TCACTCCGAC	CGCTGCGCTC	TTGAGGGGTA	ACTATCGTCC	TGAGTCCGAC	840
841	CCCGCTAAGA	CACCACGTTA	TACGCCACTG	TGACGACGCT	TACTGSTAAC	ACGGATTACC	ACAGACGAGG	910
911	TATGCTAGTG	CGGTGTCTAC	AGAGATCTTG	GAAGTGTITG	GACGTATCTA	CCGGTACTCT	AACGAAAAAC	980
981	CGCAATAGGT	ATCCGGCTAC	GTCTGCGACG	CAGTTTCCCTI	CGGTAATAAG	AAGTGAATRA	GATCGTTGAT	1050
1051	CCTGGCAAA	CAAGTAGCC	GCTTGTITAGC	GGGAGAGTTI	TTCTTGTITG	CCACTATTTT	GTATTATCTT	1120
1121	TTCAGAAATA	CAATSCATCT	CAAGTATGCA	TCCATGATC	TTATTCACC	GTGGATCAAG	AGCCTCAATT	1190
1191	GAAACTGAAA	ATCGCAGTAA	ATGGATTATIT	GTTACATGAI	ACTAGTCCAC	ACGTAATCTI	CCACCTAGAT	1260
1261	CCTTTAAGT	CATAATGATT	TGTCACCTCAG	TCTAACGGTA	GTGTATGATT	TAAACATTGG	CCTGGAACTT	1330
1331	TATCCGAGGG	CCTAATCGGT	AGCATCGAAC	TCCGATGCTAC	ATAGCCGATA	CGAATCCTCC	TA	1392



### Signal Intensity

Average raw signal intensities can still be in the acceptable range even if the data is poor quality.

A(826),C(1333),G(465),T(1528)

### Summary

This example demonstrates the results if two primers are added to a Sanger reaction. Similar results can be seen when primers are not cleaned from a PCR product. The quality of this sequence is very poor and cannot be used.

To achieve usable results, prepare a fresh sample and add only one primer. If sequencing is needed in both the forward and reverse directions, two reactions are necessary.

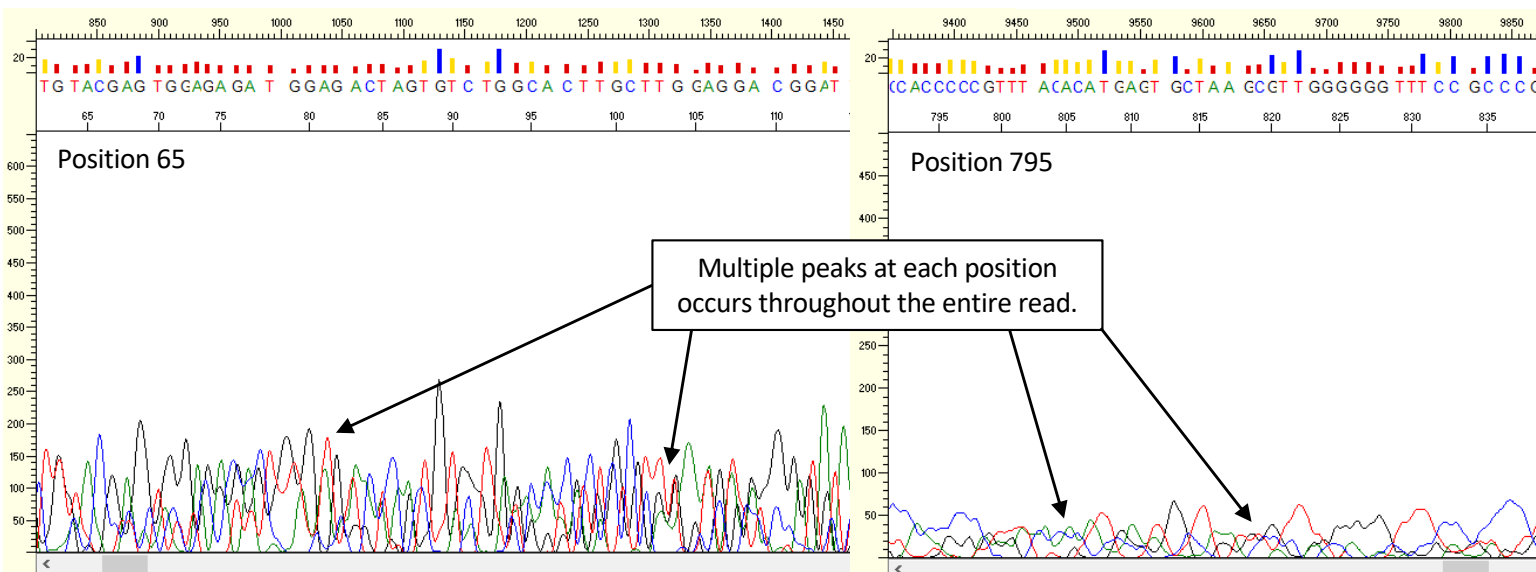
If mixed template is observed in PCR products, visualize the products on an agarose gel to confirm that only one product is present. If there are multiple products, the sample will need to be purified so that only one product remains before Sanger sequencing.

## Example 10: Mixed template but correct primer

The template source is the ZymoBIOMICS Microbial Community DNA Standard (Zymo Cat# D6306), a mock community composed of genomic DNA from eight bacteria plus two fungi. Full length 16S primers (27F and 1492R) were used to amplify the 16S rRNA gene. The product was purified with ExoSAP-IT. The product size is 1465 bp.

Template Mass	Number of Primers Added	Concentration and volume of primer	Total Volume	File Name
20 ng	1	3 µl forward primer (10µM)	12 µl	10_mixed_template_PCR_product.ab1

This sequence has multiple peaks (base calls) at each position throughout the read indicating a mixed template. This sequence is poor and should not be used.



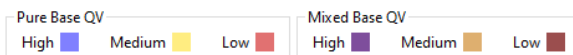
### Sequence Annotation and Quality

1	TTTCCCGGG	ATAACTGTG	CTCATGTATG	CCTACGTGTT	GAGAATCTAA	TGGTGTCTT	GTGTACGAGT	70
71	GGAGAGATGG	AGACTAGTGT	CTGGCACTTG	CTTGGAGGAC	GGATTTCCTC	CTGACAGCGG	CGCTTATCAC	140
141	CGATTATGTT	CTGAAGACCA	TGCAGGAGAA	CTGACGTGCC	TCCTGCCATC	ACATTATCCC	AGATGCCATT	210
211	ATCTTTTGG	TGATGTAACG	GGTCACCGGC	GCCACGATGC	CTATCTGGTA	TCAAACCATG	AAAGGSCACA	280
281	CTGCAACTGG	GACACGGACA	CCAGTCTTAC	ACCCTGCGGC	GGGGAGGAAT	ATTGCACTCT	TCCCGCAGGC	350
351	CTGATGCTGG	CATGCCCCCC	GCCCGAGGGT	GGCCTTAGGG	TTCTACTGTA	CTTCTTTTGT	GGATGAAGGA	420
421	AAATAAGTTA	ATAACGTTGC	TGGTTGACCC	TTGCCCGCAG	CTAACAGAAA	GCTACCTCCT	AGCTACCTGC	490
491	CCCGATACIG	CGGGAATACG	TAAGTGTCAA	TCGTTGTTC	TGAATTATTG	GGGGCAAGCG	GGCGCTCTGC	560
561	GGTTTCTTTA	TGCTCGATGT	GAAAGCTCCC	CCTGTCAAAC	TGGATATGAT	CCTGGAAAGC	TTGAAACTTG	630
631	TAGTGCAAA	TAGAATCTG	GATGTCCAGC	GTGTAACGCT	GAAATGCTA	GATATATTGC	AGGAACACCA	700
701	ATGCGGACCG	CTGCTCTACT	ACTCTGTACT	CTGACGCTGA	AGGCCTGAAG	CGCCTGACAA	GCCAAAGAGG	770
771	ATTGAGATAC	CTCTGTTACT	CCACCCCGGT	TTACACATGA	GTGCTTAGCG	TTGGGGGGTT	TCCGCCCGTA	840
841	ACTGCTGCGC	TAACCTCTTA	CCCTCTCCAC	CTGGGGAGTA	CGATCGAAGG	TTGAACATTG	AAGGAATTCC	910
911	CGGGGCTCAC	ACAACATGGG	ATCATGTGGT	ATAATTCGAA	ACAACGCGAG	AGCTTACAAG	TCTTGACATC	980
981	CTTTGACATC	TCTAGAAATA	GATCTTTCCG	TTGAGACAG	AATAGACGGT	GCATCGTGTG	TCACCTCAGG	1050
1051	TCTGAAAAAT	TGCTAAGTCC	GTAACACTCG	ACTCTTTACT	TCGTTCCCTG	TACTTGGACC	CTATAGGCAC	1120
1121	ATCCGTGAAG	ACGGTGGAGA	GGGGGAGAGA	CTCATGCCCT	GCGCCCTTTA	TCTCTGCTAC	CCCAGGTCC	1190
1191	CATGAAAGAA	AACGGCCGCA	AACGCGCGGA	CACAAGTCGA	AATGTTCTGC	ATTGCAATTG	ATTGAGCTCG	1260
1261	CAGCCGAACT	GATTCCTTGA	ATCCGAGAAC	TGACTACGTA	AACCGTCTCG	CACTGCACGC	TAGCGATCAT	1330
1331	TGACGTGATC	GCGTCTGTGA	TCGTGCA					1357

### Signal Intensity

Average raw signal intensities are in the acceptable range for this sample even though the quality is very poor.

A(497),C(449),G(370),T(455)



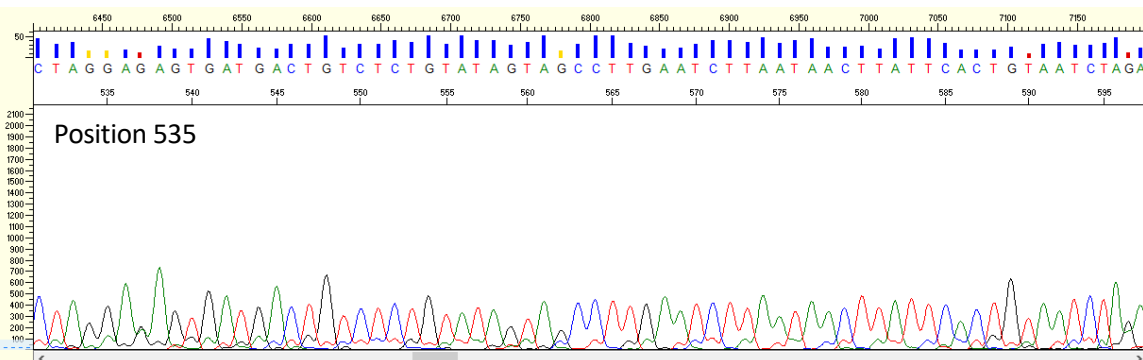
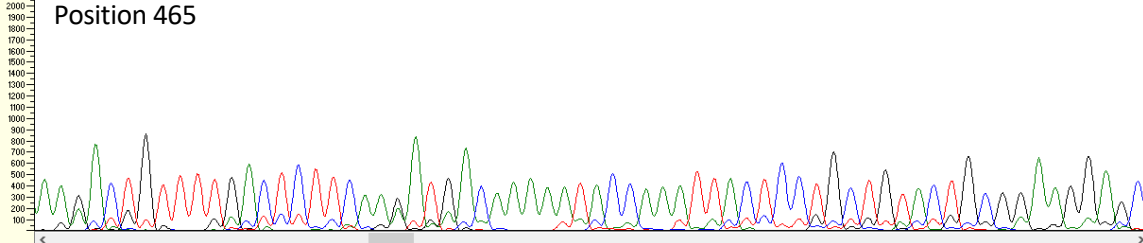
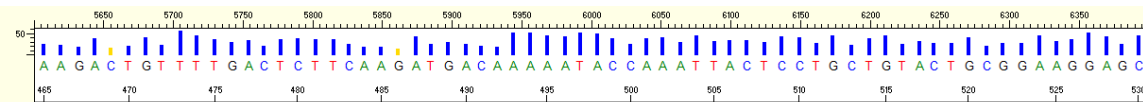
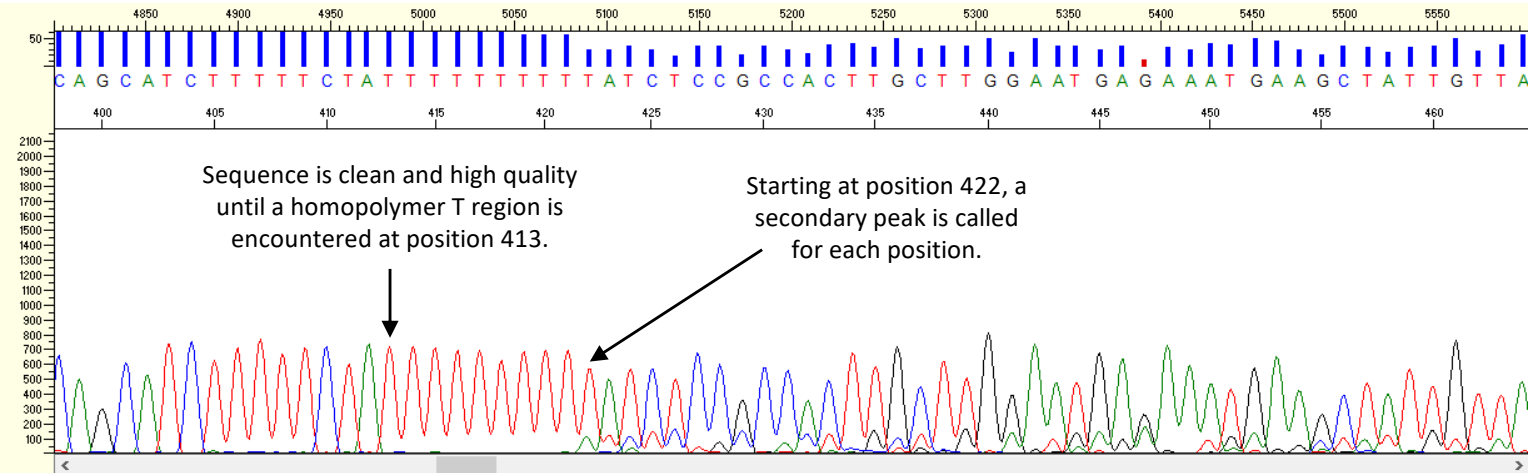
### Summary

This example demonstrates the results if one primer is added to a Sanger reaction that contains multiple targets. In this case, the target is the 16S rRNA gene from multiple bacterial species. The quality of this sequence is very poor and cannot be used. For this example, it is not possible to use Sanger sequencing. Either short read or long read sequencing is required.

# Example 11: Slippage (PCR product)

The template was added at the correct concentration. The primer was added at the correct concentration.  
The template source is a 510 bp double stranded PCR product that was purified with ExoSAP-it.

Template Mass	Number of Primers Added	Concentration and volume of primer	Total Volume	File Name
20 ng	1	3 $\mu$ l forward primer (10 $\mu$ M)	12 $\mu$ l	11_slippage_PCR_product.ab1



**Summary**

Secondary peaks continue through the entire read.

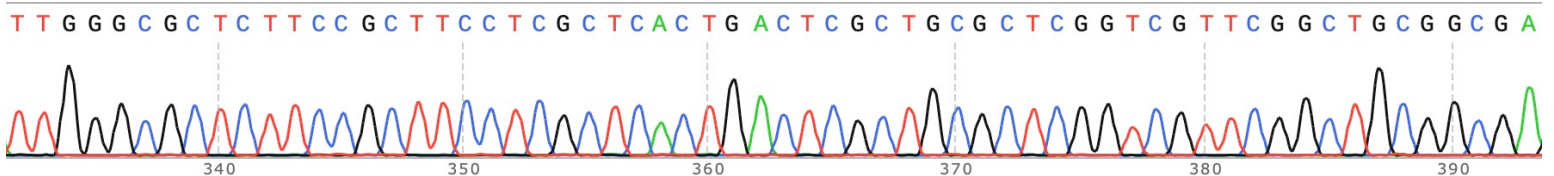
If the secondary peaks are too extreme, it may be necessary to sequence the opposite strand up to the homopolymer that is causing the slippage. Or, a new primer can be used to continue sequencing immediately after the homopolymer that caused the slippage.

In some cases, it may not be possible to satisfactorily sequence around homopolymers.

# Software for viewing Sanger Sequencing Data

Below is a non-exhaustive list of software available for viewing Sanger Sequencing data. Some of the software is free and some is paid. The images used in this guide were captured while viewing data in Sequence Scanner 2.

- Sequence Scanner 2
- SnapGene Viewer
- 4Peaks
- Chromas
- GeneStudio
- UGENE
- Chromaseq
- Geneious
- DNASTAR Lasergene
- Sequencher
- CodonCode
- DNA Baser



# File Descriptions & Where to download

File names and descriptions are in the table below. All .ab1 files presented in this guide are available to download from the Genomics Core's website at:

<https://rtsf.natsci.msu.edu/genomics/technical-documents/sanger-sequencing-best-and-worst-practices.aspx>

Page	File Name	Description
9	1_correct_plasmid.ab1	Correct template and primer concentration (plasmid)
10	2a_0.1_template_plasmid.ab1	1/10th template concentration and correct primer concentration (plasmid)
11	2b_0.01_template_plasmid.ab1	1/100th template concentration and correct primer concentration (plasmid)
13	3_correct_PCR_product.ab1	Correct template concentration and primer concentration (PCR product)
14	4a_0.1_template_PCR_product.ab1	1/10th template concentration and correct primer concentration (PCR product)
15	4b_0.01_template_PCR_product.ab1	1/100th template concentration and correct primer concentration (PCR product)
17	5_5X_template_plasmid.ab1	5X template concentration and correct primer concentration (plasmid)
18	6_5X_template_PCR_product.ab1	5X template concentration and correct primer concentration (PCR product)
19	7a_0.1_primer_plasmid.ab1	1/10th primer concentration and correct template concentration (plasmid)
20	7b_0.01_primer_plasmid.ab1	1/100th primer concentration and correct template concentration (plasmid)
22	8a_10X_primer_plasmid.ab1	10X primer concentration and correct template concentration (plasmid)
22	8b_40X_primer_plasmid.ab1	40X primer concentration and correct template concentration (plasmid)
23	9a_two_primers_1X_plasmid.ab1	Two primers added at the recommended concentration. The correct template concentration was used (plasmid)
24	9b_two_primers_1X_0.1_plasmid.ab1	Two primers added. One at the recommended concentration and the other at 1/10th the recommended concentration. The correct template concentration was used (plasmid)
25	10_mixed_template_PCR_product.ab1	A microbial community standard was amplified. The correct template mass and primer concentration were used.
26	11_slippage_PCR_product.ab1	This example demonstrates slippage in a PCR product. The correct template mass and primer concentration was used.

## Questions?

Contact the RTSF Genomics Core at [gtsf@msu.edu](mailto:gtsf@msu.edu)