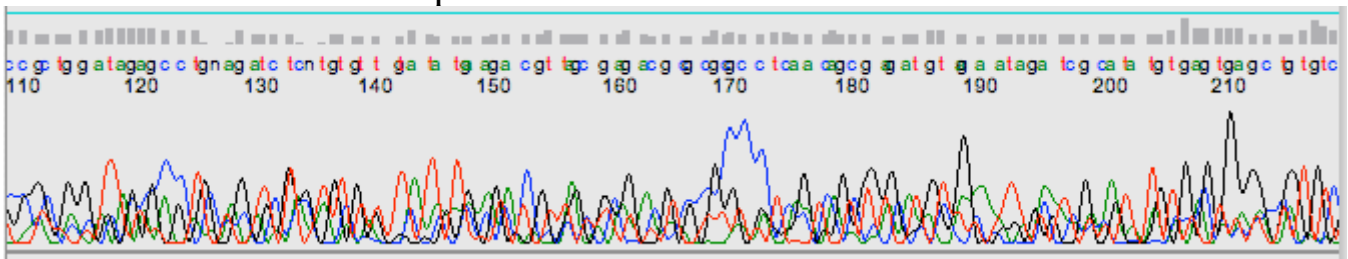


Troubleshooting Suggestions

The DNA Sequencing Facility is happy to consult with any of its customers having difficulty with DNA sequencing. Please don't hesitate to contact us with any questions you may have.

1 Blank Samples

On the chromatogram view you will see data with undefined peaks close to the baseline. See example below:



Further the signal strength will be low. Signal strengths below 200 are considered blank. You can view your signal strength on finch by clicking on your data link. Once you do so the chromatogram details page will come up. Then notice the signal str. See below

Format	ABIF
Plate Label	n/a
Sample ID	n/a
Instr. Name	3730-2-15105-004
Run Stop	2006-04-25 18:34:26
# Lanes	48
Signal Strs	A=20,C=21,G=16,T=19
Matrix	n/a

Listed below are the most common reasons for blank samples.

- a) Incorrect primer used or no primer annealing site in the template. Even a one base mismatch between the primer and the template can result in a

blank sample. The best way to test this possibility is to perform a regular PCR reaction using the sequencing primer and a second primer. Use 1 ng template and 100 ng each primer for 25 cycles. If you don't see a strong band of your correct product, you know your primer(s) aren't annealing. Getting a PCR product does not, however, guarantee a successful sequencing reaction.

b) Not enough template.

Accurate quantitation of your template is critical. Dirty template preps will often give inaccurate high Abs. 260nm readings leading you to think you have more DNA than you really do. If you believe you didn't have enough template you can double the amount per reaction.

c) Impurities in the template prep or primer.

This rarely results in a completely blank lane. Rather, impurities will most often cause weak, short, poor quality sequence that starts strong and fades quickly. The easiest solution is to reprep your template using a high quality commercial kit. Impurities in primers can be removed by purifying or remaking the primer.

d) Extremely high GC content or template secondary structure.

Once again, this rarely results in a completely blank lane. Typically you will see normal sequence until the high GC region. See below for solutions to sequencing through regions of high GC content and secondary structure.

2 Weak samples

Caused by the same reasons as blank lanes, only less severe. Also can be caused by using too little primer or a weakly annealing primer.

3 Short reads

If your peaks start off strong and then rapidly decrease, you either have impurities inhibiting the sequencing reaction or a large excess of template. Doublecheck the amount of template in your reactions or reprep your template or purify your primer and requantitate them. .

4 BAC or other large templates

The two major problems with sequencing very large templates are getting enough signal strength and denaturing the template completely. On a molar basis 1 μ g of a 100kB BAC clone contains 5% as many template molecules as 1 μ g of a 5kB plasmid. Since increased amounts of template are often required to sequence BAC clones, using high purity template is especially important. The

sequencing facility uses special PCR conditions when sequencing large templates. Be sure to let us know when you are sequencing a large (>15kB) template.

5 **High GC content / secondary structure.**

Regions with high GC content or secondary structure are difficult to sequence through. The most common problem seen is weak signal (or no signal) when the high GC or secondary structure region is encountered. SiRNA plasmids are a common example. The DNA Sequencing Facility recommends a three-step approach for sequencing through difficult regions. It is usually impossible for us to predict which, if any, approach will work best. We have a sequencing option that uses DG/DI.

a) Alter the PCR conditions.

This is the easiest approach since the template and primer remain the same.

Increase the denaturation temperature to 98°C.

Add DMSO to a final concentration of 5% v/v.

Add betaine to a final concentration of 1.0 M.

Increase the annealing temperature or eliminate the primer annealing step of the PCR program.

Contact us and we can devise a strategy to try with your sample.

b) Linearize the template or select a different primer. Since linear DNA denatures more efficiently than supercoiled DNA, digesting your template outside the region of interest sometimes helps. Alternatively, selecting a different primer even though it may anneal very near your original primer sometimes results in improved sequence due to differences in hybridization efficiency.

c) Subclone the insert into smaller fragments (<200 bp) or prepare ssDNA.

Subcloning the insert into smaller fragments minimizes the amount of high GC template and reduces the opportunity for stable secondary structures to form. Generating ssDNA for use as template is the most reliable method for sequencing through difficult regions but also the most labor-intensive.

6 **Homopolymer Regions**

Long poly A or T regions can cause problems in DNA sequencing reactions due to "slippage". The exact mechanism of "slippage" is not known, but is hypothesized to occur because the two DNA strands do not stay paired correctly during polymerization through the homopolymer region. The

sequence is usually fine up to the homopolymer region but then degrades to multiple peaks. The longer the homopolymer region, the more likely "slippage" is to occur. Good sequence immediately past the homopolymer region can usually be obtained by sequencing in the opposite direction or using an anchored sequencing primer.

7 Slippage with PCR fragments.

Slippage also occurs when sequencing PCR products. Usually the best strategy is to clone the PCR fragment and then sequence the clone.

8 Repetitive elements

Templates containing short repetitive regions (200-300 bp) are generally not too difficult to sequence unless the base composition is problematic. When the length of the repeat is more than 500 bp it can be difficult to get good sequence. In the absence of unique sequence, primer walking is not an option. Directed deletions or in vitro transposon insertion may be needed to sequence these regions.