Sanger Sequencing Services

In this issue, the DNA Core will focus on Sanger sequencing services. Next generation sequencing has certainly impacted research the past several years and has been a large part of expansion in core services. Sanger sequencing, however, continues to be an essential tool in the research lab with the DNA Core performing ~40,000 reactions per year. As a mature technology, obtaining results is often straightforward for most templates but problematic templates can result in frustration for even the experienced user. This newsletter attempts to offer best practices for submitting samples, evaluating data, and troubleshooting common sequencing issues.

Sanger Sequencing

- Improper Tube – A Tecan Freedom Evo is used to automate the transfer of sample and addition of BigDye for reaction set-up. The incorrect tube may result in incorrect volume transfers and damage to our robot.
- Incorrect Volumes– < 16ul can result in poor sequence.
- Correct Labels on Tubes– Be sure to follow guidelines on website for labeling tubes. [http://biotech.missouri.edu/dnacore/sangersequencing.html#labeltube](http://biotech.missouri.edu/dnacore/sangersequencing.html#labeltube)

Fragment Analysis

- Overloaded Sample– PCR amplification with labeled primers often yields amounts greater than can be loaded directly to the instrument. Diluting final products by 10 to 25-fold is typical for most samples. Not diluting samples can result in off-scale peaks and even lead to a damaged instrument array (~$5,300 replacement cost). Contact Ellen Kesler (keslere@missouri.edu; 884-8973) and receive guidance on proper loading amounts.

Cautionary Notes on Submission Requirements

Common Question

Several samples have been submitted to the dnaLIMs but some of the samples in the order will not be delivered to the core. Can I delete the individual samples from the order?

Answer

Any sample can be deleted from an order. Begin by logging in to your dnaLIMs account. Select "View Your Requests". The next window will allow you to delete the sample by entering the requisition number and selecting "Delete Request". Click on the "Submit" tab and the sample has been removed from the order. The same can be done for an entire order. Caution!...deleting an order removes all the samples in that order!
Best Practices for Evaluation of Sanger Data

Although automation has simplified DNA sequencing allowing large amounts of data to be quickly generated and analysis performed, errors still occur in the sequencing process. The researcher should not trust entirely the associated text file that supplies data in a text-only format. Many problems with sequencing results are not recognized by viewing the text file alone. The quality of sequence data should always be evaluated by studying the accompanying chromatogram to identify problem data and check base calls made by the analysis software.

There are situations in troubleshooting sequencing results when potentially noisy data must be distinguished from a failed reaction with only background noise. This scenario can only be sorted out by viewing the chromatogram. Sequence Scanner v1.0 is freeware provided by Applied Biosystems for viewing trace files. Open the file within the Sequence Scanner software. Select the Annotation tab at the bottom of the open chromatogram window. The "Average Raw Signal Intensity" will be >1000 units for sequence with strong signal (Figure 1). Signal intensities <100 units indicates background signal only.

A sequence reaction that yields more than one sequence product results in multiple peaks at each nucleotide position. The reaction has worked well but multiple templates result in overlapping peaks. Viewing only the text file, the wrong conclusion about the sequence reaction may be reached. The tex would look very similar to a failed reaction with many N's. The chromatogram would quickly confirm overlapping sequencing data.

Good sequence reactions can be expected to yield read lengths of at least 800-900 bases. Peaks should be evenly spaced with minimal background noise (Figure 2). Review of the chromatogram will also identify the trim position based on quality score.

Poor sequencing reactions that give weak signal strengths can be expected to yield shorter read lengths with ambiguous base calls. Smaller peaks beneath larger peaks are often due to background noise being amplified by the analysis software. The amplification of background signal occurs when the software detects low signal strength, typically 200-500 intensity units, and attempts to normalize sequence signal for the entire read length.

Common problems encountered are:

- Template quantity - excess template typically yields sequence data with short read lengths and excess signal at the start of the sequence.
- Excess salt - Salts can be introduced in a sample through poor elution from a column or a poor wash step during the precipitation method. Excess salts severely inhibit polymerase activity. The effect of salt on a reaction can often be observed by viewing a “ski” slope effect in the raw signal intensity plot as the signal drops with read length.
- EDTA in buffer - EDTA is a potent inhibitor. Use of EB buffer or 10mM Tris-Cl (pH 8.5) is recommended.
- Ethanol - The presence of ethanol will inhibit polymerase activity. Ethanol contamination most commonly occurs to samples that have been precipitated with ethanol and residual ethanol remaining from an incomplete drying step.
Tech Tips for Handling the Problematic Template

Technical Tip #1 — Avoiding contaminants when using spin columns in the isolation method.

The presence of inhibitors (i.e., salts, ethanol, phenol) can be introduced during the isolation of the plasmid. A common source can be the silica based commercial spin columns found in various kits from Qiagen, Promega, and others. Binding of the DNA is carried out in the presence of an alcohol solution containing chaotropic salts. The wash step prior to the elution step can be problematic if all of the alcohol and associated salts are not removed. The geometry of the columns with the use of an angle rotor can leave residual binding buffer which in even small quantities can inhibit the sequencing reaction.

You can confirm that residual buffer remains by transferring the column to a new tube after the wash step and spin at the suggested g-force for an additional 5 minutes. You will likely observe an additional small volume. A couple of simple steps that will improve your signal intensities and reaction success.

1. Spin the column 3 minutes longer than the recommended time at the wash step to ensure removal of binding buffer.
2. After decanting the buffer, repeat the spin for 2 minutes to dry the filter.
3. Rotate the column 180 degrees to remove any buffer trapped on the retaining ring. (Zymo Research columns typically don’t have a problem with trapped liquid on the retaining ring due to their design)

Technical Tip #2 — Strategies for Sequencing of secondary structures.

Secondary structure can be very stubborn to sequence. The core’s initial recommendation is to use DMSO in a 5-10% concentration in the sequencing reaction. To obtain a 5-10% final concentration in the sequencing reaction, add 1.4 - 2.6ul of 100% DMSO in your 16ul of submitted sample. The next method to attempt is the addition a 5 minute heat denaturation step (98oC) prior to the addition of Taq and normal cycling parameters. Shown to be quite effective in an earlier study.


Strong hairpin structures can be resistant to a high heat-denaturizing step alone. Designing a primer with a high Tm directly upstream of the hairpin structure (20-30 bases upstream) and cycling the reaction with a high denaturizing and annealing step has also been effective in our hands in sequencing difficult hairpin structures. The close proximity of the polymerase initiation complex is thought to aid in keeping the hairpin structure denatured during the extension step. Cycling conditions used under these circumstances are:

**Cycling Parameters:**
- Step 1 - 96oC for 1 minute
- Step 2 - 96oC for 30 seconds
- Step 3 - 70oC for 20 seconds
- Step 4 - 60oC for 4 minute
- 45 cycle’s total

The addition of other additives such as betaine, glycerol, etc. have not been shown to be very effective.
Why Did My Reaction Fail?

None of us enjoys receiving a sequence trace which displays only background noise indicating a failed reaction. Unlike other sequencing issues, a failed reaction provides few clues to the reason for the lack of sequence. The failure can involve an issue with either the template or primer. Therefore, a systematic approach to distinguishing between the two can save time and reduce costs. The following flow chart provides a step-wise process to help determine the cause of a failed reaction.

Is the DNA Suspended in TE Buffer?  
*EDTA will chelate the Mg$^{2+}$; a necessary cofactor for the polymerase

- Yes → Perform an ethanol precipitation with 70% ethanol wash. Resuspend in nuclease-free water and repeat reaction. Was the reaction successful?
- No → No

Was the template submitted at the correct DNA concentration? Don’t trust your nanodrop; confirm by agarose gel. PCR product (50—275 ng) dsDNA (800-2000 ng)

- Yes → Adjust the DNA concentration and submit the sample again. Was the reaction successful?
- No → No

Does the sequencing primer have a Tm $\geq$ 60°C?  
*reactions are annealed at 50°C

- Yes → Redesign primer with Tm greater than 60°C and submit the sample again. Was the reaction successful?
- No → No

Is there an alternate primer that will anneal to the template?  
*An alternate primer can demonstrate that the template is good and the original primer is the problem

- Yes → Submit template with the new primer. Was the reaction successful?
- No → No

Contact the DNA Core staff for further troubleshooting.

Success!