Troubleshooting Guide

This guide identifies some of the most common sequencing problems, as well as possible solutions. The guide will also include more general tips for improving DNA sequencing quality.

Identifying the cause of a poor DNA sequencing result can often be difficult, as a particular sequencing problem may have many different underlying causes, or be the result of multiple interacting factors. Often the only way to work out the real cause of a particular problem is to perform a process of elimination.

Template purity and concentration are the two most important factors in obtaining good quality sequence data. Fluorescent sequencing is very sensitive to certain DNA contaminants and DNA that is considered clean enough for many other molecular biological procedures such as PCR, cloning, or even manual radioactive sequencing, is NOT necessarily clean enough for fluorescent sequencing, and can lead to poor quality data or, sometimes, no data at all.

Sample quantitation: quantitation of your DNA by spec is probably the least accurate as both RNA and contaminating DNA will also absorb at 260nm and can potentially lead to inaccurate values. Quantitating by agarose gel electrophoresis tends to be more accurate as you can visualize any contaminating DNA or RNA. Perhaps the most accurate measurement method is the use of a fluorometer; fluorometric measurements can detect DNA levels as low as 5 ng/ul.

Note: Do not use Tris/EDTA (TE) buffer to dissolve DNA or primers. EDTA chelates Mg++, which is required for the sequencing reactions. If possible, use water. If water is not feasible, make sure the buffer does not contain EDTA.

Key DNA Sequencing Terms:

Vector: DNA molecule used to replicate DNA, i.e. plasmid or BAC (see below)

Plasmid: small, circular piece of DNA often found in bacteria; isolated, target DNA is spliced in, plasmid is put back into bacteria where it replicates and yields billions of copies of target DNA

BAC (Bacterial Artificial Chromosome): used like a plasmid, BAC's are constructed with target DNA, inserted into bacterium, replicated; used to replicate large pieces of DNA (100-400kb)

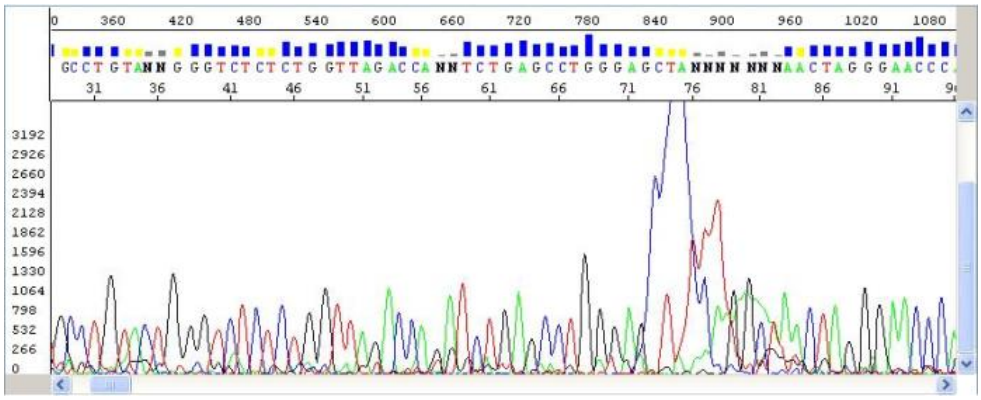
Insert: piece of DNA put into a vector and replicated; target DNA being sequenced

Shotgun Sequencing: large segments of DNA are fragmented, replicated in a vector and sequenced; sequences are then analyzed to determine the sequence for the entire DNA segment

Primer: small piece of ssDNA that can hybridize to one strand of template DNA

Sequencing Instrumentation Errors:

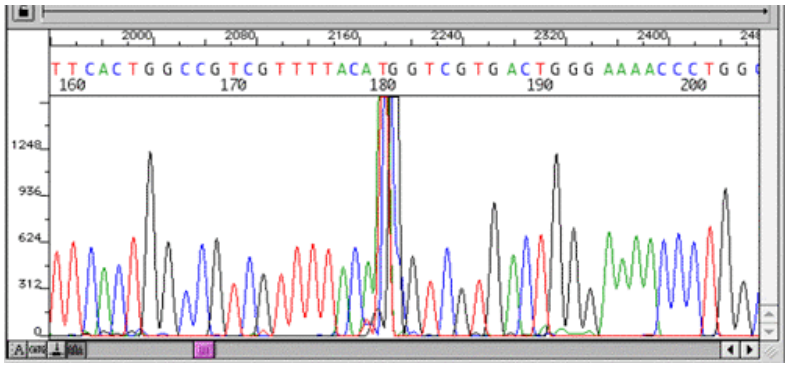
Dye Blob: due to the co-precipitation of dye-labeled ddNTPs with the labeled DNA



- Causes:
    - Poor post-sequencing cleanup
    - Lost pellet
    - Failed reaction
    - Sample itself may have contaminant that binds unincorporated dyes

NOTE: If this dye artifact affects the sequencing, we will re-sequence the sample. If we do not repeat it, and this causes problems with your data, just let us know and we will re-sequence the sample.

Bubble: Bubbles in the capillary cause the chromatogram below. Re-sequencing generally solves the problem. If you see this and we have not repeated the sequencing, let us know and we will re-sequence immediately.

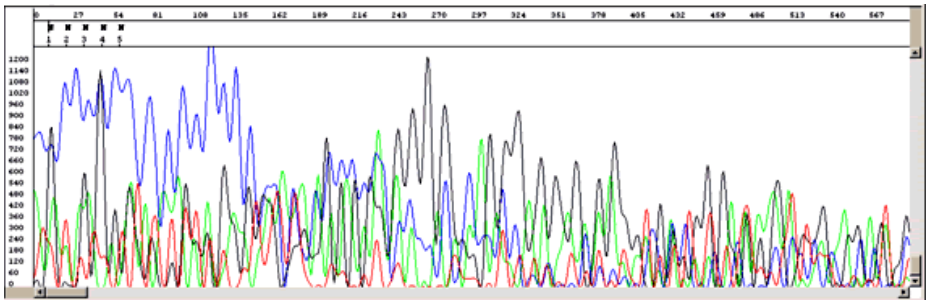


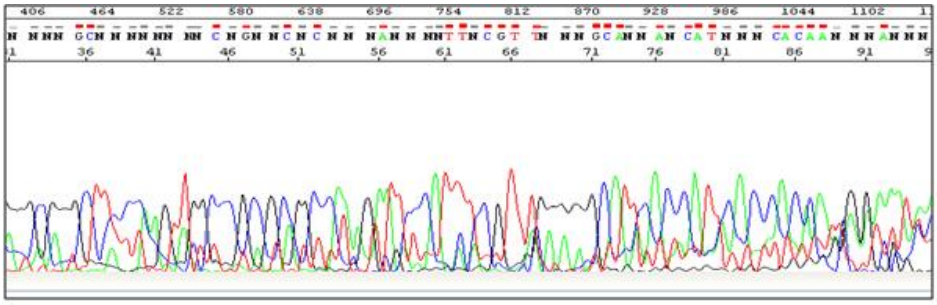
Primer Notes:

- Make sure they are at the correct concentration
    - A 40nm primer diluted in 1mL H2O results in 4uM primer concentration
- Make sure there is only one primer binding site
- Melting temperature of 50-55C
- Length should be 18 – 24 bases
- Use a G or C (G/C “clamp”) on the 3’ end
- Avoid strings of 4 or more of the same base in the primer
- The PCR primer does not always work for sequencing and a new primer must be designed

Chromatogram Interpretation:

No Sequencing Data





Cause:

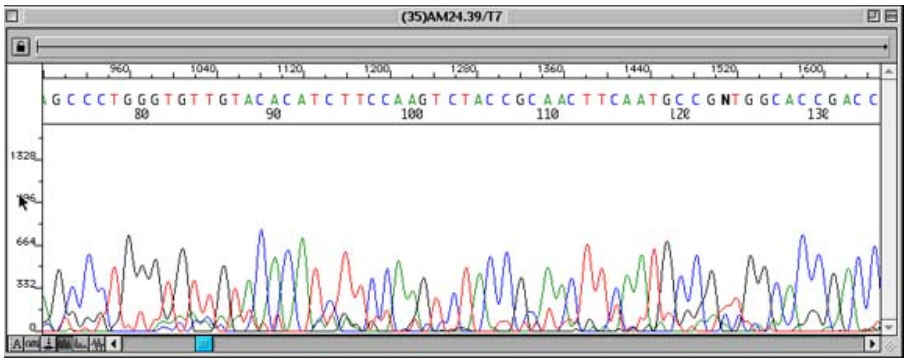
- Priming site not present
- Inefficient primer annealing
- PCR primer did not work as sequencing primer
- Not enough or no DNA/primer in the tube

- Contaminants inhibiting sequencing
- Secondary structure

Solution:

- Redesign primer to contain priming site on vector
- Carefully quantitate both DNA and primer – make sure both are being added to the tube according to the submission policies found at: http://medicine.yale.edu/keck/dna/protocols/index.aspx
- Use a high quality purification method that will remove contaminants.
- Make sure reagents are not expired.
- If template has secondary structure, high GC content, high Tm, etc., try our Difficult Template protocol.

Noise



Throughout the sequence

- Very low signal strength (not enough DNA)
- Degraded DNA
- High signal strength saturated the detector
- Multiple templates in the reaction
- Multiple priming sites
- Multiple primers
- No primer
- Inhibitory contaminant
- Inefficient primer annealing.

After a point in the sequence

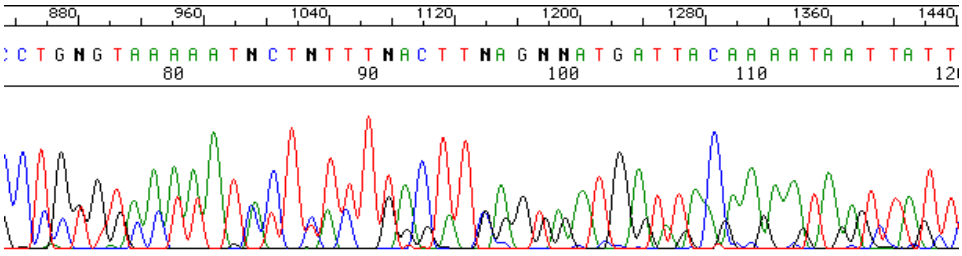
- Mixed plasmid or PCR prep
- Frameshift mutation

- Repeat region in template
- Slippage after a homopolymer region in template
- Primer – dimer contamination

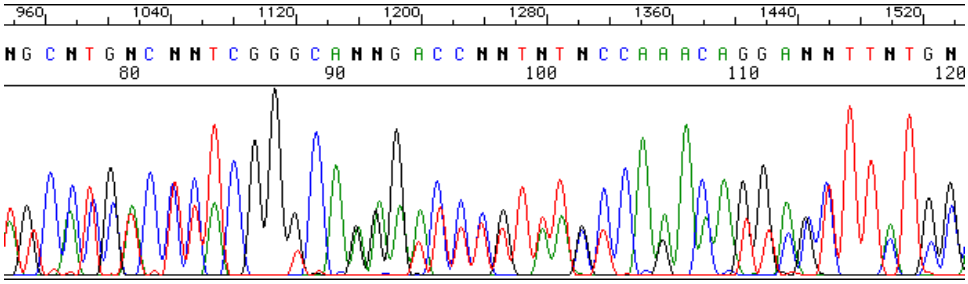
Mixed Sequence

Occurs with both plasmids and PCR. Two or more templates present in the sequencing reaction. Characterized by one major sequence, second set of peaks exactly under the first set, can result in "N" readings. *Make sure only one clone is selected and grown up, or check PCR products on a gel.*

Mixed Sequence (unrelated templates)



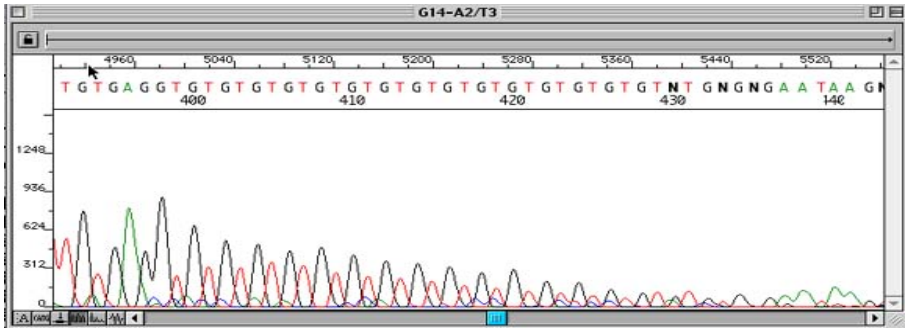
Mixed Sequence (related templates)



Causes:

- Multiple priming sites
- PCR primers acting as both forward and reverse
- Residual primers and/or dNTPs
- Primers with high Tm
- Primers with n-1 population
- Mixed templates or multiple PCR products
- Frame shift mutation

Gradual Early Termination of Sequencing Signal



Problem:

Premature termination of sequence due to repetitive structure(s) of DNA.

How to identify:

Repetitive stretches of DNA sequence (usually mono- or dinucleotide repeats) that show strong sequencing signals at the beginning of the repeat and then gradually taper off to an unreadable signal.

Cause:

- Slippage of the DNA polymerase on the template strand during elongation

- Formation of secondary structure due to the repeat.

- Depletion of dNTPs.

Solution:

- Sequence the template from the other side of the repeat in the reverse direction to obtain sequence on both sides of the repeat. Be aware that it will be difficult to determine the precise length of the repeat if neither sequencing reaction manages to span the entire length of the repeat.
- Use a poly-mononucleotide primer with a degenerate base (wobble) at the 3′ end.

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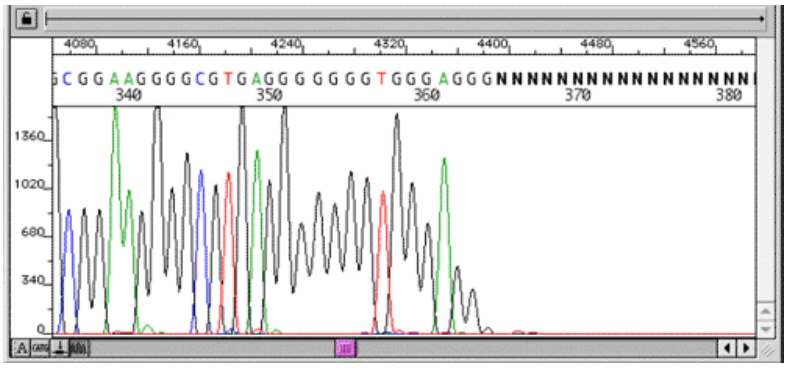
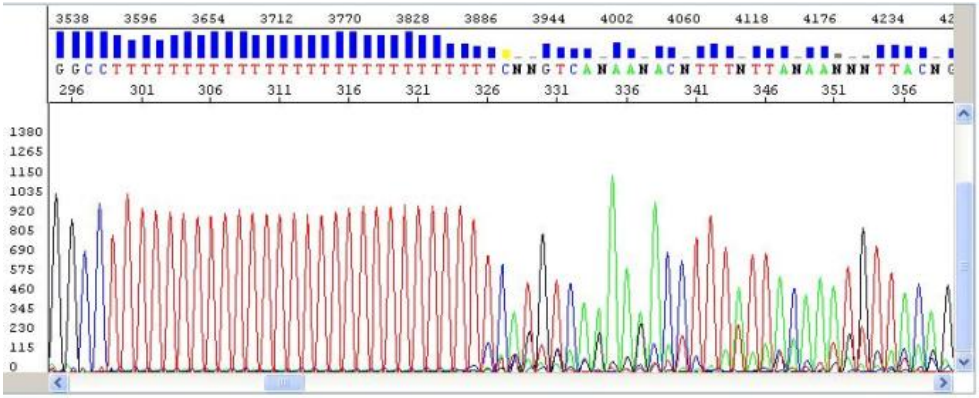
Mid Sequence Stop or Drop-Off



Causes:

- Secondary structure
- DNA is digested
- Too much DNA
- Salts or other contaminants
- GC or GT rich regions
- Inaccurate primer concentrations

Homopolymer Region



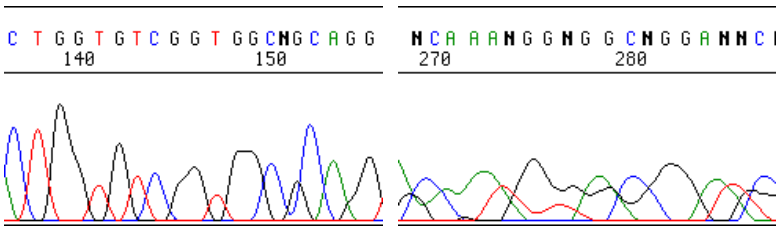
Causes:

This is called "polymerase slip". It happens when the growing strand temporarily dissociates from the template, then re-associates at a different spot - say, one nucleotide forward or back from where it started. If this happens often enough (as it will on polyA or polyT templates), every individual band becomes a family of closely-spaced peaks giving a 'roller coaster' look to the chromatogram.

Solutions:

- Try sequencing in the other direction from the opposite strand
- Try another primer either closer or further from the homopolymer region.
- Use a poly-mononucleotide primer with a degenerate base at the 3’ end

Loss of Resolution



Causes:

- High salt concentration
- Too much DNA
- Poor template quality
- Unknown contaminant

Solutions:

- Perform further clean up steps on template
- Verify concentration of DNA