SEQUENCING TROUBLESHOOTING

No sequence or very weak sequence

Possible explanations:

There was no DNA in your tube (or far less DNA than necessary).

- **Did you quantitate the DNA using a spectrophotometer?**
  - Make sure your reading was at least 0.1 AU (or as low as 0.05 might be acceptable if the spec was carefully calibrated).
  - Mini-preps, PCR reactions and gel-eluted fragments usually cannot be measured reliably only with a microliter-scale spec.
- **Did you estimate the concentration from a gel?**
  - If you are sequencing PCR reactions, you will almost always have to use an analytical gel to estimate DNA concentration.
  - Estimation by gel is difficult. If you are not experienced at this, find someone who is more experienced to help you. You should run the gel on the *same* tube of DNA you are sending us ... i.e. don't gel elute the band and assume 100% recovery.
- **Double-check all calculations!!**

There was no primer in your primer tube.

- Double-check the primer concentrations. (pMol/ul - *picomoles per microliter* **not** 'picomolar'.

The primer did not interact efficiently with the template.

Things to consider:

- Identity of your plasmid? A very common error is to submit an erroneous plasmid due to a cloning mistake, rearranged plasmid or damaged priming site. (Make a complete restriction map, look for unexplained bands or unexpected band sizes. Cutting out the insert or PCR a fragment as verification of your construct may not be enough.)
- Did you design the primer from accurate sequence information?
- Are you sure the priming site is present in *this* template?
- Did you design the primer to function at *our* annealing temperatures (We anneal at 50, 55 and 60 degrees C)? Because we handle so many samples, we must process them all at consensus cycling conditions. Exceptions can be made if requested, but NOT routinely.
- Might the priming site have been accidently damaged?

Occasionally, cloning artifacts can create a deletion near the insertion site, or a deletion that removes the priming site. Careful restriction mapping can detect the latter, and using an alternative primer will usually work in the former situation.

- Was this template a PCR product? Did it amplify correctly?

The lane was blank THIS time, but the exact same sample worked fine before!

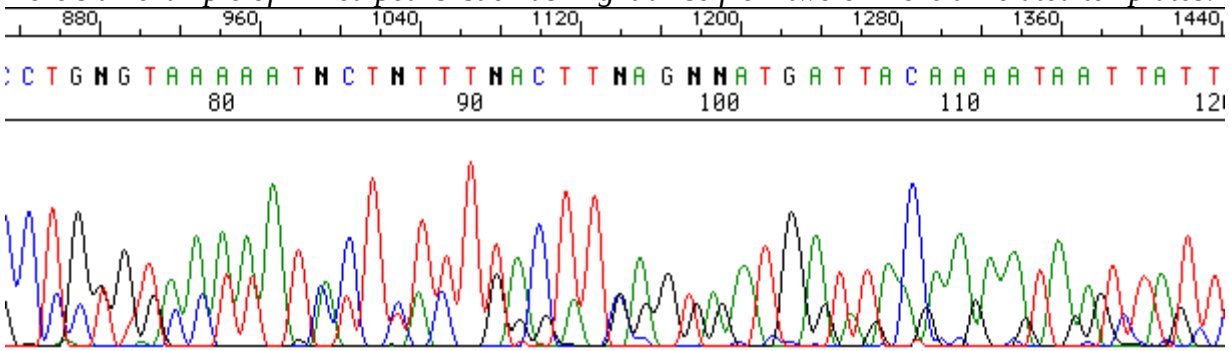
- Your sample may have been weak before when you say it "worked".
  A weak lane, when repeated, will often be blank, and vice-versa. Which one you get is a matter of luck, depending on how noise-free is the lane to which your sample was assigned. Check the signal strength of the previous "good" lane and if it was below 100, you probably just got lucky before.

Poor quality sequence

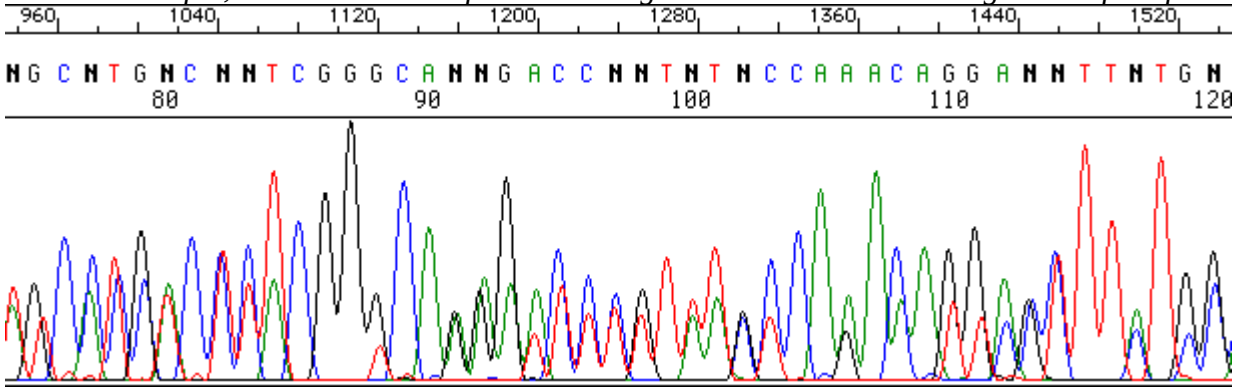
Please consult the following table.

There are bands, but they are weak and noisy, or even uninterpretable.	Check your chromatogram for text like: "Signal G:23 A:28 T:17 C:30". On a printed chromatogram, it's near the top. On Applied Biosystems' free program Sequence scanner v.1.0 (download from http://www.appliedbiosystems.com/support/software/) you can view the signals from opened trace (sequence) on a Annotation sheet. Please, ask if you need help with this program. These signal-strength numbers are an indication of the relative fluorescent strength of the bands in your lane. Good samples will have a G signal of 100-1000, and ideally it will be around 500. If your signal strength is below 100, background bands that are normally too low to see will become very evident and will interfere with basecalling. If your signal is below about 40, your peaks may get lost in the background noise. You might get lucky sometimes. Baseline noise varies from lane to lane, run to run, and instrument-to-instrument. If you happen to get a low-noise situation, even a sample with a G signal of 25 will give great sequence. The next time, that exact same sample will bomb, because the noise was too high.
Poor resolution from the start.	You probably have a contaminant in your template that caused a loss of resolution in our capillary electrophoresis.
The bands are present and strong, but irregularly spaced, or with mixed colors.	If you see this, you usually have two sequences superimposed on each other. There are several common causes: The sequencing primer binds to two (or more) sites on the template. There are two (or more) templates present. This was a PCR reaction, and you didn't remove the original primers. This was a PCR reaction, and one primer generated *both* ends. This was a PCR reaction, and there is more than one amplified species present. A similar outcome is often seen in which the bands start out fine, but later on become superimposed.

Here's an example of 'mixed peaks' such as might arise from two or more unrelated templates:

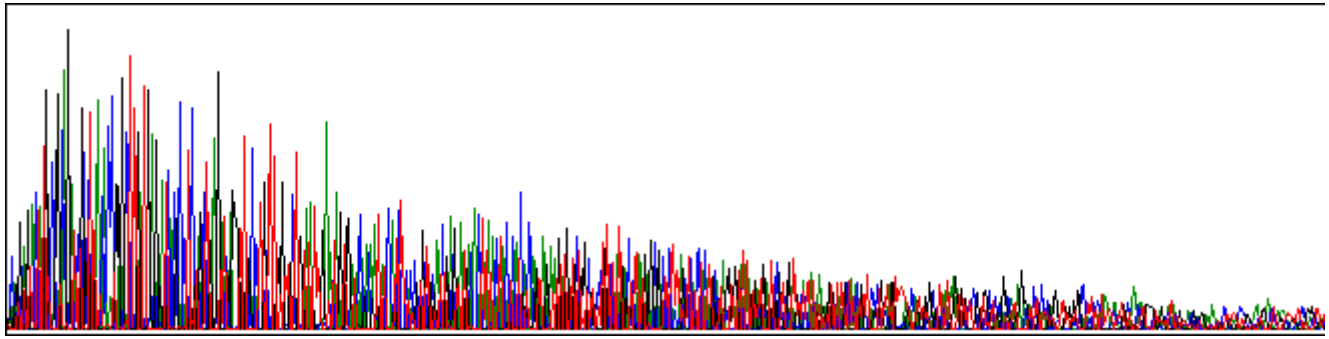


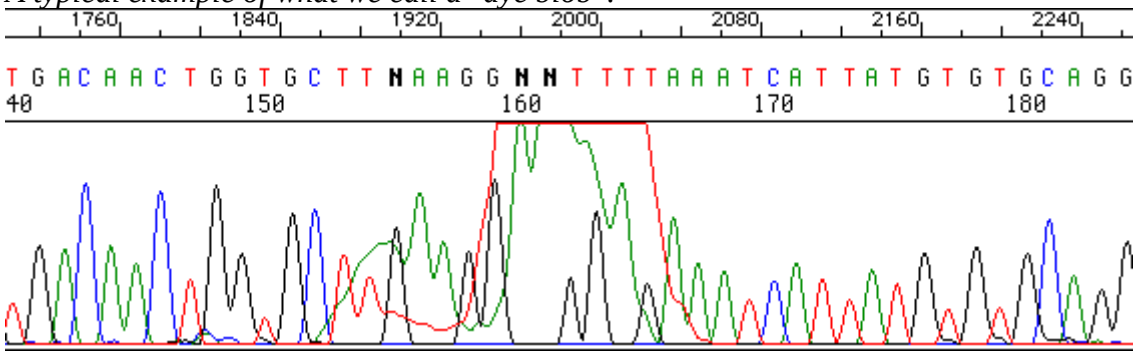
Another example, this time with templates that might be related. Note the alignment of the peaks:

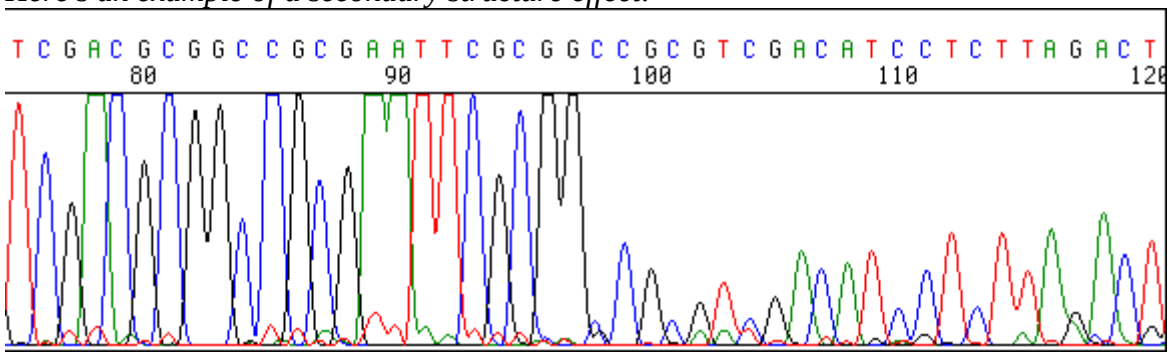


The sequence looks OK in some spots, but not others.

Please consult the following table:

Bands start out normal (or even over-sized) but decline rapidly - "ski slope" effect.	The 'ski-slope' effect can be viewed using the Sequence Scanner program from opened trace (sequence) on a Raw data sheet. Below is an example (a fairly mild example of a ski-slope): This is not well understood, but here are four possible explanations: (i) salt in the DNA, (ii) too much DNA in the reaction, (iii) an unknown impurity "poisoning" the Taq processivity, or (iv) an unknown contaminant increasing the binding of dyes in the enzyme's active site. The latter effect can arise from free NTP's in the sample, and *perhaps* from a contaminant that disturbs the divalent cation concentration (EDTA, Mg++ etc). Capillary electrophoresis instruments such as our ABI3100/3130XL sequencer are quite sensitive to the presence of excess salt. 

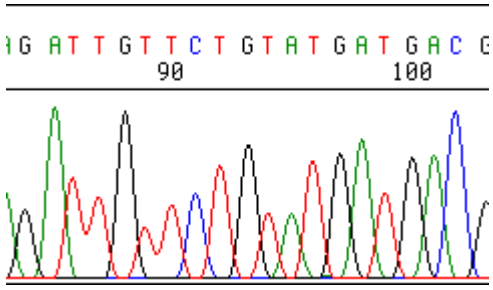
The sequence is generally good, but there's one place where a huge green (or red or black) peak obscures everything under it. The peak shape is clearly abnormal.	This is a common artefact of automated sequencing that arises from complexes formed between the sequencing dyes and unknown other components (often contaminants). There are two things that cause this artefact: First, this may be due to the sample cleanup and there might excess unincorporated dyes left in the sample. We clean the sequencing reactions by ethanol purification and these artefacts are more typical to this purification method. We are currently not able to perform other purifications. Second, your sample itself may have a contaminant that binds unincorporated dyes. *A typical example of what we call a "dye blob":* 

Your sequence proceeds normally, then the bands abruptly become much smaller.	Secondary structure in the template is the most likely cause of this problem. The polymerase is presumably unable to progress through some stem-loop form. A couple possible solutions: (1) increase the annealing temperature in sequencing, (2) try to sequence from another primer at a different position (closer or further); (3) sequence the other strand, or (4) add DMSO (5 % of the final volume) in your seq reaction. . *Here's an example of a secondary structure effect:* 
Your sequence proceeds normally, then the bands abruptly vanish.	This usually happens when the template DNA has simply stopped, for example if it was restricted at a downstream site or if the template was a PCR product. This may also be caused by an extremely stable secondary structure. See the section above for suggestions on how to sequence your template.
Some peaks seem to be missing. The machine called an 'N'.	If the peak just *before* the missing one is green, this is normal. The enzyme we use has difficulty adding a 'G' immediately after 'A', with the result that the peak will be much smaller. Check to see if your 'N' has a small black band below it and an 'A' immediately before. If so, it's a 'G'-after-'A' dropout. *The chemistries we use are no longer likely to cause "dropouts" as described in the preceding paragraph. Band intensities are much less variable with these newest dyes.*

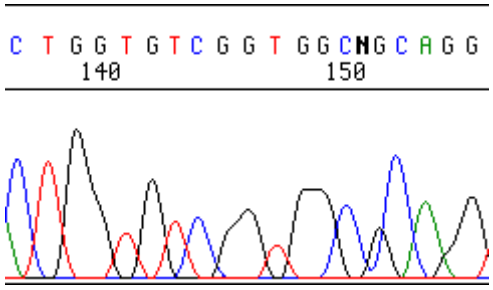
Your chromatogram proceeds normally, but the bands become broad and low after only a couple hundred nucleotides.	The resolution of the gel normally decreases after perhaps 600-700 nucleotides, which is normal for a ABI3130 sequencer when 50 cm capillary and POP7 polymer are used (max length of read is 700 to 850 depending on the run module: rast vs. standard. If the peak resolution decreases earlier, however, you may have a contaminant in your template that caused a loss of resolution in our capillary electrophoresis instruments.

A typical example of a "loss of resolution" artifact:

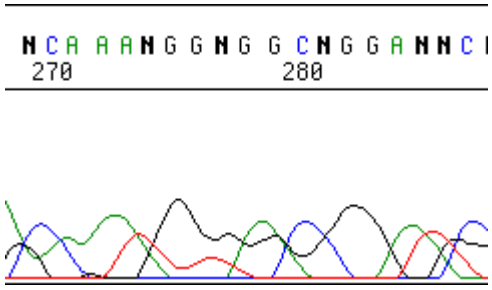
Early on, the sequence is fine:



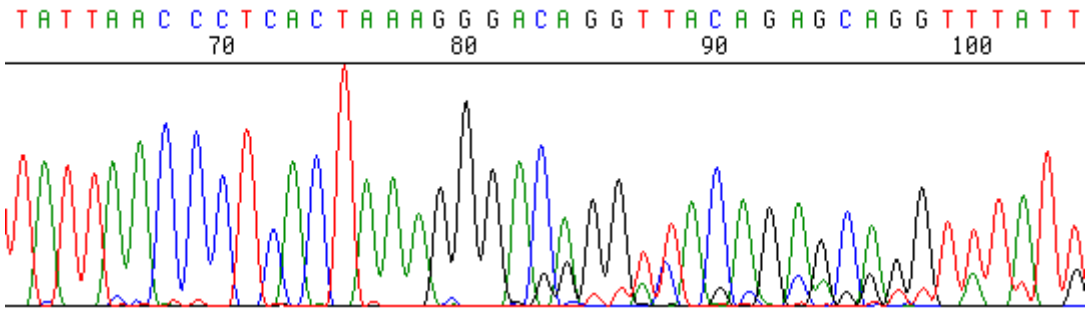
Before long, problems are evident:



Too soon, resolution is lost completely:



The first 10-20 nucleotides are obscured by huge, trashy-looking peaks, then normal sequence is seen thereafter.	The most likely explanation is that your primer is formed self-dimers and the 'trash' peaks are from sequencing on itself. All primers should be designed using a computer, in order to avoid such artifacts. Most common primer design programs will avoid primers that form self-dimers. Alternatively, if your sample is a PCR product, these large peaks may arise from a small PCR product contaminating your main band. You wouldn't even see such a product on an agarose gel, if it is small enough.

The first 20-50 nucleotides are fine, but suddenly the chromatogram shows mixed peaks or terrible background.	This may be due to the fact that the template DNA is actually a mixture of two clones that are identical up to the cloning site and diverge thereafter. Alternatively, your primer could be sitting down on two independent sites within the construct, and generating identical sequence on those two sites up until the point where the two sequences diverge, whereupon you get the peaks-on-peaks effect. This is common when you're priming inside an insert and you've accidently inserted *two* copies of that insert. Other structural errors can produce this type of effect as well. When searching for mutations, this is good news, you probably have found a deletion or insertion☺ *Here's an example of two mixed clones, identical in sequence until they hit the cloning site:* 

The sequence looks great until it hits a polyA (or polyT), and then the bands rise and fall in waves.	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 that 'roller'coaster' look to the chromatogram. Try sequencing in the other direction from the opposite strand, or try another primer either closer or further from the homopolymer region. *The following is an excellent example of 'polymerase slip' on a homopolymeric tract:*