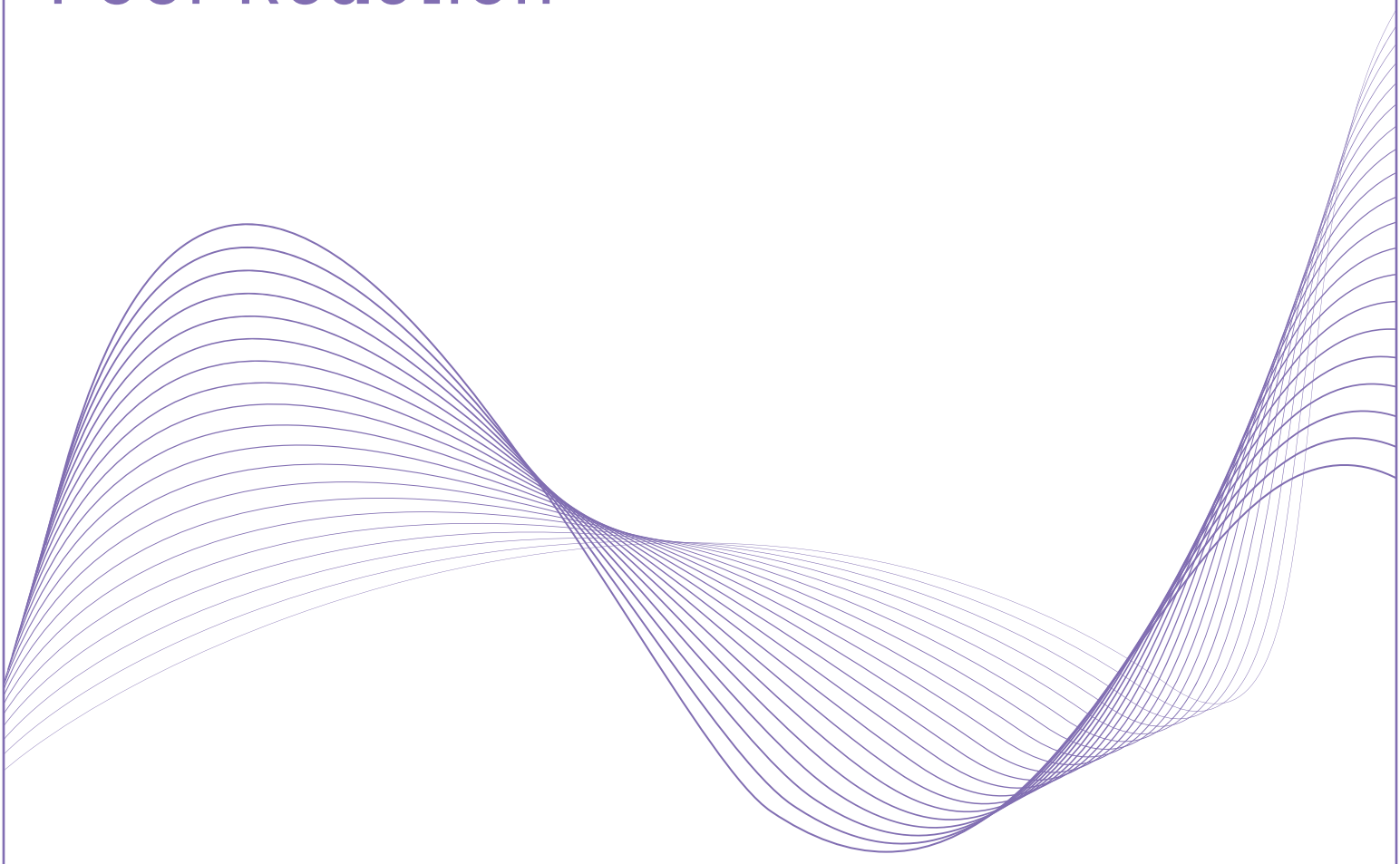
GUIDE

Volume 5: Sanger Quick Tips for Sequencing Purified PCR Products Troubleshooting a Poor Reaction



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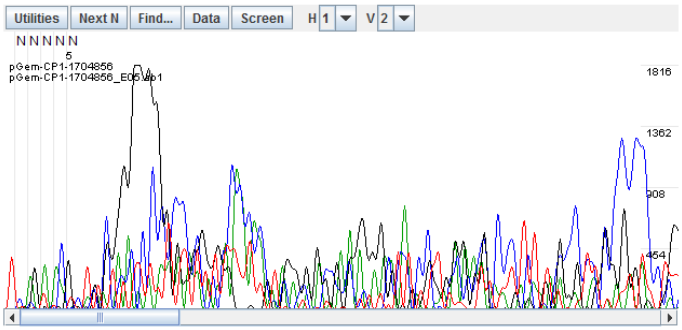
Sometimes, a sequencing reaction might fail despite your diligent efforts at following a protocol to isolate and purify a PCR product. Fortunately, Azenta scientists have many years of experience helping customers rescue failed sequencing reactions. The most common categories of failed sequencing are explained below, accompanied by representative chromatograms and tried-and-tested solutions.

Failed Reaction (No Priming)

A chromatogram with a signal intensity of <100 without a definable trace suggests a failed (no priming) reaction.

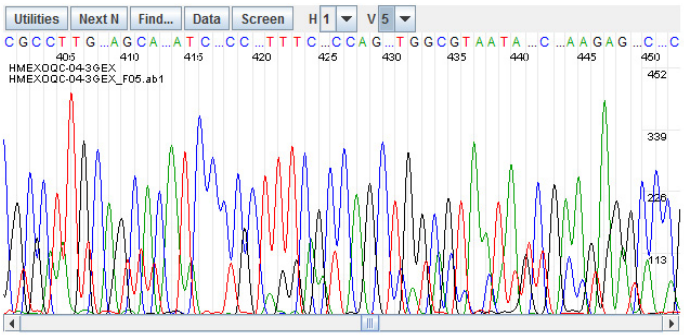


Follow the recommendations below when you encounter failed sequencing reactions with PCR products:

- If you purified the PCR product using a DNA-binding column or beads, use a spectrophotometer to determine the $OD_{260/230}$ and $OD_{260/280}$ ratios of the eluate (optimal values should be >1.8). An $OD_{260/230}$ ratio below 1.8 suggests the presence of contaminants such as residual salts or organic compounds. Perform an additional wash or precipitation/wash step in order to improve the DNA purity. Please note that the yield will decrease with each additional wash step. For spectrophotometers, it can be difficult to measure concentrations below 10 ng/µl accurately.
- When performing enzymatic cleanup of PCR products, run an aliquot of the original sample on a gel to check the size and band intensity. The concentration of product can be estimated by comparing the band intensity with a mass ladder. Fluorometric quantitation is another option. However, please note that a spectrophotometer cannot be used to measure the concentration of enzymatically cleaned PCR products. The degradation products of primers and dNTPs still absorb UV light and will cause spectrophotometers to significantly overestimate the amount of dsDNA in the sample. If you find that your PCR products were likely over-diluted, repeat the sequencing reaction with a higher sample concentration.
- Has the primer ever been used in a successful sequencing reaction? If not, the lack of priming may be due to improper primer design. Although primers that were originally designed for PCR may be used for sequencing, the results may be suboptimal. A good solution is to follow the Azenta primer design guidelines (i.e., $_{Tm}$ of 50-60°C, 18-24 nucleotides in length, without a high degree of self-complementarity) to design and synthesize nested primers for sequencing.

Nonspecific Reaction

Nonspecific results are characterized by two or more overlapping traces in the chromatogram, representing different populations of sequencing products. The competing peaks may be of equal height or one of the traces may appear dominant. Extreme caution must be used when using sequence data from nonspecific reactions, as the base calling software often cannot differentiate between the competing traces.

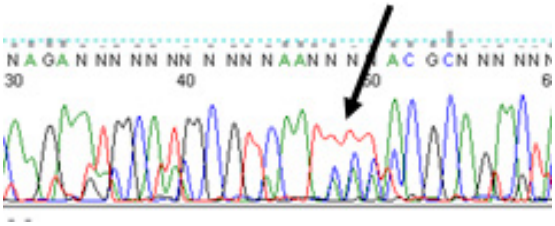


Some of the common causes and solutions for nonspecific reactions are:

- Incomplete removal of unincorporated PCR primers prior to sequencing. This can occur when an excessive amount of primers is added to the original PCR and the subsequent enzymatic cleanup is inefficient.
- The presence of multiple PCR products within the sequencing reaction. Occasionally, what appears to be a single band on an agarose gel may actually be multiple amplicons of similar or identical sizes. You can often overcome this problem by sequencing with a nested primer or cloning the PCR product into a vector and sequencing several colonies.
- The addition of two primers instead of one to the PCR sequencing reaction. Please note that all sequencing reactions require only one primer.

Spectral Pull-Up

Spectral pull-up occurs when the signal intensity of the sequencing reaction is so strong that the fluorescence signal effectively spills over into the other collection channels. This phenomenon typically manifests itself as C and A peaks under T peaks. The fluorescent dyes used in sequencing have emission spectra that overlap, and the analysis software uses an algorithm to interpret the contribution of each dye. Very high intensities in one color, however, can overwhelm this calibration, leading to artificial peaks in other colors. Spectral pull-up is mainly seen with short PCR products.



Although at a first glance a chromatogram with spectral pull-up resembles that from a nonspecific sequencing reaction, the former is characterized by very high intensities (usually greater than 10,000) and the distinctive pattern of C and A peaks underneath T peaks. At Azenta, we will automatically perform a modified reload for reactions that exhibit spectral pull-up at no cost to you. For reloads, the sequencing product is diluted and rerun on the sequencer, which usually corrects the spectral pull-up.

If you suspect that your sample contains spectral pull-up, and Azenta does not automatically reload the sample free of charge, please contact our Technical Support department at dnaseq@azenta.com or 877-436-3949, option 2. We will be more than happy to arrange a free reload. Since spectral pull-up is caused by excessive template in the sequencing reaction, resubmission of your sample at a lower concentration is recommended for high-quality sequencing results.