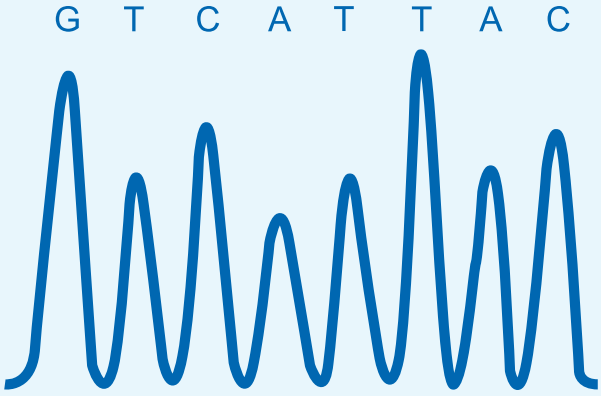

Diagnosis Sanger

Interpreting and Troubleshooting Chromatograms

Volume 1: Help! No Data!

GENEWIZ®

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Troubleshooting

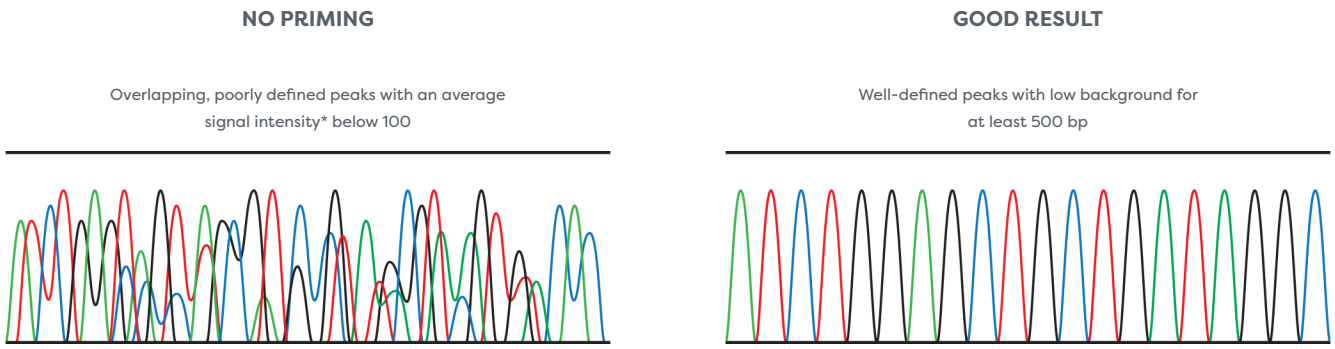
This troubleshooting guide is based on common issues seen from samples within GENEWIZ laboratories in the United States. There are several possible causes for a "no priming" result. It does not necessarily indicate that no primer was added to the reaction or that the primer does not bind to the template. The list of potential causes is not exhaustive, but represents the most common issues. For more assistance, please contact Technical Support at 877-436-3949 ext. 2 or dnaseq@genewiz.com. The following sections are broken out by DNA template types and discusses many causes for "no priming" failures.

Introduction

One of the hardest problems to troubleshoot is a lack of data. This is called “no priming” in the GENEWIZ failure cause guide, but it can also be interpreted to mean a failed sequencing reaction. A “no priming” failure cause indicates that the primer annealed to the template with poor efficiency and/or extension from the bound primer was inhibited. As a result, little or no sequencing product was generated.

To identify a “no priming” reaction, the chromatogram should be opened and examined. Typically, “no priming” reactions have an average signal intensity of less than 100. Often the signal will be garbled, with no clear or uniform peaks. Very low intensity traces can be pulled up by the analysis program through normalization/rescaling, making it difficult to distinguish true signal from background noise (which typically has an intensity of 40 to 50). Due to the lack of a sequencing product, base calling in these chromatograms is unreliable and mostly inaccurate. GENEWIZ technical support scientists recommend that any data with signal intensity under 100 should not be used for further downstream analysis. Please note that the distinction between “poor quality” and “no priming” results is sometimes arbitrary; however, the former may provide some useful data whereas the latter usually does not.

Figure 1. Illustration of a “no priming” reaction vs. a successful reaction.



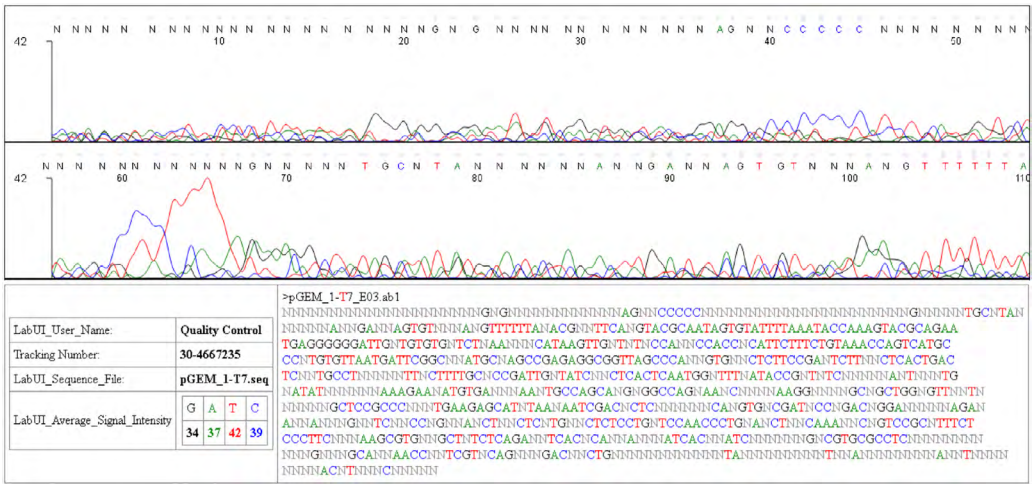
*The average signal intensity of each base (A, T, G, C) is displayed at the bottom of the online trace viewer.

Example Chromatograms

Figure 2. "No priming" chromatogram examples

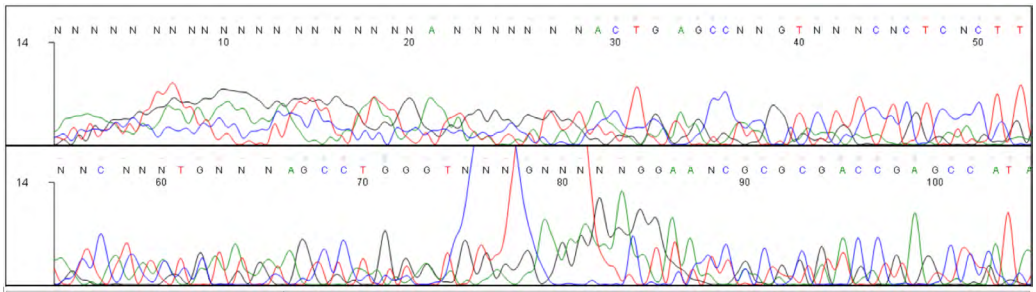
(A) Notice the signal intensities for each nucleotide (A, T, G, C) are below 100. The chromatogram peaks are unpatterned and appear garbled; a good quality sequence will have regular, patterned chromatogram peaks with well-defined peaks and troughs. Another indication of a poor-quality reaction is the appearance of many "N" residues distributed through the .txt file sequence. A high occurrence of "N" residues in the middle portion of the .txt file sequence indicates a problem with the sequencing reaction and requires careful examination of the chromatogram file. (B) Occasionally, the .txt file sequence will actually terminate after only a handful of "N" residues. This means the algorithm did not detect sufficient usable sequence in the trace file and automatically stopped reading the file.

A.



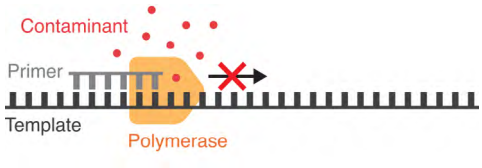
LabUI_User_Name:	**Quality Control**
Tracking Number:	**30-4667235**
LabUI_Sequence_File:	**pGEM_1-T7.seq**
LabUI_Average_Signal_Intensity	G: **34** A: **37** T: **42** C: **39**

B.

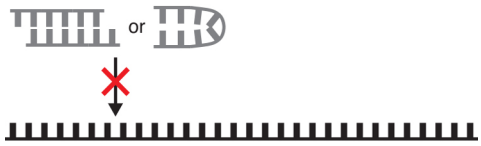
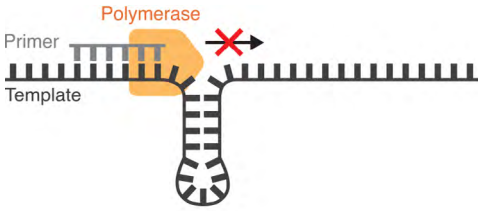


LabUI_User_Name:	**Quality Control**
Tracking Number:	**30-4477427**
LabUI_Sequence_File:	**pGem-3-T7.seq**
LabUI_Average_Signal_Intensity	G: **60** A: **59** T: **73** C: **69**

Common causes for many template types: "no priming" failures

Possible Cause	Solution
DNA template concentration is too low or high.	Check that the DNA concentration is in the correct range. Review our Sample Submission Guidelines.
• Primer concentration is too low or high.	Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer). • Premix reaction: Add 5 µL of 5 pmol/µL primer to your DNA samples • Pre-Defined reaction: Send your primer separately at 5 pmol/µL • Note: 5 pmol/µL = 5 µM = (1.65 ng/µL)×(# of bases in oligo)
• Primer not added.	• Make sure that the primer has been added if sending a • "premix" order and not using a GENEWIZ universal primer.
Poor-quality DNA template, including contamination with: • Guanidine or other chaotropic salts • Phenol or chloroform • EDTA • Ethanol or isopropanol 	Check the absorbance ratios on a spectrophotometer. Aim for values greater than 1.8. o A260/280 < 1.8 may indicate protein contamintion. o A260/230 < 1.8 may indicate contamination with organic chemicals (e.g. guanidine, phenol or EDTA). This ratio is critical for successful sequencing. We recommend eluting your DNA in molecular biology grade water and avoiding buffers containing EDTA, such as TE, which can inhibit the sequencing reaction. Tris-Cl, sometimes called EB buffer, is safe for sequencing. For preps or cleanups, be sure to dry samples well prior to elution to avoid reagent carryover. Check the integrity of your DNA on a gel. Sheared or fragmented DNA can lead to poor sequencing results. For PCR products, run your product on an agarose gel to confirm that it produces a single band of the correct size. For additional tips on how to optimize PCR for sequencing, see our Sanger Tips & Tricks series.

Common causes for many template types: "no priming" failures (cont'd)

Possible Cause	Solution
• Primer binding site is not present.	Check that the primer binding site is present. If using a GENEWIZ universal primer, check the sequence to make sure it matches your vector. Perform a diagnostic restriction digest to verify plasmid identity and the presence of an insert.
Primer binding site not present. • Primer Tm is too low.  • Primer forms a dimer or hairpin. 	Use oligo analysis software to verify the following: • Melting temperature (Tm) of the primer is 50-60ºC. • Primer does not have significant self-complementary, leading to self-dimersor hairpins. o Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).
Template forms secondary structure near the primer binding site. 	Use GENEWIZ's "Alternative Protocol", designed for difficult templates. This protocol helps to denature secondary structure that impedes polymerization. If you know your template is prone to forming secondary structure, simply choose "Difficult Template, Hairpin, RNAi, or GC-rich" from the "Special Request" column when placing your order. All options refer to the "Alternative Protocol." Please note there is an additional charge for this service. You can repeat failed reactions with the "Alternative Protocol" at half price by choosing "1/2 Price with Alt. Protocol" from the Repeat column dropdown menu on the "Order Results" page. Try sequencing with a primer that is further upstream or downstream of the current primer location, or try sequencing from the other direction. Sometimes changing the primer location can help sequence through a difficult region.

Problems for purified plasmids: "no priming" failures

Possible Cause	Solution
Plasmid is greater than 20 kb. Larger plasmids can be difficult to sequence efficiently.	For >20 kb plasmids, ensure you submit highly pure samples at 100-150 ng/µL. For plasmids over 50 kb, our BAC protocol may yield better results. Alternatively, use PCR to amplify the region of interest and submit PCR products for sequencing.

Specific problems for Purified PCR: "no priming" failures

Possible Cause	Solution
PCR primer is not compatible with sequencing. Occasionally, a primer used in PCR amplification will not be compatible to use as a sequencing primer. When using a PCR primer for sequencing, please note that the cycling conditions of sequencing may differ from that of your PCR. For example, our sequencing protocol uses an annealing temperature of 50°C. Thus, a primer that works well in your PCR may not be appropriate for sequencing.	Designing a nested primer slightly downstream of the original primer binding sequence can yield better sequencing results.
PCR reaction loading dye not compatible with sequencing reaction.	Most PCR loading dyes are compatible with Sanger sequencing. However, if the sequencing reaction fails and there is a loading dye in the reaction, a good troubleshooting step to take is to test the reaction using a different system without loading dye.
Failure to inactivate enzymatic PCR clean-up reaction post-clean-up.	If the PCR reaction is purified using an enzymatic purification protocol, the enzyme will need to be inactivated prior to use in the sequencing reaction. Make sure that the heat inactivation step recommended by the protocol/kit manufacturer is followed prior to setting up the sequencing reaction.

Specific problems for unpurified PCR products: “no priming” failures

Possible Cause	Solution
Low-copy plasmid used. These plasmids may not provide sufficient input DNA for efficient amplification.	Use a vector with a high-copy origin of replication. Prep the DNA and submit purified plasmids. Use PCR to amplify the region of interest and submit PCR products for sequencing.
PCR reaction loading dye not compatible with sequencing reaction.	Most PCR loading dyes are compatible with Sanger sequencing, particularly those which are included in commercial PCR master mixes. However, if the sequencing reaction fails and there is a loading dye in the reaction, a good troubleshooting step to take is to test the reaction using a different system without loading dye. Loading dyes containing EDTA can potentially inhibit the sequencing reaction.

Specific problems for bacterial colonies: "no priming" failures

Possible	Solution
Low-copy plasmid used. These plasmids may not provide sufficient input DNA for efficient amplification.	Use a vector with a high-copy origin of replication. Prep the DNA and submit purified plasmids. Use PCR to amplify the region of interest and submit PCR products for sequencing.
EndA+ strain used. Certain strains of E. coli (e.g. BL21, Stbl3) contain a non-specific endonuclease in the periplasmic space that can cleave plasmid DNA when cells are lysed, leading to inefficient amplification.	Use a strain with the *endA* mutation (e.g. DH5α, TOP10). Prep the DNA and submit purified plasmids. Use PCR to amplify the region of interest and submit PCR products for sequencing.
Plasmid is greater than 20 kb. Rolling circle amplification is less efficient on larger templates.	Use PCR to amplify the region of interest and submit PCR products for sequencing.
Cells do not contain a plasmid. Only small circular templates (i.e. plasmids) can be used successfully with rolling circle amplification. Bacterial chromosomes are not efficiently amplified by the process and cannot be directly sequenced with our Sanger service.	To sequence genomic DNA, use PCR to amplify the region of interest and submit the PCR products.
Appropriate antibiotic selection was not used. If the selection antibiotic has degraded or was not added, the bacteria will often not retain the plasmid.	Check the plates used for colony prep for antibiotic addition and expiration date. When in doubt, it is a good idea to throw out any old sleeves of plates nearing their expiration date and make a fresh batch.

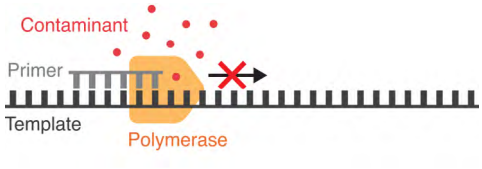
Specific problems for glycerol stocks: "no priming" failures

Possible Cause	Solution
Low-copy plasmid used. These plasmids may not provide sufficient input DNA for efficient amplification.	Use a vector with a high-copy origin of replication. Prep the DNA and submit purified plasmids. Use PCR to amplify the region of interest and submit PCR products for sequencing.
EndA+ strain used. Certain strains of E. coli (e.g. BL21, Stbl3) contain a non-specific endonuclease in the periplasmic space that can cleave plasmid DNA when cells are lysed, leading to inefficient amplification.	Use a strain with the *endA* mutation (e.g. DH5α, TOP10). Prep the DNA and submit purified plasmids. Use PCR to amplify the region of interest and submit PCR products for sequencing.
Rich media used. Certain media (e.g. TB, SOB, 2YT) contain high salt concentration or ingredients that can inhibit polymerase activity during rolling circle amplification.	Use LB medium. Submit bacterial colonies.
Plasmid is greater than 20 kb. Rolling circle amplification is less efficient on larger templates.	Use PCR to amplify the region of interest and submit PCR products for sequencing.
Cells do not contain a plasmid. Only small circular templates (i.e. plasmids) can be used successfully with rolling circle amplification. Bacterial chromosomes are not efficiently amplified by the process and cannot be directly sequenced with our Sanger service.	To sequence genomic DNA, use PCR to amplify the region of interest and submit the PCR products.
Appropriate antibiotic selection was not used. If the selection antibiotic has degraded or was not added, the bacteria will often not retain the plasmid	Check the plates used for colony prep for antibiotic addition and expiration date. When in doubt, it is a good idea to throw out any old sleeves of plates nearing their expiration date and make a fresh batch.

Specific problems for phage: "no priming" failures

Possible Cause	Solution
Titer is too low or high.	For phage supernatant, titers of 108 to 1012 PFU/mL can produce good results. However, you may need to perform a dilution series to determine the optimal titer for your samples. Alternatively, you can submit phage plaques.
Phage with linear genome used. Only small circular templates can be used successfully with rolling circle amplification.	If your phage has a linear genome, we recommend using PCR to amplify your region of interest and submitting the PCR products for sequencing.
Phage genome is greater than 20 kb. Rolling circle amplification is less efficient on larger templates.	Use PCR to amplify the region of interest and submit PCR products for sequencing.

Specific problems for BAC DNA: "no priming" failures

Possible Cause	Solution
DNA template concentration is too low. Most BAC DNA sequencing failures arise from templates that have either too low of a concentration or are low-quality.	Double check that the DNA concentration is in the correct range. Review our Sample Submission Guidelines.
Poor quality DNA template, including contamination with: • Guanidine or other chaotropic salts • Phenol or chloroform • EDTA • Ethanol or isopropanol 	Check the absorbance ratios on a spectrophotometer. Aim for values greater than 1.8. o A260/280 < 1.8 may indicate protein contamination. o 260/230 < 1.8 may indicate contamination with organic chemicals (e.g. guanidine, phenol or EDTA). This ratio is critical for successful sequencing. We recommend eluting your DNA in molecular biology grade water and avoiding buffers containing EDTA, such as TE, which can inhibit the sequencing reaction. Tris-Cl, sometimes called EB buffer, is safe for sequencing. For preps or cleanups, be sure to dry samples well prior to elution to avoid reagent carryover. Check the integrity of your DNA on a gel. Sheared or fragmented DNA can lead to poor sequencing results.