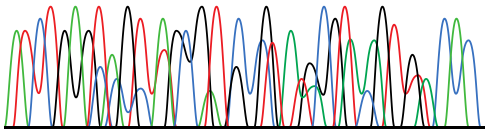
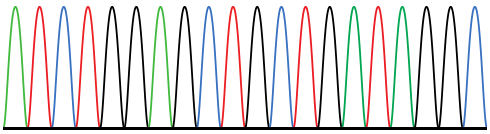
Description

No priming results indicate that little or no sequencing product was generated. Base calling in these chromatograms is therefore unreliable. Very low intensity traces are 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). 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.

NO PRIMING	**GOOD RESULT**
Overlapping, poorly defined peaks with an average signal intensity* below 100	Well defined peaks with low background for at least 500 bp
	

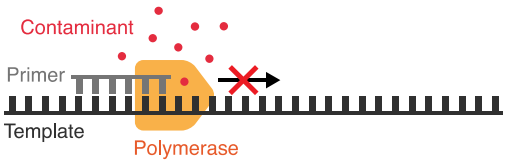
*The average signal intensity of each base (G, A, T, C) is displayed at the bottom of the online trace viewer.

Troubleshooting

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. Select your DNA type below to jump to the appropriate section in the guide. 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.

DNA Type **Page**

1. Plasmids

POSSIBLE CAUSE	SOLUTION
DNA template concentration is too low or high.	Double 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. ○ A260/280 < 1.8 may indicate protein contamination. ○ **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.
Primer binding site is not present.	• Double 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.

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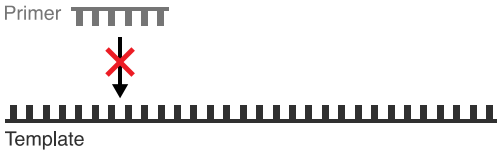
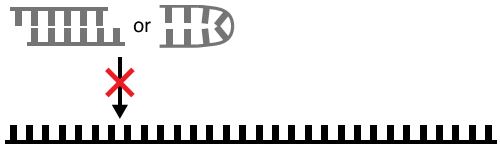
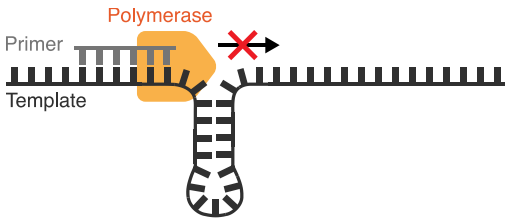
1. Plasmids

POSSIBLE CAUSE	SOLUTION
Poorly designed primer: • 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-dimers or hairpins. ○ 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.
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.

2. Purified PCR Products

POSSIBLE CAUSE	SOLUTION
DNA template concentration is too low or high.	Double check that the DNA concentration is in the correct range. Review our Sample Submission Guidelines. • For PCR products purified with a **DNA-binding matrix** (e.g. column or beads), a spectrophotometer can be used to measure the concentration; however, accuracy declines below 10 ng/µL. For best results, measure the concentration of the eluate before diluting it. Because DNA loss is inevitable with bind-wash-elute systems, it is highly recommended to optimize your PCR so that it produces a strong band on a gel. • For PCR products purified with an **enzymatic cleanup** protocol (e.g. ExoSAP-IT), a spectrophotometer cannot be used for accurate DNA quantitation. Reaction components, such as degraded primers and nucleotides, will absorb UV light and inflate the calculated DNA concentration. Instead, use the band intensity on an agarose gel relative to that of mass standards to estimate DNA concentration. Alternatively, a fluorometer, which employs a dye that specifically binds dsDNA, can provide accurate quantitation. • In our experience, enzymatic cleanup is the most convenient and effective method of preparing PCR products for sequencing. It uses a cocktail of enzymes that degrades ssDNA (i.e. leftover oligos) and dNTPs, leaving intact the dsDNA product. This method allows for full recovery of your PCR product and avoids the use of reagents, such as guanidine and ethanol, that may inhibit the sequencing reaction. For your convenience, GENEWIZ provides enzymatic PCR cleanup for unpurified PCR products. • For additional tips on how to optimize PCR for sequencing, see our Sanger Tips & Tricks series.
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 (5 µM) primer to your DNA samples. • Pre-Defined reaction: Send your primer separately at 5 pmol/µL (5 µM). Note: 5 pmol/µL = 5 µM = (1.65 ng/µL)×(# of bases in oligo)

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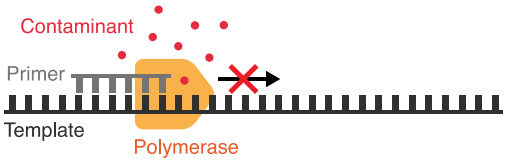
2. Purified PCR Products

POSSIBLE CAUSE	SOLUTION
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 • Agarose • Phenol or chloroform • EDTA • Ethanol or isopropanol  	• For PCR products purified with a DNA-binding matrix, check the absorbance ratios on a spectrophotometer. Aim for values greater than 1.8. Note that ratios may be inaccurate for samples with a concentration lower than 10 ng/µL. ○ A260/280 < 1.8 may indicate protein contamination. ○ **A260/230 < 1.8** may indicate contamination with organic chemicals (e.g. guanidine, agarose, phenol or EDTA). **This ratio is critical for successful sequencing.** Reagent carryover is a common issue with gel purification and methods that use a DNA-binding material. Note that enzymatic cleanup can avoid the use of such chemicals. • 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. • 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.

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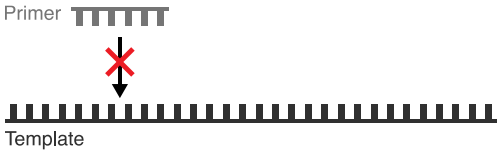
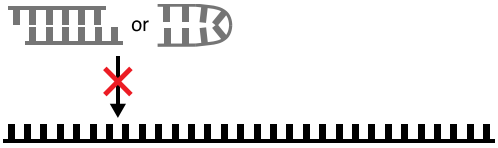
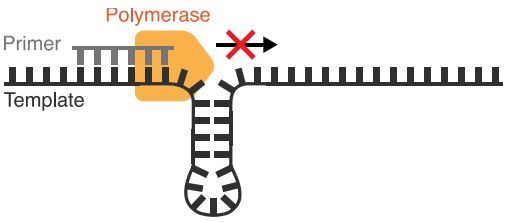
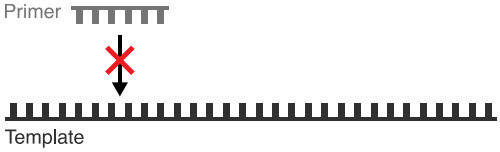
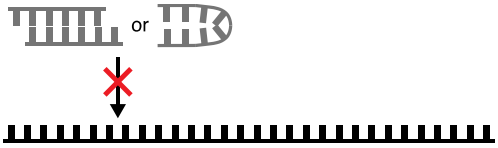
2. Purified PCR Products

POSSIBLE CAUSE	SOLUTION
Poorly designed primer: • 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-dimers or hairpins. ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007). 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.
Primer binding site is not present.	• Double check that the primer binding site is present. • If using a GENEWIZ universal primer, check the sequence to make sure it matches your vector.
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.

3. Unpurified PCR Products

POSSIBLE CAUSE	SOLUTION
DNA template concentration is too low or high.	• Run your product on an agarose gel to confirm that it produces a strong single band of expected size. If not, optimize your PCR conditions. • We recommend submitting a gel image that shows your product run alongside DNA standards. Our scientists can estimate the concentration of your PCR product based on the relative band intensity and perform an appropriate dilution after cleanup. • For additional tips on how to optimize PCR for sequencing, see our Sanger Tips & Tricks series.
Primer concentration is too low or high.	Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer).
Poorly designed primer: • 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-dimers or hairpins. ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007). 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.
Primer binding site is not present.	• Double check that the primer binding site is present. • If using a GENEWIZ universal primer, check the sequence to make sure it matches your vector.

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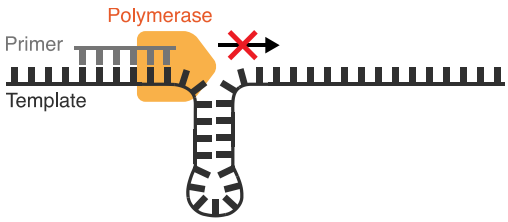
3. Unpurified PCR Products

POSSIBLE CAUSE	SOLUTION
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.

4. Bacterial Colonies

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.
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.
Primer concentration is too low or high.	Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer). Note: 5 pmol/µL = 5 µM = (1.65 ng/µL)×(# of bases in oligo)

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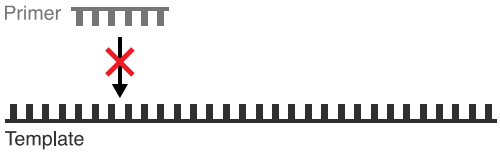
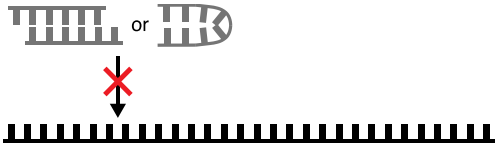
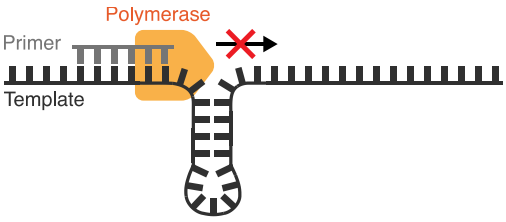
4. Bacterial Colonies

POSSIBLE CAUSE	SOLUTION
Poorly designed primer: • 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-dimers or hairpins. ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).
Primer binding site is not present.	• Double check that the primer binding site is present. • If using a GENEWIZ universal primer, check the sequence to make sure it matches your vector.
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.

5. Glycerol Stocks

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.
Primer concentration is too low or high.	Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer). Note: 5 pmol/µL = 5 µM = (1.65 ng/µL)×(# of bases in oligo)

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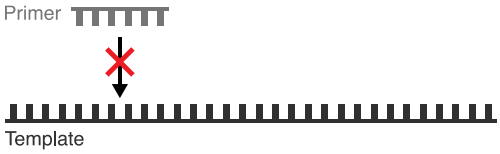
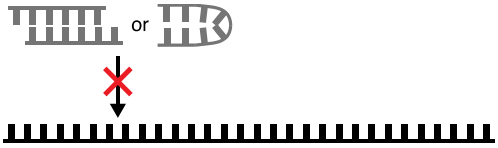
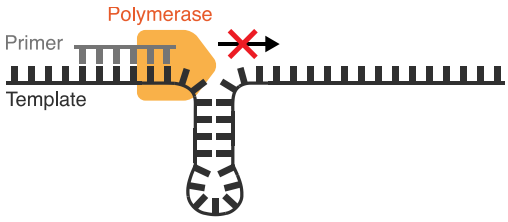
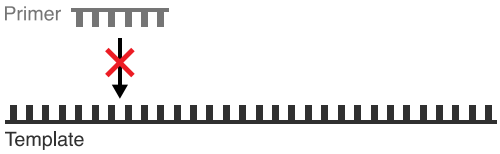
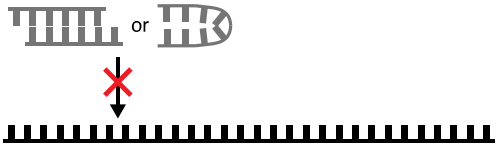
5. Glycerol Stocks

POSSIBLE CAUSE	SOLUTION
Poorly designed primer: • 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-dimers or hairpins. ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).
Primer binding site is not present.	• Double check that the primer binding site is present. • If using a GENEWIZ universal primer, check the sequence to make sure it matches your vector.
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.

6. Phage

POSSIBLE CAUSE	SOLUTION
Titer is too low or high.	For phage supernatant, titers of 10^8 to 10^{12} 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.
Primer concentration is too low or high.	Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer). Note: 5 pmol/µL = 5 µM = (1.65 ng/µL)×(# of bases in oligo)
Poorly designed primer: • 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-dimers or hairpins. ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).
Primer binding site is not present.	• Double check that the primer binding site is present. • If using a GENEWIZ universal primer, check the sequence to make sure it matches your vector.

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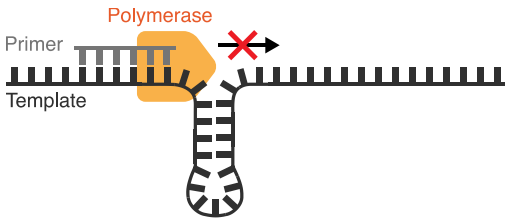
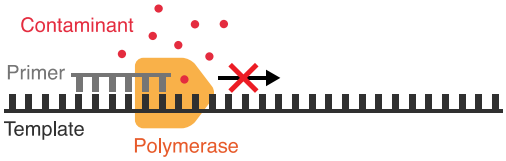
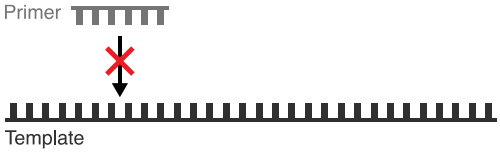
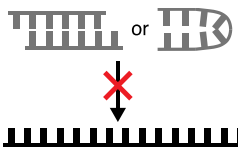
6. Phage

POSSIBLE CAUSE	SOLUTION
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.

7. BAC DNA

POSSIBLE CAUSE	SOLUTION
DNA template concentration is too low.	Double 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). Note: 10 pmol/µL = 10 µM = (3.3 ng/µL)×(# bases in oligo)
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. ○ A260/280 < 1.8 may indicate protein contamination. ○ **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.
Poorly designed primer: • 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-dimers or hairpins. ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).
Primer binding site is not present.	• Double check that the primer binding site is present. • If using a GENEWIZ universal primer, check the sequence to make sure it matches your vector.