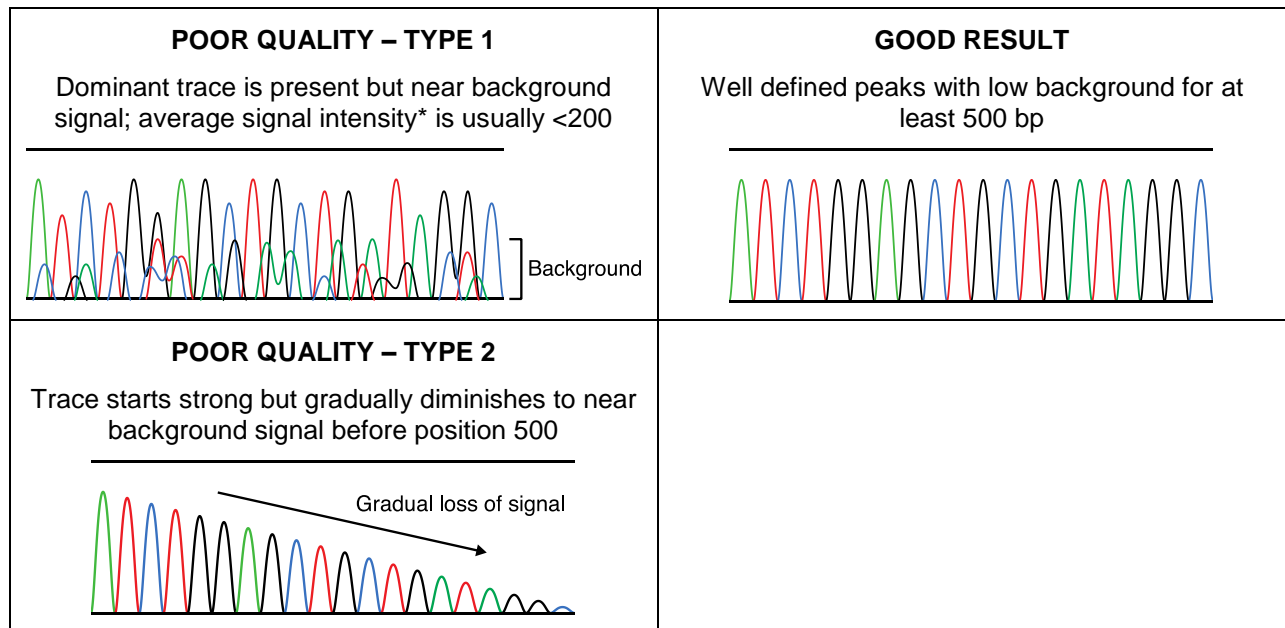




Solutions Guide for Sanger Sequencing Poor Quality

Description

Poor quality results are typically due to an inefficient reaction. In general, some or all of the trace before position 500 is close to background signal, which makes base calling in these regions unreliable. Furthermore, results are scored as “poor quality” if they fail to pass quality control and cannot be well classified as another failure type. 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.



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

Troubleshooting

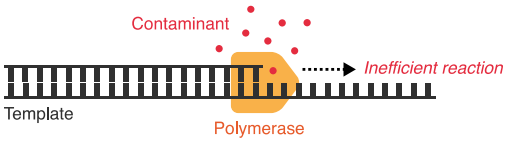
There are several possible causes for a poor quality result. Select your DNA type below to jump to the appropriate section in the guide. The list of potential causes is not exhaustive but represents the most common issues. For more assistance, please contact Technical Support at 877-436-3949 ext. 2 or dnaseq@genewiz.com.

DNA Type	Page
1. Plasmids	2
2. Purified PCR Products	4
3. Unpurified PCR Products	7
4. Bacterial Colonies	9
5. Glycerol Stocks	11
6. Phage	13
7. BAC DNA	14



Solutions Guide for Sanger Sequencing
Poor Quality

1. Plasmids

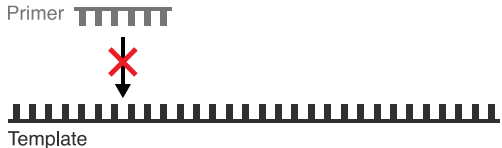
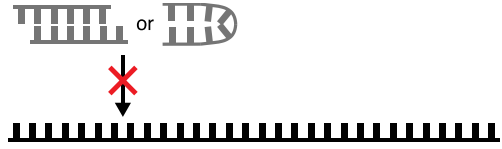
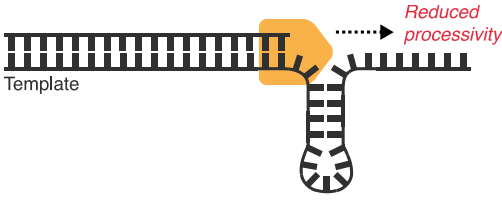
POSSIBLE CAUSE	SOLUTION
DNA template concentration is too low or high.	Double check that the DNA concentration is in the correct range. Review our Sample Submission Guidelines .
Primer concentration is too low or high.	Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer). <ul style="list-style-type: none"> • 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) \times (# of bases in oligo)
Poor quality DNA template, including contamination with <ul style="list-style-type: none"> • Guanidine or other chaotropic salts • Phenol or chloroform • EDTA • Ethanol or isopropanol 	<ul style="list-style-type: none"> • Check the absorbance ratios on a spectrophotometer. Aim for values greater than 1.8. <ul style="list-style-type: none"> ○ $A_{260}/280 < 1.8$ may indicate protein contamination. ○ $A_{260}/230 < 1.8$ 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.

(Continued on next page)



Solutions Guide for Sanger Sequencing Poor Quality

1. Plasmids

POSSIBLE CAUSE	SOLUTION
<p>Poorly designed primer:</p> <ul style="list-style-type: none"> Primer T_m is too low.  <ul style="list-style-type: none"> Primer forms a dimer or hairpin. 	<p>Use oligo analysis software to verify the following:</p> <ul style="list-style-type: none"> Melting temperature (T_m) of the primer is 50-60°C. Primer does not have significant self-complementary, leading to self-dimers or hairpins. <ul style="list-style-type: none"> Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. <p>Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).</p>
<p>Template forms secondary structure.</p> 	<ul style="list-style-type: none"> Use GENEWIZ's "Alternative Protocol", designed for difficult templates. This protocol helps to denature secondary structure that impedes polymerization. <ul style="list-style-type: none"> 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.
<p>Plasmid is greater than 20 kb. Larger plasmids can be difficult to sequence efficiently.</p>	<ul style="list-style-type: none"> 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.



Solutions Guide for Sanger Sequencing
Poor Quality

2. Purified PCR Products

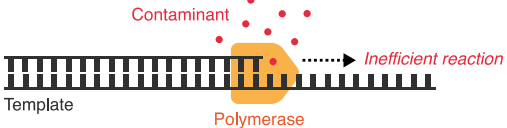
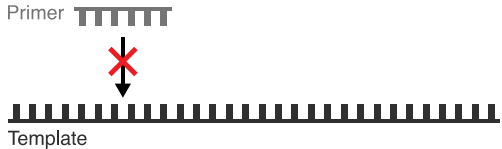
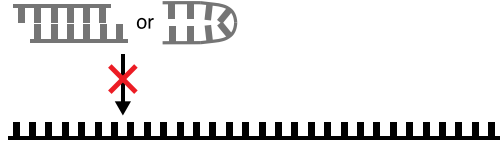
POSSIBLE CAUSE	SOLUTION
<p>DNA template concentration is too low or high.</p>	<p>Double check that the DNA concentration is in the correct range. Review our Sample Submission Guidelines.</p> <ul style="list-style-type: none"> For PCR products purified with a DNA-binding matrix (e.g. column or beads), 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.
<p>Primer concentration is too low or high.</p>	<p>Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer).</p> <ul style="list-style-type: none"> 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. <p>Note: 5 pmol/μL = 5 μM = (1.65 ng/μL) × (# of bases in oligo)</p>

(Continued on next page)



Solutions Guide for Sanger Sequencing Poor Quality

2. Purified PCR Products

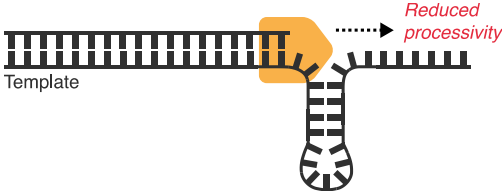
POSSIBLE CAUSE	SOLUTION
<p>Poor quality DNA template, including contamination with</p> <ul style="list-style-type: none"> • Guanidine or other chaotropic salts • Agarose • Phenol or chloroform • EDTA • Ethanol or isopropanol 	<ul style="list-style-type: none"> • For PCR products purified with a DNA-binding 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. <ul style="list-style-type: none"> ○ 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.
<p>Poorly designed primer:</p> <ul style="list-style-type: none"> • Primer T_m is too low.  <ul style="list-style-type: none"> • Primer forms a dimer or hairpin. 	<p>Use oligo analysis software to verify the following:</p> <ul style="list-style-type: none"> • Melting temperature (T_m) of the primer is 50-60°C. • Primer does not have significant self-complementary, leading to self-dimers or hairpins. <ul style="list-style-type: none"> ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. <p>Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).</p> <p>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.</p>

(Continued on next page)



Solutions Guide for Sanger Sequencing Poor Quality

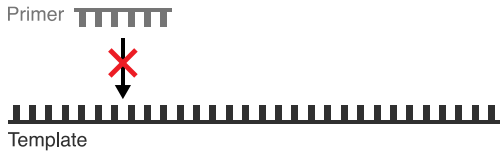
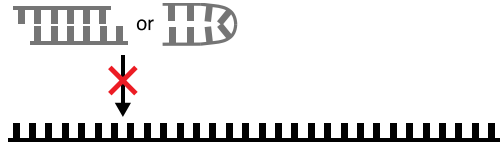
2. Purified PCR Products

POSSIBLE CAUSE	SOLUTION
<p>Template forms secondary structure.</p>  <p>Template</p>	<ul style="list-style-type: none"> • Use GENEWIZ’s “Alternative Protocol”, designed for difficult templates. This protocol helps to denature secondary structure that impedes polymerization. <ul style="list-style-type: none"> ○ 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.



Solutions Guide for Sanger Sequencing
Poor Quality

3. Unpurified PCR Products

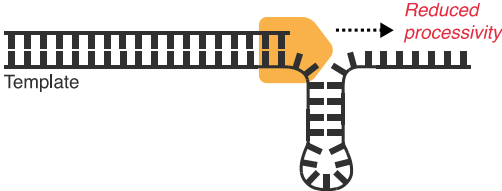
POSSIBLE CAUSE	SOLUTION
DNA template concentration is too low or high.	<ul style="list-style-type: none"> Run your product on an agarose gel to confirm that it produces a strong single band of expected size. If not, optimize your PCR conditions. We recommend submitting a gel image that shows your product 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). Note: $5 \text{ pmol}/\mu\text{L} = 5 \text{ }\mu\text{M} = (1.65 \text{ ng}/\mu\text{L}) \times (\# \text{ of bases in oligo})$
<p>Poorly designed primer:</p> <ul style="list-style-type: none"> Primer T_m is too low.  <ul style="list-style-type: none"> Primer forms a dimer or hairpin. 	<p>Use oligo analysis software to verify the following:</p> <ul style="list-style-type: none"> Melting temperature (T_m) of the primer is 50-60°C. Primer does not have significant self-complementary, leading to self-dimers or hairpins. <ul style="list-style-type: none"> Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. <p>Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).</p> <p>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.</p>

(Continued on next page)



Solutions Guide for Sanger Sequencing Poor Quality

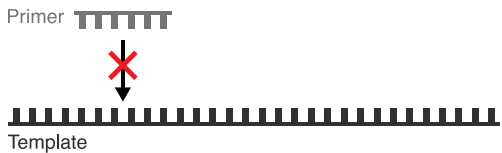
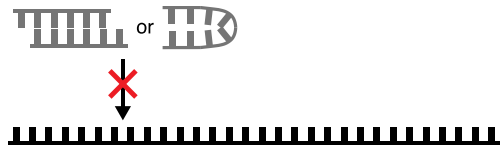
3. Unpurified PCR Products

POSSIBLE CAUSE	SOLUTION
<p>Template forms secondary structure.</p>  <p>Template</p>	<ul style="list-style-type: none"> • Use GENEWIZ’s “Alternative Protocol”, designed for difficult templates. This protocol helps to denature secondary structure that impedes polymerization. <ul style="list-style-type: none"> ○ 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.



Solutions Guide for Sanger Sequencing
Poor Quality

4. Bacterial Colonies

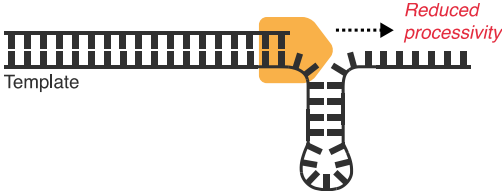
POSSIBLE CAUSE	SOLUTION
<p>Low copy plasmid used. These plasmids may not provide sufficient input DNA for efficient amplification.</p>	<ul style="list-style-type: none"> • Use a vector with a high copy origin of replication. • Prep the DNA and submit purified plasmids. • Use PCR to amplify the region of interest and submit PCR products for sequencing.
<p><i>EndA+</i> strain used. Certain strains of <i>E. coli</i> (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.</p>	<ul style="list-style-type: none"> • Use a strain with the <i>endA</i> 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.
<p>Plasmid is greater than 20 kb. Rolling circle amplification is less efficient on larger templates.</p>	<ul style="list-style-type: none"> • Use PCR to amplify the region of interest and submit PCR products for sequencing.
<p>Primer concentration is too low or high.</p>	<p>Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer).</p> <p>Note: 5 pmol/μL = 5 μM = (1.65 ng/μL)\times(# of bases in oligo)</p>
<p>Poorly designed primer:</p> <ul style="list-style-type: none"> • Primer T_m is too low.  <ul style="list-style-type: none"> • Primer forms a dimer or hairpin. 	<p>Use oligo analysis software to verify the following:</p> <ul style="list-style-type: none"> • Melting temperature (T_m) of the primer is 50-60°C. • Primer does not have significant self-complementary, leading to self-dimers or hairpins. <ul style="list-style-type: none"> ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. <p>Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).</p>

(Continued on next page)



Solutions Guide for Sanger Sequencing
Poor Quality

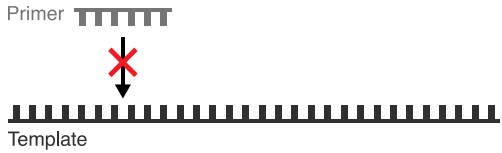
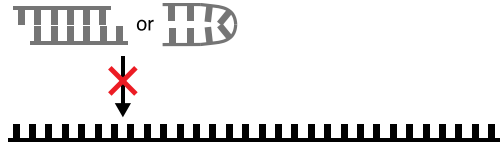
4. Bacterial Colonies

POSSIBLE CAUSE	SOLUTION
<p>Template forms secondary structure.</p>  <p>Template</p>	<ul style="list-style-type: none"> • Use GENEWIZ’s “Alternative Protocol”, designed for difficult templates. This protocol helps to denature secondary structure that impedes polymerization. <ul style="list-style-type: none"> ○ 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.



Solutions Guide for Sanger Sequencing Poor Quality

5. Glycerol Stocks

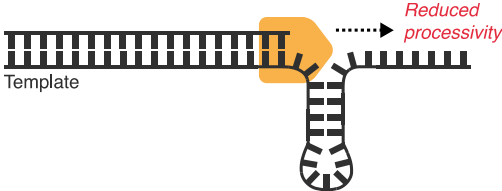
POSSIBLE CAUSE	SOLUTION
<p>Low copy plasmid used. These plasmids may not provide sufficient input DNA for efficient amplification.</p>	<ul style="list-style-type: none"> • Use a vector with a high copy origin of replication. • Prep the DNA and submit purified plasmids. • Use PCR to amplify the region of interest and submit PCR products for sequencing.
<p><i>EndA+</i> strain used. Certain strains of <i>E. coli</i> (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.</p>	<ul style="list-style-type: none"> • Use a strain with the <i>endA</i> 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.
<p>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.</p>	<ul style="list-style-type: none"> • Use LB medium. • Submit bacterial colonies.
<p>Plasmid is greater than 20 kb. Rolling circle amplification is less efficient on larger templates.</p>	<p>Use PCR to amplify the region of interest and submit PCR products for sequencing.</p>
<p>Primer concentration is too low or high.</p>	<p>Check that the primer concentration is in the correct range (if not using a GENEWIZ universal primer).</p> <p>Note: 5 pmol/μL = 5 μM = (1.65 ng/μL)\times(# of bases in oligo)</p>
<p>Poorly designed primer:</p> <ul style="list-style-type: none"> • Primer T_m is too low.  <ul style="list-style-type: none"> • Primer forms a dimer or hairpin. 	<p>Use oligo analysis software to verify the following:</p> <ul style="list-style-type: none"> • Melting temperature (T_m) of the primer is 50-60°C. • Primer does not have significant self-complementary, leading to self-dimers or hairpins. <ul style="list-style-type: none"> ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. <p>Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).</p>

(Continued on next page)



Solutions Guide for Sanger Sequencing
Poor Quality

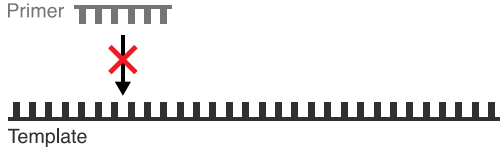
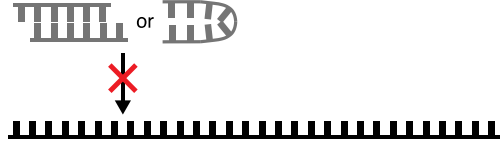
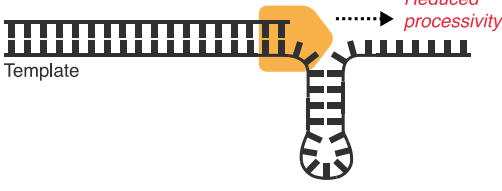
5. Glycerol Stocks

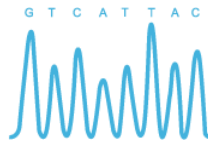
POSSIBLE CAUSE	SOLUTION
<p>Template forms secondary structure.</p>  <p>Template</p>	<ul style="list-style-type: none"> • Use GENEWIZ’s “Alternative Protocol”, designed for difficult templates. This protocol helps to denature secondary structure that impedes polymerization. <ul style="list-style-type: none"> ○ 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.



Solutions Guide for Sanger Sequencing Poor Quality

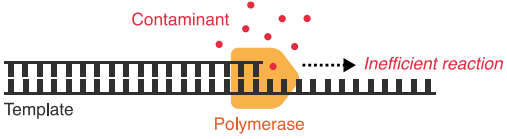
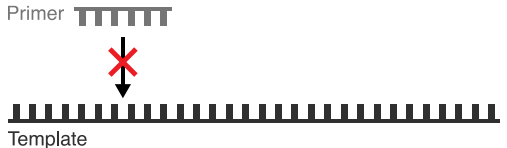
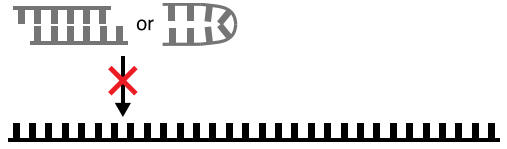
6. Phage

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.
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: $5 \text{ pmol}/\mu\text{L} = 5 \text{ }\mu\text{M} = (1.65 \text{ ng}/\mu\text{L}) \times (\# \text{ of bases in oligo})$
Poorly designed primer: <ul style="list-style-type: none"> Primer T_m is too low.  <ul style="list-style-type: none"> Primer forms a dimer or hairpin. 	Use oligo analysis software to verify the following: <ul style="list-style-type: none"> Melting temperature (T_m) of the primer is 50-60°C. Primer does not have significant self-complementary, leading to self-dimers or hairpins. <ul style="list-style-type: none"> 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. 	<ul style="list-style-type: none"> Use GENEWIZ's "Alternative Protocol", designed for difficult templates. This protocol helps to denature secondary structure that impedes polymerization. <ul style="list-style-type: none"> 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.



Solutions Guide for Sanger Sequencing Poor Quality

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: $10 \text{ pmol}/\mu\text{L} = 10 \text{ }\mu\text{M} = (3.3 \text{ ng}/\mu\text{L}) \times (\# \text{ bases in oligo})$
<p>Poor quality DNA template, including contamination with</p> <ul style="list-style-type: none"> • Guanidine or other chaotropic salts • Phenol or chloroform • EDTA • Ethanol or isopropanol 	<ul style="list-style-type: none"> • Check the absorbance ratios on a spectrophotometer. Aim for values greater than 1.8. <ul style="list-style-type: none"> ○ $A_{260}/280 < 1.8$ may indicate protein contamination. ○ $A_{260}/230 < 1.8$ 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.
<p>Poorly designed primer:</p> <ul style="list-style-type: none"> • Primer T_m is too low.  <ul style="list-style-type: none"> • Primer forms a dimer or hairpin. 	<p>Use oligo analysis software to verify the following:</p> <ul style="list-style-type: none"> • Melting temperature (T_m) of the primer is 50-60°C. • Primer does not have significant self-complementary, leading to self-dimers or hairpins. <ul style="list-style-type: none"> ○ Problems arise at dimer/hairpin conformations more negative than -10 kcal/mol. <p>Free online tools include OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007).</p>