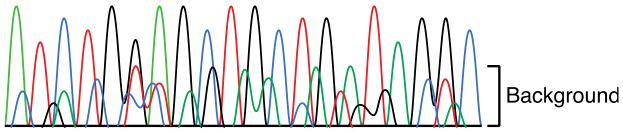
Solutions Guide for Sanger Sequencing
Poor Quality

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, “poor quality” data may provide some useful data whereas “no priming” usually does not.

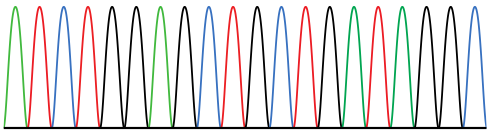
POOR QUALITY – TYPE 1

Dominant trace is present but near background signal; average signal intensity* is usually <200



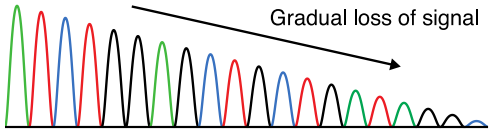
GOOD RESULT

Well defined peaks with low background for at least 500 bp



POOR QUALITY – TYPE 2

Trace starts strong but gradually diminishes to near background signal before position 500



*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. Explanations and solutions are provided in the table below. The list of potential causes is not exhaustive but represents the most common issues. For more assistance, please contact Technical Support team at +49-341-520122-41 or sanger.europe@genewiz.com.

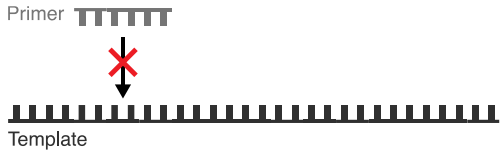
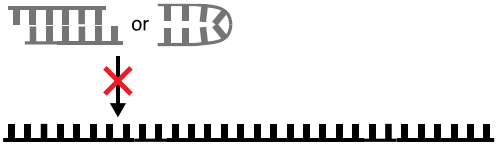
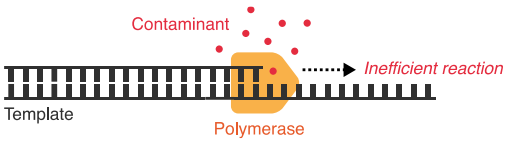
POSSIBLE CAUSE	SOLUTION

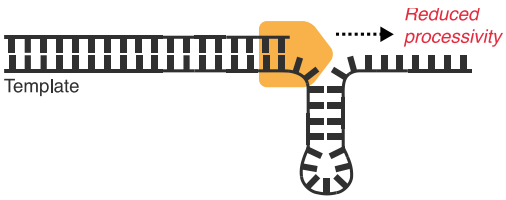
Primer concentration is not in the range of our guidelines. The primer concentration could be too low or high.	• Check that the primer concentration is in the correct range (If not using a GENEWIZ universal primer)
	  ○ Premix reaction: Add 5 µL of 5 pmol/µL primer to your DNA samples.
	  ○ Pre-Defined reaction: Send your primer separately at 10 pmol/µL (20 µL for tube reactions, 60 µL for reactions submitted in plates).

POSSIBLE CAUSE	SOLUTION

Concentration of DNA template is not in the range of our guidelines. The DNA template concentration could be too low or high.	• Double check that the DNA concentration is in the correct range. Review our Sample Submission Guidelines.
	• For **PCR purified products** please apply the appropriate method to determine concentration:
	  ○ 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.
	  ○ 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.
	• For **unpurified PCR products** the following can be performed to verify the concentration:
	  ○ 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.
	• For additional tips on how to optimize PCR for sequencing, see our Sanger Tips & Tricks series.
	• We can repeat reactions with an alternative protocol using a less or more template volume, this could improve the data quality (applies only to our predefined service type).

Plasmid is greater than 20 kb. Larger plasmids can be difficult to be sequenced efficiently and rolling circle amplification is less efficient on larger templates.	• For **plasmids** greater than 20 kb, ensure you submit highly pure samples at 100-150 ng/µL. • Use PCR to amplify the region of interest and submit PCR products for sequencing.

POSSIBLE CAUSE	SOLUTION
Poorly designed primer. The primer design could be the reason for a no priming result. • 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 are for example OligoAnalyzer (IDT) and OligoCalc (Kibbe 2007). • In case you are sending **PCR products** and using a PCR primer for sequencing, please note that the cycling conditions of sequencing may differ from that of your PCR. A primer that works well in your PCR may not be appropriate for sequencing. We do not recommend using the same primer for sequencing as used for the PCR. Using a nested primer usually provides a better data quality.
Poor quality DNA template. The sample could be contaminated, which can prevent an efficient reaction. Contaminations can be: • 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. • When sending **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.

POSSIBLE CAUSE	SOLUTION
Secondary structures. Template forms secondary structure 	• 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. • We can repeat the reaction using GENEWIZ's "Alternative Protocol", designed for difficult templates. This protocol helps to denature secondary structure that impedes polymerization.
Low copy plasmid used. In case Bacterial Colonies or Glycerol Stocks were send 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**. In case Bacterial Colonies or Glycerol Stocks were send, please note that 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. When sending Glycerol stocks or liquid bacterial cultures, the media used could interfere with the sequencing reaction. 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.