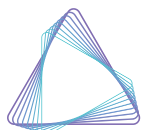
GUIDE

# Sanger Quick Tips

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PART 1

# Sanger Quick Tips for Sequencing PCR Products

## Introduction

PCR allows you to amplify specific regions of DNA quickly and accurately. PCR followed by Sanger sequencing can be used for a wide number of applications, including colony screening, genomic DNA sequencing, transcript sequencing, and a lot more. Although PCR product sequencing is widespread and frequently used, it can be more technically challenging than sequencing from purified plasmid DNA. Azenta Life Sciences Technical Support scientists have put together some tips and tricks to take the pain out of Sanger sequencing from PCR.

## Highly Intense Single-Band PCR Products Yield High-Quality Sequencing Results

The first mantra of Sanger sequencing PCR products is to start from a single and robust PCR band. Azenta recommends investing time and energy in the setup of the reaction in order to minimize time spent troubleshooting a sub-par PCR sequencing reaction. It is always recommended to run any PCR on an agarose gel to check for amplicon size and yield before proceeding with clean-up and sequencing.

## Tips For Producing a Robust, Single-Band PCR Product

### 1. PCR PRIMER DESIGN

You can use several freeware tools on the internet to design your PCR primers. One such tool that Azenta scientists have had good results with is Primer3, which now is available via NCBI. (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

The NCBI version of Primer3 also allows you to check the target DNA against your primer design for any potential nonspecific binding activity. It is important to reduce the likelihood of nonspecific amplifications so that you can avoid multiple rounds of primer design and optimization because it will save you time and money. In addition, check for the propensity of your primer design to form secondary structures and primer dimers.

### 2. POLYMERASE SELECTION

***Taq* DNA Polymerase:** This is still the enzyme of choice for many researchers who perform PCR amplifications plus sequencing. Although it is relatively affordable, some of the drawbacks of *Taq* include higher activity at low temperatures (sometimes resulting in unwanted, nonspecific amplification at resting temperatures), as well as slow processivity for longer amplicons. If you get multiple PCR bands from the use of *Taq*, you may want to increase the annealing temperature or switch to highfidelity enzyme.

**Hot-start *Taq* enzymes:** There are several commercially available hot-start *Taq* enzymes, which contain blocking antibodies that prevent enzyme activity at low/resting temperatures, thus reducing nonspecific product amplification. The blocking antibodies are detached from the polymerase after the first PCR heat activation step, and this restricts the enzyme activity to just the thermocycling process. The use of a hot-start *Taq* tends to produce reactions with higher fidelity.

***Pfu* DNA polymerase:** This enzyme, which was isolated from *Pyrococcus furiosus,* has an additional 3' to 5' proofreading capability that produces less errors during PCR amplifications. *Pfu* polymerase is also widely available from a variety of commercial providers and it is a good choice for high-fidelity PCR amplifications.

**Phusion® High-Fidelity DNA Polymerase:** This is a good choice if you want a combination of high fidelity and high processivity in a PCR enzyme. Phusion polymerases are a fusion of the 3' to 5' proofreading capabilities of *Pyrococcus* with a DNA-binding domain, which enhances enzyme processivity to produce a very fast and highly accurate polymerase.

## 3. BUFFER CONDITIONS

Although most scientists resort to the default buffer that is shipped with a particular polymerase enzyme, it is helpful to know that you can adjust both the pH and $MgCl_2$ concentrations in order to achieve optimal primer binding plus more robust amplification. If a first pass with standard buffer conditions yields poor-quality results, you may adjust the pH between 8.0 to 9.5. $MgCl_2$ concentration can also be adjusted from a final concentration of 1.0 mM to 4.0 mM. Note that higher $MgCl_2$ concentrations and pH may have adverse effects on the fidelity of the reaction. You may perform pH or $MgCl_2$ gradients to test the effect of these variables on the robustness and specificity of the PCR.

## 4. CYCLING PARAMETERS

Before you use a new set of primers for PCR, we recommend that you run a temperature gradient to determine the optimum annealing temperature conditions. Typically, an annealing temperature gradient can be started at 5°C below the $T_m$ of your primer set, but no lower than 40°C. The extension time varies according to the requirements of the polymerase and the size of the target amplicon. Increasing the extension time would allow slower polymerases to complete the extension of longer products. The number of PCR cycles typically starts around 30 but that can be increased for difficult (e.g., high-GC) or longer (> 2 kb) constructs.

## 5. PCR ADJUNCTS

Occasionally, certain amplicons would prove difficult to amplify for unknown reasons. In such cases, the addition of PCR adjuncts may alter the chemistry of the amplification reaction so that you can achieve a more robust reaction, or enhance the ability of the polymerase to sequence through difficult amplicons (e.g., regions with repetitive sequences or areas with secondary structures). We have found that it is usually more effective to troubleshoot all other conditions before turning to the addition of PCR adjuncts. While the inclusion of PCR adjuncts may optimize a suboptimal reaction, please note that the addition of an adjunct rarely transforms a failed PCR into a robust one. Our experience at Azenta has shown that the following adjuncts can be useful.

- **DMSO, 1-10% (v/v):** DMSO is a cosolvent, which facilitates PCR amplification by denaturing amplicon regions with secondary structures or high-GC content. DMSO is also useful for generating long PCR products. Since DMSO may reduce the $T_m$ of the primer set, we recommend that you run a temperature gradient in order to evaluate the impact of DMSO on the $T_m$ of your primers.
- **BSA, 0.1 mg/ml:** BSA enhances PCR by stabilizing the polymerase enzyme. It is also a useful additive when you are dealing with an impure DNA template.
- **Formamide, 1-5% (v/v):** Formamide is another cosolvent whose mode of action is similar to that of DMSO. It decreases the $T_m$ of a dsDNA in order to overcome secondary structure or high-GC content.
- **Glycerol, 5-20% (v/v):** This is another adjunct for templates with high-GC content or secondary structures.
- **Commercial PCR additives:** Several commercial companies offer PCR additives for the amplification of difficult PCR targets. While these can be effective options, they are often just proprietary combinations of the adjuncts listed above sold at a higher price. The most cost-effective option is to try the standard adjuncts listed above first (as they are likely already in most molecular biology labs), and turn to a commercial alternative only if needed.

PART 2

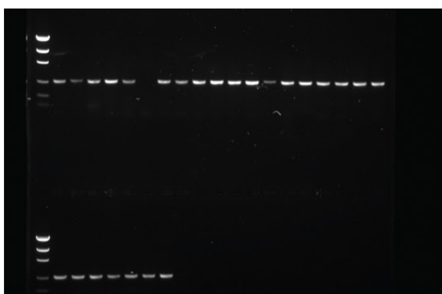
# Sanger Quick Tips for PCR Clean-up and Template Preparation for Sanger Sequencing

## Clean-up of Single-Band PCR Products

After confirming the size and intensity of the single-band PCR product via agarose gel electrophoresis, you can proceed with pre-sequencing clean-up. This process removes excess oligonucleotides and unincorporated dNTPs from the sample so that the cycle sequencing reaction can occur with optimal concentrations of primer, dNTPs, and fluorescently labeled terminators. There are a number of protocols available to accomplish this task.

### 1. ENZYMATIC PCR PURIFICATION

In our experience, an enzymatic PCR clean-up protocol is the most convenient method for producing highquality sequencing results. In general, these methods use a cocktail of enzymes that degrade ssDNA and dNTPs, leaving intact the dsDNA PCR product. As there is no resin or column to bind DNA, an enzymatic PCR clean-up protocol allows for the full recovery of your PCR product. It has the added benefit of eliminating the use of reagents like guanidine and ethanol that may inhibit the sequencing reaction. For your convenience, Azenta Life Sciences provides enzymatic PCR clean-up (per customer request) for unpurified Sanger PCR product reactions. This service is only available when you submit a single-band PCR product. You also have the option of buying and using any commercially available enzymatic clean-up kit.



## Tips for Success

- To prevent adverse effects during sequencing, inactivate the clean-up enzymes via heat denaturation prior to sample submission.
- A spectrophotometer cannot be used for accurate quantitation of a PCR product. Reaction components such as primers and nucleotides (whether degraded or intact) will absorb UV light and inflate the calculated DNA concentration. Band intensity on an agarose gel evaluated relative to standard dsDNA markers is the preferred method.
- Although DNA markers differ, you may use the following dilutions as a rule of thumb when you are preparing samples for sequencing:
  — For bands of moderate intensity (i.e., roughly equal to that of the standard DNA marker), dilute 5 to 10X with water.
  — For strong bands (i.e., significantly brighter than the standard DNA marker), dilute 10 to 15X.
  — For weak bands (i.e., significantly fainter than the standard DNA marker), dilute 2 to 3X.
- Primer dimers (i.e., short double-stranded byproducts of PCR) are not removed during enzymatic clean-up.

## 2. PURIFICATION WITH A DNA-BINDING MATRIX

Another common option for single-band PCR purification involves DNA binding onto columns, beads, or resins followed by washing and elution steps. However, these protocols tend to be more tedious and time-consuming than enzymatic purifications. Another disadvantage is that the binding and elution steps may allow salt or ethanol carryover, which can inhibit the sequencing reaction.

In practice, dsDNA over 40 to 100 bp in the PCR binds to a matrix (i.e., column, beads, or resin). The bound DNA is then washed to remove excess primer, buffer, and dNTPs. After washing and drying, the DNA is eluted and ready for downstream applications such as Sanger sequencing.

If you start with a single-band PCR product, you can obtain high-quality DNA from a kit that uses DNA-binding matrix technology with the following recommendations:

### Tips for Success

- Optimize your PCR amplification so that it produces a strong band on a gel. High yield PCR mitigates several risks:
  — DNA loss is inevitable with any bind-wash-elute purification system.
  — The accuracy of DNA concentration measurements with a spectrophotometer declines below 10 ng/µl.
  — Carryover contaminants in the purified product may interfere with the sequencing reaction.
- Include any added “optional” wash steps described in the manufacturer’s protocol, especially for bands purified from agarose gels.
- Dry the DNA-binding material well after the ethanol wash step in order to minimize the carryover of alcohol, which may inhibit downstream applications.
- After purification, measure the DNA concentration using a spectrophotometer, fluorometer, or by running some of your sample on a gel alongside a standard DNA marker.
- The 260/230 absorbance ratio is useful for detecting carryover contamination from the purification. A value $<1.8$, due to strong absorbance at 230 nm, may indicate the presence of compounds that can inhibit the sequencing reaction.
- For better accuracy, measure your sample concentration before diluting it for submission– the best approach is to purify and measure, then dilute.

## Salvaging Nonspecific PCR Products

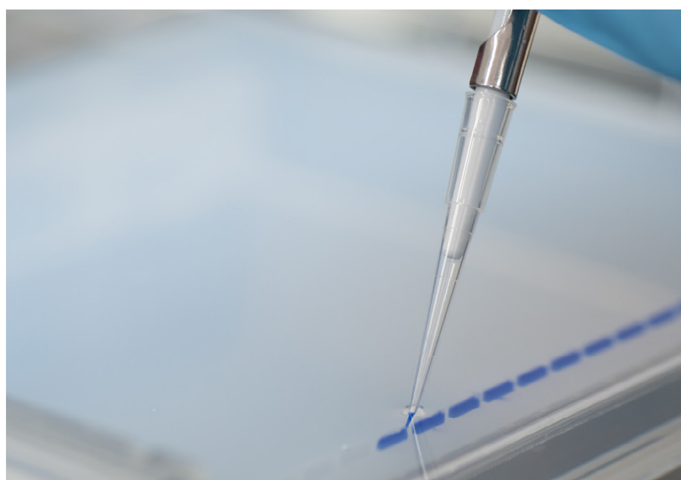
Sometimes, a nonspecific band or two will persist despite your best efforts at optimizing the reaction to a single-band PCR product. Although it is possible to sequence the target PCR product from a mixed population of amplicons, the quality of the sequencing results may fail to match those from a single-band product. As with single-band PCR purifications, you can choose from several commonly used protocols to purify the target band for sequencing.

### 1. GEL PURIFICATION

Although it can be arduous and tedious, gel purification has become the method of choice for purifying a well-defined band from a reaction mixture containing other nontarget products. Despite other disadvantages such as, the need to buy relatively expensive kits, use of spin columns, a vacuum manifold or centrifuge, low yields, and low purity, gel purification remains a feasible method to isolate and purify a target PCR band. Start by running the reaction products on an agarose gel and then identify the PCR band of interest using either fluorescent DNA dye and blue light illumination or an ethidium bromide stain with a UV transilluminator. Using a clean razor blade, excise the band and transfer the gel slice to a microcentrifuge tube. Dissolve the agarose gel containing the DNA with a chaotropic buffer and heat, followed by DNA isolation from the solution by silica adsorption (typically column-based).

## Tips for Success

- Select a robust band of high signal intensity.
- Trim the agarose band carefully in order to minimize the amount of agarose carryover into the purification reaction.
- Do not use TE buffer to elute the DNA because the EDTA will interfere with the Sanger sequencing reaction.
- The $OD_{260/280}$ and $OD_{260/230}$ ratios of the sample should be at least 1.8. If you get a ratio below 1.2, we strongly recommend an additional wash step to remove excess salts or ethanol/ organics.



## 2. BAND-STAB PCR

The band-stab approach to gel purification is becoming increasingly popular because it avoids the tedium of gel extraction protocols. However, it requires a second PCR amplification step for product enrichment. As with traditional gel purification, you start by running the nonspecific PCR products on an agarose gel followed by the identification of the target band via transilluminator. Instead of excising the entire band of interest, you can use a pipette tip or small glass capillary tube to stab a small portion of the band. Next, set up another PCR with either the original or nested (internal) primers. Release the template DNA into the PCR mix by swirling the pipette or capillary with the stabbed agarose plug into the new reaction. Prepare and run a sample of the second reaction on an agarose gel to confirm the presence of a single band. The PCR mixture can then be added to a DNA-binding matrix for purification or cleaned up by the addition of enzymes that degrade unincorporated primers and dNTPs.

### Tips for Success

- Select a robust band with a high signal intensity.
- Avoid ejecting agarose into the second PCR setup.
- If the original primer set still generates multiple products during the second round of PCR, consider using nested primers for increased specificity.

## 3. NESTED PRIMERS

To increase the specificity of PCR, a second round of amplification can be performed using a nested primer set. In practice, a highly diluted sample of the original PCR is used as the template in a second PCR containing primers designed to anneal at sites internal to the original primer set.

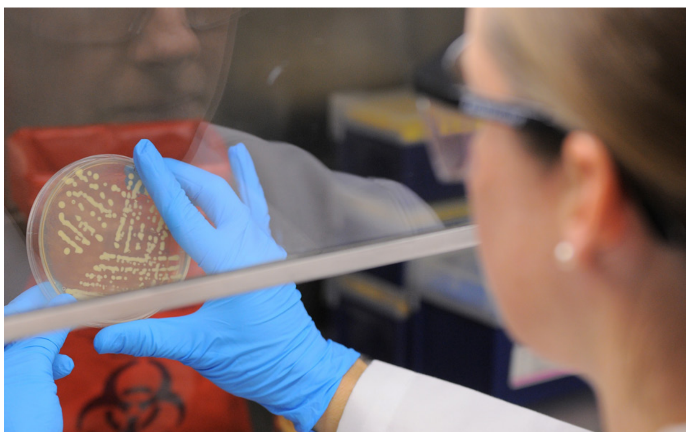
### Tips for Success

- Use a minute amount of the original PCR as the template for the second PCR to avoid significant carryover of the original template and unwanted products.
- Ensure that the sequences of the nested primers are devoid of similarities to that of the original primers used for the nonspecific reaction.
- Be sure to adjust the annealing temperature for the second PCR based on the melting temperature ($T_m$) of the nested primer set.

## 4. TA CLONING AND SEQUENCING OF COLONIES

If you cannot isolate the target PCR product from a nonspecific mixture, then subcloning is a good strategy for sequencing a representative sample of amplicons contained within the mixed population. After inserting the linear product into a vector and transforming bacteria cells, select a random set of colonies and either sequence them directly (via Azenta's colony sequencing service) or prepare mini-preps and submit the purified plasmid for sequencing. There are several commercially available kits for this cloning strategy.

One popular method, called TA cloning, takes advantage of the 3'A-overhangs left by *Taq* polymerase during PCR. The A-tailed product binds to complementary "T" overhangs on the ends of a linearized vector thus facilitating ligation.



Next-generation sequencing (NGS) may be a good option if you need high resolution and quantitative data for a mixed population of PCR products. Additional factors to consider for NGS are depth of coverage, number of samples, and amplicon frequency. For some of your projects, NGS may be more cost effective than Sanger sequencing. To determine if NGS is right for you, please contact the Azenta Project Management team (PM@azenta.com) for a free consultation and a comparative cost analysis for our NGS Amplicon and Sanger sequencing services.

## Tips for Success

- Standard *Taq* lacks 3' to 5' exonuclease activity, and it is ideal for generating an amplicon for TA cloning.
- Perform TA cloning as soon as possible after generating the PCR product because of A-tail degradation.
- Proofreading polymerases typically have exonuclease activity, which results in PCR amplicons with blunt ends. Blunt-ended products can be cloned using blunt-end ligation or A-tailed via the addition of *Taq* polymerase and dATP after PCR.
- Commercially available topoisomerase-based TA and blunt cloning kits are a fast and efficient alternative to classic TA cloning.
- You can submit bacteria colonies on agar directly to Azenta for sequencing. We recommend that you keep the original agar plate and submit a duplicate for sequencing. Once you receive your data, you can pursue any downstream work with the original plate immediately.
- Determining how many clones to sequence depends on several factors, such as the number and relative intensity of bands produced by the PCR and the depth of information you require. For example, sequencing five clones should be sufficient if your goal is simply to identify the prominent products in a reaction containing three bands of roughly equal intensity. For a complete characterization of the products in a nonspecific PCR, approximately 20 or more clones may be required.

PART 3

# Sanger Quick Tips for Running an Informative Gel for PCR Visualization by Gel Electrophoresis

Accurate assessment of PCR success depends on a well-resolved gel image with sufficient band separation and definition. There are several simple and effective steps you can take to get the most information out of your gels.

## Running Buffer

When choosing a running buffer, it is key to consider downstream applications. Common electrophoresis buffers include:

- **TBE:** Tris-borate-EDTA (TBE) is the recommended electrophoresis running buffer for fragments less than 1.5 kb. TBE is a good choice for standard gel visualization and can be run at moderate voltages (no more than 10V/cm gel box length, cathode to anode). A note of caution about TBE buffer: borate can impede enzymatic reactions, so if you aim to recover the DNA from your gel for downstream use, a buffer lacking borate is recommended (e.g., TAE). TBE is normally used at a 0.5X working concentration.
- **TAE:** Tris-acetate-EDTA (TAE) is another common electrophoresis running buffer. TAE has less buffering capacity than TBE, so gels must be run for a slightly longer time at lower voltage, and the buffer should be replaced after every usage for best results. However, DNA isolated from a TAE gel has the advantage of being suitable for downstream enzymatic reactions. Thus, TAE is the buffer of choice for DNA recovery. TAE is also better for larger fragments (>1.5 kb), as well as for genomic DNA and supercoiled DNA. TAE is normally used at a 0.5X-1X working concentration. NOTE: The same supercoiled DNA sample would run differently in TBE and TAE buffers, and this should be taken into account when comparing results between the two buffering systems.
- **Alternatives to TAE/TBE:** Over the last several years, bench scientists have determined several alternatives to the classic TBE/TAE electrophoresis buffers, namely Sodium Borate (SB) and Lithium Borate (LB). They are viable for quick visualization of DNA molecules. Gels in these buffers can be run at higher voltages (typically 10-50 V/cm) without overheating, thus decreasing the time needed for sufficient band separation. Like TBE, both SB and LB contain borate, therefore they are not suitable for band extraction and downstream enzymatic reactions with recovered DNA.

### Tips for Success

Always make sure the agarose is dissolved in the same buffer used in the electrophoresis chamber.

## 1. AGAROSE GEL CONCENTRATION

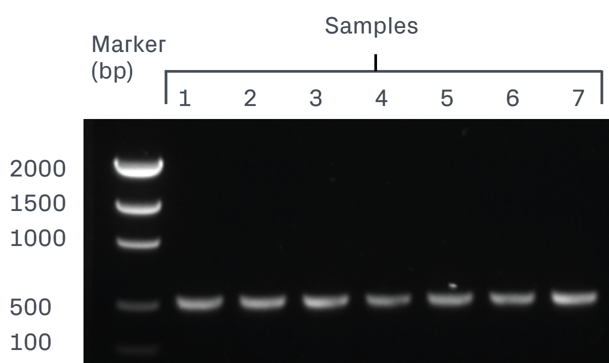
Agarose concentration affects gel porosity, which in turn determines its resolving power. The concentration of agarose in the prepared gel should be based on the size of DNA molecules to be resolved (Table 1). Note that gel rigs often come with two types of combs; thick combs create higher-volume wells that are appropriate for preparative work and thin combs create lower-volume wells for analytical work.

| DNA Band Size (bp) | % Agarose in Gel (w/v) |
|---|---|
| <250 | Not Recommended |
| 250 - 1,500 | 2.00 |
| 300 - 4,000 | 1.50 |
| 400 - 7,000 | 1.20 |
| 500 - 9,000 | 1.00 |
| 800 - 11,000 | 0.75 |
| 1,000 - 30,000 | 0.50 |

**Table 1.** Agarose Gel Percentage Guidelines by DNA Size

## 2. LADDER

Make sure you include at least one lane of DNA standards, known as a ladder, on each gel. Ladders are available in many sizes and configurations; the best way to select the appropriate ladder is to identify the size range of DNA molecules you wish to resolve. A 100-bp ladder typically contains bands of 100-bp increments ranging from about 100 bp to 1500 bp. A 1-kb ladder contains 1,000-bp increments covering a range from about 500 bp to 10 kb. A 2-Log ladder is also a popular option, as it spans both the lower size range (from 100 bp onwards) through to the larger 10 kb range. Many labs stock both the 100 bp and the 1 kb ladder. Note that for gel consistency and size accuracy, it is best to use the same loading buffer for both your ladder and samples.




## 3. AMOUNT OF TEMPLATE TO LOAD

The mass of DNA required to visualize your band on an agarose gel tends to be in the nanogram range. The minimum recommended amount per band is about 10 ng; anything less than that will be very difficult to detect on a gel. For crisp bands with no distortion (e.g., “smiley faces”) or smearing, ~150 ng per band should be the highest mass loaded into a lane. The appropriate amount of sample volume to load depends on the volume of the well in the gel; at a minimum, the sample should occupy at least a third of the well’s capacity.

## 4. LOADING BUFFER SELECTION

There are a variety of different sample loading buffers available. The easiest approach is to use the same loading buffer as that used for the ladder. Loading buffers can also be prepared from reagents normally stocked in a standard molecular biology lab. You can either obtain commercially available loading buffers from several vendors or prepare one from reagents normally stocked in a standard molecular biology lab. They usually contain a high density compound, such as Ficoll® or glycerol, and one or more dyes, such as Orange G, xylene cyanol, and bromophenol blue. These buffers are normally prepared at a 6X concentration and run at a 1/6 final concentration (or 1X) in your sample (Table 2).

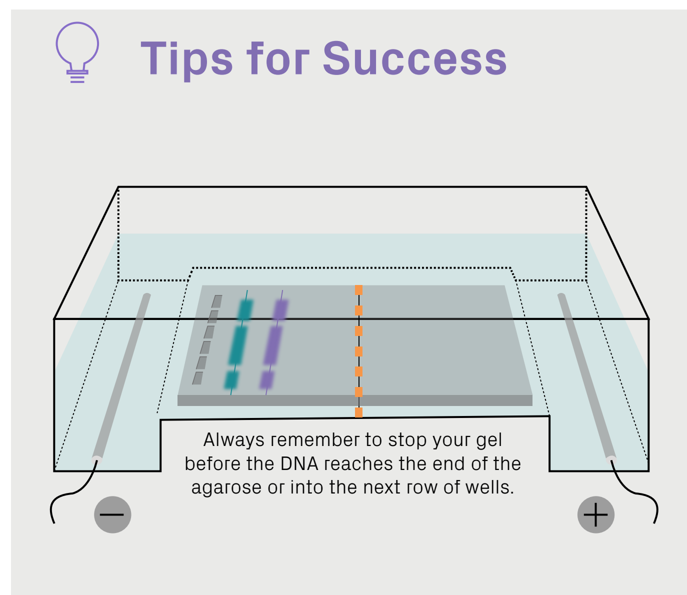
| Recipe (6X) | High-Density Compound |
|---|---|
| 40% (w/v) Sucrose<br>0.25% Bromophenol Blue<br>0.25% Xylene cyanol FF | Sucrose |
| 30% Glycerol in distilled water<br>0.25% Bromophenol Blue<br>0.25% Xylene cyanol FF | Glycerol |
| 15% Ficoll®<br>0.25% Bromophenol Blue<br>0.25% Xylene cyanol FF | Ficoll |

**Table 2.** Common Loading Buffers

## 5. VOLTAGE AND RUNNING TIME

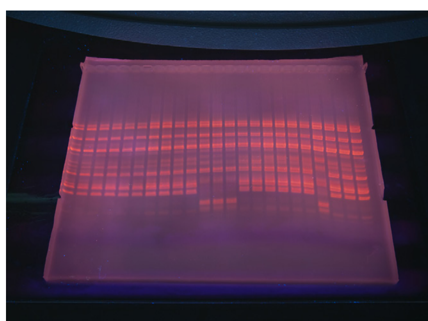
In general, gel electrophoresis should be run with sufficient time to clearly separate your bands of interest as well as those of the ladder. Under such conditions, you can accurately determine the number of DNA molecules present in your sample and estimate their size. In general, the minimum running distance for the gel should be about 5 cm, but it can be as long as needed for your specific purposes. The recommended voltage depends on the type of running buffer and agarose being used. For example, standard agarose gels with TBE buffer are usually run at 5-10V/cm (distance measured between electrodes). Using low-melt agarose or less stable buffers may need a lower voltage and longer running time for optimal results. Solutions with high buffering capacities can be run at a higher voltage, and this requires less time for sufficient band separation. Be sure to check the recommendations of your agarose supplier for more specific guidance on running conditions. The loading buffer dye front will enable you to easily visualize the progress of electrophoresis; keep in mind that some dye fronts may actually migrate behind your band of interest, so check the electrophoretic properties of your dye (i.e., the size of DNA which co-migrates with the dye).

### Tips for Success



## 6. CHOOSING AN APPROPRIATE GEL STAIN

Ethidium bromide (EtBr) is a widely used stain for DNA detection. EtBr is a long-lasting stain that can be mixed directly into your agarose gel before it is poured, obviating the need for a subsequent gel staining step. It is cost effective and has good sensitivity for band visualization. The major drawback of EtBr is toxicity - it is a mutagen. Therefore, many labs choose safer alternatives, such as SYBR® Gold and SYBR® Safe. Compared to EtBr, the SYBR® series is less stable and more expensive but has equal or better sensitivity. Due to their instability, these types of dyes can require loading directly into the sample, or post-staining prior to visualization. Follow the recommendations of the manufacturer in order to determine optimal stain application techniques for your gel.



# Gel Interpretation and Troubleshooting Tips

## 1. BAND DISTORTION

A common problem encountered when running gels is band distortion, often observed as a U-shape or "smiley face". These banding patterns usually occur when there is too much sample loaded on your gel and/or the gel is run at too high a voltage; most of the time if you reduce the amount loaded on the gel and reduce the voltage, you can eliminate this issue. If this does not resolve the problem, high salt or contamination with organic compounds could be the problem. An extra precipitation and wash step might help.

## 2. OVERHEATING

Overheating can cause bands in the middle lanes of the gel to run faster than those on the edge. Under these conditions, it can be difficult to estimate the molecular size of the band or make comparisons between samples on the gel. If this occurs, you should reduce the voltage or, alternatively, run the gels in a cold room or chill the electrophoresis tank with cold packs.

## 3. SMEARING

If you are expecting a nice, clean band but end up with a disseminated smear on your gel, check for the following causes:

- High voltage
- Concentration and/or type of buffer used in the gel is different than that in the chamber
- Heterogeneous gel, caused by incomplete dissolution of the agarose prior to casting
- High salt or organic compound concentration in the template
- Suboptimal PCR - have these primers worked in the past?

Refer to the Sanger QuickTips guide Volume 1: Tips for a Robust, Single-band PCR product for troubleshooting and optimization ideas.

## 4. BLURRY BANDS

In addition to the issues discussed above, blurry or fuzzy bands can be caused by a poorly focused image. To obtain sharpness, manually adjust the focus on your gel imaging system (or use autofocus, if available) every time you capture an image. Note that an image can become out-of-focus when changing the zoom of the lens, so adjust the zoom first, then focus.

## 5. EXTREMELY BRIGHT OR FAINT BANDS

To capture a good gel image, the exposure must be adjusted properly. Too long an exposure can result in blown-out bands, which may cause two nearby bands to appear as one. If the exposure is too short, you may fail to detect fainter bands. It is often helpful to try a series of exposures to make sure the gel image is informative and accurate.

## 6. MULTIPLE BANDS

The presence of multiple bands indicates that your primer set amplified more than one unique product. Take a peek at our Tip Sheet section on PCR tips for a high-intensity single-band product.

> Refer to the Sanger QuickTips guide Volume 1: Tips for a Robust, Single-band PCR product for troubleshooting and optimization ideas.

## 7. PRIMER DIMER

Primer self-annealing often shows up as a small smear at the front edge of the gel (around 50 bp in length). This indicates that the primer set is annealing to itself and needs to be redesigned. There are online tools available to check the propensity for primers to form homo- or heterodimers; Azenta scientists recommend that you use these tools before ordering any newly-designed primer set.

## 8. INSUFFICIENT BAND SEPARATION

If you fail to achieve good resolution even after using the full length of the gel, follow the following suggestions:

- Adjust the agarose percentage.
- Use a longer gel.
- Run the gel at a lower voltage for a longer length of time; this can help increase band resolution. The gel rig can even be set up in a cold room or refrigerator to maximize the crispness of your bands.
- Make sure buffer composition and concentration is the same in the gel and in the buffer chamber.
- Investigate possible sources of contamination (e.g., salt, organic compounds) in your template.

## 9. MISSING BANDS

Before panic sets in, set up the PCR again and make sure all reaction components are added correctly. Always include a positive and negative control, if available. It helps to keep a checklist in your lab notebook and physically check off each component as it is added. If, after a gel re-run, all the bands are still missing, you can investigate the following possibilities:

- Did the bands migrate off the gel? (i.e., the gel was run too long). Look for the position of the dye front and the ladder to confirm.
- Did the bands migrate down into another gel space (if you have a multi-row gel set up)?
- Check PCR design - have these primers worked in the past?

Refer to the Sanger QuickTips guide
Volume 1: Tips for a Robust, Single-band PCR product for troubleshooting and optimization ideas.

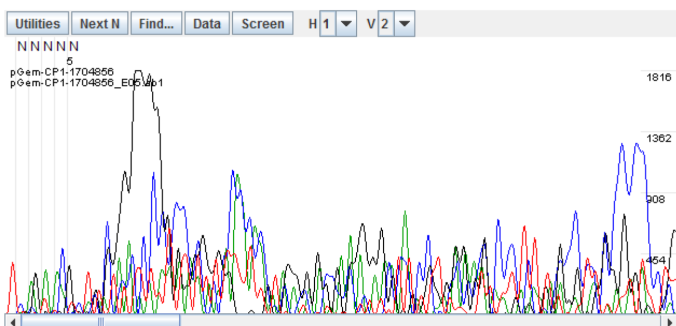
PART 4

# Sanger Quick Tips for Sequencing Purified PCR Products Troubleshooting a Poor Reaction

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.



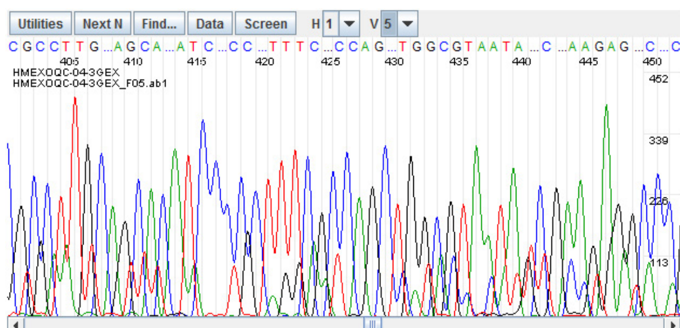

Follow the recommendations below when you encounter failed sequencing reactions with PCR products:

- 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., $T_m$ 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.

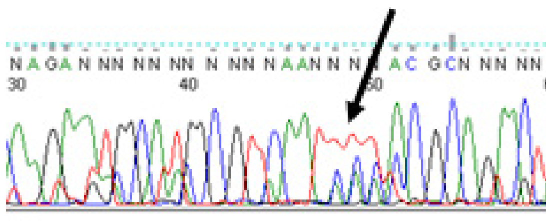



Some of the common causes and solutions for nonspecific reactions are:

- 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.



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