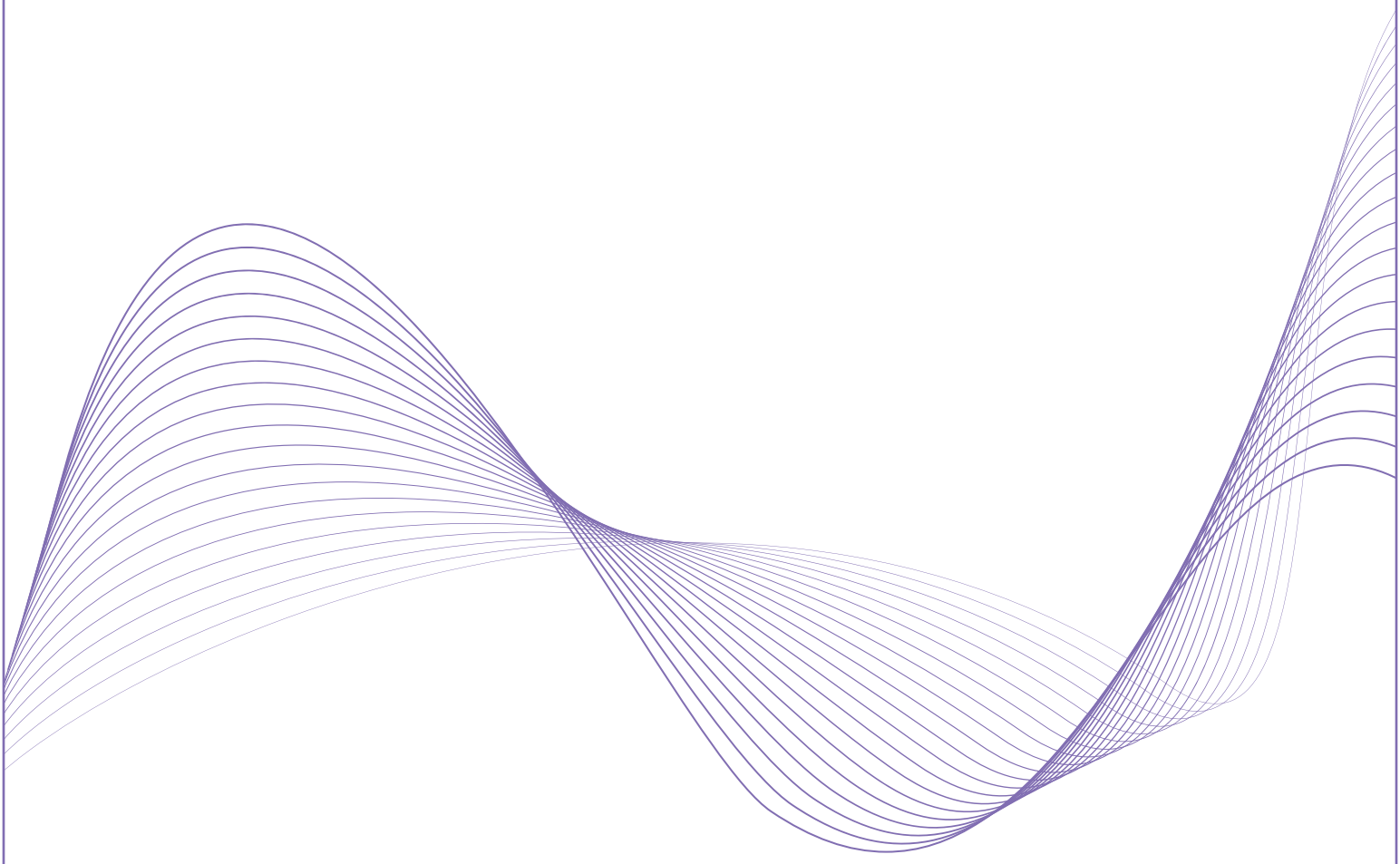
GUIDE

Volume 1: Sanger Quick Tips for Sequencing PCR Products



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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.