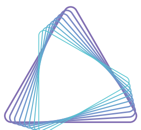
GUIDE

Volume 3: Sanger Quick Tips for PCR Clean-up and Template Preparation for Sanger Sequencing



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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.

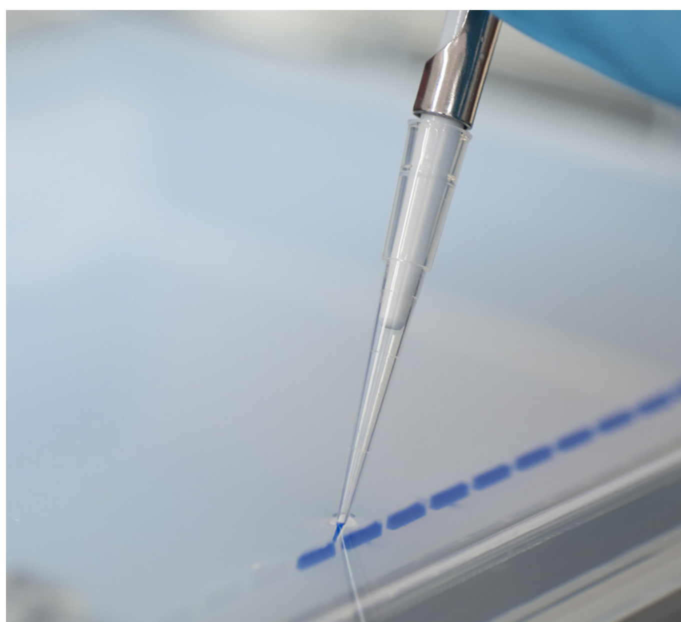
1. GEL PURIFICATION

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.

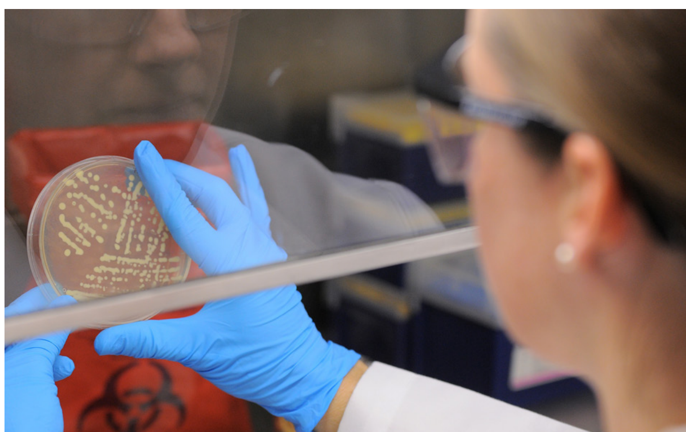
Tips for Success

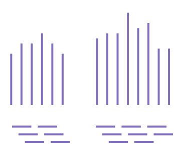
- Use a minute amount of the original PCR as the template for the second PCR to avoid significant carryover of the original template and unwanted products.
- Ensure that the sequences of the nested primers are devoid of similarities to that of the original primers used for the nonspecific reaction.
- 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.

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