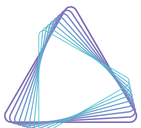
GUIDE

Volume 2: Sanger Quick Tips for PCR Clean-up and Template Preparation for Sanger Sequencing



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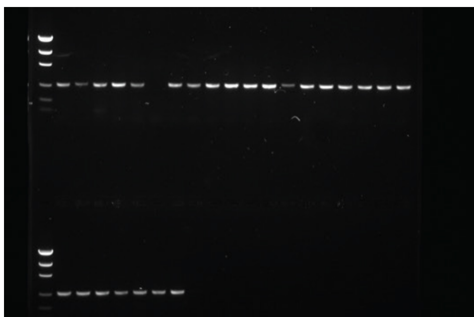
Clean-up of Single-Band PCR Products

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 high-quality 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.

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