Exonuclease I, Shrimp Alkaline Phosphatase

PCR Purification Protocol

This protocol features a method for preparing the products of symmetric (double-stranded) PCR for sequencing. This method requires no purification or separation steps at all. It is designed to require a minimum of "hands-on" time and could readily be automated by automatic pipetting devices. All gel purification, sedimentation, filtration and magnetic separations are eliminated by the use of two enzymes that effectively remove the excess dNTPs and primers from DNA produced by PCR amplification. The method consists of a few simple steps.

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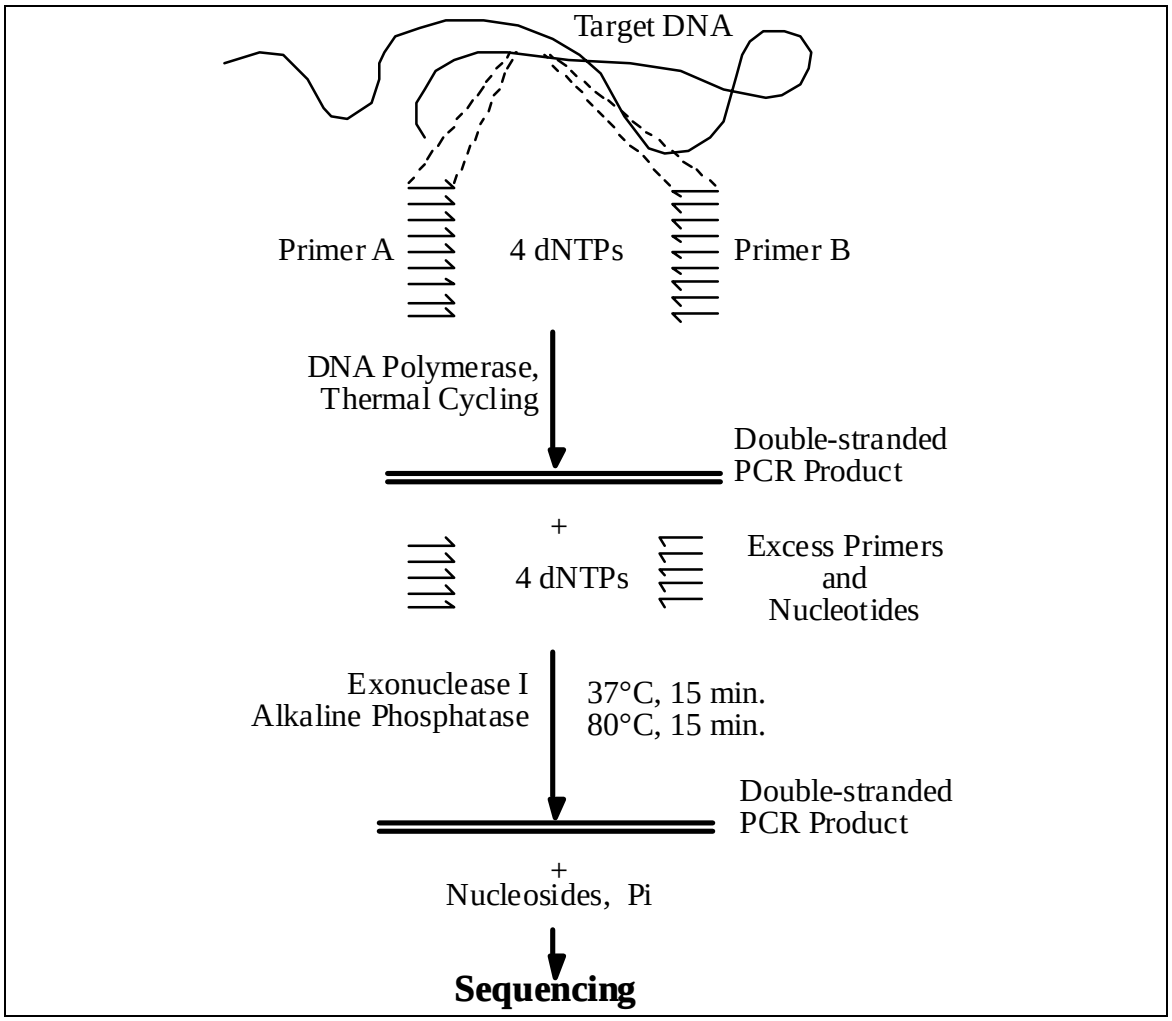
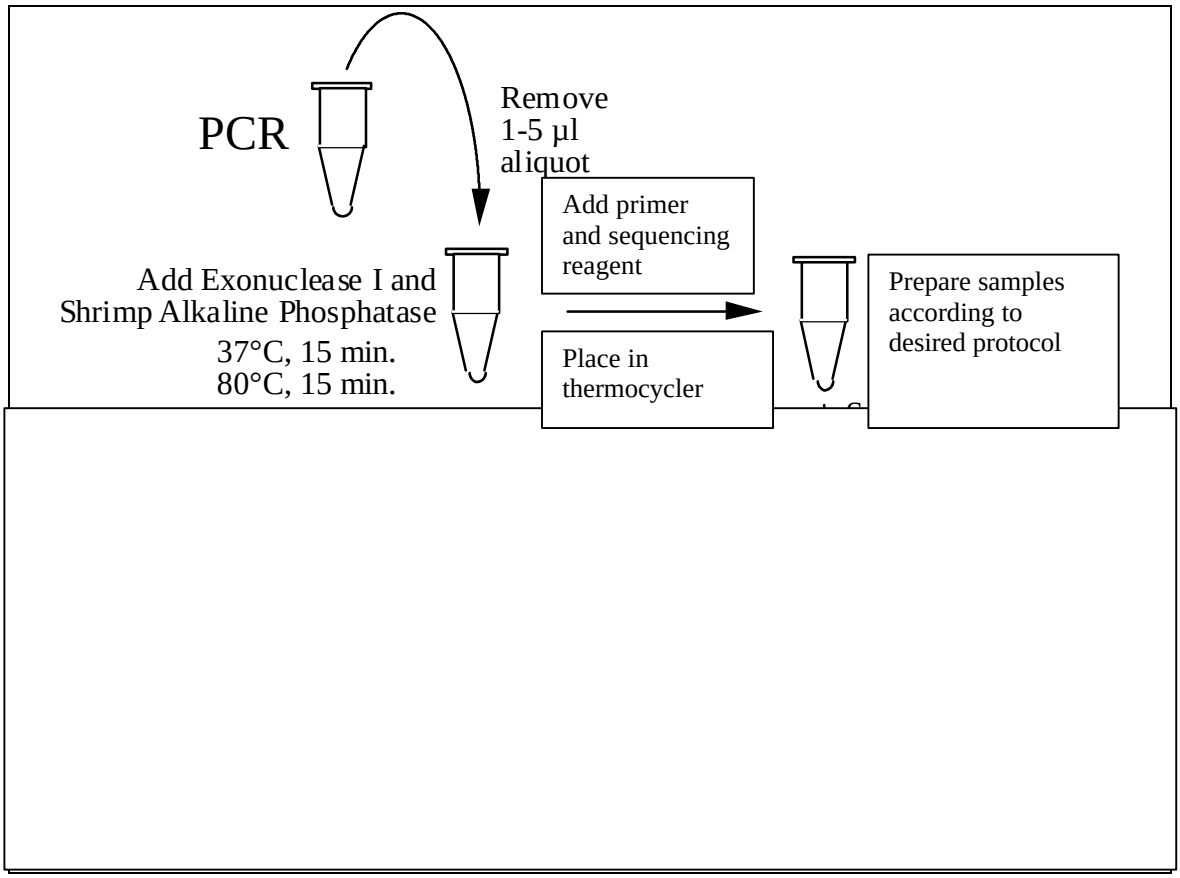


The polymerase chain reaction (PCR) makes use of two primers, deoxynucleoside triphosphates (dNTPs) and DNA polymerase to produce multiple copies of a specific DNA sequence. When complete most of the dNTPs and primers remain intact, and these will interfere with normal sequencing methods that also make use of primers and nucleotides. There are two hydrolytic enzymes; alkaline phosphatase and exonuclease I used in this protocol which can be used to remove these unwanted materials. These enzymes are added to an aliquot (1-5 µl) of the PCR reaction mixture. This is incubated (typically in the thermal cycling instrument) at 37°C for 15 min. Then heating the mixture to 80°C for an additional 15 minutes inactivates these two enzymes.



Sequencing is accomplished normally. The template is denatured and annealed to primer using a heating and snap-cooling procedure. Since only simple pipette transfers are required, many samples can be processed at once, and very simple automation devices such as multiple-head pipettors can be used to speed the process.

MATERIALS REQUIRED FOR THIS PROTOCOL

The solutions required for the PCR product sequencing kit have been carefully prepared to yield the best possible sequencing results. Each reagent has been tested extensively and its concentration adjusted to meet high quality standards.

E70073Z Exonuclease I

10 unit/µl in
20 mM Tris.HCl (pH 7.5)
5 mM 2-mercaptoethanol
50% glycerol

E70092X Shrimp Alkaline Phosphatase

2 units/µl in
25 mM Tris.HCl (pH 7.6)
1 mM $MgCl_2$
0.1 mM $ZnCl_2$
50% glycerol

Supplied with 10X SAP Dilution Buffer
200 mM Tris pH 8.0
100 mM $MgCl_2$

All nucleotide mixtures should be stored frozen at -20°C and for longest life be kept on ice when thawed for use.

PCR PROTOCOLS

It is essential that high quality and good yield PCR be performed to obtain high quality sequence information. Problems with high background low signal intensity and ambiguities can often be traced to the PCR step. Not every PCR will yield a product that can be sequenced. Analysis of the PCR product on agarose gels and optimization of the PCR may be necessary to obtain good-quality sequences. The sequencing protocols presented in this protocol are appropriate for the products of the PCR. Detailed protocols for the PCR steps are beyond the scope of this manual, but the following reaction mixture is typical.

Final Concentration
10mM Tris-HCl pH 8.3
1.5mM $MgCl_2$
50mM KCl
200µM each dATP, dGTP, dCTP, dTTP
1µM each (100 pmol/100 µl) Primer
2.5units/100 µl Taq DNA Polymerase

Amplification is carried out in a volume of 25-100 µl in tubes or plates (96 or 384) in a thermal cycler. A total of 20-25 cycles for 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C is usually appropriate using primers that are 20-25 nucleotides long. The yield of PCR can be measured by fluorescence in the presence of ethidium bromide or other fluorescent dye using a fluorometer or estimated by agarose gel electrophoresis. The minimum volume of PCR for sequencing is about 25 µl. Of this volume, 10 µl is used to run an agarose gel to check quality and quantity of DNA amplified and an additional 2-5 µl used for sequencing. Generally, the presence of a single amplified band which is readily visible by ethidium bromide staining indicates that sufficient template has been produced. If necessary, re-amplification of PCR products using the same or internal primers may be carried out to improve yield. Simply dilute 0.1-1% of the PCR product into fresh reaction mixture and cycle. NOTE: **When multiple amplified bands are observed, or when the yield is low, DNA sequences will usually be poor. It is essential to check the quality of amplified DNA prior to sequencing. The method described here cannot be expected to yield flawless sequence with every PCR amplification. For difficult cases, purification of the product using gels or other methods may be required.**

NESTED PCR

When the sequence of interest is present in a large, complex background such as a gene fragment from a complex genome it is very often desirable to run sequential PCRs using nested pairs of primers. First, PCR amplification is done with an "outer" pair of primers. Then the product of this amplification (which is often too heterogeneous and dilute for sequencing) is diluted 100- to 1000-fold into a second PCR amplification mixture containing inner (or nested) primers. The product of the second amplification is usually a single amplified product DNA suitable for sequencing, even from very small samples of DNA from a complex mammalian genome. Amplification of DNA cloned in bacteria may not require repeated amplification using nested pairs of primers.

ASYMMETRIC PCR

Single-stranded DNA for sequencing can be produced by a variation of the PCR procedure. One way is to use unequal concentrations of the two amplification primers, a method known as asymmetric PCR. An equally effective method is to simply subject a symmetric-PCR product to a second round of amplification using only a single primer. For example, the target sequence is first amplified using two primers. Then the product DNA is diluted approximately 100-fold into a second PCR amplification reaction mixture that contains only one of the two original primer at a final concentration of 0.5µM. After 30-50 cycles of amplification, sufficient single-stranded DNA for sequencing is usually produced. Sequencing of this DNA can be primed using the primer omitted from the second amplification. Note: the yield of single-stranded product during this kind of amplification increases only linearly with the number of cycles, so yield should be checked carefully. **Single stranded DNA cannot be treated with Exonuclease I prior to sequencing. When using the products of asymmetric PCR, the DNA should be treated ONLY with shrimp alkaline phosphatase prior to sequencing.**

ENZYMATIC PRE-TREATMENT OF PCR PRODUCT

The key step in this method for sequencing PCR products consists of treating the PCR product with a combination of Exonuclease I and Shrimp Alkaline Phosphatase. Both of these enzymes are active in the buffer used for PCR, so no change in buffer is required. The Exonuclease I removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The shrimp alkaline phosphatase removes the remaining dNTPs from the PCR mixture that would interfere with the labeling step of the sequencing process. Simply heating to 80°C for 15 min inactivates the exonuclease and phosphatase. Combine the following:

PCR amplification mixture	5 µl
Exonuclease I (10.0 unit/µl)	1 µl
Shrimp Alkaline Phosphatase (2.0 units/µl)	1 µl
TOTAL	7 µl

1. **Mix** and incubate at 37°C 15 min.
2. **Inactivate** Exonuclease and phosphatase by heating to 80°C for 15 minutes.

The DNA is now ready for direct sequencing using standard techniques.

Alternately, if automation requires the use of larger volumes to ensure accurate pipetting prepare the following ExoI/SAP cocktail:

Exonuclease I (10.0 units/µl)	100 l
Shrimp Alkaline Phosphatase (2.0 units/µl)	100 l
10X SAP Dilution Buffer	50 l
De-ionized water	250 l
Total	**500 l**

Combine the following:

PCR amplification mixture 5–25 µl

ExoI/SAP cocktail 5 µl

1. **Mix** and incubate at 37°C, 15-30 minutes.
2. Inactivate the exonuclease and the phosphatase by heating at 80°C for 15 minutes.

The DNA is now ready for direct sequencing using standard techniques.

DETERMINING HOW MUCH TEMPLATE DNA TO USE

The amount of template used is very important. Experience has shown that the use of the minimum possible amount of template that gives reasonable exposure times is best. The volume of PCR product (after treatment with exonuclease and alkaline phosphatase) depends on the concentration of the PCR product expressed in molar (pmol/µl) terms. Ideally, 0.05-0.1 pmol of template should be used for sequencing. This volume of PCR product required to give this amount of template can be calculated from the size of the PCR product and the concentration (in ng/µl) estimated from an agarose gel by comparison with known DNA standards. The following table gives an approximate minimum volume of PCR product to use for sequencing. Good amplifications result in the production of 10-20 ng of product DNA per µl but yields vary greatly depending on numerous factors.

* If the yield is so low that much more than 5 µl may be required for sequencing, it is better to re-amplify (using the same or nested primers as required for specificity). The recommended protocol calls for a maximum of 5 µl of template, but up to 10 µl can be used if required. The use of Mn buffer (see below) may give better results than increasing the amount of template.

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