USB Corporation
26111 Miles Road
Cleveland, Ohio 44128 USA
www.usbweb.com

P-70995A
rev 02/07

PCR Product Pre-Sequencing Kit

Product Number 70995 (100 reactions)
70996 (500 reactions)
70997 (2,000 reactions)

STORAGE

Store at -15°C to -30°C.

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

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CONTENTS

COMPONENTS OF THE KIT

Exonuclease I
10 units/µl in 20mM Tris-HCl, pH 7.5, 5mM 2-mercaptoethanol, 0.5mM EDTA, 50% glycerol

Shrimp Alkaline Phosphatase
2 units/µl in 25mM Tris-HCl, pH 7.6, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 50% glycerol

QUALITY CONTROL

All kit batches are functionally tested by USB® using radiolabeled-dATP and a control M13 clone single-stranded DNA template as described in the Sequenase™ PCR Product Sequencing Kit (PN 70170) protocol. Release specifications are based on sequence length, band intensity and sequence quality. The entire PCR product sequence must be readable on a standardized gel with less than 24 hours exposure. The sequence must also be free of background bands strong enough to interfere with sequence interpretation.

SAFETY WARNINGS AND PRECAUTIONS

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Caution: All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as lab coat, safety glasses, and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water.

INTRODUCTION

The PCR Product Pre-Sequencing Kit features a method for preparing the products of symmetric (double-stranded) Polymerase Chain Reaction (PCR) for sequencing. This method is designed to require a minimum of 'hands-on' time and could readily be automated by robotic pipetting devices. All gel or column purifications, sedimentations, filtrations and magnetic separations are eliminated by the use of two enzymes which effectively remove the excess deoxynucleoside triphosphates (dNTPs) and primers from DNA produced by PCR amplification. The method consists of a few simple steps. 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.

PCR makes use of two primers, dNTPs and DNA polymerase to produce multiple copies of a specific DNA sequence. When complete, any unconsumed dNTPs and primers remaining in the PCR product mixture will interfere with subsequent DNA sequencing methods. Two hydrolytic enzymes in this kit, Shrimp Alkaline Phosphatase and Exonuclease I, remove these unwanted dNTPs and primers (Figure 1). 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 which would interfere with the sequencing reaction. The exonuclease and phosphatase are inactivated simply by heating to 80°C for 15 minutes.

The PCR Product Pre-Sequencing Kit prepares PCR products for sequencing by either radioactive or fluorescent detection methods. No alterations in the procedure are required for either method. After the clean-up procedure is complete the sequencing protocol of choice should be followed as described in the specific sequencing kit protocol booklet (see below for recommendations). It has been found that PCR product clean-up is not always necessary when sequencing with dye-primer fluorescent sequencing methods, however, in many cases it is beneficial. When sequencing with fluorescent dye-terminators, it is essential that the PCR product be cleaned-up. For more detailed information, contact USB Technical Support.

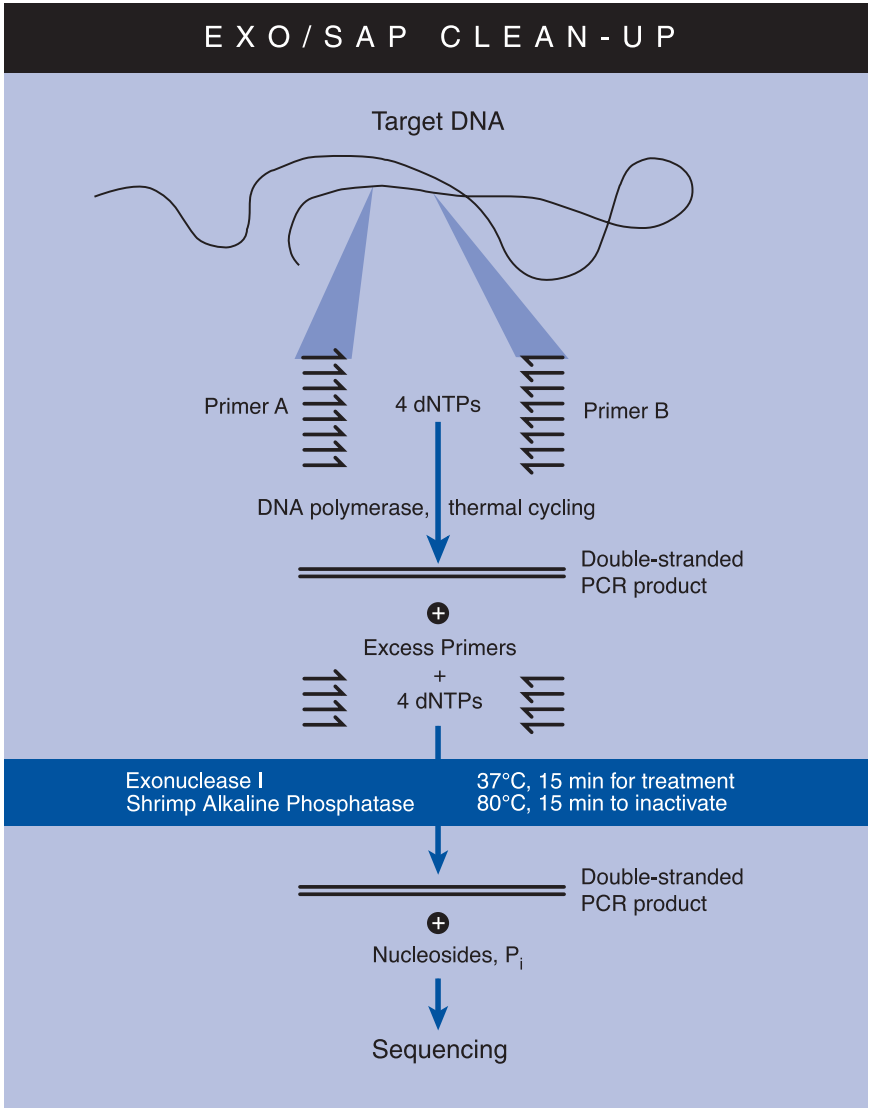

Figure 1: Schematic of the PCR Product Pre-Sequencing Kit method.

PCR PROTOCOLS

Detailed protocols for the PCR steps are beyond the scope of this manual, but the following reaction mixture is a recommended starting point when using Taq DNA Polymerase or other thermostable enzymes.

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
2.5 units/100 µl Taq DNA Polymerase

Note: When multiple amplified bands are observed, or when the yield is low, DNA sequences will usually be poor. The use of an internal primer for sequencing (instead of one of the amplification primers) is often successful when multiple PCR products are encountered. It is essential to check the quality of amplified DNA prior to sequencing. The methods available with this kit 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.

When using the products of asymmetric PCR, the DNA should be treated ONLY with Shrimp Alkaline Phosphatase prior to sequencing.

ENZYMATIC PRE-SEQUENCING TREATMENT OF PCR PRODUCTS

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

   Mix and incubate at 37°C 15 minutes. (It is convenient to do this in a thermal cycler.) **Note**: When treating more than 10 µl of PCR product, increase the amount of exonuclease and phosphatase proportionally.

2. Inactivate Exonuclease I and Shrimp Alkaline Phosphatase by heating to 80°C for 15 minutes. (It is also convenient to do this in a thermal cycler.)

   The DNA is now ready for direct sequencing with Sequenase Version 2.0 or Thermo Sequenase™ DNA Polymerase using the protocols supplied with the individual sequencing kit. The following sequencing kits are recommended for use in sequencing PCR products.

   Sequenase Version 2.0 DNA Sequencing Kit (PN 70770)

   Thermo Sequenase Cycle Sequencing Kit (PN 78500)

   Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit
   PN 188403 - 50 reactions - Includes ^{33}P ddNTPs
   PN 79750 - 50 reactions - Without ^{33}P ddNTPs

Note: When sequencing the treated PCR product with USB's Sequenase Version 2.0 DNA Sequencing Kit (PN 70770) the following primer annealing protocol should be used:

Annealing primer and template

Treated PCR product	__ µl	(up to 9 µl)
Primer (5-10 pmol/µl)	1 µl	
H_2O	__ µl	(to adjust volume to 10 µl)
Total	10 µl	

Incubate 2-3 minutes at 100°C, preferably in a thermal cycler. Cool as quickly as possible by placing the vial directly in an ice/water bath for 5 minutes. Keep the tube on ice prior to sequencing.

DETERMINING HOW MUCH TEMPLATE DNA TO USE FOR DNA SEQUENCING

The amount of template used is very important. Experience has shown that the use of the minimum possible amount of template which gives reasonable exposure times is best. Ideally, 0.2-0.5 pmol of template should be used for sequencing. 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 microliter, but yields vary greatly depending on numerous factors.

	Yield (ng/µl)				
Length	2	5	10	20	50
100 bp	7 µl*	3 µl	1 µl	1 µl	1 µl
200 bp	*	5 µl	3 µl	1 µl	1 µl
300 bp	*	8 µl*	4 µl	2 µl	1 µl
400 bp	*	*	5 µl	3 µl	1 µl
500 bp	*	*	7 µl*	3 µl	1 µl
1000 bp	*	*	*	7 µl*	3 µl

*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 with Sequenase DNA Polymerase may give better results than increasing the amount of template.

RELATED PRODUCTS

PCR Reagents

Product	Application	Pack size	Product number
Taq DNA Polymerase	For use in PCR	50 units 250 units 1,000 units 5 x 250 units 5,000 units	71160
Taq PCR Kit	For use in PCR	100 rctns	71161
Taq PCR Master Mix (2X)	PCR reaction mix (2X), ready to use	100 rctns (125 units)	71162/3
FideliTaq™ DNA Polymerase	For high fidelity PCR Long and Accurate PCR	50 units 250 units 1,000 units 5 x 250 units 5,000 units	71180
FideliTaq PCR Master Mix (2X)	PCR reaction mix (2X), ready to use	100 rctns	71182/3
HotStart-IT™ Taq DNA Polymerase	For hot start PCR	50 units 250 units 1,000 units 5 x 250 units 5,000 units	71195
HotStart-IT Taq Master Mix (2X)	PCR reaction mix (2x), ready-to-use	25 rctns 100 rctns 500 rctns	71196
HotStart-IT™ FideliTaq™ DNA Polymerase	Hot start for high fidelity PCR	50 units 250 units 1,000 units 5,000 units	71155
HotStart-IT™ FideliTaq™ PCR Master Mix	Hot start PCR reaction mix (2X) for high fidelity PCR	25 rctns 100 rctns 500 rctns	71156
ExoSAP-IT®	Clean-up of PCR products	100 rctns 500 rctns 2,000 rctns 5,000 rctns	78200 78201 78202 78205
PCR Nucleotide Mixes	Functionally tested in long PCR	10mM, 500 µl 25mM, 500 µl	77212 77119

RT-PCR and cDNA Synthesis Kits

Product	Application	Pack size	Product number
RT-PCR Master Mix (2X)	RT-PCR reaction mix	100 rctns	78370
FideliTaq RT-PCR Master Mix (2X)	RT-PCR reaction mix	100 rctns	71185
One-Step RT-PCR Kit	RT-PCR, optimization, analysis of multiple genes	50 rctns (50 µl/rctn)	78350

Product	Application	Pack size	Product number
Two-Step RT-PCR Kit	RT-PCR, optimization, analysis of multiple genes	50 RT/100 PCR rctns (25 µl/RT rctn) (50 µl/PCR rctn)	78355
RT Script Kit	RT synthesis of cDNA for cloning, arrays and RT-PCR	50 rctns (25 µl/rctn)	78360

Sequencing Kits

Product	Application	Pack size	Product number
Sequenase™ Version 2.0 DNA Sequencing Kit	For non-cycle radioactive sequencing	100 rctns	70770
Sequenase PCR Product Sequencing Kit	Complete kit for rapid sequencing of PCR products	100 rctns	70170
Thermo Sequenase™ Cycle Sequencing Kit	For radioactive cycle sequencing	100 rctns	78500
Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit	For cycle radioactive sequencing	50 rctns 50 rctns 500 rctns	188403 (includes ^{33}P ddNTPs) 79750 (without ^{33}P ddNTPs) 79770 (without ^{33}P ddNTPs)

USB Ultrapure Reagents

Product	Application	Pack size	Product number
Agarose – LE	Gel electrophoresis	25 gm 100 gm 250 gm 500 gm	32802
Agarose – Separation ≥ 500 bp, Genetic Performance Certified™	Gel electrophoresis	25 gm 100 gm 250 gm 500 gm	75817
Mineral Oil	PCR	10 ml 25 ml 1 L	71600
RapidGel-XL-6%	Gel electrophoresis	500 ml	75861
RapidGel-XL-40%	Gel electrophoresis	100 ml 500 ml	75863
RapidRun Agarose Buffer, 20X Solution	Gel electrophoresis	1 L 5 L	77523
TBE Buffer, 5X Solution	Gel electrophoresis	1 L 5 L	75891
TBE Buffer, 10X Powder	Gel electrophoresis	6 x 200 ml	70454
TEMED	Gel electrophoresis	100 gm 500 gm	76320

Product	Application	Pack size	Product number
X-Gal	Cloning	100 mg 250 mg 1 gm 2 gm 5 gm	10077
Water, Nuclease-Free		10 x 1 ml 100 ml 500 ml 1 L 5 L	71786
Water, PCR-Qualified		10 x 1 ml	71785

USB CORPORATION

USA
Cleveland, Ohio
(800) 321-9322
www.usbweb.com

USB Europe GmbH
Staufen, Germany
+49(0)76 33 - 933 400
www.usbweb.de

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