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P 70775A
rev 04/14

USB® Sequenase™ Version 2.0 DNA Polymerase

Protocols for DNA Sequencing

Product number 70775Y/Z

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 supplied

Sequenase Version 2.0 DNA Polymerase (PN 70775Y/Z): 20 mM potassium phosphate buffer, pH 7.4, 1 mM DTT, 0.1 mM EDTA and 50% glycerol.

5X Sequenase Version 2.0 Reaction Buffer (PN 70702): 200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl.

Sequenase Version 2.0 Dilution Buffer: 10 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.1 mM EDTA.

Quality control

All kit batches are functionally tested using radiolabeled-dATP and M13mp18 single-stranded DNA template as described in this protocol. Release specifications are based on sequence length, band intensity and sequence quality. The sequence must be visible up to 300 base pairs 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: For use with radioactive material. Please follow the manufacturer's instructions relating to the handling, use, storage, and disposal of such material.

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 and eyes. In the case of contact with skin or eyes, wash immediately with water.

Introduction

Sequenase Version 2.0 DNA Polymerase is derived from bacteriophage T7 DNA polymerase, genetically modified to improve its properties for sequencing[1,2]. These properties include high processivity, no 3'→5' exonuclease activity, high speed and the ability to incorporate the popular nucleotide analogs used for sequencing (ddNTPs, α-thio dNTPs, dITP, 7-deaza-dGTP, etc.). This booklet briefly describes reaction conditions which have proved to be successful (the same protocols are recommended for the Sequenase DNA Sequencing Kits).

Materials not supplied

Necessary reagents:

α-Labeled dATP*:

[α-^{33}P]dATP
[α-^{35}S]dATP
[α-^{32}P]dATP

*The specific activity: 1,000-1,500 Ci/mmol.

DTT Solution (PN 70726): 0.1 M

Labeling Mix (dGTP) (PN 70710) 5X concentrate, for use with radiolabeled dATP: 7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP

Termination Nucleotide Mixes (one for each dideoxynucleotide): Each mix contains 80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP and 50 mM NaCl. In addition, the 'A' mix (PN 70714) contains 8 µM ddATP; the 'C' mix (PN 70716), 8 µM ddCTP; the 'G' mix (PN 70718), 8 µM ddGTP; and the 'T' mix (PN 70720), 8 µM ddTTP.

Stop Solution (PN 70724): 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF

Mn Buffer (PN 72600) (optional): 100 mM $MnCl_2$, 150 mM sodium isocitrate

Pyrophosphatase, Inorganic, Recombinant (PN 78450) (optional): 4 units/ml in 10 mM Tris•HCl, 0.1 mM EDTA, 50% glycerol. Add to stock polymerase (0.33 µl per 1 µl of Sequenase Version 2.0 DNA Polymerase) or use in enzyme dilution (below) to eliminate faint band problems, especially with dITP.

Glycerol Enzyme Dilution Buffer (optional): 20 mM Tris•HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA, 50% glycerol.

Water—Only deionized, distilled water should be used for sequencing reactions.

Tris-EDTA (TE) Buffer (PN 75834)—10 mM Tris•HCl, pH 7.5, 1 mM EDTA. It is used for template preparation.

Gel reagents—Sequencing gels should be made from fresh solutions of acrylamide and bis-acrylamide. Other reagents should be ultrapure or electrophoresis grade materials. For convenience, RapidGel™ gel mixes are strongly recommended. RapidGel-XL formulations yield up to 40% more readable sequence per gel. See 'Related Products' section for range of USB Ultrapure gel products.

Specialized sequencing primers—Some sequencing projects will require the use of primers which are specific to the project. For most sequencing applications, 0.5-2.0 pmol of primer should be used for each set of sequencing reactions. Always determine the concentration of the primer by reading the optical density at 260 nm (OD_{260}). If the primer has N bases, the concentration (pmol/µl) is given by the following formula: Concentration (pmol/µl)=OD_{260}/(0.01 x N).

Necessary equipment:

Constant temperature bath—Sequencing will require incubations at room temperature, 4°C, 37°C and 100°C (boiling H_2O bath or thermal cycler). Denaturing the samples before the gel is loaded will require heating at 75°C.

Electrophoresis equipment—While standard, non-gradient sequencing gel apparatus is sufficient for much sequencing work, the use of field-gradient ('wedge') or salt-gradient gels will allow much greater reading capacity on the gel[3]. A power supply offering constant voltage operation at 2000V or greater is essential.

Gel handling—If ^{35}S or ^{33}P sequencing is desired, a large tray for washing the gel (to remove urea) and a gel drying apparatus are necessary. Gels containing ^{35}S or ^{33}P must be exposed dry in direct contact with the film at room temperature. If RapidGel-XL is used the gel does not need to be soaked. In fact, soaking RapidGel-XL gels will cause swelling thereby affecting band resolution in the final result.

Autoradiography—Any large format autoradiography film can be used. Develop according to the manufacturer's instructions.

Sequencing reaction protocols

Annealing template and primer

1. For each template (either single-stranded DNA or denatured double-stranded DNA), a single annealing (and subsequent labeling) reaction is used.

   Combine:

Primer	0.5 pmol	
DNA	0.5-1.0 pmol	(For *e.g.*, 1 µg of M13)
Sequenase Reaction Buffer	2 µl	
H_2O	__ µl	(Adjust total volume to 10 µl)
Total	10 µl	

2. Warm the capped tube to 65°C for 2 minutes, then allow the mixture to cool slowly to room temperature over a period of about 30 minutes. Chill on ice.
3. Dilute Sequenase enzyme 1:8 in dilution buffer with the optional addition of Pyrophosphatase (0.04 units per 1 µl of undiluted polymerase).

Labeling reaction

To the annealed template-primer add the following:

Annealing reaction	(10 µl)	
DTT (0.1 M)	1 µl	
Labeling mix (diluted 1:5)	2 µl	
[α-^{35}S], [α-^{33}P] or [α-^{32}P]dATP	0.5 µl	(5 µCi)
Sequenase DNA Polymerase	3.25 units	(2 µl of 1:8 dilution)
Total	15.5 µl	

Mix thoroughly and incubate for 2-5 minutes at room temperature or cooler (4-20°C).

Termination reactions

1. Label 4 tubes 'G', 'A', 'T', 'C'. Fill each with 2.5 µl of the appropriate dideoxy termination mix. Pre-warm these tubes to 37°C (it may be helpful to use temperatures up to 45°C; avoid temperatures below 37°C).
2. When the labeling reaction is complete, transfer 3.5 µl of it to the tube (pre-warmed to 37°C) labeled 'G'. Similarly transfer 3.5 µl of the labeling reaction to each of the other three tubes ('A', 'T', 'C').
3. After 5 minutes incubation at 37°C, add 4 µl of Stop Solution to each termination reaction, mix and store on ice.
4. To load the gel, heat the samples to 75-80°C for 2 minutes and load 2-4 µl in each lane.

Supplementary protocols

Protocols for sequencing double-stranded plasmids

Two simple and efficient denaturation protocols of plasmid DNA allow for the rapid denaturation of plasmid templates for sequencing. For more details see instructions provided with the Sequenase Quick-Denature Plasmid Sequencing Kit (PN 70140).

Glycol Plasmid Denaturation Method[4,5]

Plasmid denaturing reagent	5 µl	
Primer (1-5 pmol)	1 µl	
dsDNA (3-4 kb plasmid) (0.5-3 µg)	up to 7 µl	
H_2O	__ µl	(to adjust total volume to 15 µl)

Incubate at 95°-100°C for 5 minutes (thermal cycler or boiling water bath). Chill on packed ice for 5 minutes. Briefly spin. Add:

Plasmid reaction buffer	2 µl
Total	15 µl

Mix well. Incubate at 37°C for 10-15 minutes to anneal. Proceed to labeling reaction as described above. Using this protocol, the volume of the labeling reaction will be larger than outlined above. Therefore, transfer 4.5 µl of labeling reaction to each termination tube ('G', 'A', 'T', 'C'), and continue with the protocol. See Note[+] below (page 8) for gel conditions.

Fast Alkaline Denaturation Method[6]

dsDNA (3-4 kb plasmid) (0.5-3 µg)	8 µl
Primer (1-5 pmol)	1 µl
NaOH (1 N)	2 µl

Incubate at 37°C for 10 minutes. Add:

HCl (1 N)	2 µl
Plasmid reaction buffer	2 µl
Total	15 µl

Incubate at 37°C for 10 minutes to anneal. Proceed to labeling reaction as outlined above. Using this protocol, the volume of the labeling reaction will be larger than outlined above. Therefore, transfer 4.5 µl of labeling reaction to each termination tube ('G', 'A', 'T', 'C'), and continue with the protocol. **Note**: The concentrations and volumes of NaOH and HCl used must be accurate to avoid pH extremes and failed sequences.

Sequencing with glycerol

Using Glycerol Enzyme Dilution Buffer permits the entire stock of Sequenase Version 2.0 DNA Polymerase to be diluted (1:8) and stored. This eliminates the enzyme dilution step prior to every sequencing reaction. When enzyme is used in this manner, the sequencing reactions will contain about 5% glycerol. This has been observed to improve sequences by permitting wider tolerances in reaction times and temperatures, and can reduce the incidence of sequencing artifacts such as 'stops'. The temperature of the labeling reaction can be increased (as high as 37°C), increasing the stringency of primer annealing. Termination reactions can be run for longer periods of time (10-30 minutes) and at warmer temperatures (37°-50°C), making sequencing more convenient. Polymerization will be more rapid at the elevated temperatures, and some template secondary structures may be eliminated at temperatures above 45°C.

†**Note**: Glycerol may severely distort ordinary sequencing gels. Use Glycerol Tolerant Gel Buffer in both the gel and the running buffer chambers to eliminate this problem. 20X Glycerol Tolerant Gel Buffer is available, PN 75827.

Sequencing close to the primer

The addition of 1 µl of Mn Buffer (0.1 M $MnCl_2$, 0.15 M sodium isocitrate) to the labeling reaction will alter the relative reactivities of the deoxy- and dideoxynucleotides[7]. This results in emphasizing sequences very close to the primer, but sacrifices sequences distant (>250) from the primer. Mn Buffer (PN 72600) is available from Affymetrix.

Elimination of compressions

7-deaza-2'-deoxyguanosine triphosphate can be substituted for dGTP in the labeling and termination mixes at exactly the same concentration as dGTP. Deoxyinosine-5'-triphosphate can be substituted for dGTP at a concentration twice that usually used for dGTP. In addition, the concentration of ddGTP in the termination mix should be 1.6 µM (one-fifth of the usual concentration). When these mixes are substituted for those containing dGTP, secondary structures on the gels are greatly reduced. It is always a good practice to run both dGTP and dITP reactions on difficult templates. While 7-deaza-dGTP eliminates many compressions, dITP may be required for the most difficult cases.

References

1. Tabor, S. and Richardson, C. C. (1989) *J. Biol. Chem.* **264**, 6447-6458.
2. Tabor, S. and Richardson, C. C. (1987) *Proc. Nat. Acad. Sci. USA* **84**, 4767-4771.
3. Ansorge, W. and Labeit, S. (1984) *J. Biochem. Biophys. Methods* **10**, 237-243.
4. Pisa-Williamson, D. and Fuller, C. W. (1992) *Comments* **19**, No. 2, 1-7, United States Biochemical Corp., Cleveland OH.
5. *Sequenase Quick-Denature Plasmid Sequencing Kit Protocol*, (1996) United States Biochemical Corp., Cleveland, OH.
6. Hsiao, K. (1991) *Nucleic Acids Research* **19**, 2787.
7. Tabor, S. and Richardson, C. C. (1998) *Proc. Nat. Acad. Sci. USA* **86**, 4076-4080.

Related products

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	For rapid sequencing of PCR products	100 rctns	70170
Sequenase Quick-Denature Plasmid DNA Sequencing Kit	For rapid denaturation and sequencing of plasmid DNA	100 rctns	70140
Thermo Sequenase™ Cycle Sequencing Kit	For radioactive cycle sequencing	100 rctns	78500

Sequencing reagents

Product	Application	Pack size	Product number
Acrylamide, Ultrapure	Gel electrophoresis	100 gm 500 gm 1 kg	75820
Ammonium Persulfate	Gel electrophoresis	10 gm 100 gm 1 kg	76322
Glycerol Tolerant Gel Buffer, 20X Solution	Gel electrophoresis	1 L	75827
N,N'-Methylene-Bis Acrylamide, Ultrapure	Gel electrophoresis	25 gm 50 gm 100 gm 1 kg	75821
RapidGel-40% Liquid Acrylamide Stock Solution	Gel electrophoresis	500 ml	75848
RapidGel-XL Liquid Acrylamide-6% TBE for long reads	Gel electrophoresis	500 ml	75861

Sequencing reagents

Product	Application	Pack size	Product number
TEMED, Ultrapure	Gel electrophoresis	100 gm 500 gm	76320
Tris, Ultrapure	Gel electrophoresis	500 gm 1 kg 5 kg 10 kg	75825
Urea, Ultrapure	Gel electrophoresis	1 kg 5 kg	75826

A full range of sequencing kits and reagents can be found in the USB Products Catalog and at usb.affymetrix.com.

PCR enzymes and related products

Product	Application	Pack size	Product number
Taq DNA Polymerase	For use in PCR	50 units 250 units 1,000 units 5,000 units	71160
Taq PCR Master Mix (2X)	PCR reaction mix (2X), ready to use	100 rctns (125 units)	71162
RubyTaq™ DNA Polymerase	For use in PCR, with tracking dyes	50 units 250 units 1,000 units 5,000 units	71190
RubyTaq PCR Master Mix (2X)	PCR reaction mix (2X), ready to use	100 rctns 500 rctns	71191
FideliTaq™ DNA Polymerase	For high fidelity 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
HotStart-IT® Taq DNA Polymerase	For high specificity & high sensitivity 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	For high specificity and high sensitivity PCR	50 units 250 units 1,000 units 5,000 units	71155
HotStart-IT FideliTaq PCR Master Mix (2X)	PCR reaction mix (2X), ready to use	25 units 100 units 500 units	71156

PCR enzymes and related products

Product	Application	Pack size	Product number
ExoSAP-IT® PCR Product Cleanup	Cleanup of PCR products	20 rctns 100 rctns 500 rctns 2,000 rctns 5,000 rctns	78250 78200 78201 78202 78205
HT ExoSAP-IT High-Throughput PCR Product Cleanup	High-throughput cleanup of PCR products in 8-tube strip format	480 rctns x 8-tube strip 5,760 rctns x 1 plate (12 x 8-tube strips) 23,040 rctns - 4 plates	78395
PCR Nucleotide Mixes	Functionally tested in long PCR	10 mM, 500 µl 2 x 500 µl 25 mM, 500 µl	77212 77119
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

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