Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit

Product Number 79260
50 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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COMPONENTS OF THE KIT

This kit contains sufficient reagents to sequence 50 templates.

Kit Reagents:

Reaction Buffer (concentrate): 150mM Tris-HCl pH9.5, 35mM $MgCl_2$

Control DNA: pUC19, 0.03µg/µl

Control Primer: TAMRA -40 Universal Forward Primer (23mer), 1pmol/µl (1µM)

ddA Term Mix: 300µM each of dATP, dCTP, dTTP & 7-deaza-dGTP*, 3µM ddATP

ddC Term Mix: 300µM each of dATP, dCTP, dTTP & 7-deaza-dGTP*, 3µM ddCTP

ddG Term Mix: 300µM each of dATP, dCTP, dTTP & 7-deaza-dGTP*, 3µM ddGTP

ddC Term Mix: 300µM each of dATP, dCTP, dTTP & 7-deaza-dGTP*, 3µM ddTTP

Thermo Sequenase DNA Polymerase: 20U/µl with 0.03U/µl *Thermoplasma acidophilum* inorganic pyrophosphatase in 50mM Tris-HCl pH 8.0, 1mM EDTA, 1mM DTT, 0.5% Tween-20, 0.5% Nonidet™ P-40, 50% glycerol.

Formamide Loading Dye: contains 95% formamide and tracking dyes.

This kit and all enclosed reagents should be stored at -20°C (not in a frost-free freezer). When removed from freezer for use keep enzyme on ice and allow remaining reagents to thaw at room temperature (do not heat). Keep thawed reagents on ice. It is **important** to gently vortex each reagent and briefly centrifuge to collect at the bottom of the tube before use.

Note: This kit contains an enzyme mixture with high glycerol concentration. The use of Glycerol Tolerant Gels (GTG) and buffer (PN 71949 or PN 75827) to eliminate glycerol-induced distortion of sequencing bands is strongly recommended. The use of Tris-Borate-EDTA (TBE) buffer can result in severe distortion of the sequence at approximately 300-600 bases beyond the primer (1).

*To minimize gel compression problems, this kit provides 7-deaza-dGTP nucleotide mixes in place of dGTP. The substitution of analog (7-deaza-dGTP or dITP) for dGTP has been successful in eliminating many gel artifacts by forming weaker secondary structure which are more easily resolved in denaturing gels (2, 3).

MATERIALS NOT SUPPLIED

Necessary reagents and equipment:

Water—High quality deionized, distilled water must be used for the sequencing reactions.

Sequencing primer—This kit does not contain primers except for that used with the supplied control DNA. Commonly used primers are available from USB. Some sequencing projects will require primers, which are specific to that project. For most sequencing applications, 1-2pmol of primer per reaction should be used for each set of sequencing reactions. Always determine the concentration of the primer by reading the absorbance at 260nm (A_{260}) and preferably, run a wavelength scan from 220 to 650nm to ensure that the primer is fluorescently labeled. If the primer has N bases, the approximate concentration (pmol/µl) is given by the following formula:

$$\text{Concentration (pmol/µl)} = A_{260}/(0.01 \times N).$$

Fluorescently labeled primers available from USB

All primers are 1µM; 25 pmol and 100 pmol sizes.

Product Number	Description	Sequence
79263	TAMRA -40 Universal Forward Primer	5'- GTT TTC CCA GTC ACG ACG TTG TA
79270	TAMRA -28 Reverse Primer	5'- AGG AAA CAG CTA TGA CCA T
79272	TAMRA T3 Promoter Primer	5'- TAA TAC GAC TCA CTA TAG GG
79271	TAMRA T7 Promoter Primer	5'- ATT AAC CCT CAC TAA AGG GA
79273	TAMRA SP6 Promoter Primer	5'-ATT TAG GTG ACA CTA TAG

Liquid handling supplies such as vials, pipetters (accurate to 1µl)—all sequencing reactions are run in plastic microcentrifuge tubes (0.2 or 0.5ml) suitable for thermal cycling.

Microcentrifuge

Thermal cycler

Sequencing gel box—The choice of gel apparatus depends on the kind of imager to be used. Scanning areas vary from imager to imager, be sure to follow manufacture’s specification. Standard glass sequencing plates are used routinely at USB. Quartz plates have not been used.

Power supply—a sequencing grade constant current model.

Fluorescent scanner—This kit has been developed for use with a fluorescence scanning imager equipped with a YAG (532mm) laser. When deciding on the kind of scanner to use, please refer to the specific instrument manual to determine its limit of detection. Performance of scanners varies from manufacturer to manufacturer.

Note: Figures in this protocol book were obtained using standard glass sequencing plates (33x41cm) and a Hitachi® Genetic Systems FMBIO® II fluorescence imaging system.

QUALITY CONTROL

All lots of reagents are functionally tested using TAMRA-40 forward primer and pUC19 double-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. 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: This product may be used with radioactive material (p. 21). Please follow the manufacturer's instructions relating to the handling, use, storage, and disposal of such materials.

Warning: Loading Dye contains formamide. See 'Material Safety Data Sheet' on page 24.

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 a 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 (see 'Material Safety Data Sheet' for specific advice).

INTRODUCTION

Traditional manual DNA sequencing by the dideoxy chain termination method of Sanger et. al. (4) is associated with the use of either radioactively labeled nucleotides (deoxy or dideoxynucleotide triphosphates) or primers. The reaction products are then resolved by gel electrophoresis followed by autoradiography. However, with the availability of high-resolution fluorescence imaging systems and a wide variety of fluorescent dyes, fluorescently tagged sequencing products can be detected following electrophoresis by simply scanning the gel.

Sequencing using fluorescently labeled primers provide two main advantages over traditional radiolabeled sequencing. First, high-resolution fluorescence imaging instruments permit more consistent direct sequencing from single bacterial colonies or single M13 or lambda phage plaques. The ability to quickly screen these templates without any purification or pre-amplification allows individual colonies or plaques to be sequenced and analyzed during a normal working day. Second, the gel can be scanned in a fluorescent imager multiple times during electrophoresis, allowing longer reads.

This kit contains all the reagents necessary for sequencing with a fluorescently labeled primer. It provides protocols for routine sequencing from purified DNA templates (single-stranded or double-stranded) or PCR products and protocols to sequence directly from single bacterial colonies or phage plaques without DNA purification. The methods for sequencing directly from single bacterial colonies or phage plaques are direct and no alcohol precipitation of the reaction products is required. Simply load samples on to a gel and analyze. Some scanners, such as the Hitachi FMBIO II and Molecular Dynamics' Typhoon, permit scanning the gel without opening the glass plates. Thus, gel electrophoresis can be stopped at any point for scanning and further electrophoresis may be resumed if longer read length is needed.

The choice of fluorescent dye depends on the scanner used. TAMRA, ROX (Rhodamine dye), FAM and HEX (fluorescein dye) labeled primers have been tested with fluorescent scanners having a YAG (532nm) laser and have yielded excellent results in our hands. Keep in mind, however, that optimal reaction and scanning conditions are very important when dealing with low abundance DNA templates such as those obtained directly from bacterial colonies and phage plaques.

The components in this kit can also be used with radioactively labeled ^{33}P and ^{32}P primers. However, this kit does not provide reagents (T4 Polynucleotide Kinase, PN 70033) for 5' end labeling of the primers. The protocol for sequencing with radioactive labeled-primer is essentially the same as the protocol for sequencing with fluorescently labeled-primers, except additional template DNA is required. The protocols differ in that after gel electrophoresis, the gel is dried and exposed to X-ray Film.

PROTOCOL FOR CYCLE SEQUENCING

Sequencing reactions are run in small plastic microcentrifuge tubes (typically 0.5ml) suitable for thermal cycling. The solution must be thoroughly mixed after addition of each component. At any stage where the possibility exists for some solution to cling to the walls of the tube, it should be collected by brief centrifugation.

1. Have on ice 4 tubes labeled ‘G’, ‘A’, ‘T’ and ‘C’.
2. Prepare a master reaction mix as follows:

Template DNA	up to 13.8µl	
Reaction Buffer (conc.)	2.2µl	
Labeled primer	1.0µl	(0.5 to 2pmol)
Thermo Sequenase (20U/µl)	1.0µl	(add last)
H_2O	__µl*	
Total volume	18µl	

*The volume of H_2O and template DNA must sum to 13.8µl.

Aliquot 4µl of the master reaction mix to each of the tubes labeled ‘G’, ‘A’, ‘T’ and ‘C’.

Note: If using the control primer and DNA template provided in the kit, use 1µl of the TAMRA-40 forward primer (1pmol) and 5 to 10µl (75-150fmol) of the pUC19 DNA template per sequencing reaction.

3. Add 1µl of the ‘ddGTP’ termination mix in the tube labeled ‘G’, mix thoroughly, briefly centrifuge if some solution clings to the walls. Similarly fill the ‘A’, ‘T’ and ‘C’ tubes with 1µl of the ‘ddATP’, ‘ddTTP’ and ‘ddCTP’ termination mixes respectively. Cap the tubes and briefly centrifuge if some solution clings to the walls. Overlay with mineral oil is not necessary if the thermal cycler is fitted with a heated lid. Place the samples in the thermal cycler.
4. Start the cycling program. Note: The specific cycling parameters used depend on the primer sequence and the amount and purity of the template DNA. For the control primer (TAMRA-GTTTTCCCCAGTCACGACGTTGTA) and DNA template (pUC19) provided in the kit, the following cycling parameters are recommended:

95°C	30 sec
50-55°C	30 sec
72°C	60 sec

30-60 cycles (number of cycles depend on template source, see supplementary information section).

5. After cycling is complete, centrifuge briefly to collect any condensate and place on ice. Add 2µl of the formamide loading dye to each of the termination reactions (see supplementary information on the loading dye), mix thoroughly and centrifuge briefly to collect the sample at the bottom of the tube**. Note:** If reactions are not to be run immediately, either store at -20°C w/o loading dye or store reactions w/loading dye at -80°C.
6. Heat samples to 75°C for 2 minutes to denature, then place on ice. Do not heat samples above 80°C in the presence of formamide loading dye. DNA sequencing reactions with 7-deaza dGTP may give inferior results when over heated.
7. Load 3-5µl of the reaction onto each lane of the sequencing gel. We recommend loading 5µl when dealing with single bacterial colonies or single phage plaques.
8. Gel electrophoresis can be stopped at any point for scanning. Scanners, such as the Hitach FMBIO II and Molecular Dynamics' Typhoon, allow the gel to be scanned without opening the glass plates. Thus, electrophoresis can be resumed if a longer read length is needed.

Note: The termination mixes provided in the kit will typically yield sequences 350-400 bases from the primer. This is as much sequence as most users will be able to read using the current standard slab gel electrophoresis technology.

SUPPLEMENTARY INFORMATION

Formamide Loading Dye

The 'Formamide Loading Dye' contains two dyes that migrate toward the anode. In an 8% gel, the faster migrating dye (at free primer area) fluoresces at a wavelength greater than 649nm. The slower migrating dye (at ~100 bases area) fluoresces at a wavelength greater than 600nm. If using dyes with an emission wavelength around the second dye region, please be aware that the fluorescence from the tracking dye will obscure part of the DNA sequence. For a 532mm laser, we find TAMRA and HEX with excitation and emission of 559/578 (nm) and 529/560 (nm) work very well.

Thermo Sequenase DNA polymerase

This kit contains Thermo Sequenase DNA polymerase, an enzyme engineered for DNA sequencing (5). This enzyme is thermostable and exonuclease-free. Like T7 Sequenase DNA polymerase, it readily incorporates dideoxynucleotide triphosphates, resulting in much more uniform band intensities. It is stable at 90°C for at least 1 hour and retains 50% of its activity when incubated at 95°C

for 1 hour. The enzyme is formulated as a mixture with thermostable inorganic pyrophosphatase (TAP) cloned from the *Thermoplasma acidophilum.* TAP hydrolyzes the inorganic pyrophosphate product of nucleotide polymerization preventing pyrophosphorolysis from occurring. Pyrophosphorolysis can result in faint bands, which can affect the accuracy of base calling.

Cycling temperatures

The temperatures used for cycling should be determined from the characteristics of the sequencing primer and the quality of the template DNA. A routine three temperature cycling is recommended, especially with unpurified DNA templates such as that derived directly from bacterial colonies and phage plaques. A denaturing temperature of 95°C for 30 seconds, an annealing temperature of 50-55°C for 15-30 seconds followed by an extension at 70-72°C for 60-120 seconds is recommended as a starting point for further optimization. The optimum temperature for polymerization for Thermo Sequenase is 70-75°C. It is best to determine the melting temperature of the primer used with one of the many available software packages or do an approximate calculation of two degrees for A-T and four degrees for each G-C base-pair. The annealing temperature should be at least 5 degrees less than the melting temperature of the primer.

Number of cycles and quantity of templates

The number of cycles will depend primarily upon the amount of template DNA (in fmol) used for sequencing. It will also depend on the purity of the DNA. The minimum quantities of highly purified DNA which we have been able to sequence using TAMRA, HEX, FAM or ROX labeled primers are approximately 10 fmol (20ng) of M13mp18 DNA and approximately 35 fmol (60ng) of pUC19 DNA (Figure 1,2). We have had very good success with sequencing directly from single bacterial colonies when high to medium copy-number vectors are used and from both single M13 and lambda phage plaque. When sequencing very small amounts of template, increasing the number of cycles from 30 to 60 or 70 (if necessary) will increase the signal significantly especially when sequencing directly from single plaques. Increasing the number of cycles when more than 200 fmol of DNA is present is of little benefit, and may even produce background sequence. When sequencing from single bacterial colonies or plaques, we recommend starting with 50 cycles and then optimizing the number of cycles as needed.

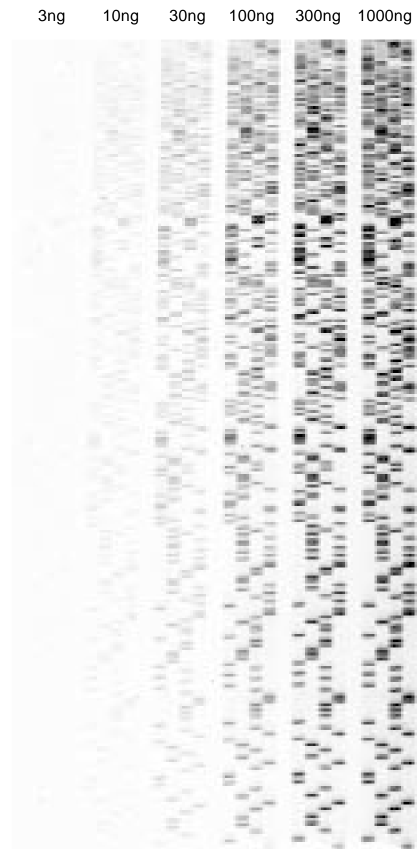

Figure 1. M13mp18 DNA template titration. Cycle sequencing with varying amount of single-stranded M13mp18 template DNA. Sequencing reactions were performed with TAMRA labeled -40 universal forward primer and cycled 40 cycles. The amount of template DNA used is indicated at the top of each sequence.

Figure 2. pUC19 DNA template titration. Cycle sequencing with varying amount of double-stranded pUC19 template DNA. Sequencing reactions were performed with TAMRA labeled -40 universal forward primer and cycled 40 cycles. The amount of template DNA used is indicated at the top of each sequence.

TEMPLATE PREPARATION FROM SINGLE BACTERIAL COLONIES

Our experience with sequencing directly from single bacterial colonies has been very good (Figure 3). This protocol provides a guide which, when followed carefully, results in good sequences. Sequencing will require the use of high to medium copy-number vectors (e.g. pBS, pUC, pGEM, pRE). To have strong band intensities, we highly recommend colonies that are ~2mm in diameter. Preparing plates with single bacterial colonies is beyond the scope of this protocol book. 'Molecular Cloning—A Laboratory Manual' by Sambrook, Fritch and Maniatis is a good reference.

The techniques of picking single colonies is very important, care should be taken to recover the whole colony.

1. With a sterile pipette tip (e.g. 20-200µl) transfer a single bacterial colony (~2mm) from an overnight culture to a sterile 0.5ml microcentrifuge tube containing 14µl of distilled H_2O. Care should be taken not to include agar.
2. Pipet up and down several times to resuspend the bacteria. Make sure all the colony mass is resuspended. Centrifuge briefly to collect the sample at the bottom of tube, but do not pellet. Remove 1µl, spot on a plate and incubate to serve as a master plate for future analysis.
3. Heat the cell suspension at 100°C (best done in thermal cycler) for 5 minutes, followed by cooling in ice.
4. Centrifuge at ~6000rpm (4000 x g) for 2-3 minutes.
5. Remove 12µl or all of the supernatant for sequencing, avoid pipeting the pellet. If some pelleted material is included, repeat step 4. If the sequencing reaction can not be done on the same day, store supernatant at -20°C and run the sequencing reaction the following day.

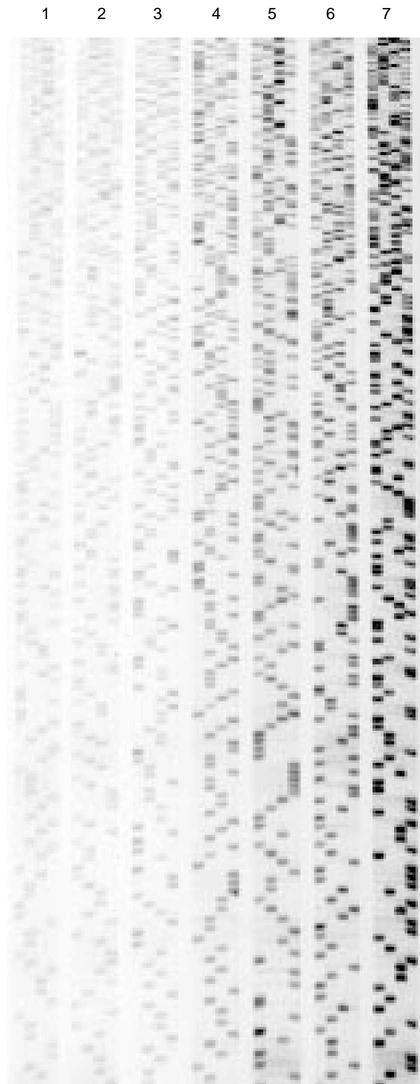

Figure 3. Direct single bacterial colony sequencing. Sequencing reactions were performed on seven different bacterial colonies (1-7), each harboring a different cloning vector. The band intensities reflect copy-number of vectors. A fresh colony ~2mm was suspended in 14µl of ddH_2O, heated at 100°C for 5 minutes followed by centrifugation. The supernatant was removed for sequencing with TAMRA labeled primers and cycled 50 cycles.

TEMPLATE PREPARATION FROM PLAQUES

Using the protocol outlined below, very good results can be obtained by sequencing directly from M13 or lambda plaques (Figure 4, 5). Protocols for plating bacteriophage M13 or lambda can be found in any standard molecular biology laboratory manual.

A bacteriophage M13 plaque contains between 10^6 and 10^8 pfu. To have strong band intensities, we highly recommend the use of large well separated plaques, ~2mm in size. The plaque size can be increased by reducing the amount of the bacterial inoculum used when setting the bacterial lawn.

An average lambda plaque yields 10^6-10^7 infectious bacteriophage particles. Plaques vary in size with different strains of bacteriophage lambda. Since vigorous bacterial growth favors small plaques, to obtain larger plaque sizes (~2mm), it is necessary to reduce the bacterial inoculum. Bacteriophage lambda plaques do not increase in size after the bacterial lawn is fully grown and the cells have reached stationary phase. We have had good success with plaques of lambda FIXII library without the excision step (figure 5).

Important—Because of phage diffusion, only freshly prepared plaques will have a sufficient amount of template. Do not store plaques overnight before use.

1. Cut off pipette tips (200µl) with a fresh razor blade so that the bore diameter is about 2mm. Autoclave and keep sterile for picking plaques.
2. Select an isolated plaque (~2mm). With a cut off sterile pipette tip, apply mild suction, punch through the top agar and gently lift the plaque together with the top agar. Be careful not to transfer too much of the bottom agar. Transfer the plaque to 14µl TE buffer in a sterile 0.5ml microcentrifuge tube. (Note: it has been shown that many microbiological agars contain an inhibitor of Taq DNA polymerase. Thus minimize the amount of agar transferred).
3. Carefully pipet up and down several times to release the agar, briefly spin if agar sticks to side of the tube. Care should be taken to remove all the agar from the pipette tip.
4. Incubate at room temperature for 30 minutes (M13) or 60-120 minutes (lambda) to allow the bacteriophage particles to diffuse out of the agar. At the end of the incubation, remove 1µl and store at 4°C for future analysis.
5. Heat the remaining suspension at 100°C (best done in thermal cycler) for 5 minutes, followed by cooling in ice.
6. Centrifuge at ~6,000 rpm (4000xg) for 5 minutes to pellet the agar.
7. Remove 12µl or all of the supernatant for sequencing, avoid pipeting the agar. If some pelleted agar is included, repeat step 6. If the sequencing reaction can not be done on the same day, store supernatant at -20°C and run the sequencing reaction the following day. See supplementary section for recommended cycling temperature and number of cycles.

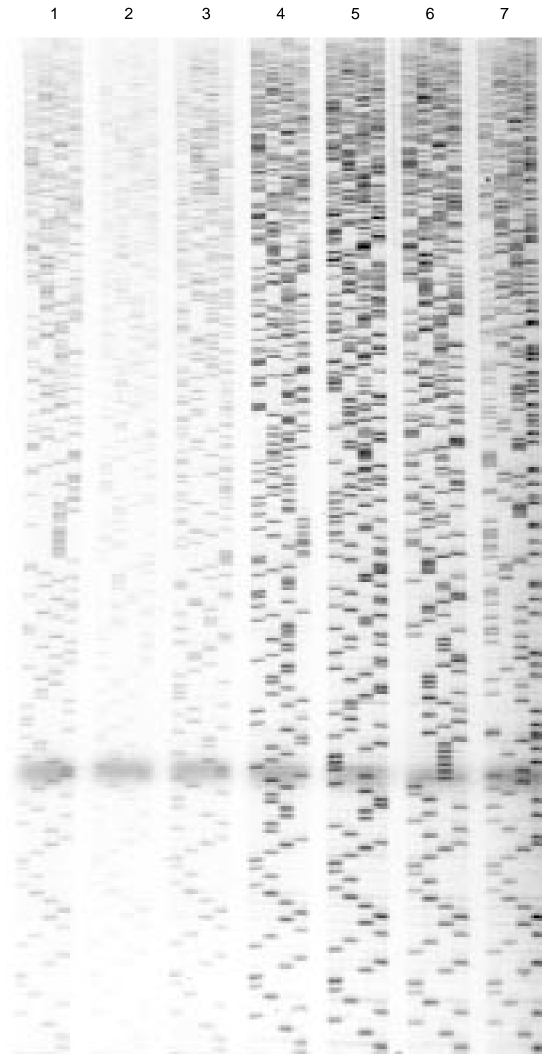

Figure 4. Direct sequencing from single M13 plaques (Clones derived from *Sau*3A I fragments of bacteriophage lambda DNA inserted at the BamHI site of M13mp18). Fresh ~2mm plaques (1-7) were lifted with a sterile cut-off pipette tip, transferred to 14µl TE buffer and incubated at room temperature for 30 minutes to 1 hour to elute the phage from the top agar. 1µl was removed for future analysis and the remaining suspension heated at 100°C for 5 minutes followed by centrifugation. The supernatant was removed for sequencing with TAMRA labeled -40 universal forward primer and cycled 60 cycles.

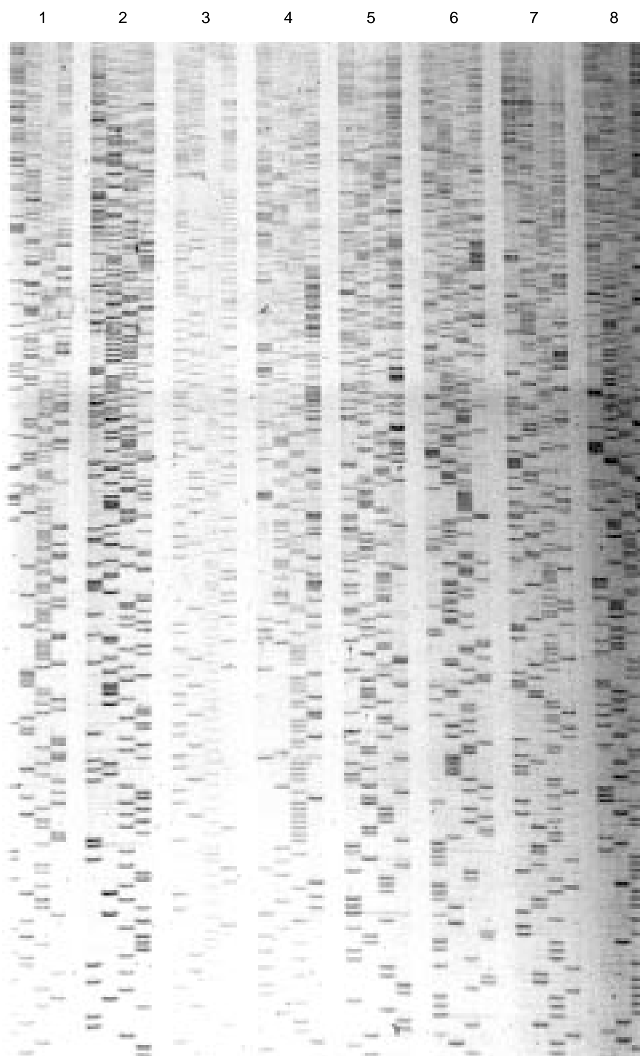

Figure 5. Direct sequencing from single lambda plaques (lambda FIXII genomic library). No preliminary screening or phage minipreps were performed. Fresh ~2mm plaques (1-8) was lifted with a sterile cut-off pipette tip, transferred to 14µl TE buffer, incubated at room temperature for 1 to 2 hours to allow the phage particles to diffuse out of the top agar. 1µl was removed for future analysis and the remaining suspension heated at 100°C for 5 minutes followed by centrifugation. The supernatant was removed for sequencing with TAMRA labeled T3 promoter primer and cycled 50 cycles.

SEQUENCING PCR PRODUCTS

In addition to sequencing directly from bacterial colonies or phage plaques, this kit may be used for sequencing PCR products. All PCR products need to be purified before sequencing. Methods of removing residual primers and dNTPs ranges from alcohol precipitation to column purification or a simple enzymatic method provided by USB Corporation.

It is essential that PCR products are of high quality and quantity in order 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 amplification will yield a product which can be sequenced. Analysis of the PCR product on agarose gels and optimization of the PCR may be necessary to obtain quality sequences. If the PCR product is a single band on agarose gel, the use of ExoSAP-IT (PN 78200) can efficiently remove the excess dNTPs and primers. If the PCR reaction contains multiple bands, either a second round of amplification with nested primers or gel purification is needed. The following is a simple protocol for enzymatic treatment of the PCR product.

Enzymatic pre-treatment of PCR products

1. | | |
   |---|---|
   | PCR amplification mixture | 5µl |
   | ExoSAP-IT (PN 78200) | 2µl |
   | Total | 7µl |

   Mix and incubate at 37°C for 15 minutes. (It is convenient to do this in the thermal cycler.) Note: When treating more than 10µl of PCR product, increase the amount of ExoSAP-IT proportionally.
2. Inactivate ExoSAP-IT by heating to 80°C for 15 minutes (it is also convenient to do this in the thermal cycler).
3. The DNA is now ready for direct sequencing.

Determining how much template DNA to use

The amount of template per sequencing reaction is very important; excess template leads to sequencing artifacts. Ideally, 10-100fmol of template should be used per sequencing reaction. Thus the *volume* of PCR product per sequencing reaction will depend on the PCR product concentration. The guidelines given in Table 1 give an approximate volume of PCR product to use for sequencing:

Table 1. Volume of PCR Product to Use Per Sequencing Reaction

	PCR Product Concentration Estimated From Agarose Gel				
Length	2 ng/µl	5 ng/µl	10 ng/µl	20 ng/µl	50 ng/µl
100bp	1µl	0.3µl	0.1µl	0.1µl	0.1µl
200bp	1µl	0.5µl	0.3µl	0.1µl	0.1µl
300bp	1µl	1µl	0.4µl	0.2µl	0.1µl
400bp	1µl	1µl	0.5µl	0.3µl	0.1µl
500bp	2µl	1µl	1µl	0.3µl	0.1µl
1000bp	5µl	2µl	1µl	1µl	0.3µl

Molar concentration of DNA template can be calculated by the following formula:

Concentration (fmol/µl) = [(ng/µl) / (N bases x 660)] x 10^6

ng/µl = PCR product concentration estimated from agarose gel
N bases = PCR size
660 = mol. wt. of nucleotides (double stranded)
N bases x 660 = mol. wt. of PCR product

PCR protocols

Detailed protocols for the PCR steps are beyond the scope of this booklet, but the following reaction mixture is typical.

Final concentrations for PCR

10mM Tris-HCl, pH 8.3
1.5mM $MgCl_2$
50mM KCl
200µM each dATP, dGTP, dCTP, dTTP
1µM (100 pmol/100µl) each primer
2.5U Taq DNA polymerase (for a 100µl reaction)

Amplification is carried out in a volume of 25-100µl. Start with an initial denaturation step at 95°C for 1 minute followed by 20-25 cycles at 95°C for 30 seconds; 50-55°C for 30 seconds; 72°C for 1-2 minutes and a final extension step at 72°C for 5 minutes. The precise annealing temperature used depends upon the melting temperature of the primer used.

The yield can be approximated on an agarose gel. The minimum volume of PCR for sequencing is about 25µl. Of this volume, 10µl is used to run an agarose gel to check quality and quantity of DNA amplified and an additional 0.1-10µl is used for sequencing. Generally, the presence of a single amplified band on a gel 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.

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 ExoSAP-IT treatment works best when the PCR product is a single band. For difficult cases, purification of the product using gels or other methods may be required.

GEL ELECTROPHORESIS REAGENTS

The quality of the gel electrophoresis is often the factor which limits the extent of sequence information that can be determined in a single sequencing experiment. The length of time the gel is run will determine the region of sequence that is readable. Under optimal conditions, 300 or more bases can be read starting at the bottom of a gel. Unfortunately, many factors can reduce this resolution. Among these are the quality of reagents used, the polymerization and the temperature of the gel during electrophoresis. In short, the greatest care should be given to the pouring and running of sequencing gels. The specifics of running the electrophoresis will depend on the apparatus used. The following suggestions for reagent compositions and procedures are intended as guidelines. For specific instructions contact the manufacturer of the gel apparatus used.

Note: This kit contains an enzyme mixture with high glycerol concentration. The use of Glycerol Tolerant Gels (GTG) and buffer (PN 71949 or PN 75827) to eliminate glycerol-induced distortion of sequencing bands is strongly recommended. The use of Tris-Borate-EDTA (TBE) buffer can results in severe distortion of gel at approximately 300-600 bases beyond the primer (1).

The following are recipes for typical sequencing gel reagents. There are many variations in current use, but these are among the most common.

Buffers

20X Glycerol Tolerant Gel Buffer (PN 71949 or PN 75827)

Tris	216g
Taurine	72g
$Na_2EDTA \bullet 2H_2O$	4g
H_2O to 1000ml, filter (may be autoclaved)	

This buffer can be used with samples containing glycerol at any concentration (1). If gels seem to run a bit slower with this buffer at 1X strength, use it more dilute—approximately 0.8X strength. Be certain to run glycerol tolerant gels at the same power (wattage) as TBE-buffered gels so the gel temperature is normal.

10X Tris-Borate-EDTA buffer (PN 70454)

Tris	108g
Boric acid	55g
$Na_2EDTA \cdot 2H_2O$	9.3g

H_2O to 1000ml, filter (may be autoclaved)

TBE is a traditional sequencing gel buffer. However, the enzyme mixture in this kit contains high glycerol concentration and it will result in glycerol-induced distortion of bands at approximately 350-600 bases beyond the primer. If this region is beyond the required reading frame, TBE buffer can be used.

Gel recipes

Standard gel

Gel conc. (%)	Acrylamide/ bis-acrylamide	Urea* (7-8.3M)	20X Gly. Tol. Gel Buffer	H_2O
6%	5.7g/0.3g	42-50g	5ml**	~45ml
8%	7.6g/0.4g	42-50g	5ml**	~45ml

Dissolve, adjust volume to 100ml with H_2O, filter and de-gas. When ready to pour, add 1ml of 10% ammonium persulfate and 25µl TEMED (N, N, N', N'-tetramethylethylenediamine).

Formamide gel (for resolution of compressions)

Gel conc. (%)	Acrylamide/ bis-acrylamide	Urea* (7M)	20X Gly. Tol. Gel Buffer	Formamide	H_2O
6%	5.7g/0.3g	42g	5ml**	30-40ml	~15ml
8%	7.6g/0.4g	42g	5ml**	30-40ml	~15ml

Adjust volume to 100ml with H_2O, filter and de-gas. When ready to pour add 1ml of 10% ammonium persulfate and 80-100µl TEMED. The temperature of the mixture should be 30-35°C—warmer mixtures will polymerize too fast while mixtures below 25°C may precipitate urea. Formamide gels require higher running voltage and run slower than urea-only gels.

RapidGel Acrylamide Stock Solutions

Gels can also be prepared by using 40% RapidGel-XL (PN 75863) or 40% RapidGel (PN 75848).

Gel conc. (%)	40% RapidGel XL/ 40% RapidGel	Urea (7M)	20X Gly. Tol. Gel Buffer	H_2O
6%	15ml	42g	5ml*	ml
8%	20ml	42g	5ml*	ml

*Warming to 35-45°C may be required to dissolve urea completely.
**Use 4ml for faster gel migration.

General guidelines for gel electrophoresis

1. Glass plates should be thoroughly washed and dried with 70% methanol.
2. Electrophoresis grade reagents should be used.
3. Solutions of monomers should be made fresh and used at once. Storage longer than one week in the dark at 4°C is not recommended. Commercial preparations of acrylamide gel mixes in liquid or powder (RapidGel XL and RapidGel-see 'Related Products' section) should be used according to manufacturers recommendations.
4. Gels should be prepared 2-20 hours prior to use, and pre-run for ~15 minutes.

Note: For radioactive labeled primers continue with the following instruction.

1. Gels other than RapidGel-XL need to be soaked. Soak gels in 5% acetic acid, 15% methanol to remove the urea. Soaking time depends on gel thickness. Approximate minimum times are 5 minutes for 0.2mm gels, 15 minutes for 0.4mm gels and 60 minutes for field gradient (0.4-1.2mm wedge) gels. For Formamide gel, increase the methanol to 20%. Drying should be done at moderate temperature (80°C) to preserve resolution.
2. For ^{33}P gels, exposure must be done with direct contact between the dried gel and the emulsion side of the film. Gels dried without prior soaking will require longer drying and exposure times but give sufficient resolution for most purposes. Leaving plastic-wrap on helps to prevent the film from sticking to incompletely-dried gels.
3. Good autoradiography film can improve image contrast and resolution. We recommend Hyperfilm™ or Kodak Biomax™ autoradiography films.
4. In general, overnight to 36 hour exposures are sufficient when using film such as Hyperfilm-MP.
5. The use of tapered spacers ('wedge' gels) improves overall resolution and allows more nucleotides to be read from a single loading (6).

RADIOLABELED PRIMER CYCLE SEQUENCING

5'-end labeling of primer

While this kit has been designed to utilize fluorescently labeled primers, traditional radiolabeled primers can also be used. However, mini-prep of plasmid or phage DNA is necessary for optimal results. The core reagents will perform equally well if radiolabeled primers are used.

To label primer, obtain a supply of T4 Polynucleotide Kinase (PNK) and suitable reaction buffer and dilution buffer. The following method is optimized for use with USB T4 Polynucleotide Kinase (PN 70031). Reaction buffer and dilution buffer are included.

A common difficulty with labeling primers comes from the use of too much T4 Polynucleotide Kinase. Whenever 20 or more units are used in a 10µl reaction volume, no usable labeled product is formed. It is essential that the T4 Polynucleotide Kinase be diluted to approximately 10units/µl so that no more than 20 units are used in a labeling reaction.

Dilute T4 Polynucleotide Kinase to 10units/µl:

T4 Polynucleotide Kinase (30U/µl)	5µl
Kinase dilution buffer	10µl
Total	15µl

Mix well, Store at -20°C. The dilution buffer is suitable for storage of the diluted kinase for up to 3 months at -20°C.

Primers are labeled using T4 Polynucleotide Kinase as follows:

γ-Labeled ATP*	25pmol *	
Primer, 20µM	20pmol	(1µl)
Kinase reaction Buffer, 10X	1X	(1µl)
Kinase, 10U/µl (add last)	10 units	(1µl)
H_2O	___µl	
	10-20µl	

Mix well and incubate at 37°C for 10 minutes.

Heat at 95°C for 2-5 minutes to inactivate the kinase. Centrifuge the mixture briefly to collect any condensate. Store at -20°C. The labeled primer may be used for 2-3 weeks.

Following labeling, the primer concentration is about 2pmol/µl. This concentration is suitable for ^{33}P-labeled primers but dilution of fresh ^{32}P-labeled primers (4-fold) may be recommended for some situations. No further purification is required for most sequencing.

*γ-Labeled ATP	Specific radioactivity	Conc.	Volume for 25pmol
[γ-^{32}P]ATP	3000Ci/mmol	10mCi/ml	7µl
[γ-^{32}P]ATP	6000Ci/mmol	10mCi/ml	15.6µl
[γ-^{33}P]ATP	1700Ci/mmol	10mCi/ml	4.5µl

CONTROL DNA SEQUENCE

The control DNA included in the kit is pUC19, a double-stranded circular DNA of 3.0Kb. A partial sequence of this DNA is given below.

```
          (-40 Forward Primer)
         G TTTTCCCAGT CACGACGTTG TA—>                0         10
AACGCCAGGG TTTTCCCAGT CACGACGTTG TAAAACGACG GCCAGTGAAT TCGAGCTCGG
        20         30         40         50         60         70
TACCCGGGGA TCCTCTAGAG TCGACCTGCA GGCATGCAAG CTTGGCGTAA TCATGGTCAT
        80         90        100        110        120        130
AGCTGTTTCC TGTGTGAAAT TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGGAA
       140        150        160        170        180        190
GCATAAAGTG TAAAGCCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC
       200        210        220        230        240        250
GCTCACTGCC CGCTTTCCAG TCGGGAAACC TGTCGTGCCA GCTGCATTAA TGAATCGGCC
       260        270        280        290        300        310
AACGCGCGGG GAGAGGCGGT TTGCGTATTG GGCGCTCTTC CGCTTCCTCG CTCACTGACT
       320        330        340        350        360        370
CGCTGCGCTC GGTCGTTCGG CTGCGGCGAG CGGTATCAGC TCACTCAAAG GCGGTAATAC
       380        390        400        410        420        430
GGTTATCCAC AGAATCAGGG GATAACGCAG GAAAGAACAT GTGAGCAAAA GGCCAGCAAA
       440        450        460        470        480        490
AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG
       500        510        520        530        540        550
ACGAGCATCA CAAAAATCGA CGCTCAAGTC AGAGGTGGCG AAACCCGACA GGACTATAAA
       560        570        580        590        600        610
GATACCAGGC GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG ACCCTGCCGC
```

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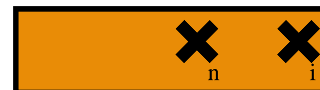
Material Safety Data Sheet



Revision: 07/24/2000

Hazard information is provided for compliance with both the UK Chemicals (Hazard Information and Packaging) (CHIP) Regulations and the US Hazard Communication Standard (HCS)

IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND COMPANY	**PRODUCT NAME**	**PRODUCT CODE**	**EEC NUMBER**
	Fluorescent Thermo Sequenase Cycle Sequencing Kit	79260	None

SUPPLIER:
USB Corporation
26111 Miles Road, Cleveland, OH 44128
Phone: (216) 765-5000

EMERGENCY CONTACT:
Chemtrec (800) 424-9300
Outside USA and Canada (703) 527-3887

COMPOSITION/ HAZARDOUS COMPONENTS	**HAZARD**	**CAS NO.**	**%WT**	**TLV**	**CHIP R & S PHRASES**
	Formamide in 79269	75-12-7	95%	10ppm (ACGIH)	R:62 Possible risk of impaired fertility R:63 Possible risk of harm to the unborn child S:24/25 Avoid contact with skin and eyes
	Glycerol in 79268	56-81-5	50	10mg/m3 (ACGIH)	R:36/37/38 Irritating to eyes, respiratory system and skin S:26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
	Tris-HCl in 79261	1185-53-1	2.4%	—	No applicable information

HAZARDS IDENTIFICATION

CHIP
Formamide: Toxic to Reproduction, Category 3. Glycerol: Irritant.
HCS
Formamide: Teratogen. Glycerol, Tris-HCl: Irritant.

FIRST-AID MEASURES

EYES: Flush with water for 15 minutes. Seek medical advice if irritation persists.
SKIN: Flush with water, then wash thoroughly with soap and water. Remove contaminated clothing and wash before reuse. Seek medical advice if irritation persists.
INHALATION: Remove the victim from exposure and move to fresh air. If breathing is difficult, give oxygen. If not breathing, give artificial respiration. Keep victim quiet and warm. Seek immediate medical attention.
INGESTION: Drink water and seek immediate medical attention. Avoid alcoholic beverages. Never give anything by mouth to an unconscious person.

FIRE-FIGHTING INFORMATION

Use media suitable to extinguish the supporting or surrounding fire. Wear NIOSH (or equivalent) approved self contained breathing apparatus. Fires involving Formamide are likely to produce very toxic gases such as carbon monoxide and ammonia, which should not be inhaled. Flash point = 310°F (154°C) for Formamide; 379.4°F (193°C) for Glycerol; No data available for Tris-HCl.

ACCIDENTAL RELEASE MEASURES

Wear appropriate protective equipment and clothing including lab coat, safety glasses and gloves. Dilute the material with a suitable inert absorbent. Place the waste in an appropriate container. Avoid contact of material with skin or eyes. Use adequate ventilation.

HANDLING AND STORAGE

Wear suitable protective clothing including lab coat, safety glasses and gloves. Wash thoroughly after handling. Use with adequate ventilation. Store at -20°C.

PERSONAL PROTECTION

Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, Neoprene or other chemical resistant gloves and a NIOSH-approved respirator. A qualified industrial hygienist should evaluate the need for respiratory protection. Use respiratory protection approved by NIOSH (or equivalent) and appropriate to the hazard. Avoid contact of material with skin or eyes. Mechanical ventilation or local exhaust as needed to control exposure to dust, vapors or mists. Access to a safety shower and eye wash. Pregnant women or women of child bearing age should minimize contact and exposure to Formamide.

PHYSICAL AND CHEMICAL PROPERTIES

Appearance: Kit containing vials of solutions. **Boiling Point:** Formamide = 210°C; Glycerol = 288°C; Tris-HCl = No information available. **Melting Point:** Formamide = 1.55; Glycerol = 3.17; Tris-HCl = No information available. **Vapor Pressure:** formamide = 0.08mm at 20°C; Glycerol = .0025mm at 5°C; Tris-HCl = No information available. **Vapor Density:** Formamide = 1.55; Glycerol = 1.26; Tris-HCl = No information available. **Specific Gravity:** Formamide = 1.134; Glycerol = 1.26; Tris-HCl = No information available. **Evaporation Rate:** No information available. **Solubility (water):** Formamide = insoluble in water; Glycerol = Miscible in water; Tris-HCl = No information available. **Solubility (other):** No information available.

STABILITY AND REACTIVITY

Product is stable. Formamide decomposes at temperatures above 180°C and is incompatible with alkali and acids. Hazardous decomposition products may include carbon monoxide and nitrogen oxides. Hazardous polymerization will not occur. Glycerol is stable under normal conditions. Avoid strong oxidizing agents including mixtures with hydrogen peroxide, potassium permanganate, calcium hypochlorite, nitric acid, sulfuric acid, perchloric acid and lead oxide. Tris-HCl: Avoid excessive heat. Incompatible with oxidizing materials and may produce toxic fumes upon decomposition.

TOXICOLOGICAL INFORMATION

Formamide: May cause irritation to skin, eyes and mucous membranes. Vapors are irritating to nose, throat and lungs. In animal studies, Formamide has caused embryotoxicity and birth defects at levels high enough to cause maternal toxicity. Defects and fetal deaths were seen. Formamide may be absorbed through the skin. Irritation, mutation, reproductive effects and toxicity effects data are listed

under RTECS LQO525000. Oral rat LD50 = 5677 mg/kg (1967). **Glycerol:** May cause irritation to skin, eyes and mucous membranes. Ingestion may cause irritation of the gastrointestinal tract and diarrhea. Ingestion of large quantities may cause nausea and vomiting. Irritation, mutation, reproductive effects and toxicity data for Glycerol is listed in RTECS under MA8050000. Oral rat LD50 = 12600 mg/kg (1945). **Tris-HCl:** May cause irritation to eyes, skin and mucous membranes. Contact may cause redness, swelling and pain. Excessive inhalation may cause irritation, cough and shortness of breathe. Ingestion or excessive exposure may lead to nausea, vomiting and diarrhea. Large amounts may cause weakness, collapse and coma.

ECOLOGICAL INFORMATION

No information available

DISPOSAL CONSIDERATIONS

Dispose of material in accordance with applicable local, state, and federal regulations.

TRANSPORTATION INFORMATION

No applicable information.

REGULATORY INFORMATION

RCRA - No applicable information.
SARA 302 - No applicable information.
SARA 313 - No applicable information. EPA TSCA Section 8(b) - For Formamide and Glycerol: Chemical inventory. 8(d) - For Formamide and Glycerol: unpublished health/safety studies.
Exposure Limits - For Formamide: ACGIH TLV-TWA: 10 ppm (skin). NIOSH REL to Formamide-air: 10H TWA 10 ppm (Sk). For Glycerol: ACHIH TLV-TWA: 10 mg/m3. OSHA PEL (Gen Indu): 8H TWA 15 mg/m3, total dust. OSHA PEL (Gen Indu): 8H TWA 5 mg/m3, respirable fraction.
California Proposition 65 - No applicable information.

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The purchase of this kit (reagent) includes a limited non-exclusive sublicense under certain patents* to use the kit (reagent) to perform one or more patented DNA sequencing methods in those patents solely for use with Thermo Sequenase DNA polymerase purchased from Amersham Pharmacia Biotech Limited and/or its subsidiaries for research activities. No other license is granted expressly, impliedly or by estoppel. For information concerning availability of additional licenses to practice the patented methodologies, contact Amersham Pharmacia Biotech, Inc., Director, VP Corporate Development, Amersham Place, Little Chalfont, Buckinghamshire, HP79NA England. *US Patent numbers 4,962,020, 5,173,411, 5,409,811, 5,498,523, 5,614,365 and 5,674,716. Patents pending.

*Thermo Sequenase DNA Polymerase—This reagent (kit) is covered by or suitable for use under one or more US Patent numbers: 4,962,020; 5,173,411; 5,409,811; 5,498,523; 5,614,365 and 5,674,716. Patents pending in US and other countries.

**Pyrophosphatase—This product and/or its method of use is covered by one or more of the following patent(s): US Patent number 5,498,523 and foreign equivalents.

[8]Glycerol Tolerant Gel Buffer—This product and/or its method of use is covered by US Patent number 5,314,595.

The Polymerase Chain Reaction (PCR) is covered by patents owned by Roche Molecular Systems and F. Hoffmann-La Roche Ltd.

Exonuclease I/Shrimp Alkaline Phosphatase method of use covered by one or more of the following US patents: 5,756,285 and 5,741,676. ExoSAP-IT patent pending.

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Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit

Product Number 79260
50 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.