

Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit

Product Number 79750, 50 reactions
79760, 100 reactions
79770, 500 reactions

Product Number 188403 includes:
79750, 50 reactions
AH9539, ³³P-labeled
terminators

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.



GE imagination at work



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COMPONENTS OF THE KIT

The solutions included in the Thermo Sequenase™ Radiolabeled Terminator Cycle 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 USB™ standards. It is strongly recommended that the reagents supplied in the kit be used as directed.

The following solutions are included in the kit:

Thermo Sequenase DNA Polymerase: 4U/μl, 0.0006U/μl *Thermoplasma acidophilum* inorganic pyrophosphatase**; 50mM Tris·HCl, pH 8.0, 1mM dithiothreitol (DTT), 0.1mM ethylenediamine tetraacetic acid (EDTA), 0.5% Tween™-20, 0.5% Nonidet™ P-40, 50% glycerol

Reaction Buffer (concentrate): 260mM Tris·HCl, pH 9.5, 65mM MgCl₂

dGTP Nucleotide Master Mix: 7.5μM dATP, dCTP, dGTP, dTTP

dITP Nucleotide Master Mix: 7.5μM dATP, dCTP, dTTP, 37.5μM dITP

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

Control DNA: double-stranded pUC18, 0.02μg/μl

Control Primer (-40 M13 forward; 23-mer): 2.0pmol/μl
5'-GTTTTCCCAGTCACGACGTTGTA-3'

This kit and all the enclosed reagents should be stored at -15°C to -30°C (NOT in a frost-free freezer). Keep all reagents on ice when removed from storage for use. The kit can conveniently be stored at 2°C to 4°C for periods of up to 3 months with no loss of performance, but this should be avoided if it is expected that the reagents will not be completely consumed within 3 months.

Note: The formulation of Thermo Sequenase DNA polymerase in this kit necessitates the use of a glycerol tolerant⁸ DNA sequencing gel. See 'Supplementary Information, denaturing gel electrophoresis' section.

³³P-labeled Terminators: A package of four ³³P-labeled terminators must be purchased for use with the kit. They may be ordered separately from GE Healthcare using product number AH9539. In the US, the terminators may be ordered together with the sequencing kit from USB using product number 188403.

ddGTP, 0.3μM [α-³³P]ddGTP (1500Ci/mmol, 450μCi/ml), Redivue™

ddATP, 0.3μM [α-³³P]ddATP (1500Ci/mmol, 450μCi/ml), Redivue

ddTTP, 0.3μM [α-³³P]ddTTP (1500Ci/mmol, 450μCi/ml), Redivue

ddCTP, 0.3μM [α-³³P]ddCTP (1500Ci/mmol, 450μCi/ml), Redivue

Redivue nucleotides can be stored at 4°C for up to 1 week after receipt, or at a constant -20°C if longer storage is desired. Care must be taken to prevent evaporation of these small volumes of material. Tightly cap the vials after use. Store at -20°C between uses if frequency of use is less than every 1-3 days. If condensation is observed on the walls of the vial or in the cap, return the liquid to the bottom of the vial and mix well before use.

QUALITY CONTROL

All kit batches are functionally tested using ³³P labeled terminators and pUC18 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 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: This product is to be used with radioactive material. Please follow the manufacturer's instructions relating to the handling, use, storage, and disposal of such materials.

Warning: Contains formamide. See 'Material Safety Data Sheet' on page 26.

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

This sequencing kit combines two revolutionary innovations for sequencing DNA using radioactive labels. First, the label is incorporated into the DNA sequencing reaction products by the use of four [α - ^{33}P]dideoxynucleotide (ddNTP) terminators (G,A,T,C). The labeled ddNTPs are more efficient for labeling sequencing experiments than other labeled nucleotides because they specifically label only the properly terminated DNA chains. Also, since prematurely terminated chains are not labeled, 'stop' artifacts and most background bands are eliminated. As an additional benefit, the absence of artifact bands allows the routine use of dITP, which can eliminate even very strong compression artifacts.

The second innovation is the use of Thermo Sequenase DNA polymerase⁺. This enzyme has been engineered to efficiently incorporate dideoxynucleotides, allowing the use of very low amounts of isotope ([α - ^{33}P]ddNTP) for the termination reactions. Thermo Sequenase DNA polymerase is also thermostable and performs very well in convenient and sensitive cycle or non-cycle sequencing methods. This polymerase produces very uniform band intensities (with dGTP), so mixed sequences (such as those of heterozygotes) can be easily identified.

Thus, the kit offers:

- Clean, background-free sequences
- Complete elimination of compressions
- Efficient use of labeled nucleotides, less than 1 μCi per sequence
- Convenient single-step protocol
- Uniform band intensities for identification of mixed sequences (*e.g.* heterozygotes)
- Sensitive cycle-sequencing protocols for sequencing 20fmol or less of template
- Overnight exposures with ordinary autoradiography film—same day results possible with fast films
- Exceptionally easy-to-read sequences
- ^{33}P for sharp autoradiogram resolution
- Sample storage for 1-2 days prior to running on gel

Chain termination sequencing

This kit is designed to eliminate sequencing artifacts such as stops (or BAFLs—bands across four lanes) and background bands. BAFLs can result from the enzyme pausing at regions of secondary structures in GC-rich templates, producing prematurely aborted primer extension products of the same length in all four termination reactions. Background bands can be caused by primer extensions aborting prematurely at random positions, such as when a template is rich in a certain base and the complementary nucleotide in the reaction becomes depleted.

Traditional chain termination sequencing methods (1) involve the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA template. Synthesis is initiated at the site where a primer anneals to the template. Elongation of the 3' end of the annealed primer is catalyzed by a DNA polymerase in the presence of 2'-deoxynucleoside-5'-triphosphates (dNTPs), and is terminated by the incorporation of a 2',3'-dideoxynucleoside-5'-triphosphate nucleotide analog (ddNTP) that will not support continued DNA elongation (hence the name 'chain termination'). Four separate reactions, each with a different ddNTP, (ddG, ddA, ddT, or ddC), give complete sequence information. A radiolabeled dNTP (2,3) or primer is normally included in the synthesis, so the labeled chains of various lengths can be visualized after separation by high-resolution gel electrophoresis (4,5). In this kit, a radioactive label is incorporated into the sequencing reaction products at the 3' end by the use of an [α -³³P]ddNTP, thus ensuring that only properly terminated DNA strands are labeled and are visible in the sequence. This results in a cleaner, more reliable and easier to read sequence with fewer background bands and virtually no BAFLs.

The accuracy and readability of the sequence obtained depends strongly on the properties of the polymerase used for chain termination. Some polymerases, such as Sequenase™ Version 2.0 DNA polymerase, generate much more uniform, readable bands than others like Klenow and *Taq* DNA polymerase (6,7,8). Thermostable polymerases, such as *Taq* polymerase, can be used for multiple rounds (cycles) of DNA synthesis, generating stronger signals. Tabor and Richardson (9) have discovered that DNA polymerases can be modified to accept dideoxynucleotides as readily as the normal deoxynucleotide substrates. Using this technology, a new DNA polymerase for DNA sequencing was developed. This enzyme, called Thermo Sequenase DNA polymerase, is thermostable and possesses many of the excellent DNA sequencing qualities of Sequenase DNA polymerase. The properties of this DNA polymerase include activity at high temperature and absence of associated exonuclease activity. Like Sequenase DNA polymerase, derived from T7 bacteriophage, it readily

uses dideoxynucleoside triphosphates, generating uniform band intensities in sequencing experiments (with dGTP). These properties make the enzyme ideal for generating high-quality DNA sequences using cycle-sequencing methods. It is stable at 90°C for at least 1 hour and retains 50% of its activity when incubated at 95°C for 60 minutes. The Thermo Sequenase polymerase in this kit combines the advantages of both Sequenase DNA polymerase and *Taq* DNA polymerase. It produces bands (with Mg²⁺) that are nearly as uniform as those produced with Sequenase DNA polymerase with Mn²⁺ (10), yet is thermostable like *Taq* DNA polymerase.

Cycle sequencing is the name given to the process of using repeated cycles of thermal denaturation, primer annealing, and polymerization to produce greater amounts of product in a DNA sequencing reaction. This amplification process employs a single primer so the amount of product DNA increases linearly with the number of cycles. (This distinguishes it from PCR* which uses 2 primers so that the amount of product can increase exponentially with the number of cycles.)

The earliest examples of cycle sequencing used ³²P-labeled primers and a non-thermostable polymerase which was added after each denaturation cycle (11,12). Later improvements included the use of thermostable *Taq* polymerase (13,14) and the use of alpha-labeled dNTPs in place of the labeled primer using mixtures of nucleotides similar to those used originally by Sanger (15,16). The labeled-primer methods make efficient use of ³²P giving a sequence with as little as 4μCi of [γ -³²P]ATP (14). The methods using internally-labeled products were less efficient, requiring either 10μCi of [α -³³P]dATP or 20μCi of [α -³⁵S]dATP for a sequence. This is a consequence of the relatively low specific radioactivity and the small number of labeled bases in short product molecules. This kit makes very efficient use of [α -³³P]ddNTP, requiring less than 1μCi of ³³P per sequence. Cycle sequencing is necessary with this kit when using less than 0.2-0.5pmol of template DNA. Non-cycle (or very few cycle) protocols may be used with more than ~0.5pmol of template.

*See license information on back cover.

MATERIALS NOT SUPPLIED

Necessary reagents:

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

Specialized sequencing primers—Some sequencing projects will require the use of primers which are specific to the project. For most sequencing applications, 0.5-2.5pmol of primer should be used for each set of sequencing reactions. Always determine the concentration of the primer by reading the optical density at 260nm (OD_{260}). If the primer has N bases, the approximate concentration (pmol/ μ l) is given by the following formula:

Concentration (pmol/ μ l) = $OD_{260} / (0.01 \times N)$ where N is the number of bases.

Gel reagents—Sequencing gels should be made from fresh solutions of acrylamide and bis-acrylamide. Other reagents should be 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.

Necessary equipment:

Liquid handling supplies such as vials, pipettes and a microcentrifuge—All sequencing reactions are run in plastic microcentrifuge tubes (typically 0.5ml) suitable for thermal cycling.

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 (4,5,17). A power supply offering constant voltage operation at 2000V or greater is essential.

Gel handling—For ^{33}P sequencing, a large tray for washing the gel (to remove urea) and a gel drying apparatus are highly recommended. For best results, gels containing ^{33}P must be exposed dry in direct contact with the film at room temperature.

Autoradiography—Any large format autoradiography film such as the BioMax™ MR, and a large film cassette.

Thermal cycler—Sequencing will require thermally cycled incubations between 50°C and 95°C (1-100 cycles).

PROTOCOL

1. **Termination mixes**—Prepare the termination mixes on ice. Mix 2 μ l of Nucleotide Master Mix (either dGTP or dTTP—see note below) and 0.5 μ l of [α -³³P]ddNTP (G, A, T, or C—one of each per sequence) to produce a termination mix for each ddNTP. Label, fill and cap four tubes ('G', 'A', 'T', 'C') with 2.5 μ l of each termination mix. **It is more accurate and convenient to prepare batches of termination mixes sufficient for all sequences to be performed, then dispense 2.5 μ l from this batch to each vial for the termination reactions.** It is recommended that these batches of termination mixes be made up routinely.

To prepare termination mixes for (n) reactions, mix:

	G	A	T	C
Nucleotide Master Mix	(2 x n) μ l	(2 x n) μ l	(2 x n) μ l	(2 x n) μ l
[α - ³³ P]ddNTP	(0.5 x n) μ l	(0.5 x n) μ l	(0.5 x n) μ l	(0.5 x n) μ l
Total	(2.5 x n) μ l	(2.5 x n) μ l	(2.5 x n) μ l	(2.5 x n) μ l

Note: The termination tubes can be left uncapped until all reagents have been added if the tubes are kept on ice and the reaction mixture is added within a few minutes. For determination of new sequences, or of sequences with high G-C content, the dTTP Nucleotide Master Mix is recommended. This will eliminate all compression artifacts but will result in somewhat uneven band intensities, especially in the 'G' lane. When perfectly uniform band intensities are desired, such as when examining sequences from potentially heterozygous individuals, the dGTP Nucleotide Master Mix should be used.

2. Reaction mixture:

For multiple (n) reactions with different primers and/or templates, prepare a n+1 batch of reaction buffer, water, polymerase and aliquot; then add the unique primer and/or template in the appropriate concentration and volume to the aliquots.

Reaction Buffer	2 μ l
DNA	_ μ l* (50-500ng or 25-250fmol)
Primer	_ μ l* (0.5-2.5pmol)
H ₂ O	_ μ l (To adjust total volume to 20 μ l)
Thermo Sequenase polymerase (4U/ μ l)	2 μ l (8 units polymerase—add LAST)
Total	20 μ l

*For the control reaction, use 10 μ l of control DNA and 1 μ l of control primer.

3. Cycling termination reactions

Transfer 4.5 μ l of reaction mixture (prepared in step 2) to each termination tube ('G', 'A', 'T' and 'C') from step 1. Mix well and overlay with 10-20 μ l of mineral oil (if needed). Cap and place the tube in the thermal cycling instrument.

Note: When sequencing single-stranded DNA, the primer may anneal to the template with reduced specificity while the tubes are on ice, and extension of these primers can occur as the thermal cycler heats up during the first cycle. To minimize nonspecific extension products, the cycler can be pre-heated to 85-95°C or pre-cooled to 4°C.

4. Start the cycling program. **Note:** The specific cycling parameters used will depend on the primer sequence and the amount and purity of the template DNA. For the primers included in the kit and the suggested amount of purified DNA (25-250fmol), cycle 30-60 times as follows:

dGTP	dITP
95°C, 30s	95°C, 30s
55°C, 30s	50°C, 30s
72°C, 60-120s	60°C, 5-10min

(typically 30 cycles taking 2-3hr) (typically 30 cycles taking 3-5hr)

Fewer (1-10) cycles may produce better results when using 250-500fmol DNA.

5. Add 4 μ l of Stop Solution to each of the termination reactions, mix thoroughly and centrifuge briefly to separate the oil from the aqueous phase. Alternatively, remove 6 μ l from each termination reaction and transfer to a fresh tube containing 3-4 μ l of Stop Solution. Samples should be kept on ice for same day loading or may be stored frozen up to 3 days before loading onto gel.
6. When the gel is ready for loading, heat the samples to 70°C for 2-10 minutes and load immediately on the gel—3-5 μ l in each gel lane. **Note:** Heating in open vials will promote evaporation of water from the formamide-reaction mixture. This is not normally necessary, but will increase the signal by concentrating the isotope and will promote more complete denaturation of the DNA. This may improve results when using older ³³P-ddNTPs. Avoid complete evaporation to dryness by prolonged heating.

SUPPLEMENTARY INFORMATION

General guidelines

- Since the popular multiple cloning sites all derive from similar sequences, one primer can serve for the sequencing of insert DNA in most of the common vectors. Among the vectors compatible with the primer supplied in the Thermo Sequenase radiolabeled terminator cycle sequencing kit are M13mp8, M13mp9, M13mp12, M13mp13, M13mp18, M13mp19, mWB2348, mWB3295, mWB3225, pUC18, pUC19, and virtually any vector featuring blue/white screening with β -galactosidase activity.
- Good sequences can be obtained using as little as 0.05 μ g of M13 DNA, 0.1 μ g of plasmid DNA, or 50fmol of PCR product. Mix reagents by gently 'pumping' the pipettor. The total volume of the reaction mix should be 20 μ l—the volumes of DNA and primer added will depend on their concentration. Adjust the amount of distilled water so that the total volume of DNA, primer and water is 16 μ l.
- The specific cycling parameters used will depend on the primer sequence and the amount and purity of the template DNA. See 'Supplementary Information, cycle conditions and template quantity'.
- The dGTP Nucleotide Master Mix should be used if the sequence is already known to be free of compression artifacts and the benefits of uniform band intensities are desired. The uniform band intensities can aid in finding heterozygotes or in other cases where mixed sequence may be present. If compressions are a problem when using dGTP, gels containing formamide can be used as described in the 'Supplementary Information, denaturing gel electrophoresis' section of this booklet.
- For running sequences where compressions are a problem, the dITP Nucleotide Master Mix included in this kit can be substituted for the dGTP Nucleotide Master Mix. See 'Supplementary Information, elimination of compressions' section for details. **Note:** When using dITP, use an 'extension' temperature of 60°C with a duration of at least 4 minutes.
- Whenever possible, tubes should be kept capped and on ice to minimize evaporation of the small volumes employed. Additions should be made with disposable-tip micropipettes and care should be taken not to contaminate stock solutions. The solutions must be thoroughly mixed after each addition, typically by 'pumping' the solution two or three times with a micropipette, avoiding the creation of air bubbles. At any stage where the possibility exists for some solution to cling to the walls of the tubes, the tubes should be centrifuged. With care and experience these reactions can be set-up in 15-20 minutes.

Preparation of template DNA

Since cycle sequencing can be performed using very little template DNA, only very small amounts of detrimental impurities are likely to be carried along with the DNA. Therefore, though still important, template purity may not be as crucial for cycle sequencing as it is for non-cycle sequencing.

Preparation of single-stranded template DNA

Single-stranded template DNA of good purity is essential for excellent sequencing results. Several popular plasmid cloning vectors contain the same lac-derived cloning region as the M13mp vectors and a single-stranded phage replication origin. Production of single-stranded DNA from these vectors is similar to that of the M13 phage and the single-stranded DNA produced can also be used as template for sequencing.

Preparation of double-stranded plasmid DNA

Sequencing double-stranded templates with the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit works effectively with no changes in the reaction protocol. Alkaline denaturation is not required for plasmid DNA templates. For best results, purified plasmid DNA should be used—CsCl gradients, PEG precipitation, adsorption to glass, columns, and other common DNA purification methods all produce suitable DNA. (However, since such small quantities of DNA are added to the reactions, even impure DNA samples can sometimes yield acceptable sequence data.) There are many popular protocols for purifying plasmid DNA from 2-10ml cultures. We have had consistent success with 'boiling' (21) and 'alkaline' (22) mini-prep methods.

Cycle conditions and template quantity

The temperatures used for cycling the termination reactions should be determined from the characteristics of the sequencing primer, the template, and the length of the termination product desired. The number of cycles required will depend on the quantity and quality of the template DNA used. The following guidelines should assist in choosing cycling parameters.

Cycling temperatures

The melting temperature of the primer should be kept in mind when choosing cycle temperatures. The control primer included in the kit is moderately long (23 bases) with 50% G/C content. The melting temperature of this primer is ~73°C under sequencing reaction conditions, and excellent results are achieved by cycling between 60°C and 95°C. The duration of the steps does not seem to be critical, and even brief pauses (1-10 seconds) at these temperatures seem to be effective (except with dITP as described above).

As another example, when using the universal -40 17-mer, which has a melting temperature of about 50°C, cycling between 45°C and 95°C is effective. If in doubt, choose a wide temperature range with pauses (15-30 seconds) at the extremes of temperature.

The termination reaction cycles should always have a denaturation temperature of 95-98°C (however, avoid extended steps at 98°C since at this temperature the enzyme has a half-life of less than one hour). Since the optimum temperature for polymerization is about 70-75°C, 72°C is a good choice for the termination step (except when using dTTP, which requires a maximum temperature of 55-60°C). An annealing step (*e.g.* <60°C) is required only with primers less than ~24 bases.

Number of cycles and quantity of template

The number of cycles required will primarily depend on the amount of template DNA (in fmols) used for sequencing. It will also depend on the purity of the DNA, and the sensitivity of autoradiographic detection. The minimum quantities of highly-purified DNA which we have been able to sequence using these methods are about 5fmol of M13mp18 DNA and about 15fmol of pUC18 DNA. (For routine sequencing, we recommend 25fmol of M13 and 75fmol of plasmid DNA). When sequencing very small amounts of template, it has been observed that the number of cycles has a strong influence on sequence intensity. Increasing the number of cycles from 30 to 60 will increase the signal significantly when using less than ~50fmol of template DNA, whereas increasing the number of cycles with more than ~100fmol is of little benefit, and may even produce background sequence. So in general, use more cycles when template amounts are limited. Also, a modest improvement can sometimes be achieved by increasing the amount of primer 2-5 fold. It is undesirable to use too much template as the result will be a shortened sequence extension. Figure 1 shows the result of increasing template quantities to an excess.

Designing a new sequencing primer

The length of the primer (and its sequence) will determine the melting temperature and specificity. For the cycling temperatures normally used, the primer should be about 18-25 nucleotides long. It is also a good idea to check the sequence of the primer for possible self-annealing (dimer formation could result) and for potential 'hairpin' formation, especially those involving the 3' end of the primer. Finally, check for possible sites of false priming in the vector or other known sequence if possible, again stressing matches which include the 3' end of the primer.

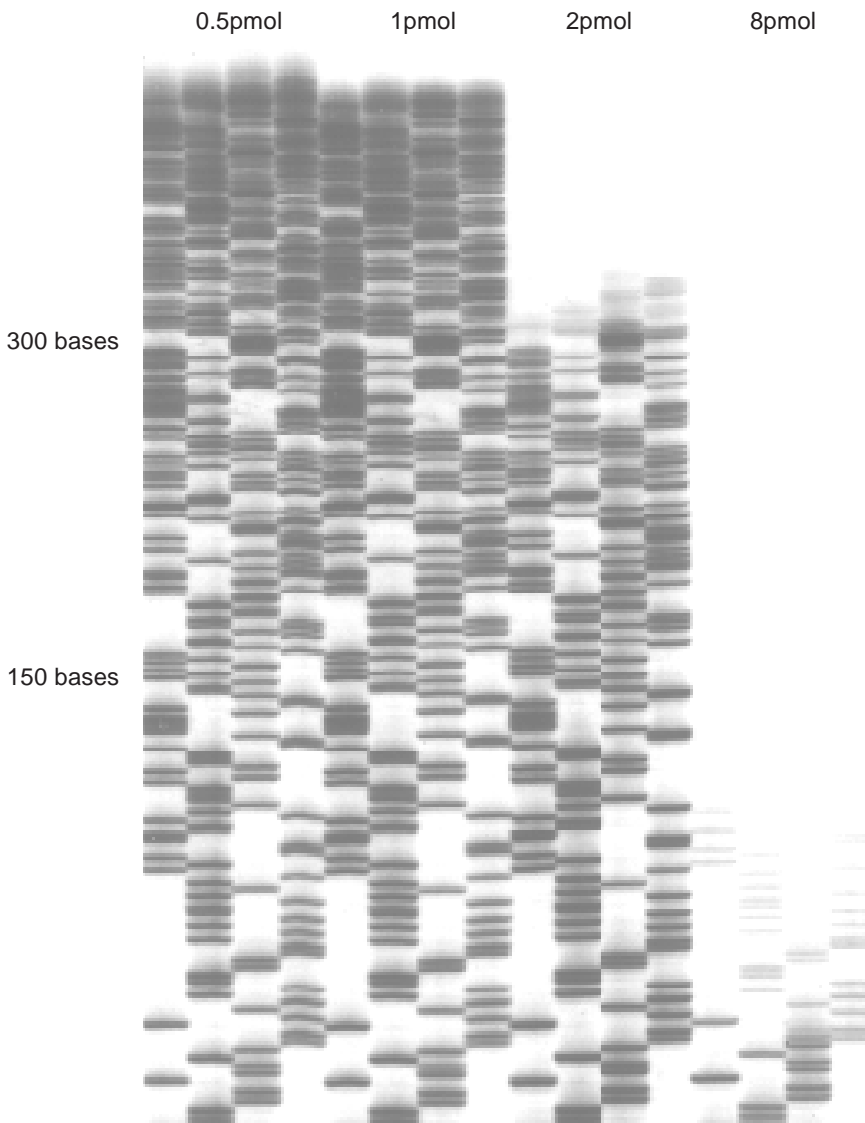


Figure 1. Excess template DNA can reduce sequence extension lengths. In cases where 2pmol or more template DNA are sequenced, the supply of nucleotides can be exhausted before extensions reach suitable length for optimal sequencing. These sequences were run using up to 16 μ g (8pmol) of M13mp18 DNA template.

Sequencing PCR Products

The products of Polymerase Chain Reaction (PCR) can have structures which make them difficult to sequence. One of the most common problems associated with sequencing of PCR products is the presence of stops or BAFs, where the sequence pauses or stops at artifactual ends in the template (actually the ends of truncated PCR product). This kit incorporates label by way of a radiolabeled dideoxy terminator so that only the fragments which were properly terminated are visible in the sequence. No labeled bands are formed at 'ends' in the template, eliminating many of these artifacts and enabling sequences to extend to essentially the last base of a PCR product. Artifacts caused by appearance of double-stranded PCR product on denaturing gels are similarly eliminated since they are not labeled. Following is information which should assist in producing high quality, reliable sequence information even with PCR product templates which have been very difficult to sequence with standard methods.

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 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.

Enzymatic pre-treatment of PCR products

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[®] to eliminate any primer or dNTPs which were not incorporated into the PCR product. These enzymes are available from USB in a reagent pack (70995) or pre-mixed (ExoSAP-IT[™], 78200) with detailed protocols for their usage. It is recommended that this enzymatic clean-up of the PCR product be used with this sequencing method.

Elimination of compressions

Some DNA sequences, especially those with dyad symmetries containing dG and dC residues, are not fully denatured during electrophoresis. When this occurs, the regular pattern of migration of DNA fragments is interrupted; bands are spaced closer than normal (compressed together) or sometimes farther apart than normal and sequence information is lost. The substitution of a nucleotide analog (dITP) for dGTP which forms weaker secondary structure has been successful in eliminating most of these gel artifacts (18, 19). Two examples are shown in figure 2 in the sequences run with dGTP.

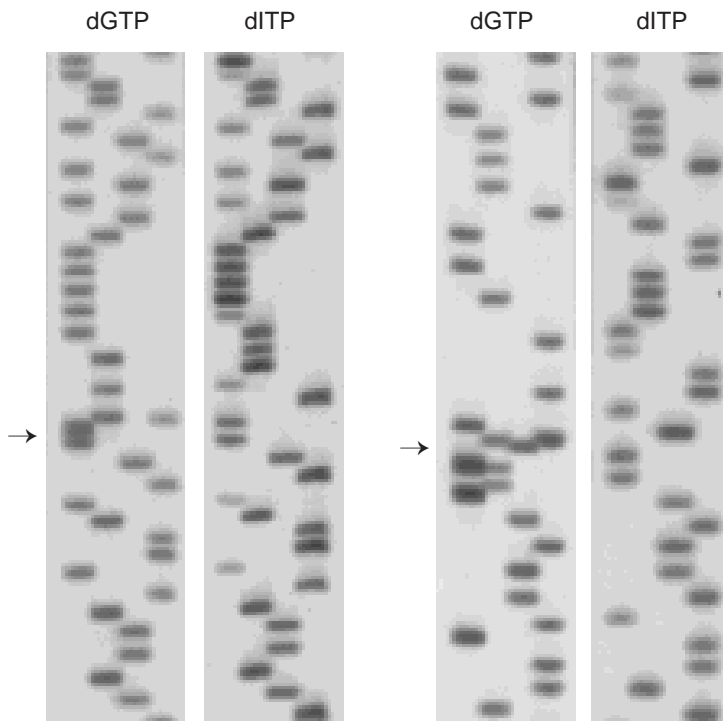


Figure 2. Compression artifacts can be eliminated using dITP in place of dGTP without interference by stops or other artifact bands. Shown are two severely 'compressed' regions of secondary structure (see arrows). The sequences run using dITP in place of dGTP are accurate and unambiguous.

A suitable nucleotide mixture containing dITP is included in the kit for use with templates prone to gel compression artifacts. To use dITP simply substitute the dITP Nucleotide Mix for the dGTP Nucleotide Mix. All other aspects of the sequencing protocol remain unchanged except that when using dITP, reduce the termination temperature from 72°C to 60°C and increase the time to approximately 5 minutes or longer (see figure 3). The use of dITP will result in less uniform band intensities, but will completely resolve even the strongest compressions. A 40% formamide gel will also eliminate almost all compressions (see 'Denaturing gel electrophoresis' section).

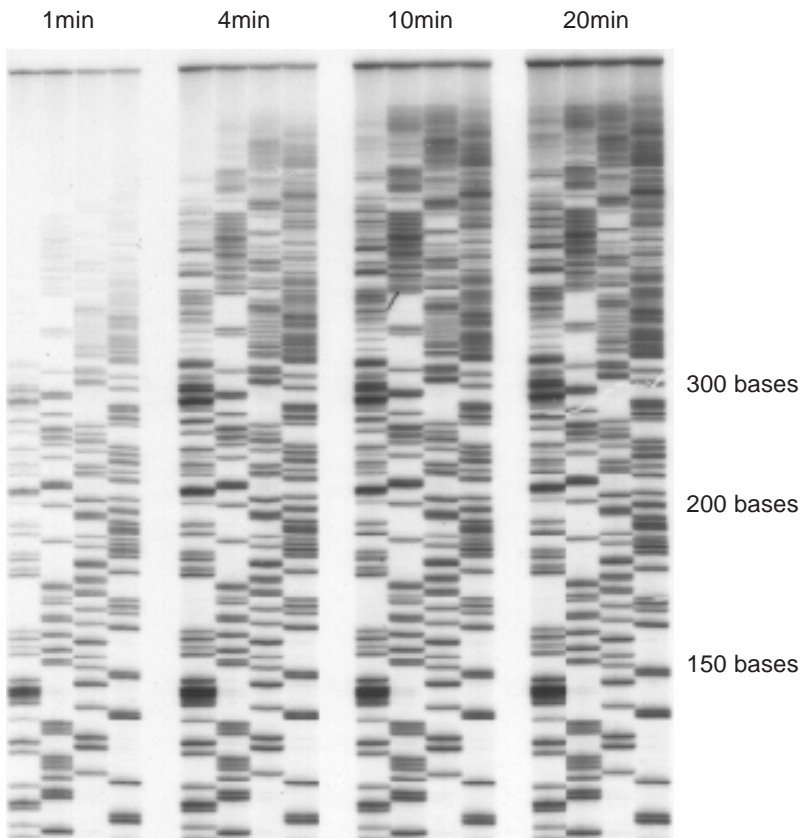


Figure 3. Use of dITP requires longer extension times at 60°C. Shown are four sequences of plasmid pUC18 obtained using cycles with 1, 4, 10 and 20 minute extension steps in the cycles. Extension steps of 4-5 minutes or longer are necessary for reading beyond 200 bases.

Reading farther from or closer to the primer

The termination mixes described in the protocol will typically yield sequencing data from the first base to over 500 bases from the primer. This is as much sequence as most users will be able to read using current standard electrophoresis technology. If it is desired to obtain sequence >500 bases from the primer, the dNTP:ddNTP ratios can be easily altered to shift the distribution of sequencing reaction products by adding more dNTPs to the termination reaction. Adding 3 μ l instead of 2 μ l dNTPs will increase the dNTP:ddNTP ratio

by 50%, thus increasing the average extension length of each primer before a ddNTP is incorporated. Conversely, adding 1 μ l of [α -³³P]ddNTP will decrease the ratio by 50%, thus decreasing the average extension length of each primer.

Running sequencing gels which resolve more than 600 nucleotides requires high quality apparatus, chemicals and attention to many details. While specific instructions are beyond the scope of this manual, following are some general guidelines: The gel should be loaded with 8 adjacent lanes (GATCGTAC or see 'Supplementary Information, denaturing gel electrophoresis' section) with a sharktooth comb and be run 4 to 10 times longer than usual. For this kind of experiment, gradient (or 'wedge') gels or very long gels (80-100cm) are almost a necessity. The highest resolution gels appear to be approximately 6-8% acrylamide and are run relatively cool (40°C).

Denaturing gel electrophoresis

Under optimal gel electrophoresis conditions, 250-300 bases can be read from the bottom of a standard size sequencing gel. The length of time the gel is run will determine the region of sequence that is readable. Many factors can limit the sequence information which can be determined in a single experiment. Among these are the quality of reagents used, the polymerization, the temperature of the gel during electrophoresis, and proper drying of the gel after running. 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.

Gel electrophoresis reagents

This kit contains a prediluted enzyme mixture which contains a high glycerol concentration, requiring the use of a glycerol tolerant gel buffer. The use of other buffers such as TBE can result in severe distortion of sequencing bands in the upper third of the gel. The following recipe is for typical sequencing gel reagents.

Buffers

20X Glycerol Tolerant Gel Buffer (71949 or 75827)

Tris base	216g
Taurine	72g
Na ₂ EDTA·2H ₂ O	4g
H ₂ O to 1000ml, filter (may be autoclaved)	

This buffer can be used with samples containing glycerol at any concentration (20). 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 TBE Buffer (70454)

Tris base 108g
 Boric acid 55g
 Na₂EDTA·2H₂O 9.3g
 H₂O to 1000ml, filter (may be autoclaved)

This is the traditional sequencing gel buffer. It should NOT be used with the polymerase supplied in this kit (Glycerol Tolerant Gel Buffer should be used).

Gel recipes (for 100ml of gel solution)

Standard gel

Gel conc. (%)	Acrylamide/ bis-acrylamide	Urea (7-8.3M)	20X Gly. Tol. Gel Buffer	OR	10X TBE Buffer	H ₂ O
6%	5.7g/0.3g	42-50g	5ml*		-	~45ml
8%	7.6g/0.4g	42-50g	5ml*		-	~45ml
6%	5.7g/0.3g	42-50g	-		10ml	~40ml
8%	7.6g/0.4g	42-50g	-		10ml	~40ml

Dissolve, adjust volume to 100ml with H₂O, filter and de-gas. When ready to pour, add 1ml of 10% ammonium persulfate and 25µl TEMED (N, N, N', N'-tetramethylethylenediamine).

*Use 4ml for faster gel migration.

Formamide gel (for resolution of compressions)

Gel conc. (%)	Acrylamide/ bis-acrylamide	Urea* (7M)	20X Gly. Tol. Gel Buffer	OR	10X TBE Buffer	Formamide	H ₂ O
6%	5.7g/0.3g	42g	5ml		-	40ml	~10ml
8%	7.6g/0.4g	42g	5ml		-	40ml	~10ml
6%	5.7g/0.3g	42g	-		10ml	40ml	~5ml
8%	7.6g/0.4g	42g	-		10ml	40ml	~5ml

*Warming to 35-45°C may be required to dissolve urea completely.

Adjust volume to 100ml with H₂O, filter and de-gas. When ready to pour add 1ml of 10% ammonium persulfate and 100-150µl TEMED. The temperature of the mixture should be 25-35°C—warmer mixtures will polymerize too fast while mixtures below 20°C may precipitate urea. They will require higher running voltage and run slower than urea-only gels. Prior to drying, these gels should be soaked in 5% acetic acid, 20% methanol to prevent swelling. For more detailed information, refer to TechTip #200 available from USB Technical Support or the Technical Library at usbweb.com.

General guidelines for electrophoresis

1. Ultrapure or electrophoresis grade reagents should be used.
2. Sequencing gels should be made fresh. Store solutions no longer than one week in the dark at 4°C. Commercial preparations of acrylamide gel mixes in liquid or powder form (RapidGel gel mixes—see 'Related Products') should be used according to manufacturers recommendations.
3. Gels should be prepared 2-20 hours prior to use, and pre-run for ~15 minutes.
4. When reading longer sequences, it is usually convenient to run gels overnight with a timer-controlled power supply. Gel runs of 18-24 hours at 40-50 watts are often necessary for reading in the 400-600bp range.
5. Loading 8 adjacent lanes in a pattern that abuts all pairs of lanes (*e.g.* GATCGTAC) aids reading closely spaced bands.
6. Gels should be soaked 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) or formamide gels. Drying should be done at moderate temperature (80°C) to preserve resolution.
7. 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.
8. For ³²P gels, autoradiography must be done with direct contact between the dried gel and the emulsion side of the film. Gels dried without prior soaking (leaving plastic-wrap on helps to prevent the film from sticking to the incompletely-dried gels) will require longer drying and exposure times but give sufficient resolution for most purposes.
9. Good autoradiography film can improve image contrast and resolution. We recommend Kodak Biomax™ MR or Hyperfilm™-bmax autoradiography film.
10. In general, overnight to 36 hour exposures are sufficient when using fast film such as Hyperfilm™-MP.
11. The use of tapered spacers ('wedge' gels) improves overall resolution and allows more nucleotides to be read from a single loading (4).

TROUBLESHOOTING

Problem Possible causes and solutions.

Extensions appear short (read length limited to less than 200 bases)

1. If using dITP, increase time of extension step in cycles to 5-10 minutes and decrease temperature to 60°C. See figure 2.
2. Too much template DNA. In some cases, the use of too much DNA, especially PCR product DNA, can exhaust the supply of ddNTPs. Use less

than 1pmol of template DNA for each sequence (0.25pmol per reaction).
See figure 1.

3. G-C rich template producing strong secondary structure. Try less DNA, longer extension times, more cycles, more enzyme, 5% DMSO, or a 96°C denaturation temperature.

Film blank or very faint

1. If using single-sided film, the emulsion side must be placed facing the dried gel.
2. DNA preparation may be bad. Try the control DNA supplied in the kit.
3. Labeled dideoxynucleotide too old. Try longer exposure.
4. Some component missing.
5. Enzyme lost activity.
6. Insufficient template DNA or insufficient number of cycles. Try more DNA, more cycles or longer film exposure.
7. Incorrect temperatures for primers used. Try a lower temperature for cycling (e.g. 50°-95°C), especially when using dITP.
8. Incorrect termination time or temperature for dITP. Termination should be 5-10 minutes at 55-60°C.
8. Too little primer used. The recommended amount of primer is 0.5-2.5pmol.
9. Primer bad. Some primers form dimers, hairpins etc., interfering with annealing with the template. Try a different primer.
10. Wrong amounts of dNTP or [α -³³P]ddNTP used. Check volumes added.
11. Large excess of primer and DNA used. Check quantities added to reaction.

Bands faint near the primer

1. Too much dNTP or too little [α -³³P]ddNTP used. Check volumes added.

Bands smeared

1. Contaminated DNA preparation. Try control DNA. Thermo Sequenase DNA polymerase is sensitive to salt concentration, especially above 75mM.
2. Gel may be bad. Gels should be cast with fresh acrylamide solutions and should polymerize rapidly, within 15 minutes of pouring. Try running a second gel with the same samples.
3. Gel run too cold. Sequencing gels should be run at 40-55°C.
4. Gel dried too hot or not flat enough to be evenly exposed to film.
5. Samples not denatured. Make sure samples are always heated to 70°C for at least 2 minutes (longer in an air-filled heat block) immediately prior to loading on gel. When re-loading a sample (e.g. for a second gel or a double-loaded gel) the heating step should be repeated.

Bands appear across all 4 lanes

1. Gel compression artifacts. Sometimes a band in all 4 lanes indicates a severe gel compression caused by secondary structures not completely denatured during electrophoresis. If the gel has a region where the bands are very closely spaced, followed by a region where the bands are widely

spaced, a compression artifact is indicated. Try using the dITP reaction mixture or a formamide gel.

Bands in 2 or 3 lanes

1. Heterogeneous template DNA (2 bands) caused by spontaneous deletions arising during M13 phage growth. Try control DNA and limit phage growth to less than 6-8 hours.
2. Insufficient mixing of reaction mixtures.
3. The sequence may be prone to compression artifacts in the gel. Compressions occur when the DNA (usually G-C rich) synthesized by the DNA polymerase does not remain fully denatured during electrophoresis. Try using the dITP reaction mixture, or a 30-40% formamide gel.

If problems persist please contact USB Technical Support for assistance at (800) 321-9322 or techsupport@usbweb.com in the United States. For your authorized distributor and support staff outside the United States, contact your local GE Healthcare office. Contact information is listed in the back of this protocol booklet.

CONTROL DNA SEQUENCE

The control DNA included in the kit is from pUC18, a double-stranded circular DNA of 2.7kb. A partial sequence of this DNA is given below (14).

(Universal cycle primer)

```

5' -G TTTTCCCAGT CACGACGTTG TA->
AACGCCAGGG TTTTCCCAGT CACGACGTTG TAAAACGACG GCCAGTGCCA
    10           20           30           40           50
AGCTTGCATG CCTGCAGGTC GACTCTAGAG GATCCCCGGG TACCGAGCTC
    60           70           80           90          100
GAATTCGTAA TCATGTCATA GCTGTTTCTT GTGTGAAATT GTTATCCGCT
                                <--CTTTAA CAATAGGCGA
    110          120          130          140          150
CACAAATCCA CACAACATAC GAGCCGGAAG CATAAAGTGT AAAGCCTGGG
GTGTT-5' (Reverse cycle primer)
    160          170          180          190          200
GTGCCTAATG AGTGAGCTAA CTCACATTAA TTGCGTTGCG CTCACTGCC
    210          220          230          240          250
GCTTCCAGT  CGGGAAACCT GTCGTGCCAG CTGCATTAAT GAATCGGCCA
    260          270          280          290          300
ACGCGCGGGG AGAGGCGGTT TGCGTATTGG GCGCTCTTCC GCTTCTTCG
    310          320          330          340          350
TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC GGTATCAGCT
    360          370          380          390          400
CACTCAAAGG CGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGC

```

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RELATED PRODUCTS

Kits and Enzymes

Product	Application	Pack size	Product number
Sequenase PCR Product Sequencing Kit	For rapid sequencing of PCR products	100 templates	70170
Sequenase Quick-Denature Plasmid Sequencing Kit	For rapid denaturation and sequencing of plasmid DNA	100 templates	70140
Sequenase Version 2.0 DNA polymerase	For non-cycle radioactive sequencing	200 units 1000 units	70775Y 70775Z
Sequenase Version 2.0 DNA Sequencing Kit	For non-cycle radioactive sequencing	100 templates	70770
Thermo Sequenase™ Cycle Sequencing Kit	For radioactive cycle sequencing	100 templates	78500

PCR Clean-up

Product	Application	Pack size	Product number
ExoSAP-IT	Removes primers and dNTPs from PCR products	100 rctns 500 rctns 2000 rctns	78200 78201 78202

USB Ultrapure reagents for DNA sequencing

Product	Application	Pack size	Product number
Agarose, high efficiency separation >1000bp		25g	10132-25g
		100g	10132-100g
Agarose, high efficiency separation >500bp		25g	10133-25g
		100g	10133-100g
Ammonium Persulfate	Gel electrophoresis	1kg	76322-1kg
		100g	76322-100g
Antibiotic G418	Cloning	1g	11379-1g
		100mg	11379-100mg
Glycerol Tolerant Gel Buffer, pre-mixed powder	Gel electrophoresis	6 bottles	71949
Glycerol Tolerant Gel Buffer, 20X solution	Gel electrophoresis	1 liter	75827-1L
IPTG	Cloning	1g	10078-1g
		1g	10078-5g
LB Broth	Cloning	250g	75852-250g
		1kg	75852-1kg
LB Agar	Cloning	250g	75851-250g
		1kg	75851-1kg
Mineral Oil	Sequencing/PCR	10ml	71600-10ml
		25ml	71600-25ml
RapidGel-6%	Gel electrophoresis	500ml	75843-500ml
RapidGel-8%	Gel electrophoresis	500ml	75844-500ml
RapidGel-GTG-6%	Gel electrophoresis	500ml	75846-500ml

Product	Application	Pack size	Product number
RapidGel-GTG-8%	Gel electrophoresis	500ml	75847-500ml
RapidGel-40%	Gel electrophoresis	500ml	75848-500ml
RapidGel-XL-6%	Gel electrophoresis	500ml	75861-500ml
RapidGel-XL-8%	Gel electrophoresis	500ml	75862-500ml
RapidGel-XL-40%	Gel electrophoresis	500ml	75863-500ml
TBE Buffer, 10X	Gel electrophoresis	6 bottles	70454-1pk
TEMED	Gel electrophoresis	100g 500g	76320-100g 76320-500g
Tris	Gel electrophoresis	5kg 1kg	75825-5kg 75825-1kg
Urea	Gel electrophoresis	1kg 500g	75826-1kg 75826-500g
Water, RNase-free		500ml 1 liter	70783-500ml 70783-1L
X-Gal	Cloning	250mg	10077-250mg
Xylene cyanol	Gel electrophoresis	25g	23513-25g

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Material safety data sheet

Revision: 10/30/00

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****SUPPLIER:**

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

PRODUCT NAME

Thermo Sequenase Radiolabeled
 Terminator Cycle Sequencing Kit

EMERGENCY CONTACT:

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

PRODUCT CODE/EC NUMBER

79750, 79760 None

COMPOSITION/**HAZARDOUS****COMPONENTS**

<u>HAZARD</u>	<u>CAS NO.</u>	<u>%WT</u>	<u>TLV</u>	<u>CHIP R & S PHRASES</u>
Formamide in 70724	75-12-7	96%	10ppm	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
Tris-HCl in Part 79802	1185-53-1	4.1%	—	No applicable information
Tris in Part 71949	77-86-1	74%	—	
EDTA in Part 71949 For the Part 71949 mixture/preparation	6381-92-6	1.4%	—	R:36/38 Irritating to eyes and skin S:26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.



Formamide

HAZARDS IDENTIFICATION

CHIP

Formamide: Toxic to reproduction, category 3. Tris: Irritant.

HCS

Formamide: Teratogen. Tris-HCl, Tris and EDTA: Irritant.

Remove from exposure. Flush from skin or eyes with water. If irritation is evident or if ingested or inhaled, seek medical advice.

For small fires only: Use carbon dioxide, dry powder or foam.

Wear suitable protective clothing including lab coat, safety glasses and gloves to clean small releases.

Wear suitable protective clothing including lab coat, safety glasses and gloves. Store at -20°C.

Same as handling and storage information. Pregnant women or women of child bearing age should minimize contact and exposure to formamide.

Molecular biology sequencing kit containing separate vials and solutions of reagents.

Product is stable. Avoid freeze-thaw cycles. Store at -20°C.

Formamide: Has caused embryotoxicity and birth defects in animal studies; may cause damage to liver and denatures proteins; may be absorbed through the skin. Tris-HCl: May cause irritation to eyes, skin and mucous membranes; may be harmful if ingested in large quantities. Tris and EDTA may cause irritation to skin, eyes and mucous membranes.

No information available

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

No applicable information.

No applicable information.

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*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.

§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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