Sequenase Quick-Denature Plasmid Sequencing Kit

Product Number 70140
100 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.

usb®

CONTENTS

COMPONENTS OF THE KIT

The solutions included in the Sequenase™* Quick-Denature Plasmid 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's standards. It is strongly recommended that the reagents supplied in the kit be used.

The following solutions are included in the kit:

Plasmid Reaction Buffer (concentrate), 300µl;
1.0M Tris-HCl, pH 7.5, 100mM $MgCl_2$, 250mM NaCl

Control DNA, pUC19, 0.15µg/µl

Primer (-40 forward, 23-mer), 2µM; 2.0pmol/µl
5'-GTTTTCCCAGTCACGACGTTGTA-3'

Primer (-50 reverse, 21-mer), 2µM; 2.0pmol/µl
5'-TTGTGAGCGGATAACAATTTC-3'

Plasmid Denaturing Reagent (**yellow-capped** tube), 1000µl
10mM Tris-HCl, pH 7.5, 1mM ethylenediamine tetraacetic acid (EDTA), 50% glycerol, 50% ethylene glycol

NaOH (**yellow-capped** tube), 250µl; 1.0M

HCl (**yellow-capped** tube), 250µl; 1.0M

Dithiothreitol (DTT) solution, 150µl; 0.1M

Labeling Mix (7-deaza-dGTP*) (5X concentrate; **green-capped** tube), 100µl; 7.5µM 7-deaza-dGTP, 7.5µM dCTP, 7.5µM dTTP

ddG Termination Mix (for 7-deaza-dGTP, **red-capped** tube), 250µl; 80µM 7-deaza-dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddGTP, 40mM Tris-HCl, pH 7.6, 50mM NaCl

ddA Termination Mix (for 7-deaza-dGTP, **red-capped** tube), 250µl; 80µM 7-deaza-dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddATP, 40mM Tris-HCl, pH 7.6, 50mM NaCl

ddT Termination Mix (for 7-deaza-dGTP, **red-capped** tube), 250µl; 80µM 7-deaza-dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddTTP, 40mM Tris-HCl, pH 7.6, 50mM NaCl

ddC Termination Mix (for 7-deaza-dGTP, **red-capped** tube), 250µl; 80µM 7-deaza-dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddCTP, 40mM Tris-HCl, pH 7.6, 50mM NaCl

*See license information on back cover.

Mn Buffer** (Not for dITP), 100µl;
0.15M Sodium isocitrate, 0.1M $MnCl_2$

Stop Solution, 2 x 1.25ml
95% Formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF

Sequenase Plasmid Sequencing Formulation (**blue-capped** tube), 200µl; preblended Sequenase Version 2.0 DNA polymerase and inorganic pyrophosphatase$^{\infty}$ in 20mM Tris-HCl, pH 7.5, 2mM DTT, 0.1mM EDTA and 50% glycerol.

This kit and all the enclosed reagents should be stored frozen at -20°C (NOT in a frost-free freezer). Keep all reagents on ice when removed from storage for use. Sequenase Version 2.0 enzyme must be stored at -20°C. Never store Sequenase enzyme in a frost-free freezer since the temperature rises above 0°C daily.

QUALITY CONTROL

All kit batches are functionally tested using radiolabeled dATP 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 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 material.

Warning: Contains formamide. See Material Safety Data Sheet on page 32.

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).

PROPERTIES OF SEQUENASE VERSION 2.0 DNA POLYMERASE

Sequenase Version 2.0 DNA polymerase, like the original Sequenase DNA polymerase, as described by Tabor and Richardson (1), is a superior enzyme for deoxyribonucleic acid (DNA) sequencing. It is a genetic variant of bacteriophage T7 DNA polymerase created by *in vitro* genetic manipulation (2). The genetic modifications of Sequenase Version 2.0 completely remove the 3' $\rightarrow$ 5' exonuclease activity of native, wild-type T7 DNA polymerase. Its properties also include high processivity, high speed, and the ability to incorporate the popular nucleotide analogs used for sequencing (ddNTPs, alpha-thio dNTPs, 7-deaza-dGTP, dITP, etc.). Compared with original Sequenase, version 2.0 enzyme has lower exonuclease activity, higher processivity, higher purity, higher specific activity, and somewhat better stability. The same, simple reaction conditions which have been used with the original Sequenase polymerase can be used with Sequenase Version 2.0 for DNA sequencing. Protocols are included for making use of the properties of Sequenase Version 2.0 polymerase when used in the presence of Mn^{2+} (3). The addition of manganese can greatly improve band uniformity and the ability to read sequence close to the priming site.

INTRODUCTION TO DNA SEQUENCING

Sequencing of plasmid DNA has undergone rapid improvement since the introduction of the chain-termination DNA sequencing method (4). The chain-termination method involves the *synthesis* of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA template. Synthesis is initiated at only the one site where an oligonucleotide primer anneals to the template. The synthesis reaction is terminated by the incorporation of a nucleotide analog that will not support continued DNA elongation (hence the name chain-termination). The chain-terminating nucleotide analogs are the 2',3'-dideoxynucleoside-5'-triphosphates (ddNTPs). These lack the 3'-OH group necessary for DNA chain elongation. When proper mixtures of dNTPs and one of the four ddNTPs are used, enzyme-catalyzed polymerization will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP, give complete sequence information. A radioactively labeled nucleotide is also included in the synthesis, so the labeled chains of various lengths can be visualized by autoradiography after separation by high-resolution electrophoresis.

SEQUENCING PLASMID DNA

Originally, chain-termination methods were limited to using single-stranded template DNAs. This requirement was met elegantly through the use of bacteriophage M13 as a cloning vector. Bacteriophage M13 can yield sufficient DNA for most sequencing purposes in cultures as small as 0.5ml. Plasmid vectors such as pBR322 and later, pUC vectors, gained popularity for cloning, in part because they are easy to handle, provide convenient selection schemes, and can yield 5µg of double-stranded DNA, suitable for restriction analysis and ligation from 1ml cultures.

Several early methods for sequencing plasmid DNAs were developed, but none proved satisfactory until the introduction of alkaline denaturation methods (5-9). This method takes advantage of the fact that covalently closed circular DNAs will form special, 'collapsed' structures when denatured which do not readily re-anneal and which contain regions which behave like single-stranded DNA. DNA denatures at high pH because the guanine and thymine bases become negatively charged above pH 10. Denaturation with alkali was favored over heat denaturation because most circular plasmid DNAs have melting temperatures above 100°C. Some workers have reported success in sequencing plasmid DNA simply by boiling to denature (10). Presumably this method works by denaturing the fraction of the plasmid DNA preparation that is nicked or linear, but not the covalently closed circular forms. Our own experience is that simple heat denaturation works well but only with relatively large quantities of template DNA.

The original alkaline denaturation methods involved four steps—mixing the DNA with alkali, neutralizing with concentrated acetate buffer, precipitation with ethanol and re-dissolving the DNA in reaction buffer. The precipitation step serves both to concentrate the DNA and to separate it from the salts added in prior steps. These steps work quite well to generate single-strand template suitable for sequencing, but the precipitation and re-dissolving steps are time consuming and laborious.

Two simple and efficient denaturation methods that do not require precipitation of plasmid DNA are recommended for use with this kit. Both allow for the rapid generation of denatured template DNA suitable for generating high-quality sequence data.

One method involves the addition of a reagent that destabilizes DNA secondary structure. The plasmid denaturing reagent in this kit is a mixture of 50% glycerol and 50% ethylene glycol[Δ]. Like urea and formamide, these glycols decrease the melting temperature of DNA, but they do not interfere with the polymerase activity (11). Double-stranded plasmid DNA and primer are mixed with this glycol reagent so that the final concentration of glycols is

approximately 40%. This concentration is sufficient to decrease the melting temperature of most plasmids to less than 90°C. The mixture is then incubated at 90-100°C for five minutes, denaturing the plasmid. Following denaturation, buffer is added and the mixture is incubated briefly at 37°C to allow the primer to anneal to the appropriate sequence within the template. The glycols used for denaturation in this procedure will cause distortions in sequencing gels run with Tris-boric acid-ethylenediamine tetraacetic acid (TBE) buffer in the 300-400 base region. Appropriate glycerol tolerant sequencing gel recipes are described in the electrophoresis section.

The second method involves the addition of NaOH directly to a mixture of purified plasmid DNA and primer, denaturing the double-stranded DNA (12). After a short incubation at 37°C, an equimolar amount of HCl is added to the mixture, thereby neutralizing the alkali. Plasmid reaction buffer is added (fixing the pH at an appropriate value). The mixture is then incubated briefly at 37°C to allow the primer to anneal to the appropriate sequence within the template. The NaOH and HCl combine to form NaCl which is normally a component of the DNA sequencing reaction mixtures. As long as the concentration of NaCl is kept below approximately 0.2M, the polymerase works well and high-quality sequence is obtained.

A major determinant of success for sequencing plasmid DNA is the purity and quantity of the plasmid DNA template. Good results can be obtained using traditional methods of purification such as CsCl gradients, polyethylene glycol (PEG) precipitations and both boiling and alkaline miniprep procedures. Any of these methods may work in your laboratory, and choice may be simply a matter of personal preference and finding a method which works well for you.

SEQUENCING WITH SEQUENASE VERSION 2.0 DNA POLYMERASE

The unique properties of Sequenase Version 2.0 DNA polymerase (high processivity, no 3' $\rightarrow$ 5' exonuclease activity, and the efficient use of nucleotide analogs important for sequencing) produce radioactive bands of uniform intensity and low background radioactivity. Template DNA is purified by standard techniques, and is annealed to a synthetic oligonucleotide primer. DNA synthesis is carried out in two steps. The first is the labeling step and the second is the chain-termination step using dideoxynucleotides. In the first step, the primer is extended using limiting concentrations of the deoxynucleoside triphosphates, including radioactively labeled dATP. This step continues to virtual complete incorporation of labeled nucleotide into DNA chains which are distributed randomly in length from several nucleotides to hundreds of

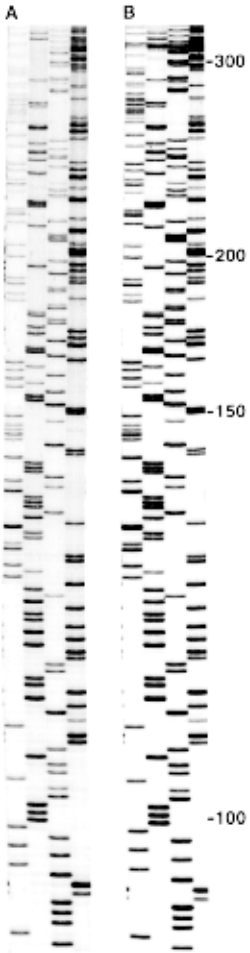

Figure 1. **Comparison of glycol/heat denaturation (A) with alkaline denaturation without precipitation (B).** Glycol denaturation will sometimes have an even, gray background (A) and requires the use of glycerol tolerant gel buffer for reading beyond 250-300 bases. Alkaline denaturation typically has lower background (B) but often exhibits 'bands-in-all-lanes' artifacts as seen in this example just above 300 nucleotides.

nucleotides. In the second step, the concentration of all the deoxynucleoside triphosphates is increased and a dideoxynucleoside triphosphate is added. Processive DNA synthesis occurs until all growing chains are terminated by a dideoxynucleotide. During this step, the chains are extended on the average only several dozen nucleotides. The reactions are terminated by the addition of EDTA and formamide, denatured by heating and run on electrophoretic gels. The entire synthesis process can be completed in as little as ten minutes. Some alterations of the standard conditions are suggested if the focus of a particular experiment demands it. **Note**: The concentrations of nucleotides in these steps must be appropriate for Sequenase Version 2.0 enzyme. Mixtures designed for other DNA polymerases will not work with Sequenase Version 2.0 DNA polymerase.

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. When this occurs, sequence information is lost. The substitution of a nucleotide analog for dGTP (7-deaza-dGTP or dITP) which forms weaker secondary structure is often successful in eliminating these gel artifacts (13-15). Both dITP and 7-deaza-dGTP are incorporated into DNA by Sequenase polymerase. When 7-deaza-dGTP is used, most compressions are eliminated. This kit conatains 7-deaza-dGTP in the labeling and termination mixes.

Many factors limit the ability to determine long, accurate sequences by the chain-termination method. The resolution of autoradiography is limited by the use of high-energy ^{32}P which gives diffuse bands on film. The use of lower energy ^{35}S or ^{33}P labeled nucleotides greatly improves autoradiographic resolution (16, 17). The use of gradient electrophoresis gels (both buffer gradient and field gradient) increases the number of bands visible on a single gel run (18, 19).

Another limitation to resolution is the quality of the DNA polymerization. Ideal polymerization would give equal numbers of chains terminated at each correct nucleotide (or at least equal signal intensity) and no 'false' terminations at incorrect nucleotides. This would result in uniform intensities of bands and the absence of background bands. Sequenase Version 2.0 DNA polymerase produces very even band intensities, especially with Mn^{+2} Buffer, and low backgrounds.

USE OF PYROPHOSPHATASE FOR SEQUENCING

When running sequencing reactions (especially with dITP), some of the bands on the resulting sequencing gel can appear to be weak. This is particularly noticeable if the termination reactions are run for 30 minutes or longer. This band-weakening is caused by slow, sequence-dependent reversal of the DNA polymerase reaction, pyrophosphorolysis (20, 21). This can be eliminated by adding pyrophosphatase to the sequencing reaction. Pyrophosphatase is formulated with the Sequenase polymerase for optimum performance when sequencing with dGTP or its analogs (dITP, 7-deaza-dGTP, etc.) and is effective in the presence of Mg^{2+}, Mn^{2+}, or both.

MATERIALS NOT SUPPLIED

Necessary reagents:

α Labeled dATP*	Product codes†
[α-^{33}P]dATP	AH 9904/BF 1001
[α-^{35}S]dATP	AG 1000/SJ 1304
[α-^{32}P]dATP	AA 0004/PB 10204

*The specific activity should be 1000-1500Ci/mmol.

†Codes correspond to radiolabeled nucleotides available from Amersham Pharmacia Biotech.

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

Tris-EDTA (TE) buffer—This buffer is 10mM Tris-HCl, 1mM EDTA, pH 7.5. It is used for template preparation.

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

Specialized sequencing primers—Some sequencing projects will require the use of primers which are specific to the project. For most sequencing applications, 0.5-2.0pmol 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 concentration (pmol/µl) is given by the following formula:

Concentration (pmol/µl)=OD_{260}/(0.01 x N)

Necessary equipment:

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

Electrophoresis equipment—While standard, non-gradient sequencing gel apparatus is sufficient for much sequencing work, the use of field-gradient ('wedge') or salt-gradient gels will allow much greater reading capacity on the gel (18, 19). A power supply offering constant power operation and 2000V or greater is essential.

Gel handling—If ^{35}S or ^{33}P sequencing is desired, a large tray for washing the gel (to remove urea) and a gel drying apparatus are necessary. Gels containing ^{35}S or ^{33}P must be exposed dry in direct contact with the film at room temperature.

Autoradiography—Any large format autoradiography film and film cassette, such as Hyperfilm™ and Hypercassettes™, can be used. Development of films is performed according to the film manufacturer's instructions.

PROTOCOLS

Sequencing Reaction Protocols

All sequencing reactions are run in small plastic centrifuge tubes (typically 0.5ml), which should be kept capped 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 the micropipette, avoiding the creation of air bubbles. At any stage where the possibility exists for some solution to cling to the walls of the tube, it should be centrifuged. With care and experience these reactions can be completed in 30-40 minutes, including the plasmid denaturation. Previous methods requiring ethanol precipitation could take up to 2 hours just for plasmid denaturation.

Brief protocols

1. Denature double-stranded templates with either of the quick denaturing protocols below:

A. **Glycol/heat denaturation**

Combine the following in a microcentrifuge tube:

DNA (0.5-3µg)	_µl	(Up to 7µl)
H_2O	_µl	(To adjust total volume)
Plasmid denaturing reagent	5µl	
Primer (2-4pmol)	1µl	
Total	13µl	

Mix thoroughly (the mixture is viscous). Incubate at 90-100°C for 5 minutes. Chill the mixture in an ice water bath.
Then add:

Plasmid reaction buffer	2µl	
Total	15µl	

Continue with annealing in step 2 below.

B. **Alkaline denaturation (without precipitation)**

Combine the following in a microcentrifuge tube:

DNA (0.5-3µg)	_µl	(Up to 8µl)
H_2O	_µl	(To adjust total volume)
1.0M NaOH	2µl	
Primer (2-4pmol)	1µl	
Total	11µl	

Mix thoroughly, incubate for 10 minutes at 37°C. Place the mixture on ice.
Then add:

HCl, 1.0M	2µl
Plasmid reaction buffer	2µl
Total	15µl

Continue with annealing in step 2 below.

2. **Annealing**: Incubate the template/primer/buffer mixture at 37°C for 10 minutes. Chill on ice.
3. While annealing, label, fill and cap tubes with 2.5µl of each termination mixture (G, A, T and C, red-capped vials). Keep covered on ice for steps 5 and 7.
4. Dilute labeling mix 5-fold to working concentration. Retain for use in step 6.

Labeling mix	_µl	(typically 2µl)
H_2O	_µl	(typically 8µl)

5. Pre-warm 4 termination tubes from step 3 (G, A, T and C) in 37°C bath.
6. **Labeling reaction**

To ice-cold annealed DNA mixture add:	(15µl)
DTT, 0.1M	1µl
Diluted labeling mix	2µl
[α-^{35}S], [α-^{33}P] or[α-^{32}P]dATP (5µCi)	0.5µl
Sequenase plasmid sequencing formulation	2µl
Total	20.5µl

   Mix and incubate at room temperature 2-5 minutes (5-10 minutes for glycol-denatured plasmid).
7. **Termination reactions**

   Transfer 4.5µl of labeling reaction to each termination tube (G, A, T and C), mix and continue incubation of the termination reactions at 37°C for 5 minutes.
8. Stop the reactions by adding 4µl of stop solution.
9. Heat samples to 75°C, 2 minutes immediately before loading onto sequencing gel. Load 2-4µl in each lane.

Detailed notes on protocols

Denaturing plasmid template DNA

1. It is important to use high-quality plasmid DNA for best sequencing results. Preparations which contain large fractions of nicked or cut plasmid DNAs may produce sequences with high backgrounds and false bands resulting from the strand breaks. Double-stranded plasmid DNA must be denatured prior to sequencing. Denaturation of covalently closed circular DNA cannot usually be achieved by boiling in annealing or reaction buffers since the melting temperature is typically above 105°C. Two convenient and rapid methods of denaturing plasmids are offered with this kit. One makes use of the fact that glycerol and other glycols decrease the melting temperature of plasmid DNAs. The other makes use of alkali to denature the DNA. Both methods are simple and rapid and we have found that both methods work well for a majority of templates. If one method fails it may be that the other will yield good sequence information.

   For both of these denaturation protocols we recommend using 0.5-3µg (0.5-1.0pmol) of template DNA and 2-4pmol of primer. If a smaller volume of DNA solution is used, the balance should be made up with distilled water. We have had good results with a molar stoichiometry (primer:template) of 4:1.

This stoichiometry should be maintained when using templates of higher or lower molecular weight. The use of too little template (or primer) will narrow the effective sequencing range, resulting in faint bands near the bottom of the gel. This kit requires less plasmid template DNA than the Sequenase Version 2.0 kit since no DNA is lost during ethanol precipitation.

2. **Denaturation by heating in the presence of glycols.** The Plasmid Denaturing Reagent contained in this kit is a buffered, 50-50 mixture of glycerol and ethylene glycol. Addition of this mixture to a DNA solution in the proportions recommended (approx. 40% glycol v/v) decreases the melting temperature of DNA by about 20°C. In fact, we have found that heating the DNA in this mixture to temperatures as low as 75°C can denature the DNA in some cases. **Note:** The use of the glycol reagent will cause distortions on sequencing gels buffered with TBE in the region 300-400 bases from the primer. The use of glycerol tolerant gel buffer‡ (which replaces boric acid with taurine) will eliminate this distortion (see 'Denaturing gel electrophoresis' section).

   For the control DNA included in the kit use:

DNA	5µl
H_2O	2µl
Plasmid Denaturing Reagent	5µl
Primer	1µl
Total	13µl

   Place the tube in a water bath or thermal cycler which is >90°C. After 5 minutes, quick chill the tube on ice for 5 minutes.

   Add:

Plasmid Reaction Buffer	2µl
Total	15µl

   Proceed with annealing.

3. **Denaturation with alkali.** Plasmid DNA will denature (at any temperature) when exposed to pH 13. The method outlined in this protocol requires that the alkali used to denature the DNA be precisely neutralized by the addition of HCl. Thus, it is best to use the same pipetting device at the same setting to measure both the NaOH and HCl used for this procedure. The NaOH and HCl solutions used in the kit are carefully adjusted to have equal concentrations of 1.0M. If you must substitute your own NaOH or HCl solutions, be sure they are within the range of 0.95-1.05M. The alkaline denaturation methods which follow denaturation by ethanol precipitation (6-9) will also work with the reagents in this kit.

For the control DNA included in the kit use:

DNA	5µl
H_2O	3µl
NaOH, 1M	2µl
Primer	1µl
Total	11µl

Place the tube at 37°C for 10 minutes. Return the tube to ice.

Add:

HCl, 1M	2µl
Plasmid reaction buffer	2µl
Total	15µl

Proceed with annealing below.

4. For sequencing single-stranded DNA such as clones in M13 vectors, the denaturation step should be omitted. Simply mix single-stranded DNA (0.5pmol; approx. 1µg of an M13 clone) with primer, 2µl of plasmid reaction buffer and water to a final volume of 15µl and proceed with annealing below.

Annealing template and primer

1. For each denatured template, a single annealing (and subsequent labeling) reaction is used. The total volume from either of the denaturing methods above should be 15µl.

   Warm the capped tube to 37°C for 10 minutes to promote annealing (other times and temperatures have been used successfully). Place the tube on ice. Annealed template should be used within about 4 hours.

Labeling reaction

1. For standard reactions (reading sequences up to 400 or so bases from the primer), dilute the labeling mix (7-deaza-dGTP, **green-capped** tube) 5-fold with distilled water (e.g. 4µl of mix combined with 16µl of water). This diluted nucleotide solution should be stable for several weeks if stored frozen at -20°C. **Note:** For sequencing within 30 bases of the primer, dilution should be about 15-fold and the amount of template DNA must be greater than 0.5pmol (preferably 2µg of plasmid). Insufficient DNA (or primer) will reduce the labeling of the first few nucleotides from the primer. A better alternative for obtaining sequence close to the primer is to use Mn Buffer (see below).
2. **Never** add Sequenase Plasmid Sequencing Formulation to labeling mix, DTT solution or other non-buffered solutions. Enzyme may be added to pre-mixed cocktails only after all other materials are added.

3. To the annealed template-primer add the following (on ice):

Template-primer (above)	15.0µl	
DTT, 0.1M	1.0µl	
Diluted labeling mix	2.0µl	
Radiolabeled dATP	0.5µl	(See note below)
Sequenase plasmid sequencing formulation (with pyrophosphatase)	2.0µl	(Always add enzyme separately, as the last component)
Total	20.5µl	

Mix thoroughly (avoiding bubbles) and incubate for 2-5 minutes at room temperature (or cooler-sometimes incubation for too long or too warm will give sequence artifacts within 100 bases of the primer). **The reactions contain more than 5% glycerol, therefore, incubations can be extended to 30 minutes at room temperature or at 37°C for 5 minutes. This may be both more convenient and help reduce the possibility of false-priming (11). Note:** The amount of labeled nucleotide can be adjusted according to the needs of the experiment. Either [α-^{33}P]dATP (17), [α-^{35}S]dATP or [α-^{32}P]dATP can be used. Nominally, 0.5µl of 10µCi/µl and 10µM (1000Ci/mmol) dATP should be used. Larger amounts have little effect on the reactions unless higher concentrations of the other dNTPs are used. As little as 0.1µl (1µCi) can be used for many experiments.

Note: Mn Buffer (1µl) can be added to the labeling reaction after the 5 minute incubation at room temperature, if desired, to emphasize bands close to the primer (see 'Reading sequences close to the primer' section).

Termination reactions

1. Have on hand 4 tubes labeled G, A, T and C.
2. Place 2.5µl of the ddGTP termination mix in the tube labeled G. Similarly fill the A, T and C tubes with 2.5µl of the ddATP, ddTTP and ddCTP termination mixes respectively. Cap the tubes to prevent evaporation. (This is best done before beginning the labeling reaction.)
3. Pre-warm the tubes at 37°C at least 1 minute.
4. When the labeling incubation is complete, remove 4.5µl and transfer it to the tube labeled G. Mix, and continue incubation of the G tube at 37°C. Similarly transfer 4.5µl of the labeling reaction to the A, T and C tubes, mixing and returning them to the 37°C bath. If necessary, centrifuge briefly to collect the solution at the tube bottom.
5. Continue the incubations for a total of 5 minutes. (Incubations can usually be extended to 30 minutes without problems.)

6. Add 4µl of stop solution to each of the termination reactions, mix thoroughly and store on ice until ready to load the sequencing gel. Best results are obtained if the gel is run on the same day as the reactions.
7. When the gel is ready for loading, heat the samples to 75-80°C for 2 minutes and load immediately on the gel. Use 2-4µl in each lane. Samples containing glycerol are more easily loaded using standard 'yellow' pipet tips.

Preparation of double-stranded templates

A major determinant of success for sequencing plasmid DNA is the purity and quantity of the plasmid DNA template. Good results can be obtained using various methods of purification such as CsCl gradients, polyethylene glycol (PEG) precipitations, both boiling and alkaline mini-prep procedures and other commercially available plasmid preparation devices.

Denaturing gel electrophoresis

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, the temperature of the gel during electrophoresis, and proper drying of the gel after running. 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.

Gel electrophoresis reagents

Note: TBE should NOT be used with this kit since the enzyme formulation used contains sufficient glycerol to distort TBE gels above 300 bases.

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

Buffer

20X Glycerol Tolerant Gel Buffer (1 liter)

216gm	Tris base
72gm	Taurine
4gm	$Na_2EDTA{\cdot}2H_2O$
	H_2O to 1000ml, filter (may be autoclaved)

This buffer can be used with samples containing glycerol at any concentration (11). If gels seem to run a bit slower with this buffer at 1X strength, use it more

dilute—approximately 0.8X strength. Available in liquid and powder pre-mix forms, product numbers 75827 and 71949, respectively. Be certain to run glycerol tolerant gels at the same power (wattage) as TBE-buffered gels so the gel temperature is normal.

Gel recipes (for 100ml of gel solution)

Gel conc. (%)	Acrylamide/ bis-acrylamide	Urea (7-8.3M)	20X Gly. tol. gel buffer	H_2O
6%	5.7gm/0.3gm	42-50gm	5ml*	~45ml
8%	7.6gm/0.4gm	42-50gm	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 N, N, N', N' tetramethyl-ethylenediamine (TEMED).
*Use 4ml for faster gel migration.

Formamide-containing gels may be used for very strong compressions not resolved by 7-deaza-dGTP. They will require higher running voltage and run more slowly than urea-only gels. Prior to drying, these gels should be soaked in 5% acetic acid, 20% methanol to prevent swelling.

Gel recipes for formamide gels (for 100ml of gel solution)

Gel conc. (%)	Acrylamide/ bis-acrylamide	Urea (7M)	20X Gly. tol. gel buffer	Formamide	H_2O
6%	5.7gm/0.3gm	42gm	5ml	30-40ml	~10ml
8%	7.6gm/0.4gm	42gm	5ml	30-40ml	~10ml

Warming to 35-45°C may be required to dissolve completely. Adjust volume to 100ml with H_2O, filter and de-gas. When ready to pour add 1ml of 10% ammonium persulfate and 100–150µl TEMED.

RapidGel Information

USB Ultrapure RapidGels, ready-to-use liquid acrylamide, makes DNA sequencing simpler and more convenient. Gels can be prepared in minutes without the need to weigh harmful reagents. RapidGel gel mixes are available in 4%, 5%, 6% or 8% solutions with 7M urea; or a 40% stock solution containing 19:1 acrylamide to bis-acrylamide may be used for a customized percentage. TBE and Glycerol Tolerant Gel formulations are offered.

General guidelines for electrophoresis

1. Electrophoresis grade reagents should be used.
2. Solutions of monomers should be made fresh. Storage longer than one week in the dark at 4°C is not recommended.
3. Gels should be prepared 2–20 hours prior to use, and pre-run for 15-60 minutes.

4. It is usually convenient to run gels for reading longer sequences overnight (with a timer). Gel runs of 18–24 hours at 40–50 watts are often necessary for reading in the 400-600 range. Resolution on these gels will be higher if they are run at 40–45°C surface temperature (cooler than typical gel runs).
5. Loading 8 adjacent lanes in a pattern that abuts all pairs of lanes (e.g. GATCGTAC) aids reading closely spaced bands. In this manner, all lanes are adjacent to all others.
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) 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 ^{35}S or ^{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 (leaving plastic-wrap on helps to prevent the film from sticking to incompletely-dried gels) will require longer drying and exposure times but give sufficient resolution for most purposes.
9. High quality autoradiography film can improve image contrast and resolution.
10. In general, overnight to 36 hour exposures are sufficient when using fast film (Hyperfilm MP).
11. The use of tapered spacers ('wedge' gels) or salt-gradients improves overall resolution and allows more nucleotides to be read from a single loading (18, 19).

Alterations of reaction conditions

Reading short or very long sequences

Depending on the region of DNA to be sequenced in a particular experiment, reaction conditions can be altered to provide maximum information of sequences close to the primer, or alternatively those more distant from the primer. These can be best understood by describing the events that take place during each phase of the sequencing reactions.

During the labeling reaction, the enzyme extends the primers and consumes the available 7-deaza-dGTP, dCTP, dTTP and labeled dATP. The extensions are heterogeneous and range in size from several nucleotides to 80-150 nucleotides. The longer extensions are much less common, but they contain proportionately more label so they still appear on gels. In the termination step,

the addition of high levels of dNTPs and the ddNTP at 37°C result in the further elongation of each chain at a very rapid rate (hundreds per second) until a dideoxynucleotide is incorporated. Since the enzyme does not discriminate strongly between dNTPs and ddNTPs, and the ratio of deoxynucleotide to dideoxynucleotide is 10 to 1, the extensions in this phase are limited to about 150 additional nucleotides on the average (although the observed range is quite wide). The addition of Mn^{2+} to the reaction mixture makes the dideoxynucleotides much more potent chain-terminating nucleotides (3). Thus, the addition of Mn Buffer to the reactions will decrease the extensions in the termination reactions. This also improves the ability to read sequence close to the primer.

Reading sequences close to the primer

There are two methods for specifically emphasizing sequence very close to the primer. One is to use less nucleotide in the labeling step and the other is to use Mn Buffer which affects the reactions in the termination step. With either of these methods, the gel should be run only until the first blue dye runs about 80% of the length of the gel (typically 1-2 hours).

Labeling Step Method—The general conditions described in this manual should be followed for sequencing from the primer up to 300-400 nucleotides. If the interest is only in sequences close to the primer (<200 nucleotides), it is possible to dilute the labeling mix further (a 1:10 or 1:20 dilution of the stock reagent) and keep both reaction times to 3-5 minutes. When reading sequences within 20 nucleotides of the 3' end of the primer, it is essential that sufficient template DNA and primer be present. It is a good practice to double the usual amounts of each for optimal results.

Mn Buffer Method—The general conditions will generate sequencing ladders which are faint or absent for nucleotides close to the primer if limited amounts of DNA (less than 0.5pmol or approximately 1µg of M13) are used for the reactions (Figure 2). A solution to this situation is to add the Mn Buffer provided with the Sequenase Quick-Denature plasmid sequencing kit. This reagent takes advantage of the activity of Sequenase Version 2.0 DNA polymerase in the presence of Mn^{2+} ions (3). The addition of Mn^{2+} to normal (Mg^{2+}) sequencing reactions (with fixed deoxy- to dideoxy- ratios) reduces the average length of DNA synthesized in the termination step, intensifying bands corresponding to sequences close to the primer. With Mn^{2+}, sequences from less than 20 nucleotides from the primer up to approximately 200 nucleotides can be observed even with reduced amounts of template (Figure 2).

Mn Buffer is a buffered solution of $MnCl_2$ which can be added to normal sequencing reactions. To use this reagent, simply add 1µl of Mn Buffer to the labeling reaction after incubation (immediately before dividing the mixture among termination reaction vials). No other changes are necessary. The normal

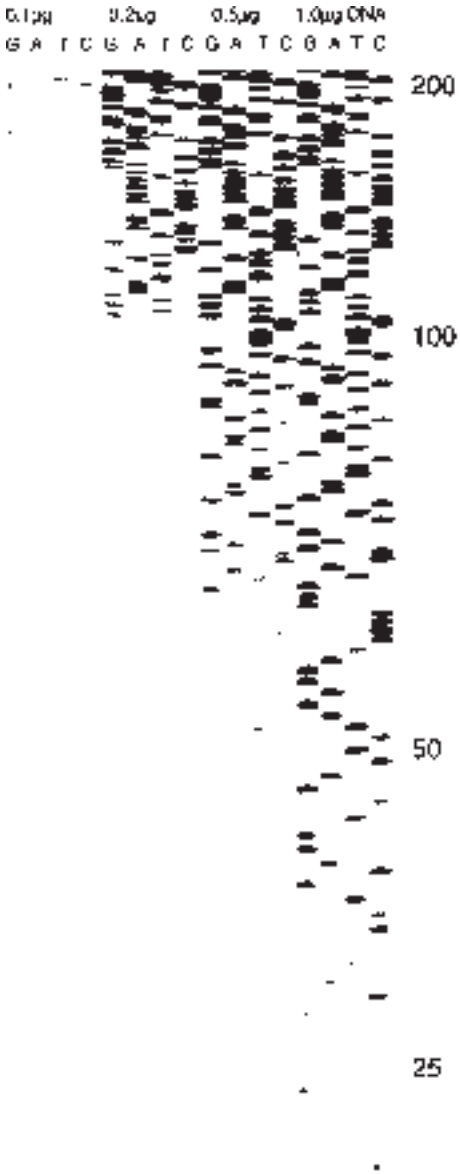

Figure 2. **Normal sequences with Mg^{2+}**

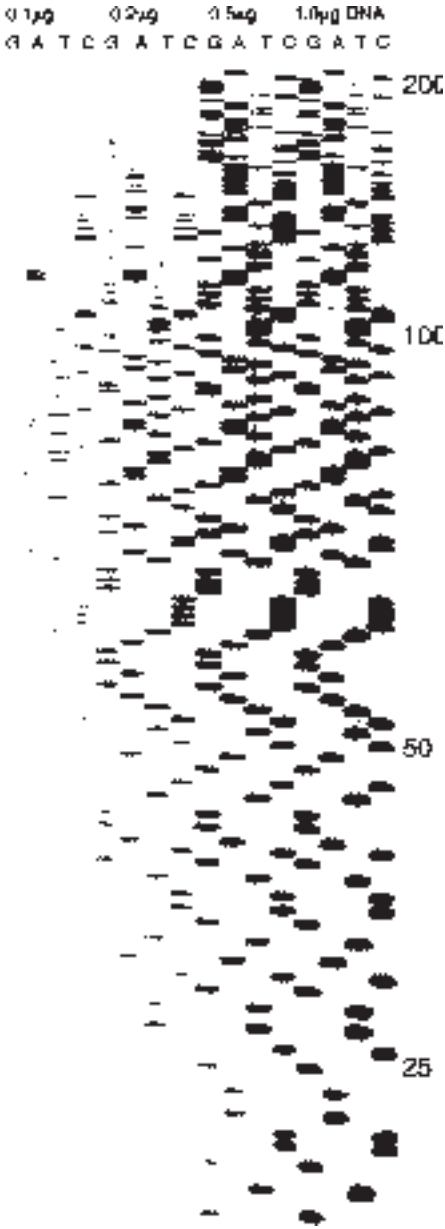

Figure 3. **Sequences with Mn buffer**

Plasmid Reaction Buffer and other reagents are included as usual.

A typical reaction using Mn Buffer would begin with a normal annealing step:

To annealed template and primer (15µl) add:

DTT solution	1µl
Labeling mix (diluted 1:5)	2µl
[α-^{35}S], [α-^{33}P] or [α-^{32}P]dATP	0.5µl
Sequenase DNA plasmid sequencing formulation	2µl
Total	20.5µl

Incubate 5 minutes at room temperature (20°C). Add:

Mn Buffer	1µl
Total	21.5µl

Then proceed with normal termination reactions, transferring 4.5µl portions to each of the four termination reaction tubes.

Notes:

1. It is not recommended to pre-mix the Mn Buffer with any other reagents prior to use. It may oxidize, forming a yellow-brown precipitate.
2. Mn Buffer is effective for sequences generated using dGTP and 7-deaza-dGTP. It is not recommended for dITP sequences because the ddG lane will be faint.
3. The bromophenol blue dye in gel lanes containing Mn Buffer will appear somewhat different from normal during electrophoresis. This does not interfere with gel resolution or readability.
4. The amount of Mn Buffer added to the reactions is not critical. Comparable results will be obtained if 0.2-2.0µl of Mn Buffer are added to the reaction. Intermediate effects are not observed since Mn Buffer has an 'all-or-none' effect.
5. Reaction products should be run on gels within 2-3 days of preparation.

Extending sequences farther from the primer

When using high-resolution electrophoresis gels, it is possible to see that the sequence-specific bands generated by the normal sequencing protocol begin to fade at about 600-800 nucleotides from the primer. (The precise point where sequence information fades out depends on many factors including the template DNA concentration, the primer concentration, the label concentration and the film exposure conditions.) This is entirely normal; the Sequenase Quick-Denature plasmid sequencing kit was designed to limit extensions to the more easily resolved range up to 500-600 bases (extensions beyond this point

are just wasted radioactivity if they cannot be read). If your sequencing gel protocol is capable of resolving sequence bands beyond 400-600 bases, you may want to further extend the polymerization reactions to obtain sequence information beyond this point.

For reading beyond 300-400 nucleotides, the concentrations of the dNTPs in the labeling reaction can be increased 3 to 5-fold (i.e. use the 7-deaza-dGTP labeling mixture undiluted) and the labeling reaction lengthened to 5 minutes. This increase in concentration applies to dATP as well, so additional labeled dATP must be added to the labeling reaction (1-2µl of 10µM, 10µCi/µl). This will result in a sequence ladder which emphasizes (darkens) the bands in the 200-800 nucleotide range at the expense of the bands in the 20-100 nucleotide range. It has been our experience that sequences run using 7-deaza-dGTP do not have sharp bands beyond 350-400 nucleotides, restricting the length of sequence that can be read with this analog. For best results with long gels, dGTP or dITP usually give sharper bands. Reagents for sequencing with dGTP and dITP are available separately.

Running sequencing gels which resolve more than 400 nucleotides requires high quality apparatus, chemicals and attention to many details. While specific instructions are beyond the scope of this manual, here are some general guidelines. The gel should be loaded with 8 adjacent lanes (GATCGTAC, or see 'Denaturing gel electrophoresis' section) with a sharkstooth comb and be run 4 to 10 times longer than usual. For this kind of experiment, very long gels (80-100cm) are almost a necessity. The highest resolution gels appear to be approximately 6-8% acrylamide and run relatively cool (40°C). This means they should be run at lower power than normal gels. Reactions should be run with ^{35}S or ^{33}P and gels dried down at a temperature of no more than 80°C.

Elimination of compressions

Regions of secondary structure in the product DNA can produce several kinds of gel artifacts, some of which may go unnoticed when reading a gel. One way to ensure that the correct sequence is determined is to sequence both strands in opposite directions. This practice is a very good one, but requires additional work. It has been observed that the substitution of dI for dG in DNA eliminates the secondary structures that produce gel compression artifacts (13, 14). Similarly, substitution of 7-deaza-dGTP has also been used (15). Either of these substitutions can be made with Sequenase polymerase. 7-deaza-dGTP has been found to work effectively and is the analog used in this kit. Suitable nucleotide mixtures containing dGTP and dITP are available in other kits. Alternatively, the inclusion of up to 40% formamide in sequencing gels (along with 7M urea) has been successful for eliminating very strong compressions (see 'Denaturing gel electrophoresis' section for methods).

Glycerol enables higher reaction temperatures

Sequenase DNA polymerase, like many enzymes, is stabilized by glycerol. Unfortunately, the boric acid present in most sequencing gels interacts with glycerol, so loading of more than a few micrograms of glycerol onto sequencing gels results in severe distortions. If a sequencing gel is prepared without boric acid using a glycerol tolerant gel buffer, then higher concentrations of glycerol can be present in sequencing reaction mixtures (11). The polymerase/ pyrophosphatase formulation in 50% glycerol buffer included with this kit will increase the concentration of glycerol present in the labeling reaction from 0.8% to 5%. When the plasmid denaturing reagent is used, the glycerol concentration in the labeling reaction will be increased by 12%. The glycerol concentration is high enough to stabilize the polymerase so that the labeling reactions can be incubated up to 30 minutes at room temperature (20°C) or even up to 5 minutes at 37°C. Increasing the concentration of glycerol to 20% in the labeling step will further stabilize the polymerase so that even longer labeling reactions can be tolerated or brief reactions at even higher temperature (1 minute, pre-warmed to 45°C, adding polymerase last) can be run. Higher temperature (stringency) labeling reactions can be beneficial since primer specificity will be greater (11).

Termination reaction temperatures can also be reliably increased when the glycerol concentration of the termination reaction mixture is increased. For instance, with 25% glycerol, termination reactions pre-warmed to 60°C or even higher can be run (11). Be sure to run glycerol tolerant gels at the same power (wattage) as the TBE-buffered gels so that the gel temperature is normal.

Note: When using increased glycerol concentrations, a glycerol tolerant gel buffer should be used for the sequencing gel. See 'Denaturing gel electrophoresis' section.

TROUBLESHOOTING

Problem **Possible causes and solutions**

Film blank or nearly blank

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 nucleotide too old.
4. Some component missing.
5. Enzyme lost activity.
6. No priming, try control DNA and primer in the kit.

Bands smeared

1. Contaminated DNA preparation; try control DNA.
2. Gel may be bad. Gels should be cast with freshly made 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 or too hot. 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 75°C for at least 2 minutes (longer in a heat block) immediately prior to loading on gel.

Autoradiogram appears distorted in upper third

1. Too much glycerol present in samples. Use glycerol tolerant gel buffer (see 'Denaturing gel electrophoresis' section) or ethanol precipitate samples to remove glycerol. Precipitated samples should be re-dissolved in stop solution and heat-denatured as usual.

Sequence faint near the primer

1. Insufficient DNA in the sequencing reaction. A minimum of 0.5pmol of DNA is required for sequencing close to the primer. This usually corresponds to about 2-3µg of plasmid DNA. Try increasing the amount of DNA or try adding 1µl of the Mn Buffer to the labeling reaction.
2. Insufficient primer. Use a minimum of 0.5pmol. Primer to template molar ratio should be 1:1 to 5:1.
3. Labeling reaction time too short. When using the plasmid denaturing reagent, the labeling reaction has a half-time for synthesis of about 2.5 minutes. Incubation of this reaction step for 10-15 minutes will increase the intensity of the sequence.

Bands appear across all 4 lanes

1. DNA preparation may be bad. Try the control DNA in the kit and re-purify the template DNA.
2. Reagents not mixed thoroughly during the reactions. Mix carefully after each addition, avoiding bubbles and centrifuging to bring all solution to the tip of the tube.
3. Be sure that the annealing step is not run too long or too hot. It is usually sufficient to heat the mixture to 37°C for 10 minutes.
4. The labeling step should not be run warmer than 20°C or longer than 5 minutes without added glycerol. Doing so will often result in many 'pause' sites in the first 100 bases from the primer. The addition of glycerol to the labeling step (as with the use of the Plasmid Sequencing Formulation of the Sequenase/pyrophosphatase enzyme mix provided in this kit) can greatly

improve enzyme stability during this step, allowing longer and warmer labeling reaction incubations (up to 30 minutes at 20°C or 5 minutes at 37°C). The use of glycerol will require the use of a glycerol tolerant electrophoresis gel (see 'Denaturing gel electrophoresis' section).

5. The termination step should not be run cooler than 37°C or longer than 15 minutes. Room temperature termination reactions (even ones where the tubes are not pre-warmed) will promote this problem above 100 bases from the primer. Termination reactions can be run up to 50°C (especially with the addition of glycerol as in 4 above), which may improve results for some templates.
6. Sequences with strong secondary structure. Sequenase Version 2.0 polymerase will pause at sites of exceptional secondary structure, especially when dITP is used. Try reducing the concentration of nucleotides in the labeling step to keep extensions during this step from reaching the pause site or using slightly more Sequenase Version 2.0 enzyme on difficult templates. If the problem persists, the addition of 0.5µg of single-stranded DNA binding protein (SSB) (70032Y/Z) during the labeling reaction usually eliminates the problem. When using SSB, it is necessary to inactivate it prior to running the gel. Add 0.1µg of proteinase K (76230Y/Z) and incubate at 65°C for 20 minutes after adding the stop solution.

Bands in 2 or 3 lanes

1. Heterogeneous template DNA caused by isolation of multiple plasmids. Re-purify the plasmid-containing strain on selective media.
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. Some compression artifacts are not completely resolved by the use of 7-deaza-dGTP. Try using a formamide-containing electrophoresis gel (see 'Denaturing gel electrophoresis' section) or using dITP-containing reaction mixtures (available separately) to eliminate gel compressions.

Sequence fades early in one lane

1. Template DNA has a biased nucleotide composition. This is common for cDNA templates which have poly-A sequences. In this case, the 'T' lane does not extend as far as the others. This is caused by early exhaustion of dTTP and ddTTP in the reactions. Try adding a solution of dTTP (120µM) to the 'T' reaction only (use 2µl of 120µM dTTP and 1µl 'T' termination mix.) This situation may also be improved by adding extra dTTP to the labeling reaction (1µl of 500µM dTTP).

If problems persist, please contact Technical Service for assistance.

CONTROL DNA SEQUENCE

The control DNA included in the kit is pUC19, a double-stranded circular DNA of 3.0 Kb. A partial sequence of this DNA is given below (22).

```
          (-40 Forward 23-mer 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
             <—CTTTA ACAATAGGCG AGTGTT (-50 Reverse 21-mer Primer)
       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
       620         630         640         650         660         670
TTACCGGATA CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT CAATGCTCAC
```

REFERENCES

1. TABOR, S., and RICHARDSON, C. C. (1987) *Proc. Nat. Acad. Sci. USA* **84**, 4767-4771.
2. TABOR, S., and RICHARDSON, C. C. (1989) *J. Biol. Chem.* **264**, 6447-6458.
3. TABOR, S., and RICHARDSON, C. C. (1989) *Proc. Nat. Acad. Sci. USA* **86**, 4076-4080.
4. SANGER, F., NIKLEN, S., and COULSON, A. R. (1977) *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467.
5. TONEGUZZO, F., GLYNN, S., LEVI, E., MJOLSNESS, S., and HAYDAY, A. (1988) *BioTechniques* **6**, 460-469.
6. CHEN, E. J., and SEEBURG, P. H. (1985) *DNA* **4**, 165-170.
7. HALTINER, M., KEMPE, T., and TJIAN, R. (1985) *Nucleic Acids Research* **13**, 1015-1026.
8. HATTORI, M., and SAKAKI, Y. (1986) *Anal. Biochem.* **152**, 232-238.
9. LIM, H. M., and PENE, J. J. (1988) *Gene Anal. Techniques* **5**, 32-39.
10. ANDERSON, A. S., PETTERSSON, A. F., and Kjeldsen, T. B. (1992) *BioTechniques* **13**, 678-679.
11. PISA-WILLIAMSON, D., and FULLER, C. W. (1992) *Comments* **19** No. 2, p.1, 7 United States Biochemical Corp., Cleveland, OH.
12. HSIAO, K. (1991) *Nucleic Acids Research* **19**, 2787.
13. BARNES, W. M., BEVAN, M., and SON, P. H. (1983) *Methods in Enzymology* **101**, 98-122.
14. GOUGH, J. A., and MURRAY, N .E. (1983) *J. Mol. Biol.* **166**, 1-19.
15. MIZUSAWA, S., NISHIMURA, S., and SEELA, F. (1986) *Nucleic Acids Research* **14**, 1319-1324.
16. BIGGIN, M. D., GIBSON, T. J., and HONG, G. F. (1983) *Proc. Nat. Acad. Sci. USA* **80**, 3963-3965.
17. ZAGURSKY, R. J., CONWAY, P. S., and KASHDAN, M. A. (1991) *BioTechniques* **11**, 36-38.
18. ANSORGE, W., and LABEIT, S. (1984) *J. Biochem. and Biophys. Method.* **10**, 237-243.
19. SHEEN, J., and SEED, B. (1988) *BioTechniques* **6**.
20. TABOR, S., and RICHARDSON, C. C. (1990) *J. Biol. Chem.* **265**, 8322-8328.
21. RUAN, C. C., SAMOLS, S. B., and FULLER, C. W. (1990) *Comments* **17** No. 1, p. 1, United States Biochemical Corp., Cleveland, OH.
22. YANISCH-PERRON, C., VIEIRA, J., and MESSING, J. (1985) *Gene* **33**, 103-119.

RELATED PRODUCTS

Sequencing kits

Product	Application	Pack size	Product Number
Sequenase Version 2.0 DNA Sequencing Kit	For non-cycle radioactive sequencing	100 templates	70770
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
Thermo Sequenase™ Cycle Sequencing Kit	For cycle radioactive sequencing	100 templates	78500

Sequencing enzymes

Product	Application	Pack size	Product Number
Sequenase Version 2.0 DNA Polymerase	For non-cycle radioactive sequencing	200 units 1000 units	70775Y 70775Z

Sequencing reagents

Product	Application	Pack size	Product Number
Ammonium Persulfate	Gel electrophoresis	1kg 100g	32810-1KG 32810-100G
DNA Sequencing Gel Mix, 6%, Ultrapure	Supplied with TBE. Each bottle makes 125ml of gel solution	5 bottles	72990-5BTL
DNA Sequencing Gel Mix, 8%, Ultrapure	Supplied with TBE. Each bottle makes 125ml of gel solution	5 bottles	70090-5BTL
DNA Sequencing Gel Mix, 6%, Glycerol Tolerant, Ultrapure	Supplied with Glycerol Tolerant running buffer. Each bottle makes 125ml of gel solution	5 bottles	72994-5BTL
DNA Sequencing Gel Mix, 8%, Glycerol Tolerant, ultrapure	Supplied with Glycerol Tolerant running buffer. Each bottle makes 125ml of gel solution	5 bottles	72992-5BTL
Glycerol Tolerant Gel Buffer, pre-mixed powder	Gel electorphoresis	6 bottles	71949
Glycerol Tolerant Gel Buffer, 20X solution	Gel electorphoresis	1 liter	75827
RapidGel™-6%	Gel electrophoresis	100ml 500ml	75843-100ml 75843-500ml
RapidGel-8%	Gel electrophoresis	100ml 500ml	75844-100ml 75844-500ml
RapidGel-GTG-6%	Gel electrophoresis	100ml 500ml	75846-100ml 75846-500ml

Product	Application	Pack size	Product Number
RapidGel-GTG-8%	Gel electrophoresis	100ml 500ml	75847-100ml 75847-500ml
RapidGel-40%	Gel electrophoresis	500ml	75848-500ml
RapidGel-XL-6%	Gel electrophoresis	100ml 500ml	75861-100ml 75861-500ml
RapidGel-XL-8%	Gel electrophoresis	100ml 500ml	75862-100ml 75862-500ml
RapidGel-XL-40%	Gel electrophoresis	100ml 500ml	75863-100ml 75863-500ml
Taurine, Ultrapure	Gel electrophoresis	1kg	75824-1KG
TEMED	Gel electrophoresis	500g 100g	32825-500G 32825-100G

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Material Safety Data Sheet

Revision: 9/26/95

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
	Sequenase Quick-Denature plasmid sequencing kit	US 70140	None

SUPPLIER:

Amersham International plc
Amersham Place Little Chalfont
Buckinghamshire England HP7 9NA 01494 544000

U.S. CONTACT:

Chemtrec: 1-800-424-9300

COMPOSITION/ HAZARDOUS COMPONENTS

HAZARD	CAS NO.	%WT	TLV	CHIP R & S PHRASES
Formamide in US 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
Ethylene glycol in 70141	107-21-1	50%	50ppm	R:22 Harmful if swallowed S:02 Keep out of reach of children
Sodium hydroxide in 70136	1310-73-2	4%	2mg/m^3	R:34 Causes burns
Tris-HCl in 70142	1185-53-1	15.8%	—	No applicable information
Dithiothreitol in 70726	27565-41-9	1.5%	—	No applicable information
Hydrochloric acid in 70135	7647-01-0	3%	5ppm	No applicable information

HAZARDS IDENTIFICATION

CHIP

Formamide: Toxic to reproduction, category 3. Ethylene glycol: Harmful. Sodium hydroxide: Corrosive

	HCS Formamide: Teratogen. Tris-HCl & dithiothreitol: Irritant. Ethylene glycol: Ingestion hazard. Hydrochloric acid & sodium hydroxide: Corrosive.
FIRST-AID MEASURES	Remove from exposure. Flush from skin or eyes with water. If irritation is evident or if ingested or inhaled, seek medical advice.
FIRE-FIGHTING INFORMATION	For small fires only: Use carbon dioxide, dry powder or foam.
ACCIDENTAL RELEASE MEASURES	Wear suitable protective clothing including lab coat, safety glasses and gloves to clean small releases.
HANDLING AND STORAGE	Wear suitable protective clothing including lab coat, safety glasses and gloves. Store at -20°C.
PERSONAL PROTECTION	Same as handling and storage information. Pregnant women or women of child bearing age should minimize contact and exposure to formamide.
PHYSICAL AND CHEMICAL PROPERTIES	Kit containing vials of solutions.
STABILITY AND REACTIVITY	Product is stable. Avoid freeze-thaw cycles. Store at -20°C.
TOXICOLOGICAL INFORMATION	Formamide: Has caused embryotoxicity and birth defects in animal studies; may cause damage to liver and denatures proteins; may be absorbed through the skin. Ethylene Glycol: Harmful if ingested, inhaled or absorbed through the skin; may be a reproductive hazard; causes irritation to skin, eyes and mucous membranes; may cause nervous system disturbances; prolonged exposure can cause nausea, headache and mucous membranes and upper respiratory tract and eyes and skin. Tris-HCl: May cause irritation to eyes, skin and mucous membranes; may be harmful if ingested in large quantities. Hydrochloric acid: May cause severe irritation to skin, eyes and mucous membranes; ingestion and inhalation are harmful to respiratory and gastrointestinal tract. Dithiothreitol: May cause irritation to skin, eyes and mucous membranes.
ECOLOGICAL INFORMATION	No information available
DISPOSAL CONSIDERATIONS	Dispose of material in accordance with applicable local, state, federal regulations.
TRANSPORTATION INFORMATION	No applicable information.
REGULATORY INFORMATION	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 a patented DNA sequencing method in those patents solely for use with Sequenase DNA polymerase purchased from USB Corporation for research activities. Use of this kit (reagent) for commercial activities is not licensed. By way of example, but not by way of limitation, this license does not grant rights to use the kit (reagent) (1) for any procedure involving the diagnosis, treatment, prevention or mitigation of disease or other health-related condition or involving the detection of pathogens or genetic traits for medical uses; (2) for agriculture or veterinary medicine; (3) for cloning or producing nucleotide sequences for commercial sale. 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, HP7 9NA England.

*Sequenase DNA polymerase—This reagent (kit) is covered by or suitable for use under one or more US Patent numbers: 4,795,699, 4,946,786, 4,942,130, 4,962,020, 4,994,372, 5,145,776, 5,173,411, 5,266,466, 5,409,811, 5,498,523, 5,639,608 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.

**Mn Buffer—Purchase includes a non-exclusive sublicense solely for use with Sequenase DNA polymerase. No other license is granted expressly, impliedly or by estoppel.

ΔGlycol/heat denaturation method—This product and/or its method of use is covered by one or more of the following patent(s): US Patent number 5,500,339 and foreign equivalents.

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

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