Sequenase Version 2.0 DNA Sequencing Kit

Product Number 70770
100 reactions

Also for use with:

Sequenase dGTP Nucleotide Reagent Kit
Product Number 70754

Sequenase dITP Nucleotide Reagent Kit
Product Number 70752

Sequenase Reagent Kit
Product Number 70721

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

CONTENTS

COMPONENTS OF THE KIT

The solutions included in the Sequenase™ Version 2.0 DNA sequencing kit from USB™ have been carefully prepared to yield the best possible sequencing results. Each reagent has been tested extensively to meet rigorous standards. It is strongly recommended that the reagents supplied in the kit be used as directed.

The following solutions are included in the kit:

Sequenase Version 2.0 DNA Polymerase (**blue-capped** tube), 25µl;
13U/µl in 20mM KPO_4, pH 7.4, 1mM DTT, 0.1mM EDTA, 50% glycerol

Inorganic Pyrophosphatase (**purple-capped** tube), 25µl;
4U/ml in 10mM Tris·HCl, pH 7.5, 0.1mM EDTA, 50% glycerol

Sequenase Reaction Buffer (5X concentrate), 1ml;
200mM Tris·HCl, pH 7.5, 100mM $MgCl_2$, 250mM NaCl

Control DNA, M13mp18, 50µl; 0.2µg/µl

Primer (-40 M13), 100µl; 0.5pmol/µl
5'-GTTTTCCCAGTCACGAC-3'

Dithiothreitol (DTT) Solution, 150µl; 0.1M

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

Labeling Mix (dITP) (5X concentrate, **yellow-capped** tube), 100µl;
15µM dITP, 7.5µM dCTP, 7.5µM dTTP

ddG Termination Mix (for dGTP, **red-capped** tube), 250µl;
80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddGTP, 50mM NaCl

ddA Termination Mix (for dGTP, **red-capped** tube), 250µl;
80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddATP, 50mM NaCl

ddT Termination Mix (for dGTP, **red-capped** tube), 250µl;
80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddTTP, 50mM NaCl

ddC Termination Mix (for dGTP, **red-capped** tube), 250µl;
80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddCTP, 50mM NaCl

Sequence Extending Mix (for dGTP), 100µl;
180µM each dGTP, dATP, dCTP, dTTP, 50mM NaCl

ddG Termination Mix (for dITP, **orange-capped** tube), 125µl;
160µM dITP, 80µM dATP, 80µM dCTP, 80µM dTTP, 1.6µM ddGTP, 50mM NaCl

ddA Termination Mix (for dITP, **orange-capped** tube), 125µl;
160µM dITP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddATP, 50mM NaCl

ddT Termination Mix (for dITP, **orange-capped** tube), 125µl;
160µM dITP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddTTP, 50mM NaCl

ddC Termination Mix (for dITP, **orange-capped** tube), 125µl;
160µM dITP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddCTP, 50mM NaCl

Sequence Extending Mix (for dITP), 50µl;
360µM dITP, 180µM each dATP, dCTP, dTTP, 50mM NaCl

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

Stop Solution, 2 x 1.2ml;
95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF

Enzyme Dilution Buffer, 1ml; 10mM Tris·HCl, pH 7.5, 5mM DTT

Glycerol Enzyme Dilution Buffer (**pink-capped** tube), 250µl;
20mM Tris·HCl, pH 7.5, 2mM DTT, 0.1mM EDTA, 50% glycerol

All kit components should be stored frozen at -20°C and for longest life be kept on ice when thawed for use. Never store Sequenase Version 2.0 enzyme in a frost-free freezer (the temperature rises above 0°C daily).

QUALITY CONTROL

All kit batches are functionally tested using radiolabeled-dATP and M13mp18 single-stranded DNA template as described in this protocol. Release specifications are based on sequence length, band intensity and sequence quality. The sequence must be visible up to 300 base pairs on a standardized gel with less than 24 hours exposure. The sequence must also be free of background bands strong enough to interfere with sequence interpretation.

SAFETY WARNINGS AND PRECAUTIONS

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

Caution: 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 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 TO CHAIN-TERMINATION SEQUENCING

The Sequenase chain-termination DNA sequencing method (1,2) involves the *in vitro* synthesis of a DNA strand by a DNA polymerase using a specifically primed single-stranded DNA template. DNA synthesis is carried out in two steps. The first is the labeling step in which 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. These initial primer extensions are distributed randomly in length from several nucleotides to hundreds of nucleotides. In the second step, the concentration of all the deoxynucleoside triphosphates is increased and a chain-terminating nucleotide analog is added. These 2',3'-dideoxynucleoside-5'-triphosphates (ddNTPs) lack the 3'-OH group necessary for DNA chain elongation. Processive DNA synthesis occurs, with extensions on the average of only several dozen nucleotides, until all growing chains are terminated by a ddNTP. 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. The sequencing reactions are stopped by the addition of EDTA and formamide, denatured by heating, separated by high-resolution denaturing acrylamide gel electrophoresis and visualized by autoradiography.

Sequencing with Sequenase Version 2.0 DNA polymerase

Sequenase Version 2.0 DNA polymerase, as described by Tabor and Richardson (2), is a superior enzyme for isothermal DNA sequencing. It is a genetic variant of bacteriophage T7 DNA polymerase created by in vitro genetic manipulation (3). The genetic modifications of Sequenase Version 2.0 completely remove the 3'->5' exonuclease activity of native, wild-type T7 DNA polymerase. Its properties also include high processivity, high speed and the ability to incorporate nucleotide analogs needed for sequencing (ddNTPs, α-thio dNTPs, dITP, 7-deaza-dGTP, etc.) (4).

Note: The concentrations of nucleotides in these reactions must be appropriate for Sequenase Version 2.0 enzyme. Mixtures designed for thermostable DNA polymerases, Klenow, or reverse transcriptase will not work with Sequenase Version 2.0 DNA polymerase.

MATERIALS NOT SUPPLIED

Necessary reagents:

For 3-dNTP protocol:

α Labeled dATP*	Product codes†
[α-^{33}P]dATP	AH9904/BF1001
[α-^{35}S]dATP	AG1000/SJ1304
[α-^{32}P]dATP	AA0004/PB10204

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

†Codes correspond to radiolabeled nucleotides available from GE Healthcare.

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, 0.5-1.0pmol of primer should be used for each set of sequencing reactions. See ‘Supplementary Information, Quantity of template, primer and primer:template ratio’ for details.

Necessary equipment:

Constant temperature bath—Sequencing will require incubations at room temperature, 37°C, 65°C and 75°C. The annealing step will require slow cooling from 65°C to room temperature.

Electrophoresis equipment—While a standard, non-gradient sequencing gel apparatus is sufficient for most sequencing work, the use of field-gradient (‘wedge’) gels will allow greater reading capacity on the gel (5). A power supply offering constant power operation at 2000V or greater is essential.

Gel handling—If ^{35}S or ^{33}P sequencing is desired, a large tray for soaking 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, such as Kodak Biomax™ MR and film cassette, can be used. Development of films is performed according to the film manufacturer's instructions.

PROTOCOLS

Recommended pre-dilution of polymerase

This kit contains two reagents which can be used to provide increased convenience and reliability to sequencing experiments by pre-diluting the Sequenase DNA polymerase before use. These are the Inorganic Pyrophosphatase$^{\infty}$ (**purple-capped** tube) and the Glycerol Enzyme Dilution Buffer (**pink-capped** tube). The addition of pyrophosphatase will eliminate the possibility of occasional weak bands which can occur with prolonged reaction times due to the reverse polymerization reaction, pyrophosphorolysis (6,7). Dilution of Sequenase DNA polymerase in Glycerol Enzyme Dilution Buffer will eliminate the necessity of diluting the polymerase fresh for each sequencing experiment and allow more flexibility in the labeling reaction incubation time and temperature (8) (see 'Supplementary Information, Glycerol enables higher reaction temperatures'). The use of the Glycerol Enzyme Dilution Buffer will necessitate the use of a Glycerol Tolerant Gel Buffer‡ (71949) in the sequencing gel since glycerol severely distorts the upper third of ordinary TBE buffered sequencing gels. (see 'Supplementary Information, Denaturing gel electrophoresis').

First, briefly centrifuge the 3 vials to collect the contents. Add the pyrophosphatase and Glycerol Enzyme Dilution Buffer to the Sequenase Version 2.0 DNA polymerase vial.

Inorganic Pyrophosphatase	25µl	
Glycerol Enzyme Dilution Buffer	150µl	
Sequenase DNA polymerase	25µl	(Add other reagents to this vial)
Total	200µl	(For 100 sequences, 2µl each)

Store frozen at -20°C.

Never dilute Sequenase Version 2.0 DNA polymerase in labeling mix, DTT solution or other non-buffered solutions. Enzyme may be added to pre-mixed cocktails only after dilution with Glycerol Enzyme Dilution Buffer or enzyme dilution buffer.

Sequencing reactions

Sequencing reactions are run in microcentrifuge tubes (typically 0.5ml) or microtiter plates. These 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 10-15 minutes.

1. **Denature double-stranded templates**, see 'Supplementary Information'.
2. **Annealing mixture**:

DNA	_µl	(Up to 7µl)
H_2O	_µl	(To adjust total volume)
Sequenase Reaction Buffer	2µl	
Primer	1µl	
Total	10µl	

   Anneal by heating 2 minutes at 65°C, then cool slowly to <35°C over 15-30 minutes. Centrifuge briefly and chill on ice for use in step 6.
3. While the annealing mixture is cooling, label, fill and cap tubes with 2.5µl of each termination mix (G, A, T and C). Use mixes from **red-capped** tubes for dGTP or **orange-capped** tubes for dITP. Keep covered at room temperature for use in steps 5 and 7.
4. Dilute labeling mix 5-fold to working concentration if needed; dGTP (**green-capped** tube) or dITP (**yellow-capped** tube). 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) for 1-5 minutes at 37°C.
6. **Labeling reaction**

   To ice-cold annealed DNA mixture (10µl), add:

DTT, 0.1M	1µl
Diluted labeling mix	2µl
[α-^{35}S or α-^{33}P]dATP	0.5µl
Diluted Sequenase polymerase	2µl
Total	15.5µl

   Mix and incubate at room temperature 2-5 minutes.
7. **Termination reactions**

   Transfer 3.5µl of labeling reaction to each termination tube (G, A, T and C), mix well 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 at 75°C for 2 minutes immediately before loading onto sequencing gel. Load 2-3µl in each lane.

SUPPLEMENTARY INFORMATION

Important to note

1. The 1:5 dilution of the labeling mix nucleotide solution should be good for several weeks if stored frozen at -20°C.
2. If the polymerase was not pre-diluted in Glycerol Enzyme Dilution Buffer (e.g. if using TBE gels), it must be diluted 1:8 in ice-cold enzyme dilution buffer. Only enough enzyme for immediate use should be diluted (2µl per reaction); diluted enzyme should be stored on ice for no more than 60 minutes.
3. The amount of labeled nucleotide can be adjusted according to the needs of the experiment. Either [α-^{35}S]dATP, [α-^{33}P]dATP (9) or [α-^{32}P]dATP (10) 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 four unlabeled dNTPs are used. As little as 0.1µl (1µCi) can be used for many experiments.

Preparation of template DNA

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. There are several published methods for preparing single-stranded DNA from clones in M13 vectors and hybrid plasmid-phage vectors (11,12).

Preparation of double-stranded template DNA

For good results, purified, RNA-free DNA must be used. Purification of plasmid DNA on CsCl gradients and PEG precipitation methods produce excellent quality DNA for sequencing. Other, more convenient methods such as alkaline lysis or boiling mini-preps, adsorption to glass and common resin and bead DNA purification methods yield sequence quality DNA. PCR products must be free of excessive primers and nucleotides. ExoSAP-IT™, 78200, from USB, provides an easy, one-tube enzymatic method for PCR clean-up. See ‘Related Products’.

Quantity of template, primer and primer:template ratio

The recommended quantity of DNA template for a reaction is approximately 1µg of single-stranded M13 or 3-5µg of plasmid DNA. Typically 0.5-1pmole of primer should be used. This is approximately a 1:1 (primer:template) molar stoichiometry. The concentration of a primer specifically synthesized for use in

sequencing should always be determined. The concentration of the primer is determined by measuring the optical density at 260nm (OD_{260}). If the primer has N bases, the approximate concentration (pmol/µl) is given by the following formula:

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

This stoichiometry should be maintained when using larger or smaller templates. The use of too little template or primer will narrow the effective sequencing range, resulting in faint bands near the bottom of the gel. When suboptimal amounts of DNA must be used data can be generated beginning near the primer by using the Mn buffer supplied with this kit (see 'Reading sequences close to the primer').

Denaturing double-stranded DNA

Plasmid DNA will denature (at any temperature) when exposed to pH 13. DNA is denatured by adding 0.1 volumes of 2M NaOH, 2mM EDTA and incubating 10-30 minutes at 37°C (13). The mixture is neutralized by adding 0.1 volumes of 3M sodium acetate (pH 4.5-5.5) and the DNA precipitated with 2 1/2 volumes of ethanol (-70°C, 15 minutes). After washing the pelleted DNA with 70% ethanol, it is redissolved in 7µl of distilled water, and 2µl of Sequenase reaction buffer and 1µl of primer are added. Annealing can be done in exactly the same manner as for single-stranded DNA or by warming to 37°C for 15-30 minutes.

Fast alkaline denaturation methods are also effective. They depend on the use of carefully calibrated 1M NaOH and 1M HCl. For dedicated plasmid sequencing projects we recommend the use of the Sequenase Quick-Denature Plasmid Sequencing Kit (70140) which contains the above reagents.

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 for dGTP (dITP or 7-deaza-dGTP) which forms weaker secondary structure has been successful in eliminating most of these gel artifacts (14-16). Both dITP and 7-deaza-dGTP are incorporated into DNA by Sequenase Version 2.0 enzyme. When dITP is used, bands are somewhat sharper and all compressions tested were eliminated while the use of 7-deaza-dGTP eliminates some (but not all) compressions tested. The substitution of dITP for dGTP is simple with this kit, and recommended for all sequences which may contain ambiguous, compressed regions. Reactions containing dITP should be performed with the Sequenase polymerase and pyrophosphatase mixture which will prevent the formation of 'holes' in the sequence due to

pyrophosphorolysis (see ‘Protocols, Recommended pre-dilution of polymerase’). 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’ for details).

To use dITP, simply substitute the dITP labeling mixture (**yellow-capped** tube) for the dGTP labeling mixture and substitute the dITP termination mixtures (**orange-capped** tubes) for the dGTP termination mixtures. Use the suggested mixture of polymerase and pyrophosphatase (see Recommended pre-dilution of polymerase). All other aspects of the sequencing protocol (dilutions, etc.) remain unchanged. Sequenase Version 2.0 DNA polymerase will sometimes pause at sites of exceptional secondary structure when dITP is used generating BAFLs (Bands Across Four Lanes). These pause sites should not be confused with gel compressions. Since the use of dITP accentuates pauses, dGTP reactions should be run in parallel with dITP reactions.

To alleviate BAFL artifacts in dITP reactions and obviate the need to run parallel dITP and dGTP reactions, a chase step can be added in which Terminal Deoxynucleotidyl Transferase (TdT) and high concentration of dNTPs are added to the termination reaction at its completion and incubated at 37° for 10-30 minutes. Stop solution is then added as usual. See TechTip #201 available from USB. Contact USB Technical Support or visit the Technical Library at www.usbweb.com. Terminal Deoxynucleotidyl Transferase is available from USB (70033).

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 so that the primer extension is more limited than normal. The other is to use Mn Buffer** which renders the ddNTPs more potent chain terminators thereby reducing the average extension during 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 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), dilute the labeling mix further (1:10 dilution of the 5X stock reagent) and keep both labeling and termination reaction times to 3-5 minutes. For sequencing within 20 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 M13). Insufficient DNA (or primer) will reduce the labeling of the first few nucleotides from the primer. 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 1). A solution to this situation is to add the Mn Buffer. This reagent takes advantage of the activity of Sequenase Version 2.0 DNA polymerase in the presence of Mn^{2+} ions (17). 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 prior to distribution to the termination reaction tube. No other changes are necessary. The normal Sequenase Reaction Buffer and other reagents are included as usual.

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 very narrow 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 as Mn Buffer has an ‘all-or-none’ effect.
5. Mn Buffer is equally effective when sequencing M13 or denatured plasmid DNA.

Reading 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 Version 2.0 DNA sequencing kit was designed to limit extensions to the more

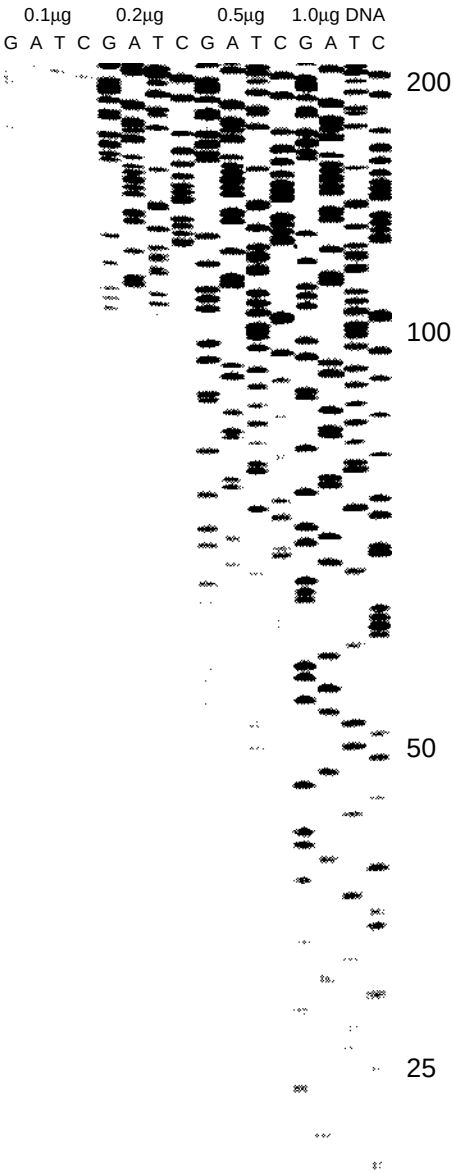

Figure 1. **Normal sequences with Mg^{2+}**

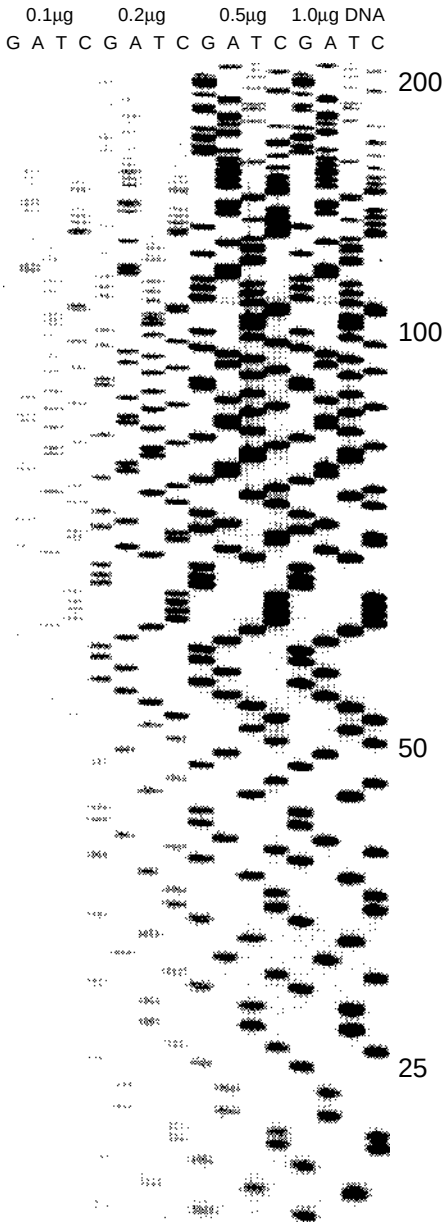

Figure 2. **Sequences with Mn buffer**

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 600 bases, you may want to further extend the polymerization reactions to obtain sequence information beyond this point. There are two simple ways to do this using the materials available in the Sequenase Version 2.0 DNA sequencing kit. One involves using more nucleotide in the labeling step and the other involves alteration of the nucleotide mixture used in the termination step.

Extending reactions in the labeling step

For reading beyond 400 nucleotides, the concentrations of the dNTPs in the labeling reaction can be increased 3-5 fold (i.e. use the dGTP or dITP 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.

Extending reactions in the termination step with sequence extending mix

There are two sequence extending mixes supplied in the Sequenase Version 2.0 DNA sequencing kit. The dGTP sequence extending mix is used with the dGTP termination mixes in the **red-capped** tubes, the dITP sequence extending mix is used with the dITP termination mixes in the **orange-capped** tubes. These mixes work equally well for sequencing single-stranded M13 templates and denatured, double-stranded plasmid templates.

To use the sequence extending mix, the usual 2.5µl volume of dideoxy-termination mix is replaced by a mixture of termination mix and sequence extending mix. The required amounts of each can be chosen with the aid of the table below; a good general choice is 1.5µl of sequence extending mix and 1µl of termination mix. Thus, when preparing the termination tubes for your extended sequencing reactions, first label 4 tubes ‘G’, ‘A’, ‘T’ and ‘C’ and put 1.5µl (or other selected volume) of sequence extending mix into each termination tube. Then add 1µl (or other selected volume) of the ddGTP termination mix to the ‘G’ tube, 1µl of ddATP termination mix to the ‘A’ tube, and similarly fill the ‘T’ and ‘C’ tubes. Cap the tubes to prevent evaporation (This is best done before beginning the labeling reaction.) Use these tubes in exactly the same manner as ‘normal’ termination reaction tubes; pre-warm the termination tubes at 1-5 minutes at 37°C, and add 3.5µl of labeling reaction product to each to begin the 5 minute termination reaction. There is no need to prolong incubations using this method. Note: If large numbers of extended sequencing reactions are to be performed, the sequence extending mix and the ddNTP termination mixes can be pre-mixed to make stocks.

Table 1. Termination mix/extending mix volumes to achieve approximate extensions.

Volume of term. mix (μl)	Volume of seq. extending mix (μl)	Total volume (μl)	Approximate relative extension*
2.5	0.0	2.5	1.0
2.0	0.5	2.5	1.5
1.5	1.0	2.5	2.5
1.0	1.5	2.5	4.0
1.0	2.0	3.0	6.0
0.5	2.0	2.5	10.0
0.25	2.25	2.5	20.0

*If sequences are visible to 700 nucleotides with no added sequence extending mix, a relative extension of 4.0 would give bands visible to approximately 700 x 4=2800 nucleotides.

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 a few general guidelines are offered. 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, 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 run relatively cool (40°C). Reactions should be run with ^{35}S or ^{33}P and gels dried down at a temperature of no more than 80°C.

Glycerol enables higher reaction temperatures

Sequenase Version 2.0 DNA polymerase, like many enzymes, is stabilized by glycerol. The recommended pre-dilution of the polymerase in the Glycerol Enzyme Dilution Buffer included with this kit will increase the concentration of glycerol present in the labeling reaction from 0.8% to 6%. This allows convenient use of the polymerase without diluting it immediately before use. It also allows higher temperature (stringency) labeling reactions to be run which can increase primer specificity (8). The polymerase is stabilized sufficiently by the use of the Glycerol Enzyme Dilution Buffer such that the labeling reactions can be incubated up to 5 minutes at 37°C or even up to 30 minutes at room temperature (20°C). The increased primer specificity can allow the use of a primer generated for a 'primer walking' sequencing scheme that inadvertently anneals at a secondary (unknown) location(s).

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 (8). This can be of aid in sequencing templates with high secondary structure.

Note: When using increased glycerol concentrations, a Glycerol Tolerant Gel Buffer should be used for the sequencing gel. See 'Denaturing gel electrophoresis' section.

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. Also, the specifics of gel 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

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

Buffers

20X Glycerol Tolerant Gel Buffer (71949 or 75827)

Tris base	216g
Taurine	72g
$Na_2EDTA{\cdot}2H_2O$	4g
H_2O to 1,000ml, filter (may be autoclaved)	

This buffer can be used with samples containing glycerol at any concentration (8). 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
$Na_2EDTA{\cdot}2H_2O$	9.3g
H_2O to 1,000ml, filter (may be autoclaved)	

This is the traditional sequencing gel buffer. It should NOT be used when the polymerase is pre-diluted in the Glycerol Enzyme Dilution Buffer (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_2O
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 H_2O, 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. OR Gel Buffer	10X TBE Buffer	Formamide	H_2O
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 H_2O, 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. Refer to TechTip #200 available from USB. Contact USB Technical Support or visit the Technical Library at www.usbweb.com.

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. 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. 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 400-600bp 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 ^{35}S or ^{33}P 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 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 Hyperfilm™-βmax or Kodak Biomax™ MR autoradiography films.
10. In general, overnight to 36 hour exposures are sufficient when using fast film such as Hyperfilm-MP from GE Healthcare.
11. The use of tapered spacers ('wedge' gels) improves overall resolution and allows more nucleotides to be read from a single loading (5).

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. Quality of DNA preparation may be poor, 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 fresh acrylamide solutions and should polymerize rapidly, within 15 minutes of pouring. Try running a second gel with the same samples, or for convenience, try RapidGels.
3. Gel run too cold or too hot; sequencing gels should be run at 45-50°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.

Upper third of autoradiogram appears distorted

1. Glycerol present in samples. If polymerase is pre-diluted in Glycerol Enzyme Dilution Buffer or glycerol is otherwise introduced into the reactions, a glycerol tolerant sequencing gel must be used. Use Glycerol Tolerant Gel Buffer (see 'Supplementary Information, 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 1μg of single-stranded M13 DNA and 3-4μg of plasmid DNA. Try increasing the amount of DNA or use the Mn Buffer (see 'Supplementary Information, Reading close to the primer').
2. Insufficient primer; use a minimum of 0.5pmol. Primer to template mole ratio should be 1:1 to 5:1.

Bands appear across all 4 lanes

1. Quality of DNA preparation may be poor; try the control DNA in the kit and repurify the template.
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 65°C and cool to room temperature within 15-30 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 (e.g. by using the Glycerol Enzyme Dilution Buffer) 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 'Supplementary Information, Denaturing gel electrophoresis' section).
5. The termination step should not be run cooler than 37°C or longer than 5 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 DNA 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.
7. Terminal Deoxynucleotidyl Transferase and a concentrated stock of the deoxynucleotides (dATP, dCTP, dGTP, dTTP) may be used in a chase step following the termination reaction to extend fragments which are terminated with a dNTP instead of a ddNTP. For a detailed protocol for this procedure see TechTip #201 (R962477/S939) available from USB. Contact Technical Support or visit the Technical Library at www.usbweb.com.

Bands in 2 or 3 lanes

1. Heterogeneous template DNA caused by spontaneous deletions arising during M13 phage growth or isolation of multiple plasmids or PCR products. Try control DNA and limit phage growth to less than 6-8 hours for M13 vectors. For plasmids and PCR products, consider repurification.
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-containing reaction mixtures to eliminate gel compressions or try using a formamide-containing electrophoresis gel (see 'Supplementary Information, Denaturing gel electrophoresis' section). For a detailed protocol on the use of formamide gels, see TechTip #200 available from USB. Contact USB Technical Support or visit the Technical Library at www.usbweb.com.

Some bands faint

1. Termination reaction time too long. If the termination reaction is allowed to continue too long, the synthesized DNA may be degraded at specific sequences, especially when dITP is used. Try adding pyrophosphatase (0.5µl, **purple-capped** tube) to the labeling step or reducing the termination reaction time (1 minute is usually sufficient). It is a good practice to pre-dilute the polymerase and pyrophosphatase together using the Glycerol Enzyme Dilution Buffer so that pyrophosphatase is used in all reactions. This will require the use of a glycerol tolerant sequencing gel (see 'Supplementary Information, Denaturing gel electrophoresis' section).

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 sequence extending mix to the 'T' reaction only (use 2µl Sequence extending mix 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 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 bacteriophage M13mp18, a single-stranded circular DNA of 7.3Kb. A partial sequence of this DNA, beginning at the priming site, is given below (18).

```
            -40 primer
          G TTTTCCCAGT CACGAC—>                       0          10
 AACGCCAGGG TTTTCCCAGT CACGACGTTG TAAAACGACG GCCAGTGCCA AGCTTGCATG
         20          30          40          50          60          70
 CCTGCAGGTC GACTCTAGAG GATCCCCGGG TACCGAGCTC GAATTCGTAA TCATGGTCAT
         80          90         100         110         120         130
 AGCTGTTTCC TGTGTGAAAT TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGGAA
        140         150         160         170         180         190
 GCATAAAGTG TAAAGCCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC
        200         210         220         230         240         250
 GCTCACTGCC CGCTTTCCAG TCGGGAAACC TGTCGTGCCA GCTGCATTAA TGAATCGGCC
        260         270         280         290         300         310
 AACGCGCGGG GAGAGGCGGT TTGCGTATTG GGCGCCAGGG TGGTTTTTCT TTTCACCAGT
        320         330         340         350         360         370
 GAGACGGGCA ACAGCTGATT GCCCTTCACC GCCTGGCCCT GAGAGAGTTG CAGCAAGCGG
 Ava II 380         390         400         410         420         430
 TCCACGCTGG TTTGCCCCAG CAGGCGAAAA TCCTGTTTGA TGGTGGTTCC GAAATCGGCA
        440         450         460  Ava I 470         480         490
 AAATCCCTTA TAAATCAAAA GAATAGCCCG AGATAGGGTT GAGTGTTGTT CCAGTTTGGA
        500         510         520         530         540         550
 ACAAGAGTCC ACTATTAAAG AACGTGGACT CCAACGTCAA AGGGCGAAAA ACCGTCTATC
        560Dra III 570         580         590         600         610
 AGGGCGATGG CCCACTCAGT GAACCATCAC CCAAATCAAG TTTTTTGGGG TCGAGGTGCC
        620         630         640 Ban II 650         660         670
 GTAAAGCACT AAATCGGAAC CCTAAAGGGA GCCCCCGATT TAGAGCTTGA CGGGGAAAGC
```

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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
Thermo Sequenase™ Cycle Sequencing Kit	For radioactive cycle sequencing	100 templates	78500
Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit	For radioactive terminator cycle sequencing	50 templates	In USA, order 188403 Outside USA, order 79750
Sequenase Version 2.0 DNA polymerase	For non-cycle radioactive sequencing	200 units 1000 units	70775Y 70775Z
Terminal Deoxynucleotidyl Transferase (TdT)	For BAFL (Bands Across Four Lanes) resolution	500 units 2500 units	70033Y 70033Z

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 100g	10132-25g 10132-100g
Agarose, high efficiency separation >500bp		25g 100g	10133-25g 10133-100g
Ammonium Persulfate	Gel electrophoresis	100g	76322-100g
Antibiotic G418	Cloning	1g 100mg	11379-1g 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
IPTG	Cloning	1g 1g	10078-1g 10078-5g
LB Broth	Cloning	250g 1kg	75852-250g 75852-1kg
LB Agar	Cloning	250g 1kg	75851-250g 75851-1kg
Mineral Oil	Sequencing/PCR	25ml	71600-25ml
RapidGel™-6%	Gel electrophoresis	500ml	75843-500ml
RapidGel-8%	Gel electrophoresis	500ml	75844-500ml

Product	Application	Pack size	Product number
RapidGel-GTG-6%	Gel electrophoresis	500ml	75846-500ml
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
TEMED	Gel electrophoresis	100g	76320-100g
TBE Buffer, 10X	Gel electrophoresis	6 bottles	70454-1pk
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

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Material Safety Data Sheet

Revision: 10/07/00

Formamide

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 Version 2.0 DNA Sequencing Kit	70770	None

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

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

COMPOSITION/ HAZARDOUS COMPONENTS

HAZARD	CAS NO.	%WT	TLV	CHIP R & S PHRASES
Formamide in 70724	75-12-7	95%	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 70702	1185-53-1	3.2%	—	No applicable information
Dithiothreitol in 70726	27565-41-9	1.5%	—	No applicable information

HAZARDS IDENTIFICATION

CHIP
Formamide: Toxic to reproduction, Category 3
HCS
Formamide: Teratogen. Tris-HCl & Dithiothreitol: Irritant

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.
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. Tris-HCl & 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.

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*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.

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