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.
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 KPO₄, 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 MgCl₂, 250mM NaCl

**Control DNA**, M13mp18, 50μl; 0.2mg/ml

**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 MnCl₂

**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).

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**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.

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**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:

<table>
<thead>
<tr>
<th>α Labeled dATP*</th>
<th>Product codes†</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\alpha^{32}\text{P}]d\text{ATP}$</td>
<td>AH9904/BF1001</td>
</tr>
<tr>
<td>$[\alpha^{35}\text{S}]d\text{ATP}$</td>
<td>AG1000/SJ1304</td>
</tr>
<tr>
<td>$[\alpha^{32}\text{P}]d\text{ATP}$</td>
<td>AA0004/PB10204</td>
</tr>
</tbody>
</table>

*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 $^{35}\text{S}$ or $^{33}\text{P}$ sequencing is desired, a large tray for soaking the gel (to remove urea) and a gel drying apparatus are necessary. Gels containing $^{35}\text{S}$ or $^{33}\text{P}$ 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 (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.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic Pyrophosphatase</td>
<td>25µl</td>
</tr>
<tr>
<td>Glycerol Enzyme Dilution Buffer</td>
<td>150µl</td>
</tr>
<tr>
<td>Sequenase DNA polymerase</td>
<td>25µl   (Add other reagents to this vial)</td>
</tr>
<tr>
<td>Total</td>
<td>200µl  (For 100 sequences, 2µl each)</td>
</tr>
</tbody>
</table>

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)
   - H₂O: _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)
   - H₂O: _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-<sup>35</sup>S or α-<sup>33</sup>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-35S]dATP, [α-33P]dATP (9) or [α-32P]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\textsubscript{260}). If the primer has
N bases, the approximate concentration (pmol/μl) is given by the following
formula:

\[
\text{Concentration (pmol/μl)} = \frac{\text{OD}_{260}}{(0.01 \times N)}
\]

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 Mn2+ ions (17). The addition of Mn2+ to normal (Mg2+) 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 Mn2+, 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 MnCl₂ 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.

<table>
<thead>
<tr>
<th>Volume of term. mix (µl)</th>
<th>Volume of seq. extending mix (µl)</th>
<th>Total volume (µl)</th>
<th>Approximate relative extension*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.0</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
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<td>2.5</td>
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<tr>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>2.5</td>
<td>10.0</td>
</tr>
<tr>
<td>0.25</td>
<td>2.25</td>
<td>2.5</td>
<td>20.0</td>
</tr>
</tbody>
</table>

*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
- Na₂EDTA·2H₂O 4g
- H₂O 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
- Na₂EDTA·2H₂O 9.3g
- H₂O 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**

<table>
<thead>
<tr>
<th>Gel conc. (%)</th>
<th>Acrylamide/bis-acrylamide</th>
<th>Urea (7-8.3M)</th>
<th>20X Gly. Tol. Gel Buffer</th>
<th>OR 10X TBE Buffer</th>
<th>H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>6%</td>
<td>5.7g/0.3g</td>
<td>42-50g</td>
<td>5ml*</td>
<td>-</td>
<td>~45ml</td>
</tr>
<tr>
<td>8%</td>
<td>7.6g/0.4g</td>
<td>42-50g</td>
<td>5ml*</td>
<td>-</td>
<td>~45ml</td>
</tr>
<tr>
<td>6%</td>
<td>5.7g/0.3g</td>
<td>42-50g</td>
<td>-</td>
<td>10ml</td>
<td>~40ml</td>
</tr>
<tr>
<td>8%</td>
<td>7.6g/0.4g</td>
<td>42-50g</td>
<td>-</td>
<td>10ml</td>
<td>~40ml</td>
</tr>
</tbody>
</table>

Dissolve, adjust volume to 100ml with H$_2$O, 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)**

<table>
<thead>
<tr>
<th>Gel conc. (%)</th>
<th>Acrylamide/bis-acrylamide</th>
<th>Urea* (7M)</th>
<th>20X Gly. Tol. OR 10X TBE Buffer</th>
<th>Formamide</th>
<th>H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>6%</td>
<td>5.7g/0.3g</td>
<td>42g</td>
<td>5ml</td>
<td>-</td>
<td>40ml</td>
</tr>
<tr>
<td>8%</td>
<td>7.6g/0.4g</td>
<td>42g</td>
<td>5ml</td>
<td>-</td>
<td>40ml</td>
</tr>
<tr>
<td>6%</td>
<td>5.7g/0.3g</td>
<td>42g</td>
<td>-</td>
<td>10ml</td>
<td>40ml</td>
</tr>
<tr>
<td>8%</td>
<td>7.6g/0.4g</td>
<td>42g</td>
<td>-</td>
<td>10ml</td>
<td>40ml</td>
</tr>
</tbody>
</table>

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

Adjust volume to 100ml with H$_2$O, 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 ³⁵S or ³³P 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->

AACGCCAGGG TTTTCCCAGT CACGACGTGG TAAAACGACG GCCAGTGCAC ACGTTCGATG
20 30 40 50 60 70

CCTGCAGGTC GACTCTAGAG GATCCCCGGG TACCGAGCTC GAATTCTAA TCAATGTCAT
80 90 100 110 120 130

AGCTGTTTTC TGTGTGAAAT TGTTATCCGC TCACAATTCC ACACAAACATA CGAGGCGGAA
140 150 160 170 180 190

GCTAAAGTGT AAGACCTGAG GGTGCCAAT GAGTGAGCTA ACTCACATTA ATGGCGGATGC
200 210 220 230 240 250

GCTCAGTGGC CGCTTTCCAG TCGGGAAACC TGGTCTGCCA GCTGCATTAA TGAATCGGACC
260 270 280 290 300 310

AAGCAGCCGGG GAGAGCGGCT TGGTCTGATG GGGGCCAGGG TGGTTTCTT TTCACACAGT
320 330 340 350 360 370

GAGACGGGCA ACAAGTGAATG GCCCTTACC ACCCTGGCCT GAGAGTTGGG CAGCAAGCGG
380 390 400 410 420 430

Ava II 380 420 440 450 460 Ava I 470 480 490

TCCACGCTGG TTTGCCCCAG CAGGCGAAAA TCTGTGTTGG TGGTGGTTCC GAAATCGGCA
440 450 460 470 480 490

AAATCCCTTA TAAATCAAAA GAATGGCCCG AGATAGGTTT GAGTGGTTGT CGAGTGGTCA
500 510 520 530 540 550

AACAAGTGGT ACTATTTAAC AAGCGAGCCT TCAACGCTCA AAGGCGAAAA ACCGTCTATC
560Dra III 570 580 590 600 610

AGGGCGATTTG CCCACTTCAG TGGTGTGCTC CCTTAGTGGGA TCAGGGCTGC
620 630 640 Ban II 650 660 670

GTAAAGCAGT AAATCGGAAC CCTAAAGGGA GCCCCGATT TAGAGGTTGA CGGGGAAAGC
REFERENCES

### RELATED PRODUCTS

#### Kits and Enzymes

<table>
<thead>
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<th>Product</th>
<th>Application</th>
<th>Pack size</th>
<th>Product number</th>
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<tbody>
<tr>
<td>Sequenase PCR™ Product Sequencing Kit</td>
<td>For rapid sequencing of PCR products</td>
<td>100 templates</td>
<td>70170</td>
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<td>For radioactive cycle sequencing</td>
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<td>Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit</td>
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<td>50 templates</td>
<td>In USA, order 188403 Outside USA, order 9750</td>
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<tr>
<td>Terminal Deoxynucleotidyl Transferase (TdT)</td>
<td>For BAFL (Bands Across Four Lanes) resolution</td>
<td>500 units 2500 units</td>
<td>70033Y 70033Z</td>
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#### PCR Clean-up

<table>
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<td>100 rctns 500 rctns 2000 rctns</td>
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#### USB Ultrapure reagents for DNA sequencing

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USB CORPORATION

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www.usbweb.com

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Tel: 0800 73888

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Tel: 1 800 463 5800

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Sweden
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Switzerland
Tel: 01 802 81 50

UK
Tel: 0800 616 928

USA
Tel: 1 800 526 3593
# Material Safety Data Sheet

Revision: 10/07/00

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

## IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND COMPANY

**PRODUCT NAME**
- Sequenase Version 2.0
- DNA Sequencing Kit

**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

<table>
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<tr>
<th>HAZARD</th>
<th>CAS NO.</th>
<th>%WT</th>
<th>TLV</th>
<th>CHIP R &amp; S PHRASES</th>
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<td>75-12-7</td>
<td>95%</td>
<td>10ppm</td>
<td>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</td>
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## 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**  
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