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This product is sublicensed from GE Healthcare. The purchase of this kit (reagent) includes a
limited nonexclusive sublicense under certain patents to use the kit (reagent) to perform a
patented DNA sequencing method in those patents solely for use with Sequenase DNA Polymerase
purchased from USB Corporation for research activities. Use of this kit (reagent) for commercial
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prevention or mitigation of disease or other health-related condition or involving the detection of
pathogens or genetic traits for medical uses; (2) for agriculture or veterinary medicine; (3) for
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Systems, F Hoffmann-La Roche Ltd and the Perkin-Elmer Corporation. Purchase of this product is
accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process for
research in conjunction with a thermal cycler whose use in the automated performance of the PCR
process is covered by the up-front license fee, either by payment to Perkin-Elmer or as purchased,
ie., an authorized thermal cycler.

‡Glycerol Tolerant Gel Buffer—This product and/or its method of use is covered by US Patent No.
5,314,595.

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Exonuclease I/Shrimp Alkaline Phosphatase—This product is licensed under US Patent Nos.
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ExoSAP-IT—is covered by US Patent Nos. 6,379,940 and 6,387,634.


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Components of the kit

The solutions included in the Sequenase PCR Product Sequencing Kit have been carefully prepared to yield the best possible sequencing results. Each reagent has been tested extensively and its concentration adjusted to meet rigorous standards. It is strongly recommended that the reagents supplied in the kit be used.

The following solutions are included in the kit:

**Sequenase Version 2.0 DNA Polymerase** (blue-capped tube), 325 units; 1.6 units/µl with inorganic pyrophosphatase (2 units/ml) in 20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 0.1 mM (EDTA), 50% glycerol

**Sequenase Reaction Buffer** (5X concentrate), 1 ml; 200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl

**Control DNA** (M13 clone), 5 µg; 0.2 µg/µl

**Primer** (-40 forward 23-mer), 25 µl; 20 pmol/µl, 5’-GTTTTCCCAGTCACGACGTTGTA-3’

**Primer** (-50 reverse 21-mer), 25 µl; 20 pmol/µl, 5’-TTGTGAGCGGATAACAATTTC-3’

**Dithiothreitol (DTT) Solution**, 150 µl; 0.1 M

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

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

**dGTP** (3 µM dGTP for labeling) (green-capped tube), 100 µl

**dATP** (3 µM dATP for labeling) (green-capped tube), 100 µl

**dTTP** (3 µM dTTP for labeling) (green-capped tube), 100 µl

**ddCTP** (3 µM ddCTP for labeling) (green-capped tube), 100 µl

**7-deaza-dGTP** (3 µM 7-deaza-dGTP for labeling), (green-capped tube), 100 µl

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

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

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

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

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

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

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

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

Mn Buffer, 100 µl; 0.15 M sodium isocitrate, 0.1 M MnCl₂

Stop Solution, 2 X 1.25 ml; 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF

Exonuclease I (green-capped tube) 1,000 units; 10 units/µl in 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 50% glycerol

rShrimp Alkaline Phosphatase (green-capped tube) 200 units; 2 units/µl in 25 mM Tris-HCl, pH 7.6, 1 mM MgCl₂, 0.1 mM ZnCl₂, 50% glycerol

All nucleotide mixtures should be stored frozen at -20°C and for longest life be kept on ice when thawed for use. The buffers, control DNA, primer and stop solutions can be stored for 4-8 weeks at 4°C. The enzymes must be stored at -20°C and never be allowed to warm up above -20°C. Never store enzymes in a frost-free freezer (the temperature rises above 0°C daily).

Quality control

All kit batches are functionally tested using radiolabeled dATP and a control M13 clone single-stranded DNA template as described in this protocol. Release specifications are based on sequence length, band intensity and sequence quality. The entire PCR product sequence must be visible 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 25.

Caution: 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 lab coat, safety glasses, and gloves. Care should be taken to avoid contact with skin and eyes. In the case of contact with skin or eyes, wash immediately with water. See MSDS (Material Safety Data Sheet) for specific advice.

Introduction to sequencing PCR products

This kit features a method for preparing the products of symmetric (double-stranded) polymerase chain reaction (PCR) for sequencing. This method requires a minimum of ‘hands-on’ time and could readily be automated by automatic pipetting devices. All gel or column purifications, sedimentations, filtrations and magnetic separations are eliminated by the use of two enzymes which effectively remove the excess dNTPs and primers from DNA produced by PCR amplification.

PCR(1) makes use of two primers, deoxynucleoside triphosphates (dNTPs) and DNA polymerase to produce multiple copies of a specific DNA sequence. When the PCR is complete, most of the dNTPs and primers remain and will interfere with normal sequencing methods. Two hydrolytic enzymes provided in this kit, rShrimp Alkaline Phosphatase and Exonuclease I, can be used to remove these unwanted materials. The steps involved for this enzymatic cleanup method are described in Figure 1.

Sequencing is then performed as usual. The template is denatured and annealed to the primer using a heating and snap-cooling procedure followed by a labeling step and a termination step. In some cases, a special labeling procedure (the 3-dNTP method-described below) may be used to further enhance the specificity of the sequencing experiment since more than one sequence is often amplified during PCR.
Chain-termination sequencing

The Sequenase chain-termination method\(^2\)\(^\text{-}\)\(^3\) involves the in vitro synthesis of a DNA strand by a DNA polymerase using a specifically primed single-stranded DNA template. The synthesis reaction is terminated by the incorporation of a nucleotide analog that will not support continued DNA elongation (hence the name chain-termination). These chain-terminating 2',3'-dideoxynucleoside-5'-triphosphates (ddNTPs) lack the 3'-OH group necessary for DNA chain elongation. When proper mixtures of dNTPs and one of the four ddNTPs are used, enzyme-catalyzed polymerization will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP, give complete sequence information. 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\(^3\), is a superior enzyme for isothermal DNA sequencing. It is a genetic variant of bacteriophage T7 DNA polymerase created by an 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, rapid polymerization rate, and the ability to incorporate nucleotide analogs useful for DNA sequencing (ddNTPs, alpha-thio dNTPs, dITP, 7-deaza-dGTP, etc.)\(^4\).

Note: The concentrations of nucleotides in sequencing 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:

- α-Labeled dATP*
  - \([\alpha^3\text{P}]\text{dATP}\)
  - \([\alpha^3\text{S}]\text{dATP}\)
  - \([\alpha^{32}\text{P}]\text{dATP}\)
  - *The specific activity should be 1,000-1,500 Ci/mmol.

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

- Gel reagents—Sequencing gels should be made from fresh solutions of acrylamide and bis-acrylamide. Other reagents should be ultrapure or electrophoresis grade. For convenience, RapidGel™ gel mixes are strongly recommended. RapidGel-XL formulations yield up to 40% more readable sequence per gel. See both ‘Denaturing gel electrophoresis’ and ‘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, 5-10 pmol of primer should be used for each set of sequencing reactions. See ‘Supplementary information’, ‘Determining how much PCR product and primer to use for sequencing’ for details.

Necessary equipment:

- Constant temperature bath—PCR product cleanup and sequencing will require incubations at room temperature, 37°C, 75°C, 80°C and 100°C.

- Thermal cycler—Not only is this required for PCR, but it is useful for performing sequencing reaction steps as well.

\[\text{Exonuclease I} \quad \text{rShrimp Alkaline Phosphatase} \quad 37\text{C}, 15\text{ minutes for treatment} \quad 80\text{C}, 15\text{ minutes to inactivate} \]

\[\text{Nucleotides, P} \quad \text{Sequencing} \]

Figure 1. Enzymatic cleanup of PCR products.
Electrophoresis equipment—While standard, non-gradient sequencing gel apparatus is sufficient for much sequencing work, the use of field-gradient (‘wedge’) gels will allow much greater reading capacity on the gel[5]. A power supply offering constant power operation and 2,000V or greater is essential.

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

Autoradiography—Any large format autoradiography film can be used. Develop films according to the manufacturer’s instructions.

Protocols

PCR and sequencing reactions are typically run in microcentrifuge tubes (0.5 ml) or 96-well 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 briefly. With care and experience these reactions can be completed in 10-15 minutes.

1. **Enzymatic pre-treatment of PCR product**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR amplification mixture</td>
<td>5 µl</td>
</tr>
<tr>
<td>Exonuclease I (10.0 units/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>rShrimp Alkaline Phosphatase (2.0 units/µl)</td>
<td>1 µl</td>
</tr>
</tbody>
</table>
   **Total**                        | 7 µl   |

   Mix and incubate at 37°C for 15 minutes. (It is convenient to do this in a thermal cycler.) Note: When treating more than 10 µl of PCR product, increase the amount of Exonuclease I and rShrimp Alkaline Phosphatase proportionally. See ‘Supplementary information’ for details concerning the amount of PCR product to use.

2. **Inactivate Exonuclease I and rShrimp Alkaline Phosphatase by heating to 80°C for 15 minutes.** (It is convenient to do this step in a thermal cycler.)

3. While PCR cleanup reactions are incubating, label, fill and cap tubes with 2.5 µl of each termination mixture (G, A, T and C). Keep covered at room temperature for step 6.

4. **Annealing template and primer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated PCR product DNA</td>
<td></td>
</tr>
<tr>
<td>Primer (5-10 pmol/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>H$_2$O</td>
<td></td>
</tr>
<tr>
<td>(Up to 9 µl)</td>
<td></td>
</tr>
<tr>
<td>(To adjust total volume to 10 µl)</td>
<td></td>
</tr>
</tbody>
</table>
   **Total**                        | 10 µl  |

   Incubate 2-3 minutes, 100°C (preferably in the thermal cycler, boiling water baths may not reach 100°C) then cool as quickly as possible by placing the vial directly in an ice/water bath for 5 minutes. **Keep the tube on ice.**

   **Note:** Rapid cooling helps promote primer annealing over template re-annealing. Annealed template should be used within about 4 hours. If using a thermocycler without a heated lid, overlay the 10 µl annealing mixture with 8 µl of mineral oil, heat and cool as above. Then transfer the lower (aqueous) mixture to a fresh 0.5 ml microcentrifuge vial for subsequent steps.

5. **Labeling reaction**

   **Method I:** Labeling with 4 dNTPs—

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealed DNA mixture (ice-cold)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Sequenase Reaction Buffer (5X)</td>
<td>2 µl</td>
</tr>
<tr>
<td>DTT, 0.1 M</td>
<td>1 µl</td>
</tr>
<tr>
<td>1:5 diluted labeling mix*</td>
<td>2 µl</td>
</tr>
<tr>
<td>[33P] or [35S]dATP</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>[33P] or [35S]dATP</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Sequenase Version 2.0 DNA</td>
<td>2 µl</td>
</tr>
<tr>
<td>Polymerase</td>
<td></td>
</tr>
</tbody>
</table>
   **Total**                        | 17.5 µl|

   Mix and incubate at room temperature for 2-5 minutes (or cooler).

   **Note:** The appropriate dilution of the labeling mix is determined by the amount (pmol) of PCR product used for sequencing. With less than 0.3 pmol of template, the sequences may be faint near the primer. If this occurs, further dilution of the labeling mix (1:10 or 1:20) may be tried or Mn Buffer (1 µl) can be added after the labeling reaction to help emphasize bands close to the primer (see ‘Reading sequences close to the primer’ in the ‘Supplementary information’ below).

   **Method II:** Labeling with 3 dNTPs—

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealed DNA mixture (ice-cold)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Sequenase Reaction Buffer (5X)</td>
<td>2 µl</td>
</tr>
<tr>
<td>DTT, 0.1 M</td>
<td>1 µl</td>
</tr>
<tr>
<td>[33P] or [35S]dATP</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Sequenase Version 2.0 DNA</td>
<td>2 µl</td>
</tr>
<tr>
<td>Polymerase</td>
<td></td>
</tr>
<tr>
<td>dNTP (3 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>dNTP (3 µM)</td>
<td>1 µl</td>
</tr>
</tbody>
</table>
   **Total**                        | 17.5 µl|

   Mix and incubate at room temperature for 2-5 minutes (or cooler).

   **Note:** Actual dNTPs used will depend on sequence, see ‘Supplementary information.’

6. **Termination reactions**

   Transfer 3.5 µl of labeling reaction to each termination tube (G, A, T and C—step 3), mix and continue incubation of the termination reactions at 37°C for 5-10 minutes.
7. Stop the reactions by adding 4 µl of Stop Solution.
8. Heat samples to 75°C for 2 minutes immediately before loading onto sequencing gel. Load 2-3 µl of each reaction per lane.

Supplementary information

Enzymatic pre-treatment of PCR product

The key step in this method for sequencing PCR products consists of treating the PCR product with a combination of Exonuclease I and Shrimp Alkaline Phosphatase. Both of these enzymes are active in the buffer used for PCR, so no change in buffer is required. The Exonuclease I removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The Shrimp Alkaline Phosphatase removes the remaining dNTPs from the PCR mixture which would interfere with the labeling step of the sequencing process. The Exonuclease I and Shrimp Alkaline Phosphatase are inactivated easily by heating to 80°C for 15 minutes.

Labeling methods for sequencing PCR products

Normally, the labeling step is performed using all four dNTPs, one of which is α-labeled. This labeling method works well for most PCR product sequencing and is recommended for any PCR which has high yield and high specificity.

Unfortunately, sometimes PCR yields more than one product (revealed as multiple bands on agarose gels) or simply has low yield. The use of an internal primer (instead of one of the amplification primers) is one good way to sequence when multiple PCR products are encountered.

This kit introduces an alternative labeling method which may improve the specificity of sequencing reactions without using internal primers or sacrificing the convenience of labeling with α-dNTPs. This labeling method is carried out using only three of the four deoxynucleotides present, so extensions are terminated when the fourth (omitted) nucleotide is needed. The position of the primer is chosen so that 3 or more labeled nucleotides are incorporated during this limited extension. For example, the sequence of the -40 forward 23-mer primer and its corresponding site in M13 or pUC cloning vectors is as follows:

Primer

5’-GTTTTCCACGTACGACGTTGTA-3’

Template

CCCAAAAGGTCATGTGCAACATTTTGCTGCCGGTCACGG

Polymerization in the presence of dGTP, dCTP and labeled dATP (omitting dTTP) will result in extension of the primer by 13 nucleotides as shown below:

Primer

5’-GTTTTCCACGTACGACGTTGTAAGAAGCAgGACGACC-3’

Template

CCCAAAAGGTCATGTGCAACATTTTGCTGCCGGTCACGG

Synthesis during this step will result in the production of a labeled extended primer of limited length. Synthesis at unwanted sites (which have different down-stream sequences) will usually yield products with fewer labeled bases and thus much lower specific radioactivity so they will interfere only weakly with resulting sequence. Furthermore, since the length of primer extension is limited, sequencing results can be obtained even when the PCR has low yield. The use of this method requires careful choice of the priming site.

Designing primers for 3-dNTP labeling

A primer sequence is chosen for both amplification and sequencing so that it becomes strongly labeled even when extended in the presence of only 3-dNTPs. This is done by using the known sequence immediately downstream of the primer as described below.

Within typical, non-repetitive DNA sequences, it is usually easy to find a suitable combination of primer, label and nucleotides to give sensitive sequences. The primary object in designing the primer is to have as many molecules of labeled nucleotide as possible incorporated into the extended primer during the labeling step.

First, decide which label (e.g. α-dATP or α-dCTP) is to be used. Second, write down the sequence (30-50 bases) of the intended newly-synthesized strand around the priming site in the 5’→3’ orientation. For example:

5’-ACAAGAGGTCAGATTAAAGAAGAGTGACTCACCACTCGTAAGG-3’

Now, find an area in the sequence where preferably 3-4 or more of these labeled nucleotides will be incorporated close together. For example, using α-dCTP label and the following sequence, the underlined stretch contains four potential labeling sites:

5’-ACAAGAGGTCAGATTAAAGAAGAGTGACTCACCACTCGTAAGG-3’

Now scan this sequence region to find one nucleotide which is not required for the incorporation of the nearby labeled nucleotides. In the example above, A, C and T are all underlined, but synthesis of this 8-base stretch will not require dGTP. This is the nucleotide which will be omitted from the labeling step.

You are now ready to pick the primer sequence. Any of the ‘omitted’ nucleotides (G in the example) upstream from the desired extension/labeling region must be included within the primer sequence so ‘minus G’ primers (such as the one in this example) will typically have a ‘G’ at their 3’ terminus. The length of the primer (and its sequence) will determine the melting temperature and specificity. Choice of the following primer (double underlined) will allow labeling with either dCTP (4 sites) or dATP (3 sites) (underlined).

5’-ACAAGAGGTCAGATTAAAGAAGAGTGACTCACCACTCGTAAGG-3’
It is also a good idea to check the sequence of the primer for possible self-annealing (dimer formation could result) and for potential ‘hairpin’ formation, especially those involving the 3’ end of the primer. Finally, check for possible sites of false priming in the vector or other known sequence, if possible, again stressing matches which include the 3’ end of the primer. Since the sequencing primer will often be used as a PCR primer, its melting temperature must also be considered.

The 20-mer primer in this example will have the sequence

5’-TCCAGTATTTAAGAAGTCG-3’

An alternative primer for the above sequence could have the following sequence (double underlined) incorporating 5 ‘A’ bases before the first ‘T’ base:

5’-ACAAGAGTCCAGTATTAAAGAACGTCGACTCCAACGTCAAAGG-3’

**PCR protocols**

Detailed protocols for the PCR steps are beyond the scope of this manual, but the following reaction mixture is a recommended starting point.

**Final concentration**

- 10 mM Tris-HCl, pH 8.3
- 1.5 mM MgCl₂
- 50 mM KCl
- 200 µM each dATP, dGTP, dCTP, dTTP
- 1 µM each (100 pmol/100 µl) primer
- 2.5 units/100 µl Taq DNA Polymerase

*Note: When multiple amplified bands are observed, or when the yield is low, DNA sequences will usually be poor. The use of an internal primer (instead of one of the amplification primers) is often successful when multiple PCR products are encountered. It is essential to check the quality of amplified DNA prior to sequencing. The methods available with this kit cannot be expected to yield flawless sequence with every PCR amplification. For difficult cases, purification of the product using gels or other methods may be required.*

When using the products of asymmetric PCR, the DNA should be treated ONLY with rShrimp Alkaline Phosphatase prior to sequencing.

### Determining how much PCR product and primer to use for sequencing

The amount of template used is very important. Experience has shown that the use of the minimum possible amount of template which gives reasonable exposure times is best. Good amplifications result in the production of 10-20 ng of product DNA per µl but yields vary greatly depending on numerous factors.

Ideally, 0.2-0.5 pmol of template should be used for non-cycle sequencing. The following table gives an approximate volume of PCR product to sequence when using Sequenase DNA Polymerase for radioactive sequencing:

<table>
<thead>
<tr>
<th>Length</th>
<th>100 bp</th>
<th>200 bp</th>
<th>300 bp</th>
<th>400 bp</th>
<th>500 bp</th>
<th>1,000 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of PCR product needed</td>
<td>7 µl</td>
<td>5 µl</td>
<td>8 µl</td>
<td>5 µl</td>
<td>7 µl</td>
<td>7 µl</td>
</tr>
<tr>
<td>PCR product concentration* (ng/µl)</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*If the yield is so low that much more than 5 µl may be required for sequencing, it is better to re-amplify (using the same or nested primers as required for specificity). The recommended protocol calls for a maximum of 5 µl of template, but up to 10 µl can be used if required. The use of Mn Buffer (see below) may give better results than increasing the amount of template.*

The total volume for the PCR product cleanup reaction should be 10 µl. If a smaller volume of DNA solution is used, the balance should be made up with distilled water. The molar ratio of primer to template should be 5:1 to 20:1. The excess primer helps promote priming over re-annealing of the template DNA. The use of too little template will narrow the effective sequencing range, resulting in faint bands near the bottom of the gel. The use of too much template may increase background problems with the appearance of ambiguous bands in all lanes of the sequencing gel.

Always determine the concentration of the primer by reading the absorbance at 260nm (A₂₆₀). If the primer has N bases, the approximate concentration (pmol/µl) is given by the following formula: Concentration (pmol/µl) = A₂₆₀ / (0.01 X N).
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 (7-deaza-dGTP) which forms weaker secondary structure has been somewhat successful in eliminating these gel artifacts. The use of 7-deaza-dGTP eliminates some (but not all) compressions tested. The substitution of 7-deaza-dGTP for dGTP is simple, and recommended for all sequences which may contain ambiguous, compressed regions and is included in the kit. Regions of secondary structure in the product DNA can produce several kinds of gel artifacts, some of which may go unnoticed when reading a gel. One way to ensure that the correct sequence is determined is to sequence both strands in opposite directions. Alternatively, the inclusion of up to 40% formamide in sequencing gels (along with 7 M urea) has been successful for eliminating very strong compressions (see ‘Supplementary information-Denaturing gel electrophoresis’ for details).

To use 7-deaza-dGTP, simply substitute the 7-deaza-dGTP labeling mixture (green-capped tube) for the dGTP labeling mixture and substitute the 7-deaza-dGTP termination mixtures (red-capped tube) for the dGTP termination mixtures. All other aspects of the sequencing protocol (dilutions, etc.) remain unchanged.

Reading sequences close to the primer

When using the 3-dNTP labeling reaction, all sequence information prior to the base where the labeling step extension stops will be lost. Sequence bands 2-3 bases beyond this base should be strong and readable.

Labeling step method—The general conditions described in this manual should be followed for sequencing from the primer up to 300-400 nucleotides. If the interest is only in sequences close to the primer (<50 nucleotides) or if PCR yield is low, it is possible to dilute the labeling mix further (a 1:10 dilution of the 5X stock reagent). When reading sequences within 20 nucleotides of the 3′-end of the primer, it is essential that sufficient template DNA and primer be present. The best alternative is to label the primer or to use the 3-dNTP labeling method.

Mn Buffer method—Another solution to this situation is to add the Mn Buffer provided with the kit. This reagent takes advantage of the activity of Sequenase Version 2.0 enzyme in the presence of Mn⁺⁺ ions. The addition of Mn⁺⁺ to normal (Mg⁺⁺) sequencing reactions (with fixed deoxynucleotide to dideoxynucleotide ratios) reduces the average length of DNA synthesized in the termination step, intensifying bands corresponding to sequences close to the primer. With Mn⁺⁺, sequences from less than 20 nucleotides from the primer up to approximately 200 nucleotides can be observed even with reduced amounts of template.

Mn Buffer is a buffered solution of MnCl₂, which can be added to normal sequencing reactions. To use this reagent, simply add 1 µl of Mn Buffer after the labeling reaction is complete (immediately prior to dividing the labeling reaction mixture among the four termination reactions). 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.
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 0.2-1.0 µl of Mn Buffer are added to the reaction. Intermediate effects are not observed since Mn Buffer has an ‘all-or-none’ effect.

Glycerol enables higher reaction temperatures

Sequenase Version 2.0 DNA Polymerase, like many enzymes, is stabilized by glycerol. The polymerase in 50% glycerol buffer provided with this kit produces a glycerol concentration in the labeling reaction of 6%. This stabilizes the polymerase enough that the labeling reactions can be incubated up to 30 minutes at room temperature (20°C) or even up to 5 minutes at 37°C. Increasing the concentration of glycerol to 20% in the labeling step will further stabilize the polymerase so that even longer labeling reactions can be tolerated or brief reactions at even higher temperature (1 minute, pre-warmed to 45°C, adding polymerase last) can be run. Higher temperature (stringency) labeling reactions can be beneficial since primer specificity will be greater.

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. This can be of aid in sequencing templates with strong 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 conditions, 250-300 bases can be read starting at the bottom of a standard size sequencing gel. The length of time the gel is run will determine the region of sequence that is readable. Many factors can limit the sequence information which can be determined in a single experiment. Among these are the quality of reagents used, the polymerization, the temperature of the gel during electrophoresis, and proper drying of the gel after running. The greatest care should be given to the pouring and running of sequencing gels. The specifics of running the electrophoresis will depend on the apparatus used. The following suggestions for reagent compositions and procedures are intended as guidelines. For specific instructions contact the manufacturer of the gel apparatus used.

Gel electrophoresis reagents

Note: TBE should NOT be used with this kit if sequence information beyond 300 bases is required. The enzymes used with this kit contain sufficient glycerol to distort TBE gels above 300 bases.

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

Buffers

20X Glycerol Tolerant Gel Buffer (1 liter)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>216 g</td>
</tr>
<tr>
<td>Taurine</td>
<td>72 g</td>
</tr>
<tr>
<td>Na₂EDTA.2H₂O</td>
<td>4 g</td>
</tr>
<tr>
<td>H₂O to 1,000 ml, filter (may be autoclaved)</td>
<td></td>
</tr>
</tbody>
</table>

This buffer can be used with samples containing glycerol at any concentration(19). 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.

10X TBE Buffer (1 liter)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>Tris base</td>
<td>108 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>55 g</td>
</tr>
<tr>
<td>Na₂EDTA.2H₂O</td>
<td>9.3 g</td>
</tr>
<tr>
<td>H₂O to 1,000 ml, filter (may be autoclaved)</td>
<td></td>
</tr>
</tbody>
</table>

This is the traditional sequencing gel buffer. It may be used if sequence information is not required beyond 300 bases. The enzyme used in this kit is supplied in a high glycerol buffer which will distort TBE gels above 300 bases.

Gel recipes (for 100 ml of gel solution)

<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>10X TBE Buffer</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>6%</td>
<td>5.7 g/0.3 g</td>
<td>42-50 g</td>
<td>5 ml*</td>
<td>-</td>
<td>~45 ml</td>
</tr>
<tr>
<td>8%</td>
<td>7.6 g/0.4 g</td>
<td>42-50 g</td>
<td>5 ml*</td>
<td>-</td>
<td>~45 ml</td>
</tr>
<tr>
<td>6%</td>
<td>5.7 g/0.3 g</td>
<td>42-50 g</td>
<td>-</td>
<td>10 ml</td>
<td>~40 ml</td>
</tr>
<tr>
<td>8%</td>
<td>7.6 g/0.4 g</td>
<td>42-50 g</td>
<td>-</td>
<td>10 ml</td>
<td>~40 ml</td>
</tr>
</tbody>
</table>

Dissolve, adjust volume to 100 ml with H₂O, filter and degas. When ready to pour, add 1 ml of 10% ammonium persulfate and 25 µl TEMED (N, N’, N’, N’-tetramethylethylene-diamine).

*Use 4 ml 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. Gel Buffer</th>
<th>10X TBE Buffer</th>
<th>Formamide</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>6%</td>
<td>5.7 g/0.3 g</td>
<td>42 g</td>
<td>5 ml</td>
<td>-</td>
<td>40 ml</td>
<td>~10 ml</td>
</tr>
<tr>
<td>8%</td>
<td>7.6 g/0.4 g</td>
<td>42 g</td>
<td>5 ml</td>
<td>-</td>
<td>40 ml</td>
<td>~10 ml</td>
</tr>
<tr>
<td>6%</td>
<td>5.7 g/0.3 g</td>
<td>42 g</td>
<td>-</td>
<td>10 ml</td>
<td>40 ml</td>
<td>~5 ml</td>
</tr>
<tr>
<td>8%</td>
<td>7.6 g/0.4 g</td>
<td>42 g</td>
<td>-</td>
<td>10 ml</td>
<td>40 ml</td>
<td>~5 ml</td>
</tr>
</tbody>
</table>

Warming to 35-45°C may be required to dissolve completely. Adjust volume to 100 ml with H₂O, filter and degas. When ready to pour add 1 ml of 10% ammonium persulfate and 100-150 µl 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. These formamide-containing gels may be used for very strong compressions not resolved by 7-deaza-dGTP. 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. Avoid pre-running of formamide gels more than 15 minutes.

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%, 6% or 8% solutions with buffer and urea; or a 40% stock solution containing 19:1 acrylamide to bis-acrylamide may be used for a customized percentage. TBE formulation is available.
General guidelines for electrophoresis

- Electrophoresis grade reagents should be used.
- Fresh solutions of monomers should be used. Store no longer than one week in the dark at 4°C. Commercial preparations of acrylamide gel mixes in liquid or powder form (RapidGel—see ‘Related products’) should be used according to manufacturer’s recommendations.
- Gels should be prepared 2-20 hours prior to use, and pre-run for ~15 minutes.
- It is usually convenient to run gels for reading longer sequences overnight (with a timer). Gel runs of 18-24 hours at 40-50 watts are often necessary for reading in the 400-600 bp range.
- Loading 8 adjacent lanes in a pattern that abuts all pairs of lanes (e.g. GATCGTAC) aids reading closely spaced bands.
- 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 0.2 mm gels, 15 minutes for 0.4 mm gels and 60 minutes for field gradient (0.4-1.2 mm wedge) gels. Drying should be done at moderate temperature (80°C) to preserve resolution.
- 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.
- For 35S or 33P gels, exposure must be done with direct contact between the dried gel and the emulsion side of the film. Gels dried without prior soaking (leaving plastic-wrap on helps to prevent the film from sticking to incompletely-dried gels) will require longer drying and exposure times but give sufficient resolution for most purposes.
- Good autoradiography film can improve image contrast and resolution.
- The use of tapered spacers (‘wedge’ gels) improves overall resolution and allows more nucleotides to be read from a single loading[5].

Troubleshooting

Control DNA—Many of the problems encountered when using this kit can be better identified by using a control DNA template. The control DNA included in this kit is single-stranded M13 DNA (M13mp18 with a 262-base insert at the BamHI site) that can be used two ways. First, if sequencing reagents are suspected to be faulty, it can be used directly for sequencing. Simply use 5 µl (1 µg) for annealing along with the -40 forward 23-mer primer included with the kit (1 µl of kit primer diluted 1:5 with H2O).

The control DNA can also be used to test the entire PCR sequencing process by first amplifying it using PCR, treating with Exonuclease I and rShrimp Alkaline Phosphatase and then using the PCR product as template for sequencing. To amplify, use 50 pmol (2.5 µl) of each kit primer along with 5 ng (5 µl of DNA diluted 200:1) in a 50 µl PCR amplification. Then run a 10 µl aliquot on a 1.5% agarose gel (with standards) to check for amplification of a 260-base pair product. Yield should be such that the 10 µl sample should contain 50-100 ng of DNA product. Take an additional 2-5 µl of the PCR product for sequencing. Treat this aliquot with Exonuclease I and rShrimp Alkaline Phosphatase as directed in the protocols and sequence using 5 pmol of the -40 forward 23-mer primer (1 µl of kit primer diluted 1:4) labeling with 4 dNTPs (5-fold dilution of the labeling mix).

Problem Possible causes and solutions

Film blank or nearly blank
1. If using single-sided film, the emulsion side must be placed facing the dried gel.
2. DNA template 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 freshly made acrylamide solutions and should polymerize rapidly, within 15 minutes of pouring. Try running a second gel with the same samples.
3. Gel run too cold or too hot; sequencing gels should be run at 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 ‘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 when using 4 dNTPs in the labeling step. A minimum of 0.2-0.5 pmol of DNA is required for sequencing close to the primer, this usually corresponds to about 0.1 µg of a 750 base pair PCR product (see ‘Determining how much PCR product and primer to use for sequencing’ in the ‘Supplementary information’ above). Try using a primer suitable for 3-dNTP labeling or increasing the amount of DNA or using the Mn Buffer (see ‘Reading sequence close to the primer’ in the ‘Supplementary information’ above).

2. Insufficient primer—use a minimum of 2.5 pmol. Primer to template molar ratio should be 5:1 to 20:1. Be sure that annealing includes a very rapid cooling of the template-primer mixture in ice water. (Simply placing the vial in a cold block does not ensure rapid cooling).

**Sequence missing but strong bands (smears) near the top of the gel**

1. The rShrimp Alkaline Phosphatase did not remove all dNTPs from the PCR product. Usually the best remedy for this problem is to use less PCR product DNA. Try using half as much. Alternatively, try using twice the amount of rShrimp Alkaline Phosphatase.

A single strong band appears in all four lanes at the mid-point of the PCR product sequence (typically with PCR products shorter than 600 bases)

1. This artifact band is labeled, double-stranded PCR product DNA. On gels containing 7 M urea, this DNA migrates about twice as fast as single-stranded DNA of the same length. This band is eliminated when gels contain 40% formamide (see ‘Denaturing gel electrophoresis’ in the ‘Supplementary information’ above).

**Bands appear across all 4 lanes**

1. Too much PCR product used. Try using less DNA. For example if 5 µl was used originally, try sequencing using 1-2 µl of PCR product.

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. The labeling step should not be run longer than 15 minutes or warmer than 20°C. This step can be run at 0°C (on ice) 15-30 minutes.

4. The termination step should not be run cooler than 37°C or longer than 45 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), which may improve results for some templates.

5. Sequences with strong secondary structure. Sequenase Version 2.0 DNA Polymerase will pause at sites of exceptional secondary structure. 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 0.5 µg of single-stranded DNA binding protein (SSB) (70032) during the labeling reaction usually eliminates the problem. When using SSB, it is necessary to inactivate it prior to running the gel. Add 0.1 µg of Proteinase K (76230) and incubate at 65°C for 20 minutes after adding the stop solution.

**Bands in 2 or 3 lanes**

1. Heterogeneous template DNA caused by PCR artifacts. This problem is often seen when the PCR amplification is not completely specific so that more than one sequence is present in the product DNA. Check to see if more than one band of amplified DNA is present in the PCR product (including so-called ‘primer-dimer’). If more than one band is present, or if the sequence looks as if more than one sequence is being exhibited, the specificity of the sequencing process must be increased. This can be achieved by purifying the PCR product prior to sequencing (for example by agarose gel electrophoresis), using a sequencing primer internal to the amplified DNA, using specific 3-dNTP labeling, by sequencing the opposite strand or by using a new primer (or primers) to more-specifically amplify the desired sequence especially by ‘nested’ PCR methods. In addition, the following suggestions may help minor problems.

2. Exonuclease I digestion may not have completely removed one of the PCR primers. We have seen results where sequencing with one PCR primer works well but the other gives double sequence, as if reading from both ends, suggesting that some primers are resistant to Exonuclease I digestion. Try digesting with Exonuclease I again or using a different primer for PCR.

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 7-deaza-dGTP-containing reaction mixtures to eliminate gel compressions or try using a formamide-containing electrophoresis gel (see ‘Denaturing gel electrophoresis’ in the ‘Supplementary information’ 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 extra dTTP to the labeling reaction (1 µl of 50 µM dTTP).

If problems persist please contact Technical Support for assistance at (888) 362-2447 or USBTechsupport@affymetrix.com. For technical support outside the U.S., please visit our website for up-to-date contact information within your area.
Control DNA sequence
The control DNA included in the kit is single-stranded phage DNA from a clone of a Sau3AI fragment of bacteriophage λ DNA inserted at the BamHI site of M13mp18. A partial sequence of this DNA, including the priming sites, is given below. Amplification using the primers included in the kit results in the production of a DNA product 262 bases long. The single-stranded DNA can also be sequenced without amplification using the forward primer (but NOT the reverse primer).

-40 Forward 23-mer primer
5' -G TTTCCCAGTT CGACGCTGGTA TA-- 0 10
AACGCCAGGG TTTCCCAGTT CGACGCTGGTA TAAAACGACA GCCAGTGCCA AGCTTGCATG
20 30 40 50 60 70
CCTGCAGGTCT GACTCTAGAG GATCAATTAA TACGATACCT GCGTCATAAT TGATTATTGG
80 90 100 110 120 130
ACGTGGTTTG ATGGCCTCCA CGCACGTTGT GATATGTAGA TGATAATCAT TATCACTTTAG
140 150 160 170 180 190
CGGGTCCTTT CCGGTGATCC CCGGGTACCG AGCTCGAATT CGTAATCATG GTCATAGCTGG
200 210 220 230 240 250
TTTCCTGTGT GAAATTGTTA TCCGCCACA ATTCACACA CACATCGAGG CGGAA
<---CTTTAACAAAT AGGCCAGTGT T-5'

References
## Ultrapure reagents for DNA sequencing

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<thead>
<tr>
<th>Product</th>
<th>Application</th>
<th>Pack size</th>
<th>Product number</th>
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</thead>
<tbody>
<tr>
<td>Ammonium Persulfate</td>
<td>Gel electrophoresis</td>
<td>10 gm, 100 gm, 1 kg</td>
<td>76322</td>
</tr>
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<td>Glycerol Tolerant Gel Buffer, 20X, Pre-mixed powder</td>
<td>Gel electrophoresis</td>
<td>6 bottles</td>
<td>71949</td>
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<tr>
<td>Glycerol Tolerant Gel Buffer, 20X solution</td>
<td>Gel electrophoresis</td>
<td>1 L</td>
<td>75827</td>
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<tr>
<td>RapidGel-6%, TBE</td>
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<td>75843</td>
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<tr>
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<td>RapidGel-40% Liquid Acrylamide</td>
<td>Gel electrophoresis</td>
<td>500 ml</td>
<td>75848</td>
</tr>
<tr>
<td>RapidGel-XL-6%, TBE</td>
<td>Gel electrophoresis; long</td>
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<td>75861</td>
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<tr>
<td>RapidGel-XL-40% Liquid Acrylamide</td>
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<td>Gel electrophoresis</td>
<td>100 gm, 500 gm</td>
<td>76320</td>
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<td>TBE Buffer, 10X Powder</td>
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</tr>
</tbody>
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**Affymetrix, Inc.**

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Please visit our website at usb.affymetrix.com for up-to-date contact information within your area.
FIRST-AID MEASURES

EYES: Flush with water for 15 minutes. Seek medical advice if irritation persists.

SKIN: Flush with water, then wash thoroughly with soap and water. Remove contaminated clothing and wash before reuse. Seek medical attention if irritation persists.

INHALATION: Remove the victim from exposure and move to fresh air. If breathing is difficult, give oxygen. If not breathing, give artificial respiration. Keep victim quiet and warm. Seek immediate medical attention.

INGESTION: Drink water and seek immediate medical attention. Avoid alcoholic beverages. Never give anything by mouth to an unconscious person.

FIRE-FIGHTING INFORMATION

Use media suitable to extinguish the supporting or surrounding fire. Wear NIOSH (or equivalent) approved self contained breathing apparatus. For small fires only: use carbon dioxide, dry powder or foam. Formamide decomposes at temperatures > 180°C. Formamide is incompatible with strong oxidizing agents, alkali and acids. Thermal decomposition of Formamide may produce carbon monoxide, ammonia and nitrogen oxides. Toxic fumes of sulfur oxides, carbon oxides and hydrogen sulfide gas may be produced upon decomposition of Dithiothreitol. Flash Point = 154°C (309°F) Open Cup for Formamide.

ACCIDENTAL RELEASE MEASURES

Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, gloves and NIOSH-approved respirator. Collect in a manner that does not create dust and place in a suitable waste container. Avoid contact of material with skin or eyes. Use adequate ventilation.

HANDLING AND STORAGE

Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, gloves and NIOSH-approved respirator. Use adequate ventilation. Avoid contact of material with skin or eyes. Store at -20°C away from incompatible material.

PERSONAL PROTECTION

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

PHYSICAL AND CHEMICAL PROPERTIES

Appearance: Kit containing vials of solutions

Vapor Pressure: 0.08 mmHg @ 20°C (for Formamide)

Solubility (Water): All components are soluble

Percent Volatile: No data available

Evaporation Rate: <1 (for Formamide)

Chemical Formula: Not applicable

Melting Point: 2-3°C (for Formamide)

STABILITY AND REACTIVITY

Product is stable. Absorbs moisture from air. Burning may produce ammonia, carbon monoxide and nitrogen oxides. At boiling point may produce ammonia, carbon monoxide and hydrogen cyanide. Hazardous polymerization will not occur. Incompatibilities: Strong oxidizing agents, isocyanates, acids, alkalines, iodine, pyridine and sulfur trioxide. Copper, brass, lead and rubber are attacked by Formamide. Decomposes at temperatures above 180°C. Toxic fumes of sulfur oxides, carbon oxides and hydrogen sulfide gas may be produced upon decomposition of Dithiothreitol. Avoid freeze-thaw cycles.

TOXICOLOGICAL INFORMATION

EFFECTS OF OVEREXPOSURE:

FOR FORMAMIDE:

EYES: Contact causes irritation.

SKIN: Contact causes irritation. May be absorbed through the skin. Symptoms may parallel ingestion.

INHALATION: Causes irritation to the respiratory tract. Symptoms may include coughing and shortness of breath. Excessive inhalation of vapor may cause symptoms that parallel ingestion.

INGESTION: Chronic ingestion or excessive dosage may cause central nervous system disorders, headache, dizziness, nausea, vomiting, abdominal pain, and unconsciousness. May affect the reproductive system. May cause damage to liver & denatures proteins. Has caused embryo toxicity and birth defects in animal studies. TARGET ORGAN(S): Central Nervous System, Liver, Kidneys, Eyes, Reproductive System and Skin.

ADDITIONAL INFORMATION: Harmful if swallowed, inhaled or absorbed through skin. Reproductive effects, irritation, mutation and toxicity listed in RTECS under LQ0250000. Oral Rat LD50 = 5577 mg/kg (Moscow, USSR - 1967). Toxic effects may include incontinence and ataxia.

FOR TRIS-HCL:

EYES: Contact may cause irritation.

SKIN: Contact may cause irritation.

INHALATION: May cause irritation to mucous membranes and upper respiratory tract.

INGESTION: Chronic ingestion or excessive dosage may cause irritation to gastrointestinal tract.

ADDITIONAL INFORMATION: RTECS: No data available.

FOR DITHIOHREITOL:

EYES: Contact may cause irritation.

SKIN: Contact may cause irritation.

INHALATION: May cause irritation to mucous membranes and upper respiratory tract.

INGESTION: Harmful if swallowed, inhaled or absorbed through skin. Reproductive effects, irritation, mutation and toxicity listed in RTECS under EK1610000. Intraperitoneal Mouse LD50 = 154 mg/kg (1974). Details of toxic effects not reported other than lethal dose value.

ECOLOGICAL INFORMATION

No information available.

DISPOSAL CONSIDERATIONS

US DOT / IATA: No applicable information.

TRANSPORTATION INFORMATION

RCRA - No applicable information.

SARA 302 - No applicable information.

SARA 313 - No applicable information.

EPA TSCA Section 8(b) - For Formamide, DTT and Tris-HCl: Chemical Inventory.

Exposure Limits - For Formamide: ACGIH TLV-TWA 18 mg/m3 (10 ppm) Skin.

DISPOSAL REL TO FORMAMIDE - air: 10H TWA 10 ppm (Sk).

California Proposition 65 - No applicable information.

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