

# T7 Sequencing Kit

**Product number 27168201**  
**100 reactions**

## T7 Sequencing Mixes\*

<b>Standard</b>	<b>27167401</b> <b>100 reactions</b>
<b>7-Deaza</b>	<b>27168601</b> <b>50 reactions</b>

\*Used to replenish “Read Short” mixes in 27168201 kit.

## 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.



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The T7 Sequencing™ Kit from USB Corporation provides all of the solutions and reagents required for 100 dideoxy sequencing reactions using T7 DNA polymerase. With these reagents, chain-terminated fragments representing as much as 1 kilobase of DNA sequence may be generated. In addition to cloned T7 DNA polymerase, the kit includes optimized mixtures of ultrapure deoxy- and dideoxynucleotides, and labeling mixes which allow the use of <sup>33</sup>P, <sup>32</sup>P and <sup>35</sup>S labeled dATP and dCTP. Detailed protocols are provided for all stages of DNA sequencing.

## COMPONENTS OF THE KIT

<i>'A' Mix-Short:</i>	840μM each dCTP, dGTP and dTTP; 93.5μM dATP; 14μM ddATP; 40mM Tris-HCl (pH 7.5) and 50mM NaCl.
<i>'C' Mix-Short:</i>	840μM each dATP, dGTP and dTTP; 93.5μM dCTP; 17μM ddCTP; 40mM Tris-HCl (pH 7.5) and 50mM NaCl.
<i>'G' Mix-Short:</i>	840μM each dATP, dCTP and dTTP; 93.5μM dGTP; 14μM ddGTP; 40mM Tris-HCl (pH 7.5) and 50mM NaCl.
<i>'T' Mix-Short:</i>	840μM each dATP, dCTP and dGTP; 93.5μM dTTP; 14μM ddTTP; 40mM Tris-HCl (pH 7.5) and 50mM NaCl.
<i>'A' Mix-Long:</i>	840μM each dCTP, dGTP and dTTP; 93.5μM dATP; 2.1μM ddATP; 40mM Tris-HCl (pH 7.5) and 50mM NaCl.
<i>'C' Mix-Long:</i>	840μM each dATP, dGTP and dTTP; 93.5μM dCTP; 2.8μM ddCTP; 40mM Tris-HCl (pH 7.5) and 50mM NaCl.
<i>'G' Mix-Long:</i>	840μM each dATP, dCTP and dTTP; 93.5μM dGTP; 2.8μM ddGTP; 40mM Tris-HCl (pH 7.5) and 50mM NaCl.
<i>'T' Mix-Long:</i>	840μM each dATP, dCTP and dGTP; 93.5μM dTTP; 2.8μM ddTTP; 40mM Tris-HCl (pH 7.5) and 50mM NaCl.
<i>T7 DNA Polymerase:</i>	8 units/μl in buffered glycerol solution.
<i>Enzyme Dilution Buffer:</i>	25mM Tris-HCl (pH 7.5), 5mM DTT, 100μg BSA/ml and 5% glycerol.

<i>Universal Primer:</i>	5'-d[GTAAAACGACGGCCAGT]-3' in aqueous solution, 0.86 A <sub>260</sub> units/ml (5pmol/μl).
<i>Annealing Buffer:</i>	1M Tris-HCl (pH 7.5), 100mM MgCl <sub>2</sub> and 160mM DTT.
<i>Labeling Mix-dATP:</i>	1.375μM each dCTP, dGTP and dTTP and 333.5mM NaCl.
<i>Labeling Mix-dCTP:</i>	1.375μM each dATP, dGTP and dTTP and 333.5mM NaCl.
<i>Stop Solution:</i>	0.3% each Bromophenol Blue and Xylene Cyanol FF; 10mM EDTA (pH 7.5) and 97.5% deionized formamide.
<i>Control Template:</i>	10μg of single-stranded M13mp18 DNA in 50μl of Tris-EDTA buffer.

**NOTE:** Remove the stock of T7 DNA Polymerase from storage at -20°C only momentarily to remove an aliquot. During use, keep all other reagents on ice until required. Additional reagents required are listed in Appendix 1, page 19.

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 solution can be stored for 4-8 weeks at 4°C. The T7 DNA Polymerase must be stored at -20°C and never be allowed to warm up above -20°C. Never store T7 DNA Polymerase in a frost-free freezer (the temperature rises above 0°C daily).

## QUALITY CONTROL

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

## SAFETY WARNINGS AND PRECAUTIONS

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

Caution: This product is to be used with radioactive material. Please follow the manufacturer's instructions relating to the handling, use, storage, and disposal of such material.

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

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as a lab coat, safety glasses, and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water (see material safety data sheet for specific advice).

# INTRODUCTION

Dideoxy sequencing depends upon base-specific termination of enzyme-catalyzed primer-extension reactions (1). Four separate reactions are performed, all containing primer, template, and the four deoxynucleotides, but each including a different chain-terminating dideoxynucleotide. In each reaction, a mixture of fragments is generated, each terminated with the particular dideoxynucleotide present in that reaction. Thus, the chain-terminated fragments in each reaction represent the occurrence of the corresponding deoxynucleotide in the sequence. When the products of the four reactions are electrophoresed side-by-side, the sequence in which nucleotides are added to the primer can be deduced from the sequence in which successively larger fragments occur in the four lanes. The positions of the separated fragments are detected by virtue of a label (radioactive or fluorescent) introduced either before or during the primer-extension reactions.

In the original procedure, primer extension was catalyzed by the Klenow fragment of *E. coli* DNA polymerase I. T7 DNA polymerase does, however, offer significant advantages over Klenow fragment for sequencing:

- Because of its processivity and high rate of polymerization, longer chain-terminated fragments ( $\geq$  one kilobase in length) can be generated very rapidly, with a more even distribution of label between fragments. This allows a greater length of sequence to be determined reliably from a single set of sequencing reactions.
- Because of its tolerance for substrate analogues, the same set of sequencing mixes may be used with either  $^{33}\text{P}$ ,  $^{32}\text{P}$  or  $^{35}\text{S}$ . In contrast, the Klenow fragment requires separate mixes for the two labels.

For successful sequencing, the enzyme used to catalyze primer extension must also be low in exonuclease activity, so that all chain-terminated fragments have the same 5'-end, and so that (di)deoxynucleotides are not removed from the 3'-ends of these fragments. Under the reaction conditions specified in this booklet, T7 DNA polymerase is suitably low in exonuclease activity.

The major practical difference when using T7 DNA polymerase rather than Klenow fragment is that the primer-extension reactions are performed in two stages, a "labeling" reaction and a "termination" reaction. The two stages are required because the enzyme uses dideoxynucleotides very readily. To permit synthesis of long chain-terminated fragments, dideoxynucleotides are therefore

excluded during the first stage, then added for the second. Even so, the total time required for these reactions is significantly less than the time required for reactions with the Klenow fragment. The steps involved in using T7 DNA polymerase to sequence DNA using a radioactive label are as follows:

- *Isolation of template DNA*, either single-stranded or double-stranded, containing the sequence to be determined (the “target” sequence). Protocols for culturing recombinant M13 or phagemids are given in Appendix 2. Protocols for isolating single-stranded and double-stranded DNA templates are found in Appendices 3 and 4. Other rapid procedures (2, 3) may also be used, if particular care is taken to remove all proteins.
- *Annealing of a primer* to the template, adjacent to the target sequence. Instructions for annealing primers to single-stranded templates are given in Procedure A. Two protocols, Procedures B and C, are given for annealing primers to double-stranded templates.
- *Labeling reaction* where enzyme-catalyzed extension of the primer is initiated in the presence of limiting concentrations of all four deoxynucleotides, one of which is radiolabeled.
- *Termination reactions* where the labeled and extended primer from the labeling reaction is terminated in four separate reactions, each containing a specific dideoxynucleotide in addition to non-limiting concentrations of all four deoxynucleotides.

*NOTE:* The labeling and termination reactions are performed in the same way for both single- and double-stranded templates, as described in Procedure D. If template or labeled nucleotide is limited, amounts of these reagents can be reduced with some sacrifice in performance. Such modifications to Procedure D are discussed in Appendix 5.

- *Electrophoresis* of chain-terminated reaction products in four adjacent lanes of a thin polyacrylamide gel, under denaturing conditions. Optimal results are obtained using wedge-shaped gels. Instructions for gel preparation and use are given in Appendix 6.
- *Autoradiography* to detect electrophoretically separated fragments. Appendix 7 contains instructions for autoradiography and guidelines for interpreting the results.

Common problems encountered in sequencing with T7 DNA polymerase are discussed in the Troubleshooting Guide following Appendix 7.

# PROTOCOL

## Introduction

### Choice of Label

Unlike the Klenow fragment, T7 DNA polymerase utilizes  $^{35}\text{S}$ -labeled nucleotides as efficiently as  $^{33}\text{P}$  or  $^{32}\text{P}$ -labeled nucleotides. Therefore, the same protocol and set of nucleotide mixes can be used with either radionucleotide. To allow even greater flexibility in choice of label, two labeling mixes are included: Labeling Mix-dATP for use with  $[\alpha\text{-}^{33}\text{P}]\text{dATP}$ ,  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$  or  $[\alpha\text{-}^{35}\text{S}]\text{dATP}\alpha\text{S}$  and Labeling Mix-dCTP for use with  $[\alpha\text{-}^{33}\text{P}]\text{dCTP}$ ,  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  or  $[\alpha\text{-}^{35}\text{S}]\text{dCTP}\alpha\text{S}$ . The choice of radiolabel should be based primarily on the following considerations:

- *With  $^{32}\text{P}$* , the sequencing gel can be autoradiographed without drying, and with shorter exposure times than required for  $^{35}\text{S}$ .
- *With  $^{33}\text{P}$ ,  $^{35}\text{S}$* , autoradiographic resolution is better than with  $^{32}\text{P}$ , exposure to radiation is reduced, and the labeled nucleotide has a longer “shelf-life”. (Half lives are 25 days for  $^{33}\text{P}$ ; 87 days for  $^{35}\text{S}$ .)

### Reactions to “Read Short” and “Read Long”

The sequencing reactions can be performed using either the “Read Short” or “Read Long” conditions:

- The “Read Short” conditions allow sequence to be read up to approximately 500 nucleotides from the primer, with either  $^{33}\text{P}$ ,  $^{32}\text{P}$  or  $^{35}\text{S}$ . (The four nucleotide mixes with suffix “-Short” are designed for such reactions.)
- The “Read Long” conditions generate fragments covering the sequence up to 1000 nucleotides or more from the primer. (The four nucleotide mixes with suffix “-Long” are designed for such reactions.) The full value of these conditions can of course only be realized if a gel system capable of resolving very large chain-terminated fragments is available. Because of differences in autoradiographic detection efficiencies, the starting point for readable sequence will depend on the radiolabel used. With overnight exposures, the sequence will generally start 10-50 bases from the primer with  $^{32}\text{P}$  and 50-100 bases from the primer with  $^{35}\text{S}$ .

In practice, the “Read Short” conditions can be used for most routine sequencing. If a high-resolution gel system is available, the “Read Long” reactions should be run as well, to reveal additional sequence information.

## Procedure A: Annealing of Primer to Single-Stranded Template

Single-stranded DNA can be isolated as described in Appendix 3.

- Adjust the concentration of the template so that 10 $\mu$ l contain 1.5-2 $\mu$ g of DNA.
- Using the Universal Primer provided in the kit, dilute an appropriate amount of the stock 1:5 with sterile distilled water. If using a primer other than the Universal Primer, adjust its concentration to 0.8 $\mu$ M (2 $\mu$ l should contain 1-2 $\mu$ mol); for a 17-base primer, this will be 4.44 $\mu$ g/ml.
- Add the following to a 1.5ml microcentrifuge tube:

Template DNA	10 $\mu$ l
Primer	2 $\mu$ l
Annealing Buffer	2 $\mu$ l
<i>Total Volume</i>	<u>14<math>\mu</math>l</u>

- Vortex the tube gently, then centrifuge briefly. Incubate at 60°C for 10 minutes.
- Place the tube at room temperature for at least 10 minutes, then centrifuge briefly.
- Proceed immediately with the sequencing reactions, Procedure D. (It is better to use freshly annealed primer/template, rather than to store the solution at room temperature or -20°C prior to use.)

## Procedure B: Quick Annealing of Primer to Double-Stranded Template

Double-stranded DNA can be isolated as described in Appendix 4.

The quick annealing protocol is very sensitive to even slight changes in pH. Calibrated 1.000  $\pm$  0.005M NaOH and 1.000  $\pm$  0.005M HCl are essential for the success of this procedure. A simple dilution of concentrated NaOH and HCl is not sufficient. Use the same micropipette to add both the NaOH and HCl since slight variations in pipetting can adversely affect the results. If results utilizing this protocol are unsatisfactory, repeat the sequencing reactions using the template/primer prepared according to the standard annealing protocol, Procedure C.

- Adjust the concentration of the template so that 8 $\mu$ l contain 1.5-2 $\mu$ g of DNA.
- With the Universal Primer, use 2 $\mu$ l of *undiluted* stock. If using a primer other than the Universal Primer, adjust its concentration to 2.5-5 $\mu$ M (2 $\mu$ l should contain 5-10 $\mu$ mol); for a 17-base primer, this would equal 15-30 $\mu$ g/ml.

- Add the following to a 1.5ml microcentrifuge tube:

Template DNA	8 $\mu$ l
Primer	2 $\mu$ l
1.000 $\pm$ 0.005M NaOH	<u>1.5<math>\mu</math>l</u>
<i>Total Volume</i>	11.5 $\mu$ l

- Vortex the tube gently, then centrifuge briefly. Incubate at 65°C for 5 minutes.
- Transfer the tube to a 37°C water bath and quickly add 1.5 $\mu$ l of 1.000  $\pm$  0.005M HCl and 2 $\mu$ l of Annealing Buffer. Remove the tube briefly and vortex gently.
- Immediately return the tube to 37°C and incubate for an additional 10 minutes.
- Place at room temperature for 5 minutes, then centrifuge briefly.
- Proceed immediately with the sequencing reactions, Procedure D. (It is better to use freshly denatured and annealed primer/template, rather than to store the solution at room temperature or -20°C prior to use.)

## Procedure C: Standard Annealing of Primer to Double-Stranded Template

- Adjust the concentration of the template so that 32 $\mu$ l contain 1.5-2 $\mu$ g.
- To denature the double-stranded template, add the following to a 1.5ml microcentrifuge tube:

Template	32 $\mu$ l
2M NaOH	<u>8<math>\mu</math>l</u>
<i>Total Volume</i>	40 $\mu$ l

- Vortex the tube gently, then centrifuge briefly. Incubate at room temperature for 10 minutes.
- Add 7 $\mu$ l of 3M sodium acetate (pH 4.8) and 4 $\mu$ l of distilled water.
- Add 120 $\mu$ l of 100% ethanol, mix, and place on Dry Ice for 15 minutes. Collect the precipitated DNA by centrifuging for 15 minutes. Carefully remove and discard the supernatant, then gently wash the pellet with ice-cold 70% ethanol. Recentrifuge for 10 minutes, and remove the supernatant. Dry the pellet briefly under vacuum, and redissolve it in 10 $\mu$ l of distilled water.
- With the Universal Primer, use 2 $\mu$ l of *undiluted* stock. If using a primer other than the Universal Primer, adjust its concentration to 2.5-5 $\mu$ M (2 $\mu$ l should contain 5-10pmol); for a 17-base primer, this would equal 15-30 $\mu$ g/ml.

- Add Primer and Annealing Buffer to the resuspended template, as indicated below:

Template DNA	10 $\mu$ l
Primer	2 $\mu$ l
Annealing Buffer	2 $\mu$ l
<i>Total Volume</i>	<u>14<math>\mu</math>l</u>

- Vortex the tube gently, then centrifuge briefly. Incubate at 65°C for 5 minutes.
- Quickly transfer the tube to a 37°C water bath and incubate for 10 minutes. Place at room temperature for at least 5 minutes, then centrifuge briefly.
- Proceed immediately to the sequencing reactions, Procedure D. (It is better to use freshly denatured and annealed primer/template, rather than to store the solution at room temperature or -20°C prior to use.)

## Procedure D: Sequencing Reactions

### Essential Preliminaries

- Choice of Labeling Mix: With an unlabeled primer, use Labeling Mix-dATP with [ $\alpha$ -<sup>33</sup>P]dATP, [ $\alpha$ -<sup>32</sup>P]dATP or [ $\alpha$ -<sup>35</sup>S]dATP $\alpha$ S and Labeling Mix-dCTP with [ $\alpha$ -<sup>33</sup>P]dCTP, [ $\alpha$ -<sup>32</sup>P]dCTP or [ $\alpha$ -<sup>35</sup>S]dCTP $\alpha$ S.
- Label four microcentrifuge tubes 'A', 'C', 'G' and 'T' respectively.
- To "*Read Short*" (up to 500 nucleotides with <sup>33</sup>P, <sup>32</sup>P or <sup>35</sup>S): Pipette 2.5 $\mu$ l respectively of the 'A' Mix-Short, 'C' Mix-Short, 'G' Mix-Short and 'T' Mix-Short into the corresponding tube.
- To "*Read Long*" (50-1000 nucleotides with <sup>32</sup>P, 100-1000 nucleotides with <sup>35</sup>S; see page 9): Pipette 2.5 $\mu$ l respectively of the 'A' Mix-Long, 'C' Mix-Long, 'G' Mix-Long and 'T' Mix-Long into the corresponding tube.
- Using cold Enzyme Dilution Buffer, dilute enough of the stock T7 DNA Polymerase\* for your immediate needs using the table provided opposite. Two microliters of diluted T7 DNA Polymerase will be required for each template to be sequenced. Mix by gentle pipetting and keep on ice until required.

\*NOTE: For maximum stability, remove the stock of T7 DNA Polymerase from storage at -20°C only momentarily to remove an aliquot.

Number of Templates	Volume of T7 DNA Polymerase	Volume of Dilution Buffer	Total Volume
2	1.0µl	4.0µl	5.0µl
3	1.5µl	6.0µl	7.5µl
4	2.0µl	8.0µl	10.0µl
5	2.5µl	10.0µl	12.5µl
6	3.0µl	12.0µl	15.0µl
7	3.5µl	14.0µl	17.5µl
8	4.0µl	16.0µl	20.0µl
9	4.5µl	18.0µl	22.5µl
10	5.0µl	20.0µl	25.0µl
11	5.5µl	22.0µl	27.5µl
12	6.0µl	24.0µl	30.0µl

### Labeling Reaction

- To the tube containing the annealed template and primer (from Procedures A, B or C), add the following:

Annealed template/primer	14-15µl
Labeling Mix	3µl
Labeled dNTP	1µl
<i>Diluted</i> T7 DNA Polymerase	<u>2µl</u>
<i>Total Volume</i>	20-21µl

- Mix the components by gentle pipetting, and collect the contents at the bottom of the tube by a brief centrifugation. Incubate at room temperature for 5 minutes.
- While this incubation is in progress, warm the four sequencing mixes just dispensed by placing the microcentrifuge tubes or at 37°C for at least 1 minute.
- After the 5-minute incubation of the labeling reaction, proceed immediately to the termination reactions.

### Termination Reactions

- After the labeling reaction has been incubated for 5 minutes, transfer 4.5µl of this reaction into each of the four pre-warmed sequencing mixes, using a fresh pipette tip for each transfer. Mix the components by gentle pipetting. Incubate at 37°C for 5 minutes.
- Add 5µl of Stop Solution to each tube, and mix gently. Spin briefly to collect the contents at the bottom.

- Transfer an aliquot (approximately 3 $\mu$ l) of each stopped reaction to a separate microcentrifuge tube, then heat it at 75-80°C for 2 minutes. Immediately load 1.5-2 $\mu$ l of each heated sample into the appropriate well of a sequencing gel.
- Store the remaining material at -20°C. If further aliquots are to be loaded (e.g. if there is a problem with the gel and it must be rerun), remove the samples from the freezer several minutes in advance, take aliquots (approximately 3 $\mu$ l), and heat them at 75-80°C for 2 minutes. Load 1.5-2 $\mu$ l of each as before.

## Appendix 1: Additional Reagents Required

**For culturing recombinant microorganisms** (Appendix 2):

- *M9 minimal medium agar*: Prepare a 5X M9 salt solution by dissolving 64g of Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 15g of KH<sub>2</sub>PO<sub>4</sub>, 2.5g of NaCl and 5.0g of NH<sub>4</sub>Cl in deionized water to a final volume of 1 liter. Divide the solution into 200ml aliquots and sterilize by autoclaving. Prepare the following and autoclave simultaneously: 750ml of deionized water containing 15g of agar; 100ml of deionized water; a 1M MgCl<sub>2</sub> solution; and a 1M CaCl<sub>2</sub> solution. Prepare a 20% (w/v) solution of glucose and a 1M solution of thiamine-HCl and sterilize both by filtration. After sterilizing the agar solution, cool to 50°C. Add 200ml of the 5X M9 salt solution and sterile water to a final volume of 1 liter. Add 1ml of 1M MgCl<sub>2</sub>, 1ml of 1M CaCl<sub>2</sub>, 1ml of 1M thiamine-HCl and 20ml of 20% glucose.
- *2X YT medium*: Dissolve 16g of Bactotryptone, 10g of yeast extract and 5g of NaCl in 1 liter of distilled water. Mix and autoclave.
- *LB medium*: Dissolve 10g of Bactotryptone, 5g of yeast extract and 10g of NaCl in 1 liter of distilled water. Mix and autoclave.
- *Plating agar*: Add 6g of agar to 1 liter of LB medium. Mix and autoclave.
- *LB agar*: Add 15g of agar to 1 liter of LB medium. Mix, autoclave, cool to 50-55°C and pour plates.
- *Ampicillin*: Dissolve 100 mg of the sodium salt of ampicillin in 4ml of distilled water. Sterilize by filtration and store in aliquots at -20°C.
- *Kanamycin*: Dissolve 280 mg of kanamycin in 4ml of distilled water. Sterilize by filtration and store in aliquots at -20°C.

**For DNA template isolation** (Appendices 3 and 4):

- *3.5M ammonium acetate (pH 7.4)/20% polyethylene glycol*: Aqueous solution.
- *Phenol*: Redistilled phenol saturated with TE buffer containing 8-hydroxy quinoline (3).

- *Chloroform/isoamyl alcohol*: Reagent-grade chloroform and isoamyl alcohol, mixed 24:1.
- *Phenol/chloroform*: Equal parts of redistilled phenol and chloroform/isoamyl alcohol (24:1), each prepared as described above.
- *3M sodium acetate (pH 7.5)*: Aqueous solution.
- *Tris buffer*: 10mM Tris-HCl (pH 7.5).
- *TE buffer*: 10mM Tris-HCl (pH 7.5), 1mM EDTA.
- *Solution I*: 100mM Tris-HCl (pH 7.5), 10mM EDTA, 400µg of heat-treated RNase I/ml.
- *Solution II*: 0.2M NaOH, 1% (w/v) SDS.
- *Solution III*: 3M potassium, 5M acetate. To prepare 100ml, mix 60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml of distilled water.

**For quick denaturation of double-stranded templates (Procedure B):**

- $1.000 \pm 0.005M$  NaOH.
- $1.000 \pm 0.005M$  HCl.

**For standard denaturation of double-stranded templates (Procedure C):**

- *2M NaOH*: Aqueous solution.
- *3M sodium acetate (pH 4.8)*: Aqueous solution.

**For sequencing reactions (Procedure D):**

- $[\alpha\text{-}^{32}\text{P}]\text{dATP}/[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ : 10mCi/ml, 3000 Ci/mmol,  $[\alpha\text{-}^{33}\text{P}]\text{dATP}/[\alpha\text{-}^{33}\text{P}]\text{dCTP}$ : 10mCi/ml or  $[\alpha\text{-}^{35}\text{S}]\text{dATP}\alpha\text{S}/[\alpha\text{-}^{35}\text{S}]\text{dCTP}\alpha\text{S}$ : 10mCi/ml, >1000 Ci/mmol.

**For gel electrophoresis (Appendix 6):**

- *Ammonium persulfate*.
- *TEMED*.
- *20% acrylamide solution*: Dissolve 193g of acrylamide, 6.7g of N,N'-methylenebisacrylamide, and 467g of urea (ultrapure) in distilled water to a final volume of 1 liter. Add 20-30g of mixed bed resin (1:1 mixture of strong cation and anion exchange resin). Stir for 30 minutes. Filter and store at room temperature.
- *RapidGels™-ready-to-use acrylamide gel solutions* (see Related Products Section)
- *46.7% urea solution*: Dissolve 467g of urea (ultrapure) in distilled water to a final volume of 1 liter. Add 20-30g of mixed bed resin. Stir for 30 minutes. Filter and store at room temperature.
- *10X TBE buffer*: Dissolve 121g of Tris base, 7.4g of EDTA and 53.4g of boric acid in distilled water to a final volume of 1 liter. Check the pH, which should be 8.3.

## Appendix 2: Culture of Recombinant Phage

The DNA to be sequenced should be cloned into an M13 or phagemid vector using standard procedures (3). M13 clones should be cultured according to the first procedure below, phagemids according to the second.

### Culture of M13 Recombinants

This procedure requires freshly grown M13 plaques, and *E. coli* cells which have been streaked out on minimal medium.

- Pick a single *E. coli* colony into 3ml of LB medium. Incubate with shaking at 37°C and grow until the  $A_{600}$  reaches 0.8-1.0.
- Transfer 200 $\mu$ l of this culture into a tube containing 2ml of 2X YT medium. Inoculate with a single colorless M13 plaque. Shake vigorously at 37°C for at least 5 hours, but no more than 8 hours.
- Remove all cells from the culture by two rounds of centrifugation. Carefully transfer the supernatant from the second round to a clean tube.
- At this point the clarified supernatant can be stored at 4°C before proceeding with the isolation of single-stranded DNA.

### Culture of Phagemid Recombinants

This procedure requires single colonies containing recombinant phagemids, a stock of M13KO7 helper phage, and both LB and 2X YT media with ampicillin at 100 $\mu$ g/ml.

- Pick a single phagemid colony into a tube containing 2ml of LB/ampicillin medium. Incubate overnight at 37°C.
- Transfer 50 $\mu$ l of this culture into a 50ml culture tube containing 1ml of 2X YT/ampicillin medium. Incubate at 37°C for 1-2 hours, until the  $A_{600}$  reaches 0.5-1.0.
- Assuming that an  $A_{600}$  of 1 is equivalent to  $8 \times 10^8$  cells/ml, add M13KO7 helper phage to a multiplicity of infection of 10. For example, if the phagemid culture has an  $A_{600}$  of 0.8, add  $6.4 \times 10^9$  helper phage, or 128 $\mu$ l from a stock at  $5 \times 10^{10}$  pfu/ml.
- Incubate with vigorous aeration at 37°C for 1 hour, then add 9ml of 2X YT broth and 10 $\mu$ l of kanamycin solution (70 $\mu$ g/ml). Continue incubation at 37°C with rapid shaking for 5-6 hours.
- Remove all cells from the culture by two rounds of centrifugation. Carefully transfer the supernatant from the second round to a clean tube.
- At this point the clarified supernatant can be stored at 4°C before proceeding with the isolation of single-stranded DNA.

### Appendix 3: Isolation of Single-Stranded Template

- To precipitate phage, add 0.25 volume of a 3.5M ammonium acetate/20% polyethylene glycol solution, invert several times to mix, and place on ice for 30 minutes.
- To collect precipitated phage, centrifuge for 15-30 minutes at 11,000 x g. Check for a visible white phage pellet and carefully remove the supernatant. Drain thoroughly, then remove any excess liquid by aspiration.
- Resuspend the pellet in TE buffer (100µl for M13 pellets, 400µl for phagemids), with gentle vortexing. All subsequent steps can be performed in microcentrifuge tubes.
- Add an equal volume of phenol/chloroform and vortex for 30 seconds. Centrifuge for 1 minute to separate the phases. Carefully remove the upper aqueous layer and transfer it to a clean tube. Repeat the extraction, and again recover and transfer the aqueous phase.
- Extract with an equal volume of chloroform, separate the phases by centrifugation, and transfer the upper phase to a clean tube. Repeat this extraction and recovery once.
- Add 0.1 volume of 3M sodium acetate (pH 7.5) and two volumes of 100% ethanol, mix and place on dry ice for 15 minutes. Pellet the DNA by centrifugation for 10 minutes in a microcentrifuge. Remove the supernatant. Add 1ml of ice-cold 70% ethanol and recentrifuge. Remove the supernatant and briefly dry the pellet under vacuum.
- Dissolve the DNA pellet in 20µl of Tris buffer and store at -20°C. The  $A_{260}/A_{280}$  ratio should be at least 1.7 for DNA sequencing.

### Appendix 4: Isolation of Double-Stranded Template

A procedure for purifying double-stranded DNA is described below. See Appendix 1, page 19, for required reagents.

- Transfer 1.5ml of an overnight culture of *E. coli* to a microcentrifuge tube and centrifuge at full speed for 30 seconds to pellet the cells.
- Remove the supernatant by aspiration without disturbing the cell pellet, leaving the pellet as dry as possible.
- Resuspend the pellet in 200µl of Solution I by vigorously vortexing.
- Add 200µl of Solution II and mix by inverting the tube several times. Incubate at room temperature for 5 minutes.
- Add 200µl of Solution III and mix by inverting the tube several times. Place on ice for 5 minutes.
- Centrifuge at full speed for 5 minutes at room temperature.
- Carefully decant the supernatant into a clean centrifuge tube.
- Add 420µl (0.7 volume) of ambient-temperature isopropanol to the supernatant and vortex to mix. Incubate for 5 minutes at room temperature.

- Centrifuge at full speed for 10 minutes. Decant the supernatant and invert the tube to drain.
- Resuspend the DNA pellet in 200 $\mu$ l of TE buffer by vortexing.
- Add 200 $\mu$ l of phenol to the aqueous sample. Vortex for 1 minute and centrifuge for 5 minutes at full speed to separate the phases.
- Transfer the upper aqueous phase to a fresh tube and add 200 $\mu$ l of chloroform/isoamyl alcohol. Vortex for 1 minute, then centrifuge for 5 minutes at full speed to separate the phases.
- Transfer the upper aqueous phase to a fresh tube and add 20 $\mu$ l of 3M sodium acetate and 500 $\mu$ l of absolute ethanol. Mix and place at -20°C for 10 minutes.
- Centrifuge at 4°C for 10 minutes, remove the supernatant, and wash the pellet with 1ml of 70% ethanol.
- Recentrifuge for 2 minutes, drain thoroughly, and dry the DNA pellet under vacuum.
- Dissolve the DNA pellet in 20 $\mu$ l of TE buffer and proceed immediately with denaturing (Procedure B or C) or store at -20°C for later use.

## **Appendix 5. Reducing the Amount of Template and Labeled Nucleotide**

Procedure D has been developed to give optimum performance with minimum exposure times and even band intensities. All of the required reagents except the template and labeled nucleotide are provided in the kit. In cases where the template is available in limited quantity or labeled nucleotide is at a premium, the amounts of these reagents can be reduced with some sacrifice in performance.

### **Template**

Using the kit, the best sequencing results are achieved with 2 $\mu$ g (~1pmol) of template. Decreasing the amount of template below this level produces a “two-tone effect” on the autoradiograph, with bands representing chain-terminated fragments less than 50 bases in length reduced in intensity by about one-half. This effect is caused by a reduction in the concentration of the primer/template complex relative to that of the nucleotides in the labeling reaction. By decreasing the nucleotide concentrations in the labeling reaction, even band patterns may be obtained from reactions using as little as 0.5 $\mu$ g of template, with some extension of exposure time.

Options:

- Follow the instructions in Procedure D using less than 2 $\mu$ g of template and accept a two-tone effect on the autoradiograph. If the sequence less than 50

bases from the primer is not legible, increase the gel exposure time by a factor of two.

- To restore even band intensities for reactions using less than 2 $\mu$ g of template, dilute the labeling mix by a factor of four with 330mM NaCl. This will eliminate the two-tone effect but will also increase the exposure time by a factor of two, requiring overnight exposure. (This dilution step is recommended for use only when  $^{32}\text{P}$  is the label.)

## Labeled Nucleotide

If the cost of the labeled nucleotide is critical, amounts can be reduced provided longer autoradiographic exposure times are acceptable.

Option:

- Use 3-5 $\mu$ Ci of [ $\alpha$ - $^{32}\text{P}$ ]dATP/[ $\alpha$ - $^{32}\text{P}$ ]dCTP in Procedure D and increase the gel exposure time by a factor of two. Generally, legible autoradiographs can be obtained overnight (16 hrs).

Since  $^{35}\text{S}$ -gels require a minimum of overnight exposure, we do not recommend reducing the amount of [ $\alpha$ - $^{35}\text{S}$ ]dATP $\alpha$ S/[ $\alpha$ - $^{35}\text{S}$ ]dCTP $\alpha$ S used in Procedure D.

## Appendix 6: Electrophoresis

To increase the amount of information obtained from each set of sequencing reactions, two or three separate samples from each reaction should be electrophoresed on the same sequencing gel, with a period of electrophoresis between loadings. Each loading should yield up to 200 nucleotides of sequence. When planning your gel requirements, remember that for this approach you will need to allocate four adjacent lanes for *each* loading, or 8-12 lanes per set of reactions.

The following general protocol is provided for the preparation and use of 8% polyacrylamide wedge gels. Wedge gels can increase the number of readable bands on a DNA sequencing gel by compressing the normally more widely spaced smaller sequencing fragments.

- Clean the glass gel plates with soap, rinse them thoroughly with water, and dry them. Rinse with ethanol and dry.
- Arrange the glass plates for assembly, with the Silane-treated sides of the plates facing inward, and with the bottom edges of the plates *slightly* offset. Insert 0.2mM (top)-0.4mM (bottom) wedge spacers between the long edges of the plates. Alternatively, use uniform 0.2mM spacers, and insert an extra piece of the same material, 2cm long, at the bottom of the long edges on each side. If the plates are to be clamped together, place a thin bead of petroleum jelly between the plates and the spacers before clamping.
- Tape or clamp the plates together.

- Insert the sample application comb at the top of the plates, or the surface-former if a shark's-tooth comb is to be used. Set the plates at a 45° angle with the *bottom* of the plates elevated so that the polymerizing solution can be introduced from this end.
- Mix 20ml of 20% acrylamide solution, 5ml of 10X TBE buffer, and 25ml of 46.7% urea solution (total = 50ml; 8% acrylamide).
- Filter the mixture and degas under low vacuum for 5 minutes.
- To initiate gel formation, add 250µl of 10% ammonium persulfate and 50µl of TEMED to the mixture, and swirl to mix.
- Immediately pour the solution into a 50ml syringe while blocking the spout. Insert the plunger into the syringe, invert, fit with an 18-gauge needle, and eject any air. Inject the solution between the plates, taking care to avoid introducing air bubbles. Any bubbles which do form can be dislodged by standing the plates upright (bottom uppermost), and tapping gently. Insert comb.
- After pouring the gel, lay the plates in a horizontal position for 45-60 minutes to allow the gel to polymerize.
- Wet the area around the sample comb or surface former with distilled water, and carefully remove the comb or former. Immediately rinse the sample wells or surface with distilled water, to remove unpolymerized acrylamide.
- Place the gel in the electrophoresis apparatus and add buffer, making sure that the sample wells are filled with buffer and free of air bubbles.
- Pre-run the gel at 40 W constant power for 45-60 minutes.
- If using a shark's-tooth comb, switch off the power supply and insert the comb several minutes prior to denaturing the samples. Insert it so that the points just touch the surface of the gel. Reconnect the gel to the power supply.
- After the gel has pre-electrophoresed and the samples are denaturing, switch off the power supply and wash out the wells with buffer. (*NOTE:* If this wash is omitted, loading will be difficult, and the bands may smear.) Load 1.5-2µl aliquots of denatured sequencing reactions (from Procedure D) into adjacent wells, in sets of four. If you will be running multiple samples from each set of sequencing reactions, remember to allocate four lanes of the gel for *each* loading.
- Reconnect the gel to the power supply and electrophorese at 40 W constant power. If you plan to load additional samples of the same sequencing reactions, continue electrophoresis until the xylene cyanol in the most recently loaded set is 4-5cm from the bottom of the gel (about 2 hours for a 40cm gel). At this point, switch off the power, wash out the wells in an adjacent group of four empty lanes, load the next set of samples, and continue electrophoresis as before.

- Stop the final period of electrophoresis when the bromophenol blue in the samples loaded last reaches the bottom of the gel.

## Appendix 7: Autoradiography and Analysis

- Switch off the power, then remove the gel/plate assembly from the electrophoresis apparatus. Lay the assembly in a horizontal position with the notched plate uppermost. Carefully separate the plates so that the gel remains attached to one of them.
- *For a gel containing  $^{32}P$ :*

To dry the gel (optional): Transfer it carefully to a supporting sheet of filter paper. Cover with high-quality plastic wrap, and dry using a vacuum gel dryer.

For an undried gel: Transfer the gel to a support (e.g. an old X-ray film) and cover it with high-quality plastic wrap. Fold the plastic wrap around the edges of the support.

In a darkroom, place the covered gel/support in a film cassette with an appropriately sized sheet of X-ray film next to the gel. Expose for 4-16 hours at  $-70^{\circ}C$  (undried gel) or room temperature (dried gel), then develop the film according to the manufacturer's instructions.

- *For a gel containing  $^{35}S$  or  $^{33}P$ :*

Place the gel/plate in a suitable tray containing 1-2 liter of 10% acetic acid/10% methanol in distilled water. Allow the gel to soak for 20 minutes. Remove the solution by aspiration, taking particular care if the gel has become detached from the plate. Reposition the gel on the glass plate if necessary, then transfer it carefully to a supporting sheet of filter paper. Cover with high-quality plastic wrap, and dry using a vacuum gel dryer.

When the gel is dry, carefully remove the plastic wrap and transfer the gel, on its filter-paper support, to a film cassette. In a darkroom, place an appropriately sized sheet of X-ray film next to the gel. Expose overnight at room temperature, then develop the film according to the manufacturer's instructions.

Read the sequence of the target DNA from the pattern of bands on the autoradiograph. To help locate the start of the target sequence, identify any known sequence between it and the primer site, such as the polylinker sequence in an M13-related vector. If multiple samples were loaded from a single set of reactions, look for overlaps of at least 20 bands between the patterns from successive sets of samples. With this approach, it should be possible to read at least 600 nucleotides from each set of reactions.

# TROUBLESHOOTING GUIDE

## ***“Band compressions” mask the correct sequence in a particular region of the gel***

- Fragments differing in size by one or a few nucleotides migrated with similar mobilities, because residues had formed stable intrastrand secondary structures which were not fully denatured during electrophoresis. Repeat the sequencing reactions with analogues less able to form intrastrand base pairs. Deaza G/A T7 Sequencing Mixes (27-1686-01) are designed for this purpose. Two purine analogues, 7-deaza dGTP and 7-deaza dATP, are combined in one set of mixes to eliminate the majority of compressions.

## ***Bands within 50 bases of the primer are faint, creating a “two-tone effect”***

- The template:nucleotide ratio in the labeling reaction was too low, resulting in the synthesis of longer labeled fragments. This reduces the relative number of smaller chain-terminated fragments, producing weaker band intensities in the lower section of the gel. Increase the amount of template and primer by a factor of two. Alternatively, decrease the nucleotide concentration in the labeling reaction (see Appendix 5).

## ***Band intensities in the uppermost portion of the sequence pattern are very light, creating a “two-tone effect”***

- A high concentration of protein in the electrophoresis sample may have affected the band pattern. Decrease the amount of enzyme added to the sequencing reactions by a factor of two. **Caution:** Using too little enzyme per set of reactions may cause the appearance of parallel bands in sequencing autoradiographs.

## ***The pattern is distorted in the 400-600 base region***

- The concentration of glycerol in the electrophoresis sample was too high. Dilute the enzyme in TE buffer instead of dilution buffer and then repeat the sequencing reactions. Alternatively, use a Glycerol Tolerant Gel (see Related Products Section) to resolve bands in this region.

## ***Specific bands are very light in intensity or missing***

- Certain chain-terminated fragments may be susceptible to attack by inorganic diphosphate generated during formation of the 5'-3' bond. This attack cleaves the ddNTP molecule from the end of the fragment and regenerates the triphosphate group on the remaining base, allowing further extension of the fragment by the polymerase. This phenomenon may often be reduced by limiting the time of the termination reactions to 2-3 minutes. Alternatively, inorganic pyrophosphatase may be used.

**Parallel bands are present in all four lanes above 200 bases from the primer**

- The enzyme activity may have been reduced if reaction temperatures above 37°C were used. Make sure that the temperature of the termination reaction is 37°C.
- The enzyme may have lost activity. Increase the amount of enzyme in the reaction two-fold.
- The template may have strong secondary structures that cause the polymerase to pause. Following the annealing step, incubate the primer/template solution at 60-70°C for 4 minutes, return the solution to room temperature for 2 minutes and then immediately proceed to the labeling reaction.

**Sequencing “troublesome” double-stranded templates**

- When sequencing troublesome double-stranded templates, it may help to include dimethyl sulfoxide (DMSO) in the annealing reaction. Prepare the template again so that 7µl contains 1.5-2µg of denatured double-stranded template. Modify the annealing reaction (page 15) as follows:

Denatured template	7µl
Primer	2µl
Annealing Buffer	2µl
DMSO	3µl
<i>Total Volume</i>	14µl

- Incubate at 37°C for 20 minutes to anneal the primer.
- Place the tube at room temperature for at least 10 minutes, then centrifuge it briefly.
- Proceed with the sequencing reactions, Procedure D. (It is better to use freshly denatured and annealed primer/template, rather than to store the solution at room temperature or -20°C prior to use.)

## REFERENCES

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2. Haltiner, M., Kempe, T. and Tjian, R., *Nucl. Acids Res.* **13**, 1015 (1985).
3. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboratory, second edition (1989).

## RELATED PRODUCTS

### Sequencing kits

Product	Application	Pack size	Product number
Sequenase™ PCR <sup>™</sup> Product Sequencing Kit	For rapid sequencing of PCR products	100 templates	70170
Sequenase Quick-Denature Plasmid Sequencing Kit	For rapid denaturation and sequencing of plasmid DNA	100 templates	70140
Thermo Sequenase™ Cycle Sequencing Kit	For radioactive cycle sequencing	100 templates	78500
Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit	For radioactive terminator cycle sequencing	50 templates 100 templates	79750 79760

### Sequencing enzymes

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

### USB Ultrapure reagents for DNA sequencing

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

## USB Ultrapure reagents for DNA sequencing continued...

<b>Product</b>	<b>Application</b>	<b>Pack size</b>	<b>Product number</b>
RapidGel™-6%	Gel electrophoresis	100ml 500ml	75843-100ml 75843-500ml
RapidGel-8%	Gel electrophoresis	100ml 500ml	75844-100ml 75844-500ml
RapidGel-GTG-6%	Gel electrophoresis	100ml 500ml	75846-100ml 75846-500ml
RapidGel-GTG-8%	Gel electrophoresis	100ml 500ml	75847-100ml 75847-500ml
RapidGel-40%	Gel electrophoresis	500ml	75848-500ml
RapidGel-XL-6%	Gel electrophoresis	100ml 500ml	75861-100ml 75861-500ml
RapidGel-XL-8%	Gel electrophoresis	100ml 500ml	75862-100ml 75862-500ml
RapidGel-XL-40%	Gel electrophoresis	100ml 500ml	75863-100ml 75863-500ml
TEMED	Gel electrophoresis	100g 500g	76320-100g 76320-500g
TBE Buffer, 10X	Gel electrophoresis	6 bottles	70454-1pk
Water, RNase-free		500ml 1 liter	70783-500ml 70783-1l
X-Gal	Cloning	100mg 250mg 1g 2g	10077-100mg 10077-250mg 10077-1g 10077-2g

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**Material Safety Data Sheet**

Revision: 05/05/99

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  
Stop Solution (in T7  
Sequencing Kit 27168201)

SUPPLIER:

USB Corporation  
26111 Miles Road  
Cleveland, Ohio 44128 (216) 765-5000

PRODUCT CODE

1682H

EEC NUMBER

None

EMERGENCY CONTACT:

Chemtrec; 1-800-424-9300  
Outside USA & Canada 703-527-3887

COMPOSITION/HAZARDOUS  
COMPONENTS

HAZARD  
Formamide

CAS.NO.  
75-12-7

%WT 97.5%  
TLV 10ppm  
(AcGIH)

CHIP R & S PHRASES  
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

HAZARDS IDENTIFICATION

CHIP

Formamide: Toxic to reproduction, Category 3

HCS

Formamide: Teratogen.

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. Flash Point=310°F.  
Fire or excessive heat may produce hazardous oxides of carbon and nitrogen.



Formamide

## 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

Pregnant women or women of child bearing age should minimize contact and exposure to formamide.

## PHYSICAL AND CHEMICAL PROPERTIES

Kit containing vials of solutions.

Colorless liquid. Faint odor of ammonia. Soluble in water.

Boiling Point=210°C

Melting Point=3°C

Density=1.13

Vapor Density=1.55 (Air=1)

Specific Gravity=1.13

Flash Point=310°F

## STABILITY AND REACTIVITY

Product is stable. Fire or excessive heat may produce hazardous oxides of carbon and nitrogen.

## 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.

## ECOLOGICAL INFORMATION

No information available

## DISPOSAL CONSIDERATIONS

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

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