

T7Sequencing™ Kit from Amersham Biosciences provides all of the solutions and reagents required for 100 sets of 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 labelling mixes which allow the use of both 32P- and 35S-labelled dATP and dCTP. Detailed protocols are provided for all stages of DNA sequencing.

P H A R M A C I A B I O T E C H

T7Sequencing™ Kit INSTRUCTIONS

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Rev. 3

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Molecular Biology Reagents Division

COMPONENTS

'A' Mix-Short: 840 μ M each dCTP, dGTP and dTTP; 93.5 μ M dATP; 14 μ M ddATP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.

'C' Mix-Short: 840 μ M each dATP, dGTP and dTTP; 93.5 μ M dCTP; 17 μ M ddCTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.

'G' Mix-Short: 840 μ M each dATP, dCTP and dTTP; 93.5 μ M dGTP; 14 μ M ddGTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl.

'T' Mix-Short: 840 μ M each dATP, dCTP and dGTP; 93.5 μ M dTTP; 14 μ M ddTTP; 40 mM Tris-HCl (pH 7.6)

and 50 mM NaCl.

'A' Mix-Long: 840 μ M each dCTP, dGTP and dTTP; 93.5 μ M

dATP; 2.1 μ M ddATP; 40 mM Tris-HCl (pH 7.6)

and 50 mM NaCl.

'C' Mix-Long: 840 μ M each dATP, dGTP and dTTP; 93.5 μ M

dCTP; 2.8 μ M ddCTP; 40 mM Tris-HCl (pH 7.6)

and 50 mM NaCl.

'G' Mix-Long: 840 μ M each dATP, dCTP and dTTP; 93.5 μ M

dGTP; 2.8 μ M ddGTP; 40 mM Tris-HCl (pH 7.6)

and 50 mM NaCl.

'T' Mix-Long: 840 μ M each dATP, dCTP and dGTP; 93.5 μ M

dTTP; 2.8 μ M ddTTP; 40 mM Tris-HCl (pH 7.6)

and 50 mM NaCl.

(continued)

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T7 DNA Polymerase: 8 units/ μ l in buffered glycerol solution.

Enzyme Dilution Buffer: 20 mM Tris-HCl (pH 7.5), 5 mM DTT, 100 μ g BSA/ml

and 5% glycerol.

Universal Primer: 5'-d[GTAAAACGACGGCCAGT]-3' in aqueous solution,

0.86 A260 units/ml (5 pmol/ μ l).

Annealing Buffer: 1 M Tris-HCl (pH 7.6), 100 mM MgCl₂ and 160 mM DTT.

Labelling Mix-dATP: 1.375 μ M each dCTP, dGTP and dTTP and 333.5 mM NaCl.

Labelling Mix-dCTP: 1.375 μ M each dATP, dGTP and dTTP and 333.5 mM NaCl.

Stop Solution: 0.3% each Bromophenol Blue and Xylene Cyanol FF;
10 mM EDTA (pH 7.5) and 97.5% deionized formamide.

Control Template: 10 µg of single-stranded M13mp18 DNA in 50 µl 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.

NOTICE TO PURCHASERS

This kit is sold pursuant to a limited sublicense from United States Biochemical Corporation under U.S. Patent Nos. 4,795,699; 4,921,794; 4,942,130; 4,946,786; 4,962,020 and 4,994,372 and corresponding foreign patents and patent applications. The purchase of this kit includes a limited nonexclusive sublicense (without the right to resell, repackage or further sublicense) under such patent rights to use the kit for manual DNA sequencing. No license under the above-recited patents is hereby granted for use of this kit within the United States. Such sublicense is granted solely for research and legally approved uses. No other license under the above-recited patents is granted expressly, impliedly, or by estoppel.

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OVERVIEW

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 ^{32}P or ^{35}S . In contrast, the Klenow fragment requires separate mixes for the two labels.

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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, as purified by Amersham Biosciences, 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 “labelling” 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 doublestranded DNA templates are found in Appendices 3 and 4. Alternatively, Sephaglas™ PhagePrep Kit from Amersham Biosciences (27-9284-01) may be used to extract and purify single-stranded DNA while FlexiPrep Kit (27-9281-01) is recommended for isolating double-stranded templates. Other rapid procedures (2, 3) may also be used, if particular care is taken to remove all proteins.

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- *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.

- *Labelling reaction* where enzyme-catalyzed extension of the primer is initiated in the presence of limiting concentrations of all four deoxynucleotides, one of which is radiolabelled.

- *Termination reactions* where the labelled and extended primer from the labelling reaction is terminated in four separate reactions, each containing a specific dideoxynucleotide in addition to non-limiting concentrations of all four deoxynucleotides.

NOTE: The labelling and termination reactions are performed in the same way for both single- and double-stranded templates, as described in Procedure D. If template or labelled 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.

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PROTOCOL

Introduction

Choice of Label

Unlike the Klenow fragment, T7 DNA polymerase utilizes 35S-labelled nucleotides as efficiently as 32P-labelled 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: Labelling Mix-dATP for use with [α -32P]dATP or [α -35S]dATP α S and Labelling Mix-dCTP for use with [α -32P]dCTP or [α -35S]dCTP α S. The choice of radiolabel should be based primarily on the following considerations:

- *With 32P*, the sequencing gel can be autoradiographed without drying, and with shorter exposure times than required for 35S.
- *With 35S*, autoradiographic resolution is better than with 32P, exposure to radiation is reduced, and the labelled nucleotide has a longer “shelf-life”.

For automated sequencing, Amersham Biosciences provides AutoRead™ Sequencing Kit (27-1690-04), AutoRead™ 1000 Sequencing Kit (27-1791-01) and AutoCycle™ Sequencing Kit (27-1693-03) for use with Automated Laser Fluorescent ALF™ DNA Sequencer. For producing fluorescent-labelled primers on a DNA synthesizer such as Gene Assembler® Plus, Amersham Biosciences offers the fluorescein amidite, FluorePrime™ (27-1796-01).

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 ^{32}P or ^{35}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}P and 50-100 bases from the primer with ^{35}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.

Timetable for Sequencing

When planning to perform and analyze sequencing reactions, it may be helpful to allocate time for the various stages, as follows:

1. Pour gel (45 minutes).

2. While gel sets (1 hour), plan reactions in detail and thaw reagents.
3. While gel pre-runs (45 minutes-1 hour), perform sequencing reactions.
4. Load first set of samples on gel (10-15 minutes).
5. Run gel (2-3 hours, depending upon gel system).
6. Load second set of samples (10-15 minutes).
7. Run gel (2-3 hours).
8. Load third set of samples (10-15 minutes).
9. Run gel (2-3 hours).
10. Fix and dry gel (optional for ³²P label; about 1 hour).
11. Set up autoradiography (10 minutes).
12. Expose autoradiograph (4 hours for ³²P; 16-24 hours for ³⁵S).
13. Develop and dry autoradiograph (up to 1 hour).

In the procedures which follow, components provided with the kit are highlighted by the use of **boldface type**.

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Procedure A: Annealing of Primer to Single-

Stranded Template

Single-stranded DNA can be isolated as described in Appendix 3. Alternatively, Sephaglas™ PhagePrep Kit from Amersham Biosciences (27-9284-01) may be used for the rapid extraction and purification of single-stranded DNA which is suitable for direct use in sequencing.

- Adjust the concentration of the template so that 10 µl contain

1.5-2 μg 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 μM (2 μl should contain 1-2 pmol); for a 17-base primer, this will be 4.44 $\mu\text{g}/\text{ml}$.

- Add the following to a 1.5 ml microcentrifuge tube:

Template DNA 10 μl

Primer 2 μl

Annealing Buffer 2 μl

Total Volume 14 μl

- 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.)

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Procedure B: Quick Annealing of Primer to Double-

Stranded Template

Double-stranded DNA can be isolated as described in Appendix 4. Alternatively, FlexiPrep Kit from Amersham Biosciences (27-9281-01) may be used for rapid extraction and purification of double-stranded DNA.

The quick annealing protocol is very sensitive to even slight changes in pH. Calibrated 1.000 + 0.005 M NaOH and 1.000 + 0.005 M 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 μ l contain 1.5-2 μ g of DNA.
- With the **Universal Primer**, use 2 μ l of *undiluted* stock. If using a primer other than the Universal Primer, adjust its concentration to 2.5-5 μ M (2 μ l should contain 5-10 pmol); for a 17-base primer, this would equal 15-30 μ g/ml.
- Add the following to a 1.5 ml microcentrifuge tube:

Template DNA 8 μ l

Primer 2 μ l

1.000 + 0.005 M NaOH 1.5 μ l

Total Volume 11.5 μ l

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- 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 μ l of 1.000 + 0.005 M HCl and 2 μ 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 μl contain 1.5-2 μg .

- To denature the double-stranded template, add the following to a

1.5 ml microcentrifuge tube:

Template 32 μl

2 M NaOH 8 μl

Total Volume 40 μl

- Vortex the tube gently, then centrifuge briefly. Incubate at room temperature for 10 minutes.

- Add 7 μl of 3 M sodium acetate (pH 4.8) and 4 μl of distilled water.

- Add 120 μl 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 μl of distilled water.

- With the **Universal Primer**, use 2 μl of *undiluted* stock. If using a primer other than the Universal Primer, adjust its concentration to 2.5-5 μM (2 μl should contain 5-10 pmol); for a 17-base primer, this would equal 15-30 $\mu\text{g/ml}$.

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

Template DNA 10 μl

Primer 2 μ l

Annealing Buffer 2 μ l

Total Volume 14 μ l

- 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.)

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Procedure D: Sequencing Reactions

Essential Preliminaries

- Choice of Labelling Mix: With an unlabelled primer, use **Labelling Mix-dATP** with [α -32P]dATP or [α -35S]dATP α S and **Labelling MixdCTP** with [α -32P]dCTP or [α -35S]dCTP α S.
- Label four microcentrifuge tubes 'A', 'C', 'G' and 'T' respectively.

Alternatively, label four wells of a MicroSample Plate (18-1013-69) in corresponding fashion.

- *To "Read Short"* (up to 500 nucleotides with 32P or 35S): Pipette 2.5 μ l respectively of the '**A**' **Mix-Short**, '**C**' **Mix-Short**, '**G**' **Mix-Short** and '**T**' **Mix-Short** into the corresponding tube or well.

• To “*Read Long*” (50-1000 nucleotides with 32P, 100-1000 nucleotides with 35S; see page 9): Pipette 2.5 µl respectively of the ‘**A**’ **Mix-Long**, ‘**C**’ **Mix-Long**, ‘**G**’ **Mix-Long** and ‘**T**’ **Mix-Long** into the corresponding tube or well.

• 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. The diluted stock may be kept at 4°C for up to one week and still give good sequencing results.

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Number of Volume of T7 Volume of Total Templates DNA Polymerase Dilution Buffer
Volume

2 1.0 µl 4.0 µl 5.0 µl

3 1.5 µl 6.0 µl 7.5 µl

4 2.0 µl 8.0 µl 10.0 µl

5 2.5 µl 10.0 µl 12.5 µl

6 3.0 µl 12.0 µl 15.0 µl

7 3.5 µl 14.0 µl 17.5 µl

8 4.0 µl 16.0 µl 20.0 µl

9 4.5 µl 18.0 µl 22.5 µl

10 5.0 µl 20.0 µl 25.0 µl

11 5.5 µl 22.0 µl 27.5 µl

12 6.0 μ l 24.0 μ l 30.0 μ l

Labelling Reaction

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

[Annealed template/primer 14-15 μ l]

Labelling Mix 3 μ l

Labelled dNTP 1 μ l

Diluted T7 DNA Polymerase 2 μ l

Total Volume 20-21 μ l

- 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.

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- While this incubation is in progress, warm the four sequencing mixes just dispensed (page 16), by placing the microcentrifuge tubes or MicroSample Plate at 37°C for at least 1 minute (the plate may safely be floated without its lid in a water bath).
- After the 5-minute incubation of the labelling reaction, proceed immediately to the termination reactions.

Termination Reactions

- After the labelling reaction has been incubated for 5 minutes, transfer 4.5 μ l 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 5 μ l of **Stop Solution** to each tube or well, and mix gently. If using microcentrifuge tubes, spin these briefly to collect the contents at the bottom.

- Transfer an aliquot (approximately 3 μ l) of each stopped reaction to a separate microcentrifuge tube or new MicroSample Plate, then heat it at 75-80°C for 2 minutes (again, the MicroSample Plate may be floated without its lid in a water bath). Immediately load 1.5-2 μ 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 (see rationale on page 29), remove the samples from the freezer several minutes in advance, take aliquots (approximately 3 μ l), and heat them at 75-80°C for 2 minutes. Load 1.5-2 μ l of each as before.

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Appendix 1: Additional Reagents Required

Code numbers listed below refer to products from Amersham Biosciences.

For culturing recombinant microorganisms (Appendix 2):

- *M9 minimal medium agar*: Prepare a 5X M9 salt solution by dissolving 64 g of Na₂HPO₄·7H₂O, 15 g of KH₂PO₄, 2.5 g of NaCl and 5.0 g of NH₄Cl in deionized water to a final volume of 1 liter. Divide the solution into 200 ml aliquots and sterilize by autoclaving. Prepare the following and autoclave simultaneously: 750 ml of deionized water containing 15 g of agar; 100 ml of deionized water; a 1 M MgCl₂ solution; and a 1 M CaCl₂ solution. Prepare a 20% (w/v) solution of glucose and a 1 M solution of thiamine-HCl and sterilize both by filtration. After sterilizing the agar solution, cool to 50°C. Add 200 ml of the 5X M9 salt solution and sterile water to a final volume of 1 liter. Add 1 ml of 1 M MgCl₂, 1 ml of 1 M CaCl₂, 1 ml of 1 M thiamine-HCl and 20 ml of 20% glucose.

- *2X YT medium*: Dissolve 16 g of Bactotryptone, 10 g of yeast extract and 5 g of NaCl in 1 liter of distilled water. Mix and autoclave.
- *LB medium*: Dissolve 10 g of Bactotryptone, 5 g of yeast extract and 10 g of NaCl in 1 liter of distilled water. Mix and autoclave.
- *Plating agar*: Add 6 g of agar to 1 liter of LB medium. Mix and autoclave.

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Caution:

Several of the reagents listed below (such as phenol and acrylamide) are toxic or corrosive and should be handled with great care.

- *LB agar*: Add 15 g 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 4 ml of distilled water. Sterilize by filtration and store in aliquots at -20°C.
- *Kanamycin*: Dissolve 280 mg of kanamycin in 4 ml of distilled water. Sterilize by filtration and store in aliquots at -20°C.

For DNA template isolation (Appendices 3 and 4):

- *3.5 M 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.
- *3 M sodium acetate (pH 7.5)*: Aqueous solution.
- *Tris buffer*: 10 mM Tris-HCl (pH 7.5).
- *TE buffer*: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.
- *Solution I*: 100 mM Tris-HCl (pH 7.5), 10 mM EDTA, 400 µg of heat-treated RNase I/ml.
- *Solution II*: 0.2 M NaOH, 1% (w/v) SDS.
- *Solution III*: 3 M potassium, 5 M acetate. To prepare 100 ml, mix 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of distilled water.

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

- *1.000 + 0.005 M NaOH*.
- *1.000 + 0.005 M HCl*.

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

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

For sequencing reactions (Procedure D):

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

For gel electrophoresis (Appendix 6):

- *Ammonium persulfate*: 80-1128-12.

- *Repel-Silane*: 80-1129-42.
- *TEMED*: 80-1128-13.
- *20% acrylamide solution*: Dissolve 193 g of acrylamide [80-1128-10 or 80-1128-15; the latter is Pre PAG Mix (19:1) for 500 ml], 6.7 g of N,N'-methylenebisacrylamide (80-1128-11), and 467 g of urea [80-1128-80 (ultrapure)] in distilled water to a final volume of 1 liter.

Add 20-30 g of Amberlite MB-3, and stir for 30 minutes. Filter and store at room temperature.

- *46.7% urea solution*: Dissolve 467 g of urea [80-1128-80 (ultrapure)] in distilled water to a final volume of 1 liter. Add 20-30 g of Amberlite MB-3, and stir for 30 minutes. Filter and store at room temperature.

- *10X TBE buffer*: Dissolve 121 g of Tris base, 7.4 g of EDTA and 53.4 g of boric acid in distilled water to a final volume of 1 liter.

Check the pH, which should be 8.3.

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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 3 ml of LB medium. Incubate with shaking at 37°C and grow until the A600 reaches 0.8-1.0.
- Transfer 200 µl of this culture into a tube containing 2 ml 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.

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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 µg/ml.

- Pick a single phagemid colony into a tube containing 2 ml of LB/ampicillin medium. Incubate overnight at 37°C.
- Transfer 50 µl of this culture into a 50 ml culture tube containing 1 ml of 2X YT/ampicillin medium. Incubate at 37°C for 1-2 hours, until the A600 reaches 0.5-1.0.
- Assuming that an A600 of 1 is equivalent to 8×10^8 cells/ml, add M13KO7 helper phage to a multiplicity of infection of 10. For example, if the phagemid culture has an A600 of 0.8, add 6.4×10^9 helper phage, or 128 µl from a stock at 5×10^{10} pfu/ml.
- Incubate with vigorous aeration at 37°C for 1 hour, then add 9 ml of 2X YT broth and 10 µl of kanamycin solution (70 µg/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.

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Appendix 3: Isolation of Single-Stranded Template

NOTE: Rapid extraction and purification of single-stranded DNA is greatly simplified by the use of Sephaglas PhagePrep Kit from Amersham Biosciences (27-9284-01), instead of the method described here.

- To precipitate phage, add 0.25 volume of a 3.5 M 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 (100 µl for M13 pellets, 400 µl 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 3 M 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 1 ml of ice-cold 70% ethanol and recentrifuge. Remove the supernatant and briefly dry the pellet under vacuum.

- Dissolve the DNA pellet in 20 μ l of Tris buffer and store at -20°C . The A260/A280 ratio should be at least 1.7 for DNA sequencing.

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Appendix 4: Isolation of Double-Stranded Template

NOTE: Rapid isolation of plasmid DNA is greatly simplified by the use of FlexiPrep Kit from Amersham Biosciences (27-9281-01). An alternate procedure is described below. See Appendix 1, page 19, for required reagents.

- Transfer 1.5 ml 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 200 μ l of Solution I by vigorously vortexing.
- Add 200 μ l of Solution II and mix by inverting the tube several times. Incubate at room temperature for 5 minutes.
- Add 200 μ l 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 420 μ l (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.

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- Resuspend the DNA pellet in 200 μ l of TE buffer by vortexing.
- Add 200 μ 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 μ 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 μ l of 3 M sodium acetate and 500 μ 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 1 ml of 70% ethanol.
- Recentrifuge for 2 minutes, drain thoroughly, and dry the DNA pellet under vacuum.
- Dissolve the DNA pellet in 20 μ l of TE buffer and proceed immediately with denaturing (Procedure B or C) or store at -20°C for later use.

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Appendix 5. Reducing the Amount of Template and Labelled 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 labelled 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 µg 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 labelling reaction. By decreasing the nucleotide concentrations in the labelling reaction, even band patterns may be obtained from reactions using as little as 0.5 µg of template, with some extension of exposure time.

Options:

- Follow the instructions in Procedure D using less than 2 µg 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.

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- To restore even band intensities for reactions using less than 2 µg of template, dilute the labelling mix by a factor of four with 330 mM 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 ³²P is the label.)

Labelled Nucleotide

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

Option:

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

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

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The following general protocol is provided for the preparation and use of 8% polyacrylamide wedge gels.

- Clean the glass gel plates with soap, rinse them thoroughly with water, and dry them. Rinse with ethanol and dry. Treat both plates with Repel-Silane (80-1129-42), rinse with distilled water 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 0.2 mm (top)-0.4 mm (bottom) wedge spacers (CBS Scientific, Del Mar, CA) between the long edges of the plates. Alternatively, use uniform 0.2 mm spacers, and insert an extra piece of the same material, 2 cm 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 20 ml of 20% acrylamide solution, 5 ml of 10X TBE buffer, and 25 ml of 46.7% urea solution (total = 50 ml; 8% acrylamide).
- Filter the mixture and degas under low vacuum for 5 minutes.
- To initiate gel formation, add 250 µl of 10% ammonium persulfate and 50 µl of TEMED to the mixture, and swirl to mix.
- Immediately pour the solution into a 50 ml syringe while blocking the spout. Insert the plunger into the syringe, invert, fit with an

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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 1.5-2 μ l 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.

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- 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 4-5 cm from the bottom of the gel (about 2 hours for a 40 cm 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 32P:*

To dry the gel (optional): Transfer it carefully to a supporting sheet of filter paper (Whatman Number 1). 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

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4-16 hours at -70°C (undried gel) or room temperature (dried gel), then develop the film according to the manufacturer's instructions.

- *For a gel containing 35S:*

Place the gel/plate in a suitable tray containing 1 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 (Whatman Number 1). 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.

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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 T7Sequencing™ 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 labelling reaction was too low, resulting in the synthesis of longer labelled 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 labelling reaction (see Appendix 5, page 27).

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:**

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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, reduce the amount of enzyme added to the sequencing reaction by a factor of two. **Caution:** Using too little enzyme per set of reactions may cause the appearance of parallel bands (bands in all four lanes at the same position) in sequencing autoradiographs.

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.

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.

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- 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 labelling 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 7 µl contains 1.5-2 µg of denatured double-stranded template. Modify the annealing reaction (page 15) as

follows:

Denatured template 7 µl

Primer 2 µl

Annealing Buffer 2 µl

DMSO 3 µl

Total Volume 14 µl

- 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.)

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FUNCTION TESTING

The performance of each batch of T7Sequencing™ Kit is tested by sequencing a standard M13 template.

STORAGE

Store at -20°C.

ORDERING INFORMATION

T7Sequencing™ Kit 27-1682-01

Companion Products

T7Sequencing™ Mixes1 27-1674-01

Deaza G/A T7Sequencing™ Mixes2 27-1686-01

1 Fully compatible with T7Sequencing™ Kit; may be used to replenish the “Read Short” mixes included in the kit. Sufficient for 100 sets of sequencing reactions. 2 Similar to above but includes 7-deaza dGTP and 7-deaza dATP for elimination of ambiguous sequence data caused by band compressions.

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