CHAPTER 13

Dideoxy Sequencing Reactions Using T7 Polymerase

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1. Introduction

The chain termination DNA sequencing procedure introduced by Sanger and coworkers in 1977 (1) meant a revolutionary achievement in fast, simple, and accurate deciphering of DNA fragments. Long stretches of this nucleic acid could from then on be sequenced within a relatively short time. The method makes use of the enzymatic elongation of specific oligodeoxyribonucleotide primers that are complementary to a specific sequence of the DNA template. Four different reaction mixes each contain all four conventional deoxyribonucleotide triphosphates (dNTPs) plus a small amount of one dideoxyribonucleotide triphosphate (ddNTP), an analog that leads to chain termination since the lacking 3'-hydroxyl group prevents formation of a phosphodiester bond with a succeeding nucleotide. The four assays each contain a different dideoxynucleotide triphosphate. The chain extension reaction thus competes with the infrequent but specific termination by a ddNTP. This leads to a mixture of DNA fragments of varying lengths that all possess the same 5'-end owing to the common primer. Since one of the dNTPs contains a radioactive isotope (either ^{32}P or ^{35}S) these fragments become radioactively labeled and can be detected by autoradiography after separation by gel electrophoresis. The corresponding sequence is derived from the

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band pattern of the four different reaction mixtures. Single-stranded phage and denatured double-stranded plasmid DNA can serve as a template, thus opening the possibility to use a large variety of vectors for cloning. Primers that are complementary to various regions of the multicloning sites of these vectors are commercially available from a number of molecular biological companies or can easily be synthesized in the lab (compare Chapter 11).

The sequencing reaction is catalyzed by DNA polymerases such as Klenow fragment of *Escherichia coli* DNA polymerase I, reverse transcriptase (from Avian Myeloblastosis Virus [AMV] or Moloney-Murine Leukemia Virus [M-MLV]), a number of thermostable polymerases (from *Thermus aquaticus* [Taq], *Thermus thermophilus* [Tth], *Thermococcus litoralis* [Vent_R], *Bacillus stearothermophilus* [Bst]), and T7 DNA polymerase. Originally, the Klenow fragment was used for dideoxy sequencing. However, this enzyme has a low processivity, meaning that it will randomly dissociate from the template and thus give rise to fragments that were *not* terminated by incorporation of a dideoxyribonucleotide. Thus, only relatively short sequences can be determined reliably in one run. Furthermore, chain elongation proceeds at a relatively low rate, secondary structures of the template create a problem, special nucleotides used in case of band compressions are not equally well incorporated, and the enzyme is very sensitive to contaminants in template preparations. Reverse transcriptase can better cope with problems caused by secondary structures in the template DNA, but thermostable polymerases usually are the method of choice in this case since even stable secondary structures are precluded at temperatures of 70–75°C.

The bacteriophage T7 DNA polymerase complex consists of two polypeptides, the gene 5 product encoded by the phage and thioredoxin encoded by the *E. coli* host (2,3). This enzyme is now widely used for dideoxy sequencing because of its high processivity, high rate of polymerization, and tolerance for nucleotide analogs. Since the enzyme has been reported to possess a high 3'–5' exonuclease activity (4,5) two different ways have been used to render it suitable for sequencing. One approach was to modify the enzyme either chemically by oxidation (6,7) or genetically to remove the exonuclease activity. The resulting products are known under the trade name "Sequenase" and

their use is described in Chapter 14. The other approach made use of a specific FPLC-purification of cloned T7 DNA polymerase to obtain a protein suitably low in exonuclease activity. Such a preparation proved to be similarly advantageous or even superior to modified T7 polymerase (8). This chapter describes the dideoxy sequencing procedure using unmodified T7 DNA polymerase.

2. Materials

2.1. Primers

Various primers for a number of commonly used cloning vectors are commercially available (e.g., from Amersham [Arlington Heights, IL], GIBCO BRL [Gaithersburg, MD], International Biotechnologies Inc. [New Haven, CT], New England Biolabs [Beverly, MA], Pharmacia LKB [Piscataway, NJ], Sigma [St. Louis, MO], Stratagene [La Jolla, CA], United States Biochemical Corporation [Cleveland, OH]). Alternatively, appropriate primers can be synthesized by automatic machines or by hand (*compare* Chapter 11). The concentration of primer in the annealing reaction should be approx 0.11 μM (corresponding to 0.67 $\mu\text{g/mL}$ for a 17mer). However, even much higher concentrations have been used successfully (*compare* Chapter 11).

2.2. Template Preparation (Double-Stranded Plasmid DNA)

1. STE buffer: 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.5.
2. Lysozyme solution: 50 mM glucose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0. This solution should be autoclaved or filter sterilized and stored at 4°C. Prior to use 5 mg/mL crystalline lysozyme are added and completely dissolved.
3. SDS solution: Sodium dodecyl sulfate (1%, w/v) in NaOH (0.2M). Prepare fresh before use. Store at room temperature.
4. Sodium acetate solution: 3M sodium acetate, pH 4.8. This solution is prepared by dissolving 246.1 g of waterfree CH_3COONa in 500 mL H_2O , adjusting the pH to 4.8 with glacial acetic acid, and then adjusting the volume to 1 L. Store at room temperature.
5. Isopropanol: 100% (v/v).
6. Phenol/chloroform solution: Melt crystalline phenol at approx 50°C. Equilibrate the liquid with 0.2M Tris-HCl, pH 7.8. Only distilled phenol should be used. Do not use higher concentrated buffers for equili-

- bration, since that will affect phase separation. Mix chloroform and isoamyl alcohol in a ratio of 24:1. Mix this new solution with the equilibrated phenol in a ratio of 1:1 to obtain the final reagent. Store in a brown glass bottle (if possible, under a nitrogen atmosphere) at 4°C.
7. Ethanol: 96 and 70% (v/v).
 8. TE buffer: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.5.
 9. RNase solution: 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, RNase (2 mg/mL). Dissolve enzyme completely and keep the solution at 100°C for 15 min to inactivate possible DNase contaminants. Let cool to room temperature, divide in small aliquots, and store at -20°C.
 10. Chloroform solution: Mix chloroform and isoamyl alcohol in a ratio of 24:1.

2.3. Sequencing Reactions

2.3.1. Primer Annealing

1. Template: single- or double-stranded DNA, 0.25 µg/µL.
2. NaOH solution: 2M NaOH.
3. Sodium acetate solution: 3M sodium acetate, pH 4.5. For preparation see Section 2.2., step 4.
4. Ethanol: 96 and 70% (v/v).
5. Primer solution: 0.8 µM in TE buffer or H₂O.
6. Annealing buffer: 280 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 350 mM NaCl.
7. DTE solution: 0.3M dithioerythritol.

2.3.2. Labeling Reaction

1. T7 DNA polymerase, FPLC-purified, suitable for sequencing (Pharmacia LKB). Be careful to keep the enzyme constantly at -20°C. For taking an aliquot the polymerase should be removed only briefly from the freezer. The aliquot is then diluted with dilution buffer and used for sequencing. The dilution can be kept on ice until the sequencing reactions are set up.
2. Dilution buffer: 20 mM Tris-HCl, pH 7.5, 5 mM dithioerythritol, 50 µg/mL bovine serum albumin.
3. Radioactive nucleotide: [α -³²P]dATP or [α -³⁵S]dATP α S, 370 MBq (10 mCi)/mL and 370–463 MBq (10–12.5 mCi)/mL, respectively. Both nucleotides are incorporated equally well into the growing oligodeoxyribonucleotide chain by T7 DNA polymerase. Using ³⁵S, sharper bands are obtained in the autoradiogram and, as a result of the avoidance of scattering, longer sequences can be deciphered in one run. The shelf life of this isotope is longer, and the radiation dose of the personnel is reduced (9). Advantages of ³²P are that shorter exposure times are required and that the sequencing gel does not need to be dried before autoradiography.

Table 1
Composition of Termination Mixes

Component stock solution, mM	A-Mix		C-Mix		G-Mix		T-Mix	
	μL	μM	μL	μM	μL	μM	μL	μM
dATP (1)	15	150	15	150	15	150	15	150
dCTP (1)	15	150	15	150	15	150	15	150
dGTP (1)	15	150	15	150	15	150	15	150
dTTP (1)	15	150	15	150	15	150	15	150
ddATP (0.5)	3	15	—	—	—	—	—	—
ddCTP (0.5)	—	—	3	15	—	—	—	—
ddGTP (0.5)	—	—	—	—	3	15	—	—
ddTTP (0.5)	—	—	—	—	—	—	3	15
Sequencing buffer	20	—	20	—	20	—	20	—
MgCl ₂ (50)	—	10	—	10	—	10	—	10
Tris-HCl, pH 7.5 (200)	—	40	—	40	—	40	—	40
NaCl (250)	—	50	—	50	—	50	—	50
H ₂ O	17	—	17	—	17	—	17	—

4. Labeling mix: 1.375 μM dCTP, 1.375 μM dGTP, 1.375 μM dTTP, 333.5 mM NaCl. This mix can be stored in aliquots at -20°C .
5. DTE solution: 0.3M dithioerythritol.

2.3.3. Termination Reaction

1. Termination mixes: dATP, dGTP, dCTP, dTTP, ddATP, ddCTP, ddGTP, ddTTP, concentrations *see* Table 1 (*see* Note 1).
2. Sequencing buffer: Composition *see* Table 1.
3. Stop solution: 95% (v/v) formamide, analytical grade, 20 mM EDTA, pH 7.5, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue. This solution should be stored at -20°C (if possible, under a nitrogen atmosphere).

T7 DNA polymerase is also available in a kit, together with most of the required solutions (Pharmacia LKB). The composition of these solutions was not disclosed by the manufacturer. Sequencing using the reagents described above yields results that are practically identical to those obtained by the premade sequencing kit (Fig. 1). However, use of this kit saves the time for preparation of the solutions and, furthermore, provides so-called "read long sequencing mixes" that allow sequencing of up to 1000 nucleotides per run if a high resolution sequencing equipment is available.

Fig. 1. T7 DNA sequencing using different solutions and reagent mixtures. (A) Results from sequencing using solutions and chemicals described in this chapter. (B) Results using a commercially available sequencing kit from Pharmacia LKB. A part of the DNA sequence of *Clostridium acetobutylicum*, a strictly anaerobic, sporeforming bacterium, is shown. A 6% sequencing gel has been used. G, A, T, C, products from sequencing reactions.

2.4. Gel Electrophoresis

The respective materials and methods are detailed in Chapters 16 and 17.

2.5. Autoradiography

1. Acetic acid: 10% (v/v).
2. X-ray film: Kodak (Rochester, NY) or Amersham.
3. Light-protected box for washing and autoradiography of the gel.

3. Method

3.1. Primers

Primer design and synthesis are detailed in Chapter 11.

3.2. Template Preparation

Isolation procedures of single-stranded phage DNA and double-stranded plasmid DNA suitable for sequencing are described in other chapters of this volume. In case of plasmid DNA we found a modification of the method of Birnboim and Doly (10) especially well suited. This procedure is detailed below.

1. Centrifuge 5 mL of bacterial culture grown overnight at 6,000g for 5 min at 4°C.
2. Discard supernatant and suspend the sediment in 150 μ L lysozyme solution.
3. Transfer the preparation to a sterilized microcentrifuge cup and incubate for at least 5 min at room temperature.
4. Add 300 μ L SDS solution and mix gently by hand for a few seconds.
5. Immediately add 225 μ L sodium acetate solution.
6. Mix gently by hand for a few seconds and incubate for 15 min at 0°C.
7. Centrifuge for 5 min at 4°C in a microcentrifuge at maximal speed. Use either a model with cooling system or place centrifuge in the coldroom.
8. Transfer supernatant to a new microcentrifuge cup using a microliter pipet with sterilized tip. Be very careful to take only the supernatant.
9. Add 600 μ L isopropanol to precipitate the DNA and vortex a few seconds.
10. Incubate for 5 min at room temperature.
11. Centrifuge for 5 min at room temperature in a microcentrifuge at maximal speed.
12. Remove supernatant completely and rinse sediment with 1 mL cold ethanol (70%).
13. Let the sediment air dry (3–5 min) and then suspend it in 100 μ L TE buffer.
14. Add 1 μ L RNase solution and incubate for 15 min at room temperature.
15. Add 260 μ L H₂O and 40 μ L sodium acetate solution.
16. Add 1 vol of phenol/chloroform solution. **Attention:** Wear gloves to avoid any harm to your fingers if spilling this solution.
17. Vortex a few seconds and centrifuge for 5 min at room temperature in a microcentrifuge at maximal speed.
18. Transfer upper phase to a new microcentrifuge cup and add 1 vol chloroform solution.
19. Mix gently by inverting the closed cup several times.
20. Centrifuge a few seconds for phase separation and transfer upper phase to a new microcentrifuge cup.
21. Add 2 vols of cold ethanol (96%) to precipitate the DNA and vortex a few seconds.
22. Incubate for 15 min at –70°C.

23. Centrifuge for 10 min at 4°C in a microcentrifuge at maximal speed.
24. Rinse sediment with 1 mL cold ethanol (70%) and mix briefly.
25. Centrifuge for 10 min at 4°C in a microcentrifuge at maximal speed and discard supernatant.
26. Dry the sediment under vacuum (3–5 min) and then suspend it in 20 µL TE buffer.

This procedure usually yields 10–20 µg plasmid DNA from a 5-mL culture.

3.3. Sequencing Reactions

The procedure described below represents a modification of the method of Tabor and Richardson (6).

3.3.1. Primer Annealing

Whereas single-stranded phage DNA can be used directly as a template, double-stranded plasmid DNA must be denatured first.

1. Pipet 8 µL double-stranded plasmid DNA (containing 2 µg nucleic acid) into a sterilized microcentrifuge cup.
2. Add 2 µL NaOH and vortex the cup briefly.
3. Centrifuge the cup for a few seconds in a microcentrifuge at maximal speed to concentrate the complete solution at the bottom of the cup.
4. Incubate the cup for 10 min at room temperature.
5. Add 7 µL sterilized H₂O and 3 µL of sodium acetate solution.
6. Add 60 µL of cold (–20°C) ethanol (96%), mix well and incubate the cup for 20 min at –70°C.
7. Centrifuge for 10 min at room temperature in a microcentrifuge at maximal speed and discard supernatant.
8. Add 300 µL of cold ethanol (70%) and mix briefly.
9. Centrifuge for 10 min at room temperature in a microcentrifuge at maximal speed.
10. Remove the supernatant carefully and dry the sediment under vacuum (3–5 min).
11. Suspend the pellet in 10 µL sterilized H₂O.
12. Add 2 µL annealing buffer, 2 µL of primer solution, and 1 µL of DTE solution.
13. Incubate for 20 min at 37°C.
14. Keep the cup at room temperature for at least 10 min.

If the sequencing reaction is not carried out subsequently, the solution should be stored at –20°C until needed. For single-stranded DNA the following procedure can be used:

1. Pipet 10 μL template (containing 2 μg DNA) into a sterilized microcentrifuge cup.
2. Add 2 μL of primer solution, 2 μL of annealing buffer, and 1 μL of DTE solution.
3. Vortex the cup briefly.
4. Centrifuge the cup for a few seconds in a microcentrifuge at maximal speed to concentrate the complete solution at the bottom of the cup.
5. Incubate the cup for 10 min at 60°C.
6. Keep the cup at room temperature for at least 10 min.

If the sequencing reaction is not carried out subsequently, the solution should be stored at -20°C until needed.

3.3.2. Labeling Reaction

Since T7 DNA polymerase exhibits a high processivity and a high rate of polymerization the proper sequencing reaction is divided into two parts. In the first stage (labeling reaction) low concentrations of the four deoxyribonucleotides and a relatively low temperature result in the formation of short oligodeoxyribonucleotide chains (approx 20–30 bases). Since a radioactive nucleotide is present in the reaction mixture, all fragments become uniformly labeled, which is important for equal band intensities in the autoradiography step. In the second stage (termination reaction) the four standard reactions mixtures are set up that contain high concentrations of the four deoxyribonucleotides and a single dideoxyribonucleotide each. Higher temperature and nonlimiting dNTP concentration then allow a high rate of polymerization that is only terminated by incorporation of a ddNTP.

1. Dilute T7 DNA polymerase with dilution buffer to a concentration of 1.5 U/ μL .
2. Add 0.8 – 1 μL radioactive dATP (370 μBq –10 μCi) to the microcentrifuge cup containing annealed primer/template.
3. Add 3 μL labeling mix.
4. Add 1 μL DTE solution.
5. Add 2 μL diluted T7 DNA polymerase.
6. Mix by pipeting several times up and down in the cup with a microliter pipet and a sterilized tip.
7. Incubate for exactly 5 min at room temperature and then proceed directly with the termination reaction.

3.3.3. Termination Reaction

1. Prepare the 4 termination mixes. This should be done in advance (e.g., during the incubation period of the annealing reaction).

2. Set up appropriate vials for the termination reaction (microcentrifuge cups or a microtiter plate) and mark them carefully with "A," "C," "G," and "T."
3. Pipet 2.5 μL of the respective termination mix into the corresponding cup or well.
4. Warm mixtures to 37°C in a water bath (takes approx 1 min).
5. Pipet 4.5 μL of the labeling mixture into each cup or well. Use a fresh tip for each transfer.
6. Mix briefly by pipeting several times up and down with the microliter pipet.
7. Incubate the reaction mixture for exactly 5 min at 37°C. Since 4 reactions have to be processed, use defined time intervals (e.g., 30–60 s) for pipeting to ensure that every mixture was incubated the same amount of time.
8. Add 5 μL of stop solution to each cup or well.
9. Mix briefly by pipeting several times up and down with the microliter pipet.
10. Denature template and oligodeoxynucleotides by heating the cups or microtiter plate for 3 min at 80°C.
11. Put vials on ice.
12. Load 1.8–2 μL of each mixture onto a sequencing gel. The remaining material of the termination reactions can be stored at –20°C and used for further sequencing runs if necessary. Be sure to denature the mixtures again before loading onto a gel.
13. In order to read as many bases as possible, 2 samples from each reaction mixture should be run on the same gel, with a period of electrophoresis (approx 2–3 h, until the first colored marker band reaches the end of the gel) between the two loadings.

3.4. Gel Electrophoresis

The respective methods are detailed in Chapters 16 and 17.

3.5. Autoradiography

1. After finishing the electrophoresis as detailed in the above-mentioned chapters remove the glass plates from the sequencing equipment.
2. Remove one of the plates so that the gel still sticks to one glass plate.
3. Put gel on plate into a horizontal box and cover it with 2 L acetic acid (10%) (*see* Notes 2 and 3).
4. Incubate for 30–40 min at room temperature with occasional agitation to remove the urea from the gel.
5. Dry gel on plate at 80°C for 90 min. Liquid should be wiped off the plate before putting it into the oven. Alternatively, the gel can be transferred to Whatman filter paper and dried on an ordinary gel dryer. If ^{32}P -labeled dATP is used, drying is optional. Instead, the gel can be

covered with a plastic wrap. Suitable X-ray film is then put on top of it and exposed at -20 or -70°C for approx 4 h.

6. Cover the gel directly with a suitable X-ray film and expose it overnight (^{35}S -labeling) in a light-protected box at room temperature.
7. Develop the film according to the manufacturer's instructions and determine the sequence (see Note 4).

4. Notes

1. To avoid band compressions by G-G or G-C band pairing nucleotide analogs such as 7-deaza-dGTP, 7-deaza-dITP, or dITP can be used instead of dGTP. Complete premade mixes with c^7 dGTP and c^7 dITP are available from Pharmacia LKB.
2. To remove urea from sequencing gels with a final acrylamide concentration of more than 12%, the acetic acid wash solution should contain 2% (w/v) glycerol. Otherwise, the gel might crack upon drying.
3. The acetic acid wash solution might be reused for not more than four times. This will help to reduce the amount of radioactive waste.
4. Starting with prepared template DNA and primers one round of sequencing including the autoradiography can be performed in 24 h (calculating overnight as exposure time).
5. Using this procedure we found that even PCR-products could be directly sequenced after only one phenol treatment, one chloroform extraction, and one ethanol precipitation as described in Section 3.2.
6. If multiple sequencing reactions with the same double-stranded plasmid are planned, it is recommended to use a large amount of plasmid in the denaturation reaction. This will save time for future sequencing reactions. The denatured DNA after being dissolved in $10\ \mu\text{L}$ sterilized H_2O can be stored at -20°C .

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