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T4 DNA Polymerase

Technical Bulletin 18005-2

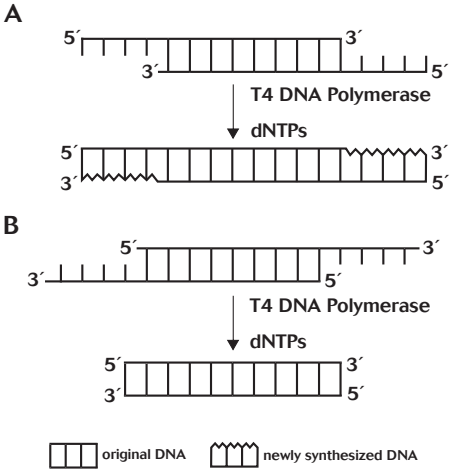
T4 DNA Polymerase is a DNA-dependent 5´➔ 3´ DNA polymerase possessing a 3´➔ 5´ exodeoxyribonuclease activity but lacking a 5´➔ 3´ exodeoxyribonuclease activity. Its two activities make T4 DNA Polymerase a useful enzyme for generating blunt ends on any duplex DNA molecule and for labeling DNA by a method known as replacement synthesis. This bulletin describes conditions for using T4 DNA Polymerase for both purposes.

The creation of blunt ends on a duplex DNA molecule is often necessary prior to adding adapters, linkers, or cloning into a blunt-ended site in a vector. T4 DNA Polymerase can be used to generate blunt ends from 3´ recessed ends, from 3´ protruding ends, or from a population containing both. The creation of blunt ends can be accomplished using either the 5´➔ 3´ polymerase or the 3´➔ 5´ exonuclease activity, depending on the structure of the DNA termini. In the presence of all four deoxyribonucleoside triphosphates (dNTPs), the polymerase reaction proceeds much more rapidly than the exonuclease reaction. Thus, a molecule with a 3´ recessed end will be rendered blunt-ended when the polymerase activity of the enzyme extends the recessed strand in the 3´ direction using the 5´ overhang of the other strand as a template (figure 1A). A molecule with a 3´ protruding end will be rendered blunt-ended when the exonuclease activity of the enzyme digests the overhang from the 3´ end until it reaches a double-stranded portion of the DNA (figure 1B). Once a blunt end is created, it is maintained as an equilibrium state because as the exonuclease activity removes nucleotides from the 3´ ends they are immediately replaced by the much more rapid polymerase activity (1).

T4 DNA Polymerase is used to generate radiolabeled DNA by replacement synthesis. Unlike nick translation, which introduces nicks into the target DNA and generates probes that are smaller than the original fragment, replacement synthesis yields intact, labeled DNA that is the same size as the original DNA fragment. This makes it a suitable procedure for labeling DNA molecular size standards for gel electrophoresis.

Replacement synthesis is a two-step reaction (figure 2). First, the linear DNA is treated with T4 DNA Polymerase in the absence of dNTPs. Without dNTPs, the exonuclease activity hydrolyzes each DNA strand in a 3´➔ 5´ direction without competition from the 5´➔ 3´ polymerase. If this process were allowed to continue indefinitely, each strand would be degraded to the point where no double-stranded portion of the DNA remained. The two strands would separate and be rapidly, completely degraded because the exonuclease hydrolyzes single-stranded DNA much more rapidly than it hydrolyzes double-stranded DNA (2). However, at a time determined by considering the rate of the exonuclease reaction and the size of the DNA fragment, all four deoxyribonucleotides, at least one of which is labeled, are added to the reaction mixture. Under these conditions, the polymerase activity is faster than the exonuclease activity. This results in the resynthesis of the 3´ portion of each strand, with the 5´ region of the other strand serving as a template. The double-stranded DNA structure is restored and the 3´ portion of each strand is labeled.

Figure 1 - Generation of blunt-ended DNA from (A) 3´ recessed ends (B) 3´ protruding ends



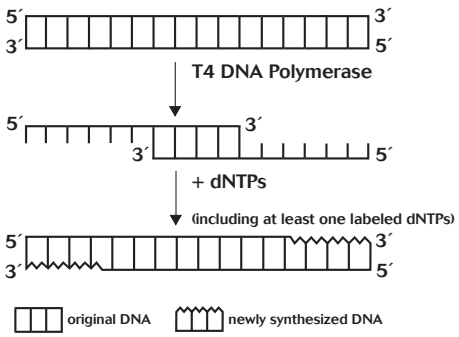
Introduction (cont.)

T4 DNA Polymerase is the product of gene 43 of the *Escherichia coli* bacteriophage T4 (3). It has a molecular weight of 114 kDa (4). T4 DNA Polymerase is purified from *E. coli* containing the plasmid clone pTL43W. It is supplied in 0.1 M potassium phosphate (pH 6.5), 10 mM 2-mercaptoethanol, 50% (v/v) glycerol. The enzyme has no detectable contaminating activity in single-stranded endodeoxyribonuclease, double-stranded endodeoxyribonuclease, or phosphatase assays. Exodeoxyribonuclease and polymerase activities are verified by replacement synthesis, followed by restriction endonuclease cleavage and urea polyacrylamide gel analysis.

Unit Definition

One unit of T4 DNA Polymerase incorporates 10 nmol of total deoxyribonucleotide into acid-precipitable material in 30 min at 37°C. In the replacement synthesis method of O'Farrell (5), the unit definition is equivalent to ~2.5 units as defined above (6).

Figure 2 - Schematic of replacement synthesis



Materials

In addition to the enzyme and DNA, the following reagents and equipment are required for the protocols described below:

For both protocols:

- Autoclaved, distilled water
- Microcentrifuge (15,000 X *g*)

For replacement synthesis or generating blunt ends with a radioactive tracer present:

- [α-^{32}P]dNTP. See protocols and *Additional Information* for information on selection of the appropriate label.
- TCA solution [10% (w/v) trichloroacetic acid, 1% (w/v) sodium pyrophosphate]. Store at 4°C.
- 95% (v/v) ethanol
- Glass fiber filters (Whatman GF/C or equivalent)
- Scintillation fluid

For generating blunt ends:

- 5X T4 DNA Polymerase blunt-ending buffer [165 mM Tris-acetate (pH 7.9), 50 mM magnesium acetate, 330 mM sodium acetate, 0.5 mM each dATP, dCTP, dGTP and dTTP]. Store at -20°C.
- Polymerase dilution buffer [200 mM KH_2PO_4 (pH 6.5), 10 mM 2-mercaptoethanol, 50% glycerol, 500 µg/ml nuclease-free BSA]
- 0.1 M dithiothreitol (DTT). Store at -20°C.
- Autoclaved, 1.5-ml microcentrifuge tubes
- 11°C water bath
- Buffer-saturated phenol
- Chloroform:isoamyl alcohol [24:1 (v/v)]
- 7.5 M ammonium acetate
- Absolute ethanol
- 70% (v/v) ethanol

For replacement synthesis:

- 5X T4 DNA Polymerase replacement synthesis buffer [165 mM Tris-acetate (pH 7.9), 50 mM magnesium acetate, 330 mM sodium acetate, 500 µg/ml nuclease-free BSA, 2.5 mM DTT]. Store at -20°C. This buffer is included with T4 DNA Polymerase.
- Autoclaved, 0.5-ml microcentrifuge tubes
- 37°C water bath

Three of the following unlabeled dNTP solutions:

- 2 mM dATP
- 2 mM dCTP
- 2 mM dGTP
- 2 mM dTTP

- Stop buffer (0.5 M EDTA, pH 8.0)

Protocol for generating blunt-end DNA

The following reaction conditions can be used for generating blunt ends on 0.5 to 2.5 µg of double-stranded, linear DNA. The efficiency of this reaction is generally > 70%. If significantly less than 70% blunt ends are obtained, see Troubleshooting. To monitor the efficiency of the reaction with a radioactive tracer, read the Analysis of the Reaction section before setting up the reaction.

1. To a sterile, 1.5-ml microcentrifuge tube on ice, add

Component	Amount
5X T4 DNA Polymerase blunt-ending buffer	20 µl
0.1 M DTT	1.0 µl
DNA	0.5 - 2.5 µg
autoclaved, distilled water	to 95 µl (total volume)

NOTE: The final concentration of each dNTP is 0.1 mM. The final concentration of DTT is 1 mM.

2. Dilute an aliquot of T4 DNA Polymerase in polymerase dilution buffer to a concentration of 2 units/µl in an autoclaved, 1.5-ml microcentrifuge tube.
3. Add 5.0 µl (10 units) of the diluted T4 DNA Polymerase to the reaction. Mix gently.
4. Incubate at 11°C for 15 min.
5. Place the reaction on ice.
6. Add 100 µl of buffer-saturated phenol, vortex, and centrifuge 5 min at 15,000 X *g* at room temperature to separate the phases. Transfer the upper, aqueous phase to a new tube.
   Note: If a radioactive tracer is present, the phenol solution will contain radiolabeled material and should be discarded properly.
7. Add 100 µl of chloroform:isoamyl alcohol [24:1(v/v)], vortex, and centrifuge 5 min at 15,000 X *g* at room temperature to separate the phases. Transfer the upper phase to a new tube, being careful not to remove any chloroform:isoamyl alcohol.
8. Precipitate the DNA by adding 0.5 volume of 7.5 M ammonium acetate followed by 2.5 volumes of absolute ethanol. Centrifuge at 15,000 X *g* at 25°C for 30 min (7). Remove the supernate.
   Note: If a radioactive tracer is present, the supernate from the ethanol precipitation will contain radiolabeled material and should be discarded properly.
9. Wash the pellet in 70% ethanol and centrifuge briefly. Remove the supernate and dry the DNA pellet.

Analysis of the blunt-ending reaction

A radioactive tracer may be included to monitor the reaction, but accurate quantitation is possible only for DNA with a 3´ recessed end of known structure. It is necessary to choose a labeled nucleotide that is complementary to at least one base in the 5´ overhang region. If more than one labeling nucleotide molecule can be incorporated, it is not possible to differentiate the partial labeling of a large number of ends from the complete labeling of a smaller number of ends since similar amounts of label would be incorporated in both cases. The ideal choice is a nucleotide that is complementary only to the final base in the 5´ overhang. Monitoring the incorporation of the final nucleotide ensures that the incorporation of a labeled nucleotide molecule represents a complete reaction.

The following additions and modifications to the *Protocol for Generating Blunt-ended DNA* can be used to determine the efficiency of the reaction.

1. Construct a TCA "washing machine" as follows: Punch 10 to 15 holes, 2 to 3 mm in diameter, in the bottom and sides of a 150-ml plastic beaker. Place a stir bar into a 250-ml beaker and place the plastic beaker in it. Filters can be washed by adding sufficient ice-cold TCA solution to cover the bottom of the inner beaker by 1 to 2 cm and placing the apparatus on a magnetic stirrer. Adjust the stirring speed so that the solution circulates through the holes. Drop the glass fiber filters into the solution and wash as described below.
2. Modify the *Protocol for Generating Blunt-ended DNA* as follows:
   a. Include 2.5 µl of [α-^{32}P]dNTP (400 Ci/mmol, 10 mCi/ml) in the reaction mixture prepared in step 1.
   b. Before adding the enzyme in step 2, remove 5.0 µl of the reaction mixture and spot it onto a glass fiber filter (filter 1). This filter will be used to measure the amount of nonspecific binding of the isotope to glass fiber filters.
   c. Remove 5.0 µl of the reaction before phenol extraction (step 6 in the protocol) and spot it onto another glass fiber filter (filter 2). Filter 2 will be used to measure the incorporation of label into acid-insoluble material. Then dilute 2.0 µl of the reaction with 18 µl of distilled water and spot 2.0 µl of the dilution onto a third glass fiber filter (filter 3). This filter will be used to measure the specific activity of the labeled nucleotide in the reaction mixture.
3. Wash filters 1 and 2 in the TCA "washing machine". Wash three times in ice-cold TCA solution for at least 5 min per wash cycle.
   Note: The TCA solution washes will contain radiolabeled material and should be discarded properly.
4. Wash filters 1 and 2 once in 95% ethanol at room temperature for 2 min.
   Note: The ethanol wash may contain radiolabeled material and should be discarded properly.
5. Dry the filters at room temperature or under a heat lamp.
6. Put each of the three filters in scintillation fluid and count them in a liquid scintillation counter.

Calculate the specific activity of the label in the reaction mixture by dividing the counts per min (cpm) obtained from filter 3 by the total amount of nucleotide. Since the amount of nucleotide contributed by the radiolabeled material is negligible compared to the amount of unlabeled nucleotide, the amount can be calculated by multiplying the amount of unlabeled material by the fraction actually counted:

$$\text{dNTP(pmol)} = \frac{2500 \text{ pmol dNTP}}{100 \text{ µl}} \times \frac{2 \text{ µl}}{20 \text{ µl}} \times 2 \text{ µl} = 5 \text{ pmol dNTP} \quad \text{[equation 1]}$$

The specific activity (SA) can be calculated:

$$\text{SA} = \frac{\text{cpm filter 3}}{5 \text{ pmol dNTP}} \quad \text{[equation 2]}$$

The amount of dNTP incorporated into the termini of the DNA can be calculated from the number of acid-precipitable counts on filter 2 after correction for nonspecific binding of the isotope to the filter, as determined by counting filter 1.

$$\text{dNTP incorporated (pmol)} = \frac{(\text{cpm filter 2} - \text{cpm filter 1}) \times 100 \text{ µl}}{\text{SA} \times 5 \text{ µl}} \quad \text{[equation 3]}$$

Calculate the number of termini made blunt and the total number of termini:

$$\text{termini made blunt (pmol)} = \frac{\text{pmol dNTP incorporated}}{\text{labeled nucleotides incorporated per end}} \quad \text{[equation 4]}$$

Analysis of the blunt-ending reaction (cont.)

The number of labeled nucleotides incorporated per end depends on the structure of the ends.

$$\text{termini (pmol)} = \frac{2 \times (\text{g of DNA})}{(\text{number of bp}) \times (660\ \text{Da/bp})} \times \frac{10^{12}\ \text{pmol}}{\text{mol}} \quad \text{[equation 5]}$$

The efficiency of blunt-ending can now be calculated

$$\text{efficiency (\%)} = 100 \times \frac{\text{pmol termini made blunt}}{\text{pmol termini}} \quad \text{[equation 6]}$$

Example: The generation of blunt ends on 2.5 µg of a 5300-bp, double-stranded DNA fragment is monitored by the incorporation of [α-^{32}P]dATP. The ends of the DNA are 5´ overhangs containing a single dTMP. Filter 1 (nonspecific binding) gave 400 cpm and filter 2 (TCA precipitable counts) gave 2,000 cpm when they were counted. The unwashed filter (filter 3) gave 155,000 cpm.

The specific activity is calculated using equations 1 and 2:

$$\text{SA} = \frac{155{,}000\ \text{cpm}}{5\ \text{pmol dATP}} = \frac{31{,}000\ \text{cpm}}{\text{pmol dATP}}$$

The amount of dATP incorporated is calculated using equation 3:

$$\text{dATP incorporated} = \frac{(2{,}000\ \text{cpm} - 400\ \text{cpm}) \times 100\ \mu\text{l}}{(31{,}000\ \text{cpm/pmol dATP}) \times 5\ \mu\text{l}} = 1.0\ \text{pmol}$$

Using equation 4:

$$\text{termini made blunt (pmol)} = \frac{1.0\ \text{pmol dATP}}{1\ \text{pmol termini/pmol dATP}} = 1.0\ \text{pmol termini made blunt}$$

From equation 5:

$$\text{termini (pmol)} = \frac{2 \times (2.5 \times 10^{-6}\ \text{g DNA})}{5300\ \text{bp} \times (660\ \text{Da/bp})} \times \frac{10^{12}\ \text{pmol}}{\text{mol}} = 1.4\ \text{pmol termini}$$

The efficiency of blunt-ending can be calculated:

$$\text{efficiency (\%)} = 100 \times \frac{1.0\ \text{pmol termini made blunt}}{1.4\ \text{pmol termini}} = 71\%$$

Protocol for replacement synthesis

Replacement synthesis is used to label molecular size standards for gel electrophoresis because it yields full-length labeled fragments (8). Since it is usually unnecessary to label size standards to a very high specific activity, conditions under which 50 nucleotides are removed and replaced from each 3´ end are generally sufficient. It is important not to allow the exonuclease step to proceed long enough to destroy the smaller fragments. For molecular size standards with small size fragments, like the 100 bp DNA Ladder, decrease the exonuclease activity by performing the reaction at 25°C. Fragments that are present in equal molar amounts will be equally labeled even if they are very different in size. Therefore, small bands will give as intense a signal as large bands on an autoradiograph.

The following procedure labels 10 µg of the 1 Kb DNA Ladder with [α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml). Under these conditions, the exonuclease will remove approximately 25 nucleotides/min. This protocol can often be used without modification to label single DNA fragments if the same amount of DNA (10 µg) and incubation time (2 min) are used to obtain DNA labeled to a specific activity of 10^6 cpm/µg. The efficiency of labeling depends on the structure of the DNA. For information on modifying the procedure to label different amounts of DNA or to label DNA to high specific activity, see *Additional Information*.

Protocol for replacement synthesis (cont.)

It is possible to do the exonuclease reaction and freeze the reaction mixture at -70°C in aliquots (9). These can be thawed and the resynthesis reaction performed whenever needed.

Exonuclease reaction

1. To an autoclaved, 1.5-ml microcentrifuge tube on ice, add the following:

Component	Amount
5X T4 DNA polymerase replacement synthesis buffer	4.4 µl
1 Kb DNA Ladder	10 µl
T4 DNA Polymerase (40 units)	8 µl
autoclaved, distilled water	to 22.4 µl

2. Mix gently. Centrifuge briefly at 4°C to collect the reaction to the bottom of the tube.
3. Incubate for 2 min at 37°C.
4. Place the reaction tube in ice.

Resynthesis reaction (fill-in)

5. Add the following to the reaction tube on ice after the exonuclease reaction:

Component	Amount
5X T4 DNA Polymerase replacement synthesis buffer	6.0 µl
autoclaved, distilled water	8.0 µl
2 mM dCTP	5.0 µl
2 mM dGTP	5.0 µl
2 mM dTTP	5.0 µl
[α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml)	1 µl

NOTE: The final concentration of each dNTP is 0.2 mM. The final concentration of DTT is 0.5 mM. The final concentration of 2-mercaptoethanol is 1.5 mM.

6. Incubate for 2 min at 37°C.
7. Mix gently. Centrifuge briefly. Add 5 µl of 2 mM dATP.
8. Add 2.5 µl of 0.5 M EDTA.

Determination of the specific activity of the replacement synthesis product

1. Dilute a 1.0 µl aliquot of the reaction in 24 µl of distilled water (a 1:25 dilution).
2. Spot a 5.0 µl aliquot of the diluted sample onto a glass fiber filter.
3. Wash the filter in ice-cold TCA solution as described in *Analysis of the Blunt-ending Reaction*.
4. Dry the filter. Put it in scintillation fluid and count in a liquid scintillation counter.
5. Calculate the specific activity as in the example below: Assume that 240,000 cpm are obtained after washing the filter.

$$\text{specific activity} = \frac{\text{cpm obtained X dilution factor X fraction of reaction counted}}{\text{quantity of DNA in the reaction}}$$

$$\text{specific activity} = \frac{(2.4 \times 10^5 \text{ cpm}) \times 25 \times (60\mu l / 5\ \mu l)}{10\ \mu g\ DNA} = 7.2 \times 10^6 \text{ cpm/µg DNA}$$

Troubleshooting

Generation of blunt ends

Failure to generate blunt ends from 3´ protruding ends or from a population of ends with various structures is inferred from the failure of a subsequent step that depends on the presence of blunt termini, such as cloning into a blunt-ended vector. Some possible causes of failure to generate blunt-ended DNA are listed below with suggested solutions.

Possible Causes	Suggested Solutions
Exonuclease activity decreased due to improper storage of enzyme	Avoid multiple freeze-thaw cycles. Avoid storage in a “frost-free” freezer.
The polymerase is inactive because of incorrect buffer conditions	Be sure that the Mg^{2+} concentration is ≥6 mM and that there is no EDTA in the reaction. Be sure that the salt concentration is < 70 mM (NaCl, KCl, NH_4Cl). Be sure that 1 mM DTT is present.
Required dNTPs are not present	Be sure that at least 100 µM each of those nucleotides required for complete fill-in are present.
Incorrect label used for monitoring reaction	Be sure that the labeled nucleotide is complementary to at least one nucleotide in the 5´ overhang.
Incorrect reaction temperature	Perform reaction at 11°C to stabilize ends.
Reaction time was too long	Maximum reaction time should be 15 min.

Troubleshooting (cont.)

Replacement synthesis

Poor labeling by replacement synthesis can be due to failure of either the exonuclease reaction or the resynthesis reaction. It may be possible to differentiate between the two cases by examining the reaction products by electrophoresis on a denaturing gel. If no change in migration is seen when comparing the reaction products to untreated DNA, the exonuclease reaction has failed. If the migration changed, but there has been poor incorporation of label, the problem is probably associated with the resynthesis reaction. Some possible causes of poor replacement synthesis are listed below with suggested solutions.

Possible Causes	Suggested Solutions
Exonuclease activity decreased due to improper storage of enzyme	Avoid multiple freeze-thaw cycles. Avoid storage in a “frost-free” freezer.
The exonuclease is blocked by GC rich regions or stretches of G’s or C’s in the template	Linearize the DNA at another site to avoid labeling the GC rich regions.
The polymerase activity is inhibited by incorrect buffer conditions	Be sure that the Mg^{2+} concentration is ≥6 mM and that there is no EDTA in the reaction. Be sure that the salt concentration is < 70 mM (NaCl, KCl, NH_4Cl).
Resynthesis cannot occur because the required dNTPs are not present	Be sure that all four dNTPs are present during replacement synthesis.
The concentration of the labeled nucleotide is too low	If using a high specific activity label, be sure to include the recommended molar concentration. Do a “chase” reaction with unlabeled nucleotide of the same species as the labeled one.
The polymerase cannot extend through template secondary structures	Add glycerol or dimethylsulfoxide to 10% (v/v) during resynthesis reaction only. Increase the resynthesis reaction time and raise the reaction temperature to 42°C. Add 1 to 2 units Large Fragment of DNA Polymerase I (Klenow fragment) during the resynthesis reaction (9). Add 2 µg T4 gene 32 protein per µg DNA during the resynthesis reaction (9).
The labeled dNTP is old, leading to inhibition of the polymerase by decay products	Use fresh labeled dNTP. ^{32}P-labeled dNTPs should be < 2 weeks old.
DNA is degraded because exonuclease reaction time was too long	Decrease the time of the exonuclease reaction, use more DNA in the reaction, or decrease temperature from 37°C to 25°C.
DNA is degraded because the template contains nicks that allow exonuclease degradation in the interior of the molecule	Use DNA that is free of single-stranded nicks.

Additional information

Reaction conditions

The polymerase activity of T4 DNA Polymerase is enhanced by the presence of a reducing agent. The enzyme exhibits 100% polymerase activity at 10 mM 2-mercaptoethanol, 36% activity at 1 mM 2-mercaptoethanol, and only 19% activity in the absence of 2-mercaptoethanol (3). Invitrogen has found that dithiothreitol (DTT) can be substituted for 2-mercaptoethanol at lower concentrations.

Polymerase activity requires Mg^{2+}. Mn^{2+} can substitute for Mg^{2+}; however, at the optimal Mn^{2+} concentration of 0.1 mM, the activity is 25% of that observed with 6 mM Mg^{2+} (3).

The K_m of T4 DNA Polymerase for dNTPs is approximately 20 µM (5). Therefore, it is recommended that the reaction mixture contain at least 20 µM of each dNTP.

High salt concentrations inhibit the polymerase. One hundred percent activity is observed at 50 mM NaCl, but only 3% activity at 200 mM NaCl. The same concentration requirements are seen with KCl and NH_4Cl (3).

The exonuclease activity is inhibited by glycerol concentrations of 10% (v/v) or greater.

Although we recommend performing T4 DNA Polymerase reactions in the Tris-acetate buffers described above, the enzyme has been found to be active in other buffers, including Tris-HCl (3,10), NH_4HCO_3 (11) and sodium glycinate (3).

Storage and stability

Store T4 DNA Polymerase at -20°C. Dilution decreases stability. Dilute the enzyme only immediately prior to use. Do not store diluted enzyme.

Inactivation

T4 DNA Polymerase can be inactivated by heating at 65°C for 15 min or by adding EDTA to 50 mM to chelate the Mg^{2+} ions in the reaction mixture.

Choice of labeled nucleotide for replacement synthesis

When the amount of labeled dNTP is limiting, the efficiency of incorporation of a species of labeled dNTP by T4 DNA Polymerase depends on the ease with which a newly added dNMP is hydrolyzed by the exonuclease activity before it can be protected by the addition of another dNMP (5). dAMP is hydrolyzed 1.5 to 3 times faster than dTMP and dGMP, and 6 to 9 times faster than dCMP (11). Thus, [α-^{32}P]dCTP yields probes of higher specific activity than [α-^{32}P]dATP. When labeling with [α-^{32}P]dCTP, up to 80% of the nucleotide may be incorporated, as compared to 50% incorporation with [α-^{32}P]dATP.

Using labeled dNTP's with specific activities of 400 Ci/mmol, probes with specific activities > 1 X 10^8 cpm/µg can be prepared. The use of nucleotides with specific activities of 3000 Ci/mmol is not recommended for generating high specific activity DNA because it requires the use of 7.5 times as much isotope in order to achieve the same molar concentration of dNTP in the reaction mixture. The use of nucleotides with specific activities of 3000 Ci/mmol can be used if additional unlabeled nucleotide is added as a chase, as in the protocol given in this bulletin to generate DNA with specific activities of 10^6 cpm/µg.

Use of dNTP analogs

The replacement synthesis method can be used to label DNA with [α-^{35}S]dATP (9). However, α-thiophosphate-containing phosphodiester bonds are resistant to the 3´➔5´ exonuclease activity of T4 DNA Polymerase. This blocking effect has been used to generate unidirectional deletions (12). T4 DNA Polymerase has been used to replace terminal dGMPs with dIMPs (13). T4 DNA Polymerase has also been used to incorporate 5-methyl-dCTP to create a hemimethylated DNA (14).

Ribonucleoside triphosphates are not substrates for T4 DNA Polymerase (3,11).

Additional information (cont.)

Modifying the replacement synthesis protocol to generate high specific activity DNA

The replacement synthesis protocol described in this bulletin can be modified to generate high specific activity DNA (5,9). There are two important parameters to adjust: the exonuclease reaction time and the quantity of dNTPs provided. The radiolabeled nucleotide used should be of a specific activity of 400 Ci/mmol and the final concentration should be 4.4 µM.

The time of the exonuclease reaction depends on the length of the DNA fragment being labeled and the rate of the reaction. It is desirable to choose conditions under which ~37% of the nucleotides are removed from each end of the molecule. This permits labeling of ~75% of the DNA fragment but minimizes the danger of removing so many nucleotides that the two strands of the DNA duplex separate. The rate at which nucleotides are removed from the 3´ ends depends on the number of ends. Increasing the number of ends decreases the rate of the reaction. Any calculation of the rate of exonuclease activity should be regarded as an estimate. The actual rate will vary with the structure of the DNA being labeled.

For example, if labeling a 4,300 bp fragment using the conditions given for replacement synthesis ~25 nucleotides are removed per minute. Removing 37% of each strand would mean removing 1,600 nucleotides. At a rate of ~25 nucleotides/min, the reaction time for the exonuclease reaction would be 64 min.

It is important to include enough labeled nucleotide during the resynthesis reaction to permit complete resynthesis of the DNA molecule (5). In order to calculate the number of moles of nucleotide removed during the exonuclease reaction, first estimate the fraction of DNA degraded using the following formula:

$$\text{fraction degraded} = \frac{\text{rate X time X 2 ends}}{\text{number of bp in fragment X 2 nucleotides per bp}}$$

The number of moles of nucleotide removed is calculated by multiplying the fraction of the DNA degraded by the number of moles of nucleotide present in the DNA originally. 1 µg of DNA contains 3 X 10^{-9} moles of nucleotides.

$$\text{moles of nucleotide removed} = \text{fraction degraded X µg DNA X } \frac{(3 \text{ X } 10^{-9} \text{ moles})}{\text{µg}}$$

If the DNA contains all four nucleotides in equal proportion, the amount of one nucleotide required for complete resynthesis will be one fourth the amount removed. However, the polymerase reaction cannot incorporate every molecule of labeled nucleotide present. Usually, the fraction of the label which is incorporated will be between 0.5 (for dATP) and 0.8 (for dCTP). Hence, the minimum amount of nucleotide that is required during the resynthesis step is:

$$\text{moles of nucleotide required} = \text{moles removed X } \frac{1}{\text{fraction incorporated}} \text{ X } \frac{1}{4}$$

Variations on blunt-ending

T4 DNA Polymerase can be used to prepare 3´ end-labeled DNA or to manipulate the structure of DNA termini. See the article by Challberg and Englund (1) for a detailed discussion.

Variations on replacement synthesis

It is possible to use T4 DNA Polymerase to simultaneously label a plasmid insert and purify it of smaller vector DNA fragments (9). This is accomplished by allowing the exonuclease to completely digest the smaller fragments before initiating resynthesis by adding dNTPs.

Single-stranded probes can be prepared by first labeling the 3´ ends of a double-stranded DNA molecule by replacement synthesis, then digesting the labeled molecule with λ exonuclease to remove the unlabeled 5´ portion of each strand (9).

Site-specific oligonucleotide-directed mutagenesis

Kunkel *et al.* employed T4 DNA Polymerase in a site-specific oligonucleotide-directed mutagenesis procedure to synthesize a second strand of DNA using a single-stranded template with an oligonucleotide as a primer (15). T4 DNA polymerase is a good choice for this purpose because it does not perform strand displacement synthesis as readily as does the large fragment of DNA polymerase I. The incorporation of 5-methyl-dCTP by T4 DNA Polymerase has been exploited for oligonuclotide-directed mutagenesis (14). An oligonucleotide is used as a primer for the synthesis of a second strand of DNA by T4 DNA Polymerase in the presence of 5-methyl-dCTP, dATP, dTTP, and dGTP resulting in a hemimethylated DNA. The parental strand is nicked by digestion with *Msp* I or *Hha* I and removed by exonuclease III digestion. Mutants are recovered by transformation of a *mcrAB* strain of *E. coli* with the nascent strand (14).

Ordering information

Description	Concentraion	Quantity	Cat. No.
T4 DNA Polymerase	5 units/µl	50 units	18005-017
	5 units/µl	250 units	18005-025
1 Kb DNA Ladder		250 µg	15615-016
		1 mg	15615-024
Bovine Serum Albumin		10 mg	15561-012
		150 mg	15561-020
dATP (10mM)		2.5 µmol	18252-015
dCTP (10mM)		2.5 µmol	18253-013
dGTP (10mM)		2.5 µmol	18254-011
dTTP (10mM)		2.5 µmol	18255-018
10 mM dNTP Mix		100 µl	18427-013
Dithiothreitol		5 g	15508-013
0.5 mM EDTA, pH 8.0		4 X 100 ml	15575-020
λ Exonuclease	1-10 units/µl	150 units	28023-018
DNA Polymerase I, Large (Klenow) Fragment	3-9 units/µl	100 units	18012-021
	3-9 units/µl	500 units	18012-039
	3-9 units/µl	2 X 500 units	18012-096
Phenol		500 g	15509-037
Buffer-Saturated Phenol		100 ml	15513-039
		400 ml	15513-047
Tris		1 kg	15504-020

For research use only. Not for diagnostic or therapeutic use in humans or animals.

References:

1. Challberg, M.D., *et al*. (1980) *Methods Enzymol.* **65:** 39.
2. Huang, W.M., *et al*. (1972) *J. Biol. Chem*. **247:** 3139.
3. Goulian, M., *et al*. (1968) *J. Biol. Chem*. **243:** 627.
4. Kornberg, A. (1974) *DNA Synthesis*, p. 138, W. H. Freeman and Company, San Francisco.
5. O'Farrell, P. (1981) *FOCUS*® **3:** 1.
6. *FOCUS*® (1981) **3:** 6.
7. Crouse, J., *et al*. (1987) *FOCUS*® **9:** 3.
8. *FOCUS*® (1984) **6:** 9.
9. Deen, K.C., *et al*. (1983) *Anal. Biochem.* **135:** 456.
10. Ausubel, F.M., *et al*. (1989) *Current Protocols in Molecular Biology*, p. 3.5.10, John Wiley and Sons, New York.
11. Hershfield, M.S., *et al*. (1972) *J. Biol. Chem*. **247:** 3393.
12. Barcak, G.J., *et al*. (1986) *Gene* **49:** 119.
13. Modrich, P., *et al*. (1977) *J. Biol. Chem*. **252:** 7273.
14. Vandeyar, M.A., *et al*. (1988) *Gene* **65:** 129.
15. Kunkel, T.A., *et al*. (1987) *Methods Enzymol.* **154:** 367.

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