Terminal Deoxynucleotidyl Transferase, Recombinant

Technical Bulletin 8008-1

Terminal Deoxynucleotidyl Transferase (TdT) is a DNA polymerase that catalyzes the addition of dNTPs to the 3´ hydroxyl terminus of DNA (1). Protruding, recessed, or blunt-ended double- or single-stranded DNA molecules with chain lengths of three or more nucleotides serve as substrates for TdT (2). When a nucleotide is added to the DNA, pyrophosphate is released.

TdT is used to add stretches of several nucleotides to the 3´ ends of DNA. These nucleotides subsequently serve as "tails" for cloning (3-6). For cDNA cloning procedures, vector and insert DNAs are joined by annealing complementary homopolymer tails (Figure 1). Tail length can vary from one to more than 1000 nucleotides and is controlled by varying the reaction conditions.

TdT is also used to end-label DNA with ^{32}P or non-radioactive reporter molecules, including biotin (See Figure 2). End-labeled DNA is used for hybridization studies to identify specific DNA sequences (7), to detect point mutations (3,8), and to locate expressed genes in *in situ* studies (9,10).

Terminal Deoxynucleotidyl Transferase is purified from a baculovirus clone of calf thymus TdT. It has a molecular weight of 58 kDa. The enzyme is supplied with 1 ml of 5X TdT Reaction Buffer [500 mM potassium cacodylate (pH 7.2), 10 mM $CoCl_2$, 1 mM DTT]. It is stored in 0.1 M potassium phosphate (pH 7.2), 200 mM KCl, 1 mM 2-mercaptoethanol, and 50% (v/v) glycerol. One unit of TdT incorporates 1 nmol dATP into acid-precipitable material in 1h at 37°C using $d(pA)_{50}$ as a primer. No detectable contaminating activity is observed in endodeoxyribonuclease, 3´- and 5´-exodeoxyribonuclease, or phosphatase assays. Levels of incorporation into both blunt-end and protruding-end DNA fragments are determined in tailing assays.

This bulletin describes conditions and considerations for adding homopolymer tails to DNA. In addition, conditions and considerations for adding biotinylated nucleotides to the 3´ ends of oligonucleotides are provided.

Tailing using Terminal Deoxynucleotidyl Transferase

When joining vector and insert DNAs for cDNA cloning, optimal annealing requires homopolymer tails of equal lengths of ~20 dGs and dCs or ~100 dAs and dTs (12). Variation between DNA substrates and enzyme preparations can affect the tailing reaction; however, the most important consideration is the nucleotide concentration. For typical tailing reactions, the concentration range of dGTP, dCTP, and dTTP is 5 to 50 µM dNTP. However, for dATP the nucleotide concentration range is 50 to 500 µM. The following method is used for determining the appropriate conditions for incorporating a specified number of dGs onto *Pst I*-cleaved pBR322 with TdT (13). The same procedure can be used to determine optimal conditions for other nucleotides and linearized DNAs.

Figure 1 - Adding nucleotide tails to vector and insert DNAs using TdT

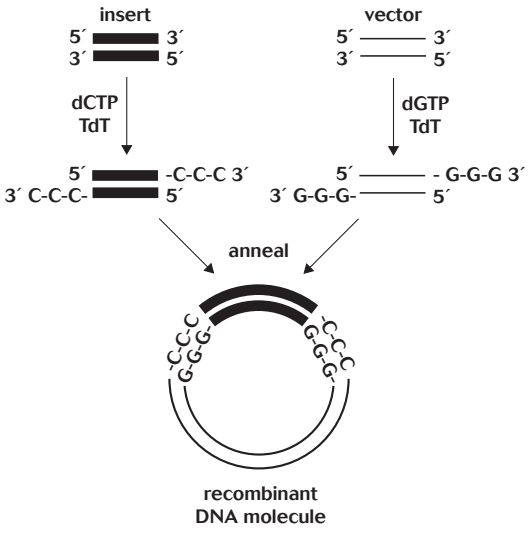
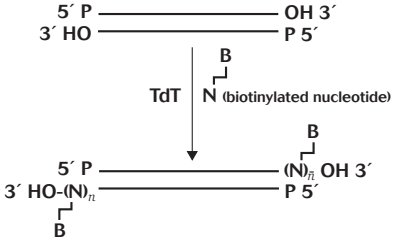


Figure 2- End-labeling Using a Biotinylated Nucleotide and TdT.



Materials

In addition to the enzyme and the buffer, the following materials are required for dG tailing using TdT:

- dGTP
- 2 µl of [^{3}H]dGTP (5-20 Ci/mmol) (1 mCi/ml)
- 1.5-ml microcentrifuge tubes
- Absolute ethanol (-20°C)
- 10 mM Tris-HCl (pH 7.5), 0.1 mM Na_2EDTA (TE)
- 0.1 M Na_2EDTA (pH 8.0)
- Microcentrifuge (15,000 X *g*)
- 2 pmol of 3´ termini of DNA (3 µg of *Pst I* cut pBR322)
- Autoclaved, distilled water
- 37°C water bath
- Buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:21(v/v/v)]
- 7.5 M ammonium acetate

For calculation of tail length the following materials are needed:

- 10% (w/v) trichloroacetic acid, 1% (w/v) sodium pyrophosphate (TCA-PPi) (4°C)
- 5% (w/v) trichloroacetic acid (TCA)
- Glass fiber filters (1 x 2 cm), Whatman GF/C or equivalent
- Scintillation fluid
- Scintillation counter

Protocol for determining the number of nucleotides added

1. Number 12, 1.5-ml microcentrifuge tubes.
2. In these microcentrifuge tubes, prepare a series of 50 µl assays containing 5 to 50 µM dGTP by combining the components as shown in Table 1. ADD THE TdT LAST.
3. Mix by gentle pipetting.
4. Centrifuge tubes briefly at 4°C to collect the reaction mixture in the bottom of the tube.
5. Incubate at 37°C for 30 min.
6. Places tubes on ice.
7. Spot 10 µl of each assay on numbered GF/C filters. A pencil or India ink pen may be used to number the filters.
8. Precipitate the DNA with cold (4°C) 10% TCA-PPi (10 ml/filter). All filters may be washed together in a beaker.
9. Wash the filters four times with 5% (w/v) TCA (10 ml/filter).
10. Wash the filters two times with absolute ethanol (5 ml/filter).
11. In addition, spot 5 µl of assays 1 and 2 on separate filters, 1T and 2T. These contain no DNA and will be used to calculate the total amount of [^{3}H]dGTP per assay.
12. Place all filters under a heat lamp to dry.
13. Place filters in vials with scintillation fluid and determine the radioactive counts (cpm) per sample.

Table 1 - TdT tailing assay

Component	1 (µl)	2 (µl)	3 (µl)	4 (µl)	5 (µl)	6 (µl)	7 (µl)	8 (µl)	9 (µl)	10 (µl)	11 (µl)	12 (µl)
5X reaction buffer	10	10	10	10	10	10	10	10	10	10	10	10
Pst I-cleaved pBR322 DNA (1 µg/µl)	--	--	3	3	3	3	3	3	3	3	3	3
^{3}H-dGTP (1 mCi/ml)	2	2	2	2	2	2	2	2	2	2	2	2
dGTP (100 µM)	25	25	2.5	2.5	5	5	10	10	15	15	25	25
sterile distilled H_2O	13	13	31.5	31.5	29	29	26	26	19	19	9	9
TdT (15 U/µl)	--	--	1	1	1	1	1	1	1	1	1	1

Protocol for determining the number of nucleotides added (cont.)

Calculation of the number of residues added

The following calculations are used to determine the length of the dG tails added at each dGTP concentration (13):

A. Number of 3´ ends.

1. Formula for double-stranded DNA

$$\text{pmoles 3´-ends/assay} = \frac{\mu\text{g DNA}}{\text{MW}} \times 2 \times \frac{10^6 \text{ pmole}}{\mu\text{mole}}$$

MW = (number of base pairs) x (660 µg/µmole)
(assume 50% A + G content)

Formula for single-stranded oligonucleotides

$$\text{pmoles 3´-ends/assay} = \frac{\mu\text{g DNA}}{\text{MW}} \times \frac{10^6 \text{ pmole}}{\mu\text{mole}}$$

MW = (number of base pairs) x (330 µg/µmole)
(assume 50% A + G content)

2. Sample Calculation

All sample calculations are from actual data for tailing double-stranded *Pst I*-cut pBR322.

$$\text{pmoles of 3´-ends/assay} = \frac{3\mu\text{g}}{2.9 \times 10^6 \ \mu\text{g/}\mu\text{mole}} \times 2 \times \frac{10^6 \text{ pmole}}{\mu\text{mole}} = 2 \text{ pmoles of 3´-ends/assay}$$

B. Specific Activity (SA) of dGTP in assay.

1. Formula

$$\text{SA (cpm/pmole)} = \frac{\text{cpm} - \text{cpm background}}{\text{pmole dGTP}}$$

a. cpm = average cpm of filter 1T and 2T
b. cpm background = average cpm of filters 1 and 2
c. pmole dGTP = (µM dGTP) x (µl spotted)
d. $\mu\text{M dGTP} = \frac{(\mu\text{M conc. of dGTP stock}) \times (\text{vol. dGT added})}{(\text{total volume of assay})}$

2. Sample Calculations

The cpms obtained from each filter were as follows:

Filter No.	1	2	1T	2T	3	4
cpm	38	38	114,313	129,783	32,660	35,436
average cpm	38		122,048		34,048	

$$\mu\text{M dGTP} = \frac{100 \ \mu\text{M} \times 2.5 \ \mu\text{l}}{50 \ \mu\text{l}} = 5 \ \mu\text{M}$$

$$\text{pmole dGTP} = 5 \ \mu\text{M} \times 5 \ \mu\text{l} = 25 \text{ pmoles}$$

$$\text{SA} = \frac{122{,}048 \text{ cpm} - 38 \text{ cpm}}{25 \text{ pmoles}} = 4{,}880 \text{ cpm/pmole}$$

C. Length of tails.

1. Formula

$$\text{Length of dG tail} = \frac{\text{cpm} - \text{cpm backgrnd.}}{\text{SA}} \times \frac{\text{total assay vol.}}{\text{volume spotted}} \times \frac{1 \text{ pmole of 3´-ends}}{\text{pmoles of 3´-ends/assay}}$$

2. Sample Calculation

$$\frac{34{,}048 - 38 \text{ cpm}}{4{,}880 \text{ cpm/pmole}} \times \frac{50 \ \mu\text{l}}{10 \ \mu\text{l}} \times \frac{1}{2} = 17 \text{ pmoles dGTP/pmoles 3´-ends}$$

Repeat calculations B and C for each dGTP concentration. Based on the number of dG tails resulting from each dGTP concentration, determine the appropriate molar concentration to yield the desired tail length. For example:

Number of dGs added/pmole 3´ end at the following dGTP concentrations				
5 µM	10 µM	20 µM	30 µM	50 µM
17	23	31	37	43

Graph pmoles dGTP added/pmole DNA termini vs. concentration of dGTP. Interpolate to find the concentration of dGTP needed to give the desired number of tails. In this example, to get ~20 dG tails, use 7.5 µM dGTP.

Protocol for tailing DNA using TdT

After determining the optimal concentration of dGTP needed to add the desired number of nucleotides to the experimental DNA, perform the tailing reaction as described below:

1. To a 1.5-ml microcentrifuge tube add the following:

Component	Amount	Final Concentration
5X TdT reaction buffer	10 µl	1X
DNA	2 pmol end	40 pmol/ml
[^{3}H]dGTP (1 mCi/ml	2 µl	tracer
dGTP (100 µM)	*	*
autoclaved, distilled water	up to 49 µl	(total volume)

* Optimal amount as determined (See "Protocol for determining the number of nucleotides added.")

2. Add 1 µl of TdT (15 units/µl)
3. Mix gently, then centrifuge briefly to collect the reaction mixture in the bottom of the tube.
4. Incubate at 37°C for 30 min.
5. Stop the reaction by placing the tube on ice and adding 10 µl of 0.1 M Na_2EDTA (pH 8.0).
6. Add 60 µl buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1(v/v/v)]. Vortex thoroughly. Centrifuge 5 min at 15,000 X *g* at room temperature to separate the phases. Transfer the upper aqueous phase to a new tube.
7. 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 room temperature for 30 min (14). Carefully remove the supernate.
8. Dissolve the pellet in 50 µl of 10 mM Tris-HCl (pH 7.5), 0.1 mM Na_2EDTA (TE).
9. Repeat steps 7 and 8.
10. Store the DNA at –20°C.

Considerations for TdT tailing

Use of [^{3}H] dNTP as a tracer

If the [^{3}H]dNTP to be added as a tracer is supplied in ethanol, remove the ethanol. Transfer the material into a microcentrifuge tube. After diluting with water, freeze it in a dry ice/ethanol bath and lyophilize, being careful not to contaminate the surrounding equipment. Dissolve the dried material in distilled water to a final concentration of 1 mCi/ml.

Characterizing tailed DNAs

To determine the number of nucleotides added to the ends of the DNA, monitor the incoporation of a radiolabeled tracer as described above. Determining the number of nucleotides added by electrophoresing the products on a 1% (w/v) agarose gel is inaccurate because interactions between the tails yield multiple high molecular weight bands (15). If the tailed fragments are small ($< \sim 200$ b), resolve them on a 6% (w/v) denaturing polyacrylamide gel and detect them by autoradiography (16).

Tailing a heterogenous population of cDNAs

The cDNA to be cloned often is composed of a population of mixed sizes; therefore, the picomoles of ends cannot be accurately determined. In this case, perform a control transformation experiment to maximize the number of clones obtained per nanogram of cDNA (15, 17). Tail a population of heterogenous fragments such as ϕX174 RF DNA/*Hae III* Fragments by taking aliquots at varying time intervals. Anneal the tailed DNA from each aliquot with a tailed vector and perform transformations to determine transformation efficiency for each time point. Use the optimal reaction time when tailing the experimental cDNA.

Optimizing the tailing reaction based on time

Instead of varying the nucleotide concentration to obtain the desired number of nucleotides added, vary the incubation time of the reaction (11). Set up a reaction with the highest recommended nucleotide concentration and take five aliquots every 15 min. Determine the tail lengths added at each time point by TCA-precipitation as described in the Protocol for determining the number of nucleotides added. Calculate the time required to yield the desired number of added nucleotides, and then set up the tailing reaction accordingly.

Preserving restriction endonuclease recognition sites

The restriction endonuclease recognition site is often eliminated when an insert DNA is tailed and annealed to a tailed vector. If necessary, precautions can be taken to preserve some of the recognition sites (18). For example, when a dG tail is added to the 3´ end of a *Pst I*-cleaved fragment, the restriction site is restored after annealing with a dC-tailed vector. Other enzyme recognition sites also may be restored, depending upon the site and the nucleotide added to the 3´ end. An alternative is to restore the restriction endonuclease recognition site by filling in the overhang, thus producing blunt-ended DNA that can be tailed.

Annealing tailed DNA

Typical conditions for annealing vector and double-stranded cDNA are as follows (19): Mix equimolar amounts of tailed cDNA and complementary tailed vector in 100 mM NaCl, 10 mM Tris-Cl (pH 7.8), and 0.1 mM Na_2EDTA at a final DNA concentration of 1 ng/µl. Heat to 65°C for 5 min and then incubate for 2 h at 57°C to allow the DNA strands to anneal. Ethanol precipitate the DNA and dissolve it in water. Perform a standard transformation procedure using the appropriate competent cells.

3´ end-labeling oligonucleotides with biotin using terminal deoxynucleotidyl transferase

TdT is used to end-label DNA with ^{32}P or nonradioactive reporter molecules (Figure 2). A common use of TdT is end-labeling synthetic oligonucleotides for use as hybridization probes (7-10). More than one nucleotide is generally added to the 3´ end of the DNA, making it possible to produce high specific activity (10 to 25 x 10^9 dpm/µg) oligonucleotides. The homopolymer tail has no effect on the specificity of dissociation temperature of the probe. Detection levels obtained with a 3´ end-labeled probe are ~10 times greater than with the same probe if it is 5´ end-labeled (20, 21).

An alternative to using radioactive probes is nonisotopic detection of biotinylated probes. Oligonucleotide probes tailed with biotinylated nucleotides provide nonradioactive detection that is more sensitive than oligonucleotides with biotin incorporated into internal nucleotides by chemical synthesis methods (22). The following protocol is for using TdT to add biotinylated nucleotides to oligonucleotides. Under these conditions, 1 to 7 nucleotides are added to the oligonucleotide (average length ~2.5 nucleotides), and ~90% of the oligonucleotide molecules are labeled (23).

Materials

In addition to the enzyme and the buffer, the following materials are required to 3´ end-label oligonucleotides with biotin-14-dATP:

- 0.25 to 2.0 nmol of oligonucleotide
- 3.0 M sodium acetate (pH 5.2)
- 0.1 M Na_2EDTA (pH 8.0)
- 10 mM Tris-HCl (pH 7.5), 0.1 mM Na_2EDTA (TE)
- Autoclaved, distilled water
- Microcentrifuge
- Biotin-14-dATP (0.4 mM)*
- Buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1(v/v/v)]
- Absolute ethanol (-20°C)
- 1.5-ml microcentrifuge tubes
- 37°C water bath

*Biotin-7-dATP (0.4 mM) may also be used.

Protocol for 3´ end-labeling DNA with biotin-14-dATP

1. Add the following components to a 1.5-ml microcentrifuge tube.

Component	Amount	Final Concentration
0.4 mM biotin-14-dATP	12.5 µl	100 µM
oligonucleotide	0.25 to 2.0 nmol	5 to 40 nmol/ml
5X TdT Reaction buffer	10 µl	1X
autoclaved, distilled water	up to 49 µl	(total volume)

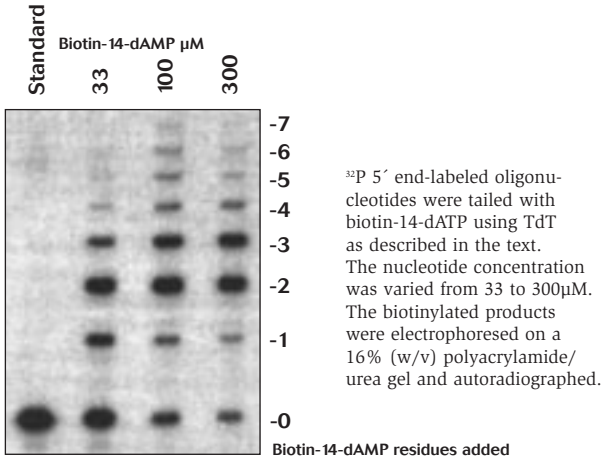
2. Add 1 µl TdT (15 units/µl). Mix by gentle pipetting.
3. Incubate at 37°C for 2 to 4 h.
4. Stop the reaction by placing the tube on ice and adding 10 µl of 0.1 M Na_2EDTA (pH 8.0).
5. Precipitate the DNA to remove unincorporated nucleotides by adding 0.1 volume of 3.0 M sodium acetate followed by 2 volumes of absolute ethanol.
6. Centrifuge at 15,000 X *g* for 30 min (14). Carefully remove the supernate.
   NOTE: Alternatively, the biotinylated DNA may be separated from unincorporated nucleotides by gel exclusion chromatography (24).
7. Dissolve the pellet in 10 mM Tris-HCl, pH 7.5, 0.1 mM Na_2EDTA (TE).
8. Store the DNA at –20°C.

Considerations for end-labeling using TdT

Monitoring the reaction

The actual number of biotin molecules incorporated onto each oligonucleotide can be determined by first 5´ end-labeling the molecule with ^{32}P using T4 Polynucleotide Kinase and then electrophoresing with biotinylated products on a polyacrylamide gel (Figure 3) (23). When using radioisotopes to end-label oligonucleotides, radiolabeled material can be bound to DE-81 filters (27). Incorporation of radiolabeled nucleotides onto larger DNA fragments can be monitored by the incorporation of the label onto TCA-precipitable counts (See "Protocol for determining the number of residues added").

Figure 3 - Tailing of oligonucleotides using TdT and biotin-14-dATP.



^{32}P 5´ end-labeled oligonucleotides were tailed with biotin-14-dATP using TdT as described in the text. The nucleotide concentration was varied from 33 to 300µM. The biotinylated products were electrophoresed on a 16% (w/v) polyacrylamide/urea gel and autoradiographed.

Nucleotide substrates for TdT

TdT will end-label DNA with [α-^{32}P]dNTPs, biotinylated nucleotides, and [α-^{35}S]dATP (3, 20), adding several nucleotides onto the 3´ end of each DNA molecule. TdT also incorporates nucleotide triphosphates with free primary amines such as 5-amino(12)-dUTP and 8-(6-aminohexyl)-amino-ATP (24, 26). These amine groups can then be tagged with activated biotin or fluorescent dyes. In addition, TdT will incorporate 1 to 4 rNTPs onto the 3´ end of a DNA molecule (27).

Additional enzyme information

Optimal reaction conditions

TdT requires the presence of a divalent metal cation such as Mg^{2+}, Mn^{2+}, or Co^{2+}. In the presence of Mg^{2+}, single-stranded DNA or DNA with a 3´ protruding end are substrates for TdT. In the presence of Co^{2+} or Mn^{2+}, blunt and recessed 3´ DNA termini, in addition to 3´ protruding ends or single-stranded DNA, are substrates for TdT (3, 29, 30). Reactions using dATP and dTTP are more efficient in the presence of Co^{2+}. Reactions with dCTP and dGTP work better in the presence of Mn^{2+}, but reaction conditions can be adjusted with either Co^{2+} or Mn^{2+} (3).

TdT is active at pH 7.2 in 100 mM potassium cacodylate buffer and requires the presence of a sulfhydryl reagent such as dithiothreitol (18, 31). Do not use ^{35}S that is supplied in more than 1 mM DTT because a high concentration of DTT causes the Co^{2+} in the reaction buffer to precipitate. Do not use phosphate buffers because cobalt phosphate and cobalt pyrophosphate are insoluble and may deplete the Co^{2+} causing the reaction to terminate (21).

WARNING: The potassium cacodylate contained in the reaction buffer is toxic. Handle it with care and dispose of it according to federal, state, and local regulations.

Inhibitors

TdT is inhibited by Na^+, NH_4^+, Tris buffer, and metal chelators such as EDTA (2, 18, 21, 31). To remove inhibitors from the DNA sample, precipitate the DNA with potassium acetate and ethanol and wash the pellet with 70% (v/v) ethanol (21). Dissolve the DNA in distilled water.

Inactivation

TdT can be inactivated by adding Na_2EDTA to a final concentration of 5 mM or by heating the reaction to 65°C for 5 min (16, 21).

Storage

Store TdT at –20°C.

Additional enzyme information (cont.)

Preparation of potassium cacodylate buffer

TdT is supplied with 1 ml of 5X of TdT Reaction Buffer. It is prepared as follows (21, 32): Titrate 1.0 M cacodylic acid (free acid form) to pH 7.2 with potassium hydroxide pellets to yield potassium cacodylic acid.

WARNING: Potassium cacodylate is toxic. Handle it with care and dispose of it according to federal, state, and local regulations.

The cacodylic acid used in the 5X TdT Reaction Buffer is purified by ion exchange chromatography to eliminate trace endonuclease impurities. To the 1.0 M potassium cacodylic acid, add water, DTT, and cobalt chloride to yield a final concentration of 500 mM potassium cacodylate (pH 7.2), 10 mM $CoCl_2$, and 1 mM DTT. Be sure to add the DTT before adding cobalt chloride; otherwise, a precipitate will be formed. Filter the solution. The solution will have a pink appearance.

Ordering information

Description	Concentration	Quantity	Cat. No.
Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT)	15 units/µl	500 units	10533-065
	15 units/µl	3 x 500 units	10533-073
Terminal Deoxynucleotidyl Transferase Buffer		2 x 0.8 ml	16314-015
Dithiothreitol (DTT)		5 g	15508-013
Phenol		500 g	15509-037
ϕX174 RF DNA/*Hae* III Fragments		40 µg	15611-015
T4 Polynucleotide Kinase	10 units/µl	200 units	18004-010
	10 units/µl	1,000 units	18004-028

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