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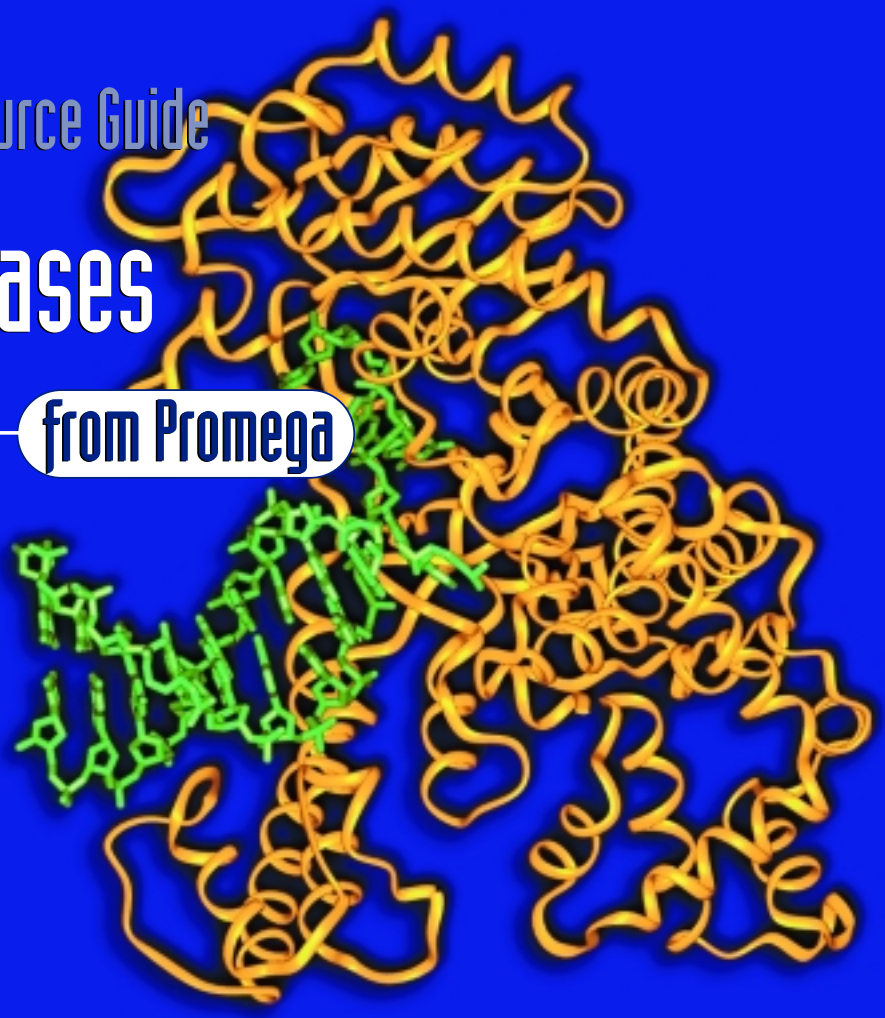
Enzyme Resource Guide

Polymerases

from Promega



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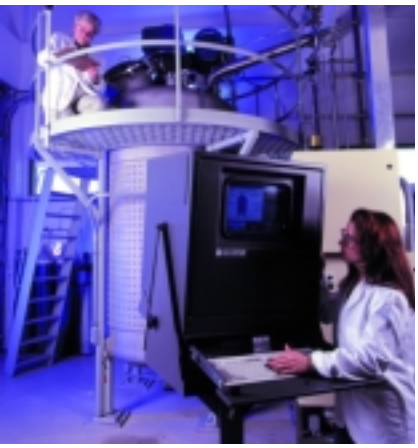

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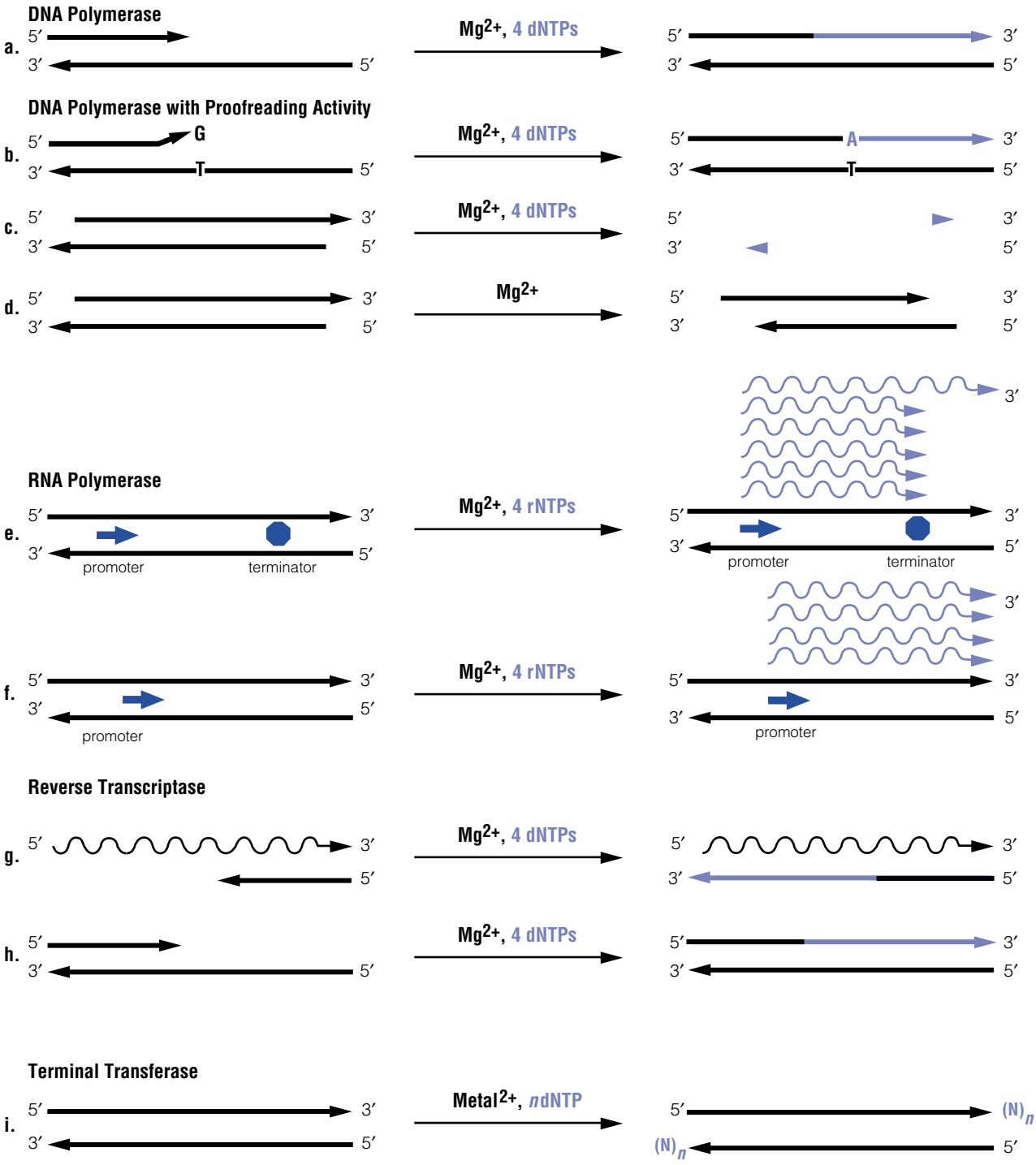

Figure 1. Diagram depicting enzymatic activities of polymerases commonly used in molecular biology applications. Straight lines indicate DNA molecules, wavy lines indicate RNA molecules and arrowheads indicate the 3′-end of a nucleic acid molecule. **Panel a:** DNA polymerases incorporate dNTPs 5′→3′ from the 3′-end of a DNA initiator annealed to ssDNA. **Panel b:** DNA polymerases possessing a 3′→5′ exonuclease (proofreading) activity can repair nucleotide misincorporations. **Panel c:** In the presence of dNTPs, polymerases possessing a 3′→5′ exonuclease activity will remove ssDNA 3′-overhangs from dsDNA. **Panel d:** In the absence of dNTPs, these enzymes will continue to remove 3′-nucleotides from dsDNA. **Panels e and f:** RNA polymerases incorporate NTPs 5′→3′ on a DNA template from a specific dsDNA promoter. **Panel e:** Polymerase-specific terminator sequences will terminate transcription, although with less than 100% efficiency. **Panel f:** Allowing the polymerase to "run-off" the end of the DNA template will produce a higher percentage of identically sized transcripts. **Panels g and h:** Reverse transcriptases incorporate dNTPs 5′→3′ from the 3′-end of a DNA initiator annealed to RNA (**Panel g**) or DNA (**Panel h**). **Panel i:** Terminal deoxynucleotidyl transferase adds one or more dNTPs to the 3′-end of ds- or ssDNA.

INTRODUCTION: The Polymerases

Enzymes that modify nucleic acids provide the foundation for many of the techniques in molecular biology. These enzymes are used to synthesize, degrade, join or remove portions of nucleic acids in a controlled and generally defined manner. Specific features of the *in vivo* functions of these enzymes have been exploited *in vitro* to provide many of the protocols currently used in nucleic acid manipulations. Figure 1 summarizes the activities of polymerases generally used for these purposes.

This chapter describes many of the commonly used polymerases and their basic characteristics, with an emphasis on their practical applications in today's laboratories. While a number of these enzymes may perform the same general function, one enzyme may be a better choice than the others for a specific purpose. This information is imparted in the individual enzyme descriptions and Table 1. The reaction conditions provided in each entry are those optimized at Promega, unless otherwise indicated. Many of the techniques using polymerases are detailed in Promega's *Protocols and Applications Guide,* Third Edition, in Promega system protocols (technical bulletins and technical manuals) and in references to these detailed protocols provided within each enzyme description in this guide.

Table 1. Recommended Applications of Polymerases.

	DNA Pol. I	Klenow	Klenow exo minus	T4 DNA Pol.	*Taq* DNA Pol.	Seq. Grade *Taq*	*Tfl* DNA Pol.	*Tth* DNA Pol.	*Tli* DNA Pol.	*Pfu* DNA Pol.	T7 RNA Pol.	T3 RNA Pol.	SP6 RNA Pol.	AMV RT	MMLV RT	MMLV RT H minus	TdT
Labeling																	
3′ addition			✓		✓	✓	✓	✓									✓
3′ fill-in	✓̸	✓	✓	✓	✓	✓	✓	✓	✓	✓					✓		
3′ replacement	✓̸	✓̸		✓					✓	✓							
Nick translation	✓																
Replacement synthesis	✓̸	✓̸		✓					✓	✓							
Random priming		✓	✓														
RNA probes											✓	✓	✓				
Cloning																	
Creating blunt ends		✓		✓					✓	✓							
5′-overhang fill-in	✓̸	✓		✓̸					✓	✓							
3′-overhang removal	✓̸	✓̸		✓					✓	✓							
Sequencing																	
DNA	✓̸	✓	✓			✓								✓	✓	✓	
RNA														✓	✓	✓	
Mutagenesis																	
Oligo-directed		✓̸		✓						✓							
Misincorporation		✓̸			✓		✓	✓									
Nested deletions				✓													
Mapping																	
Transcript														✓	✓	✓	
cDNA Synthesis																	
1st strand														✓	✓	✓	
2nd strand	✓̸	✓		✓	✓	✓̸	✓	✓	✓̸	✓				✓̸	✓		
***In vitro* transcription**											✓	✓	✓				
PCR[g]					✓		✓	✓	✓	✓							
RT-PCR[g] (one enzyme)							✓̸	✓									

✓ *Preferred enzyme for this application.*

✓̸ *Acceptable enzyme for this application (not preferred).*

✓ *Acceptable enzyme at elevated temperature (>65°C).*

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DNA Polymerases

DNA polymerases meet the cell's need for DNA-directed DNA synthesis during processes such as chromosomal replication and DNA repair. DNA polymerases have two substrates: one is the template-bound primer terminus containing a free 3′-hydroxyl (OH) group, the other is a deoxynucleotide 5′-triphosphate (dNTP) (Figure 1a; 1). A phosphodiester bridge is formed by nucleophilic attack of the 3′-OH of the primer terminus on the α-phosphate group of the dNTP and elimination of the terminal pyrophosphate (PP_i):

$$(dNMP)_n + dNTP \rightleftharpoons d(NMP)_{n+1} + PP_i$$

DNA polymerases require a primer with a free 3′-OH, otherwise new DNA chains cannot be initiated. The newly added monomer has a free 3′-OH group and can serve as a primer terminus for another round of polymerization. Chain elongation during polymerization occurs in the 5′→3′ direction; polymerization is either distributive or processive. A distributive polymerase dissociates from the template after the addition of each nucleotide, whereas a processive polymerase maintains its association with the template for a succession of nucleotide additions.

Some polymerases depend upon accessory factors for their high processivity (2,3). This may be a consideration when using these polymerases *in vitro*, as commercial preparations may not contain the required cofactors. Another consideration is the reverse reaction, which yields free dNTPs (4). In living cells, the reaction is strongly biased in the forward direction by an enzyme called pyrophosphatase, which hydrolyzes the pyrophosphate (PP_i) to inorganic phosphate.

The number of nucleotides added by a DNA polymerase before it dissociates from the primer-template is often expressed as a processivity index or value (5). Although this index can be measured, it is unclear how important processivity is in most molecular biology applications because theoretically saturating concentrations of enzyme are used in most techniques.

Other factors to consider are turnover rate and fidelity (6). The turnover rate is the maximum number of nucleotides polymerized per minute per molecule of DNA polymerase. It should not be confused with processivity; the turnover rate does not consider whether a polymerase is processive or distributive. Fidelity is the enzyme's ability to faithfully replicate a DNA molecule. Error rates are commonly expressed as the mutation rate per base pair duplicated, and accuracy as the inverse of error rate. In other words, accuracy is the average number of nucleotides the polymerase incorporates before making an error.

Some DNA polymerases have functions other than that of polymerization. The polymerase may have an associated 3′→5′ exonuclease activity, 5′→3′ exonuclease activity, the ability to use RNA rather than DNA as substrate (reverse transcription) and nontemplate-dependent addition of nucleotides. The 3′-exonuclease activity is associated with a proofreading role, where it removes an incorrectly inserted (noncomplementary) base and thereby allows the polymerase reaction to continue (Figure 1b). The proofreading and polymerization reactions are in competition (Figures 1a, c and d).

Generally, a DNA polymerase with 3′-exonuclease activity will demonstrate higher fidelity than one without, which may affect enzyme choice for some applications. The so-called 5′-exonuclease activity is involved with excision repair within a cell (7). This activity is actually that of a single-strand dependent endonuclease, so the conventional name is inaccurate (8). While this activity is necessary for applications such as nick translation (9,10), it is undesirable for DNA sequencing applications (11). Additionally, some DNA polymerases have a reverse transcriptase activity (replication of DNA from RNA templates), which is usually enhanced by altering the cation in the reaction (e.g., Mn^{2+} instead of Mg^{2+}).

DNA polymerases lacking a 3′-exonuclease may add a nontemplate-dependent nucleotide to the 3′-end, behaving, in other words, like a terminal transferase (12). However, unlike terminal transferase, only a single nucleotide is added to the 3′-end. There is a preference for the nucleotide added, often dATP, but this preference is sensitive to the sequence context near the 3′-end (12). Two strategies are used to clone fragments carrying the nucleotide added by nonproofreading polymerases. One is to "polish" or blunt the ends of the DNA fragment, for example, by using a proofreading DNA polymerase. Another approach is to use vector that compensates for the added nucleotide, for example, a T-vector (13).

Of the DNA polymerases used for molecular biology manipulations, the most common are those isolated from bacterial or bacteriophage sources. These can be divided into two groups, based on the enzyme's thermostability (i.e., the ability of the enzyme to withstand prolonged exposure to an elevated temperature). Thermostable DNA polymerases tend to have an optimum reaction temperature of approximately 74˚C, but can withstand repeated exposures to temperatures higher than the optimum. Mesophilic DNA polymerases have optimum reaction temperatures much lower than the thermostable DNA polymerases (usually 37–42˚C) and cannot withstand prolonged exposure to temperatures higher than this optimum.

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DNA Polymerase I
E.C. 2.7.7.7

Description

DNA Polymerase I (Cat.# M2055) is a DNA-dependent DNA Polymerase that has three activities: 5′→3′ polymerase, 5′→3′ exonuclease and 3′→5′ exonuclease (1,2).

DNA Polymerase I is most commonly used in combination with DNase I for generating labeled DNA probes by nick translation (3,4). The enzyme can also be used for the labeling of 3′-ends of DNA by 3′ fill-in or 3′ replacement. DNA Polymerase I can be used to convert 5′- and 3′-overhangs to blunt ends; however, the Klenow Fragment of DNA Polymerase I or T4 DNA Polymerase is preferred for these applications. DNA Polymerase I can be used for DNA sequencing, although it is not the enzyme of choice due to its exonuclease activity. The enzyme can also be used in oligo-directed mutagenesis; however, T4 DNA Polymerase is preferred for this application due to the relatively low fidelity and strand displacement activity of DNA Polymerase I. This lower fidelity makes DNA Polymerase I more suitable for mutagenesis by nucleotide misincorporation. DNA Polymerase I is also commonly used in combination with RNase H for second-strand synthesis of cDNA (5).

Applications
- Nick translation (3,4).
- 3′-end labeling.
- Conversion of 5′- and 3′- overhangs to blunt ends.
- Second-strand synthesis of cDNA (5).
- Mutagenesis by misincorporation.

DNA Polymerase I is a component of the following Promega systems:
- Nick Translation System (Cat.# U1001)
- Universal RiboClone® cDNA Synthesis System[(a)] (Cat.# C4360)

Enzyme Properties

Requirements: Divalent cations are required for both the polymerase and exonuclease activities of the enzyme (2). A concentration of 7mM Mg^{2+} is optimal (6).

Optimal Substrate: For polymerase activity, the optimal substrate is primed ssDNA. DNA Polymerase I will also extend from single-strand nicks or gaps in dsDNA. The enzyme is active on DNA:RNA and RNA:RNA hybrids, under certain conditions (7). The 3′→5′ exonuclease activity of DNA Polymerase I affects both ss- and dsDNA. The 5′→3′ exonuclease activity affects dsDNA and RNA:DNA hybrids.

Optimal pH: 7.4 with potassium phosphate buffers (6).

K_m: For dNTPs, 1–2µM (7). For DNA, 2µM (8).

Alternative Cofactors and Substrates: Mn^{2+} can fulfill the requirement for the divalent cation; however, the activity and fidelity of the enzyme is reduced (6,9). Hapten-labeled nucleotides can be incorporated by DNA Polymerase I (10), including biotinylated (11) and digoxigenin-labeled (12) nucleotides.

Inhibitors: Polymerization by DNA Polymerase I is inhibited by a variety of agents including antibiotics, DNA intercalating and alkylating agents and nucleotide analogs (2,13).

Extinction Coefficient/Absorbance: The absorbance of a 1mg/ml solution in 10mM sodium bicarbonate at 280nm is 0.85 (14).

Strand Displacement Activity: Yes.

Inactivation: 68˚C for 10 minutes (16).

Error Rate: 9 x 10^{-6} (15).

Genetic Locus: *E. coli pol*A.

Promega Product Information

Source: DNA Polymerase I is purified from the recombinant *E. coli* strain CM5199 (17), which is a lysogen carrying a lambda *pol*A transducing phage.

Molecular Weight: *E. coli* DNA Polymerase I is a monomeric protein first described as 109kDa in size (14). However, as deduced from the sequence of the *pol*A gene of *E. coli,* the enzyme has a calculated molecular weight of 103kDa (18).

Storage Conditions: Store at –20˚C. Avoid exposure to frequent temperature changes. DNA Polymerase I is supplied in storage buffer containing 50mM Tris-HCl (pH 7.5 at 25˚C), 1mM DTT, 0.1mM EDTA and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 10nmol of deoxyribonucleotides into TCA-insoluble form in 30 minutes at 37˚C. For unit definition the reaction conditions are: 67mM potassium phosphate (pH 7.5), 6.7mM $MgCl_2$, 1mM DTT, 50µg/ml activated calf thymus DNA and 33µM of each dNTP.

Purity: The purity is >90% as judged by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.

Contaminant Assay
Endonuclease Assay: To test for endonuclease activity, 1µg of Type I supercoiled plasmid DNA is incubated with 25 units of DNA Polymerase I for 5 hours at 37˚C. Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

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DNA Polymerase I Large (Klenow) Fragment
E.C. 2.7.7.7

Description

The Large (Klenow) Fragment of DNA Polymerase I (Cat.# M2206) is a DNA-dependent DNA polymerase that lacks the 5′→3′ exonuclease activity of intact *E. coli* DNA polymerase I, but retains the 5′→3′ polymerase, 3′→5′ exonuclease and strand displacement activities.

The Large (Klenow) Fragment of DNA Polymerase I is used in several applications including dideoxy sequencing (1), second-strand cDNA synthesis (2) and random primer labeling (3–5). The enzyme can also be used to fill 5′-overhangs with labeled or unlabeled dNTPs (6). The 3′→5′ exonuclease activity of the enzyme can be used to generate blunt ends from a 3′-overhang (7); however, the stronger 3′→5′ exonuclease activity of T4 DNA Polymerase makes it the enzyme of choice for this application (8). The Large (Klenow) Fragment of DNA Polymerase I is also used for *in vitro* mutagenesis experiments using synthetic oligonucleotides (9). One problem with using the Klenow Fragment for this application is that the strand displacement activity of the enzyme tends to displace the mutagenic primer, thereby removing the intended mutation. As such, T4 DNA Polymerase, which has no strand displacement activity, is a superior enzyme for this application.

Applications
- Dideoxy sequencing (1).
- Second-strand cDNA synthesis (2).
- Random primer labeling (3–5).

Enzyme Properties

Requirements: Mg^{2+}.

Optimal Substrate: For 5′→3′ polymerase activity, the optimal substrate is a DNA primer with a 3′-OH terminus annealed to ssDNA. The enzyme will also extend from a nick or gap in double-stranded DNA but will not translate the nick due to the enzyme's lack of 5′→3′ exonuclease activity. For 3′→5′ exonuclease activity, the optimal substrate is ss- or dsDNA with a 3′-OH terminus.

Optimal pH: 7.4 (phosphate), 8.4 (Tris) (11).

K_m: For dNTPs, 2µM (10).

Inhibitors: DNA Polymerase I Large (Klenow) Fragment is inhibited by the adenosine analog adenosine-2′, 2′-riboexpoxide 5′-triphosphate (12). Figures 2, 3 and 4 provide information on the effects of various restriction enzyme buffers, salt concentrations and temperatures, respectively, on DNA Polymerase I Large (Klenow) Fragment.

Inactivation: 75˚C for 10 minutes.

Error Rate: 4 x 10^{-5} (13).

Strand Displacement Activity: Yes.

Genetic Locus: *E. coli pol*A.

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone (14).

Molecular Weight: The Large (Klenow) Fragment of DNA Polymerase I is a 68kDa monomer. The enzyme is a C-terminal fragment of DNA Polymerase I (103kDa).

Typical Working Conditions: For optimal activity, use the Klenow 10X Buffer supplied with the enzyme (Table 12). DNA Polymerase I Large (Klenow) Fragment is also active in many restriction enzyme buffers; some users may choose to perform the fill-in reaction directly in the restriction buffer. For other applications, such as sequencing, the optimal reaction conditions are described in the Promega Product Information sheet provided with the enzyme.

Storage Conditions: Store at –20˚C. DNA Polymerase I Large (Klenow) Fragment is supplied in storage buffer containing 50mM Tris-HCl (pH 7.5 at 25˚C), 1mM DTT, 0.1mM EDTA and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 10nmol of total deoxy-ribo-nucleotides into TCA-insoluble form in 30 minutes at 37˚C. For unit definition the reaction conditions are: 67mM potassium phosphate (pH 7.5), 6.7mM $MgCl_2$, 1mM DTT, 50µg/ml activated calf thymus DNA and 33µM each of dATP, dCTP, dGTP and radiolabeled dTTP.

Purity: The DNA Polymerase I Large (Klenow) Fragment preparation is >90% pure by SDS-polyacrylamide gel electrophoresis. Klenow Fragment is free from intact DNA polymerase and small fragment, as indicated by SDS-polyacrylamide gel electrophoresis.

Contaminant Assay

Endonuclease Assay: To test for endonuclease activity, 1µg of Type I (supercoiled) plasmid DNA is incubated with 25 units of DNA Polymerase I Large (Klenow) Fragment in Klenow 1X Buffer (Table 12) for 16 hours at 37˚C. Following incubation, the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

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DNA Polymerase I Large (Klenow) Fragment, Exonuclease Minus

E.C. 2.7.7.7

Description

The Large (Klenow) Fragment, Exonuclease Minus, of DNA Polymerase I (Cat.# M2185) is a DNA-dependent DNA polymerase that lacks both the 5′→3′ and 3′→5′ exonuclease activities of intact *E. coli* DNA polymerase I (1,2). The enzyme is primarily used for dideoxy sequencing (3). It is also used for random primer labeling (4,5) and in strand displacement amplification (SDA) (6). Klenow Fragment, Exonuclease Minus, will leave a single-base 3′-overhang on a significant proportion of DNA fragments during fill-in of 5′-overhangs (7). Therefore, this enzyme is not recommended for preparation of blunt-end fragments for ligation.

Applications

- Dideoxy sequencing (3).
- Random primer labeling (4,5).
- Strand displacement amplification (6).

Enzyme Properties

Requirements: Mg^{2+}.

Optimal Substrate: The enzyme is active on a DNA primer having a 3′-OH terminus annealed to ssDNA.

Inhibitors: Figures 2, 3 and 4 provide information on the effects of various restriction enzyme buffers, salt concentrations and temperatures, respectively, on DNA Polymerase I Large (Klenow) Fragment, Exonuclease Minus, activity.

Inactivation: 75˚C for 10 minutes.

Promega Product Information

Source: DNA Polymerase I Large (Klenow) Fragment, Exonuclease Minus, is purified from an *E. coli* strain expressing a recombinant clone (7).

Molecular Weight: 68kDa.

Storage Conditions: Store at –20˚C. DNA Polymerase I Large (Klenow) Fragment, Exonuclease Minus, is supplied in storage buffer containing 50mM Tris-HCl (pH 7.5), 1mM DTT, 0.1mM EDTA and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 10nmol of deoxynucleotide into acid-precipitable material in 30 minutes at 37˚C. For unit definition the reaction conditions are: 67mM potassium phosphate (pH 7.5), 6.7mM $MgCl_2$, 1mM DTT, 50µg/ml activated calf thymus DNA and 33µM each of dCTP, dATP, dGTP and [^{3}H]dTTP.

Purity: The purity is >90% as judged by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.

Contaminant Assays

Endonuclease Assay: To test for endonuclease activity, 1µg of Type I (supercoiled) plasmid DNA is incubated with 25 units of Klenow Fragment, Exonuclease Minus, in Klenow 1X Buffer for 16 hours at 37˚C (Table 12). Following incubation, the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

Exonuclease Assay: To test for DNase activity, 50ng of radiolabeled ssDNA and dsDNA are incubated with 30 units of Klenow Fragment, Exonuclease Minus, in Klenow 1X Buffer (Table 12) for 16 hours at 37˚C. The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for ssDNA and <2% release for dsDNA.

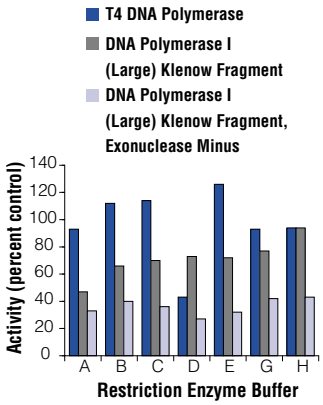

Figure 2. Activity of DNA polymerases in Promega Restriction Enzyme Core Buffers (see also Table 11). Control activity was determined for each polymerase using its standard reaction conditions, buffer composition and temperature, specified for Unit Definition.

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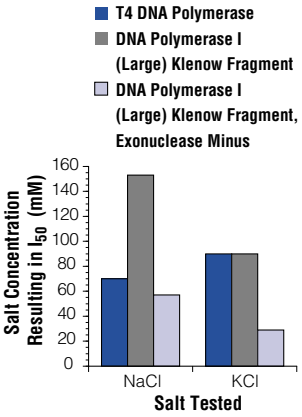

Figure 3. The concentrations of NaCl and KCl that result in 50% inhibition (I_{50}) of DNA polymerase activity. Activity of each polymerase at these salt concentrations was compared to its activity at reaction conditions specified for Unit Definition.

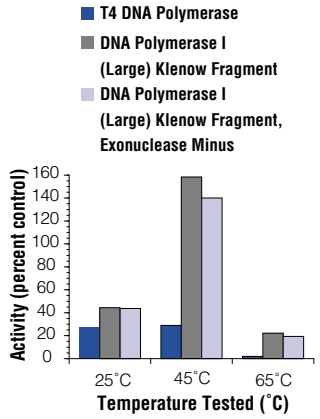

Figure 4. The influence of various temperatures on DNA polymerase activity. Control activity was determined for each polymerase, using its standard reaction conditions, buffer and temperature, specified for Unit Definition.

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T4 DNA Polymerase

E.C. 2.7.7.7

Description

T4 DNA Polymerase (Cat.# M4211 and M4215) is a DNA-dependent DNA polymerase that catalyzes the repetitive addition of nucleotide triphosphates to the 3′-OH of a DNA initiator (i.e., primer) bound to ssDNA. T4 DNA Polymerase also possesses a potent 3′→5′ exonuclease activity on both ssDNA and dsDNA templates (1,2). T4 DNA Polymerase is the highest fidelity polymerase available.

T4 DNA Polymerase can be used for labeling of 3′ ends of DNA by 5′ fill-in or the 3′ replacement or exchange reaction (1,3). The enzyme is also commonly used for fill-in of 5′-overhangs and removal of 3′-overhangs prior to ligation (1,3). T4 DNA Polymerase is preferred over DNA Polymerase I Large (Klenow) Fragment for the conversion of 3′-overhangs to blunt ends because of the superior 3′→5′ exonuclease activity of T4 DNA Polymerase. T4 DNA Polymerase is also the enzyme of choice for site-directed mutagenesis, because of the enzyme's high fidelity and lack of strand displacement activity (1,3).

Applications

- Polishing or fill-in of 5′-protruding ends with labeled or unlabeled dNTPs (1,3).
- Blunting 3′-overhangs (1,3).
- Labeling 3′-termini of DNA fragments (replacement reaction) (1,3).
- *In vitro* mutagenesis (1,3).

T4 DNA Polymerase is a component of the following Promega systems:

- Subcloning Applications Pack (Cat.# M7670)
- Universal RiboClone® cDNA Synthesis System[(a)] (Cat.# C4360)
- Altered Sites® II *in vitro* Mutagenesis System[(b)] (Cat.# Q5590, Q6600, Q6511, Q6080, Q6090, Q6501, Q6210, Q6491)
- GeneEditor™ *in vitro* Site-Directed Mutagenesis System[(c)] (Cat.# Q9280)

Enzyme Properties

Requirements: Mg^{2+}.

Cofactor Concentration: Mg^{2+}, 6mM optimal (6).

Optimal Substrate: Polymerase activity: primed ssDNA. T4 DNA Polymerase will fill a gap in dsDNA but will not extend from a nick. Exonuclease activity: ssDNA and dsDNA.

Typical Working Concentration: For most applications, use approximately five units T4 DNA Polymerase per microgram DNA and 100µM dNTPs (5).

Optimal pH: 8.0–9.0 (50% activity at pH 7.5 and pH 9.7) (6).

K_m: For dNTPs, 2µM (4).

Stimulators: Two-fold stimulation at 1mM spermidine. Approximately 25% stimulation at 50mM sodium acetate*. Approximately 20% stimulation at 100mM ammonium acetate. T4 DNA Polymerase activity is increased in the presence of the T4 DNA Polymerase accessory proteins T4 Gene 45 protein and the T4 Gene 44/62 protein complex (2,7,8).

Alternative Cofactors and Substrates: Mn^{2+}, 0.1mM optimal (Mn^{2+} at 0.1mM results in activity 4X lower than Mg^{2+} at 6mM) (6); biotin-11-dUTP (15).

Inhibitors: T4 DNA Polymerase is reported to be inhibited by -SH blocking reagents (7). T4 DNA Polymerase is sensitive to the concentration of NaCl (20mM, 90% activity; 200mM, 5% activity), KCl (20mM, 75% activity; 200mM, 5% activity), Tris (67mM, 100% activity; 200mM, 50% activity), sodium acetate (50mM, 130% activity; 200mM 40% activity), potassium phosphate (20mM, 100% activity; 100mM, 20% activity) and ammonium sulfate (20mM, 100% activity; 100mM, 20% activity)*. The presence of 20% glycerol results in 40% reduction in activity*. The presence of 1% Tween® 20 or Triton® X-100 results in a 5% decrease in activity*. T4 DNA Polymerase is sensitive to inhibition by various nucleotide/nucleoside analogs (9–12) and aphidicolin (9–12). Pyrophosphate analogs phosphoroacetate (PAA) and phosphoroformate (PFA) are weak inhibitors of T4 DNA Polymerase (11). Figures 2, 3 and 4 provide information on the effects of various restriction enzyme buffers, salt concentrations and temperatures, respectively, on T4 DNA Polymerase.

K_i: For aphidicolin and nucleotides BuAdATP and BuPdGTP, refer to references 9 and 10.

Temperature Stability: Optimal temperature is 37˚C (25% polymerase activity at 25˚C and 45˚C). Some sources recommend use of the enzyme at 11–12˚C in 3′-end labeling applications to maximize the ratio of polymerase activity to exonuclease activity (13,14).

Inactivation: 75˚C, 10 minutes.

Error Rate: 1×10^{-7} (1,16).

Processivity: 100–800 nucleotides per binding event. Processivity is template- and enzyme-concentration dependent (1).

Polymerization Rate: Approximately 250 nucleotides per second (17).

Strand Displacement Activity: No.

Genetic Locus: T4 gene 43.

* Data was generated at Promega, using unit definition reaction conditions.

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone.

Molecular Weight: T4 DNA Polymerase is a monomeric protein with a calculated molecular weight of 103kDa (18). Molecular weights of 112–114kDa have been reported (13,14).

Storage Conditions: Store at –20˚C. Avoid exposure to multiple freeze/thaw cycles. T4 DNA Polymerase is supplied in storage buffer containing: 200mM potassium phosphate (pH 6.5), 2mM DTT and 50% glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 55nmol of dTTP into acid-precipitable material in 30 minutes at 39˚C using poly(dA):oligo(dT) as a substrate. See the unit concentration on the Product Information Label. One unit as defined by these conditions is equivalent to one unit under the previous unit definition for this product.

Purity: Purity is >95% as determined by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.

Contaminant Assays

Endonuclease Assay: To test for endonuclease activity, 1µg of Type I supercoiled plasmid DNA is incubated with 40 units of T4 DNA Polymerase for one hour at 37˚C in 1X restriction enzyme Buffer D. Following incubation, the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

Single-Strand Endonuclease Assay: There must be no endonuclease activity detected when 1µg of M13mp18 DNA is incubated with 40 units of T4 DNA Polymerase for 1 hour at 37˚C.

Table 2. The Effect of Promega Restriction Enzyme Core Buffers on T4 DNA Polymerase Activity (see also Figure 2).

Buffer	Activity (% Control)
A	≥ 50%
B	≥ 50%
C	≥ 50%
D	< 50%
E	≥ 50%
G	≥ 50%
H	≥ 30%

Control activity was determined for T4 DNA Polymerase using reaction conditions specified for Unit Definition.

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RNA Polymerases

The RNA polymerases of bacteriophages T7, T3 and SP6 are single-subunit DNA-dependent RNA polymerases that exhibit extremely high specificity for their respective promoter sequences (1,2). For example, T3 and T7 are closely related bacteriophages, but T3 RNA Polymerase will not utilize a T7 promoter, nor will T7 RNA Polymerase recognize a T3 promoter (3).

Of the three commonly used enzymes within this group, T7 RNA Polymerase has been the most extensively studied. T7 RNA Polymerase transcribes the class II and III T7 phage genes, so named for the time of their expression during the phage growth cycle. Class III promoters appear stronger *in vitro* and *in vivo* than class II promoters (4) and are the promoters commonly used for *in vitro* transcription vectors. Table 9 provides the phage promoter and leader sequences present in some commonly used *in vitro* transcription vectors.

The bacteriophage RNA polymerases require a DNA template with the appropriate phage promoter, but synthesis does not require a primer as with the DNA polymerases. *In vitro* transcripts can be produced from a circularized plasmid template, but messages of varying length will result. Discrete RNA transcripts without vector sequences can be produced in two ways. First, transcription can be terminated by the presence of the specific phage RNA polymerase terminator sequence, although some read-through may occur (Figure 1e). The other more common method is to generate run-off transcripts from a template linearized by restriction enzyme digestion (Figure 1f). One complication can arise using the latter procedure if a restriction enzyme that generates 3′-overhangs is used (5). In this instance, transcripts complementary to the opposite strand and vector sequences will be produced, as the polymerase wraps around and begins transcription of the complementary strand. To avoid this, the 3′-overhangs should be converted to blunt ends using any of a number of standard methods (6).

Because of the polymerases' promoter specificity, only DNA downstream of the specific phage promoter sequence will be transcribed. Highly specific, single-stranded RNA produced *in vitro* by this method can be used in a wide variety of molecular biology applications. If a radiolabeled or tagged (e.g., biotinylated) rNTP is present, RNA probes can be produced. These probes provide hybrids with a higher melting temperature (RNA:RNA > RNA:DNA > DNA:DNA) that may be used in blotting and *in situ* hybridization procedures. By adjusting reaction conditions, large amounts of RNA can be made, making this a useful means to produce substrates for translation, processing and antisense RNA studies or to study ribozymes, tRNAs or rRNAs. Cap analogs can be easily added to the *in vitro* transcripts, which may enhance translation of these messages *in vitro* (7).

Table 9. Commonly Used Bacteriophage Promoter and Leader Sequences. The actual start site of transcription is indicated by ^ and the 6-base leader following the ^ will appear in the transcript. Changes to the leader sequence can also impact *in vivo* and *in vitro* transcriptional levels.

SP6 Promoter/Leader Sequence	Vectors
5′-ATT TAGGT GACAC TATA^G AATAC-3′	pGEM®-Zf Vectors[i]; pGEM®-3Z and 4Z Vectors[i]; pSP64, pSP65, pSP64 Poly(A), pSP70, pSP71 and pSP72 Vectors[i]; pGEMEX®-1 and -2 Vectors[i,j,z]; pALTER® Vectors; LambdaGEM® Vectors[i]
T7 Promoter/Leader Sequence	**Vectors**
5′-TAA TACGA CTCAC TATA^G GGCGA-3′ 5′-TAA TACGA CTCAC TATA^G GGAGA-3′	pGEM®-Zf Vectors; pGEM®-3Z and 4Z Vectors; pALTER® Vectors; pET Vectors[i,j,z]; RiboMAX™[a,k,l] and TnT® Luciferase Control DNA[l]; pGEMEX®-1 Vector; pGEM®-2 Vector; LambdaGEM® Vectors
T3 Promoter/Leader Sequence	**Vectors**
5′-TTA TTAAC CCTCA CTAAA^GGGAAG-3′ 5′-AAA TTAAC CCTCA CTAAA^GGGAAT-3′	pGEMEX®-1 and -2 Vectors; RiboMAX™ and TnT® Luciferase Control DNA

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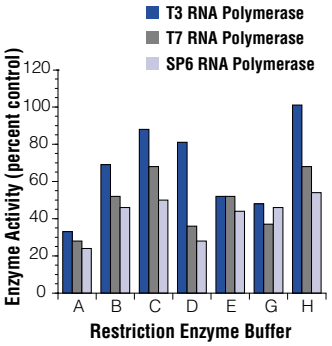

Figure 9. RNA Polymerase activity in Promega Restriction Enzyme Core Buffers (see Table 11). Control activity was determined for each polymerase, using its standard reaction conditions, including buffer composition and temperature, as specified for Unit Definition.

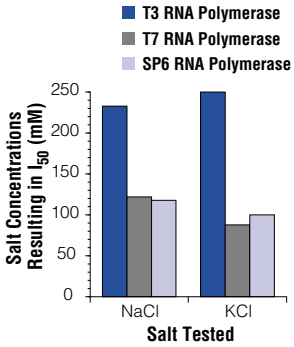

Figure 10. The concentrations of NaCl and KCl that result in 50% inhibition (I_{50}) of RNA Polymerase activity. Activity of each polymerase at these salt concentrations was compared to its activity at standard reaction conditions, specified for Unit Definition.

T7 RNA Polymerase

E.C. 2.7.7.6

Description

T7 RNA Polymerase (Cat.# P2075, P2077, P4074) is a DNA-dependent RNA polymerase with an extremely high specificity for its cognate promoter sequence. The enzyme catalyzes the repetitive addition of nucleotide triphosphates on a ss- or dsDNA template. The enzyme does not require a primer to initiate polymerization, but does require that the promoter sequence be double-stranded (1,2).

T7 RNA Polymerase is used for the *in vitro* synthesis of labeled RNA for use as hybridization probes and unlabeled RNA for *in vivo* or *in vitro* translation (3–5).

Applications

- Synthesis of RNA transcripts for hybridization probes (3,4).
- Synthesis of large amounts of nonlabeled RNA (3,5).
- Synthesis of *in vitro* capped RNA transcripts (3–5).

T7 RNA Polymerase is a component of the following Promega systems:

- Riboprobe® System-T7[a] (Cat.# P1440)
- Riboprobe® Combination System-SP6/T7 RNA Polymerase[a] (Cat.# P1460)
- Riboprobe® Combination System-T3/T7 RNA Polymerase[a] (Cat.# P1450)
- RiboMAX™ Large Scale RNA Production System-T7[a,k,l] (Cat.# P1300)
- TNT® T7 Coupled Wheat Germ Extract System[l,m,n] (Cat.# L4140)
- TNT® T7/SP6 Coupled Wheat Germ Extract System[l,m,n] (Cat.# L5030)
- TNT® T7/T3 Coupled Wheat Germ Extract System[l,m,n] (Cat.# L5040)

Enzyme Properties

Requirements: Mg^{2+}; thiol-reducing agent such as DTT (6).

Optimal Substrate: Both ss- and dsDNA can be used; however, the promoter sequence must be double-stranded (1,2). T7 DNA Polymerase can use both supercoiled and linear dsDNA as a template. Extraneous transcripts complementary to the opposite strand are generated from dsDNA templates with 3′-overhanging ends. Use linear templates with blunt ends or 5′-overhangs (3,7).

Optimal pH: 7.7–8.3 (6).

Alternative Cofactors and Substrates: T7 RNA Polymerase can incorporate biotinylated (8) and digoxigenin-modified nucleotides (9).

Inhibitors: T7 RNA Polymerase is inhibited by actinomycin D, heparin, poly r(U) and high salt concentrations (Figure 10; 10).
The presence of 2.5% PEG results in a 20% reduction in transcription activity (11).
The presence of 5% ethanol results in a 10% reduction in transcription activity (11).
T7 RNA Polymerase has variable activity in Promega Restriction Enzyme Core Buffers (Figure 9).

K_m: ATP, 47µM; CTP, 81µM; GTP, 160µM; UTP, 60µM (6).

Temperature Stability: Optimal temperature is 37˚C. In some cases, transcription at temperatures lower than 37˚C may result in a higher proportion of full-length transcripts produced (12). T7 RNA Polymerase is inactivated at temperatures greater than 50˚C (11).

Inactivation: Phenol:chloroform extraction or 70˚C, 10 minutes (11).

Polymerization Rate: 200–300 nucleotides per second at 37˚C (13).

Strand Displacement Activity: Yes.

Genetic Locus: T7 gene 1.

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone.

Molecular Weight: T7 RNA Polymerase is a monomeric protein with a calculated molecular weight of 98kDa (14). The protein has an apparent molecular weight of 107–110kDa on SDS-polyacrylamide gels (6,13).

Storage Conditions: Store at –20˚C. T7 RNA Polymerase is supplied in storage buffer containing 20mM potassium phosphate buffer (pH 7.7), 1mM EDTA, 10mM DTT, 0.1M NaCl, 0.1% Triton® X-100 and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 5nmol of CTP into acid-insoluble product in one hour at 37˚C (15). For unit definition the reaction conditions are: 40mM Tris-HCl (pH 7.9), 10mM NaCl, 6mM $MgCl_2$, 10mM DTT, 2mM spermidine, 0.05% Tween®20, 0.5mM each of ATP, GTP, CTP and UTP, 0.5µCi [^{3}H]CTP and 2µg of supercoiled pGEM®-5Zf(+) Vector DNA[i] (Cat.# P2241). The final volume is 100µl.

Purity: The purity is >90% as judged by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.

Activity Assays

RNA Synthesis Assay: T7 RNA Polymerase is tested for RNA synthesis using the same conditions as for Unit Definition except unlabeled CTP is limited to 12µM. pGEM® Express Positive Control DNA[i] (20ng/µl; Cat.# P2561) is used as template. Separate reactions are performed using 1, 2, 5, 10 and 20 units of enzyme for 1 hour at 37˚C. Minimum passing specification is >65% incorporation of [^{3}H]CTP using 20 units of enzyme.

Transcription Assay: T7 RNA Polymerase is tested in a transcription assay (20ng/µl) using pGEM® Express Positive Control DNA[i] incubated for 1 hour at 37˚C with 5 or 10 units of enzyme. Transcripts are denatured by heating at 65˚C for 10 minutes in formamide/formaldehyde buffer and resolved on a 1% agarose gel in TAE buffer. Specification is to obtain intact transcripts of the correct size with no degradation.

Contaminant Assay

Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 100 units of T7 RNA Polymerase in transcription optimized 1X Buffer for one hour at 37˚C (Table 12). The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for DNase and RNase activity.

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T3 RNA Polymerase
E.C. 2.7.7.6

Description

T3 RNA Polymerase (Cat.# P2083, P4024) is a DNA-dependent RNA polymerase with an extremely high specificity for its cognate promoter sequence. The enzyme catalyzes the repetitive addition of nucleotide triphosphates on a DNA template. T3 RNA Polymerase does not require a primer to initiate polymerization, but does require that the promoter sequence be double-stranded.

T3 RNA Polymerase is used for the *in vitro* synthesis of labeled RNA for use as hybridization probes and unlabeled RNA for *in vivo* and *in vitro* translation (1–3).

Applications
- Synthesis of RNA transcripts for hybridization probes (1,2).
- Synthesis of large amounts of nonlabeled RNA (1,3).
- Synthesis of *in vitro* capped RNA transcripts (1–4).

T3 RNA Polymerase is a component of the following Promega systems:
- Riboprobe® System-T3[a] (Cat.# P1430)
- Riboprobe® Combination System-T3/T7 RNA Polymerase[a] (Cat.# P1450)
- RiboMAX™ Large Scale RNA Production System-T3[a,k,l] (Cat.# P1290)
- TNT® T3 Coupled Wheat Germ Extract System[l,m,n] (Cat.# L4120)
- TNT® T7/T3 Coupled Wheat Germ Extract System[l,m,n] (Cat.# L5040)

Enzyme Properties

Requirements: Mg^{2+}.

Optimal Substrate: T3 DNA Polymerase can use both supercoiled and linear dsDNA as a template. Extraneous transcripts complementary to the opposite strand are generated from dsDNA templates with 3′ overhanging ends. Use linear templates with blunt ends or 5′-overhangs (5,6).

Alternative Substrates: T3 RNA Polymerase can incorporate biotinylated and digoxigenin-modified nucleotides (4,7).

Inhibitors: T3 RNA Polymerase is inhibited by salts (Figure 10). 2.5% PEG results in a 40% reduction in transcription activity (6). The presence of 5% ethanol does not result in reduction in transcription activity (6). T3 RNA Polymerase has variable activity in Promega Restriction Enzyme Core Buffers (Figure 9).

Temperature Stability: Optimal temperature is 37˚C. T3 RNA Polymerase is inactivated at temperatures greater than 50˚C (1).

Inactivation: Phenol:chloroform extraction or 70˚C, 10 minutes (6).

Polymerization Rate: 200–300 nucleotides per second (8) at 37˚C.

Strand Displacement Activity: Yes.

Genetic Locus: T3 gene 1.

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone.

Molecular Weight: T3 RNA Polymerase is a monomeric protein with a calculated molecular weight of approximately 98.8kDa (9).

Storage Conditions: Store at –20˚C. T3 RNA Polymerase is supplied in storage buffer containing 20mM potassium phosphate buffer (pH 7.7), 1mM EDTA, 10mM DTT, 0.1M NaCl, 0.1% Triton® X-100 and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 5nmol of CTP into acid-insoluble product in one hour at 37˚C. For unit definition the reaction conditions are: 40mM Tris-HCl (pH 7.9), 10mM NaCl, 6mM $MgCl_2$, 10mM DTT, 2mM spermidine, 0.05% Tween® 20, 0.5mM each of ATP, GTP, CTP and UTP, 0.5µCi [^{3}H]CTP and 2µg of supercoiled pSP6/T3 vector DNA. The final volume is 100µl.

Purity:T3 RNA Polymerase is judged to be greater than 90% pure as indicated by SDS-polyacrylamide gel electrophoresis and Coomassie® blue staining.

Activity Assays
RNA Synthesis Assay: T3 RNA Polymerase is tested for RNA synthesis using the same conditions as for unit definition (above) except unlabeled CTP is limited to 12µM. pGEM® Express Positive Control DNA[i] (20ng/µl; Cat.# P2561) is used as template. Separate reactions are performed using 1, 2, 5, 10 and 20 units of enzyme for 1 hour at 37˚C. Minimum passing specification is ≥65% incorporation of [^{3}H]CTP, using 20 units of enzyme.

Transcription Assay: T3 RNA Polymerase is tested in a transcription assay using pGEM® Express Positive Control DNA (20ng/µl) incubated for 1 hour at 37˚C with 5 or 10 units of enzyme. Transcripts are denatured by heating at 65˚C for 10 minutes in formamide/formaldehyde buffer and resolved in a 1% agarose gel in TAE buffer. Specification is to obtain intact transcripts of the correct size with no degradation.

Contaminant Assay
Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 100 units of T3 RNA Polymerase in Transcription Optimized 1X Buffer for one hour at 37˚C (Table 12). The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for DNase and RNase activity.

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SP6 RNA Polymerase
E.C. 2.7.7.6

Description

SP6 RNA Polymerase (Cat.# P1081, P1085, P4084) is a DNA-dependent RNA polymerase with an extremely high specificity for its cognate promoter sequence. The enzyme catalyzes the repetitive addition of nucleotide triphosphates on a ss- or dsDNA template. The enzyme does not require a primer to initiate polymerization but does require the promoter sequence to be double-stranded (1).

SP6 RNA Polymerase is used for the *in vitro* synthesis of labeled RNA for use as hybridization probes and unlabeled RNA for *in vivo* or *in vitro* translation (2–4).

Applications
- Synthesis of RNA transcripts for hybridization probes (2,3).
- Synthesis of large amounts of nonlabeled RNA (2,4).
- Synthesis of *in vitro* capped RNA transcripts (2,4).

SP6 RNA Polymerase is a component of the following Promega systems:

- Riboprobe® System-SP6[a] (Cat.# P1420)
- Riboprobe® Combination System-SP6/T7 RNA Polymerase[a] (Cat.# P1460)
- RiboMAX™ Large Scale RNA Production System-SP6[a,k,l] (Cat.# P1280)
- TNT® SP6 Coupled Wheat Germ Extract System[l,m,n] (Cat.# L4130)
- TNT® T7/SP6 Coupled Wheat Germ Extract System[l,m,n] (Cat.# L5030)

Enzyme Properties

Requirements: Mg^{2+}; thiol-reducing reagents, such as DTT (5).

Optimal Substrate: Transcription is most efficient using dsDNA, although ssDNA can be used, provided that the promoter sequence is double-stranded (2). SP6 RNA Polymerase can use both supercoiled and linear dsDNA as a template. Extraneous transcripts complementary to the opposite strand are generated from dsDNA templates with 3′ overhanging ends. Use linear templates with blunt ends or 5′-overhangs (6).

Optimal pH: 7.5 in Tris-HCl buffer; optimal range is pH 7–8.5 (5).

K_m: ATP, 67µM; CTP, 30µM; GTP, 50µM; UTP, 31µM (5).

Inhibitors: SP6 RNA Polymerase is inhibited by salts (Figure 10); activity decreases up to 50% in 20mM $(NH_4)_2SO_4$ (5). The presence of 2.5% PEG results in a 40% reduction in transcription activity (7). The presence of 5% ethanol results in no reduction in transcriptional activity (7). SP6 RNA Polymerase has variable activity in Promega Restriction Enzyme Core Buffers (Figure 9).

Temperature Stability: Optimal temperature is 37˚C. SP6 RNA Polymerase is inactivated at temperatures greater than 50˚C (7).

Inactivation: Phenol:chloroform extraction or heat-inactivation at 70˚C for 10 minutes (7).

Strand Displacement Activity: Yes.

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone.

Molecular Weight: SP6 RNA polymerase is a monomeric protein with a calculated molecular weight of 98.6kDa (8).

Storage Conditions: Store at –20˚C. SP6 RNA Polymerase is supplied in storage buffer containing 20mM potassium phosphate buffer (pH 7.7 at 25˚C), 1mM EDTA, 10mM DTT, 0.1M NaCl, 0.1% Triton® X-100 and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the incorporation of 5nmol of CTP into acid-insoluble product in one hour at 37˚C. For unit definition the reaction conditions are: 40mM Tris-HCl (pH 7.9), 10mM NaCl, 6mM $MgCl_2$, 10mM DTT, 2mM spermidine, 0.05% Tween®-20, 0.5mM each of ATP, GTP, CTP and UTP, 0.5µCi [^{3}H]CTP and 2µg of supercoiled pGEM®-5Zf(+) Vector DNA[i] (Cat.# P2241). The final volume is100µl.

Purity: The purity is judged to be greater than 90% as indicated by SDS-polyacrylamide gel electrophoresis and Coomassie® blue staining.

Activity Assays

RNA Synthesis Assay: SP6 RNA Polymerase is tested for RNA synthesis using the same conditions as for Unit Definition (above), except that the unlabeled CTP is limited to 12µM. pGEM® Express Positive Control DNA[i] (20ng/µl; Cat.# P2561) is used as the template; the reaction volume is 50µl. Separate reactions are performed using 1, 2, 5, 10 and 20 units of enzyme for 1 hour at 37˚C. Minimum passing specification is >65% incorporation of [^{3}H]CTP using 20 units of enzyme.

Transcription Assay: SP6 RNA Polymerase is tested in a transcription assay using pGEM® Express Positive Control DNA incubated for 1 hour at 37˚C with 5 or 10 units of enzyme. Transcripts are denatured by heating at 65˚C for 10 minutes in formamide/formaldehyde buffer and resolved in a 1% agarose gel in TAE buffer. Specification is to obtain intact transcripts of the correct size with no degradation.

Contaminant Assay

Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 100 units of SP6 RNA Polymerase in transcription optimized 1X Buffer for one hour at 37˚C (Table 12). The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for DNase and RNase activity.

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Reverse Transcriptases

The discovery of reverse transcriptases, or RNA-dependent DNA polymerases, and their role in retrovirus infection (1,2) altered molecular biology's central dogma of DNA→RNA→protein. These findings revealed that reverse transcriptases, which are found in the virion, catalyze the synthesis of a proviral DNA from the virion's RNA genome at the onset of the infection process. The primary *in vitro* application of reverse transcriptase is in the production of cDNA libraries in which the enzyme is used to copy RNA into the first strand of the cDNA product to be cloned. Similarly, reverse transcriptases are used in the first step of two-enzyme reverse transcriptase-polymerase chain reaction (RT-PCR) systems to generate the DNA template required for thermostable DNA polymerases. They also are used to map the 5′-ends of mRNAs in RNA sequencing and cDNA probe production.

Reverse transcriptases require a primer for synthesis, like other DNA polymerases. For *in vitro* applications, these primers can be either oligo(dT), which hybridizes to the poly(A)+ tails of eukaryotic mRNAs; random hexamers, which prime the synthesis from internal sites of the RNA template; or a sequence-specific primer, which hybridizes to a known sequence within the RNA template (3). Polymerization from a primer then proceeds as for DNA-dependent DNA polymerases (Figure 1g and 1h). The two commonly used reverse transcriptases, Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) perform the same reaction, but at different optimum temperatures (AMV, 42˚C; MMLV, 37˚C). AMV RT is the enzyme of choice for templates with high degrees of secondary structure, as more structure can be melted at 42˚C than at 37˚C.

Some reverse transcriptases also possess intrinsic 3′- and/or 5′-exoribonuclease (RNase H) activity, similar to the exonuclease activities associated with some DNA-dependent DNA polymerases. These activities are generally used to degrade the RNA template after the first strand of a cDNA is produced. Absence of the 5′-exoribonuclease (RNase H) activity may aid in the production of longer cDNAs (4).

Some DNA-dependent DNA polymerases also possess a reverse transcriptase activity, which can be favored under certain conditions. For example, the thermostable DNA-dependent DNA Polymerase, *Tth* DNA Polymerase[d,e], exhibits reverse transcriptase activity when Mn^{2+} is substituted for Mg^{2+} in a reaction.

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AMV Reverse Transcriptase
E.C. 2.7.7.49

Description

AMV Reverse Transcriptase (Cat.# M5101, M5108, M9004) catalyzes the polymerization of DNA using template DNA, RNA or RNA:DNA hybrids (1,2). AMV Reverse Transcriptase is the preferred reverse transcriptase for templates with high secondary structure, due to its higher reaction temperature. AMV Reverse Transcriptase is used in a wide variety of applications including first- and second-strand DNA synthesis (2,3), sequencing of RNA (4,5) and DNA (6,7), RT-PCR(g) and 5′-rapid amplification of cDNA ends (RACE) (8). The enzyme can also be used for primer extension (9), filling in 5′-overhangs (10) and 3′ end-labeling (11). Although the high optimal temperature makes it the enzyme of choice for cDNA synthesis using templates with complex secondary structure, its relatively high RNase H activity limits its usefulness for generation of long cDNAs (>5kb). For these templates M-MLV Reverse Transcriptase may be a better choice.

Applications

- First-strand/second-strand synthesis of cDNA (2,3).
- RT-PCR.
- RNA sequencing (4,5).
- DNA sequencing (6,7).
- Filling in 5′-overhangs where Klenow is not satisfactory (10).
- 5′-RACE (rapid amplification of cDNA ends) (8).
- Primer extension (9).
- 3′-end labeling (11).
- Studies on secondary structure of RNA (12).

AMV Reverse Transcriptase is available in the following Promega systems:

- Access RT-PCR System(g) (Cat.# A1250, A1260, A1280)
- Universal RiboClone® cDNA Synthesis System(a) (Cat.# C4360)
- Reverse Transcription System(a) (Cat.# A3500)

Enzyme Properties

Requirements: A DNA primer is required for efficient reverse transcription of RNA. Primers of greater than 8 nucleotides are most effective. Divalent cations such as Mg^{2+} or Mn^{2+} at 6–10mM are also required (13). Mn^{2+} has been reported to increase the DNA endonuclease activity of AMV Reverse Transcriptase.

Cofactors: Sodium pyrophosphate and spermidine help drive the DNA polymerase activity forward and denature RNA secondary structure, respectively (11).

Optimal Substrate: ssDNA or RNA. Fifteen units of enzyme per microgram of RNA is commonly used for first-strand synthesis (13).

Optimal pH: 8.3 in Tris-HCl buffer.

Inhibitors: DNA-dependent DNA polymerase activity is strongly inhibited by actinomycin D at 50µg/ml final concentration (9,14). Nucleic acids rRNA and tRNA (15) may also inhibit the enzyme. High amounts of secondary structure in the RNA being transcribed can inhibit chain elongation. Glycerol concentrations >10% may inhibit up to 50% of activity. Ribonucleoside-vanadyl complexes at 2mM or above can inhibit AMV Reverse Transcriptase activity, unless all four dNTPs exceed approximately 1mM. N-ethyl maleimide inactivates AMV RT(10).

Temperature Stability: 37–58˚C. Typical working conditions are at 42˚C, but for RT-PCR applications, temperatures as high as 58˚C will yield acceptable results (16).

Inactivation: Addition of 50mM EDTA (pH 8.0) heating to 75˚C for 15 minutes. Alternatively, 100˚C for 5 minutes followed by a 5-minute incubation on ice. If analyzing reverse transcription on native gels, do not heat as this may cause RNA/cDNA hybrids to form, making interpretation difficult.

Error Rate: Due to the lack of proofreading 3′→5′ exonuclease activity, error rates for all reverse transcriptases are high. The average error rate for AMV RT is 4.9×10^{-4}, based on misincorporation studies (17). In addition, AMV RT will only resume faithful incorporation 25% of the time after a misinsertion.

Promega Product Information

Source: Purified from Avian myeloblastosis virus particles.

Molecular Weight: AMV Reverse Transcriptase is a 170kDa heterodimer with an α-subunit of 65kDa and a β-subunit of 94kDa (18). The smaller subunit is a proteolytic product of the larger subunit (1,2).

Typical Working Conditions: 50mM Tris-HCl (pH 8.3 at 25˚C), 50mM KCl, 5mM $MgCl_2$, 5mM DTT, 40µM each dNTP, 0.1mg/ml BSA.

Storage Conditions: Store at –20˚C. Avoid exposure to frequent temperature changes. AMV Reverse Transcriptase is supplied in storage buffer containing 200mM potassium phosphate (pH 7.2), 0.2% Triton® X-100, 2mM DTT and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the transfer of 1nmol of deoxynucleotide into acid-precipitable material in 10 minutes at 37˚C. For unit definition the reaction conditions are: 50mM Tris-HCl (pH 8.3), 40mM KCl, 8.75mM $MgCl_2$, 10mM DTT, 0.1mg/ml BSA, 1mM [^{3}H]-labeled dTTP, 0.25mM poly(A)$_{400}$ and 0.25mM oligo(dT)$_{50}$.

Purity: The enzyme is greater than 80% pure, as judged by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.

Activity Assay

Functional Assay: First-strand cDNA of a 1.2kb Control RNA (Cat.# C1381) is synthesized using 15 units of AMV Reverse Transcriptase, 1µg of template, 0.5µg of oligo(dT)$_{15}$ primer and 1mM each dNTP for 60 minutes at 42˚C. The minimum specification is the production of >120ng of first-strand cDNA. Full-length cDNA must be observed following gel electrophoresis and autoradiography.

Contaminant Assays

Endonuclease Assay: To test for endonuclease activity, 0.5µg of Type I supercoiled plasmid DNA is incubated with 25 units of AMV Reverse Transcriptase in 50mM Tris (pH 8.3), 40mM KCl, 7mM $MgCl_2$ and 10mM DTT for one hour at 37˚C. Following incubation, the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of nicking or cutting.

Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabled DNA or radiolabeled RNA is incubated with 25 units of AMV Reverse Transcriptase in 50mM Tris (pH 8.3), 40mM KCl, 7mM $MgCl_2$ and 10mM DTT for one hour at 37˚C. The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for DNase and 3% release for RNase.

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M-MLV Reverse Transcriptase (M-MLV RT)

E.C. 2.7.7.49

Description

M-MLV RT (Cat.# M1701, M1705) is a single polypeptide RNA-dependent DNA polymerase. The enzyme also has DNA-dependent DNA polymerase activity at higher enzyme levels (1,2). M-MLV RT is used in a variety of applications including cDNA synthesis, RNA sequencing (3), RT-PCR(g) and 5′-RACE (4). The enzyme can be used for primer extension (3) and filling in 5′-overhangs (2). Its relatively low RNase H activity compared to AMV RT (5) makes M-MLV RT the choice for generation of long cDNAs (>5kb). However, for short templates with complex secondary structure, AMV RT may be a better choice due to its higher optimal temperature. M-MLV RT is less processive than AMV RT; more units of M-MLV RT may be required to generate the same amount of cDNA (6).

Applications

- cDNA synthesis of mRNA.
- RT-PCR.
- RACE (rapid amplification cDNA ends) (4).
- mRNA 5′-end mapping by primer extension analysis (3).
- 3′-termini fill-in of dsDNA with 5′-protruding ends (2).
- RNA sequencing using ddNTPs (3).

Enzyme Properties

Requirements: Mg^{2+} and/or Mn^{2+}.

Optimal Substrate: RNA template and DNA primer of 9–15 bases.

Optimal pH: 8.0 at 37˚C with Tris-HCl.

K_m: The K_m varies considerably for the individual dNTPs, from 0.5 to 80µM, depending upon the nucleotide and template sequence. A K_m of 18µM was determined for all 4 dNTPs combined, using the HIV p25 *gag* template (7).

Alternative Cofactors and Substrates: RNA can also act as a primer, but is much less efficient than DNA (2); 6mM $MnCl_2$ (1); ddNTPs (3).

Inhibitors: Inorganic phosphate and pyrophosphate (1): 20mM inorganic phosphate or 0.5mM sodium pyrophosphate results in nearly 50% loss of activity; spermidine (8); 50µg/ml actinomycin D inhibits second-strand synthesis (3).

Half-Life: 6.5 minutes at 45˚C, with or without template (2); 15 minutes at 42˚C (1).

Inactivation: The addition of 50mM EDTA or heating to 70˚C for 10 minutes will inactivate M-MLV RT.

Error Rate: Due to the lack of a proofreading 3′→5′ exonuclease activity, error rates for all reverse transcriptases are high. An average error rate of 1 in 1,000 bases has been shown with Mg^{2+}(6,7). M-MLV will only resume faithful incorporation 50% of the time after a misinsertion (7).

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone (1).

Molecular Weight: 71kDa (1).

Typical Working Conditions: 3mM $MgCl_2$ is optimal, while 2–4mM results in >80% activity and >10mM results in <50% activity; 75mM KCl and NaCl is optimal, while 0–125 results in >70% activity (1); 10mM DTT, 0.5–2mM each dNTP; 0.1mg/ml BSA.

Storage Conditions: Store at –20˚C. Avoid exposure to frequent temperature changes. M-MLV RT is supplied in storage buffer containing 20mM Tris-HCl (pH 7.5), 200mM NaCl, 0.1mM EDTA, 1mM DTT, 0.01% Nonidet® P-40 and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the transfer of 1nmol of deoxynucleotide into acid-precipitable material in 10 minutes at 37˚C. For unit definition the reaction conditions are: 0.5mM [^{3}H]dTTP, 0.025mM $oligo(dT)_{50}$ and 0.25mM $poly(A)_{400}$ in 50mM Tris-HCl (pH 8.3), 7mM $MgCl_2$, 40mM KCl, 10mM DTT and 0.1mg/ml BSA.

Purity: Purity is >90% as judged by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.

Activity Assay

Functional Assay: First-strand cDNAs, of 1.2kb and 7.5kb control RNAs, are synthesized using 200 units of M-MLV RT, 1µg of each template, 0.5µg $oligo(dT)_{15}$ primer 0.5mM each dNTP and a radiolabeled dCTP at 37˚C for 1 hour in 25µl. The minimum specification is the production of 120ng of first-strand cDNA. In addition, >90% full-length cDNA for the 1.2kb RNA must be observed by gel electrophoresis and autoradiography.

Contaminant Assays

Endonuclease Assay: To test for endonuclease activity, 1µg of Type I supercoiled plasmid DNA is incubated with 500 units of M-MLV Reverse Transcriptase in 1X reaction buffer for 1 hour at 37˚C. Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained gel to verify the absence of visible nicking or cutting.

Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 200 units of M-MLV Reverse Transcriptase in 1X reaction buffer for 1 hour at 37˚C (Table 12). The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for DNase and <3% for RNase.

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M-MLV Reverse Transcriptase, RNase H Minus (M-MLV RT, H Minus)

E.C. 2.7.7.49

Description

M-MLV Reverse Transcriptase, RNase H Minus (Cat.# M5301*), is an RNA-dependent 5′→3′ DNA polymerase that has been genetically altered to remove the associated ribonuclease H activity (1). M-MLV Reverse Transcriptase, RNase H Minus, is used in a wide variety of applications including synthesis of cDNA, RT-PCR(g) and sequencing of RNA (2). The enzyme can also be used for primer extension (2) and filling in 5′-overhangs (3). The absence of RNase H activity of the enzyme makes it the enzyme of choice for generation of long cDNAs (>5kb). However, for shorter templates with complex secondary structure AMV Reverse Transcriptase may be a better choice due to its higher optimal temperature.

*Product is only available in certain countries.

Applications

- cDNA synthesis from mRNA.
- RT-PCR.
- Primer extension.
- mRNA 5′-end mapping by primer extension analysis (2).
- 3′-termini fill-in of dsDNA with 5′-protruding ends (3).
- RNA sequencing using ddNTP (2).

Enzyme Properties

Requirements: Mg^{2+} and/or Mn^{2+}.

Optimal Substrate: RNA template and DNA primer of 9–15 bases.

Optimal pH: 8.0 at 37˚C with Tris-HCl.

K_m: The K_m varies considerably for the individual dNTPs, from 0.5 to 80µM, depending on the nucleotide and template sequence. A K_m of 18µM was determined for all 4 dNTPs combined, using the HIV p25 *gag* template (4).

Alternative Cofactors and Substrates: RNA can act as a primer also, but is much less efficient than DNA (3), 6mM $MnCl_2$ (5), ddNTPs (2).

Inhibitors: Inorganic phosphate and pyrophosphate (1): 20mM inorganic phosphate or 0.5mM sodium pyrophosphate results in nearly 50% loss of activity; spermidine (3); 50µg/ml actinomycin D inhibits second-strand synthesis (2).

Half-Life: 6.5 minutes at 45˚C, with or without template (3); 15 minutes at 42˚C (5).

Inactivation: Addition of 50mM EDTA or heating to 70˚C for 10 minutes.

Error Rate: Due to the lack of a proofreading 3′→5′ exonuclease activity, error rates for all reverse transcriptases are high. As high as 1 in 500 bases is misincorporated by M-MLV RT, H Minus, in the presence of Mn^{2+} and high concentrations of dNTPs (6). An average error rate of 1 in 1,000 bases has been shown with Mg^{2+}. M-MLV RT, H Minus, will only resume faithful incorporation 50% of the time after a mismatch (4).

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone (5).

Molecular Weight: 71kDa (5).

Typical Working Conditions: 3mM $MgCl_2$ is optimal, while 2–4mM results in >80% activity and >10mM results in <50% activity; 75mM KCl and NaCl is optimal, while 0–125mM results in >70% activity (1); 10mM DTT; 0.5–2mM each dNTP; 0.1mg/ml BSA.

Storage Conditions: Store at –20˚C. Avoid exposure to frequent temperature changes. M-MLV RT, H Minus, is supplied in storage buffer containing 20mM Tris-HCl (pH 7.5), 200mM NaCl, 0.1mM EDTA, 1mM DTT, 0.01% Nonidet® P-40 and 50% glycerol.

Unit Definition: One unit is defined as the amount of enzyme that incorporates 1nmol of dTTP into acid-insoluble form in 10 minutes at 37˚C. For unit definition the reaction conditions are: 50mM Tris-HCl (pH 8.3), 40mM KCl, 10mM DTT, 7mM $MgCl_2$, 0.1mg/ml BSA, 0.5mM radiolabeled dTTP, 0.25mM $poly(A)_{400}$ and 0.025mM $oligo(dT)_{50}$.

Purity: Purity is >90% as judged by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.

Activity Assay

Functional Assay: First-strand cDNA of a 1.2kb Control RNA is synthesized using 200 units of M-MLV RT, H Minus. By autoradiography the cDNA product must be observed as a single, full-length band. The minimum passing specification is 12% conversion of RNA into cDNA. The product is labeled with [^{32}P]dCTP.

Contaminant Assays

Endonuclease Assay: To test for endonuclease activity, 1µg of Type I supercoiled plasmid DNA is incubated with 200 units of M-MLV RT, H Minus, in 1X reaction buffer for 1 hour at 37˚C (Table 12). Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained gel to verify the absence of visible nicking or cutting.

Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 200 units of M-MLV Reverse Transcriptase, H Minus, in 1X reaction buffer for 1 hour at 37˚C (Table 12). The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for DNase and <3% for RNase.

RNase H Activity: Two hundred units of enzyme is incubated with polyA:poly(d)T for 1 hour at 37˚C. The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release.

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Terminal Deoxynucleotidyl Transferase

E.C. 2.7.7.31

Terminal deoxynucleotidyl transferase (TdT) is the sole member of this final group of polymerases. Unlike all other polymerases, TdT-directed synthesis does not copy or require a template, and appends nucleotides to the 3′-ends of oligo- and polynucleotide primers in the following manner (see also Figure 1i):

$$n\text{dNTP} + \text{d(pX)}_m \xrightarrow{\text{Metal}^{2+}} \text{d(pX)}_m\text{(pN)}_n + n\text{PPi}$$

The enzyme is distributive and does not exhibit PPi exchange, pyrophosphorolysis or exonuclease activities. TdT has been used widely for synthesis of homo- and heteropolymers; it can be used to synthesize a wide variety of polymers, since it is very tolerant of nucleotide derivatives (1). TdT is closely related to the repair enzyme DNA Polymerase B (2), but the biological role of TdT remains a mystery. Enzyme activity is only found in pre-T and pre-B lymphocyte nuclei during immunopoiesis, suggesting the enzyme could play a role in generating somatic diversity of immonoglobulin and T-cell receptor genes (3). While its mutator role has not been directly demonstrated, TdT has proven a valuable marker in the diagnosis of leukemia (4,5).

Description

Terminal Deoxynucleotidyl Transferase (Cat.# M1871) catalyzes the repetitive addition of mononucleotides from deoxynucleotide triphosphates to the 3′-OH of a DNA initiator, accompanied by the release of inorganic phosphate. Polymerization is not template dependent. ssDNA is preferred as the initiator. The addition of 1mM Co^{2+} (as $CoCl_2$) in the reaction buffer allows the tailing of any type of 3′-end, with various efficiencies. Of all dsDNA initiators, those with protruding 3′-ends are most efficiently tailed. TdT demonstrates DNA acceptor preference in the following order: ssDNA>dsDNA w/3′-overhangs >dsDNA with 5′-overhangs or blunt ends (6). TdT is used for 3′ end-labeling with labeled or unlabeled nucleotides (2–4). The addition of nucleotides can be limited to a single nucleotide with the use of cordycepin-5′-triphosphate (deoxyadenosine-5′-triphosphate). Tailing DNA with radiolabeled nucleotides will generate probes with higher specific activities than 5′-end-labeling with T4 polynucleotide kinase. However, the addition of multiple nucleotides will change the sequence of the probe and perhaps its specificity. Another common application for TdT is its use in the TUNEL assay (TdT-mediated dUTP Nick End Labeling assay) for apoptosis detection (5,18).

Applications

- Tailing reactions to add homopolymer tails to DNA vectors and cDNA for cloning (7).
- DNA 3′-end labeling, isotope and non-radioactive (8,9).
- Apoptosis detection (TUNEL assay (10,22,23).

TdT is a component of the following Promega systems:

- 3′-End Labeling Application Pack (Cat.# M7710)
- Apoptosis Detection System, Fluorescein(n) (Cat.# G3250)
- DeadEnd™ Colorimetric Apoptosis Detection System (Cat.# G7130)

Enzyme Properties

Requirements: A divalent metal is required. Mg^{2+} is preferred for adding purines and cobalt is preferred for adding pyrimidines or rNTPs. Acceptor nucleic acid must be ss- or dsDNA with a free 3′-OH and a minimum of 3 nucleotides in length (7).

Cofactor Concentration: 1–2mM $CoCl_2$ (2mM is optimal for dsDNA) or 4–10mM $MgCl_2$ are commonly reported.

Optimal Substrate: ssDNA is preferred, but dsDNA with 3′-overhangs, 5′-overhangs or blunt ends will also work with varying efficiency.

Optimal Substrate Concentration: The ratio of acceptor DNA to the NTP used will determine, approximately, the number of nucleotides added, i.e., 1pmol 3′-ends to 10pmol nucleotide will add an average of 10 nucleotides per acceptor. For cordycepin triphosphate, the ratio of acceptor to label should be at least 1:1.5 to 1:2. A minimum of 5 units of enzyme should be used for each pmol of acceptor for uniform incorporation (8).

Optimal pH: 6.8–7.2 (11,12).

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K_m: 100µM for dATP and dGTP; 500µM for dTTP and dCTP; up to 1mM for homopolymer acceptors (11); approximately 1µM for oligonucleotide acceptors (13,16).

Stimulator: If using Mg^{2+} as the primary divalent metal, $ZnSO_4$ in concentrations from 0.33mM to 0.8mM have been shown to improve activity (12,13).

Alternative Cofactors and Substrates: Mn^{2+} (6), [^{35}S]-labeled nucleotide triphosphates (17), ribonucleotide triphosphates, ddNTPs, cordycepin triphosphate, biotinylated nucleotide triphosphates (18), digoxygenin-conjugated nucleotide triphosphates (19), fluorescent nucleotide triphosphates and amino-modified triphosphates [NH_2-rATP] (20).

Inhibitor: Phosphate buffers and high dNTP concentrations, which subsequently produce high pyrophosphate concentrations, should be avoided, as cobalt phosphate and cobalt pyrophosphate are insoluble (14). DTT concentrations above 5mM will also precipitate cobalt. If DNA is ethanol-precipitated prior to TdT tailing, sodium is a better cation than ammonium for enzyme activity. Chloride and iodide are also inhibitory and therefore cacodylate buffers are typically used (15). Tris has the potential to form a complex with Zn^{2+} and should be avoided (13).

Temperature Stability: Avoid temperatures over 40˚C (11).

Inactivation: 70˚C for 10 minutes or addition of 20mM EDTA in a reaction containing 1mM $CoCl_2$ and 10 units of TdT (12).

Promega Product Information

Source: Calf thymus.

Molecular Weight: 32,360 daltons with an α-subunit estimated at 8,000 daltons and a β-subunit estimated at 26,500 daltons (21).

Storage Conditions: Long-term (use of 1–2 times per month): –70˚C. For daily/weekly use: –20˚C. TdT is supplied in storage buffer containing 50mM potassium phosphate (pH 7.4), 1mM β-mercaptoethanol and 50% glycerol.

Unit Definition: One unit of activity catalyzes the transfer of 1 nanomole of dATP to $p(dA)_{50}$ per hour at 37˚C. For unit definition the reaction conditions are: 40mM cacodylate buffer (pH 6.8 with KOH), containing 8mM $MgCl_2$, 0.33mM $ZnSO_4$, 1mM dATP, 10µM $p(dA)_{50}$ and 1µCi of [^{32}P]dATP in a 50µl total reaction volume.

Reaction Buffer: TdT is provided with a 5X assay buffer consisting of 500mM cacodylate (pH 6.8), 5mM $CoCl_2$ and 0.5mM DTT.

Activity Assay
Functional Assay: 3′-end labeling is accomplished by incubating 8ng of the positive control $oligo(dT)_{15}$ primer in 1X assay buffer with 16µCi [α-^{32}P]dATP and 20 units of TdT (final volume 20µl) for 60 minutes at 37˚C. Minimum passing specification is >50% incorporation.

Contaminant Assays
Endonuclease Assay: To test for endonuclease activity, 1µg of Type I supercoiled plasmid DNA is incubated with 5 units of TdT for one hour at 37˚C in 1X reaction buffer (Table 12) with 10mM $MgCl_2$ added. Following incubation, the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 25 units of Terminal Deoxynucleotidyl Transferase for one hour at 37˚C in 1X reaction buffer with 10mM $MgCl_2$ added (Table 12). The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <3% for both DNase and RNase.

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Table 10. Promega's Quality Acceptance Criteria for Enzymes.

ENZYME	CONCENTRATION (u/µl)	SDS-PAGE PURITY	PCR (g,*)	CONTAMINANT ACTIVITY		
				Endonuclease: Supercoiled DNA, 1µg	dsDNase: 50ng Radiolabeled DNA	RNase: 50ng Radiolabeled RNA
DNA Polymerase I	5–10	>90%		Gel analysis, absence of visible nicking or cutting, 25u/5hr at 37°C	N/A	N/A
DNA Polymerase I Large (Klenow) Fragment	5–10	>90%, no Pol I		Gel analysis, absence of visible nicking or cutting, 25u/16hr at 37°C	N/A	N/A
DNA Polymerase I Large (Klenow) Fragment, Exo Minus	5-10	>90%		Gel analysis, absence of visible nicking or cutting, 25u/16hr at 37˚C	<2% release, 30u/16hr at 37˚C	N/A
T4 DNA Polymerase[1]	5–10	>95%		Gel analysis, absence of visible nicking or cutting, 40u/1hr at 37°C	N/A	N/A
Taq DNA Polymerase[2 (d)]	5	>90%	✓	Gel analysis, absence of visible cutting, 5u/8 hr at 45°C and 8hr at 70°C	N/A	N/A
Tfl DNA Polymerase [(d)]	5	>90%	✓	Gel analysis, absence of visible nicking or cutting, 5u/16hr at 74°C	<3% release, 25u/1hr at 74°C	<3% release, 25u/1hr at 48°C
Tli DNA Polymerase [(d)]	3	N/A	✓	Gel analysis, absence of visible nicking or cutting,10u/6hr at 74°C	N/A 2–10	N/A N/A
Tth DNA Polymerase [(d,e)]	5	>95%	✓	>90% Form I, 5u/16hr at 70°C also tested on lambda DNA	<1% release, 5u/1hr at 70°C	<3% release, 5u/1hr at 37°C
Pfu DNA Polymerase [3 (d)]	2–3	N/A	✓	N/A	N/A	N/A
T7 RNA Polymerase[4]	10–20 80	>90%		N/A	<1% release, 100u/1hr at 37°C	<1% release,
T3 RNA Polymerase[4]	10–20 80	>90%		N/A	<1% release, 100u/1hr at 37°C	<1% release,
SP6 RNA Polymerase[4]	10–20 80	>90%		N/A	<1% release, 100u/1hr at 37°C	<1% release,
AMV Reverse Transcriptase[5]	5–10 20–25	>80%		Gel analysis, absence of visible nicking or cutting, 25u/1hr at 37°C	<1% release, 25u/1hr at 37°C	<3% release, 25u/1hr at 37°C
M-MLV RT	200	>90%		Gel analysis, absence of visible nicking or cutting, 500u/1hr at 37°C	<1% release, 200u/1hr at 37°C	<3% release, 200u/1hr at 37°C
M-MLV Reverse Transcriptase, RNase H Minus	100–200	>90%		Gel analysis, absence of visible nicking or cutting, 200u/1hr at 37°C	<1% release, 200u/1hr at 37°C	<3% release, 200u/1hr at 37°C
Terminal Deoxynucleotidyl Transferase	15–30	N/A		Gel analysis, absence of visible nicking or cutting, 5u/1hr at 37°C	<1% release, 25u/1hr at 37°C	<3% release, 25u/1hr at 37°C

N/A – Not Applicable

Special Assays: Performance Testing

[1]T4 DNA Polymerase: Free of endonuclease on single-strand circular M13mp18 DNA.

[2]*Taq* DNA Polymerase: Free of contaminating exonucleases on lambda/*Hin*d III digested DNA, 5u/8hr at 45˚C and 8hr at 72˚C.

[3]*Pfu* DNA Polymerase: Free of contaminating exonucleases on lambda/*Hin*d III digested DNA, 5u/8hr at 45˚C and 8hr at 72˚C. Free of endonucleases on lambda DNA, 12.5u/8hr at 45˚C and 8hr at 72˚C.

[4]RNA Polymerases: Using a transcription plasmid with the appropriate promoter, an incorporation assay with limiting CTP must result in >65% incorporation of label using 20 units of polymerase. The denatured transcription products are analyzed on agarose gels.

[5]AMV RT: First-strand cDNA of 1.2kb is synthesized and analyzed by autoradiography.

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Table 11. Composition of Promega Restriction Enzyme Buffers (at 10X) .

Promega's Restriction Enzyme Reaction Buffers are provided as 10X stocks.

Buffer	pH (at 37°C)	Tris-HCl (mM)	$MgCl_2$ (mM)	NaCl (M)	KCl (M)	DTT (mM)
A	7.5	60	60	0.06	–	10
B	7.5	60	60	0.5	–	10
C	7.9	100	100	0.5	–	10
D	7.9	60	60	1.5	–	10
E	7.5	60	60	1.0	–	10
F	8.5	100	100	1.0	–	100
G	8.2	500	50	–	–	–
H	7.5	900	100	0.5	–	–
J	7.5	100	70	–	0.5	10
K	7.4	100	100	–	1.5	–
L	9.0	100	30	1.0	–	–

MULTI-CORE™ Buffer (10X) = .25M Tris-Acetate (pH 7.8 at 25°C), 1M potassium acetate, 100mM magnesium acetate, 10mM DTT.

Notes: 1. For each 10°C rise in temperature between 0°C and 25°C, the pH of Tris buffers decreases 0.31 pH units.
2. For each 10°C rise in temperature between 25°C and 37°C, the pH of Tris buffers decreases 0.25 pH units.

Table 12. Polymerase Reaction Buffer Formulations.

Enzyme	Dilution Factor	Formulation
DNA Polymerase I Klenow Klenow Exo Minus	10X	500mM Tris-HCl (pH 7.2 at 25˚C), 100mM $MgSO_4$, 1mM DTT
Taq DNA Polymerase[1,2 (d)] *Tth* DNA Polymerase[1,3(d,e)] *Tli* DNA Polymerase[1(d)]	10X	500mM KCl, 100mM Tris-HCl (pH 9.0 at 25˚C) and 1.0% Triton® X-100
Tfl DNA Polymerase[4 (d)]	10X	200mM Tris-Acetate (pH 9.0 at 25˚C), 100mM ammonium sulfate, 750mM potassium acetate, 0.5% Tween® 20
Pfu DNA Polymerase [(d)]	10X	200mM Tris-HCl (pH 8.8 at 25˚C), 100mM KCl, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1% Triton® X-100 and 1mg/ml nuclease-free BSA
T7 RNA Polymerase[5] T3 RNA Polymerase[5] SP6 RNA Polymerase[5]	5X	200mM Tris-HCl (pH 7.9), 30mM $MgCl_2$, 10mM spermidine and 50mM NaCl
AMV Reverse Transcriptase	5X	250mM Tris-HCl (pH 8.3 at 25˚C), 250mM KCl, 50mM $MgCl_2$, 2.5mM spermidine, 50mM DTT
M-MLV Reverse Transcriptase M-MLV Reverse Transcriptase, RNase H Minus	5X	250mM Tris-HCl (pH 8.3 at 25˚C), 375mM KCl, 15mM $MgCl_2$, 50mM DTT
Terminal Deoxynucleotidyl Transferase (TdT)	5X	500mM cacodylate buffer (pH 6.8), 5mM $CoCl_2$, 0.5mM DTT

[1]Also provided with 25mM $MgCl_2$.
[2]Also available with a similar buffer containing 15mM $MgCl_2$.
[3]*Tth* DNA Polymerase is also provided with a 10X Reverse Transcription Buffer [100mM Tris-HCl (pH 8.3), 900mM KCl] and a 10X Chelate Buffer [100mM Tris-HCl (pH 8.3), 1mM KCl, 7.5mM EGTA, 0.5% Tween® 20, 50% glycerol].
[4]Also provided with 25mM $MgSO_4$.
[5]Also provided with 100mM DTT.

Glossary

Accuracy: The average number of nucleotides the polymerase incorporates before making an error.
Activated calf thymus DNA: Nicked and gapped dsDNA prepared by treatment with DNase I; a substrate for many DNA polymerases.
BuAdATP: 2-(p-n-butylanilino)-2′-deoxyadenosine 5′-triphosphate. Also known as butylanilino dATP.
BuPdGTP: N^2-(p-n-butylphenyl)-2′-deoxyguanosine 5-triphosphate. Also known as butylphenyl dGTP.
dNTP: Deoxyribonucleotide 5′-triphosphate.
Distributive: Enzyme dissociates from template after a single nucleotide addition.
ds: Double-stranded.
DTT: Dithiothreitol.
Error rate: The mutation rate per base pair duplicated; accuracy is the inverse of error rate.
Fidelity: A polymerase's ability to faithfully replicate a DNA molecule.
I_{50}: Concentration of a substance that causes 50% inhibition in an enzyme's activity.
ID_{min}: The minimum inhibitor concentration causing inhibition of enzyme activity.
ID_{50}: See I_{50}.
kb: Kilobase or kilobase pairs.
k_{cat}: Maximum number of substrate molecules converted to products per active site per unit time.
kDa: KiloDalton.
K_m: The Michaelis constant is the concentration of substrate at which an enzyme can convert to product at half its maximal rate.
Misinsertion: A rare event in which a polymerase adds a nucleotide to the 3′-terminus of a primer that does not conform to AT and GC base pairing with the template. Misinsertion usually leads to a mutation after duplication of the cDNA.
Mismatch: When bases on opposite strands of dsDNA are in the same position but do not conform to AT and GC base pairs.
Mesophilic: Optimal activity at temperatures around that of a warm blooded animal (i.e., 37˚C) but includes the temperature range 15–45˚C .
Nick translation: Polymerization at a DNA nick with concurrent hydrolysis of the 5′-end at the nick, so as to translate (move) the nick without a change in mass of the DNA.
PCR(g): Polymerase Chain Reaction.
PEG: Polyethylene glycol.
PP_i: Inorganic pyrophosphate.
Primer: Short ssDNA that binds to a longer, complementary template strand; the 3′-terminus serves as the site of polymerization.
Processive: Repetitive enzyme action without dissociation between steps; nondistributive.
Promoter: DNA sequence for the initiation of RNA transcription by RNA polymerase.
Read-through transcript: Describes either those RNA transcripts not properly terminated by RNA polymerase or RNA transcripts that proceed from the promoter to the end of a linear template.
ss: Single-stranded.
Strand displacement: Ability of a polymerase to replicate at a nick by melting the dsDNA and displacing the 5′-chain as it polymerizes a new copy of the displaced ssDNA.
5′-RACE: Rapid amplification of cDNA 5′ ends.
rNTP: Ribonucleotide 5′-triphosphate.
TAE: Tris Acetate EDTA .
TCA: Trichloroacetic acid.
Template: Complementary DNA chain providing the sequence that directs nucleotide additions to the primer strand.
Terminator: DNA sequence for the termination of RNA transcription by RNA polymerase.
Thermostable: The ability to withstand prolonged exposure to an elevated temperature, e.g., 95˚C.
Thermophilic: Optimal activity at elevated temperatures, usually above 50–60˚C.
TUNEL Assay: TdT-mediated dUTP Nick End Labeling Assay.
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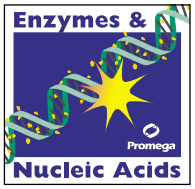

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