



**Just fine Molecular Biology**

## Usage Guide for Tth DNA polymerase

### I. Description

Tth DNA Polymerase is a thermostable enzyme of 94kDa isolated from *Thermus thermophilus* HB-8 (1). Tth DNA Polymerase catalyzes the DNA-dependent polymerization of nucleotides into duplex DNA in the 5'→3' direction in the presence of magnesium chloride. The enzyme also catalyzes the polymerization of DNA using an RNA template in the presence of manganese chloride (1,2). The ability of Tth DNA Polymerase to reverse transcribe at elevated temperatures minimizes the problems encountered with strong secondary structures in RNA since they are unstable at higher reaction temperatures. Higher temperatures also result in increased specificity of primer hybridization and extension. Applications of Tth DNA Polymerase include:

- Primer extension.
- Reverse transcription. cDNA
- z synthesis.
- z

### II. Standard Applications

#### Reagents to Be Supplied by the User

(Solution compositions are provided in Section III.)

- z [ $\alpha$ -<sup>32</sup>P]dCTP (>400Ci/mmol; optional)
- z dNTP mix
- z downstream primer
- z upstream primer
- z Nuclease-Free Water
- z EGTA, 0.5M (optional)

#### A. First-Strand Synthesis of cDNA

Successful cDNA synthesis is dependent on the integrity of the mRNA used as the template. Procedures for creating an RNase-free laboratory have been described (3). We also recommend the use of Recombinant RNasin® Ribonuclease Inhibitor for the protection of mRNA samples.

First-strand cDNA synthesis relies on the RNA-dependent DNA polymerase activity of Tth DNA Polymerase in the presence of manganese. The following procedure requires 5pmol (2 x 2.5pmol) of starting mRNA for first-strand synthesis. Total RNA (1-1,000ng) may be used as template. The amount of total RNA to use is greatly dependent upon the relative abundance of the target message. Since reaction conditions are template-dependent perform pilot reactions to optimize conditions for each template.

Optimize the primer:template ratio for each template. Mix in a sterile, RNase-free microcentrifuge tube

RTx10 buffer

MnCl <sub>2</sub> , 10mM Solution	2μl
dNTP mix, 2mM each	2μl
[ $\alpha$ - <sup>32</sup> P]dCTP (>400Ci/mmol)	2μl
downstream primer	2-5μCi
Tth DNA Polymerase	*15pmol
mRNA template	4-6 units
Nuclease-Free Water to final volume	<u>2.5pmol</u> 20μl

Note: Since the entire 20μl first-strand reaction must be used for second-strand synthesis, prepare a duplicate reaction for quantitating first-strand synthesis by TCA precipitation or alkaline agarose gel analysis (4).

Mix gently by flicking the tube and incubate at 70°C for 20 minutes.

Note: Oligo(dT) or random hexamer primers will not anneal to the RNA template at 70°C. Therefore, we recommend the use of gene-specific primers designed with a melting temperature high enough to anneal at 70°C.

If second-strand synthesis is to be performed, place the first-strand reaction on ice and proceed to Section II.B. Otherwise, add EGTA to a final concentration of 20mM to terminate the reaction and place the sample on ice.

### B. Second-Strand Synthesis of cDNA

Second-strand cDNA synthesis relies on the DNA-dependent DNA polymerase activity of Tth DNA Polymerase, which requires the removal of manganese and the addition of magnesium. Removal of manganese is accomplished by chelation with EGTA, which is present in the Chelate 10X Buffer.

1. Prepare the following second-strand reaction mix:
 

upstream primer	*15pmol
Chelate 10X Buffer	8μl
MgCl <sub>2</sub> , 25mM Solution	8μl
Nuclease-Free Water to final volume	80μl
2. Centrifuge the first-strand reaction from Section II.A in a microcentrifuge for 5 seconds to collect any condensate.
3. Add 80μl of the second-strand reaction mix to 20μl of the first-strand reaction. Mix gently by flicking the tube. Heat at 95°C for 5 minutes to denature the RNA:DNA hybrids.
4. Incubate the reactions at 70°C for 20 minutes. Alternatively, second-strand synthesis can be accomplished by a cycled reaction.
5. The results of the second-strand reaction can be quantitated by measuring the incorporation of [ $\alpha$ -<sup>32</sup>P]dCTP by TCA precipitation or by agarose gel analysis (4). Purify the reaction products using the PCR DNA Purification kits.

### III. Composition of Buffers and Solutions

#### Chelate 10X Buffer

100mM	Tris-HCl (pH 8.3)
1M	KCl
7.5mM	EGTA
0.5%	Tween® 20
50%	glycerol

#### dNTP mix

2mM	each of dATP, dCTP, dGTP and dTTP in water
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#### EGTA, 0.5M

Dissolve 19.2g of EGTA in 70ml of deionized water, adjust the pH to 8.0 with 10N NaOH and add deionized water to 100ml final volume. Filter-sterilize (0.22μm) and store at room temperature.

#### RT 10X Buffer

100mM	Tris-HCl (pH 8.3 at 25°C)
900mM	KCl

#### Thermophilic DNA Polymerase 10X Buffer

500mM	KCl
100mM	Tris-HCl (pH 9.0 at 25°C)
1%	Triton® X-100

Buffer is optimized for use with the dNTP mix (0.2mM eachdNTP).

### IV. References

1. Rüttimann, C. et al. (1985) Eur. J. Biochem. 149, 41-6.
2. Myers, T.W. and Gelfand, D.H. (1991) Biochemistry 30, 7661-6.
3. Blumberg, D.D. (1987) Meth. Enzymol. 152, 20-4.
4. Protocols and Applications Guide, Third Edition (1996) Promega Corporation. Wizard
5. PCR Preps DNA Purification System Technical Bulletin #TB118, Promega Corporation.

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