

# Genaxxon BioScience

## *Tth* DNA Polymerase

Isolated from eubacterium *Thermus thermophilus*, strain HB8

Deoxynucleoside-triphosphate: DNA deoxynucleotidyl-transferase (DNA-directed); EC 2.7.7.7

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Product	Cat#	Package size
Tth DNA Polymerase (H minus)	M3005.0500	500 units
Tth DNA Polymerase (H minus)	M3005.2500	5 x 500 units

### Description

*Tth* DNA Polymerase is a thermostable enzyme of approximately 94kDa isolated from eubacterium *Thermus thermophilus* strain HB8. This enzyme replicates DNA at 74°C, and reveals RNA-dependent DNA-polymerase activity in the presence of Mn<sup>2+</sup> ions without RNase H activity (6.). The error rate of *Tth* DNA polymerase increase in the present of Mn<sup>2+</sup> ions (5, 8, 12 and 13).

*Tth* DNA polymerase can reverse transcribe and amplify fragments up to 2-3 kb. however, the fragment should be ideally smaller 1 kb. The error rate is 3.0 x 10<sup>-5</sup> in PCR reaction(8.). For effective reverse transcription of template RNA with *Tth* DNA polymerase the RNA concentration should be higher compared to MMuLV or AMV. Although *Tth* DNA polymerase adds 3'da overhangs, it is not recommended for PCR product cloning because the high error rate.

### Applications

**Incorporation of modified dNTPs:** *Tth* DNA polymerase accepts modified dNTPs and can therefore be used to label DNA fragments with dNTPs labeled with digoxigenin, biotin or fluorescein.

**RT-PCR:** 1-step and 2-step RT-PCR (5) with *Tth*-polymerase. One step means reverse transcription and amplification is done in one tube. Two steps means that the reverse transcription reaction (first strand synthesis of the cDNA) is done in one tube (with Mn<sup>2+</sup> in the reaction buffer), followed by the PCR reaction in a second tube (with a PCR buffer (Mg<sup>2+</sup>)). In both cases, the concentration of RNA template for effective reverse transcription with *Tth* DNA polymerase should be higher than that for reaction directed by Reverse Transcriptases (M-MuLV, AMV).

**Presence of phenol:** *Tth* DNA polymerase displays the unique property of maintaining both DNA- and RNA-dependent DNA polymerase activities in the presence of 2%-5% (v/v) of phenol-saturated PBS buffer. *Tth* DNA polymerase mediated reverse transcriptase activity is unaffected by phenol-saturated phosphate-buffered saline concentrations as high as 15% (v/v). In contrast, Taq DNA Polymerase is inactive under these conditions. The ability to function in the presence of phenol can greatly simplify reverse transcriptase, PCR and reverse transcription-PCR protocols since the phenol-saturated aqueous phase of a phenol partition can be added directly to the reaction mixtures (9.).

### Supplied material

- Tth Polymerase (5 units/μL)
- 5X RT-PCR buffer without MgCl<sub>2</sub> for "one step RT-PCR"
- 10X RT buffer for Reverse Transcription
- 10X Tth-PCR buffer with 15 mM MgCl<sub>2</sub>
- Magnesium stock solution (25 mM)
- Manganese stock solution (100 mM)

### Storage and Dilution Buffer

10 mM Tris-HCl (pH 7.5), 300 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Nonidet P40 and 0.5% Tween 20.

### One-step RT-PCR Reaction Buffer (5X)

250 mM bicine/KOH, pH 8.2 (25°C), 575 mM K-acetate, 40% glycerol (v/v)

### Reverse Transcription Reaction Buffer (10X)

100 mM Tris-HCl, pH 8.9 (25°C), 900 mM KCl

### Tth Polymerase PCR Reaction Buffer (10X)

100 mM Tris-HCl, pH 8.9 (25°C), 1 mM KCl, 500 μg/mL BSA, 0.5% Tween 20, 15 mM MgCl<sub>2</sub>

- Tth concentration:** 5 units/ $\mu$ L
- Mg<sup>2+</sup> concentration:** a final concentration of 3 - 6 mM Mg<sup>2+</sup> is recommended.
- Mn<sup>2+</sup> concentration:** a final concentration of 1-2 mM MnCl<sub>2</sub> for RNA-dependent cDNA synthesis is recommended.

### Unit definition

One unit is defined as the amount of enzyme that incorporates 10 nmoles of dNTP's into acid-insoluble fraction in 30 minutes at 74°C under the standard assay conditions: 25 mM TAPS (tris-(hydroxymethyl)-methyl-amino-propansulfonic acid, sodium salt) pH 9.3 (at 25°C), 50 mM KCl, 2 mM 50 mM MgCl<sub>2</sub>, 1 mM  $\beta$ -mercapto-ethanol, 200  $\mu$ M each dATP, dGTP, dTTP, 100  $\mu$ M dCTP (a mix of cold and P32-labelled), 12.5  $\mu$ g activated salmon sperm DNA, in a final volume of 50  $\mu$ L.

### Stability

The enzyme is stable for more than 12 months if stored at -20°C.  
 The enzyme is also stable for some days at temperatures above 20°C.

### Notes:

- Drops should be collected by centrifugation and 50  $\mu$ L of mineral oil should be layered upon the reaction mixture.
- Program the thermal cycler according to the manufacturer's instructions. Each programme should start with an initial heat incubation step at 94°C for 3-5 minutes!
- **Recommended elongation time is 1 minute per 1kb of target!**
- For maximum yield and specificity, temperatures (annealing) and cycling times should be optimised for each new template target or primer pair.

## General Note for RNA preparation

Successful RT - PCR depends on the quality of the RNA. Use highest purity of RNA ( $A_{260}/A_{280}$  ratio of 1.7 or higher). RNA should be DNA free. Total RNA, messenger RNA or viral RNA can be used. The quality of template RNA can be assessed using a positive control primer pair for a housekeeping gene ( $\beta$ -actin, GAPDH). The 16S rRNA gene from various bacterial cultures was amplified by the polymerase chain reaction without DNA purification, and sequenced directly by using a laser fluorescent DNA sequencer and *Tth* polymerase with a cycle sequencing protocol. The described procedures provide almost complete 16S rDNA sequence data within a couple of days and facilitate systematic studies (10).

### Reference:

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## Protocols

### Part A: Reverse transcription polymerase chain reaction (RT-PCR)

The use of *Tth*-DNA polymerase, which is also a reverse transcriptase in the presence of manganese and accepts both RNA and DNA as template, enables the whole reaction to be performed as a "one-step/one-tube" RT-PCR analysis (6). This experimental approach includes the amplification of fragments only to a maximum of 1 kb and a relatively high error rate for DNA polymerase as a result of the manganese ion concentration (7).

This one-step procedure minimizes the risk of cross-contamination of the sample.

Reverse transcription can be performed at 60° C minimizing problems from RNA secondary structure and high G/C content!

#### 1. One step RT - PCR (One step reaction eliminates the risk of cross contaminations associated with two step RT-PCR.)

"One-step" RT-PCR means reverse transcription and amplification is mediated by one enzyme in one tube.

The "one enzyme/one step" *Tth* DNA polymerase assay uses bicine buffers containing Mn<sup>2+</sup> ions that are compatible with both RT and subsequent PCR (6, 11). NOTE: For RT-PCR amplification (reverse transcription and amplification in one step), the concentration of Mn<sup>2+</sup> needs to be determined by testing Mn<sup>2+</sup> concentrations from 1- 4 mM for each reaction.

#### Setting up "one-step" RT-PCR Reaction

"One-step" RT-PCR Reaction Buffer (5X):	250 mM bicine/KOH, pH 8.2 (25° C); 575 mM K-acetate, 40% glycerol (v/v).
MnCl <sub>2</sub> :	2.5 mM (test 1 - 4 mM)
<i>Tth</i> DNA polymerase:	5 units
Template:	up to 1 µg (dilute 1:10 and analyse 1ng, 10 ng, 100 ng and 1µg)
Primers (forward and reverse):	450 nM each
dNTPs:	200-300 µM
sterile Water:	up to 20 µL

Table 1: Possible 1-tube RT-PCR Cycle Protocol

Step	Time	Temperature	Comments
<b>RT-Reaction</b>			
Initial Denaturation	1-2 minutes	94° C	
RT-Reaction	30 minutes	60° - 70° C *	
<b>PCR-Reaction</b>			
Initial Denaturation	0.5 - 1 min.	94° C	
<b>Cycling (1-10 step)</b>			
Denaturation	0.5 - 1 min.	94° C	
Annealing	30-60 sec.	50-70° C *	Approximately 5° C to 8° C below T <sub>m</sub> of primers
Elongation	45-90 sec.	60-70° C	
<b>Cycling (11-&gt;30 step) **</b>			
Denaturation	30 sec.	94° C	
Annealing	30 sec.	50-70° C *	Approximately 5° C to 8° C below T <sub>m</sub> of primers
Elongation	45 sec.	60-70° C	
Final elongation	7 minutes	72° C	

\*depends on your primers, 70 - 75° C is the optimal reaction temperature for *Tth* DNA polymerase. *Tth* DNA polymerase is resistant to prolonged incubations (20 min Half - Life time at 95° C) at high temperatures (94° C) and can therefore be used for PCR.

\*\* Number of cycles ranges from 30 - 50 in the literature. If the template is limited, increased cycle numbers may result in nonspecific product yield.

## Protocols

### Part B: Two-step RT-PCR

In the two-step protocol the reversed transcription abilities of the *Tth* polymerase are used to perform a "standard" reverse transcription reaction for synthesis of cDNA. For this step the reverse primer is used together with the special RT-buffer system plus  $Mn^{2+}$ . The RT reaction is followed by a PCR reaction by adding PCR buffer,  $MgCl_2$  and forward primer.

As the error rate of *Tth* is increased at higher  $Mn^{2+}$  concentration (5), a two-step reaction is recommended if PCR products shall be used for subsequent cloning and/or sequencing experiments.

#### 2. Two step RT - PCR:

##### Reverse Transcription Reaction:

10X Reverse transcription buffer:	100 mM Tris-HCl, pH 8.9 (25°C), 900 mM KCl
$MnCl_2$ :	1 - 2 mM final concentration.
dNTPs:	each 200 $\mu$ M final concentration
reverse primer:	750 nM
template RNA:	200 ng
<i>Tth</i> DNA polymerase:	5 units
Sterile Water:	up to 20 $\mu$ L

##### PCR reaction:

10X PCR Reaction Buffer:	0.8 $\mu$ L of 10X Reaction buffer
EGTA:	0.75 mM final concentration (10 $\mu$ L of a 7.5 mM EGTA *** solution).
Forward primer:	750 nM
Sterile H <sub>2</sub> O:	up to 80 $\mu$ L

Table 2: Possible 2-step Protocol

Step	Time	Temperature	Comments
Prepare RT reaction on ice. Final volume = 20 $\mu$ L.			
<b>RT-Reaction</b>			
Initial Denaturation	1 - 2 minutes	94°C	
RT-Reaction	30 minutes	60° - 70°C *	
The RT : PCR reaction volume ratio has to be <b>NOT</b> higher than 20% (For example: 20 $\mu$ L RT up to 100 $\mu$ L PCR). PCR reaction can made up on ice or at room temperature. Mix well, centrifuge and place the PCR reaction on a thermocycler.			
<b>PCR-Reaction</b>			
Initial Denaturation	2 minutes	94°C	
<b>Cycling (1-10 step)</b>			
Denaturation	0.5 - 1 min.	94°C	
Annealing	30-60 sec.	50-70°C *	Approximately 5°C to 8°C below T <sub>m</sub> of primers
Elongation	45-90 sec.	60-70°C	
<b>Cycling (11-&gt;30 step) **</b>			
Denaturation	30 sec.	94°C	
Annealing	30 sec.	50-70°C *	Approximately 5°C to 8°C below T <sub>m</sub> of primers
Elongation	45 sec.	60-70°C	
<b>Final elongation</b>	7 minutes	72°C	

\*depends on your primers, 70 - 75° C is the optimal reaction temperature for *Tth* DNA polymerase. *Tth* DNA polymerase is resistant to prolonged incubations (20 min Half - Life time at 95° C) at high temperatures (94° C) and can therefore be used for PCR.

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\*\*\* For a 0.5 M EGTA stock: dissolve 19.2 g EGTA in 70 ml deionized water, adjust pH 8.0 with NaOH (10 M). Add deionized water to 100 mL final volume. Filter sterilize with 0.22  $\mu$ m and store at room temperature).

## Protocols

### Part C: "Standard PCR

*Tth*-polymerase can be used like *Taq*-polymerase for a standard PCR. As *Tth* is more unspecific for nucleotides than the other DNA-polymerases, *Tth* can be ideally used in the case of incorporation of modified nucleotides (digoxigenin, biotin or fluorescein). In all cases MgCl<sub>2</sub>-concentration as well as the enzyme concentration in the PCR reaction has to be optimized. Typical MgCl<sub>2</sub> concentrations are in the range of 1 - 6 mM. The supplied standard concentration is 1.5 mM MgCl<sub>2</sub>. Optimal enzyme concentrations are in the range of 1.0 - 5.0 units. The standard concentration is 2.5 units per reaction.

#### PCR reaction set-up:

Table 3: PCR reaction components (50 µL reaction)

Components	Quantities
Template DNA	1 ng - 10 ng plasmid DNA or 5 ng - 500 ng genomic DNA
Nucleotides	1 µL (10 mM) each of dATP, dCTP, dGTP, dTTP
10X amplification buffer (25 mM MgCl <sub>2</sub> )	5 µL 1.5 µL (if no complete buffer is used)
primer 1:	4-7 µL of 3 µM solution (10 - 20 pmole absolute)
primer 2:	4-7 µL of 3 µM solution (10 - 20 pmole absolute)
sterile, bidistilled water	up to 50 µL
<i>Tth</i> DNA-Polymerase	0.25 - 1.0 µL (1.25 - 5 units)

Table 4: MgCl<sub>2</sub> concentration in a 50 µL reaction

Final MgCl <sub>2</sub> conc. in reaction (mM)	1.5	2.0	2.5	3.0	3.5	4.0	4.5
Additional volume of 25 mM MgCl <sub>2</sub> per reaction (µL)	0	1	2	3	4	5	6

Table 5: Possible PCR-Protocol

Step	Time	Temperature	Comments
<b>Initial Denaturation</b>	2-5 minutes	94°C	
<b>Cycling (1-10 step)</b>			
Denaturation	0.5 - 1 min.	94°C	
Annealing	30-60 sec.	50-70°C *	Approximately 5°C to 8°C below T <sub>m</sub> of primers
Elongation	45-90 sec.	60-70°C	
<b>Cycling (11-&gt;30 step) **</b>			
Denaturation	30 sec.	94°C	
Annealing	30 sec.	50-70°C *	Approximately 5°C to 8°C below T <sub>m</sub> of primers
Elongation	45 sec.	60-70°C	
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