

fon:
 +49 (0)731 - 3608 123
 fax:
 +49 (0)731 - 3608 962
 eMail:
info@genaxxon.com
 internet:
www.genaxxon.com

Tth DNA Polymerase

Isolated from eubacterium *Thermus thermophilus*, strain HB8
 Deoxynucleoside-triphosphate: DNA deoxynucleotidyl-transferase (DNA-directed); EC 2.7.7.7

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 Mn²⁺ ions without RNase H activity (6.). The error rate of *Tth* DNA polymerase increase in the present of Mn²⁺ 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 1kb. The error rate is 3.0 x 10⁻⁵ 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-step** (optional) means that the reverse transcription reaction (first strand synthesis of the cDNA) is done in one tube, followed by the PCR reaction in a second tube (with a PCR buffer). 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 MgCl₂ for "one step RT-PCR"
- 10X *Tth*-PCR buffer with 15mM MgCl₂
- Manganese stock solution (25mM)

Storage and Dilution Buffer

10mM Tris-HCl (pH7.5), 300mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 0.5% Nonidet P40 and 0.5% Tween 20.

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

250mM bicine/KOH, pH8.2 (25°C), 575mM K-acetate, 40% glycerol (v/v)

Tth Polymerase PCR Reaction Buffer (10X)

100mM Tris-HCl, pH8.9 (25°C), 500μg/mL BSA, 0.5% Tween 20, 15mM MgSO₄, 800mM (NH₄)₂SO₄

Mn²⁺ concentration: a final concentration of 1-2 mM MnCl₂ for RNA-dependent cDNA synthesis is recommended.

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internet:
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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: 25mM TAPS (tris-(hydroxymethyl)-methyl-amino-propansulfonic acid, sodium salt) pH9.3 (at 25°C), 50mM KCl, 50mM MgCl₂, 1mM β-mercapto-ethanol, 200μM each dATP, dGTP, dTTP, 100μM dCTP (a mix of cold and P32-labelled), 12.5μg activated salmon sperm DNA, in a final volume of 50μL.

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 50μL 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 (β-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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Protocol

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^{2+} ions that are compatible with both RT and subsequent PCR (6, 11). **NOTE:** For RT-PCR amplification (reverse transcription and amplification in one step), it might be necessary to determine concentration of Mn^{2+} first by testing Mn^{2+} concentrations from 1- 4mM for each reaction.

Setting up “one-step” RT-PCR Reaction

Prepare two mastermixes 25µL each before starting.

Table 1: Mix I for “one-step” RT-PCR

Component	Volume	Final concentration
dNTP-Mix (10mM each)	1.5µL	300µM
Forward primer	Var.	450µM
Reverse primer	Var.	450µM
Template RNA	Var.	Up to 1µg (in steps of 1ng; 10ng; 100ng; 1µg)
Sterile water	Up to 25µL	
Total	25µL	

Table 2: Mix II for “one-step” RT-PCR

Component	Volume	Final concentration
5X RT-PCR buffer	10µL	1-time
Mn(OAc) ₂	5µL	2.5mM
<i>Tth</i> DNA Polymerase	0.5-1.0µL	2.5 - 5 units
Sterile water	Var.	
Total	25µL	

NOTE: Combine Mix I and Mix II on ice and gently vortex the final mixture in a PCR-tube. Place tube in Thermal cycler and run below mentioned (page 4) cycling programme immediately.

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

Step	Time	Temperature	Comments
RT-Reaction			
Initial Denaturation	1-3 minutes	95 °C	
RT-Reaction*	30 minutes	60° - 70 °C *	
PCR-Reaction			
Initial Denaturation	0.5 - 1 min.	95 °C	
Cycling (1-10 step)			
Denaturation	30-60 sec.	94 °C	
Annealing	30-60 sec.	50-70 °C	Approximately 5 °C to 8 °C below Tm of primers
Elongation	45-90 sec.	72-74 °C	
Cycling (11->30 step) **			
Denaturation	30 sec.	94 °C	
Annealing	30 sec.	50-70 °C	Approximately 5 °C to 8 °C below Tm of primers
Elongation	45 sec.	72-74 °C	
Final elongation	7 minutes	72-74 °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.

Part B: Two-step RT-PCR (optional - material not supplied)

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:

Table 3: “two-step” RT-PCR reaction mixture - Components for RT-reaction

Component	Volume	Final concentration
10X Rev. Transcription buffer	2 μ L	1-time
Mn(OAc) ₂	2 μ L	0.9mM
dNTP-Mix (10mM each)	0.4 μ L	200 μ M
Reverse primer	Var.	450 μ M
Template RNA	Var.	up to 200ng
Tth-Polymerase	0.8 μ L	4 units
Sterile water	up to 20 μ L	
Total	20μL	For RT-reaction denature at 95 °C for 1-3 minutes, then incubate at 60-70 °C for 10-30 min.

2. Two step RT - PCR:

Table 4: “two-step” RT-PCR reaction mixture - PCR-reaction

Component	Volume	Final concentration
10X PCR reaction buffer	8 μ L	1-time
dNTP-Mix (10mM each)	0.4 μ L	200 μ M
Reverse primer	Var.	450 μ M
Forward primer	Var.	150nM
EGTA (7.5mM)*	10 μ L	0.75nM
Tth-Polymerase	0.8 μ L	4 units
Sterile water	up to 80 μ L	
Total	80μL	Gently vortex and add the 80 μ L PCR mastermix to the 20 μ L rev. transcriptase mixture after reverse transcriptase reaction. Total PCR volume: 100μL Continue cycling immediately!!!

*** For a 0.5M EGTA stock: dissolve 19.2g EGTA in 70mL deionized water, adjust pH8.0 with NaOH (10M). Add deionized water to 100mL final volume. Filter sterile with 0.22 μ m and store at room temperature

Table 5: Two-step PCR Protocol

Step	Time	Temperature	Comments
Initial Denaturation	1-2 minutes	95 °C	
Cycling (1-10 step)			
Denaturation	30-60 sec.	95 °C	
Annealing	30-60 sec.	50-70 °C	Approximately 5 °C to 8 °C below T _m of primers
Elongation	45-90 sec.	72-74 °C	
Cycling (11->30 step)**			
Denaturation	30 sec.	94 °C	
Annealing	30 sec.	50-70 °C	Approximately 5 °C to 8 °C below T _m of primers
Elongation	45 sec.	72-74 °C	
Final elongation	7 minutes	72-74 °C	

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

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 $MgCl_2$ -concentration as well as the enzyme concentration in the PCR reaction has to be optimized. Typical $MgCl_2$ concentrations are in the range of 1 - 6mM. The supplied standard concentration is 1.5mM $MgCl_2$. Optimal enzyme concentrations are in the range of 1.0 - 5.0 units. The standard concentration is 2.5 units per reaction.

Setting up PCR Reaction mixtures

Prepare two mastermixes 50 μ L each before starting.

Table 6: Mix I for standard PCR

Component	Volume	Final concentration
dNTP-Mix (10mM each)	0.4 μ L	200 μ M
Forward primer	Var.	400 μ M
Reverse primer	Var.	400 μ M
Template DNA	Var.	up to 0.5 μ g
Sterile water	up to 50 μ L	
Total	50μL	

Table 7: Mix I for standard PCR

Component	Volume	Final concentration
10X PCR-buffer	10 μ L	1-time
<i>Tth</i> DNA Polymerase	0.5-1.0 μ L	2.5 - 5 units
Sterile water	up to 50 μ L	
Total	50μL	

NOTE: Combine Mix I and Mix II on ice and gently vortex the final mixture in a PCR-tube. Place tube in Thermal cycler and run below mentioned cycling programme immediately.

Table 8: PCR Cycling Protocol

Step	Time	Temperature	Comments
PCR-Reaction			
Initial Denaturation	1 - 2 min.	95 °C	
Cycling (1-10 step)			
Denaturation	30-60 sec.	94 °C	Approximately 5 °C to 8 °C below T_m of primers
Annealing	30-60 sec.	50-70 °C	
Elongation	45-90 sec.	72-74 °C	
Cycling (11->30 step) *			
Denaturation	30 sec.	94 °C	Approximately 5 °C to 8 °C below T_m of primers
Annealing	30 sec.	50-70 °C	
Elongation	45 sec.	72-74 °C	
Final elongation	7 minutes	72-74 °C	

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