EURx® MOLECULAR BIOLOGY PRODUCTS

T4 DNA Polymerase

(bacteriophage T4 of Escherichia coli)

Cat. No.	size
E1100-01	200 units
E1100-02	1 000 units

Unit Definition: One unit is the amount of enzyme that catalyzes the incorporation of 10 nmoles of total nucleotide into acid-insoluble product in 30 min at 37°C.

Storage Conditions: Store at -20°C.

Enzyme Properties:

Feature	Value	Remarks
Template	ssDNA	Poor activity on dsDNA
5'-3' exonuclease	-	
3'-5' exonuclease	+	strong
Proofreading	+	strong
Strand displacement	-	
Error rate	$<10^{-7}$	dNTP conc. dependent
pH range / pH optimum	7 – 9 / ~8	
Temperature range	0 - 42°C	~15 min half life at 42°C
Heat inactivation	75°C, 10 min	
Buffer requirements	6 mM $MgCl_2$, 0 – 0.1 mM salt, 10 mM sulfhydryl reagents	

References:

1. *Goulian, M., Lucas, Z.J. and Kornberg, A. (1968) J. Biol. Chem. 243, 627-638.*
2. *Lehman, I.R. (1981) Enzymes 14, 51-65.*
3. *Tabor, S. and Struhl, K (1989) in Current Protocols in Molecular Biology (Ausubel, F. M., et al., eds) pp. 3.5.10-3.5.12, John Wiley&Sons, New York.*
4. *Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, second edition, pp. 5.44-5.47, Cold Spring Harbour.*
5. *Dale, R., McClure, B. and Houchins, J., (1985) Plasmid 13, 31-40.*
6. *Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) Methods Enzymol.154, 367-382.*
7. *Kunkel, T.A., Loeb, L.A. and Goodman M.F. (1984) J.Biol.Chem 59 (3) 1539-1545*
8. *Aslanidis, C., de Jong, P.J. (1990) Nucleic Acids Research 18 (20) 6069-6074*

T4 DNA Polymerase is an extremely precise, mesophilic DNA polymerase, exhibiting very strong 3'→5' exonuclease activity.

Description:

- Exhibits 5'→3' polymerase and 3'→5' exonuclease activities (1, 2).
- Removes 3 – protruding termini (exonuclease) and fills in 3'– recessed (5'- protruding) ends (DNA polymerase), generating blunt ended DNA. Adds labeled nucleotides to recessed 3'-ends of DNA fragments.
- Polymerase activity requires the presence of a single-stranded DNA template and a primer.
- Exonuclease, stronger than that found in DNA Polymerase I, is more active on single-stranded DNA than on double-stranded DNA.
- Switching between exonuclease and polymerase activity is triggered by dNTP concentrations. At high dNTP concentrations, DNA polymerase activity is preferred. The optimum dNTP concentration for favoring polymerase over exonuclease activity is 100 µM (or, at least, equivalent to nucleotide equivalents of template DNA), but is reduced in labeling experiments to 1-2 µM. Low dNTP concentrations (below 1 µM) favor exonuclease activity.
- Exonuclease activity can be used to remove one or a few nucleotides from 3'-end of double–stranded DNA.
- Extremely precise and accurate DNA polymerase with exceptionally high fidelity (error rate <10E-7). Proofreading activity is dNTP concentration dependent and decreases at extremely high dNTP concentrations (1000 µM). Higher accuracies are obtained with 100, 50 and 20 mM, respectively (6).
- Enzyme suitable for:
  - 3' overhang removal to form blunt ends (3,4),
  - 5' fill-in to form blunt ends (3,4),
  - probe labeling using replacement synthesis (3,4),
  - single strand deletion subcloning (5),
  - second strand synthesis in site-directed mutagenesis (6),
  - Ligation-independent cloning of PCR products (8),
  - Sample preparation for Next Generation Sequencing (NGS).
- Compatible with common restriction enzyme buffers, e.g. EURx L, M, H and A, respectively.
- Ultrapure recombinant enzyme.

Fig 1: Endogenous *cis*-priming: Occasionally the 3'-terminus of template DNA loops back and forms transient base pairs between 3'-terminal nucleotides and positions on the same strand. The loop, normally unstable, is used by T4 DNA polymerase as initiation point for DNA synthesis and is fixed irreversibly with progressing DNA synthesis (1).

Storage Buffer:

20 mM potassium phosphate (pH 6.5), 5 mM dithiothreitol and 50% (v/v) glycerol.

Assay Conditions:

67 mM Tris-HCl (pH 8.8 at 22°C), 6.7 mM $MgCl_2$, 10 mM dithiothreitol, 16.6 mM ammonium sulfate, 6.7 µM EDTA, 20 µg bovine serum albumin, 45 µg activated calf thymus DNA and 0.033 mM each of dCTP, dGTP, dTTP and [α-^{32}P]dATP. Incubation is at 37°C for 30 min in a reaction volume of 100 µl (1).

Quality Control:

All preparations are tested for contaminating endonuclease activity.

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Protocol: T4 DNA Polymerase - Exonuclease Digestion of 3'-Protruding (Sticky) Ends:

3'-exonuclease activity is favored to DNA polymerase activity in the absence of dNTPs.

Component	Volume / reaction	Final concentration
RE digested DNA (3'-protruding ends, e.g.	1 – 2 µg	50 – 100 ng / µl
10 x T4 DNA Polymerase Buffer*	2 µl	1 x
T4 DNA Polymerase	0.5 - 1 µl / reaction	0.25 – 0.5 U / µl
H_2O, DNA and DNase free	@20 µl	~ 55.5 M
Total Reaction Volume	20 µl	

*T4 DNA Polymerase is approx. 50% active in most restriction enzyme buffers and can be added direct to RE reactions, if accompanied by high dNTP concentrations.

Incubate for 15 min at 12°C (or at room temperature for no longer than 5 min).

Following digestion, add 0.5 µl of dNTPs (5 mM each) to the reaction mixture (final concentration 125 µM) to prevent any further exonuclease activity.

Inactivate T4 DNA Polymerase by heating to 65°C for 10 min (or higher, e.g. 5 min at 85°C, if active restriction enzymes remained in the assay). Else purify DNA with EURx PCR/DNA Clean Up Kit (E3520) or by phenol-chloroform extraction. For ligation, purification is not necessarily required, since T4 DNA Ligase is not inhibited by dNTPs and is compatible with most restriction buffers, if 1 mM ATP (final) is added to the reaction.

Note: T4 DNA Polymerase removes single-stranded 3'-protruding ends. Addition of dNTPs establishes a balance between exonuclease and DNA polymerase activities. Since at 37°C 3'-exonuclease activity is approx. threefold higher as compared to DNA polymerase activity (resulting in further net digestion), the reaction is carried out at 12°C, where the difference between both activities is much less pronounced.

Protocol: T4 DNA Polymerase - End-labelling of Blunt or 3'-Recessed Termini:

Component	Volume / reaction	Final concentration
RE digested DNA (3'-protruding ends, e.g. EcoRI or HindIII)	0.1 – 2 µg	5 – 100 ng / µl
10 x T4 DNA Polymerase Buffer*	2 µl	1 x
Three unlabeled dNTPs [5 mM each]	0.5 µl	125 µM
Fourth labeled dNTP [5 mM]	0.25 µl	62.5 µM
T4 DNA Polymerase	0.5 - 1 µl / reaction	0.25 – 0.5 U / µl
H_2O, DNA and DNase free	@20 µl	~ 55.5 M
Total Reaction Volume	20 µl	

Incubate for 5 min at 37°C.

Add 0.25 µl of unlabeled fourth dNTP, then continue incubation for 10 min at 37°C.

Following digestion, add 0.5 µl of dNTPs (5 mM each) to the reaction mixture (final concentration 125 µM) to prevent any further exonuclease activity.

Inactivate T4 DNA Polymerase by heating to 65°C for 10 min. Else purify DNA with EURx PCR/DNA Clean Up Kit (E3520) or by phenol-chloroform extraction.

Note: This will add labels to or in close vicinity to 3'-termini. Additional amounts of 3'-label can be added using Terminal Deoxynucleodityl Transferase (TdT, Cat. No. E1390), which efficiently incorporates various modified or fluorescent labeled nucleotides. For labeling of 5'-termini use T4 Polynucleotide Kinase (Cat. No. E1261) or conduct PCR with 5'-labeled primers.

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Protocol: T4 DNA Polymerase - Filling of 3'-Recessed (5'-Protruding) Termini:

DNA polymerase activity is favored to 3'-exonuclease activity in the presence of high dNTP concentrations.

Component	Volume / reaction	Final concentration
RE digested DNA (3'-protruding ends, e.g.	0.1 – 2 µg	5 – 100 ng / µl
10 x T4 DNA Polymerase	2 µl	1 x
dNTPs [5 mM each]	0.5 µl	125 µM
T4 DNA Polymerase	0.5 - 1 µl /	0.25 – 0.5 U / µl
H_2O, DNA and DNase free	@20 µl	~ 55.5 M
Total Reaction Volume	20 µl	

Incubate for 15 min at 37°C.

Inactivate T4 DNA Polymerase by heating to 65°C for 10 min. Else purify DNA with EURx PCR/DNA Clean Up Kit (E3520) or by phenol-chloroform extraction. For ligation, purification is not necessarily required, since T4 DNA ligase is not inhibited by dNTPs and is compatible with most restriction buffers, if 1 mM ATP (final) is added to the reaction.

Application Note: T4 DNA Polymerase - Ligation independent cloning:

Assay preparation is as described for exonuclease digestion of 3'-protruding (sticky) ends. Additionally, only one dNTP (e.g. dGTP) is added to template DNA in high concentrations (e.g. 500 µM) and all other three dNTPs are entirely omitted from the assay. In the presence of high dGTP concentrations, exonuclease will digest DNA from the 3'-end until occurrence of the first dGMP-residue. At this position, polymerase and exonuclease activities are balanced and exonuclease digestion halts. If the first G position is positioned 12 bp upstream the 3'-end, digestion proceeds to this position and a defined single stranded 12 bp sticky 5'-end sequence stretch is generated (8).

Primers are designed to ensure complementary ends of insert DNA to each respective side of the vectors multiple cloning site. Furthermore, sticky ends of vector and of insert DNA respectively, are not self-complementary to prevent intramolecular self-ligation. If – and only if - annealing of insert to vector DNA occurs, "hybrid products" are formed. “Hybrid products” are circles containing non-covalently bound single insert and vector molecules. These cyclic nucleic acids are attached solely via annealing of their complementary sticky ends and are directly used for transformation without prior ligation. Since non-annealed linear DNA fragments are not transformed efficiently, few background recombinants are generated. A detailed cloning strategy is described in (8).

Protocol: T4 DNA Polymerase - Replacement Synthesis:

For generation of extensively labeled hybridization probes. This protocol switches between 3'-exonuclease and DNA polymerase activity by varying dNTP concentration.

Component	Volume / reaction	Final concetration
RE digested DNA (3'-protruding ends, e.g. EcoRI or HindIII)	0.1 – 0.5 µg	5 – 25 ng / µl
10 x T4 DNA Polymerase Buffer*	2 µl	1 x
H_2O, DNA and DNase free	@20 µl**	~ 55.5 M
Total Reaction Volume	20 µl	

**after addition of enzyme.

Exonuclease step: Add an appropriate amount of T4 DNA Polymerase. For a ratio of 0.62 U enzyme per µg DNA, av. 10 nt per min are removed from 3'-termini. For ratios of 1.25, 1.75 and 2.50, respectively, 20, 30 and 40 nt per min are removed, respectively (4).

DNA Polymerase step: Add 0.5 µl of three unlabeled dNTPs [5 mM each] (final 125 µM each), and the fourth labeled dNTP (final 100 µM, but min. 1 nmol) and incubate for 1 h at 37°C.

Add 0.5 µl of the fourth unlabeled dNTP [5 mM] (final 0.125 mM) and incubate for 15 min at 37°C.

Heat inactivate enzyme (10 min, 65°C) or purify DNA.

Note: First, dsDNA is partially digested to ssDNA by 3'-exonuclease activity. Second, upon subsequent addition of labeled and unlabeled dNTPs, 3'-single strands are extended to full length dsDNA by polymerase activity. Optimal labeling is achieved when the terminal 30-40% of each strand are labeled.

Note: 3'-exonuclease activity acts on both termini of DNA strands. Therefor, digestion must be stopped before the midpoint of the DNA molecule is reached. Else, the DNA will dissociate into two ssDNA molecules, which are rapidly degraded by 3'-exonuclease activity.

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Protocol: T4 DNA Polymerase - Primer Extension:

In this protocol, rather high dNTP concentrations are used to prevent pronounced exonuclease activity, which would otherwise act preferentially and rapidly on single-stranded oligonucleotides.

Component	Volume / reaction	Final concentration
Template DNA	0.1 – 2 µg	1 – 20 ng / µl
10 x T4 DNA Polymerase Buffer	10 µl	1 x
dNTPs [5 mM each]	10 µl	500 µM
Primer (expressed as molar ratio of primer to template DNA)	2:1 – 10:1	
T4 DNA Polymerase	0.5 - 1 µl / reaction	0.25 – 0.5 U / µl
H_2O, DNA and DNase free	@100 µl	~ 55.5 M
Total Reaction Volume	100 µl	

Incubate for 5 min at 0°C, followed by 5 min at room temperature and 30 min - 2 h at 37°C.

Note: At low temperatures (0°C and RT), T4 DNA Polymerase adds only a small number of bases to the 3'- ends of primers. This stabilizes the initial complex between primer and template DNA. Incubation at 37°C improves template DNA utilization and provides optimal conditions for primer extension (7).

Note: An extended version of this protocol describes site-directed mutagnesis (7).

Note: For dsDNA templates, do not add T4 DNA Polymerase prior to heat denaturation and annealing.

Note: In case primer extension is negatively affected by the presence of strong pause sites for T4 DNA Polymerase, add T4 GP32 single stranded binding protein (T4 gene 32 protein; EURx Cat. No. E1170) to the reaction.

Calculating pmol *ds*DNA from µg:

dsDNA [pmol] =

$$\frac{DNA\ amount\ [\mu g]}{DNA\ length[bp] \cdot 616[g\ mol^{-1} bp^{-1}]} \cdot \frac{10^{12}[pmol\ mol^{-1}]}{10^{6}[\mu g\ g^{-1}]}$$

Calculating pmol *ss*DNA from µg:

ssDNA [pmol] =

$$\frac{DNA\ amount\ [\mu g]}{DNA\ length\ [bp] \cdot 308\ [gmol^{-1}\ bp^{-1}]} \cdot \frac{10^{12}[pmol\ mol^{-1}]}{10^{6}[\mu g\ g^{-1}]}$$

Protocol: T4 DNA Polymerase - Blunting DNA Fragments for Next Generation Sequencing:

Set up the DNA polishing reaction mix on ice by combining the following components in the order shown:

Component	Volume /	Final concentration
Sonicated DNA	25 ng –	250 pg – 100 ng / µl
10 x T4 DNA Ligase Buffer (+ 10 mM ATP) (part of Cat. No. E1060)	10 µl	1 x (+ 1 mM ATP)
Bovine Serum Albumin (BSA), acetylated (Cat.	2.5 µg	25 µg / ml
dNTPs [10 mM] (Cat. No. E0501)	4 µl each	0.4 mM each
T4 DNA Polymerase	15 U	
Klenow Fragment (*E. coli* DNA Polymerase I) (Cat. No. E1091)	5 U	
T4 Polynucleotide Kinase (Cat. No. E1261)	50 U	
H_2O, DNA and DNase free	@ 100 µl*	~ 55.5 M
Total Reaction Volume	100 µl	

Incubate at 20°C for 30 min.

Purify DNA with EURx PCR/DNA Clean Up Kit (E3520) and elute to a volume of approx. 32 µl.

Add a single adenine (A) overhang to blunt ended 3' ends. Set up the DNA adenylation reaction mix on ice by combining the following components in the order shown:

Component	Volume / reaction	Final concentration
Blunt ended DNA	25 ng – 10 µg in 32 µl	250 pg – 100 ng / µl
10 x Klenow exo- buffer	5 µl	1 x
dATP (10 mM) (Cat. No. E0501)	1 µl	200 µM
Klenow Fragment exo- (*E. coli* DNA Polymerase I) (Cat. No. E1092)	15 U	~ 55.5 M
Total Reaction Volume	50 µl	

Add T4 DNA Ligase (Cat. No. 1060) for ligating DNA fragments to the proprietary adaptors according to the protocol supplied by the manufacturer of the NGS device.

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