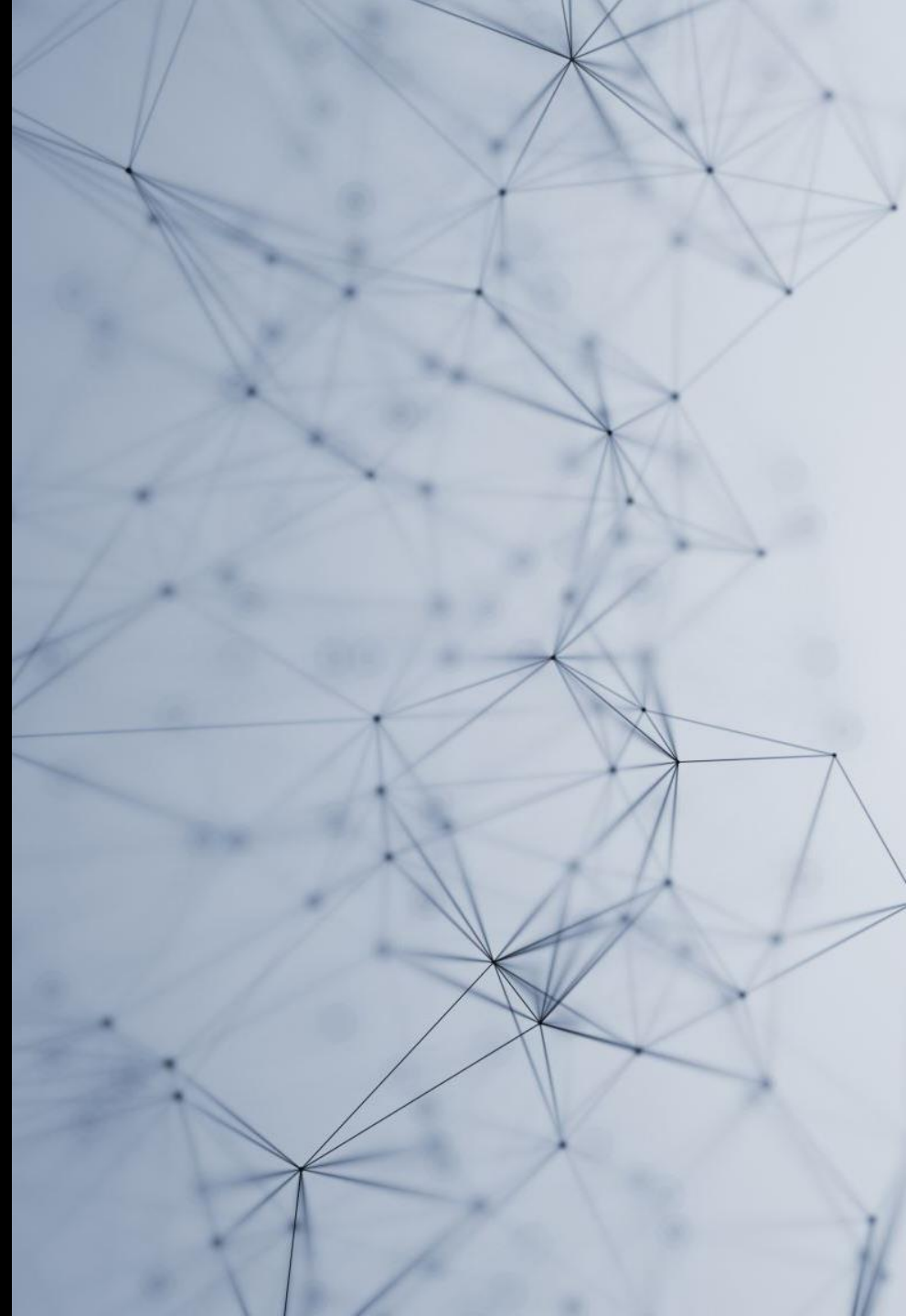
The main enzymes used in DNA recombination technologies

Lecturer: Senior Lecturer, Department of Molecular Biology and Genetics, PhD, Smekenov I.T.
Subject: Genetic engineering

(Lecture 2)



Lecture Goal:
To explore the main enzymes used in DNA recombination technologies, their roles, and mechanisms of action.

Tasks:

1. Discuss restriction enzymes, their classification, and the types of DNA fragment ends they produce.
2. Explain the function of DNA ligase, phosphatase (FastAP), and protein kinases in DNA manipulation.
3. Describe the roles of T4 DNA polymerase, Klenow fragment, DNA polymerase I, and reverse transcriptase in DNA recombination and synthesis.

Keywords: *Restriction enzymes, blunt ends, sticky ends, DNA ligase, phosphatase, FastAP, protein kinases, T4 DNA polymerase, Klenow fragment, DNA polymerase I, reverse transcriptase*

Restriction enzymes

- A **restriction enzyme**, restriction endonuclease, or *restrictase* is an enzyme that cleaves DNA into fragments at or near specific recognition sites within molecules known as restriction sites.
- Over **3,000 restriction enzymes** have been studied in detail, and more than 600 of these are available commercially.
- These enzymes are routinely used for DNA modification in laboratories, and they are a vital tool in molecular cloning.

Summary of the discovery of restriction enzymes

Year	Restrictase	Prototype
1976	84	22
1980	227	54
1984	503	103
1986	710	126
1988	839	152
2002	3516	211

TYPES AND ACTIVITIES OF RESTRICTION ENZYMES

Type I
Cleaves DNA at random sites far from its recognition sequence

Type II
Cleaves DNA at defined positions close to or within its recognition sequence

Type IIG
Cleaves outside its recognition sequence with both REase and MTase enzymatic activities in the same protein

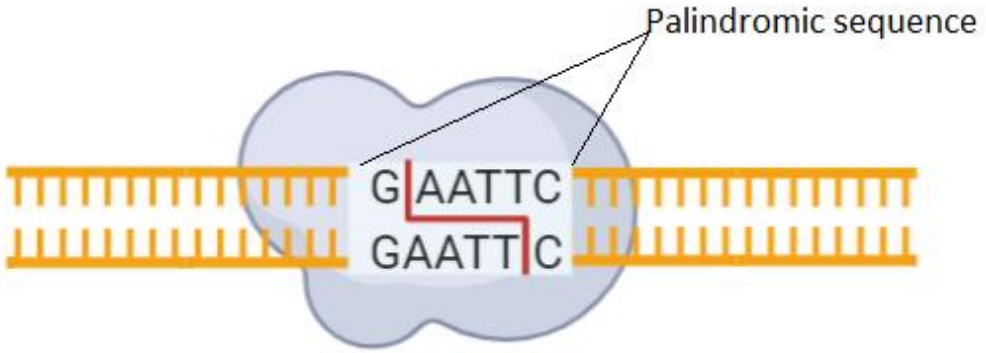
Type IIP
Cleaves symmetric targets and cleavage sites

Type IIS
Recognizes asymmetric sequences

Type III
Cleaves outside its recognition sequence and require two sequences in opposite orientations within the same DNA

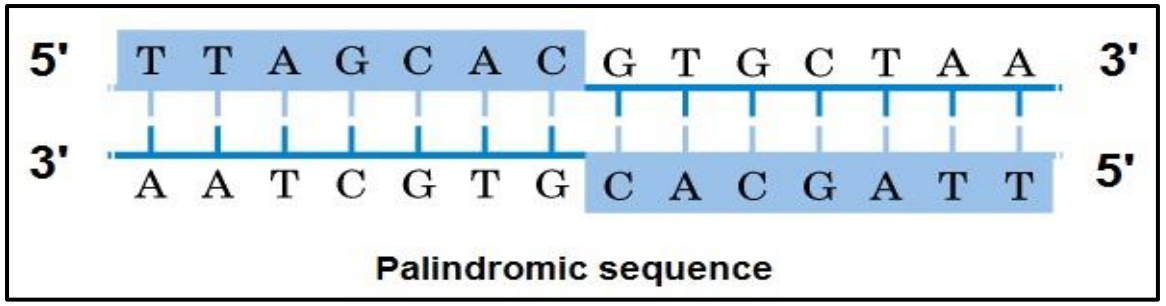
Type IV
Cleaves modified (e.g., methylated) DNA

	Cleavage site	Location of methylase	Examples
Type I	Random Around 1000bp away from recognition site	Endonuclease and methylase located on a single protein molecule	EcoK I EcoA I CfrA I
Type II	Specific Within the recognition site	Endonuclease and methylase are separate entities	EcoR I BamH I Hind III
Type III	Random 24-26 bp away from recognition site	Endonuclease and methylase located on a single protein molecule	EcoP I Hinf III EcoP15 I

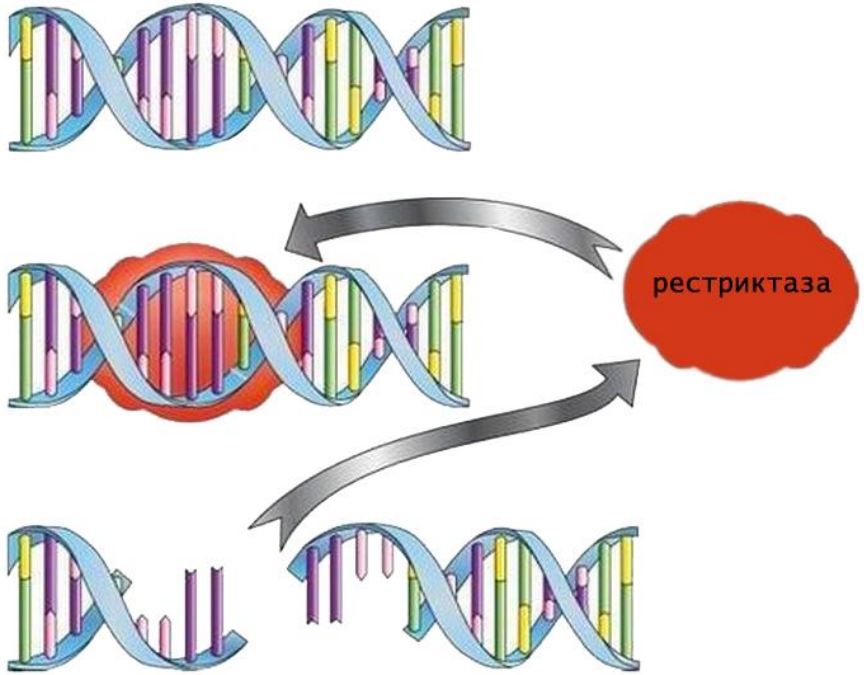


Restriction Endonuclease enzyme

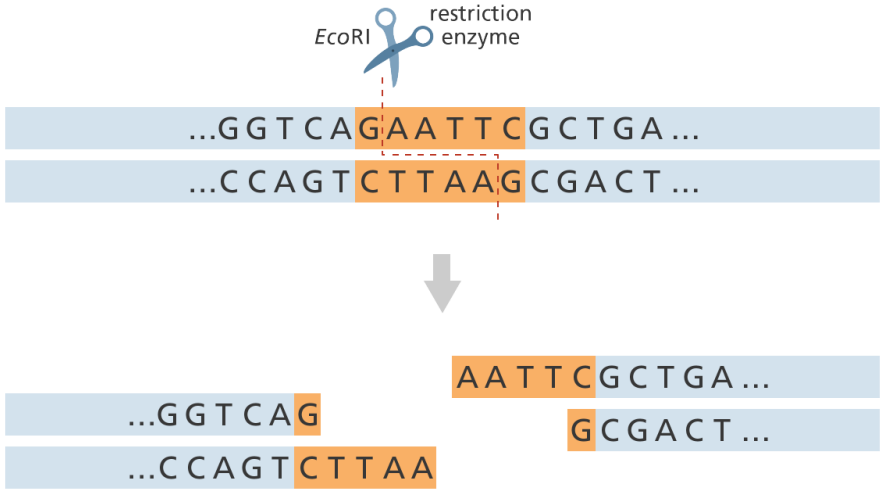
	Type I	Type II	Type III	Type IV
Nuclease structure	Multimer; heterotrimer	Homodimer	Homodimer	
Recognition site pattern	Two sites, in any orientation	Small (4–8 bp); usually palindromic	Two sites, in head-to-head orientation; non-palindromic	Weak specificity
Cleavage site	Variable distance from recognition site; non-specific cleavage	Cleavage within (Type IIP) or outside (Type IIS) the recognition site	Cleavage of one strand (nicking activity) 24–25 bp from recognition site	Methylated only
Cofactor	ATP, Mg2+, SAM	Mg2+	ATP, Mg2+, SAM	ATP, GTP



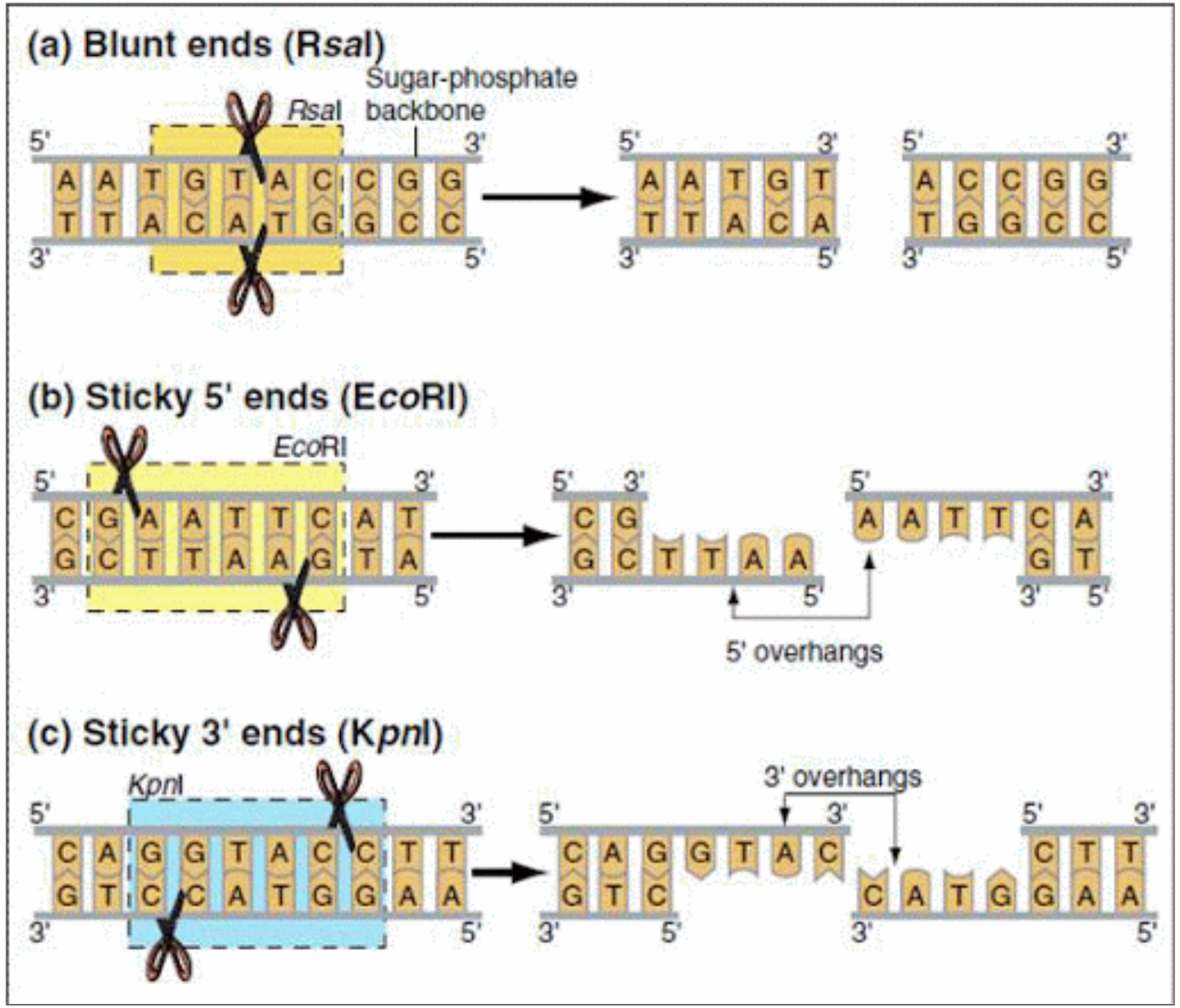
A **palindromic recognition site** reads the same on the reverse strand as it does on the forward strand when both are read in the same orientation



- **In 1968** M. Mezolson and R. Yuan for the first time isolated the restriction enzyme from E. coli K12 strain (EcoK and EcoB).
- **In 1970**, X. Smith and K. Wilvox isolated the HindII enzyme specifically for recognizing and cutting DNA for the first time.
- **In 1970** X. Smith and D. Nathans discovered EcoRI and EcoRII



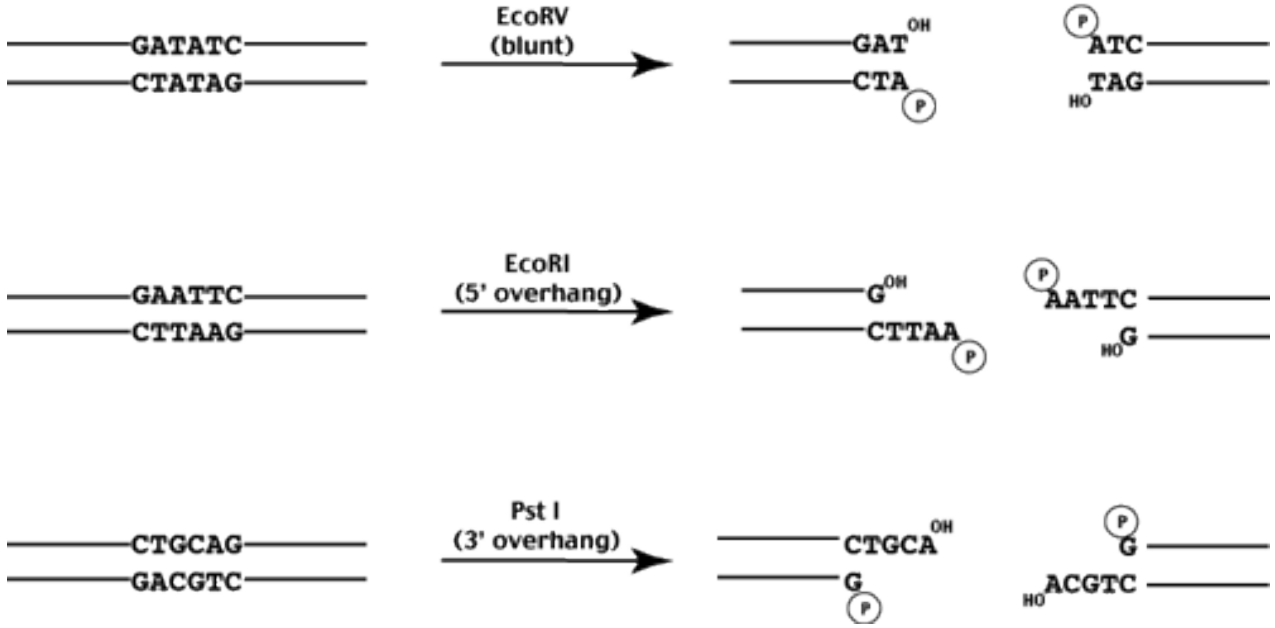
Types of fragment ends

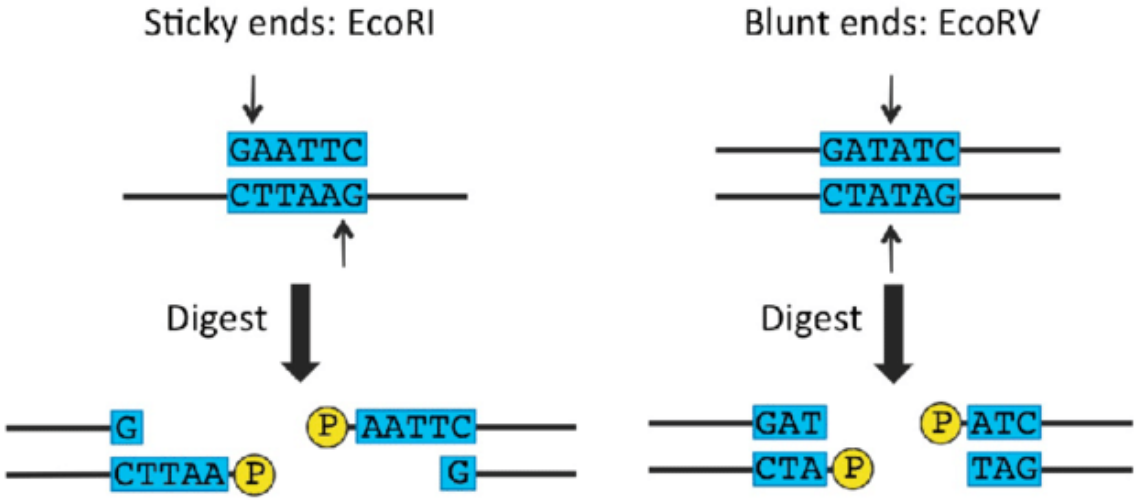


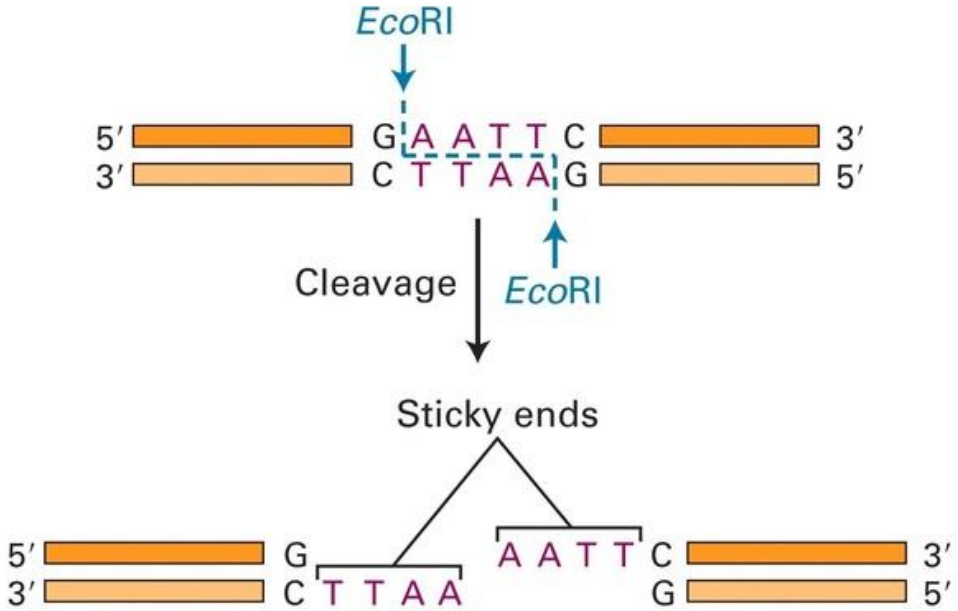
The prototype is the first discovered enzyme that recognizes a new sequence.

Isoschisomers are pairs of restriction endonucleases that have specificity for recognizing the same sequences, but sometimes differing in the presence of methylated nucleotide residues, and cut these sequences in the same places.

Examples of restrictase







Derivation of the EcoRI name

Abbreviation	Meaning	Description
E	*Escherichia*	Genus
co	*Coli*	species
R	RY13	Strain
I	First identified	order of identification

Microorganism of origin	Enzyme	Recognition site	After restriction enzyme digestion
Escherichia coli	*Eco*RI	5′-GAATTC-3′ 3′-CTTAAG-5′	5′-G AATTC-3′ 3′-CTTAA G-5′
Serratia marcescens	*Sma*I	5′-GGGCCC-3′ 3′-CCCGGG-5′	5′-GGG CCC-3′ 3′-CCC GGG-5′
Arthrobacter luteus	*Alu*I	5′-AGCT-3′ 3′-TCGA-5′	5′-AG CT-3′ 3′-TC GA-5′
Streptomyces albus	*Sal* I	5′-GTCGAC-3′ 3′-CAGGTG-5′	5′-G TCGAC-3′ 3′-CAGGT G-5′
Haemophilus influenzae	*Hin*dIII	5′-AAGCTT-3′ 3′-TTCGAA-5′	5′-A AGCTT-3′ 3′-TTCGA A-5′

Microorganism	Restriction Enzyme Name	Restriction Site
Bacillus amyloliquefaciens H	*Bam*HI	G|G A T C C C C T A G|G
Brevibacterium albidum	*Bal*I	T G G|C C A A C C|G G T
Escherichia coli RY13	*Eco*RI	G|A A T T C C T T A A|G
Haemophilus aegyptius	*Hae*II	Pu G C G C|Py Py|C G G C Pu
Haemophilus aegyptius	*Hae*III	G G|C C C C|G G
Haemophilus influenzae R_d	*Hin*dII	G T Py|Pu A C C A Pu|Py T G
Haemophilus influenzae R_d	*Hin*dIII	A|A G C T T T T C G A|A
Haemophilus parainfluenzae	*Hpa*I	G T T|A A C G A A|T T G
Haemophilus parinfluenzae	*Hpa*II	C|C G G G G C|C
Providencia stuartii 164	*Pst*I	C T G C A|G G|A C G T C
Streptomyces albus G	*Sal*I	G|T C G A C C A G C T|G

Table 2.1: *Restriction enzymes*

Restriction nomenclature

In 1973, Smith and Nathans proposed a nomenclature for restrictases, including the following items:

1. The abbreviation of the name of each enzyme is derived from the binary name of the microorganism containing this methylase-restriction system. Compose according to the rule: the first two lowercase letters of the species are added to the first capital letter of the genus name.

   Streptomyces albus - Sal, *Escherichia coli* – Eco.

2. If necessary, add a serotype or strain designation, e.g. Eco B.

3. Various restriction systems - modifications encoded by one bacterial cell are indicated by Roman numerals: Hind II, Hind I, Hind III (*Haemophilus influenzae*).

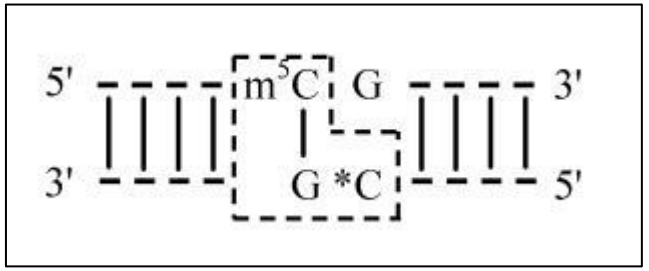
4. Restriction enzymes are denoted by the letter R (R Hind III), метилазы - M (M Hind III).

The discovery of new restrictases forced Roberts in 1978 to make additions to the system of rational designations of enzymes: if the abbreviated name is the same for several enzymes, then the first 2 letters of the abbreviation remain unchanged, and the third is taken from the subsequent letters of the species name:
Haemophilus parainfluenzae - Hpa I *Haemophilus parahaemolyticus* - Hph I.

DNA methyltransferases are a group of enzymes that catalyze the methylation of nucleotide residues in DNA.The activity of methyltransferases, which consists in the transfer of methyl (CH3-) groups to the nitrogenous base cytosine in DNA, leads to a change in the properties of DNA, while the activity, functions of the corresponding genes, as well as the spatial structure of the nucleic acid (conformation) change

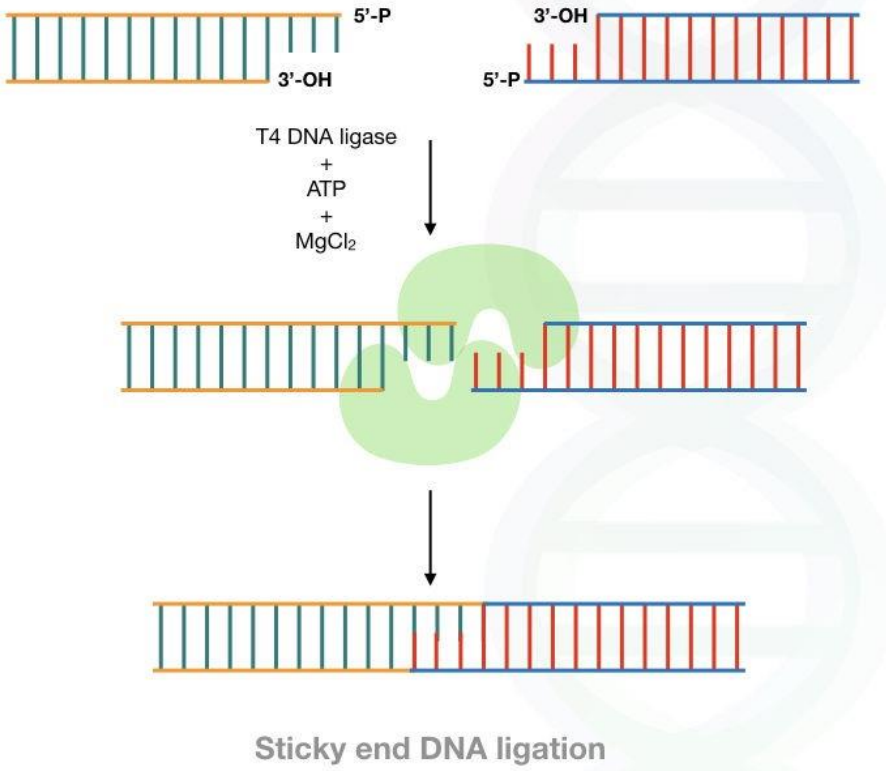
A quick review of relevant DNA methylation restriction enzymes.

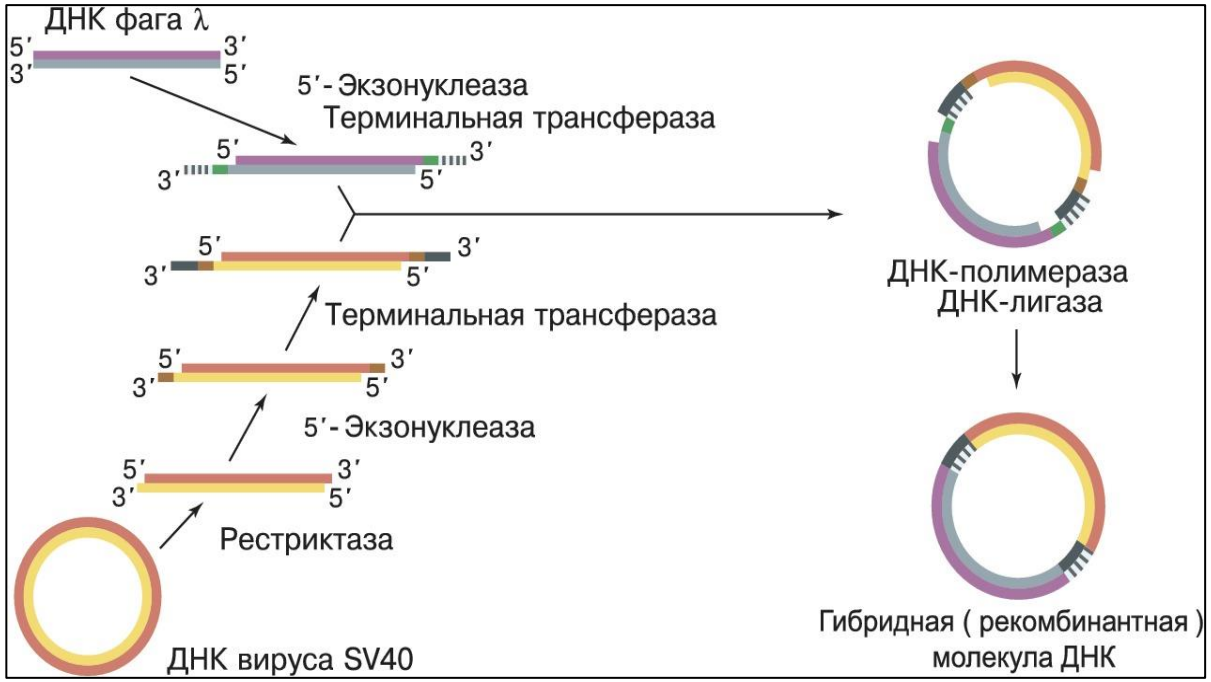
Product	DNA Methylation Application	Recognition Site
MspJI	Identify 5-hmC and 5-mC	5′... ^{m}CNNR(n)$_{9}$ ▼...3′ 3′... GNNY(N)$_{11}$▲...5′
LpnPI	Identify 5-hmC and 5-mC	5′... C^{m}CDG(N)$_{10}$ ▼...3′ 3′... GGHC(N)$_{14}$▲...5′
FspEI	Identify 5-hmC and 5-mC	5′... C^{m}C(N)$_{12}$ ▼...3′ 3′... GG(N)$_{16}$▲...5′
DpnI	Identify 5-mA; used with DpnII	CH$_3$ 5′... GA▼TC...3′ 3′... CT▲AG...5′ CH$_3$
DpnII	Identify 5-mA; used with DpnI	5′... ▼GATC ...3′ 3′... CTAG▲...5′
McrBC	Identify 5-mC	5′...Pu^{m}C(N$_{40\text{-}3000}$)Pu^{m}C...3′
MspI	Identify 5-mC; used with HpaII	5′... C▼CGG ...3′ 3′... GGC▲C...5′
HpaII	Identify 5-mC; used with MspI	5′... C▼CGG ...3′ 3′... GGC▲C...5′



DNA ligase

DNA ligases (68 kDa) are enzymes (EC 6.5.1.1) that catalyze the covalent fusion of DNA strands in a duplex during replication, repair and recombination. They form phosphodiester bridges between the 5'-phosphoryl and 3'-hydroxyl groups of adjacent deoxynucleotides at DNA breaks or between two DNA molecules (discovered in 1967).





Thermo Scientific T4 DNA Ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA. The enzyme repairs single-strand nicks in duplex DNA, RNA, or DNA/RNA hybrids. It also joins DNA fragments with either cohesive or blunt termini, but has no activity on single-stranded nucleic acids.

T4 DNA Ligase requires ATP as a cofactor.

Highlights

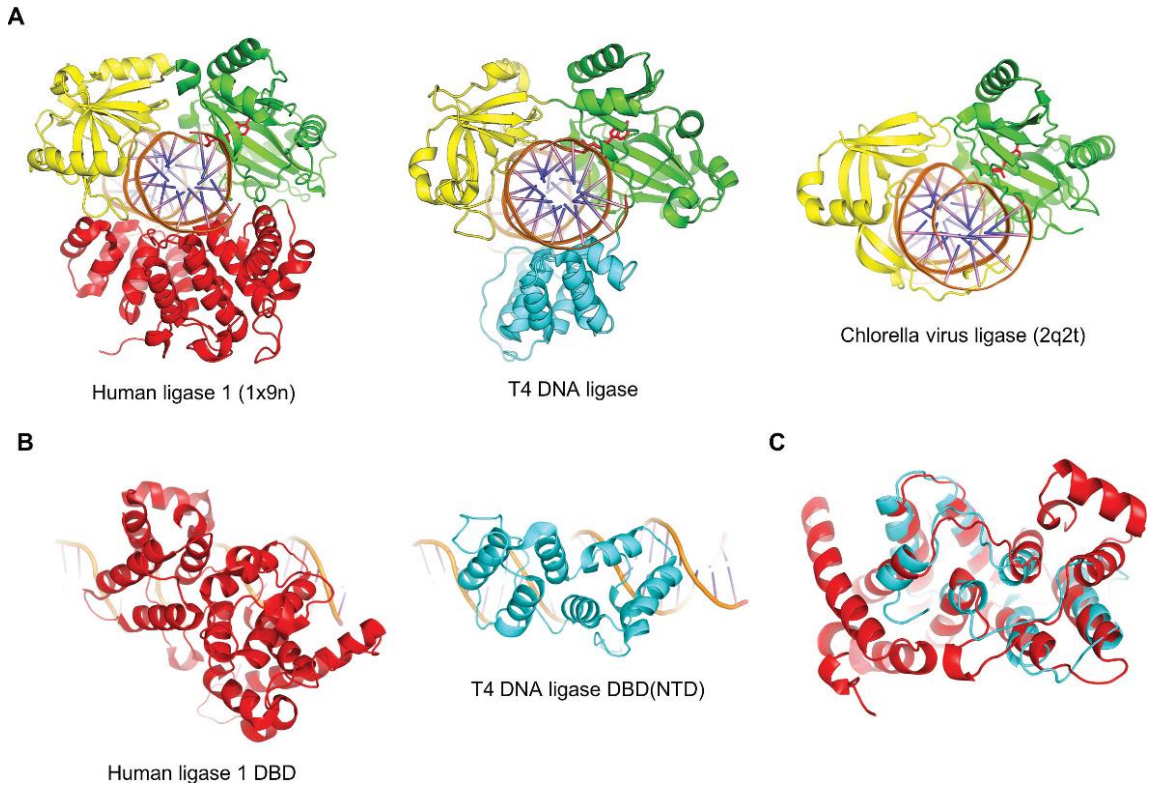
• Active in Themo Scientific restriction enzyme, PCR, and RT buffers (when supplemented with ATP)
• Fast—sticky-end ligation is completed in 10 minutes at room temperature
• Supplied with PEG solution for efficient blunt-end ligation

Applications

• Cloning of restriction enzyme generated DNA fragments
• Cloning of PCR products
• Joining of double-stranded oligonucleotide linkers or adaptors to DNA
• Site-directed mutagenesis
• Amplified fragment length polymorphism (AFLP)
• Ligase-mediated RNA detection (*see* Reference 3)
• Nick repair in duplex DNA, RNA or DNA/RNA hybrids
• Self-circularization of linear DNA.

Includes

• T4 DNA Ligase
• 10X T4 DNA Ligase Buffer
• 50% PEG Solution

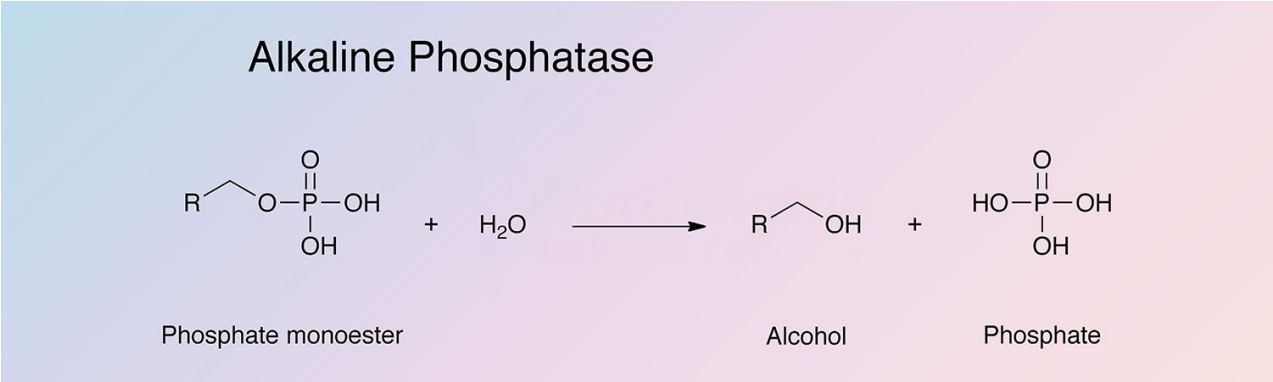


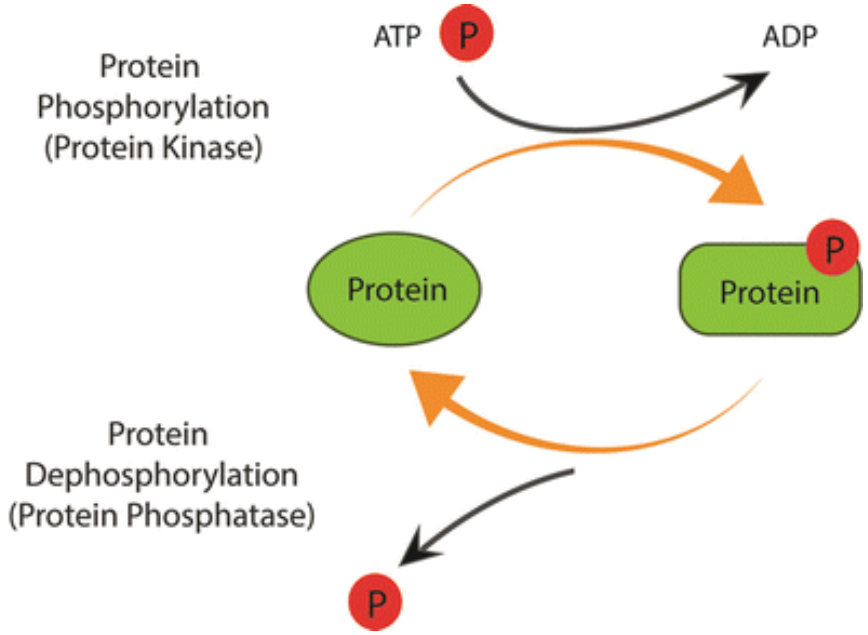
Specifications

Concentration	5 U/µL
Enzyme	DNA Ligase
Compatible Buffer	10X T4 DNA Ligase Buffer
Quantity	200 U
Product Type	T4 DNA Ligase
Unit Size	200 units

Additional Enzymes

- **Phosphatase** is an enzyme that catalyzes the dephosphorylation of a substrate (usually another protein) by hydrolysis of the phosphoric acid ester bond. In this case, a phosphate anion and a product molecule with a hydroxyl group are formed. According to its catalytic and physiological action, phosphatase is an antagonist of phosphorylase and kinase, which attach a phosphate group to the substrate.





FastAP Thermosensitive Alkaline Phosphatase (1 U/µL)

Thermo Scientific FastAP Thermosensitive Alkaline Phosphatase catalyzes the release of 5'- and 3'-phosphate groups from DNA, RNA, and nucleotides. This enzyme also removes phosphate groups from proteins.

FastAP is a novel alkaline phosphatase, which is active in all Thermo Scientific restriction enzyme buffers as well as in PCR buffers. It dephosphorylates all types of DNA ends (blunt, 5'- and 3'-overhangs) in 10 minutes at 37°C. The enzyme is inactivated in 5 minutes at 75°C (*see* Figure 1 in Supporting Data). Therefore, removal of alkaline phosphatase is not required prior to ligation.

Highlights

- **Recombinant enzyme**
- **Fast dephosphorylation**—10 minutes at 37°C
- **Fast and complete inactivation**—5 minutes at 75°C
- **Simultaneous digestion and dephosphorylation of vector DNA**
- **100% active** in restriction enzyme and PCR buffers
- **PCR clean-up in conjunction with Exo I**
- **Protein dephosphorylation**

One protocol for all types of DNA ends:

- 5'-overhangs
- 3'-overhangs
- blunt-ends
- single nucleotides

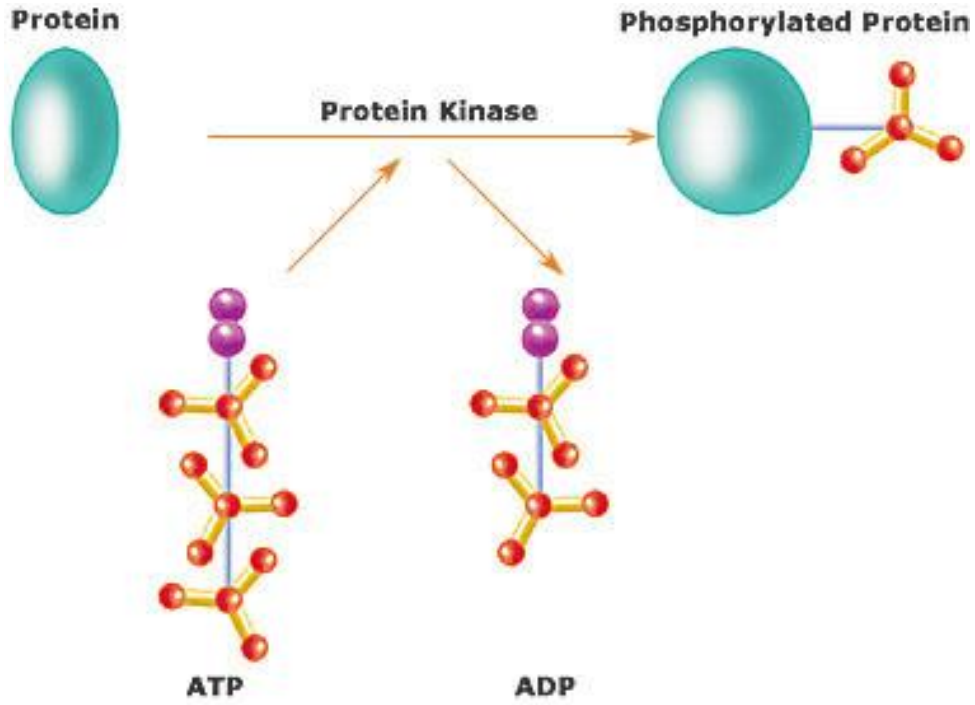
Specifications

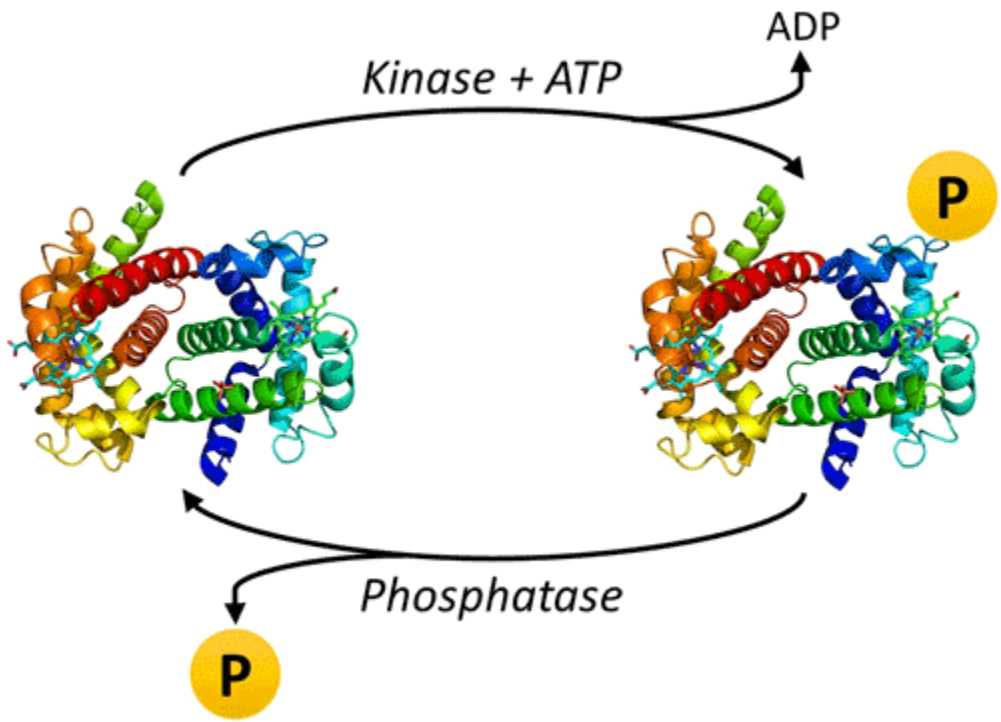
Concentration	1 U/µL
Enzyme	Phosphatase
Compatible Buffer	PCR Buffer, Enzyme Buffer
Quantity	300 U
Product Type	Alkaline Phosphatase
Unit Size	300 units

Applications

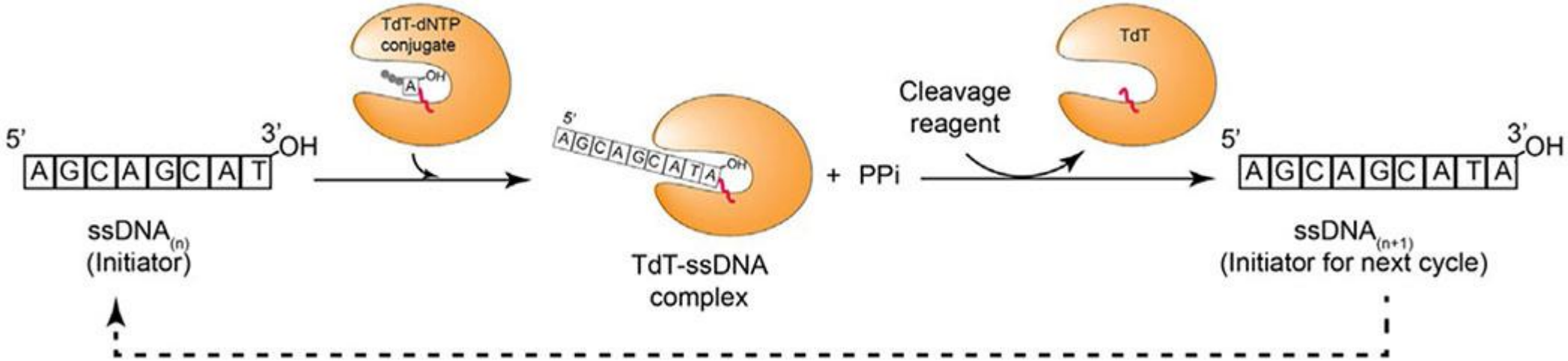
- Dephosphorylation of cloning vector DNA to prevent recircularization during ligation
- Simultaneous digestion and dephosphorylation of vector DNA
- PCR product clean-up: nucleotide degradation prior to sequencing of PCR product
- Dephosphorylation of nucleic acid 5'-termini prior to labeling with T4 Polynucleotide Kinase
- Other applications where dephosphorylation of DNA and RNA substrates is necessary
- Protein dephosphorylation

- **Protein kinases** are a subclass of kinase enzymes (phosphotransferases). Protein kinases modify other proteins by phosphorylation of amino acid residues that have hydroxyl groups (serine, threonine, and tyrosine) or the heterocyclic amino group of histidine.





- **Terminal deoxynucleotidyl transferase (TdT),** also known as DNA nucleotidyl exotransferase (DNET) or terminal transferase, is a specialized DNA polymerase expressed in immature, pre-B, pre-T lymphocytes, and in acute lymphoblastic leukemia.



Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT) is a DNA polymerase that catalyzes the addition of deoxynucleotides to the 3fi hydroxyl terminus of DNA. A TdT Technical Bulletin is available.

Applications: Homopolymer tailing of vector and insert for cloning. Labeling oligonucleotides with biotin (1,2), ^{32}P- or ^{35}S-label (3), or in apoptosis (TUNEL) (4,5).

Source: Purified from E. coli clone of calf thymus TdT.

Performance and Quality Testing: Endonuclease, 3´ and 5´ exodeoxyribonuclease, and levels of incorporation tested.

Unit Definition: One unit incorporates 1 nmol dATP into acid-precipitable material in 1 h at 37°C, using d(pA)$_{50}$ as a primer.

Hazard Warning: Toxic; potassium cacodylate contained in reaction buffer. Also contains cobalt chloride, a highly toxic chemical. See MSDS.

Unit Reaction Conditions: 0.2 M potassium cacodylate (pH 7.2), 10 mM MgO_4 C_4 H_6 , 1 mM 2-mercaptoethanol, 0.5 mg/ml BSA, 100 flM d(pA)$_{50}$, 1 mM [^{3}H]dATP, and enzyme in 0.15 ml for 1 h at 37°C.

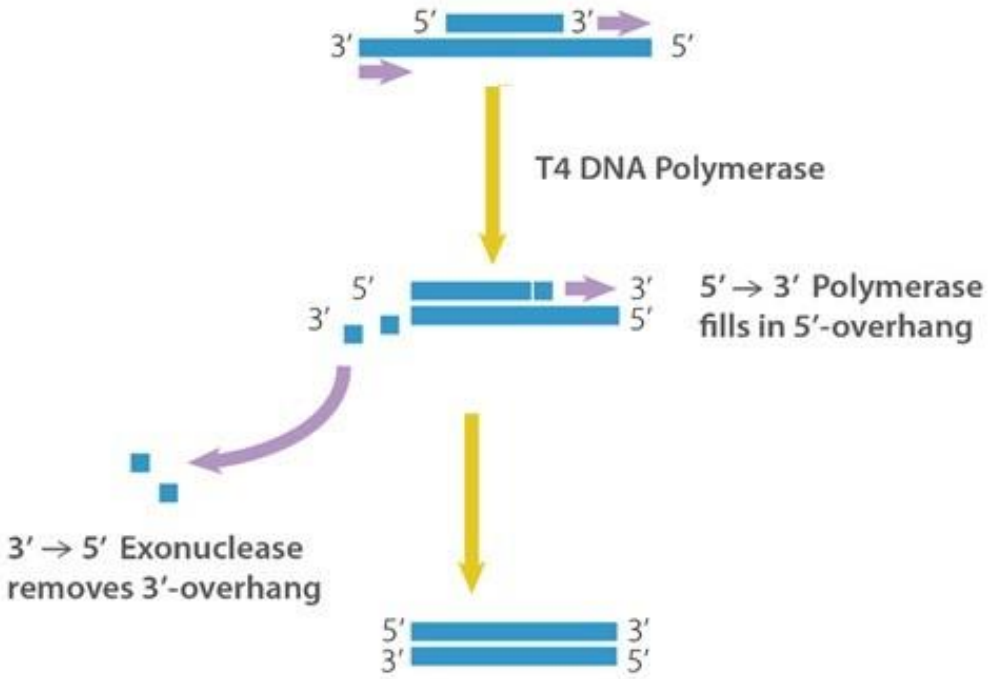
Specifications

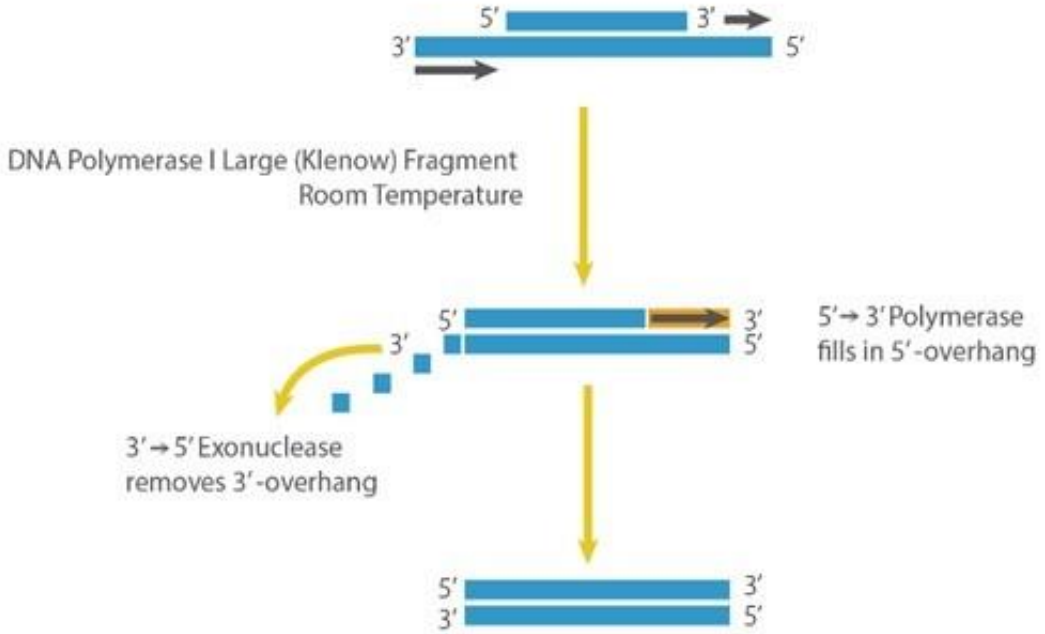
Shipping Condition	Approved for shipment on Wet or Dry Ice
Enzyme	Terminal Deoxynucleotidyl Transferase
Compatible Buffer	5X Buffer, Reaction Buffer
Quantity	500 U
Product Type	TdT
Unit Size	500 units

Contents & Storage

Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT) is supplied with vial of 5X buffer [500 mM potassium cacodylate (pH 7.2), 10 mM $CoCl_2$, 1 mM DTT]. Store at -20°C.

- **T4 DNA polymerase and Klenov fragment** are DNA blunting enzymes.





Thermo Scientific T4 DNA Polymerase is a template-dependent DNA polymerase that catalyzes 5'-3' synthesis from primed single-stranded DNA. The enzyme has a 3'-5' exonuclease activity, but lacks 5'-3' exonuclease activity.

Highlights

- **Stronger 3'-5' exonuclease activity** on single-stranded than on double-stranded DNA and greater (more than 200 times) than DNA polymerase I, *E. coli,* and Klenow fragment
- Active in Thermo Scientific restriction enzyme, PCR, RT and T4 DNA Ligase buffers

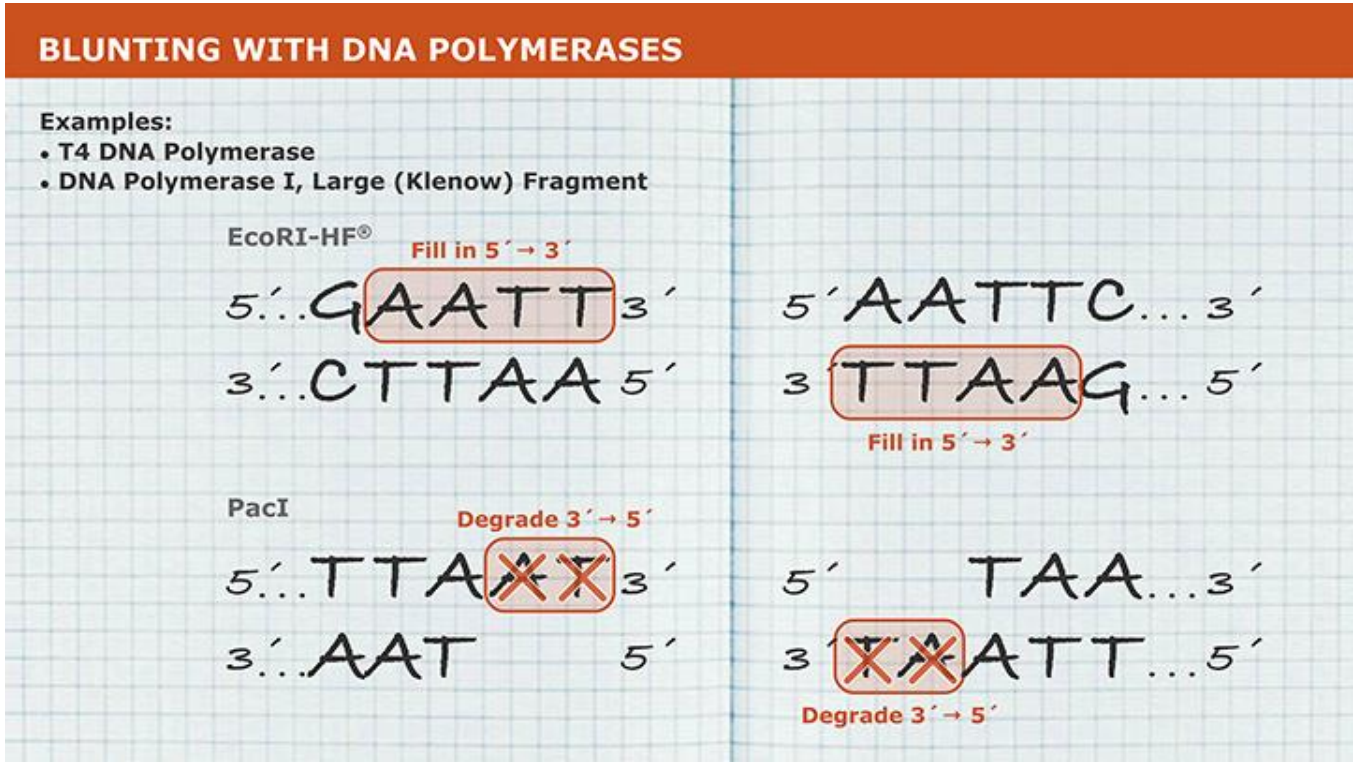
Applications

- Blunting of DNA ends: fill-in of 5'-overhangs or/and removal of 3'-overhangs(*see* References1, 2)
- Blunting of PCR products with 3'-dA overhangs
- Synthesis of labeled DNA probes by the replacement reaction(*see* Reference3)
- Oligonucleotide-directed site-specific mutagenesis(*see* Reference4)
- Ligation-independent cloning of PCR products

DNA Polymerase I, Large (Klenow) Fragment

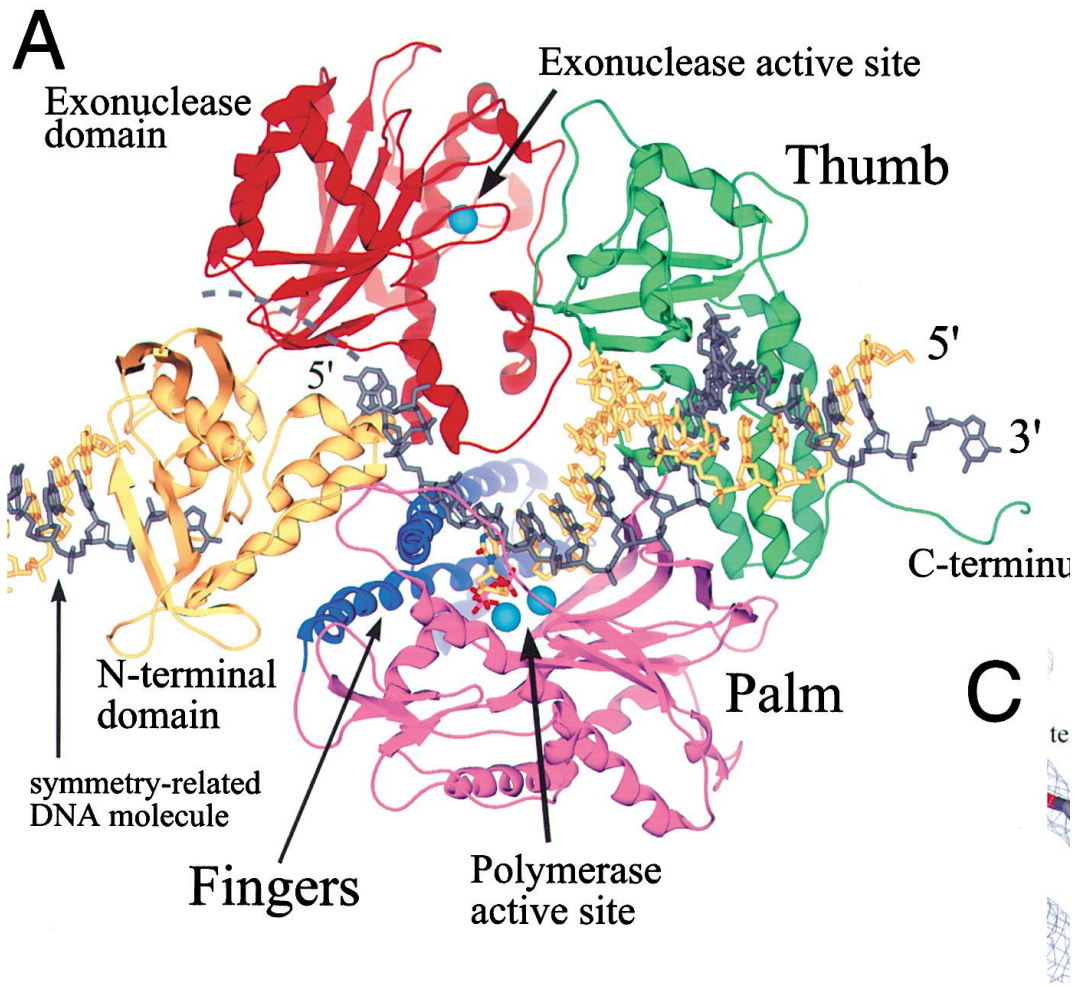
DNA Polymerase I, Large (Klenow) fragment was originally derived as a proteolytic product of *E.coli* DNA polymerase that retains polymerase and 3' —> 5' exonuclease activity

- Removal of 3' overhangs or fill-in of 5' overhangs to form blunt ends
- Lacks 5' —> 3' exonuclease activity
- Generates probes using random primers
- Second strand cDNA synthesis



DNA polymerase I (103 kDa) - polI

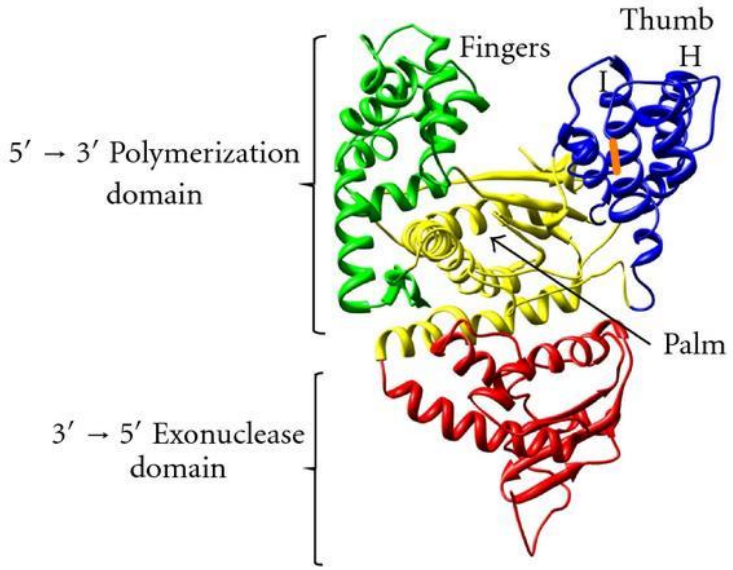
- 1958 Kornberg discovered this enzyme in E. coli.
- **Domains**:
- N-terminus: 5'-3' - exoculase activity
- C-terminus: 5'-3' - polymerase activity
- Middle domain: - 3'-5' - exonuclease activity



Thermo Scientific *Taq* DNA Polymerase is a highly thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus*. The enzyme catalyzes 5' to 3' synthesis of DNA, has no detectable 3' to 5' exonuclease (proofreading) activity, and possesses low 5' to 3' exonuclease activity. In addition, *Taq* DNA Polymerase exhibits deoxynucleotidyl transferase activity, which frequently results in the addition of extra adenines at the 3'-end of PCR products. Recombinant *Taq* DNA Polymerase is the ideal tool for standard PCR of templates 5 kb or shorter.

Features
- Thermostable—half life is more than 40 min at 95°C
- Generates PCR products with 3'-dA overhangs
- Supplied with two buffers: 10X *Taq* Buffer with KCl and 10X *Taq* Buffer with $(NH_4)_2SO_4$
- Incorporates modified nucleotides (e.g., biotin-, digoxigenin-, fluorescent-labeled nucleotides)

Applications
- Routine PCR amplification of DNA fragments up to 5 kb
- High throughput PCR
- DNA labeling



Specifications

GC-Rich PCR Performance	Low
Polymerase	*Taq* DNA Polymerase
Reaction Speed	Standard
Product Type	*Taq* DNA Polymerase (Recombinant)
Quantity	100 Units
Shipping Condition	Dry Ice
For Use With (Application)	Standard PCR
Concentration	5 U/µL
Fidelity (vs. Taq)	1X
Hot Start	No
Overhang	3'-A
Reaction Format	Separate Components
Unit Size	100 units

- Thermo Scientific **Phusion high quality DNA polymerases** set the gold standard for high throughput PCR. With an error rate 50 times lower than Taq and 6 times lower than Pfu , Phusion's high fidelity DNA polymerase is an excellent choice for cloning and other high precision applications.
- Phusion DNA polymerases provide reliable performance with short protocol times even in the presence of PCR inhibitors and provide higher yields with lower enzyme levels than other DNA polymerases.• Fast PCR due to short expansion time (15-30 s/kb)

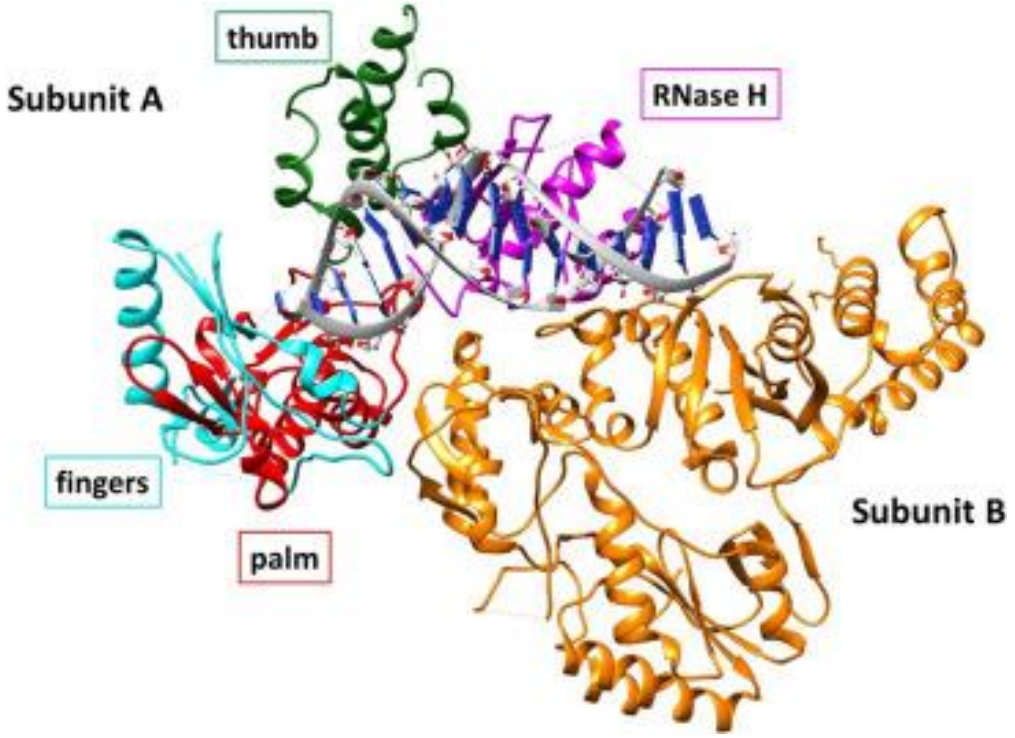
Features
•High fidelity (52X *Taq*)
•No non-specific amplification and primer degradation during reaction setup
•Fast PCR due to short extension times (15–30 s/kb)
•Increased product yields with minimal enzyme amounts
Applications
•Standard PCR
•qPCR
•High fidelity and long PCR
•LAMP-PCR
•cDNA synthesis
•RT-PCR, RDA, MDA
•DNA labeling and sequencing

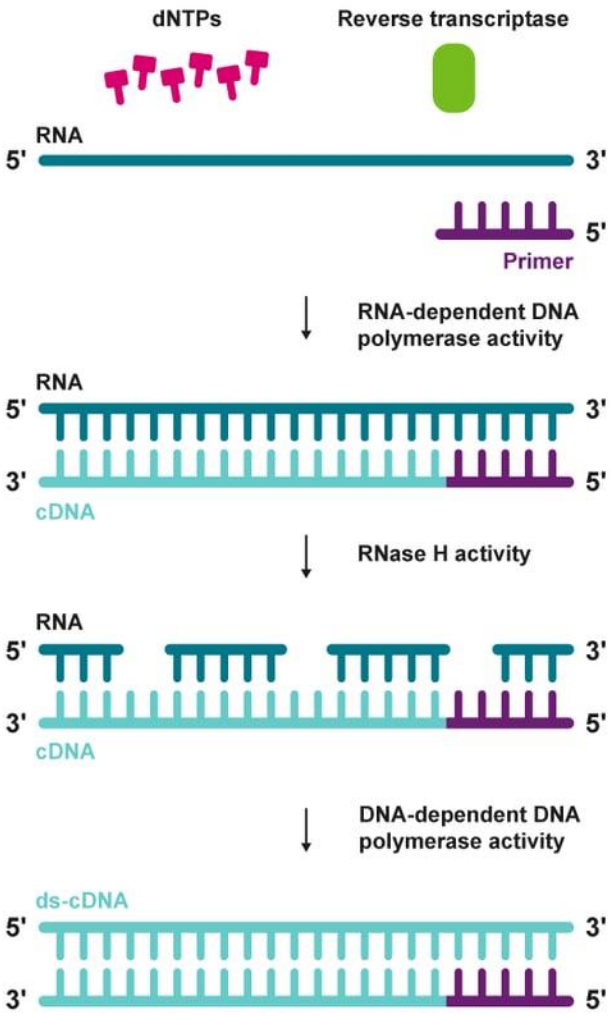
Specifications

Detection Method	Primer-probe
GC-Rich PCR Performance	High
Polymerase	Phusion Hot Start II DNA Polymerase
Reaction Speed	Fast
Product Type	Hot Start DNA Polymerase and dNTP Mix
Shipping Condition	Dry Ice
For Use With (Application)	Hot-start PCR, High-fidelity PCR
Concentration	2 U/µL
Fidelity (vs. Taq)	52X
Hot Start	Built-In Hot Start
No. of Reactions	2000 Reactions
Overhang	Blunt
Reaction Format	Separate Components
Size (Final Product)	20 kb or less
Unit Size	2000 reactions

Reverse transcriptase

- **Description**
- RevertAid (RT) reverse transcriptase has lower RNase H activity compared to AMV reverse transcriptase.
- The enzyme remains active at 42-50°C and is suitable for the synthesis of cDNA up to 13 kb.





- The Thermo Scientific RevertAid RT Kit is a complete system for efficient synthesis of first strand cDNA from mRNA or total RNA templates. The kit uses RevertAid Reverse Transcriptase (RT), which has lower RNase H activity compared to AMV reverse transcriptase. The enzyme maintains activity at 42–50°C and is suitable for synthesis of cDNA up to 13 kb. The recombinant RiboLock RNase Inhibitor, supplied with the kit, effectively protects RNA from degradation at temperatures up to 55°C.

  First strand cDNA synthesized with this system can be directly used as a template in PCR or real-time PCR. It is also ideal as a first step for second strand cDNA synthesis or linear RNA amplification. Radioactively and non-radioactively labeled nucleotides can be incorporated into first strand cDNA for use as a probe in hybridization experiments, including microarrays.

  Features of the RevertAid RT Kit include:
  • Full-length first strand cDNA up to 13 kb
  • Optimum reaction temperature 42°C
  • Complete kit includes all the components for RT reactions

  Applications
  • First strand cDNA synthesis for RT-PCR and RT-qPCR
  • Construction of full length cDNA libraries
  • Antisense RNA synthesis

Specifications

Format	Kit
Reaction Speed	60 min.
Technique	Reverse Transcription
Optimal Reaction Temperature	42°C
Reverse Transcriptase	RevertAid
Ribonuclease H Activity	Yes
Shipping Condition	Dry Ice
For Use With (Application)	Real Time PCR (qPCR)
Final Product Type	First-Strand cDNA
No. of Reactions	500 Reactions
Reaction Format	Separate components
Reagent Type	Reverse Transcription
Size (Final Product)	Up to 13 kb
Starting Material	RNA
Unit Size	500 reactions