Manipulation of DNA- I

ENZYMES USED IN MOLECULAR BIOLOGY EXPERIMENTS

- **Nucleases** are enzymes that cut, shorten, or degrade nucleic acid molecules.
- **Ligases** join nucleic acid molecules together.
- **Polymerases** make copies of molecules.
- **Modifying enzymes** remove or add chemical groups.

Nucleases

- Exonucleases remove nucleotides one at a time from the end of a DNA molecule.
- Endonucleases are able to break internal phosphodiester bonds within a DNA molecule.

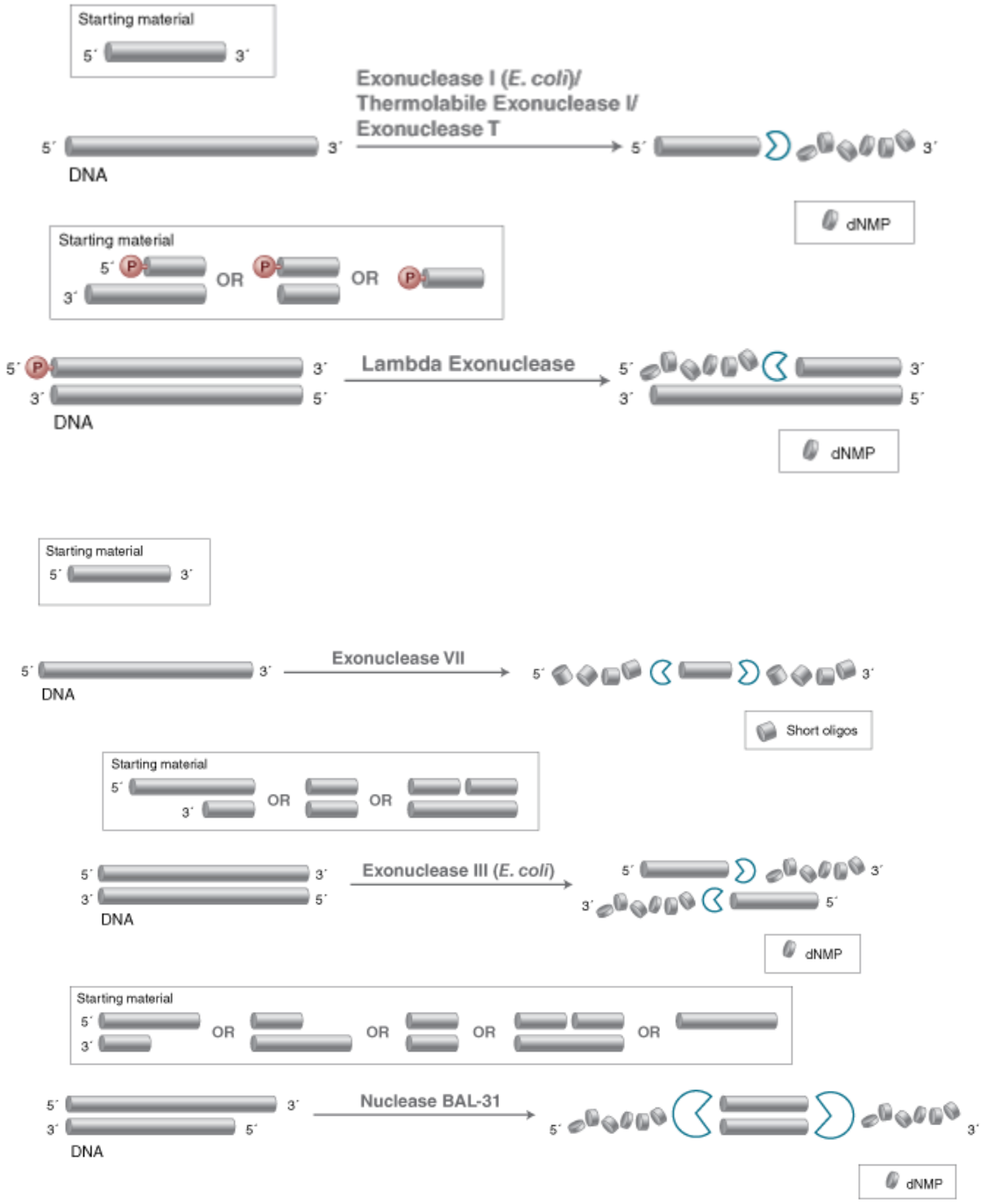
Exonuclease I

Lambda Exonuclease

Exonuclease VII

Exonuclease III

Nuclease Bal -31



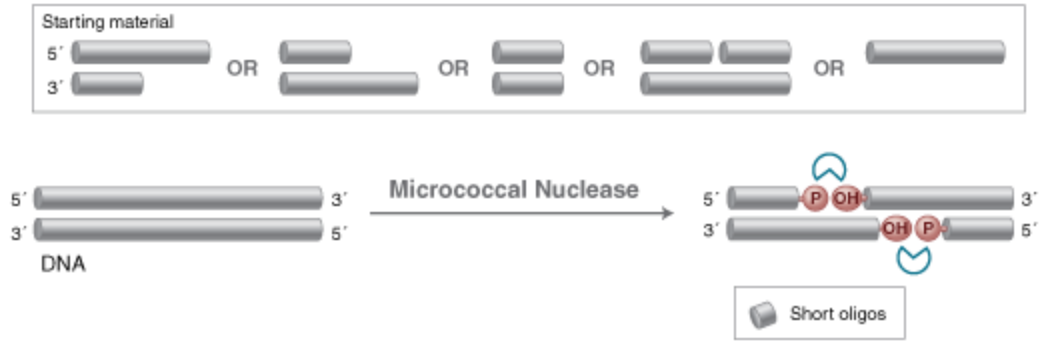
- DNA specific exonuclease that also acts as an endonuclease on single-strand DNA
- Initiates at both 5' and 3' termini of linear double-stranded DNA
- Cleaves linear double-stranded DNA in both the 3' to 5' and 5' to 3' directions
- Requires ATP in the reaction

Exonuclease V is ideal for:

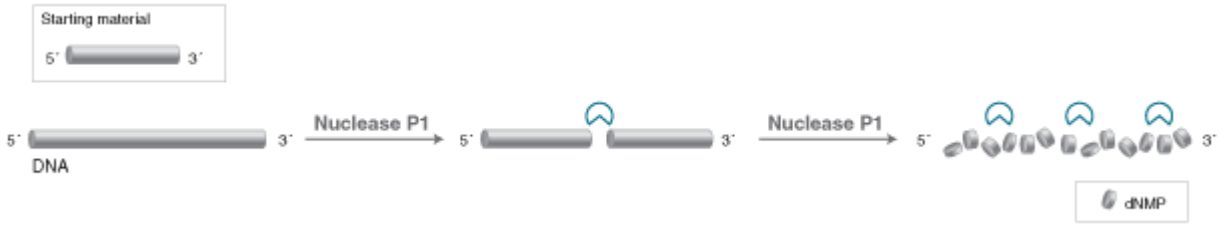
- Degradation of contaminating linear DNA in plasmid samples
- Removal of residual gDNA after purification of low copy plasmid

Dnase I

Micrococcal Nuclease I



P1 or S1 Nuclease I



Nucleases

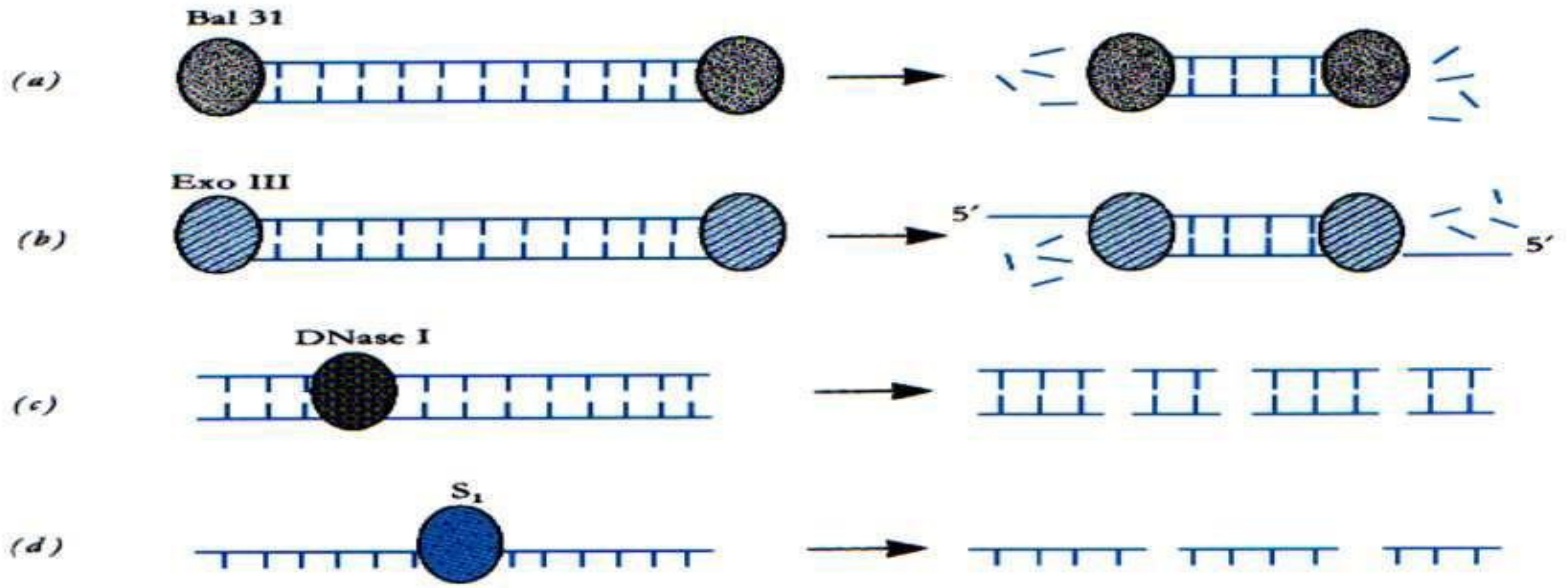
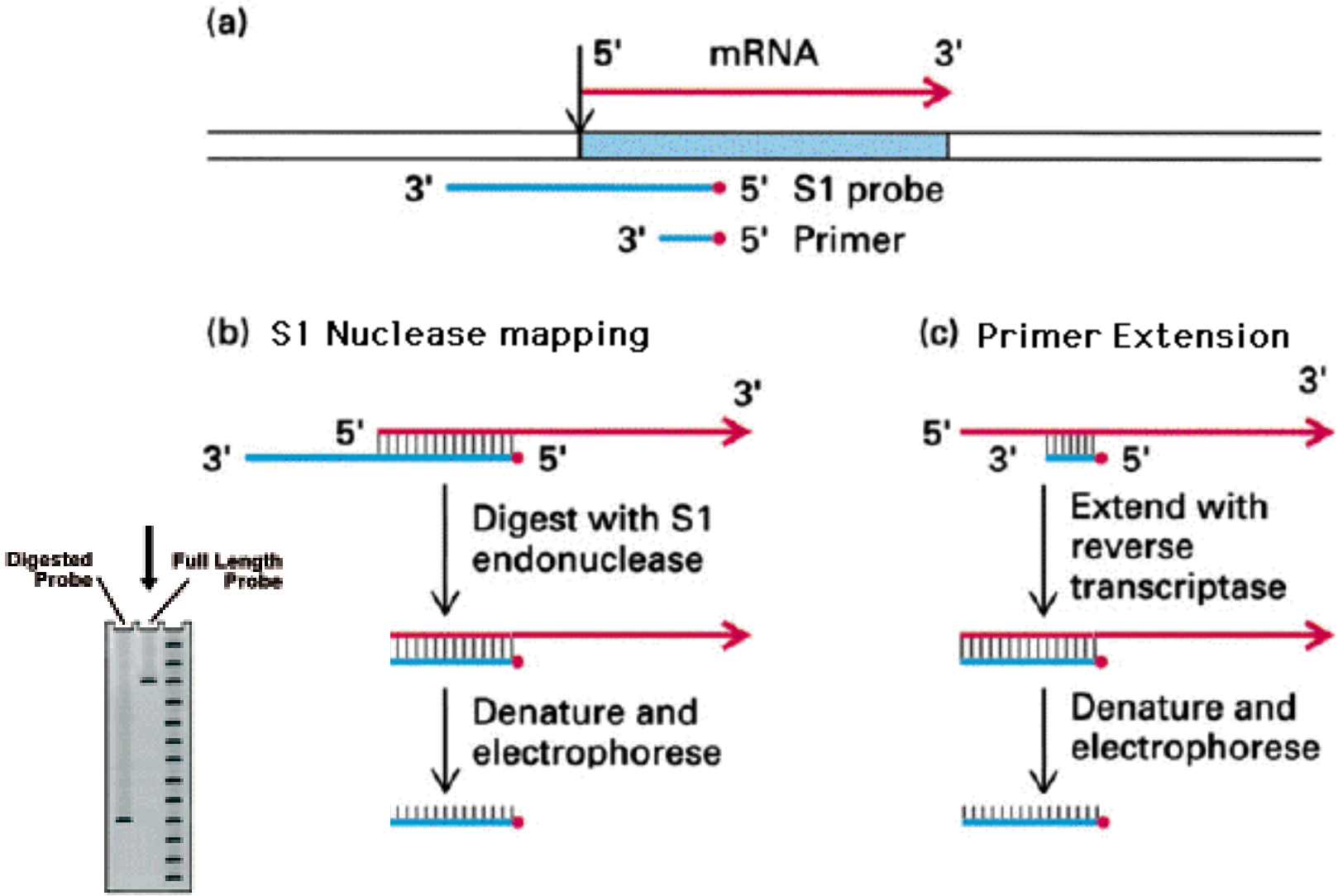


Fig. 4.5 Mode of action of various nucleases. (*a*) Nuclease Bal 31 is a complex enzyme. Its primary activity is a fast-acting 3′ exonuclease, which is coupled with a slow-acting endonuclease. When Bal 31 is present at a high concentration these activities effectively shorten DNA molecules from both termini. (*b*) Exonuclease III is a 3′ exonuclease that generates molecules with protruding 5′ termini. (*c*) DNase I cuts either single-stranded or double-stranded DNA at essentially random sites. (*d*) Nuclease S_1 is specific for single-stranded RNA or DNA. Modified from Brown (1990), *Gene Cloning*, Chapman and Hall; and Williams and Patient (1988), *Genetic Engineering*, IRL Press. Reproduced with permission.

For a detailed list of exo and endonucleases refer
https://international.neb.com/tools-and-resources/selection-charts/properties-of-exonucleases-and-nonspecific-endonucleases
For their specific uses
https://international.neb.com/tools-and-resources/selection-charts/common-applications-for-exonucleases-and-endonucleases

S1 Nuclease Use- Nuclease Protection Assay

Nuclease protection assays are used to map introns and 5' and 3' ends of transcribed gene regions.



RNAses

RNase A- obtained from bovine pancreas- RNase commonly used in research (cuts after C or U)

RNAse H- RNAse that cleaves the RNA in DNA/RNA hybrid
(uses- eg after cDNA synthesis)

Note- Reverse transcriptases lacking RNase H activity provide another option to prepare long cDNAs and libraries containing a high percentage of full-length cDNA.

RNase III-
Various classes- Some cleave precursors to make mature rRNA, snRNA or snoRNA. Also have examples like DROSHA , or DICER required for miRNA biogenesis

RNase T1- derived from Aspergillus oryzae (cleaves next to guanosine) in ssRNA

RNase R can degrade all linear RNAs

RNase P- ribozyme, a RNA that acts as a catalyst. Its function is to cleave off extra bases on tRNA molecules.

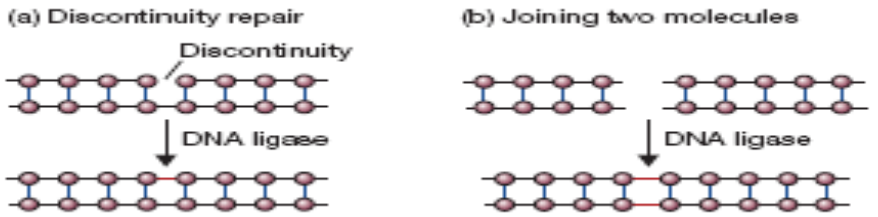
Restriction Endonucleases will be covered in the next class

Ligases

It is an important cellular enzyme as its function is to repair the broken phosphodiester bonds that may occur at random or as consequence of DNA replication or recombination

Figure 4.4

The two reactions catalyzed by DNA ligase. (a) Repair of a discontinuity—a missing phosphodiester bond in one strand of a double-stranded molecule. (b) Joining two molecules together.



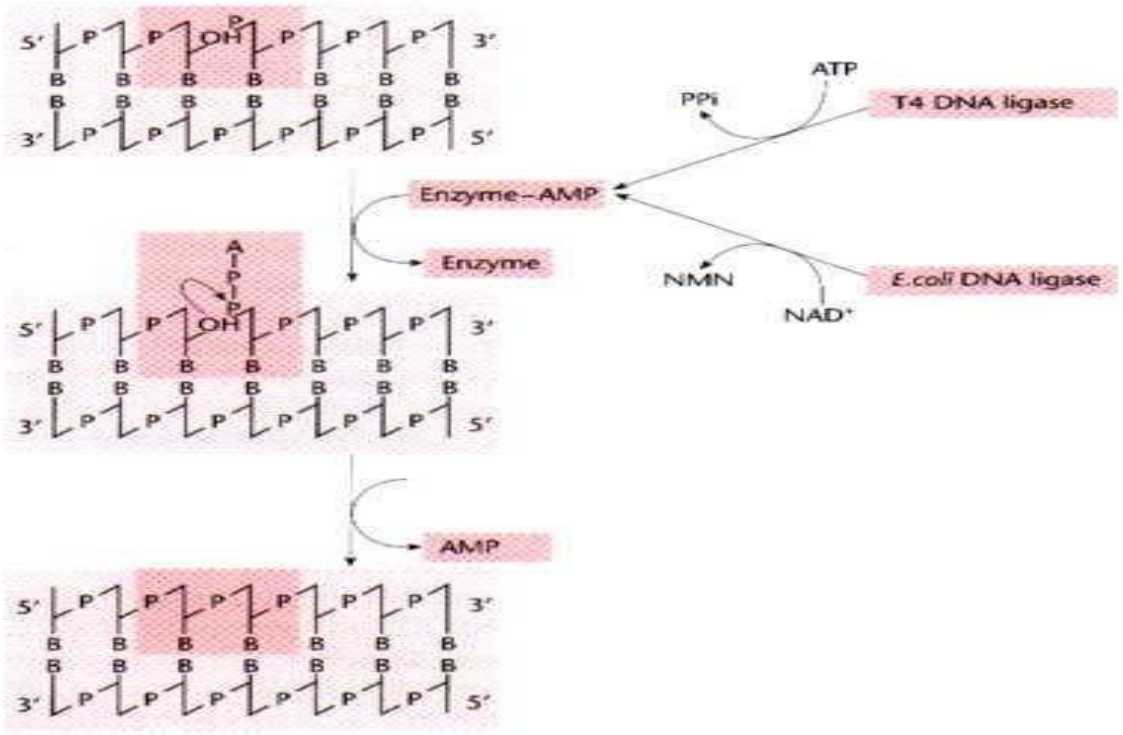


Fig. 3.6 Action of DNA ligase. An enzyme – AMP complex binds to a nick bearing 3′ OH and 5′ P groups. The AMP reacts with the phosphate group. Attack by the 3′ OH group on this moiety generates a new phosphodiester bond, which seals the nick.

DNA LIGASE SELECTION CHART

	Blunt/TA Ligase Master Mix	Instant Sticky-end Master Mix	ElectroLigase®	T4 DNA Ligase	Hi-T4™ DNA Ligase	Salt-T4® DNA Ligase	Quick Ligation™ Kit	T3 DNA Ligase	T7 DNA Ligase	HiFi *Taq* DNA Ligase	*Taq* DNA Ligase	9°N™ DNA Ligase	NEBNext Quick Ligation™ Module	SplintR® Ligase	*E.coli* DNA Ligase
DNA APPLICATIONS															
Ligation of sticky ends	★★	★★★	★★	★★	★★	★★	★★★	★★	★★	★	★	★			★
Ligation of blunt ends	★★★	★	★★	★★	★★	★★	★★★	★★							
T/A cloning	★★★	★	★★	★★	★★	★★	★★	★	★						
Electroporation			★★★	★★	★★										
Ligation of sticky ends only									★★★						
Repair of nicks in dsDNA	★★	★★	★★	★★★	★★★	★★★	★★	★★	★★	★★	★★	★★		★★	★★
High-complexity library cloning	★★	★★	★★	★★★			★★								
Adapter Ligation ▲	★★★	★★	★★	★			★★	★					▲		

Refer to DNA ligase selection Chart
https://international.neb.com/tools-and-resources/selection-charts/dna-ligase-selection-chart

Ligation Temperature

Note- The DNA ligase enzyme has optimal activity at 25°C so the ligation reaction is carried out at a temperature that is a trade-off between the optimal temperatures for bringing the DNA ends together (1°C) and the enzymatic reaction (25°C). Normally 1hr at 16°C is fine but since bringing the DNA ends together is the least efficient part of the reaction favoring this by lowering the temperature to 8°C/4°C can give even more efficiency. However, the enzyme will work very slowly at this temperature so a long (e.g. overnight) incubation time is required.

RNA LIGASE

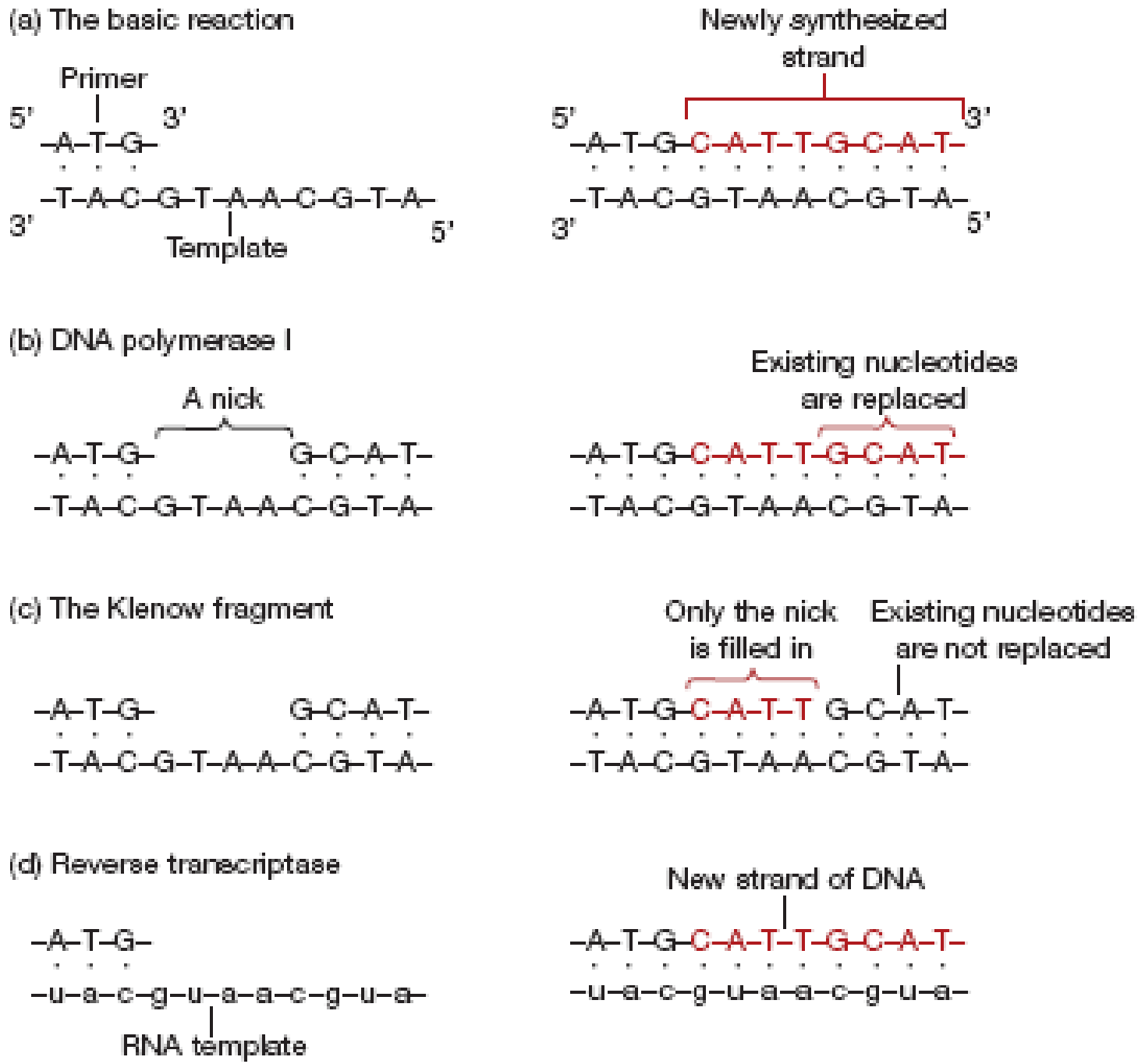
Ligase	Product Number	Recommended Reaction Temp. (Useful Range)	Heat inact.	Co-factor	Main Applications	Other notes
T4 RNA Ligase	M0204S/L	37°C (4-37°C)	Y (65°C)	ATP	ssRNA/ssRNA ligation and ssRNA circularization	
T4 RNA Ligase 2	M0239S/L	37°C (4-37°C)	N	ATP	Ligation of dsRNA nicks and ssRNA to ssRNA	Can also ligate the 3' end of RNA or DNA to a 5'-pDNA when annealed to an RNA complement, and the 3' end of RNA to a 5'-pRNA when annealed to an DNA complement, with reduced efficiency.
T4 RNA Ligase 2, Truncated	M0242S/L	25°C (4-37°C)	Y (65°C)	N/A	Ligation of the 3'-end of ssRNA to 5' adenylated ssDNA and 5' adenylated ssRNA	
T4 RNA Ligase 2, Truncated K227Q	M0351S/L	25°C (4-37°C)	Y (65°C)	N/A	Ligation of the 3'-end of ssRNA to 5' adenylated ssDNA and 5' adenylated ssRNA	Reduction of side products vs M0242.
T4 RNA Ligase 2, Truncated KQ	M0373S/L	25°C (4-37°C)	Y (65°C)	N/A	Ligation of the 3'-end of ssRNA to 5' adenylated ssDNA and 5' adenylated ssRNA	Preferred choice for ligation of ssRNA to preadenylated adapters. Reduction of side products vs M0242.
RtcB Ligase	M0458S	37°C	Y (65°C)	GTP	Ligation of ssRNA with 3'phosphate or 2',3'- cyclic phosphate to the 5'- OH of ssRNA.	

T4 RNA Ligase 1 catalyzes the ligation of a 5´ phosphoryl-terminated nucleic acid donor to a 3´ hydroxyl-terminated nucleic acid acceptor through the formation of a 3´ → 5´ phosphodiester bond with hydrolysis of ATP to AMP and PPi. Substrates include single-stranded RNA and DNA as well as dinucleoside pyrophosphates.

Polymerases

- When describing a polymerase enzyme, the terms DNA- dependent or RNA-dependent may be used to indicate the type of nucleic acid template that the enzyme uses.

  - DNA-dependent DNA polymerase copies DNA into DNA
  - RNA-dependant DNA polymerase copies RNA into DNA
  - DNA-dependant RNA polymerase transcribes DNA into RNA


(a) The basic reaction
Primer
5'
3'
–A–T–G–
–T–A–C–G–T–A–A–C–G–T–A–
3'
5'
Template
Newly synthesized strand
5'
3'
–A–T–G–C–A–T–T–G–C–A–T–
–T–A–C–G–T–A–A–C–G–T–A–
3'
5'
(b) DNA polymerase I
A nick
–A–T–G–
G–C–A–T–
–T–A–C–G–T–A–A–C–G–T–A–
Existing nucleotides are replaced
–A–T–G–C–A–T–T–G–C–A–T–
–T–A–C–G–T–A–A–C–G–T–A–
(c) The Klenow fragment
–A–T–G–
G–C–A–T–
–T–A–C–G–T–A–A–C–G–T–A–
Only the nick is filled in
Existing nucleotides are not replaced
–A–T–G–C–A–T–T G–C–A–T–
–T–A–C–G–T–A–A–C–G–T–A–
(d) Reverse tranecriptase
–A–T–G–
–u–a–c–g–u–a–a–c–g–u–a–
RNA template
New strand of DNA
–A–T–G–C–A–T–T–G–C–A–T–
–u–a–c–g–u–a–a–c–g–u–a–

Klenow Fragment

The Klenow fragment is a large protein fragment produced when DNA polymerase I from E. coli is enzymatically cleaved by the protease subtilisin.

It retains the 5' → 3' polymerase activity and the 3’ → 5’ exonuclease activity for removal of preceding nucleotides and proofreading, but loses its 5' → 3' exonuclease activity.

Because the 5' → 3' exonuclease activity of DNA polymerase I from *E. coli* makes it unsuitable for many applications, the Klenow fragment, which lacks this activity, can be very useful in research. The Klenow fragment is extremely useful for research-based tasks such as:

- Removal of 3’ overhangs or fill-in of 5’ overhangs to form blunt ends
- Generates probes using random primers
- Second strand cDNA synthesis

Exo-Klenow Fragment

- Just as the 5' → 3' exonuclease activity of DNA polymerase I from *E.coli* can be undesirable, the 3' → 5' exonuclease activity of Klenow fragment can also be undesirable for certain applications. This problem can be overcome by introducing mutations in the gene that encodes Klenow. This results in forms of the enzyme being expressed that retain 5' → 3' polymerase activity, but lack any exonuclease activity (5' → 3' or 3' → 5'). This form of the enzyme is called the **exo- Klenow fragment**.
- The exo-Klenow fragment is used in some fluorescent labeling reactions for microarray, random primer labeling, DNA sequencing by the dideoxy method.
- Note-This enzyme will leave a single-base 3´ overhang on a significant proportion of DNA fragments during fill-in of 5´-overhangs. Therefore, Klenow Fragment, Exonuclease Minus, is not recommended for preparation of blunt-ended fragments for ligation
-

T4 DNA Polymerase

- **T4 DNA Polymerase** catalyzes the synthesis of DNA in the 5´→ 3´ direction and requires the presence of template and primer. This enzyme has a 3´→ 5´ exonuclease activity which is much more active than that found in DNA Polymerase I (*E. coli*). Unlike *E. coli* DNA Polymerase I, T4 DNA Polymerase does not have a 5´→ 3´ exonuclease function.

Highlights

- Extreme fidelity
- Gap filling (no strand displacement activity)
- Best enzyme for creating blunt ends
- Isolated from a recombinant source
- **Product Source**
- Purified from a strain of *E. coli* that carries the T4 DNA Polymerase gene.

- **Applications**
- 3´ overhang removal to form blunt ends
- 5´ overhang fill-in to form blunt ends
- Second strand synthesis in site-directed mutagenesis
- Probe labeling using replacement synthesis

Polymerase comparison Chart

Polymerases for DNA Manipulation	3'–>5' Exonuclease	Error Rate (x 10^{-6})	5'–>3' Exonuclease	Strand Displacement	Nick Translation	Extend RNA Primer	Extension from Nick	dU Tolerance	Resulting Ends	Popular Formats Available	Applications
T7 DNA Polymerase (unmodified)	++++	15[h]	No	_	No	Yes	No		Blunt	T7 DNA Polymerase (unmodified)	
Sulfolobus DNA Polymerase IV	_		No	_	No				3'A	Sulfolobus DNA Polymerase IV	Trans lesion bypass
Therminator™ DNA Polymerase	_		No	+	No	Yes	Yes	Yes	3'A	Therminator™ DNA Polymerase	Chain terminator
DNA Polymerase I (E. coli)	++	9[f]	Yes	_[j]	Yes	Yes	Yes	Yes	Blunt	DNA Polymerase I (E. coli)	Second strand synthesis, nick translation
DNA Polymerase I, Large (Klenow) Fragment'	++	18[g]	No	+	No	Yes	Yes	Yes	Blunt	DNA Polymerase I, Large (Klenow) Fragment'	Blunting, primer extension
Klenow Fragment (3'→5' exo_)	_	100[g]	No	+++	No	Yes	Yes	Yes	3'A	Klenow Fragment (3'→5' exo_)	A tailing, random priming labeling
T4 DNA Polymerase	++++	<1[f]	No	_	No	Yes	No		Blunt	T4 DNA Polymerase	Blunting

Taq DNA polymerase

- **Taq** polymerase is a thermostable **DNA polymerase** I named after the thermophilic bacterium ... **Taq** makes DNA products that have A (adenine) **overhangs** at their **3'** ends.

DNA MODIFYING ENZYMES

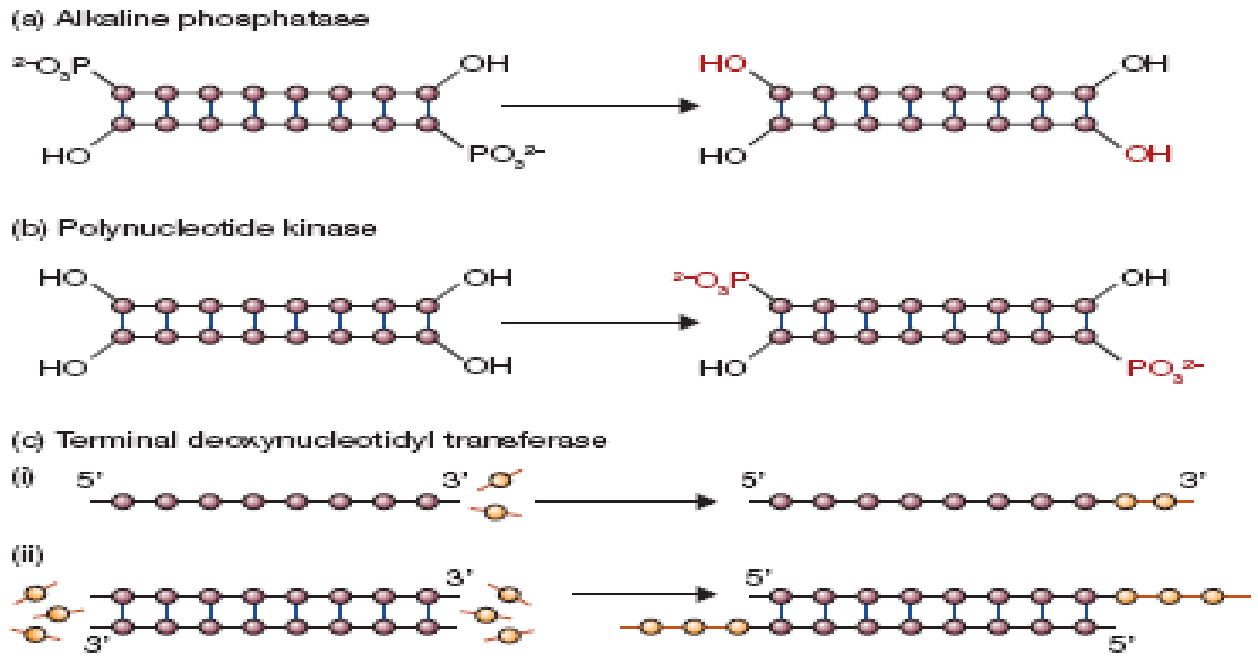


Figure 4.6

The reactions catalyzed by DNA modifying enzymes. (a) Alkaline phosphatase, which removes 5′-phosphate groups. (b) Polynucleotide kinase, which attaches 5′-phosphate groups. (c) Terminal deoxynucleotidyl transferase, which attaches deoxyribonucleotides to the 3′ termini of polynucleotides in either (i) single-stranded or (ii) double-stranded molecules.

- **Alkaline phosphatase** (from *E. coli*, calf intestinal tissue, or arctic shrimp), which removes the phosphate group present at the **5′ terminus** of a DNA molecule (Figure 4.6a).
- **Polynucleotide kinase** (from *E. coli* infected with T4 phage), which has the reverse effect to alkaline phosphatase, adding phosphate groups onto free 5′ termini (Figure 4.6b).
- **Terminal deoxynucleotidyl transferase** (from calf thymus tissue), which adds one or more deoxyribonucleotides onto the **3′ terminus** of a DNA molecule (Figure 4.6c).

Polynucleotide Kinase

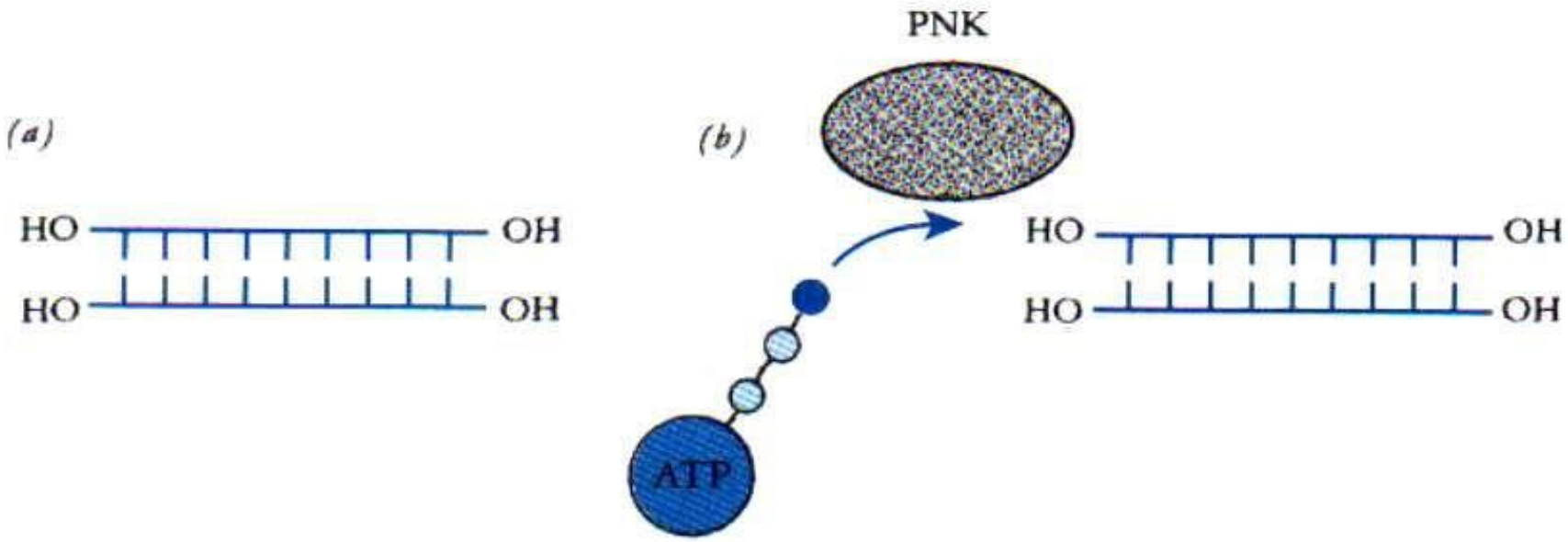


Fig. 3.2 End labelling DNA using polynucleotide kinase (PNK). (*a*) DNA is dephosphorylated using phosphatase to generate 5′-OH groups. (*b*) The terminal phosphate of [γ-^{32}P]ATP (solid circle) is then transferred to the 5′ terminus by PNK. The reaction can also occur as an exchange reaction with 5′-phosphate termini.

Terminal Transferase

Figure 4.26

Homopolymer tailing: (a) synthesis of a homopolymer tail; (b) construction of a recombinant DNA molecule from a tailed vector plus tailed insert DNA; (c) repair of the recombinant DNA molecule.