The FIC has referred the following resources for preparation of the ppt for the Topic "Enzymes" in Genetic Engineering:

1. Principles of gene manipulation : an introduction to genetic engineering ,R.W. Old, S.B. Primrose

2. J. SAMBROCK, E. F. FRITSCH and T. MANIATIS, Molecular Cloning, A Laboratory Manual, Volumes 1, 2 and 3.

3.https://www.ncbi.nlm.nih.gov/

4.Nucleic Acids Res. 2010 Jan; 38(Database issue): D234–D236,Published online 2009 Oct 21. doi: 10.1093/nar/gkp874,PMCID: PMC2808884, REBASE—a database for DNA restriction and modification: enzymes, genes and genomes, Richard J. Roberts, Tamas Vincze, Janos Posfai, and Dana Macelis

❖ ***SAVITHRI BHAT***

Nucleases

Classified

1.functionally (Exo and Endo nucleases)

2.Substrate (DNA/RNA, SS OR DS)

Eg of both Exo and Endo nucleases :

S1 nuclease, mung bean nuclease

Eg of Exo: Bal 31, exo III, exo VII

Endonuclease: RE

Exonucleases and Endonucleases

1. **S1 nuclease**: A.oryzae, 30 kda, ss DNA/RNA from 5 ‘ Work in extreme conditions such as high ionic strength and low Ph (4.5)

2. **Mung bean nuclease**: ss DNA/RNA from 5 ‘,

35 kda, mung bean.

Exonucleases

1.Bal 31: Alteromonas espejiana, 3 to 5 and 5 to 3 exo,

Kinetically 2 forms: S (slow, 85 kda)and F(fast 105 kda), S derived from F

2.Exo III:E coli, 31 kda, coded by x gene , from 5 ‘, multifunctional

a.Phosphomonoesterase : removes 3’ modified ends such as 3’ phosphoglycolate, 3’ phosphoglyceraldehyde.

b.Nucleotidyl hydrolase: remove apurinic/apyrimidinic sites

c.Rnase H : cleaves RNA from RNA-DNA hybrid

3.Exo VIII: Ecoli, removes from 5’ and 3’ both, 2 subunit, 10 kda xse A, 54 kda xse B, cant cleave blunt ends.

Restriction endonucleases

- Restriction endonucleases RESTRICT viruses
  - Viral genome is destroyed upon entry
- Restriction endonuclease = Restriction enzymes
  - Endo (inside), nuclease (cuts nucleic acid)
- Restriction endonuclease recognizes a short and specific DNA sequence and cuts it from inside.
- The specific DNA sequence is called recognition sequence

RESTRICTION

- 1952-53: Luria and Human discovered the phenomenon of restriction and modification
- Named as host-induced, or host-controlled, variation.
- Bacteriophages varied in their ability to grow on different strains of *E.coli*.
- Once growth was achieved on one host strain, the phages could continue to grow happily on this strain.
- However, the phages were now restricted in their ability to grow on other strains.

NOMENCLATURE

- Smith and Nathans (1973) proposed enzyme naming scheme(SMITH, NATHAN, ARBER WON NOBEL PRIZE, 1978)
  - three-letter acronym for each enzyme derived from the source organism
  - First letter from genus
  - Next two letters represent species
  - Additional letter or number represent the strain and order discovered.
- 1.Example *Hind*II was isolated from *Haemophilus influenzae* strain d, Order discovered II

  2.Example: EcoR1

  Genus: Escherichia
  Species: coli
  Strain: R
  Order discovered: 1

CLASSIFICATION

- Synonymous to Restriction Endonuclease
- Endonuclease: Cut DNA from inside
- Evolved independently rather than diverging form a common ancestor
- Broadly classified into four Type

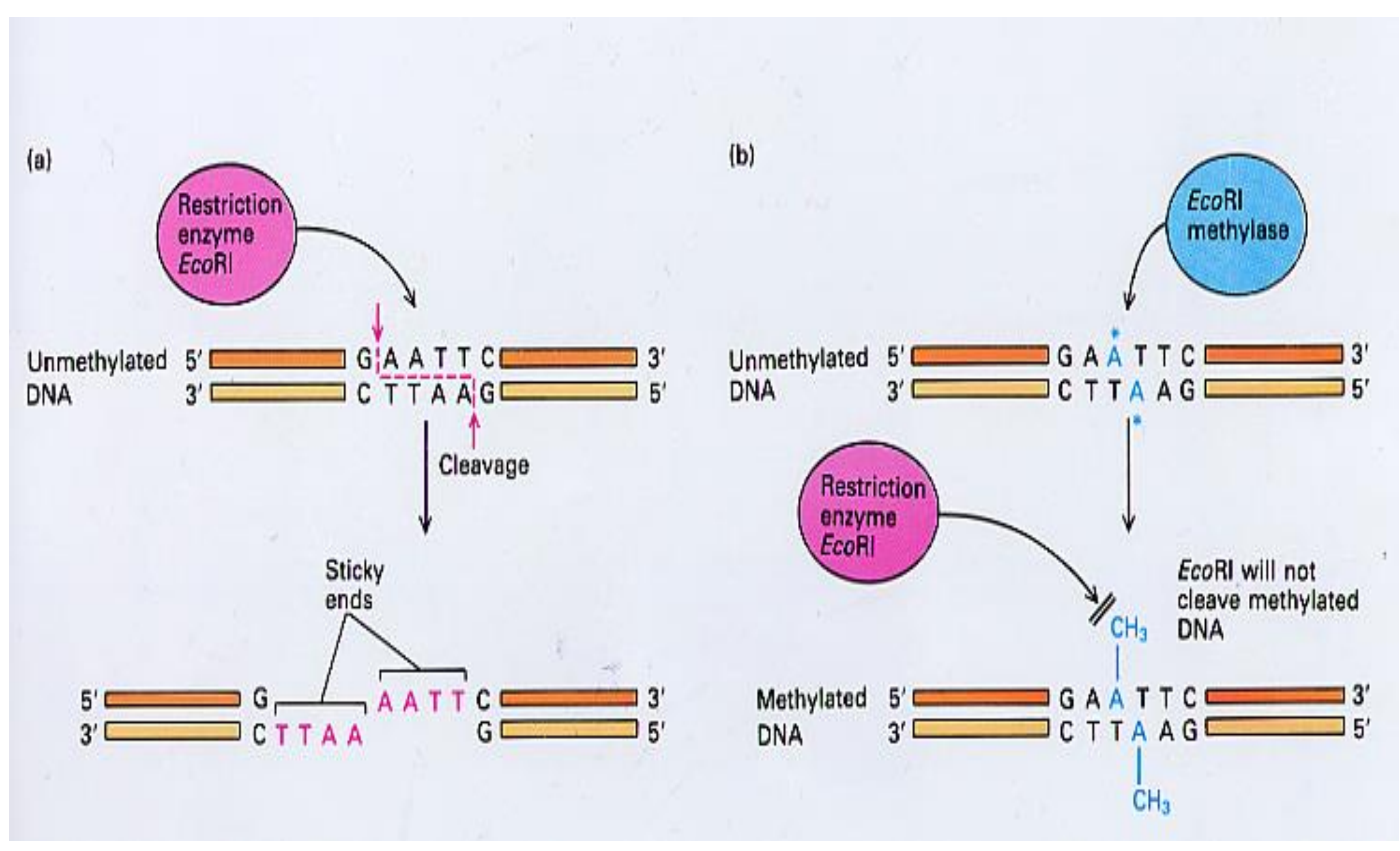
(R-M) system

- Restriction-modification (R-M) system
  - Endonuclease activity: cuts foreign DNA at the recognition site
  - Methyltransferase activity: protects host DNA from cleavage by the restriction enzyme.
  - Methyleate one of the bases in each strand

SELF PROTECTION

- Bacteria protect their self DNA from restriction digestion by methylation of its recognition site.
- Methylation is adding a methyl group (CH_3) to DNA.
- Restriction enzymes are classified based on recognition sequence and methylation pattern.

Methylation



TYPE I

- Multi-subunit proteins
- Function as a single protein complex
- Contain
    - two R (restriction) subunits,
    - two M (methylation) subunits and
    - one S (specificity) subunit
- Cleave DNA at random length from recognition site

TYPEIII

- Large enzymes
- Combination restriction-and-modification
- Cleave outside of their recognition sequences
- Require two recognition sequences in opposite orientations within the same DNA molecule
- No commercial use or availability

TYPE IV

- Cleave only modified DNA (methylated, hydroxymethylated and glucosyl-hydroxymethylated bases).
- Recognition sequences have not been well defined
- Cleavage takes place ~30 bp away from one of the sites.
- Sequence similarity suggests many such systems in other bacteria and archaea.

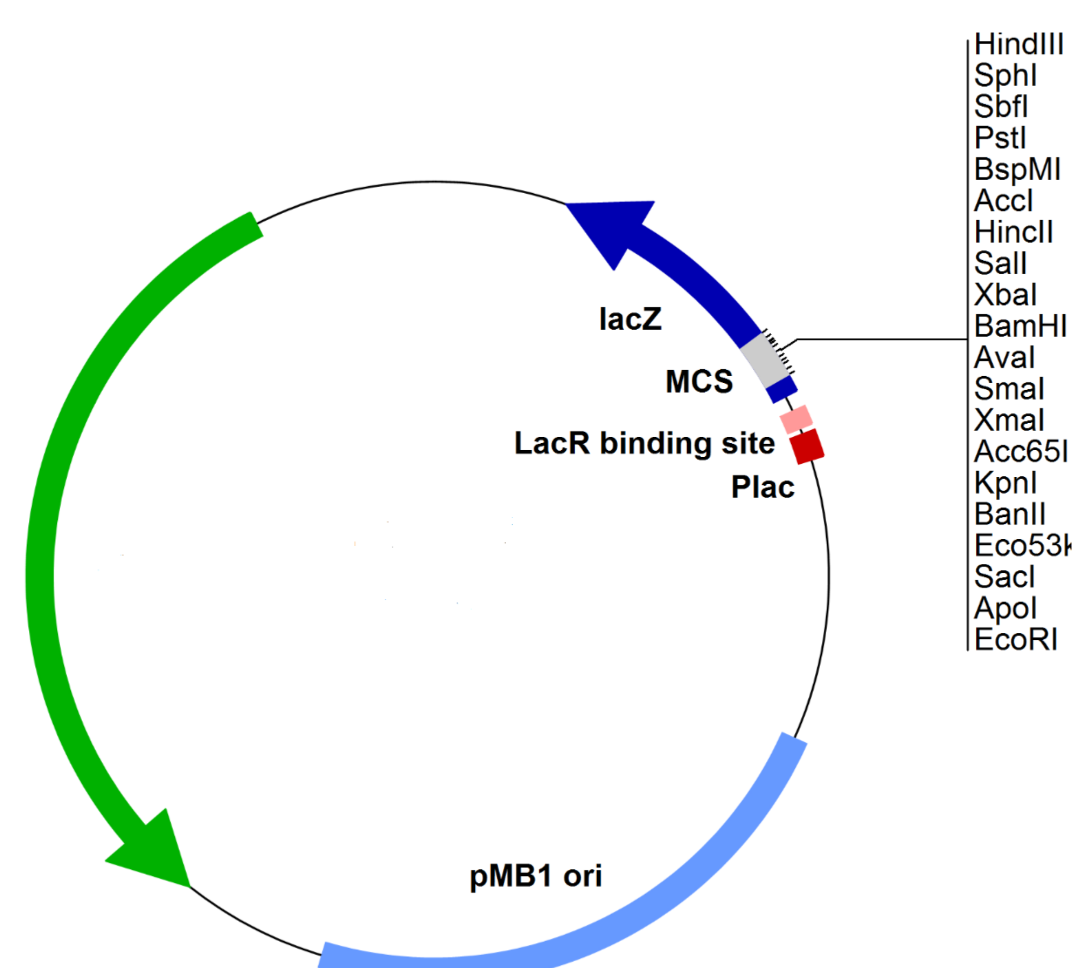
TYPE II

- Most useful for gene analysis and cloning
- More than 3500 REs
- Recognize 4-8 bp sequences
- Need Mg^{2+} as cofactor
- Cut in close proximity of the recognition site
- Homodimers
- ATP hydrolysis is not required

TYPE II

- Each restriction enzyme always cuts at the same recognition sequence.
- Produce the same gel banding pattern (fingerprint)
- Many restriction sequences are palindromic. For example,
  - 5’ GAATTC 3’
  - 3’ CTTAAG 5

Sl No	Enzyme	Organism from which derived	Target sequence (cut at *) 5' -->3'
1	Bam HI	*Bacillus amyloliquefaciens*	G* G A T C C
2	Eco RI	*Escherichia coli RY 13*	G* A A T T C
3	Hind III	*Haemophilus inflenzae Rd*	A* A G C T T
4	Mbo I	*Moraxella bovis*	*G A T C
5	Pst I	*Providencia stuartii*	C T G C A * G
6	Sma I	*Serratia marcescens*	C C C * G G G
7	Taq I	*Thermophilus aquaticus*	T * C G A
8	Xma I	*Xanthamonas malvacearum*	C * C C G G G



HindIII
SphI
SbfI
PstI
BspMI
AccI
HincII
SalI
XbaI
BamHI
AvaI
SmaI
XmaI
Acc65I
KpnI
BanII
SacI
ApoI
EcoRI
lacZ
MCS
LacR binding site
Plac
pMB1 ori

Enzymes recognize specific 4-8 bp sequences

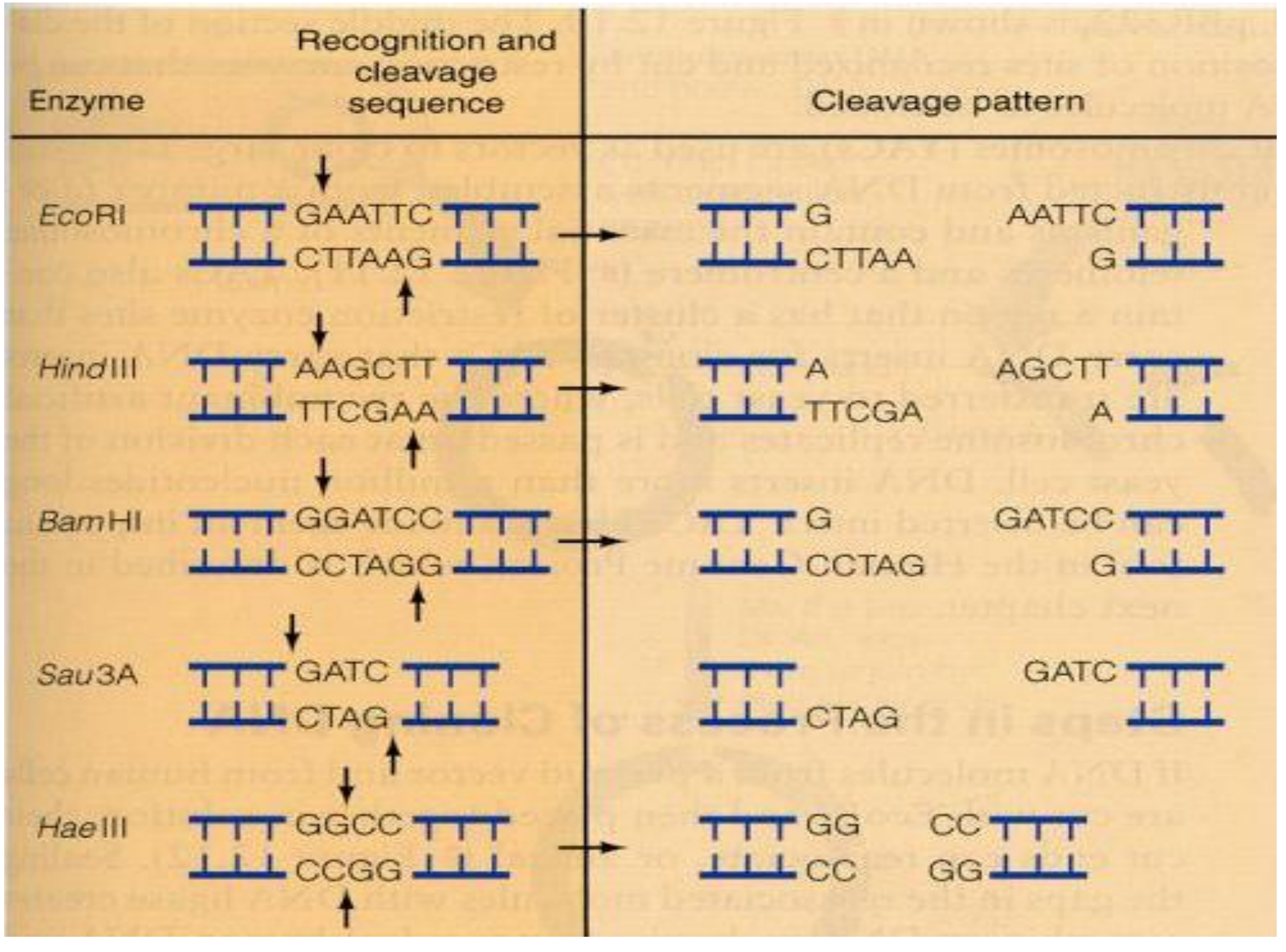
Some enzymes cut in a staggered fashion - “sticky ends”

EcoRI 5’…GAATTC…3’
3’…CTTAAG…5’

Some enzymes cut in a direct fashion – “blunt ends”

PvuII 5’…CAGCTG…3’
3’…GTCGAC…5’

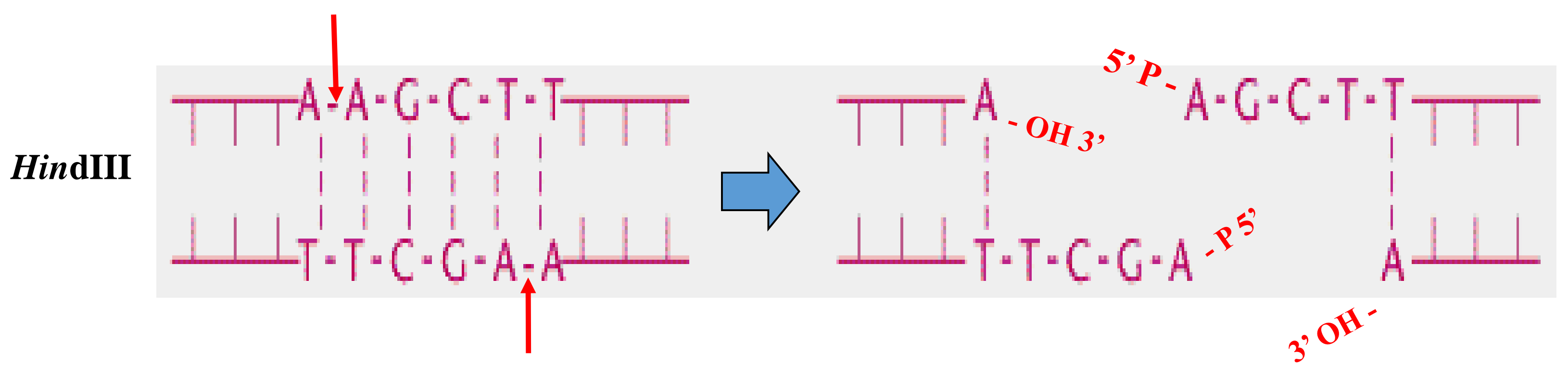
CLEAVAGE PATTERN

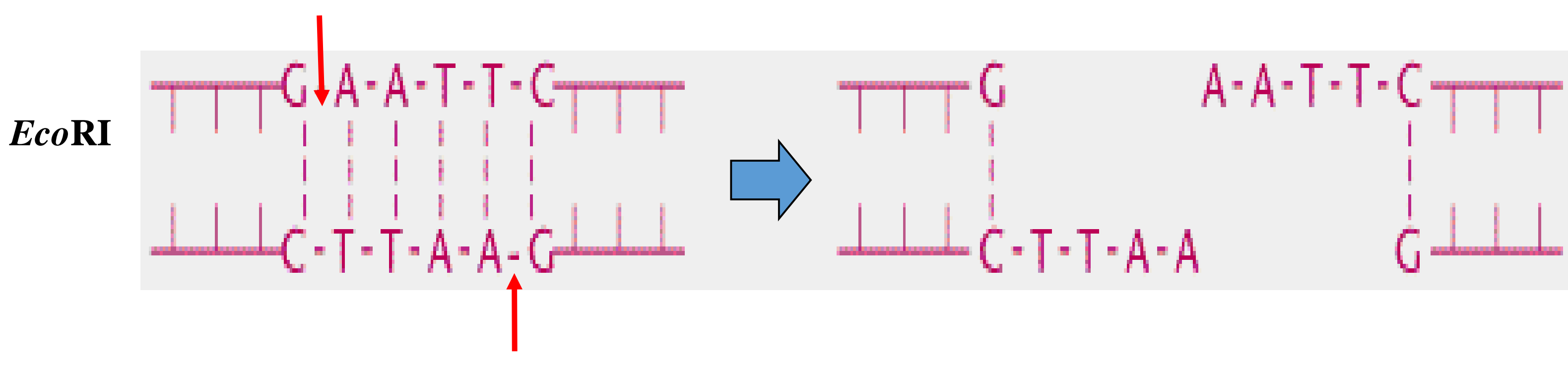


Staggered cuts -sticky-ends

- Most restriction enzymes make staggered cuts
- Staggered cuts produce single stranded “sticky-ends”
- DNA from different sources can be spliced easily because of sticky-end overhangs

Staggered cuts -sticky-ends

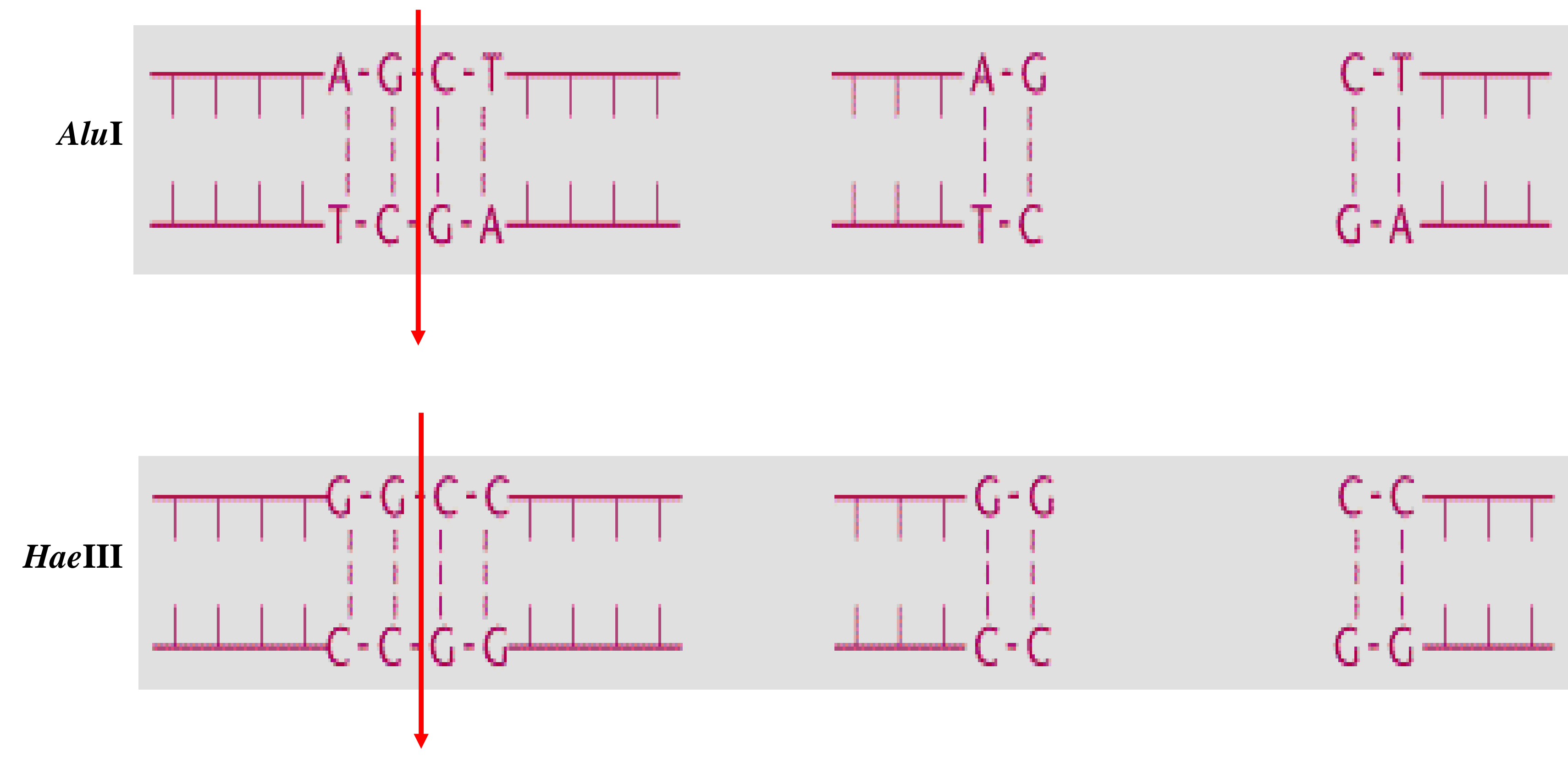




Blunt end

- Some restriction enzymes cut DNA at opposite base
- They leave blunt ended DNA fragments
- These are called blunt end cutters

Blunt end



Restriction Enzyme Use

Discovery of enzymes that cut and paste DNA make genetic engineering possible.DNA fragments from different species can be ligated (joined) to create Recombinant DNA

Application:

RFLP analysis (Restriction Fragment Length Polymorphism)

DNA sequencing

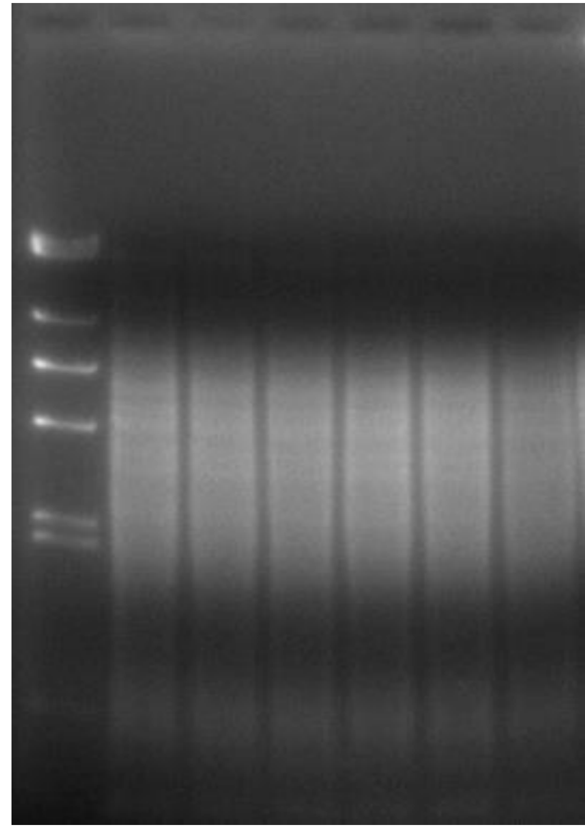
DNA storage – libraries

Transformation

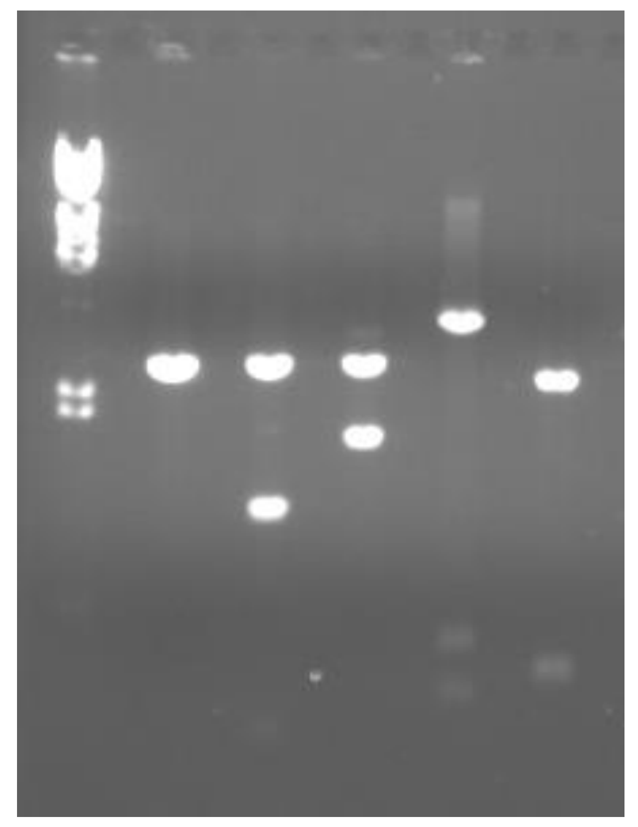
Large scale analysis – gene chips

Typical restriction digestion expt. gel

Genomic DNA Digest



Plasmid DNA Digest



Star activity

- **Star activity** is a relaxation or alteration of the specificity of restriction enzyme mediated cleavage of DNA that can occur under reaction conditions that differ significantly from those optimum for the enzyme.
- Differences which can lead to star activity include low ionic strength, high pH, and high glycerol concentrations

Methylase

- ***Dam-Dcm and CpG Methylation***
- DNA methyltransferases (MTases) that transfer a methyl group from S-adenosylmethionine to either adenine or cytosine residues, are found in a wide variety of prokaryotes and eukaryotes.
- Methylation should be considered when digesting DNA with restriction endonucleases because cleavage can be blocked or impaired when a particular base in the recognition site is methylated.
- ***Prokaryotic Methylation***
- In prokaryotes, MTases have most often been identified as elements of restriction/modification systems that act to protect host DNA from cleavage by the corresponding restriction endonuclease. Most laboratory strains of *E. coli* contain three site-specific DNA methylases.
- Dam methylase–methylation at the N^6 position of the adenine in the sequence GATC .
- Dcm methyltransferases–methylation at the C5 position of the second cytosine in the sequences CCAGG and CCTGG .
- EcoKI methylase–methylation of adenine in the sequences AAC(N^6)GTGC and GCAC(N^6)GTT

- Some or all of the sites for a restriction endonuclease may be resistant to cleavage when isolated from strains expressing the Dam or Dcm methylases if the methylase recognition site overlaps the endonuclease recognition site. For example, plasmid DNA isolated from *dam+ E. coli* is completely resistant to cleavage by MboI (*Morexella bovis),* which cleaves at GATC sites.

- Not all DNA isolated from *E. coli* is methylated to the same extent. While pBR322 DNA is fully modified (and is therefore completely resistant to MboI digestion), only about 50% of λ DNA Dam sites are methylated, presumably because the methylase does not have the opportunity to methylate the DNA fully before it is packaged into the phage head. As a result, enzymes blocked by Dam or Dcm modification will yield partial digestion patterns with λ DNA.

- Restriction sites that are blocked by Dam or Dcm methylation can be un-methylated by cloning your DNA into a *dam⁻, dcm⁻* strain of *E. coli*, such as *dam⁻/dcm⁻* Competent *E. coli* .Restriction sites can also be blocked if an overlapping site is present.

- ***Eukaryotic Methylation***
- CpG MTases, found in higher eukaryotes (e.g., Dnmt1), transfer a methyl group to the C^5 position of cytosine residues. Patterns of CpG methylation are heritable, tissue specific and correlate with gene expression. Consequently, CpG methylation has been postulated to play a role in differentiation and gene expression .
- The effects of CpG methylation are mainly a concern when digesting eukaryotic genomic DNA. CpG methylation patterns are not retained once the DNA is cloned into a bacterial host.
- ***Methylation Sensitivity***
- The table below summarizes methylation sensitivity for NEB restriction enzymes, indicating whether or not cleavage is blocked or impaired by Dam, Dcm or CpG methylation if or when it overlaps each recognition site. This table should be viewed as a guide to the behavior of the enzymes listed rather than an absolute indicator.
- **REBASE,** the restriction enzyme database, for more detailed information and specific examples upon which these guidelines are based

Dam Methylation: G^{m}ATC	
Blocked by Overlapping Dam	
AlwI	GGATC
BcgI[1]	CGATCNNNNNTGC
BclI	TGATCA
BsaBI	GATCNNNNATC
BspDI	ATCGATC
BspEI	TCCGGATC
BspHI	TCATGATC
ClaI	ATCGATC
DpnII	GATC
HphI	GGTGATC
Hpy188I	TCNGATC
Hpy188III	TCNNGATC
MboI	GATC
MboII	GAAGATC
NruI	TCGCGATC
Taq$^{\alpha}$ I	TCGATC
XbaI	TCTAGATC

Not Blocked by Overlapping Dam			
AsiSI	BglII	BstYI	Sau3AI
BamHI	BsaWI	PvuI	

Dcm Methylation: C^{m}CWGG	
Blocked by Overlapping Dcm	
Acc65I[2]	GGTACCWGG
AlwNI	CAGNNCCTGG
ApaI	GGGCCCWGG
AvaII	GGWCCWGG
BanI	GGYRCCWGG
BsaI	GAGACCWGG
BsaHI[2]	GRCGCCWGG
BslI[2]	CCWGGNNNNGG
BsmFI	GGGACT
BssKI	CCWGG
BstXI	CCAGGNNNNTGG
EaeI	YGGCCAGG
EcoO109I	RGGNCCTGG
FseI	GGCCGGCCWGG
MscI	TGGCCAGG
NlaIV	GGNNCCWGG
PflMI	CCAGGNNNTGG
PpuMI	RGGWCCTGG
PspGI	CCWGG
PspOMI	GGGCCCWGG
Sau96I	GGNCCWGG
ScrFI	CCWGG
SexAI	ACCWGGT

DNA ligase

DNA ligase is a specific type of enzyme that facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond. It plays a role in repairing single-strand breaks in duplex DNA in living organisms, but some forms (such as DNA ligase IV) may specifically repair double-strand breaks (i.e. a break in both complementary strands of DNA).

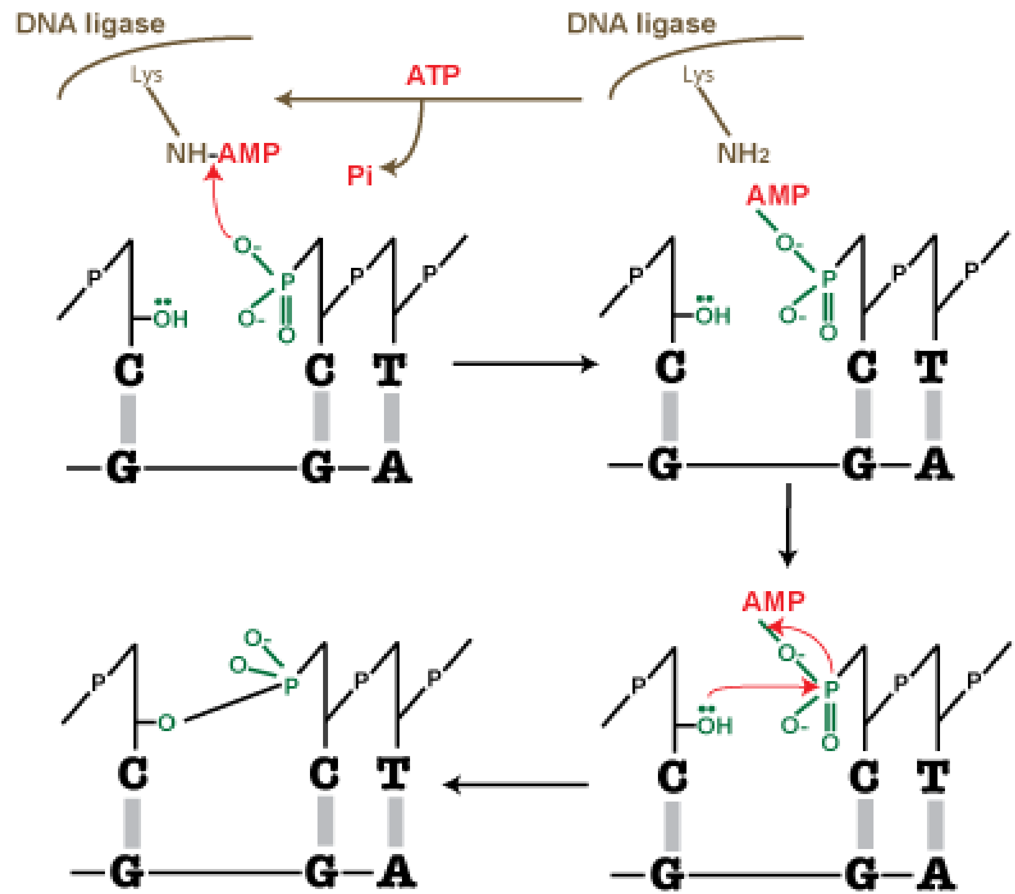
Working condition for ligase

- Ligase works best with sticky or cohesive ends.
- Ligase will also work with blunt ends, although higher enzyme concentrations and different reaction conditions are required.

Ligase mechanism

The mechanism of DNA ligase is to form two covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide, ("acceptor") with the 5' phosphate end of another ("donor"). ATP is required for the ligase reaction, which proceeds in three steps:

1. Adenylation (addition of AMP) of a residue in the active center of the enzyme.
2. Transfer of the AMP to the 5' phosphate of the donor, formation of a pyrophosphate bond.
3. Formation of a phosphodiester bond between the 5' phosphate of the donor and the 3' hydroxyl of the acceptor.



Ligases types

In mammals, there are four specific types of ligase.

- DNA ligase I: ligates the DNA of the lagging strand after the Ribonuclease H has removed the RNA primer from the Okazaki fragments.
- DNA ligase II: alternatively spliced form of DNA ligase III found in non-dividing cells.
- DNA ligase III: complexes with DNA repair protein to aid in sealing DNA during the process of nucleotide excision repair and recombinant fragments.
- DNA ligase IV: complexes with DNA repair protein . It catalyzes the final step in the non-homologous end joining DNA double-strand break repair pathway

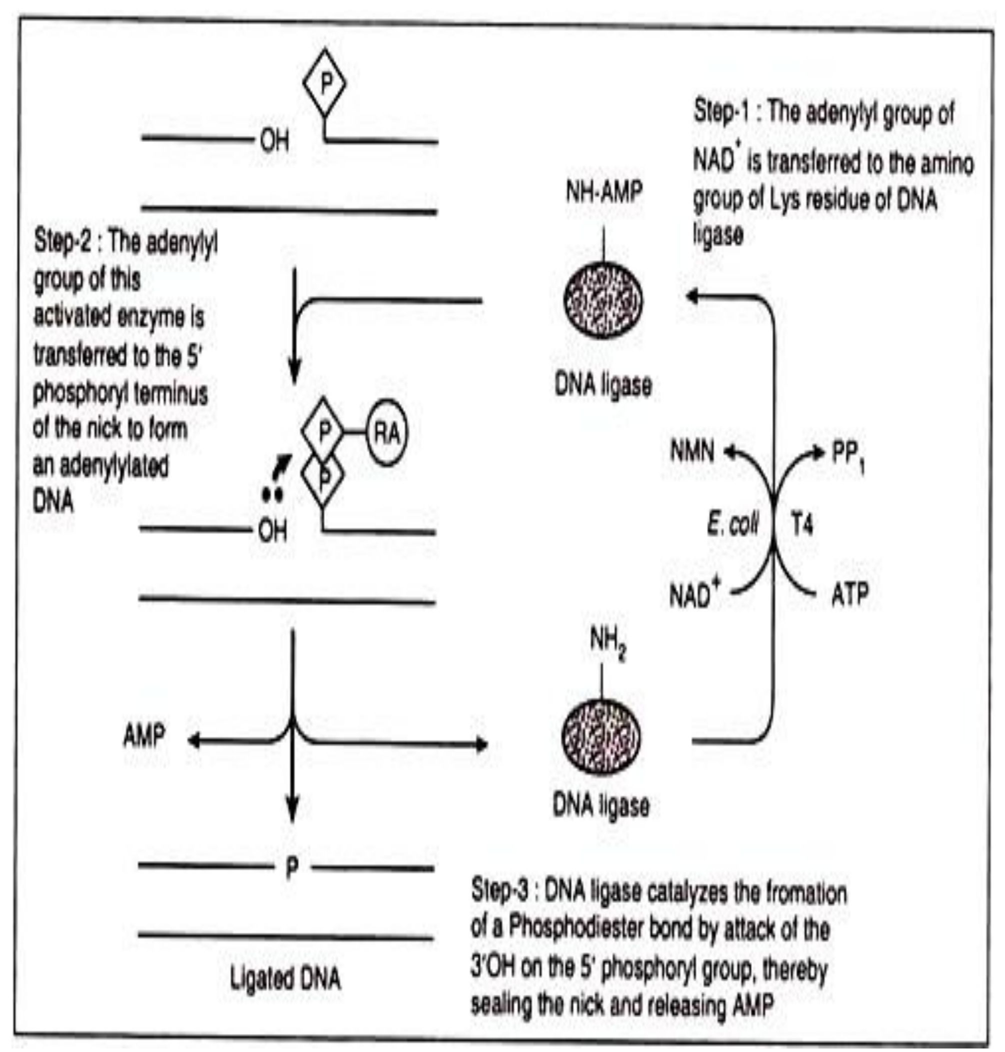
DNA ligase from eukaryotes and some other microbes uses adenosine triphosphate (ATP).

E coli ligases

- Source:Purified from recombinant *E. coli* strain
- It is heat Inactivated at 65°C for 20 minutes
- Catalyzes the formation of a phosphodiester bond between 5'-phosphate and 3'-hydroxyl termini in duplex DNA containing cohesive ends.

E coli ligases

- Does not ligate RNA to DNA. This enzyme ligates only DNA fragments with cohesive termini.
- Requires NAD^+ (nicotinamide adenine dinucleotide) as a cofactor, in contrast to other ligases which use ATP.
- Ligation of blunt-ended fragments is extremely inefficient. For ligation of blunt-ended fragments T4 DNA Ligase is better.



T4 ligases

- Catalyzes the formation of a phosphodiester bond between 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA.
- Obtained from T4 phage.
- This enzyme can join blunt end and cohesive end termini as well as repair single stranded nicks in duplex DNA, RNA or DNA/RNA hybrids
- It is heat Inactivated at 65°C for 10 minutes.

T4 ligases

- ATP is an essential cofactor for the reaction. This contrasts with *E. coli* DNA ligase which requires NAD.
- Room Temperature Ligation:
  For convenience, ligations may be done at room temperature (20-25°C). For cohesive (sticky) ends, reaction is carried out for 10 minutes. For blunt ends, for 2 hours.
- Alternatively, NEB's Quick Ligation Kit is uniquely formulated to ligate both blunt and cohesive (sticky) ends in 5 minutes at room temperature.

Thermophilic ligases

- Thermophilic and thermostable DNA ligase was purified to near homogeneity from the extract of Thermus thermophilus
- The purified enzyme consists of a single polypeptide of about 79 kda in molecular weight.
- The enzyme requires divalent cations, Mg2+ or Mn2+, and the optimum concentration of these ions .
- The enzyme also requires NAD as a cofactor.
- The pH optimum is 7.4-7.6
- The nick-closing activity of the enzyme was observed over a wide range of the temperature from 15 to 85 degrees C and the optimum temperature is 65-72 degrees C
- The enzyme was stable for 1 week at 37 degrees C, its activity dropped by 50% within 2 days at 65 degrees C.

Applications in molecular biology research

DNA ligases have become an indispensable tool in modern molecular biology research for generating recombinant DNA sequences. For example, DNA ligases are used with restriction enzymes to insert DNA fragments, often genes, into plasmids.

Ribonuclease

Ribonuclease (**RNase**) is a type of nuclease that catalyzes the degradation of RNA into smaller components. Ribonucleases can be divided into endoribonucleases and exoribonucleases

Major types of endoribonucleases

- **RNase A** is an RNase that is commonly used in research. RNase A (e.g., bovine pancreatic ribonuclease A is one of the hardiest enzymes in common laboratory usage; one method of isolating it is to boil a crude cellular extract until all enzymes other than RNase A are denatured. It is specific for single-stranded RNAs. It cleaves 3'end of unpaired C and U residues.
- **RNase H** is a ribonuclease that cleaves the RNA in a DNA/RNA duplex to produce ssDNA. RNase H leaves a 5'-phosphorylated product.
- **RNase I** cleaves 3'-end of ssRNA at all dinucleotide bonds leaving a 5' hydroxyl, and 3' phosphate.
- **RNase III** is a type of ribonuclease that cleaves rRNA (16s rRNA and 23s rRNA). It also digests double strands RNA .
- **RNase L** upon activation, destroys all RNA within the cell.

Major types of endoribonucleases

- **RNase PhyM** is sequence specific for single-stranded RNAs. It cleaves 3'-end of unpaired A and U residues.
- **RNase T1** is sequence specific for single-stranded RNAs. It cleaves 3'-end of unpaired G residues.
- R**Nase T2** is sequence specific for single-stranded RNAs. It cleaves 3'-end of all 4 residues, but preferentially 3'-end of As.
- **RNase U2** is sequence specific for single-stranded RNAs. It cleaves 3'-end of unpaired A residues.
- **RNase V1** is non-sequence specific for double-stranded RNAs. It cleaves base-paired nucleotide residues.

Major types of exoribonucleases

- **Polynucleotide Phosphorylase (PNPase)** functions as an exonuclease as well as a nucleotidyltransferase .
- R**ase PH** functions as an exonuclease as well as a nucleotidyltransferase.
- **RNase II** is responsible for the processive 3'-to-5' degradation of single-stranded RNA.
- **RNase R** is a close homolog of RNase II, but it can, unlike RNase II, degrade RNA with secondary structures without help of accessory factors.
- **RNase D** is involved in the 3'-to-5' processing of pre-tRNAs
- **Oligoribonuclease** degrades short oligonucleotides to mononucleotides.
- **Exoribonuclease I** degrades single-stranded RNA from 5'-to-3', exists only in eukaryotes.

Precautions to be taken while working with RNAses

- When working with RNA, care must be taken to create a ribonuclease-free environment. As they are ubiquitous.
- RNases are very stable and difficult to inactivate. To ensure success, it is important to maintain an RNase-free environment starting with RNA purification and continuing through analysis

Precautions to be taken while working with RNAses

1. The most common sources of Rnase contamination are hands (skin) and bacteria or mold that may be present on airborne dust particles or laboratory glassware. To prevent contamination from these sources, wear gloves at all times and use sterile technique when handling the reagents used for RNA isolation or analysis.
2. Whenever possible, use sterile, disposable plasticware for handling RNA. These materials are generally RNase-free and do not require pretreatment to inactivate RNase.
3. Treat non-disposable glassware and plasticware before use to ensure that it is RNase-free. Bake glassware at 250°C overnight. Thoroughly rinse plasticware with 0.1N NaOH/1mM EDTA and then with diethyl pyrocarbonate (DEPC)-treated water.

Precautions to be taken while working with RNAses

4. While most sources of fresh, deionized water are free of contaminating RNases, deionized water can be a contributor of RNase activity. If degradation of the target or probe RNA occurs, it may be necessary to test the water source for RNase activity by incubating an RNA sample with the water and checking for degradation by gel electrophoresis.

5. Chemicals for use in RNA isolation and analysis should be reserved for RNA applications and kept separate from chemicals for other uses. Wear gloves when handling the chemicals, and use only

baked spatulas and untouched weigh boats or weighing paper.

6. Autoclaving alone is not sufficient to inactivate RNases. Solutions prepared in the lab should be treated by adding DEPC to 0.05% and incubating overnight at room temperature. The treated solutions should be autoclaved for 30 minutes to remove any trace of DEPC

RNASES INHIBITORs: Diethylpyrocarbonate (DEPC)

- **Diethylpyrocarbonate** (DEPC), also called diethyl dicarbonate , diethyl oxydiformate, ethoxyformic anhydride, or pyrocarbonic acid diethyl ester, is used in the laboratory to inactivate RNase enzymes in water and on laboratory wares. It does so by the covalent modification of histidine, lysine, cysteine tyrosine residues of RNAses.
- DEPC-treated (and therefore RNase-free) water is used in handling of RNA in the laboratory, to reduce the risk of RNA being degraded by RNases.
- Water is usually treated with 0.1% v/v diethylpyrocarbonate for at least 2 hours at 37 °C and then autoclaved (at least 15 min) to inactivate traces of DEPC.
- Inactivation of DEPC in this manner yields CO_2, H_2O and ethanol. Higher concentrations of DEPC are capable of deactivating larger amounts of RNase, but remaining traces or byproducts may inhibit further biochemical reactions such as in vitro translation.
- Furthermore, chemical modification of RNA such as carboxymethylation is possible when traces of DEPC or its byproducts are present, resulting in impaired recovery of intact RNA

RNASES INHIBITORs: protein from Human Placenta

RNase Inhibitor from Human Placenta expressed in *E. coli* strain (recombinant strain that carries the Ribonuclease Inhibitor gene from human placenta. The 50 kDa protein inhibits RNases by binding noncovalently in a 1:1 ratio.

Specifically inhibits ribonucleases (RNases) A, B and C . It is not effective against RNase 1, RNase T1, S1 Nuclease, RNase H or RNase from *Aspergillus*.

In addition, no inhibition of polymerase activity is observed when RNase Inhibitor, Human Placenta is used with *Taq* DNA Polymerase, Reverse Transcriptases, or Phage RNA Polymerases .

RNase Inhibitor : RNasinPlus

- Rnasin Plus RNase Inhibitor is a recombinant mammalian RNase inhibitor that is expressed as a soluble protein in *E. coli*.
- The protein is capable of inhibiting eukaryotic RNases (e.g., RNase A and RNase B) similarly to human placental RNase inhibitor. It doesnot inhibit Reverse Transcriptases or *Taq* DNA Polymerases.
- The inhibitor offers increased resistance to oxidation over the human version of the protein. Two cysteines in the human protein have been identified as especially sensitive to oxidation and react by forming a disulfide bond that can block the active site of the inhibitor. RNasin Plus, through natural amino acid diversity, lacks the ability to form this site-blocking disulfide.

RNASES INHIBITORs :Ribonucleoside-vanadyl complex

- Ribonucleoside-vanadyl complex is a potent inhibitor of various ribonucleases

- The ribonucleoside-vanadyl complex should be added to the buffers not containing EDTA .
- Doesnot work in translation systems and with reverse transcriptase . The vanadyl complex can be used in the selective degradation of DNA while preserving RNA .

- The toxological properties of this compound have not been fully investigated-avoid contact with skin.

DNA Modifying Enzymes & DNA polymerases

- DNA Modifying Enzymes

  Nucleases: DNase and RNase
  DNA Ligase
  Alkaline Phosphatase
  Polynucleotide Kinase

- DNA polymerases

  -E. coli DNA Polymerase I
  - **Klenow Fragment of E. coli DNA Polymerase I**
  - **T4 DNA Polymerase**
  - **T7 DNA Polymerase**
  - **Terminal Transferase**
  - **Thermostable DNA Polymerases (Taq, Pfu, Vent, etc.)**
  - **Reverse Transcriptases**
  - **Bacteriophage RNA Polymerases**

DNA Modifying Enzymes

Alkaline Phosphatase

Polynucleotide Kinase

Terminal Transferase

Alkaline phosphatase (ALP, ALKP)

Alkaline phosphatase (**ALP**, **ALKP**) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called *dephosphorylation*. As the name suggests, alkaline phosphatases are most effective in an alkaline environment.

Alkaline phosphatase (ALP, ALKP)…

- **There are several sources of alkaline phosphatase that differ in how easily they can be inactivated:**
- Bacterial alkaline phosphatase (BAP) is the most active of the enzymes, but also the most difficult to destroy at the end of the dephosphorylation reaction.
- Calf intestinal alkaline phosphatase (CIP) is purified from bovine intestine. This is phosphatase most widely used in molecular biology labs because, although less active than BAP, it can be effectively destroyed by protease digestion or heat (75C for 10 minutes in the presence of 5 mM EDTA).
- Placental alkaline phosphatase (PLAP) and its C terminally truncated version that lacks the last 24 amino acids- the secreted alkaline phosphatase (SEAP)
- Shrimp alkaline phosphatase is derived from a cold-water shrimp and is promoted for being readily destroyed by heat (65C for 15 minutes).

Alkaline phosphatase (ALP, ALKP)…

There are two primary uses for alkaline phosphatase in DNA manipulations:

- **Removing 5' phosphates from plasmid and bacteriophage vectors** that have been cut with a restriction enzyme. In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby greatly facilitates ligation of other DNA fragments into the vector (e.g.subcloning).

- **Removing 5' phosphates from fragments of DNA prior to labeling with radioactive phosphate**. Polynucleotide kinase is much more effective in phosphorylating DNA if the 5' phosphate has previously been removed.
- It is usually recommended that dephosphorylation of DNAs with blunt or 5'-recessed ends be conducted using a higher concentration alkaline phosphatase or at higher temperatures than for DNAs with 5' overhangs.

Alkaline phosphatase Inhibitors

All mammalian alkaline phosphatase except placental (PALP and SEAP) are inhibited by homoarginine, and, in similar manner, all except the intestinal and placental ones are blocked by levamisole. Heating for ~2 hours at 65°C inactivated most ALP except Placental (PALP and SEAP).

Terminal transferase

- Terminal transferase catalyzes the addition of nucleotides to the 3' terminus of DNA. Interestingly, it works on single-stranded DNA, including 3' overhangs of double-stranded DNA, and is thus an example of a DNA polymerase that does not require a primer. It can also add homopolymers of ribonucleotides to the 3' end of DNA.
- The much preferred substrate for this enzyme is protruding 3' ends, but it will also, less efficiently, add nucleotides to blunt and 3'-recessed ends of DNA fragments. Cobalt is a necessary cofactor for activity of this enzyme.

Terminal transferase

Terminal transferase is useful for at least two procedures:

Labeling the 3' ends of DNA: Most commonly, the substrate for this reaction is a fragment of DNA generated by digestion with a restriction enzyme that leaves a 3' overhang, but oligodeoxynucleotides can also be used. When such DNA is incubated with tagged nucleotides and terminal transferase, a string of the tagged nucleotides will be added to the 3' overhang or to the 3' end of the oligonucleotide .

Polynucleotide kinase (PNK)

- **Polynucleotide kinase (PNK) is an enzyme that catalyzes the transfer of a phosphate from ATP to the 5' end of either DNA or RNA.** It is a product of the T4 bacteriophage, and commercial preparations are usually products of the cloned phage gene expressed in E. coli.
- **The enzymatic activity of PNK is utilized in two types of reactions:**

- In the "**forward reaction**", PNK transfers the gamma phosphate from ATP to the 5' end of a polynucleotide (DNA or RNA). The target nucleotide is lacking a 5' phosphate either because it has been dephorphorylated or has been synthesized chemically.

- In the "**exchange reaction**", target DNA or RNA that has a 5' phosphate is incubated with an excess of ADP - in this setting, PNK will first transfer the phosphate from the nucleic acid onto an ADP, forming ATP and leaving a dephosphorylated target. PNK will then perform a forward reaction and transfer a phosphate from ATP onto the target nucleic acid.

Polynucleotide kinase (PNK)

- The efficiency of phosphorylating via the exchange reaction is considerably less than for the forward reaction. In addition to its phosphorylating activity, PNK also has 3' phosphatase activity, although this has little significance to molecular technologists.
- **There are two major indications for phosphorylating nucleic acids and hence use of PNK are:**
- Phosphorylating linkers and adaptors (CONVERSION OF BLUNT ENDS TO STICKY)or fragments of DNA as a prelude to ligation which requires a 5' phosphate. This includes products of polymerase chain reaction which are typically generated using non-phosphorylated primers.
- Radiolabeling oligonucleotides, usually with ^{32}P, for use as hybridization probes.
- **PNK is inhibited** by small amounts of ammonium ions, so ammonium acetate should not be used to precipitate nucleic acids prior to phosphorylation. Low concentrations of phosphate ions, or NaCl concentrations greater than about 50 mM, also inhibit this enzyme.

Linkers, Adaptors &Homopolymer tailing

When same sticky end creating enzyme used for cleavage of vector and gene of interest, then DNA ligase seals the nick between gene of interest and vector and creates recombinant vector. Whereas when blunt end creating enzyme used then resealing become difficult.

These situations are overcome by using

i) Linkers

ii) Adaptors

iii) Homopolymer tailing

Linkers

Linker molecules are used to ligate the blunt end gene of interest with cohesive end vectors. They are normally synthesized decameric oligonucleotides, which contain sites for one or more restriction endonucleases which will create sticky ends.

The linker can be ligated to both ends of the foreign gene to be cloned, and then treated with restriction endonuclease to produce a sticky ended fragment which can be incorporated into a vector molecule that has been cut with the same restriction endonuclease.

Insertion by means of the linkers creates restriction enzyme target sites at each end of the foreign gene and so enables the foreign gene to be excised and recovered after cloning and amplification in the host bacterium.

Linkers…

Blunt-ended DNA molecule

5′ 3′

3′ 5′

Linkers

5′ GGATCC 3′

3′ CCTAGG 5′

BamHI recognition sequence

5′ 3′

3′ 5′

Linkers attached to the ends of the DNA molecule

BamHI

5′ 3′

3′ 5′

BamHI sticky end

G 3′

CCTAG 5′

Adaptors

When linkers added to link at the end of blunt end of gene interest, then there is an possibility of joining of multiple linkers at the end. This makes some time larger genes and waste of linker molecules. This problem is overcome by using adapters.

Adapter is a synthetic, double stranded oligonucleotide used to attach sticky ends to a blunt ended molecule. It contain normal 5' and 3' end at blunt end and the sticky end of adapter molecule is modified in such manner that it contain OH group on both 5' and 3' ends.

This is achieved by using alkaline phosphatases. In contrast to linkers, adapters contain preformed sticky ends and joining blunt ends. Because of lack of 5' phosphate group on sticky end prevents adapter polymer formation.

After the adaptors have been attached, the 5'OH terminus is converted to the natural 5'P form by treatment with the enzyme … icky ended fragment t… ector.

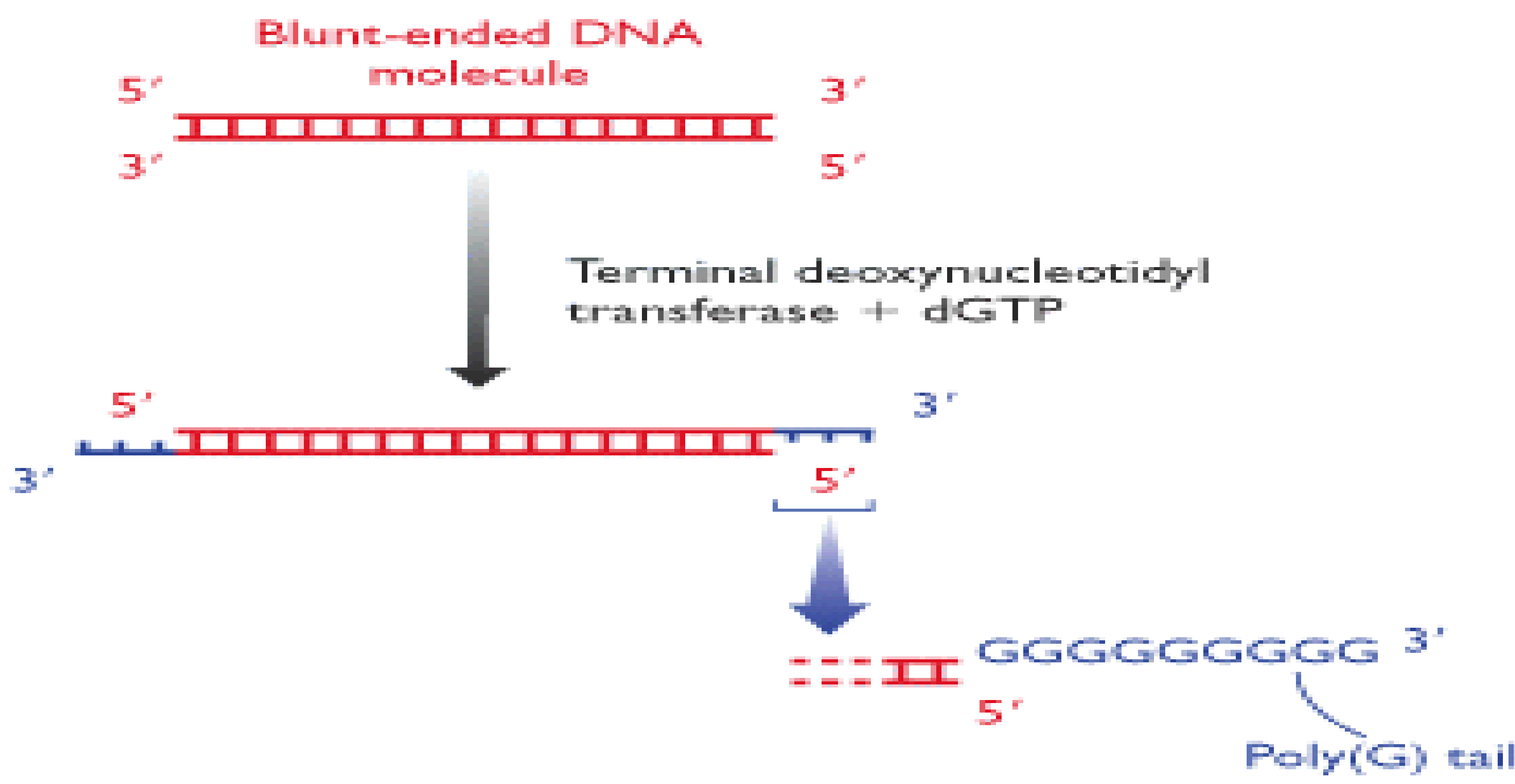
5' - OH - GATCCCCGGG - OH - 3'
3' -GGGCCC - P -5'
OH- -OH
OH- -P

Homopolymer tailing

A homopolymer is simply a polymer in which all the subunits are the same. Tailing involves using the enzyme terminal deoxynucleotidyl transferase, to add a series of nucleotides on to the 3'-OH termini of a double stranded DNA molecule.

If this reaction is carried out in the presence of just one deoxynucleotide, then a homopolymer tail will be produced. In this method, gene of interest is tailed with one nucleotide and vector is tailed with an complementary base and when they are combined then only vector recombined with gene of interest.

Homopolymer tailing..



DNA Polymerase

-E. coli DNA Polymerase I

- **Klenow Fragment of E. coli DNA Polymerase I**
- **T4 DNA Polymerase**
- **T7 DNA Polymerase**
- **Terminal Transferase**
- **Thermostable DNA Polymerases (Taq, Pfu, Vent, etc.)**
- **Reverse Transcriptases**
- **Bacteriophage RNA Polymerases**

E. coli DNA Polymerase I

- Nick translation of DNA and Second strand cDNA synthesis

DNA Polymerase I (*E coli*) is a DNA-dependent DNA polymerase with inherent 3´→ 5´ and 5´→ 3´ exonuclease activities . The 5´→ 3´ exonuclease activity removes nucleotides ahead of the growing DNA chain, allowing nick-translation. Source: An *E. coli* strain that carries an overexpressed copy of the *polA* gene.DNA polymerase I obtained from *E. coli* is used extensively for molecular biology research. However, the 5' -> 3' exonuclease activity makes it unsuitable for many applications. Fortunately this undesirable enzymatic activity can be simply removed from the holoenzyme to leave a useful molecule called the Klenow fragment, widely used in molecular biology. Exposure of DNA polymerase I to the protease subtilisin cleaves the molecule into a smaller fragment, which retains only the DNA polymerase and proofreading activities

T7 DNA polymerase

- **The DNA polymerase of T7 bacteriophage has DNA polymerase and 3' -> 5' exonuclease activities, but lacks a 5' -> 3' exonuclease domain.** It is thus very similar in activity to Klenow fragment and T4 DNA polymerase.
- **The claim to fame for T7 DNA polymerase is it's processivity.** That is average length of DNA synthesized before the enzyme dissociates from the template is considerably greater than for other enzymes. Due to this talent, **the principle use of T7 DNA polymerase is in DNA sequencing by the chain termination technique.**
- T7 DNA polymerase can be chemically-treated or genetically engineered to abolish it's 3' -> 5' exonuclease activity. These forms of the enzyme are marketed under the name **Sequenase and Sequenase 2.0**, and are widely used for DNA sequencing reactions.

T4 DNA polymerase

- T4 is a bacteriophage of E. coli. **The activities of T4 DNA polymerase are very similar to Klenow fragment** of DNA polymerase I - it functions as a 5' -> 3' DNA polymerase and a 3' -> 5' exonuclease, but does not have 5' -> 3' exonuclease activity.
- In general, T4 DNA polymerase is used for the same types of reactions as Klenow fragment, particularly in blunting the ends of DNA with 5' or 3' overhangs. *There is however, difference between the two enzymes that have practical significance:*
- The 3' -> 5' exonuclease activity of T4 DNA polymerase is roughly 200 times that of Klenow fragment making it preferred by many investigators for blunting DNAs with 3'overhangs.

Klenow fragment

- Because the 5' → 3' exonuclease activity of DNA polymerase I from *E. coli m*akes 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:
- Synthesis of double-stranded DNA from single-stranded templates
- Filling in (meaning removal of overhangs to create blunt ends) recessed 3' ends of DNA fragments
- Digesting away protruding 3' overhangs
- Preparation of radioactive DNA probes
- The Klenow fragment was also the original enzyme used for greatly amplifying segments of DNA in the polymerase chain reaction(PCR) process,before being replaced by thermostable/thermophilic enzymes such as Taq polymerase

Thermophilic DNA polymerases

- The original report of Taq DNA polymerase enzyme, purified from the hot springs bacterium *Thermus aquaticus*, was published in 1976. Roughly 10 years later, the polymerase chain reaction was developed and shortly thereafter "Taq" became a household word in molecular biology circles. Currently, the world market for Taq polymerase is in the hundreds of millions of dollars each year.
- **The thermophilic DNA polymerases, like other DNA polymerases, catalyze template-directed synthesis of DNA from nucleotide triphosphates.** A primer having a free 3' hydroxyl is required to initiate synthesis and magnesium ion is necessary. In general, they have maximal catalytic activity at 75 to 80C, and substantially reduced activites at lower temperatures. At 37C, Taq polymerase has only about 10% of its maximal activity.

Thermophilic DNA polymerases..

In addition to Taq DNA polymerase, several other thermostable DNA polymerases have been isolated and expressed from cloned genes. Three of the most-used polymerases are described in the following table:

Polymerase	3'->5' Exonuclease	Source and Properties
Taq	No	From *Thermus aquaticus*. Halflife at 95C is 1.6 hours.
Pfu	Yes	From *Pyrococcus furiosus*. Appears to have the lowest error rate of known thermophilic DNA polymerases.
Vent	Yes	From *Thermococcus litoralis*; also known as Tli polymerase. Halflife at 95 C is approximately 7 hours.

Thermophilic DNA polymerases..

- In addition to the native polymerases listed in the table above, a number of mutants have been generated and are available, for example, a form of Vent polymerase that lacks the 3'->5' exonuclease and is thereby more similar to Taq.
- One of the most discussed characteristics of thermostable polymerases is their error rate. Error rates are measured using several different assays, and as a result, estimates of error rate vary, particularly when the assays are performed by different labs. As would be expected from first principles, polymerases lacking 3'->5' exonuclease activity generally have higher error rates than the polymerases with exonuclease activity. The total error rate of Taq polymerase has been variously reported between 1×10^{-4} to 2×10^{-5} errors per base pair. Pfu polymerase appears to have the lowest error rate at roughly 1.5×10^{-6} error per base pair, and Vent is probably intermediate between Taq and Pfu.
- Error rate is not the only consideration in chosing a polymerase for PCR, otherwise Taq polymerase would not continue to be the most widely used enzyme by far for the PCR applications..

Reverse transcriptase

Reverse transcriptase is a common name for an enzyme that functions as a RNA-dependent DNA polymerase. They are encoded by retroviruses, where they copy the viral RNA genome into DNA prior to its integration into host cells.

Reverse transcriptases have two activities:

- **DNA polymerase activity:** In the retroviral life cycle, reverse transcriptase copies only RNA, but, as used in the laboratory, it will transcribe both single-stranded RNA and single-stranded DNA templates with essentially equivalent efficiency. In both cases, an RNA or DNA primer is required to initiate synthesis.

- **RNase H activity:** RNase H is a ribonuclease that degrades the RNA from RNA-DNA hybrids, such as are formed during reverse transcription of an RNA template. This enzyme functions as both an endonuclease and exonuclease in hydrolyzing its target.

Reverse transcriptase..

All retroviruses have a reverse transcriptase, but the enzymes that are available commercially are derived from one of two retroviruses, either by purification from the virus or expression in E. coli:

- Moloney murine leukemia virus: a single polypeptide
- Avian myeloblastosis virus: composed of two peptide chains
- Both enzymes have the same fundamental activities, but differ in a number of characteristics, including temperature and pH optima. Most importantly, the murine leukemia virus enzyme has very weak RNase H activity compared to the avian myeloblastosis enzyme, which makes it the clear choice when trying to synthesize complementary DNAs for long messenger RNAs.

Reverse transcriptase..

Reverse transcriptase is used to copy RNA into DNA. This task is integral to cloning complementary DNAs (cDNAs), which are DNA copies of mature messenger RNAs. Cloning cDNAs is the technique usually initiated by mixing short (12-18 base) polymers of thymidine (oligo dT) with messenger RNA such that they anneal to the RNA's polyadenylate tail. Reverse transcriptase is then added and uses the oligo dT as a primer to synthesize so-called first-strand cDNA.

ACGGCUAUACCGCUAGCCUAAGCAAAAAAA

TTTTTTT primer

TTTTTTT
ACGGCUAUACCGCUAGCCUAAGCAAAAAAA

reverse transcriptase

dATP, dCTP, dGTP, dCTP

TGCCGATATGGCGATCGGATTCGTTTTTTT
ACGGCUAUACCGCUAGCCUAAGCAAAAAAA

Reverse transcriptase…

Another common use for reverse transcriptase is to generate DNA copies of RNAs prior to amplifying that DNA by polymerase chain reaction (PCR). Reverse transcription PCR usually called simply RTPCR, is a useful tool for cloning cDNAs.In most cases, standard preparations of reverse transcriptase are used for RTPCR, but mutated forms with relatively high thermal stability have been developed to facilitate the process.

RNA Polymerases

- Bacteriophage RNA Polymerases:Phage-encoded DNA-dependent RNA polymerases are used for in vitro transcription to generate defined RNAs. Most commonly, the reaction utilizes ribonucleotides that are labeled with radionuclides or some other tag, and the resulting labeled RNA is used as a probe for hybridization. Other applications of in vitro transcription including making RNAs for in vitro translation or to study RNA structure and function.
- Several bacteriophage RNA polymerases are commercially available. They are named after the phage that encodes them, and either purified from phage-infected bacteria or produced as recombinant proteins.

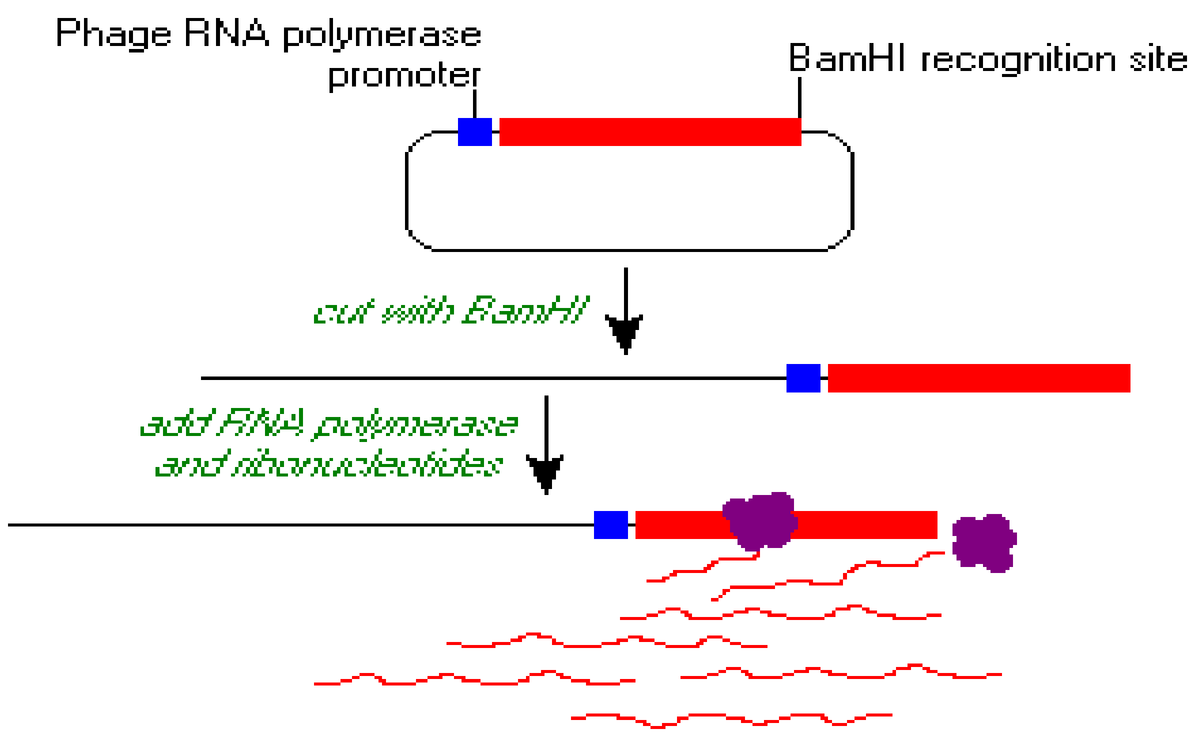
RNA Polymerases…

Polymerase Name	Host of Encoding Phage	Promoter Sequence
T7 RNA polymerase	*E. coli*	**TAATA**CGA**CTCACTATAGGG**
T3 RNA polymerase	*E. coli*	**AATTA**ACC**CTCACTAAAGGG**
SP6 RNA polymerase	*Salmonella typhimurium*	**AATTTA**GGTGACACTATAGAA

RNA Polymerases…

Many of the plasmids used for carrying cloned DNA incorporate promoters for bacteriophage RNA polymerases adjacent to the cloning site. This allows one to readily obtain either mRNA-sense or antisense transcripts from the inserted DNA. The process is often called run-off transcription, because the plasmid is cut with a restriction enzyme downstream of the inserted DNA, which causes the polymerase to fall off the template when it reaches that spot.

RNA Polymerases…



RNA Polymerases…

If we assume that the RNA transcribed in the figure above has the polarity of a mRNA (e.g. sense), it is easy to modify the construct to express an antisense RNA - simply reverse the orientation of the transcribed region. Indeed, most plasmids used for in vitro transcription have two different phage polymerase promoters flanking the insertion site, which allows transcription of sense RNA with one polymerase and antisense with the other.