Enzymes used in Gene Manipulation
Enzymes used in gene manipulation

- Aid in recombinant DNA technology.
- Originally identified and isolated from different bacteria strains.
- Commercially available as highly purified recombinant enzymes.
Enzymes used in gene manipulation

- Enzymes used in gene manipulation, based on their functions – 5 classes:
  1) Nuclease – cut or degrades DNA molecules
  2) Polymerase – copy or make new strands of DNA
  3) Ligase – joins pieces of DNA fragments together
  4) Modifying enzymes – modify the DNA by adding or removing chemical groups
  5) Topoisomerase – remove or introduce supercoils from covalently closed circular DNA
Nucleases

Degrade DNA at either ends

- Exonuclease III (Exo III) - cleave dsDNA
- Exo III (Exo VII) – cleave ssDNA

Degrade DNA within DNA

- Exonucleases
- Endonucleases

a) Cleave DNA at nonspecific cleavage sites:
   - DNAase I (isolated from bovine pancreas) – digest dsDNA
   - mung bean nuclease (from sprouts of mung bean) – digest ssDNA

b) Cleave DNA at sites specified by specific DNA sequences
   - Restriction endonucleases (RE)

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Polymerases

- Enzymes that can synthesize **new strands of nucleic acids** that are complementary to an existing DNA/RNA strand.

- Only when template has an existing **double-stranded region** that act as a primer to initiate synthesis.
DNA Polymerase

- DNA polymerase adds free nucleotides to the 3’ end of the new strand
- so, the new strand grows in a 5’-3’ direction
- requires a “primer” (short sequence) to initiate extension

- New strands grow in 5’-3’ direction
Polymerases

3 main type of polymerases frequently used

DNA polymerase I
- attaches to the s-stranded portion of a largely dsDNA and initiates synthesis from 5’ to 3’.
- possess inherent 3’ to 5’ and 5’ to 3’ exonuclease activity, removing nucleotides ahead of the growing DNA chain
- Used second strand synthesis of cDNA and nick-translation

Klenow fragment
- derived from DNA polymerase I.
- synthesize DNA strand complementary to the s-stranded portion.
- lacks the both nuclease activity
- major application in DNA sequencing and random priming labeling

Reverse transcriptase
- uses RNA as template.
- synthesize a DNA strand complementary to a RNA template
- used in cDNA cloning (generating cDNA from RNA).
The major polymerases used in DNA cloning

a) **Basic reaction**

5′ -G-A-T-T-G-C-A-T-C-3′

3′ -T-A-G-5′

Primer

5′ -G-A-T-T-G-C-A-T-C-3′


Newly synthesized strand

b) **DNA polymerase I**

5′ -G-A-T-T-G-C-A-T-C-3′

3′ -C-T-A - T-A-G-5′

nick

5′ -G-A-T-T-G-C-A-T-C-3′


nucleotides are replaced

b) **Klenow fragment**

5′ -G-A-T-T-G-C-A-T-C-3′

3′ -C-T-A - T-A-G-5′

5′ -G-A-T-T-G-C-A-T-C-3′

3′ -C-T-A A-C-G-T-A-G-5′

only the nick is filled

b) **Reverse transcriptase**

**RNA**

5′ -G-A-U-U-G-C-A-U-C-3′

3′ T-A-G-5′

5′ -G-A-U-U-G-C-A-U-C-3′


new strand of DNA

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Other DNA polymerases

- **T4 DNA polymerase**
  - strong 3’ to 5’ exonuclease activity but deficient in 5’to3’ exo activity
  - use to form blunt ends by either – removal of 3’ overhangs or fill-in 5’ overhang.

- **T7 DNA polymerase**
  - strong 3’ to 5’ exonuclease activity but deficient in 5’to3’ exo activity
  - rapid extension rate and high fidelity
  - usage: site-directed mutagenesis, and copying long stretches of DNA

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Ligases

- DNA ligases
  - Catalyze the formation of phosphodiester bonds between juxtaposed 5’ phosphate and a 3’ – hydroxyl terminus in duplex DNA
DNA ligases

- Two DNA ligases are used for nucleic acid research – *E. coli* ligase and T4 ligase.
- These enzymes differ in two important properties.
  - Source of energy: T4 ligase uses ATP, while *E. coli* ligase uses NAD.
  - Ability to ligate blunt ends.

Link DNA strand together by forming phosphodiester bonds between the 5'-phosphate and the 3'-OH termini of the discontinuous strands.

Repair single-stranded nicks (breaks) in cell, that arise during DNA replication.

Commonly used = T4 DNA ligase purified from *E. coli* infected with the T4 bacteriophage.

Used in DNA cloning to join two individual pieces of DNA.
RNA ligases

- T4 RNA ligase
- Catalyzes the ATP-dependent covalent joining of single-stranded DNA @ RNA termini.

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Topoisomerases

- Changes conformation of a closed circular DNA by adding or removing supercoils.
- Usage: in the study of DNA replication, not so in DNA cloning
Modifying Enzymes

- Modify the DNA by either **adding or removing** a chemical group.
- 3 most commonly used are:
  
  a) **Alkaline phosphatase**
     - removes a phosphate group from the 5’ end of the DNA.
     - used to prevent vector re-ligation.
  
  b) **Polynucleotide kinase (eg. T4 polynucleotide kinase)**
     - acts in reverse of the alkaline phosphatase by adding a phosphate group (phosphorylation) to the 5’-terminus of a DNA.
  
  c) **Terminal transferase**
     - adds on one or more nucleotides on the 3’ end of a DNA.
Molecular Scissors

Restriction enzymes are molecular scissors

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- Restriction Enzymes scan the DNA code
- Find a very specific set of nucleotides
- Make a specific cut
Restriction endonucleases are enzymes that cleave the sugar-phosphate backbone of DNA.

In practice, a given enzyme cuts both strands of duplex DNA within a stretch of just a few bases.

Several thousand different REs have been isolated, which collectively exhibit a few hundred different sequence (substrate) specificities.
Picking a palindrome

Words that read the same forwards as backwards

Hannah
leveL
Madam

hannaH
deveL
madaM
Palindromes in DNA sequences

Genetic palindromes are similar to verbal palindromes. A palindromic sequence in DNA is one in which the 5’ to 3’ base pair sequence is identical on both strands (the 5’ and 3’ ends refers to the chemical structure of the DNA).

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Each of the double strands of the DNA molecule is complimentary to the other; thus adenine pairs with thymine, and guanine with cytosine.
HaeIII

HaeIII is a restriction enzyme that searches the DNA molecule until it finds this sequence of four nitrogen bases.

5’ TGACGGGTTTCGAGGCCAG 3’
3’ ACTGCCCCAAGGTCCGGTC 5’

5’ TGACGGGTTTCGA\textcolor{red}{GGCC}CAG 3’
3’ ACTGCCCCAAGGT\textcolor{red}{CCGG}TC 5’
Once the recognition site was found HaeIII could go to work cutting (cleaving) the DNA

5’ TGACGCGGGTTTCGAAGGCC AG 3’
3’ ACTGCCCAGGGGTTCGGTC 5’
These cuts produce what scientists call “blunt ends”

5’ TGACGGGGTTCGA**GG** CCAG 3’
3’ ACTGCCCCAAAGG**GG** GGTC 5’
The names for restriction enzymes come from:

- the type of bacteria in which the enzyme is found
- the order in which the restriction enzyme was identified and isolated.

*EcoRI* for example

*R* strain of *E. coli* bacteria

* I as it is was the first *E. coli* restriction enzyme to be discovered.
“blunt ends” and “sticky ends”

Remember how *HaeIII* produced a “blunt end”? *EcoRI*, for instance, makes a staggered cut and produces a “sticky end”

5’ GAATTC 3’
3’ CTTAAG 5’

5’ **GA**ATTC 3’
3’ CTTA**AG** 5’

5’ G AATTC 3’
3’ CTTAA**G** 5’

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blunt end  
sticky end
Some more examples of restriction sites of restriction enzymes with their cut sites:

- **HindIII**: 5’ AAGCTT 3’
  3’ TTCGAA 5’

- **BamHI**: 5’ GGATCC 3’
  3’ CCTAGG 5’

- **AluI**: 5’ AGCT 3’
  3’ TCGA 5’

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“sticky ends” are useful

DNA fragments with complimentary sticky ends can be combined to create new molecules which allows the creation and manipulation of DNA sequences from different sources.
Characteristics of Restriction Endonucleases

By convention, RE are named after their host of origin.
eg. Eco RI was isolated from *Escherichia coli* (strain RY13)
    Hind II and Hind III from *Haemophilus influenzae*
    Xho I from *Xanthomonas horticola*

Restriction Enzyme Recognition Sequences

The substrates for REs are specific sequences of double-stranded DNA called recognition sequences.

The length of restriction recognition sites varies, determines the frequency of RE cut in a sequence of DNA:

4 base pairs /base cutters (eg. Sau 3AI)
6 base pairs (eg. Eco RI, Sac I and Sst I)
8 base pairs (eg. Not I)
Shorter recognition site, higher frequency of cut.

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Different REs can have the same recognition site - such enzymes are called isoschizomers (eg. Sac I and Sst I have identical RE site)

Isoschizomers often have different optimum reaction conditions, stabilities and costs, which may influence the decision of which to purchase.

RE sites can be unambiguous or ambiguous:

eg. Unambiguous – Bam HI recognizes the sequence GGATCC

Ambiguous – Hinf I GANTC ("N" = any nucleotide)

- Xho II Pu GATC Py (Py = pyrimidine (T or C) and Pu = purine (A or G), so Xho II will recognize and cut sequences of AGATCT, AGATCC, GGATCT and GGATCC.

The recognition site for one enzyme may contain the restriction site for another:

eg. BamHI recognition site contains the recognition site for Sau3AI, thus all BamHI sites will cut with Sau3AI. Similarly, one of the four possible Xho II sites will also be a recognition site for Bam HI and all four will cut with Sau3AI.

Most recognition sequences are palindromes - they read the same forward (5' to 3' on the top strand) and backward (5' to 3' on the bottom strand).

Most, but certainly not all recognition sites for commonly-used restriction enzymes are palindromes.
Restriction enzymes cut the backbone of DNA between deoxyribose and phosphate groups, resulting in a phosphate group on the 5' ends and a hydroxyl on the 3' ends of both strands.

RE can generate one of three different types of ends:

**5' overhangs:** The enzyme cuts asymmetrically within the recognition site such that a short single-stranded segment extends from the 5' ends (eg. BamHI).

**3' overhangs:** Again, we see asymmetrical cutting within the recognition site, but the result is a single-stranded overhang from the two 3' ends (eg. KpnI)

The 5' or 3' overhangs are called **sticky ends** or **cohesive ends**, because they will readily stick or anneal with their partner by base pairing.

**Blunts:** Enzymes that cut at precisely opposite sites in the two strands of DNA generate blunt ends without overhangs (eg. SmaI)
HindII restriction digest results in blunt ends
Eco RI restriction digestion

- A ‘sticky’ or cohesive ends produced by EcoRI digestion can anneal to any other ‘sticky’ ends produced by EcoRI cleavage.
Restriction enzyme – scissor
DNA ligase - glue

Restriction Enzyme
Action of EcoRI

- The enzyme cuts both DNA strands at the same site.
- DNA fragments gain of sticky ends.
- Foreign DNA
- DNA is cut with EcoRI at arrows.
- Resulting DNAs have sticky (complementary) ends.
- DNA is spliced by complementary base pairing and sealed with DNA ligase.
- Recombinant DNA.

Inserting a DNA Sample into a Plasmid

- DNA to be inserted
- Plasmid
- Recombinant DNA

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Cloning involves 'cut-and-paste'.
Cloning gene of interest

Plasmid Insertion

Gene for antibiotic resistance
Foreign DNA with gene of interest
Cutting sites for endonuclease
Sticky ends
Mismatch
Desired recombinant plasmid
Mismatch
Bacterial chromosome
Recombinant plasmid

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Restriction Fragment Length Polymorphism (RFLP)

- Sites are closely linked to DMD (Duchenne Muscular Dystrophy) locus, encoding dystrophin.

  Absence of polymorphic site, a 30kb fragment produced by cleavage with BglII.

  A polymorphic site is present, 22kb and 8kb fragments are produced.

  Southern blot analysis using a probe hybridizes to the 30kb and 8kb fragment.
Partial Restriction Endonuclease Digestion

- If a fragment is considered partially digested with an restriction endonuclease, it means RE digestion is incomplete.
- Eg. If you radioactively label the DNA fragment at one end, and partially digest a fragment containing 3 Eco RI sites,
  a) how many different fragments which are labeled?
  b) how many different fragments which are unlabeled?

\[
\begin{array}{ccc}
E & E & E \\
\text{labeled fragments} & \text{unlabeled fragments} \\
\end{array}
\]

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Complete restriction endonuclease digestion:
Separate or combination of restriction digests by different restriction enzymes can be used to provide markers or restriction map on a chromosome.
DNA polymerases

- DNA polymerase III
  - main **DNA builder**
- DNA polymerase I
  - **editing, repair & primer removal**

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Restriction endonucleases

Type 1
- They recognize some specific sequences like all other Res, but they are not particularly useful in gene manipulation since their cleavage site is non-specific. In addition, they have DNA methylases, ATPases activity.

Type 2
- e.g. EcoR1, they are Mg²⁺ dependent with specific recognition site. They have no unusual properties, recognize particular target site to give rise discrete DNA fragments of defined length. So these enzymes are useful to make DNA recombinants.

Type 3
- Contains nuclease and methylase activity.
- Recognition sites are not symmetrical.
Application of RE

- Construction of an endonuclease map of a plasmid @ bacteriophage clone;
- Fragmentation of genomic DNA prior to electrophoretic separation & Southern blotting;
- Generation of fragments that can be subcloned in appropriate vectors;
- Generation of fragments to be used as labeled probes in both Southern & northern blotting, as well as in nuclease protection analysis.
Exonucleases

- Single-stranded 5’ → 3’ & 3’ → 5’
  - Exonucleases- Exonucleases VII (exo VII)
- Does not requires Mg $^{2+}$
- For mapping the position of introns in genomic DNA
- To excise segments of DNA that have been inserted into plasmid vectors by the poly(dA-dT) tailing method
- Double-stranded 5’ → 3’ Exonucleases
  - Lambda Exonuclease (λ exo)
  - T7 Gene 6 Exonuclease
Exonucleases

- Double-Stranded 3’ → 5’ Exonuclease
  - Exonuclease III (exo III)

Applications
- Utilize the nonprocessive 3’ → 5’ ds exonuclease activity of exo III to generate uniform single-stranded regions in ds DNA.
Endonucleases

S1 Nuclease
- Aspergillus oryzae, a highly specific single-stranded endonuclease.
- Most applications of S1 nuclease make use of its ability to trim protruding single-stranded ends of DNA & RNA without significant nibbling of blunt duplex ends.

Deoxyribonuclease I (DNase I)
- From bovine pancreas, degrades dsDNA to produce 3’-hydroxyl oligonucleotides.
- Use to produce nick translocation and also for random cloning of DNA fragments.
Ribonucleases

- Ribonucleases (RNases) with different sequence specificities are used for a variety of analytical purposes, including RNA sequencing, mapping, & quantitation.

- Ribonuclease A (from bovine pancreas is an endoribonuclease, cleave after C and U.
  - Can be inhibit by RNase inhibitor from human placenta.
  - Very persist and active in wide range of condition.
  - Generally remove from the solution using proteinase K followed by multiplied phenol extraction.

- Ribonuclease H (RNase H) [From E. coli is an endoribonuclease
  - Specifically hydrolyzes the phosphodiester binds of RNA in RNA: DNA duplexes.
  - It will not degrade ss or ds DNA @ RNA. RNase H cleavage can be directed to specific sites by hybridizing short deoxyoligonucleotides to the RNA.
Application

**RNase A & RNase T1**: mapping & quantitating RNA species using the ribonuclease protection assay.

**RNase A**: hydrolyzing RNA that contaminates DNA preparations.

**RNase A & RNase T1**: RNA sequencing.

**RNase H**: Remove RNA from RNA:DNA duplex.

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One component of the bacterial restriction-modification system, a natural defense mechanism of bacteria to against the introduction of foreign DNA into the cell

- **Restriction endonuclease**: recognize a short, symmetrical DNA sequence, and cut DNA backbone in each strand at a specific site within that sequence (kill foreign DNA)

- **Methylase**: methylates C or A of the cellular DNA
Restriction sequences & Cohesive ends

5' protruding ends

3' protruding ends

Cohensive ends

5'-CCC-GGG-3'
3'-GGG-CCC-5'

SmaI

5'-CCC-OH + 3'-GGG-\(\bullet\) + OH-CCC-5'

5'-CCC-GGG-3'
3'-GGG-\(\bullet\) OH-CCC-5'

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Restriction digestion

Fig. 2. The digestion of a plasmid with two different restriction enzymes.

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