Manipulation of Purified DNA

To produce the recombinant DNA molecule, the vector, as well as the DNA to be cloned, must be cut at specific points and then joined together in a controlled manner by DNA manipulative enzymes.

- **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.

The cutting and joining manipulations that underlie gene cloning are carried out by enzymes called restriction endonucleases (for cutting) and ligases (for joining).
Enzymes for cutting DNA—restriction endonucleases

Each vector molecule must be cleaved at a single position, to open up the circle so that new DNA can be inserted: a molecule that is cut more than once will be broken into two or more separate fragments and will be of no use as a cloning vector. Furthermore, each vector molecule must be cut at exactly the same position on the circle. Thus a very special type of nuclease is needed to carry out this manipulation.
Function of restriction endonucleases

Restriction endonucleases are synthesized by many, perhaps all, species of bacteria: over 2500 different ones have been isolated and more than 300 are available for use in the laboratory.

Type II restriction endonucleases cut DNA at specific nucleotide sequences

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>ORGANISM</th>
<th>RECOGNITION SEQUENCE*</th>
<th>BLUNT OR STICKY END</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td><em>Escherichia coli</em></td>
<td>GAATTC</td>
<td>Sticky</td>
</tr>
<tr>
<td>BamHI</td>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>GGATCC</td>
<td>Sticky</td>
</tr>
<tr>
<td>BglII</td>
<td><em>Bacillus globigii</em></td>
<td>AGATCT</td>
<td>Sticky</td>
</tr>
<tr>
<td>PvuII</td>
<td><em>Proteus vulgaris</em></td>
<td>CGATCG</td>
<td>Sticky</td>
</tr>
<tr>
<td>PvuII</td>
<td><em>Proteus vulgaris</em></td>
<td>CAGCTG</td>
<td>Blunt</td>
</tr>
<tr>
<td>HindIII</td>
<td><em>Haemophilus influenzae R_d</em></td>
<td>AAGCTT</td>
<td>Sticky</td>
</tr>
<tr>
<td>Hinfl</td>
<td><em>Haemophilus influenzae R_f</em></td>
<td>GANTC</td>
<td>Sticky</td>
</tr>
<tr>
<td>Sau3A</td>
<td><em>Staphylococcus aureus</em></td>
<td>GATC</td>
<td>Sticky</td>
</tr>
<tr>
<td>Alul</td>
<td><em>Arthrobacter luteus</em></td>
<td>AGCT</td>
<td>Blunt</td>
</tr>
<tr>
<td>TaqI</td>
<td><em>Thermus aquaticus</em></td>
<td>TCGA</td>
<td>Sticky</td>
</tr>
<tr>
<td>HaeIII</td>
<td><em>Haemophilus aegyptius</em></td>
<td>GGCC</td>
<td>Blunt</td>
</tr>
<tr>
<td>NotI</td>
<td><em>Nocardia otitidis-caviarum</em></td>
<td>GCGGCGC</td>
<td>Sticky</td>
</tr>
<tr>
<td>SII</td>
<td><em>Streptomyces fimbriatus</em></td>
<td>GGCCNNNNGGGCC</td>
<td>Sticky</td>
</tr>
</tbody>
</table>
Some examples:

The ends produced by cleavage of DNA with different restriction endonucleases.

A blunt end produced by *AluI*.

A sticky end produced by *EcoRI*.

The same sticky ends produced by *BamHI*, *BglII* and *Sau3A*. 
Performing a restriction digest in the laboratory

How to digest a sample of λ DNA?

(a) Cleavage sites on λ DNA

What is the number of fragments produced by cleavage with each of these restriction endonucleases?
Digest a sample of λ DNA with BglIII:

1 unit of enzyme is defined as the quantity needed to cut 1 µg of DNA in 1 hour.

Here we need 2 units of BglIII to cut 2 µg of e DNA.
Analyzing the result of restriction endonuclease cleavage

A way of determining the number and sizes of the fragments is needed if restriction endonucleases are to be of use in gene cloning.

Using gel electrophoresis

Separation of molecules by gel electrophoresis:

This technique uses differences in electrical charge to separate the molecules in a mixture. DNA molecules have negative charges, and so when placed in an electric field they migrate toward the positive pole.

The smaller the DNA molecule, the faster it can migrate through the gel. Gel electrophoresis therefore separates DNA molecules according to their size.
Visualizing DNA molecules in an agarose gel

Visualizing DNA bands in an agarose gel by EtBr staining and ultraviolet (UV) irradiation:

Soak in 0.5 μg/ml solution of EtBr, 15 min
Estimation of the sizes of DNA molecules

More accurate measurement of fragment size is gained by using the mobilities of the HindIII–λ fragments to construct a calibration curve; the sizes of the unknown fragments can then be determined from the distances they have migrated.

The relevant formula is:

\[ D = a - b \log M \]
Mapping the positions of different restriction sites in a DNA molecule

Only when a restriction map is available can the correct restriction endonucleases be selected for the particular cutting manipulation that is required.

What restriction enzyme should be used to obtain gene B and D?
Ligation – joining DNA molecules together

The final step in construction of a recombinant DNA molecule is the joining together of the vector molecule and the DNA to be cloned. This process is referred to as ligation, and the enzyme that catalyzes the reaction is called DNA ligase.
The different joining reactions catalysed by DNA ligase:

(a) Ligating blunt ends

(b) Ligating sticky ends

Transient base-paired structure

DNA ligase seals the discontinuities
Introduction of DNA into Living Cells

The next step in a gene cloning experiment is to introduce these molecules into living cells, usually bacteria, which then grow and divide to produce clones.

Cloning serves two main purposes:

- Amplification
- Purification
Cloning can supply large amounts of recombinant DNA:
Cloning is analogous to purification. From a mixture of different molecules, clones containing copies of just one molecule can be obtained: