Exploring genes -
Analysing, constructing and cloning genes
Lecture structure

- Lecture 1: Gene expression in bacterial hosts
  - Isolating specific genes

- Lecture 2: DNA Technology
  - Genetic engineering tools

- Lecture 3: Gene cloning, recombinant DNA
  - Introducing DNA into host cells
What is gene cloning?

1. The gene is isolated (DNA)
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2. Inserted into vector - Recombinant DNA
What is gene cloning?

1. The gene is isolated (DNA)
2. Inserted into vector
What is gene cloning?

1. The gene is isolated (DNA)
2. Inserted into vector
3. Transported into host cell

Typically an easy-to-grow, benign, laboratory strain of E. coli bacteria

Gene encoding human protein

Cloning Vector

Host = bacterium
What is gene cloning?

1. The gene is isolated (DNA)
2. Inserted into vector
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Gene encoding human protein

Cloning Vector

Host = bacterium

10^6 copies

Gene structure and sequence
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Basic genetic engineering tools

1. Plasmid vectors
2. Restriction enzymes to cut DNA
3. Gel electrophoresis to separate DNA fragments
4. DNA ligase to join DNA fragments
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Plasmid vectors

Simple cloning vectors

- To generate large amounts of DNA
- Derived from small circular DNAs (PLASMIDS) found in bacteria
  - They can replicate independently of the host cell
Transport into host bacterium

Cloning Vector

Recombinant DNA molecule
Transport into host bacterium

Cloning Vector

Recombinant DNA molecule

Multiplication of recombinant plasmid

200 copies

Division of host cell
Plasmid vector - pBR322

p stands for plasmid
BR for Bolivar and Rodriguez
- created it in 1977
322 identifies it

4,363bp

Ori - origin of replication

High copy number
- Increase to ~1000s with amplification & protein synthesis inhibitors

Less than 10,000bp
- Easily purified
- Avoid DNA breakdown
Plasmid vector - pBR322

amp<sup>r</sup> - ampicillin resistance gene
tet<sup>r</sup> - tetracycline resistance gene

Ori - origin of replication
Plasmid vector - pBR322

- EcoRI
- BamHI
- SalI
- ampr - ampicillin resistance gene
- tetr - tetracyclin resistance gene
- pBR322 4363bp
- Ori - origin of replication

amp<sup>r</sup> - ampicillin resistance gene
tet<sup>r</sup> - tetracyclin resistance gene
Basic genetic engineering tools

1. Plasmid

2. Restriction enzymes to cut DNA

3. Gel electrophoresis to separate DNA fragments

4. DNA ligase to join DNA fragments
Restriction enzymes: 

- In bacteria to protect them from viruses
- Recognise specific sequences of bases in the DNA
- Bind to these sites
- Cut the double-stranded DNA by hydrolysing the phosphodiester bonds
The discovery of restriction enzymes

- Discovered in the 1960s
  - Werner Arber, Hamilton Smith and Daniel Nathans
  - 1978 Nobel Prize

- It was shown that some bacteria are immune to bacteriophage infection

- This is known as host-controlled restriction
Phage injects DNA into bacterium

Restriction enzymes bind to DNA from phage

Phage DNA is cleaved and inactivated
Restriction enzymes

Escherichia coli bacteria

Contains a restriction enzyme

ECO RI

Contains a ECO RI methylase

 Methylates the sequence GAATTC

DNA

GAATTC
Why don't restriction enzymes cleave bacterial DNA?

Bacterial DNA

Contains a ECO RI methylase

- Methylates the sequence GAATTC
Why don't restriction enzymes cleave bacterial DNA?

Contains a ECO RI methylase

- Methylates the sequence GAATTC
Why don't restriction enzymes cleave bacterial DNA?

Bacterial DNA  Me

Recognition sequence methylated  Me

ECO RI
DNA Methylation

DNA is replicated through semi-conservative replication

DNA methylase selectively methylates hemi-methylated DNA
Why don't restriction enzymes cleave bacterial DNA?

Bacterial DNA

Recognition sequence methylated

ECO RI
Types of restriction enzymes

- **Exonucleases** - removing nucleotides from the ends
Types of restriction enzymes

- **Exonucleases** - removing nucleotides from the ends
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DNA
Types of restriction enzymes

- **Exonucleases** - removing nucleotides from the ends

- **Endonucleases** - break nucleic acid chains in the interior
Types of restriction enzymes

- **Exonucleases** - removing nucleotides from the ends

- **Endonucleases** - break nucleic acid chains in the interior

```
   DNA
```

Types of restriction endonucleases

- **Type I** - cuts DNA 1000 bases from a recognition site

- **Type 2** - cuts DNA at the recognition site

- **Type 3** - cuts DNA 25 bases from the recognition site
Types of restriction endonucleases

- **Type I** - cuts DNA 1000 bases from a recognition site

  ![Diagram of Type I restriction endonuclease](image)

- **Type 2** - cuts DNA at the recognition site

  ![Diagram of Type 2 restriction endonuclease](image)

- **Type 3** - cuts DNA 25 bases from the recognition site

  ![Diagram of Type 3 restriction endonuclease](image)
**Type II Restriction Enzymes**

<table>
<thead>
<tr>
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<th>Recognition sequence</th>
<th>Blunt or sticky end</th>
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<tbody>
<tr>
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<td>GAATTC</td>
<td>sticky</td>
</tr>
<tr>
<td>BamHI</td>
<td>GGATCC</td>
<td>sticky</td>
</tr>
<tr>
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<td>AGATCT</td>
<td>sticky</td>
</tr>
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<td>CGATCG</td>
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</tr>
<tr>
<td>PvuII</td>
<td>CAGCTG</td>
<td>blunt</td>
</tr>
<tr>
<td>HindIII</td>
<td>AAGCTT</td>
<td>sticky</td>
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<tr>
<td>HinfI</td>
<td>GANTC</td>
<td>sticky</td>
</tr>
<tr>
<td>Sau3A</td>
<td>GATC</td>
<td>sticky</td>
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<tr>
<td>AluI</td>
<td>AGCT</td>
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<td>TCGA</td>
<td>sticky</td>
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<tr>
<td>HaeIII</td>
<td>GGCC</td>
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</tr>
<tr>
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<td>GCGGCCGC</td>
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- First letter - genus
- Second two letters - species of prokaryotic cell from which they were isolated
- Numbers indicate the order in which enzymes were isolated from single strains of bacteria
For example, EcoRI comes from *Escherichia coli* RY13 bacteria.

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Many recognise hexanucleotide sites

E.g. EcoR1

Other restriction enzymes recognise 4, 5, or 8 nucleotide sequences
Blunt ends or Sticky ends

Blunt end cutters

\[ \text{SmaI} \]  
\[ \text{--- CCCGGGG} \] 
\[ \text{--- GGGCCC} \]

\[ \text{EcoRI} \]  
\[ \text{--- GAATTC} \] 
\[ \text{--- CTTAAG} \]

\[ \rightarrow \]

\[ \text{--- CCC} \]  
\[ \text{--- GGG} \]
\[ \text{--- GGG} \]
\[ \text{--- CCC} \]

Sticky end cutters

\[ \text{SmaI} \]  
\[ \text{--- CCCGGGG} \] 
\[ \text{--- GGGCCC} \]

\[ \text{EcoRI} \]  
\[ \text{--- GAATTC} \] 
\[ \text{--- CTTAAG} \]

\[ \rightarrow \]

\[ \text{--- G} \]  
\[ \text{--- CTTAA} \]
\[ \text{--- AATTC} \]
\[ \text{--- G} \]

Sticky or cohesive ends
Basic genetic engineering tools

1. Plasmid

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4. DNA ligase to join DNA fragments
Gel electrophoresis

Analyzing the result of restriction endonuclease cleavage

a) Separation of DNA fragments by gel electrophoresis
Analysing the result of restriction endonuclease cleavage

b) Visualising DNA in a gel with Ethidium Bromide and UV light
Analysing the result of restriction endonuclease cleavage
Basic genetic engineering tools

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Joining DNA fragments together - ligation

- This reaction is catalysed by the enzyme DNA ligase
  - Discovered by Gellert in 1967

- All living cells contain DNA ligase which is normally used in the replication process
DNA Ligation - joining DNA molecules together

- DNA ligase catalyses a phosphodiester bond
- Most experiments use T4 DNA ligase (isolated from bacteriophage T4)
- This requires ATP
Ligation

Blunt end ligation

\[ \text{(blue)} + \text{(red)} \rightarrow \text{(blue + red)} \]

Sticky end ligation

\[ \text{(blue)} + \text{(red)} \rightarrow \text{(blue + red)} \]

DNA ligase catalyzes a phosphodiester bond between adjacent bases

➢ Recombinant DNA
Ligation

EcoRI

DNA fragments join at sticky ends

Sticky end

Sticky end

Recombinant DNA
Major steps in gene cloning

1. Construction of recombinant DNA molecule
   - DNA fragment is joined to a DNA vector

2. Introduce gene + vehicle into host cell

3. Selection

4. Grow up host cells
1. Construction of a recombinant DNA molecule
Type II Restriction Enzymes

Blunt end cutters

*Smal*  ---CCCGGG---  ---GGGCCC---

**EcoRI**  ---GAATTC---  ---CTTAAG---

Sticky end cutters

*EcoRI*  ---GAATTC---  ---CTTAAG---

Sticky or cohesive ends

Cleave at specific recognition site in DNA

DNA
Cloning into a cloning vehicle

**Option 1:** Cut vector and gene with same restriction enzyme

- **Gene** cut with **BamHI**
- **Vector** cut with **BamHI**
- **BamHI sticky ends**
Option 2: Cut vector and gene with different restriction enzyme
BamHI

GGATCC
CCTAGG

Gene

BamHI + SalI

GATCC
G

SalI

GTCGAC
CAGCTG

SalI

Gene
GGATCC -- GTGCAC
CCTAGG -- CAGCTG
Option 3: Gene and vector do not contain compatible enzymes

A) Using nucleases to create blunt ends

- **Gene** (compatible with **BglII** and **NotI**)
- **Vector** (compatible with **BamHI**)
- **Nuclease** treatment results in **blunt ends**
A) Using nuclease to create blunt ends
e.g. Mung Bean nuclease

![Diagram of blunt end cloning]

**Blunt end cloning**
B) Using DNA Pol to fill in ends

\[
\begin{array}{c}
\text{-----A} \\
\text{-----TTCTGA} \\
\end{array}
\quad \rightarrow \\
\begin{array}{c}
\text{-----AAGCT} \\
\text{-----TTCTGA} \\
\end{array}
\]
Option 4: using linkers/adaptors

BamHI sticky ends
1. The gene is isolated (DNA)
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Gene encoding human protein

Cloning Vector

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10^6 copies

Gene structure and sequence
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