Enzymes

Restriction Enzymes (Site-Specific Endonuclease)

- Enzymes that recognize and cleave dsDNA in a highly sequence specific manner.
- Generally recognize an inverted repeat sequence 4, 6, or 8 base pairs in length (occurrences average every $4^n$, $4^6$, or $4^8$ in length)
- Cleavage occurs to leave blunt ends, 3' or 5' over-hanging ends.

**Hind III**

```
5'---AAGCTT---3'
3'---TTCGAA---5'
```

```
5'---A 3'  5'---AGCTT---3'
3'---TTCGA 5' 3'---A---5'
```

**Pst I**

```
5'---CTGCAG---3'
3'---GACGTC---5'
```

```
5'---CTGCA 3'  G---3'
3'---GACGT---5'
```

**Eco RV**

```
5'---GATATC---3'
3'---CTATAG---5'
```

```
5'---GAT 3'  5'---ATC---3'
3'---CTA 5'  3'---TAG---5'
```

**Bam-HI**

```
5'---GGATCC---3'
3'---CCTAGG---5'
```

```
5'---G 3'  5'---GGATC---3'
3'---CCTAG 5'  3'---G---5'
```

**Bgl-II**

```
5'---AGATCT---3'
3'---TCTAGA---5'
```

```
5'---A 3'  5'---GGATT---3'
3'---TCTAG 5'  3'---A---5'
```
Enzymes

Restriction Enzymes (Site-Specific Endonuclease)

Bam-HI

5' ↔  G G A T C C ↔ 3'
3' ↔  C C T A G G ↔ 5'

5' ↔  G  3'  5' ↔  G G A T C ↔ 3'
3' ↔  5'  5' ↔  C C T A G ↔ 5'

Bgl-II

5' ↔  A G A T C T ↔ 3'
3' ↔  T C T A G A ↔ 5'

5' ↔  A 3'  5' ↔  G G A T T ↔ 3'
3' ↔  5'  5' ↔  T C T A G ↔ 5'

Ligation

5' ↔  G G A T C T ↔ 3'
3' ↔  C C T A G A ↔ 5'

No longer a restriction site
Cannot cleave by Bam-HI or BglII
Enzymes

Restriction Enzymes (Site-Specific Endonuclease)

Modifying a restriction site:

\[
\begin{align*}
5' & \quad GAATTC & 3' \\
3' & \quad CTTAAG & 5' \\
5' \quad & \quad GAATTC & 3' \quad \text{Eco RI} \\
3' \quad & \quad CTTAAG & 5' \\
\end{align*}
\]

Klenow

\[
\begin{align*}
5' & \quad G & 3' \\
3' & \quad AATTC & 5' \\
5' \quad & \quad G & 3' \\
3' \quad & \quad CTTAA & 5' \\
\end{align*}
\]
dNTPs

\[
\begin{align*}
5' & \quad GAATT & 3' \\
3' & \quad CTTA & 5' \\
5' \quad & \quad GAATT & 3' \quad \text{Ase I} \\
3' \quad & \quad CTTA & 5' \\
\end{align*}
\]

DNA Ligase

Generated New Restriction Site

Transformation

Insertion of DNA of interest

Amplification

1. Isolation of DNA.
2. Ligating the DNA fragment into a vector.
3. Transformation of a host cell with the recombinant DNA.
4. Selection of host cells containing the recombinant DNA.
5. Screening cells for those with recombinant DNA or producing a protein of interest.
Molecular Cloning

1. **Construction of recombinant DNA molecules** by in vitro ligation of the desired DNA fragments (target DNA) to a plasmid vector. This step is facilitated by cutting the target DNA and plasmid molecules with specific restriction endonucleases before joining the different DNA fragments using the enzyme DNA ligase.

2. **Transformation.** The recombinant DNA molecules are transferred into host cells (often bacterial or yeast cells) in which the chosen plasmid can undergo DNA replication independently of the host cell chromosome(s).

3. **Selective propagation of cell clones** involves two stages. Initially the transformed cells are plated out by spreading on an agar surface in order to encourage the growth of well-separated cell colonies. These are cell clones (populations of identical cells all descended from a single cell). Subsequently, individual colonies can be picked from a plate and the cells can be further expanded in liquid culture.

4. **Isolation of recombinant DNA clones** by harvesting expanded cell cultures and selectively isolating the recombinant DNA.
Molecular Cloning

Inserting DNA fragment into a vector.

Cloning vector needs to have the following characteristics:

1. Have an origin of replication so that the DNA can be replicated within a host cell.

2. Have one or more selectable markers for determining whether the cloning vehicle has been transferred into cells and to indicate whether the foreign DNA has been inserted into the vector.

3. Generally have several unique restriction sites for cloning a DNA fragment (called a "multiple cloning site," or "MCS") so that the vector will be cut with desired restriction enzyme.
Molecular Cloning

Inserting DNA fragment into a vector.

pRB322 provides two selectable marker:

- If one of the antibiotic resistance genes is broken with the DNA of interest, then the bacteria receiving the plasmid will be sensitive to the antibiotic and die if treated with the antibiotic.

- A method that determines if a recombinant plasmid was created correctly and inserted correctly into the bacteria.

Cloning vector needs to have the following characteristics:

1. Have an origin of replication so that the DNA can be replicated within a host cell.

2. Have one or more selectable markers for determining whether the cloning vehicle has been transferred into cells and to indicate whether the foreign DNA has been inserted into the vector.

3. Modern vector generally have several unique restriction sites for cloning a DNA fragment (called a “multiple cloning site,” or “MCS”) so that the vector will be cut with desired restriction enzyme.
Molecular Cloning

Inserting DNA fragment into a vector.

α (alpha)-complementation:

- Involves a portion of the lacZ gene that is present in pUC plasmids and encodes for the enzyme beta-galactosidase.

- The selection is based on the interruption of the lacZ gene by the DNA of interest:

- If the lacZ gene is not interrupted, the bacteria will produce beta-galactosidase. The bacteria will break down a chemical in the plate, called "X-gal," to produce a blue product. The recombinant DNA was not successfully made.

- If the lacZ gene is interrupted, the bacteria will not produce beta-galactosidase. The bacteria will not break down X-gal and will remain a white color. The recombinant plasmid was successfully made.

X-gal:
Molecular Cloning

Troubleshooting:

- High background: vector ligating without insert.
  
  a) Use two different restriction enzymes to generate different ends.
  
  b) Dephosphorylate the vector with phosphatase (unable to circularize without phosphate at 5’ end).

- Insert ligate in opposite orientation.
  
  - Use two different restriction enzymes to generate different ends.
  
  - Alter one end of restriction site (by phosphatase, Mungbean Nuclease, DNA pol) to achieve directional cloning.

- Multiple insert in single vector.
  
  - Reduce amount of insert.
  
  - Dephosphorylate the insert.

Molecular Cloning

1. Genomic DNA Library.
2. cDNA Library.
3. Expression Library.
4. Use of reporter gene construct.
Molecular Cloning

Genomic DNA Library:

Contains DNA fragments that represent an entire genome.

a) Total nuclear DNA is isolated and cut with a restriction enzyme.

b) A cloning vector is also cut with the same enzyme (or enzyme that generates compatible ends).

c) The two DNAs are ligated in a test tube and transformed into host cells.

d) The host cells are selected for the recombinant DNA by antibiotics.

e) A collection of colonies represents a library.

f) Can calculate how many clones are needed to represent a genome.

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Molecular Cloning

cDNA Library

1. Made from mRNA: RNA that is used has already been processed and does not contain regulatory elements such as promoters or introns.

2. Will be able to deduce the protein sequence based on cDNA sequence.

3. Reduces the amount of DNA to be cloned because the entire genome is not being used.

4. Fewer clones represent a library, making screening less labor-intensive.

5. May represent only genes expressed by a cell at a given time.
1. mRNA is isolated from cells, and the enzyme reverse transcriptase makes one strand of DNA from the mRNA.

2. mRNA is degraded with a ribonuclease (an enzyme that breaks down RNA) or an alkaline (basic) solution.

3. DNA polymerase makes the second DNA strand.

4. Double-stranded DNA pieces, called “DNA linkers,” are added to the newly-made DNA, and the recombinant DNA is inserted into a vector.

Expression Library

- Made with a cloning vector that contains the required regulatory elements for gene expression, such as the promoter region.

- Can insert into host cells to produce a protein or create a library.

- Useful for identifying a clone containing the gene or cDNA of interest.
Molecular Cloning

Use of Reporter (marker) gene:

Identify regulatory elements:

- The regulatory sequence of a cloned eukaryotic gene is ligated to a reporter gene that encodes an easily detectable enzyme.
- The resulting plasmid is then introduced into cultured recipient cells by transfection.
- An active regulatory sequence directs transcription of the reporter gene, expression of which is then detected in the transfected cells.

- β-galactosidase gene - Break down X-gal to produce blue color.
- Luciferase gene—found in firefly and the bacteria, the protein produces light in response to the molecules luciferin and ATP.
- Green fluorescent protein (GFP)—produced by the jellyfish and interacts with the protein aequorin to produce fluorescence.
- β-glucuronidase gene (GUS)—encodes an enzyme that breaks down chemicals called β-D-glucuronides. Can produce a blue or fluorescent color.
Molecular Cloning

Use of Reporter gene:

Identify regulatory elements:

- The regulatory sequence of a cloned eukaryotic gene is ligated to a reporter gene that encodes an easily detectable enzyme.
- The resulting plasmid is then introduced into cultured recipient cells by transfection.
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DNA Sequencing

Developed by two groups in 1977:

1. Allan Maxam and Walter Gilbert developed a chemical method that modifies and removes a specific base from the DNA strand. The pieces that are generated can be separated by electrophoresis.

2. Frederick Sanger developed a DNA sequencing method using DNA polymerase and 2', 3'-dideoxynucleotides (nucleotide analogue). DNA synthesis is terminated by incorporation of dideoxynucleotides specifically at A, T, C or G to cause chain-termination and fragments can be separated by electrophoresis.
DNA Sequencing

1) A DNA primer is annealed to the desired DNA.

2) DNA polymerase extends to primer, and labeled nucleotides incorporate in the newly made DNA.

3) 2', 3'-dideoxynucleotides are incorporated and stop DNA synthesis in four test tubes, with each tube containing one dideoxynucleotide such as ddATP, ddGTP, ddCTP, and ddTTP. For example, ddATP will stop DNA synthesis wherever an adenine nucleotide needs to be inserted.

4) Each resulting strand is a different length, and is separated by electrophoresis.
DNA Sequencing

Automated sequencing: many samples and up to 600-650 bases:

1. Mixing dideoxynucleotides labeled with difference dyes in one tube.

2. Electrophoresis performed in a capillary tube.

3. A laser stimulates the DNA piece, and a recorder sends the information to a computer for analysis.
DNA Sequencing

FROM PROTEIN TO GENE

1. Isolate protein on the basis of its molecular function (e.g., enzymatic or hormonal activity)
2. Determine partial amino acid sequence of the protein
3. Synthesize oligonucleotides that correspond to portions of the amino acid sequence
4. Use oligonucleotides as probes to select cDNA or genomic clone encoding the protein from library
5. Sequence isolated gene

FROM GENE TO PROTEIN

1. Isolate genomic clone corresponding to an altered trait in mutants (e.g., nutritional auxotrophy, inherited disease, developmental defect)
2. Use genomic DNA to isolate a cDNA for the mRNA encoded by the gene
3. Sequence the cDNA to deduce amino acid sequence of the encoded protein
4. Compare deduced amino acid sequence with that of known proteins to gain insight into function of the protein
5. Use expression vector to produce the encoded protein