Restriction Endonucleases,
(cutting dna)
(ligation)
ligases & phosphatases

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Is an enzyme that cuts DNA at or near specific recognition nucleotides sequences known as **Restriction site**.

**Idea:**

These enzymes are found in bacteria and provide a defense mechanism against invading viruses.

To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.
The phenomenon was first identified in work done in the laboratories of Salvador Luria and Giuseppe Bertani in early 1950s.

It was found that a bacteriophage λ that can grow well in one strain of Escherichia coli, for example E. coli C, when grown in another strain, for example E. coli K, its yields can drop significantly.

The E. coli K host cell, known as the restricting host, appears to have the ability to reduce the biological activity of the phage λ.
Restriction enzyme = Restriction Endonuclease

In the 1960s, it was shown in work done in the laboratories of Werner Arber and Matthew Meselson and they defined that:

The restriction is caused by an enzymatic cleavage of the phage DNA, and the enzyme involved was therefore termed a restriction enzyme.
Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially.
Restriction site

Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA. The recognition sequences usually vary between 4 and 8 nucleotides, and many of them are palindromic, meaning the base sequence reads the same backwards and forwards.
Restriction site:

In theory, there are two types of palindromic sequences that can be possible in DNA:

The *mirror-like palindrome* is similar to those found in ordinary text, in which a sequence reads the same forward and backwards on a single strand of DNA strand.
Restriction site:

The inverted repeat palindrome is also a sequence that reads the same forward and backwards, but the forward and backward sequences are found in complementary DNA strands. Inverted repeat palindromes are more common and have greater biological importance than mirror-like palindromes.
Restriction site:

- EcoRI digestion produces "sticky" ends:
  
  ![EcoRI sequence]

- whereas SmaI restriction enzyme cleavage produces "blunt" ends:
  
  ![SmaI sequence]
Restriction endonucleases (restriction enzymes)

- sticky ends
- blunt ends

**Nomenclature**

\[ \text{EcoRI} \]

- \( E = \text{genus (Escherichia)} \)
- \( co = \text{species (coli)} \)
- \( R = \text{strain} \)
- \( I = \# \text{ of enzyme} \)
**Restriction Endonucleases:**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Recognition Sequence</th>
<th>Cut</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>Escherichia coli</td>
<td>5'GAATTC 3'CTTAAG</td>
<td>5'---G AATTC---3' 3'---CTTAA G---5'</td>
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<tr>
<td>EcoRII</td>
<td>Escherichia coli</td>
<td>5'CCWGG 3'GGWCC</td>
<td>5'--- CCWGG---3' 3'---GGWCC ---5'</td>
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<td>BamHI</td>
<td>Bacillus amyloboliquefaciens</td>
<td>5'GGATCC 3'CCTAGG</td>
<td>5'---G GATCC---3' 3'---CCTAG G---5'</td>
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<td>HindIII</td>
<td>Haemophilus influenzae</td>
<td>5'AAGCTT 3'TTCGAA</td>
<td>5'---A AGCTT---3' 3'---TTCGA A---5'</td>
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<td>TaqI</td>
<td>Thermus aquaticus</td>
<td>5'TCGA 3'AGCT</td>
<td>5'---T CGA---3' 3'---AGC T---5'</td>
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<tr>
<td>SmaI*</td>
<td>Serratia marcescens</td>
<td>5'CCCGGGG 3'GGGCC</td>
<td>5'---CCC GGG---3' 3'---GGG CCC---5'</td>
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</tbody>
</table>
Which enzymes I should use

PubMed

```
gene 1..642
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CDS 1..642
  /gene="omp25"
  /codon_start=1
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  /db_xref="GOA:BSU6Y0"
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  /db_xref="UniProtKB/TrEMBL:BSU6Y0"
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ORIGIN
  1 atgcgcacct ttaagtctct cgtaatcgtc tccggtgcgc tgcgtgcgtt ctctgccgac
  61 gcttttgctg cgcaccccat ccagcaacag ccctccggttc cggtcccggt tgaatagctt
  121 cccccagtata gctgggtctgg tggctatacc gttctttacc tggcgtcagc ctggaacaag
  181 gccagagcgg gacacctttgc cagcaacggt cggagcctgg gaaggtcgct ggttgggtgt
  241 ggttggaactt tccagacagg ccagatcgtta tattgtgtgg aagtgtaagc agttatcca
  301 tggcccaaga agttcagagg cgccctggacag tcaagagcag gtttggaagg ctcgctcgcgg
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  541 taacccaggt acggcacaaga gaactagcttg ccgctgctgtga caaatgtcgc cacaagcgtg
  601 gcacagcagg atatcgcggt ccggcattgc tacaagttct aa
```
NEBcutter V2.0

This tool will take a DNA sequence and find the large, non-overlapping open reading frames using the E.coli genetic code and the sites for all Type II and commercially available Type restriction enzymes that cut the sequence just once. By default, only enzymes available from NEB are used, but other sets may be chosen. Just enter your sequence and "submit". Further options will appear with the output. The maximum size of the input file is 1 MByte, and the maximum sequence length is 300 KBases.

What's new in V2.0  Citing NEBcutter
### Restriction Map


<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Site Length</th>
<th>Overhang</th>
<th>Frequency</th>
<th>Cut Positions</th>
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<tbody>
<tr>
<td>EcoRV</td>
<td>GATATC</td>
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<td>bhunt</td>
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<td>Avall</td>
<td>GGWCC</td>
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<td>five_prime</td>
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<td>five_prime</td>
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</table>
Ligation (Ligases & Phosphatases)

**Ligation** in molecular biology is the joining of two nucleic acid fragments through the action of an enzyme. It is an essential laboratory procedure in the molecular cloning by which DNA fragments are joined together to create recombinant DNA molecules.

When a foreign DNA fragment is inserted into a plasmid. The ends of DNA fragments are joined together by the formation of phosphodiester bonds between the 3'-hydroxyl of one DNA termini with the 5'-phosphoryl of another.
Pasting DNA

- Complementary ends (sticky ends) H–bond
- Ligase forms phosphodiester bond to seal strands together.
Sticky-end ligation:

In cloning experiments most commonly-used restriction enzymes generate a 4-base single-stranded overhang called:

The sticky or cohesive end.

These sticky ends can anneal to other compatible ends and become ligated in a sticky-end (or cohesive end) ligation. For most restriction enzymes, the overhangs generated have a $T_m$ that is around 15°C. For practical purposes, sticky end ligations are performed at 12-16°C.
Blunt-end ligation:

Blunt end may be ligated to another blunt end, Blunt ends may be generated by restriction enzymes such as *SmaI* and *EcoRV*. However a major advantage of blunt-end cloning is that the desired insert does not require any restriction sites in its sequence as blunt-ends are usually generated in a PCR, and the PCR generated blunt-ended DNA fragment may then be ligated into a blunt-ended vector generated from restriction digest.
Disadvantages of blunt-end ligation:

1- ligation is much less efficient than sticky end ligation, typically the reaction is 100X slower than sticky-end ligation.

2- The concentration of ligase used is higher than sticky end ligation (10x or more).

3- The concentration of DNA used in blunt-end ligation is also higher to increase the likelihood of collisions between ends.

4- Longer incubation time may also be used for blunt-end ligations.
Method of Ligation:

Add 1 µl of the PDrive vector of the ligation in 0.2 µl or 0.5 µl PCR tubes,

Add 1:4 µl of the purified PCR product,

Add 5 µl of ligation master mix & Variable µl of water then put in the thermo cycler for four hours at 16°C.
Alkaline phosphatase dephosphorylation

Removing phosphate group to prevent self ligation of the vector
**Alkaline phosphatase:**
Removes 5' phosphate groups from DNA and RNA.
It will also remove phosphates from nucleotides and proteins.
These enzymes are most active at alkaline pH - hence the name.
There are several sources of alkaline phosphatase that differ in how easily they can be inactivated:

1. **Bacterial alkaline phosphatase (BAP)** is the most active one, but also the most difficult to destroy at the end of the dephosphorylation reaction.

2. **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 (75°C for 10 minutes in the presence of 5 mM EDTA).

3. **Shrimp alkaline phosphatase** is derived from a cold-water shrimp and is promoted for being readily destroyed by heat (65°C for 15 minutes).
Trouble-shooting

Sometimes ligation fail to produce the desired ligated products, and some of the possible reasons may be:

1- Damaged DNA - over-exposure to UV radiation during preparation of DNA for ligation can damage the DNA.
2- Excessive amount of DNA used.
3- Incomplete DNA digest, The vector DNA that is incompletely digested will give rise to a high background. Insert that is not completely digested will also not ligate properly and circularize.
4- Incomplete ligation. Blunt-ends DNA and some sticky-ends DNA that have low-melting temperature require more ligase and longer incubation time.
Molecular cloning

Plasmid DNA

Insert new DNA

New DNA

Replicate inside bacteria

Bacterial colony

Many copies of DNA

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Thanks a lot

with my Best Regards and My Best wishes

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