Restriction endonucleases and ligases

Mohamed N. Seleem
Big picture:
Gene cloning

• From Gene to Protein
• Generally use bacteria as the “factory”
What we have done so far?

- Decide which gene to clone
- Designing primers
- PCR amplification (xx billions copies)
- Gel electrophoresis
- Cutting our gene from gel
What is next

- Move my gene to the bacteria to purify protein as a final product.

**Problem**: Gene by itself does not go inside bacteria, it needs a carrier (vector or plasmid)
Cloning Tools

- Restriction endonucleases
- Ligase
- Vectors
- Host
- Methods for introducing DNA into a host cell
Restriction endonucleases enzymes

Immune system of Bacteria

Bacterial host cell  Host DNA
**Haemophilus influenzae**

To date well over 3000 diff REs to choose from, which were found from screening >10000 bacteria

Hamilton Smith
1973 Nobel Prize
Cutting DNA

- Restriction endonucleases (restriction enzymes)
  - sticky ends
  - blunt ends

- Nomenclature
  - *EcoRI*
  - *E* = genus (*Escherichia*)
  - *co* = species (*coli*)
  - *R* = strain
  - *I* = # of enzyme
Some restriction enzymes

- 100’s of restriction enzymes (RE’s) are commercially available
- Artificial RE sites can be inserted at ends of any gene
- RE’s and ligase allow precise cutting and pasting of any DNA sequences

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bacterial Source</th>
<th>Restriction Site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td><em>Bacillus amyloliquefaciens</em> H</td>
<td>G(^4)GATCC&lt;br&gt;CCTAG(_3)G</td>
</tr>
<tr>
<td>EcoRI</td>
<td><em>Escherichia coli</em> RY13</td>
<td>G(^4)AATTC&lt;br&gt;CTTAA(_3)G</td>
</tr>
<tr>
<td>EcoRII</td>
<td><em>E. coli</em> R245</td>
<td>CC(^4)GG&lt;br&gt;GG(_3)CC</td>
</tr>
<tr>
<td>HindII</td>
<td><em>Haemophilus influenzae</em> Rd</td>
<td>GTP(_6)PuAC&lt;br&gt;CAPu(_3)PyTG</td>
</tr>
<tr>
<td>HindIII</td>
<td><em>H. influenzae</em> Rd</td>
<td>A(^4)AGCTT&lt;br&gt;TTCGA(_3)A</td>
</tr>
<tr>
<td>HiriI</td>
<td><em>H. influenzae</em> Rf</td>
<td>G(^4)ANTC&lt;br&gt;CNTA(_3)G</td>
</tr>
<tr>
<td>Hpal</td>
<td><em>H. parainfluenzae</em></td>
<td>GTT(^4)AAC&lt;br&gt;CAA(_3)TTG</td>
</tr>
<tr>
<td>Mspl</td>
<td><em>Moraxella sp.</em></td>
<td>CC(^4)GG&lt;br&gt;GG(_3)CC</td>
</tr>
<tr>
<td>Smal</td>
<td><em>Serratia marcescens</em></td>
<td>CCC(^4)GGG&lt;br&gt;GGG(_3)CCC</td>
</tr>
</tbody>
</table>

* Arrows indicate sites of cleavage; Py = pyrimidine (either T or C); Pu = purine (either A or G); N = any nucleotide (A, T, G, or C).

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Which enzymes I should use

Pubmed


Omp25 Brucella

gene
   1..642
   /gene="omp25"
   1..642
   /gene="omp25"
   /isdn_start=1
   /product="outer membrane protein 25"
   /protein_id="CA068333.1"
   /db_xref="GI:206596726"
   /db_xref="GOA:B5U6Y0"
   /db_xref="InterPro:IPR011250"
   /db_xref="UniProtKB/TrEMBL:B5U6Y0"
   /translation="MRTLKSLVIVSAAILPFSATAFAADAIEQPQPVPAPVEVAPQYS
WAGGYTQLYLYGYNKAKTSTVGSIKPDWKAAGAFAGWNFQQLQIVYGVEGDAAGYWSA
KKSKDGEVOKYEFSRLARVGYDLNPVYLTAGIASQIKLNNGLDDSKRFVGTST
AGACLEAKLTILGRVEYRTQYGNKNYDLAGTNVRKLDRTQDIRVGIGYKF"

ORIGIN

1  atgcgcacaat ttaaggctct cgtatatgcc tggctgccgg ttcttggcag ac
61  gcttttgctg ccagcggcct ccaagaaacc ccccggtttc cgcttcgcgt tgaagttgct
121  cccgcaattt atttggcttc ggctcatacc ggctctcttg tgggtaacgg ctggaaacag
181  gcccaacacc gcaccctttg cacatcagct ccgagcatgt qgaaggcac ggcgttggct
241  dqchtgcaact ttccacacagca ccagatcgg taattgtggt aagttgactgc agtttacctc
301  tggcccaaga agtcgccagga cgctctggaa tgtcaacgag gttttgagg cttgctggct
361  gcctgcgttc gcctagacct gaaaccgggt atctcgtacc tcacggctcg tattgcccgt
421  tgcggacagtac agcttacaaa ccggctggcg acagaaaccg atgcctcggct ttggtgacg
481  gctgtgctcc gcctggaaga ccagctgacg gcaacactcc tggccggctg tgattctcct
541  tacacccagggagcggag aagactcgg atctgccggta cgaatgtccg ccagcagcgtg
601  gcacgcaagg atatcgcgtg ogggctggtg tttagaattc 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
Copy and paste sequence

**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 III 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 "minibutl". 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.

**DNA Sequence**

```
GAGLEAKLTDILGLREVETYQTGYNKXNYLHAGTVRNLKDITQDIRVGIGYRE
```

**NEBcutter Options**

- Local sequence file: 
- GenBank number: 
- or paste your DNA sequence: (plain or FASTA format)

**Standard sequences:**
- # Plasmid vectors
- # Viral + phage

**Sequence Settings:**
- The sequence is: 
- Linear
- Circular
- Enzymes to use: 
- NEB enzymes
- All commercially available specificities
- Any specificities
- All + defined oligonucleotide sequences
- Only defined oligonucleotide sequences
- Minimum ORF length to display: 100 n.a.
## 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>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRV</td>
<td>GATATC</td>
<td>6</td>
<td>blunt</td>
<td>1</td>
<td>612</td>
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<tr>
<td>FspAI</td>
<td>RTGCGCAY</td>
<td>8</td>
<td>blunt</td>
<td>1</td>
<td>4</td>
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<tr>
<td>Acyl</td>
<td>GRCGYC</td>
<td>6</td>
<td>five_prime</td>
<td>1</td>
<td>74</td>
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<td>AfII</td>
<td>CTAAAG</td>
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<td>1</td>
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<td>ACCGCT</td>
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<td>five_prime</td>
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<td>CCATC</td>
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<td>five_prime</td>
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<td>CCCAGC</td>
<td>6</td>
<td>five_prime</td>
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<td>131</td>
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<td>1</td>
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<td>Eco3II</td>
<td>GGTCTC</td>
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<td>five_prime</td>
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</tr>
<tr>
<td>Esp3I</td>
<td>CGTCTC</td>
<td>6</td>
<td>five_prime</td>
<td>1</td>
<td>33</td>
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<tr>
<td>FauI</td>
<td>CCCGC</td>
<td>5</td>
<td>five_prime</td>
<td>1</td>
<td>370</td>
</tr>
</tbody>
</table>
Put sequence of Non cutting enzymes at both ends of the gene

How? PCR and Primers
Gene

Adding sequence to the ends by PCR

linkers

Vector
Pasting DNA

- Complementary ends (sticky ends) H-bond

- Ligase forms phosphodiester bond to seal strands together.
How to chose your enzymes

• Non cutting enzymes
• Enzymes on Vector
• Enzymes that work together (Double digest)
• Price
• Company (I highly recommend Promega)

Don’t waste clean thinking on dirty enzymes

Efraim Racker, Cornell University
How use your enzymes

• Ice
• No Vortex
• Set up reaction of 10 μl at least
  • 1 μl (10 units) enzyme 1 μl 10xbuffer and 8 μl DNA (you need 1 unit to cut 1 μg of DNA)
• Incubate (usually 37 °C for at least 1 hour)
• Clean your DNA after cutting
Joining DNA

Ligase enzyme
anneal two pieces of compatible ends of DNA together

Repair Mechanism in biological system
How to join DNA

Get rid of restriction enzymes

1 µl (3 units) enzyme
1 µl 10x buffer
4 µl gene
4 µl vector (plasmid)
Incubation ligation reaction

- 4 °C over night or 16 °C over night
- 25 °C 3 hours
- TCL (temperature cycle ligations) Lund et al., 1996
  99 cycles (1 min 30 °C 1 min 10 °C) 4 fold increase ligation
- 5 minutes ligation buffer (Polyethylene glycol PEG 6000)
How to check for successful ligation reaction

- Transformation and count number of good colonies
Alkaline phosphatase
dephosphorylation

Removing phosphate group to prevent self ligation of the vector

Add it only to the vector
Questions!!!!

Suddenly, Bobby felt very alone in the world.