Lecture 7: Molecular techniques I

Restriction mapping
Molecular cloning
Library construction and screen)

Read 279-294

Fig. 9.2; 9.5b; 9.6; 9.7; 9.8; 9.9. 9.10; 9.11; 9.12

Table 9-1; 9-2
Restriction enzymes

(a) Blunt ends (*RsaI*)

Sugar-phosphate backbone

(b) Sticky 5' ends (*EcoRI*)

5' overhangs

(c) Sticky 3' ends (*KpnI*)

3' overhangs
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sequence of Recognition Site</th>
<th>Microbial Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqI</td>
<td>5' TCGA 3' 3' AGCT 5'</td>
<td>Thermus aquaticus YTI</td>
</tr>
<tr>
<td>RsaI</td>
<td>5' GTAC 3' 3' CATG 5'</td>
<td>Rhodopseudomonas sphaeroides</td>
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<tr>
<td>Sau3AI</td>
<td>5' GATC 3' 3' CTA G 5'</td>
<td>Staphylococcus aureus 3A</td>
</tr>
<tr>
<td>EcoRI</td>
<td>5' GAATT 3' 3' CTTAA 5'</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>BamHI</td>
<td>5' GGA1CC 3' 3' GTAG 5'</td>
<td>Bacillus amylobacter faciens H.</td>
</tr>
<tr>
<td>HindII</td>
<td>5' AAGCT 3' 3' TCGAA 5'</td>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>KpnI</td>
<td>5' GGTACC 3' 3' CCAAG 5'</td>
<td>Klebsiella pneumoniae OK8</td>
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<tr>
<td>ClaI</td>
<td>5' ATCGAT 3' 3' TACGTA 5'</td>
<td>Caryophanum latum</td>
</tr>
<tr>
<td>BssHII</td>
<td>5' CSGC 3' 3' CCGCGG 5'</td>
<td>Bacillus stearothermophilus</td>
</tr>
<tr>
<td>Ncol</td>
<td>5' CGCGCCGC 3' 3' CGCGGGG 5'</td>
<td>Nocardia otitidiscaviarum</td>
</tr>
</tbody>
</table>
Restiction mapping

Cloned linear DNA segment

Sample 1
Cut with EcoRI

Sample 2
Cut with Bam

Sample 3
Cut with EcoRI and Bam

Load each sample into gel. Run gel.

Restriction map
Gel electrophoresis
# Molecular cloning

1. Inserts and Vectors
2. Restriction enzymes and ligase

## Table 9.2 Various Vectors and the Size of the Inserts They Carry

<table>
<thead>
<tr>
<th>Vector</th>
<th>Form of Vector</th>
<th>Host</th>
<th>Typical Carrying Capacity (Size of Insert Accepted)</th>
<th>Major Uses</th>
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<tbody>
<tr>
<td>Plasmid</td>
<td>Double-stranded circular DNA</td>
<td><em>E. coli</em></td>
<td>Up to 15 kb</td>
<td>cDNA libraries; subcloning</td>
</tr>
<tr>
<td>Bacteriophage lambda</td>
<td>Virus (linear DNA)</td>
<td><em>E. coli</em></td>
<td>Up to 25 kb</td>
<td>Genomic and cDNA libraries</td>
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<tr>
<td>Cosmid</td>
<td>Double-stranded circular DNA</td>
<td><em>E. coli</em></td>
<td>30–45 kb</td>
<td>Genomic libraries</td>
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<tr>
<td>Bacteriophage P1</td>
<td>Virus (circular DNA)</td>
<td><em>E. coli</em></td>
<td>70–90 kb</td>
<td>Genomic libraries</td>
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<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
<td><em>E. coli</em></td>
<td>100–500 kb</td>
<td>Genomic libraries</td>
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<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
<td>Yeast</td>
<td>250–2000 kb (2 megabases)</td>
<td>Genomic libraries</td>
</tr>
</tbody>
</table>
Restriction digestion movie
(a) Human DNA is cut with EcoRI. DNA and plasmid vectors are cut with EcoRI. Cleaved fragments and vectors are combined in the presence of ligase.

(b) Recombinant plasmids are added to a population of E. coli cells.

E. coli plated onto medium containing ampicillin. Cells containing recombinant plasmids are able to grow.
Fig. 9.8

(c) Distinguishing cells carrying insert-containing recombinant molecules from cells carrying vectors without inserts

(a) Transformation: foreign DNA enters the host cell

(b) Selecting cells that have received a plasmid

Intact vector, no insert

Vector with insert

Disrupted lacZ gene

lacZ gene intact

lacZ gene transcript

X-Gal

Blue pigment

No product
(a) Separating plasmid from bacterial chromosome

Lyse cells, extract DNA.

Treat with ethidium bromide.

Add to solution of CsCl and centrifuge.

CsCl forms density gradient. DNA settles according to its density.

(b) Separating insert from plasmid vector

Cut with EcoRI.

Gel electrophoresis

Insert blue

Vector

Cutout

Purified cloned fragment

(c) 1 mb YAC insert

20 kb lambda insert

1 kb Plasmid insert

2 kb Plasmid insert
Genomic Library
Human: 3,000,000kb/haploid genome
150 kb per insert--20,000 clones equals a genome

cDNA Library

Expression Libraries
Fig. 9.10

(a) Red blood cell precursors

Release mRNA from cytoplasm and purify.

5' mRNA
A A A A

3' mRNA

5' mRNA
A A A A

3' mRNA

5' mRNA
A A A A

3' mRNA

(b) Add oligo(dT) primer. Treat with reverse transcriptase in presence of four nucleotides.

1. Primer
T T T T

5' mRNA

3' mRNA
A A A A

2. Growing cDNA
T T T T

5' mRNA

3' RNA
A A A A

3. Reverse transcriptase

5' cDNA

3' mRNA

(c) Denature cDNA-mRNA hybrids and digest mRNA with RNase. cDNA acts as template for synthesis of second cDNA strand in the presence of four nucleotides and DNA polymerase.

Growing second strand

5' cDNA

S1 nuclease cuts hairpin loop

DNA polymerase

3' cDNA double helix

(d) Insert cDNA into vector.
Fig. 9.11

Random 100 kb genomic region

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<tr>
<th>kb</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
<th>72</th>
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<tr>
<td>Intron</td>
<td>Exon</td>
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<td></td>
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</table>

Gene A expressed only in brain
Gene B expressed in all tissues
Gene C expressed only in liver

Clones from a genomic library with 20 kb inserts that are homologous to this region
- Contains part of gene A
- Contains parts of genes B and C
- Contains all of gene C
- Contains only last exon of gene A

Clones from cDNA libraries

**Brain cDNA library**

<table>
<thead>
<tr>
<th>A : B</th>
<th>1:9</th>
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<tbody>
<tr>
<td>B</td>
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<tr>
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</table>

**Liver cDNA library**

<table>
<thead>
<tr>
<th>B : C</th>
<th>4:7</th>
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<tbody>
<tr>
<td>B</td>
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</tr>
<tr>
<td>C</td>
<td>B</td>
</tr>
<tr>
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<td>C</td>
<td>C</td>
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</table>
(a) An expression vector allows production of specific polypeptide

Expression library

(b) Screening for insulin gene expression

Overlay plate with nitrocellulose paper. Pick up cells.
Lyse cell. Treat with NaOH. Proteins adhere to paper.
Incubate paper in solution of labeled insulin antibody. Antibodies will bind to insulin protein.
Wash filter. Expose to UV light and identify fluorescent spots. Compare with original plate in order to find bacterial clone containing human insulin gene.
Hybridization (Southern blot & Northern blot)
PCR (polymerase chain reaction)
DNA sequencing

Read 294-307

Fig. 9.13 9.15 9.16 9.17 9.18 9.19
In each genomic DNA sample, the H2K probe hybridizes to all 20–30 related major histocompatibility genes present within the mouse genome.
Southern Hybridization Movie
Colony hybridization

Overlay a nitrocellulose disk to make a replica of the plate.

Remove disk from plate and lyse cells on it and denature DNA with NaOH. Bake and treat with UV light to bind DNA strands to disk.

Add labeled probe. Colonies with complementary DNA sequences hybridize to probe and restrain it.

Wash disk, expose to X-ray film.

Compare with original plate to locate bacterial clone with desired genomic fragment.
Colony hybridization movie
The polymerase chain reaction, part 1

1. 94°C for 5 minutes
2. 50–60°C for 2 minutes
3. 72°C for 2–5 minutes

Denatures genomic DNA

Primers base pair at sites flanking target sequence of genomic DNA

Elongation of primers with Taq polymerase

Target sequence

3' Mutant
5' β-globin allele
Fig. 9.16

The polymerase chain reaction, part 2

Denature strands → Base pairing of primers → Elongation of primers

1 copy → 2 copies → 4 copies → 8 copies → 16 copies → 32 copies → 64 copies

animation
PCR (polymerase chain reaction) Movie
Hierarchical sequencing

1. Generate & align large BAC or P1 clones
2. Fragment and sequence a subset of clones

Shotgun sequencing

1. Fragment and sequence entire genome

Adapted from Fig. 2.7 Gibson and Muse
Sanger sequencing

DNA polymerase, ATP, GTP, CTP, and TTP

+ddA  +ddG  +ddC  +ddT
Fig. 9.17c

Gel analysis of fragments

ddA  ddG  ddC  ddT

Sequence of synthesized DNA

G
A
A
T
C
C
G
T

Sequence of template DNA

5'
C
T
T
A
G
G
C
A

3'
(a) Automated sequencing

1. Generate nested array of fragments; each with a fluorescent label corresponding to the terminating 3' base.

2. Fragments separated by electrophoresis in a single vertical gel lane.

3. As migrating fragments pass through the scanning laser, they fluoresce. A fluorescent detector records the color order of the passing bands. That order is translated into sequence data by a computer.

(c)
DNA Sequencing Movie

Deoxyribonucleotide

\[
\text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} \\
\text{H}_2\text{O} \quad \text{PO}_{4} \quad \text{PO}_{4} \quad \text{PO}_{4} \quad \text{CH}_2 \quad \text{O} \\
\text{O} \quad \text{O} \quad \text{O} \quad \text{O} \\
\text{H} \quad \text{H} \quad \text{H} \quad \text{H} \\
\text{OH} \\
\text{H}_2
\]

Dideoxyribonucleotide

\[
\text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} \\
\text{H}_2\text{O} \quad \text{PO}_{4} \quad \text{PO}_{4} \quad \text{PO}_{4} \quad \text{CH}_2 \quad \text{O} \\
\text{O} \quad \text{O} \quad \text{O} \quad \text{O} \\
\text{H} \quad \text{H} \quad \text{H} \quad \text{H} \\
\text{H}_2 \quad \text{H}_2
\]