DNA methods summary

1. **Restriction enzymes** cut at specific DNA sites. (N)
2. Vectors allow genes to be “cloned” and proteins “expressed”. (N)
3. Gel electrophoresis separates DNA on the basis of size.
4. DNAs can be synthesized (up to ~100 bases commercially). (N)
5. PCR amplifies any target DNA sequence. (N)
6. Genes and genomes can be sequenced by chain termination. (N)
7. Oligonucleotides can be used to change bases by “site-directed mutagenesis”. (N)
8. “Southern” blotting detects sequences by hybridization.
9. Genes can be knocked out (deleted) or replaced in prokaryotes and eukaryotes. (N)
10. Microarrays detect gene expression patterns over the genome.
Restriction enzymes cut DNA at specific sites

Palindrome

Restriction enzymes cut DNA at specific sites

- 3 types of ends: 5’ overhang, blunt and 3’ overhang
- Cognate methyl transferases protect host genome from digestion. Restriction-modification systems degrade “foreign” DNA.

Average frequency of restriction sites in “random” DNA sequences

<table>
<thead>
<tr>
<th>Site size</th>
<th>Average Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1/256</td>
</tr>
<tr>
<td></td>
<td>(1/4 x 1/4 x 1/4 x 1/4)</td>
</tr>
<tr>
<td>6</td>
<td>1/4,096</td>
</tr>
<tr>
<td>8</td>
<td>1/65,536</td>
</tr>
</tbody>
</table>

The average occurrence of each sequence = 1/4ⁿ, where n = the site length and all bases are equally represented.
Lots of different recognition sites known

Core four bases

Flanking bases

None

A----T

C----G

G----C

T----A

A simple cloning procedure

1. Cut “insert” and “vector” DNA with a restriction enzyme

2. Mix and join ends with DNA ligase. The ends should match for efficient ligation.
Cloning without DNA ligase

**Ligation-independent cloning**

1. Prepare open vector and insert with the same long “sticky” ends
   No dT in template

   + Pol I Klenow fragment + dATP

2. Mix and let the ends anneal.

3. Transform the nicked plasmid. The plasmid is repaired in vivo.

**“Gateway” cloning**

1. Prepare an insert flanked by sites for a site-specific DNA recombinase.

2. Mix insert with the closed vector containing the recipient recombination site and recombinase enzyme.

3. (Have lunch.) Transform.

“Vectors” allow DNA sequences to be cloned - 1

**Ori + selectable marker + cloning site (polylinker)**

**Phage λ for cloning big (7-25 kb) DNA pieces**

- Region into which exogenous DNA can be inserted

Sticky end

Package in vitro

Recombinant λ virions
"Vectors" allow DNA sequences to be cloned - 2

Shuttle vectors: move genes between organisms

Expression vectors:
Make your favorite protein

"Reporter" genes:
β-gal, GFP . . .

"Vectors" allow DNA sequences to be cloned - 3

Transient transfection: eukaryotes

Stable transfection

Plasmid is unstable -- Expression variable

Plasmid integrated in large tandem arrays -- protein overexpressed
Gel electrophoresis separates DNA on the basis of size

**Agarose:** big fragments (>300 bp)
**Acrylamide:** smaller fragments, higher resolution

Mobility proportional to log MW.

Chemical DNA synthesis

Sequential rounds of coupling, oxidation and deprotection of the 5’ OH build up the oligonucleotide.
Frontiers in DNA synthesis

Currently: 100–200 nucleotides routine (Assemble 5 kW)
10,000 = largest.

Primer set for the human genome (30,000 genes) ~ $10^4

Goal 1: Make yeast chromosome 3: 300 kW without errors!
(Jeff Boeke; $300,000)

Goal 2: Assemble a total of 16 x 10^6 w/o errors for ~$1000
(George Church)

PCR (Polymerase Chain Reaction): isolate and amplify any DNA sequence

N cycles amplifies the target sequence 2^N-fold.
Quantitative PCR (QPCR) defines amount of starting template.
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DNA sequencing by partial chain termination

ddNTPs terminate the chain
DNA sequencing by partial chain termination

1. All fragments start at the primer
2. All fragments ending in a particular base have a different length and a different color tag
3. Separating the mixture of products by size reveals the sequence.
4. <1000 bases/reaction
Two strategies for genome sequencing

Hierarchical Sequencing
1. Genetic (linkage) mapping (distances in centimorgans)
2. Physical mapping (distances in nucleotides bases)
3. DNA sequencing

Shotgun Sequencing

Genome resequencing -- Many short reads

1. Prepare sample: Shear, repair, ligate adapters to both ends
2. Create clusters: Attach one end to a solid support, PCR in situ with one primer attached to support

3. Sequence-by-synthesis:
   • Denature, add primer + all 4 fluorescent dNTPs with blocked 3’ OH to add 1 base to each cluster.
     • Read each cluster with laser.
     • Deblock 3’ OH and remove color.
     • Repeat synthesis of next base.
     • Read.
     • Repeat 30 x for 10⁶ clusters!
4. “Assemble” genome sequence by finding overlaps of 30mers and comparing to known genome sequence.
Genome resequencing

Currently:
10^9 reads ~ $5,000

X-Prize:
Human genome < $1,000
(3.2 x 10^9 bases (x 8 reads))

Site-directed mutagenesis

1. Denature methylated template and anneal divergent mutagenic primers.

2. PCR amplify the entire plasmid with a DNA pol lacking 5'-->3' exonuclease.

3. Select against parental strands with DpnI restriction enzyme, which cuts methylated and hemimethylated DNA.

   Transform
Gene replacement in mice -- make donor cells

1. Insert drug markers into genome of ES cells
   - Insert drug markers into genome of ES cells
   - Select to enrich for homologous recombinants

Check insertion site by Southern blotting

| Neo<sup>r</sup> confers resistance to G-418. | tk<supHSV</sup> confers sensitivity to ganciclovir. |

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“Southern” blotting detects DNA sequences by hybridization

1. Digest DNA using restriction enzyme(s)
2. Run gel
3. Transfer DNA from gel to (nitrocellulose) paper.
4. Denature DNA, hybridize probe DNA, and wash off excess probe.
5. Detect the probe on the paper. E.g. by autoradiography.

“Northern” blotting detects RNA on the gel.
Gene replacement in mice -- germline incorporation

Transgenic mice express a new gene

Which mouse expresses extra copies of the growth hormone gene?

1. Inject ES cells into early embryos,
2. Transfer embryos to foster mother,
3. Breed chimeric mice and screen for progeny with mutant germ line,
4. Screen progeny DNA for mutation,
5. Mate heterozygotes (X+/X-),
6. Screen progeny DNA for KO genotype (X-/X-).

Entire process takes a year.

Gene replacement in plants -- engineered crops

E.g. "Golden rice" synthesizes β-carotene
Microarrays detect expressed genes by hybridization

1. Label cDNAs with red fluorophore in one condition and green fluorophore in another reference condition.
2. Mix red and green DNA and hybridize to a “microarray”.
3. Relative to the reference, Red=enriched, yellow = =, green = depleted.

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