DNA Sequencing and Genetic Testing Technology

- Dideoxynucleoside sequencing
- New sequencing methods
- Detecting mutations
- High-throughput genotyping approaches
- DNA chip formats
- cDNA sequencing
- Karyotyping
Steps in DNA Sequencing and Genotyping

- Selecting part of genome to sequence or genotype
- Amplifying nucleic acid
- Determine sequence of one or a succession of nucleic acids:
  - Chemical reaction format
  - Read out format
- Resequencing vs de novo sequencing

Dideoxynucleoside Sequencing

Key:
- Phosphate group

Figure 3.1: Human Molecular Genetics, 3rd ed. W.B. Saunders Company (1989)
Dideoxynucleoside Sequencing

Figure 7-2 (part 1 of 2) Human Molecular Genetics, 3rd. (G. Garland Science 2004)

Dideoxynucleoside Sequencing

Figure 7-3 (part 1 of 2) Human Molecular Genetics, 3rd. (G. Garland Science 2004)
Genome Sequencing Approaches

Applications of Ultra-Low-Cost Sequencing

- Sequencing individual human genomes as component of preventive care.
- Genotype-phenotype associations
- Comprehensive gene expression profiling in vitro and in situ at all stages of development of a multicellular organism
- Comprehensive analysis of mutations present in cancer clones.
- Mitochondrial heteroplasmy
- Microbial diversity (metagenomic studies)

Shendure N. Advanced sequencing technology: methods and goals. Nat Rev Gen 2004
Oligonucleotide Array Synthesis

- Photolithography
- In situ synthesis
- Photolabile protective groups (photomasking)

High-Density Oligonucleotide Arrays

Chee M. Assessing genetic information with high-density oligonucleotide arrays. Science 1996
High-Density Oligonucleotide Arrays

Chee M. Assessing genetic information with high-density oligonucleotide arrays. Science 1996

Pyrosequencing

(NA)_n + Nucleotide $\xrightarrow{\text{Polymerase}}$ (NA)_{n+1} + PPI
PPI + APS $\xrightarrow{\text{ATP sulfurylase}}$ ATP + SO$_4^{2-}$
ATP + Luciferin + O$_2$ $\xrightarrow{\text{Luciferase}}$ AMP + PPI + Oxy luciferin + CO$_2$ + Light

Ronaghi M. Pyrosequencing sheds light on DNA sequencing. Genome Res 2001
Pyrosequencing - Solid Phase

Ronaghi M. Pyrosequencing sheds light on DNA sequencing. Genome Res 2001

Pyrosequencing - Liquid Phase

Ronaghi M. Pyrosequencing sheds light on DNA sequencing. Genome Res 2001
Pyrogram

Ronaghi M. Pyrosequencing sheds light on DNA sequencing. Genome Res 2001

454 LifeSciences Sequencer

PicoTiter™ Plates

- Multiple optical fibers are fused to form an optical array
- Selective removal of core material leaves wells that serve as "test tubes"
- Reactions occurring in the "test tubes" can be monitored optically through the remaining fiber
- Well diameter: 3μ - 250μ, 44μ typical
- Plate contains 1.6 million wells
454 LifeSciences Sequencer - Process Overview

1) Prepare Adapter Ligated ssDNA Library

2) Gel purification and cloning

3) Load beads and enzymes in PicoTiter Plate™

4) Perform Sequencing by synthesis on the 454 Instrument

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454 LifeSciences Sequencer - Process Overview

emPCR

A) Anneal Single Stranded template to an excess of DNA Capture beads

B) Emulsify beads and PCR reagents in water-in-oil microreactors

C) Break Microreactors, Enrich for DNA positive beads, Load DNA beads
Depositing DNA Beads into the PicoTiter™ Plate

Load beads into PicoTiter™ Plate

Load Enzyme Beads

Centrifuge Step

454 Technology - Sequencing Instrument

Sequencing and Basecalling Results for 191-base Read
### 454 LifeSciences Sequencer

#### Read Results: Run Statistics

<table>
<thead>
<tr>
<th>Organism</th>
<th>Genome Size</th>
<th>GC Content</th>
<th>Reads</th>
<th>Bases</th>
<th>Coverage</th>
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</thead>
<tbody>
<tr>
<td><em>C. jejuni (NCTC 11168)</em></td>
<td>1.6MB</td>
<td>31%</td>
<td>226,429</td>
<td>24,521,527</td>
<td>13.1</td>
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<td><em>Escherichia coli (K12)</em></td>
<td>4.6MB</td>
<td>51%</td>
<td>904,858</td>
<td>86,135,933</td>
<td>18.6</td>
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<tr>
<td><em>H. salinarum (NRC-1)</em></td>
<td>2.0MB</td>
<td>68%</td>
<td>213,774</td>
<td>21,309,326</td>
<td>10.4</td>
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</tbody>
</table>

#### Read Results: Read Length

- **C. jejuni (NCTC 11168)**
  - 1.6MB Genome
  - 31% GC Content
  - 24.5 Million Bases

- **Escherichia coli (K12)**
  - 4.6MB Genome
  - 51% GC Content
  - 86 Million Bases

- **H. salinarum (NRC-1)**
  - 2.0MB Genome
  - 68% GC Content
  - 21 Million Bases
A brave new future that makes old technology redundant

Nanopore sequencing  AFM sequencing

Rationale for Genotyping

- SNP discovery
  - High throughput

- Diagnostic testing
  - High reliability
  - Distinguishing homozygosity vs heterozygosity
  - Rare mutations
  - Wider variety of mutations
### General Approaches to Diagnostic Testing

- Testing for specific mutation(s)
- Scanning a gene
  - DNA sequencing
  - Methods based on heteroduplex or single-strand conformation analysis
  - Protein truncation test
- Gene tracking
  - Large multi-exon gene; pedigree structure

### General Approaches to Genotyping

- Methods of amplification
General Approaches to Genotyping

PCR
Cloning of PCR Products

1. Use PCR primers P1, P2 to amplify alleles in genomic DNA samples.

   - Allele 1 = (CA)_{14}
     - 40 bp
     - PCR product = 80 + 32 = 112 bp
   - Allele 2 = (CA)_{14}
     - 40 bp
     - PCR product = 80 + 20 = 108 bp
   - Allele 3 = (CA)_{11}
     - 40 bp
     - PCR product = 80 + 22 = 102 bp

2. Denature PCR products and size-fractionate by polyacrylamide gel electrophoresis.

3. Autoradiography

Microsatellite Repeat Typing
Allele-Specific Oligonucleotide (ASO) Dot Blot Hybridization

- Dot blot: aqueous soln of target DNA denatured and allowed to dry on nitrocellulose membrane,
- Single-stranded labeled probe with central mismatch

Allele-Specific PCR

- 3’ mismatch
- High stringency
- Certain mismatches are more unstable than others
Real-Time PCR (TaqMan)

- Depends on 5’ to 3’ nuclease activity of Taq DNA polymerase.

- Uses:
  - Gene expression
  - Mutation detection using allele-specific amplification
  - Detecting minor variants (e.g. “minimal residual disease”)

Litvak KJ. Allelic discrimination using fluorogenic probes and 5’ nuclease activity. Genet Anal 1999
**LightCycler**

A. Probe/Probe design

1. Primer attachment and extension

2. Product separation from template

3. Probe hybridization and FRET

- **Fluorescence resonance energy transfer (FRET)**
- **Donor fluorophore excited photometrically and transfers its energy to the acceptor fluorophore.**
- **Melting curve analysis indicates presence of heterozygous vs homozygous mutations, indels, and additional mismatches.**


**Molecular Beacons**

- **Molecular Beacon** + **Target** \[\rightarrow\] **Hybrid**

Homogenous Wild-type  Heterozygote  Homogenous Mutant

<table>
<thead>
<tr>
<th>Thermal Cycles</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>10</td>
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<tr>
<td>20</td>
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<tr>
<td>30</td>
<td>0.9</td>
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<td>40</td>
<td>1.2</td>
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Primer Extension ("Minisequencing")

- TaqMan (a,b)
- Molecular beacons
- FRET (Fluorescent energy resonance transfer)