Objectives

- Compare phenotypic consequences of point mutations.
- Distinguish detection of known mutations from scanning for unknown mutations.
- Discuss methods used to detect point mutations.
- Describe mutation nomenclature for expressing sequence changes at the DNA, RNA, and protein levels.
**Mutation Nomenclature**

- **5162 G→A**
  - Base position
  - Original base
  - Replacement

- **Q197G**
  - Original aa
  - aa position
  - Replacement

**Deletion:** 197delAG

**Insertion:** 2552insT

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**Point Mutations**

- Gene mutations involving one or few base pairs
- Not detectable by the cytogenetic method
- Detected at the DNA sequence level
Point mutations do not always have phenotypic effect.

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>Amino Acid Sequence</th>
<th>Type of Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG CAG GTG ACC TCA GTG</td>
<td>M Q V T S V</td>
<td>none</td>
</tr>
<tr>
<td>ATG CAG GTI ACC TCA GTG</td>
<td>M Q V T S V</td>
<td>silent</td>
</tr>
<tr>
<td>CAG CTG TCA GTG</td>
<td>M Q L T S V</td>
<td>conservative</td>
</tr>
<tr>
<td>ATG CG GTG ACC TCA GTG</td>
<td>M P V T S V</td>
<td>non-conservative</td>
</tr>
<tr>
<td>ATG CAG GTG ACC TGA GTG</td>
<td>M Q V T ter</td>
<td>nonsense</td>
</tr>
<tr>
<td>ATG CAG GTG AAC CTC AGT G</td>
<td>M Q V N L S</td>
<td>frameshift</td>
</tr>
</tbody>
</table>

Types of Mutation Detection Methods

- Hybridization based
- Sequencing (polymerization) based
- Cleavage based
Hybridization-Based Methods

- SSCP
- ASO
- Melt curves
- Array technology

Single-Strand Conformation Polymorphism

- Scans several hundred base pairs
- Based on intra-strand folding
  - Single strands will fold based on sequence.
  - One base difference will affect folding.
- Folded single strands (conformers) can be resolved by size and shape.
- Strict temperature requirements
Single-Strand Conformation Polymorphism (SSCP)

1. Amplify region to be scanned using PCR.

2. Denature and dilute the PCR products.

3. Separate conformers by PAGE or CGE.

4. Analyze results by comparison to reference normal control (+).
5. Detect PAGE bands by silver staining.

<table>
<thead>
<tr>
<th>T1</th>
<th>T2</th>
<th>NC</th>
</tr>
</thead>
</table>

- T1: test sample without mutation
- T2: test sample with mutation
- NC: normal control

**Allele-Specific Oligomer Hybridization (ASO)**

- Dot blot method
- Relies on binding effects of nucleotide mismatches.
- Specimen in solution is spotted on nitrocellulose.
- Labeled oligonucleotide probe is hybridized to immobilized specimen.
Allele-Specific Oligomer Hybridization (ASO)

- Three specimens spotted on duplicate membranes
- One membrane exposed to probe complementary to the normal sequence (+ probe)
- One membrane exposed to probe complementary to the mutant sequence (m probe)

\[ m/+ \quad +/+ \quad m/m \quad \text{+ probe} \]
\[ m/+ \quad +/+ \quad m/m \quad \text{m probe} \]

Allele-Specific Oligomer Hybridization (ASO)

- Chromogenic probe detection
  - 1: normal (+/+)
  - 2: heterozygous (m/+)
  - m: heterozygous mutant control
  - +: normal control
  - N: negative control

\[ 1 \quad 2 \quad m \quad + \quad N \quad \text{+ probe} \]
\[ 1 \quad 2 \quad m \quad + \quad N \quad \text{m probe} \]
Melt Curve Analysis

- Based on sequence effect on $T_m$
- Can be performed with or without probes
- Requires double-strand DNA–specific dyes
  - Ethidium bromide
  - SyBrGreen
- Also performed with fluorescence resonance energy transfer (FRET) probes

Melt Curve Analysis

- Double-stranded DNA–specific dye (SyBrGreen) will fluoresce when bound to DNA.
- Denaturation of DNA to single strands will result in loss of fluorescence.
Melt Curve Analysis

- Every sequence has a characteristic $T_m$.
- Melt curve $T_m$ indicates which sequence is present.

Detection instrument software may convert the melt curve to a derivative of fluorescence (speed of drop vs. temperature).
Array Technology

- **Reverse dot blot** methods
- Used to investigate multiple genomic sites simultaneously
- Unlabeled probes are bound to substrate.
- Specimen DNA is labeled and hybridized to immobilized probes.

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**Array Technologies**

<table>
<thead>
<tr>
<th>Method</th>
<th>Substrate</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>macroarray</td>
<td>nitrocellulose</td>
<td>radioactive, chemiluminescent, chromogenic</td>
</tr>
<tr>
<td>microarray</td>
<td>glass, nitrocellulose on glass</td>
<td>fluorescent</td>
</tr>
<tr>
<td>high-density oligonucleotide arrays</td>
<td>glass</td>
<td>fluorescent</td>
</tr>
<tr>
<td>microelectronic arrays</td>
<td>electrode grid</td>
<td>fluorescent</td>
</tr>
</tbody>
</table>
## Microarray Technologies

<table>
<thead>
<tr>
<th>Method</th>
<th>Array</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>comparative genomic hybridization (CGH)</td>
<td>microarray, macroarray</td>
<td>detection of genomic amplifications and deletions</td>
</tr>
<tr>
<td>expression array</td>
<td>microarray, macroarray</td>
<td>detection of relative changes in gene expression</td>
</tr>
<tr>
<td>SNP detection, mutation analysis, sequencing</td>
<td>high-density oligonucleotide array</td>
<td>detection of single-base differences in DNA</td>
</tr>
</tbody>
</table>

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## High-Density Oligonucleotide Arrays

- Interrogate thousands of genes simultaneously
- Requires a new array for each sample
- Unlabeled probes are synthesized on the substrate.

![Oligonucleotide array diagram](attachment:image)

(10–25mers)
High-Density Oligonucleotide Arrays

- Test DNA is fragmented before hybridization.
- Short fragments will bind specifically to complementary sequences on the array.
- Tiling (overlapping probe sequences) is used to blanket detection of nucleotide changes in the sample.

Fluorescent signal indicates which sample hybridized DNA to probe.
Fluorescence is detected, normalized, and averaged by array readers and software.
High-Density Oligonucleotide Arrays

Results displayed in graphical form.

Normal sequence (TCG)  

Heterozygous (TCG>TAG)  

Represents five probes, each carrying the indicated base or deletion at the same position.

Sequencing (Polymerization)-Based Methods

- Sequence-specific PCR (SSP-PCR)
- Allelic discrimination
- Direct sequencing
Sequence-Specific Primer PCR (SSP-PCR)

- PCR primer extension requires that the 3’ base of the primer is complementary to the template.

- Primer design is used to detect mutations in DNA.
- Generation of PCR product indicates the presence of mutation or polymorphism in the template.
Detection of BRCA1 187delAG by SSP-PCR

(1) GAAGTTGCATTTTATAAACCTT->
   AAAATGAAGTTGTCAATTTTATAACCTTTTTTTAAAGATATATATA
   TGTTTTTTCTAATGTGTTAAGTTCTGGGAACAGAAAAATGGAT
   TTATGTGCTGTTCGTTGAAAGAAGGTACAAAAT

(2) ATTAATGCTATGCAGAAAATGTTAG-> (only in normal)
   GTCATTATGCTATGCAAGAAAATGTTAG[AG]TGCCCCATCTGTAA
   (only in mutant)<--ATC - - ACAGGTAGACCATT
   GTGAGCACAAGAATGTGATTTGGGATCTGATTATATCTCTA
   CAGT (3)

   TGCAATGAACAGAATTTGGATCTAGATATCTCTA
   <- CTGTCTTTAACTGGATAT (4)

Detection of BRCA1 185delAG by SSP-PCR

230 bp (1) and (4)
180 bp (1) and (3)
120 bp (2) and (4)

Product of primers (1) and (3) is specific for mutation.
Allelic Discrimination

- Uses fluorescently labeled probes
- Similar to TaqMan technology
- Generates “color” signal for mutant or normal sequence
- Performed on real-time PCR instruments

- Probe complementary to normal sequence labeled with FAM fluorescent dye
- Probe complementary to normal sequence labeled with VIC fluorescent dye

Normal probe (FAM)  Mutant probe (VIC)

Normal: Green signal
Mutant: Red signal
Allelic Discrimination

- Signals are detected and analyzed by the instrument software.
- Multiple samples are analyzed simultaneously.

Cleavage-Based Methods

- Restriction fragment length polymorphism (RFLP)
- Nuclease cleavage
- Invader (Hologic)
Restriction Fragment Length Polymorphism (RFLP)

- Restriction enzyme site recognition detects presence of sequence changes.
  
  e.g., G→A change creates EcoRI site:

<table>
<thead>
<tr>
<th></th>
<th>NL:</th>
<th>GTCA GGGTCC</th>
<th>GTGC...</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mut:</td>
<td></td>
<td>GTCA GGATCC</td>
<td>CTGC...</td>
</tr>
<tr>
<td>NL</td>
<td>U</td>
<td>U</td>
<td>C</td>
</tr>
<tr>
<td>Mut</td>
<td>C</td>
<td>C</td>
<td>U</td>
</tr>
</tbody>
</table>

Agarose gel:
- U: uncut
- C: cut

Heteroduplex Analysis with Single-Strand-Specific Nucleases

- Uses nucleases that cut single-stranded bubbles in heteroduplexes.
- Region of interest is amplified by PCR.
- PCR product is denatured and renatured with or without added normal PCR product.
- Renatured duplexes are digested with nuclease; e.g., S1 nuclease.
- Products are observed by gel electrophoresis.
Heteroduplex Analysis with Single-Strand-Specific Nucleases

Mix, denature → Renature

Homoduplexes cleaved by enzyme
Heteroduplexes not cleaved by enzyme

Cleaved fragments indicate presence of mutation.
Invader Technology

- Mutation detection with proprietary Cleavase enzyme
- Sample is mixed with probes and enzyme.
- Enzyme cleavage of probe-test sample hybrid will yield fluorescent signal.
- Signal will only occur if probe and test sample sequence are complementary.

Probes and enzyme are provided.

- 96-well plate format
Summary

- Mutations and polymorphisms are changes in the DNA sequence.
- DNA sequence changes have varying effects on the phenotype.
- Molecular detection of mutations include hybridization-, sequence-, or cleavage-based methods.