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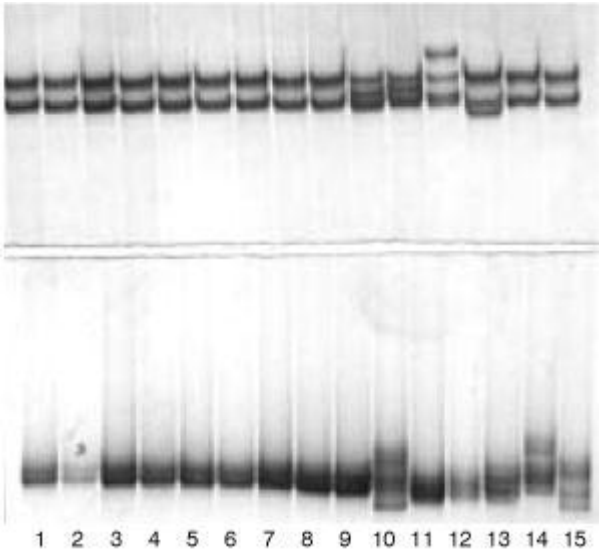

Cystic Fibrosis screening gel

http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=hmg&part=A2178

American Society of Cytopathology Core Curriculum in Molecular Biology

Chapter 3

Molecular Techniques
Other Techniques to Detect Mutations

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Objectives

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Discuss the principles and uses for denaturing gradient gel, heteroduplex analysis, SSCP, denaturing HPLC, and melting curve analysis.

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Detecting Mutations

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- Mutations include deletions, insertions, inversions, and translocations
- These may or may not affect the type of protein created and/or the effect seen
- The larger the number of bp affected, the more likely the type/function of the protein will be affected
- Mutations at the beginning of a coding sequence are likely to have severe effects

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List of Other Techniques

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- Denaturing gradient gel
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- Denaturing HPLC
- Melting curve analysis

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Denaturing gradient gel

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- **DGGE** – Denaturing gradient gel electrophoresis can detect differences in denaturation between a normal and mutated DNA molecule down to one nucleotide
- ds DNA fragments 200-700 in length are prepared from PCR or restriction digestion
- Uses polyacrimide gels with low denaturation concentrations at the top and high at the bottom
- Usually 10% - 20% difference between the two

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Denaturing Gradient Gel Electrophoresis

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- As ds DNA moves through the gel, denaturing conditions increase, and separation begins to take place
- As sections of the ds DNA with different melting temperatures begin to denature, they begin to separate, widen, and slow the progress of the DNA through the gel
- This causes normal vs. mutated DNA to travel different distances through the gel, shifting the abnormal band to a different position than the normal.

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Denaturing Gradient Gel Electrophoresis (DGGE)

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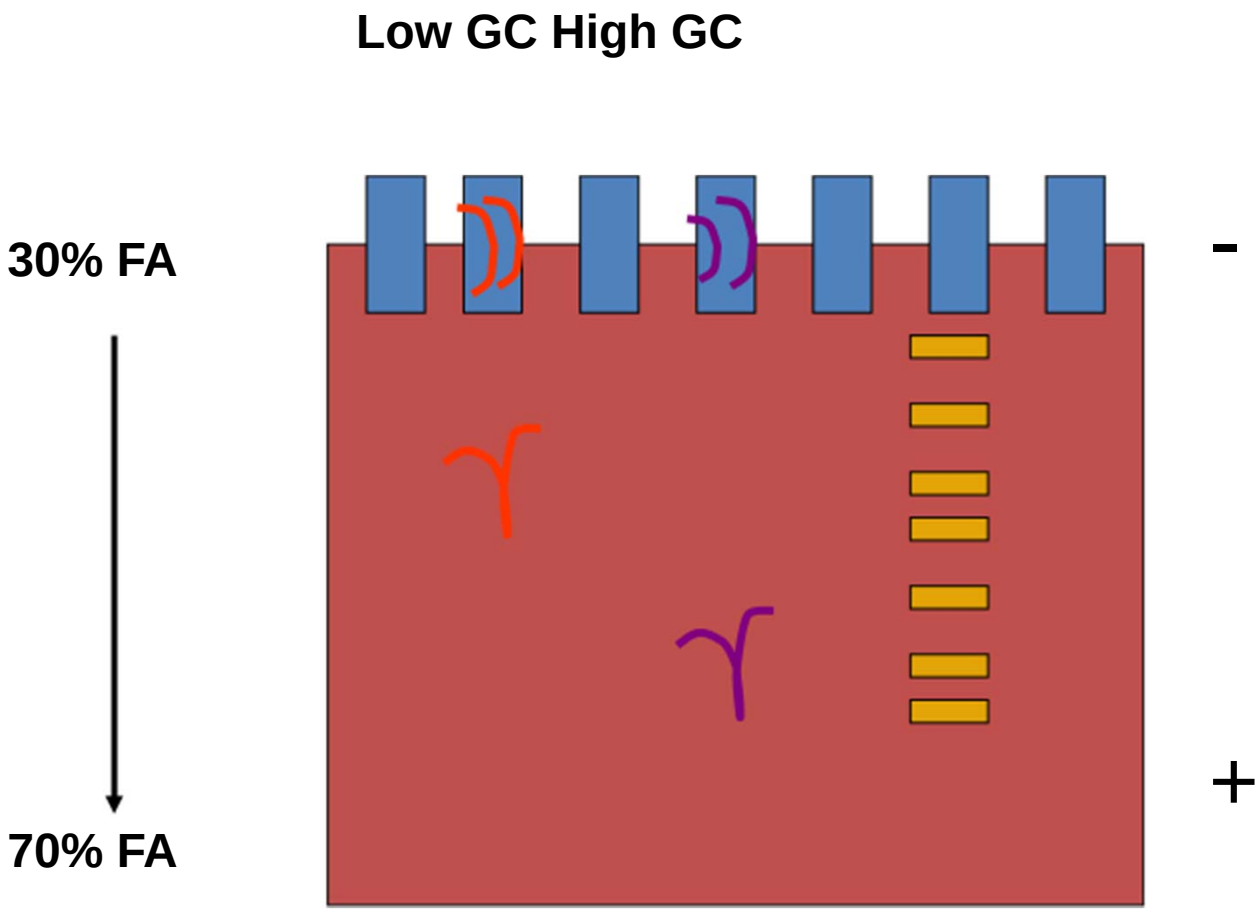

http://www.soest.hawaii.edu/marinefungi/2007_01_25_lect06.pdf

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DGGE

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- Complete strand separation is prevented by the use of CG rich sequences which may be attached to the ends of PCR products using primers with a 40 bp 5' CG tail
- These are called "**GC clamps**" and should be positioned next to the area of the highest melting domain.

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DGGE-PCR: PCR with GCclamp

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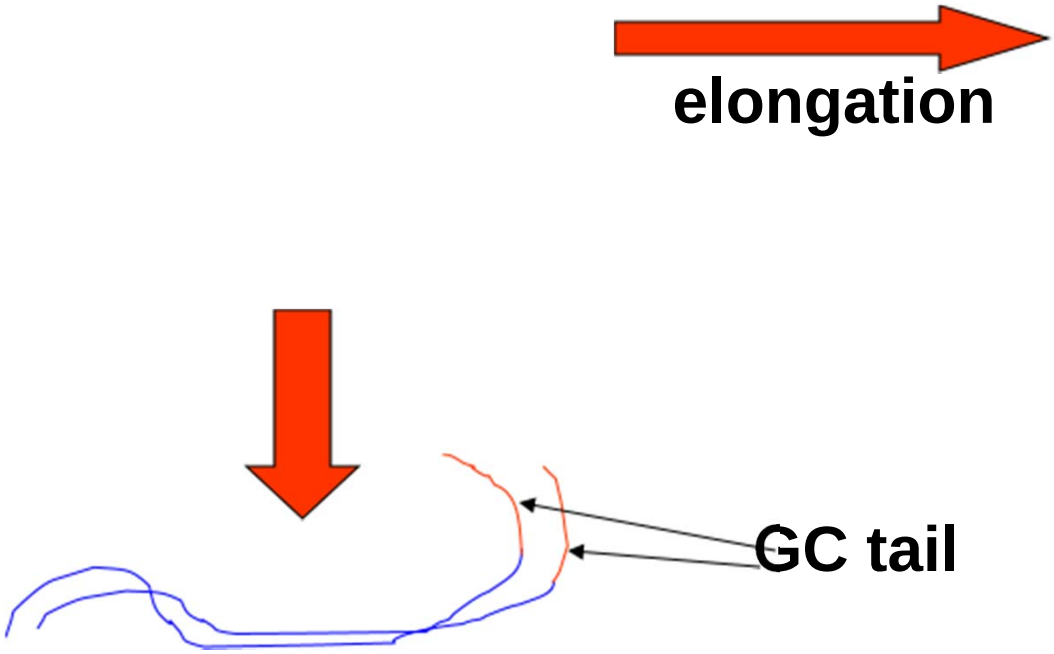
Supply PCR oligonucleotide primers with a GC-tail:

5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3'
3'-NNNNNNNNNGGATGCCCTCCGTCGTCNNNNNN….-5'



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Two gradient orientations: Perpendicular and Parallel

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- **Perpendicular** – gradient increases horizontally across gel, perpendicular to direction of current
- **Parallel** – gradient increases vertically, parallel to the direction of sample migration
- Perpendicular results in a sigmoid curve. One well is used for samples. It is used to define smaller gradient conditions for a subsequent parallel gradient.
- Parallel uses separate lanes and compares the bands (see p. 178, MD)

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Uses of DGGE

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To detect:

- Tumor suppressor genes
- Clonality
- Population polymorphisms
- Can also have variants which work on similar principles (CDGE = constant gradient gel electrophoresis, used for known mutations) (TTGE = temporal temperature gradient gel electrophoresis, where gel is run at gradually increasing temperatures to widen differences between fragments, used for cancer, genetic, and industrial applications)

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Example of various organisms at different depths in the ocean by DGGE

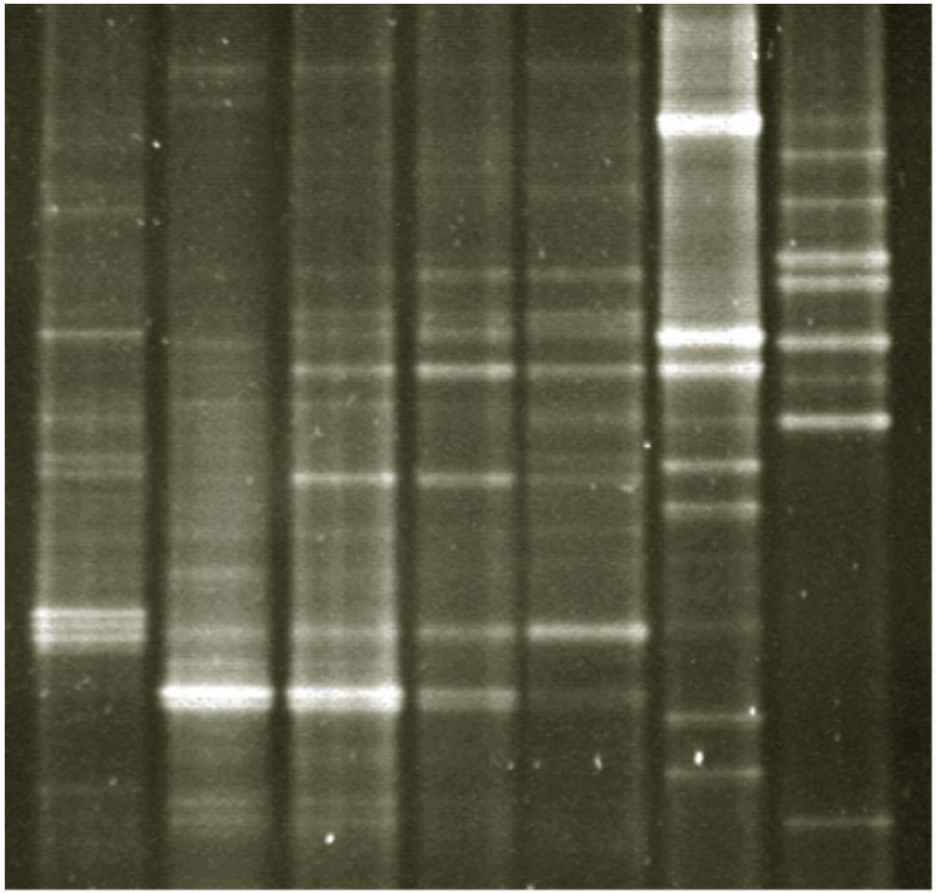
Band patterns highly variable:

Different communities in each depth

Increasing concentrations of denaturants

40% FA

70% FA



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Heteroduplex Analysis

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- Test amplicons are mixed with reference amplicons to hybridize and form **heteroduplex** structures
- These structures are not completely complementary to one another and may also be formed when a heterozygous sample is denatured and renatured
- Migration through gel differs from homoduplexes
- Presence of bands different from homozygous reference control indicates mutations

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Principle of gel-based heteroduplex analysis

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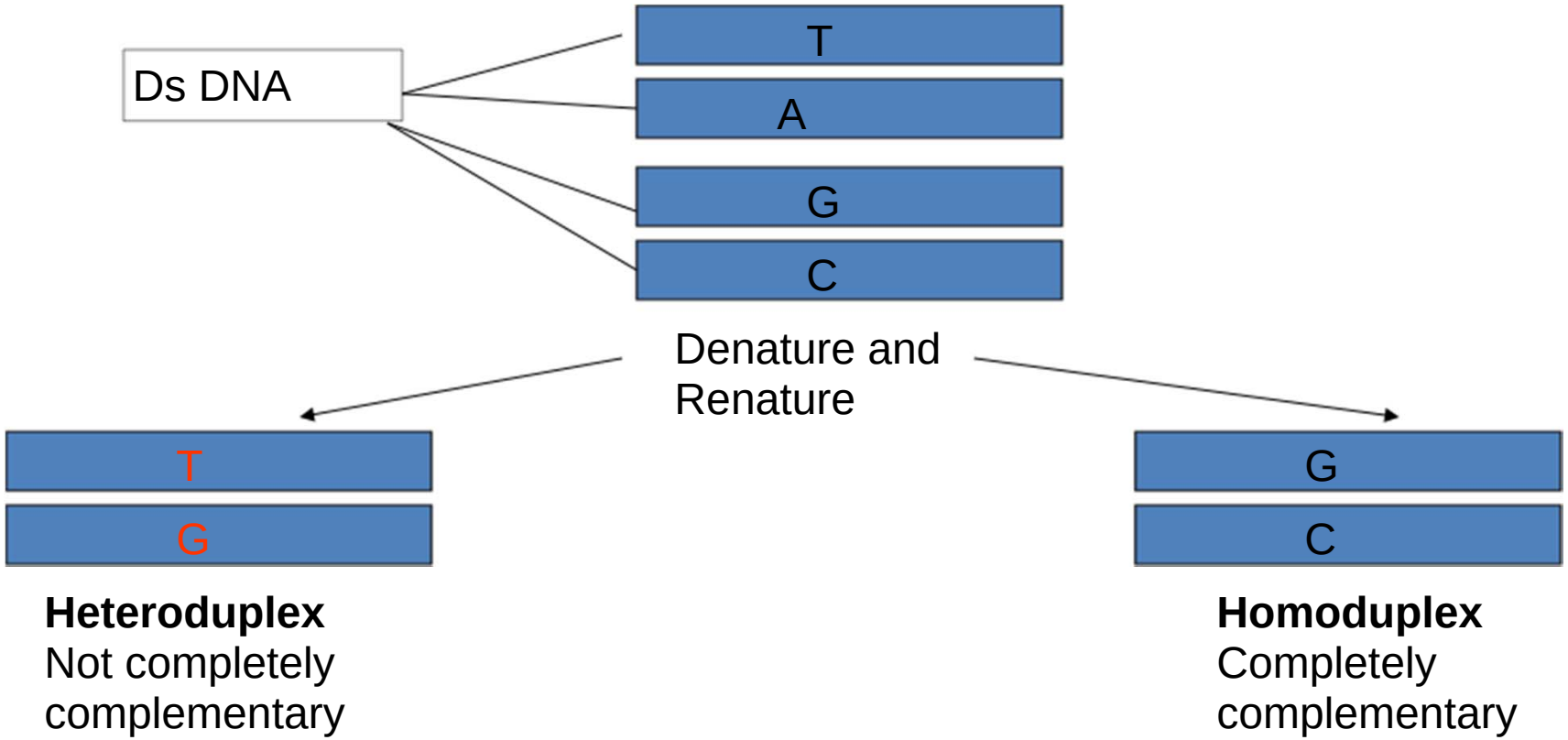

See p. 183, MD

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Uses of heteroduplex analysis (HA)

- HIV typing
- Hematologic testing

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Single-strand conformation polymorphism (SSCP)

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- A frequently used mutation analysis method
- DNA (and RNA) prefers the double stranded state
- If no complementary strand, will anneal to itself in an intrastrand duplex to form 3-D structure, a **conformer**

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Principles of SSCP

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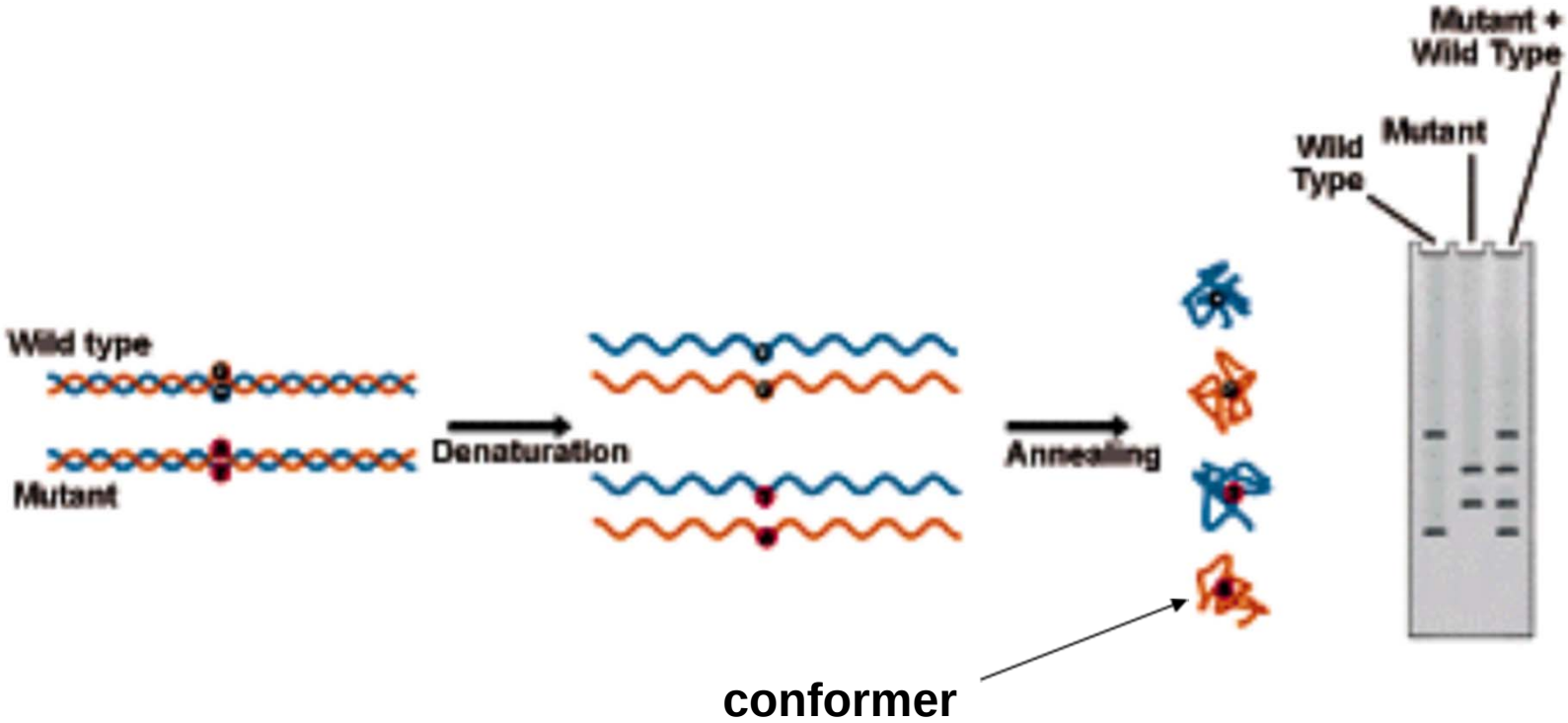

http://nationaldiagnostics.com/article_info.php/articles_id/43

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SSCP

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- Shape of conformer is determined by the primary sequence in the strand
- Denaturing and temperature conditions are tightly controlled to optimize intrastrand hybridization
- A single bp difference will cause a variation is the shape of the folded conformer

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SSCP

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- Resolved on polyacrylamide gel or by capillary electrophoresis
- Speed of migration depends on shape as well as size
- Band patterns varying from normal indicate the presence of mutations
- Most sensitive at concentrations of at least 30% mutant cells (inherited mutations will be 50%)

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SSCP

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- For analysis of tumor cells, may need a microdissection of solid tumor to obtain at least 30% mutated cells
- Selectively obtaining areas from slides or paraffin blocks to process for SSCP will aid in obtaining 30% tumor cells for analysis

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Comparison of SSCP and HA

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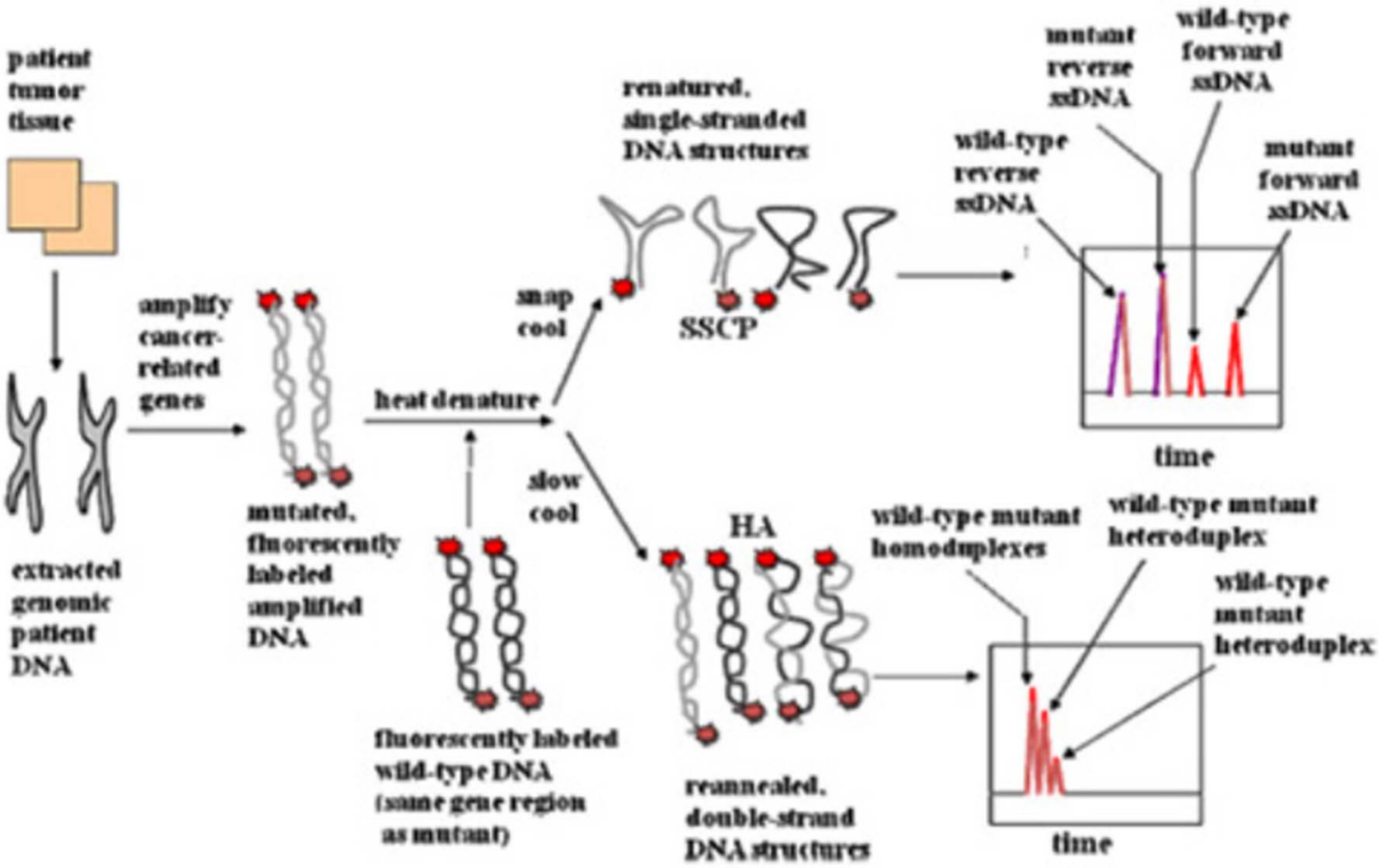

www.stanford.edu/group/barronlab/ResBioSep.htm

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Conformation-sensitive gel electrophoresis

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- Heteroduplex analysis method
- To screen large genes for mutations and polymorphisms
- Gel uses 1,4-bis (acrolyl) piperazine with ethylene glycol and formamide to weakly denature and show conformational differences
- Used to detect mutations

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Heteroduplex analysis with single-strand specific nucleases

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- Sensitivity can be increased over DHPLC by cleaving heteroduplexes at the mispaired bases.
- After cooling, a digestion is run which cleaves only the unmatched site
- Therefore, the mutant DNA is broken into smaller pieces than the homozygous normal DNA which remains intact

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Denaturing HPLC

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- **DHPLC** = denaturing high-performance liquid chromatography
- Performed on PCR products 150-450 bp in length
- Products are heated to denature, then slowly cooled
- Separation performed at melting temperature of PCR product

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DHPLC

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- Heteroduplexes elute ahead of homoduplexes
- Detected by absorbance at 260nm or fluorescence
- More sensitive than gel methods, better for large numbers of samples
- Detection of mutations occurs when bands differ from homozygous control

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Melting Temperatures

The analysis of the temperature at which 50% of the probe is double stranded and 50% is single stranded is called the **melting temperature**.

As the temperature increases during hybridization, more of the probe will become single stranded.

This can be calculated for short probes (14-20 bases) using a simplified formula for dsDNA:

$Tm = 4^{o}$ C x # GC pairs + 2° x # AT pairs

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Melting Temperatures

For longer dsDNA taking into account the sodium concentration in mol/L and the # base pairs in the shortest duplex, the formula is:

$Tm=81.5^{o}$ C +16.6 logM + 0.41 (%G+C) – 0.61 (%formamide) – (600/n)

M = sodium concentration in mol/L

n = # base pairs in the shortest duplex

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Melting Temperatures

RNA:RNA hybrids and DNA:RNA hybrids are more stable than the dsDNA probes and have slightly different formulae for Tm. (Chpt. 6, Molecular Diagnostics, p. 108.0

The ideal hybridization conditions can be estimated using the melting temperature of the probe sequence.

Specimens with genetic variation can be analyzed using melting curve analysis.

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Melting Curve Analysis

- Used to detect mutations based on differences in denaturation characteristics
- Uses PCR amplicons created with addition of dsDNA-specific dye, such as SYBR Green or ethidium bromide
- As temperature begins to rise, high levels of dye fluoresce because most DNA is still ds DNA

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Melting Curve Analysis

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- But as temperature rises, fluorescence decreases as dsDNA becomes ssDNA
- Identical DNA should have identical melting temperatures (Tm = the temperature at which ½ the DNA is ds and ½ is ss)
- Curves of amount of fluorescence plotted against temperature will reveal variations in DNA

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Melting Curve Analysis

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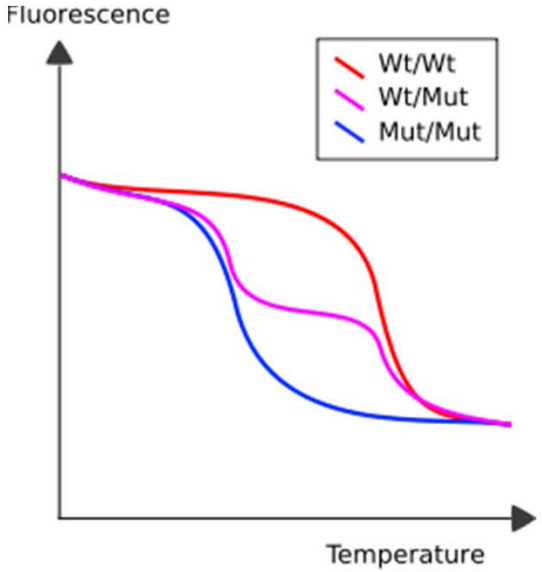
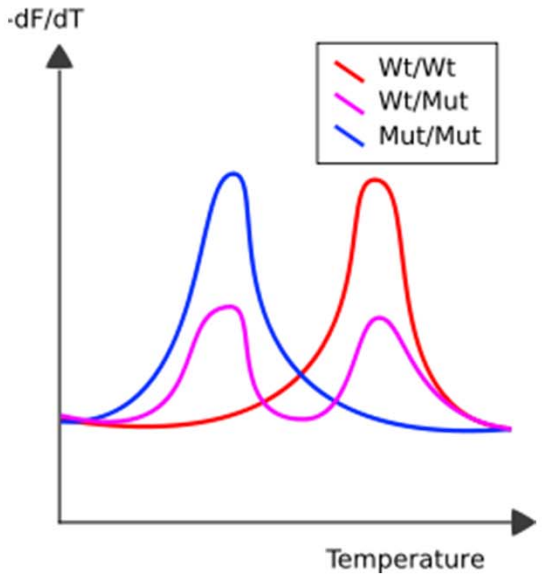


This graph shows the 3 curves from melting curve analysis, WT = wild type homozygous, WT/Mut = wild type and mutant heterozygous, and Mut/Mut is the homozygous mutant. Note that as the temperature increases the amount of dsDNA decreases.



This graph shows the derivative of the fluorescence data (df/dt) vs. temperature. The Tm (melting point) varies depending on which DNA type is present due to the variations in the DNA sequence.

http://en.wikipedia.org/wiki/File:Melting_Curve_Analysis_Graphs.svg

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HR-MCA

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- High resolution melting curve analysis (HR-MCA) can increase specificity
- Uses fluorescent resonance energy transfer (FRET) which fluoresces only when bound to target sequence
- Mismatch decreases the dissociation temperature compared to complementary DNA, indicating mutation

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Detecting mutations

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- All of these techniques can be used to detect mutations for disease detection or to detect carriers of a mutation.
- They can also be used in microbiologic applications to differentiate between various organisms
- Investigation of tumor genetics is another use for these techniques

Reference: Chapter 9, Molecular Diagnostics, Buckingham and Flaws, p. 173 - 202

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