DNA Detection

Chapter 13
Detecting DNA molecules

• Once you have your DNA separated by size
• Now you need to be able to visualize the DNA on the gel somehow
• Original techniques:
  – Radioactive label, silver staining
• Current techniques:
  – Fluorescent dyes
## Detection Methods

<table>
<thead>
<tr>
<th>Technique</th>
<th>Method</th>
<th>Colors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoradiography</td>
<td>P32 labeled dCTP incorporated during PCR; Photographic film exposed</td>
<td>One</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>Intercalating dye; exposed with UV light</td>
<td>One</td>
</tr>
<tr>
<td>SYBR green</td>
<td>Fluorescent intercalating dye; gel scanned with laser</td>
<td>One</td>
</tr>
<tr>
<td>Silver Staining</td>
<td>Gel soaked in silver solution and photo developed; gel permentantly stained</td>
<td>One</td>
</tr>
<tr>
<td>Fluorescent Dyes</td>
<td>Fluorescent dye incorporated into DNA during PCR; Gel scanned with laser</td>
<td>Up to five</td>
</tr>
</tbody>
</table>
Radioisotopes

- Incorporate P32 into one of the dNTPs
- The DNA strand that is made from PCR reaction will contain radiolabel
- Either blot the gel or dry the gel itself
- Expose the gel or the blot to photographic film – in the freezer, over night
- Radiation will expose photographic film leaving dark bands on the film wherever there are DNA bands in the gel
Radioisotopes

• Can detect the bands – very sensitive

Disadvantages:
• Radioactivity is dangerous
  – Expensive and wasteful
• Takes long time
• Only have one color band
  – Therefore need to have everything separated completely based on size first
Photographic Film:

Imagine interpreting these genotypes!
Silver Staining

• Run your PCR products through a gel to separate based on size
• Then expose gel to silver solution
  – Silver will bind to DNA within the gel matrix
• Photo develop the gel until silver becomes dark and visible with the naked eye
• Take a picture of the gel for permanent record of the DNA bands
Silver Stained Gel:

Bad Gel

Good Gel
Fluorescence

- Currently – everyone uses fluorescence to visualize DNA

Main reasons:
1. More than one colored dye can be used
   - Allowing multiplexing
2. Detection can be automated
   - The laser that excites the dye can also read the band off the gel
Fluorescent Dyes

- A specific color of fluorescent dye is attached to one of the PCR primers.
- This way the dye is incorporated into the PCR product.
- PCR products are separated on a gel or capillary based on size.
- Laser excites the dye within each product.
  - Also measures the light being emitted by each dye.
Fluorescence

• Molecule that is capable of fluorescence is called a fluorophore
• DNA labeling we use fluorophores that emit light in the visible range – 400 to 600 nm
• Photon from the laser excites the molecule
• Then as the molecule releases a photon as it descends back to its ground state
Fluorophore

- Laser Excitation
- Fluorophore Emission

Energy

Fluorescence

Wavelength (nm)
Laser Detection

- Photon emitted from fluorophore has less energy than photon delivered from laser.
- Therefore wavelength of photon emitted from fluorophore is different than wavelength of the photon delivered.
- Optical filters can be fitted onto laser so that only emitted light passes through and is measured.
  - All excitation light is filtered out.
Fluorophore Selection

• Choose different fluorophores whose emission spectra can be resolved from the emission spectra of the other dyes

• This is why dyes look like different colors
  – Emit with different wavelengths

• Also need to consider concentrations of each dye used
  – Some dyes emit more intense light
  – Need to adjust concentrations so that all dyes are fairly equivalent
Fluorescence

Four factors that will affect how well a fluorophore will emit light

1. Molar Extinction Coefficient
   • Ability of dye to absorb light

2. Quantum Yield
   • Ability to convert absorbed light into emission

3. Photo stability
   • Ability to undergo repeated excitations

4. Dye Environment
   • pH, temperature, solvent all affect fluorescence
Fluorophores

• Different fluorophores have different combinations of these attributes
• This is why some fluorophores are better at fluorescing than others
• Optimal conditions:
  – You want to choose dyes that have different emission spectra
  – Similar fluorescent intensities
Otherwise one dye may “bleed” into another
Optimization

When setting up fluorescent DNA detection must consider the following:

- Specific Dyes
- Laser
- Optical filters
- Matrix
  - Algorithm that separates the dyes’ emissions
- Fluorescence detector
Methods to Label DNA

Three different methods to label DNA with fluorescence:

1. Use an fluorescent intercalating dye to bind the DNA
   • Will bind DNA within the gel matrix

2. Using fluorescently labeled dNTPs
   • dNTPs will be incorporated into PCR product

3. Incorporating the dye into the 5’ end primer
Fluorescent dNTPs are incorporated into both strands of PCR product.

Ethidium bromide

SYBR Green

Unlabeled DNA

Intercalator inserts between base pairs on double-stranded DNA

DNA labeled with intercalating dye

Fluorescent dNTPs are incorporated into both strands of PCR product.

One strand of PCR product is labeled with fluorescent dye.

Intercalating Dyes and dNTPs

Advantages:
• Intercalating can be done after PCR
• Less expensive

Disadvantages:
• Will label DNA but not differentiate between different amplicons
• Only one color can be used
  – Simply labels any DNA
5’ Labeled Primers

Advantages

• More than one color can be used
• Amplicons can be separated
  – Each primer has a different color dye
• Only label one strand of DNA
  – Allow easier genotyping of heterozygotes

Disadvantages

• More expensive
## Specific Dyes

Most commonly used dyes:

<table>
<thead>
<tr>
<th>Name</th>
<th>Color</th>
<th>Number of Colors</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>Blue</td>
<td>1</td>
</tr>
<tr>
<td>JOE</td>
<td>Green</td>
<td>2</td>
</tr>
<tr>
<td>VIC</td>
<td>Green</td>
<td>2</td>
</tr>
<tr>
<td>HEX</td>
<td>Green</td>
<td>2</td>
</tr>
<tr>
<td>NED</td>
<td>Yellow</td>
<td>3</td>
</tr>
<tr>
<td>TMR</td>
<td>Yellow</td>
<td>3</td>
</tr>
<tr>
<td>PET</td>
<td>Orange</td>
<td>4</td>
</tr>
<tr>
<td>LIZ</td>
<td>Red</td>
<td>Size standard</td>
</tr>
<tr>
<td>ROX</td>
<td>Red</td>
<td>Size standard</td>
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Example of Dyes for Multiplex

- Our lab used 4 dyes:
  - This means three different colors for the PCR products

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</tr>
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- ABI kit now uses 5 dyes:
  - Four different colors for PCR products

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Overlapping Spectra

- Although each dye emits its maximum fluorescence at a different wavelength, they do overlap quite a bit.
Overlapping Spectra

- Less Dyes = less overlap
- Also means need more separation by size
Matrixes

- Computer algorithm that subtracts out the suspected contribution of the other dyes
- Raw data is collected from laser
- Computer software analyzes the amount of overlap between dye standards
- Then these numbers are used to subtract out certain amounts of fluorescence from each wavelength
Matrix Example

Here is an example of a matrix for a four dye machine.

Overlap is highest between green and blue.

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<th>Red</th>
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<tr>
<td>Blue</td>
<td>1.00</td>
<td>0.85</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>Green</td>
<td>0.83</td>
<td>1.00</td>
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<tr>
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<td>0.51</td>
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Matrixes

• Conditions (pH, temp, etc) will change the amount of fluorescence
  – Therefore change the amount of overlap
• Therefore need more than one matrix file
  – One for each major condition
  – Re-run matrixes standards often

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Bleeding

- Book calls this “pull up”
- When one dye’s fluorescence is picked up in another wavelength’s spectra
- Think you are seeing a small green band
- Reality the blue band is so strong that it is bleeding into the green spectra
- Worse for dyes that have a lot of overlap
- Can be decreased with matrixes and filters
Off-scale peaks

- When a peak is too strong
- Can tell by looking at the shape of the peak as well as its height
Automated Allele Calling

• Laser reads the fluorescence of the labeled PCR product
• Electronically records wavelength and intensity
  – The shape and color of the peak
• Also records the distance the band traveled
  – The size of the PCR product
Mixture of dye-labeled PCR products from multiplex PCR reaction

Sample Separation

Sample Detection

Size Separation

Argon ion LASER (488 nm)

Fluorescence

Color Separation

ABQ Prism spectrograph

Capillary

CCD Panel (with virtual filters)

Sample Interpretation

Processing with GeneScan/Genotyper software

Automated Allele Calling

- Filters are used to determine what wavelengths the laser is detecting
- Matrixes are used to subtract out overlap
- What is left is turned into an electronic file by the computer
  - Electropherogram
- This shows the fluorescent peaks caused by the bands of DNA separated in the gel
Profiler Plus™ multiplex STR result

COfiler™ multiplex STR result

Automated Allele Calling

• Next step is to turn those peaks into actual alleles
• Associating size and colors to each marker
  – Each PCR primer set
• Determining homozygous vs. heterozygous
• Finally determining actual allele sizes
Any Questions?

Read Chapter 15

→ Skipping Chapter 14!
Advantages to Silver Staining

• Reagents are less dangerous than radioactivity
  – Less expensive than radioactivity
• In fact Silver Staining is one of the cheapest ways to detect DNA bands
• Staining is quick
  – Within 30 minutes
• Sensitivity is better than ethidium bromide
  – Not as good as radioactivity
Disadvantages to Silver Staining

• Major disadvantage is that there is again only one color
• Therefore need to have everything separated completely based on size first
• Also, silver binds to both strands of DNA
  – Heterozygotes are difficult to visualize correctly on the gel
Two Types of Lasers:

Argon ion gas laser
• Produces light:
  – 488 and 514.5 nm
• Most used laser – excites wider variety of fluorophores

Solid state Nd:YAG laser
• Produces light:
  – 532 nm
Automated Allele Calling

Can record alleles during the run through the gel/polymer
• All sized alleles travel same distance
  – From well to laser
  – Measure the amount of time to reach laser

Can record after the gel has finished run
• FMBIO gel scanner

• Shorter alleles have moved further
  – Better separation than long alleles