JS 190: Methods used to Study DNA:
DNA extraction and quantification
More on PCR

I. Pre class activities
   a. Quiz
   b. Assignment and announcements

II. Learning Objectives
   a. DNA extraction and Quantification
      a. Learn methods that are used to extract DNA
      b. Learn the methods used for quantification
   b. Overview of RFLP
   c. PCR
Assignments and Announcements

• **Next week exam 1 – Weds 22 Feb!**
• **Today Intro to DNA methods**
• **Assign student led reviews for weds 15 Feb**
  – Chapter 1 Sample collection, storage and characterization- pink pipettes
  – *Presumptive testing*- Readings- histones
  – Chapter 2 Extraction- Marissa- tandem repeats
  – Chapter 3 Quantification- incredibles
Steps in Forensic DNA typing

**Evaluation- Is it there?**

1. Start with biological sample
2. Screen- blood? Semen? Saliva, human?

**Extraction- Get and clean DNA**

3. Open cells → Get DNA

4. Methods to get DNA and purify DNA

**Quantify- Determine quality and quantity?**

5. Quantify-
   How good and how much did you get?

**Type to determine and compare alleles**

6. RFLP vs PCR

7. Determine alleles and compare DNA types
   Or alleles present in samples and references

**Interpretation of Results**
DNA Methods

1) Extract
2) Quantitate
3) Distinguish

Size
Content

RFLP: Restriction Fragment Length Polymorphisms
PCR: Polymerase Chain Reaction

RFLP methods require large amounts of undegraded DNA and the process takes 1-2 weeks. PCR methods require only small amounts of DNA, are useful on degraded DNA and require much less time (as little as 1-2 days in some cases).
DNA Extraction

After screening tests are performed, a spot of the material containing the biological sample is cut and placed into a tube.

In one type of extraction method (organic), heat and chemicals are added, and protein is removed. Then the pure DNA is recovered by filtration in which the non-DNA material goes through a sieve. (analogous to a collection of your pasta in a colander)
Different DNA Extraction Methods

The **organic method** generally yields the highest quantity and quality DNA. The main disadvantages are that it is tedious with lots of steps and utilizes corrosive chemicals. The **Chelex method** is used when the sample contains very few cells and the reduced number of handling steps is the primary advantage. The main disadvantage is that it yields crude DNA that is not as pure. **FTA paper** is used to collect reference samples. It can be stored at room temperature, requires minimal handling and no quantification is required.
Differential Extraction Method For Sexual Assault Evidence

- Isolation of DNA from mixtures of cells in sexual assault evidence
- Based on differences in cell membranes
  - Spermatozoa membranes have special cross links (sulphur-sulphur bonds)
  - These membranes are quite resistant to opening.
  - Vaginal epithelial cells do not contain these membranes and are more easily broken open
Quantification of DNA

• Following extraction, the next step is to determine the quantity of the DNA
• DNA typing methods RFLP and PCR require different amounts and different quality of DNA.
• RFLP typically required 50ng. PCR typically requires less than 0.5ng to 1 ng: 100 times less!
Quantification of DNA using Gel Electrophoresis

- Total DNA can be quantified by running the samples in a gel.
- Typically, gels are made up of agarose (a carbohydrate from seaweed).
- Known DNA quantities are included.
- Samples are then subject to an electric current and is called electrophoresis.
- DNA is negatively charged and will migrate toward the positive electrode...
- Comparisons of the results are done visually or with computer software to determine the amount of DNA in the unknown sample.

Direction of DNA fragment movement
Smaller fragments move faster and are found near the bottom of the gel.
Slot Blot quantification:

DNA-DNA Hybridization
DNA is where it’s AT

• DNA samples may contain non human DNA
• In order to quantify the amount of human DNA in a sample, a human specific test is required
• One such test is DNA-DNA hybridization using a human specific probe: D17Z1
Slot blot hybridization

- Like in yield gels, known amounts of DNA (human) are included.
- DNA hybridization of D17Z1 will occur only if the sample contains human DNA.
- Detection of the hybridized fragments is done using an enzyme linked assay - yielding light or color.
Real Time PCR

Figura 4: real-time PCR
Quantitative PCR - QPCR
http://pathmicro.med.sc.edu/RTPCR/rt-pcr.ppt

- Real-time QPCR has several advantages over the other methods in that it is extremely accurate and sensitive over a broad dynamic range, and it occurs in a closed-tube system, reducing the potential for carryover contamination.

- Using this technique, a forensic biologist can monitor and quantify the accumulation of PCR products during log phase amplification. (Heid et al., 1996).


- The assays may be performed on single targets or in multiplexes (Timken et al. 2005, Walker et al. 2005, Nicklas et al. 2006).

- Recently, the detection of degraded vs intact human DNA and PCR inhibitors has been reported (Swango et al. 2006).
RNA based quantification methods

• Different genetic expression patterns (mRNAs) exist in different tissue types.
• Body fluid identification has been reported based on their mRNA profiles (Juusola and Ballantyne 2003 and 2005, Nussbaumer et al. 2006)
• In addition, the age of a bloodstain was reported using analysis of mRNA: rRNA ratios (Anderson et al. 2005). This information may be useful in establishing the time of the crime.
• Advantages of the mRNA-based approach, versus the conventional biochemical tests, include greater specificity, simultaneous and semi-automatic analysis, rapid detection, decreased sample consumption and compatibility with DNA extraction methodologies.
• The quantification of the amounts of the mRNA species relative to housekeeping genes is a critical aspect of the assays (Juusola and Ballantyne 2003).
## Comparison of Methods used for DNA Quantification

<table>
<thead>
<tr>
<th>Method</th>
<th>Ease</th>
<th>Cost</th>
<th>Sensitivity</th>
<th>Result</th>
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<tbody>
<tr>
<td>UV Spectrophotometry</td>
<td>++++</td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Total DNA</td>
<td></td>
<td></td>
<td>Int vs deg</td>
<td></td>
</tr>
<tr>
<td>Gel electrophoresis Yield</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Int vs deg DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slot Blot</td>
<td>++</td>
<td>+++</td>
<td>Human DNA</td>
<td></td>
</tr>
<tr>
<td>Gel blot</td>
<td>+</td>
<td>+++</td>
<td>Int. vs. deg</td>
<td></td>
</tr>
<tr>
<td>Pico-green microtitre plate</td>
<td>++++</td>
<td>++</td>
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</tr>
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</table>
1. Restriction enzymes cut DNA into fragments of various length. A given restriction enzyme will cut a specific sequence of DNA.

2. A sample consists of all the DNA fragments of various lengths. The sample is loaded into a gel for electrophoresis.

3. Electrophoresis. Use voltage difference to separate DNA fragments by size. Small fragments run faster.

4. The DNA fragments are treated to make them single stranded.

5. Blotting. An alkaline solution wicks up into blotting paper, carrying DNA from gel onto nylon filter, where it becomes permanently bound.

6. Hybridization with radioactive probe. Incubate the nylon membrane with a solution containing labeled probe DNA. The radioactive probe base pairs to the fragments containing complementary sequences.

7. Autoradiography. Place membrane against X-ray film. Radioactive DNA fragments expose film, forming black bands that indicate location of target DNA.
Kary Mullis Nobel Prize - 1993
Polymerase Chain Reaction:
PCR is simply repeated rounds of DNA replication

PCR based systems are rapid, require less material than RFLP and less time for typing

• Molecular xeroxing
• Calvin and Hobbes example
PCR works for very small samples—bloodstain on hat
PCR works for very small samples—hat close-up
PCR works for degraded DNA—under the microscope, sperm appear intact.
But the yield gel shows that their DNA is degraded.
PCR: repeated rounds of DNA Replication

- 5 required ingredients (components)- primer, template, Mg, dNTPs, DNA polymerase-PTMDD-(please to make DNA doubled)

- DNA Polymerase catalyzes the template directed (A-T, G-C), incorporation of dNTPs (PP is released) forming a 3’-5’ phosphodiester linkage

- Direction of synthesis 5’→3’ using primer 3’OH to attach incoming nucleotide
DNA Amplification with the Polymerase Chain Reaction (PCR)

In 32 cycles at 100% efficiency, 1.07 billion copies of targeted DNA region are created.
PCR takes place in a Thermal Cycler
Thermal Cycling Temperatures

The denaturation time in the first cycle is lengthened to ~10 minutes when using AmpliTaq Gold to perform a “hot-start” PCR.
PCR Process

Separate strands (denature)

Add primers (anneal)

Make copies (extend primers)

Repeat Cycle, Copying DNA Exponentially

Starting DNA Template

Forward primer

Reverse primer

5' 3' 5' 3'

5' 3' 5' 3'

5' 3' 5' 3'

5' 3' 5' 3'

5' 3' 5' 3'

5' 3' 5' 3'

5' 3' 5' 3'
PCR is simply repeated rounds of DNA replication

Step 1: Denature
Separate H bonds with heat at 95°C

Step 2: Anneal
Primers bind at lower temp 55°C

Step 3: Extend
Taq polymerase extends primer 3’OH
at 72°C (dNTPs and Mg++)

Step 4: Repeated 28-30 rounds of D, A, E
# Number of Target Molecules Created

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<th>Number of Double-stranded Target Molecules</th>
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## Comparison of RFLP and PCR

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RFLP Methods</th>
<th>PCR Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time required to obtain results</td>
<td>6-8 weeks with radioactive probes; ~1 week with chemiluminescent probes</td>
<td>1-2 days</td>
</tr>
<tr>
<td>Amount of DNA needed</td>
<td>50-500 ng</td>
<td>0.1-1 ng</td>
</tr>
<tr>
<td>Condition of DNA needed</td>
<td>high molecular weight, intact DNA</td>
<td>may be highly degraded</td>
</tr>
<tr>
<td>Capable of handling sample mixtures</td>
<td>Yes (single locus probes)</td>
<td>Yes</td>
</tr>
<tr>
<td>Allele identification</td>
<td>Binning required</td>
<td>Discrete alleles obtained</td>
</tr>
<tr>
<td>Power of Discrimination</td>
<td>~1 in 1 billion with 6 loci</td>
<td>~1 in 1 billion with 8-13 loci (requires more loci)</td>
</tr>
</tbody>
</table>
## Relative power of tests

<table>
<thead>
<tr>
<th>Test type</th>
<th>time</th>
<th>power</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFLP-VNTR</td>
<td>weeks</td>
<td>++++</td>
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<tr>
<td>PCR:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQAlpha- macroarray</td>
<td>1 day</td>
<td>+</td>
</tr>
<tr>
<td>PM - macroarray</td>
<td>1 day</td>
<td>++</td>
</tr>
<tr>
<td>D1S80 - gel- VNTR</td>
<td>2 days</td>
<td>++</td>
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<tr>
<td>STRs - gel, CE, arrays</td>
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<tr>
<td>mtDNA - gel, CE, arrays</td>
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<td>+</td>
</tr>
<tr>
<td>alu - gel, CE, arrays</td>
<td>2 days</td>
<td>++</td>
</tr>
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</table>

* not useful on degraded DNA
Advantages of PCR

• Minute amounts of DNA template may be used from as little as a single cell.
• DNA degraded to fragments only a few hundred base pairs in length can serve as effective templates for amplification.
• Large numbers of copies of specific DNA sequences can be amplified simultaneously with multiplex PCR reactions.
• Contaminant DNA, such as fungal and bacterial sources, will not amplify because human-specific primers are used.
• Commercial kits are now available for easy PCR reaction setup and amplification.
Multiplex PCR

• Target 2 or more DNA regions simultaneously with multiple primer sets. Copy more than one locus at a time
• Primers for all loci are present in the tube
• Conditions are adjusted to ensure all loci will be amplified
• Multiple types obtained from 1-2 ng DNA
• Greater discrimination
• Advantages:
  – more information in the same amount of time
  – less expensive (lower reagents and labor)
• Challenge lies in designing PCR primers that are compatible with one another
Primer Design

• Typically performed with assistance of computer program to identify possible primer that are then tested empirically

• Various computer programs:
  – Gene Runner (PC), Oligo (PC/Mac), Primer Express (Mac)
  – Primer 3 (web based)

• Critical parameters examined:
  – Predicted $T_m$ (melting temperature)- $T_m=4(G+C) + 2(A+T)$
  – Primer dimer and hairpin formation
  – Contiguous base runs (usually <5 bases)
  – GC content (number of G and C nucleotides within primer)
Schematic of Multiplex PCR

Locus A

Locus B

Locus C

small → large

A

B

C
Over 15 Markers Can Be Copied at Once

Sensitivities to levels less than 1 ng of DNA

Ability to Handle Mixtures and Degraded Samples

Different Fluorescent Dyes Used to Distinguish STR Alleles with Overlapping Size Ranges
Important PCR facts

- DNA polymerase is taq polymerase from a hot springs (can survive denaturation boiling temperatures)
- Taq likes to add an extra base (non template directed nucleotide addition to the 3’ end). Amplification of DNA fragments of 100bp in size are 101 in length.
- PCR amplification sometimes “stutters” on STRs resulting in an extra PCR product called a stutter product. Eg. Both 100 (correct type) and 96 base pair fragments are present. The stutter product is usually represented at less than 10% of the real allele.
Potential Pitfalls of PCR

- The target DNA template may not amplify due to the presence of PCR inhibitors in the extracted DNA.

- Amplification may fail due to sequence changes in the primer binding region of the genomic DNA template.

- Contamination from other human DNA sources besides the forensic evidence at hand or previously amplified DNA samples is possible without careful laboratory technique and validated protocols.
Contamination in the lab

From sample with high level of DNA

To sample with low level of DNA

10 \mu g/ml

\frac{1}{1000}\ \mu g/ml

net = \frac{1}{1000} \mu g of a

+ \frac{1}{1000} \mu g of b
PCR Product Contamination—
the Thousand to One Nightmare

It only takes a minuscule amount of amplified product

\[ 10^{13} \text{ copies/ml} \]

\[ 0.1 \mu l \text{ transfer} = 10^9 \text{ copies} \]

\[ 10 \mu g/ml = 1.4 \times 10^6 \text{ copies/ml} \]

net = \( 10^9 \) copies of a + \( 10^6 \) copies of b

...to cause a typing disaster
Tips for Avoiding Contamination

• Pre- and post-PCR sample processing areas should be physically separated.
• Do not move from PCR area into non PCR area without decontamination
• Process one sample at a time, Avoid splashing
• Separate reference samples from evidence
• Wear protective gear and reagent prep care
• Equipment, such as pipettors, and reagents for setting up PCR should be kept separate from other lab supplies, especially those used for analysis of PCR products.
• Disposable gloves should be worn and changed frequently.
• Reactions may also be set up in a laminar flow hood, if available.
• Aerosol-resistant pipet tips should be used and changed on every new sample to prevent cross-contamination during liquid transfers.
• Reagents should be carefully prepared to avoid the presence of any contaminating DNA or nucleases.
• Ultraviolet irradiation of laboratory PCR set-up space when the area is not in use and cleaning workspaces and instruments with isopropanol and/or 10% bleach solutions help to insure that extraneous DNA molecules are destroyed prior to DNA extraction or PCR set-up
• Controls: Negative, Positive, Stochastic, Substrate
Monitoring for Contamination—Controls ‘R’ Us

Bloodstain (Evidence)

Substrate Control

Reagent Blank—for Evidence

Victim’s Reference Sample

Reagent Blank—for References

Negative Amplification Control

Quality Control Sample

Positive Amplification Control
PCR ‘quiz’

- Template =
  - 5’ GGACTCCTATGTATGTATGCTTTTAAGGCA 3’
  - 3’ CCTGAGGATACATACATACGAAATTCCGT 5’
- **Design two primers** (five bases long): Remember-the 3’ OH end will be extended and DNA is antiparallel
- Be sure to amplify the entire template.
- **List the other required components, materials and procedure** needed to conduct a successful PCR reaction
- Assume this is an STR locus. **What is the repeat unit?**
  **What is the type (number of repeats for this allele)?**
Reverse Dot blot hybridization

deg. DQ alpha and Polymarker

Figure 6.10 Detection of PCR product on a reverse dot blot.
Once amplified detection can be done by DNA battleship

DNA probes can detect specific fragments by base pairing (complementation: hybridization)

PROBE  
JB  AAAGAAAGCCAG 
JM  AAAGAAAGGCC 
MW  AAAGAAACCC 
LG  AAAGAAACCC 
SL  AAAGAATT 
Plant  AAATTC CCC

Plant DNA

SL DNA

JB DNA
Summary

- RFLP (old) required approximately 50ng of DNA at a minimum. PCR requires as little at 500pg or 100 times less!
- One method to examine variation of variable number of tandem repeats (VNTRs) is RFLP= restriction fragment length polymorphisms
- RFLP requires many steps, undegraded DNA and takes days to weeks to complete
- In contrast, typing of STRs using PCR can be performed on very small amounts of degraded DNA and takes hours to a day to complete.
Summary 2

- PCR is polymerase chain reaction and is repeated rounds of DNA synthesis.
- There are 5 components needed, PTMDD.
- PCR takes place in a thermal cycler
- Multiplex PCR permits amplification of many loci simultaneously and saves time
- Avoid contamination and use controls
- Other markers that have been used in forensic PCR assays include, dot blot assays of DQ alpha, polymarker, and D1S80.
- Mitochondrial DNA sequencing and Y chromosome STR markers are also being used.