JSONException: Cannot construct a JSON value from null
Outline

• What is PCR?
  – DNA Background
  – PCR
  – Real Time PCR

• Why PCR?
  – Applications

• Technologies Present and Future
  – Standard Systems
  – Micro-technology
Structure of DNA

• DNA deoxyribonucleic acid
  – Two long polynucleotide chains with 4 nucleotide subunits

• Nucleotides subunits
  – Sugar, phosphate groups, base
  – Adenosine (A), Guanine (G), Cytosine (C), Thymine (T)

• “Backbone”
  – Covalently linked nucleotides

• Hydrogen bonds hold two chains together
DNA Structure – Double Helix

- Single stranded DNA will form double stranded DNA only with its complement

- Polarity – 3’ (3’ hydroxyl) and 5’ (5’ phosphate)

- Complementary base pairing G-C and T-A

- Hydrogen Bonding holds strands together

- “Double Helix”
PCR – What is it?

• Once DNA has been extracted & isolated, the sequences of interest must be amplified to a detectable concentration

• Polymerase Chain Reaction (PCR) is the most common method of **DNA amplification**

• It involves thermocycling of the sample to sequentially denature the strands, allow primers to bind, and allow polymerase to replicate
How PCR Works

Step 1 - Denaturation (optimal temperature is 94°C)
By heating the DNA, the double strand melts and open to single stranded DNA.
How PCR Works

Step 2 - Annealing (optimal temperature is 60°C)
Add single stranded primers that match to sequences of the target DNA. The single-stranded primers bind to their complementary single-stranded bases on the denaturated DNA.
How PCR Works

Step 3 - Extension (72°C is the ideal temperature)
Add single DNA nucleotides and an enzyme that reads opposing strains sequence and extend primers sequence to match (complementary). *Taq polymerase* is used because it can withstand high temperature necessary for DNA strand separation and can be left in reaction to attach and start copying the template. The result is two new helixes in place of the first.
How PCR Works

Replication – Two exact copies of the original DNA are created. The process can be repeated again and again to achieve suitable levels of detection. Since both the old and new DNA can be copied the amplification grows.

\( y = e^x \)
Review

• DNA heated – paired strands separate

• Large excess of primers added, some are complementary to sequences on target DNA. Cool the reaction mixture to allow binding to take place.

• Add single DNA nucleotides and enzyme that reads opposing strains sequence and extend primers sequence to match (complementary).

• Enzyme synthesizes new DNA. Process is repeated both new DNA and old are amplified
How Real-Time PCR Works

Used to amplify and simultaneously quantify targeted DNA molecule.

PCR reaction prepared as usual with added reporter probe. During annealing both probe and primer anneal to target DNA.

Polymerization of new DNA is initiated from primers, once reaches probe it degrades it.

The fluorescent reporter is physically separated from the quencher, results in increased fluorescence.

Fluorescence is detected and measured in a real time PCR thermocycler.

Diagram taken from TaqMan Gold RT-PCR kit protocol (PE Applied Biosys.
Real-Time PCR Data

Fluorescence curves correspond to concentrations
Real Time PCR Video: http://www.youtube.com/watch?v=8-dsnlNsCao
The bacterium *Thermus aquaticus* was first discovered in several springs in the Great Fountain area of the Lower Geyser Basin at Yellowstone National Park. Taq polymerase or Taq was isolated from the bacteria.

The taq-polymerase needs 30-60s to synthesise a 1 kbp strand of DNA. So the synthesis time depends on the length of your product.
Benefits and Applications of PCR

• ~30 cycles of reaction are required for effective DNA amplification with each cycle serving as DNA templates for the next
• Extremely sensitive – can detect single DNA molecule in sample
• Easily automated
• Depending on conc and volumes cycles can range from minutes (traditional) to seconds (new technologies)

• DNA/RNA analysis
  – Human Genomic DNA
    • Mutations (SNP), Deletions, Identification (STR)
  – Organism Detection
    • Pathogens (virus, bacteria)
  – RNA
    • Gene expression
  – Microarrays
Standard PCR Thermocyclers

PTC-100®
Peltier Thermal Cycler
MJ Research

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<th>Thermal Uniformity:</th>
<th>±0.5°C within 30 seconds of arrival at 60°C</th>
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<td>Ramping Speed:</td>
<td>60: up to 1°C/second, 96: up to 1.4°C/second, 96AgV: up to 2.5°C/second, 16MS: up to 0.5°C/second</td>
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How long to complete?
Fast PCR

Idaho Technologies

• Heat block cyclers can take hours to do 30- three temperature cycles.
• This slow rate leads to long lines for the machine and sign up sheets that force you to start your reactions at all hours.
• The RapidCycler™ can complete a 30 cycle reaction in less than 10 minutes. Finally, a machine that can keep up with the speed of the biochemistry. No more waiting in line. You can throw away those sign up sheets.

RapidCycler's unique design allows heat to enter and exit quickly to enable maximum reaction time. Air flow through the machine is indicated by the colored arrows.
Fast PCR

I-CORE® Modules

The “Core” of Cepheid’s Amplification Systems

The Smart Cycler® II System

Real Time Thermal Cycling

- 16 independently programmable reaction sites
- Faster and more sensitive than reference systems
- 4-color real time optical detection in each site
- 1-6 processing blocks
Miniaturization of PCR

• ST Lab-On-Chip

Advantages?

Disadvantages?
DNA sequencing

Sangers’ dideoxymethod

5’-labelled oligonucleotide ssDNA

+ DNA-polymerase + dNTPs

+ ddATP + ddCTP + ddGTP + ddTTP

A
C
G
T

TTT G C C A G G A G A T G G A R AC C

T
C
G
T

A
C
G
T

A
C
G
T

DNA sequencing

2’,3’-dideoxyanalog
PCR (Polymerase Chain Reaction)

DNA-template

$\downarrow 94^\circ C$ separation of DNA-strands

$\downarrow 45-65^\circ C$ annealing of specific primers

$\downarrow 72^\circ C$ extension by Taq DNA polymerase

Repeat 25-40 cycles (exponential increase of product)
SNP - single nucleotide polymorphism

Affect of SNP:

- **Coding**
  - different protein properties

- **Regulatory**
  - different amounts of mRNA (protein)

- **Stability**
  - stability of mRNA (or protein)

- **Silent**
  - neutral variations

One SNP per 1000 bp

Responsible for phenotypic differences between individuals

Can be identified by
- Mass spectrometry
- Hybridization
- Pyrosequencing
  etc

Allele 1

Allele 2

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Molecular Based Methods of Diagnosis

DNA based molecular methods
Why use a molecular test to diagnose an infectious disease?

- Need an accurate and timely diagnosis
  - Important for initiating the proper treatment
  - Important for preventing the spread of a contagious disease
Leading uses for nucleic acid based tests

- Nonculturable agents
  - Human papilloma virus
  - Hepatitis B virus
- Fastidious, slow-growing agents
  - Mycobacterium tuberculosis
  - Legionella pneumophila
- Highly infectious agents that are dangerous to culture
  - Francisella tularensis
  - Brucella species
  - Coccidioidis immitis
Leading uses for nucleic acid based tests

- In situ detection of infectious agents
  - Helicobacter pylori
  - Toxoplasma gondii
- Agents present in low numbers
  - HIV in antibody negative patients
  - CMV in transplanted organs
- Organisms present in small volume specimens
  - Intra-ocular fluid
  - Forensic samples
Leading uses for nucleic acid based tests

- Differentiation of antigenically similar agents
  - May be important for detecting specific virus genotypes associated with human cancers (Papilloma viruses)

- Antiviral drug susceptibility testing
  - May be important in helping to decide anti-viral therapy to use in HIV infections
Leading uses for nucleic acid based tests

- Molecular epidemiology
  - To identify point sources for hospital and community-based outbreaks
  - To predict virulence
- Culture confirmation
What are the different types of nucleic acid molecular techniques that are used?

- Direct probe testing – better for identification than for detection because it is not as sensitive as amplification methods
- Amplification methods – used to improve the sensitivity of the nucleic acid testing technique
  - Target amplification
  - Probe amplification
  - Signal amplification
  - Combinations of the above
Direct probe testing

• Hybridization – to come together through complementary base-pairing.
  – Can be used in identification.
  – In colony hybridization the colony is treated to release the nucleic acid which is then denatured to single strands.
    • Labeled single-stranded DNA (a probe) unique to the organism you are testing for is added and hybridization is allowed to occur.
    • Unbound probe is washed away and the presence of bound probe is determined by the presence of the label.
Direct probe testing

(a) *Salmonella* DNA fragment cloned in *E. coli*

(b) Cloned DNA fragments marked with fluorescent dye and separated into single strands, forming DNA probes

(c) Unknown bacteria collected on filter

DNA probes added to DNA from unknown bacteria

(d) DNA probes hybridize with *Salmonella* DNA from sample; excess probe is washed off; fluorescence indicates presence of *Salmonella*
Target amplification

- Target amplification requires that the DNA to be tested for be amplified, i.e., the number of copies of the DNA is increased.
- To understand this we must first review the activity of the enzyme, DNA polymerase, that is used to amplify the DNA.
Polymerase template and primer requirements

- DNA polymerase cannot initiate synthesis on its own.
  - It needs a **primer** to prime or start the reaction.
  - The primer is a single stranded piece of DNA that is complementary to a **unique** region of the sequence to be amplified.
Polymerase template and primer requirements
DNA synthesis

- Synthesis can occur only in the 5’ to 3’ direction.
Target amplification – The PCR reaction

- Polymerase chain reaction – used to amplify something found in such small amounts that without PCR it would be undetectable.
  - Uses two primers, one that binds to one strand of a double-stranded DNA molecule, and the other which binds to the other strand of the DNA molecule,
  - all four nucleotides and
  - a thermostable DNA polymerase.
  - The primers must be unique to the DNA being amplified and they flank the region of the DNA to be amplified.
The PCR reaction has three basic steps

- Denature – when you denature DNA, you separate it into single strands (SS).
  - In the PCR reaction, this is accomplished by heating at 95°C for 15 seconds to 1 minute.
  - The SS DNA generated will serve as templates for DNA synthesis.

- Anneal – to anneal is to come together through complementary base-pairing (hybridization).
  - During this stage in the PCR reaction the primers base-pair with their complementary sequences on the SS template DNA generated in the denaturation step of the reaction.
PCR

- The primer concentration is in excess of the template concentration.
- The excess primer concentration ensures that the chances of the primers base-pairing with their complementary sequences on the template DNA are higher than that of the complementary SS DNA templates base-pairing back together.
- The annealing temperature used should ensure that annealing will occur only with DNA sequences that are completely complementary. WHY?
- The annealing temperature depends upon the lengths and sequences of the primers. The longer the primers and the more Gs and Cs in the sequence, the higher the annealing temperature. WHY?
- The annealing time is usually 15 seconds to 1 minute.
PCR

- Most PCR reactions use 25 to 30 of these cycles to amplify the target DNA up to a million times the starting concentration.
What are the advantages of using a molecular test?

- **High sensitivity**
  - Can theoretically detect the presence of a single organism

- **High specificity**
  - Can detect specific genotypes
  - Can determine drug resistance
  - Can predict virulence

- **Speed**
  - Quicker than traditional culturing for certain organisms
What are the advantages of using a molecular test?

- Simplicity
  - Some assays are now automated
What are the disadvantages of using a molecular test?

- Expensive
- So specific that must have good clinical data to support infection by that organism before testing is initiated.
- Will miss new organisms unless sequencing is done (not practical in a clinical setting).
- May be a problem with mixed cultures – would have to assay for all organisms causing the infection.
What are the disadvantages of using a molecular test?

- Too sensitive? Are the results clinically relevant?