Polymerase Chain Reaction

Workshop
Phoenix, AZ
October 4, 2004
Mechanism of PCR
Reaction Components
Optimization & Role of Reactants
Forensic PCR: Considerations
The PCR Laboratory
Set-up
Contamination
Recommendations
IMPACT OF PCR

Research

Forensics

Medicine
- HIV detection
- Disease diagnosis
- Prenatal & carrier detection

Dinner conversation
Development of PCR

- 1969  Brock & Freeze: *Thermus aquaticus*
- 1976  Characteristics of Taq polymerase
- 1980  Temperature optimum for DNA synthesis
- 1985  Mullis: PCR using thermostable polymerase
- 1985  Cetus: automated thermocycling
PCR Defined...

- An *in vitro* process
- Produces millions/billions of copies of target DNA fragments (subanalytical to analytical)
- Cyclical enzymatic reaction
- Replicates DNA - products of previous cycles are used as templates in subsequent cycles
PCR!

$2^{30}$ Copies

1 original, 1X
= 1 copy

1 original, 30X
= 30 copies
POWER OF PCR

- Subanalytical levels of DNA amplified to analytical levels
- Exponential increase in the amount of product: $2^n$
  where $n = \text{the number of cycles}$
- **Theoretically:**
  - $n = 20$ yields a million-fold increase
  - $n = 30$ yields a billion-fold increase
CYCLING STEPS

1. EXTEND primers
2. DENATURE DNA
3. ANNEAL primers
4. EXTEND primers
5. DENATURE DNA
6. ANNEAL primers
7. EXTEND primers
8. DENATURE DNA

Repeat steps 1-7.
CYCLING STEPS

EXTEND primers

DENATURE DNA

ANNEAL primers
CYCLING STEPS

EXTEND primers

DENATURE DNA

ANNEAL primers

ANNEAL primers
THREE-TEMPERATURE PCR

95°C

70°C

(55°C)

Cycle

1 2 3 4 5...

...
Primers

- Short oligonucleotides
- Linear
- Single Stranded
- Sequence complementary to target

5'-ATACC-3'

3'-GATGG-5'
Primer Selection

Polymorphism
STR, SNP, etc.
Primer Annealing

5' CTAAGT 3'
3' GATTCA 5'

5' AATCTT 3'
3' TTAGAA 5'
Defined Target Region

nucleotide difference, insertion, deletion, VNTR

i.e., 230 bp
Cycle 1, Step 1
DENATURATION
Cycle 1, Step 1
DENATURATION
Cycle 1, Step 1
DENATURE
Cycle 1, Step 1
DENATURATION
Cycle 1, Step 1
DENATURATION
Cycle 1, Step 2
ANNEALING
Cycle 1, Step 3
EXTENSION
Cycle 2, Step 1
DENATURATION
Cycle 2, Step 1

DENATURATION
Cycle 2, Step 1
DENATURATION
Cycle 2, Step 1

DENATURATION
Cycle 2, Step 2
ANEALING
Cycle 2, Step 3
EXTENSION
Accumulation of Target Fragment
Fluorescent Detection
Fluorescent Detection
EXAMPLE:
3-Temperature PCR

• Pre-Cycling Denaturation:
  – Taq 95°C 1 min
  – AmpliTaq Gold 95°C 11 min

• Cycle 28 times:
  – Denaturation 95°C 1 min
  – Annealing 60°C 1 min
  – Extension 72°C 1 min

• Final Extension
  – Extension 60°C 45 min
Final Extension

Complete truncated strands

Template-independent nucleotide addition
Non-Templated Nucletide Addition ("Plus A")

- Many DNA polymerases add an extra nucleotide to the blunt end of a DNA product without the assistance of a template.
- Usually A is added.
- The resulting band/peak is 1-bp larger in size than that predicted from the DNA sequence and primer locations.
Non-Templated Nucletide Addition ("Plus A")

- Occurs during each PCR cycle and during final extension
- A is not added as well to some blunt ends
  - The sequence at the end of the DNA has an influence
5' end of primer affects adenylation

- 5' AXX
- 5' GXX
- 5' CGT
- 5' CCT
- 5' CAT
5' end of primer affects adenylation

5' TXX

5' CTT

Adenylation reduced
Non-Templated Nucletide Addition ("Plus A")

Double-stranded DNA / PCR Product
Non-Templated Nucleotide Addition ("Plus A")

Temperature effects whether the ends remain together

60°C

70°C

"Breathing"
Non-Templated Nucletide Addition (“Plus A”)

Final Extension time can been increased (e.g., 30 min to 60 min)
Taq DNA Polymerase

- Thermostable DNA Polymerase
- Isolated from *Thermus aquaticus* YT1
- 94 kDa protein
- $T_{1/2} = 40$ minutes at 95°C***
- 5'-3'exonuclease activity
- no 3'-5' exonuclease activity
- processivity = 50-60 nucleotides
- extension rate = 4-26kb/ min at 70-80°C
Taq Polymerase Activity

Extension Rate at:

- 70 °C  = 60 nt/sec
- 55 °C  = 24
- 36 °C  = 1.5
- 22 °C  = 0.25

Processivity:

- 70 °C  = 50-60 nt
AmpliTaq Gold
DNA Polymerase

• Inactive until heated prior to thermocycling
• Simulates “Hot Start” PCR
• Increases yield & specificity of PCR
  – reduces non-specific products
  – reduces primer dimers
• Facilitates multiplexing
  – reduces primer design requirements
• Automation
However, little difference in performance between Taq & Gold if primers are well-designed & if the system is well-optimized.
Taq Polymerase Fidelity

5'-3' synthesis
Taq Polymerase Fidelity

5'-3' synthesis
Taq Polymerase Fidelity

5'-3' synthesis
Taq Polymerase Fidelity

5'-3' synthesis
Taq Polymerase Fidelity

5'-3' synthesis
No Proofreading Ability

5'-3' synthesis

3'-5' exonuclease activity

<1 Error per 10K-15K bases

Stochastic implications
No 3' - 5' exonuclease activity
5' → 3' Exonuclease Activity
Template

• Sensitive to a single copy of template
• Various types
  – DNA: genomic, cloned, bacterial, viral, mitochondrial
  – RNA / cDNA
• Various sources
  – Blood, semen, saliva, hair, bone, teeth, etc.
  – Stained/unstained slides, parafin-embedded tissue
• Highly purified or crude lysate
Primers

- **Sequence**
  - random base distribution
  - no poly-purine or pyrimidine stretches
  - ~50% G+C content

- **Length**
  - typically 18-28 bases

- **Balance** $T_m$ of all primers in a reaction
  - $T_m = 2 \times (A+T) + 4 \times (G+C)$ - simplistic
Primers

• Avoid 3' complementarity among primers, which can result in ‘primer dimer’ formation

• Primer dimer = template-independent duplex PCR product comprised of primers
Primers

• Inter-primer 3' complementarity (two different primers)

5’-TTTTTTTTTCCCCC
      GGGGGGTTTTTTTTTTTTT-5’

• Intra-primer 3’ complementarity (one primer complementary to itself)

5’-TTTTTTTTTCCCCGGGG
      GGGGCCCCCTTTTTTTTTTTT-5’
Primers

• Avoid runs of >3 G’s or C’s at 3’ end
  – may misprime in G+C-rich regions
• Empirically determine ability to adenylate
• 5’ additions to primers
  – restriction site, M13 site, promoter sequence
  – biotin, fluorophore, radioisotope
• Avoid palindromic sequences and internal complementarity
Hairpin due to
Internal Complementarity
Typical Reaction (25-100 ml)

- 50 mM KCl
- 10 mM Tris-HCl
- 1.25-1.50 mM MgCl$_2$
- 100 mg/ml Gelatin
- 0.25 mM Each primer
- 200 mM Each dNTP
- 1-5 units Taq Polymerase
DNA CONCENTRATION

Too Much DNA:
- Non-specific products
- Lower yield (WHY?)

Too Little DNA:
- Lower yield
- Stochastic effects

Depends on the method of detection
Stochastic Effects
TAQ CONCENTRATION

- Additional Taq may be recommended to overcome the effects of Taq polymerase inhibitors that may co-extract with DNA from forensic biospecimens
- However, too much Taq may affect specificity
- Too little Taq may result in lower yields
5 units per reaction is typical for many forensic kits
Divalent Cations

Magnesium is the cation of choice for Taq polymerase

Cofactor in the catalytic addition of deoxynucleoside monophosphates to the 3’ end of the growing DNA chain

So why 1.5 mM magnesium?
0.8 mM Mg$^{2+}$ is bound by dNTPs (200 uM x 4 = 0.8 mM)

0.7 mM Mg$^{2+}$ is needed by Taq

0.7 mM + 0.8 mM = 1.5 mM
MgCl$_2$ Concentration

Typically 1.25 - 1.50 mM

Too High:
- Non-specific products
- Inhibits Taq (WHY?)

Too Low:
- Lower yield
PRIMER CONCENTRATION

[Too Low]

Low yield

[Too High]

Non-specific products

Primer dimer
Deoxynucleoside Triphosphates (dNTPs)

The four dNTPs are present in the PCR

Concentrations range from 20 to 200 µM each

A concentration of 200 µM of each dNTP is theoretically sufficient to synthesize 25µg
dNTP CONCENTRATION

Pick me!

Pick me!

Pick me!

Pick me!

Pick me!
CYCLE NUMBER

Non-specific products and primer dimers may be detected if too many cycles are used.

Depends on target DNA quantity

<table>
<thead>
<tr>
<th>Copy Number</th>
<th>Cycle Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>300,000</td>
<td>25-30</td>
</tr>
<tr>
<td>15,000</td>
<td>30-35</td>
</tr>
<tr>
<td>1,000</td>
<td>35-40</td>
</tr>
<tr>
<td>50</td>
<td>40-45</td>
</tr>
</tbody>
</table>
CYCLE NUMBER & PLATEAU

Mixture w/ major & minor components
OR
Heterozygote?

YIELD
1 5 10 15 20 25 30 35 40 45 50 55 60
DENATURATION

If temperature is too low:

- GC-rich regions may fail to denature.
- Primers may not bind.

**HLA-DQA1**

**“snapback”**

GC-rich regions may fail to denature. Primers may not bind.

**Taq falls off**
ANNEALING TEMPERATURE

- Too Low: Mispriming
- Just Right: Good Specificity
- Too High: Failure to Anneal
EXTENSION

70-72°C recommended
FORENSIC PCR
Template Integrity

• Forensic samples may be:
  – old, degraded
  – exposed to environmental / chemical insults
  – contain PCR inhibitors

• However, typically in forensic PCR, small regions of DNA that can be amplified from degraded samples are targeted.
FORENSIC PCR

PCR Inhibitors

• Act by binding DNA target or inhibiting Taq.

• DNA sources & substrates that may contain inhibitors:
  – Blood... Heme
  – Heparin (anticoagulant)
  – Hair... Melanin (pigment)
  – Fabrics... Dyes (i.e., indigo)
  – Soil... Humic acid (organic breakdown product)
  – Urine... Metabolic substances, drugs
Strategies for overcoming the effects of inhibitors

- Dilute out inhibitor by increasing the total PCR volume (but less DNA)
- Chelex extract DNA (or other method)
- Use microconcentrator
- Increase concentration of Taq
- Add BSA to PCR
Bovine Serum Albumin

- Does not affect PCR when no inhibitor
- Helpful with samples containing Taq inhibitors
- Degree of affect depends on manufacturer and fraction of BSA
FORENSIC PCR

Use at a concentration of 160 ug / ml

Sigma Fraction V (#A3350 or #A2153)

= 16 ug for 100 ul reactions
= 8 ug for 50 ul reactions
FORENSIC PCR
Multiplexing

• Amplification of more than one locus in a single PCR tube.

• Decreases:
  – number of manipulations
  – chance of sample mix-up
  – chance of contamination
  – DNA and reagent consumption
FORENSIC PCR Contamination

- Sensitivity of PCR: single DNA copy
- FROM THE ENVIRONMENT:
  - nature of the crime
  - handling in the field or lab
- BETWEEN SAMPLES:
  - during preparation
- PCR-PRODUCT CARRY-OVER:
  - a serious concern because amplicons are usually in high concentration and are ideal substrates.
PCR Product Carry-Over

0.1 µl carry-over of amplicon can contain a MILLION COPIES of DNA
Real-Time PCR

• Monitor the accumulation of PCR product during amplification

• Detection of changes in fluorescent signal generated during cycles of PCR

* The fewer cycles to detect fluorescence signal the greater amount of DNA in the sample

Simultaneous amplification and detection in the same well
Real Time Definitions

**Threshold** – The level of detection or the point at which a reaction reaches a fluorescent signal above baseline (7000 and 7900HT SDS default settings are 0.2)

$C_T$ (Cycle Threshold) – The cycle at which a sample amplification curve crosses the threshold
Example of Accumulation of PCR Product (fluorescence) per Cycle
5’ Nuclease Assay

R = Reporter (FAM™ or VIC® Dyes)

Q = Non Fluorescent Quencher (NFQ)

Acts as energy transfer acceptor that does not emit a detectable fluorescent signal
5’ Nuclease Assay

Excitation

Forward Primer

R

hv

Q

Hybridization

Reverse Primer
5' Nuclease Assay

Displacement
5' Nuclease Assay

Cleavage
5' Nuclease Assay

Polymerization completed
Advantages of Using the 5′-Nuclease Assay

1. The probe provides specificity

2. Low background noise increases sensitivity

3. The fluorescent signal is directly proportional to the number of amplicons generated
Monitoring Real-Time PCR
[three phases]

- Phase 1, Geometric
- Phase 2, Linear
- Phase 3, Plateau
Quantifiler Primer Mix: Pre-Formulated as A Duplex Assay

Target Probe to Autosomal or Y-Chromosome Specific Region

Internal PCR Control
Amplification Plots for DNA Concentration Standards

Eight 3-fold serial dilutions

Smaller the $C_T$ number the more DNA detected

50 ng/µL

Used to Calculate $C_T$

0.023 ng/µL
Standard Curve from DNA Concentration Standards
Example of sample positive for DNA

FAM™ dye signal from sample

VIC® dye signal from PCR control

Threshold
Example of sample with no detectable DNA

- VIC® dye signal from PCR control
- FAM™ dye signal from sample
Example of sample with inhibition

FAM™ dye signal from sample below threshold

VIC® dye signal from PCR control below threshold
Example of sample with partial inhibition
Application

• Quantitation
• SNP detection