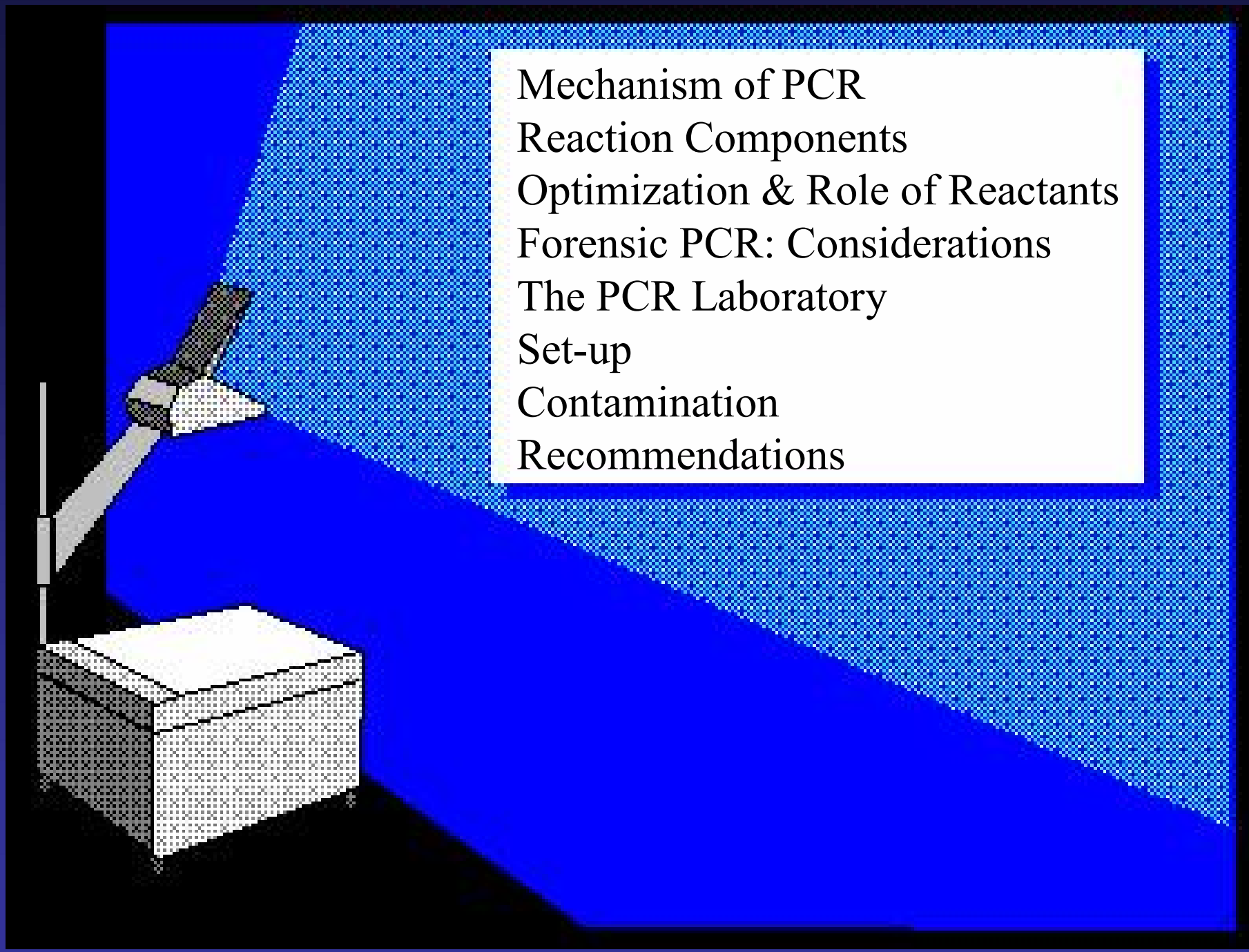
Polymerase Chain Reaction

Workshop

Phoenix, AZ

October 4, 2004

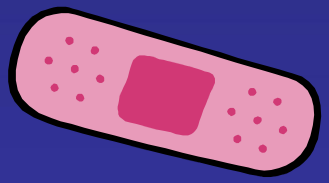

Mechanism of PCR
Reaction Components
Optimization & Role of Reactants
Forensic PCR: Considerations
The PCR Laboratory
Set-up
Contamination
Recommendations

IMPACT OF PCR

Research

Forensics

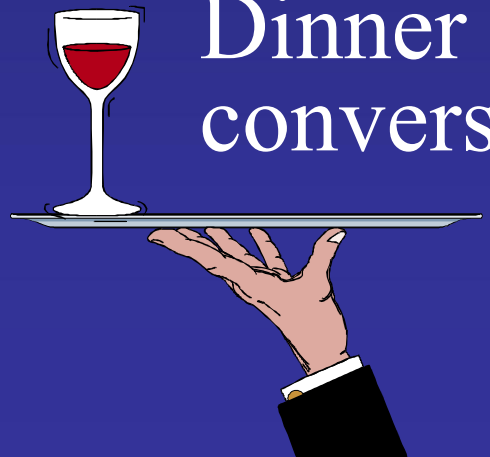
Medicine



HIV detection

Disease diagnosis

Prenatal & carrier detection



Dinner conversation

Development of PCR

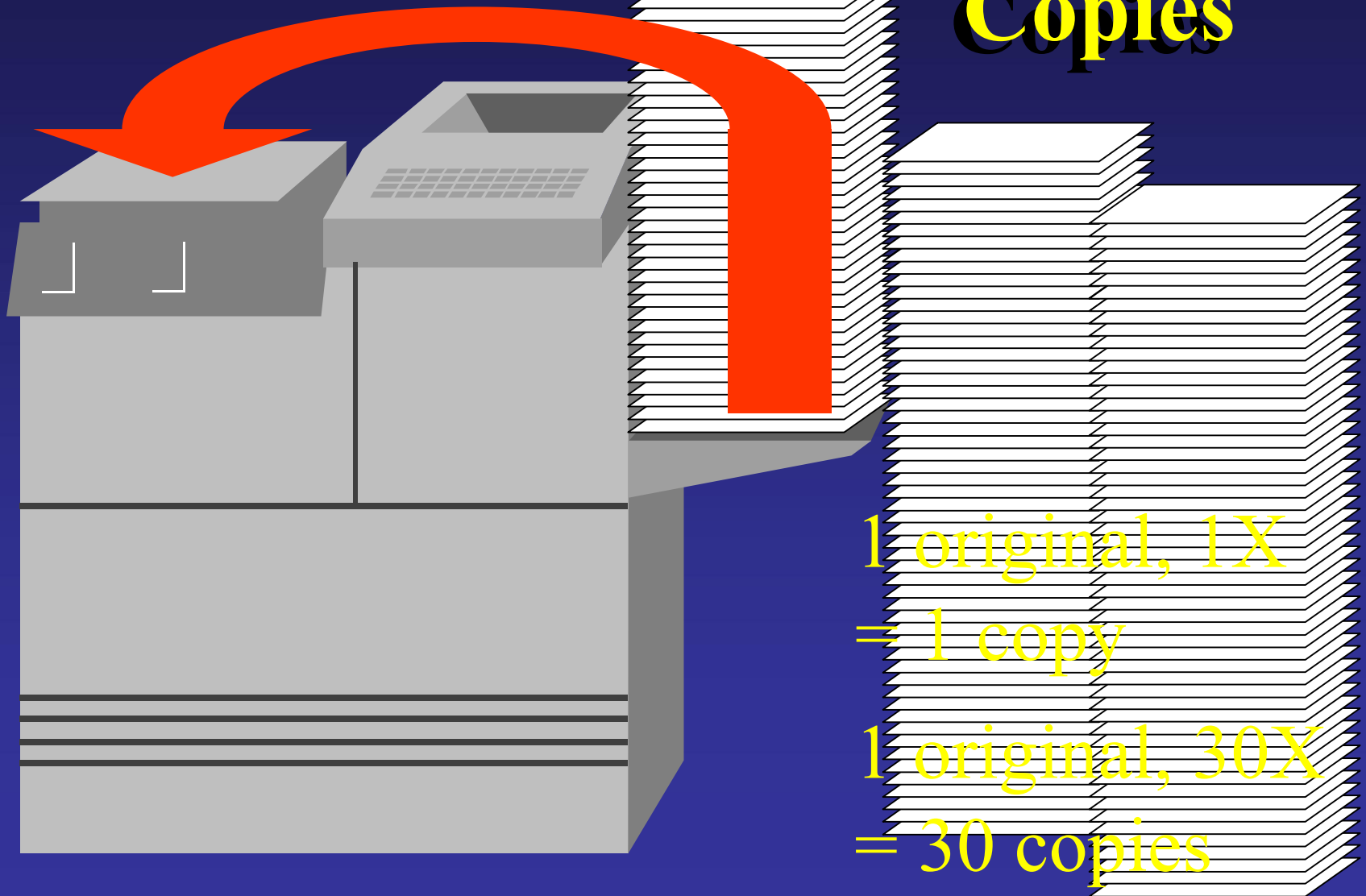
- 1969 Brock & Freeze: *Thermus aquaticus*
- 1976 Characteristics of Taq polymerase
- 1980 Temperature optimum for DNA synthesis
- 1985 Mullis: PCR using thermostable polymerase
- 1985 Cetus: automated thermocycling

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 = the number of cycles
- Theoretically:
  - n = 20 yields a million-fold increase
  - n = 30 yields a billion-fold increase

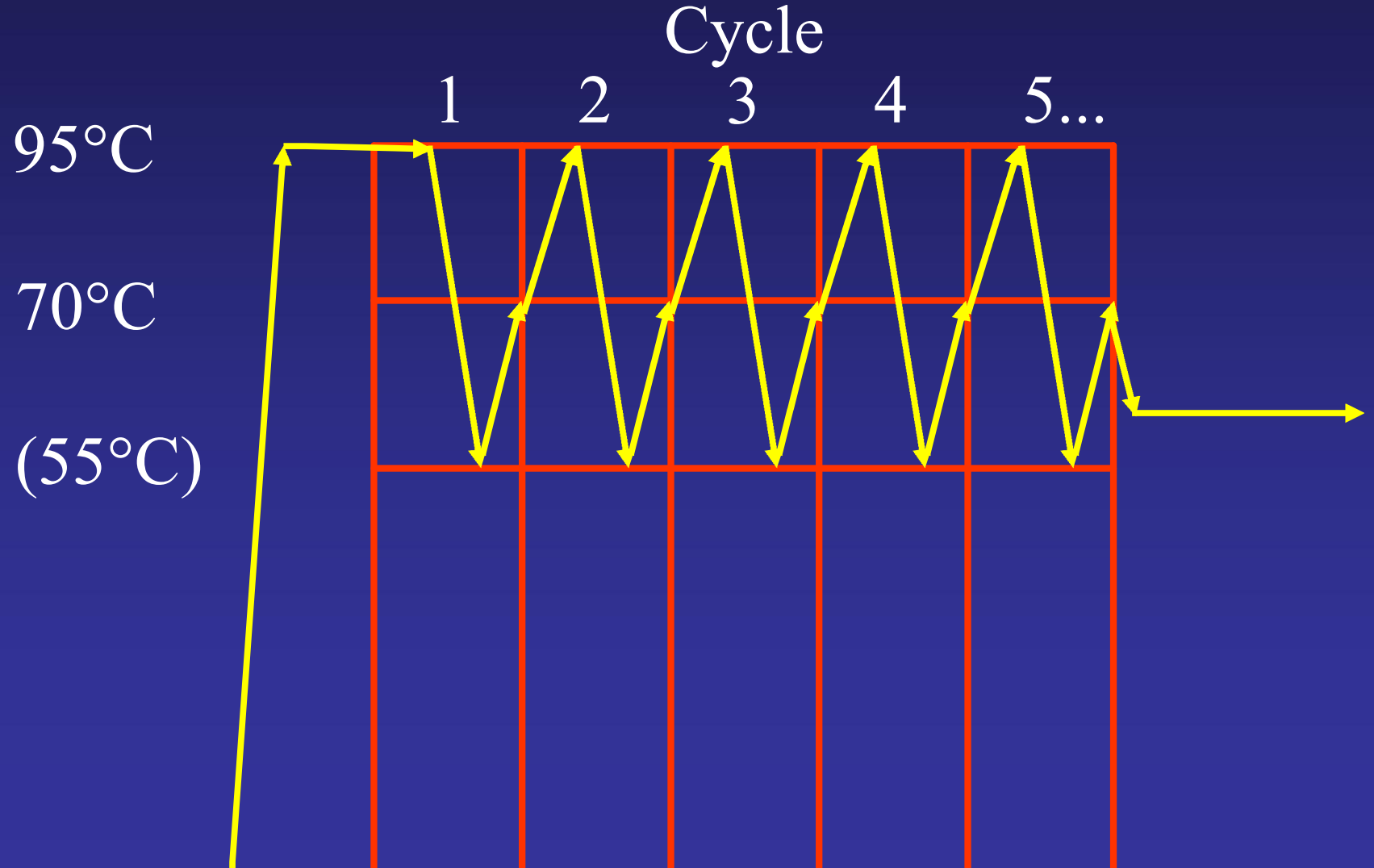
CYCLING STEPS

CYCLING STEPS

CYCLING STEPS

CYCLING STEPS

THREE-TEMPERATURE PCR



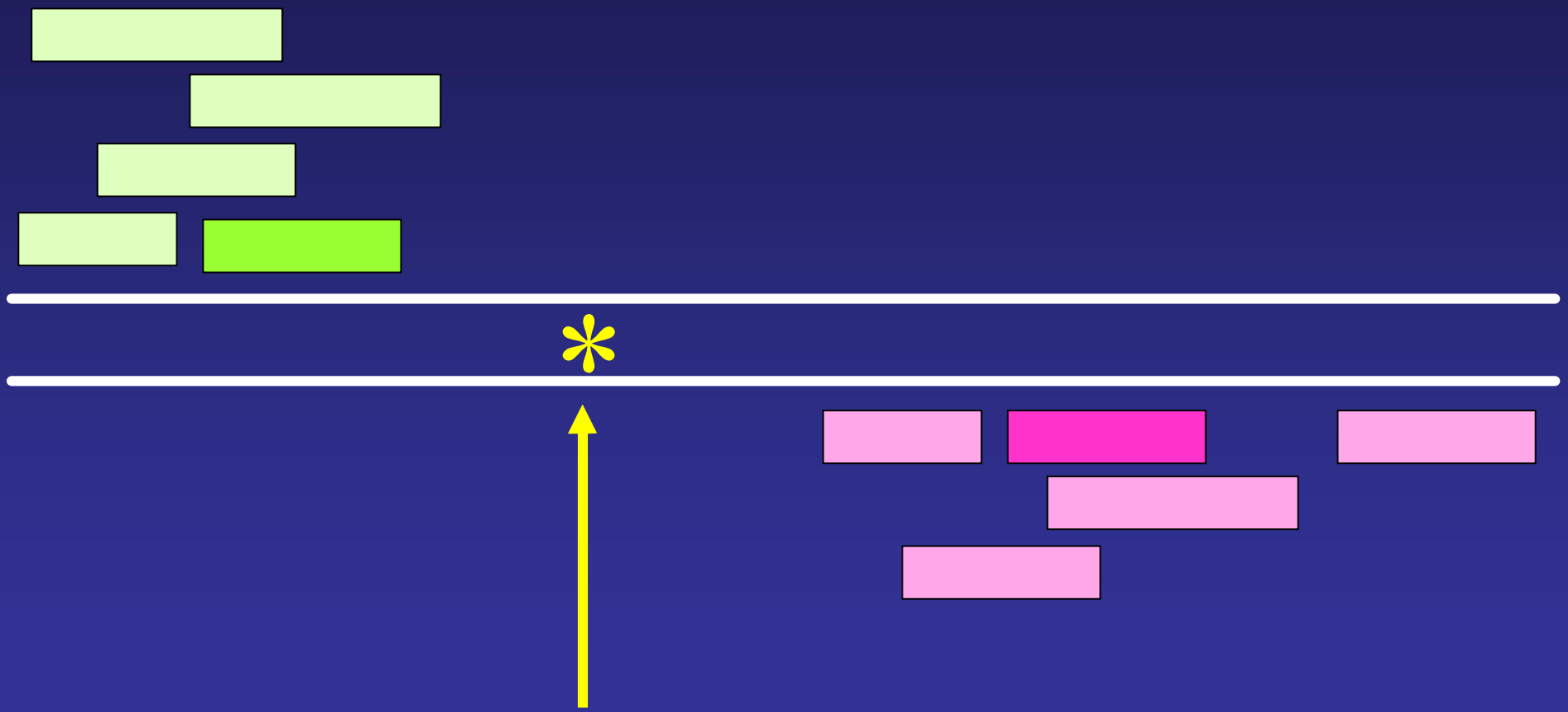
Primers

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

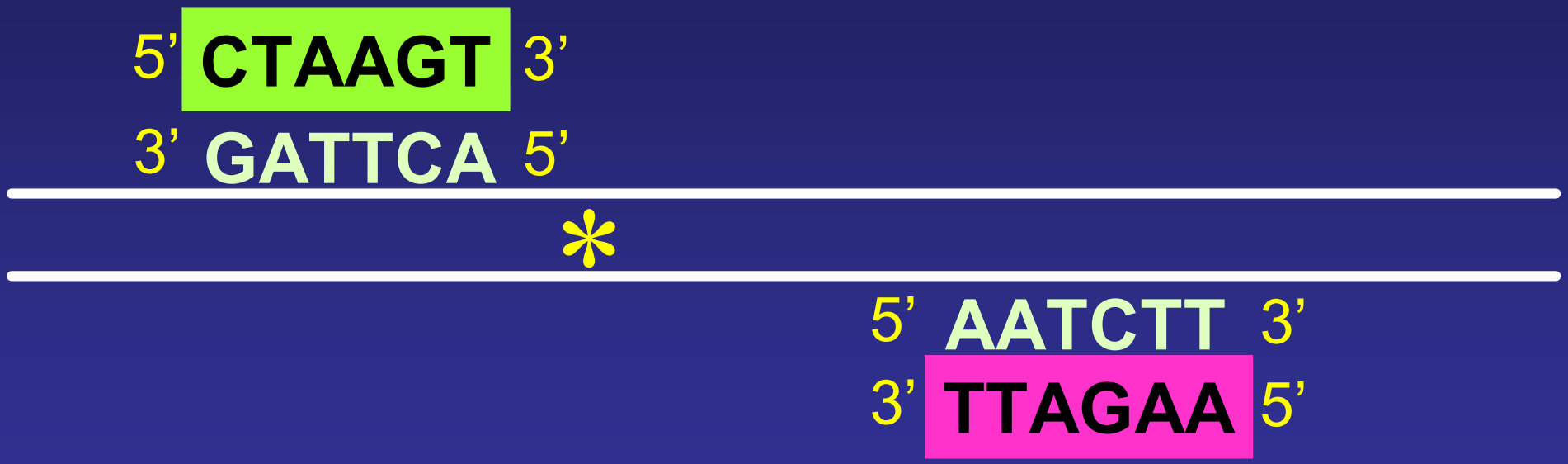
5'-ATACC-3'

3'-GATGG-5'

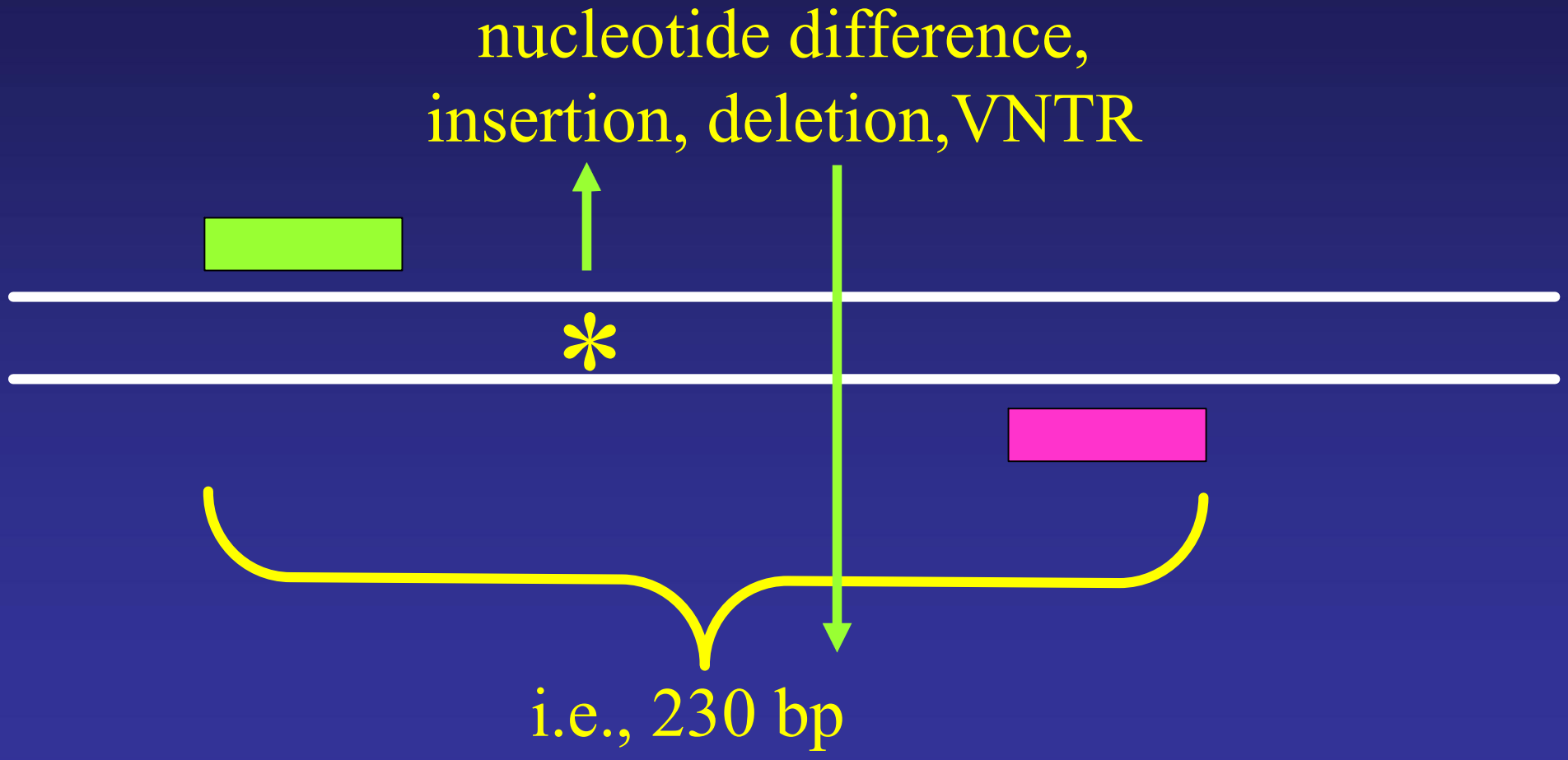
Primer Selection



Primer Annealing



Defined Target Region



Cycle 1, Step 1
DENATURATION

Cycle 1, Step 1
DENATURATION

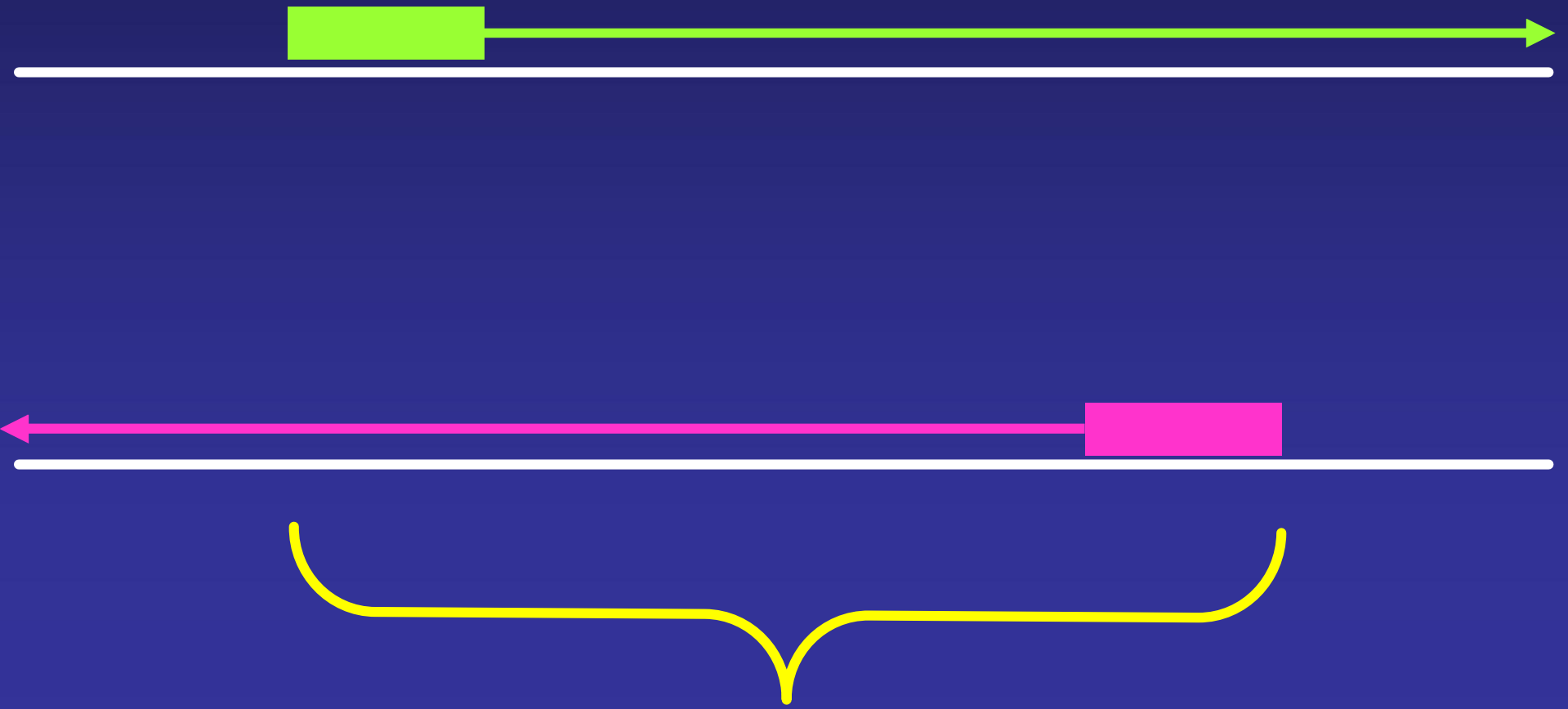
Cycle 1, Step 1
DENATURATION

Cycle 1, Step 1
DENATURATION

Cycle 1, Step 1
DENATURATION

Cycle 1, Step 2
ANNEALING

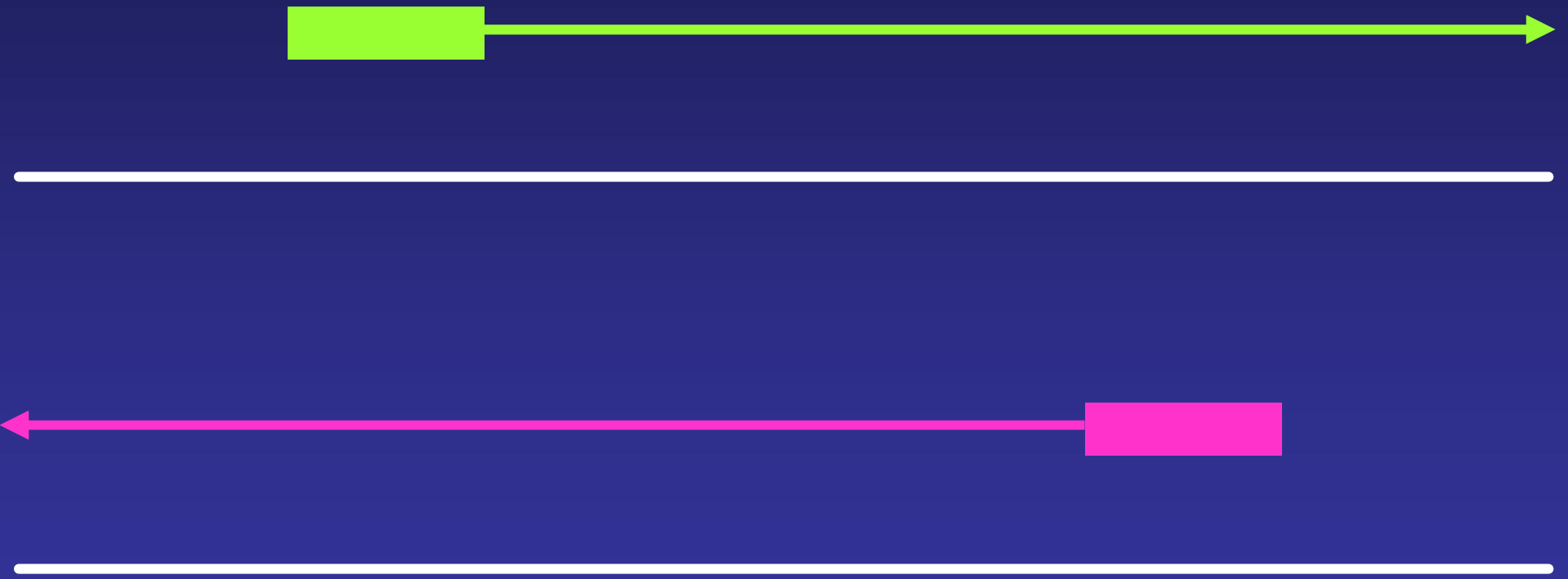
Cycle 1, Step 3
EXTENSION



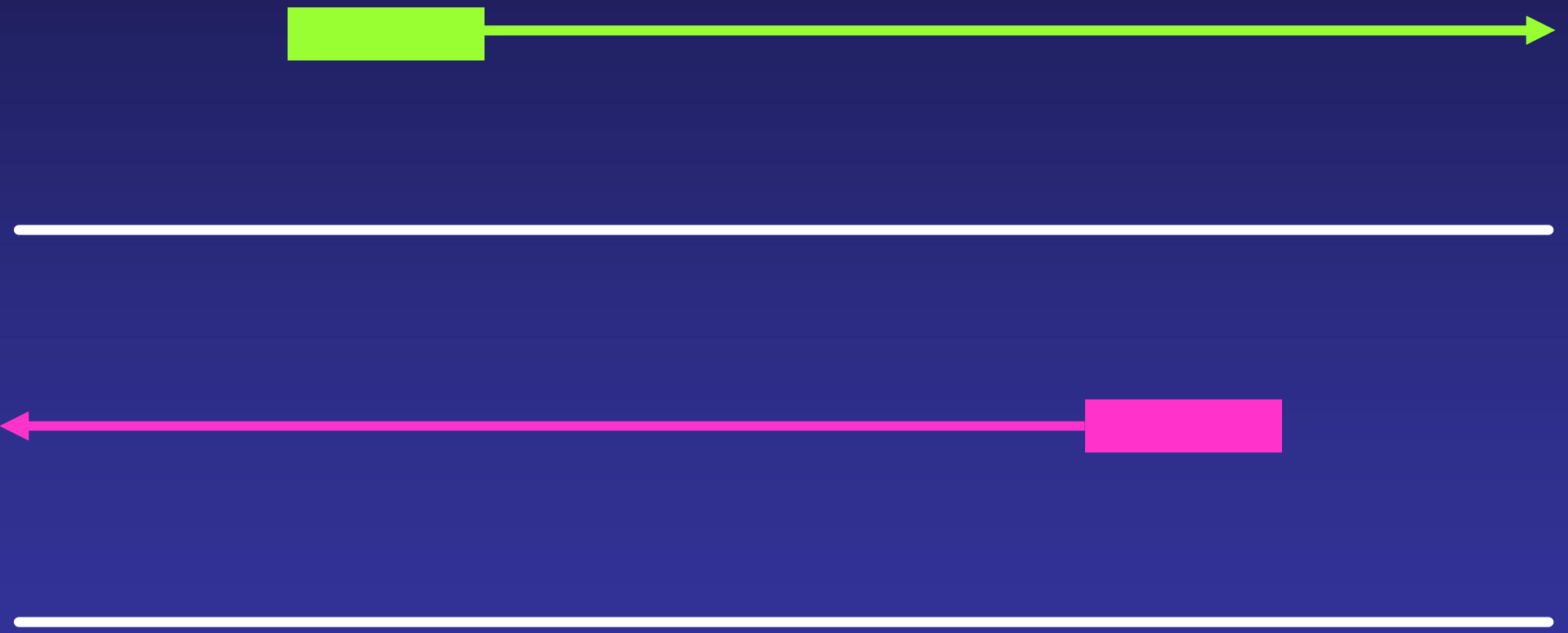
Cycle 2, Step 1
DENATURATION

Cycle 2, Step 1
DENATURATION

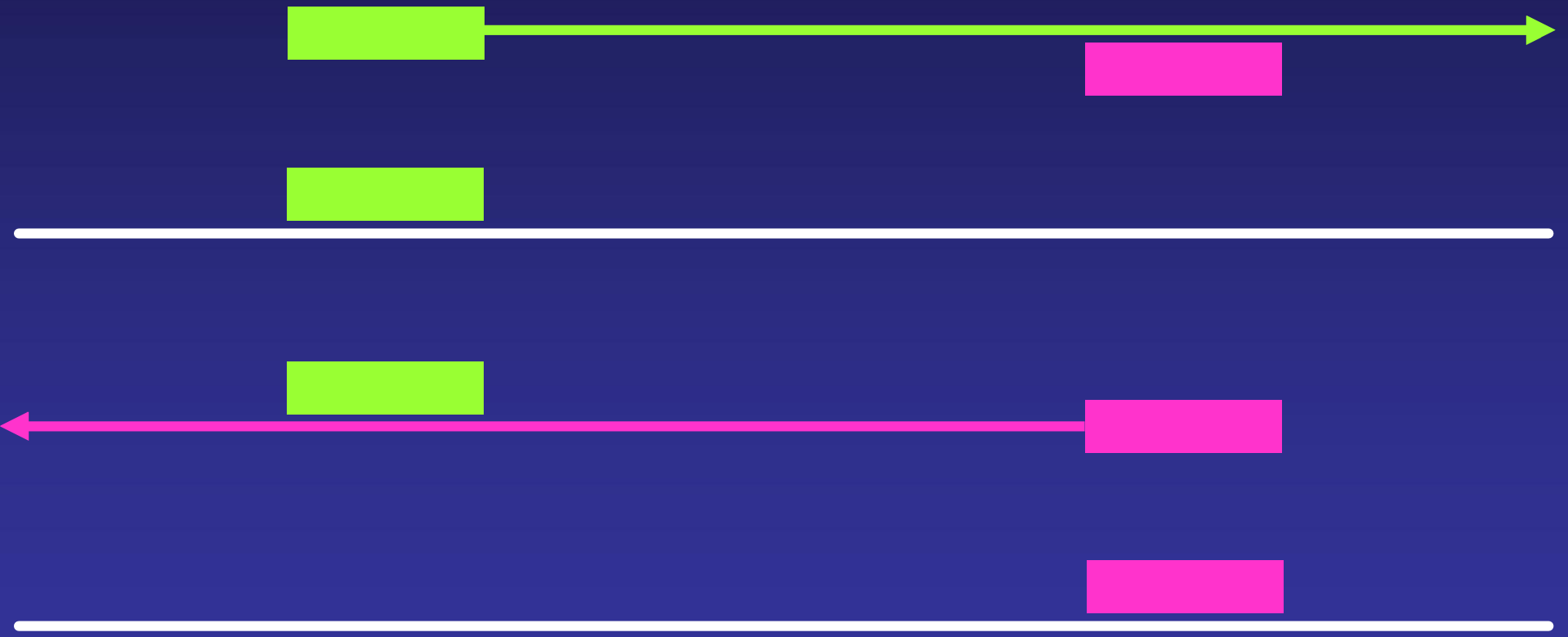
Cycle 2, Step 1
DENATURATION



Cycle 2, Step 1
DENATURATION

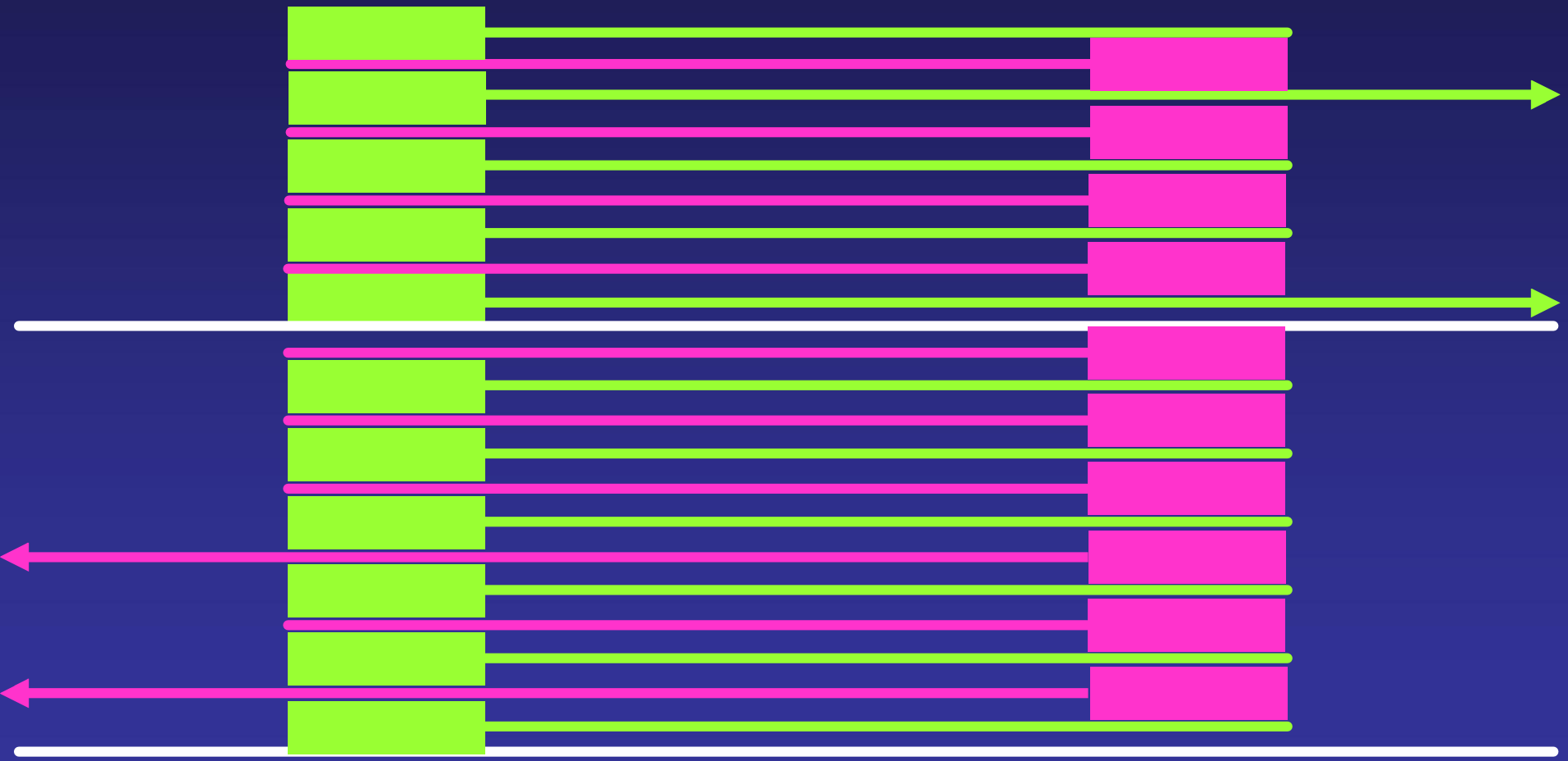


Cycle 2, Step 2
ANNEALING

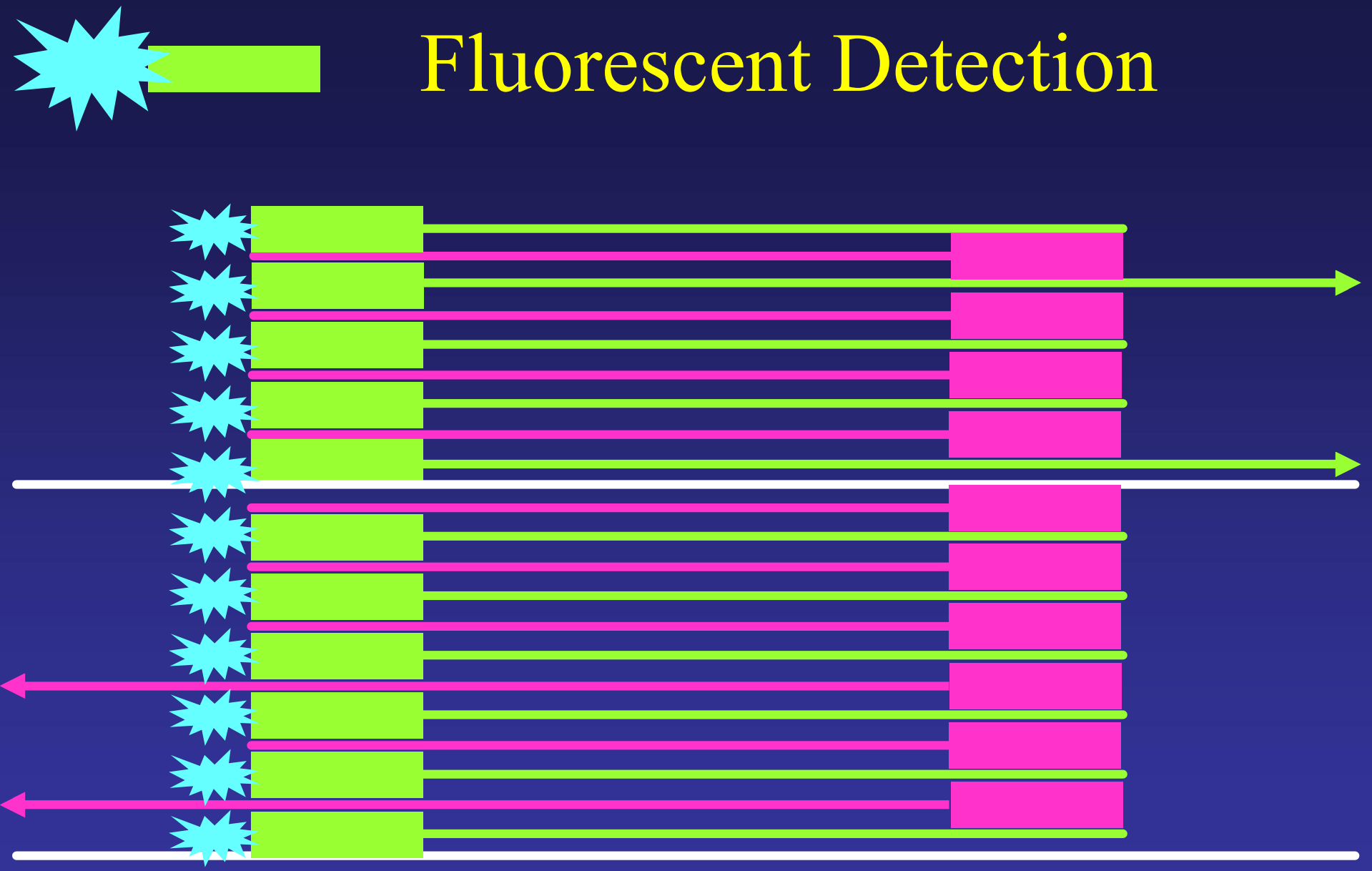


Cycle 2, Step 3
EXTENSION

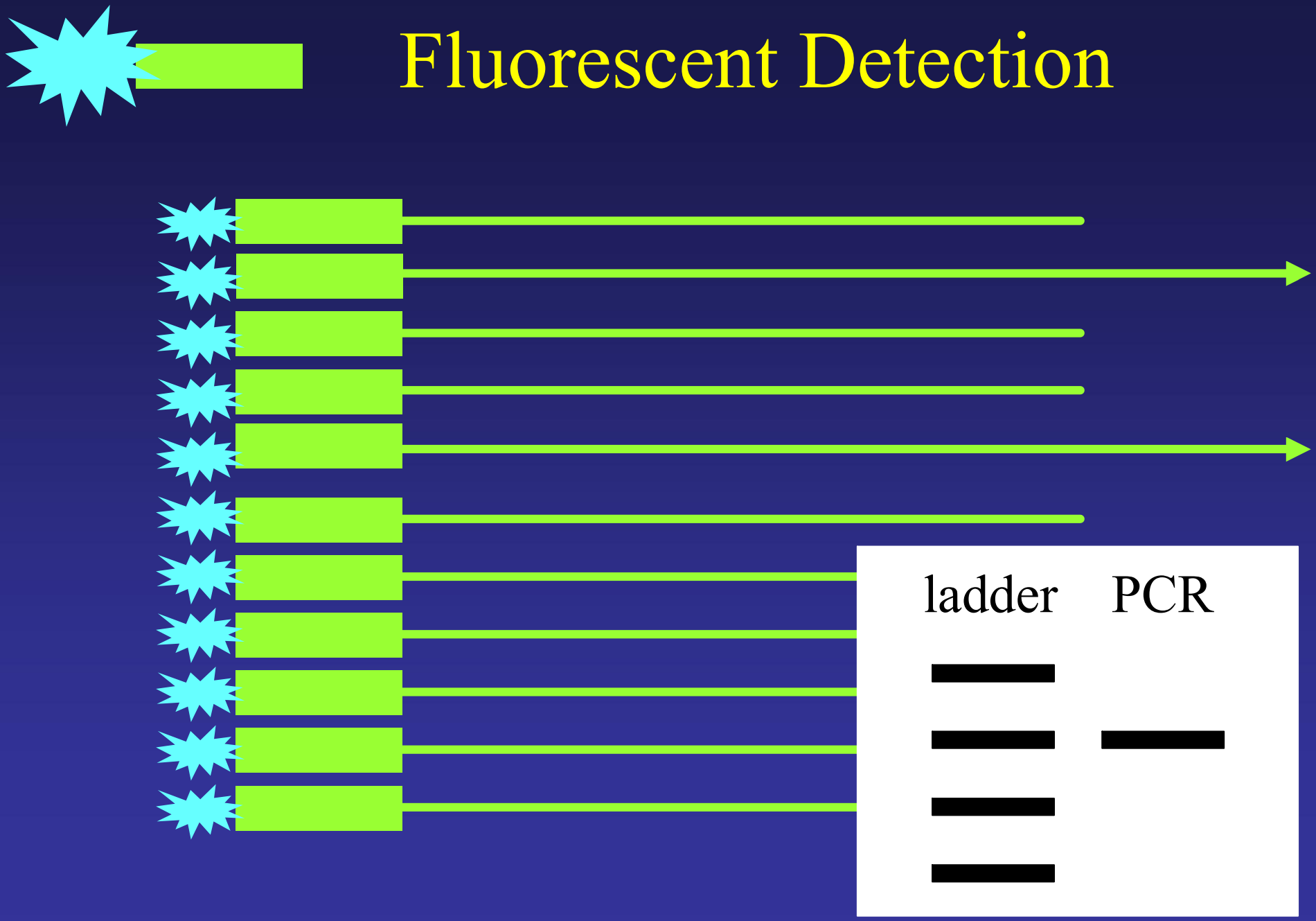
Accumulation of Target Fragment



Fluorescent Detection

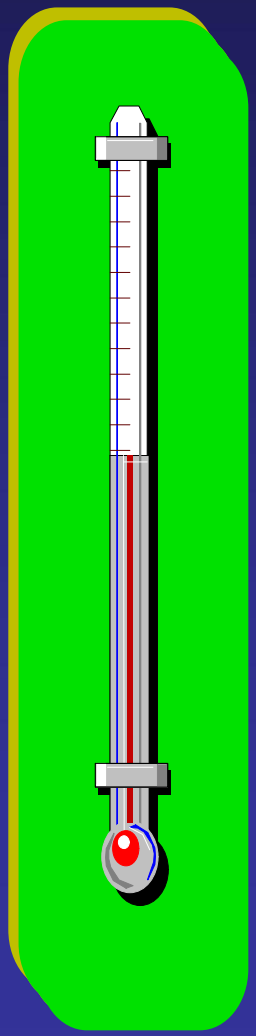


Fluorescent Detection



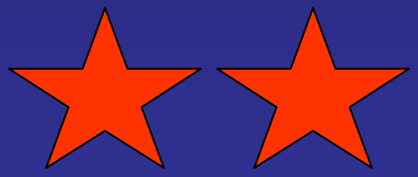
EXAMPLE: 3-Temperature PCR

- 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 Nucleotide Addition ("Plus A")

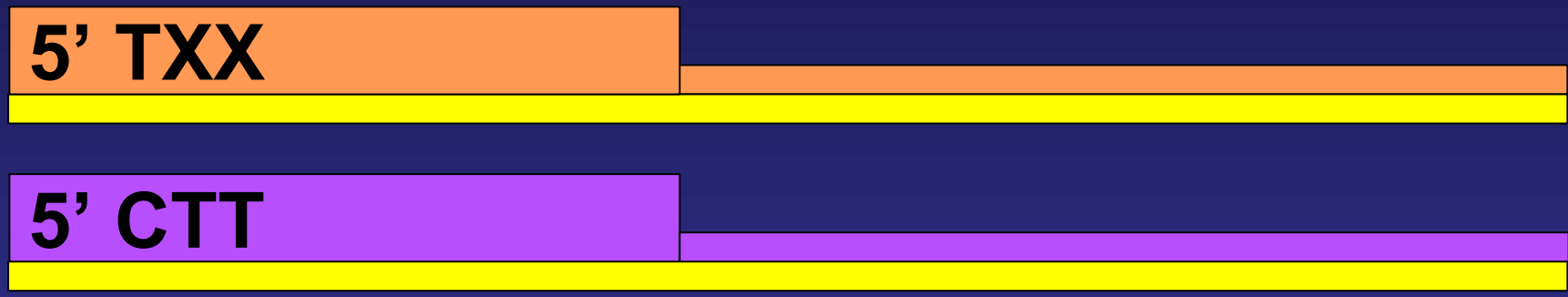
- Many DNA polymerases add an extra nucleotide to the blunt end of a DNA product *without* the assistance of a template
- Usually A is added
- The resulting band/peak is 1-bp larger in size than that predicted from the DNA sequence and primer locations

Non-Templated Nucleotide 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

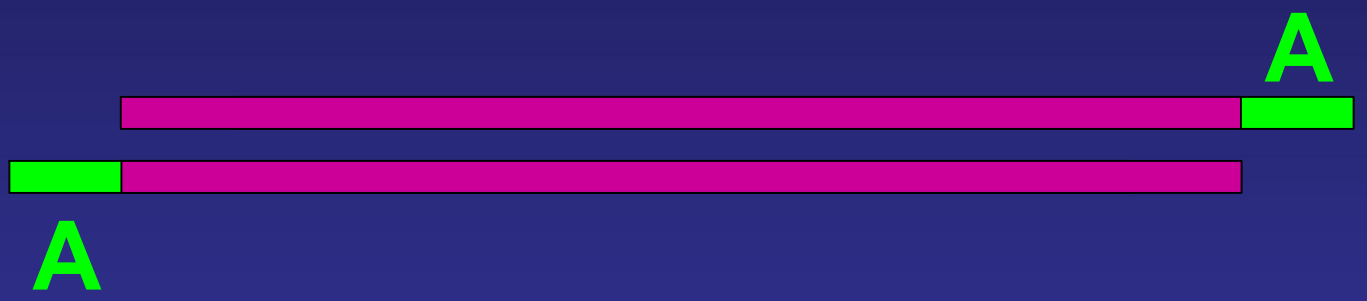
5' end of primer affects adenylation



Adenylation reduced

Non-Templated Nucleotide Addition ("Plus A")

Double-stranded DNA / PCR Product

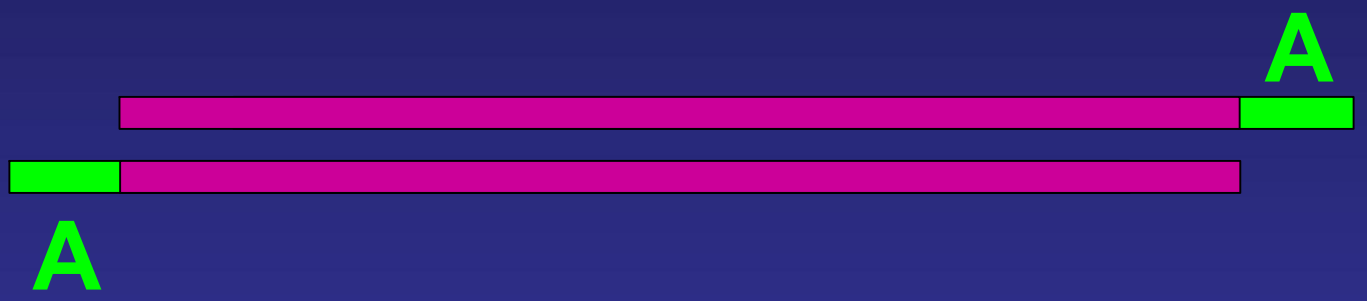


Non-Templated Nucleotide Addition ("Plus A")

Temperature effects whether the ends remain together

Non-Templated Nucleotide Addition ("Plus A")

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

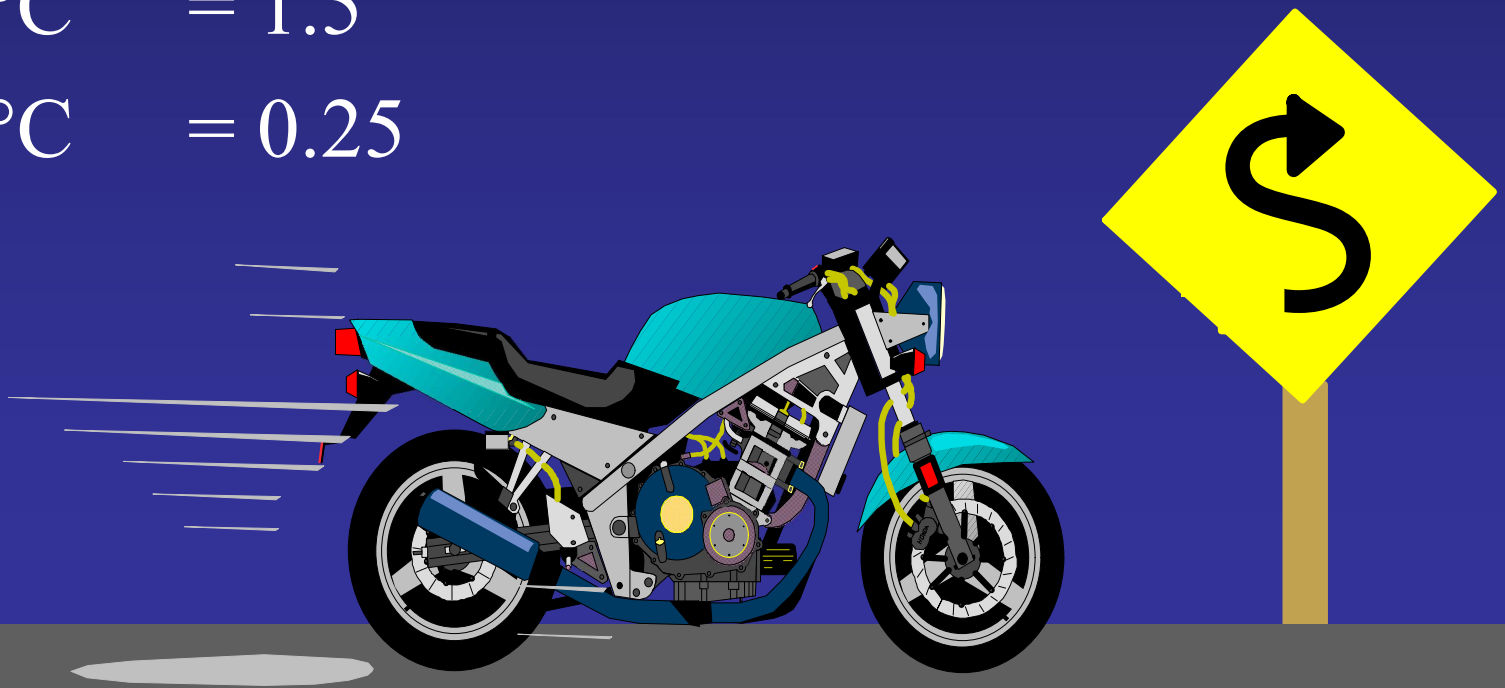


Taq DNA Polymerase

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

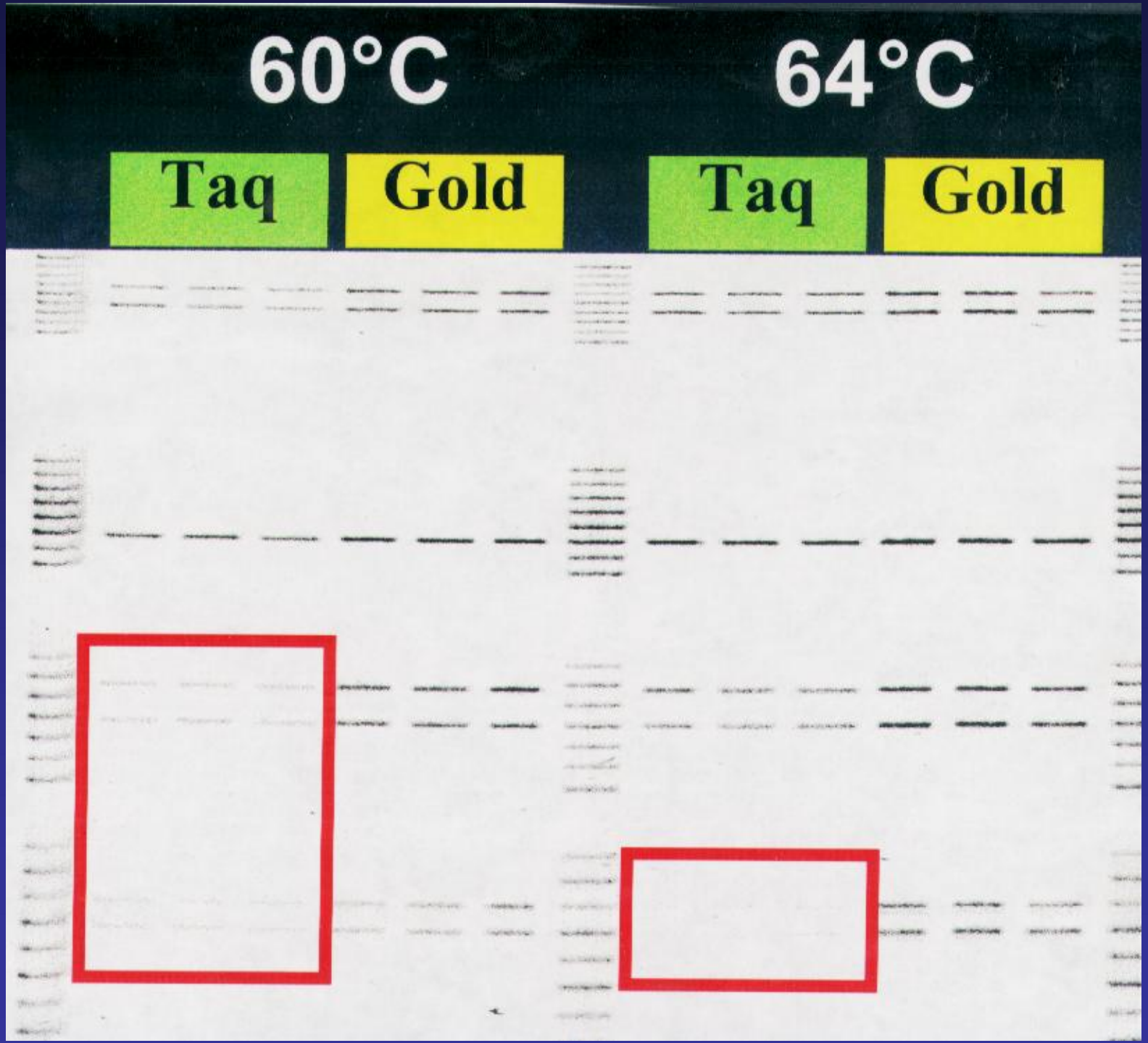
Taq Polymerase Activity

Extension Rate at:	Processivity:
• 70 °C = 60 nt/sec	• 70 °C = 50-60 nt
• 55 °C = 24	
• 36 °C = 1.5	
• 22 °C = 0.25	



AmpliTaq Gold DNA Polymerase

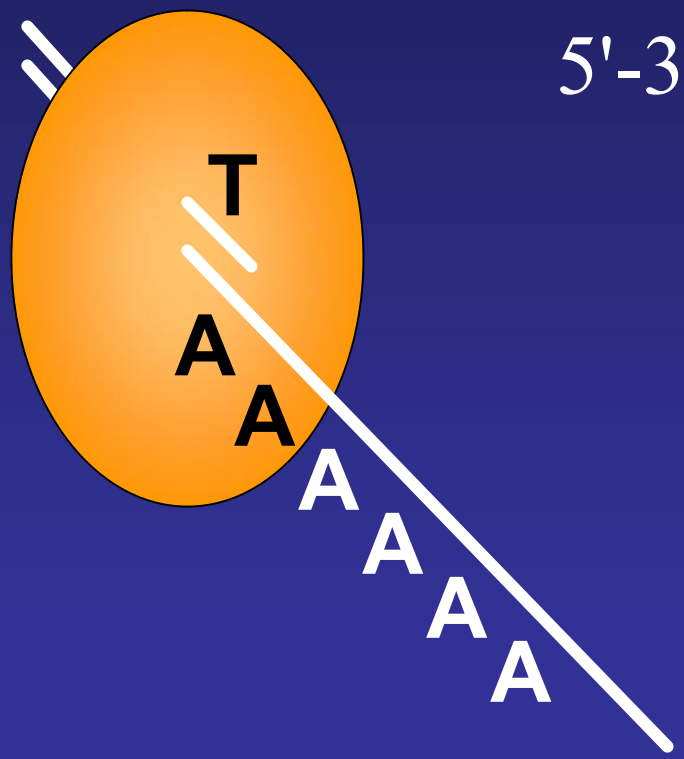
- Inactive until heated prior to thermocycling
- Simulates “Hot Start” PCR
- Increases yield & specificity of PCR
  - reduces non-specific products
  - reduces primer dimers
- Facilitates multiplexing
  - reduces primer design requirements
- Automation


60°C
64°C
Taq
Gold
Taq
Gold

However

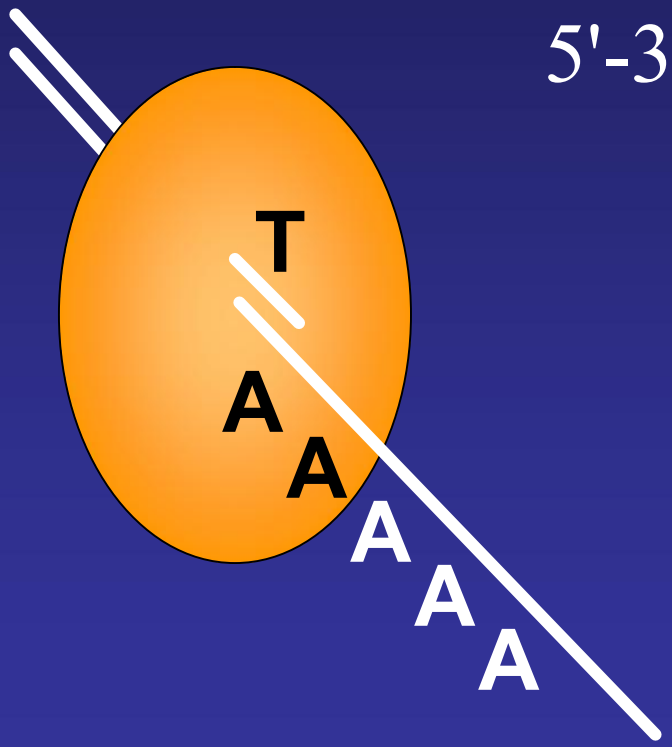

Taq
Taq Hot Start
Gold
A1 A2 A3 A4 A5 A6 A7 A8
B1 B2 B3 B4 B5 B6 B7 B8
C1 C2 C3 C4 C5 C6 C7 C8
Little difference in performance
between Taq & Gold
if primers are well-designed &
if the system is well-optimized

Taq Polymerase Fidelity



5'-3' synthesis

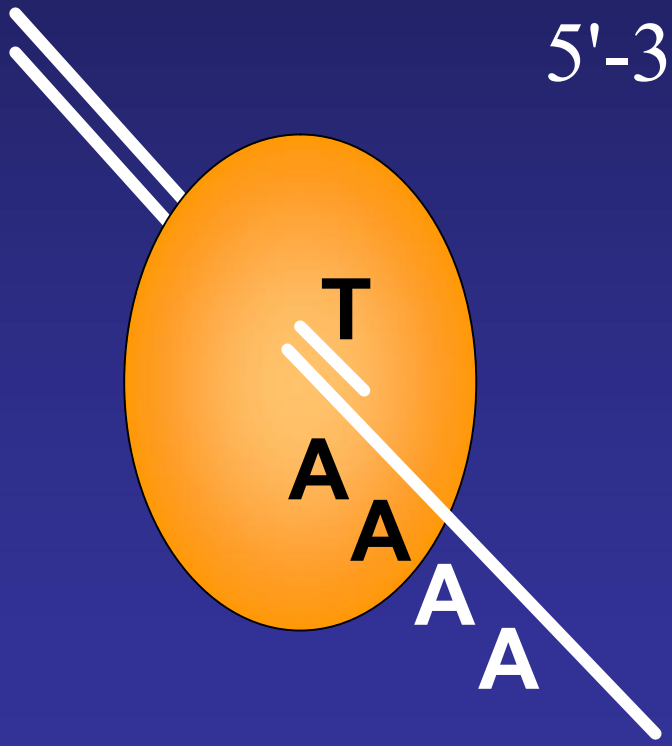
Taq Polymerase Fidelity



5'-3' synthesis

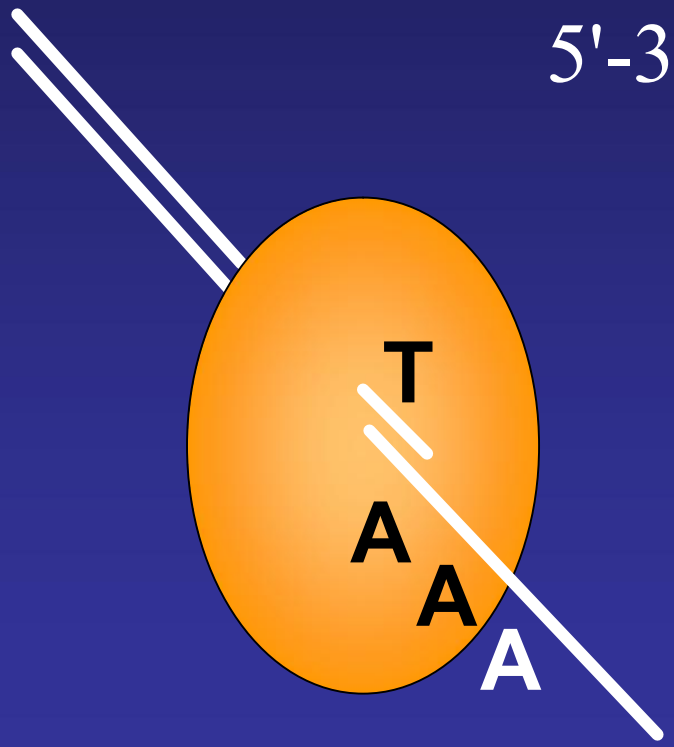
Taq Polymerase Fidelity

5'-3' synthesis



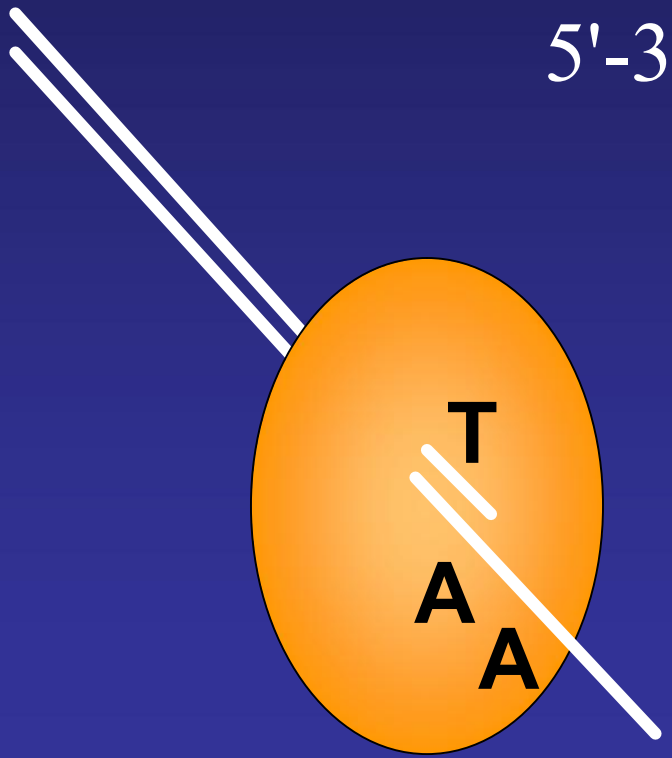
Taq Polymerase Fidelity

5'-3' synthesis

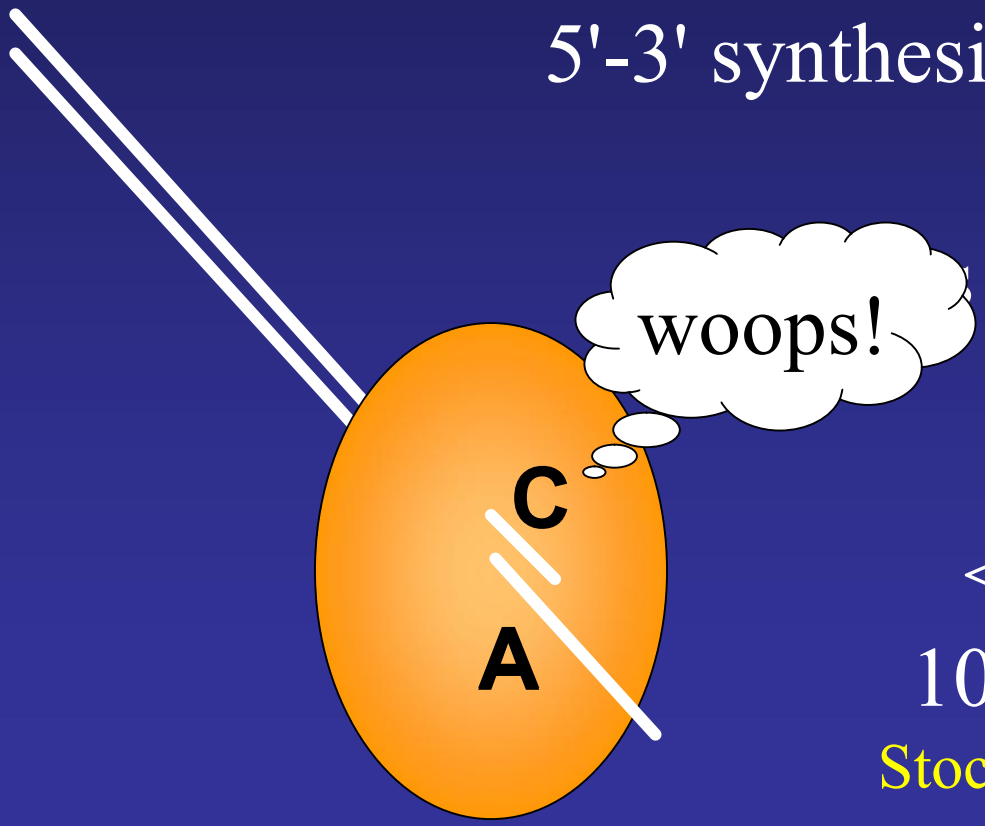


Taq Polymerase Fidelity

5'-3' synthesis



No Proofreading Ability



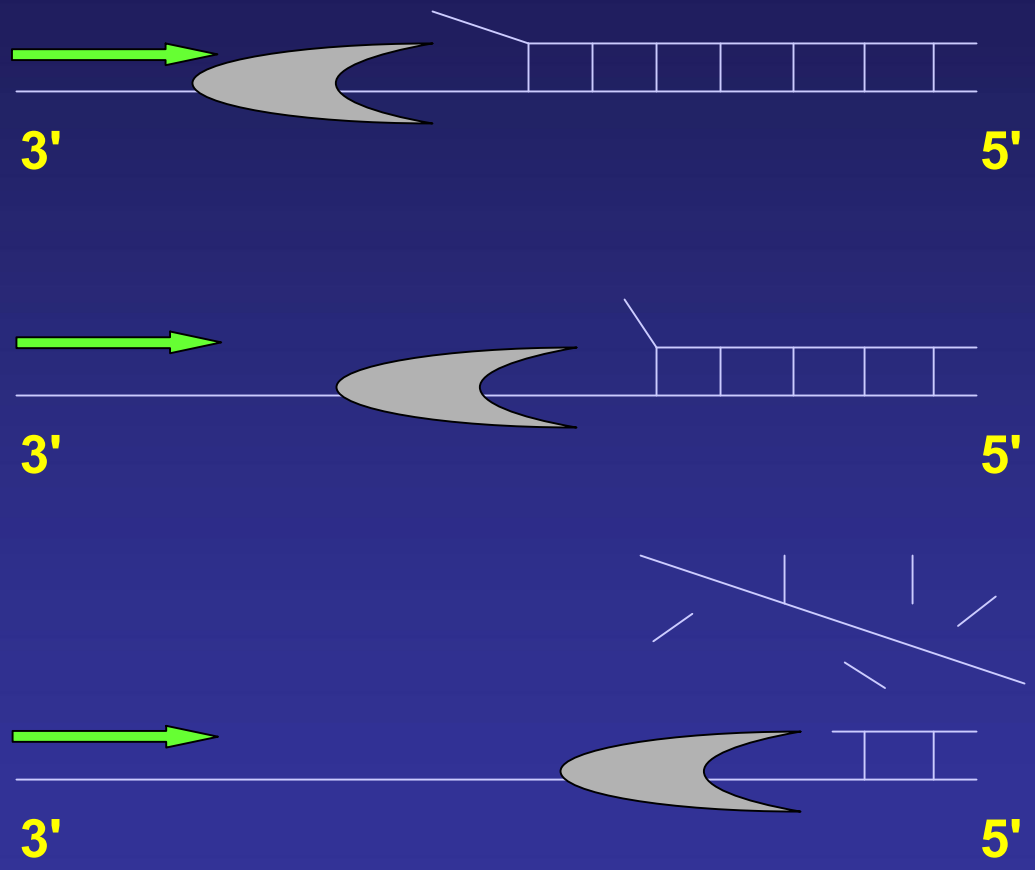
3'-5' exonuclease activity

<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

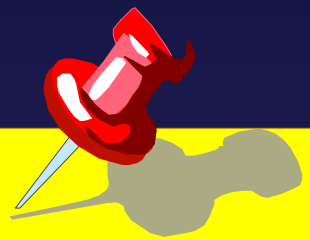
Primers

- 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’-TTTTTTTTCCCCC
  GGGGGTTTTTTTTT-5’

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

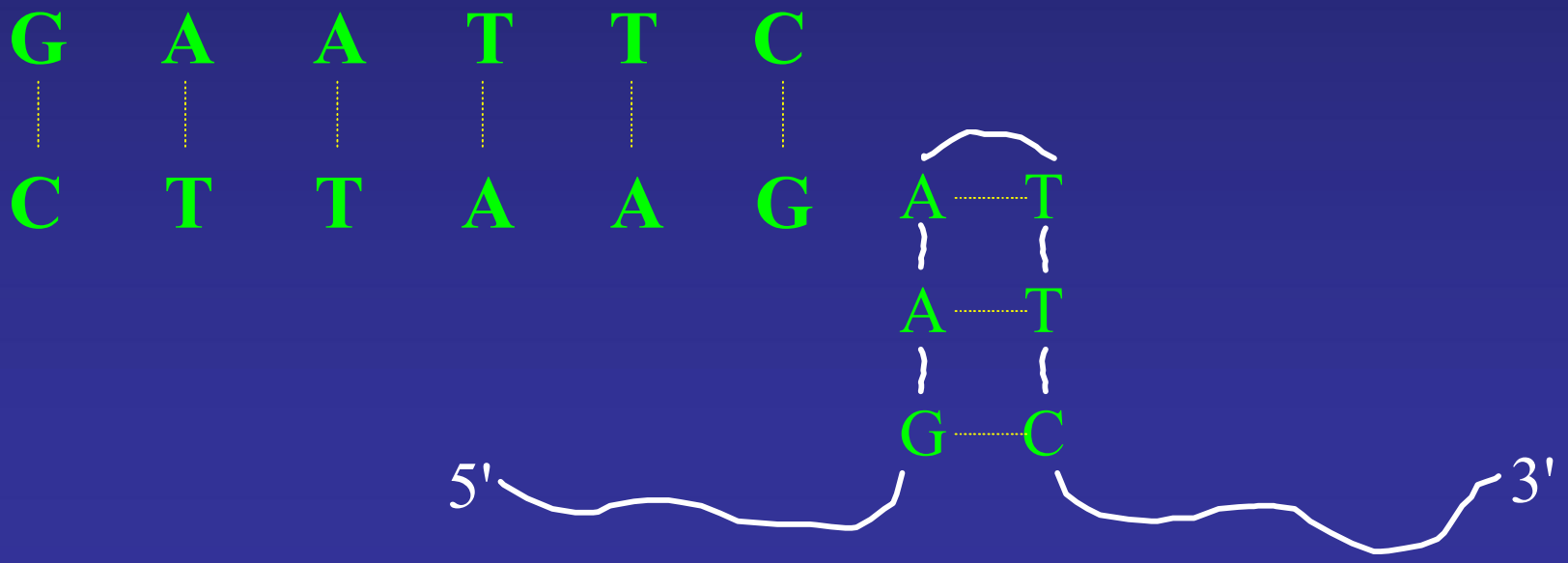
  5’-TTTTTTTCCCGGG
  GGGCCCTTTTTTTT-5’

Primers

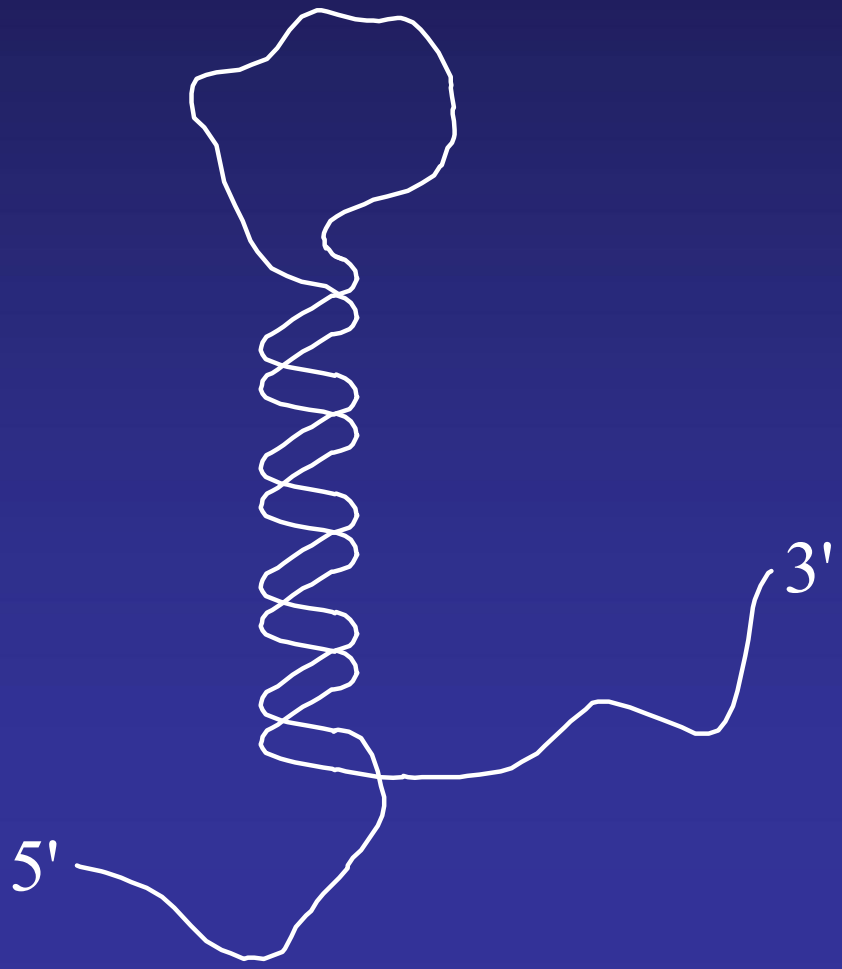
- Avoid runs of >3 G's or C's at 3' end
  - may misprime in G+C-rich regions
- Empirically determine ability to adenylate
- 5' additions to primers
  - restriction site, M13 site, promoter sequence
  - biotin, fluorophore, radioisotope

Primers

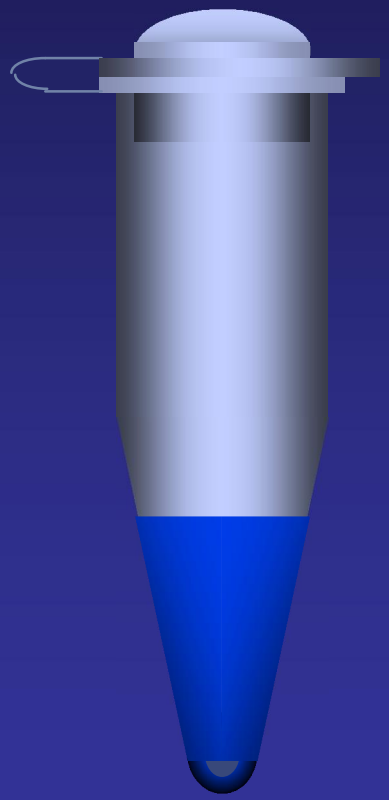
- Avoid palindromic sequences and internal complementarity



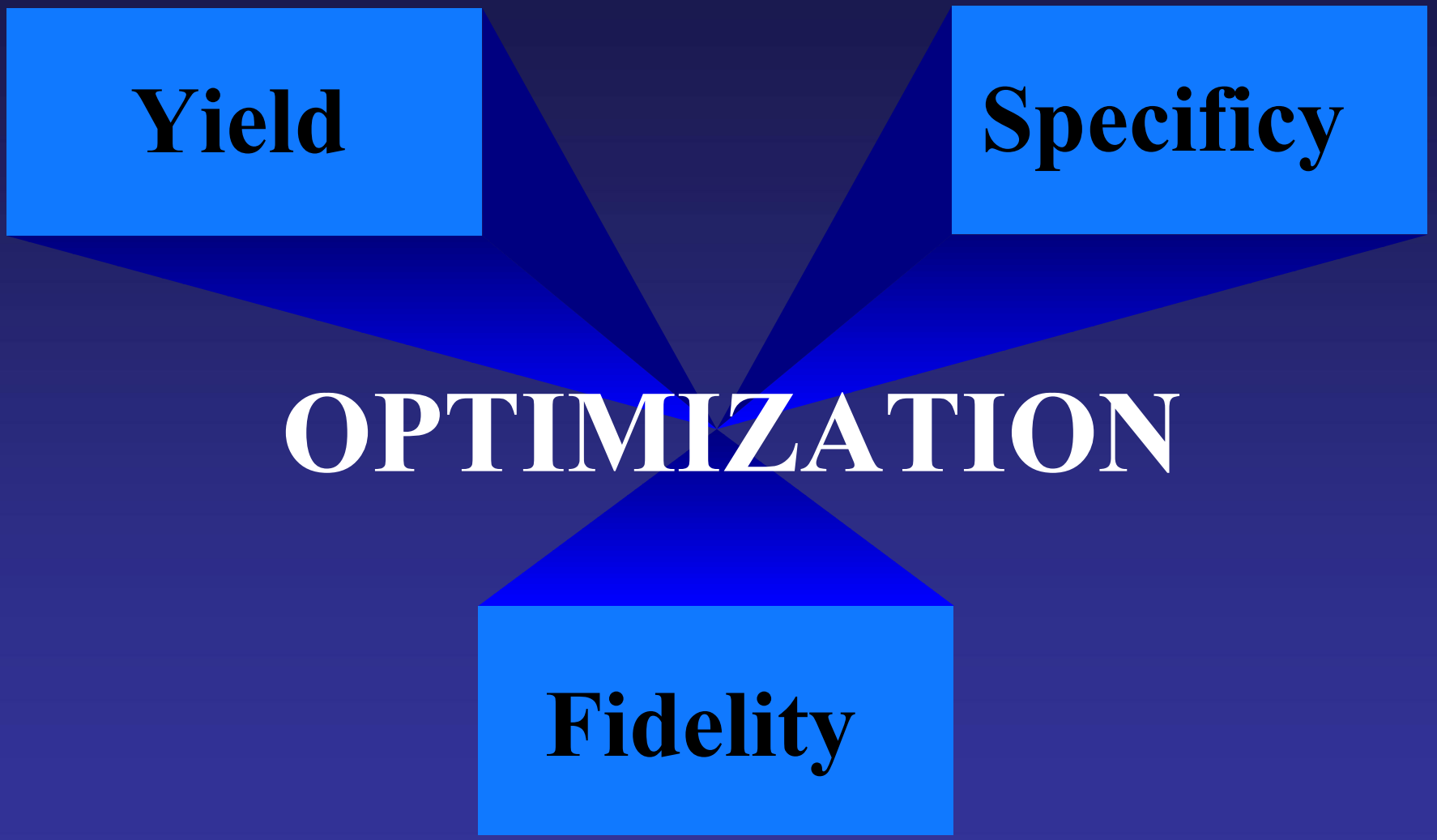
Hairpin due to Internal Complementarity



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


Yield
Specificy
OPTIMIZATION
Fidelity

DNA CONCENTRATION

Depends of method of detection

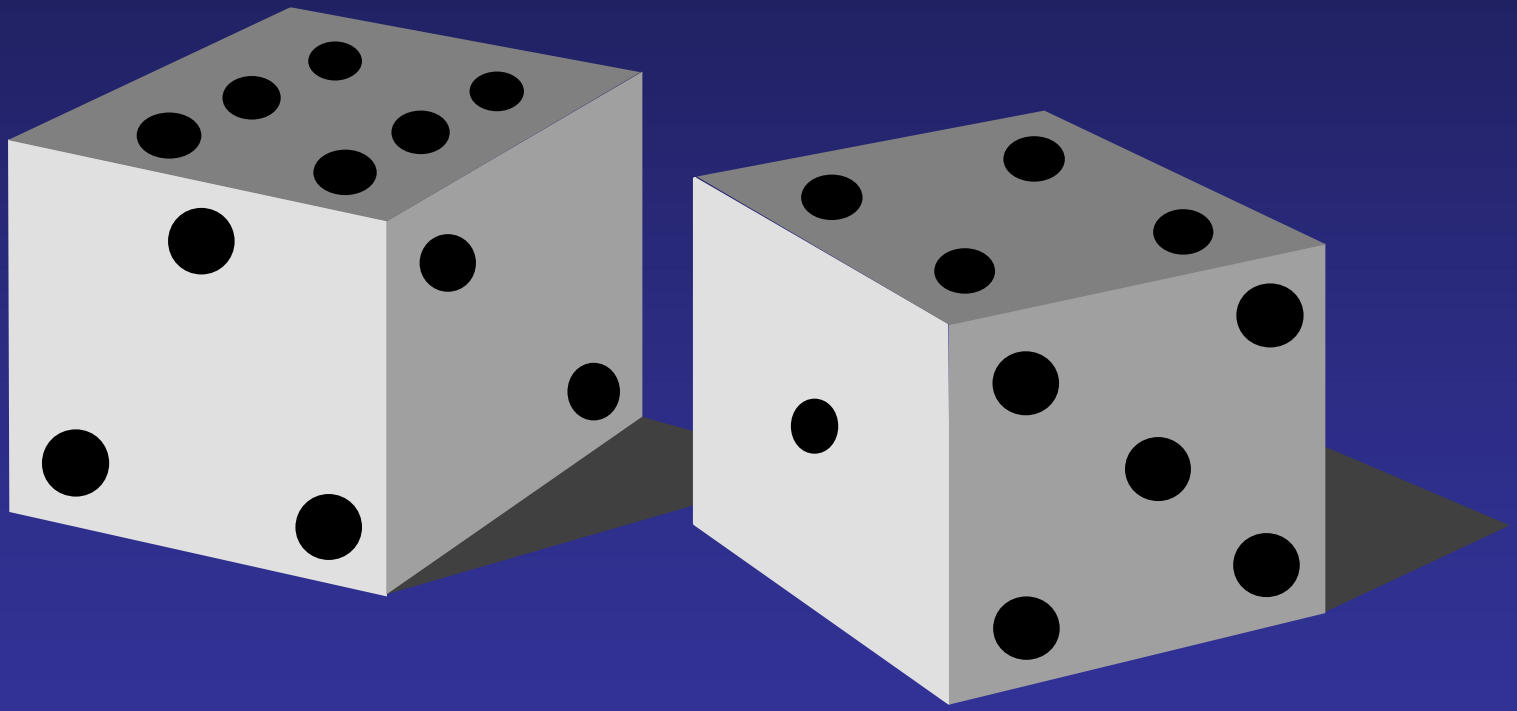
Too Much DNA:

Non-specific products
Lower yield
(WHY?)

Too Little DNA:

Lower yield
Stochiastic effects

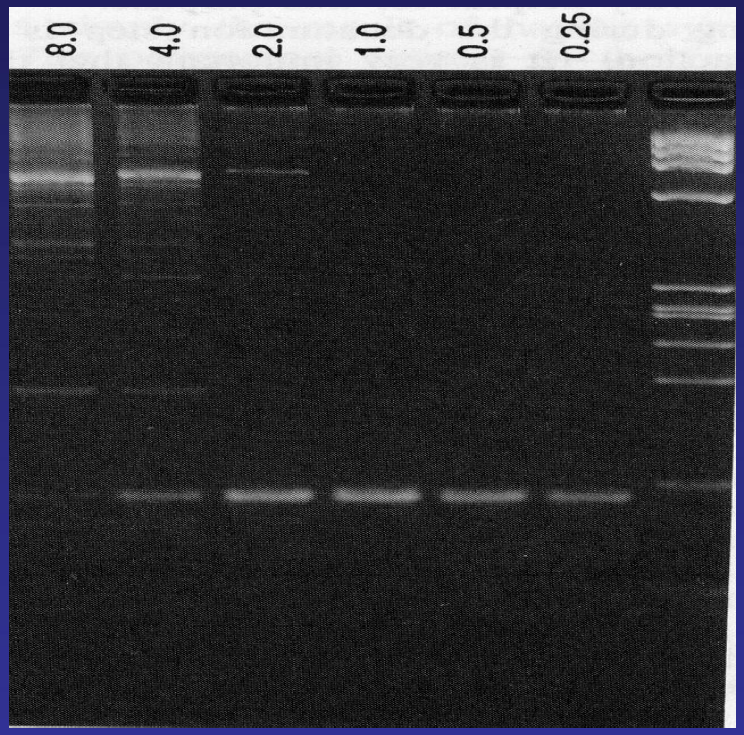
Stochastic Effects



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

VARYING TAQ POLYMERASE CONCENTRATION



5 units per reaction is typical
for many forensic kits

Divalent Cations

Magnesium is the cation of choice for Taq polymerase

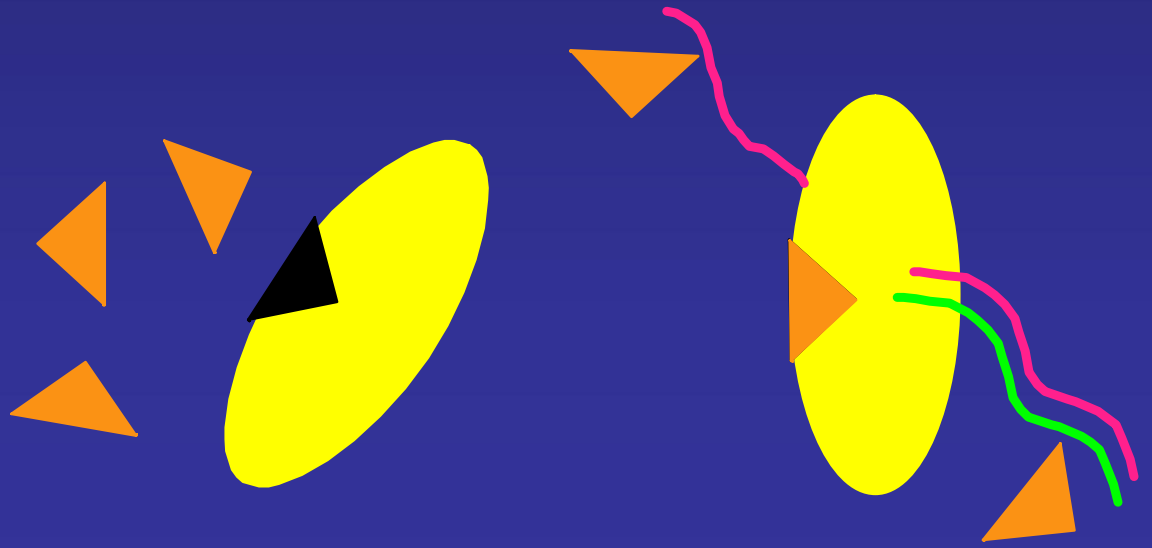
Cofactor in the catalytic addition of deoxynucleoside monophosphates to the 3’ end of the growing DNA chain

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

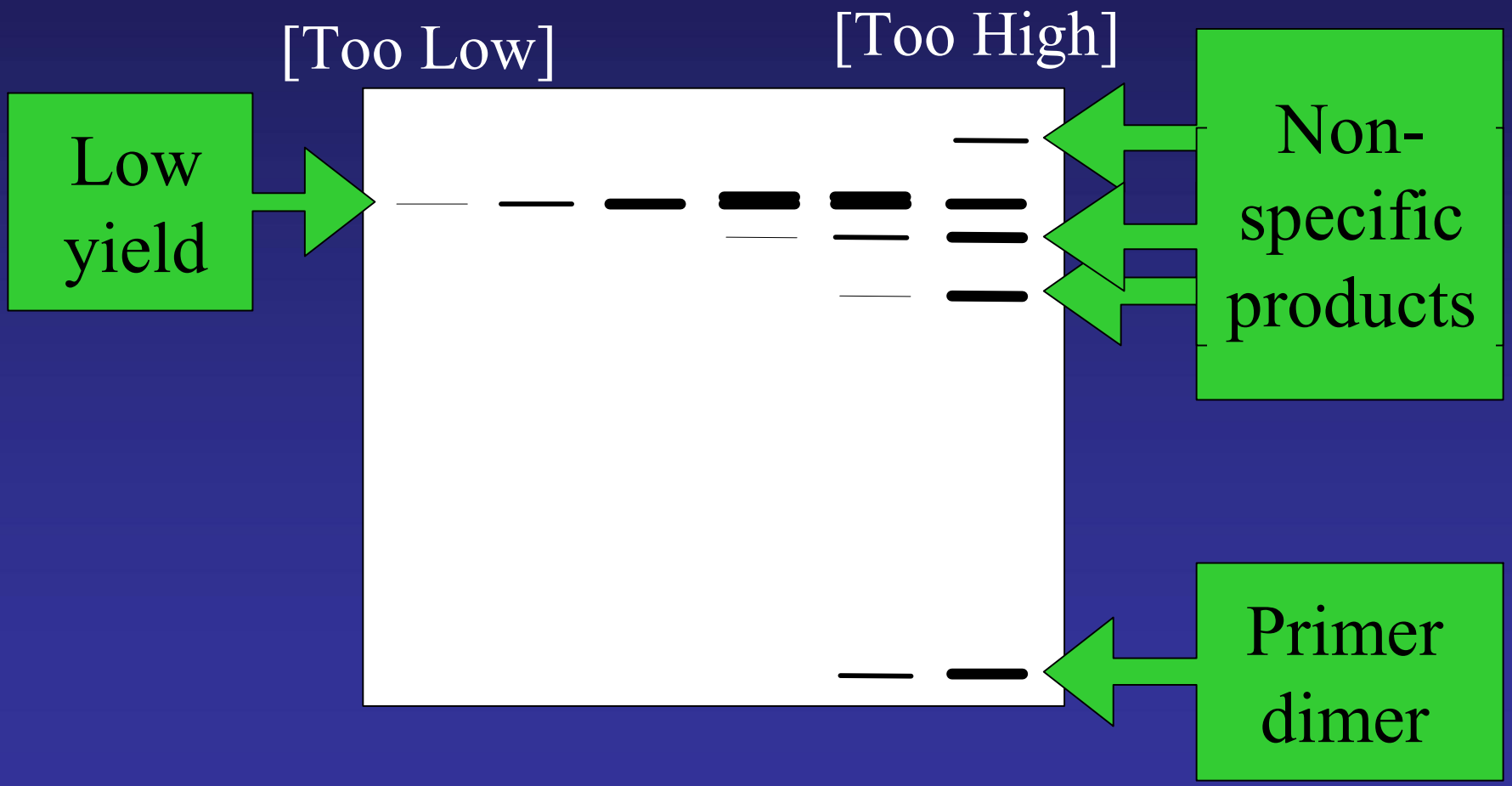
Too High:

Non-specific products
Inhibits Taq
(WHY?)

Too Low:

Lower yield

PRIMER CONCENTRATION



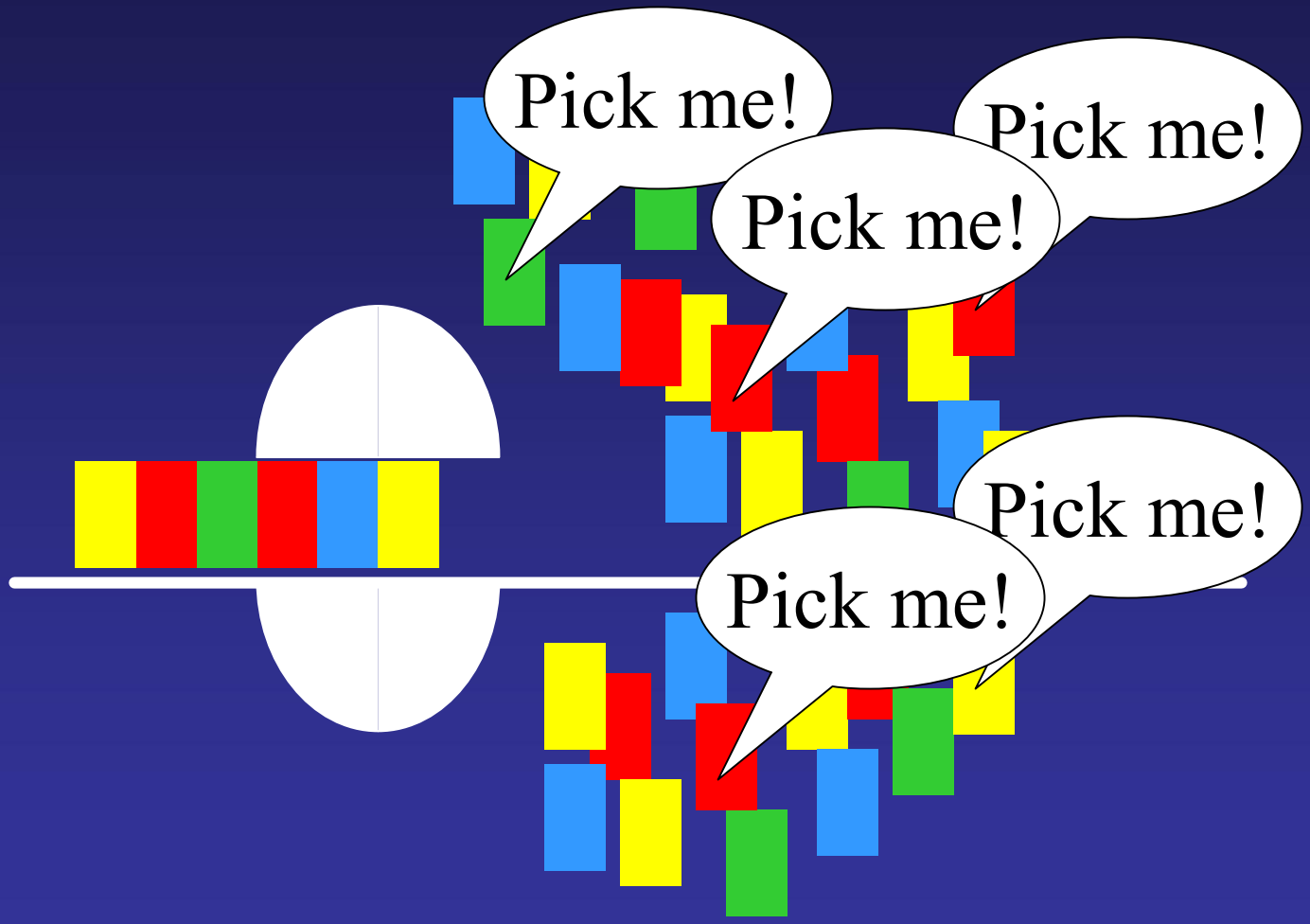
Deoxynucleoside Triphosphates (dNTPs)

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



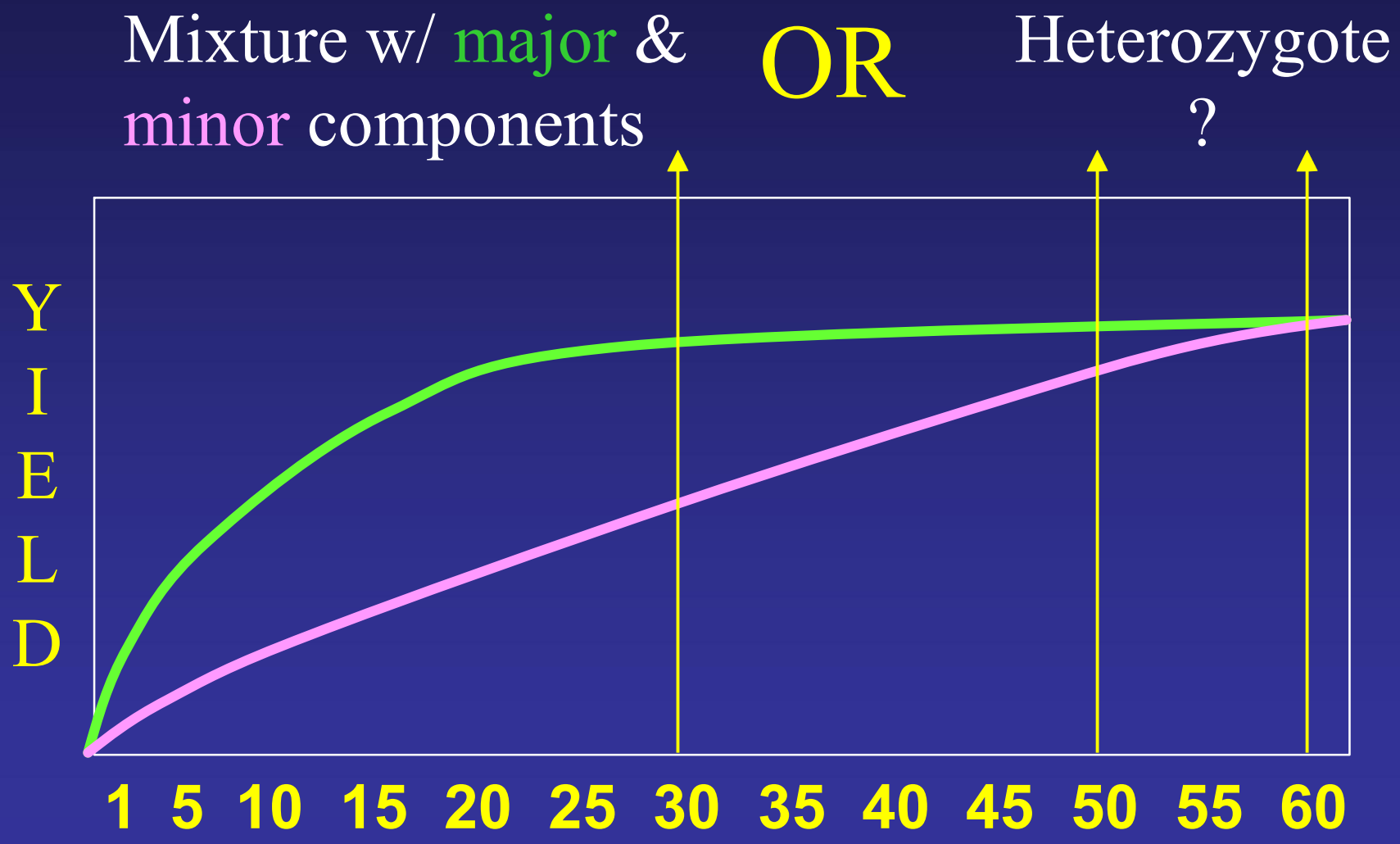
CYCLE NUMBER

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

Depends on target DNA quantity

Copy Number	Cycle Number
300,000	25-30
15,000	30-35
1,000	35-40
50	40-45

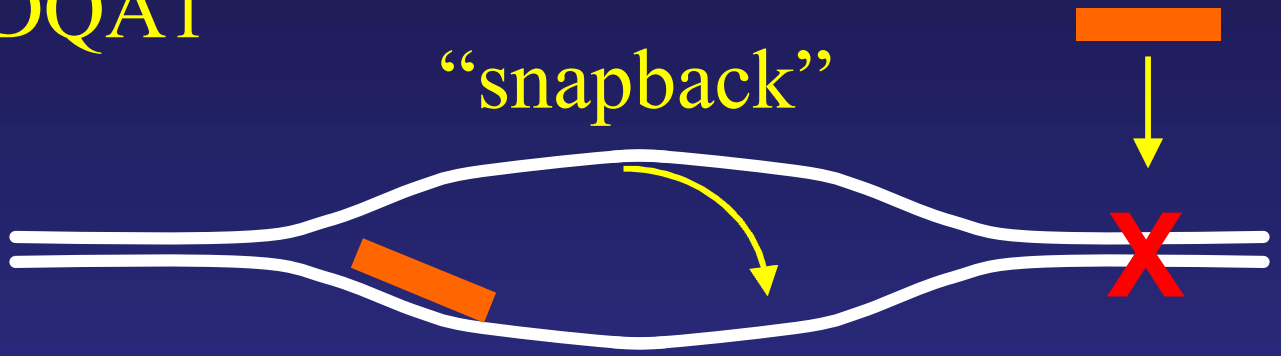
CYCLE NUMBER & PLATEAU



DENATURATION

If temperature is too low:

HLA-DQA1



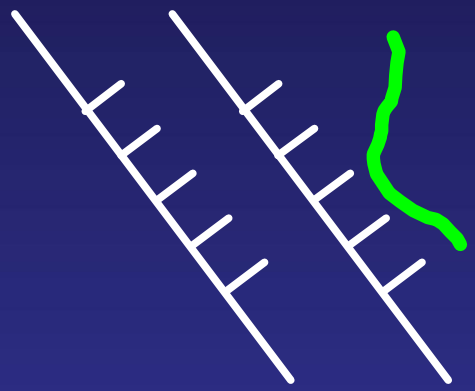
GC-rich regions may fail to denature.
Primers may not bind.

ANNEALING TEMPERATURE

Too Low

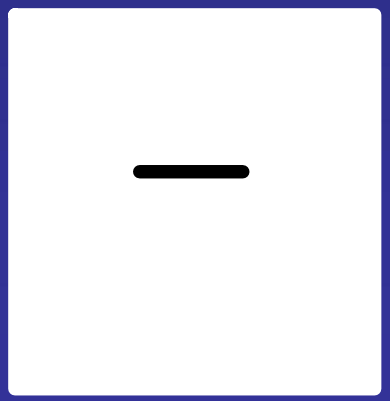
Just Right

Too High

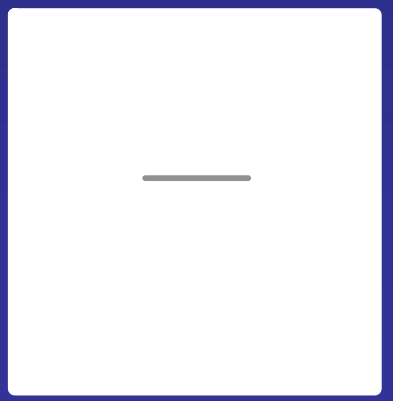


Mispriming

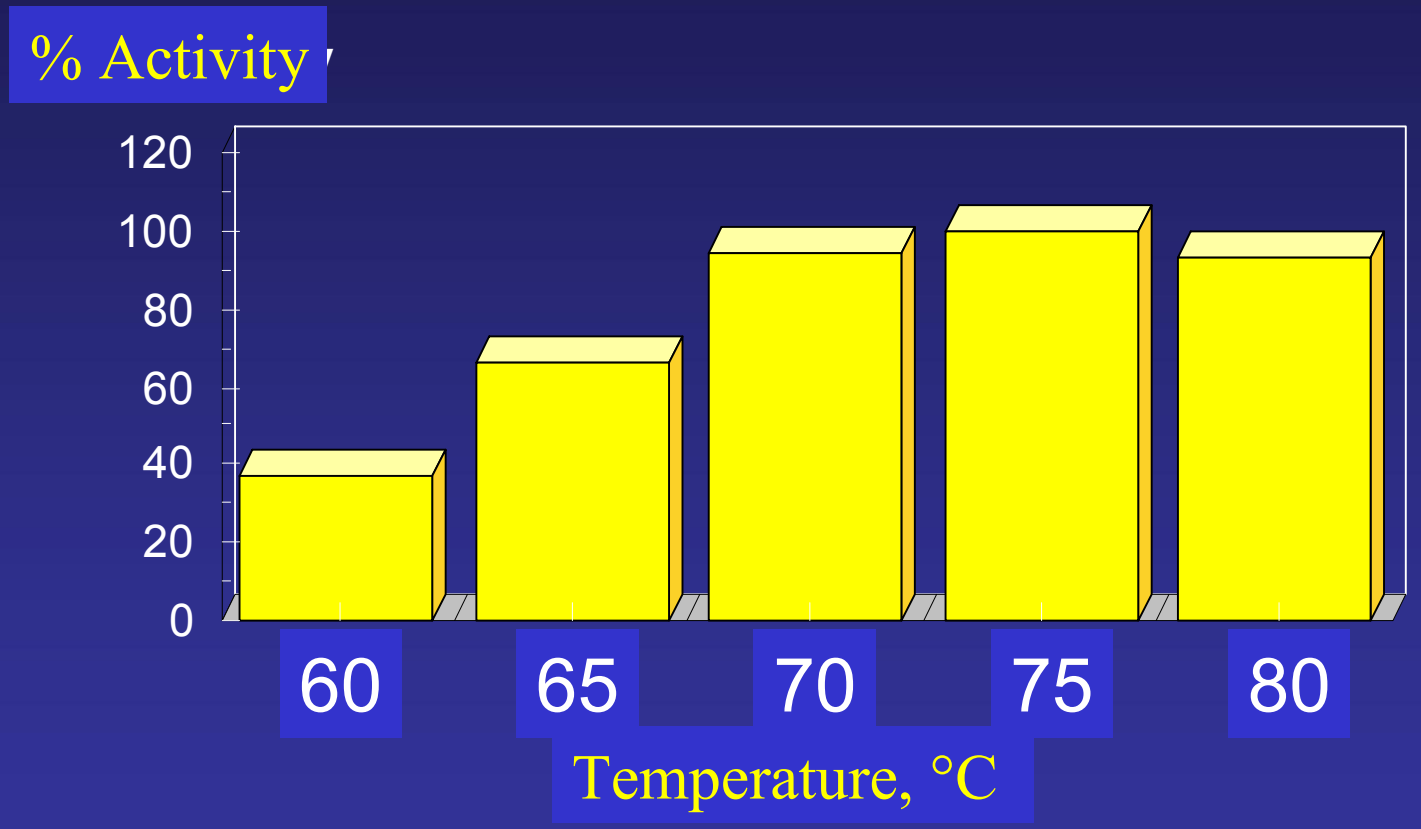
Good Specificity



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

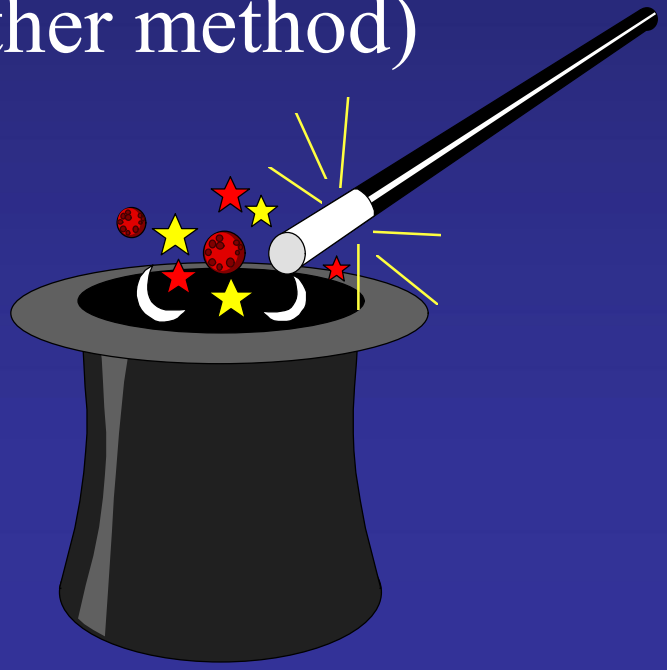
- However, typically in forensic PCR, small regions of DNA that can be amplified from degraded samples are targeted.

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

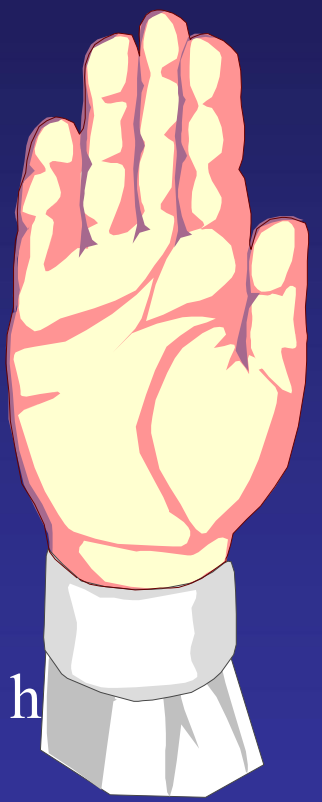
FORENSIC PCR
Multiplexing

- 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

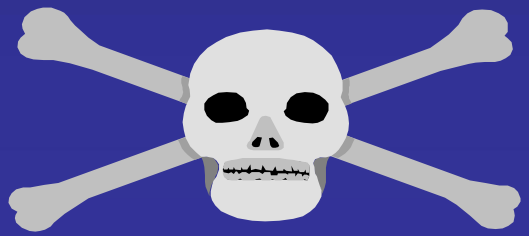
FORENSIC PCR Contamination

- 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 h
    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

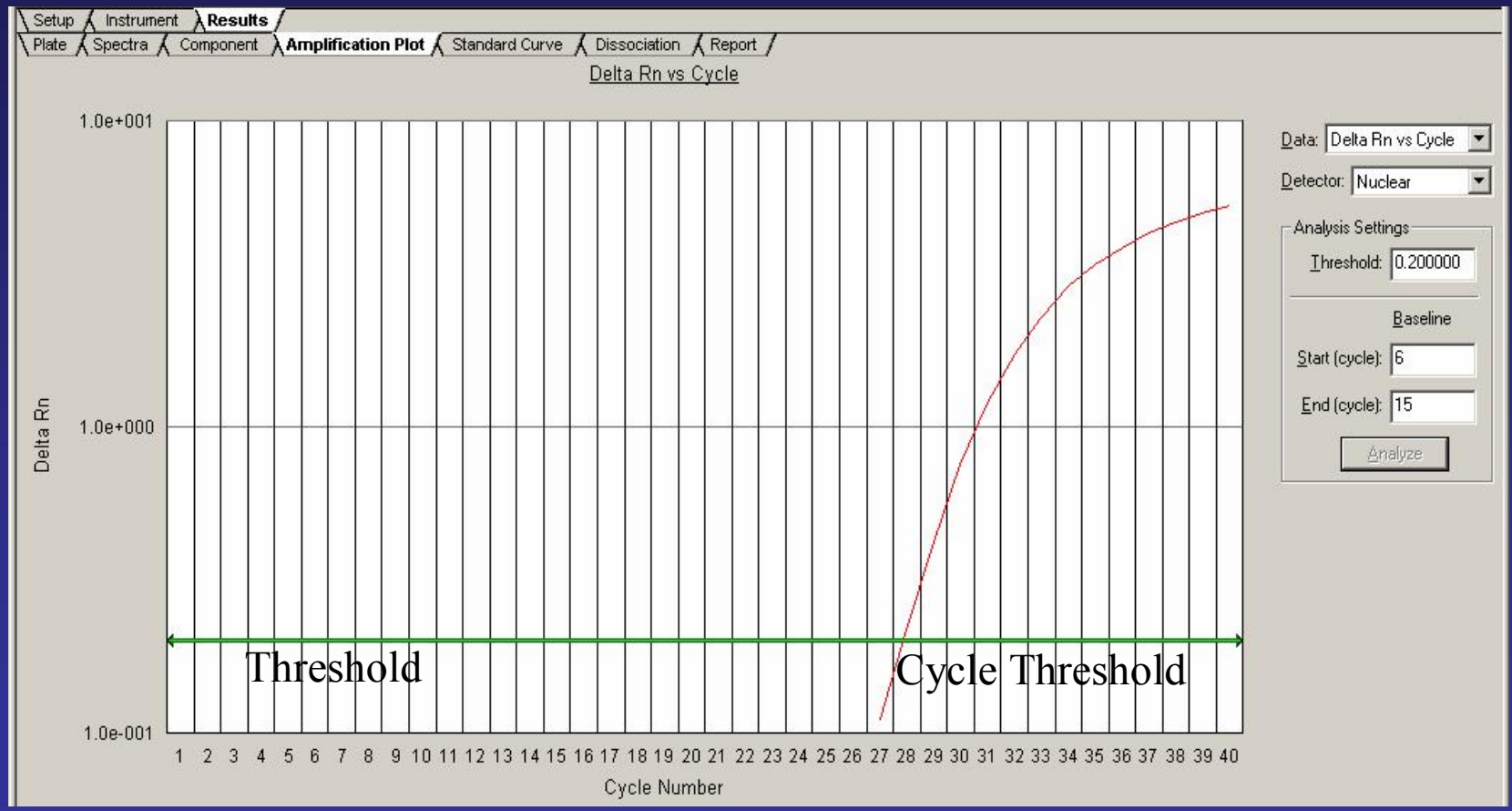
Simultaneous amplification and detection in the same well

Real Time Definitions

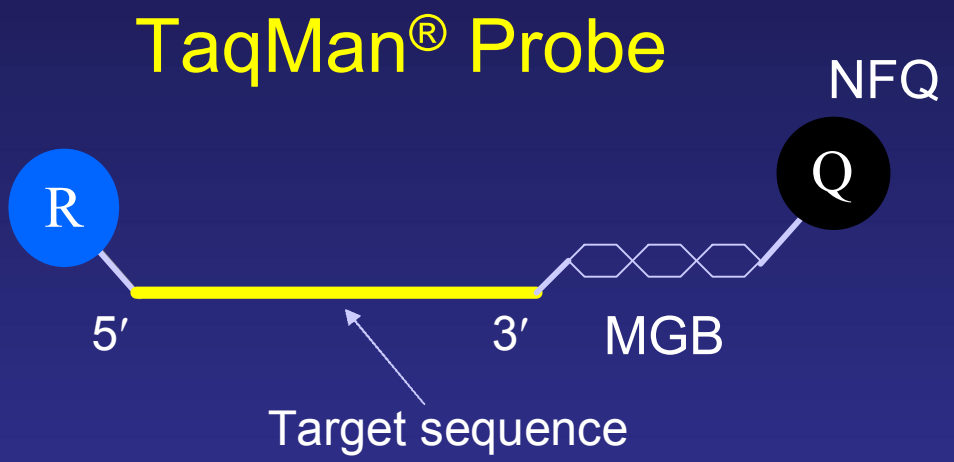
Threshold – The level of detection or the point at which a reaction reaches a fluorescent signal above baseline (7000 and 7900HT SDS default settings are 0.2)

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

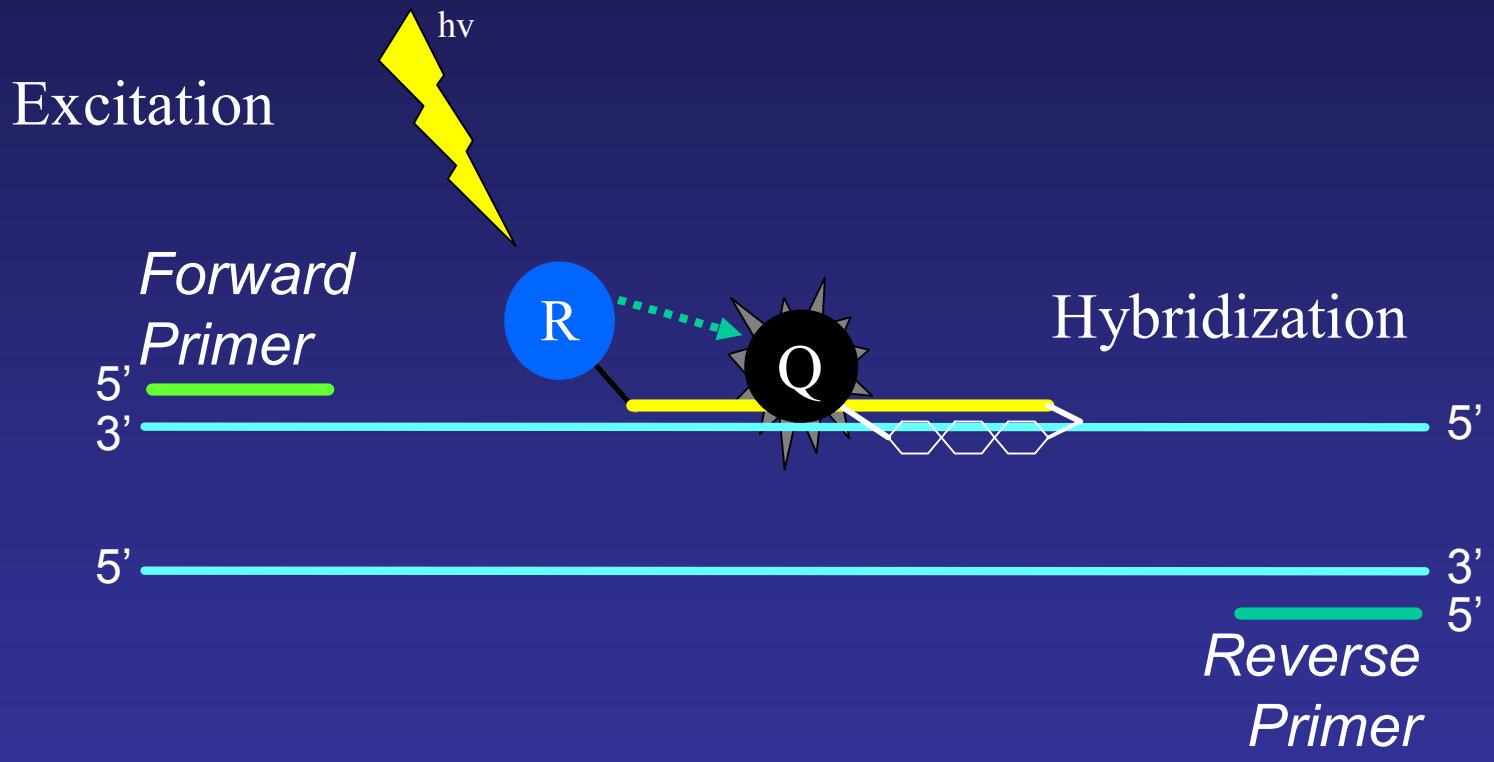


R = Reporter (FAM™ or VIC® Dyes)

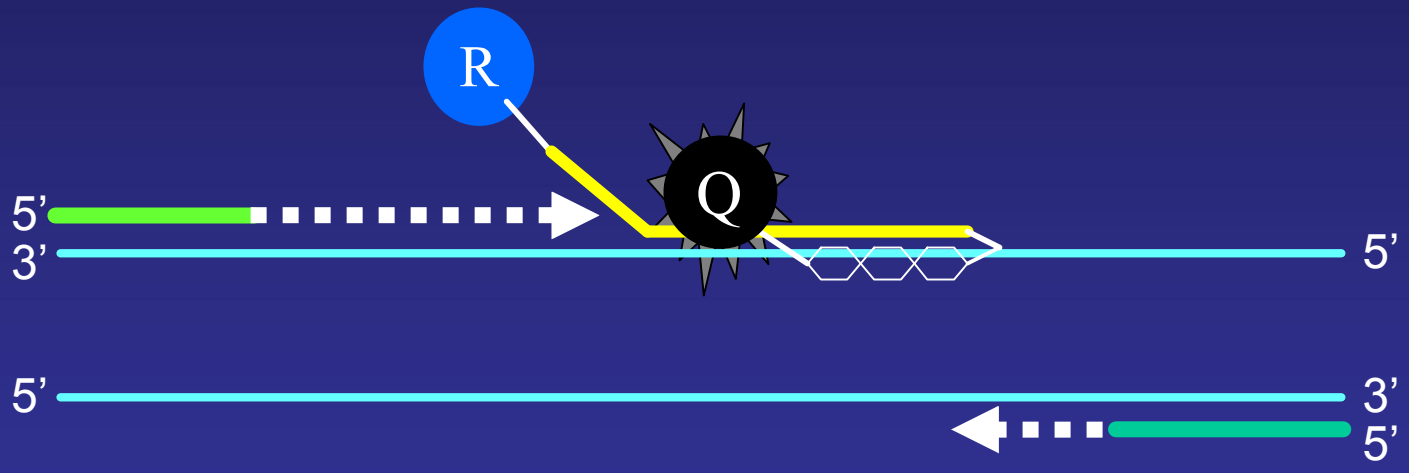
Q = Non Fluorescent Quencher (NFQ)

Acts as energy transfer acceptor that does not emit a detectable fluorescent signal

5′ Nuclease Assay

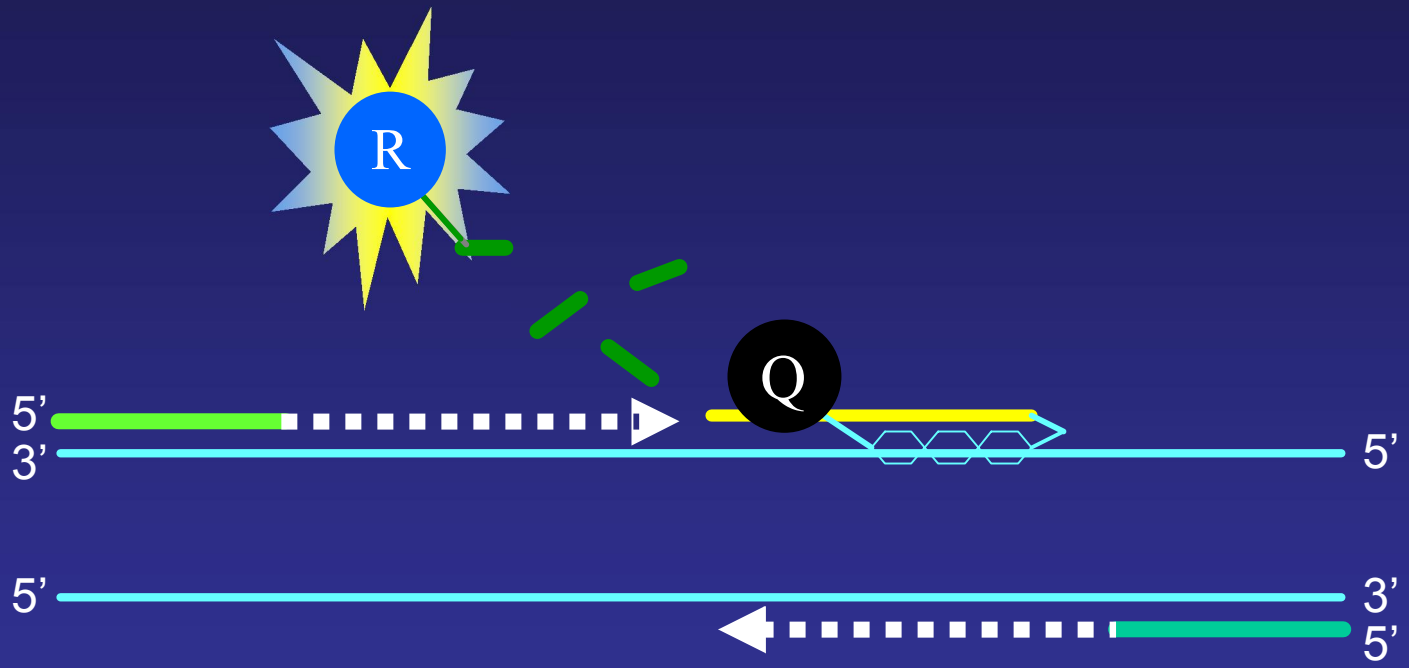


5′ Nuclease Assay



Displacement

5′ Nuclease Assay



Cleavage

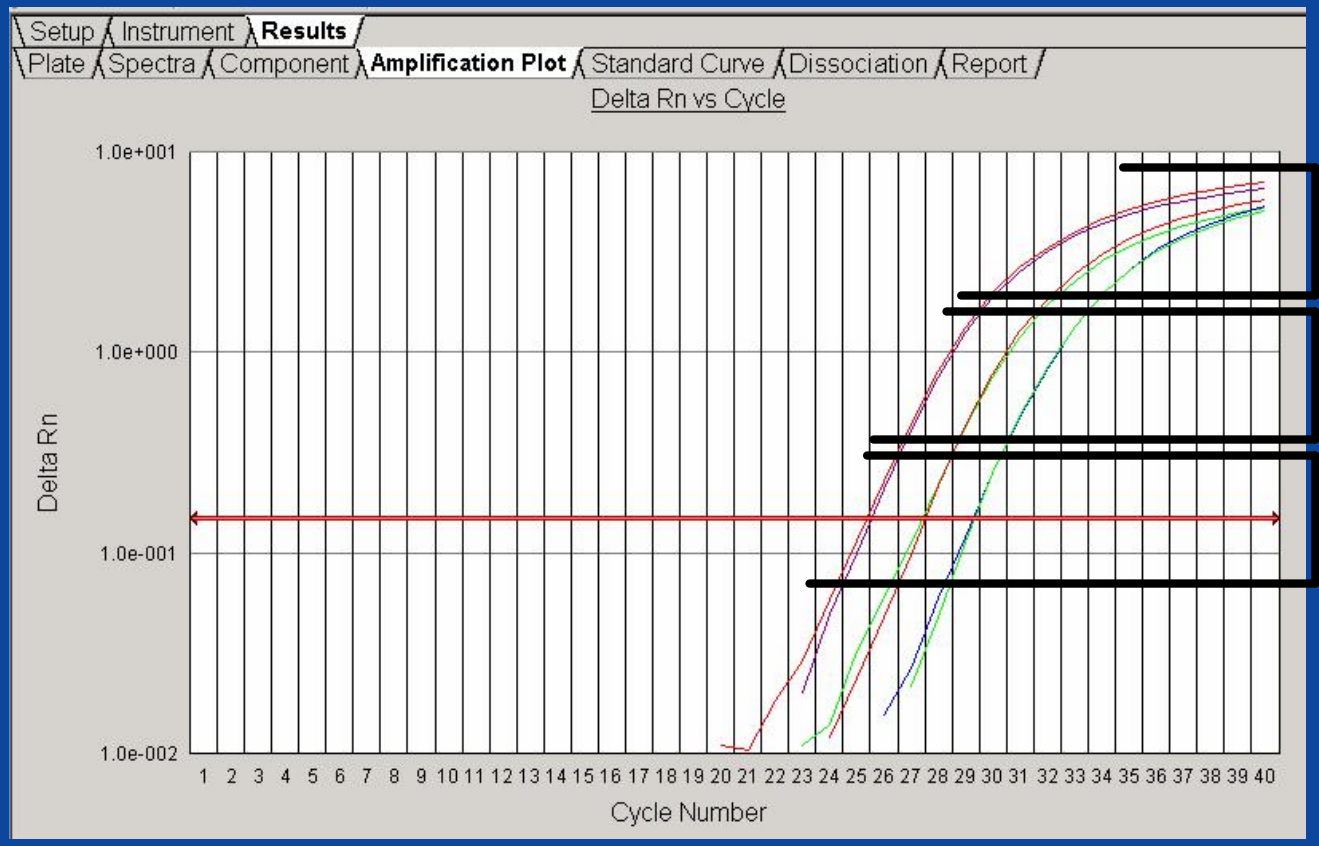
5′ Nuclease Assay

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]

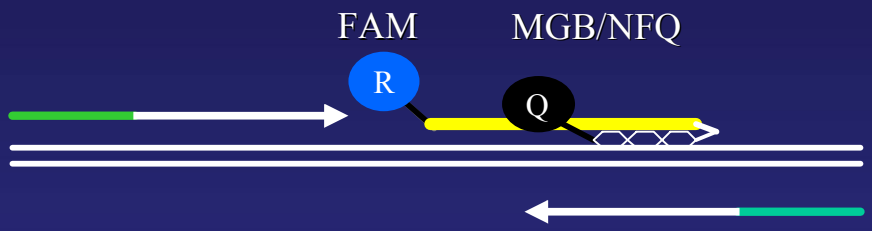


Phase 3, Plateau

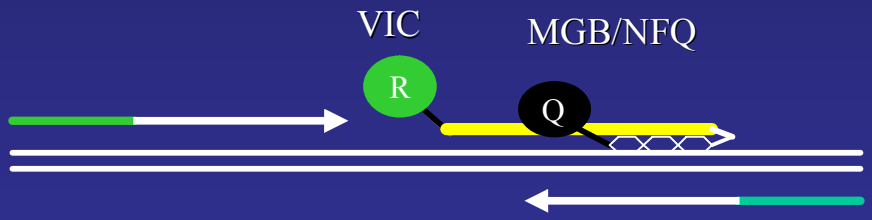
Phase 2, Linear

Phase 1, Geometric

Quantifiler Primer Mix: Pre-Formulated as A Duplex Assay



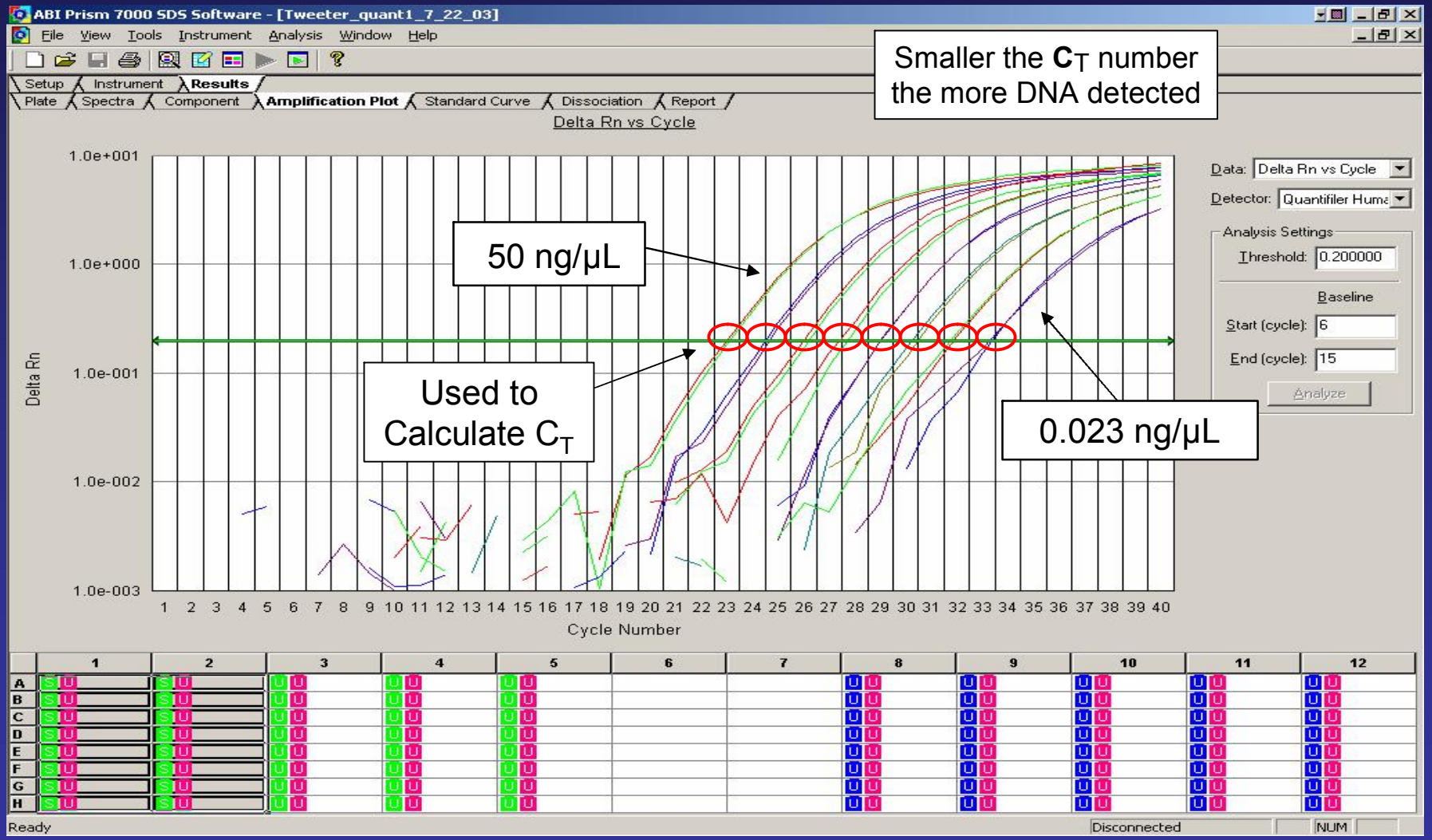
Target Probe to
Autosomal or Y-Chromosome
Specific Region



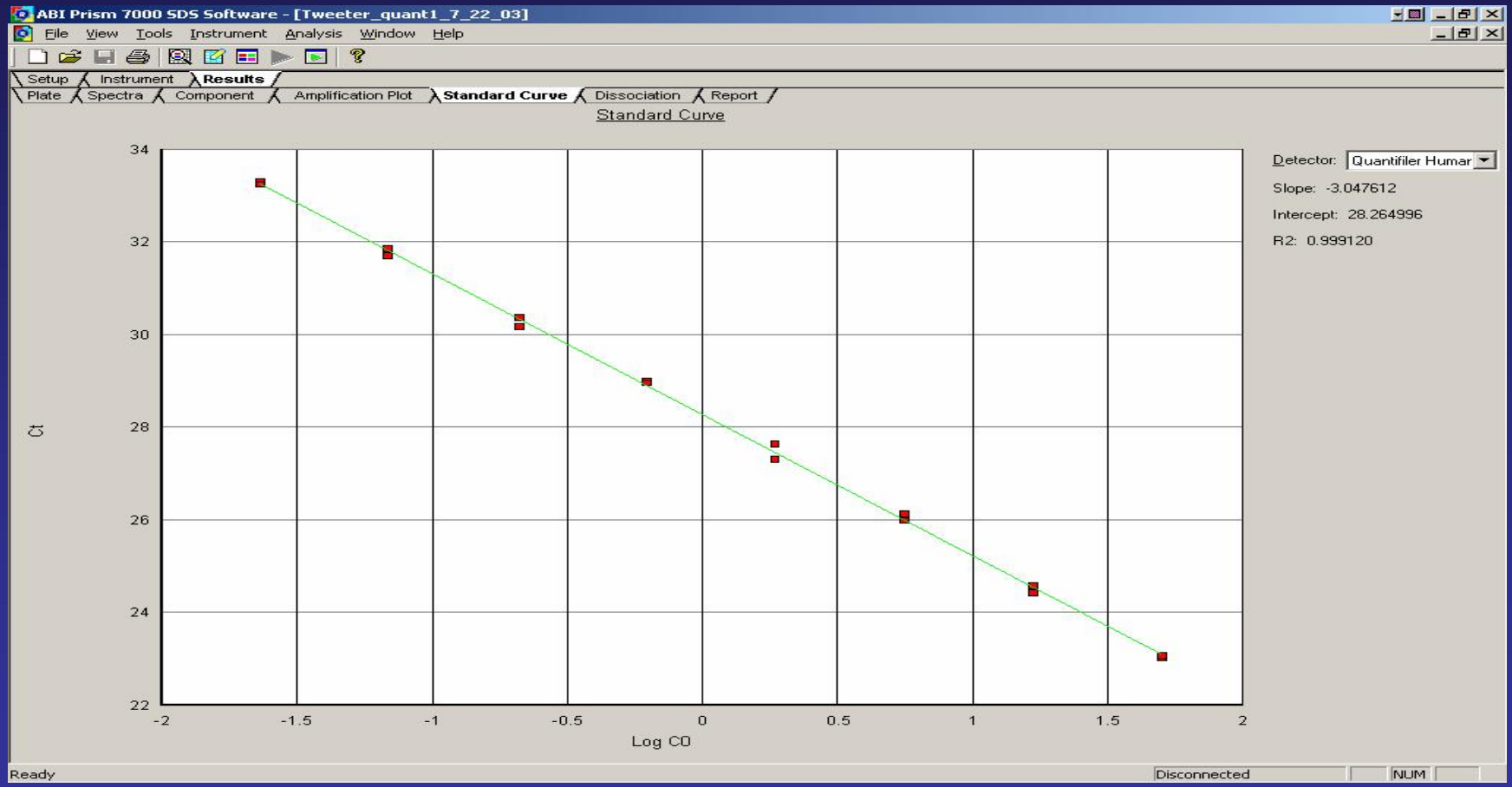
Internal PCR Control

Amplification Plots for DNA Concentration Standards

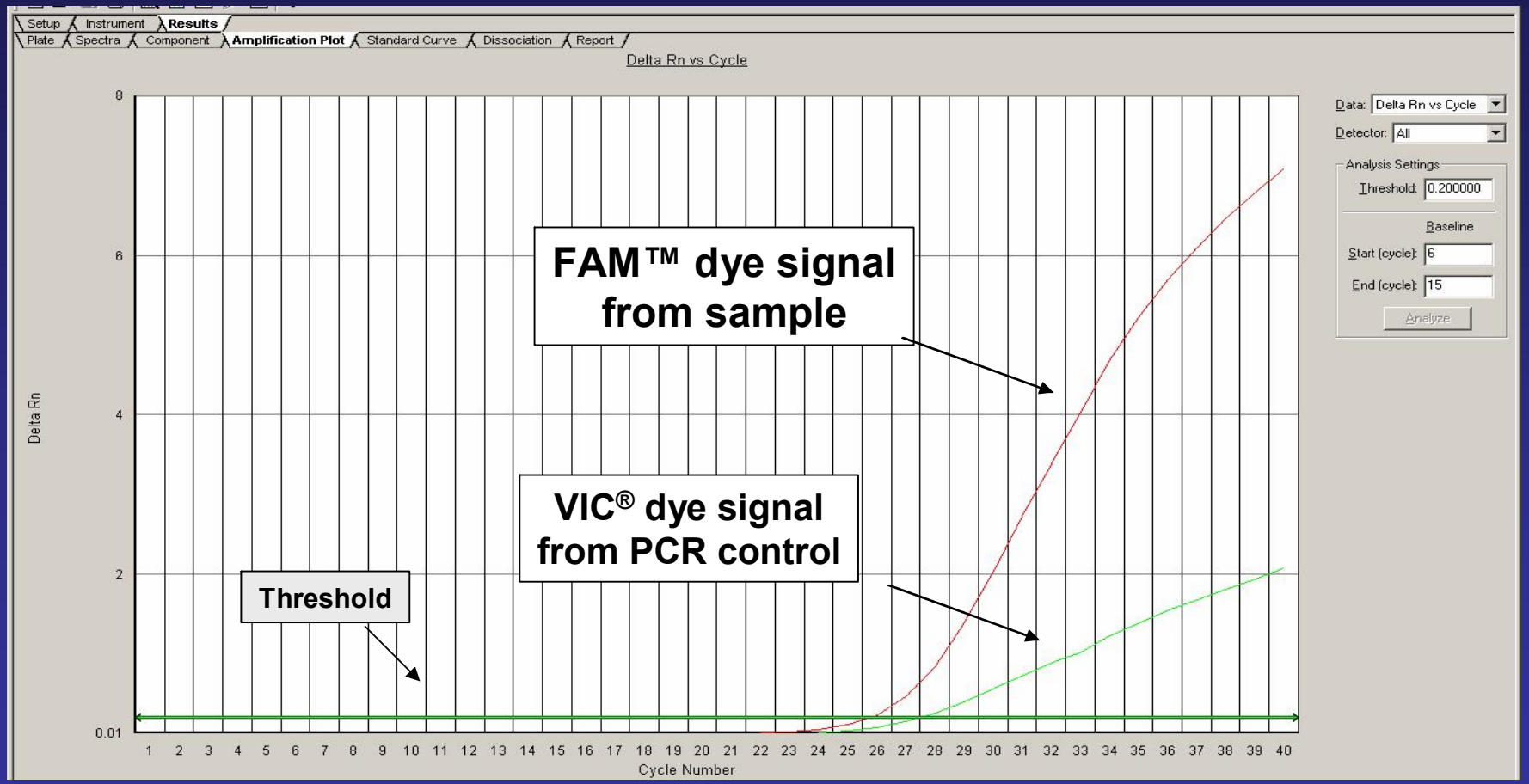
Eight 3-fold serial dilutions



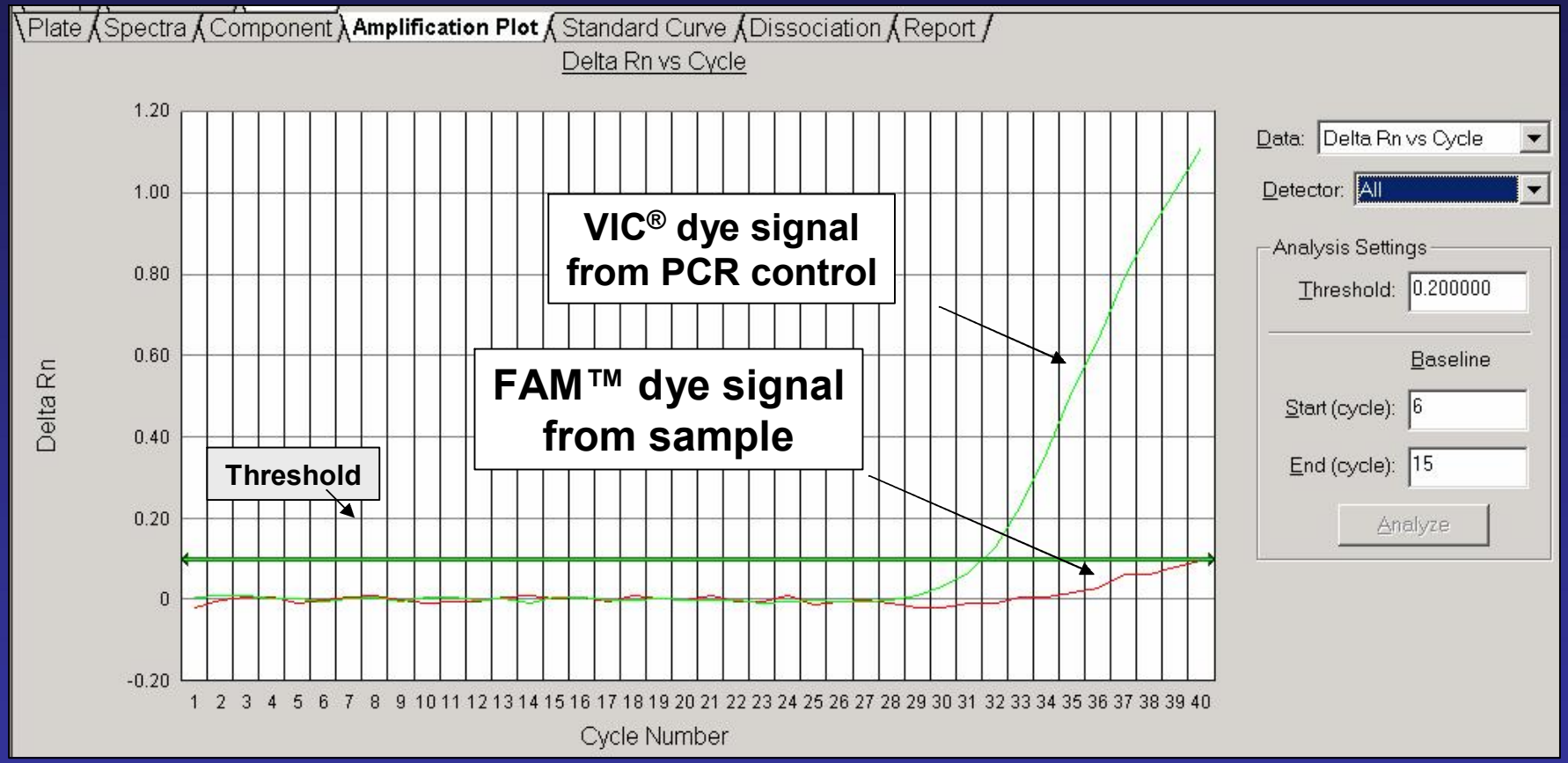
Standard Curve from DNA Concentration Standards



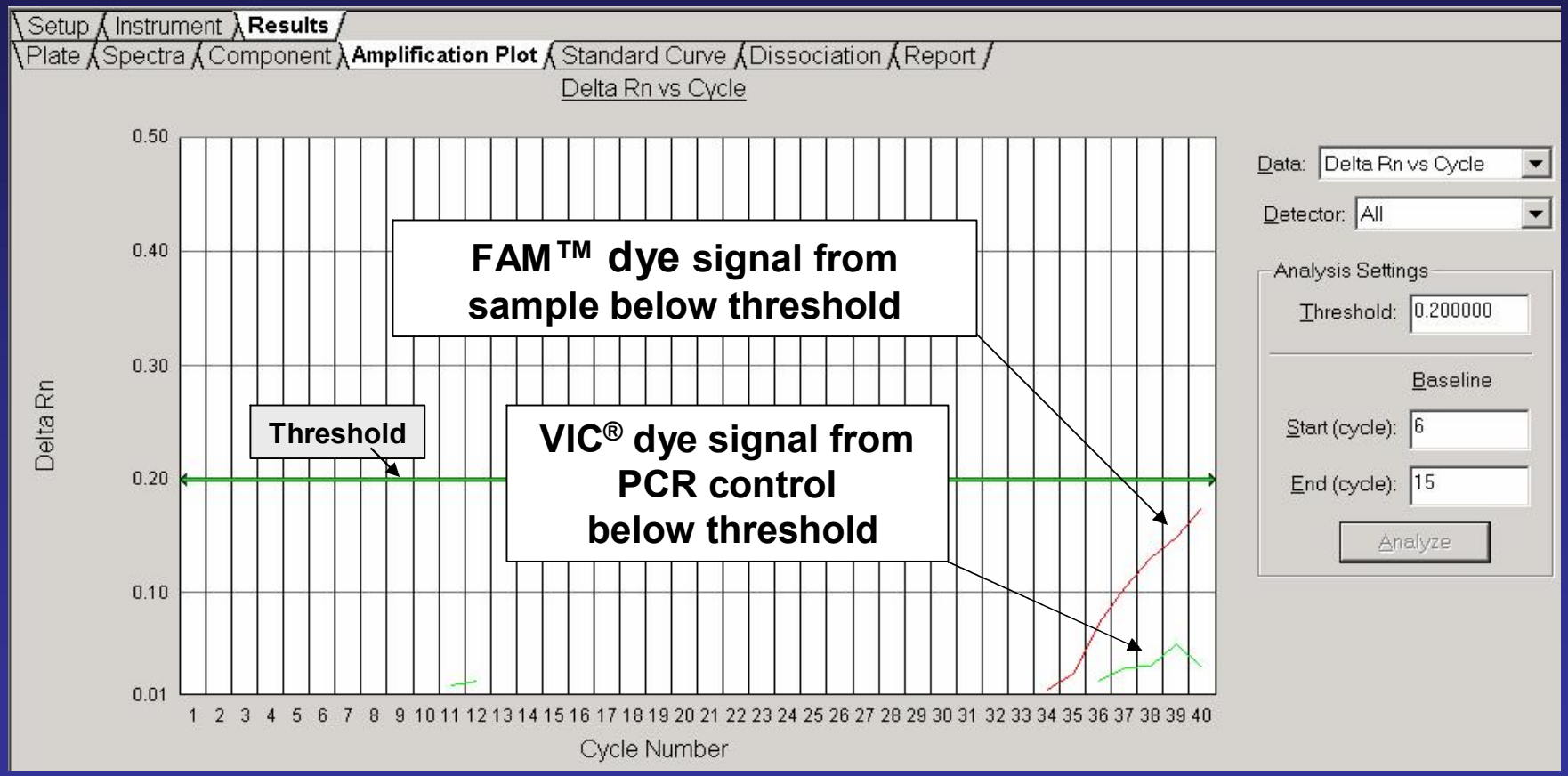
Example of sample positive for DNA



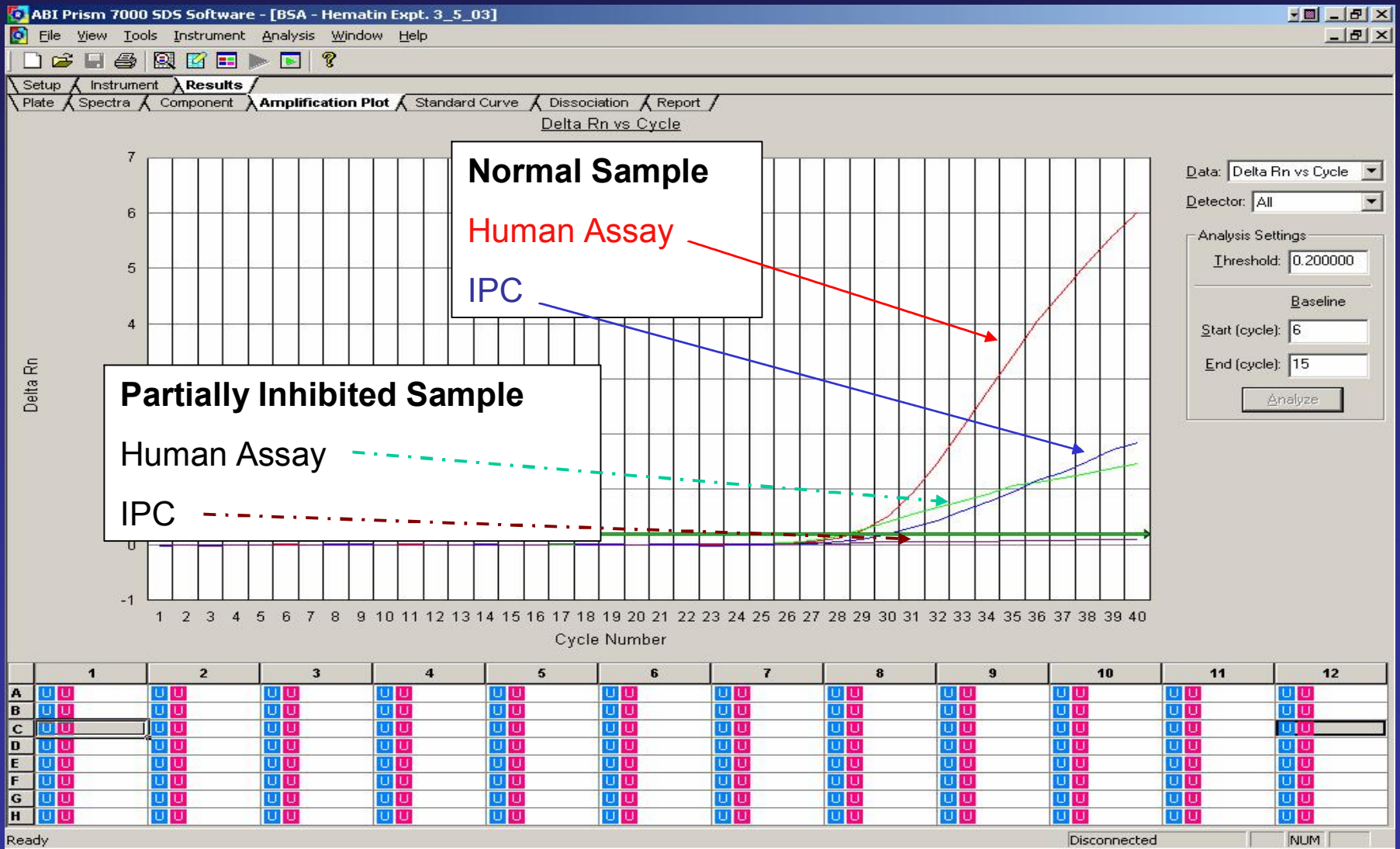
Example of sample with no detectable DNA



Example of sample with inhibition



Example of sample with partial inhibition



Application

- Quantitation
- SNP detection