

# Real Time PCR

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# Real-Time PCR

- ▶ Theory
- ▶ Chemistry
  - ▶ Non-specific DNA binding dye
  - ▶ Specific Hybridization Probe
    - ▶ Dual-labeled FRET Pairs
  - ▶ Advantages & Disadvantages

# The Applied Biosystems Real Time PCR



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# Smart Cycler by Cepheid



# iCycler iQ™ - Real Time PCR Detection System

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# Real-Time PCR

Through the use of fluorescent molecules, real-time PCR has the ability to directly measure the reaction while amplification is taking place.

# Real-Time PCR

- ▶ The fluorescent molecules can be
  - ▶ Non-specific DNA binding dyes
    - ▶ SYBR® Green I
    - ▶ Ethidium Bromide
  - ▶ Specific Hybridization Probes
    - ▶ Dual-labeled FRET pairs
      - ▶ TaqMan™
      - ▶ Pulsar™ LightCycler™
      - ▶ Molecular Beacons
      - ▶ Scorpions™
      - ▶ Amplifluor Direct™

# Cycles and number of copies

No of copies	Cycles	No of copies	Cycles	No of copies	Cycles
1	1	1024	11	1,048,576	*
2	2	2048	12	*	*
4	3	4096	13	*	30
8	4	8192	14	1,073,741,824	*
16	5	16384	15	*	*
32	6	32768	16	*	40
64	7	65536	17	1,099,511,627,776	
128	8	131072	18		
256	9	262144	19		
512	10	524288	20		
1024		1,048,576			

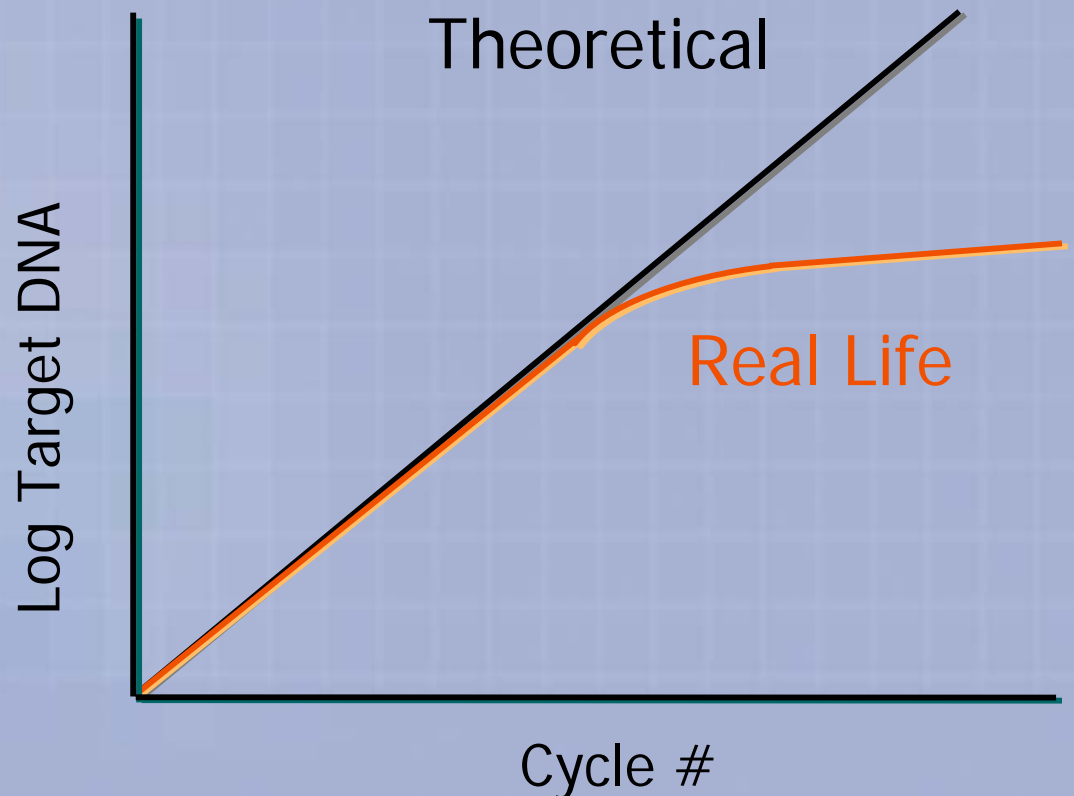
# Reality vs. Theory

Amplification is exponential, but the exponential increase is limited:

- A linear increase follows exponential
- Eventually plateaus

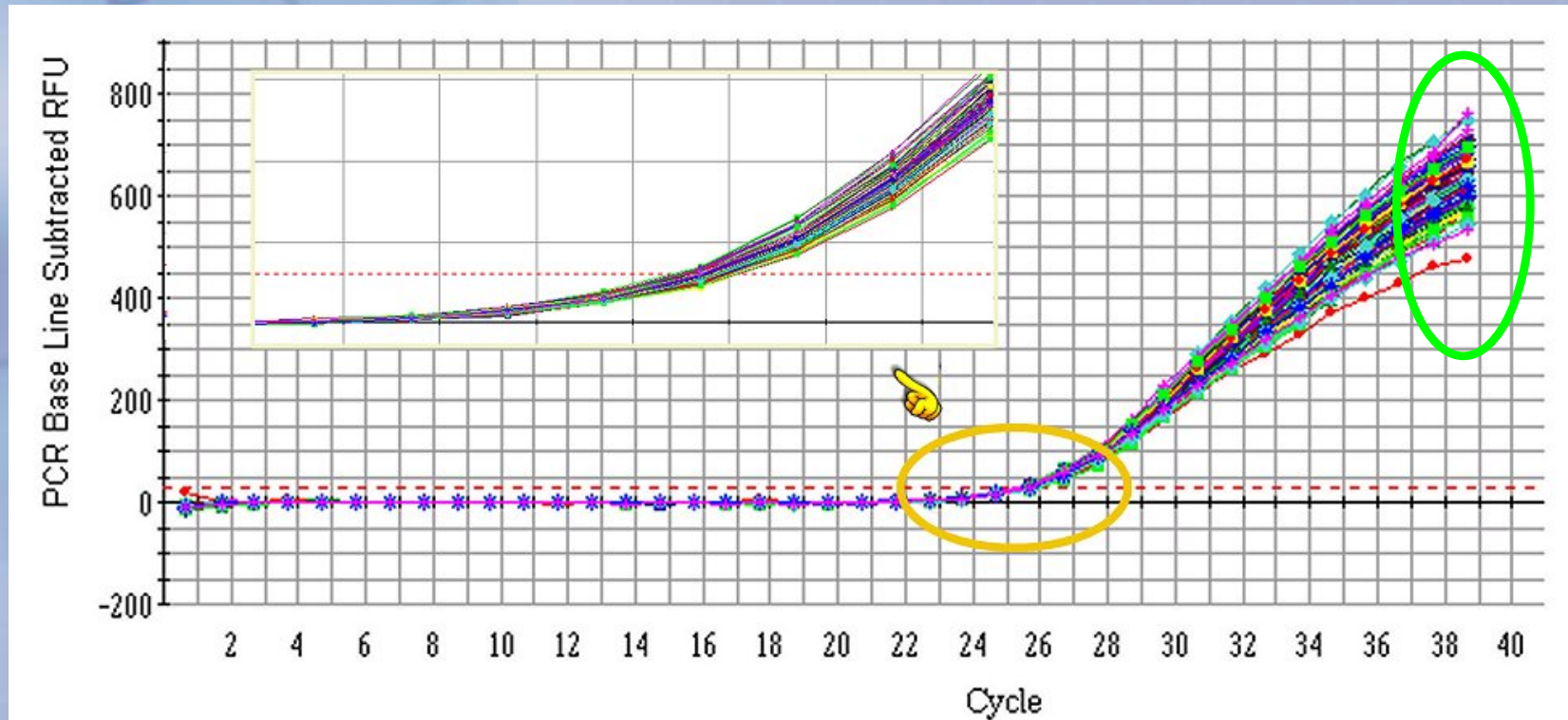
Real-Time PCR allows us to 'see' the exponential phase so we can figure out how much we started with.

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# End Point Measurements

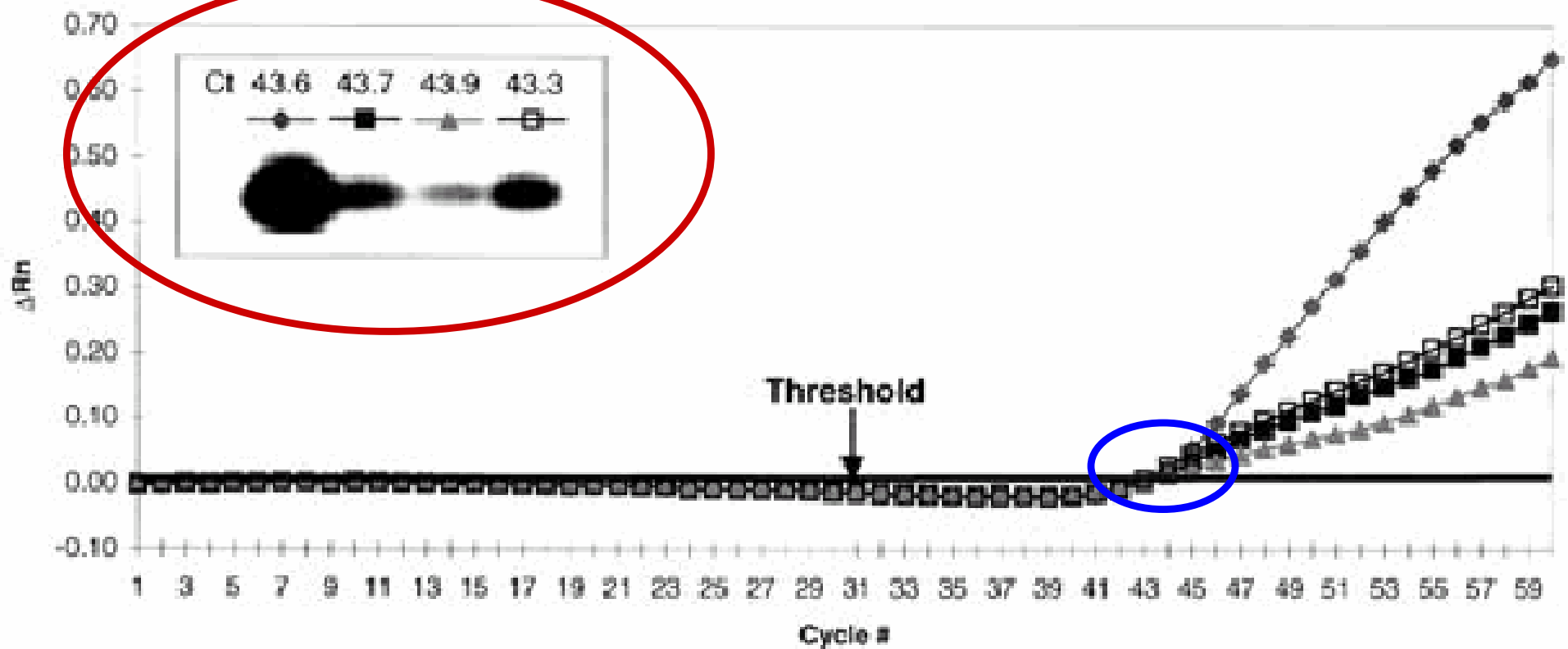
96 replicates of the **identical** reaction have very different final amounts of fluorescence



# End-point vs. Real-Time results

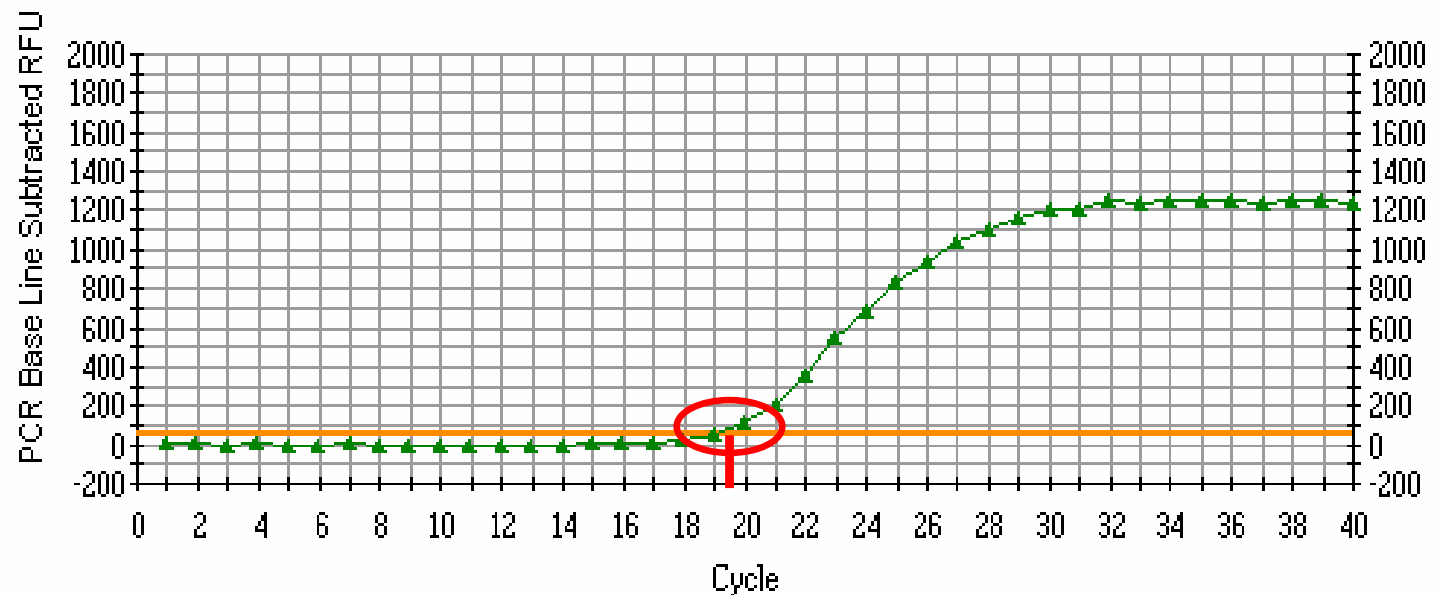
Lockey *et al.* (1998) *Biotechniques* 24:744-6

Panel C: Amplification Plots and Hybridization Results of Ten-Copy Replicates



# Threshold Cycle ( $C_t$ )

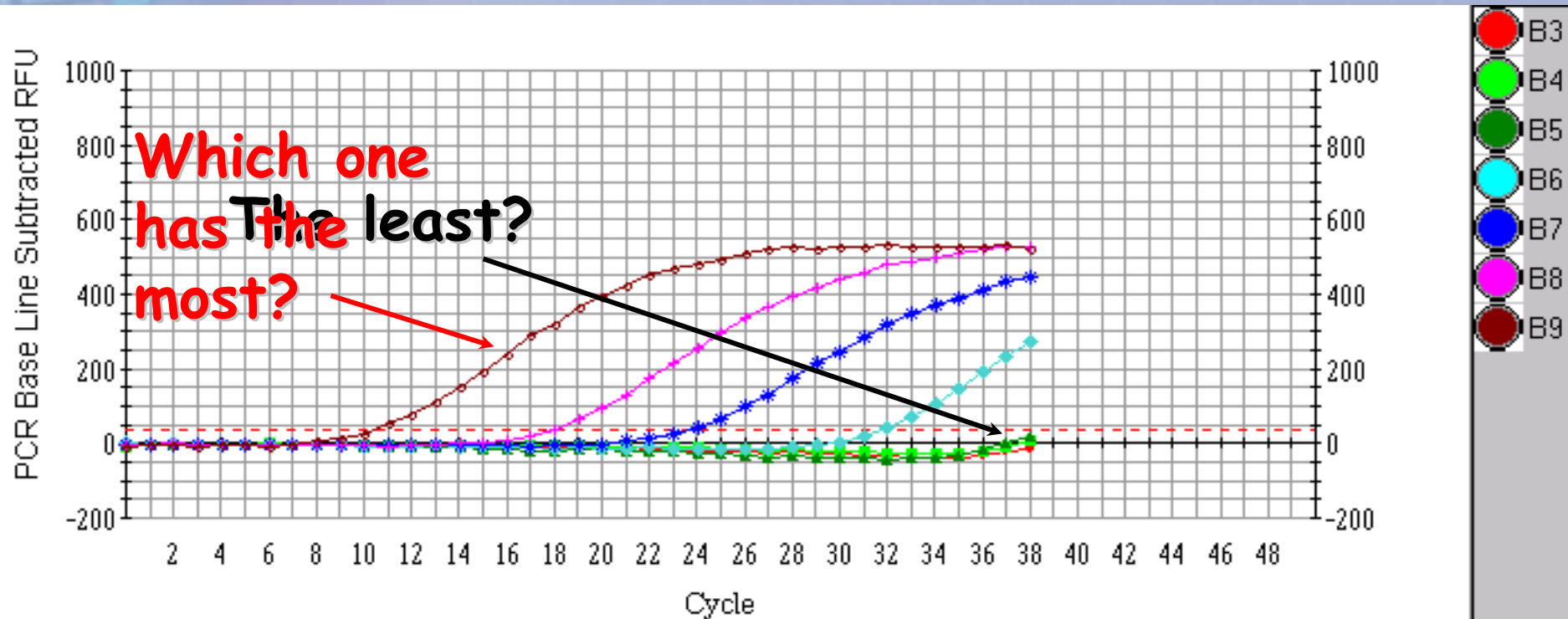
The point at which the fluorescence rises appreciably above background



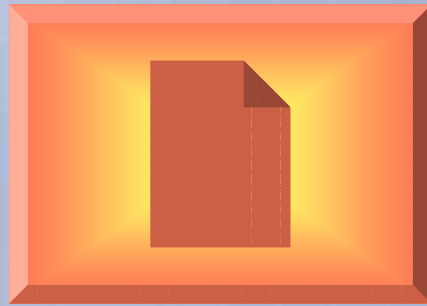
PCR Amplification vs Cycle: C:\My Documents\customer's opds\jkb1-26-01b.opd

# Threshold Cycle, $C_t$

- ▶ Correlates strongly with the starting copy number
  - ▶ If you have high [DNA] you get to  $C_t$  cycle earlier
  - ▶ If you have low [DNA] you reach  $C_t$  cycle later
- ▶ Is linear with the log of starting copy number over 6 or more orders

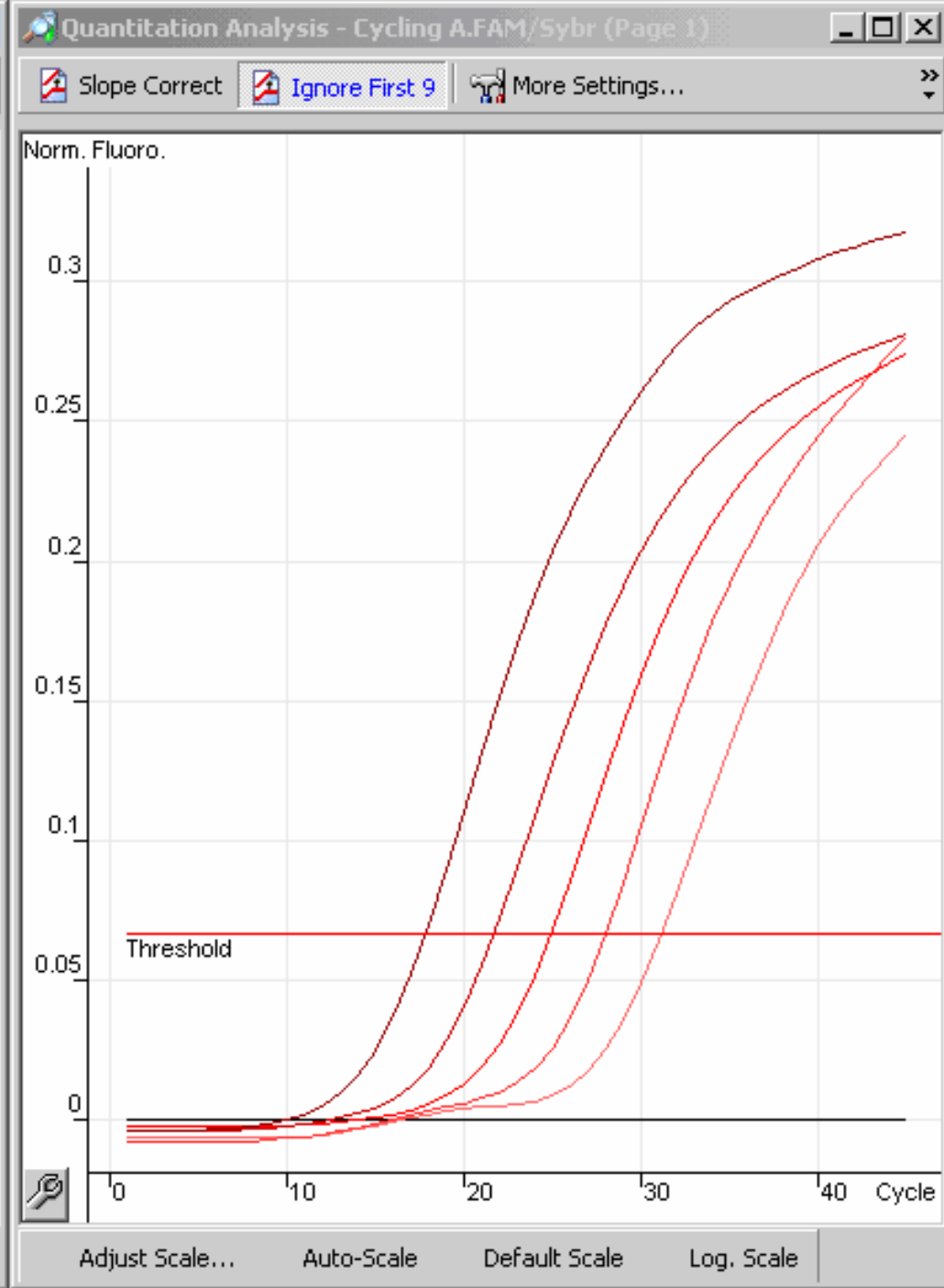
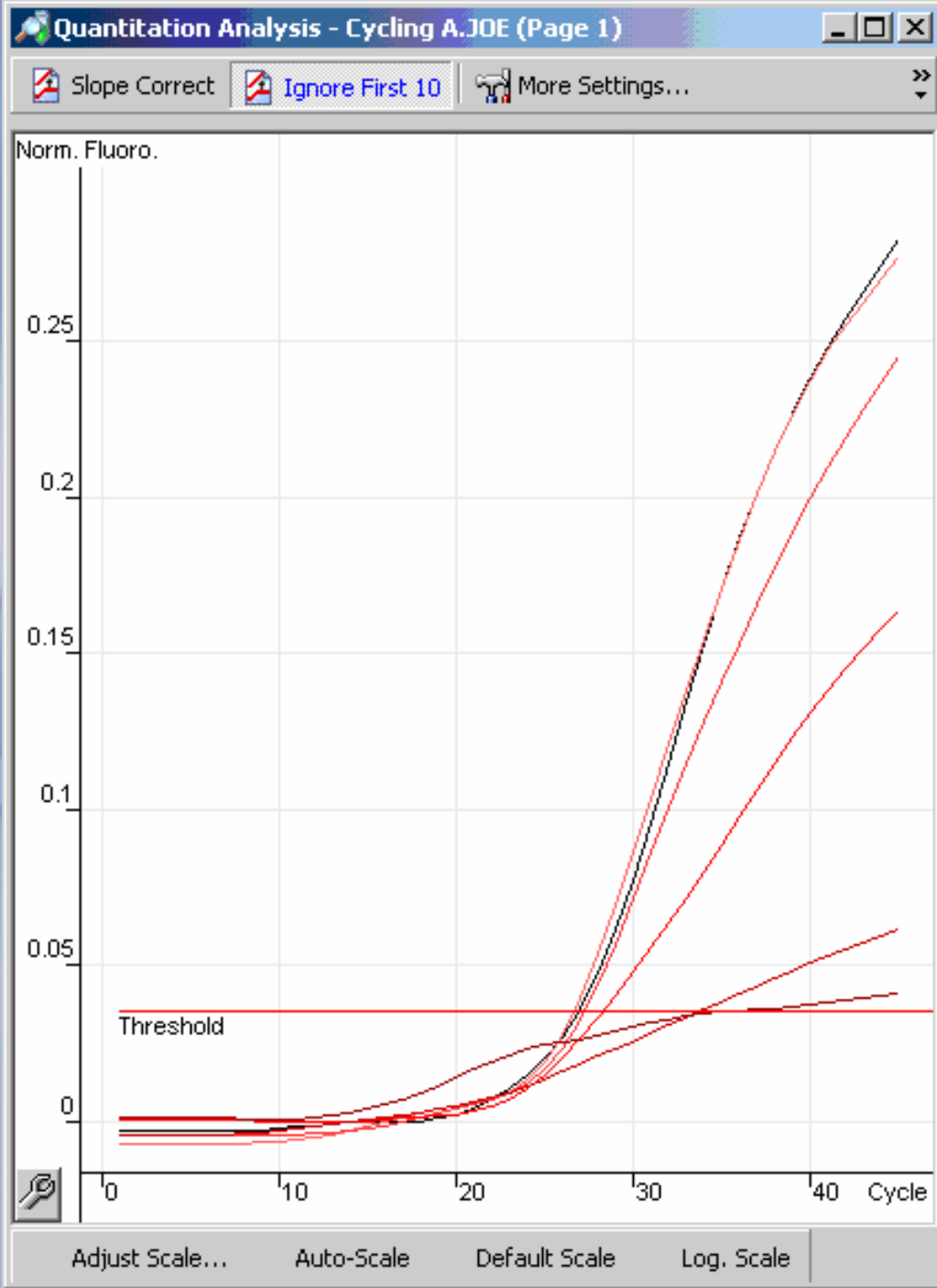


# HCV Real Time PCR Quantification



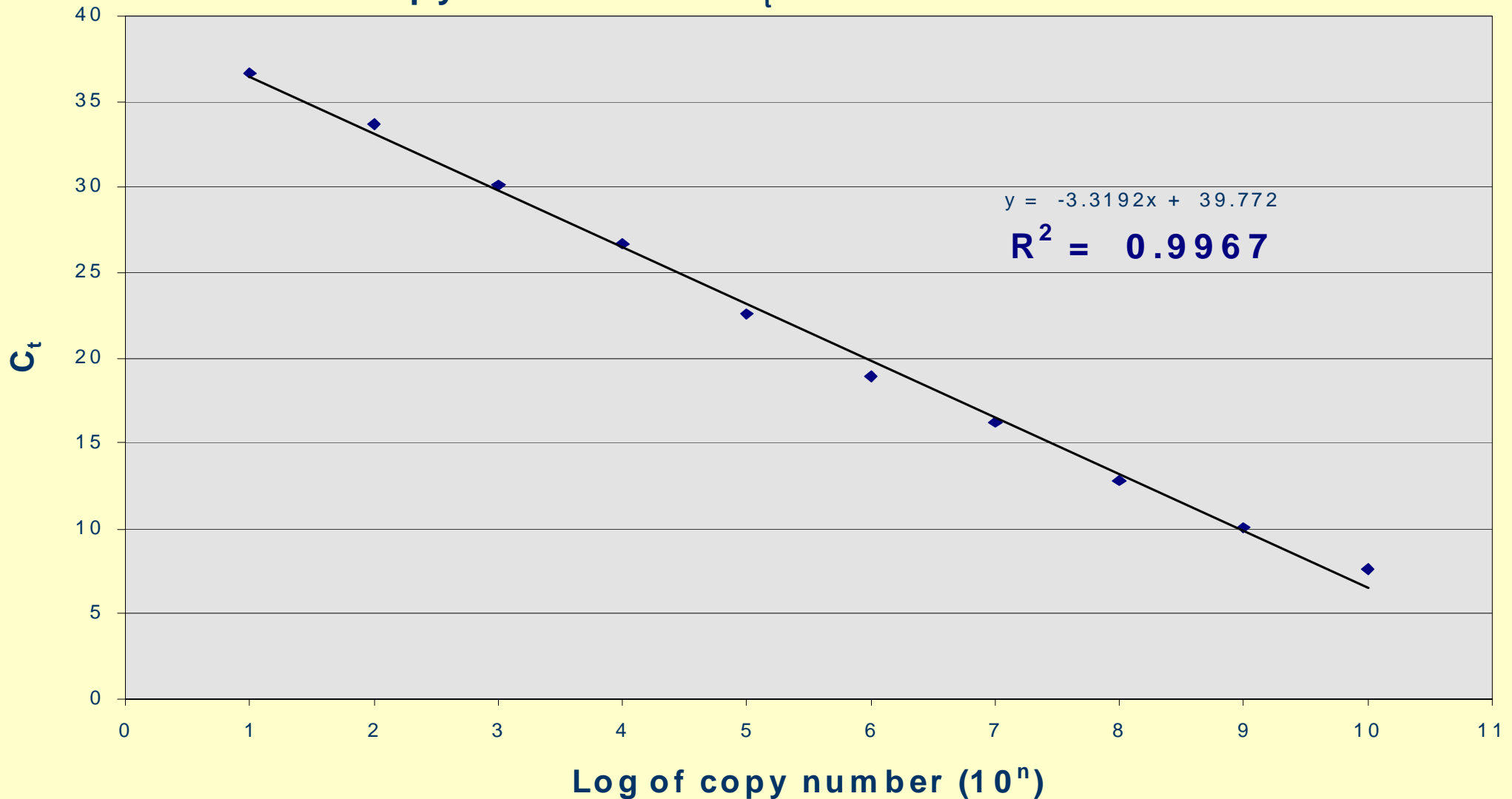
HCV Standards

# HBV Real Time PCR Quantification

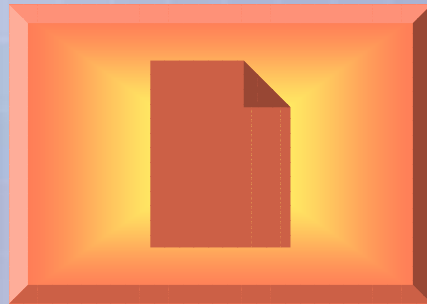


# Threshold Cycle, $C_t$ vs initial copy number

Copy Number vs.  $C_t$  - Standard Curve

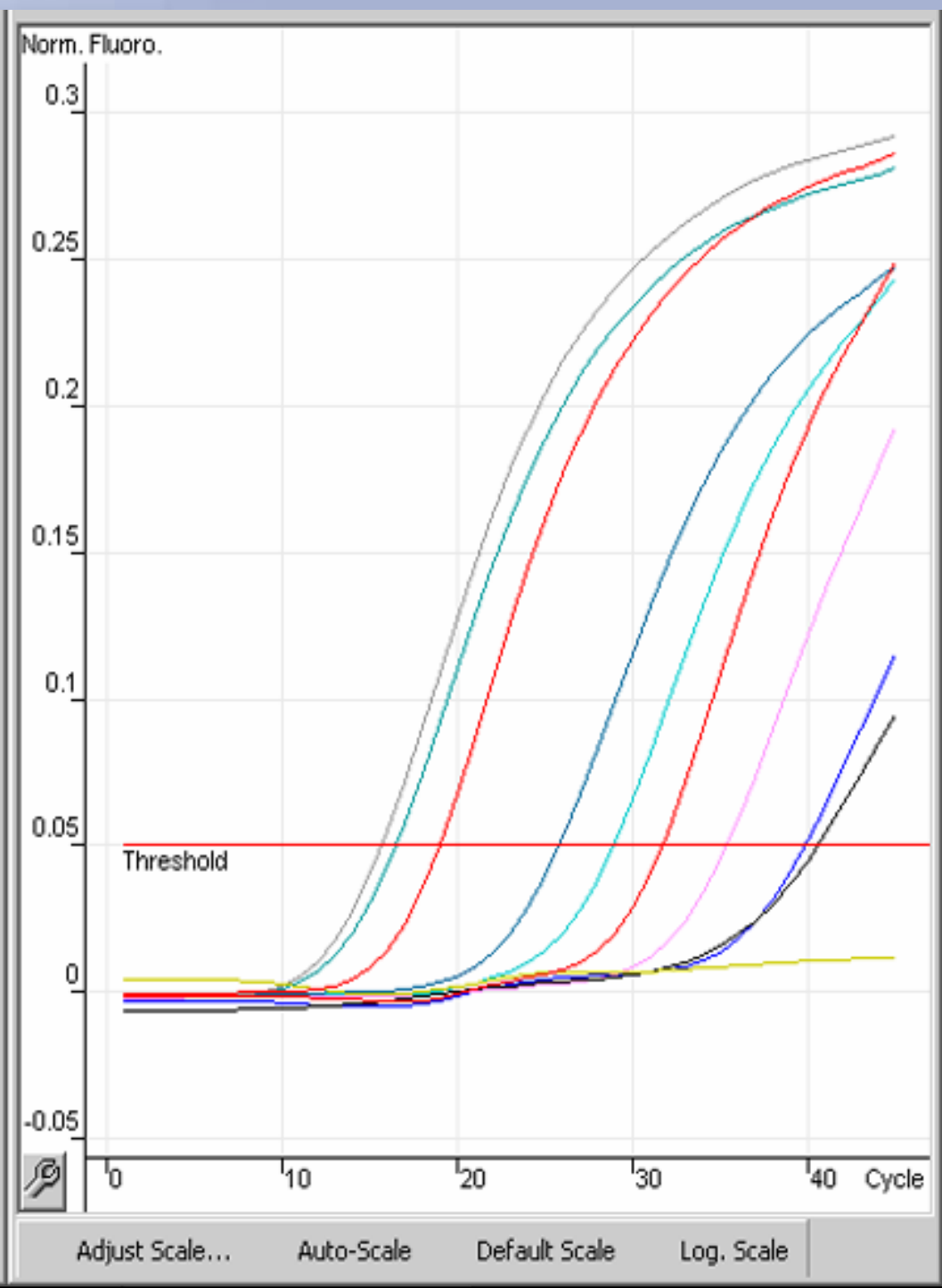
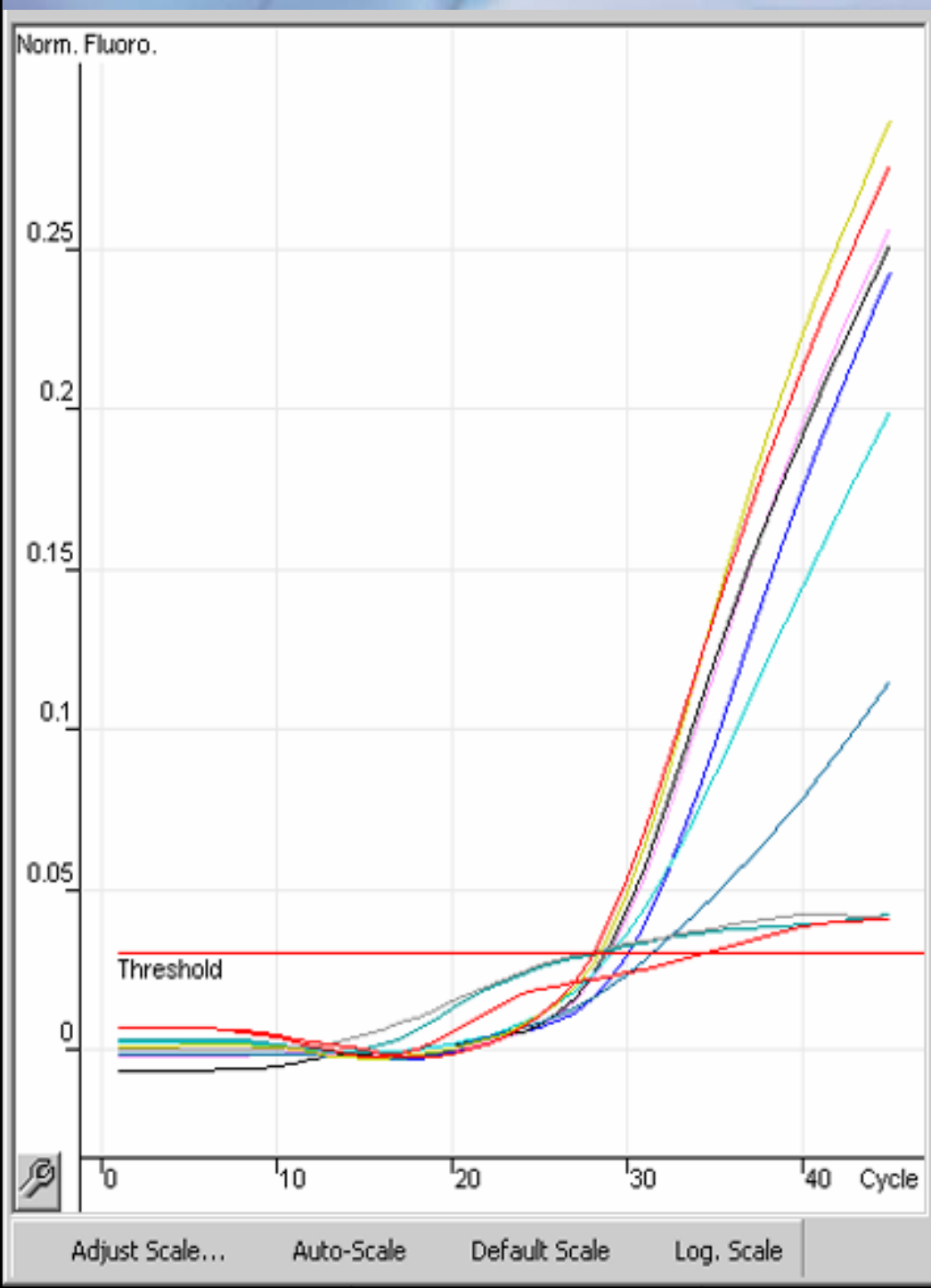


# HCV Real Time PCR Quantification



HCV samples & standard curve

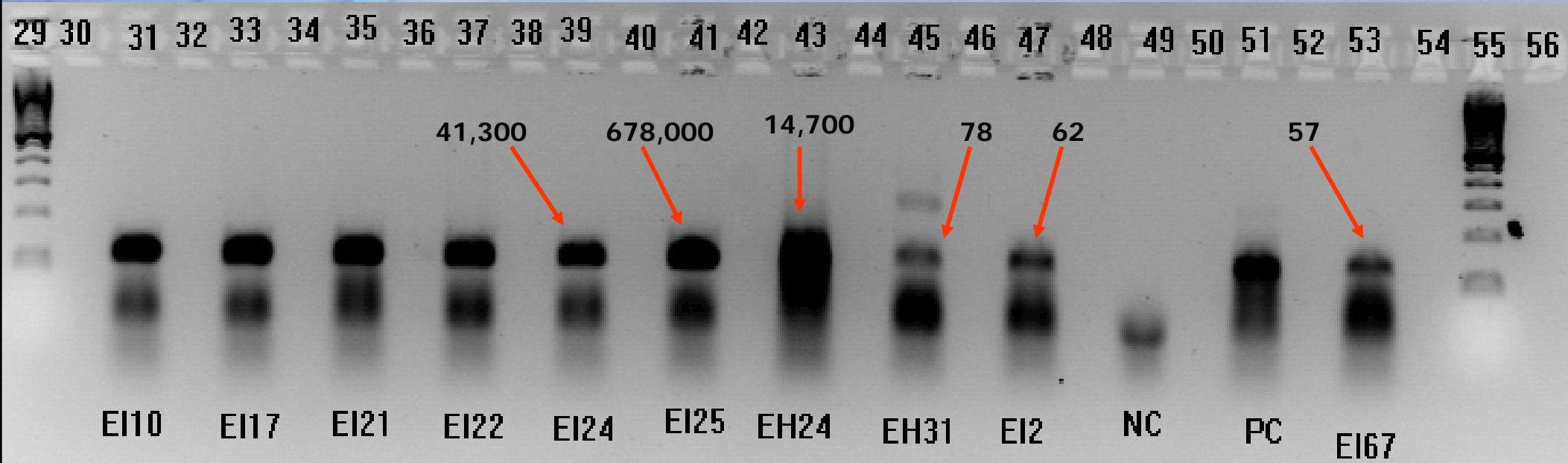
# Borderline Samples



# Results of Retesting of Borderline Samples

- ▶ 69 borderline samples
- ▶ 27 (39%) became positive upon retesting
- ▶ 27 (39%) became negative upon retesting
- ▶ 6 (9%) remained borderline upon retesting
- ▶ 9 (13%) did not come back for retesting

# Real Time PCR run on Agarose gel



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# What is Real Time PCR?

- **Monitoring of an amplification reaction in progress.**
- **HOW?**
- **By tracking the amplification via an increase in fluorescent light**

# Advantages of Real Time PCR

Data evaluated rapidly—**INCREASING** Throughput

No Post-PCR processing—**REDUCING** Contamination

Stop reactions during faulty runs—**SAVE** Time

# Applications of Quantitative PCR

- **Viral Particle concentration - Viral Load**
- **mRNA expression levels - Gene Therapy Efficacy, Drug Development, Disease State Monitoring**
- **Gene Amplification - Cancer Research**
- **Melting Temperature Behaviour - Mutation Detection in Heterozygosity (Allelic Discrimination)**
- **End Point Detection - Qualitative, screening of known mutations (SNP Analysis)**

# What are the options for Real Time PCR?

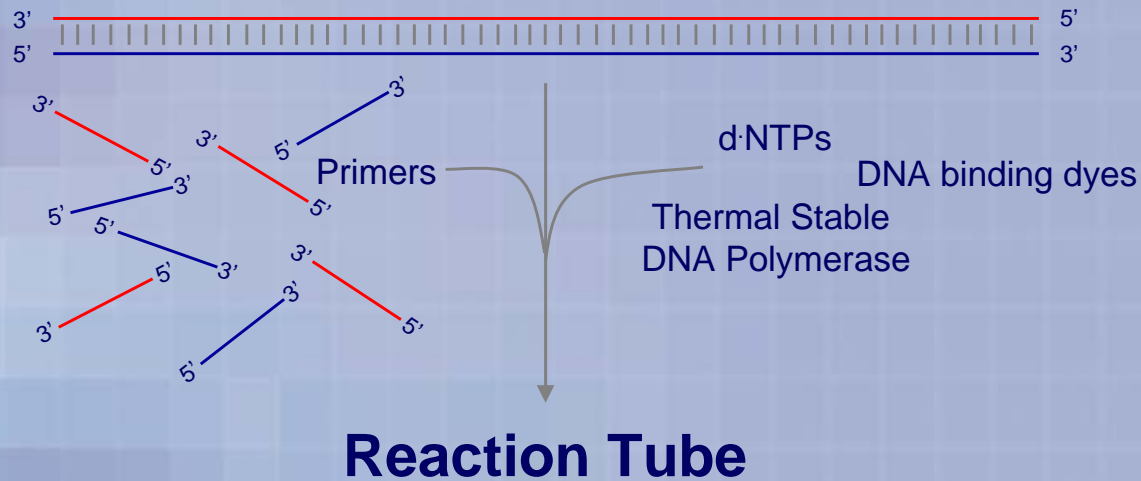
- Chemistry strategies to measure Real Time PCR are:
  - ◆ Intercalating Dyes, e.g. Ethidium Bromide and SYBR Green
  - ◆ Hybridization Probes - offer probe specificity

# Detection Strategies - DNA Binding Dyes

- ▶ DNA binding dyes are inexpensive compared to hybridization probes.
- ▶ A dye based strategy allows one to get a general confirmation of amplification.
- ▶ Higuchi demonstrated the key principle of real-time PCR using Ethidium Bromide -
  - ▶ EtBr fluoresces 25 times more brightly when bound to dsDNA
- ▶ SYBR® Green I, a more sensitive DNA binding dye, is an even more powerful approach
  - ▶ SYBR Green I fluoresces 200 times more brightly when bound to dsDNA

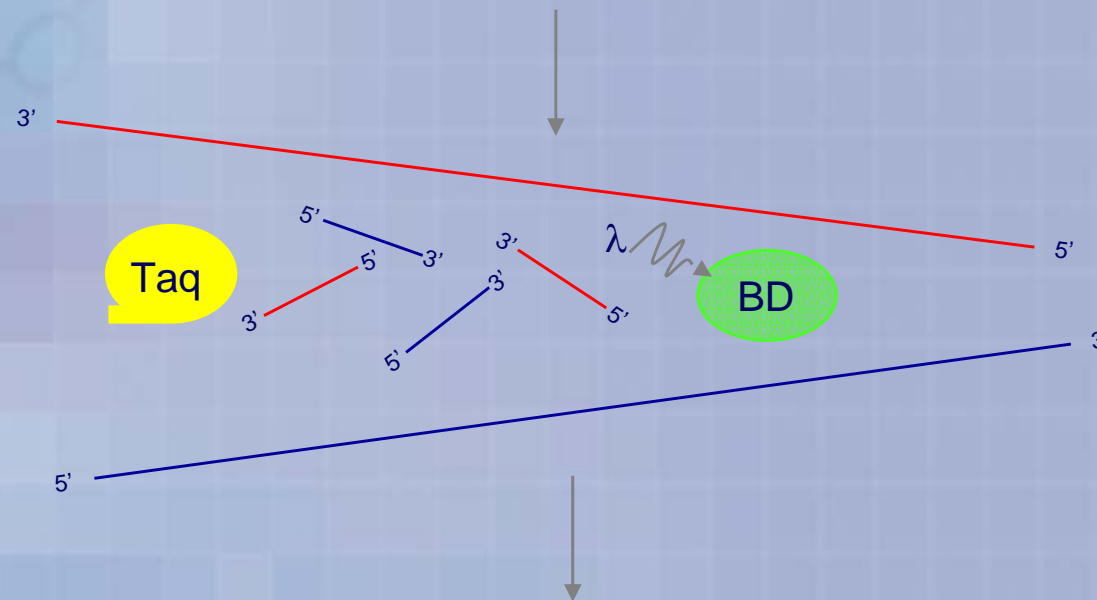
# DNA Binding Dyes

- ▶ Prepare reaction mixture



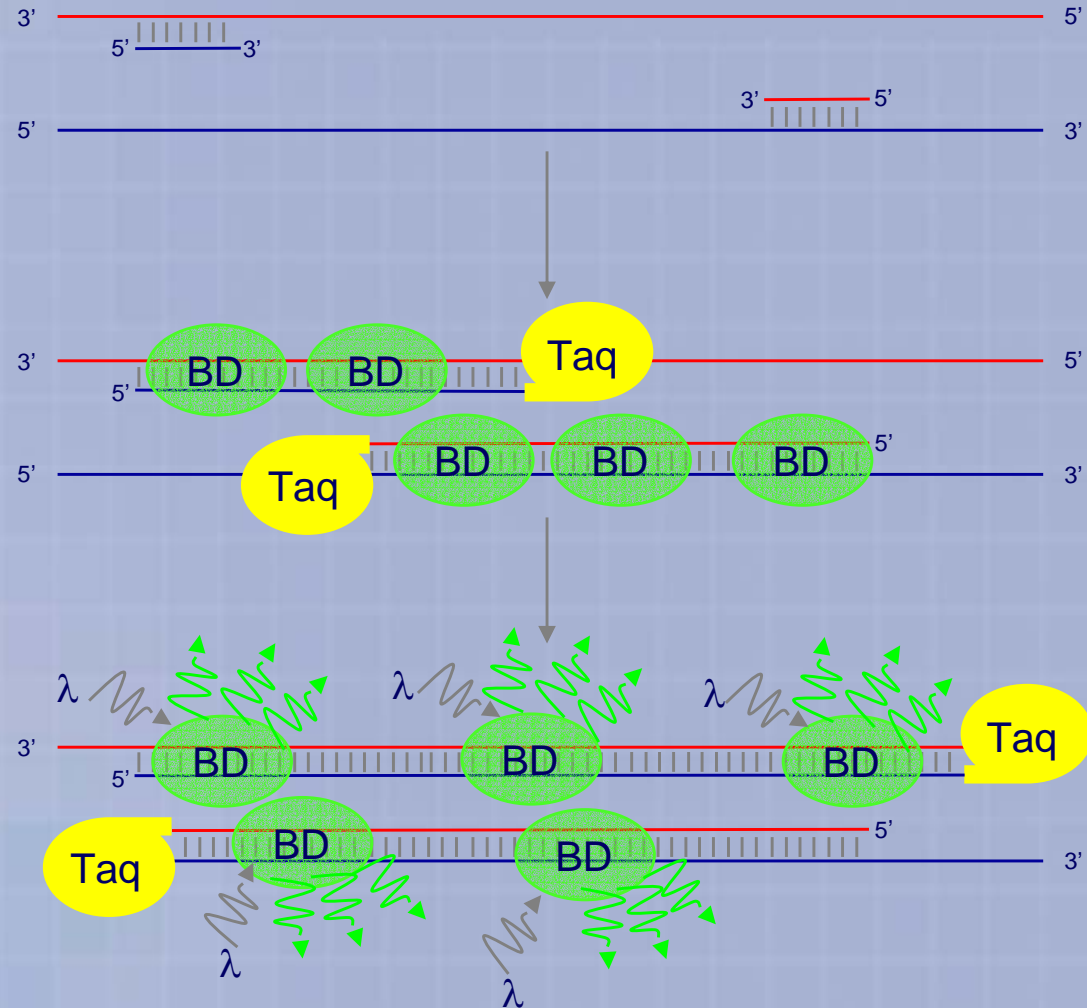
# DNA Binding Dyes

▶ Denaturation



# DNA Binding Dyes

- ▶ Annealing and extension



# DNA Binding Dyes

- ▶ Advantages

- ▶ Cheap
- ▶ Quick
- ▶ Easy

- ▶ Disadvantages

- ▶ Less specific
- ▶ Significant optimisation required to minimise primer-dimer formation and amplification of non-specific product

# Hybridization Probes

## Hybridization Probe Strategies :

- ▶ **Dual-labeled FRET probes**
  - ▶ **Cleavage Based Assay - TaqMan™ Assays**
  - ▶ **Displaceable Probe Assays**
    - ▶ **Molecular Beacons**
    - ▶ **Black Hole Scorpions**

# TaqMan :

↓ **Fluorescein is excited at 488nm and emits light around 520nm**

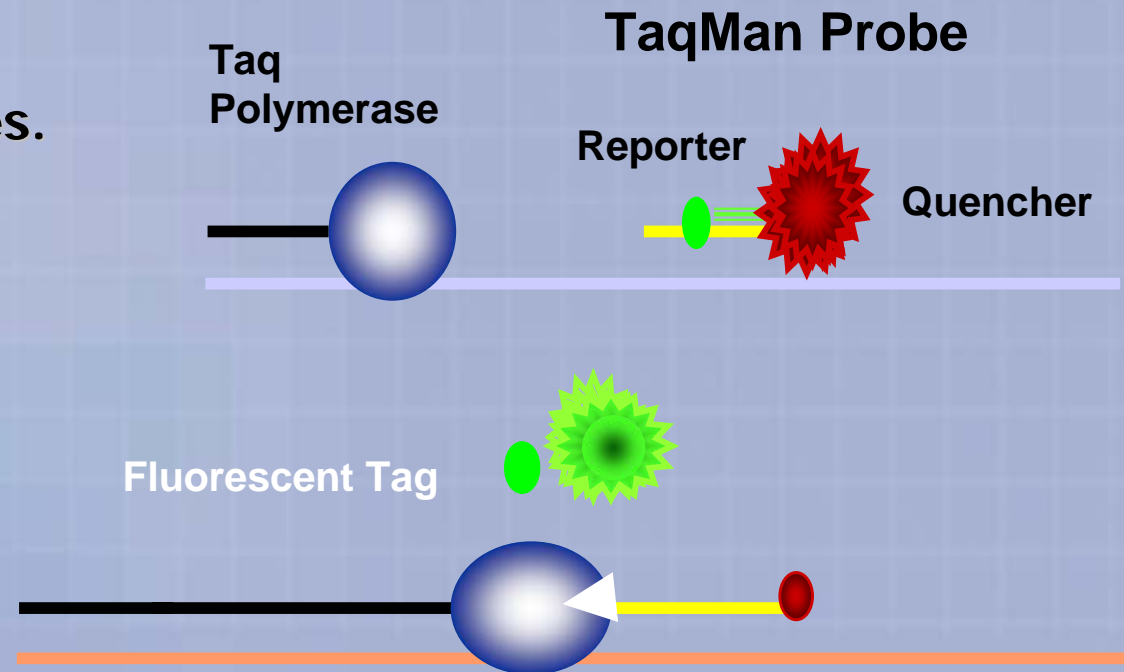
↓ **The 520nm light excites the TAMRA which emits light around 570nm**

- Reporter emitted light increases.

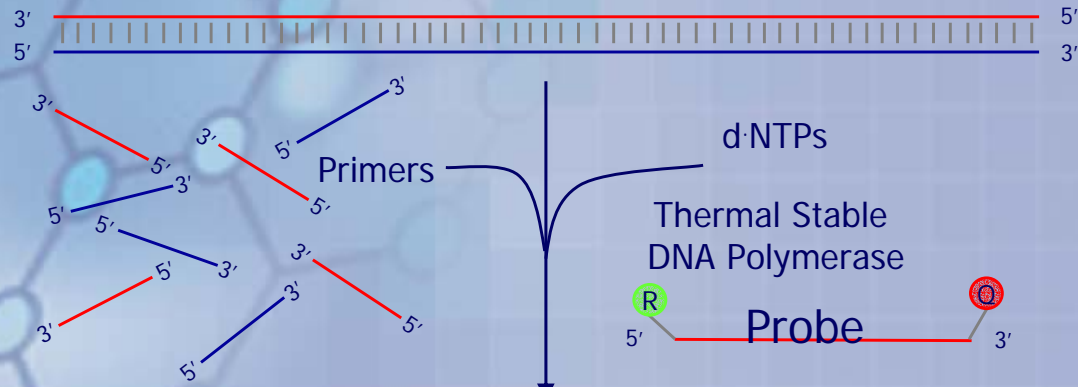
- Cleavage of probe occurs when probe has found target.

- increase in reporter emission represents increase in the amount of amplification product

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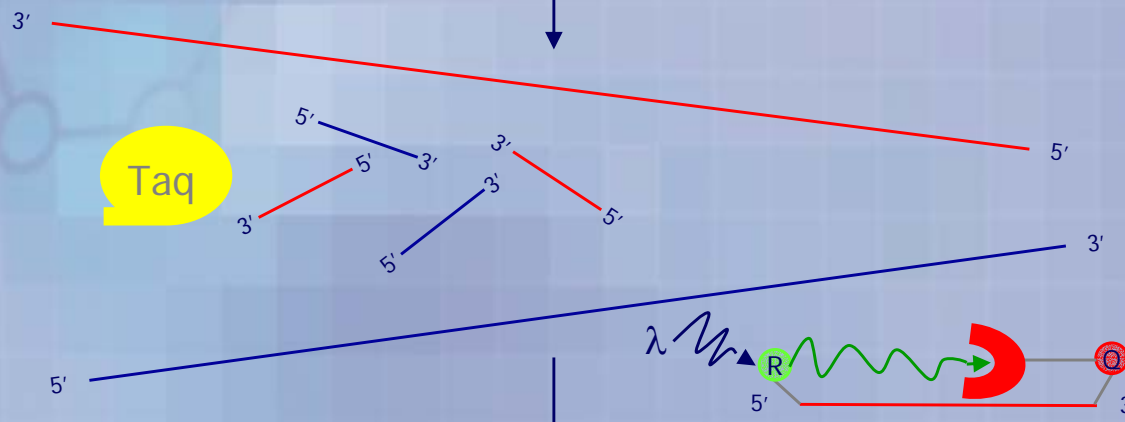


# Cleavage-based assay: TaqMan



**Add Master Mix  
and Sample**

**Reaction Tube**

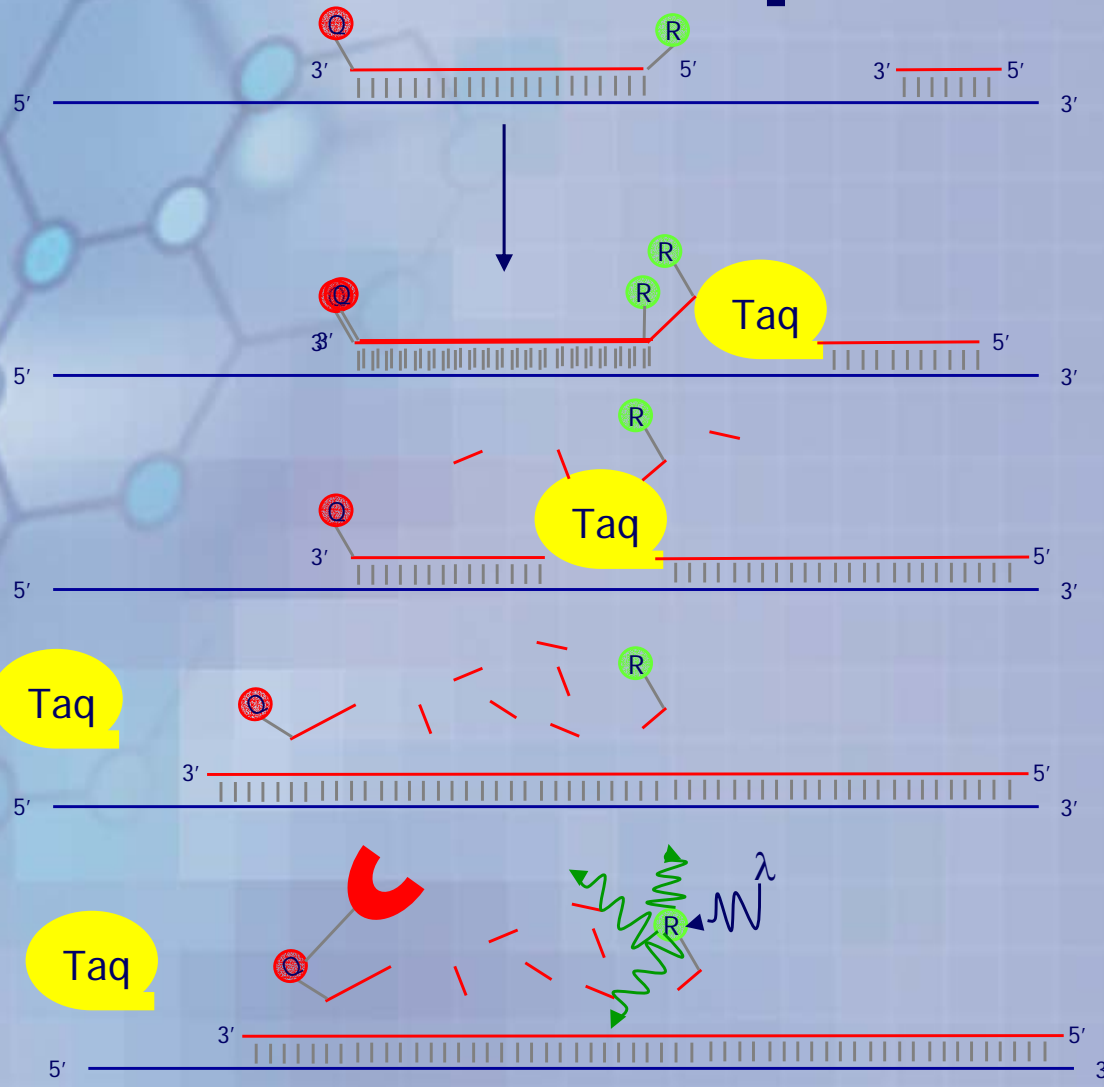


**Denaturation**

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**Annealing**

# Cleavage-based assay: TaqMan



## Extension Step

1. Strand Displacement

2. Cleavage

3. Polymerization Complete




4. Detection



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# Choosing a Fluorophore & Quencher

## Fluorophores:

	FAM
	TET, VIC
	HEX
	JOE
	CY3
	TAMRA
	CY3.5, Redmond Red
	Texas Red, ROX
	CY5 LC640
	CY5.5 LC705

## Quenchers:

BHQ-1  
DABCYL  
Eclipse  
TAMRA  
QSY-7  
BHQ-2  
BHQ-3

# Features of Real Time PCR

- ▶ **Sensitive** = good detection capability at low concentrations...
- ▶ **Dynamic Range** = good detection of wide range of sample concentrations
- ▶ **Accurate relationship between fluorescence and PCR product produced**
- ▶ **Multiplexing**

# TaqMan

## Advantages

- ▶ Amplification in signal due to large no of reporters generated:
  - ▶ 1st cycle = **1x** reporters.
  - ▶ 2nd cycle = **3x** reporters.
  - ▶ 3rd cycle = **7x** reporters.
  - ▶ 25th cycle = **33,554,431x** reporters.
- ▶ Predominant assay in Real Time PCR literature
- ▶ Many reagents available for assay targets.

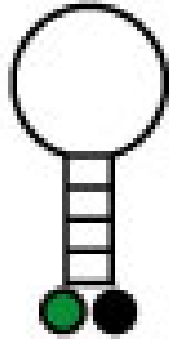
# TaqMan

## Disadvantages

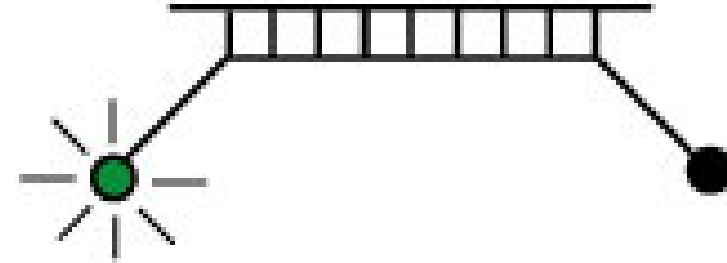
- ▶ **Quencher always fluoresces.**
  - ▶ Adds extra signal
  - ▶ Reporter must have emission spectra distinct from quencher
  - ▶ Requires that reporters emit light in the range that will excite the quencher
  - ▶ Multiplexing limited.

**Probe design options limited.**

# Molecular Beacons



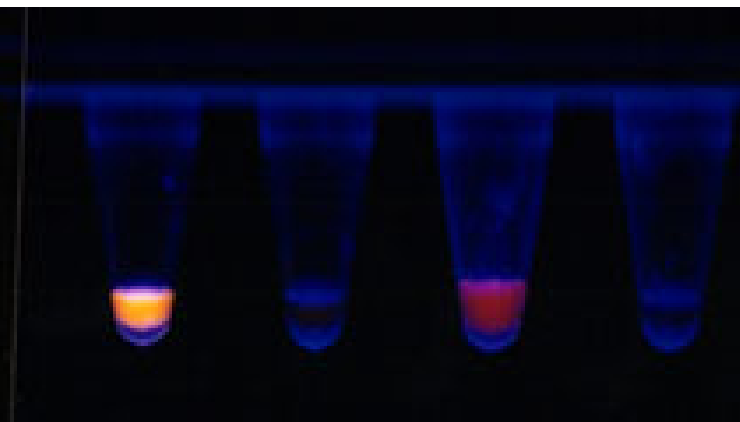
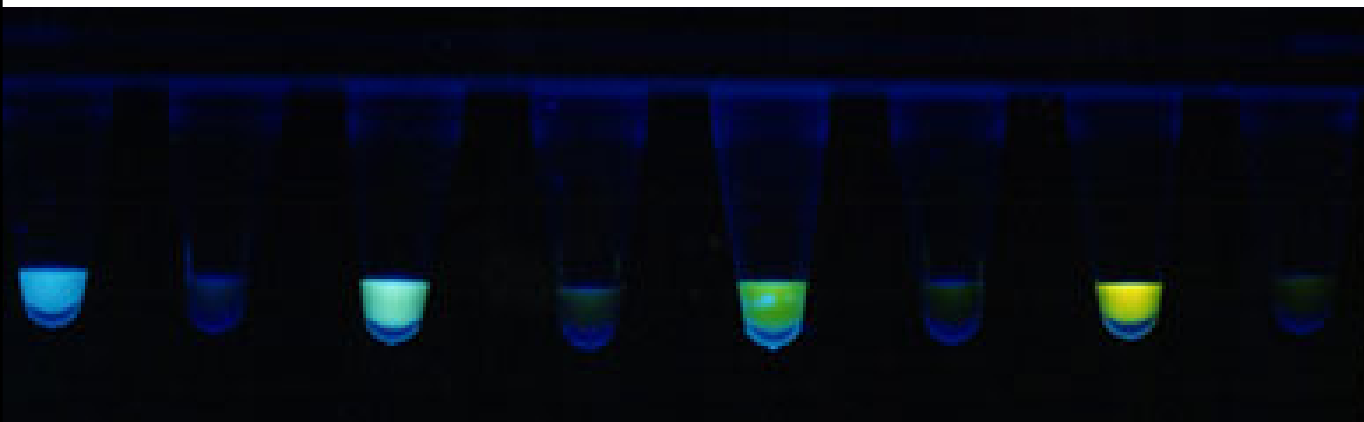
+



Molecular  
Beacon

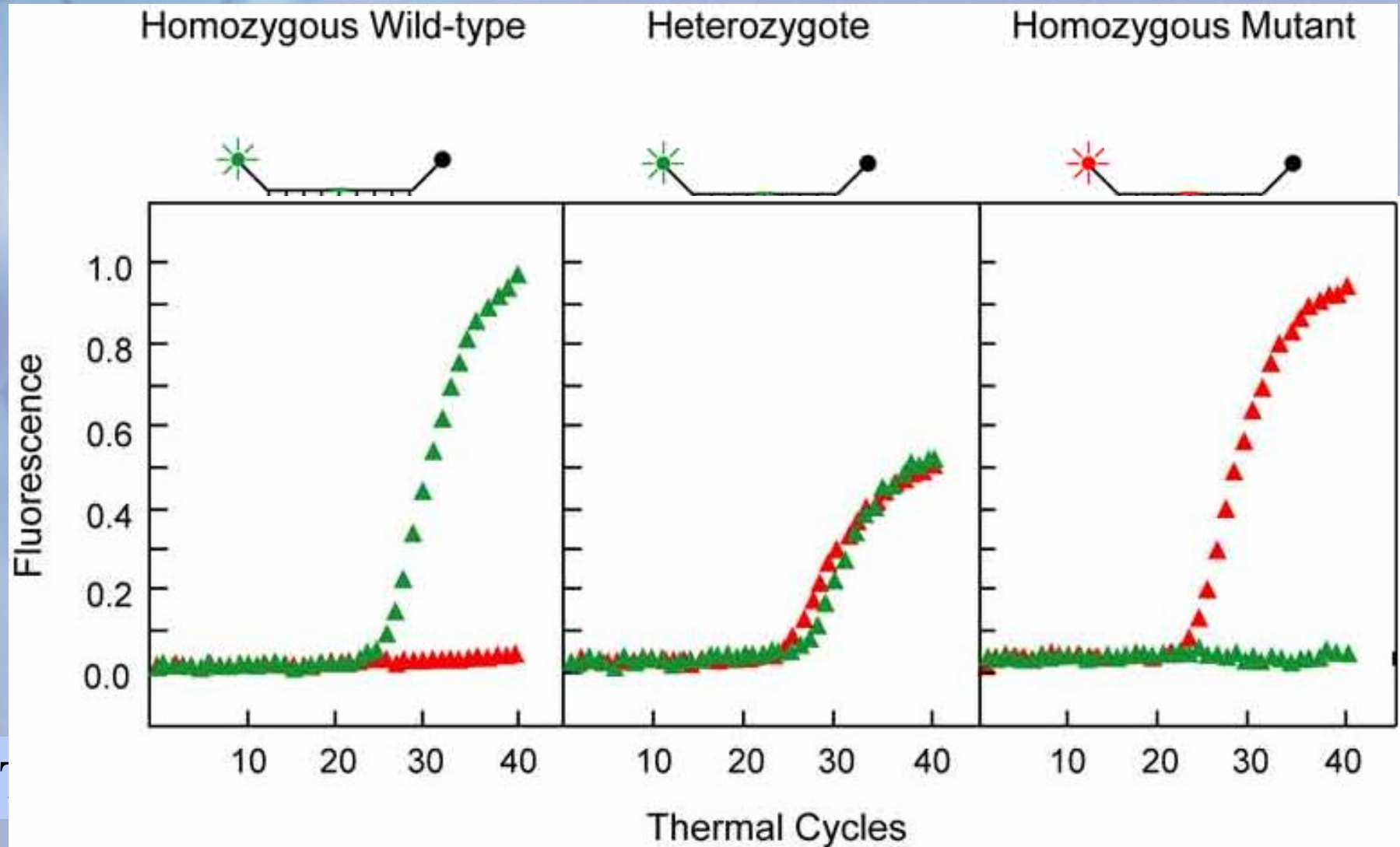
Target

Hybrid

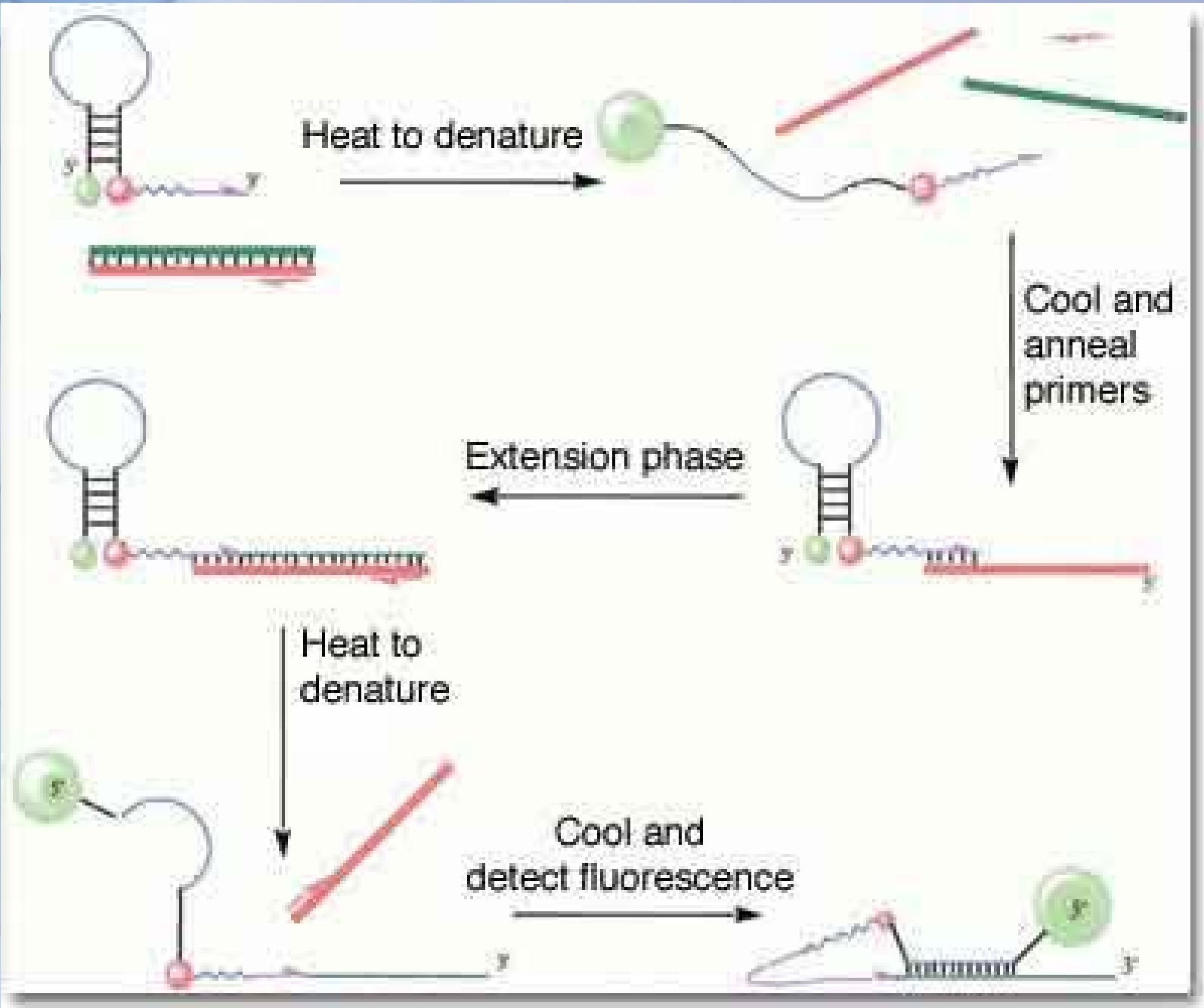


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# Molecular Beacons SNP Analysis



# Black Hole Scorpions



# Real-Time PCR

- ▶ Theory
- ▶ Chemistry
  - ▶ Non-specific DNA binding dye
    - ▶ SYBR® Green I
  - ▶ Specific Hybridization Probe
    - ▶ TaqMan™
    - ▶ Molecular Beacons
    - ▶ Black Hole Scorpions
  - ▶ Advantages & Disadvantages