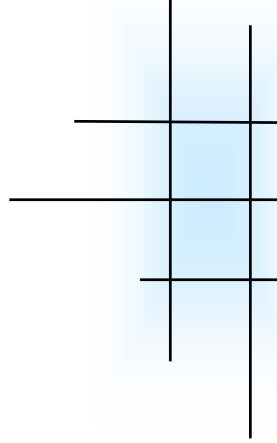
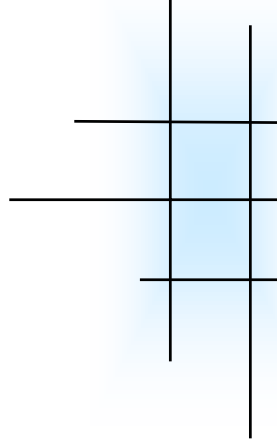


# Essentials of Real-Time PCR



Course Instructor: Dr. Man Bock Gu

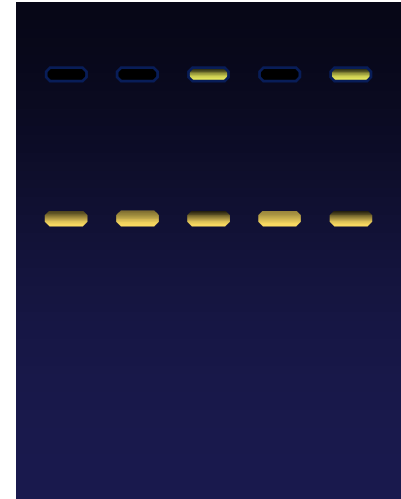
# Real-Time PCR:



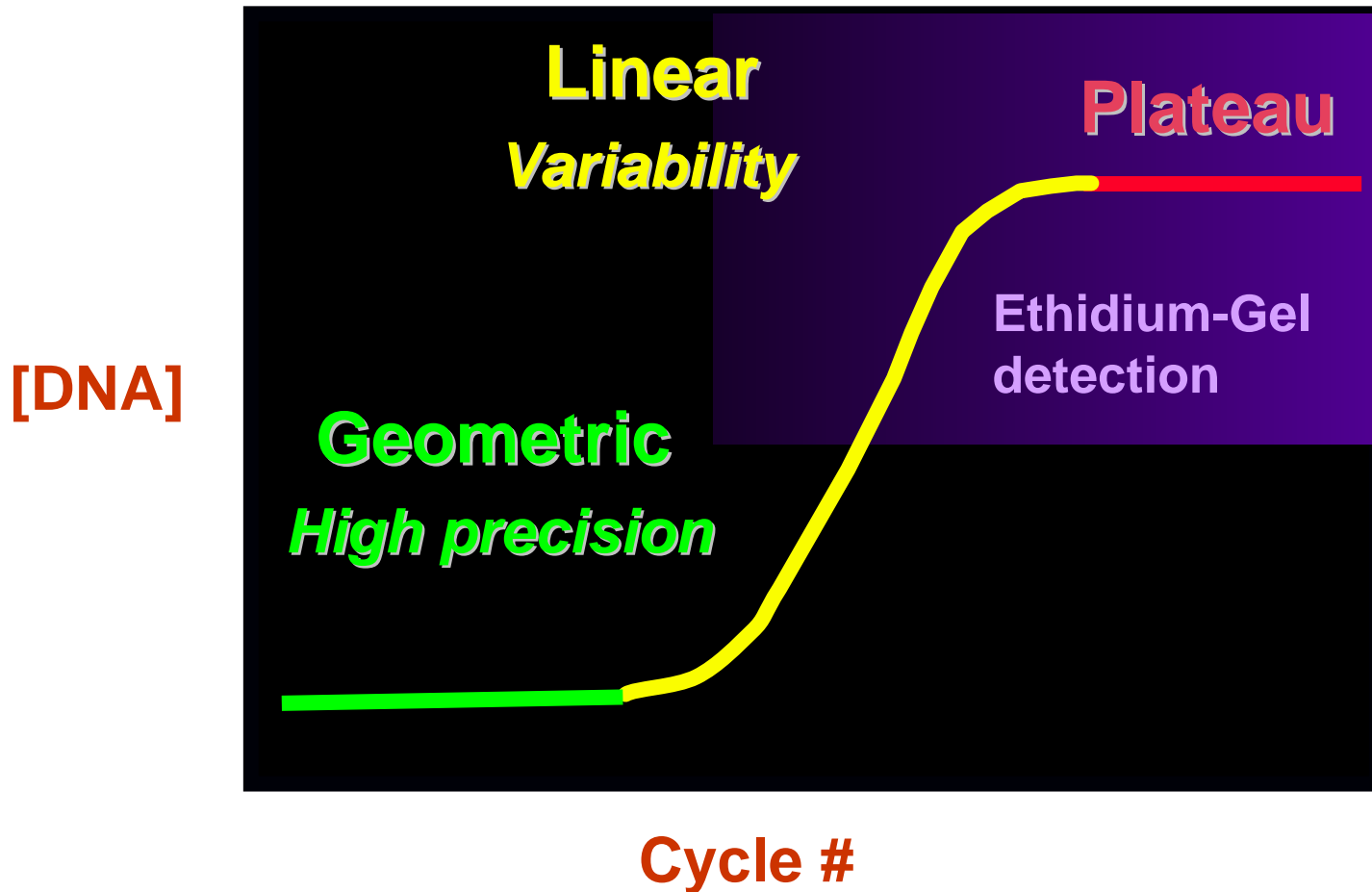
## 1. Problems with PCRs

# What's Wrong with Agarose Gels?

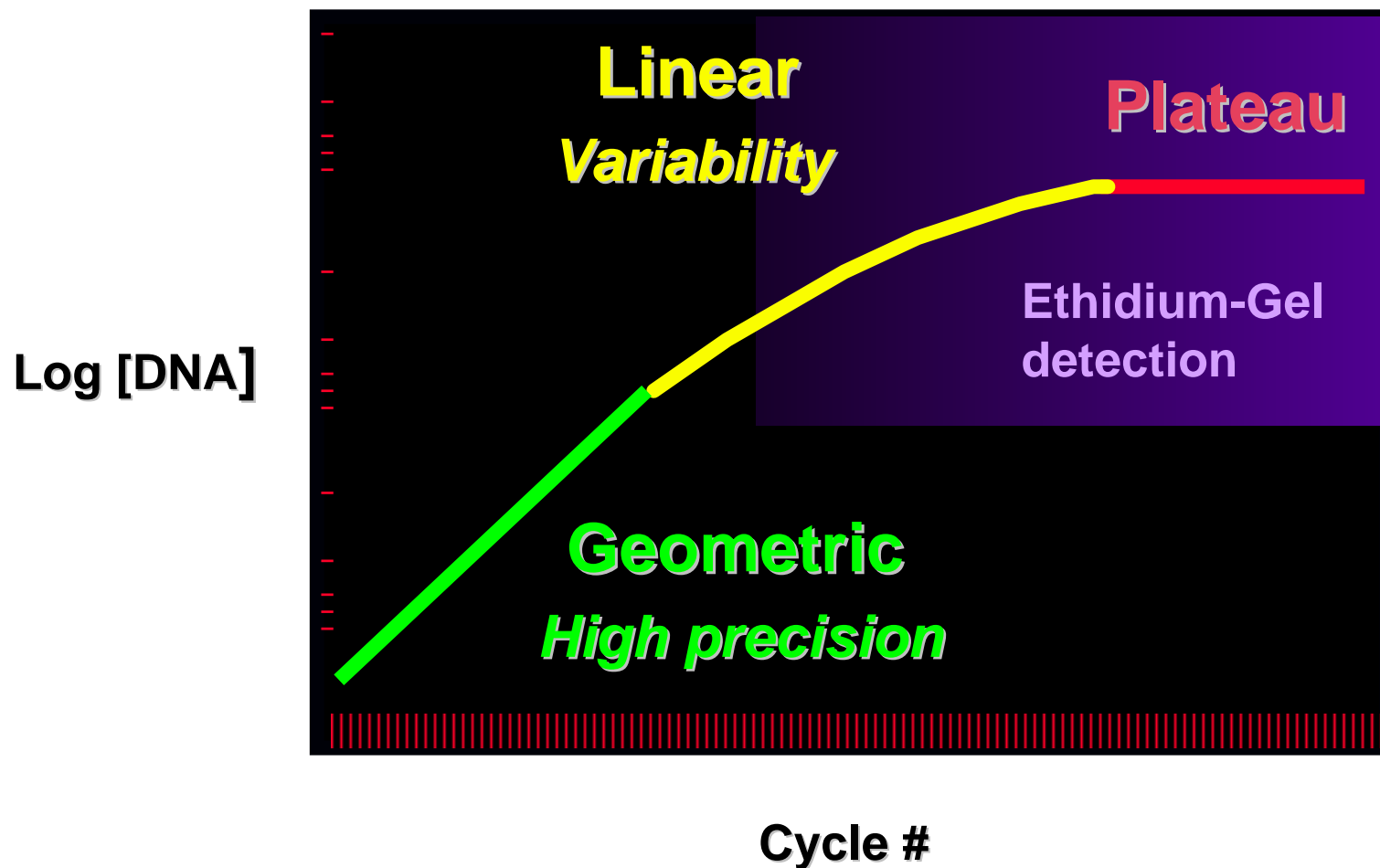
- Poor precision.
- Low sensitivity.
- Short dynamic range  $< 2$  logs.
- Low resolution.
- Non-automated.
- Size-based discrimination only.
- Results are not expressed as numbers.
- Ethidium bromide staining is not very quantitative.



# PCR Phases: Linear Plot

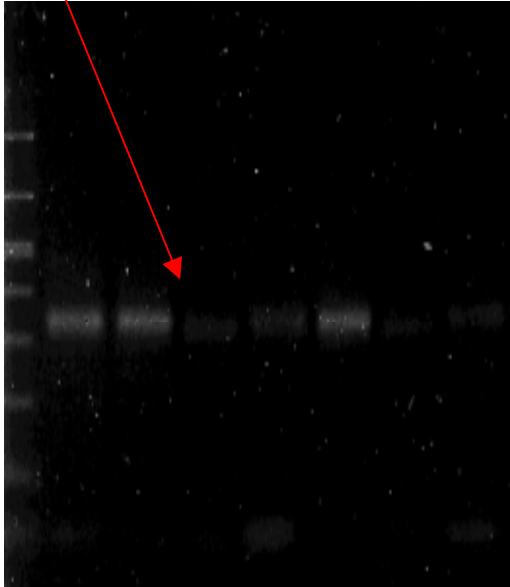
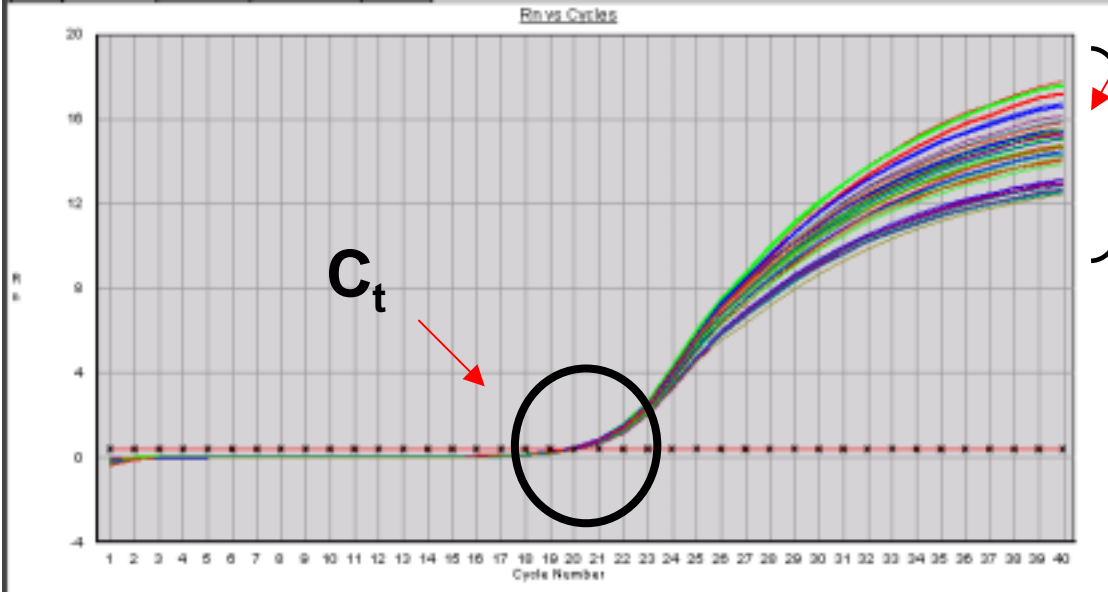


# PCR Phases: Semi-log Plot



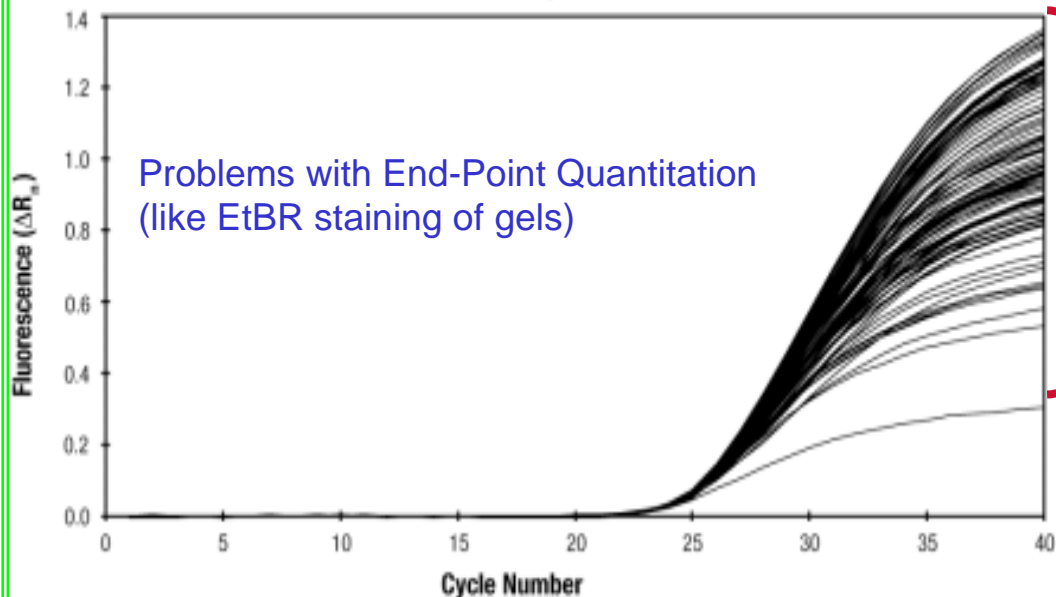
# Classic quantitation PCR product

Problems associated with endpoint analysis  
Variable PCR Plateau



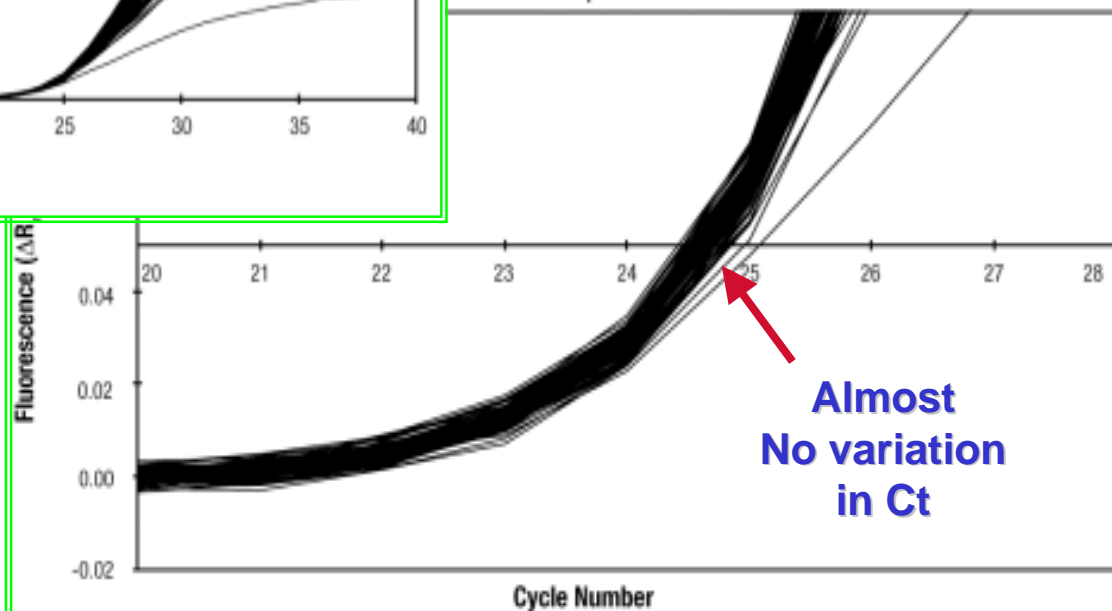
Amplification Plot of 96 Sample Replicates

**Variable PCR Plateau**  
96 replicates



**Wide variation in  
the final amount of  
PCR product**

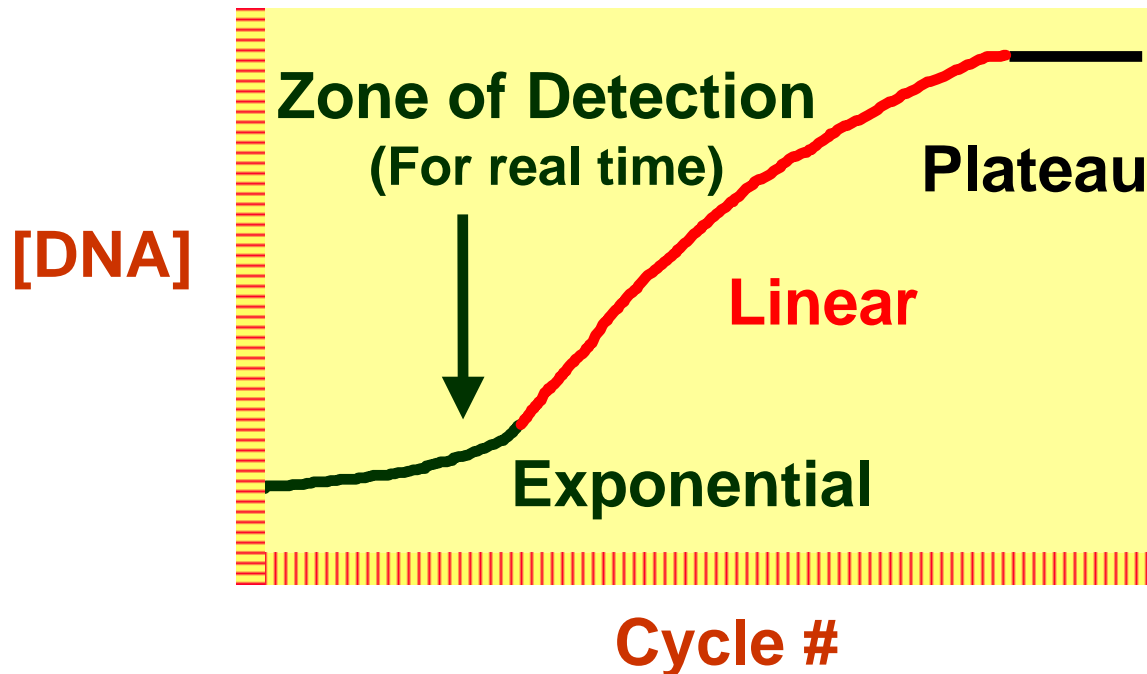
**Variable PCR Plateau**  
96 replicates



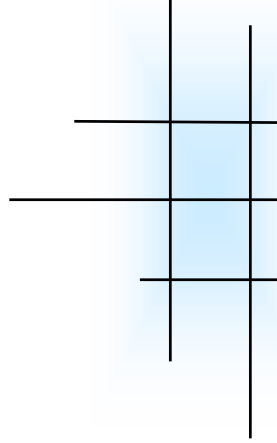
# PCR Quantitation and Phases

The optimal point for quantitative data collection is late exponential phase.

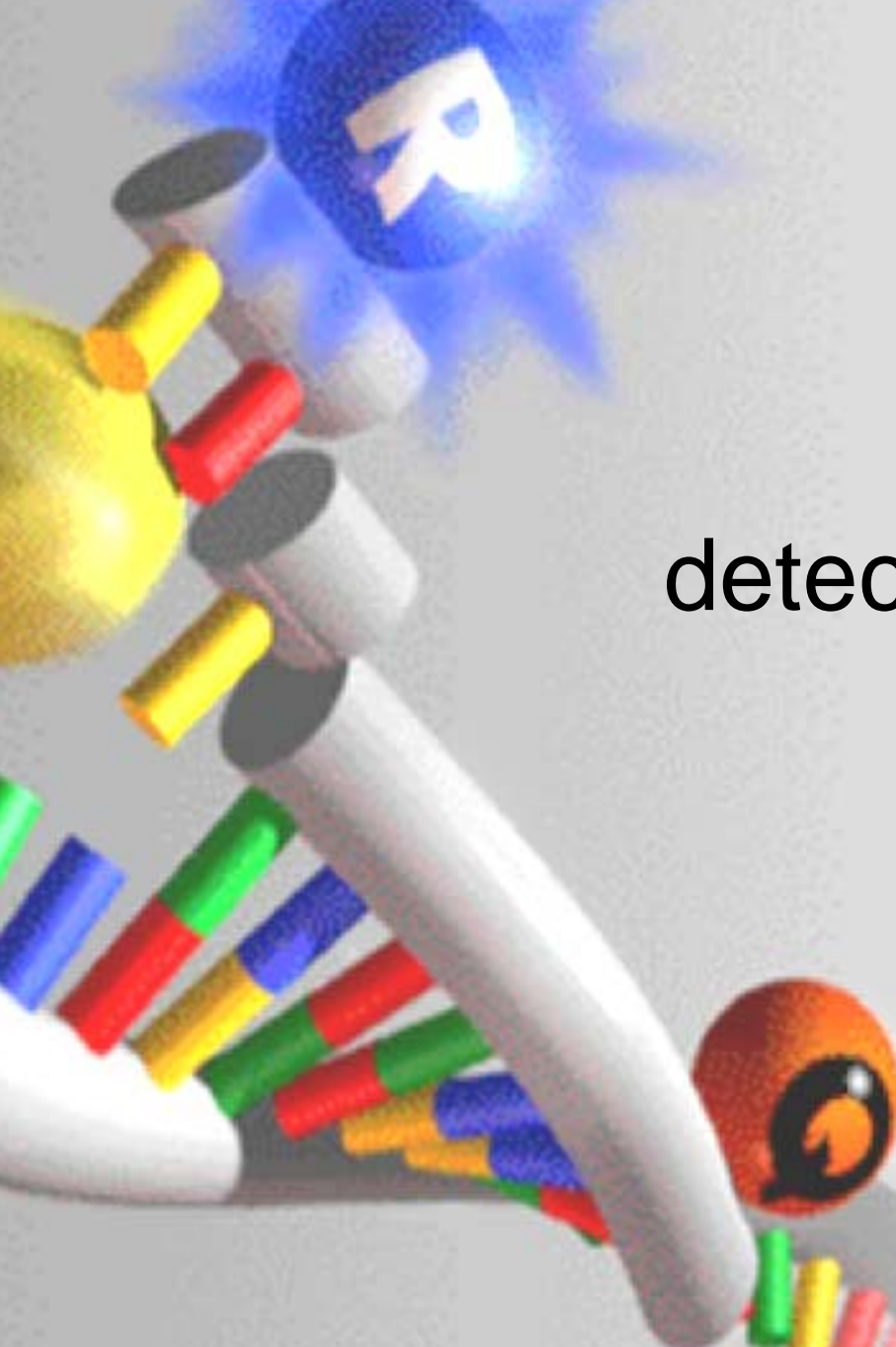
End point detection necessitates data collection at a fixed cycle number: variable signal.



# Real-Time PCR:



## 2. Dyes



detection chemistries

# two basic chemistry classes

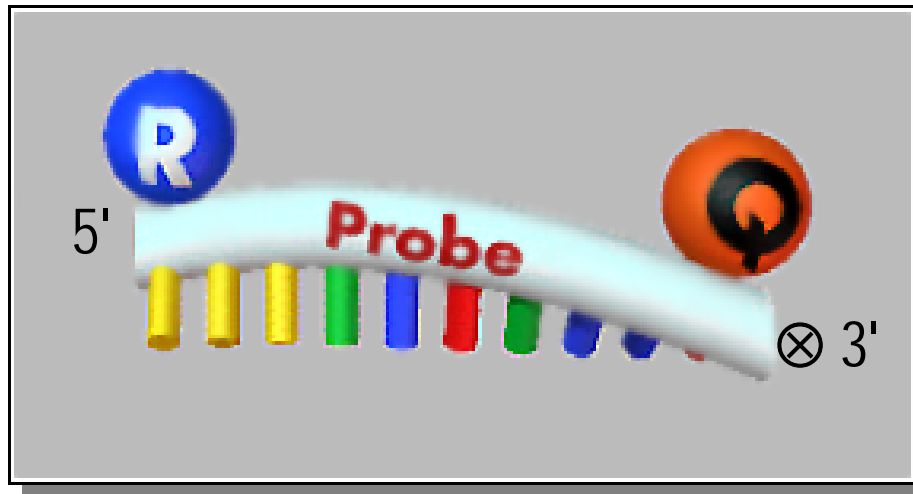
## 1 Probe based:

- *TaqMan probes (fluorogenic 5' nuclease assay)*
  - *TaqMan MGB probes*
  - *Three star (ARMS/TaqMan universal probe)*
  - *Molecular beacons*
  - *AmpliDet RNA (molecular beacons + NASBA)*
  - *Adjacent probes/ HybProbes (hybridisation probes)*
  - *DOL (dye-labeled oligonucleotide ligation)*
  - *Scorpions (labelled primer amplification)*
  - *DNA invader*
- etc*

## 2 Generic dye based:

- *SYBR Green 1 dye*
  - *Ethidium bromide*
- etc*

# TaqMan<sup>®</sup> probe

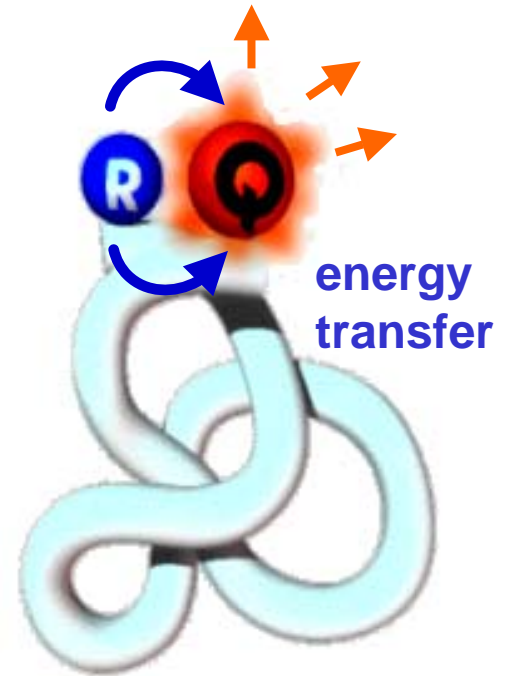
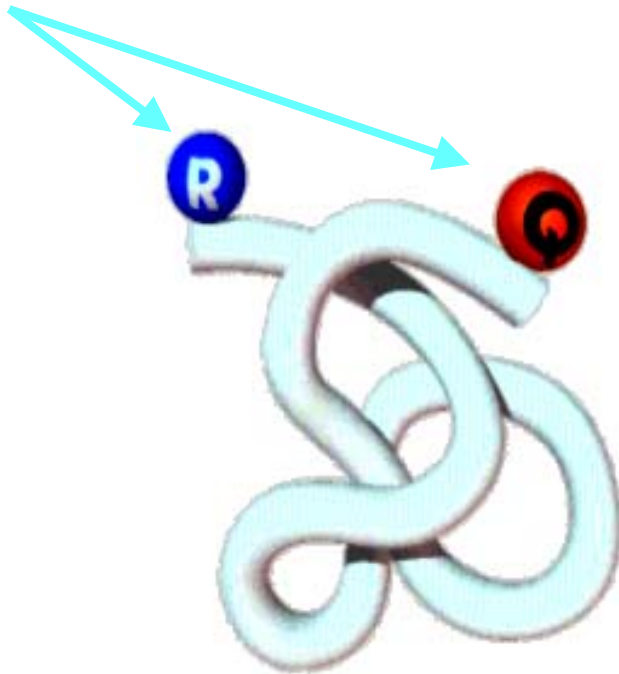


- hybridizes to target DNA sequence
- is 3' terminally blocked (cannot be extended by the polymerase)
- has two fluorescent dyes attached: **R** = reporter dye  
**Q** = quencher dye

# FRET

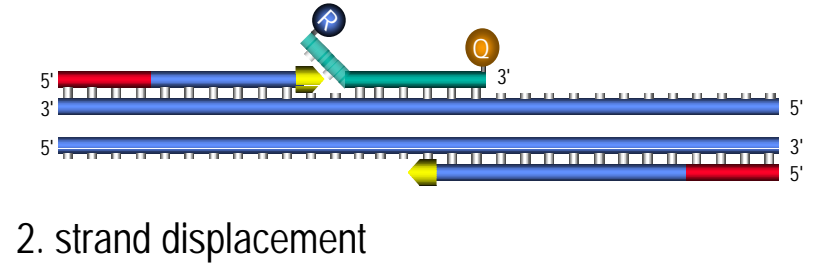
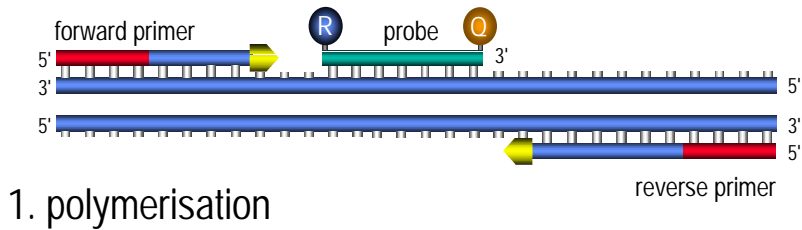
Förster resonance *energy transfer* through space (Förster *Ann. Phys.* 1948)

external  
light  
energy



R = reporter    Q = quencher

# TaqMan<sup>®</sup> chemistry in the PCR (5' nuclease assay)



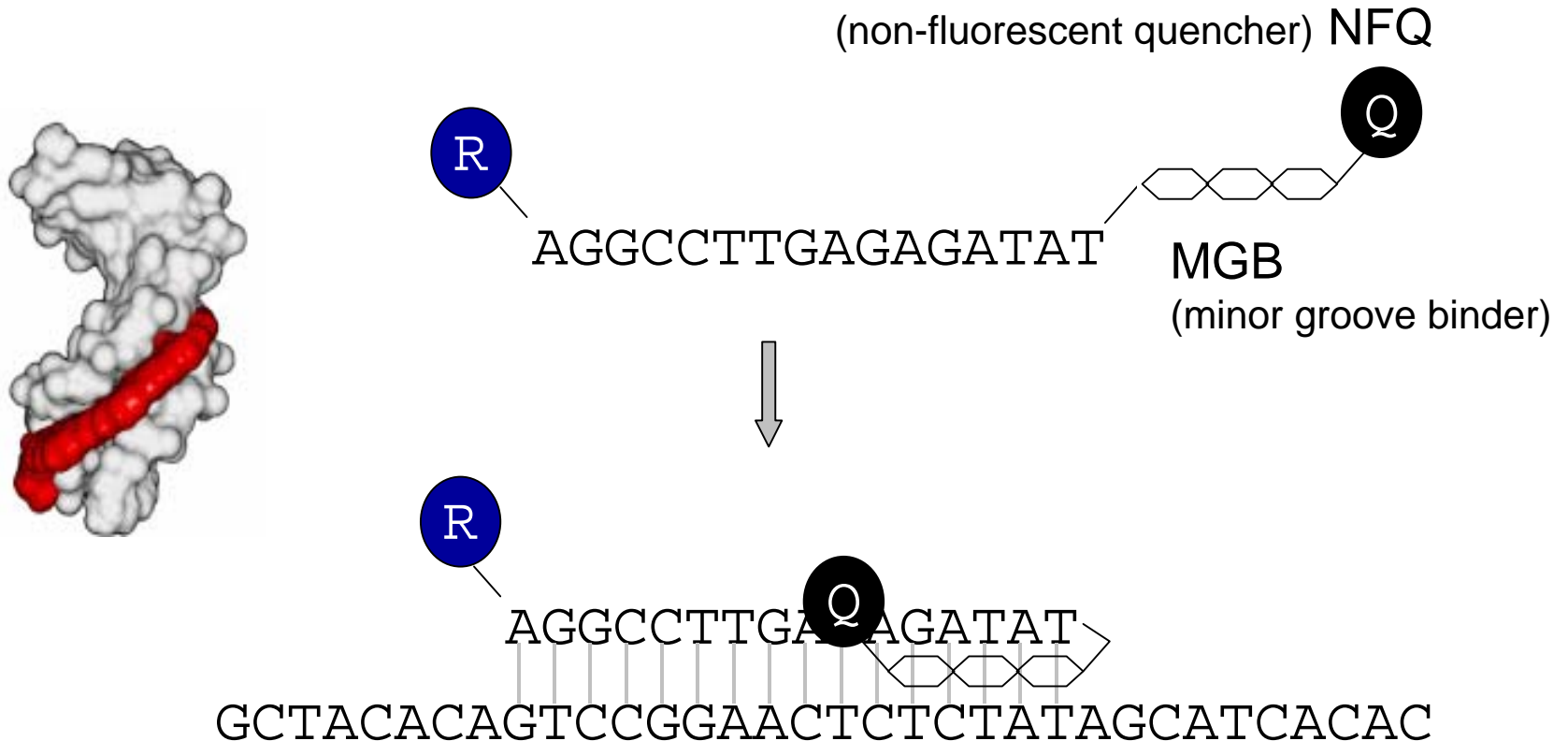
R = reporter dye  
Q = quencher dye

# advantages of TaqMan chemistry

- noiseless data  
due to the second level of specificity provided by the probe
- multiplex compatible  
each probe can be differently coloured and thereby mixed with others
- signal proportional to product  
signal related to amount of amplified product (not mass/length)
- irreversible signal accumulation  
other probe chemistries use reversible hybridisation to generate signal

Nevertheless, some situations are challenging!

# next-generation chemistry: TaqMan<sup>®</sup> MGB Probes



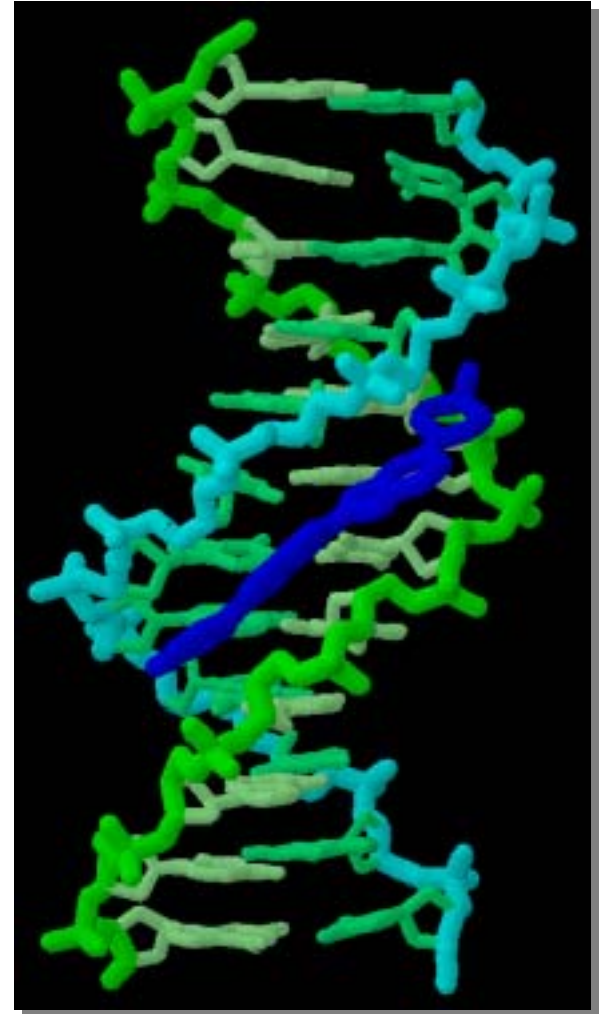
# TaqMan<sup>®</sup> MGB Probes

- clearer less complicated signals with no background fluorescence
- increased flexibility for future dye advancements
- give excellent discrimination
- easier assay design; shorter probes allow more scope
  - better use of small sequence variations
  - easier to fit into conserved sequence "windows"

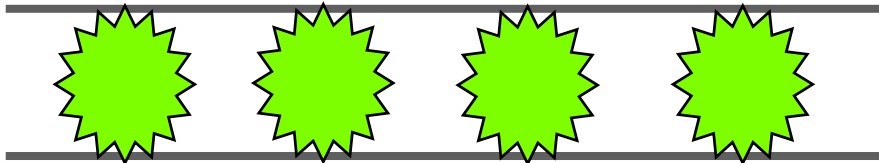
a universal homogeneous chemistry

# SYBR Green 1<sup>®</sup>

double-stranded DNA  
minor-groove binding dye

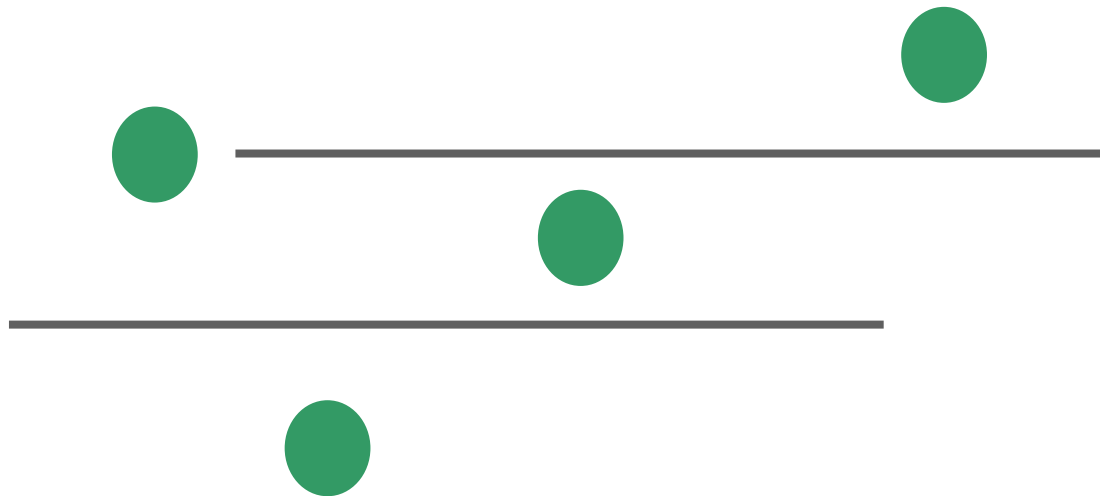


# SYBR<sup>®</sup> Green 1 Dye Assay Chemistry

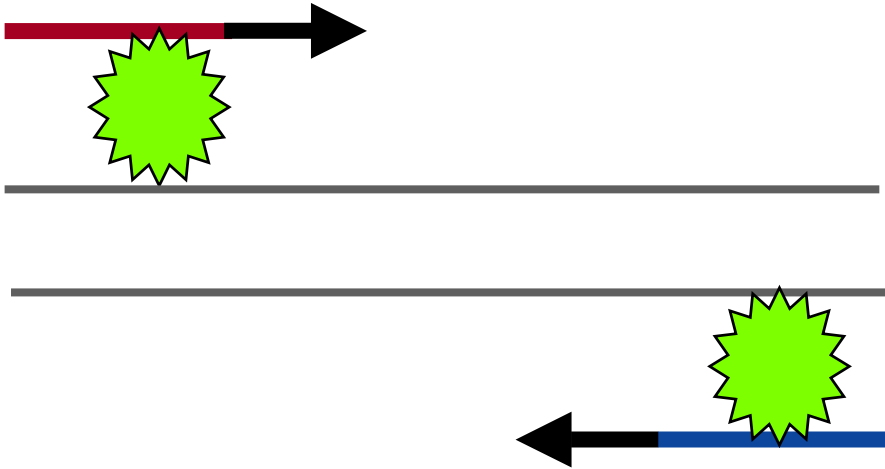


DNA Target Sequence

Denaturation

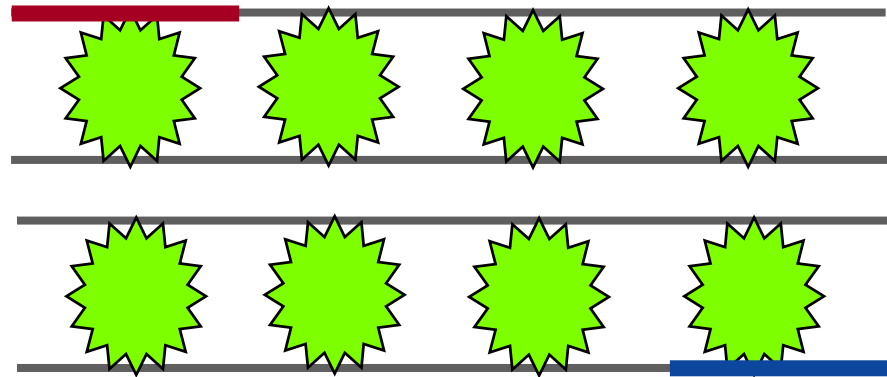


# SYBR<sup>®</sup> Green 1 Dye Assay Chemistry

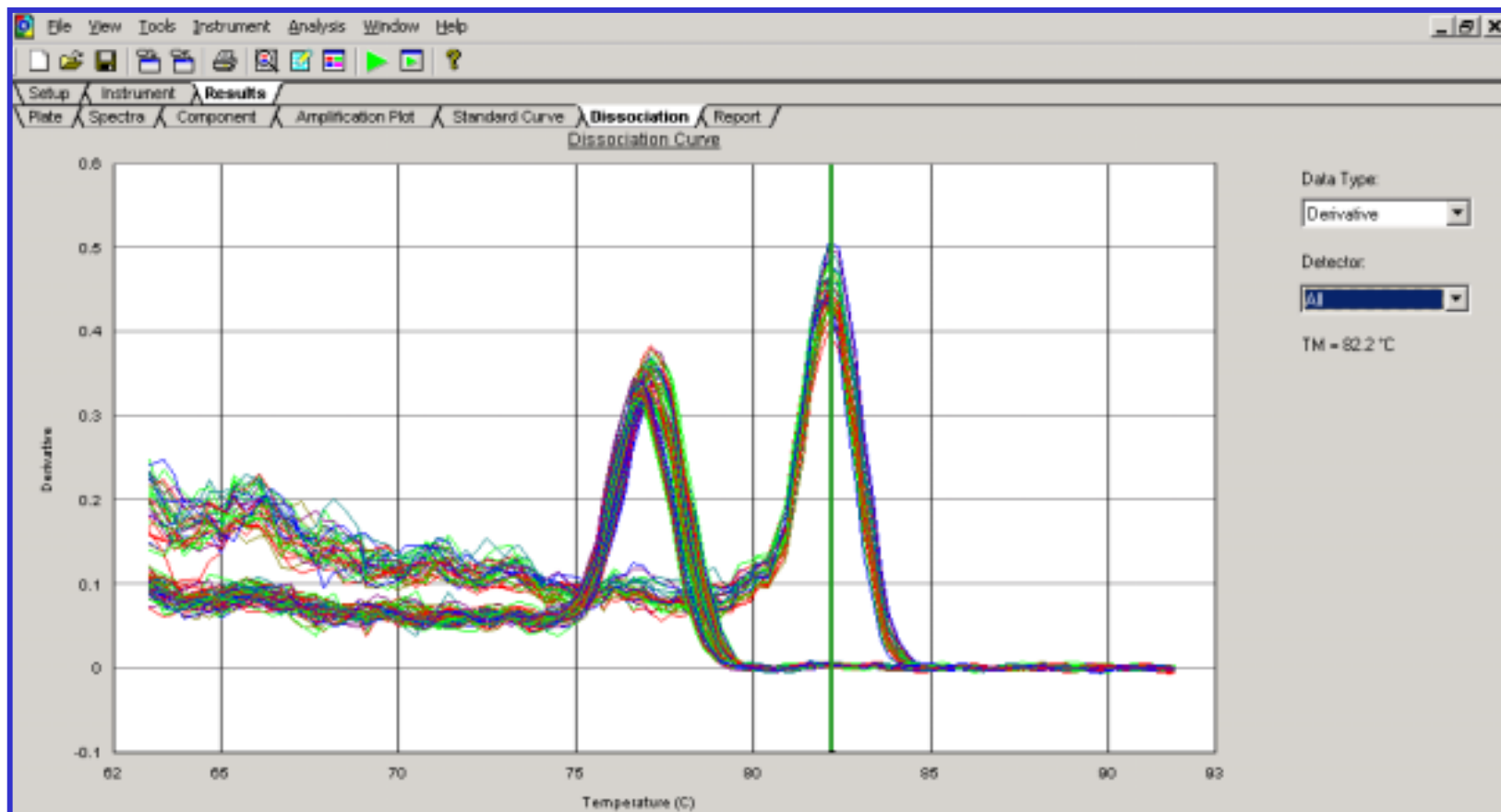


Polymerization

Polymerization Complete



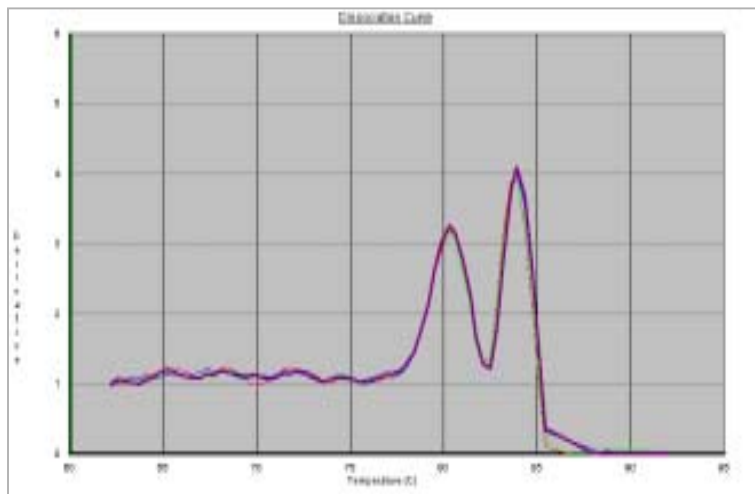
# SYBR<sup>®</sup> dissociation curve data



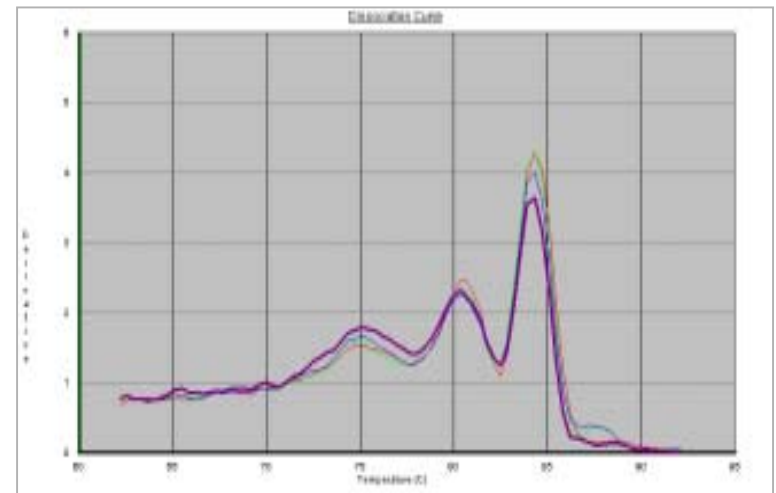
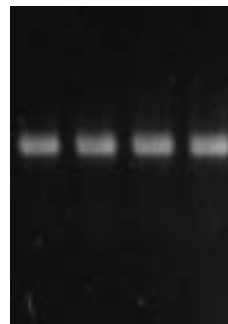
# SYBR<sup>®</sup> dissociation curves

detection of alleles

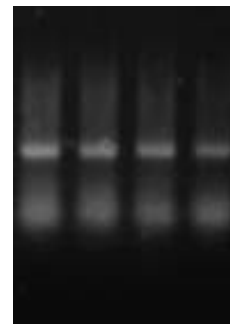
Discriminating summer barley alleles— data from Germany



Primer  
combination:  
50-50 nM



Primer  
combination:  
900-900 nM



# summary of SYBR<sup>®</sup> Green I chemistry

- Inexpensive

No probes are needed

- Ideal screening tool

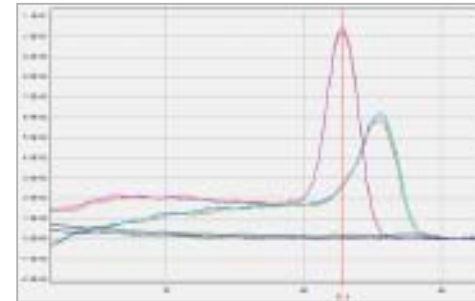
Targets can be examined first with SYBR and assays converted to TaqMan as needed

- Compatible with dissociation curve analysis

- Not compatible with multiplexing

- Low target levels may produce false signal

ALL products are detected specific and non-specific!



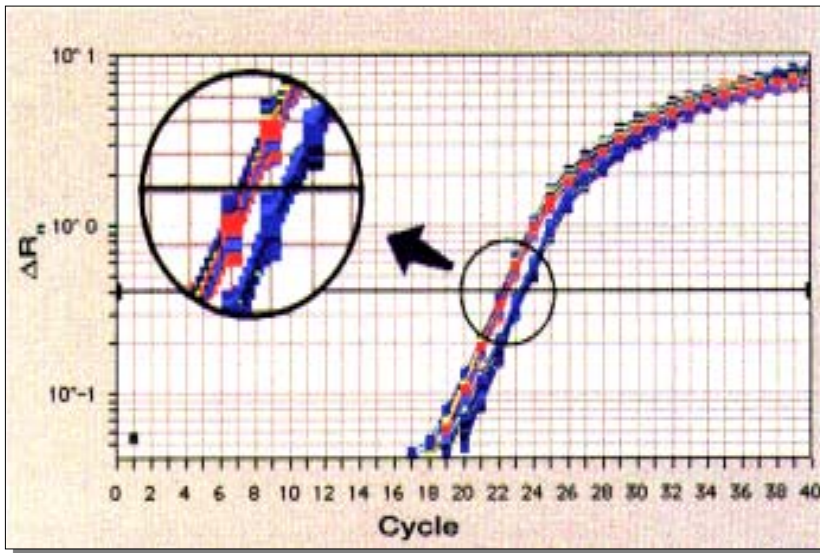
# Removing well-to-well signal variation

- Raw fluorescent signal from each well must be corrected for variation due to:
  - the amount of light reaching each well (optical system)
  - the number of fluorescent molecules in each well
  - the autofluorescence and optical properties of each plastic tube
- Signal can be normalised effectively using a “passive reference dye” (= ROX dye)
- ROX signal is PCR-independent
- Divide PCR signal by ROX signal to normalize

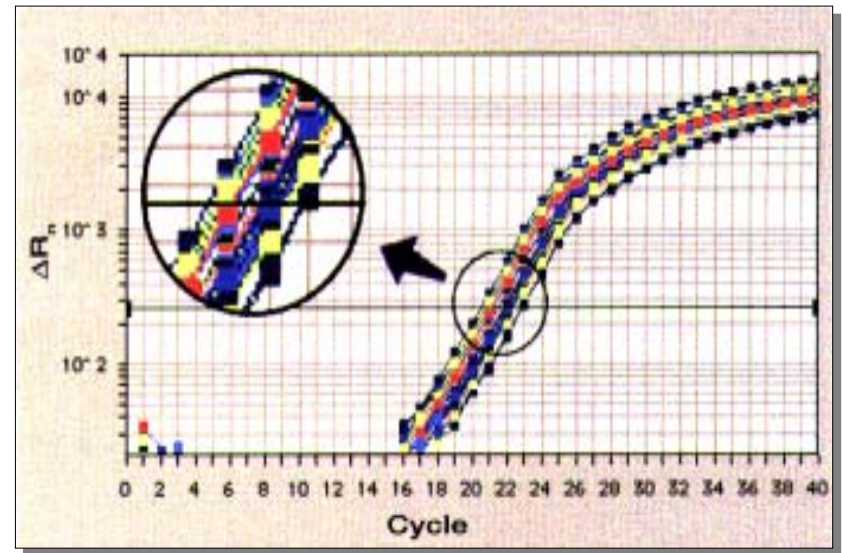
# Normalising SYBR Green to the ROX reference

—distinguishing 5,000 and 10,000 copies

(a 2-fold difference = 1 cycle of the PCR)



ROX reference

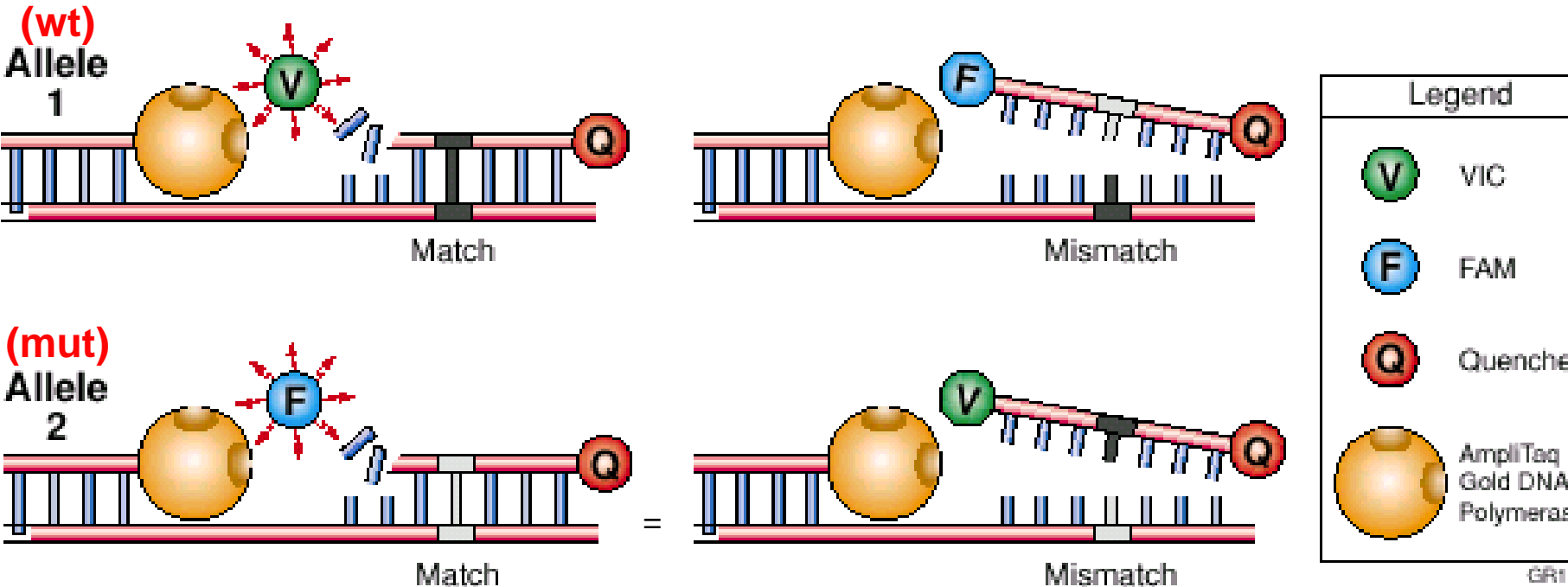


No ROX reference

# **End-Point Detection Qualitative PCR**

# genotyping—competing probes

discriminating alleles and single nucleotide polymorphisms (SNPs)

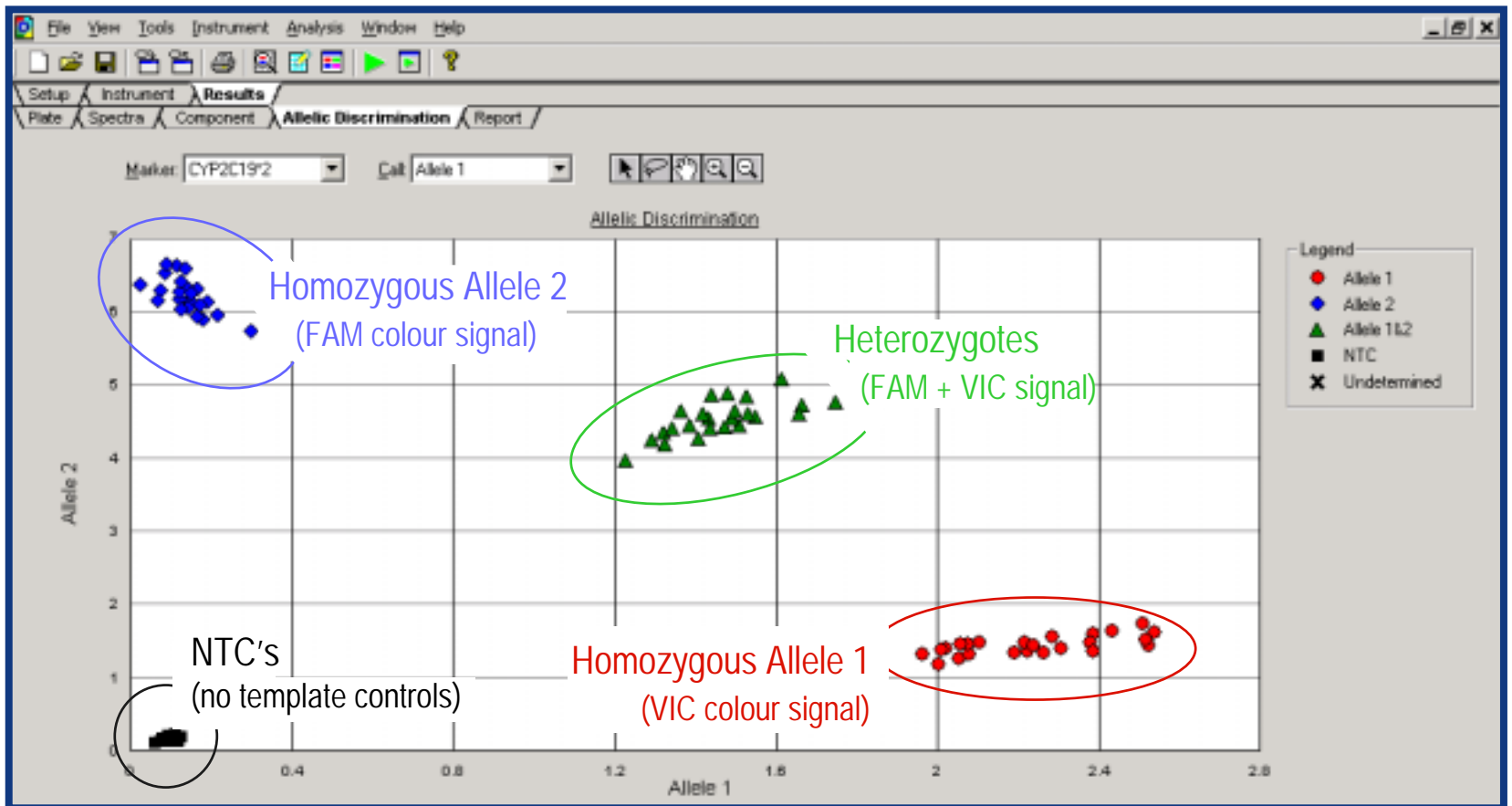


↑ VIC = homozygous allele A

↑ FAM = homozygous allele B

↑ ↑ FAM & VIC = heterozygous (A & B)

# allelic discrimination (SNP) data



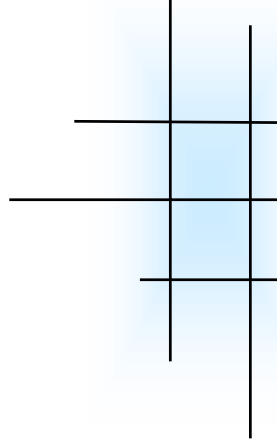
qualitative +/- assay

end-point PCR with control  
e.g. detecting a particular pathogen

|  | target | IPC    | conclusion |                  |
|--|--------|--------|------------|------------------|
|  | +      | + or - | YES        |                  |
|  | -      | +      | NO         | ← True negative  |
|  | -      | -      | RETEST     | ← False negative |

**Green signal = *Salmonella***  
**Red signal = IPC (Internal Positive Control)**

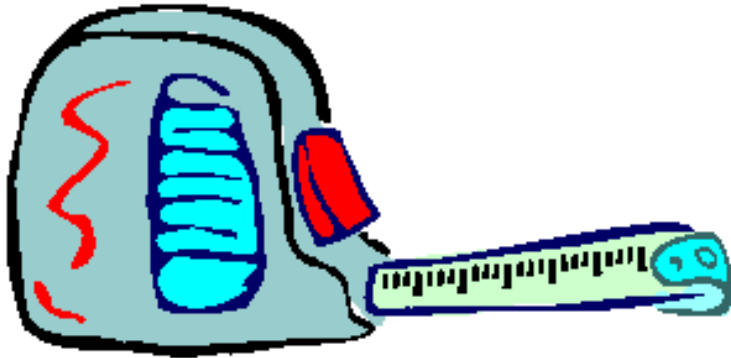
# Real-Time PCR:



## 3. Quantification

# Quantitative Assay

- A method to measure the amount of nucleic acid target during each amplification cycle of the PCR

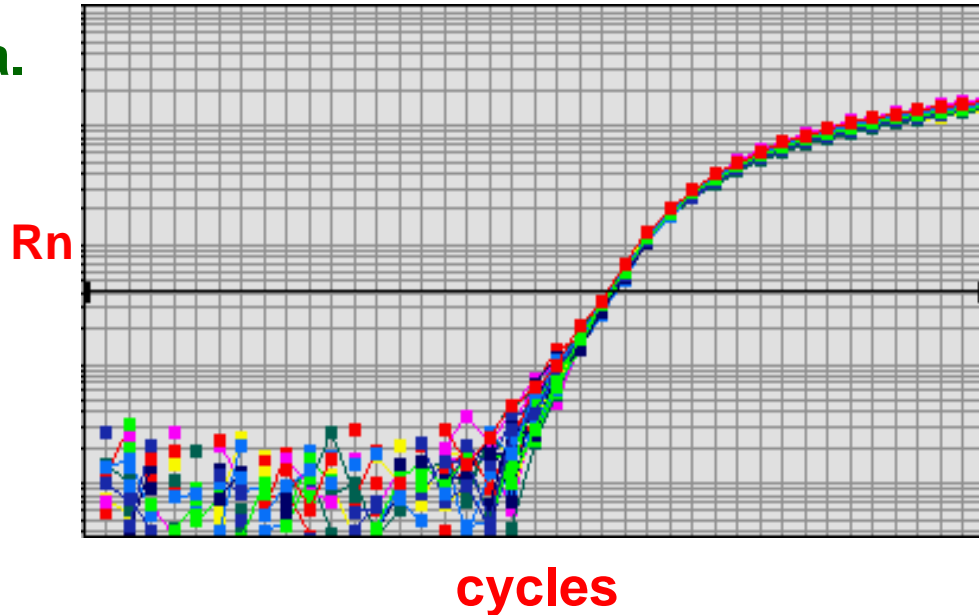


- DNA/cDNA
- RNA
- One-Step
- Two-Step

# Real-Time PCR Detection

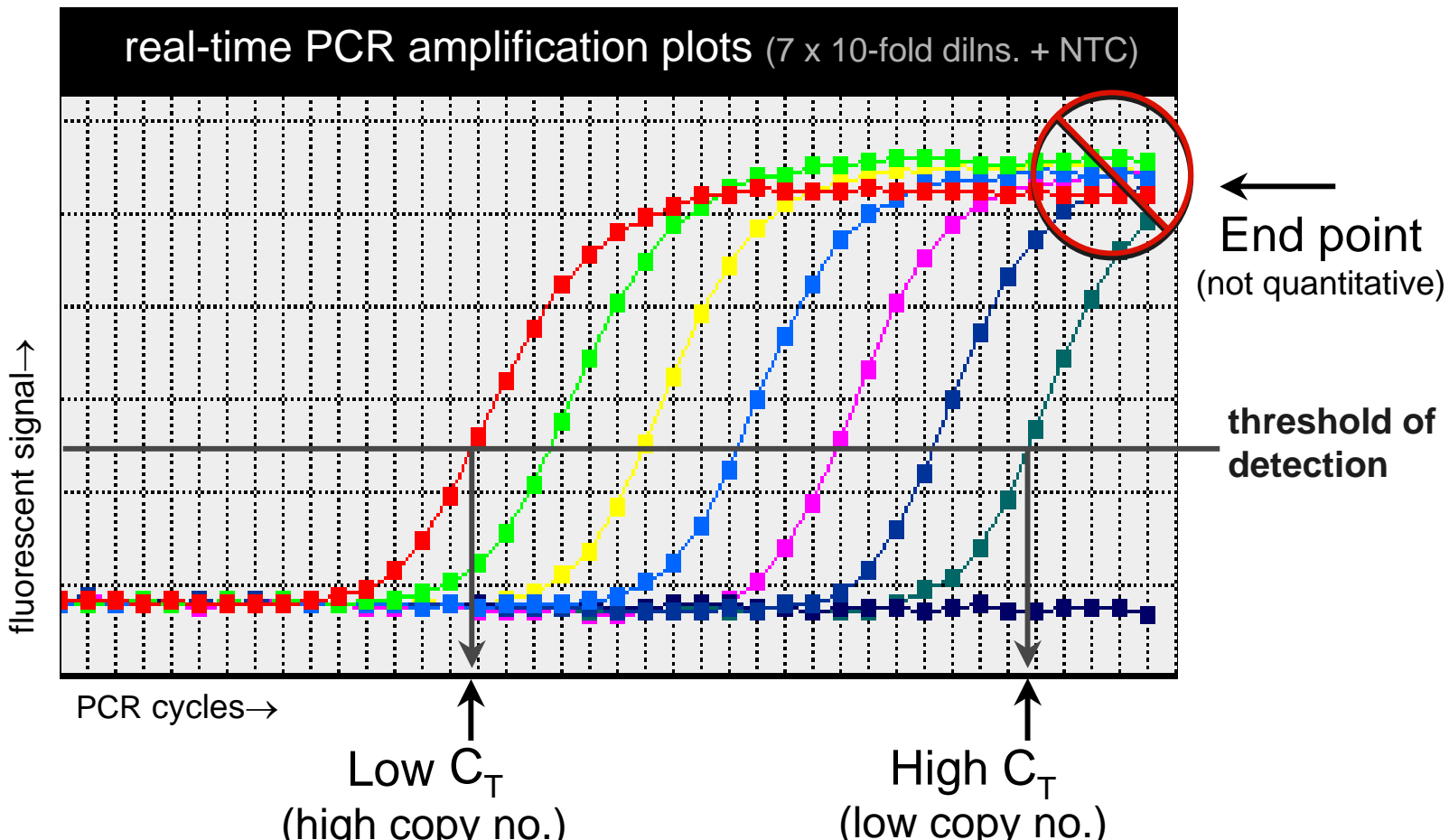
The automatic detection of PCR product growth throughout the amplification process.

- Collect geometric phase data.
- No post-PCR steps.
- Enables high precision and high throughput.

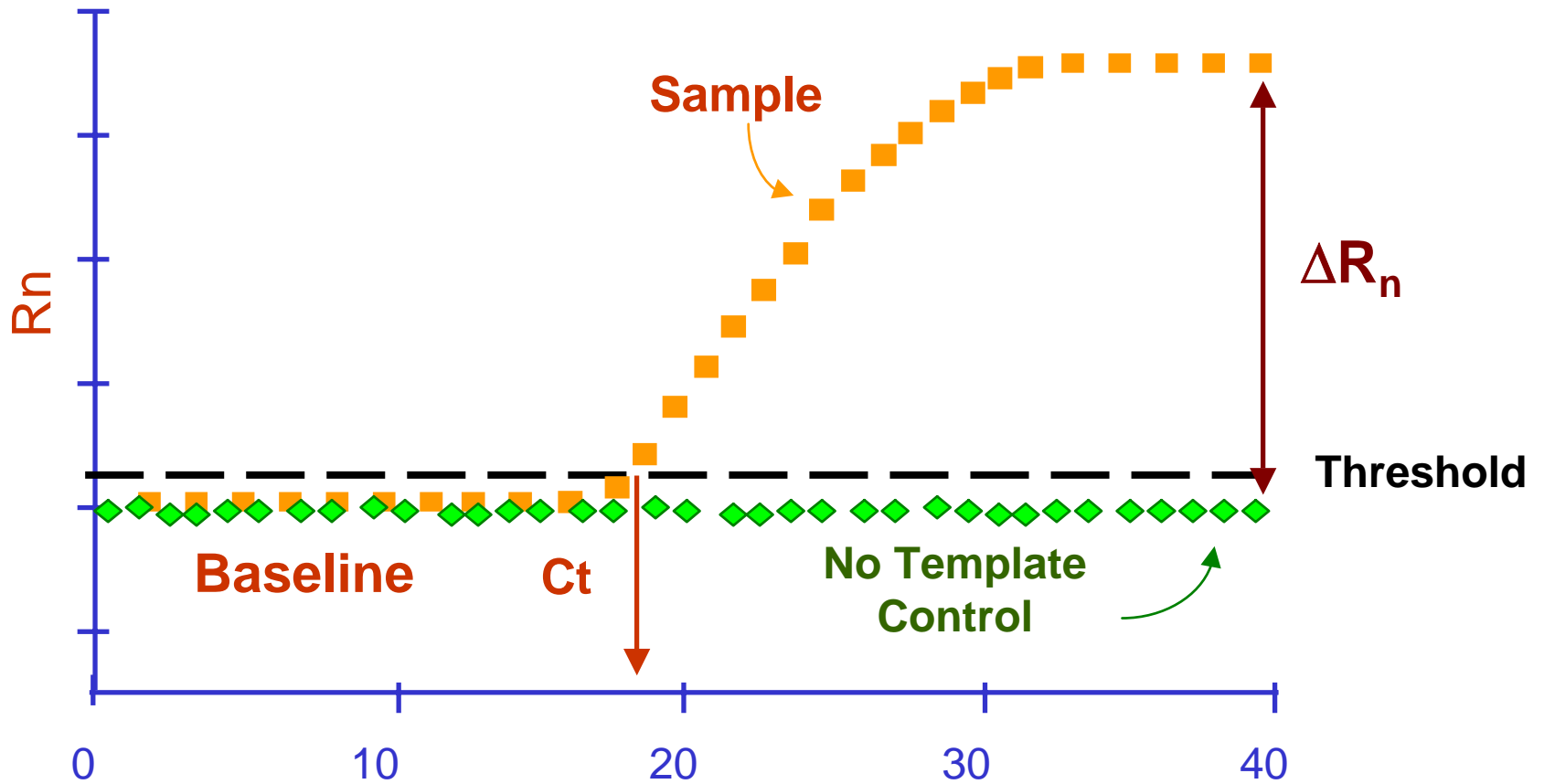


# principle of quantitative real-time PCR...

use *when* rather than *how much*



# Data Review



# Terms Used in Quantitation Analysis

- **Amplicon**
  - A short segment of DNA generated by the PCR process
- **Amplification plot**
  - The plot of Fluorescence signal versus cycle number
- **NTC(no template control)**
  - A sample that does not contain template
- **Passive reference**
  - A dye that provides an internal reference to which the reporter dye signal can be normalized during data analysis
  - To correct for fluctuations cause by change in concentration or volume

# Terms Used in Quantitation Analysis (Cont'd)

- **Standard**
  - A sample of known concentration
- **Threshold**
  - The average standard deviation of  $R_n$  for the early PCR cycles, multiplied by an adjustable factor.
  - Level of fluorescence in which reactions are in the exponential phase of amplification.
- **$C_T$ (threshold cycle)**
  - The fractional cycle number at which the fluorescence passes the fixed threshold
- **Unknown**
  - A sample containing an unknown quantity of template

# Terms Used in Quantitation Analysis (Cont'd)

$$\Delta R_n = (R_{n+}) - (R_{n-})$$

$$R_{n+} = \frac{\text{Emission intensity of Reporter}}{\text{Emission intensity of passive reference}} \quad \text{PCR with template}$$

$$R_{n-} = \frac{\text{Emission intensity of Reporter}}{\text{Emission intensity of passive reference}} \quad \begin{array}{l} \text{PCR without template} \\ \text{Early cycle of real-time} \\ \text{PCR run} \end{array}$$

# Theory of Quantitative PCR

$$y = x(1+e)^n$$

$x$  = starting quantity

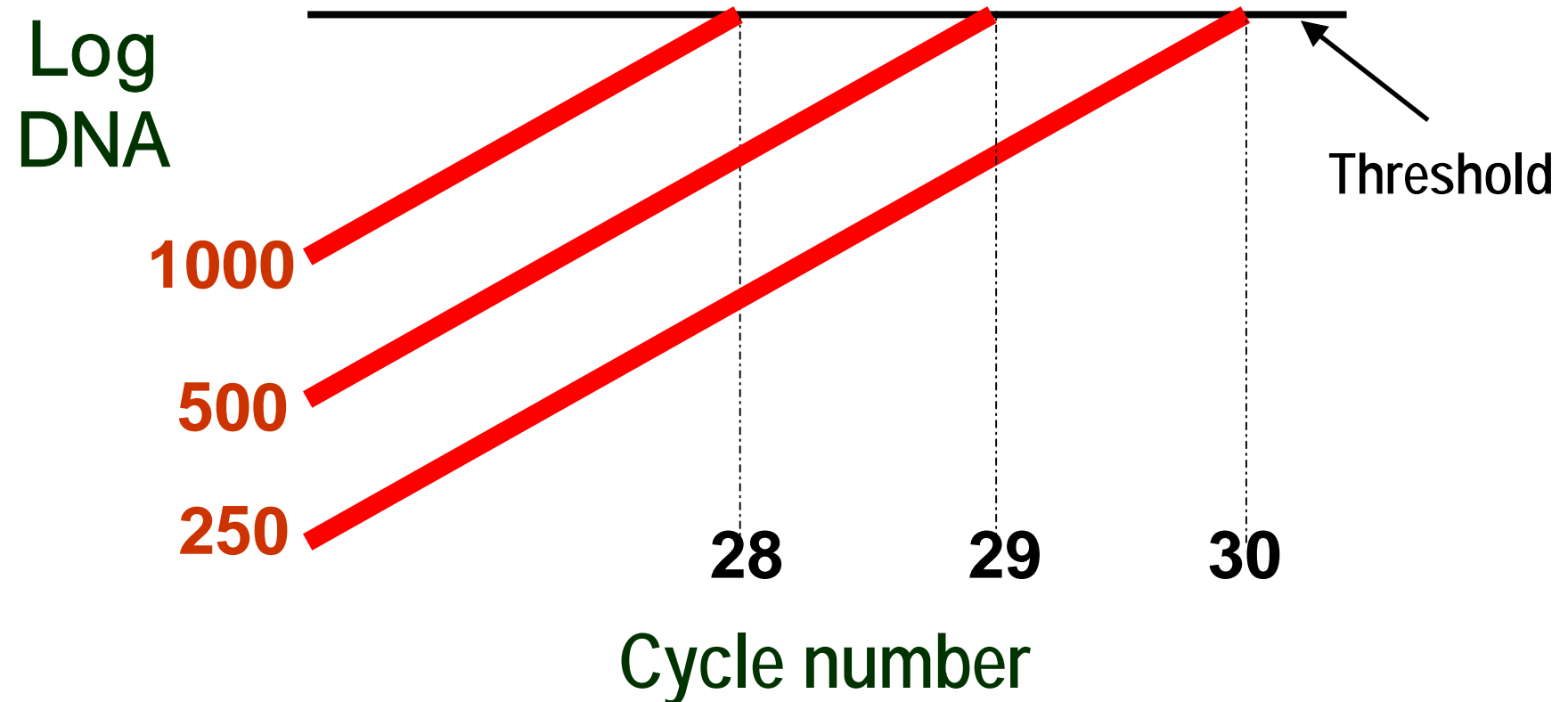
$y$  = yield

$n$  = number of cycles

$e$  = efficiency

# Relationship between Initial Copy Number and Cycle Number

100% efficiency would be a doubling of product at each cycle.



# Effect of Amplification Efficiency

$$y = x(1+e)^n$$

Case 1: e = 0.9

$$y = 100 (1+0.9)^{30}$$

$$y = 2.3 \times 10^{10}$$

Case 2: e = 0.8

$$y = 100 (1+0.8)^{30}$$

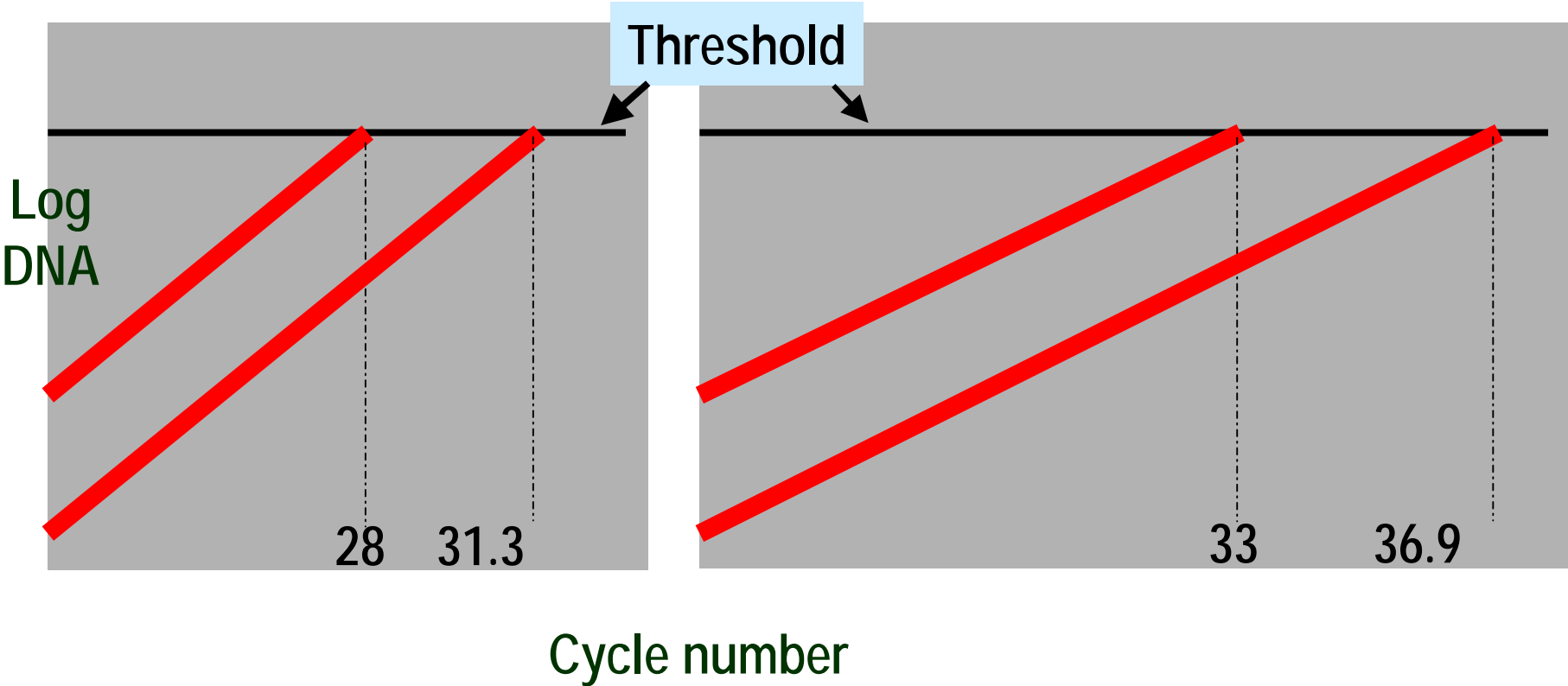
$$y = 4.6 \times 10^9$$

Result: A difference of 0.1 in amplification efficiencies created a 5-fold difference in the final ratio of PCR products.

# Amplification Efficiency

100%  
10-fold = 3.3 cycles

80%  
10-fold = 3.9 cycles



# sequence detection applications

## End-point PCR

qualitative  
simple +/- results

- PCR product detection  
eg. pathogens  
transgenes
- Genotyping  
eg. allelic discrimination (zygosity)  
single nucleotide polymorphisms (SNPs)

## Real-time PCR

quantitative  
complex results

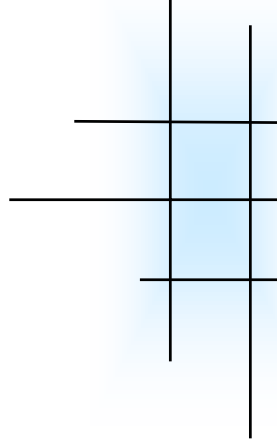
- Absolute quantitation
- Relative quantitation
- PCR interrogation  
eg. optimisation

## Hybridisation analysis

complex results

- Dissociation analysis  
eg. DNA melting/annealing curves (SYBR Green)
- Probe hybridisation  
Monitor hybridisation behaviour of a specific sequence (probe)

# Real-Time PCR:

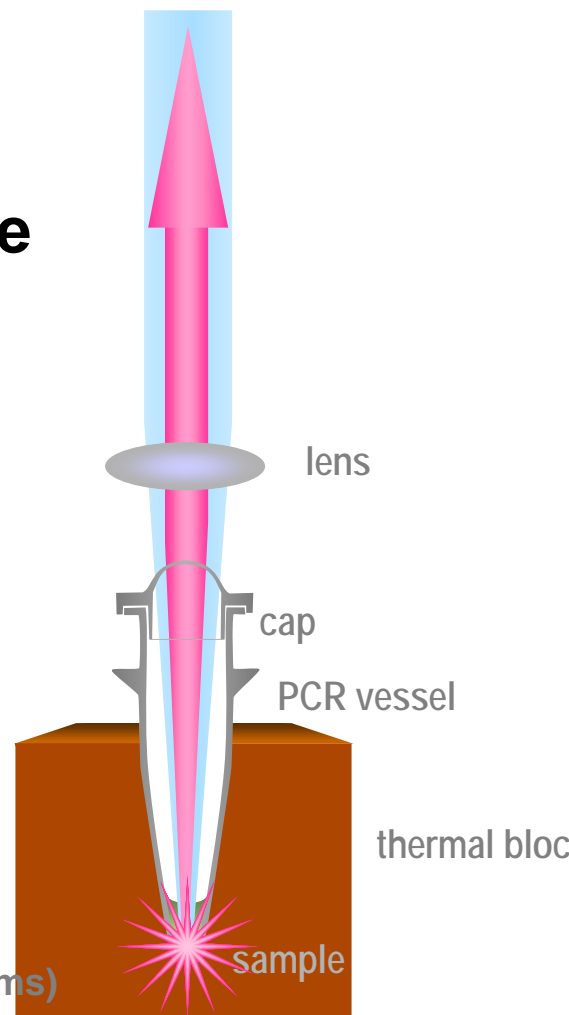


## 4. Optics

# “real-time PCR”

is *sequence detection* in a closed-tube

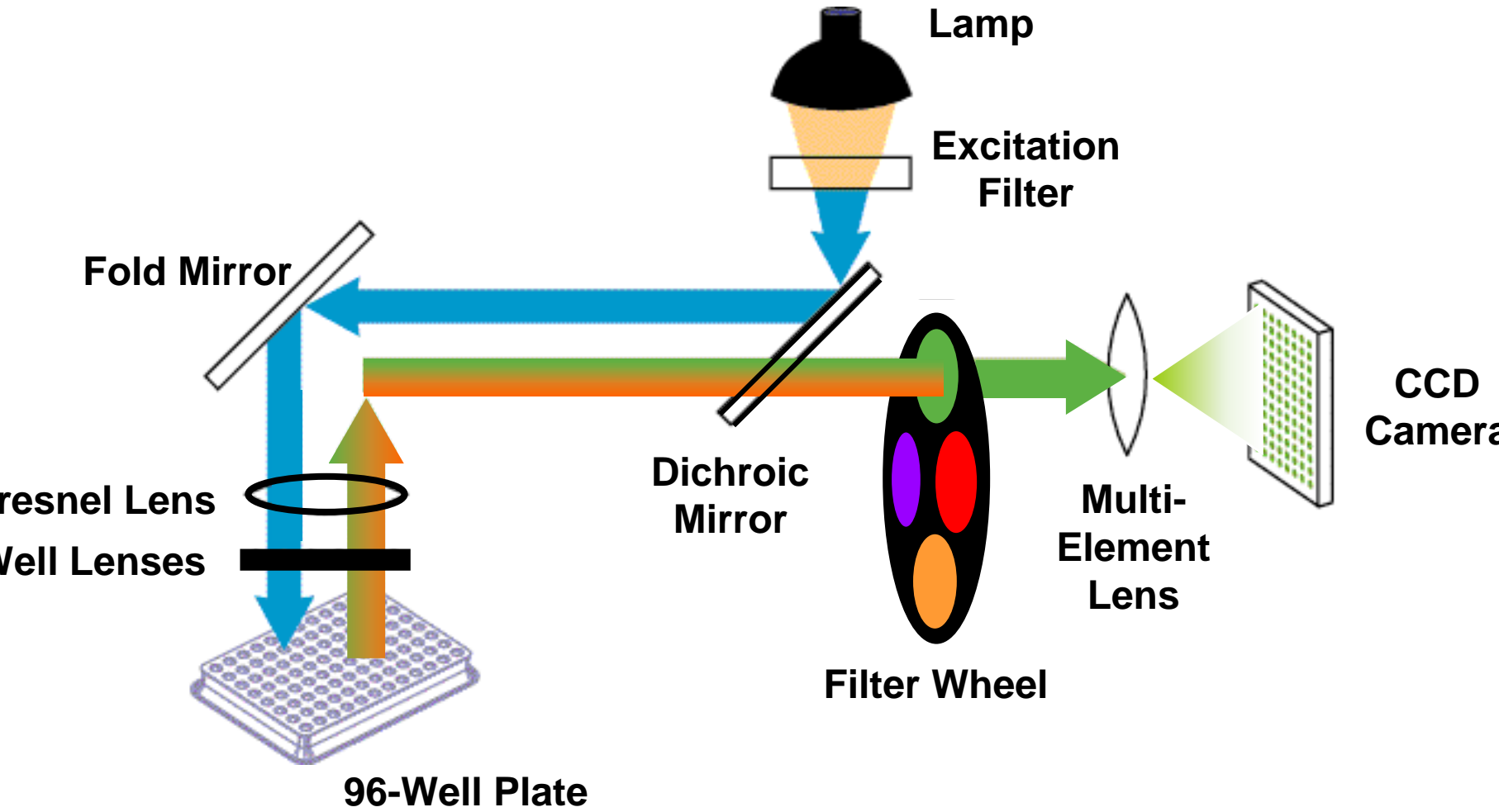
- closed-tube chemistry (reduced contamination risk)
- no post-PCR processing (no gels, films etc)
- automated, objective analysis
- 0.2 mL tube format is standard
- qualitative; single data reading
- quantitative; many data readings
- genotyping (& SNPs) (single nucleotide polymorphisms)



# Hardware Features

- Tungsten-halogen lamp excitation source
- Cooled CCD camera
- Four position fluorescence emission filter wheel
- Peltier-based 96-well block thermal cycling system
- Reduced instrument footprint
- Novel instrument door design

# 7000 System Optical Schematic



# Features and Benefits

- Multi-color detection capability provides application flexibility:
  - multiplex nucleic acid quantitation
  - allelic discrimination assays (SNP detection) using TaqMan<sup>®</sup> MGB probes
  - plus/minus assays using an Internal Positive Control (IPC)

# Real-Time PCR:

**End.**

