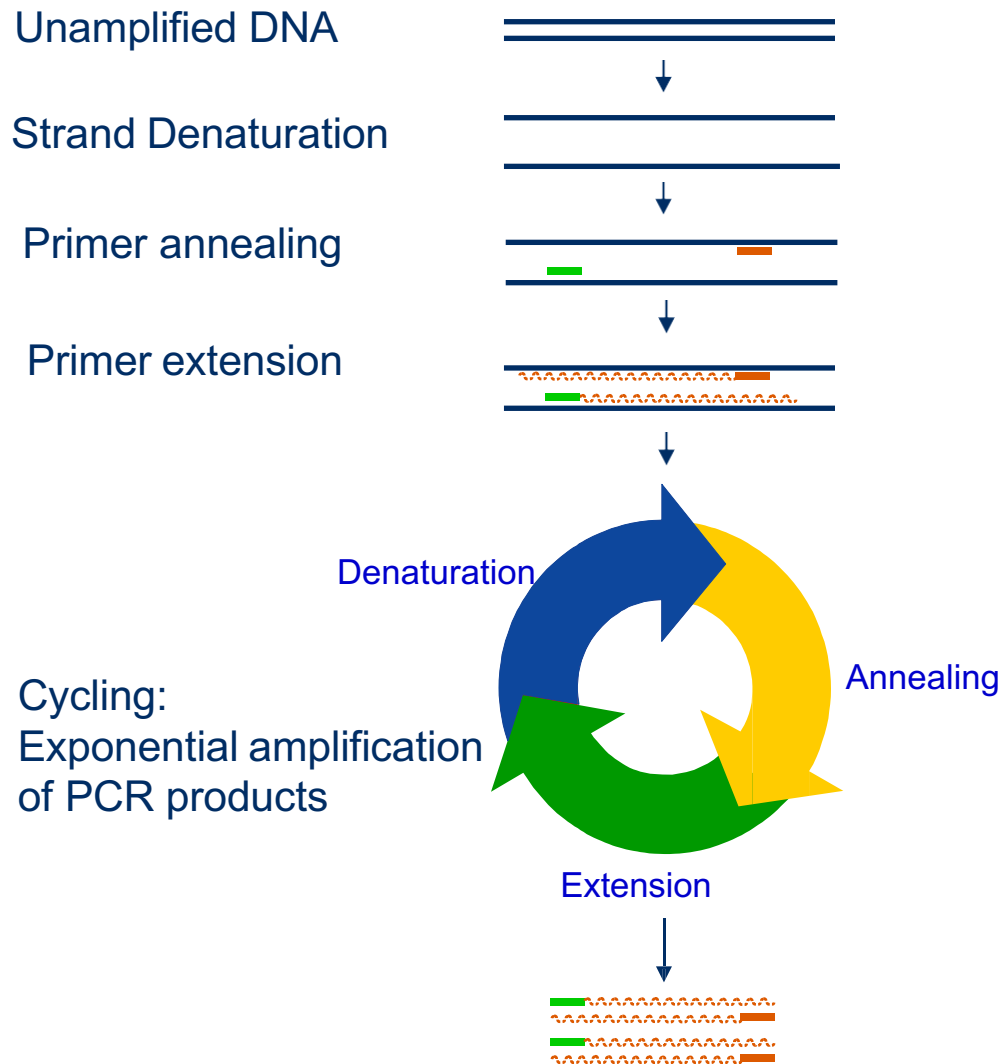


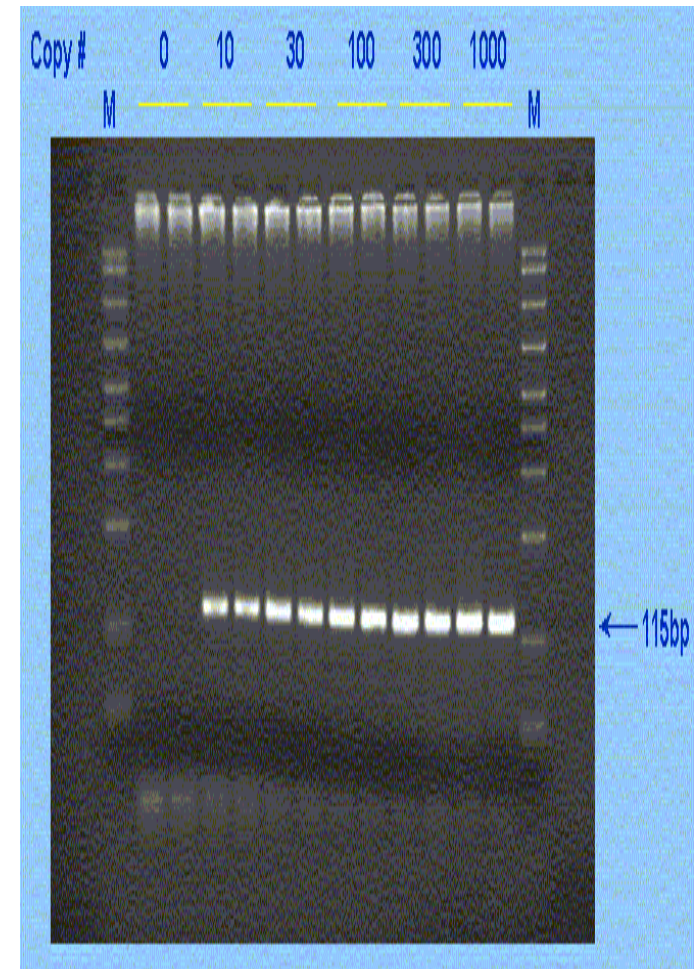
Fundamentals of Real-Time PCR

Polymerase Chain Reaction (PCR)



Limitations of Traditional End-Point PCR

- Low sensitivity
- Poor precision
- Results are not expressed as numbers
- Ethidium bromide staining is not quantitative
- Post-PCR processing required
- Narrow dynamic range (<2 logs)



Alternative Quantitative Methods

- ◆ Northern Blots
- ◆ RNase protection assays
- ◆ In Situ hybridization
- ◆ Competitive PCR
- ◆ cDNA arrays

Problems Associated With These Alternative Methods

- ◆ Difficulty achieving high throughput
- ◆ Using large RNA/DNA quantities
- ◆ Limited dynamic range
- ◆ Threat of contamination
- ◆ Difficulty designing controls
- ◆ Difficulty creating and optimizing quantitative assays

Goals For Improvement of Quantitative PCR

- ◆ Eliminate use of gel electrophoresis
- ◆ Increase reproducibility
- ◆ Enable use of internal controls/standards
- ◆ Reduce turnaround time
- ◆ Increase throughput
- ◆ Reduce sample amount usage

Quantitative Real-Time PCR

Detection of PCR product growth throughout the amplification process

- No post-PCR processing required**
- Collects data during high-precision exponential phase**

3 Phases of PCR

Exponential:

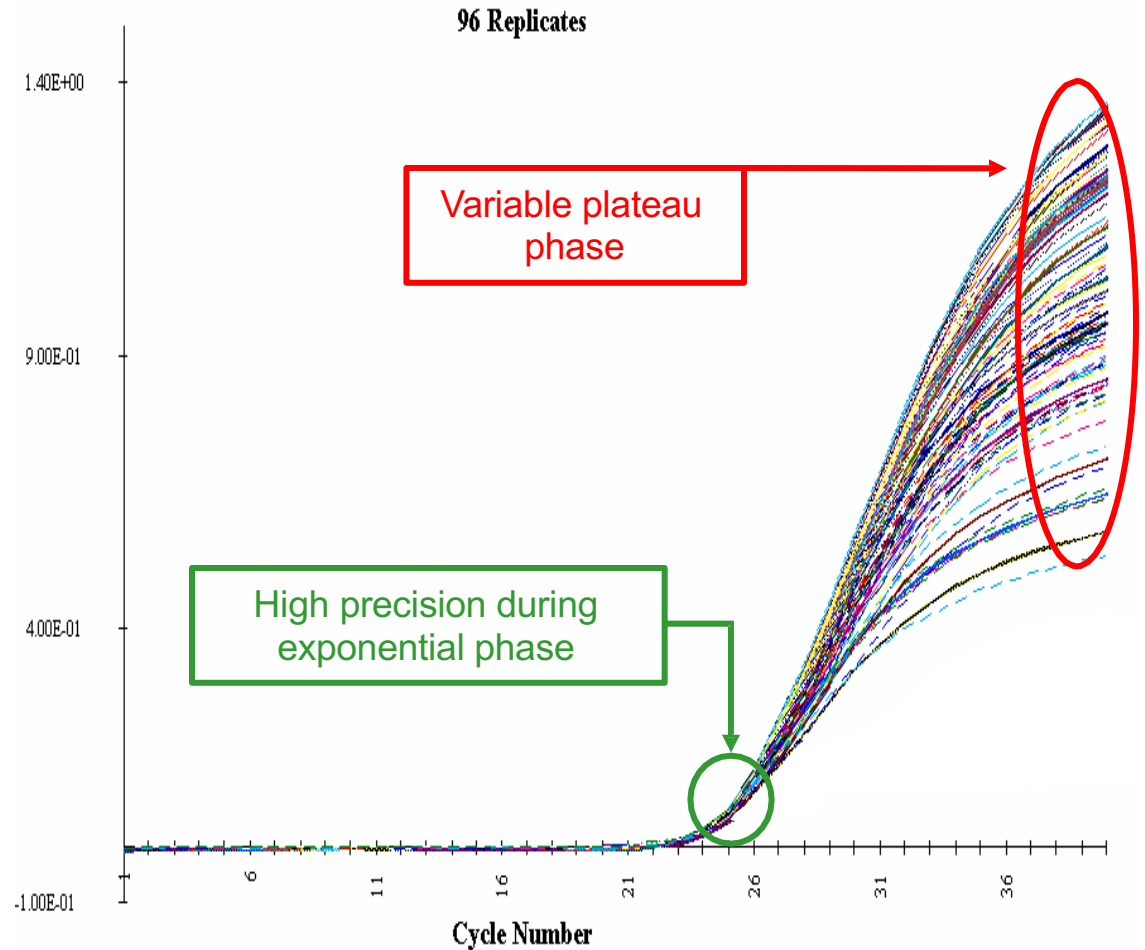
- Exact doubling of product
- Reaction is very precise and specific

Linear:

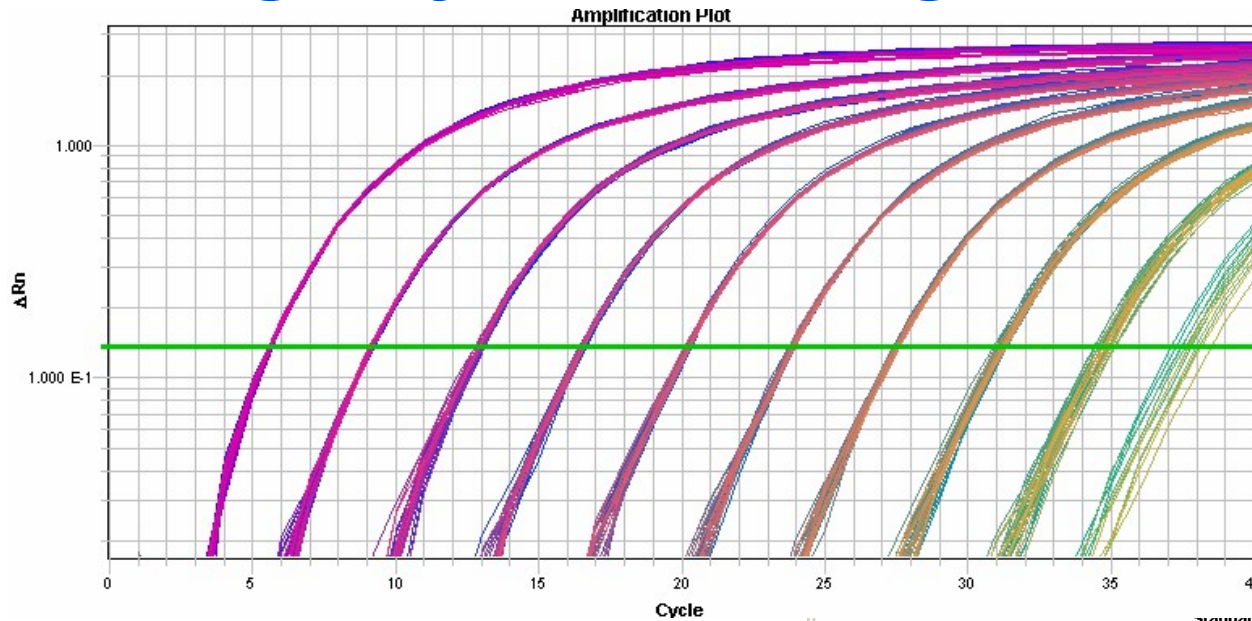
- The reaction components are becoming limited
- The reaction efficiency is dropping

Plateau:

- The reaction has stopped
- No more products are being made

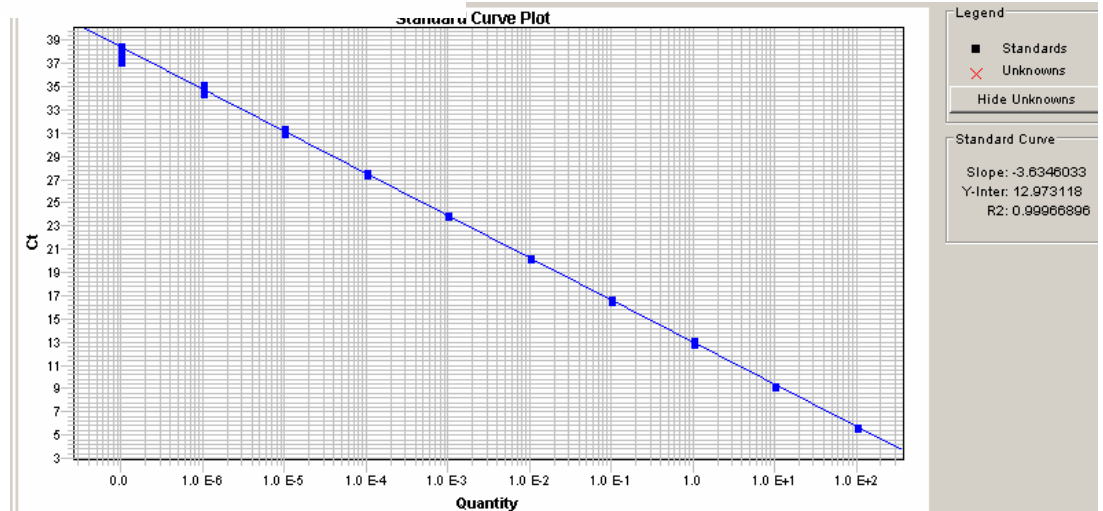


Large Dynamic Range



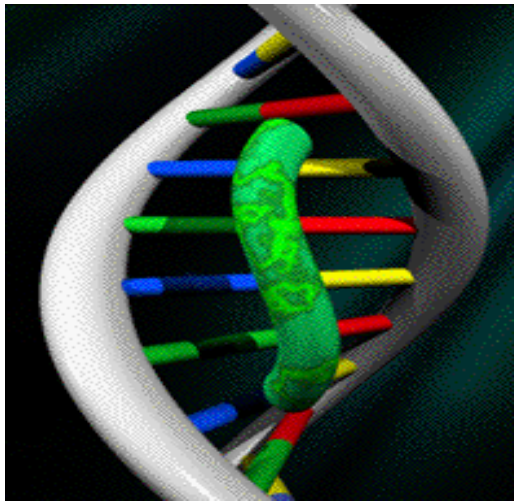
Amplification of serial dilutions of 18S rRNA target in 16 replicates

Standard curve showing 9 logs of linear dynamic range



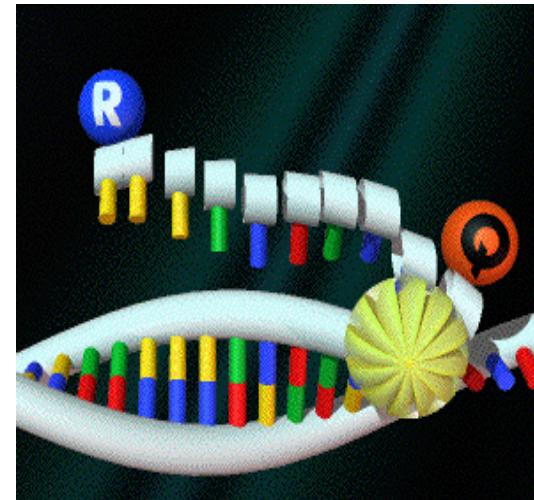
Real-Time PCR Chemistries

SYBR[®] Green I dye



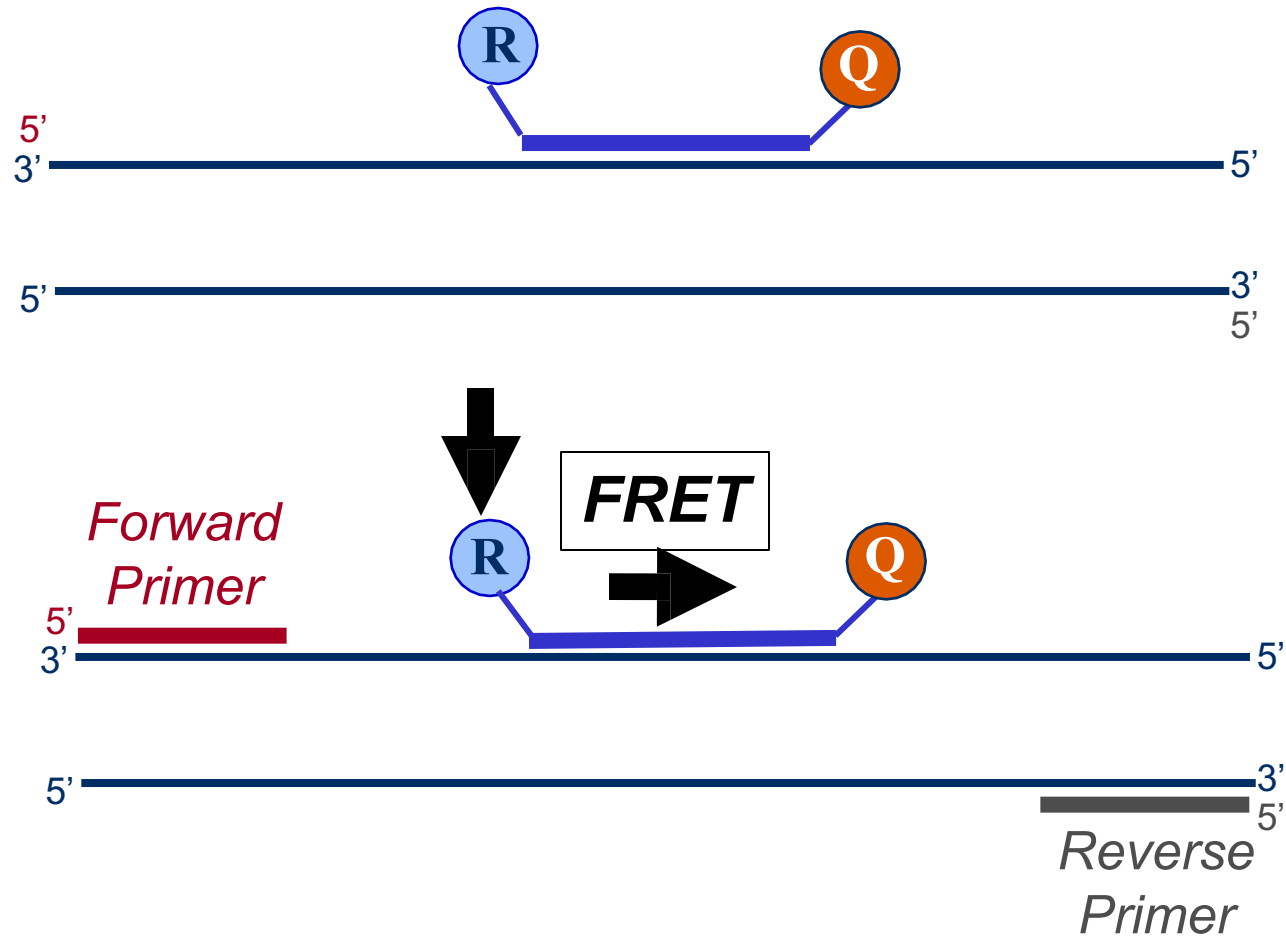
Binds double
stranded DNA

Fluorogenic 5' Nuclease Assay



Uses a TaqMan[®] probe

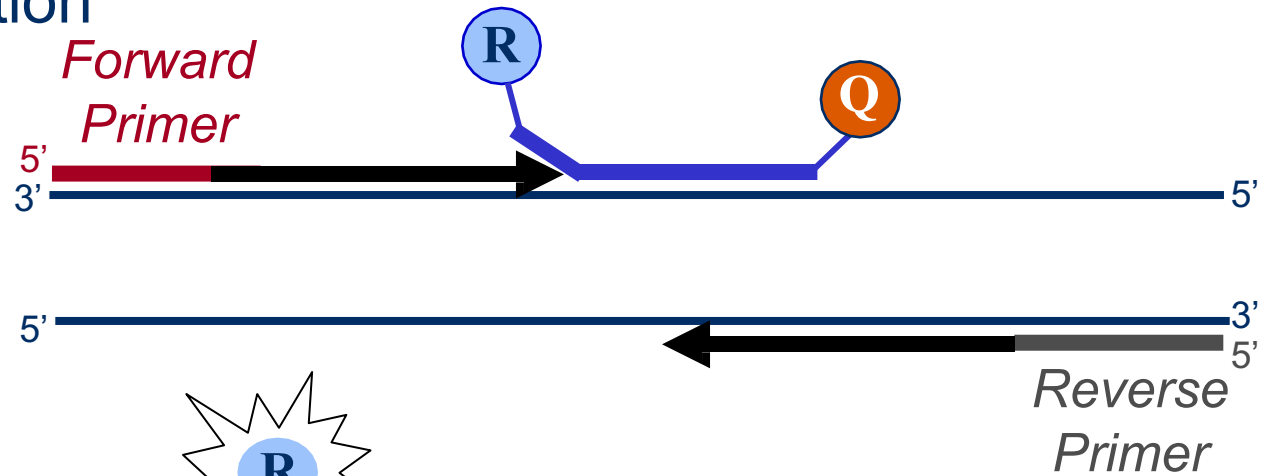
Fluorogenic 5' Nuclease Assay



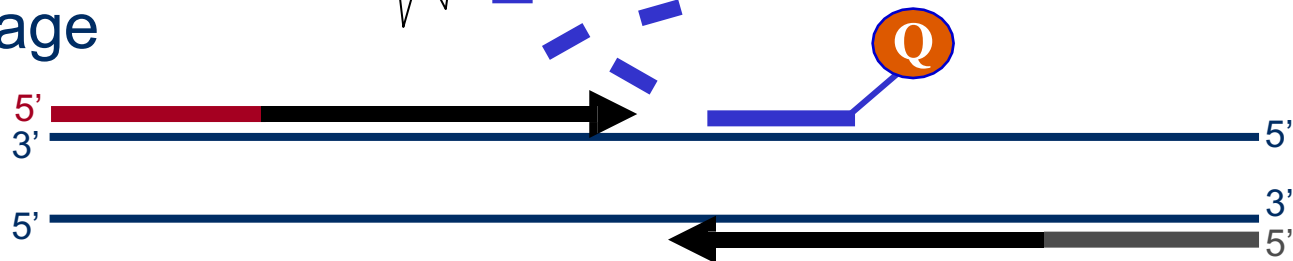
***FRET= Fluorescence Resonance Energy Transfer**

Fluorogenic 5' Nuclease Assay

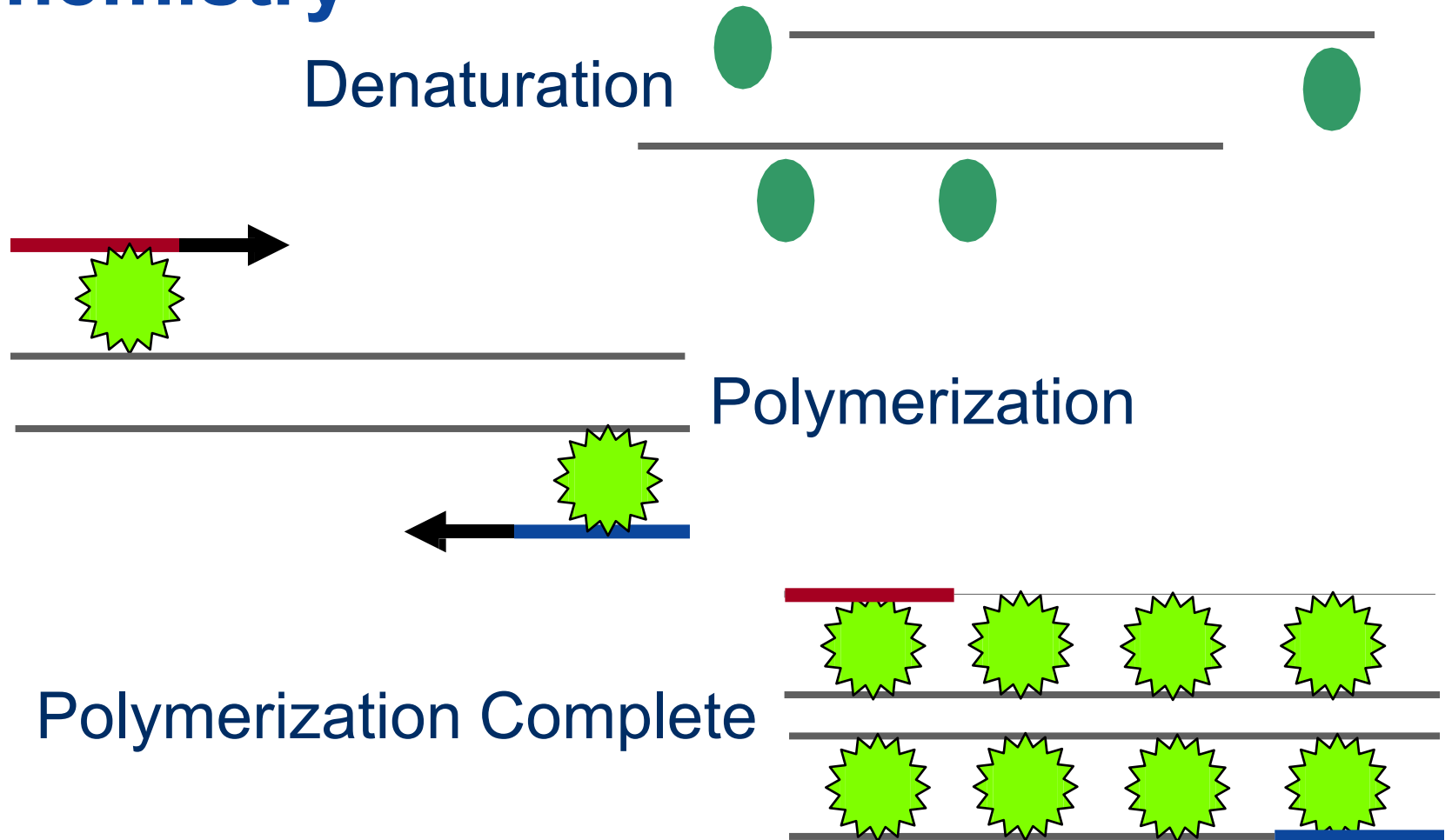
Displacement during
Polymerization



Cleavage



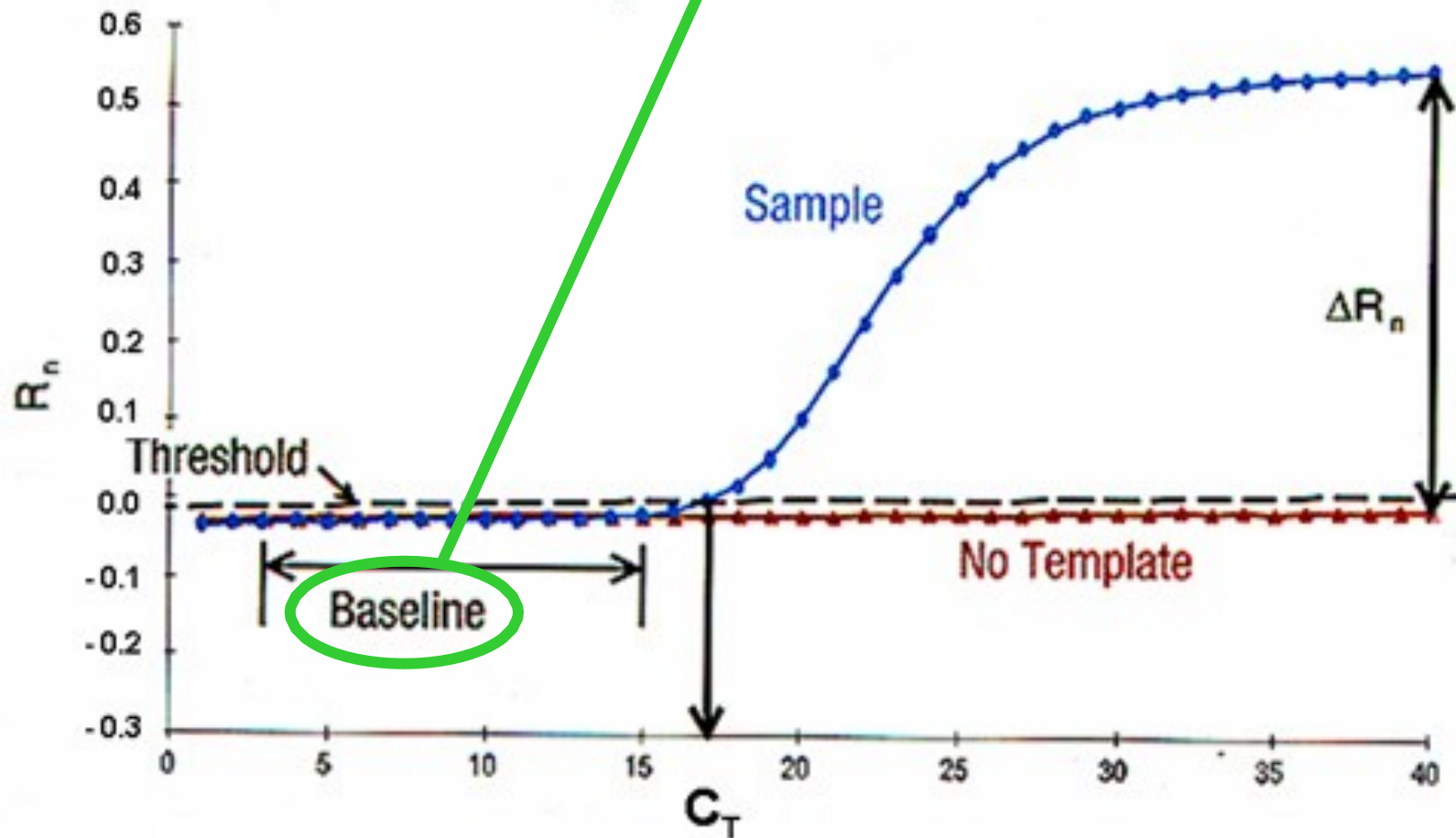
SYBR[®] Green I Dye Assay Chemistry



Terminology

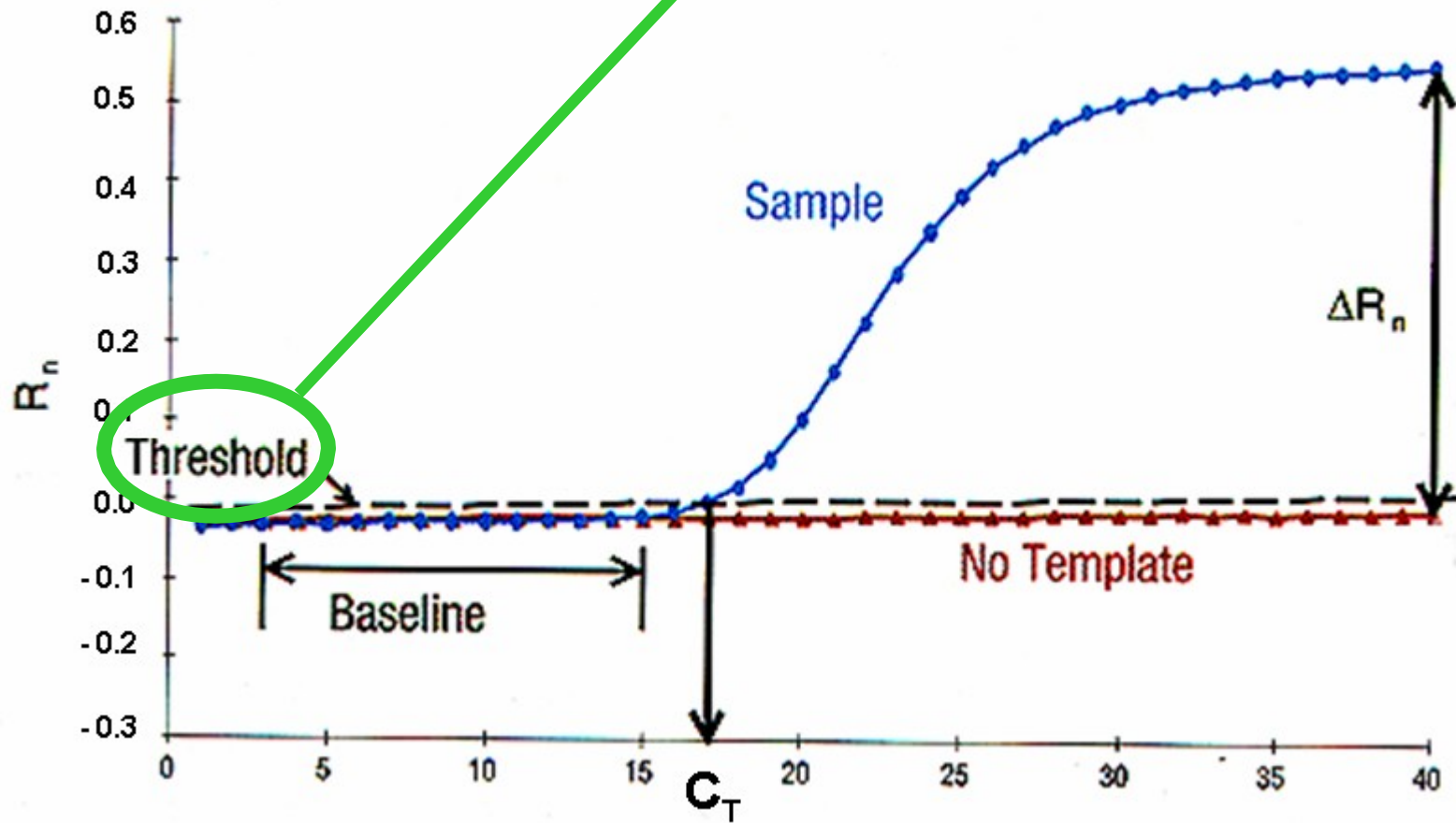
Baseline:

The initial cycles prior to any detectable amplification, in which there is little change in fluorescent signal.



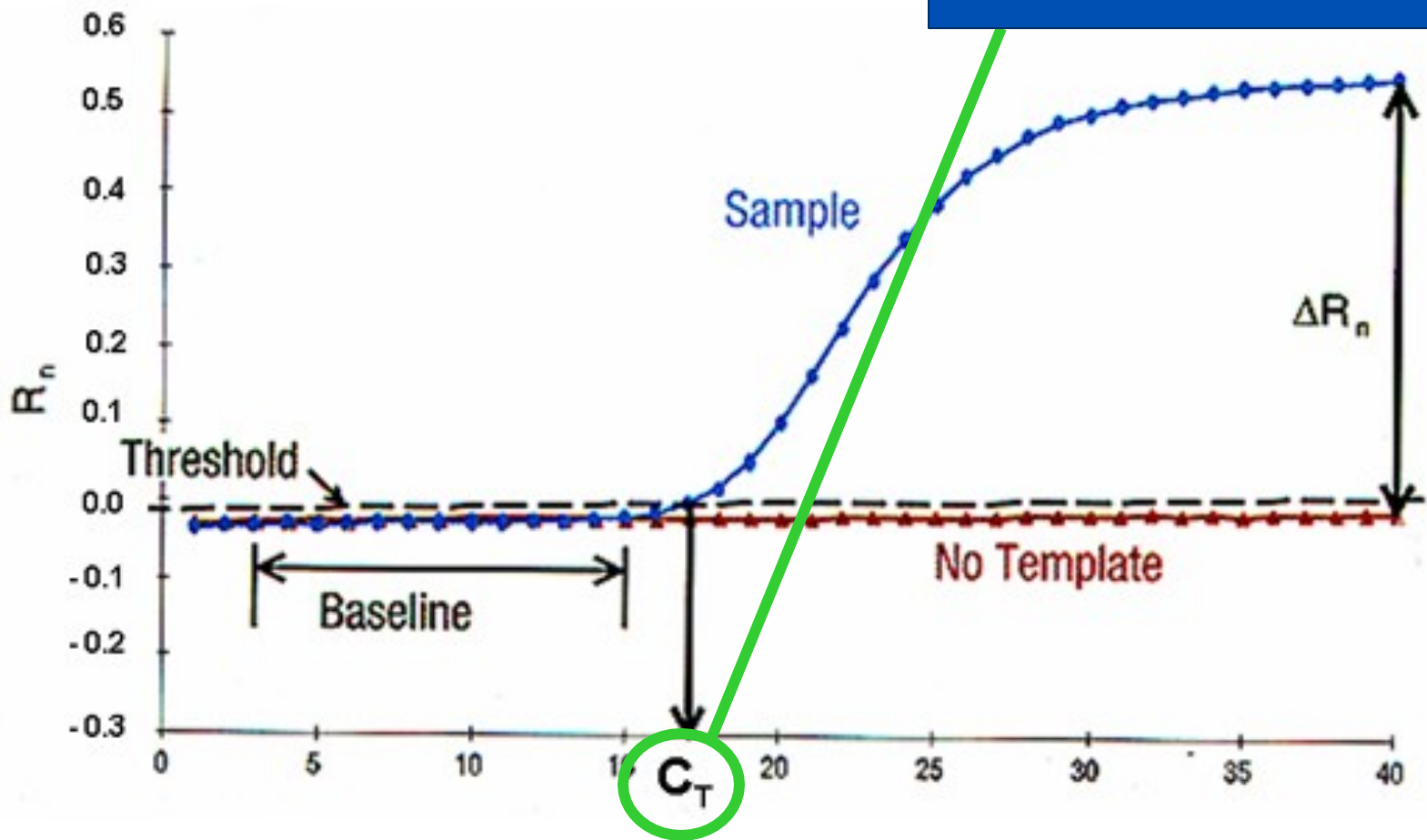
Threshold:

Level at which fluorescence is detected in reactions during the exponential phase of PCR



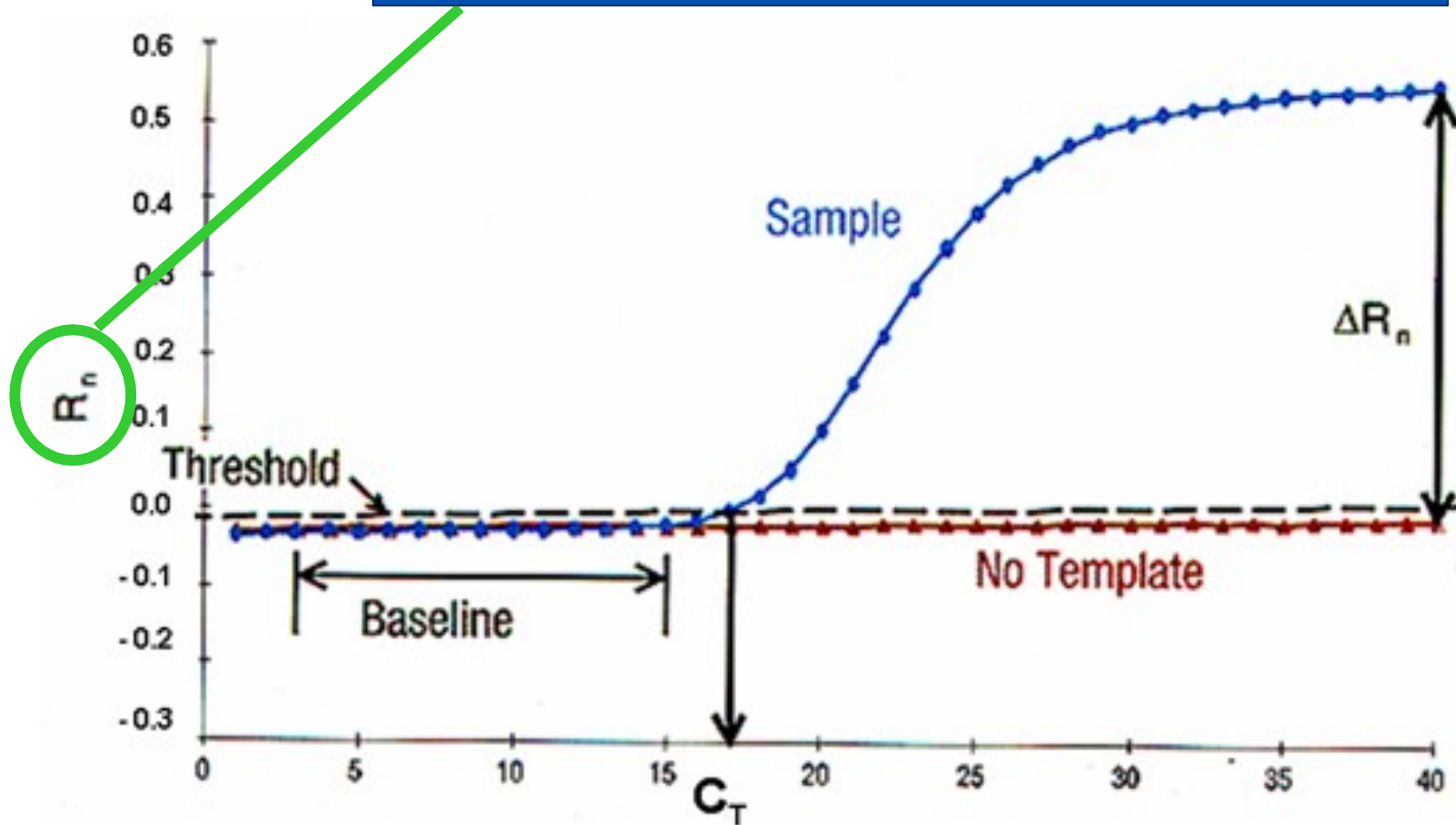
Cycle Threshold (C_T):

The cycle (point in time) at which the PCR product crosses the threshold of detection.

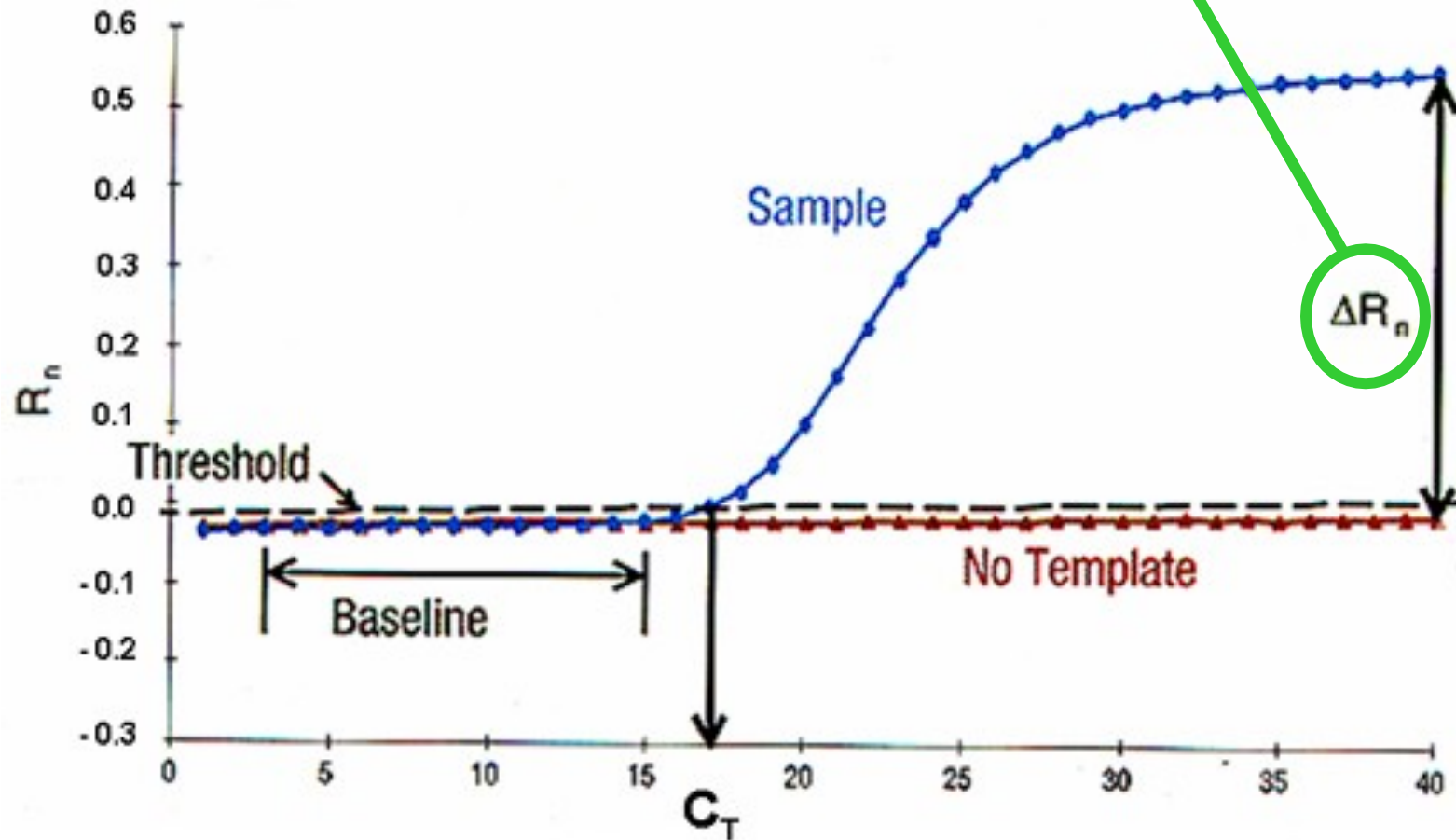


R_n :

Reporter signal divided by the passive reference ROX™
Dye signal. Normalized to account for pipetting
variation.



ΔR_n : Normalized reporter signal minus background (baseline level).



Types of Quantitation Assays

```
graph TD; A[Types of Quantitation Assays] --> B[Absolute quantitation]; A --> C[Relative quantitation]; B --> D[Provides absolute measurement of starting copy number]; D --> E[-Requires standards of known quantity]; D --> F[-e.g. Detecting DNA copy number for forensics purposes];
```

Absolute quantitation

Relative quantitation

Provides absolute measurement
of starting copy number

- Requires standards of known quantity
- e.g. Detecting DNA copy number for forensics purposes

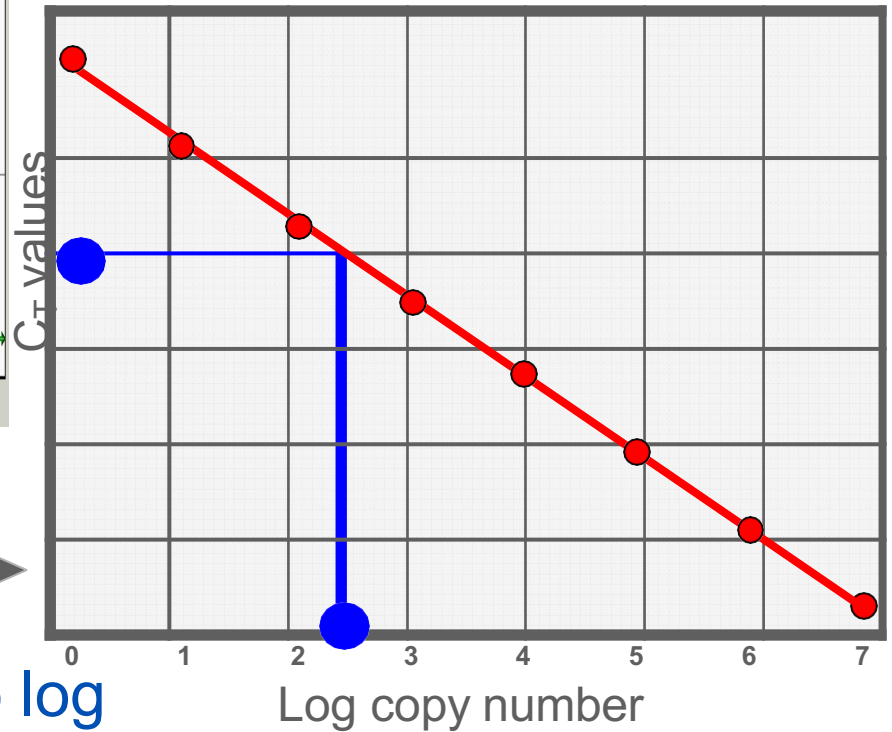
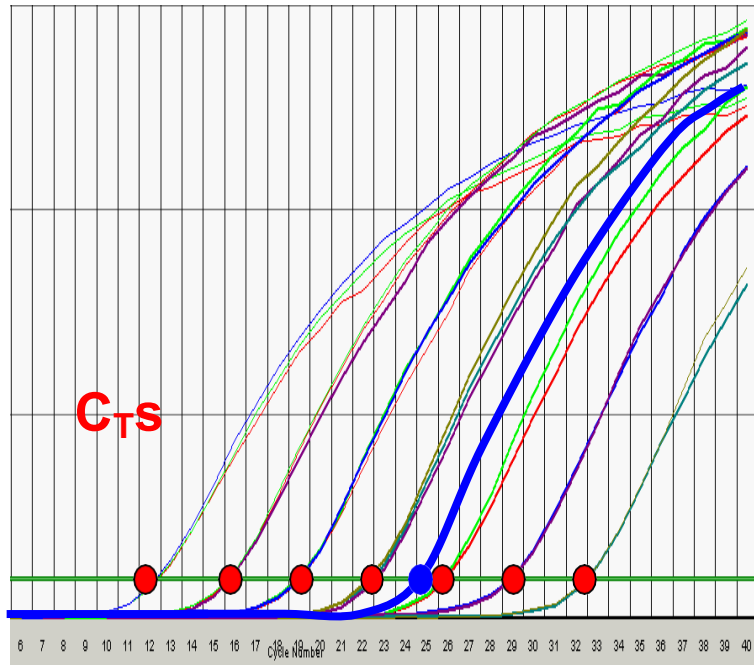
Forensic Applications

Is there any
(amplifiable) DNA?



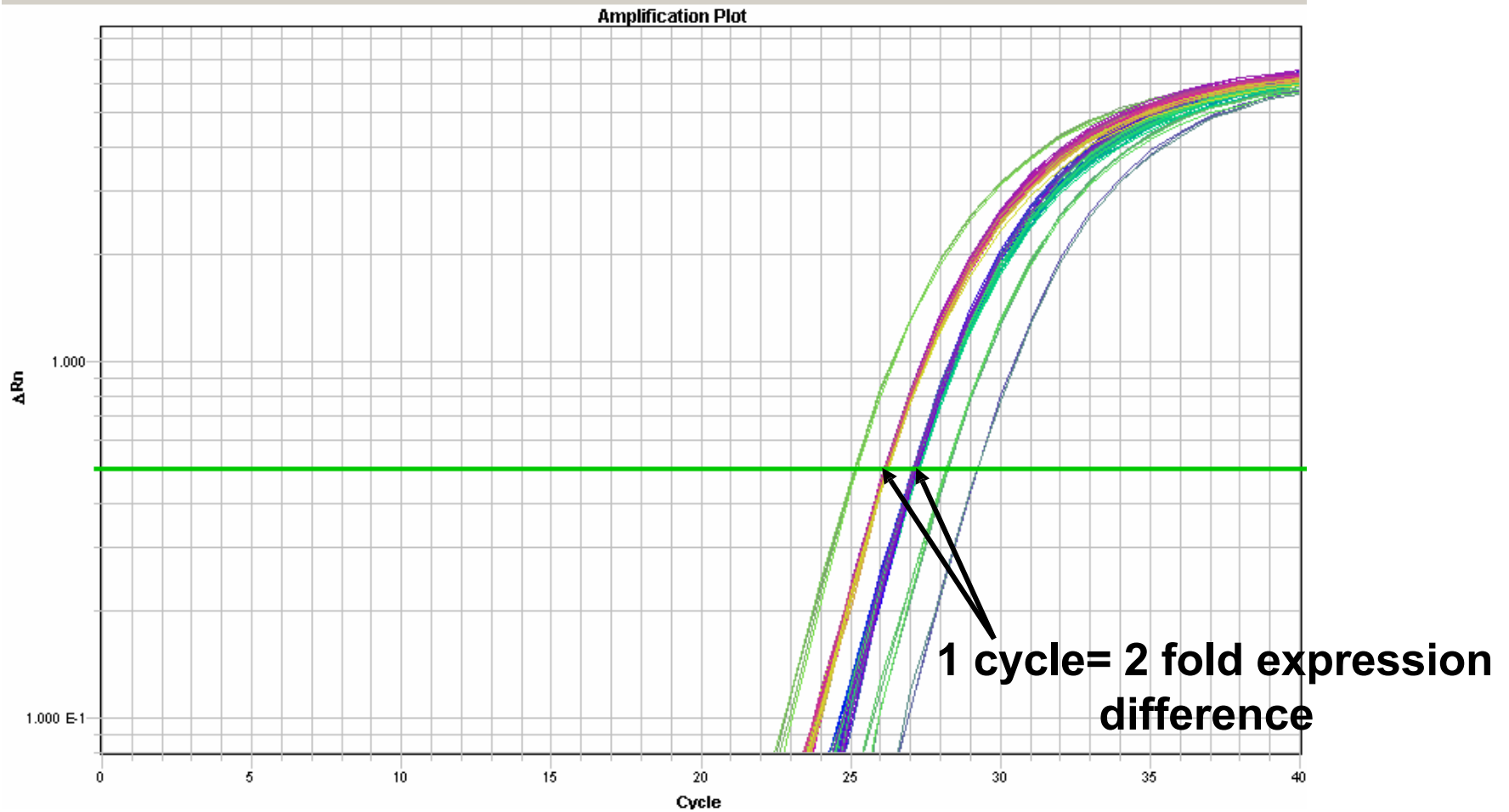
How much is there?

From Fluorescence to Copy Number



C_T is directly proportional to \log of amount of input template

High (100%) Amplification Efficiency



Types of Quantitation Assays

```
graph TD; A[Types of Quantitation Assays] --> B[Absolute quantitation]; A --> C[Relative quantitation]; C --> D[Provides accurate discrimination between relative amounts of starting material]; D --> E["-e.g. Comparing expression levels of wildtype vs. mutated alleles"]; D --> F["-e.g. Comparing expression levels of a gene across different tissues or between different biological conditions"]; D --> G["-e.g. Validating array results"];
```

Absolute quantitation

Relative quantitation

Provides accurate discrimination between relative amounts of starting material

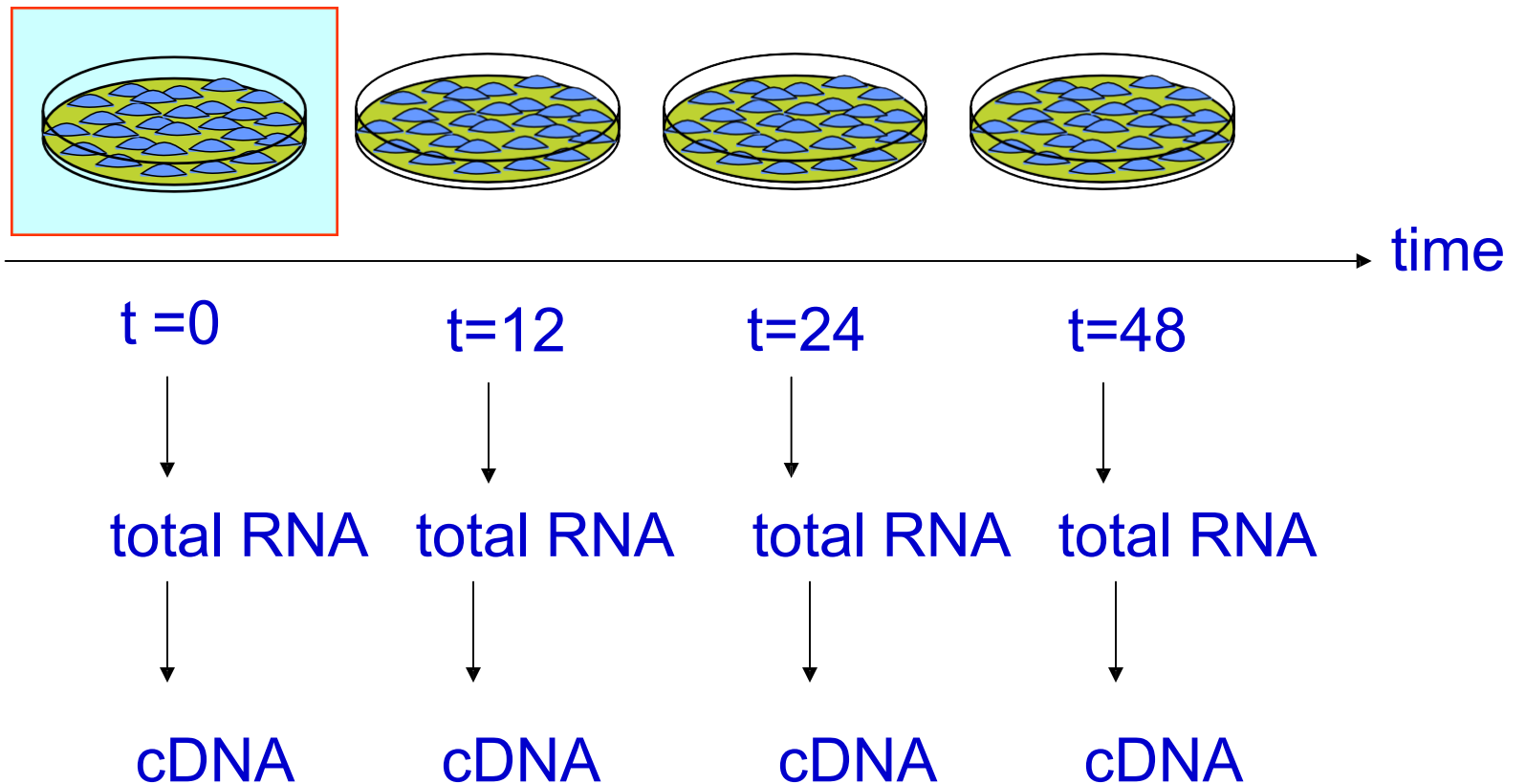
–e.g. Comparing expression levels of wildtype vs. mutated alleles

–e.g. Comparing expression levels of a gene across different tissues or between different biological conditions

–e.g. Validating array results

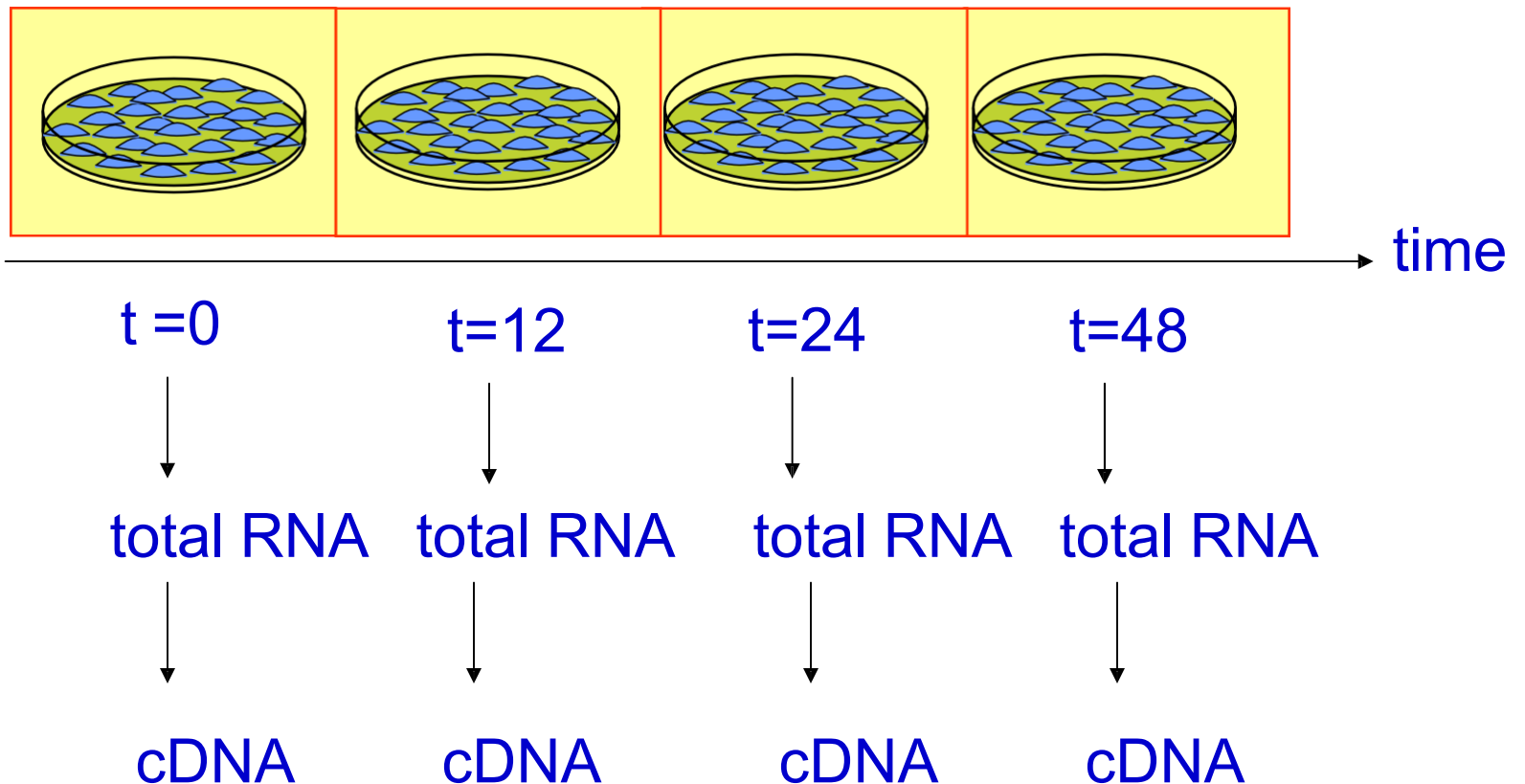
Relative Quantitation

Calibrator= The sample used as the basis for comparative results

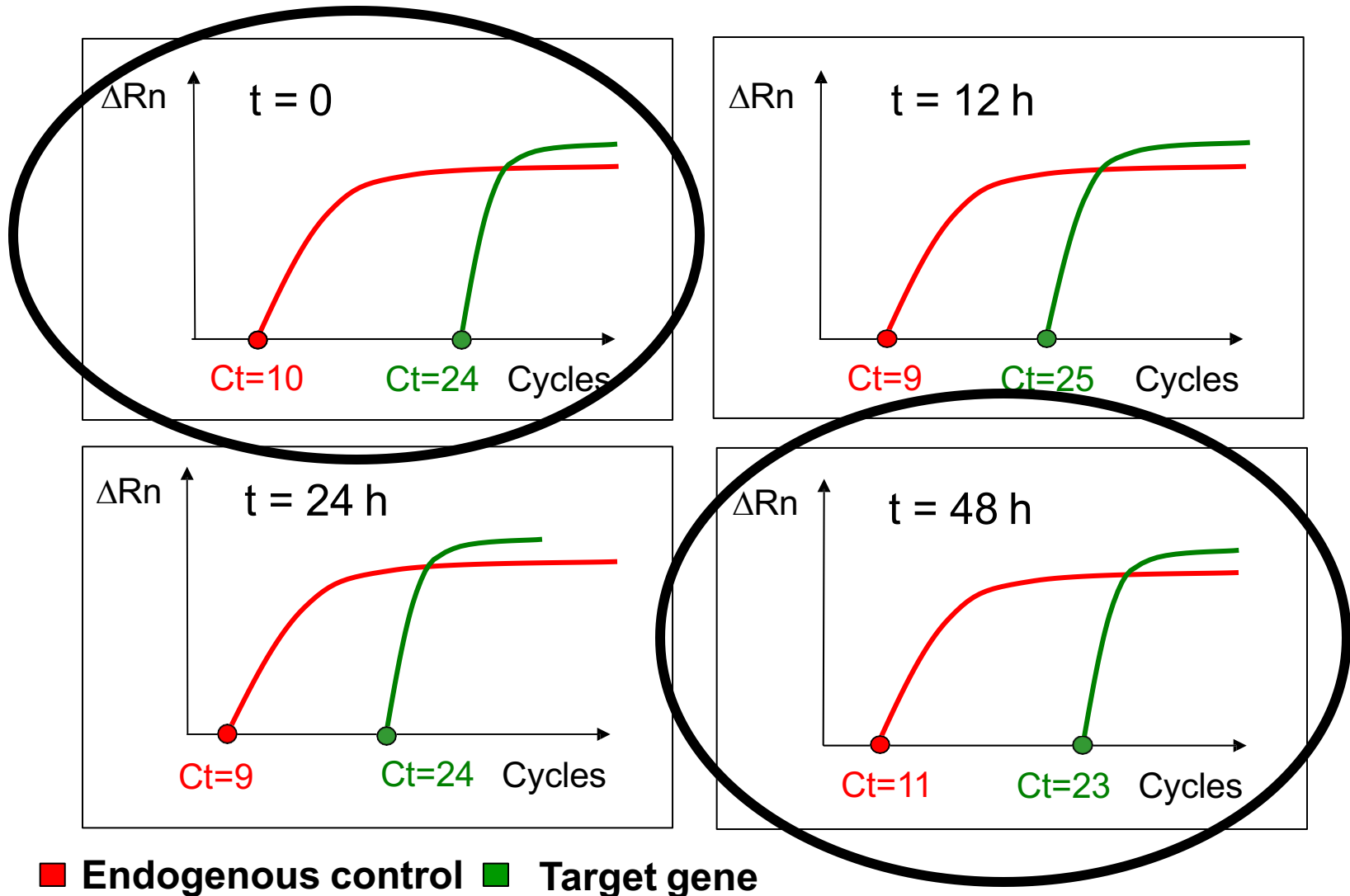


Relative Quantitation

Endogenous Control = Target used to normalize for sample handling
(e.g. 18S rRNA, GAPDH, β -actin)



Comparative C_T Method



Comparative C_T Method Calculation:

Normalized to endogenous control:

$$C_{T\ 48hrs} \text{ (23)} - C_{T\ Endo.\ Control} \text{ (11)} = \Delta C_{T\ 48hrs} \text{ (12)}$$

$$C_{T\ 0hrs, Calibrator} \text{ (24)} - C_{T\ Endo.\ Control} \text{ (10)} = \Delta C_{T\ 0hrs} \text{ (14)}$$

Normalized to calibrator sample:

$$\Delta C_{T\ 48hrs} \text{ (12)} - \Delta C_{T\ 0hrs, Calibrator} \text{ (14)} = \Delta\Delta C_{T} \text{ (-2)}$$

Relative fold change:

$$2^{-\Delta\Delta C_T} = 2^{\text{(2)}} = \text{(4)}$$

There is a 4-fold overexpression of my gene at T=48h compared T=0h...48hrs after drug treatment!

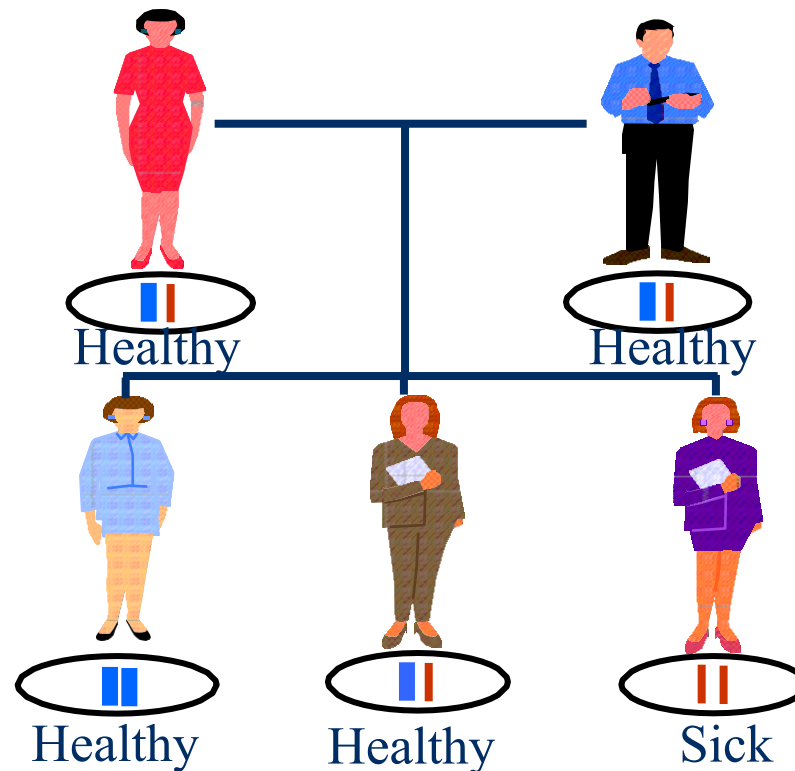
Applications

- ◆ Real-Time Detection
 - Absolute Quantitation
 - Relative Quantitation
- ◆ End Point Detection
 - Allelic Discrimination (SNP)
 - +/- Assay (IPC)
 - Pathogen Detection

Allelic Discrimination (SNP)

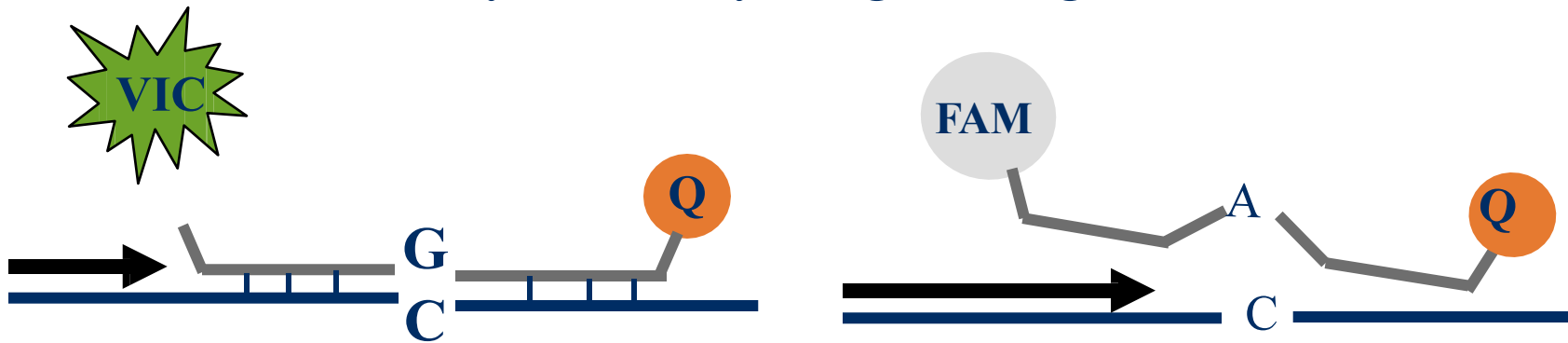
◆ Determines the genotype of samples

- Possible to differentiate a single nucleotide polymorphism (SNP)

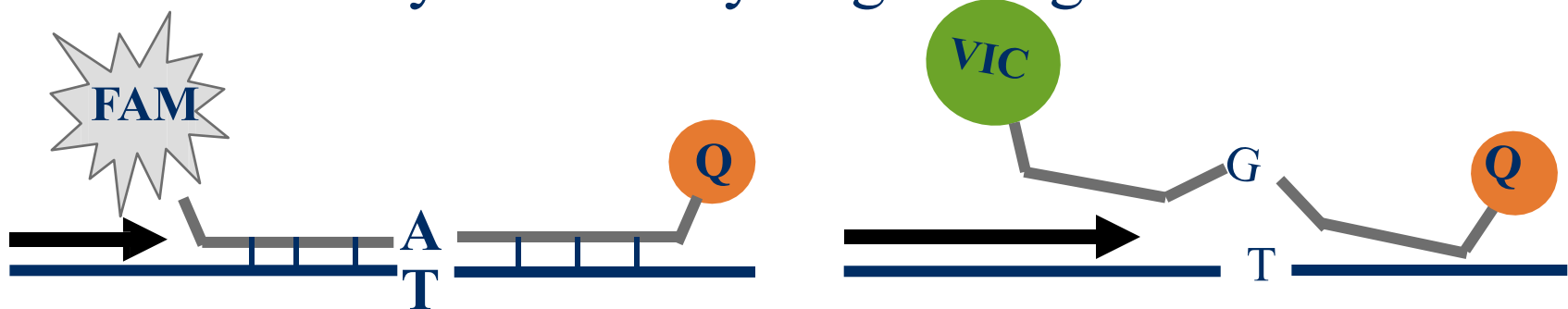


Allelic Discrimination Assay

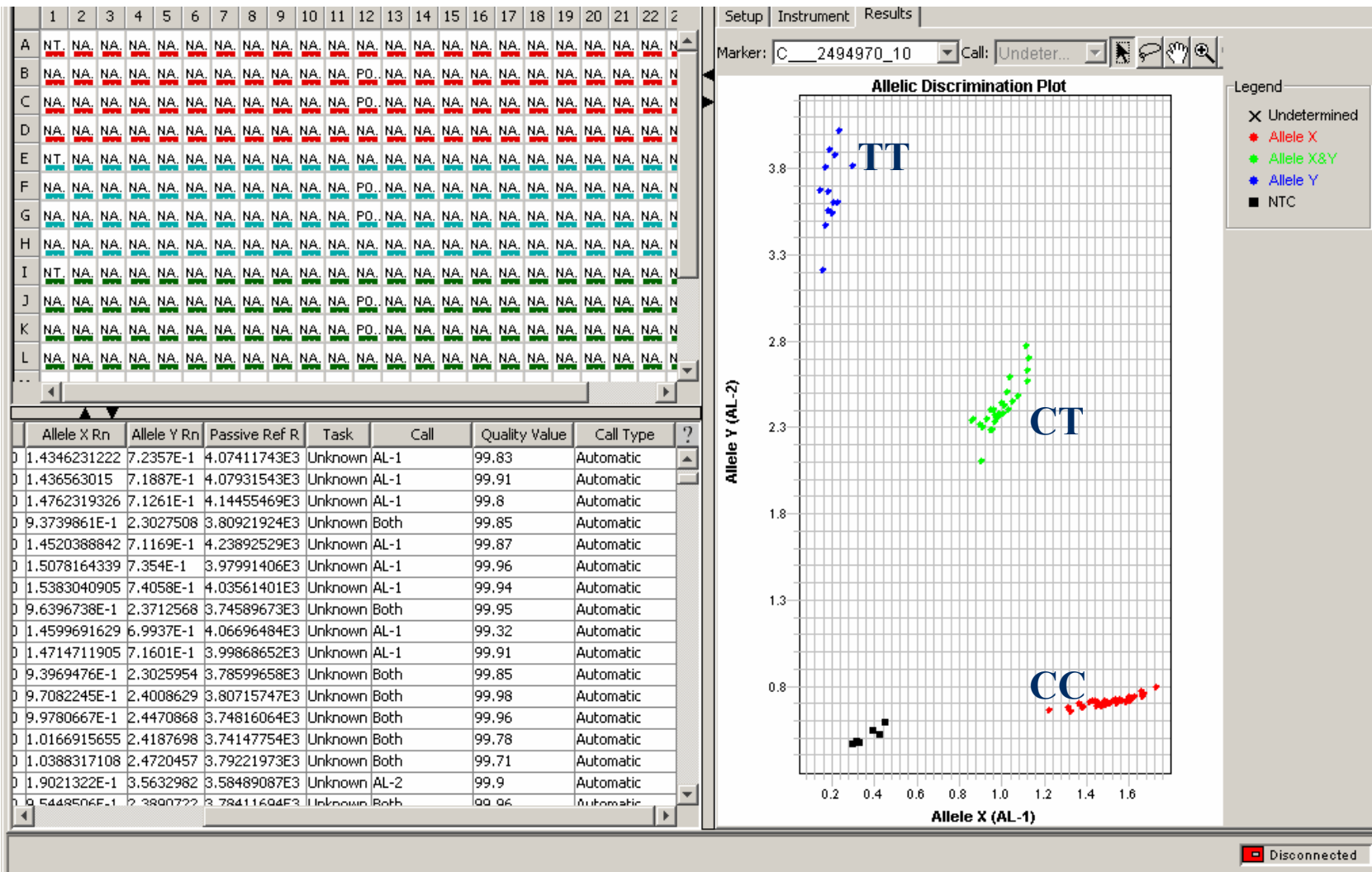
Allele C - only VIC^R dye signal is generated



Allele T - only FAMTM dye signal is generated



Allelic Discrimination (SNP)



Applications

- ◆ Real Time Detection

- Absolute Quantitation
- Relative Quantitation

- ◆ End Point Detection

- Allele Detection (SNP)
- +/- Assay (IPC)
 - Pathogen Detection

Internal Positive Control (IPC)

- ◆ Distinguish true target negative from PCR inhibition
- ◆ Co-amplified with target DNA without compromising amplification of the target sequence

Plus/Minus assay with IPC

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Disconnected NUM

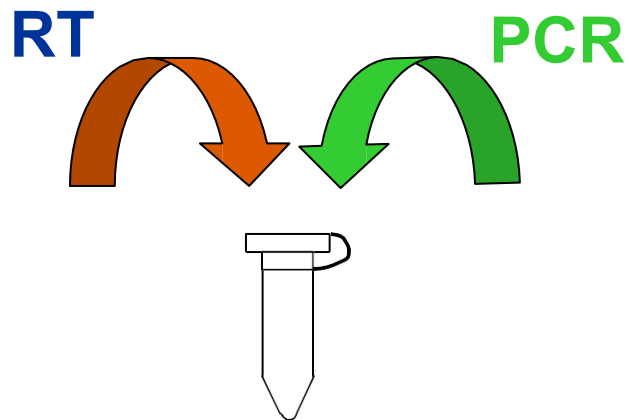
Important Considerations

- **Reagents**
- **Chemistry**
- **Assay**
- **Instrument**
- **Software**



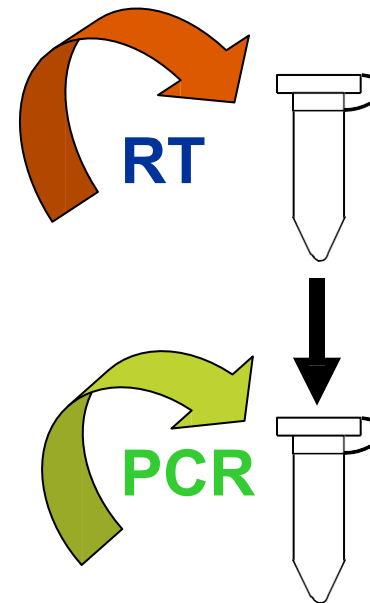
RNA to Amplified cDNA: 1-Step vs. 2-Step

1-STEP



- Closed tube (no contamination)
- Easy-to-use

2-STEP



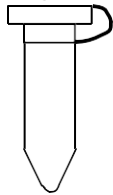
- Archive-ready sample

Formats: Master Mix vs. Core Reagent

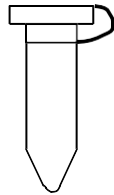


Core reagents allow flexibility and optimization

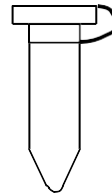
AmpliTaq Gold[®]
DNA Polymerase



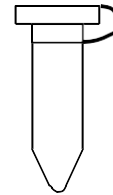
AmpErase[®]
UNG



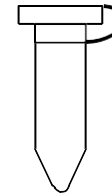
10X Buffer



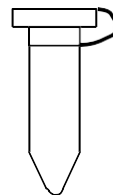
dNTPs



MgCl₂



Master mixes are easy-to-use and convenient



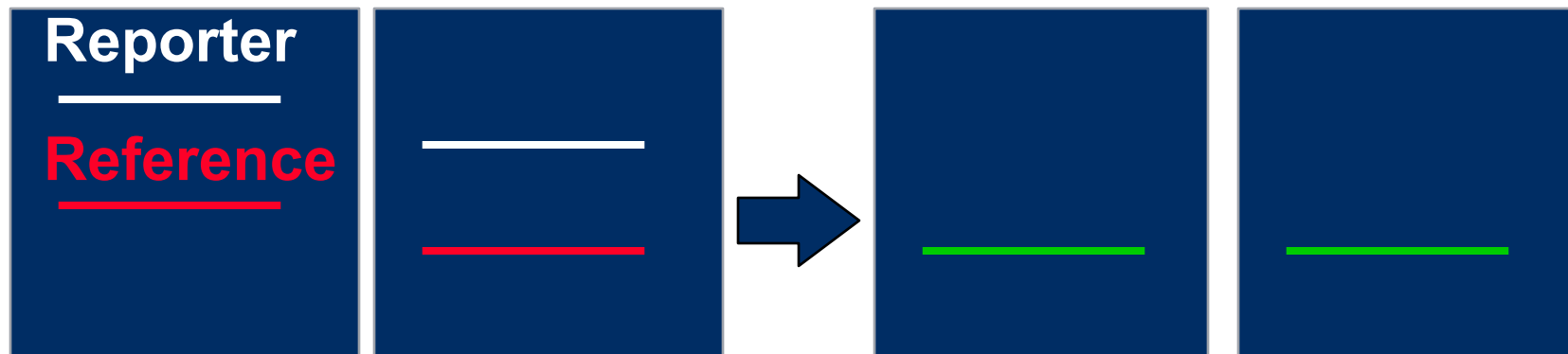
All components in one tube!

Advantage of Using a ROX™ Dye Normalizer

Improves precision

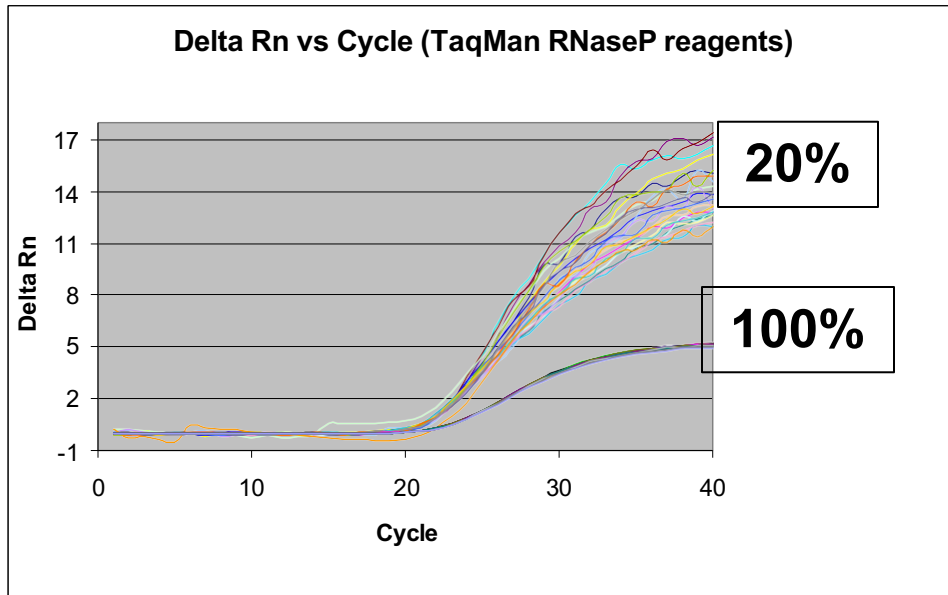
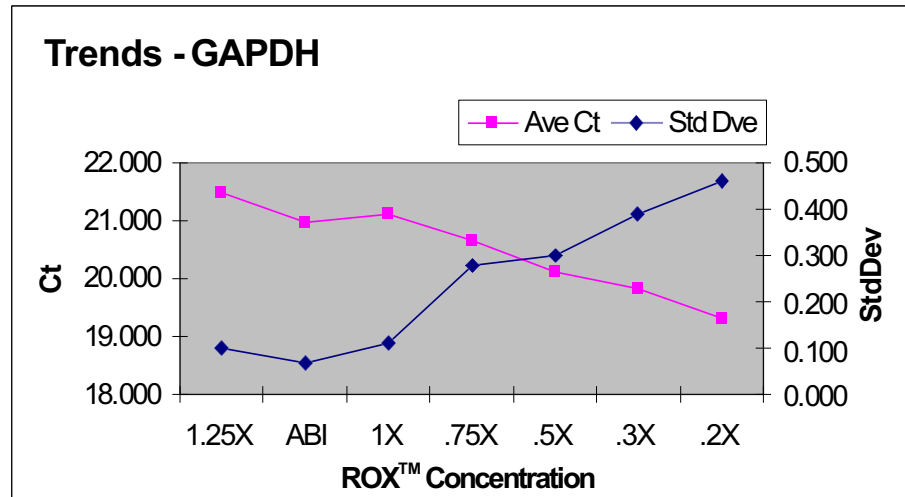
Compensates for small fluorescent fluctuations that can occur from well-to-well

Reporter / Reference



Precision with ROX™ Dye

As the concentration of passive reference decreases, the st. dev. increases; thus decreasing precision

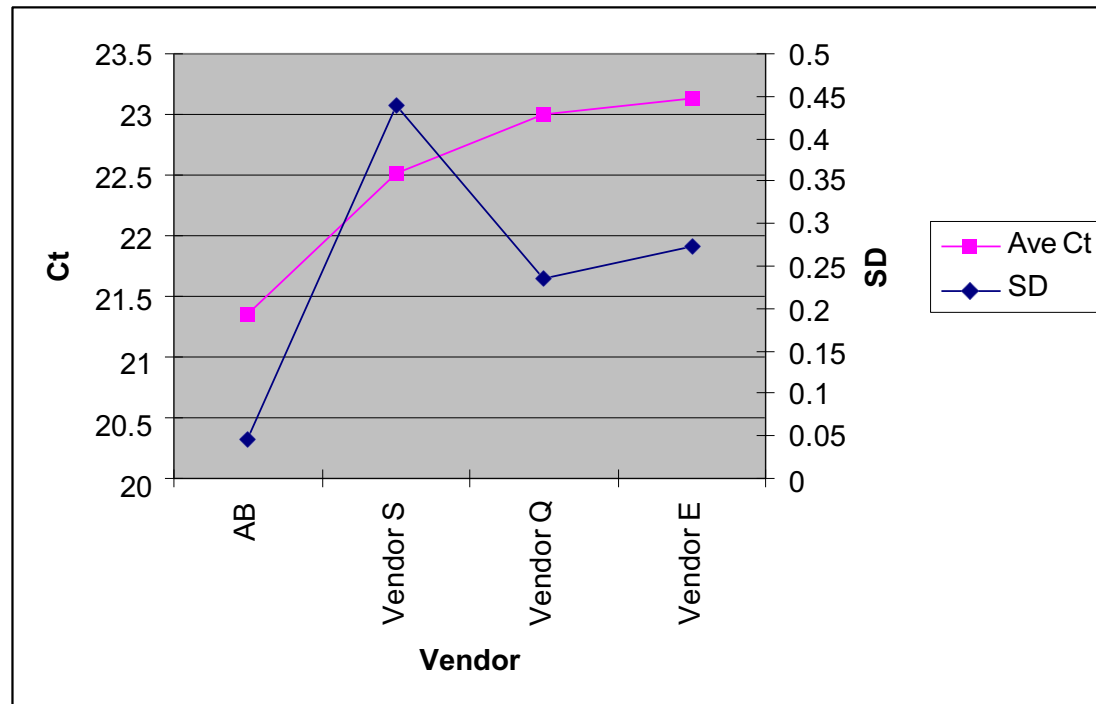


With only 20% of the Passive Reference Dye I, amplification becomes noisy with broad C_T spread.

At 100% of the Passive Reference Dye I, C_T replicates are tight and precise.

Not all ROX Dyes are Rock Solid!

Side-by-side comparison of four Master Mixes with comparable Passive Reference Dye I concentration



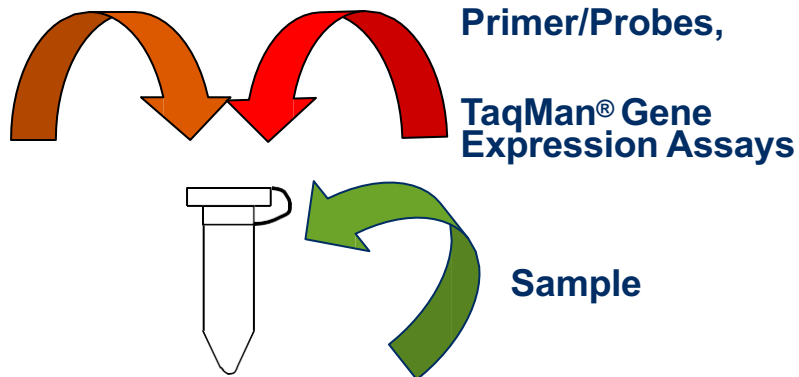
Applied Biosystems TaqMan[®] Universal PCR Master Mix produces the lowest standard deviation, therefore the most precise results!



Reaction Setup

TaqMan® Kit

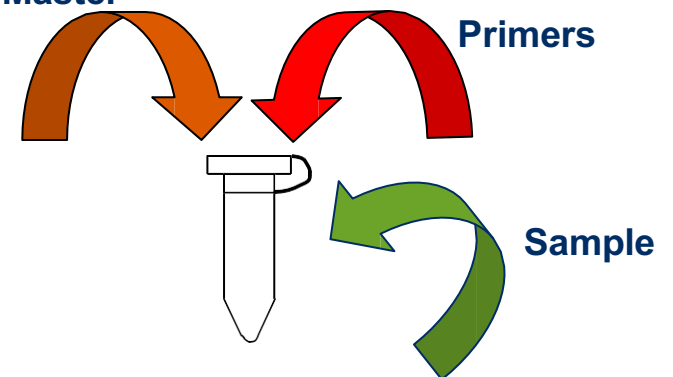
TaqMan®
Universal PCR
MasterMix



- High specificity
- Multiplexing capability
- End-point assay detection
- Rare transcript and low level pathogen detection

SYBR® Green Kit

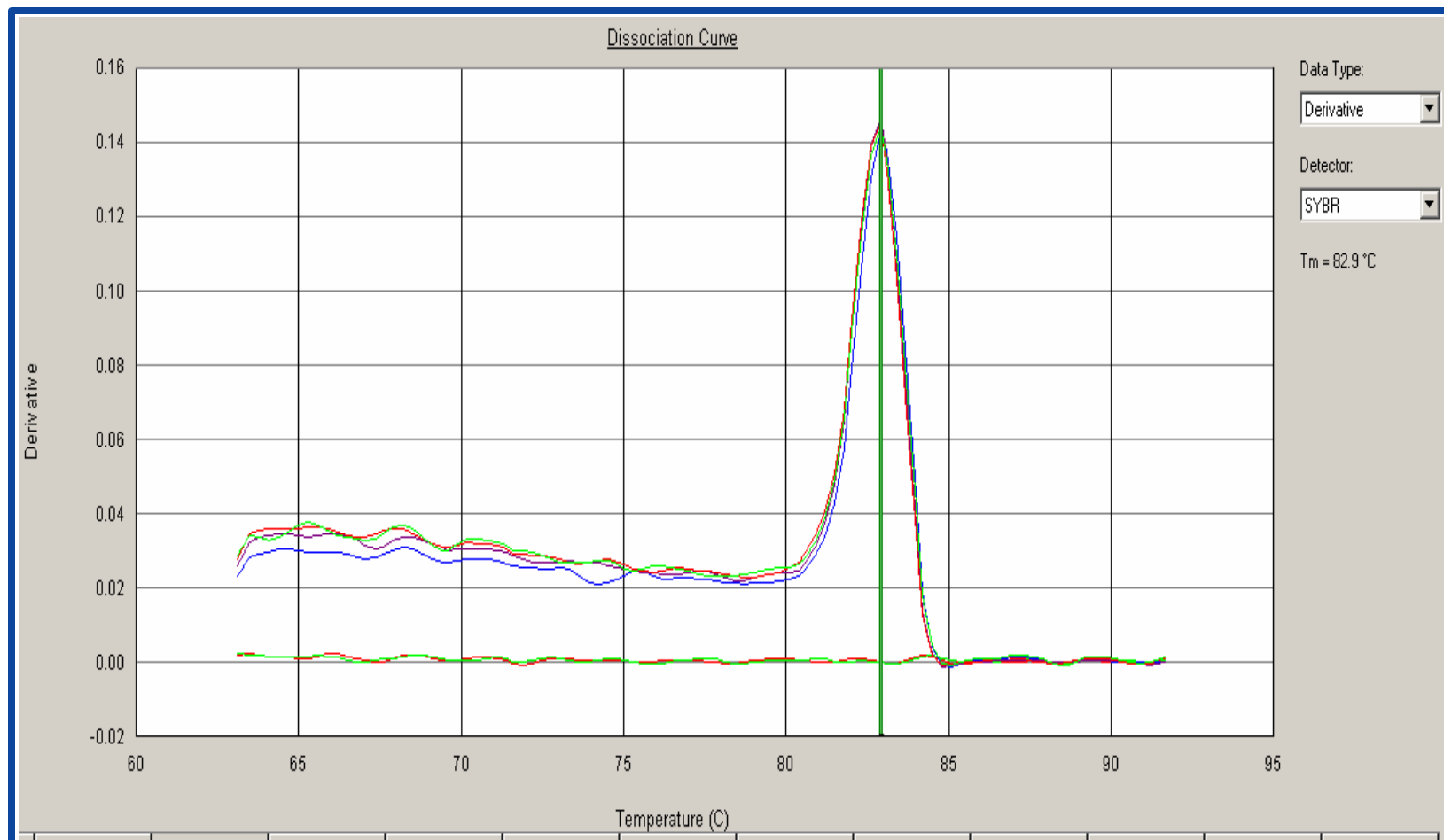
SYBR® Green
PCR Master
Mix



- Economical
- TaqMan® probe sensitivity not required
- Pre-screening targets

Dissociation Curve Analysis

- Displays melting temperature of the product generated in SYBR[®] Green assays

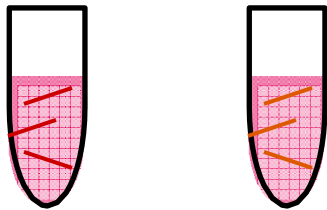


Gold Standard: AB Real-Time PCR reagent line

- TaqMan[®] Master Mix
 - Universal Master Mix
 - Fast TaqMan Master Mix
 - Improves time to result from 2 hours to about 35 minutes
- Power SYBR[®] Green Master Mix
 - Provide high sensitivity with less than 10 copies
 - High quality manufacturing ensure consistent lot-to-lot performance
- RT-Master Mix and core reagent
 - One-step or two-step RT reactions



SINGLEPLEX



- Reduced assay optimization time
- Reduced experimental validation

MULTIPLEX



- Reduced running time
- Reduced dependency on accurate pipetting
- More extensive validation required

Your Choice of Assays

- TaqMan[®] Gene Expression Assays
 - **An extensive list of pre-designed and qualified TaqMan[®] probes and primers ready for order**
 - Inventoried (off-the-shelf)
 - >40,000 gene expression assays for human, mouse, and rat
 - Non-inventoried (made-to-order)
 - >600,000 assays for human, mouse, rat, arabidopsis, and drosophila
 - Bioinformatics and information content
 - www.allgenes.com
- Custom TaqMan[®] Gene Expression Assays
 - **Submit your sequence and Applied Biosystems will design and synthesize your assay**
 - Custom made, single tube, ready-to-use
 - Same format as inventoried TaqMan Gene Expression Assays
 - For all species
- Support for user designed assays
 - **Rapid Assay Development Guidelines**

Rapid Assay Development Guidelines

- Primer and probe design using Primer Express[®] software
- The use of TaqMan[®] Universal PCR Master Mix or SYBR[®] Green PCR Master Mix
- Universal thermal cycling parameters
- Default primer and probe concentrations eliminate assay optimization



Attributes	Applied Biosystems 7900HT Fast Real-Time PCR System	Applied Biosystems 7500 Fast Real-Time PCR System	Applied Biosystems 7500 Real-Time PCR System	Applied Biosystems 7300 Real-Time PCR System
Block format	96-well, 384-well, Fast 96-well, TaqMan® Low Density Array	Fast 96-well	96-well 0.2 mL tubes	
Automation compatibility	Custom Zymark® twister robot	No		
Bar code plate tracking	Hand-held and fixed mount bar code reader	No		
Reaction volume	Variable, depending on block format	10-30 µL	25–100µL	
Excitation source	488 nm argon laser	Tungsten Halogen Lamp 5 Excitation Filters		Tungsten Halogen Lamp 1 Excitation Filter
Detection	Spectrograph Continuous 500–660 nm	5 Emission Filters		4 Emission Filters
Footprint size	924 sq. inch 1,617 sq.inch (with automation)	237 sq. inch		
Installation specification	2-fold discrimination with 99.7% confidence level			



Attributes	Applied Biosystems 7900HT Fast Real-Time PCR System	Applied Biosystems 7500 Fast Real-Time PCR System	Applied Biosystems 7500 Real-Time PCR System	Applied Biosystems 7300 Real-Time PCR System
Computer	Desktop	Laptop or Desktop		
Applications	Quantitation Allelic Discrimination Plus/Minus Detection			
Real-Time throughput	Up to 5,000 wells per day (unattended operation) with Automation Accessory	Over 1000 wells per 8 hour work day	Up to 480 wells per 8 hour work day	
Thermal cycling system	Peltier			
Software	-Standard with RQ -Paid Options: -Enterprise -RQ Manager -SNP Manager	-Standard with RQ		-Standard -Paid RQ option

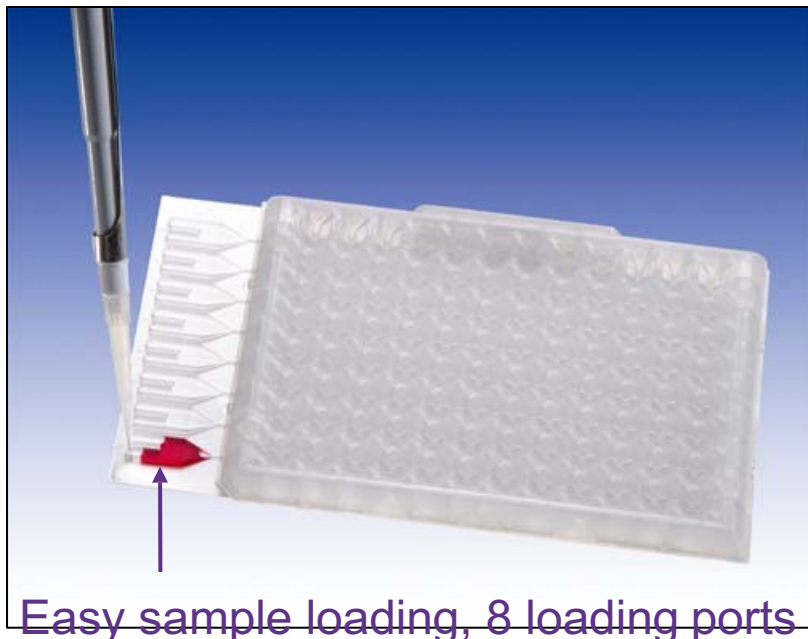
Applied Biosystems 7900HT and 7500 Fast Real-Time PCR Systems

- Complete systems designed to run fast in a standard 96-well configuration
- Can perform absolute or relative quantitation assays in about 35 minutes
- Increase productivity by providing faster time to result
- Includes a complete Fast system: hardware, software, reagents and consumables
- Comparable data on both fast and standard



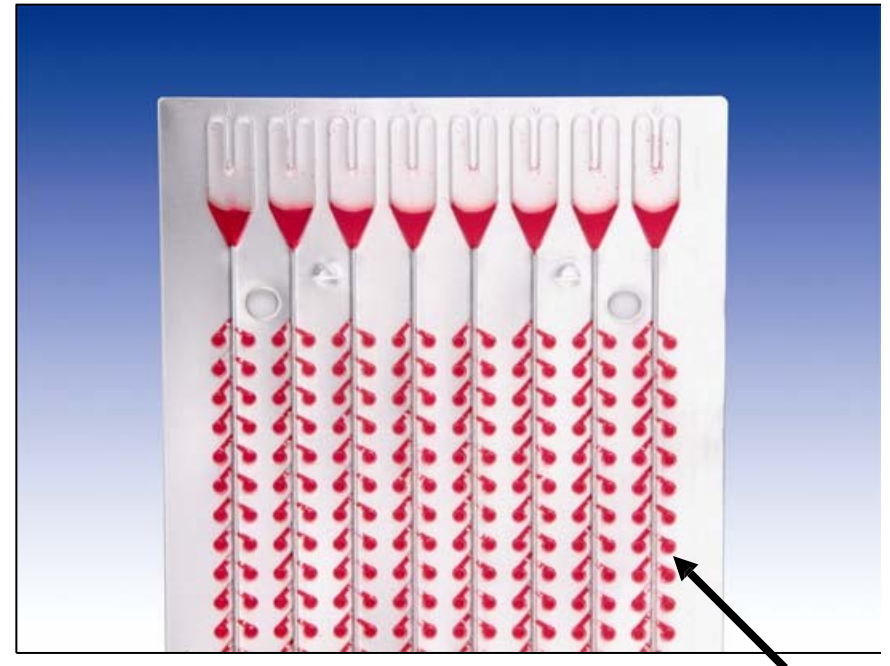
TaqMan[®] Low Density Array

- Convenient new consumable format
- Seamlessly integrates Applied Biosystems wide selection of assay products with the Applied Biosystems 7900HT Fast Real-Time PCR System



Easy sample loading, 8 loading ports

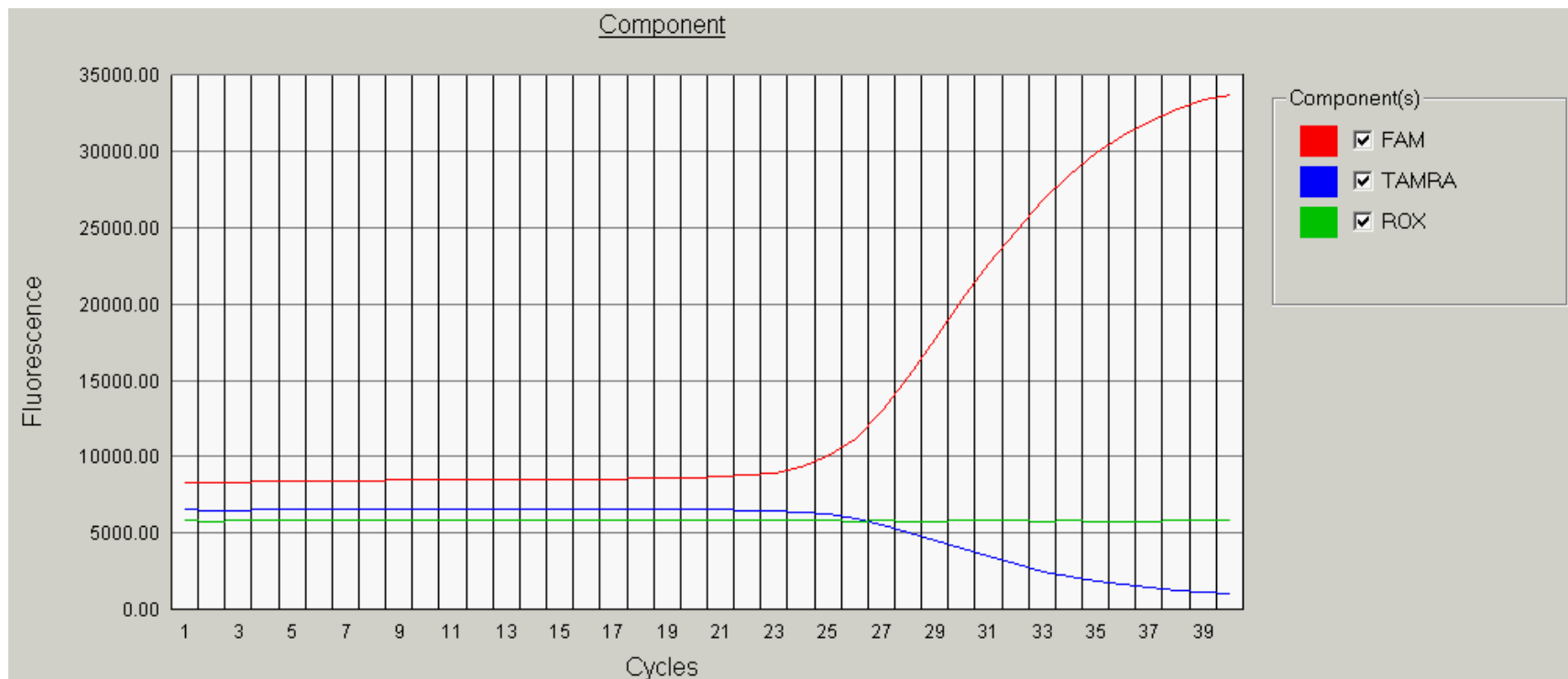
- No need for robotics
- Standardization between experiments and labs



- 8 channels each with 48 reaction chambers
- 384 reaction chambers

What is Multicomponent?

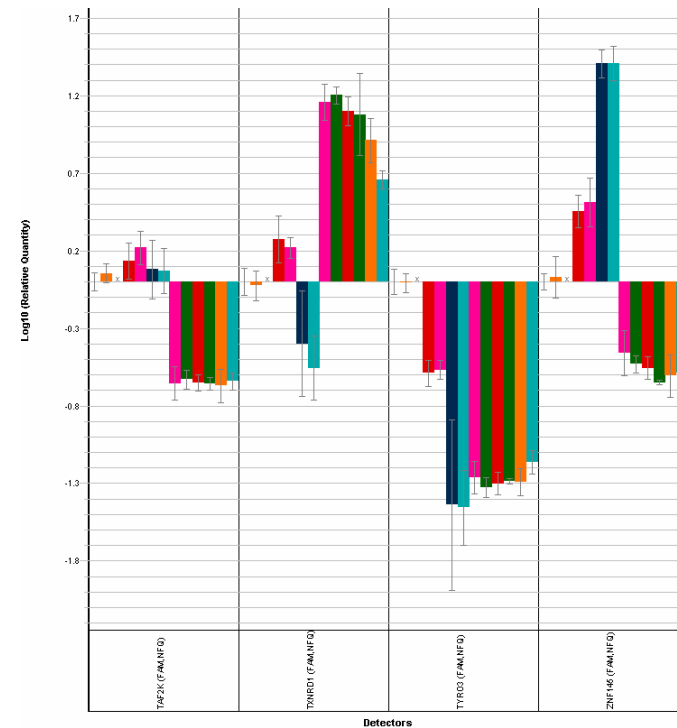
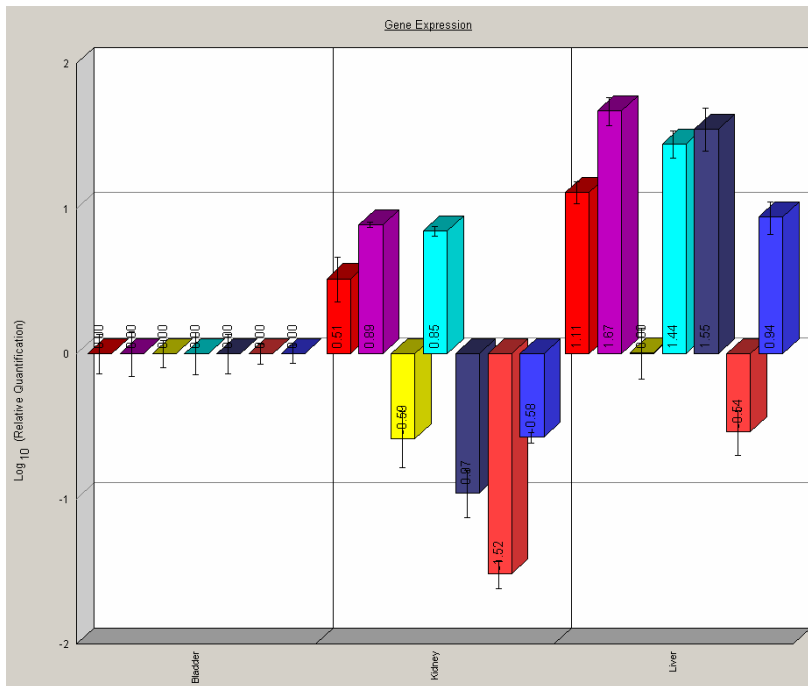
-Contribution of individual dye component is displayed throughout the PCR cycle



Software Highlights

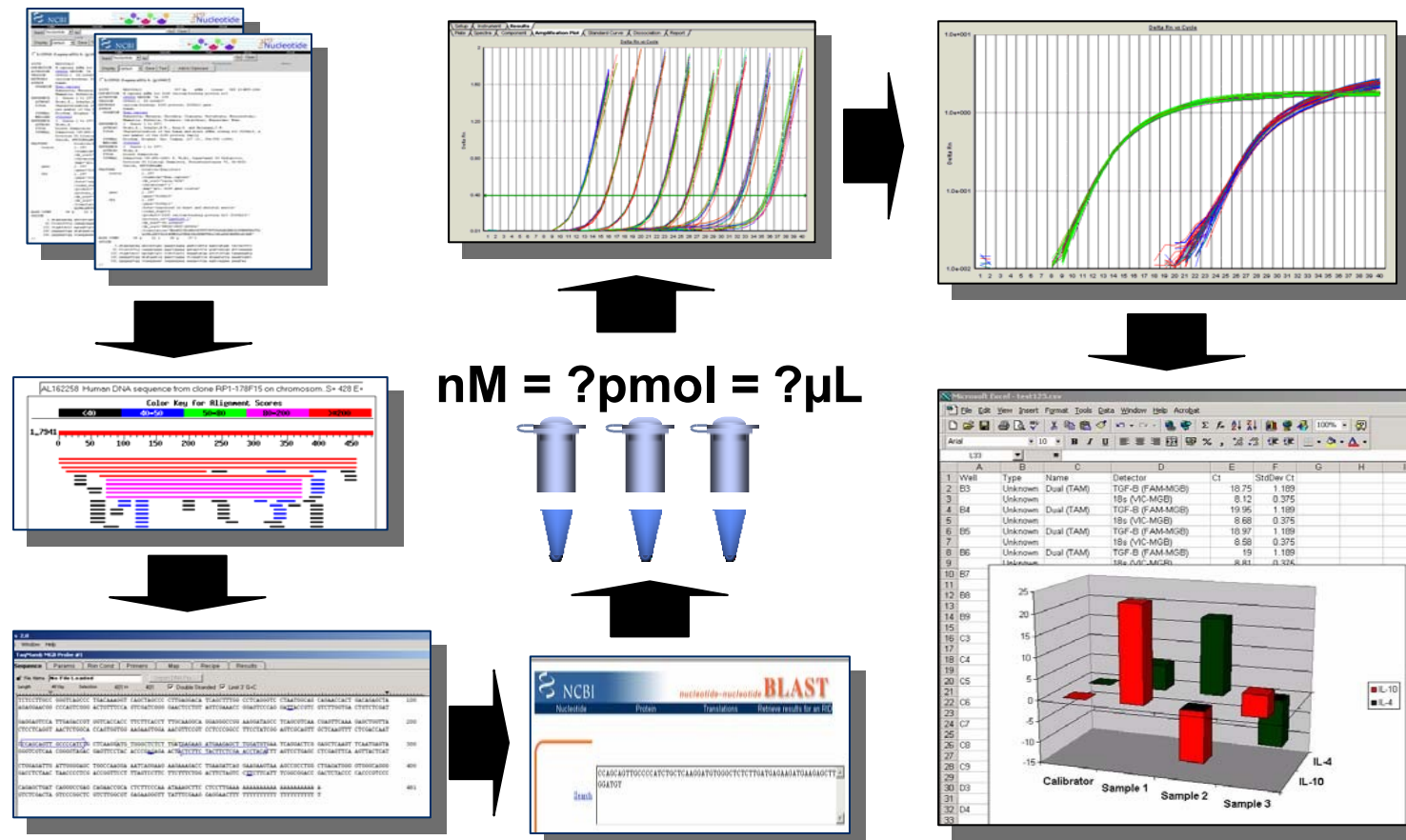
- **Gene Expression**

- Fully automated data analysis (baseline and threshold for all assays)
- Automated calculation of relative quantitation
- Data from up to 10 plates integrated into a single study



Gene Expression 2002

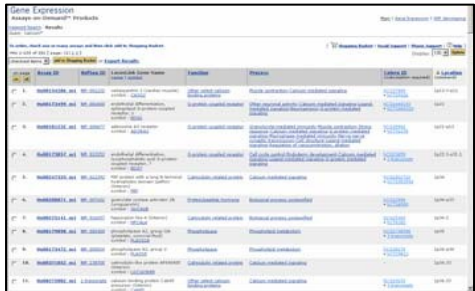
Real-time PCR and its bottlenecks



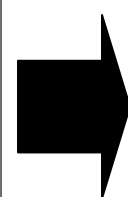
Gene Expression Today

Most bottlenecks of real-time PCR removed

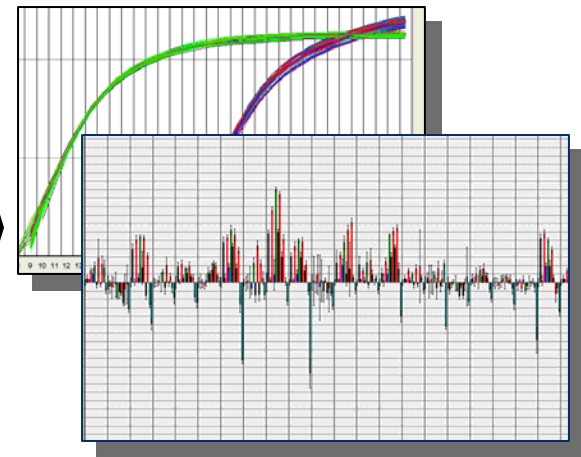
TaqMan Gene Expression Assays
Custom TaqMan Gene Expression Assays
Online Ordering Catalog



Assay ID	Assay Name	Function	Protein	Accession	Linkage
AB012345.A1	Human IL-6	Interleukin-6	IL6	U12345	Link 1
AB012346.A1	Human IL-10	Interleukin-10	IL10	U12346	Link 2
AB012347.A1	Human IL-12	Interleukin-12	IL12	U12347	Link 3
AB012348.A1	Human IL-17	Interleukin-17	IL17	U12348	Link 4
AB012349.A1	Human IL-18	Interleukin-18	IL18	U12349	Link 5
AB012350.A1	Human IL-20	Interleukin-20	IL20	U12350	Link 6
AB012351.A1	Human IL-21	Interleukin-21	IL21	U12351	Link 7
AB012352.A1	Human IL-22	Interleukin-22	IL22	U12352	Link 8
AB012353.A1	Human IL-23	Interleukin-23	IL23	U12353	Link 9
AB012354.A1	Human IL-24	Interleukin-24	IL24	U12354	Link 10
AB012355.A1	Human IL-25	Interleukin-25	IL25	U12355	Link 11
AB012356.A1	Human IL-26	Interleukin-26	IL26	U12356	Link 12
AB012357.A1	Human IL-27	Interleukin-27	IL27	U12357	Link 13
AB012358.A1	Human IL-28	Interleukin-28	IL28	U12358	Link 14
AB012359.A1	Human IL-29	Interleukin-29	IL29	U12359	Link 15
AB012360.A1	Human IL-30	Interleukin-30	IL30	U12360	Link 16



Automated Gene Expression Analysis Software



Expectations in Gene Expression Studies

- Reproducibility ✓
- Accuracy ✓
- Flexibility (Scalability) ✓
- Standardization ✓
- High Throughput ✓
- Informative Data Sets ✓
- Convenience ✓

Complete Integrated Solution

- Complete line of **REAGENTS** and consumables
- +
- Your choice of **ASSAYS**
- +
- High-quality Real-time PCR **INSTRUMENTS**
- +
- Easy-to-use **SOFTWARE** for setup and complete data analysis
- =

Enabling scientific discovery!

Questions & Discussion...

Thank You!!!

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