Introduction to Real-Time PCR:
Basic Principles and Chemistries

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Promega Corporation

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Outline

I. Real-Time PCR overview

- Basics of Real-Time PCR
- Understanding the data

II. Chemistries and Instrumentation

- Dye-based chemistries (e.g. SYBR)
- Label-based chemistries (e.g. TaqMan)
- Reverse Transcription qPCR
- Instrumentation

III. Quantification – an intro to the math

IV. The MIQE Guidelines

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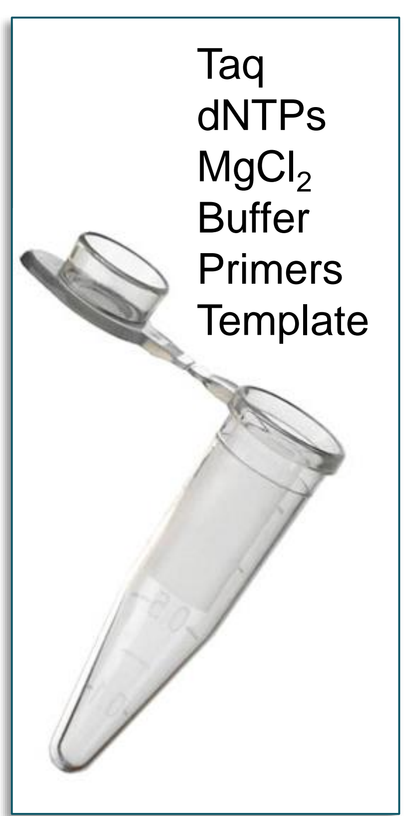
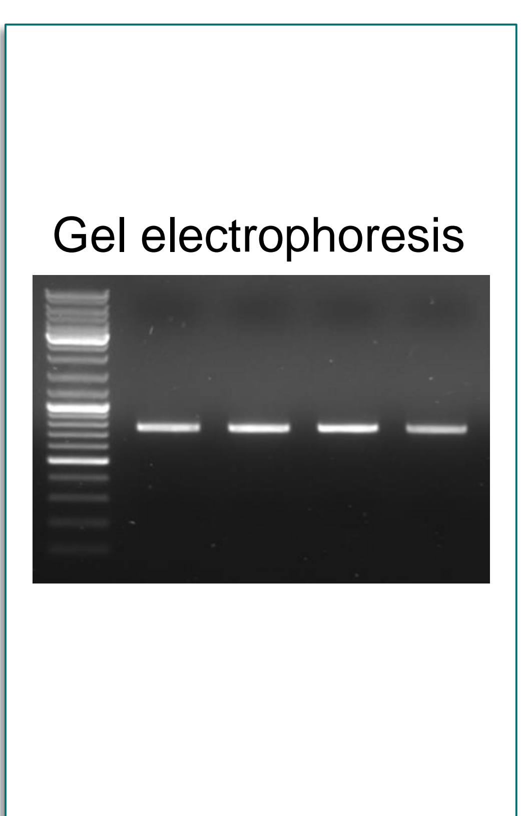
PCR Refresher



Stage	Temp	Time	Cycles
Activation	95°C	2 min	1
Dissociation	95°C	15 s	25 - 40
Annealing	60°C	15 s	
Extension	72°C	1 min	



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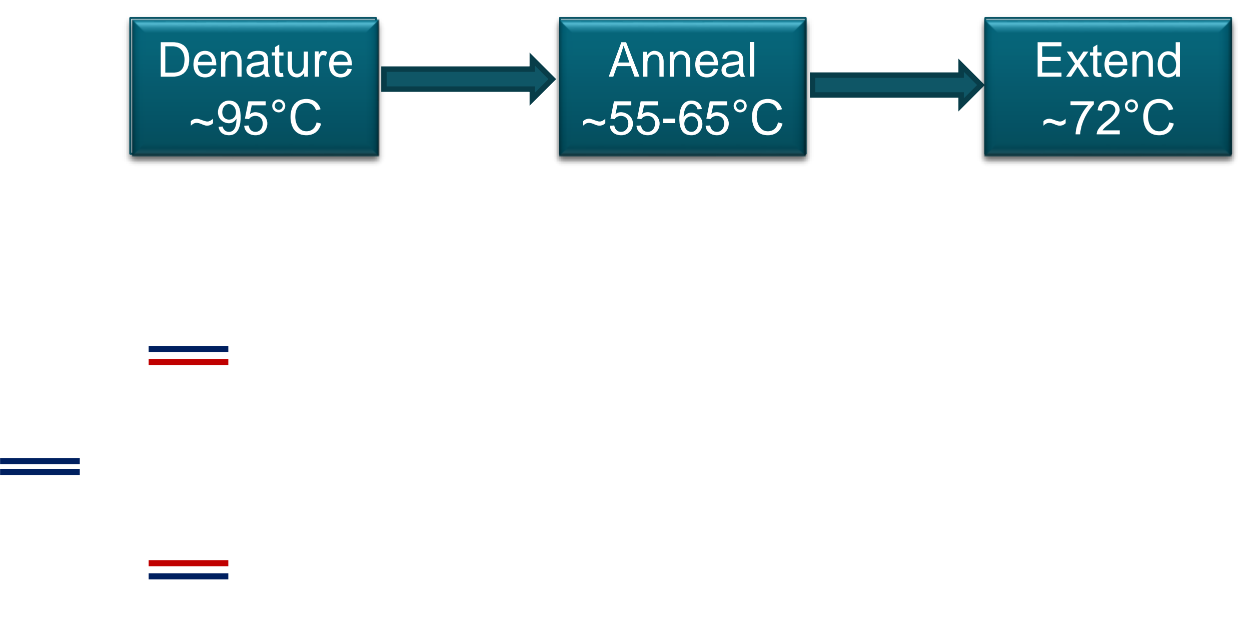
PCR Refresher



Start #	Cycle1	Cycle2	Cycle3	Cycle4	Cycle5	Cycle6	Cycle n
1	2	4	8	16	32	64	$1*2^n$

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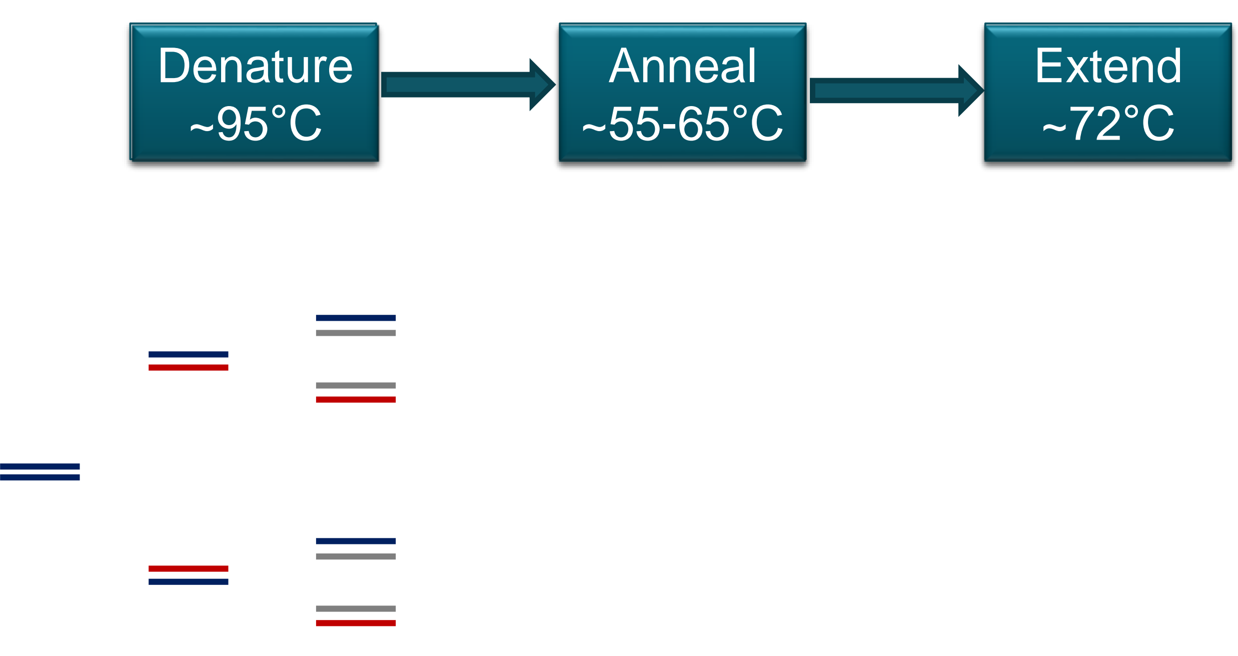
PCR Refresher



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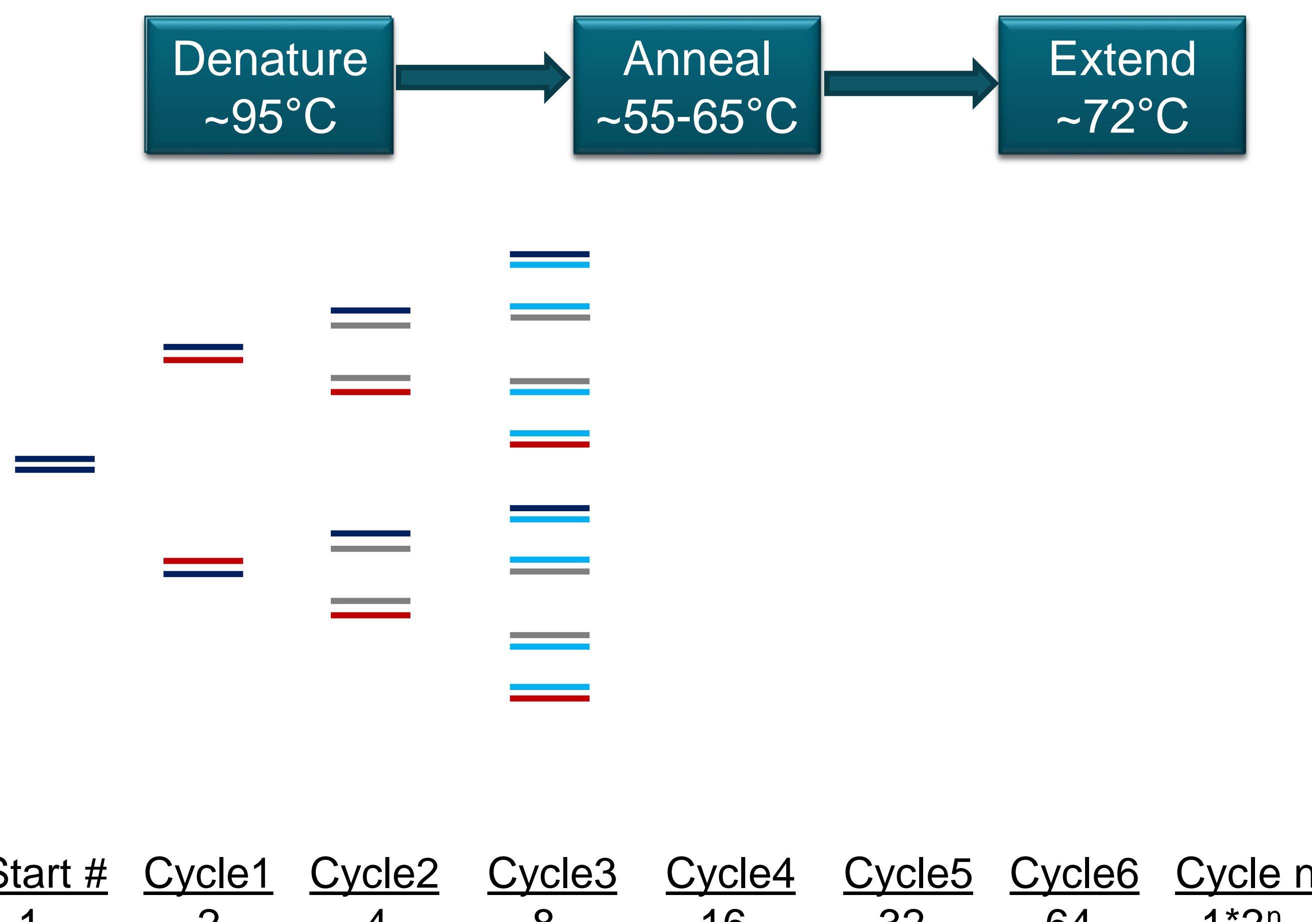

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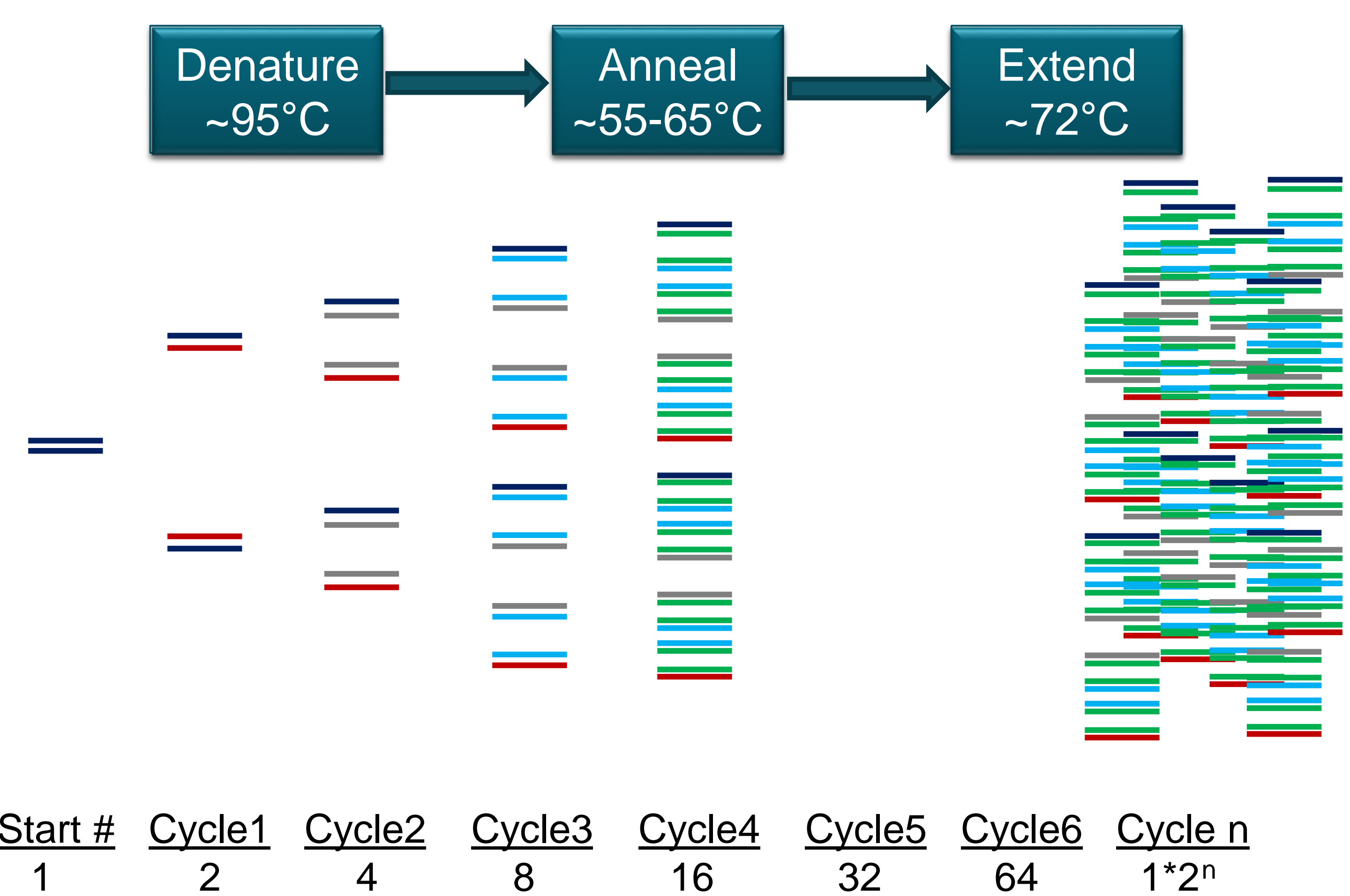
PCR Refresher



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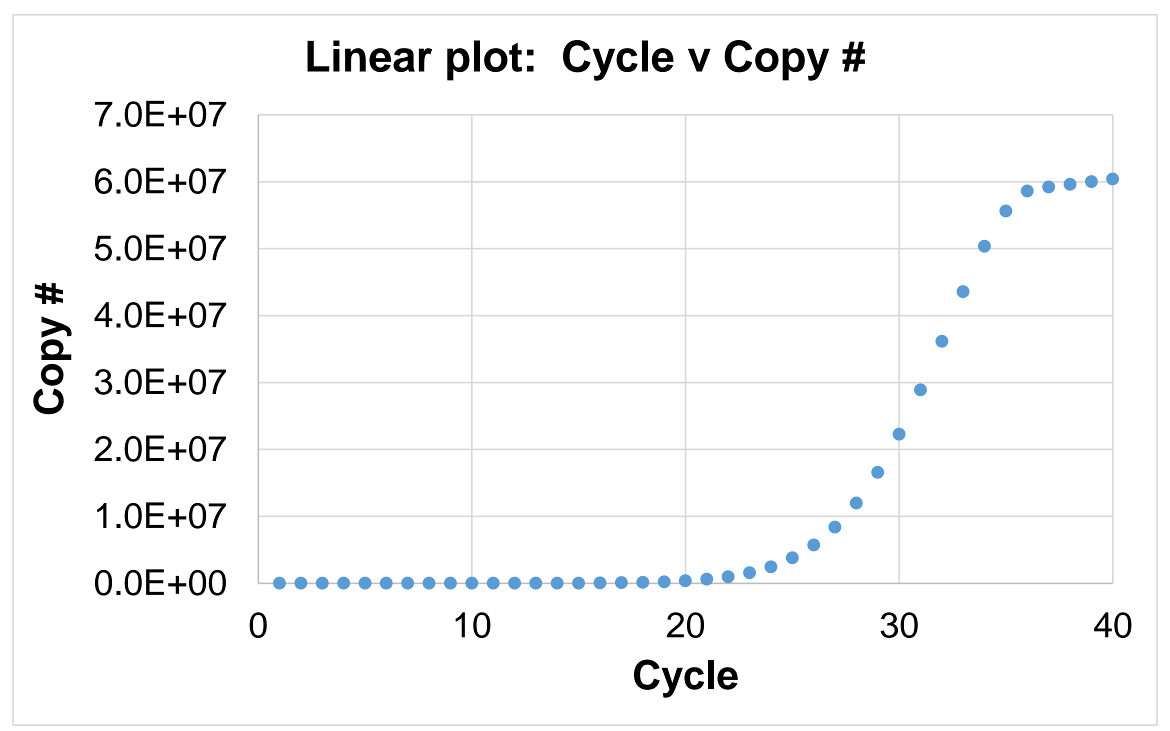
Traditional PCR
Dynamics of amplification



Start #	Cycle1	Cycle2	Cycle3	Cycle4	Cycle5	Cycle6	Cycle n
1	2	4	8	16	32	64	$1*2^n$

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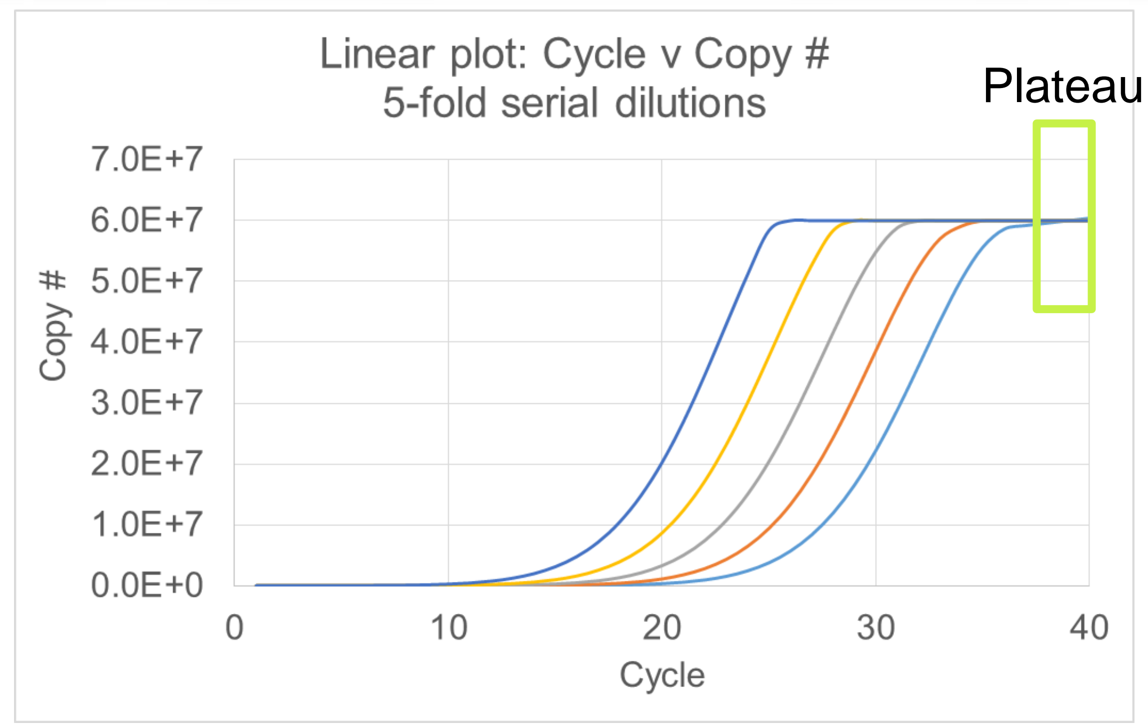
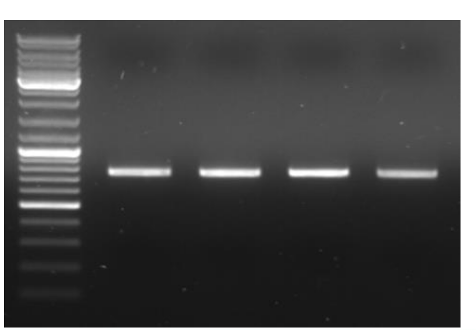
Endpoint PCR
Analyze reactions at plateau



<u>Endpoint PCR</u>
- Assayed at plateau
- Not quantitative
- Size & number
- Limited by gel sensitivity



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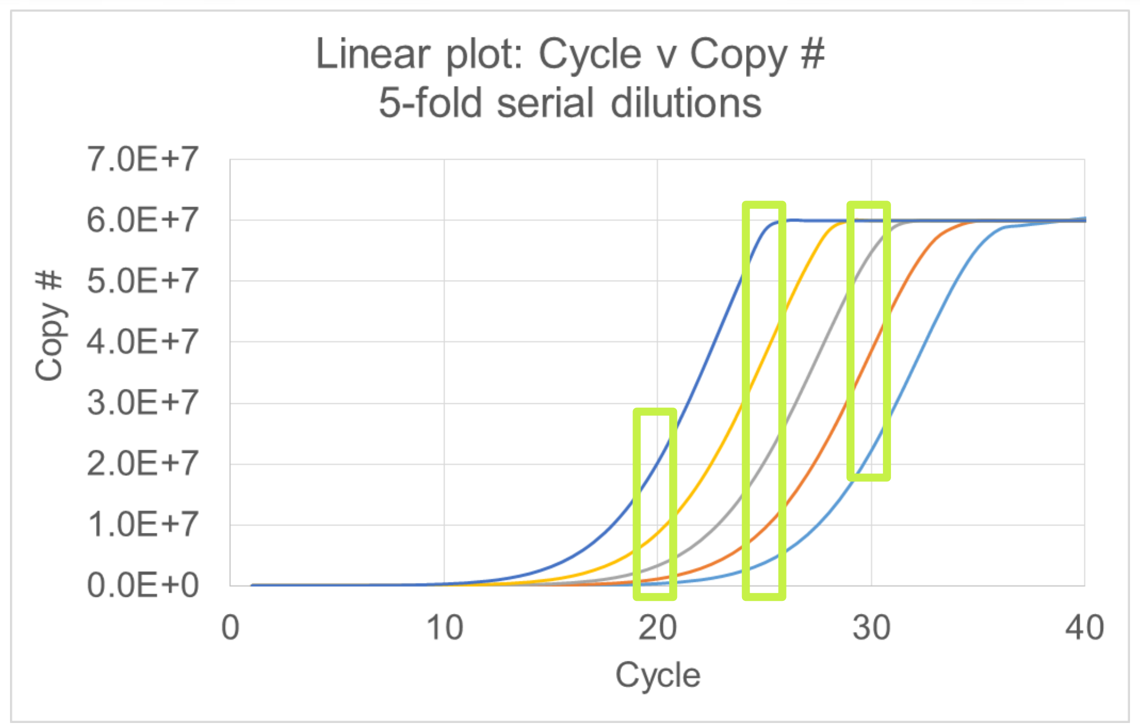
Semi-Quantitative PCR

Analyze reactions at an intermediate cycle



Semi-quantitative PCR

- Assayed before plateau
- Not linear
- Limited dynamic range

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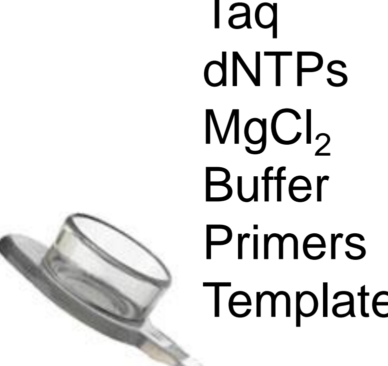
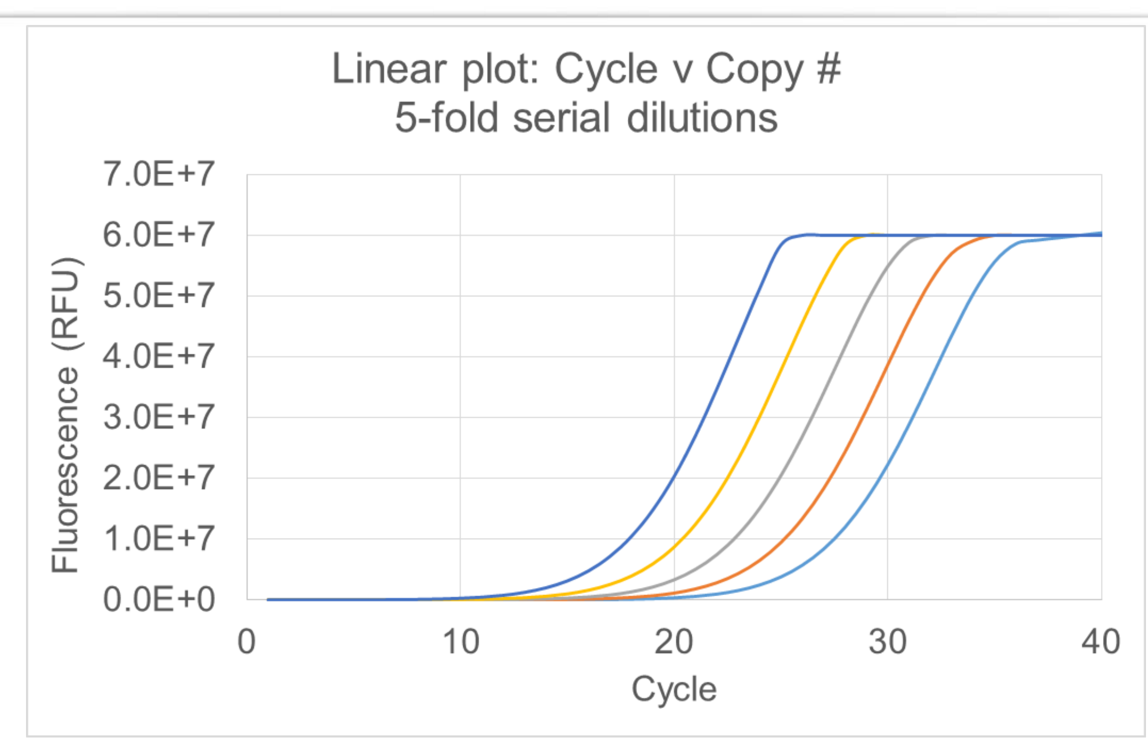
Real-time PCR
Measure product at every cycle!

Taq
dNTPs
$MgCl_2$
Buffer
Primers
Template



Fluorescent DNA marker



<u>Real-time PCR, quantitative PCR, qPCR</u>

- Assayed at every cycle
- Requires specialized instrument
- No additional sample handling
- Broad dynamic range (10^6-10^8)
- Quantitative
- High-throughput capable
- Multiplex capable
- Typically used for <250bp

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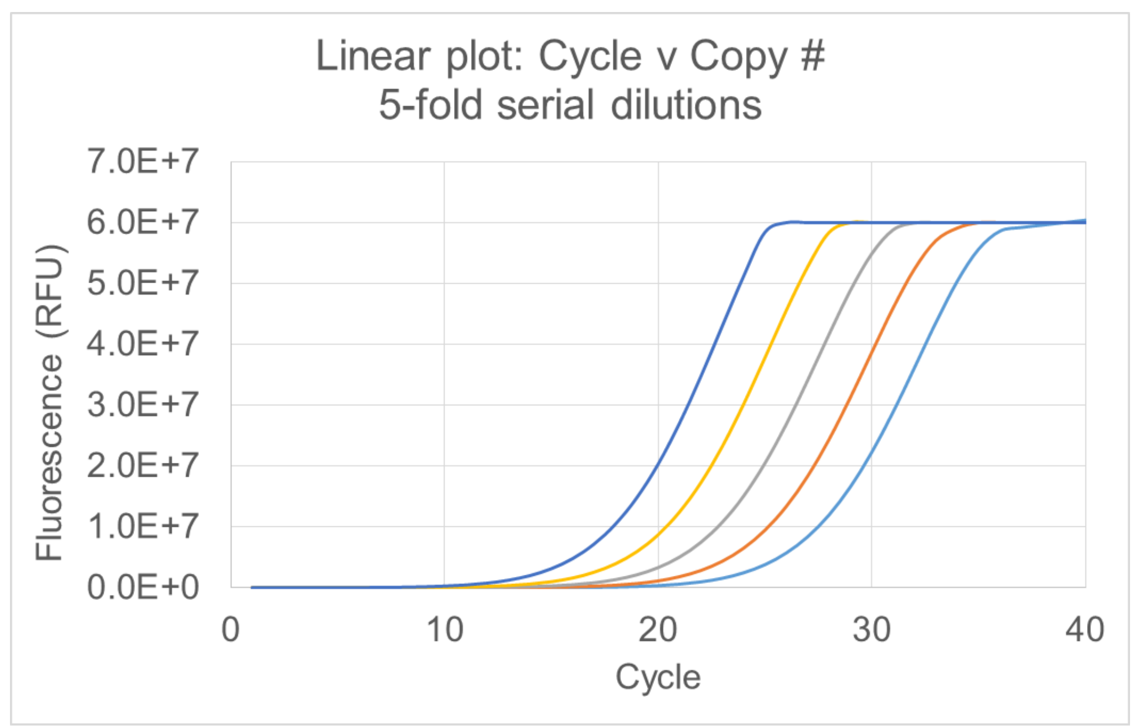
Think About the Data

QUESTION 1: Which sample has a higher target concentration? Explain.



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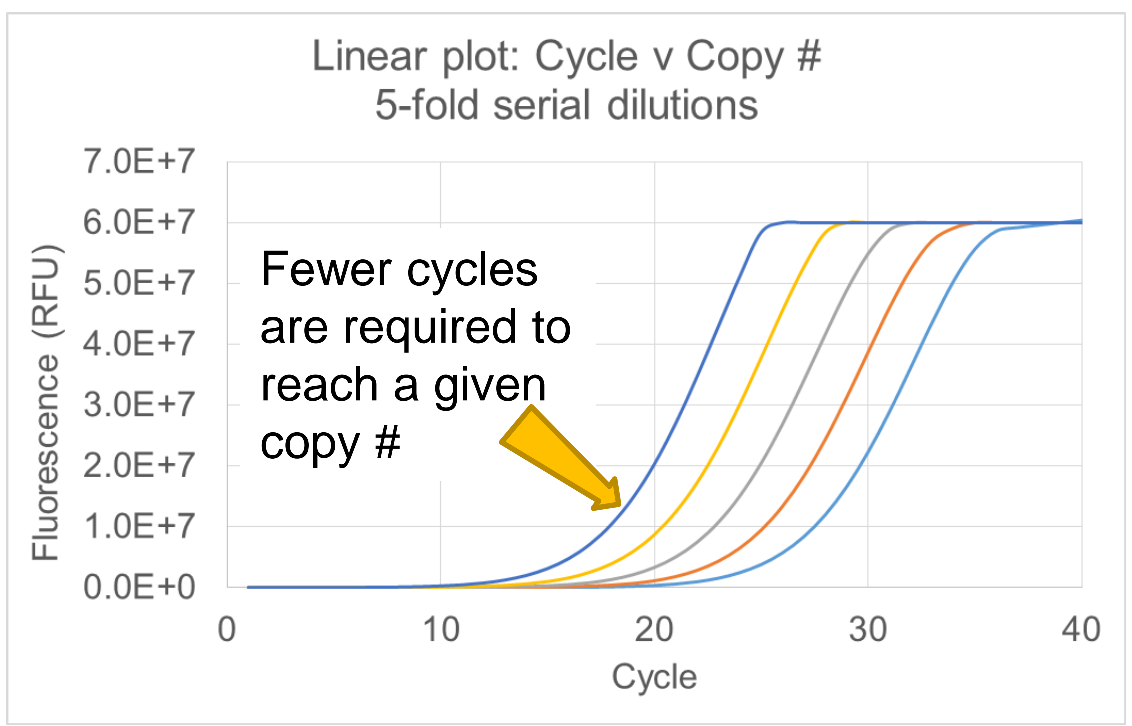

Amplification is inversely proportional to starting concentration!

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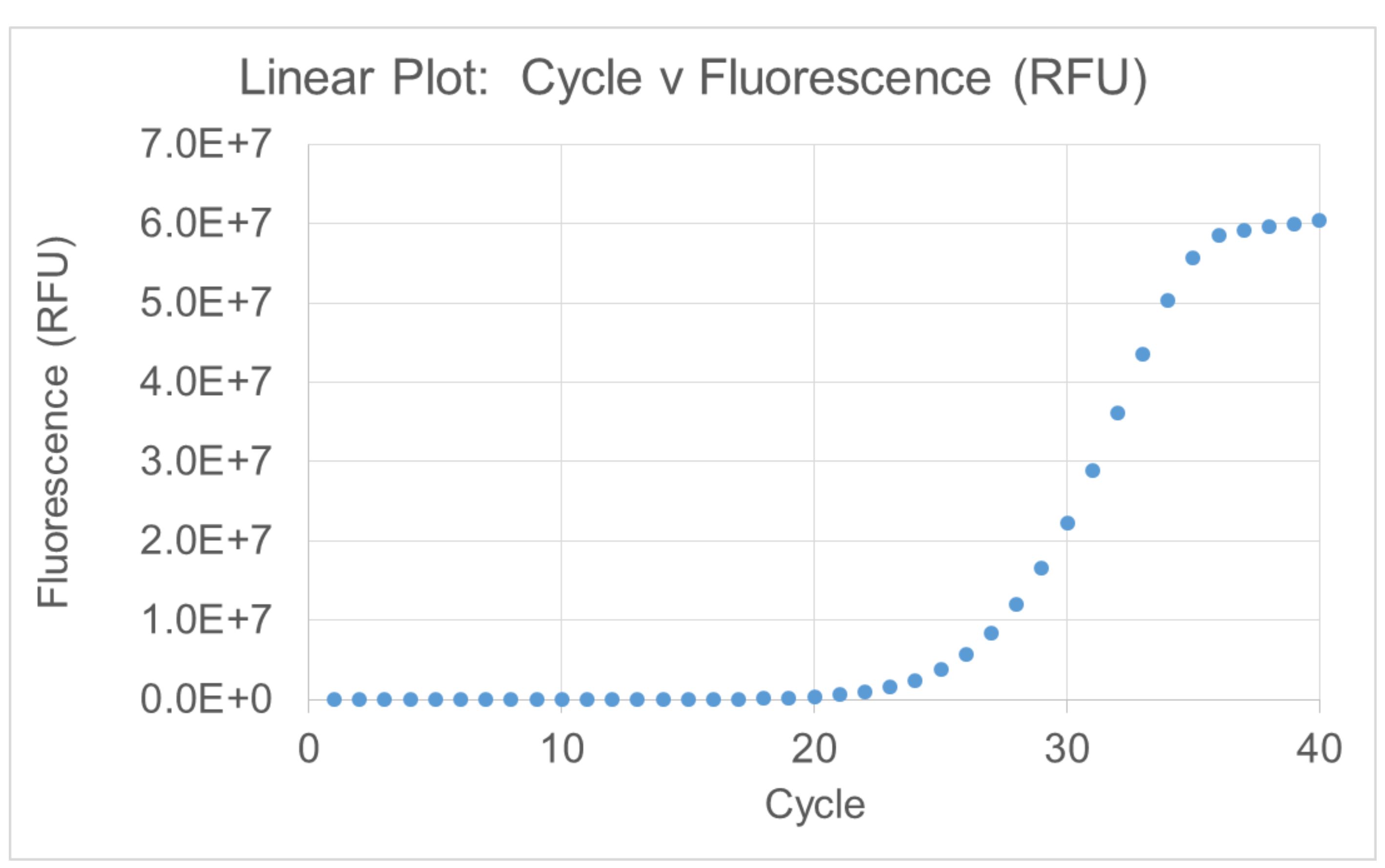
Viewing the Data



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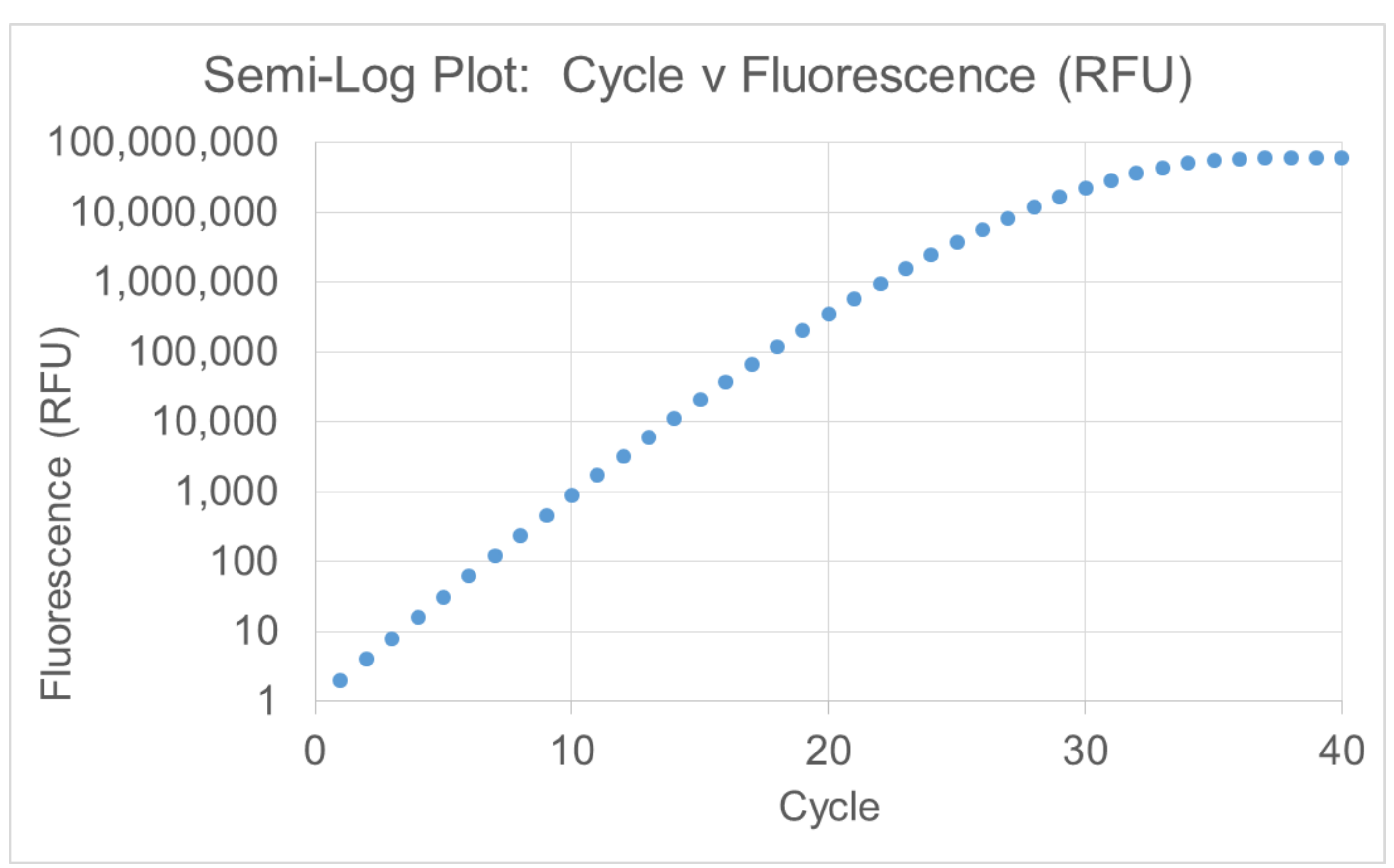
Viewing the Data



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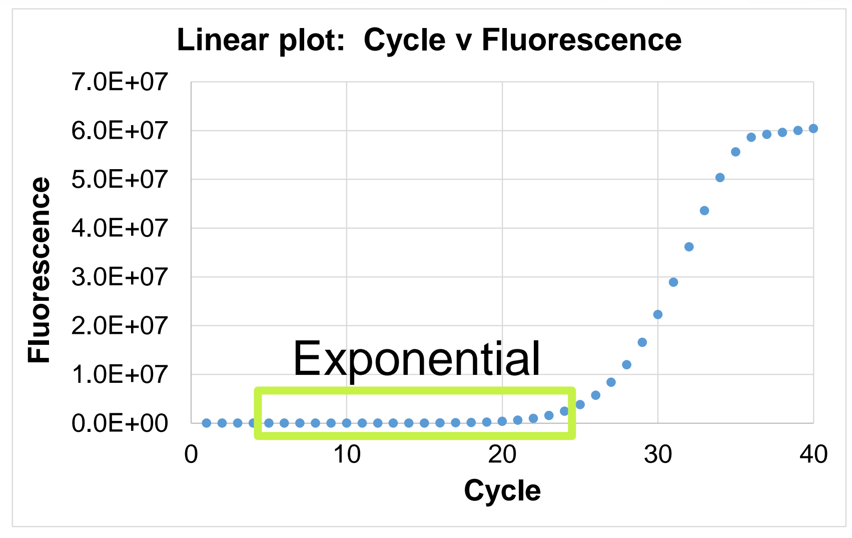
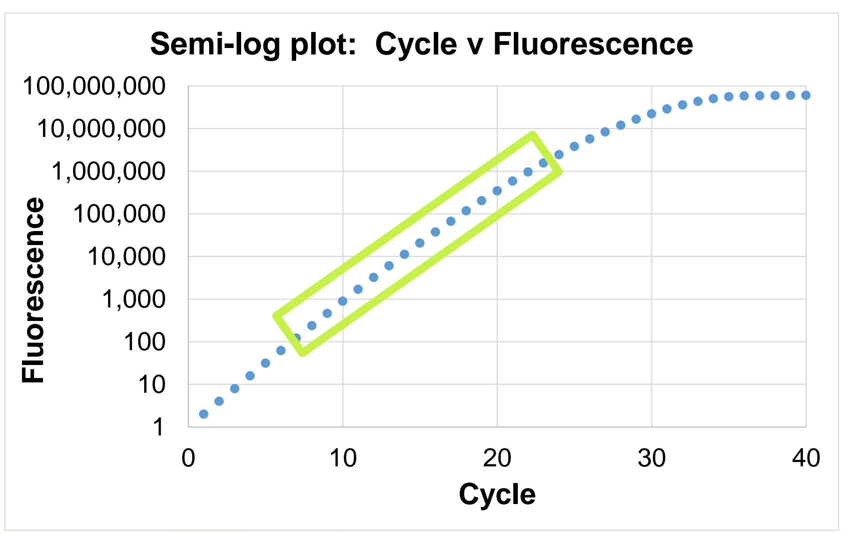
Viewing the Data

Quantification is most robust in the exponential phase of amplification

The exponential phase is best viewed in a semi-log plot





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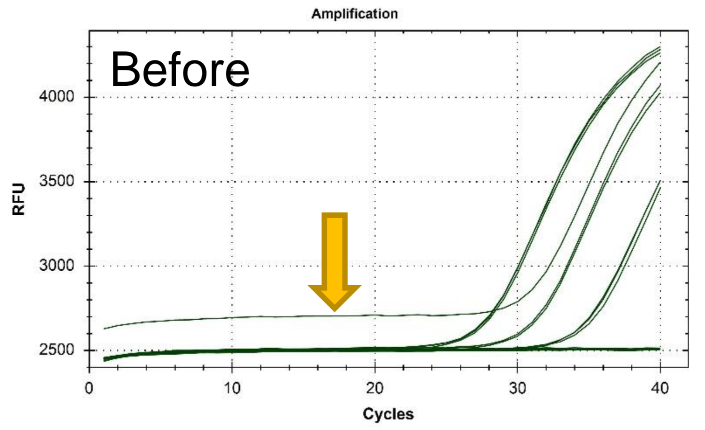
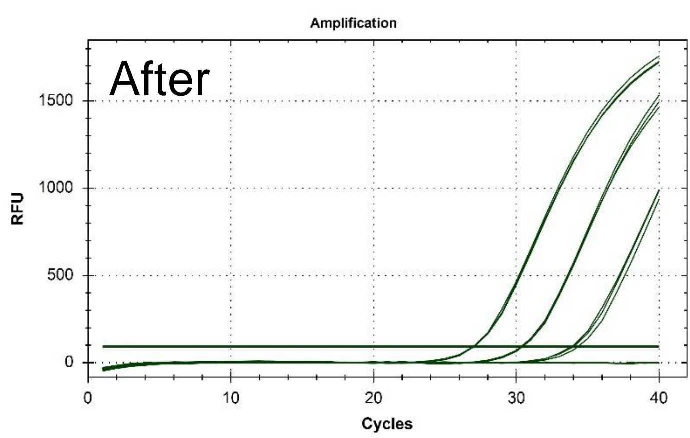
From Raw Data to Quantification

Software processing

Step 0: Normalize to passive reference (Rn, normalized RFU)
- Only required on some platforms
- Use a second fluorescent dye, not influenced by DNA
- Divide reporter dye fluorescence by passive reference

Step 1: Subtract background fluorescence (RFU, ΔR, dR, dRn)
- Appropriate background cycles determined automatically
- Average background fluorescence for each reaction
- Subtract avg background fluorescence at each cycle
- De-trend data based on background fluorescence slope





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- Average background fluorescence for each reaction
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- De-trend data based on background fluorescence slope
- Appropriate background cycles determined automatically

Step 2: Fit a curve to the data

Step 3: Determine a value (C_q) for each sample

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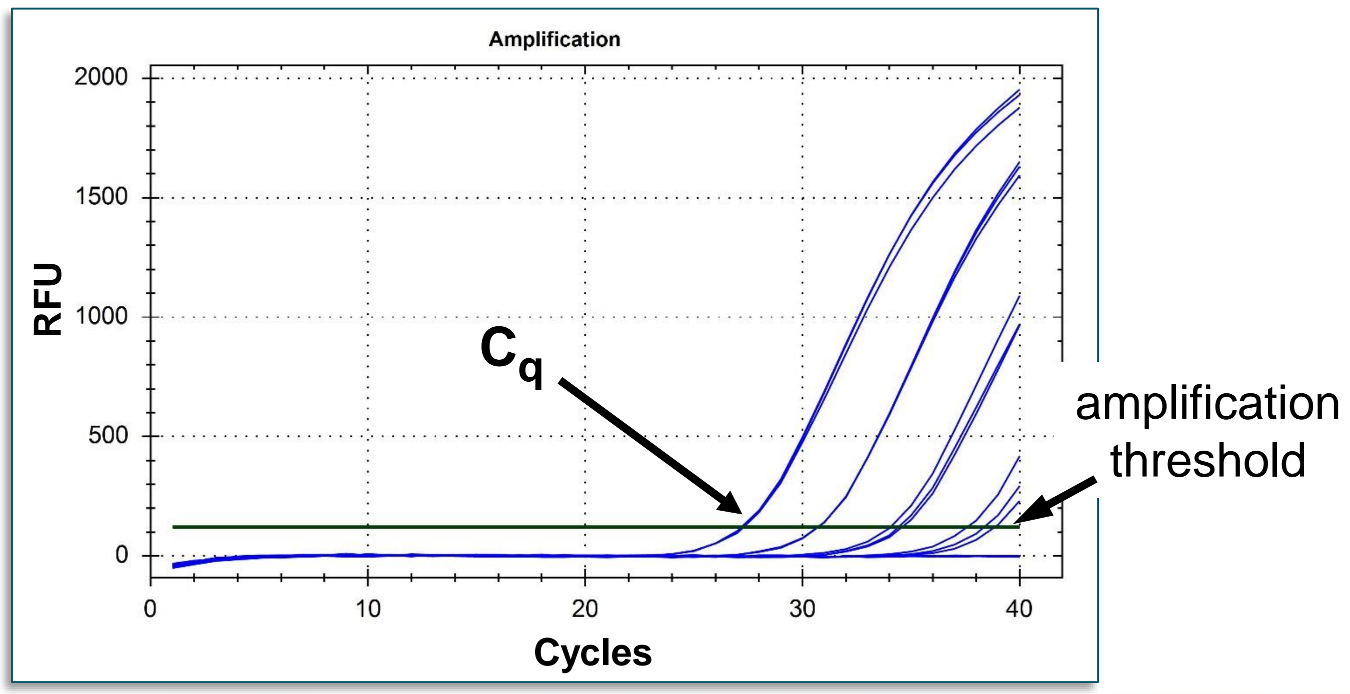
From Raw Data to Quantification

Quantification Cycle, Cq

Threshold method

- Cycle at which fluorescence crosses threshold
- Threshold set at ~10 StDev above background
- Threshold should be drawn in exponential phase
- Threshold variable between assays; must analyze separately!



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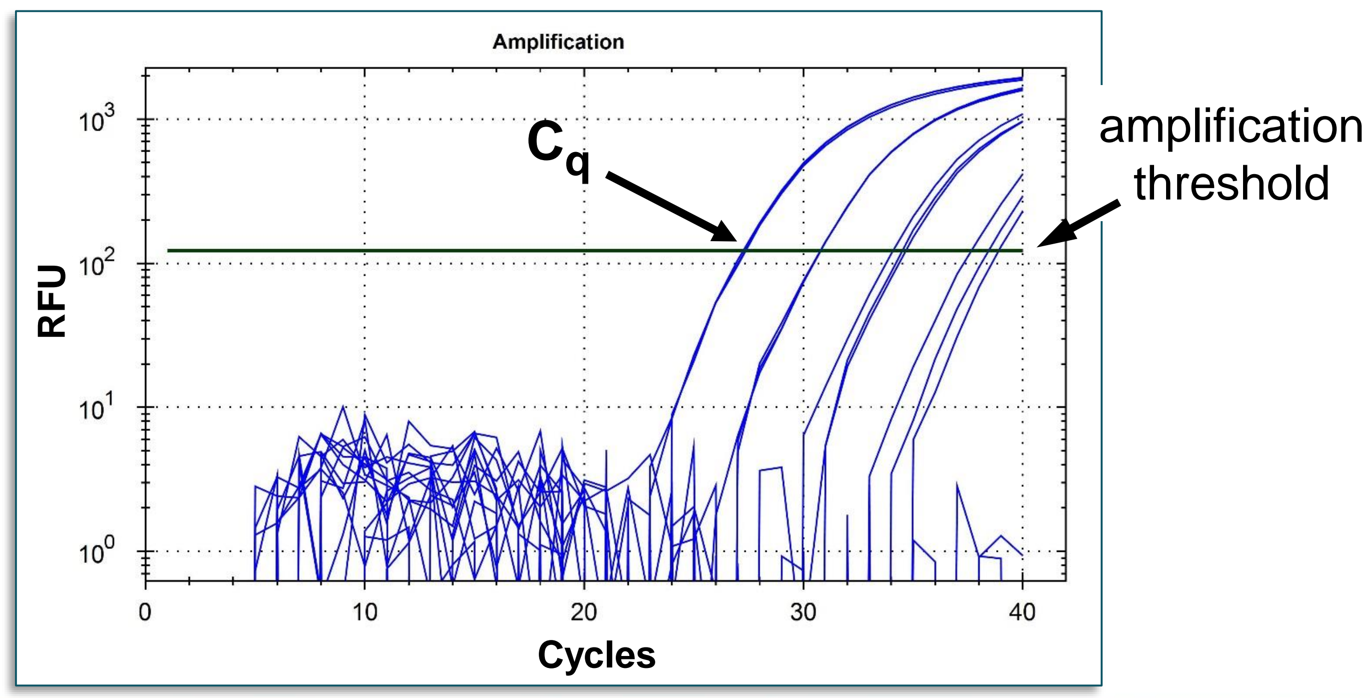

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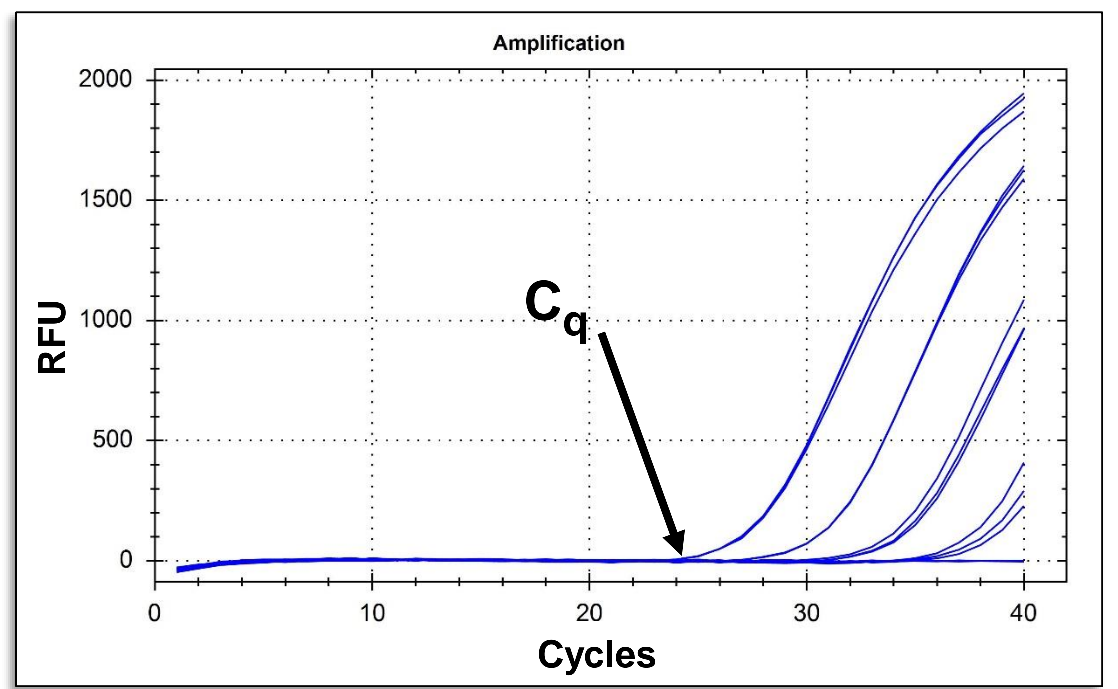
From Raw Data to Quantification

Quantification Cycle, Cq

Regression method, or 2nd derivative maximum

- Point of maximum fluorescence increase
- Independent of other wells or assays on plate
- More consistent from plate to plate



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Real-Time PCR Chemistries & Instrumentation

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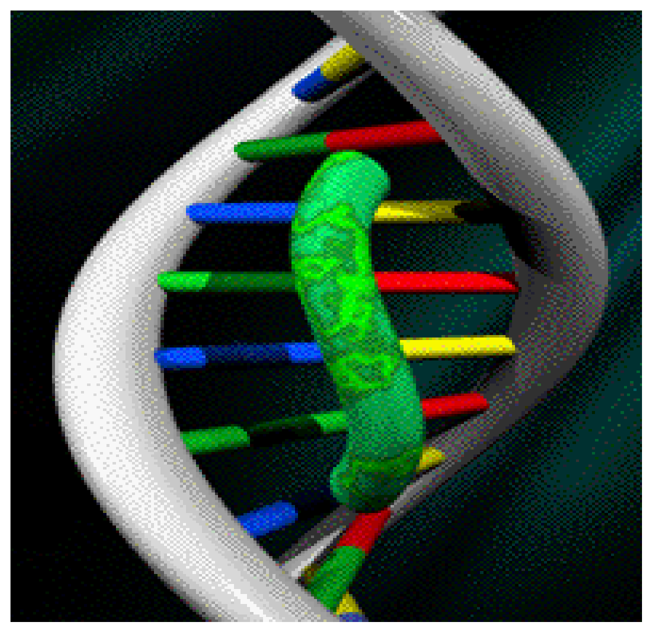
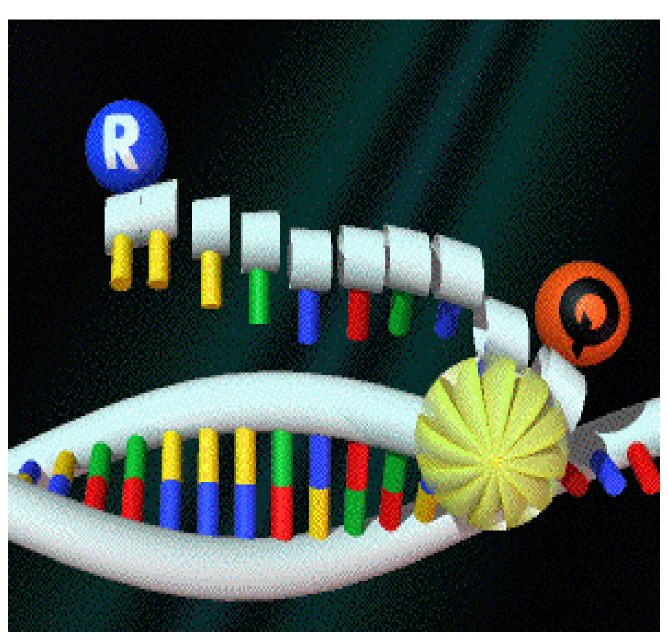
Leading Real-Time PCR Chemistries

Dye-based



Binds double stranded DNA

Probe-based



Labelled probe

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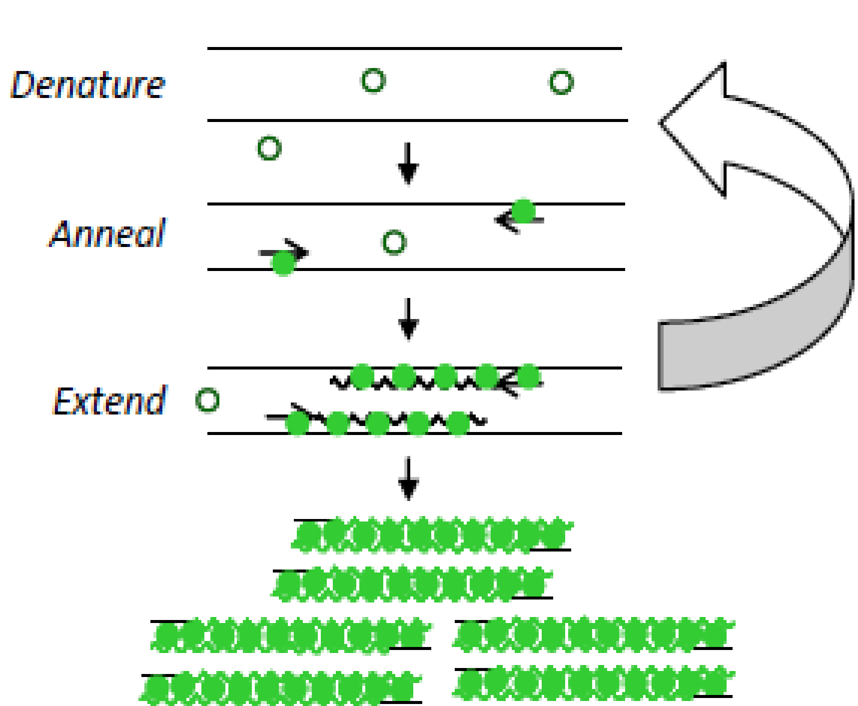
Dye-based qPCR

Uses a dsDNA-binding dye

- Low fluorescence of unbound dye
- Dye binds dsDNA stoichiometrically
- Measures all dsDNA in reaction
- Existing PCR assays can easily be adapted*
- Less expensive
- Not multiplexed
- Allows melt analysis for QC or genotyping



Examples:
SYBR Green, BRYT Green, LC Green, Eva Green

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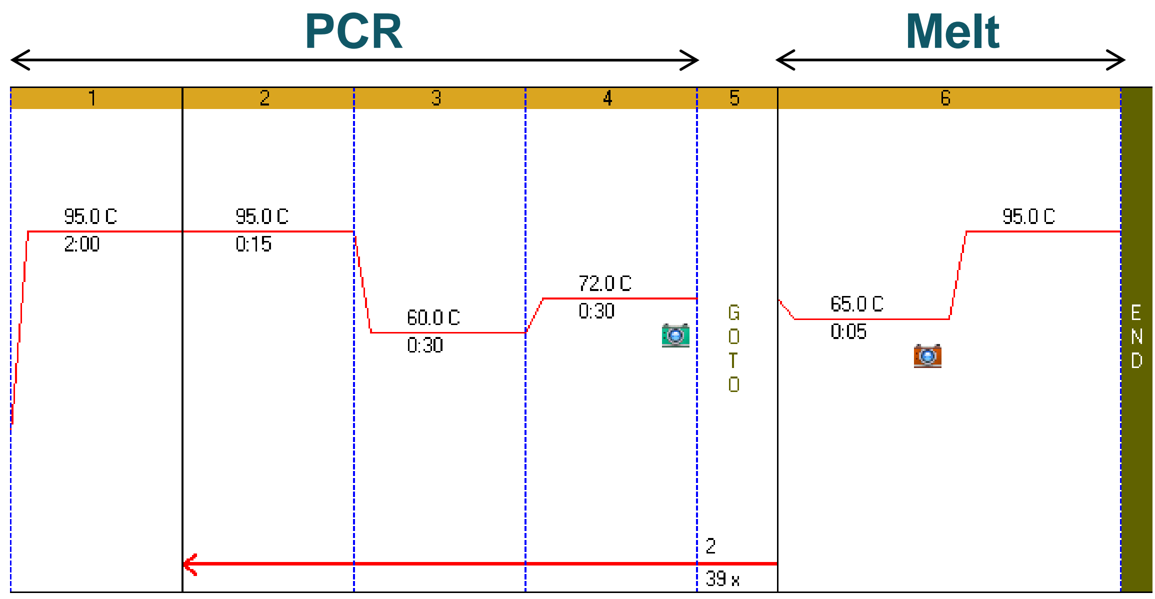
Melt Analysis: An internal QC

Melt or Dissociation Curve

- After amplification, product is heated slowly, signal is continually measured
- As dsDNA amplicon denatures, dsDNA dye is displaced, signal decreases
- Melt peak ≈ Tm of product
- Impacted by # of amplicons, size & base composition



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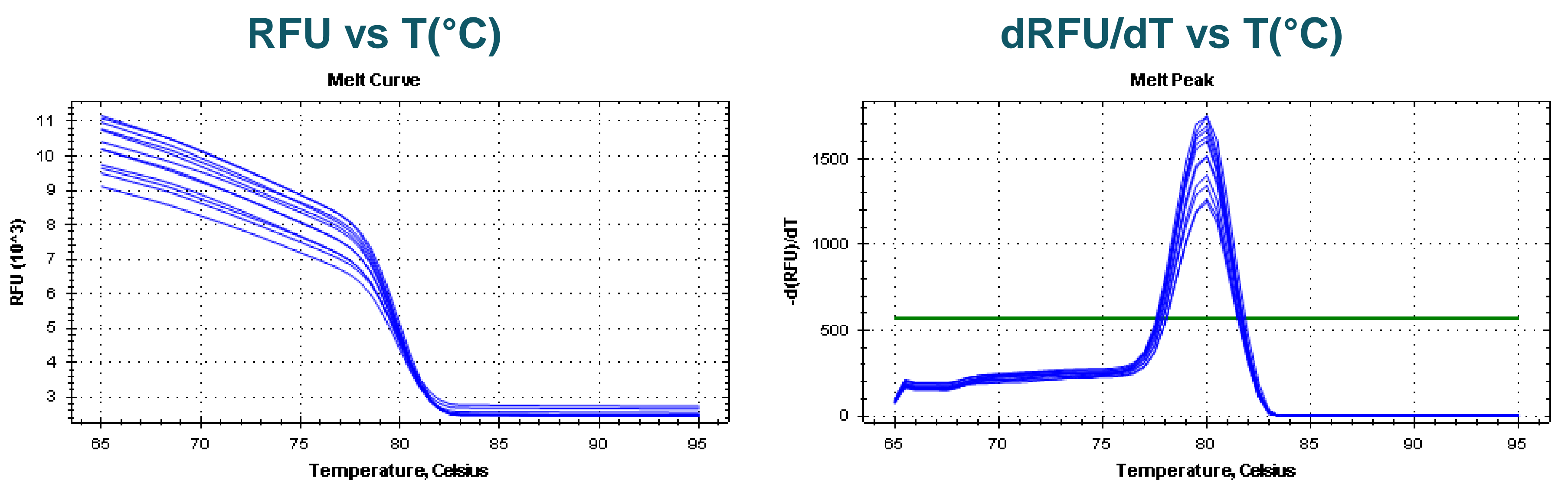

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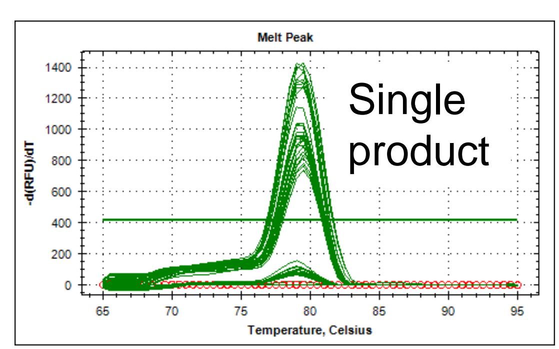
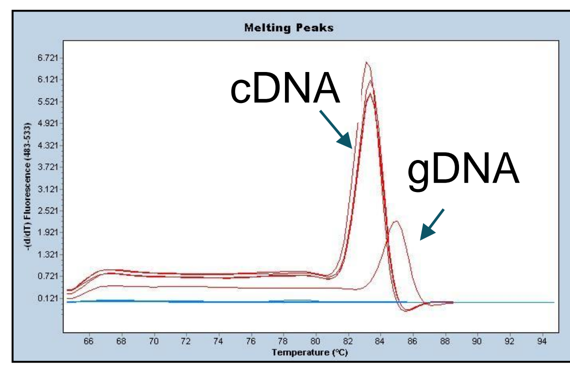
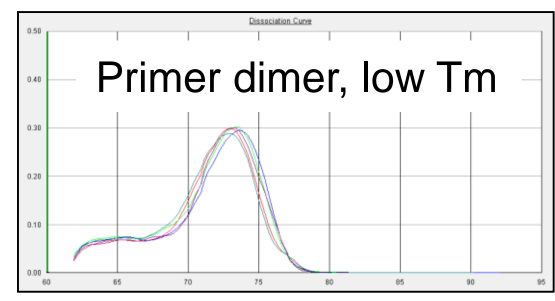
Melt Analysis: An Internal QC

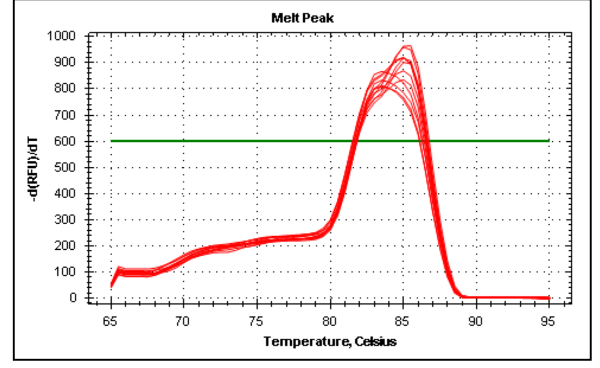
Quality control

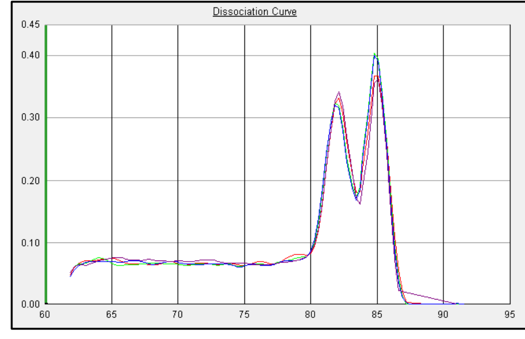


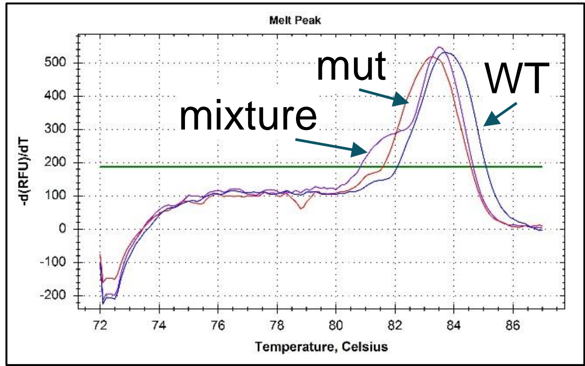




Multiple products







High Resolution Melt (HRM)

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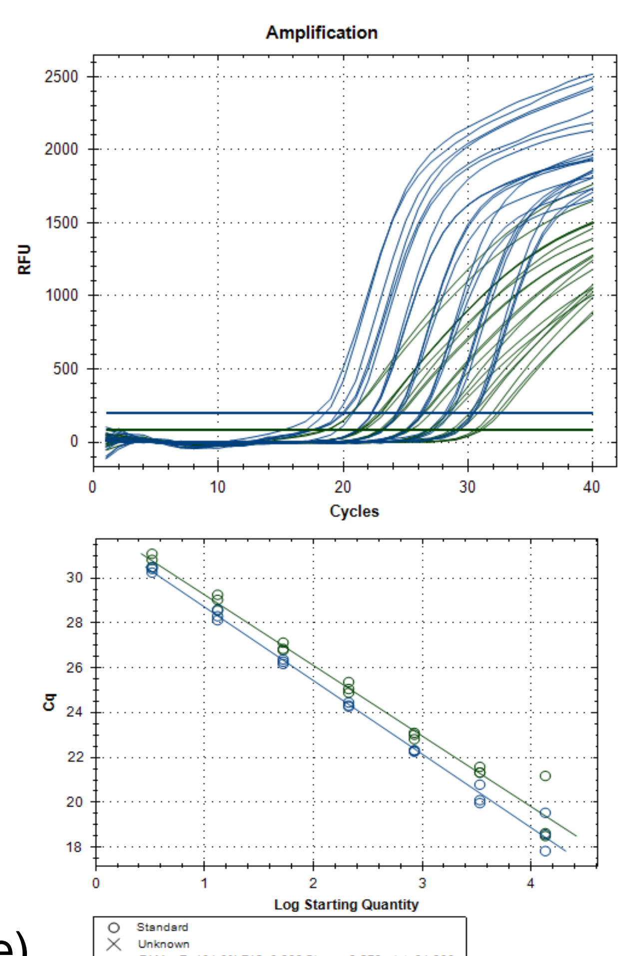
Probe-based qPCR

Uses primers and 1-2 dye-labeled probes

- Increases specificity of target detection
- gDNA, psuedogenes still detected if amplified
- More expensive
- Allows for detection of multiple targets
- May require more optimization
- TaqMan® doesn't allow melt analysis



Examples

- TaqMan® (hydrolysis probe)
- Molecular Beacons® (stem-loop FRET probe)
- Dual Hybridization Probes (donor-acceptor probe)

See *Real-time PCR Detection Chemistries, Navarro et al. (2015)*

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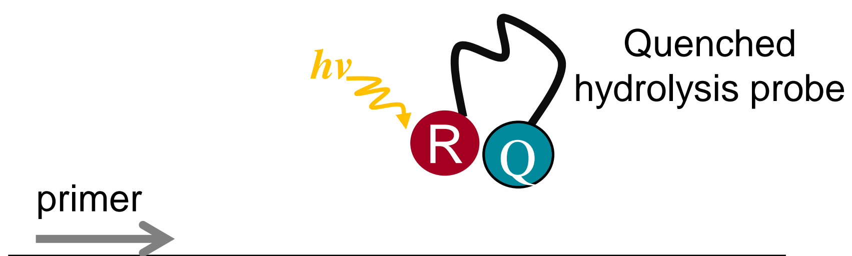
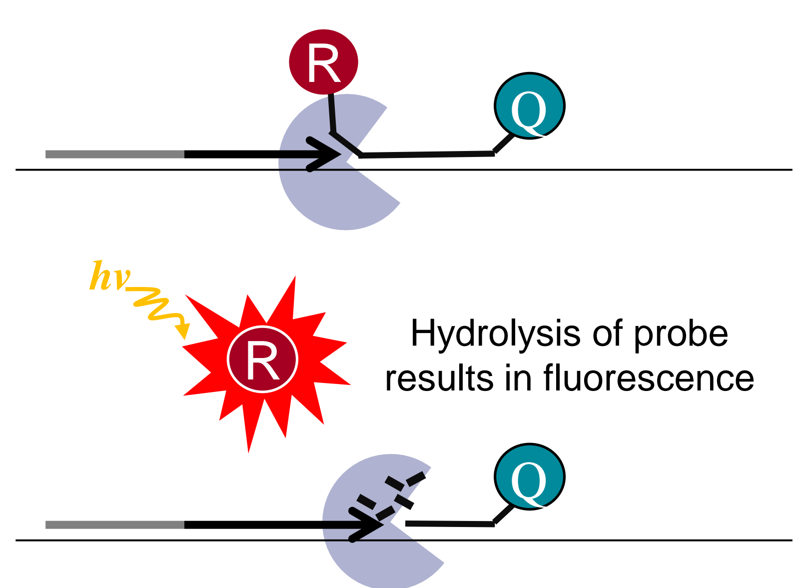
Probe-based qPCR:
TaqMan Fluorogenic 5' Nuclease Assay

- Probe contains reporter fluorophore and quencher
- Intact probe is quenched
- Taq degrades quenched probe during extension
- Requires 5' nuclease activity of Taq
- Irreversibly releases reporter dye from quenching





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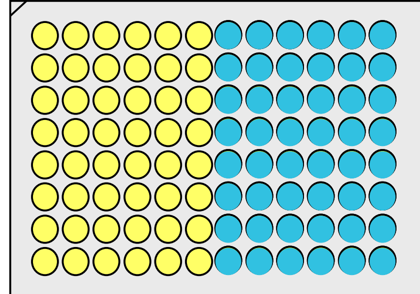
Multiplex Analysis

Analysis of multiple targets in the same well

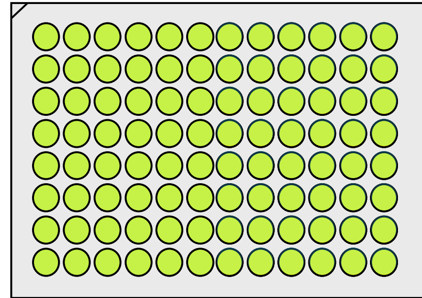
- Each target labeled with a different fluorophore
- Requires fewer wells for the same amount of data
- Requires less sample
- Better normalization
  - Same sample is assayed for all targets
- Requires more assay design & optimization
- Limited by instrumentation and dyes

Target 1
Target 2

Single target



Multiple targets



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qPCR Chemistries
Dye-based vs. Probe-based

	Dye-based qPCR	Probe-based qPCR
Amplicon labeling	dsDNA-binding dye	Fluorescently labeled probes
Cost	Lower cost	Higher cost
Flexibility	All optimized assays	Single assay
Instrumentation	All qPCR instruments	Must match probes to filters
Specificity	Measures all dsDNA	Measures amplicon with probe sequence
Multiplexing	No	Yes – different dyes/filters
Melt analysis *QC and genotyping*	Yes	No (TaqMan)
Throughput	High	Highest (multiplexed)
Sample required	Low	Lowest (multiplexed)
Requires validation	Yes	Yes

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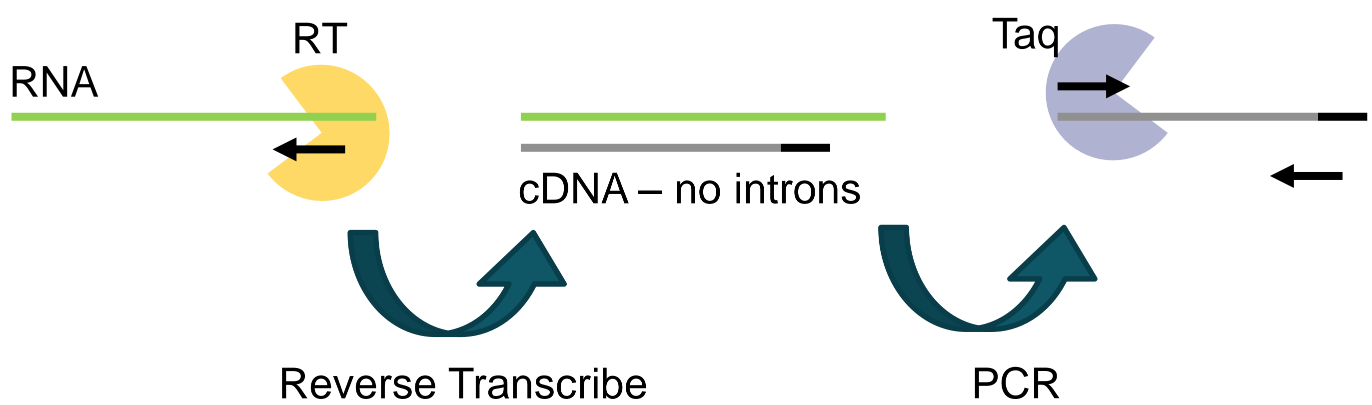
Reverse Transcription qPCR (RT-qPCR)



Applications

- Gene expression
- Biomarker discovery
- RNA Sequencing
- RNA viruses
- cDNA cloning

Reverse Transcriptase

- RNA-directed DNA polymerase
- Requires priming, Mg^{2+} or Mn^{2+} cofactor
- RNase H activity in wildtype
- Inhibits Taq polymerase
- AMV, MMLV

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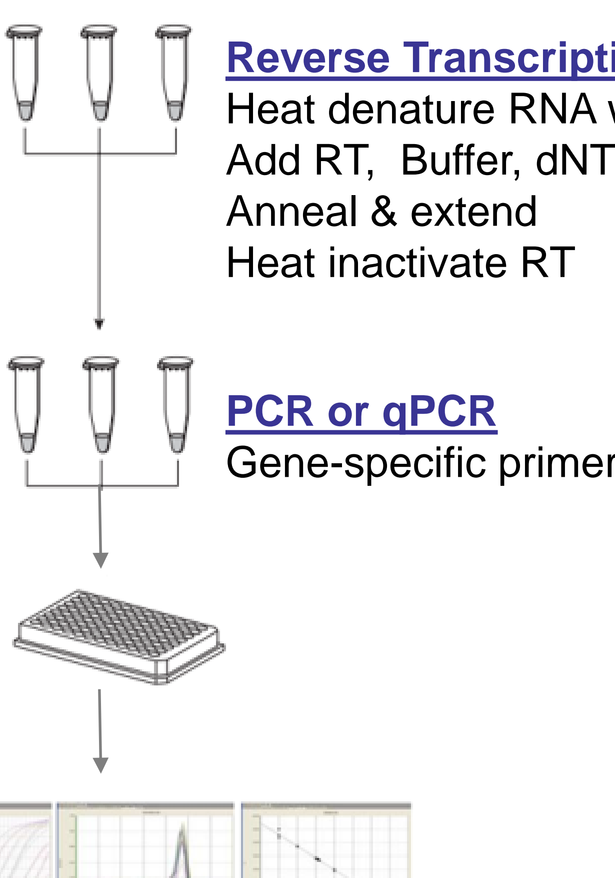
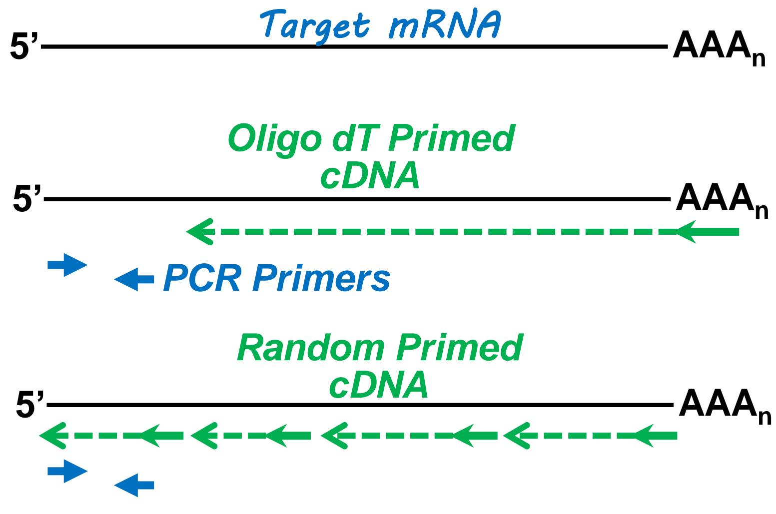
Reverse Transcription

Two-step RT-qPCR—Making a pool of cDNA

Two Step RT-qPCR





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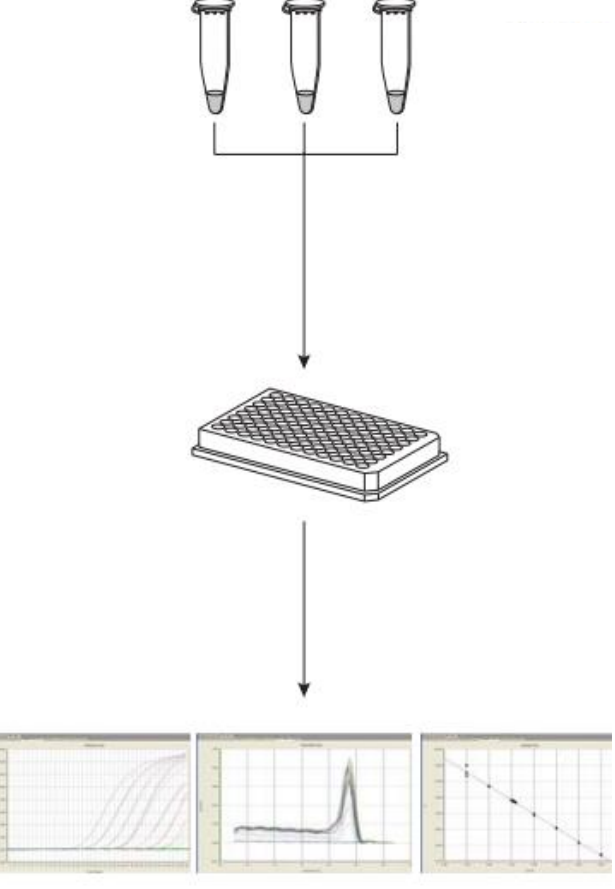
Reverse Transcription

One-step RT-qPCR—Amplification of a single target

One Step RT-qPCR



RT & qPCR
Set up as for qPCR
Add RT and RNasin
Use gene-specific primers

Cycling considerations
Perform RT first
Inactivate RT/ Activate Taq
Standard qPCR cycling

Benefits
Uses less sample
Replicates over both steps
Quant & QC for FFPE RNA

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Real-Time Instruments

Examples:

Bio-Rad
CFX96 Touch

ABI
7500 Fast

Roche
LightCycler 480

Stratagene
Mx3005p

Hardware Differences

- Excitation source (lamp, LED, LASER)
- Detection method (CCD camera, PMT)
- Filters (Filter wheel, # of filters)
- Specialized (Gradient block, # of wells, interchangeable blocks, fast cycling)

Software Differences

- Analysis methods
- Analysis flexibility
- Ease of use
- Specialized (HRM, bar-coding, etc.)
- Traceability, *in vitro* Diagnostic use

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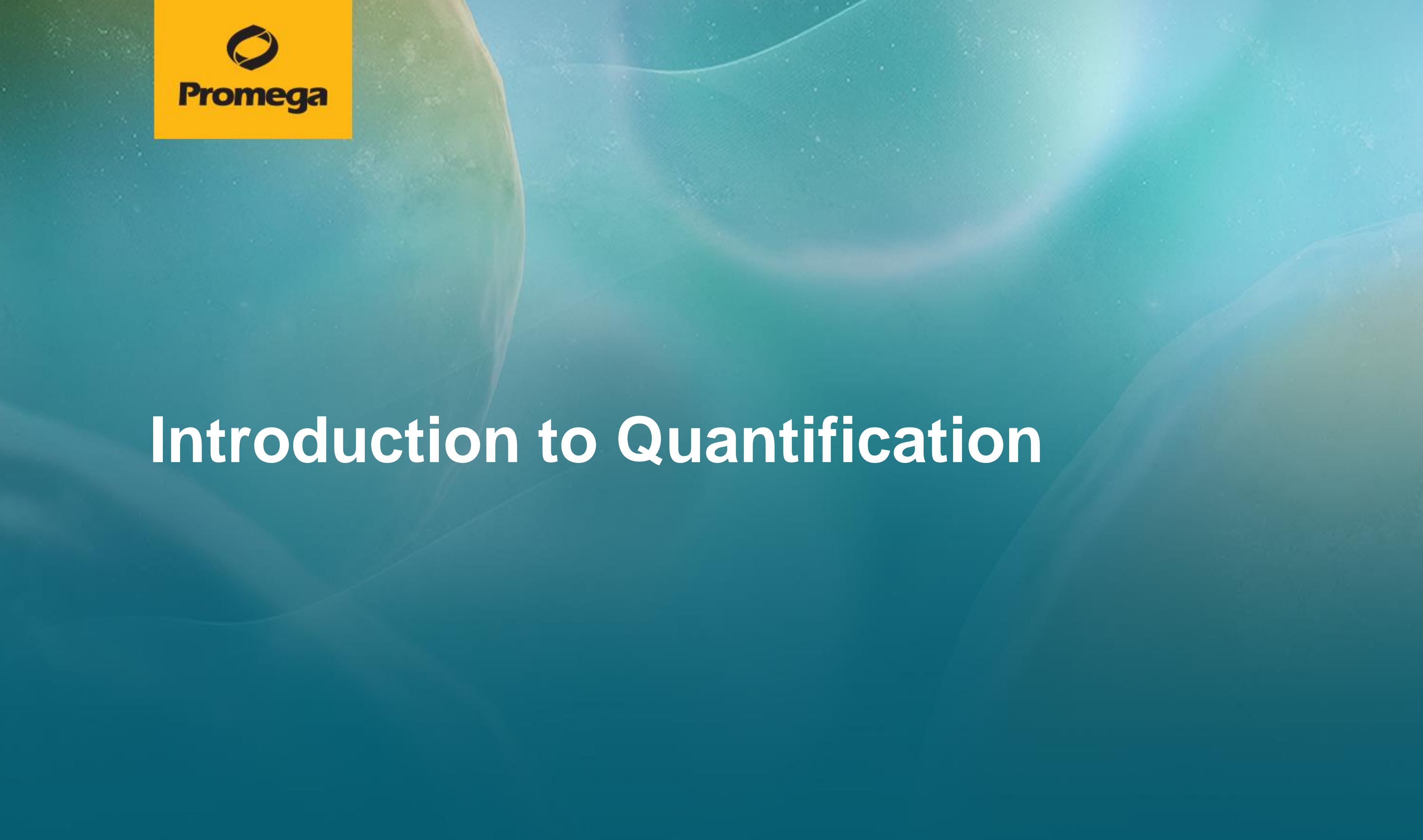

Introduction to Quantification

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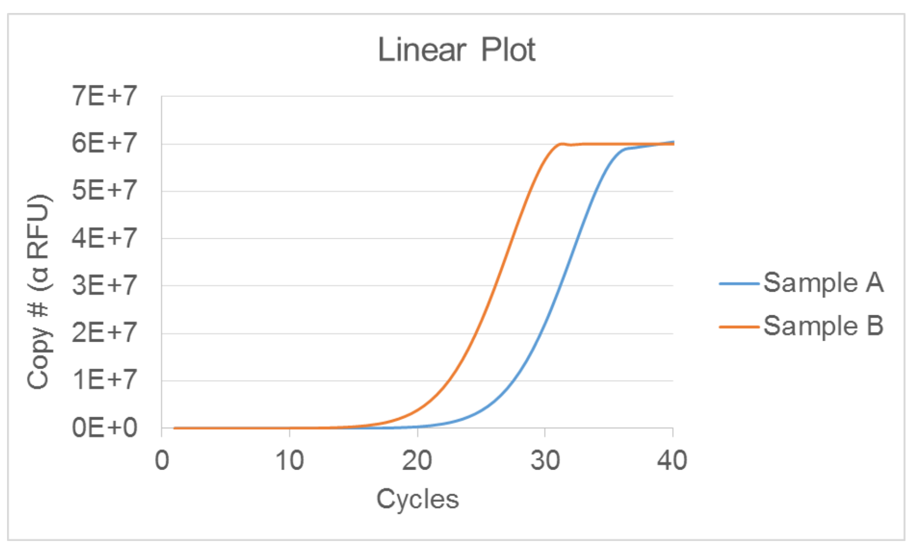
Comparing Samples

Assuming identical input amounts



Sample	Copy#	Cycle1	Cycle2	Cycle3	Cycle4	Cycle5	Cycle6	Cycle n
A	1	2	4	8	16	32	64	$1*2^n$
B	10	20	40	80	160	320	640	$10*2^n$

Quantity at a given cycle "n" is expressed as $a * 2^n$
where a is starting quantity

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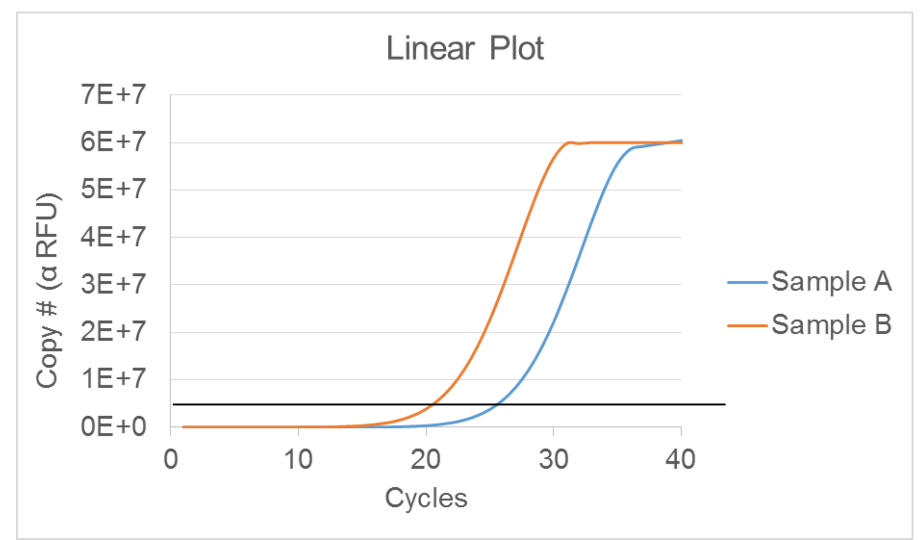
Comparing Samples

Assuming identical input amounts

<u>ΔCq method</u>

$$\frac{b}{a} = 2^{\Delta Cq}$$

Not recommended!



Sample A will reach the threshold T $= a * 2^{x}$ at cycle x
Sample B will reach the threshold T $= b * 2^{y}$ at cycle y

Since the threshold T is the same for both,

$$a * 2^{x} = b * 2^{y}$$

If we rearrange to determine fold difference in starting quantities,

$$\frac{b}{a} = \frac{2^{x}}{2^{y}} = \frac{2*2*2*2*\cdots x\ times}{2*2*2*2*\cdots y\ times} = 2^{x-y} = 2^{\Delta Cq}$$

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Common Assumptions in Calculations

- ΔCq method
  - **ASSUMPTION 1**: Amplification efficiency is perfect

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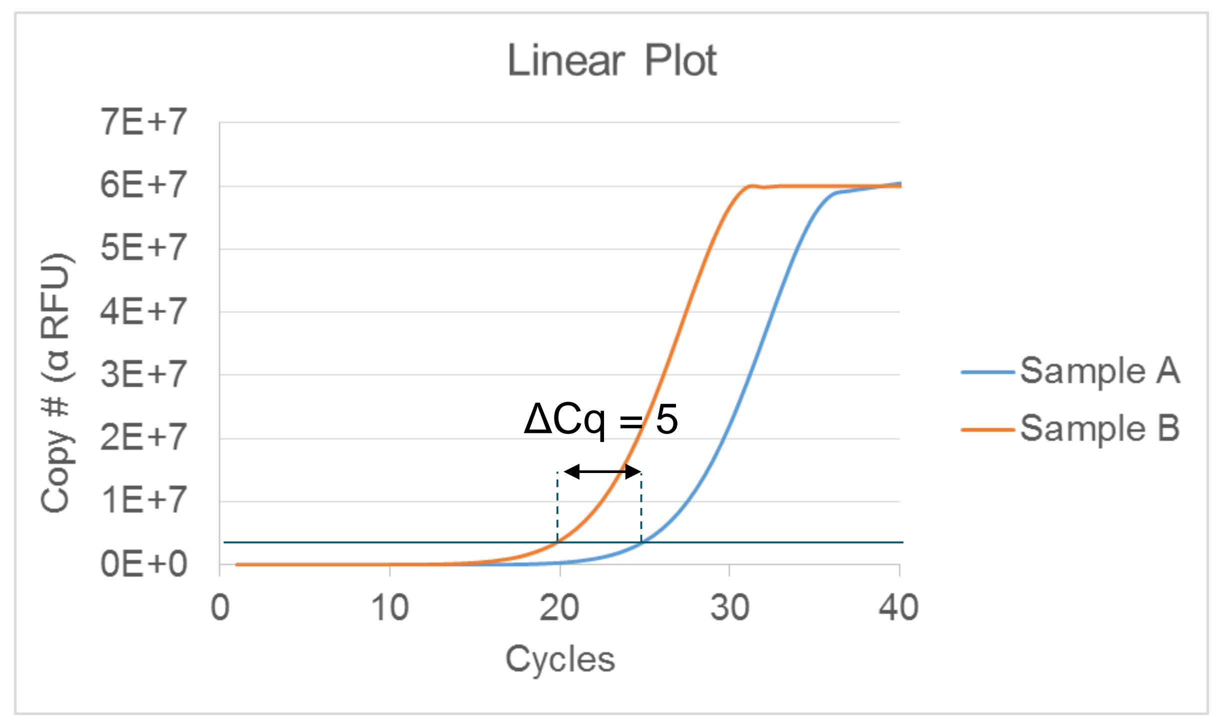
Common Assumptions in Calculations



QUESTION 2: What is the difference in target concentration between samples A & B if the assay has 100% amplification efficiency?

$$\frac{b}{a} = 2^{\Delta Cq} = 2^5$$

32-fold different

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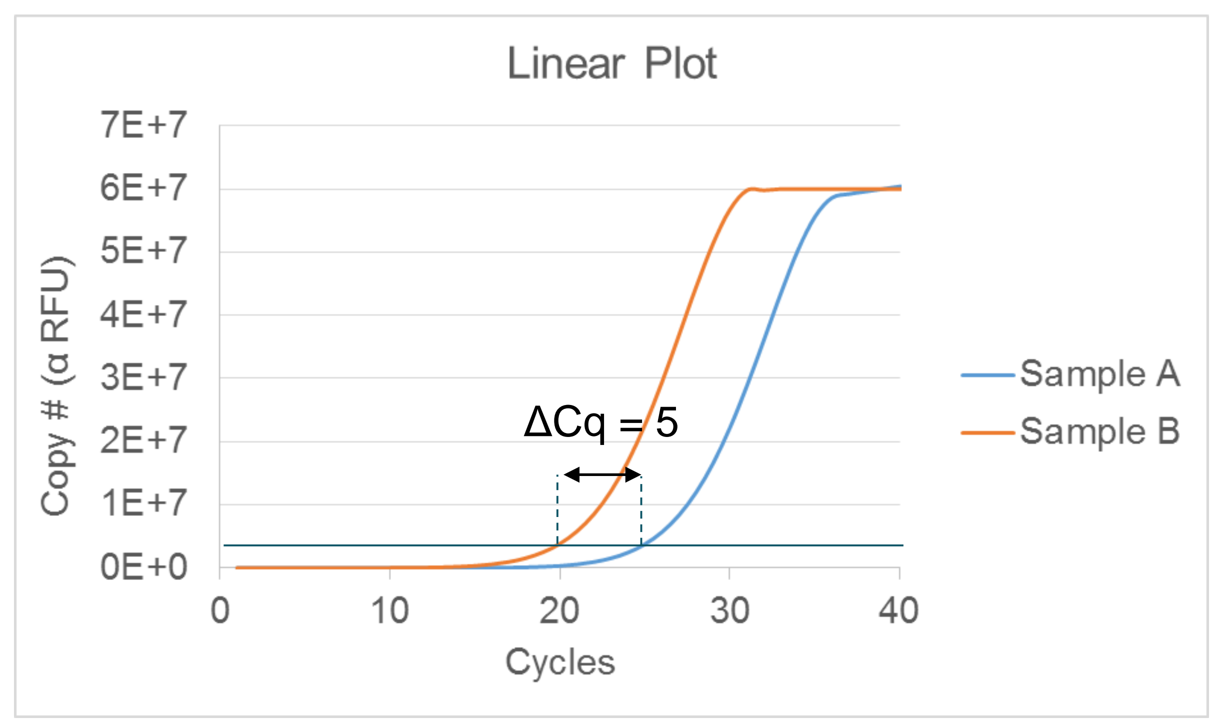
Common Assumptions in Calculations



QUESTION 3: What is the difference in target concentration between samples A & B if the assay has 80% amplification efficiency?

$$\frac{b}{a} = 1.8^{\Delta Cq} = 1.8^5$$

18.9-fold different

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Common Assumptions in Calculations

- ΔCq method
  - **ASSUMPTION 1**: Amplification efficiency is perfect
    - Instead of $2^{\Delta Cq}$, measure the assay efficiency experimentally and use it!
    - Use your samples (or very similar ones) to determine efficiency
  - **ASSUMPTION 2**: Identical inputs were used from each sample
    - Quantification accuracy really matters!
    - Normalize to a reference gene that is expressed at a constant concentration
- ΔΔCq method
  - Normalize $\frac{b}{a}$ between GOI and reference gene in the same sample
  - **ASSUMPTION 3:** Reference gene expression is constant
    - Use multiple reference genes and verify expression is constant
    - See *Hellemans & Vandesompele (2014)!*

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The MIQE Guidelines—*Read BEFORE You Plan!*

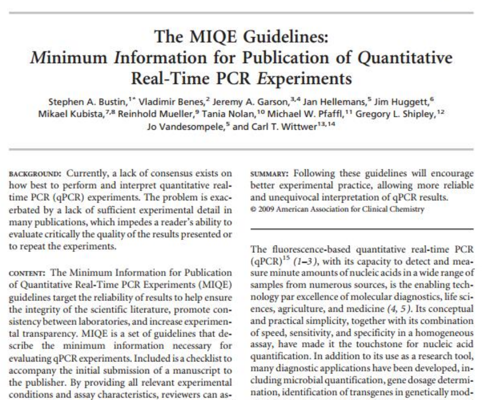
The MIQE Guidelines

***M**inimum **I**nformation for Publication of **Q**uantitative Real-Time PCR **E**xperiments*

Bustin, et al., Clinical Chemistry 55:4, 611–622 (2009)

- Goal is to improve repeatability between labs
- Outlines common vocabulary to use
- Includes a checklist for reporting & planning
- Describes minimal reporting requirements

1. Description of sample manipulation
   storage, extraction, quantification, integrity, etc.
2. Description of assay reagents, protocols, and controls
   standard curves, ≥2 reference genes, -RT controls, NTCs
3. Assay validation—Required even for purchased assays!
   sensitivity, specificity, and efficiency; reference gene stability
4. Description of calculations


Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines:
*M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

BACKGROUND: Currently, a lack of consensus exists on how best to perform and interpret quantitative real-time PCR (qPCR) experiments. The problem is exacerbated by a lack of sufficient experimental detail in many publications, which impedes a reader's ability to evaluate critically the quality of the results presented or to repeat the experiments.

CONTENT: The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. MIQE is a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments. Included is a checklist to accompany the initial submission of a manuscript to the publisher. By providing all relevant experimental conditions and assay characteristics, reviewers can assess the validity of the protocols used. Full disclosure of all reagents, sequences, and analysis methods is necessary to enable other investigators to reproduce results. MIQE details should be published either in abbreviated form or as an online supplement.

SUMMARY: Following these guidelines will encourage better experimental practice, allowing more reliable and unequivocal interpretation of qPCR results.
© 2009 American Association for Clinical Chemistry

The fluorescence-based quantitative real-time PCR (qPCR)[15] *(1–3)*, with its capacity to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources, is the enabling technology par excellence of molecular diagnostics, life sciences, agriculture, and medicine *(4, 5)*. Its conceptual and practical simplicity, together with its combination of speed, sensitivity, and specificity in a homogeneous assay, have made it the touchstone for nucleic acid quantification. In addition to its use as a research tool, many diagnostic applications have been developed, including microbial quantification, gene dosage determination, identification of transgenes in genetically modified foods, risk assessment of cancer recurrence, and applications for forensic use *(6–11)*.

This popularity is reflected in the prodigious number of publications reporting qPCR data, which invariably use diverse reagents, protocols, analysis methods, and reporting formats. This remarkable lack of consensus on how best to perform qPCR experiments has the adverse consequence of perpetuating a string [illegible] its status as [illegible] cal deficiencies [illegible] the following: (*a*) inadequate sample storage, prep-

[1] Centre for Academic Surgery, Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London, UK; [2] Genomics Core Facility, EMBL Heidelberg, Heidelberg, Germany; [3] Centre for Virology, Department of Infection, University College London, London, UK; [4] Department of

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Real-Time PCR Resources On-line

General Real-Time PCR:

MIQE Guidelines:	Bustin *et al.*, *Clin Chem* (2009) http://miqe-press.gene-quantification.info/
Gene Quantification:	www.gene-quantification.info
Real-time chemistries:	Navarro *et al.*, *Clinica Chimica Acta* (2015)
Reference genes:	Hellemans and Vandesompele, *Methods Mol Biol* (2014) http://www.gene-quantification.com/Bio-Rad_2008_Rethink_PCR_Conference_Hellemans_Vandesompele.pdf geNorm and qBase+ software

Primer design software & Pre-designed assays:

Primer3 - http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

Primer-BLAST - http://www.ncbi.nlm.nih.gov/tools/primer-blast/

IDT PrimerQuest - http://biotools.idtdna.com/Primerquest/

Primer Bank - http://pga.mgh.harvard.edu/primerbank/

RTPrimerDB - http://medgen.ugent.be/rtprimerdb

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