



Introduction to the QuantStudio™ 6 Pro Real-Time PCR System

Lauren Neves, PhD
Field Application Scientist

What is quantitative real-time PCR?

Real-time PCR is the technology to sensitively detect and monitor the progress of PCR amplification of nucleic acid targets as it occurs.



Standard Curve

Viral load analysis

Relative Standard Curve

Gene expression analysis

Comparative Ct ($\Delta\Delta$ Ct)

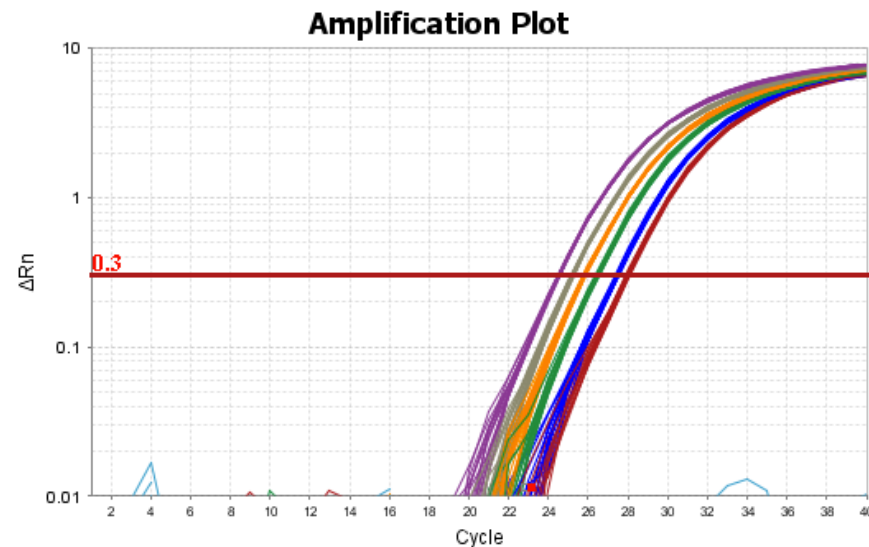
Gene expression
miRNA expression
Copy number variation
Mutation detection

Genotyping

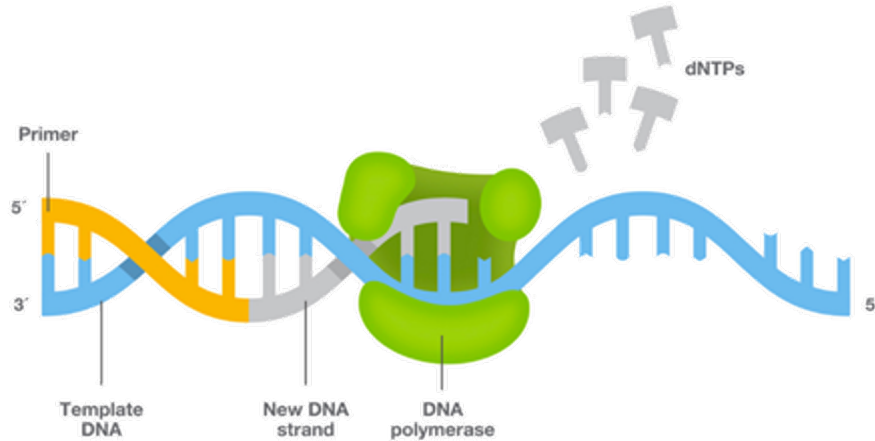
Allelic discrimination/SNPs

Presence/Absence

Pathogen detection



How is qPCR similar to traditional PCR?



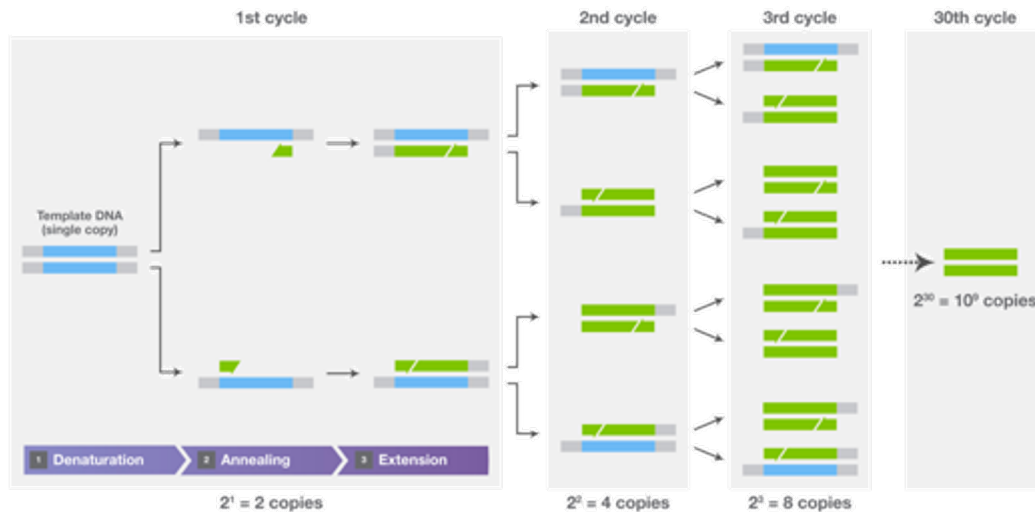
Similar reagents:

dNTPs

Mg²⁺ buffer

Taq polymerase

Primers

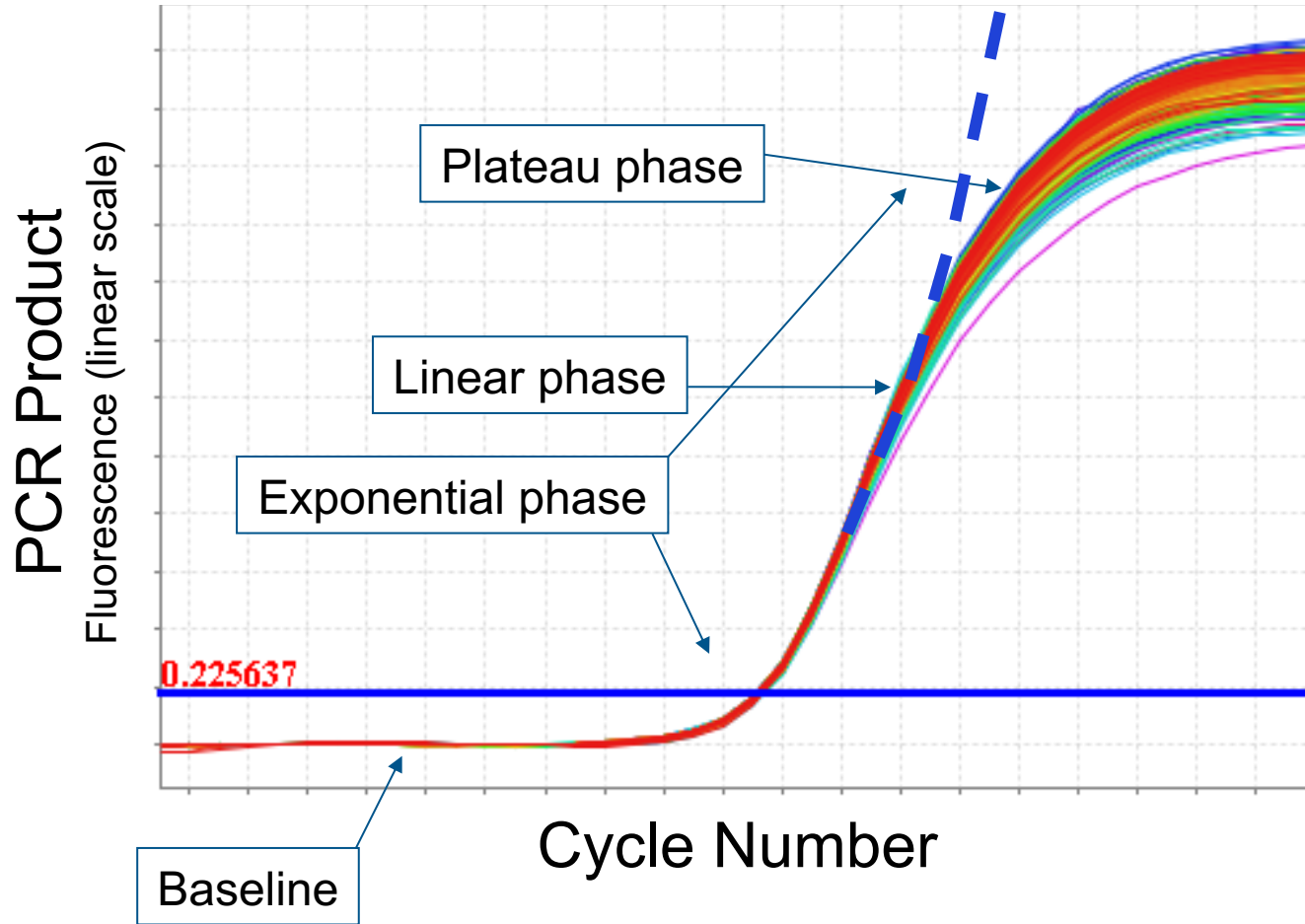


Similar reactions:

Rely on thermocycling to achieve:

- Denaturation of dsDNA template
- Annealing of primers to template
- Extension of new amplicon

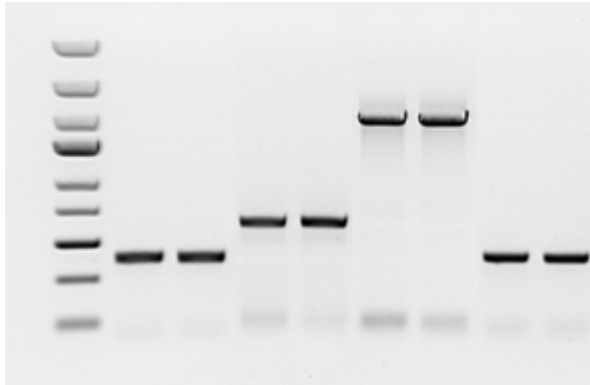
The phases of PCR amplification



Traditional PCR versus real-time PCR

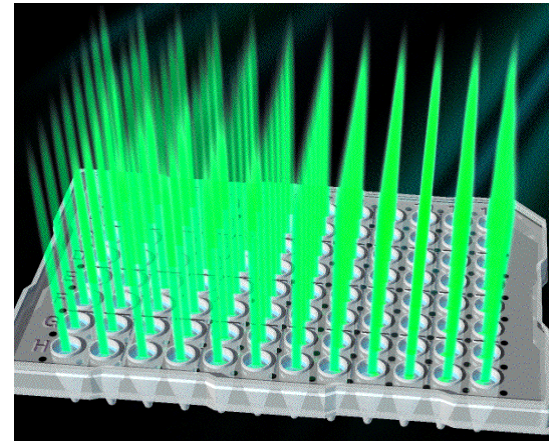
Traditional PCR

- Product is detected only after amplification cycles are complete
- Often followed by gel electrophoresis



Real-time PCR

- Fluorescent molecules added PCR before cycling
- Fluorescent signal is proportional to PCR product
- Fluorescence is read and recorded after each cycle

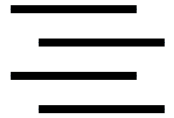


How does real-time quantitative PCR work?

PCR target



PCR



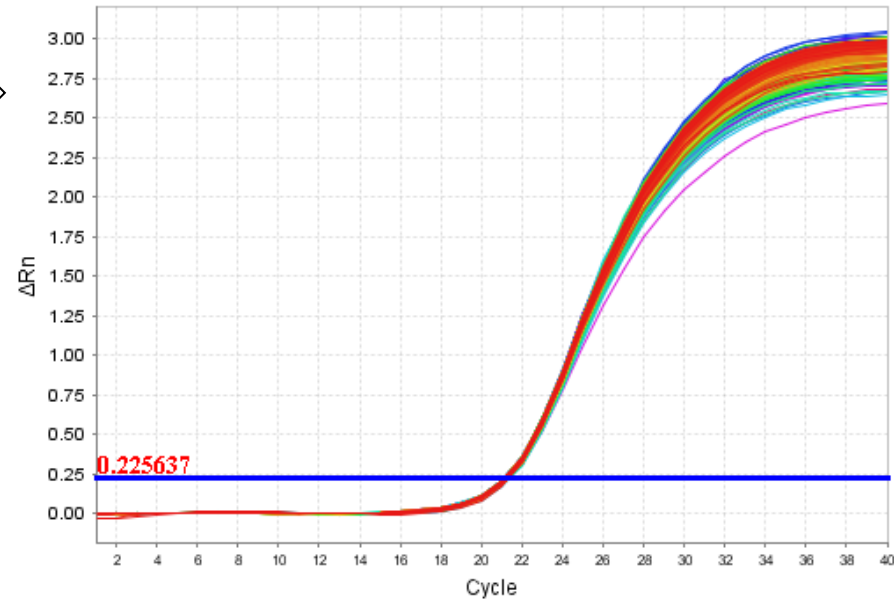
PCR



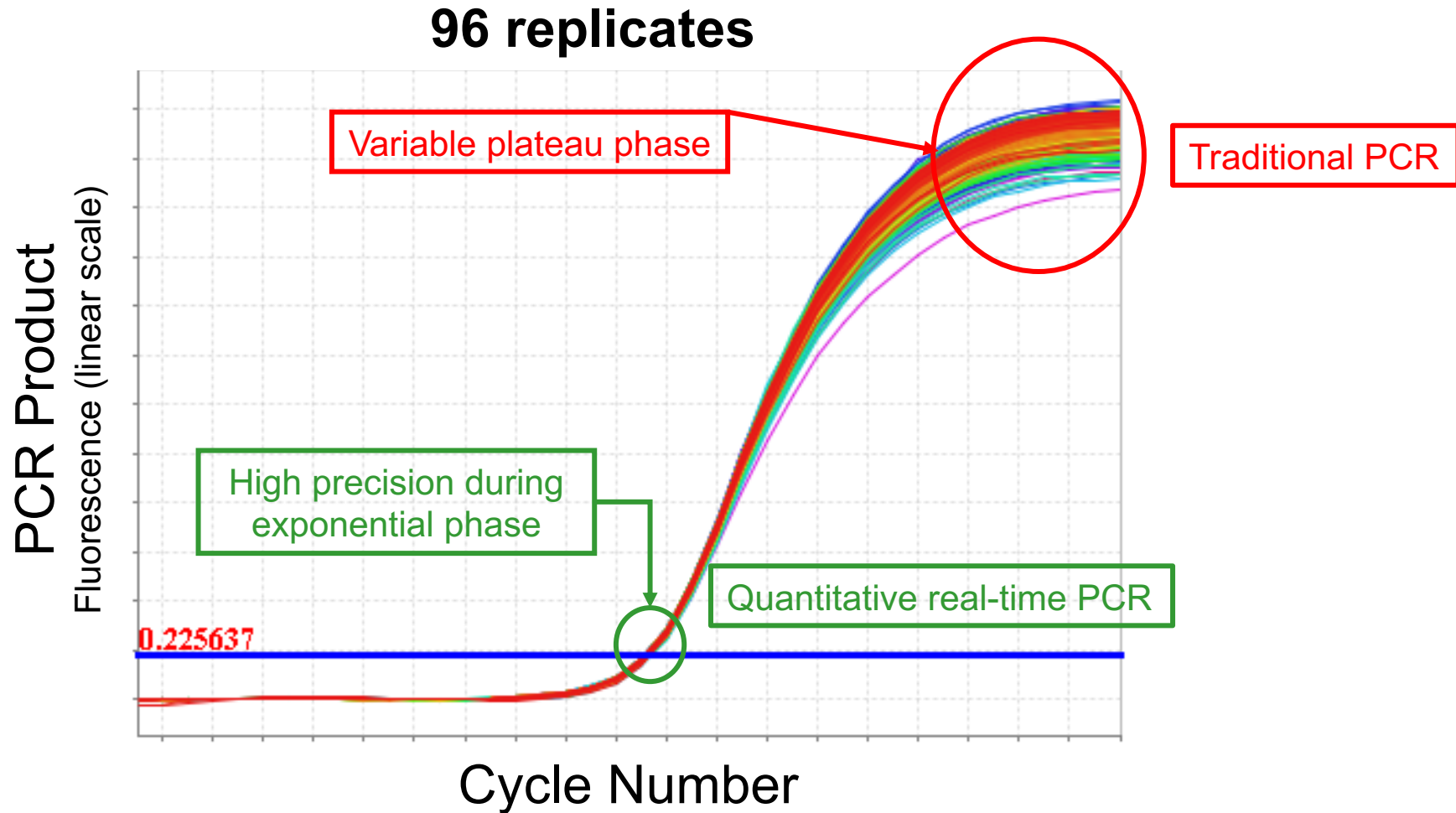
Fluorescence recorded



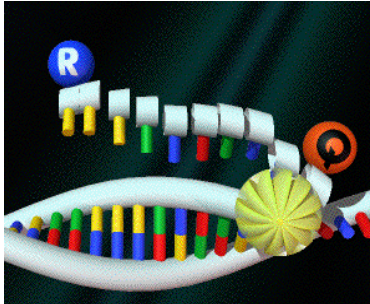
Amplification Plot



How does real-time quantitative PCR work?



Real-time PCR requirements



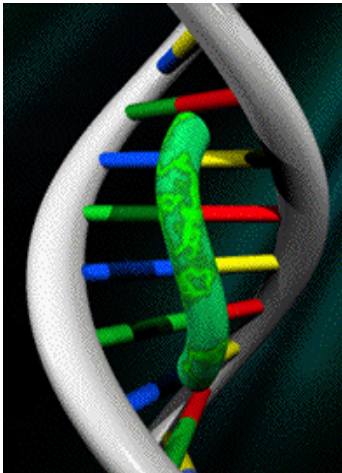
Real-time chemistry:
Fluorescent signal automatically
emitted each cycle



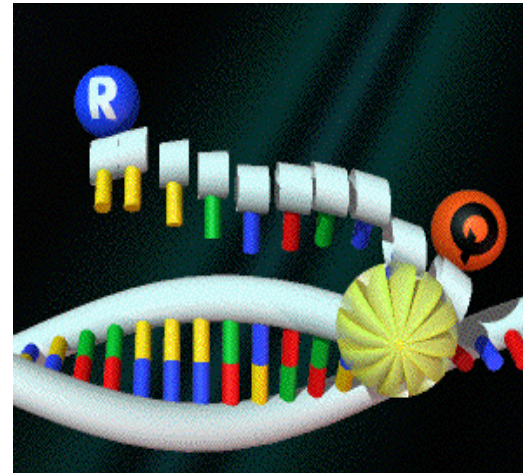
Real-time instrument:
Thermal cycler & optical detector

Chemistry of real-time qPCR

SYBR[®] Green I dye

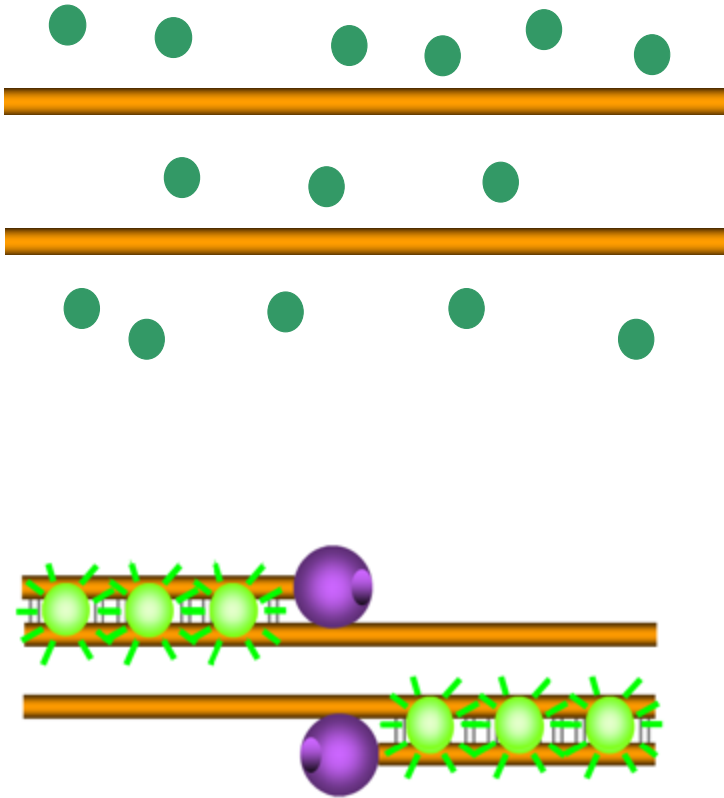


5'-nuclease chemistry

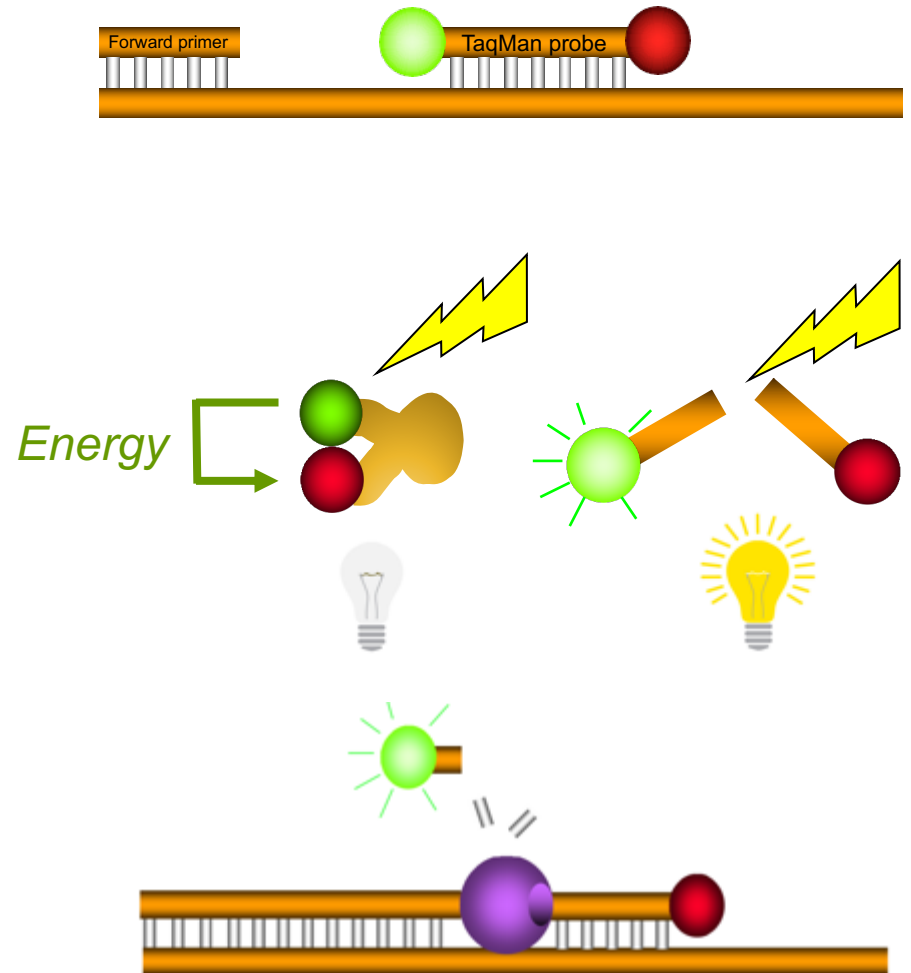


Chemistries of real-time PCR

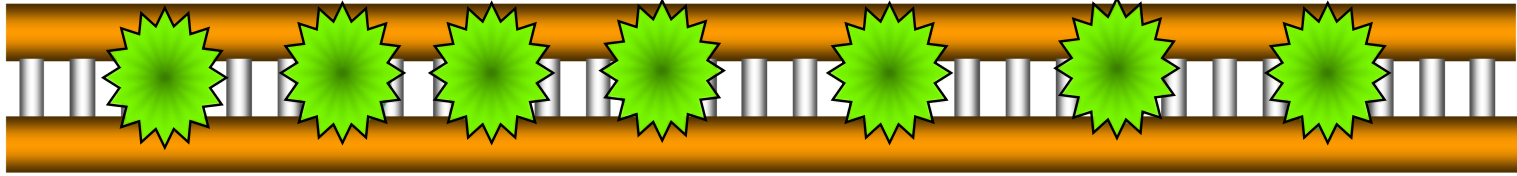
SYBR[®] chemistry



TaqMan[®] chemistry



What's the problem with SYBR® Green I dye?



Binds non-specifically to *any* double-stranded DNA

Signal from non-specific products

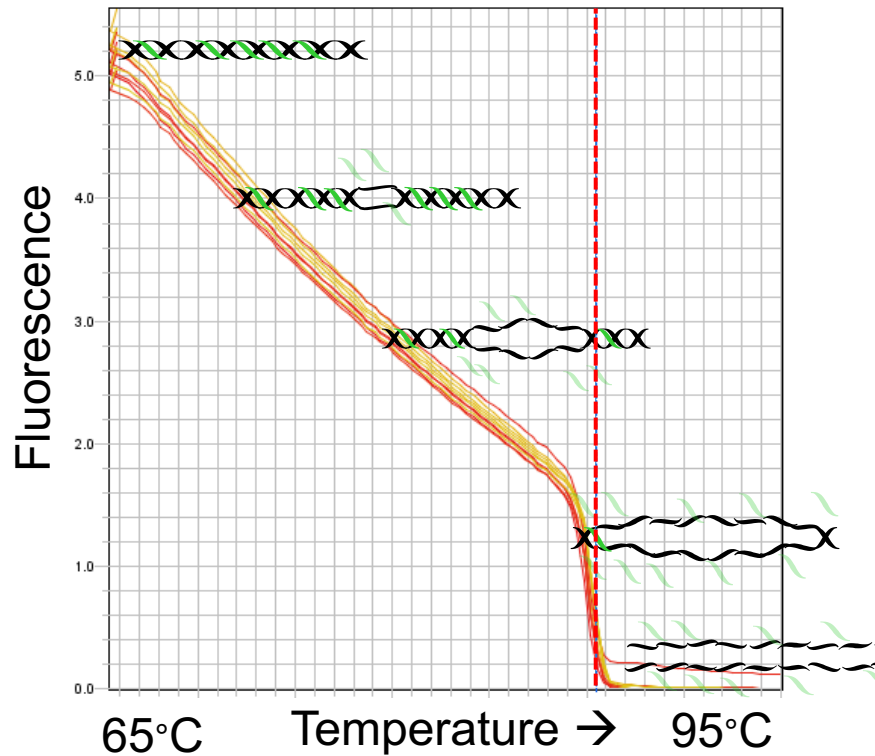


Incorrect quantitative results

Checking specificity with a melt curve

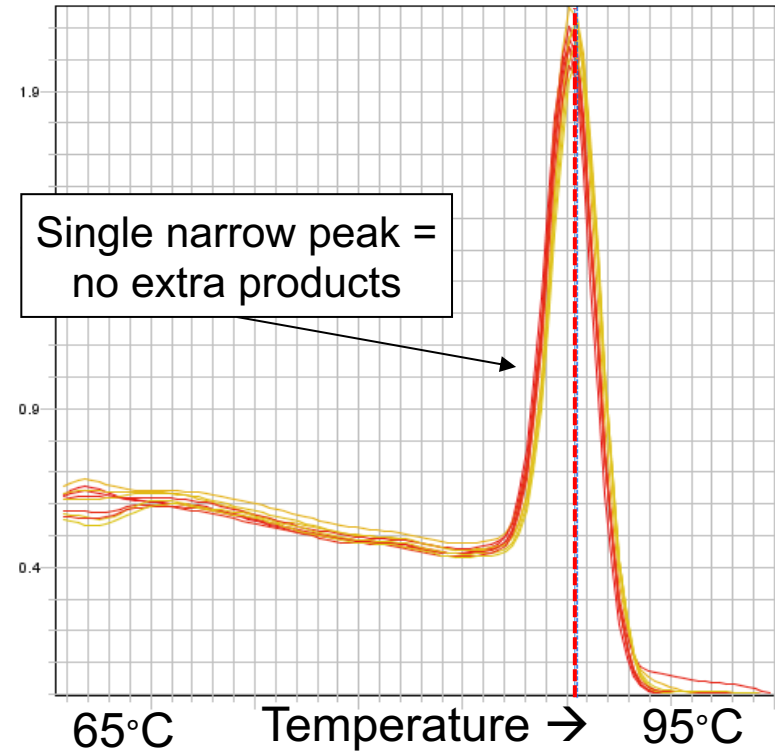
Normalized reporter view

Melt Curve



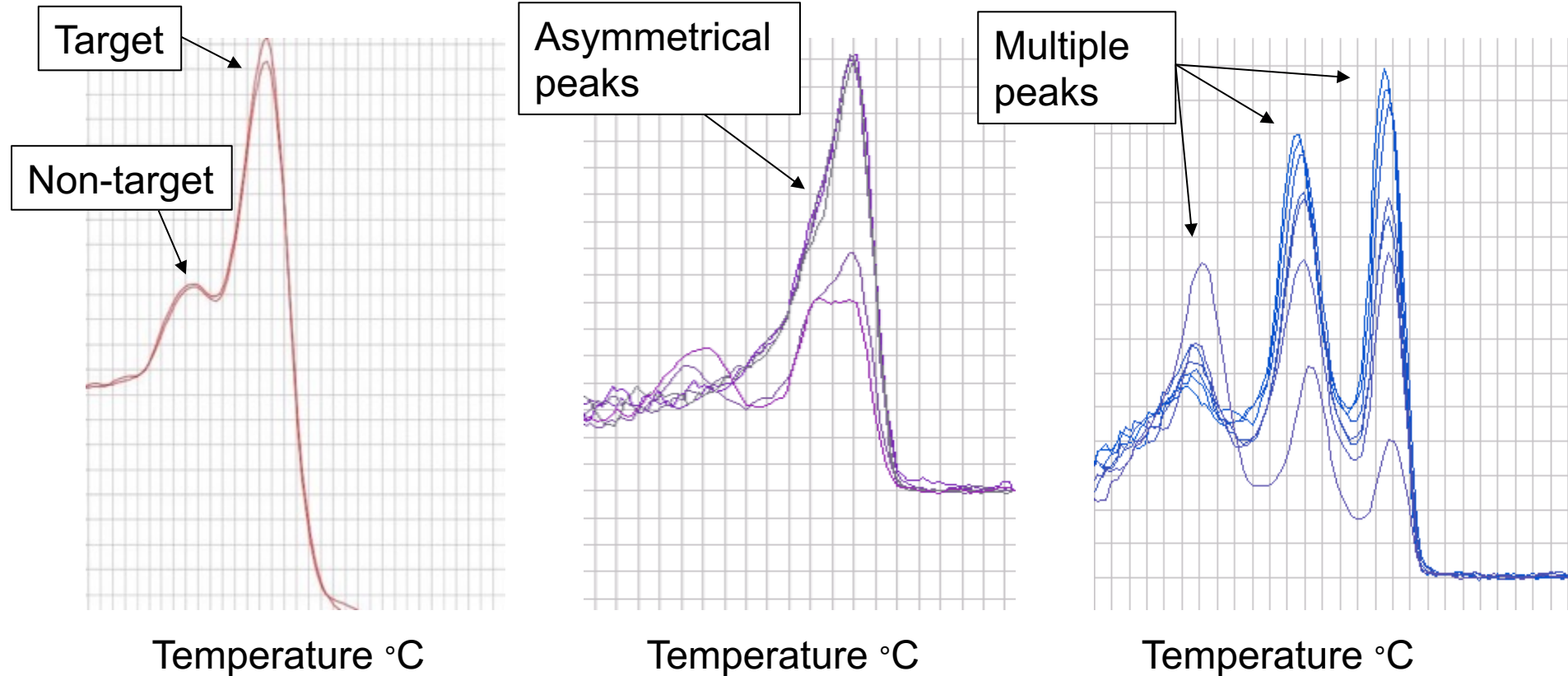
First derivative view

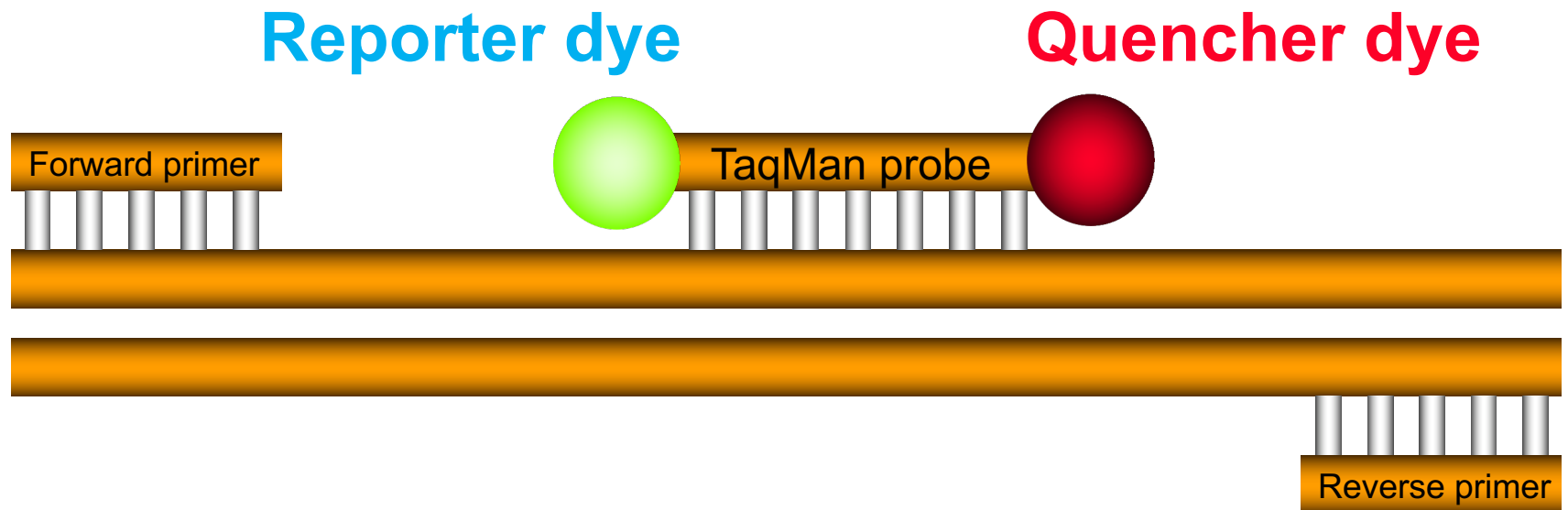
Melt Curve



SYBR® Green non-target melt peaks

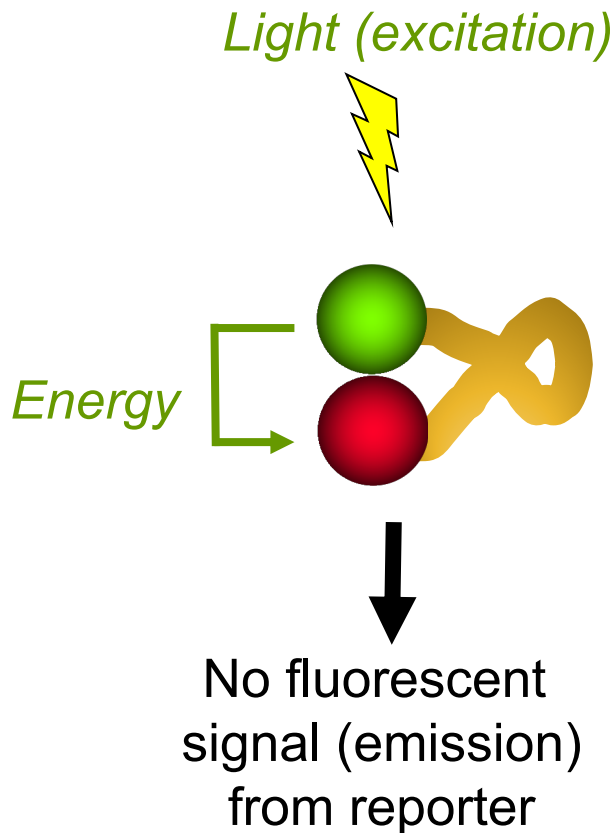
Primer-dimer or amplification of multiple products



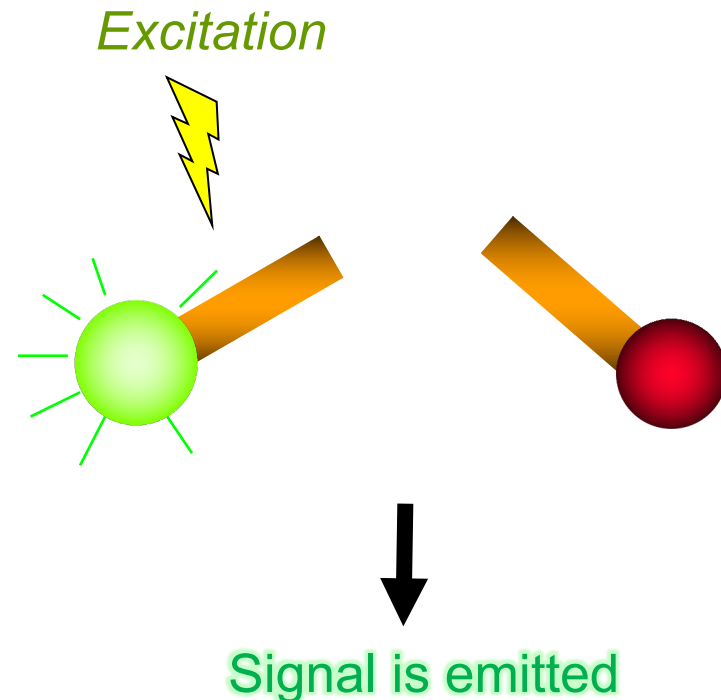


Fluorescent Resonance Energy Transfer

Intact probe



...if probe is split...



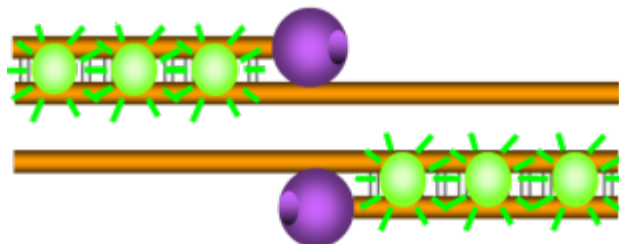
How TaqMan™ chemistry works



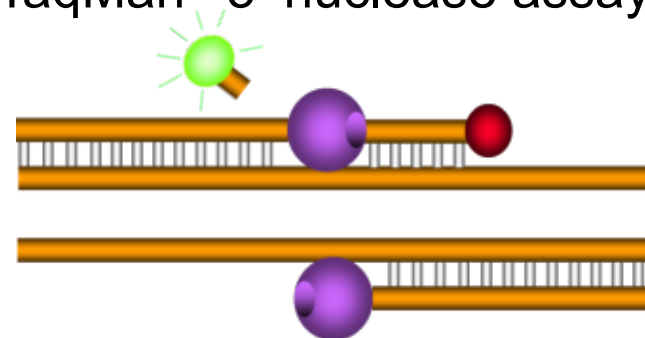
[How TaqMan™ chemistry works](#)

Supported fluorescent chemistries

SYBR® Green I dye



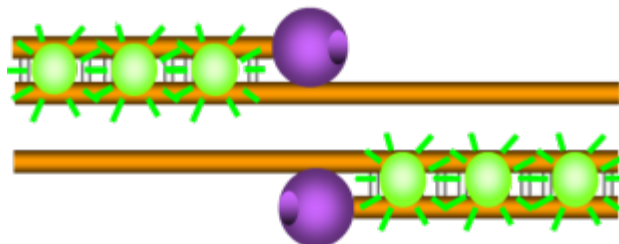
TaqMan® 5'-nuclease assay



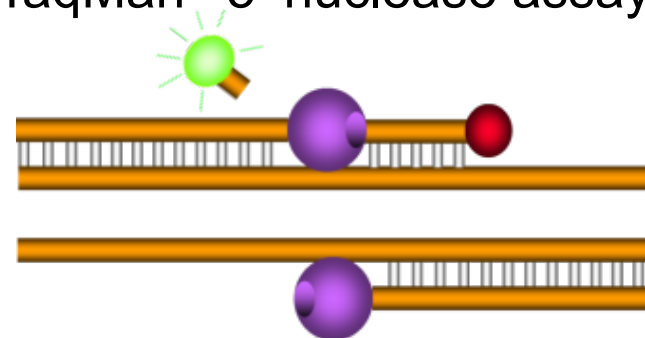
	SYBR® Green	TaqMan®
Detection	dsDNA binding dye	Target-specific fluorescent probe
Target definition	Two primers	Two primers and one probe
Specificity	Non-target products produce signal	<i>Only products from target produce signal</i>
Melt curve	Necessary to assay specificity	No melt curve needed
Multiplexing	No	Yes
Optimization	Required to ensure specificity and efficiency	Little to none required (pre-designed TaqMan® assays)

Supported fluorescent chemistries

SYBR® Green I dye



TaqMan® 5'-nuclease assay



	SYBR® Green	TaqMan®
Detection	dsDNA binding dye	Target-specific fluorescent probe
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Singleplex versus multiplex experiments

Singleplex: one assay per well

	1	2	3
A	Sample 1 U Target 1	Sample 1 U Target 1	Sample 1 U Target 1
B	Sample 2 U Target 1	Sample 2 U Target 1	Sample 2 U Target 1
C	Sample 1 U Normalizer	Sample 1 U Normalizer	Sample 1 U Normalizer
D	Sample 2 U Normalizer	Sample 2 U Normalizer	Sample 2 U Normalizer

Multiplex: two or more assays per well

	1	2	3
A	Sample 1 U Normalizer U Target 1	Sample 1 U Normalizer U Target 1	Sample 1 U Normalizer U Target 1
B	Sample 2 U Normalizer U Target 1	Sample 2 U Normalizer U Target 1	Sample 2 U Normalizer U Target 1

- ✓ Increased throughput
- ✓ Reduced reagent consumption
- ✓ Reduced sample consumption
- ✓ Improved precision

Drawbacks?

Requires more optimization

Pre-designed TaqMan[®] assays

21,685,182 TaqMan® pre-developed real-time PCR assays

Type	Number available	Species available
Gene expression	>2.6 million	36 species
SNP genotyping	>17 million	Mouse, human
Copy number variation	>1.8 million	Mouse, human
microRNA	>23 million	Mouse, human, rat

WHITE PAPER
TaqMan® Assays QPCR Guarantee Program



Gene Expression Assay Performance Guaranteed
With the TaqMan® Assays QPCR Guarantee Program

TaqMan Real-time PCR Assays

Applied Biosystems TaqMan real-time PCR assays consist of target-specific primers and one or more probes optimized for specific types of measurements. The mechanisms of action of different types of assays are described below.

Search Applied Biosystems TaqMan Assays

TaqMan Assays ▼

Search TaqMan® Assays and Arrays

Search

Try Advanced Search

Gene Expression

SNP Genotyping

Copy Number

MicroRNA

Mutation Detection

Gene symbol
Gene name
RefSeq accession
Assay ID
...and many more!

Learn more about real-time applications



Gene expression analysis



SNP genotyping analysis



MicroRNA analysis



How do TaqMan Assays work?

Additional products & services

Applied Biosystems Custom Primers & TaqMan Probes

Custom Assay Design Tool

Custom TaqMan Array Plates

Custom TaqMan Array Cards



TaqMan® assay search results

Search TaqMan® Assays and Arrays

TaqMan Assays
Gene expression

gapdh

Q Build a search

Filter your results

Assay attributes Species Gene GAPDH, +2 Assay Design Cross Reactivity ?

Home > TaqMan® Gene Expression Assays > Search Tool > All Results > Search Results

Assays | Array | **Pathways (3)**

Assay ID and Catalog Number are required to order

Assay ID: sequence

Catalog Number: configuration (e.g., dye, size)

Hs02786624_g1

Catalog Number: 4331182

Target Species: Human

GAPDH

4 RefSeq (NM)

Both primers
and probe map
within a single
exon

157

Dye:

FAM-MGB

Size:

S: 250 rxns

Availability:

Inventoried

Price (USD):

173.00

[Check your price](#)

Add to cart

[View Details](#)

[Related Reagents](#)

[Related Controls](#)

[View assay map](#)



99 / 100
Bioz Stars

Erythropoietin Receptor Expression Is a Potential Prognostic... (PLoS_One_2...) .. β-actin: Hs03023880_g1; GAPDH: Hs02786624_g1 (Applied Biosystems, Foster City, CA). (more...)

Expandable publications list for each assay ID

Hs04420697_g1

Catalog Number: 4351372

Target Species: Human

GAPDH

4 RefSeq (NM)

Both primers
and probe map
within a single
exon

130

Dye:

FAM-MGB

Size:

S: 360 rxns

Availability:

Made to Order

Price (USD):

268.00

[Check your price](#)

Add to cart

[View Details](#)

[Related Reagents](#)

[Related Controls](#)

[View assay map](#)

Pathways search mode:

Click on proteins on the pathway image to search for assay products related to that gene



TaqMan[®] assay details

☐ Select
 Assay ID
 Gene
 Transcripts
 Assay Design
 Amplicon Length
 Price (USD)

Best coverage

☐ **Hs00174122_m1** IL4 2 RefSeq (NM) Probe spans exons 70

Catalog Number: 4331182
Target Species: Human

Dye: FAM-MGB
Size: S: 250 rxns
Availability: Inventoried
Price (USD): 173.00
[Check your price](#)

[View Details](#) [Related Reagents](#) [Related Controls](#) [View assay map](#)

Add to cart

Assay Design

The assay probe spans an exon junction.

Gene Transcripts

Gene Symbol: IL4
Entrez Gene ID: 3585
Gene Name: interleukin 4
Gene Aliases: BCGF-1; BCGF1; BSF-1; BSF1; IL-4
Chromosome Location: Chr.5: 132673986 - 132682678 on Build GRCh38
UniGene: Hs.73917

Interrogated Sequence	Translated Protein	Exon Boundary	Assay Location	Amplicon Length
RefSeq				
NM_000589.3	NP_000580.1	3-4	427	70
NM_172348.2	NP_758858.1	2-3	379	70
GenBankmRNA				
AB102862.1	BAD89396.1	2-3	314	70
AF043336.1	AAC03537.1	2-3	245	70
AM937235.1		3-4	402	70
BC066277.1	AAH66277.1	3-4	426	70
BC066278.1		3-4	427	70
BC067514.1	AAH67514.1	3-4	427	70
BC067515.1	AAH67515.1	3-4	427	70
BC070123.1	AAH70123.1	3-4	424	70

Confirm Size & Dye CLOSE X

Please confirm your Size & Dye selections before adding to your cart.

Dye: FAM-MGB
Size: XS: 75 rxns
Availability: Inventoried
Catalog #: 4453320
Price (USD): 77.00

Add To Cart

Change Size & Dye for All

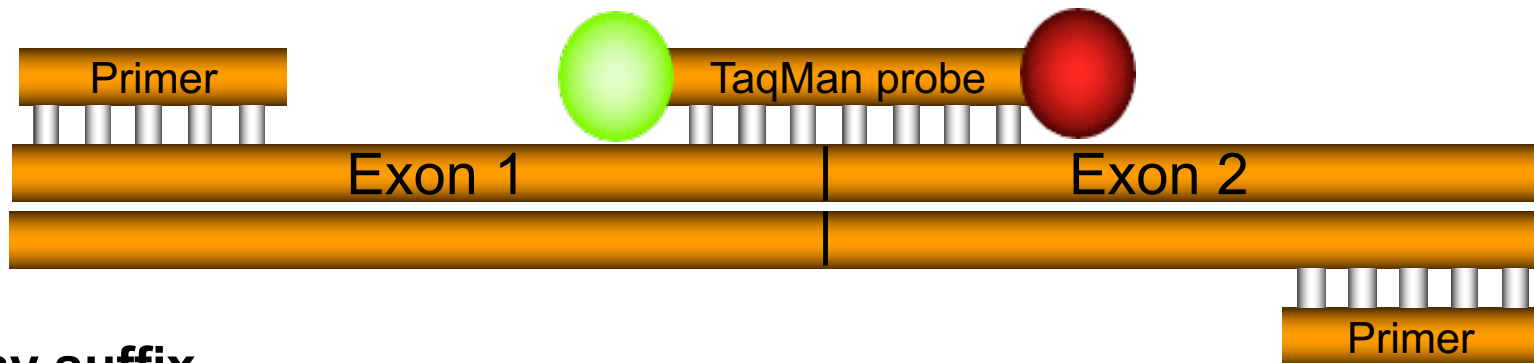
FAM-MGB | XS
FAM-MGB | S
FAM-MGB | M
FAM-MGB | L
VIC-MGB_PL | S
VIC-MGB_PL | M
VIC-MGB_PL | L
VIC-MGB | S
VIC-MGB | M
VIC-MGB | L

What's in an Assay ID?

Unique Assay ID

Species Number Suffix

Hs99999030_m1



Assay suffix

- m1 **M**ulti-exon spanning probe: mRNA only
- s1 **S**ingle-exon probe: mRNA and genomic DNA
- g1 May detect **G**enomic DNA
- U May detect **U**nspliced transcripts: probe left or right of exon junction
- H Region of high homology, homolog Ct 10-15 < target
- ft Fusion transcripts, does not detect genomic DNA

Assays specificity of target >30,000X* of nearest homolog

TaqMan[®] assay formats

Tubes



96 well plates



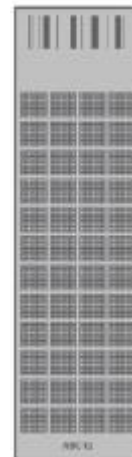
384 well plates



Low density array cards



OpenArray cards

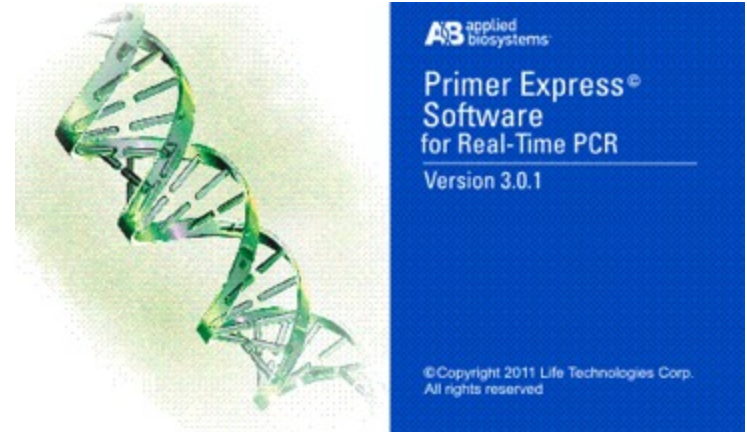


What if we don't have your assay?

Primer Express[®] Software v3.0.1

Desktop software

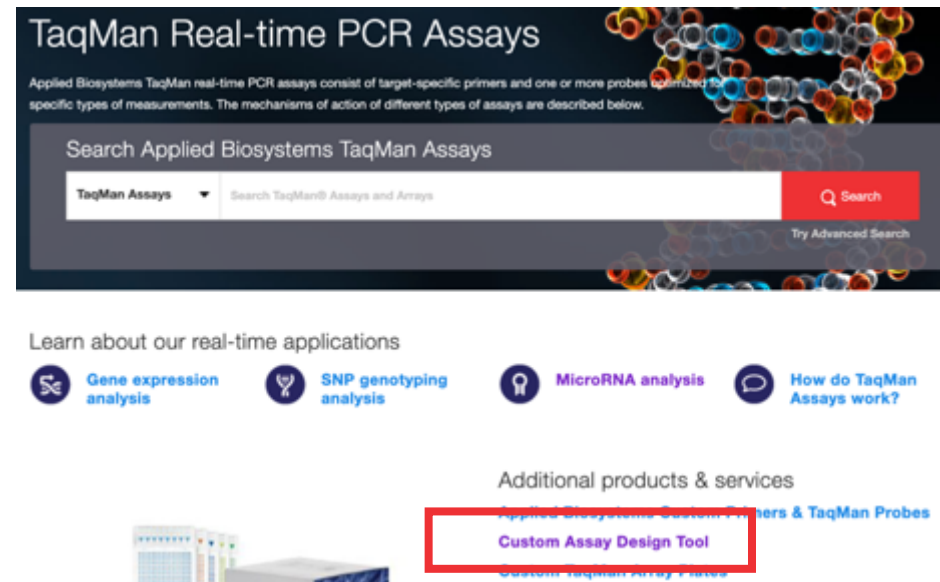
TaqMan[®] and SYBR



Custom Assay Design Tool

Web-based software

TaqMan[®]



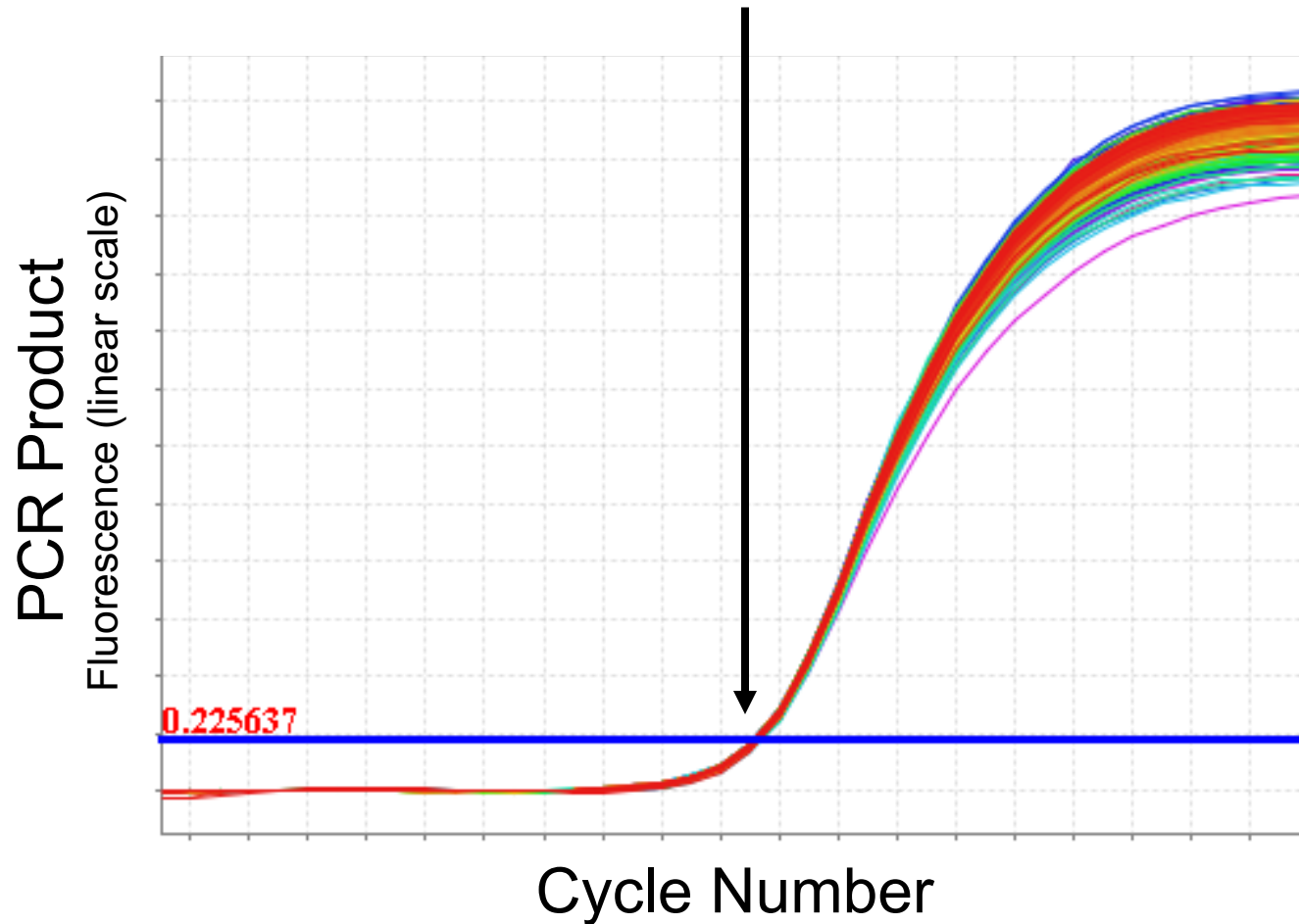
Applied Biosystems Master Mixes

Chemistry	Application	Suggested Master Mix	Why?
TaqMan®	Gene Expression	TaqMan® Fast Advanced Master Mix <i>for 2-step</i> TaqMan® Fast Virus Master Mix <i>for 1-step virus detection and gene expression</i>	Accurate and high dynamic range High accuracy with challenging samples
TaqPath (Clinical-Grade) <i>For laboratory use</i>	Genotyping	TaqPath ProAmp Master Mix <i>for human and animal samples</i>	Accurate calling in crude and pure samples
SYBR®	Gene Expression	PowerUp SYBR™ Green Master Mix <i>for 2-step</i> Power SYBR™ Green Master Mix <i>for 2-step with SuperScript IV VILO</i>	Increased specificity and less primer dimer formation compared to competitors

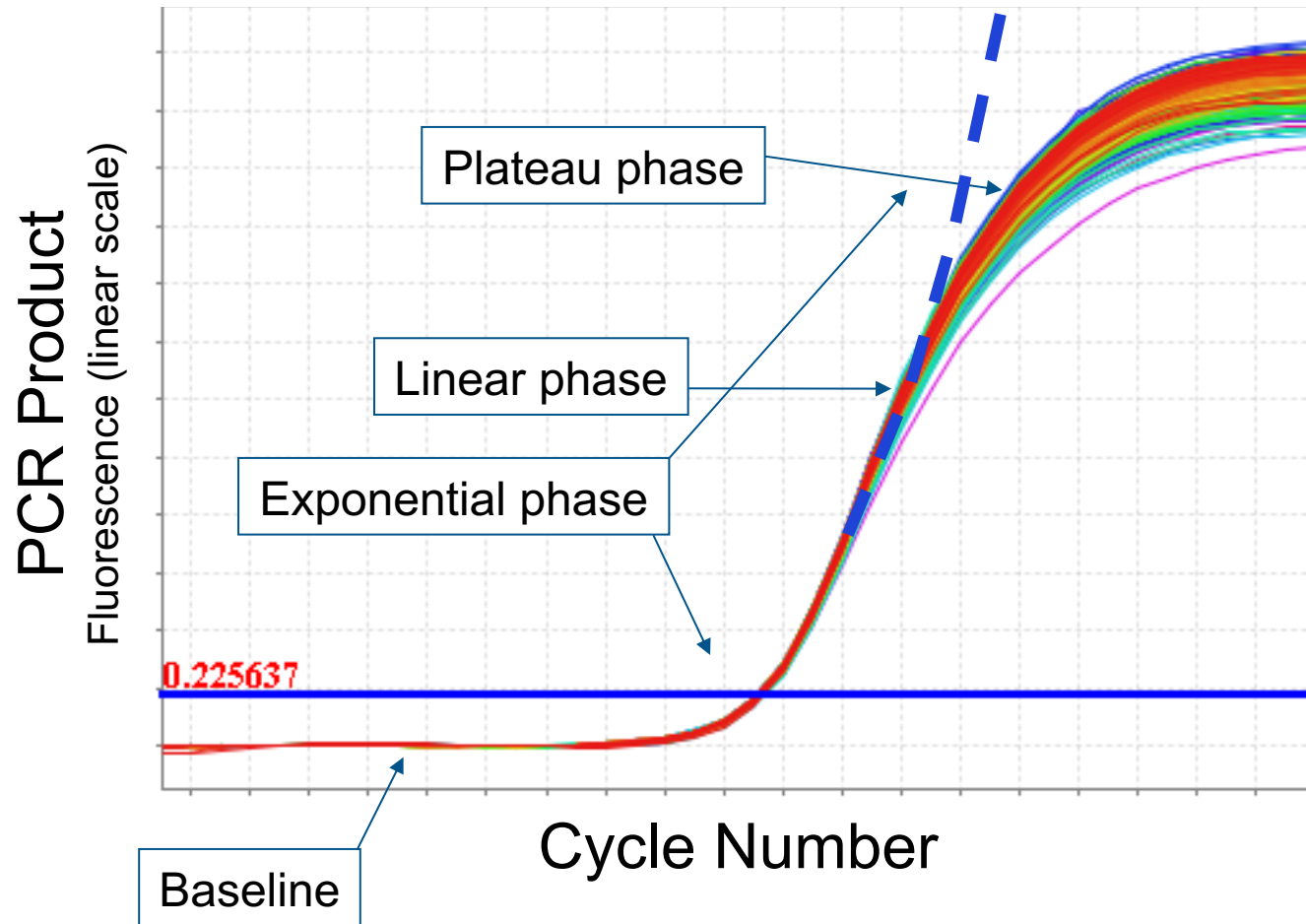
What is a Ct and what can it tell us?

What is a Ct?

Threshold Cycle (Ct) is the **fractional cycle number** at which each amplification curve crosses the threshold

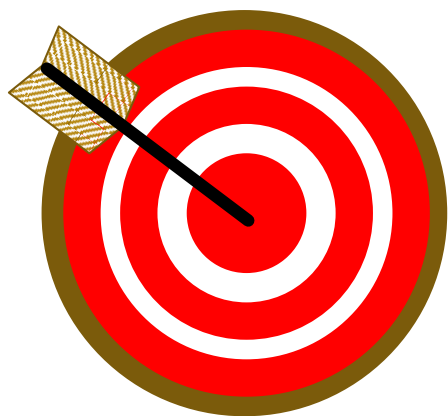


Remember the phases of PCR amplification



Criteria for generating good quantitative data

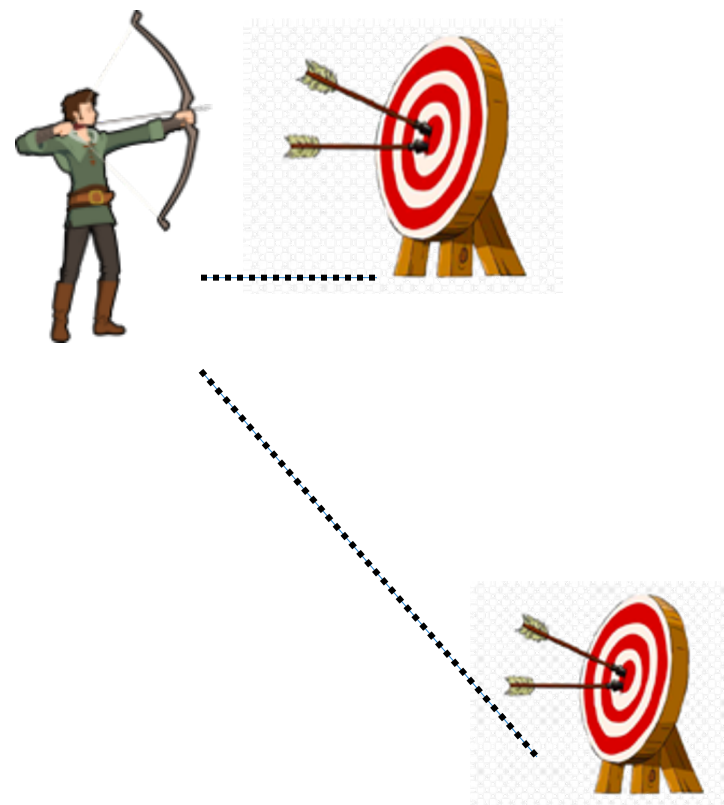
1. Accuracy



2. Precision

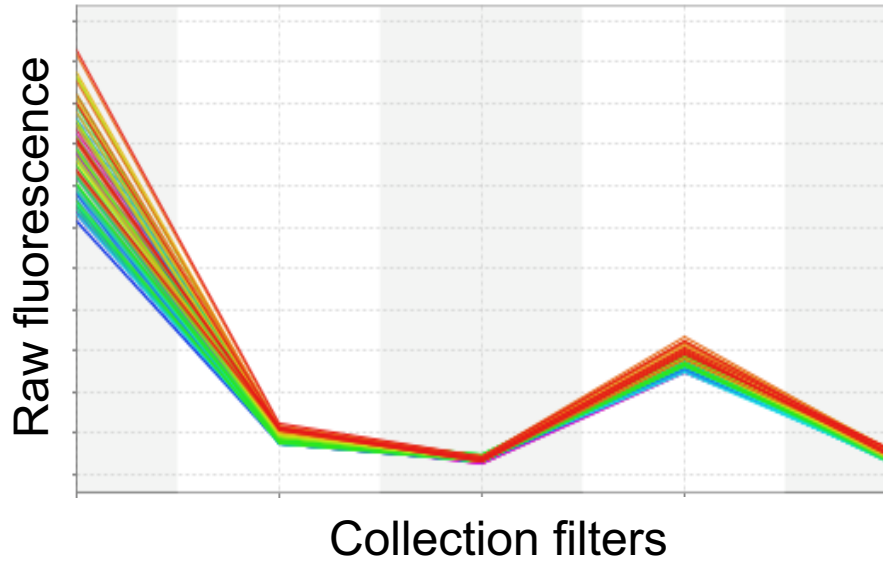


3. Dynamic range

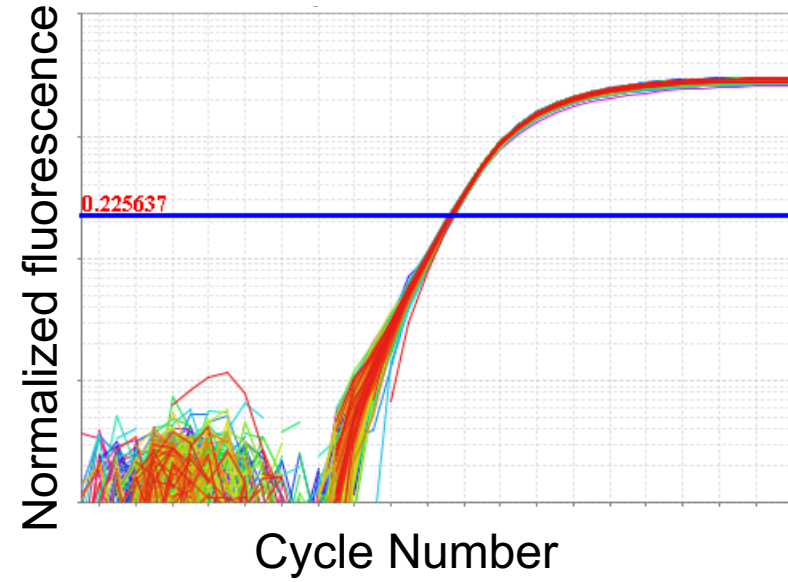


From raw data to Ct

Raw data plot



Amplification plot

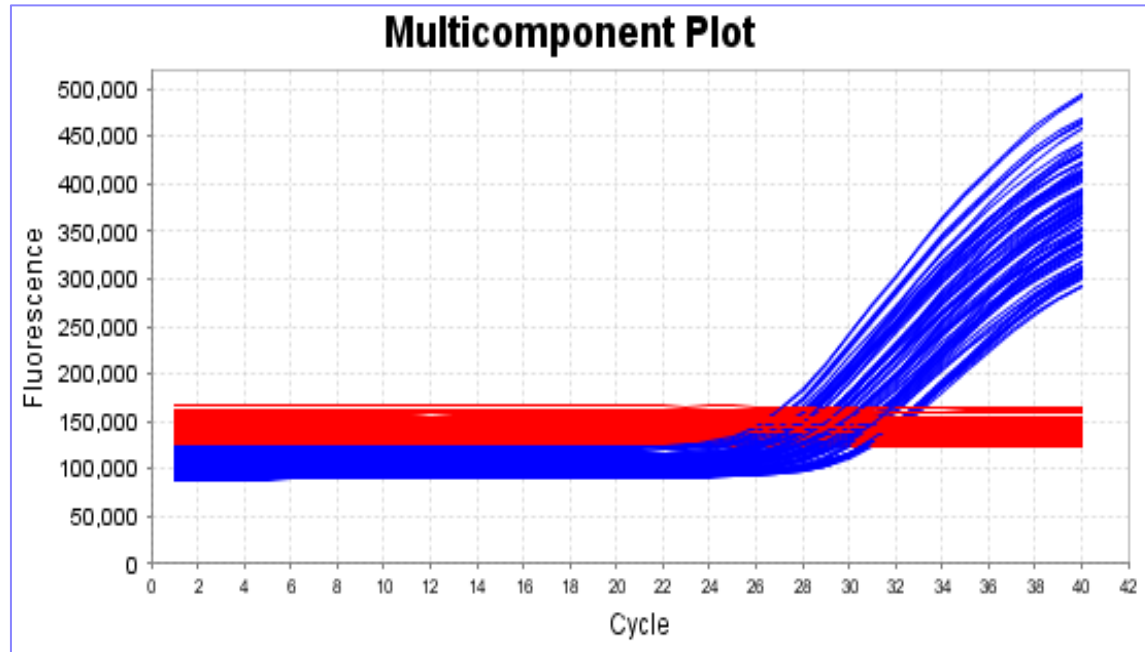
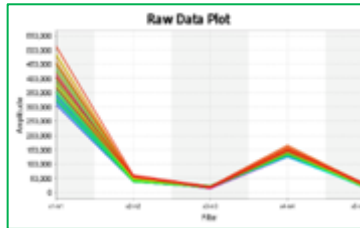


System normalizations – passive reference

Raw data
(Fluorescence)



Multicomponent
(Fluorescence)



Ct

The multicomponenting algorithm incorporates
pure dye and **background calibrations**

System normalizations – passive reference

Raw data
(Fluorescence)

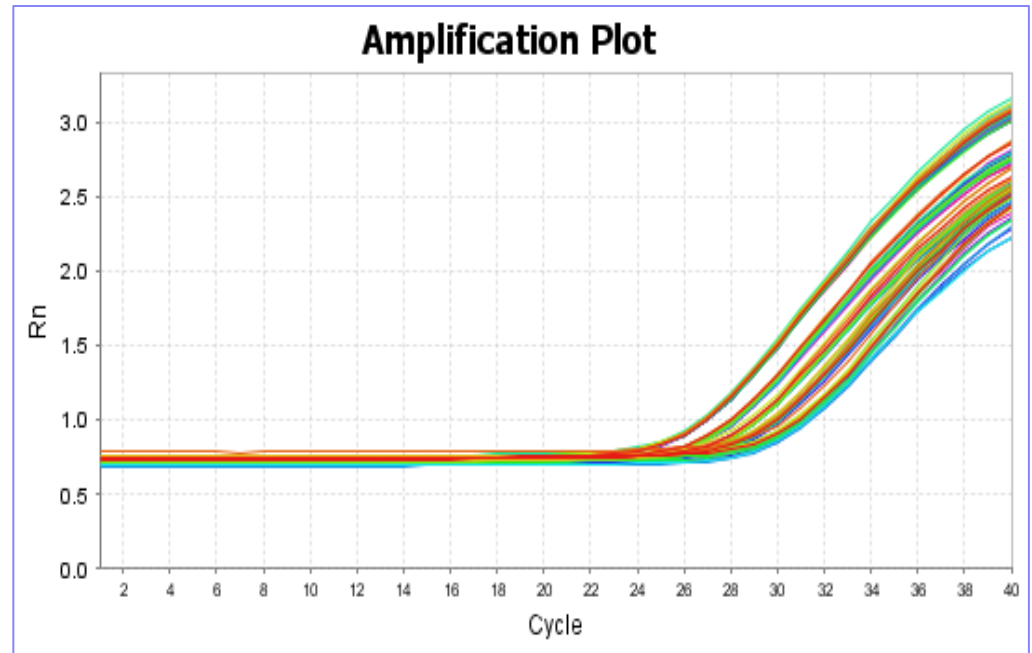
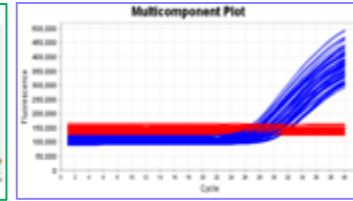
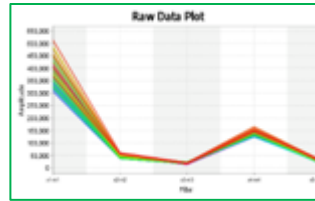


Multicomponent
(Fluorescence)



Passive Reference Dye
normalization (R_n)

Ct

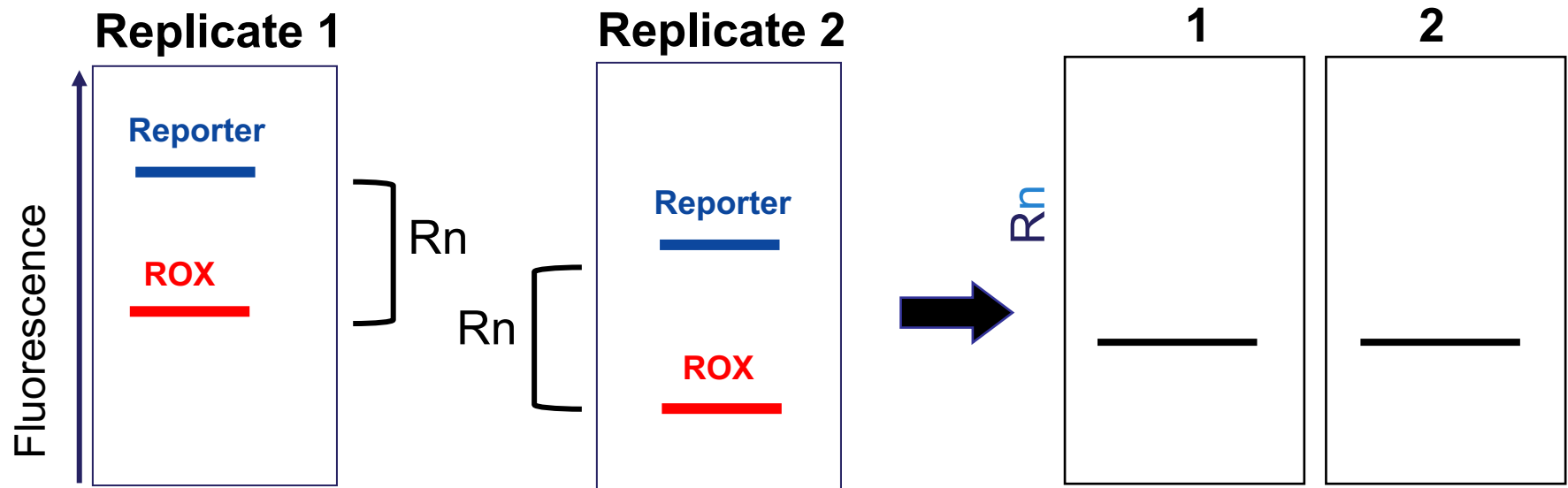
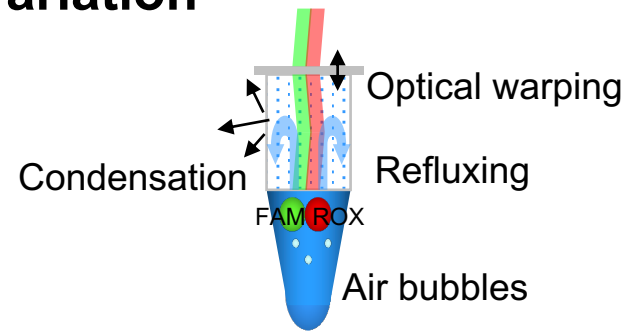


Passive reference dye normalization accounts for **non-PCR** fluctuations in fluorescence

ROX passive reference normalizes for fluorescent variation

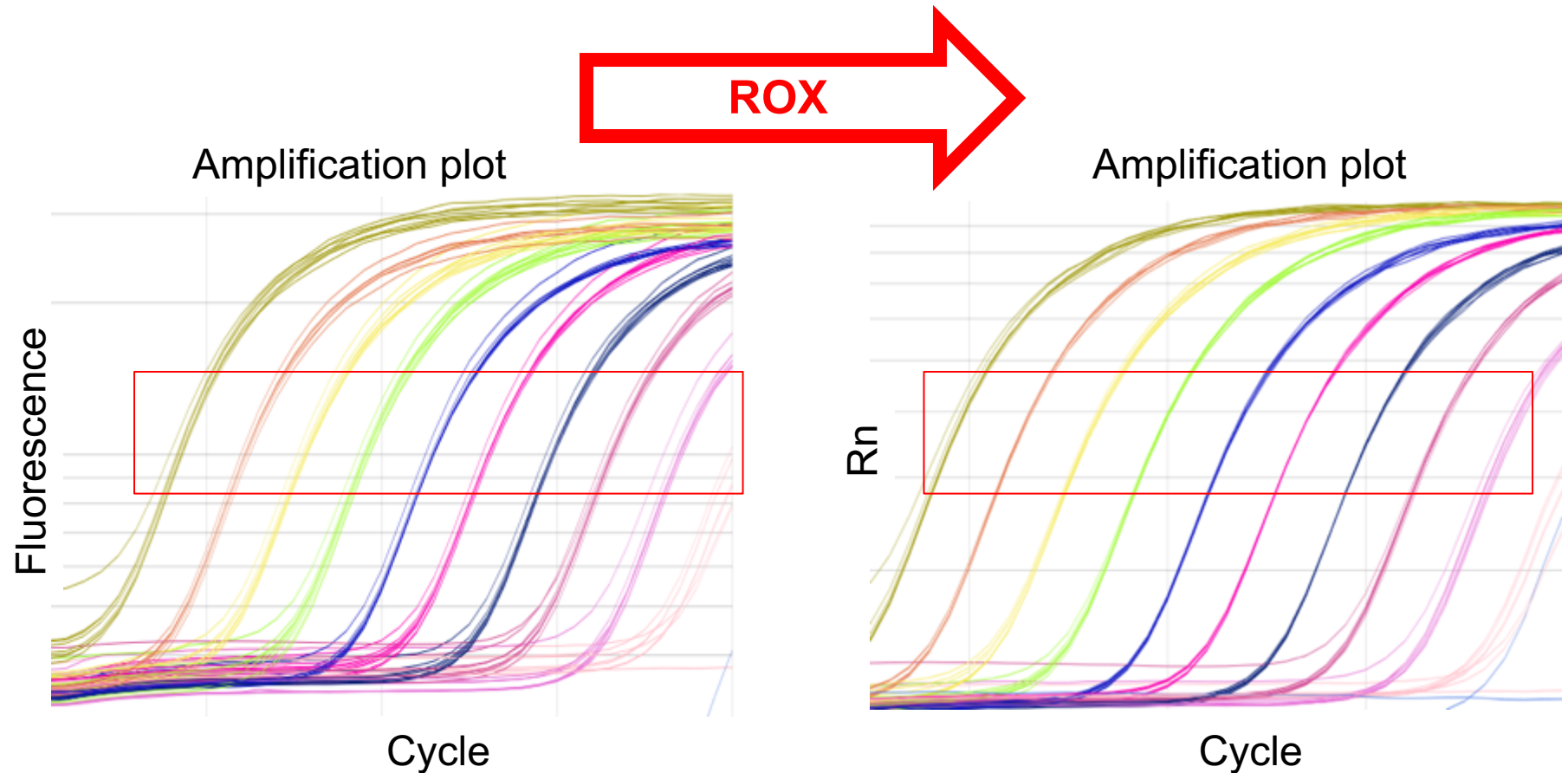
Common sources of fluorescent variation

- Water vapor reflux
- Condensation
- Air bubbles
- Evaporation



Normalized reporter (R_n) = Reporter dye / passive reference

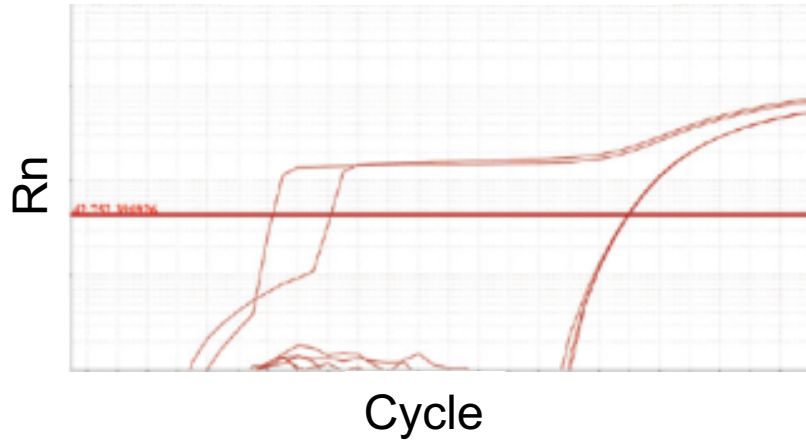
ROX passive reference dye improves precision



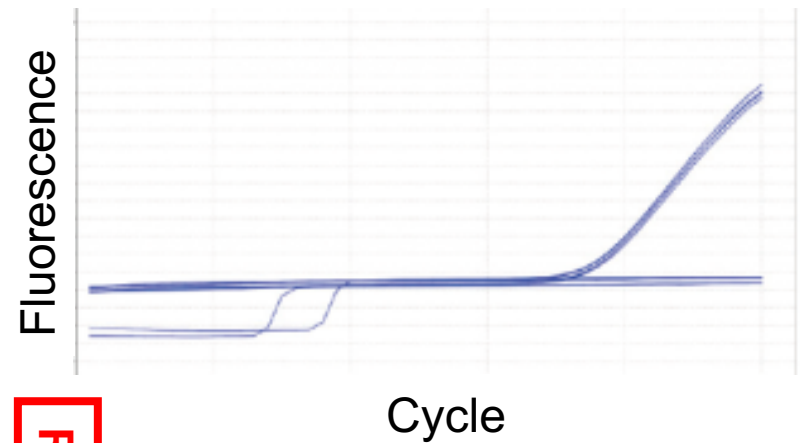
All Applied Biosystems real-time PCR master mixes from contain ROX™ passive reference dye.

ROX passive reference dye can help troubleshoot

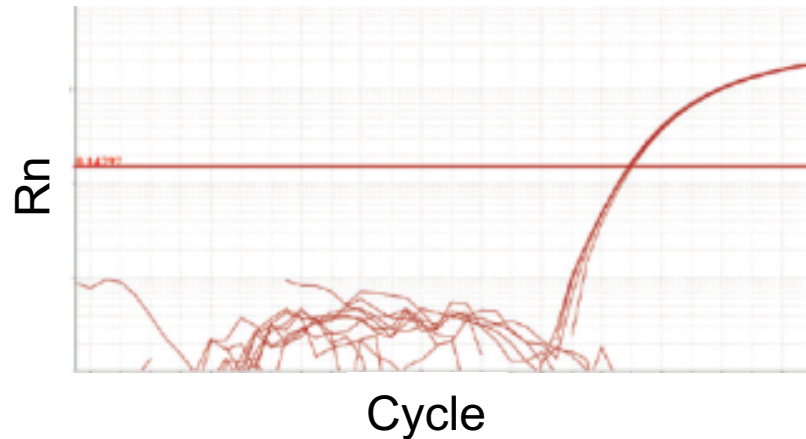
Amplification plot



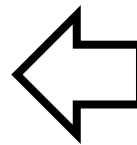
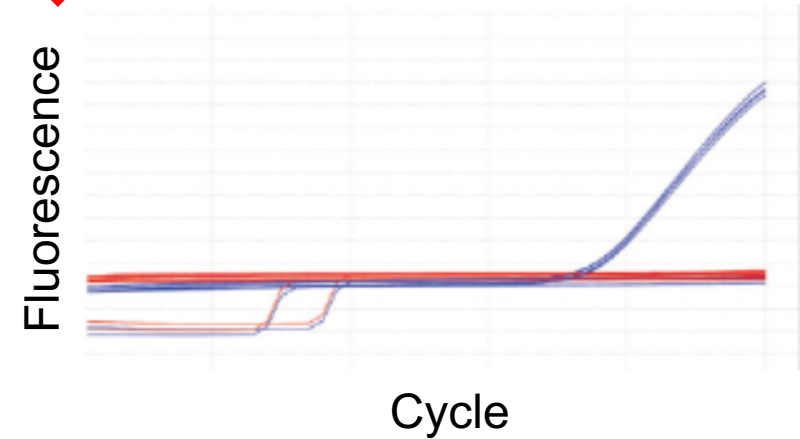
Multicomponent plot



Amplification plot

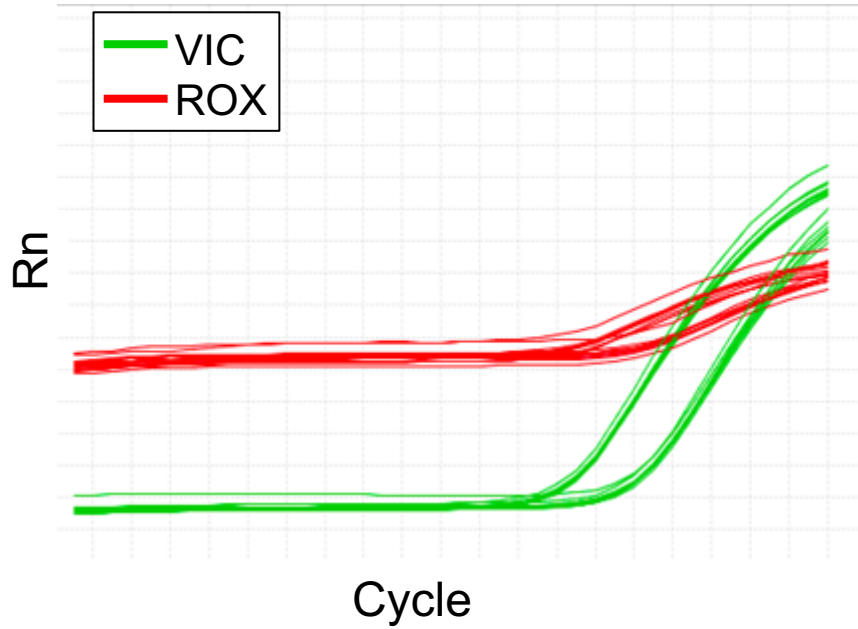


Multicomponent plot

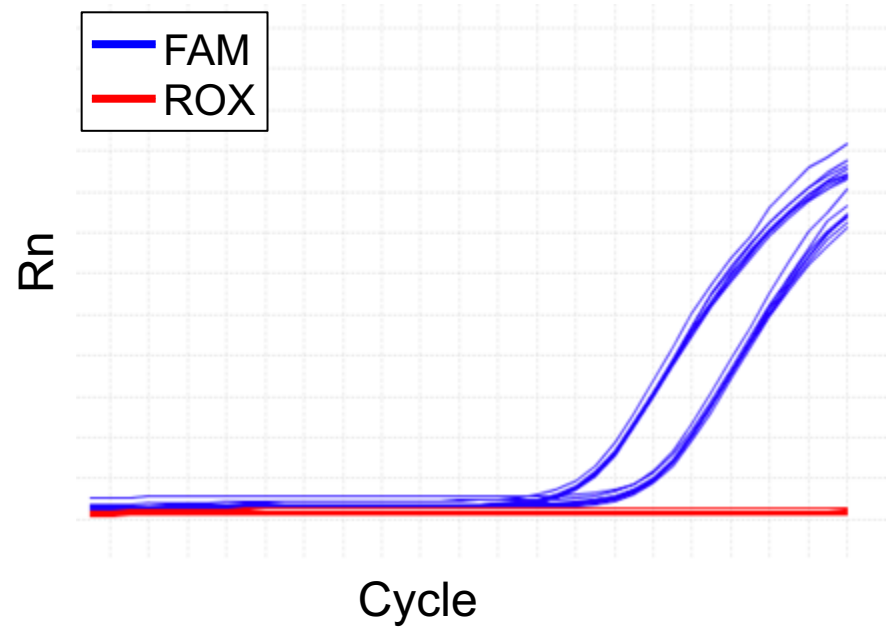


ROX passive reference dye can help troubleshoot

Amplification plot



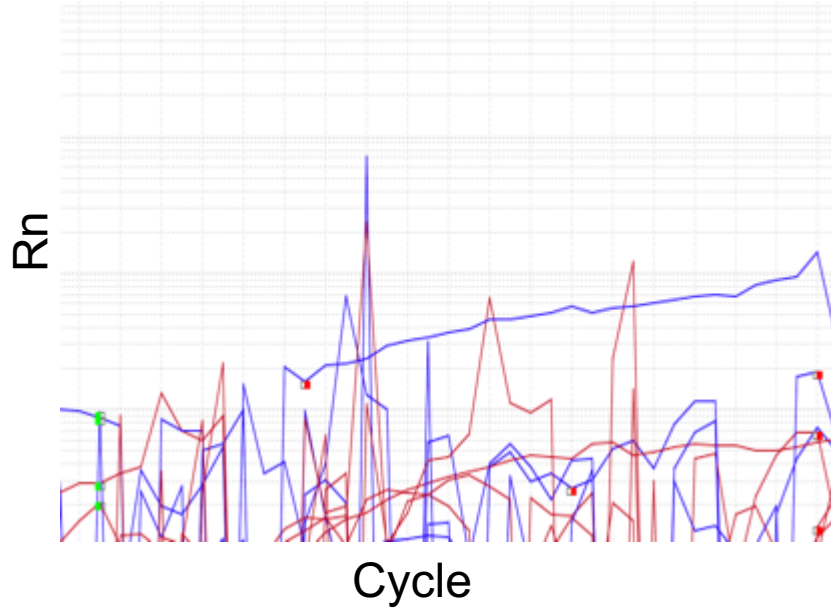
Amplification plot



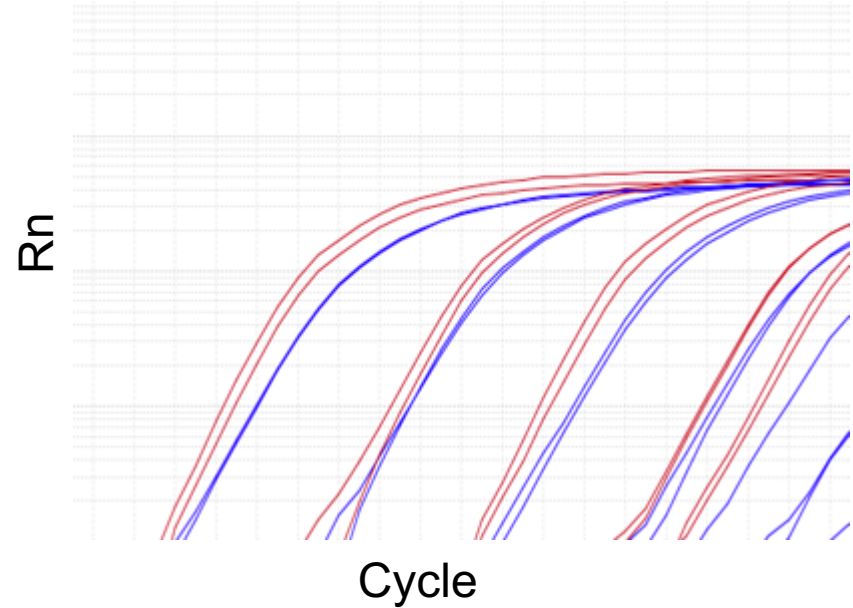
Relabel as FAM dye

No-ROX master mixes can be used on AB instruments

Amplification plot



Amplification plot



Turn off ROX normalization

System normalizations – baseline normalization

**Raw data
(Fluorescence)**



**Multicomponent
(Fluorescence)**

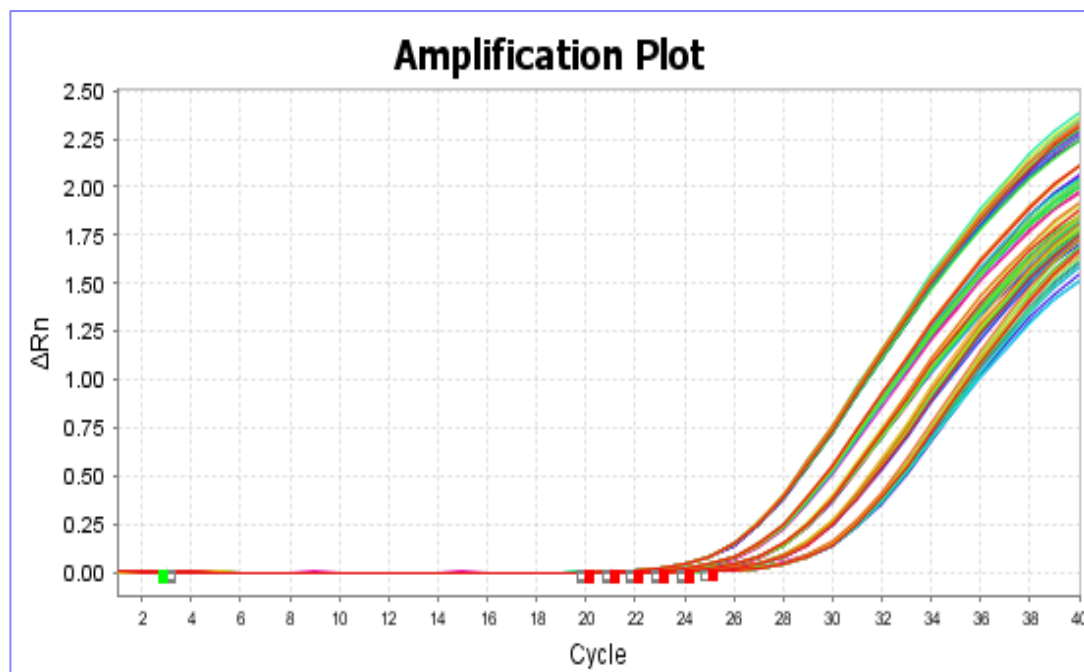
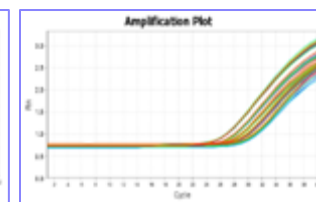
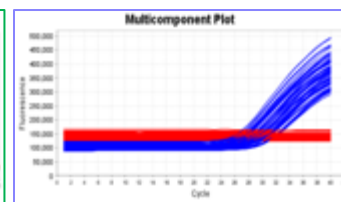
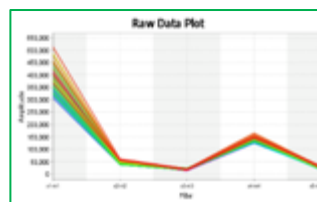


**Passive Reference Dye
normalization (R_n)**



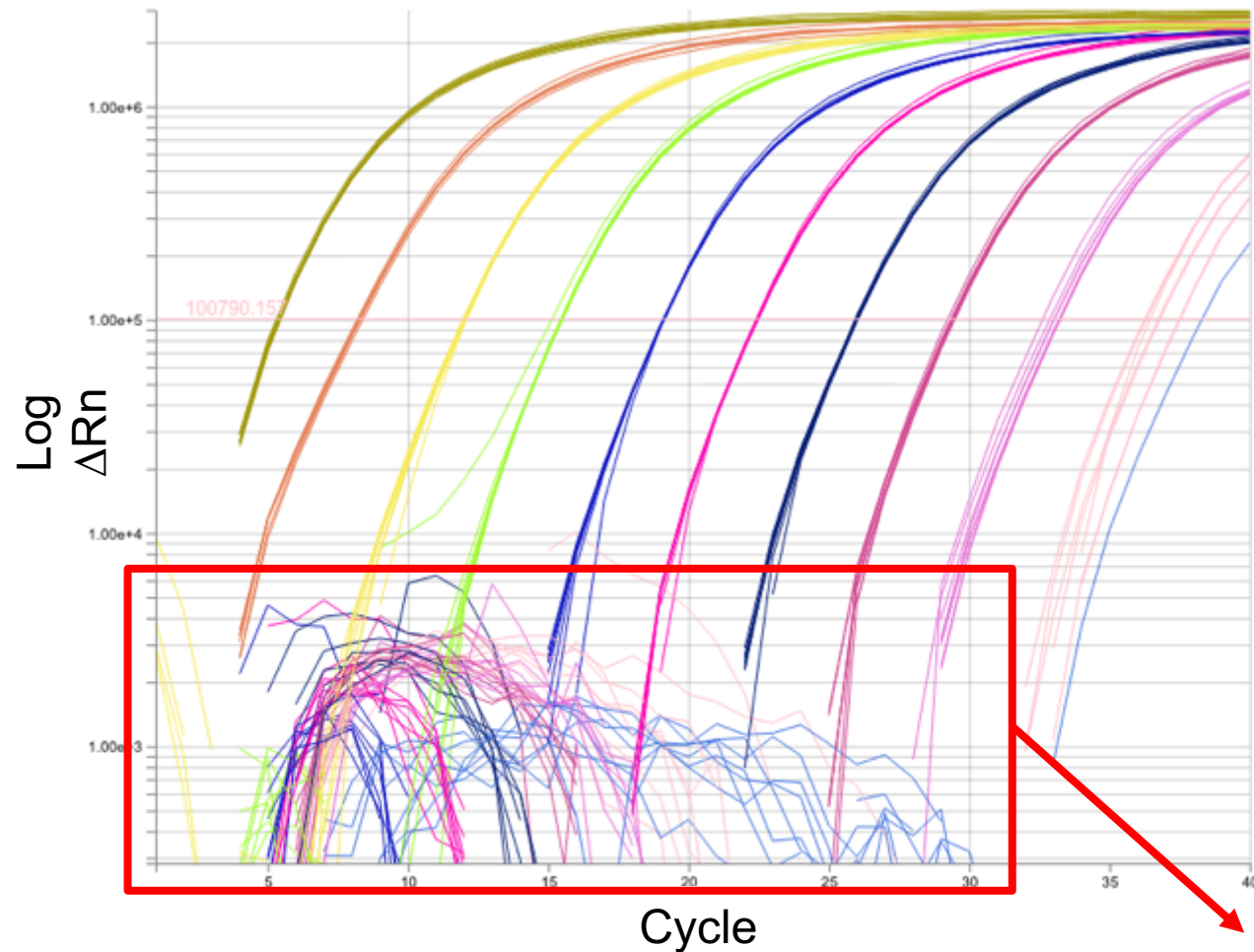
**Baseline normalization
(ΔR_n)**
automatic or manual

Ct



Baseline normalization accounts for
the different background of each well

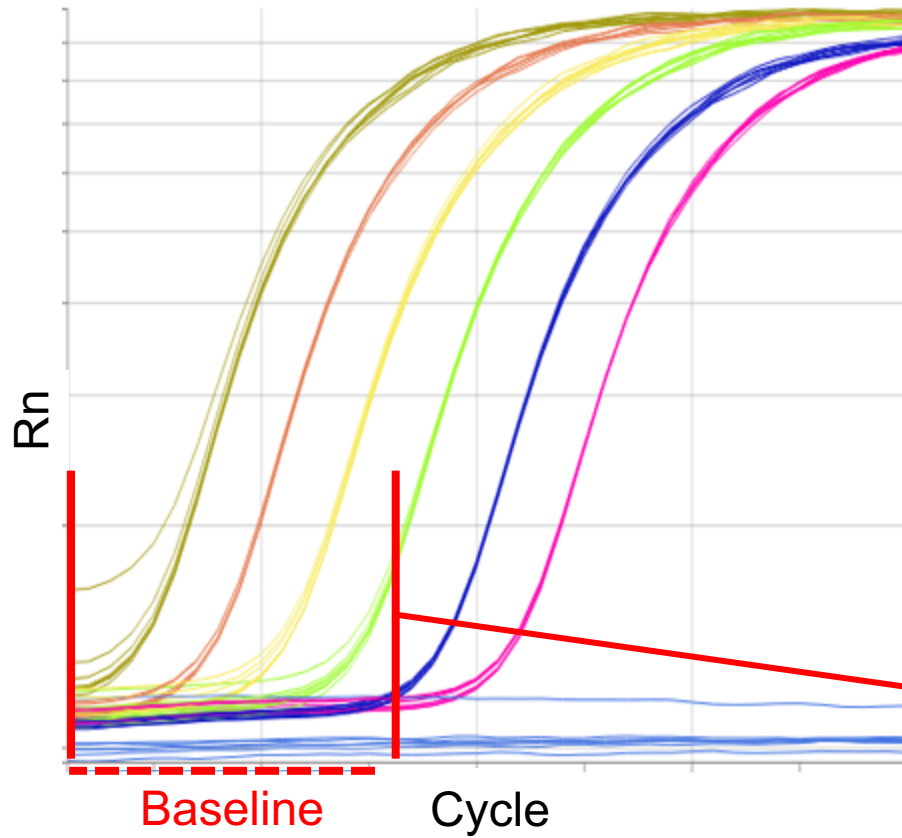
Amplification plot



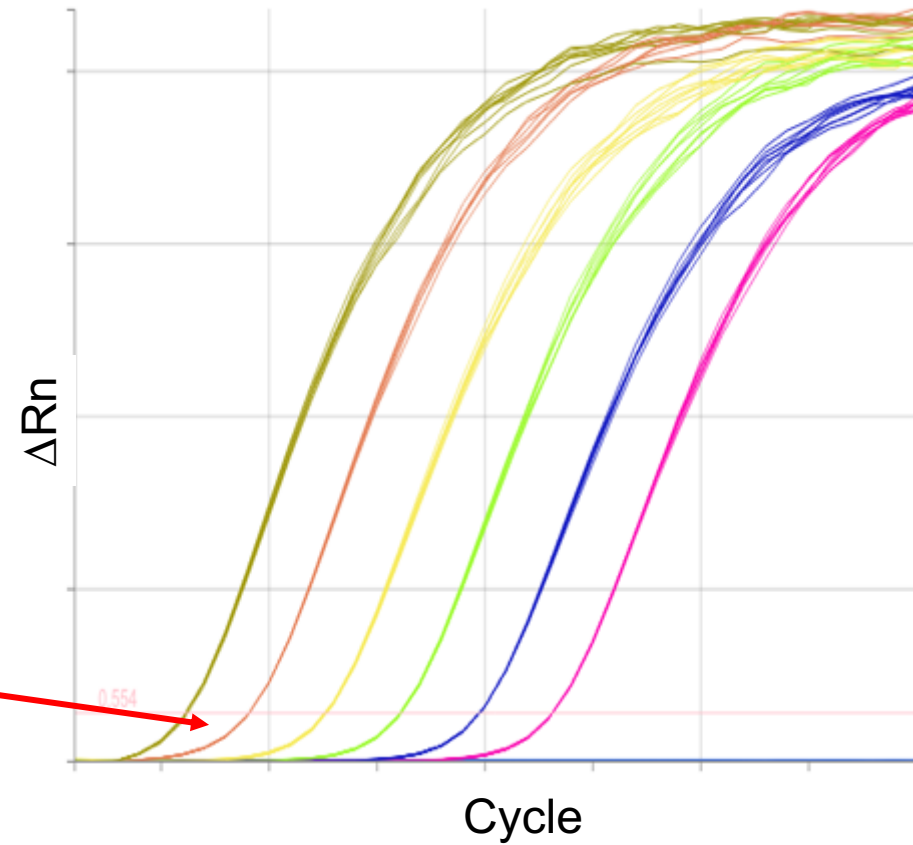
**During early cycles of PCR,
very low fluorescent signal::noise**

Baseline normalization

Amplification plot



Amplification plot

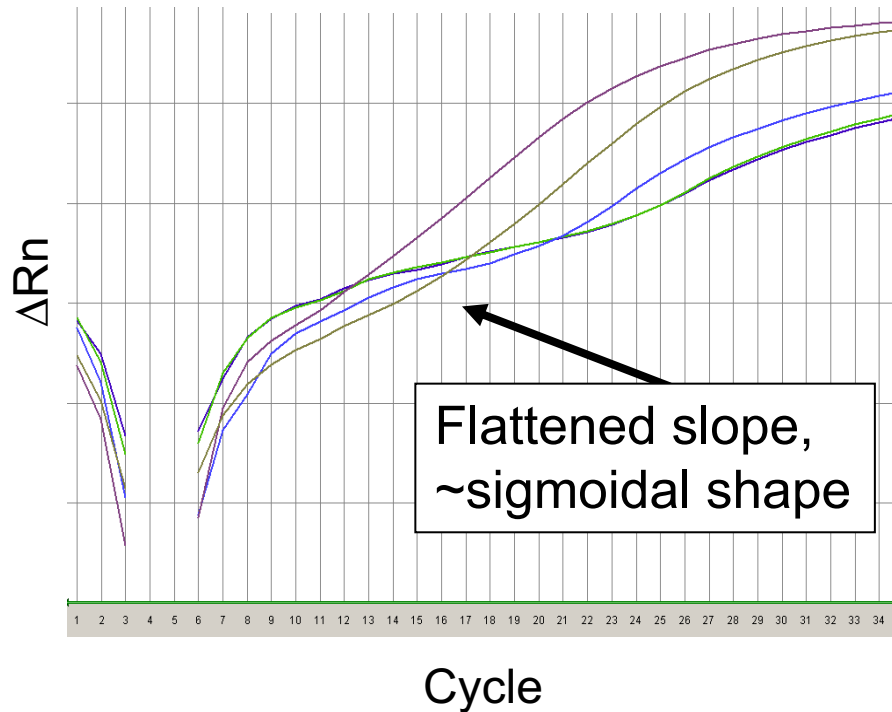


The **baseline** defined the region of background for *each reaction*.

What happens if your baseline is too low or too high?

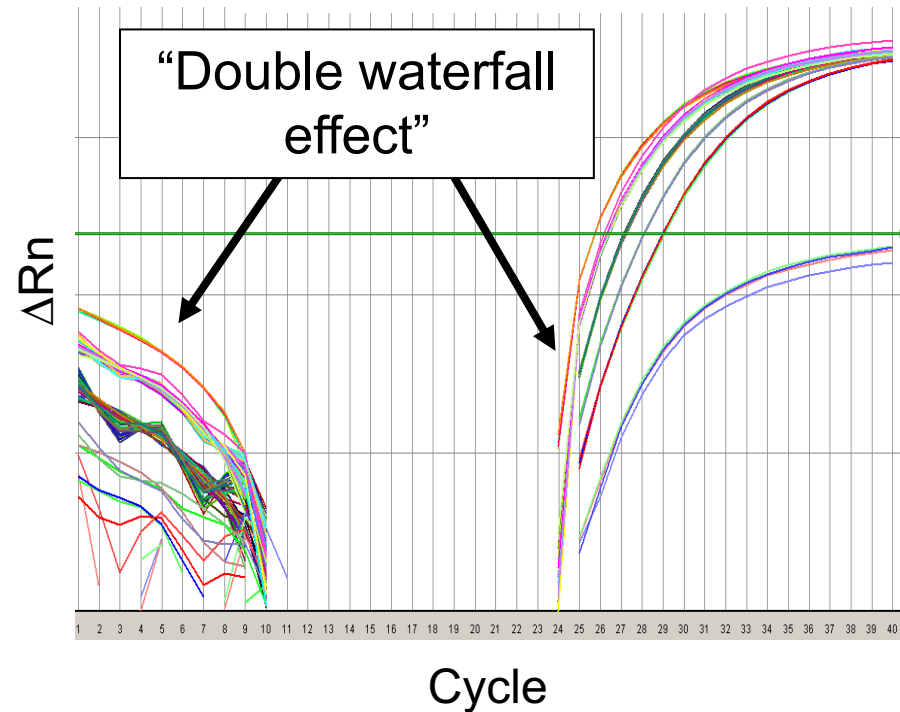
Too low – cycles 3-6

Amplification plot

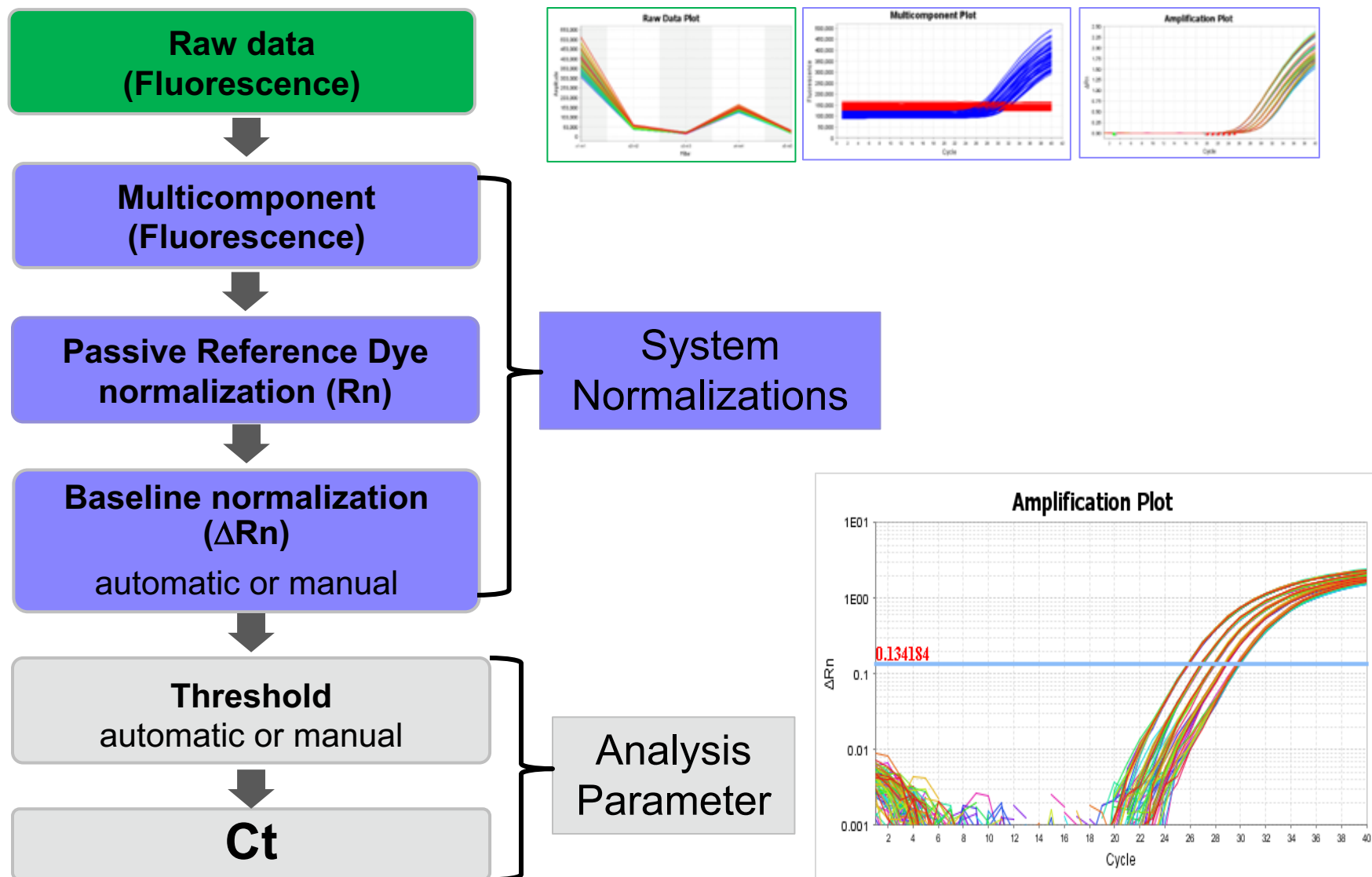


Too high – cycles 3-26

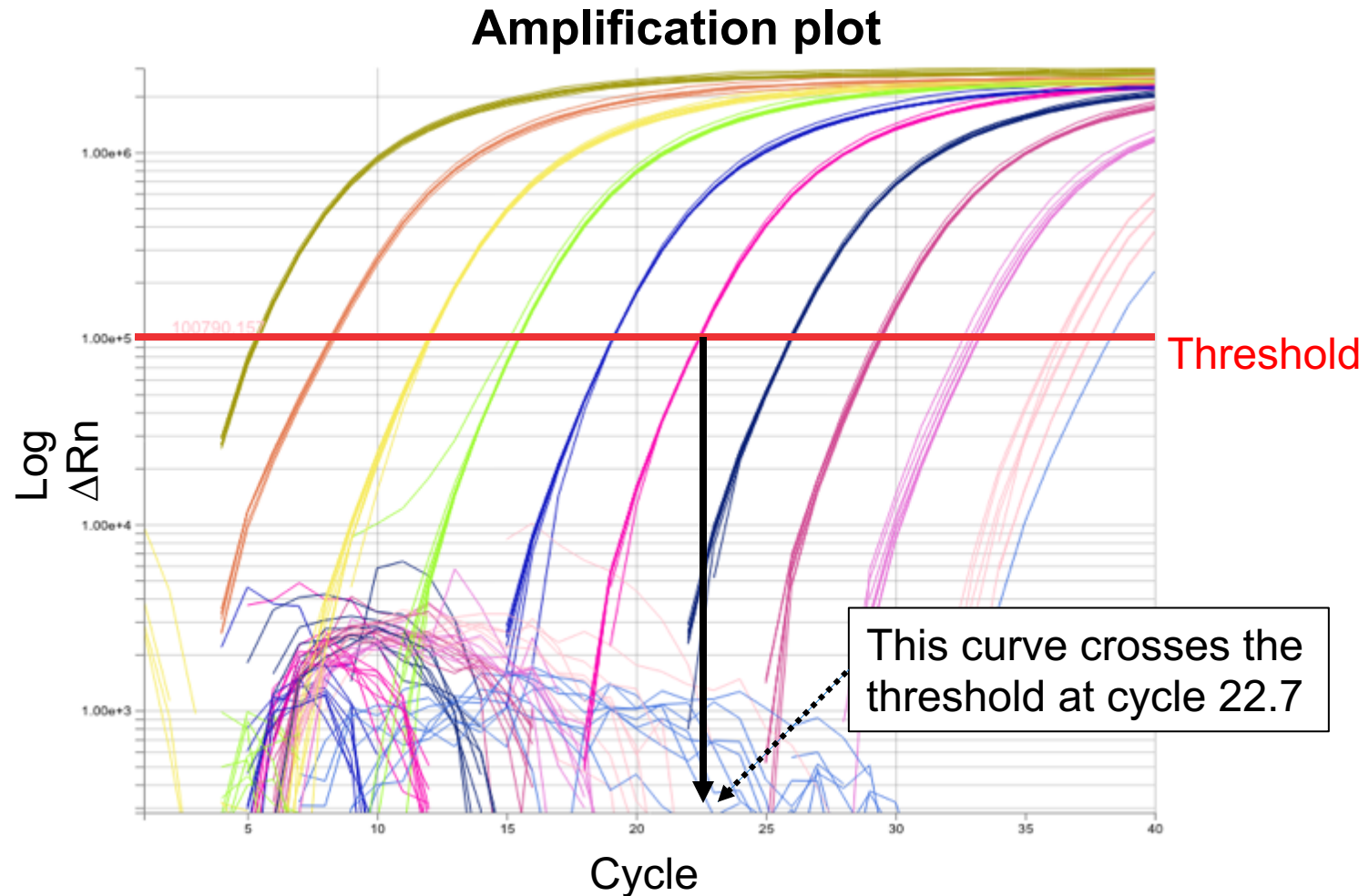
Amplification plot



From Raw Data to Ct – Analysis Parameter



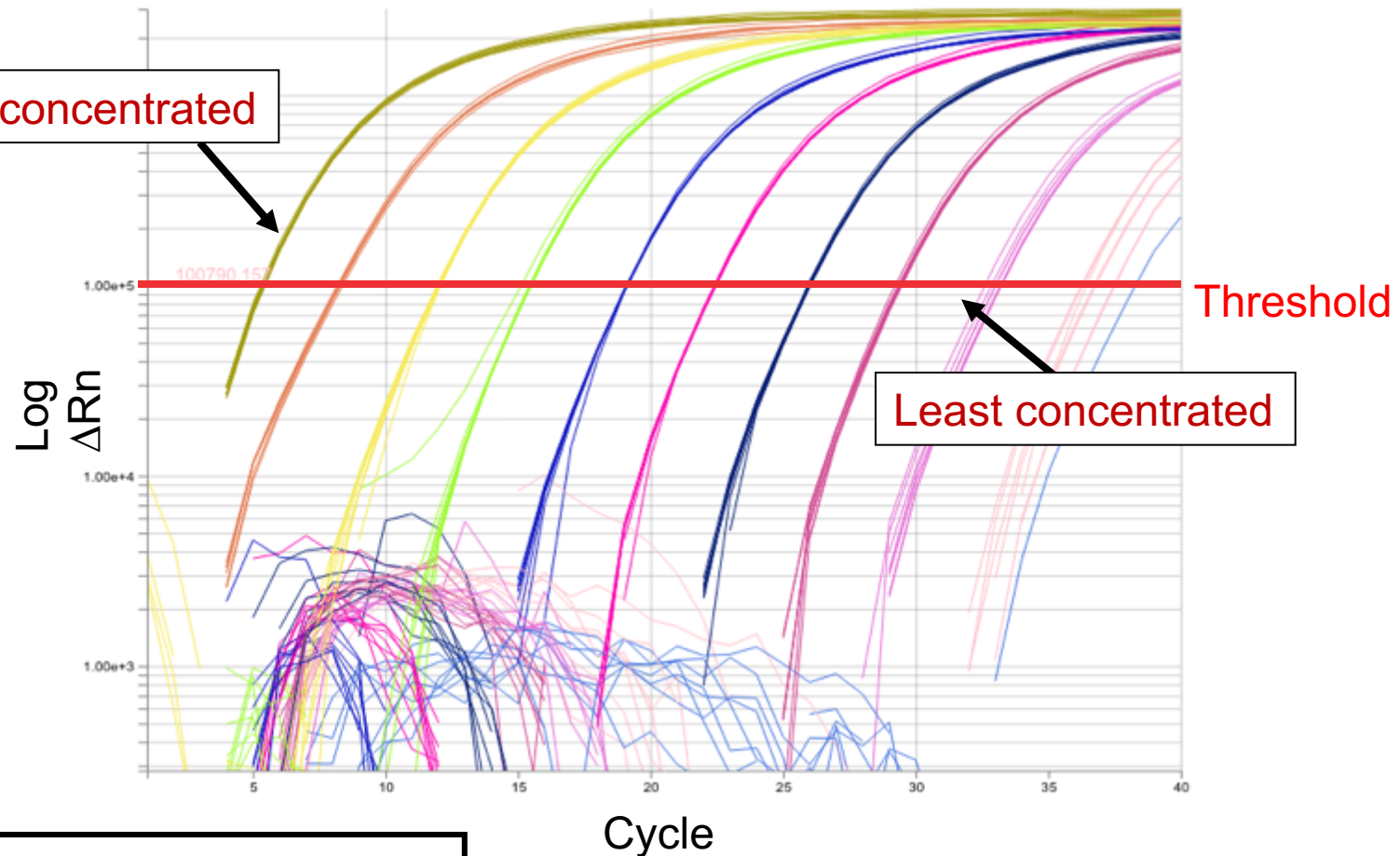
Defining the threshold



The **threshold** is an adjustable horizontal line defined for *each target* that tells the software where to collect data

Defining the threshold

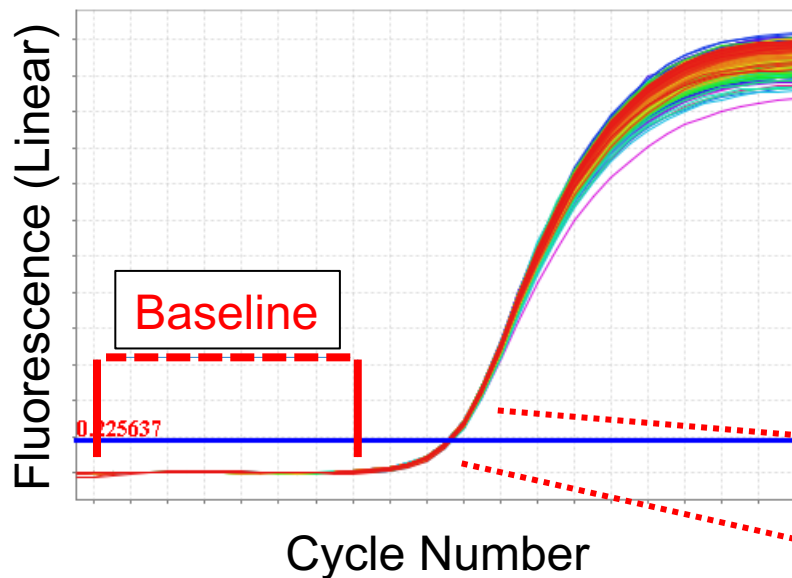
Amplification plot



The earlier we detect a sample for a given target, the higher the concentration.

Linear and log views for baseline and threshold

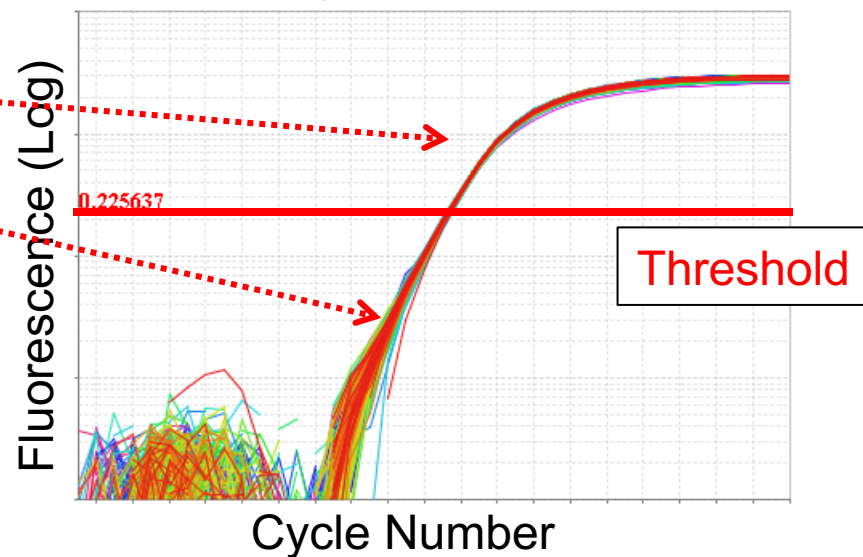
Linear view



Optimal for viewing baseline

Optimal for viewing threshold

Logarithmic view



Real-Time qPCR Instruments

QuantStudio 6 and 7 Pro Real-time PCR systems

Innovative technology to enable smart qPCR workflow for greater productivity

- **Smart qPCR workflow**—ultimate traceability to help reduce manual errors and hands-on time
- **Hands-free features**—greater ease and convenience
- **Smart Help**—maximum uptime and convenience
- **Format flexibility**—interchangeable blocks (96-well; 384-well and TaqMan Array card)
- **Automation compatibility**—Ability to run plates 24/7 with no manual intervention



Cutting edge technology— access, analyze and share



**ThermoFisher
Connect**



Monitor



Analyze



Share

- Works with both MAC and PC
- Access & analyze data any time anywhere
- IoT enabled features
- Real time run monitoring on smart phones
- Thermo Scientific PC not required

Wifi or
Ethernet



Link instrument to the
Connect cloud-enabled
platform via wifi or ethernet

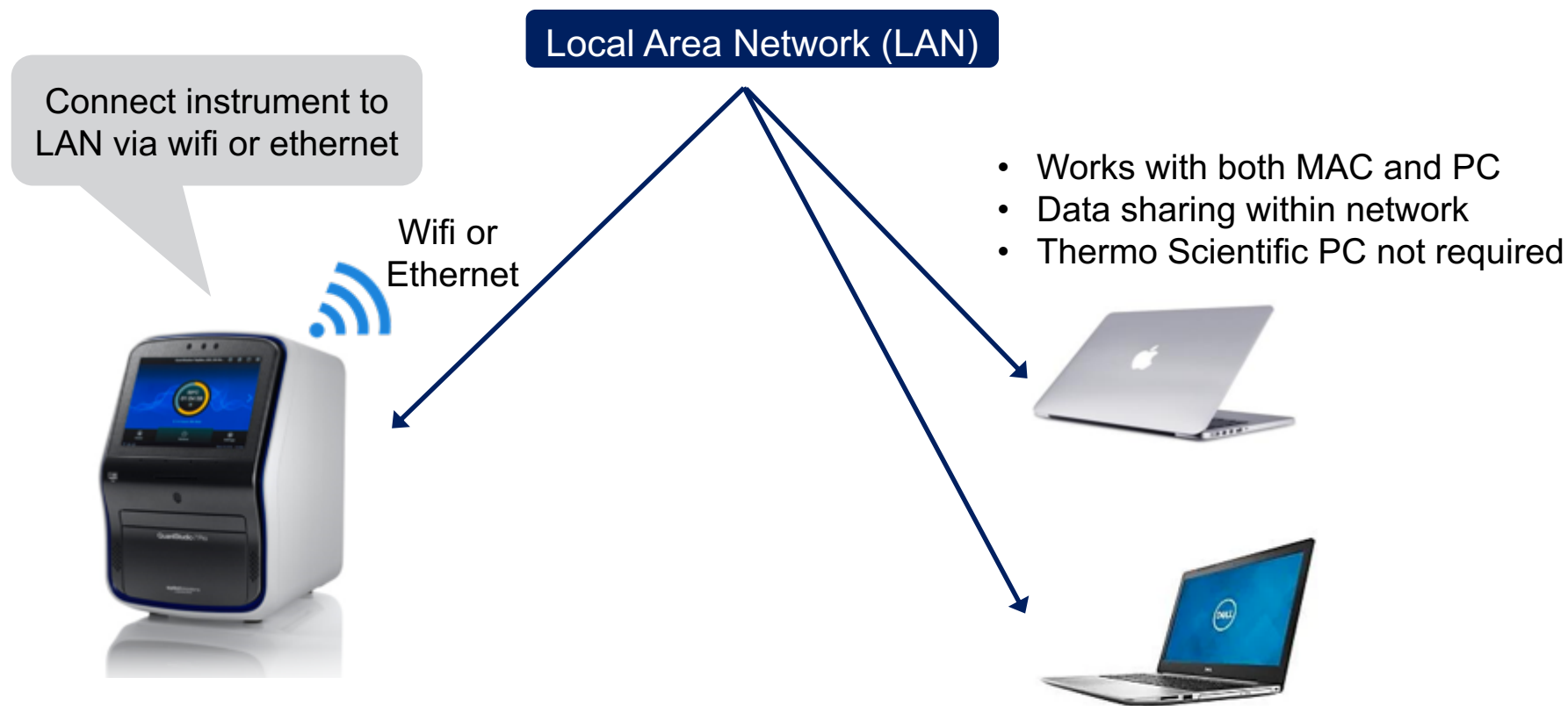


**Instrument
Connect app**

Need Online Connectivity



Flexibility for networking



Flexible system setup

Instrument connected to
dedicated PC offline

- Thermo Scientific PC required
- No networking requirement



← Ethernet →



Feature comparison for different configuration options

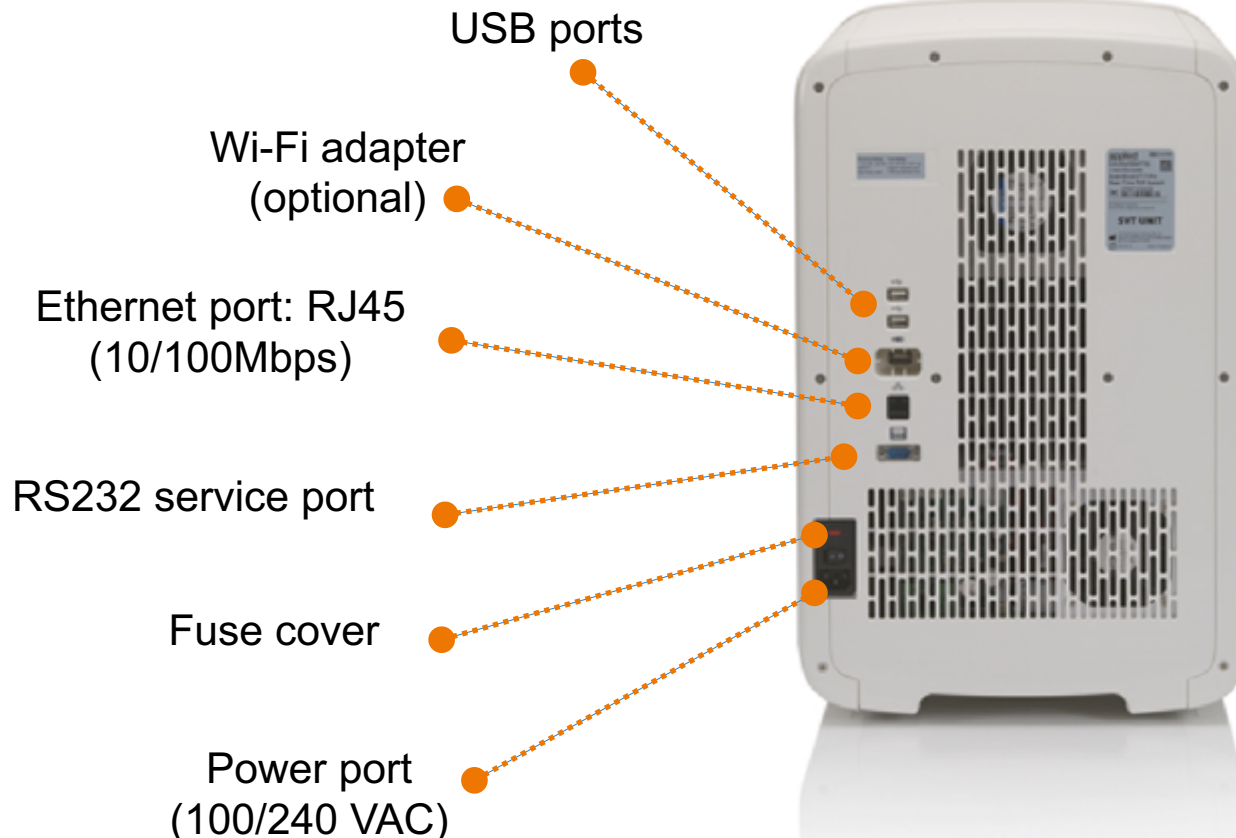
New Features when connected the QuantStudio Pro device to...	Thermo Fisher Connect	Local Network	Standalone Computer*
Facial Authentication	✓	✓	✓
TaqMan Array Plates with RFID	✓	✓	✓
Voice Commands	✓	✗	✗
Smart Help	✓	✗	✗
Instrument Scheduler	✓	✗	✗
MAC Support	✓	✓	✗
Remote Run Monitoring	Smart phone InstrumentConnect	✗	✗
Online Data Sharing	Thermo Fisher Connect	✗	✗
Online Data Analysis	Thermo Fisher Connect	✗	✗

* Thermo Fisher PC required

QuantStudio Pro system



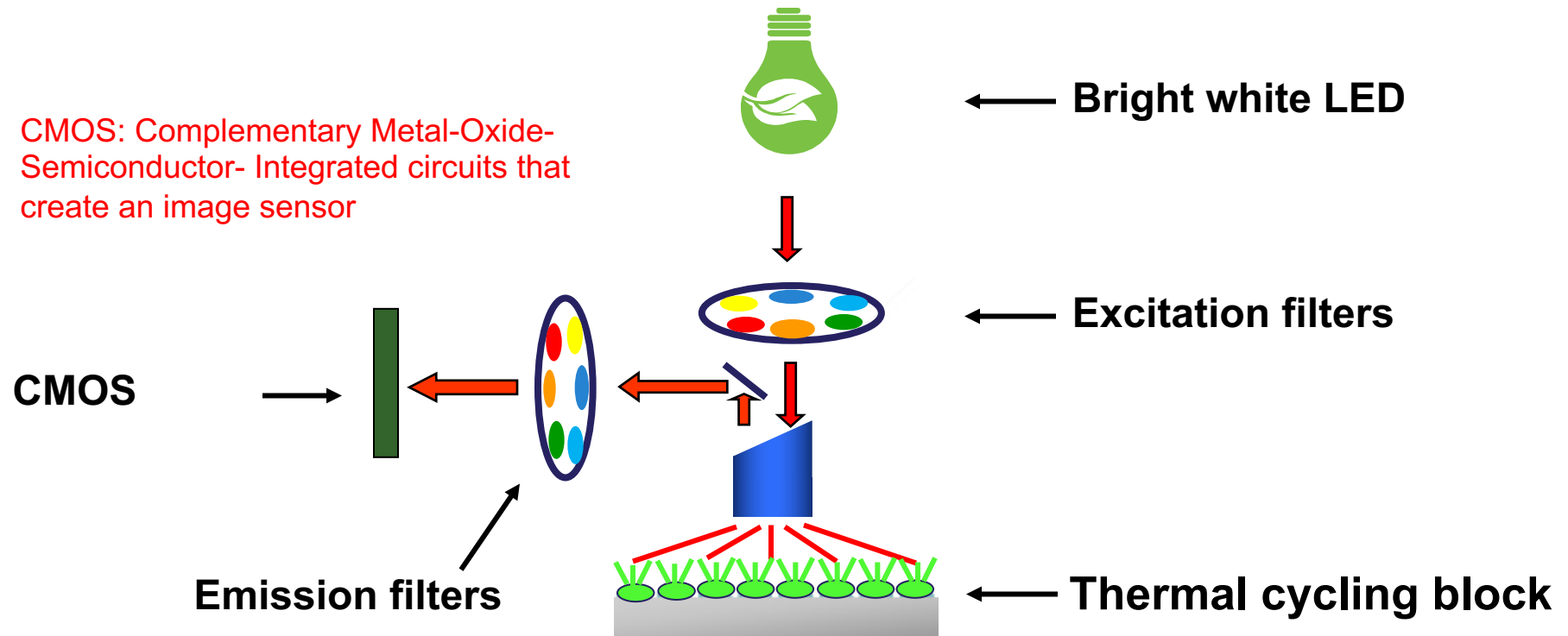
QuantStudio Pro system



How do QuantStudio Real-Time PCR instruments work?

- Thermal block
- Bright white LED to excite fluorescence
- Filters
- High-resolution CMOS to detect signal.

CMOS: Complementary Metal-Oxide-Semiconductor- Integrated circuits that create an image sensor



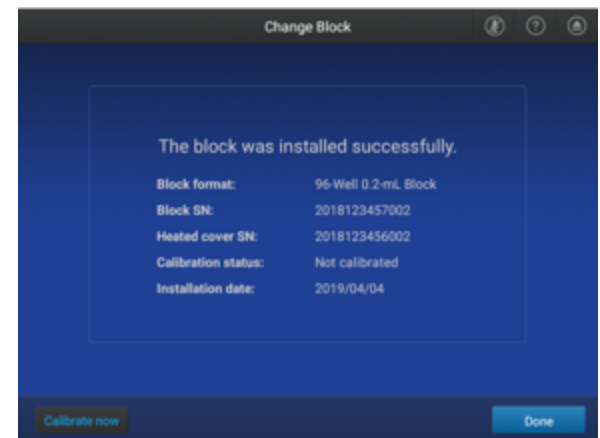
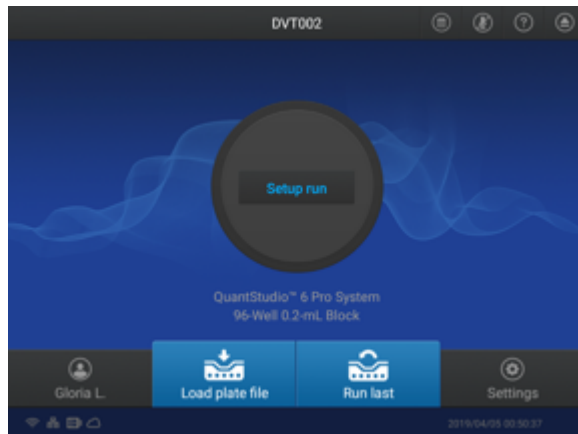
Interchangeable block flexibility

- Motorized block-change
- Small, light-weight block/heated cover with grip and storage box
- Veriflex™ zones for faster optimization of assays
 - 3 zones for the QuantStudio 6 Pro
 - 6 zones for the QuantStudio 7 Pro



Block change explained

- System shows current block status on home page
- System tracks block calibration status independently by serial number
- Any user can change the block of the system
- Upon request to change block, system verifies and informs user of current block installed
- Walks through block change step by step
- No need to be power down for block change



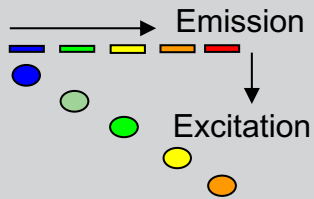
Video: How to change the block for the QuantStudio Pro system



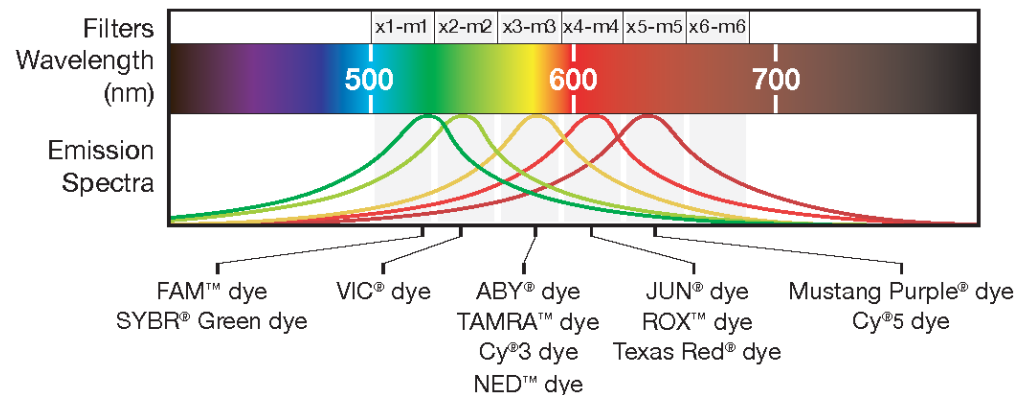
Click Here to View Video: [QuantStudio Pro Block Change](#)

QuantStudio excitation and emission filters

QuantStudio™ 6 Pro



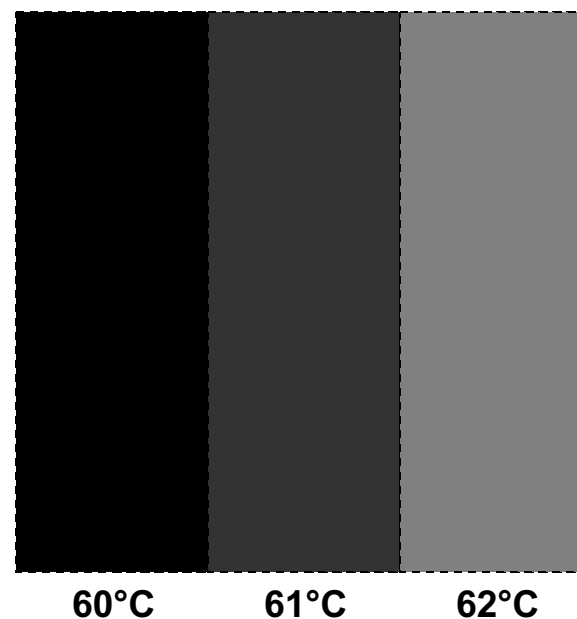
Peak channel	Color	Filter wavelength (nm) ^[1]		Pre-calibrated dyes	Example custom dyes
		Excitation	Emission		
x1-m1	Blue	470 ± 15	520 ± 15	FAM™ and SYBR® Green	SYT09
x2-m2	Green	520 ± 10	558 ± 12	VIC®	HEX™, TET™ and JOE™
x3-m3	Yellow	550 ± 10	587 ± 10	ABY®, NED™, and TAMRA™	Cy®3
x4-m4	Orange	580 ± 10	623 ± 14	JUN® and ROX™	Texas Red®
x5-m5	Red	640 ± 10	682 ± 14	Cy®5 and MUSTANG PURPLE®	LIZ®
x6-m6	Deep-Red	662 ± 10	711 ± 12	None*	Cy®5.5



VeriFlex™ Blocks

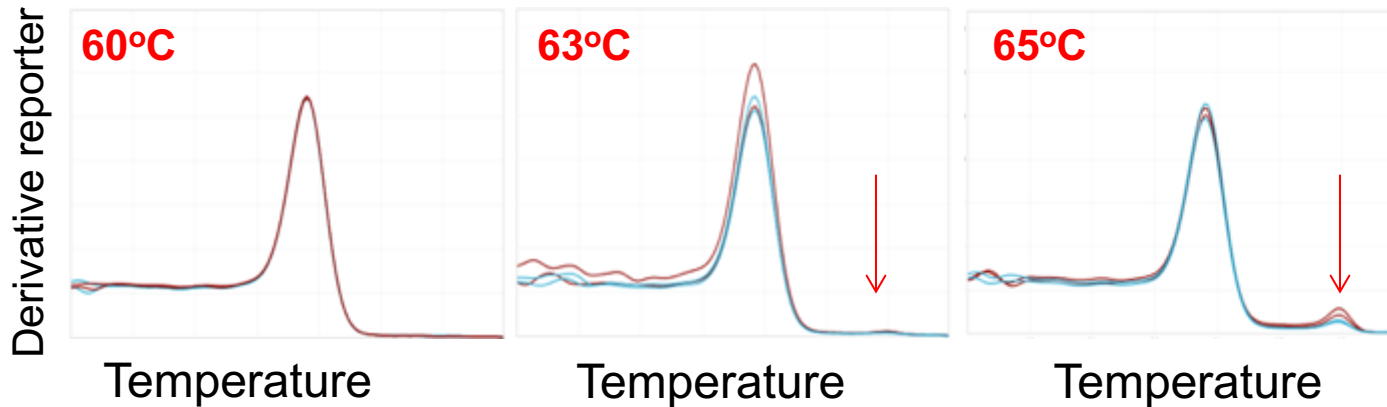
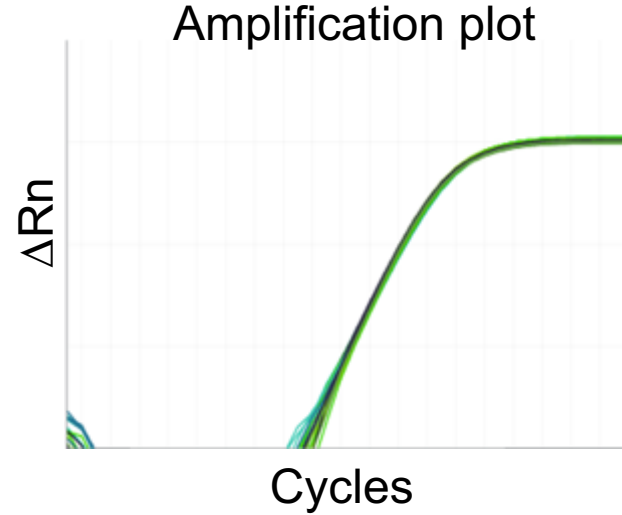
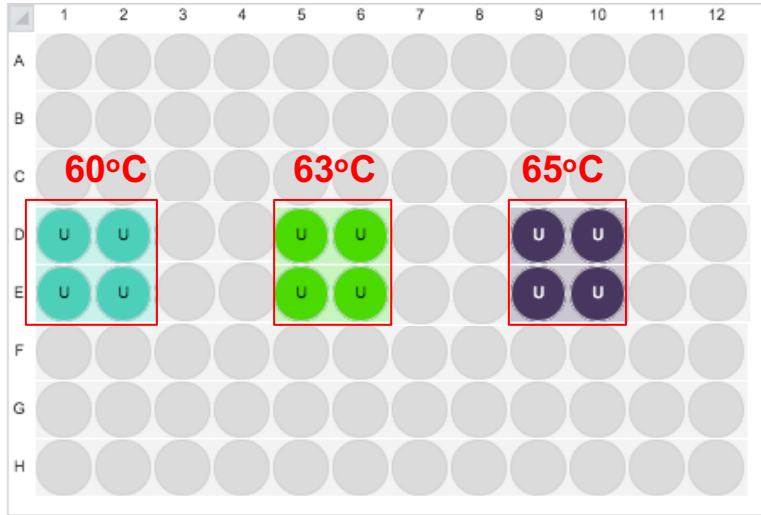
3 programmable zones

- Independent temperature control in each zone
 - More precise than gradient
- Great for optimization and running multiple assays



Veriflex™ blocks: temperature optimization

Annealing temperature optimization for SYBR Green assays



Data courtesy of Verification and Validation

TCF3 Assay: 4 replicates for each temperature

QuantStudio Pro Smart Features

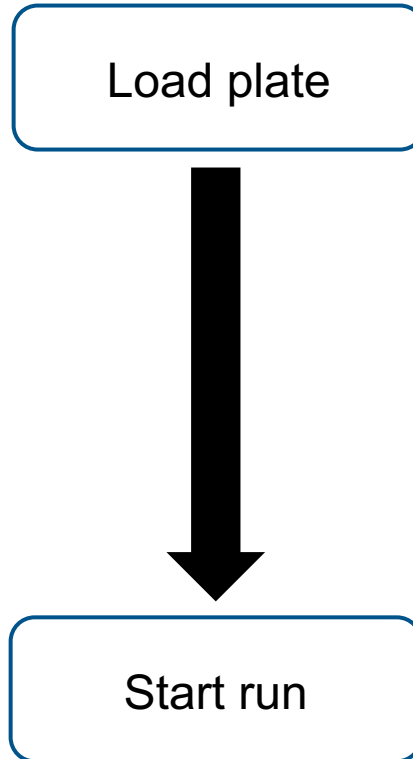
- **RFID enabled**
- *Smart Help*
- **Facial recognition**
- *Voice command*
- *Calendar*

Applied Biosystems TaqMan Array Plates with RFID

RFID tag contains:

- **Plate layout – Assay IDs in each well**
- Plate type
- Expiration date
- Catalog number
- Lot number
- Reaction volume per well
- Passive reference dye
- Thermal protocol

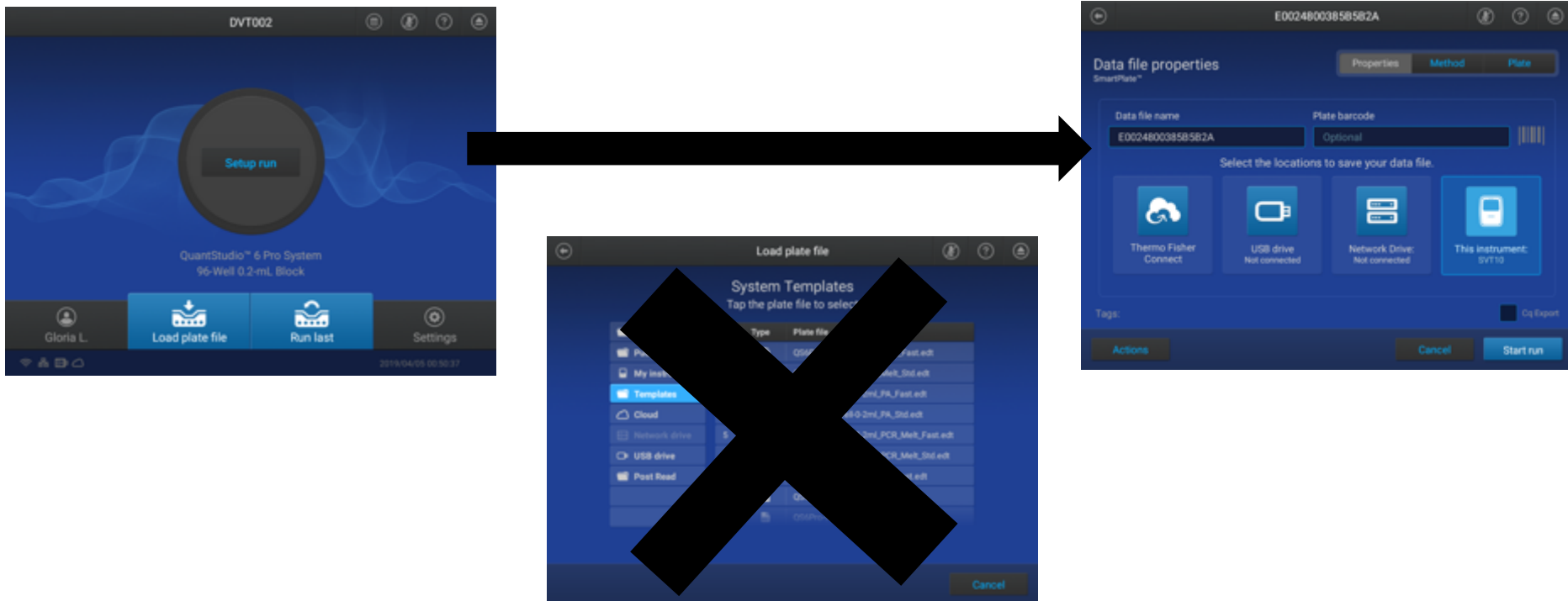
Smart qPCR workflow



- Eliminate manual steps
- Reduce errors for high-throughput and routine analysis

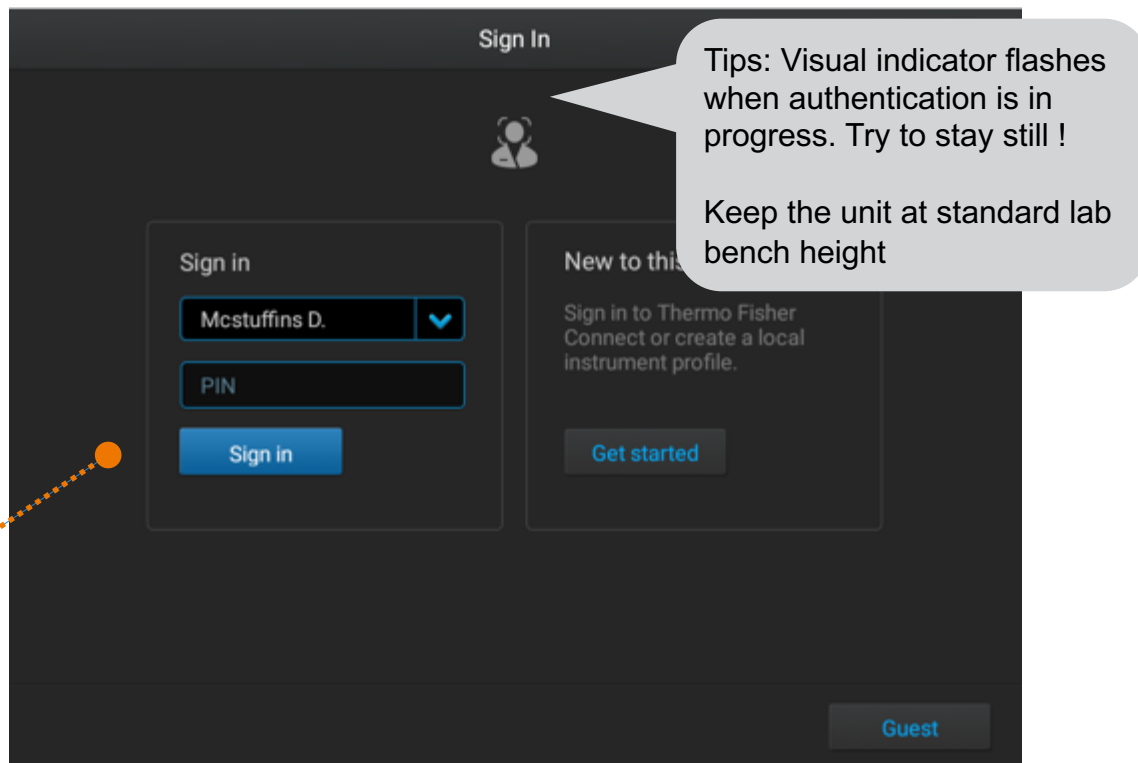
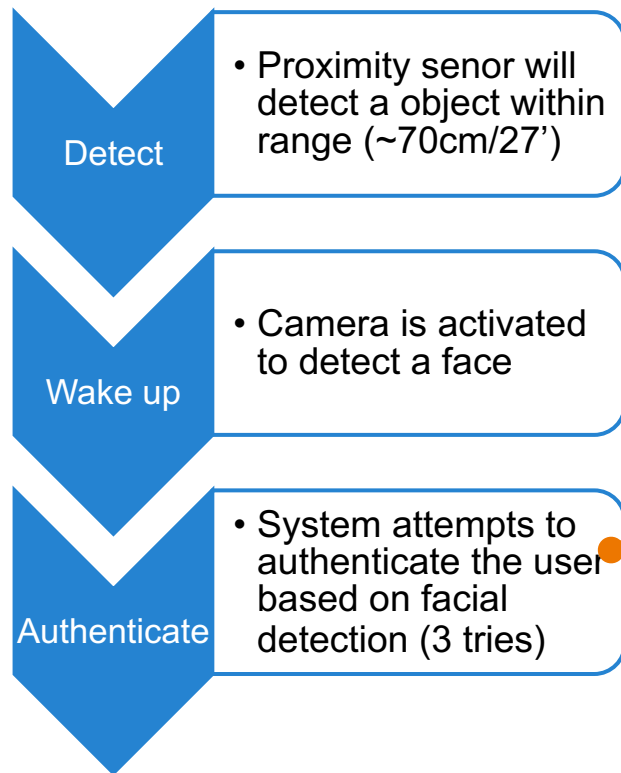


Smart workflow for TAP with RFID



- Insert TAP with RFID Tag.
- Thermal protocol and plate layout are loaded to screen for review & editing
- User can manually enter or import sample setup
- User can overwrite RFID protocol and apply different plate files(.EDT) of their choice for the run
 - i.e. validated template for the particular panel

Sign-in with facial authentication



Camera is deactivated when not in use
No surveillance!

Recommended calibration and verification

Calibration	Recommended frequency
ROI/uniformity	Every two years
	• Always perform new Background and Dye calibrations after an ROI/Uniformity calibration.
Dye	Every two years
	• During a Dye calibration, only the dyes on the given spectral calibration plate are calibrated.
Background	Every month
	• As needed: To check for contamination
	• As needed: To obtain the most accurate data for the removal of background fluorescence
RNase P verification	After installing or moving the instrument.
	After performing instrument or block calibrations.
	As needed to confirm instrument performance.

Service contract options for QuantStudio 6/7 Pro System

	AB Maintenance Plus	AB Assurance 1PM	AB Assurance 2PM
Smart Remote Support	Not included	Unlimited	Unlimited
Smart Help	Not included	Included	Included
On-site repair coverage	10% DC on T&M	Comprehensive	Comprehensive
On-site response	Priority, target 2-3 business day	Guaranteed 2 business days*	Guaranteed 2 business days*
Annual PM	1	1	2

QuantStudio Pro systems features

Description	Specification
Dye compatibility	FAM™/SYBR™ Green, VIC™/JOE™/HEX™/TET™, ABY™/NED™/TAMRA™/Cy®3, JUN™/ROX™/Texas Red™, Mustang Purple™/Cy®5/LIZ™, Cy®5.5
Multiplexing	5 or 6 targets
Dynamic range	10 logs
Sensitivity	Detect down to 1 copy
Resolution	1.5-fold resolution
Light source	Bright white LED
Data acquisition	Whole-plate imaging
Sample capacity	96-well 0.2 mL block
Reaction volume	10-100 µL for 0.2 mL block
Maximum ramp rate	6.5°C/sec
Average sample ramp rate	3.66°C/sec
Temperature uniformity	0.4°C
Temperature range	4-100°C
Veriflex™ block	3 (QS6 Pro) or 6 (QS7 Pro) independent temperature zones

Tutorials: QuantStudio 7 Pro and Design and Analysis 2.x

Video tutorials:

[Create and sign into your profile](#)

[Change the block](#)

[Create a new plate file](#)

[Edit the run method](#)

[Edit plate setup information](#)

[Analyze data](#)

[Export results](#)

Import plate layout from .csv

1. In Plate Setup tab > ... > Export Plate Setup
2. Edit exported .csv file and save
(do not change headers)
3. In Plate Setup tab > ... > Import Plate Setup
Open edited .csv file

Block Type = 96-Well 0.2-mL Block
Date Created = Wed Jul 15 08:11:44 PDT 2020
Passive Reference = ROX

Well	Well Position	Sample Name	Sample Color	Biogroup Name	Biogroup Color	Target Name	Target Color	Task	Reporter	Quencher
1 A1		Brain	RGB(216,27,96)			ACTB	RGB(216,27, UNKNOWN	FAM	NFQ-MGB	
2 A2		Brain	RGB(216,27,96)			ACTB	RGB(216,27, UNKNOWN	FAM	NFQ-MGB	
3 A3		Brain	RGB(216,27,96)			ACTB	RGB(216,27, UNKNOWN	FAM	NFQ-MGB	
4 A4										
5 A5		Brain	RGB(216,27,96)			STMN1	RGB(142,36, UNKNOWN	FAM	NFQ-MGB	
6 A6		Brain	RGB(216,27,96)			STMN1	RGB(142,36, UNKNOWN	FAM	NFQ-MGB	
7 A7		Brain	RGB(216,27,96)			STMN1	RGB(142,36, UNKNOWN	FAM	NFQ-MGB	

Need help?

[Real-Time qPCR Application Support Center](#)

[Real-Time PCR Learning Center](#)

Technical Support:

1-800-955-6288, option 2, option 1, option 2

TAC (Instrument Support):

1-800-955-6288, option 3, option 1

Lauren Neves

Lauren.Neves@thermofisher.com



Webinar series: real-time PCR fundamentals

Webinar 1	<u>Fundamentals of Real-Time PCR Webinar</u>
Webinar 2	<u>Assay Design</u>
Webinar 3	<u>Normalization</u>
Webinar 4	<u>Multiplexing</u>
Webinar 5	<u>Reagent Options</u>
Webinar 6	<u>Absolute and Relative (ddCt) Quantification</u>

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