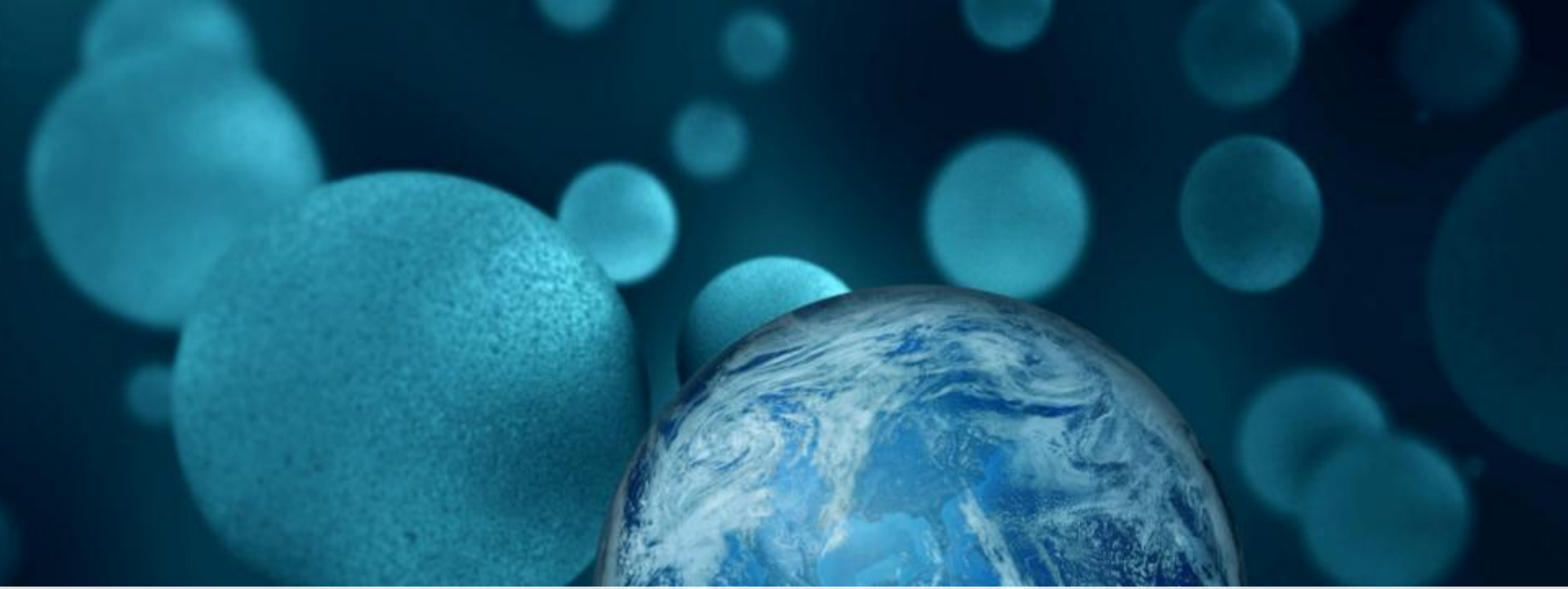

ThermoFisher SCIENTIFIC

Introduction to Real-Time PCR and Applications

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Field Application Scientist
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How is Real-Time PCR similar to Traditional PCR?

- Reactions are cycled in a temperature block:
  - **Denaturation** of dsDNA template
  - **Annealing** of primers to template
  - **Extension** of primers → new amplicon

40 cycles

Example:

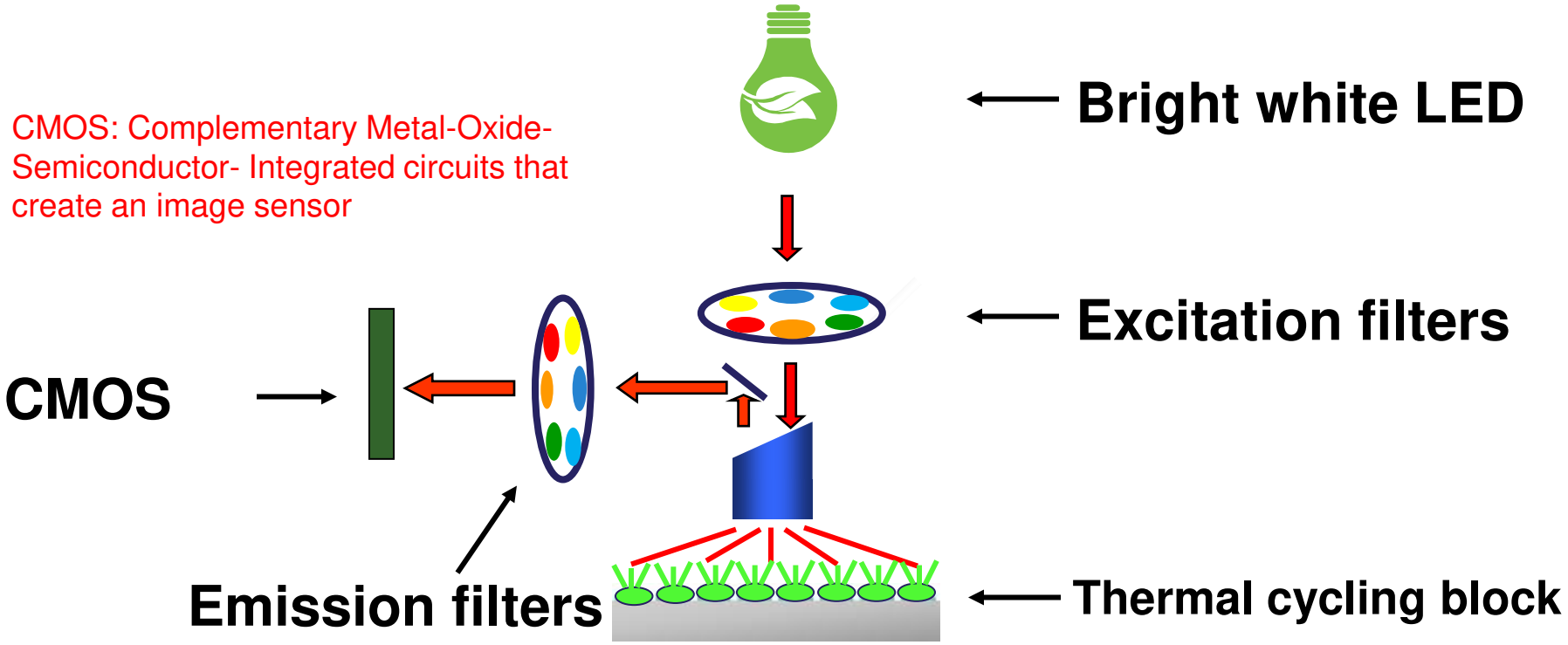
1 cycle of PCR

PCR theoretically **doubles** target after each cycle

How does Real-Time PCR work?

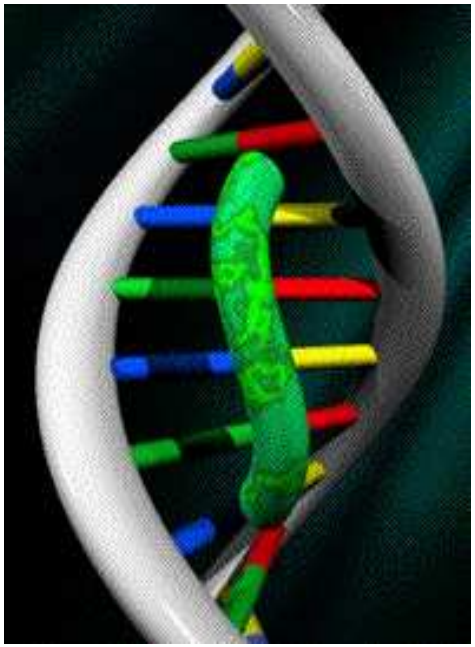
- The QuantStudio™ has a thermal block, a bright white LED to excite fluorescence, filters, and a high-resolution CMOS to detect signal.

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

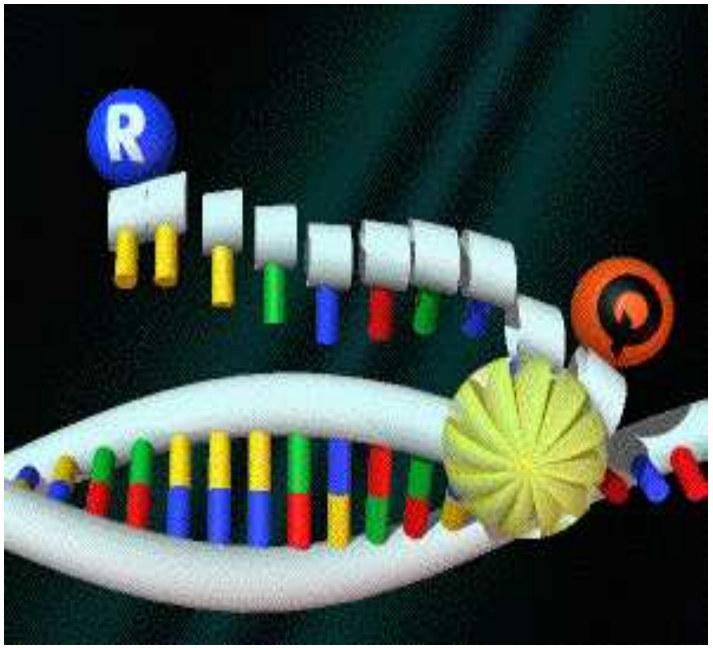


Supported Fluorescent Chemistries

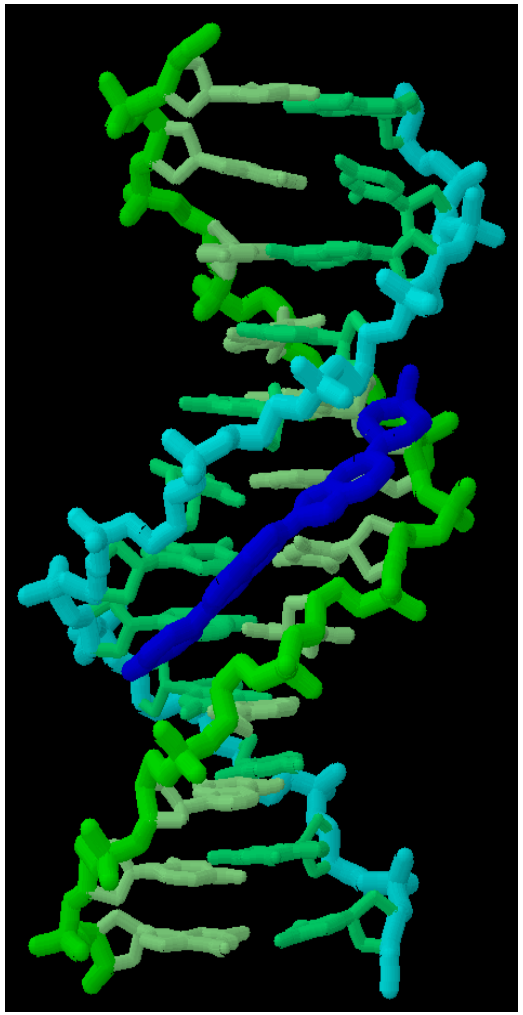
SYBR® Green I Dye



5’-Nuclease Chemistry



SYBR® Green I

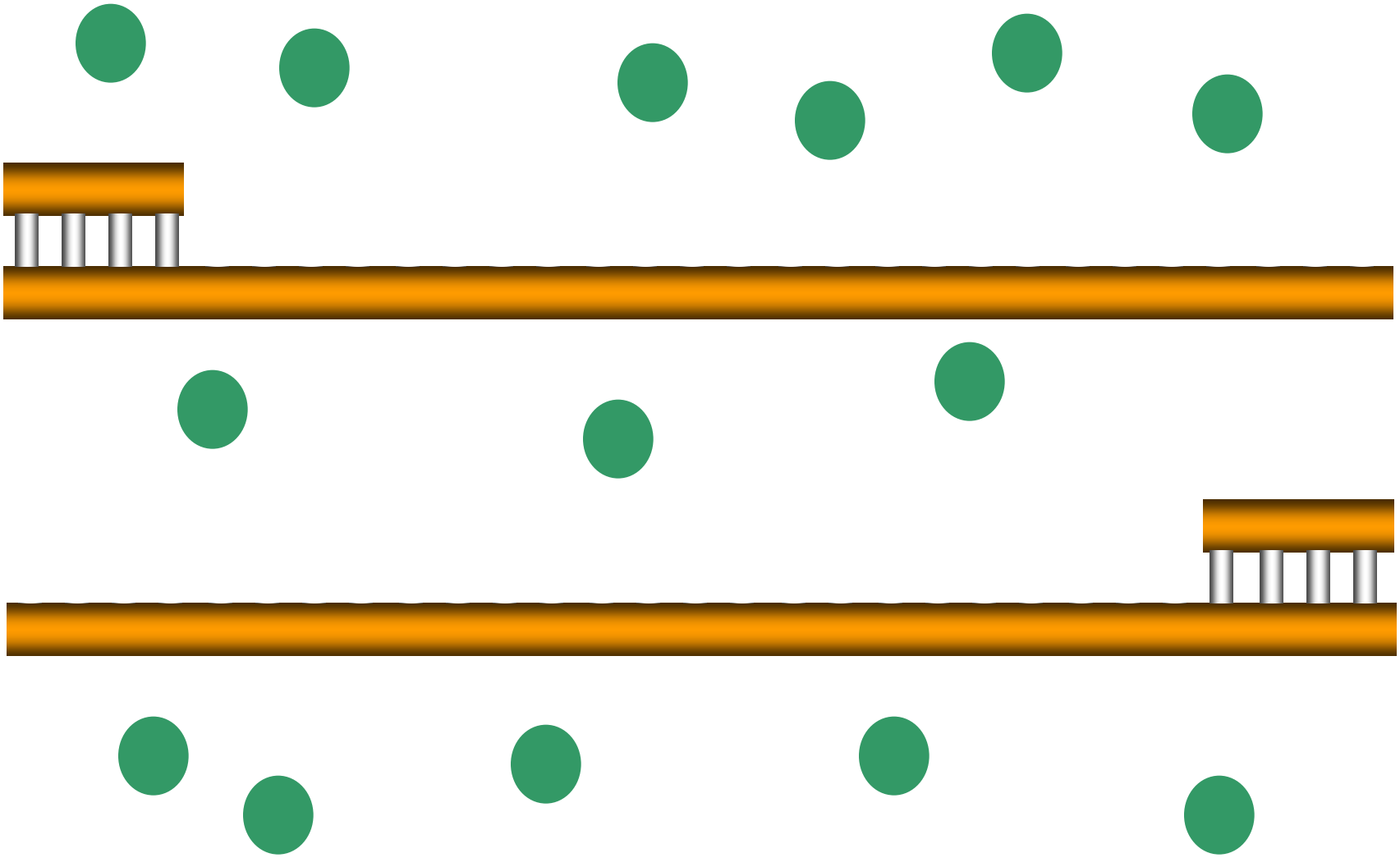


- SYBR® Green I is a dye that binds to the minor groove of double-stranded DNA.
- Like gel-based PCR, SYBR® Green assays consist of only two primers without a probe.

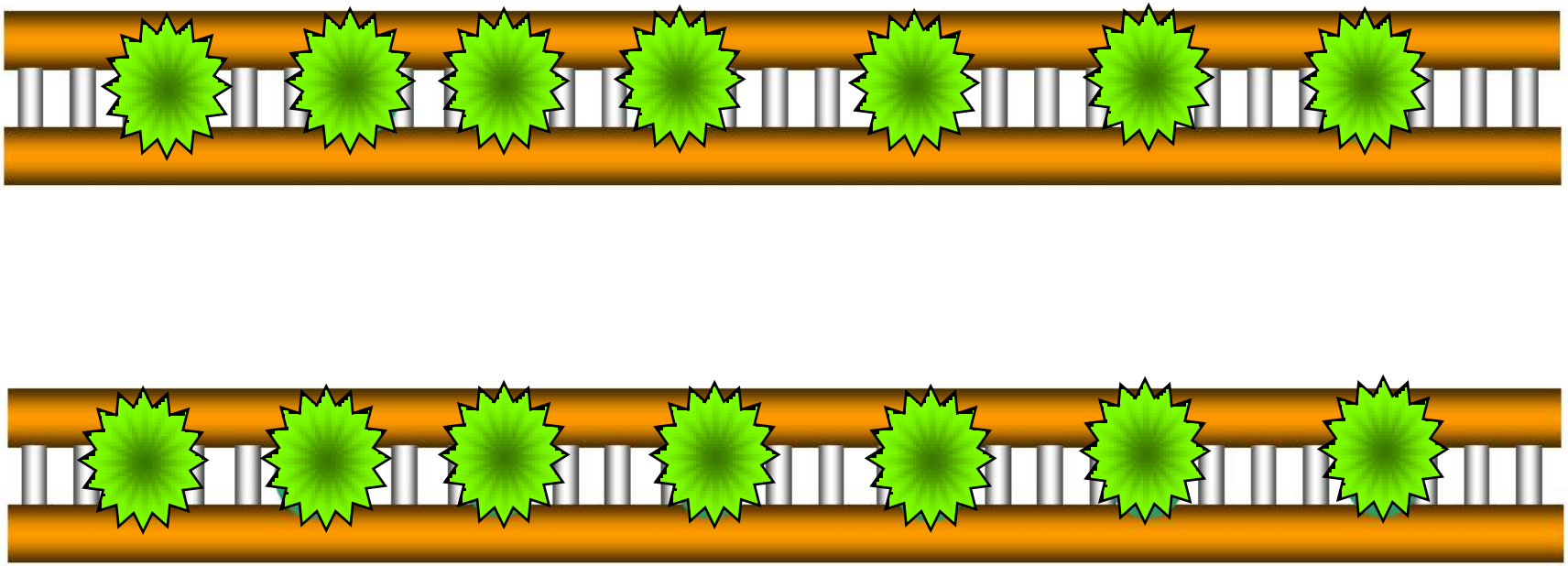
forward primer

reverse primer

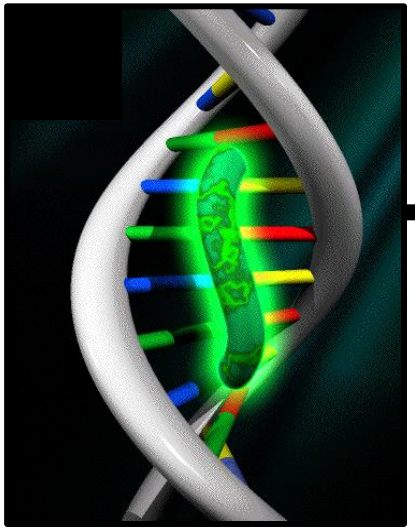
SYBR® Green I dye



SYBR® Green I dye



Problem with SYBR® Green I Dye



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

Signal from non-specific products

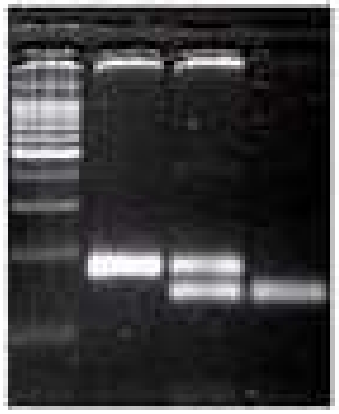
Incorrect quantitative results

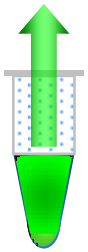
- If not well designed and optimized, SYBR® Green assay results will include signal from all PCR products, target or non-target.

Non-Target PCR Amplification

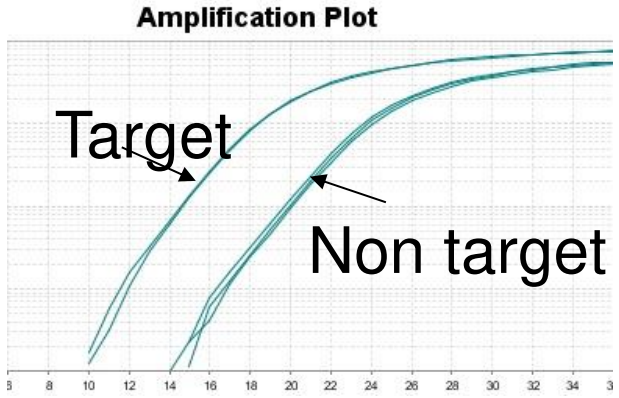
- Non-target amplification is commonly observed in gel-based PCR.
- SYBR® Green amplification plots usually look the same whether they are derived from target, non-target or a mixture of the two.
- Additional data are required to determine whether SYBR® Green assay results are derived solely from target or not.

Gel electrophoresis separates non-target products from target if there is a size difference.

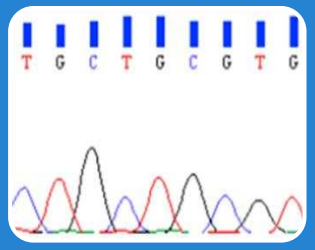




There is no opportunity in real-time PCR to separate non-target products from target.

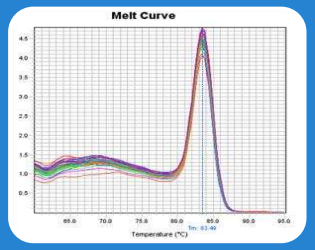


Additional data are required to determine whether SYBR® Green assay results are derived from target or not



Sequencing the assay products

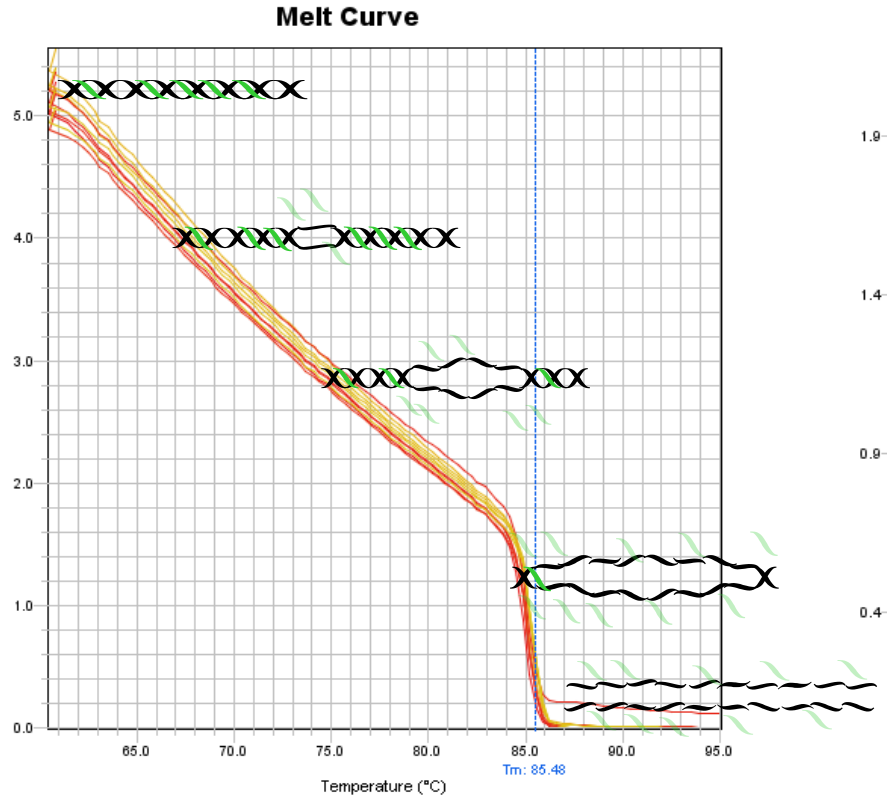
Gel analysis



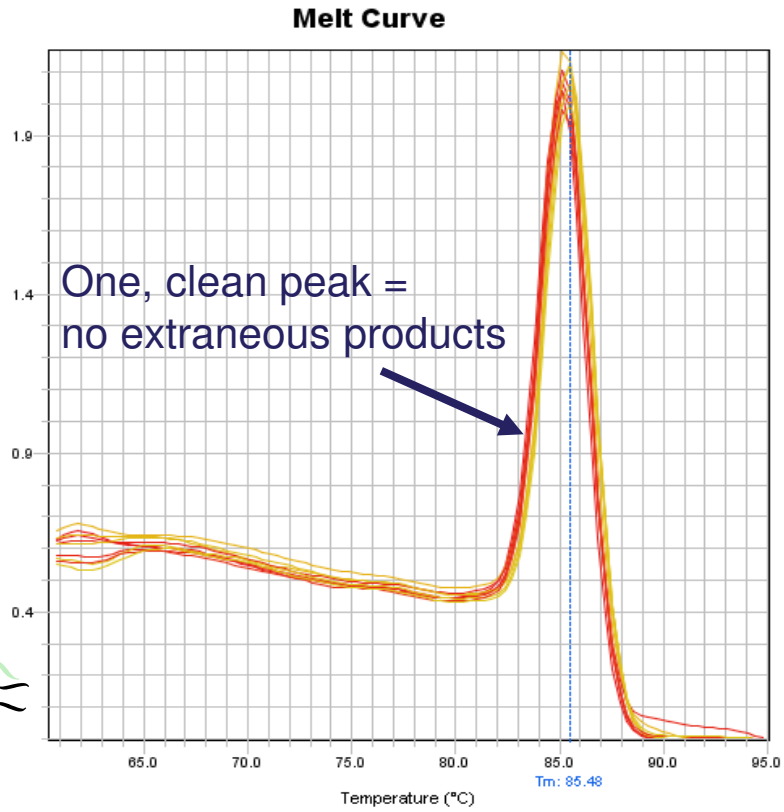
Dissociation analysis

Check Specificity of Reactions Using a Melt Curve

Fluorescence



First negative derivative view

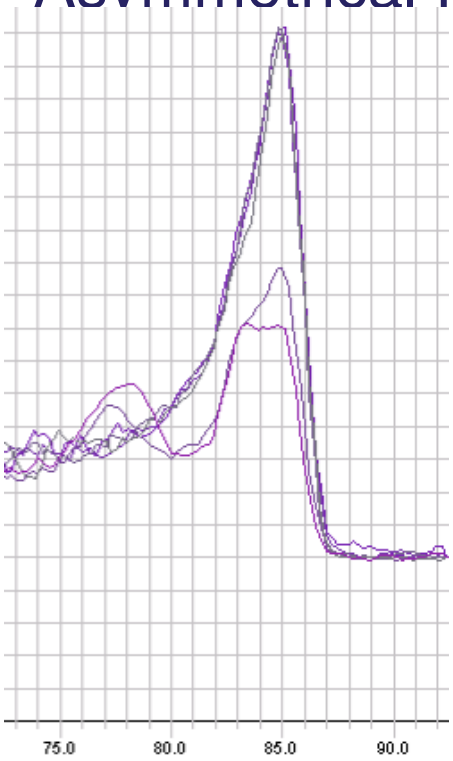


Temperature →

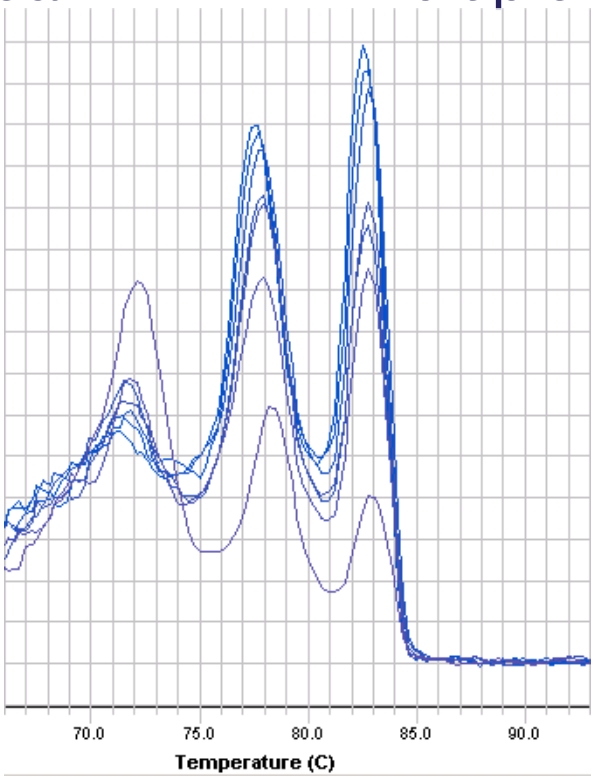
Mixed Product Peaks

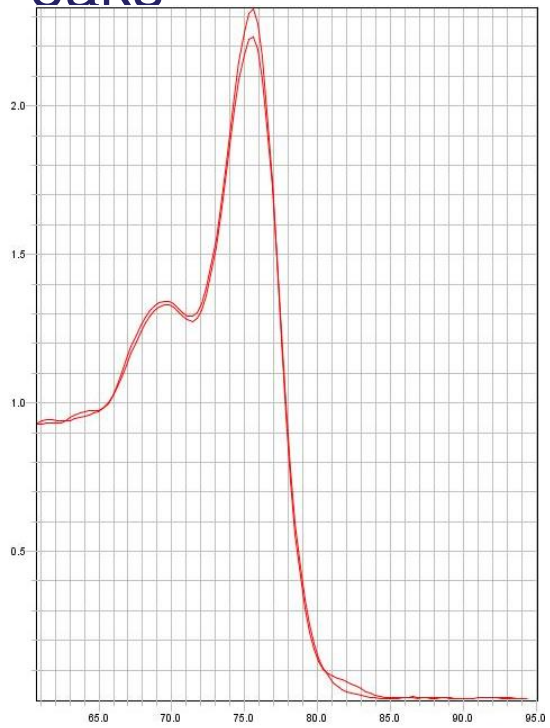
- Examples of melt peaks indicating multiple products were amplified.

Asymmetrical Peak



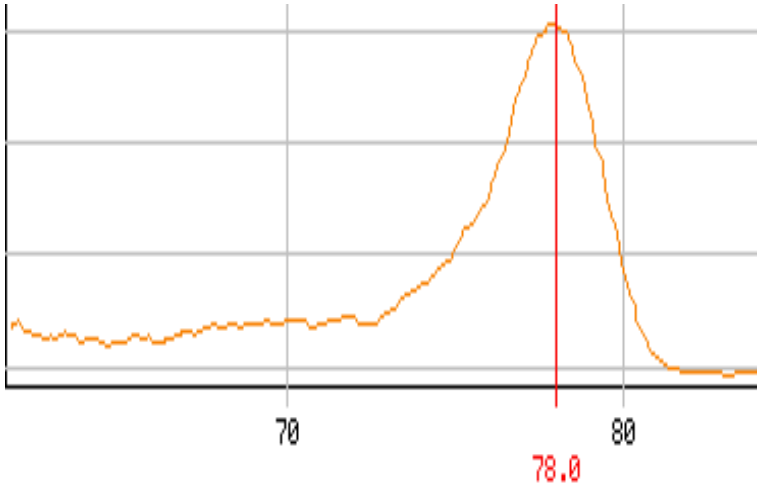
Multiple Peaks





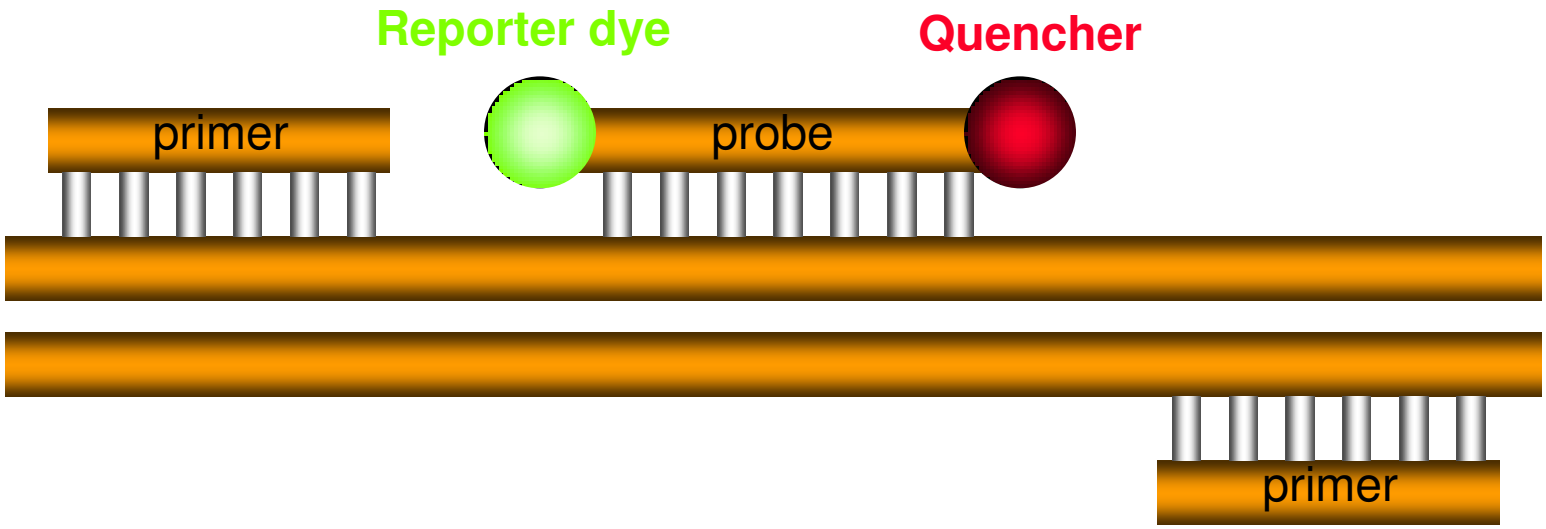
SYBR® Green Melt is Low Resolution

- SYBR® Green melt analysis is low resolution and may not discriminate target and products of similar Tm's, such as homologs.
- A single peak may not be the target, nor one product.
- Below is an example of a SYBR® Green assay that produced one apparent peak, but was amplifying multiple products based on gel electrophoresis results.



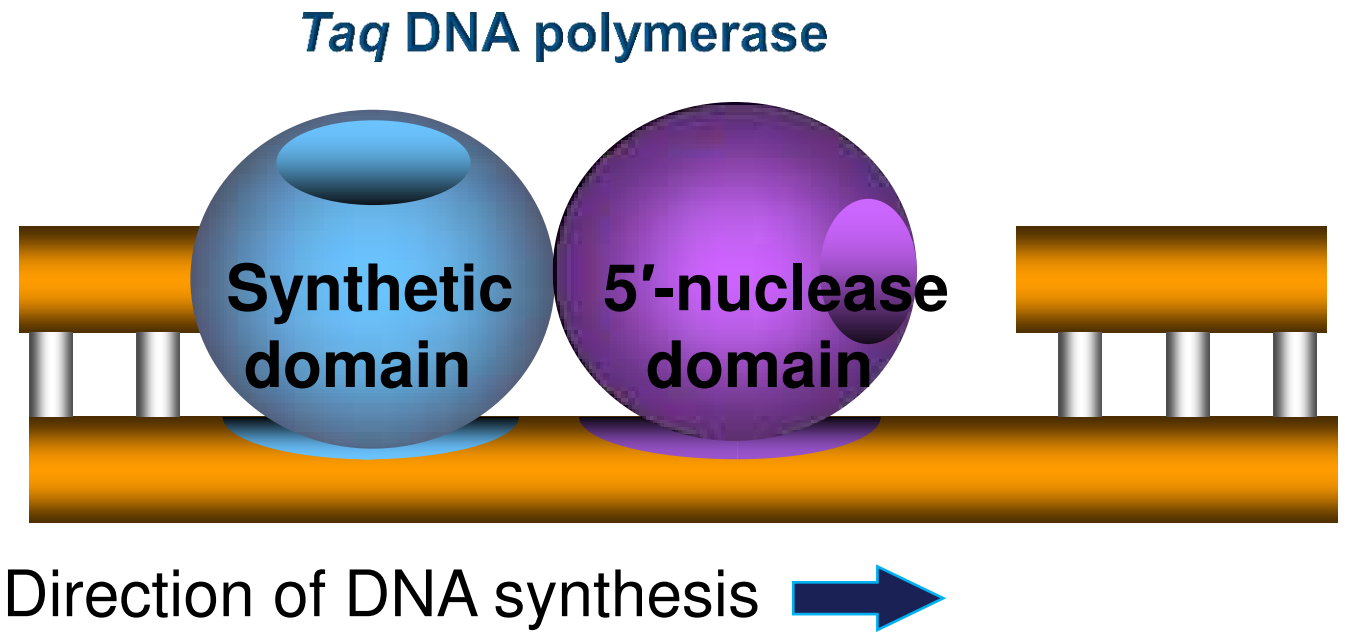
5′-Nuclease Assay

- The 5′-Nuclease Assay uses a gene specific TaqMan probe.
- The probe has a Reporter dye on the 5′-end and a Quencher on the 3′-end, which also blocks extension.

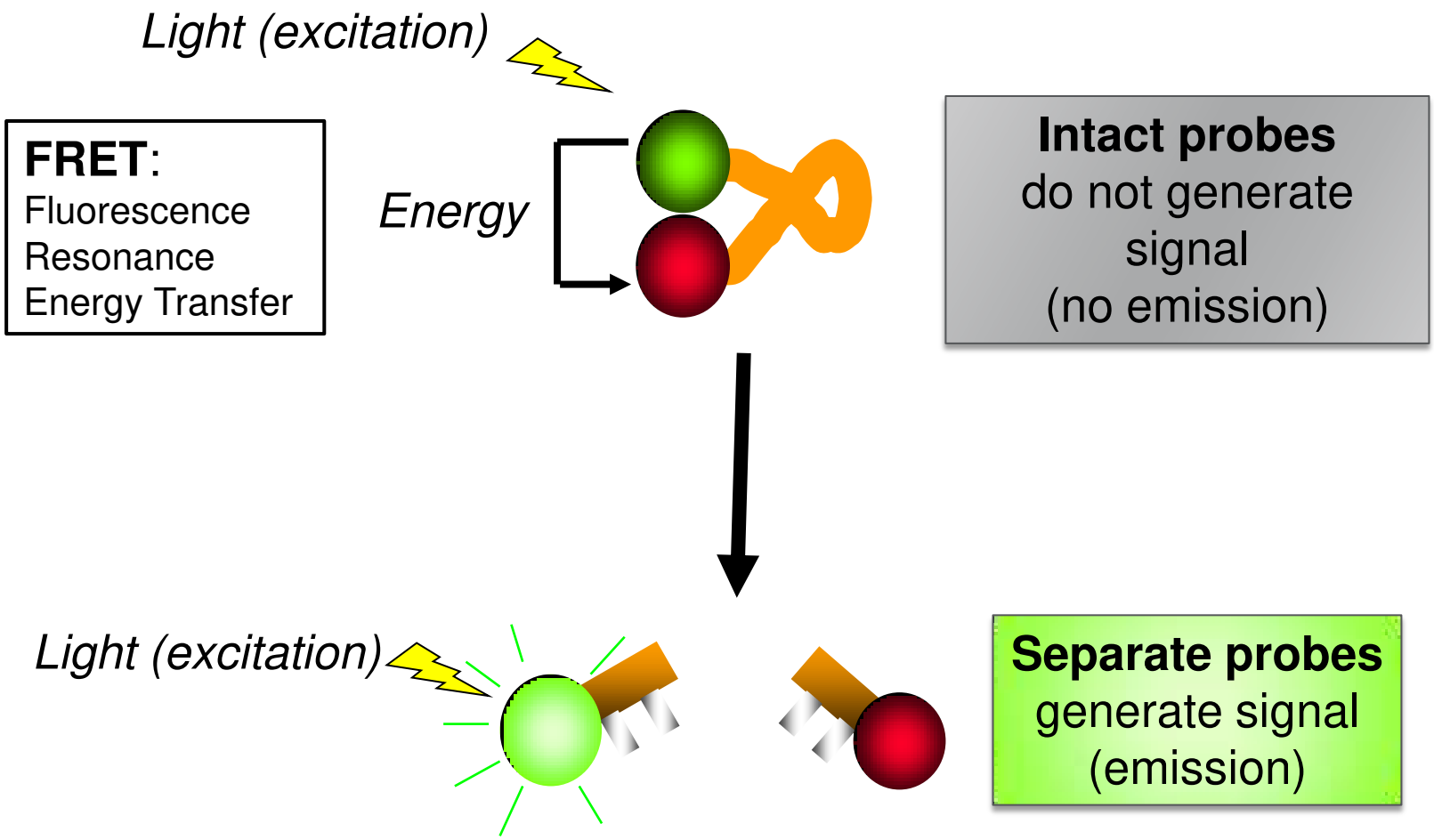


5′-Nuclease Assay

- The 5′-Nuclease Assay gets its name from the 5′-nuclease activity of Taq DNA polymerase.
- The nuclease domain can degrade DNA bound downstream of DNA synthesis.

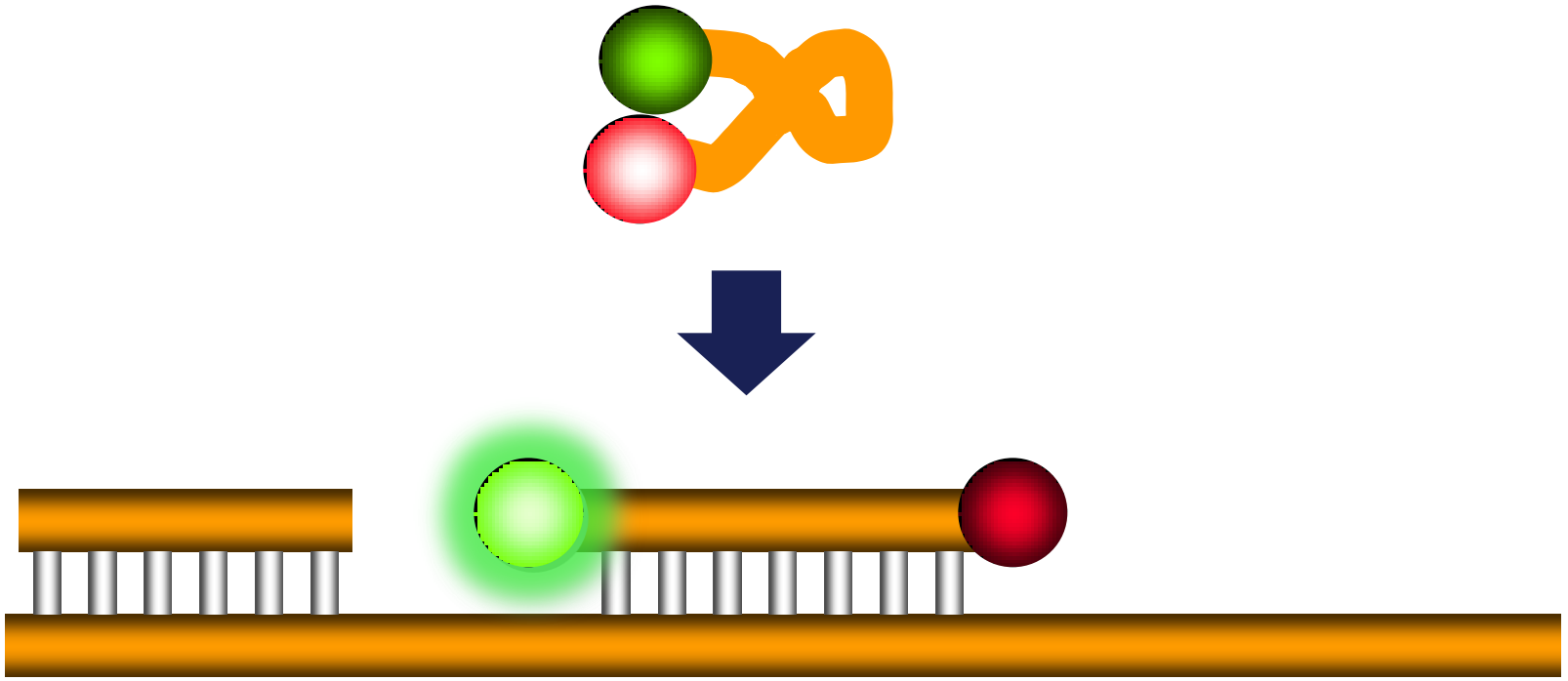


5′-Nuclease Assay



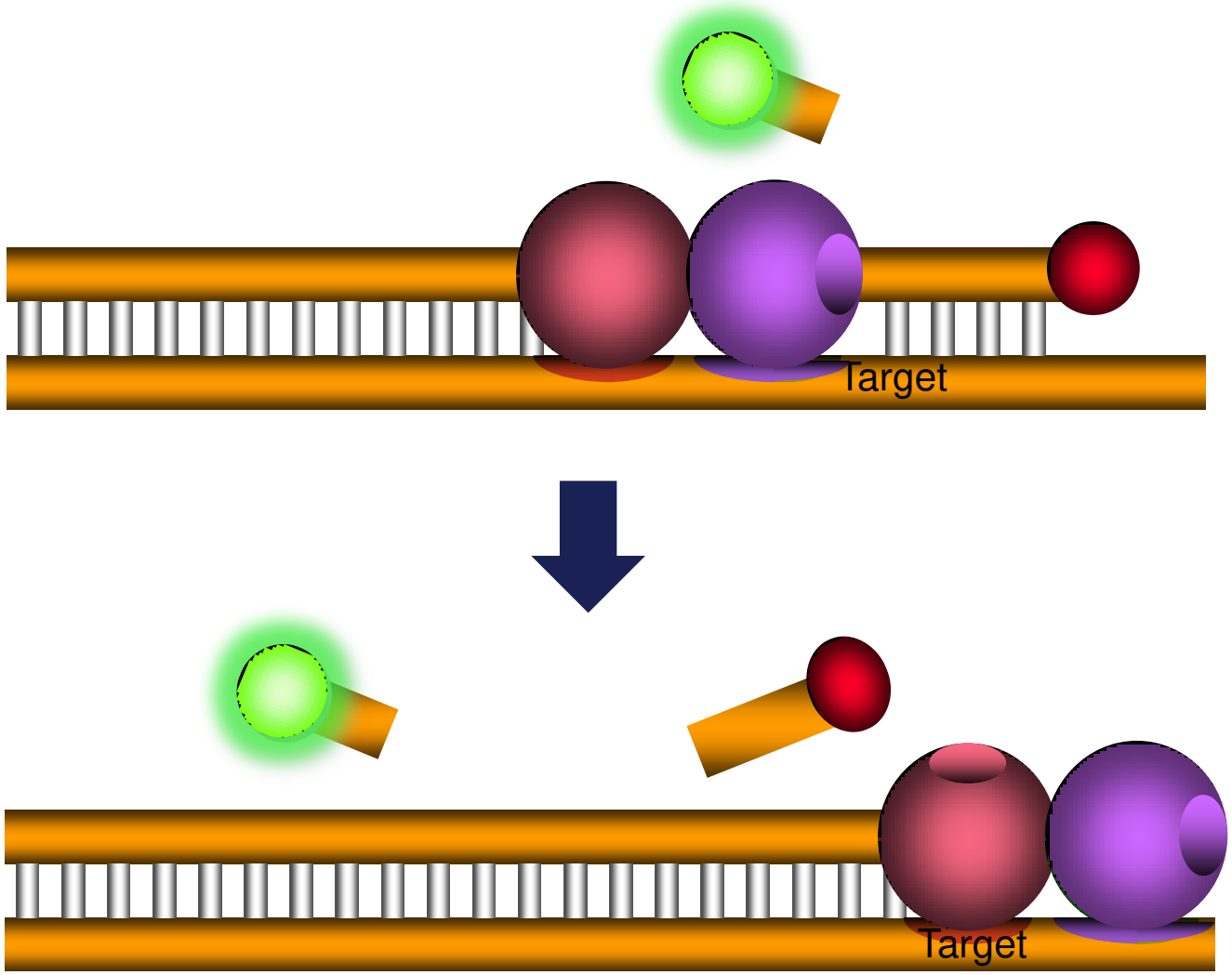
PCR Annealing Step

- During the annealing step, the primers and probe anneal to the template.



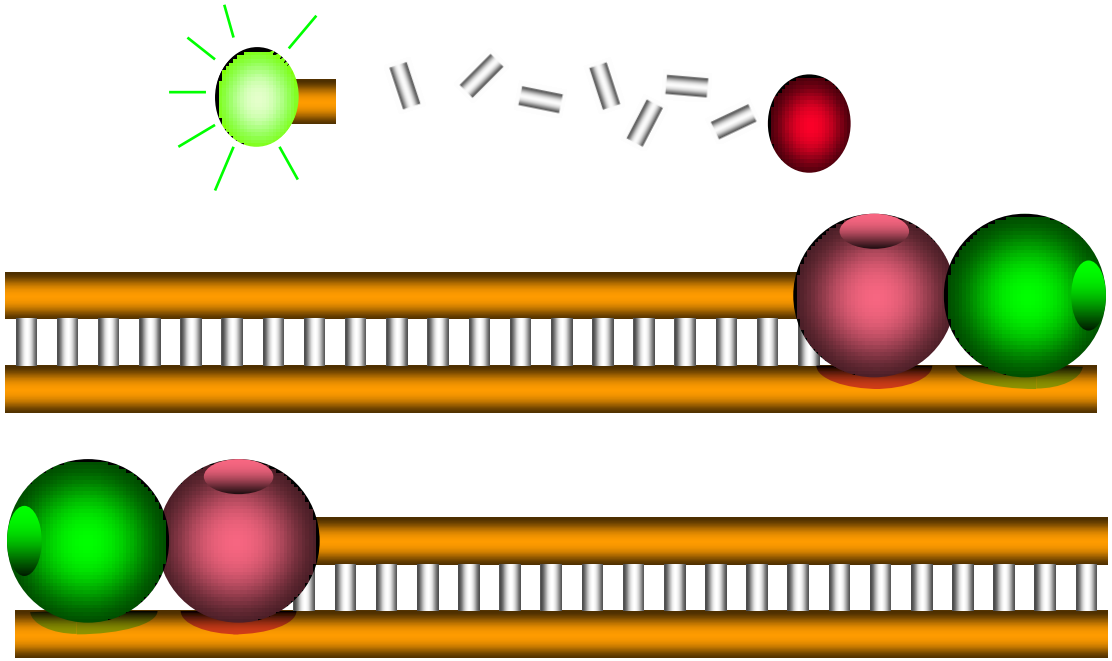
5′-Nuclease Activity Cleaves the Probe

- Following probe cleavage, the Reporter is permanently de-quenched.



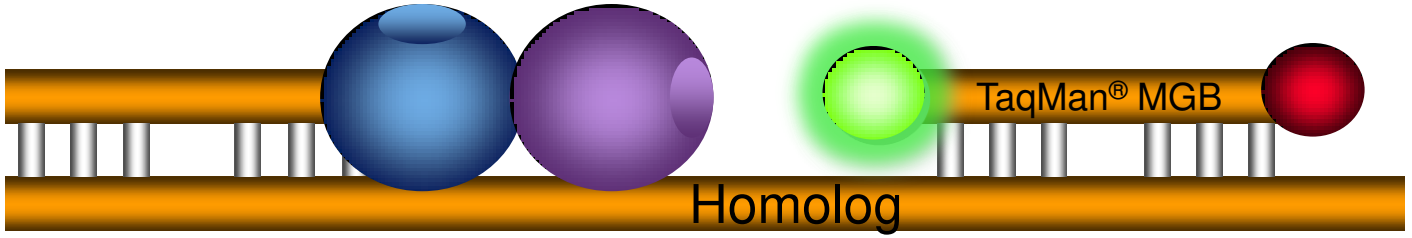
After each cycle

- Amount of PCR product has theoretically doubled.
- Level of fluorescence has increased in a “permanent” manner by a proportional amount.

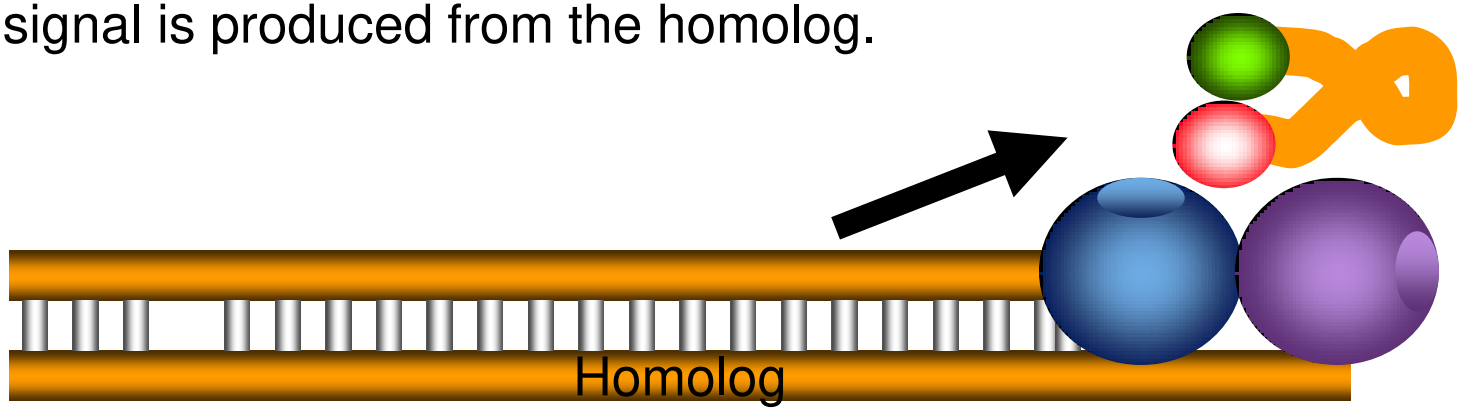


No Mismatch Extension for TaqMan® Probes

- The destabilizing effect of probe mismatches cannot be mitigated by *Taq* polymerase because extension is blocked by the quencher.

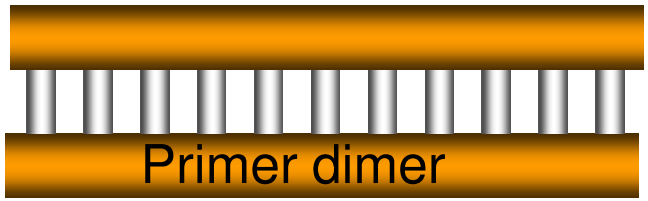


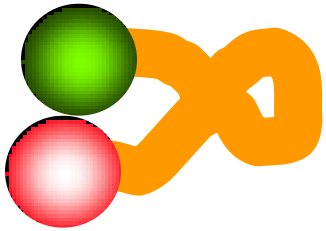
- *Taq* polymerase displaces the probe, rather than cleaving it.
- The probe returns to the quenched configuration
- No signal is produced from the homolog.



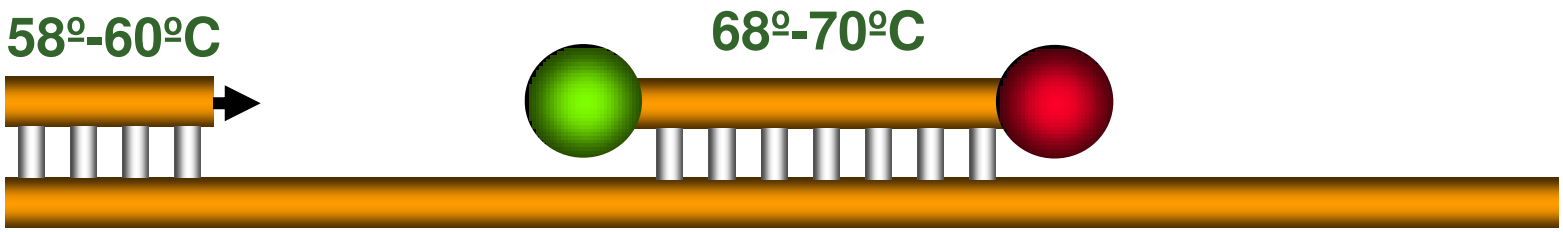
Non-Specific Product Exclusion

- Non-specific PCR products, a.k.a., primer-dimers, are amplified products unrelated to the target.
- They are often formed when primers bind to other template sequences in the sample and by chance are able to form a PCR product.
- TaqMan probes cannot bind to nor be cleaved by non-specific products, so all such products are automatically excluded from assay results.

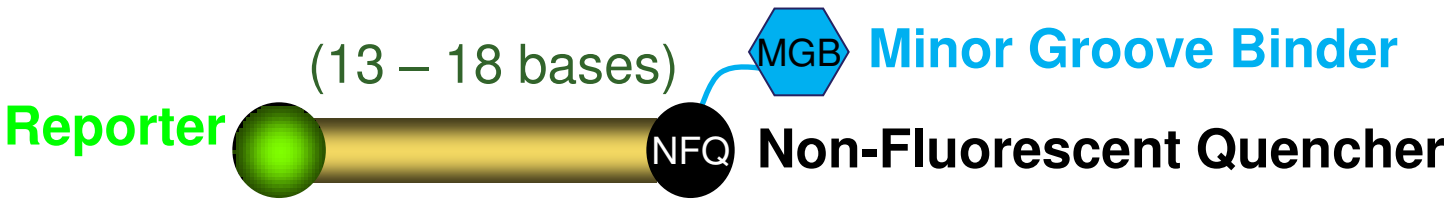




Important Note on Oligo Tms



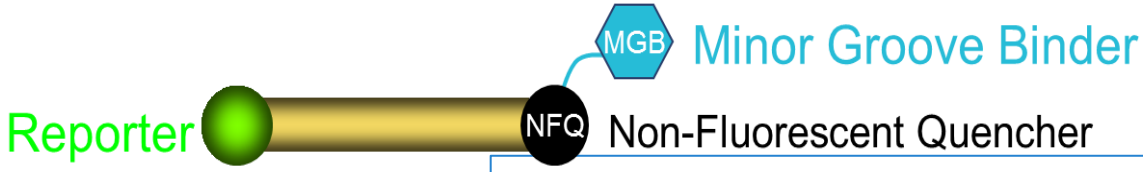
- Probe binds to the template before primer binding and stay tightly bound during extension.
- Primer Tms should be about 58 – 60 degree.
- Probe Tms should be 8-10 degrees higher than primer Tms.



Quenchers: TAMRA™ Dye vs. MGB

Fluorescent = Uses up one filter/dye spot

Long (28-38bp) = less specificity



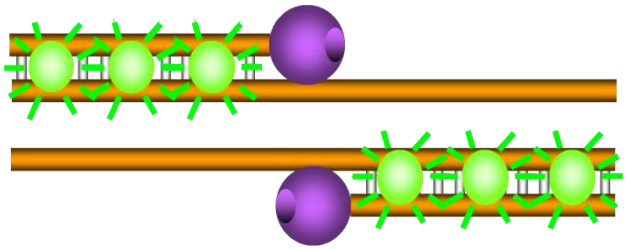
Non-fluorescent

Shorter (13-18bp) = higher specificity

Sensitive to single base mismatch!

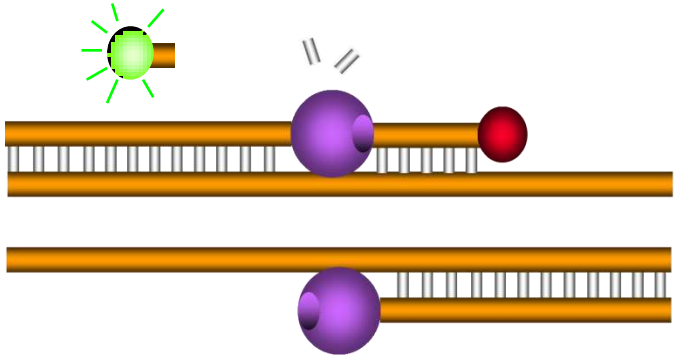
Real-time PCR Fluorescent Chemistries

SYBR™ Green Assay

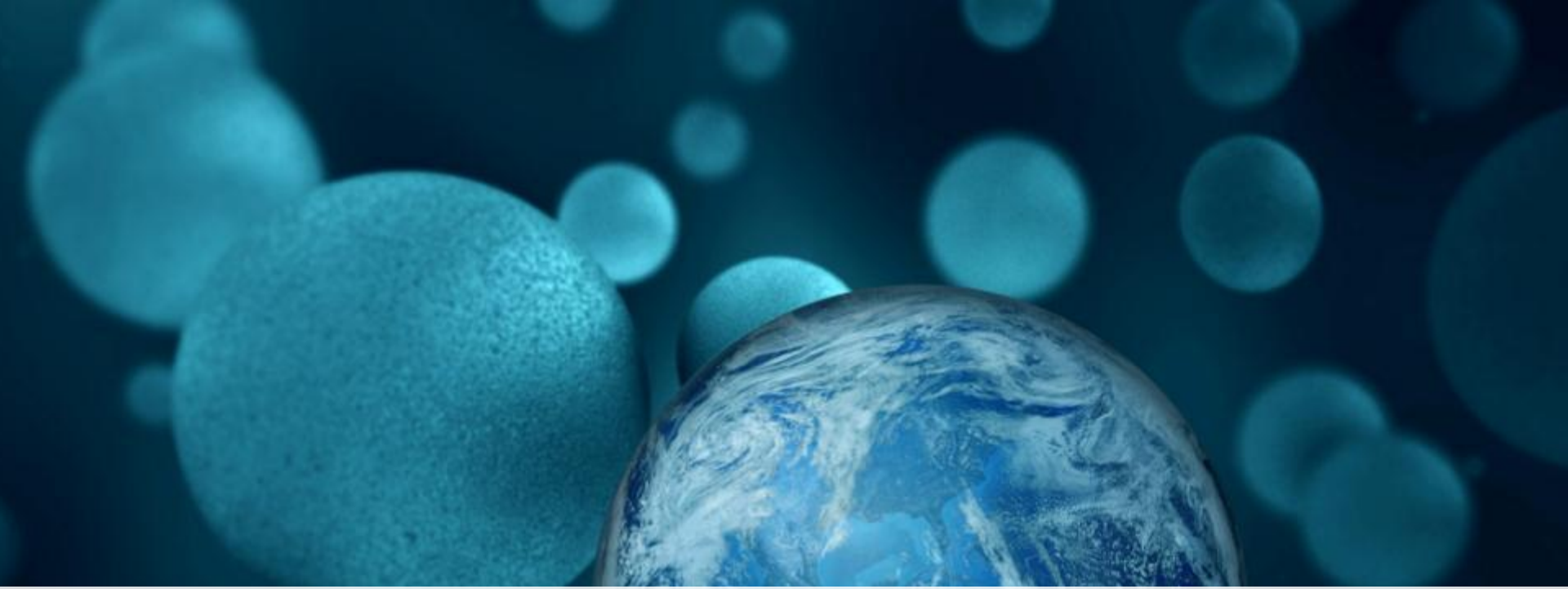


- Uses a dsDNA binding dye to detect accumulating PCR product
- Only primers are needed
- Multiplexing is not possible

5′-Nuclease Assay



- Uses a target-specific fluorogenic probe to detect accumulating PCR product
- Assay consists of forward and reverse primers and probe
- **Multiplexing**



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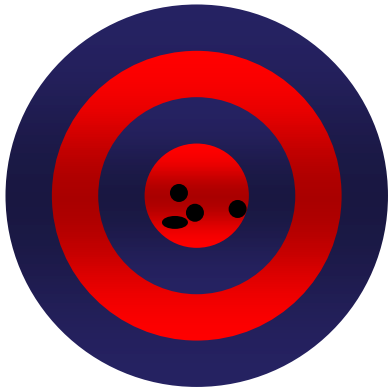
Fundamentals of Data Analysis

Note: For Research Use Only. Not for use in diagnostic procedures.

Criteria for generating good quantitative data

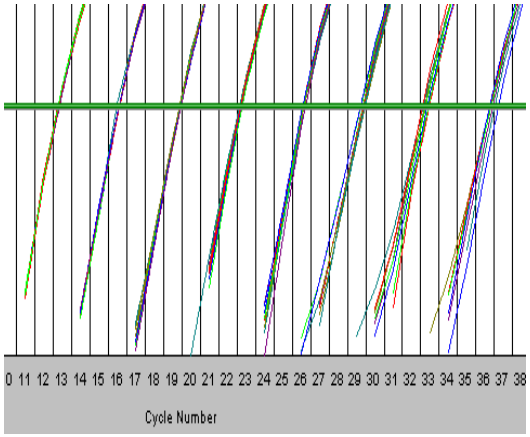
1. Precision & Accuracy



Reproducibility
Tight replicates

2. Efficiency

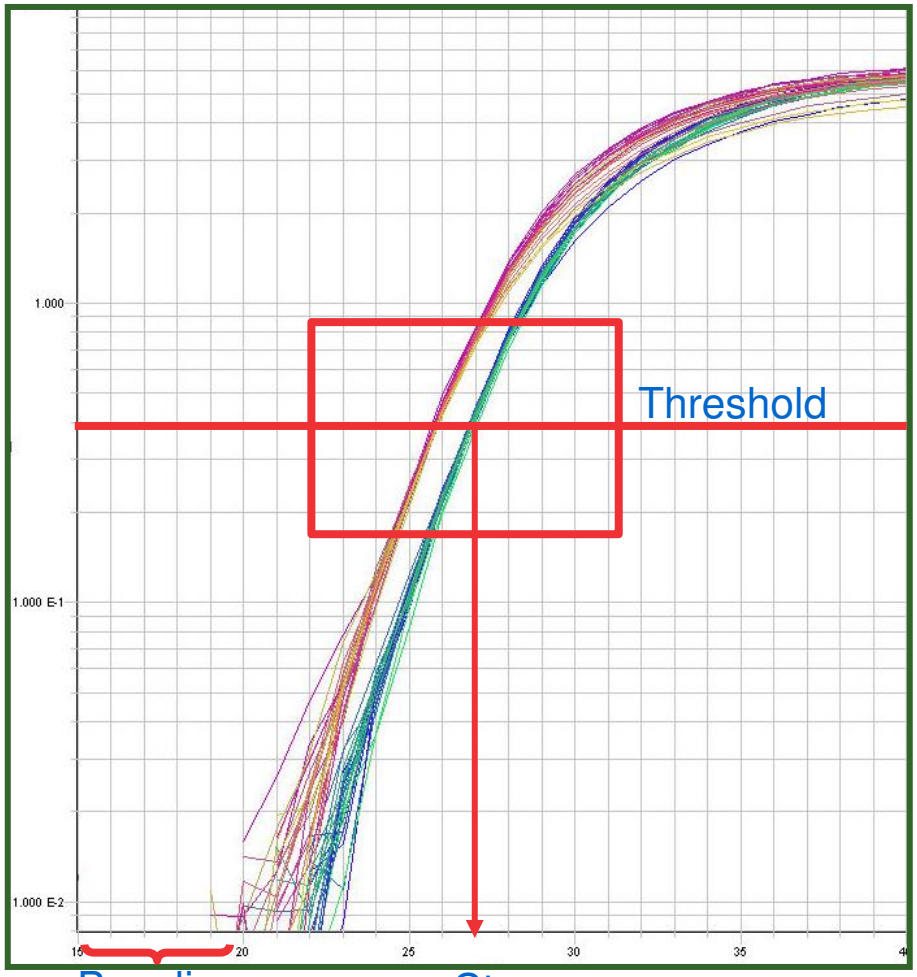
3. Dynamic range



Good data doesn't happen automatically

… we must **NORMALIZE**

Basic Real-Time PCR Terminology



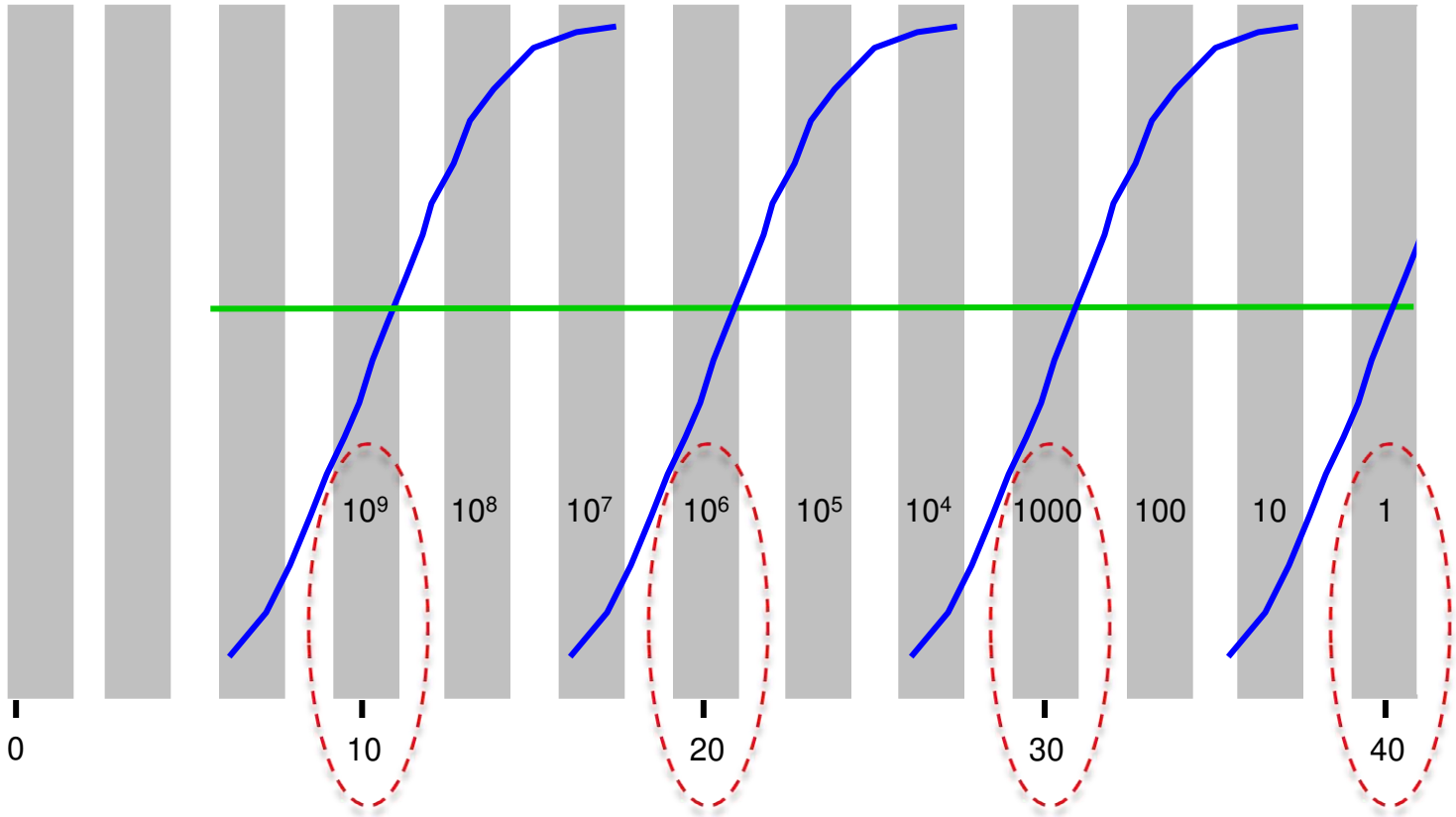
Baseline: background noise

Threshold: adjustable line that tells software where to take the data

Ct: cycle threshold

ΔRn: magnitude of the fluorescence signal generated during the PCR at each time point

With real-time PCR, Ct or Cq values indicate approx. copy



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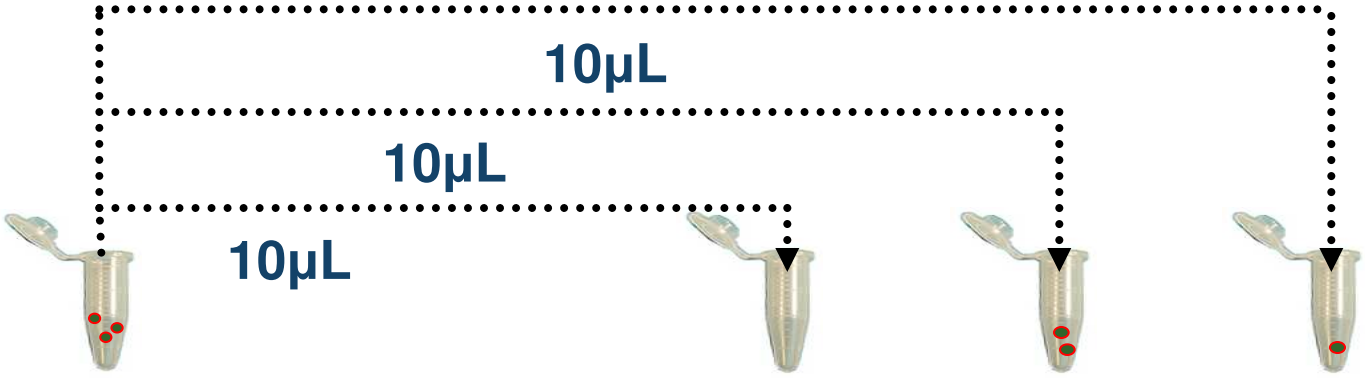
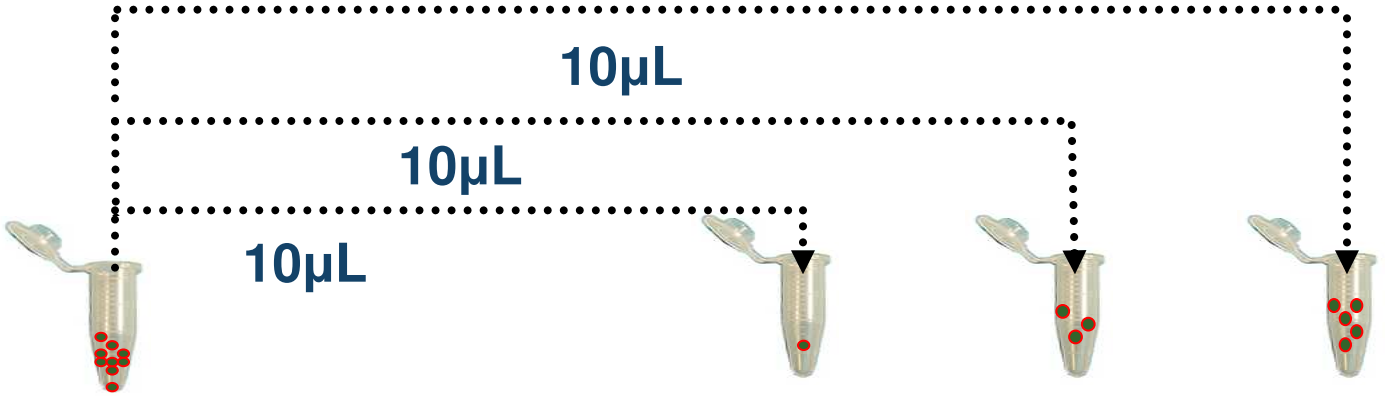
Limit of detection – Poisson distribution

How often do we capture at least 1 molecule in each tube?



30 uL / 3 molecules

Answer: ~ 63%

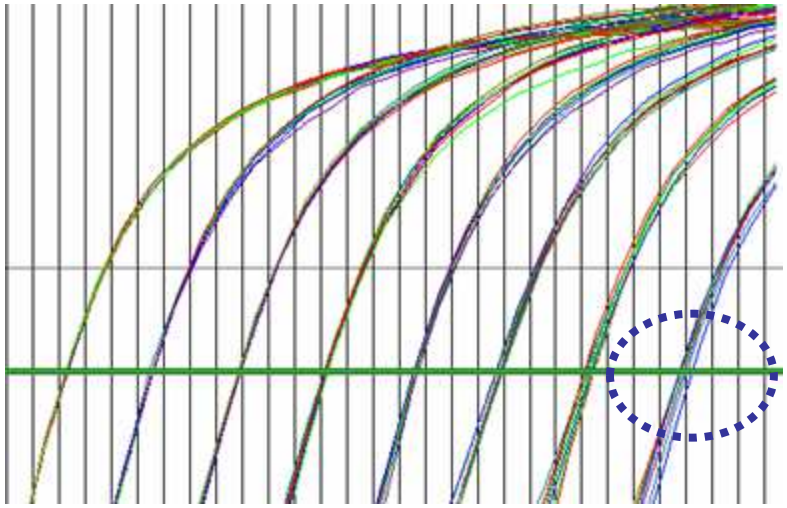


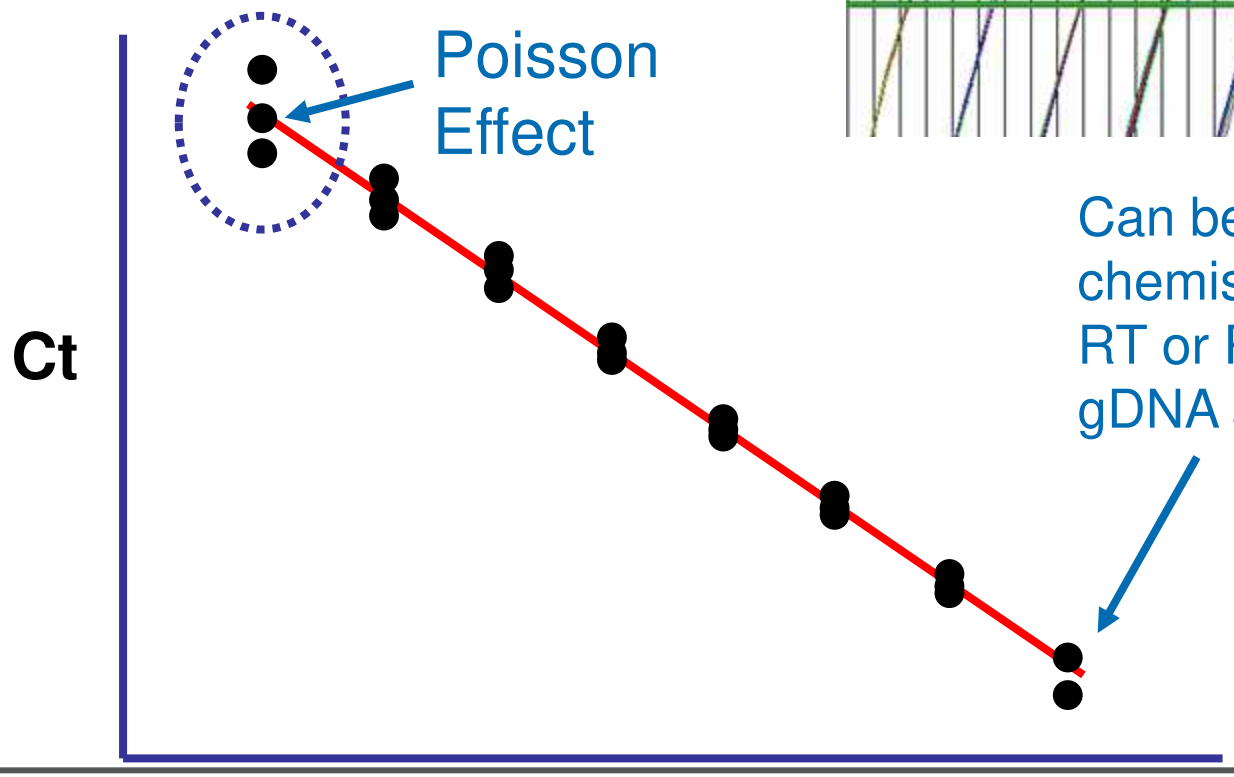
30 uL / 9 molecules

Answer: ~ 95%

How do we determine dynamic range?

- Standard curve
  - Should span 7-8 orders of magnitude for plasmid DNA and at least a 4-log range for cDNA or genomic DNA





Can be affected by RT chemistry saturation, RT or PCR inhibitors, gDNA solubility

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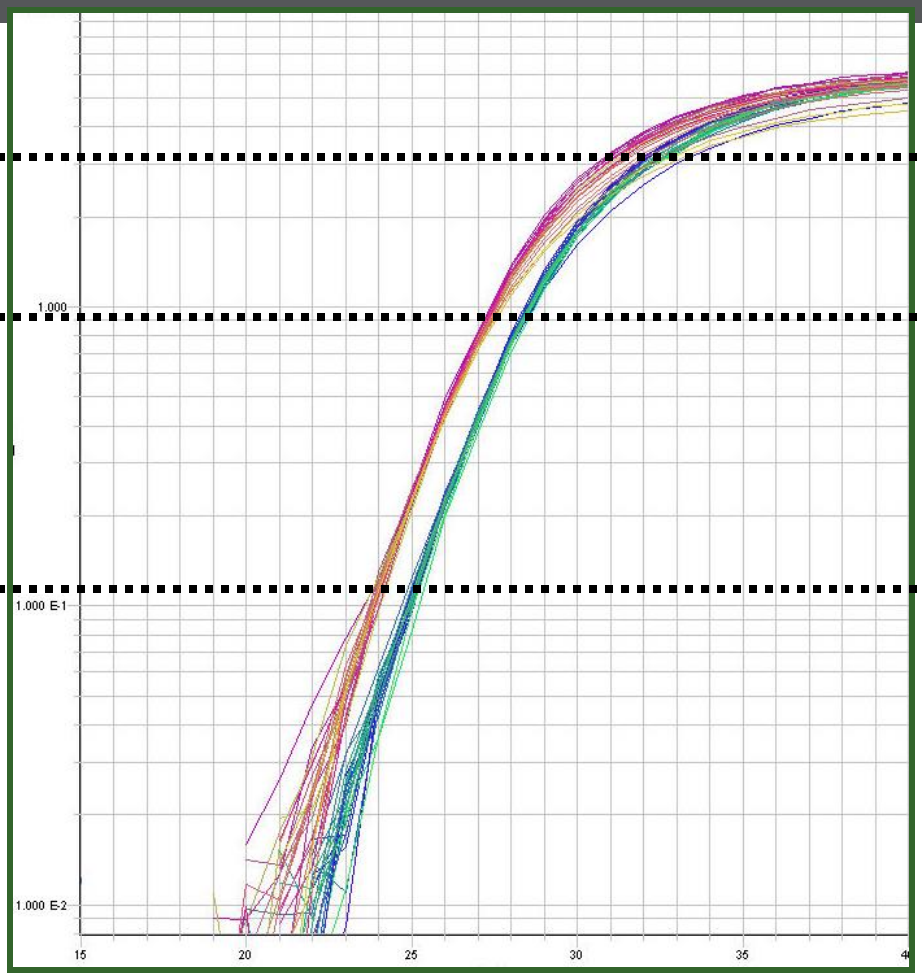
What is efficiency?

PCR product theoretically doubles after each cycle in exponential phase



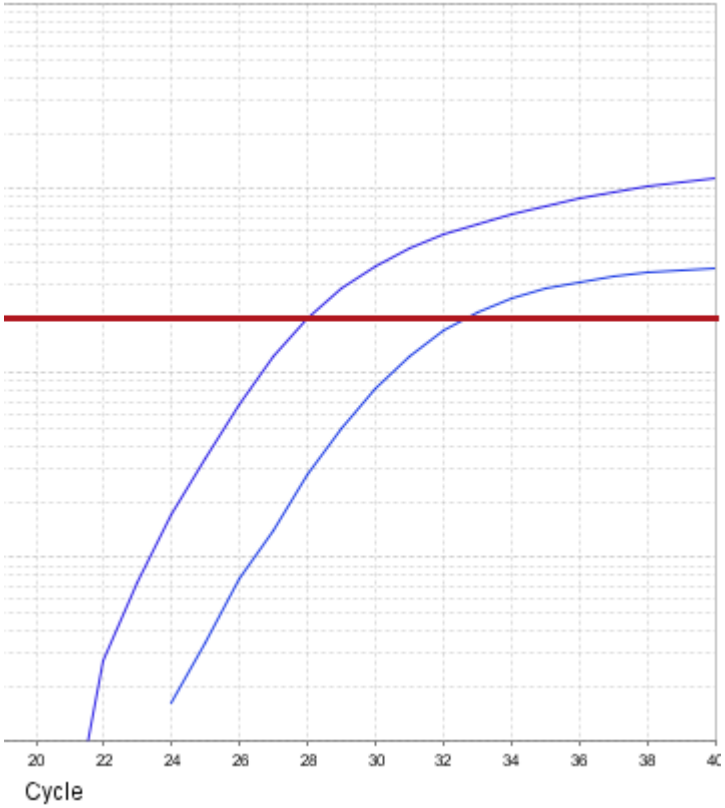
Plateau phase
<100% efficiency

Linear phase
<100% efficiency

Exponential phase
100% efficiency (theoretical)

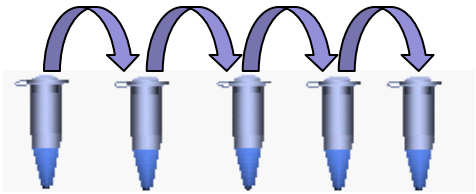
Important message

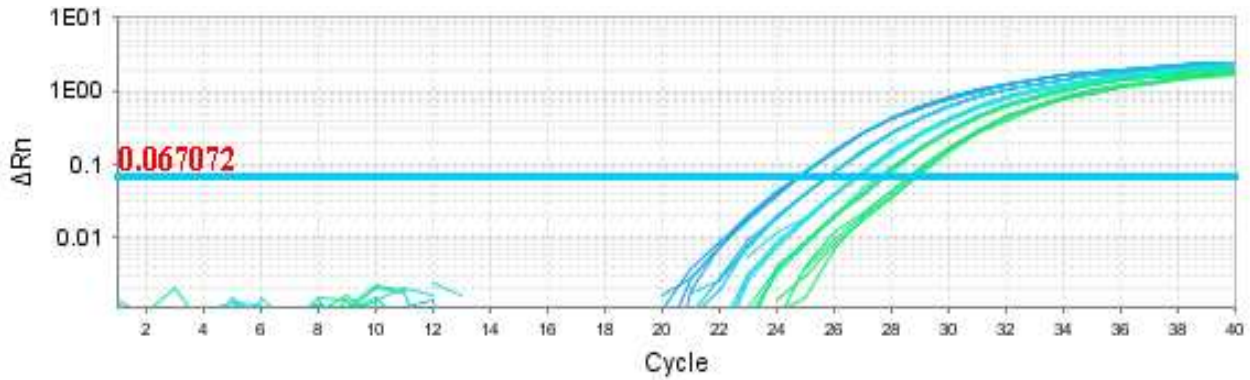
- NEVER trust Cts or quantities for samples whose geometric phases are not parallel with the other samples and standards for that same assay!



How is efficiency measured?

Serial dilutions





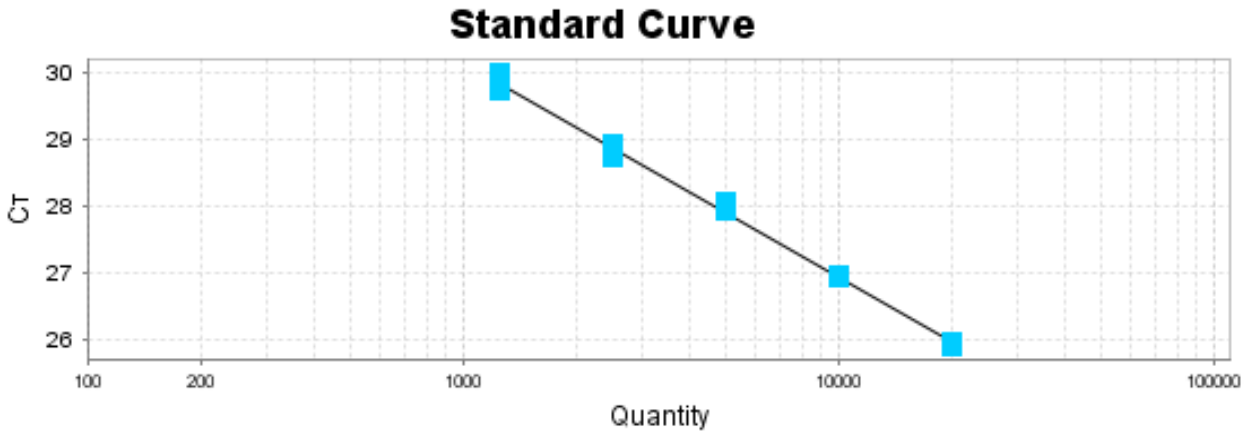
Efficiency = $10^{(-1/slope)}$-1

Ideally, slope = -3.32

$E = 10^{(-1/-3.32)}-1$

$E = 10^{(0.301)}-1$

$E = 2-1 = 1 \rightarrow 100\%$



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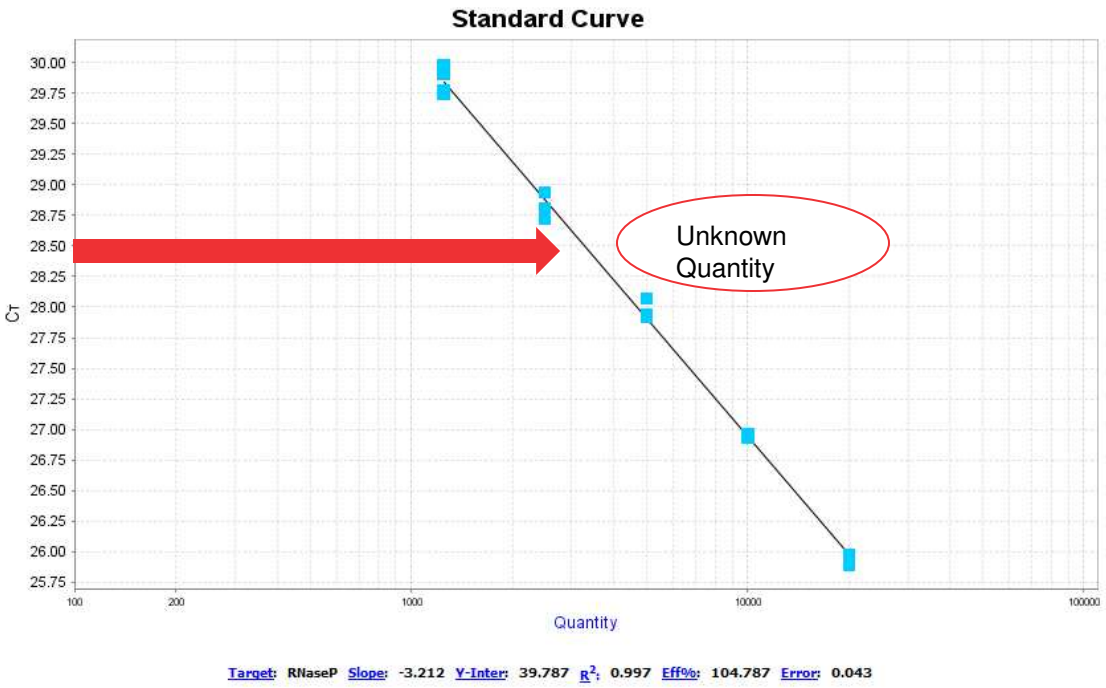
Standard Curve Plot Features

- **Slope** should be between -3.58 and -3.10 (corresponding to E = 100% ±10%)
- Ideally, **R^2** ≥ 0.99 (R^2 suggests poor pipetting of standards)
- **Y-intercept** is the theoretical limit of detection of reaction
- **Error** provides the standard error of the slope



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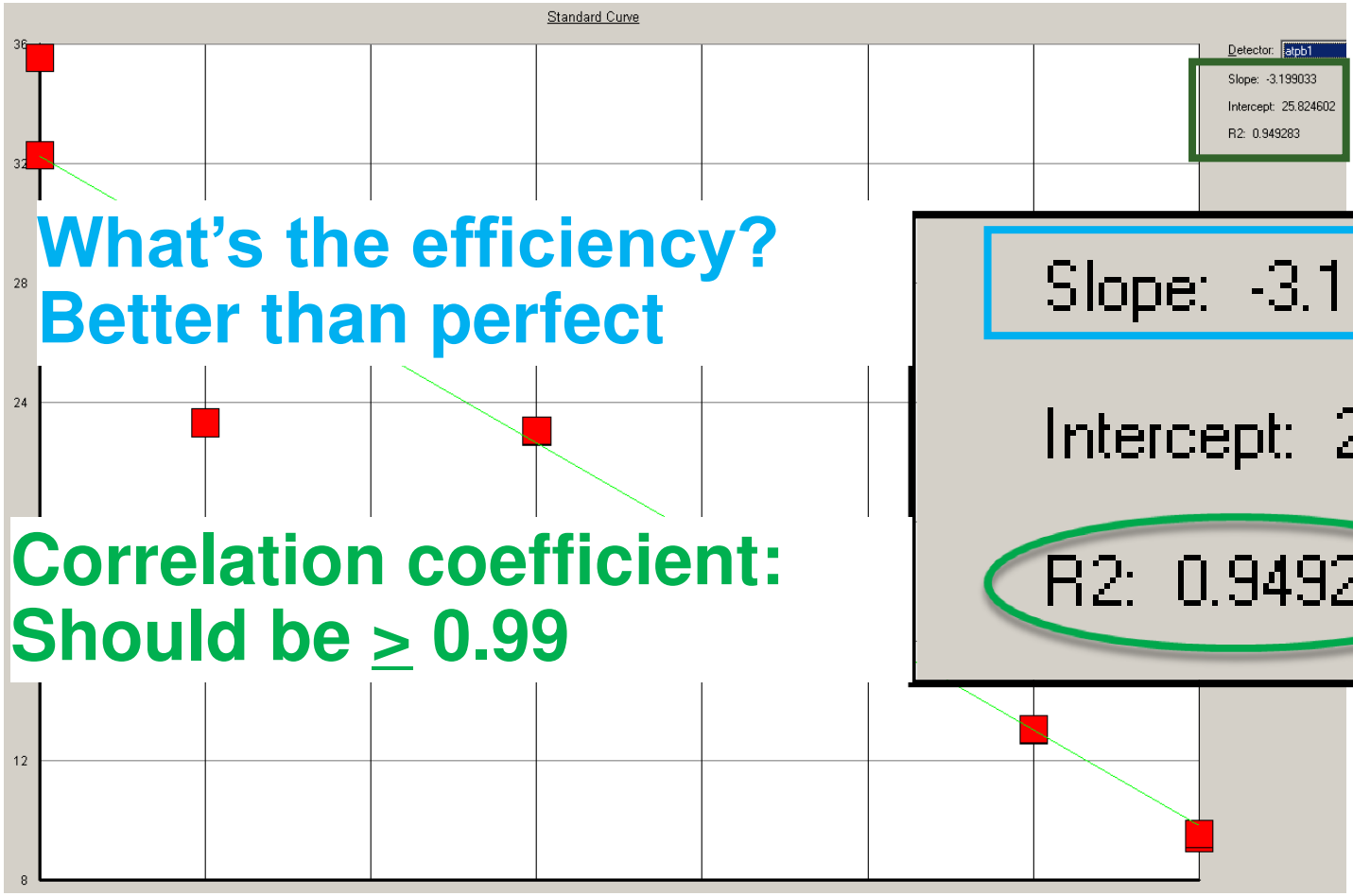
Relationship for calculating relative starting amounts

2^n = fold change, where n is number of cycles

1 cycle	=	2-fold difference
2 cycles	=	4-fold difference
3 cycles	=	8-fold difference
3.32 cycles	=	10-fold difference

Dilution curve with outliers



What's the efficiency?
Better than perfect

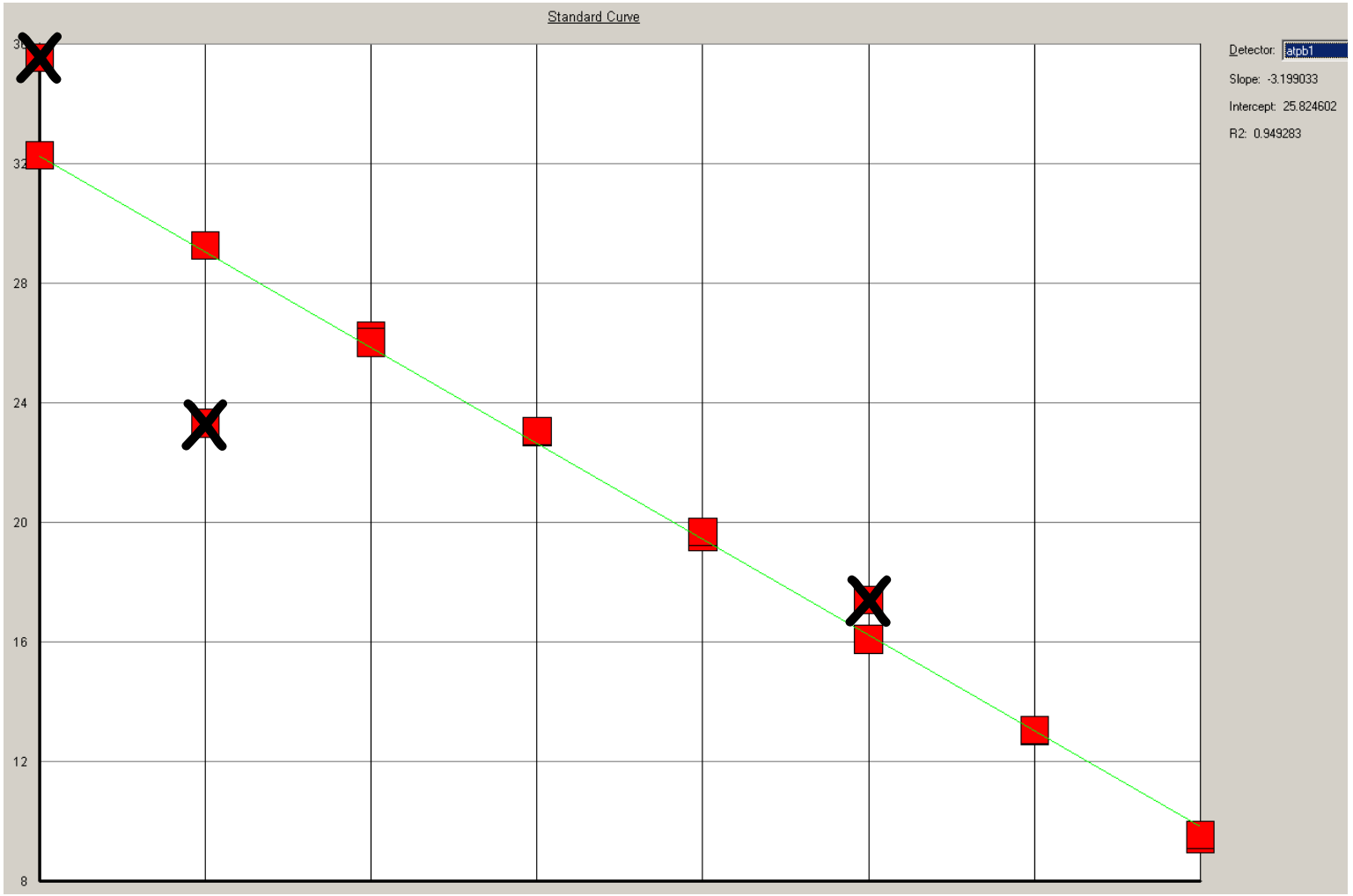
Correlation coefficient:
Should be ≥ 0.99

Slope: -3.199033

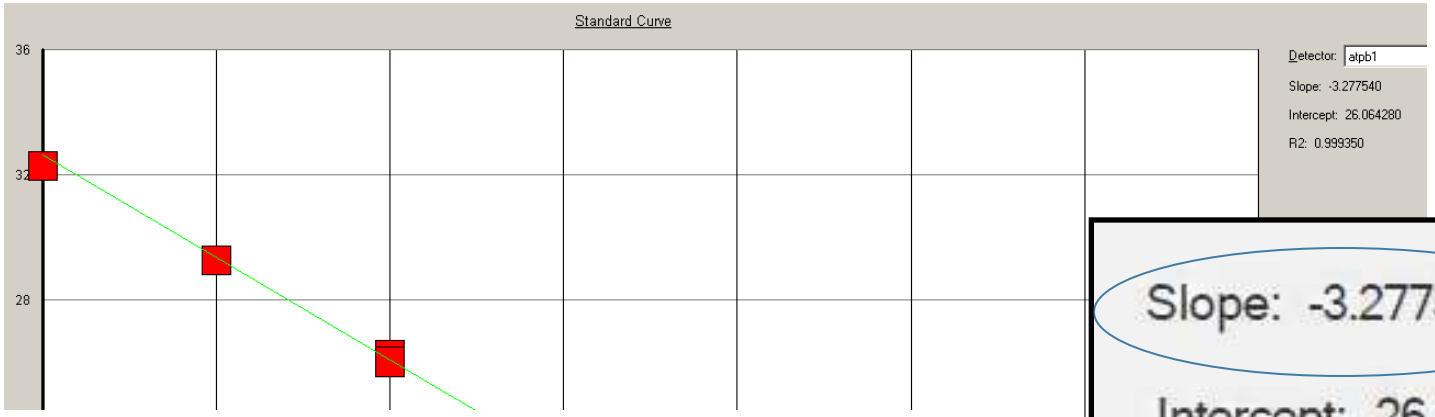
Intercept: 25.824602

R2: 0.949283

Omit outliers



Removal of outliers

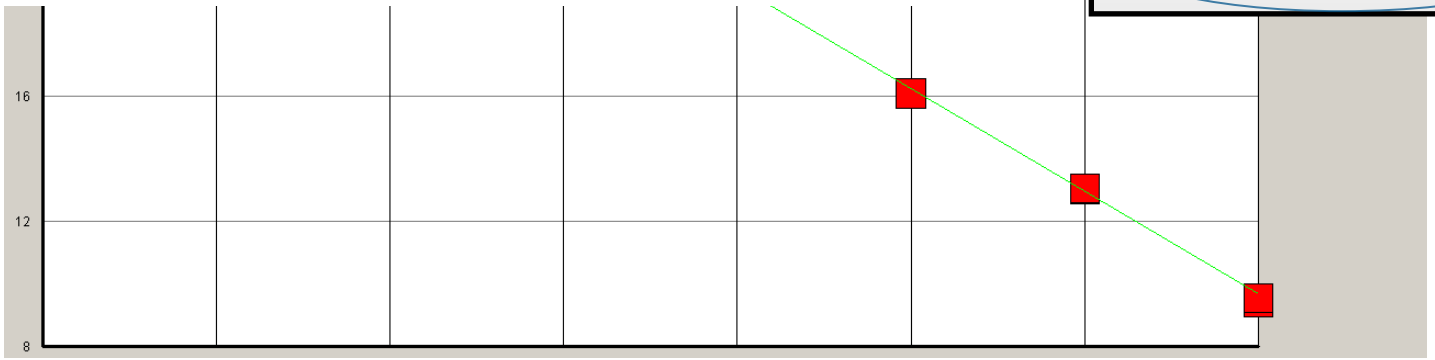


Slope: -3.2775040

Intercept: 26.06428

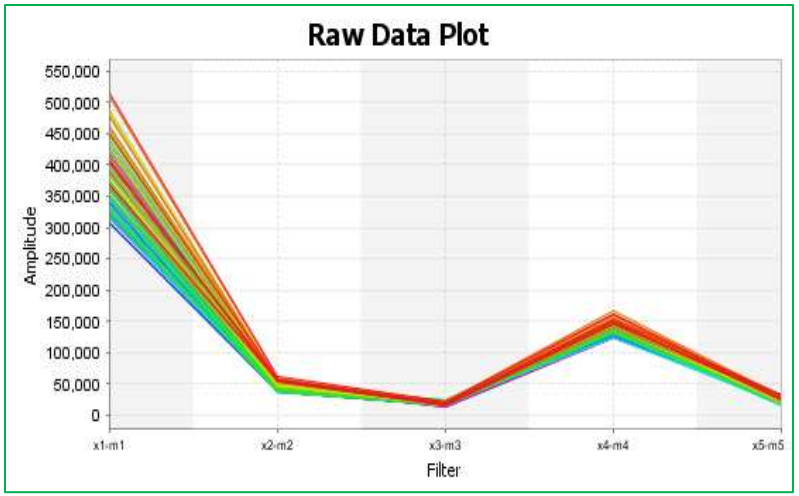
R2: 0.998746

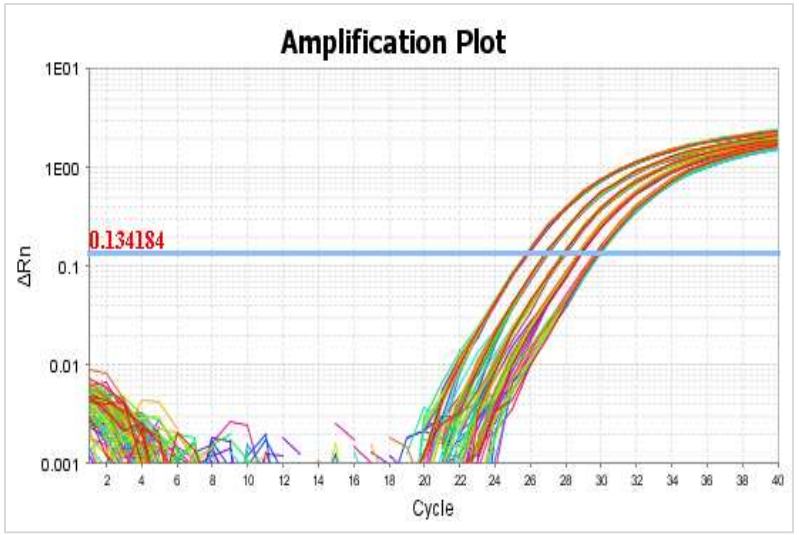
Correlation and slope now good



From Raw Data to Ct – System Normalizations

Raw data (Fluorescence)





Ct

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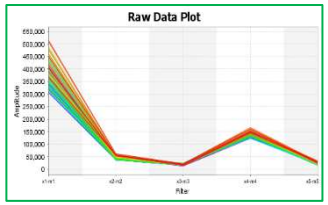
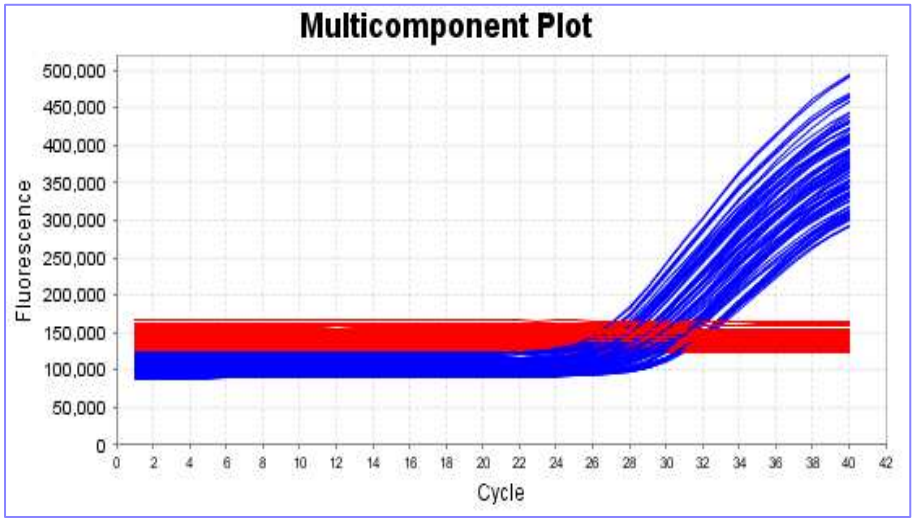
From Raw Data to Ct – System Normalizations

Raw data (Fluorescence)



Multicomponent (Fluorescence)



The multicomponenting algorithm incorporates **pure dye calibrations** as well as **Background** calibration

Ct

From Raw Data to Ct – System Normalizations

Raw data (Fluorescence)

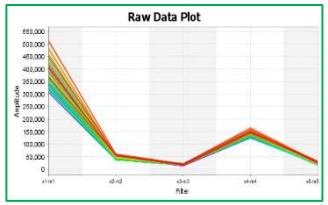
↓

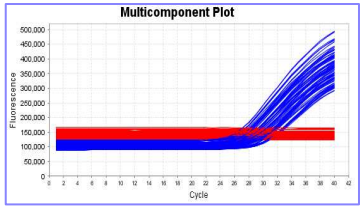
Multicomponent (Fluorescence)

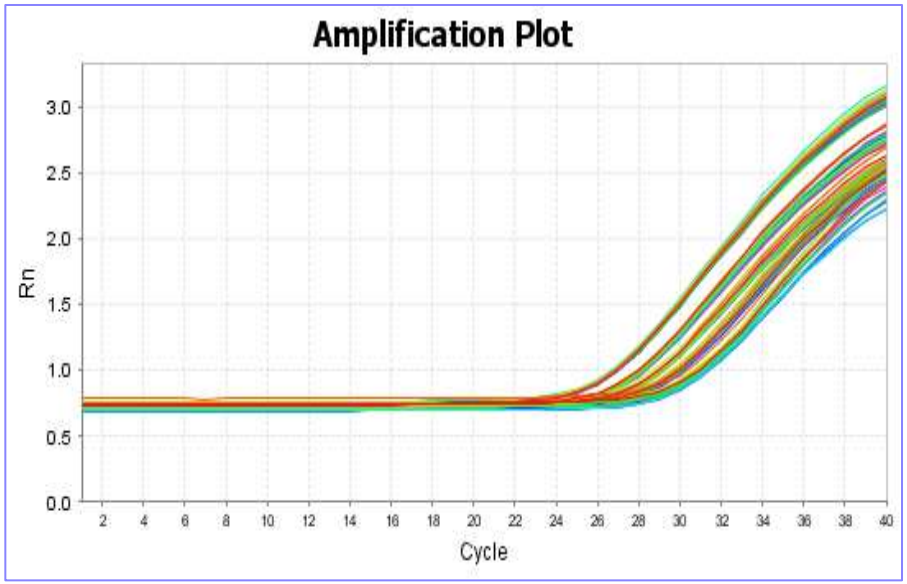
↓

Passive Reference Dye normalization (Rn)

Ct



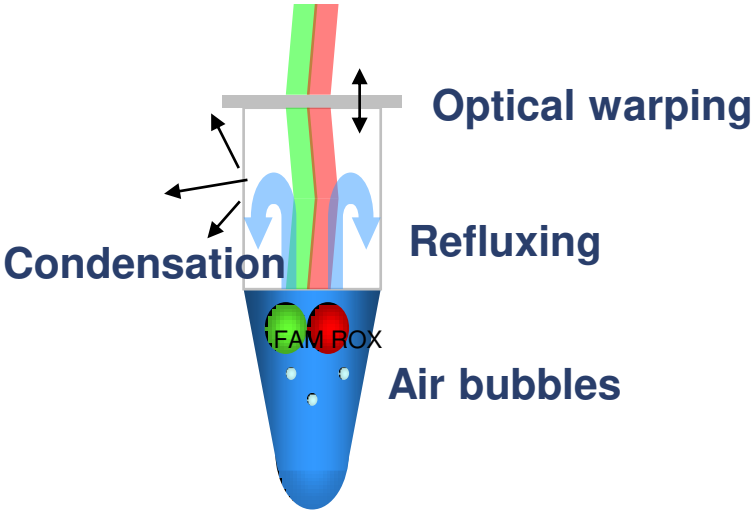




Passive reference dye normalization accounts for **non-PCR** related fluctuations in fluorescence

Common Sources of Fluorescent Variation

- Heating of the reaction to 95°C each cycle causes phenomena such as water vapor refluxing and air bubble formation.
- These phenomena are in the light path and create fluctuations in the emissions.
- The Reporter and ROX™ dye are affected to the same degree, so that normalization to ROX™ dye mathematically corrects the fluctuations.

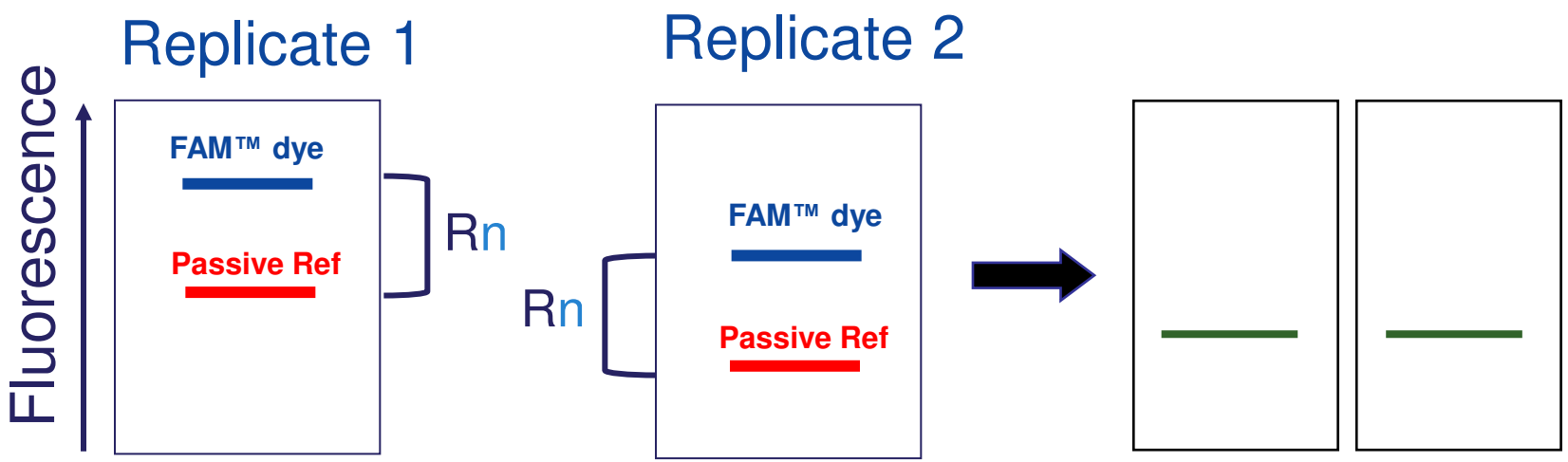


ROX™

ALL real-time PCR master mixes from ABI contain ROX™ passive reference dye.

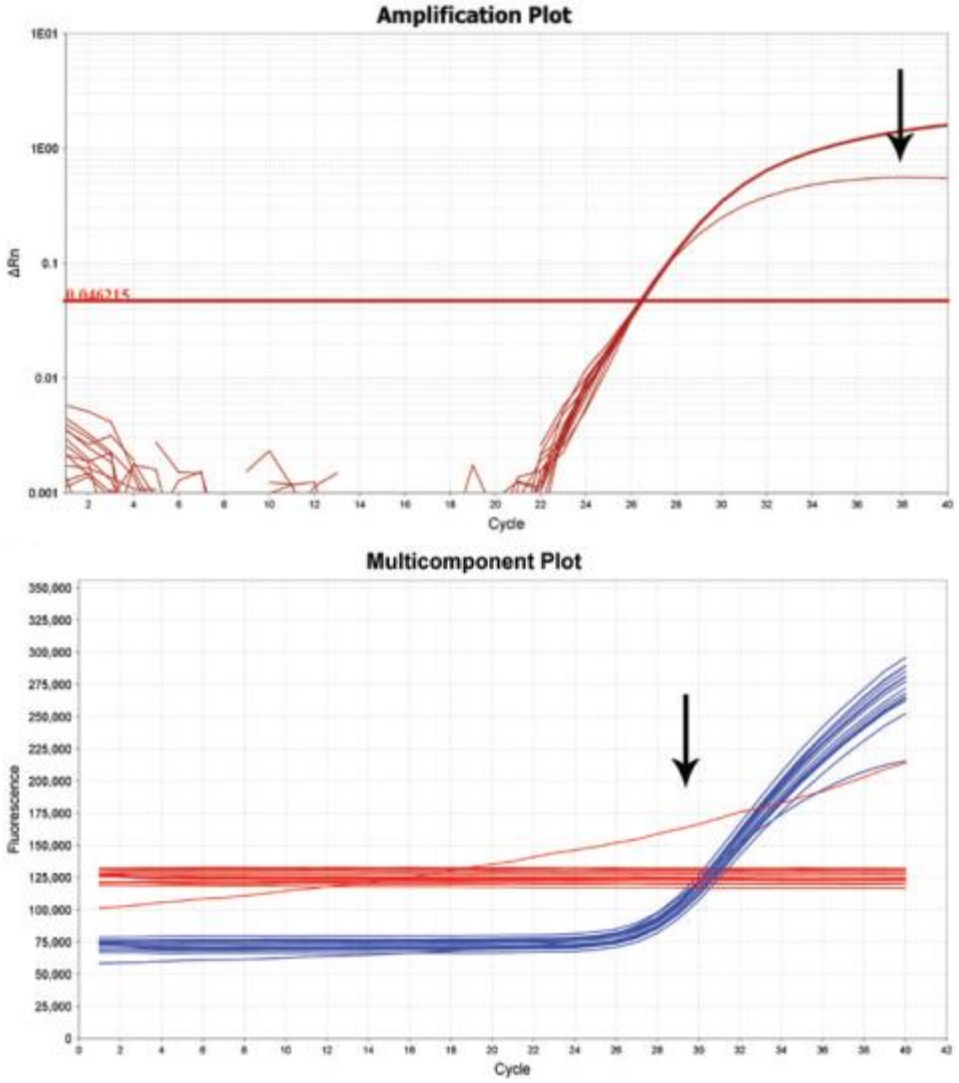
Passive Reference

- Small fluorescent fluctuations can occur from well-to-well
- Passive reference dye (ROX™) normalizes for **non-PCR related** fluorescence variations



Normalized reporter (Rn) = Reporter / Passive Reference

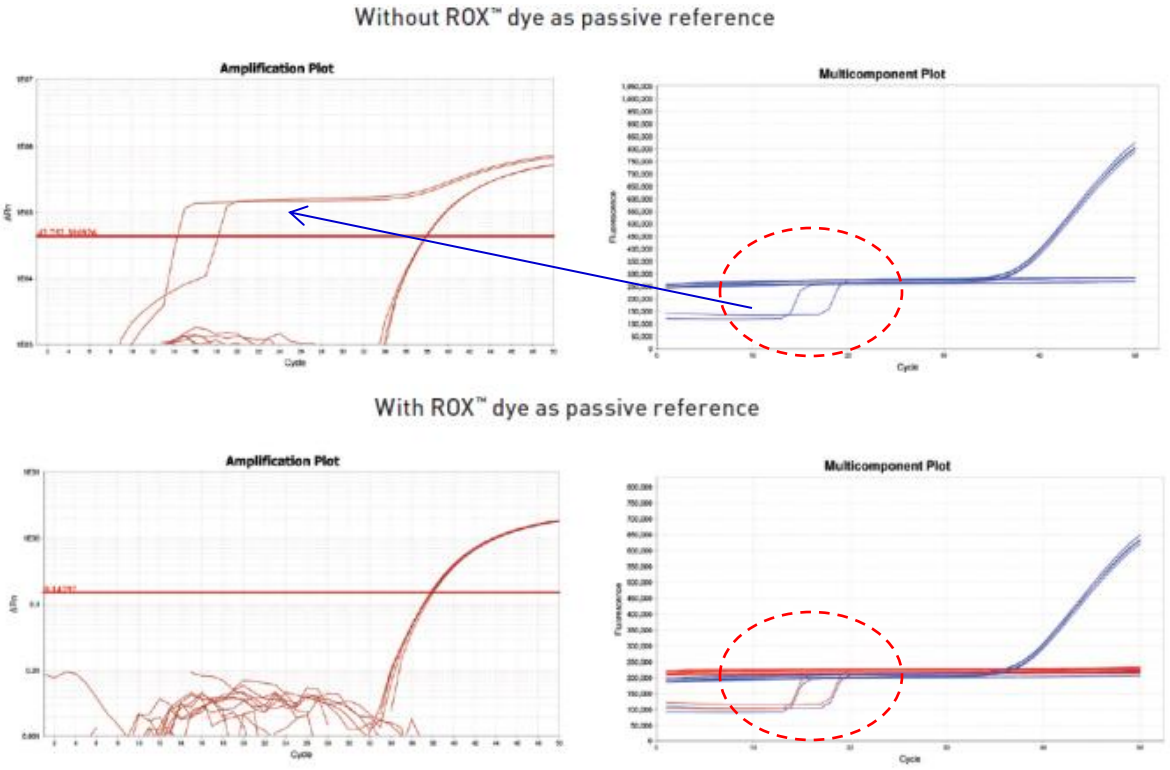
Troubleshooting Errors using ROX Dye



ROX dye makes it easier to distinguish between PCR-related and non-PCR-related events, such as evaporation in a well.

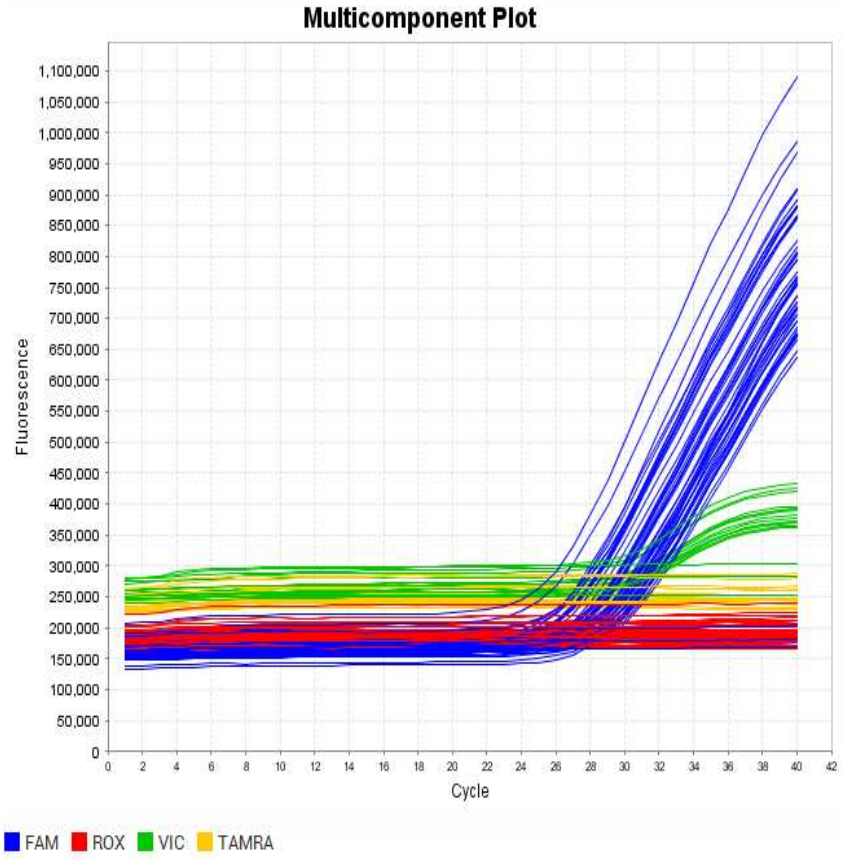
Atypical Optical Fluctuations

- Atypical optical fluctuations are those that occur only occasionally.
- The anomaly in this example could have been caused by a bubble bursting or shift in the adhesive film.

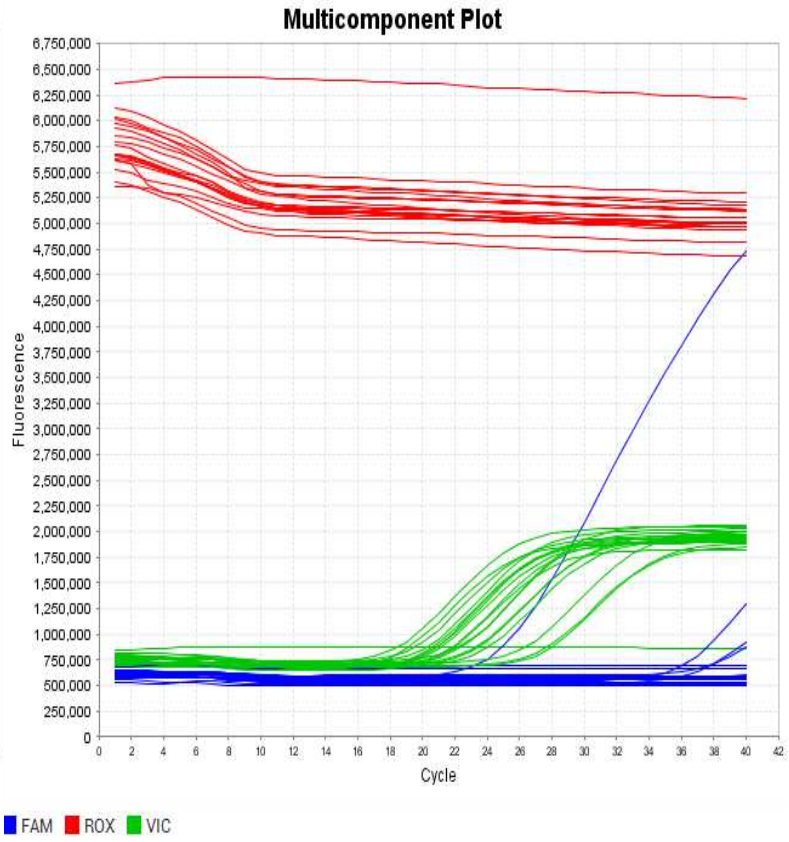


Example of Master Mix with High ROX

Normal ROX signals.



High ROX signals.



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From Raw Data to Ct – System Normalizations

Raw data (Fluorescence)

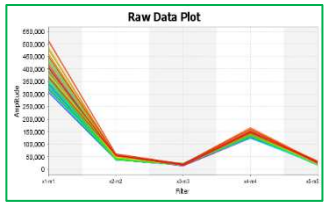
↓

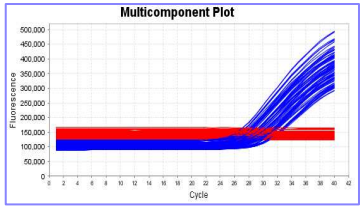
Multicomponent (Fluorescence)

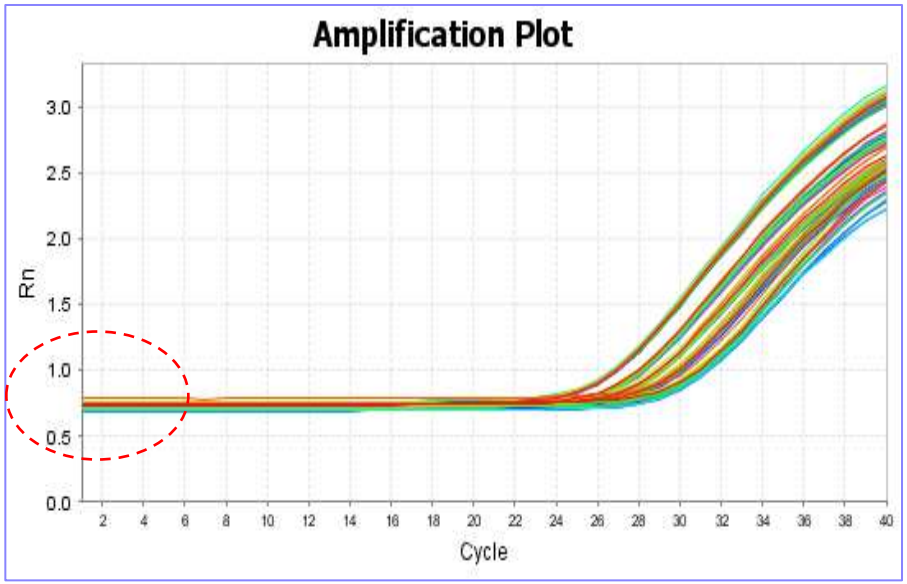
↓

Passive Reference Dye normalization (Rn)

Ct







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

From Raw Data to Ct – System Normalizations

Raw data (Fluorescence)

↓

Multicomponent (Fluorescence)

↓

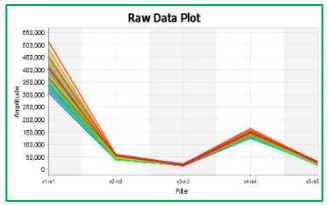
Passive Reference Dye normalization (Rn)

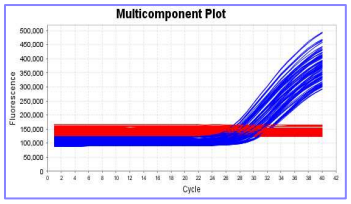
↓

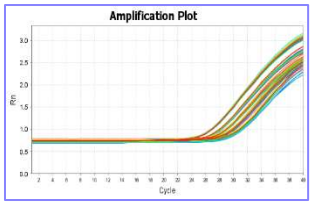
Baseline normalization (ΔRn)

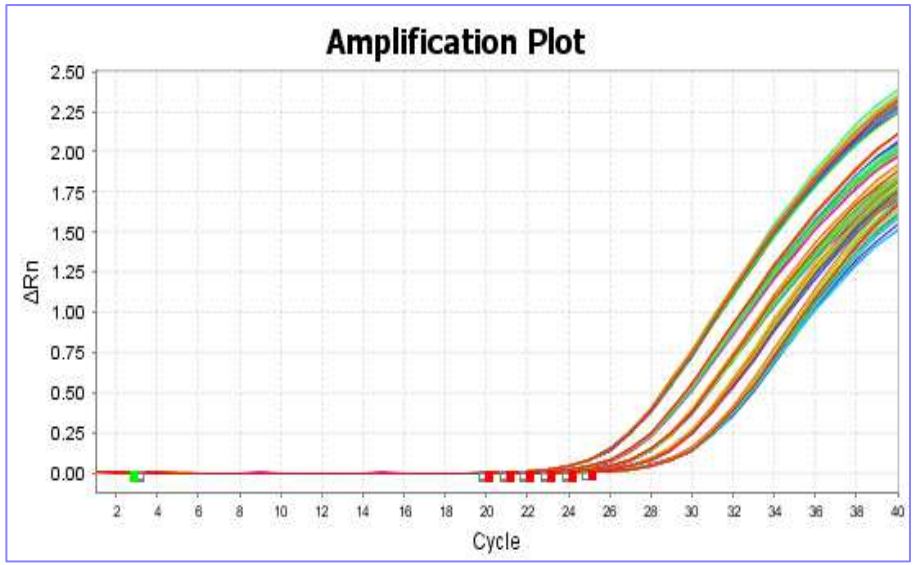
automatic or manual

Ct



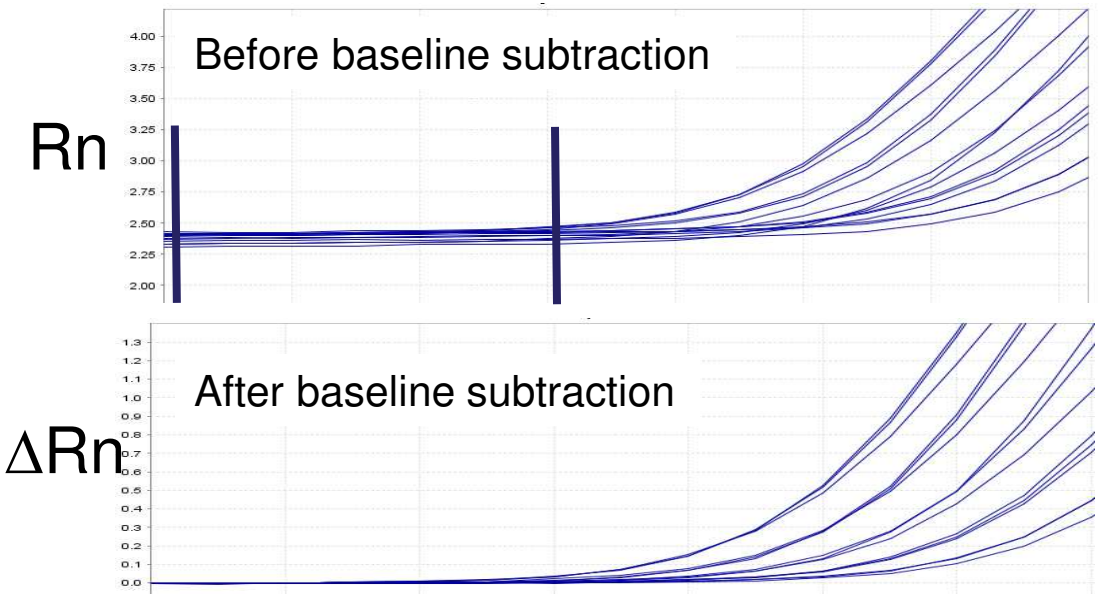






Baseline normalization accounts for the different background of each well

Importance of Baseline



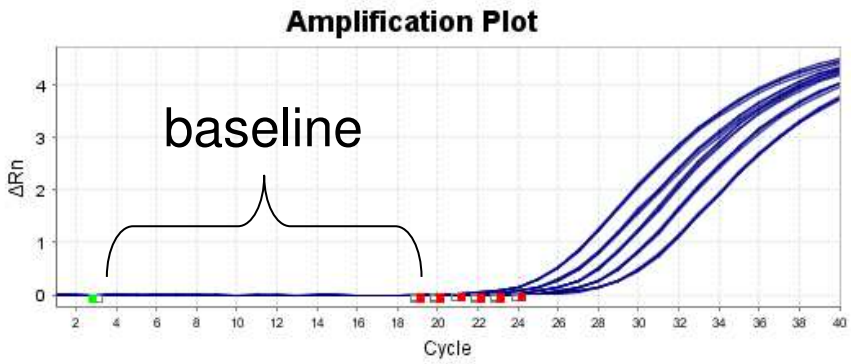
Baseline subtracted normalized reporter (ΔRn) = Rn - Bn

- Once the baseline range is established, each sample's starting point is set to "0" on the Y-axis (fluorescence).
- The effect is that all samples start from the same point, thus improving accuracy and precision.

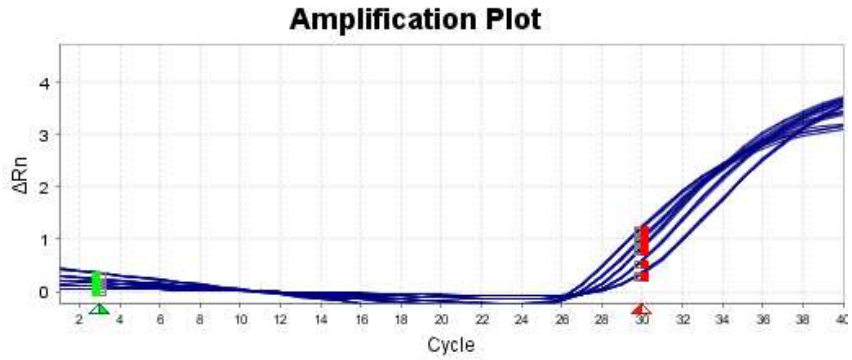
Setting Baseline

- Evaluate the baseline, which should be flat. If tilted, the baseline needs adjustment.
- The baseline may be set automatically or manually. Manual baseline defaults to 3-15, but is adjustable.

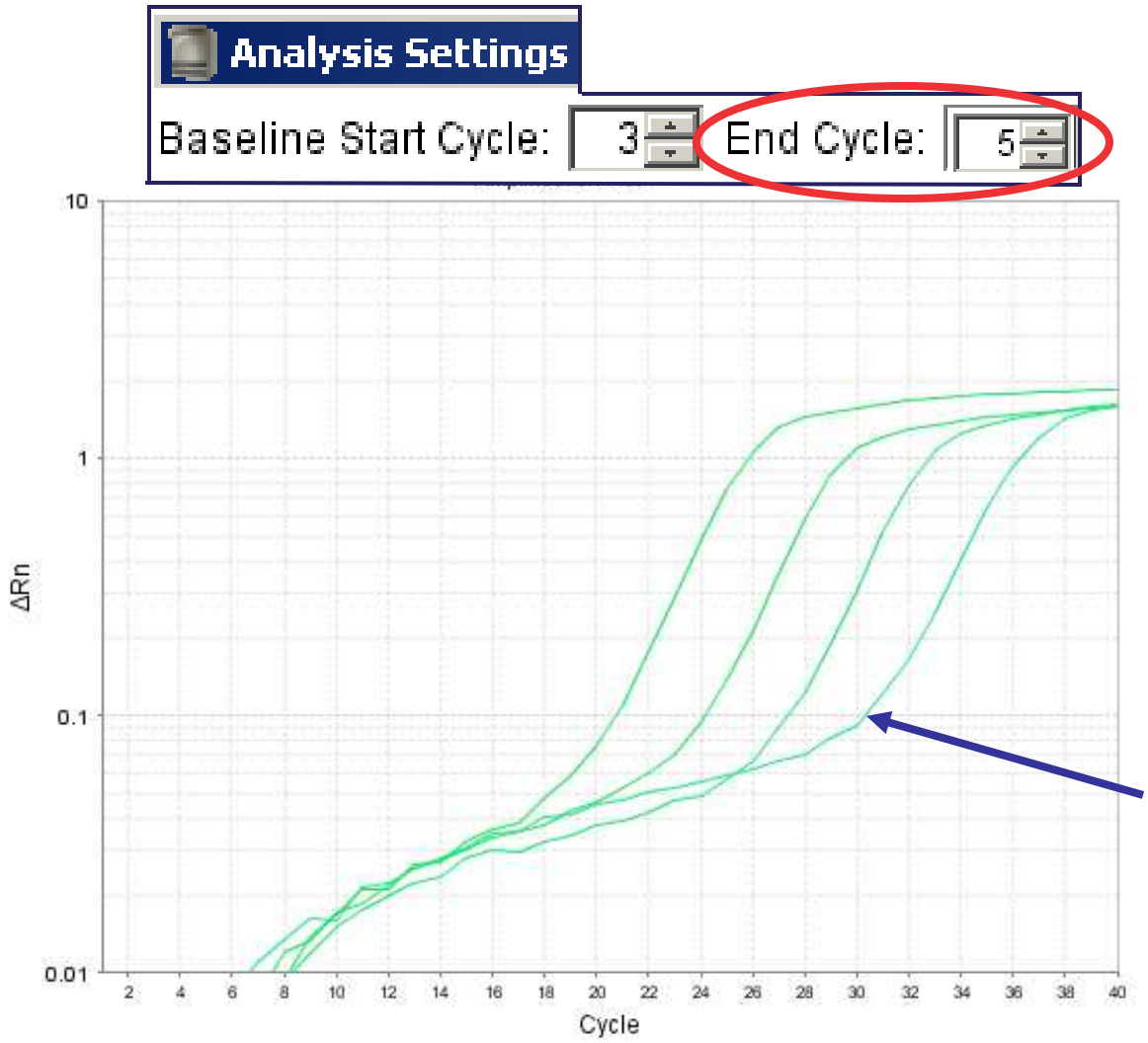
Good baseline setting



Poor baseline setting

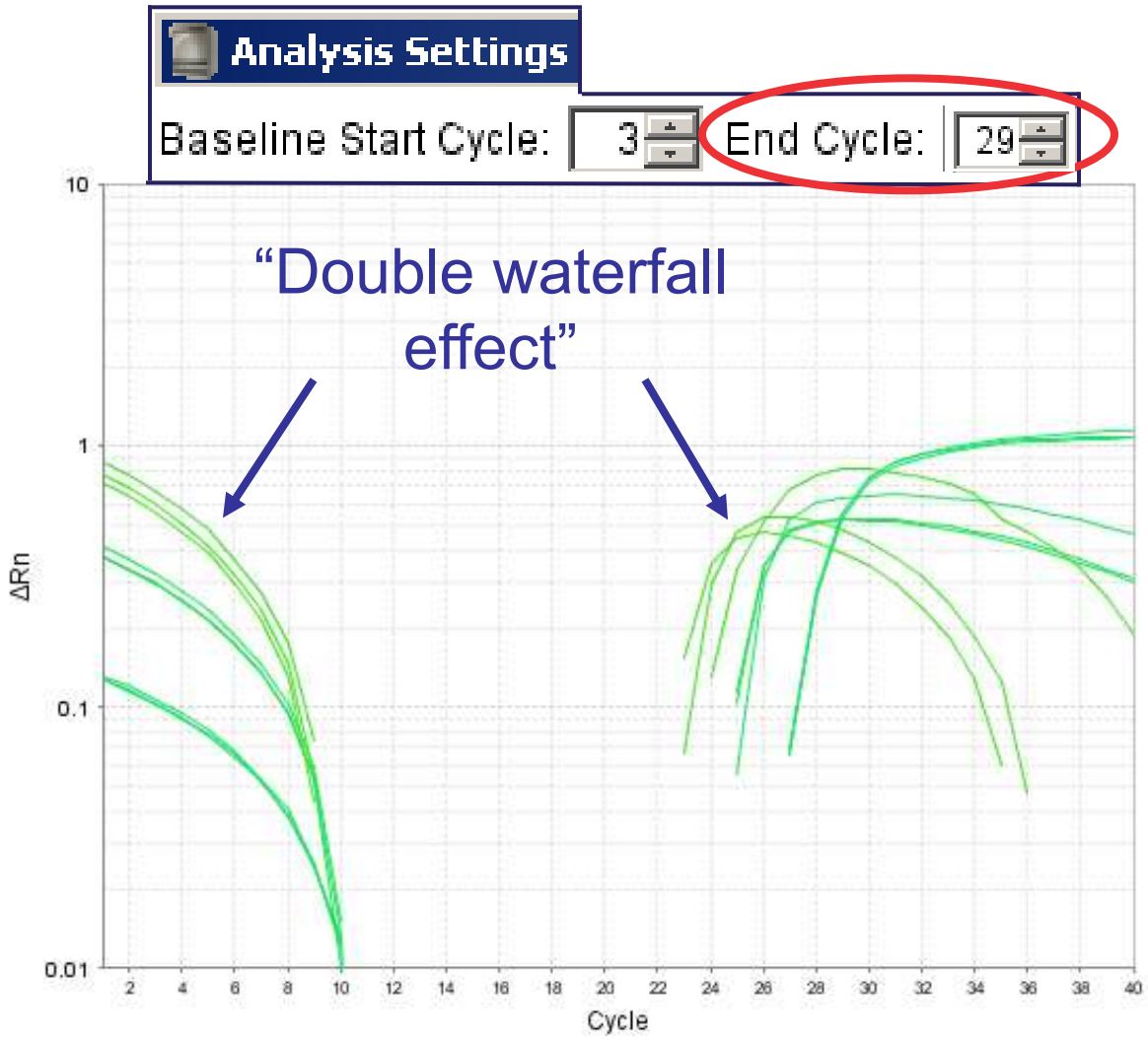


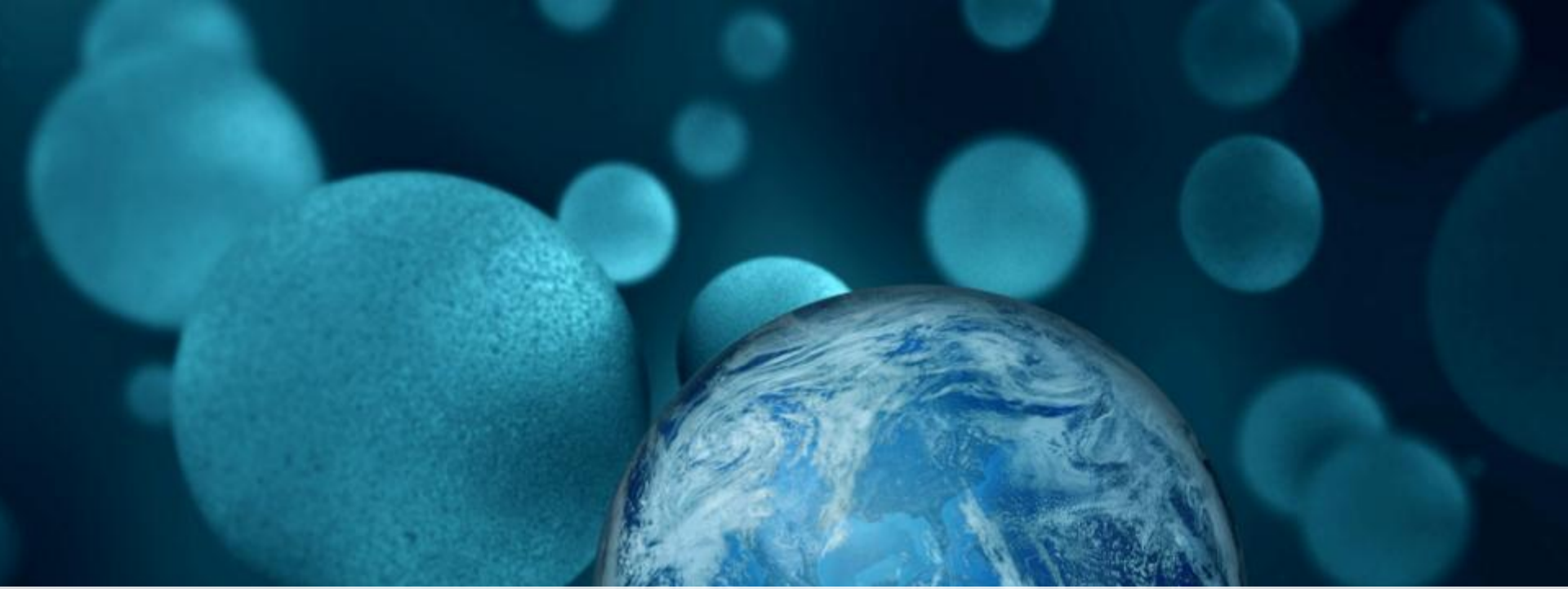
What if baseline is set too low?



Sigmoidal shape

What if baseline is set too high?





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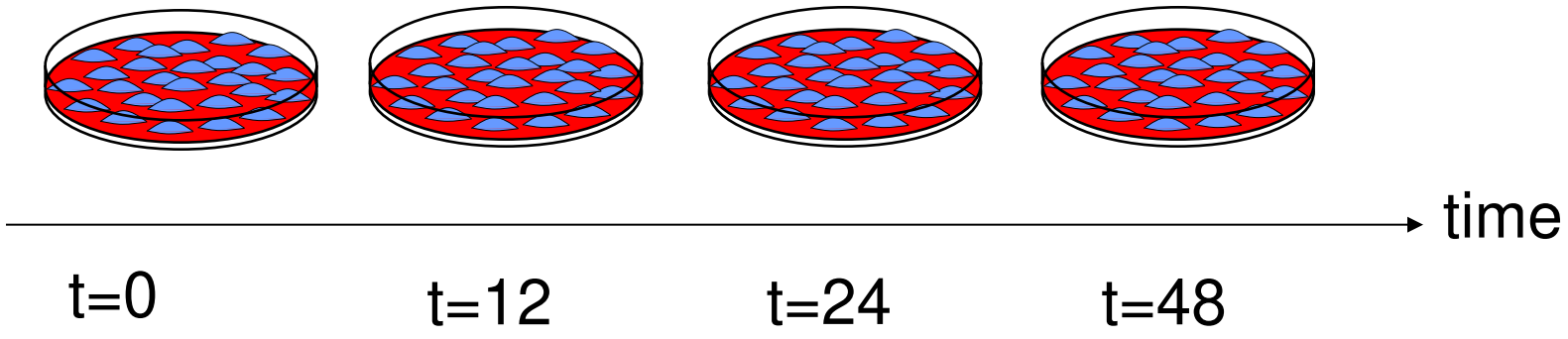
Gene Expression

Note: For Research Use Only. Not for use in diagnostic procedures.

The world leader in serving science

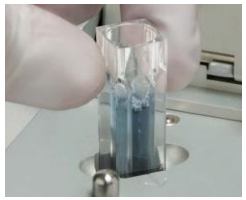
Example experiment

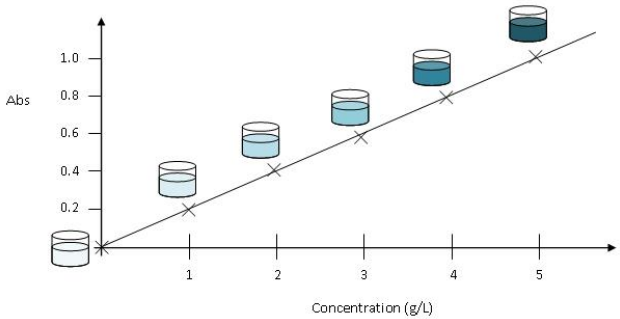
- We decide to test the difference in expression levels of the *STMN1* gene in cells treated with a certain drug over time → *STMN1* is our **target**
- We are using β-actin as our **endogenous control**
- Treated samples will be compared to our cells at 0 h (**reference** sample)



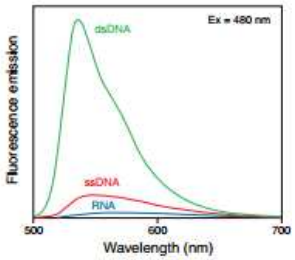
Comparison of quantitation methods

- Spectrophotometer
  - Measure absorbance at 260 nm vs. standard curve
    - Pro: Simple, economical.
    - Con: Not specific, DNA/RNA/Protein all absorb in this range





- Fluorometer
  - Measures fluorescence when a dye binds to specific molecules in a sample
    - Pro: Dyes are specific to each molecule type, very sensitive.
    - Con: More expensive, no data on other possible contaminant molecules in background.

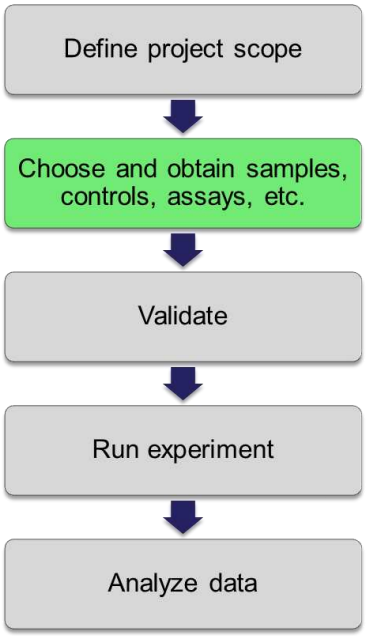


Comparison of quantitation methods

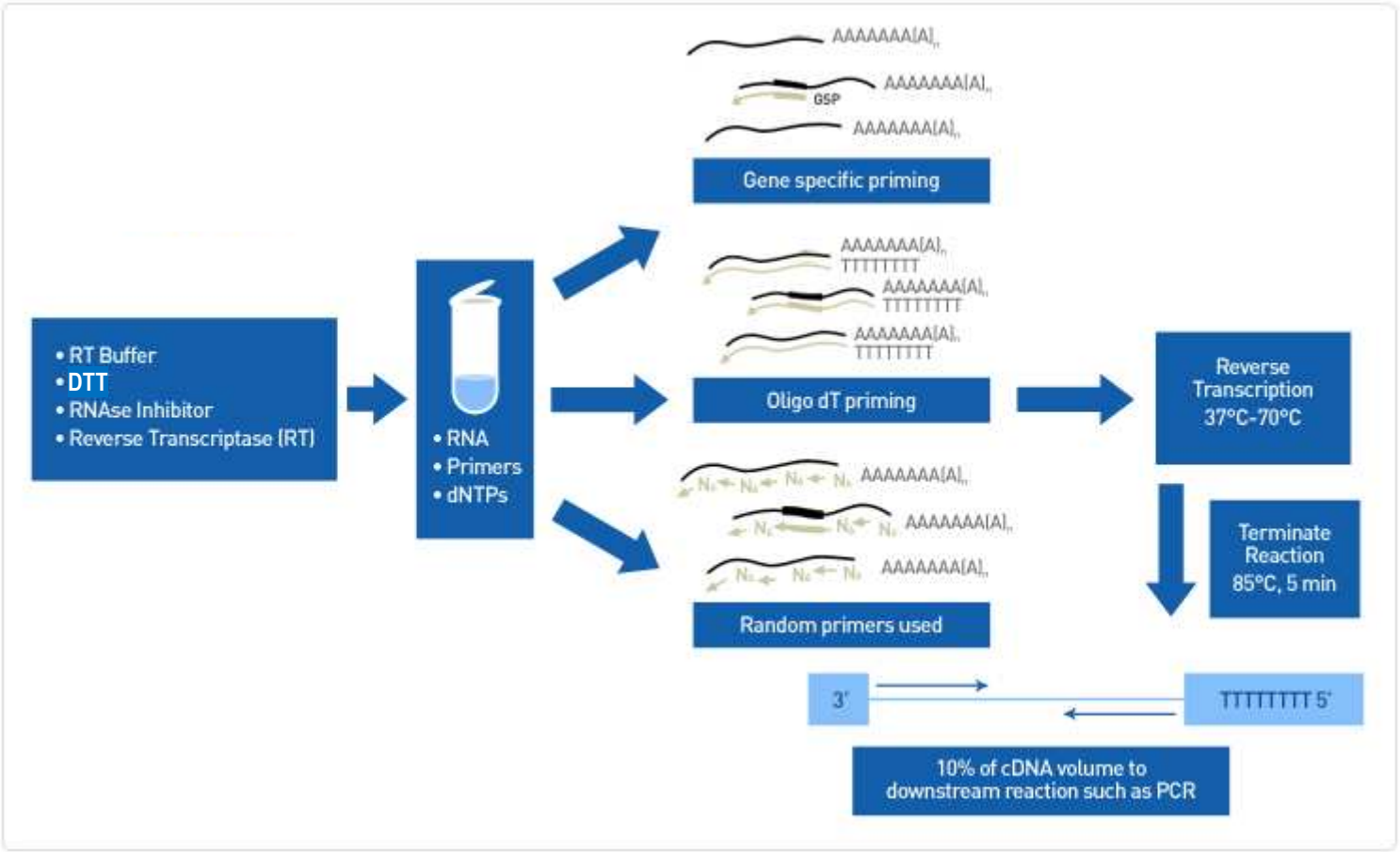
	Fluorometer	Spectrophotometer	Comparison to Previous Standard Curve
Quantification method	Fluorescence-based dyes that bind specifically to DNA, RNA, or protein	UV absorbance measurements (measures absorbance at 260 nm and 260 nm/280 nm ratio)	Comparison to previously generated standard curve data
Selectivity for DNA or RNA	Accurately measure both DNA and RNA in the same sample	Results for samples containing both DNA and RNA are nondiscriminatory—you cannot distinguish one from the other	Selectivity for DNA
Accuracy and precision at low concentrations	Accurately quantifies DNA in samples with concentrations as low as 10 pg/µL	Not recommended for concentrations under 2 ng/µL; variation for sample concentrations <10 ng/µL is often high	Dependent on dynamic range of previous standard curve
Sensitivity and range	The effective range covers a sample concentration range of 10 pg/µL to 1 µg/µL DNA	Covers a sample concentration range of 2 ng/µL to 15 µg/µL; uses 0.5–2 µL of sample	Dependent on dynamic range of previous standard curve
Can indicate contamination	No	Gives peaks revealing the presence of contaminants	No

Choices in Experimental Design

- Sample and preparation method
- Chemistry
  - Reverse transcription (RT): One-step or two-step
  - Real-Time PCR: TaqMan Assay or SYBR Green I Dye
  - Real-Time PCR: Master Mix



Review of Reverse Transcription



https://www.thermofisher.com/us/en/home/life-science/pcr/elevate-pcr-research/reverse-transcription-essentials.html

One-step vs. two-step RT-PCR

One-step RT-PCR

Isolate RNA

↓

Add RNA directly to qPCR sample plate

↓

Add master mix containing both reverse transcriptase and polymerase

↓

Reaction undergoes RT, immediately followed by PCR

Advantage: less pipetting, decreased risk of contamination
Disadvantage: repeated freezing and thawing of samples can degrade RNA (need small aliquots)

Two-step RT-PCR

Isolate RNA

↓

Convert RNA into cDNA via a reverse transcription reaction

↓

Dilute cDNAs 1:2–1:10

↓

Add to sample plate and amplify cDNA by qPCR

Advantage: cDNA far more stable than RNA – can go through more freeze-thaw cycles
Disadvantage: extra pipetting steps, bigger risk of contamination

How do they differ?

Comparative Ct (ΔΔCt) Method

- No standard curves for each experiment
- Easier, cheaper, higher throughput

Relative Standard Curve Method

- Run standard curves with each gene in each experiment
- More work, costlier, lower throughput

One big requirement of ΔΔCt

Your two genes (target and normalizer) must have approximately the *same amplification efficiencies!*

ΔΔCt is generally the method of choice

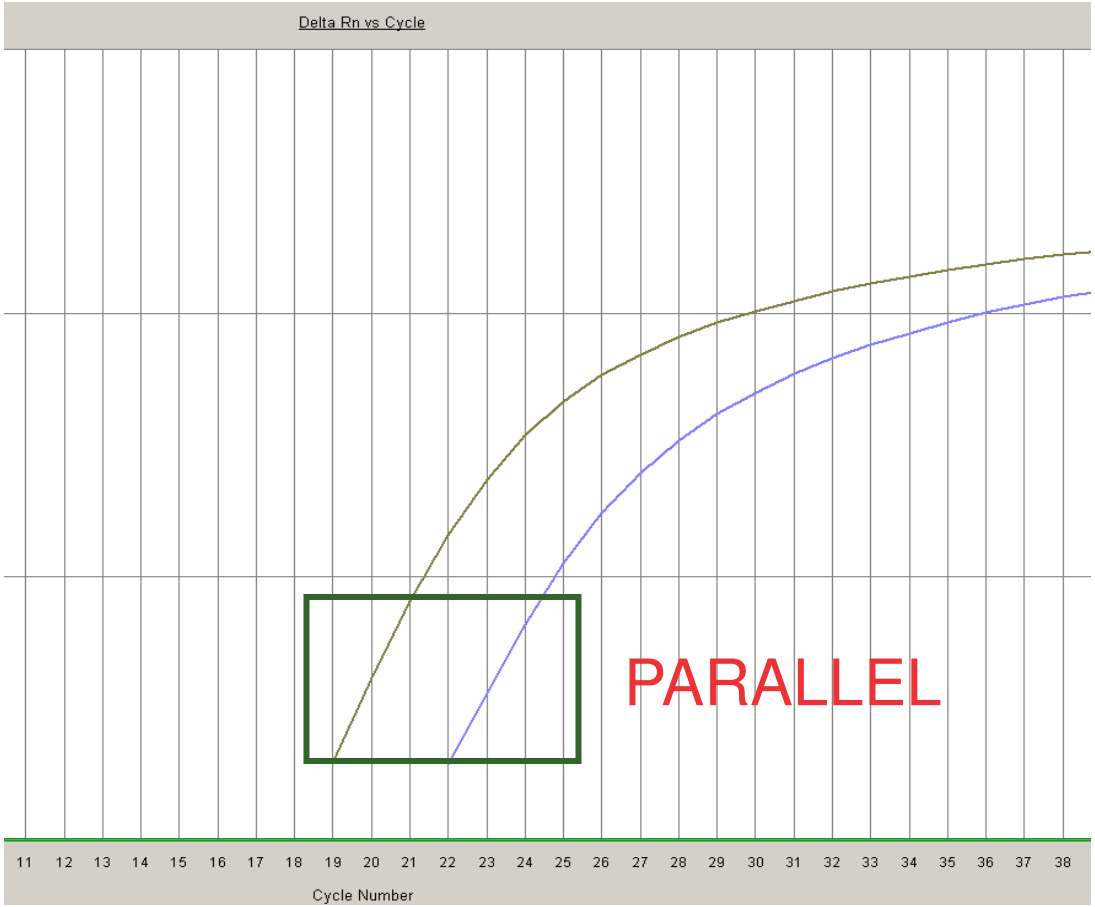
Comparative Ct (ΔΔCt) Method

- No standard curves for each experiment
- Easier, cheaper, higher throughput

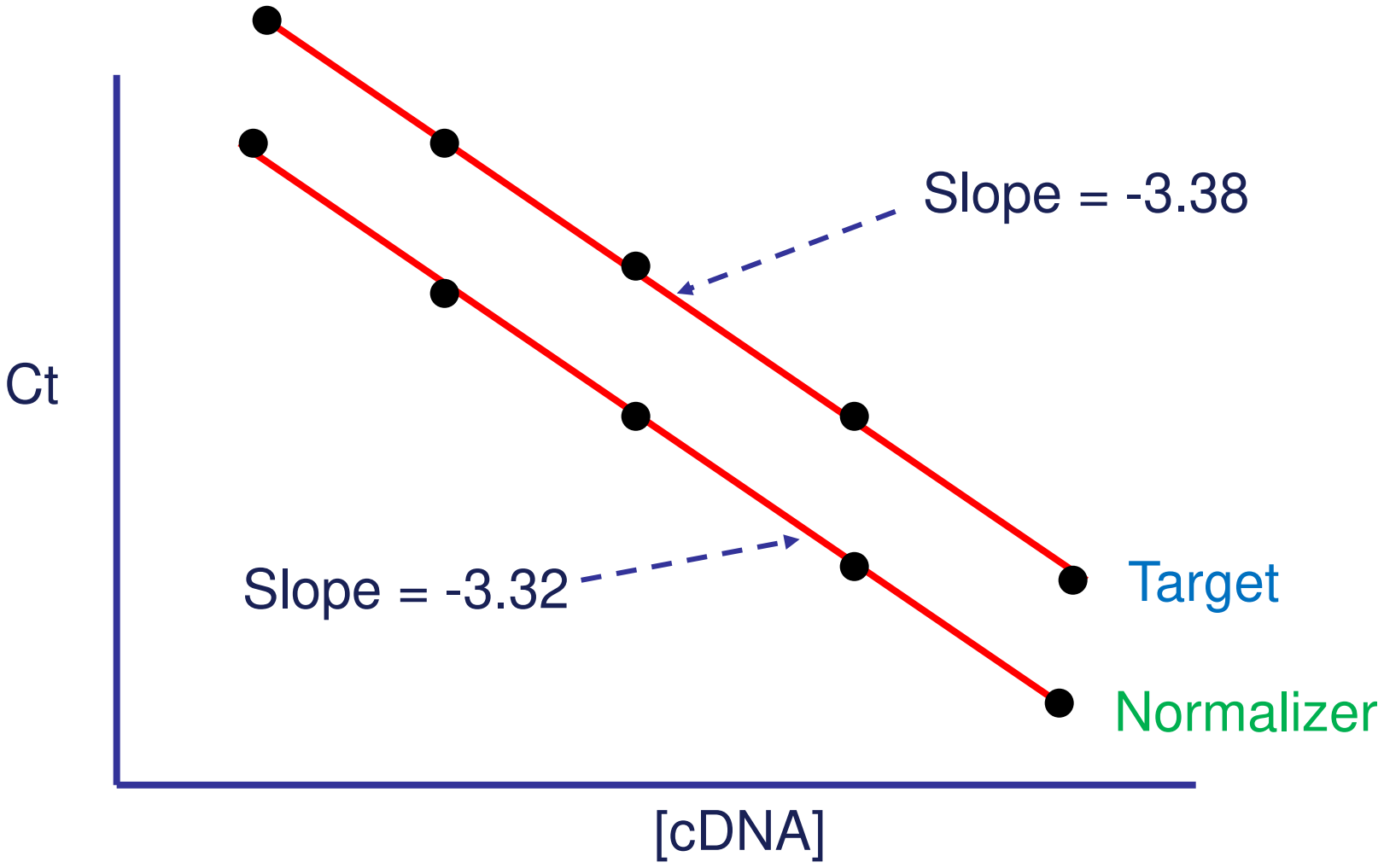
Relative Standard Curve Method

- Run standard curves with each gene in each experiment
- More work, costlier, lower throughput

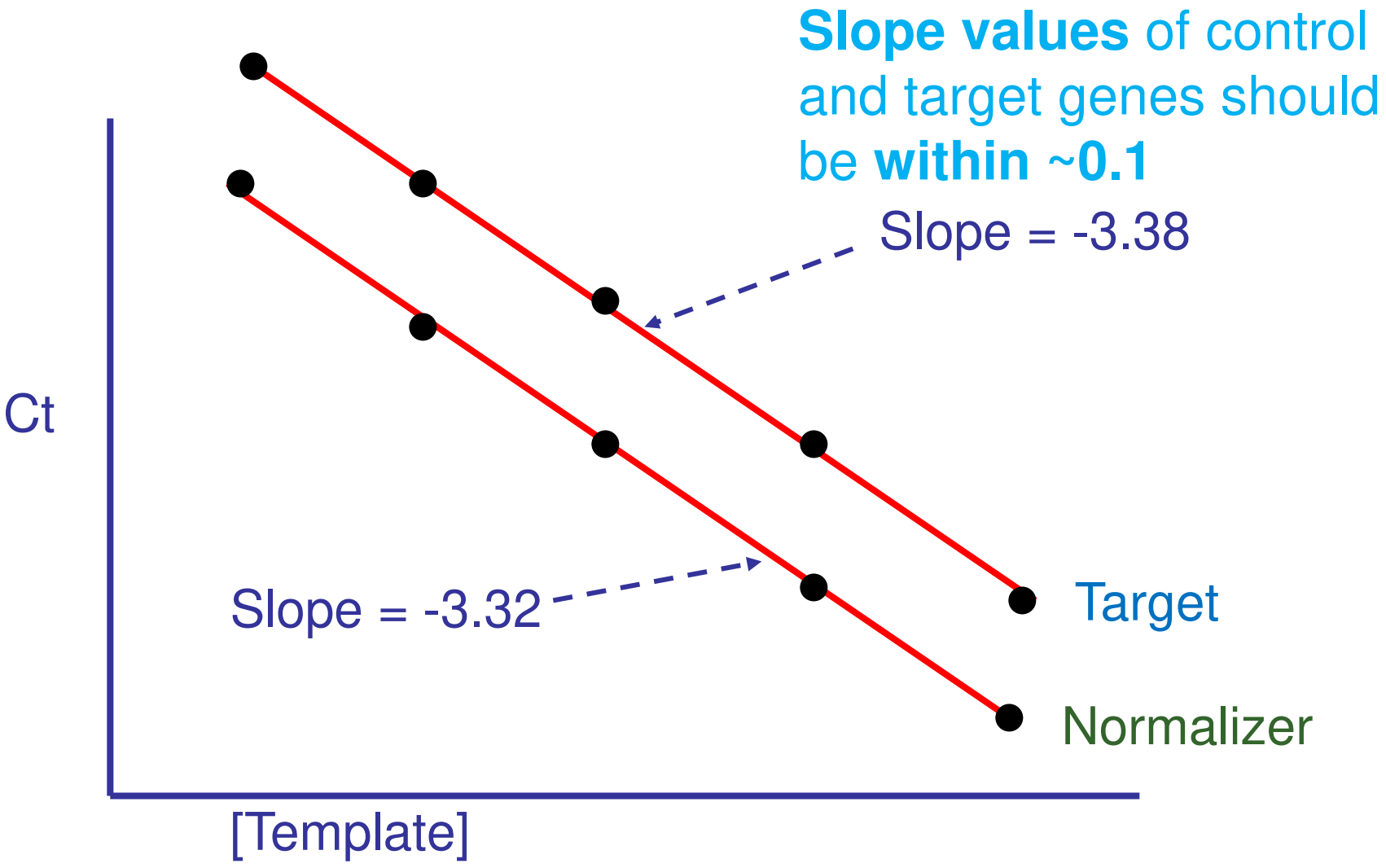
Target and normalizer have equal efficiencies in geometric phase



Run dilution curves and compare slopes

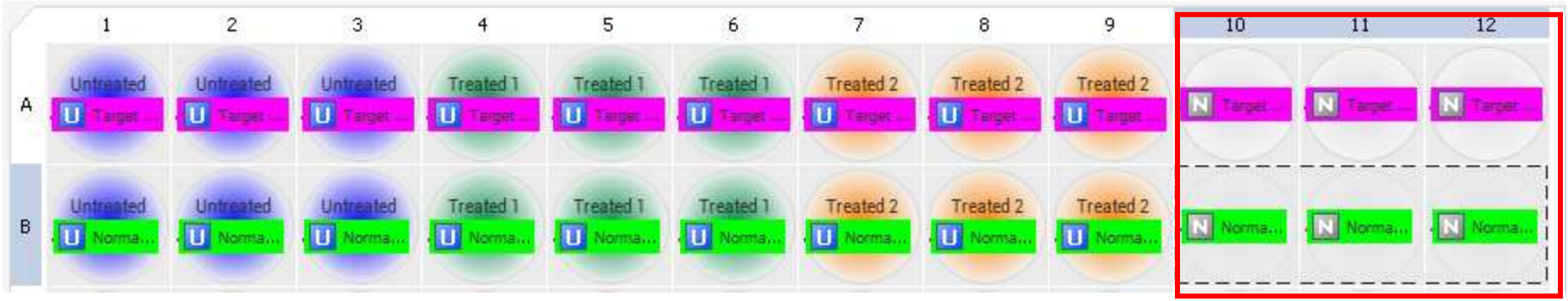


Dilution curves and slope comparison



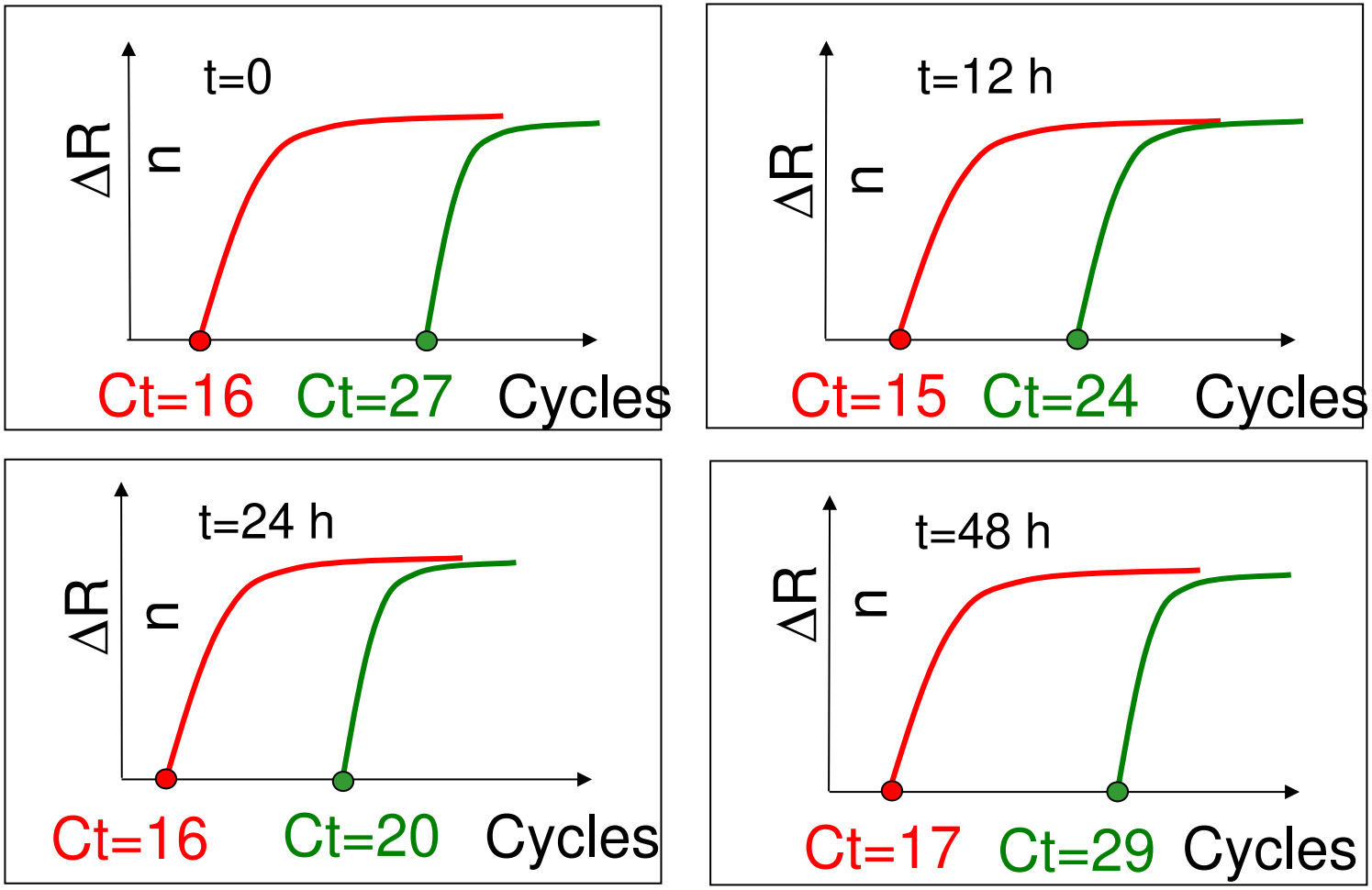
Steps for ΔΔCt

Perform real-time run using control gene and target gene on unknown samples (use at least triplicates per sample for each gene).



No-template controls (NTCs) – contamination check

Comparative Ct Method



Fold change calculation-Comparative Ct Method

step 1: Normalization to endogenous control

$$Ct_{\text{Target gene}} - Ct_{\text{Endogenous control}} = \Delta Ct$$

step 2: Normalization to calibrator sample

$$\Delta Ct_{\text{Sample}} - \Delta Ct_{\text{Calibrator (Time Point 0)}} = \Delta\Delta Ct$$

step 3: use the formula

$$\text{Fold Change} = 2^{-\Delta\Delta Ct}$$

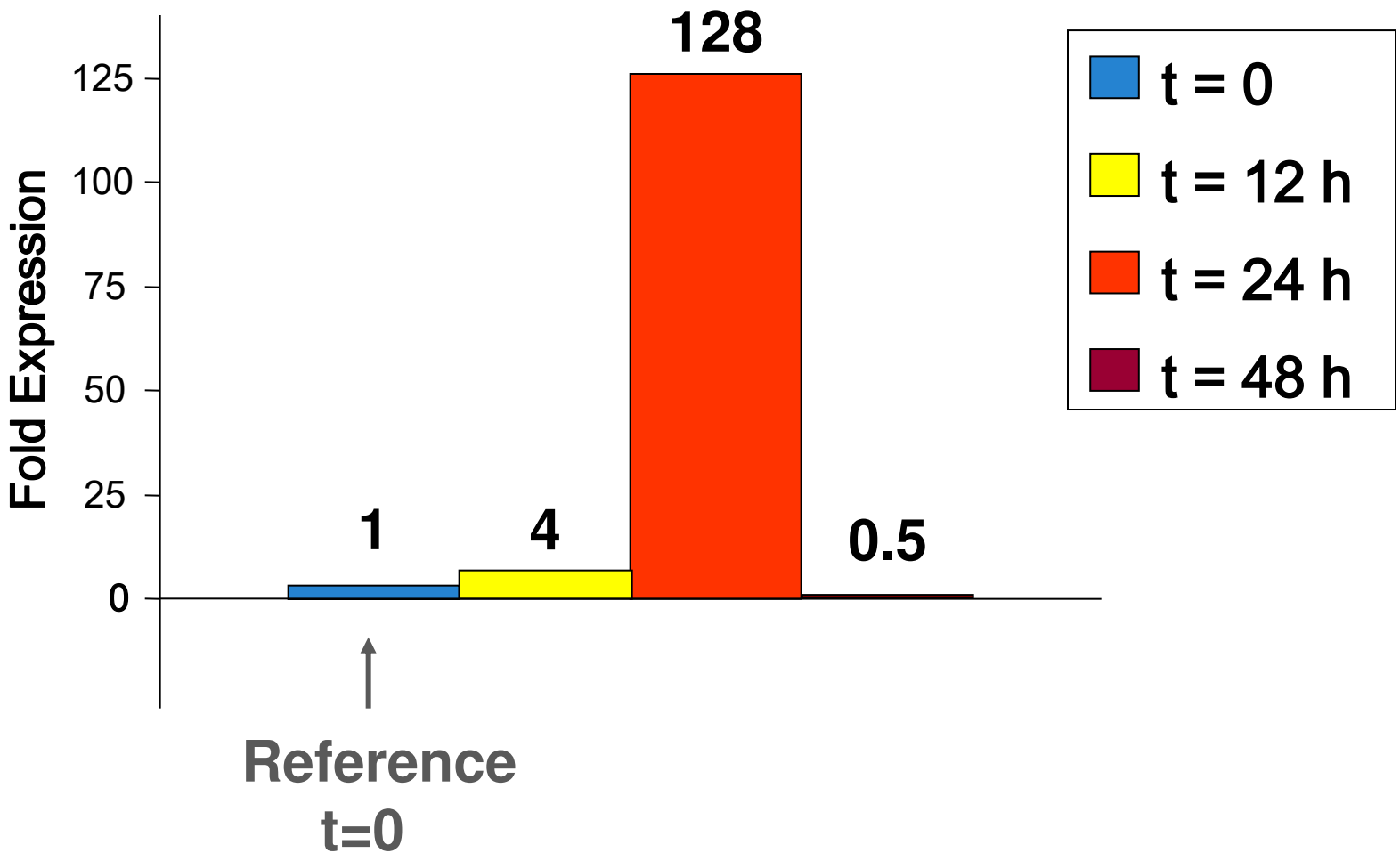
Back to our experiment… Example of ΔΔCt calculation

	Ct Target – Ct Endo		ΔCt Sample – ΔCt ctrl		$2^{-\Delta\Delta Ct}$
sample	**IL-4 (target gene)**	**18S (endo ctrl)**	**ΔCt**	**ΔΔCt**	**RQ**
0 Hr	27	16	11	0	1
12 Hrs	24	15	9	-2	4
24 Hrs	20	16	4	-7	128
48 Hrs	29	17	12	1	0.5

1/0.5=2

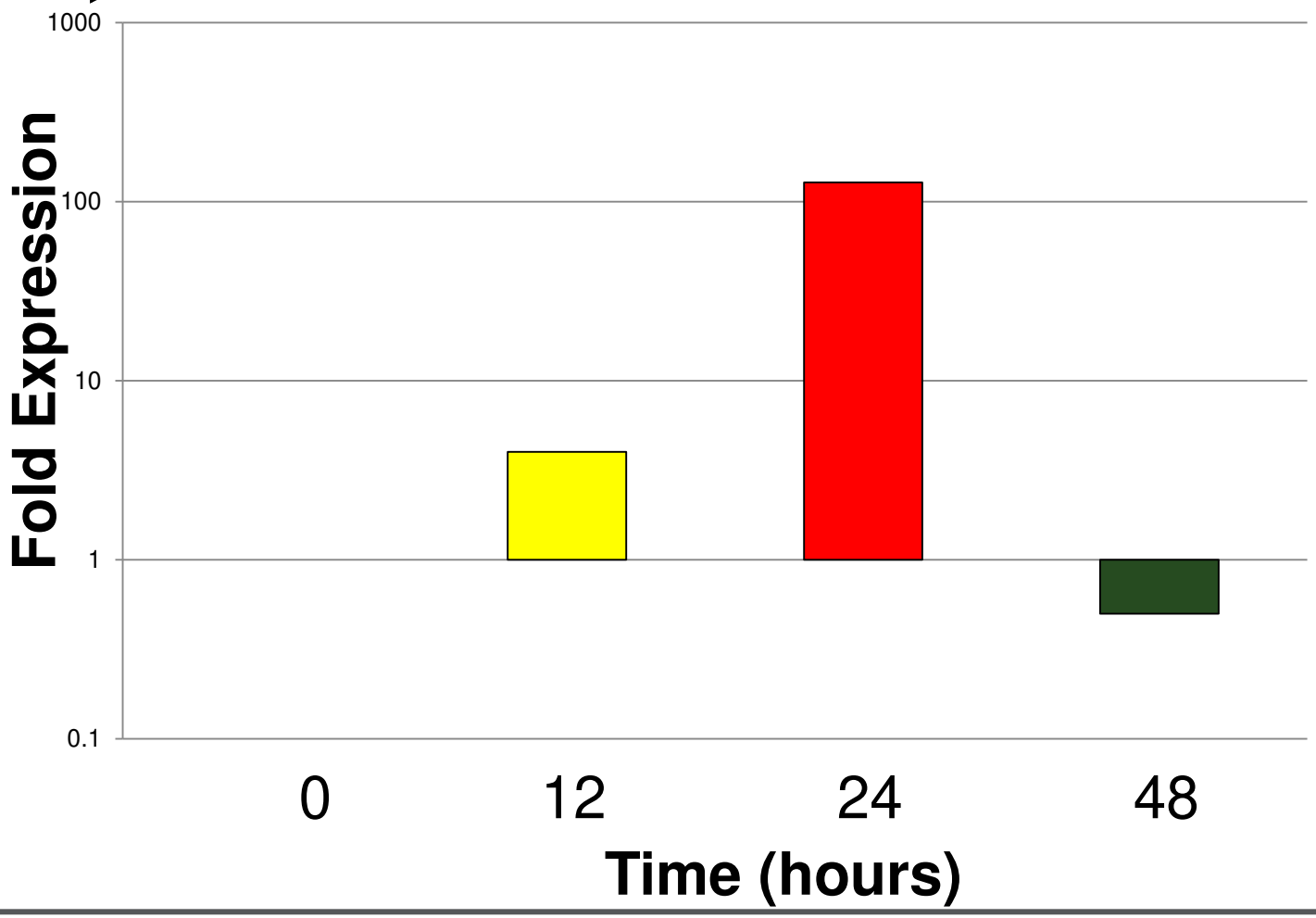
ThermoFisher SCIENTIFIC

Relative Quantification Result



Relative Quantification Result

Note that this is logarithmic scale



ThermoFisher SCIENTIFIC

Recall: used when efficiency validation of target and control genes failed

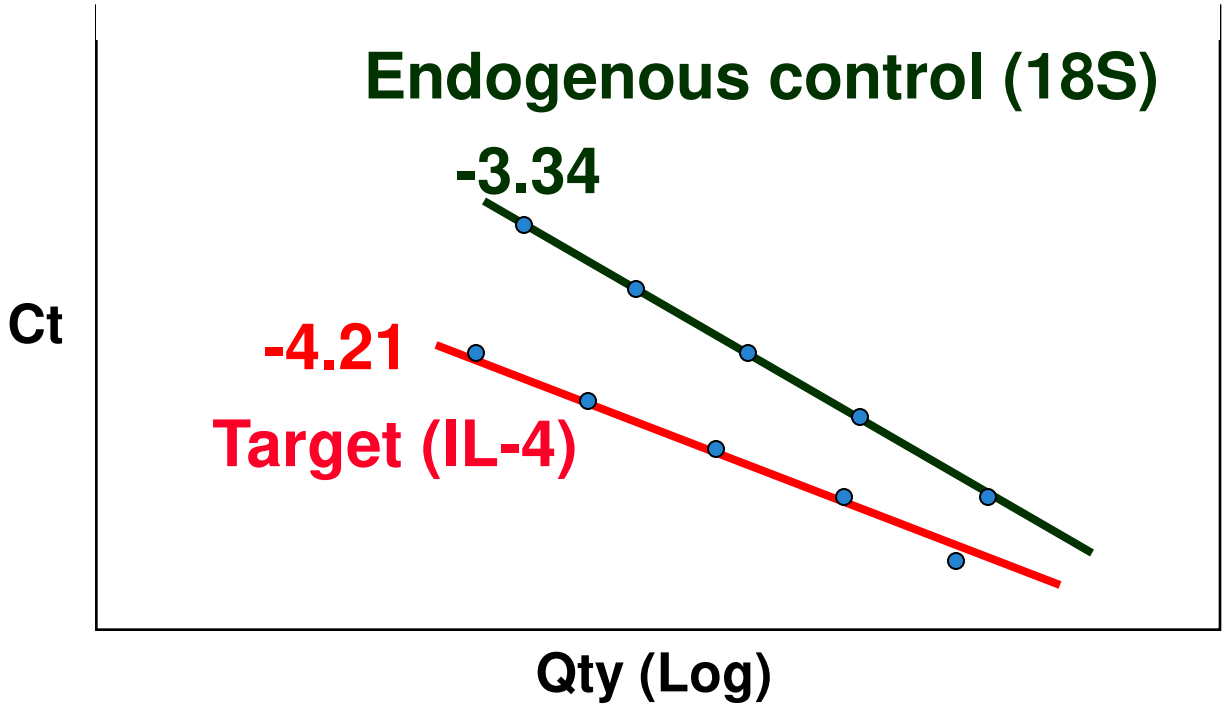


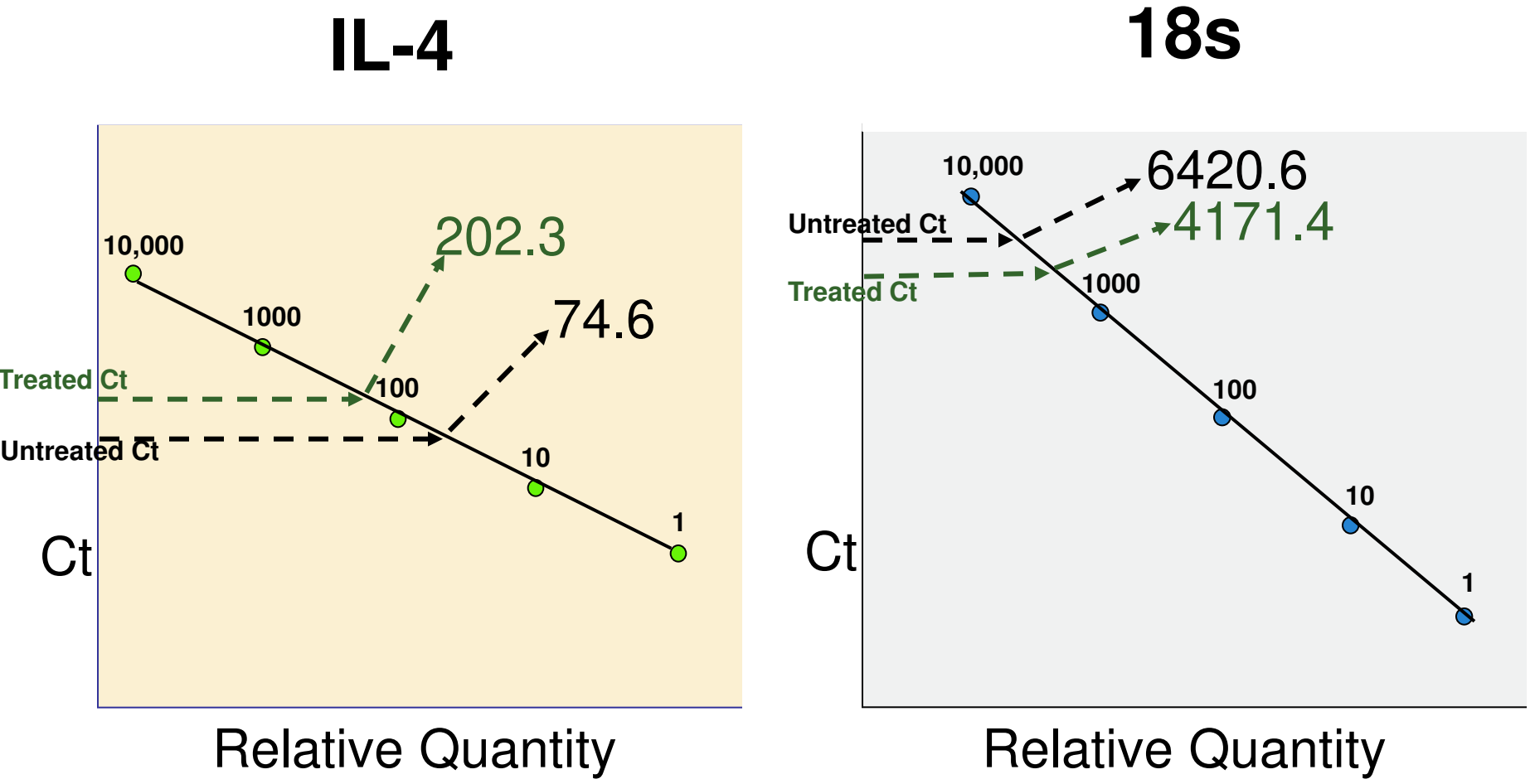
Plate set-up

What sample should you dilute to make the curves?

- Any concentrated cDNA sample that you have in abundance and is known to contain your target(s) and normalizer

- Standard sample does *not* have to be of a known concentration; only a known dilution factor

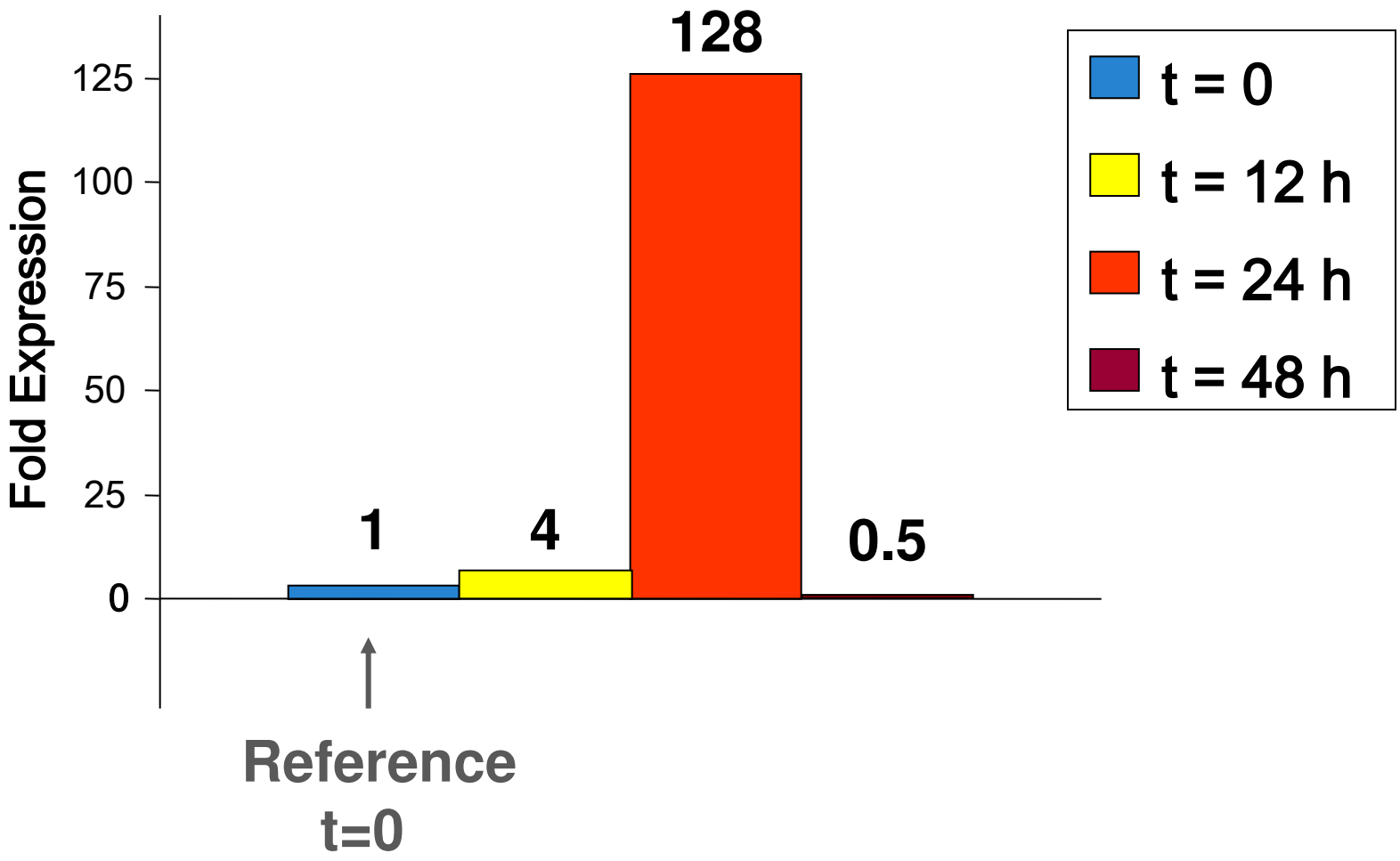
Each gene has a dilution curve



Example of Relative Standard Curve Math

sample	IL-4 quant. (target gene)	18S quant. (endo ctrl)	Target normalized to endo ctrl $(Q_{target} \div Q_{EC})$	Sample normalized to calibrator (RQ) $(Q_{Sample} \div Q_{Calibrator.})$
0 h (calibrator)	308.75	1235	0.25	1
12 h	1295	1295	1	4
24 h	41824	1307	32	128
48 h	137.88	1263	0.125	0.5

Relative Quantification Result



Instrument Features

- Touchscreen
- 10 GB of Onboard Memory (2,000-5,000 run files)
- Wi-Fi connectivity, enabling remote monitoring
- Low maintenance
- Factory calibrated for Applied Biosystems™ reagents
- Browser Based Software (Cloud), enabling PC/MAC compatibility

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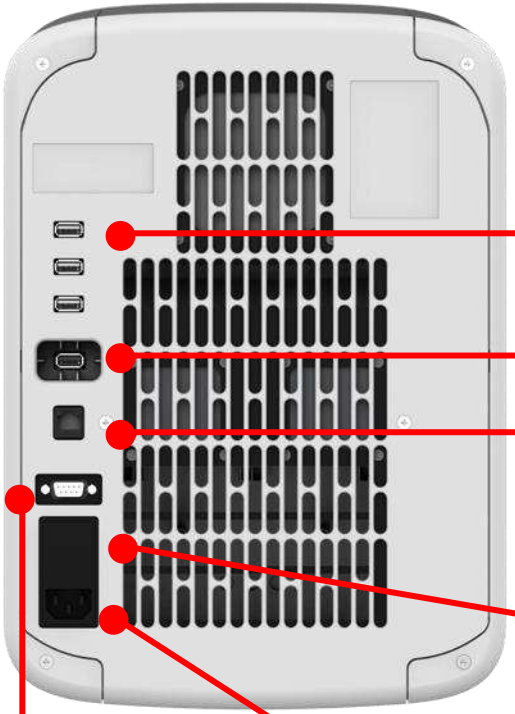
Instrument Front & Rear Features

Touchscreen (stand-alone capabilities, PIN-protected user accounts, and dye calibration/RNaseP functionality)

USB port for template upload and data download

Motorized block drawer (controlled by touchscreen)



USB ports

WiFi adapter port (optional use)

Ethernet port : RJ45 (10/100Mbps)

Fuse cover

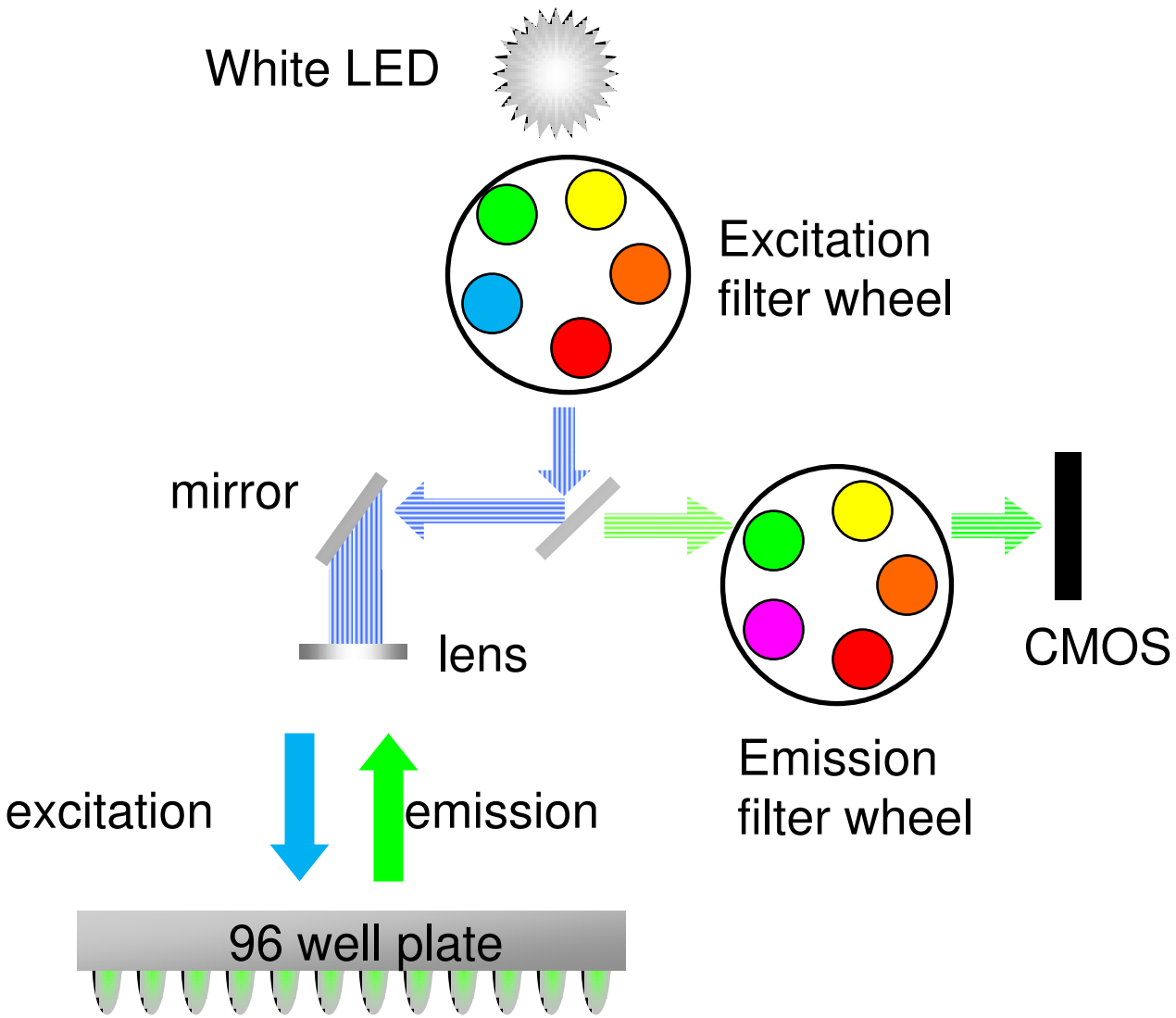
Power port: 100/240 VAC

RS232 port (Service only)

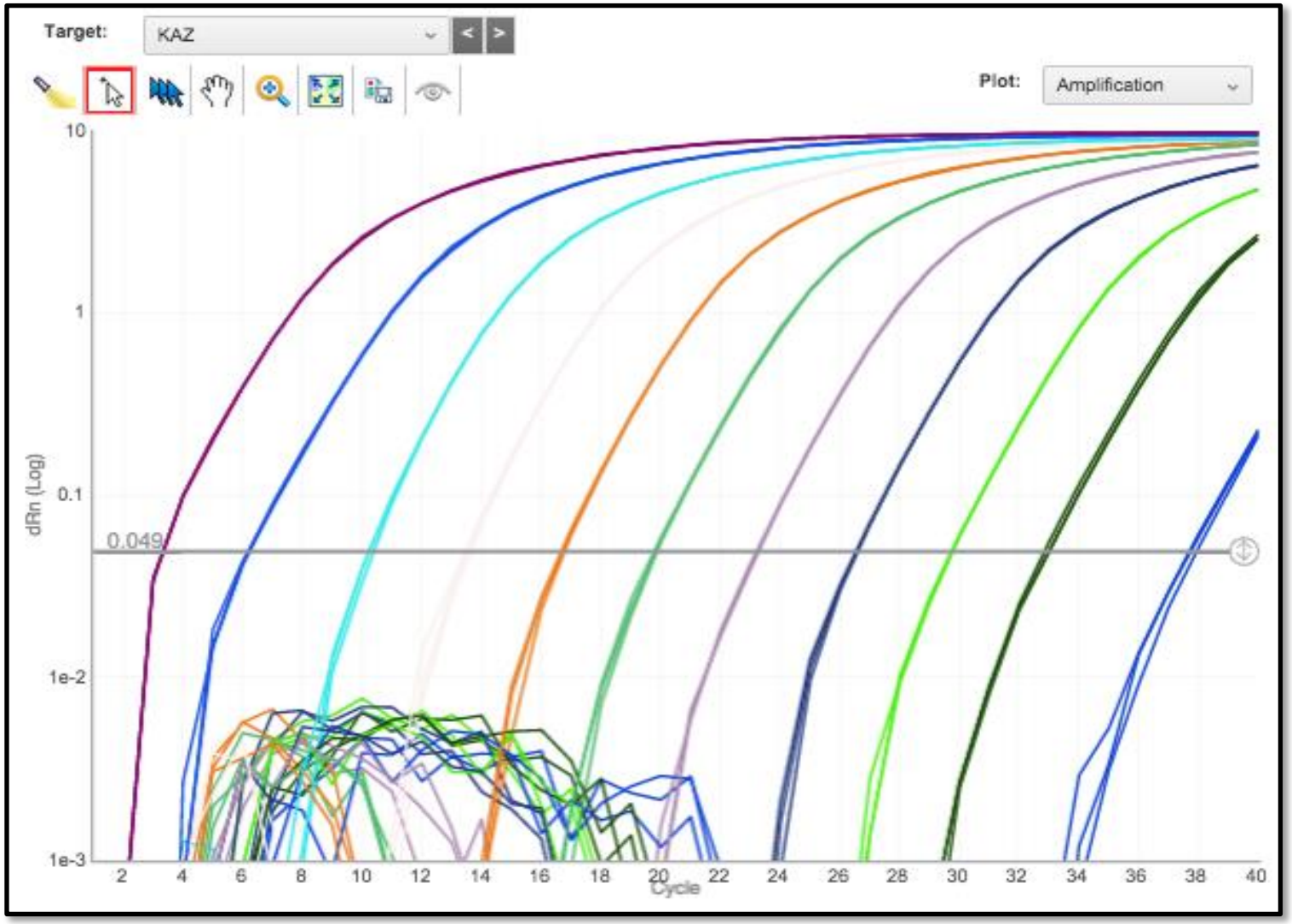
Technical Specifications

	QuantStudio™ 3 Real Time PCR System	QuantStudio™ 5 Real Time PCR System
Block configurations	96-well 0.1 ml block :10-30 µl 96-well 0.2 ml block : 10-100 µl	96-well 0.1 ml block :10-30 µl 96-well 0.2 ml block : 10-100 µl 384-well: 5-20 µl
Run time	<30 minutes	96-well block: <30 minutes 384-well block: <35 minutes
Excitation source	Bright white LED	Bright white LED
Optical Detection	4 coupled filters	96-well block: 6 decoupled filters 384-well block: 5 coupled filters
Temperature Zone Function	3 VeriFlex zones	96-well block: 6 VeriFlex zones 384-well block: N/A
Temperature Accuracy and Uniformity	± 0.25°C	± 0.25°C
Max block ramp rate	96-well 0.1 ml block: 9 ^{0}C/sec 96-well 0.2 ml block: 6.5^{0}C/sec	96-well 0.1 ml block: 9 ^{0}C/sec 96-well 0.2 ml block: 6.5^{0}C/sec 384-well block: 6.0 ^{0}C/sec
21 CFR p11 enablement	No	Yes, with no additional fees
Detection Sensitivity	10 log dynamic range sensitivity 1 copy 1.5 fold differences in target quantities	10 log dynamic range sensitivity 1 copy 1.5 fold differences in target quantities

OptiFlex™ System with Bright White LED



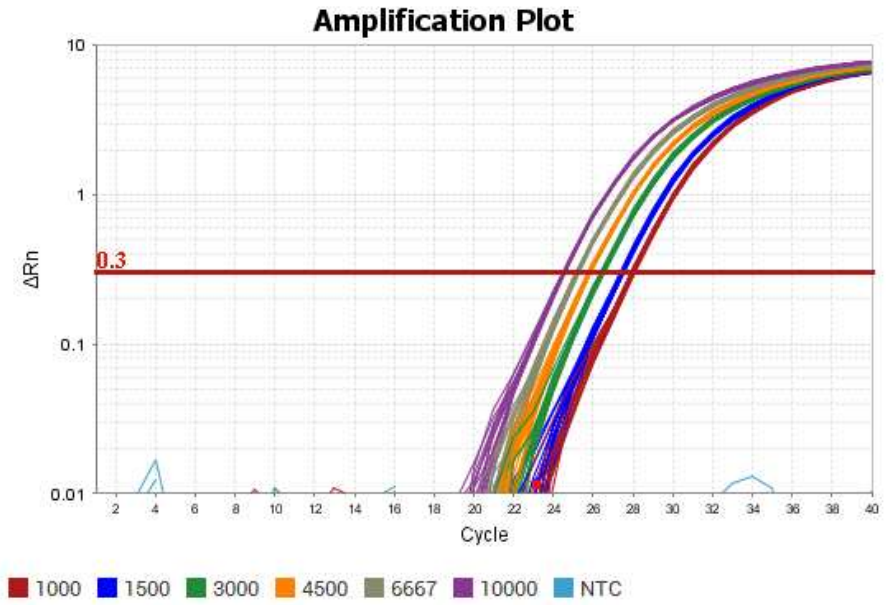
10-Log Dynamic Range Sensitivity



Data courtesy of System Verification and Validation

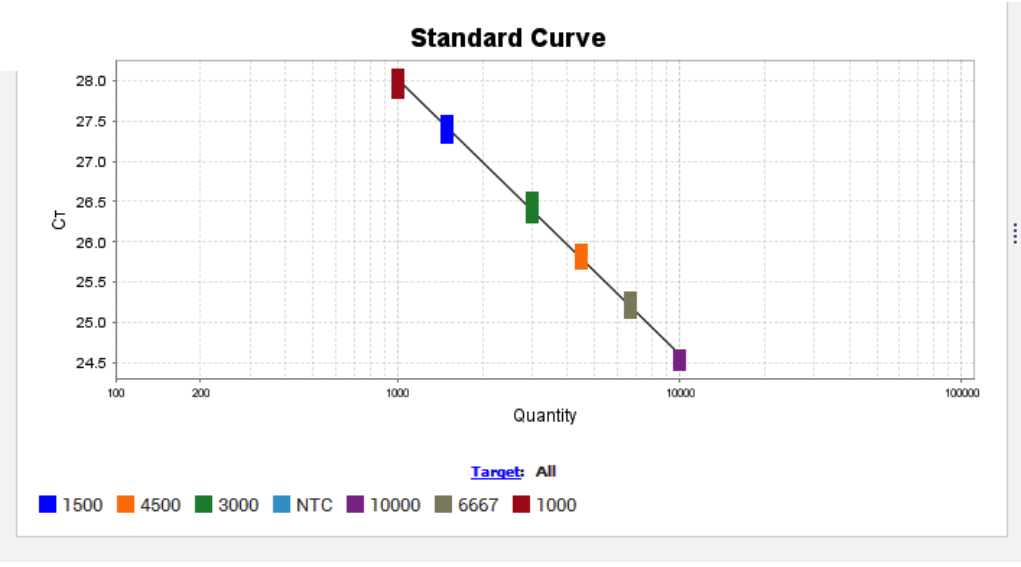
ThermoFisher SCIENTIFIC

QuantStudio™ 3 & 5 Enable 1.5-Fold Discrimination



Amplification plots for 1.5-fold dilutions of KAZ plasmid amplified with PE2 TaqMan™ assay under standard Fast run conditions using the TaqMan Fast Advanced Master Mix.

Quantity	C_T	Std Dev
1000	27.9	0.063
1500	27.45	0.059
3000	26.40	0.060
4500	25.80	0.047
6667	25.20	0.049
10000	24.50	0.041



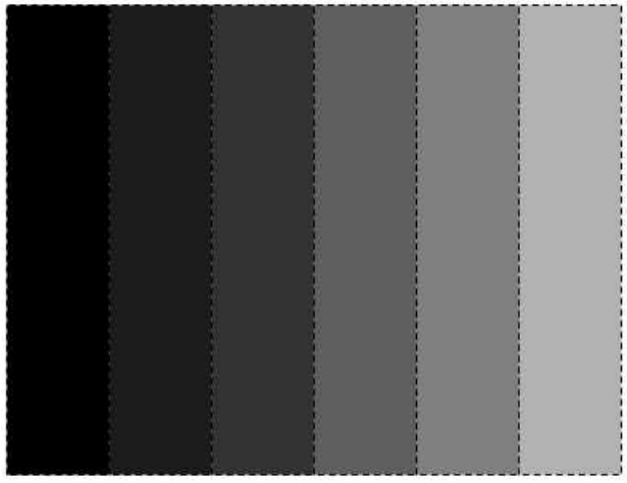
ThermoFisher SCIENTIFIC

VeriFlex™ Blocks

- Independent temperature control in each zone (more precise than gradient)
- Can program at will, including multiple zones with same temp (Temp. difference between adjacent zones ≤5°C)
- Great for optimization and also running multiple assays at the same time

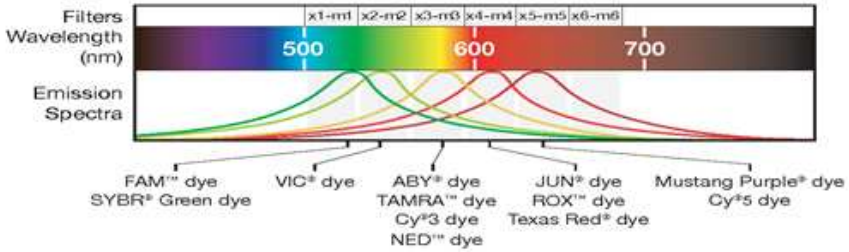
QuantStudio™ 3

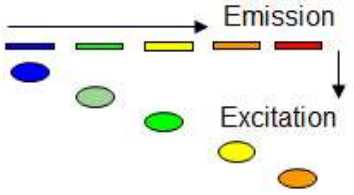
QuantStudio™ 5 96-well block

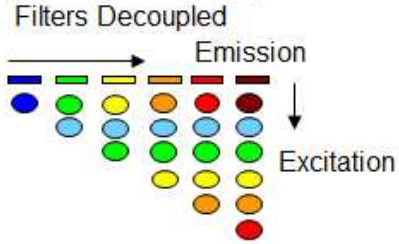


Multiplex Capabilities

Channel	Dye Examples	Excitation Filter	Emission Filter	QuantStudio™3	QuantStudio™5 384-block	QuantStudio™5 96-block
x1-m1	FAM™ and SYBR Green	470 ± 15nm	520 ± 15nm	✔	✔	✔
x2-m2	VIC™, JOE™, TET™, HEX™	520 ± 10nm	558 ± 12nm	✔	✔	✔
x3-m3	TAMRA™, NED™, ABY™, Cy3™	550 ± 10nm	586 ± 10nm	✔	✔	✔
x4-m4	ROX™, JUN™, Texas Red™	580 ± 10nm	623 ± 14nm	✔	✔	✔
x5-m5	Mustang Purple™, LIZ™, Cy®5	640 ± 10nm	682 ± 14nm		✔	✔
x6-m6	Cy®5.5, Alexa Fluor™	662 ± 10nm	711 ± 12nm			✔







ThermoFisher SCIENTIFIC

Instrument Configurations: Stand-alone, Desktop, or Online

Connected Laptop with QuantStudio Design and Analysis desktop software

ThermoFisherCloud

WiFi

LAN

TCP Port 80/433
Must be active!

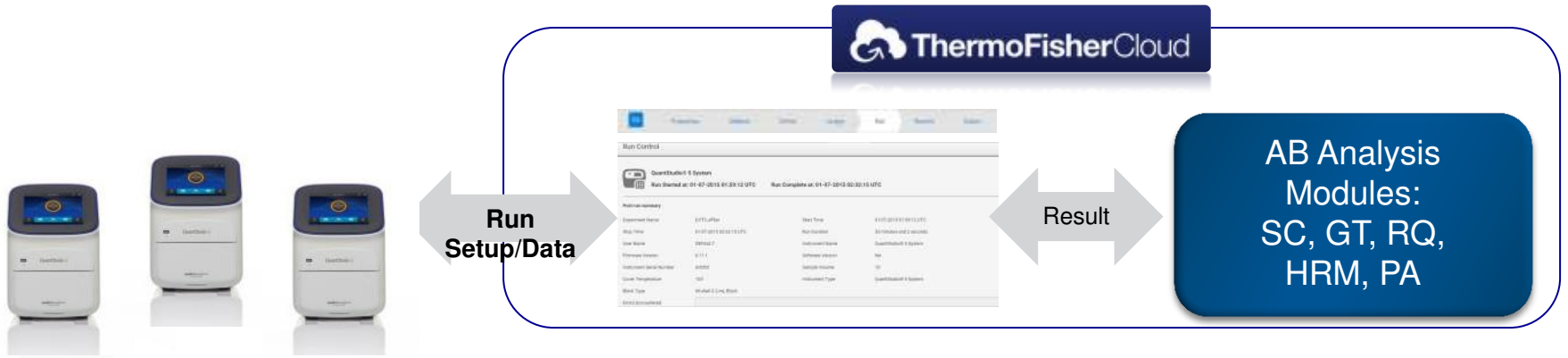
Connect to the Design and Analysis Cloud software using any device with a compatible web browser

USB

Note: You can start an experiment run only from the instrument touchscreen or from the Desktop Software

Ability to Connect Multiple Instruments

- Single software to connect and control all QuantStudio 3 and 5 instruments in the lab
- Seamless integration with instruments that helps minimize manual data transfer



How the Cloud is utilized with QuantStudio™ 3 & 5

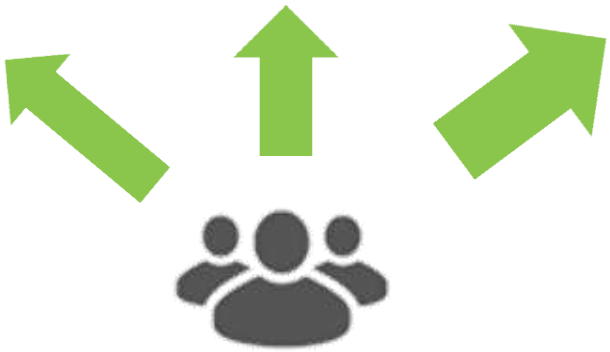
ThermoFisherCloud

- Access/share data
- Analysis Apps
- Browser Based
- Storage and Backup

- Create Studies
- Analyze Data with Apps
- Setup run/plate
- Monitor Run
- View Results

- Load run/plate
- Perform Run
- Monitor Run
- View Results
- Calibration

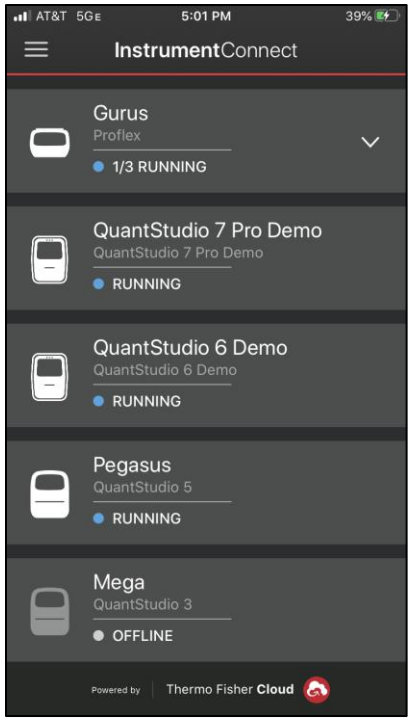
- Monitor Run
- View Results

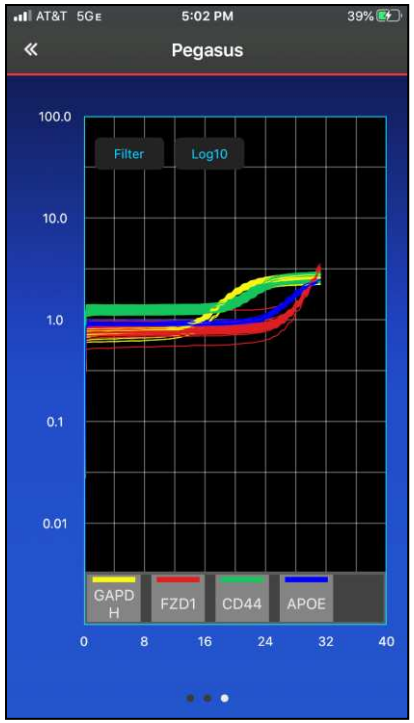


ThermoFisher SCIENTIFIC

Instrument Connect Mobile App

Monitor your runs on multiple devices





ThermoFisher SCIENTIFIC

Interactive Touch Screen

Open/Close door

Help

Sign In or Create a Local User Account

Connection type

Settings:
- Calibrations
- Runs
- Logs
- Ship Prep

Instrument Local User Accounts

Create local pin-protected account on instrument

Connect to online Thermo Fisher Cloud account

ThermoFisherCloud

Optional but highly recommended for full capabilities

- Create individual accounts for multiple users
  - PIN-protected accounts help keep protocols and data safe and stop “accidental” run interruptions
- Instrument users can be designated as “Admin” or “Standard” users
  - First user defaults to “Admin” status but can create other Admins, as needed

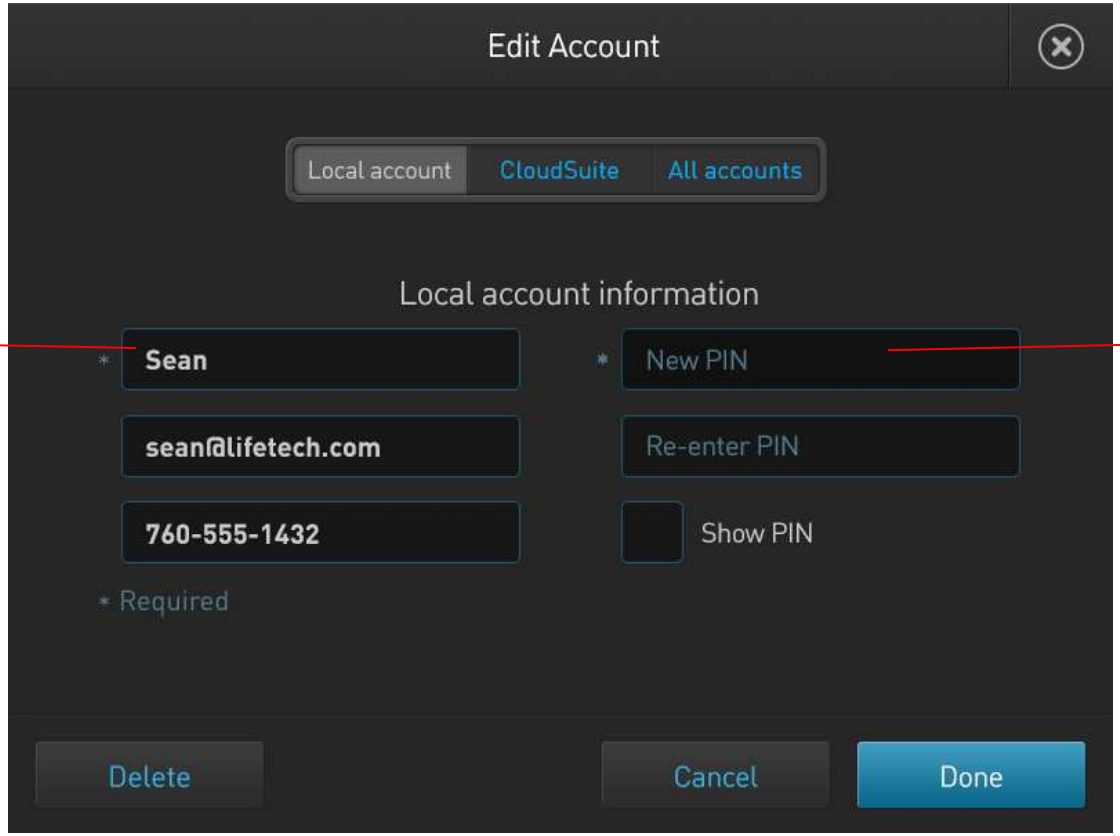
Administrator Only Tasks

- Enable SAE module (QS 5 only)
- Require Sign-In
- Enable Remote Instrument Monitoring
- Update Instrument Software
- Manage/View all Instrument Profiles
- Select Cloud Region
- Manage Sign-Out Timer and Instrument Name

After logging in, Standard Accounts start and save run files in their own folders.

Create Local User Account and Link to Cloud

Enter User Name

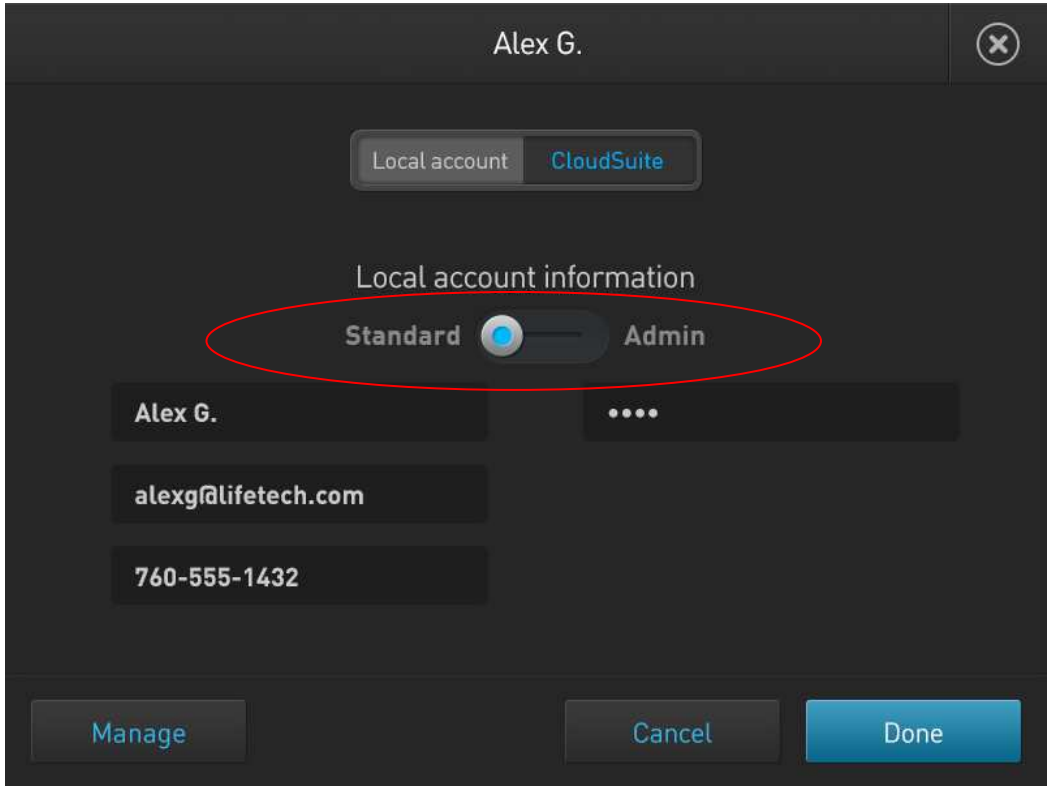


Set PIN
(and remember it!)

Why link to the Cloud?
The Cloud enables you to download run files from the cloud and automatically upload them when complete

ThermoFisher SCIENTIFIC

Admin and Standard User Accounts

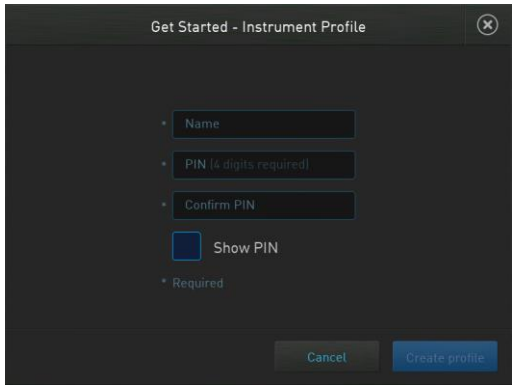


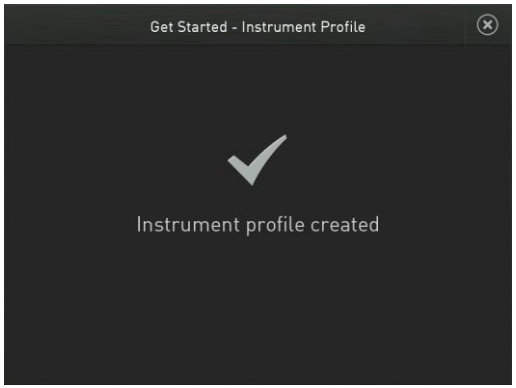
Note: the first user with “Admin” status to connect to the Cloud will be the Cloud Admin for that linked instrument

Account Setup on Touchscreen

One Step
Instrument Profile Setup

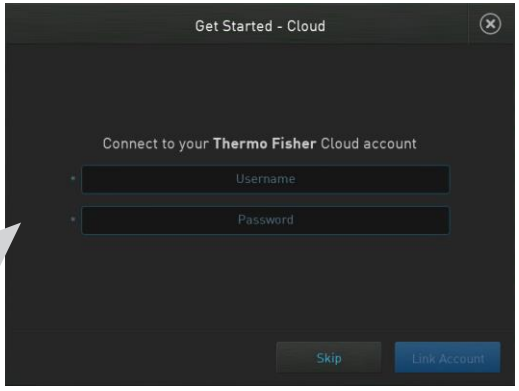
- Enter Name &PIN
- Select “Create Profile”

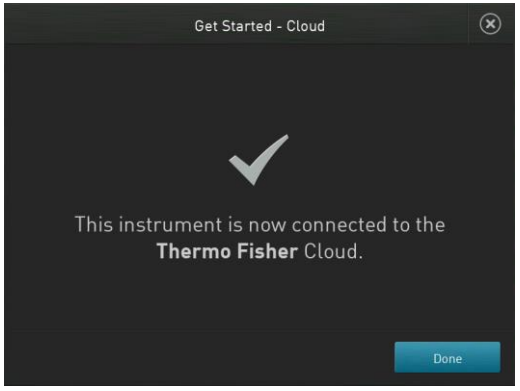




Optional Step to link to Thermo Fisher Cloud Account

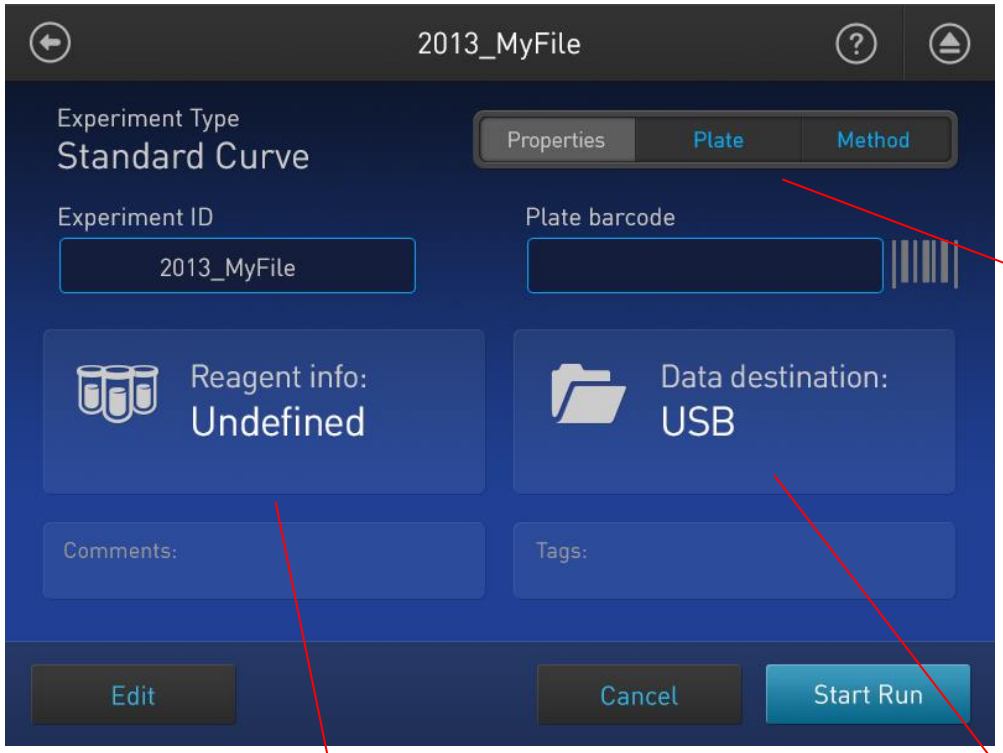
- Enter Thermo Fisher Cloud account credentials & select “Link Account”
- Or select “Skip” to complete setup without linking to cloud account

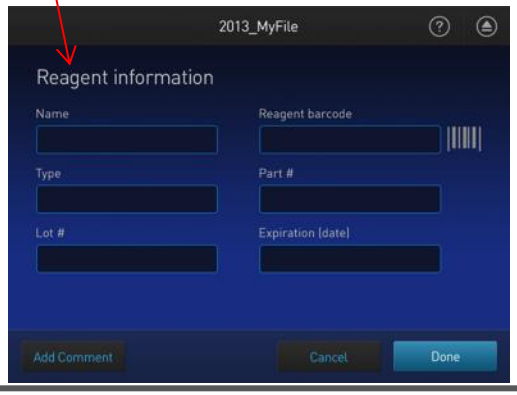


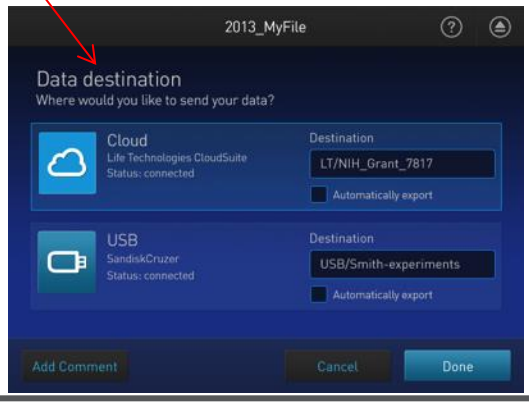


ThermoFisher SCIENTIFIC

Use touchscreen to edit reagent info, destination, and plate setup







2D reagent barcodes supported for Applied Biosystems reagents!

Edit run protocol

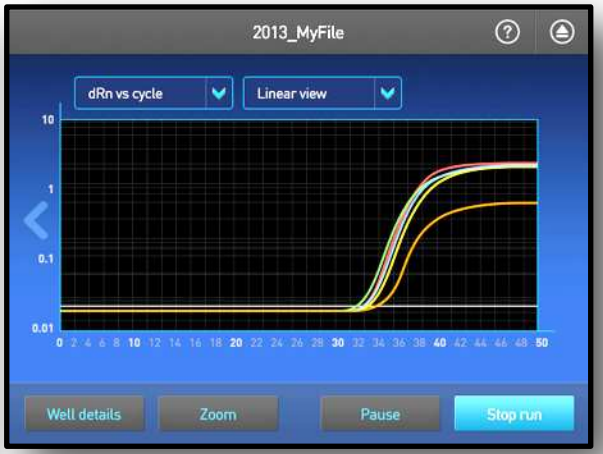
Full method editing capabilities on the touch screen, including VeriFlex, Pause, and Melt

Monitor Progress During the Run

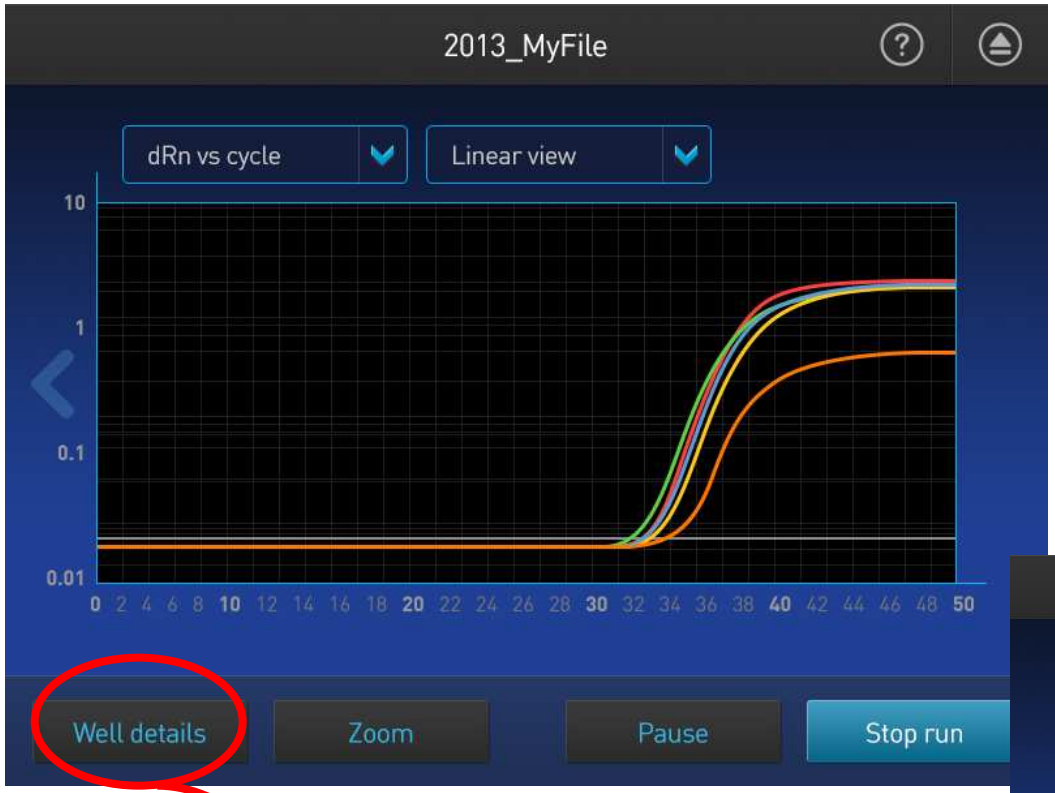
Time Remaining

Thermal Protocol Status

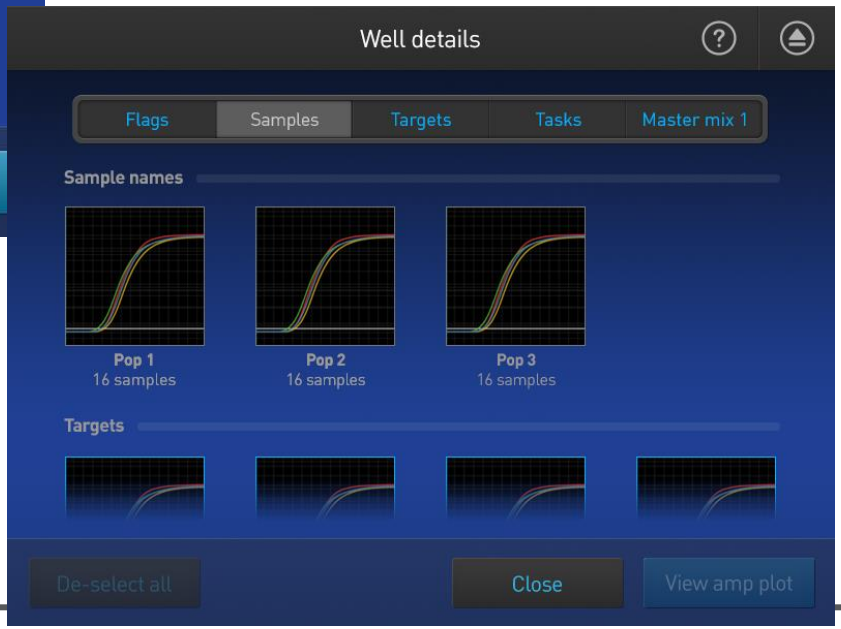
Live Amplification Curves



Review amp plots in real time



Review well details and select amplification plots to view by Target, Sample, Task, or Master Mix



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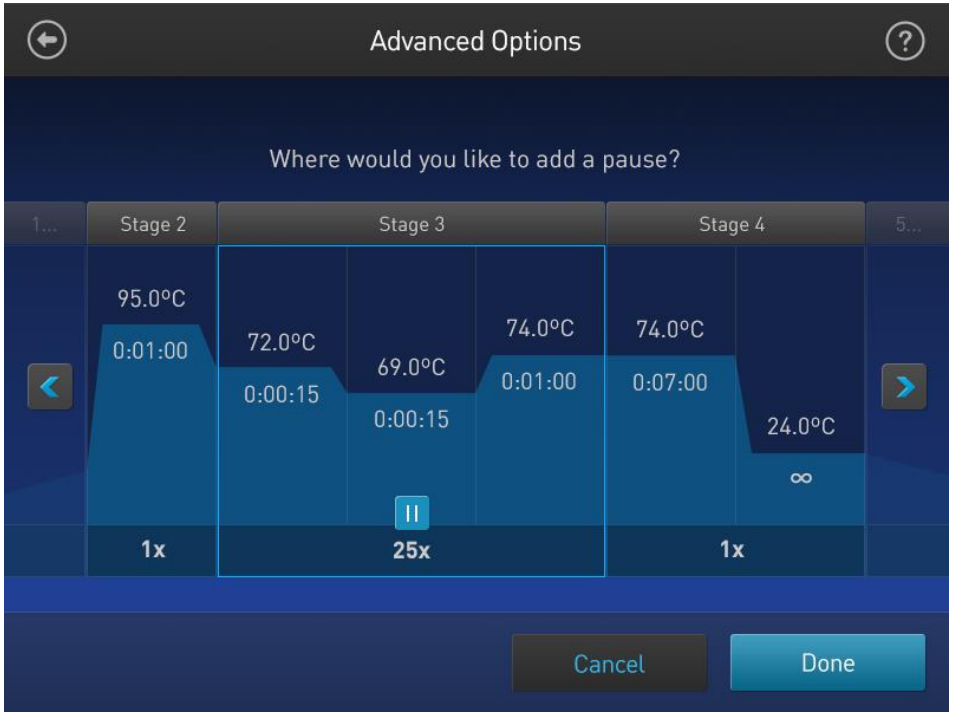
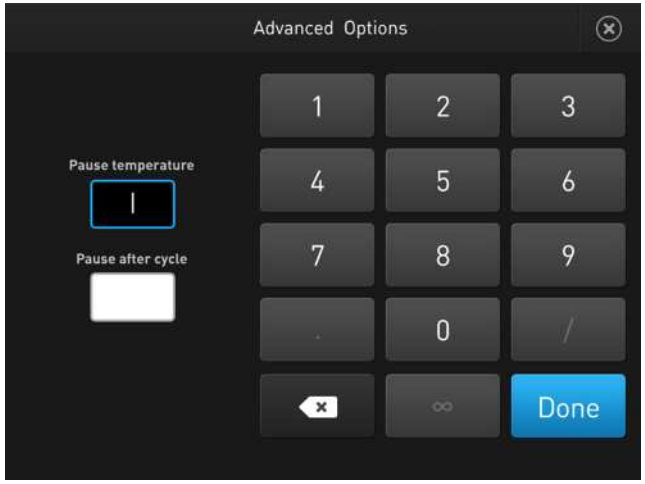
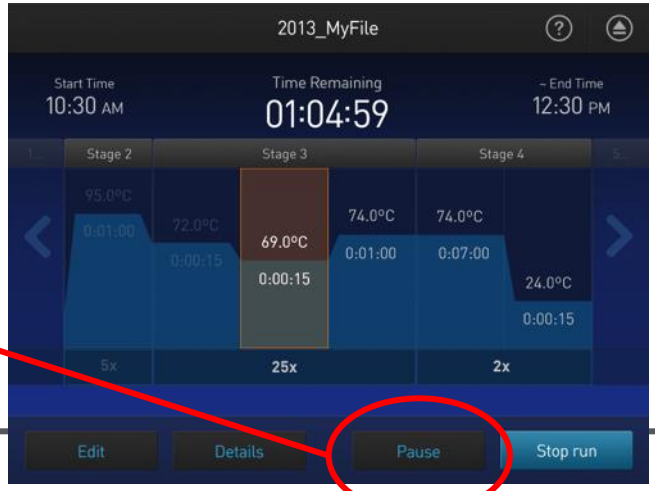
New Feature: Run Pause



Program a Pause into the run: define which step and at what temperature to pause



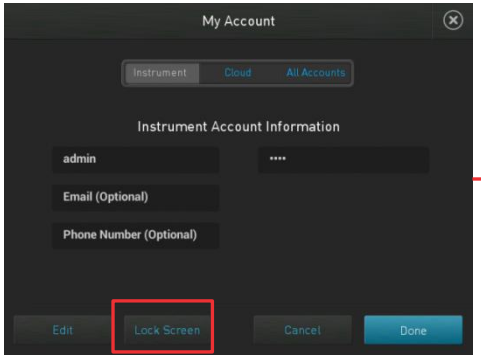
Or pause a run on the fly

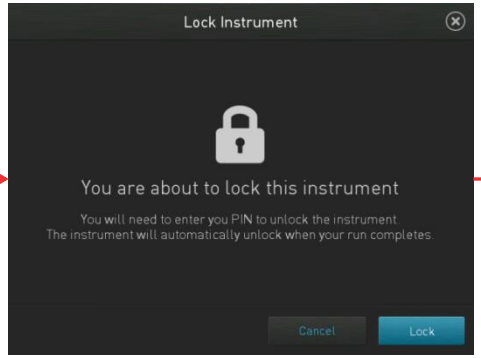


ThermoFisher SCIENTIFIC

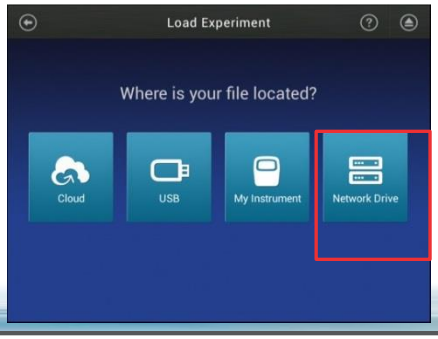
Enhanced Instrument Touchscreen

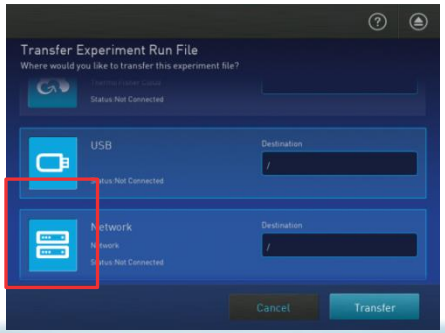
- Ability to lock instrument touchscreen during run to prevent run interruptions
  - Only current user and admin can unlock during the run.
  - Anyone can unlock and access instrument after run is completed.





Ability to transfer run data to/from a ‘Network Location”

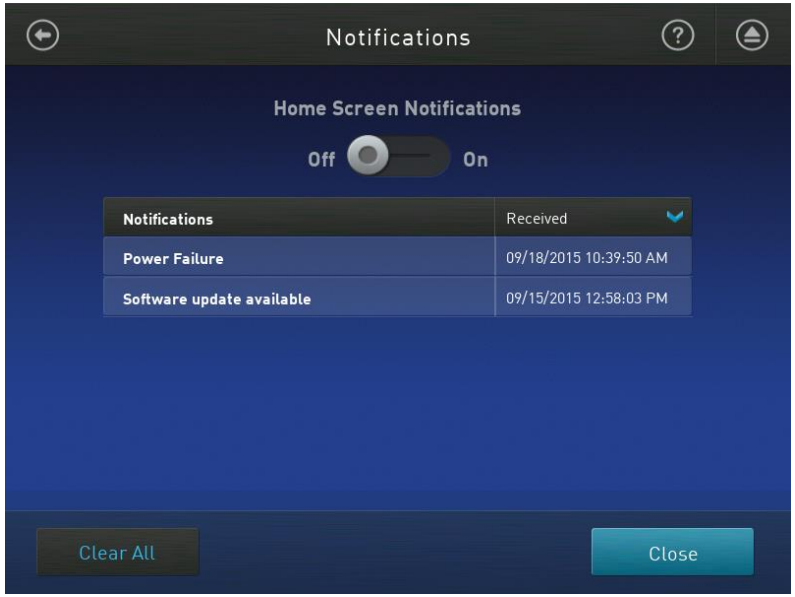


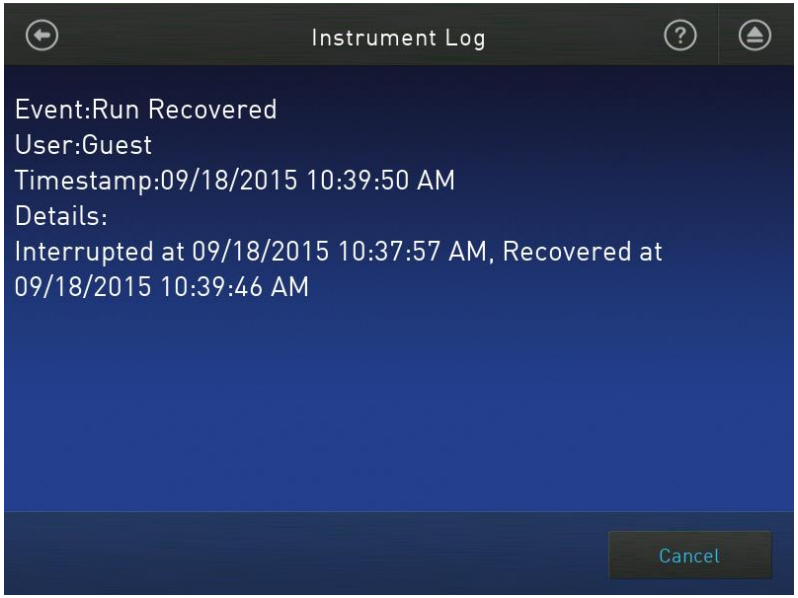


ThermoFisher SCIENTIFIC

Enhanced Instrument Touchscreen (2)

- Power Failure Mode
  - On-going run resumed automatically within 30’
  - On screen notifications, Run log & instrument log



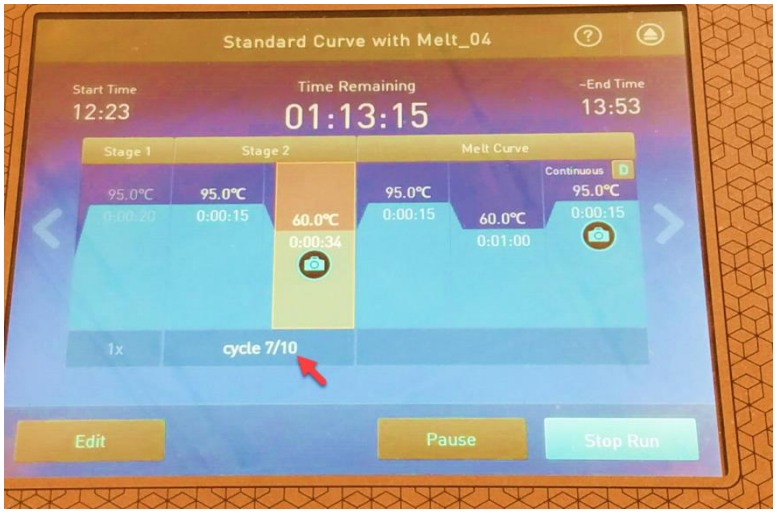


ThermoFisher SCIENTIFIC

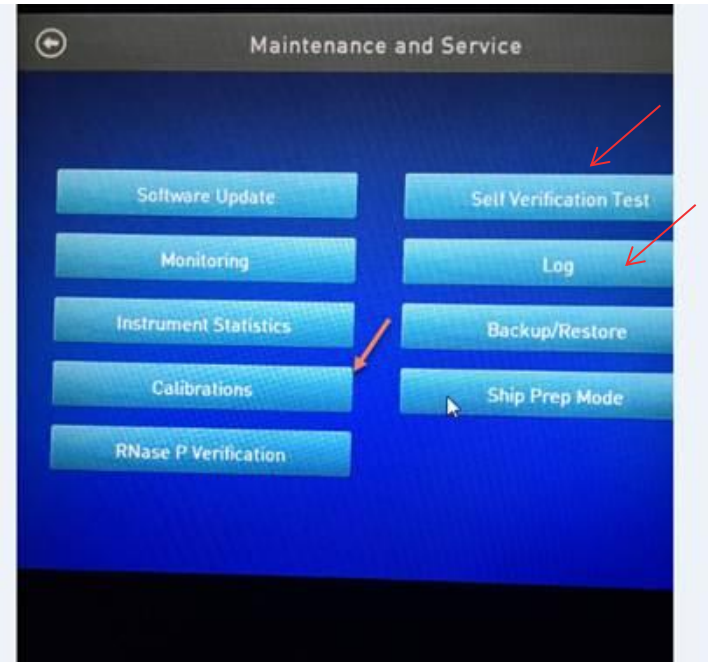
Skip Cycles During a qPCR run

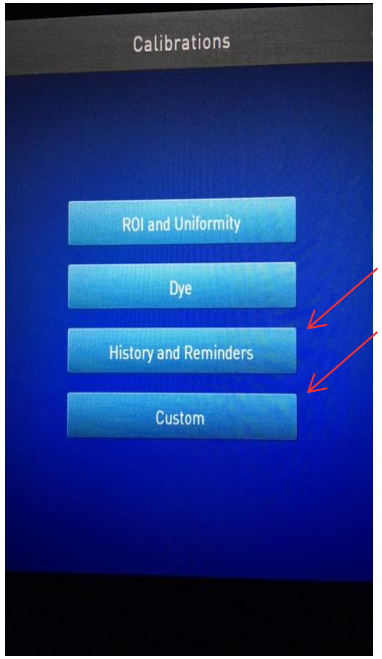
Step 1: Tap Edit

Step 2: Tap Cycle Number Box and Enter New Cycle Number



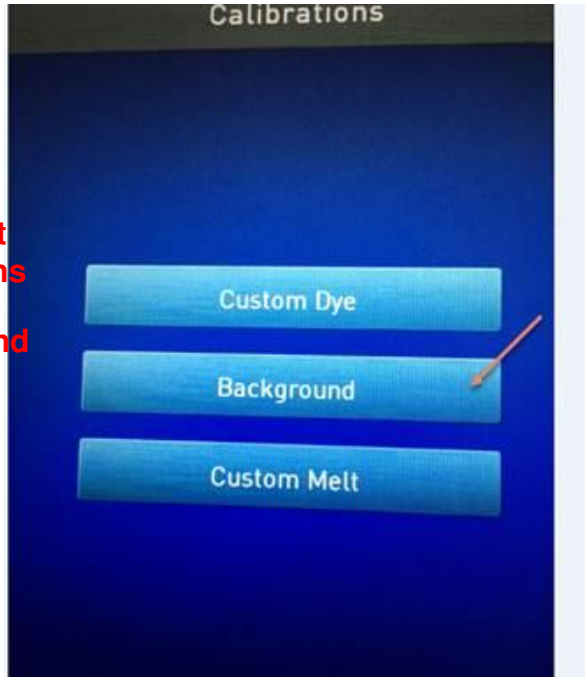
Background, Dye calibrations and Log files





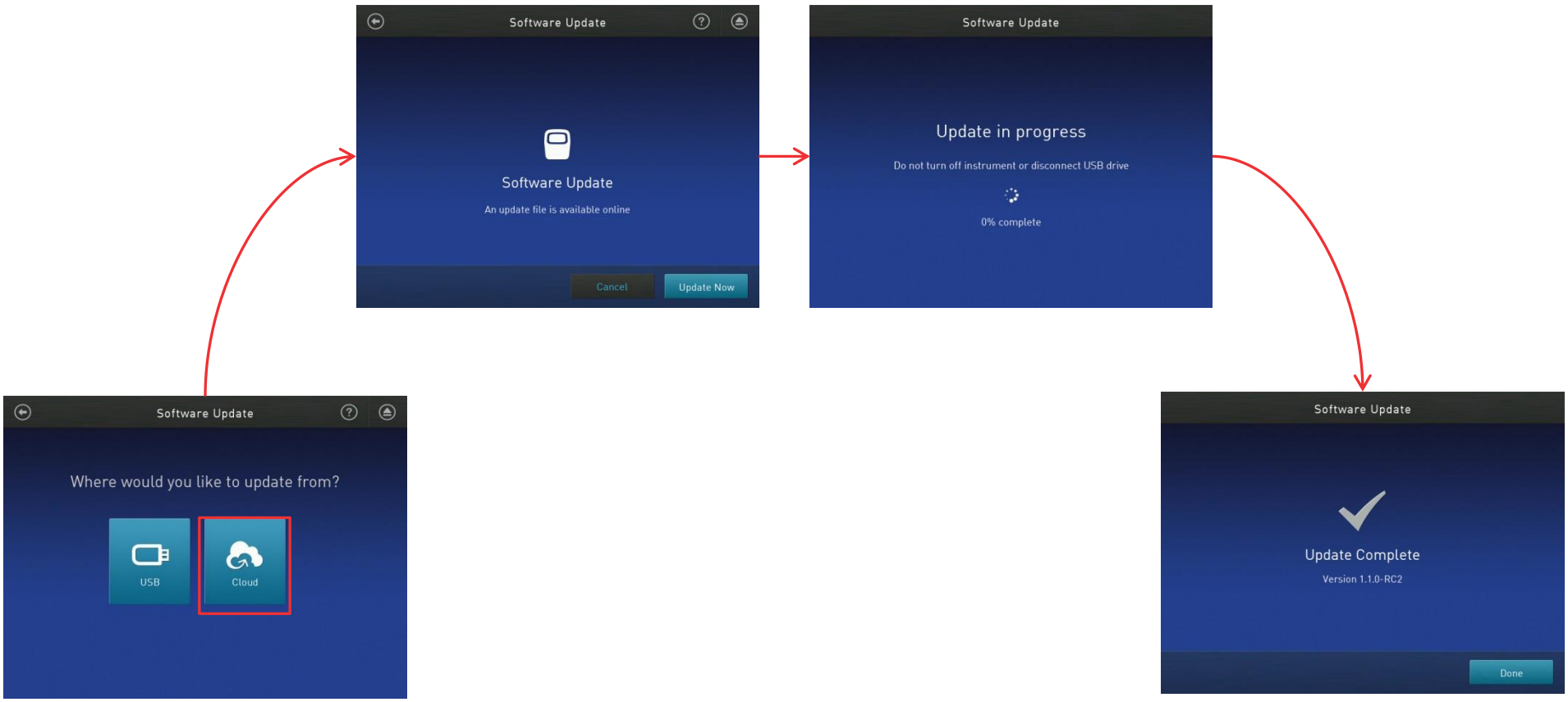
Instrument Calibrations

Background



- Perform the Self Verification Test and export the log files if you experience Instrument issues.

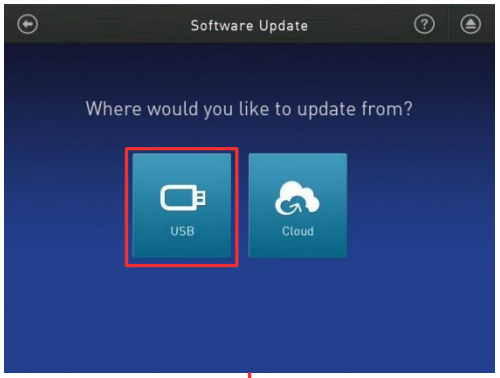
Firmware upgrade from the cloud

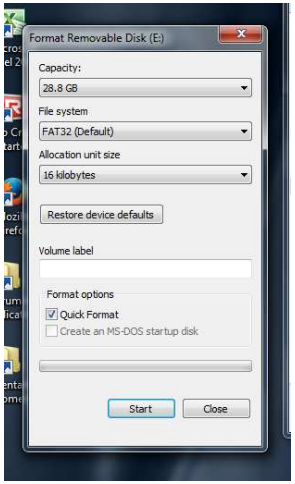


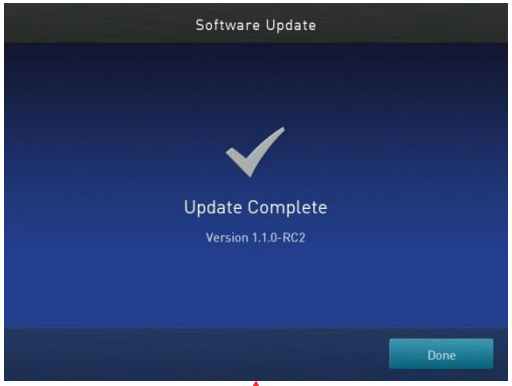
On eGUI, Firmware Upgrade for a cloud connected instrument: Settings>Maintenance and Service>Software Update

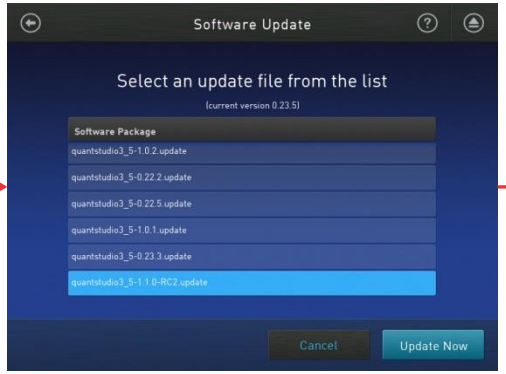
Firmware upgrade via USB

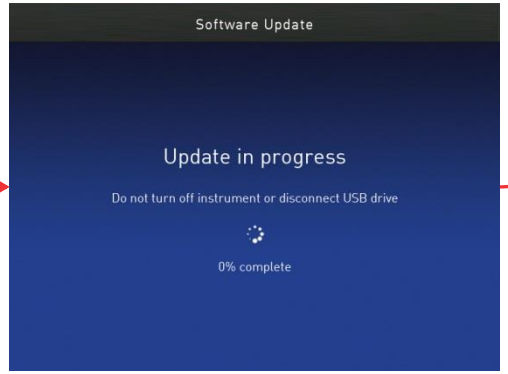
- Go to [QS D& A Software Download Webpage]
- Click on “Download” next to the “Firmware” option and download file to USB drive
- Attach USB drive to instrument and, on eGUI, Settings>Maintenance and Service>Software Update











Recommended Maintenance and Calibration

Frequency	User-performed maintenance task
Weekly	Check disk space and power off the instrument for at least 30 seconds
	Clean the instrument surface with a lint-free cloth
Monthly	Perform a background calibration (to check for thermal block contamination)
	Run disk cleanup and defragmentation
	Perform instrument self-test
Every 2 years	Perform ROI, uniformity, dye, and normalization calibrations
As needed	Perform an RNase P instrument verification run
	Replace the instrument lamp