Introduction to Real-Time PCR and Applications

Sergio Vaira, Ph.D.
Field Application Scientist
Sergio.vaira@thermofisher.com
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

Example:

- PCR theoretically doubles target after each cycle

1 cycle of PCR

40 cycles
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.

**Bright white LED**

**Excitation filters**

**Emission filters**

**Thermal cycling block**
Supported Fluorescent Chemistries

SYBR® Green I Dye

5’-Nuclease Chemistry
• 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.

Amplification Plot

Target

Non target
Additional data are required to determine whether SYBR® Green assay results are derived from target or not.
Check Specificity of Reactions Using a Melt Curve

First negative derivative view

One, clean peak = no extraneous products

Temperature →
Examples of melt peaks indicating multiple products were amplified.
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.
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

**FRET:**
Fluorescence
Resonance
Energy Transfer

*Light (excitation)*

Energy

Intact probes
do not generate
signal
(no emission)

*Light (excitation)*

Separate probes
generate signal
(emission)
During the annealing step, the primers and probe anneal to the template.
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.

NFQ MGB Reporter
(13 – 18 bases) Non-Fluorescent Quencher
Quenchers: TAMRA™ Dye vs. MGB

**TAMRA™**
Fluorescent = Uses up one filter/dye spot
Long (28-38bp) = less specificity

**Minor Groove Binder**
Non-Fluorescent Quencher
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**
Fundamentals of Data Analysis

Note: For Research Use Only. Not for use in diagnostic procedures.
Criteria for generating good quantitative data

1. Precision & Accuracy
2. Efficiency
3. Dynamic range

Reproducibility
Tight replicates

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 #
Limit of detection – Poisson distribution

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

Answer: ~ 63%

30 μL / 3 molecules

Answer: ~ 95%

30 μL / 9 molecules
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.
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)
NEVER trust Cts or quantities for samples whose geometric phases are not parallel with the other samples and standards for that same assay!

Bad curve! (not the same efficiency as the others)
How is efficiency measured?

Serial dilutions

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

Ideally, slope = -3.32

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

$E = 10^{0.301}-1$

$E = 2-1 = 1 \rightarrow 100\%$
Standard Curve Plot Features

- **Slope** should be between -3.58 and -3.10 (corresponding to E = 100% ± 10%)

- Ideally, $R^2 \geq 0.99$ ($R^2$ suggests poor pipetting of standards)

- **Y-intercept** is the theoretical limit of detection of reaction

- **Error** provides the standard error of the slope
Relationship for calculating relative starting amounts

$2^n = \text{fold change, where } n \text{ is number of cycles}$

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
$R^2$: 0.949283
Omit outliers

Standard Curve

Detector: [detector name]
Slope: 3.158633
Intercept: 25.624602
R²: 0.948333
Removal of outliers

Correlation and slope now good
From Raw Data to Ct – System Normalizations

Raw data (Fluorescence)

Ct
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)

Amplification Plot

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

ThermoFisher SCIENTIFIC
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.

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.
From Raw Data to Ct – System Normalizations

Raw data (Fluorescence) → Multicomponent (Fluorescence) → Passive Reference Dye normalization (Rn)

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)**
  - automatic or manual
- **Baseline normalization (ΔRn)**

Baseline normalization accounts for the different background of each well.
Importance of Baseline

Before baseline subtraction

After baseline subtraction

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?

"Double waterfall effect"
Gene Expression

Note: For Research Use Only. Not for use in diagnostic procedures.
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 $\beta$-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

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

- RT Buffer
- DTT
- RNase Inhibitor
- Reverse Transcriptase (RT)

- RNA
- Primers
- dNTPs

Gene specific priming

Oligo dT priming

Reverse Transcription 37°C-70°C

Terminate Reaction 85°C, 5 min

Random primers used

10% of cDNA volume to downstream reaction such as PCR

## 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
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

Slope = -3.38

Slope = -3.32
Dilution curves and slope comparison

**Slope values** of control and target genes should be within ~0.1

- **Slope = -3.38**
- **Slope = -3.32**

[Ct](#) vs. [Template]
Steps for $\Delta \Delta Ct$

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

- **Ct=16 Cycles**
  - t=0
  - ΔR
  - n
  - Ct=16
  - Ct=27

- **Ct=15 Cycles**
  - t=12 h
  - ΔR
  - n
  - Ct=15
  - Ct=24

- **Ct=16 Cycles**
  - t=24 h
  - ΔR
  - n
  - Ct=16
  - Ct=20

- **Ct=17 Cycles**
  - t=48 h
  - ΔR
  - n
  - Ct=17
  - Ct=29

**Endogenous control** - Red
**Target gene** - Green

ThermoFisher Scientific
**Fold change calculation - Comparative Ct Method**

**step 1: Normalization to endogenous control**

\[ \text{Ct Target gene} - \text{Ct Endogenous control} = \Delta \text{Ct} \]

**step 2: Normalization to calibrator sample**

\[ \Delta \text{Ct Sample} - \Delta \text{Ct Calibrator (Time Point 0)} = \Delta \Delta \text{Ct} \]

**step 3: use the formula**

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

**Back to our experiment... Example of $\Delta \Delta Ct$ calculation**

<table>
<thead>
<tr>
<th>sample</th>
<th>IL-4 (target gene)</th>
<th>18S (endo ctrl)</th>
<th>$\Delta Ct$</th>
<th>$\Delta \Delta Ct$</th>
<th>RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hr</td>
<td>27</td>
<td>16</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>12 Hrs</td>
<td>24</td>
<td>15</td>
<td>9</td>
<td>-2</td>
<td>4</td>
</tr>
<tr>
<td>24 Hrs</td>
<td>20</td>
<td>16</td>
<td>4</td>
<td>-7</td>
<td>128</td>
</tr>
<tr>
<td>48 Hrs</td>
<td>29</td>
<td>17</td>
<td>12</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

$2^{-\Delta \Delta Ct} = 1/0.5 = 2$
Relative Quantification Result

- t = 0:
  - Fold Expression: 1

- t = 12 h:
  - Fold Expression: 4

- t = 24 h:
  - Fold Expression: 128

- t = 48 h:
  - Fold Expression: 0.5
Note that this is logarithmic scale
Recall: used when efficiency validation of target and control genes failed

Endogenous control (18S)

Ct

-3.34

-4.21

Target (IL-4)

Qty (Log)
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

IL-4

\[
\begin{array}{c}
\text{Treated Ct} \\
\text{Untreated Ct}
\end{array}
\]

Relative Quantity

Treated Ct

Untreated Ct

Relative Quantity

18s

\[
\begin{array}{c}
\text{Treated Ct} \\
\text{Untreated Ct}
\end{array}
\]

Relative Quantity

10,000

1000

100

10

1

10,000

1000

100

10

1
**Example of Relative Standard Curve Math**

\[
\left( \frac{Q_{\text{target}}}{Q_{\text{EC}}} \right) \quad \left( \frac{Q_{\text{Sample}}}{Q_{\text{Calibrator}}} \right)
\]

<table>
<thead>
<tr>
<th>sample</th>
<th>IL-4 quant. (target gene)</th>
<th>18S quant. (endo ctrl)</th>
<th>Target normalized to endo ctrl</th>
<th>Sample normalized to calibrator (RQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h (calibrator)</td>
<td>308.75</td>
<td>1235</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>12 h</td>
<td>1295</td>
<td>1295</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>24 h</td>
<td>41824</td>
<td>1307</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>48 h</td>
<td>137.88</td>
<td>1263</td>
<td>0.125</td>
<td>0.5</td>
</tr>
</tbody>
</table>
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
**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)
- **RS232 port** (Service only)
- **Fuse cover**
- **Power port**: 100/240 VAC

*ThermoFisher Scientific*
## Technical Specifications

| Block configurations | QuantStudio™ 3 Real Time PCR System | 96-well 0.1 ml block: 10-30 µl  
96-well 0.2 ml block: 10-100 µl | QuantStudio™ 5 Real Time PCR System | 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 | Optical Detection | 4 coupled filters | 96-well block: 6 decoupled filters  
384-well block: 5 coupled filters | Excitation source | Bright white LED | Bright white LED | Optical Detection | 96-well block: 6 VeriFlex zones  
384-well block: N/A | Temperature Zone Function | 3 VeriFlex zones | Temperature Accuracy and Uniformity | ± 0.25°C | ± 0.25°C | Max block ramp rate | 96-well 0.1 ml block: 9 °C/sec  
96-well 0.2 ml block: 6.5°C/sec | 96-well 0.1 ml block: 9 °C/sec  
96-well 0.2 ml block: 6.5°C/sec  
384-well block: 6.0 °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

White LED

Excitation filter wheel

mirror

lens

Emission filter wheel

CMOS

96 well plate

excitation

emission
10-Log Dynamic Range Sensitivity

Data courtesy of System Verification and Validation
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.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>$C_T$</th>
<th>Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>27.9</td>
<td>0.063</td>
</tr>
<tr>
<td>1500</td>
<td>27.45</td>
<td>0.059</td>
</tr>
<tr>
<td>3000</td>
<td>26.40</td>
<td>0.060</td>
</tr>
<tr>
<td>4500</td>
<td>25.80</td>
<td>0.047</td>
</tr>
<tr>
<td>6667</td>
<td>25.20</td>
<td>0.049</td>
</tr>
<tr>
<td>10000</td>
<td>24.50</td>
<td>0.041</td>
</tr>
</tbody>
</table>
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
## Multiplex Capabilities

<table>
<thead>
<tr>
<th>Channel</th>
<th>Dye Examples</th>
<th>Excitation Filter</th>
<th>Emission Filter</th>
<th>QuantStudio™3</th>
<th>QuantStudio™5 384-block</th>
<th>QuantStudio™5 96-block</th>
</tr>
</thead>
<tbody>
<tr>
<td>x1-m1</td>
<td><strong>FAM™ and SYBR Green</strong></td>
<td>470 ± 15nm</td>
<td>520 ± 15nm</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>x2-m2</td>
<td><strong>VIC™, JOE™, TET™, HEX™</strong></td>
<td>520 ± 10nm</td>
<td>558 ± 12nm</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>x3-m3</td>
<td><strong>TAMRA™, NED™, ABY™, Cy3™</strong></td>
<td>550 ± 10nm</td>
<td>586 ± 10nm</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>x4-m4</td>
<td><strong>ROX™, JUN™, Texas Red™</strong></td>
<td>580 ± 10nm</td>
<td>623 ± 14nm</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>x5-m5</td>
<td><strong>Mustang Purple™, LIZ™, Cy5™</strong></td>
<td>640 ± 10nm</td>
<td>682 ± 14nm</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>x6-m6</td>
<td>Cy@5.5, Alexa Fluor™</td>
<td>662 ± 10nm</td>
<td>711 ± 12nm</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

**Filters Decoupled**

![Filters Diagram](image-url)
Instrument Configurations: Stand-alone, Desktop, or Online

Connected Laptop with QuantStudio Design and Analysis desktop software

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

TCP Port 80/433 Must be active!

USB

LAN

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

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

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

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

- Monitor Run
- View Results
Instrument Connect Mobile App

Monitor your runs on multiple devices
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

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.
Why link to the Cloud?
The Cloud enables you to download run files from the cloud and automatically upload them when complete.
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
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.
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
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’
Enhanced Instrument Touchscreen (2)

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

![Notifications and Instrument Log Screenshots]
Skip Cycles During a qPCR run

Step 1: Tap Edit

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

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

**Note:**
- Weekly tasks should be performed on a regular basis.
- Monthly tasks are recommended for regular monitoring.
- Every 2 years, perform comprehensive calibrations.
- As needed tasks should be addressed based on operational requirements.