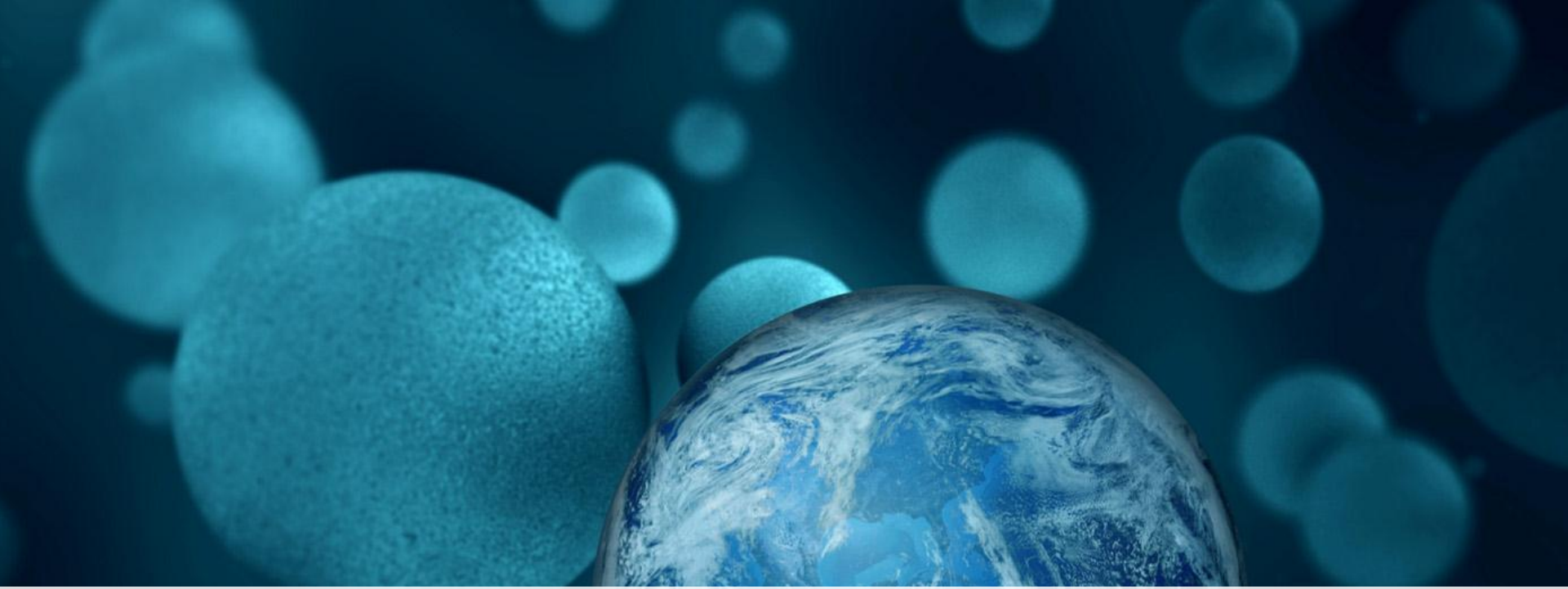

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定量PCR數據分析之常見問題與Troubleshooting

蔡如芸 (Judy Tsai, Ph.D.)
Field Application Scientist

Troubleshooting and FAQ

- Pre-PCR
  - Primer design: bioinformatics
  - Sample Preparation
- Post-PCR
  - Software
  - Operations
  - Instrument
  - Reagents and Consumables

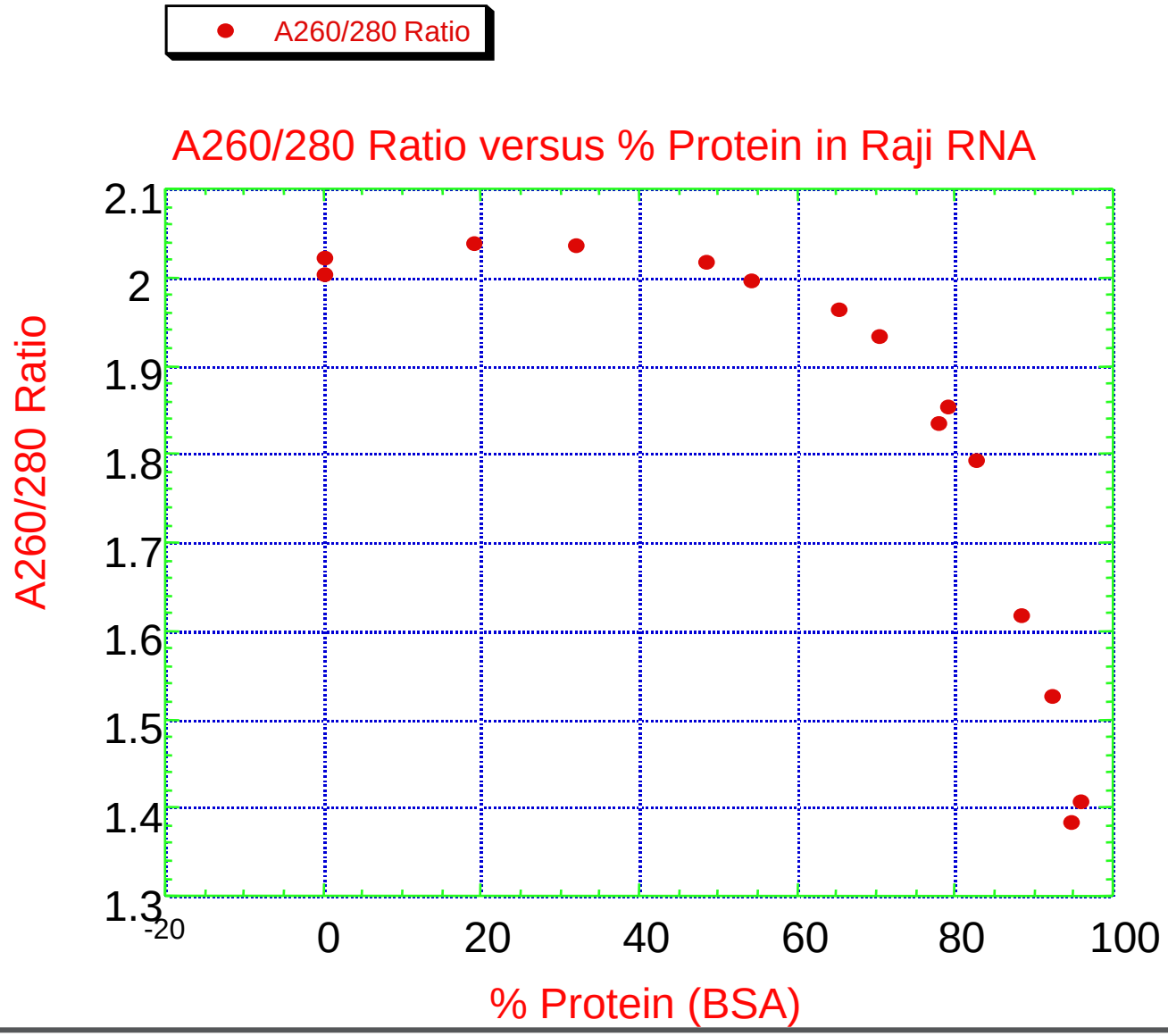
Troubleshooting and FAQ

- Pre-PCR
  - Primer design: bioinformatics evaluations
    - Quality of sequence
    - RepeatMasker (http://www.repeatmasker.org)
    - BLAST in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/)
    - Design tools
      - Custom TaqMan® Assay Design Tool (https://www.thermofisher.com/order/custom-genomic-products/tools/cadt/)
      - Primer Express® 3.0.1
  - Sample Preparation

Troubleshooting and FAQ

- Pre-PCR
  - Primer design: bioinformatics
  - Sample Preparation
    - Purity of DNA/RNA
    - Quantity of DNA/RNA
    - Reverse Transcription for RNA

Protein Contamination Affecting A260/280



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PCR Inhibitors

Sample related:

Heparin > 0.15 mg/ml

Hemoglobin > 1 mg/ml

Melanin, humidic acids, chlorophyll, polysaccharides

Extraction related:

SDS	> 0.01% (w/v)
Phenol	> 0.2% (v/v)
Ethanol	> 1%
Sod. acetate	> 5 mM

PCR additives:

DTT	> 1 mM
DMSO	> 5 %
EDTA	> 50 mM
Mercaptoethanol	

標準曲線檢查樣本品質

- 以一個高濃度的樣本(cDNA or gDNA)作為起始樣本
- 對樣本進行梯度稀釋
- 每個稀釋度重複三次進行絕對定量實驗
- 檢查標準曲線的線性
  - 所有的點都應該落在同一條線上 (R^2)
  - PCR efficiency

Troubleshooting and FAQ

- Pre-PCR
  - Primer design: bioinformatics
  - Sample Preparation
- Post-PCR
  - Software
  - Operations
  - Instrument
  - Reagents and Consumables

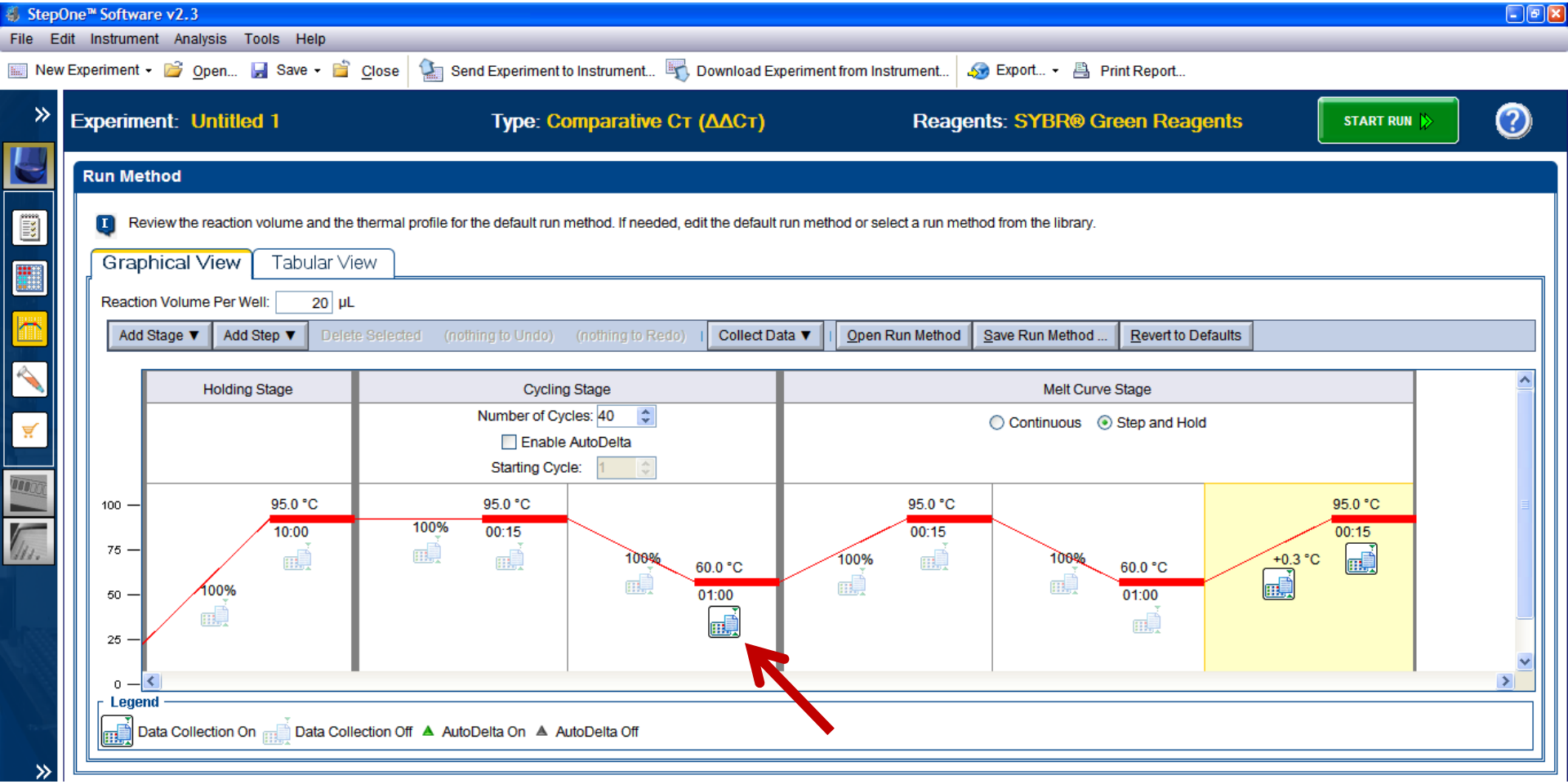
Troubleshooting and FAQ: Error Messages

- “Java.lang.IllegalArgumentException: One of the raw spectra is null”
  - Illegal characters (*e.g.* ~)
  - Double-clicking “Start Run” button
  - Interrupting analysis or saving process (*e.g.* closing experiment or unplugging USB before process completes)

- “Cannot calculate pure dye matrix”
  - Run file has lost raw data (*e.g.* run was aborted)

- “Analysis failed due to GExSession doesn't exist in context”
  - Data collection was turned off during cycling stage

Run Method: Data Collection



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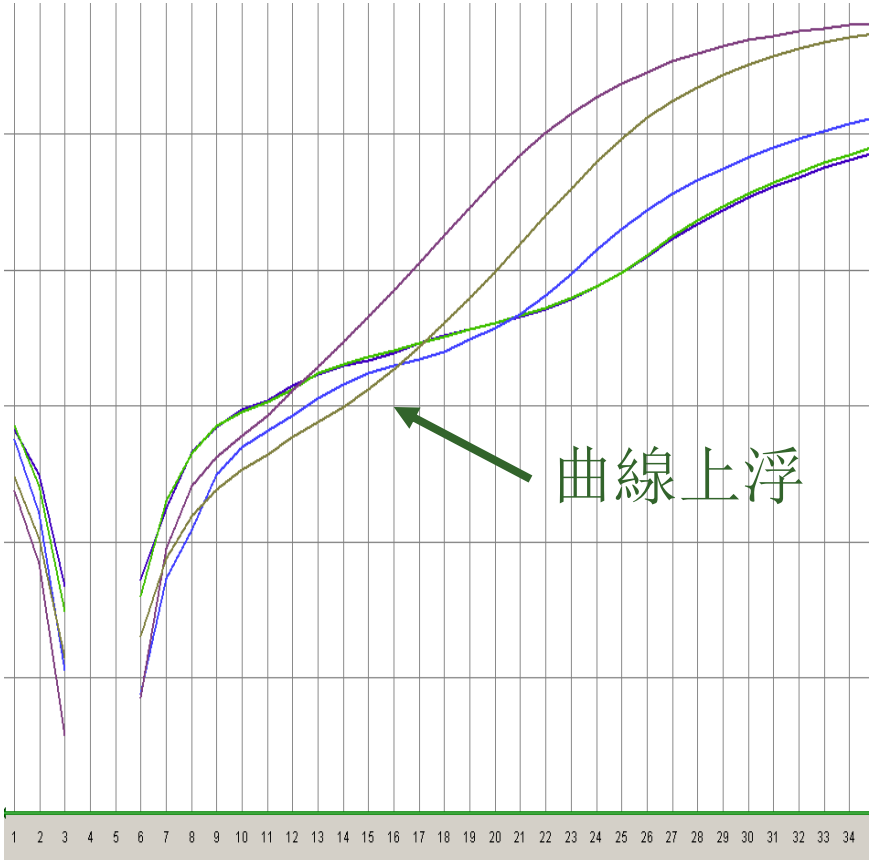
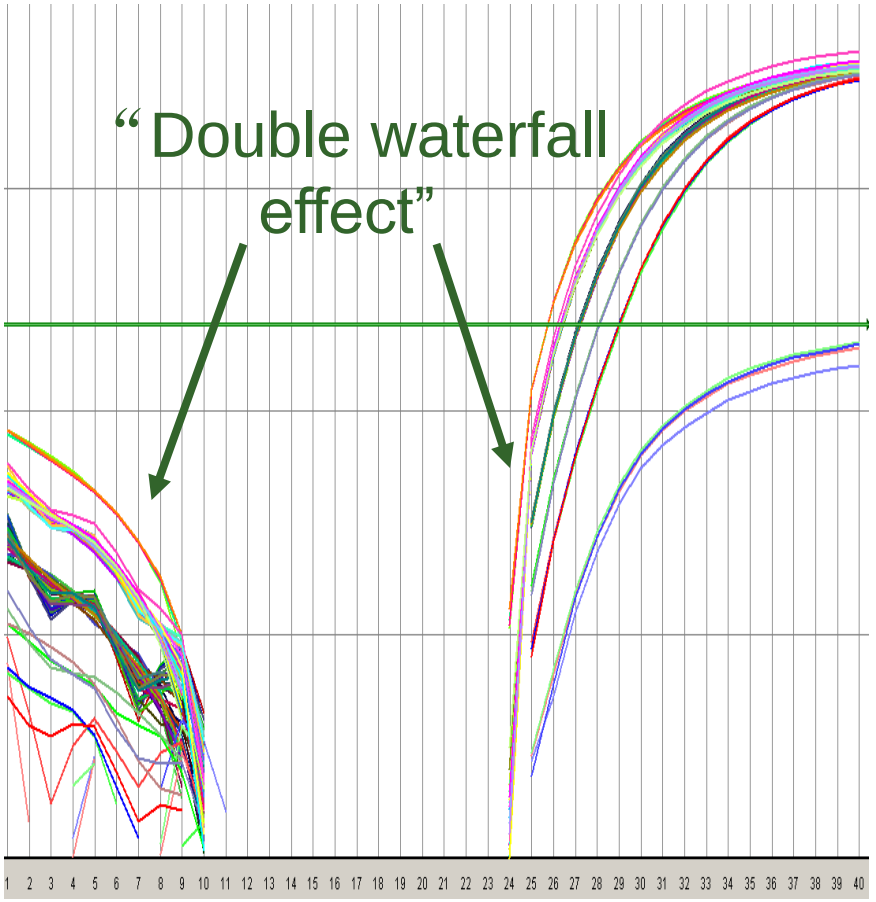
Troubleshooting and FAQ: “Weird” Amplification Plots

- Baseline設置太低

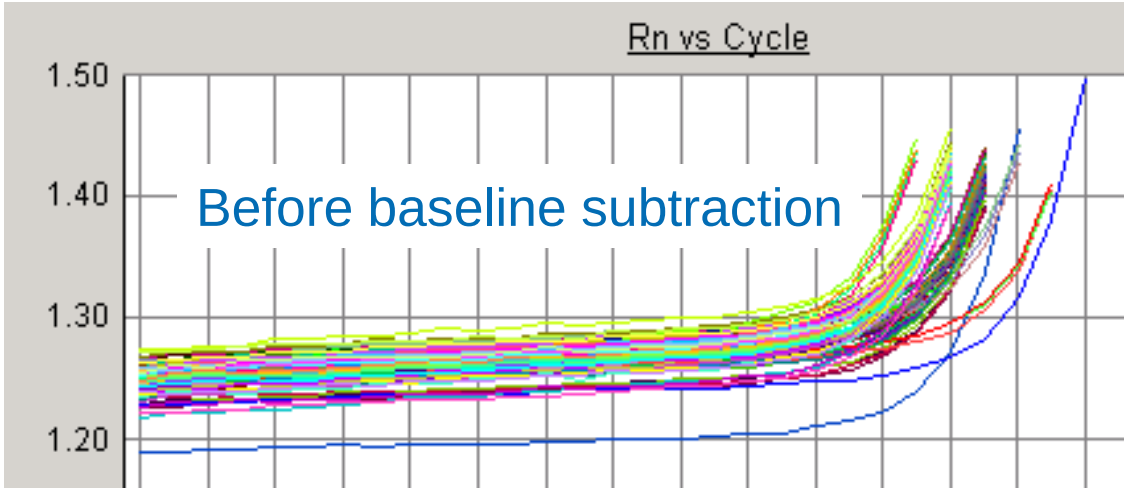


- Baseline設置太高

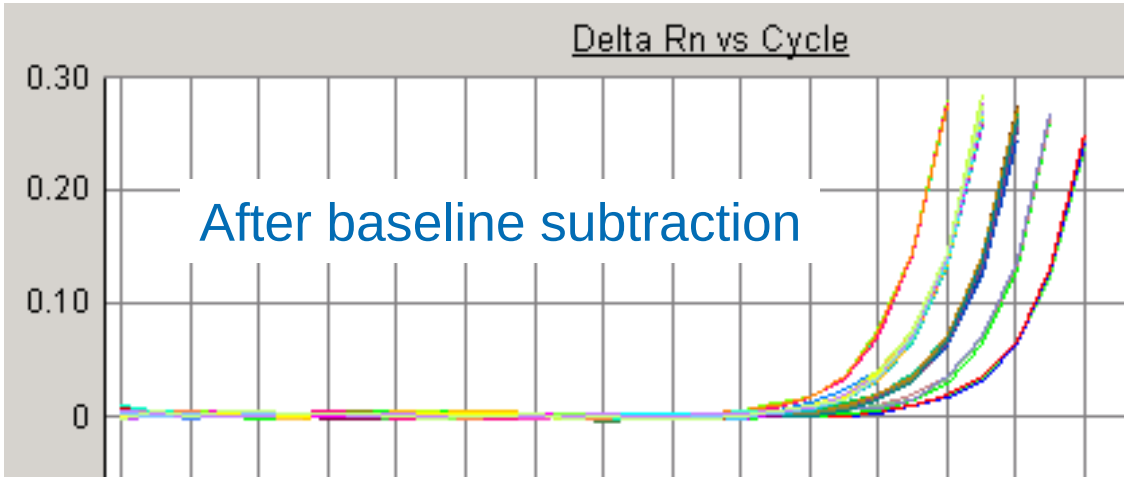


Baseline的作用

Rn

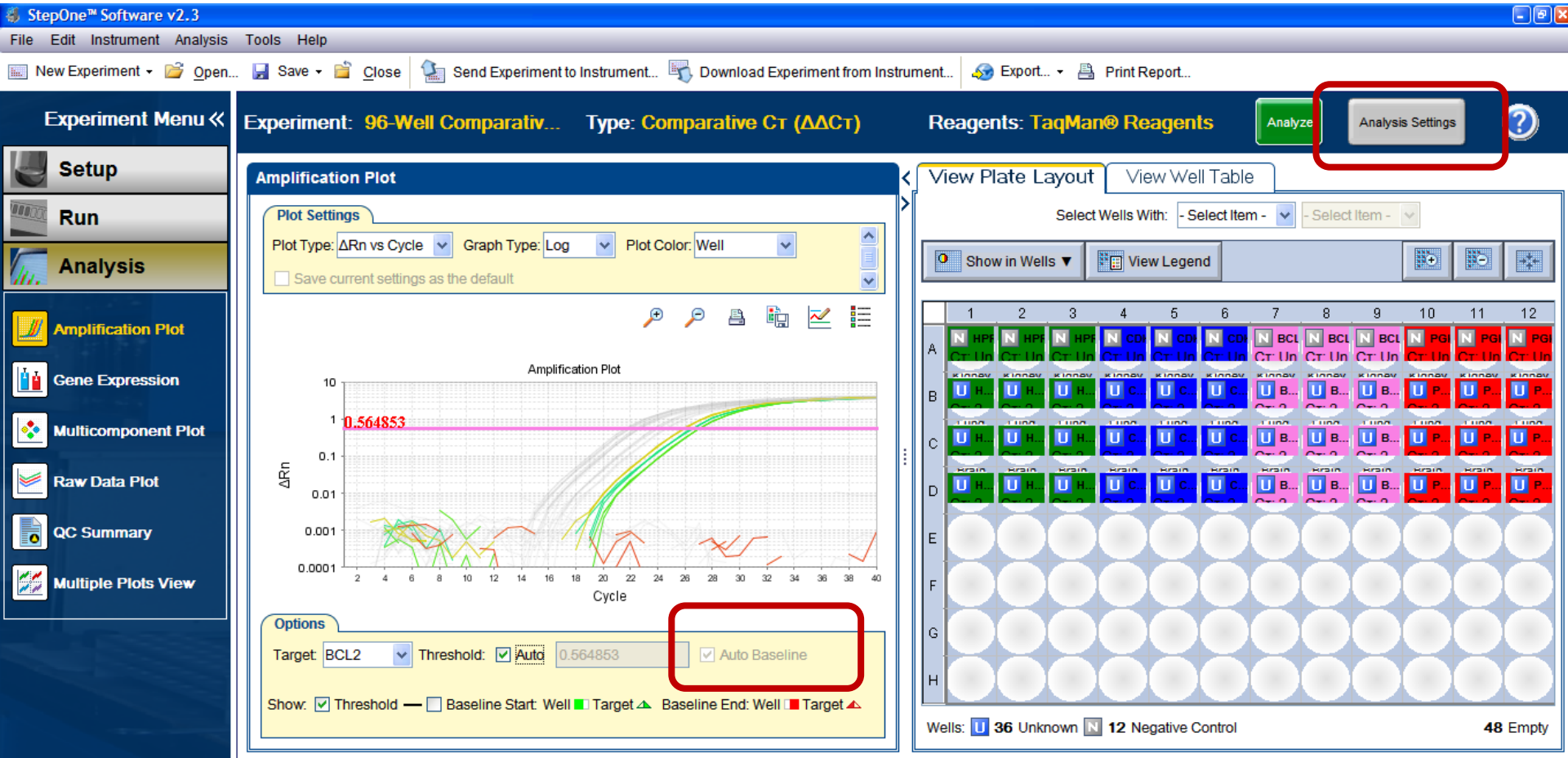


ΔRn



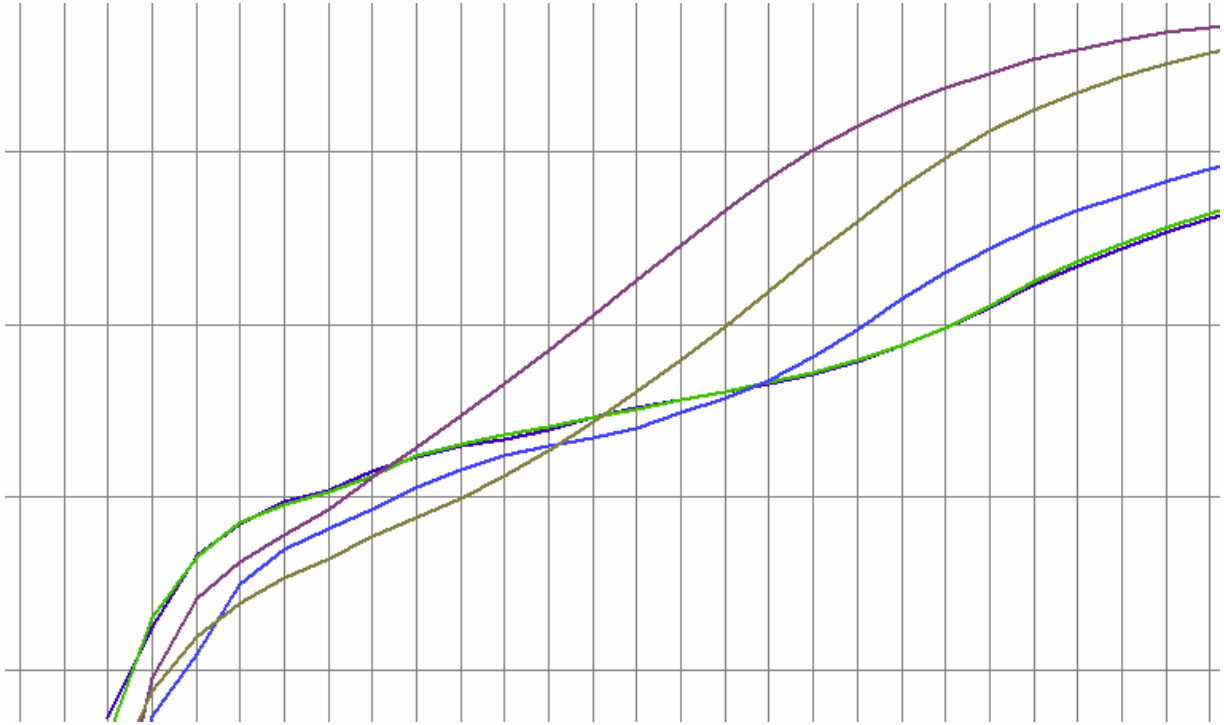
軟體自動設置baseline

- 軟體為每個well單獨設置baseline
- 使每條擴增曲線都能有最佳的baseline設置
- 同時包含高濃度和低濃度樣品的實驗也能準確設置baseline



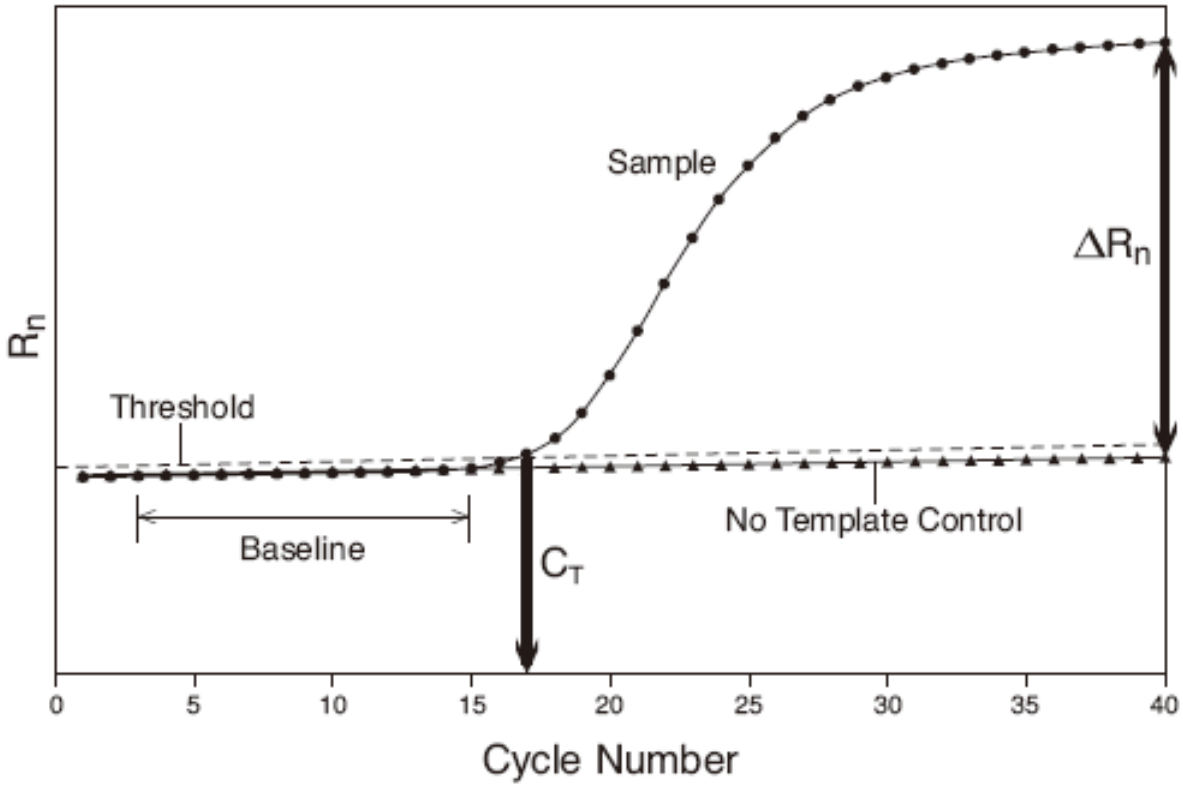
自動baseline設置會出錯嗎?

- Yes . . .
  - 如果試劑的背景值太高，有時會被軟體錯誤地識別為擴增的信號。導致某些樣本的baseline範圍設置過小。
  - 解決方案:手動設置baseline



如何正確手動設置baseline?

- 將Y軸轉為線性 (Linear)
- Baseline需設置在訊號上升之前



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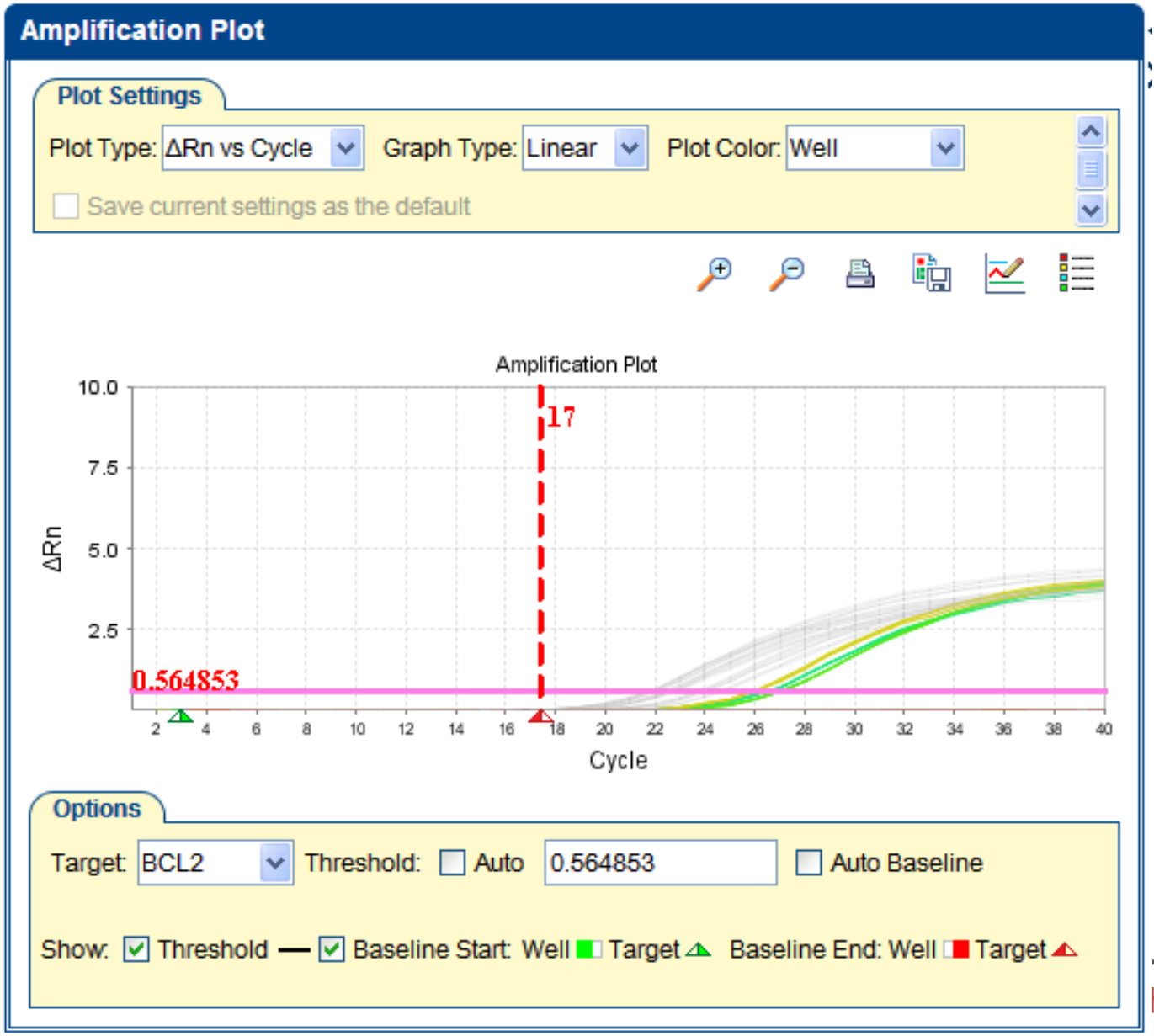
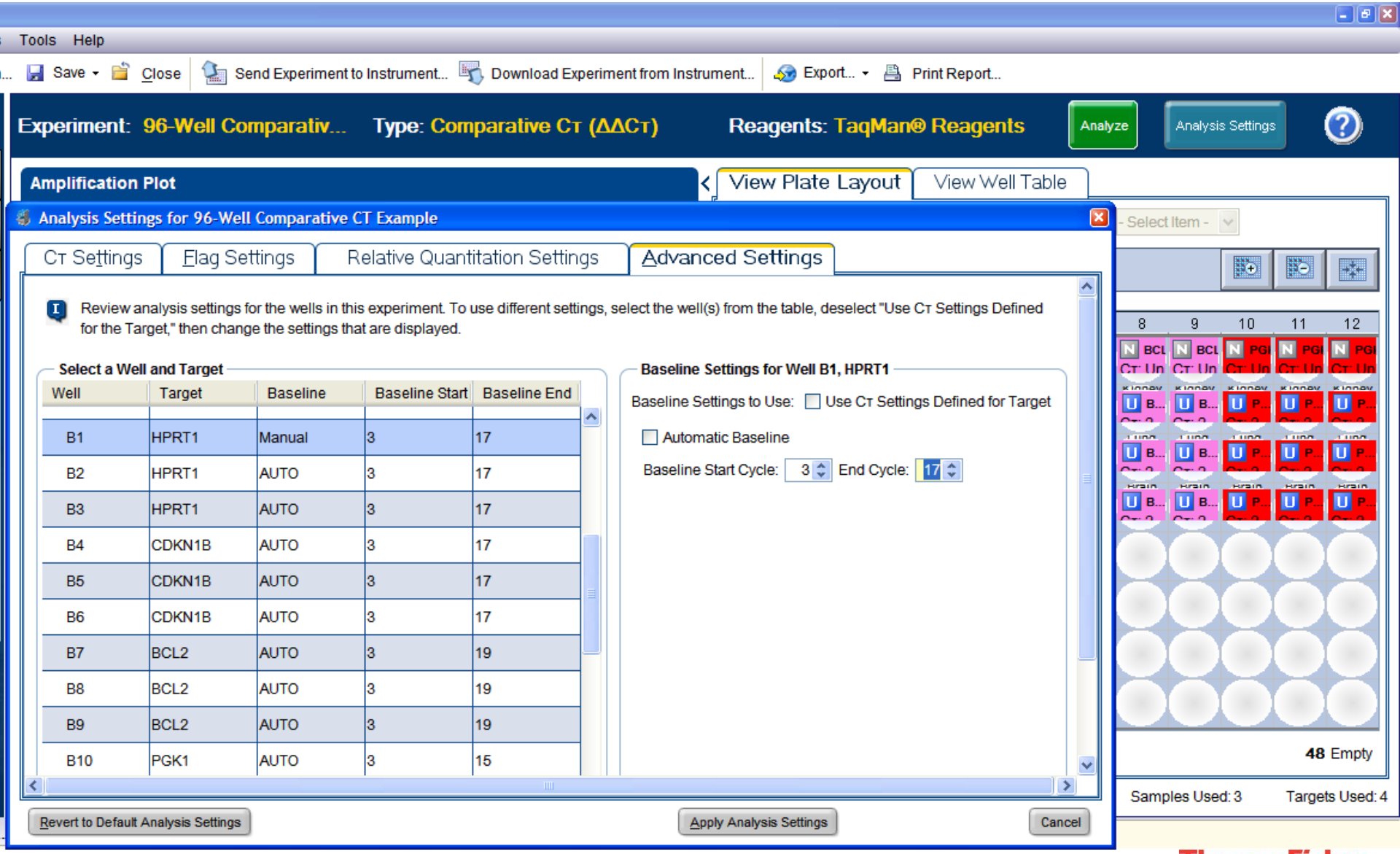
如何正確手動設置baseline?



如何正確手動設置baseline?



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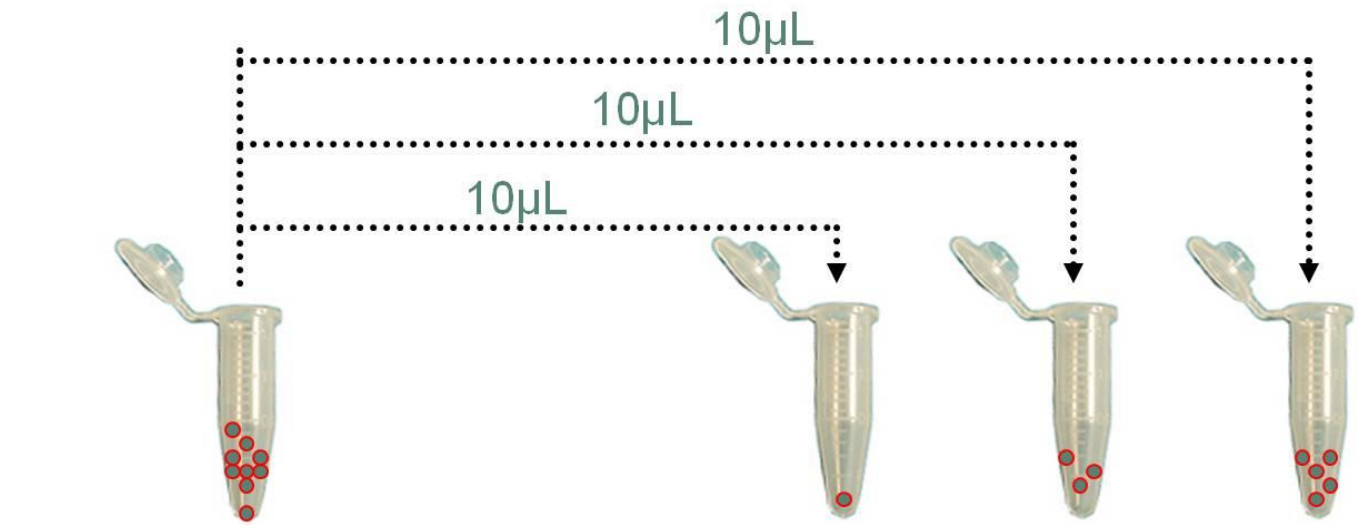
Troubleshooting and FAQ

- Pre-PCR
  - Primer design: bioinformatics
  - Sample Preparation
- Post-PCR
  - Software
  - Operations
  - Instrument
  - Reagents and Consumables
    - High standard deviations in replicates
    - Amplification in NTC

Troubleshooting: High Standard Deviations in Replicates

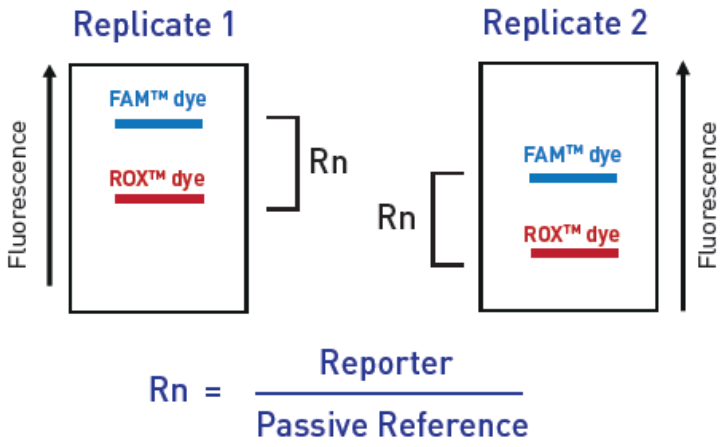
- 加樣誤差(建議每次pipetting體積至少5µl)
- 沒有將試劑和樣品充分混勻
- 低拷貝 (low copy) 的樣品: Poisson distribution



每個反應管均勻的分配到3個templates的幾率有多高?

Passive Reference: ROX™ Dye

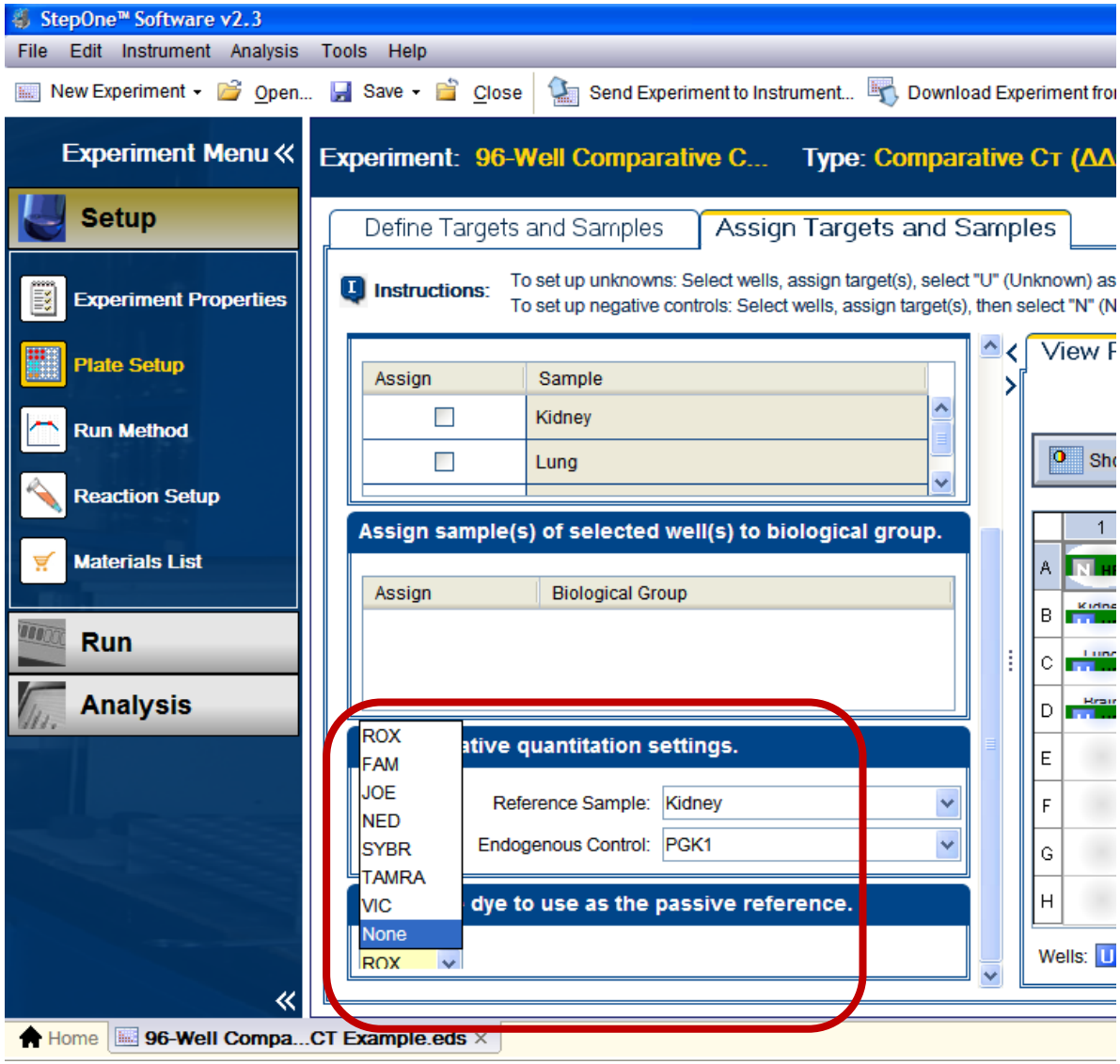
- 哪些原因會影響螢光強度的一致性?
  - Reaction是否充分混和
  - 蒸發
  - 氣泡
  - Pipetting 誤差
  - Condensation
  - Plastic consumables



- ROX™ dye normalizes for non-PCR related fluorescence variations (volume, sample…) →提高重複性
- Only the ROX dye can dynamically correct for fluorescence fluctuations during the PCR reaction
- It is impossible to apply the Ct-calling algorithm of software to correct any variables listed above during data analysis
- An excellent troubleshooting tool

ROX™ Dye Normalization

- Normalization to ROX™ dye is performed automatically by SO software
- Some non-AB PCR master mixes do not contain ROX™ dye; if these are used, the passive reference option should be turned off prior to data analysis



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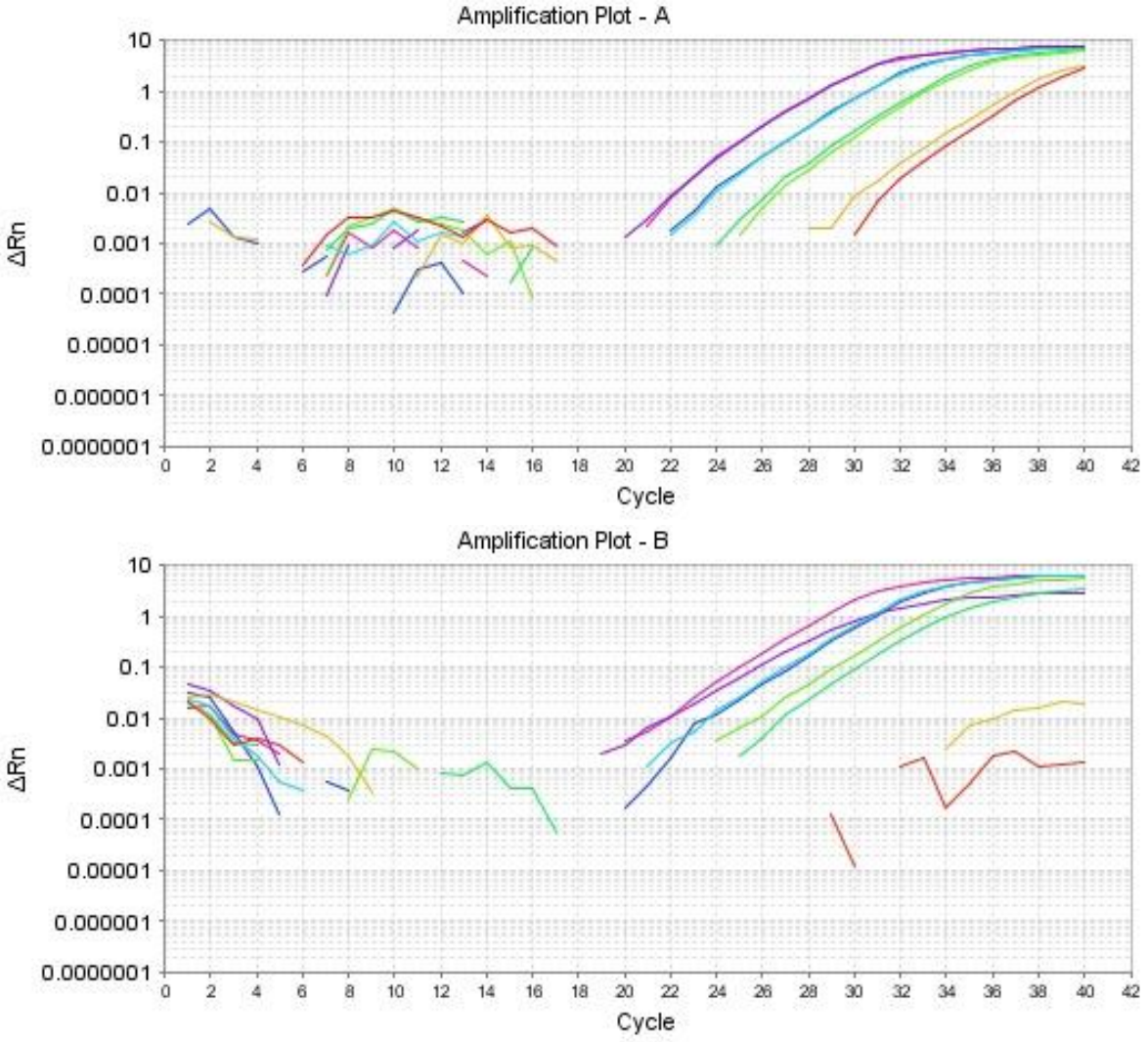
Troubleshooting and FAQ: Consumables

- Exact same reactions using 8-tube strips from different providers on the same run



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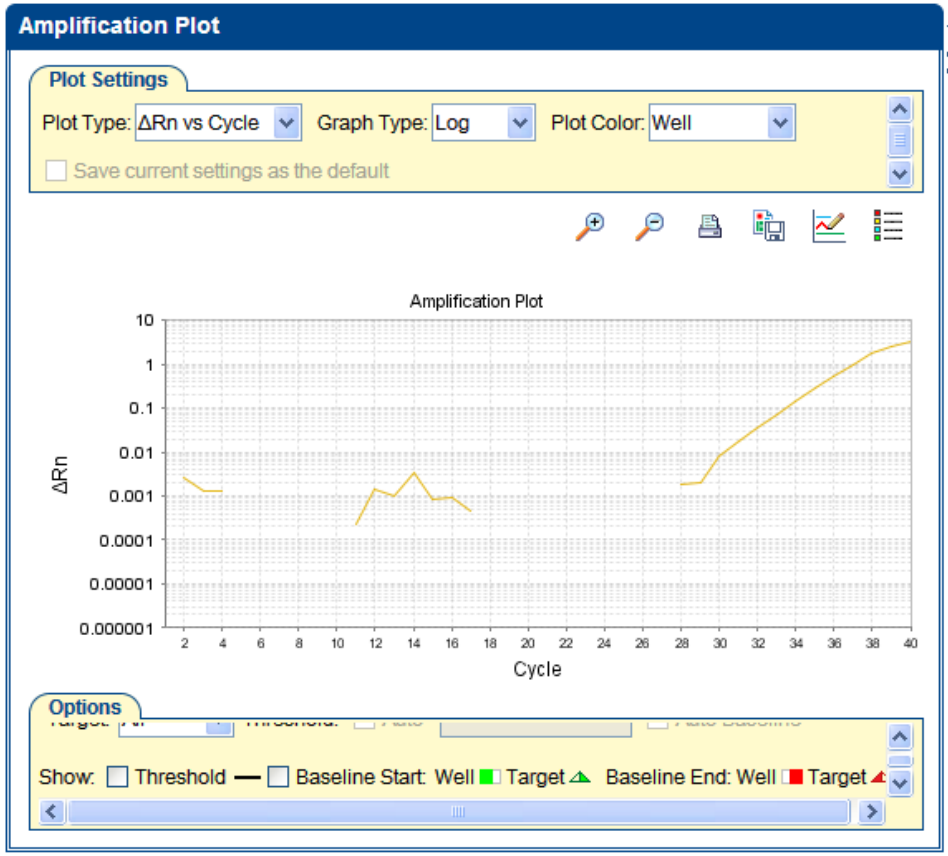
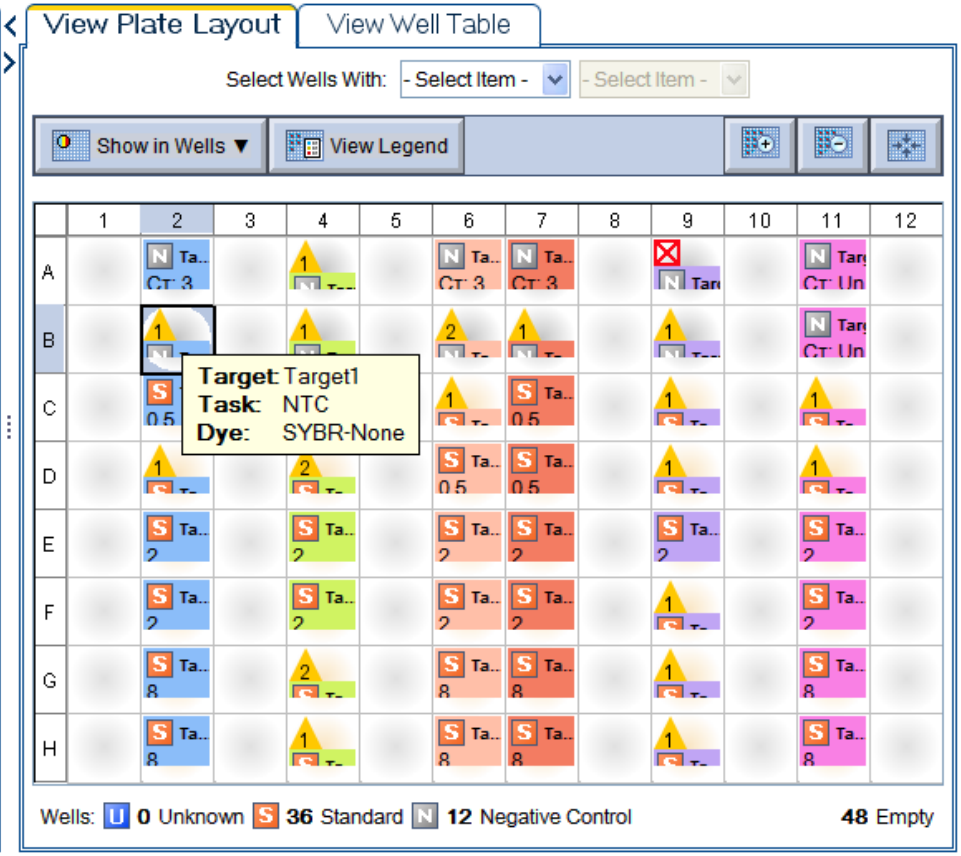
Troubleshooting and FAQ: Amplification in NTC

- Contaminations
- Primer dimer (SYBR green experiments)





Analysis Summary: Total Wells in Plate: 96 | Wells Set Up: 48 | Wells Omitted Manually: 1 | Wells Flagged: 19 | Wells Omitted by Analysis: 0 | Samples Used: 3 | Targets Used: 6

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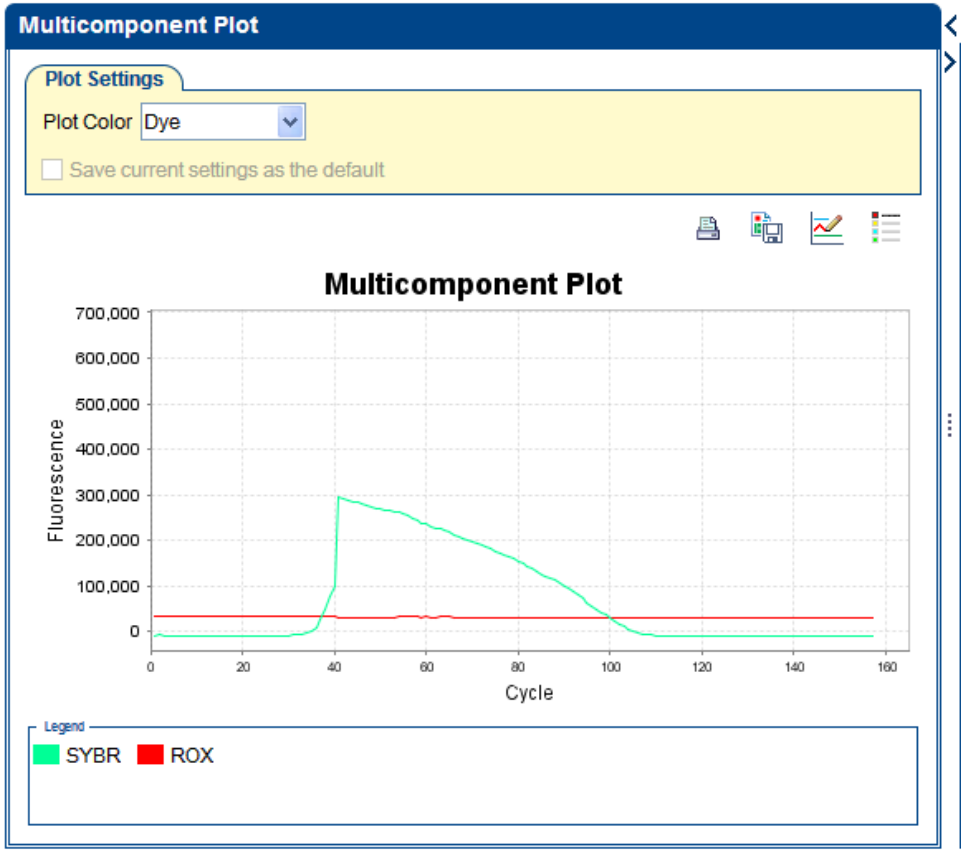
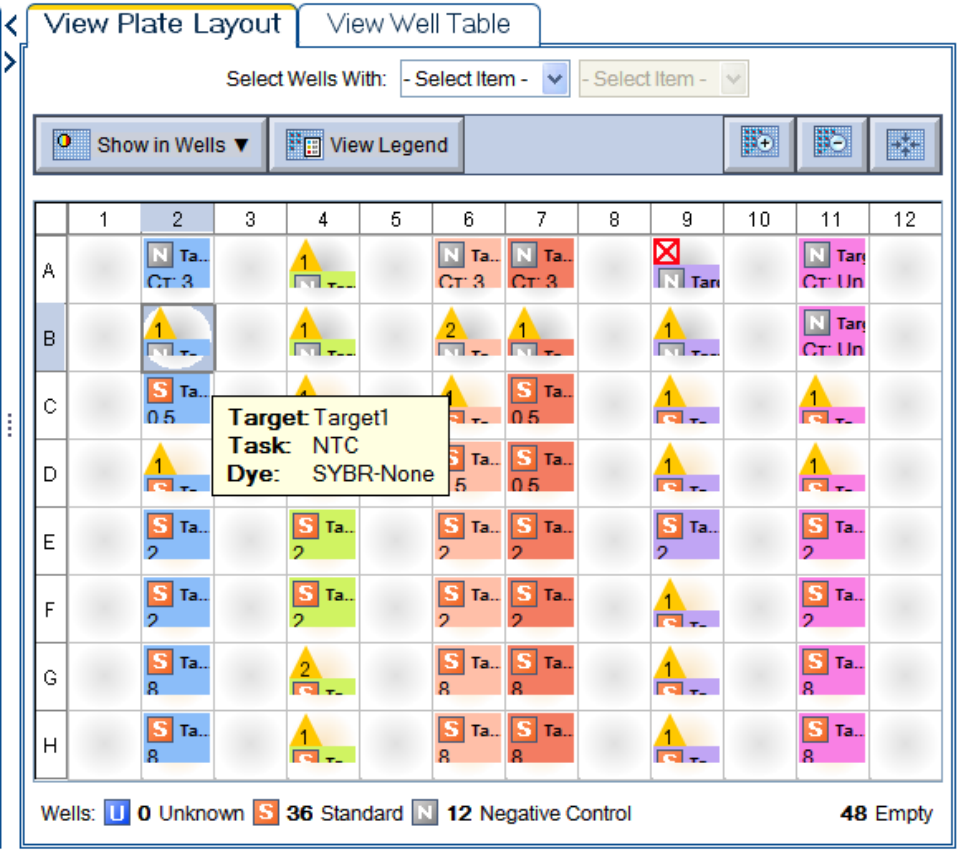
從Multicomponent Plot 確認Report Dye是否有上升





Analysis Summary: Total Wells in Plate: 96 | Wells Set Up: 48 | Wells Omitted Manually: 1 | Wells Flagged: 19 | Wells Omitted by Analysis: 0 | Samples Used: 3 | Targets Used: 6

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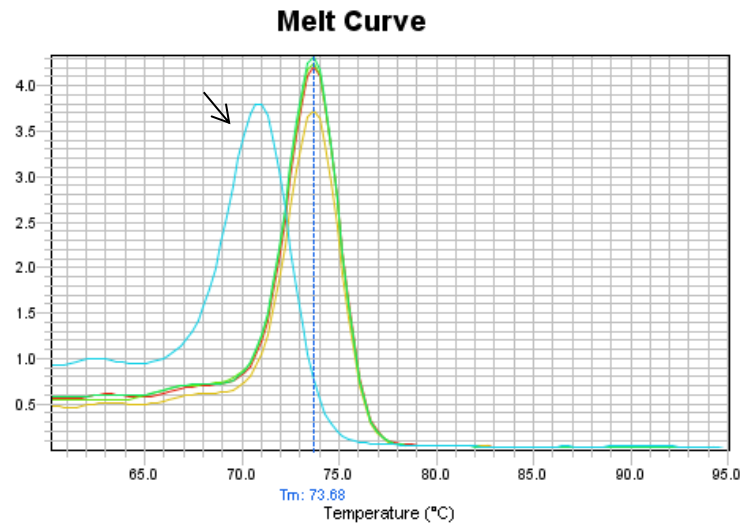
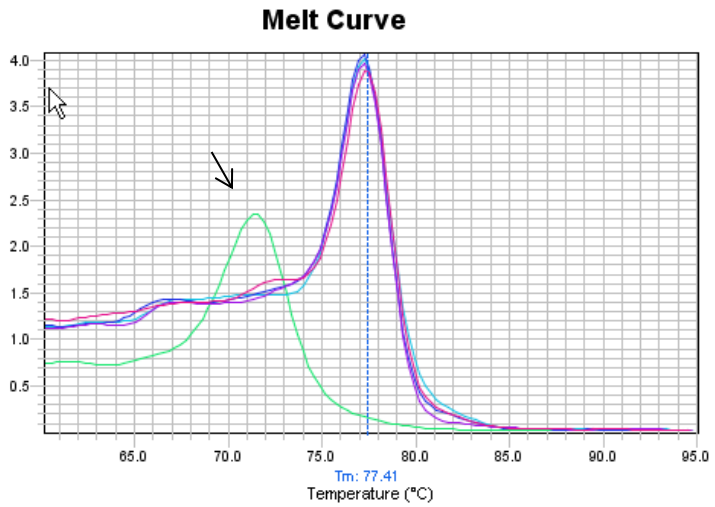
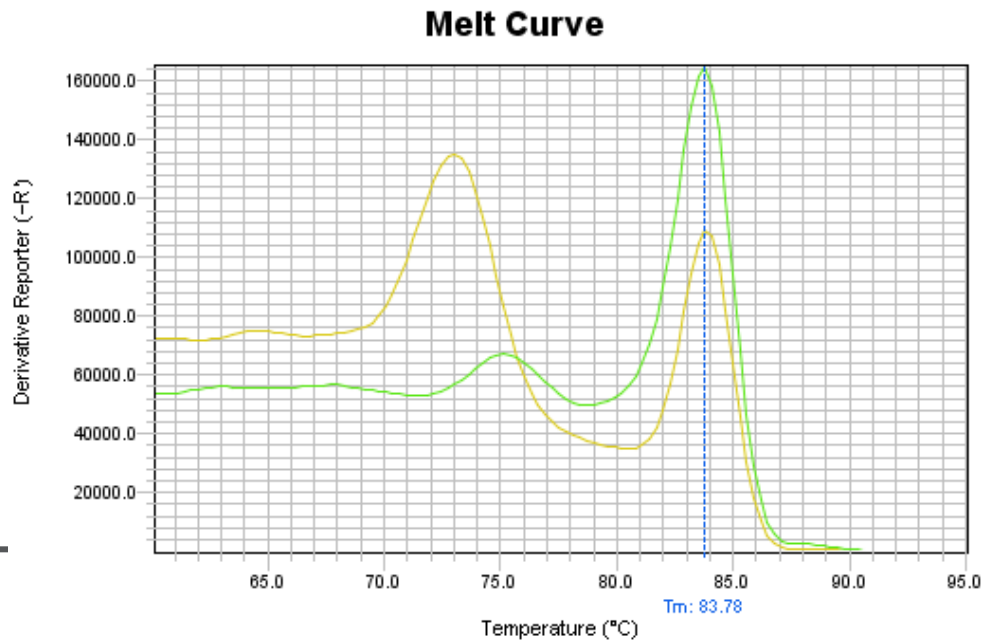
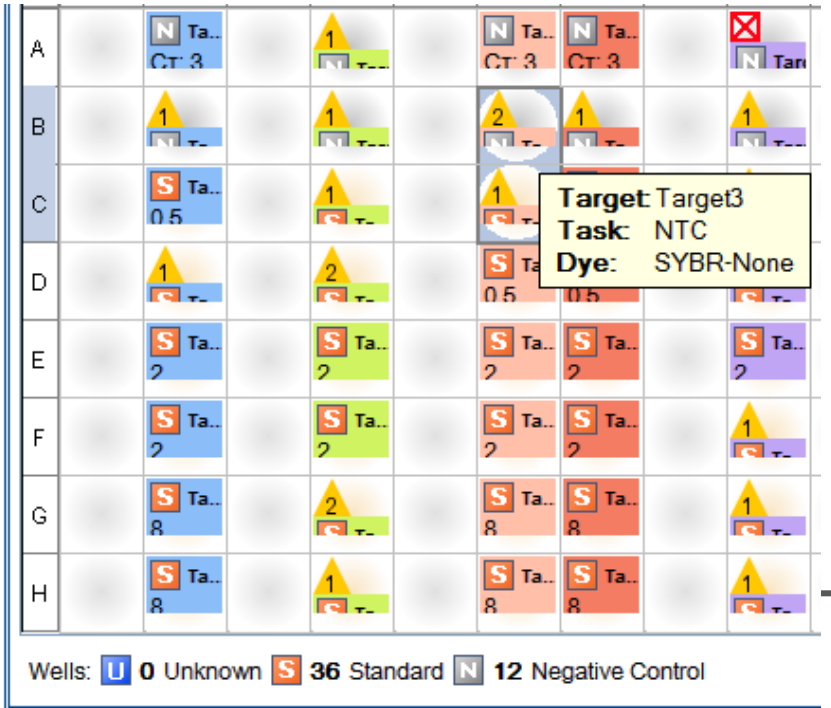
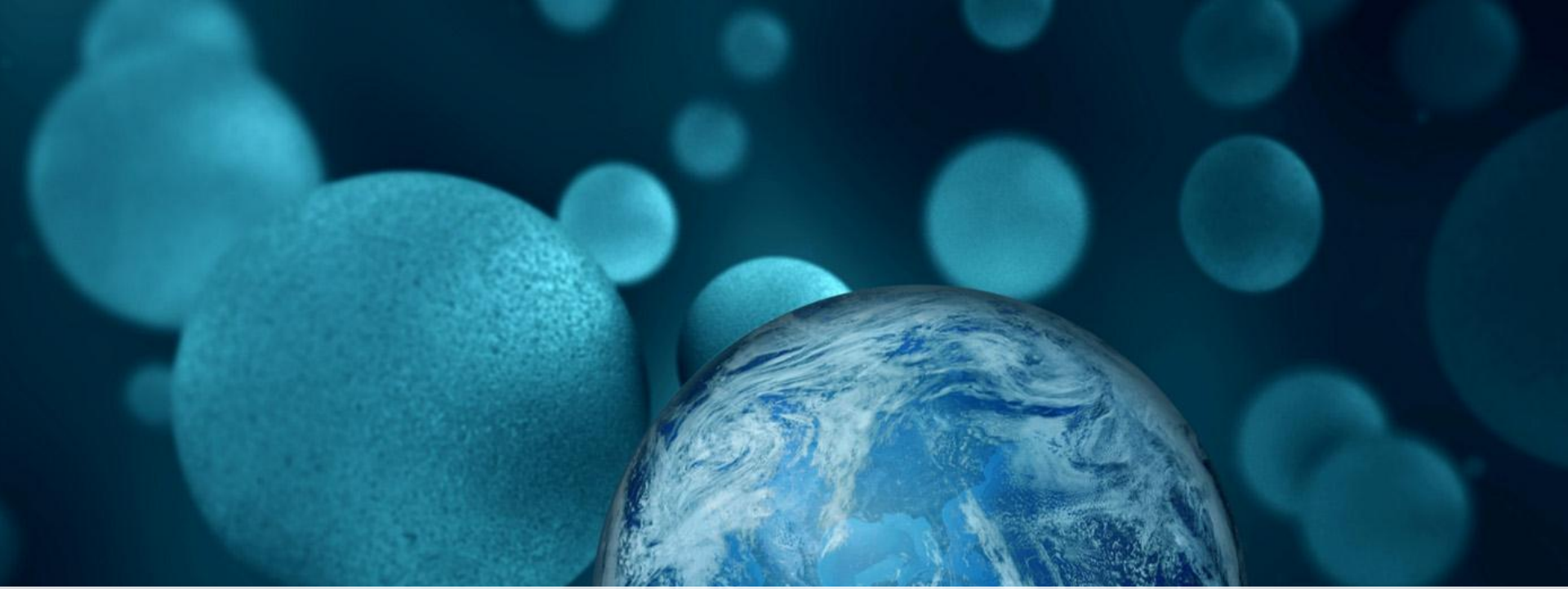
從Melt Curve確認是因為Primer Dimer或是有汙染











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Introducing Thermo Fisher Cloud

蔡如芸 (Judy Tsai, Ph.D.)
Field Application Scientist

Analysis Modules - Compatible Instrument Systems

Real-Time PCR System	Supported software version(s)	File extension
Applied Biosystems™ 7900HT Fast Real-Time PCR System	v2.4 or later	*.sds
Applied Biosystems™ 7500 Real-Time PCR System Applied Biosystems™ 7500 Fast Real-Time PCR System	v2.0.5 or later	*.eds
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR Systems	v2.0.1, v2.1, or later	
Applied Biosystems™ ViiA™ 7 Real-Time PCR System	v1.1 or later	
Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System	v1.1.1 or later	
Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System	v1.0 or later	
Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System		

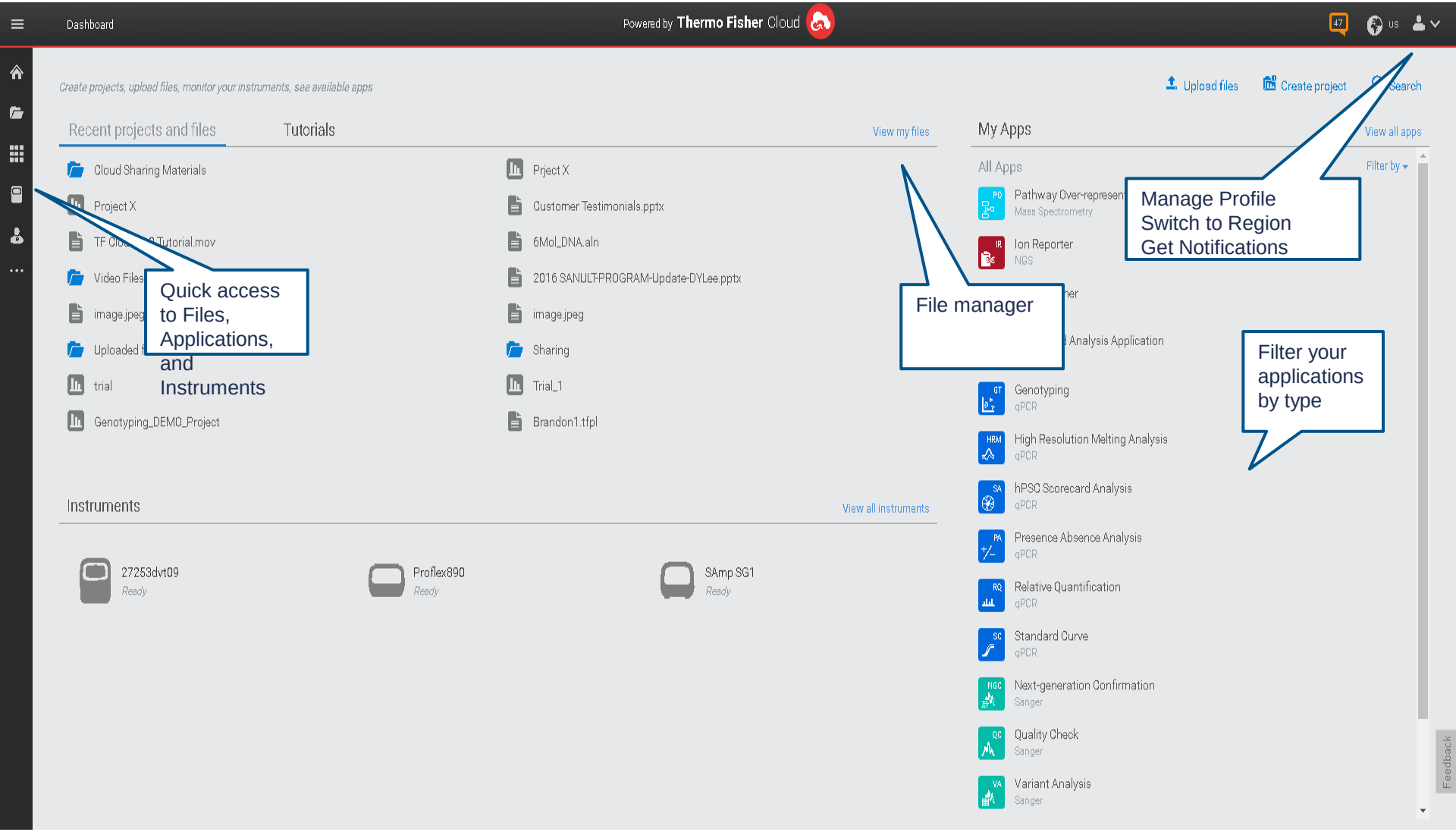
Thermo Fisher Cloud Storage

- Start with **10 GB free**, purchase more as you need it
- How much storage do I need?

PCR	Plates per day	Storage needed annually
96 well	5	1.3 Gb
	10	2.5 Gb
	20	5 Gb
384 well	5	5 Gb
	10	10 Gb
	20	20 Gb
OpenArray®	5	90 Gb
	10	180 Gb
	20	360 Gb
Sanger	Plates per day	Storage needed annually
96 well	1	10 Gb
	2	20 Gb
	4	40 Gb
	8	80 Gb
	12	120 Gb

- Terms and Conditions for Thermo Fisher Cloud Storage Plans
  - http://www.thermofisher.com/tw/en/home/cloud/data-storage.html

Dashboard



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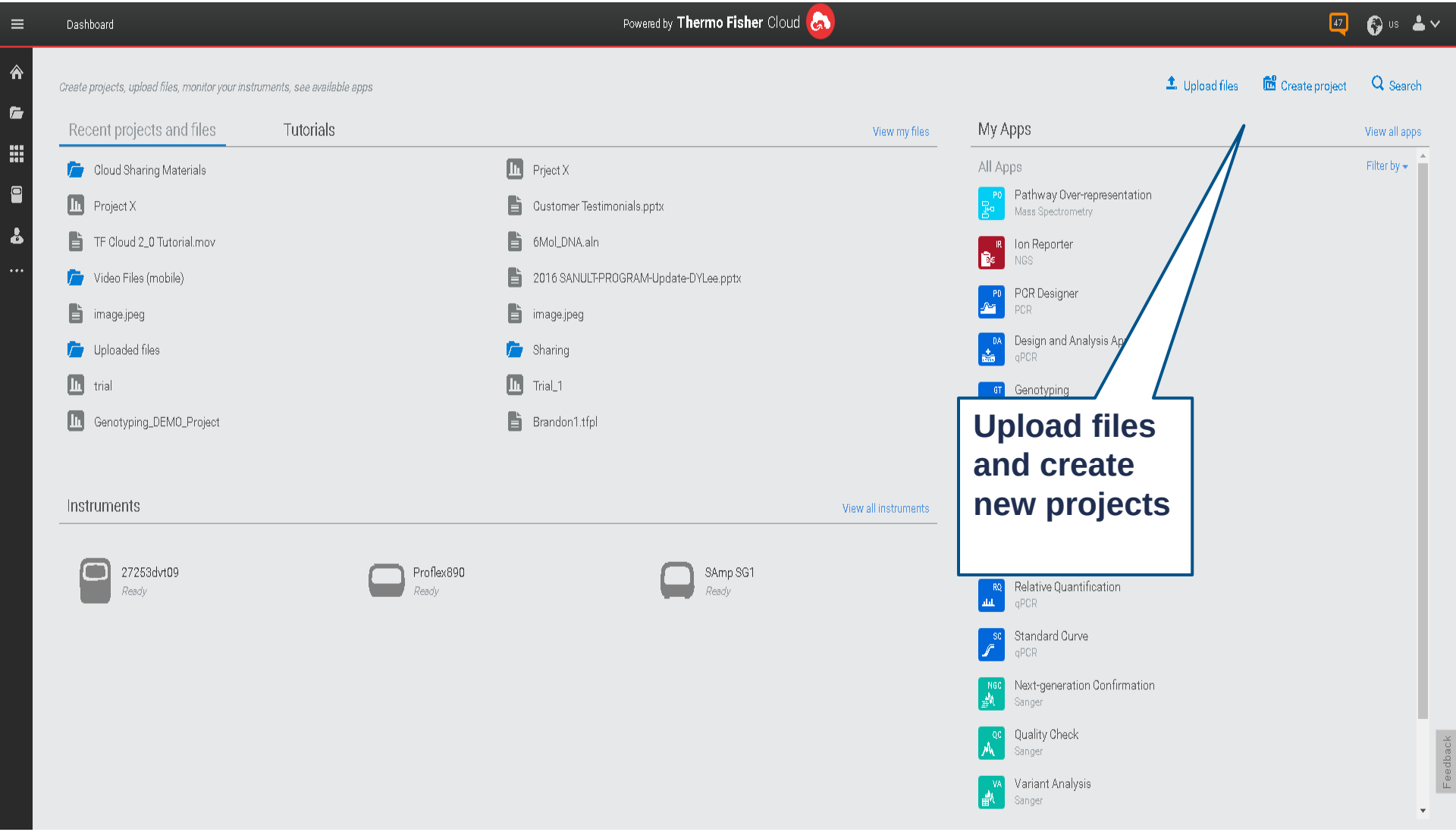
Dashboard



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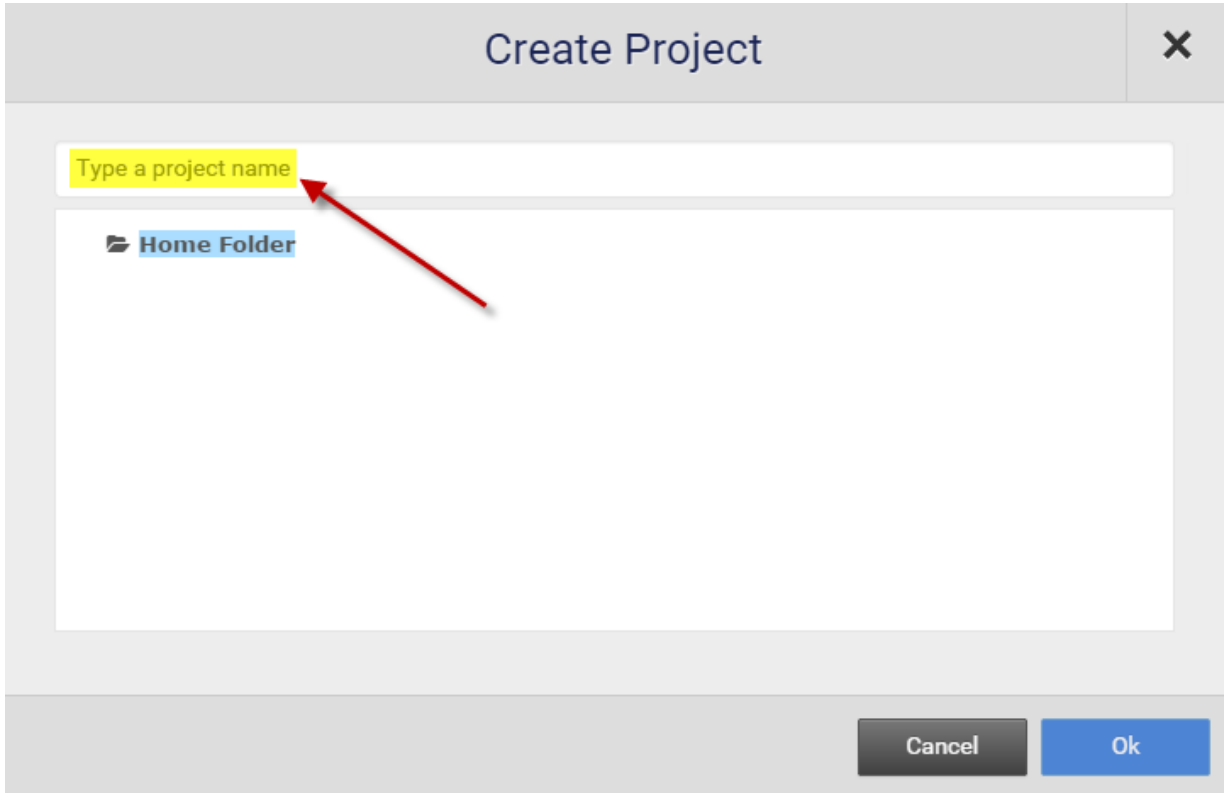
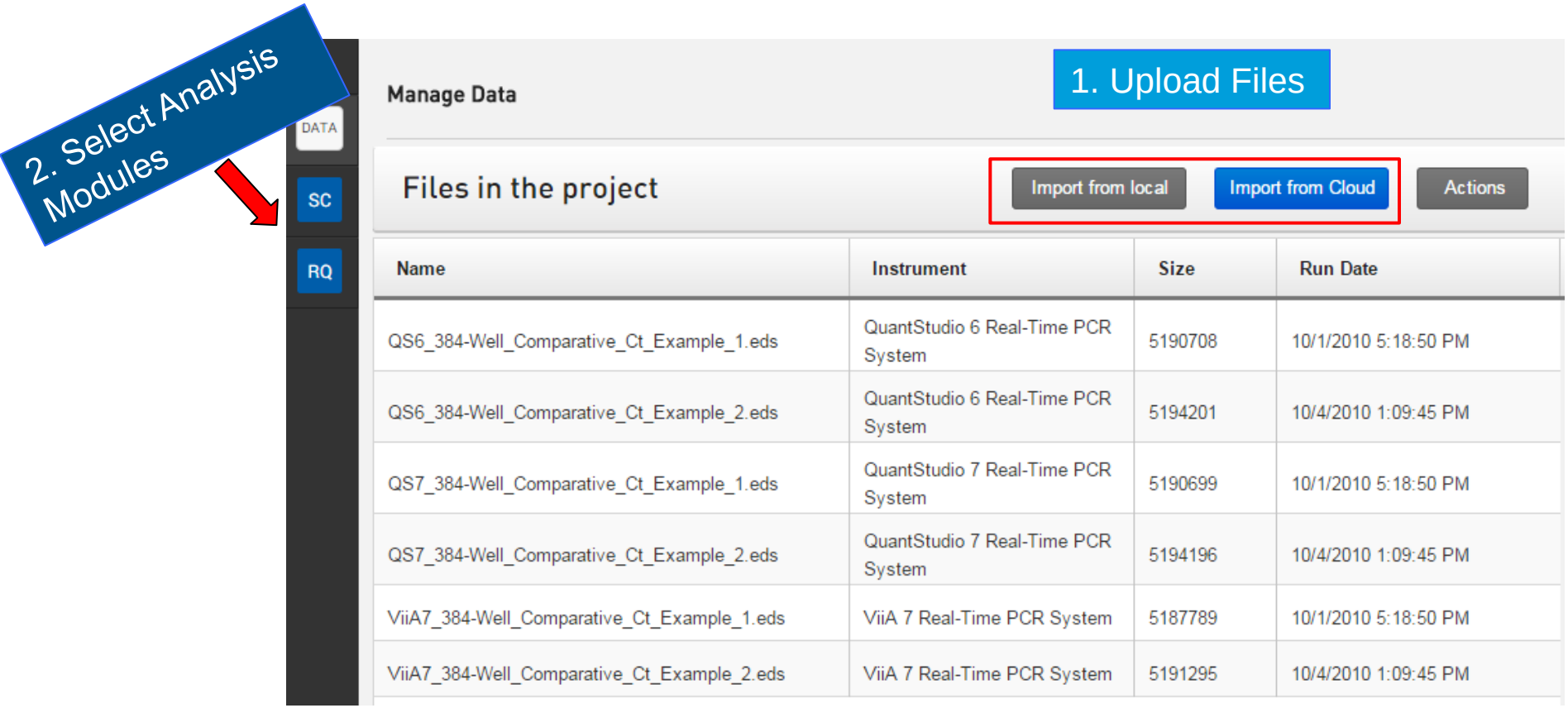
Getting Started



In a Project

- Upload eds/sds files either from data manager or local drive
- Select Analysis Module of choice: AQ, RQ or GT

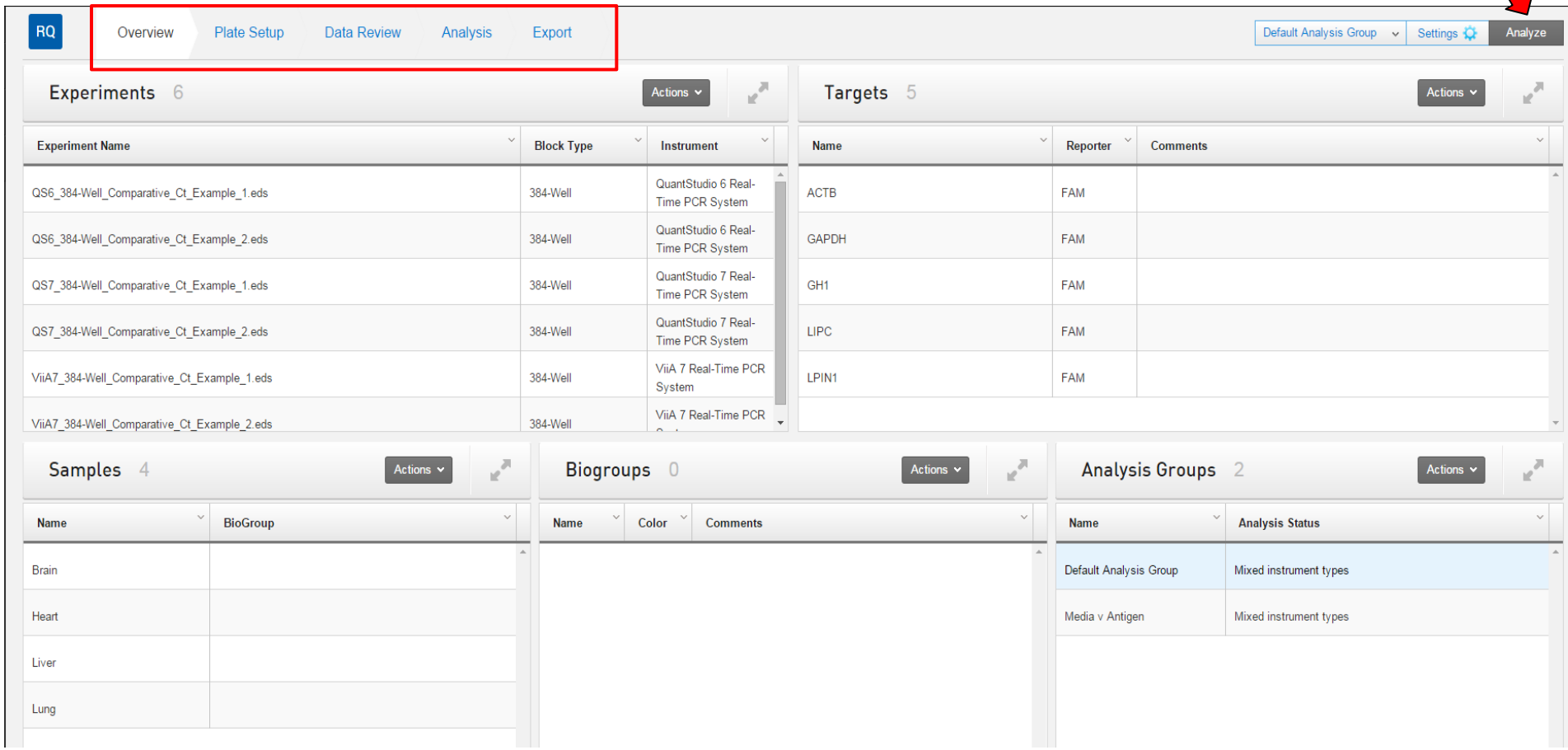


Relative Quantification Analysis Module

Project Overview

Analyze Button

Basic navigation across the top



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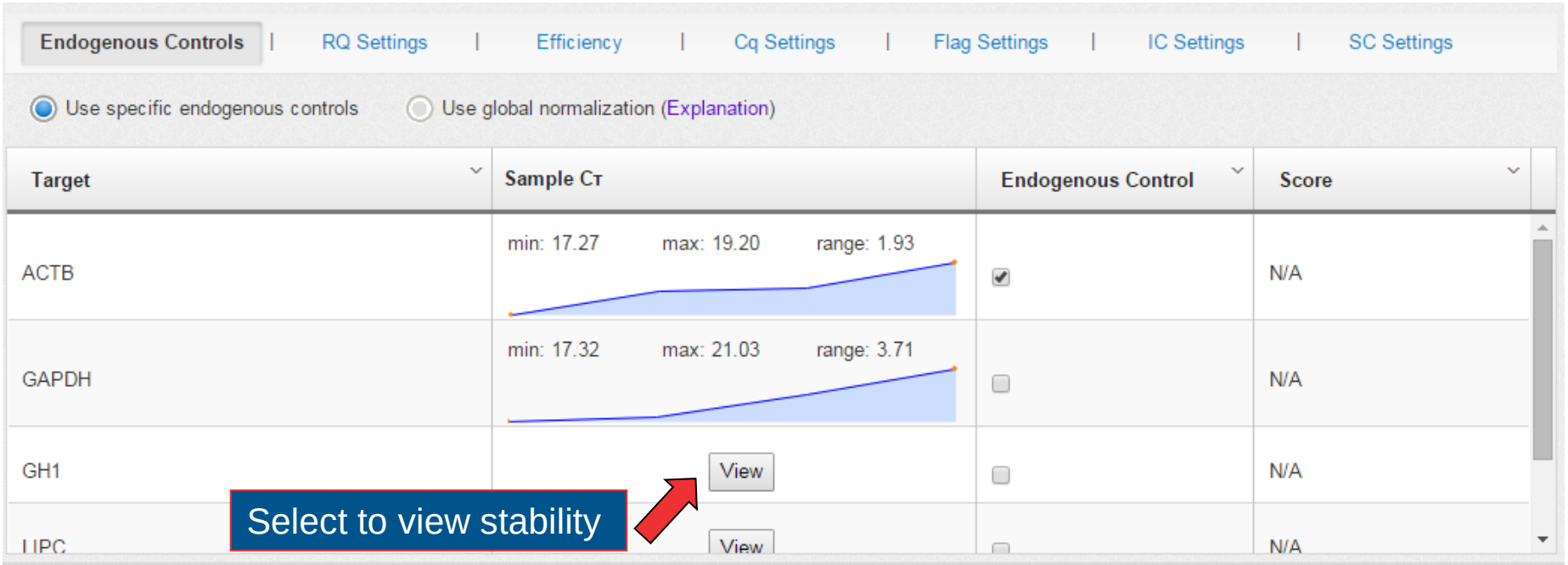
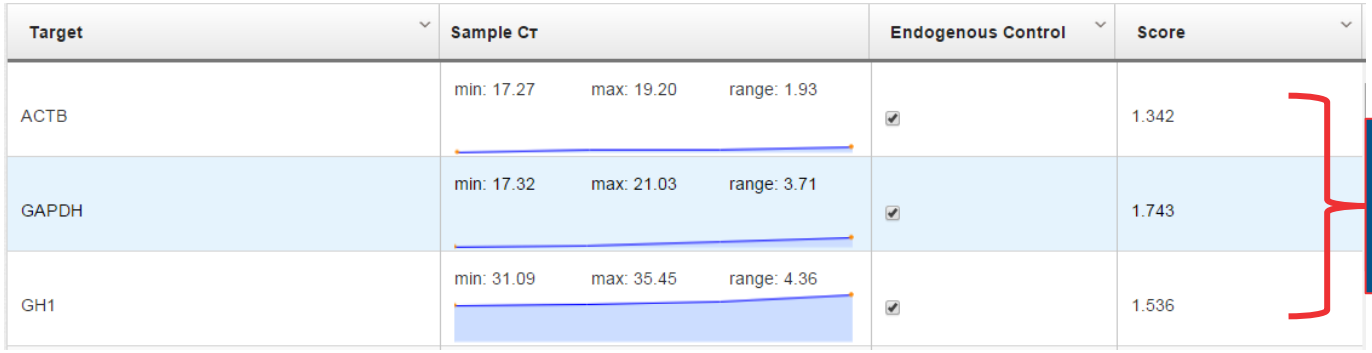
Analysis Settings: Endogenous Controls





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Analysis Settings: Inter-Plate Calibrator Setting

- No need for endogenous control on every plate
- Required to have same sample and target combination in a particular well for each plate in a project
- Select “Allow calculation as dCT” allows all plates to be analyzed together as one plate
- If checkbox is NOT selected, and no inter-plate calibrator is present, this will result in typical endogenous control calculation

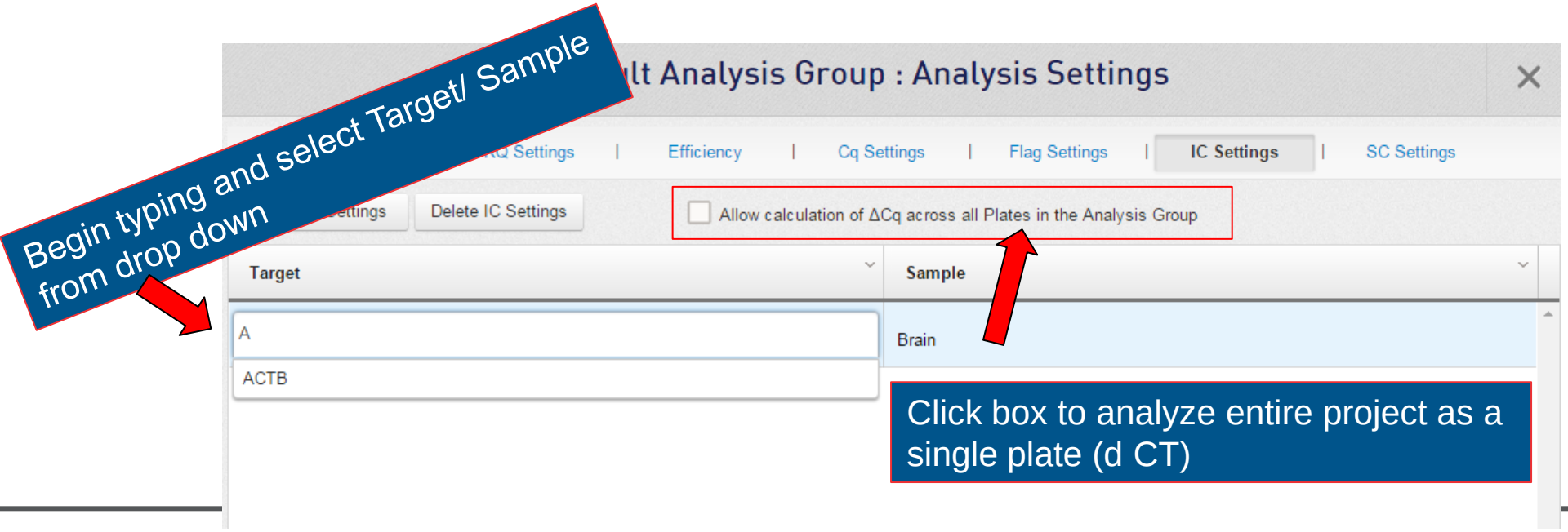

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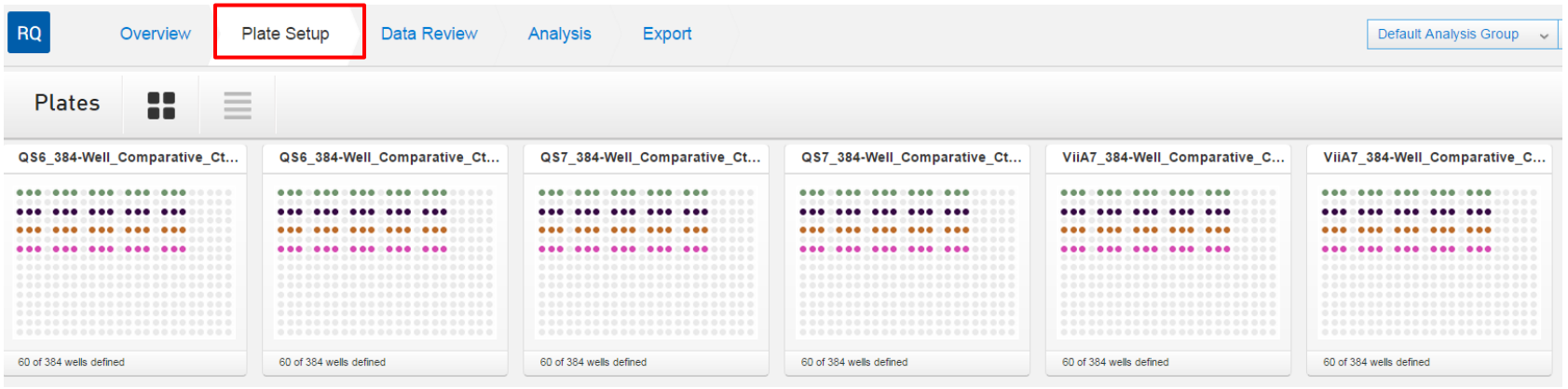
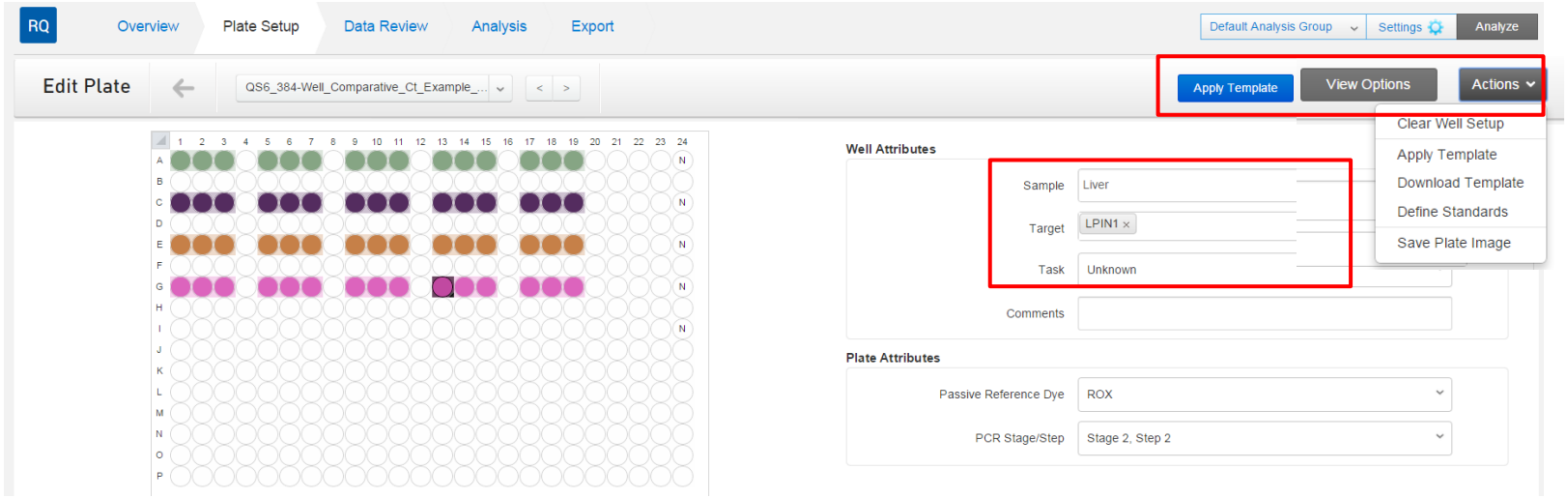
Plate Set Up: Change sample/ target layout here!

No need to go back to instrument software!!!





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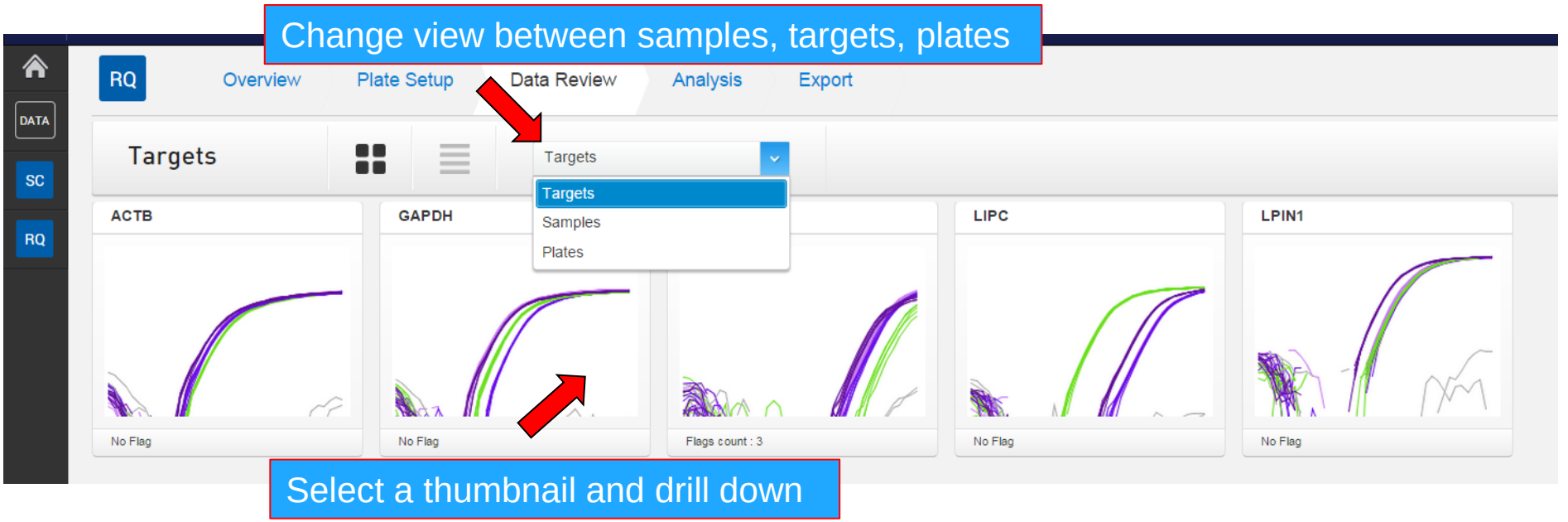
Data Review: Quick View of Samples, Targets, Plates



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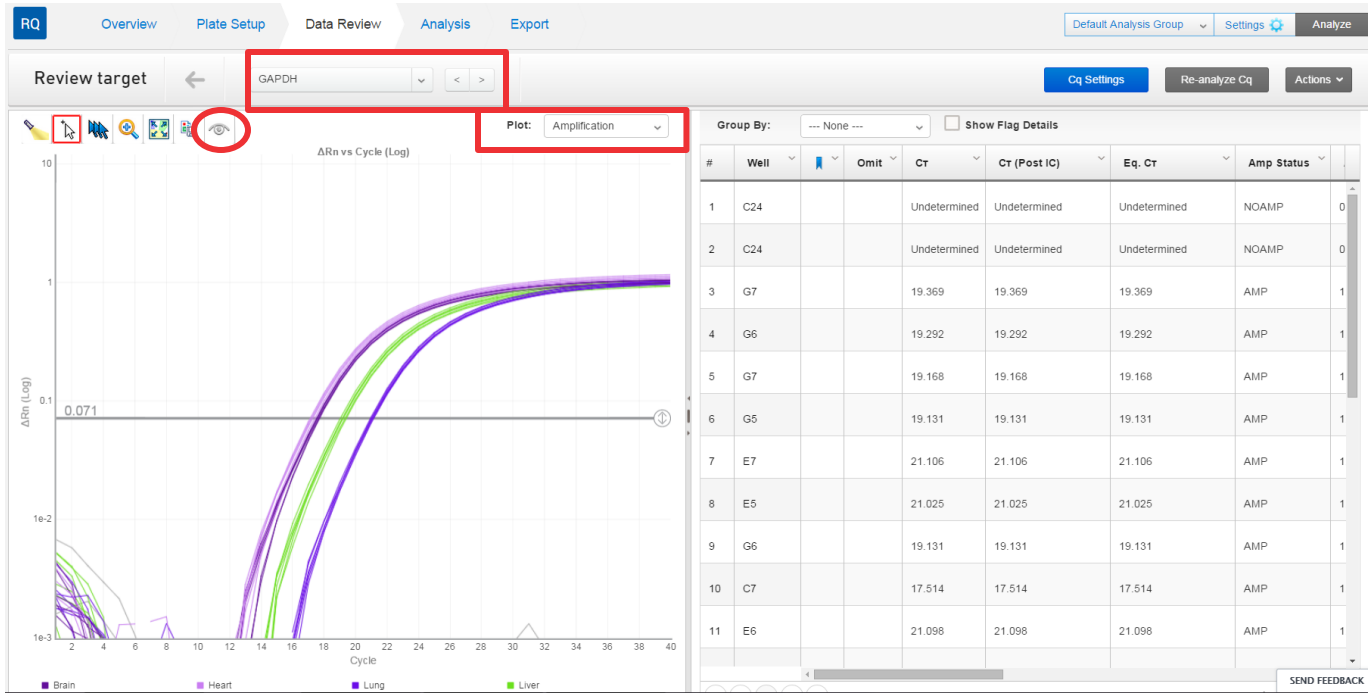
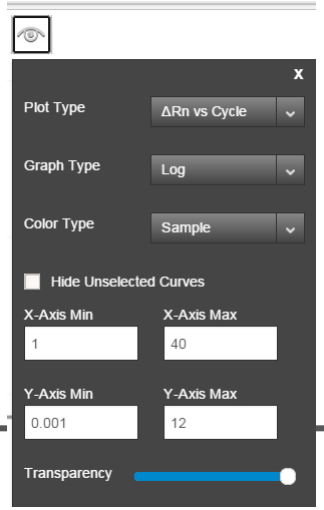
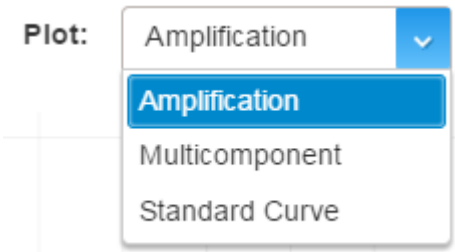
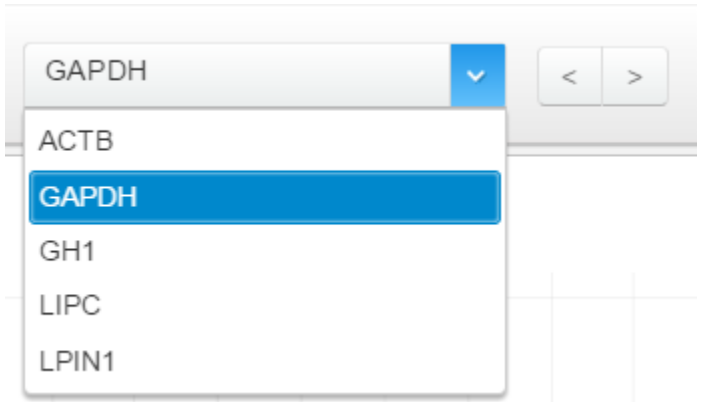
Data Review: Detailed Amplification Curves









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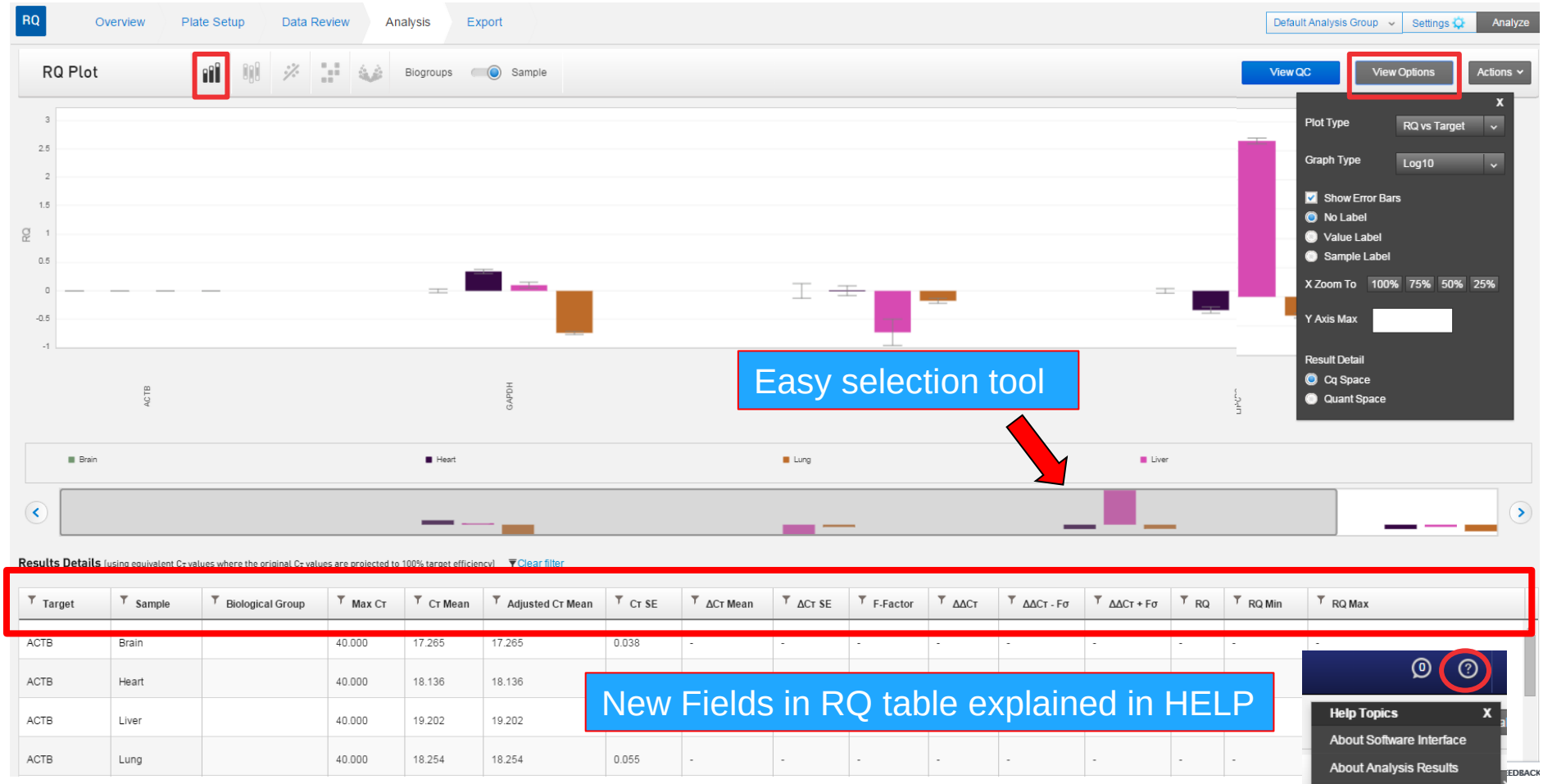
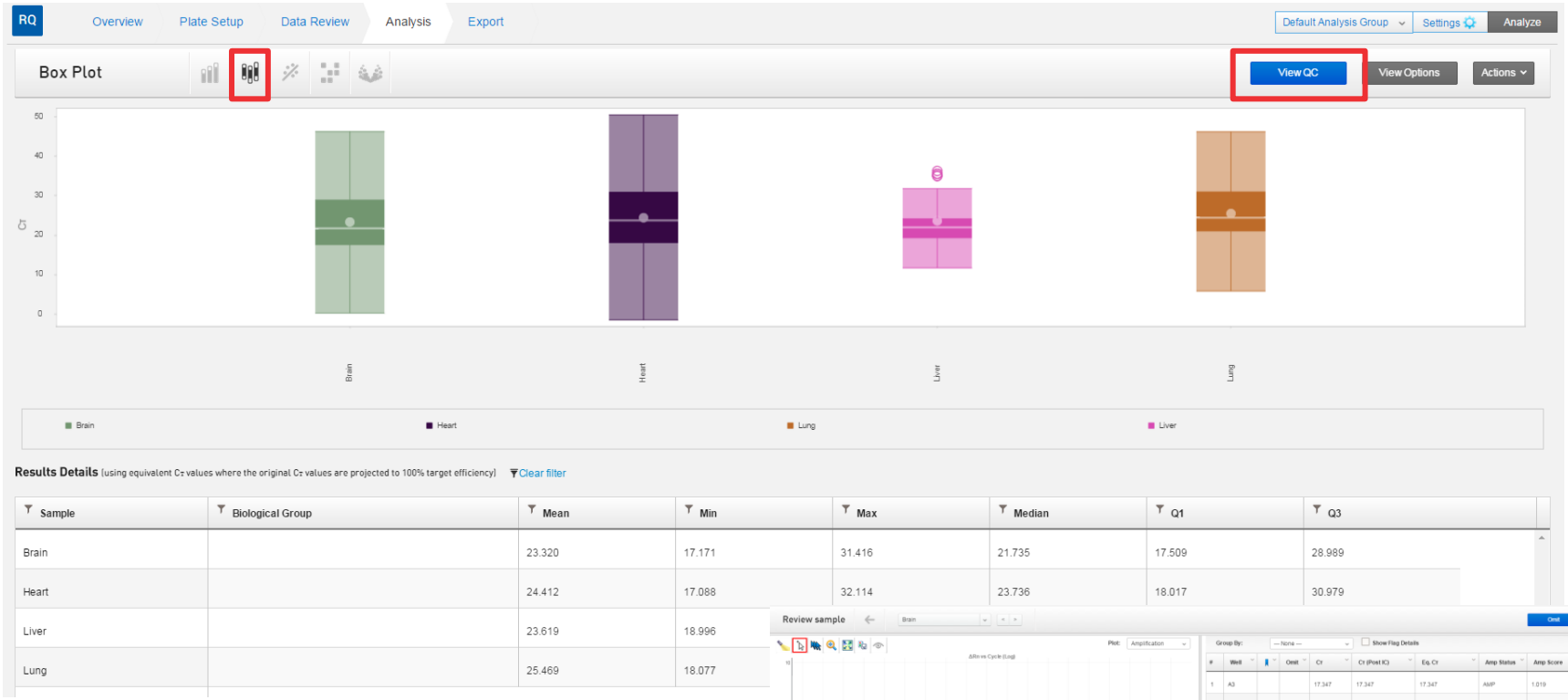
Gene Expression Plots in Analysis Section



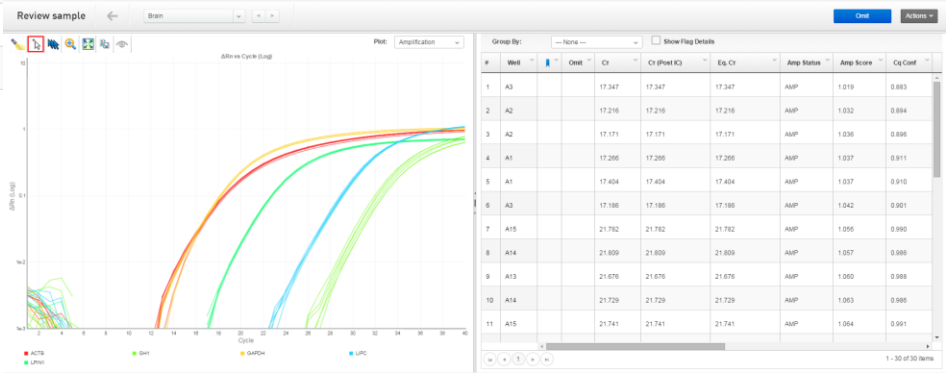
Box Plots – Quality Checks

- Assess CT distribution among biological replicates, samples, and targets



Results Details (using equivalent C_T values where the original C_T values are projected to 100% target efficiency) Clear filter

Sample	Biological Group	Mean	Min	Max	Median	Q1	Q3
Brain		23.320	17.171	31.416	21.735	17.509	28.989
Heart		24.412	17.088	32.114	23.736	18.017	30.979
Liver		23.619	18.996				
Lung		25.469	18.077				



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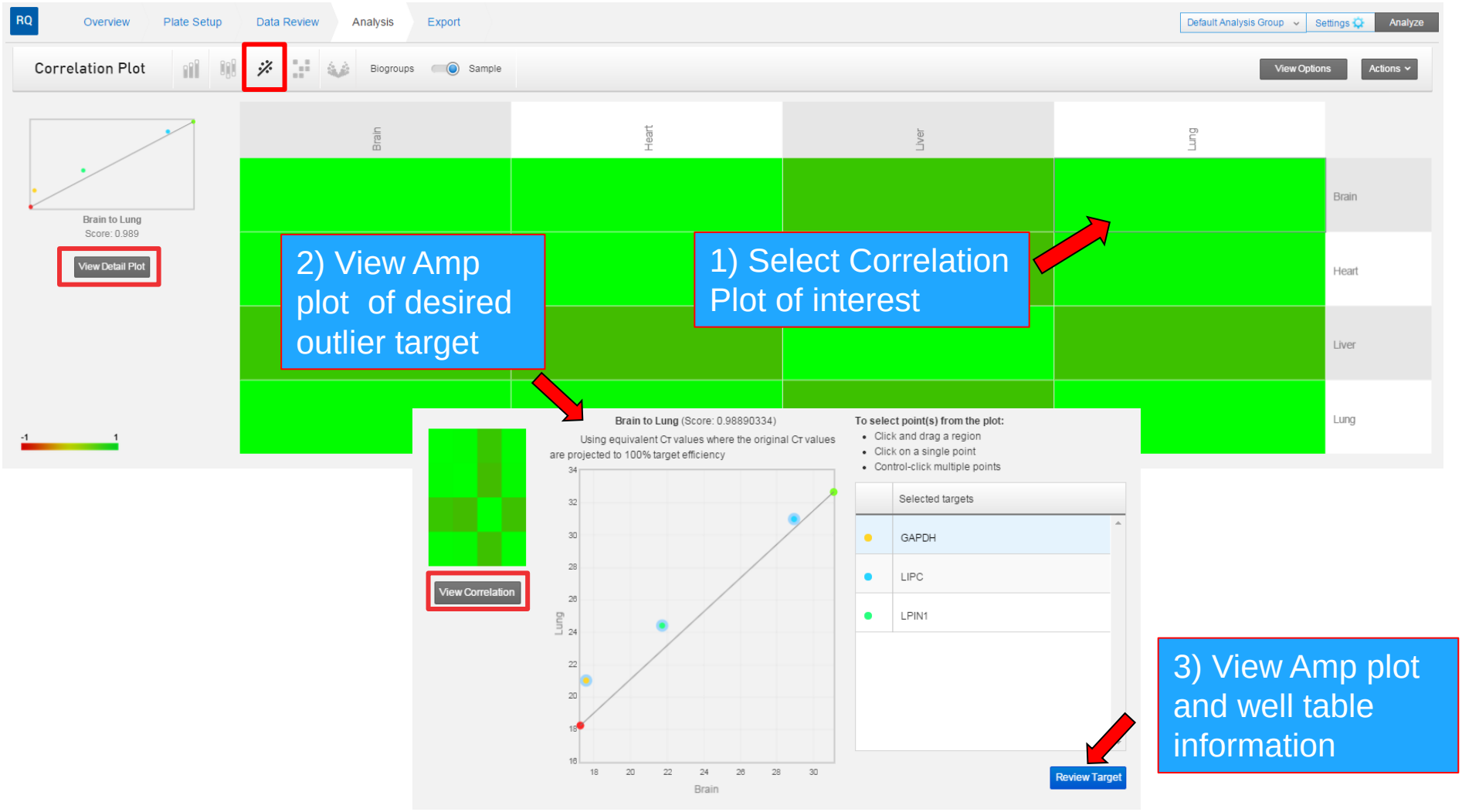
Correlation Plots – Quality Checks

- Select Correlation Plot of interest, then examine outliers



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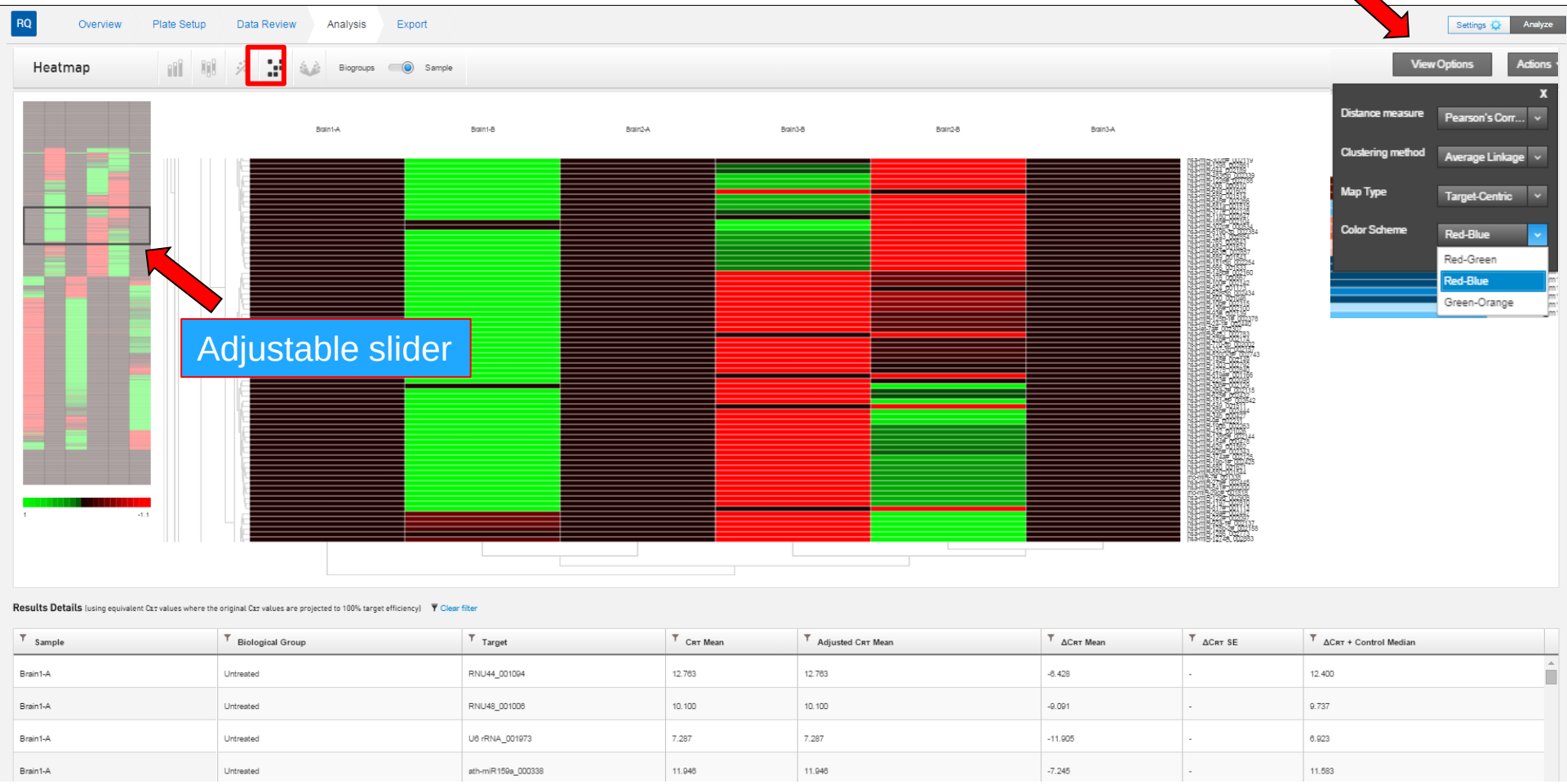
Heat Map

View Options to change settings



Results Details (using equivalent C_{RT} values where the original C_{RT} values are projected to 100% target efficiency) Clear filter

Sample	Biological Group	Target	C_{RT} Mean	Adjusted C_{RT} Mean	ΔC_{RT} Mean	ΔC_{RT} SE	ΔC_{RT} + Control Median
Brain1-A	Untreated	RNU44_001094	12.763	12.763	-6.428	-	12.400
Brain1-A	Untreated	RNU48_001006	10.100	10.100	-9.091	-	9.737
Brain1-A	Untreated	U6 rRNA_001973	7.287	7.287	-11.905	-	6.923
Brain1-A	Untreated	ath-miR159a_000338	11.946	11.946	-7.245	-	11.583

Human miRNA Starter Kit

*Slider only present with very large data sets

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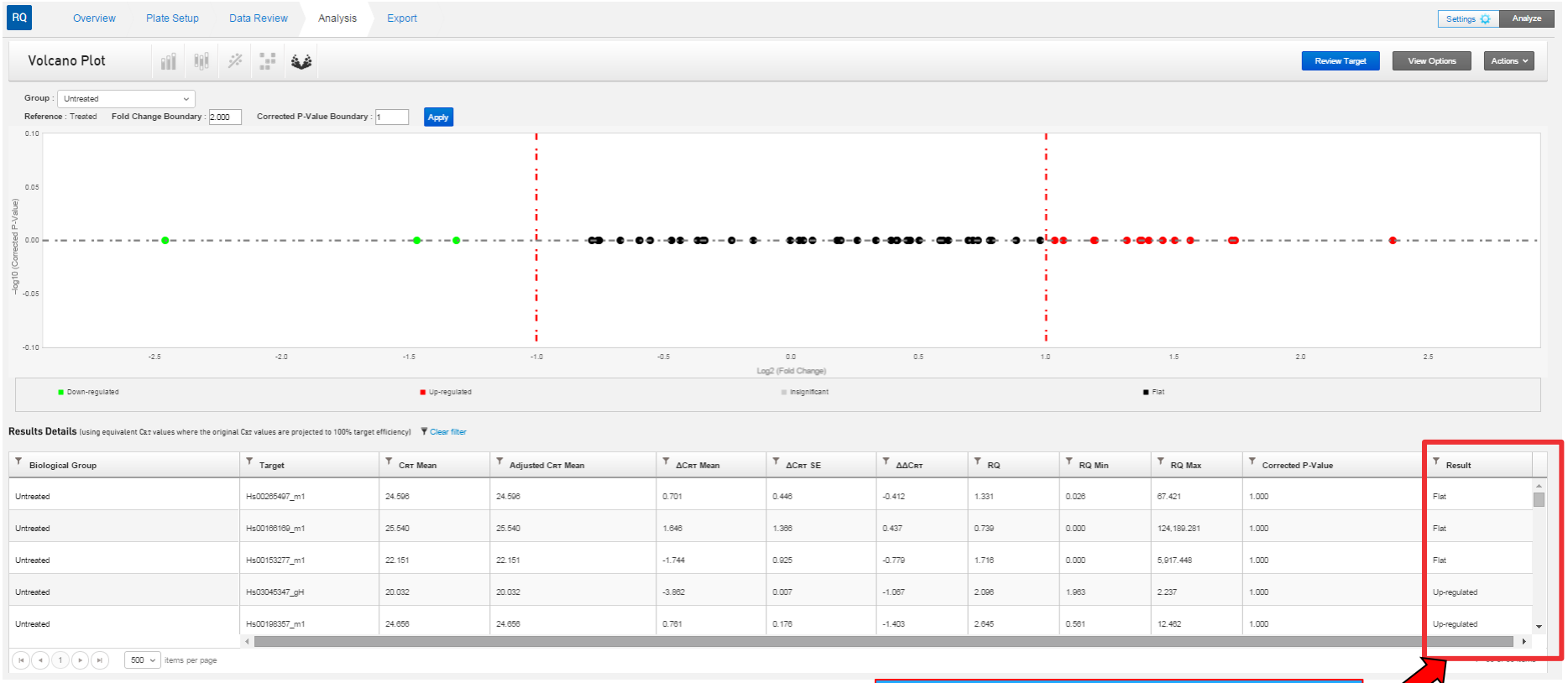
Volcano Plots: New Functionality

- Well table information with gene expression states



Gene expression states: flat, insignificant, upregulated, downregulated

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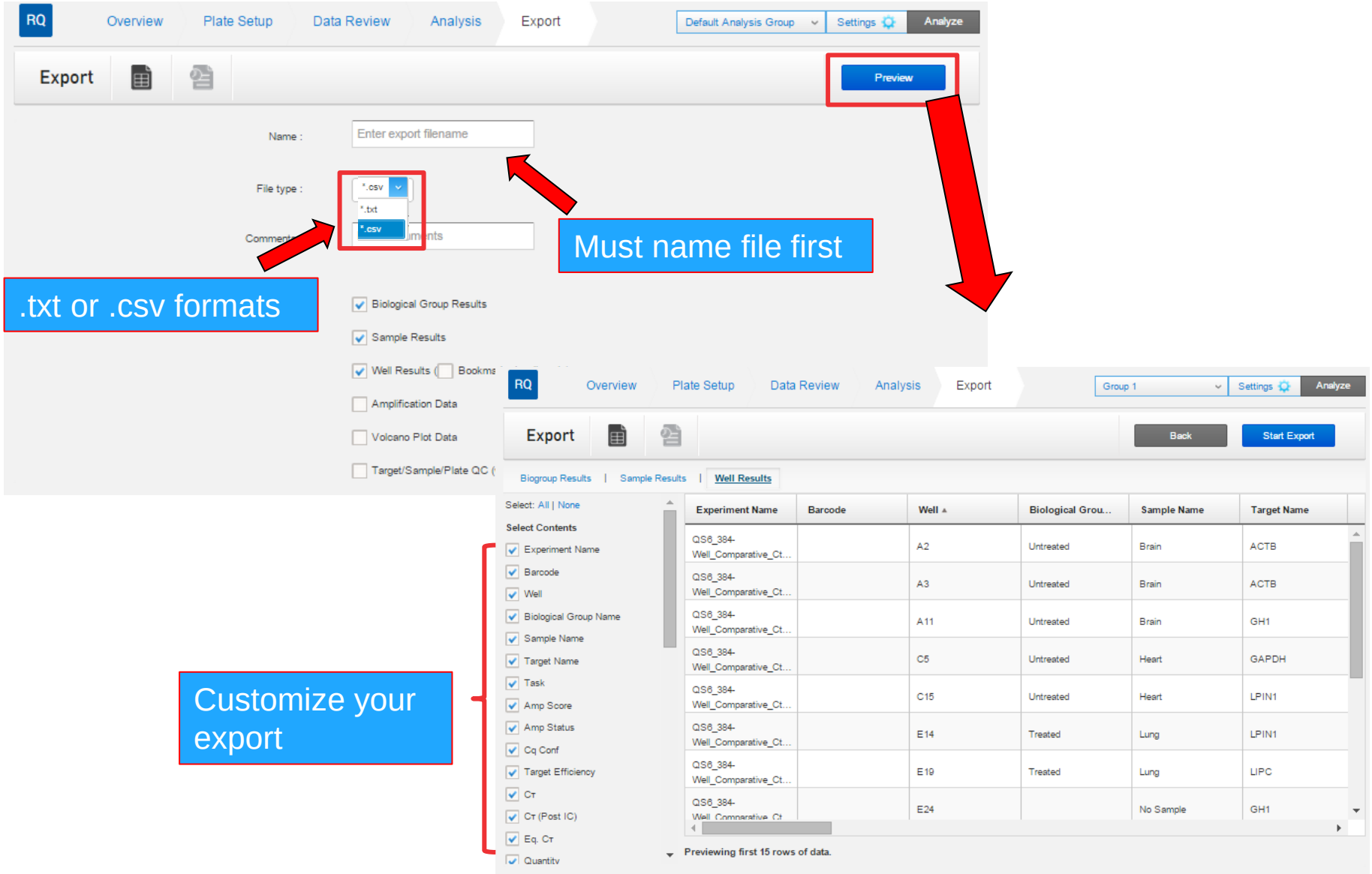
Exporting Data



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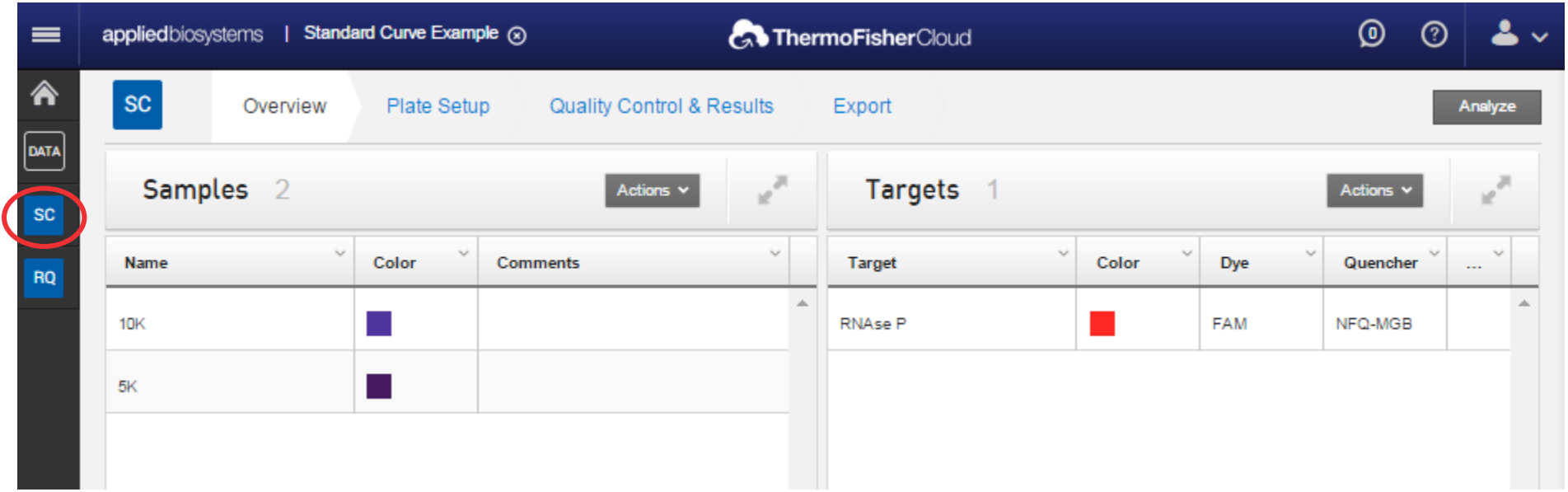
Standard Curve Analysis Module

Standard Curve Analysis Module

- No Analysis Groups because this is a single plate analysis module



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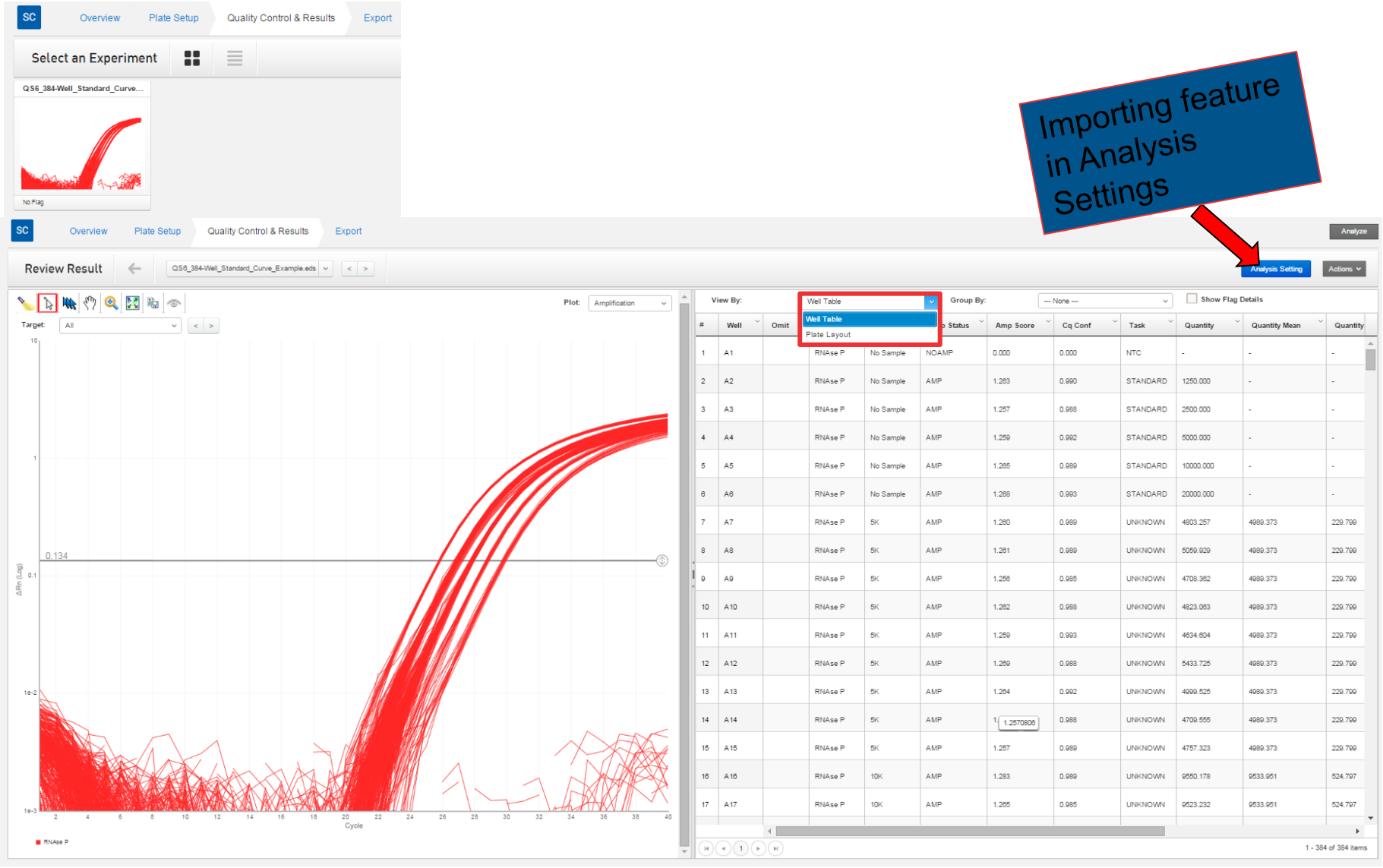
Standard Curve Features



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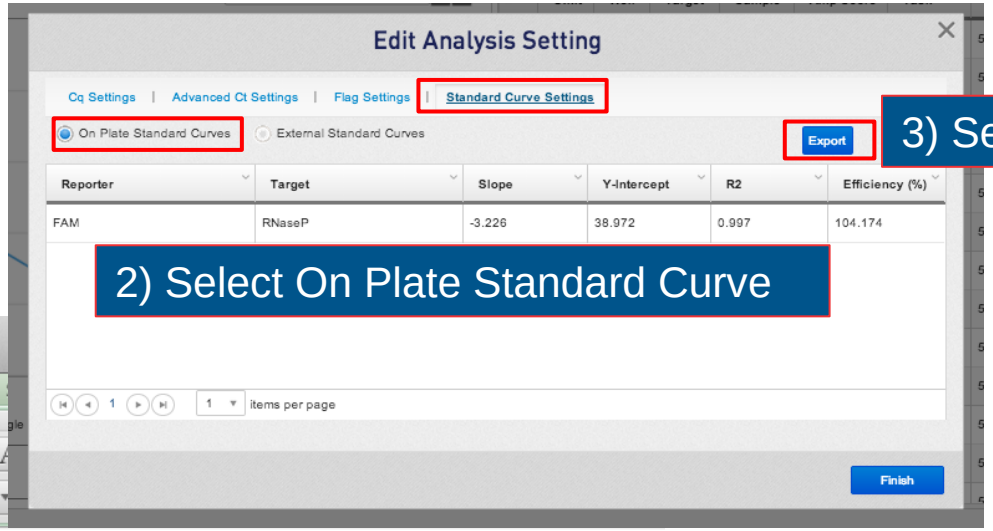
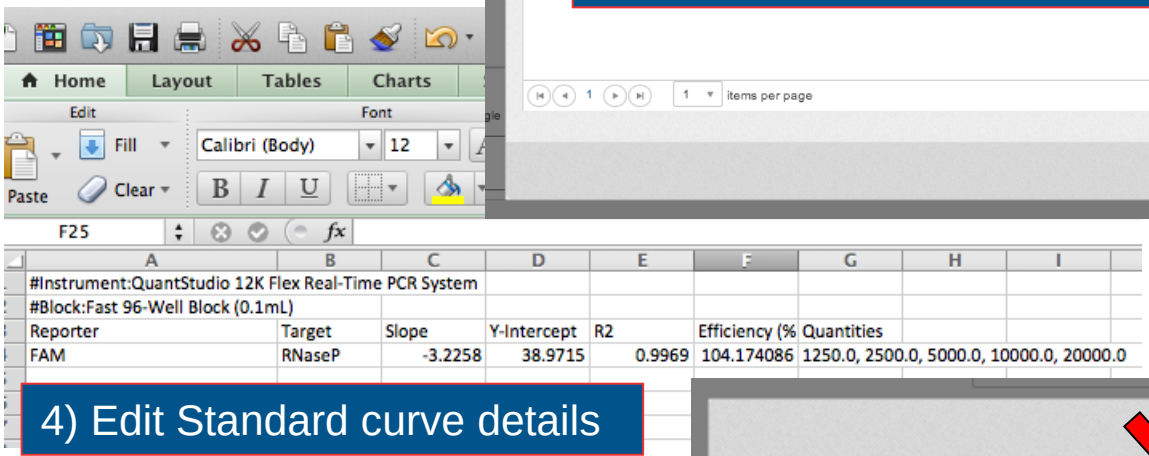
Improved Feature: Importing Standard Curves

1) Select Standard Curve Settings in top navigation pane

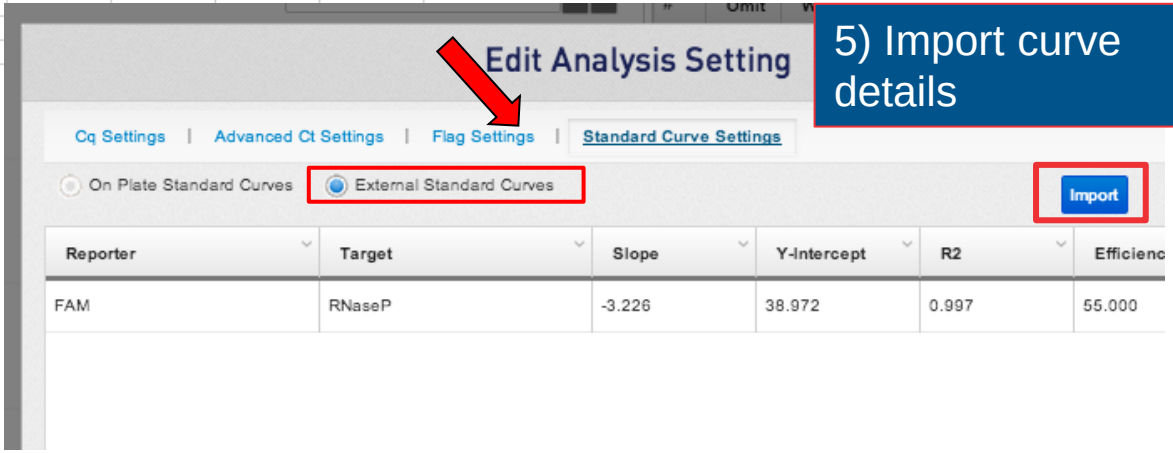


3) Select export

2) Select On Plate Standard Curve



4) Edit Standard curve details



5) Import curve details

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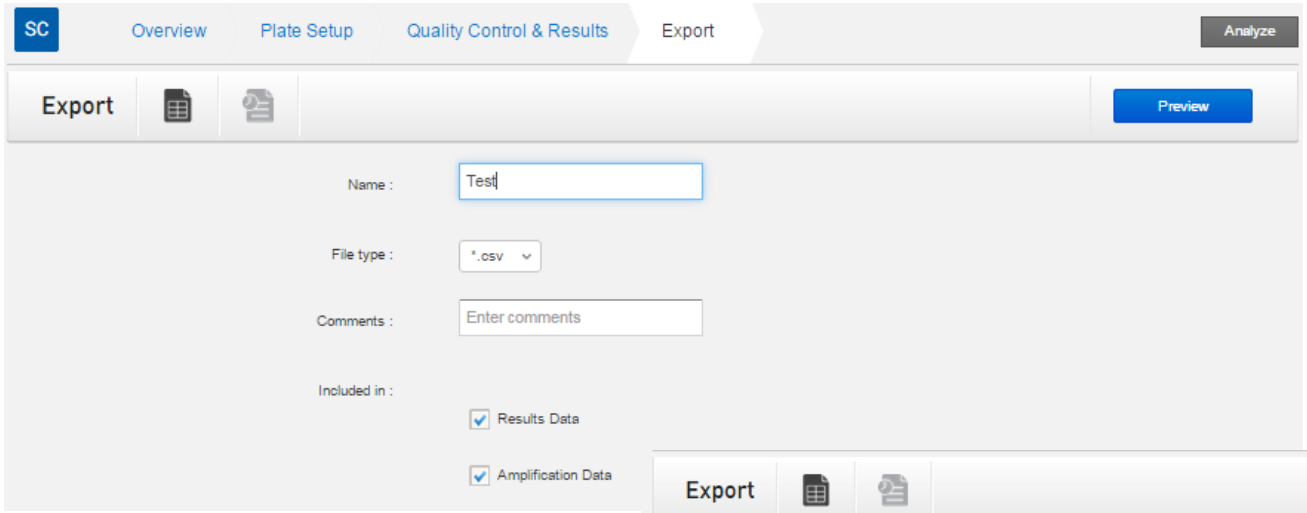
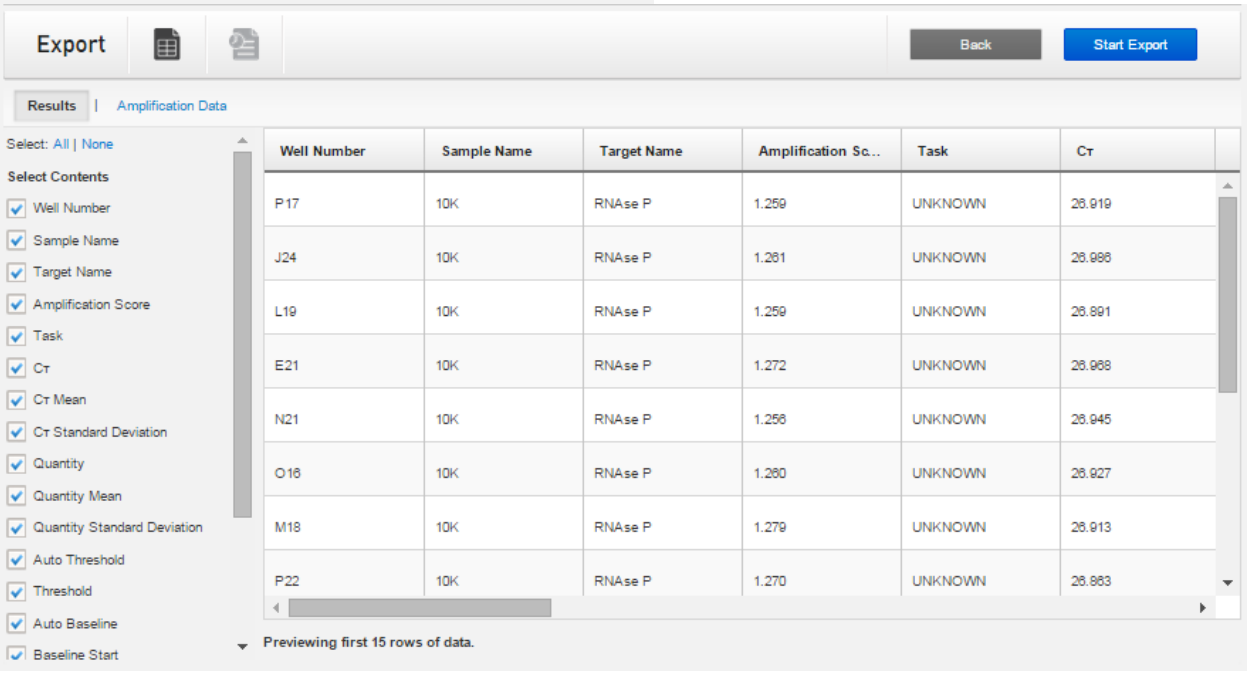
Exporting Data





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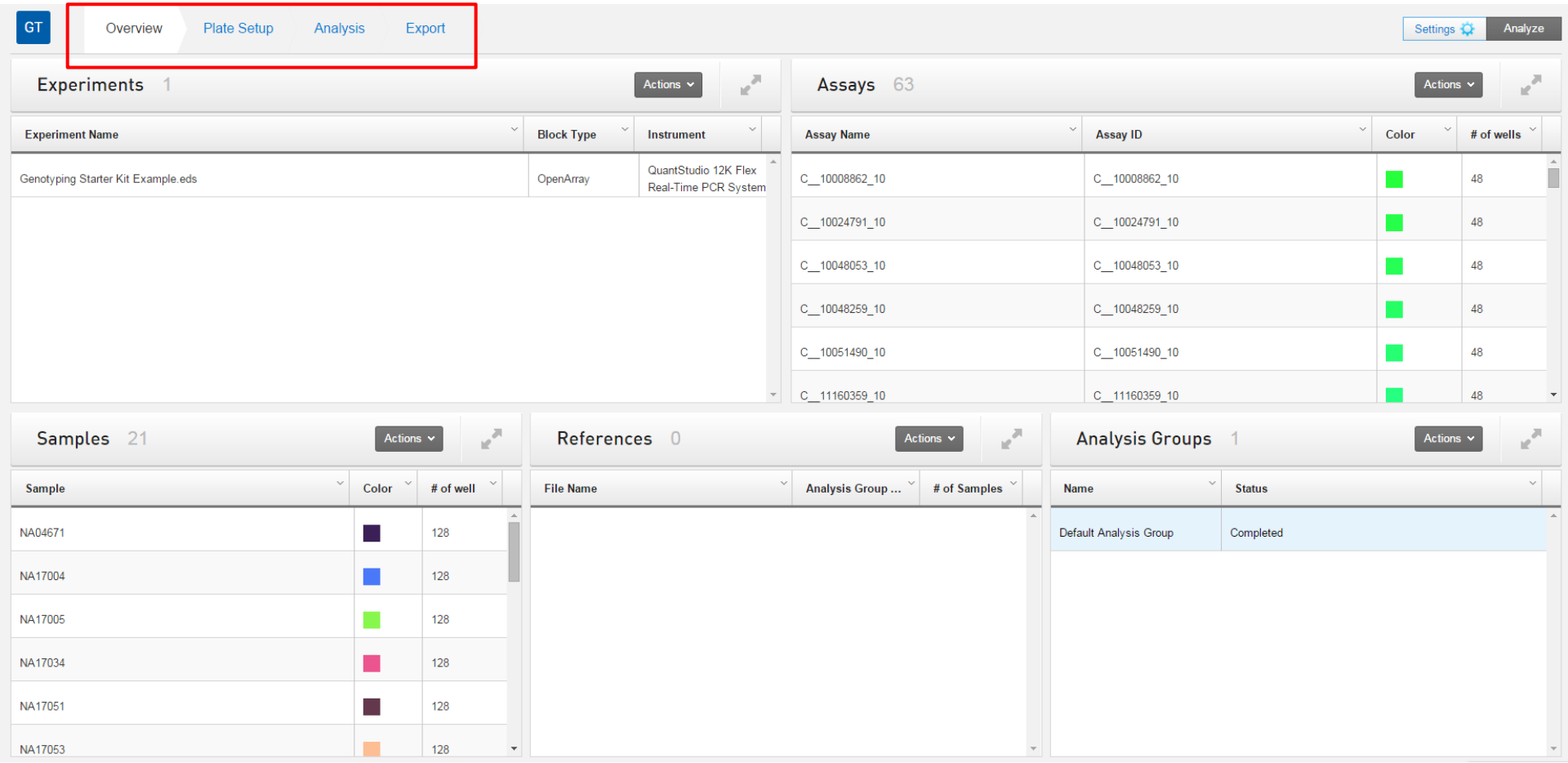
Genotyping Analysis Module

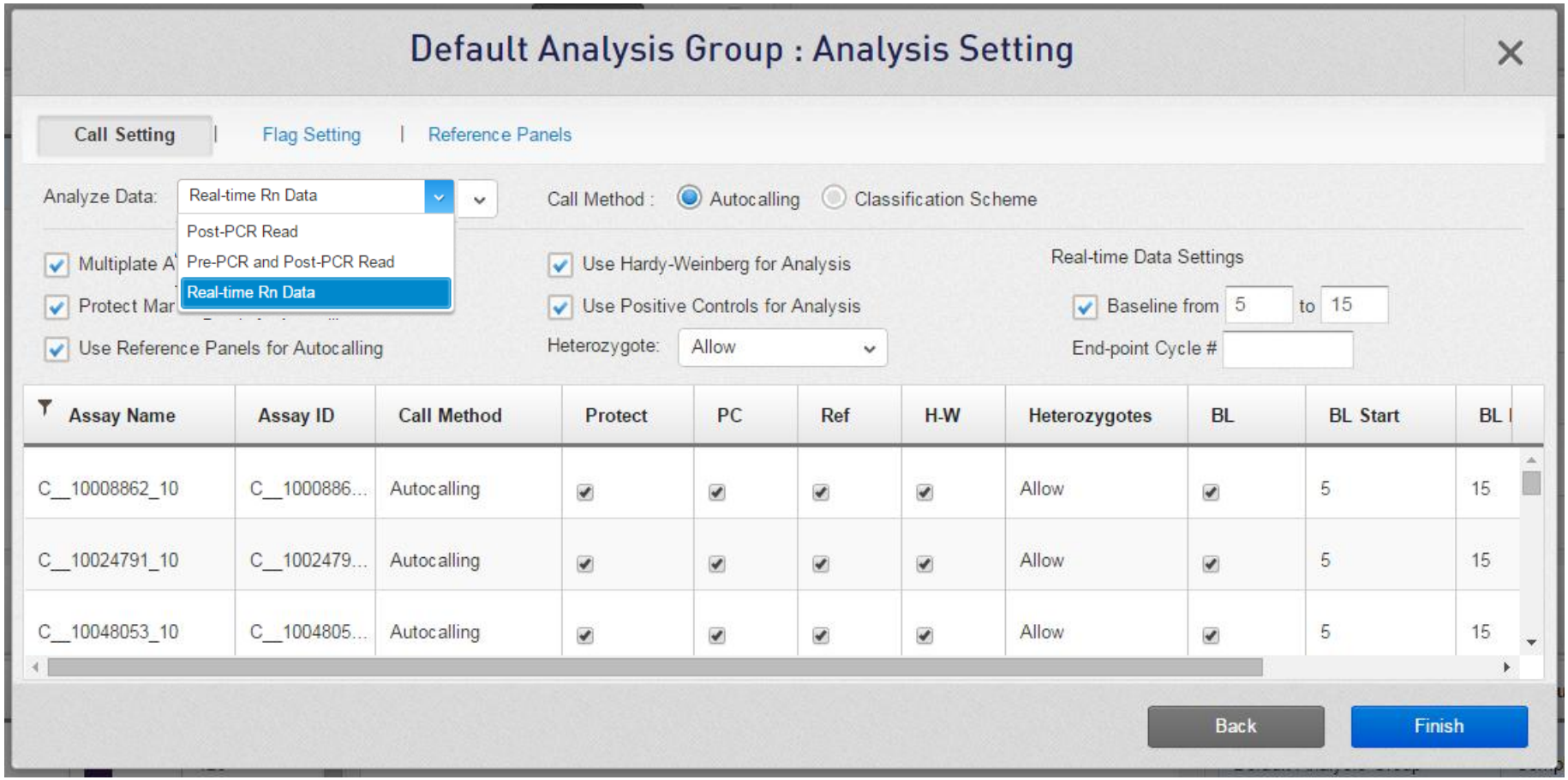
Genotyping Analysis Module

Basic navigation across the top



General View of Assay, Samples, References

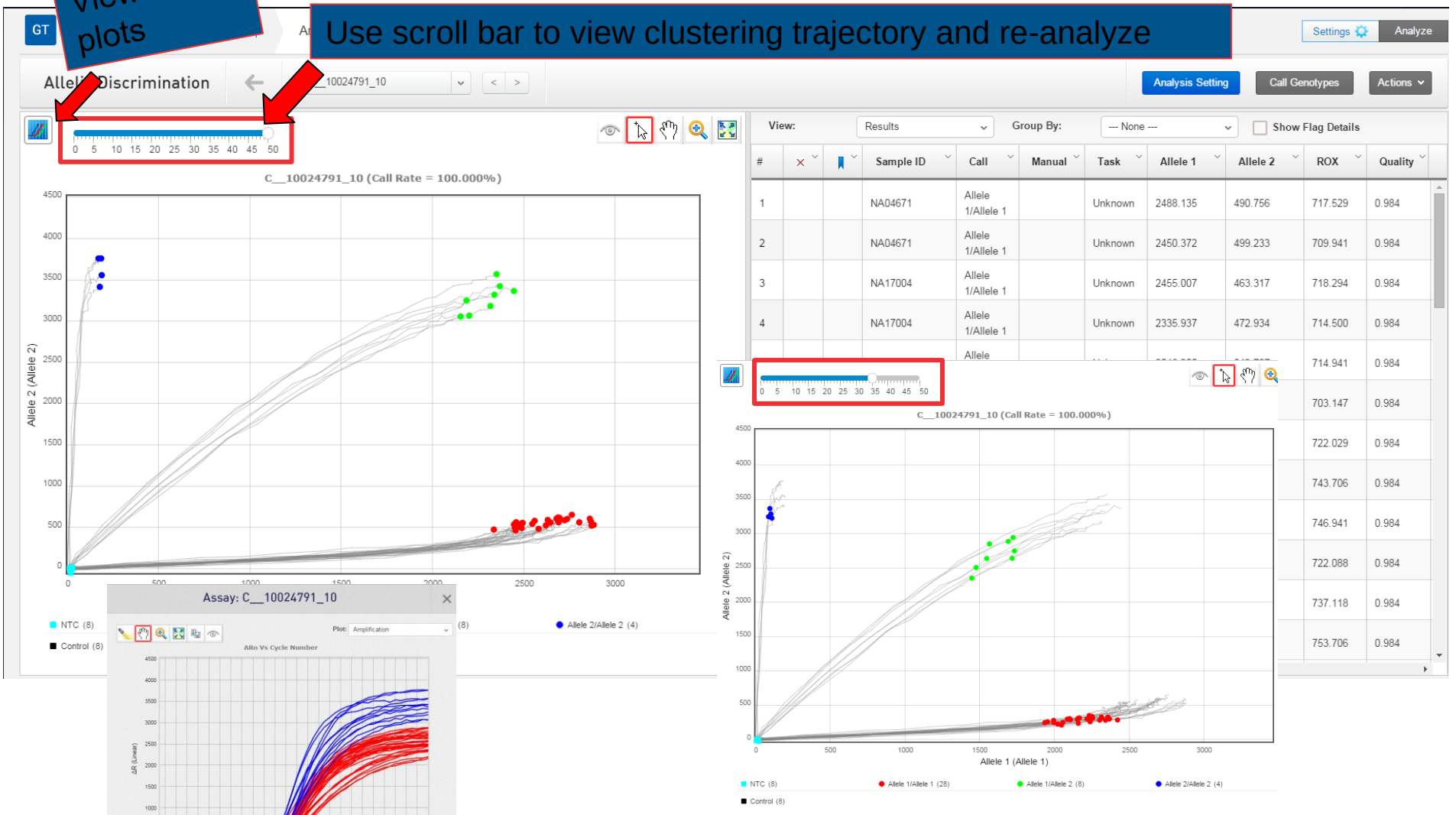
Analysis Groups and Settings



Real Time Traces to Optimize Cycling Conditions

View Amp plots

Use scroll bar to view clustering trajectory and re-analyze



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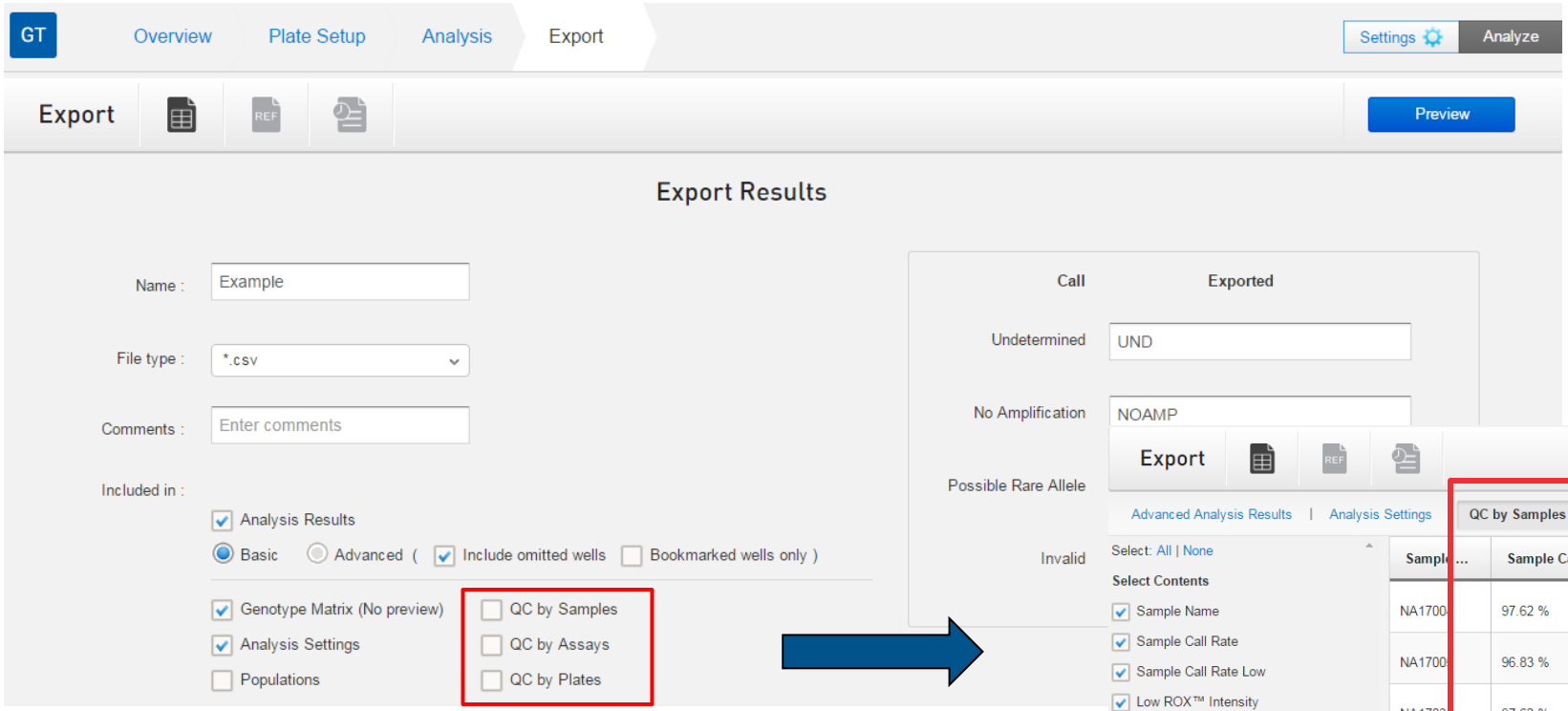
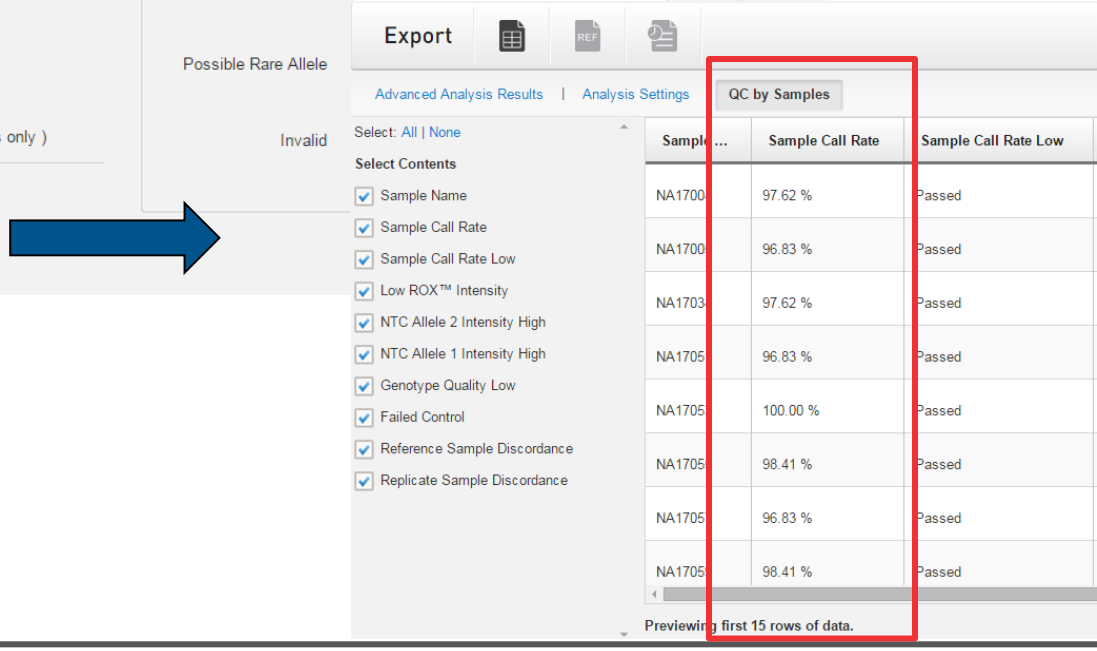
Exporting Genotyping Data

- New Export Files: Sample Call Rates





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Thank You!

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