

Session 7

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Data Analysis and Troubleshooting

Designing and setting up an experiment

Analysis software: AB 7500 v1.2.3

Examples with Quantifiler

Common problems and troubleshooting

Working with data

- Quantifiler
- Alu SYBR Green



Designing an Experiment

7 samples need to be evaluated

Experiments will be performed in duplicate

The experiment will require 2 x 2 µL of extract

An appropriate Calibrant will be serially diluted

Designing an Experiment

The experiment plate may look something like:

A	10 ng	10 ng	1a	1b
B	4	4	2a	2b
C	1.6	1.6	3a	3b
D	0.64	0.64	4a	4b
E	0.256	0.256	5a	5b
F	0.102	0.102	6a	6b
G	0.041	0.041	7a	7b
H	NTC	NTC	NTC	NTC

Standards Samples

May vary:
Range of dilutions
Spacing of dilutions

Designing an Experiment

Or the experiment plate may look something like:

	1	2	3	4	5	6	7	8	9	
A										
B	10	4	1.6	0.64	0.256	0.102	0.04	NTC		Standards
C	10	4	1.6	0.64	0.256	0.102	0.04	NTC		
D										
E		1a	2b	3c	etc					Samples
F		1a	2b	3c						
G										
H										

Exact plate setup may vary
Sometimes the perimeter of the plate is avoided
(evaporation, variations in cycler block heating)

Designing an Experiment

Preparing the serial dilution (7 dilutions)

- Will need 4 µl to run dilution series in duplicate
- Use volumes that are reasonable to pipette
- Prepare fresh that day

	Stock (ng/µL)	200		5	Buffer	Total
1		10	4	4	95	100
2		4	1.6	4	6	10
		1.6	0.64	4	6	10
		0.64	0.256	4	6	10
		0.256	0.102	4	6	10
		0.102	0.041	4	6	10
		0.041		4	6	10

Use Tris EDTA buffer
10 mM Tris-HCl (pH 8.0)
0.1 mM Na₂EDTA
Not Water

Designing an Experiment

Quantifiler Kit example

The kit comes with

- PCR Reaction Mix (dNTPs, buffer, Taq Gold, ROX)
- Human DNA Standard (200 ng/uL)
- Primer mix (hTERT-FAM, IPC template, and IPC-VIC)

Total reaction volume of 25 μ L

Designing an Experiment

- 10.5 μ L of Primer Mix
- 12.5 μ L of PCR Reaction Mix
- 2.0 μ L of extract/unknown

Add 23 μ L of the Master Mix to plate/tubes
 Add 2 μ L of template
 Cover with clear plastic (centrifuge to remove air bubbles)

Designing an Experiment

Alu assay example

Purchase

- PCR Master Mix (NTPs, buffer, Taq Gold, ROX, **SYBR Green I**)
- Commercial Human DNA Standard (e.g. Promega Human DNA Standard, 163 ng/uL)
- Primer mix (unlabeled 0.4 μ M each primer)

Total reaction volume of 25 μ L (this can vary)
 - Can add more than 2 μ L of sample

Data Analysis - Quantifiler

- Duplex assay
- hTERT (FAM)
 - IPC (VIC)

Open data

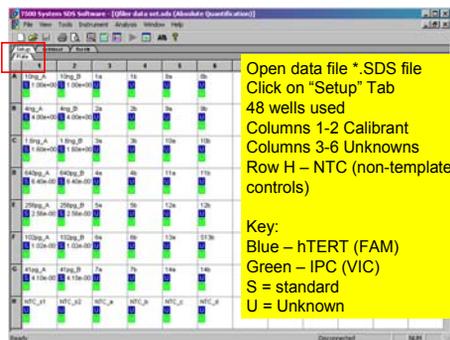
Review curves

Set **Baseline** and **Thresholds**

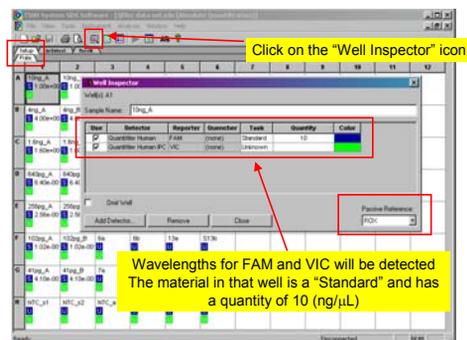
Review Standard Curve

Review and Export data

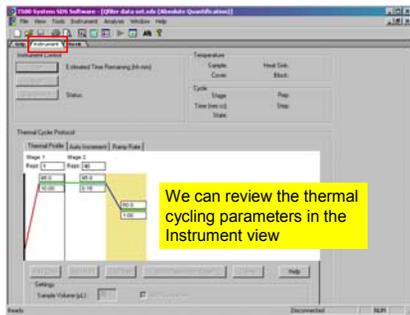
Data Analysis - Plate



Data Analysis - Well

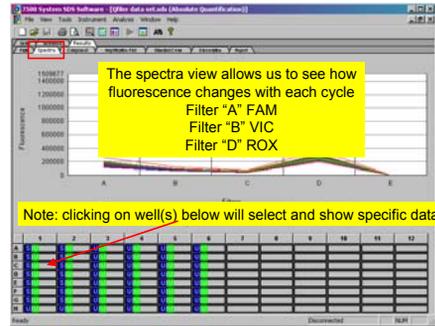


Data Analysis - Instrument



We can review the thermal cycling parameters in the Instrument view

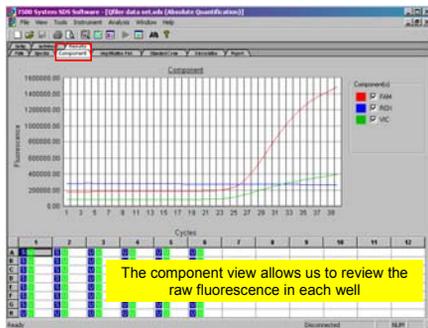
Data Analysis - Spectra



The spectra view allows us to see how fluorescence changes with each cycle
 Filter "A" FAM
 Filter "B" VIC
 Filter "D" ROX

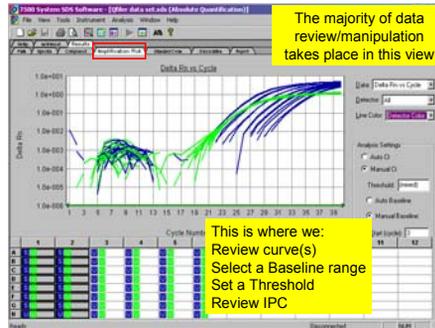
Note: clicking on well(s) below will select and show specific data

Data Analysis - Component



The component view allows us to review the raw fluorescence in each well

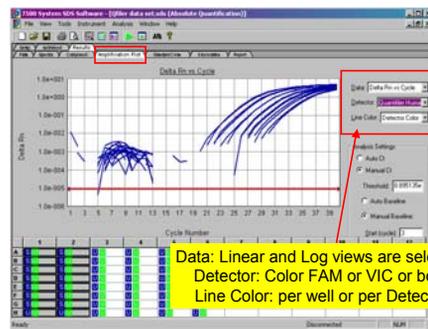
Data Analysis - Amp Plot



The majority of data review/manipulation takes place in this view

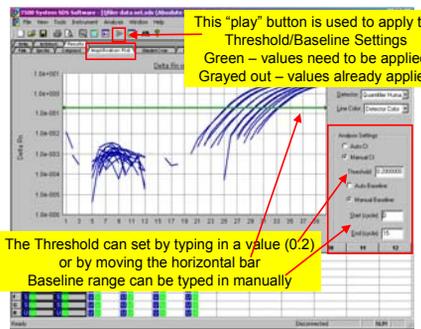
This is where we:
 Review curve(s)
 Select a Baseline range
 Set a Threshold
 Review IPC

Data Analysis - Amp Plot



Data: Linear and Log views are selected
 Detector: Color FAM or VIC or both
 Line Color: per well or per Detector

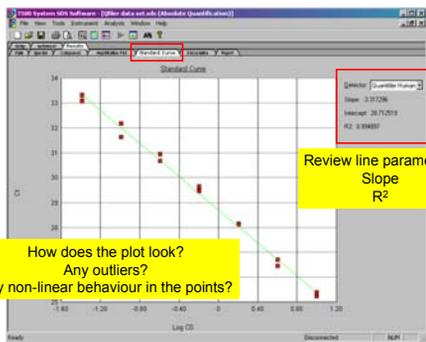
Data Analysis - Amp Plot



This "play" button is used to apply the Threshold/Baseline Settings
 Green – values need to be applied
 Grayed out – values already applied

The Threshold can be set by typing in a value (0.2) or by moving the horizontal bar
 Baseline range can be typed in manually

Data Analysis – Std Curve



Review line parameters
 Slope
 R²

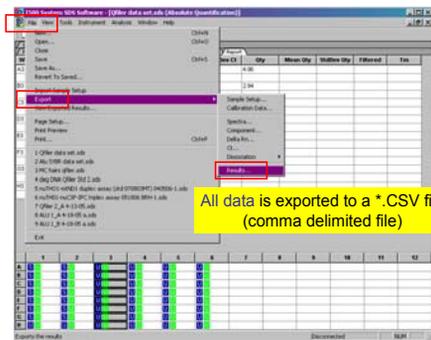
How does the plot look?
 Any outliers?
 Any non-linear behaviour in the points?

Data Analysis – Report

Well	Sample Name	Detector	Task	Ct	Mean C ₀	StdDev C ₀	Mean C _q	StdDev C _q	Efficient	Inv.
12.1	Quantifier Human	Human	27.85	4.36						
12.2	Quantifier Human	Human	27.85	3.84						
12.3	Quantifier Human	Human	27.85	3.32						
12.4	Quantifier Human	Human	27.85	2.80						
12.5	Quantifier Human	Human	27.85	2.28						
12.6	Quantifier Human	Human	27.85	1.76						
12.7	Quantifier Human	Human	27.85	1.24						
12.8	Quantifier Human	Human	27.85	0.72						
12.9	Quantifier Human	Human	27.85	0.20						
12.10	Quantifier Human	Human	27.85	0.68						
12.11	Quantifier Human	Human	27.85	1.16						
12.12	Quantifier Human	Human	27.85	1.64						
12.13	Quantifier Human	Human	27.85	2.12						
12.14	Quantifier Human	Human	27.85	2.60						
12.15	Quantifier Human	Human	27.85	3.08						
12.16	Quantifier Human	Human	27.85	3.56						
12.17	Quantifier Human	Human	27.85	4.04						
12.18	Quantifier Human	Human	27.85	4.52						
12.19	Quantifier Human	Human	27.85	5.00						
12.20	Quantifier Human	Human	27.85	5.48						
12.21	Quantifier Human	Human	27.85	5.96						
12.22	Quantifier Human	Human	27.85	6.44						
12.23	Quantifier Human	Human	27.85	6.92						
12.24	Quantifier Human	Human	27.85	7.40						
12.25	Quantifier Human	Human	27.85	7.88						
12.26	Quantifier Human	Human	27.85	8.36						
12.27	Quantifier Human	Human	27.85	8.84						
12.28	Quantifier Human	Human	27.85	9.32						
12.29	Quantifier Human	Human	27.85	9.80						
12.30	Quantifier Human	Human	27.85	10.28						

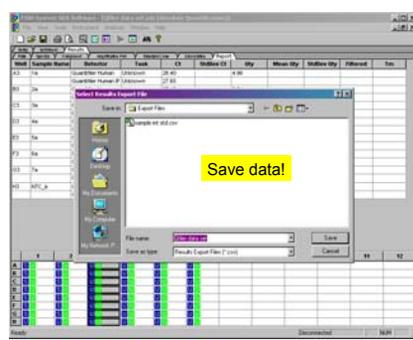
Selecting wells will give the estimated concentration of that sample. For the Standards that value will be the concentration you set (no estimate made)

Data Analysis – Export



All data is exported to a *.CSV file (comma delimited file)

Data Analysis – Save CSV



Save data!

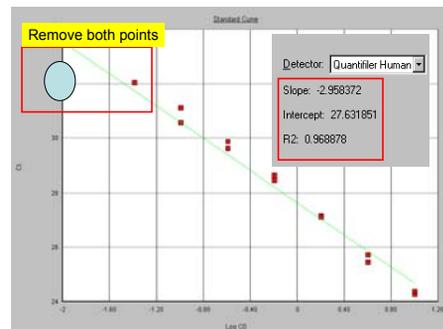
Troubleshooting

$R^2 < 0.99$

The low (or high) concentration point(s) of the dilution series can sometimes be removed to improve the R² value

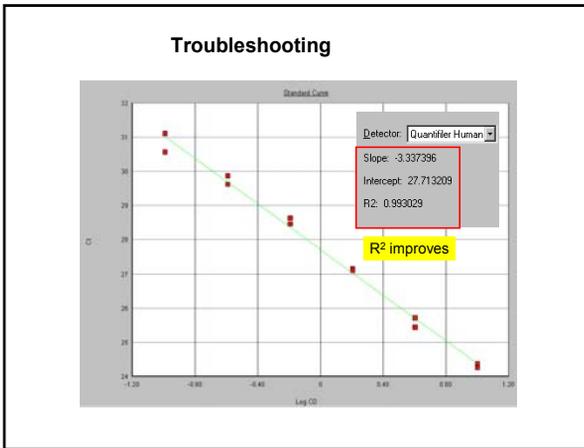
If your unknowns fall in this low range you may want to repeat the experiment

Troubleshooting



Remove both points

Detector: Quantifier Human
 Slope: -2.958372
 Intercept: 27.631851
 R2: 0.968878

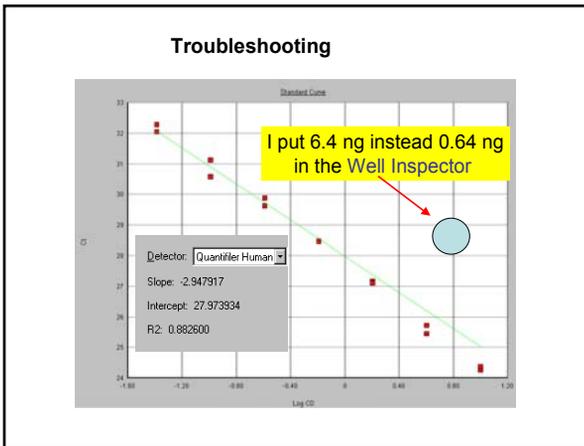


Troubleshooting

If a point(s) in the standard curve “looks off”

Make sure the **correct concentrations** are put into the plate view

- Example (6.4 ng vs 0.64 ng)

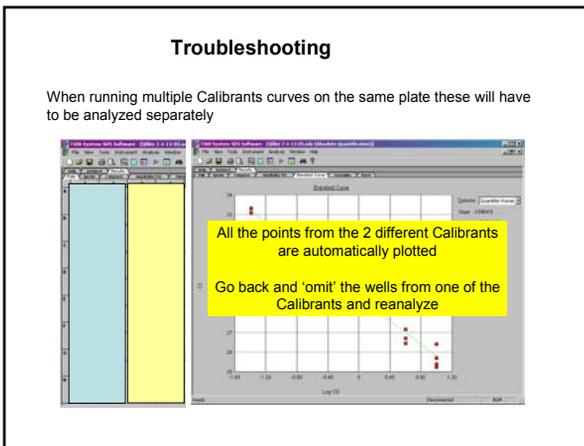


Troubleshooting

Make sure that the proper dye/detector is selected for the appropriate target

- Don't task the IPC as a “Standard”

When running multiple Calibrant curves on the same plate these will have to be analyzed separately



Troubleshooting

Replicates are inconsistent

- Evaporation of wells?
- Do you have different volumes in the wells?
- Volumes should all be the same
- Review wells post-run

Very noisy curves (observed at all [DNA])

Spikes in the signal

- Lamp going bad
- Optics misaligned
- Some technical issue (mechanical, electronics)

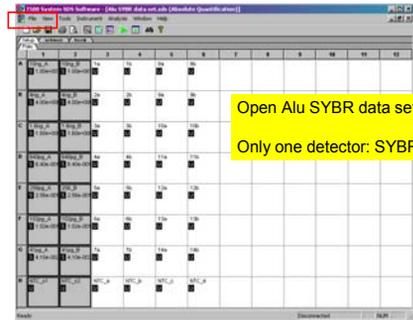
Troubleshooting

The manual for any Real Time PCR instrument should probably have a section on troubleshooting

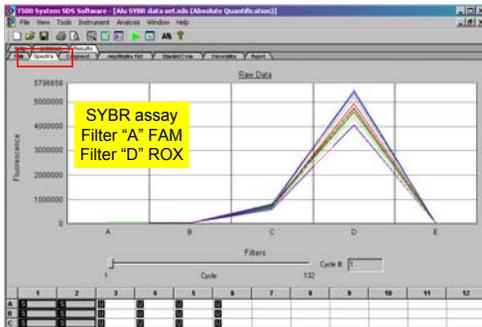
Commercial assays typically come with a manual and literature containing details/troubleshooting tips

For an assay taken from the literature you may want to contact the [authors](#) or [other labs](#) that are running that qPCR method

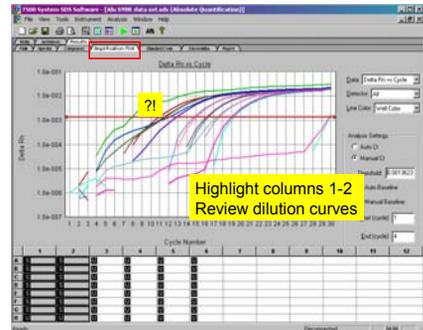
Data Analysis - Alu



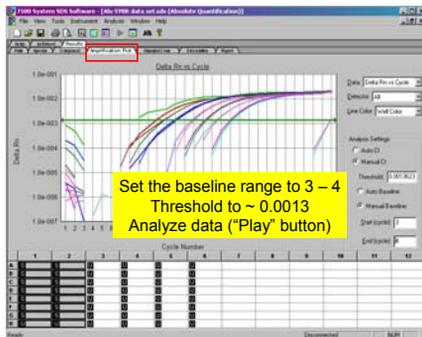
Data Analysis - Alu



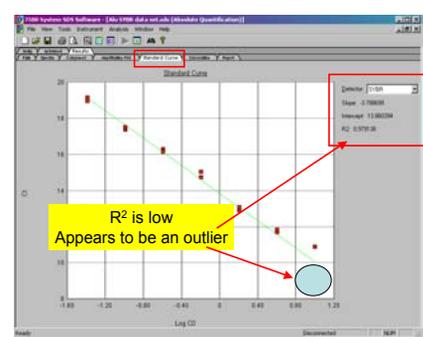
Data Analysis - Alu

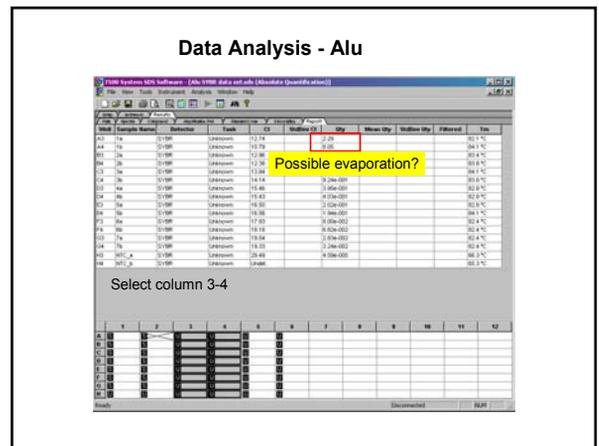
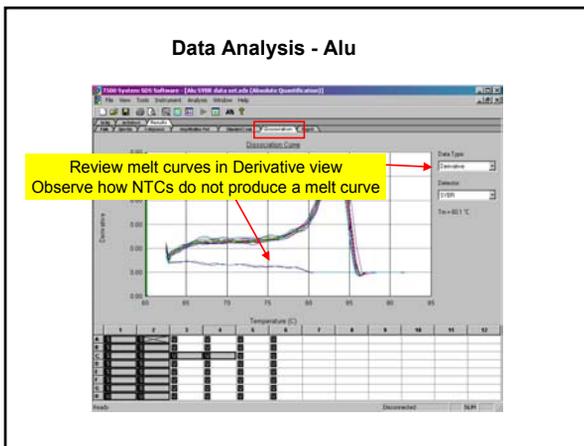
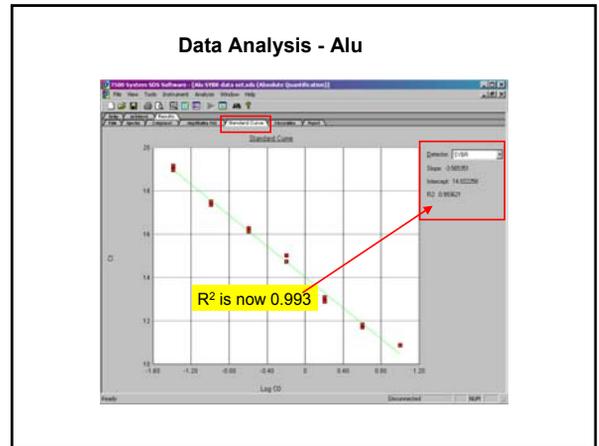
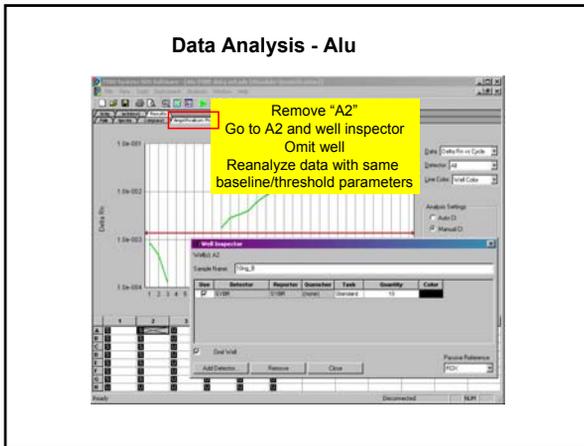


Data Analysis - Alu



Data Analysis - Alu





Summary

General guidelines for designing and setting up a qPCR experiment

Analysis software: AB 7500 v1.2.3

- Opening data
- Setting Baselines/Threshold
- Review curves/plots (R^2 , slope)
- Export estimated quantitation data
- Quantifier and Alu data