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/uL)	200		Buffer	Total
1		10	5	95	100
2		4	4	6	10
		1.6	4	6	10
		0.64	4	6	10
		0.256	4	6	10
		0.102	4	6	10
		0.041	4	6	10

Use Tris EDTA buffer
10 mM Tris-HCl (pH 8.0)
0.1 mM Na_2EDTA

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 μL

Designing an Experiment

10.5 μL of Primer Mix
12.5 μL of PCR Reaction Mix
2.0 μL of extract/unknown

Add 23 μL of the Master Mix to plate/tubes
Add 2 μL 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 μM each primer)

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

Data Analysis - Quantifiler

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

Open data

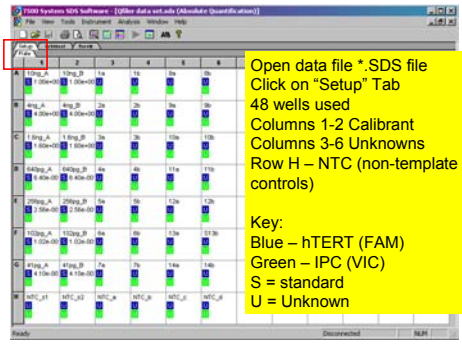
Review curves

Set Baseline and Thresholds

Review Standard Curve

Review and Export data

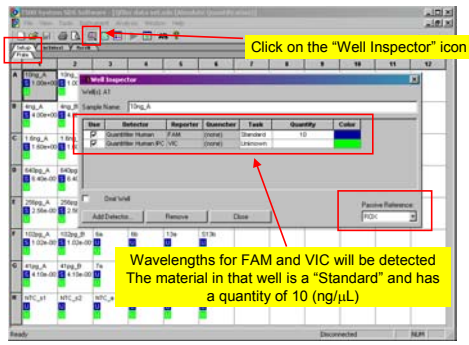
Data Analysis - Plate



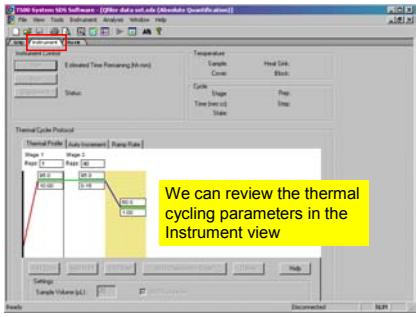
Open data file *.SDS file
Click on "Setup" Tab
48 wells used
Columns 1-2 Calibrant
Columns 3-6 Unknowns
Row H – NTC (non-template controls)

Key:
Blue – hTERT (FAM)
Green – IPC (VIC)
S = standard
U = Unknown

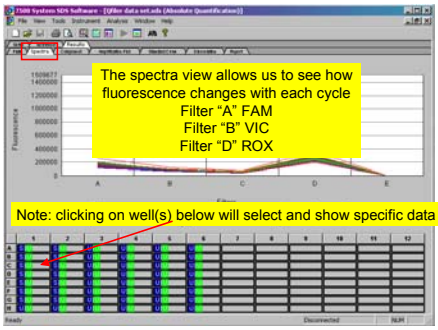
Data Analysis - Well



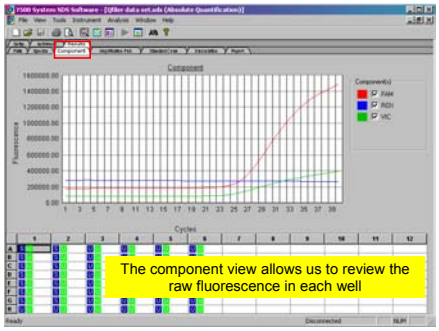
Data Analysis - Instrument



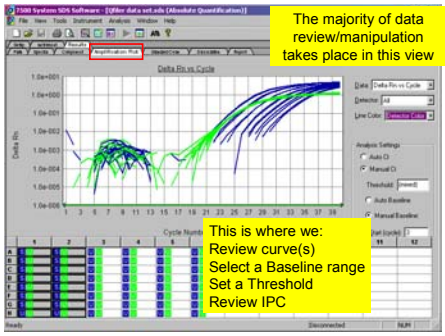
Data Analysis - Spectra



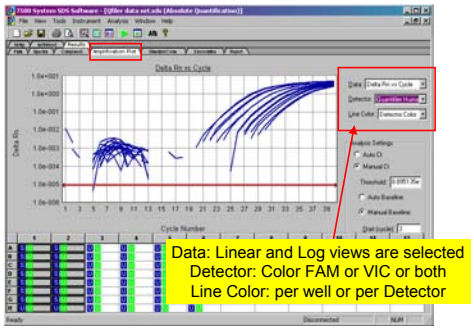
Data Analysis - Component



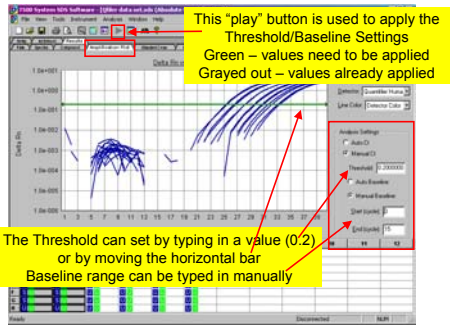
Data Analysis – Amp Plot



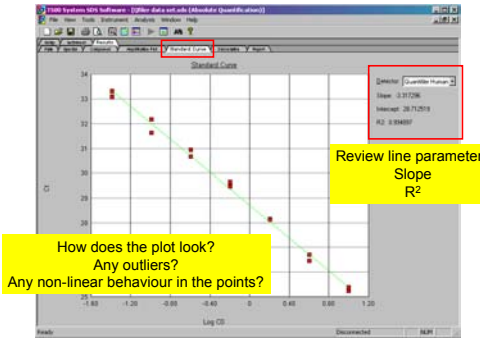
Data Analysis – Amp Plot



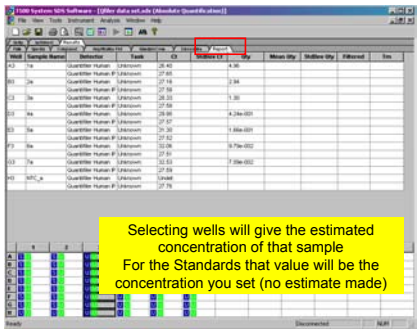
Data Analysis – Amp Plot



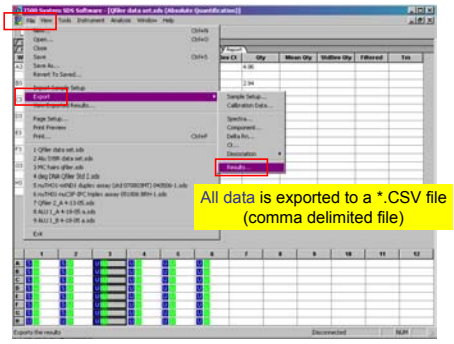
Data Analysis – Std Curve



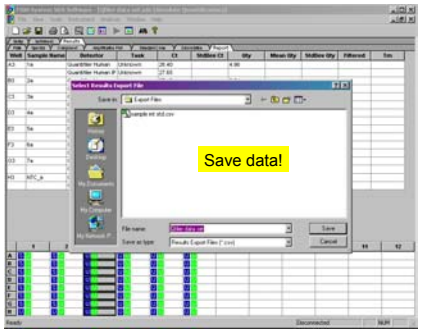
Data Analysis – Report



Data Analysis – Export



Data Analysis – Save CSV



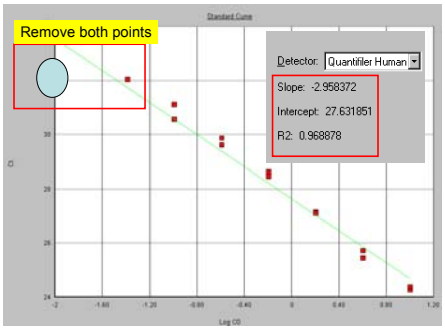
Troubleshooting

$R^2 < 0.99$

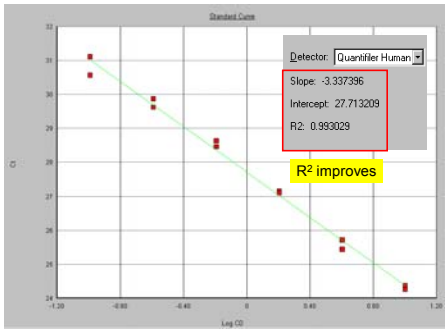
The low (or high) concentration point(s) of the dilution series can sometimes be removed to improve the R^2 value

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

Troubleshooting



Troubleshooting



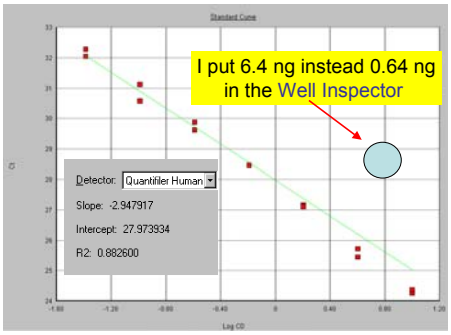
Troubleshooting

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

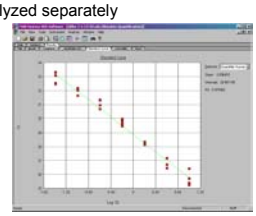


Troubleshooting

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

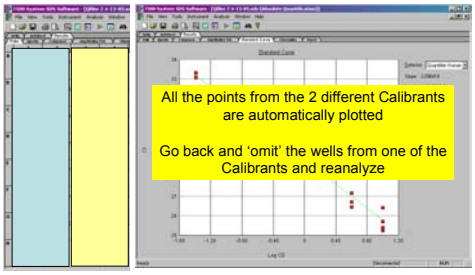
- Don't task the IPC as a "Standard"

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



Troubleshooting

When running multiple Calibrants 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)

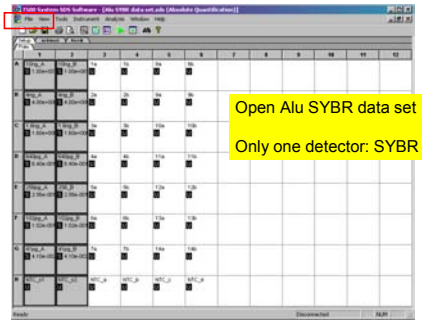
Troubleshooting

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

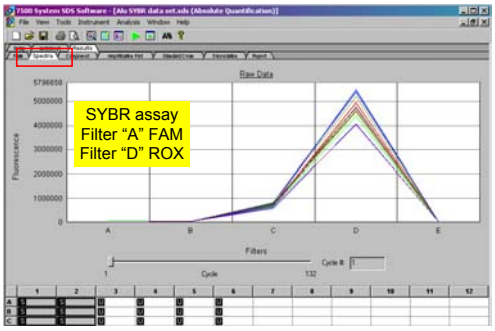
Commercial assays typically come with a manual and literature containing details/troubleshooting tips

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

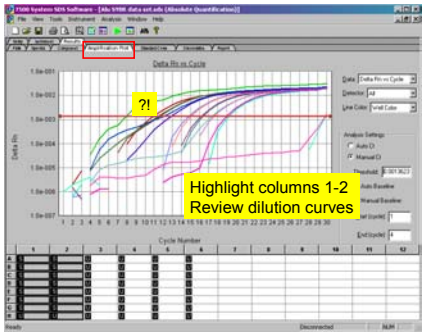
Data Analysis - Alu



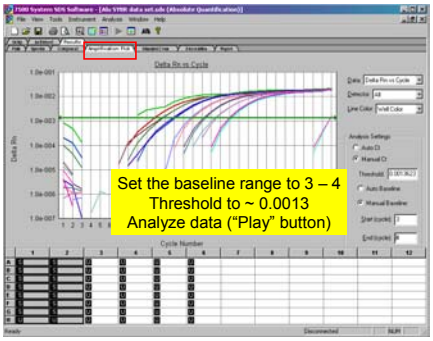
Data Analysis - Alu



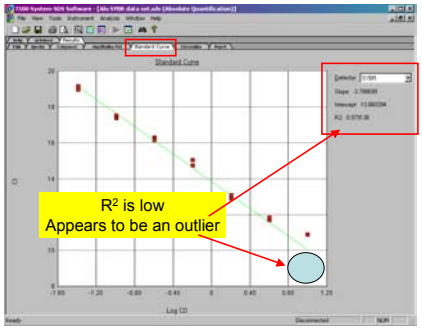
Data Analysis - Alu



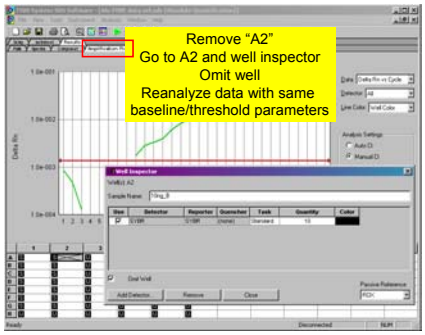
Data Analysis - Alu



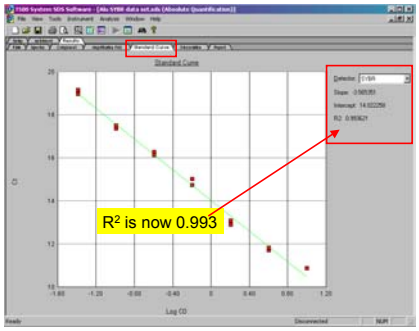
Data Analysis - Alu



Data Analysis - Alu



Data Analysis - Alu



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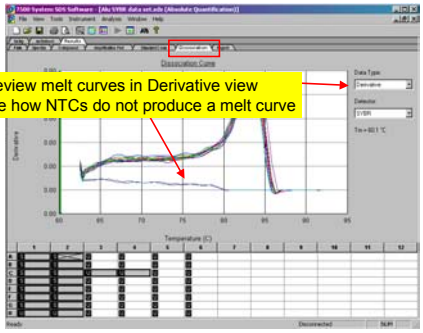
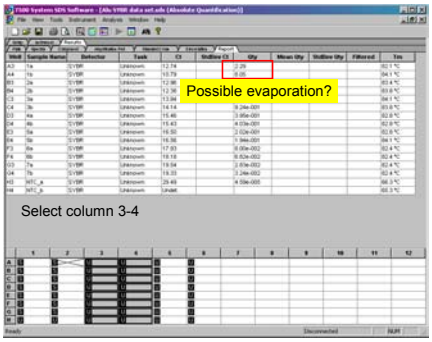


Data Analysis - Alu



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
- Quantifiler and Alu data