

MPCL PROTOCOL

Title: QuantStudio 6 Pro Relative Quantification Experiments

SCOPE:

This protocol covers how to utilize QuantStudio 6 Pro Real-Time PCR System to perform relative quantification experiments.

BACKGROUND

The Applied Biosystems QuantStudio 6 Pro Real-Time PCR System uses fluorescent-based polymerase chain reaction (PCR) reagents to provide quantitative research detection of target nucleic acid sequences (targets) using real-time analysis, post-PCR (endpoint) analysis and analysis of the PCR product (achieved by melt curve analysis that occurs post-PCR). The Applied Biosystems QuantStudio 6 Pro Real-Time PCR System features a six-color filter set that supports all Applied Biosystems dyes.

- The QuantStudio 6 Pro supports Comparative C_T experiments using TaqMan or SYBR Green Reagents, Singlex or Multiplex PCR and 1- and 2-Step PCR.

IMPORTANT! SYBR Green reagents cannot be used for multiplex PCR.

- For more information on the principle of Comparative C_T , refer to the Applied Biosystems QuantStudio 6 Pro System Getting Started Guide for Comparative C_T Experiments (Booklet 3, Part 2).
- The QuantStudio 6 Pro Real-Time PCR system consists of
 - QuantStudio 6 Pro Instrument
 - Computer containing QuantStudio 6 Pro Software
- The QuantStudio 6 Pro Real-Time PCR system is available in Centre for Heart Lung Innovation's Core 1 facility and features a sample block module suited for 384 well plates.

RESPONSIBILITIES

- If you have any questions regarding the proper operations of the QuantStudio 6 Pro instrument, please contact the Core 1 technicians (ext. 63535). The Applied Biosystems representatives can be contacted at: 1-800-831-6844.
- Please use the online equipment booking system to sign your name, local and time slot of use prior to using the QuantStudio 6 Pro. After each use, please sign your name under the appropriate lab on the usage log sheet.

SAFETY PRECAUTIONS

- The QuantStudio 6 Pro Real-Time PCR system features moving parts. Moving parts can crush and cut. Keep hands clear of moving parts while operating the instrument. Disconnect power before servicing the instrument.
- For more information refer to the Applied Biosystems QuantStudio 6 Pro Real-Time PCR System User Guide

MATERIALS and/or EQUIPMENT LIST

- 384-well clear optical reaction plates (Applied Biosystems: Part number: 4309849) containing sample, reference sample (calibrator), endogenous control, replicates and negative control and reaction mixture as dictated by your protocol.
- Sealed 384-well plate containing 20uL ddH₂O for background validation.

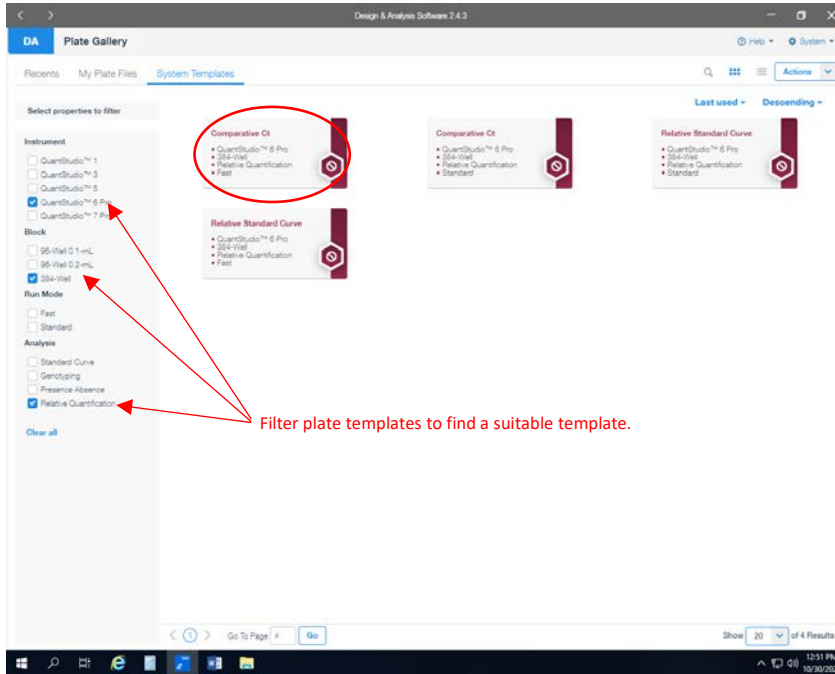
PROCEDURE

1. USING THE DESIGN AND ANALYSIS SOFTWARE TO RUN A PLATE:

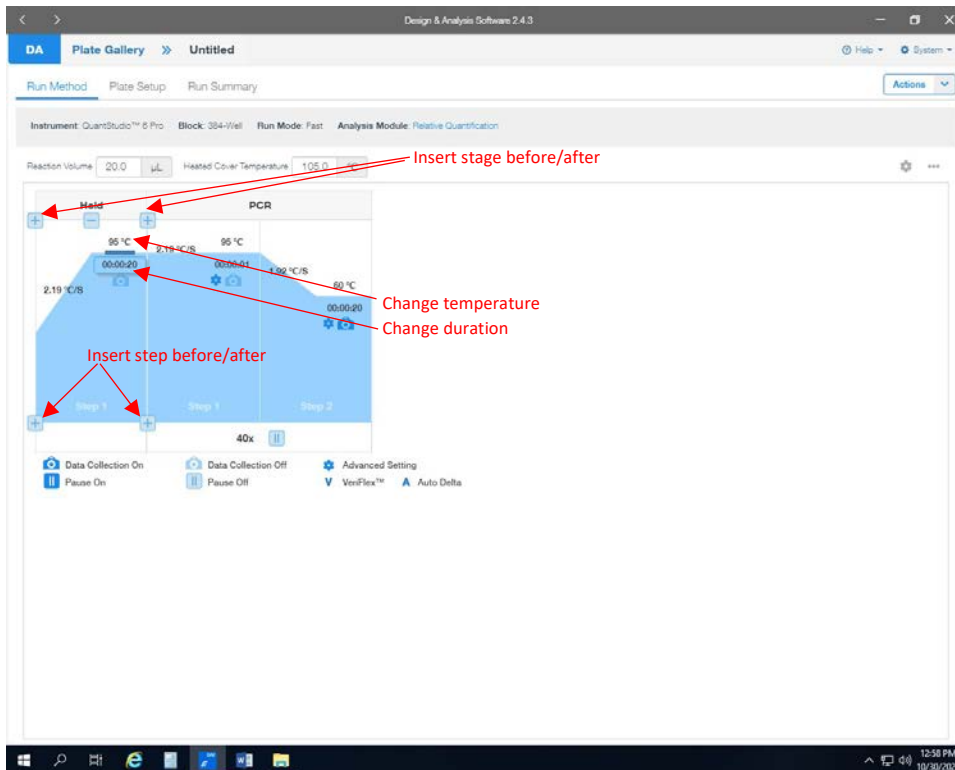
1. Start the QuantStudio Design and Analysis Software from the desktop.
2. Perform a routine background check (See MPCL Procedures for How to run a background plate)
3. Click SET UP PLATE
4. Using the filter list on the left, filter for QuantStudio 6 Pro under instruments, 384 well under block and Relative quantification under analysis. **→See Appendix A for an example**
5. Choose the appropriate template for your experiment setup and mastermix (Fast means the cycling conditions are 1sec at 95C and 20sec at 60C while standard is 15sec and 1min respectively.) **→See Appendix A for an example**
6. In the **Run method** screen adjust the run method according to the instructions for your mastermix. **→See Appendix A for an example**
 - a. To change the temperature or time, simply click on the value to be edited.
 - b. To add a step click the (+) at the bottom of the stage you want to add to;
 - c. To add a stage click the (+) at the top of the stage you want to add before (left top) or after (right top).
 - i. To add a melt curve, simply add a stage and click melt curve
 - d. Adjust the volume in your wells.
7. In the **Plate Setup** screen; **→See Appendix A for an example**
 - a. Highlight the wells you would like to process, add a sample and a target by pressing the (+) on the right panel and assign by clicking the check box next to the sample/target name.
 - b. You can enter Biogroup information by clicking the cell underneath biogroup. Once a value is entered, it will appear in the drop down menu.
 - c. You can change the Reporter and Quencher by clicking in the cell underneath and scrolling through the drop down menu.
8. Check the Passive Reference is configured correctly according to your mastermix instructions. **→See Appendix A for an example**
 - a. You can also import Plate setup, Taqman assay plate, export plate setup or flip the plate setup from the dropdown menu at the right top of plate panel or print the layout.
 - b. You can copy and paste sample information directly from Excel as long as the headings are identical.
9. Review your setup in the Run Summary screen and save your plate file. **→See Appendix A for an example**
10. Select the QuantStudio 6 Pro and send your plate to Run Queue **→See Appendix A for an example**
11. On the instrument screen,
 - a. Click Set up run, Run Queue and choose your file to run.
 - b. You can also select Network drive and find the saved plate file under your own folder.
 - c. Choose a data file name, choose the network drive location you would like to save your data file.
 - d. Check your Method and Plate and press **Start run**.

Appendix A: Examples using the Design and Analysis software to run a plate

5. Choose the appropriate template for your experiment setup and mastermix



6. Setup run method:



7. Plate Setup:

Design & Analysis Software 2.4.3

DA Plate Gallery Untitled

Run Method Plate Setup Run Summary

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Samples (4) Biogroup (3) Add a sample

Name#	Color	Type#	Quantity#	Biogroup
Sample 1	●	Unknown		Control
Sample 2	●	Unknown		treatment 1
Sample 3	●	Unknown		treatment 2
Sample 4	●	Unknown		Enter biogroup

Choose the wells to process

Assign sample and target to selected wells

Define a Biogroup

Targets (1) Reagents (0) Add a target

Target#	Color	Reporter#	Quencher#	Task
Target 1	●	SYBR	None	Unknown

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8. Passive reference and plate setup import/export

Design & Analysis Software 2.4.3

DA Plate Gallery Untitled

Run Method Plate Setup Run Summary

50%

Samples (4) Biogroup (3)

Name#	Color	Type#	Quant
Sample 1	●	Unknown	
Sample 2	●	Unknown	
Sample 3	●	Unknown	
Sample 4	●	Unknown	

Check passive reference dye

Import/export plate setup

Target#	Color	Reporter#
Target 1	●	SYBR

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9. Run summary and plate queue:

Design & Analysis Software 2.4.3

DA Plate Gallery >> Untitled

Run Method Plate Setup **Run Summary** Actions

1. Review Method

Run Properties Instrument: QuantStudio™ 6 Pro Block: 384-Well Mode: FAST Volume: 20µL Temperature

Hold PCR

95 °C 2.19 °C/S 95 °C 1.32 °C/S 60 °C

00:00:20 00:00:01 00:00:20

Step 1 Step 1 Step 2

40x

2. Plate setup

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	●	●	●																					
B	●	●	●																					
C	●	●	●																					
D	●	●	●																					
E																								
F																								
G																								
H																								
I																								
J																								

SAMPLES
4
UNKNOWN
12
STANDARDS
0
NTCs
0

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Design & Analysis Software 2.4.3

DA Plate Gallery >> Untitled

Run Method Plate Setup **Run Summary** Actions

M N O P

SNP ASSAYS
0

3. Save Plate File

Barcode
Optional

Add to My Plates Gallery

006Pro-2778620090
QuantStudio™ 6 Pro
.dat

Send to Run Queue

4. Start and monitor a run

1. Go to instrument and load this file.
2. Insert your plate, then start the run.
3. If you want to monitor your run, visit the [Manage Instruments](#) page.
4. If you want to start another run, go to the [Plate Gallery](#) page.

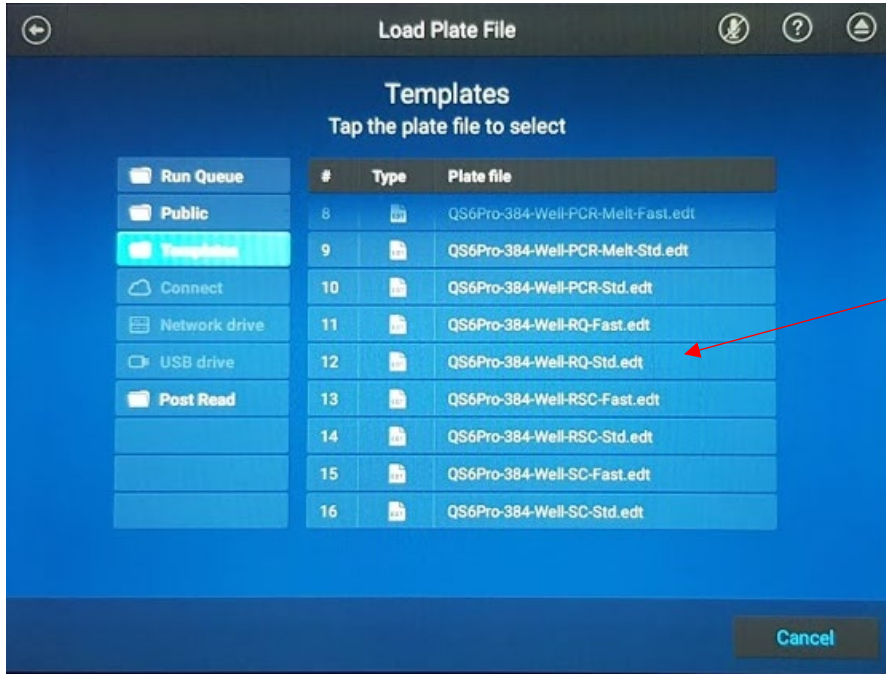
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2. USING THE QUANTSTUDIO 6 PRO INSTRUMENT INTERFACE TO RUN A PLATE:

1. Bring the Instrument out of sleep more by tapping the touch screen.
2. Tap **Set up Run**
3. Under **Templates**, tap on QS6Pro-384-Well-RQ-Fast.edt or QS6Pro-384-Well-RQ-Fast.edt →**See Appendix B for an example**
4. Determine a Data File Name and the save location (Set the save location to xe3-iot-2019/PRO/Experiments/YourFolder) →**See Appendix B for an example**
The credentials for the Network Drive are:
xe3-iot-2019/PRO
username: **tmp**
password: **tmp77**
 - a. Tap the checkbox “Export run results with...” to disable auto export.
5. Tap on the **Method** tab to set up run method according to the instructions for your mastermix.
 - a. To change the temperature or time, simply tap on the value to be edited.
 - b. To add a step first tap advanced, then tap add/remove step, then tap the (+) where you want to add your step.
 - c. To add a stage first tap advanced, then tap add/remove stage tap the then tap the (+) where you want to add your stage.
 - d. To add a melt curve, tap (+) to add stage and select melt curve.
 - e. Adjust the volume in your wells. →**See Appendix B for an example**
6. Tap on the **Plate** tab to configure your plate.
 - a. Tap on Actions to import sample setup, edit reference dye or import AIF file. →**See Appendix B for an example**
 - b. **Optional:** Tap and drag the wells to assign samples and targets. →**See Appendix B for an example**
(Tap the right panel to get to the ample/target menu)
7. Tap start run.

Appendix B: Examples using the QuantStudio 6 Pro Interface to run a plate

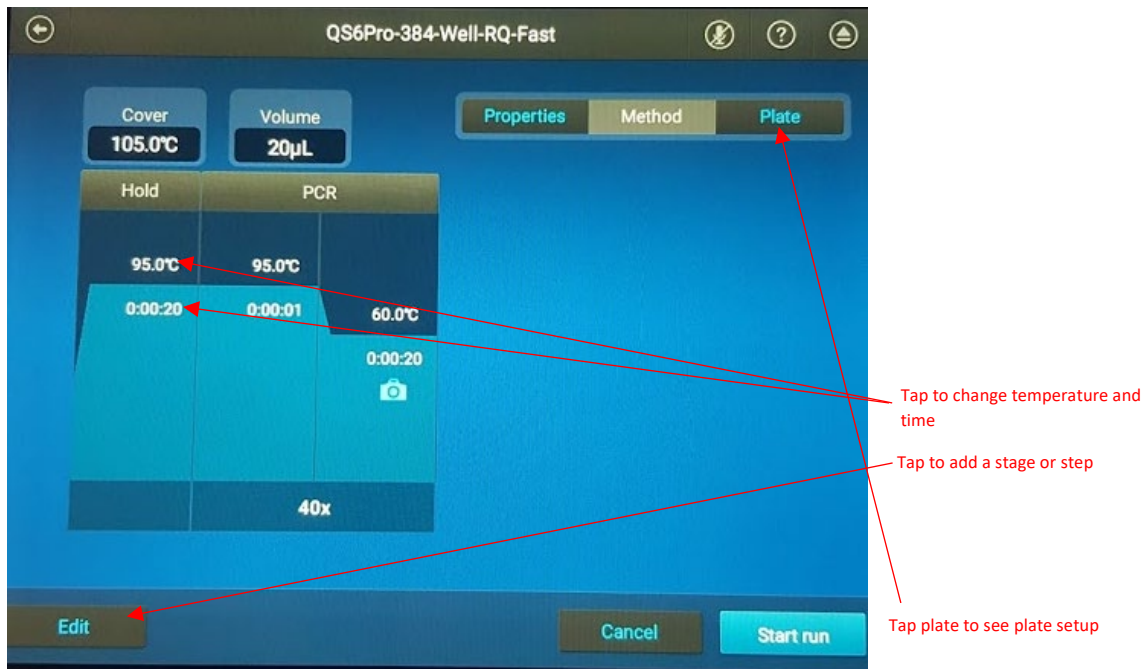
3. Choose a template:



4. Set file name and save location:



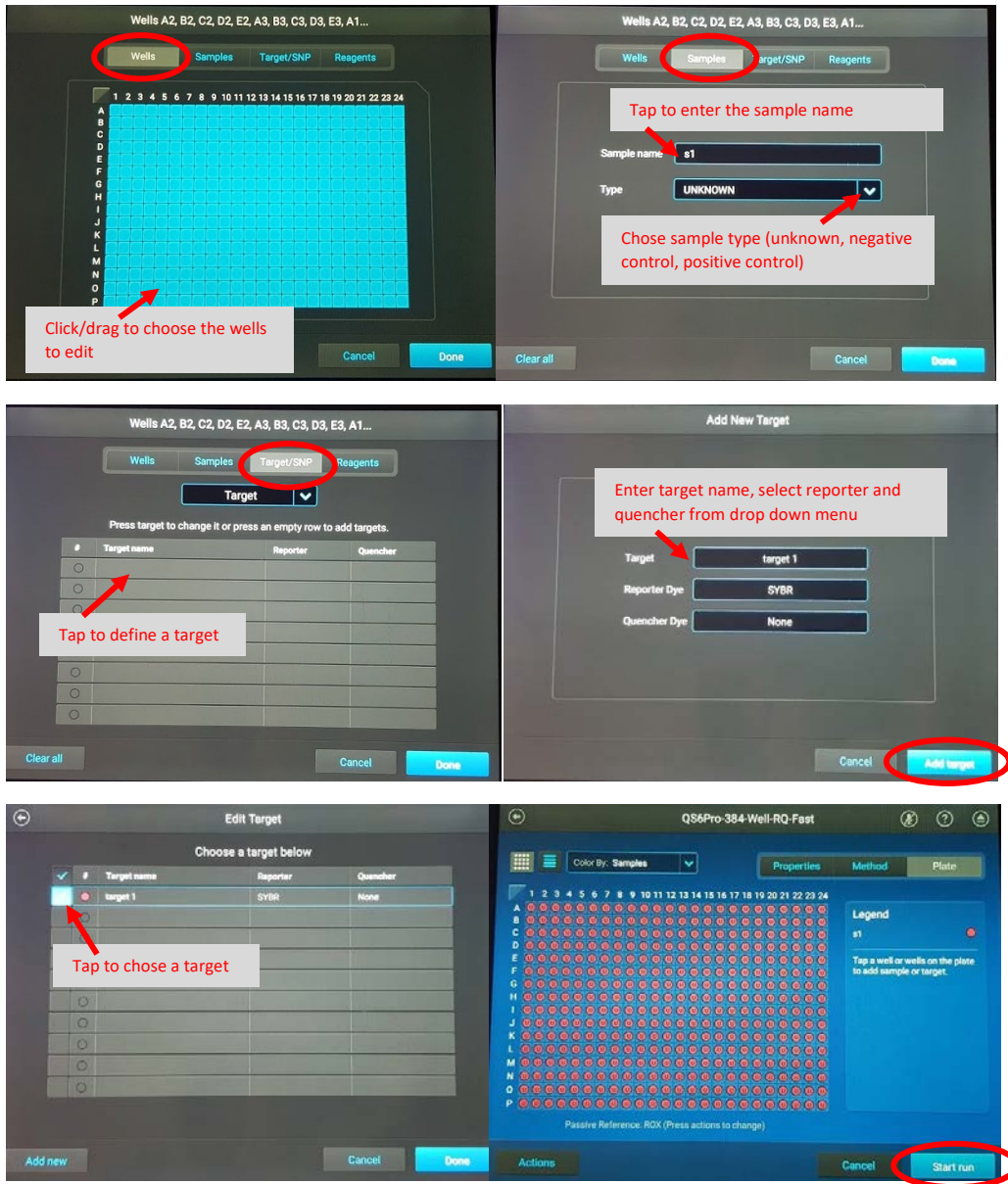
5. Set up run method:



6. a. Plate set up:



6. b. **Optional:** Defining and assigning samples and targets:



3. DATA ANALYSIS USING DESIGN AND ANALYSIS SOFTWARE:

1. When the run is complete your data will be saved in the specified location. You can now log into the OES network to carry your data to your HLI Network folder.
2. Open the .eds file with the Design and Analysis Software.
 - a. You can download the free software at: www.thermofisher.com/qpcrsoftware
3. In the **Plate Setup** screen; →See Appendix A –7. for an example
 - a. Highlight the wells you would like to process, add a sample and a target by pressing the (+) on the right panel and assign by clicking the check box next to the sample/target name.
 - b. You can enter Biogroup information by clicking the cell underneath biogroup. Once a value is entered, it will appear in the drop down menu.
 - c. You can change the Reporter and Quencher by clicking in the cell underneath and scrolling through the drop down menu.
4. Check the **Passive Reference** is configured correctly according to your mastermix instructions.
→See Appendix A – 8. for an example
 - a. You can also import Plate setup, Taqman assay plate, export plate setup or flip the plate setup from the dropdown menu at the right top of plate panel or print the layout.
 - b. You can copy and paste sample information directly from Excel as long as the headings are identical.
5. In the **Quality Check** screen:
 - a. On the left panel click to filter by sample type, name or target to view your data.
 - b. Hovering over a well or curve will highlight it in the other panels and clicking on it will filter the results. You can also click and drag.
 - c. On the plate outline you can color by different attributes like sample, target, Cq, Cq confidence, Amplification score and Amplification status.
 - d. On the Well table, click on the checkbox to omit samples that fail the QC.
 - e. Click **analyze** to re-run the analysis.
→See Appendix C for an example
6. In the **Relative Quantification** screen:
 - a. View your results (RQ) by sample or Biological group.
 - b. On the plate outline you can color by different attributes like sample, Cq, Bio group.
 - c. Click on the (...) on the RQ graph panel or Plate outline panel to save an image.
 - d. Click on the (...) on the results table panel to export results only as a .csv file.
 - e. Click **Actions** -> **Export** to export all the parameters like QC, Cq, Results into a .csv, .xls or another format.
 - f. Click Actions -> Generate report to generate a .pdf report with all of the run parameters.
→See Appendix C for an example

***NOTE: If you chose PCR and not RQ as a template, you will need to set the analysis mode by clicking **Actions -> Analysis modules -> Relative Quantification**

Appendix C: Examples for data analysis

5. Quality Check tab

Choose sample type to view

Filter by sample or target to view

Hovering over a well will highlight the well in Amplification plot and well table

Clicking on a well will select the well to view

Click analyze to see modified results

Click to omit specific wells

Design & Analysis Software 2.4.3

2019-12-19 DICER-GAPDH-ACTB- bio groups.eds

Run Method Plate Setup Run Summary **Quality Check** Relative Quantification

Amplification Plot

Amplification Plot

Well Table

Well#	Omit#	Sample#	Target#	Task#
N9	<input type="checkbox"/>	283	GAPDH	Unknown
N10	<input type="checkbox"/>	283	GAPDH	Unknown
N11	<input type="checkbox"/>	283	GAPDH	Unknown
N13	<input type="checkbox"/>	298	GAPDH	Unknown
N14	<input type="checkbox"/>	298	GAPDH	Unknown
N15	<input type="checkbox"/>	298	GAPDH	Unknown
N17	<input type="checkbox"/>	283	ACTB	Unknown
N18	<input type="checkbox"/>	283	ACTB	Unknown
N19	<input type="checkbox"/>	298	ACTB	Unknown
N21	<input type="checkbox"/>	298	ACTB	Unknown
N22	<input type="checkbox"/>	298	ACTB	Unknown
N23	<input type="checkbox"/>	298	ACTB	Unknown
O1	<input type="checkbox"/>	294	DICER	Unknown
O2	<input type="checkbox"/>	294	DICER	Unknown
O3	<input type="checkbox"/>	294	DICER	Unknown
O9	<input type="checkbox"/>	294	GAPDH	Unknown
O10	<input type="checkbox"/>	294	GAPDH	Unknown
O11	<input type="checkbox"/>	294	GAPDH	Unknown

Color By: Sample

Total of 31 legends are hidden. Try resizing the panel vertically to show legends.

Show 500 of 279 Results

Select to color by different attributes like sample, target, Cq, Cq confidence, Amplification score and Amplification status

Design & Analysis Software 2.4.3

2019-12-19 DICER-GAPDH-ACTB- bio groups.eds

Run Method Plate Setup Run Summary **Quality Check** Relative Quantification

Amplification Plot

Well Table

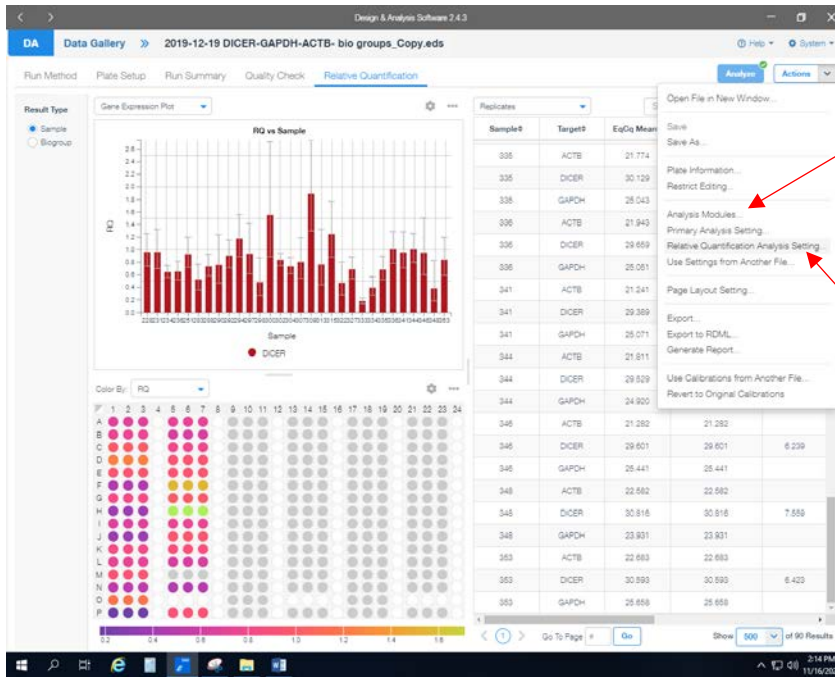
Well#	Omit#	Sample#	Target#	Task#
M10	<input type="checkbox"/>	281	GAPDH	Unknown
M11	<input type="checkbox"/>	281	GAPDH	Unknown
M13	<input type="checkbox"/>	NC	GAPDH	Negative Control
M14	<input type="checkbox"/>	NC	GAPDH	Negative Control
M15	<input type="checkbox"/>	NC	GAPDH	Negative Control
M17	<input type="checkbox"/>	281	ACTB	Unknown
M18	<input type="checkbox"/>	281	ACTB	Unknown
M19	<input type="checkbox"/>	281	ACTB	Unknown
M21	<input type="checkbox"/>	NC	ACTB	Negative Control
M22	<input type="checkbox"/>	NC	ACTB	Negative Control
M23	<input type="checkbox"/>	NC	ACTB	Negative Control
N1	<input type="checkbox"/>	283	DICER	Unknown
N2	<input type="checkbox"/>	283	DICER	Unknown
N2	<input type="checkbox"/>	283	DICER	Unknown
N5	<input type="checkbox"/>	298	DICER	Unknown
N6	<input type="checkbox"/>	298	DICER	Unknown
N7	<input type="checkbox"/>	298	DICER	Unknown
N8	<input type="checkbox"/>	293	GAPDH	Unknown

Color By: Cq

- Sample
- Target
- Cq
- Cq Confidence
- Amp Score
- Amp Status

Show 500 of 279 Results

6. Relative Quantification tab



Set the analysis mode – Relative quantification

Set the Primary analysis settings and Relative quantification analysis settings (Housekeeper, multiplex vs singleplex etc).

Set confidence level and analysis type

The 'Relative Quantification Analysis Setting' dialog box is shown in the 'General' tab. It includes fields for 'Confidence Level' (set to 95.0) and 'Analysis Type' (set to Singleplex). There are also options for 'Standard Deviation' and 'Maximum Allowed EqCo Mean'.

Set Normalization methods and Endogenous controls

The 'Relative Quantification Analysis Setting' dialog box is shown in the 'Endo Controls' tab. It includes a 'Normalization Type' section with radio buttons for 'Specific endogenous controls', 'Global normalization', and 'Skip normalization'. Below this is a table for selecting endogenous controls.

Target	Endogenous Control	Auto	Efficiency
ACTB	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	AUTO
DICER	<input type="checkbox"/>	<input checked="" type="checkbox"/>	AUTO
GAPDH	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	AUTO

Set reference sample/group

The 'Relative Quantification Analysis Setting' dialog box is shown in the 'References' tab. It includes a 'Reference Sample' dropdown (set to 336) and a 'Reference Group' dropdown (set to Control). Below these is a table for specifying reference samples for each biogroup.

Group	Use Default	Reference Sample
Control	<input checked="" type="checkbox"/>	DEFAULT
IPF	<input checked="" type="checkbox"/>	DEFAULT
dcSSc	<input checked="" type="checkbox"/>	DEFAULT
dcSScviLD	<input checked="" type="checkbox"/>	DEFAULT
lcSSc	<input checked="" type="checkbox"/>	DEFAULT
lcSScviLD	<input checked="" type="checkbox"/>	DEFAULT

6. Relative Quantification tab - continued

Select how you would like to view your results

Select to color by different attributes like sample, Cq, Bio group

Export result table only as a.csv file

Biogroup#	Target#	Eq/Dq Mean#	Adjusted Eq/Dq	Export
Control	ACTB	21.872	21.872	
Control	DICER	29.978	29.978	6.394
Control	GAPDH	25.293	25.293	
PF	ACTB	21.529	21.529	
PF	DICER	29.842	29.842	7.061
PF	GAPDH	24.034	24.034	
dc5Sc	ACTB	21.878	21.878	
dc5Sc	DICER	29.771	29.771	6.265
dc5Sc	GAPDH	25.136	25.136	
dc5ScwLD	ACTB	21.362	21.362	
dc5ScwLD	DICER	28.908	28.908	6.593
dc5ScwLD	GAPDH	24.269	24.269	
ic5Sc	ACTB	21.878	21.878	
ic5Sc	DICER	29.750	29.750	6.294
ic5Sc	GAPDH	25.038	25.038	
ic5ScwLD	ACTB	22.487	22.487	
ic5ScwLD	DICER	30.586	30.586	7.032
ic5ScwLD	GAPDH	24.651	24.651	

Save the image

Export all data into .csv, .xls or another format

Generate a .pdf report