RT-qPCR experiment and analysis using LinRegPCR

(Maurice Overzier; last modified April 19, 2018)

Purpose

The following protocol can be used for determining RNA expression using an RT-qPCR detection method, with the use of the LightCycler 480 and LinRegPCR software.

Protocol

Register the LightCycler 480 at https://calendar.yahoo.com/ and log in using 'rochlc' and password 'LightCycler-12'; LGTC passwords can be found at https://www.lumc.nl/org/lgtc/equipment/. Also, pre-book the pre-PCR and the post-PCR.

After cDNA synthesis (cDNA is stable for a few years at -20°C, or a few weeks at 4°C; but try to use fresh cDNA for RT-qPCR), dilute your cDNA 10x, to prevent inhibitors to affect the PCR efficiency. Also, be sure to prepare your master mix for RT-qPCR in the pre-PCR room. No DNA is allowed inside this room. **Use gloves and a lab coat at all times.** Prepare your 384 well plate as follows (**Always take along <u>at least two</u> reference genes**):

		1	2	3	4	5	6	7	8	9
mSMA 1	A	1	2	3	4	-RT	H2O			
mSMA 1	B	1	2	3	4	-RT	H2O			
mSMA 1	C	1	2	3	4	-RT	H2O			
mSMA 2	D	1	2	3	4	-RT	H2O			
mSMA 2	E	1	2	3	4	-RT	H2O			
mSMA 2	F	1	2	3	4	-RT	H2O			
mMyocardin 1	G	1	2	3	4	-RT	H2O			
mMyocardin 1	H	1	2	3	4	-RT	H2O			
mMyocardin 1	I	1	2	3	4	-RT	H2O			
mMyocardin 2	J	1	2	3	4	-RT	H2O			
mMycocardin 2	K	1	2	3	4	-RT	H2O			
mMyocardin 2	L	1	2	3	4	-RT	H2O			
mSM22 1	M	1	2	3	4	-RT	H2O			
mSM22 1	N	1	2	3	4	-RT	H2O			
mSM22 1	O	1	2	3	4	-RT	H2O			
	P									

Primermix per well		Total per primerset		Total qPCR	
2 x SENSI mix	4 µl	92	µl	828	µl
F primer	1 µl	23	µl	207	µl
R primer	1 µl	23	µl	207	µl
Total	6 µl	138	µl	1242	µl

cDNA dilutions

cDNA samples	short name
Brain	
7214475	1
7214477	2
7216430	3
7219311	4

Primers: dilute 1 µl of 100 µM stock solution primer in 99 µl MQ

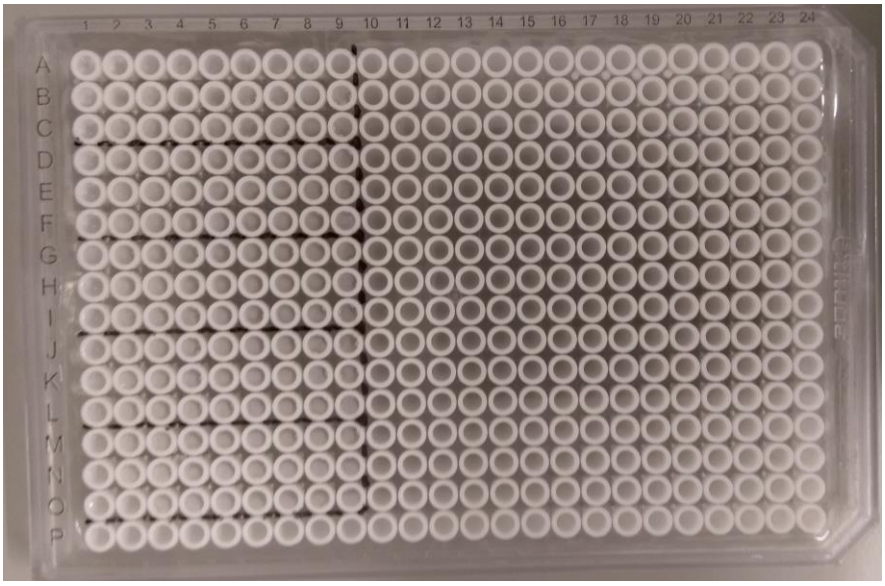
Before preparing, thaw your primer stocks (100 µM) and dilute them 50-200x (generally 100x). This depends on the primer specificity. Also thaw the 2x SYBR SensiMix HI-ROX. This SensiMix contains SYBR Green I, a relatively cheap dye that binds to all ds DNA, a hot-start Taq polymerase that is activated after a 10 minute incubation step at 95°C, a highly concentrated ROX that acts as a baseline fluorescence level that compensates well-to-well variation and variation introduced by air bubbles. The SensiMix also contains stabilisers and dNTPs.

For the master mix per primer set, use:

- 4 µl 2x SensiMix
- 1 µl Forward primer (1 µM)
- 1 µl Reverse primer (1 µM)

Make a little bit more of master mix (5% more) to compensate for pipetting errors. When choosing primer pairs, make sure the amplicon's size is between 50 and 150 bp.

For pipetting the plate, it's useful to draw a line around the group of wells you are planning to use, so you won't mix up any wells.



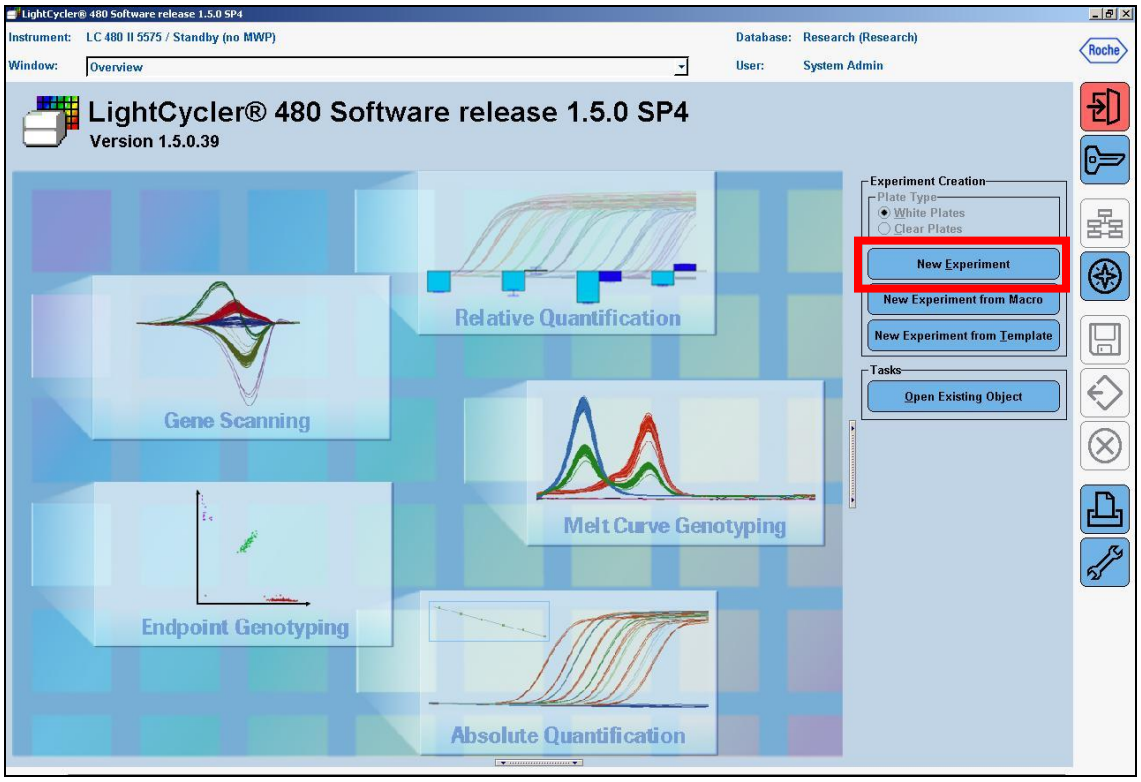
The wells are filled with 6 µl of master mix, still in the pre-PCR room. After each desired well contains your SensiMix/primers mix, move the plate to the post-PCR hood in the DMD. Make sure you cover the plate when you switch to the DMD lab **and** make sure you've cleaned the fume hood with DNAzap and turned on the UV light before you start working inside the fume hood. After working in the post-PCR hood, also clean with DNAzap.

Inside the post-PCR hood (use gloves and a special lab coat, can be found left of the post-PCR), pipette 2 µl of cDNA to each designated well (by **reversed** pipetting) and cover

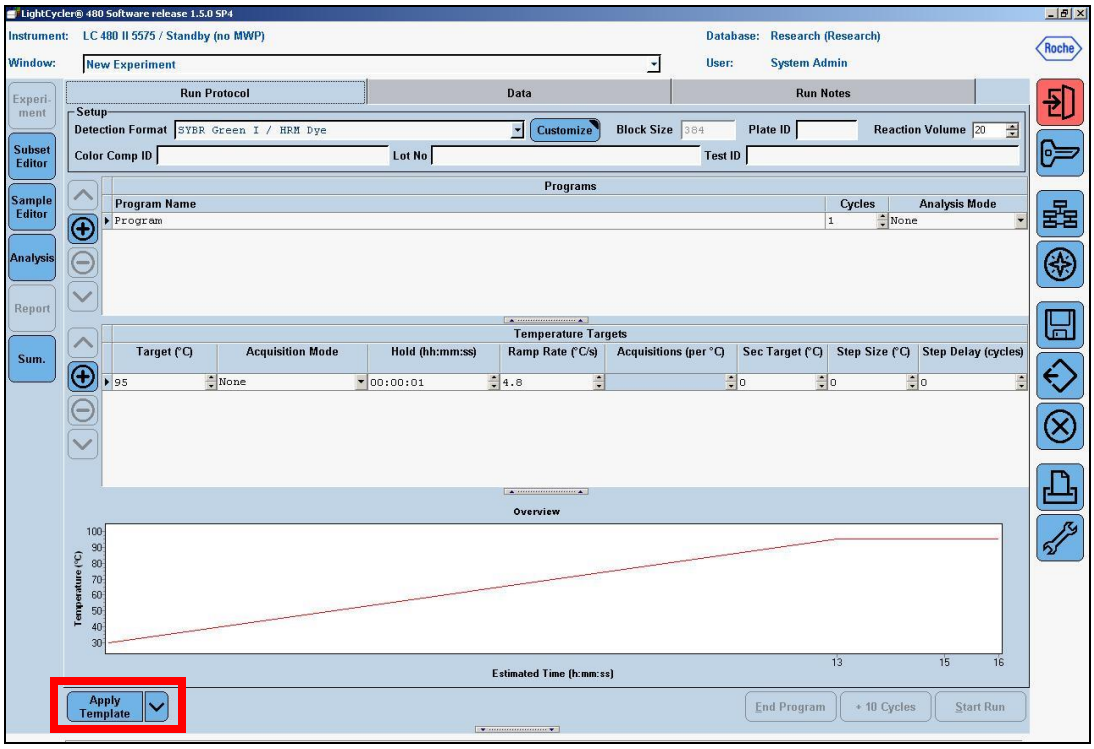
the plate with the plastic seal after all the wells contain cDNA (or negative controls, i.e. –RT and H_2O). **Take along a –RT control to check for the presence of gDNA, and take along a water control to test for primer-dimer formations.** Check if the seal is clear from smudges, **if not; take a new seal**. Make sure that the wells are all covered tightly by the plastic seal, this avoids evaporation during the PCR cycles.

When the plate is sealed, you can use the centrifuge in R4-02 to spin down the plate. Next to the centrifuge you'll find a 384 well plate to **balance the centrifuge**. Make sure to use the correct rotor inserts (i.e. the plateaus that fit the plate) and place them accordingly; check the numbers on the rotor and on the plateaus. Spin the plate down at approximately 2200 rpm for a few seconds.

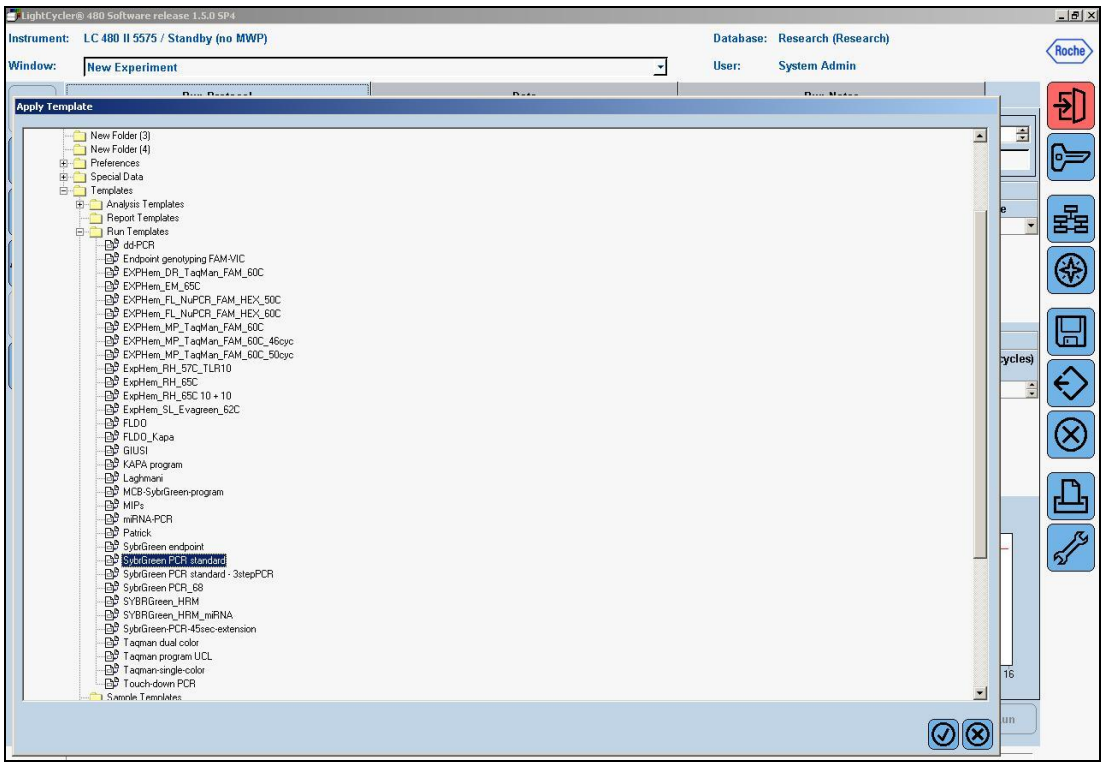
Take the plate to R4-21 and turn on the PC and the LightCycler 480, this can be done by simply flipping the switch on the right side of the machine. Use the credentials, found on the computer itself, to log in. Start the LightCycler 480 software and log in also by using the credentials found on the computer. At the bottom of the screen of the LightCycler 480 software there will be a notification when the machine is warmed up and ready for use.



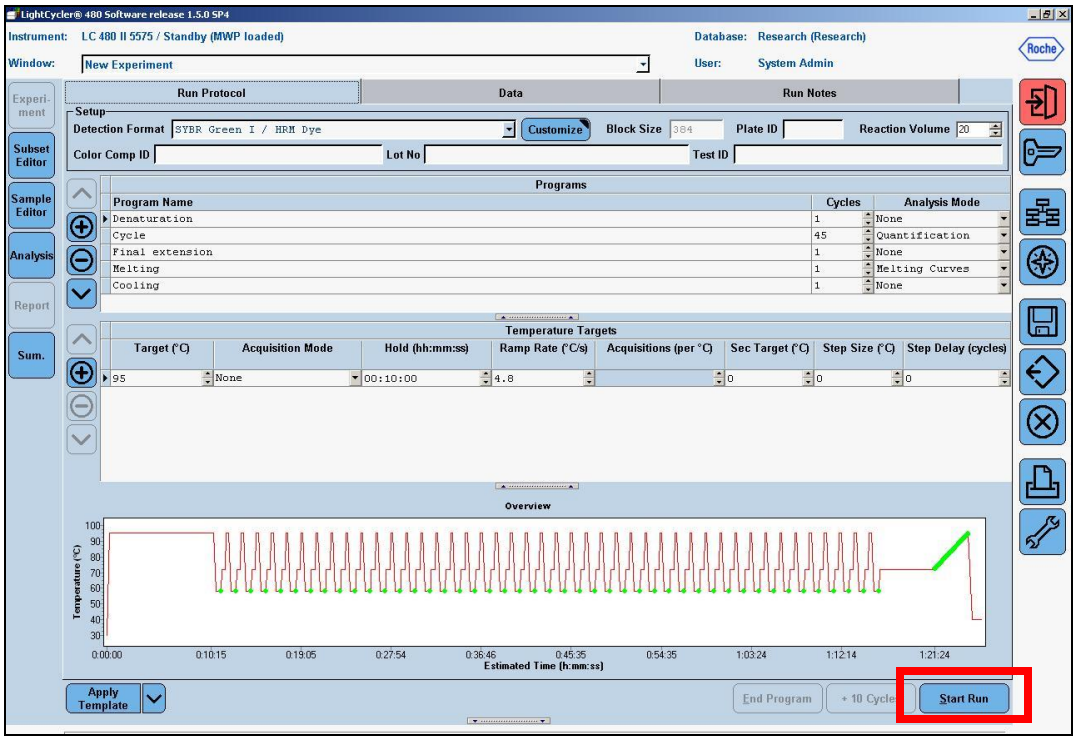
Start a new experiment by pressing 'New Experiment'.



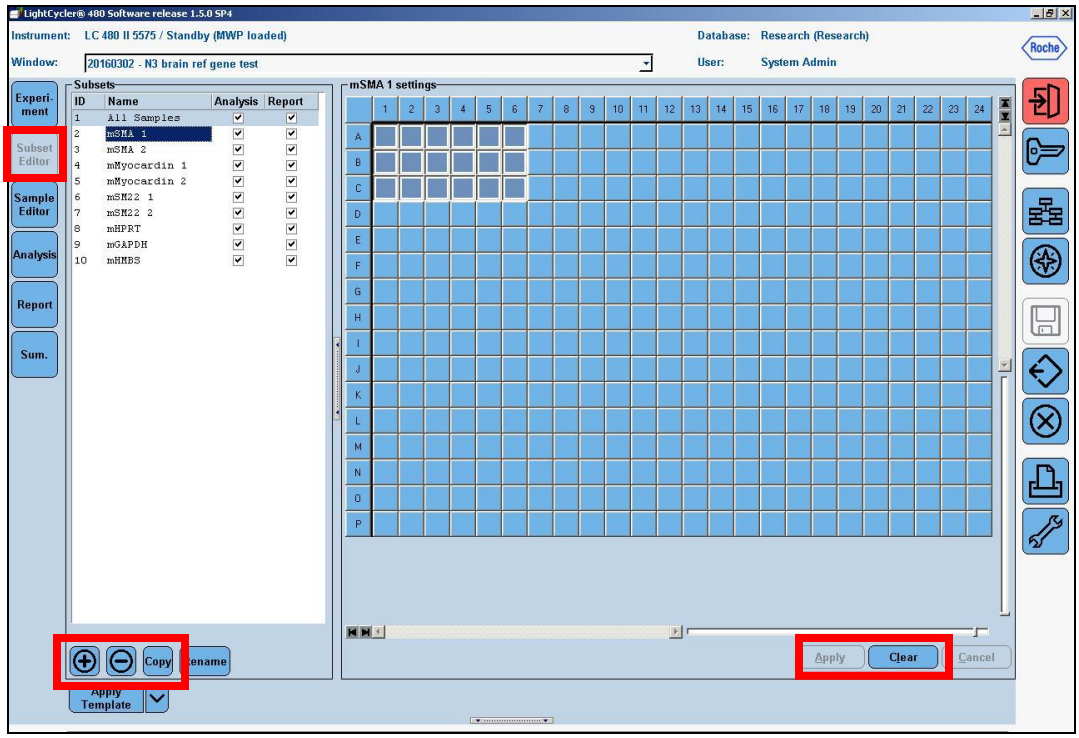
A new screen will pop up. Click ‘Apply Template’.



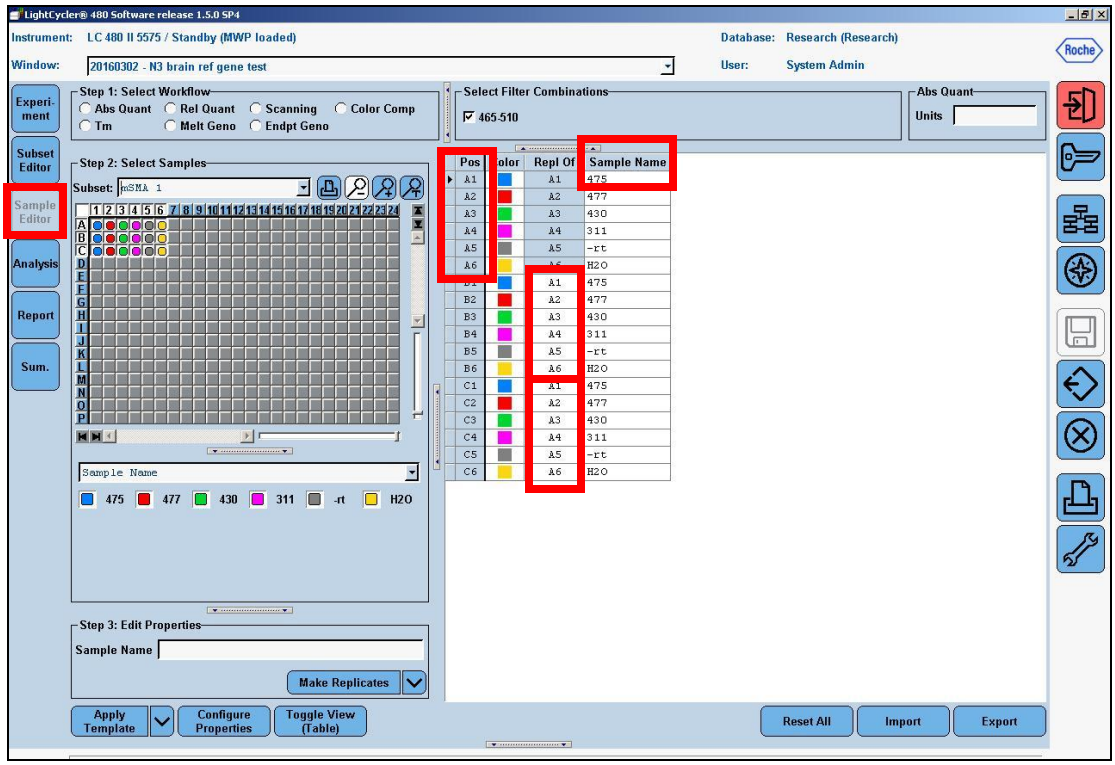
Choose the template you desire, usually ‘SybrGreen PCR standard’ will suffice. Press √.



Here you can view the RT-qPCR protocol. Press ‘Start Run’. There will be a new window asking you where to save the .ixo file. Select the preferred directory.



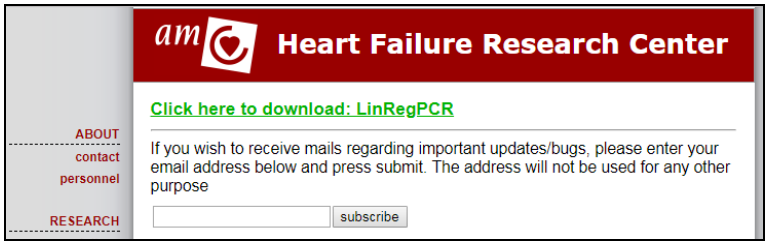
After the RT-qPCR is started, you can fill out the plate layout in the software. Go to the ‘Subset Editor’ button and press the + sign at the bottom. Select the cells on the right, representing the location of the group of wells on the 384 well plate. Do this per primer set. After selecting the cells per primer set, press ‘Apply’ in the bottom right corner.



In the ‘Sample Editor’ button, you can select which wells represent which sample. Enter a recognisable sample name for the first set of your triplicate (in this case A1-A6). Then, select the positions (in this case by pressing A1 and Shift+A6), copy (Ctrl+c) and paste (Ctrl+v) then in the cells of ‘Repl Of’ to insert these cells as replicates (in this case from B1-B6 and again for C1-C6).

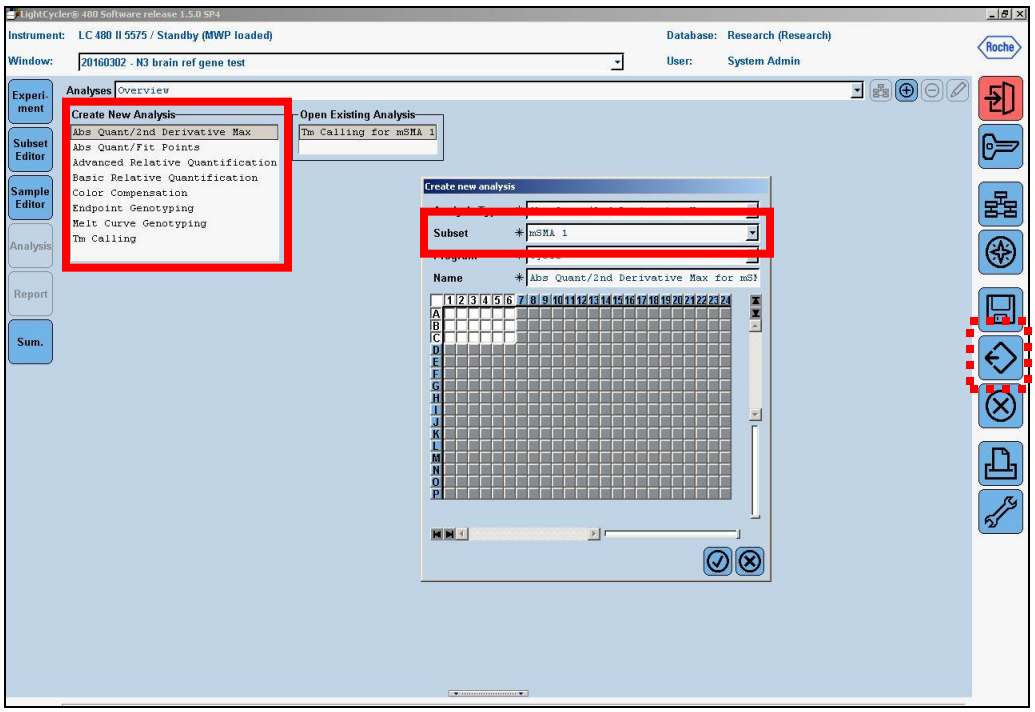
While you’re waiting for the RT-qPCR to finish, you can use the time to clean up the pre-PCR and post-PCR fume. Also, you can download the LinRegPCR software from http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LinRegPCR.zip&sub=LinRegPCR and follow the next steps.

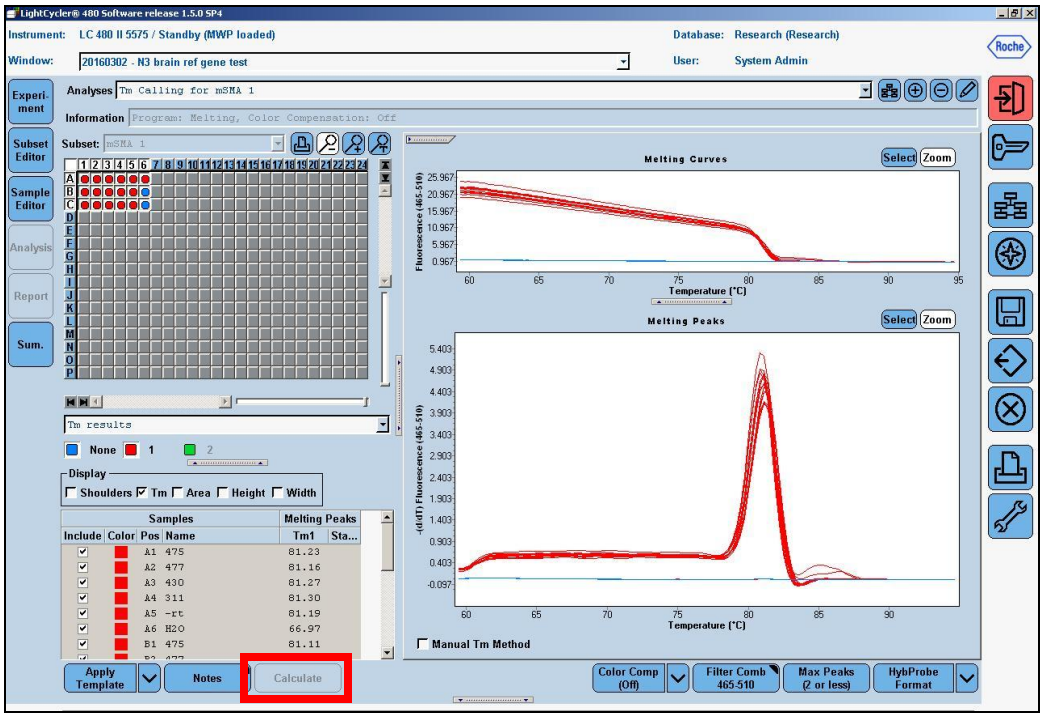
Download the software by entering the CAPTCHA text and pressing enter.



Your download will start after clicking the text ‘Click here to download: LinRegPCR’. Install the software accordingly.

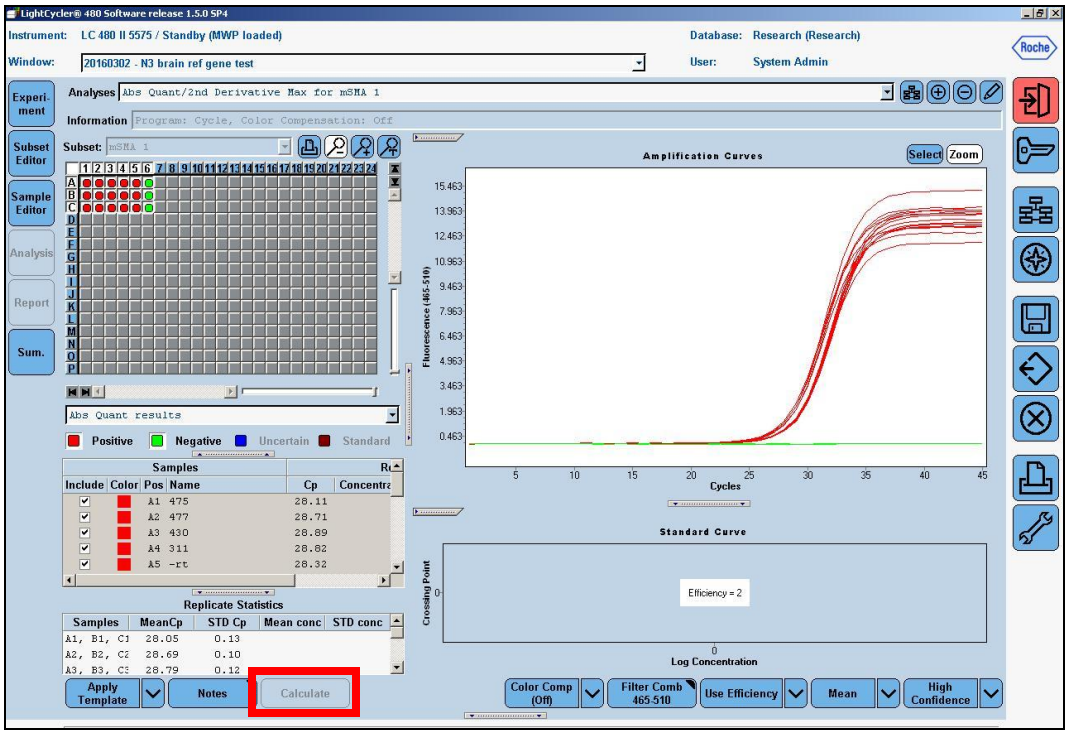
When the RT-qPCR has finished its run (using the ‘Sybr Green standard’ protocol, approximately after 1 hour and 20 minutes), go through the following steps (can be done at the R4-21 PC or export the .ixo file by clicking the ‘export’ button, below the ‘save’ button; dashed square in the picture below, and analyse on your own PC).



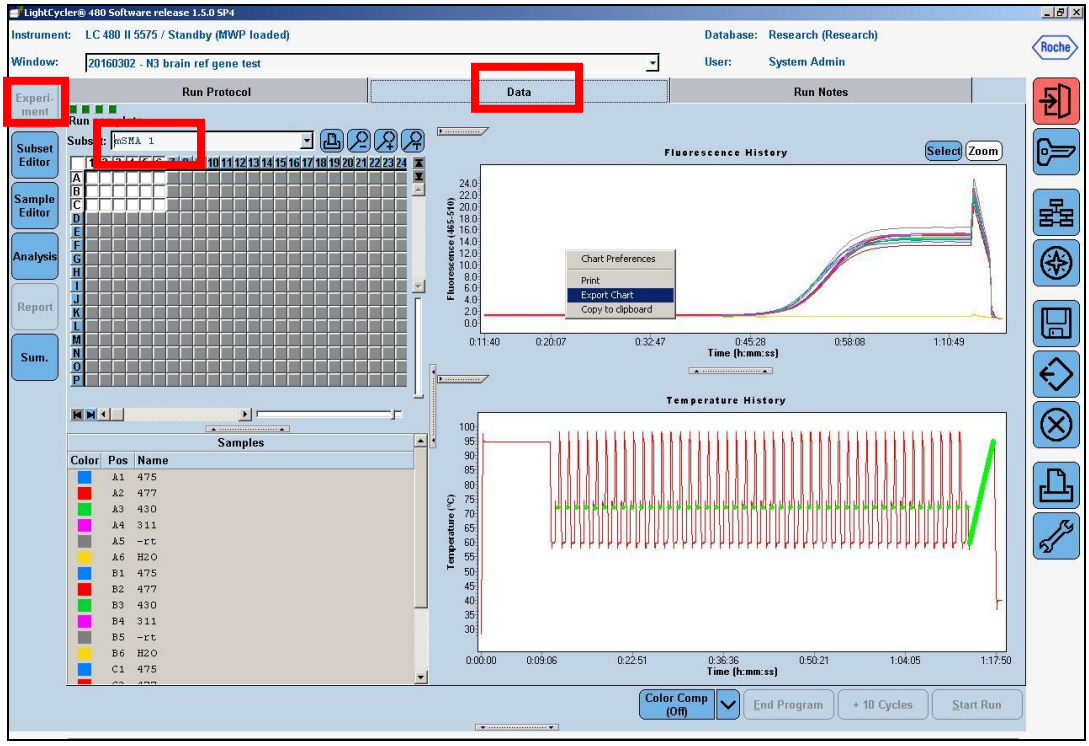


Check your primer specificity by clicking the button ‘Analysis’ followed by clicking ‘Tm calling’ and select your primer set. Click the ‘Calculate’ button to let LightCycler 480 calculate the Tm values. During PCR amplification the amplicon becomes incorporated with an intercalating dye which increases fluorescence of the reaction. Upon melting, DNA denaturation releases this dye which decreases fluorescence of the reaction. This decrease in fluorescence is converted into melt curve data that is then used to generate melt peaks (as seen above). When one peak is observed in the ‘Melting Peaks’ chart, it suggests that only one product is formed. Multiple peaks could be caused by the target sequence having multiple isoforms, a non-specific PCR product or primer-dimers. If either of these causes is the case, try to design new primers. As a check; your negative controls should not show a peak. If this is not the case, there might me a contamination in your master mix. You can check this for every subset.

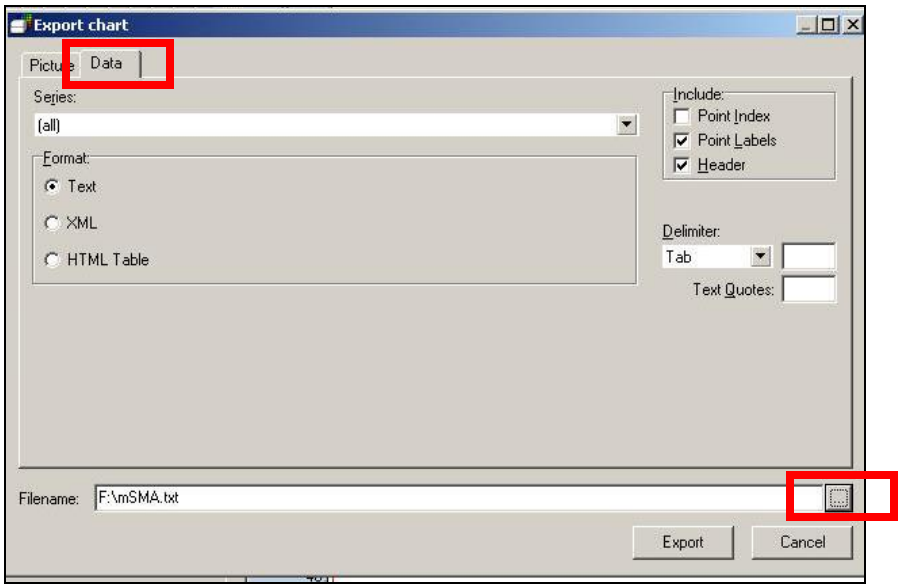
To visualise the amplification curves, go back to the ‘Analysis’ button and select ‘Abs Quant/2nd Derivative Max’ and choose your primer set of interest in the ‘Subset’ drop down menu.



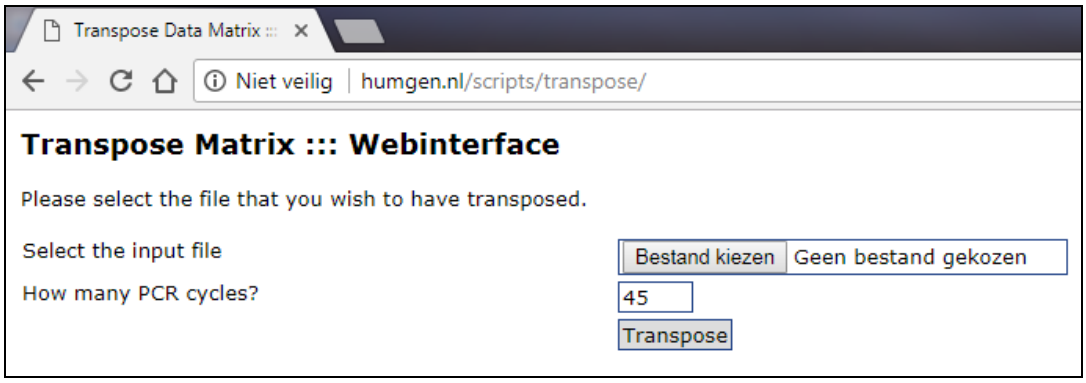
Press the ‘Calculate’ button to let LightCycler 480 calculate the Cq (Cp or Ct) values. The curves should be smooth during the exponential phase of the RT-qPCR. Any spikes in the curves may be the result of unstable light sources from the instrument, or sample preparation problems, such as the presence of bubbles in the reaction wells. You can check this for every subset.



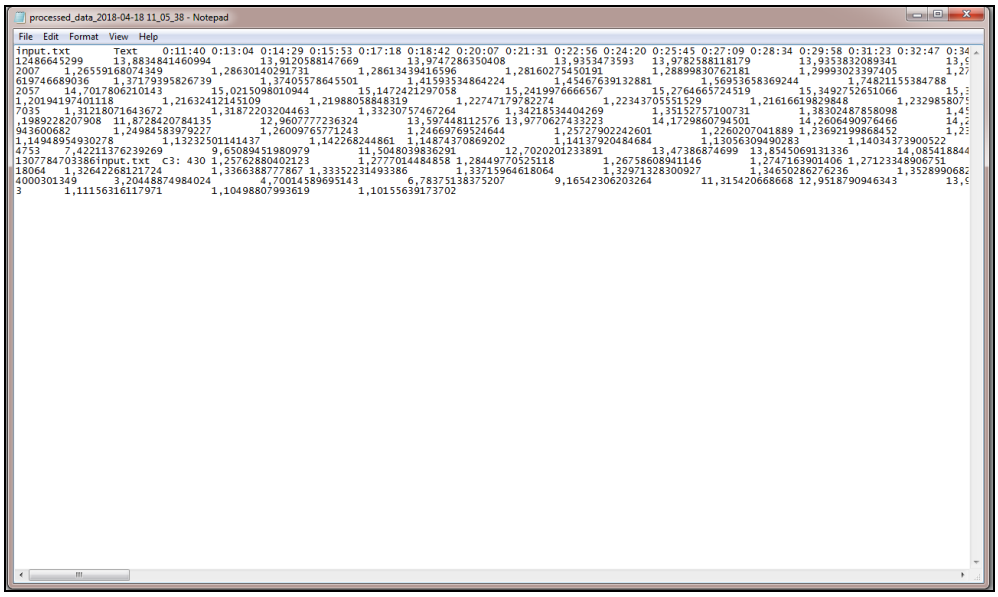
To export the data for further analysis, go back to the ‘Experiment’ button and select the ‘Data’ tab. Select your primer set in the ‘Subset’ drop down menu. Right click the graph on the right and select ‘Export Chart’.



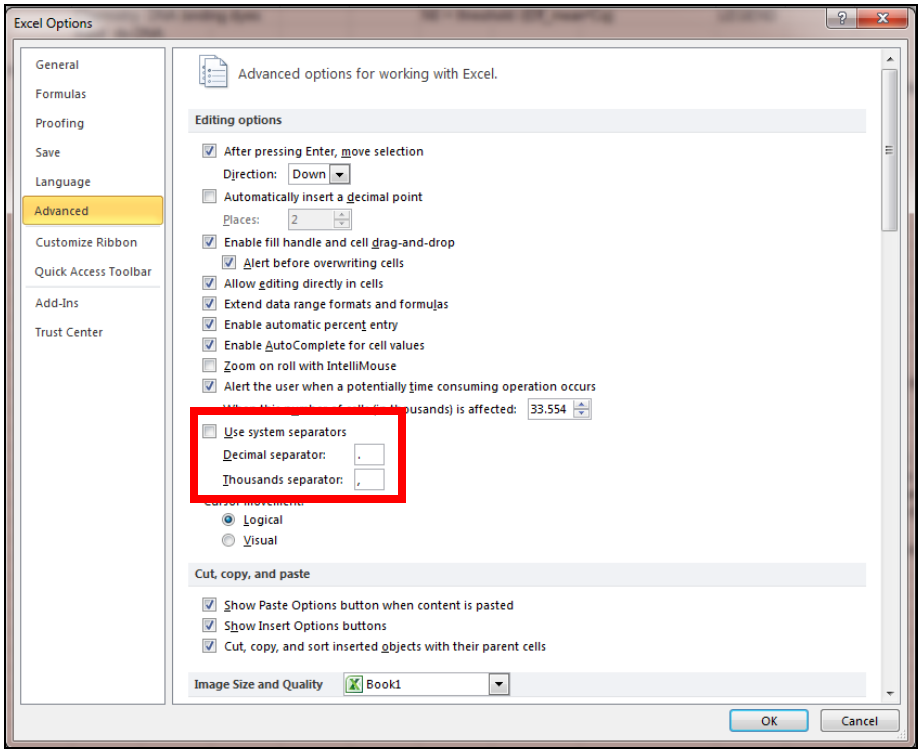
Click the ‘Data’ tab and select where you want to save the data file and name it (a USB flash drive when you’re using the computer at R4-21 or your personal file when you’re using the LightCycler 480 software on your own PC). Perform this action for every subset.



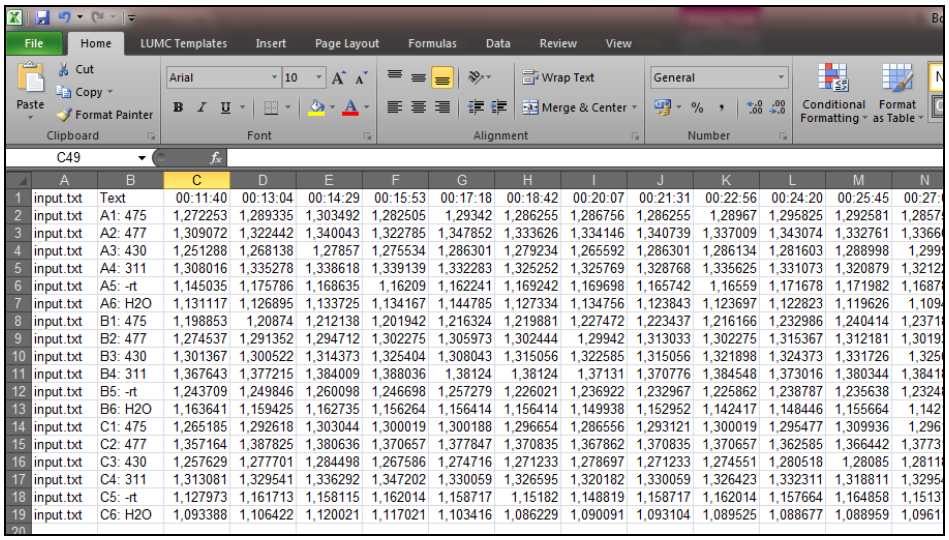
Go to http://humgen.nl/scripts/transpose/ and upload the .txt file that was obtained by retrieving the data from LightCycler 480 by pressing ‘Bestand kiezen’, set the amount of PCR cycles to 45 (or another amount if you used a different RT-qPCR protocol) and press ‘Transpose’. This step is to switch the rows and columns that are represented in the text file, and it will be easier to work with later on in Excel.



Select the complete text (Ctrl+a) and copy (Ctrl+c).

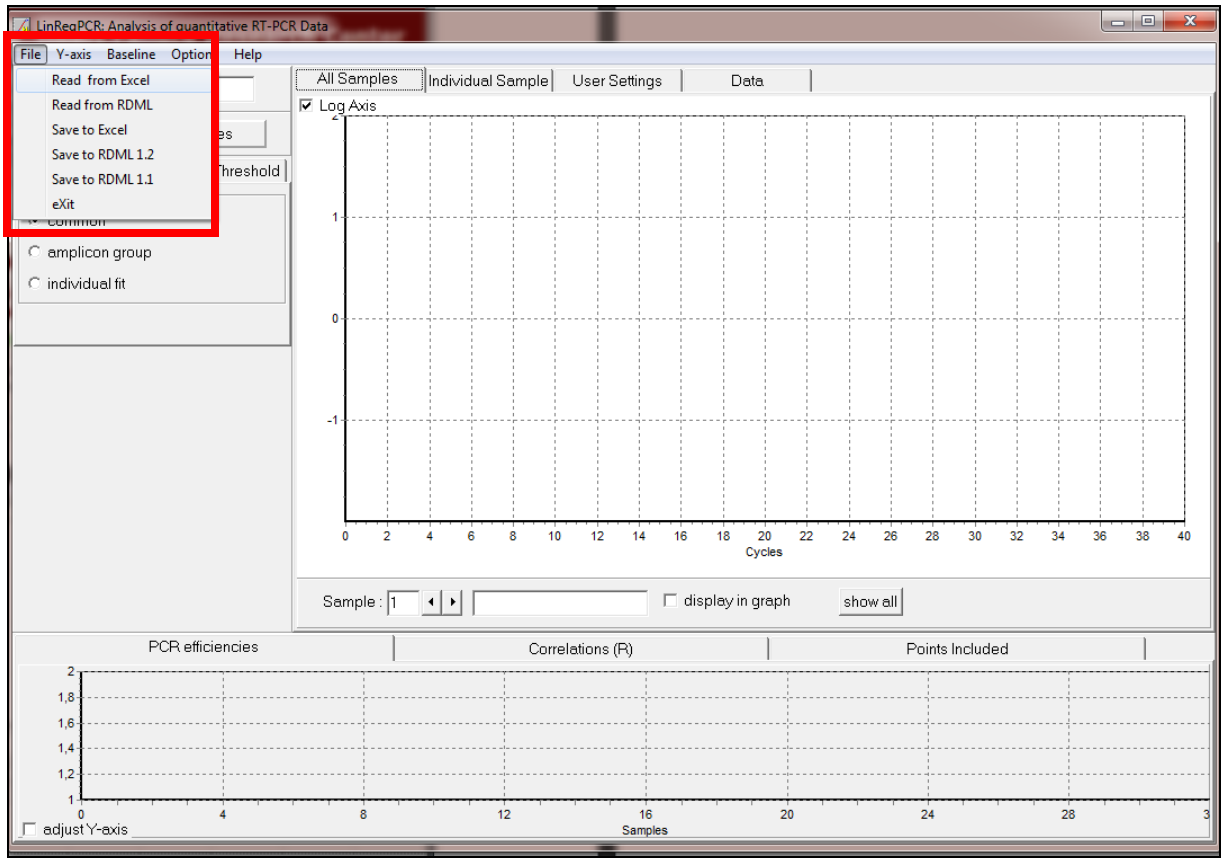


Open a new Excel file and make sure to first change the system separators to the settings as shown above. If the box is ticked, untick the box and change the decimal and thousands separators. Restart Excel after this change.

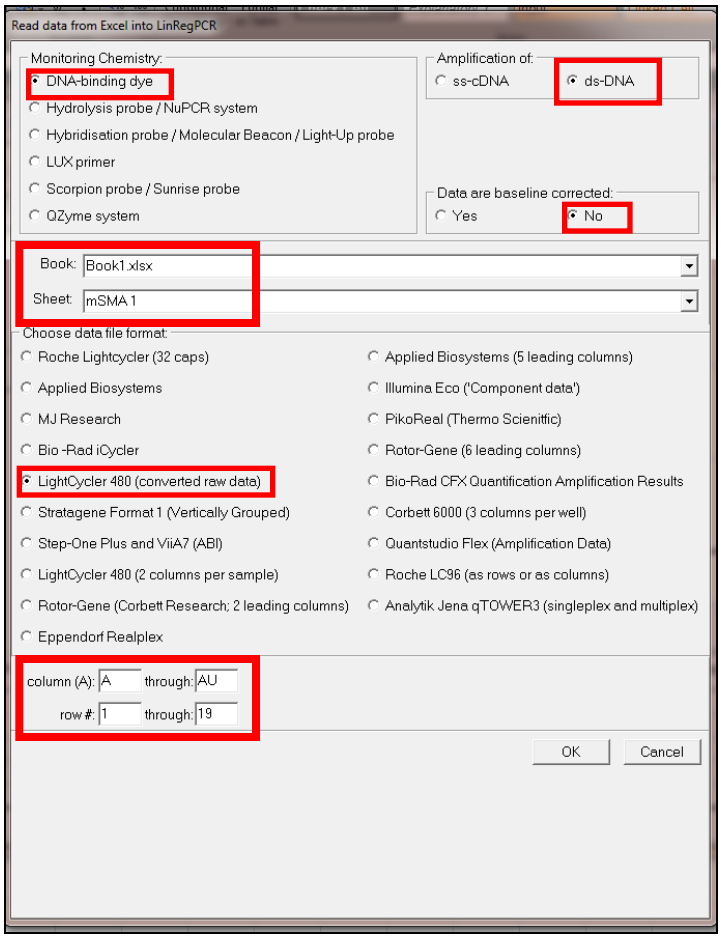


	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	input.txt	Text	00:11:40	00:13:04	00:14:29	00:15:53	00:17:18	00:18:42	00:20:07	00:21:31	00:22:56	00:24:20	00:25:45	00:27:
2	input.txt	A1: 475	1,272253	1,289335	1,303492	1,282505	1,29342	1,286255	1,286756	1,286255	1,28967	1,295825	1,292581	1,2857
3	input.txt	A2: 477	1,309072	1,322442	1,340043	1,322785	1,347852	1,333626	1,334146	1,340739	1,337009	1,343074	1,332761	1,3366
4	input.txt	A3: 430	1,251288	1,268138	1,27857	1,275534	1,286301	1,279234	1,265592	1,286301	1,286134	1,281603	1,288998	1,299
5	input.txt	A4: 311	1,308016	1,335278	1,338618	1,339139	1,332283	1,325252	1,325769	1,328768	1,335625	1,331073	1,320879	1,3212
6	input.txt	A5: -rt	1,145035	1,175786	1,168635	1,16209	1,162241	1,169242	1,169698	1,165742	1,16559	1,171678	1,171982	1,1687
7	input.txt	A6: H2O	1,131117	1,126895	1,133725	1,134167	1,144785	1,127334	1,134756	1,123843	1,123697	1,122823	1,119626	1,109
8	input.txt	B1: 475	1,198853	1,20874	1,212138	1,201942	1,216324	1,219881	1,227472	1,223437	1,216166	1,232986	1,240414	1,2371
9	input.txt	B2: 477	1,274537	1,291352	1,294712	1,302275	1,305973	1,302444	1,29942	1,313033	1,302275	1,315367	1,312181	1,3019
10	input.txt	B3: 430	1,301367	1,300522	1,314373	1,325404	1,308043	1,315056	1,322585	1,315056	1,321898	1,324373	1,331726	1,325
11	input.txt	B4: 311	1,367643	1,377215	1,384009	1,388036	1,38124	1,38124	1,37131	1,370776	1,384548	1,373016	1,380344	1,3841
12	input.txt	B5: -rt	1,243709	1,249846	1,260098	1,246698	1,257279	1,226021	1,236922	1,232967	1,225862	1,238787	1,235638	1,2324
13	input.txt	B6: H2O	1,163641	1,159425	1,162735	1,156264	1,156414	1,156414	1,149938	1,152952	1,142417	1,148446	1,155664	1,142
14	input.txt	C1: 475	1,265185	1,292618	1,303044	1,300019	1,300188	1,296654	1,286556	1,293121	1,300019	1,295477	1,309936	1,296
15	input.txt	C2: 477	1,357164	1,387825	1,380636	1,370657	1,377847	1,370835	1,367862	1,370835	1,370657	1,362585	1,366442	1,3773
16	input.txt	C3: 430	1,257629	1,277701	1,284498	1,267586	1,274716	1,271233	1,278697	1,271233	1,274551	1,280518	1,28085	1,2811
17	input.txt	C4: 311	1,313081	1,329541	1,336292	1,347202	1,330059	1,326595	1,320182	1,330059	1,326423	1,332311	1,318811	1,3295
18	input.txt	C5: -rt	1,127973	1,161713	1,158115	1,162014	1,158717	1,15182	1,148819	1,158717	1,162014	1,157664	1,164858	1,1513
19	input.txt	C6: H2O	1,093388	1,106422	1,120021	1,117021	1,103416	1,086229	1,090091	1,093104	1,089525	1,088677	1,088959	1,0961

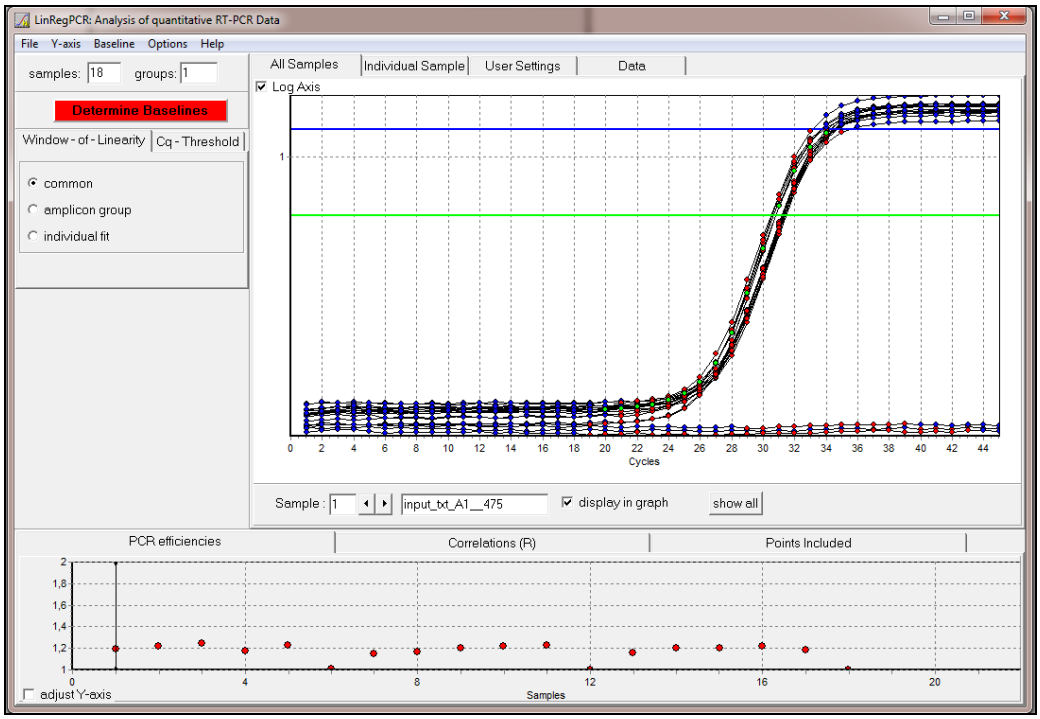
Paste (Ctrl+v) the data into a clean Excel tab. Change the name of the tab to the name of the primer target (gene name). Make sure the data is placed from the A1 cell. Repeat these steps (from transposing the data) for any other subsets and paste these data in new tabs of the same Excel file. For the next steps, it's important that this Excel file is the only file that is opened, so save and close other Excel files you're working in.



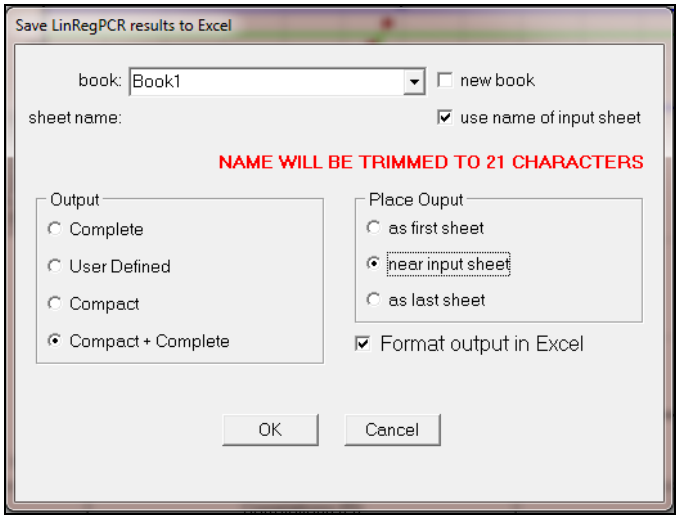
Open LinRegPCR and click 'File', then 'Read from Excel'.



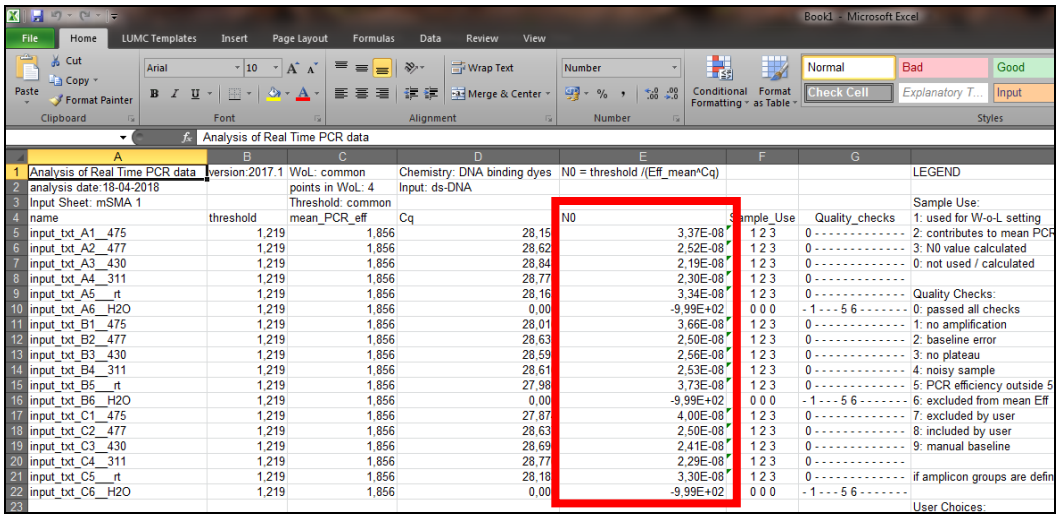
A new window opens and select the boxes as shown above. During RT-qPCR a DNA-binding dye was used (Sybr Green), ds-DNA was used as amplification and a LightCycler 480 was used to convert the raw data files. In the ‘Book’ drop down menu, you’ll see the name of the Excel file you’re working in. LinRegPCR can only read from one Excel file at a time, so it’s important to close all other Excel files. In the ‘Sheet’ drop down menu, select the tab containing the raw data from the Excel file. At the bottom, enter the columns running from ‘A’ through ‘AU’ (this is always the case) and enter the amount of rows (from ‘1’ through x, check your Excel file). Press ‘OK’.



The data will be loaded into LinRegPCR and show curves comparable as shown above. Click ‘Determine Baselines’.



Select the output as ‘Compact + Complete’ and the place of the output as ‘near input sheet’. Press ‘OK’ and LinRegPCR will start calculating. Repeat this for every subset.

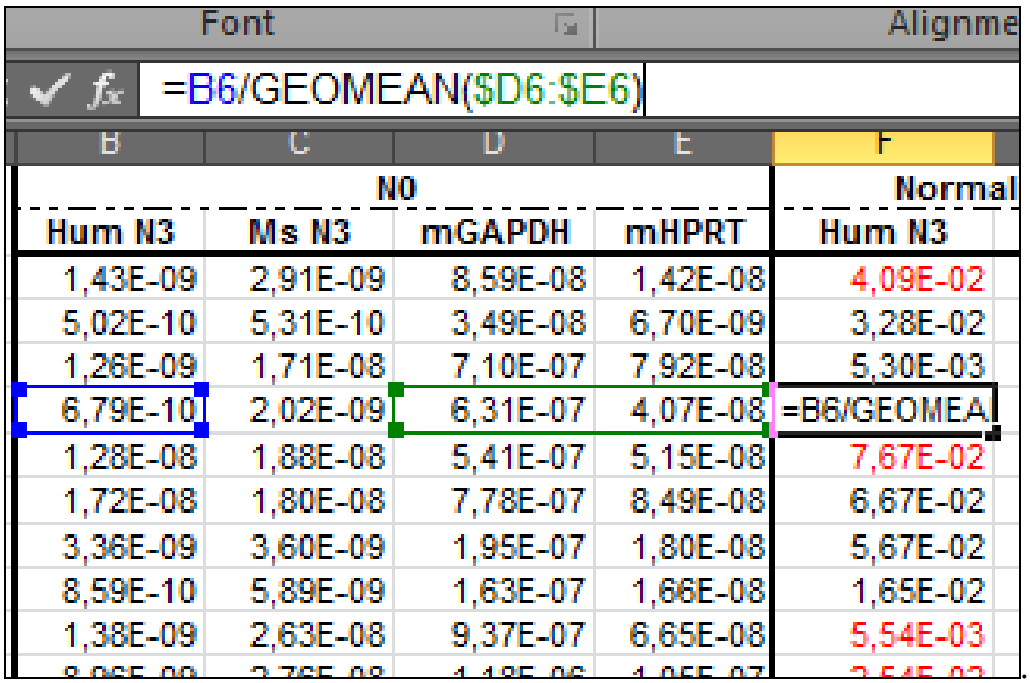


	A	B	C	D	E	F	G	
1	Analysis of Real Time PCR data	version:2017.1	WoL: common	Chemistry: DNA binding dyes	N0 = threshold /(Eff_mean^Cq)			LEGEND
2	analysis date:18-04-2018		points in WoL: 4	Input: ds-DNA				
3	Input Sheet: mSMA 1		Threshold: common					Sample Use:
4	name	threshold	mean_PCR_eff	Cq	N0	Sample_Use	Quality_checks	1: used for W-o-L setting
5	input_txt_A1__475	1,219	1,856	28,15	3,37E-08	1 2 3	0 - - - - - - - - - - - -	2: contributes to mean PCR
6	input_txt_A2__477	1,219	1,856	28,62	2,52E-08	1 2 3	0 - - - - - - - - - - - -	3: N0 value calculated
7	input_txt_A3__430	1,219	1,856	28,84	2,19E-08	1 2 3	0 - - - - - - - - - - - -	0: not used / calculated
8	input_txt_A4__311	1,219	1,856	28,77	2,30E-08	1 2 3	0 - - - - - - - - - - - -	
9	input_txt_A5___rt	1,219	1,856	28,16	3,34E-08	1 2 3	0 - - - - - - - - - - - -	Quality Checks:
10	input_txt_A6__H2O	1,219	1,856	0,00	-9,99E+02	0 0 0	- 1 - - - 5 6 - - - - - - -	0: passed all checks
11	input_txt_B1__475	1,219	1,856	28,01	3,66E-08	1 2 3	0 - - - - - - - - - - - -	1: no amplification
12	input_txt_B2__477	1,219	1,856	28,63	2,50E-08	1 2 3	0 - - - - - - - - - - - -	2: baseline error
13	input_txt_B3__430	1,219	1,856	28,59	2,56E-08	1 2 3	0 - - - - - - - - - - - -	3: no plateau
14	input_txt_B4__311	1,219	1,856	28,61	2,53E-08	1 2 3	0 - - - - - - - - - - - -	4: noisy sample
15	input_txt_B5___rt	1,219	1,856	27,98	3,73E-08	1 2 3	0 - - - - - - - - - - - -	5: PCR efficiency outside 5
16	input_txt_B6__H2O	1,219	1,856	0,00	-9,99E+02	0 0 0	- 1 - - - 5 6 - - - - - - -	6: excluded from mean Eff
17	input_txt_C1__475	1,219	1,856	27,87	4,00E-08	1 2 3	0 - - - - - - - - - - - -	7: excluded by user
18	input_txt_C2__477	1,219	1,856	28,63	2,50E-08	1 2 3	0 - - - - - - - - - - - -	8: included by user
19	input_txt_C3__430	1,219	1,856	28,69	2,41E-08	1 2 3	0 - - - - - - - - - - - -	9: manual baseline
20	input_txt_C4__311	1,219	1,856	28,77	2,29E-08	1 2 3	0 - - - - - - - - - - - -	
21	input_txt_C5___rt	1,219	1,856	28,18	3,30E-08	1 2 3	0 - - - - - - - - - - - -	if amplicon groups are defin
22	input_txt_C6__H2O	1,219	1,856	0,00	-9,99E+02	0 0 0	- 1 - - - 5 6 - - - - - - -	
23								User Choices:

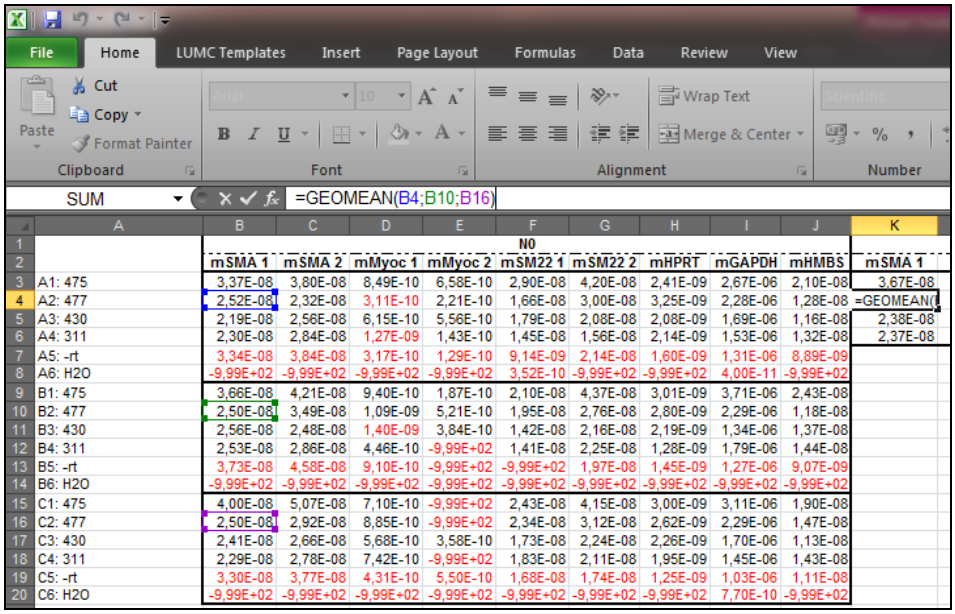
After calculating, LinRegPCR adds two new tabs to your Excel file; a ‘compact’ tab (shown above) and an ‘output’ tab. In the output tab, under ‘indiv PCR eff’ you can find the efficiencies of the primers per sample. This efficiency is calculated by quantifying the amount of newly formed amplicon after each PCR cycle. So in theory, this number should be 2.00, because you expect that after each cycle, the amount of PCR product is doubled. In practice, the PCR efficiency is somewhere between 1.80-1.90. If the efficiency is lower than 1.80, one of the following could be the cause: the samples may contain PCR inhibitors (if so, use cDNA that is more diluted), primer design is not optimal or inaccurate sample/master mix pipetting. In the ‘compact’ tab you’ll still see averaged PCR efficiency values. For easy analysis, we’re interested in the ‘N0’ values. Copy (Ctrl+c) and paste (Ctrl+v) these values to an empty tab (called ‘Analysis’). The format of these values can differ, depending on whether or not the value is set to ‘scientific’.

	A	B	C	D	E	F	G	H	I	J
1		N0								
2		mSMA 1	mSMA 2	mMyoc 1	mMyoc 2	mSM22 1	mSM22 2	mHPRT	mGAPDH	mHMBS
3	A1: 475	3,37E-08	3,80E-08	8,49E-10	6,58E-10	2,90E-08	4,20E-08	2,41E-09	2,67E-06	2,10E-08
4	A2: 477	2,52E-08	2,32E-08	3,11E-10	2,21E-10	1,66E-08	3,00E-08	3,25E-09	2,28E-06	1,28E-08
5	A3: 430	2,19E-08	2,56E-08	6,15E-10	5,56E-10	1,79E-08	2,08E-08	2,08E-09	1,69E-06	1,16E-08
6	A4: 311	2,30E-08	2,84E-08	1,27E-09	1,43E-10	1,45E-08	1,56E-08	2,14E-09	1,53E-06	1,32E-08
7	A5: -rt	3,34E-08	3,84E-08	3,17E-10	1,29E-10	9,14E-09	2,14E-08	1,60E-09	1,31E-06	8,89E-09
8	A6: H2O	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	3,52E-10	-9,99E+02	-9,99E+02	4,00E-11	-9,99E+02
9	B1: 475	3,66E-08	4,21E-08	9,40E-10	1,87E-10	2,10E-08	4,37E-08	3,01E-09	3,71E-06	2,43E-08
10	B2: 477	2,50E-08	3,49E-08	1,09E-09	5,21E-10	1,95E-08	2,76E-08	2,80E-09	2,29E-06	1,18E-08
11	B3: 430	2,56E-08	2,48E-08	1,40E-09	3,84E-10	1,42E-08	2,16E-08	2,19E-09	1,34E-06	1,37E-08
12	B4: 311	2,53E-08	2,86E-08	4,46E-10	-9,99E+02	1,41E-08	2,25E-08	1,28E-09	1,79E-06	1,44E-08
13	B5: -rt	3,73E-08	4,58E-08	9,10E-10	-9,99E+02	-9,99E+02	1,97E-08	1,45E-09	1,27E-06	9,07E-09
14	B6: H2O	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02
15	C1: 475	4,00E-08	5,07E-08	7,10E-10	-9,99E+02	2,43E-08	4,15E-08	3,00E-09	3,11E-06	1,90E-08
16	C2: 477	2,50E-08	2,92E-08	8,85E-10	-9,99E+02	2,34E-08	3,12E-08	2,62E-09	2,29E-06	1,47E-08
17	C3: 430	2,41E-08	2,66E-08	5,68E-10	3,58E-10	1,73E-08	2,24E-08	2,26E-09	1,70E-06	1,13E-08
18	C4: 311	2,29E-08	2,78E-08	7,42E-10	-9,99E+02	1,83E-08	2,11E-08	1,95E-09	1,45E-06	1,43E-08
19	C5: -rt	3,30E-08	3,77E-08	4,31E-10	5,50E-10	1,68E-08	1,74E-08	1,25E-09	1,03E-06	1,11E-08
20	C6: H2O	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	-9,99E+02	7,70E-10	-9,99E+02

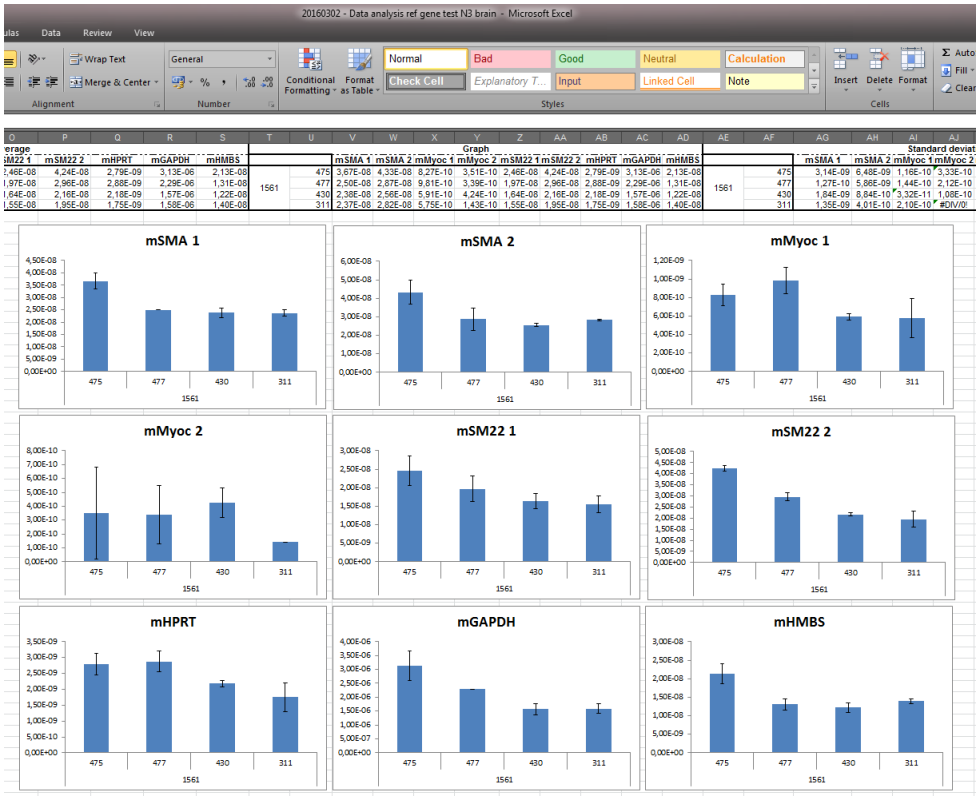
Repeat this for the other subsets as well. In your triplicates, you can exclude the outliers and mark them as red.



The next step would be to normalise your raw expression values for reference gene expression (the screen shot from above is taken from a different experiment). This is done by dividing the N0 value of the gene of interest by the N0 value of the reference gene. If using multiple reference genes, you divide by the '**geomean**' of these multiple N0 values. For calculations with Cq values and log values, the arithmetic mean is used. These values can be placed under a header called 'Normalised'. Normalising for reference gene expression is done to compensate for the differences of starting material.



After normalising (in this example only reference genes were tested, so no normalisation has been done), a geomean is calculated from your technical triplicates.



Your normalised and averaged samples can be put in a graph. The standard deviation can be calculated on the (normalised) technical triplicates. Be aware that the calculated SD only represents the variation in technical replicates and not the biological replicates and should be treated as such.

Ct value						Average					
6-7F_7R	PDGFRß	HES1	HEYL	JAG1	GAPDH	6-7F_7R	PDGFRß	HES1	HEYL	JAG1	GAPDH
29,04	28,8	30,07	29,98	27,15	27,07	29,21	28,92	30,55	29,89	27,19	27,22
26,67	27,61	30,31	29,68	25,86	27,12	27,28	27,57	30,51	29,72	25,90	27,31
27,35	27,79	30,81	30,62	27,52	28,26	27,77	28,12	30,80	30,59	27,35	28,33
25,3	26,98	29,77	30,2	26,78	27,78	25,93	26,95	30,45	30,36	26,77	27,94
20,86	26,88	31,55	35,8	32	28,02	21,09	26,99	31,08		32,03	28,06
25,14	30,08	33,31	40	34,05	30,47	24,66	30,01	33,06		34,18	30,58
25,77	28,26	31,66	35,1	30,87	30,66	25,83	28,34	31,47	33,12	30,90	31,22
33,98	31,76	33,3	40		31,92	34,09	31,85	34,02		34,83	31,95
29,59	28,81	31,12	31,44	27,03	29,77	29,67	28,77	30,97	30,87	27,04	29,64
37,85	38										
	40		36,03								
28,87	28,96	30,84	29,78	27,24	27,27						
27,42	27,59	30,89	29,85	25,9	27,62						
27,92	28,01	30,5	30,64	27,19	28,53						
26,21	26,85	30,95	30,75	26,84	28,26						
21,15	27,08	30,89	40	31,95	27,96						
24,28	29,98	33,03	35,49	33,94	30,69						
25,75	28,33	31,23	33,49	30,9	31,24						
34,55	31,75	34,73	40		32						
29,6	28,76	30,76	30,59	27,04	29,23						
40	40										
	40		36,23								
29,71	28,99	30,73	29,9	27,18	27,33						
27,74	27,5	30,32	29,62	25,94	27,2						
28,05	28,56	31,08	30,51	27,34	28,2						
26,28	27,01	30,64	30,14	26,69	27,79						
21,26	27,02	30,8	40	32,14	28,21						
24,57	29,97	32,85	40	34,56	30,57						
25,97	28,44	31,53	32,75	30,94	31,76						
33,73	32,04	35,93	35,73	34,83	31,92						
29,81	28,75	31,03	30,57	27,04	29,93						
	40				40						
	40										

In case you want to calculate the expression levels with the use of Cq values, export the raw Cq values from the LightCycler 480 software (go to the ‘Analysis’ button and select

'Abs Quant/2nd Derivative Max' and choose your primer set of interest in the 'Subset' drop down menu, right click the grey table at the bottom left corner and export), or copy them from the LinRegPCR created Excel 'compact' tab. These Cq values can then be averaged (do **not** take the geomean) and used for ΔΔCq calculation.

	H	I	J	K	L	M	N	O	P
	Average						Delta Ct GOI		
	6-7F_7R	PDGFRß	HES1	HEYL	JAG1	GAPDH	6-7F_7R	PDGFRß	HES1
7	29,21	28,92	30,55	29,89	27,19	27,22	=H3-$M3	1,69	3,32
2	27,28	27,57	30,51	29,72	25,90	27,31	-0,04	0,25	3,19
6	27,77	28,12	30,80	30,59	27,35	28,33	-0,56	-0,21	2,47
8	25,93	26,95	30,45	30,36	26,77	27,94	-2,01	-1,00	2,51
2	21,09	26,99	31,08		32,03	28,06	-6,97	-1,07	3,02
7	24,66	30,01	33,06		34,18	30,58	-5,91	-0,57	2,49
6	25,83	28,34	31,47	33,12	30,90	31,22	-5,39	-2,88	0,25
2	34,09	31,85	34,02		34,83	31,95	2,14	-0,10	2,07
7	29,67	28,77	30,97	30,87	27,04	29,64	0,02	-0,87	1,33

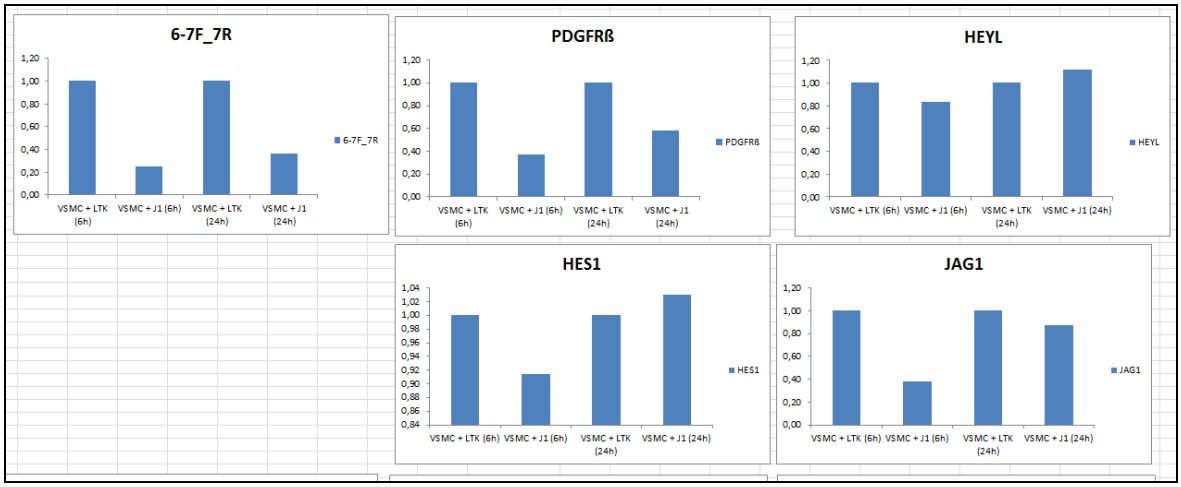
First, the ΔCq value is calculated by subtracting the reference gene Cq value (GAPDH, in this case) from the Cq value of the gene of interest. It's possible that the result is a negative value.

	N	O	P	Q	R	S	T	U	V	W
	Delta Ct GOI					delta delta CT				
	6-7F_7R	PDGFRß	HES1	HEYL	JAG1	6-7F_7R	PDGFRß	HES1	HEYL	JAG1
2	1,98	1,69	3,32	2,66	-0,03	2,02	1,44	0,13	0,26	1,38
1	-0,04	0,25	3,19	2,40	-1,41	0,00	0,00	0,00	0,00	0,00
3	-0,56	-0,21	2,47	2,26	-0,98	1,46	0,79	-0,04	-0,16	0,19
4	-2,01	-1,00	2,51	2,42	-1,17	0,00	0,00	0,00	0,00	0,00
6	-6,97	-1,07	3,02		3,97	0,00	0,00	0,00	0,00	0,00
8	-5,91	-0,57	2,49		3,61	1,06	0,50	-0,53	0,00	-0,36
2	-5,39	-2,88	0,25	1,90	-0,32	=N9-N$7	-1,81	-2,76	1,90	-4,28
5	2,14	-0,10	2,07		2,88	9,11	0,97	-0,95	0,00	-1,08
4	0,02	-0,87	1,33	1,22	-2,61	7,00	0,20	-1,69	1,22	-6,57

To calculate the ΔΔCq value, you subtract the ΔCq of your gene of interest from the ΔCq value from your experimental control (a WT or a control therapeutic sample).

	delta delta CT					relative expression				
	6-7F_7R	PDGFRß	HES1	HEYL	JAG1	6-7F_7R	PDGFRß	HES1	HEYL	JAG1
3	2,02	1,44	0,13	0,26	1,38	0,25	0,37	0,91	0,84	0,38
1	0,00	0,00	0,00	0,00	0,00	1,00	1,00	1,00	1,00	1,00
8	1,46	0,79	-0,04	-0,16	0,19	0,36	0,58	1,03	1,12	0,87
7	0,00	0,00	0,00	0,00	0,00	1,00	1,00	1,00	1,00	1,00
7	0,00	0,00	0,00	0,00	0,00	1,00	1,00	1,00	1,00	1,00
1	1,06	0,50	-0,53	0,00	-0,36	=2^(-S8)	0,71	1,44	1,00	1,28
2	1,58	-1,81	-2,76	1,90	-4,28	0,33	3,50	6,79	0,27	19,47
8	9,11	0,97	-0,95	0,00	-1,08	0,00	0,51	1,93	1,00	2,12
1	7,00	0,20	-1,69	1,22	-6,57	0,01	0,87	3,23	0,43	95,23

The relative ΔΔCq value is calculated by $2^{-\Delta\Delta Cq}$. Be aware of the minus sign in front of 'ΔΔCq'.



These results can be plotted, since the calculation is performed relative to the control group.

For publishing RT-qPCR data, **MIQE** (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines have been introduced. These guidelines can be found in the following paper:
http://clinchem.aaccjnls.org/content/clinchem/55/4/611.full.pdf

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Materials

- Bioline SYBR SensiMix HI-ROX
- Forward primers (100 µM; dilute to 0.5-2.0 µl)
- Reverse primers (100 µM; dilute to 0.5-2.0 µl)
- cDNA (10x diluted)
- 4titude FrameStar 480, 384 well, clear frame white wells
- 4titude adhesive seals (135 x 80mm)
- Roche LightCycler 480 Real-Time PCR Machine
- Roche LightCycler480 Software V1.5
- LinRegPCR 2017.1
- Microsoft Excel 2010