RT-qPCR experiment and analysis using LinRegPCR

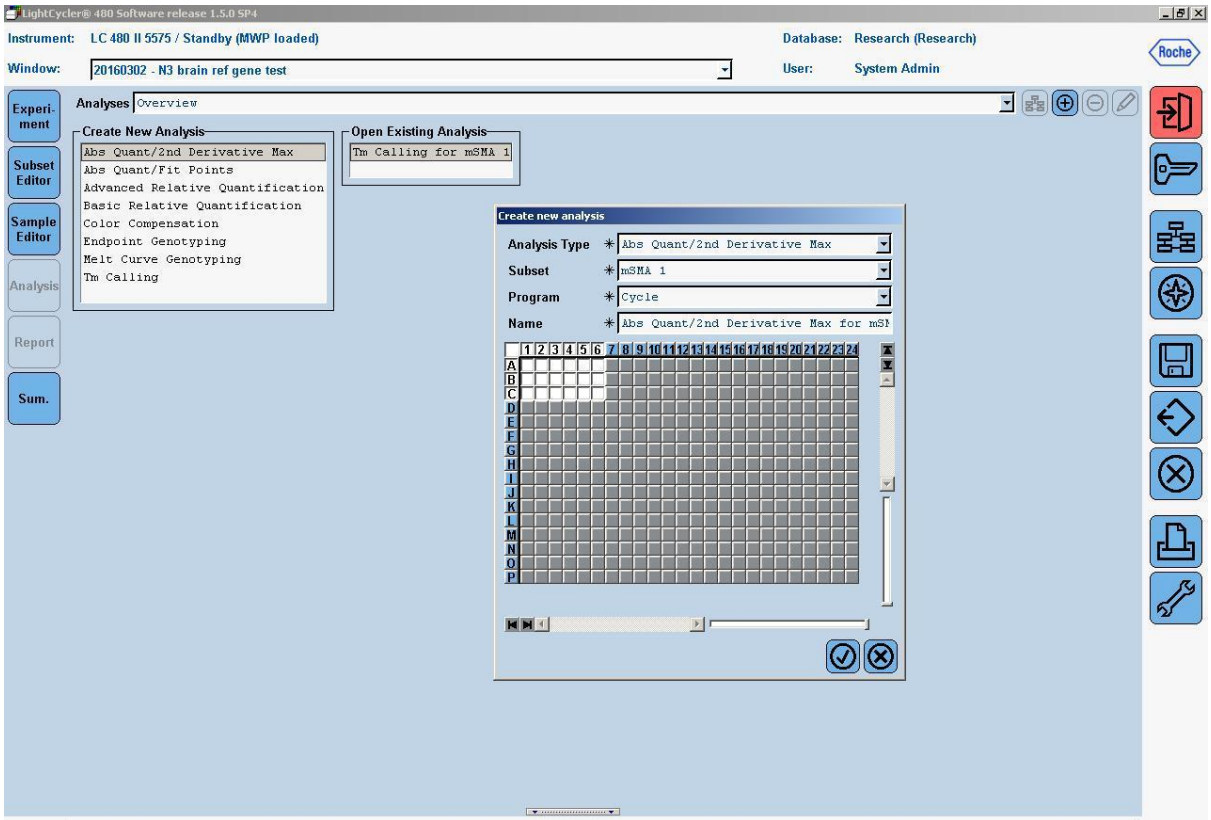
(Modified from Maurice Overzier, 2018)

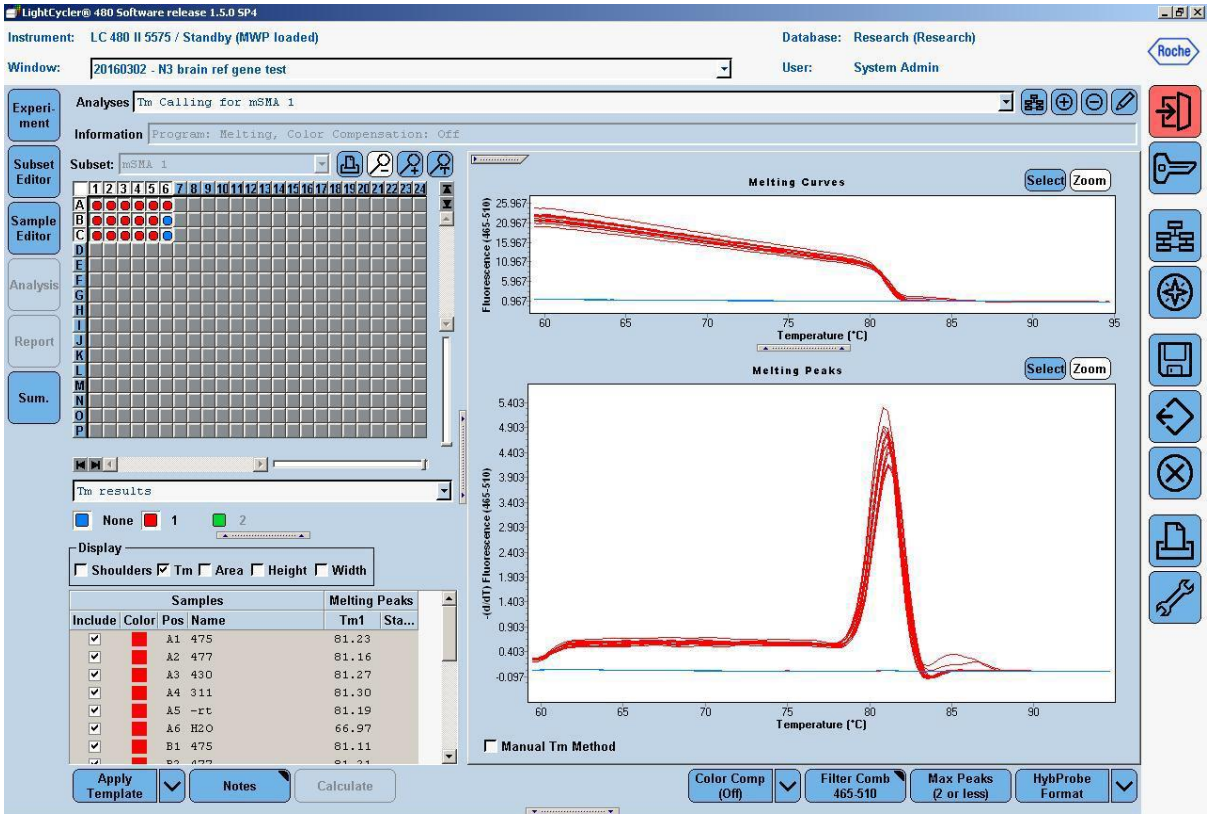
Purpose

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.

Exporting data from the LightCycler 480

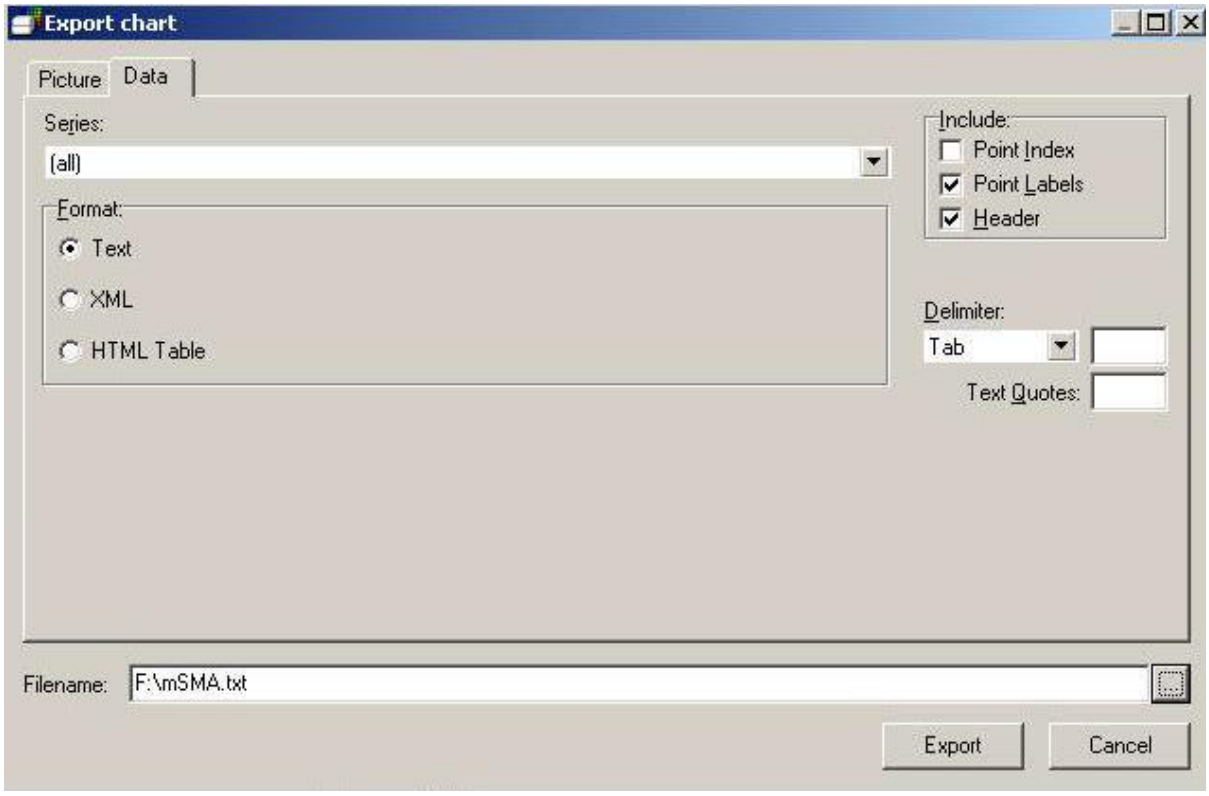
When the RT-qPCR has finished its run (using the 'Sybr Green standard' protocol, approximately after 1 hour and 20 minutes), go through the following steps (can be done at the qPCR PC or export the .ixo file by clicking the 'export' button, below the 'save' button; dashed square in the picture below,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

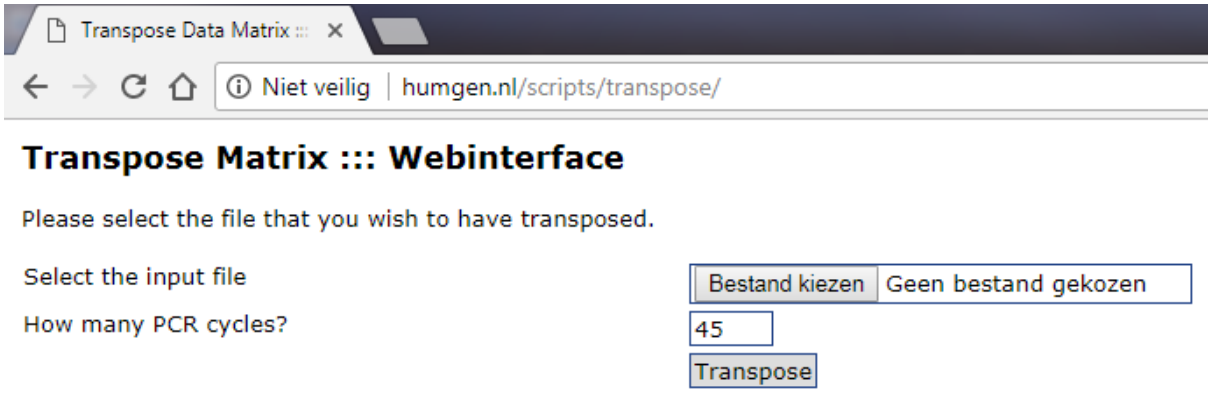
To export the data for further analysis, go back to the ‘Experiment’ button and select the ‘Data’ tab. Select your primer set in the ‘Subset’ drop down menu. Right click the graph on the right and select ‘Export Chart’.

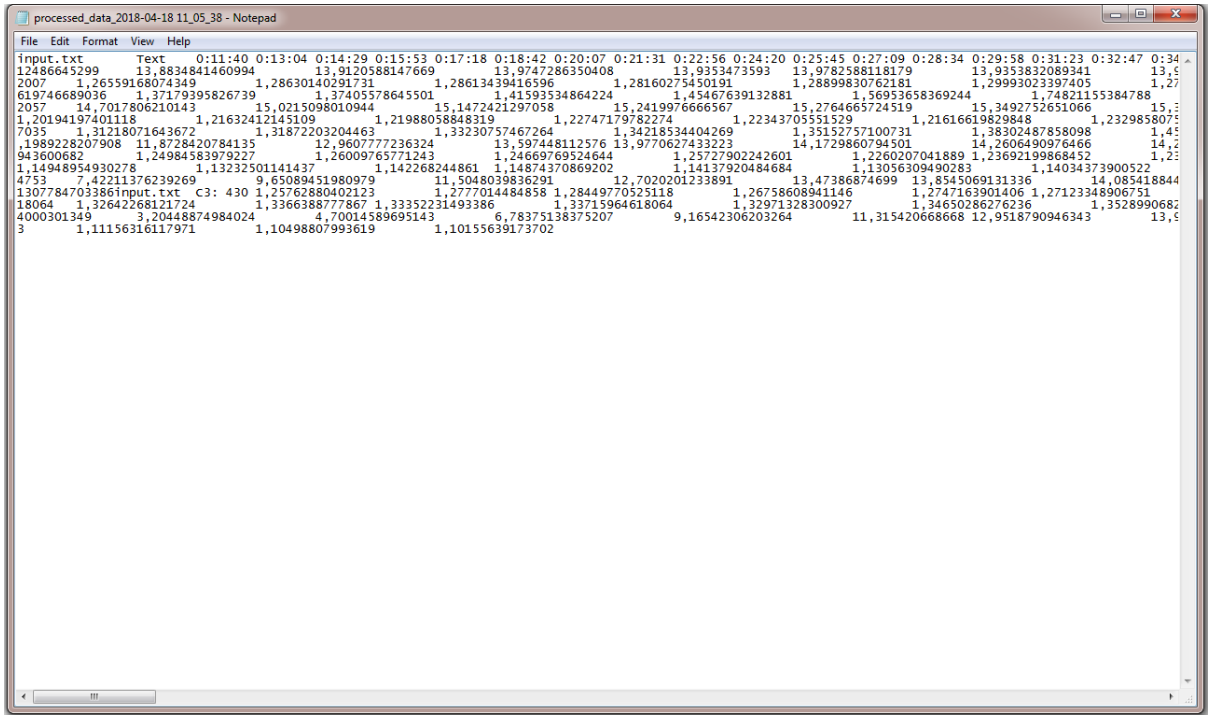


Click the ‘Data’ tab and select where you want to save the data file and name it (a USB flash drive)

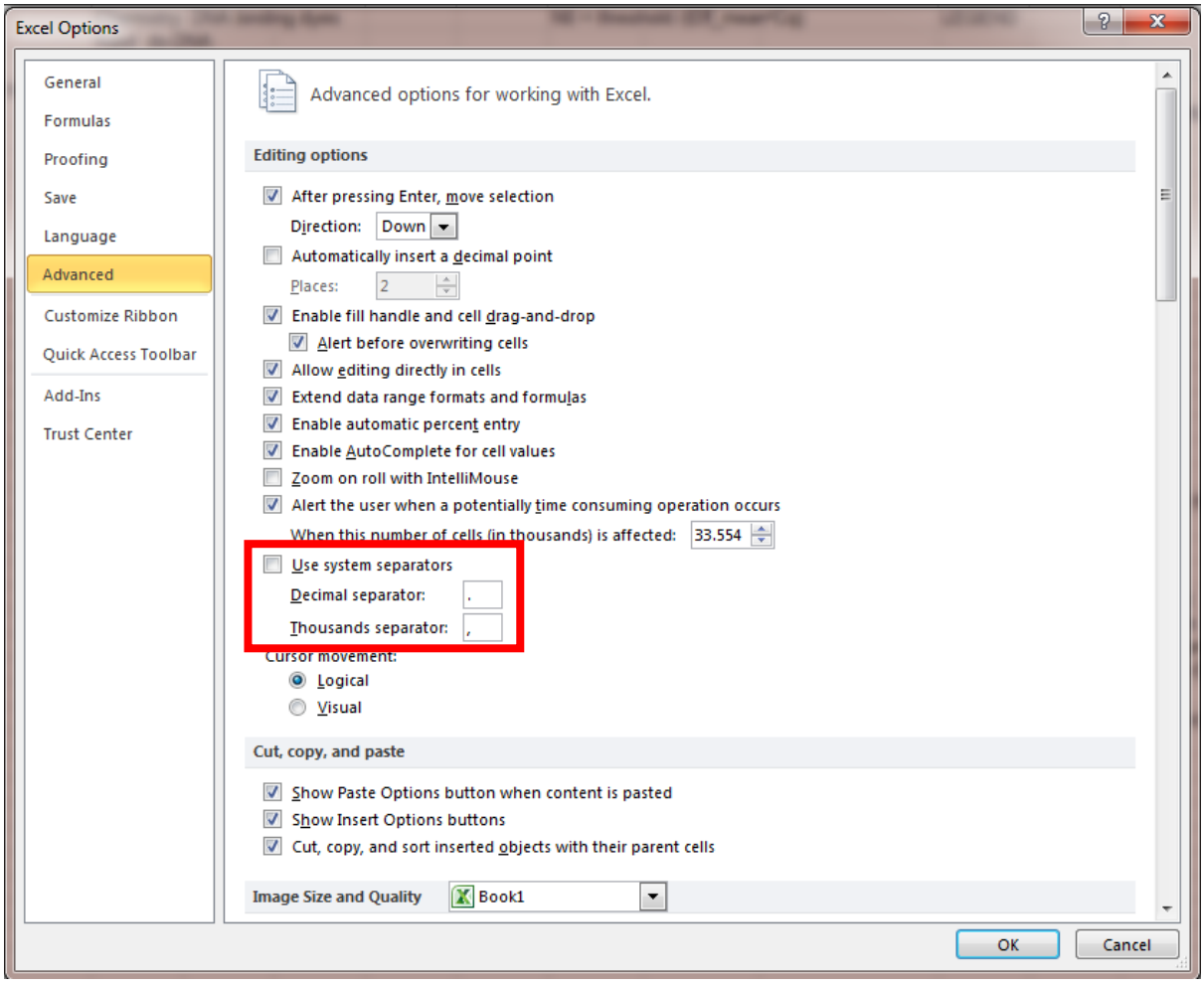
Preparation of LightCycler480 output for the qPCR Web Interface

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.

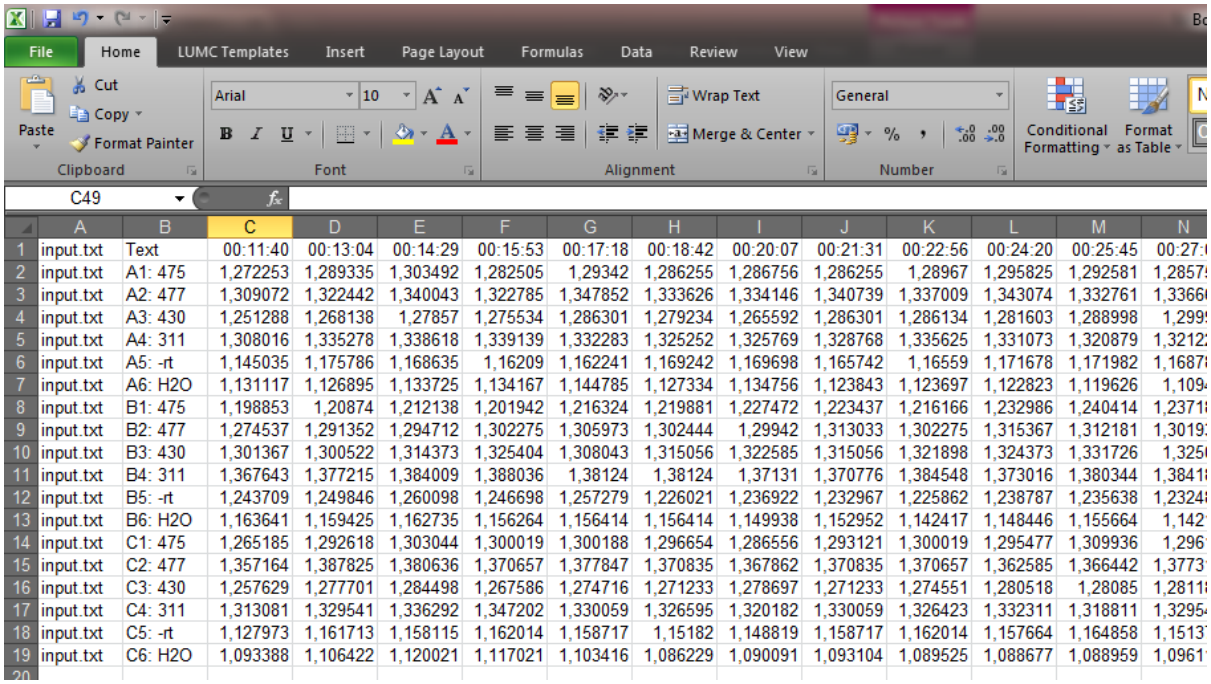




Select the complete text (Ctrl+a) and copy (Ctrl+c).

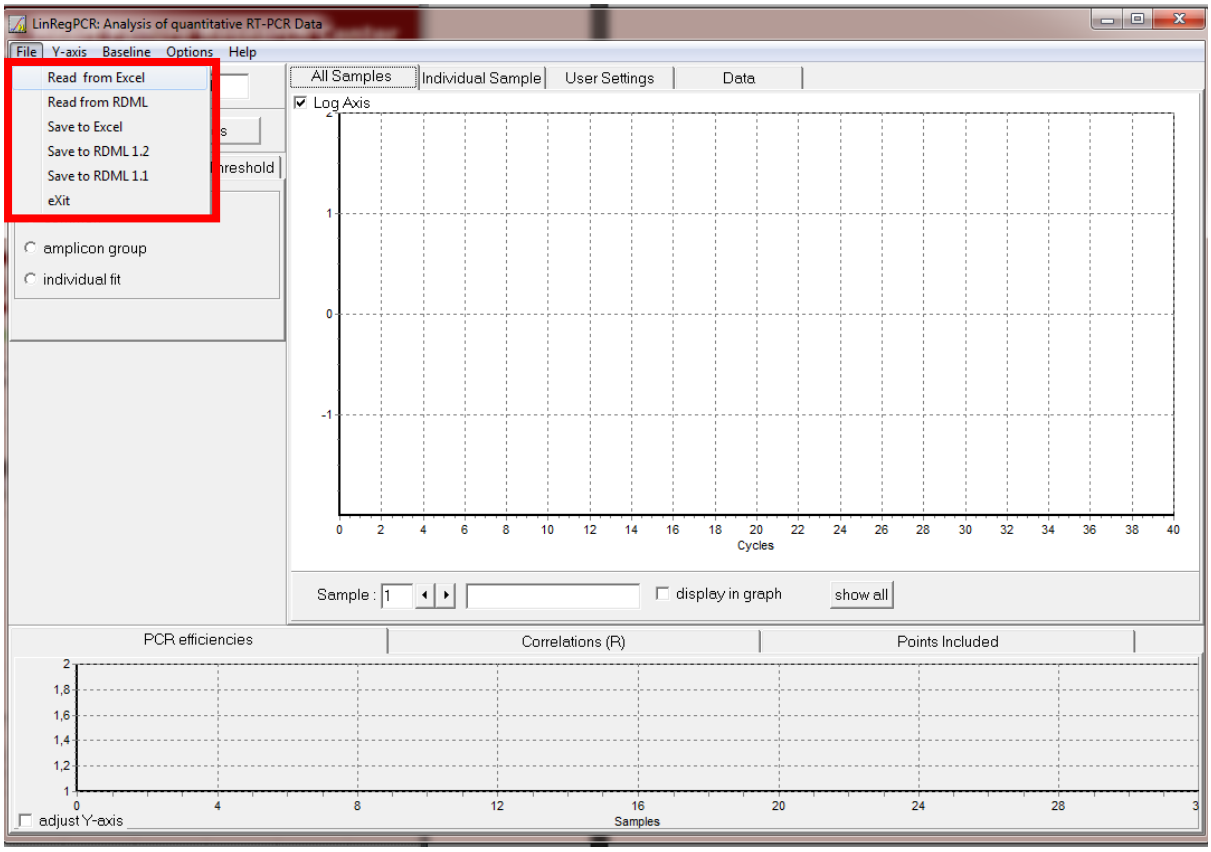


Open a new Excel file and make sure to first change the system separators to the settings as shown above. If the box is ticked, untick the box and change the decimal and thousands separators. Restart Excel after this change.

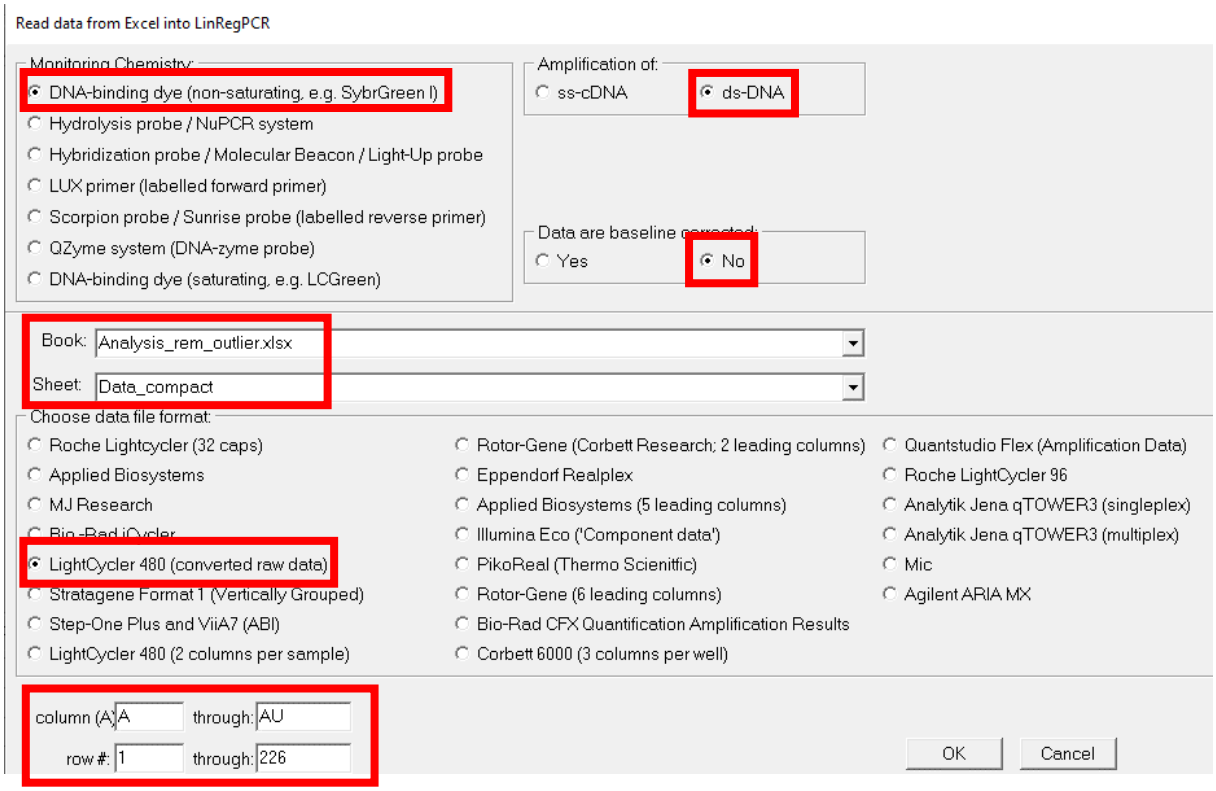


	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	input.txt	Text	00:11:40	00:13:04	00:14:29	00:15:53	00:17:18	00:18:42	00:20:07	00:21:31	00:22:56	00:24:20	00:25:45	00:27:
2	input.txt	A1: 475	1,272253	1,289335	1,303492	1,282505	1,29342	1,286255	1,286756	1,286255	1,28967	1,295825	1,292581	1,2857
3	input.txt	A2: 477	1,309072	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
20														

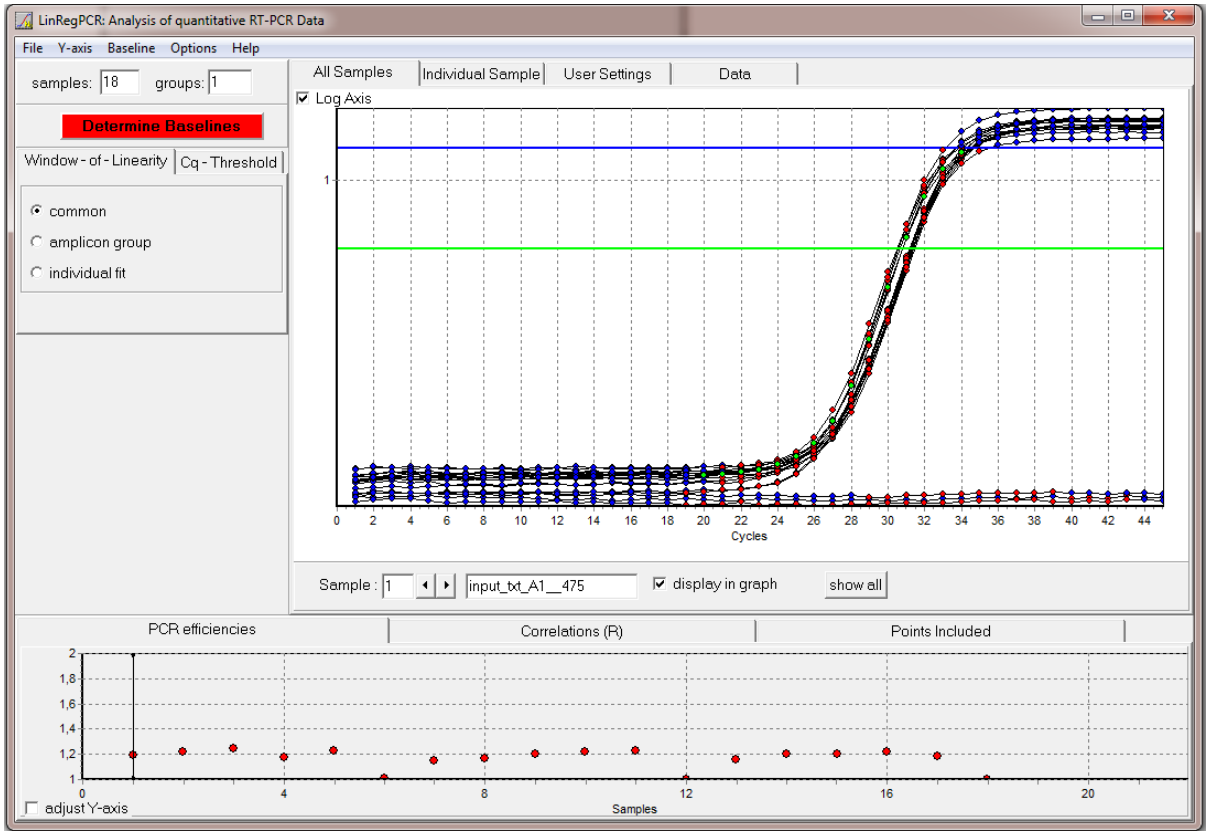
Paste (Ctrl+v) the data into a clean Excel tab. Change the name of the tab to ‘Data’. Remove data from empty wells (i.e. data belonging to wells where nothing was pipetted into) and name the samples in the column ‘Text’ as following: cell1_primer1 (e.g. Control1_GAPDH). Make sure the data is placed from the A1 cell. 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.



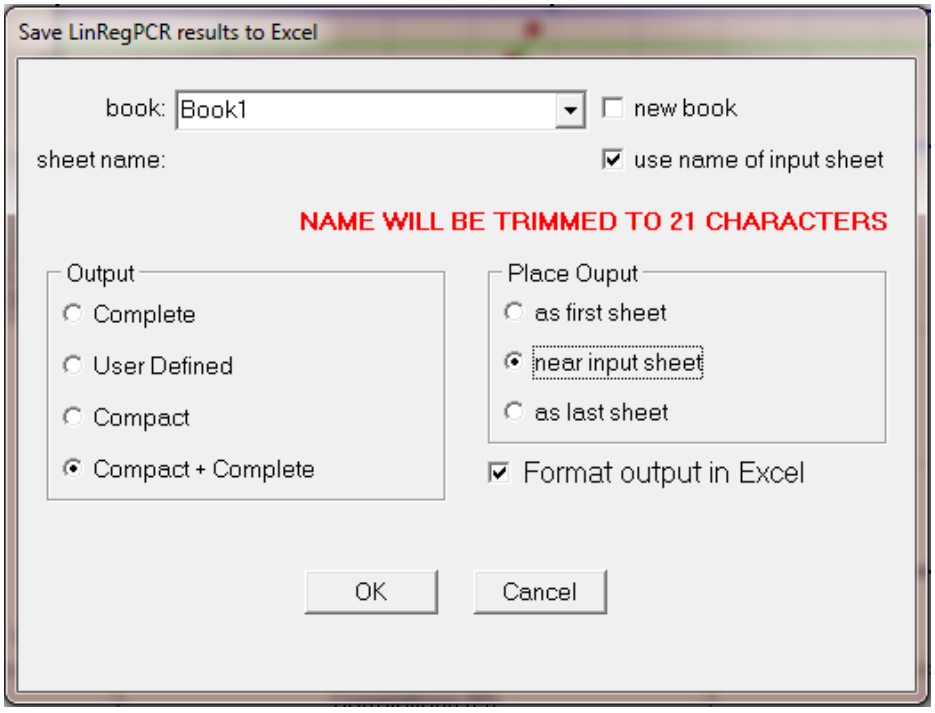
Open LinRegPCR and click ‘File’, then ‘Read from Excel’.



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 (‘Data’). 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.

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.

Calculations (performed by the qPCR Web Interface)

This section describes how the relative expression levels are calculated. You don't have to do this yourself. Once you upload your Excel file to the web interface, these calculations will be done automatically.

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

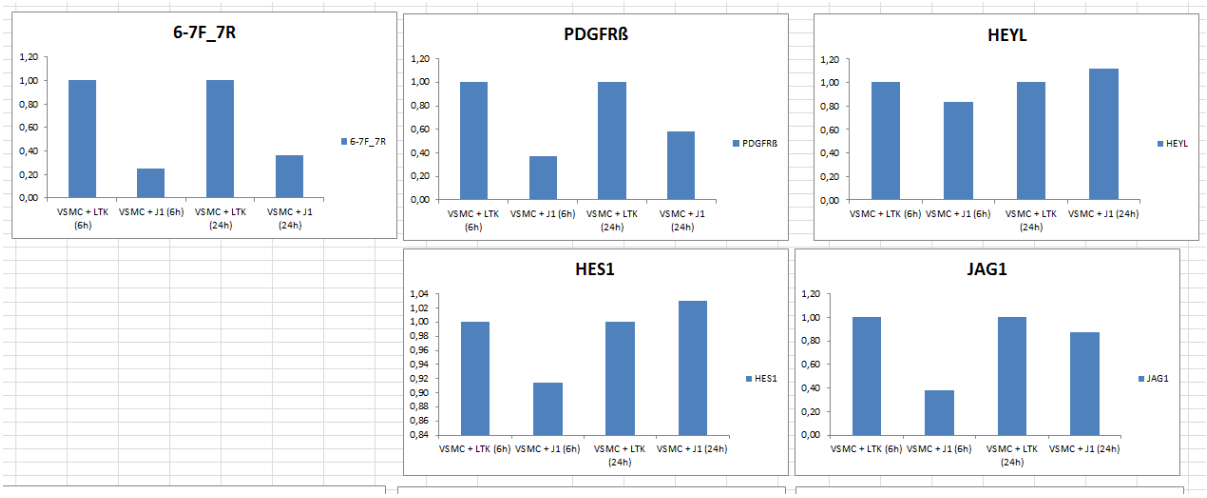
First, the ΔCq value is calculated by subtracting the reference gene Cq value (GAPDH, in this case) from the Cq value of the gene of interest. It's possible that the result is a negative value.

	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.