# qPCR data analysis

I.QC (quality control of your qPCR)

1. Check the melt curve

- if reactions with the same primer pair give only one peak -> proceed with the analysis

- if you see more than one peak, consider how high is the additional peak(s), will it contribute a lot to the fluorescence in

  the reaction:

    - if it is very low -> proceed with the analysis

    - if it is not low -> what conclusions can you make from its melting temperature:

      - is it a primer dimer? -> change annealing T, adjust concentration of primers, redesign primers

      - is it an additional product? -> increase annealing T, get rid of genomic DNA contamination, redesign the primers

results for only one target (one primer pair) are shown

primer-dimer peak of a neglectable height
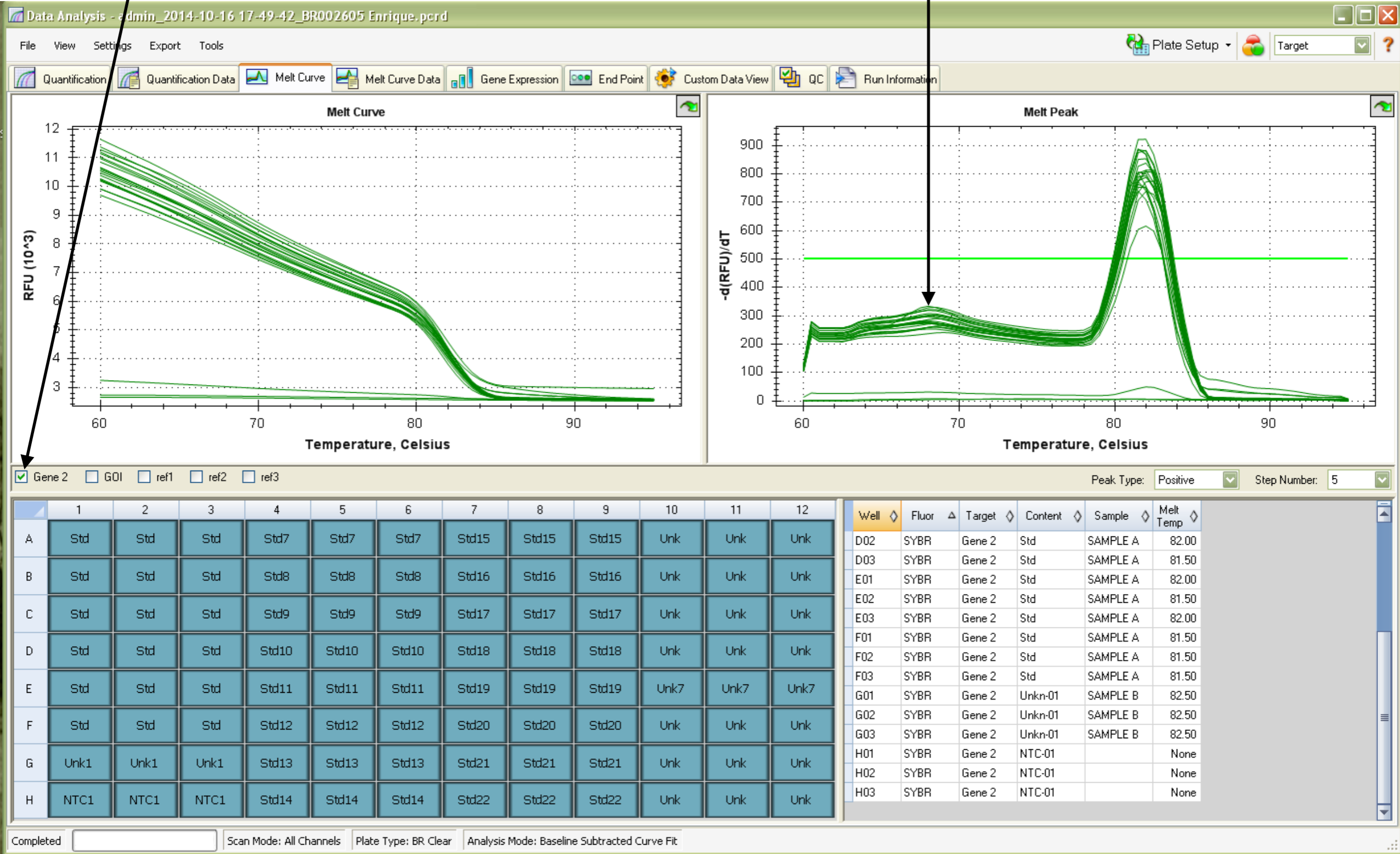
I.QC (quality control of your qPCR)

2. Check no template control

- if you see amplification in NTC, check what is the level of fluorescence intensity in NTC samples-> go to melt curve and compare the hight of peak in NTC and in your sample:

  - if NTC reactions show a melt curve peak with very low height-> ignore it and proceed with analysis

  - if NTC samples have a nice melt curve with of a peak of a considerable height-> there is contamination! change all your dilutions and rerun qPCR, pay attention to not contaminating your reactions while pipetting

not significant contamination in one out of 3 technical replicates -> proceed with analysis, but also take it into consideration while pipetting the next qPCR
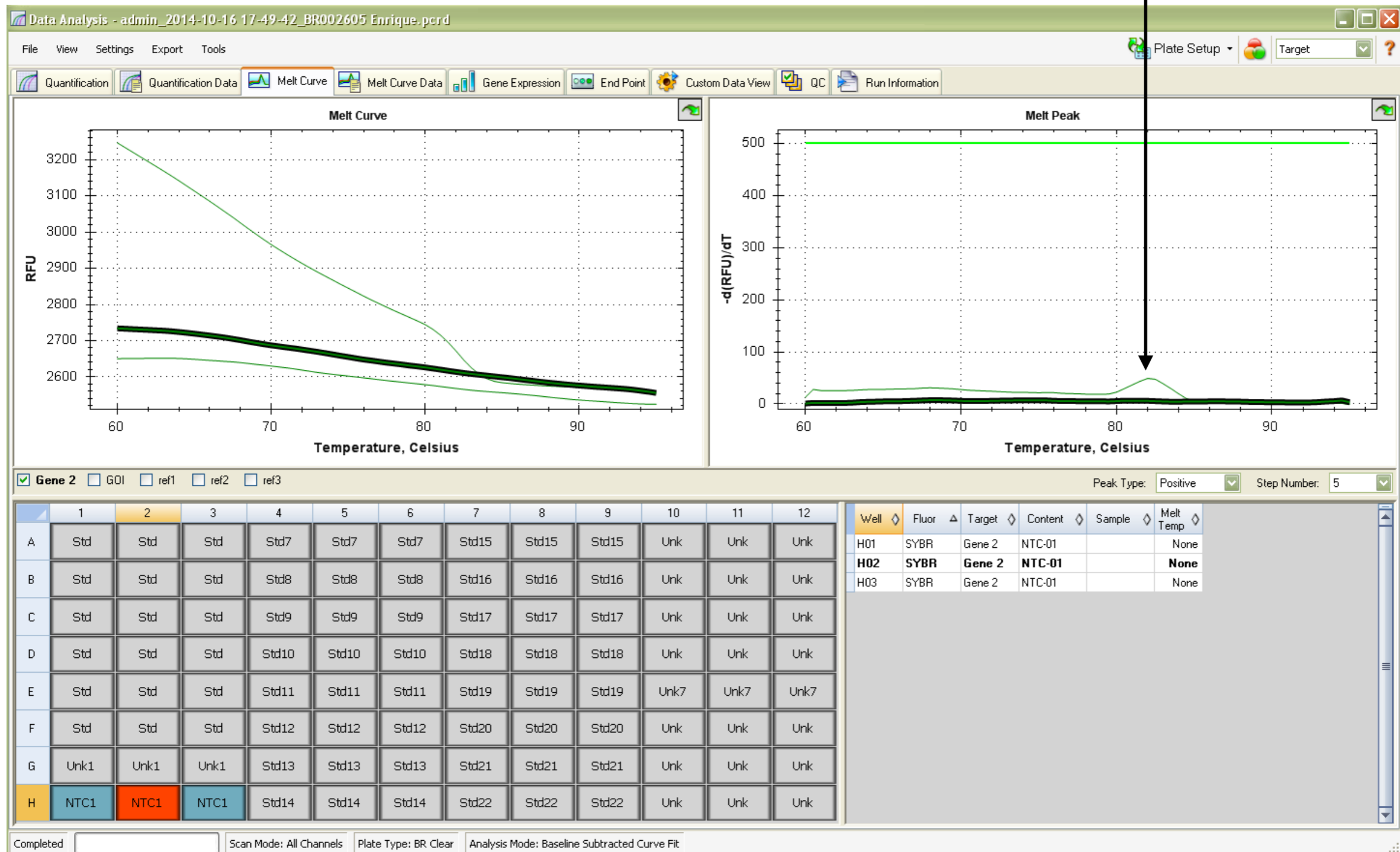
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3.Check Standard Deviation for your mean Ct values

- if you see StDev 0.1 or higher:

  - look if one of the technical replicate is a very obvious outliers, remove it and

    consider the amount of technical replicates in your qPCR = 2

  - if you can't find obvious outliers try to find where the pipetting error comes

    from: check you pipettes precision, rethink you strategy for adding

    components to the wells ->and then rerun qPCR

not ok   ok   sic! a software bug

Data Analysis - admin_2014-10-16 17-49-42_BR002605 Enrique.pcrd

File   View   Settings   Export   Tools

Plate Setup ▾   Target

Quantification   Quantification Data   Melt Curve   Melt Curve Data   Gene Expression   End Point   Custom Data View   QC   Run Information

Results   Step Number: 3

| Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log Starting Quantity	SQ Mean	SQ Std. Dev
E07	SYBR	GOI	Std-19	DNA POOL	37.33	36.14	1.034	1.000E+00	0.000	1.00E+00	0.00E+00
E08	SYBR	GOI	Std-19	DNA POOL	35.46	36.14	1.034	1.000E+00	0.000	1.00E+00	0.00E+00
E09	SYBR	GOI	Std-19	DNA POOL	35.63	36.14	1.034	1.000E+00	0.000	1.00E+00	0.00E+00
E10	SYBR	ref1	Unkn-07	SAMPLE2	34.18	33.77	0.364	1.273E-01	-0.895	1.65E-01	3.3 E-02
E11	SYBR	ref1	Unkn-07	SAMPLE2	33.50	33.77	0.364	1.903E-01	-0.720	1.65E-01	31E-02
E12	SYBR	ref1	Unkn-07	SAMPLE2	33.63	33.77	0.364	1.760E-0	-0.754	1.65E-01	3.31E-02
F01	SYBR	Gene 2	Std	SAMPLE A	25.14	25.14	0.000	1.000 05	-5.000	1.00E-05	0.00E+00
F02	SYBR	Gene 2	Std	SAMPLE A	25.01	25.01	0.000	1.00 E-05	-5.000	1.00E-05	0.00E+00
F03	SYBR	Gene 2	Std	SAMPLE A	25.06	25.06	0.000	1. 00E-05	-5.000	1.00E-05	0.00E+00
F04	SYBR	ref2	Std-12	DNA POOL	30.33	30.14	0.218	000E-01	-0.699	2.00E 1	3.40E-17
F05	SYBR	ref2	Std-12	DNA POOL	29.90	30.14	0.218	2.000E-01	-0.699	2.0 E-01	3.40E-17
F06	SYBR	ref2	Std-12	DNA POOL	30.20	30.14	0.218	2.000E-01	-0.699	00E-01	3.40E-17
F07	SYBR	GOI	Std-20	DNA POOL	N/A	0.00	0.000	2.000E-01	-0.699	0.00E+00	0.00E+00
F08	SYBR	GOI	Std-20	DNA POOL	N/A	0.00	0.000	2.000E-01	-0.699	0.00E+00	0.00E+00
F09	SYBR	GOI	Std-20	DNA POOL	N/A	0.00	0.000	2.000E-01	-0.699	0.00E+00	0.00E+00
F10	SYBR	ref2	Unkn	SAMPLE2	30.06	30.06	0.000	2.097E-01	-0.678	2.10E-01	0.00E+00
F11	SYBR	ref2	Unkn	SAMPLE2	30.26	30.26	0.000	1.904E-01	-0.720	1.90E-01	0.00E+00
F12	SYBR	ref2	Unkn	SAMPLE2	30.17	30.17	0.000	1.989E-01	-0.701	1.99E-01	0.00E+00
G01	SYBR	Gene 2	Unkn-01	SAMPLE B	9.48	9.41	0.064	3.232E-01	-0.491	3.40E-01	1.49E-02
G02	SYBR	Gene 2	Unkn-01	SAMPLE B	9.37	9.41	0.064	3.505E-01	-0.455	3.40E-01	1.49E-02
G03	SYBR	Gene 2	Unkn-01	SAMPLE B	9.38	9.41	0.064	3.470E-01	-0.460	3.40E-01	1.49E-02
G04	SYBR	ref2	Std-13	DNA POOL	33.29	33.98	0.611	4.000E-02	-1.398	4.00E-02	0.00E+00
G05	SYBR	ref2	Std-13	DNA POOL	34.46	33.98	0.611	4.000E-02	-1.398	4.00E-02	0.00E+00
G06	SYBR	ref2	Std-13	DNA POOL	34.18	33.98	0.611	4.000E-02	-1.398	4.00E-02	0.00E+00
G07	SYBR	GOI	Std-21	DNA POOL	38.83	38.83	0.000	4.000E-02	-1.398	4.00E-02	0.00E+00
G08	SYBR	GOI	Std-21	DNA POOL	N/A	0.00	0.000	4.000E-02	-1.398	0.00E+00	0.00E+00
G09	SYBR	GOI	Std-21	DNA POOL	N/A	0.00	0.000	4.000E-02	-1.398	0.00E+00	0.00E+00
G10	SYBR	ref3	Unkn	SAMPLE2	29.84	29.84	0.000	2.668E-01	-0.574	2.67E-01	0.00E+00
G11	SYBR	ref3	Unkn	SAMPLE2	30.74	30.74	0.000	1.736E-01	-0.760	1.74E-01	0.00E+00
G12	SYBR	ref3	Unkn	SAMPLE2	29.67	29.67	0.000	2.896E-01	-0.538	2.90E-01	0.00E+00
H01	SYBR	Gene 2	NTC-01		N/A	0.00	0.000	N/A	N/A	0.00E+00	0.00E+00
H02	SYBR	Gene 2	NTC-01		N/A	0.00	0.000	N/A	N/A	0.00E+00	0.00E+00
H03	SYBR	Gene 2	NTC-01		N/A	0.00	0.000	N/A	N/A	0.00E+00	0.00E+00
H04	SYBR	ref2	Std-14	DNA POOL	36.28	36.44	0.151	8.000E-03	-2.097	8.00E-03	0.00E+00

Completed   Scan Mode: All Channels   Plate Type: BR Clear   Analysis Mode: Baseline Subtracted Curve Fit

I.QC (quality control of your qPCR)

4. Check R^2 of your Standard curve(s)

- if your R^2 is less than 0.98, do not trust it:

  - if the curve look absolutely crazy, check the plate layout and make sure the software has correct data

    about your targets names, standards names, dilutions factors etc.

  - do you get sudden increase in amplification at higher dilutions? ->indicator of an inhibitor present in

    your template-> optimise your protocol for template purification/if possible use higher dilutions for

    qPCR

  - you do not get increase at higher dilutions, but R^2 is still shitty => the problem is in something else:

    rethink the way you made your serial dilution, consider how to optimise the pipetting and rerun qPCR
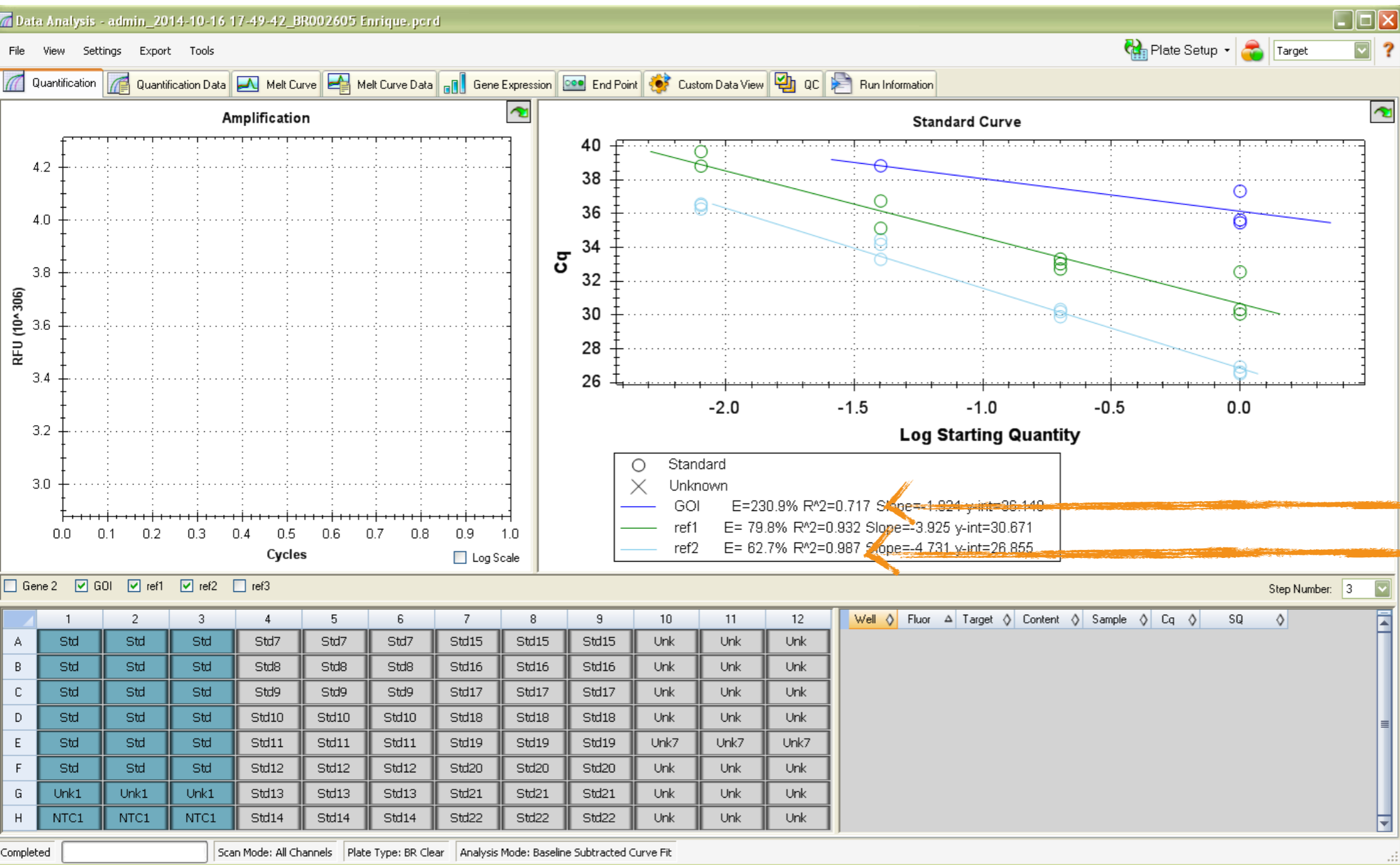
bonkers!

an ok R²

II. Absolute quantification

1. Absolute quantification will be automatically done for you by the software

- Make sure that concentration of your sample fits into the range of your standard curve, if it doesn't -> rerun with a different standard curve or make dilutions of your sample

III. Relative Gene Expression

1. Before running the $\Delta\Delta$Ct test, check the stability of your reference genes

- for example you can find a target-stability value (M-value) it should be less than 0.5

  - if it is not less than 0.5 -> pick other reference genes for your sample and rerun the experiment
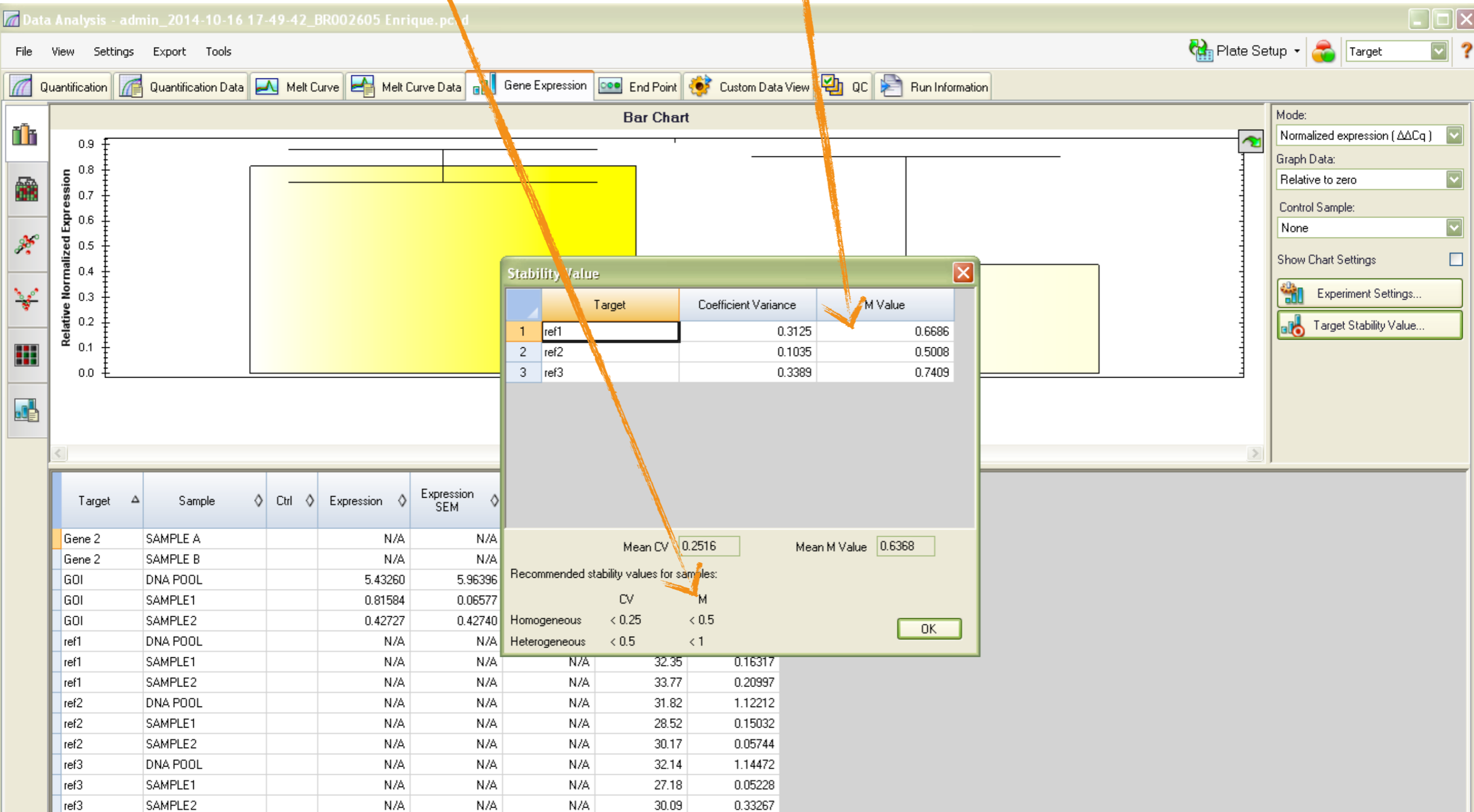
III. Relative Gene Expression

2. For more obvious comparison between your samples

- you can normalize all values to the control sample (control sample expression value will be set to 1)

- Please note, that the standard errors you see on the chart represent the stability of your pipetting!! and have very little to do with the future statistical analysis of biological replicates

it is easier to see now, that sample 2 has twice lower expression of GOI (in a very statistically insignificant way)