Using AccuCaL-D™ and the RealCount™ software for robust quantitation of gene copy number

AccuCal-D™, ACCUGEN's improved method for absolute quantitation of gene copy number

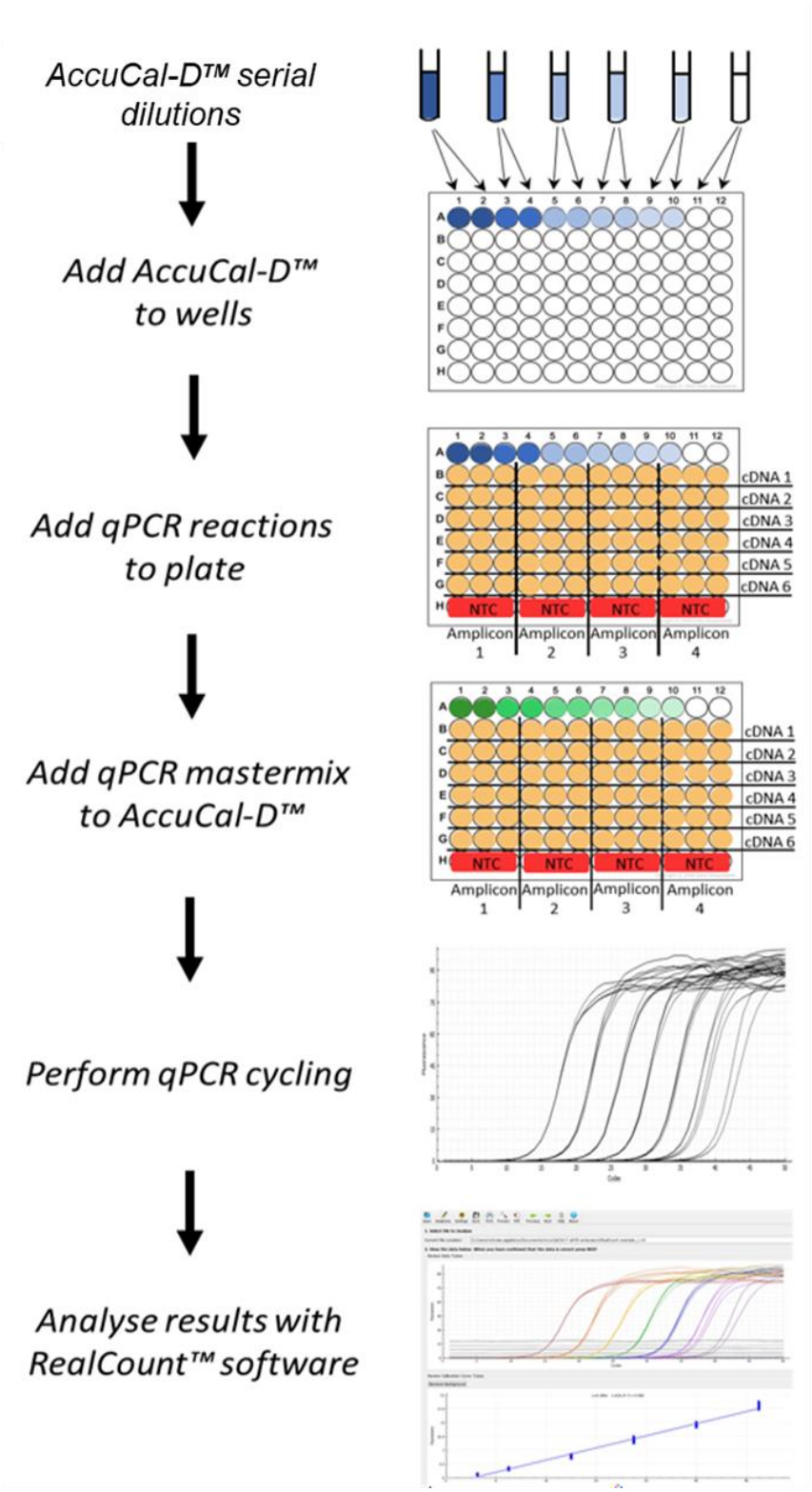
Quantitative real-time PCR (qPCR) is used extensively in research and diagnostic laboratories to determine expression levels of genes of interest (GOIs). Specific amplification of a target DNA sequence occurs during iterative qPCR cycling, which can be monitored over time by the addition of a double-stranded DNA dye, such as SYBR green, into the reaction mastermix. By examining the kinetics of fluorescence (FL) increase over time for a given amplicon, the expression level of the GOI can be determined.

To compare the expression levels of multiple GOIs, most methods of qPCR compare the cycle numbers of a GOI at an arbitrarily determined FL to that of a reference gene. Thus, the expression level of the GOI is expressed as a value relative to the reference gene and is called relative expression. In theory, this method is robust. However, there is an underlying assumption that the expression of the reference gene itself does not change. In many experimental conditions and/or disease states this assumption is wrong, and changes in the expression of the reference gene can occur. This in turn can result in the generation of gene expression data where the actual changes in GOI expression are masked or under/overstated.

AccuCal-D™ was developed to overcome the shortcomings of using relative gene expression methods for qPCR . In contrast to relative quantification, the AccuCal-D™ method uses FL data from a qPCR run to calculate the starting copy number of a GOI in a sample to give called absolute quantification. Traditionally, to perform absolute quantification, a standard curve must be generated from known copy number inputs of serially-diluted template DNA for every GOI and reference gene. This is both time consuming and requires many additional runs prior to the experiment. Using the AccuCal-D™ calibrator standard curve method removes the requirement of standard curve generation (primer efficiency must be still validated) and provides absolute quantification results, i.e. concentration in copy number/input sample. In fact, using AccuCal-D™ for absolute quantification of starting copy number also eliminates the need for a reference gene altogether!

This manual details the correct use of both the AccuCal-D™ consumable and RealCount™ software for the accurate determination of the starting copy number of GOIs using qPCR.

Overview: The process of copy number determination using AccuCal-D™ and RealCount™



Generating an AccuCal-D™ calibration curve for qPCR

AccuCal-D™ is supplied as series of dilutions (50, 40, 30, 20, 10 and 0ng/µLin $TE_{0.1}$ solution (10mM Tris pH8.0, 0.1mM EDTA) to ensure longterm stability at 4°C. The AccuCal-D™ calibration curve must be constructed using at least six dilutions and AccuCal-D™ 0, with each dilution in duplicate. For enhanced accuracy, triplicates or higher of dilutions can be used, depending on the number of available wells

The range of AccuCal-D™ dilutions is dependent on both the DNA intercalating dye present in the qPCR mastermix and the lower limit of FL detection of the qPCR machine. qPCR mastermix must contain a 'hot-start' polymerase. The qPCR instrument must be able to discriminate between the FL of the last sample of the serial dilution and AccuCal-D™ 0 (diluent only). The known copy numbers of AccuCal-D™ dilution covers the range of copy numbers most commonly observed in experimental samples.

Plating template

For ease of analysis, **you should pipette the DNA samples row by row** as the RealCount™ labels the wells from A1 to A12, followed by B1 to B12. The default standard calibrator occupies the wells from A1 to B6. Below figure shows a quick example of plating template.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	50ng AccuCal	50ng AccuCal	50ng AccuCal	40ng AccuCal	40ng AccuCal	40ng AccuCal	30ng AccuCal	30ng AccuCal	30ng AccuCal	20ng AccuCal	20ng AccuCal	20ng AccuCal	A
B	10ng AccuCal	10ng AccuCal	10ng AccuCal	0ng AccuCal	0ng AccuCal	0ng AccuCal	sample1 Gene X	sample1 Gene X	sample1 Gene X	sample2 Gene X	sample2 Gene X	sample2 Gene X	B
C	sample3 Gene X	sample3 Gene X	sample3 Gene X	sample4 Gene X	sample4 Gene X	sample4 Gene X	sample5 Gene X	sample5 Gene X	sample5 Gene X	sample6 Gene X	sample6 Gene X	sample6 Gene X	C
D	sample7 Gene X	sample7 Gene X	sample7 Gene X	sample8 Gene X	sample8 Gene X	sample8 Gene X	sample9 Gene X	sample9 Gene X	sample9 Gene X	sample10 Gene X	sample10 Gene X	sample10 Gene X	D
E	sample1 Gene Y	sample1 Gene Y	sample1 Gene Y	sample2 Gene Y	sample2 Gene Y	sample2 Gene Y	sample3 Gene Y	sample3 Gene Y	sample3 Gene Y	sample4 Gene Y	sample4 Gene Y	sample4 Gene Y	E
F	sample5 Gene Y	sample5 Gene Y	sample5 Gene Y	sample6 Gene Y	sample6 Gene Y	sample6 Gene Y	sample7 Gene Y	sample7 Gene Y	sample7 Gene Y	sample8 Gene Y	sample8 Gene Y	sample8 Gene Y	F
G	sample9 Gene Y	sample9 Gene Y	sample9 Gene Y	sample10 Gene Y	sample10 Gene Y	sample10 Gene Y							G
H													H
	1	2	3	4	5	6	7	8	9	10	11	12	

Adding AccuCal-D™ to a qPCR run

1. Vortex supplied solutions for 10s, then spin in a benchtop centrifuge at high speed.
2. Add 5µL of each AccuCal-D™ dilution in duplicate or triplicate to the qPCR plate/disc.

3. Add the mastermix containing a DNA intercalating dye as per manufacturer's instructions and make up to the final reaction volume with an appropriate diluent such as DNase/RNase-free water. Examples of 10µL and 20µL qPCR reactions are:

   10µL reaction: 5µL AccuCal-D™ dilution + 5µL 2x mastermix

   20µL reaction: 5µL AccuCal-D™ dilution + 5µL H_2O + 10µL 2x mastermix

4. Check that the FL from all wells including AccuCal-D™ will be acquired, and start the qPCR run as per usual. Note, calibrator dilutions much be labelled in the format 'AccuCal 10' for correct identification by the RealCount™ software.

Exporting raw fluorescence data into RealCount™

The RealCount™ software can directly read Rotor Gene qPCR run files (.rex files). For files from other qPCR machines, export the FL vs cycle number data for each well, making sure to export raw FL without background subtraction. The data must be arranged in the format illustrated below, keeping first 4 rows empty, 5th raw dedicated to headings and cycle number, while column A dedicated to well numbers. Insure to label your samples, including AccuCal-D™. It is important to keep the format of "AccuCal 10" for AccuCal-D™ labelling, this way the software will recognise these wells as a calibrator. Save the data as an Excel workbook.

Cycle number

4 blanc rows

Well

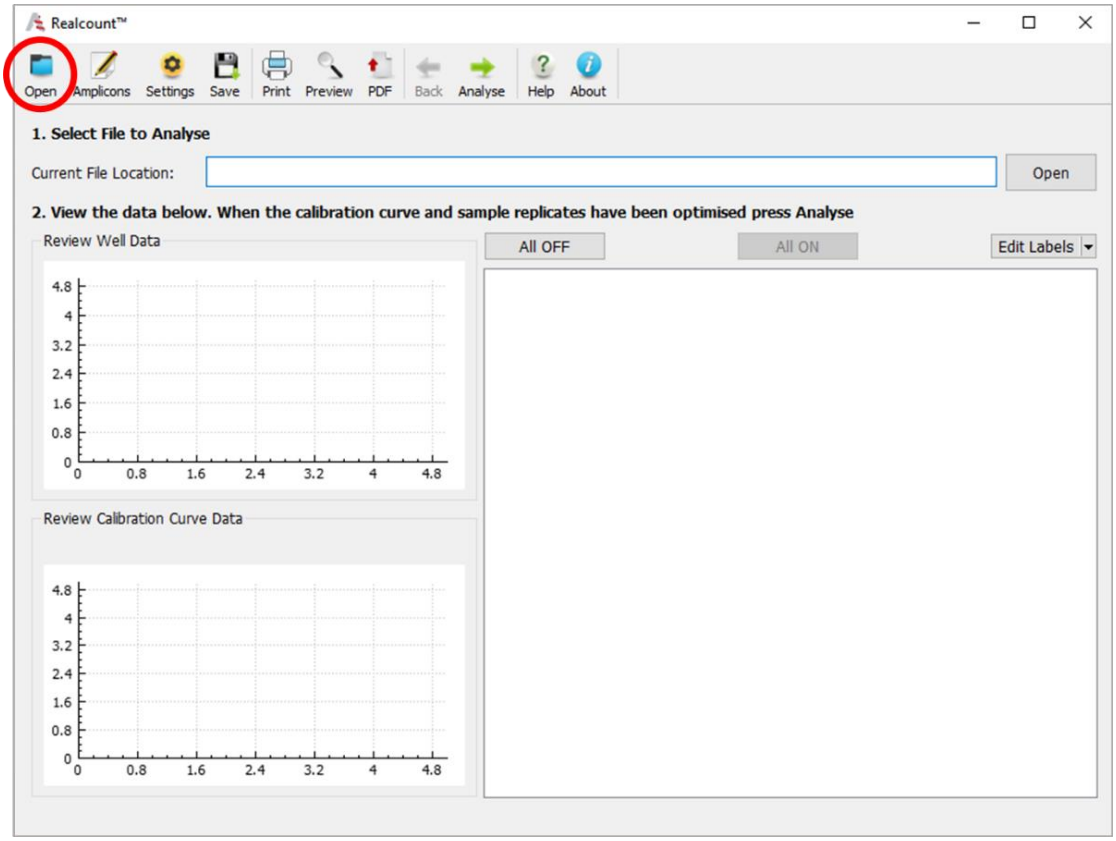
B105

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1																
2																
3																
4																
5	ID	Page 1	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6	A1	AccuCal 50	2.733908	2.710824	2.732891	2.781954	2.737977	2.747823	2.749771	2.809695	2.781611	2.720466	2.794472	2.753948	2.788528	2.754947
7	A2	AccuCal 50	2.707256	2.716615	2.719158	2.788645	2.755779	2.757305	2.758424	2.828671	2.803693	2.768597	2.865263	2.78528	2.775289	2.748695
8	A3	AccuCal 50	2.717823	2.729722	2.744159	2.820739	2.791202	2.807863	2.800249	2.845269	2.850855	2.838051	2.914855	2.862266	2.873376	2.784663
9	A4	AccuCal 40	1.9735	1.981384	1.980856	2.051016	2.01752	2.020412	2.032736	2.085718	2.096386	2.066707	2.100862	2.061611	2.066987	2.036272
10	A5	AccuCal 40	1.9744	1.988556	1.979095	2.051684	2.017134	2.008205	1.998932	2.063648	2.08718	2.046438	2.050507	2.009232	2.026507	2.002638
11	A6	AccuCal 40	2.078606	2.079587	2.061901	2.144597	2.122225	2.139853	2.149691	2.183852	2.202112	2.095827	2.162203	2.114087	2.124643	2.104982
12	A7	AccuCal 30	1.395731	1.381377	1.404644	1.442486	1.428789	1.435111	1.43849	1.462196	1.469008	1.478192	1.497782	1.478364	1.463951	1.473259
13	A8	AccuCal 30	1.38977	1.388336	1.400969	1.454878	1.418435	1.435976	1.435397	1.450663	1.451743	1.457542	1.505862	1.488191	1.483613	1.489853
14	A9	AccuCal 30	1.394566	1.38695	1.385301	1.415274	1.410035	1.406446	1.412516	1.43974	1.468121	1.42241	1.437686	1.404402	1.42308	1.412431
15	A10	Accucal 20	1.070929	1.064099	1.063276	1.086912	1.074032	1.072491	1.092097	1.113322	1.102682	1.092194	1.110857	1.091732	1.092292	1.089494
16	A11	Accucal 20	1.048731	1.060883	1.071946	1.087616	1.08072	1.086061	1.069568	1.089259	1.102719	1.089612	1.109331	1.090448	1.100175	1.097429
17	A12	Accucal 20	1.052328	1.055706	1.045659	1.075049	1.080448	1.099603	1.088186	1.11297	1.118397	1.114519	1.128252	1.105298	1.127364	1.10914
18	B1	Accucal 10	0.555524	0.552377	0.550374	0.559879	0.553616	0.55581	0.569263	0.573974	0.573103	0.571232	0.568617	0.571777	0.570115	0.565601
19	B2	Accucal 10	0.553089	0.532464	0.547447	0.559396	0.551723	0.550978	0.558385	0.576355	0.571501	0.563821	0.573194	0.566227	0.578099	0.572722
20	B3	Accucal 10	0.55355	0.545217	0.553668	0.549172	0.555238	0.559192	0.554883	0.569597	0.578953	0.573536	0.568907	0.568875	0.579843	0.579029
21	B4	Accucal 0	0.453295	0.451972	0.465291	0.462675	0.465654	0.467798	0.469562	0.470169	0.472921	0.472678	0.483295	0.471981	0.475612	0.477825
22	B5	Accucal 0	0.460009	0.460118	0.464746	0.469215	0.467278	0.461292	0.463588	0.468166	0.468706	0.47479	0.472812	0.483584	0.475847	0.475223
23	B6	Accucal 0	0.474556	0.478879	0.478261	0.479769	0.482302	0.480798	0.489624	0.479235	0.489488	0.484583	0.490832	0.474203	0.490754	0.483007
24	B7	Tubb3 5x10^6 70bp	0.510632	0.511076	0.51318	0.517909	0.519214	0.525346	0.524529	0.533521	0.530887	0.518807	0.534066	0.537931	0.561096	0.585007
25	B8	Tubb3 5x10^6 70bp	0.518095	0.509957	0.514933	0.526654	0.520567	0.533714	0.52491	0.528471	0.533596	0.531366	0.534352	0.54442	0.555101	0.563494
26	B9	Tubb3 5x10^6 70bp	0.513085	0.510669	0.520714	0.513466	0.511357	0.515183	0.52196	0.523384	0.52402	0.525564	0.531718	0.536246	0.554774	0.579139
27	B10	Tubb3 5x10^5 70bp	0.513371	0.505728	0.505661	0.507656	0.506365	0.516518	0.510356	0.520551	0.516391	0.517662	0.521205	0.524793	0.532124	0.513902
28	B11	Tubb3 5x10^5 70bp	0.49719	0.495046	0.495104	0.503548	0.501083	0.508125	0.506382	0.51545	0.504856	0.503357	0.514112	0.517408	0.51585	0.524474
29	B12	Tubb3 5x10^5 70bp	0.495918	0.500261	0.503751	0.505543	0.503643	0.504158	0.50115	0.509855	0.510121	0.519461	0.525346	0.507293	0.509064	0.519188
30	C1	Tubb3 5x10^4 70bp	0.509753	0.498383	0.505628	0.502022	0.503344	0.502843	0.515537	0.515755	0.509957	0.511895	0.507798	0.512703	0.512049	0.518924

Importing qPCR data files

1. Start the RealCount™ program. When prompted select the software licence (.lic file) provided by ACCUGEN.

2. Once the user interface (UI) has opened, select the 'Open' icon from the Menu.

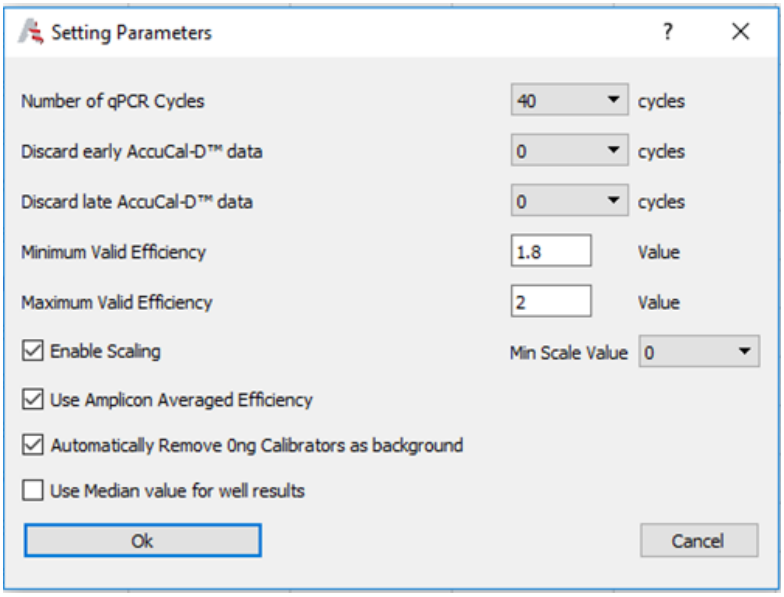


3. Select either a .rex file or the Excel workbook (.xls or .xlsx) with the correctly formatted FL data

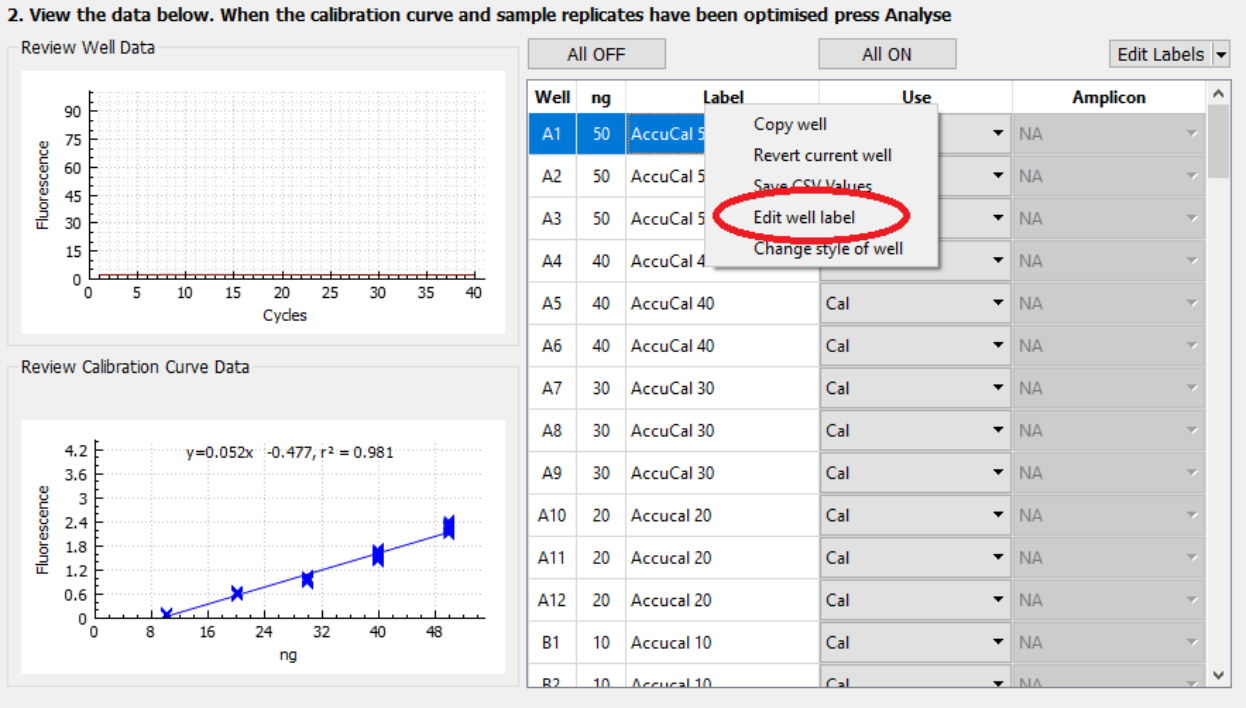
4. Files can be saved at any time to RealCount™ files (.rct) by clicking on the 'Save' icon in the Menu bar.

5. Once the data has loaded, select 'Settings' from the Menu bar.

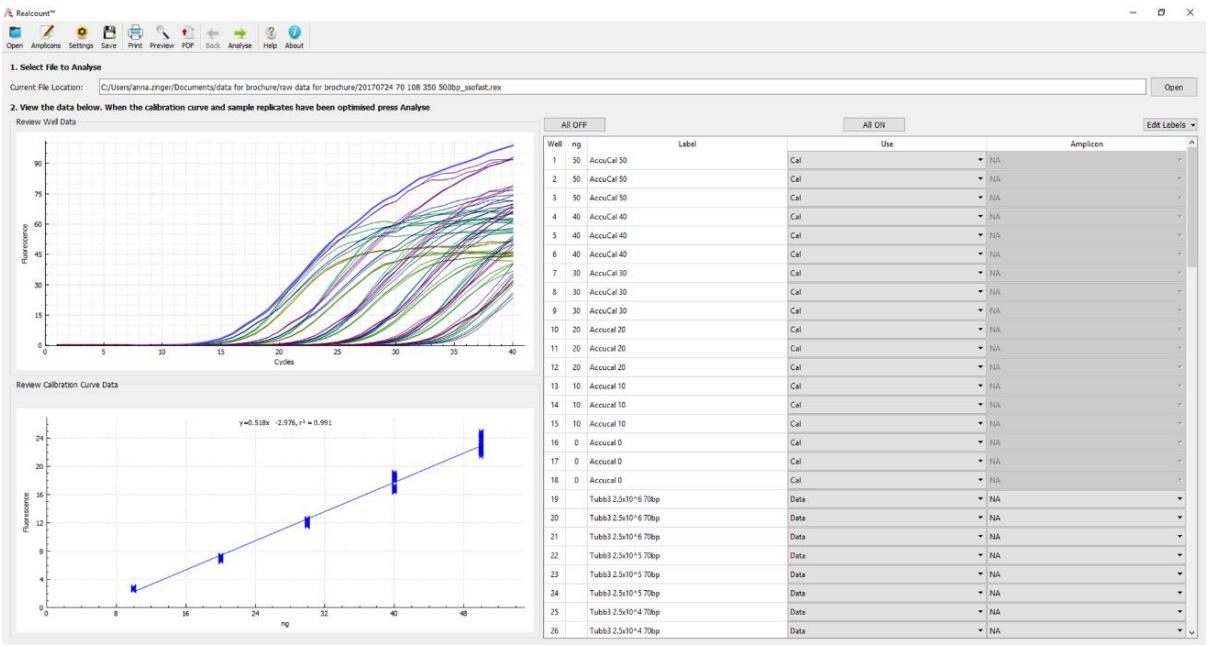
Make sure the 'Enable Scaling' box is checked. This ensures that the subtract background calculation is optimal[1], which reduces between-platform and between-mastermix variation in FL. Click 'Ok' to continue.



6. For data imported using Excel template, it is recommended to label each AccuCal-D™ dilution and sample whilst in the template format. Labels can also be edited later using the RealCount interface. Wells can be labelled individually, or as a group (i.e. replicates), by left clicking on the samples to be labelled (click and scroll or hold Ctrl for multiple selections) which will now be highlighted in blue. Right click a sample in the group for the edit popup menu. Select 'Edit well labels' and type the new label. Push 'Enter' to apply to all replicates.



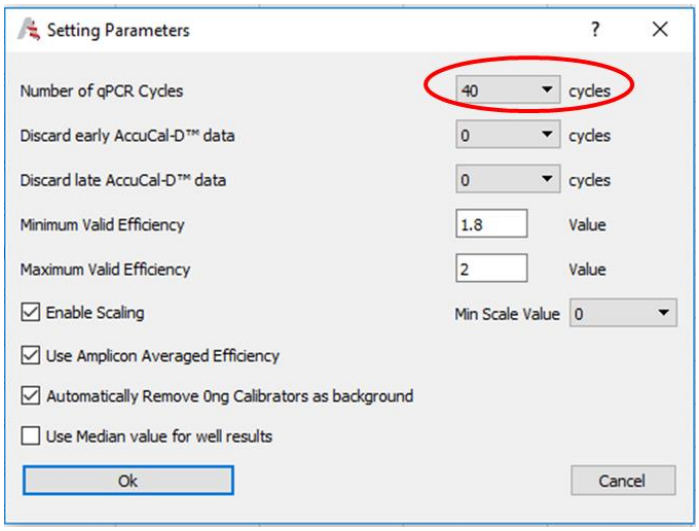
7. For manually labelled data, or direct import of a .rex file, the UI should look similar to this when all qPCR wells are selected.



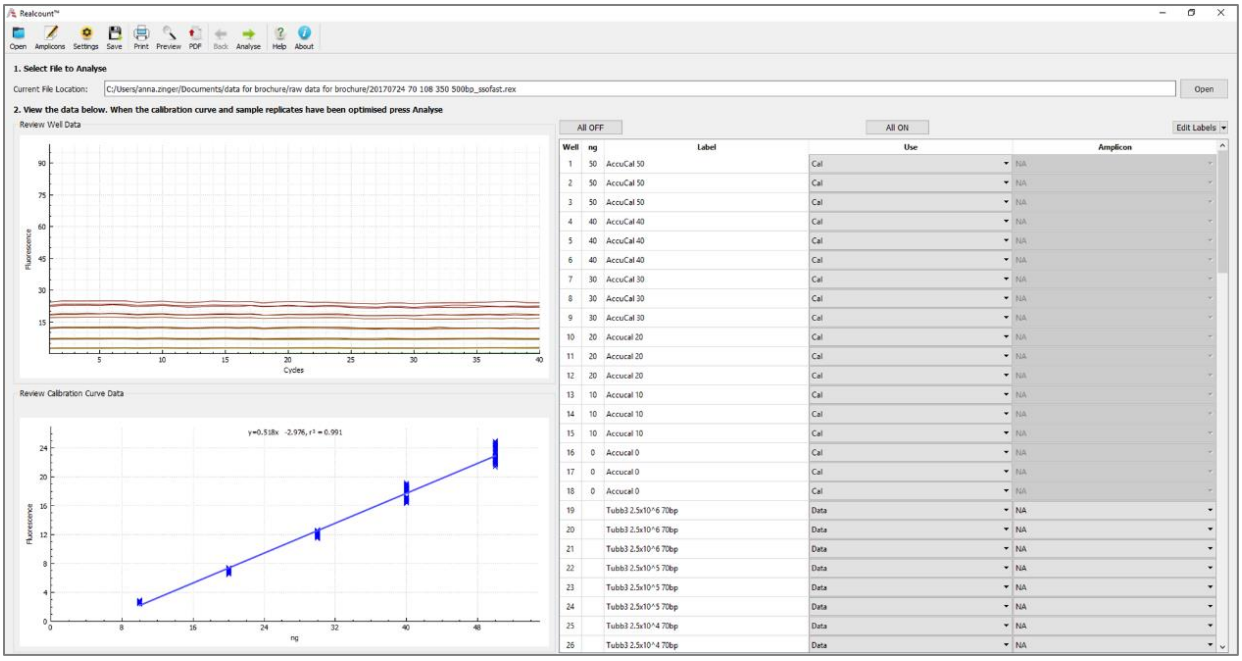
Optimising the AccuCal-D™ calibration curve

You should now see a calibration curve at the bottom left window. You have an option to optimize this curve by amending setting parameters.

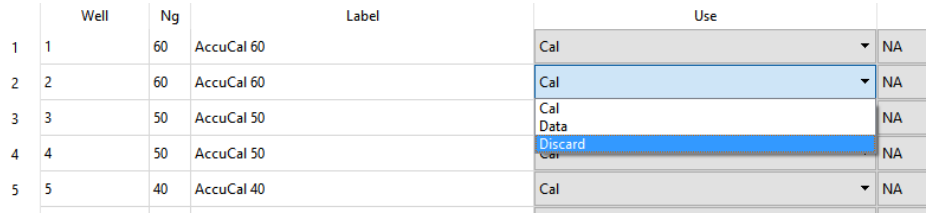
8. Open 'Settings' from the Menu. The default setting is at 40 cycles and you may select the correct number of qPCR cycles from the dropdown menu.



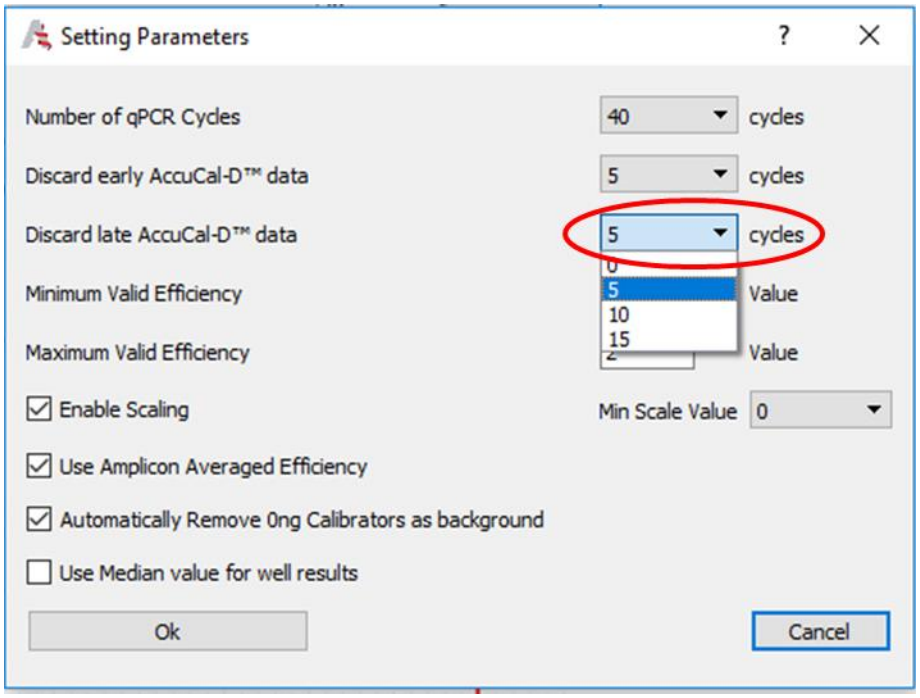
9. Select wells with AccuCal-D™ dilution series. These wells will be highlighted in blue. The AccuCal-D™ dilutions should be a series of flat lines. Note that if the wells have been correctly labelled in the format 'AccuCal 20' etc, the ng value will automatically appear in the 'Ng' column and will be designated 'Cal' for calibrator in the 'Use' column.
The AccuCal-D™ calibration curve present in the lower left panel of the interface plots the 'Ng' column on the x-axis against the FL value of that dilution for every cycle. For example, for a qPCR of 40 cycles, each well with an AccuCal-D™ dilution will have 40 FL values. A larger spread for each calibrator suggests either variable FL for a well over the qPCR cycles (which can be mastermix specific), or poor concordance within replicates of the same AccuCal-D™ dilution.

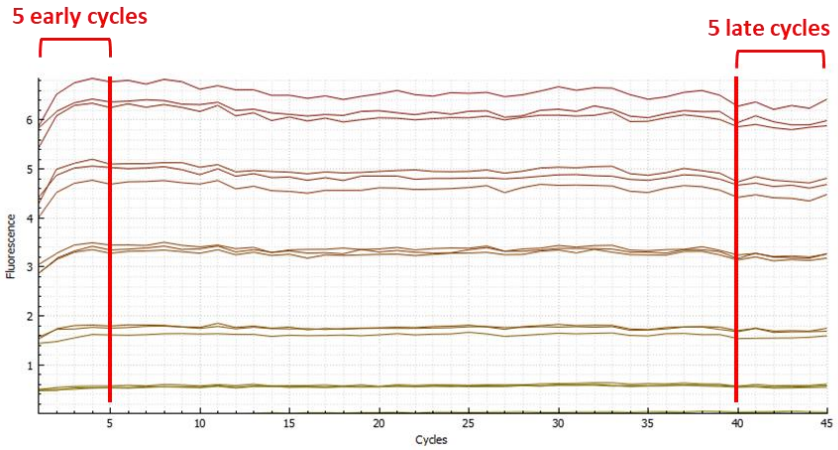


10. If more than duplicates of each AccuCal-D™ dilution exist, assess if all replicates have similar flat lines. If one is markedly different, it can be manually discarded by selecting 'Discard' from the dropdown menu under the 'Use' column.

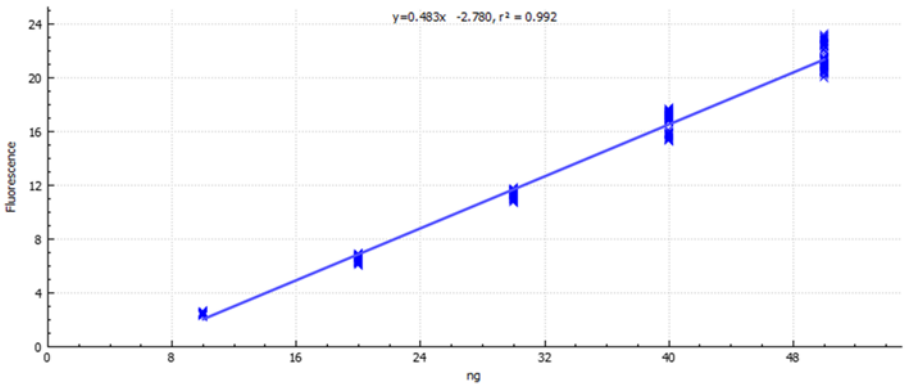


11. Unstable FL of AccuCal-D™ dilutions may occur in the first few cycles of a qPCR run, which then becomes stable after10-15 cycles. To remove these aberrant values and increase the accuracy of the AccuCal-D™ calibrator curve, early or late values for the calibrator can be discarded.
    To discard variable FL values, open the 'Settings' menu. Choose the number of early or late cycles to discard from the calibrator plot from the pulldown menu. No more than 20 cycles can be discarded. Click 'Ok' to continue.





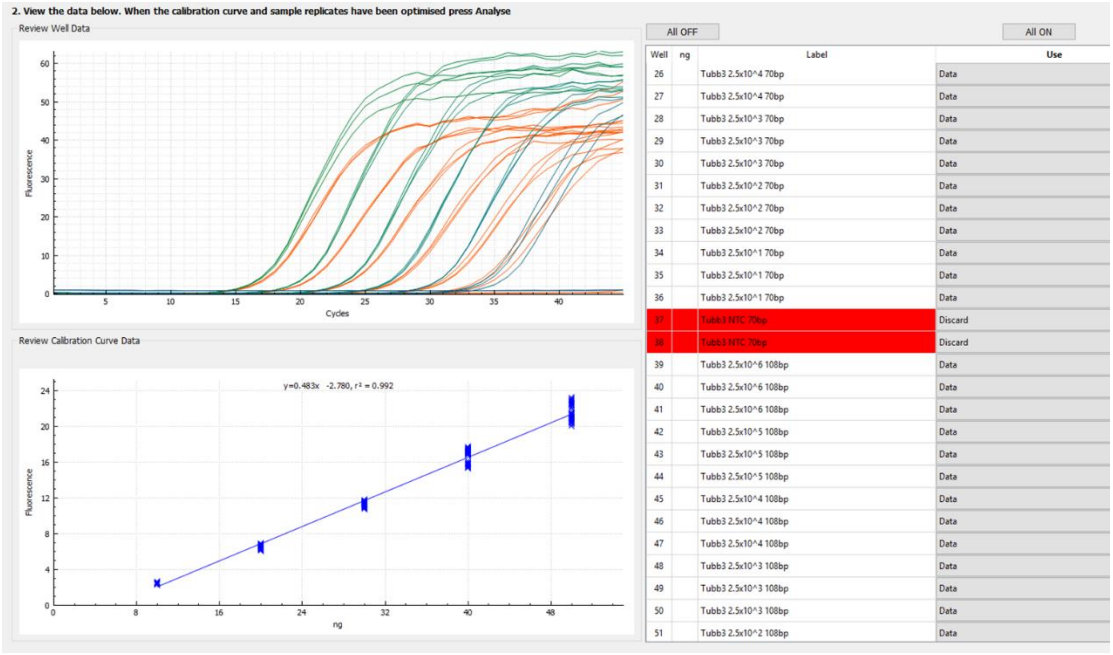
12. The calibration curve will have less spread of FL for each AccuCal-D™ dilution, and the r^2 value for the linear regression should have improved towards 1.00. The background FL is removed automatically from the calibration curve, by ticking the box 'Automatically remove 0ng calibrators as background'.



Well selection, and grouping of samples into amplicons

13. To group wells using colour, select the wells of interest, right click and select 'Change style of wells'. Choose a colour from the palette or define a custom colour. Click 'Ok'.

Grouping by colour allows easy identification of amplicons e.g. red curves for the 70bp amplicons and green for the 108bp amplicon in the figure below.

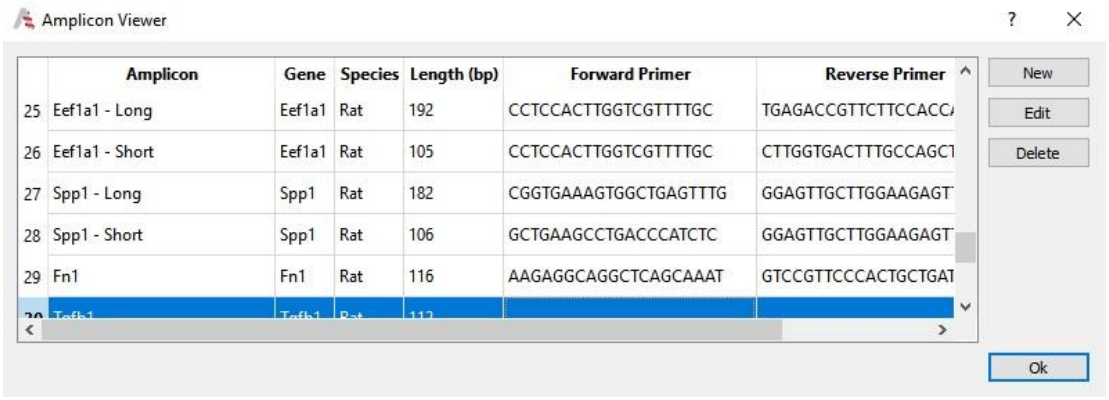


14. Check that replicates of a sample are similarly grouped. Note that as starting copy number decreases (i.e. amplification curves appearing at cycles 30+) the spread of replicates will increase. For very low copy number e.g. 10 copies, amplification may occur in only a fraction of the replicates – a sampling effect that adheres to the Poisson distribution. It is advisable to increase the number of replicates if a low starting copy number is observed. Flat lines (no amplification) or curves that didn't have "region of linearity" will be automatically discarded by the RealCount™ software.

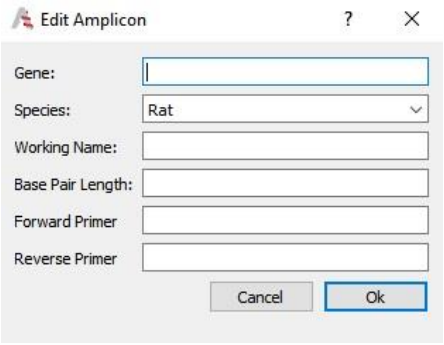
15. When a replicate has an aberrant amplification curve, discard the replicate. It is also advisable to discard samples that are failing melting curve analysis, as is usually recommended in trouble shooting guides for Master Mix that you have been using. The replicate will subsequently be highlighted red in the UI.

16. Next, samples must be grouped into amplicons. To add a new amplicon to the database select click on the 'Amplicons' icon in the Menu.

This will display the 'Amplicon Viewer'.

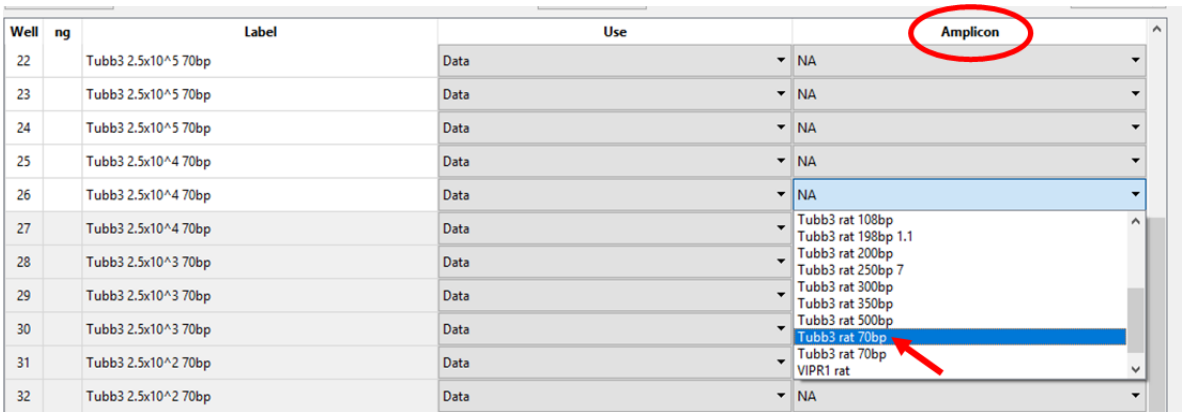


17. Select 'New' to enter an amplicon.



It is imperative that 'Base Pair Length' be completed, as this is a key parameter used to determine the number of moles of the amplicon. Complete the empty fields and click 'Ok'. These parameters can be changed by selecting 'Edit' in the 'Amplicon Viewer'.

18. Add samples to an amplicon group from the dropdown menu under the 'Amplicon' column.



19. All samples must be assigned to the correct amplicon. This is required for calculating the reaction efficiencies, which can be determined for individual curves or for the amplicon as a whole (Amplicon Average Efficiency). Amplicon Average Efficiency was shown to be more accurate for determining the efficiency of a particular primer set[2], and its application is controlled through 'Setting Parameters" menu.

20. Final check:

- Check 'Enable Scaling' and 'Automatically Remove 0ng Calibrator Background' box (Settings)
- Correct number of qPCR cycles (Settings)
- Correct labelling of AccuCal-D™ and samples (main UI)
- If using ≥triplicates, check suitability of calibrator flat lines (main UI)
- Discard early or late cycles of calibrator to improve calibration curve (Settings)
- Edit wells to group by colour (optional; main UI)
- Discard aberrant replicates of samples (main UI)
- Group samples into amplicons (Amplicon Viewer & main UI)
- Save file to .rct format

Calculating starting copy number

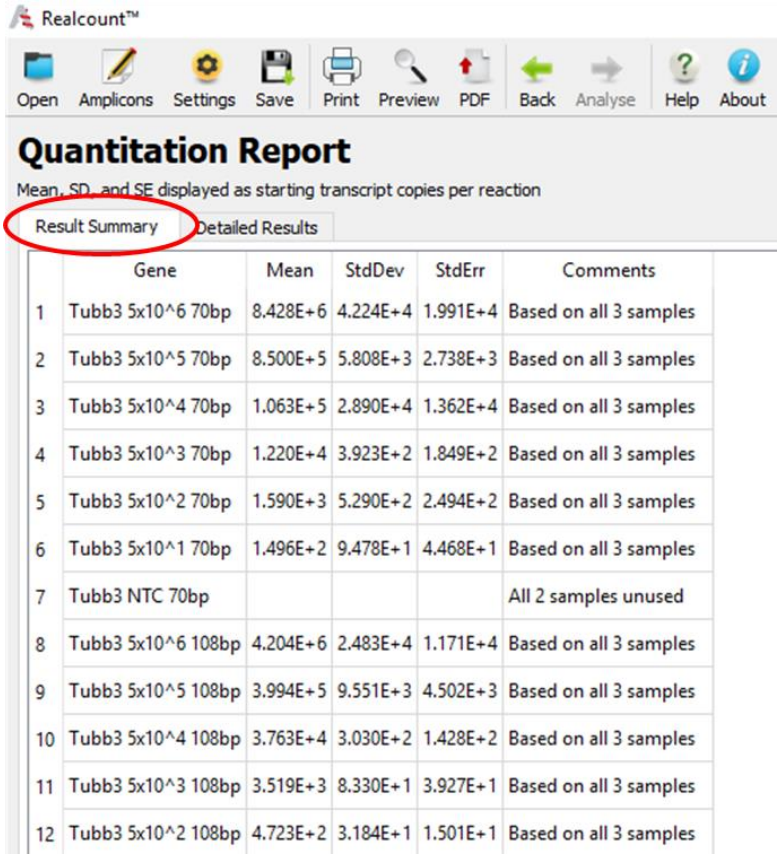
21. Click the 'Next' icon in the Menu bar to start the analysis

This will take you to the 'Quantitation Report' which has two tabs: (1) the 'Results Summary' and (2) the 'Detailed Results'.

(1) The 'Results Summary' shows the mean starting copy number for a sample as determined from the replicates.

The standard deviation (StDev) and standard error of the mean (StdErr) columns show variation between replicates of a sample.

The 'Comments' column highlights how many replicates for each sample are used in the calculations.



	Gene	Mean	StdDev	StdErr	Comments
1	Tubb3 5x10^6 70bp	8.428E+6	4.224E+4	1.991E+4	Based on all 3 samples
2	Tubb3 5x10^5 70bp	8.500E+5	5.808E+3	2.738E+3	Based on all 3 samples
3	Tubb3 5x10^4 70bp	1.063E+5	2.890E+4	1.362E+4	Based on all 3 samples
4	Tubb3 5x10^3 70bp	1.220E+4	3.923E+2	1.849E+2	Based on all 3 samples
5	Tubb3 5x10^2 70bp	1.590E+3	5.290E+2	2.494E+2	Based on all 3 samples
6	Tubb3 5x10^1 70bp	1.496E+2	9.478E+1	4.468E+1	Based on all 3 samples
7	Tubb3 NTC 70bp				All 2 samples unused
8	Tubb3 5x10^6 108bp	4.204E+6	2.483E+4	1.171E+4	Based on all 3 samples
9	Tubb3 5x10^5 108bp	3.994E+5	9.551E+3	4.502E+3	Based on all 3 samples
10	Tubb3 5x10^4 108bp	3.763E+4	3.030E+2	1.428E+2	Based on all 3 samples
11	Tubb3 5x10^3 108bp	3.519E+3	8.330E+1	3.927E+1	Based on all 3 samples
12	Tubb3 5x10^2 108bp	4.723E+2	3.184E+1	1.501E+1	Based on all 3 samples

(2) The 'Detailed Results' section shows how starting copy number is calculated at four positions along the amplification curve (Columns A-D) for individual replicates of a sample (Rep column).

The 'Eff' column denotes the calculated reaction efficiency for set of samples, grouped by amplicons (in the event that the 'Use Amplicon Averaged Efficiency' box is checked in the Setting menu, see section 19). You have the option to use individual efficiency of each sample by unticking the box, however it was demonstrated by Rojter et al. that amplicon average efficiency method is more accurate. If primer set efficiency is not known or has not been validated before, use primer sets that give rise to regular sigmoidal curves and RealCount™ will calculate the PCR efficiency for a given amplicon.

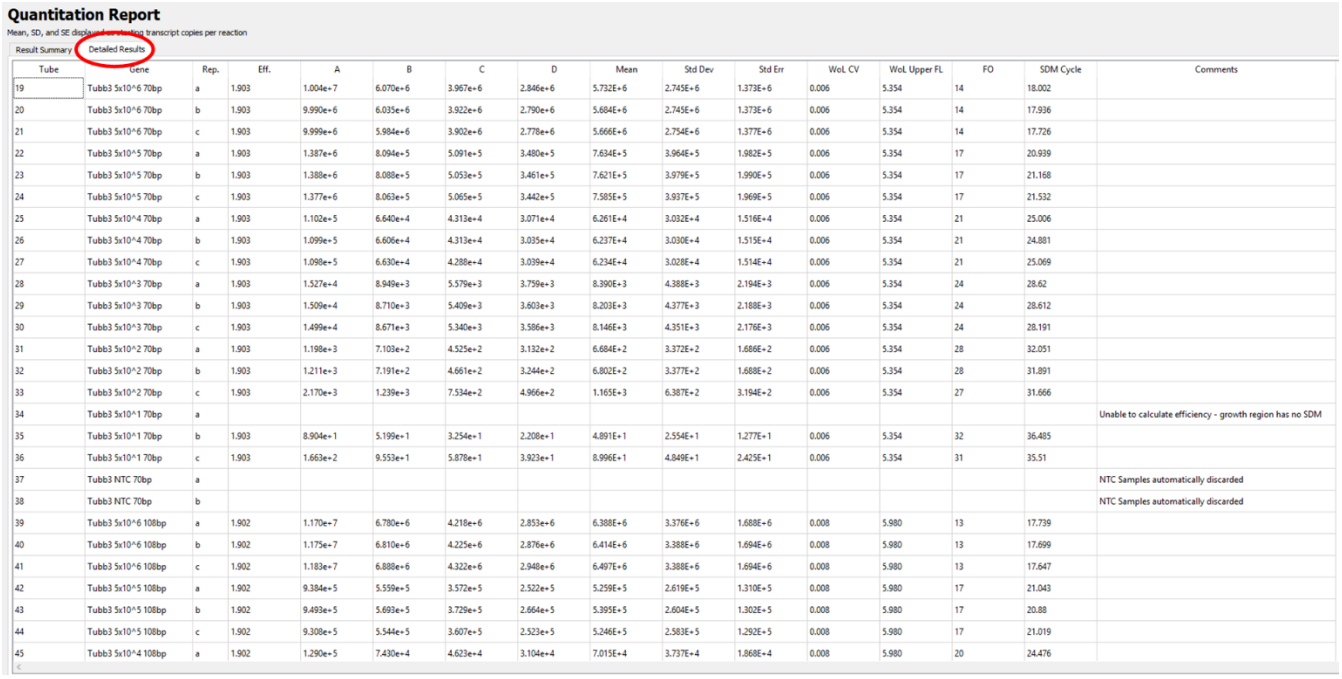
The 'Comments' column describes if a sample has been manually or automatically discarded by RealCount™ and may provide an error message in regards to where in the copy number calculation the sample failed.

Examples of error messages:

"Efficiency is not in the range", appears when amplification efficiency is lower or higher than the range specified in 'Settings'.
"Unable to determine efficiency", appears when efficiency is less than 0 and amplification failed.
"Unable to calculate efficiency-growth region has no SDM", appears when growth curve does not have inflection point or there is no valid growth curve.
"NTC samples are automatically discarded", appears when samples are discarded by the software when 'NTC' word is being recognised in the name of the sample.
"Unable to determine starting concentration", appears when the mean calculated concentration value is below 0.
"Unable to determine WOL", appears when amplification curve does not have sufficient linear space for the calculations in the window of linearity (WOL).

In the care of error message, it is recommended verifying primer efficiency and specificity by standard curve using serial dilutions of cDNA and ensuring single product amplification (single band on a gel). Dye concentration, PCR inhibitors in the sample or running parameters may also cause reaction inhibition or inefficient amplification. It is recommended optimising sample preparation, dye concentration or run settings to achieve valid sigmoid growth curve.

Columns 'WoL CV', 'WoL Upper FL', 'FO' and 'SDM Cycle' are amplicon and sample-specific parameters used by RealCount™ to determine copy number.



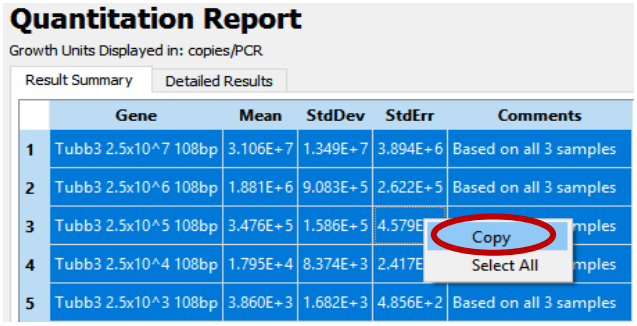
Quantitation Report

Mean, SD, and SE displayed for starting transcript copies per reaction

Result Summary | Detailed Results

Tube	Gene	Rep.	Eff.	A	B	C	D	Mean	Std Dev	Std Err	WoL CV	WoL Upper FL	FO	SDM Cycle	Comments
19	Tubb3 5x10^6 70bp	a	1.903	1.004e+7	6.070e+6	3.967e+6	2.846e+6	5.732E+6	2.745E+6	1.373E+6	0.006	5.354	14	18.002	
20	Tubb3 5x10^6 70bp	b	1.903	9.990e+6	6.035e+6	3.922e+6	2.790e+6	5.684E+6	2.745E+6	1.373E+6	0.006	5.354	14	17.936	
21	Tubb3 5x10^6 70bp	c	1.903	9.999e+6	5.984e+6	3.902e+6	2.778e+6	5.666E+6	2.754E+6	1.377E+6	0.006	5.354	14	17.726	
22	Tubb3 5x10^5 70bp	a	1.903	1.387e+6	8.094e+5	5.091e+5	3.480e+5	7.634E+5	3.964E+5	1.982E+5	0.006	5.354	17	20.939	
23	Tubb3 5x10^5 70bp	b	1.903	1.388e+6	8.088e+5	5.053e+5	3.461e+5	7.621E+5	3.979E+5	1.990E+5	0.006	5.354	17	21.168	
24	Tubb3 5x10^5 70bp	c	1.903	1.377e+6	8.063e+5	5.065e+5	3.442e+5	7.585E+5	3.937E+5	1.969E+5	0.006	5.354	17	21.532	
25	Tubb3 5x10^4 70bp	a	1.903	1.102e+5	6.640e+4	4.313e+4	3.071e+4	6.261E+4	3.032E+4	1.516E+4	0.006	5.354	21	25.006	
26	Tubb3 5x10^4 70bp	b	1.903	1.099e+5	6.606e+4	4.313e+4	3.035e+4	6.237E+4	3.030E+4	1.515E+4	0.006	5.354	21	24.881	
27	Tubb3 5x10^4 70bp	c	1.903	1.098e+5	6.630e+4	4.288e+4	3.039e+4	6.234E+4	3.028E+4	1.514E+4	0.006	5.354	21	25.069	
28	Tubb3 5x10^3 70bp	a	1.903	1.527e+4	8.949e+3	5.579e+3	3.759e+3	8.390E+3	4.388E+3	2.194E+3	0.006	5.354	24	28.62	
29	Tubb3 5x10^3 70bp	b	1.903	1.509e+4	8.710e+3	5.409e+3	3.603e+3	8.203E+3	4.377E+3	2.188E+3	0.006	5.354	24	28.612	
30	Tubb3 5x10^3 70bp	c	1.903	1.499e+4	8.671e+3	5.340e+3	3.586e+3	8.146E+3	4.351E+3	2.176E+3	0.006	5.354	24	28.191	
31	Tubb3 5x10^2 70bp	a	1.903	1.198e+3	7.103e+2	4.525e+2	3.132e+2	6.684E+2	3.372E+2	1.686E+2	0.006	5.354	28	32.051	
32	Tubb3 5x10^2 70bp	b	1.903	1.211e+3	7.191e+2	4.661e+2	3.244e+2	6.802E+2	3.377E+2	1.688E+2	0.006	5.354	28	31.891	
33	Tubb3 5x10^2 70bp	c	1.903	2.170e+3	1.239e+3	7.534e+2	4.966e+2	1.165E+3	6.387E+2	3.194E+2	0.006	5.354	27	31.666	
34	Tubb3 5x10^1 70bp	a													Unable to calculate efficiency - growth region has no SDM
35	Tubb3 5x10^1 70bp	b	1.903	8.904e+1	5.199e+1	3.254e+1	2.208e+1	4.891E+1	2.554E+1	1.277E+1	0.006	5.354	32	36.485	
36	Tubb3 5x10^1 70bp	c	1.903	1.663e+2	9.553e+1	5.878e+1	3.923e+1	8.996E+1	4.849E+1	2.425E+1	0.006	5.354	31	35.51	
37	Tubb3 NTC 70bp	a													NTC Samples automatically discarded
38	Tubb3 NTC 70bp	b													NTC Samples automatically discarded
39	Tubb3 5x10^6 108bp	a	1.902	1.170e+7	6.780e+6	4.218e+6	2.853e+6	6.388E+6	3.376E+6	1.688E+6	0.008	5.980	13	17.739	
40	Tubb3 5x10^6 108bp	b	1.902	1.175e+7	6.810e+6	4.225e+6	2.876e+6	6.414E+6	3.388E+6	1.694E+6	0.008	5.980	13	17.699	
41	Tubb3 5x10^6 108bp	c	1.902	1.183e+7	6.888e+6	4.322e+6	2.948e+6	6.497E+6	3.388E+6	1.694E+6	0.008	5.980	13	17.647	
42	Tubb3 5x10^5 108bp	a	1.902	9.384e+5	5.559e+5	3.572e+5	2.522e+5	5.259E+5	2.619E+5	1.310E+5	0.008	5.980	17	21.043	
43	Tubb3 5x10^5 108bp	b	1.902	9.493e+5	5.693e+5	3.729e+5	2.664e+5	5.395E+5	2.604E+5	1.302E+5	0.008	5.980	17	20.88	
44	Tubb3 5x10^5 108bp	c	1.902	9.308e+5	5.544e+5	3.607e+5	2.523e+5	5.246E+5	2.583E+5	1.292E+5	0.008	5.980	17	21.019	
45	Tubb3 5x10^4 108bp	a	1.902	1.290e+5	7.430e+4	4.623e+4	3.104e+4	7.015E+4	3.737E+4	1.868E+4	0.008	5.980	20	24.476	

22. Quantitation data can be copied from either the 'Results Summary' or the 'Detailed Results' tabs, by selecting and right-clicking, then choosing 'Copy'. Alternatively, this can be achieved by right-clicking on a sample and choosing 'Select All' before selecting 'Copy'. The data can then be pasted into Excel workbook for further analysis.



23. Save the file to .rct format.

References

[1] Ruijter *et al.* (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res, 37*(6). doi: 10.1093/nar/gkp045

[2] Boulter *et al.* (2016). A simple, accurate and universal method for quantification of PCR. *BMC Biotechnology, 16*(27). doi: 10.1186/s12896-016-0256-y