Avoiding qPCR Inhibition

Proper dilution of RNA isolates is key to truly relative log-linear quantitative analysis for one-step fluorogenic real-time qPCR

in brief …

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Forms of qPCR Inhibition to be aware of:

Based on experimental observations of the dynamics of numerous real-time qPCR reactions, we have been able to label and organize qPCR inhibitory phenomena into five different categories; Types 1-5:

1.) Inhibition of reverse transcriptase (RT) enzyme(s) and/or Taq DNA polymerase(s) by excessive rRNA and possibly tRNA in concentrated RNA samples (sample concentration-related template inhibition),

2.) Method of RNA isolation resulting in the carryover of inhibitory biological components or molecules (sample isolation-related inhibition),

3.) Inhibition arising from the type of tissue or cell that sample RNA has been isolated from (sample-specific inhibition),

4.) Inhibition resulting from interaction of a specific qPCR template with its specific probe and primer(s) (target-specific template inhibition),

5.) Inhibition caused by compounds such as EDTA, GIT, TRIS, glycogen, or any other user-introduced reagents (chemical inhibition)

Some qPCR-inhibitory (carryover) biological contaminants are thought to be: hemoglobin, heme, porphyrin, heparin (from peritoneal mast cells), glycogen (>2 mg/mL), polysaccharides, other unknown cell constituents, Ca^{2+}, DNA or RNA concentration, and DNA (possibly RNA) binding proteins, or other proteins (Pfaffl, et.al, Bustin [A-Z of Quantitative PCR] p. 167).

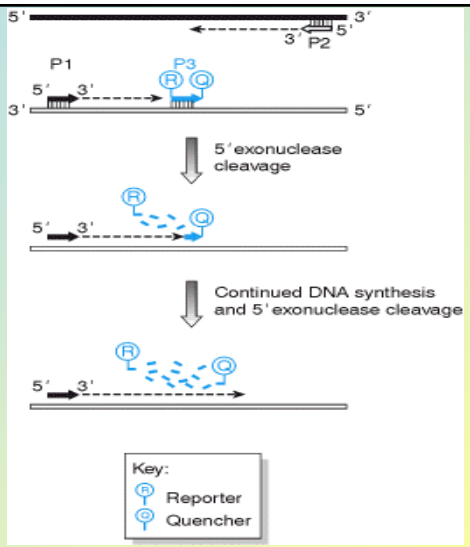
MicroRNA (miRNA) is not thought to be a contributing factor to qPCR inhibition since high thermocylcing temperatures (94-95°C) most likely disallow the formation of stable RNA-binding RSK complexes which might associate with template RNA (Ambion technical services comment).

Type 1 inhibition: inhibition of reverse transcriptase enzymes by rRNA and tRNA is not understood well, but has been noted in Invitrogen product literature (ref: Instruction Manual: **SuperScript™ III CellsDirect, cDNA Synthesis System Catalog Nos. 18080-200 and 18080-300, Version A, 14 May 2004, *25-0731*, page vi) …** Invitrogen literature

Understandably, inhibition Types 2 and 3 will always be a function of one another as method of RNA isolation and tissue or cell type from which the RNA is isolated will always affect one another distinctly. Similarly, inhibition types 1&2, 1&3, 1&4 and 1&5 are all sample-dilution dependent; a lessening of all types of inhibition is expected with increasing sample dilutions … logically

Since our qPCR studies involve the sole use of the TaqMan® (hydrolysis) probe method (which includes the use of sequence-specific forward and reverse primers), we discuss here only observations we have made with this approach using total RNA as template in fluorogenic one-step real-time qPCR … (for all targets we use 1000 nM primers and 150 nM probe concentrations) …

The TaqMan 5' exonuclease assay



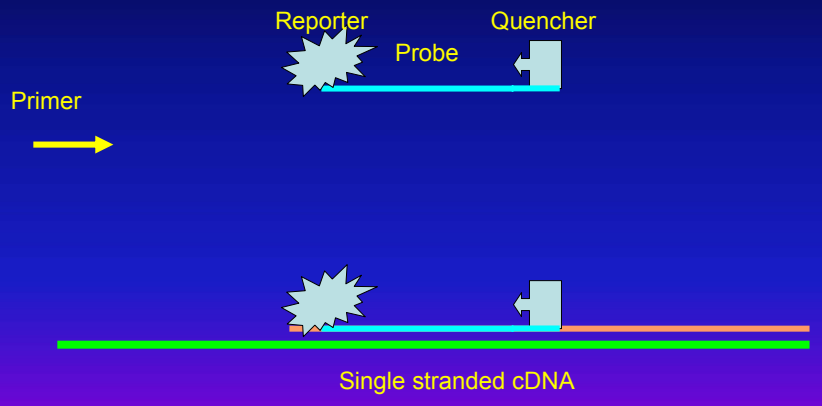
P1 = Forward Primer

P2 = Reverse Primer

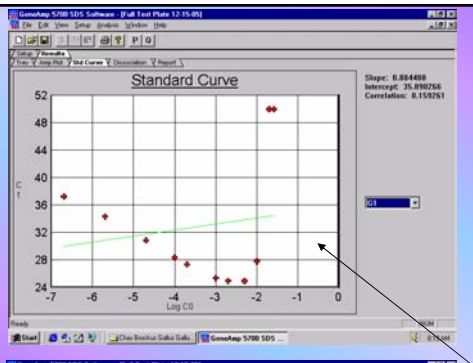
P3 = Fluorogenic TaqMan hydrolysis probe

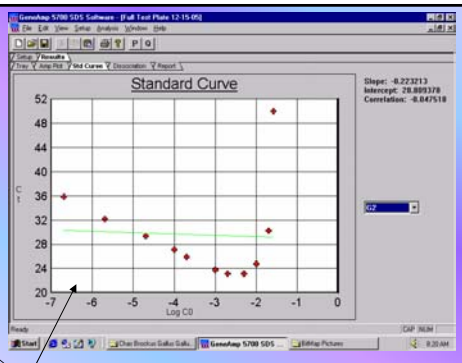
In addition to two conventional PCR primers, P1 and P2, which are specific for the target sequence, a third primer, P3 (called the 'probe'), is designed to bind specifically to a site on the target sequence downstream of the forward primer binding site. The probe is labelled with two fluorophores, a reporter dye (R) is attached at the 5' end while a quencher dye (D), which has a different emission wavelength to the reporter dye, is attached at its 3' end. Because the 3' end is blocked (by the quencher), the probe cannot by itself prime any new DNA synthesis. During the PCR reaction, *Taq* DNA polymerase synthesizes a new DNA strand primed by the forward primer, and as the enzyme approaches the probe, its 5' to 3' exonuclease activity progressively degrades the probe from its 5' end. The end result is that the nascent DNA strand extends beyond the probe binding site and the reporter and quencher dyes are no longer bound to the same molecule. As the reporter dye is no longer in close proximity to the quencher, the resulting increase in reporter emission intensity becomes easily detectable. This all occurs in "real time" as monitored by the photomultiplier tube(s) in each qPCR instrument.

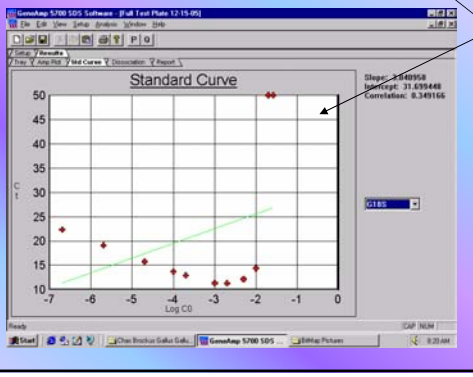
Fluorescing Real-Time qPCR



by Charles Brockus







Common inhibitory profiles observed during one-step real-time qPCR using Trizol-isolated RNA from tissues and cell culture samples …

One must identify the optimal dilution range for each qPCR target per RNA-isolation method used. This is the range over which a target shows a log linear relationship between Ct and template dilution while also exhibiting a high efficiency of reaction (>80%)

OUR GOAL HERE IS TO FIND THE TEMPLATE DILUTION RANGE (FOR EACH DIFFERENT TARGET) WHICH EXHIBITS LINEARITY AND HIGH EFFICIENCY WHILE AVOIDING ALL qPCR INHIBITORY PHENOMENA

Machine Factors:	0.026	0.02	0.01	0.005	0.002	0.001	0.0002	0.0001	0.00002	0.000002	0.0000002	
	1	2	3	4	5	6	7	8	9	10	11	12	
A	NTC	138.46	150	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000	hRSV
B	NTC	138.46	150	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000	SBD-1
C	NTC	138.46	150	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000	SP-D
D	NTC	138.46	150	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000	SP-A
E	NTC	138.46	150	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000	TTF-1
F	NTC	138.46	150	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000	ovRPS15
G	NTC	138.46	150	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000	h18S
H													

Tested Concentrations to see where inhibition lets up for each different target

Initial RNA is already at 1: 10 → in well will actually be a 1: 38.46

After DNAse treatment:
samples are diluted 1: 10
And samples, after further dilutions, are then used in-well at a proportion of:
7.80 uL sample
30.00 uL well size
sample fraction is thus: 0.26

Desired final in-well test dilution 1: 50
Desired final in-well test dilution 1: 100
Desired final in-well test dilution 1: 200
Desired final in-well test dilution 1: 500
Desired final in-well test dilution 1: 1000
Desired final in-well test dilution 1: 5000

Desired final in-well test dilution 1: 10000
Desired final in-well test dilution 1: 50000
Desired final in-well test dilution 1: 500000
Desired final in-well test dilution 1: 5000000

Given that our Stock I Solution RNA mixture is calculated to be: 48.18545 ng/uL (comprised of 1:10 RNAs)

This Test Plate dilution series thus represents:

Stock I Range tested

12.52822	ng/uL in well
9.637091	ng/uL in well
4.818545	ng/uL in well
2.409273	ng/uL in well
0.963709	ng/uL in well
0.481855	ng/uL in well
0.096371	ng/uL in well
0.048185	ng/uL in well
0.009637	ng/uL in well
0.000964	ng/uL in well
9.64E-05	ng/uL in well

Custom file

EXAMPLE 7-TARGET TEST PLATE SET-UP

Using a standard RNA (containing all your targets of interest), run a test plate, testing various dilutions of the RNA until it no longer exhibits template or chemical inhibition of each qPCR rxn … Decide where your standard curves should start for each target (e.g. always after the point of template or chemical inhibition for each target) …

FROM YOUR OBSERVATIONS OF THE TEST PLATE RESULTS, DECIDE THE FOLLOWING:
(Decide what target needs the most concentrated RNA in order to be found adequately by qPCR. Enter that target and its apparent 1st useful dilution in the yellow area below):

List in order of abundance from weakest to strongest (as observed from Cts on your Test Plate)

Enter values of apparent 1st useful dilution 1: — This serves as the 1st point (dilution) in the standard curve for this target

TEST PLATE OBSERVATIONS (least abundant target: "limiting" factor)			ng/uL (in-well) that this dilution actually corresponds to (info from Sheet 3 used)	Apparent useful serial dilution factor for each Stnd Crve 1:
Have to have at least 3 samples here, and for any samples that share identical dilutions, be sure to alter 1 of them slightly -- i.e. 1:300 and 1:300.000001 etc. -- or else the file will not work	Target 1	G-1	1000	0.2085 ng/uL in-well	10
	Target 2	G-2	1000.00001	0.2085 ng/uL in-well	10
	Target 3	ch18S	50000	0.0042 ng/uL in-well	5
	Target 4	?	?	#VALUE! ng/uL in-well	?
	Target 5	?	?	#VALUE! ng/uL in-well	?
	Target 6	?	?	#VALUE! ng/uL in-well	?
	Target 7	?	?	#VALUE! ng/uL in-well	?

G-1	G-2	ch18S
1000	1000.00001	50000
10000	10000.0001	250000
100000	100000.001	1250000
1000000	1000000.01	6250000

Or, in final ng/uL (in well values):

G-1	G-2	ch18S
0.2085	0.2085	0.0042
0.0209	0.0209	0.0008
0.0021	0.0021	0.0002
0.0002	0.0002	0.0000

To fit within stnd curve:	Name	Apparent useful Unknown dilution 1:	(in-well)			
Target 1	G-1	5500	0.03792 ng/uL in-well			
Target 2	G-2	5500.000055	0.03792 ng/uL in-well	1.00000001	1.00000001	1.00000001
Target 3	ch18S	150000	0.00139 ng/uL in-well	27.27272727	27.27272727	27.27272727

These factors are used in Sheet 2 (serialdilutionx)

1	1.00000001
27.27273	27.27272727

Type into 5700 as factors for Stnd curve relative dilutions:

G-1	G-2	ch18S
1	1	1
0.1	0.1	0.2
0.01	0.01	0.04
0.001	0.001	0.008

EXCEL FILES TO HELP SPEED UP YOUR CALCULATIONS

(in-well) 1st-sample Dilutions incurred Post DNase 1:	(in-well) 1st-sample Dilutions incurred Since isolation 1:	1st tier check in ng/uL	Sample	1:10 RNA	Water	eqMtn	from previous	Water	+15 uL / 0 uL	from previous	Water	15 uL / 386 uL
9314.98	14637.82	0.037915	BoneM1	20.0 uL	4823.79 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
31536.66	49557.61	0.037915	Crop1	6.0 uL	1427.76 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
9190.78	14442.65	0.037915	Testes1	2.0 uL	1303.99 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
25115.20	39466.74	0.037915	Lung1	2.0 uL	1267.16 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
24406.92	38353.74	0.037915	Skin1	2.0 uL	1101.69 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
21224.73	33353.14	0.037915	Spleen1	2.0 uL	1014.76 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
19553.06	30726.24	0.037915	Liver1	1.6 uL	1546.04 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
37202.85	58461.62	0.037915	Kidny1	1.0 uL	1183.85 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
45571.23	71611.93	0.037915	Bursa1	2.0 uL	1140.26 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
21966.57	34518.89	0.037915	Trach1	5.0 uL	1049.29 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
8109.91	12744.14	0.037915	Conj1	4.0 uL	1403.58 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
13534.41	21268.36	0.037915	Tongue1	2.0 uL	1144.63 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
22050.49	34650.76	0.037915	BoneM2	8.0 uL	1274.60 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
6166.35	9689.98	0.037915	Jej2	2.0 uL	1095.75 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
21110.60	33173.79	0.037915	Crop2	2.0 uL	1051.07 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
20251.27	31823.42	0.037915	Ovid2	16.0 uL	1402.75 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
3410.46	5359.29	0.037915	Lung2	2.0 uL	1072.54 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
20664.15	32472.23	0.037915	Skin2	10.0 uL	1002.40 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
3893.83	6118.87	0.037915	Spleen2	2.0 uL	1018.77 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
19630.27	30847.57	0.037915	Liver2	2.0 uL	1074.46 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
20701.07	32530.26	0.037915	Kidny2	1.4 uL	1359.63 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
37390.83	58757.02	0.037915	Bursa2	0.6 uL	1090.12 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
69917.73	109870.72	0.037915	Trach2	16.0 uL	1247.75 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
3037.86	4773.78	0.037915	Conj2	4.0 uL	1454.90 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
14027.85	22043.77	0.037915	Tongue2	2.0 uL	1091.04 uL	750 uL	414.67 uL	0.00 uL		14.67 uL	385.33 uL	
21019.96	33031.37	0.037915										

The Final print-out for final sample serial dilutions to get them into the appropriate useful One-Step real-time qPCR ranges without inhibition ...

PROGRESSIVE SERIAL DILUTION WORKSHEET

jmg/6-24-2005/8-22-05

This program allows one to make up to 20 serial dilutions using the smallest possible amount of starting reagent

Enter: Cntrl+d after adjusted

Master Volume Adjust 1X

(Print out p. 1 or pp. 2, 3 and 4 when finished)

Reset . mL (use this value for volume -- to use at least 1 uL of starting reagent)

DONE!

How To Use

1.) In Column 1: Type in in desired final dilutions starting with A as the most concentrated

2.) Type the number "1" in all Column 1 cells that you are not using for dilution calculations

3.) In Column 2: Type in all desired final volumes for each respective serial solutions Type "0" in unused cells

4.) Activate calculation:

Enter: Cntrl+d

5.) Print page 1 and use table at bottom, or print out pages 2, 3 & 4, or just those pages you will need in the lab ...

Also:

	Adjust Column 1 values to desired dilutions (type in "1" otherwise)	Column 1: Desired final ending dilution values	Column 2: Desired Final Vol.s (mL)		Adjustable Total Starting Volumes	Original ng/mL 5400
A	1:	26.00 Most dilute	0.500	662.2 uL copy	662.2 uL	207.69
B	1:	143.00	0.500	892.2 uL copy	892.2 uL	37.76
C	1:	260.00	0.500	713.1 uL copy	713.1 uL	20.77
D	1:	1300.00	0.500	1065.7 uL copy	1065.7 uL	4.15
E	1:	2600.00	0.500	1131.3 uL copy	1131.3 uL	2.08
F	1:	3900.00	0.500	947.0 uL copy	947.0 uL	1.38
G	1:	6500.00	0.500	745.0 uL copy	745.0 uL	0.83
H	1:	26000.00	0.500	980.0 uL copy	980.0 uL	0.21
I	1:	32500.00	0.500	600.0 uL copy	600.0 uL	0.17
J	1:	162500.00	0.500	500.0 uL copy	500.0 uL	0.03
K	1:	1.00	0.000	0.0 uL copy	0.0 uL	0.00
L	1:	1.00	0.000	0.0 uL copy	0.0 uL	0.00
M	1:	1.00	0.000	0.0 uL copy	0.0 uL	0.00
N	1:	1.00	0.000	0.0 uL copy	0.0 uL	0.00
O	1:	1.0	0.000	0.0 uL copy	0.0 uL	0.00
P	1:	1.0	0.000	0.0 uL copy	0.0 uL	0.00
Q	1:	1.0	0.000	0.0 uL copy	0.0 uL	0.00
R	1:	1.0	0.000	0.0 uL copy	0.0 uL	0.00
S	1:	1.0	0.000	0.0 uL copy	0.0 uL	0.00
T	1:	1.0	0.000	0.0 uL copy	0.0 uL	0.00
U	1:	1.0	0.000	0.0 uL copy	0.0 uL	0.00

Total starting reagent stock needed: 25.470 uL (achieved ng/mL)

Total diluent needed for this series of dilutions: 4.975 mL

(Some common dilution scenarios)

Stock I

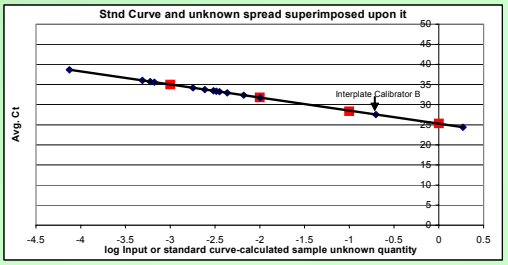
COMPREHESIVE SERIAL DILUTION TABLE

	total made	"reagent"	diluent	to next	(FINAL VOL.)	Achieved Dilutions 1:	Actual Final Dilutions Achieved for Final Plates after used in-well	
A	662.2 uL	25.5 uL	636.8 uL	162.2 uL	500.0 uL	26.0	1: 1000	A
B	892.2 uL	162.2 uL	730.0 uL	392.2 uL	500.0 uL	143.0	1: 5500	B
C	713.1 uL	392.2 uL	320.9 uL	213.1 uL	500.0 uL	260.0	1: 10000	C
D	1065.7 uL	213.1 uL	852.5 uL	565.7 uL	500.0 uL	1300.0	1: 50000	D
E	1131.3 uL	565.7 uL	565.7 uL	631.3 uL	500.0 uL	2600.0	1: 100000	E
F	947.0 uL	631.3 uL	315.7 uL	447.0 uL	500.0 uL	3900.0	1: 150000	F
G	745.0 uL	447.0 uL	298.0 uL	245.0 uL	500.0 uL	6500.0	1: 250000	G
H	980.0 uL	245.0 uL	735.0 uL	480.0 uL	500.0 uL	26000.0	1: 1000000	H
I	600.0 uL	480.0 uL	120.0 uL	100.0 uL	500.0 uL	32500.0	1: 1250000	I
J	500.0 uL	100.0 uL	400.0 uL	0.0 uL	500.0 uL	162500.0	1: 6250000	J

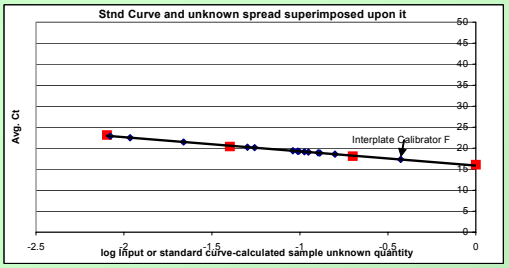
Calibrator G-1 & G-2

Calibrator Ch18S

	G1 NTC		50.00
		Log of input or Q	Av. Ct
	Stnd1	0	25.33
	Stnd2	-1	28.34
	Stnd3	-2	31.80
	Stnd4	-3	34.99
0.198088	CALB	-0.703142	27.53
1.859791	14	0.269464	24.37
0.000489	15	-3.310705	35.99
7.5E-05	16	-4.124997	38.65
0.004335	17	-2.362966	32.92
0.006612	18	-2.179648	32.32
0.000598	19	-3.223083	35.70
0.001791	20	-2.746849	34.16
0.010095	21	-1.995907	31.72
0.002431	22	-2.614143	33.73
0.000665	23	-3.176984	35.57
0.003542	24	-2.450765	33.20
0.00303	25	-2.518545	33.43
0.003265	26	-2.486163	33.31



	18S NTC		41.93
		Log input or Q_0	Av. Ct
	Stnd1	0	16.03
	Stnd2	-0.69897	18.09
	Stnd3	-1.39794	20.36
	Stnd4	-2.09691	23.10
0.374137	CALF	-0.426969	17.31
0.091145	14	-1.040266	19.37
0.021801	15	-1.661515	21.46
0.008362	16	-2.077668	22.85
0.09862	17	-1.006035	19.25
0.01085	18	-1.964578	22.47
0.097286	19	-1.011947	19.27
0.158263	20	-0.80062	18.56
0.129757	21	-0.886868	18.85
0.055264	22	-1.257557	20.10
0.127101	23	-0.895849	18.88
0.050382	24	-1.297727	20.23
0.111636	25	-0.952196	19.07
0.10612	26	-0.974204	19.15



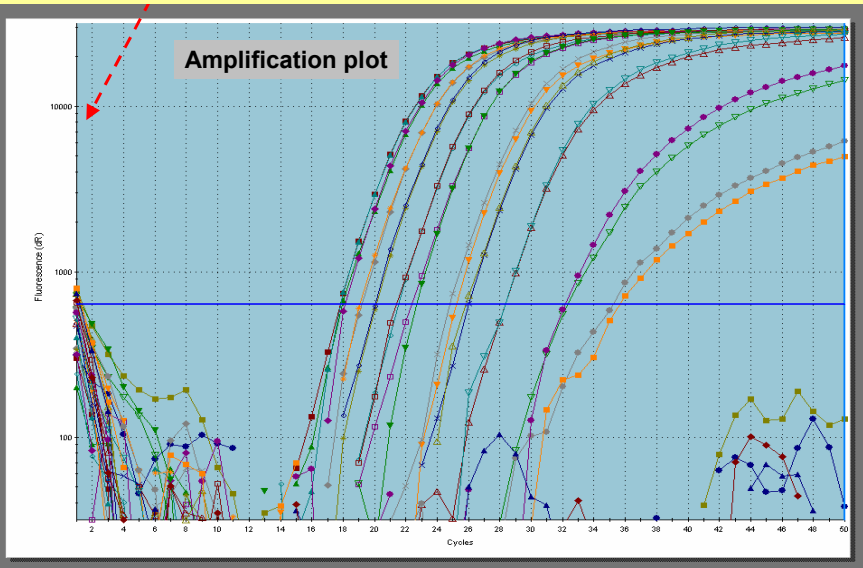
PICTURE OF A TYPICAL 1.5 mL TUBE SET-UP

PICTURE OF THE Eppendorf epMotion 5070 ROBOT IN ACTION

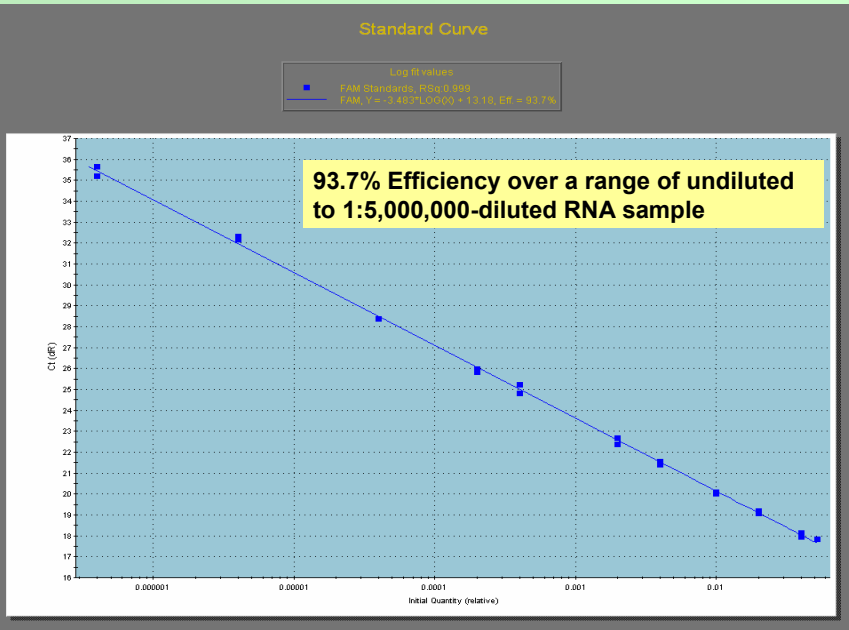
TYPICAL STORAGE OF qPCR PLATES AT 4°C BEFORE USE

Stratagene Mx3005P depiction of recent Test Plate results

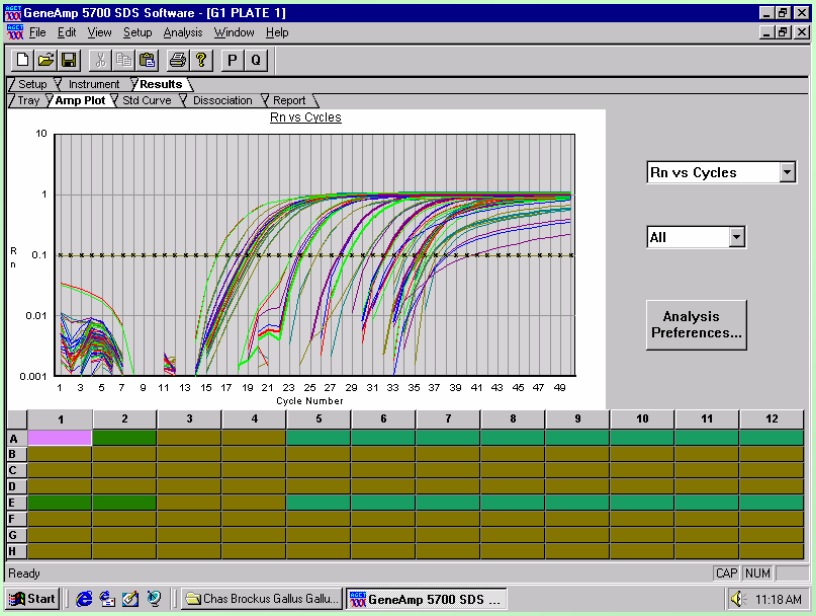
Y-axis is shown in log units here – much easier to interpret results visually this way



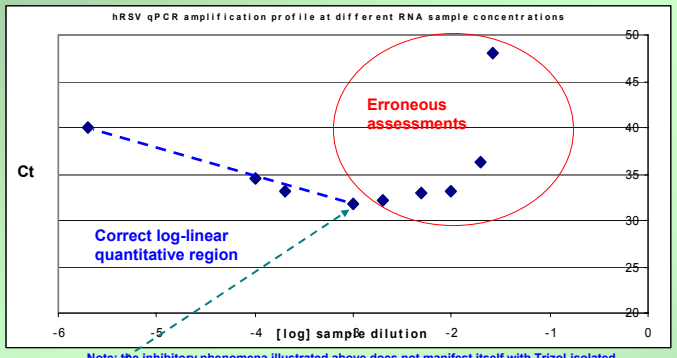
Standard Curve



Depiction of Amplification Curves on ABI 5700 sds software



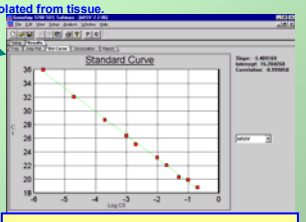
Example showing how hRSV RNA virus gives faulty values when tissue total RNA sample is used too concentrated in the qPCR application:



Note; the inhibitory phenomena illustrated above does not manifest itself with Trizol-isolated viral RNA from purified viral inoculum; only with RNA Trizol-isolated from tissue.

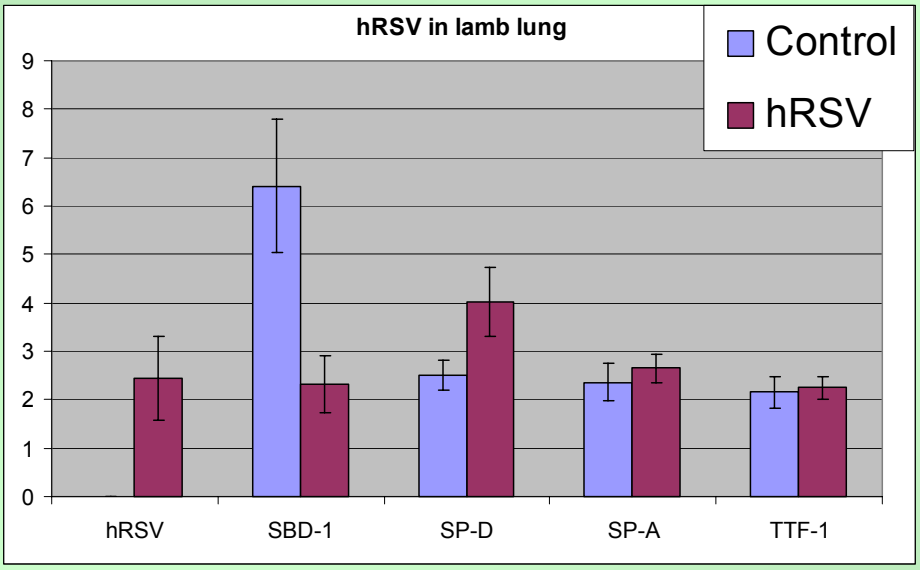
The red-circled points are erroneous and suggest much lower viral presence than is actually the case. The blue line indicates those dilutions of the RNA sample which yield true quantitative results in the assay. The first blue-lined point which begins to behave in the desired fashion represents an in-well [RNA] of 0.1248 ng/uL. For three viral signals so far (bRSV, hRSV and PCV-2, a DNA virus), we have found 0.083 ng/uL to be a very good concentration at which to start using total tissue RNA (containing virus) for qPCR analyses.

The goal is to let this assay be as sensitive as possible. Diluting RNA samples out beyond their ability to generate qPCR signal at all is just as bad as not diluting RNA samples far enough.



qPCR dilution profile generated from purified viral (hRSV) inoculum: no inhibitory phenomena evident. …

Trizol RNA isolation is considerably cheaper than alternative methods. In cases where cost is not a factor, and tissues are being extracted for RNA, we would highly suggest using the Qiazol RNEasy Lipid Tissue Mini kit #74804 from Qiagen. For non-tissue samples (i.e. swabs and lavages) other RNA column-based isolation kits from Qiagen can be employed. A nice feature of these column-based RNA isolations is that inhibitory qPCR phenomena typically disappears from qPCR reactions when the RNA isolates are used after an in-well dilution of 1:50. Trizol-isolated RNA requires in-well dilutions of at least 1:200 before one can be confident that most tissue-related qPCR inhibitory phenomena is held at bay. (But again, remember that specific target template inhibition still needs to be elimintaed by dilution as well; i.e. especially housekeepers).
Marligen Rapid Total RNA purification system No. 11502-050 (Sandra Clark)
The Trizol approach costs roughly $1.50 per each RNA sample isolated, while the Qiazol approach costs $5.40 per sample. So, realize that if you choose methods other than Trizol, your cost will increase by a factor of 3.5 or more.



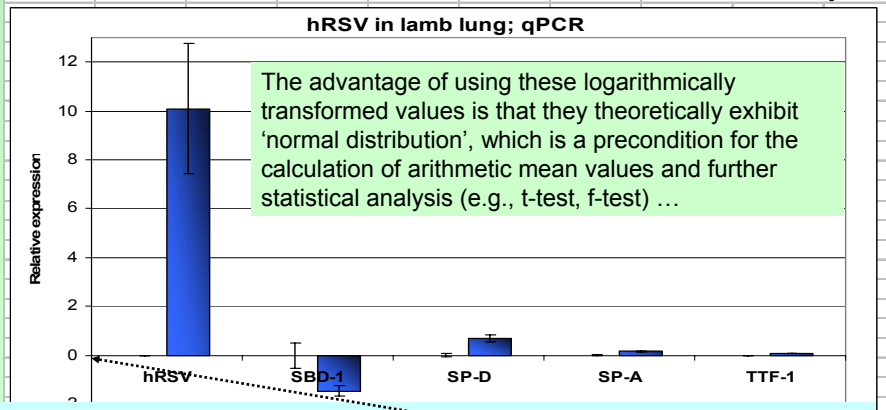
Example of results: target values in each case normalised to their respective housekeeper values. (Control animal qPCR target levels versus hRSV-infected animal levels are compared).

Log_2 Data Tranformations:

	hRSV	SBD-1	SP-D	SP-A	TTF-1
Control lambs	0	0	0	0	0
hRSV-infected lambs	10.09416	-1.46161	0.691547	0.167954	0.064018

sem	hRSV	SBD-1	SP-D	SP-A	TTF-1
Control lambs	0.004608	-0.51937	0.055431	0.029679	0.00445
hRSV-infected lambs	2.664622	-0.2191	0.128381	0.023485	0.003287

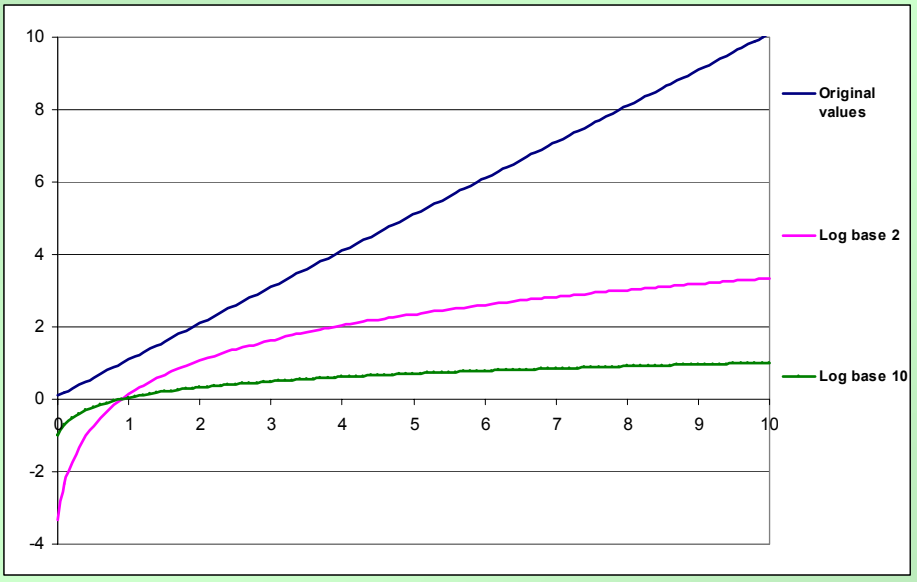
Alicia's 10-lamb study



The advantage of using these logarithmically transformed values is that they theoretically exhibit 'normal distribution', which is a precondition for the calculation of arithmetic mean values and further statistical analysis (e.g., t-test, f-test) …

Control animal target expression levels become "zero" here (as a result of normalising all housekeeper-normalised target values to control animal levels and then log base 2 transfroming those values). The result of this is then that infected animal target levels thus are shown as being above or below "zero"

Log base 2 versus Log base 10 transformed values



Log_2 transformation study file ← click → The "Bonn" Paper

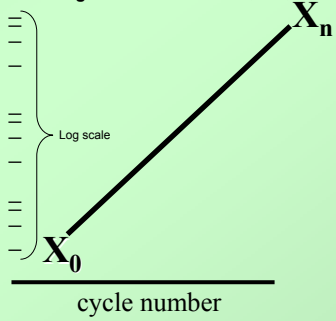
The PCR Equation: a 2^n process

Note here that the expression (1 + E) = "exponential amplification" – which tells you how close the qPCR reaction comes to doubling the template every cycle. If so, the reaction is 100% efficient and has attained the ideal "exponential amplification" value of 2.

$X_0(2)^n$

$X_n = X_0(1 + E)^n$

Amplifications are assessed only during the phase of the reaction when the relationship between detected fluorescence and cycle number is log linear in nature: ideally for 3 cycles too…



X_n = PCR product after cycle n
X_0 = initial template number
E = amplification efficiency
n = cycle number

And, Exponential Amplification = $10^{[-1/slope]}$

Efficiency = $10^{[-1/slope]} - 1$

Ideal slope is always = -3.32192809488 or -1/log(2)

Slope? Slope of what? Answer: Any user-known sample dilution series tested for a qPCR target – also called "standard curves" or "dilution curves" or "calibration curves". So, "slope" = the slope of the line describing log of sample dilution versus Ct. The user knows the dilutions she or he used.

After a long while, I realized that: $\log_{10}(f)/\log_{10}(2) = \Delta Ct_i$

• Where "f" = the sample dilution factor between any successive Cts of any progressive dilution series,

• And, where "ΔCt_i" = the expected ideal number of cycles between any successive Cts of samples differing in concentration by dilution factor "f" (this assumes that the qPCR amplification reaction is 100% efficient; or "ideal").

And, interestingly …

After applying some mathematical rules of logs to the above equation, we are able to further deduce that: $2^{\Delta Ct_i} = f$

So, with these two equations, assuming 100% reaction efficiency, we can calculate any expected qPCR Ct series; i.e. we can predict where each subsequent amplification should cross threshold.

Any deviations from these (ideal) predictions, thus, means that the qPCR amplification reaction at hand is other than 100% efficient … either lower or higher … (higher than 100%? – template or chemical inhibition, lower than 100%? – suboptimal reaction conditions including inappropriate primer, probe or template concentrations)

I find that qPCR Math boils down to a very simple equation from which all else can be derived:

$2^{\lambda} = f$ (in idealty) or $\lambda \log(2) = \log(f)$ (in idealty) or $\lambda = \log(f)/\log(2)$

where: $\lambda = \Delta C_{Ti}$ = the ideal expected frequency of appearance of Cts for any dilution series between or among samples

and f = the known dilution factor of the dilution between or among samples

Do not be afraid to dig through qPCR Math;

It is fairly straight-forward, interesting and enjoyable

Real-time qPCR Math Practice File

Click on this file to explore ideal and non-ideal qPCR mathematical situations

The Future of qPCR Math: The "Swillens Equation" may be able to interpret a single amplification curve like a fingerprint …

The Swillens, et al. paper

In the Swillins Manuscript:
(Nucleic Acids Reasearch, 2004, Vol. 32, No. 6 e53):

The Equation becomes:

$$(\text{copy number})_{initial} = [(\Delta R_n[probe]_{total})/(\Delta R_{n,plateau}E^n)]VN_o$$

(During the log linear phase only; From Baseline to final plateau)

for any single sample

Where: **here, "E" = Exponential Amplification**

ΔR_n = change in fluorescence during the linear log phase only

$\Delta R_{n,plateau}$ = total change in fluorescence from baseline to plateau

E^n = the amplification factor in the exponential phase (E_{AMP})

$[probe]_{total}$ = initially added concentration of fluorogenic probe

V = sample volume

N_o = Avogadro's number (6.0221367×10^{23})

Standard Curve still required here to estimate E reliably, however …

Good reference to read:

Tichopad A, Didier A, Pfaffl MW. Inhibition of real-time RT–PCR quantification due to tissue-specific contaminants Molecular Cellular Probes 18 (2004) 45–50.

~fin~

Thank you