qPCR Quantification Methods

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What is qPCR quantification?

- The method by which the amount of a DNA template initially present can be measured by monitoring qPCR amplification curves

- quantification may be either:

"RELATIVE" - relative to a sample or pool of samples *or*

"ABSOLUTE" - relative to standards of known copy number

Quantification is often confused with the detection strategy

- Detection is the method by which the fluorescence of the accumulating amplicon is generated and detected.

- It is independent of the quantification method

The two fluorescence detection strategies are:

- Sequence-independent DNA binding dyes e.g. SYBR Green, Eva Green, LC Green, SYTO9 etc.

or

- Sequence-specific probes e.g. hydrolysis probes, molecular beacons etc.

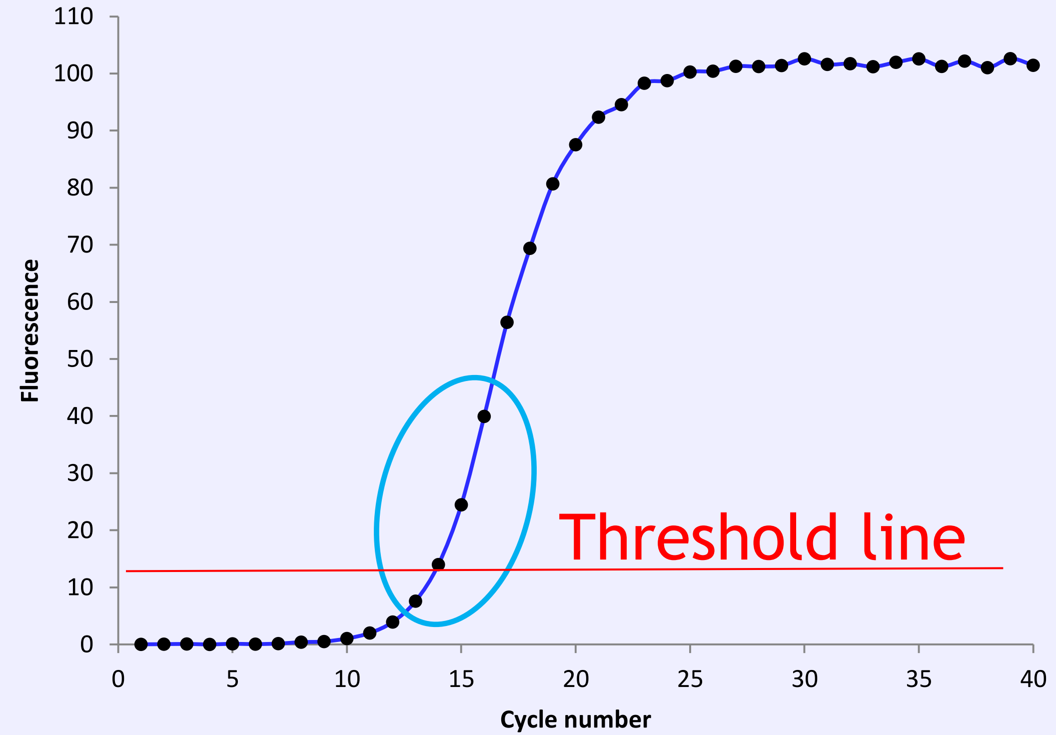
The quantification cycle (Cq)

- Whichever fluorescence detection strategy is selected, DNA binding dyes or probes, a Cq value is derived from the fluorescence data of the amplification curve.

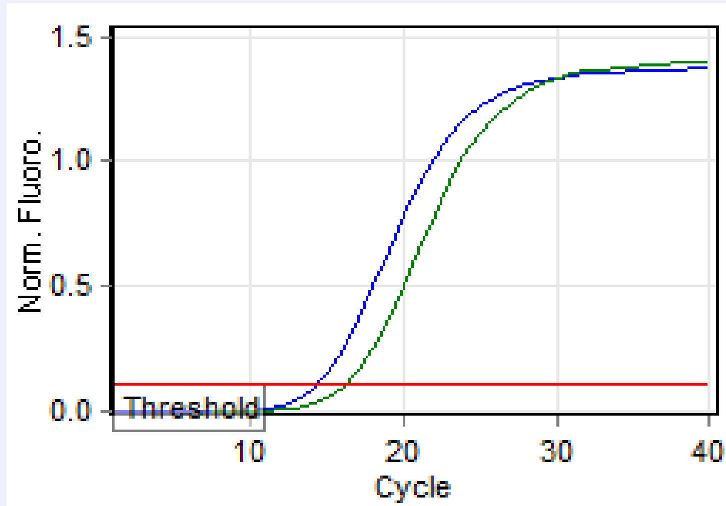
- It is the processing of this Cq value that determines the quantification method.

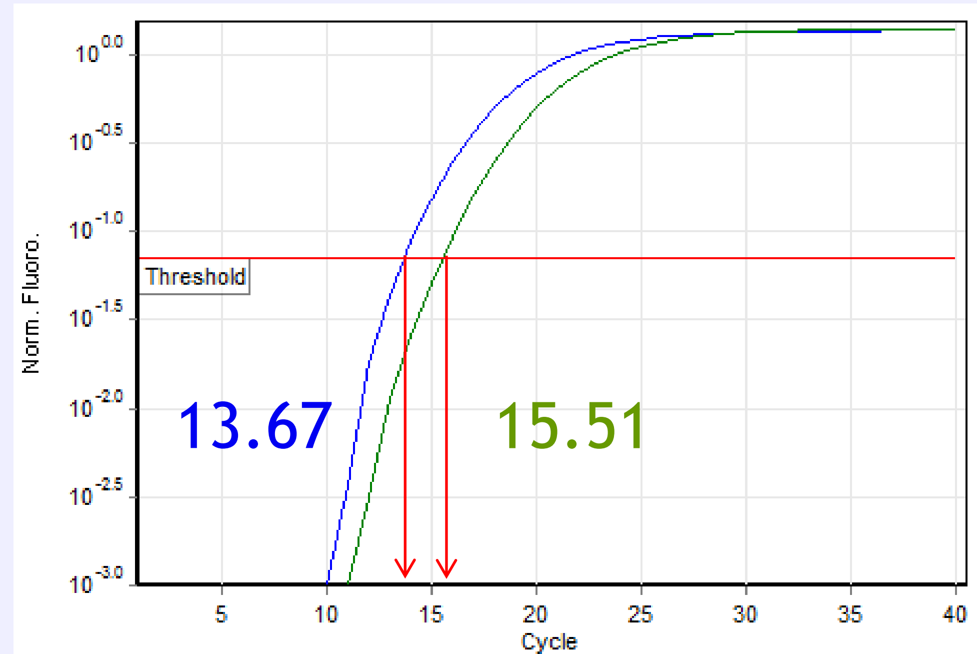
The Cq continued…

- The cycle at which the fluorescence of a sample first increases above baseline fluorescence or crosses a threshold line



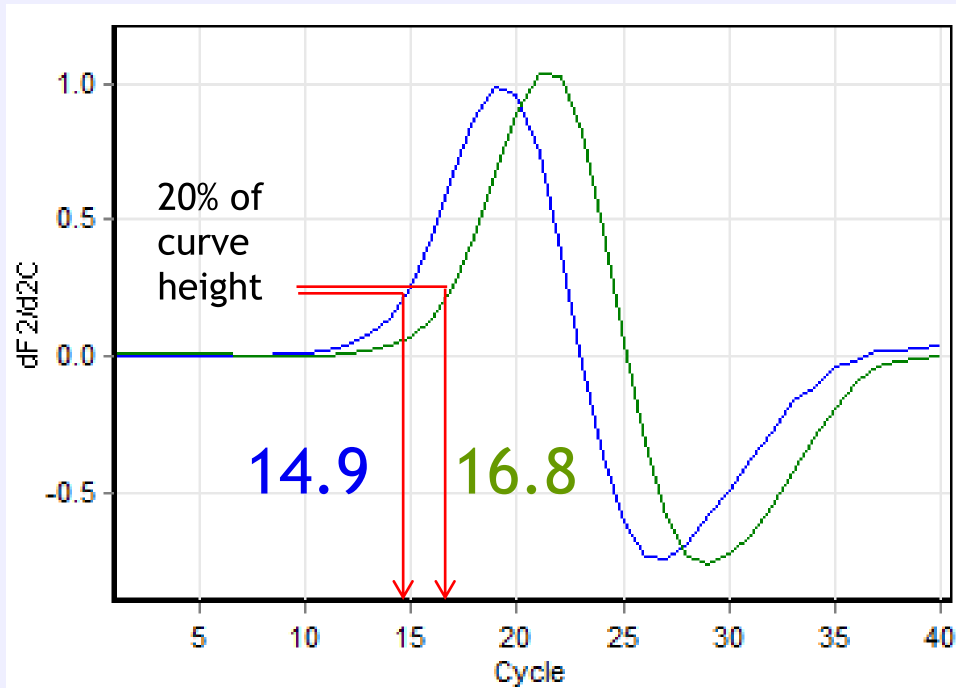
Cq determination - cycle threshold

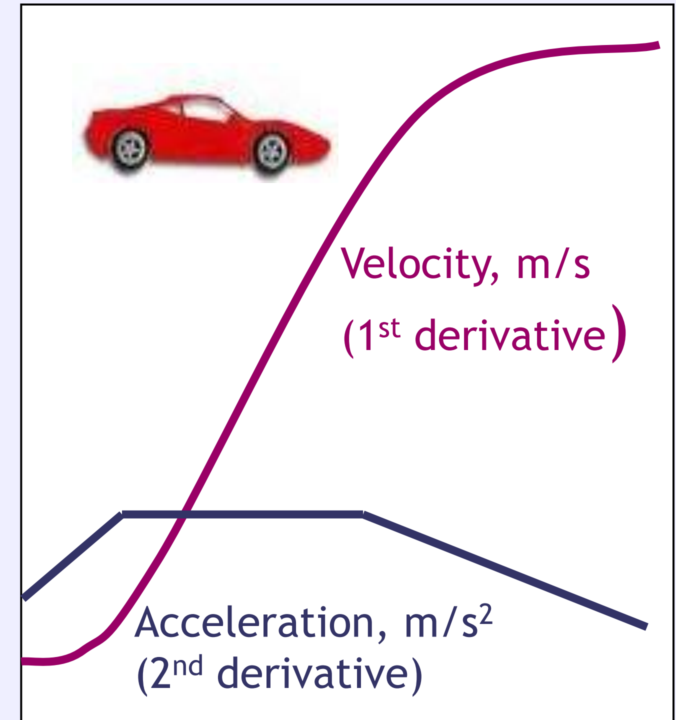




- For a good assay (efficient, linear etc.) placement of the threshold line is not critical as long as it is above baseline and in the log-linear portion of the curve.
- Threshold must be constant for all samples in a run

Cq determination – second derivative maximum





Second derivative maximum method : mathematical transformation of the amplification curve to a second derivative curve and Cq is at 20% of the peak height

Conversion of Cq to quantity

- To be useful as a measure of quantity, the Cq of a sample must be related to either:

  The Cq of another sample (often named the calibrator) = relative quantification

  or

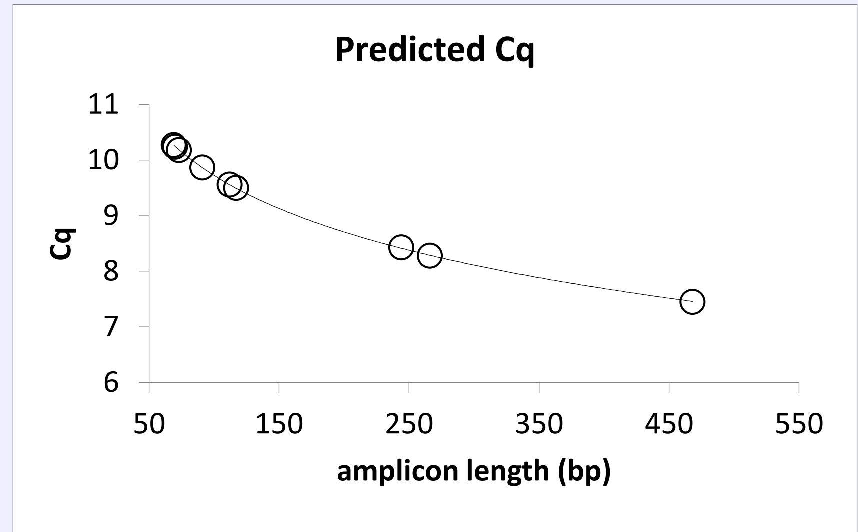
  The Cq of a set of known copy number standards = absolute quantification

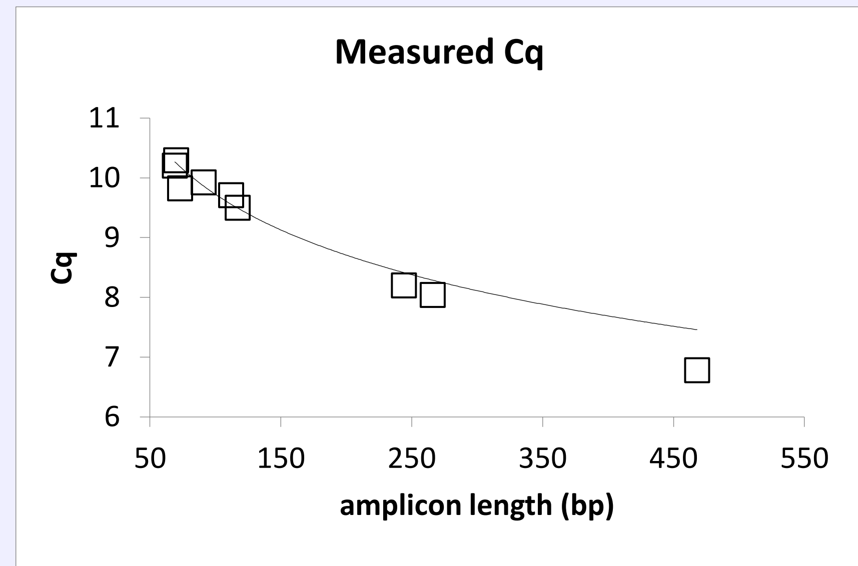
- Cq alone as a measure of quantity is meaningless - why?

For SYBR assays amplicon length affects Cq

Nine qPCR assays, all efficiencies >98%

	Amplicon length
Gene 1	69
Gene 2	70
Gene 3	73
Gene 4	91
Gene 5	112
Gene 6	117
Gene 7	244
Gene 8	266
Gene 9	468





Detection of fluorescence by the hardware

10^7 copies of mmu Actb standard run on RG6000 and LightCycler1.2

Fluorescence

0 10 20 30 40

Cycle

RG is
more sensitive
by ~2-3 cycles

Cycle threshold

Norm. Fluoro.

$10^{0.0}$ $10^{-0.5}$ $10^{-1.0}$ $10^{-1.5}$ $10^{-2.0}$ $10^{-2.5}$ $10^{-3.0}$

5 10 15 20 25 30 35 40

Cycle

Placement of threshold line affects Cq

PCR efficiency is also important

- When PCR efficiency is 100%, the amount of product doubles with each cycle
- But lower efficiency means that less product is made each cycle and so the Cq is delayed

PCR efficiency

Threshold

E=100% lower

lowest

Norm. Fluoro.

$10^{0.0}$

$10^{-0.5}$

$10^{-1.0}$

$10^{-1.5}$

$10^{-2.0}$

$10^{-2.5}$

$10^{-3.0}$

5 10 15 20 25 30 35 40

Cycle

PCR efficiency

- Can be expressed in three ways:

1) As a percentage: 0-100%
2) As a proportion: 0-1
3) As a fold increase: 1-2

Effect of efficiency on copy number

cycle	100%	98%	96%	94%	92%	90%	80%	70%
0	1	1	1	1	1	1	1	1
1	2	2	2	2	2	2	2	2
2	4	4	4	4	4	4	3	3
3	8	8	8	7	7	7	6	5
4	16	15	15	14	14	13	10	8
5	32	30	29	27	26	25	19	14
6	64	60	57	53	50	47	34	24
7	128	119	111	103	96	89	61	41
8	256	236	218	201	185	170	110	70
9	512	468	427	389	355	323	198	119
10	1024	926	837	755	681	613	357	202
11	2048	1834	1640	1465	1307	1165	643	343
12	4096	3631	3214	2842	2510	2213	1157	583
13	8192	7189	6300	5513	4819	4205	2082	990
14	16384	14234	12348	10696	9252	7990	3748	1684
15	32768	28182	24201	20750	17763	15181	6747	2862
16	65536	55801	47435	40256	34105	28844	12144	4866
17	131072	110486	92972	78096	65482	54804	21859	8272
18	262144	218763	182226	151506	125725	104127	39346	14063
19	524288	433150	357162	293923	241392	197842	70824	23907
20	1048576	857638	700038	570210	463473	375900	127482	40642
21	2097152	1698122	1372074	1106207	889868	714209	229468	69092
22	4194304	3362282	2689265	2146041	1708547	1356998	413043	117456
23	8388608	6657319	5270959	4163320	3280411	2578296	743477	199676
24	16777216	13181492	10331080	8076841	6298389	4898763	1338259	339449
25	33554432	26099354	20248916	15669071	12092907	9307650	2408866	577063
26	67108864	51676721	39687876	30397998	23218382	17684534	4335959	981007
27	134217728	102319907	77788237	58972116	44579293	33600615	7804726	1667711
28	268435456	202593416	152464944	114405904	85592242	63841168	14048506	2835109
29	536870912	401134964	298831290	221947454	164337105	121298220	25287311	4819686
30	1073741824	794247228	585709328	430578061	315527242	230466618	45517160	8193466
proportion	**1**	**0.74**	**0.55**	**0.40**	**0.29**	**0.21**	**0.04**	**0.01**

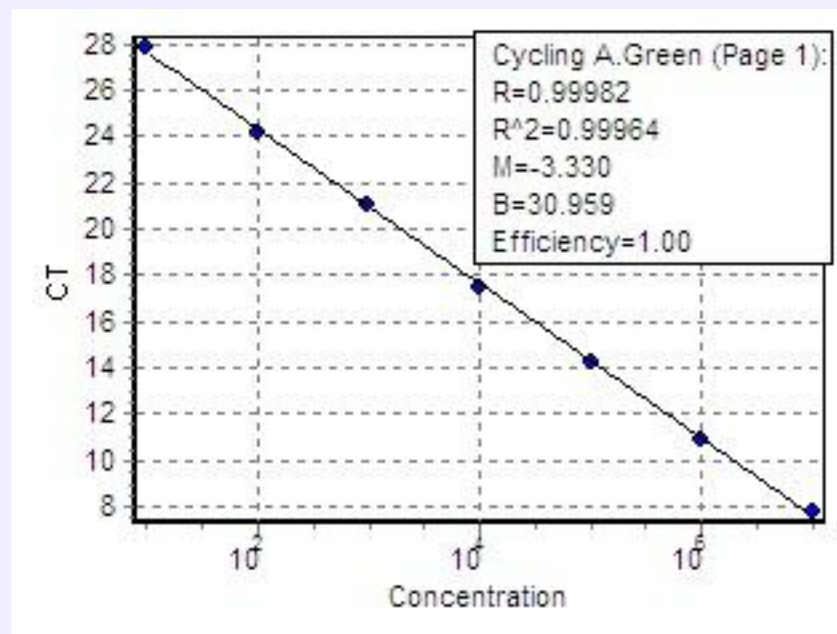
What affects efficiency?

qPCR efficiency can be affected by many factors such as:

- Some primer pairs may be more efficient than others
- Temperature differences between wells
- Presence of PCR inhibitors in a sample
- Inaccurate pipetting e.g. less master mix in one sample than another

Determination of PCR efficiency: copy number standard curve

- Provides an overall assay efficiency
- Useful for checking assay quality
- May not be the same as efficiency in individual cDNA or DNA samples
- Is a direct measure of efficiency



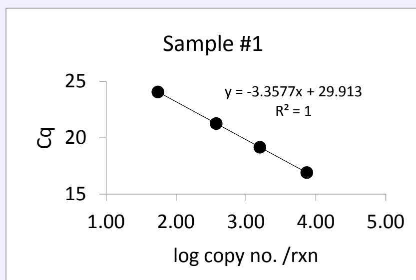
In this example:

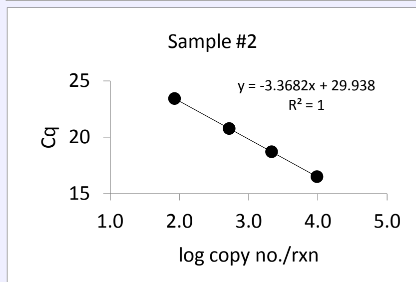
$E = 10^{(-1/slope)}$

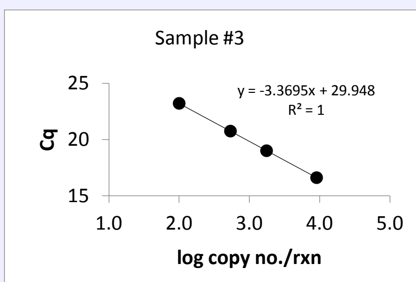
Efficiency $=10^{(-1/-3.330)}$

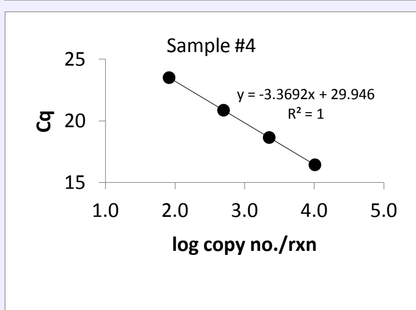
$=1.0$ or 100%

Determination of PCR efficiency: cDNA dilution series









- Four 5-fold dilutions of each cDNA prepared
- Provides individual sample efficiency – direct measure
- Standard curve efficiency was 98%

Slope	Efficiency, $E = 10^{(-1/slope)}$	Efficiency (%)
-3.3577	1.9853	98.53
-3.3682	1.9810	98.10
-3.3695	1.9805	98.05
-3.3509	1.9880	98.80
-3.3682	1.9810	98.10
-3.3637	1.9829	98.29
-3.3692	1.9806	98.06
-3.3658	1.9820	98.20

Determination of PCR efficiency: Indirect methods

- These use mathematical models to determine efficiency from individual amplification curves
- Several methods available including:
  - second derivative maximum (Tichopad/Pfaffl)
  - mid-point slope determinations (Peirson)
  - linear regression using ‘window of linearity’ (Ramakers)

Efficiency can be measured to an accuracy of only a few percent

qPCR efficiency differs from cycle to cycle

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

2.04 (104%)

1.94 (94%)

1.96 (96%)

1.93 (93%)

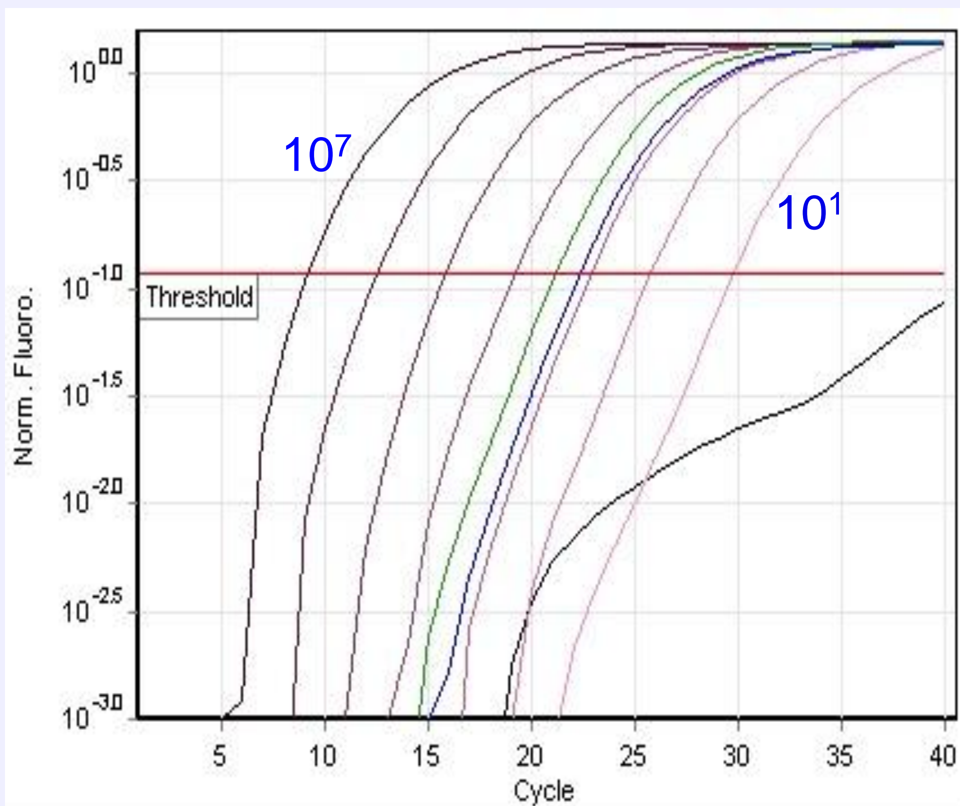
1.84 (84%)

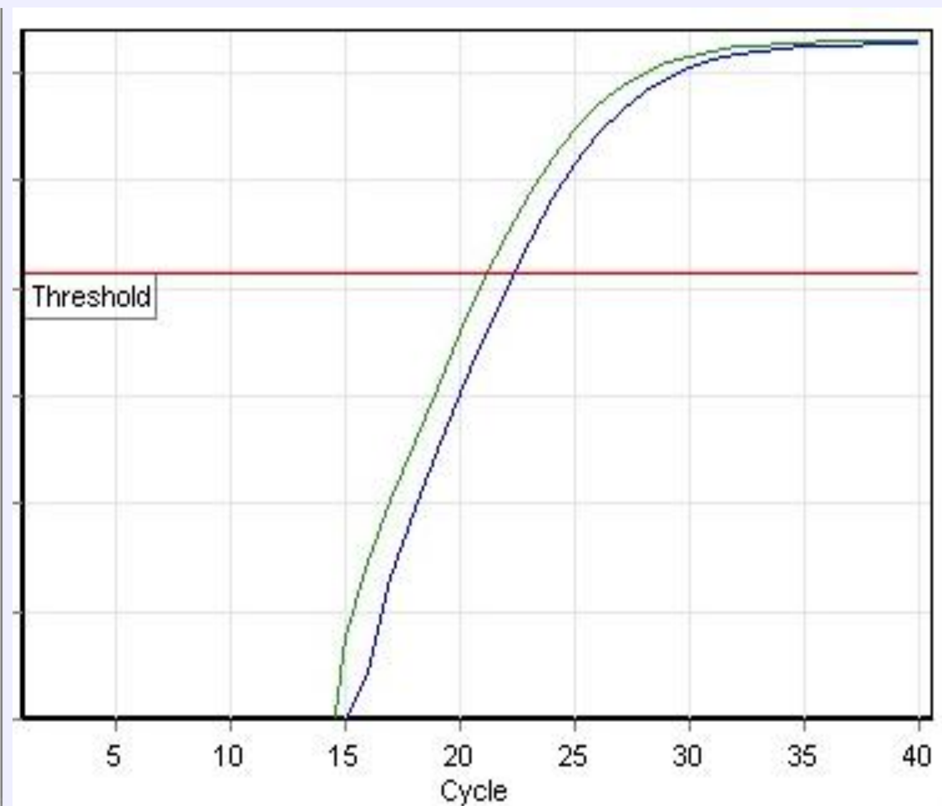
Why worry about efficiency?

- In absolute quantification, the difference in efficiency between GOI and reference genes does not affect results, as long as it is similar between reactions within one assay
- In relative quantification, the efficiency may have a marked effect on quantification where efficiencies differ between the GOI and reference genes

Absolute

Relative





Compares Cq of a sample with those of a series of standards (E is derived from standard curve)

Compares Cq of one sample with that of another (with or without correction for efficiency)

Absolute: typical qPCR output

Sample name	Type	Cq	Given Conc	Calc Conc
CAV1	Standard	10.81	10,000,000	9,619,940
CAV1	Standard	13.99	1,000,000	1,139,850
CAV1	Standard	17.55	100,000	105,341
CAV1	Standard	21.01	10,000	10,331
CAV1	Standard	24.65	1,000	896
CAV1	Standard	28.04	100	93
CAV1	Standard	31.47	10	9
water	NTC	-		0
water	NTC	34.73		1
sample1	Unknown	16.10		278,237
sample2	Unknown	17.13		138,951
sample3	Unknown	17.08		143,474
sample4	Unknown	16.41		230,076

Normalisation for absolute quantification

	Ref 1	Ref 2	Ref 3	Geomean	NormFact
Sample1	1001	9870	722	1925	1925/*1267* = 1.52
Sample2	967	4060	268	1017	1017/*1267* = 0.80
Sample3	522	4211	343	910	910/*1267* = 0.72
Sample4	877	5841	591	1447	1447/*1267* = 1.14
		Grand geomean		*1267*	

GOI copy number /rxn	Normalised copy number/rxn	
278,237	278,237 / 1.52 =	**183,148**
138,951	138,951 / 0.80 =	**173,108**
143,474	143,474 / 0.72 =	**199,744**
230,076	230,076 / 1.14 =	**201,525**

	Before	After
Mean	197,685	189,381
s	68,133	13,645
%CV	34.5	7.2

Relative quantification - $\Delta\Delta C_t$

- The classic relative quantification model, "delta-delta C_t" subtracts the Cq of a sample from that of a calibrator, and 2 is then raised to the power of this value:

$$\text{Normalised Relative Quantity} = \frac{2^{\Delta C_t \text{GOI} \, (calibrator\text{-}sample)}}{2^{\Delta C_t \text{refgene} \, (calibrator\text{-}sample)}}$$

Worked example

$$\text{NRQ} = \frac{2^{26.0-21.0}}{2^{20.0-16.5}} \frac{=}{=} \frac{2^{5}}{2^{3.5}} \frac{=}{=} \frac{32}{11.31} = 2.82$$

- **Assumes efficiency = 100% i.e.=2**

The effect of different assay efficiency on the NRQ of a sample

Relative quantification (Pfaffl method)

- Pfaffl (2001) modified the delta-delta Ct method to include the assay efficiency for each gene. E can be determined from a dilution series of pooled cDNA or by an indirect method

$$\text{Normalised Relative Quantity} = \frac{(E_{GOI})^{\Delta C_T(\text{GOI } \textit{control-sample})}}{(E_{refgene})^{\Delta C_T(\text{refgene } \textit{control-sample})}}$$

Worked example

$$NRQ = \frac{2^{26.0-21.0}}{1.89^{20.0-16.5}} = \frac{2^{5}}{1.89^{3.5}} = \frac{32}{9.28} = 3.44$$

- **BUT can't be used with multiple reference genes**

Relative quantification (qBase method)

- Hellemans (2007) modified the Pfaffl method to permit normalisation with multiple reference genes.

$$\text{Normalised Relative Quantity} = \frac{(E_{GOI})^{\Delta C_T(GOI\ \textit{control-sample})}}{\sqrt{\prod (E_{refgene})^{\Delta C_T(refgene\ \textit{control-sample})}}}$$

- Calculations more complicated – software developed to perform them (qBase^{PLUS})

Fold changes can obscure data

The fold-change between 20,000 and 40,000 copies is two-fold

BUT

the fold-change between 20 copies and 40 copies is also two-fold.

Which is more reliable?

Sampling: abundant target

Sampling: rare target

Absolute versus Relative

Absolute	Relative
Quantity as copies/rxn or fold	Quantity as fold change only

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