Methods for qPCR Analysis

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Date: Wed, 23 Apr 2003
From: "Dr Stephen A Bustin"
To: "Renee Horner"
Subject: Re: UK NA quantification meeting

Fab. Absolute vs relative is a great idea, although you must bear in mind that as conference organiser if I do not agree with any speaker's opinion they will be bundled off to the Tower of London.

S.

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Methods of Analysis

- Absolute quantitation
- Relative quantitation
- Comparative quantitation

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Why absolute quantitation?

- Gives a measure of copy number
- Viral load determination
- FDA filing
- Inter-lab comparisons

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Why is absolute quantitation not currently feasible?

- There is no reliable method for preparing, quantitating and storing RNA standards
- No NIST traceable standards

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Next Best Alternatives?

- Synthetic templates known to come up at a certain Ct value- “semi quantitative PCR”

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Why relative quantitation?

- Does not require that you know the copy numbers for the standard curve
- Can be used to determine fold increases and decreases in gene expression
- There is no need to “over optimize” the efficiencies

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What is needed for relative quantitation?

- Any sample that can be used as a comparison for other samples- “calibrator”
- A serial dilution of the calibrator to give a standard curve in terms of 1x, 2x, 10x, etc

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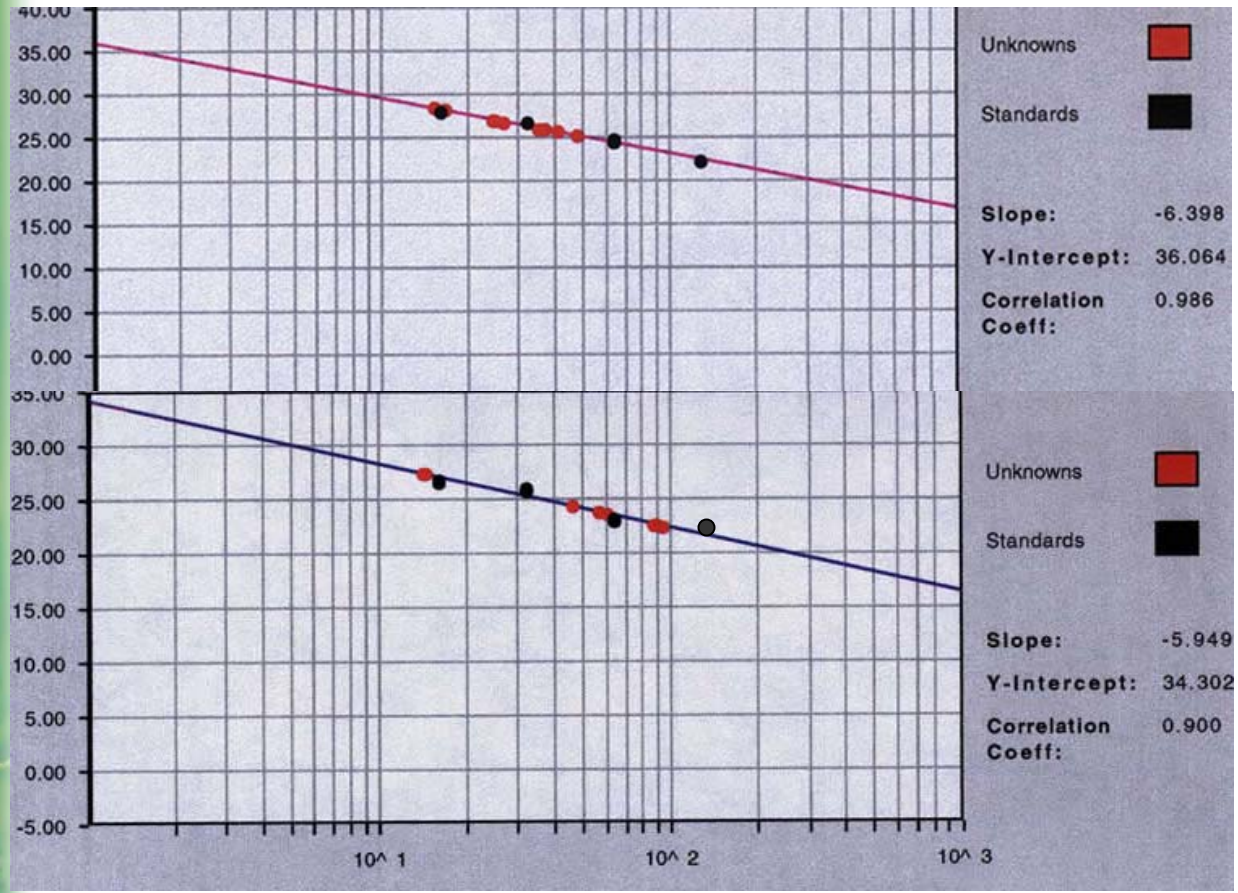
Relative qPCR Data



- GOI
- E= 43%

- Normalizer
- E= 68%

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qPCR Gene Expression Analysis

Sample	GOI	Norm	GOI/Norm	Treated/Untreated
Untreated 1	25.01	45.99	0.54	1.00
Treated 1	16.05	14.26	1.13	2.07
Untreated 2	35.40	89.10	0.40	1.00
Treated 2	42.75	57.72	0.74	1.86

- In both animals, the GOI is expressed twice as much as in the treated areas as the untreated areas. This data verifies the array data.

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Why comparative quantitation?

- Mathematical determination of relative quantities
- No standard curve needed
- Higher throughput
- Best used when particular ratios are expected or are verifying a “trend”

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What is needed for comparative quantitation?

- Calibrator sample used as a 1x standard
- Samples that are prepared identically
- Ideally, if normalizing the results, your GOI and the normalizer will have the same efficiency

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Comparative Quantitation

$$Ct_{GOI} - Ct_{norm} = \Delta Ct$$

$$\Delta Ct_{Sample} - \Delta Ct_{Calibrator} = \Delta\Delta Ct$$

$$\text{Relative quantity} = 2^{-\Delta\Delta Ct}$$

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Genotyping Experimental Rationale

Sample Type	Genome Equivalents GOI	Genome Equivalents norm	Normalized Equivalents
Homozygous	2	2	1.0
Heterozygous	1	2	0.5
Null	0	2	0.0

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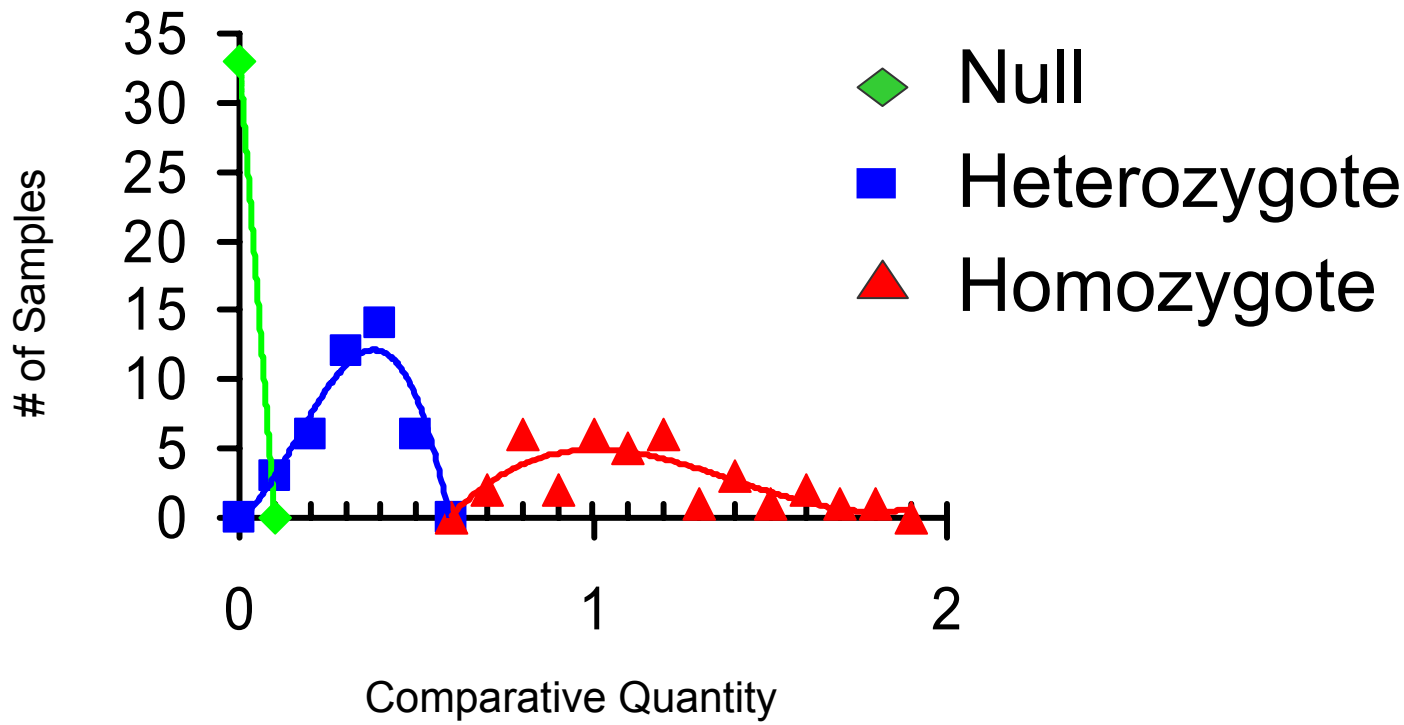
qPCR Genotype Analysis

Well	Dye	Replicate	Ct	
E1	FAM	b	22.26	wt Calibrator
F1	FAM	b	22.29	
E1	HEX	b	26.05	
F1	HEX	b	26.03	
A3	FAM	c	40	Sample MC305
A4	FAM	c	40	
A3	HEX	c	24.84	
A4	HEX	c	24.17	
A7	FAM	s	19.52	Sample AS103
A8	FAM	s	19.1	
A7	HEX	s	23.92	
A8	HEX	s	22.33	
H11	FAM	zp	40	Sample TH600
H12	FAM	zp	40	
H11	HEX	zp	24.88	
H12	HEX	zp	26.04	

	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	Genotype
wt Calibrator	-3.77	0.00	1.0	wt
Sample AS103	-3.82	-0.05	1.0	hm
Sample TH600	-2.33	1.44	0.4	ht
Sample MC305	15.50	19.26	0.0	null

Distribution of Genotype Results



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Comparative quantitation

$$Ct_{\text{Sample}} - Ct_{\text{Calibrator}} = \Delta Ct$$

$$\text{Relative quantity} = 2^{-\Delta Ct}$$

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Gene Expression Results

	Ave Ct	dct	2^-dct	1.8^-dct
WT	26.87	0	1.00	1.00
TG 1	28.45	1.58	0.33	0.40
TG 2	29.32	2.45	0.18	0.24
TG 3	27.25	0.38	0.77	0.80
TG 4	28.36	1.49	0.36	0.42

The expression of Gene X is repressed in the transgenic mouse lines relative to wild type mice

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From:"Rudy Spangler"
To:"Renee Horner"
Subject:_ comparative measures
Date: Wed, 21 May 2003

Renee
attached is a slide that i use to describe how i analyze data the example has only 4 samples so it will fit on a slide, 2 controls and 2 experimentals the geometric Ct values are transformed to arithmetic emissions values by 1/2^CT this number for me is multiplied by 10^7 ... because then actin (Ct about 16) is equal to 100 then i transform the emission values to logs for logs, ratios are created by subtracting rather than division every value is transformed to a ratio with respect to the average of the 4 samples this removes the differences in the absolute emission from gene to gene the averages of the ratios for all the genes in each sample are determined and used as a "normalizer“ alternatively, the averages of the ratios of selected genes can be used as a normalizer
rudy

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- the geometric Ct values are transformed to arithmetic emissions values by 1/2^CT multiplied by 10^7
- transform the emission values to logs for logs, ratios are created by subtracting rather than division
- every value is transformed to a ratio with respect to the average
- the averages of the ratios for all the genes in each sample are determined and used as a "normalizer“
- alternatively, the averages of the ratios of selected genes can be used as a normalizer

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	g01	g02	g03	g04	g05	g06	
C1							
C2							
S1							
S2							

Emission = 2^-Ct x 10^7

	g01	g02	g03	g04	g05	g06	
C1							
C2							
S1							
S2							

Log of Emission Value

	Avg g01	**Avg g02**	**Avg g03**	**Avg g04**	**Avg g05**	**Avg g06**	

	g01	g02	g03	g04	g05	g06	
C1							**Avg C1**
C2							**Avg C2**
S1							**Avg S1**
S2							**Avg S2**
							Grand Avg

Log of Emission Value - gene Avg

	g01	g02	g03	g04	g05	g06	
C1							
C2							
S1							
S2							

(Log of Emission Value - gene Avg) - Sample Avg - Grand Avg

Conclusions

- Absolute quantitation
  - Standard curve
  - Standards must be accurately quantitated
  - Best used for viral load determination
- Relative quantitation
  - Standard curve
  - Standards are serial dilutions of a calibrator template
  - Best used for gene expression studies
- Comparative quantitation
  - Mathematical determination
  - Calibrator sample used as a 1x standard
  - Best used when particular ratios are expected or to verify trends

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