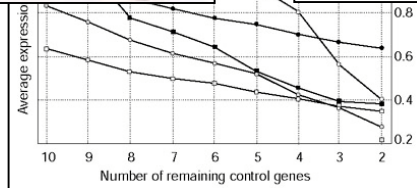
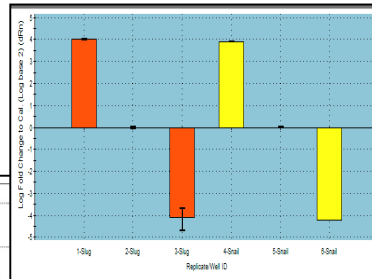


Integrated Sciences QPCR meeting 2007

Inter-sample normalisation in QPCR experiments



Fabrice Magnino, PhD

QPCR Specialist

Integrated Sciences

fabrice@qpcr.com.au



The most comprehensive QPCR solution



Normalisation is not only GAPDH or β -actin

β -Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels

E M Glare, M Divjak

Thorax 2002;57:765

Decreased β -actin mRNA expression in hyperglycemic focal cerebral ischemia in the rat

J. Dairy Sci. 86:3423–3429

© American Dairy Science Association, 2003.

The Housekeeping Genes GAPDH and Cyclophilin Are Regulated by Metabolic State in the Liver of Dairy Cows¹

zell^b, Luc Vachon^c, Roger Butterw

Peptidylprolyl Isomerase A (PPIA) as a Preferred Internal Control Over GAPDH and β -Actin in Quantitative RNA Analyses

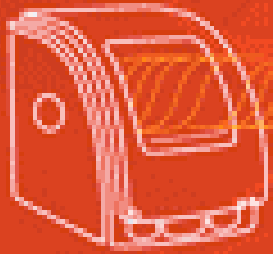
BioTechniques 32:776-782 (April 2002)

Differential expression of GAPDH and β -actin in growing collateral arteries

Elisabeth Deindl¹ ✉, Kerstin Boengler¹, Niels van Royen¹ and Wolfgang Schaper¹

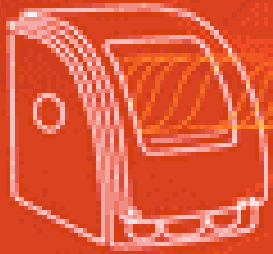
**Integrated
Sciences**

The most comprehensive qPCR solution



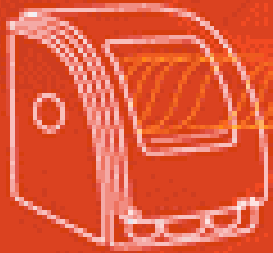
Overview/ Aims

- Why normalise?
- Overview of the different approaches
 - Applicable contexts
 - Pros
 - Cons
- New alternative



Why normalise?

- Overview of sources of variation
 - 1) Technical error
 - User, pipettes, instrument, sample quality, inhibitors, RT efficiency, QPCR efficiency
 - 2) Biological – covered by biological reps
 - **3) Effect of studied factor - what you want!**



Normalisation of Gene Quantitation

- Ideally normalise for

RNA Prep



Sample size error
Sample quality
RNA quality/quantity

RT Step



RNA quant error
Pipetting error
RT efficiency

QPCR Step



Pipetting error
QPCR inhibition



How can we achieve this?

Endogenous controls

Housekeeping genes:

GAPDH

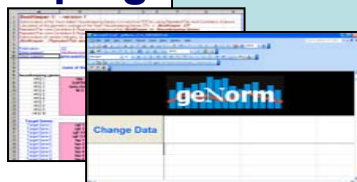
18S

β -Actin

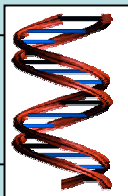
Cyclophilin

1 HKG gene

Multiple HKGs



Genomic DNA

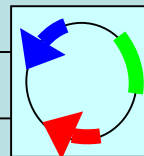


Exogenous controls

Alien RNA control

cDNA control

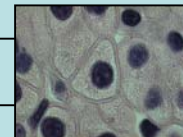
Plasmid control



Sampling size

Total RNA

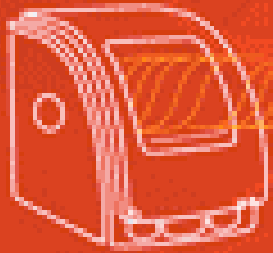
Cell number



Sample volume

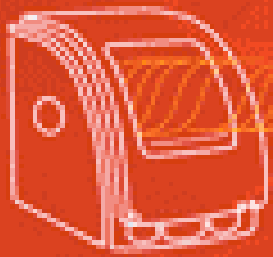
Tissue mass





Strategies for normalisation

Endogenous controls



Endogenous Reference Genes

Sample extraction

Π

RNA quant

Π

Pipetting

Π

RT

(Π)

QPCR

Π



Endogenous Reference genes

- **Pros:**

Considers all sources of technical error:

- RNA prep
- RNA quant
- RT step
- QPCR step

- **Cons:**

- Requires RNA quant
- Requires assay for each Norm
- Requires stable genes
- Requires validation
- Assumes equal RT for GOI and norm



Endogenous Genomic DNA

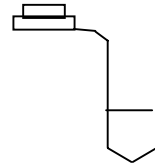
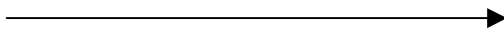
- Principle: Normalise to genomic equivalents
- When: if [RNA] not possible, eg LCM or embryo samples



Genomic DNA Work flow

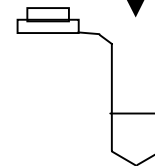


RNA & DNA extraction

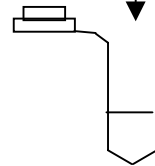


RNA + DNA
prep

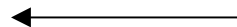
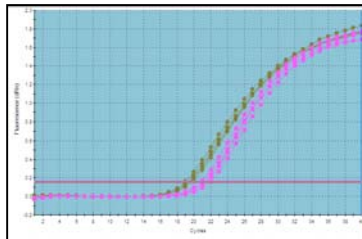
Eg: **SideStep Lysis
and Stabilisation
buffer**



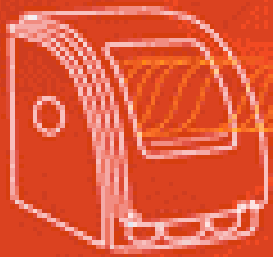
RT PCR



QPCR – assays for gDNA
and mRNA targets



Normalisation of target
Ct to gDNA Ct



Endogenous-gDNA Variation captured

Sample extraction

Π

RNA quant

X

Pipetting

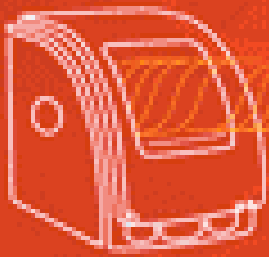
Π

RT

X

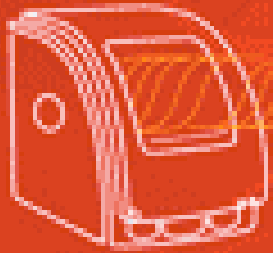
QPCR

Π



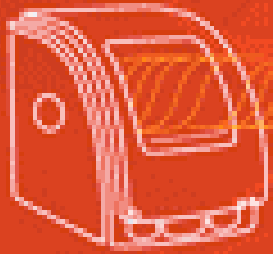
Genomic DNA Note

- Extraction method must recover RNA and gDNA, **eg: SideStep Lysis & Stabilization buffer**
- Exon-spanning primers essential
- Designed to a stable copy number region (SSLB 2nd generation)
- RT variability not captured (Need reproducible RT)



Strategies for normalisation

Exogenous controls



Strategies for normalisation

Exogenous controls

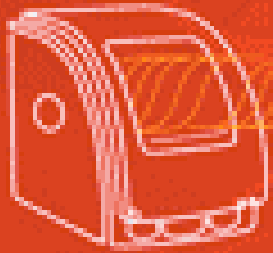
- Principle: “Spiked-in” sequences at known concⁿ.
 - RNA or DNA templates
- When: No stable normaliser genes



Exogenous controls

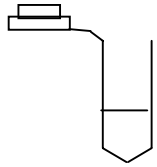
Variation captured

Spike in...	RNA	DNA
Sample extraction	X	X
RNA quant	X	X
Pipetting	(II)	(II)
RT	(II)	X
QPCR	II	II

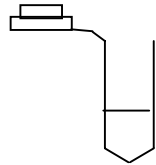


Exogenous controls RNA spikes

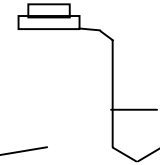
**Sample RNA
extraction and
quantification**



RT rxn

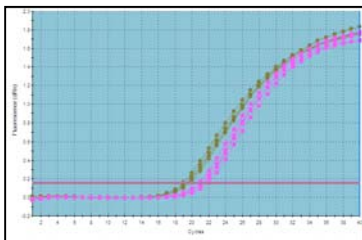


**Alien RNA
preparation**

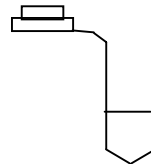


**Eg: Alien QRT-
PCR Inhibitor
Alert**

**Normalisation of target
Ct to spike Ct**



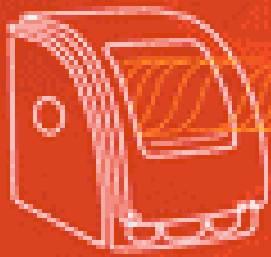
QPCR rxn





Strategies for normalisation

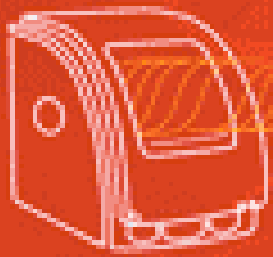
Normalisation using sampling size



Strategies for normalisation

Sampling size

- Eg: number of cells, volume or mass of tissue.
- Many assumptions:
 - Cellularity
 - Sample extraction uniformity
 - RNA quality consistency
 - RT efficiencies
 - QPCR efficiencies
 - Pipetting error not captured



Sampling size Variation captured

Sample extraction

X

RNA quant

X

Pipetting

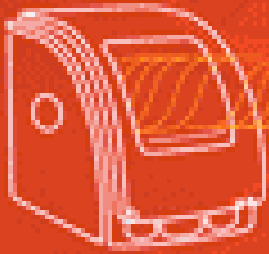
X

RT

X

QPCR

X



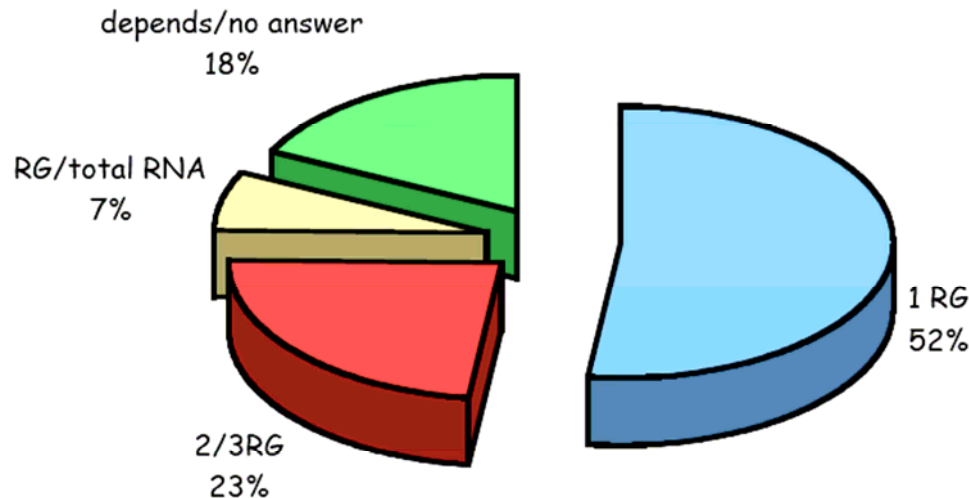
Strategies for normalisation

Total RNA

- Improvement on sampling size
- Does not capture variation post-RNA extraction step
- Requires accurate RNA quantitation
- Total RNA may not reflect mRNA fraction (1 – 5% of total RNA)



What's the most common choice?



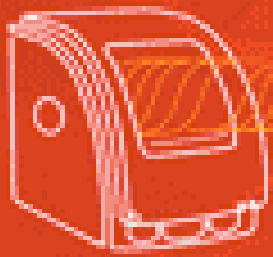
Data from survey at 3rd London qPCR Symposium / EMBO 2005 qPCR course



What was new in 2006?

Expressed Alu Repeat (EAR) Normalisation

Jo Vandesompele and colleagues,
Center for Medical Genetics
Ghent University Hospital, Belgium



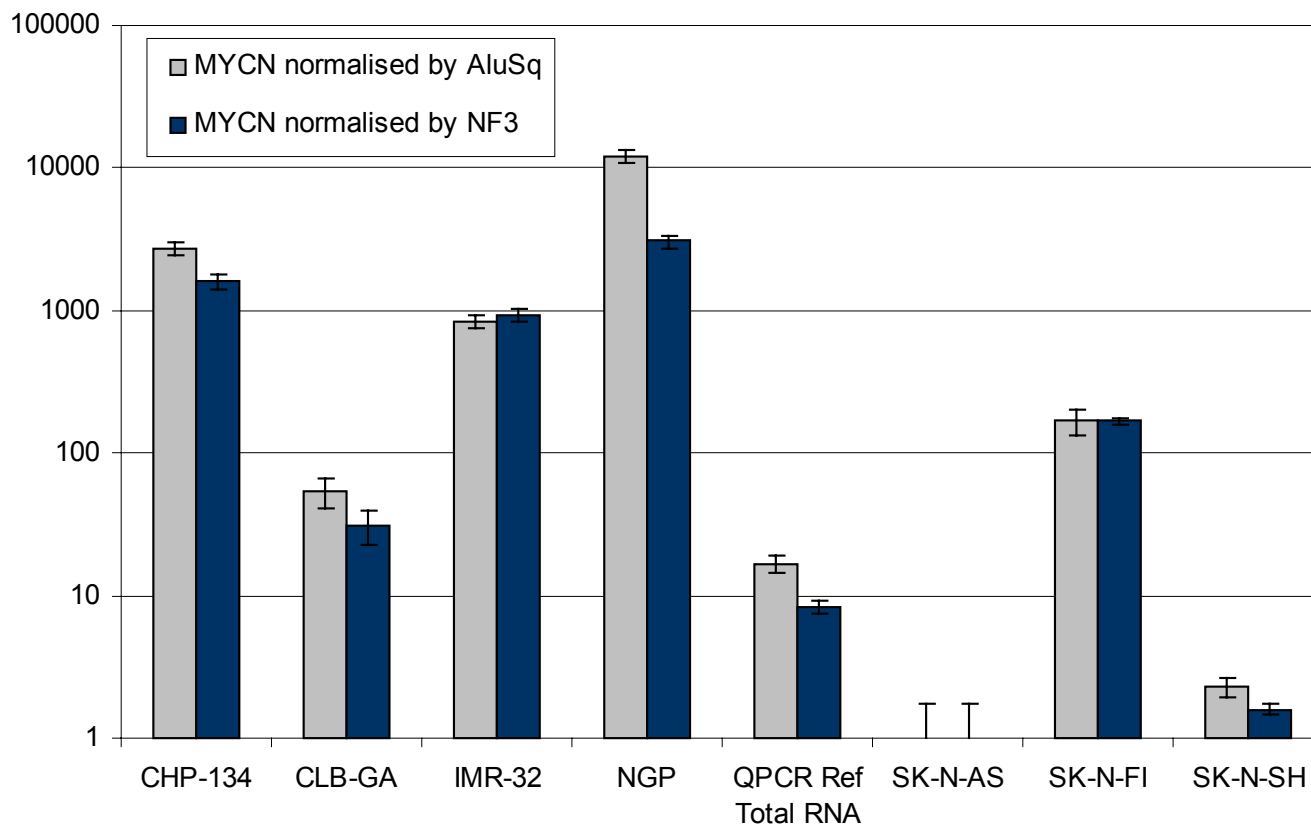
EAR Normalisation Principle

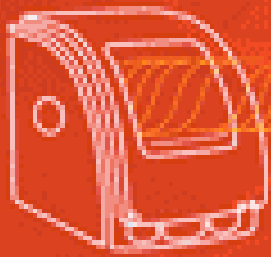
- Alu repeats:
 - Abundant in mammalian genome
 - 1 million copies, 10% of human genome
 - ~ 1,500 HSA genes contain an Alu repeat

 - 31 conserved subfamilies
 - ~280 bp long



EAR Normalisation Comparison to NF



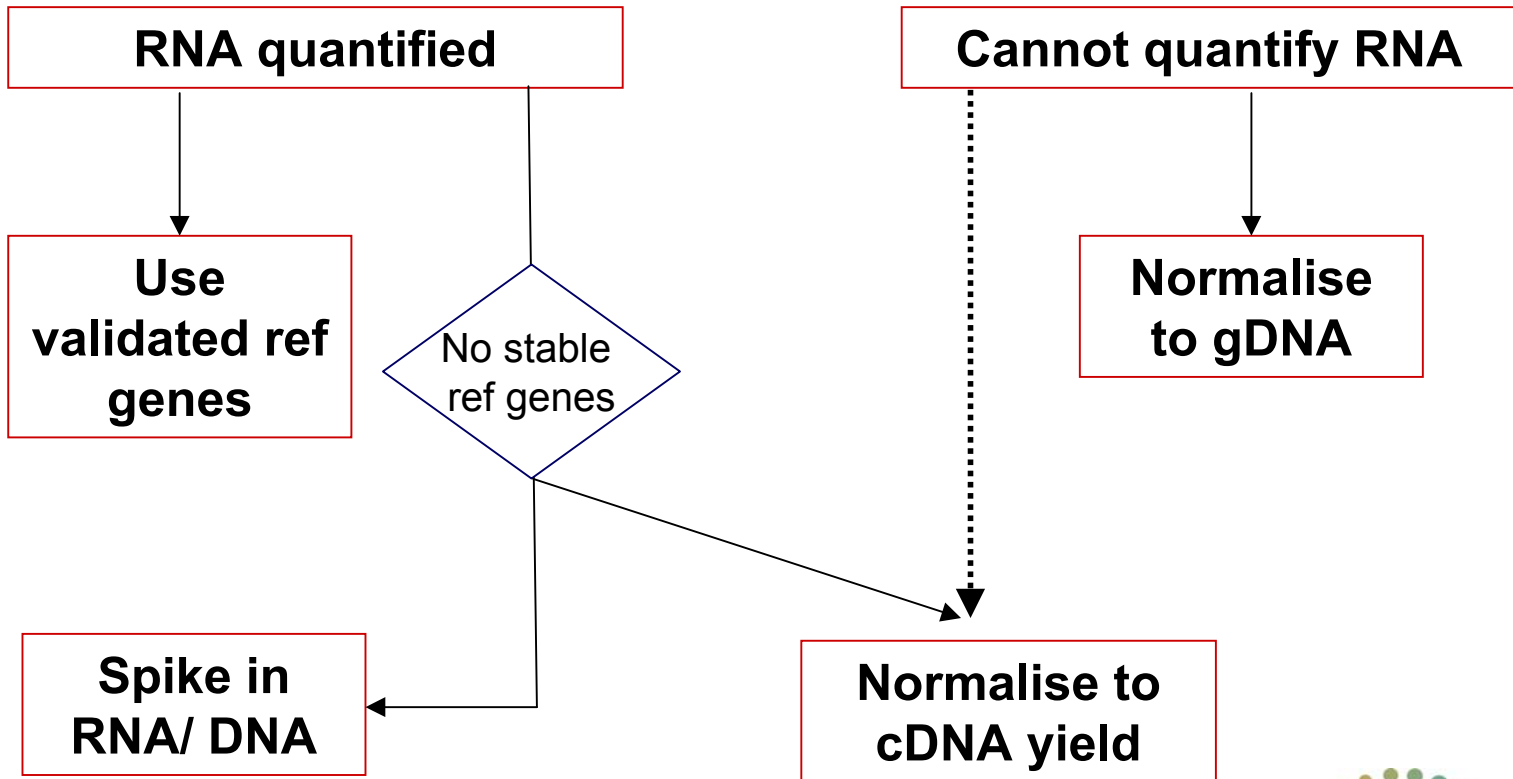


EAR Normalisation Summary

- Preliminary data were promising
- Although since then the data has not been published
- It remains a good idea but DNA is a pb



Choosing your method





cDNA Yield Normalisation

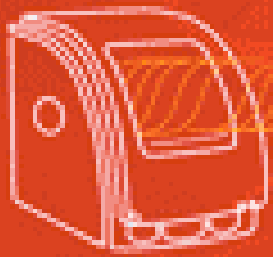
Quantification of cDNA generated by reverse transcription of total RNA provides a simple alternative tool for quantitative RT-PCR normalization

Jiří Libus and Helena Štorchová

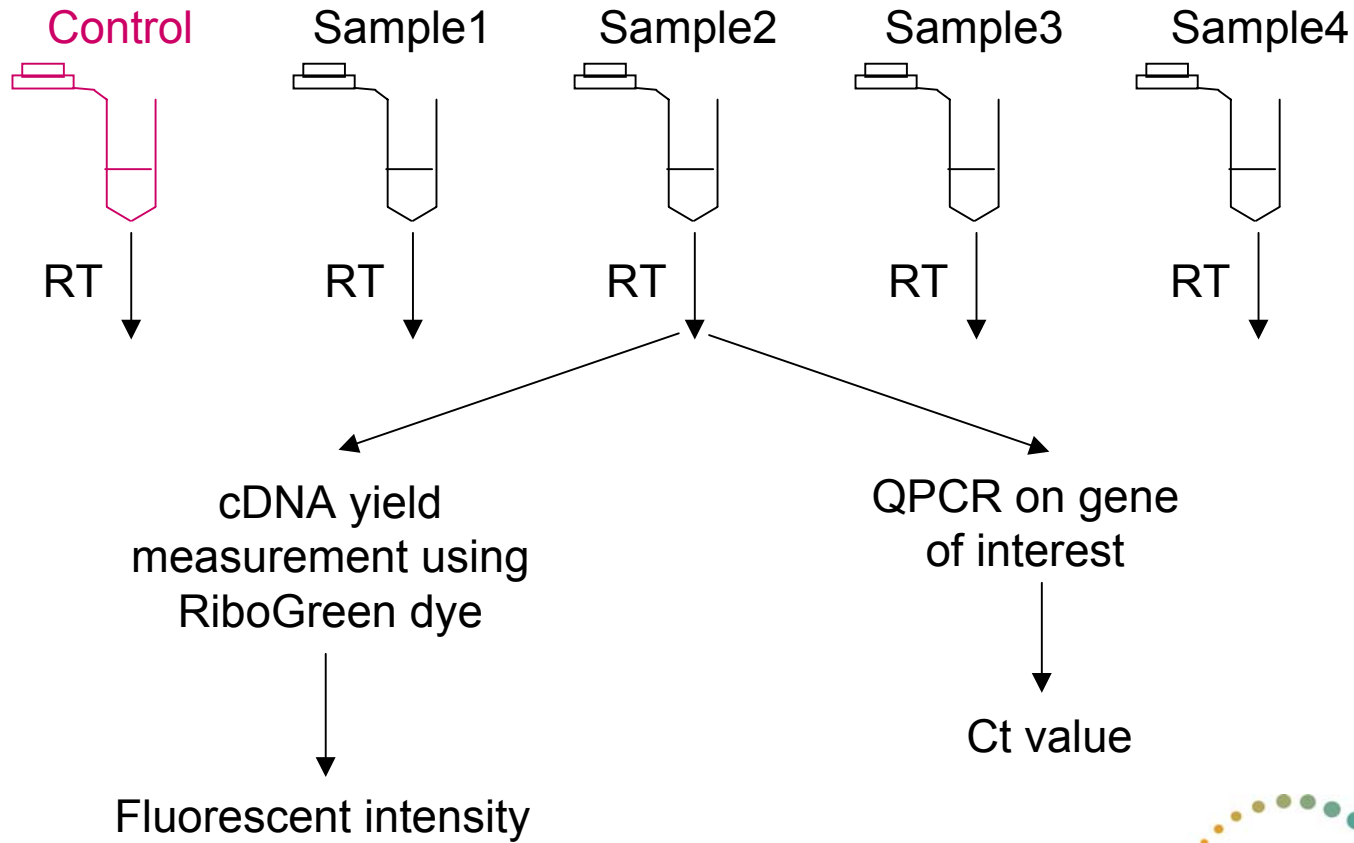
Institute of Experimental Botany, Prague, Czech Republic

BioTechniques 41:156-164 (August 2006)

doi 10.2144/000112232



cDNA Yield Normalisation Principle





cDNA Yield Normalisation Principle

$$FC = \frac{(1 + \text{Eff}_{\text{GOI}})^{-(Ct_{\text{sample}} - Ct_{\text{control}})}}{(1 + \text{Eff}_{\text{NG}})^{-(Ct_{\text{sample}} - Ct_{\text{control}})}}$$

$$FC = \frac{(1 + \text{Eff}_{\text{GOI}})^{-(Ct_{\text{sample}} - Ct_{\text{control}})}}{\left(\frac{\text{Fluo}_{\text{sample}}}{\text{Fluo}_{\text{control}}} \right)}$$

Numerical example

	Fluorescence intensity	Fluo sample/Control	Ct values GOI	FC GOI w/o correct.	FC GOI /correct. Factor
Control	6050	1.000	23.5	1.00	1.00
Sample 1	5010	0.828	21.3	4.59	5.55
Sample 2	2560	0.423	21.5	4.00	9.45
Sample 3	10180	1.683	23	1.41	0.84
Sample 4	6130	1.013	19.5	16.00	15.79

$$=(1 + \text{Eff})^{-(Ct_{\text{sample}} - Ct_{\text{control}})}$$



cDNA Yield Normalisation Pro & Cons

- **Pros:**

Considered sources of technical error:

- RNA prep

- RNA quant

- RT step

- QPCR step (can't assess PCR inhibition)

Does not require:

- RNA quant

- assay for each Norm

- stable genes

- **Cons:**

Does not assess mRNA population variation in total RNA when using random primers

Require extra steps in sample preparation. Could be an issue for large sample batch



cDNA Yield Normalisation

Other application

Reference gene validation

Actual validation usually requires multiple normalisation genes to be tested in order to find the most stable one. This is usually done using statistical software like Bestkeeper and GeNorm.

Using the cDNA yield assay, it is possible to validate your reference gene, at least to get an idea of its stability and how precise your quantification can be. This could be a nice feature for publications.



Thanks you!

QPCR technical support:

Web: www.qpcr.com.au

Email: qpcr@integratedsci.com.au

Tel: **1800 252 204**



The most comprehensive QPCR solution