Advanced quantitative real-time PCR in clinical diagnostics and cDNA microarray validation

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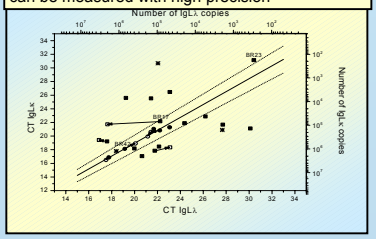
In situ calibration for clinical diagnostics and cDNA microarray validation

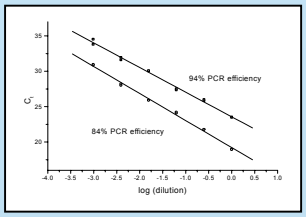
Introduction

Expression of genes reflects both the genetic predisposition and physiologic condition of the individual. From gene expression measurements it should be possile to diagnose indiviuals' state of health and also to monitor how individuals respond to medication, treatment and altered living condition. The development of powerful techniques to analyze nucleic acids opens new possibilities for gene expression studies. The expression of virtually all genes in a sample can be roughly assessed using microarrays and by real-time PCR the expression of selected genes can be measured with high precision



Lymphoma diagnostics

Healthy individuals have a kappa/lambda expression ratio of 60:40 (within the dotted lines) while individuals with B cell lymphoma have an altered ratio (outside the dotted lines). Arrows indicate more accurate classification by *in situ* calibration.



In situ calibration for two different biological samples

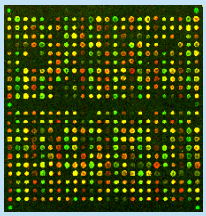
Lymphoma diagnostics

Here we demonstrate the first clinical application based on the LightUp probe. It is a three step real-time PCR assay for the detection of B-cell non Hodgkin lymphoma. Each B-lymphocyte decides early in its development which of two versions, kappa or lambda, immunoglobulin light chain to produce. This results in a kappa:lambda gene expression ratio of about 60:40 in healthy individuals. Lymphomas, like all malignant tumors, are clonal and arise from one transformed cell. Tumor cells dominate in lymphoma tissues which results in an altered kappa:lambda expression ratio.

In situ calibration

We have developed an *in situ* calibration method based on either addition of known amount of target DNA or dilution of the test sample to determine sample specific PCR efficiencies. *In situ* calibration improves accuracy and is particular suitable when a small number of samples shall be analyzed in greater detail, for example: cDNA microarray validation and relative gene expression in clinical samples.

The correlation factor was 0.99 when comparing quantitative gene expression data measured by cDNA microarray and real-time PCR



cDNA validation

The genetic profile of a mouse model for pancreatic tumors, where the neural cell adhesion molecule (NCAM) was deleted is compared with the normal tumor model by cDNA microarray. Twenty genes involved in different biological processes and with different expression levels were validated with quantitative real-time PCR

Reverse transcription studies using real-time PCR

	Hexamer	Oligo	Comb	Specific	No Prim
B-tub	19.49	18.09	19.07	18.80	27.57
CaV1D	26.53	28.79	27.94	28.73	33.74
Gapdh	15.82	16.63	16.16	16.37	23.59
Ins2	16.89	15.91	16.64	17.43	23.10
Glut2	27.46	28.35	29.28	31.81	32.61

Hexamer; random hexamer, Oligo; Oligo(dT$_{12-18}$), Comb; Five gene specific primers combined, Specific; Gene specific primer, No Prim; No primer

RT efficiencies with different priming strategies

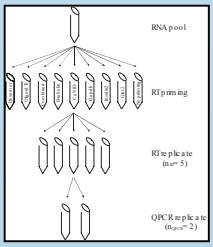
Low Ct value corresponds to high RT efficiency (best priming strategy is underlined for each gene). Largest difference in RT efficiency was found for Glut2 and is about $2^{31.81-27.46}$=20 fold. This indicates that RT efficiency is highly dependent on priming strategy.

	β-actin (Ct)	HTR1a (Ct)
MMLVH	20.6	27.8
Omniscript	22.6	28.4
AMV	*	27.7
MMLV	20.9	27.4
Improm	21.0	27.9
cAMV	20.9	27.5
Termoscript	22.9	27.7
Superscript	20.6	28.6

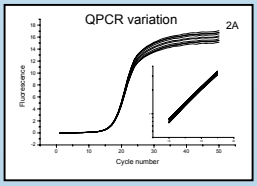
* No product

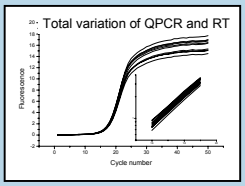
RT efficiency with different enzymes

RT efficiency is enzyme and gene target dependent. A difference of one in Ct corresponds to 100% difference in RT efficiency



Experimental set-up to analyze reproducibility and efficiency of RT. Same pool of RNA was used in all experiments. The following priming strategies were tested; random hexamers, oligo(dT$_{12-18}$), gene specific primer, combination of five gene specific primers and no primers.





QPCR and RT variation	QPCR	Hexamer	Oligo	Comb.	Specific
Btub	0.007	0.16	0.14	0.28	0.11
CaV1D	0.115	0.24	0.14	0.44	0.24
Gapdh	0.024	0.13	0.12	0.47	0.11
Ins2	0.016	0.05	0.11	0.05	0.06
Glut2	0.360	0.70	0.60	0.49	0.23

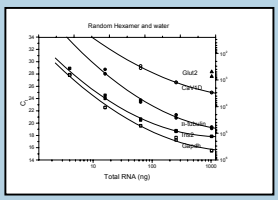
All values are expressed as standard deviation of Ct

Hexamer; random hexamer, Oligo; Oligo(dT$_{12-18}$), Comb; five gene specific primers combined, Specific; gene specific primer

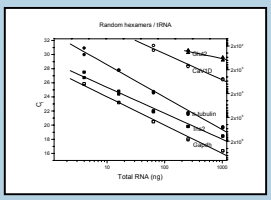
RT reproducibility

The variation in RT (SD_{RT}) is higher than that in QPCR(SD_{QPCR}). From our data, using the equation below, we conclude that the error in the estimation of mRNA expression is mainly due to uncertainty in the RT step and any replicates should therefore be made at the RT level. Our data suggest that expression of a specific mRNA can be quantified with less than 50% error if the RT reaction is run as duplicate and the QPCR as a single reaction.

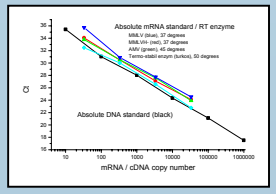
$$e = t\left(\frac{SD_{RT}^{\,2}}{n_{RT}} + \frac{SD_{QPCR}^{\,2}}{n_{QPCR} * n_{RT}}\right)^{\frac{1}{2}}$$



Dynamic range of RT using different amounts of mRNA



Dynamic range of RT at different amounts of mRNA at constant amount of total RNA.



Absolute quantification

By spiking with both RNA and DNA standards, the cDNA recovery can be determined for different RT enzymes. Using well defined RNA and DNA standards it is possible to compare gene expression levels between different tissues.

Conclusions

- In situ calibration is efficient to account for PCR inhibition in biological samples.
- Real-time PCR and cDNA microarray expression data correlate well.
- RT efficiency depends on target gene, priming strategy and choice of enzyme.
- Real-time PCR reproducibility is substantially higher than RT reproducibility.
- The dynamic range of RT is higher at constant total RNA concentration.
- Absolute quantification can be improved by spiking with RNA and DNA standards.