A Quantitative RT-PCR-based Approach to Assess cDNA Quality for Comparative Gene Expression Analysis

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Background and Objective

Quantitative RT-PCR (qRT-PCR) is a widely used technique to detect differential gene expression in toxicological and patho-physiological studies. High quality cDNA synthesis, which is essential for reliable and reproducible qRT-PCR data, depends on:

- RNA integrity,
- Reverse transcriptase efficiency,
- Priming strategy

The cDNA quality can be assessed by measuring the 3'/5' expression ratio of a house-keeping gene, where values closer to 1 indicate equal representation.

Most genes in mammals produce several transcripts by "alternative splicing". Measurement of these alternatively spliced transcripts may be more sensitive to cDNA quality, as non-homogenous representation across mRNA span may lead to measurement biases.

The homogeneity of cDNA synthesis depends upon:

1. Priming strategy - Oligo-dT, hexamer or a mixture of Oligo-dT+Hexamer.

2. Reverse transcriptase efficiency - which varies depending on sources and manufacturing processes.

Although cDNA quality and homogeneity will contribute to the reliability of gene expression analysis, it is not generally assessed due to a lack of easily adaptable quality control measures.

Rat is the predominant model in toxicological studies, and qRT-PCR method has been used to assess toxicant-induced perturbation of gene expression.

The objective of this study is to develop an easily adaptable SYBR1-dye based qRT-PCR method for cDNA quality assessment in the rat model in order to further improve the reproducibility and reliability of toxicogenomic studies.

Materials and Methods

A. Gene selection criteria and primer design:

- Ubiquitously expressed across species and tissues.
- Well-characterized structure and sequence information.
- Multiple exons, but no alternative splicing.

Some house-keeping genes meet all these criteria and are suitable for mRNA/cDNA quality assessment.

Two sets of PCR primers per gene – one towards the 5' end, and another towards the 3' end – were designed along with two sets of internal primers for several house keeping genes using Primer3 on-line program (http://frodo.wi.mit.edu/). The *Pgk1* gene is one of the house-keeping genes that proved useful.

Table 1: Primer sets for *Pgk1* gene in rat

Primer name	Accession	Primer sequence	Amplicon size (bp)	Distance 3'-end	Exon Location
Pgk1_5F	NM_053291	TCGTGATGAGGGTGGACTT	109	1517 nt	1-2
Pgk1_5R		GCTCCATTGTCCAAGCAGA			3
Pgk1_E4-5F	NM_053291	TGGGAACAAGGTTAAAGCTGA	107	1167 nt	4-5
Pgk1_E4-5R		CTCTGTGTGCAGTCCCAAAA			5
Pgk1_E6-7F	NM_053291	GAAGGCTGGTGGATTTTTGA	105	1028 nt	6
Pgk1_E6-7R		GCAACTTTAGCTCCTCCCAAG			6-7
Pgk1_3F	NM_053291	TGGGGTATTTGAATGGGAAG	107	555 nt	9
Pgk1_3R		TGTCTCCGCCTCCTATGATAGT			9-10

B. RNA samples, cDNA synthesis and qRT-PCR analysis:

RNA was isolated from a C6 rat cell line by RNeasy kit (Qiagen). RNAs from various rat, mouse and man tissues were purchased from Zyagen and Clontech.

RNA quantity and quality were assessed using Nanodrop spectrophotometer and Agilent Bioanalyzer, respectively. The qRT-PCR assays were performed using SYBR1 dye in a BioRad iQ5 real-time PCR machine.

The expression of 3' and 5'-end amplicons were normalized using two internal sets of primers for the same gene presenting similar efficiencies and annealing temperatures (Fig. 1).

The cDNA quality was assessed by measuring the normalized expression ratios of 3':5'-end amplicons.

C. Impact of heat treatment on cDNA integrity:

Total RNAs were subjected to heat treatment at 90ºC for 5 min, 10 min, 20 min and 30 min.

The RNA quality was judged by Bioanalyzer analysis and a RIN value for each group was obtained.

The 3':5' ratio was compared for different heat treatment groups.

Results and Discussion

A. Gene primer specificity and efficiency testing:

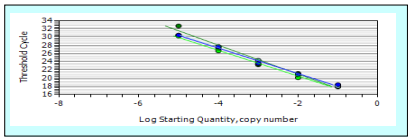

Figure 1: PCR efficiencies of four primer sets for rat *Pgk1* gene.

B. Impact of RNA quality on the cDNA 3'/5' ratio:

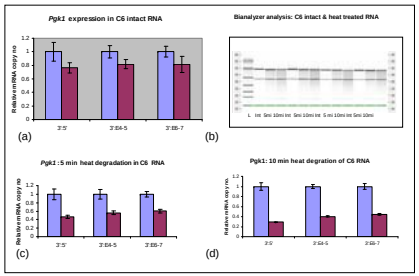

Figure 2: Impact of heat treatment on cDNA integrity.

Table 2: cDNA integrity assay using Superscript III and Oligo-dT.

Samples	RIN	Amplicons (see Fig 3)	Normalized expression values of 3' & 5' amplicons (t-test p-value)	3':5' ratio (High-lighted)
C6-intact	10	3' & 5'	1.296 & 0.988 (0.1)	1.31
C6-intact	10	3' & E4-5	1.141 & 0.932 (0.11)	1.22
C6-intact	10	3' & E6-7	1.050 & 0.8556 (0.17)	1.23
C6-Heat-degraded 5min	8.2	3' & 5'	1.011 & 0.472 (0.000219)	2.14
C6-Heat-degraded 5min	8.2	3' & E4-5	0.954 & 0.534 (0.00065)	1.78
C6-Heat-degraded 5min	8.2	3' & E6-7	1.057 & 0.635 (0.00177)	1.67
C6-Heat-degraded10min	6.9	3' & 5'	0.818 & 0.238 (0.0000198)	3.44
C6-Heat-degraded10min	6.9	3' & E4-5	0.904 & 0.364 (0.000020)	2.49
C6-Heat-degraded10min	6.9	3' & E6-7	0.763 & 0.340 (0.000198)	2.25
C6-Heat-degraded 20min	3.7	3' & 5'	0.860 & 0.0838 (0.00065)	10.3
C6-Heat-degraded 30min	2.9	3' & 5'	1.578 & 0.0196 (0.0037)	80.6
C6-Heat-degraded 30min	2.9	3' & E4-5	1.662 & 0.0534 (0.00919)	31.1
C6-Heat-degraded 30min	2.9	3' & E6-7	1.822 & 0.1022 (0.0277)	17.8

Acknowledgements

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C. Impact of Reverse Transcriptase and priming strategy on cDNA quality:

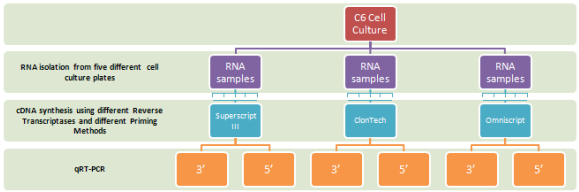

Figure 3: Experimental design for cDNA integrity assay using different Reverse Transcriptase enzymes.

Table 3: Impact of Reverse Transcriptase and Priming Strategy on 3'/5' ratio using C6 cell line (RIN – 10)

Reverse Transcriptase	Priming Strategy	3'/5'	p-value
SuperscriptIII	Oligo-dT	1.31	0.1
	Hexamer	0.63	0.1
	Oligo-dT+Hexamer	1.2	0.2
Omniscript	Oligo-dT	1.82	0.003
	Hexamer	1.25	0.3
	Oligo-dT+Hexamer	2.1	0.0001
ClonTech	Oligo-dT	1.88	0.001
	Hexamer	0.8	0.02
	Oligo-dT+Hexamer	1.69	0.002

The 3'/5' ratio of the amplicons at both ends of *Pgk1* cDNA varies depending upon the cDNA synthesis protocol.

3':5' gene expression ratio depends on the distance between the primer location. The larger the distance between the locations of amplicons, the higher the ratio.

Heat treatment studies revealed that as the RNA quality (RIN value) decreases, the 3':5' ratio of cDNA increases (Fig. 2 and Table 2).

The homogeneity of cDNA synthesis depends on priming strategy and Reverse Transcriptase source.

In general, the oligo-dT+ Hexamer priming method resulted in 3':5' ratios that are closer to 1, while Oligo-dT priming method shows a 3'-end representation bias, and random hexamer priming method shows a 5'-end representation bias (Table 3).

Conclusions and Further Studies

We have developed an easily adaptable method for assessing cDNA integrity that can be applied across various rat strains and can be adopted to other mammalian species.

This method is useful to check the quality of cDNA prepared from RNA or tissue samples preserved for years at -80ºC.

Based on our data, we suggest that for quantification of alternative splice variants, Oligo-dT+ random hexamer priming strategy would result in more homogenous representation of cDNA molecules.

Our preliminary study suggests that the cDNA samples to be used for differential expression analysis (such as toxicant treated vs control), should present similar 3':5' ratio for obtaining a reliable data.

Since different Reverse transcriptase enzymes and priming strategies used for cDNA synthesis produce different 3':5' amplicon ratios, we are working on the development of an approach to harmonize cDNA quality control assessment.