Gene Ther Mol Biol Vol 12, 15-24, 2008

Validation of the comparative quantification method of real-time PCR analysis and a cautionary tale of housekeeping gene selection

Research Article

Richard D. McCurdy[1,#,*], John J. McGrath[2,3], Alan Mackay-Sim[1]

[1]Eskitis Institute for Cell and Molecular Therapies, and School of Biomedical and Biomolecular Science, Griffith University, Brisbane, QLD 4111, Australia

[2]Queensland Centre for Mental Health Research, The Park Centre for Mental Health, Wacol QLD 4076, Australia

[3]Department of Psychiatry, University of Queensland, St Lucia QLD 4072, Australia

---

***Correspondence**: Dr. Richard D. McCurdy, Sick Kids Research Institute, Program in Genetics and Genomic Biology, TMDT Building, East Tower, 15th Floor, 101 College St., Toronto, Ontario CANADA M5G 1L7; Tel: 1-416-813-7654 X 8164; Fax: 1-416-813-4931; e-mail: richard.mccurdy@sickkids.ca

#Present Address: Sick Kids Research Institute, Genetics and Genomic Biology, TMDT Building, 101 College St., 15th Floor, East Tower, Toronto, ON M5G 1L7, Canada

Key words: Relative quantification, housekeeping gene, reference gene, SYBR® Green I, linear regression, second derivative maximum, schizophrenia, Rotor Gene

Abbreviations: beta-2-microglobulin, (β2M); Comparative Quantification, (CQ); glyceraldehyde-3-phosphate dehydrogenase, (GAPDH); optical density reading, (OD); Real-time reverse transcription PCR, (RT-PCR); Relative Quantity, (RQ); Take Off Point, (TOP); housekeeping gene (HKG)

Received: 6 March 2008; Revised: 19 March 2008
Accepted: 25 March 2008; electronically published: June 2008

Summary

Real-time reverse transcription PCR (RT-PCR) is now widely used for quantifying levels of expressed gene transcripts. The present study validates the use of a new RT-PCR analysis method, Comparative Quantification, by comparing it against the 'gold standard' Comparative Threshold Cycle method. The former method calculates individual PCR reaction efficiencies, obviating the need for multiple PCRs to generate standard curves from serial dilutions of sample. Real-time reverse transcription PCR was used to verify expression of 18 genes suggested by microarray analysis of schizophrenia versus control fibroblasts. A high correlation (R=0.853) was observed between the two methods, validating Comparative Quantification as a method of RT-PCR data analysis, with the advantage that it is also a quicker and cheaper method. Also, RT-PCR compares the relative expression of target genes to the expression of a reference or "housekeeping" gene in the sample, which is assumed to have stable expression across all samples. Variable expression of the reference gene would reveal itself as a false change in expression in the target gene. The present study investigates the expression of "housekeeping" genes in fibroblast cultures from patients with schizophrenia and matched healthy controls. The results reveal consistent patient versus control differences in expression of commonly used "housekeeping" genes, including GAPDH. We propose that researchers derive housekeeping genes from stable expression data in the system studied and disregard previously published housekeeping genes when designing their real-time PCR experiments.

I. Introduction

Real-time reverse transcription PCR (RT-PCR) is a widely used, semi-automated method for deriving quantitative estimates of the expression of a gene of interest (i.e. levels of a specific mRNA) compared to a reference gene. In RT-PCR, a fluorescent dye binds to the double-stranded DNA as it is formed, providing continuous monitoring of amplified cDNA levels over the course of the PCR (Higuchi et al, 1993). Currently, the most accepted method for analysing RT-PCR data is the comparative threshold cycle method (Pfaffl, 2001; Pfaffl et al, 2002), which requires determining a fractional cycle number (the crossing point; Cp, also called the threshold cycle; Ct) (Rasmussen, 2001) during the exponential phase of DNA amplification to generate a value for the amount of gene transcript. One drawback of this method is that the

efficiency of the PCR reaction must be calculated for each gene of interest by generating a separate standard curve for each. This greatly increases the time and cost required for RT-PCR verification of multiple genes from microarray expression screening.

Recently a new method was proposed by the manufacturers of the Rotor Gene, a real time PCR thermal cycler (Rotor Gene 5.0 software, Corbett Research, Australia). This method of RT-PCR analysis, "Comparative Quantification", calculates the efficiencies of each gene for each individual PCR reaction and is based on the second differential maximum method (Rasmussen, 2001) to calculate single reaction efficiencies. The comparative quantification method does not require any extra RT-PCR reactions to calculate PCR efficiencies is cheaper, less time consuming and uses fewer reagents compared to the more commonly used comparative threshold cycle method (Pfaffl, 2001; Pfaffl et al, 2002). The major aim of this study was to directly compare these two methods of RT-PCR analysis and assess their concordance using the same cDNA samples.

PCR-based methods of quantifying gene transcription levels depend on the comparison of expression of another gene in the same sample against which the expression of the target or gene of interest is normalised. These reference genes are usually genes thought to be expressed equally in all cells and tissues and are often known as "housekeeping" genes giving the impression that they have the same function in all cells under all conditions. The most commonly used housekeeping gene is GAPDH (glyceraldehyde-3-phosphate dehydrogenase). During a microarray study of gene expression in fibroblasts derived from participants with schizophrenia and from healthy controls we noted differences in the expression of GAPDH between participant groups (unpublished observations). Similar findings were recently reported by an independent group in a microarray study of post-mortem prefrontal cortex from schizophrenia patients and healthy controls (Prabakaran et al, 2004). Furthermore, a review of GAPDH expression variability concluded its continued use as an internal reference was "a mystery" (Bustin, 2000; Rajeevan et al, 2001; Rasmussen, 2001).

Differential expression of reference genes across groups can introduce false differences, or obscure true differences in the expression of the gene(s) being studied. The expression level of reference genes can be modulated by several factors including: experimental treatment (Zhong and Simons, 1999; Schmittgen and Zakrajsek, 2000), pathology (Bhatia et al, 1994; Lupberger et al, 2002), individuality (de Leeuw et al, 1989) and even i*n vitro* cell culturing (Hamalainen et al, 2001). Therefore, validation of endogenous housekeeping genes must be carefully undertaken to ensure they exhibit stable expression across all individual samples and groups in the experimental system under investigation. A second aim of this study was to identify potential housekeeping genes with stable expression for use in comparing gene expression among control samples and those from persons with schizophrenia.

II. Materials and Methods

A. Human tissue collection, cell culture, total RNA extraction and reverse transcription

Seventeen participants (9 with schizophrenia, 8 healthy controls) were recruited from consumer groups and through a research participant register maintained by the Queensland Centre for Mental Health Research. Written informed consent was obtained from all participants and the study was approved by the West Moreton Hospital Ethics Committee. Skin biopsies were collected and skin fibroblast cultures generated as per Mahadik and colleagues in 1991. Fibroblasts were harvested and cryogenically stored in liquid nitrogen. When needed, fibroblasts were thawed and cultured in DMEM, supplemented with 10 % foetal bovine serum, penicillin and streptomycin (1 %; GibcoBRL) plus 0.2 % Fungizone™ (amphotericin B, GibcoBRL) at 37°C under 5% CO_2, using 500 cm^2 Nunclon™ Triple flasks (Nunc). Cultures were grown simultaneously until 80 % confluency was reached. There were no group differences in the time taken to reach 80 % confluency. Cultures were harvested by washing 3 times in Hank's Buffered Saline Solution (HBSS, GibcoBRL) followed by a five-minute incubation at 37 °C in 0.025 % trypsin (GibcoBRL). Trypsin was inactivated by suspending the cells in 50 ml of serum-containing media, pelleting the cells by centrifugation, aspiration of supernatant and resuspension in 50 ml HBSS (GibcoBRL) followed by another centrifugation. The supernatant was removed and cells were prepared for RNA extraction.

Cells were homogenized and total RNA extracted using an RNeasy® kit (Qiagen; Clifton Hill, Australia) with an on-column RNase-Free DNase Set (Qiagen) as per the manufacturer's instructions. Purity of RNA was determined by an optical density reading (OD) $OD_{260/280}$ ratio greater than 1.75 and by 2% agarose gel electrophoresis. Integrity of the 18S and 28S ribosomal RNA bands was visually verified by ethidium bromide staining. Five micrograms RNA was reverse-transcribed by using a SuperScript III RNase H$^-$ Reverse Transcriptase Kit (300 U; Invitrogen) with 0.5 U oligo (dT_{12-18}) primers (Invitrogen) for 90 min. at 50 °C. Resultant cDNA was treated with 1U RNase H (Invitrogen) for 30 min. at 37 °C.

B. Selection of reference genes for comparison

Candidate reference genes were selected from those represented on the microarray. These included four of the most widely used housekeeping genes, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ; NM_003406), beta-2-microglobulin (β2M; NM_004048), H3 histone, family 3A (H3F3A; NM_002107), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_002046); and three 'newly' suggested housekeeping genes not commonly or previously used (Warrington et al, 2000; Hsiao et al, 2001; Lee et al, 2002), dullard (Xenopus laevis) homolog (DULLARD; NM_015343), non-POU domain containing, octamer-binding (NONO; NM_007363), DnaJ (Hsp40) homolog, subfamily B member 1 (DNAJB1; NM_006145). Normalized microarray expression data (not shown) demonstrated that two of the four widely used housekeeping genes had significantly different group expression levels (2-tailed t-test, $p< 0.05$; see **Table 1**) supporting the contention that stable expression of housekeeping genes must be verified in each experimental system. However, with the exception of GAPDH, the standard deviation and variance of the normalized expression data was small (under 0.5, less than 0.3; **Table 1**). Normalized microarray expression values of the three 'new' housekeeping genes, were not significantly different

between patient and control groups and exhibited small standard deviation, and low variability (under 0.2; less than 0.03; range 0.027-0.004; **Table 1**) across all individuals from both groups. The expression levels of these genes as shown by microarray analysis of cultured skin fibroblasts from schizophrenia patients and healthy controls are displayed in **Figure 1**.

Table 1. Normalized microarray expression data for housekeeping genes

	Gene ID	Standard Deviation	Variance	P-Value
"New" HKGs	DULLARD	0.0630	0.0040	0.1585
	DNAJB1	0.1349	0.0182	0.0649
	NONO	0.1648	0.0272	0.4313
Common HKGs	H3F3A	0.2466	0.0608	0.2755
	YWHAZ	0.2844	0.0809	0.0072
	B2M	0.4816	0.2320	0.0534
	GAPDH	2.7588	7.6112	0.0034

Standard deviation and variance was calculated using all samples across groups. A two tailed t-test determined any significant difference in the expression level of housekeeping genes between schizophrenia patients and healthy controls. HKGs = housekeeping genes.

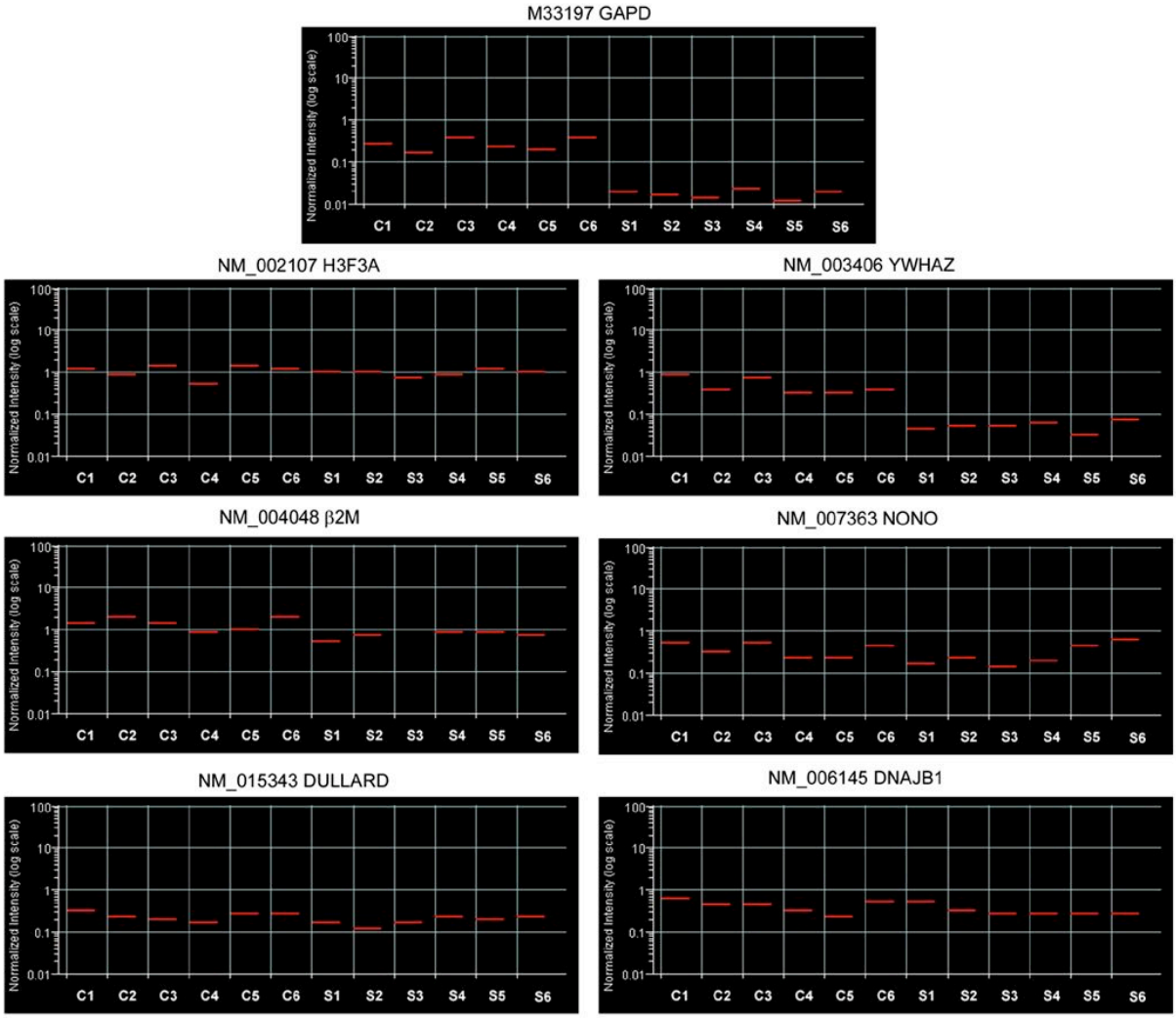

Figure 1. Reference genes expression levels from microarray data. Red lines represent the log of normalized expression values for each individual participant. Microarray data includes a subpopulation only of subjects included in the RT-PCR experiments. C = healthy control, S = schizophrenia patient.

C. Primers

Primers sets (20-21 bases in length) were initially designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/) with the criteria of: product size range 85-150 bases, GC-content 45-60 %, primer Tm of 57-61 °C. Selection of primer sets generated by the software matching these criteria was restricted to those that encompassed an amplicon spanning known exon boundaries within the gene-coding domain. Most primer sets had a minimal difference in Tm (range 0.01-0.5 °C) between primers. Querying the NCBI BLAST database ensured the specificity of primer set sequences. Furthermore, primers had insignificant or no complementary sequences between them to avoid dimerization. Primer sequences are presented in **Tables 2 and 3**. All primer sets were synthesized by Genset Oligos (*E@sy Oligo*™; Lismore, Australia). PCR reactions were performed on a PTC-200 Peltier Thermal Cycler (MJ Research) using a HotStarTaq® DNA polymerase kit (Qiagen) according the manufacturer's instructions. Annealing temperatures for each primer set were optimized by evaluating a temperature gradient of 57-61 °C in 1 °C increments. An optimal annealing temperature was defined as the temperature that gave the largest quantity of specific amplified product without accumulation of primer-dimer, determined by agarose gel visualization. PCR products were visualized by 2 % agarose gel electrophoresis and ethidium bromide staining. Product bands of appropriate length were excised and extracted using QIAquick® gel extraction kit (Qiagen). Purified cDNA was then prepared for sequencing with Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Half-reactions were prepared under the following conditions: 12 µl of cDNA template, 4 µl Big Dye® Terminator, 4 µl 2.5X sequencing buffer (200 mM Tris-HCl, 5 mM $MgCl_2$; pH 9.6) and 0.33 µl each of the appropriate forward and reverse primers. The thermal cycler sequencing program was as follows: 25 cycles of 30 sec. at 96 °C, 15 sec. at 50 °C, 4 min. at 60 °C, with 1 °C / sec. ramping between each step of the cycle. Sequencing was performed using an ABI Model 377 DNA sequencer at the Griffith University sequencing facility. Primer sets producing amplified sequences with a minimum of 98 % homology to their corresponding DNA sequences and without similarities to any other gene, as per the NCBI BLAST database, were deemed acceptable for use.

D. Real-time PCR

RT-PCR was performed using a Rotor-Gene 2000 fluorometric thermal cycler (Corbett Research; Sydney, Australia). Samples of cDNA (5 µl, diluted 1/10) were made up into 20 µl reactions using a QuantiTect™ SYBR® Green PCR Kit (Qiagen) containing HotStar Taq®, QuantiTect™ SYBR® Green PCR buffer, dNTP mix, SYBR Green I, and 5 mM $MgCl_2$. To minimize pipetting error and maintain volume consistency between samples, master mixes and individual samples were aliquotted with a CAS-1200 robotic liquid handling system (Corbett Robotics; Brisbane, Australia). The CAS-1200 can handle a 1µL - 200µL range of volumes, uses graphite tips with automatic liquid level sensing and is highly precise (< 1 % C.V. on volumes ≥ 5 µl according to manufacturer). The Rotor-Gene RT-PCR program was as follows: 15 min. at 95 °C to activate the Taq polymerase and then 40 cycles of 30 sec. at 95 °C, 30 sec. at 58 °C, and 30 sec. at 72 °C. A melt curve analysis with a temperature range of 65 °C to 95 °C ramping at 0.5 °C / 5 sec. was performed to determine product specificity for each sample (Ririe et al, 1997). PCR reaction efficiency was determined by generating relative standard curves with five quadruplicate 10-fold serial dilutions of control cDNA.

E. Comparison of RT-PCR data analysis methods

Eighteen genes were selected from existing microarray data on skin fibroblasts that were expressed differently between the schizophrenia and control groups (unpublished observations). These genes were quantified by RT-PCR using the selected stably expressed reference gene (see **Table 3**). The relative amount of each gene product in each sample was determined determined using two different quantification methods, the comparative threshold cycle method (Pfaffl, 2001) using REST© XL (Relative Expression Software Tool) (Pfaffl et al, 2002) and the Comparative Quantification (CQ) method supplied as part of the Rotor Gene 5.0 software (Corbett Research). The REST© XL method used the Pair Wise Fixed Reallocation Randomisation Test© and 2000 randomizations were performed as recommended by the authors of this method (Pfaffl et al, 2002).

The comparative threshold cycle method sets an arbitrary threshold level subjectively set by the researcher within the exponential phase of amplification on a plot of normalized fluorescence values to determine a fractional Ct value. While this method yields robust results (Rasmussen, 2001) the threshold may inadvertently be set outside the exponential phase of product amplification. The CQ method differs from this in that it uses the second derivative of raw fluorescence values to help calculate the point at which the exponential phase of amplification begins. This point, termed the Take Off Point (TOP), is used in the equivalent manner as the Ct value. The TOP is described as the point 80% below the second derivative plot peak (**Figure 2**). The CQ method calculates reaction efficiencies for each individual sample, obviating the need for standard curves to generate efficiencies and controlling for efficiency differences between reactions. RT-PCR correlates the fluorescence levels to levels of synthesized product. The increase in fluorescence (R) is represented by the exponential growth model during the PCR: $R_{n+1} = R_n * (A)$, where n is the cycle number and A is the measure of the efficiency of the reaction (amplification value).

Table 2. Primer sequences of housekeeping genes investigated.

Gene Symbol	Accession Number	Sense Primer	Antisense Primer	mRNA Range	Product Size
YWHAZ	NM_003406	5'-TGAAGCCATTGCTGAACTTG-3'	5'-CTTCAGCTTCGTCTCCTTGG-3'	675-799	126
β2M	NM_004048	5'-TGACTTTGTCACAGCCCAAG-3'	5'-AGCAAGCAAGCAGAATTTGG-3'	374-487	114
H3F3A	NM_002107	5'-ACTGGAGGGGTGAAGAAACC-3'	5'-AGCAATTTCTCGCACCAGAC-3'	212-343	132
GAPDH	NM_002046	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'	529-615	87
DULLARD	NM_015343	5'-CCGAAACCTTCACCAACATC-3'	5'-AGGCAGTCCTCACATTGGAC-3'	1151-1277	127
NONO	NM_007363	5'-AGATTCGGATGGGTCAGATG-3'	5'-CATAGTGGCAGGTCCTGGAG-3'	1313-1428	116
DNAJB1	NM_006145	5'-TTCCCCAGACATCAAGAACC-3'	5'-ACCCTCTCATGGTCCACAAC-3'	1017-1152	136

Table 3. Primer sequences of genes found to be differentially expressed by cDNA microarray analysis (unpublished observations) and used to evaluate the CQ method of RT-PCR analysis against the comparative threshold cycle method.

Gene Symbol	Accession Number	Sense Primer	Antisense Primer	mRNA Range	Product Size
JAK1	NM_002227	5'-CCAATCAGAGGCCTTTCTTC-3'	5'-AAATGTGTGGGGTCCACTTC-3'	2534-2651	127
GHR	NM_000163	5'-GACTTTTTCATGCCACTGGAC-3'	5'-TCAGGGCATTCTTTCCATTC-3'	229-351	123
NFKB1	NM_003998	5'-ACTCTGGCGCAGAAATTAGG-3'	5'-TGACTGTACCCCCAGAGACC-3'	2970-3078	109
CDK6	NM_001259	5'-GCATCGCGATCTAAAACCAC-3'	5'-CTGTACCACAGCGTGACGAC-3'	541-672	132
CDK4	NM_000075	5'-TCAGCACAGTTCGTGAGGTG-3'	5'-TACCTTGATCTCCCGGTCAG-3'	379-494	116
CCNB1	BC006510	5'-TGTGGATGCAGAAGATGGAG-3'	5'-GTGACTTCCCGACCCAGTAG-3'	560-688	129
PCNA	NM_002592	5'-CTGAGGGCTTCGACACCTAC-3'	5'-GCGTTATCTTCGGCCCTTAG-3'	400-526	127
CCND1	BC014078	5'-TCTACACCGACAACTCCATCC-3'	5'-GGCATTTTGGAGAGGAAGTG-3'	520-649	130
GADD45A	NM_001924	5'-GATCACTGTCGGGGTGTACG-3'	5'-TGCAGAGCCACATCTCTGTC-3'	400-510	111
SSTR3	NM_001051	5'-GTGTCCACGACCTCAGAACC-3'	5'-AGGTAGACCAGGGGGATCAG-3'	27-153	131
DDX5	NM_004396	5'-CTCCAGAGGGCTAGATGTGG-3'	5'-GTATGCTGTGCCTGTTTTGG-3'	1373-1496	124
HDAC6	NM_006044	5'-CCCAATCTAGCGGAGGTAAAG-3'	5'-GTGCTTCAGCCTCAAGGTTC-3'	226-340	115
HDAC4	NM_006037	5'-AGATCCTCATCGTGGACTGG-3'	5'-GAAGTTCCCATCGTCGTAGC-3'	3290-3302	113
CALM1	NM_006888	5'-AGCTGACCGAAGAACAGATTG-3'	5'-GGTTCTGACCCAGTGACCTC-3'	213-332	120
ITGAE	NM_002208	5'-AGACCCATGCTTTCAAGGTG-3'	5'-CTGGTAGTGAAGGGCGTCTC-3'	972-1078	113
CCNDBP1	NM_012142	5'-AGGATGCACATGAAGAAATGG-3'	5'-GAAACCCCAACACATCATCC-3'	711-833	123
VDR	NM_000376	5'-GCCCACCATAAGACCTACGA-3'	5'-AGATTGGAGAAGCTGGACGA-3'	526-729	203
GAPDH	NM_002046	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'	529-615	87
CAPON	NM_014697	5'-ACATCTCCCTGCTGGTCAAG-3'	5'-GAAGGTGATCTCCAGCAAGC-3'	1463-1669	98

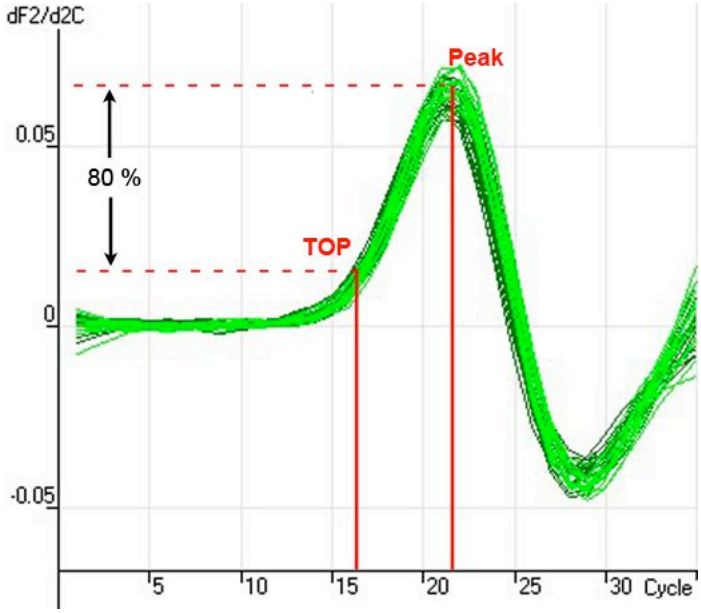

Figure 2. DNAJB1 second differential of raw data plot for each sample. Fluorescent readings were taken from all samples (schizophrenia and healthy control). The peak is equivalent to the maximum rate of exponential amplification and the take off point (TOP) is defined as 20 % of the peak (or 80 % less). The TOP in the CQ method is equivalent to C_t value in other real time PCR analysis methods.

Background fluorescence was removed by taking the first differential of the normalized fluorescence values. Monitoring the exponential phase increase in fluorescence was accomplished by rearranging the above formula to give an observed amplification (A_n) at each point within the exponential phase of a reaction: $(A_n) = R_{n+1}/R_n$. Averaging the amplification over these points produced an amplification value for the sample (A_s). The amount of gene product in any given sample relative to a designated reference sample was calculated using the formula: Relative Quantity (RQ) = (A_s) ^ (Control TOP – Sample TOP). Since each sample was performed in triplicate, the mean RQ of the replicates for each sample was determined. The mean RQ values for the gene of interest (RQ_{GOI}) were then transformed into a ratio of the reference gene (RQ_{REF}) values for each individual sample. Group differences (fold changes) were calculated by taking the mean of all RQ_{GOI}/RQ_{REF} values for

schizophrenia and control samples and expressing the schizophrenia values as a ratio of the control value. Calculated group expression level differences from both the comparative threshold cycle and CQ methods were assessed for correlation by linear regression analysis using XLSTAT-Pro 7.0 (Addinsoft, NY).

F. Reference gene analysis

RT-PCR was performed on all samples with each primer set to determine which of the reference genes was stable across control and patient groups. All samples, in triplicate, were subjected to RT-PCR as above and the PCR runs were repeated 3 times. Data (Ct values) were collected and viewed by using Rotor Gene 5.0 software (Corbett Research). The statistical procedure Proc Mixed in SAS 8.02 (SAS Institute, Cary, NC) was used with a nested random effects model for subjects, replicate, and run between the two groups, with post-hoc planned comparisons (t tests) between the schizophrenia versus healthy control groups for each of the seven genes. Statistical tests used an α level of 0.05 and tests were two tailed. Results are expressed as Mean ± S.E.M (**Table 4**). Genes with expression levels that were not different between groups ($p > 0.05$) were deemed stably expressed. The gene with the highest p-value was selected as the reference gene for the remainder of the study.

III. Results

A. Specificity and linearity of the RT-PCR reactions

Specificity of the RT-PCR reactions for each reference gene primer set was analysed on a 2 % agarose gel and confirmed by the observation of a single amplified band of the correct length. Nucleotide sequencing of these products demonstrated a minimum 98 % homology to their corresponding DNA sequences, with no significant similarity to other gene sequences as per the NCBI BLAST database. Melt curve analysis of the RT-PCR amplicons, did not detect any non-specific amplification products. Serial dilutions (1/10) of human skin fibroblast cDNA over a 10 000 fold range were prepared for each gene and standard curves were generated using five dilutions of this cDNA. The Rotor Gene 5.0 software calculated correlation coefficients and reaction efficiencies from these curves. The mean correlation coefficient for the seven reference genes was 0.998 (SD = 0.001, range 0.996 - 0.999), and the average efficiency was 93 % (SD = 3.4 %, range 89 – 99 %). The efficiencies generated by these standard curves were used in the comparative threshold cycle calculations.

B. Comparative quantification versus comparative threshold cycle

Eighteen genes were analysed for differential expression between patient and control groups to provide ratios of gene expression between the genes of interest and the reference gene. The absolute values of the ratios from each analysis method were subjected to linear regression analysis. The two methods gave very similar results and were strongly correlated ($R = 0.853$; **Figure 3**). The equation of the fitted line is Comparative Threshold Cycle Analysis = (0.101 ± 0.133) + (0.899 ± 0.138)*CQ. The fitted line does not differ significantly from a ray through the origin with unit slope (i.e. perfect correlation ($R=1$); $F_{2, 16} = 0.29$, $p = 0.75$).

C. Differential expression of reference genes in schizophrenia

In order to test the stability of expression in patient and control samples of the seven reference genes a total of 1071 individual RT-PCR reactions were performed (17 samples, by 3 replicates each, by 3 runs, by 7 genes). Ct values were collected and collated by gene and participant and then analysed for differences between Ct value within and between subjects and between groups. Analysis of variance showed significant effects of gene ($F_{6, 960} = 8450.10$, $P < 0.001$), and participant group ($F_{1, 18} = 9.48$, $P = 0.007$), plus a strong gene by participant group interaction ($F_{6, 960} = 4.46$, $P < 0.001$). Post-hoc analysis revealed significant differences between groups for five of the seven genes tested (**Table 4**). Of the two genes without significant differences between groups, DNAJB1 demonstrated the most stable expression level (highest p-value) and as such was the reference gene used for comparing the quantification methods (**Table 4**).

IV. Discussion

Our data show that the Comparative Quantification method for calculating RT-PCR ratios provides comparable data to that of the well-accepted Comparative Threshold Cycle method. This is important because the cost of the latter is considerably greater than the former when multiple genes of interest are under analysis, such as in verifying gene expression microarray data.

Table 4. Mean Ct values (± S.E.M.) for each reference gene listed by group.

GENE	STATUS	MEAN	SEM	P-VALUE
β2M	Sz	14.34	0.088	< 0.001
	HC	14.80	0.088	
H3F3A	Sz	11.95	0.104	0.003
	HC	12.41	0.104	
YWHAZ	Sz	15.44	0.087	0.05
	HC	15.69	0.087	
GAPDH	Sz	9.35	0.094	0.02
	HC	9.67	0.094	
DNAJB1	Sz	16.13	0.102	0.19
	HC	16.32	0.102	
DULLARD	Sz	15.10	0.127	> 0.05
	HC	15.44	0.127	
NONO	Sz	13.53	0.116	0.008
	HC	13.97	0.116	

Differential expression for all but 2 genes, DNAJB1 and DULLARD, was observed between schizophrenia patients and healthy controls. The gene with the highest p-value (DNAJB1); and hence selected as the reference gene for this study is highlighted in yellow. (Sz = schizophrenia; HC = healthy control).

A)

Gene Symbol	Comparative Threshold Cycle	Comparative Quantification
VDR*	1.292	1.260
JAK1	0.987	0.872
GHR	0.682	0.709
NFKB1*	0.839	0.787
GADD45A	0.991	0.966
CCND1	1.197	1.015
PCNA*	0.713	0.722
CCNB1	1.257	1.084
CDK4	0.934	0.935
CDK6	0.943	0.968
CCNDBP1	0.942	0.969
ITGAE	0.701	0.844
CALM1	1.024	1.007
HDAC4*	1.113	1.323
HDAC6*	0.781	0.821
GAPDH	0.984	0.931
DDX5*	0.884	0.775
CAPON	0.892	0.902

B)

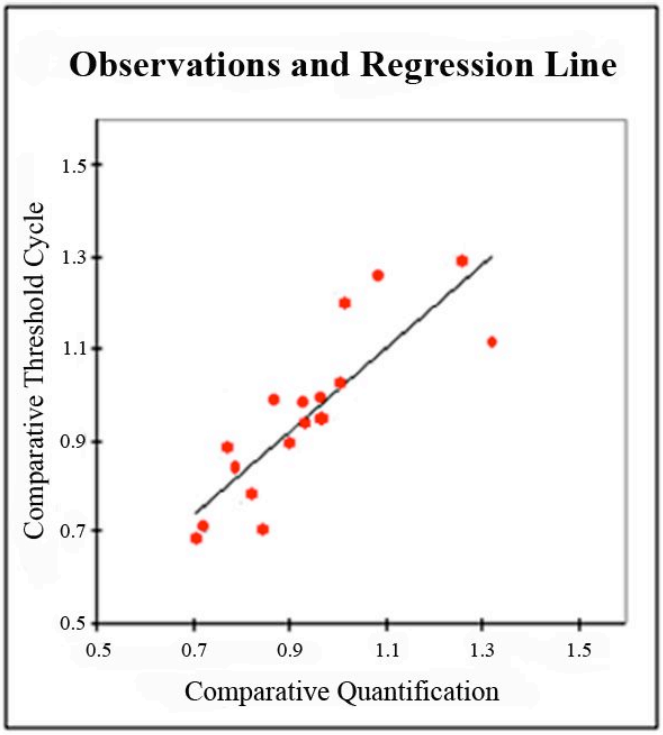

Figure 3. Validation of CQ analysis. A) Relative change in gene expression level (Sz vs HC) of the 18 genes selected from the microarray data (unpublished observations) as calculated by the two methods of RT-PCR data analysis. *Genes found to be differentially expressed by RT-PCR analysis. B) Linear regression analysis of comparative threshold cycle analysis versus CQ analysis; R = 0.853.

The second finding of this study is that some commonly used reference genes are differentially expressed among individuals with schizophrenia and healthy controls. These findings are consistent with the fact that expression levels of certain normalizing genes can be modulated *in vivo* or *in vitro* by treatment or disease state (Thellin et al, 1999; Bustin, 2000; Schmittgen and Zakrajsek, 2000; Suzuki et al, 2000). This difference in gene expression level indicates the necessity to identify a reference gene prior to its use as such in RT-PCR. The best reference gene in our samples was DNAJB1, recently suggested as a reference gene (Eisenberg and Levanon, 2003), but not yet tested for its stability of expression in any experimental model. We found that in fixed quantities of total RNA from skin fibroblast cell lines DNAJB1 had consistent expression levels between individuals and across groups. These results demonstrate that so-called "housekeeping" genes are not always appropriate as reference genes for all tissues and suggest that reference gene expression level should be confirmed in the samples under investigation.

A. Comparison of RT-PCR data analysis methods

Quantification of PCR products is estimated by various methods, all with the assumption that the amount of amplified product is a function of the amount of cDNA at the start and the efficiency of the PCR amplification. Different methods use different ways to estimate the amount of amplified product and different ways to estimate the efficiency of the PCR reaction. A widely accepted method for RT-PCR analysis is the Comparative Threshold Cycle method (Pfaffl, 2001; Pfaffl et al, 2002). This method uses a fractional cycle number (the threshold cycle; Ct) during the exponential phase of DNA amplification to generate a value for the amount of PCR product. The efficiency of the PCR reaction is estimated via a standard curve generated by multiple PCR reactions of serial dilutions of starting material with the same primers (Saiki et al, 1988).

The Comparative Threshold Cycle method loses accuracy because it assumes that all individual reactions have the same rate of amplification (Ramakers et al, 2003). Since PCR efficiency is estimated from the gradient of the line of best fit from multiple separate PCR reactions for each primer set, it may not accurately reflect the efficiency in the sample of interest because efficiencies change between samples due to systematic and random effects in their preparation (Freeman et al, 1999; Ramakers et al, 2003). This shortcoming is recognised and recently two methods have been described in which the same sample is used to estimate both the amount of PCR product and the efficiency of the PCR reaction (Liu and Saint, 2002; Tichopad et al, 2003). Liu and Saint described in 2002 a method in which the experimenter subjectively determines the PCR efficiency from the slope of a regression line fitted to the log of the rate of the PCR reaction during the exponential phase. The method requires the researcher to determine the exponential phase of the PCR reaction. A second method (Tichopad et al,

2003), obviates this subjective influence by using statistical methods to determine the exponential phase to estimate the efficiency.

The present study describes a third method for calculating the amount of PCR product and the efficiency of the PCR reaction in a single sample, Comparative Quantification (CQ; Rotor Gene 5.0 software, Corbett Research) using the second differential maximum method (Rasmussen, 2001) to calculate reaction efficiencies and a set percentage of the maximum fluorescence value to calculate the beginning of the exponential phase. This makes the method simpler than described previously and less reliant on complex statistical criteria to determine the exponential phase. The second differential of raw fluorescence values from a RT-PCR reaction describes a parabola with peak as the point of maximum exponential growth of amplified product. By taking a point 80 % back from this maximum the CQ method ensures that our data points used for quantity estimation are within the exponential phase of amplification yet still well above the background noise level. Also, by avoiding the assumption of constant reaction efficiency, a more accurate estimate of relative quantity of PCR product is obtained (Freeman et al, 1999; Rasmussen, 2001; Tichopad et al, 2003). Our data show no significant difference in accuracy between either the CQ or Comparative Threshold Cycle methods of RT-PCR data analysis. The primary advantages of the CQ method are that all quantitative information is located within the exponential phase (Rasmussen, 2001) and that there is no need to run standard curves to estimate efficiencies, saving time and reducing costs. Importantly, the CQ method also reduces the amount of nucleic acid required, a most valuable commodity when the original amounts of starting material are restricted, as in the case of tissue biopsies.

B. Housekeeping gene selection

Consistent with the microarray data the real time PCR data showed stable expression of DNAJB1 and DULLARD and significant group differences in the expression of GAPDH and YWHAZ. However, the real-time PCR data showed significant group differences in H3F3A, β2M and NONO in opposition to the microarray findings. With the exception of GAPDH, (investigated solely based on its ubiquitous use as a housekeeping gene), the expression of all genes selected for testing varied little (low standard deviation) across all samples and groups. These data indicate that, while using the criteria of gene expression data with low variance across all samples may be supportive for the selection of a housekeeping gene, researchers should not rely on microarray data exclusively for this purpose. Instead, the stability of housekeeping gene expression across all samples and groups should be verified using similar methods outlined here. Moreover, use of a normalization factor derived from multiple housekeeping genes provides a more accurate description of gene expression (Vandesompele et al, 2002). However, in multi-housekeeping gene strategies, the stability of each housekeeping gene should be validated in the system to be tested prior to their use in RT-PCR expression profiling.

In the present study, DNAJB1 was the most stably expressed housekeeping gene across all samples used. DNAJB1, a mammalian homologue of bacterial heat shock protein 40 (Ohtsuka, 1993), is an ideal candidate as a housekeeping gene (Eisenberg and Levanon, 2003) because of its constitutive expression (Ohtsuka and Suzuki, 2000), compact gene length (Ohtsuka, 1993; Hata et al, 1996), and its requirement for cell maintenance (Abdul et al, 2002; Farinha et al, 2002). However, DNAJB1 expression can be modulated by ischemia (Tanaka et al, 2002) and heat treatment (Abdul et al, 2002). Therefore caution must be taken before using DNAJB1 as a housekeeping gene. Potential homeostatic effects on DNAJB1 expression were minimized in the present culture system because constant levels of oxygen, carbon dioxide and temperature were maintained across all samples in both groups.

In conclusion, the stable expression of a reference gene must be validated in the system of interest prior to its use in RT-PCR. This strategy is being recommended by more and more researchers (Aerts et al, 2004; Dheda et al, 2004) using RT-PCR to investigate gene expression in disease. Our study showed DNAJB1 to be a suitable reference gene in a cultured skin fibroblast model to study gene expression differences in schizophrenia. Also, we showed the validity of the more efficient, and inexpensive Comparative Quantification method of RT-PCR data analysis.

Acknowledgements

We would like to thank Dr. François Féron, and Mr. Duncan McLean for assistance with cell culturing and participant care respectively. Special thanks go to Dr. David Chant for his advice on statistical analysis. This project was supported by grants from the Stanley Medical Research Institute (USA) and the Garnett Passe and Rodney Williams Memorial Foundation.

References

Abdul KM, Terada K, Gotoh T, Hafizur RM, Mori M **(2002)** Characterization and functional analysis of a heart-enriched DnaJ/ Hsp40 homolog dj4/DjA4. **Cell Stress Chaperones** 7, 156-166.

Aerts JL, Gonzales MI, Topalian SL **(2004)** Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. **Biotechniques** 36, 84-86, 88, 90-81.

Bhatia P, Taylor WR, Greenberg AH, Wright JA **(1994)** Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28S-ribosomal RNA gene expression as RNA loading controls for northern blot analysis of cell lines of varying malignant potential. **Anal Biochem** 216, 223-226.

Bustin SA **(2000)** Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. **J Mol Endocrinol** 25, 169-193.

de Leeuw WJ, Slagboom PE, Vijg J **(1989)** Quantitative comparison of mRNA levels in mammalian tissues: 28S ribosomal RNA level as an accurate internal control. **Nucleic Acids Res** 17, 10137-10138.

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A **(2004)** Validation of housekeeping genes for normalizing

RNA expression in real-time PCR. **Biotechniques** 37, 112-114, 116, 118-119.

Eisenberg E, Levanon EY **(2003)** Human housekeeping genes are compact. **Trends Genet** 19, 362-365.

Farinha CM, Nogueira P, Mendes F, Penque D, Amaral MD **(2002)** The human DnaJ homologue (Hdj)-1/heat-shock protein (Hsp) 40 co-chaperone is required for the in vivo stabilization of the cystic fibrosis transmembrane conductance regulator by Hsp70. **Biochem J** 366, 797-806.

Freeman WM, Walker SJ, Vrana KE **(1999)** Quantitative RT-PCR: pitfalls and potential. **Biotechniques** 26, 112-122, 124-115.

Hamalainen HK, Tubman JC, Vikman S, Kyrölä T, Ylikoski E, Warrington JA, Lahesmaa R **(2001)** Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. **Anal Biochem** 299, 63-70.

Hata M, Okumura K, Seto M, Ohtsuka K **(1996)** Genomic cloning of a human heat shock protein 40 (Hsp40) gene (HSPF1) and its chromosomal localization to 19p13.2. **Genomics** 38, 446-449.

Higuchi R, Fockler C, Dollinger G, Watson R **(1993)** Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. **Biotechnology (N Y)** 11, 1026-1030.

Hsiao LL, Dangond F, Yoshida T, Hong R, Jensen RV, Misra J, Dillon W, Lee KF, Clark KE, Haverty P, Weng Z, Mutter GL, Frosch MP, Macdonald ME, Milford EL, Crum CP, Bueno R, Pratt RE, Mahadevappa M, Warrington JA, Stephanopoulos G, Gullans SR **(2001)** A compendium of gene expression in normal human tissues. **Physiol Genomics** 7, 97-104.

Lee PD, Sladek R, Greenwood CM, Hudson TJ **(2002)** Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. **Genome Res** 12, 292-297.

Liu W, Saint DA **(2002)** A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. **Anal Biochem** 302, 52-59.

Lupberger J, Kreuzer KA, Baskaynak G, Peters UR, le Coutre P, Schmidt CA **(2002)** Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR. **Mol Cell Probes** 16, 25-30.

Mahadik SP, Mukherjee S, Laev H, Reddy R, Schnur DB **(1991)** Abnormal growth of skin fibroblasts from schizophrenic patients. **Psychiatry Research** 37, 309-320.

Ohtsuka K **(1993)** Cloning of a cDNA for heat-shock protein hsp40, a human homologue of bacterial DnaJ. **Biochem Biophys Res Commun** 197, 235-240.

Ohtsuka K, Suzuki T **(2000)** Roles of molecular chaperones in the nervous system. **Brain Res Bull** 53, 141-146.

Pfaffl MW **(2001)** A new mathematical model for relative quantification in real-time RT-PCR. **Nucleic Acids Res** 29, e45.

Pfaffl MW, Horgan GW, Dempfle L **(2002)** Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. **Nucleic Acids Res** 30, e36.

Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JJ, Griffin JL, Wayland M, Freeman T, Dudbridge F, Lilley KS, Karp NA, Hester S, Tkachev D, Mimmack ML, Yolken RH, Webster MJ, Torrey EF, Bahn S **(2004)** Mitochondrial dysfunction in Schizophrenia: evidence for compromised brain metabolism and oxidative stress. **Mol Psychiatry** 9, 684-697.

Rajeevan MS, Ranamukhaarachchi DG, Vernon SD, Unger ER **(2001)** Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. **Methods** 25, 443-451.

Ramakers C, Ruijter JM, Deprez RH, Moorman AF **(2003)** Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. **Neurosci Lett** 339, 62-66.

Rasmussen R: Quantification on the LightCycler, in Meuer S, Wittwer C, Nakagawara K (eds): Rapid Cycle Real-Time PCR Methods and Applications. Berlin: Springer-Verlag, 2001, pp 21-34

Ririe KM, Rasmussen RP, Wittwer CT **(1997)** Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. **Anal Biochem** 245, 154-160.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA **(1988)** Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. **Science** 239, 487-491.

Schmittgen TD, Zakrajsek BA **(2000)** Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. **J Biochem Biophys Methods** 46, 69-81.

Suzuki T, Higgins PJ, Crawford DR **(2000)** Control selection for RNA Quantification. **Biotechniques** 29, 332-337.

Tanaka S, Kitagawa K, Ohtsuki T, Yagita Y, Takasawa K, Hori M, Matsumoto M **(2002)** Synergistic induction of HSP40 and HSC70 in the mouse hippocampal neurons after cerebral ischemia and ischemic tolerance in gerbil hippocampus. **J Neurosci Res** 67, 37-47.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E **(1999)** Housekeeping genes as internal standards: use and limits. **J Biotechnol** 75, 291-295.

Tichopad A, Dilger M, Schwarz G, Pfaffl MW **(2003)** Standardized determination of real-time PCR efficiency from a single reaction set-up. **Nucleic Acids Res** 31, e122.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F **(2002)** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. **Genome Biol** 3, RESEARCH0034.

Warrington JA, Nair A, Mahadevappa M, Tsyganskaya M **(2000)** Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. **Physiol Genomics** 2, 143-147.

Zhong H, Simons JW **(1999)** Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. **Biochem Biophys Res Commun** 259, 523-526.

Richard D. McCurdy