Validation of rat reference genes for improved quantitative gene expression analysis using low density arrays

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BioTechniques 42:503-512 (April 2007)
doi 10.2144/000112400

Real-time PCR has become increasingly important in gene expression profiling research, and it is widely agreed that normalized data are required for accurate estimates of messenger RNA (mRNA) expression. With increased gene expression profiling in preclinical research and toxicogenomics, a need for reference genes in the rat has emerged, and the studies in this area have not yet been thoroughly evaluated. The purpose of our study was to evaluate a panel of rat reference genes for variation of gene expression in different tissue types. We selected 48 known target genes based on their putative invariability. The gene expression of all targets was examined in 11 types of rat tissues using TaqMan® low density array (LDA) technology. The variability of each gene was assessed using a two-step statistical model. The analysis of mean expression using multiple reference genes was shown to provide accurate and reliable normalized expression data. The least five variable genes from each specific tissue were recommended for future tissue-specific studies. Finally, a subset of investigated rat reference genes showing the least variation is recommended for further evaluation using the LDA platform. Our work should considerably enhance a researcher's ability to simply and efficiently identify appropriate reference genes for given experiments.

INTRODUCTION

Understanding gene expression patterns may provide insight into complex biological and pathological processes, as well as be predictive of disease outcome or therapeutic treatment (1,2). In recent years, microarray and real-time reverse transcription PCR (RT-PCR) analyses have gained popularity in evaluating messenger RNA (mRNA) expression. Microarray analysis is a genome-wide screening assay based on competitive dual-color hybridization that results in the simultaneous interrogation of thousands of mRNA species. Although microarray analysis is a powerful screening tool for establishing mRNA expression patterns, the extensive replicate sampling can be labor-intensive, sensitivity and dynamic range are small, and analysis of thousands of data points can be technically challenging. The high sensitivity, reproducibility, and large dynamic range of RT-PCR provides high-throughput and accurate differential expression profiling of usually 10–20 select genes (3,4). RT-PCR is extensively applied to functional genomics, molecular medicine, diagnostics, forensics, virology, microbiology, and other biotechnology applications when simultaneous measurement of gene expression in many different samples from small amounts of starting material is required.

Although quantitative RT-PCR is a powerful tool in mRNA expression analysis, there are several variables that need to be controlled, such as RNA quality and quantity and enzyme efficiencies. Therefore, mRNA expression data are often normalized to internal reference genes. Some housekeeping genes are used as reference controls for RT-PCR because they tend to be ubiquitously expressed (5–7). Since the expression of the target gene is normalized to such reference genes it is essential to choose the appropriate reference gene(s) for accurate and reliable data analysis (8–10).

Commonly used housekeeping genes in real-time RT-PCR assays are β-actin (*ACTB*), glycerolaldehydphosphate dehydrogenase (*GAPDH*), ribosome small subunit (*18S*) ribosomal RNA (rRNA), β-2 microglobulin (B_2M), and hypoxanthine phosphoribosyltransferase (*HPRT*) (7,10). It is reported that the expression of housekeeping genes can vary considerably under experimental conditions and thus pose problems when interpreting expression data (3,6,11). Suzuki and colleagues discussed the advantages and pitfalls of *GAPDH* and β-actin as control genes and emphasized precautions associated with using these as normalizers (12). Vandesompele et al. showed that normalization based on a single housekeeping gene led to erroneous quantification with gene expression changes varying up to 3-

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fold in 25% of cases and 6.4-fold in 10% of cases, while sporadic cases showed errors greater than 20-fold (13). *18S* rRNA shows high sequence conservation among eukaryotes and prokaryotes and is relatively abundant compared with most other mRNA transcripts. This high abundance can make it difficult to accurately subtract the baseline value in real-time RT-PCR data analysis, therefore attenuation of the concentration of *18S* primers/probe might be needed when quantification of weakly expressed genes is conducted (6,11,14). Without appropriate normalization, expression profiles of target genes will likely be misrepresented (15). With increased gene expression profiling in preclinical research and toxicogenomics, a need for reference genes in rat has emerged; however, extensive studies in this area have not yet been conducted.

In this study, we selected 48 target genes based on putative invariability and examined their expression patterns in 11 rat tissues using the low density array (LDA) platform from Applied Biosystems. This platform allows the simultaneous assay of mRNA gene expression of up to 384 targets on a single card using only a small amount of RNA sample input and a fast setup procedure, therefore large numbers of transcripts can be expeditiously investigated and assessed relatively simply.

MATERIALS AND METHODS

Tissue Collection, RNA Preparation, and cDNA Synthesis

Tissues from normal adult rats (three males, three females) were evaluated in this study. Following CO_2 asphyxiation and exsanguinations, animals were sacrificed in compliance with The Institutional Animal Care and Use Committee. Tissues were snap-frozen in liquid nitrogen and then stored at -80°C. Collected tissues included liver, adrenals, kidney, spleen, jejunum, thymus, lung, heart, brain, gastrocnemius muscle, pancreas, testis, and ovaries for a total of 72 test samples (six animals by eleven common tissues and two sex-related tissues). To insure that tissue samples were collected expeditiously, the six animals were processed sequentially and to completion, and the problematic pancreatic tissue was always taken first. Total RNA was extracted from 30 mg each tissue using the RNeasy® Mini kit (Qiagen, Valencia, CA, USA). The tissues were first homogenized using the MagNA Lyser Green Bead tube (Roche Diagnostics, Indianapolis, IN, USA) with 1 mL ice-cold lysis buffer on a FastPrep FP120 homogenizer (Thermo Fisher Scientific, Waltham, MA, USA) three times (40 s at max speed). A QIAshredder™ (Qiagen) was used to filter the homogenate to prevent clogging on the RNeasy column, and then the manufacturer's recommended RNeasy protocol was followed to completion. All of the RNA samples were treated with DNase as the standard protocol at room temperature for 15 min. RNA was then quantified using the NanoDrop® ND-1000 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). RNA quality was assessed on the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip® kit (Agilent Technologies), and the RNA integrity number (RIN) was calculated based on the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products (16). RNA is considered to be of high quality if no degradation products are observed in the electrophoretic trace. Subsequently, 1 µg high-quality total RNA from samples was reverse-transcribed to cDNA using BD Sprint™ PowerScript™ Hexamer PrePrimed 6 × 8 well (BD Biosciences, San Jose, CA, USA) in 20 µL volume at 42°C for 90 min followed by 70°C for 10 min to inactivate the reverse transcriptase, according to the supplier's protocol. One hundred nanograms reversed-transcribed RNA were then loaded into each LDA port and used in real-time PCR assays.

Target Selection

An internal bioinformatics search [body map and GeneChip® data (Affymetrix, Santa Clara, CA, USA) on human and rat control tissues] identified several genes with low mRNA variability. Additionally, targets were selected from the literature among housekeeping and reference genes commonly used to normalize mRNA expression data. A total of 48 genes were selected and spanned a range of expression levels (high, medium, and low) in a given tissue.

TaqMan® LDA

TaqMan LDA microfluidic card technology from Applied Biosystems (Foster City, CA, USA) allows the simultaneous assay of mRNA gene expression of up to 384 targets on a single card. The LDA used in this study was custom designed to consist of 48 TaqMan Gene Expression Assays (Applied Biosystems) per loading port (48 genes × 8 samples = 384). Each reaction well contained all reagents specific for a given assay. Each target assay consisted of a forward primer, a reverse primer, and a TaqMan MGB probe (6-FAM dye-labeled) and are detailed in Table 1. Although most of the gene assays (36 out of 48) target exon-exon junctions to be mRNA-specific, there are some assays that amplify genomic DNA (12 out of 48). Therefore, we treated all RNA samples with DNase. Several other controls were examined in these assays. No reverse transcriptase negative controls were performed for each RNA sample, to ensure that genomic DNA was removed and not amplified. The positive control was a commercial rat total RNA (BD Rat Universal reference total RNA, 1 µg/µL; BD Biosciences) and was reverse-transcribed to cDNA along with the test samples. The negative controls consisted of no template (water).

For each tissue sample, 100 ng reverse-transcribed RNA were diluted to 50 µL with sterile water, combined with an equal volume of TaqMan Universal PCR Master Mix (2×; Applied Biosystems), mixed by inversion, and spun briefly in an Eppendorf® 5415C microcentrifuge (Brinkmann Instruments, Westbury, NY, USA). After TaqMan LDAs were brought to room temperature, 100 µL master mix were loaded into each port connected to 48 reaction wells. LDAs were placed in Sorvall®/Heraeus® Custom Buckets (Applied Biosystems) and centrifuged in a Sorvall Legend™ centrifuge (Kendro Scientific, Asheville, NC, USA) for

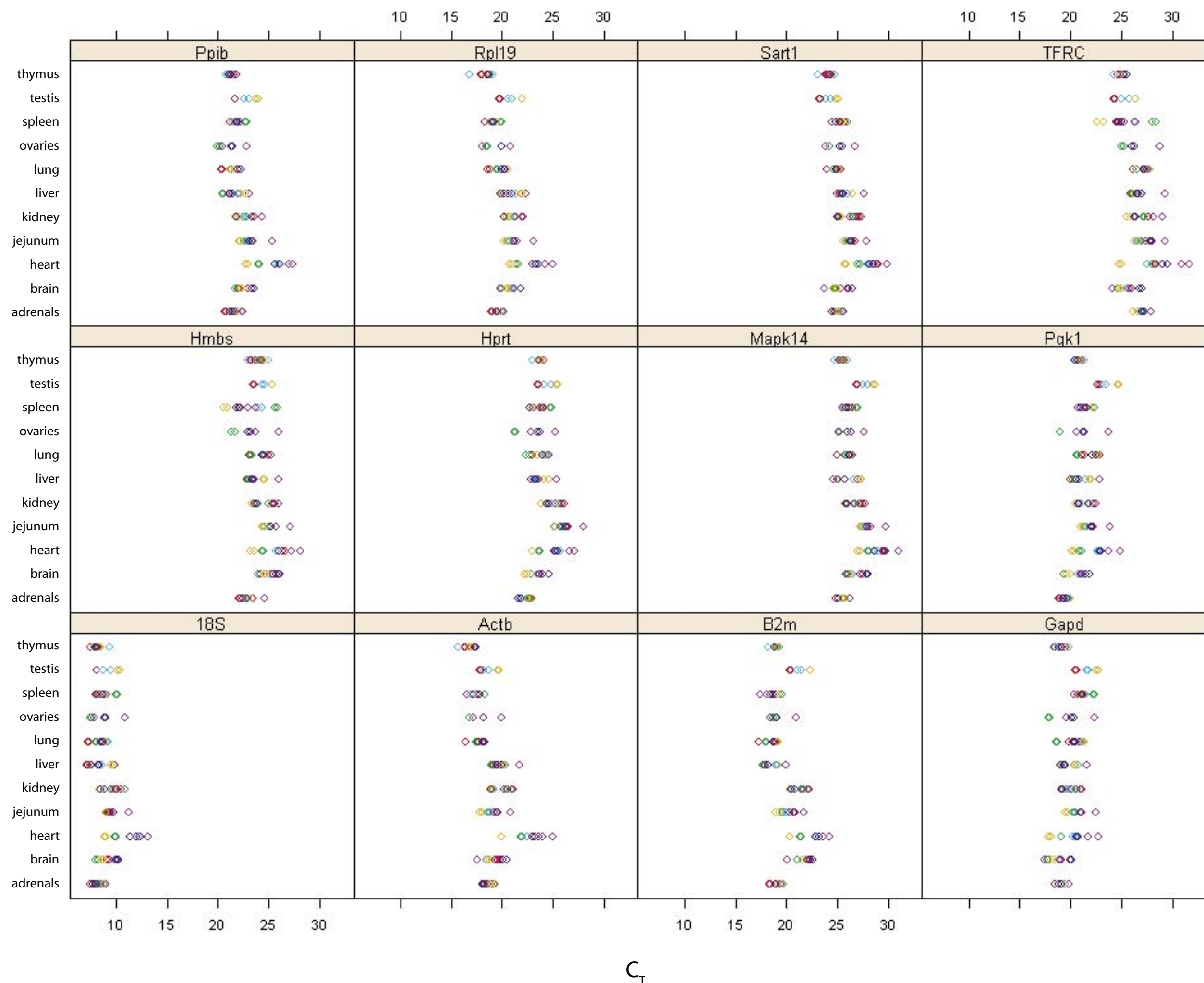

Figure 1. Strip plots representing cycle threshold (C_T) values for tissues and messenger RNA (mRNA) targets for 12 selected genes. The plots representing the remaining 36 genes can be found in Supplementary Figure S1 available online at www.BioTechniques.com. Each circle represents a C_T value from a sample replicate, so there are 12 circles for each tissue (six animals times two replicates, but six data points for sex tissues), unless data was treated as missing because the sample C_T values exceeded 40. Each color represents an animal. Through visual inspection of the strip plots, it is clear that certain mRNA targets show greater tissue variability than do others. Additionally, variability among animals and between replicates is evident.

1 min at 331× *g* followed closely by a second 1-min centrifugation at 331× *g*. Cards with excess sample in the fill reservoir were spun for an additional 1 min. Immediately following centrifugation, the cards were sealed with a TaqMan LDA Stylus Staker (Applied Biosystems), and the loading ports excised. The final volume in each well after centrifugation was <1.5 µL; thus, the final reverse-transcribed RNA concentration was approximately 1.5 ng/reaction. Real-time RT-PCR amplifications were run on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems) with a TaqMan LDA cycling block and an automation accessory upgrade. Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each test sample was processed in duplicate on individual LDA cards, thus allowing four samples to be processed on each card. There were a total of 20 LDA cards processed in three runs, and controls were included in each of the runs.

Analysis of Real-Time RT-PCR Data

RT-PCR TaqMan instrumentation monitors gene-specific products with fluorescent dye chemistry. A cycle threshold (C_T) for each reaction is the number of cycles at which the reaction crosses a selected threshold. The threshold is defined as a straight line drawn above noise/baseline and positioned within the linear region of the semi-log amplification plot. The fewer cycles required to reach threshold fluorescence intensity, the lower the C_T value and the greater the initial amount of input target (3). All samples for a given detector were analyzed concurrently using the ABI Relative Quantity Manager software (Applied Biosystems) automated algorithms for background, baseline, and threshold detection (see ABI 7900 User's Guide and Reference 5). Manual confirmation of threshold detection was conducted

for quality control purposes. We utilized C_T number as input for our variability analysis among tissue samples for each target. Since we conducted 40 cycles of PCR, assays that did not yield a $C_T < 40$ cycles were treated as negative results and not included in further analysis. Results for each target on LDAs were quantified concurrently using the same baseline and threshold for a target gene in order to limit interplate errors in the analysis.

Variability of C_T values was first examined through graphical visualizations (see Figure 2). To quantitatively assess the variability in the C_T data, different sources of variability were identified and fitted into a linear random effect model as described below. Based on the design of the experiment, variability was partitioned among animals, tissues, and replicates for a given target. Equation 1 represents the components that contribute to the observed C_T values

$$Ct_{ijk\lg} = \mu_g + C_k + T_{ig} + A_{jg(i)} + e_{ijk\lg}$$

[Eq. 1]

where $Ct_{ijk\lg}$ is the observed C_T value of the ith tissue, jth animal, kth card, and lth replicate for the gene g. Parameter μ_g is the intercept, the true C_T value of gene g for a reference group. All parameters are gene-specific except C_k. C_k is a card-specific effect that is added to all genes on the same card and is a systematic effect for which we would like to adjust through normalization. T_{ig} is the effect of tissue i, $A_{jg(i)}$ is the effect of animal j for tissue i, and $e_{ijk\lg}$ is the random error for the lth replication.

The card-specific variability C_k explains a small part (due to card) of the overall systematic variability that the reference control genes are intended to remove. The systematic variability due to sample is still included in the gene-specific sample terms in Equation 1. Since the systematic sample variability will be the same for all genes, it should have very limited effect for the comparison of the gene-specific sample variability's between genes.

The model in Equation 1 is commonly fitted with a three-way analysis of variance (ANOVA) model where effects are assumed to be fixed parameters, and the variability of each factor is derived from the sum of squares associated with each term. However, this fixed effect model does not provide a direct estimate of variability associated with each term; it either depends on the order of the effect entered in the model or does not sum up to the total. So, we fit Equation 1 with a two-step approach, similar to what Littell, Wolfinger, and coworkers (17) used for microarray gene expression data. In the first step, a fixed effect model 1 is fitted to obtain the card-specific effect estimate $\hat{C}_k$, and subsequently the card-specific effect is removed from the data to obtain a card-normalized C_T value $Ct'_{ijk\lg}$

$$Ct'_{ijk\lg} = Ct_{ijk\lg} - \hat{C}_k$$

[Eq. 2]

In the second step, we fit a random effect model to the normalized C_T values for each gene target separately.

$$Ct'_{ijk\lg} = \mu_g + T_{ig} + A_{jg(i)} + e_{ijk\lg}$$

[Eq. 3]

Effects A and T are assumed from normal distributions, $T_{ig} \sim N(0,\sigma^2_{Tg})$ and $A_{jg(i)} \sim N(0,\sigma^2_{Ag})$. The error e is assumed to be normally distributed, $e_{ijk\lg} \sim N(0,\sigma^2_g)$. The parameters σ^2_{Ag}, σ^2_{Tg}, and σ^2_g in this model describe the variability associated with animal, tissue, and replicate effect, respectively, and are estimated using the restricted maximum likelihood (REML) method (17). Furthermore, we assume that the terms in Equation 3 are independent of one another. Under these assumptions, the variability of C_T is a sum of variability of all its components.

$$Var(Ct'_{ijk\lg}) = \sigma^2_{Ag} + \sigma^2_{Tg} + \sigma^2_g$$

[Eq. 4]

Compared with a fixed effect model approach, the random effect model has several advantages: (*i*) it provides a direct estimate of the variability from each source; (*ii*) it does not depend on how the effects are ordered in the model; and (*iii*) the total variability is the sum of variability as in Equation 4. Although the assumption of normality may not hold for some effects, in those cases the estimated variance parameters still provide a descriptive measure of the variability associated with the effects. Random effect models are fitted using SAS® PROC MIXED.

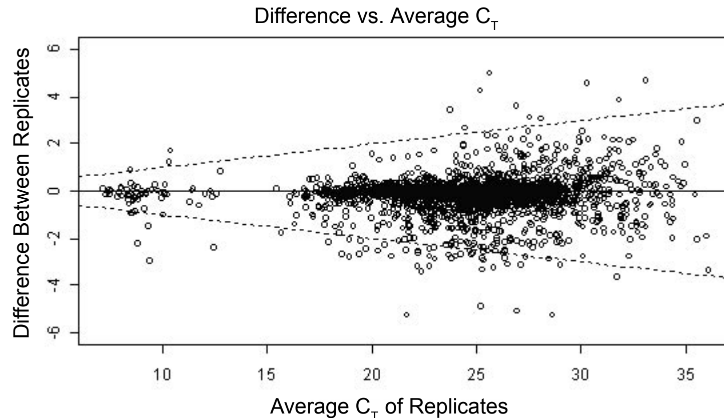

Figure 2. Difference versus mean cycle threshold (C_T) of replicates. The dashed lines correspond to a coefficient of variation (CV) of 10%.

RESULTS

RNA Quality and Yield

Most RNA samples from rat tissues in this study were of high quality and yield with the exceptions of pancreas and gastrocnemius muscle (data not shown). Most of the tissue samples generated a range of 20–100 µg total RNA from 30 mg of each tissue. The 260/280 ratios of the RNA samples were approximately 2, and RIN numbers were near 9 for most samples. RIN normally ranges from 1–10, with an RIN above 7 considered to be indicative of high-quality RNA. All RNA samples from pancreas were extremely degraded (RIN was around 2); this is a common issue in this tissue due to its inherently high RNase activity. RNA quality from gastrocnemius muscle was

high, but yield was low (<1 µg RNA for most samples). This was most likely due to difficulties in tissue lysis from incomplete disruption of the connective components in this tissue and thus made it impossible to fairly evaluate the expression of all 48 gene targets. Therefore, the pancreas and gastrocnemius muscle samples were excluded from comparative expression analysis. In all of the no reverse transcriptase control reactions, no amplification of the 48 gene targets was measured, implying a lack of contribution of genomic DNA to the final expression data.

mRNA Expression Analysis

Expression analysis was conducted on the remaining 60 samples from 11 tissues and 6 animals (two tissues are sex-specific). The RT-PCR expression data for the 48 genes were acquired and quantified as described. The strip plot (Figure 1) illustrates the dynamic range and variability of C_T measurements among samples and animals for all mRNA targets. The display of C_T values indicates that certain genes have greater expression variability among tissues than do others.

To examine the reproducibility of our results, duplicates of each sample were run simultaneously on the same card. Figure 2 displays the difference in C_T values of replicates relative to their means, most of which (>95% of data) fall within a 10% coefficient of variation (CV). This is an acceptable level of intra-assay variability, therefore LDA assay precision was considered high for the 48 mRNA targets (18). C_T values of 40 (e.g., some data from *Aqp2*, *Hspa4*, *S100a3*) were treated as missing data and excluded from further analysis.

A two-step modeling approach was used to evaluate sources of variability. Originally we included a gene-specific gender effect in the model, since there are three male and three female rats used in the study. The results indicate a very small gender effect for all mRNAs (data not shown). The variability of C_T values due to gender is <1% of the variability due to tissues for almost all mRNAs when comparing the sum of squares in a fixed effect model. The variability estimate of the gender effect is mostly zero or close to zero in the random effect model. Therefore, the negligible gene-specific sex effect was not included in the model for simplicity. Figure 3 represents the variance component estimation based on normal theory for each of the 48 mRNAs with total C_T variance partitioned among the three sources and represented in bar graph format. The tissue component accounted for the majority of variability in the data for a given mRNA. In most cases, the animal component accounted for the second most variability followed by the replicate component (tissues > animals > replicates). The fact that the replicate component contributed relatively little to the total variance for a given mRNA further confirmed acceptable assay precision (19). These results are consistent with the strip plots (Figure 1) that visually depict the raw data. Most genes have comparable overall variability, with standard deviations between 1 and 2 (data not shown). The majority of genes display medium levels of expression with C_T values between 20 and 30. The *18S* mRNA is the most abundant and least variable target. In contrast, *S100a3* shows low variability but also low abundance. As would be expected with low expresser targets such as *S100a3*, there were missing data points for some of the tissues.

As an indirect way to validate our approach above, we grouped the 48 genes into three equal-sized groups based on their tissue C_T variability. The first group has the 16 genes with smallest tissue variability, and the third group has the largest tissue variability, with the second group in between. If the three groups of genes are used as an endogenous control (pooled for each group) to normalize all data, the ΔC_T variability of the 48 genes should be the smallest when the first group is used as the normalizer, while the ΔC_T variability should be largest if the data are normalized by the third group. When the three groups of genes are used as normalizers, the median standard deviations of ΔC_T of all genes are 1.18, 1.31, and 2.94, respectively, which in some degree validates our assessment of variability in the housekeeping genes based on their C_T values.

The level of expression among putative endogenous controls varies widely in different tissues and therefore presents a problem for data analysis. For example, examination of the average C_T values among the 48 targets (Figure 3) reveals an approximate 16,000-fold difference between the most (*18S*) and least abundant (*S100a3*) transcripts. Furthermore, examination of the strip plots (Figure 1) indicates that some mRNAs are expressed at a relatively high and constant levels in select tissues (e.g., *Alb*:liver; *Gfap*:brain; *Aqp2*:kidney) while variable and low expressing in other tissues. Some of this variability may be due to systematic sample variability; however, the majority is not, because other genes do not show similar changes among these tissues.

DISCUSSION

There are a number of reviews and research papers evaluating the selection and effect of controls on normalized gene expression data, however, most of them derive from expression analysis in human samples (4,7,15). With increased gene expression profiling in preclinical research and toxicogenomics, there is an urgent need for rigorous validation of reference genes in experimental animal model systems. A recent paper has discussed the validation of canine reference genes for gene expression in that species (20). The study of rat reference genes for gene expression has not yet been thoroughly evaluated. Routinely, high-quality quantitative gene expression data are often normalized relative to a reference control gene. Therefore, it is critical to choose an appropriate reference control gene(s) for normalization so as not to misrepresent the expression profile of a target gene (11). Since no single reference gene is optimal for all studies, the selection of an appropriate control gene for a given study is key, especially among complex multiple tissue and treatment regimens (9). In our study, we evaluated the expression levels for 48 genes across a panel of rat tissues to select potential reference genes for normalization of mRNA expression data.

The first step in any mRNA expression analysis study is to confirm the quality and yield of RNA from the

Table 1. 48 mRNA Targets Included on the Low Density Array

Gene No.	Gene Symbol	Gene Name	Accession No.	Assay Location (nt)	ABI Gene Expression Assay No.
1	*FLJ20445*	Hypothetical protein	XM_215286	1100	Rn01454146_m1
2	*Sart1*	Squamous cell carcinoma antigen recognized by T-cells 1	NM_031596	481	Rn00580979_m1
3	*FLJ10587*	Hypothetical protein	BF411321	2400	Rn01456650_m1
4	*COP9*	COP9 constitutive photomorphogenic homolog subunit 6 (Arabidopsis)	XM_222002; AI409481	589	Rn01528050_g1
5	*RNA-bp*	RNA binding protein	BG375054; BI294261	473	Rn01482572_m1
6	*Hspa4*	Likely ortholog of mouse heat shock protein, 70 kDa 4	BE101732	439	Rn01477779_m1
7	*FLJ10498*	Hypothetical protein	BF394953	388	Rn01414061_m1
8	*Epo*	Brain Zn-finger protein (LOC362154)	BF550329	463	Rn01515220_g1
9	*AP82*	Acidic 82 kDa protein mRNA	BF410997	1551	Rn01521658_m1
10	*Ring1*	Ring finger protein 1	BI300772	1041	Rn01429212_g1
11	*SRP14*	Signal recognition particle 14 kDa (homologous Alu RNA binding protein)	AA799994	106	Rn01485827_g1
12	*HP1-BP74*	*Rattus norvegicus* Unknown (protein for MGC:72624), mRNA (cDNA clone MGC:72624 IMAGE:5599940), complete cds.	BC061837	1197	Rn01519754_m1
13	*Stau*	Staufen	NM_053436	169	Rn00584855_m1
14	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	X02231	327-377	Rn99999916_s1
15	*RPL10a*	Ribosomal protein L10A	NM_031065	334	Rn00821239_g1
16	*Ppib*	Cyclophilin B	NM_022536	251	Rn00574762_m1
17	*L32*	Ribosomal protein L32(Rpl32)	est594385; X06483	421	Rn00820748_g1
18	*PPIA*	Peptidylprolyl isomerase A (Cyclophilin A)	M19533	228	Rn00690933_m1
19	*ActB*	Actin, β	NM_031144	888	Rn00667869_m1
20	*RPL19*	Ribosomal protein L19	AA800054; NM_031103	266	Rn00821265_g1
21	*Nedd4a*	Neural precursor cell expressed, developmentally down-regulated gene 4A	U50842	2564	Rn01530544_m1
22	*Slit1*	Slit homolog 1	AB017170	232	Rn01444575_m1
23	*Rabin3*	Rabin 3	NM_017313	531	Rn00568959_m1
24	*Ggcx*	γ-Glutamyl carboxylase	NM_031756	803	Rn00582138_m1
25	*Mapk14*	Mitogen activated protein kinase 14	NM_031020	125	Rn00578842_m1
26	*Map2k5*	Mitogen activated protein kinase kinase 5	NM_017246	412	Rn00568384_m1
27	*Hmbs*	Hydroxymethylbilane synthase	NM_013168	356	Rn00565886_m1
28	*Gapds*	Glyceraldehyde-3-phosphate dehydrogenase type 2	NM_023964	530	Rn00576699_m1
29	*B2m*	β-2 microglobulin	NM_012512	71	Rn00560865_m1
30	*Jak2*	Janus kinase 2	NM_031514	133	Rn00580452_m1
31	*Gusb*	Glucuronidase, β	NM_017015	1390	Rn00566655_m1
32	*Map3k1*	Mitogen activated protein kinase kinase kinase 1	NM_053887	1316	Rn00588007_m1
33	*Pde3a*	Phosphodiesterase 3A	NM_017337	1004	Rn00569192_m1
34	*Mapk6*	Mitogen-activated protein kinase 6	NM-031622	1089	Rn00581152_m1
35	*S100a3*	S100 calcium binding protein A3	NM_053681	39	Rn00586633_m1
36	*Rpn1*	Ribophorin I	NM_013067	328	Rn00565052_m1
37	*Tabu1*	Tubulin	NM_022298	71	Rn01532518_g1
38	*TFRC*	Transferrin receptor	rCT52708	569	Rn01474701_m1
39	*HPRT1*	Hypoxanthine guanine phosphoribosyl transferase	M63983	486	Rn01527838_g1
40	*Ubd*	Ubiquitin D	NM_053229	79	Rn00583977_m1
41	*alb*	α Albumin	NM_134326	716	Rn00592480_m1
42	*GFAP*	Glial fibrillary acidic protein	NM_017009	1189	Rn00566603_m1
43	*Tnni1*	Troponin I, slow isoform	NM_017184	235	Rn00567843_m1
44	*AQP2*	Aquaporin 2	NM_012909	486	Rn00563755_m1
45	*PGK1*	Phosphoglycerate kinase 1	NM_053291	1019	Rn00821429_g1
46	*Rpl13a*	Ribosomal protein L13A	NM_173340	272	Rn00821946_g1
47	*TBP*	TAF9-like RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31 kDa (Taf9l)	NM_133615	478	Rn00592425_m1
48	*18S*	Eukaryotic 18S rRNA	X03205		Hs99999901_s1

mRNA, messenger RNA; nt, nucleotide; rRNA, ribosomal RNA.

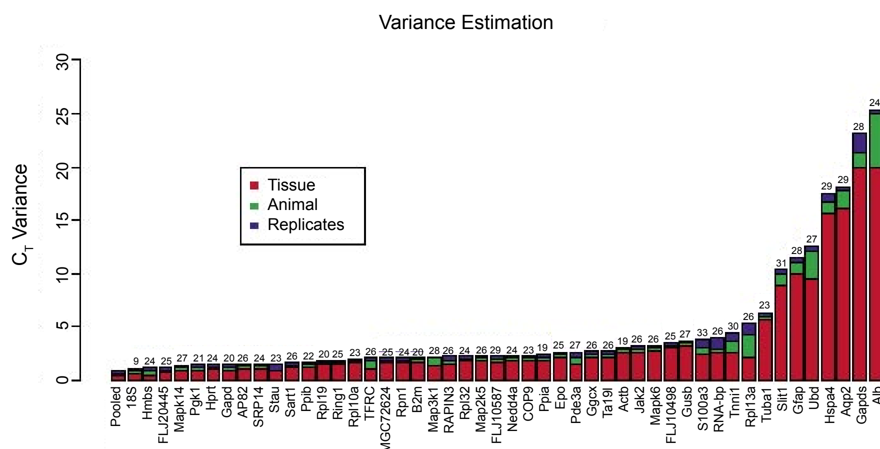

Figure 3. Variance component estimation based on normal theory for each messenger RNA (mRNA). Total cycle threshold (C_T) variance is partitioned among three sources. The bar graphs are arranged from left to right to show lowest to highest C_T variance. Through visual inspection of the data, it is evident that in most cases the majority of C_T variance is attributable to differences among tissues. The number on top of each bar represents the mean C_T for all analyzable samples for a given target. The first bar to the left was derived from pooling data among the four mRNA targets showing the lowest total C_T variance.

processed tissues, because RNA is less stable than DNA in normal working conditions. RNA degradation and transcriptional induction can occur immediately after harvesting of the biological sample (21). Due to poor quality and low yield of RNA, pancreas and gastrocnemius muscle samples were excluded from comparative expression analysis. In the future, alternative extraction methods may be evaluated to improve RNA quality and yield from muscle and pancreas tissues.

Other considerations for accurate real-time PCR also include evaluations of assay efficiency, assay variation, and potential assay inhibition (22). A recent report on the evaluation of LDA technology indicated that gene expression measurements conducted with LDAs are highly reproducible and precise, both within and across arrays. Comparisons between LDAs reveal low variability, with correlation coefficients close to 1.0 (19). Although our study was not designed to look at the assay inhibition, it is interesting to note the breakdown of the variance analysis by tissue, animal, and replicate-specific components reports indirectly on any influence by an assay inhibitor. Figure 3 clearly shows that the tissue variance outweighs any assay-specific effects.

In normal rat tissues, nearly two-thirds of the 48 mRNA targets showed relatively low expression variability among replicates, tissues, and animals; and thus, are considered good candidates to be selected as reference controls. However, it is impossible to predict how different experimental conditions may affect the expression of putative normalization genes. When measuring gene expression under different experimental conditions (e.g., drug treatments, diseases, etc.), this subset of mRNAs should be further evaluated so as to select the best reference gene(s) for specific experimental conditions. When examining gene expression patterns among multiple tissues/treatment groups, it is imperative that the same normalization strategy be applied to all tissues types, in order to reduce the number of experimental variables and allow direct comparison between samples. In other words, the same set of control genes should be used for normalization among tissues and groups, if possible (13).

We analyzed tissue-specific gene expression profiles and listed the five control genes for each tissue showing the least ΔC_T variability within the tissue (Table 2). The rank is based on the standard deviation of ΔC_T (data not shown) calculated by normalizing to a pooled average of the 36 genes (see Figure 3) showing the least variable expression in C_T. For example, B_2M is a good potential reference gene for liver, but not a good one for heart. This suggests that different reference gene(s) or biomarkers may be used when a given tissue is studied. The reference gene should also be of similar abundance to the gene of interest. For *18S*, which is shown as the most stable gene in the overall variability assessment in Figure 1, but is not in any of the top organ-specific gene lists in Table 2, the abundance is so high that it in fact explains its overall stability. This can also be seen from Figure 1. Assay attenuation of *18S* is not possible due to the nature of LDA technology, as all the gene assays are preloaded on the plate and there is no chance to further optimize the abundance of the gene assay to the target gene level. Therefore, *18S* is decidedly not the best reference gene within a tissue or in its potential ability to respond to experimental conditions, due to its high abundance.

Normalization based on a single reference gene without validation can lead to erroneous results (11).

Table 2. Top Five Control Genes Recommended for Each Tissue

Organ	Selection 1	Selection 2	Selection 3	Selection 4	Selection 5
Liver	B2M	Pgk1	Rpn1	Ggcx	FLJ20445
Kidney	Pgk1	Ring1	ActB	Gapd	Mapk14
Brain	Mapk6	Gapd	Rp10a	TFRC	Map2k5
Heart	Rpl10a	COP9	Gapd	Ppib	Hmbs
Lung	Rpl10a	Mapk14	FLJ20445	Taf91	Map2k5
Spleen	Ppib	Tuba1	COP9	Taf91	Rpl19
Jejunum	FLJ20445	Rpl10a	MGC72624	Tuba1	FLJ10498
Adrenals	Rpl19	Mapk14	Nedd4a	FLJ10586	Ggcx
Thymus	Rpl10a	Hprt	Pgk1	Gusb	COP9
Testis	B2M	FLJ10498	RABIN3	Rpl10a	SRP14
Ovaries	Actb	Ppib	COP9	FLJ20445	Epo

Different methods for identifying the most suitable combination of control genes have been proposed. Recent studies using the geometric mean and pair-wise comparison from multiple housekeeping genes suggest that to accurately measure gene expression, normalization to multiple housekeeping genes is essential when many target genes are assayed (13). A recent report by de Kok and colleagues details the calculation and application of the mean expression from multiple housekeeping genes (7). Evaluating the approach of using multiple reference controls, our analysis has shown that by pooling the four least variable genes (*18S*, *Hmbs*, *FLJ20445*, *Mapk14*), variation within the pooled data was less than any one of the four genes (Figure 3). However, underpinning the increased confidence achieved with pooling of a small number of reference genes for normalization is the critical fact that each and every reference control gene should be experimentally validated in the test system under study. Our approach represents a first step in that direction.

Under conditions where samples are limited, such as in an early developing embryo or a minute biopsy sample, it might not be prudent to prescreen experimental material to select a reference gene to use in normalizing expression data. In such a case, preselection of a battery of reference genes known to vary minimally among tissues would reduce the likelihood of the expression levels of target mRNAs being skewed by a single gene, the expression of which varies under the experimental conditions in question. By normalizing across several select reference genes and estimating the inherent component variance, the potential negative impact on the accuracy of relative gene expression data would be minimized. This approach would allow more rapid gene expression data collection in the pharmaceutical setting and supply a downstream data analysis tool to support the selection of the best reference genes for the study in question.

The number of genes used to normalize mRNA expression data are a trade-off between practical considerations and assay validity within an experimental design. For example, it might not be reasonable to use numerous reference controls when only a few mRNAs are to be studied in normal tissues, especially when sample template availability is limiting. If the goal is to select a single normalization gene, the control and mRNA(s) of interest should be expressed at similar levels, and the control should not vary under experimental conditions (6,11,23). However, if the expression of many genes is to be evaluated among numerous tissue and/or treatment groups, it may then become necessary to assay multiple reference controls for normalization of the data.

Although the primary purpose of this study was to assess the variation of reference control genes in various tissues, the same rules can also be applied to other experimental conditions, such as in vitro assays. We have several in-house programs utilizing our rat reference LDA cards that have successfully uncovered the reference genes that remained stable under each study's complicated conditions. The commonly used housekeeping genes, like β-actin and *GAPDH*, were not appropriate selections for the experiments in question. We have successfully applied one to six internal controls for gene expression analysis (data not shown) for a number of programs, each based on experimental design. From our experience, we feel strongly that the use of a battery of reference genes within the LDA format not only expedites the selection of custom endogenous controls but also improves the quality of the gene expression data. Therefore, we recommend that the use of the reference control LDA card and the analysis of component variance, within an appropriately normalized real-time experiment, is the first step to confident quantitative RT-PCR gene expression data.

ACKNOWLEDGMENTS

J.H.C., D.S.G., and A.R. participated in hypothesis design. J.H.C. designed the experiment, and J.H.C., S.W.K., and P.A.L. carried out the experiments. P.Z. provided the bioinformatics search on the invariable genes. S.D. supported all statistical analysis. J.H.C., S.D., and D.S.G. drafted the manuscript. A.R. was a critical reviewer. All authors read and approved the final manuscript. We thank James Yan for contributing the ideas on study design and Gary Seitis for providing necropsy assistance and collecting study tissue materials.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 7 September 2006; accepted 15 December 2006.

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