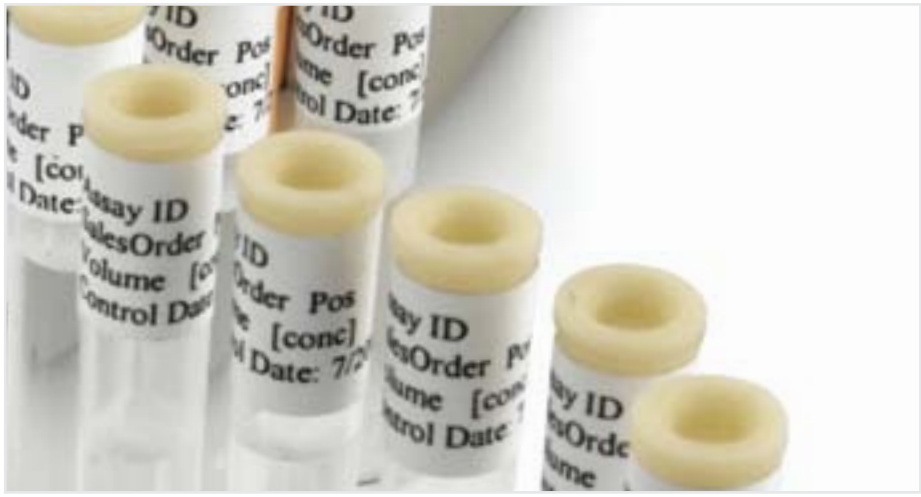
Using TaqMan® Endogenous Control Assays to select an endogenous control for experimental studies

Introduction

Quantitative real-time PCR (qPCR) allows for highly sensitive, rapid, and reproducible quantification of mRNA. In any gene expression study, selection of a valid normalization or endogenous control to correct for differences in RNA sampling is critical to avoid misinterpretation of results. Inter-sample variation due to inherent sample differences, sample collection, RNA preparation and quality, reverse transcription efficiency, and pipetting errors are common sources of variability.



The ideal endogenous control should have a constant RNA transcription level under different experimental conditions and be sufficiently abundant across different tissues and cell types. Although any gene that is stably expressed under the defined experimental conditions can serve as a normalization gene, the selection is most commonly made from constitutively expressed mRNA housekeeping genes, or ribosomal RNAs such as 18S rRNA.

Generally, the mRNA transcripts are moderately abundant and are involved in basic cellular functions such as glycolytic pathway (GAPD, PGK1) and protein folding (PIPA); or structural components such as cytoskeleton (ACTB). Several genes, including those for 18S rRNA, ACTB, and GAPD, have been widely used to normalize expression, often without experimental validation.

However, numerous studies have shown that these genes display varying expression levels in different tissues and under different experimental conditions, and need to be used with caution [1,2]. Other genes such as HPRT [3], GUS, and B2M have shown relative stability across a number of tissues. Thus, there is no universal control gene and it is important to identify the most appropriate endogenous control for a particular cell type and experimental condition.

In a study of normal lymphocytes and lymphomas, Lossos et al. provided guidelines for using quantitative real-time PCR to identify the appropriate endogenous control gene [4]. In their study, which included evaluation of RNA quality and quantity, the expression of 11 common human housekeeping genes was compared across a large number of cell lines and tissues to identify the appropriate endogenous control to use for RNA normalization.

Following their guidelines and using available statistical methods, we used TaqMan® Endogenous Controls from human and mouse to easily identify the best candidate genes for normalization in an experimental study. We analyzed the RNA expression of 11 human and 8 mouse housekeeping genes in peripheral blood samples isolated from human and mouse. In addition, we used the TaqMan® Array Endogenous Control Card (Cat. No. 4367563), which contains 16 human control genes (11 housekeeping genes and 5 additional genes are included on the card) to identify genes with the most stable expression across 16 different human tissues.

Methods

TaqMan® Endogenous Controls are included as inventoried TaqMan® Gene Expression Assays (Cat. No. 4331182) and are identified by an assay-specific Life Technologies assay ID. These assays include 11 human, 8 mouse, and 4 rat housekeeping genes listed in Table 1 with gene name, RNA accession number, and Life Technologies assay ID.

The five additional TaqMan® Assays available on the TaqMan® Array Endogenous Control Card are noted with an asterisk in Table 1. Each assay on the Array Card is spotted in triplicate.

Note: TaqMan® Endogenous Controls are also available individually in larger volumes as a separate part number with a choice of FAM™ or VIC® dye labels. Assays can also be ordered as primer-limited with a VIC® dye label.

Real-time PCR

Each TaqMan® Gene Expression Assay consists of a fluorogenic FAM™ dye–labeled MGB probe and two amplification primers (forward and reverse) provided in a preformulated 20X mix; 1X final concentrations are 250 nM for the probe and 900 nM for each primer. Each assay has an amplification efficiency of 100% ±10% [5].

Total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Cat. No. 4322171) to generate cDNA. For each assay, 50 ng of cDNA (as total input RNA) was amplified with TaqMan® Universal PCR Master Mix (Cat. No. 4324018) and the TaqMan® Endogenous Control. The real-time PCR reactions were run for 40 cycles using universal cycling conditions (95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min) on an Applied Biosystems® 7900HT Fast Real-Time PCR System.

Each cDNA prepared from total RNA from 16 different tissues (Clontech) was diluted with TaqMan® Universal PCR Master Mix to a final concentration of 1 ng/µL cDNA. Next, 100 µL of a single-tissue sample mixture was loaded into one of 8 sample ports of a TaqMan® Array Endogenous Control Card. The cards were processed according to the TaqMan® Array Card User's Guide and run using universal thermal cycling conditions on a 7900HT Fast Real-Time PCR System fitted with a 7900HT TaqMan® Array Card Upgrade (Cat. No. 43290124).

Table 1. TaqMan® Endogenous Control Assays.

Gene ID	Gene name	Accession number	Life Technologies assay ID
RPLP0	Ribosomal protein, large, P0	NM_001002	Hs99999902_m1
ACTB	Actin, beta	NM_001101	Hs99999903_m1
PPIA	Peptidylpropyl isomerase A (cyclophilin A)	NM_021330	Hs99999904_m1
PGK1	Phosphoglycerate kinase	NM_000291	Hs99999906_m1
B2M	Beta-2-microglobulin	NM_004048	Hs99999907_m1
GUSB	Glucuronidase, beta	NM_000181	Hs99999908_m1
HPRT	Hypoxanthine phosphoribosyl transferase	NM_000194	Hs99999909_m1
TBP	TATA box–binding protein	M34960	Hs99999910_m1
18S	Eukaryotic 18S ribosomal RNA	X03205	Hs99999901_s1
GAPD	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Hs99999905_m1
TFRC	Transferrin receptor (P90, CD71)	NM_003234	Hs99999911_m1
IPO8*	Importin 8	NM_006390	Hs00183533_m1
POLR2A*	Polymerase (RNA) II (DNA-directed) polypeptide A	NM_000937	Hs00172178_m1
UBC*	Ubiquitin C	NM_021009	Hs00824723_m1
YWHAZ*	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_003406	Hs00237047_m1
HMBS*	Hydroxymethylbilane synthase	NM_000190	Hs00609297_m1

*For this study, these assays were run only on the TaqMan® Array Card.

Results and discussion

To identify the best candidate genes to use as endogenous controls for normalization, the expression profile of each gene was determined for ten individual peripheral blood samples for human and mouse. Each TaqMan® Assay was run in quadruplicate for each sample.

The average C_t with standard deviation (StDev) for each sample and endogenous control assay is shown in Figure 1 for human samples, Figure 2 for mouse, and Figure 3 for the TaqMan® Array Human Endogenous Control Card. The average C_t was then used to determine the average C_t and StDev of C_t for each gene across the different samples. The StDev was used to identify the gene with the least variation among the 11 genes examined (Figure 4).

From these data, the human endogenous control genes with the lowest StDev, and therefore, the lowest variation across samples, are 18S (0.18), TBP (0.82), and GUSB (0.86). From these data, 18S appears to be the best candidate to serve as an endogenous control under these conditions.

18S is a ribosomal RNA subunit and is highly abundant, making up >80% of total cellular RNA and serving as a reliable reference gene for total RNA mass. Since ribosomal RNAs are transcribed by a distinct RNA polymerase, and their synthesis is independent of mRNA, their expression levels might be regulated by different biological conditions compared to mRNA.

Lassos et al. also found that 18S had the least variability among the genes they tested. However, because it was resistant to degradation compared to other genes and did not correct for RNA quality differences, they decided it was not useful for their samples. In parallel, they had identified PGK1 and/or TBP—two housekeeping genes that also displayed very low variability across their samples—and decided to use these instead of 18S.

Therefore, although 18S often shows the least variability across samples, it still needs to be evaluated in parallel with other candidate genes. In this example, then, 18S with its very low variability can serve as a good normalization gene for total RNA. Although TBP and GUSB show more variability, they can perform as endogenous controls since they display the most stable expression of the housekeeping

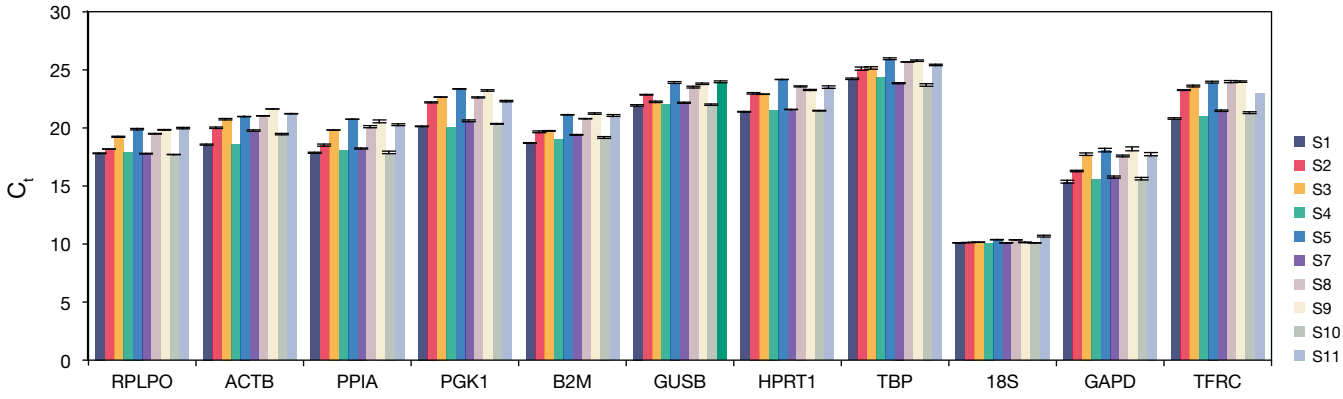


Figure 1. mRNA Expression of 11 putative human endogenous control genes in peripheral blood samples. Each bar represents one individual (S1–S5, S7–S11) and represents the average C_t (n = 4); error bars show StDev.

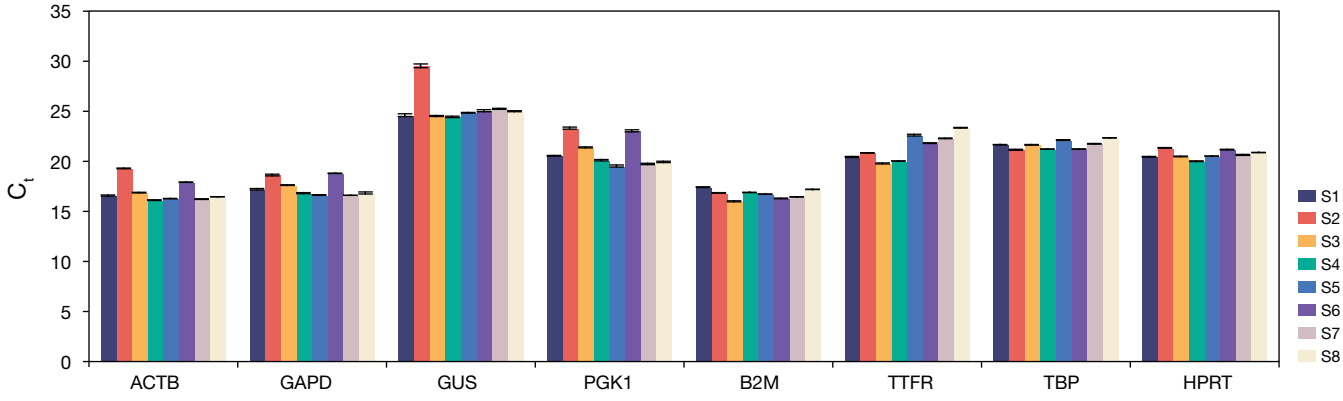


Figure 2. mRNA expression of 8 mouse endogenous control genes in peripheral blood samples. Each bar represents one individual and represents the average C_t (n = 4); error bars are StDev.

genes across the samples.

The expression profiles for the mouse TaqMan® Endogenous Controls are shown in Figure 2. The mouse genes HPRT, TBP, and B2M had the lowest StDev of C_t and thus the least variability across samples (Figure 5). These data indicate that these three genes are the best control candidates for this experimental study with very similar StDev of C_t at 0.43, 0.44, and 0.46, respectively. GUS and PGK1 gave the highest variance (2.86 and 2.21, respectively) and are not recommended.

In both human and mouse studies, there was no correlation between C_t values (expression level) and variability; that is, high expressors with C_t values less than 20 were no more or less variable than moderate expressors with C_t values greater than 20.

Note: C_t average and StDev calculations were performed using Microsoft® Excel® software. Analysis for the TaqMan® Array Card was done with SDS software v.2.1.

TaqMan® Array Cards—A convenient screening tool

The TaqMan® Array Endogenous Control Card allows for rapid screening of many samples across many genes (Figure 3). We screened 16 human housekeeping genes (Table 1) with 16 different tissues using two cards. Each assay and sample was run in triplicate and as described above, the average C_t of each gene for each sample was used to calculate the average C_t and StDev of C_t for each gene across the sample set. Figure 3 shows the average C_t of each sample for the 16 different TaqMan® Assays and Figure 6 shows the StDev of C_t for each assay across the 16 tissues.

RPLPO (0.68), 18S (0.71), and HMBS (0.72) were the most stable, and showed the least variation in transcription across the set of tissues.

GAPD and ACTB, which are commonly used as endogenous controls often without testing (as well as 18S), showed much higher variability across human and mouse samples and human tissues than any of the best candidates. These data show that GAPD and ACTB are not appropriate control genes for these studies, and confirm the need to screen a series of genes to identify the appropriate control.

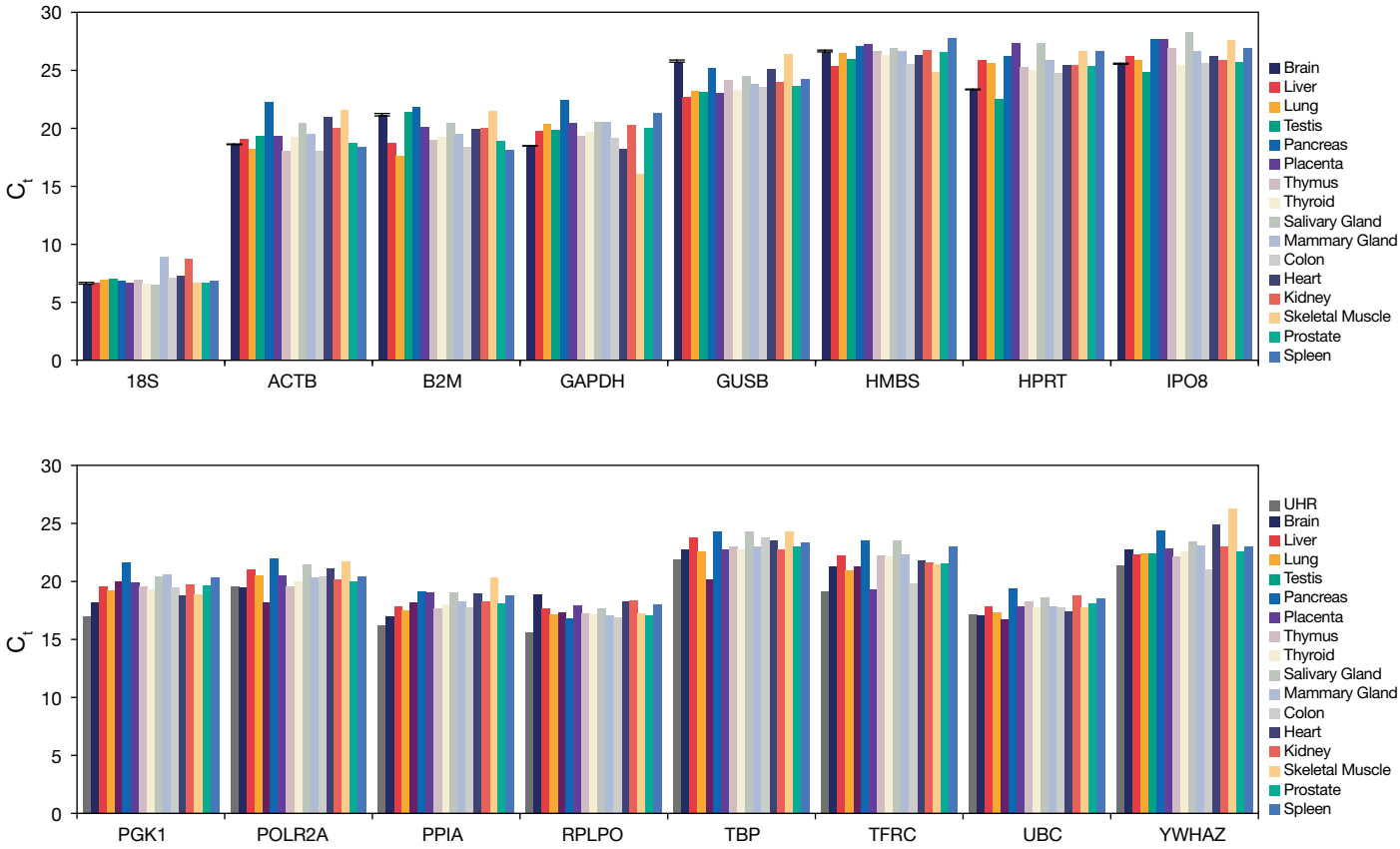


Figure 3. TaqMan® Array Endogenous Control Card. mRNA expression of 16 putative human endogenous controls across 16 tissues. Each bar represents one individual and measures the average C_t (n = 3); error bars are StDev.

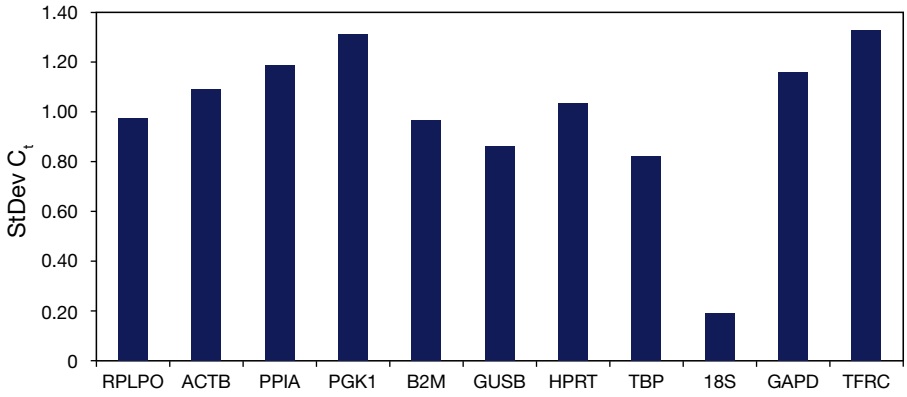


Figure 4. Human endogenous controls. Variation of 11 human endogenous controls across 11 samples as measured by StDev of C_t.

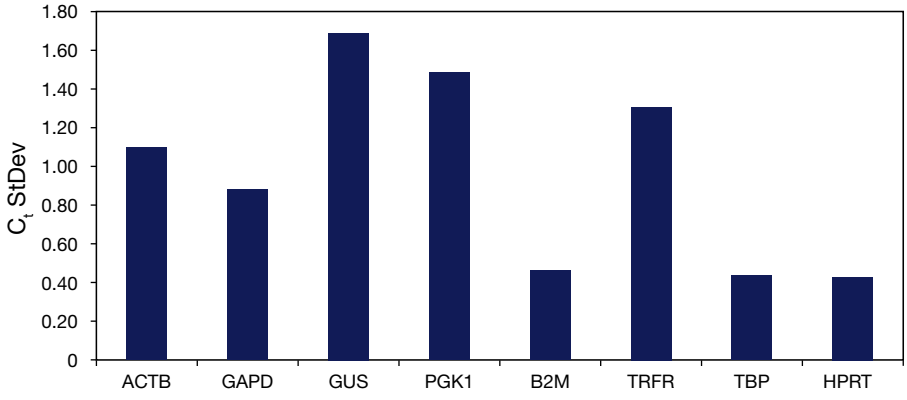


Figure 5. Mouse endogenous controls. Variation of eight mouse endogenous controls across eight samples as measured by StDev of C_t.

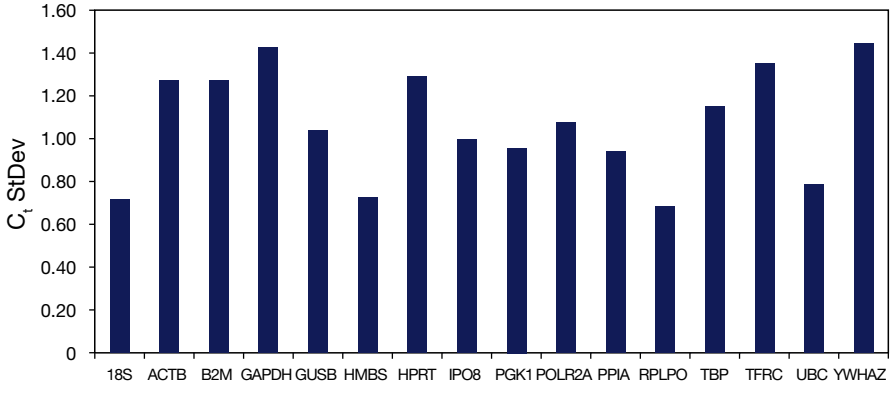


Figure 6. Human endogenous control panel. Variation of 16 human endogenous controls across 16 samples as measured by StDev of C_t.

Conclusion

For meaningful gene expression measurements, an internal or endogenous control gene with a constant expression level is essential to control for differences between samples. TaqMan® Endogenous Controls consist of the most commonly used housekeeping genes in human, mouse, and rat, and are provided as a preformulated set of predesigned probe and amplification primers.

The data presented here demonstrate the use of TaqMan® Endogenous Controls to quickly and easily identify multiple candidate endogenous control genes that can be used to normalize gene expression data within a defined experimental study.

References

1. Radonic A, Thulke S, Mackay IM et al. (2004) Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Comm* 313:856–862.
2. Schmid H, Cohen CF, Henger A et al. (2003) Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies. *Kidney Int* 64:356–360.
3. de Kok JB, Roelofs RW, Giesendorf BA et al. (2005) Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Laboratory Invest* 85:154–159.
4. Lossos IS, Czerwinski DK, Wechser MA et al. (2003) Optimization of quantitative real-time RT-PCR parameters for the study of lymphoid malignancies. *Leukemia* 17:789–795.
5. Life Technologies Application Note: Amplification Efficiency of TaqMan® Gene Expression Assays.