Using TaqMan Endogenous Control Assays to select an endogenous control

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. Inherent differences between samples, 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 of 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 the glycolytic pathway (e.g., GAPDH, PGK1) and protein folding (e.g., PPIA) or structural components such as the cytoskeleton (ACTB). Several genes, including those for 18S rRNA, ACTB, and GAPDH, have been widely used to normalize expression, but 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 HPRT1 [3], GUSB, 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 qPCR 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 Applied Biosystems™ 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 Applied Biosystems™ TaqMan® Array Human Endogenous Control Panel (Cat. No. 4367563), which contains 16 human endogenous control candidates, to identify genes with the most stable expression across 16 different human tissues.

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Methods

TaqMan Endogenous Controls are available as inventoried Applied Biosystems™ TaqMan® Gene Expression Assays (Cat. No. 4331182) and are identified by a specific TaqMan Assay ID. These assays include the 16 listed in Table 1, which are also the ones that are included on the TaqMan Array Human Endogenous Control Panel. Each assay on the array card is spotted in triplicate.

Note: The TaqMan Endogenous Control Assays are also available individually in larger volumes, with separate part numbers and with a choice of FAM™ (medium, Cat. No. 4351370; large, Cat. No. 4351368) or VIC™ (small, Cat. No. 4448489; medium, Cat. No. 4448490; large, Cat. No. 4448491) dye labels. Assays can also be ordered as primer-limited with a VIC dye label (small, Cat. No. 4448484; medium, Cat. No. 4448485; large, Cat. No. 4448486).

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 from human and mouse peripheral blood samples was reverse transcribed using the Applied Biosystems™ High Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814) to generate cDNA. For each assay, 50 ng of cDNA was amplified with Applied Biosystems™ TaqMan® Universal PCR Master Mix (Cat. No. 4324018) and the TaqMan Endogenous Control Assay. 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 cDNA sample was loaded into one of 8 sample ports of a TaqMan Array Human Endogenous Control Panel. 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.

Table 1. TaqMan Gene Expression Assays featured in this study.

Gene ID	Gene name	Accession number	Assay ID
RPLP0	Ribosomal protein lateral stalk subunit P0	NM_001002	Hs99999902_m1
ACTB	Actin, beta	NM_001101	Hs99999903_m1
PPIA	Peptidylpropyl isomerase A	NM_021130	Hs99999904_m1
PGK1	Phosphoglycerate kinase 1	NM_000291	Hs99999906_m1
B2M	Beta-2-microglobulin	NM_004048	Hs99999907_m1
GUSB	Glucuronidase, beta	NM_000181	Hs99999908_m1
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_000194	Hs99999909_m1
TBP	TATA box–binding protein	M34960	Hs99999910_m1
18S	Eukaryotic 18S ribosomal RNA	X03205	Hs99999901_s1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Hs99999905_m1
TFRC	Transferrin receptor	NM_003234	Hs99999911_m1
IPO8*	Importin 8	NM_006390	Hs00183533_m1
POLR2A*	Polymerase (RNA) II (DNA-directed) polypeptide A	NM_000937	Hs00172187_m1
UBC*	Ubiquitin C	NM_021009	Hs00824723_m1
YWHAZ*	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	NM_003406	Hs00237047_m1
HMBS*	Hydroxymethylbilane synthase	NM_000190	Hs00609297_m1

*For this study, these assays were run only on the TaqMan Array Human Endogenous Control Panel.

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 up to 10 individual peripheral blood samples from human and mouse. Each TaqMan Assay was run in quadruplicate for each sample.

C_t average and standard deviation (StDev) calculations were performed using Microsoft™ Excel™ software. Analysis for the TaqMan Array Card was done with SDS software v.2.1. The average C_t with StDev for each sample and endogenous control assay is shown in Figure 1 for human samples, Figure 2 for mouse samples, and Figure 3 for the TaqMan Array Human Endogenous Control Panel. 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 variability among the 11 genes examined (Figure 4).

From these data, the human endogenous control genes with the lowest StDev, and therefore, the lowest variability 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 rRNA is a component of a ribosomal 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.

Lossos 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 the resulting C_t values did not correct for RNA quality differences, they decided it was not useful for their samples. In parallel, they had identified PGK1 and 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 genes across the samples.

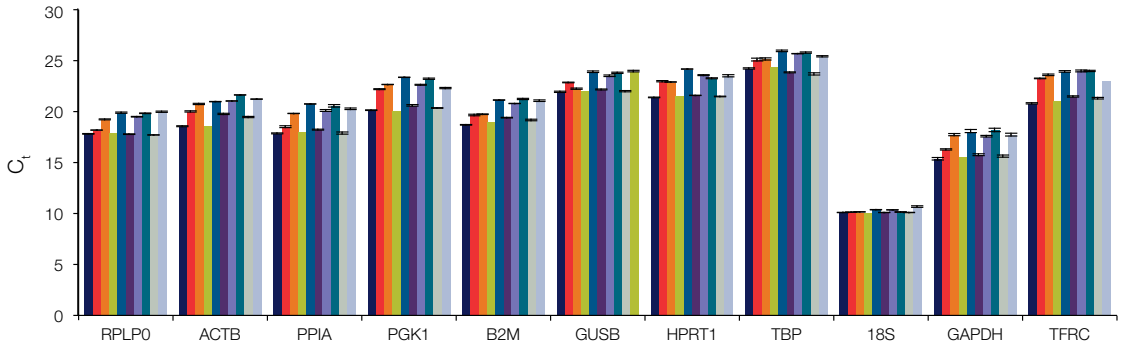

Figure 1. mRNA expression of 11 candidate human endogenous control genes in peripheral blood samples. Each color represents one individual. C_t values are averages of 4 replicates; error bars are StDev.

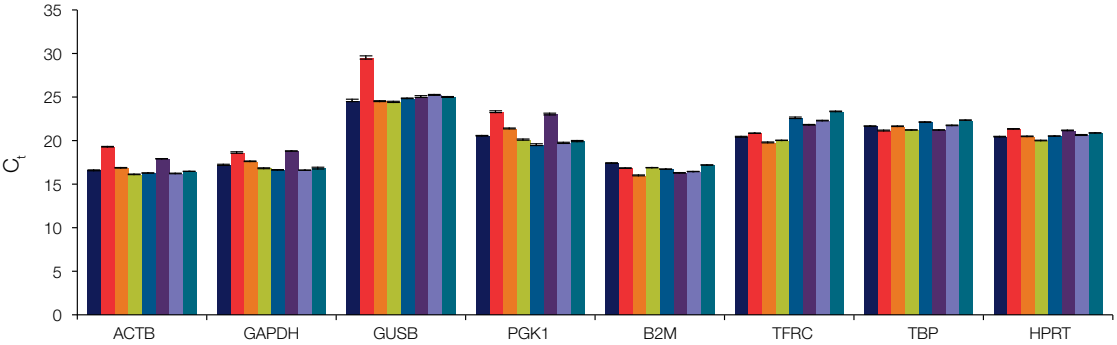

Figure 2. mRNA expression of 8 candidate mouse endogenous control genes in peripheral blood samples. Each color represents one individual. C_t values are averages of 4 replicates; error bars are StDev.

The expression profiles of the mouse TaqMan Endogenous Controls are shown in Figure 2. The mouse genes HPRT, TBP, and B2M had the lowest StDev of C_t of 0.43, 0.44, and 0.46, respectively (Figure 5). These data indicate that these three genes are the best control candidates for this experimental study. GUSB and PGK1 gave the highest StDev values (2.86 and 2.21, respectively) and are not recommended.

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

TaqMan Array Cards—a convenient screening tool

The TaqMan Array Human Endogenous Control Panel 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 as described above, and 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.

RPLP0 (0.68), 18S (0.71), and HMBS (0.72) were the least variable in transcription across the set of tissues.

GAPDH 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 indicated by this study. These data show that GAPDH and ACTB may be inappropriate control genes, and confirm the need to screen a series of genes to identify an appropriate endogenous control.

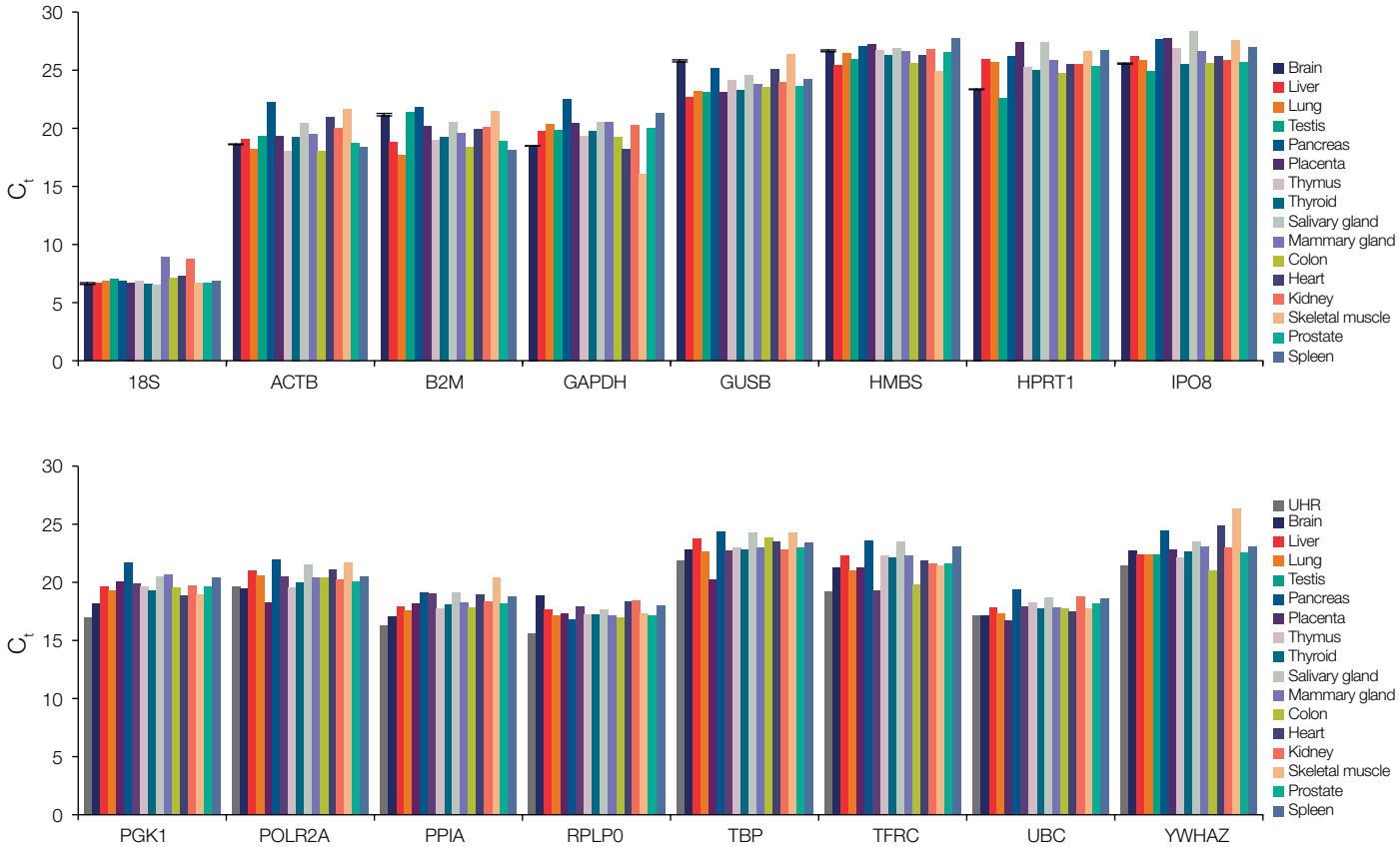

Figure 3. mRNA expression of 16 candidate endogenous controls comprising the TaqMan Array Human Endogenous Control Panel, across 16 tissue types. Each color represents one tissue type. C_t values are averages of 3 replicates; error bars are StDev. UHR = universal human reference RNA.

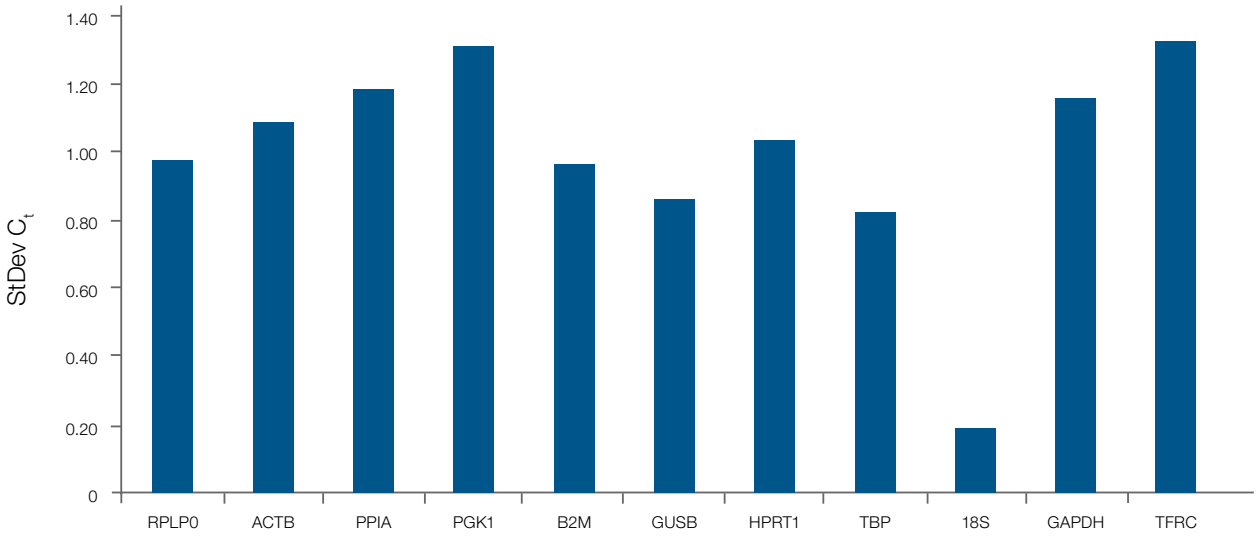

Figure 4. Human endogenous controls. Variability of 11 human endogenous controls across 10 samples, as measured by StDev of C_t.

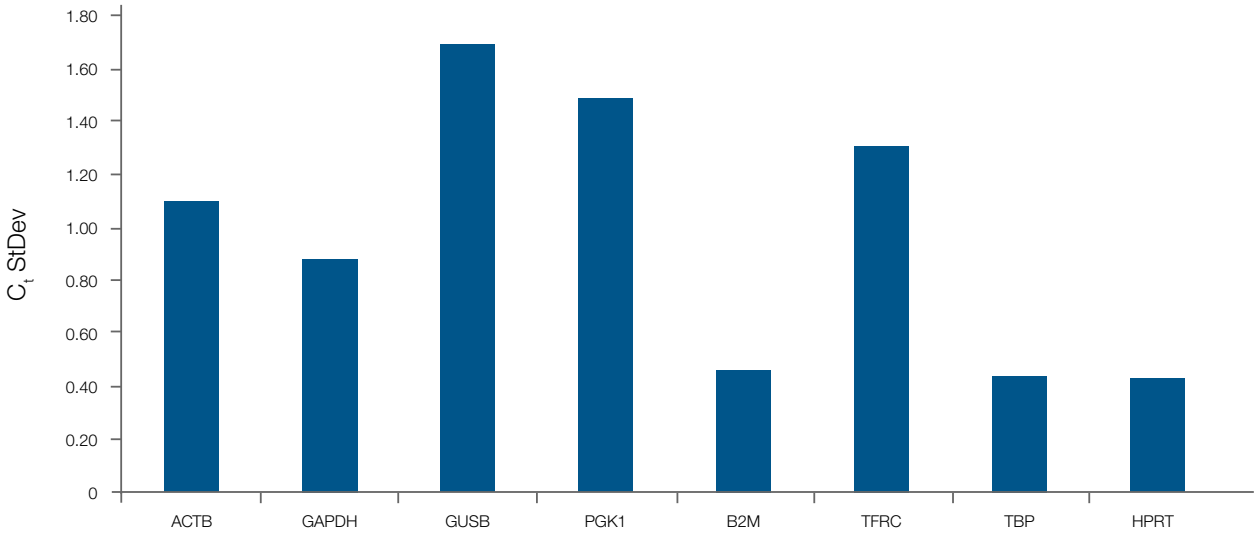

Figure 5. Mouse endogenous controls. Variability of 8 mouse endogenous controls across 8 samples, as measured by StDev of C_t.

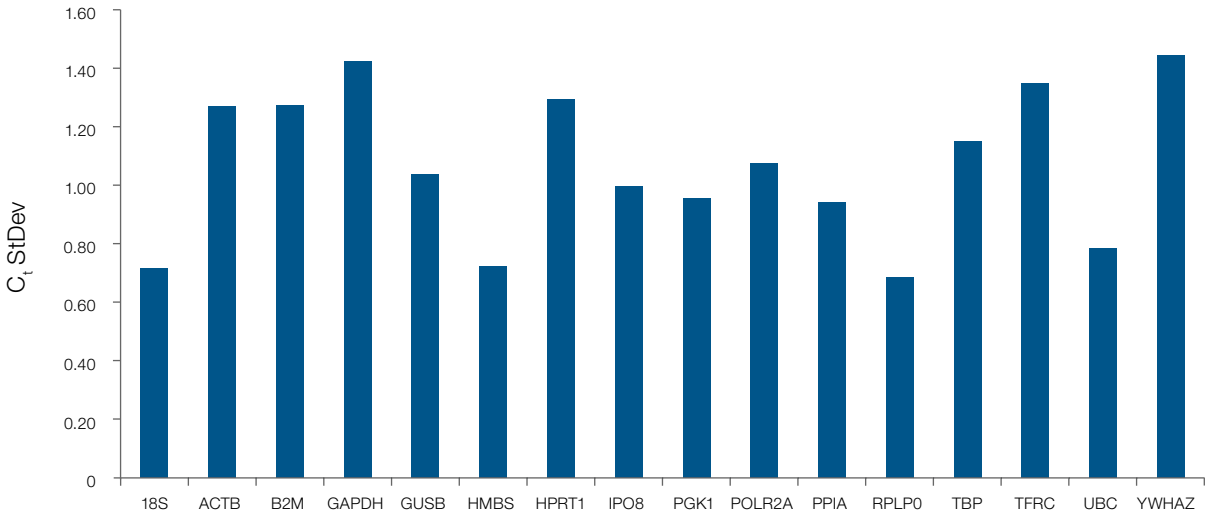

Figure 6. TaqMan Array Human Endogenous Control Panel. Variability of 16 human endogenous controls across 16 tissue types, as measured by StDev of C_t.

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Conclusion

For meaningful gene expression measurements, an internal or endogenous control gene with a constant expression level is essential to normalize for differences between samples. TaqMan Endogenous Controls represent 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 candidates for endogenous control genes that can be used to normalize gene expression data within a defined experimental study.

Ordering information

Product	Format	Cat. No.
TaqMan Human Endogenous Control Panel	0.1 mL 96-well plate	4426700
TaqMan Human Endogenous Control Panel	0.2 mL 96-well plate	4396840
TaqMan Human Endogenous Control Panel	384-well plate	4398985*
TaqMan Human Endogenous Control Panel	384-well array card	4367563
TaqMan Mouse Endogenous Control Panel	0.1 mL 96-well plate	4426699
TaqMan Mouse Endogenous Control Panel	0.2 mL 96-well plate	4426701
TaqMan Mouse Endogenous Control Panel	384-well array card	4378702
TaqMan Rat Endogenous Control Panel	0.1 mL 96-well plate	4426697
TaqMan Rat Endogenous Control Panel	0.2 mL 96-well plate	4426698
TaqMan Rat Endogenous Control Panel	384-well array card	4378704

To order, contact **specialty_plates@thermofisher.com**

References

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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.
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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. Thermo Fisher Scientific application note: Amplification efficiency of TaqMan Gene Expression Assays.

Find out more at **thermofisher.com/taqmancontrols**

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