Quick notes

Control variables that may introduce errors such as quantity and quality of starting material.

Validate the stability of a panel of different housekeeping (HK) genes for the cells under investigation, or in response to experimental treatment.

Confirm consistent, high qPCR efficiencies (should be 95% – 105%) for all housekeeping genes and gene of interest (GOI) by the CT slope method.

Use geNorm[5] to determine the most suitable stably expressed housekeeping genes for use in the study.

Use KAPA SYBR® FAST qPCR Kits to ensure high amplification efficiencies across all genes.

Microarrays and quantitative real-time PCR (qPCR) are common methods for investigating differential patterns of gene expression.

Relative quantification using qPCR measures the changes in steady-state mRNA levels of a gene across multiple samples normalized to a reference gene(s). In theory, the expression levels of the reference gene (often referred to as the housekeeping gene) should remain stable in the tissues or cells under investigation or in response to the experimental treatment. In practice, there is considerable evidence that housekeeping gene expression varies significantly.[1–6]

Introduction

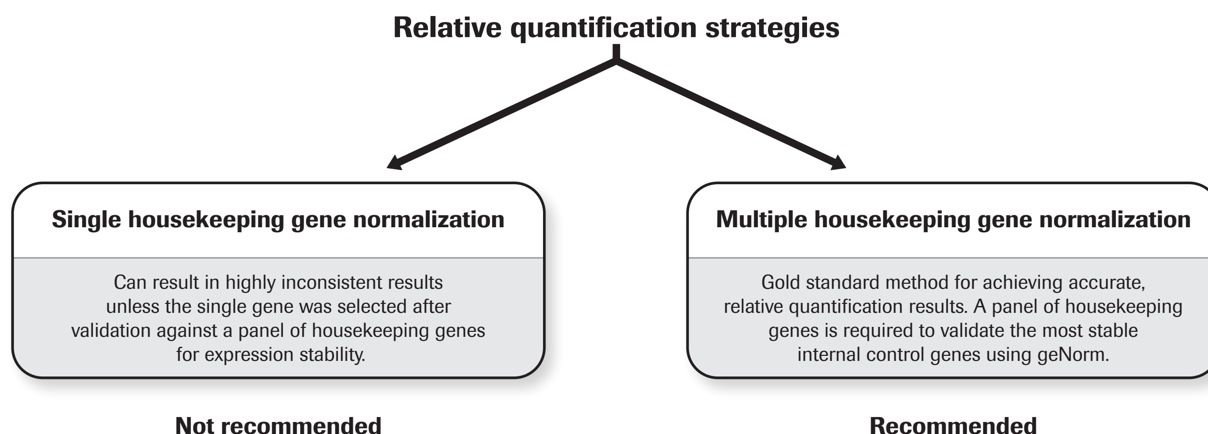
Despite this fact, many gene expression studies still make use of internal control gene(s) without validation of the presumed stability of expression. The geNorm algorithm developed by Vandosompele, et al. (2002)[5] enables rapid and accurate determination of the most stable reference genes from a set of tested genes in a given cDNA sample and is considered the gold standard for determining the most suitable set and number of housekeeping genes to use for accurate relative quantification.

One challenge when using multiple housekeeping genes for relative quantification is the requirement for high-amplification efficiencies (95 – 105%) across all genes, regardless of amplicon length, complexity, or GC content. KAPA SYBR FAST qPCR Kits contain the first DNA polymerase engineered specifically for SYBR Green I-based qPCR through a process of molecular evolution. The KAPA SYBR DNA Polymerase exhibits improved speed, processivity and robustness, resulting in consistently high amplification efficiencies required for accurate relative quantification using a panel of diverse housekeeping genes (see Application Note: **Gene Expression I: Housekeeping Genes**).

The aim of this Application Note is to highlight the potential drawbacks of using single housekeeping genes in relative quantification analysis using the ΔΔCT method. The multiple housekeeping gene approach using geNorm is presented as the preferred method against which relative quantification results from different methods are compared. The benefits of using the novel KAPA SYBR FAST qPCR Kit in the context of gene expression analysis using multiple housekeeping genes are also highlighted.

Experimental model and typical results

In this study, the expression levels of T-box factor 2 (*Tbx2*) in the human breast cancer cell line, MCF-7, were monitored at three different time points (t=0 hrs, t=8 hrs, and 24 hrs) after treatment with a specific drug. To demonstrate the dramatic effect on relative quantification results using different housekeeping genes either as single or multiple normalization control genes, ten commonly used housekeeping genes were selected. In an effort to reduce the chances that genes might be co-regulated, special attention was given to selecting genes that belong to different functional classes. Amplicon, primer, and qPCR efficiency details for all primers used in this study can be found in the KAPA SYBR® FAST Application Note **Gene Expression I: Housekeeping Genes**.



Two workflows were followed to illustrate the potential problems associated with performing relative gene expression analysis using single and multiple housekeeping gene normalization methods, respectively.

In **Workflow A**, relative quantification results were compared using two different housekeeping genes. When the stable (as determined by geNorm analysis) housekeeping gene, *HMBS*, was used for normalization, the relative expression levels of *Tbx2* decreased from 1.00 to 0.75, to 0.76 at time points 0 hrs, 8 hrs, and 24 hrs post-treatment, respectively. When the relatively unstable housekeeping gene, *SDHA*, was used for normalization, the relative expression levels of *Tbx2* decreased from 1.00 to 0.84 to 0.45 at time points 0 hrs, 8 hrs, and 24 hrs post-treatment, respectively.

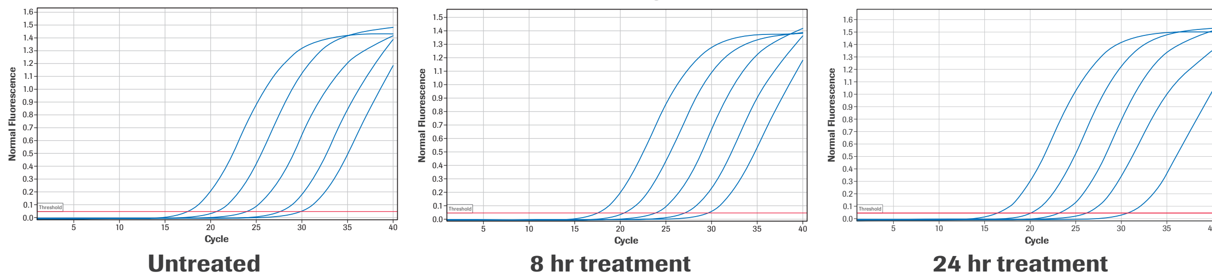
If these housekeeping genes had not been validated for expression stability, it would have been impossible to determine which of these two results was accurate.

In **Workflow B**—the current gold standard method for relative quantification—using a combination of the most stable housekeeping genes for the cell under investigation and in response to the experimental conditions, is presented. All ten housekeeping genes are systematically compared with each other resulting in an average expression stability plot. This output ranks each housekeeping gene in order of expression stability. A combination of the most stable housekeeping genes is then used in relative quantification analysis. Using this multiple, validated housekeeping gene approach the relative expression levels of *Tbx2* decreased from 1.00 to 0.75 to 0.71 at time points 0 hrs, 8 hrs, and 24 hrs post-treatment, respectively. As expected, these results are very similar to those obtained when the single most stable gene, *HMBS*, was used for relative quantification analysis.

Workflow A: Application of a single housekeeping gene to calculate relative gene expression levels ($2^{-\Delta\Delta CT}$ method)

Step 1:
- Confirm qPCR efficiencies (should be 95% – 105%) for housekeeping (HK) gene and gene of interest (GOI)
- Perform five $\log_{10}$-fold dilutions of cDNA for each HK gene and GOI to determine PCR efficiency (100 ng – 10 pg/reaction)

Amplification curves for *SDHA* gene for each cDNA sample



Step 2:
- Use 100 ng cDNA/reaction C_T replicate values for ΔC_T calculation
- Select C_T of each cDNA sample at 100 ng/reaction in triplicate for HK gene and GOI

Average replicate C_T values for each gene at 100 ng/reaction

	Tbx2 (GOI)	***HMBS*** (HK1)	***SDHA*** (HK2)
t=0 hrs	19.81	19.54	17.47
t=8 hrs	20.14	19.45	17.55
t=24 hrs	20.08	19.41	16.59

Step 3:
- Use the $\Delta\Delta C_T$ method for calculating relative quantification
- Relative fold change in gene expression = $2^{-\Delta\Delta C_T}$
- Where: $\Delta\Delta C_T = \Delta C_{T\ time\ x} - \Delta C_{T\ time\ 0}$, and $\Delta C_T = (C_{T\ GOI} - C_{T\ HK})$

Relative fold change in *Tbx2* expression when two different HK genes are used

	HMBS (most stable)	***SDHA*** (least stable)
t=0 hrs	1.00	1.00
t=8 hrs	0.75	0.84
t=24 hrs	0.76	0.45

Workflow B: Application of multiple housekeeping genes to calculate gene expression levels

Step 1:
- Confirm qPCR efficiencies (should be 95 – 105%) for HK and GOI
- Perform five log_{10}-fold dilutions of cDNA for each HK gene and GOI to determine PCR efficiency (100 ng – 10 pg/reaction)

Step 2:
- Use 100 ng cDNA/reaction C_T replicate values for ΔC_T calculation
- Select C_T of each cDNA sample at 100 ng/reaction in triplicate for HK gene and GOI

Average replicate C_T values for each gene at 100 ng/reaction

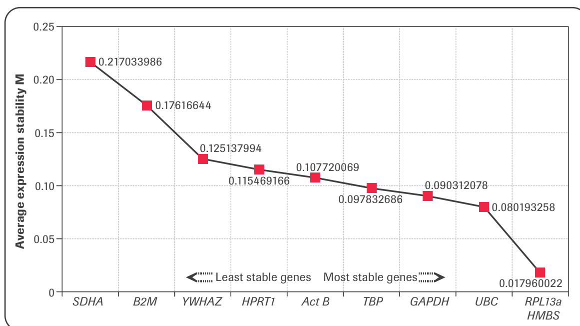
	Tbx2 (GOI)	*RPL13a* (HK1)	*HMBS* (HK2)	*UBC* (HK3)	*SDHA* (HK4)	*HPRT1* (HK5)	*ActB* (HK6)	*YWHAZ* (HK7)	*TBP* (HK8)	*B2M* (HK9)	*GAPDH* (HK10)
t=0 hrs	19.81	12.94	19.54	14.79	17.47	18.89	12.14	14.90	20.21	16.03	15.40
t=8 hrs	20.14	12.85	19.45	14.67	17.55	19.02	12.00	14.79	20.26	16.22	15.24
t=24 hrs	20.08	12.77	19.41	14.43	16.59	18.67	11.65	14.84	19.97	15.34	14.98

Step 3:
- Convert C_T values to relative quantities for geNorm input
- Relative quantities for each gene=$E^{(\text{Minimum } C_T \text{ - Sample } C_T)}$, E=2 for 100% efficiency

Relative quantities of each gene at each time point

	Tbx2 (GOI)	*RPL13a* (HK1)	*HMBS* (HK2)	*UBC* (HK3)	*SDHA* (HK4)	*HPRT1* (HK5)	*ActB* (HK6)	*YWHAZ* (HK7)	*TBP* (HK8)	*B2M* (HK9)	*GAPDH* (HK10)
t=0 hrs	1.00	0.89	0.91	0.78	0.54	0.86	0.71	0.93	0.85	0.62	0.75
t=8 hrs	0.80	0.95	0.97	0.85	0.51	0.78	0.78	1.00	0.82	0.54	0.84
t=24 hrs	0.83	1.00	1.00	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00

Step 4:
- Use geNorm to calculate the geometric mean of the most stable reference genes to obtain the normalization factor



Calculation of normalization factors for most stable reference genes

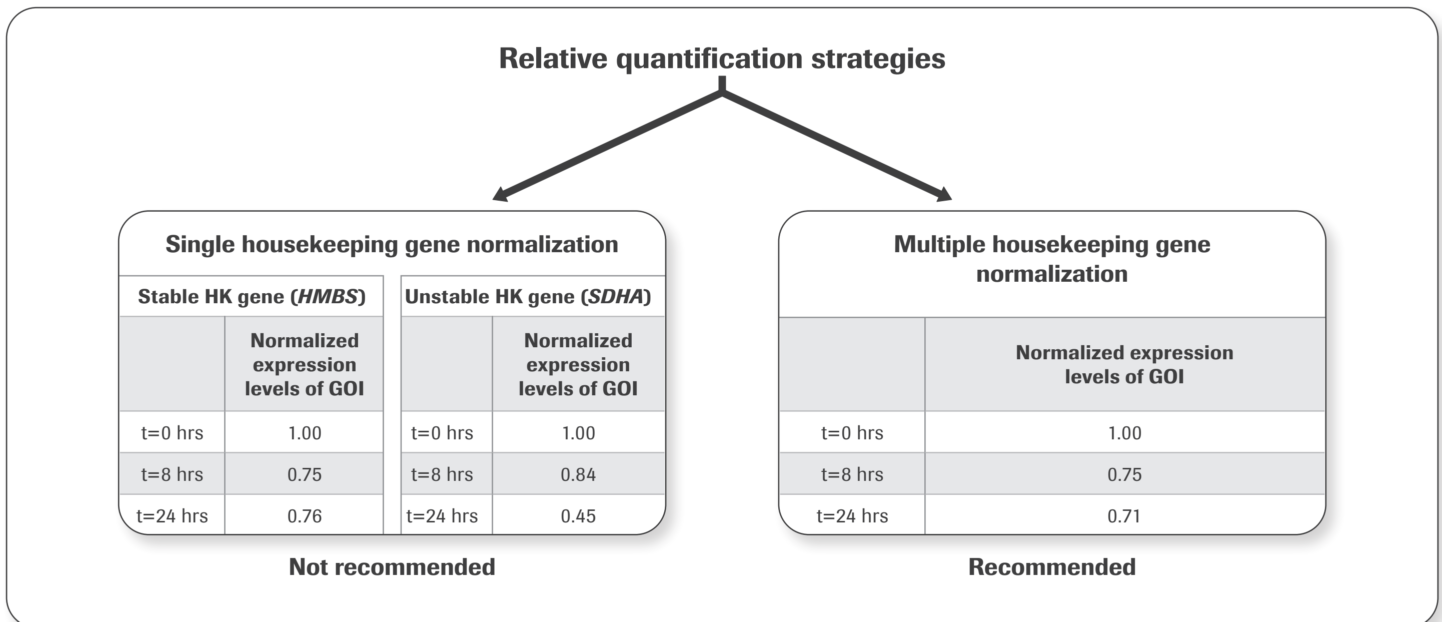
	RPL13a	*UBC*	*HMBS*	Norm. factor
t=0 hrs	0.89	0.78	0.92	**0.86**
t=8 hrs	0.95	0.84	0.97	**0.92**
t=24 hrs	1.00	1.00	1.00	**1.00**
M <1.5	**0.06**	**0.11**	**0.069**	

Step 5:
- Calculate relative GOI expression levels by dividing the GOI quantity by the normalization factor at each time point
- Normalize the results

	Tbx2 (GOI)	Normalization factors	Relative expression levels	Relative fold change in *Tbx2* expression
t=0 hrs	1.00	0.86	1.16	**1.00**
t=8 hrs	0.80	0.92	0.86	**0.75**
t=24 hrs	0.83	1.00	0.83	**0.71**

Conclusions

Accurate normalization of gene expression levels is an absolute prerequisite for reliable results, especially when the biological significance of subtle gene expression differences is studied. Vandersompele, et al.[5] showed that by using conventional normalization strategies based on a single housekeeping gene, erroneous normalization of up to 3.0- and 6.4-fold in 25% and 10% of cases, respectively, were observed. Certain cases showed error values in excess of 20-fold.



The data presented in this Application Note demonstrates a similar trend, although the differences observed were less dramatic due to the fact that all housekeeping genes displayed relatively high stabilities under the specific experimental conditions. When relative quantification was performed using a combination of the most stable housekeeping genes (as determined by geNorm), the relative expression levels of *Tbx2* decreased from 1.00 to 0.75 to 0.71 at time points t=0 hrs, 8 hrs, and 24 hrs respectively. As expected, when the least stable gene (*SDHA*) was used for single housekeeping gene normalization, the greatest variation in expression ratio was obtained, namely 0.84 (t=8 hrs) and 0.45 (t=24 hrs), relative to 1.00 at t=0 hrs. Conversely, when the most stable gene (*HMBS*) was used for single housekeeping gene normalization, the greatest concordance with multiple gene normalization was obtained (0.75 and 0.76 at t=8 hrs and t=24 hrs, respectively, relative to 1.00 at t=0 hrs). This data confirms the findings of other large-scale studies,[6] namely that ideal and universal control genes do not exist. Normalization against multiple housekeeping genes is therefore a prerequisite for reliable relative gene expression analysis.

Methods

Total RNA was isolated from the human breast cancer cell line, MCF-7, using the NucleoSpin® RNA II kit (Macherey-Nagel). RNA was digested with DNase I to remove contaminating genomic DNA. The ImProm-II™ Reverse Transcription System (Promega) was used to generate cDNA from 1 µg RNA with oligo(dT) primers. The cDNA was used as template to determine the amplification efficiency for each housekeeping gene and the gene of interest, *Tbx2*, using the KAPA SYBR® FAST Universal qPCR Kit according to standard protocols. The reaction efficiency achieved for each gene was calculated using the C_T slope method, with five data points corresponding to log_{10}-fold MCF-7 cDNA serial dilutions (100 ng – 10 pg/reaction).

Consistent, high amplification efficiencies (95 – 104%) were achieved in all cases. For relative quantification calculations, C_T scores for the 100 ng MCF-7 cDNA/reaction at each of the three time points (average of triplicate determinations) were used.

References and acknowledgements

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