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Critical selection of internal control genes for quantitative real-time RT-PCR studies in lipopolysaccharide-stimulated human THP-1 and K562 cells ☆

Xi-mei Cao [a,1], Xu-guang Luo [b,1], Jun-hong Liang [c], Chao Zhang [c], Xiao-ping Meng [c], Da-wei Guo [c,*]

[a] Department of Histology and Embryology, Shanxi Medical University, Taiyuan, China
[b] Department of Microbiology and Immunology, Shanxi Medical University, Taiyuan, China
[c] Department of Forensic Science, Shanxi Medical University, Taiyuan, China

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ABSTRACT

The choice of internal control genes is important since it may affect the study outcome in RT-qPCR. Indeed, it is well-known that expression levels of traditional internal control genes can vary across tissue types and across experimental settings within one specific tissue type. The aim of this study is an evaluation of a set of housekeeping genes (HKGs) to be used in the normalization of gene expression *in vitro* different cultured cells, THP-1 and K562. The transcriptional stability of eleven potential internal control genes (RPL37A, ACTB, GAPDH, B_2M, PPIB, PGK1, PPIA, SDHA, TBP, HPRT1 and RPL13A) were evaluated using RT-qPCR and were compared in different treatment, that was un-stimulated or LPS-stimulated cells. The raw Ct values were determined for each candidate gene at different time points following LPS-stimulated or unstimulated cells. Furthermore, all data were analyzed by the geNorm, BestKeeper, and NormFinder validation programs. Results indicated that PPIB and PGK1 were the most stable internal control genes in this study. RPL13A was found to be the least stable. This study provides the comprehensive reported assessment of internal control genes for use in expression studies *in vitro* cultured cells. These findings further emphasize the need to accurately validate candidate internal control genes in the study before use in gene expression studies using RT-qPCR.

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1. Introduction

Gene-expression analysis is increasingly important in many fields of biological research. Understanding patterns of expressed genes is expected to provide insight into complex regulatory networks and will most probably lead to the identification of genes relevant to new biological processes, or implicated in disease [1]. Quantitative real-time reverse transcription PCR (RT-qPCR) is often considered the gold standard for quantifying gene expression, and is commonly used to validate techniques with greater throughput but less sensitivity, such as microarray analysis [2]. However, there are limitations to this technique including the inherent variability of RNA expression, RNA instability, and the need for high-quality starting material, variability in the efficiencies of enzymes and primers, and improper normalization and analysis techniques that can all lead to misinterpretation of results. Until now, various strategies have been applied to normalize these variations [3]. Internal control genes are most frequently used to normalize RT-qPCR data. This internal control – often referred to as a housekeeping gene (HKG) – should not vary in the tissues or cells under investigation, or in response to experimental treatment. Choosing an internal control gene to normalize gene expression data is also one of the most crucial steps in the experimental design [4]. And this crucial step can minimize the influence of confounding factors and improve the fidelity of the quantification process with respect to the specific biological conditions. However, many studies make use of these constitutively expressed control genes without proper validation of their presumed stability of expression.

Stimulation of monocytes with lipopolysaccharide (LPS), a cell membrane component of gram-negative bacteria, is a frequently employed model system to study inflammatory responses [5,6]. A thorough review of the literature of the last years indicated that most of the published RT-qPCR results in LPS-stimulated monocytes were normalized to either ACTB or GAPDH alone, although normalization to a single internal control gene is only rarely justified [7]. However, different experiments should have different selection criteria. To investigate whether traditional internal control genes are stably expressed in LPS-stimulated cells apart from monocytes, we have assessed and evaluated gene expression stability of eleven candidate internal control genes in the present study. And we apply several novel criteria, including (1) how

☆ All authors provided acquisition of data and gave final approval of the manuscript submitted.

* Corresponding author. Address: Department of Forensic Science, Shanxi Medical University, 56 South Xinjian Nan Road, Taiyuan 030001, Shanxi, China.
E-mail address: guo8dawei@yahoo.com.cn (D.-w. Guo).

[1] These two authors contributed equally to this work.

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internal control genes perform on different sample testing, (2) whether internal control genes expression change significantly with inflammatory stimuli. mRNA expression levels of eleven candidate internal control gene were assessed by RT-qPCR in human THP-1 monocytic leukemia cell line and human erythroleukemic cell line K562 under various experimental conditions. The quality assessments described, are in line with the recently published MIQE guides (Minimum Information for Publications of Quantitative Real-Time PCR Experiments) [7]. Thus, critical selection of internal control genes for quantitative real-time RT-PCR studies in THP-1 and K562 may be important for understanding the pathophysiological process of inflammation.

2. Material and methods

2.1. Cells culture and inflammatory stimuli

Human THP-1 monocytic leukemia cell line and human erythroleukemic cell line K562 were obtained from Shanghai Institutes for Biological Sciences. THP-1 and K562 cells were cultured in RPMI 1640 (Hyclone, SH30809.01B) supplemented with 10% fetal calf serum (Gibco, 10099-141) and 100 U/ml penicillin–streptomycin (Beijing Solarbio Science & Technology Co., Ltd., P1400–100). Cells were incubated at 37 °C under 5%CO_2 and subcultured 1:4 every 96 h. Parts of THP-1 and K562 (2×10^5/ml) cells were stimulated with 10 μg/ml lipopolysaccharide (LPS,Sigma-L2880) at different time points, from 2 to 24 h. Finally, THP-1 and K562 were collected for RT-PCR analysis before and at various times after stimulation.

2.2. Isolation of total RNA

Total cellular RNA was extracted using *TransZol* UP (Beijing TransGen Biotech Co., Ltd., ET111–01) from unstimulated or LPS-stimulated THP-1 and K562 cells according to the manufacturer's instructions. The concentration of freshly extracted total RNA was quantitated (in nanograms per microliter) using a UV-visible spectrophotometer (UItrospec 4300 pro, Biochrom Ltd., Cambridge, UK). The integrity of the isolated RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) by loading samples onto a eukaryote total RNA nano-chip.

2.3. Internal control genes primer design

Eleven potential internal control genes were chosen based on their common use as endogenous control genes in gene expression studies. Primers were designed based on sequences obtained from GenBank and imported into Allele ID6 software (Premier Biosoft International), a program designed to generate primer pairs suitable for real-time PCR. All assays were designed to span at least one intron. And the assays setting "SYBR Green Design" were chosen to limit primer sequences to regions with little secondary template structure. The designed primer sequences were validated on BLAST in order to ensure high efficiency. Primers were synthesized by Invitrogen Biotechnology. The sequences, length of products, and source sequences are listed in Table 1.

2.4. Real-time fluorescent quantitative PCR

The first-strand cDNA was synthesized using the Prime Script RT-PCR Kit (TakaRa Biotechnology Co. Ltd., Dalian, China) according to the standard protocol. For reverse transcription, 0.4 μg of total RNA was used in a reaction volume of 10 μL. Real-time PCR amplification was performed in a 20 μL reaction mixture that included 10 μL 2×SYBR Premix Ex Taq, 0.2 μL ROX, 0.4 μL (10 μM) of each primer, 1 μL cDNA and 8.4 μL ddH_2O as recommended in the manufacturer's instructions provided with SYBR® Premix Ex Taq™ (TaKaRa Code:DDR820A, Takara Biotechnology Co. Ltd., Dalian, China). Amplification was performed by one round of predenaturation at 95 °C for 5 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 55 °C for 30 s. Fluorescence signals were detected at the end of every cycle. All reactions were performed using the Mx3005P Real-Time PCR System (Stratagene Inc., La Jolla, CA). A standard curve was constructed to calculate the genespecific PCR efficiency over a 5-fold dilution series ($1:10^1$, $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$) of template cDNA for each primer pair. The efficiency values (Eff.) were obtained from the standard curves.

2.5. Data analysis

The PCR results were analyzed by Stratagene MxPro software. Baseline and threshold values were automatically set by the program. The number of PCR cycles required to reach the fluorescence threshold in each sample was defined as the Ct value, and each sample was analyzed in duplicate to obtain an average Ct for each sample. To confirm results, three different Microsoft Excel-based applets, geNorm [1], NormFinder [8], and BestKeeper [9], were also used. These bioinformatics packages calculate a stability value where a lower value indicates a higher stability (lower variability) in gene expression. The genes were ranked according to these gene stability values.

3. Results

3.1. Integrity of total RNA

When dealing with *in vitro* cell culture, it can also be difficult to estimate sample size (cell number) because cells will often clump up or have different morphologies, particularly when cultured as a monolayer. While ensuring a similar sample size is important it clearly is not sufficient on its own [3]. In this study, cells can be treated with 0.4% Trypan Blue to statistic cell viability and assist counting. Every samples, about 2×10^5 cells, were harvested and performed on an Agilent 2100 Bioanalyzer using the RNA 6000 LabChipsKit. Approximately 0.1–0.4 μg of total cellular RNA was extracted using the SV Total RNA Isolation System. Only those RNA samples with a RNA Integrity Number above 8.0 and with clearly visible 28/18S peaks were used for real-time PCR.

3.2. Calculation of the amplification efficiency of potential internal control genes

The linear regression for 10-fold dilution series of standard samples shows that the squared correlation coefficients (R^2) of the eleven potential internal control genes are greater than 99%. The dissociation curves of all eleven genes exhibit signal cusps, and the dissociation temperatures are all greater than 80 °C (Fig. 1A). The amplification efficiency of the eleven genes are $Eff._{ACTB}$ = 101.7%, $Eff._{GAPDH}$ = 102.6%, $Eff._{RPL37A}$ = 107.5%, $Eff._{B2M}$ = 106.4%, $Eff._{PPIB}$ = 94.4%, $Eff._{PGK1}$ = 106.5%, $Eff._{PPIA}$ = 101.9%, $Eff._{SDHA}$ = 115.3%, $Eff._{TBP}$ = 136.7%, $Eff._{HPRT1}$ = 122.8%, $Eff._{RPL13A}$ = 114.9%, respectively.

3.3. RT-qPCR and statistical analysis

3.3.1. Validation of potential internal control genes

The raw Ct values for the eleven primer pair from the different samples are analyzed by the Stratagene MxPro software and range from 11.66 to 35.36 which is acceptable for reliable RT-PCR

Table 1
Candidate internal control genes evaluated in this study.

Symbol	Accession number	Name	Strand	Nucleotide sequence	Position	Amplicon size (bp)
ACTB	NM_001101.3	Beta actin	Sense	5′-TTGGCAATGAGCGGTTCC-3′	833–850	92
			Antisense	5′-GTTGAAGGTAGTTTCGTGGATG-3′	924–945	
GAPDH	NM_002046.3	Glyceraldehyde-3-phosphate dehydrogenase	Sense	5′-CAACAGCGACACCCACTCCT-3′	960–979	115
			Antisense	5′-CACCCTGTTGCTGTAGCCAAA-3′	1054–1074	
RPL37A	NM_000998.4	Ribosomal protein L37a	Sense	5′-ATTGAAATCAGCCAGCACGC-3′	163–182	96
			Antisense	5′-GCAGGAACCACAGTGCCAGATCC-3′	236–258	
B_2M	NM_004048.2	Beta-2-microglobulin	Sense	5′-CGCTACTCTCTCTTTCTGG-3′	92–110	92
			Antisense	5′-ATTTGACTTTCCATTCTCTGC-3′	183–203	
PPIB	NM_000942.4	Peptidylprolyl isomerase B	Sense	5′-GTCCGTCTTCTTCCTGCTG-3′	229–247	110
			Antisense	5′-CATCTTCATCTCCAATTCGTAGG-3′	338–360	
PGK1	NM_000291.3	Phosphoglycerate kinase 1	Sense	5′-AAGAACAACCAGATAACAAACAAC-3′	260–283	102
			Antisense	5′-GTGGCTCATAAGGACTACCG-3′	361–380	
PPIA	NM_021130.3	Peptidylprolyl isomerase A	Sense	5′-ACTGGAGAGAAAGGATTTGG-3′	204–223	94
			Antisense	5′-CATTATGGCGTGTGAAGTC-3′	297–315	
SDHA	NM_004168.2	Succinate dehydrogenase complex subunit A	Sense	5′-GCCAGGACCTAGAGTTTGTTC-3′	975–995	110
			Antisense	5′-GCCTTGACTGTTAATGAGAATGC-3′	1084–1106	
TBP	NM_003194.4	TATA box binding protein	Sense	5′-AGAGTTCTGGGATTGTACC-3′	547–565	110
	NM_001172085.1		Antisense	5′-ATTATATTCGGCGTTTCGG-3′	656–674	
HPRT1	NM_000194.2	Hypoxanthine phosphoribosyl-transferase 1	Sense	5′-AGGATTTGGAAAGGGTGTTTATTC-3′	256–279	109
			Antisense	5′-CAGAGGGCTACAATGTGATGG-3′	364–384	
RPL13A	NM_012423.2	Ribosomal protein L13a	Sense	5′-GTTCCTGCTGCCCTCAAG-3′	380–397	101
			Antisense	5′-GTCACTGCCTGGTACTTCC-3′	480–498	

quantification. Fig. 1B presents the Ct values for each primer pair from different samples. However, the Ct values of B_2M from different samples show highest variation and should be excluded from further analysis in this study.

3.3.2. Determination of the stability of potential internal control genes by Normfinder

Normfinder analysis is a Visual Basic applet and a RT-qPCR data normalization tool that ranks the expression values of each of the potential internal control genes. The program calculates a stability value that is inversely correlated with the stability of gene expression. Therefore, a higher stability value indicates lower stability. All raw Ct values were transformed to relative quantities using the △△Ct method excepting B_2M (Table 2), and each point was calculated and ranked using Normfinder (Table 6). The results of the NormFinder analysis are presented in Fig. 2A and 2B.

3.3.3. Determination of the stability of potential internal control genes by geNorm

The geNorm program is a Visual Basic Application (VBA) applet for Microsoft Excel which defines the expression stability of a possible internal control gene by allocating a so-called *M* value to each gene in a pool of potential internal control gene [10]. An *M* value is the mean pairwise variation between an individual gene and the other putative reference genes tested [1]. Genes with the lowest *M* values have the most stable expression. The geNorm program also has a cutoff limit of variability suggesting that any gene with an $M < 1.5$ should be considered reliable as a stable internal control gene. For geNorm, all raw Ct values were transformed to relative quantities using the △△Ct method (Table 3), and each data point was calculated using geNorm. From the transformed data, geNorm produced a graph based on expression stability values (*M* values) (Fig. 3). The most stably expressed genes were PGK1 and GAPDH ($M = 0.2339$), followed by (from most to least stable) PPIB, SDHA, RPL37A, TBP, HPRT1, PPIA, ACTB and RPL13A. From these results, we suggest that the most commonly used ACTB should not be considered as reliable internal control genes for RT-qPCR analysis in this study.

3.3.4. Determination of the stability of potential internal control genes by BestKeeper

BestKeeper, another Excel-based tool, determines the variability in expression of a set of internal control genes by analyzing quantification cycle (Cq) values and classifying variability by the coefficient of variance (CV) and the standard deviation (SD) [10]. Raw Ct values are imported directly into the Microsoft Excel-based BestKeeper program. To define the most stable internal control gene, the software generates a BestKeeper index which finally is compared to each candidate gene resulting in a value for the Pearson correlation coefficient (*r*) and probability which are then allocated to each candidate internal control gene. And BestKeeper determines the optimal internal control genes by employing a pairwise correlational analysis of all pairs of candidate genes and calculates the geometric mean of the best choice [9]. It is recommended that any gene with a standard deviation of greater than 1 ($SD > 1$) should be excluded from consideration as a stable internal control genes. The descriptive statistics from our research are presented in Table 4. A range of standard deviations were observed, from 0.68 for ACTB to 2.19 for RPL13A. Those with a standard deviation less than 1 were listed in the Table 5. Using the internal control gene data sets from the remaining candidates for a new BestKeeper index calculation, ACTB was shown to be the most correlated ($r = 0.898$, $p = 0.001$). PPIA followed by ACTB. However, the remaining potential internal control genes should not be considered as reliable internal control genes for RT-qPCR analysis in this study.

When taking geNorm, BestKeeper and NormFinder-analyzed data of all samples together, positions for top and bottom ranked genes were fairly constant (Table 6). The top two ranked genes by NormFinder and geNorm included PPIB and PGK1, while RPL13A was listed at the bottom of the ranking in both softwares. Ranking slightly differed when using BestKeeper, but this applet also assigned ACTB and PPIA as reliable genes when all samples were analyzed.

4. Discussion

RT-PCR is often considered the gold standard for quantifying gene expression, and is commonly used to validate techniques with greater throughput but less sensitivity, such as microarray

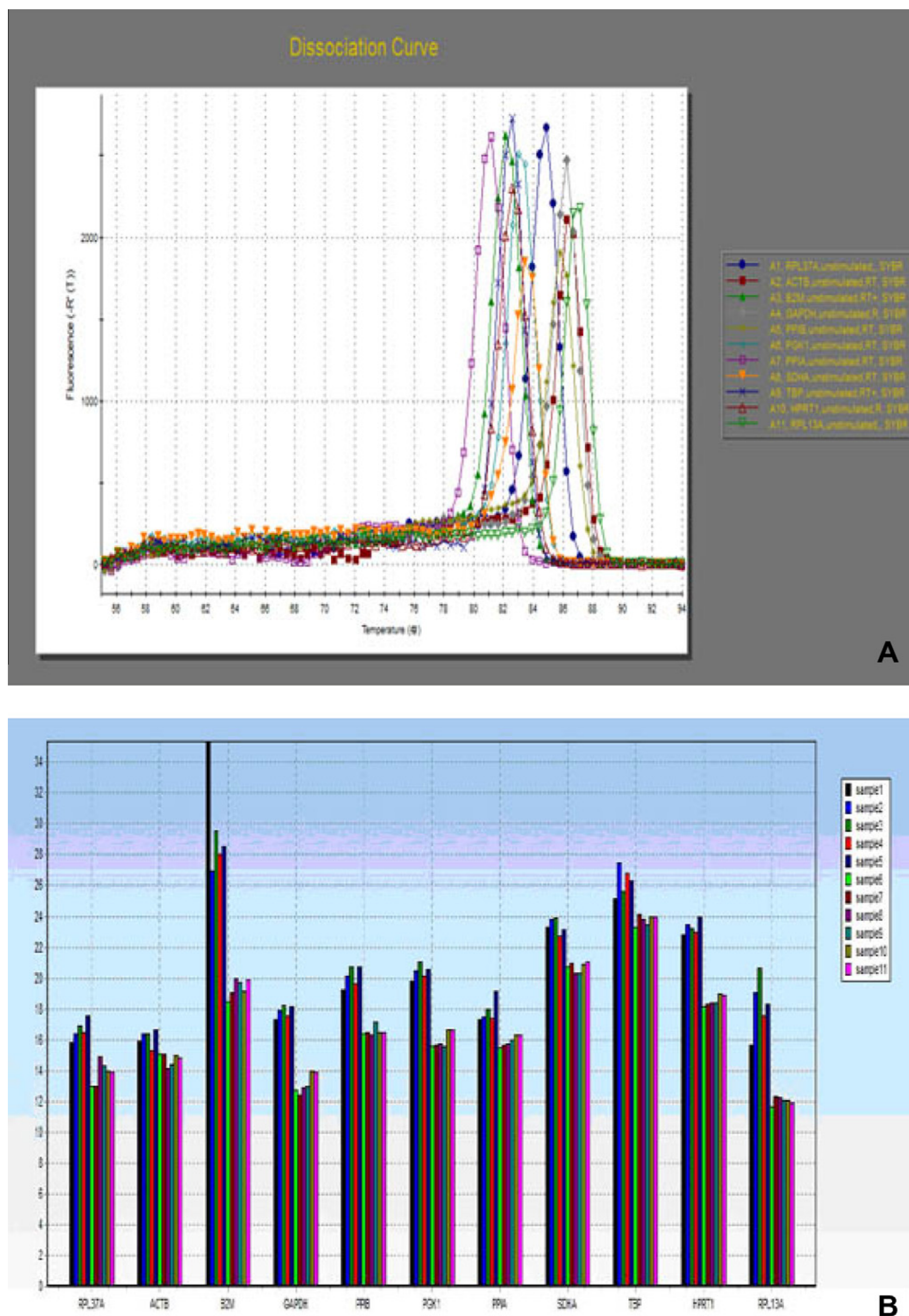

Fig. 1. Graph of the dissociation curves of potential internal control genes(A). *Notes:* The dissociation curves of eleven potential internal control genes showed single cusps, and the dissociation temperatures were Tm_{ACTB} = 86.3 °C, Tm_{GAPDH} = 86.3 °C, Tm_{RPL37A} = 84.9 °C, Tm_{B2M} = 82.1 °C, Tm_{PPIB} = 85.8 °C, Tm_{PGK1} = 83.0 °C, Tm_{PPIA} = 81.2 °C, Tm_{SDHA} = 83.5 °C, Tm_{TBP} = 82.6 °C, Tm_{HPRT1} = 82.6 °C, Tm_{RPL13A} = 87.2 °C, respectively. The results could be explained by the fact that there were no contamination, mispriming, primer-dimer artifact, etc. Distribution of the expression (Ct) of the eleven potential internal control genes (B). *Notes:* Ct values for each primer pair from the eleven samples (two controls without stimulation, nine at LPS-stimulated THP-1 and K562 cells). The Ct values of B_2M show highest variation and should be excluded from further analysis in this study.

analysis [2]. The sensitivity of RT-PCR makes it a powerful tool for gene expression measurement, especially when sample quantities are limited or a transcript is expressed at a low level. However, this sensitivity also means that a great deal of care must be taken with regards to experimental design and implementation of the procedure [4]. Various strategies have been applied to normalize these variations [3]. The establishment of a clear-cut set of reliable internal control genes is an essential prerequisite for analyzing gene expression using RT-PCR as shown by several studies [1]. Of course, choosing an internal control gene to normalize gene expression data is also one of the most crucial steps in the experimental design [4]. And this crucial step can minimize the influence of confounding factors and improve the fidelity of the quantification process with respect to the specific biological conditions. However,

Table 2
Relative quantities of each potential internal control gene.

Sample	Potential internal control genes									
	RPL37A	ACTB	GAPDH	PPIB	PGK1	PPIA	SDHA	TBP	HPRT1	RPL13A
Sample1	0.126718	0.294993	0.032569	0.143551	0.045545	0.278389	0.096431	0.203111	0.025095	0.047976
Sample2	0.081182	0.20055	0.021472	0.078919	0.029053	0.252309	0.066225	0.027755	0.013983	0.003587
Sample3	0.058027	0.207711	0.01713	0.054389	0.018003	0.177568	0.062764	0.133161	0.018069	0.001023
Sample4	0.076577	0.44314	0.027492	0.110034	0.036907	0.25588	0.153947	0.048175	0.022076	0.0113
Sample5	0.03637	0.178001	0.017621	0.055117	0.027021	0.079151	0.109858	0.074759	0.009596	0.006175
Sample6	1	0.539335	0.803418	0.980255	0.978481	1	0.724639	1	1	1
Sample7	0.992727	0.528102	1	0.923329	0.930054	0.919144	0.584614	0.48912	0.872679	0.617577
Sample8	0.24443	1	0.732957	1	0.85875	0.827205	0.992361	0.678595	0.858808	0.641657
Sample9	0.373272	0.833252	0.68783	0.583655	1	0.693951	1	0.856336	0.865716	0.730774
Sample10	0.471489	0.550808	0.330047	0.935687	0.450389	0.562067	0.636069	0.571178	0.518453	0.753481
Sample11	0.526046	0.629353	0.364339	0.929487	0.456969	0.550344	0.562622	0.566277	0.561696	0.813385

many studies make use of these constitutively expressed control genes without proper validation of their presumed stability of expression. And few studies have compared the stability of internal control genes.

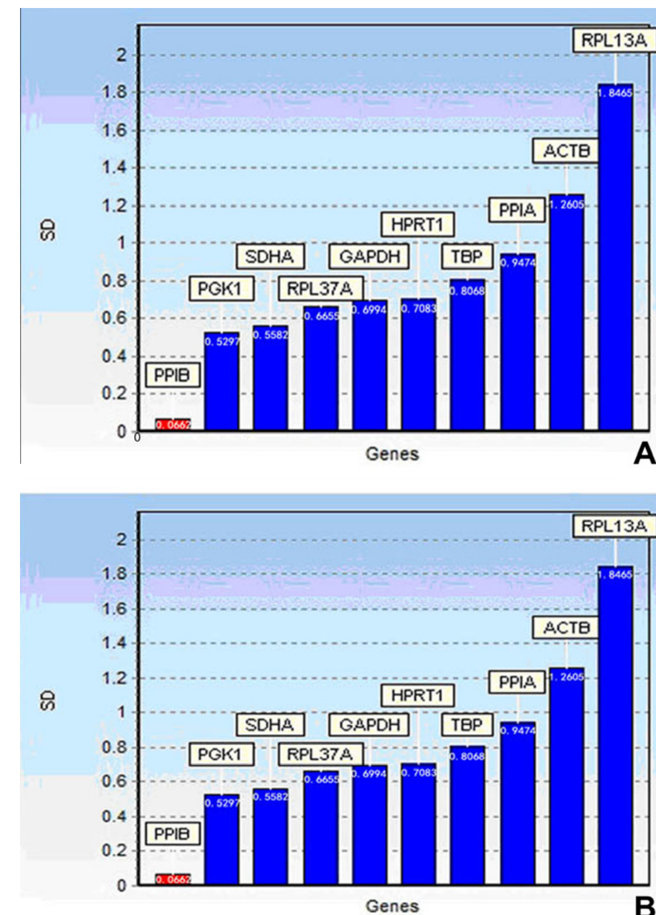

Fig. 2. Stability analysis of candidate reference genes by NormFinder (A). *Note:* The variability of candidate reference genes was calculated by NormFinder. The stability values of PPIB was 0.0662, lower than the other candidates, including the ubiquitous internal control genes GAPDH (SD = 0.6994) and ACTB (SD = 1.2605), indicating that PPIB was the most stably expressed gene in this study. Determination of the optimal number of internal control genes for normalization based on the calculation of the Acc. SD (B). *Note:* NormFinder algorithm also allowed for the determination of the optimal number of internal control genes to be used in the normalization processes through the calculation of the accumulated standard deviation (Acc. SD). The lowest value for the Acc. SD was achieved when using PPIB (Acc.SD = 0.0662).

As we all know, reliable internal control genes are a vital prerequisite for any functional study employing RT-qPCR for analyzing gene expression. GAPDH and ACTB are commonly used endogenous control genes. However, these common internal control genes were not stably expressed under all experimental conditions [11]. It is worth mentioning that LPS stimulation may also induce cell type specific alterations in gene expression and cause abnormalities and alterations of immune responses and cellular functions. In addition, recently published studies further highlighted the importance of individual validation of internal control genes even if comparable cell models are used [12,13]. To diminish the menace of erroneous results because of the use of invalid internal control genes, we validated eleven potential internal control genes, using geNorm, BestKeeper and NormFinder algorithms, suitable for RT-PCR profiling experiments in a broad range of cells including THP-1 and K562. Human THP-1 monocytic leukemia cell line is a well-established model in toxicology, immunology and atherosclerosis research, regarding monocyte and macrophage function and biology. And human erythroleukemic cell line K562 is the chronic myelogenous leukemia-derived cell line. These leukemia cell lines are widely used and have properties similar to those of human monocytes-derived macrophages and lymphoblast-like cells. Lipopolysaccharide (LPS), a cell membrane component of gram-negative bacteria, is a potent stimulator of immune responses of the mononuclear phagocyte system. Stimulation of monocytes with LPS is also a frequently employed model system to study inflammatory responses [6].

In LPS-stimulated monocytes, ACTB and GAPDH are mainly used as reference genes. However, one of the main findings from our study is that several genes, such as GAPDH, ACTB or RPL13A, which probably due to traditional reasons are still frequently used for normalization of RT-qPCR data, exhibit substantial variation in LPS-stimulated and non-stimulated THP-1 and K562 cells. PPIB and PGK1 were the most stable and reliable option and were sufficient for an accurate assessment of relative changes in gene expression in the present study. In contrast to this, other very frequently used reference genes, such as RPL13A, were much less reliable. Thus, the frequently used internal control genes ACTB and GAPDH were actually not suitable for normalization and indeed may cause misinterpretations of results. These findings clearly suggest that these genes disqualify as single internal control genes in LPS-stimulated THP-1 and K562 cells.

The evaluation of potential internal control gene stability depends on the method used. In our study, all data were analyzed by the NormFinder, geNorm and BestKeeper validation programs. geNorm calculates the average pairwise variation for a candidate reference gene with all other tested genes and BestKeeper focuses on the coefficient of variation of a gene across all samples, whereas Normfinder employs a model-based approach which, in addition to the overall expression level variation, also takes intra- and inter-

Table 3
The *M* values of the remaining potential internal control gene.

Sample	Potential internal control genes										
	RPL37A	ACTB	GAPDH	PPIB	PGK1	PPIA	SDHA	TBP	HPRT1	RPL13A	Normalization factor
Sample1	1.27E−01	2.95E−01	3.26E−02	1.44E−01	4.55E−02	2.78E−01	9.64E−02	2.03E−01	2.51E−02	4.80E−02	0.3999
Sample2	8.12E−02	2.01E−01	2.15E−02	7.89E−02	2.91E−02	2.52E−01	6.62E−02	2.78E−02	1.40E−02	3.59E−03	0.2152
Sample3	5.80E−02	2.08E−01	1.71E−02	5.44E−02	1.80E−02	1.78E−01	6.28E−02	1.33E−01	1.81E−02	1.02E−03	0.2154
Sample4	7.66E−02	4.43E−01	2.75E−02	1.10E−01	3.69E−02	2.56E−01	1.54E−01	4.82E−02	2.21E−02	1.13E−02	0.2988
Sample5	3.64E−02	1.78E−01	1.76E−02	5.51E−02	2.70E−02	7.92E−02	1.10E−01	7.48E−02	9.60E−03	6.17E−03	0.1790
Sample6	1.00E+00	5.39E−01	8.03E−01	9.80E−01	9.78E−01	1.00E+00	7.25E−01	1.00E+00	1.00E+00	1.00E+00	4.2117
Sample7	9.93E−01	5.28E−01	1.00E+00	9.23E−01	9.30E−01	9.19E−01	5.85E−01	4.89E−01	8.73E−01	6.18E−01	3.6940
Sample8	2.44E−01	1.00E+00	7.33E−01	1.00E+00	8.59E−01	8.27E−01	9.92E−01	6.79E−01	8.59E−01	6.42E−01	3.2693
Sample9	3.73E−01	8.33E−01	6.88E−01	5.84E−01	1.00E+00	6.94E−01	1.00E+00	8.56E−01	8.66E−01	7.31E−01	3.2883
Sample10	4.71E−01	5.51E−01	3.30E−01	9.36E−01	4.50E−01	5.62E−01	6.36E−01	5.71E−01	5.18E−01	7.53E−01	2.4338
Sample11	5.26E−01	6.29E−01	3.64E−01	9.29E−01	4.57E−01	5.50E−01	5.63E−01	5.66E−01	5.62E−01	8.13E−01	2.4780
$M < 1.5$	1.184	1.547	1.110	1.007	1.076	1.325	1.102	1.244	1.337	2.184	

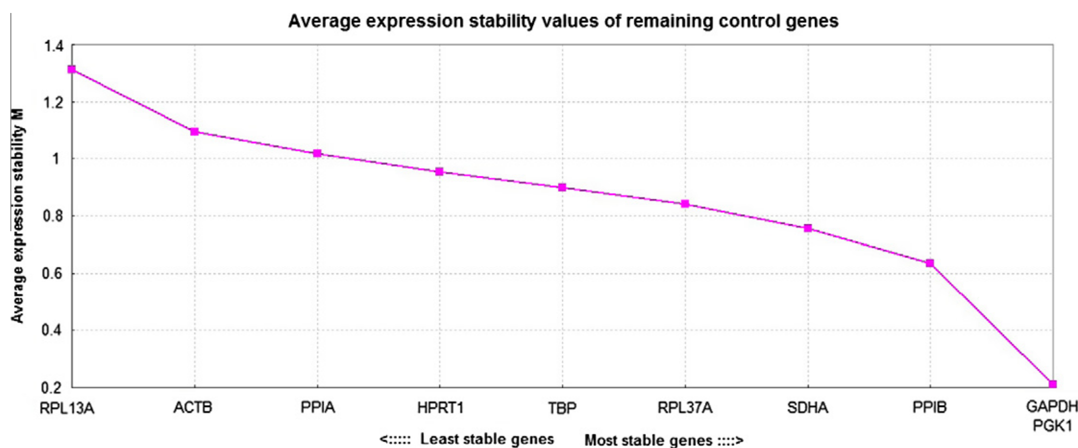

Fig. 3. Average expression stability values of remaining potential internal control genes. *Note:* Average expression stability values (*M*) during stepwise exclusion of the least stable internal control genes by geNorm application. The highest *M* values corresponded to least stable genes. The highest *M* values corresponded to least stable genes, RPL13A and ACTB, while the lowest *M* values corresponded to the most stable genes, GAPDH and PGK1.

Table 4
BestKeeper description statistics.

	RPL37A	ACTB	GAPDH	PPIB	PGK1	PPIA	SDHA	TBP	HPRT1	RPL13A
n	12	12	12	12	12	12	12	12	12	12
geo Mean [CP]	15.48	15.52	16.13	18.26	18.49	17.31	22.33	23.87	20.42	15.43
ar Mean [CP]	15.53	15.54	16.26	18.34	18.60	17.34	22.36	23.98	20.56	15.67
min [CP]	12.98	14.25	12.57	16.41	15.61	15.55	20.27	20.12	17.48	11.87
max [CP]	17.52	16.60	18.19	20.72	21.09	19.31	23.88	27.47	23.97	20.66
STD dev [±CP]	1.05	0.68	1.65	1.57	1.85	0.89	1.13	1.92	2.25	2.19
CV [%CP]	6.76	4.35	10.16	8.56	9.96	5.15	5.07	8.02	10.97	14.00

Standard deviation was used to evaluate reference gene stability. A standard deviation > 1 represented an unstable reference gene.

Table 5
BestKeeper index comparing genes which had a standard deviation < 1.

BestKeeper vs.	RPL37A	ACTB	GAPDH	PPIB	PGK1	PPIA	SDHA	TBP	HPRT1	RPL13A
Coeff. of corr. [*r*]		0.898				0.936				
p-Value		0.001				0.001				

group variation into account [14]. In the present study, the commonly used ACTB and GAPDH were found to have highly variable expression in LPS-stimulated THP-1 and K562 cells. Because they had high SDs (±Ct) compared to other candidates. They were deemed unsuitable for normalization of RT-qPCR (Fig. 2A).

In conclusion, this study analyzed a set of internal control genes for normalization of gene expression profiles in LPS-stimulated and non-stimulated THP-1 and K562 cells using RT-qPCR. Our goal was not to suggest a "best" approach for all conditions, but rather to define the most stably expressed genes in THP-1 and K562 cells. Thus, the identified pair of stable internal control genes may help to improve the accuracy of gene expression studies aimed at understanding the pathophysiological process of inflammation. At the same time, these findings further emphasize the need to accurately validate candidate internal control genes in the study before use in gene expression studies using RT-qPCR.

Table 6
Stability ranking of potential internal control genes in THP-1 cells and K562 cells by NormFinder, geNorm and BestKeeper.

THP-1 and K562			
Rank	NormFinder	geNorm	BestKeeper
1	PPIB	PGK1/GAPDH	ACTB
2	PGK1		PPIA
3	SDHA	HPRT1	
4	RPL37A	PPIB	
5	GAPDH	SDHA	
6	HPRT1	RPL37A	
7	TBP	TBP	
8	PPIA	PPIA	
9	ACTB	ACTB	
10	RPL13A	RPL13A	

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