METHODS MANUSCRIPT

Identification of stable reference genes for lipopolysaccharide-stimulated macrophage gene expression studies

Roshini Kalagara, Weimin Gao, Honor L. Glenn, Colleen Ziegler, Laura Belmont, Deirdre R. Meldrum*

Center for Biosignatures Discovery Automation, The Biodesign Institute, Arizona State University, Tempe, AZ 85287, USA

*Correspondence address: Deirdre R. Meldrum, Center for Biosignatures Discovery Automation, The Biodesign Institute, Arizona State University, 1001 S. McAllister Ave., P.O. Box 877101, Tempe, AZ 85287-7101, USA. Tel: +480 727 9397; Fax: +480 965 2337; E-mail: deirdre.meldrum@asu.edu

Abstract

Gene expression studies which utilize lipopolysaccharide (LPS)-stimulated macrophages to model immune signaling are widely used for elucidating the mechanisms of inflammation-related disease. When expression levels of target genes are quantified using Real-Time quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR), they are analyzed in comparison to reference genes, which should have stable expression. Judicious selection of reference genes is, therefore, critical to interpretation of qRT-PCR results. Ideal reference genes must be identified for each experimental system and demonstrated to remain constant under the experimental conditions. In this study, we evaluated the stability of eight common reference genes: Beta-2-microglobulin (B2M), Cyclophilin A/Peptidylprolyl isomerase A, glyceraldehyde-3-phosphatedehydrogenase (GAPDH), Hypoxanthine Phosphoribosyltransferase 1, Large Ribosomal Protein P0, TATA box binding protein, Ubiquitin C (UBC), and Ribosomal protein L13A. Expression stability of each gene was tested under different conditions of LPS stimulation and compared to untreated controls. Reference gene stabilities were analyzed using C_t value comparison, NormFinder, and geNorm. We found that UBC, closely followed by B2M, is the most stable gene, while the commonly used reference gene GAPDH is the least stable. Thus, for improved accuracy in evaluating gene expression levels, we propose the use of UBC to normalize PCR data from LPS-stimulated macrophages.

Keywords: PCR; inflammation; standardization; murine; J774A.1; housekeeping genes

Introduction

Accurate normalization of quantitative real-time PCR data is critical for obtaining meaningful gene expression results [1]. This is most simply accomplished by scaling raw expression data from genes of interest to a stably expressed standard gene. However, it is well understood that there is no single gene with invariant expression in all cell types under all experimental conditions. Therefore, it is necessary to evaluate potential normalization targets for each experimental protocol. Once a reliable gene or gene panel has been rigorously substantiated, it may then be employed as a standard for the specific experimental approach, allowing better reproducibility of results both within and between laboratories. We sought to address this need in a widely used model of inflammation signaling. A

robust inflammatory response, including inflammasome activation and release of cytokines, can be stimulated in the mouse monocyte/macrophage cell line J774A.1 by treatment with lipopolysaccharide (LPS) [2, 3]. Various reference genes have been used in this popular model [4–7], but, to our knowledge, there are no published studies on the relative stability of these genes under typical experimental conditions. We analyzed the expression stability of eight commonly used reference genes: Ribosomal protein L13A (RPL13A), Beta-2-microglobulin (B2M), Ubiquitin C (UBC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine Phosphoribosyltransferase 1 (HPRT1), Cyclophilin A (Peptidylprolyl isomerase A) (PPIA), TATA box binding protein (TBP), and large ribosomal protein P0 (RPLP0).

Investigation of inflammation signaling in macrophages relies heavily upon real-time quantitative reverse transcription PCR (qRT-PCR) (RRID: SCR_003089), which measures gene expression based on mRNA quantity [8–10]. This approach is particularly relevant for establishing signaling pathways and networks. These gene expression measurements often provide the basis for the development of diagnostic and therapeutic applications. Normalization of qRT-PCR is accomplished by using a reference gene, ideally a ubiquitously expressed housekeeping gene, as an internal control. However, normalization can become problematic due to unanticipated variability in expression of the reference gene, particularly in response to the experimental conditions under study. Indeed, the expression level of reference genes can vary tremendously between different experimental designs and cell types. Because different studies use a variety of reference genes with varying expression stabilities, target gene expression levels, and trends can be misinterpreted or unreproducible. Thus, one major task for gene expression analysis is to identify the most stable reference genes for each study system and to normalize data accordingly [11].

Macrophages were first documented in the context of their ability to engulf foreign particles via phagocytosis. Subsequent studies demonstrated that these cells play an important role in innate immunity and are the primary mediators of inflammation. Increasing appreciation for the role of inflammation in the progression of cancer, heart disease, diabetes, and other clinically relevant conditions has spurred interest in this cell type [12–17]. Initially, these cells exist as small, circulating monocytes in the bloodstream. However, when stimulated, they enter the tissues and undergo a series of significant transformations, eventually differentiating into macrophages [18]. Fully mature macrophages are found in all mammalian tissues and exhibit important functions in embryonic development and tissue repair, in addition to immune system activation [19].

Murine J774A.1 cells (RRID:CVCL_0358) are a popular model in gene expression studies because they recapitulate in vivo macrophage activation behavior with high fidelity [20]. In addition, it is common to use LPS to stimulate macrophage differentiation and cytokine secretion. This in turn triggers pyroptosis, a type of programmed cell death invoked in macrophages during cytokine-facilitated inflammation [3, 21, 22]. Extensive gene expression analysis has been conducted using these LPS-stimulated macrophages, employing qRT-PCR with various reference genes [23–25]. There has been work done to evaluate reference genes in a number of systems, such as in rat oligodendrocytes [26] and bovine muscular tissues [27]. Reference genes have also been explored for J774A.1 cells under conditions of stimulation with laminin [28]. However, to our knowledge, there are no similar reports focused on the stability of reference genes in LPS-treated macrophages.

In this study, following the guidance of Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE), we have evaluated the stability of various reference genes in LPS-stimulated J774A.1 mouse macrophages [1]. We carefully reviewed the literature to determine the most widely used reference genes in this and similar systems. From this search, we chose to analyze the following reference genes: RPL13A, B2M, UBC, GAPDH, HPRT1, PPIA, TBP, and RPLP0 (Table 1). Then, we evaluated the expression of these genes in macrophages under different conditions of LPS stimulation. We then analyzed the expression data using statistical models such as cycle threshold (C_t) value comparison, geNorm [29] (RRID:SCR_006763), and NormFinder [30] (RRID:SCR_003387). Both geNorm and NormFinder are commonly used programs for identifying the most stable reference gene among a group of candidates. Both tools rank a set of candidate genes by stability, but they employ different approaches. GeNorm ranks via sequential, pairwise comparisons between each gene and all other genes in the test set. NormFinder generates a stability value for each gene based on its variability both within-sample groups and between groups. We found that the three methods yielded similar results and all identified the same gene as the best reference for the system. Finally, we evaluated the performance of the reference genes for evaluating the expression levels of NF-κB1, an exemplar target gene that plays a role in the inflammatory response in macrophages [31].

Materials and Methods

Cell culture and LPS treatments

J774A.1 macrophage cells (ATCC TIB-67™, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 m/ml penicillin G and 100 mg/ml streptomycin sulfate (all from Gibco, Grand Island, NY, USA) in T25 flasks (Sigma-Aldrich, St. Louis,

Table 1: Reference genes and functions

Gene	Full name	RefSeq	Function	References
B2M	Beta-2-microglobulin	NM_009735.3	Beta-chain of major histocompatibility complex class I molecules	32
PPIA	Cyclophilin A (peptidylprolyl isomerase A)	NM_008907.1	Protein metabolism and modification	33
GAPDH	Glyceraldehyde-3-phosphatedehydrogenase	NM_008084.2	Carbohydrate metabolism	34
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_013556.2	Purine synthesis	35
RPLP0	Large ribosomal protein P0	NM_007475.5	Ribosome production and assembly	36
TBP	TATA box binding protein	NM_013684.3	RNA polymerase II transcription factor	37
UBC	Ubiquitin C	NM_019639.4	Protein degradation	38
RPL13A	Ribosomal protein L13A	NM_009438.5	Structural component of ribosomal subunit	39

MO, USA) at 37˚C with 5% CO_2 in a humidified incubator. Tissue culture flasks were passaged every 3 or 4 days by scraping and cells were counted for density and viability with a Countess® Automated Cell Counter (Life Technologies, Eugene, OR, USA) using the trypan blue dye exclusion assay. For LPS treatment assay, 1×10^6 cells were suspended in 1 ml DMEM, supplemented with 10% FBS (Sigma-Aldrich). Cells were exposed to LPS (Sigma-Aldrich) at 10 ng/ml, 1 µg/ml, or left untreated, and then incubated for 1 h or 4 h at 37˚C with 5% CO_2. After the LPS treatment, the macrophages were transferred into Corning® 15-milliliter tubes (Sigma-Aldrich), followed by centrifugation at $300 \times g$ for 3 min. After discard the supernatants, the left cell pellet was used for Ribonucleic acid (RNA) extraction as below.

RNA extraction and reverse transcription

RNA was extracted from the cells using the RNeasy Mini Kit (Cat No. 74104, QIAGEN, Hilden, Germany) according to the following modified version of the manufacturer's protocol. First, 350 µl of RLT and 350 µl of 70% ethanol were added into a Corning® 15-milliliter tube containing 1×10^6 cells, followed by vortexing for homogenization for 1 min. Then, 700 µl of lysate was transferred to an RNeasy Mini Spin column sitting on a 2 ml collection tube and centrifuged at $8000 \times g$ for 1 min. The flow-through was discarded and the spin column was replaced. Then 700 µl of RW1 was added in the spin column followed by centrifugation at $8000 \times g$ for 1 min. Again, the flow-through was discarded and the spin column was replaced. Five hundred microliters of RPE was added into the spin column, followed by centrifugation at $8000 \times g$ for 1 min. The flow-through was then discarded and the spin column was replaced. The RPE washing procedure was repeated. Lastly, the spin column was transferred to a new 1.5 ml Eppendorf tube and 50 µl of RNase-free water was added, followed by centrifugation at $8000 \times g$ for 1 min. After RNA was collected, it was quantified using the NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA) and RNA concentration was adjusted to 1 µg/ml. Complementary DNA (cDNA) was synthesized using the QuantiTect reverse transcription protocol (Cat No. 205311, QIAGEN). For each reaction, 4 µl of iScript reaction mix (5×), 1 µl of iScript reverse transcriptase, and 15 µl of the adjusted RNA were added together and mixed well with a pipette. Afterward, the RT was run with the following program at a thermal cycler (MJ Mini Personal Thermal Cycler, Bio-Rad, Hercules, CA, USA): 5 mins at 25°C, 30 mins at 42°C, and 5 mins at 85°C. The obtained cDNA was stored at −20°C prior to use in qPCR.

Primer design and validation

Primers were designed and evaluated using two online genomic information databases to ensure validity: UCSC Genome Bioinformatics and NCBI Primer Blast (Table 2). Designed PCR primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Before use in analyzing reference gene expression, primers were evaluated for their efficiency and specificity. Primers were tested by qPCR using the ABI Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixture used was: 5 µl of SYBR GreenER™ qPCR SuperMix Universal (2×) (Cat No. 11762100, Thermo Fisher Scientific), 1.0 µl of forward primer (4 µM), 1.0 µl of reverse primer (4 µM), 1 µl of cDNA template (1/20 dilution of RT reaction), and 2.0 µl of Nuclease-free water (Cat No. AM 9930, Thermo Fisher Scientific). The PCR amplification profile consisted of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 72°C for 30 s, and ending with a melt curve analysis according to the defaulted program of ABI. Following qPCR, the samples were purified using QIAquick Gel Extraction Kit (Cat No. 28704, QIAGEN), and then sequenced. The resulting chromatograms were compared to known sequences and the specificity of the primers was verified.

qPCR

After primer validation, qPCR was conducted using template cDNA prepared from J774A.1 cells that were exposed to various LPS treatments. Reactions were prepared using reagent quantities similar to those used in primer design and validation: 5 µl of SYBR GreenER SuperMix Universal (2×), 1.0 µl of forward primer (4 µM), 1.0 µ of reverse primer (4 µM), 1 µl of cDNA template (1/20 dilution of RT reaction), and 2.0 µl of nuclease-free water. An ABI Step One Plus Real-Time PCR System (Applied Biosystems) was used with an amplification profile of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 72°C for 30 s, and a step and hold melt curve analysis. Each gene was analyzed in two independent experiments, each conducted in triplicate and done on different days. Moreover, qPCR using the same protocols and amplification methods were run on NF-κB1, an example target gene.

Statistical analysis

Expression data for NF-κB1 was analyzed by two-way ANOVA with Dunnett's post-hoc test for multiple comparisons, using GraphPad Prism (GraphPad Software, San Diego, CA, USA) (RRID:SCR_002798). $P < 0.05$ were considered significant.

Results

Data were collected from qPCR runs and each gene was analyzed for utility as a normalization standard for LPS-stimulated macrophages. J774A.1 macrophages were treated with LPS

Table 2: Reference and target gene primers

| Gene	Forward Primer 5'–3'	Reverse Primer 5'–3'
B2M	ACCGTCTACTGGGATCGAGA	TGCTATTTCTTTCTGCGTGCAT
GAPDH	AAGGGCTCATGACCACAGTC	CAGGGATGATGTTCTGGGCA
HPRT1	GATCAGTCAACGGGGGACAT	ATCCAACAAAGTCTGGCCTGT
PPIA	CCAAGACTGAATGGCTGGATG	TGTCCACAGTCGGAAATGGTG
RPL13A	GAAGCAGATCTTGAGGTTACGGA	GCAGGCATGAGGCAAACAGT
RPLP0	TCACTGTGCCAGCTCAGAAC	ATCAGCTGCACATCACTCAGA
TBP	AAACTCTGACCACTGCACCG	CTGCAGCAAATCGCTTGGGA
UBC	CCCAGTGTTACCACCAAGAAG	CCCCATCACACCCAAGAACA
NF-κB1	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG
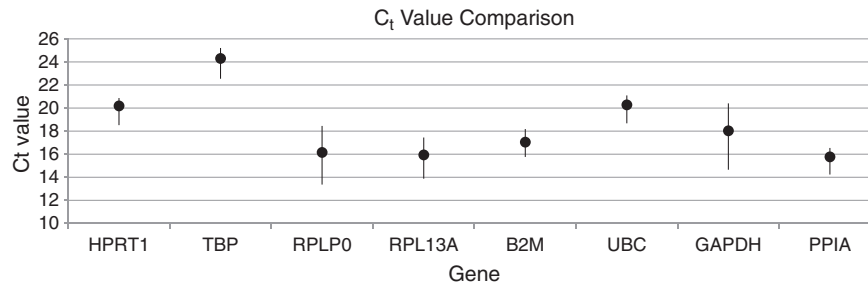
Figure 1: C$_t$ value comparison. Relative expression levels from all treatment conditions for each reference gene, obtained through qRT-PCR, were combined and averaged. Each point represents the average of 30 C$_t$ values with six replicates of each treatment. Error bars indicate the range of C$_t$ values.

under the conditions of 10 ng/ml for 1 h, 10 ng/ml for 4 h, 1 µg/ml for 1 h, and 1 µg/ml for 4 h, while control cells were left untreated. The success of this LPS activation protocol was previously shown in our laboratory by both western blotting and cell death assay [40, 41]. Expression values from each condition were run a total of six times; two qPCR experiments were conducted in triplicate on two different days.

C$_t$ value comparison of reference genes

C$_t$ values for eight genes from the J774A.1 cells were calculated using raw PCR data and found to range from 13.9 to 25.2 (Fig. 1). The values presented for each gene are the average C$_t$ from all treatment conditions for each of the genes. The genes PPIA and RPLP0 were determined to have the highest expression levels. A delta C$_t$ value comparison of the variation in C$_t$ values revealed that B2M and UBC had the lowest standard deviation in expression while RPLP0 and GAPDH had the highest deviation. The error bars in Fig. 1 indicate the range of C$_t$ values and, therefore, the variability of each gene across the panel of control and LPS treatments. These results highlight the need to validate stability of reference genes in this experimental system since LPS treatments caused large variations in some of the observed C$_t$ values.

Gene expression stability determined by NormFinder

NormFinder is an algorithm that measures the stability of various reference genes and produces raw stability values for genes, where a lower stability value indicates a more stable gene. NormFinder calculates this value by combining the approximation of group expression variations of target reference genes [30, 42]. The resulting numbers represent the variation in expression across samples and between groups [43]. Data was entered in the form of linear efficiency corrected quantities (Q). The equation used for this calculation is [44]: $Q = E^{\,C_t\,(min)\,-\,C_t\,(sample)}$. C$_t$ (min) corresponds to the lowest C$_t$ value for an assay and C$_t$ (sample) refers to the sample in question. NormFinder was used as a Microsoft Excel add-in and two analyses were conducted. In the first analysis, data were grouped based on the day the qRT-PCR was done. In the second analysis, data were organized based on experimental conditions, such as LPS concentration and treatment time. In the first test, the average stability value was 0.055. The most stable gene was UBC followed closely by TBP, which had stability values of 0.015 and 0.020, respectively. GAPDH, with a value of 0.087, and RPL13A, with a value of 0.084, were the least stable reference genes (Fig. 2A). In the second analysis, which separated the LPS treatments as different samples, an average stability value of 0.042 was calculated. UBC and TBP were again found to be the most stable genes in the panel with stability values of 0.002 and 0.005, respectively (Fig. 2B). However, in this analysis, RPL13A was the least stable gene with

a stability value of 0.081, followed by RPLP0 and PPIA. Together, these analyses highlight the stability of UBC and TBP, as well as the inconsistency of GAPDH and RPL13A as normalization genes in LPS-stimulated macrophages.

Gene expression stability determined by geNorm

Data were also analyzed using geNorm, an algorithm that determines the most stable reference gene from a panel by calculating M-values for each gene. M-values are determined by averaging the pairwise variation between the gene of interest and all other reference genes [29]. Similar to the NormFinder stability values, a lower M-score indicates more stable gene expression. In untreated control cells, the average M-value for this data was 0.685. GAPDH was the least stable gene with an M-value of 0.698, and UBC was the most stable with an M-value of 0.124 (Fig. 3A). Combining data across all treatments for each gene resulted in similar ranking with M-values of 0.746 and 0.311 for GAPDH and UBC, respectively (Fig. 3B). Data were also combined based on the concentration of LPS used for stimulation. At 10 ng/ml LPS, the average stability value was 0.256. HPRT1, RPL13A, and RPLP0 were the most stable, with M-values of 0.083, 0.075, and 0.062 (Fig. 3C). GAPDH was the least stable gene in this condition with an M-value of 0.728. At 1 µg/ml LPS, the average gene stability value was 0.1375. UBC, B2M, and HPRT1 proved to be the most stable genes, with M-values of 0.043, 0.033, and 0.033, respectively (Fig. 3D). GAPDH again proved to be the least stable gene with an M-value of 0.508. Finally, data were grouped by duration of LPS treatment. In the 1-h subcategory, the average M-value was 0.338 and the most stable reference genes were PPIA and UBC, with M-values of 0.116 and 0.133, respectively (Fig. 3E). The least stable gene was GAPDH, with an M-value of 0.978. In the 4-h subcategory, the average M-value was 0.148 and the most stable reference genes were B2M and UBC, both with M-values of 0.010 (Fig. 3F). The least stable reference gene, again, was GAPDH with an M-value of 0.446.

Comparison of most and least stable reference genes

GAPDH was generally the least stable reference gene, while UBC was the most stable. GeNorm was used to compare the M-values of these two genes for each condition (Fig. 4). The M-values of UBC, which were the lowest of any of the tested genes, ranged from 0.100 to 0.311. The M-values of GAPDH, which were consistently higher, ranged from 0.446 to 0.978.

Effects of reference genes on calculated NF-κB1 expression

In order to show how selection of different reference genes impacts gene expression results, we evaluated the expression of
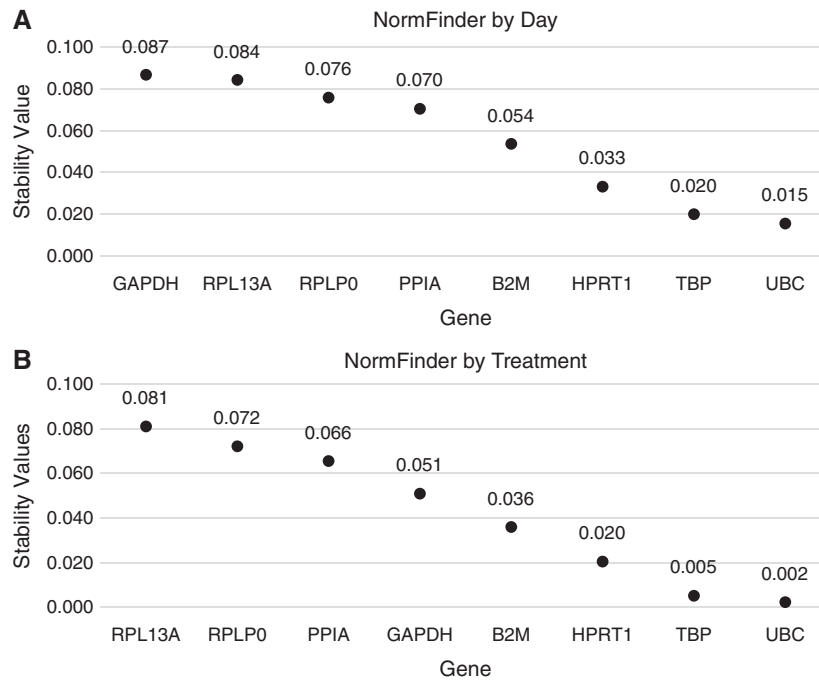
Figure 2: NormFinder Analysis. **(A)** NormFinder analysis of reference genes by day. Gene stability values and accumulated standard deviation analysis using NormFinder. Data were grouped together by the day the experiment was conducted. **(B)** NormFinder analysis of reference genes by treatment. Gene stability values and accumulated standard deviation analysis using NormFinder grouped together by treatment. Stability values were calculated between data sets from control cells and four LPS treatment protocols: 10 ng/ml for 1 h, 10 ng/ml for 4 h, 1 μg/ml for 1 h, and 1 μg/ml for 4 h. Genes are ordered by increasing stability from left to right.
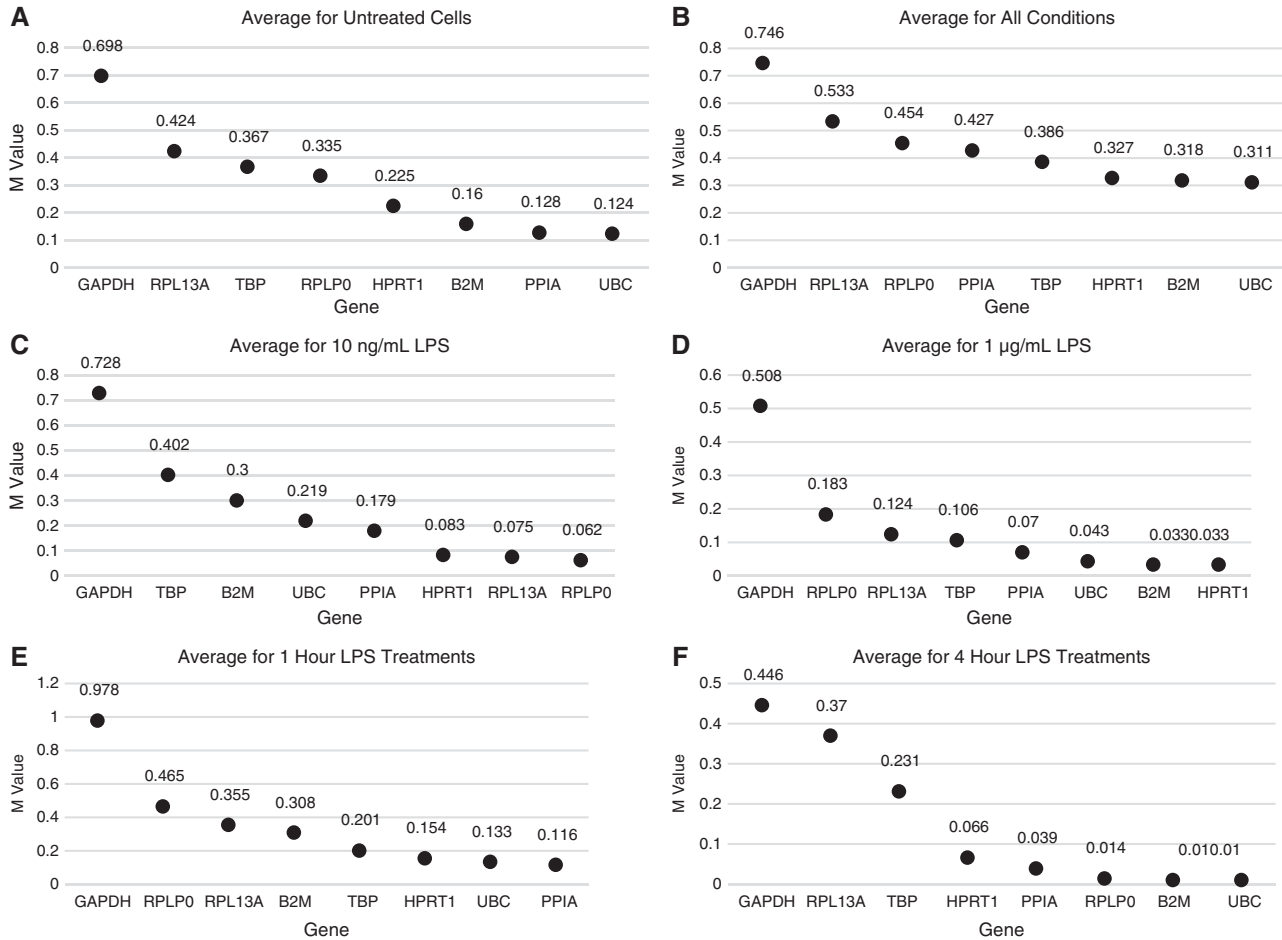

Figure 3: GeNorm analysis. Summary of geNorm analysis of reference genes grouped by LPS treatment. GeNorm was used to calculate M-values for the reference genes from qRT-PCR data for: **(A)** untreated controls, **(B)** all conditions combined, **(C)** 10 ng/ml LPS treatments, **(D)** 1 μg/ml LPS treatments, **(E)** 1 h LPS treatments, and **(F)** 4 h LPS treatments. Genes are ordered by increasing stability from left to right.
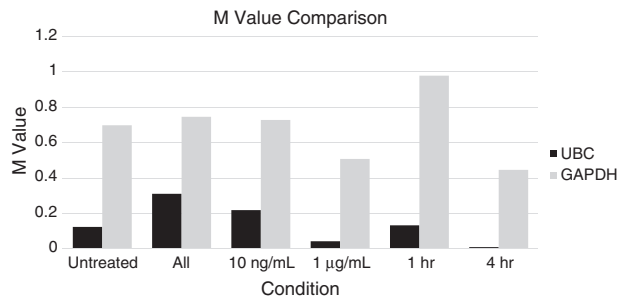
Figure 4: GeNorm analysis of UBC and GAPDH stability. Data are the *M*-values calculated by geNorm of data from: untreated control samples, all conditions, LPS concentrations, and LPS treatment times.
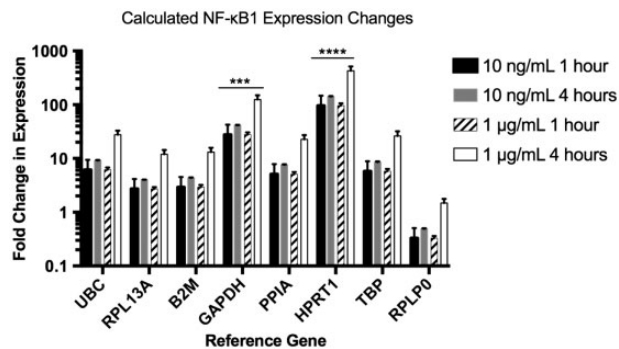

Figure 5: Calculated NF-κB1 expression changes. Values of NF-κB1 expression for each LPS treatment were calculated using the delta-delta C_t method. Expression was normalized to each of the eight reference genes. The resulting values are expressed as fold change in expression relative to untreated control. Calculated expression values were compared to the most stable reference gene, UBC. Data are the mean of two experiments, error bars represent standard deviation. ***$P < 0.001$, ****$P < 0.0001$.

NF-κB1 in our experimental system. NF-κB1 is a key mediator of inflammation signaling and is highly upregulated in macrophage in response to LPS stimulation [31]. We used qRT-PCR and the $2^{-\Delta\Delta CT}$ method to compare expression of this target gene when normalized to each reference gene (Fig. 5). Expression values are presented relative to the untreated control. We found that the use of either GAPDH or HPRT1 resulted in significantly different expression values, across the panel of treatments, compared to the most stable reference gene UBC. Results from normalization to the other reference genes were not significantly different from UBC for NF-κB1 expression.

Discussion

Macrophages have been extensively studied because of their importance in a wide range of normal and pathological processes, but the evaluation of reference genes used for normalization in macrophage studies has been less well explored. An exhaustive search of the literature revealed no published reports evaluating reference genes for the LPS-stimulated J774A.1 macrophage model. This is an important area of investigation, due to the known pitfalls in reference gene selection. For instance, previous studies have shown that reference genes like GAPDH and ACTB contain many pseudogenes, which skew the measured expression levels [45]. Generally, reference genes need to be evaluated independently for each experimental system to ensure stability in the cell type of interest and under the specific experimental conditions to be tested [46].

Therefore, we set out to determine the stability of prevalent reference genes used in this LPS-stimulated, macrophage system. Macrophage gene expression has been studied in contexts such as autoimmune-related inflammation, coronary artery dilation, cancer progression, and wound healing [7, 47–51]. In each case, macrophage gene expression was critically linked to pathology. In these studies, reference genes with a wide range of stability were used, including UBC, GAPDH, 18S, HPRT1, and PPIA. For example, GAPDH normalization was used in a study that sought to characterize LPS-stimulated monocyte-to-macrophage differentiation in terms of gene expression [50]. Our data suggest that GAPDH and PPIA are particularly poor references for LPS-stimulated J774A.1 gene expression analysis. In contrast, we find that UBC is a reliable reference gene for this model. This conclusion is supported by the results from both NormFinder and geNorm analyses. Because these two analyses rank using different methods, we have a high level of confidence in this finding. Further, C_t value analysis also shows that UBC expression remains consistent under the treatment conditions.

In addition to calculating *M*-values, geNorm analysis also includes a determination of the optimal number of reference genes needed to ensure accurate data normalization. Often the average expression of a panel of genes provides more reliable normalization than any single gene. However, this analysis showed that there was no subset of genes in the tested panel that would be expected to outperform UBC alone under our treatment conditions.

We demonstrate here a comprehensive approach for determining the best reference gene to use in any cell system. Our results substantiate the importance of evaluating potential reference genes for use in specific gene expression studies. We show that there are measureable differences in stability among a number of commonly used housekeeping genes in an inflammation model of LPS-stimulated macrophages. We used expression of the inflammation mediator NF-κB1 to show that use of the less stable reference genes produced significantly altered results relative to the most stable gene.

Here, we evaluated eight metabolic housekeeping genes for their suitability as reference genes. It would be valuable to expand this analysis to a larger pool of possible reference genes including targets such as the ribosomal protein, 18S. It is also important to note that we evaluated reference gene stability on the bulk cell level. Single-cell gene expression measurements are becoming increasingly accessible [52, 53]. It is likely that genes shown to be acceptably stable across cell populations would not be suitable as references at the single-cell level. Indeed, for this application, larger panels of standard genes may be required to produce robust datasets.

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Author Contributions

R.K., L.B., and C.Z. devised the project. R.K. and L.B. conducted experiments. R.K. analyzed the data. R.K., H.L.G., and W.G. prepared the manuscript. D.R.M., W.G., and H.L.G. directed the work and edited the manuscript.

Conflict of interest statement. None declared.

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