Validation of housekeeping genes for normalizing RNA expression in real-time PCR

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Analysis of RNA expression using techniques like real-time PCR has traditionally used reference or housekeeping genes to control for error between samples. This practice is being questioned as it becomes increasingly clear that some housekeeping genes may vary considerably in certain biological samples. We used real-time reverse transcription PCR (RT-PCR) to assess the levels of 13 housekeeping genes expressed in peripheral blood mononuclear cell culture and whole blood from healthy individuals and those with tuberculosis. Housekeeping genes were selected from conventionally used ones and from genes reported to be invariant in human T cell culture. None of the commonly used housekeeping genes [e.g., glyceraldehyde-phosphate-dehydrogenase (GAPDH)] were found to be suitable as internal references, as they were highly variable (>30-fold maximal variability). Furthermore, genes previously found to be invariant in human T cell culture also showed large variation in RNA expression (>34-fold maximal variability). Genes that were invariant in blood were highly variable in peripheral blood mononuclear cell culture. Our data show that RNA specifying human acidic ribosomal protein was the most suitable housekeeping gene for normalizing mRNA levels in human pulmonary tuberculosis. Validations of housekeeping genes are highly specific for a particular experimental model and are a crucial component in assessing any new model.

INTRODUCTION

It is essential to control for error between samples when measuring RNA expression. This error can be introduced at a number of stages throughout the experimental protocol (input sample, RNA extraction, reverse transcription, etc.). There are many methods to control for this error. One approach is to normalize to total RNA. However, this requires a reliable RNA quantification method and fails to take into account the variability of the reverse transcription and other steps. A widely used alternative is to normalize RNA levels to an internal reference or housekeeping gene (1).

The expression levels of reference genes should remain constant between the cells of different tissues and under different experimental conditions (2). If these requirements are not fulfilled, then normalization to varying internal references can lead to increased "noise" or erroneous results (3). If the chosen housekeeping gene fluctuates randomly between samples, then small differences between genes of interest will be missed. Furthermore, if the experimental condition causes a directional change in the housekeeping gene, the subsequent normalization will cause an erroneous result. This was shown in a study of human asthma, where target gene expression between experimental groups became falsely different when β-actin was used as a normalizer (4). It was in fact the β-actin rather than the target gene that was changing (4). More recently, it has become clear that housekeeping genes like β-actin and glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) may be inappropriate as internal references because of their variability (5). Appropriate validation of internal references is therefore crucial to avoid misinterpretations of study findings.

Our experimental protocols will be transferred to sites in the developing world as part of a program to build research capacity (http://www.eubusiness.com/funding/research/rtd01_en.htm). We therefore required a method with simplicity and relatively low cost. The housekeeping gene method of normalization was chosen for this reason. Our main genes of interest specify cytokines that have low RNA expression (6). Consequently, we anticipate that differences between study groups may be small. Therefore it was important to find an internal reference that had minimal variability. Previous reports have used conventional housekeeping genes in models of tuberculosis (TB) to normalize for differing amounts of input RNA (7–9), but there has been no report of studies to check the validity of these housekeeping genes in TB or in in vitro lymphocyte cultures. We used real-time reverse transcription PCR (RT-PCR) to study the levels of 13 housekeeping genes expressed in whole blood and peripheral blood mononuclear cell (PBMC) culture of healthy volunteers and patients with TB. Housekeeping genes were selected from those usually used and others found to be invariant in human

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Table 1. Housekeeping Genes Selected for Group 1 and Group 2 Panels

Group 1 Housekeeping Genes	Group 2 Housekeeping Genes
Human acidic ribosomal protein (*HuPO*)	Human acidic ribosomal protein (*HuPO*)
β-Actin (*BA*)	Elongation factor-1-α (*EF-1-α*)
Cyclophylin (*CYC*)	Metastatic lymph node 51 (*MLN51*)
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	Ubiquitin conjugating enzyme (*UbcH5B*)
Phosphoglycerokinase (*PGK*)	
β_2-Microglobulin (*B2M*)	
β-Glucuronidase (*GUS*)	
Hypoxanthine phosphoribosyltransferase (*HPRT*)	
Transcription factor IID TATA binding protein (*TBP*)	
Transferrin receptor (*TfR*)	

T cell culture (10). Here we report a validation exercise to identify the most suitable housekeeping gene in studies of pulmonary tuberculosis (PTB).

MATERIALS AND METHODS

Patients and Samples

Clinical samples ($n = 28$) were taken from four patients with smear-positive PTB, four healthy individuals, and the cells harvested from PBMC cultures of four additional patients with PTB. In order to maximize variability, we chose subjects of different ages (from 26 to 50 years), sex, and ethnicity (Caucasian, Somalian, Indian, Chinese, Filipino, and Black African). Informed consent was obtained from patients, and the relevant hospital ethics committees approved the study.

Whole blood (20 mL) was taken, and 2.5 mL was immediately transferred into PreAnalytiX PAXgene™ blood RNA tubes (Qiagen, Valencia, CA, USA) to fix the mRNA expression profile (11). The remaining blood was transferred to a heparinized container and transported in a thermo flask at 37°C. Heparinized blood was incubated at 37°C for a further 4 h on reaching the laboratory, after which a further 2.5-mL sample was transferred to a PAXgene tube.

Blood taken from four of the patients with PTB was layered over a Ficoll-Paque® gradient (Amersham Biosciences, Piscataway, NJ, USA) and the PBMCs isolated. PBMCs were cultured in triplicate in RPMI (supplemented with 5% human AB serum, glutamine, and penicillin-streptomycin) at a cell concentration of 1×10^6 cells/mL (final

volume of 0.5 mL) in 24-well plates. Cells stimulated with TB antigen (12) and harvested at baseline and days 2/3. Days 4/5 were used for housekeeping gene expression studies. Control wells were challenged with phytohemagglutinin, *Mycobacterium vaccae* sonicate, or no antigen. Cell viability was assessed at each harvest with trypan blue, and a proliferation assay was performed on days 4/5 to quantify the proliferative response to antigen challenge [bromodeoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (ELISA); Roche Applied Science, Indianapolis, IN, USA].

Selection of Housekeeping Genes

We first studied the gene expression levels of 10 housekeeping genes (designated group 1) using a commercially available assay (TaqMan® human endogenous control plate; Applied Biosystems, Foster City, CA, USA) (13). The 10 genes investigated in this assay are shown in Table 1. We selected group 2 genes as they were among 47 out of 535 maintenance genes found by microarray data to be relatively stable in 11 different human tissues (14), and since they were found to be invariant in human CD4 T cell cultures from cord blood (10).

Our aim was to identify a housekeeping gene with minimal variability under our different experimental conditions. As some of the genes of interest are low-copy number cytokines, we anticipated that a significant change between study groups was likely to be small. Therefore, a standard deviation of less than 2-fold from the mean expression level of the gene was chosen as a requirement for suitability as a reference gene.

We did not use ribosomal subunit RNAs as housekeeping genes, as we used oligo(dT) as a primer for cDNA synthesis, and compared to specific downstream primers, random hexamer primers have been shown to overestimate mRNA copy numbers by up to 19-fold (15).

Isolation of RNA and cDNA Synthesis

RNA was isolated from whole blood collected in PAXgene tubes using the PreAnalytiX PAXgene blood RNA kit (Qiagen) and from harvested PBMCs using the RNeasy® Mini kit (Qiagen). All samples were DNase (Qiagen) treated. The RNA template was qualitatively assessed and quantified using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Labchip® kit for blood-derived RNA and the RNA 6000 Pico Labchip kit for culture-derived RNA (all from Agilent Technologies, Palo Alto, CA, USA). Total RNA extraction varied from approximately 1–5 µg for blood and 0.1–0.8 µg from 5×10^5 cells. To study the effect on housekeeping gene expression, we used a fixed amount of input RNA for each cDNA reaction. Limited RNA quantities dictated input RNA amounts to be 600 ng for PTB patients, 400 ng for healthy volunteers, and 3 ng for the PBMC culture reactions. Reverse transcription reactions were performed following the manufacturer's instructions using Omniscript®

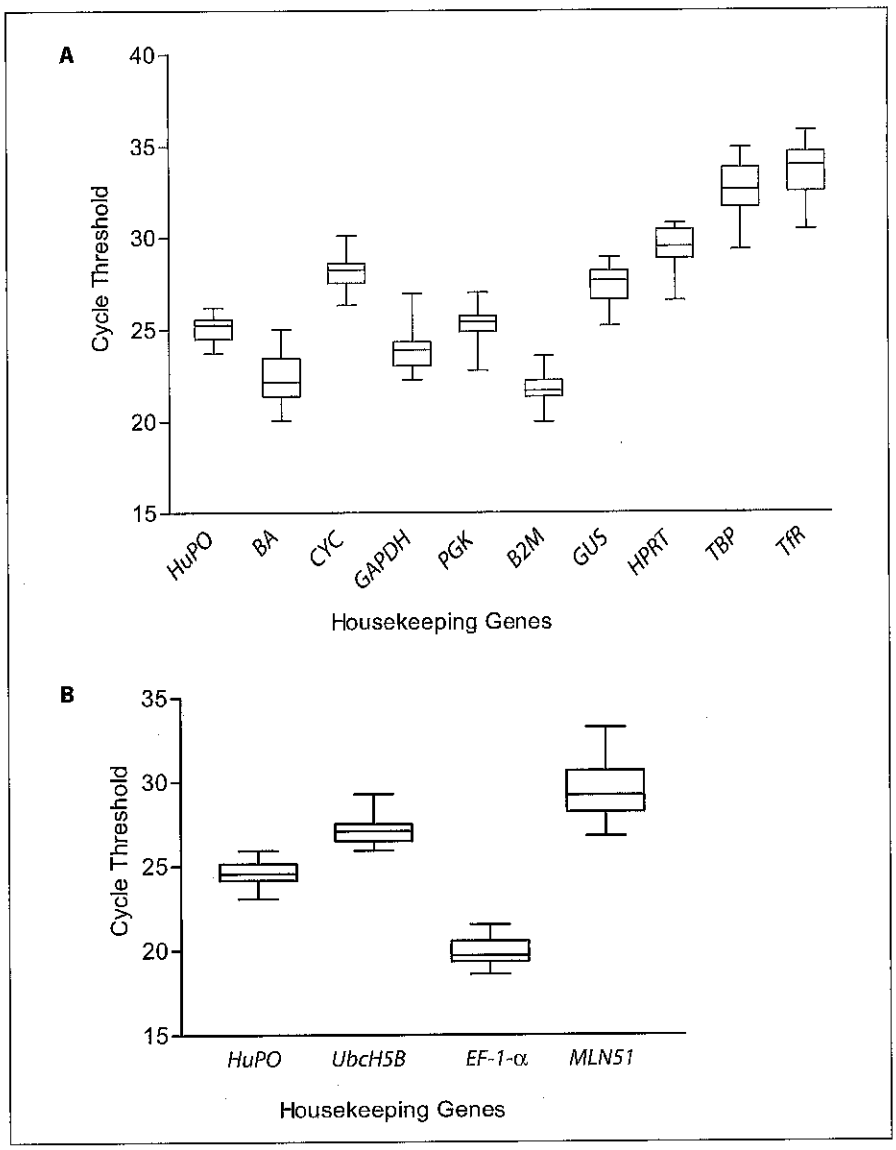

Figure 1. Real-time PCR cycle threshold values in blood samples. Expression levels of group 1 genes (A) and group 2 genes (B) are shown as medians (lines), 25th percentile to the 75th percentile (boxes) and ranges (whiskers) for 16 human blood samples (4 healthy and 4 tuberculosis patients at two time points 4 h apart). *HuPO*, human acidic ribosomal protein; *BA*, β-actin; *CYC*, cyclophylin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PGK*, phosphoglycerokinase; *B2M*, β_2-microglobulin; *GUS*, β-glucuronidase; *HPRT*, hypoxanthine phosphoribosyltransferase; *TBP*, transcription factor IID TATA binding protein; *TfR*, transferrin receptorl; *UbcH5B*, ubiquitin conjugating enzyme; *EF-1-α*, elongation factor-1-α; *MLN51*, metastatic lymph node 51.

Table 2. Primer and Probe Sequences Used to Quantify Gene Expression by Real-Time PCR

Genes	Sequences	Product Size (bp)	R^2
EF-1-α	probe: 5′-(FAM-AGCGCCGGCTATGCCCCTG-TAMRA)-3′ primer 1: 5′-CTGAACCATCCAGGCCAAAT-3′ primer 2: 5′-GCCGTGTGGCAATCCAAT-3′	59	0.992
MLN51	probe: 5′-(FAM-AGGCCTGTGGAAGCTGGTGGGC-TAMRA)-3′ primer 1: 5′-CAAGGAAGGTCGTGCTGGTT-3′ primer 2: 5′-ACCAGACCGGCCACCAT-3′	72	0.999
UbcH5B	probe: 5′-(FAM-TGATCTGGCACGGGACCCTCCA-TAMRA)-3′ primer 1: 5′-TGAAGAGAATCCACAAGGAATTGA-3′ primer 2: 5′-CAACAGGACCTGCTGAACACTG-3′	64	0.999
HuPO	Assays-on-Demand (Applied Biosystems)	110	0.999
Group 1	TaqMan Human Endogenous Control Plate (Applied Biosystems)	N.A.	N.A.

EF-1-α, elongation factor-1-α; *MLN51*, metastatic lymph node 51; *UbcH5B*, ubiquitin conjugating enzyme; *HuPO*, human acidic ribosomal protein; N.A., not applicable.

Reverse Transcriptase (Qiagen) for blood-derived RNA and Sensiscript® Reverse Transcriptase (Qiagen) for culture-derived RNA in 60-µL reactions.

Real-Time PCR

The PCRs for group 1 genes were performed using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems). In each reaction, approximately 15 and 0.1 ng of reverse-transcribed RNA (based on the initial RNA concentration) was used for blood and cell culture PCRs, respectively. The TaqMan endogenous control plate assay was used according to the manufacturer's instructions, with the exception of the 18S ribosomal RNA reaction, which was omitted (initial step of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, in a 50-µL reaction volume). Using these parameters, the reaction efficiency approaches 100%.

The primer and probe sequences for group 2 were obtained from previously published work (10). These sequences are shown in Table 2. Primers and probes were synthesized by MWG (Ebersberg, Germany) and Sigma-Genosys Ltd. (Cambridgeshire, UK), respectively, with the exception of human acidic ribosomal protein (*HuPO*), which was purchased from Applied Biosystems (Assays-on-Demand™). Primers and probes were used at 500 and 300 nM, respectively, in a 50-µL reaction. The reactions were performed on the 7700 Sequence Detection System with the same parameters as the group 1 genes. Reaction efficiencies for group 2 genes (range of 96%–100%) were derived from serial dilutions of purified PCR product. Amplification of the correct product was confirmed by using the Agilent 2100 Bioanalyzer (with the DNA 500 Labchip Kit; Agilent Technologies) (16). All reactions were run in duplicate, and cycle threshold (C_t) values for group 1 genes were normalized to the internal positive control (IPC) to control for interplate variability. *HuPO* measurements were used to compare the results between groups 1 and 2. Nontemplate controls were used as recommended (5).

Data Presentation and Calculations

In PCRs with efficiencies approaching 100%, the amount of internal reference gene relative to a calibrator (fold change between two C_t values) is given by the equation (17):

$$\text{Fold difference} = 2^{-\Delta Ct}$$

At a reaction efficiency of 100%, 1 cycle (expressed as C_t in real-time PCR) corresponds to a 2-fold change. The variability of individual housekeeping genes were reflected as standard deviation and ranges, expressed as an average fold change from the mean or as a maximum fold change (maximum variability), respectively.

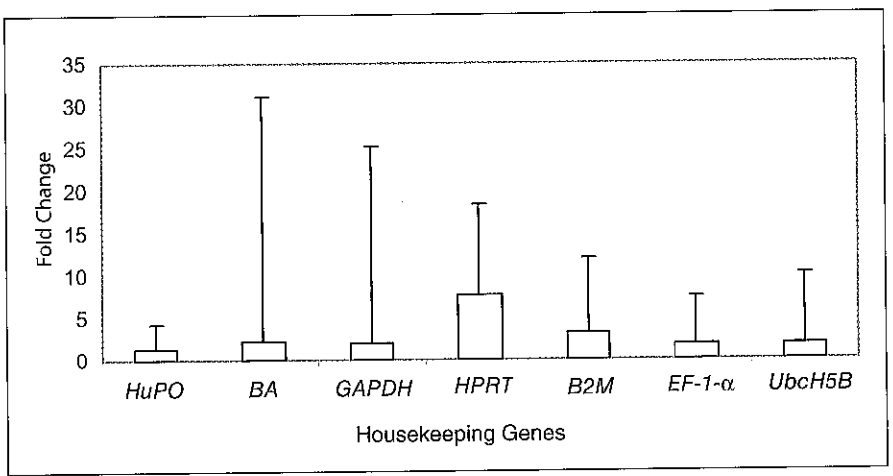

Figure 2. Fold change in gene expression. Variability of selected group 1 genes and group 2 genes in human whole blood shown as an average fold change from the mean (columns) and maximum fold change (error bars). *HuPO*, human acidic ribosomal protein; *BA*, β-actin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT*, hypoxanthine phosphoribosyltransferase; *B2M*, β_2-microglobulin; *EF-1-α*, elongation factor-1-α; *UbcH5B*, ubiquitin conjugating enzyme.

RESULTS

Housekeeping Gene Expression in Whole Blood

The median expression level (C_t value) of group 1 and group 2 housekeeping genes for whole blood ($n = 16$) are shown in Figure 1 (A and B). Also

shown are C_t 25th and 75th percentile values and ranges for each housekeeping gene. Figure 2 shows the standard deviation expressed as a fold change from the mean and range expressed as maximum variability for selected housekeeping genes in whole blood.

The most stable housekeeping gene in whole blood was *HuPO*, with an average fold change of <2 and a maximal variability of <5-fold. There was considerably greater variability for *GAPDH*, β-actin, and hypoxanthine phosphoribosyltransferase (*HPRT*) (maximum variability of 20- to 25-fold). When gene expression at 4 h was compared to baseline, there was little difference in the expression levels of all 13 genes (average fold change for all genes over 4 h = $1.37 \pm 0.33x$).

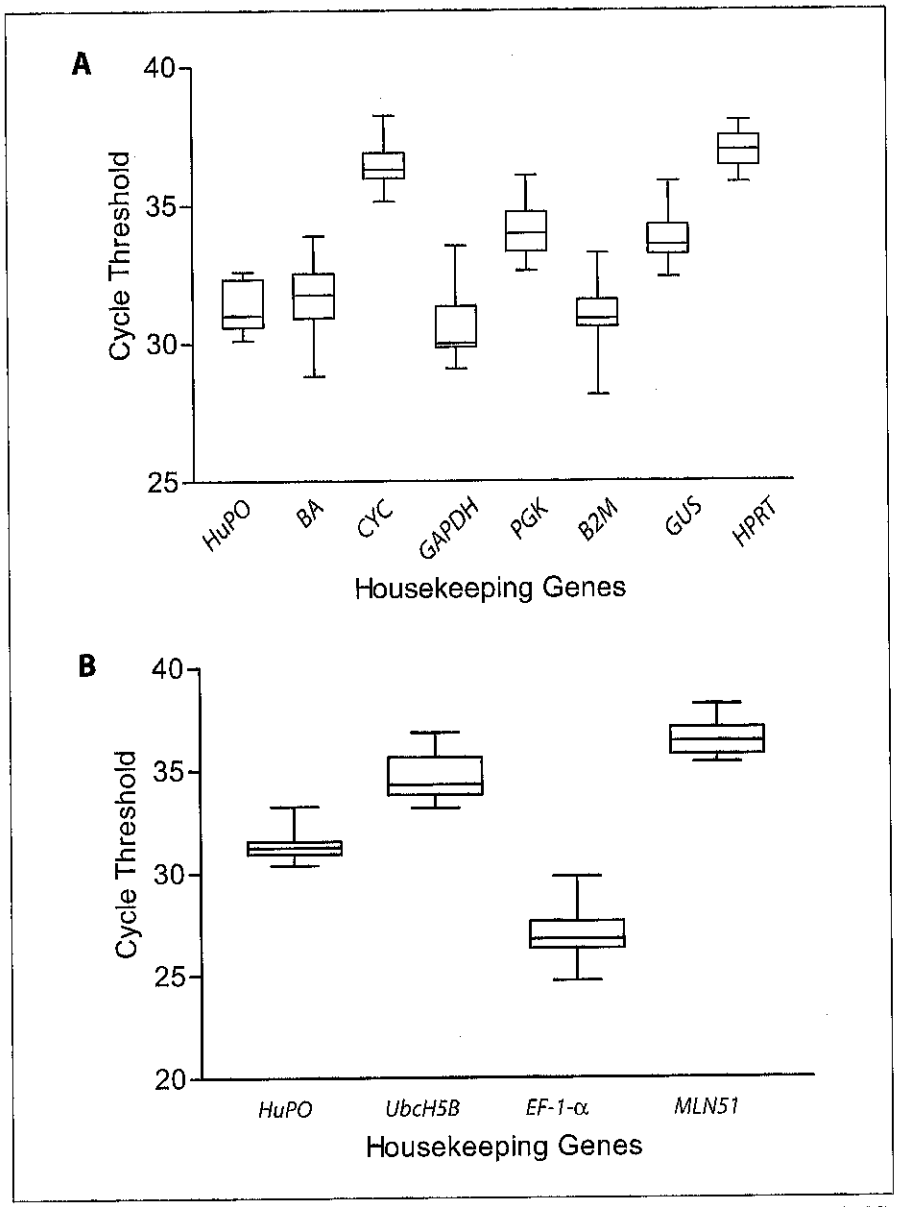

Figure 3. Real-time PCR cycle threshold values in peripheral blood mononuclear cell (PBMC) culture samples. Expression levels of group 1 genes (A) and group 2 genes (B) are shown as medians (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers) for 12 human PBMC culture samples (4 tuberculosis patients at 3 time points). *HuPO*, human acidic ribosomal protein; *BA*, β-actin; *CYC*, cyclophylin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PGK*, phosphoglycerokinase; *B2M*, β_2-microglobulin; *GUS*, β-glucuronidase; *HPRT*, hypoxanthine phosphoribosyltransferase; *UbcH5B*, ubiquitin conjugating enzyme; *EF-1-α*, elongation factor-1-α; *MLN51*, metastatic lymph node 51.

Housekeeping Gene Expression in PBMC Culture

The median expression levels (C_t value) of group 1 and group 2 housekeeping genes for PBMC culture (n = 12) are shown in Figure 3 (A and B). Figure 4 shows the C_t values expressed as fold changes. The most stable housekeeping genes in PBMC culture were *HuPO* and *HPRT* with an average fold change of <2 and a maximal variability of approximately 5-fold each. The most variable genes were *GAPDH*, β_2-microglobulin, β-actin, and elongation factor 1-α (*EF-1-α*) with an average fold of >2 and a maximum variability of 20- to 35-fold.

DISCUSSION

A study of the expression levels of 13 housekeeping genes in patients with TB revealed only one gene suitable for normalization of RNA levels. According to our selection criteria of <2-fold, *HuPO* was the most suitable gene overall (blood and PBMC culture). *GAPDH* and β-actin did not satisfy our suitability criteria and had an unacceptably high maximal variability. Moreover, genes found to be invariant in mitogen-stimulated human T cell cultures (10) were found to be unsuitable when studied in human PBMC cultures stimulated with TB antigen. Other genes like *HPRT* were more variable in whole blood than in proliferating PBMC cultures. This report shows that housekeeping genes are highly specific for a particular experimental model, and validation for each situation, on an individual basis, is a crucial requirement.

Housekeeping genes can be variable and prone to directional shifts induced by experimental conditions, thereby causing problems for reliable normalization. An alternative is normalization to total RNA. This approach avoids the controversies and validation of housekeeping genes. However, it does not control for error introduced by the reverse transcription and other steps and requires significant amounts of RNA. More importantly, there must be an accurate and reliable method of RNA quantification. This can be problematic when a spectrophotometer is used

because of instrument insensitivity, signal contribution by contaminants, residual DNA despite DNase treatment, and unreliability at concentrations below 100 ng/μL (3,5). We have found the Agilent 2100 Bioanalyzer to give consistent results between group 1 and group 2 genes, and it is generally considered to be more reliable than a spectrophotometer (5). The analyzer, however, is expensive and may not be widely available, especially to laboratories in developing countries where the burden of TB is the highest, and there is an urgent need to build research capacity.

The data presented here describe a practical alternative to RNA normalization and address some of the problems with housekeeping genes. For our model of cytokine study in human TB, finding a suitable housekeeping gene created a convenient way to normalize for differing input amounts of RNA and avoided the errors associated with *GAPDH* and β-actin. We also defined the limits of the *HuPO* gene's variability, which is helpful for data interpretation when normalizing low-copy number cytokines. The disadvantage of HK gene validation is that considerable effort and cost is expended to perform an exercise like the one presented here. This may not be feasible in small studies or those with limited budgets. Similar constraints apply to normalization with an average of three housekeeping genes (18). Our findings will facilitate the use of the housekeeping gene method in small laboratories or those from resource-poor settings when studying TB host gene expression in blood and PBMC culture.

In order to create maximum variability, we used subjects of different ages, sex, and ethnic backgrounds. We also used different tissues or cell types sampled at different time points, which included cells challenged with TB antigen. Our selection of subjects and time points mirrored experimental protocols that we propose to use in our laboratory to study cytokines. We found that the housekeeping gene variability in blood was largely due to inter-individual differences. There was surprisingly little difference in gene expression levels at 4 h in the samples of both healthy and TB patients, considering that these samples had been subjected to in vitro storage conditions for several hours. It is possible that the expression of highly regulated genes [e.g., cyclooxygenase-2 (*COX2*)] would have shown a large variability between time points. Compared to blood, the variability in culture was due to both inter-individual differences and differences between different culture time points. These differences were not patient specific.

We found that using the Applied Biosystems' commercial plate was a convenient way to do our initial screen because these genes vary in expression levels and cover a wide range of biologi-

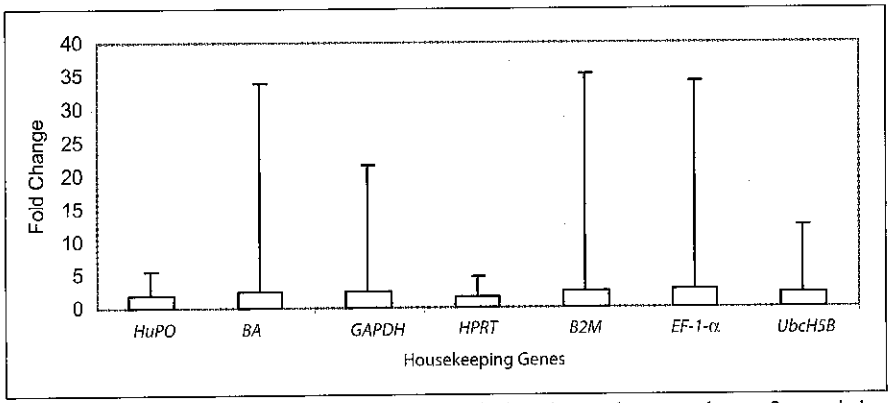

Figure 4. Fold change in gene expression. Variability of selected group 1 genes and group 2 genes in human peripheral blood mononuclear cell (PBMC) culture shown as an average fold change from the mean (columns) and maximum fold change (error bars). *HuPO*, human acidic ribosomal protein; *BA*, β-actin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT*, hypoxanthine phosphoribosyltransferase; *B2M*, β_2-microglobulin; *EF-1-α*, elongation factor-1-α; *UbcH5B*, ubiquitin conjugating enzyme.

cal functions. We selected group 2 genes to increase the number of genes considered in our validation exercise. A variability of 2-fold was chosen, as some cytokines [e.g., interleukin 4 (IL-4)] are both expressed and biologically functional at low levels (17). A one-log difference is significant in human models of TB (19). A housekeeping gene with wider variability would increase the assay noise, hence limiting sensitivity.

In conclusion, *HuPO* is suitable for use as a housekeeping gene in models of human PTB when gene expression is studied in whole blood and PBMC cultures. *GAPDH* and β-actin are unsuitable for this purpose. Whatever strategy is used to control for differences in input RNA it must be validated for a particular experimental model on an individual basis. This is to avoid significant inaccuracies when quantifying target gene expression.

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REFERENCES

1. **Karge III, W.H., E.J. Schaefer, and J.M. Ordovas.** 1998. Quantification of mRNA by polymerase chain reaction (PCR) using an internal standard and a nonradioactive detection method. Methods Mol. Biol. *110*:43-61.
2. **Thellin, O., W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen, T. Grisar, A. Igout, and E. Heinen.** 1999. Housekeeping genes as internal standards: use and limits. J. Biotechnol. *75*:291-295.
3. **Bustin, S.A.** 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. *2*:169-193.
4. **Glare, E.M., M. Divjak, M.J. Bailey, and E.H. Walters.** 2002. β-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. Thorax *57*:765-770.
5. **Bustin, S.A.** 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. *1*:23-39.
6. **Lewis, D.B., K.S. Prickett, A. Larsen, K. Grabstein, M. Weaver, and C.B. Wilson.** 1988. Restricted production of interleukin 4 by activated human T cells. Proc. Natl. Acad. Sci. USA *85*:9743-9747.
7. **Zhu, G., H. Xiao, V.P. Mohan, K. Tanaka, S. Tyagi, F. Tsen, P. Salgame, and J. Chan.** 2003. Gene expression in the tuberculous granuloma: analysis by laser capture microdissection and real-time PCR. Cell Microbiol. *5*:445-453.
8. **Shi, L., Y.J. Jung, S. Tyagi, M.L. Gennaro, and R.J. North.** 2003. Expression of Th1-mediated immunity in mouse lungs induces a Mycobacterium tuberculosis transcription pattern characteristic of nonreplicating persistence. Proc. Natl. Acad. Sci. USA *100*:241-246.
9. **Kawahara, M., T. Nakasone, and M. Honda.** 2002. Dynamics of gamma interferon, interleukin-12 (IL-12), IL-10, and transforming growth factor beta mRNA expression in primary Mycobacterium bovis BCG infection in guinea pigs measured by a real-time fluorogenic reverse transcription-PCR assay. Infect. Immun. *70*:6614-6620.
10. **Hamalainen, H.K., J.C. Tubman, S. Vikman, T. Kyrola, E. Ylikoski, J.A. Warrington, and R. Lahesmaa.** 2001. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. Anal. Biochem. *299*:63-70.
11. **Rainen, L., U. Oelmueller, S. Jurgensen, R. Wyrich, C. Ballas, J. Schram, C. Herdman, and D. Bankaitis-Davis, et al.** 2002. Stabilization of mRNA expression in whole blood samples. Clin. Chem. *48*:1883-1890.
12. **Seah, GT., and G.A. Rook.** 2001. IL-4 influences apoptosis of mycobacterium-reactive lymphocytes in the presence of TNF-alpha. J. Immunol. *167*:1230-1237.
13. **Tricarico, C., P. Pinzani, S. Bianchi, M. Paglierani, V. Distante, M. Pazzagli, S.A. Bustin, and C. Orlando.** 2002. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal. Biochem. *309*:293-300.
14. **Warrington, J.A., A. Nair, M. Mahadevappa, and M. Tsyganskaya.** 2000. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. Physiol. Genomics *2*:143-147.
15. **Zhang, J. and C.D. Byrne.** 1999. Differential priming of RNA templates during cDNA synthesis markedly affects both accuracy and reproducibility of quantitative competitive reverse-transcriptase PCR. Biochem. J. *337*:231-241.
16. **Panaro, N.J., P.K. Yuen, T. Sakazume, P. Fortina, L.J. Kricka, and P. Wilding.** 2000. Evaluation of DNA fragment sizing and quantification by the agilent 2100 bioanalyzer. Clin. Chem. *46*:1851-1853.
17. **Livak, K.J. and T.D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods *25*:402-408.
18. **Vandesompele, J., K. Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. *3*:RESEARCH0034.
19. **Seah, G.T., G.M. Scott, and G.A. Rook.** 2000. Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis. J. Infect. Dis. *181*:385-389.

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