Validation of Reference Genes in Cervical Cell Samples from Human Papillomavirus-Infected and -Uninfected Women for Quantitative Reverse Transcription-PCR Assays

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Reference genes for quantitative reverse transcription-PCR (qRT-PCR) studies must be validated for the cell type studied and should be stable between the groups that represent the independent variable in an experimental design. We sought to identify the reference genes in cervical cell specimens showing the most stable expression between human papillomavirus (HPV)-infected and -uninfected women without high-grade cervical intraepithelial neoplasia. Using endocervical cells collected by cytology brush and Sybr green-based qRT-PCR, eight candidate genes were screened for amplification efficiency, specificity, and overall stability (by use of geNorm software). The five most stable genes were then further evaluated both for overall stability (geNorm) and intergroup stability (by use of NormFinder software) in specimens from HPV-negative and HPV-positive women. The combination of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) and RPLP0 was the most stable overall, with a geNorm stability measure of 0.603. The intergroup analysis showed GAPDH to be the most stable single gene and RPLP0 to be second most stable and also showed that these genes represent the most stable two-gene combination, with a NormFinder stability value of 0.130. The fact that these two distinct approaches identified the same pair of genes provides added confidence that, when the focus is on HPV infection, a normalization factor derived from these two genes is likely to be appropriate.

An ideal universal reference gene for quantitative reverse transcription-PCR (qRT-PCR) studies would be stably expressed across tissue and cell types and independent of disease state, therapeutic intervention, physiologic covariates, or ex vivo manipulation; unfortunately, such a gene has never been identified and may not exist. Because of exponential amplification, validation of the expression stability of a candidate reference gene carries the same requirement for an independently stable reference against which to normalize input differences as does quantitation of a gene of interest (GOI). Recently, several algorithms have been developed to circumvent this problem (1, 8, 14, 18). The approach of Vandesompele et al. starts with the proposition that the expression ratio of two suitable reference genes should be constant across samples to be studied and thus uses a pairwise evaluation strategy to identify the most stable genes from a pool of candidates (18). In contrast, that of Andersen et al. addresses directly the requirement for stable expression of the reference between the groups that represent the independent variable in an experimental design (e.g., pre- and posttreatment or infected versus uninfected) (1). Both algorithms can be automated using Microsoft Excel-based applications available from the respective authors.

One area where studies of gene expression have been gaining momentum is in the investigation of diseases of the female genital tract. When the focus of such work is on the cervical mucosa, as in studies of cervical neoplasia or sexually transmitted infections, cellular specimens are often obtained from the cervical mucosa via cytology brush. Steinau et al. recently advanced the state of the art in such studies by critically examining reference gene stability in cervical samples, using a combination of the aforementioned approaches, with particular attention to studies of cervical intraepithelial neoplasia (CIN) (17). They identified the genes that were most stable overall in a cohort including women with and without intraepithelial lesions and also the most stable choices when stratifying by CIN status and grade. Because our group and others are interested, in contrast, in the early host response to human papillomavirus (HPV) infection in women without premalignant lesions of the cervix, we sought here to use the same approaches to identify reference genes showing the most stable expression, in cervical specimens, between HPV-infected and -uninfected women without high-grade CIN.

MATERIALS AND METHODS

Study subjects. Cervical samples were collected in the course of a prospective study of the natural history of HPV infection described previously (15). Informed consent was obtained according to the guidelines set forth and approved by the Committee for Human Research at The University of California, San Francisco. Briefly, women aged 13 to 21 with less than 5 years of sexual experience were recruited for the prospective study at two study sites, without selection for HPV status at the time of recruitment. Study visits, conducted at 4-month intervals, included face-to-face interviews and collection of samples for monitoring of sexually transmitted infections, HPV detection and typing, cytokine gene expression measurement by qRT-PCR, and cytology. HPV testing was performed as previously described (15), using PCR amplification with PGMY09/11 primers and a reverse line blot assay. The endocervical cell samples collected for cytokine gene expression measurement provided the RNA for the present reference gene stability examination; only samples from visits where women had normal cytology and were negative for Chlamydia trachomatis, Neisseria gonorrhoeae, and

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Trichomonas vaginalis were used. The 28 samples used in this study included 19 from women who were HPV negative and 9 from women who were HPV positive. Of the latter, three were positive for a single HPV type each and six were positive for multiple types, as is often seen in HPV infection (Table 1). Both oncogenic types (16, 26, 39, 51, 52, 53, 59, 66, 68, and 73) and nononcogenic types (6, 40, 55, and 62) were represented (12).

### Specimen collection and RNA isolation for qRT-PCR
Endocervical samples were collected by use of a cytology brush prior to other cervical samples to minimize contamination by peripheral blood. The brush was rotated counterclockwise in the cervical os through two full turns and then placed in RNAlater (Ambion, Austin, TX) as the monophase lysing reagent. Purified total RNA was measured and assessed for purity by determining absorbance at 260 and 280 nm.

### Table 1. HPV type distribution in study subjects

| Subject | HPV type(s) |
|---------|-------------|
| 1       | 53*         |
| 2       | 59*         |
| 3       | 73*         |
| 4       | 16, 52*     |
| 5       | 51, 66*     |
| 6       | 55, 68*     |
| 7       | 6, 16, 39*  |
| 8       | 16, 26, 52, 68* |
| 9       | 39, 40, 62, 68* |

* Oncogenic or probable oncogenic type (12).

### Table 2. Genes and primer sequences

| Gene symbol | GenBank accession no. | Protein name | ID* | Primer sequences (5’→3’)* | Amplicon length (bp) | Exon(s)* | Functional class |
|-------------|-----------------------|--------------|-----|---------------------------|---------------------|---------|------------------|
| ACTB        | NM_001101             | β-Actin      | 1   | CTGGAACGGTGAAAGGTGACA (F) AAGGGACCTTCTGTAAACATGCA (R) | 140                | 5       | Cytoskeletal structural protein |
| GAPDH       | NM_002046             | Glyceraldehyde-3-phosphate dehydrogenase | 2065 | AAGGTCGAGGTCACAACGGATTT (F) ACCAGAGTAAAAGACGCGCTT (R) | 66                | 2/3     | Glycolysis and gluconeogenesis |
| HPRT1       | NM_000194             | Hypoxanthine phosphoribosyltransferase 1 | 2989 | TGGTCGAGCAGTATAACTCAGGA (F) TCAAATCTCAACAAGTCTGCTT (R) | 100               | 6       | Purine synthesis in salvage pathway |
| RPLP0       | NM_001002             | Large ribosomal protein P0 | 2507 | CTCCTATATCCGGGAGGATG (F) GCAGCACTGGACCTTATTTG (R) | 95                 | 4/5     | Ribosomal protein |
| PGK1        | NM_000291             | Phosphoglycerate kinase 1 | 2387 | AAGTGAAGCTCGGAAAAGCTTCT (F) AAGGAAAAGATGCTTCATGG (R) | 71                 | 4       | Glycolysis |
| TBP         | NM_003194             | TATA box binding protein | 2630 | TGCCACAGGCCACAGAGTTGAA (F) TACATCACAGCCCTCCCACCA (R) | 132                | 5/6     | RNA polymerase II transcription factor |
| GUSB        | NM_000181             | β-Glucuronidase   | 2929 | CTCATTGGAAATTTTGGCGATT (F) CCGGTGGAAGATCCCTTTTAT (R) | 81                 | 11, 12  | Lysosomal exoglycosidase |
| B2M         | NM_004048             | β2-Microglobulin  | 1535 | TGAATTTGTCACAGGCCAAGGATA (F) CGGATCTCTCAACCTCCCACCA (R) | 75                 | 2       | Beta chain of major histocompatibility complex class I molecule |

* Identification number of primer sequence entry in RTPrimerDB.

b F, forward; R, reverse.

c Exon (or, for splice site-spanning primers, exons) in which primer binds.
TABLE 3. Amplification characteristics and geNorm ranking of candidate reference genes

| Gene   | Amplification efficiency<sup>a</sup> | Dissociation curve analysis<sup>c</sup> | Expression stability<sup>d</sup> | geNorm stepwise exclusion ranking | Average expression stability (M) |
|--------|-----------------------------------|----------------------------------|----------------------------|-------------------------------|---------------------------------
| ACTB   | 99.7                              | s                                | 1                          | 0.347                         |
| B2M    | 98.4                              | s                                | 1                          | 0.347                         |
| PGK1   | 100.5                             | s                                | 3                          | 0.537                         |
| GAPDH  | 100.1                             | s                                | 4                          | 0.723                         |
| RPLP0  | 101.1                             | s                                | 6                          | 0.931                         |
| GUSB   | 108.7                             | m                                | 10                         | 1.044                         |
| HPRT1  | 100.5                             | s                                | 7                          | 1.102                         |
| TBP    | 104.4                             | s                                | 8                          | 1.294                         |

<sup>a</sup> qPCR was performed using RNA isolated from cytology brush samples from 10 HPV-negative women. One sample was excluded from analysis due to failed amplification reactions.

<sup>b</sup> Calculated as $E = \left(10^{-\text{slope} - 1}\right) \times 100$, where slope is the slope of the standard curve dilution series. Regression coefficients ($r^2$) for all standard curves were 0.99.

<sup>c</sup> Results from negative first derivative plots of dissociation curves (temperature versus fluorescence intensity): s, single peak observed (exclusive of late-amplifying primer-dimer peaks seen only in no-template control samples); m, multiple peaks observed.

<sup>d</sup> Expression stability ranking and average expression stability measure (M) of remaining reference genes as each successive lowest ranking (least stable) gene is eliminated in stepwise fashion, starting with TBP, and the stability of remaining genes is recalculated, using geNorm software. Lower M values indicate greater stability. The two most stable genes ($ACTB$ and $B2M$) cannot be further ranked.

RESULTS AND DISCUSSION

We first evaluated the amplification efficiency, primer specificity, and overall stability of candidate reference genes. Although the number of potential candidate genes is virtually limitless, practical considerations inevitably constrain how many can be evaluated; Andersen et al. recommend evaluating a minimum of five genes (1). In order to test genes in triplicate in a single 384-well plate, eliminating potential interplate variation, this preliminary evaluation included eight genes (Table 2) and was carried out using cervical samples from 10 HPV-negative women. The eight genes included the three previously identified as most stable in cervical cells (17) plus additional common housekeeping genes. Because Andersen et al. recommend including a minimum of eight samples per group in their model-based approach (1), intergroup stability assessment using that approach was not a goal of this preliminary evaluation. One sample had multiple failed amplifications and was eliminated from analysis. Except for $GUSB$ and $TBP$, all genes showed amplification efficiencies (derived from the slope of the standard curve) of between 98% and 101% (Table 3) and standard curve regression coefficients of 0.99, meeting standard guidelines of acceptability (2, 3). Except for $GUSB$, all candidates showed only a single peak in the negative first derivative plot of the dissociation curves. (Distinct primer-dimer-associated peaks seen only in the no-template controls, and with threshold cycle values greater than 30 cycles and at least 10 cycles later than the lowest dilution in the standard curve, were disregarded.) $GUSB$ showed multiple peaks and was not considered further. The stability ranking by geNorm’s stepwise exclusion algorithm is shown in Table 3.

Based on the minimum sample and gene number recommendations of Andersen et al. (1), the five most stable genes from the preliminary evaluation were carried into further evaluation using samples from nine HPV-positive and nine HPV-negative women. Our goal was again to run all genes and samples in triplicate reactions within a single plate. Overall stability was assessed using the stepwise exclusion algorithm of geNorm, and intergroup stability, with stratification by HPV infection status, was assessed using NormFinder (Table 4). The former showed the combination of $GAPDH$ and $RPLP0$ to be the most stable overall, while the latter showed $GAPDH$ to be the most stable single gene and $RPLP0$ to be second most stable single gene in an infection status model. NormFinder furthermore determined that these same two genes represent the most stable two-gene combination. In order to increase the power of the model-based NormFinder approach, the data from the two runs were combined, for a total of 18 HPV-negative and 9 HPV-positive cases. The NormFinder rankings were unchanged by the inclusion of additional samples, and stability values were nearly identical (data not shown).

The circular problem that investigators have historically faced in evaluating housekeeping gene stability has been largely overcome, although probably not definitively settled, by the recent development of mathematical approaches such as the ones employed in this study (4, 5). While concerns remain about the influence of gene coregulation on the outcome of the geNorm algorithm and debate continues over whether and when it is advantageous to use multiple reference genes for normalizing GOI expression (1), these algorithms have nonetheless allowed development of improved normalization strat-
...egies for a number of cell types and experimental settings (6, 11, 17). To the best of our knowledge, this is the first report employing these contemporary approaches to identify a suitable normalization strategy for examination of cervical gene expression in studies of cervical HPV infection. The fortunate result that these two very distinct approaches identified the same pair of genes as being the most stable provides added confidence that, when the focus is on HPV infection, a normalization factor derived from GAPDH and RPLP0 is likely to be most appropriate. These genes represent unrelated functional classes, essentially abrogating concerns that the pairwise stability rankings from geNorm arose artificially from coregulation of expression. We considered, however, the possibility that inclusion in the analysis of two genes involved in the same metabolic pathway (GAPDH and PGK1) may have skewed the geNorm analysis due to some degree of unappreciated coregulation. To investigate this possibility, we performed separate geNorm analyses excluding one or the other of these genes a priori. When PGK1 was excluded, the same two genes (GAPDH and RPLP0) emerged on top, with the same stability measure as in the full analysis (M = 0.603). Upon exclusion of GAPDH, on the other hand, ACTB and B2M rose to top ranking, but with a less favorable stability measure (M = 0.846), supporting the finding of the GAPDH and RPLP0 pair being the most stable among evaluated genes.

Regarding the optimal number of reference genes to be used for calculating a normalization factor when analyzing GOI expression data, Vandesompele et al. recommend generally using the three most stable genes (tempered, of course, by the practical limitations of cost, sample volumes, and throughput needs) (18). In this case, however, we would recommend using the top two genes, on which the two algorithms agree, for three reasons. The first is that the third overall most stable gene per geNorm, PGK1, fared much more poorly in the NormFinder model-based algorithm, with a fifth-place rank and a relatively unfavorable stability value of 0.302 (Table 4). The second reason is that, although we could identify no reports indicating known coregulation of GAPDH and PGK1, it seems reasonable to avoid, when possible, including two genes from a single metabolic pathway in the calculation of a normalization factor. Lastly, expression of PGK1 has been shown to be increased by tissue hypoxia (16), regulated by signaling through the CXCR4 chemokine receptor (19), and elevated in HPV-associated respiratory papillomas (J. A. DeVoti, personal communication) as well as in various human cancers (9, 10, 20, 21).

The observation that our results, focusing on HPV infection status, differed somewhat from those of Steinau et al. (17) is consistent with the contention of Andersen et al. that candidate genes must be evaluated not just for overall expression variation but also for systematic variation across the subgroups that are relevant to an investigation (1). Employing the same two algorithms, Steinau et al. identified ACTB as the single most stable gene, both overall and in their CIN disease model approach. The combination of ACTB, RPLP0, and PGK1 was identified as the best overall multigene combination by geNorm. For studies with attention to intraepithelial lesions, the combination of PGK1 and the ribosomal protein L4 gene was the one recommended by NormFinder, with a stability value of 0.181 (compared with 0.244 for ACTB alone). They concluded that ACTB is a good single reference for most situations, while the combination of PGK1 and the ribosomal protein L4 gene is more appropriate for studies focusing on intraepithelial lesions. We included from the start the three overall most stable genes from Steinau’s findings (ACTB, RPLP0, and PGK1) in our panel of candidates. The different result for overall stability is likely due to differences in sample populations, highlighting the importance of evaluating reference gene stability using samples that are most representative of those to be ultimately studied for GOI expression. In their case, the focus was on CIN grade, so while the samples “represented the spectrum of HPV infection,” their sample choice deliberately and appropriately included many specimens representing all grades of CIN. In contrast, we sought not to include those but rather to ensure that HPV-positive and -negative women were equally represented. Our data suggest that the use of a normalization factor derived from the relatively stable combination of GAPDH and RPLP0 represents an advance in the measurement of cervical gene expression in studies focusing on HPV infection, although one must recognize that future methodologic advances may identify yet-superior reference genes or normalization strategies. Also, as this study adds to the literature showing the exquisite dependence of normalization strategy on the specimen types and populations under study, investigators with different goals or sample types remain advised to validate reference genes accordingly before proceeding.

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