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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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TABLE 1. HPV type distribution in study subjects

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

[a] Oncogenic or probable oncogenic type (12).

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 RNA stabilization solution (Ambion, Austin, TX) for transport and storage. RNA isolation was performed as previously described (15), using a modified acid-phenol method (7) and TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) as the monophase lysing reagent. Purified total RNA was measured and assessed for purity by determining absorbance at 260 and 280 nm and then was stored at −80°C until testing.

Candidate reference gene primers. Eight candidate reference genes were chosen for preliminary evaluation based on previously reported overall stability in cervical samples (17), as well as our own prior experience and that of colleagues studying gene expression in cervical samples. To the best of our knowledge, none of these genes are known to be coregulated. Primer sequences (Table 2) for these genes were chosen from the RTPrimerDB online real-time PCR primer database (http://medgen.ugent.be/rtprimerdb) (13) and synthesized by Integrated DNA Technologies (Coralville, IA). Preference was given to primers meeting the following criteria: amplicon length of between 50 and 150 bp, GC content of between 40 and 60%, maximum of 2 Cs or Gs at the 3′ end, annealing in different exons or having no more than 5 bp of 3′ overhang into the same exon, and no runs of the same nucleotide (3).

RT and qPCR. One microgram of total RNA was DNase treated with Turbo DNA-free (Ambion) following the manufacturer's instructions. RT was performed using 0.5 μg DNase-treated RNA in a 20-μl reaction volume with random hexamer (37-ng/μl final concentration; Promega, Madison, WI) and random nonamer (25 nM; Gene Link, Hawthorne, NY) priming, RNasin RNase inhibitor (Promega), and the Omniscript RT kit (Qiagen, Valencia, CA) at 37°C for 60 min, following the manufacturer's instructions.

qPCR was performed in 384-well optical reaction plates (Applied Biosystems, Foster City, CA) in triplicate 15-μl reaction volumes containing Power Sybr green PCR master mix (1× final concentration; Applied Biosystems), forward and reverse primers at a 150 nM final concentration, and either cervical unknown cDNA template (12.5 ng/reaction volume), standard, or water for the no-template controls. The template (unknown or standard) was premixed with the Sybr green master mix prior to pipetting into the plate, to minimize the influence of pipetting errors through normalization of reporter fluorescence to the included passive reference. Standard curves for each plate were prepared from Stratagene QPCR Human Reference Total RNA (Stratagene, La Jolla, CA), reverse transcribed identically to the cervical RNA samples, and diluted in fivefold steps from 50 ng/reaction volume (final concentration) to 0.08 ng/reaction volume. The plates were run on an ABI Prism 7900HT sequence detection system (Applied Biosystems), using the following program: an enzyme activation step at 95°C for

TABLE 2. Genes and primer sequences

Gene symbol	GenBank accession no.	Protein name	ID[a]	Primer sequences (5′→3′)[b]	Amplicon length (bp)	Exon(s)[c]	Functional class
ACTB	NM_001101	β-Actin	1	CTGGAACGGTGAAGGTGACA (F) AAGGGACTTCCTGTAACAATGCA (R)	140	5 5	Cytoskeletal structural protein
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	2065	AAGGTCGGAGTCAACGGATTT (F) ACCAGAGTTAAAAGCAGCCCTG (R)	66	2/3 3	Glycolysis and gluconeogenesis
HPRT1	NM_000194	Hypoxanthine phosphoribosyltransferase 1	2989	TGGTCAGGCAGTATAATCCAAAGA (F) TCAAATCCAACAAAGTCTGGCTTA (R)	100	6 7/8	Purine synthesis in salvage pathway
RPLP0	NM_001002	Large ribosomal protein P0	2507	CCTCATATCCGGGGGAATGTG (F) GCAGCAGCTGGCACCTTATTG (R)	95	4 4/5	Ribosomal protein
PGK1	NM_000291	Phosphoglycerate kinase 1	2387	AAGTGAAGCTCGGAAAGCTTCTAT (F) AGGGAAAAGATGCTTCTGGG (R)	71	4 5	Glycolysis
TBP	NM_003194	TATA box binding protein	2630	TGCACAGGAGCCAAGAGTGAA (F) CACATCACAGCTCCCCACCA (R)	132	5/6 6	RNA polymerase II transcription factor
GUSB	NM_000181	β-Glucuronidase	2929	CTCATTTGGAATTTTGCCGATT (F) CCGAGTGAAGATCCCCTTTTTA (R)	81	11 12	Lysosomal exoglycosidase
B2M	NM_004048	β_2-Microglobulin	1535	TGACTTTGTCACAGCCCAAGATA (F) CGGCATCTTCAAACCTCCA (R)	75	2 3/4	Beta chain of major histocompatibility complex class I molecule

[a] 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[a]

Gene	Amplification efficiency[b]	Dissociation curve analysis[c]	Expression stability[d]	
			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	100.1	s	5	0.931
GUSB	108.7	m	6	1.044
HPRT1	100.5	s	7	1.102
TBP	104.4	s	8	1.294

[a] 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.

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

[c] 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.

[d] 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.

10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min, and a final dissociation curve analysis step using default program settings.

Gene stability analysis. Two Microsoft Excel-based applications, the geNorm VBA applet for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm) (18) and the NormFinder MS Excel Add-in (http://www.mdl.dk/publicationsnormfinder.htm) (1) were used, following instructions included with the software downloads, to analyze candidate reference gene quantities in cervical samples (interpolated from the standard curves, in arbitrary units) for expression stability. The former application tests for overall stability using a pairwise comparison method, while the latter tests stability in a model-based approach which attempts to minimize estimated intra- and intergroup variation.

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.

TABLE 4. geNorm and NormFinder rankings of the top five candidate reference genes[a]

Rank[b]	geNorm analysis		NormFinder analysis	
	Gene	Avg M[c]	Gene	Stability value[d]
1	*GAPDH-RPLP0*	0.603	*GAPDH*	0.088
2			*RPLP0*	0.238
3	*PGK1*	0.747	*B2M*	0.265
4	*ACTB*	0.878	*ACTB*	0.289
5	*B2M*	0.949	*PGK1*	0.302

[a] qPCR was performed using RNA isolated from cytology brush samples from 18 women (9 HPV negative and 9 HPV positive).

[b] Stability ranking, from most to least stable, of candidate housekeeping genes by the two respective approaches.

[c] Average overall expression stability measure (M) of remaining reference genes as each successive lowest ranking (least stable) gene is eliminated in stepwise fashion, starting with *B2M*, and the stability of remaining genes is recalculated, using geNorm software. Lower values indicate greater stability. The two most stable genes (*GAPDH* and *RPLP0*) cannot be further ranked.

[d] Stability value determined by NormFinder software, using HPV status as the independent grouping variable. Lower values indicate greater stability. The software algorithm also determined the combination of *GAPDH* and *RPLP0* to be the best combination of two genes, with a stability value of 0.130.

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 artifactually 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 to not 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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