JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, July 2012, Vol. 57, No. 4
doi: 10.1111/j.1556-4029.2012.02086.x
Available online at: onlinelibrary.wiley.com

TECHNICAL NOTE

CRIMINALISTICS

Lilliana I. Moreno,[1,2] M.A., M.FS.; Courtney M. Tate,[2] Ph.D.; Erika L. Knott,[3] B.S.; Jade E. McDaniel,[2] B.S.; Stephanie S. Rogers,[2] B.S.; Barbara W. Koons,[2] M.S.; Mark F. Kavlick,[2] B.S.; Rhonda L. Craig,[1] M.S.; and James M. Robertson,[2] Ph.D.

Determination of an Effective Housekeeping Gene for the Quantification of mRNA for Forensic Applications*

ABSTRACT: The potential application of mRNA for the identification of biological fluids using molecular techniques has been a recent development in forensic serology. Constitutively expressed housekeeping genes can assess the amount of mRNA recovered from a sample, establish its suitability for downstream applications, and provide a reference point to corroborate the identity of the fluid. qPCR was utilized to compare the expression levels of housekeeping genes from forensic-like body fluid stains to establish the most appropriate assessment of human mRNA quantity prior to profiling. Although variability was observed between fluids and individuals, results indicated that beta-2 microglobulin exhibited the highest expression for all body fluids examined and across donors. A one-way analysis of variance was performed for housekeeping gene variability between donors (at the α, 0.05, significance level), and the results indicated significant differences for semen, vaginal secretions, and menstrual blood.

KEYWORDS: forensic science, forensic biology, serology, messenger RNA, real-time polymerase chain reaction, glyceraldehyde-3-phosphate dehydrogenase, beta-actin, beta-2 microglobulin, cyclophilin A, phosphoglycerate kinase 1, ribosomal protein, large, P0

RNA profiling has been under scrutiny over the past couple of years for its potential ability to identify a variety of body fluids in forensic samples and thus complement or even substitute current serological techniques (1–5). Within cells, DNA is transcribed into messenger RNA (mRNA), which is translated into proteins that carry out cell-specific processes. Exploitation of the unique genes expressed by specific cell types gives insight into which biological fluid(s) or cells are present in a given stain. As with DNA, quantification of the recovered genetic material is important for the quality assurance of the mRNA profiling process. In addition to determining sufficiency, the relation of the quantity recovered as translated to expression and weighed against the expression of a confirmatory gene could serve as an internal guide to substantiate the identity of the fluid in question. To measure the recovered mRNA, the levels of ubiquitously expressed housekeeping gene transcripts are determined and compared with the levels of a chosen reference gene transcript. Determining the best reference gene is the focus of this study.

The assessment of mRNA recovery after extraction can prove useful in determining the approach for downstream applications. Too little mRNA material prevents downstream analysis, whereas too much compromises the final results. A series of technologies that exploit different properties of the molecule under investigation are available for the quantification of nucleic acids. Systems commonly used for the quantification of RNA include spectrophotometric UV–VIS assays, which determine the amount of total RNA present in any given sample based on absorbance readings, and fluorometric assays, which determine the concentration of the molecule based on the amount of intercalated dye (6–8). These systems provide good estimates of total RNA, but will often overestimate the amount of material suitable for molecular typing because mRNA typically constitutes only ~3% of total cellular RNA (9). Alternatively, the presence and quantity of total mRNA in a sample may be determined based on the transcript level of a constitutively expressed housekeeping gene(s) (10,11). In turn, tissue-/cell-specific mRNA transcripts may be quantified relative to the housekeeping gene(s) using quantitative polymerase chain reaction (qPCR or real-time PCR), which is based on the amount of fluorescence emitted during amplification (10). This sensitive and specific technique can be used to quantify both transcript types simultaneously, to establish the level of mRNA, and to determine the tissue source of the sample. Studies have suggested several housekeeping genes as potential candidates for this purpose (11,12); however, variation in expression has been reported for

[1]Nuclear DNA Unit, FBI Laboratory Division, Quantico, VA 22135.
[2]Counterterrorism and Forensic Science Research Unit, FBI Academy, Quantico, VA 22135.
[3]Arkansas State Crime Laboratory, Little Rock, AR 72205.

*This is publication number 10-21 of the Federal Bureau of Investigation (FBI). All samples were collected from consenting individuals according to FBI's institutional standards. Names of commercial manufacturers are provided for identification purposes only, and inclusion does not imply endorsement of the manufacturer, its products, or services by the FBI. The views expressed are those of the authors and do not necessarily reflect the official policy or position of the FBI or the U.S. Government. This research was supported in part by the FBI's Visiting Scientist Program, an educational opportunity offered by the Oak Ridge Institute for Science and Education (ORISE).

Received 13 Dec. 2010; and in revised form 31 Mar. 2011; accepted 10 June 2011.

certain housekeeping genes among different sources and sample types (13–15). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most widely used housekeeping genes in gene expression studies, despite the knowledge of the existence of pseudogenes and a myriad of other factors that affect its expression levels (16–19). However, GAPDH can be a good reference candidate and serve as a normalization factor when assessing a variety of target genes.

The expression stability of the gene of choice is important for obtaining reliable quantification results. Housekeeping gene evaluation studies have focused on gene expression level variation depending on tissue degradation time, specific tissue type, or nonhuman samples (20–22). However, housekeeping gene evaluation in forensic-like samples has not been addressed. After an *in silico* query of various housekeeping genes, six were chosen to investigate their expression levels in human body fluid samples to determine which one was most appropriate for the quantification of low-level, human-specific mRNA. Genes were selected based on their commercial availability, the consistency of their expression among different tissues, and the size of their qPCR amplicons (Table 1). The chosen genes included GAPDH, beta-actin (ACTB), beta-2 microglobulin (B2M), cyclophilin A (PPIA), phosphoglycerate kinase 1 (PGK1), and large/acidic ribosomal protein P0 (RPLP0), all of which have important functions in the cell (23–31). To investigate differences in gene expression levels of the aforementioned markers, semen, saliva, blood, vaginal secretion, and menstrual blood samples were analyzed by qPCR. Although not addressed in this work, it should be noted that the queried genes are affected by circadian rhythm and are usually up-regulated during the nighttime (32–36). However, gene expression variation among individuals was assessed to determine whether the chosen marker was consistently and highly expressed in the various contributors of the sample/stain in question.

Materials and Methods

Sample Collection

Biological fluids were donated by five healthy, consenting volunteers. Volunteers ranged in age from 28 to 55 years. Samples were collected and processed during the same time of the day to minimize any circadian rhythm–based expression differences. Owing to the nature of semen samples, which were purchased from the Fairfax Cryobank (Fairfax, VA), samples were collected during daytime hours. Saliva, blood, menstrual blood, and vaginal secretions were obtained using sterile techniques. Blood, semen, and saliva samples were spotted onto sterile swabs in 50-μL sample volumes and allowed to dry overnight. Menstrual blood and vaginal secretions were collected directly from the vaginal area onto sterile cotton swabs; thus, the volumes of the stains varied between samples. A second set of samples obtained from the same donors were exposed outdoors (environmental samples) to an average temperature of 25.5°C (month of June) in the absence of precipitation for 24 h before RNA extraction.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from each of the stains utilizing the RNAqueous®-4 PCR kit (Ambion, Austin, TX) following the manufacturer's recommendations, except for an adaptation of the lysis step for forensic stains spotted on cotton swabs (1). To determine the highest-expressed housekeeping gene, seven replicate RNA extractions were obtained from each individual for each body fluid. RNA was extracted in triplicate from the samples provided by each of the five donors to assess individual-to-individual differences in mean mRNA levels. RNA was converted into cDNA after heating the sample at 75°C for 3 min to eliminate any potential mRNA secondary structure (1). The samples were then snap-cooled on ice for 3 min before the addition of 10 mM dNTPs (2.5 mM each), 10× first-strand synthesis buffer (500 mM Tris–HCl pH 8.3, 750 mM KCl, 30 mM $MgCl_2$, 50 mM DTT), 1.33 mM random decamers, 30 units (U) SUPERase-In™ RNAse inhibitor, and 150 U Moloney murine leukemia virus reverse transcriptase (all from Ambion) in a final reaction volume of 30 μL. The mixture was then incubated at 42°C for 60 min followed by reverse transcriptase enzyme inactivation at 95°C for 10 min.

Assessment of Commercial GAPDH Quantification Kits Using Forensic-Like Samples

Two commercially available kits were used to assess the reliability of GAPDH as a potential indicator of the presence of mRNA in forensic-like sample extracts. The GAPDH JOE (226-bp amplicon) TAMRA probe kit (Applied Biosystems, Foster City, CA) was used in combination with the one-step TaqMan® EZ RT-PCR kit (Applied Biosystems). For this one-step reaction, 1 μL of total RNA sample was added to a 24-μL mixture of 1× TaqMan EZ Buffer, 3 mM manganese acetate, 300 μM each dATP, dCTP, and dGTP, 600 μM dUTP, 200 nM GAPDH forward primer, 200 nM GAPDH reverse primer, 100 nM GAPDH probe, 2.5 U rT*th* DNA polymerase, and 0.25 U AMPErase UNG (Applied Biosystems). Samples were amplified using a 7900HT Real-Time PCR instrument (Applied Biosystems) under the following thermal cycling conditions: 2 min at 50°C, a 30-min hold at 60°C, followed by a 5-min hold at 95°C, and 40 cycles of 20 sec at 94°C alternating with 1 min at 62°C.

A two-step assay using the GAPDH FAM (122-bp amplicon) minor groove binder (MGB) probe (Applied Biosystems) was also investigated to determine whether there were any differences in the quantification results obtained owing to the amplicon size and method of cDNA synthesis disparities. For this assay, the RNA extracts were first converted to cDNA as described in the previous section. Following reverse transcription, 2 μL of cDNA was added to a mixture of 2× Fast PCR Master Mix and 20× GAPDH

TABLE 1—*Housekeeping genes utilized in two-step real-time PCR.*

Gene	Amplicon Length (bp) (https://products.appliedbiosystems.com/)	Target Gene Size (bp) (http://www.ncbi.nlm.nih.gov)
GAPDH (glyceraldehyde-3-phosphate)	122	1310
ACTB (beta-actin)	171	1793
B2M (beta-2 microglobulin)	75	987
PGK1 (phophoglycerate kinase 1)	75	2338
PPIA (cyclophilin A)	98	2276
RPLP0 (large/acidic ribosomal protein P0)	105	1289

primers/probe mix (Applied Biosystems) for a final reaction volume of 25 μL per sample. Thermal cycling conditions were set to a 20-sec hold at 95°C followed by 40 cycles of 1 sec at 95°C alternating with 20 sec at 60°C.

Concentration Variability Effect on Target and Reference Gene Relationship

Gene expression for each marker was determined by relative quantification using the comparative cycle threshold (C_t) method. This method enables the comparison of C_t values without the use of a standard curve. To use this technique, a validation experiment must be performed to demonstrate that the amplification efficiencies of the target and reference genes are approximately equal. GAPDH was chosen as the reference gene to normalize the data because it is a commonly used housekeeping gene. Using each marker, a human control RNA (Raji) sample was assayed in duplicate at 5, 2, 1, 0.5, and 0.1 ng/μL. All target genes were monitored by qPCR using commercially available kits with a probe containing an FAM reporter and an MGB quencher (Applied Biosystems). The study was performed in a single plate to eliminate plate-to-plate and run-to-run variability. Thermal cycling conditions were 95°C for 20 sec followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. The duplicate C_t values were averaged, and the difference between the C_t of the various target genes and that of the reference gene (GAPDH) was calculated (ΔC_t). The log of RNA sample input (ng) versus ΔC_t was plotted, and the slopes were used to calculate amplification efficiency (37).

Gene Expression Assay on Environmentally Exposed Forensic-Like Samples and Evaluation Among Different Biological Fluid Donors

Relative quantification assays were performed on the previously described biological fluids to establish the levels of gene expression of the aforementioned markers (Table 1). In addition, to assess the level of expression of the various markers among different biological fluid donors, RNA was isolated in triplicate from five donors for each body fluid sample. Briefly, 12.5 μL 2× Fast PCR Master Mix, 1.25 μL 20× GAPDH primers/probe mix (all obtained from Applied Biosystems), 9.25 μL of DEPC water, and 2 μL of cDNA were mixed in a final reaction volume of 25 μL per sample. To eliminate run-to-run variability in environmental samples, each fluid was examined for all six markers in a single plate, with each of the fluid extraction replicates run in duplicate. In addition, a commercial human Raji cDNA control (Applied Biosystems) was amplified in duplicate for each of the genes in question, and the results were used as a calibrator for the data (37). Samples were amplified using a 7900HT Fast Real-Time PCR System (Applied Biosystems) under the following cycling conditions: 95°C for 20 sec, followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. Cycle threshold (C_t) data obtained for each individual fluid type were averaged and used to calculate the relative quantification value (37). The comparative C_t method calculates the relative quantification value expressed by a target using the formula $2^{-\Delta\Delta Ct}$ in which the amount of target is normalized to an endogenous reference (GAPDH) and then compared to a calibrator (Raji cDNA).

Data and Statistical Analysis

All data were analyzed using Microsoft's Excel software (Microsoft Inc., Redmond, WA). C_t values were averaged for the replicate samples, and a relative quantification value was calculated using the aforementioned formula ($2^{-\Delta\Delta Ct}$). To assess the variability of gene expression among individuals, a one-way analysis of variance (ANOVA) was performed on each average C_t result for each gene and group of individuals per body fluid. The *F*-test was used to assess whether there were significant differences between the mean C_t values obtained for a specific gene across sample donors at an $\alpha = 0.05$ significance level.

Results

GAPDH JOE-TAMRA One-Step Assay Proves Inconsistent Depending on Sample Source

A GAPDH JOE-TAMRA one-step commercial assay kit was used to quantify GAPDH mRNA in semen, saliva, blood, menstrual blood, and vaginal secretion samples. This assay requires RNA input into a one-step (single well, single enzyme) procedure that combines reverse transcription of RNA and qPCR amplification utilizing GAPDH primers and probes (226-bp amplicon) within a single reaction. A human control RNA template of known concentration is included in the kit for the generation of a standard curve for absolute quantification. The one-step assay successfully detected GAPDH mRNA in vaginal secretions, semen, and menstrual blood samples (Fig. 1). In contrast, the detection of GAPDH mRNA in saliva and blood samples was not as successful. Saliva and blood samples consistently amplified below the lowest concentration point in the standard curve (corresponding to 0.001 ng/μL), which is below the assay fluorescent threshold (Fig. 1). Points outside the standard curve cannot be accurately assessed; thus, the amount of GAPDH mRNA from saliva and blood recovered from the samples was, based on these results, not sufficient to undergo a successful amplification. Despite these negative results, the nondetectable RNA samples were converted to cDNA and successfully amplified above the threshold intensities with a traditional PCR using GAPDH-specific primers and separation by capillary electrophoresis (data not shown).

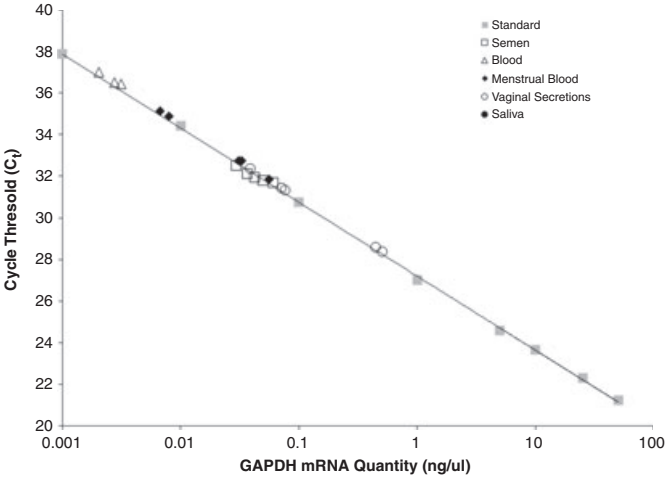

FIG. 1—*One-step real-time quantitative PCR assay analyzing GAPDH mRNA expression in body fluid samples. Graph represents GAPDH amplification results using a one-step GAPDH JOE-TAMRA commercial assay. The standard curve plot was generated using human control RNA diluted to various concentrations (50, 25, 10, 5, 1, 0.1, 0.01, and 0.001 ng/μL) (black squares). Data points represent the amplification results for each of the body fluids examined. All saliva samples and two blood samples consistently amplified at or above the 36 cycle threshold (i.e., below 0.001 ng/μL) and are therefore outside the limits of the graph.*

Failure to detect GAPDH mRNA in saliva and some blood samples prompted the use of an alternative two-step quantification assay using GAPDH FAM-MGB primers and probe (122-bp amplicon). This two-step assay requires reverse transcription of RNA into cDNA prior to analysis by qPCR. In contrast to the one-step assay, the two-step assay successfully amplified GAPDH mRNA above the detection threshold for all but one of the body fluid cDNA samples examined. Only one saliva sample amplified below the lowest concentration point in the standard curve (corresponding to 0.001 ng/μL), which is below the fluorescent threshold of the assay (Fig. 2). These results corresponded to an overall 96% increase in recovery yield of the body fluids as compared to those obtained with the single-step assay (Fig. 2). Because of the increased detection of GAPDH mRNA in the body fluids, the two-step assay was utilized for subsequent studies.

Similar Efficiencies were Attained Between Reference and Target Genes Regardless of Initial RNA Concentration

Prior to performing a gene expression comparison study utilizing a comparative C_t method, it is important to demonstrate that concentration has no significant effect on the amplification efficiency of any given marker. Therefore, the variation in efficiency between the chosen reference gene (GAPDH) and the various target genes (PGK1, RPLP0, PPIA, ACTB, and B2M) was examined at various input concentrations of cDNA (Fig. 3). Delta C_t (ΔC_t) values and the difference between the cycle threshold of the reference gene (GAPDH) and that of the chosen target gene at a specific concentration were calculated and plotted (Fig. 3). The slope of the various plots corresponds to the log of the input amount of total RNA versus ΔC_t values. All slopes were consistently close to zero (B2M: −0.0189, ACTB: −0.086, PGK1: 0.0491, RPLP0: −0.0083, and PP1A: −0.0083). A slope with an absolute value of <0.1 indicates that the efficiencies of the target and reference genes are approximately equal; therefore, a comparative C_t method can be used to compare the relative levels of gene expression.

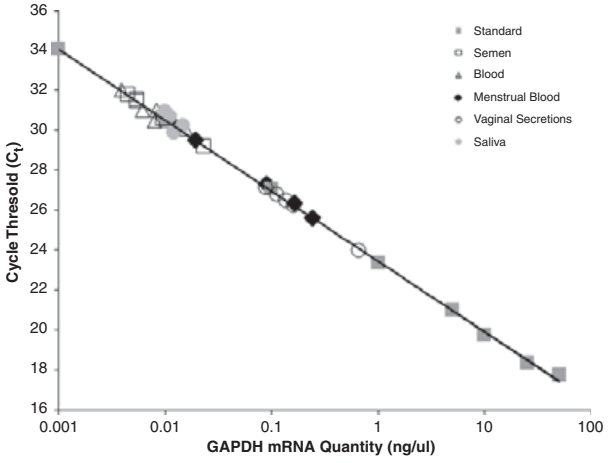

FIG. 2—*Two-step real-time quantitative PCR assay analyzing GAPDH mRNA expression in body fluid samples. Graph represents the amplification of GAPDH utilizing a two-step cDNA conversion/quantification process. The standard curve plot was generated using human control cDNA diluted to various concentrations (50, 25, 10, 5, 1, 0.1, 0.01 and 0.001 ng/μL) (black squares). Data points represent the amplification results of saliva, semen, blood, menstrual blood, and vaginal secretion samples. One saliva sample amplified above the 36 cycle threshold (i.e., below 0.001 ng/μL) and is therefore outside the limits of the graph.*

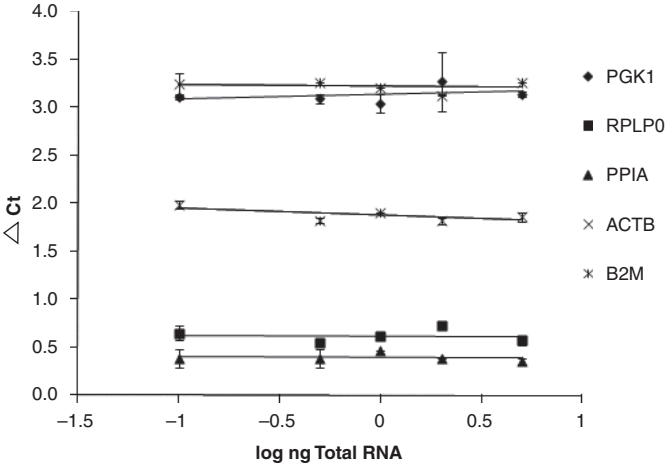

FIG. 3—*Concentration-based efficiency comparison between target and reference (GAPDH) genes. The difference between the average C_t values obtained for each target gene (PGK1, RPLP0, PP1A, ACTB, and B2M) and that of the reference gene (GAPDH) for different corresponding concentrations of human control RNA was calculated and plotted. Numbers represent slopes, and error bars represent standard deviation.*

Beta-2 Microglobulin Appears to Provide the Strongest Overall Expression Pattern in Forensic-Like Stains Examined After 1-Day Environmental Exposure

To determine the housekeeping gene expression after a period of environmental exposure, body fluid samples from a single donor were spotted on cotton swabs and exposed to the external environment (average temperature, 25.5°C, no precipitation) for 24 h before RNA extraction and cDNA synthesis. The two-step quantification assay and comparative C_t method were used to compare the relative gene expression levels in all body fluids. Overall, B2M, ACTB, and PGK1 were consistently among the highest-expressed genes analyzed in saliva, blood, vaginal secretion, and menstrual blood samples (Fig. 4). In contrast, PPIA, PGK1, and B2M exhibited similar expression in the semen samples examined, all of which were higher than ACTB, GAPDH, and RLPL0 expression. GAPDH showed the lowest expression level for most of the fluids examined, followed by RPLP0 and PPIA (Fig. 4). Although consistently low, GAPDH demonstrated higher expression than PPIA in vaginal secretion samples and less variation than RPLP0 for the examined semen donor.

B2M Consistently Exhibits the Highest Expression in Forensic-Like Samples Across Individuals

To compare gene expression differences between individuals, housekeeping gene expression levels were determined using each of the body fluids from five individual donors. In agreement with the single-donor study, the results indicated that B2M was among the most actively expressed housekeeping genes between individuals in saliva, blood, menstrual blood, and vaginal secretion samples (Fig. 5*A–E*). In addition, PGK1 along with ACTB showed fairly robust expression in menstrual blood, vaginal secretion, saliva, and semen samples. B2M clearly displayed the highest expression in blood samples. In semen samples, PPIA, PGK1, B2M, RPLP0, and ACTB showed similar expression levels, all of which were much higher than GAPDH expression. However, in terms of consistency across individuals, it appears that ACTB, although not as actively expressed as B2M, remained the most

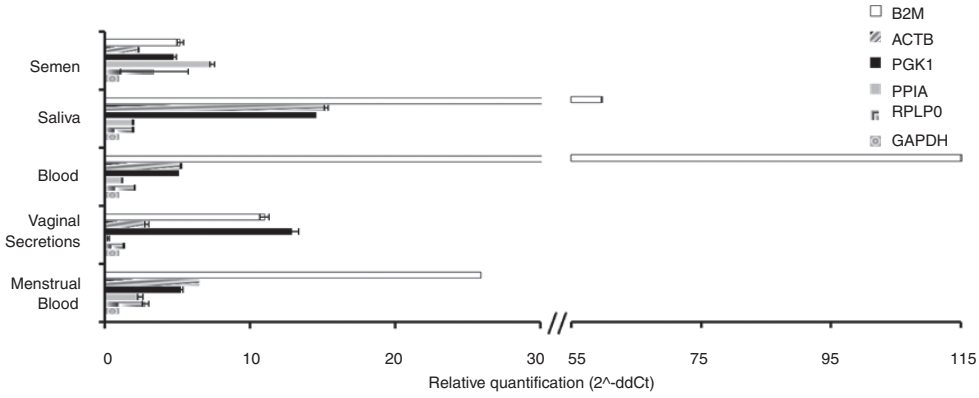

FIG. 4—*Two-step real-time PCR quantification of housekeeping gene expression in body fluid samples from one donor. The graph represents the relative quantification of the indicated target gene to the chosen internal reference gene (GAPDH). Each fluid was donated by a single, different individual (men or women). The average data from seven body fluid extract replicates and their corresponding standard deviation error bars are depicted.*

consistently expressed across all body fluids analyzed, with the exception of vaginal secretions (Fig. 5*B*). Because of the consistently high level of expression of ACTB and B2M across all fluids, these genes were used for an absolute quantification of various-sized forensic-like stains utilizing a control cDNA of known concentration as a standard curve. Results indicated that B2M and ACTB were successfully detected above the threshold limit of detection in all samples analyzed, including 1 μL saliva, blood, and semen stains (data not shown).

To assess whether the mean gene expression level differed in samples from individual donors, a one-way ANOVA was performed on mean C_t values measured for each housekeeping gene and group of individuals per body fluid. The *F*-ratio was used to test the equality of the mean C_t value for each sample from all donors. In this analysis, with number of samples (donors) $n = 5$, replicate size = 3, and $\alpha = 0.05$, the critical *F*-value was determined to be equal to 3.48. The *F*-value for each sample from each body fluid is provided in Table 2. Values greater than the critical *F*-value indicate samples in which mean values of expression level differed significantly between donors (Table 2).

Discussion

Prior to mRNA profiling, a method to quantify the nucleic acid is essential to determine the approach for downstream forensic analysis. qPCR can be used to establish the presence and the quantity of human-specific mRNA in a given sample. Previous forensic studies (1,38) have based the quantity of mRNA in a given sample on the results obtained using a GAPDH amplicon that could be too long when assessing samples that are inherently low in concentration and prone to degradation. Our results indicate that shortening the length of the target amplicon from 226 to 122 bp, coupled with the two-step amplification kit, significantly improves the amount of GAPDH mRNA identified. The lack of full recovery of GAPDH observed with the one-step kit could be due to a lower transcribing efficiency of RNA as compared to the two-step kit. Alternatively, GAPDH regulation is affected by an overwhelming number of factors (16–19), any of which could increase the likelihood of obtaining false-negative results, as was observed in our experiments. These negative results would prevent a sample from being carried through the downstream analysis process.

Many researchers widely accept and utilize GAPDH as the housekeeping gene of choice in gene expression studies because it is a glycolytic intermediate expected to be present in all cells and displays limited variation. However, a number of research articles show that although GAPDH is constitutively expressed throughout tissues, it has numerous pitfalls as a standard (39–42), including the overwhelming occurrence of pseudogenes (19,43). In this study, rather than focusing on GAPDH as the gene of choice, it was used as an endogenous reference gene to normalize the expression levels of other housekeeping genes. Owing to expected variations in housekeeping gene expression between different body fluid types, the expression of the housekeeping genes, ACTB, B2M, GAPDH, PGK1, PP1A, or RPLP0, was compared in semen, saliva, blood, menstrual blood, and vaginal secretion samples. To ensure that gene expression comparison could be carried out using relative quantification, a validation study was performed in which the efficiencies of amplification at various concentrations of cDNA were determined for each housekeeping gene. Results revealed that the efficiency of amplification for all housekeeping genes was independent of concentration; therefore, a reliable comparison between gene expression levels could be performed given that the starting concentration of the samples analyzed was unknown.

Gene expression results, based on relative quantification, revealed that B2M exhibits the highest relative expression as compared to all other genes analyzed in saliva, blood, menstrual blood, and vaginal secretion stains (Fig. 5*A–E*). In addition, PGK1 has similar levels of expression as ACTB in blood, saliva, menstrual blood, and vaginal secretion stains. In semen samples, fairly similar levels of expression were observed for PP1A, PGK1, RPLP0, and ACTB. However, caution must be observed when considering the use of B2M as a housekeeping gene because B2M expression levels have been shown to be down- or over-regulated when specific tumor cells are present (44,45) and increased when multidrug resistance cells are present or when the immune system is compromised or triggered by the excessive production of interferons (46,47).

Because of the uncertain nature of biological stains deposited at crime scenes, there is a slim possibility of underestimation of the expression results if the chosen housekeeping gene is not as highly expressed as others in the particular unknown fluid. Underestimations could possibly hinder downstream applications. However, given the vast amount of expression in the case of B2M (Figs 4 and 5*A–E*), it is unlikely that an underestimation of quantity in a particular fluid will yield too low a result as to be considered a false negative. Indeed, our studies showed successful detection of B2M and ACTB from minute volumes (1 μL, neat) of semen, saliva, and blood stains. In addition, B2M and ACTB were successfully detected from 25 μL saliva and semen stains exposed for 1 or

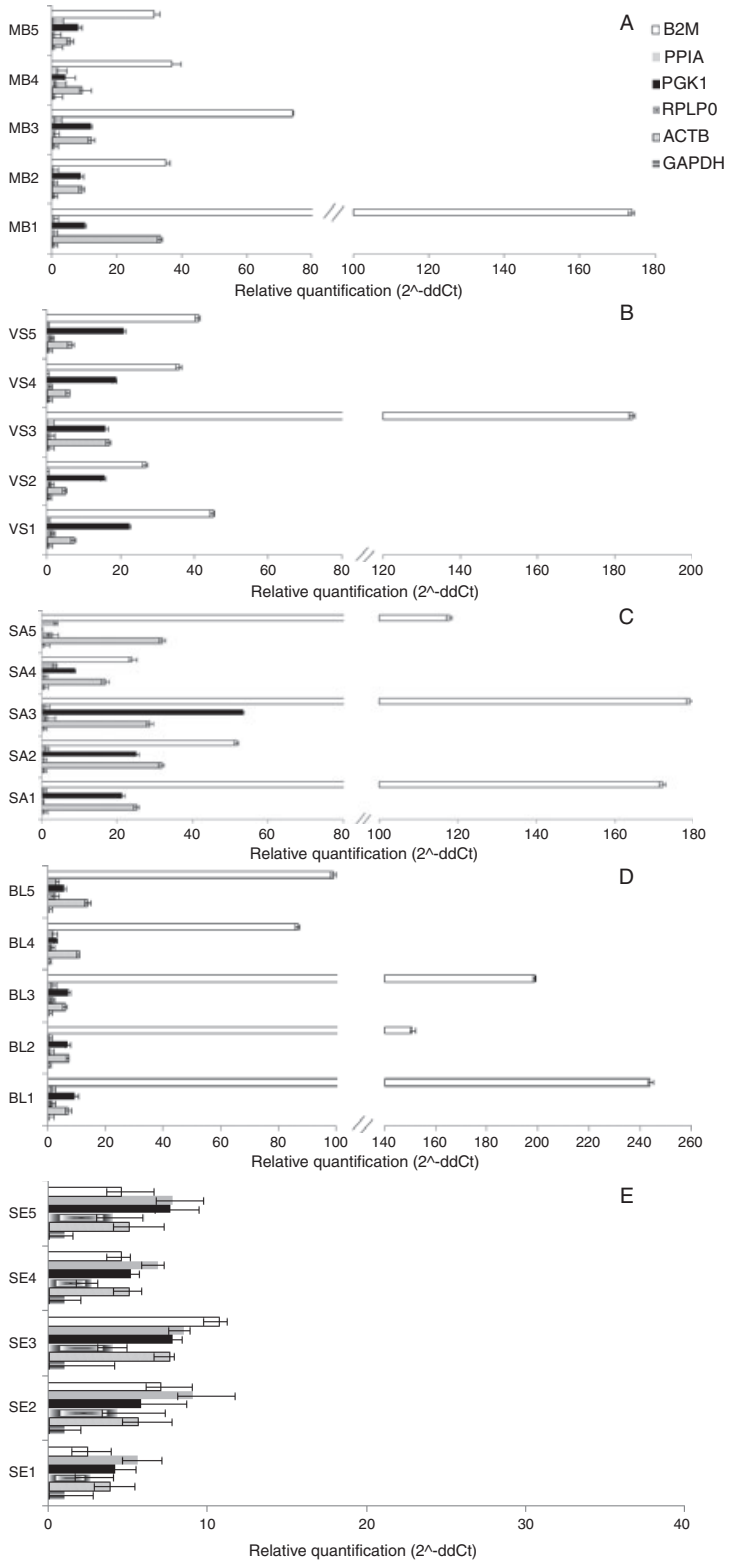

FIG. 5—*Two-step real-time PCR quantification of housekeeping gene expression in body fluid samples from five donors. The graphs represent the relative quantification of the indicated target gene relative to the chosen internal reference gene (GAPDH). Each of five individuals (women or men) contributed the samples. The average data from three body fluid extract replicates isolated from five donors and their corresponding standard deviation error bars are depicted. Each graph represents a body fluid (A—menstrual blood, B—vaginal secretions, C—saliva, D—blood, E—semen).*

2 weeks to the environment (data not shown). Therefore, detection of B2M and ACTB mRNA in forensic samples appears promising. For forensic applications, recovering enough material to identify the body fluid origin of a stain is required. As a result, overestimations, as defined by assessing the relation to an individual's normal basal level, are not a concern but can prove beneficial in this type of comparative (presence/absence) assay. Nevertheless, it is important to ensure that the level of expression of the

TABLE 2—*ANOVA test results for same fluid expression variability between donors ($\alpha = 0.05$).*

	F					
	Blood	Semen	Saliva	VS	MB	*F* Critical
GAPDH	1.99	**5.14**	0.720	**23.2**	**4.65**	3.48
ACTB	0.752	**6.11**	0.730	**61.6**	**5.52**	
RLPL0	1.73	**5.34**	**3.60**	**9.46**	**6.33**	
PGK1	0.736	**6.19**	**7.98**	**32.1**	**4.94**	
PPIA	1.67	**6.08**	1.82	**21.5**	**5.51**	
B2M	2.38	**4.77**	**18.3**	**82.2**	**4.24**	

Values in bold face represent those values that are greater than the critical *F*-value, indicating that the mean value of gene expression differed significantly between individual donors at the $\alpha = 0.05$ significance level.

housekeeping gene of choice is similar among individuals, excluding those rare instances in which large positive or negative fluctuations are expected because of uncontrollable "flaws" (e.g., disease) in the various gene-"controlling" systems.

As vaginal secretion and menstrual blood samples were collected directly, there is an inherent variability in the initial input of such samples. Consequently, high intra- and inter-individual variability was expected. In addition, as with any fluid, the secretion level of the different donors is not expected to be the same; therefore, variability between individuals will always be expected. This consideration may account for the high variability observed in semen stains and in some saliva samples (Table 2). With blood, on the other hand, similar cell counts are expected if the same amount of material from healthy individuals is given; therefore, the level of variability in gene expression of this fluid was expected to be low and was observed to be minimal. The levels of disparity of GAPDH and ACTB between individuals were found to be relatively low and similar (Table 2); however, the expression of ACTB was consistently higher than that of GAPDH, and GAPDH expression was consistently among the lowest (Figs 4 and 5*A–E*).

For mRNA profiling applications, it is not imperative to have a completely uniform expression of the gene of choice. However, it is crucial to select a gene that is known to be actively expressed throughout the various fluids of interest, and it is essential to ensure that the variability in expression between individuals is not too high as this could provide erroneous interpretations when more than one fluid is present. Therefore, GAPDH might not be the optimal housekeeping gene for the assessment of mRNA recovery from forensic stains, and it seems appropriate to search for an alternative to GAPDH for these purposes. In conclusion, our results indicate higher expression levels of ACTB (beta-actin) and B2M (beta-2 microglobulin) as compared to GAPDH in the human body fluid samples examined. In addition to having high levels of expression and commercially available probes, B2M also exhibited fairly consistent expression among the body fluids examined, an observation made previously for the cell line K562 and leukocytes from both healthy individuals and cancer patients (48). Furthermore, B2M has a small target amplicon (75 bp) and has been reported to be the least unstable control mRNA (20), both of which may aid in the detection of small or degraded samples. Also, B2M has limited occurrences of pseudogenes (as recorded in NCBI), has a probe across a splice junction, and is not linked to many metabolic activities that could affect its expression. Consequently, B2M appears to be the best candidate among the housekeeping genes evaluated for human-specific mRNA quantification prior to mRNA profiling for forensic applications. Given that B2M expression is not exempt from being affected by various factors, the use of additional housekeeping genes (in addition to B2M) for forensic mRNA sample quantitation normalization should be considered as suggested for real-time quantitative RT-PCR in general (49).

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Additional information and reprint requests:
James M. Robertson, Ph.D.
Research Biologist
FBI Laboratory
2501 Investigation Parkway, Rm 4250
Quantico, VA 22135
E-mail: james.m.robertson@ic.fbi.gov