J Forensic Sci, March 2008, Vol. 53, No. 2
doi: 10.1111/j.1556-4029.2007.00652.x
Available online at: www.blackwell-synergy.com

Mindy Setzer,[1,2] *M.S.; Jane Juusola,*[2,3] *Ph.D.; and Jack Ballantyne,*[1,2,4] *Ph.D.*

Recovery and Stability of RNA in Vaginal Swabs and Blood, Semen, and Saliva Stains

ABSTRACT: RNA expression patterns, including the presence and relative abundance of particular mRNA species, provide cell and tissue specific information that could be used for body fluid identification. In this report, we address perceived concerns on the stability, and hence recoverability, of RNA in forensic samples. Stains were prepared from blood, saliva, semen, and vaginal secretions and exposed to a range of environmental conditions from 1 to 547 days. The persistence and stability of RNA within each type of body fluid stain were determined by quantitation of total RNA, and reverse transcriptase-polymerase chain reaction (RT-PCR) using eight different mRNA transcripts from selected housekeeping and tissue-specific genes. The results demonstrate that RNA can be recovered from biological stains in sufficient quantity and quality for mRNA analysis. On average, several hundred nanograms of total RNA was recovered from 50-μL-sized blood and saliva stains, 1 μg from a 50-μL semen stain and nearly 70 μg from a whole vaginal swab. Messenger RNA is detectable in some samples stored at room temperature for at least 547 days. The environmental samples that were protected from direct rain impact exhibited housekeeping and tissue specific mRNA recoverability up to 7 days (saliva and semen), 30 days (blood), or 180 days (vaginal swab). Additionally, rain had a detrimental effect on the recoverability of blood (3 days), saliva (1 day), semen (7 days), and vaginal secretions (3 days) specific transcripts, with one of the mRNA species (the semen marker PRM2) not being detectable after 1 day.

KEYWORDS: forensic science, mRNA profiling, RNA, body fluid identification, RNA stability

Conventional methods of body fluid identification use a variety of technologically diverse techniques that are performed in a series, not parallel, manner (1). Moreover, for some frequently encountered body fluids no confirmatory test exists (1). There is no definitive test, for example, for the presence of saliva or vaginal secretions (1,2). Increasingly, classical methods for body fluid identification are being supplanted by the routine identification of human DNA in the sample. However this approach, while practical and convenient, is fraught with potential danger as there are a number of casework scenarios whereby the tissue origin of the sample itself is of immense probative value.

To develop a parallel analysis procedure for body fluid identification that is compatible with current DNA analysis procedures, we have developed assays based upon messenger RNA (mRNA), the molecular intermediate between DNA and protein that is expressed in a tissue-specific manner (3–6). Each cell type that makes up a body fluid or tissue has a unique pattern of gene expression called a transcriptome (7) that is defined by the presence and relative abundance of specific mRNAs. The potential forensic application of the transcriptome is determination of the type and abundance of gene transcripts present in a sample recovered at the crime scene which would permit the definitive identification of the body fluid or tissue present. Advantages of an mRNA-based approach, compared to conventional biochemical analysis, include greater specificity, simultaneous and semi-automated analysis through a common assay format, improved timeliness, decreased sample consumption, and compatibility with DNA extraction methodologies (8,9).

[1]Graduate Program in Industrial Chemistry Forensic Track, University of Central Florida, P.O. Box 162366, Orlando, FL 32816-2366.
[2]National Center for Forensic Science, P.O. Box 162367, Orlando, FL 32816-2367.
[3]Graduate Program in Biomolecular Sciences, University of Central Florida, P.O. Box 162366, Orlando, FL 32816-2366.
[4]Department of Chemistry, University of Central Florida, P.O. Box 162366, Orlando, FL 32816-2366.

Received 2 Dec. 2006; and in revised form 1 Aug. 2007; accepted 19 Aug. 2007.

Previously we have reported that it is possible to isolate total RNA of sufficient quality and quantity from dried blood, semen, and saliva stains to enable subsequent detection of particular mRNA species using reverse transcription-polymerase chain reaction (RT-PCR) (5). To address perceived concerns over RNA stability in forensic samples, we have conducted an in-depth study on the persistence of "analyzable" RNA in biological stains. Previous studies have either been limited in the type of sample and/or by the scope of the environmental conditions to which the samples were exposed (5,10). Vaginal secretion swabs and blood, saliva, and semen stains were prepared and exposed to a range of environmental conditions and the ability to recover total RNA and perform mRNA profiling analysis assessed. The results indicate that mRNA profiling can be performed successfully on environmentally impacted biological stains and is therefore potentially useful for forensic casework analysis.

Material and Methods

Body Fluid Samples

Body fluids were collected using procedures approved by the University's Institutional Review Board. Blood was collected by venipuncture and 50 μL aliquots were placed onto cotton cloth and dried at room temperature. Saliva was obtained in a sterile 50-mL centrifuge tube and 50 μL aliquots were placed onto cotton cloth and dried at room temperature. Freshly ejaculated semen was collected in plastic cups, and 50 μL aliquots were placed onto cotton cloth and dried at room temperature. Vaginal secretions were collected using sterile polyester-tip swabs and allowed to dry at room temperature. Recovery and sensitivity samples were placed at −47°C and environmental samples were placed at their appropriate condition.

© 2008 American Academy of Forensic Sciences

For the recovery study extractions, 50 μL blood, saliva, and semen stains were cut into five different sizes (1/8 = 6.25 μL, 1/4 = 12.5 μL, 1/2 = 25 μL, 3/4 = 37.5 μL, and 1 = 50 μL). Vaginal secretion swabs were evaluated in three sizes including 1/4, 1/2, and a whole swab. Cutting the samples into various sizes allowed us to determine the least amount of sample consumption needed to achieve a sufficient amount of RNA recovery for analysis. For the sensitivity studies, 50 μL of blood, saliva, and semen stains was extracted, and varying amounts of total extracted RNA (100 pg–200 ng) were used for analysis. Similarly, total RNA was isolated from whole vaginal secretion swabs and varying amounts (100 pg–4000 ng) were used for analysis.

To evaluate the environmental effects on all four body fluids, one sample from each of the four body fluids was placed at each of the different temperature, light, and environmental conditions. Some of the samples were placed at room temperature protected from light in envelopes and plastic bags as both wet and dry stains. Other samples were exposed to ultraviolet and luminescent light at room temperature. Additional samples were placed outside under two conditions; outside (heat, humidity, light, and rain) and outside no rain (heat, humidity, and light). Blood, saliva, semen, and vaginal secretion samples were exposed for 1, 3, 7, 30, 90, 180, 365, and 547 days. For RNA isolation from the environmental samples, the following stain or swab sizes were used: blood and saliva 1/2 of a stain (equivalent to 25 μL), for semen 1/4 of a stain (equivalent to 12.5 μL), and for vaginal secretions 1/4 of a swab.

RNA Isolation

Total RNA was extracted from vaginal secretion swabs and blood, saliva, and semen stains with guanidine isothiocyanate-phenol:chloroform and precipitated with isopropanol (11), as previously described (5). Briefly, 500 μL of denaturing solution (4 M guanidine isothiocyanate, 0.02 M sodium citrate, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) was added to each extraction tube (Fisher, Suwanee, GA). The stain/swab was placed into the extraction tube with the preheated denaturing solution and incubated for 30 min in a 56°C water bath. The cotton swatch or polyester swab was removed and placed into a spin-basket (Promega, Madison, WI) and centrifuged at $8160 \times g$ for 10 min. After centrifugation, the basket with associated substrate was discarded. Fifty microliter of 2 M sodium acetate and 600 μL of acid phenol chloroform 5:1 (pH: 4.5) were added to the extract, vortexed briefly, placed at 4°C for 1 h, and then centrifuged for 20 min at $16{,}000 \times g$. The RNA containing aqueous layer was removed to a new sterile 1.5 mL tube. Two microliter of GlycoBlue™ glycogen carrier (Ambion Inc., Austin, TX) and 500 μL of isopropanol were added to the aqueous layer. RNA was precipitated at −20°C for 2 h, after which the samples were centrifuged for 20 min at $16{,}000 \times g$. After centrifugation, the supernatant was removed and the pellet was washed once with 1 mL of 75% ethanol/25% diethylpyrocarbonate (DEPC) treated water. The samples were then centrifuged for 10 min at $16{,}000 \times g$, supernatant discarded, and dried in a vacuum centrifuge for 5 min. The pellet was re-suspended in 12 or 20 μL of RNA Secure Resuspension Solution (Ambion, Inc.) and heated at 60°C for 10 min. The samples were DNase-treated immediately or stored at −20°C until further use.

DNase Treatment

Six units of RNase-free DNase I (2U/μL) (Ambion, Inc.) and digestion buffer provided (10 mM Tris–HCl, pH 7.5, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$) were added to each extract. The samples were incubated at 37°C for 1 h. The DNase was inactivated at 75°C for 10 min (12,13). The samples were stored at −20°C until further use.

RNA Quantitation

Each sample was quantitated using a sensitive fluorescence assay based upon the binding of the unsymmetrical cyanine dye Ribogreen® (Molecular Probes, Eugene, OR) (14), which detects human and nonhuman RNA. The manufacturer's instructions were followed for the high-range assay, which detects RNA in a range from 20 ng/mL to 1 μg/mL.

Briefly, 200 μL assay volumes were used with 96-well microplates. The final mixture in each sample well consisted of 2 μL DNase I-treated RNA extract, 98 μL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5 in nuclease-free water), and 100 μL of 750 nM Ribogreen® reagent (diluted 200-fold from the concentrated stock provided in kit). After a 2-min incubation of the samples at room temperature protected from light, the samples were analyzed with a Wallac Victor^2 microplate reader (Perkin Elmer Life Sciences, Boston, MA) at a fluorescence emission at 535 nm (excited at 485 nm). The RNA concentrations in the samples were calculated using an appropriate standard curve as described by the manufacturer.

cDNA Synthesis

For the reverse transcription (RT) reaction, RNA template, 0.5 mM each dNTP (Applied Biosystems, Foster City, CA), 5 μM random decamers, and nuclease-free water (Ambion, Inc.) were combined to a final volume of 16 μL. This mixture was heated at 75°C for 3 min to eliminate secondary structure of target mRNA and snap-cooled on ice. To the mixture, 2 μL of 10x first-strand buffer (500 mM Tris–HCl, pH 8.3, 500 mM KCl, 30 mM $MgCl_2$, 50 mM dithiothreitol [DTT]) (Ambion, Inc.), 1 μL of SUPERase-In™ RNase Inhibitor (20 U/μL) (Ambion, Inc.), and 1 μL of Moloney Murine Leukemia Virus-Reverse Transcriptase (100 U/μL) (Ambion, Inc.) were added to yield a final reaction volume of 20 μL. This reaction mixture was incubated at 42°C for 1 h. The enzyme was inactivated by incubating the reaction mixture at 95°C for 10 min. The samples were then placed at −20°C until further use.

Polymerase Chain Reaction

A volume of 2 μL of the RT-reaction was amplified in a final reaction volume of 25 μL. The reaction mixture included buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (Applied Biosystems), 0.125 mM each dNTP (Applied Biosystems), 0.8 μM PCR primer (Invitrogen, Grand Island, NY), and 1.25 units AmpliTaq Gold® DNA Polymerase (5 U/μL) (Applied Biosystems). PCR primer sequences for GAPDH and β-actin were obtained from Strategene (LaJolla, CA). The PCR primer sequence for S15 was obtained from Ambion and the sequences for PBGD, PRM1, and PRM2 were obtained from published sources (15,16). The PCR primer sequences for STATH and HTN3 have been previously reported (5). PCR primers for the other blood-, semen-, and vaginal-secretion specific genes were designed using either Oligo® Primer Analysis Software, Version 6 (Lifescience Software Resource, Long Lake, MN) or Primer3 Online primer design software (17). Candidate genes that were not used in the stability study are designated with the type of body fluid and a numerical number (i.e.,

TABLE 1—*Housekeeping- and tissue-specific gene PCR primer sequences and the predicted sizes of the RT-PCR amplified products.*

Body Fluid	Gene	Primer Sequences/Dyes	Size (bp)	Reference
Housekeeping	S15	5′-TTC CGC AAG TTC ACC TAC C 5′-CGG GCC GGC CAT GCT TTA CG	361	Ambion (18)
	β-actin	5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG	661	Stratagene
	GAPDH	5′-CCA CCC ATG GCA AAT TCC ATG GCA 5′-TCT AGA CGG CAG GTC AGG TCC ACC	600	Stratagene
Blood	SPTB	5′-AGG ATG GCT TGG CCT TTA AT 5′-ACT GCC AGC ACC TTC ATC TT	247	Primer 3
	PBGD	5′- TGG ATC CCT GAG GAG GGC AGA AG 5′-TCT TGT CCC CTG TGG TGG ACA TAG CAA T	177	Gubin and Miller (15)
Saliva	STATH	5′-CTT CTG TAG TCT CAT CTT G 5′-TGG TTG TGG GTA TAG TGG TTG TTC	198	Oligo 6
	HTN3	5′-GCA AAG AGA CAT CAT GGG TA 5′-GCC AGT CAA ACC TCC ATA ATC	134	Oligo 6
Semen	PRM1	5′-GCC AGG TAC AGA TGC TGT CGC AG 5′-TTA GTG TCT TCT ACA TCT CGG TCT	153	Steger et al. (16)
	PRM2	5′-GTG AGG AGC CTG AGC GAA CGC 5′-TTA GTG CCT TCT GCA TGT TCT CTT C	294	Steger et al. (16)
Vaginal secretions	MUC4	5′-GGA CCA CAT TTT ATC AGG AA 5′-TAG AGA AAC AGG GCA TAG GA	235	Primer 3

Blood 1 or abbreviated BL1). Primers were custom synthesized by Invitrogen. Table 1 shows the PCR primer sequences and the expected product sizes for selected genes. PCR conditions consisted of a denaturing step (95°C, 11 min) followed by 35 cycles (94°C, 20 sec; 55°C, 30 sec; 72°C, 40 sec) and a final extension step (72°C, 5 min) (18–20).

Gel Electrophoresis

RT-PCR products were separated on 2.5% NuSieve agarose gels (Cambrex, Rockland, ME). Electrophoresis was carried out at 100 volts for 60 min in TAE buffer (0.04 M Tris–acetate, 1 mM EDTA). The gel was stained with SYBR® Gold nucleic acid stain (Molecular Probes) and photographed under UV transillumination.

Results

Unexposed control swabs were prepared from vaginal secretions and stains were prepared from blood, saliva, and semen. The amount of total RNA recoverable from these unexposed control samples was determined as was the sensitivity of mRNA detection, using both housekeeping- and tissue-specific genes. Subsequently, the samples were exposed to a range of environmental conditions for varying time periods so that the effects of different light sources, different storage conditions at room temperature and the outside environment could be assessed. An overview of the experimental schema is provided in Fig. 1.

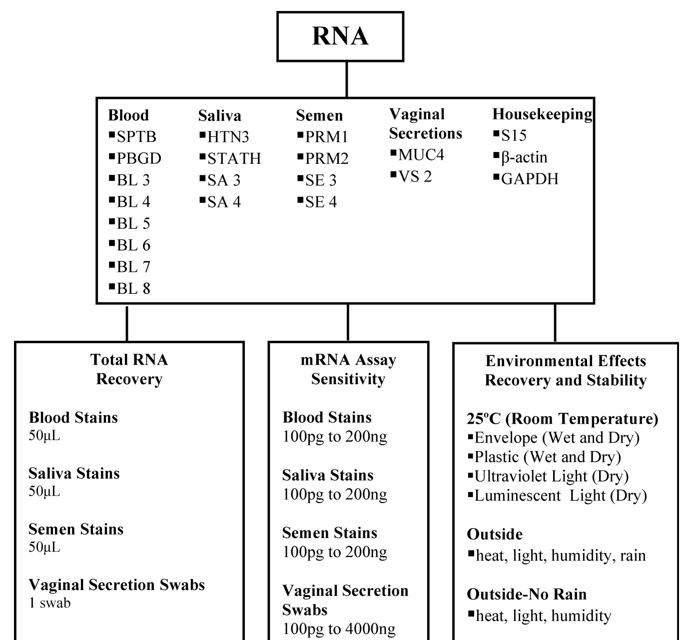

FIG. 1—*Overview of experimental scheme to assess the recovery and stability of RNA from body fluid stains. The body fluid-specific and housekeeping gene transcripts tested are listed. BL, SA, SE, and VS refer to blood-, saliva-, semen-, and vaginal-secretions candidate genes, respectively.*

Total RNA Recovery (Unexposed Control Samples)

The first objective was to determine the amount of total RNA that could be recovered from unexposed control samples. For blood, saliva, and semen, 50 µL stains were cut into five different sizes (1/8 = 6.25 µL, 1/4 = 12.5 µL, 1/2 = 25 µL, 3/4 = 37.5 µL, and 1 = 50 µL). Vaginal secretion swabs were evaluated in three sizes including 1/4, 1/2, and the whole swab. Cutting the samples into various sizes allowed us to determine the least amount of sample consumption needed to achieve a sufficient amount of RNA recovery for analysis. After the different size samples were extracted and quantitated, the amount of total RNA in the extract and the original 50 µL stain was calculated (Table 2).

The quantity of total RNA recovered from unexposed control biological samples was determined using the Ribogreen® fluorescence assay (14). Table 2 shows the average, standard deviation, minimum, and maximum amount of total RNA (nanogram) recovered from a 50 µL stain or whole swab. The average recovery of total RNA from these freshly prepared biological samples was as follows: 450 ng for 50 µL bloodstains, 430 ng for 50 µL saliva stains, 1100 ng for 50 µL semen stains, and 68 µg for a vaginal swab. The variance in the recovered RNA was extremely high as can be seen from the associated standard deviation metrics. The causes of such wide variation were not determined but may be due to both inter- and intra-individual variation in the number of cells deposited in each separate aliquot of body fluid secretion.

TABLE 2—*Total RNA recovery from unexposed control and environmentally challenged samples.*

Body Fluid	Conditions	*n*	Average (ng)	Standard Deviation (ng)	Min. (ng)	Max. (ng)
Blood	Unexposed control	63	450	140	160	900
	25°C Envelope dry	8	500	120	280	680
	25°C Envelope wet	8	530	120	350	730
	25°C Plastic dry	8	540	115	410	750
	25°C Plastic wet	8	530	40	500	600
	25°C Ultraviolet	8	530	135	350	820
	25°C Luminescent	8	610	90	490	730
	Outside	7	700	185	500	1100
	Outside no rain	7	1900	1755	490	4600
Saliva	Unexposed control	60	430	230	90	1000
	25°C Envelope dry	8	400	140	240	620
	25°C Envelope wet	8	490	150	280	780
	25°C Plastic dry	8	460	100	360	690
	25°C Plastic wet	8	580	330	270	1400
	25°C Ultraviolet	8	700	300	350	1100
	25°C Luminescent	8	590	350	120	1300
	Outside	7	800	535	500	2000
	Outside no rain	7	900	850	300	2200
Semen	Unexposed control	21	1100	680	550	2500
	25°C Envelope dry	6	700	200	460	950
	25°C Envelope wet	6	950	370	670	1400
	25°C Plastic dry	6	760	225	420	910
	25°C Plastic wet	6	660	205	580	800
	25°C Ultraviolet	6	2200	800	1500	3000
	25°C Luminescent	6	2250	870	1600	3400
	Outside	6	2000	1500	520	4500
	Outside no rain	6	3800	2860	1000	8100
Vaginal secretions	Unexposed control	36	68,000	26,735	13,000	108,000
	25°C Envelope dry	8	29,000	19,750	7000	65,000
	25°C Envelope wet	8	100,000	45,960	16,500	168,000
	25°C Plastic dry	8	53,000	24,860	26,000	87,000
	25°C Plastic wet	8	84,000	46,435	30,000	149,000
	25°C Ultraviolet	8	15,000	5700	6500	23,000
	25°C Luminescent	8	89,000	39,765	42,000	148,000
	Outside	7	21,200	19,110	850	40,200
	Outside no rain	7	80,100	67,385	1600	154,000

mRNA Assay Sensitivity (Unexposed Control Samples)

We have previously identified and tested sets of blood-, saliva-, semen-, and vaginal secretion-specific genes using a combination of literature and public database searches (5,6). To determine whether the gene transcripts were present in sufficient quantities for forensic purposes in fresh (unexposed control) samples, the analytical sensitivity of each of the tissue-specific and housekeeping genes was assessed by adding varying specific amounts of total RNA into the reverse transcription reaction. The presence or absence of an RT-PCR product was assessed by gel electrophoresis (Fig. 2). Based on the sensitivity studies, the genes (both tissue specific and housekeeping) were classified as high (≤5 ng of total RNA required), medium (5–30 ng RNA), or low (≥31 ng RNA) abundance genes (Table 3).

Eight tissue-specific genes were tested in blood stains (Table 3). The detection sensitivities ranged from 0.5 to 80 ng and from 0.2 to 1.0 ng for the blood specific and the three housekeeping genes, respectively. Four tissue-specific genes were tested in saliva stains (Table 3). The detection sensitivities ranged from 2 to 53 ng and from 2 to 20 ng for the saliva specific and housekeeping genes, respectively. Four tissue-specific genes were tested in semen stains (Table 3). The detection sensitivities ranged from 1 to 75 ng for the semen specific genes and were 0.5 ng for all three housekeeping genes. Two tissue-specific genes were tested in vaginal secretion swabs (Table 3). The detection sensitivities ranged from 2 to 1900 ng and from 0.5 to 30 ng for the vaginal fluid specific and housekeeping genes, respectively. Figures 2*a*–2*d* illustrate the results of sensitivity testing of some of the more abundant blood-, saliva-, semen- and vaginal-secretions genes, respectively. Figure 2*a* shows variability with product detected at 2, 4, and >10 ng and no product detected at 3 and 5 ng.

Every body fluid tested possessed tissue specific mRNAs that exhibited relatively high, medium, or low levels of expression. The abundance of different housekeeping genes within a body fluid also varied but to a lesser degree. Interestingly the abundance of the same housekeeping gene varied somewhat between different body fluids with the commonly used GAPDH demonstrating the most variance (0.5–30 ng sensitivity, compared with 0.2–5 ng for S15 and 0.2–2 ng for β-actin).

Environmental Effects

The sensitivity results described above were used as the basis for choosing high/medium abundance tissue-specific transcripts from each body fluid for subsequent environmental impact studies. The genes selected for this study are not present in bacteria and therefore were good candidates for the environmental studies. The tissue-specific genes selected for further study included β-spectrin (SPTB) and porphobilinogen deaminase (PBGD) for blood, histatin 3 (HTN3) and statherin (STATH) for saliva, protamine 1 (PRM1) and protamine 2 (PRM2) for saliva, and mucin 4 (MUC4) for vaginal secretions. The S15 housekeeping gene was also determined for each of the four body fluids. With the exception of MUC4, which

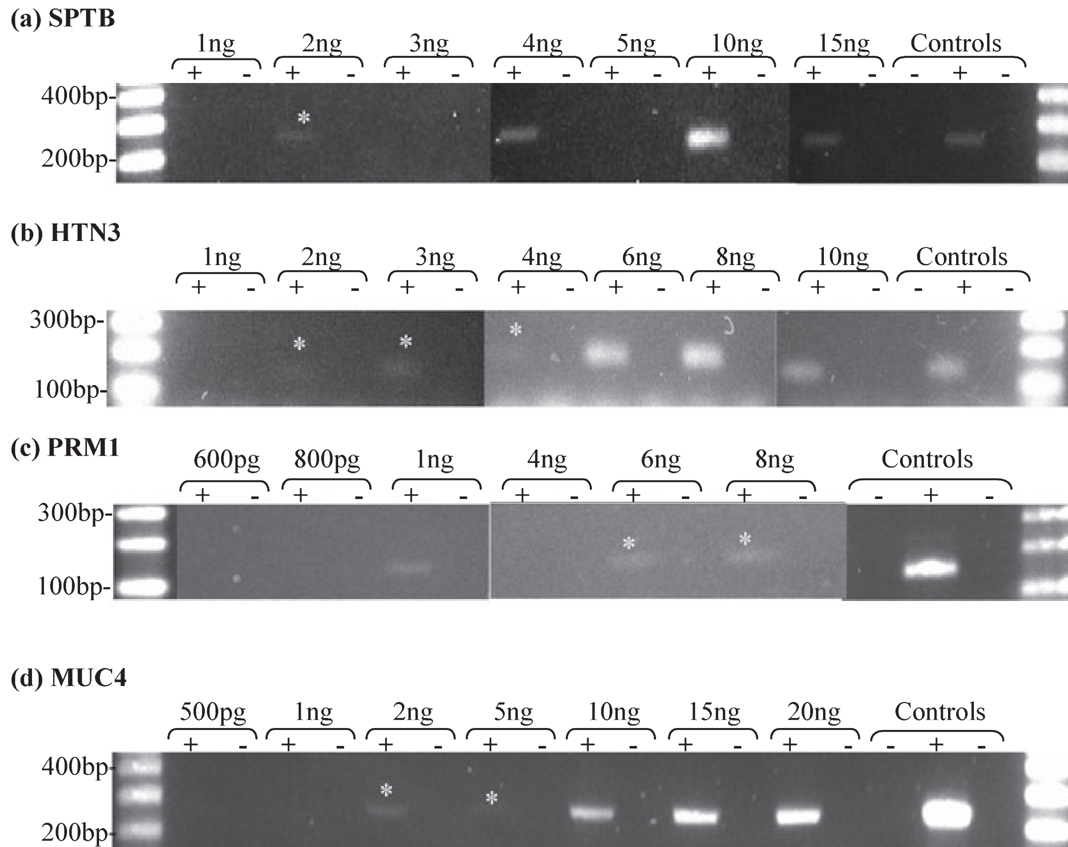

FIG. 2—*Sensitivity of gel based mRNA detection by RT-PCR. The gel images show RT-PCR products for SPTB (a), HTN3 (b), PRM1 (c), and MUC4 (d) using unexposed control total RNA samples and a range of total RNA input. Each sample is shown with (+) and without (−) the use of reverse transcriptase (RT). Additional controls for each gene tested (shown on the extreme right of each panel) include no RNA added (−) and amplification of the gene using a well-characterized sample with (+) and without (−) RT. PCR products were separated on a 2.5% agarose gel and visualized with SYBR® Gold. The asterisk (*) indicates the presence of a difficult-to-visualize amplimer.*

TABLE 3—*mRNA assay sensitivity (unexposed control samples).*

Body Fluid	Gene	Sensitivity	Abundance
Blood	S15	200 pg	High
	β-actin	200 pg	High
	GAPDH	1 ng	High
	SPTB	2 ng	High
	PBGD	3 ng	High
	Blood #3	500 pg	High
	Blood #4	800 pg	High
	Blood #5	6 ng	Medium
	Blood #6	60 ng	Low
	Blood #7	80 ng	Low
	Blood #8	70 ng	Low
Saliva	S15	3 ng	High
	β-actin	2 ng	High
	GAPDH	20 ng	Medium
	HTN3	2 ng	High
	STATH	15 ng	Medium
	Saliva #3	20 ng (P)	Medium
	Saliva #4	53 ng	Low
Semen	S15	500 pg	High
	β-actin	500 pg (P)	High
	GAPDH	500 pg	High
	PRM1	1 ng	High
	PRM2	1 ng	High
	Semen #3	15 ng	Medium
	Semen #4	75 ng	Low
Vaginal secretions	S15	5 ng	High
	β-actin	500 pg (P)	High
	GAPDH	30 ng	Medium
	MUC4	2 ng	High
	Vaginal #4	1912 ng	Low

required 500 ng of input RNA, 50 ng of total RNA was used for the analysis of each of the mRNA types.

In this validation study, vaginal secretion swabs and blood, saliva, and semen stains were exposed to a variety of environmental conditions to which evidence could potentially be exposed. Some samples were placed at room temperature (25°C) protected from light in envelopes and plastic bags as both wet and dry stains. Other samples were exposed to short wave ultraviolet (λ_{max} = 254) and luminescent light (λ_{max} = 440 nm, 585 nm; range = 400–700 nm) at room temperature. Additional samples were placed outside exposed to the natural (Florida) environment under two different conditions; outside (i.e., heat, humidity, light, and rain) and outside protected from rain (heat, humidity, and light). Samples were exposed for 1, 3, 7, 30, 90, 180, 365, and 547 days. The amount of total RNA recovered and the presence of housekeeping and tissue specific mRNAs was determined for each environmental condition.

Total RNA Recovery (Environmental Samples)

Blood—The amount of total RNA recoverable from 50-μL sized bloodstains stored over time at room temperature (25°C) in envelopes or plastic bags (protected from light and either wet or dry) or exposed to UV or luminescent light was similar to that expected based upon unexposed control samples (∼450 ng) (Table 2). Increased amounts of RNA were recovered from samples exposed to the elements, particularly those that were protected from rain (∼1900 ng) (Table 2).

Saliva—The amount of total RNA recoverable from room temperature-exposed 50-μL-sized saliva stains was similar to that described for bloodstains above with a concomitant increase seen in samples exposed to the elements (Table 2).

Semen—The total RNA recovery values for 50-μL-sized semen stains stored at room temperature protected from light ranged were slightly diminished (~700 ng) compared with unexposed control samples (~1100 ng). Increased amounts of RNA were recovered from samples exposed to luminescent and UV light as well as those samples exposed to the elements, particularly those that were protected from rain (Table 2).

Vaginal Secretions—Tens of micrograms of total RNA were recovered from single semen free vaginal swabs (Table 2) and such RNA is expected to comprise a significant amount of microbiota-derived RNA in addition to that originating from the vaginal epithelia (or other tissues of the female reproductive tract) (21). The results were consistent with this hypothesis in that those samples exposed to conditions that would be expected to promote microbial growth (i.e., samples that were wet prior to storage in plastic or envelopes, exposure to luminescent versus UV light and outside exposure to the elements [without rain being allowed to wash out the stain]) yielded more total RNA than identical samples treated in a manner less suited for such growth (Table 2).

mRNA Stability (Environmental Samples)

The presence of particular mRNA species was determined in the total RNA extracts in a manner similar to that described above for the unexposed control samples. The presence or the absence of the mRNA as determined by gel electrophoresis was scored. A result was deemed positive when a band of the correct molecular weight was observed in the gel with no band seen when the same sample was analyzed without reverse transcriptase. The results for the stability studies are summarized in Figs. 3*a*–3*h*, and an example of the gel data obtained from the differently exposed samples is provided for MUC4 mRNA, a vaginal secretions marker (Fig. 4).

Blood—The SPTB transcripts appeared to be stable for at least 365 days in bloodstains stored in all room temperature and light conditions (Figs. 3*a*–3*f*), and were detectable after 3 days in the outside samples and after 30 days in outside–no rain samples (Figs. 3*g*–3*h*). Similarly PBGD transcripts were detectable after at least 365 days in the room temperature envelope-dry, plastic-dry, plastic-wet, and ultraviolet light samples (Figs. 3*a*, 3*c*, 3*d*, and 3*f*), and were detectable up to 180 days in the envelope-wet and luminescent light samples (Figs. 3*b* and 3*e*). PBGD transcripts, like SPTB, were detectable after 3 days in outside samples and 30 days in outside–no rain samples (Figs. 3*g*–3*h*). The housekeeping gene S15 was detected in bloodstains for at least 547 days in all room temperature and light conditions (Figs. 3*a*–3*f*). It was detected in the outside samples after 90 and 30 days for outside–no rain samples (Figs. 3*g*–3*h*).

Saliva—The results from the saliva stains indicated that HTN3 was stable for at least 365 days in all of the room temperature samples except for the ultraviolet light samples (Fig. 3*e*), in which it was detectable for up to 180 days. In contrast, STATH exhibited variation in recovery success with the room temperature samples (Figs. 3*a*–3*d*). Specifically, the samples that were placed wet into the envelope and plastic bag demonstrated the presence of STATH transcripts up to 30 (Fig. 3*b*) and 3 days (Fig. 3*d*), respectively, compared with 365 days (Fig. 3*a*) and 180 days (Fig. 3*c*) when placed in the same containers dry. This is consistent with a heightened sensitivity of STATH transcripts to hydrolytic damage. Both HTN3 and STATH showed significantly decreased stability in the outside and outside–no rain samples (Figs. 3*g*–3*h*) with positive results only obtainable up to 1 and 7 days, respectively. S15 was stable in saliva stains for at least 365 days in all room temperature conditions except for the envelope-wet samples, where it was recoverable after 180 days (Fig. 3*b*). Decreased recoverability of S15 in the luminescent and UV light-exposed samples was observed, with positive mRNA signals being obtained up to 30 and 90 days, respectively (Figs. 3*e*–3*f*). In the outside–no rain samples, S15 showed similar results to HTN3 and STATH (i.e., up to 7 days) (Fig. 3*g*); however, S15 was detectable up to 30 days in the outside samples (Fig. 3*h*), a considerable improvement over the 1-day stability observed with the HTN3 and STATH transcripts.

Semen—In general, mRNA markers were recovered with less efficiency from semen stains than that from other body fluids over time when stored at room temperature in envelopes or plastic bags, at least when placed therein in the dried state (Figs. 3*a*–3*d*). PRM1 appeared to be stable for at least 180 days at all of the room temperature and light conditions (Figs. 3*a*–3*f*) but was recoverable for at least 547 days after room temperature storage in envelope and plastic when placed wet therein. PRM2 was detectable between 30 and 365 days in the room temperature samples, with the exception of the samples stored dry in a plastic bag at room temperature, which gave positive results only up to 3 days (Fig. 3*c*). For the samples stored outside and outside–no rain, PRM1 was detectable up to 7 days (Figs. 3*g*–3*h*). PRM2 was not detectable in the outside samples, but was detectable up to 7 days in the outside–no rain samples (Figs. 3*g*–3*h*). In the room temperature and light samples, S15 was stable up to 90 days with the exception of both envelope-wet and plastic-wet samples, where S15 appeared to be stable for at least 180 days (Figs. 3*a*–3*f*). S15 exhibited decreased recovery in the outside and outside–no rain samples with positive results at 1 and 7 days, respectively (Figs. 3*g*–3*h*).

Vaginal Secretions—For the vaginal secretion samples, MUC4 was detectable for at least 547 days with the envelope (wet) and plastic (wet) as well as the luminescent light conditions (Figs. 3*b*, 3*d*, and 3*e*; Figs. 4*b*, 4*d*, and 4*f*). MUC4 was detectable in plastic (dry) and ultraviolet light for at least 365 days (Figs. 3*c*, 3*f* and 4*c*, 4*e*), although the samples stored in the envelope (dry) conditions were only detectable up to 90 days (Figs. 3*a* and 4*a*). S15 was detectable for at least 547 days in the envelope (wet) and plastic (dry) samples (Figs. 3*b* and 3*c*) and at least 365 days with the other room temperature conditions (Figs. 3*a* and 3*d*). S15 was also detectable for at least 365 days in luminescent light (Fig. 3*e*); however, detectability was reduced in samples exposed to ultraviolet light (Fig. 3*f*). For the outside samples, MUC4 was detectable up to 3 days (Figs. 3*h* and 4*g*) and S15 was detectable up to 30 days (Fig. 3*h*). For the outside–no rain samples MUC4 was detectable up to 180 days (Fig. 4*h*) and S15 was detectable for at least 365 days (Fig. 3*g*).

Discussion

In this work, we have conducted a study of the persistence and stability of RNA in biological samples to assess the possible use of gene expression profiling for forensic applications. Samples were prepared from vaginal secretion swabs and blood, saliva, and semen stains and exposed to a range of environmental conditions

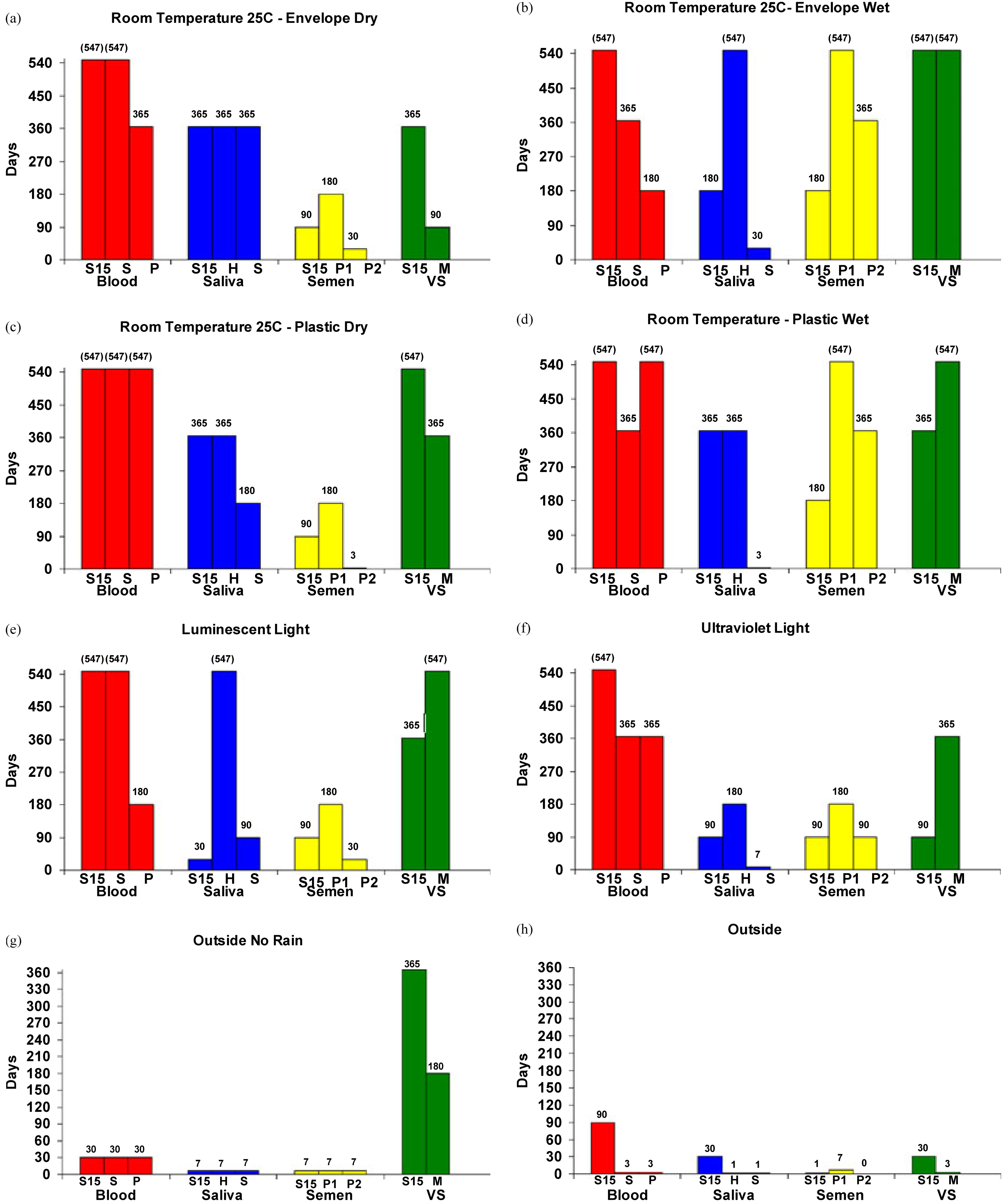

FIG. 3—*mRNA stability under a variety of environmental conditions. The graphs indicate the limits (in days) after which particular mRNA species were detected in blood, saliva, semen, and vaginal secretions after exposure to a variety of environmental conditions. These include (a) envelope (dry); (b) envelope (wet); (c) plastic (dry); (d) plastic (wet); (e) luminescent light; (f) UV light; (g) outside exposed to the elements; and (h) outside, as in (g), but protected from the rain. For each body fluid, the first gene shown is the housekeeping gene S15. The second and third genes represent the tissue-specific genes: SPTB (S) and PBGD (P) for blood, HTN3 (H) and STATH (S) for saliva, PRM1 (P1) and PRM2 (P2) for semen, and MUC4 (M) for vaginal secretions. Numbers in parentheses indicate the time of sampling but that the detection limit had not yet been reached.*

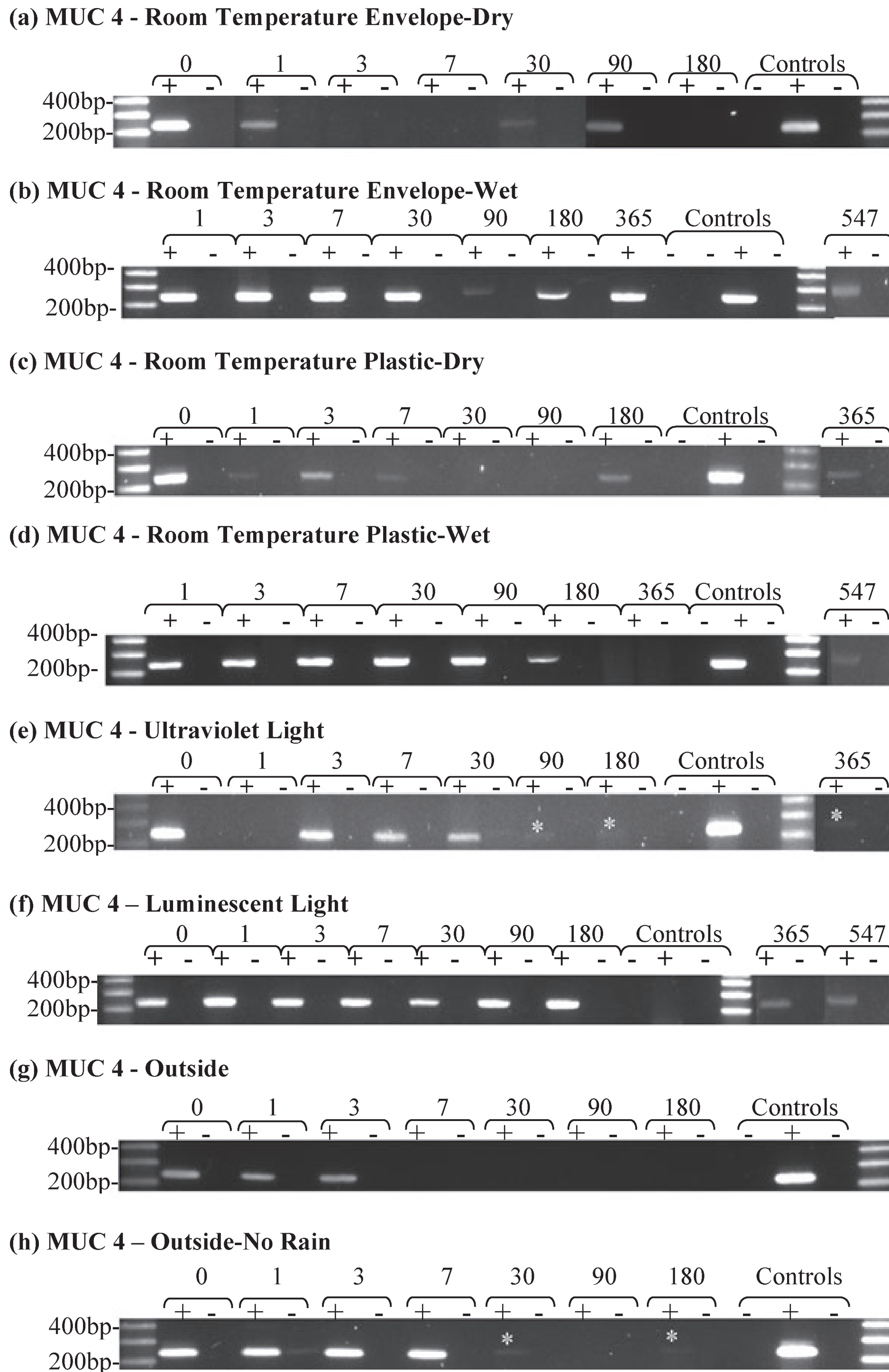

FIG. 4—*Example of mRNA stability studies using a variety of environmental conditions: Mucin 4. The gel images show RT-PCR products for MUC4 using total RNA recovered from vaginal swabs exposed to a variety of environmental conditions. Each sample is shown with (+) and without (−) the use of reverse transcriptase (RT). Additional controls for each gene tested (shown on the extreme right of each panel) include no RNA added (−) and amplification of the gene using a well-characterized sample with (+) and without (−) RT. PCR products were separated on a 2.5% agarose gel and visualized with SYBR® Gold. The asterisk (*) indicates the presence of a difficult-to-visualize amplimer.*

over varying time periods (1–547 days) so that the effects of different light sources, temperature, and environment could be assessed. The recoverability of RNA within each type of body fluid samples was determined using quantitation of total RNA and RT-PCR with selected housekeeping and tissue-specific genes. It is recognized that, due to the lack of species specificity of the Ribogreen® total RNA quantitation system, the recoverability of total RNA from the nonpristine samples is likely to comprise an admixture of human and nonhuman sources.

The first objective was to determine the amount of total RNA that could be recovered from unexposed control samples. On average, several hundred nanograms of total RNA was recovered from 50-μL-sized blood and saliva stains, 1 μg from a 50 μL semen stain, and nearly 70 μg from a whole vaginal swab (Table 2).

These results are consistent with previously reported total RNA recovery (5) and demonstrate that total RNA can be consistently recovered from dried biological samples.

The second objective was to develop assays to detect mRNA transcripts in dried samples. Using a combination of literature and public database searches, we identified and subsequently tested sets of housekeeping and candidate body fluid-specific genes. The analytical sensitivities of all the tissue-specific and housekeeping genes in freshly prepared ("unexposed control") body fluid samples were assessed by adding varying amounts of total RNA into the reverse transcription reaction. Variability was observed in analytical sensitivity at low levels of input RNA. This was probably due to a combination of the use of separate samples for each RNA input value and the stochastic nature of PCR product formation with limiting amounts of starting template. The genes were then classified into one of three abundance categories: high (≤5 ng sensitivity), medium (6–30 ng sensitivity), or low (≥31 ng sensitivity) (Table 3). Abundant gene classes were then chosen for subsequent environmental impact studies. The chosen candidates for blood included an enzyme of the heme biosynthesis pathway, PBGD (15), and the SPTB erythrocyte membrane protein (22,23). Saliva candidates included HTN3 (5) and STATH (5), which are both involved in host defense. The candidates for semen included the DNA binding proteins PRM1 (4,16) and PRM2 (4,16). Vaginal secretions candidate genes included a gene involved in antimicrobial defense (MUC4) (24,25). The housekeeping gene selected for further study was the S15 ribosomal protein (19).

The analysis strategy of the samples exposed to different environmental conditions was twofold: first, we wanted to determine, how would total RNA recovery be affected by exposure to a variety of temperature, light, and environmental conditions, and second, what effect would such exposure have on mRNA stability.

The total RNA recovered from the environmental samples was compared with the RNA recoverable from unexposed control samples. For all body fluid stains, the recovery of total RNA from samples stored at room temperature (in plastic or in envelopes, placed therein dry or wet) was generally comparable to that obtained from unexposed control samples. Although the UV- and luminescent light-impacted blood and saliva samples gave RNA recovery values similar to the corresponding unexposed control samples, the semen samples exhibited increased RNA recovery. The reason for this is unknown at present, but it is speculated that such pretreatment enhances RNA isolation from spermatozoa, perhaps by facilitating the disruption of cellular integrity during RNA isolation. The vaginal swab samples exhibited significant reduction in RNA recovery upon UV pretreatment but not with luminescent light. This may be the result of the germicidal action of UV irradiation as a proportion of the total RNA recoverable from vaginal swabs is expected to originate from the residential microbiota communities present in the female reproductive tract (21). Blood, saliva, and semen samples that were exposed to the outside environment all exhibited significant increases in RNA recovery compared with unexposed control samples, particularly those that were protected from the rain. The presence of adventitious environmentally derived microbiota was possibly responsible for this phenomenon (data not shown). Those samples that were exposed to rain exhibited less of an increase in RNA and this may have been due to partial loss of the stain by being "washed out" by the rain. The amount of total RNA recoverable from vaginal swabs was approximately 100-fold greater than that recovered from 50-μL-sized body fluid stains.

Much of the variability observed in the total RNA recoverability of the above samples was probably due to the use of the fluorescent cyanine dye Ribogreen® (14) for quantitation. This dye binds to contaminating nonhuman RNA and DNA, and some of the samples tested would have contained both of these molecular species in addition to human RNA. Although not available commercially during this work, it is recommended that a human RNA quantitation system be developed specifically for forensic samples so as to prevent underestimation of the quantity of total human RNA in a contaminated sample.

The detection of both the housekeeping- and tissue-specific gene transcripts in dried biological samples exposed to a variety of environmental conditions exhibited significant variation. In bloodstains, all the mRNAs tested were detectable up to at least 365 days in all samples stored at room temperature including those exposed to different light sources. The only exception was PBGD from samples stored wet in an envelope and from samples exposed to luminescent light: nevertheless the mRNA from these samples was still recoverable up to 180 days. Generally all of the transcripts tested in saliva stains were detected up to at least 180 days in all samples stored at room temperature, including those exposed to different light sources. Exceptions included STATH, when recovered from samples stored wet in plastic (3 days) or in envelopes (30 days) or after exposure to UV (7 days), and S15 when recovered from samples exposed to UV (90 days) or luminescent light (30 days). The semen-specific transcript PRM1 was recoverable for at least 180 days in all room temperature and light-exposed samples. In contrast, PRM2 exhibited more variable recovery (3–365 days) under the same conditions and S15 was recoverable up to 90 days. The S15 and MUC4 transcripts from vaginal swabs were detected up to 365 days in the room temperature and light-exposed samples except for S15 after UV treatment (90 days) and the envelope-dry (90 days) samples.

Samples that were exposed to the natural outside environment exhibited dramatically reduced recovery compared with the room temperature and light exposed samples. The environmental samples that were protected from direct rain impact exhibited housekeeping- and tissue-specific mRNA recoverability up to 7 days (saliva and semen), 30 days (blood), or 180 days (vaginal swab). The S15 transcripts in the latter sample were recoverable after 365 days. The vaginal swabs results were somewhat surprising but may be explicable due to the protective effect of the outer dried swab itself. It is possible that the environmental insults are confined to the outer layer of the swab thus permitting recovery of essentially undamaged RNA from the swab interior. The latter hypothesis is supported by the vaginal swab results from the outside samples that were exposed to the outside environment but permitted to be impacted directly by rain. Messenger RNA was only recoverable up to 3 days (MUC4) or 30 days (S15) in these samples. Similarly, rain had a detrimental effect on the recoverability of blood- (3 days), saliva- (1 day), and semen-specific transcripts (7 days), with one of the mRNA species (the semen marker PRM2) not being detectable after 1 day. Interestingly, the S15 transcripts exhibited significant variation in recoverability from the rain-impacted samples (from 1 day in semen to 90 days in blood). The deleterious effect of the rain was probably primarily due to the stain being simply washed out (and therefore effectively lost) from the cotton by the copious amounts of rain water impacting on the stains.

It is recognized that significant variability in the recovery of mRNA was observed in the environmental samples. Some of the potential sources of variation have already been alluded to. Another source of the variation observed could be due to inherent stability differences between the different transcripts tested. Probably a more significant cause of the observed mRNA recovery differences in some of the samples was inter-sample variation in the colonization

of the samples with both exogenous and, in the case of saliva stain and vaginal swab samples, endogenous microbiota. Unlike the tissue-specific genes chosen, the genome possesses an S15-processed pseudogene that could cause apparent S15 variation (5). Variation in the amount of minor DNA contamination of the RNA isolates could result in pseudogene amplification products that would be indistinguishable from bona fide mRNA-derived products. Such minor DNA contamination may still have been present despite the lack of significant DNA contamination that was not observed in the reverse transcriptase negative controls.

This study addresses the stability of RNA in body fluid stains as measured by its recovery and detectability. Future work will study the molecular basis of the degradation of the mRNA in dried samples including whether the 5′ end, the 3′ poly(A) tail, or the intervening sequences are specifically degraded or whether nucleolytic damage is randomly distributed.

In summary, we have demonstrated in this report that total RNA can be recovered from biological stains exposed to a variety of different environmental conditions in sufficient quantities to perform mRNA based assays. Seven different tissue-specific gene transcripts were tested in addition to a housekeeping gene. Although variation in recovery success was observed, messenger RNA was detectable in some samples stored at room temperature for at least 547 days but heat and humidity appear to be detrimental to RNA stability. Overall, the results provide a measure of confidence that mRNA instability should not in itself preclude the possible implementation of mRNA profiling in forensic science.

Acknowledgment

Support for this project was provided by the state of Florida through the UCF Center for Forensic Science.

References

1. Ballantyne J. Serology: overview. In: Siegal JA, Suakko PA, Knupfer GC, editors. Encyclopedia of forensic sciences. London: Academic Press, 2000;1322–31.
2. Gaensslen R. Sourcebook in forensic serology, immunology, and biochemistry. Washington, DC: NIJ, US Government Printing Office, 1983.
3. Bauer M, Kraus A, Patzelt D. Detection of epithelial cells in dried blood stains by reverse transcriptase-polymerase chain reaction. J Forensic Sci 1999;44(6):1232–6.
4. Bauer M, Patzelt D. Protamine mRNA as molecular marker for spermatozoa in semen stains. Int J Legal Med 2003;117(3):175–9.
5. Juusola J, Ballantyne J. Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int 2003;135(2):85–96.
6. Juusola J, Ballantyne J. Multiplex mRNA profiling for the identification of body fluids. Forensic Sci Int 2005;152(1):1–12.
7. Caron H, van Schaik B, van der Mee M, Baas F, Riggins G, van Sluis P, et al. The human transcriptome map: clustering of highly expressed genes in chromosomal domains. Science 2001;291(5507):1289–92.
8. Alvarez M, Juusola J, Ballantyne J. An mRNA and DNA co-isolation method for forensic casework samples. Anal Biochem 2004;335(2):289–98.
9. Bauer M, Patzelt D. A method for simultaneous RNA and DNA isolation from dried blood and semen stains. Forensic Sci Int 2003;136(1-3):76–8.
10. Bauer M, Polzin S, Patzelt D. Quantification of RNA degradation by semi-quantitative duplex and competitive RT-PCR: a possible indicator of the age of bloodstains? Forensic Sci Int 2003;3(1-3):94–103.
11. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162(1):156–9.
12. Huang Z, Fasco MJ, Kaminsky LS. Optimization of Dnase I removal of contaminating DNA from RNA for use in quantitative RNA-PCR. Biotechniques 1996;20(6):1012–4.
13. Wiame I, Remy S, Swennen R, Sagi L. Irreversible heat inactivation of DNase I without RNA degradation. Biotechniques 2000;29(2):252–4.
14. Jones LJ, Yue ST, Cheung CY, Singer VL. RNA quantitation by fluorescence-based solution assay: RiboGreen reagent characterization. Anal Biochem 1998;265(2):368–74.
15. Gubin AN, Miller JL. Human erythroid porphobilinogen deaminase exists in 2 splice variants. Blood 2001;97(3):815–7.
16. Steger K, Pauls K, Klonisch T, Franke FE, Bergmann M. Expression of protamine-1 and -2 mRNA during human spermiogenesis. Mol Hum Reprod 2000;6(3):219–25.
17. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–86.
18. Ambion. RETROscript™ first-strand synthesis kit for RT-PCR instruction manual. Austin, TX: Ambion, 1997.
19. Kitagawa M, Takasawa S, Kikuchi N, Itoh T, Teraoka H, Yamamoto H, et al. rig encodes ribosomal protein S15. The primary structure of mammalian ribosomal protein S15. FEBS Lett 1991;283(2):210–4.
20. Shiga K, Yamamoto H, Okamoto H. Isolation and characterization of the human homologue of rig and its pseudogenes: the functional gene has features characteristic of housekeeping genes. Proc Natl Acad Sci USA 1990;87(9):3594–8.
21. Wilson M. Microbial inhabitants of humans. London, UK: Cambridge University Press, 2005.
22. Amin KM, Scarpa AL, Winkelmann JC, Curtis PJ, Forget BG. The exon-intron organization of the human erythroid beta-spectrin gene. Genomics 1993;18(1):118–25.
23. Chu ZL, Wickrema A, Krantz SB, Winkelmann JC. Erythroid-specific processing of human beta spectrin I pre-mRNA. Blood 1994;84(6):1992–9.
24. Gipson IK, Ho SB, Spurr-Michaud SJ, Tisdale AS, Zhan Q, Torlakovic E, et al. Mucin genes expressed by human female reproductive tract epithelia. Biol Reprod 1997;56(4):999–1011.
25. Gipson IK, Spurr-Michaud S, Moccia R, Zhan Q, Toribara N, Ho SB, et al. MUC4 and MUC5B transcripts are the prevalent mucin messenger ribonucleic acids of the human endocervix. Biol Reprod 1999;60(1):58–64.

Additional information and reprint requests:
Jack Ballantyne, Ph.D
Associate Professor
Department of Chemistry
University of Central Florida
Bldg.# 5, 4000 Central Boulevard
Orlando
FL 32816-2366
E-mail: jballant@mail.ucf.edu