J Forensic Sci, Mar. 2003, Vol. 48, No. 2
Paper ID JFS2002247_482
Available online at: www.astm.org

Janice A. Nicklas,[1] Ph.D. and Eric Buel,[1] Ph.D.

Development of an *Alu*-Based, QSY 7-Labeled Primer PCR Method for Quantitation of Human DNA in Forensic Samples*

ABSTRACT: Determining the amount of human DNA extracted from a crime scene sample is an important step in DNA profiling. The forensic community relies almost entirely upon a technique (slot blot) to quantitate human DNA that is imprecise, time consuming, and labor intensive. This paper describes the development of a new technique based on PCR amplification of a repetitive *Alu* sequence. Specific primers were used to amplify a 124-bp fragment of *Alu* sequence; amplification was detected by SYBR Green I staining in a fluorescent plate reader. To reduce background in the plate reader assay, QSY-7 labeled primers were utilized. The assay was tested on animal DNAs, human blood spots, mock crime samples, and degraded DNA in comparison with the slot blot technique. The QSY *Alu* assay has a dynamic range of 10 ng to 10 pg, and is sensitive, specific, fast, quantitative, and comparable in cost to the slot blot assay.

KEYWORDS: forensic science, human DNA, DNA quantitation, *Alu* sequences, polymerase chain reaction

DNA analysis of biological material for individualization purposes has become the norm within forensic laboratories. A number of different DNA markers exist for the individualization of biological stains; however, with the establishment of the CODIS core short tandem repeat loci (STR), the forensic community is focused on STRs and on a set of loci that can be shared within the community. The STR systems currently on the market yield excellent results with small quantities of DNA and a statistical evaluation of the STR data results in numbers bordering on, if not yielding, identity. Validation studies of the STR systems currently in place in many forensic laboratories have shown that the amount of template DNA must be strictly controlled to allow proper amplification and subsequent identification of the amplified product (1–3). The STR systems are very sensitive to and intolerant of elevated concentrations of input DNA and have an optimum DNA range of approximately 0.5 to 2.0 ng. DNA amounts outside this range can result in unbalanced peaks due to stochastic effects when DNA amounts fall below the recommended range. Split peaks, known as minus A or n-1 peaks, high concentrations of amplification products that are outside the usable instrumental analysis range, and inconsistent amplifications are the results of high amounts of template DNA used in amplification. For these reasons it is important to accurately estimate the concentration of extracted human DNA prior to performing an amplification. The focus of our work is to develop a technique that is not labor intensive, uses minimal sample, and yields quick, accurate results that allow the analyst to perform amplifications of template DNA yielding usable STR analytical results.

DNA can be quantified via a number of routine analytical procedures; however, only a few methods allow for the estimation of human DNA contained in an extracted sample. A slot blot procedure using a primate specific probe has been used predominately in the forensic community to assess the amount of human DNA extracted from a sample. Commercially available kits have offered the probe and reagents necessary to perform this analysis. Briefly, in this procedure, DNA is bound to a membrane using a slot blot apparatus. The bound DNA is hybridized with a primate specific probe, and through a number of steps the bound probe can be visualized by using either a colorimetric or chemiluminescence approach. The intensity of the slot blot band is dependent upon the amount of human DNA contained in the sample. The operational concentration range for this technique is approximately 10 to 0.16 ng for colorimetric and 10 to ≈0.04 ng for chemiluminescence analysis. Samples from a crime scene or reference samples "blotted" on this membrane are compared visually by the analyst to a series of concentration standards also contained on the membrane to obtain an estimate of the amount of DNA present in the questioned sample. This visual comparison of the intensity of the "bands" is inherently imprecise and results can vary considerably depending on the reader and operator. The comparative evaluation of the observed bands can be improved through the use of a digital camera system (4). This approach eliminates subjective evaluation, but requires expensive instrumentation and still necessitates the production of the blot that is time and labor consuming. A system that would yield nonsubjective quantitative results, quickly and with minimal operator attention, would be the next logical step to assess the amount of human DNA obtained in a forensic sample.

Alu sequences are a family of repetitive elements that are found in 500 000 to 1 100 000 copies in the human genome representing 6 to 13% of the haploid genome (5,6). The consensus *Alu* sequence is ~280 bp in length and consists of two similar monomers connected by an A-rich region. *Alu* sequences are postulated to be

[1] VT Forensic Laboratory, Department of Public Safety, 103 S. Main St., Waterbury, VT.

* Supported in part under award 2000-U-CX-K012 from the Office of Justice Programs, National Institute of Justice, Department of Justice. This paper does not necessarily represent the official position of the U.S. Department of Justice.

Received 19 July 2002; and in revised form 5 Oct. 2002; accepted 5 Oct. 2002; published 3 Feb. 2003.

Copyright © 2003 by ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959.

retrotransposons derived from 7SL RNA found in all species (7,8) that have amplified immensely through the 65 million years of primate evolution. While rodents have the B1 repeats that are also derivatives of 7SL, they are entirely distinct from the primate *Alu* sequences (3). The *Alu* sequences are divided into families with the J families being the oldest (80 million years) and the Y families (3 to 4 million years old) the youngest (9). The Sx family (37 million years old) accounts for 50% of human *Alus.* Because *Alu* sequences are present in many copies and are primate specific, they make an excellent target or marker for human DNA, and they have been exploited by others to develop assays to detect human DNA. The *Alu* Quant™ Human DNA Quantitation System (Promega, Madison, WI) uses an *Alu* specific probe in a Read-It™ based system whose endpoint is luciferase-produced light. The system requires a luminometer, detects 0.1 to 50 ng of DNA as described in the manufacturer's protocol, but still requires a number of manipulations by the analyst. A recent paper reports use of an *Alu* PCR-based system (10) to quantitate human genomic DNA from 2.5 to 100 pg. The human DNA present in the sample was estimated by determining the amount of PCR product generated by using the peak heights (RFU) obtained with an ABI PRISM™ 377 Genetic Analyzer and GeneScan software.

This article reports the development of a PCR-based method to amplify and detect *Alu* sequences using only a thermocycler and a plate reader. This assay quickly, reliably, and inexpensively quantitates human DNA over a range of 10 pg to 10 ng.

Materials and Methods

DNA Samples

Most experiments were performed with a human DNA standard (250 ng/μL; G3041) purchased from Promega (Madison, WI). The DNA standard "A" (2 ng/μL) from the Quantiblot® kit (ABI, Foster City, CA) was also used for comparison. Blood spots from 13 DNA databank samples, samples from control blood spotted on five different pieces of cloth, control blood placed on eight surfaces (leaves, concrete, cardboard, denim, leather, soapy cloth, stick, metal) and taken at two time points (fresh and six weeks at RT in the dark), and blood samples from two controls that were kept either at RT in the dark for three months, at RT in the sunlight for three months, or at 37°C in the dark for three months were utilized. DNA was isolated using an organic extraction method (11) as modified by Buel et al. (12).

Animal DNAs

Animal DNA (baboon, cat, chicken, cow, deer, dog, bear, horse, moose, mouse, pig, rabbit) was isolated from samples of blood on paper or cloth (from veterinary samples or from a game warden) using the organic extraction method described above. DNA from *Drosophila* was isolated by the above method using squashed whole flies. DNA from rat was obtained from Dr. Richard Branda, University of Vermont. DNA from *Clostridium, E. coli,* and *Micrococcus* were purchased from Sigma (St. Louis, MO). DNA from chimp, macaque, gorilla, and marmoset were purchased from BIOS (New Haven, CT). Herring sperm DNA was purchased from Gibco/BRL (Bethesda, MD).

PCR Primers

The *Alu* PCR primers GTCAGGAGATCGAGACCATCCC (forward) and TCCTGCCTCAGCCTCCCAAG (reverse) were designed from the sequence of plasmid pPD39 (Ya5 subfamily) (13) using the program Oligos © 1999–2002 v.9.6 (http://wwww.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm), designed by R. Kalendar of the Institute of Biotechnology, University of Helsinki. The unlabeled primers were purchased from Synthetic Genetics (now Epoch Biosciences, San Diego, CA), while those labeled with QSY 7 (a quencher dye placed on the primer to quench the SYBR Green I fluorescence of primers and primer-dimers) on the 5′ end were purchased from Synthegen, LLC (Houston, TX).

Inhibitors

DNA was treated for various times (0.75 to 48 min) with DNAse I (0.0005U/μL final concentration) (M6101, Promega, Madison, WI). Hematin (Sigma, St. Louis, MO) was added to PCR reactions at final concentrations from 0 to 10 μ*M*. EDTA (Sigma, St. Louis, MO) was added to PCR reactions at final concentrations from 0 to 2.5 m*M*.

PCR Assay

PCR utilized the SYBR® Green JumpStart™ Taq ReadyMix™ kit (S4438, Sigma, St Louis, MO). Initially, the 25 μL reactions contained 12.5 μL of 2X Buffer (1X final), 10 pmoles of each QSY 7 labeled primer in 1 μL H_2O, 6.5 μL of distilled H_2O and 5 μL of TE (10 m*M* Tris, pH 7.5, 0.1 m*M* EDTA) containing the input DNA at various concentrations. The ReadyMix™ 2X buffer contains 20 m*M* Tris-HCl, pH 8.3, 100 m*M* KCl, 7 m*M* $MgCl_2$, 0.4 m*M* each dNTP, 0.05 U/μL Taq DNA polymerase, JumpStart Taq antibody and with a proprietary amount of SYBR® Green I and unnamed other ingredients. Various experiments added additional components such as inhibitors (see below) or extra SYBR Green or 250 μg/mL BSA (A9647, Sigma, St. Louis, MO).

A homebrew PCR mix containing SYBR Green was also utilized for some experiments. This consisted of 10 m*M* Tris, pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 200 μ*M* each dNTP, 8% DMSO, and 1/40 000 or 1/20 000 of SYBR Green I (S7563, Molecular Probes, Eugene, OR).

Initial experiments to test the PCR primers were performed in an ABI 9600 thermocycler (Foster City, CA) using the homebrew reagents except without SYBR Green. PCR cycling consisted of 10 min at 95°C (HotStart for Taq Gold) followed by 10 to 20 cycles of 95°C for 20 s and 60°C for 45 s, followed by 5 min at 60°C and a 4°C hold.

PCR for the QSY assay was performed in an ABI 9700 thermocycler in ABgene (Rochester, NY) 0900 PCR plates with flat caps (ABgene, AB0783). Immediately before PCR, initial readings of the loaded wells were taken in the fluorescent plate reader (FL_x800, Bio-Tek, Winooski, VT). PCR consisted of 95°C for 2 min (HotStart) followed by 11 to 16 cycles (depending on the experiment) of 94°C for 15 s, 60°C for 1 min, 72°C for 1 min followed by 72°C for 7 min and 20°C for 10 min. For the last 20°C 10 min step, the hot bonnet was moved off and the PCR plate was covered by a foil cover to keep out light. This step was performed to allow the sample to cool to the same temperature as the initial reading before the final reading was taken in the plate reader. Various experiments changed annealing times, temperatures, and cycle number to optimize the assay.

For those later experiments involving the homebrew assay, PCR conditions were as described above for the initial experiments in the ABI 9600 with 1/20 000 SYBR Green.

Slot Blots

The Quantiblot® kit (Applied Biosystems, Foster City, CA) was used for the slot blot detection of human DNA following manufacturer's directions except that 25 μL of probe was utilized per filter. The final readout utilized horseradish peroxidase and 3′,3′,5,5′-tetramethylbenzidine (TMB) as the chromogen and the membrane was read by visual examination.

STR Analyses

The AmpFℓSTR® COfiler™ kit (Applied Biosystems, Foster City, CA) was used according to manufacturer's recommendations (except using a 25 μL reaction with 9.55 μL of reaction mix, 5 μL of primer mix, 0.45 μL AmpliTaq Gold, and 10 μL of sample DNA at a concentration of 0.1 ng/μL) for STR analyses.

Mixing Experiments

Rat and human DNAs were mixed at ratios from 100 to 0% (rat to human), and PCRs were performed on this series of DNAs using 2.5 ng of the mixed DNAs in each reaction.

Results

Development of Assay

Initial experiments utilized *Alu* primers in a simple 20-cycle PCR with an EtBr-stained gel-based readout to check for the correct size product, sensitivity, specificity, and the presence of primer-dimer. The PCR gave the correct size product, and the amount of product was proportional to the amount of human input DNA, although the results plateaued at high input DNA concentrations (Fig. 1). Additional experiments showed that the PCR was specific for human (or other primate) DNA, that the PCR was relatively insensitive to annealing temperature (58 to 64°C), and Mg^{++} concentration (1 m*M* to 5 m*M*) (data not shown).

With these positive results, the assay was then moved to a 96 well format using the SYBR® Green JumpStart™ Taq ReadyMix™ kit (SYBR Green I is the DNA stain) and a plate reader for quantitation. Initial experiments indicated that background fluorescence from the input DNA and primers made the assay unworkable. The Molecular Probes (Eugene, OR) website suggested a patented method using QSY 7 or 9 dyes attached to the primers to quench the fluorescence of primers and primer-dimers. The QSY dye only quenches SYBR Green fluorescence in "close" vicinity to the dye; thus, it can quench the fluorescence of the short 22 base primer or of primer-dimers but not the fluorescence of the entire 124 bp PCR product. The background problem was essentially removed in the *Alu* assay by utilizing QSY 7 labeled forward and reverse primers (data not shown). The use of the QSY 7 labeled primers made this assay now feasible and allowed for further optimization.

Another assay improvement came with the realization that some of the leveling off seen at high concentration might be the result not of the PCR plateauing because of lack of primers or dNTPs but rather of insufficient SYBR Green in the reaction. Experiments were then performed adding extra SYBR Green to the reaction mix despite anecdotal evidence that it inhibited the PCR reaction. Our homebrew used a 1/40 000 final concentration of the Molecular Probes (Eugene, OR) SYBR Green I based on other protocols; however, a recent publication (14) indicated better amplification with 1/20 000 or 1/10 000 SYBR Green than 1/40 000. The amount of SYBR Green in the ReadyMix™ kit is proprietary. Therefore, the concentration was assumed to be ~1/40 000 and 1/80 000, 1/40 000 and 1/20 000 were added. Figure 2 shows the results of these experiments. The plateau effect was significantly reduced by the addition of additional SYBR Green to the reaction mix. Adding an additional 1/20 000 seemed to have a slight inhibitory effect; thus, addition of 1/40 000 SYBR Green to the ReadyMix™ kit mix was chosen for future experiments.

The variables of number of PCR cycles, annealing temperature, annealing time, extension time, denaturation time, and homebrew

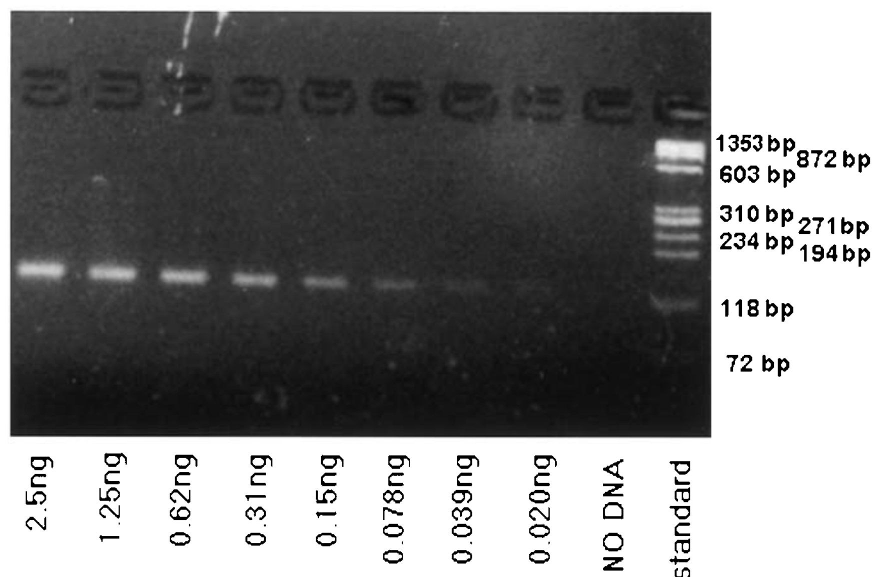

FIG. 1—*EtBr-stained gel of Alu PCR products from 16 cycles of PCR and homebrew reagents. The input DNA for each PCR is indicated below the gel. The standard is HaeIII digested Φ X174.*

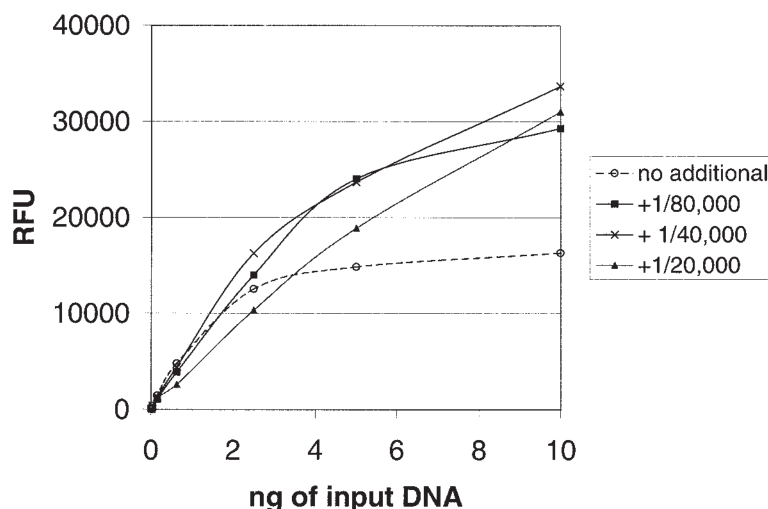

FIG. 2—*The effect of varying SYBR Green I concentration on QSY PCR using the ReadyMix™ SYBR Green Jumpstart kit. Either 1/20 000 SYBR green, 1/40 000, or 1/80 000 SYBR green final concentration was added to the Sigma mix, which already contained a proprietary amount of SYBR Green. The relative fluorescence units (RFU) are plotted against the input DNA concentration for seven concentrations of standard (10 to 0.01 ng).*

mix versus use of the ReadyMix™ kit were explored. Figure 3 shows the effect of cycle number. A cycle number below 13 lost the sensitivity for 10 pg or less, while the values plateaued over 10 ng for a cycle number over 15. Fourteen cycles were thus chosen for the final assay. Annealing temperature (56 to 62°C), denaturation time (15 or 30 s), and extension time (30 s to 2 min) (data not shown) changes had little effect. The amount of product did increase with annealing time (10 to 120 s) (data no shown), but the effect was minimal after 60 s; thus, a 60 s anneal was chosen for the assay.

The effects of changing primer concentration, dNTP, and Taq concentration were also determined (Fig. 4). Increasing the primer concentration slightly slowed the reaction speed. Increasing the dNTP concentration definitely decreased amplification. Both of these effects could be mediated by decreasing the available Mg^{++} concentration for the polymerase. Increasing the amount of Taq did increase the reaction speed and increase the plateau but did not affect the reaction sufficiently to be worth the extra cost.

Because the assay plateaus and even decreases at high input DNA, there could be concern that 5 ng and 40 ng could be confused because the final assay results would be identical; however, the zero time reading can discriminate between these amounts because the 40 ng sample would have a much higher zero time reading. The zero time reading can, in essence, be used as a SYBR Green measurement of the amount of input DNA. Figure 5 shows the zero time and 14-cycle results for a large range of input DNA concentrations (80 to 0.010 ng). The zero time readings continue to increase with increasing input DNA, while the 14-cycle readings drop after the 20 ng point.

Assay Validation

An important part of assay validation is to determine if the assay is primate specific, i.e., that the assay gives negative results with non-primate DNA. Five primates, 13 commonly encountered animals as well as three bacteria, one insect, and yeast were evaluated using approximately 10 ng of each DNA. The primates gave the expected positive result, but the animals, insect, bacteria, and yeast were all negative (data not shown).

Another important point is to show that non-primate DNAs do not interfere with detection of human DNA. A mixing experiment of rat and human DNA (Fig. 6) was performed where each well contained the same total amount of DNA (2.5 ng) but with different ratios of human and rat DNA. This figure shows that as the percentage of human DNA increases, RFUs increase accordingly. The rat DNA neither contributed to the final reading nor inhibited the PCR reaction.

Degraded human genomic DNA was created by treatment with DNAseI for varying lengths of time (0.75 to 48 min) (Fig. 7). These degraded samples were quantitated by slot blot and the QSY assay (Table 1). The samples were then diluted to 0.1 ng/μL based on the results of the QSY assay, amplified using the COfiler™ STR kit (Applied Biosystems) and analyzed on an ABI 310. All of the results for the samples diluted based on the QSY results were within acceptable ranges for peak heights (150 to 5500 RFU for each heterozygous peak) (data not shown). This indicates that the QSY assay successfully quantitated the amount of "PCRable" DNA within the degraded sample so that a correct prediction of the dilution of sample necessary for STR analysis could be made.

Results from the slot blot assay were also compared to the results from the new QSY *Alu* PCR assay on blood and semen samples. A set of 13 blood spots, 6 mock crime samples, and 24 samples of DNA placed on different surfaces with different treatments (RT, 37°C, sunlight) for different periods of time and four samples of DNA from colored cloths (denim) were studied. The middle two columns of Table 2 list the QSY assay versus slot blot results for examples of these different sample types. In general, the determined concentrations for the blood spots based on the slot blot or QSY assay results were close, but the results for the degraded, environmental samples were much lower for the QSY assay. This is to be expected if the DNA is so degraded that PCR cannot occur. The values for the denim cloth samples also deviated from expected, clearly due to the known inhibitory effects of agents within denim.

All of the above samples were analyzed with the COfiler™ STR kit. For those samples where the determined concentrations using the slot blot and QSY methods were nearly identical, dilutions were based on the concentration from the QSY *Alu* method. The reason

for this was that it would be redundant to perform STR analysis on only slightly different concentrations since minor changes in template DNA would not alter the STR results. Using 1 ng of DNA based on the readings from the QSY experiment, all of the samples gave results on the ABI 310 within the acceptable range (150 to 5500 RFU for each heterozygous peak) (Table 2, Columns 6 and 7) except for one cloth sample that was high (Cloth 1), and one sample from concrete (Concrete −1 day). Some of these samples were also subject to COfiler™ amplification but diluted based on the slot blot results (Table 2, Columns 4 and 5). One of these samples was clearly overloaded (Denim 2). For some samples, the slot blot assay and QSY assay gave widely disparate results (Table 2), but COfiler™ amplifications based on both the slot blot and QSY concentrations were within generally acceptable limits (except for the Cloth 1, Denim 2, and Concrete—1 day samples).

To address the fact that the QSY assay appeared to give low concentration values that resulted in too much DNA being added to the COfiler™ amplifications for the denim samples, a final concentration of 250 μg/mL BSA was added to the QSY assay. The addition of BSA did not change the values for the standard curve (data not shown) nor for samples such as blood spots (Table 3); however, it did raise the concentration values for difficult samples such as denim, now making COfiler™ amplifications based on the QSY+BSA assay result in STR profiles within acceptable limits (data not shown).

Two inhibitors of PCR, EDTA, and hematin were evaluated. EDTA chelates the Mg^{++} needed by the polymerase. As the EDTA concentration increased in the assay, the resulting RFU values fell accordingly, resulting in 50% inhibition at 0.75 m*M* (data not shown). The zero time point was unaffected by EDTA; thus, inhi-

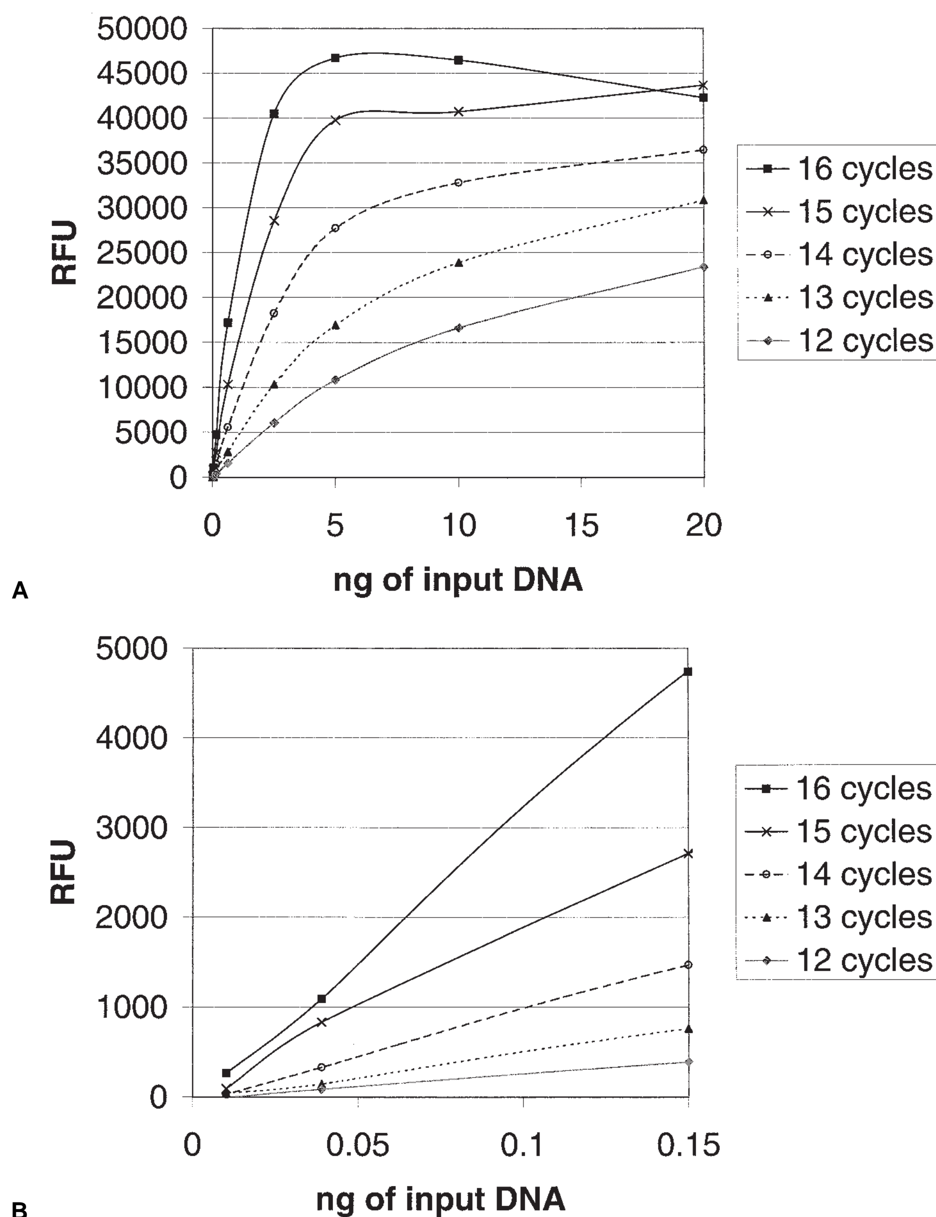

FIG. 3—*The effect of the number of PCR cycles on the QSY Alu assay was determined by performing the assay for 12, 13, 14, 15, or 16 cycles. The relative fluorescence units (RFU) are plotted against the input DNA concentration for seven concentrations of standard (10 to 0.01 ng):* (a) *full concentration range of input DNA;* (b) *closeup of lower end of concentration range.*

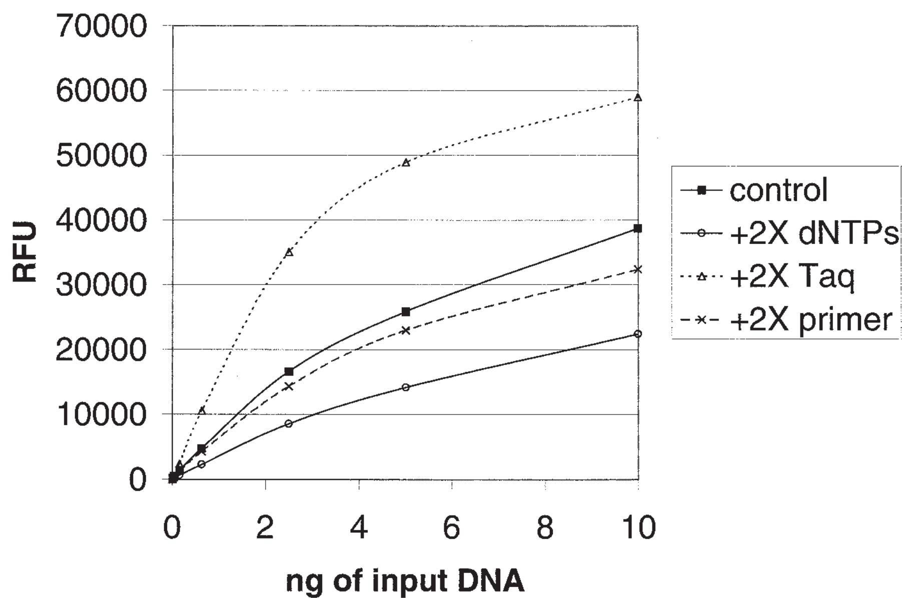

FIG. 4—*The effect of doubling primer, Taq, or dNTP concentration on the QSY Alu assay using the ReadyMix™ kit. PCR was performed using 14 cycles and 1/40 000 extra SYBR Green. The relative fluorescence units (RFU) are plotted against the input DNA concentration for seven concentrations of standard (10 to 0.01 ng).*

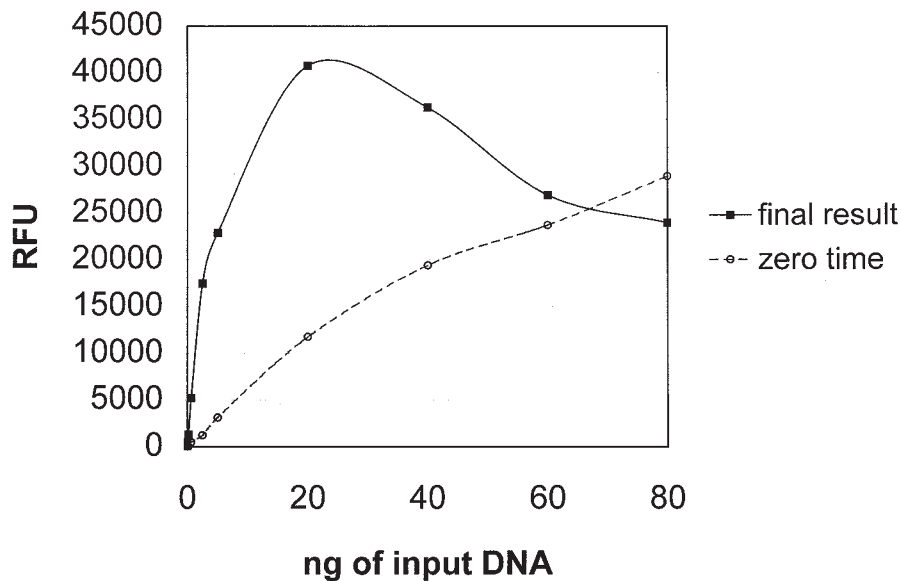

FIG. 5—*Zero time and final (14 cycle minus zero time) results of the QSY Alu assay using high concentrations of input DNA. PCR was performed using 14 cycles, and the ReadyMix™ kit with 1/40 000 extra SYBR Green. The relative fluorescence units (RFU) are plotted against the input DNA concentration for nine concentrations of standard 80 to 0.01 ng).*

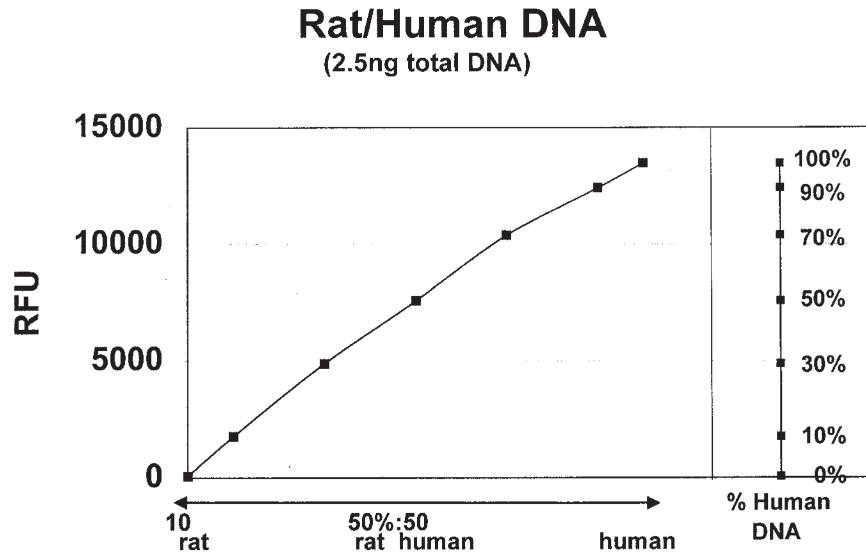

FIG. 6—*Human and rat DNA was mixed in ratios from 0 to 100% rat and QSY Alu PCR performed using 14 cycles and the ReadyMix™ kit with 1/40 000 extra SYBR Green. The relative fluorescence units (RFU) are plotted against the 100% of human DNA for seven mixture ratios. The right side shows a vertical pictorial of the results.*

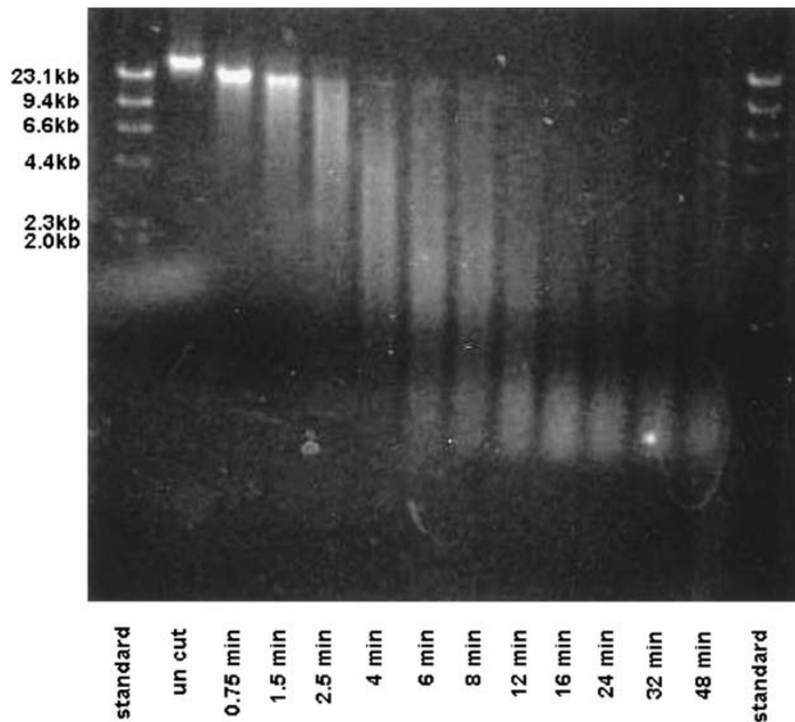

FIG. 7—*An agarose gel of Promega human DNA standard cut with 0.0005U/µL final concentration DNAseI for 0.75 to 48 min. The time of incubation with the enzyme is shown below the lane. The standard is HindIII digested lambda DNA.*

TABLE 1—*Results on DNAse I treated samples.*

Sample Treatment	Slot Blot, ng/5 μL	QSY Assay, ng/5 μL	COfiler™ Result RFU Amelogenin X	COfiler™ Result—RFU D7S820 Allele 10
None	2.25	2.77	2777	842
0.75 min	2.25	2.51	3648	886
1.5 min	1.25	2.48	3614	678
2.5 min	0.62	2.97	2090	505
4 min	0.62	2.29	2216	268
6 min	0.62	2.04	2043	245
8 min	0.31	1.06	2911	363
12 min	0.15	0.72	2665	262
16 min	0.15	0.71	2284	164
24 min	0.15	0.66	1932	165
32 min	0.15	0.87	1559	211
48 min	0.15	0.92	2094	398

TABLE 2—*Slot blot, QSY assay, and COfiler™ results on selected test samples.*

			Slot Blot-Based COfiler™ Result		QSY-Based COfiler™ Result*	
Sample	Slot Blot Result	QSY PCR Assay Result	TH01	D7S820	TH01	D7S820
MOCK CRIME SAMPLES						
Male fraction 2	1.0 ng	0.87 ng			2930	1366
Male fraction 4	<0.15 ng	<0.010 ng			<150	<150
Female fraction 2	0.8 ng	0.79 ng			3163	1644
Female fraction 4	0.06 ng	0.068 ng			1762	731
Female standard 3	0.25 ng	0.23 ng			2172	1392
BLOOD SAMPLES TREATED FOR THREE MONTHS						
No. 5–3 month sunlight	0.6 ng	0.44 ng			1386	839
No. 6–3 month sunlight	0.5 ng	0.37 ng			2039	697
No. 5–3 month dark	0.5 ng	0.43 ng			1861	968
No. 6–3 month dark	0.6 ng	0.55 ng			1494	732
No. 5–3 month 37°C, dark	0.7 ng	0.69 ng			1387	461
No. 6–3 month 37°C, dark	0.8 ng	0.68 ng			1151	437
DATABANK BLOOD SPOTS						
Databank 1	1.0 ng	0.84 ng			2734	1092
Databank 2	1.0 ng	0.93 ng			2962	1473
Databank 3	1.0 ng	0.91 ng			2587	1175
Databank 4	0.6 ng	0.48 ng			2340	1049
BLOOD SPOTS ON CLOTH						
Denim 1	3.5 ng	0.092 ng	2033	873		
Denim 2	3.5 ng	0.035 ng	7124†	2696	3385	1946
Cloth 1	3.5 ng	0.028 ng	2401	1355	5888†	5061
ENVIRONMENTAL SAMPLES						
Stick—1 day	0.2 ng	0.050 ng	1097	401	2116	587
Concrete—1 day	0.5 ng	0.17 ng	1717	853	7027†	3508
Leather—1 day	1.0 ng	0.14 ng	912	271	4057	1533
Stick—6 weeks	0.8 ng	0.17 ng	933	273	5272	1831
Concrete—6 weeks	0.32 ng	0.14 ng	871	259	2578	749
Leather—6 weeks	0.5 ng	0.18 ng	1691	537	2807	1003

*These results are based on the QSY *Alu* assay WITHOUT added BSA.
†Shaded values indicate an RFU over the accepted maximum (5500).

TABLE 3—*Comparison of QSY assays with and without added BSA results from selected blood spots (databank samples), problematic (cloth) samples, and blood spots left one day or six weeks or RT on selected surfaces. The presence of inhibitors in the problematic samples greatly decreases amplification when BSA is not added to the PCR (Column 2), while addition of BSA greatly reduces the effects of inhibitors (Column 3).*

Sample	QSY Assay—No BSA Added	QSY Assay—+ BSA
Databank 1	0.84 ng	0.85 ng
Databank 2	0.93 ng	0.98 ng
Databank 3	0.91 ng	1.22 ng
Databank 4	0.48 ng	0.47 ng
Denim 1	0.092 ng	3.0 ng
Denim 2	0.035 ng	2.0 ng
Cloth 1	0.028 ng	1.8 ng
Cloth 2	<0.010 ng	2.0 ng
Stick—6 weeks	0.17 ng	0.64 ng
Concrete—6 weeks	0.14 ng	0.27 ng
Leather—6 weeks	0.18 ng	0.36 ng
Stick—1 day	0.050 ng	0.062 ng
Concrete—1 day	0.17 ng	0.41 ng
Leather—1 day	0.14 ng	0.33 ng

bition could be detected as an incongruity between the zero time and final readings. Hematin is a derivative of heme that is known to inhibit PCR reactions. Fifty percent inhibition was observed at 0.5 μ*M* hematin without BSA in the *Alu* assay; however, it required increasing the hematin concentration to 25 μ*M* to give 50% inhibition when BSA was added to the assay as above.

While the ReadyMix™ kit worked well in this assay, it is expensive and for optimal assay performance requires the analyst to supplement the kit with additional SYBR Green. Figure 8 compares the results obtained with the ReadyMix™ kit to that of a homebrew mix that costs approximately 60% of the ReadyMix™ kit/well. Although the absolute RFU values with the homebrew are slightly lower than that obtained with the ReadyMix™ kit, the assays are comparable and the homebrew mix may even give a more linear response. Depending on the laboratory's needs, the homebrew mix would be a viable alternative with only slight in-house modifications required, e.g., perhaps the addition of more polymerase, SYBR Green, or Mg^{++}.

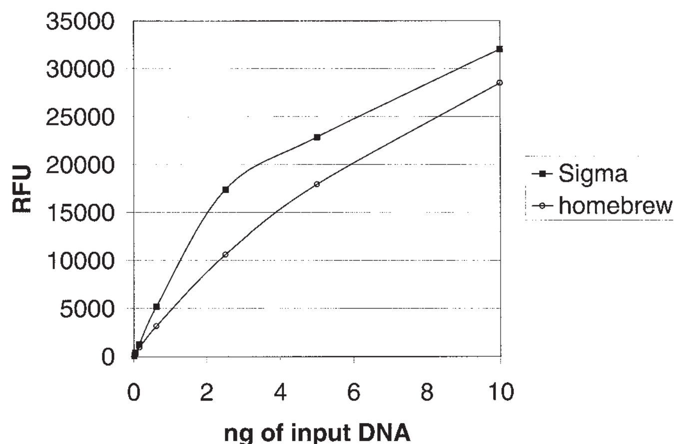

FIG. 8—*Comparison of QSY Alu PCR results using the ReadyMix™ kit with 1/40 000 extra SYBR Green with homebrew reagents with 1/20 000 SYBR Green. The Sigma reactions were a three-cycle PCR using Jumpstart Taq (95°C for 2 min followed by 14 cycles of 94°C for 15 s, 60°C for 1 min, 72°C for 1 min), while the homebrew was a two-cycle PCR using Taq Gold (10 min at 95°C, followed by 14 cycles of 95°C for 20 s and 60°C for 45 s).*

Discussion

Final optimization of the QSY assay resulted in a 25 μL reaction of: 12.5 μL of the SYBR® Green JumpStart™ Taq ReadyMix™ kit (Sigma), 1 μL of 6.25 mg/mL BSA, 0.5 μL of 20 pmoles/μL QSY 7 labeled forward primer, 0.5 μL of 20 pmoles/μL QSY 7 labeled reverse primer, 0.0625 μL of a 1/100 dilution of SYBR Green I (diluted in DMSO) and 5.4375 μL PCR amplification grade H_2O plus 5 μL of input DNA in TE. The assay as performed here allows for addition of the input DNA in 5 μL of TE (10 m*M* Tris, pH 7.5, 0.1 m*M* EDTA); however, a volume up 10.4375 μL could be added in a 25 μL reaction. However, addition of >5 μL of TE to the reaction does reduce PCR amplification (data not shown), presumably because the additional EDTA chelates the Mg^{++} needed by the polymerase. If it is necessary to add the sample in a larger volume, the sample should be resuspended in water or more Mg^{++} should be added to the mastermix.

The assay also requires that the DNA be denatured before the QSY assay is set up. Many isolation protocols (such as Chelex or many commercial kits) result in denatured DNA; therefore, no further treatment is required. However, sample DNA from organic extractions must be denatured (5 min at 95°C followed by 5 min on ice was utilized here). The DNA standard must also be denatured. The reason for this is that SYBR Green yields a higher fluorescence with double-stranded DNA than single-stranded DNA; thus, for the zero time reading the fluorescence of the input DNA will be very high if it is double stranded, while at the final reading the fluorescence of the input DNA will be low because it will be denatured.

The QSY +BSA *Alu* assay has many advantages over the current slot blot assay. It is reproducible; Table 4 shows the results of a standard curve performed in triplicate and with values and standard deviations for each datapoint. The assay is cost effective, as use of a homebrew mastermix results in a test actually costing less than the ABI Quantiblot® kit in our hands. The QSY assay is also fast requiring approximately 1/2 h of setup time, an hour of PCR amplification (during which the analyst can be performing other tasks), and then the quantitation results are immediately calculated by the plate reader software and printed out. Lastly, the dynamic range is larger than the slot blot assay using either colorimetric or luminescence readout. As discussed in the introduction, other groups have developed assays based on *Alu* sequences; however, the QSY assay developed here has several advantages over these systems in simplicity, analyst time, and sensitivity.

The QSY *Alu* assay has shown it can correctly determine the amount of human DNA in a wide range of samples and predict the

TABLE 4—*Results of triplicate QSY Alu assay experiment.*

Input DNA	Mean Result, triplicates	Standard Deviation	%CV
20 ng	36958	1597	4.32
10 ng	36100	1384	3.83
5 ng	28899	273	0.94
2.5 ng	18562	300	1.62
1.25 ng	10419	169	1.62
0.62 ng	5234	253	4.83
0.31 ng	2692	28	1.04
0.15 ng	1266	38	3.00
0.078 ng	628	2	0.31
0.039 ng	337	50	14.84
0.02 ng	238	57	23.95
0.01 ng	131	43	32.82

proper dilution for STR analysis. Because both the QSY *Alu* assay and STR analysis are PCR based, the QSY *Alu* assay should have better predictive value for STR success than the slot blot assay. The QSY assay also gives additional information that the slot blot assay does not. Study of the initial zero time reading and comparison with the final result allows for determination of the presence of too much human DNA or the presence of animal DNA or inhibitors. For example, if the initial reading indicates 40 ng of DNA, then the sample needs to be diluted. A separate adequately diluted sample may be prepared and run before the assay is started, avoiding the necessity of rerunning highly concentrated samples post assay. If the zero time results indicate 5 ng of DNA but the final results indicate no amplification, then either a PCR inhibitor is present or the DNA is nonhuman. Such results can indicate the need for sample cleanup if an inhibitor is suspected or the recognition that the sample may be nonhuman based. Dilution of the sample and a rerun may remove problems with inhibitors but will not affect results for animal/plant/bacterial contamination. Inhibitors can also be detected by spiking the sample with a known amount of human DNA. If there is only a partial incongruity between the zero time and final result, then one could proceed with STR analysis based on the final QSY result. This should result in a proper dilution since both the QSY assay and STR amplification respond similarly to inhibitors and both effectively ignore the presence of animal/plant/bacterial DNA.

There are possibilities for further improvement of the assay including changes in the primers. The primers designed here are perfect matches to the consensus *Alu* Ya5 family sequence; however, this forward primer has two mismatches from the most common family consensus sequence (Sx − 50% of *Alu*s) (at Base 9 from the 5′ end and Base 4 from the 3′ end), and the reverse primer has a single mismatch (at three bases from the 3′ end). These primers may amplify the Sx sequence albeit at lower efficiency; however, it should be possible to design Sx specific. The tradeoff using Sx specific primers may be reaction with animal DNA. More human specific primers (not reacting with other primates) could also be designed. Human specific primers will be present in lower copy number and thus lower sensitivity, but several primer sets could be combined in one assay. Batzer et al. have found that some of human specific sequences are not present in equal amounts in all races (15); such sequences would need to be avoided for a general assay. Lastly, there is the possibility of moving this assay to a real-time PCR format. These experiments are currently underway.

Acknowledgments

The authors would like to thank Marcie LaFountain and Dr. Margaret Schwartz for advice, support, and critical review of the manuscript and Dr. Mark Batzer for *Alu* sequence information and advice.

References

1. Krenke BE, Tereba A, Anderson SJ, Buel E, Culhane S, et al. Validation of a 16-locus fluorescent multiplex system. J Forensic Sci 2002;47: 773–85.
2. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Smerick JB, et al. Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. J Forensic Sci 2001;46:647–60.
3. LaFountain MJ, Schwartz MB, Svete PA, Walkinshaw MA, Buel E. TWGDAM validation of the AmpFℓSTR Profiler Plus and AmpFℓSTR COfiler STR multiplex systems using capillary electrophoresis. J Forensic Sci 2001;46:1191–8.
4. Budowle B, Hudlow WR, Lee SB, Klevan L. Using a CCD camera imaging system as a recording device to quantify human DNA by slot blot hybridization. BioTechniques 2001;30:680–5.
5. Mighell AJ, Markham AF, Robinson PA. Alu sequences. FEBS Lett 1997;417(1):1–5.
6. Schmid CW. Alu: structure, origin, evolution, significance and function of one-tenth of human DNA. Prog Nucleic Acid Res Mol Biol 1996;53: 283–319.
7. Ullu E, Tschudi C. Alu sequences are processed 7SL RNA genes. Nature 1984;312:171–2.
8. Chen PJ, Cywinski A, Taylor JM. Reverse transcription of 7S L RNA by an avian retrovirus. J Virol 1985;54:278–84.
9. Batzer MA, Deininger PL, Hellmann-Blumberg U, Jurka J, Labuda D, Rubin CM, et al. Standardized nomenclature for Alu repeats. J Mol Evol 1996;42:3–6.
10. Sifis ME, Both K, Burgoyne LA. A more sensitive method for the quantification of genomic DNA by *Alu* amplification. J Forensic Sci 2002;47: 589–92.
11. Procedure for the detection of restriction length polymorphisms in Human DNA. FBI Laboratory, Forensic Science Research Training Center, Quantico, VA, 1989.
12. Buel E, Wang G, Schwartz M. PCR amplification of animal DNA with human X-Y amelogenin primers used in gender determination. J Forensic Sci 1995;40:641–4.
13. Batzer MA, Alegria-Hartman M, Deininger PL. A consensus *Alu* repeat probe for physical mapping. Genet Anal Tech Appl 1994;11:34–8.
14. Karsai A, Muller S, Platz S, Hauser MT. Evaluation of a homemade SYBR green I reaction mixture for real-time PCR quantification of gene expression. Biotechniques 2002;32:790–6.
15. Batzer MA, Rubin CM, Hellmann-Blumberg U, Alegria-Hartman M, Leeflang EP, Stern JD, et al. Dispersion and insertion polymorphism in two small subfamilies of recently amplified human *Alu* repeats. J Mol Biol 1995;247:418–27.

Additional information and reprint requests:
Eric Buel, Ph.D.
Vermont Forensic Laboratory
P.O. Box 47
Waterbury, VT 05676
E-mail: ebuel@dps.state.vt.us