J Forensic Sci, September 2003, Vol. 48, No. 5
Paper ID JFS2002414_485
Available online at: www.astm.org

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Development of an *Alu*-based, Real-Time 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. We have previously described a method for quantitation of human DNA based on PCR amplification of a repetitive *Alu* sequence that uses a fluorescence plate reader. This manuscript describes and validates a variation of this assay using real-time PCR and SYBR® Green I for quantitation. The advantages of the real-time assay over the plate reader assay are: reduced hands-on time, lower assay cost, and a greater dynamic range. The main disadvantage is the cost of the real-time instrument. However, for those forensic laboratories with access to a real-time instrument, this *Alu*-based assay has a dynamic range of 16 ng to 1 pg, is sensitive, specific, fast, quantitative, and uses only 2 μL of sample.

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

Quantitation of the DNA extracted from a crime scene sample is essential to appropriate short tandem repeat (STR) DNA profiling. Using too little or too much template DNA will often cause problems with the STR amplification, necessitating reanalysis (1–3). The forensic community today typically relies upon a technique known as the slot blot approach to estimate human DNA (4). The technique is time consuming and labor intensive. When used in conjunction with instrumentation (5), it can offer semi-quantitative results, but without such instrumentation the analyst must estimate values based on band intensities as judged by eye.

In a previous paper, we discussed an instrumental approach to estimate human DNA extracted from samples (6). The method relied upon the polymerase chain reaction (PCR) in combination with a fluorescence plate reader. Portions of a specimen were amplified for a section of the highly repetitive, primate-specific *Alu* sequence. *Alu* sequences are found in 500,000 to 1,000,000 copies in the human genome, representing 6 to 13% of the haploid genome (7,8). The consensus *Alu* sequence is ~280 bp in length, consisting of two similar monomers connected by an A-rich region. Because *Alu* sequences are present in many copies in primates, 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 utilizing luciferase-produced light (9). Another paper reports use of an *Alu* PCR-based system (10) to quantitate human genomic DNA from 2.5 to 100 pg by determining the peak heights (RFU) obtained with an ABI PRISM™ 377 Genetic Analyzer and GeneScan software (Applied Biosystems, Foster City, CA). Lastly, Urban et al. (11) used PCR of *Alu* sequences to detect template contamination. In our assay (6), the DNA dye SYBR® Green I (Molecular Probes, Eugene, OR) was included in the amplification mix, which allowed an estimate of the PCR product when assayed in a fluorescence plate reader. The amount of input DNA was shown to be related to the quantity of PCR product produced. The dynamic range of this assay is approximately 10 pg to 10 ng of input DNA.

Another approach to DNA quantitation employs the use of a real-time PCR instrument (12–14). This instrument monitors the accumulation of PCR product with each cycle and allows assessment of each sample individually during the exponential growth phase. The final readout for each sample is the cycle threshold (*Ct*), which is defined as the point where the amplification curve crosses a set fluorescence value. *Ct* is thus inversely proportional to DNA concentration. In other words, as DNA concentration increases, amplification and therefore fluorescence increase accordingly, and thus the fluorescence threshold is crossed at a lower number of cycles (lower *Ct*). Real-time assays have the advantages of a greater dynamic quantitative range and require only limited analyst attention.

This paper describes use of *Alu*-specific PCR and SYBR® Green I staining in a real-time PCR format. This assay is validated by study of animal samples, blood spots, mock casework, and degraded DNA and is shown to quickly, reliably, and inexpensively quantitate human DNA over a range of 16 ng to 1 pg.

Materials and Methods

DNA Samples

Most experiments were performed with a human DNA standard (297 ng/μL; G3041) purchased from Promega (Madison, WI). DNA was isolated from a number of samples to validate the assay: blood spots from seven DNA databank samples (bloodspots on FTA

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* Supported under Award Number 2000-IJ-CX-K012 from the Office of Justice Programs, National Institute of Justice, Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position on of the U.S. Department of Justice.

Received 3 Dec. 2002; and in revised form 25 Jan. 2003 and 15 March 2003; accepted 15 March 2003; published 4 Aug. 2003.

paper, Whatman, Newton, MA), three samples from control blood spotted on denim, control blood placed on eight surfaces (leaves, concrete, cardboard, denim, leather, soapy cloth, stick, metal), sets of two blood spots placed either in the dark, in sunlight, or at 37°C in the dark for 3 months, male and female fractions from four sexual assault cases and one case standard, three samples from a proficiency test, six swabs from various surfaces (phones, computers, keys), swabs of three fingerprints, saliva from three envelope seals, and a blank from a DNA extraction. DNA was isolated using an organic extraction method (15) as modified in Akane et al. (16).

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) or buccal swabs of pets of laboratory personnel 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) (17) using the program Oligos © 1999–2002 version 9.6 (http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm), designed by R. Kalendar of the Institute of Biotechnology, University of Helsinki. The primers were purchased from Synthetic Genetics (now Epoch Biosciences, San Diego, CA).

Inhibitors/DNA Degradation

DNA was treated for various times (0.5 to 256 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*.

PCR Assay

PCR utilized the SYBR® Green JumpStart™ Taq ReadyMix™ kit (S4438, Sigma, St. Louis, MO). Some experiments used the Brilliant™ SYBR® Green QPCR Master Mix (600548, Stratagene, La Jolla, CA). The 10-μL reactions contained 5 μL of 2X Sigma or Stratagene Master Mix (1X final), 0.2 μL of 20 pmoles/μL each primer, 0.025 μL of 1:100 (in DMSO, D-8779 Sigma, St. Louis, MO) SYBR® Green I (S7563, Molecular Probes, Eugene, OR), 0.4 μL of 250 μg/mL BSA (A-9647, Sigma, St. Louis, MO), 2.175 μL distilled H_2O and 2 μL of TE (10mM Tris, pH 7.5, 0.1 m*M* EDTA) containing the input DNA at various concentrations. Some initial optimization experiments varied the concentrations of components such as SYBR® Green I or primers. The SYBR® Green I stock is a 10,000X concentrate. The 10,000X concentrate was diluted 1/100 in DMSO, and either 0.025 or 0.050 μL was added to the 10-μL reaction (0.25X final or 0.50X final concentration, respectively).

Real-time PCR for the *Alu* assay was performed in a Corbett Research Rotorgene (Phenix Research, Hayward, CA) using the small 0.1-mL tubes (MPCR-72, Phenix Research, Hayward, CA). PCR consisted of 95°C for 2 min ("hotstart") followed by 35 cycles of 95°C for 15 s, 68°C for 30 s, 72°C for 30 s. Various initial experiments changed annealing and/or extension times and temperatures to optimize the assay. A melt curve was also performed after the assay to check for specificity of the reaction. This consisted of 20 s at 72°C followed by a ramp up of 1° step with 5-s hold at each step.

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. A colorimetric method was used for detection as described by the manufacturer, and the membrane was read by visual examination.

STR Analyses

The AmpF*l*STR® 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 ng total of the mixed DNAs in each reaction.

Results

Development of Assay

Previous studies demonstrated the specificity of the *Alu* primers and their use in a fluorescence plate reader assay (6). With these positive results, the assay was then moved to a real-time format and a real-time PCR instrument for quantitation. Previous studies with the plate reader assay has also indicated that it probably would be necessary to add additional SYBR® Green I to the master mix because the large number of *Alu* PCR copies generated quickly bound all the available SYBR® Green I. The Sigma SYBR® Green JumpStart™ Taq ReadyMix™ contains a proprietary amount of SYBR® Green I. Additional SYBR® Green (diluted 1:100 in DMSO) was added to the reactions prior to PCR amplification to increase the amount of SYBR® Green in the final assay above the proprietary amount in the Sigma mix. Experiments using the SYBR® Green JumpStart™ Taq ReadyMix™ kit with three concentrations of SYBR® Green I were performed (no additional SYBR® Green I added to the Master Mix, additional 0.25X final concentration SYBR® Green I added and additional 0.50X final concentration SYBR® Green I added) (data not shown). Use of no additional SYBR® Green I caused loss of linearity at the higher DNA concentrations, and curve shape was significantly altered. Also, the fluorescence intensity was very low when no SYBR® Green I was added. The addition of SYBR® Green I did slow the PCR reaction (increased the cycle thresholds (*Cts*)) with the 0.50X having the greatest effect as expected. Thus, as with the plate assay, added 0.25X was chosen for the final assay. Previous studies (6) had also indicated that addition of BSA (10 μg/μL) to the PCR overcame the effects of the inhibitors examined and made the assay more reflective of results with the STR assays; thus, 10 μg/μL BSA was routinely added to the PCR mix. It did not change values for the real-time assay (data not shown).

The next studies focused on changing the annealing temperature. Two annealing temperatures, 60 and 68°C, were compared. Figure 1 shows the results on the *Ct* values and Fig. 2 shows the effects on

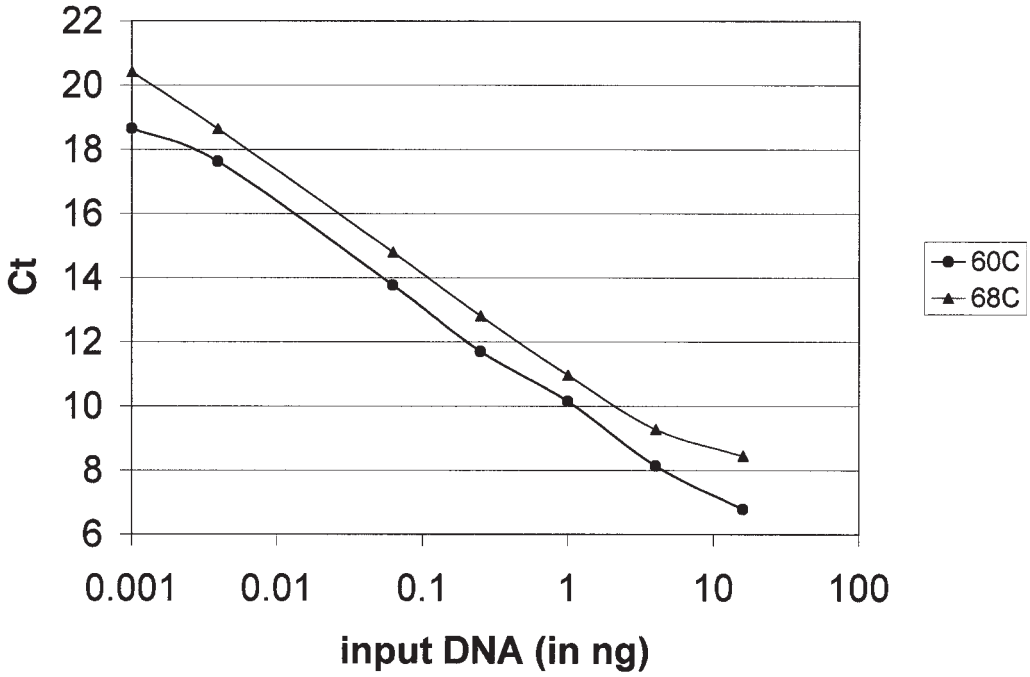

FIG. 1—*Plot of the Ct values versus input human DNA standard for annealing temperatures 60°C (black circles) versus 68°C (gray triangles). The curves are of identical shape with the Cts just slightly higher for the 68°C.*

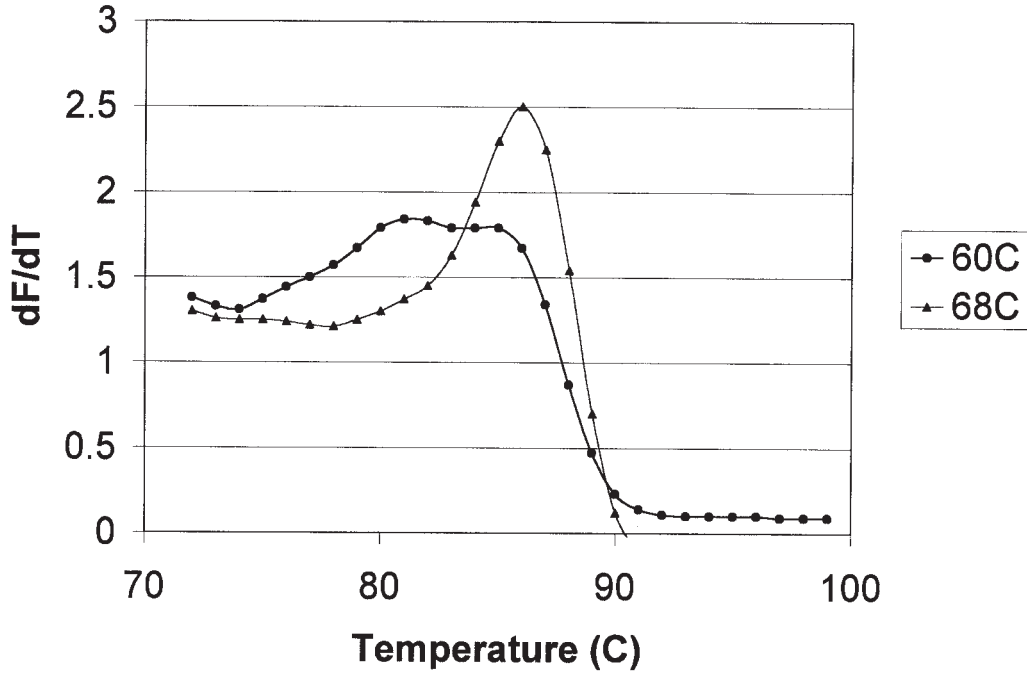

FIG. 2—*Melt curves for the experiment in Fig. 1 that compared annealing temperatures of 60°C (black circles) versus 68°C (gray triangles). The 60°C annealing temperature experiment has a much broader melt curve indicating a greater variety of subspecies of Alu sequences. The dF/dT (the first derivative of the fluorescence versus temperature) is plotted versus the temperature.*

the melt curves. As expected, 68°C increases the *Cts* slightly; however, there is a dramatic effect on the melt curve, with the higher temperature resulting in a much sharper peak. Since *Alu*s are a family with many related but not identical members, a lower annealing temperature allows amplification of many diverse sequences, while the higher temperature selects a more uniform product. The 68°C anneal was chosen for the assay for this reason.

A denaturation time of 15 s, an annealing time of 1 min, and an extension time of 1 min (1 min/1 min) were initially used for the assay. However, theoretically with a small PCR product like the 124-bp *Alu* PCR in these experiments, shorter times should be sufficient. A comparison was made between a 30-s anneal and extension with a 60-s anneal and extension (Fig. 3). The assays were very similar except that the longer anneal experiments had

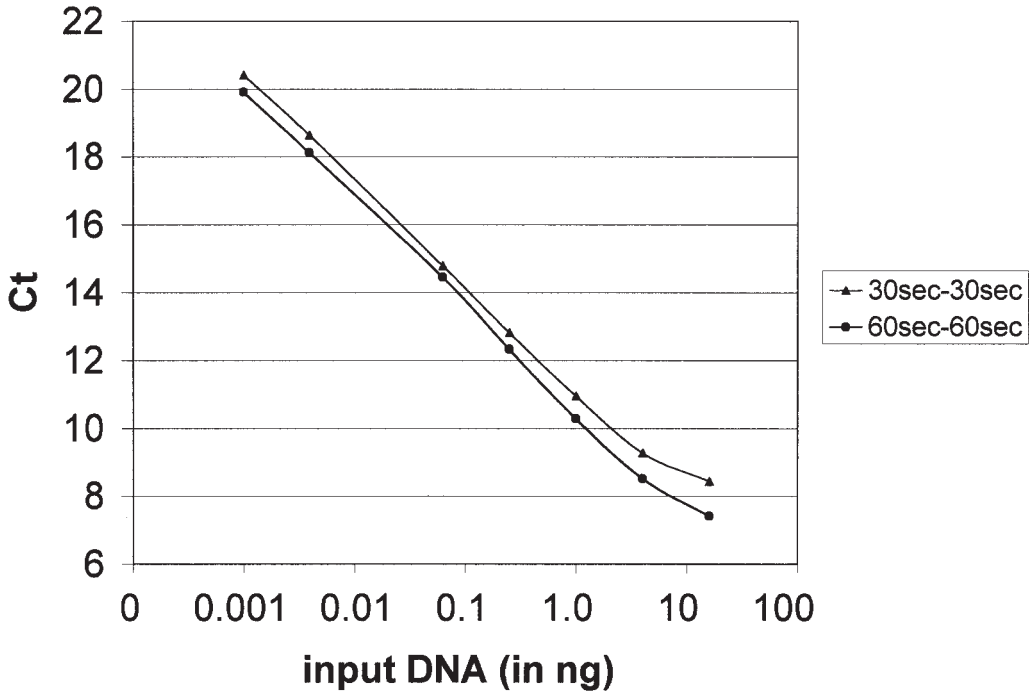

FIG. 3—*Plot of the Ct values versus input human DNA standard for 60-s anneal/60-s extension (black circles) versus 30-s anneal/30-s extension (gray triangles). The curves are of identical shape with the Cts, just slightly higher for the 30-s anneal and 30-s extension.*

TABLE 1—*Effect of primer concentration.*

Forward Primer Concentration (pmoles/μL final)	Reverse Primer Concentration (pmoles/μL final)	*Ct*
0.8	0.8	10.305
0.4	0.8	10.400
0.2	0.8	10.465
0.8	0.4	10.295
0.4	0.4	10.460
0.2	0.4	10.590
0.8	0.2	10.300
0.4	0.2	10.425
0.2	0.2	10.550

slightly lower *Cts*. Experiments using a 30-s anneal with s 60-s extension as well as a 60-s anneal with a 30-s extension were also performed with results intermediate to the 30-s anneal and the 30-s extension and the 60-s anneal and the 60-s extension (data not shown). Since the results were similar for all cases, and the 30-s anneal and 30-s extension time saved 35 min per assay over use of 60 s for each, the shorter times were chosen for the assay.

The effect of changing primer concentrations was also investigated. The forward and reverse primer concentrations were varied independently from 0.2 to 0.8 μ*M* (Table 1). While increasing the primer concentrations fourfold did decrease the *Ct* slightly (~0.4 *Ct*) with a slightly greater decrease for the forward primer, the effects were not significant and thus 0.4 μ*M* was chosen for the assay.

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. Three primates, twelve commonly encountered animals as well as three bacteria, one insect, and yeast were evaluated using approximately 10 ng of each DNA (Table 2). The primates gave the expected positive result, but the animals, insect, bacteria, and yeast were all essentially negative (i.e., gave *Cts* approximately that of the no template control (27.44) and or at least close to the *Ct* of 1 pg of human DNA (21.5)). The middle column (human equivalent) shows the concentration of human DNA that would give the observed *Ct* based on the standard curve; division of that number by the input 10 ng of DNA (X100%) gives a relative detection efficiency of the animal DNA versus human (right-hand column). Thus, while the chimp DNA gives an efficiency of 82%, rat DNA has an efficiency of only 0.005% relative to human DNA.

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. 4) was performed where each well contained the same total amount of DNA (2 ng) but with different ratios of human and rat DNA. The theoretical values for the amount of added human DNA are shown as gray triangles. This figure shows that as the percentage of human DNA increases, *Cts* decrease accordingly with very good agreement between the expected, theoretical, and the observed values. 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 (0.5 to 256 min). These degraded samples were quantitated by slot blot and the real-time *Alu* assay. The *Alu* assay gave higher values for DNA concentration, especially at longer digestion times; this is probably the result of small fragments not binding to the slot blot membrane (data not shown). The samples were then diluted to 0.1 ng/μL based on the results of the *Alu* assay, 1.0 ng of template DNA was amplified using the COfiler™ STR kit and the product analyzed on an ABI 310. All of the results for the samples diluted based on the *Alu* results were within laboratory-acceptable ranges for peak heights (150 to 5500 RFU for each heterozygous peak) for the THO1 locus; how-

TABLE 2—*Real-time Alu assay results with 10 ng each of animal DNAs.*

DNA Source	*Ct*	Human DNA Equivalent (input ng)*	Efficiency of Amplification Compared to Human DNA†
		PRIMATES	
Chimp	9.57	8.22	82%
Baboon	10.49	4.07	41%
Macaque	9.23	10.66	100%
		NON-PRIMATES ANIMALS	
Cat 1	21.16	0.0012	0.012%
Cat 2	21.16	0.0012	0.012%
Cat 3	21.70	0.0016	0.016%
Cat 4	23.11	0.0006	0.006%
Cat 5	23.45	0.0004	0.004%
Cat 6	26.98	0.0000	0%
Cat 7	27.86	0.0000	0%
Chicken	23.60	0.0002	0.002%
Cow	23.65	0.0002	0.002%
Deer	25.99	0.0000	0%
Dog 1	20.48	0.0020	0.02%
Dog 2	23.23	0.0002	0.002%
Dog 3	20.05	0.0027	0.03%
Dog 4	19.89	0.0065	0.07%
Fish (herring)	28.56	0.0000	0%
Horse	23.06	0.0003	0.003%
Moose	25.38	0.0000	0%
Mouse	22.66	0.0008	0.008%
Pig	22.02	0.0006	0.006%
Rabbit	25.37	0.0000	0%
Rat	22.27	0.0005	0.005%
		INSECT	
Drosophila	26.23	0.0000	0%
		BACTERIA/YEAST	
Clostridium	24.16	0.0001	0.001%
E. coli	28.15	0.0000	0%
Micrococcus	28.90	0.0000	0%
Yeast	25.38	0.0000	0%

*The amount of human DNA that would give the same amplification as the 10 ng of animal DNA.

†The equivalent human DNA concentration divided by the input 10 ng of animal DNA times 100%.

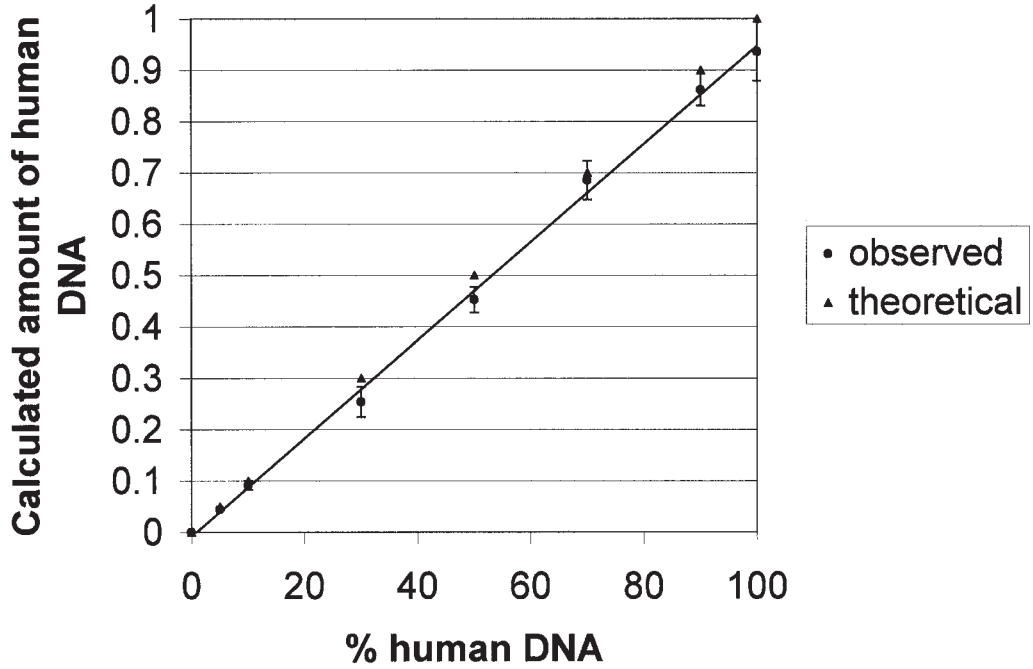

FIG. 4—*Human and rat DNAs were mixed in ratios from 0 to 100% rat (with a constant total of 1 ng) and then Alu PCR was performed. The determined human DNA concentration based on the Ct values relative to a standard curve are plotted against the percentage of human input DNA for eight mixture ratios (black circles). The theoretical results are shown in gray triangles.*

ever, the results for the highly degraded DNAs for the D7S820 locus were <150 RFU (data not shown). This is to be expected as the 124-bp *Alu* product will successfully predict amplification of similarly sized STR products such as THO1 (~175 bp) but not for the larger D7S820 product (~275 bp) in a highly degraded sample. These results indicate that the *Alu* assay successfully quantitated the amount of "PCRable" DNA within a moderately 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 real-time *Alu* assay from a wide variety of sample types. Table 3 lists the *Alu* assay versus slot blot results for these different sample types. Figure 5 plots the real-time assay results against the slot blot results for the 50 samples in Table 3. The slope of the least squares regression line is 1.0076, indicating that on average the assays give the same result although there is a bit of scatter (correlation coefficient $R = 0.698$, coefficient of determination $R^2 = 0.4874$).

Most of the above samples (those with sufficient DNA) were analyzed with the COfiler™ STR kit. For samples where the sample concentration was above the usually used 0.1 ng/μL, samples were diluted to 0.1 ng/μL and 10 μL (1.0 ng total) was used for

TABLE 3—*Results for various samples.*

Sample	Slot Blot Result (ng/μL)	*Alu* Assay Result (ng/μL)	THO1 Peak Height (RFU) (smaller allele)	D7S820 Peak Height (RFU) (smaller allele)
Databank 1	0.44	0.322	2110	715
Databank 2	0.40	0.941	(1012)*	(399)*
Databank 3	0.04	0.193	1706	449
Databank 4	0.12	0.283	1482	738
Databank 5	0.24	1.096	485	192
Databank 6	0.24	0.461	1113	413
Databank 7	0.03	0.057	IS†	IS†
Proficiency test 01-02	0.7	0.258	2380	1714
Proficiency test 01-03	0.4	0.339	1824	751
Proficiency test 01-04	1.0	0.522	1951	712
Female fraction—F‡	0.08	0.093	1193	708
Female fraction—G	0.4	0.664	1679	768
Female fraction—H	0.5	0.670	(1008)*	893
Female fraction—I (Dilution 1)	0.06	0.064	IS†	IS†
Female fraction—I (Dilution 2)	0.5	0.548	(1116)*	884
Female fraction—J	0.6	0.612	1435	809
Male fraction—E	0.48	0.425	1352	761
Male fraction—G	0.24	0.428	2194	675
Male fraction—H	0.01	0.012	IS†	IS†
Male fraction—J	0.4	0.207	2386	728
Standard—I	0.1	0.121	(2941)*	760
Standard—J	0.02	0.02	IS†	IS†
Computer keys swab 1‡	0.1	0.079	536	105
Computer keys swab 2	0.2	0.208	1510	560
Inside glove swab 3	<0.03	0.001	IS†	IS†
Phone swab 1‡	0.03	0.016	227	87
Phone swab 2	0.2	0.109	1186	575
Phone swab 3‡	<0.03	0.007	No peaks	No peaks
Envelope seal 1	0.2	0.262	1238	391
Envelope seal 2	0.16	0.195	1014	403
Envelope seal 3‡	0.06	0.046	772	279
Swab of fingerprint 1	<0.03	0.000	IS†	IS†
Swab of fingerprint 2‡	0.03	0.037	371	(193)*
Swab of fingerprint 3	<0.03	0.001	IS†	IS†
Blood spot 1–3 mo in dark	0.6	0.888	1388	(653)*
Blood spot 2–3 mo in dark	0.5	0.901	1602	830
Blood spot 1–3 mo in sunlight	1.0	1.784	1034	(290)*
Blood spot 2–3 mo in sunlight	0.2	0.377	1189	418
Blood spot 1–3 mo at 37°C	0.4	0.689	853	(236)*
Blood spot 2 to 3 mo at 37°C	0.7	1.671	958	280
Swab of blood on stick	0.5	0.325	1883	748
Swab of blood on metal	0.5	0.401	1893	883
Swab of blood on concrete	0.5	0.402	1860	945
Swab of blood on leaves‡	0.2	0.084	1540	887
Swab of blood on cardboard	0.24	0.266	1450	715
Swab of blood on soapy cloth‡	0.05	0.039	577	216
Blood on denim 1	1.0	0.245	1237	1060
Blood on denim 2	1.0	1.696	1847	874
Blood on denim, 6 weeks in dark	1.0	0.606	1274	980
Blood on denim 3	0.36	0.657	1314	676

*Numbers in parenthesis are 1/2 peak height of single peak (homozygote).
†IS = insufficient sample to perform STR analysis.
‡Used neat because original concentration was below 0.1 ng/μL.

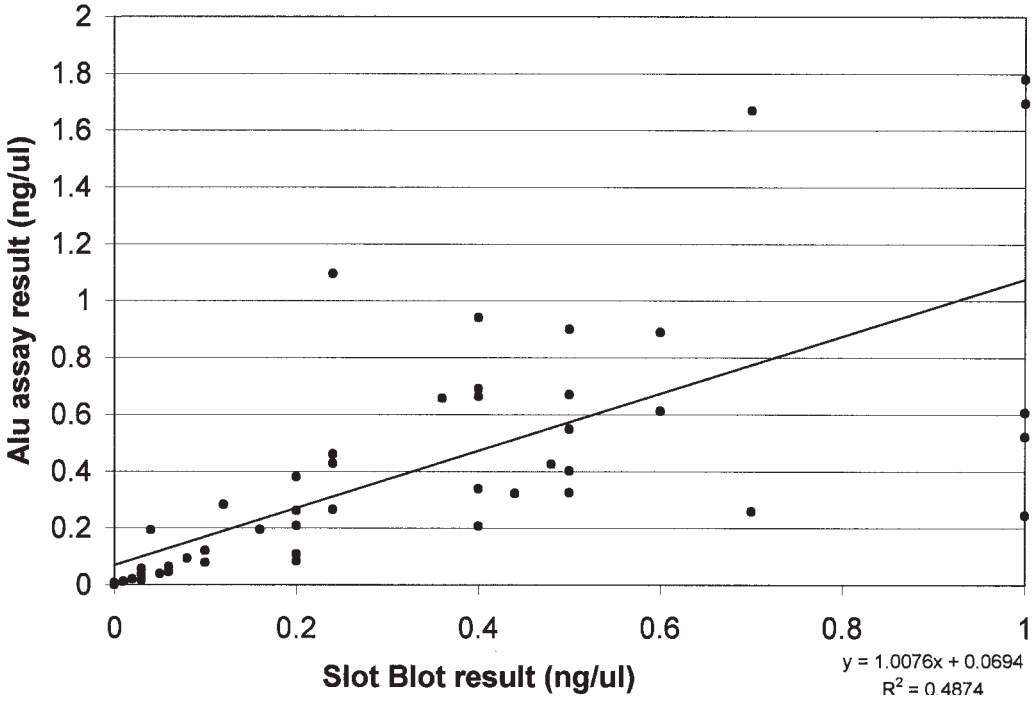

FIG. 5—*Plot of the results with the Quantiblot® assay versus the real-time Alu assay for 50 samples in Table 3. The equation of the least squares regression line and the coefficient of determination (R^2) value is shown in the lower right.*

COfiler™ STR analysis. All of these samples gave results on the ABI 310 within the laboratory's acceptable range (150 to 5500 RFU for each heterozygous peak), and Table 3 shows the results for the THO1 and D7S820 loci. For those 13 samples below the 0.1 ng/μL level, the samples were amplified using 10 μL of the neat sample. The THO1 and D7S820 results for these samples are shown in Figs. 6*a* and 6*b*, respectively. The DNaseI-treated samples are shown in gray and were not used to generate the shown trend line. A linear relationship between initial concentration and RFU was found. From these results, an *Alu* assay measurement of approximately 0.04 ng/μL (i.e., 0.4-ng input DNA) will be required to guarantee a RFU $>$ 150 (laboratory cutoff) for the D7S820 locus. These results indicate that the *Alu* assay is able to correctly quantitate DNA even at low concentrations. One sample (computer keyboard 1, concentration 0.079 ng/μL) did give unexpectedly low RFUs; this sample gave results similar to the DNAseI-treated DNAs; thus, this sample was apparently highly degraded.

The reproducibility of the assay was also investigated in several ways. Quadruplicates of the standard curve performed on the same day had percent standard deviations for the *Ct* values of less than 1.4% (except for the NTC, which was 3.8%) (Table 4). Seven samples repeated three times over 3 days had percent standard deviations for the *Ct* values less than 2% (data not shown), while the percent standard deviation for the concentration values ranged from 7 to 19% (Table 5). Lastly, an experiment testing three lots of the Sigma ReadyMix™ showed comparable results with the different lots (data not shown).

Assay performance with addition of hematin, an inhibitor of PCR, was evaluated. Hematin is a derivative of heme, which is known to inhibit PCR reactions. Fifty percent inhibition was observed at ~15 μ*M* hematin (data not shown).

The Stratagene Brilliant™ SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA) and the Sigma SYBR® Green JumpStart™ Taq ReadyMix™ kit were comparison tested on a large number of samples. For reasons that are not understood, the concentration values with the Brilliant master mix were much lower than those obtained with the Sigma master mix and much lower than those obtained with the slot blot technique (on average 0.29) (data not shown); thus, further use of the Brilliant kit was discontinued.

Discussion

The real-time *Alu* assay has many advantages over the current slot blot assay and also advantages over the *Alu* plate reader assay. The assay is cost effective, as use of 10-μL reactions results in a test actually costing less than the ABI Quantiblot® kit in our hands. The real-time *Alu* assay is also fast, requiring approximately 0.5 h of setup time, 72 min of PCR amplification (during which the analyst can be performing other tasks), and then the quantitation results are immediately calculated by the real-time instrument software and can be printed out. Also, the dynamic range is larger than the slot blot assay using a colorimetric readout. Lastly, variation of assay conditions such as annealing temperature, primer concentrations, or extension time do not have any major effect on the assay, suggesting it may be robust over the variations seen between instruments or laboratories. Advantages over the plate reader assay include: no need for QSY-labeled primers, no need to move plates back and forth between instruments, use of less reagents, and a greater dynamic range.

A difference was seen between the two different brands of mastermix that were studied, which may be due to stronger effects of inhibitors on the Stratagene mastermix. Since the mixes are proprietory, it is unclear what the cause of the difference might be, although the Stratagene mix does contain dUTP instead of dTTP, while the Sigma kit does not.

While some animal DNAs (especially from dogs and cats) appear to give low-level reaction with the real-time assay (see Table 2), this is several orders of magnitude below the results obtained

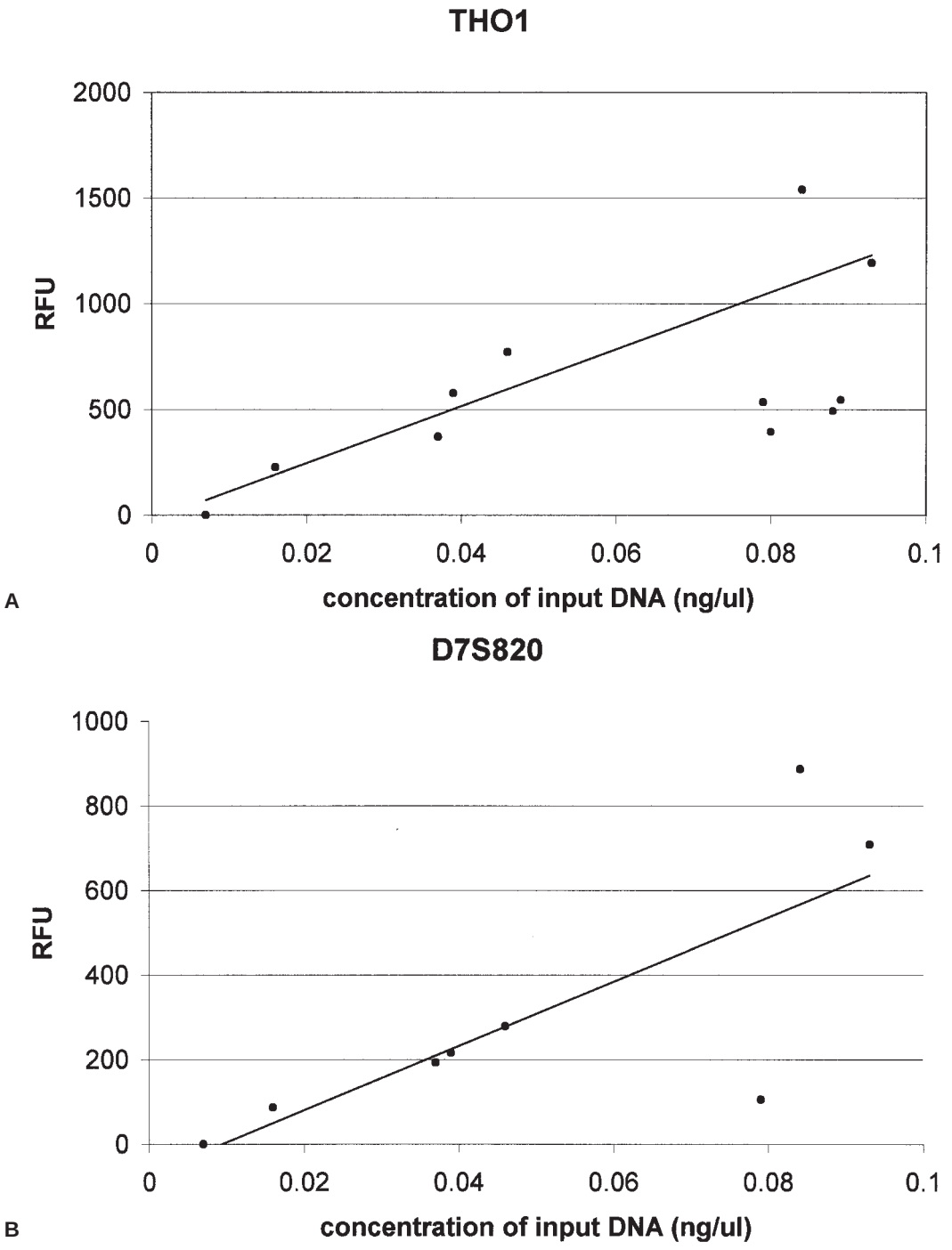

FIG. 6—*STR results for samples with concentrations less than 0.1 ng/μL. Ten microlitres of neat sample were used for COfiler™ analysis. Results for the ~180 bp THO1 product* (a) *and ~270 bp D7S820* (b) *product are shown plotted against the input DNA concentration.*

TABLE 4—*Results of quadruplicate experiment.*

Input DNA	Mean *Ct*	Std. Dev.	%Std Dev.
16 ng	10.05	0.13	1.28
4 ng	11.18	0.09	0.80
1 ng	12.96	0.08	0.59
0.25 ng	15.11	0.17	1.11
0.0625 ng	16.93	0.13	0.79
0.0156 ng	18.98	0.26	1.39
0.0039 ng	20.97	0.20	0.95
0.0010 ng	22.90	0.19	0.81
0ng (NTC)	28.06	0.92	3.28

TABLE 5—*Repeats of seven samples on 3 different days.*

Sample	Mean Concentration	Std. Dev.	%Std. Dev.
Male fraction—J	0.43	0.05	11.36
Female fraction—E	2.35	0.17	7.25
Blood on denim	0.47	0.06	12.33
Swab of blood on leather	0.18	0.02	10.25
Databank 5	0.96	0.15	15.80
Proficiency test 01-02	0.75	0.14	19.29
Envelope seal 2	0.37	0.03	8.87

with human DNA (i.e., 10 ng of some canine DNAs yielded values similar to 2 to 3 pg of human DNA). Of note, even the no template control (NTC) has a *Ct* of ~28. This could be due to primer/dimer, some form of nonspecific amplification, or the presence of extremely low levels of human DNA. The NTC product does have a melting profile essentially identical to the products obtained when 16 ng to 1 pg of human DNA is added, suggesting it is *Alu* product, although we have not sequenced it for confirmation. Since 1 pg gives a *Ct* of ~23 (5 *Cts* higher than the NTC), it can be calculated that the NTC has about 1 pg/2^5 = 0.031 pg/2μL or about 1/100 of a single cell's DNA. This would be very, very low level "contamination." This result compares favorably with the lower detection limit of 17.4 pg/mL (0.036 pg/2 μL) found by Urban et al. when determining template contamination in reagents and equipment by *Alu* PCR (11). That the results observed here were variable from animal sample to animal sample and also occurred in samples from house pets may indicate that either house pets pick up human DNA from their owners or that the owners contaminated the samples when they were taken. These results indicate the extreme sensitivity of the assay at low levels; if 4 pg and 20 cycles were to be used as an assay cutoff, this low level "contamination" would not be observed.

The real-time *Alu* assay has shown it can reproducibly determine the amount of human DNA in a wide range of samples. Standard deviations for *Ct* values were very low for the quadruplicate experiment. These values compare to the quoted 0.05 in the instrument brochure and values of 0.12 to 0.28 from other sources (18–20). For concentration values, Bustin reported variability of between 0 and 5% between different runs and %CV differences of 20 to 30% between different kits or probe lots on concentration values (14,21). In terms of the Quantiblot™ assay, in our hands for multiple dilutions on the same sample values are off by 0 to 30% between the two dilutions. Certainly, a 20% difference in concentration is well within the tolerance of the STR amplification in order to obtain reliable amplification (1–3); thus, the assay is sufficiently robust to generate successful STR analysis.

Because both the *Alu* assay and STR analyses are PCR based, the *Alu* assay should have better predictive value for STR success than the slot blot assay. This was shown in the results with DNAseI-treated DNA where the assay was very good at predicting amplification of the similarly sized THO1 but not as good at predicting amplification of the larger D7S820. Amplification based on the slot blot results would have fared no better as it underestimated the amount of DNA needed for proper STR amplification. High levels of PCR inhibitors will clearly effect the quantitation of the human DNA present even with added BSA; however, once again the PCR-based *Alu* assay should be more reflective of STR amplification than the slot blot. In addition, clues to the presence of an inhibitor are visible. First, the shape of the amplification curve is altered. It is flatter and never reaches the plateau seen in the standard curve (data not shown). Second, each sample, even if it contains no DNA, should, at least, display a curve similar to the no template control (amplify greater than or equal to the NTC). Samples that do not amplify at all would indicate the presence of an inhibitor and the need to purify the sample to avoid a probable STR amplification failure. Lastly, the baseline value in the raw data will be high if a high concentration of DNA is present but it does not amplify.

There are possibilities for further improvement of the assay including use of a molecular beacon type assay. Use of a molecular beacon should give a much larger effective concentration range because the problem of binding all of the SYBR® Green I will be avoided. 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.

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