J Forensic Sci, Sept. 2005, Vol. 50, No. 5
Paper ID JFS2004514
Available online at: www.astm.org

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An *Alu*-based, MGB Eclipse™ Real-Time PCR Method for Quantitation of Human DNA in Forensic Samples*

ABSTRACT: The forensic community needs quick, reliable methods to quantitate human DNA in crime scene samples to replace the laborious and imprecise slot blot method. A real-time PCR based method has the possibility of allowing development of a faster and more quantitative assay. *Alu* sequences are primate-specific and are found in many copies in the human genome, making these sequences an excellent target or marker for human DNA. This paper describes the development of a real-time *Alu* sequence-based assay using MGB Eclipse™ primers and probes. The advantages of this assay are simplicity, speed, less hands-on-time and automated quantitation, as well as a large dynamic range (128 ng/μL to 0.5 pg/μL).

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

DNA isolated from crime scene samples must be quantitated to determine the amount of human DNA present. Recently the forensic community has become interested in developing faster, cheaper and more quantitative methods than the widely used slot blot method utilizing a D17Z1 probe. Real-time PCR methods have the advantages of being fast and quantitative with much less hands-on time than slot blot methods. Thus, development of quantitative PCR methods to measure the amount of human DNA in the forensic setting is a logical progression. The choice of possible genomic sequences for this quantification is limited only by the obvious requirement that the sequence be human or at least, primate specific.

Several groups have developed real-time human DNA quantitation assays based on the amelogenin (*AMEL*) locus. Syn et al. (1) developed a SYBR® Green assay for the *AMEL* locus with a range of 0.25 ng to 200 ng. Alonso et al. (2) have developed a multiplex real-time TaqMan® method to detect both the X (FAM labeled) and Y linked (VIC labeled) *AMEL* gene copies, to perform both human DNA quantitation (60 pg to 10 ng) and sex determination. Andreasson and Allen (3) also report development of an *AMEL*-based quantitation (30 pg to 32 ng) and gender assay based on SYBR® Green real-time PCR and dissociation (melting curve) methodology. Quantification was by SYBR® Green fluorescence of the PCR product but gender was determined by melting differences of the 73 bp male versus 70 bp female product, respectively. The problems with these assays are that deletions for the Y-linked gene occur at appreciable frequency in some populations (4) which can decrease quantitation by 50% and confound gender determination.

Several other groups have used other single copy genes for development of quantification assays. The BODE Technology Group (5) has developed a method based on amplification of the *THO1* locus followed by Picogreen® staining and plate reader detection (0.2 ng to 40 ng). Richard et al. (6) have also developed an assay based on *THO1* but using a TaqMan®-based assay (5 pg to 10 ng). Haque et al. (7) have also developed a TaqMan®-based real-time assay using the human *BRCA1* locus (1 ng to 10 ng). In comparisons to spectrophotometric and fluorometric methods, the *BRCA1* method could detect lower amounts of human DNA but results were skewed by a number of samples giving zero values indicating possible PCR inhibition. Andréasson et al. (8) recently report the use of real-time PCR to detect human nuclear and mitochondrial DNA. They used a unique copy sequence from the retinoblastoma gene (*RB1*) for a TaqMan® assay (30 pg to 32 ng). However, the assay was not tested against non-human DNA; a BLAST search of their probe and primers suggests cross-reactivity to sequences in other animals such as cat or horse could be a problem. Applied Biosystems (Foster City, CA) has recently announced their new Quantifiler™ Human DNA Quantitation Kit based on the *TERT* gene (23 pg to 50 ng). This assay has an internal positive control to ascertain PCR amplification.

A sequence present in multiple copies would allow quantitation using less DNA template or more dilute samples. Individuals may have differences in sequence copy numbers but with high numbers of widely disseminated repeated sequences this should not be a significant problem. These differences should be masked by day to day assay fluctuations and differences in DNA quality between samples. The greater concern is in assays utilizing a single copy gene where deletion of one copy creates a 50% difference in quantitation. This is exemplified by the *AMEL* gene as discussed above.

Alu sequences may be ideal candidates for human DNA quantitation using a multicopy sequence. They are primate-specific and 500,000 to 1,000,000 copies exist in the human genome (6–13% of the haploid genome) (9,10). 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

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* This work was 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 of the U.S. Department of Justice.

Received 17 Dec. 2004; and in revised form 21 April 2005; accepted 23 April 2005; published 3 Aug. 2005.

assays to detect human DNA. An *Alu* PCR based system (11) has been used to quantitate human genomic DNA (2.5 pg 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). Urban et al. (12), used PCR of *Alu* sequences to detect template contamination (50 pg/ml-200 pg/ml). While Promega (Madison, WI) manufactures a human DNA quantitation system called the AluQuant™ Human DNA Quantitation System based on Read-It™ technology (13), this kit detects a human centromeric repeat and not an *Alu* sequence. Walker et al. (14) performed both inter and intra *Alu*SYBR® Green based real-time PCR. Intra-*Alu* Yb8 based PCR was human specific (10 ng to 1 pg).

We have developed two human DNA quantitation assays utilizing PCR of the Ya5 family of *Alu* sequence and detection with the DNA dye SYBR® Green I (Molecular Probes, Eugene, OR) (15,16). The first assay (15) uses limited DNA cycles and detection in a fluorescence plate reader. The second assay uses a real-time method which we developed for casework use (16). These methods have limits in concentration range (only up to 8 ng/μL sample) because of plateau effects probably due to binding of all available SYBR® Green I. While increasing the upper end of the range is not critical as samples can be diluted and re-run, the ability to use all samples undiluted does save time. With the SYBR® Green assay, samples are routinely diluted 1/20 before testing. Increasing sensitivity at the lower end is not needed as 0.5 pg/μL is below a usable value in terms of short tandem repeat (STR) analysis.

PCR quantitation methods that use probes (oligonucleotides with fluorescent dyes attached) have the advantage that a greater dynamic range can be achieved. Molecular beacons are oligonucleotide probes with a fluorescent dye and a quencher dye, as well as a short complementary sequence that can form a hairpin (17). When the beacon is in solution, the fluorescent dye and quencher are in close proximity due to the hairpin; however, when the beacon binds to its complementary sequence on the PCR product, the fluorescence of the fluor is released. This reaction is quantitative, the more PCR product, the more beacon molecules bind yielding increased fluorescence. MGB Eclipse™ (Epoch Biosciences, Bothell, WA) probes are a form of molecular beacon that have, in addition to a 3′ fluor and 5′ quencher, a minor groove binder (MGB) entity on the 5′ end which assists in binding of the probe to the PCR product (18). This paper describes the development and optimization of a MGB Eclipse™ assay for the quantitation of human DNA. This assay is fast, inexpensive and has a sample concentration range of 128 ng/μL to 0.5 pg/μL.

Materials and Methods

DNA Samples

Most experiments were performed with a human DNA standard (catalog number G3041) purchased from Promega (Madison, WI). DNA was isolated from a number of samples to evaluate the assay: blood spots from five DNA databank samples (bloodspots on FTA paper, Whatman), five samples from control blood spotted on denim or colored cloth, control blood placed on five surfaces (stick, leather, metal, cardboard, soapy cloth), a control blood spot placed in the dark for three months, two male and two female fractions from sexual assault cases, and two samples from a proficiency test. All DNA samples including those from FTA paper were isolated using an organic extraction method (19) as modified in Akane et al. (20).

Animal DNAs

The organic extraction method detailed above was used to isolate animal DNAs (cat, chicken, cow, deer, dog, horse, moose, mouse, pig, rabbit) from samples of blood on paper or cloth (from veterinary samples or from a game warden) or buccal swabs of pets belonging to laboratory personnel. DNA from *Drosophila* was isolated by the above method using squashed whole flies and that from yeast using dried baker's yeast. Rat DNA was obtained from Dr. Richard Branda, University of Vermont. DNAs from *Clostridium*, *E. coli* and *Micrococcus* were purchased from Sigma (St. Louis, MO). DNA from chimpanzee, macaque, gorilla, baboon and marmoset were purchased from BIOS (New Haven, CT). Herring sperm DNA was purchased from Gibco/BRL (Rockville, MD). Blood samples from cats and dogs were obtained from Steven Metz, DVM.

Eclipse™ Primers/Probe

The *Alu* PCR primers 5′-GAG ATC GAG ACC ATC CCG GCT AAA-3′ (forward) and 5′-CTC AGC CTC CCA AGT AGC TG-3′ (reverse) and the Eclipse™ probe 5′-GGG CGT AGT GGC GGG-3′ (FAM fluor-Eclipse™ quencher) were designed by and purchased from Synthetic Genetics (now Epoch Biosciences, Bothell,WA) from the sequence of plasmid p*PD39* (Ya5 subfamily) (21). These PCR primers generate a 113 bp product that was confirmed on an agarose gel (data not shown).

PCR Assay

PCR utilized JumpStart™ Taq DNA polymerase (Sigma) and the Eclipse™ 10X buffer (Epoch Biosciences). The 10 μL reactions contained 1 μL of Eclipse™ 10X buffer (1X final), 0.5 μL of primer mix (0.1 μM forward, 1 μM reverse), 0.5 μL probe, 0.4 μL of 6.25 mg/mL BSA (A-9647, Sigma), 1.6 μL of 2.5 mM each dNTP (Applied Biosystems), 0.08 μL JumpStart™ Taq DNA polymerase (D4184, Sigma, 2.5 U/μL), 3.92 μL distilled H_20 and 2 μL of TE (10 mM Tris, pH 7.5, 0.1 mM EDTA) containing the input DNA at various concentrations. Some initial optimization experiments varied the concentrations of components such as Taq polymerase, primer or probe. Some experiments tested pre-made mastermixes [Jumpstart™ Taq ReadyMix™ polymerase (P2893, Sigma), Platinum® PCR SuperMix (11306-016, Invitrogen, Carlsbad, CA), HotMasterMix (954 14 018-1, Eppendorf, Hamburg, Germany)].

Real-time PCR for the *Alu* assay was performed in a Corbett Research Rotorgene (formerly distributed in the USA by Phenix Research but now distributed by Biotage, Charlottesville, VA) using the small 0.1 ml tubes. PCR consisted of 95°C for 2 min ("hotstart") followed by 40 cycles of 95°C for 15 sec, 56°C for 20 sec, 72°C for 30 sec. 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 one cycle of 95°C for 15 sec, 56°C for 20 sec followed by a ramp up of 1 degree/step with 5 sec hold at each step.

Short Tandem Repeat (STR) Analyses

The AmpF*l*STR® CO filer™ kit (Applied Biosystems) amplifies 6 autosomal tetranucleotide repeats plus the X and Y linked *AMEL* loci using dye labeled primers. These loci are routinely used in forensic analysis and are part of the 13 loci FBI National Database Combined DNA Index System (CODIS) set. The kit was used

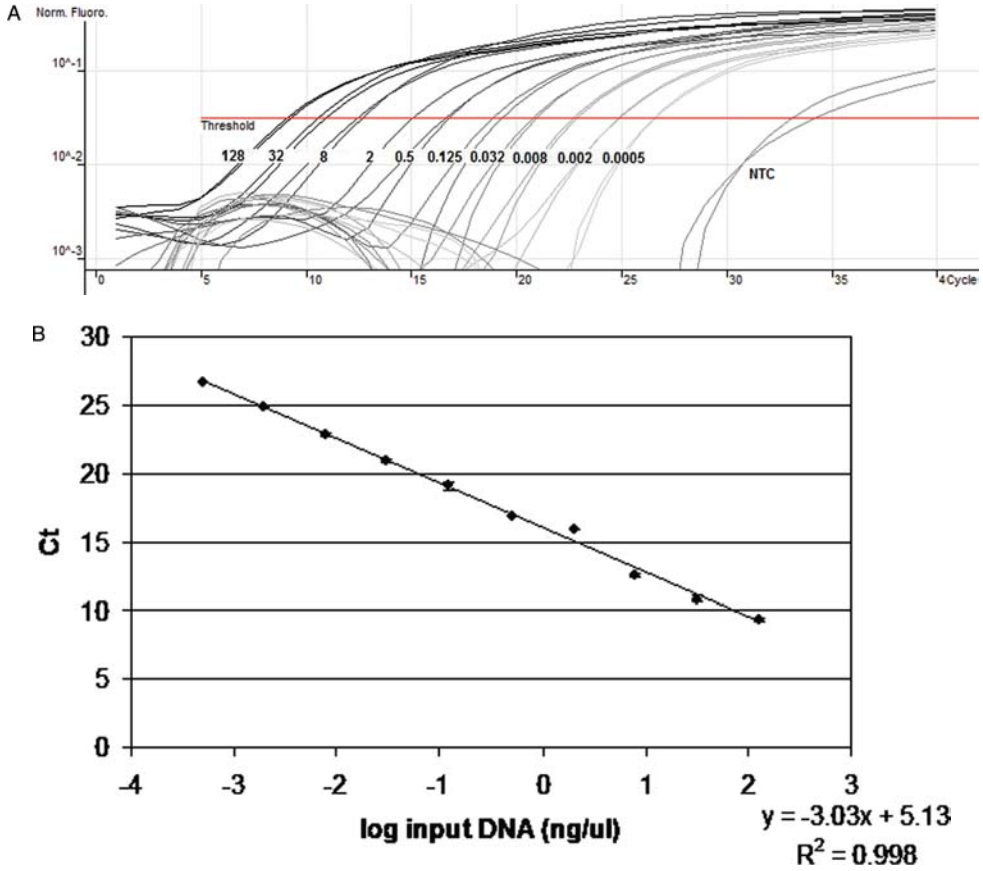

FIG. 1—*A) Eclipse™* Alu *real-time PCR assay results for standard curve dilution series of human DNA. Normalized RFU are plotted versus cycle number for 11 serial 1:4 dilutions of human DNA (128 ng/μL, 32 ng/μL, 8 ng/μL, 2 ng/μL, 0.5 ng/μL, 0.125 ng/μL, 0.032 ng/μL, 0.008 ng/μL, 0.002 ng/μL, 0.0005 ng/μL) plus a no template control (NTC). B) Using the Ct values at a threshold of 0.02 generates a standard curve ($R^2 = 0.999$). The slope of the standard curve ($m = -3.31$) indicates the reaction has an efficiency of 100%.*

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 polymerase and 10 μL of sample DNA at a concentration of 0.1 ng/μL) for STR analyses. After amplification on an Applied Biosystems GeneAmp 9600 thermal cycler according to manufacturer's recommendations, products were analyzed on an Applied Biosystems 310 Genetic Analyzer using collection software 1.2.2, Genescan® software 3.1 and GenoTyper® software 2.5 (Applied Biosystems).

Mixing Experiments

Various concentrations of rat DNA (eight 1/4 dilutions from 10 ng/μL to 0.025 ng/μL) were added to a constant concentration of human DNA (1 ng/μL). PCR was performed on this series of DNAs using 2 μL of the mixed DNAs in each 8 μL reaction.

Results

Development of Assay

PCR using the Eclipse™ primers and the conditions suggested in the Eclipse™ instruction manual gave a product of the expected size (113 bp) (data not shown). Real-time PCR using the Eclipse™ probe resulted in consistent Ct values for duplicates and large dynamic range (128 ng/μL to 0.5 pg/μL) on standard curves (Fig. 1*A* and 1*B*). The threshold of 0.031 was chosen by the Rotorgene 3000 software (v5). As with the SYBR® Green assay (16), the no template control (NTC) had a Ct of ~31, presumably because of a very low amount of ambient human DNA in the air and water (see Discussion).

The next studies attempted to improve the assay (save on reagents, shorten experiment running, increase specificity) by varying the DNA range in the standard curve, annealing time and temperature, extension time, polymerase concentration and primer concentration. The first studies focused on changing the annealing temperature. Annealing temperatures, of 52°C, 54°C, 56°C, 60°C, 64°C and 68°C were compared. In the SYBR® Green assay increased annealing temperature caused a narrowing of the melting peak, probably due to the fact that *Alu*s are a family of related sequences; lower annealing temperatures allow amplification of slightly more distantly related species that, thus, will generate a broader melting curve. In contrast, using just the Eclipse™ primers in a SYBR® Green based assay (no probe added) gave essentially equivalent amplification and melting curve profiles (87°C peak) with all of the annealing temperatures tested (data not shown). This is possibly due to increased specificity of the Eclipse™ primers. However, when the Eclipse™ probe was used in a full Eclipse™ assay, each increase in the annealing temperature decreased the RFU until the reaction essentially failed at 68°C (Fig. 2). Increasing the annealing temperature had no effect on the melting curve of the Eclipse™ product (data not shown). Differences in the mechanism of the two assays perhaps account for these findings; there is melting of the entire PCR product in the SYBR® Green assay whereas in the Eclipse™ assay, only melting of the probe with the PCR product is measured. Furthermore, the minor groove binder (MGB) may also affect the melting. The manufacturer's recommended

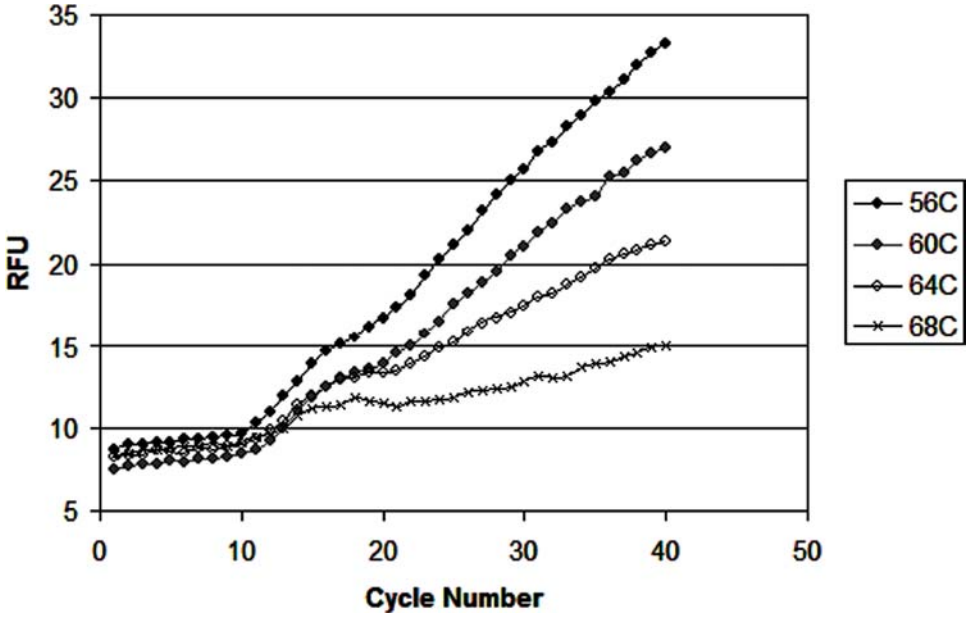

FIG. 2—*Raw RFU data for 32 ng/μL input DNA samples plotted versus cycle number for Eclipse™ real-time PCR assays performed at different anneal temperatures (56°C, 60°C, 64°C, 68°C). RFU decrease markedly with increased anneal temperature.*

56°C annealing temperature was chosen for the final assay because increasing the temperature did not enhance the specificity (SYBR® Green-based primer study) but did cause RFUs to decline. However, the annealing temperature probably could be increased to 60°C without any detriment to the assay and a possible saving in a few minutes in assay length.

A denaturation time of 15 sec, an annealing time of 20 sec and an extension time of 30 sec were initially used for the assay as per vendor recommendations. Experiments were performed comparing annealing times of 10, 20 and 40 sec. Little difference in Ct was observed between the annealing times (data not shown); therefore, the recommended 20 sec was chosen for the final assay. Experiments comparing extension times of 15, 30 and 60 sec (data not shown) revealed a slight decrease of Ct and plateau RFU with decreased extension time; again the 30 second recommended time was chosen for the final assay.

Absolute amounts of primer and probe were varied to determine optimal concentrations. Probe concentrations of 50 nM, 100 nM, and 200 nM and primer concentrations (forward:reverse) of 25 nM:250 nM, 50 nM:500 nM and 100 nM:1000 nM were independently varied for a single fixed DNA concentration (2 ng/μL). For a fixed probe concentration, as the primer concentration decreased, the Ct increased about 0.8 cycles from the highest to lowest primer concentration. For a fixed primer concentration, as the probe concentration increased, the Ct also increased about 0.4 cycles. The first is understandable as decreased primer could slow the reaction and thus increase the Ct. Why the decrease in probe causes a decrease in Ct is not so clear. The probe does come in a buffer; it is possible that this buffer may have an effect on the amplification independent of the effect of probe. This was a reproducible effect as it was observed in an additional experiment that tested the probe at three concentrations (100 nM, 200 nM, 400 nM) over a standard curve DNA dilution series. The Ct values for the 400 nM probe were on average 0.80 more than with the 200 nM probe which was on average 0.40 greater than the average for the 100 nM probe. Since the Ct increased substantially with the 400 nM probe, the recommended 200 nM probe was chosen for the final assay.

When the original primer mix was obtained from Synthetic Genetics (now Epoch Biosciences), it came as a 20X 1:10 mix (2 μM forward primer: 20 μM reverse primer). However, separately packaged primers were also obtained and tested in ratios of 0.05 μM : 1 μM, 0.1 μM : 1 μM, 0.2 μM : 1 μM, 0.5 μM : 1 μM and 1 μM : 1 μM final concentration (Fig. 3). Figure 3 shows that the RFU decrease with increased forward primer concentration. This is to be expected as extension off the forward primer will displace the Eclipse™ probe from the PCR product, reducing signal or possibly the increased primer sequesters Mg^{++}, reducing polymerase efficiency. However, the Ct values remained fairly stable across the different ratios (data not shown). The recommended 1:10 ratio was therefore chosen for the assay. Of note, currently Epoch Biosciences now ships primer mixes as a 20X 1:1 mix. An experiment comparing the 1:1 ratio versus the 1:10 ratio on eight samples was therefore performed. While the raw data had slightly different characteristics and the Ct values were perhaps slightly lower with 1:1 primer ratio, the R^2 values for the standard curves and the PCR efficiencies were identical (data not shown). The differences in calculated concentrations at 1:10 and 1:1 for the 8 samples (Table 1) were within replicate and day to day variation [compare to Tables 5*b* and Table 6 below, respectively].

TABLE 1—*Differences in Eclipse™* Alu *assay results (ng/μL) for eight samples using different ratios of forward to reverse primer [1:1 (1 μM: 1 μM) versus 1:10 (0.1 μM:1 μM)]*

Sample	1:1 F:R Primer Ratio	1:10 F:R Primer Ratio
Databank #1	0.83	0.91
Databank #4	2.97	6.01
Databank #5	4.15	5.19
Female fraction J	0.54	0.45
Quantiblot™ StdA	3.77	3.14
Quantiblot™ Cal2	0.09	0.10
Blood, 3 mo dark	2.37	1.21
ABI 9947A	0.14	0.11

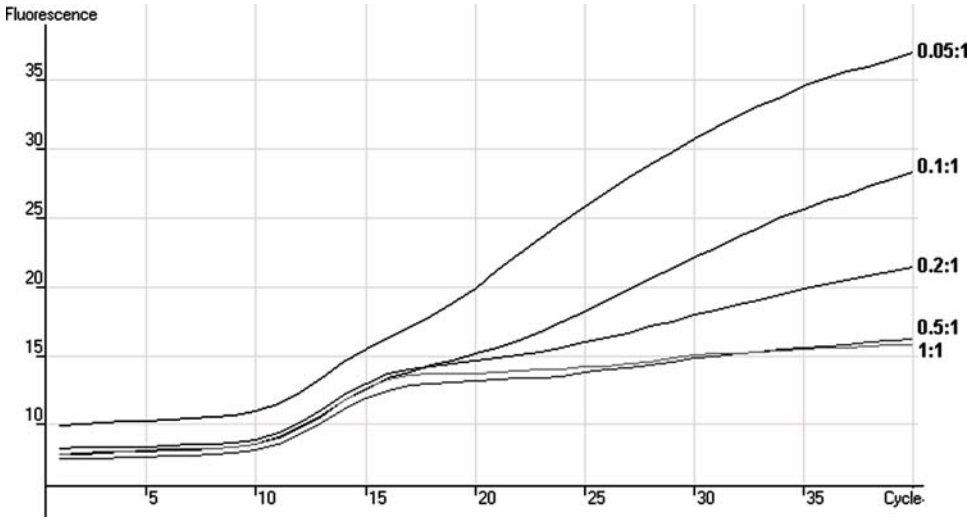

FIG. 3—*Raw RFU data for 2 ng/μL input human DNA plotted versus cycle number for Eclipse™ real-time PCR assays performed at different ratios of forward to reverse primer (0.05 μM:1 μM, 0.1 μM:1 μM, 0.2 μM:1 μM, 0.5 μM:1 μM, 1 μM:1 μM). RFU values decrease as the amount of forward primer is increased.*

TABLE 2—*Differences in Eclipse™ assay results (ng/μl) for 11 samples using varying amounts of Taq polymerase.*

Sample	0.4 U Taq/10 μL Reaction	0.2 U Taq/10 μL Reaction
Denim #1	2.07	2.96
Denim #2	0.33	0.36
Denim #3	11.8	7.8
Cloth #1	0.67	0.50
Cloth #2	0.79	0.88
Blood on soapy cloth	0.07	0.08
Blood on stick	0.77	0.71
Blood on leather	0.10	0.15
Blood on metal	0.78	0.89
Blood on cardboard	0.39	0.57
Female fraction J	0.68	0.73
Blood, 3 mo dark	0.76	1.33
Databank #1	0.45	0.89
Databank #2	0.96	0.75

In general, the above experiments showed that Epoch Biosciences suggested conditions were essentially optimal. While the experiments indicated that less probe or primer or shorter cycle times could be utilized in our laboratory with our thermal cycler, since the intent was to develop this assay for a wide audience, we chose to be conservative and pick temperatures and concentrations far from any values where the assay deteriorated, i.e., values that would be robust in any laboratory.

One important deviation from Epoch Bioscience's suggested protocol that was determined from experimental results was the use of a lower Taq concentration. The original methods received from Synthetic Genetics suggested using 0.4 U per 10 μL reaction; however, more recent directions from Epoch Biosciences suggested using 0.8 U per 10 μL reaction. Initial experiments performed here used 0.4 U/10 μL reaction; however, experiments were performed to titrate down the amount of enzyme for cost considerations. Initial experiments showed paradoxically that Ct decreased about 0.6 cycles as Taq polymerase concentration was lowered from 0.4 U/10 μL reaction to 0.05 U/10 μL reaction (data not shown). An experiment was performed to determine if Taq polymerase concentration affected the results with difficult samples (blood on denim or degraded DNA). Table 2 shows results of parallel experiments using 14 samples at 0.4 U/10 μL reaction or 0.2 U/10 μL reaction. The variations seen in Table 2 between the two concentrations were within replicate and day to day variation of the assay [see below (Tables 5*b* and Table 6, respectively)]. The higher Taq polymerase concentration did not result in higher determined concentrations for the difficult samples. Thus, 0.2 U/10 μL reaction was chosen as the assay concentration because Taq is the major cost consideration for the assay and less Taq actually seemed to decrease Ct.

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 one yeast were evaluated using approximately 5 ng/μL DNA of each DNA (Table 3). The primates gave the expected positive result while the others all yielded Ct values ranging between that obtained for the NTC to that observed for the 0.5 pg/μL human DNA sample. Table 3 shows the concentration of human DNA that would give the observed Ct based on the standard curve.

Another important point is to show that non-primate DNA does not interfere with detection of human DNA. A mixing experiment of rat and human DNA (Fig. 4) was performed (triplicates) where each well contained the same concentration of human DNA (1.0 ng/μL) but with different concentrations of rat DNA from 10 ng/μL to 0.0 ng/μL (8 fourfold dilutions from 10 ng/μL to 0.025 ng/μL plus no added rat DNA). This figure shows that the measured concentration of human DNA is constant (within well-to well deviation) as the amount of rat DNA increases. Thus, the rat DNA neither contributed to the final reading nor inhibited the PCR reaction.

The new real-time Eclipse™ assay was compared to the SYBR® Green assay on a wide variety of samples types (Table 4 and Fig. 5). Results were quite similar for both assays except for two datapoints (blood on stick and denim#2). Repeats of the blood on stick samples brought the two values much closer, 0.51 ng/μL and 0.63 ng/μL,

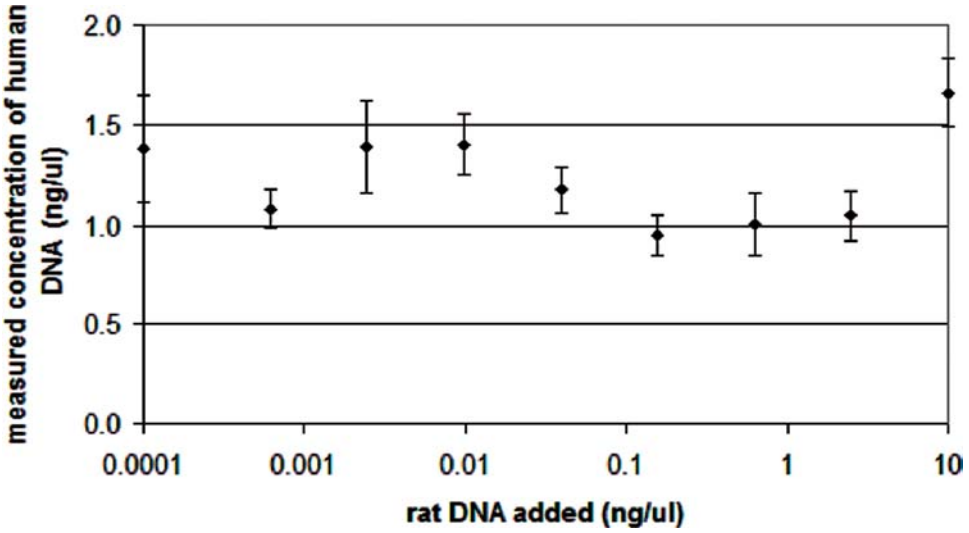

FIG. 4—*Rat and human DNA mixture Eclipse™ real-time PCR assay. Plot of measured amount of human DNA versus the input rat DNA. All assays contained the same 1.0 ng/μL of human DNA but with different concentrations of added rat DNA (0.0 ng/μL, 0.025 ng/μL, 0.01 ng/μL, 0.04 ng/μL, 0.15 ng/μL, 0.63 ng/μL, 2.5 ng/μL, 10 ng/μL). Error bars for triplicates are shown.*

TABLE 3—*Eclipse™ assay results with animal DNAs*

Animal DNA	Apparent Amount (ng/μL)
Primates	
Baboon	0.11
Chimp	0.044
Macaque	0.18
Non-primates	
Cat	0.0001
Chicken	0.0003
Clostridium	0.0001
Cow	0.0001
Deer	0.0001
Dog	0.0002
Drosophila	0.0000
E. coli	0.0001
Herring	0.0000
Horse	0.0002
Micrococcus	0.0000
Moose	0.0000
Mouse	0.0006
Pig	0.0012
Rabbit	0.0001
Rat	0.0001
Yeast	0.0000

TABLE 4—*Comparison of SYBR™ Green assay and Eclipse™ assay concentration results for samples and results of STR analysis with the CO filer™ kit. DNA dilutions for the STR analysis based on Eclipse™ assay concentration results.*

Sample	SYBR® Green *Alu* Assay Result (ng/μL)	Eclipse™ *Alu* Assay Result (ng/μL)	CO Filer™ *THO1* Peak Height	CO Filer™ D7S180 Peak Height
Databank #1	0.57	0.48	1379	703
Databank #2	0.89	0.91	(1341)[a]	462
Databank #3	0.87	1.17	(2441)[a]	1091
Databank #4	2.09	1.62	2390	901
Databank #5	2.31	2.43	1317	771
Proficiency #1	0.18	0.15	1635	(957)[a]
Proficiency #2	0.96	1.19	1523	(770)[a]
Female fraction F	0.22	0.32	1352	763
Female fraction J	0.69	0.65	1543	845
Male fraction D	0.053	0.055	724	427
Male fraction 01-6-1	0.046	0.039	not done[b]	not done[b]
Blood, 3 mo dark	0.73	0.79	1175	(462)[a]
Blood on stick	0.31, 0.51	0.78, 0.63	757	339
Blood on metal	not done[b]	0.65	1074	502
Denim #2	1.46, 2.18	4.21, 6.33	2576	1958
Cloth #1	0.47	0.55	928	681

[a] Value is half of homozygous peak.
[b] Insufficient sample.

respectively, suggesting that the original values were both just statistical extremes in opposite directions. For the denim#2 sample, repeats increased both concentration determinations (2.18 ng/μL and 6.33 ng/μL, respectively). Examination of the raw data curves showed that the curve shapes were flat, a symptom of the presence of an inhibitor which explains the inconsistent results and differences between the assays for this sample.

Fifteen of the 16 above samples (the male fraction 01-6-1 sample had insufficient DNA) were analyzed with the CO filer™ STR kit. Fourteen samples were diluted to 0.1 ng/μL and 10 μL (1.0 ng total) was used for CO filer™ STR analysis. One sample, male fraction D, was used neat because the concentration was below 0.1 ng/μL. All of these samples gave results on the ABI 310 within the laboratory's acceptable range (150-5500 RFU for each heterozygous peak) and Table 4 shows the results for two selected loci representing small and large amplicons (*THO1* and D7S820, respectively).

The question arises as to the lowest DNA concentration (as determined by the Eclipse™ assay) that will allow generation of a complete STR profile. Establishing this cutoff concentration would allow a laboratory to cease testing on those samples containing insufficient DNA to reasonably expect generation of a complete or near complete STR profile with peak heights within the acceptable RFU range (i.e., suitable for inclusion into CODIS). In other cases, where a complete profile may not be necessary, such as suspect exclusion, knowing this level would help with an expectation of results, decisions as to whether to switch to mtDNA testing or perhaps triage of multiple samples from one crime scene. To determine this cutoff concentration, six samples of approximately

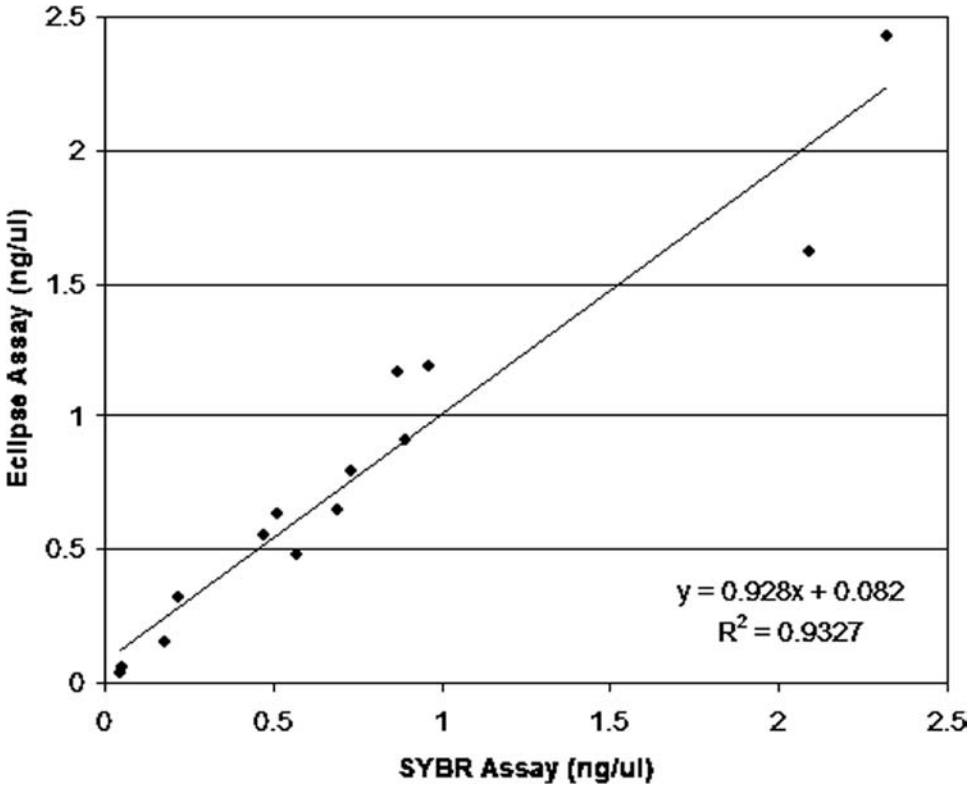

FIG. 5—*Graph of the comparison of Eclipse™* Alu *and SYBR® Green* Alu *assays from Table 4. The Eclipse™* Alu *assay results for 15 samples (denim #2 is not shown) plotted against the results for the SYBR® Green assay. The slope of the trend line is approximately 1 and the* R^2 *value is 0.93.*

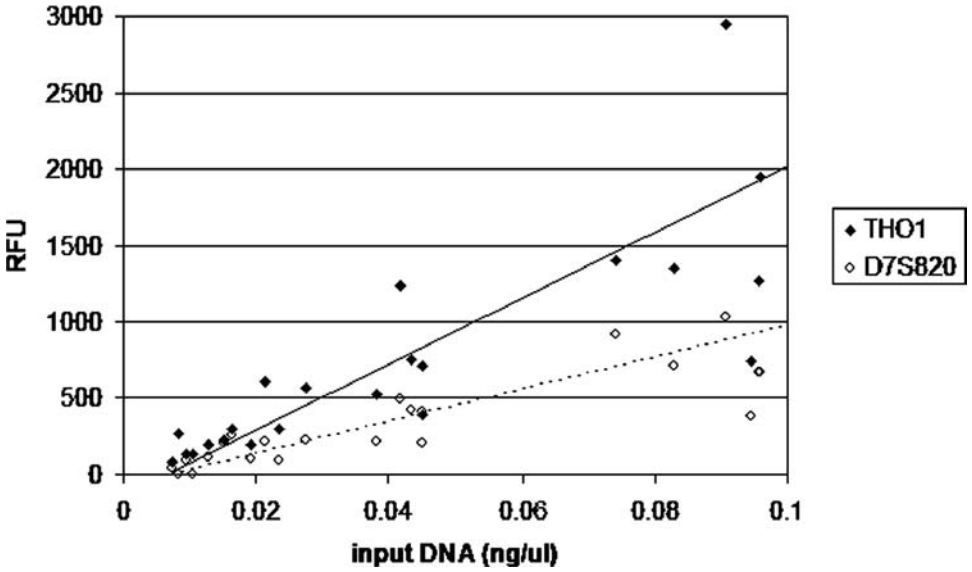

FIG. 6—*Graph of CO filer™ STR RFU versus input DNA as determined by the Eclipse™* Alu *assay. Six DNA samples were serially diluted 1:1 and the Eclipse* Alu *assay performed on all dilutions to determine DNA concentration. CO filer™ analysis was performed on neat samples. The DNA samples were isolated from three convicted offender FTA blood spots, one commercial mixture, a buccal swab and one aged blood sample.*

0.1 ng/μL were serially diluted (1:1) three times. The Eclipse™ assay was then performed on all the samples and the serial 1:1 dilutions to determine the concentration of each. STR analysis (CO filer) was also performed on each sample and dilution (all neat). The RFU results for *THO1* and D7S820 were graphed versus the input DNA concentration to determine what the lowest input concentration was (as measured by the Eclipse™ assay) that would allow successful STR analysis (Fig. 6). As can be seen, RFUs decrease linearly as input DNA decreases below 0.1 ng/μL (the recommended input DNA concentration) with RFUs lower as expected for the larger D7S820 product (~255–295 bp) than the smaller THO1 product (~165–190 bp).

An experiment was also performed to check on the amplification of a number of reagent blanks from casework. These should amplify in the range of the NTC (Ct ≈ 32) or at least below 0.5 pg/μL (Ct ≈ 25). Ten reagent blanks were tested; they had Ct values of

TABLE 5*a*—*Triplicate Eclipse™ assay standard curve Ct results.*

Input DNA	Run 1	Run 2	Run 3	Mean	st. dev.	% st. dev.
128 ng/μL	7.66	7.70	7.59	7.65	0.06	0.73
32 ng/μL	8.88	9.29	9.01	9.06	0.21	2.31
8 ng/μL	10.85	10.87	10.95	10.89	0.05	0.49
2 ng/μL	12.92	13.10	13.19	13.07	0.14	1.05
0.5 ng/μL	14.93	15.03	14.84	14.93	0.10	0.64
0.125 ng/μL	16.67	16.80	16.18	16.55	0.33	1.98
0.0312 ng/μL	18.68	18.73	18.65	18.69	0.04	0.22
0.0078 ng/μL	20.63	20.71	20.28	20.54	0.23	1.11

TABLE 5*b*—*Triplicate Eclipse™ assay sample concentration (ng/μL) results.*

Sample	Replicate 1	Replicate 2	Replicate 3	Mean	st. dev.	% st. dev.
Databank #1	0.62	0.47	0.42	0.50	0.10	20.12
Databank #4	2.25	2.46	3.52	2.74	0.68	24.77
Databank #5	3.21	2.08	2.51	2.60	0.57	21.90
Female fraction J	0.54	0.55	0.51	0.53	0.02	3.57
Quantiblot™ StdA	2.22	2.16	1.97	2.11	0.13	6.12
Quantiblot™ Cal2	0.09	0.08	0.08	0.08	0.01	8.94
Blood, 3 mo dark	0.99	0.99	1.03	1.00	0.02	2.44
ABI 9947A	0.11	0.12	0.08	0.10	0.02	19.16

TABLE 6—*Eclipse™ assay concentration (ng/μL) results on eight samples over five days compared to SYBR® Green assay results over three days.*

	Eclipse™ Assay			SYBR® Green Assay		
Sample	Mean	st. dev.	% st. dev.	Mean	st. dev.	% st. dev.
Databank #1	0.44	0.05	11.3	0.60	0.09	15.1
Databank #4	1.76	0.43	24.2	2.52	0.45	17.6
Databank #5	2.63	0.30	11.2	2.74	0.56	20.2
Female fraction J	0.54	0.08	14.4	0.66	0.04	5.8
Quantiblot™ StdA	1.87	0.25	13.5	2.78	0.58	20.9
Quantiblot™ Cal2	0.08	0.01	9.2	0.09	0.02	21.1
Blood, 3 mo dark	0.73	0.17	22.6	1.06	0.05	4.1
Cloth#1	0.48	0.06	13.1	0.49	0.04	8.6

25.21 to 37.11 which gave concentrations of 0.0000 ng/μL to 0.0005 ng/μL.

The reproducibility of the assay was also investigated in several ways. Triplicates of the standard curve performed on the same day had average percent standard deviations for the Ct values of less than 2.5% (Table 5*a*). Triplicates of concentration values for eight samples performed on the same day are shown in Table 5*b* (percent standard deviations of 3.5 to 24%). Eight samples repeated five times over different days had percent standard deviations for the concentration values from 9% to 24% (Table 6). These same 8 samples were repeated 3 times with the SYBR® Green assay (Table 6) and the percent deviations ranged from 4% to 21%. Thus, the two assays are generally similar in terms of reproducibility.

Test of Commercial Mastermixes

Use of a pre-made mastermix would simplify assay set up; therefore, the performance of several pre-made mastermixes were directly compared on a standard curve dilution series. The "homemade" mix resulted in an RFU of ~40 (for 32 ng/μL input DNA), a reaction efficiency of 1.00 and an R value of 0.998. The Invitrogen Platinum® PCR SuperMix gave a low RFU of 15, a reaction efficiency of 0.69 and a low R value of 0.784. The Sigma Jumpstart™ ReadyMix™ Taq polymerase gave an RFU of 30, a reaction efficiency of 0.98 and an R value of 0.998. Lastly, the Eppendorf® HotMasterMix gave an RFU of 20, a reaction efficiency of 0.98 and an R value of 0.998. The latter two mixes would probably be acceptable although the "homemade" mix appears to perform slightly better based on the RFU.

Discussion

The main advantage of the MGB Eclipse™ real-time *Alu* assay over the SYBR® Green *Alu* assay is its much greater dynamic range. The assay is linear from 256 ng to 1 pg input DNA (128 ng/μL to 0.5 pg/μL sample concentration). The Eclipse™ assay does have a slightly longer run time (87 min versus 72 min) and the Eclipse™ probe costs more than SYBR® Green. However, when purchased in bulk, the probe becomes a negligible cost/reaction (~$0.04). The major cost is the price of Taq polymerase and/or the mastermix kit (~$0.30). Both assays actually cost less than the Applied Biosystems Quantiblot® kit in our hands. As with the SYBR® Green assay, variation of Eclipse™ 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. Comparisons between the mastermix made in the laboratory and the three different commercial brands of mastermix that were tested showed that the mastermix made in the laboratory gave higher RFU values than the commercial mixes. The Invitrogen Platinum® PCR SuperMix gave unacceptably low RFU values and variable results.

A concern could be raised about the amplification of the no template control. This was also observed in our SYBR® Green assay (16) at about the same Ct. As discussed in this paper, other groups have observed NTC amplification when performing assays with an *Alu* sequence (12,14). For example, Walker et al. recently found their NTC gave a Ct approximately the same as their 0.0001 ng point (36 cycles) when performing a SYBR® Green based *Alu* assay (14). The amplification of the NTC is presumably a result of a very low (unavoidable) amount of ambient human DNA in the air and water. The NTC amplification seen here has a Ct 5.8 less than the Ct of the 1 pg (0.5 pg/μL) standard curve sample (Fig. 1*B*). This translates to 1 pg $\div 2^{5.8}$ or 0.018 pg; since a cell has about 6 pg of DNA, this is ~1/400 of a cell, certainly well below the amount needed for amplification by the STR kits. If appearance of the NTC amplification is troublesome to users, then its appearance can be eliminated by simply stopping the assay at 25 cycles. However, the appearance of the NTC does have value. Because the NTC (which has no deliberately added exogenous human DNA but identical PCR reagents and PCR conditions to the samples) has a Ct of ~5–7 cycles greater than the 1 pg standard, all samples, whether they have extracted human DNA present or not, must have a Ct equal to or lower than the NTC value. The reason for this is that even samples with no extracted DNA will contain the background human DNA that is present in the NTC. If the Ct for any sample is greater than the NTC or gives no result at all, then either an ingredient was missed in the PCR or PCR inhibitors must be present. Samples with an inhibitor such as phenol give a flat amplification curve out to 40 cycles and beyond (data not shown). Samples with partial inhibition give a flatter curve without the sharp exponential increase (data not shown); thus, samples with odd curves should be suspected of containing inhibitors. If the Ct value of the NTC is close to that of the Ct of the 1 pg standard then contamination of the primer or other stocks should be investigated.

The Eclipse™ assay has shown it can reproducibly determine the amount of human DNA in a wide range of samples. Standard deviations for Ct values were low for the triplicate experiment and the standard deviations for concentration values were low for the samples repeated over five days. Sample concentration values also were consistent using either the 1:10 forward to reverse primer ratio originally suggested by the vendor or the newly recommended 1:1 forward to reverse ratio. The standard deviations for the Eclipse™ assay are slightly higher than comparable results with the SYBR® Green assay but are still well within the tolerances of the STR kits. Like the STR kits, the Eclipse™ assay is also PCR-based with an amplicon size approximating that of the forensic STRs and as such it should more accurately determine the relevant size of the DNA and the presence of inhibitors. Thus, quantification using this assay should be more predictive of STR success than a non-amplification based assay such as the slot blot based assay.

The question arises with an assay using a repeated sequence as to variations in sequence number in the population. This Eclipse™ assay is based on the Ya5 *Alu* family (sequence differences on the primers and probes make the assay specific for this *Alu* family). Currently there are 2473 identified Ya5 sequences in the human genome of which 75–80% are fixed (present in all individuals) and 20 to 25% are polymorphic (Dale J. Hedges and Jerilyn A. Walker, personal communication). There are estimates of another ~557 polymorphic *Alu* sequences that are not yet discovered (Dale J. Hedges and Jerilyn A. Walker, personal communication). Therefore, there are a total of 3030 (2473 + 557) *Alu* sequences of which 1114 are polymorphic and 1916 are fixed. A single diploid cell will have 3,832 fixed *Alu* loci [1916 × 2 (diploid)] plus 2228 (1114 × 2) loci which may or may not contain an *Alu* sequence. Assuming the chance of each site containing an *Alu* is 0.5, the average number of polymorphic *Alu* sequences in a cell will be 1114. The standard deviation for this binomial expansion is $\sqrt{n/2}$ and two standard deviations is thus $\sqrt{2228}$ or 47. Two standard deviations in either direction is 94 which occurs over a total mean of 4946 *Alu* sequences (3,832 fixed +1114 mean polymorphic) or 1.9% (100% × 94/4946). This ~2% variation is well within the day to day variation of the assay.

This assay can be compared with other available assays both commercial and published. The *AMEL* assays (1–3) all suffer from the fact that the *AMEL* locus is deleted in a significant number of men from some ethnic groups. We also performed some preliminary experiments on a duplex quantitation/gender TaqMan® assay similar to Alonso et al. [2]; however, we decided that such an assay would not be viable because *AMEL* is deleted in a significant number of men from some ethnic groups and, thus, we discontinued work on this assay. The *THO1* assays (5,6) are also single copy. The BODE method (5) has many difficulties associated with an endpoint assay including a high threshold (200 pg). The *BRCA1* assay was not thoroughly investigated but appears to have a high threshold as well (7). The *RB1* assay uses 50 cycles to gain a threshold of 30 pg and there is a possible uninvestigated problem with cross reaction of the primers/probe with other animals by BLAST search (8). The two mtDNA assays published (2,8) are both very sensitive and based on TaqMan® methodology, but they are better suited for quantitation of DNA for mitochondrial DNA typing than STR analysis.

The other published assays are based on the use of *Alu* sequences. The Sifis et al. (11) and Urban et al. (12) and Nicklas and Buel (15) assays are all cumbersome requiring gels, a capillary instrument or a plate reader for analysis. They also have small dynamic ranges. Of the three assays of Walker et al. (inter, Yb8, Yd6) (14), the Yb8 assay is the most robust in terms of range and specificity. However, all of these assays are SYBR® Green assays which limits the upper range. The Nicklas and Buel SYBR® Green assay (15) has similar limitations.

Limitations exist as well in the two assays available commercially, AluQuant™ (Promega) based on a human centromeric repeat and the Quantifiler™ Human DNA Quantitation Kit (Applied Biosystems) based on the *TERT* gene. The former is a repeated sequence, however the range is limited by the READIT® technology which is non-linear and somewhat complicated. The AluQuant™ assay lacks the predictive power of amplification as it is not PCR-based and therefore, cannot detect the presence of PCR inhibitors, nor are results apparently affected by sonication of DNA (13). The Quantifiler™ kit again detects a unique sequence with a lower limit of detection of ~23 pg/μL. While the assay is PCR-based, the Quantifiler™ product is quite small (only 62 bp); thus, might not be expected to be a good predictor of success of PCR of the much longer STR products in degraded DNA samples. The size of the Eclipse™ product (113 bp) described here overlaps the sizes of the forensic STR products while the 62 bp Quantifiler™ product is much smaller. The Eclipse™ assay is thus more likely to be successful in correctly determining the amount of sample to add to STR amplifications.

The Eclipse™ assay reported here thus has the advantage of lower cost than the commercial kits and the greatest sample concentration range of the published assays (0.5 pg/μL to 128 ng/μL). There are possibilities for further development of real time assays for use by the forensic community. For example, we are currently developing *Alu*-based assays using TaqMan® chemistry. Richard et al. (6) recently described a TaqMan® assay for human quantitation; however, they use a single copy locus in intron 1 of the *THO1* gene. The use of a single copy gene limited their sensitivity to ~25 pg versus the 1 pg or lower possible in this *Alu* Eclipse™ assay. Due to the availability of MGB Eclipse™ or TaqMan® probes with several fluorophores, there is the potential for multiplexing probe-based assays which is not possible with SYBR® Green based assays. Thus, combining of several assays with markers of interest to the forensic scientist is possible and currently under development.

Acknowledgments

The authors would like to thank Marcie LaFountain, Joseph Abraham and Suzanne Newberry for advice, support and critical review of the manuscript. We would also like to thank Dr. Mark Batzer and Dale J. Hedges and Jerilyn A. Walker in his laboratory for *Alu* sequence information and advice.

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