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Human DNA quantitation using *Alu* element-based polymerase chain reaction

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Abstract

Human forensic casework requires sensitive quantitation of human nuclear DNA from complex sources. Widely used commercially available systems detect both nonhuman and human primate DNA, often require special equipment, and have a detection limit of approximately 0.1 ng. Multicopy *Alu* elements include recently integrated subfamilies that are present in the human genome but are largely absent from nonhuman primates. Here, we present two *Alu* element-based alternative methods for the rapid identification and quantitation of human DNA, inter-*Alu* PCR and intra-*Alu* PCR. Using SYBR green-based detection, the effective minimum threshold level for human DNA quantitation was 0.01 ng using inter-*Alu*- and 0.001 ng using intra-*Alu*-based PCR. Background cross-amplification with nonhuman DNA templates was detected at low levels using inter-*Alu*-based PCR, but was negligible using intra-*Alu*-based PCR. These *Alu*-based methods have several advantages over currently available systems. First, the assays are PCR based and no additional unique equipment is required. Second, the high copy number of subfamily-specific *Alu* repeats in the human genome makes these assays human specific within a very sensitive linear range. The introduction of these assays to forensic laboratories will undoubtedly increase the sensitivity and specificity of human DNA detection and quantitation from complex sources.

The quantitative detection of biomaterials in mixed forensic samples has been approached using a variety of different systems. Early approaches to identify the origin of mixed sample components involved the use of high-performance liquid chromatography (HPLC)-based methods [1]. These methods have proven useful, although the detection limits using these approaches are restrictive. The detection of single-copy nuclear DNA sequences has also been useful in this regard, but is limited as a result of the single copy. Polymerase chain reaction (PCR)-based analysis of mitochondrial DNA sequences has also been used in the analysis of complex DNA samples. The advantage of mitochondrial-based DNA analyses derives from the fact that there are many mitochondria per cell and many mitochondrial DNA molecules within each mitochondria, making mitochondrial DNA a naturally amplified source of genetic variation [2]. However, a significant proportion of human forensic casework involves the analysis of nuclear loci [3] making the identification and quantitation of human nuclear DNA a paramount issue.

Commercially available products for human DNA quantitation include the Quantiblot (Applied Biosystems, Inc.) and the AluQuant (Promega Corp.) systems. The Quantiblot system is based on the hybridization of a biotinylated oligonucleotide probe to extracted DNA, followed by visual comparison of the colorimetric or chemiluminescent sample results to the DNA standards. The AluQuant system utilizes a luciferase reaction that results in light output suitable for interpretation with a luminometer [4]. These systems can become quite costly, particularly if a luminometer needs to be purchased. The

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Quantiblot and AluQuant systems also detect both nonhuman and human primate DNA, and the detection limit for each of these systems is approximately 0.1 ng.

The use of *Alu* PCR amplification has been reported to be a more sensitive method for the quantitation of genomic DNA compared to typical blot-based procedures currently used in most forensic laboratories [5]. Note that the term "AluQuant" is a trademark of Promega Corp. and the AluQuant system may not necessarily be based on *Alu* mobile elements, per se. *Alu* elements are transposable elements which have been amplified to a copy number of over 1 million elements throughout primate evolution, producing a series of subfamilies of *Alu* elements that appear to be of different genetic ages [6]. The expansion of these elements throughout primate evolution has created several recently integrated "young" *Alu* subfamilies that are present in the human genome but are largely absent from nonhuman primates [6]. These human-specific subfamilies have only a fraction of the copy number compared to primate-specific elements, but when approached collectively, human-specific and primate-specific *Alu*-based assays provide powerful tools for sensitive human DNA identification and quantitation from mixed sources. Because of their high copy number, *Alu* elements are a naturally amplified source of human genetic information.

Recently Sifis et al. [5] reported a method whereby a fluorescently labeled oligonucleotide PCR primer pair was designed to amplify the core *Alu* sequence within primate DNA. They reported that the assay had a sensitivity of 100–2.5 pg of DNA, making it particularly useful in legal cases where the apparent absence of DNA in profile-generating samples is due merely to the poor detection limit of the quantitation procedure, which is typically 100–150 pg [5]. However, the authors acknowledge that forensic samples are often contaminated with nonhuman DNA, and they make no attempt to determine the specificity of their intra-*Alu* amplification using other mammalian DNAs as tester templates. To overcome a series of shortcomings of previously reported methods for human DNA quantitation we have designed and evaluated a series of assays based upon *Alu* mobile element amplification.

Materials and methods

Primer design and PCR amplification

Oligonucleotide primers for inter-*Alu* PCR were Alu 3- 5′GATCGCGCCACTGCACTCC 3′ and Alu 5- 5′ GGATTACAGGCGTGAGCCAC 3′ [7,8]. The intra-Yb8 primers 5′ CGAGGCGGGTGGA*T*CA*T*GAGGT 3′ (positions 48–69) and 5′ TCTGTCGCCCAGGC *CGGACT* 3′ (positions 273–254) have the diagnostic bases shown here in italics and underlined. The forward intra-Yd6 primer 5′ GAGATCGAGACCA*C*/GGT GAAA 3′ crosses the characteristic Yd subfamily deletion, marked by the slash, whereas the reverse primer 5′ TTTGAGACGGAGTCTCG*T*T 3′ contains a Yd6 subfamily-specific diagnostic mutation at the penultimate base.

Intra-*Alu* oligonucleotide primers were designed using either Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) or Primer Express software (Applied Biosystems). The need to incorporate subfamily-specific diagnostic mutations into the primer design and the high intrinsic GC content of *Alu* repeats made it challenging to identify oligonucleotide primers acceptable to the design software packages. Oligonucleotides were purchased from Sigma-Genosys, Inc. The SYBR green PCR core reagent kit was purchased from Applied Biosystems, Inc.

PCR conditions were optimized for each assay with regard to annealing temperature and concentrations of $MgCl_2$ and oligonucleotide primers. PCRs were carried out in 50 μl using 1X SYBR green buffer, 1 mM dNTPs, and 1.25 units Ampli*Taq* Gold DNA polymerase as recommended by the supplier. Inter-*Alu* PCR used 2 μM each oligonucleotide primer and 3 mM $MgCl_2$. Each sample was subjected to an initial denaturation of 12 min at 95 °C to activate the Ampli*Taq* Gold, followed by 40 amplification cycles of denaturation at 95 °C for 20 s, 56 °C to anneal for 1 min, and 1 min of extension at 72 °C. Intra-Yb8 PCR used 1 μM each oligonuclotide primer, 3 mM $MgCl_2$, an initial denaturation of 12 min at 95 °C, followed by 40 amplification cycles of 95 °C for 15 s and 74 °C for 1 min to anneal and extend. Intra-Yd6 PCR used 0.5 μM each oligonuclotide primer, 5 mM $MgCl_2$, an initial denaturation of 12 min at 95 °C, followed by 40 amplification cycles of 95 °C for 15 s and 61 °C for 1 min to anneal and extend. Each reaction contained 49 μl of PCR master mix and 1 μl of DNA template. Quantitative PCR experiments were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Inc.) or a Bio-Rad i-cycler iQ real-time PCR detection system.

Cell lines and DNA samples

The cell lines used to isolate DNA samples were HeLa (*Homo sapiens*), pygmy chimpanzee (*Pan paniscus*), and chimpanzee (*Pan troglodytes*) as reported previously [11]. DNA from nonprimate species was obtained by tissue and blood extraction using the Wizard Genomic DNA Purification kit (Promega) for samples provided by the Louisiana State University School of Veterinary Medicine. Human control DNA (HeLa) was serially diluted 10-fold in 10 mM Tris/0.1 mM EDTA such that concentrations from 100 ng to 0.1 pg were evaluated in replicates of two to four each.

Data analysis

Data from the replicate DNA standards were exported from ABI Prism 7000 SDS software into a Microsoft Excel spreadsheet where the mean value and standard deviation were calculated for each point on the standard curve. The negative control (no template; NT) was included in these calculations but was considerably lower than the last data point for inter-*Alu* and intra-Yd6 standard curves and therefore does not appear on those charts, but does appear on the intra-Yb8 chart. Using the Excel trendline option, a line of best fit was plotted with Y-error bars equal to one standard deviation. Data from the nonhuman DNA cross-hybridization/amplification experiments were exported to Excel in a similar manner and the mean and standard deviation were calculated for each of four replicates. The Excel chart wizard was used to construct bar graphs with Y-error bars equal to one standard deviation.

Results

Here, we present *Alu* element-based alternative methods for the rapid identification and quantitation of human DNA. The two approaches that we present here are inter-*Alu* and intra-*Alu* PCR-based methods (Fig. 1) in conjunction with SYBR green fluorescence detection (SYBR is a registered trademark of Molecular Probes, Inc.). Inter-*Alu* PCR was originally developed by Nelson et al. [9] to detect human DNA sequences in somatic cell hybrids. Oligonucleotide primers were designed based upon the primate *Alu* consensus sequence and used to amplify unique human DNA sequences between adjacent *Alu* repeats in an effort to isolate large regions of human DNA without the laborious task of first creating a recombinant library from the somatic cell hybrids. Later, the technique was improved by primers, Alu 5′/3′, which amplified human DNA sequences between adjacent *Alu* repeats, regardless of their orientation in the genome, and reportedly could generate PCR products detectable by ethidium bromide staining from as little as 0.001 ng of human genomic DNA [7]. Inter-*Alu* PCR generates a complex pool of PCR amplicons of different sizes [7,8].

By contrast, intra-*Alu* PCR generates a homogeneous product composed entirely of repeat core-unit DNA sequences characteristic of the element being amplified. This approach is similar to the primate *Alu* assay developed by Sifis et al. [5]. However, our assay is based on the limited amplification of members of the young *Alu* subfamilies that are present in the human genome but are largely absent from nonhuman primate genomes. The subfamilies that we selected for intra-*Alu* evaluation were Yb8 [10] and Yd6 [11] because of the large diagnostic insertion or deletion that is characteristic of these *Alu* families. The Yb8 subfamily consensus sequence contains eight individual diagnostic mutations different from the ancestral *Alu* Y subfamily and a 7-nucleotide insertion at position 253 (Fig. 2). There are an estimated 1852 Yb8 *Alu* elements in the human genome [12]. The recently reported Yd6 subfamily has six subfamily-specific diagnostic mutations and a 12-nucleotide deletion starting at position 87 that defines the Yd lineage from the draft sequence of the human genome [11]. There are an estimated 97 Yd6 *Alu* subfamily members in the human genome [11].

The inter-*Alu*-based PCR assays had a linear quantitation range of 10–0.001 ng as shown by the standard curve (Fig. 3A). The mean value of the negative control was 31.3 with a 0.4 standard deviation. This assay was expected to amplify DNA from nonhuman primates (*P. paniscus* and *P. troglodytes*) and from humans [7–9]; however, significant background amplification was also detected in other species when tested with an equivalent amount (2 ng) of nonhuman DNA (Fig. 4A). This limits the effective minimum threshold quantitation level to 0.01 ng (threshold PCR cycle 22) rather than 0.001 ng and restricts the range of the inter-*Alu*-based PCR assay to 1000-fold when testing DNA samples from complex sources. To further evaluate the potential background amplification that nonhuman DNA might have on the assay with respect to human DNA quantitation, a "domestic DNA mix" (10 ng/μl) that contained 45% canine, 45% feline, and 10% human DNA was prepared. The results of that experiment (mean of duplicates, ±

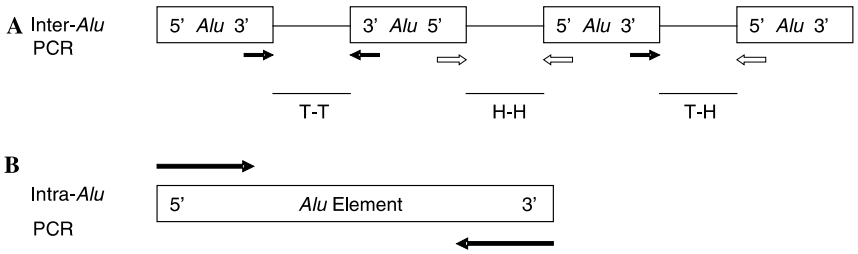

Fig. 1. Schematic of inter-*Alu* and intra-*Alu* PCR. Rectangles represent 5′–3′ or 3′–5′ orientation of *Alu* elements in the genome. (A) During inter-*Alu* PCR the 5′ primer (unshaded arrows) and the 3′ primer (small black arrows) amplify genomic DNA sequences between adjacent *Alu* elements (dark lines) in any possible orientation, "tail-to-tail" (T-T), "head-to-head" (H-H), or "tail-to-head" (T-H). (B) During intra-*Alu* PCR, primers are designed within the core body of the *Alu* element to amplify multiple copies of the element derived from locations dispersed throughout the genome.

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AluY   GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGA  50
AluYb8 ..................................................  50
AluYd6 ..................................................  50

AluY   GGCGGGCGGATCACGAGGTCAGGAGATCGAGACCATCCTGGCTAACACGG  100
AluYb8 ......T......T.................................A..  100
AluYd6 ..................................C------------..   88

AluY   TGAAACCCCGTCTCTACTAAAAATACAAAAAATTAGCCGGGCGTGGTGGC  150
AluYb8 ........................................C......      150
AluYd6 ........................................CA.....      138

AluY   GGGCGCCTGTAGTCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATGGCGT  200
AluYb8 ..................................................  200
AluYd6 ..................................................  188

AluY   GAACCCGGGAGGCGGAGCTTGCAGTGAGCCGAGATCGCGCCACTGCACTC  250
AluYb8 ..........A.......................T...........G..  250
AluYd6 ........A...................G.............A......  238

AluY   CA-------GCCTGGGCGACAGAGCGAGACTCCGTCTCAAAAAA       287
AluYb8 .GCAGTCCG.................................        294
AluYd6 .C-------............AA...................        275
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Fig. 2. Sequence alignment of *Alu* subfamilies. The consensus sequence for the *Alu* Y subfamily is shown at the top with *Alu* Yb8 and Yd6 subfamilies shown below. The dots represent the same nucleotide as the *Alu* Y consensus sequence. Deletions are shown as dashes and mutations are shown as the correct base for each subsequent subfamily. Sequences of the subfamily-specific intra-*Alu* oligonucleotide primers for amplification of the *Alu* core body sequence only are shown in boldface.

one standard deviation) are shown as data point "a" in Fig. 3A. Since 10% of 10 ng is 1 ng, the assay was able to accurately quantitate the human DNA in the mixed sample.

The intra-Yb8-based PCR assay had a low-scale linear quantitation range of 10–0.001 ng (10,000-fold) as shown by the standard curve (Fig. 3B). The value of the NT negative control was not significantly different from the last data point (0.0001 ng). Background amplification was detected in *P. paniscus* (pygmy chimpanzee), the nonhuman primate most closely related to humans, following 31 cycles of PCR and was detected in other species in trace amounts following 36 cycles of PCR using an equivalent amount of DNA template (2 ng) (Fig. 4B). This demonstrates that the intra-Yb8 assay is human specific to 0.01 ng and specific to only humans and pygmy chimpanzees to 0.001 ng (1 pg) when evaluating mixed DNA samples. If a sample is known to consist of only human DNA, the detection limit of this assay may extend between 1 and 0.1 pg but must accompany an appropriate NT control.

The intra-Yd6-based PCR assay had a high-scale linear quantitation range of 100–0.1 ng (1000-fold) as shown by the standard curve (Fig. 3C). The mean value of the negative control was 35.7 with a 1.17 standard deviation. No amplification signal was detected from any of the nonhuman species tested, making this assay absolutely human specific within its quantitation range (Fig. 4C). The intra-Yd6 assay not only extends the linear quantitative range of the intra-*Alu* assays combined, but also allows for human DNA detection and rough quantitative estimates to be performed by simple, inexpensive agarose gel electrophoresis as an initial screening tool (Fig. 5).

Discussion

The vast majority of human forensic casework involves the analysis of nuclear loci, making the quantitation of human nuclear DNA a paramount issue [3]. There are several advantages to our *Alu*-based methods for the rapid identification and quantitation of human DNA over commercially available systems and other recently reported methods. First, these assays are PCR based. If an ample amount of DNA is available for testing, human-specific DNA detection and quantitative estimates can be performed by simple agarose gel analysis using the intra-Yd6 *Alu*-based PCR assay as an initial screening tool. The addition of SYBR green PCR core reagents to the amplification protocol allows accurate quantitation using any qPCR system. No additional special expertise or unique equipment, such as a luminometer or automated DNA sequencer/genotyper, is required. This format alone minimizes the cost of performing these analyses on a large scale and gives most forensic laboratories with average resources the ability to perform these assays.

We have also systematically tested each assay for human specificity, especially with regard to closely related nonhuman primates, whereas documentation associated with other currently available methods is rather

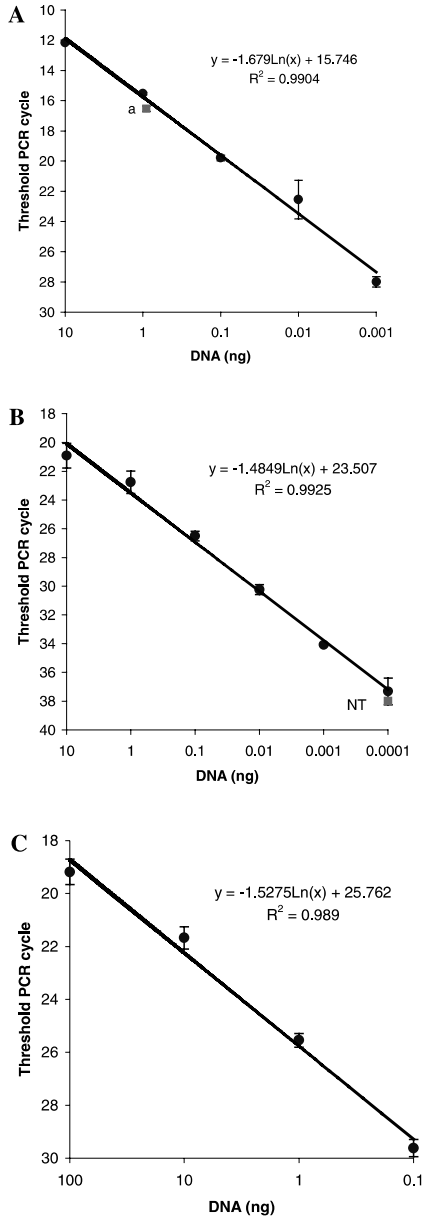

Fig. 3. Effective quantitation range of various *Alu*-based assays. The effective ranges for (A) inter-*Alu*-, (B) intra-*Alu* Yb8-, and (C) intra-*Alu* Yd6-based PCR assays using SYBR green fluorescence detection are shown. The PCR cycle at which the fluorescent signal crosses baseline is considered to be the threshold cycle, plotted on the y axis. The fluorescent signal produced by a 10-fold dilution series of human DNA is plotted as the mean of two to four replicates, ± one standard deviation. The R^2 value is at least 99% for all three standard curves.

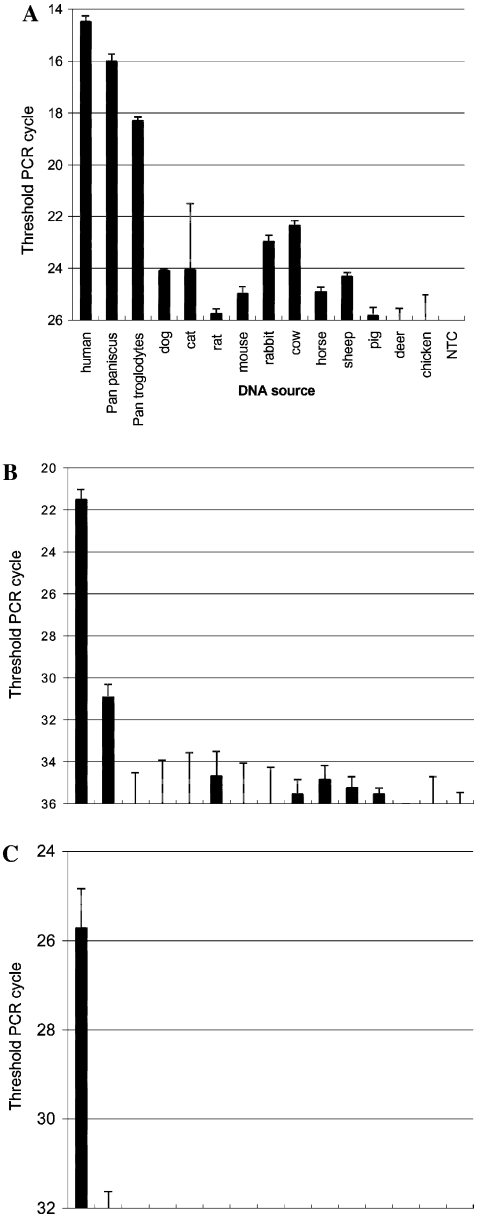

Fig. 4. Background amplification using nonhuman DNA templates. The cross-amplification of nonhuman template DNA is shown for the (A) inter-*Alu*-, (B) intra-*Alu* Yb8-, and (C) intra-*Alu* Yd6-based PCR assays using SYBR green fluorescence detection. The PCR cycle at which the fluorescent signal crosses baseline is considered to be the threshold cycle, plotted on the y axis (mean of three replicates, ± one standard deviation). The inter-*Alu* assay detected human, pygmy chimpanzee (*Pan paniscus*), and common chimpanzee (*Pan troglodytes*) DNA (2 ng per reaction), but also produced significant background amplification using DNA from other mammals as template. The intra-*Alu* Yb8 assay (2 ng DNA per reaction) and the intra-*Alu* Yd6 assay (10 ng DNA per reaction) are entirely human-specific with a small amount of background amplification using pygmy chimpanzee (the primate closest genetically to humans) DNA as a template after 30 cycles of PCR.

vague concerning mammalian cross-hybridization/amplification. In addition, the range of quantitation using the combined intra-*Alu*-based assays (Yb8 and Yd6 subfamilies) is approximately 10^5 based on our 10-fold dilution series experiments, whereas the current commercial quantitation systems AluQuant and Quantiblot have a 500-fold and 100-fold quantitation range, respectively. In other words, the low range detection limit of the intra-Yb8 assay reported here exceeds the commercial systems by a minimum of 100-fold and exceeds the method recently reported by Sifis et al. [5] by at least 2.5-fold. Since Sifis et al. [5] do not address possible

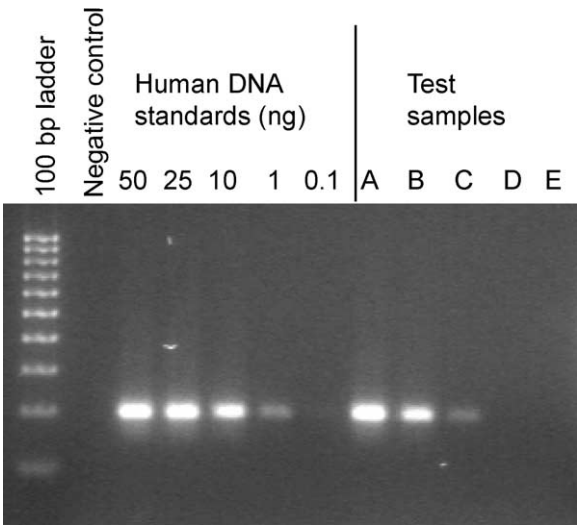

Fig. 5. Human DNA detection on an agarose gel using the intra-Yd6 *Alu* PCR assay. Following 30 cycles of conventional PCR using the intra-Yd6 oligonucleotide primers, amplicons are chromatographed on a 2% agarose gel stained with ethidium bromide. Using the human DNA standards on the left, samples A–C were positive for the presence of human DNA (generated a PCR amplicon) while samples D and E are not (10 ng chimpanzee and rat DNA template, respectively). Samples A–C contain 30, 5, and 0.5 ng of human DNA, respectively. These values are reasonably consistent with expected values based on empirical observations of fragment intensity. This demonstrates that the assay is a simple, rapid, and inexpensive means of human DNA detection that also provides quantitative estimates of human template DNA.

mammalian cross-amplification with their assay, the intra-Yb8 method reported here should prove to be even more sensitive for the identification of human DNA from complex sources.

The high copy number of *Alu* repeats in the human genome makes these assays ideal for human DNA detection and quantitation. When inter-*Alu* PCR was first developed over 15 years ago, it was revolutionary in allowing sensitive amplification and detection of human DNA from somatic cell hybrids while circumventing the laborious task of first creating a recombinant library. The detection limit reported then of 0.001 ng of human DNA [7] is consistent with our findings. However, the fact that the inter-*Alu* PCR method amplifies unique genomic DNA sequences between adjacent *Alu* repeats, creating a complex pool of various sized PCR amplicons, appears to predispose the method to greater background cross-amplification potential with DNA from other mammals compared to the intra-*Alu* methods that we evaluated. Furthermore, the inter-*Alu* PCR method requires the use of fluorescence or radioactive isotope in the PCR to be quantitative, whereas the intra-*Alu* methods are more amenable to other detection schemes such as ethidium bromide or *Taq*Man chemistry.

Although we did not evaluate the use of *Taq*Man chemistry with these intra-*Alu* PCR assays, we have designed *Taq*Man probes and primers for other quantitative assays in our laboratory using Primer Express software and compared the results to SYBR green with regard to detection sensitivity. No significant increase in sensitivity was observed with *Taq*Man-based detection as compared to SYBR green-based detection in the assays that we tested (data not shown). The reason for this is unclear. Perhaps the sensitivity of SYBR is not significantly different from that of the 5′-FAM reporter typically used with *Taq*Man probes, while other reporter dyes may be more sensitive. The use of *Taq*Man hybridization probes would also be more difficult with our intra-*Alu* assays because of the need to incorporate *Alu* subfamily-specific diagnostic mutations into the design of the probe primers and the level of sequence divergence within the *Alu* core body units that are amplified throughout the genome (e.g., as much as 1% pairwise sequence divergence between Yb8 *Alu* elements). The position of subfamily-specific diagnostic mutations in conjunction with the intrinsic high GC content of *Alu* repeats often prevented the design of primer/probe combinations by Primer Express due to the lack of an acceptable T_m range. For example, the optimal annealing temperature for the intra-Yb8 *Alu*-based PCR assay was determined empirically to be 74 °C, which is exceedingly high to permit a functional primer and probe design.

However, the high T_m of the intra-Yb8 *Alu*-based primers was essential to the elimination of artifact amplicons from DNA of other species as a result of sequence similarity to SINE elements from other species. This became evident when we attempted to design a similar intra-*Alu* PCR assay using the Ya5 subfamily consensus sequence, but were unable to eliminate background amplification. Optimization of oligonucleotide primer concentration also proved to be an important component in the development of these *Alu*-based PCR assays. Compared to single-copy PCRs, the high number of target sequences in these assays made it important to have sufficient amounts of primer without compromising PCR amplification efficiency.

We have reported two alternative methods for human DNA detection and quantitation, inter-*Alu*- and intra-*Alu*-based PCR. Although the inter-*Alu*-based PCR method revolutionized human DNA detection when it was first developed, we have demonstrated that the intra-*Alu*-based PCR method is more suited to modern forensic needs. The intra-*Alu* assays presented, Yb8 and Yd6, employ the amplification of a high copy number of target sequences to achieve very sensitive human-specific DNA detection, while simultaneously maintaining some of the same advantages of single-locus PCR. For example, the amplified products are a uniform size amplicon in each assay, conducive to easy visualization. These amplicons are also short enough (226 bp for Yb8 and 200 bp for Yd6) to tolerate sheared or degraded DNA often associated with forensic applications. An

infinite number of experiments could be run, testing various qualities of DNA along with various contaminants, in an effort to validate this assertion. However, we have demonstrated the tremendous potential of these *Alu*-based PCR assays for human DNA identification and quantitation in forensic applications. Forensic laboratories can empirically evaluate these methods with regard to their specific needs. Nevertheless, the application of these multiple-copy *Alu*-based quantitative PCR assays to modern forensic DNA assays will undoubtedly increase the sensitivity and specificity of human DNA detection and quantitation from complex sources.

Acknowledgments

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