TECHNICAL NOTE

Melanie E. Sifis;[1] *Katrin Both,*[1] *Ph.D.; and Leigh A. Burgoyne,*[2] *Ph.D.*

A More Sensitive Method for the Quantitation of Genomic DNA by *Alu* Amplification*

REFERENCE: Sifis ME, Both K, Burgoyne LA. A more sensitive method for the quantitation of genomic DNA by *Alu* amplification. J Forensic Sci 2002;47(3):589–592.

ABSTRACT: Current procedures for human DNA quantitation reach their limit at 150 pg DNA, which is above the limit of the PCR profiling range using Profiler-Plus™ (Applied Biosystems, CA). This study tested the potential for the use of primate specific *Alu* sequences in forensic science for the sensitive detection and quantitation of DNA. A fluorescently labelled primer pair was designed enabling high efficiency amplification of the core *Alu* sequence within primate DNA. Quantitation was performed by measurement of fluorescence intensity and comparison to a series of standard template DNA amounts via the construction of a standard curve. The new *Alu*-based quantitation protocol developed has shown its feasibility in more sensitively quantitating (100–2.5 pg) unknown amounts of human DNA for forensic use. The method is compatible with the use and throughput of current forensic procedures.

KEYWORDS: forensic science, DNA quantitation, primates, *Alu* elements

Alu accounts for 5–10% of the genome and is the most well-known primate short interspersed element (SINE). Discovered in 1979 by Houck et al. (1), it was named for the *Alu*I restriction endonuclease site present within its sequence. It has a 282-nucleotide consensus sequence, and is followed by a 3′ Adenosine (A) rich region resembling a poly (A) tail of varying length (2). *Alu* is found up to one million times per haploid genome, a large majority being found at the same chromosomal positions within the genome of primates (3) and for this reason above, should be a good target for the detection of human DNA.

The rapid, sensitive, and reliable quantitation of human DNA samples is important for not only providing an efficient approach to DNA typing of forensic samples, but possibly more importantly, as proof of technical stringency when challenged in court. Many of the samples involved in forensic casework may be contaminated with non-human DNA (bacterial, fungal, plant, animal). Therefore, it is desirable that any method of quantitation is also primate specific; otherwise, any substantial contamination may lead to an overestimation of the amount of primate DNA within the sample DNA extract (4). Presently, blot-based procedures are in use at the Forensic Science Centre of South Australia (and most other forensic DNA laboratories around the world), for the rapid, non-radioactive quantitation of 0.15 to 10 ng of primate DNA, in around 3 h.

The polymerase chain reaction (PCR) has revolutionized the detection of DNA and RNA (5). Theoretically, there is a quantitative relationship between the amount of starting target sequence and the amount of PCR product at any given cycle (before reaching saturation). Therefore, dye labeled primers were designed to amplify the core sequence of *Alu* repeats, as a potential method for more sensitively quantitating human (primate) DNA. The conditions of use of these primers was optimized with known amounts of genomic DNA so that a standard curve could be generated, and its stability and thus utility, assessed. Quantitation was achieved by comparing the amount of product generated from standard reactions to that generated from unknown samples.

Materials and Methods

Serial Dilution of Template DNA

Control DNA was obtained at 0.2 mg/mL (Roche Diagnostics, Basel, Switzerland) and serially diluted so that various concentrations of human genomic DNA (1 pg to 100 pg) were used in duplicate as a template source in the PCR reaction.

Oligonucleotides

The primer pair was designed using the Primer three-web interface from the Whitehead Institute for Biomedical Research. The primers termed SP were SP1 5′-TGGTGGCTCACGCCTGTAA-3′ and SP2 5′-CGATCTCGGCTCACTGCAA-3′, producing a 229 bp product from within the 282 bp consensus sequence. Purified primers were obtained from Genset Pacific Pty. Ltd., at a guaranteed 400 μM. SP1 primer was purchased with a FAM-6 phosphoramidite fluorescent label to allow detection of product by the ABI PRISM™ 377 Genetic Analyser.

Amplification of DNA

The DNA (2 μL for standards in duplicate and sample extracts) was incubated in a total volume of 50 μL containing the SP primer pair (each at 0.3 μM), 0.2 mM of each dNTP, 1X PCR Buffer II

[1] Student and senior forensic scientist respectively, Forensic Science Centre, 21 Divett Place, Adelaide 5000, SA, Australia.

[2] Professor, School of Biological Sciences, Flinders University of South Australia, Sturt Rd, Bedford Park 5042, SA, Australia.

*This work was supported by the Biotechnology Program, School of Biological Sciences, Flinders University of South Australia, Bedford Park 5042, SA, Australia.

Received 16 March 2001; and in revised form 6 June and 24 Sept. 2001; accepted 24 Sept. 2001.

(Applied Biosystems, CA, containing 500 mM KCl, 100 mM Tris-HCl pH 8.3), 1 mM $MgCl_2$, 66 μg/mL BSA, and 0.025 U/μL TaqGold (Applied Biosystems, CA). A negative control was used containing no template DNA to detect any possible contamination. PCR was carried out in a Perkin-Elmer Cetus DNA Thermal Cycler using 0.6 mL eppendorf tubes, and the reaction overlaid with oil. Optimized cycling conditions for the SP primer pair were an initial denaturation of 94°C for 4 min, followed by 40 cycles of 94°C denaturation for 30 s, 55°C annealing for 45 s, and 72°C extension for 30 s. This was followed by a 72°C extension step for 7 min. Samples were stored at 4°C until analysis.

Analysis of PCR Products

PCR products were analyzed using the ABI PRISM™ 377 Genetic Analyser and the GeneScan® Analysis Software, enabling their sizing and quantitation. Two μL denaturation buffer (consisting of Hi-di Formamide and Loading Buffer mixed 5:1) and 0.25 μL ROX 400 HD Size Standard (Applied Biosystems, CA) were mixed per sample. Two μL of the mix was added to 2 μL PCR product, centrifuged, heat denatured at 96°C for 2 min, and placed on ice immediately. Long Ranger Singel® Packs (6% acrylamide gel using 36 cm plates) designed for the ABI PRISM™ 377 Genetic Analyser were used for electrophoresis. Two μL of prepared samples were run on the gel using 1 X TBE buffer at 51°C for 2.25 h. GeneScan® Software was used to determine the peak heights for the 229 bp products.

Constructing the Standard Curve and Quantitation of Extracts

Results from replicate standard template DNA amounts were averaged and the data complied in a spreadsheet (Microsoft Excel software). The template DNA amounts added to the PCR reaction were plotted against the corresponding peak height of the 229 bp product. Microsoft Excel software via its chart wizard function was used to construct a smoothed curve showing the maximum peak height for the 229 bp product (RFU) versus the amount of template DNA added to the PCR reaction (pg). The standard deviations of the raw data about the mean values were calculated using the Excel statistical function, and they were also plotted on the chart. Using the Excel trendline option, a line of best fit was also calculated via a least squares regression. The Y-intercept of the line was determined by the peak height of the zero DNA control. The resultant equation of the line was used to determine the amount of DNA added to the PCR reaction for several "unknown" extracts by obtaining the peak height for the respective 229 bp products. From this, the concentration of the model extracts was determined.

Results and Discussion

The assay is based on the amplification of core *Alu* sequences from primate DNA and measurement of fluorescence intensities from a standard curve of reactions containing known template DNA amounts. The results have shown the feasibility for more sensitive detection and quantitation of unknown amounts of primate DNA for forensic use than are available using the current established methodologies.

The SP primer pair was designed to amplify the core sequence of *Alu* in primate DNA. Protocols were optimized enabling the sensitive detection of DNA from 2.5–100 pg, a sensitivity limit 100-fold greater than the best blot procedure. Less PCR cycles meant that the system was less sensitive than the optimized 40 cycles. More PCR cycles produced an increased amount of non-specific amplification products, which lead to an increased variability in peak heights for each template DNA amount. Template DNA amounts above 100 pg were not used since they can be measured using currently available methods. Moreover, early results showed that saturation started to occur and it was important that the standard curve generated was in the linear range.

The gel image shown in Fig. 1 is the result of *Alu*-PCR of the known template DNA amounts. A correlation between the decrease

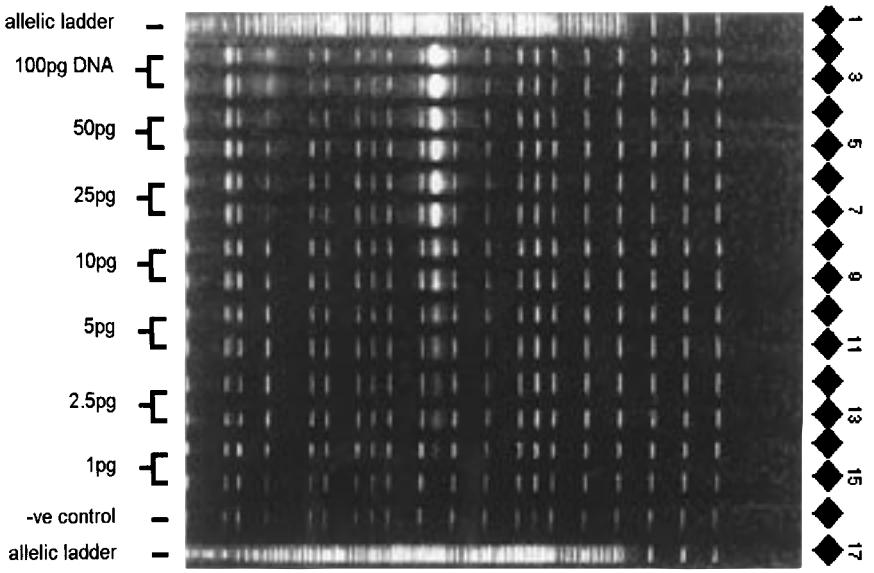

FIG. 1—*Template DNA amounts range from 1–100 pg in duplicate. Experiment was repeated three times. Horizontal axis represents the different lanes and vertical axis represents electrophoretic mobility, which is related to the product size. Internal size standards are present in each lane. Fluorescence of the PCR products is the result of the FAM-6 labeled SP1 primer. Cycling conditions were: 94°C, 4 min; (9°C, 30 s/ 5°C, 45 s/ 7°C, 30 s) × 40; 7°C, 7 min.*

TABLE 1—*Correlation between template DNA and product peak height.*

Template DNA (pg)	Replicate 1 (RFU)*	Replicate 2 (RFU)	Replicate 3 (RFU)	Average RFU	Standard Deviation
100	1545	1322	1404	1424	113
50	910	737	736	794	100
25	519	380	879	593	258
10	155	216	167	179	32
5	81	86	81	83	3
2.5	50	44	56	50	6

* Relative fluorescent unit.

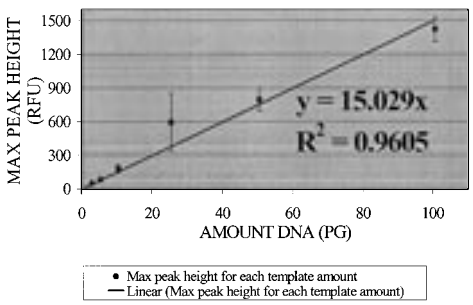

FIG. 2—*Quantitation standard curve.*

in template DNA and the peak height of the 229 bp PCR product given in relative fluorescent units (RFU) was observed. During amplification of the 229 bp product, the typical baseline background RFUs observed were generally up to 15 RFUs. It is for this reason that a threshold of 30 RFUs was used during the analysis. An example of results for one standard curve can be seen in Table 1. The large standard deviation obtained for the 25 pg template amount did not occur routinely in repeated experiments. Other template amounts amplified have also produced large standard deviations in some occasions. An explanation for this observation could be that samples weren't well mixed resulting in stochastic sample variation. These results allowed the construction of the standard curve shown in Fig. 2. The linear trendline was calculated using the Least Squares fit (Excel software), which minimises the distance between each plotted point and the trendline. When no DNA was present in a sample (i.e., negative control), the RFU was zero as no product was generated (data not shown). Because of this, the intercept for the trendline was set to pass through the origin. R^2 is the coefficient of determination, which represents the proportion of total variability of Y-values accounted for by X-values. In this case, the amount of template DNA accounts for 96.05% of the total variability in the peak heights given in RFUs.

In order to test the reliability of the proposed standard curve, the concentration of the prepared standards was "back calculated," based on the resulting RFU values obtained from the standard curve. These were found to be quite comparable as shown in Table 2. Examples of the sensitive quantitation of "unknown" model extracts unable to be quantitated using the current forensic methods are shown in Table 3.

Forensic Science Application

With the advances in molecular biology, forensic scientists can now generate DNA profiles from such low level samples as vaginal swabs, where only a few sperm cells are present, bone, urine, cigarette butts, and the inside of gloves. Sometimes samples such as these generate a full or partial DNA profile, but give a zero reading when quantitated using the slot-blot methods. However, a zero reading is only indicative of the detection limit of the quantitation procedure (i.e., typically up to 150 pg DNA). The apparent absence of DNA in profile-generating samples has posed problems in the legal system, causing DNA evidence to be challenged on the basis that if there was no DNA present in the sample, how could a profile have been generated? In court, it has been suggested that perhaps the profile was derived from contaminants or was the result of false alleles and/or allele dropout. Therefore, sensitive quantitation of extracts unable to be quantitated using the current forensic methods is needed.

The standard curve using amplification of *Alu* repeats that is observed in this paper is an example of how one may be generated. A curve using standard amounts of DNA run in duplicate at the same time, in the same machine as the sample extracts being quantitated. This involves 15 tubes per batch of estimates (7 different amounts in duplicate plus a negative control).

It should be noted that the amount of DNA obtained from an unknown sample can never be an exact value for any procedure. The *Alu*-PCR method is as sensitive to the presence of inhibitors in the reaction as any PCR reaction, but the very high sensitivity gives it utility for estimation of inhibitors by dilution. Common inhibitors include various components of body fluids and reagents encountered in forensic science (e.g., hemoglobin, urea, and heparin). Other more widespread inhibitors include constituents of bacterial cells, non-target DNA, and contaminants, and laboratory items such as glove powder and laboratory plasticware (6). Inhibition can manifest itself as complete reaction failure or as reduced sensitivity of detection. If inhibitors contained in a sample lead to reaction failure or reduced sensitivity during quantitation, it is highly probable that the same effect will be observed upon profiling since both are PCR based procedures. Therefore the quantitation result could be indicative of the profile that may or may not be produced.

TABLE 2—*Test of the reliability of the proposed standard curve by "back calculation."*

Prepared Standard DNA Amount (pg)	Resulting RFU Value	Proposed DNA Amount (pg)
2.5	50	3.3
5	83	5.5
10	179	11.9
25	593	39.5
50	794	52.8
100	1424	94.8

TABLE 3—*Examples of DNA samples tested that were unable to be quantitated using current forensic methods.*

Item Swabbed & Analyzed	RFU Obtained	Amount DNA (pg)
Glove	756	51
Knife	412	27
Cup	193	13
Computer mouse	1012	67
Elevator button	168	11

It is suggested that the method should be used not to claim an accurate quantitation value, as such a claim is probably a doubtful one to make with any method, but to explain the generation of profiles in critically important samples apparently containing no DNA using the current quantitation method. For such critically important samples there must be a slight trade-off in terms of time consideration (total time to perform the quantitation is 6 h compared to 3 h) to get the better sensitivity. The effect would be negligible in terms of casework flow through; as such samples are not typical in the day-to-day running of the laboratory.

The method can also be used to detect when DNA of useable quality and amount is in a sample but obscured by impurities. This is because *Alu* amplification requires template sizes of the order of many STR loci and that the impurities can be diluted out by approximately 100 fold while still expecting an *Alu* signal if there is large amounts of DNA of sufficient quantity. This latter is important because, if further purification is necessary, the worker needs proof that there is indeed useable DNA present before embarking on special procedures for further purification. However, if a negative result is obtained (due to reaction failure or below the detection limit) in a sample containing low level DNA, it is highly doubtful that dilution will improve the amplification. This is because it is highly unlikely the inhibitory effect will match the decrease of DNA since there is so little DNA to start with.

Nevertheless, the application of *Alu* primers to forensic samples theoretically shows potential for use in forensic quantitation analyses, as an aid to deciding the basis of profiling failures, and is compatible with the use and throughput of current forensic procedures.

References

1. Houck CM, Rinehart FP, Schmid CW. A ubiquitous family of repeated DNA sequences in the human genome. J Mol Biol 1979 Aug 15;132(3): 289–306.
2. Schmid CW. Does SINE evolution preclude *Alu* function? Nucleic Acids Res. 1998 Oct 15;26(20):4541–50.
3. Deininger PL, Batzer MA. *Alu* repeats and human disease. Mol Genet Metab 1999 Jul;67(3):183–93.
4. Waye JS, Presley LA, Budowle B, Shutler GG, Fourney RM. A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. Biotechniques. 1989 Sep;7(8):852–5.
5. Martin CS, Butler L, Bronstein I. Quantitation of PCR products with chemiluminescence. Biotechniques 1995 May;18(5):908–13.
6. Wilson IG. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 1997 Oct;63(10):3741–51.

Additional information and reprint requests:
Dr. Katrin Both
Senior Forensic Scientist
21 Divett Place, Adelaide 5000
E-mail: both.katrin@saugov.sa.gov.au