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Quantitative Polymerase Chain Reaction-based Assay for Estimating DNA Yield Extracted from Domestic Cat Specimens

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A quantitative polymerase chain reaction (PCR) assay has been developed for the quantification of genomic DNA extracted from domestic cat samples. The assay, which targets highly repetitive genomic short interspersed nuclear elements (SINE), can be performed rapidly and is highly sensitive, detecting as little as 10 fg of feline genomic DNA. The assay was linear over a 10^6 dilution range. We have recently developed a short tandem repeat (STR) multiplex panel for forensic analysis of feline specimens. The SINE assay is an integral part of the forensic typing system. The sensitivity of the assay will enable forensic examiners to determine the likelihood of success of genotyping sample extracts with the STR panel without sacrificing valuable DNA necessary to perform genotyping of samples.

Key words: *cats; forensic medicine; polymerase chain reaction; short interspersed nucleotide elements; tandem repeat sequences*

Identification of an individual pet or other animal may provide the critical piece of information in a criminal investigation. A recent survey of household pets in the United States estimated pet cat ownership at 75 million, with over 34% of all households having at least one cat (S. Payne, Pet Food Institute, Washington, DC, USA). A study on the transfer and persistence of animal hair demonstrated that it is almost impossible to enter a house where a domestic animal lives without being contaminated by its hair (1). Therefore, it is not surprising that animal hairs are frequently part of the physical evidence associated with a crime scene.

Higuchi et al (2) first demonstrated that DNA genotyping could be accomplished with single human hair specimens by using the polymerase chain reaction (PCR), and genetic individualization of animal hair specimens is increasingly being employed in forensic cases (3-7). A limiting factor in animal genetic profiling with short tandem repeats (STR), particularly from hair specimens, will be the quantity of genomic DNA available for analysis. With STR multiplex kits used for forensic analysis of human specimens, genotyping can be performed with DNA quantities as low as 0.25 ng (8). Amplification of less than 0.25 ng frequently results in stochastic amplification of only one of two alleles at a heterozygous locus, or allele "dropout" (8). DNA yields from cat hairs with excellent roots can range from 15-30 ng (4), 10 to 30 times less than is typically extracted from a human hair root.

We have developed an STR typing system for the genetic individualization of domestic cat (*Felis catus*) specimens (9,10). Given the low yield of DNA from cat hair specimens, the goal of our feline STR forensic typing system has been to develop an assay to quantify DNA to determine the likelihood of STR genotyping success without compromising yield, and to ensure maximum utilization of DNA yield, which may be required for future analyses. We have designed a method to estimate feline genomic DNA yield through a quantitative PCR-based assay targeting a class of highly repetitive nuclear elements, the short interspersed nuclear elements (SINE). These elements are in the transposable element (TE) class of nuclear elements, comprising a large fraction of the genomes of many plants and animals, estimated at 45% of the human genome (11) and 39% of the mouse genome (12).

The abundance of these elements in the human genome has led to the development of a quantitative assay of human genomic DNA targeting Alu SINE elements (AluQuant™ Human DNA Quantitation System, Promega, Madison, WI, USA), which uses an Alu-specific hybridization probe to detect nuclear DNA in the range of 0.1-50 ng (13). Walker et al (14) have recently reported on an Alu SINE-based quantitative PCR assay for identification and quantitation of human DNA. They additionally reported on species-

specific rat, mouse, guinea pig, and hamster SINE-based quantitative PCR assays, with the pig assay sensitive up to 100 fg (14,15).

We designed a quantitative assay using quantitative PCR targeting feline SINE elements, due to their abundance in the cat genome (16) and the availability of a large body of sequence information on SINE elements within felines (16,17). Feline SINE elements were previously described by Slattery et al (17) as composed of a 5′ RNA polymerase III promoter followed by a unique sequence region, a dinucleotide repeat motif area of variable length, and terminated by a 3′ poly(A) tail. Most eukaryotic SINEs are derived ancestrally from transfer RNA (tRNA) elements (18), and the described feline SINEs bear sequence similarity to tRNA-lys or tRNA-arg, similar to the B2 family of SINE elements reported in mink, dog, and harbor seal (19,20).

The recent sequencing of 800 kb of the domestic cat class II major histocompatibility complex (MHC) identified 437 SINE elements (16). The abundance of these elements identified in the largest region sequenced in the cat genome (11.8% of the extended class II region and 9.1 % of the classical class II region, ref. 16), is similar to SINE element density reported in the human (13.1%) and mouse (8.2%) genomes (11,12). The high genomic copy number of these elements in the cat genome thus offers a rich target source for quantification of trace amounts of nuclear DNA extracted from forensic samples.

Material and Methods

Genomic DNA Extraction

DNA was extracted from blood of a male domestic short-hair cat in our inventory, FCA 136, by using a Qiagen QiaAmp Mid Blood kit according to the manufacturer's suggestions. DNA was quantified by absorbance (A260) in a spectrophotometer (Molecular Devices SpectraMax PLUS 384, with Softmax PRO software, Sunnyvale, CA, USA). A serial dilution series was generated with DNA concentrations from 10 ng/μL to 1 fg/μL.

Alignment Strategy and Primer Design

One hundred SINE elements sequenced from the domestic cat MHC (16), (GenBank accession numbers, AY152825, AY152829, and AY152836), were aligned by use of CLUSTALX (21) and visually checked. Three sets of primers were designed in regions of sequence conservation with Primer 3.0 (22). These included primer set 1: (forward) TGGGTGGCTCAGTCAGTTAAG and (reverse) AGAAGCCGAAGCAGGCTC; primer set 2 (forward) GGTGGCTCAGTCAGTTAAGCA and (reverse) AGAAG CCGAAGCAGGCTC; and primer set 3 (forward) TGGGTGG CTCAGTCAGTTAAG and (reverse) GAAGCAGGCTCCAGG CTC to amplify products of 123 bp, 121 bp, and 116 bp, respectively.

PCR Amplifications

PCR amplifications were performed in 20 μL reaction volumes, with an ABI SYBR® Core Reagents Kit with 1 X SYBR Core PCR buffer containing SYBR® green; 2.0 mmol/L magnesium chloride ($MgCl_2$), 250 μmol/L of four deoxyribonucleoside 5′-triphosphates (dATP, dCTP, dGTP, and dUTP), 1.0 unit of AmpliTaq gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 0.4 μmol/L final primer concentration of forward and reverse primer (Sigma Co., St. Louis, MO, USA), 0.2 μL of AmpErase® uracil-N-glycosylase (UNG, Applied Biosystems), and 1 μL of DNA. The initial hot start procedure and the use of enzyme AmpliTaq gold minimized the generation of non-specific products during PCR. Amplifications were performed in an ABI PRISM 9700 Sequence Detector (PE Biosystems, Foster City, CA, USA) under the following conditions: 2 min at 50 °C for activation of UNG, 10 min at 95°C, followed by 35 cycles of 1 min at 95 °C, 1 min at 50 °C, and 1 min at 72 °C. The purpose of dUTP used in PCR amplifications and pre-incubation with UNG (1 U/100 μL) was to prevent contamination of reactions with prior PCR products by degrading any contaminating products before amplification. ABI PRISM 7700 Sequence Detection software (version 1.7a; Applied Byosystems) was used to calculate threshold cycle values. Three replicate assays were performed.

Results

Three sets of primer pairs designed to amplify feline SINE elements were tested for product quality and sensitivity. PCR product generated with the three separate primer pairs amplified in multiple dilution series of genomic DNA demonstrated that the three

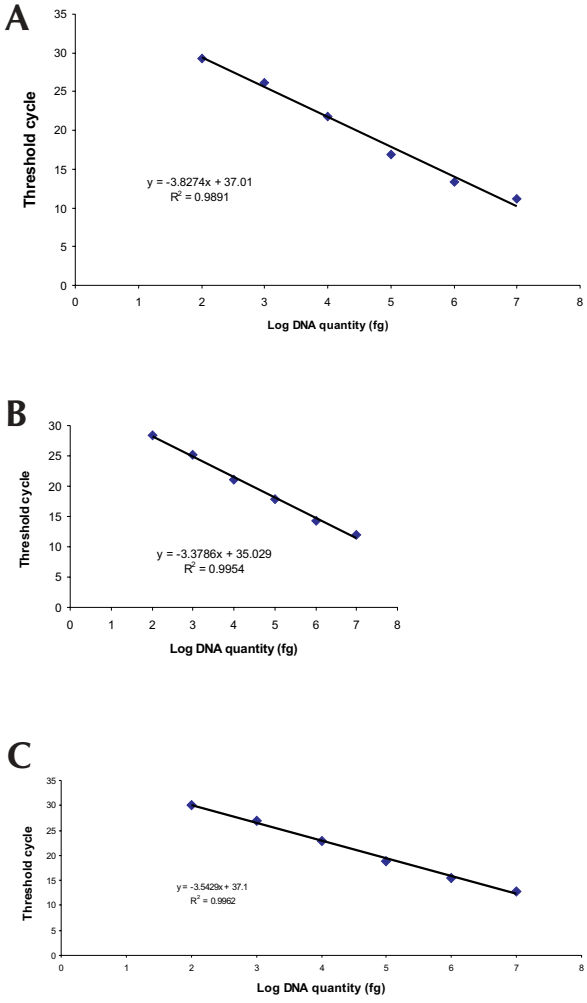

Figure 1. Relationship of DNA concentration to threshold cycle (Ct) for short interspersed nuclear elements (SINE) primer sets 1-3. Ct values observed from amplifications of SINE primer sets 1-3 are presented in the figures A-C, respectively. Amplification was performed in a domestic cat genomic DNA dilution series by using 1 μL of indicated DNA quantity in a 20 μL reaction. Thirty cycles of amplification were performed. Threshold cycle was measured as the number of cycles required to reach a ΔRn of 0.205 fluorescence units.

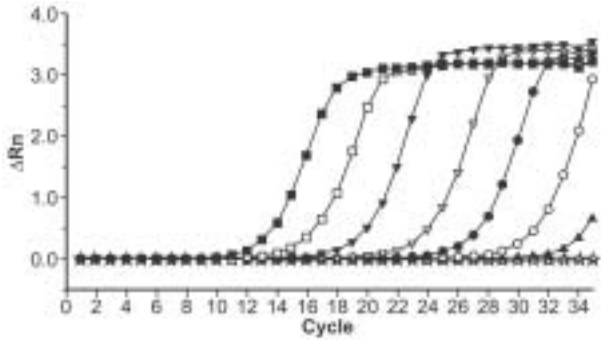

Figure 2. Fluorescence profile of short interspersed nuclear elements (SINE) primer set 2 generated in serial dilutions of cat genomic DNA. One μL of DNA was amplified in a reaction volume of 20 μL. Thirty-five cycles of amplification were performed. ΔRn – fluorescence units; cycle – polymerase chain reaction cycle number; DNA amounts (fg): 10^7 – full square; 10^6 – empty square; 10^5 – full triangle reversed; 10^4 – empty triangle reversed; 10^3 – full circle; 10^2 – empty circle; 10^1 – full triangle; 1 – empty triangle; star – no template control.

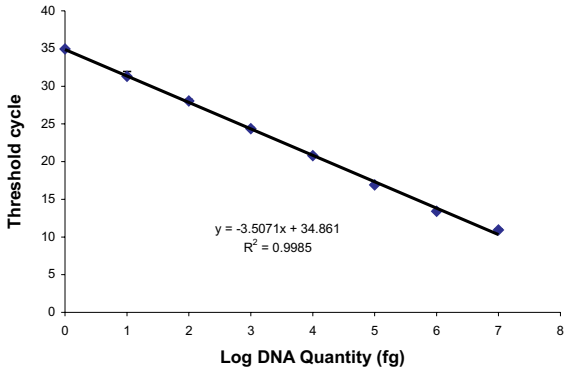

Figure 3. Relationship of DNA concentration to threshold cycle (Ct) observed for primer set 2. Average Ct values observed from three independent amplifications of short interspersed nucleotide elements (SINE) primer set 2 in a domestic cat genomic DNA dilution series using 1 FL of indicated DNA quantity in a 20 FL reaction. Bars indicate one standard deviation. Thirty-five cycles of amplification were performed. Threshold cycle was measured as the number of cycles required to reach a ΔRn of 0.205 fluorescence units.

primer pairs generated products of expected size and were highly sensitive. Quantitative PCR product profiles generated from the three sets of primers in a serial dilution of domestic cat DNA from 10 ng to 1 fg (in a 20 μL reaction volume) demonstrated equivalent sensitivity. PCR product was generated from as little as 100 fg of DNA following 30 cycles of amplification. Figures 1 A-C demonstrate standard curves of threshold cycle values generated with the three primer sets in the dilution series. Figure 2 demonstrates a fluorescence profile of products generated from SINE primer set 2 in the dilution series exhibiting higher threshold cycle values with decreasing amounts of target DNA. Figure 3 demonstrates a standard curve of threshold cycle values generated from three replicate runs amplified with primer set 2 following 35 cycles of amplification. Standard deviations for the 8 data points are presented in Table 1. The relationship between threshold cycle value and the logarithm of the DNA concentration was linear over the detection limit of the assay representing a 10^7 dilution range, with a trendline correlation coefficient of 0.9985. The domestic cat SINE-based quantitative PCR assay demonstrated sensitivity to a concentration of 10 fg of DNA in a reaction volume of 20 μL.

Table 1. Threshold cycle (Ct) values and standard deviations observed for standard curves of the feline short interspersed nucleotide elements (SINE) quantitative polymerase chain reaction PCR assay

DNA amount (fg)	Ct value (mean ± SD)
NT*	35.00 ± 0.00
1	34.94 ± 0.06
10	31.32 ± 0.64
10^2	28.03 ± 0.20
10^3	24.37 ± 0.14
10^4	20.78 ± 0.14
10^5	16.91 ± 0.12
10^6	13.41 ± 0.12
10^7	10.92 ± 0.15

*NT – no template control.

A potential problem with the use of DNA double-stranded binding dye in quantifying PCR product is that the dye binds to all double-stranded products and does not distinguish between the target sequence and non-specific products generated during PCR. The generation of primer dimers to quantify genomic DNA would be of major concern in this assay. The absence of primer dimers as visualized following electrophoresis of products in agarose gels (data not shown), the total absence of fluorescence in quantitative PCR in water controls (Fig. 2), and the linearity of values with respect to DNA concentration demonstrate the absence of primer dimers and the robustness of the assay.

To examine the specificity of the feline assay, the primers were amplified in DNA in a wide range of North American mammalian species. PCR products of anticipated size were generated in DNA of badger, bear, beaver, chipmunk, cow, coyote, deer, dog, ferret, fox, goat, guinea pig, hamster, horse, human, mink, mole, mouse, ocelot, opossum, otter, pig, puma, rabbit, raccoon, rat, sheep, skunk, and wolf (data not shown).

Discussion

We have designed a PCR assay to quantify feline nuclear DNA using amplification and detection of highly repetitive SINE elements. Three sets of primer pairs were designed from overlapping regions in an alignment of SINE elements sequenced in the MHC of the domestic cat (14). They demonstrated equivalent sensitivity in quantitative assays generated from a dilution series of domestic cat DNA. The assay was highly sensitive, could be performed rapidly by using trace amounts of DNA, and detected feline genomic DNA at a concentration of 10 fg in a 20 μL reaction.

In quantitative PCR, the quantity of the targeted product is measured by using the detection of fluorescent labeled product at the completion of each PCR

cycle. An arbitrary threshold cycle is selected where an increase in signal fluorescence is associated with the exponential growth of the product. A standard curve of threshold cycle values, generated from a dilution series of known DNA concentration, can be used to interpolate the concentration of an unknown sample. The feline assay monitors product accumulation through a fluorescent dye, which binds to double-stranded DNA (ie, SYBR® Green I) (23). We elected to use this detection method due to the high degree of sequence diversity exhibited in the 437 SINE elements sequenced in the domestic cat MHC (16). Use of double-stranded DNA binding dye maximizes sensitivity of a quantitative assay of feline genomic DNA, as it detects the population of SINE elements amplified. A second method of product detection often used in quantitative PCR employs a 5-exonuclease detection method, utilizing a fluorgenic labeled probe complementary to the amplified sequence (24). This technique requires a high degree of specificity between probe and amplified sequence and would not have been as sensitive as the method we employed, detecting a subset of SINE elements.

The assay was designed to maximize sensitivity by targeting a high proportion of SINE elements. Primers were designed in a region (polymerase region) that exhibited a high degree of sequence conservation across the aligned SINE elements. These regions also demonstrated a high degree of sequence conservation across species. We do not think that this lack of species specificity deters the utility of the assay, as DNA mixtures are unlikely to be an issue with feline samples. We would anticipate that the majority of feline samples will be hair isolates, where DNA will be extracted from a minimal fraction of hair comprising the root and washed before sample extraction.

The assay is an integral part of an STR typing system we have developed for the genetic individualization of domestic cat specimens (9,10). A set of 11 tetranucleotide STR loci have been isolated, mapped, and characterized from the cat genome (9). A multiplex amplification protocol for the 11 STR loci and a gender identifying sequence tagged site from the feline SRY gene has been optimized (10), which is robust and highly specific for the feline family. The 11-member panel exhibited relatively high heterozygosity in an initial sample collection of 28 cat breeds (n = 223) with an average composite locus heterozygosity of 0.73 across the breeds, ranging from 0.59 to 0.86. The power of discrimination of the panel is high in the sample set of cat breeds examined ($Pm = 5.5 \times 10^{-7}$-3.3×10^{-13}). The multiplex is in the final stages of genotyping in a sample collection of approximately 1,200 cats representing the major cat breeds currently recognized in the United States.

The feline STR multiplex panel has been successfully amplified and genotyped with 0.7 ng of DNA (10), although the sensitivity of the multiplex is still under examination. The sensitivity of the SINE assay will allow investigators to determine whether there is sufficient yield from single hair isolates to perform STR analysis. Consider a case scenario where the total yield of DNA from a single hair is 0.7 ng in an extraction volume of 100 μL, an adequate amount of DNA to successfully genotype the STR multiplex panel. A single μL of extract (7 pg) could be quantified with the feline SINE assay, allowing an investigator to determine that there is a sufficient quantity of DNA to perform the single amplification of the STR multiplex without compromising yield for STR analysis in the quantification process.

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