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TECHNICAL NOTE

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Real-Time Polymerase Chain Reaction Quantification of Canine DNA*

ABSTRACT: The accurate quantification of target DNA is an important step in the short tandem repeat analysis of forensic biological samples. By utilizing quantification data to control the amount of template DNA in the polymerase chain reaction (PCR), forensic scientists can optimize testing and minimize the consumption of limited samples. The ability to identify and quantify target DNA in mixed-species samples is crucial when it may be overwhelmed by nontarget DNA, as in cases of dog attack. We evaluated two quantitative real-time PCR assays for dynamic range, species specificity, and inhibition by humic acid. While both assays proved to be highly sensitive and discriminating, the *Melanocortin-1 Receptor* (*MC1R*) gene Taqman® assay had the advantages of a shorter run time, greater efficiency, and safer reagents. In its application to forensic casework, the *MC1R* assay has been advantageous for quantifying dog DNA in a variety of mixed-species samples and facilitating the successful profiling of individual dogs.

KEYWORDS: forensic science, DNA quantification, real-time PCR, QPCR, canine, dog, *MC1R*

DNA quantification of human forensic samples is routinely performed to optimize the polymerase chain reaction (PCR) of multiplexed short tandem repeat (STR) markers (1). Because commercially available genotyping kits function best within a fairly narrow range of template DNA concentration, accurate quantification promotes the fidelity of STR profiles by reducing PCR artifacts such as peak imbalance and allelic dropout in low DNA concentration samples as well as baseline pull-up and increased stutter product in high DNA concentration samples. This can prevent reanalysis of potentially limited samples.

The challenges of analyzing animal trace DNA samples are essentially the same as those faced by human forensic laboratories. In addition, evidence often contains mixtures of DNA from different species as in cases of dog attacks on people or other animals. Evidence from fatal dog maulings usually contains large amounts of human blood and tissue intermingled with canine saliva and hairs. Effective analysis of such evidence necessitates accurate quantification of the target DNA.

It has been demonstrated that real-time PCR assays are more sensitive and more accurate than dot or slot-blot assays commonly used in human forensics (2–5). Furthermore, blot-based assays require subjective assessment, while real-time PCR methods do not (2). As quantitative real-time PCR (QPCR) determines the concentration of target DNA by quantifying amplicons throughout amplification, it facilitates *a priori* determination of the success of applications utilizing PCR.

QPCR assays monitor fluorescence during PCR and record the cycle number where exponential growth is achieved. This cycle threshold (C_T) value is compared with a series of standards to determine the concentration of the unknown sample (6). One type of real-time PCR assay is the TaqMan® (6), which incorporates a probe attached to a reporter dye that fluoresces when it is cleaved by the polymerase during primer extension. A second assay utilizes SYBR® Green I dye (7) that fluoresces when bound to double-stranded DNA.

We evaluated two QPCR assays for detecting and quantifying forensic canine DNA: the canine *Melanocortin-1 Receptor* (*MC1R*) gene, and a canine short interspersed element (SINE) reported by Walker et al. (8). The *MC1R* assay was developed for the TaqMan® platform, while the SINE assay utilized SYBR® Green I dye.

Our criteria for selecting a QPCR target region were that the sequence was conserved within a species and between closely related species, that it contained enough sequence variation between taxonomic families to exclude nontarget DNA, and that sequence data were readily available for a variety of species. Because *MC1R* is involved in hair and fur coloring (9,10), it is a gene that is of interest and has been extensively characterized for a number of species. We designed our probe to target the coding region of the *MC1R* gene that codes for a seven-pass transmembrane G-protein-coupled receptor that is transcribed from a single exon. It is involved in the determination of hair color by promoting the production of eumelanin that causes the black or brown coloration of hair and fur. Our goal was to exploit the sequence information available for this gene to design a suite of QPCR assays for quan-

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tifying the DNA of a variety of species. Here, we present our findings for species specificity and inhibition studies carried out with the canid *MC1R* and the canine SINE assays. We also demonstrate the efficacy of adjusting template DNA concentration based on quantification data in the analysis of casework samples.

Methods

Primer and Probe Design

The TaqMan® probe (Applied Biosystems, Foster City, CA) and flanking primer sequences were designed using Primer Express 2.0 software (Applied Biosystems). Sequence information was obtained from GenBank, Schmutz et al. (10), and Newton et al. (9). *MC1R* sequences for Doberman, Newfoundland, Flat-coated Retriever, Black Labrador, Yellow Labrador, Irish Setter, Red Fox (*Vulpes vulpes*), and Artic Fox (*Alopex lagopus*) were aligned, and a series of candidate probes were generated for a region that was conserved between breeds and across canid species. These probes were then aligned with noncanid *MC1R* sequences (human, horse, cow, pig, zebra, sheep, goat, domestic cat, and jaguar) to determine which were canid specific. The probe that met our criteria, K9MC1R-P 5′-CTGCGTCTTTCAGAACT-3′, was labeled with a 6FAM reporter at the 5′-end and a nonfluorescent quencher that was bound to an MGB (minor groove binder) at the 3′-end (Applied Biosystems). The flanking PCR primers that were initially chosen, K9MC1R-F 5′-GCCCTCAACACCCCATCTG-3′ and K9MC1R-R 5′-TGATGAGGGTGAGGAAGAGGTT-3′, were evaluated for sensitivity and efficiency using QPCR with SYBR® Green I (Cambrex, Rockland, ME). Quantification of 183 archived dog buccal swab extracts yielded one sample with a lower than expected result. Sequencing of the amplicon region showed this dog (a Siberian Husky cross) to be heterozygous for a two base pair deletion near the 3′-end of the K9MC1R-F primer-binding site. The primer was redesigned by moving the K9MC1R-F primer eight bases (5′-TGGTCCTCTGCCCTCAACA-3′), and the quantification of the Siberian Husky cross fell within the expected range. For validation, 252 DNA samples representing both purebred dogs from 20 AKC-recognized breeds and mixed-breed dogs were quantified with this new forward primer. SINE-based quantification was performed using the published primers of Walker et al. (8).

Thermal Cycling Parameters

Real-time PCR amplification of *MC1R* was carried out in a 25 μL reaction containing 12.5 μL 1 × TaqMan® Universal Master Mix (Applied Biosystems), 250 nM probe, 300 nM primer, and 1 μL of sample. The SINE assay used 50 nM primer and a 1:25,000 dilution of SYBR® Green I Nucleic Acid Stain (Cambrex) in addition to 12.5 μL 1 × TaqMan® Universal Master Mix and 1 μL sample in a 25 μL reaction. The real-time thermal cycling parameters consisted of 10 min at 95°C, and 40 cycles of 1 min at 60°C and 15 sec at 95°C. In the experiments using SYBR® Green I for detection, an additional disassociation step was added that consisted of a slow (*c*. 1 h) ramping upward from 60 to 95°C as the instrument recorded the fluorescence. All quantification experiments were carried out on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems).

Standard Curve

To establish a standard curve, DNA was isolated by subjecting dog whole blood to proteolysis with proteinase-K, followed by phenol–chloroform extraction (11). The standard curve samples were purified by size exclusion using a Microcon YM-100 (Millipore, Bedford, MA) filter unit and further purified by ethanol precipitation. Total DNA was then diluted in series and quantified by measuring the absorbance at 260 nm on a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany) according to the manufacturer's recommendations. The concentration determined for the original extract was used as a reference for subsequent work.

Experiments—To evaluate the functional detection range of the QPCR assays, samples with known concentrations were serially diluted and then quantified using both methods. The standard curve correlation coefficients were calculated using Pearson's correlation (R^2).

To test the effects of inhibitors on assay sensitivity, we added humic acid, which is an inhibitor commonly found in decomposing organic matter (12,13). A set of mixtures containing 10 ng/μL of dog DNA and serially diluted 0.001% humic acid was prepared (14,15). One microliter of each dilution was quantified on the ABI 7300 using both the SINE and the *MC1R* assays. This experiment was conducted on DNA from four unrelated dogs.

To evaluate the species specificity of the SINE and *MC1R* assays, we tested them on 10 ng of goat, cattle, sheep, deer, human, domestic cat, bobcat, coyote, and fox DNA extracts prepared in the same manner as the dog DNA for the standard curve. To simulate the effects of admixture with a noncanid source, we combined target dog DNA with each of the following: cat, cattle, human, horse, goat, and deer in 1:1 (10 ng/μL each) and in 100:1 (10 ng/μL noncanid: 0.10 ng/μL dog) dilutions. This experiment was run on DNA from four unrelated dogs.

Results and Discussion

Sensitivity

The SINE SYBR® Green I and the *MC1R* TaqMan® assays showed sensitivity down to 5 pg of DNA (Fig. 1). For higher concentrations of DNA, the integrity of the standard curve in the TaqMan® assay was maintained throughout samples containing up to 58 ng of DNA (standard curve correlation $R^2 > 0.99$). The TaqMan® assay showed the greatest consistency on concentrated samples while displaying sensitivity to low template samples. In

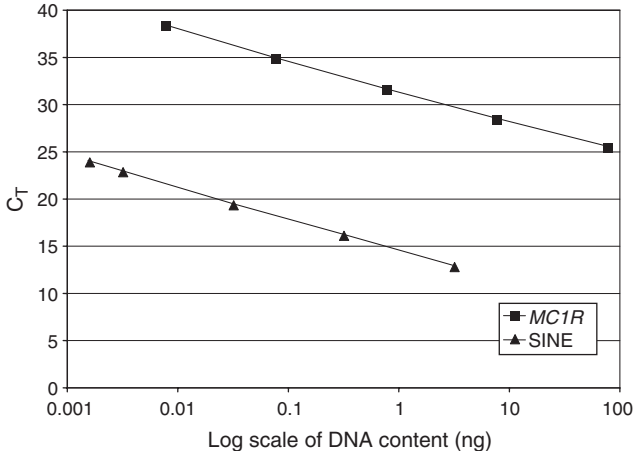

FIG. 1—*Efficiency of quantitative real-time PCR as measured by the slopes of the standard curves was closer to the theoretical maximum of −3.32 with the Melanocortin-1 Receptor (MC1R) assay (−3.45 or 95% efficiency) than with the short interspersed element (SINE) assay (−3.66 or 90% efficiency).*

addition, the TaqMan® assay has a potentially shorter run time because there is no disassociation curve.

The SINE assay showed lower than expected C_T values for samples over 14.5 ng. When the DNA samples with a higher concentration (58 and 29 ng) were included, the integrity of the standard curve for the SINE assay was reduced (standard curve correlation $R^2 < 0.99$) compared with the standard curve created when the higher concentration DNA samples were omitted (standard curve correlation $R^2 > 0.99$). It has been suggested that this loss of sensitivity is due to insufficient SYBR® Green I and that the addition of more dye to the reaction may improve the performance on high-concentration samples (16), but these experiments were not performed. The SINE assay reported DNA concentrations down to 0.5 pg of DNA, but this result varied. The multiple genomic copies of SINEs allow the quantification of a fraction of a single cell's DNA. This is of debatable use in STR applications where most STR loci are diploid. In addition, the SINE used in this study contains regions that are not well conserved (17). This can lead to sequence variation within an individual and variable copy numbers across individuals affecting the accuracy of quantification results.

Inhibition Effects

For all of the humic acid-inhibited samples, both *MC1R* and SINE assays failed to detect the sample when the humic acid concentration was greater than 0.0001% (Table 1). The reactions containing 0.0001% humic acid amplified in both assays, but they had a higher C_T value than the control sample and the plot of fluorescence versus cycle number yielded a lower exponential growth curve (Fig. 2). Quantification of the 0.00005% humic acid samples gave C_T values similar to undiluted samples.

As these assays rely on PCR, the ability to quantify samples can be affected by inhibitors. In the TaqMan® assay, an inhibited sample yields the same result as a no-template control reaction. Partial inhibition can often be inferred by examination of the plot of fluorescence versus C_T and is evidenced by an exponential growth of PCR product that is slower than the standard curve. Modification of the TaqMan® protocol to include a serial dilution of the sample consumes a very small amount of material and can restore the ability to quantify target DNA in inhibited samples. A determination can then be made whether or not additional sample purification is needed. SYBR® Green I assays have an advantage over TaqMan® assays on inhibited samples because of the characteristic PCR artifact that is expected in no-template controls during later cycles. The absence of this artifact in the disassociation curve indicates a probable PCR inhibitor (7).

Both real-time detection methodologies used here showed similar performance with partially inhibited samples. It should be noted that different polymerases respond differently to various inhibitors (18,19) and that the enzyme used in the real-time PCR may not entirely reflect inhibition of genotyping performed with a different enzyme.

TABLE 1—*Mean (n = 5) C_T values for inhibition series on 10 ng of dog DNA.*

Humic Acid Concentration (%)	C_T	
	MC1R	SINE
No inhibitor	27.6 ± 0.17	11.9 ± 0.15
0.0002	No result	No result
0.0001	30.4 ± 1.74	20.8 ± 1.97
0.00005	27.8 ± 0.26	13.1 ± 0.51

SINE, intrashort interspersed element.

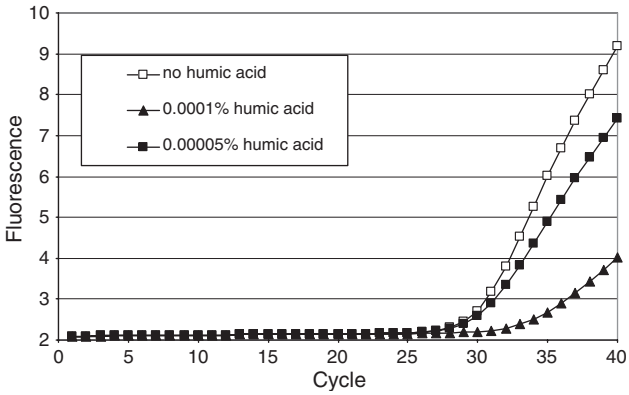

FIG. 2—*Effects of a polymerase chain reaction (PCR) inhibitor on quantification using Melanocortin-1 Receptor (short interspersed element data not shown). The lower slope of the more inhibited sample (▲) is symptomatic of weak PCR inhibition.*

Species Specificity

The K9MC1R probe did not detect DNA from goat, cattle, sheep, elk, deer, human, domestic cat, or bobcat. As expected, it detected both fox and coyote DNA (data not shown). Experiments with dog and noncanid sample mixtures (Table 2) showed that in 1:1 ratios (0.10 ng each), DNA quantification was not affected by the other species tested (cat, cattle, horse, elk, deer, goat, human). In the 100:1 experiments with *MC1R* (Column A) in which 0.10 ng of dog DNA was mixed with 10 ng of either cat or horse DNA, the plots of fluorescence versus cycle were similar to the exponential growth curves of partially inhibited samples. However, the C_T values generated at the point of exponential growth did not change despite the reduction in the steepness of the curve. These results indicate that horse and cat DNA may have a dampening effect on the ability of the K9MCIR TaqMan® probe to detect canine DNA in sample mixtures greater than 100:1.

The SINE assay detected dog DNA without any discernible interference from nontarget species DNA (Column B) as evidenced by the fluorescence v cycle number data and the disassociation curve data. The assay produced a signal at a high C_T in noncanid DNA samples that was comparable to the artifact observed in the no-template PCR control. The lower C_T values observed are a reflection of the higher copy numbers of SINEs in the genome.

Casework

We have successfully incorporated the canine *MC1R* assay into routine analysis of casework involving canine biological material.

TABLE 2—*Mean (n = 4) C_T values for dog DNA in mixed-species samples (noncanid:dog).*

Species	(A) *MC1R* (ng)		(B) SINE (ng)	
	10:10	10:0.10	10:10	10:0.10
Dog control	25.68 ± 0.10	32.92 ± 0.29	11.00 ± 1.28	20.12 ± 0.91
Cat	25.87 ± 0.21	33.11 ± 0.71	11.11 ± 1.24	18.75 ± 1.01
Cattle	25.48 ± 0.16	33.30 ± 0.41	10.31 ± 0.54	18.83 ± 0.58
Deer	25.96 ± 0.24	32.84 ± 0.56	11.32 ± 1.04	19.32 ± 0.78
Goat	26.18 ± 0.10	32.21 ± 0.94	11.18 ± 1.03	19.11 ± 0.86
Horse	26.11 ± 0.14	33.50 ± 0.13	11.54 ± 0.82	18.98 ± 0.86
Human	26.41 ± 0.27	32.50 ± 1.26	11.42 ± 1.14	19.11 ± 0.96

SINE, intrashort interspersed element.

The use of the K9MC1R probe to quantify canine DNA has allowed us to optimize the PCR for mixed-species, low copy number, and inhibited samples. Extracted material that exhibits little or no canine DNA upon quantification is set aside in favor of analyzing samples containing more canine DNA. Samples evidencing inhibition are amplified with bovine serum albumin or receive additional purification.

In one case of a fatal dog attack on a 6-year-old boy, the boy's clothing as well as swabs from his wounds were submitted for analysis. As three dogs were identified as potential offenders, numerous samples were collected and tested in an effort to identify all of the dogs involved. Areas with rips were targeted as possible bite sites; eight cuttings were taken from the boy's shirt, six from his pants, and two from his underwear. These samples were extracted using a phenol:chloroform protocol and quantified with the *MC1R* assay. Nine of the 16 extracts—including three that QPCR identified as being inhibited—yielded quantifiable DNA ranging from 23 to 0.050 ng/μL. Those nine extracts were then amplified using an in-house panel of 15 canine-specific STRs with extract volumes adjusted to provide optimal template DNA. Full DNA profiles were obtained for all nine samples. The DNA on the boy's pants matched a German Shepherd mix and the DNA from the boy's shirt matched a Pit Bull Terrier mix. Swabs of bite wounds generally yield poor results due to medical treatment and cleansing of the wounds. Of the 40 wound swabs collected, 12 were extracted and quantified, and seven of those yielded trace amounts of DNA (<0.007 ng/μL). Only one swab yielded a partial DNA profile (four markers), and it was consistent with the Pit Bull Terrier mix. None of the evidence indicated contribution from a third dog.

In dog attack cases where there is abundant evidentiary material and the dogs involved are presumably minor contributors to the total DNA content, QPCR has proven to be invaluable for increasing genotyping success by identifying samples with sufficient dog DNA and those that are inhibited.

Conclusions

Both the *MC1R* TaqMan® assay and the SINE SYBR® Green I assay are effective tools for the quantification of dog DNA; both are canid-specific and sensitive to approximately one cell's DNA mass. We chose the *MC1R* TaqMan® assay over the SINE SYBR® Green I assay for use on forensic casework because of the shorter run time, the toxicity of SYBR® Green I, and the belief that the diploid copy may be more predictive of STR genotyping success than the multiple (and variable) copy numbers of SINEs.

We have demonstrated that QPCR can promote successful STR genotyping of evidentiary samples with a low DNA content. The two primary limiting factors with respect to the accuracy and reproducibility of QPCR assays are the accuracy of pipetting and the quality of the standard curve dilutions. To counter potential pipetting discrepancies, increasing the template volume to more than 1 μL or use of a robotic system for PCR setup would improve assay precision. As the target DNA concentration is measured relative to a standard, it is imperative that the standard be accurately quantified. A stable, calibrated reference standard will enhance the accuracy of these assays.

The accurate quantification of canid template DNA using the *MC1R* assay has improved the efficacy of genotyping challenging samples by identifying those most likely to yield STR profiles. Furthermore, by optimizing DNA template in the PCR reaction, we have reduced sample consumption, turn-around time, and costs related to testing.

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