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PAPER

CRIMINALISTICS

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Developmental Validation of Feline, Bovine, Equine, and Cervid Quantitative PCR Assays*

ABSTRACT: Accurate DNA quantification is essential for optimizing DNA testing and minimizing sample consumption. Real-time quantitative polymerase chain reaction (qPCR) assays have been published for human and canine nuclear DNA, and the need for quantifying other forensically important species was evident. Following the strategy employed for the canine qPCR assay, we developed individual assays to accurately quantify feline, bovine, equine, and cervid nuclear DNA. Each TaqMan-based assay incorporates a genus-specific probe targeting the *Melanocortin-1 Receptor* gene and includes a piece of synthetic DNA that acts as an internal PCR control for detecting inhibition. Developmental validations were carried out following the revised guidelines of the Scientific Working Group on DNA Analysis Methods with modifications necessary for validation of non-human qPCR assays. All assays demonstrated the specificity, sensitivity, stability, reproducibility, accuracy, and precision required for forensic casework. The application of these assays to animal forensic DNA analysis has both conserved laboratory resources and improved genotyping results.

KEYWORDS: forensic science, DNA quantification, real-time PCR, quantitative PCR, bovine, feline, cervid, equine, cow, cat, deer, horse, *MC1R,* IPC

Quantification of DNA in sample extracts as a preliminary step to further DNA analysis by short tandem repeat (STR) typing has been used to ensure robust, reliable, and reproducible DNA results as well as to conserve valuable forensic samples (1,2). With the commercial kits available today for analyzing human DNA, there is an optimal concentration range for the amount of DNA in the reaction, usually between 0.5 and 2.5 ng (3–5). When too much target DNA is added, off-scale fluorescent peaks are seen in the electropherogram and the sample becomes difficult if not impossible to read (6,7). When too little DNA is added, stochastic effects, such as allelic dropout (5,8,9) and peak imbalance (6), are observed. DNA quantification prior to STR analysis has become an integral part of DNA analysis in forensic laboratories and is required for laboratory accreditation under ASCLD/LAB Legacy (10) and mandated by the DNA Advisory Board (11).

As yet, there are no commercial kits available for animal DNA quantification. The animal STR genotyping methods utilized currently have been developed to mimic commercial kits available for human DNA testing and have a range of optimal DNA input concentrations similar to human kits. This makes quantification necessary. DNA quantification is also important when mixtures are involved. A case may involve an attack of one species upon another, and the DNA contribution from the target animal needs to be quantified to accurately genotype it. Accurate and efficient analysis of these cases necessitates sensitive and discriminating quantification assays for the species being tested.

It has been shown that TaqMan®-based quantitative polymerase chain reaction (qPCR) assays (Applied Biosystems, Foster City, CA) provide a sensitive, accurate, and reproducible means of quantifying DNA (12–14). Unlike UV absorbance or slot-blot assays, the TaqMan assay is genus-specific and does not rely on subjective assessment (12). The multiple targets of Alu or SINE sequences have been used successfully for quantification (9,15,16), but the TaqMan assay utilizes a single target sequence that more closely mirrors the PCR that will be used in subsequent analysis. While other platforms for single target qPCR exist, including molecular beacons, Scorpion® probes (Sigma-Aldrich Corporation, St. Louis, MO), and the Plexor™ system (Promega Corporation, Madison, WI), the TaqMan platform has been successfully utilized for both dog and human qPCR assays (13,17) and has been routinely employed for animal casework by this laboratory since 2004. TaqMan-based qPCR has been thoroughly documented and is widely accepted by the forensic community (12,18,19).

The *Melanocortin-1 Receptor* (*MC1R*) gene was chosen as the target because it is conserved within each of the genera and between closely related species. It contains enough sequence variation to exclude nontarget DNA, and sequence data for the genera of interest were readily available (17). The *MC1R* gene is a single-copy gene that has been characterized extensively because of its involvement in hair and fur coloration (20–22) and has been successfully utilized as the target for our canine qPCR assay (17). In most cases, the differences between species, genera, or families are individual single nucleotide polymorphism (SNP) sites, and these were utilized in designing species and genus-specific primer and probe sets.

Included in each duplex assay, along with the *MC1R* target, is an internal PCR control (IPC; [23]). This segment of synthetic DNA along with associated primers and probe (24) is included as a

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control to verify that all reaction components are present and that the PCR occurs as expected. In addition to this function, the IPC determines the extent of inhibition in the sample. The more inhibition is present, the later in the PCR the IPC fluorescence will reach the threshold. With excessive inhibition, amplification never reaches the threshold, and the cycle at which the signal crosses the threshold (C_t) of the IPC is undetermined. This can be valuable information when analysts are making decisions as to how best to proceed with downstream analyses.

Assays were validated using the current guidelines set forth by the Scientific Working Group on DNA Analysis Methods (SWGDAM; [25]). These guidelines were developed to assist forensic DNA testing laboratories in assessing the reliability, reproducibility, and limitations of new procedures. As these assays are novel, they were tested rigorously under the developmental validation guidelines set forth by SWGDAM. Each aspect of the guidelines is addressed in turn—family/genus specificity, sensitivity, stability including inhibition, reproducibility, precision, and accuracy. The assays were also evaluated on multiple-species mixtures and simulated casework samples.

The development of qPCR assays for feline (*Felis*), bovine (*Bos*), equine (*Equus*), and cervid (*Odocoileus*) genera represents an expansion of previous research to include some of the diverse species encountered in veterinary forensic casework. Here, we present the validation of these new duplex assays.

Materials and Methods

Probe and Primer Design—MC1R and IPC

Sequences for the *MC1R* gene for the species of interest were retrieved from GenBank (26). Candidate primer and probe sequences were generated using Applied Biosystems Primer Express® software. Candidate probes were then aligned with all available *MC1R* sequence data, and only those probes that would theoretically exclude nontarget species or genera were selected. Candidate primer and FAM-labeled probe combinations were then screened for strength of PCR amplification and efficacy in qPCR. After primer sets for each assay were chosen, the optimal primer concentrations were determined (Table 1).

The IPC used in every assay is a published synthetic oligonucleotide sequence designed by Swango et al. (24). The primers and VIC-labeled probe (Table 1) were designed to avoid homology with known sequences found in the NCBI GenBank database and SNP sites that would interfere with primer or probe binding (24). The IPC template was diluted to working concentration (1:1 trillion dilution of 100 μM stock), aliquoted, and stored at −80°C until use.

Thermal Cycling Parameters

Amplification of each duplex reaction consisted of 12.5 μL TaqMan® Universal PCR Master Mix, No AmpErase® UNG, *MC1R* primer concentrations optimized for each reaction (Table 1), 250 nM *MC1R* probe, 100 nM IPC primers, 100–250 nM IPC probe, 60 million copies IPC template, and 1 μL sample for a final reaction volume of 25 μL. The IPC probe concentration was 100 nM for all assays except the bovine assay, which was 250 nM. All thermal cycling reactions were carried out on an Applied Biosystems 7300 Real-Time PCR system. The qPCR parameters were 10 min at 95°C, and 40 cycles of 1 min at 60°C and 15 sec at 95°C. Data were analyzed using the Applied Biosystems 7000 Sequence Detection Software.

Samples and Standards

Extracted tissue and blood samples were used as known-quantity high-quality DNA standards for the validation. Feline samples were both tissue (ovaries) and blood, bovine and equine samples were blood, and cervid samples included both tissue (muscle) and blood. The choice of sample source for the standards was determined by availability. All standard samples were extracted with an initial digestion in Proteinase K followed by a phenol:chloroform extraction (27) and an ethanol purification procedure (28), and then quantified independently, according to manufacturer's instructions, using a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany) monitoring absorbance at 260 nm. A standard curve was generated from a dilution series of this standard DNA. The standard curve was run in duplicate with every plate to ensure accurate quantification data.

Samples used in the validations included buccal swabs, hair samples, tissue samples, and blood samples. All sample types were extracted using the applicable extraction procedure from the Standard Operating Procedures of the Veterinary Genetics Forensic Laboratory. These procedures included phenol:chloroform for blood

TABLE 1—*Primer and probe sequences and concentrations.*

Oligonucleotide	Sequence	Concentration
Feline-F	CTT CAT CGC CTA CTA CGA TCA CA	300 nM
Feline-R	GGC CAT GAG CAC CAG CAT	600 nM
Feline-probe	FAM-CCT GCT CTG TCT CGT CA-MGBNFQ	250 nM
Bovine-F	AAT AAA TCA TAA ACC AGC CTG CTC TTC ATC AC	600 nM
Bovine-R	AAT AAA TCA TAA AGC TAT GAA GAG GCC AAC GA	600 nM
Bovine-probe	6FAM-CAC AAG GTC ATC CTG CTG TGC C-MGBNFQ	250 nM
Equine-F	CCT CTT CAT CGC TTA CTA CAA CCA	300 nM
Equine-R	CCA GCA TGG CCA CAA AGA A	300 nM
Equine-probe	FAM-CTG CTC TGT CTC GTC AC-MGBNFQ	250 nM
Cervid-F	AAT AAA TCA TAA GCA GCA GCT GGA CAA TGT CA	300 nM
Cervid-R	AAT AAA TCA TAA CGA TGG CGC CCA GGA	300 nM
Cervid-probe	FAM-CGA TGT GCT TAT CTG TG-MGBNFQ	250 nM
IPC-F	AAG CGT GAT ATT GCT CTT TCG TAT AG	100 nM
IPC-R	ACA TAG CGA CAG ATT ACA ACA TTA GTA TTG	100 nM
IPC-probe	VIC-TAC CAT GGC AAT GCT-MGBNFQ	100 or 250 nM
IPC template	AAG CGT GAT ATT GCT CTT TCG TAT AGT TAC CAT GGC AAT GCT TAG AAC AAT ACT AAT GTT GTA ATC TGT CGC TAT GT	60 million copies

F, forward; R, reverse; IPC, internal PCR control.

and tissue samples, a Protienase K digest for pulled hair samples, and a sodium hydroxide preparation (Yves Amigues, INRA, Jouey-en-Josas, personal communication) for buccal swab samples.

Upon completion of the validation studies, a total of 10 buccal swabs, 23 blood samples, 28 hair samples, and one tissue sample had been used for the feline validation; the bovine validation included 12 blood samples, 58 hair samples, and one tissue sample; the equine validation included eight blood samples and 61 hair samples; and the cervid validation included seven blood samples, 12 hair samples, 11 tissue samples, and 42 of unknown source (see Table 2). Samples were chosen that mimic the types of samples seen in forensic casework. For example, as we do not advise the collection of buccal swabs from horses or cattle because of the inhibition caused by the plant material in their mouths, this sample type was not included in the validation.

Specificity, Sensitivity, Reproducibility, Precision, and Accuracy

Family/genus specificity studies (SWGDAM 2.2) included a total of 11 different families including 23 genera and 31 species (Table 3). The feline assay was tested on 20 different breeds of the domesticated cat (*Felis catus*) as well as several other genera of felines including bobcat (*Lynx*), leopard (*Unica*), cheetah (*Acinonyx*), tiger and African lion (*Panthera*), and serval (*Leptailurus*). The bovine assay was tested on 10 different breeds of cattle (*Bos taurus*) as well as bison (*Bison bison*) and beefalo (hybrid *Bison bison/Bos taurus*). The equine assay was tested on five breeds of common horse (*Equus caballus*), as well as other species of the *Equus* genus including Grevy's zebra (*Equus grevyi*), Hartmann's mountain zebra (*Equus hartmannae*), kulan (*Equus hemionus hemionus*), onager *(Equus hemionus*), Przewalski's horse (*Equus przewalskii*), donkey (*Equus asinus*), and kiang (*Equus kiang*). The cervid assay was tested on multiple individuals from the two primary species in the United States: white-tailed deer (*Odocoileus virginianus*) and mule deer (*Odocoileus hemionus*; Tables 2 and 3).

Sensitivity studies (SWGDAM 2.3) were performed to determine the lower detection limit of each assay. For each assay, five dilution series were run and the lowest quantity that consistently yielded a detectable signal was recorded.

Reproducibility studies (SWGDAM 2.5) were carried out to confirm that uniform quantification data were obtained by multiple analysts. Two samples from each genus—*Felis*, *Bos*, *Equus*, and *Cervus*—were quantified three times each by two independent analysts to assess reproducibility.

Precision and accuracy studies (SWGDAM 2.9) were completed using the following methods (29). Precision was determined by calculating the standard deviation of the C_t value for 12 replicate quantifications of a sample run on the same plate. Five samples (ranging from 1 to 140 ng) were used for each species. Accuracy was determined by calculating the percent difference between the observed C_t for a sample and the expected C_t of the sample as determined by an independent method of quantification (spectrophotometry or fluorometry).

Inhibition and Mixtures

Even though qPCR methods have been shown to be robust regardless of sample type (24), the substrates and environmental factors commonly encountered in forensic casework were evaluated to identify their inhibitory effects. These studies assessed the ability of the IPC to detect varying amounts of inhibitors.

Environmental inhibition (SWGDAM 2.4) was evaluated using humic acid and hematin—both common inhibitors found in forensic samples. A dilution series of humic acid was prepared at 0.00005%, 0.0001%, and 0.0002% humic acid by volume. Likewise, a dilution series of hematin was prepared at 10, 15, 20, 25, 30, and 40 μM. One microliter of either humic acid or hematin dilution was added to the reaction.

Substrate inhibition (SWGDAM 2.4) was assayed by extracting and quantifying DNA recovered from common substrates. Whole blood was spotted on the following substrates: an exterior tree branch and laundered cotton fabric. After drying on the laboratory bench, swabs were taken of each genus' blood and extracted using an organic extraction protocol (27).

For each assay, source inhibition in case-type samples (SWGDAM 2.6) was assessed by extracting DNA from the common sample types for that genus. Typical case-type samples in an animal forensic laboratory vary depending on the genus. Whereas buccal swab reference samples or saliva evidentiary samples are common in cases involving domesticated cats and dogs, they are rare in cattle and horse cases. For each genus, the most common sample types were identified and used for this part of the validation.

Each assay was evaluated on common mixtures (SWGDAM 2.8) for that genus, and the precision, accuracy, and lower detection limits under mixed-source conditions were determined. Genera that have a predator–prey relationship with the target genus were tested, and human DNA admixture was tested to assess possible analyst contamination. The predator–prey mixtures chosen were those reflected by casework. For example, feline DNA mixed with bovine DNA in the case of an attack on livestock would be expected, whereas a mixed sample containing equine and bovine DNA would not. The feline assay was tested with human, dog, deer, elk, sheep, goat, and horse admixture DNA. The bovine, equine, and cervid assays were tested with bear, cat, dog, and human admixture DNA. One microliter of admixture DNA

TABLE 2—*Number of samples used.*

Organism	Scientific Name	Species Specificity	Reproducibility	Precision, Accuracy, and Inhibition	Total
Cattle	*Bos taurus*	6 (5)	58 (10)	7	71
White tail deer	*Odocoileus virginianus*	4	31	7	42
Mule deer	*Odocoileus hemionus*	3	20	7	30
Horse	*Equus caballus*	1	61 (5)	7	69
Cat	*Felis catus*	2	48 (20)	12	62
Dog	*Canis lupus familiaris*	2		15	17

Samples used in the validations of the feline, equine, bovine, cervid, and revised canine assays are indicated earlier. The number of individuals used in each study is given followed by the number of breeds (in parentheses) represented by those individuals, when applicable. Additional species tested as part of the family/genus specificity studies are detailed in Table 3.

TABLE 3—*Family/genus specificity.*

Organism	*n*	Scientific Name	Family	Feline	Equine	Bovine	Cervid
Cattle	6	*Bos taurus*	Bovidae	−	−	+	−
Duiker	1	*Cephalophus*	Bovidae	−	−	−	−
Goat	2	*Capra aegagrus hircus*	Bovidae	−	−	−	−
Sheep	6	*Ovis aries*	Bovidae	−	−	−	−
Bison	1	*Bison bison*	Bovidae	−	−	+	−
Water buffalo	4	*Bubalus bubalis*	Bovidae	−	−	−	−
Beefalo	1	*Bos taurus/Bison bison*	Bovidae	−	−	+	−
Llama	1	*Lama glama*	Camelidae	−	−	−	−
Domestic dog	2	*Canis lupus familiaris*	Canidae	−	−	−	−
White-tail deer	4	*Odocoileus virginianus*	Cervidae	−	−	−	+
Mule deer	3	*Odocoileus hemionus*	Cervidae	−	−	−	+
Elk	1	*Cervus Canadensis*	Cervidae	−	−	−	+
Horse	8	*Equus* sp.	Equidae	−	+	−	−
Bobcat	2	*Lynx rufus*	Felidae	+	−	−	−
Domestic cat	2	*Felis catus*	Felidae	+	−	−	−
Snow leopard	2	*Unica unica*	Felidae	+	−	−	−
Cheetah	1	*Acinonyx jubatus*	Felidae	+	−	−	−
Tiger	1	*Panthera tigris*	Felidae	+	−	−	−
Serval	1	*Leptailurus serval*	Felidae	+	−	−	−
Cougar	1	*Puma concolor*	Felidae	+	−	−	−
African lion	1	*Panthera leo*	Felidae	+	−	−	−
Human	1	*Homo sapiens*	Hominidae	−	−	−	−
Rabbit	1	*Oryctolagus cuniculus*	Leporidae	−	−	−	−
Mouse	1	*Mus musculus*	Muridae	−	−	−	−
River otter	1	*Lutra Canadensis*	Mustelidae	−	−	−	−
Black bear	1	*Ursus americanus*	Urisdae	−	−	−	−

Successful amplification with a particular assay is notated with (+) while no amplification is notated with (−); *n* denotes the number of individuals within that species that were tested, except in the case of horse where eight different species of horse were tested.

(10–20 ng, purified with ethanol precipitation [28]) was added to the reaction along with 1 μL of sample DNA (10–20 ng).

Canine IPC Integration

The IPC was integrated into our canine qPCR assay (17) subsequent to its publication. A validation of this duplex assay was performed in conjunction with the validation of the other four assays. Because the purpose of the IPC is to detect inhibition and provide a positive PCR control, this validation was limited to those areas that involve inhibition—specifically stability (SWGDAM 2.4) and casework-type samples (SWGDAM 2.6). These studies were carried out using the same methods as the other four assays.

While keeping the original reaction conditions and the original canine *MC1R* primers and probe the same, a set of IPC primers, probe, and IPC template were added to the reaction. This IPC set was the same as that for the other assays ([24]; Table 1) with the probe concentration at 250 nM.

Results and Discussion

While SWGDAM provides guidelines for developmental validation, the nonhuman nature of these assays required modifications and additions to those guidelines to fully evaluate the assays for forensic casework. In regards to specificity, the guidelines were written anticipating a species-specific assay, but these assays are genus or family specific. This required that a range of more distantly related samples be included in the validation as well as multiple individuals and breeds from the target species.

Family/Genus Specificity (SWGDAM 2.2)

Depending on the target organism, assays were designed to be either genus or family specific, not species specific. The feline assay showed accurate quantification throughout the Felidae family and showed no amplification in the other families. The bovine assay was specific to the Bos and Bison genera with two exceptions. Slight amplification was seen in one goat sample and in the duiker sample; however, the level of amplification in these samples was so low that the samples would be deemed insufficient for genotyping. The equine assay was shown to be genus specific and accurate for the eight species of equids tested, while no amplification was seen with any nonequid samples. The cervid assay amplified both white-tail deer and mule deer equally while showing an extremely low affinity for elk. This is to be expected as elk share a relatively recent ancestor with these common deer species ([30]; Table 3).

Sensitivity (SWGDAM 2.3)

The sensitivity studies that were performed to determine the lower detection limit of each assay established that the feline and equine assays had a lower detection limit of 15 pg (*c.* 2 copies), while the bovine and cervid assays were at 10 pg (*c.* 1 copies; Table 4). Some assays did show detection at amounts lower than these values; however, it was not repeatable between runs. It is suggested that at these levels, there are only a few copies of the target in the entire reaction volume, and the enzyme does not consistently find its target.

Reproducibility (SWGDAM 2.5)

All breeds and species tested showed successful quantification using their respective assays. Data received from each analyst were consistent showing between 3 and 37% coefficient of variation for all samples tested. The IPC indicated no inhibition and a low standard deviation between replicates of each sample (0.19–0.86; Table 5).

TABLE 4—*Precision, accuracy, and lower detection limit for each assay.*

	Feline Assay	Equine Assay	Bovine Assay	Cervid Assay
Precision	0.19/0.17	0.17/0.15	0.19/0.15	0.18/0.2
Accuracy (%)	4/4	5/5	6.5/5	6/6
Lower detection limit	15/10	15/10	10/4	10/50

Precision is measured as the standard deviation of the C_t for 12 replicates of the same sample; accuracy is measured as the percentage difference between observed and expected C_t for known quantity samples; and the detection limit is measured as the lowest quantity (pg) that the assay can consistently detect. Results are shown as results with no admixture DNA/results with admixture DNA.

TABLE 5—*Reproducibility for all assays.*

	DNA Quantity (ng/μL) Mean (%CV)	IPC C_t (cycles) Mean (SD)
Deer 1	16.28 (37%)	28.46 (0.19)
Deer 2	1.16 (19%)	28.35 (0.19)
Cow 1	9.3 (3%)	27.29 (0.25)
Cow 2	2.28 (6%)	27.20 (0.34)
Horse 1	3.47 (9%)	28.35 (0.50)
Horse 2	12.67 (12%)	28.54 (0.86)
Cat 1	0.21 (24%)	28.21 (0.27)
Cat 2	4.6 (14%)	29.10 (0.59)

Each sample was run in triplicate by each analyst and the mean quantity and coefficient of variation (%CV) determined. The mean C_t value for the IPC and the standard deviation (SD) is reported to estimate variation.

Precision and Accuracy (SWGDAM 2.9)

The precision and the accuracy of all assays were found to be within accepted limits (29) and is detailed in Table 4. For precision, the highest standard deviation of the C_t obtained from five samples of each species is reported. The standard deviation for each assay was <0.21 indicating that throughout the range of quantities tested, results were consistent. The method used for determining accuracy is completely dependent upon the accuracy of the independent method of quantification, and both independent methods produced consistent results for the sample types used. The qPCR assays yielded values that mirrored those obtained through the independent quantification methods, varying from the expected values by at most 1 C_t.

Inhibition and Mixtures (SWGDAM 2.4, 2.6, 2.8)

The IPC in the feline assay showed slight inhibition from the wood substrate and none from the cloth substrate. All other assay IPCs showed minimal inhibition with both substrate types. Because the wood substrate was taken from an uncontrolled environment, the inhibition seen in the feline assay is most likely attributable to inconsistencies of the substrate—perhaps the area used was one that had laid on soil, a source of humic acids. Some variation in IPC is also expected because of pipetting uncertainties. These variations are seen in uninhibited samples when the IPC is slightly (<0.5 C_t) different; it reaches the threshold either earlier or later than the IPC of the standard curve. As expected, the assays performed well under both substrate conditions, and the IPC indicated no significant inhibition in the samples (Table 6).

The assays demonstrated increased inhibition as the concentration of the environmental inhibitors hematin and humic acid increased. This was evidenced by depression of the C_t of the IPC as it crossed the threshold value of fluorescence at later and later points. All assays showed complete inhibition of the IPC at 40 μM hematin and 0.0002% humic acid (Table 6, Fig. 1).

TABLE 6—*Effects of inhibitors on the IPC.*

	Feline	Equine	Bovine	Cervid	Canine
10 ng mixture DNA	1.7	0.25	0.08	0.18	0.85
Buccal swab as source	0.00	NT	NT	NT	0.14
Hair as source	0.00	0.00	0.30	0.0	0.73
Blood as source	0.00	0.00	0.21	0.53	0.16
Cloth as substrate	0.00	0.00	0.00	0.28	NT
Wood as substrate	4.36	0.53	0.24	0.28	NT
0.00005% humic acid	5.43	5.11	1.20	0.71	6.90
0.0001% humic acid	—	—	1.80	2.17	—
0.0002% humic acid	—	—	—	—	—
10 μM hematin	0.4	0.16	0.43	0.00	2.57
15 μM hematin	1.11	1.20	0.83	0.42	5.25
20 μM hematin	4.15	4.50	0.21	0.80	6.51
25 μM hematin	7.73	4.65	6.49	8.41	—
30 μM hematin	11.42	8.52	—	11.28	—
40 μM hematin	—	—	—	—	—

The numbers indicate the difference in the C_t of the IPC in the presence of the listed inhibitor when compared to the C_t of the IPC when no inhibitor is present. When the difference in IPC C_t is <1.00, minimal inhibition is seen in the sample and further amplifications are not anticipated to be affected. If no result is given, the sample is completely inhibited, whereas if NT is indicated, the sample was not tested.

Sample source inhibition was estimated for the common source types for each assay (Table 6). All sample sources demonstrated minimal inhibition as determined by depression of the IPC by less than one cycle. It should be noted that the blood samples used in this validation study were purified using an ethanol purification protocol that is not a part of the standard casework protocols because of increased sample loss.

For all the mixtures tested for each assay, the genus of interest was quantified accurately in the presence of multiple DNA sources. The assays performed as expected and showed no significant deviation from precision, accuracy, and detection limit determinations performed without mixture DNA (Table 4). In some assays, the lower detection limit in the presence of admixture was observed to be lower than that for the unadulterated sample. Because the quantities being reported represent only a few cells, variation is expected at this low level.

Canine IPC Integration

Inhibition because of substrate was tested to evaluate the stability of the duplex. No difference was observed when cloth or wood was used as a substrate. Similarly, the effects of the environmental inhibitors hematin and humic acid were tested by their addition in dilution series to the reaction. These showed the expected depression of the C_t with increasing inhibitor concentration (Table 6). A total of 14 casework-type samples involving saliva, blood, and hair were tested. None of the extracts from these sample types showed inhibition (Table 6).

Casework

All four assays have been successfully integrated into the casework analysis process at the laboratory. In the same way that the canine qPCR assay has facilitated the identification (ID) of potentially probative samples and allowed determination of inhibition prior to genotyping, these four assays have enhanced casework workflow.

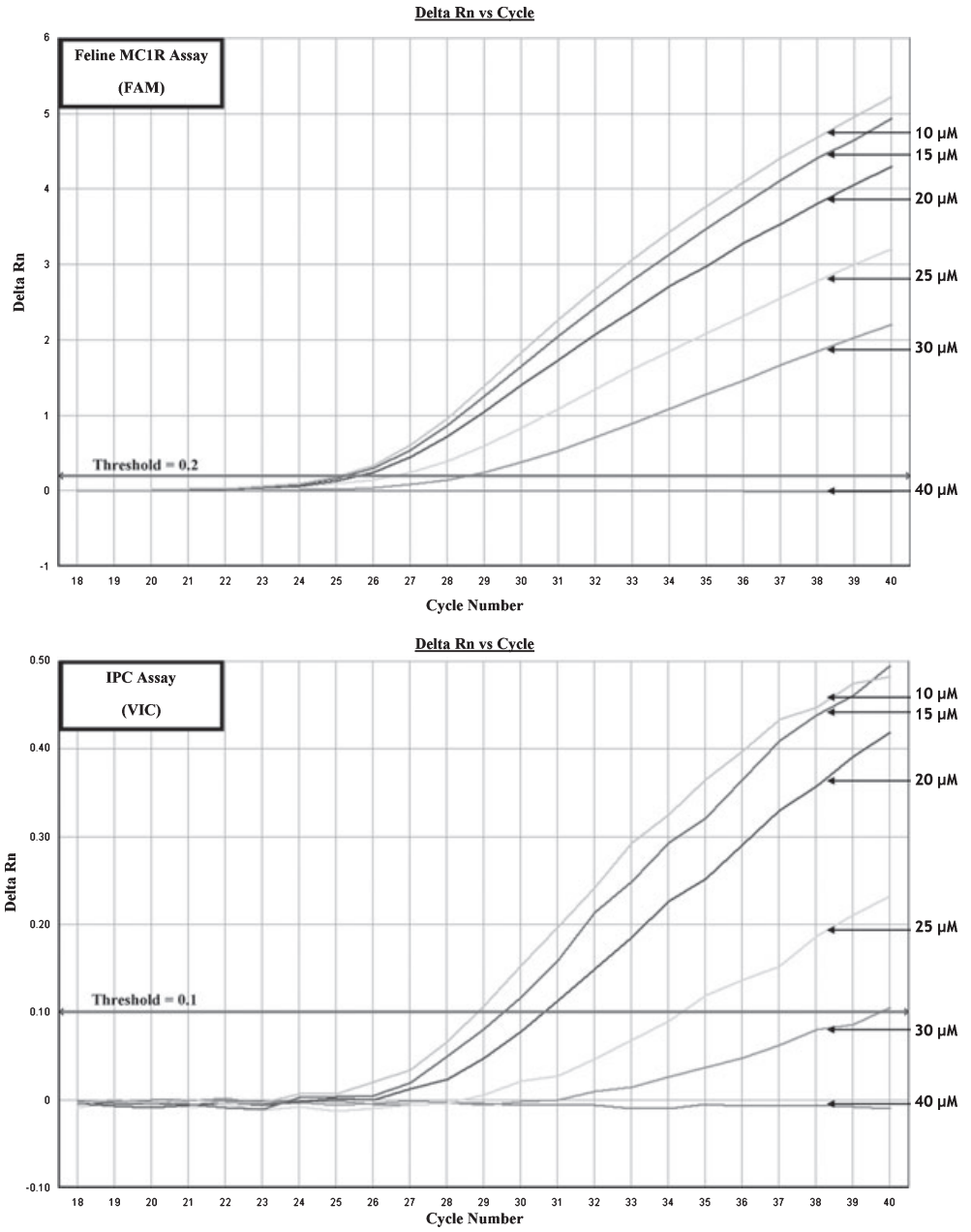

FIG. 1—*Hematin inhibition. The feline assay is pictured; however, all assays showed similar profiles in the presence of hematin.*

The equine qPCR assay has been invaluable in horse urine drug testing casework because DNA will degrade in urine samples stored under refrigeration. In a recent case, the trainer contested that the postrace urine that tested positive for a banned substance was from the horse he trained. To establish identity of the drug-positive urine, a portion was submitted along with a reference pulled mane-hair sample from the horse. Using the equine qPCR assay, the quantity of DNA in the urine was determined prior to genotyping, and a full profile was obtained from the low-quantity sample in the first round of genotyping.

The cervid assay has been used to optimize amplification of marginal samples, such as the gut pile from a poached deer. The gut pile was submitted to the laboratory for comparison with venison steak samples subsequently submitted. Using the qPCR assay to determine the optimal input volume for STR amplification, a full 19-locus genotype was garnered from this difficult sample.

The feline and canine assays were used for species ID in a case involving fecal matter from an animal cruelty investigation. A fecal sample was submitted for the purpose of determining whether it was of cat (victim) or dog (belonging to the suspect). Both the feline and canine qPCR assays were run on the sample extract that generated a signal for the feline assay but not the canine assay. No further testing was carried out because the feces were determined to have originated from the victim. Prior to the development of these qPCR assays, species ID was performed via direct sequencing of the *cytochrome b* region of the mitochondrial genome. qPCR has provided a quick and inexpensive alternative for species ID.

Conclusions

As with the canine and human qPCR assays, TaqMan real-time chemistry has demonstrated its reliability and sensitivity for

quantifying feline, bovine, equine, and cervid DNA. The assays that were developed have been shown to be suitably specific, sensitive, stable, reproducible, precise, and accurate. When applied to low-template forensic samples, these assays promote amplification of full STR profiles, and the IPC provides information that allows analysts to make informed decisions about sample testing. By implementing these assays, the laboratory has been able to improve turn-around time and quality of results and reduce consumption of valuable evidentiary samples. Furthermore, these assays can be implemented in human forensic laboratories to potentially identify sources of nonhuman DNA encountered in forensic casework.

Conflict of interest: The authors have no relevant conflicts of interest to declare.

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