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A TaqMan® multiplex assay for hoofed livestock species identification and nuclear DNA quantification

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Abstract: We have developed a TaqMan® real-time quantitative PCR (qPCR) multiplex assay that identifies and quantifies minute amounts (0.5 – 5 pg) of mixed-species nuclear DNA of cattle (*Bos taurus*), horse (*Equus caballus*), goat (*Capra hircus*)/sheep (*Ovis aries*), and pig (*Sus scrofa*) in a species-specific manner. The assay's ability to accurately detect and quantify target DNA in multi-species mixtures is crucial when the target specimen may be overwhelmed by non-target specimen. We have evaluated the assay using simulated forensic sample conditions and demonstrated that this assay is applicable for investigating criminal and civil cases including meat authentication and agri-food testing to ensure health and safety. The efficient determination of the species of origin of biological material and the accurate quantification of the species-specific template DNA will help reduce the potential risks that fraudulent/imitation and contaminated food products could pose to our food supply. Our assay's ability to accurately identify and quantify species-specific DNA present in biological samples will also facilitate downstream analyses that generate DNA profiles of the specimen for genetic identity testing and traceability. This TaqMan® qPCR assay will facilitate meat inspectors, regulatory agencies, and food safety and quality control laboratories to enforce accurate labeling of livestock meat products and prevent fraudulent manipulations of our food supply.

Keywords: Multi-species identification, multi-species DNA quantification, forensic science, food safety, food testing

Introduction

Authentication of food products by means of DNA-based species determination is a crucial part of quality control to ensure the safety and integrity of the food supply (Woolfe *et al.,* 2004; Ballin 2010; Fajardo *et al.*, 2010). This is particularly true with meat products where consumers require protection from falsely labeled food that could contain adulterated, unknown or less desirable meat species (Soares *et al*., 2010). Multiplexed end-point PCR assays have been successfully developed for the identification of fraudulent food products (Dalmasso *et al*., 2004; Ghovvati *et al*., 2009; Zha *et al*., 2010), but these protocols require amplification of target DNA by PCR reaction and gel electrophoresis for fragment sizing before species identification can be performed. TaqMan® real-time quantitative PCR (qPCR) technology allows for simultaneous amplification, identification and quantification of one or more specific DNA sequences. The use of species-specific primers and fluorescent probes in multiplexed reactions within a single well facilitates the detection of target DNA in mixed species samples, and thus simplify data collection and analysis (Kanthaswamy *et al.*, 2011). The ability to perform accurate DNA quantification has streamlined downstream genotyping analysis in addition to improving genotyping results and conserving reagents (Evans *et al*., 2007; Kanthaswamy *et al.*, 2011; Lindquist *et al*., 2011). With the reaction, detection, and data analysis completed simultaneously and in real-time, qPCR protocols minimize the risk of exposure to contamination, while increasing efficiency.

As the mitochondria occurs in high copy numbers and is able to withstand degradation and environmental challenge, end-point PCR and real-time quantitative PCR (qPCR) techniques for mitochondrial (mtDNA) analysis have already been used for species testing in food safety management and for screening for fraudulent/imitation meat (Dalmasso *et al.*, 2004; Dooley *et al.*, 2004; Woolfe *et al*., 2004; Köppel *et al*., 2009; Zha *et al.*, 2010). While mtDNA markers can reveal sufficient inter-species variation to facilitate species determination of a particular sample (Hajibabaei *et al*., 2007), mtDNA introgression can

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occur between conspecifics and can obscure the true phylogenetic relationships, particularly those among closely related species (Ferris *et al*., 1983). For these reasons, it would be desirable to verify the results obtained by mtDNA markers with those based on nuclear markers. Assays that rely on nuclear markers for species determination and DNA quantification analysis have a crucial advantage over assays that employ mtDNA that confer accurate species confirmation but without a nuclear DNA quantification feature (Kanthaswamy *et al.*, 2011). Nuclear DNA concentration is pertinent information for optimal genotyping that can be used for individual identity testing and for identifying/tracing the source of meat products throughout the entire supply chain (Alonso *et al.*, 2004; Timken *et al.*, 2005).

This multiplex assay uses species-specific hydrolysis probes and primers to target nuclear loci in common hoofed livestock animals including the Melanocortin 1 Receptor (*MC1R*) gene in cattle (*Bos taurus*) and horse (*Equus caballus*), the beta-actin (*ACTB*) gene in pig (*Sus scrofa*), and the T-cell surface glycoprotein *CD4* gene in goat (*Capra hircus*) and sheep (*Ovis aries*). The *MC1R* and *ACTB* genes have been used in studies and have been proven to be species-specific (Köppel *et al*., 2009; Lindquist *et al.*, 2011). The assay is capable of simultaneously detecting nuclear DNA templates from all five common livestock species. As the assay's species identification and nuclear DNA quantification technique interrogates single copy nuclear loci, it is thus more relevant for DNA profiling than mtDNA-based approaches that analyze non-nuclear loci that occur in multiple copies in a cell (Walker *et al.*, 2004).

Materials and Methods

DNA standards and samples

Purified and quantified high molecular weight genomic DNA extracts (Bovine: Cat. # GB-110, Sheep: GS-190, Goat: GG-150, Pig: GP-160, Equine: GE-170) were purchased from Zyagen (San Diego, CA) and used as controls and quantification standards for all experiments. Purified and quantified DNA extracts from hair, blood and meat samples (Bovine: N = 8, Pig: N = 8, Horse: N = 7, Goat: N = 7) were obtained *ex gratia* from Scidera and Questgen Forensics (Davis, CA). These DNA samples had been extracted using conventional extraction techniques. Buccal and nasal swabs from the University of California, Davis animal barns and livestock from the Davis, CA area were collected with owners' consent and were used in the population study. Buccal and nasal samples were extracted and purified using QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) according to vendor's buccal extraction protocol. The concentrations of extracted samples were quantified with the Qubit™ fluorometer (Invitrogen, Carlsbad, CA) using the Quant-it™ dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA).

Primer and probe design

The bovine and equine primers and probes were the same as those designed and published by Lindquist *et al.* (2011). The porcine primers and probes were the same as Köppel *et al*. (2009). We attempted to design species-specific primers and probes for nuclear genes in both goat and sheep, but were unsuccessful due to the limited genetic information available for these two species. Instead, a common primer and probe set that detected both goat and sheep was designed. All goat and sheep nuclear genes available in GenBank, the genetic sequence database at the National Center for Biotechnology Information (NCBI; *http://www.ncbi.nlm.nih.gov/*), were screened to identify those shared by both species. Shared gene files were downloaded from GenBank and aligned using Sequencher v.4.10.1 (GeneCodes, Corp., Ann Arbor, MI) to generate consensus sequences for *in silico* primer and probe design using Primer Express™ v.3.0 (Applied Biosystems, Carlsbad, CA). Candidate primer and probe sequences were searched against the NCBI Nucleotide and Genome (chromosomes from all organisms) databases using the Primer Basic Local Alignment Search Tool (Primer-BLAST). Search results were screened and primer pairs not specific to goat and sheep were discarded. The amplified fragments from the resulting primer pairs were located in the gene sequences and were searched against the NCBI Genomic BLAST database for *Bos taurus* (5.2), *Canis lupus familiaris, Equus caballus, Felis catus, Gallus gallus, Homo sapiens* (Build 37.2), *Ovis aries*, and *Sus scrofa* (Scrofa 9.2). Amplified fragments from primer pairs that had the longest aligned fragments and lowest percentage matches with non-specific species were selected for testing. The primers were tested using the polymerase chain reaction (PCR) and gel electrophoresis. The PCR reaction had 10.5 µL of PCR cocktail and 2 µL of 20 ng/µL DNA for a total reaction volume of 12.5 µL. The PCR cocktail for each reaction consisted of 7.59 µL nuclease free distilled deionized water, 0.25 µL 10 mM dNTPs, 1.25 µL 10x PCR reaction buffer, 0.85 µL 50 mM MgCl2, 0.25 µL 10 uM primers (Integrated DNA Technologies, San Diego, CA), and 0.06 µL Platinum® Taq Polymerase (Invitrogen, Carlsbad, CA).

Table 1. Oligonucleotide sequences and concentrations

Oligonucleotide	Final conc. (µM)	Sequence, 5' - 3'	Position	Length	GenBank accession no./source
Bovine-F	0.4	AATAAATCATAA*ACCAGCCTGCTCTTCATCAC	806 - 825	72	AF445642 (Lindquist *et al.*, 2011)
Bovine-R	0.4	AATAAATCATAA*AGCTATGAAGAGGCCAACGA	877 - 858		
Bovine-probe	0.25	**VIC**-CACAAGGTCATCCTGCTGTGCC-**MGB-NFQ**	836 - 857		
Goat/Sheep-F	0.2	CCCTCTGGGAGCAACCCTTCT	436 - 456	74	EU913093 (Goat), NM_001129902 (Sheep)
Goat/Sheep -R	0.2	GACAGGCTCTTGAGTTCTTCCTTCCT	509 - 484		
Goat/Sheep -probe	0.25	**NED**-TGCAGTGGAAGGGTCCA-**MGB-NFQ**	458 - 474		
Equine-F	0.2	CCTCTTCATCGCTTACTACAACCA	1184 - 1207	71	NM_001114534 (Lindquist *et al.*, 2011)
Equine-R	0.2	CCAGCATGGCCACAAAGAA	1254 - 1236		
Equine-probe	0.1	**Cy5**-CTGCTCTGTCTCGTCAC-I**BRQ**	1218 - 1234		
Swine-F	0.2	CGAGAGGCTGCCGTAAAGG	299 - 317	108	DQ452569 (Köppel *et al.*, 2009)
Swine-R	0.2	TGCAAGGAACACGGCTAAGTG	406 - 386		
Swine-probe	0.04	**FAM**-TCTGACGTGACTCCCCGACCTGG-**MGB-NFQ**	352 - 374		

*12 base tail added for specificity. (C. Lindquist, Personal Communication.)

The primers were used to amplify 13 samples: 12 species (*Anas platrhynchos, Anser anser, Bos taurus, Canis lupus familiaris, Capra hircus, Equus caballus, Felis catus, Gallus gallus, Homo sapiens, Meleagris gallopavo, Ovis aries, Sus scrofa*) and a negative control in singleplexes for species specificity. The PCR was run on the Eppendorf Mastercycler Pro S (Eppendorf North America, Hauppage, NY) using the following cycling conditions: Initial holding step of 95°C for 15 min and 35 cycles of 94°C for 5 s, 62°C for 1 min, and 72°C for 1 min. The PCR product was sized with a size standard using gel electrophoresis of 6% acrylamide gels (Bio-Rad Laboratories, Hercules, CA) run at 170V and stained with ethidium bromide to confirm the length of the specific target. Primer pairs with gel bands not specific to goat and sheep were discarded. The remaining primer pairs were selected for probe design. Probe sequences were obtained from Primer Express™. The probe sequences and their respective reverse complements were tested for species specificity in the same manner as primers. For the Primer-BLAST and genome BLAST searches, forward probes were treated as forward primers and reverse probes as reverse primers. Search results were screened and non-specific probes were discarded. The probe sequence was ordered as a primer oligo and tested with PCR.

qPCR multiplex assay design

The qPCR assay was designed and optimized for a qPCR cocktail volume of 15 µL and 5 µL of DNA. Each reaction cocktail consisted of 12 µL of FastStart Universal Probe Master (ROX) 2X Concentration (Roche Diagnostic Corporation, Indianapolis, IN), primers and probes (FAM, VIC, NED dyes: Applied Biosystems, Carlsbad, CA; Cy5 dye: Integrated DNA Technologies, San Diego, CA) as listed in Table 1 and distilled deionized water for a total volume of 15 µL. Five microliters of DNA consisting of 1 µL each of 10 ng/µL DNA for each of the five target hoofed animal species was used in the multiplex. The qPCR was run on an ABI 7500 Fast Real-Time PCR System using the Quantitation – Standard Curve method and Standard Run ramp speed with the following condition: Initial holding steps of 50°C for 2 min and 95°C for 10 min, and 40 cycles of 95°C for 15 s and 61°C for 1 min.

Standard curves

Standard curves for each target were generated using a ten-fold dilution with water of 500 ng to 0.05 ng of DNA and run following the conditions above. The baselines were set automatically using the 7500 Software v.2.0.5 (Applied Biosystems, Carlsbad, CA) and the thresholds averaged over the replicate runs for each target. The standard curves were graphed by plotting the quantification cycle (Cq; Bustin *et al.*, 2009) values against the log quantity of DNA and the equation of the best-fit line and R^2 values were calculated in Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA). Efficiencies were calculated using the slope (m) in the equation $E=10^{[-1/m]}-1$ (Bustin *et al.*, 2009). The standard curves facilitated the measurements of DNA quantities of unknown samples.

Validation studies

Dilution study

Dilution studies were conducted using 10 µg/µL DNA per species for a total concentration of 50 µg/µL. The DNA was serially diluted from 1:1 to 10^5 with distilled water. Another dilution study was performed following the same dilution factors but diluted with non-target DNA to test for species-specificity in the presence of non-target DNA. The non-target DNA (Cat: Cat. # GC-130F, Chicken: GC-120F, Dog: GD-150F, Duck: GD-220, Human: GH-180F, Turkey: GT-150) purchased from Zyagen was used in equal amounts for diluting. The various DNA dilutions underwent qPCR with the parameters described above to determine the dilution level detectable by the assay.

Degradation study

Degradation studies were conducted following the procedures published by Swango *et al.* (2006). The reactions contained 18 µg of DNA, 10X DNase buffer, and 2.5 µL of 1 U/µL Turbo DNase I (Applied Biosystems, Carlsbad, CA). The degradation protocol was performed three times and the average room temperature for the incubation step was 25.7°C. An additional sample at time 0 min was included in the studies without the addition of EDTA to observe the inhibitory effects of EDTA. The level of degradation was assessed with gel electrophoresis (6% acrylamide gels, ethidium bromide detection) and was added directly to the qPCR assay without dilution.

Inhibition studies

Inhibition studies were conducted using both humic acid and porcine hematin (both from Sigma Aldrich, St. Louis, MO). The humic acid was prepared following procedures by Kanthaswamy *et al*. (2011) to a final concentration to 1 mg/mL. The hematin was diluted according to Swango *et al's.,* (2006) procedure to 1 mM and both inhibitors were serially diluted from 1:1 to $1:2^8$ with distilled water. A 1:2 dilution series was chosen instead of 1:10 for a more gradual dilution to better detect the assay's inhibitory limit (Heid *et al*., 1996; Monteiro *et al.,* 1997). The multiplex reaction mixture with standard DNA concentrations was combined with 2 µL of each inhibitor dilution for a final reaction volume of 22 µL and subjected to qPCR conditions.

Population study

A sample size of N=10 for each species was obtained *ex gratia* from Scidera (Davis, California), animals housed by the UC Davis Animal Sciences department, and with consent from animal owners in the Davis, CA area. The study samples were obtained opportunistically; therefore the quality and condition of the samples were not consistent. Only samples from unrelated individuals were included in the study. The DNA from each individual was added separately into reaction wells to test the assay's specificity. The qPCR reaction cocktail was prepared as described but with increased distilled water for a total cocktail volume of 18 µL and decreased DNA volume to 2 µL per reaction well for a final volume of 20 µL. The assay was run according to qPCR conditions above.

Results and Discussion

Standard curves

Table 2 lists calculated results for each target species. Theoretically, qPCR reactions with 100% efficiency double the amount of DNA per cycle.

Table 2. Standard curves and efficiencies. The standard curves were generated by graphing the log amount of DNA against the Cq value from the run. Efficiencies are calculated using the slope of the line of best-fit and unknown sample concentrations are determined by locating the corresponding quantity with the run Cq value.

Species	Equation	R^2	Efficiency
Bovine	y = -3.3393x + 30.675	0.9991	99.28%
Goat/Sheep	y = -3.6391x + 30.449	0.9893	88.28%
Horse	y = -3.3369x + 30.015	0.9915	99.38%
Swine	y = -3.2918x + 28.472	0.9973	101.27%

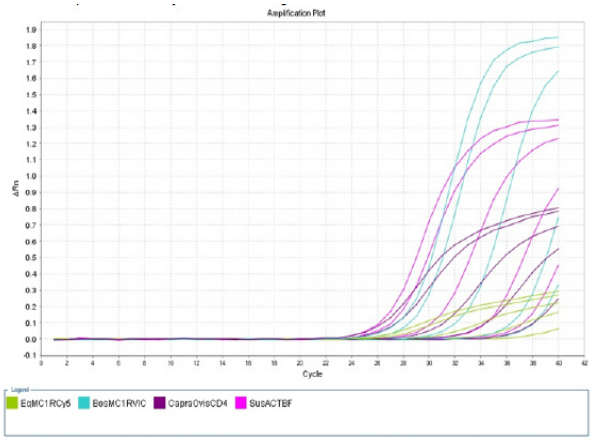

Figure 1. Dilution study multi-species amplification plot. Each color indicates a different target species (EqMC1RCy5: horse; BosMC1RVIC: cattle; CapraOvisCD4: goat/sheep; SusACTBF: pig) and each line within a color designates a different dilution. The DNA from each species was serially diluted from 1:1 to 105 with distilled water. The varying peak heights for the different colors are dependent on the dyes used for the targets.

Efficiencies greater than 100% have been observed (Clark-Langone *et al.*, 2010; da Silva Coelho *et al.,* 2010); however, these are most likely due to pipetting errors and reaction inefficiencies such as primer dimer.

Validation studies

Dilution study

The multiplex assay was run containing serial dilution mixtures with equal amounts of cattle, goat, sheep, horse, and pig DNA to test the limit of detection (LOD) of the assay. The assay was able to detect all species down to $1:10^3$ dilutions (5 pg of each target species) which is less than the amount of nuclear DNA in a diploid cell (6 pg). The assay also detected both goat and sheep at $1:10^4$ dilutions (0.5 pg). Figure 1 shows the multi-species dilution plot.

Degradation study

DNA samples not treated with DNase showed very bright, large molecular weight bands consistent with non-degraded genomic DNA. The DNA mixtures treated with DNase consistently showed smeared bands for the time intervals 2.5 to 10 minutes (Figure 2a). All species were detected by qPCR for

the digestion time intervals 0 and 5 minutes and all species except pig were detected after 10 minute's digestion. For the time intervals between 15 and 180 minutes, only cattle DNA was detected (Figure 2b).

Inhibition studies

Both hematin and humic acid completely inhibited the qPCR reactions for samples with undiluted inhibitors and those up to $1:2^5$ dilution. Only cattle, goat, and sheep were detected in combination with $1:2^6$ inhibitor dilutions and all samples were detected with $1:2^7$ and $1:2^8$ dilutions for both inhibitors.

Population study

All populations were run in duplicate and only target species were detected in each reaction (Figure 3). The reactions did not show signs of inhibition for any of the individuals. It is reasonable to postulate that the target fragments do not have intraspecific mutations in the primer and probe annealing sites that affect primer and probe binding and species specificity because all 10 individuals from each species population were detected and correctly identified.

Though few livestock assays are designed and used in forensic applications including animal theft and abuse cases (Lindquist *et al.*, 2011), these assays are readily used in the food industry for detecting food adulteration and fraudulence (Dooley *et al.*, 2004; Köppel *et al.,* 2009; Ballin 2010). The detection of meat adulteration in the form of meat substitutions, additions, and omissions has severe economical and ethical effects on the industry. This assay failed to differentiate between goat and sheep biological material because of the lack of informative target sequences. This is probably due to the fact that both these taxa hybridize resulting in the lack of species-specific nuclear sites that could be targeted by the assay. Moreover, unlike the other hoofed animals, the genomes for these two species have not yet been published. As more segments of the genomes of these taxa are completed, it is our hope there will be more candidate nuclear genes to select from to design primers and probes specific to each species.

The TaqMan® multiplex qPCR assay that we developed for the multi-species identification and DNA quantification of mixed samples of domesticated hoofed animal species is simple to use and does not require specialized expertise. The use of nuclear genes provides the ability to accurately quantify the nuclear DNA from mixed species samples on a level that is not previously possible using mitochondrial markers. Sequencing or digestion of genomic DNA samples and PCR products composed of two or more species

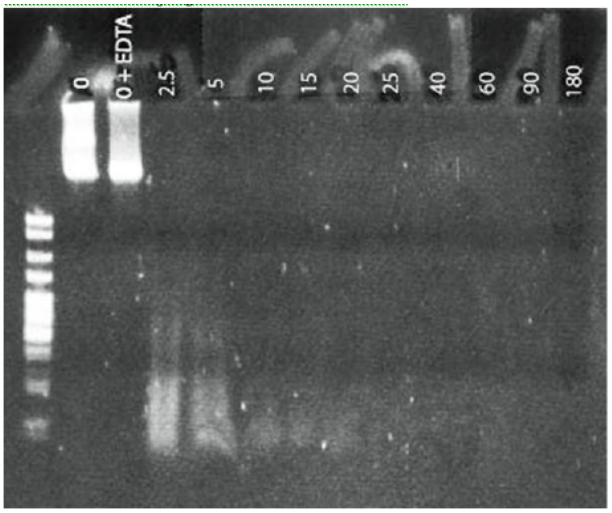

Figure 2a. Degradation gel with bright bands indicating abundant genomic DNA at 0 minutes and 0 minutes with EDTA and smeared bands indicating fragmented DNA at 2.5 to 10 minutes

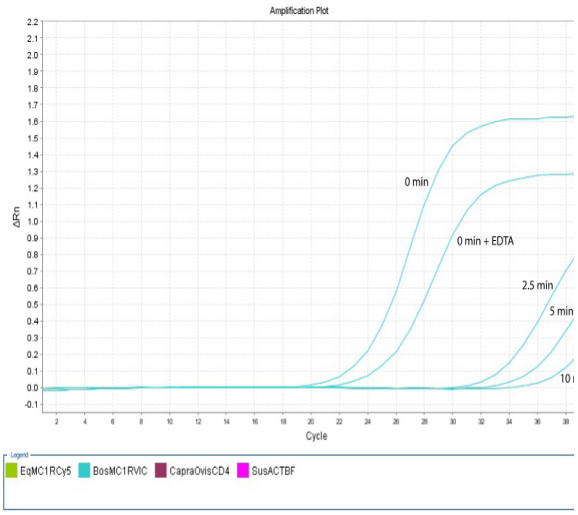

Figure 2b. For simplicity, only the Bos Taurus degradation amplification plot is shown below

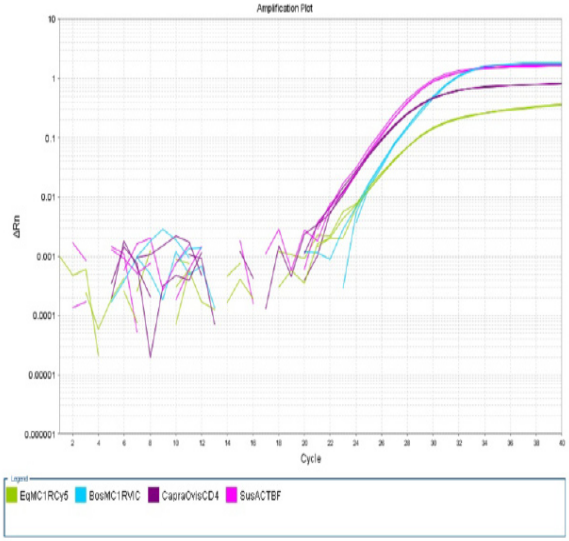

Figure 3. Multiplex amplification plot showing species-specificity

could result in a combination of different sequences or restriction patterns with all of the possible species included in the mixed sample, and therefore may be too difficult to interpret. Cooked or cured meats were not used due to the large varieties of potential cooking/curing methods that are available. As long as a questioned sample - cooked, cured or raw, has amplifiable DNA within the assay's detection range, this assay will quantify the DNA in a species-specific manner.

Our qPCR assay can detect very minute amounts of DNA with the lowest detection limit of 5 pg for cattle, horse and pig, and 0.5 pg for goat and sheep. The simplicity and sensitivity of this assay underscore its usefulness for forensic evidence analysis including successful amplification even in the presence of hematin and humic acid. Testing with t known PCR inhibitors known to be highly potent adds to the assays utility in analyzing forensically probative samples. Therefore, while contributing to the quality and safety of our food supply by facilitating downstream DNA-based identity testing and traceability assays, our robust qPCR assay also lends itself for application in the animal forensic science field.

Conclusion

The application of our TaqMan® assay-based species determination and DNA quantification assay in food regulatory control should facilitate the determination of adulterated and undeclared livestock species compositions will help to protect both consumers from fraudulent manipulations of our food supply. Although the multiplex assay was not tested on cooked or cured meat samples, mixed DNA samples from buccal cells, nasal swabs, hair, blood and raw meat representing each livestock species were tested to simulate extracted DNA from adulterated samples, and each species was successfully detected and quantified without reaction competition or confusing results. In relation to alternative techniques for species identification such as direct sequencing of PCR products or restriction analysis, qPCR using species-specific primers and hydrolysis probes offers the advantage of being cheaper, faster and more efficient for routine high-throughput analysis of large numbers of samples.

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