Report title: Development of a DNA-Based Multi-Species Identification and Quantification Assay

Award Number: National Institute of Justice (NIJ) grant number: 2008-DN-BX-K288

Authors: K'Lynn Solt, Sree Kanthaswamy

Abstract:

Species identification of forensic samples allows the analyst to determine the appropriate tests to select when evaluating a wide range of biological evidence. Since most animal short tandem repeats (STR) analyses are species-specific, it is important to know the species of origin of the sample before attempting STR typing. The ability to accurately detect and quantify target DNA in mixed-species samples is crucial when target templates may be overwhelmed by non-target DNA.

Depending on the analytical procedure, standards for quantifying DNA templates recovered by extraction are required by forensic laboratory accrediting agencies. The use of accurate species-determining tests will enable analysts to optimize the typing tests used in a case and minimize the consumption of limited samples. The analysis of non-human DNA will portend an additional means of forensic inquiry. An accurate taxonomic-identification and quantification assay of forensic DNA samples is also important for cost efficient analyses of the evidence.

For this NIJ-funded project, our goal was to combine the strengths of DNA-based taxon identification with that of a nuclear marker for DNA quantification into a simple and quick duplex single nucleotide polymorphism (SNP) TaqMan® assay. This robust and cost efficient method for taxon differentiation and DNA quantification will be developed for test commercialization. As in the development of novel forensic DNA methodologies, our speciation and quantification assays have undergone scientific

scrutiny to assure accuracy, precision, and reproducibility of the procedure, and species-specificity, sensitivity, stability, and mixture analyses as part of its validation. Based on this research and evaluation, we have published four papers on species-specific DNA quantification of human, dog, cat, livestock, and poultry (see publication list below). Our conditions for primers and probes were that these reagents would identify sequences within target species that were conserved but contained sufficient polymorphisms such as SNPs that would allow us to tell one target species apart from another. We found target regions within these species' genomes for our species-specific primers and probes. In order to produce short but sufficiently informative targets for our qPCR probes, amplicons ranged from approximately 70 to 210 bp.

Two triplexes targeting human-dog-cat and poultry species (duck, chicken and turkey) were designed and validated with simulated forensic conditions. Each target was validated for species-specificity and the limit of detection (LOD) determined through dilution, degradation, inhibition, and population studies. Two quadriplexes, the human-dog-cat triplex with the addition of an internal positive control and a livestock (cattle, goat, horse, sheep, and pig with a single target for goat and sheep), were also designed and validated. Based on the same approach designed for animal DNA testing and quantification, i.e., by targeting regions within these species' genomes for our species-specific primers and probes, we developed a marijuana or *Cannabis sativa* (*C. sativa*) species testing and DNA quantification. The underlying scientific procedures and results involving each assay have been presented to state and federal agencies via forensic meetings and peer-reviewed journals.

Table of Contents: **Page**

Executive Summary:

In the United States, human forensic evidence collected from crime scenes is frequently comingled with biomaterial of canine (dog) and feline (cat) origins. Knowledge of the concentration of nuclear DNA extracted from a crime scene biological sample and the species from which the sample originated is essential for DNA profiling. The ability to accurately detect and quantify target DNA in mixed-species samples is crucial when target DNA may be overwhelmed by non-target DNA. As a variety of DNA-based applications are introduced into forensic science, the influence of genetic characterization on criminal and civil investigations has become significant. As a science that depends on transfer and trace evidence, forensic DNA analysis has almost exclusively relied on biomaterials such as blood, semen, hair, or epithelial cells from human sources, even though animal and plant evidence has been identified in crimes for decades and the DNA testing of non-human evidence has been firmly established and used for forensic purposes.

We have designed and evaluated a conceptually straightforward and practical fluorogenic triplex assay that uses species-specific TaqMan® MGB hydrolysis primers and probes to target the short tandem repeat (STR) region of the human tyrosine hydroxylase (TH01), a locus that is widely used for human forensic identification (Timken et al. 2005) and the canine and feline Melanocortin 1 Receptor (MC1R), a nuclear gene that encodes for a seven-pass transmembrane G protein-coupled receptor protein involved in hair and fur coloring in all mammals. This MC1R gene sequence is conserved in dogs and cats as well as their closely related species (Evans et al. 2007) but has sufficient inter-specific variability that can be used for species identification of dog and cat DNA with high levels of taxonomic confidence. Since all three are nuclear markers and have unique subchromosomal locations, they make ideal and relevant

target sites to estimate the amount of human, canine, and feline nuclear templates for DNA profiling.

The combination of kinetic real-time PCR technology and fluorescent probes provides a greater functionally effective quantitative range and does not require extensive attention or expertise on the part of the analysts especially for post-PCR processing, and data collection and analysis. Our qPCR triplex assay is highly specific and sensitive to detecting minute concentrations of DNA. The lowest DNA detection limit of this triplex assay is 0.4 picograms (pg) in humans and cats, and 4.0 pg in dogs. This remarkably low amount of input template that can be detected by the triplex assay portends its role in the analysis of forensic evidence. Apart from its high sensitivity, mixture studies do not show non-specific amplification of DNA from animals other than the target species, i.e., either human, dog or cat. The presence of a strong inhibitor like humic acid in samples often tend to hinder PCR reactions, and the fact that this potent inhibitor did not prevent the triplex assay from detecting and measuring sample DNA further confers an added benefit to the assay.

While the triplex qPCR assay quantifies and identifies the presence of trace amounts of human, cat, and dog DNA in mixed or unmixed samples, the assay did not include an Internal Positive Control (IPC) due to the three-dye limit in the Applied Biosystems 7300 machine. After the purchase of a 7500 Fast Real Time PCR system, the assay is now modified to include an IPC. We used the TaqMan® Exogenous Internal Positive Control (ABI catalog number-4308335). The thermal cycling conditions are the same as previously published (Kanthaswamy et al. 2012). A volume of 0.56 µL of Exo-IPC DNA, 2.52 µl of Exo-IPC mix and 0.4 µL of molecular grade water is integrated into the assay. The qPCR cocktail volume is now reduced to 28 µL and uses 16.0 µL of Roche FastStart Universal Probe Master (ROX). The dyes on the target

probes are NED, FAM, Cy5, and VIC for human, dog, cat, and IPC, respectively. Similar studies to determine the sensitivity, specificity, and robustness of the assay were conducted in the same manner as the above-cited paper. The results of the population, inhibition, degradation, and dilution studies remain unchanged. Dilution studies were conducted using 48 ng/µl as the starting DNA concentration (mixture of 16 ng/µl human, dog and cat DNA). The quadriplex started dropping off at dilution 1:10,000. It detects DNA as low as 8 pg/µl, which is very close to the amount of DNA present in one diploid cell (approximately 6 pg). The quadriplex assay has an advantage over the triplex assay due to the inclusion of an IPC, shorter run time, reduced master mix volume, and the use of the 7500 Fast Real time system. It is essential that forensic DNA laboratories understand the importance of the robustness and specificity of the assay and validate this method at their respective laboratories for mixed-species DNA analysis.

In addition to the human-dog-cat assay, we developed a TaqMan® real-time 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*) or sheep (*Ovis aries*), and pig (*Sus scrofa*) in a species-specific manner. 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 and horse, the beta-actin (ACTB) gene in pig and the T-cell surface glycoprotein CD4 gene in goat and sheep. 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 nuclear DNA profiling than mtDNA-based approaches that analyze

non-nuclear loci that occur in multiple copies in a cell (Walker et al. 2004). 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 or 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. 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 which 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.

A multiplex assay based on TaqMan® qPCR for species-specific detection and quantification of chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), and turkey *(Meleagris gallopavo*) nuclear DNA was also designed, developed, and validated. The poultry multiplex assay utilizes the advantages of real-time qPCR technology and nuclear genes to ensure efficient poultry species identification and accurate DNA quantification. Our assay uses species-specific hydrolysis probes and primers (see Table 3) to target nuclear genes in common poultry animals including the T-cell surface glycoprotein CD4 gene in duck, the transforming growth factor, beta 3 gene (TGFB3) in chicken, and the prolactin receptor gene (PRLR) in turkey. The TFGB3 and PRLR genes have previously been used in studies and have been determined to be species-specific (Köppel et al. 2009). While this assay is sensitive and specific to chicken, turkey, and duck nuclear DNA detection and quantification, respectively, in single source samples, it is also capable of simultaneously detecting and quantifying all three poultry species' nuclear DNA in multi-species mixtures without cross-reactivity. The multiplex assay is able to accurately detect very low quantities of species-specific DNA from single or multispecies sample mixtures; its minimum effective quantification range is 5 to 50 pg of starting DNA material. In addition to its use in food fraudulence cases, we have validated the assay using simulated forensic sample conditions to demonstrate its utility in forensic investigations. Despite treatment with potent inhibitors such as hematin and humic acid, and degradation of template DNA by DNase, the assay was still able to robustly detect and quantify DNA from each of the three poultry species in mixed samples. The efficient species determination and accurate DNA quantification will help reduce fraudulent food labeling and facilitate downstream DNA analysis for genetic identification and traceability.

Our one reaction step TaqMan®-based multiplex qPCR assay for the identification and DNA quantification of mixed poultry samples including duck, chicken, and turkey has a limit of detection (LOD) of 5 pg of poultry DNA. In the presence of overwhelming non-target DNA, the LOD of the assay is 50 pg of DNA for chicken and 5 pg for duck and turkey DNA, respectively. In addition to its use in the food industry for safety and fraudulence management, the simplicity and sensitivity of this assay lends itself to be very useful in criminal or civil forensic investigations that involve biological evidence from poultry. The assay was successful in the identification and quantification of the target species even in the presence of 3.91 μM of hematin and 1.95 ng/μL of humic acid, two PCR inhibitors known to be highly potent. Therefore, while our assay can contribute to the quality and safety assurance of our food supply through the facilitation of downstream STR- and single nucleotide polymorphism (SNP)-based identity testing and traceability assays, our robust qPCR assay can similarly be applied to downstream analyses in the animal forensic science field such as evidentiary and reference sample comparisons.

Plant material is also a common source of contamination; the presence of these contaminants in assays that use ancient genes (including those found in green plant autosomal and chloroplast genomes) can be conserved in different genomes and can undermine the development of our assay. As we become more familiar with assays based on these genes we can also opportunistically develop projects for disease testing and marijuana or *Cannabis sativa* (*C. sativa*) typing; the latter is of utmost importance because most narcotics-related cases in the US involve marijuana or *C. sativa*. DNA based tests of *C. sativa* particulates is somewhat limited in the forensic community. *C. sativa* material is typically identified based on the cystolithic hairs on the leaves and with chemical tests to identify the presence of cannabinoids. Suspect seeds are germinated

into a viable plant so that morphological and chemical tests can be conducted. Seed germination, however, causes undue analytical delays. DNA analyses that involve the chloroplast and nuclear genomes have been developed for identification of *C. sativa* materials but they require several nanograms of template DNA. Using the trnL 3' exon-trnF intragenic spacer regions within the *C. sativa* chloroplast, we have developed a qPCR assay that is capable of identifying picogram (pg) amounts of chloroplast DNA for species determination of suspected *C. sativa* material. This assay provides forensic science laboratories with a quick and reliable method to identify an unknown sample as *C. sativa*.

To address simultaneously the problems of low target *C. sativa* DNA concentration and low quality DNA, we have designed and developed a real-time qPCR method that uses the TaqMan® chemistry and targets chloroplast DNA (cpDNA). Multiple chloroplasts exist per plant cell, and there are multiple copies of the chloroplast genome in each chloroplast. Additionally, the chloroplast membrane protects the DNA from degradation (Taberlet el al. 2007); therefore, cpDNA was chosen as the target for this assay to increase the probability of success of testing limited or degraded *C. sativa* samples. To further address the problem of low starting sample quantity, qPCR provides both the specificity and the sensitivity needed to identify forensic samples from all tissue sources (Kanthaswamy et al. 2012, Evans et al. 2007).

The qPCR technology has already been used to identify unknown samples of animal origin in forensic samples (Kanthaswamy et al. 2012, Evans et al. 2007) and is already widely used and accepted in forensic settings (Vallone et al. 2008). This method is not limited to those samples from which DNA of large quantity or high quality can be prepared. Unlike Sanger sequencing (Mukherjee et al. 2008), STR (Howard et al. 2008), or ABI PRISM® SNaPshot™ Multiplex System analysis of *C. sativa*, qPCR also requires

no post-PCR processing, cutting down on both the cost and time needed to identify an unknown sample, both important factors in a forensic setting. Because qPCR technology allows for the simultaneous amplification, identification, and quantification of one or more specific DNA sequences in real-time, the qPCR protocols minimize the risk of exposure to contamination. Therefore, the creation of a qPCR test that is capable of specifically identifying pg amounts of *C. sativa* DNA would further the forensic science community's ability to serve its clients in an efficient and timely manner. The novel qPCR assay for species identification of suspected *C. sativa* particulate material described in this study aims to provide such a test, while utilizing instruments and reagents either already in forensic laboratories, or ones that can be easily obtained by forensic laboratories. The goal of this assay was to design a quick and easy test to identify trace amounts of *C. sativa* cpDNA using technology, instruments, and reagents and other resources readily available in most crime laboratories. The results clearly show that our duplex qPCR assay is able to robustly and accurately identify minute amounts (5 to 50 pg/µL) of *C. sativa* cpDNA. This assay enables crime laboratories to identify suspected *C. sativa* seed samples in a timely manner that could not be done in the past.

This NIJ grant has sponsored the research of six Masters of Forensic Science graduate students at the University of California, Davis. This final report herein is about the development and validation of the four species testing and DNA quantification assays and the direct goals of the project that was proposed to NIJ.

Main Body of the Final Technical Report

I. Introduction:

Orthologs, which represented our target sites for species testing and DNA quantification are conserved regions within the genomic DNA in different species that originated by vertical descent from a single gene of the last common ancestor. Therefore, orthologs are homologous sequences separated by a speciation event, i.e., when a species diverges into two separate species. We hypothesized that if a sequence is conserved within a species and between closely related species it would contain enough variation among taxonomic families to exclude non-target DNA. Another underlying hypothesis is that it is conceivable that none of the target species exhibited any intraspecific variation within the primer and probe annealing sites that can affect the binding affinities of the primers and probes, and the sensitivity of this assay. Based on these hypotheses our goal was to exploit the sequence information available for target orthologs to design a suite of qPCR assays for quantifying the DNA of a variety of species in a species-specific manner.

Human-dog-cat assays

Human forensic evidence collected from crime scenes is frequently mixed with dog and cat biological material (Halverson et al. 2005, Smalling et al. 2010). In the United States (US), 55% of households own at least one dog (68 million dogs in the US) or one cat (73 million cats in the US [APPMA 07-08A Survey]). Not surprisingly, this makes the exchange of pet biological evidence between a crime suspect and victim at the scene of the crime very likely. Due to the vast amounts of biomaterial from dogs and cats, D'Andrea et al. (1998) showed that this material in households is easily transferred and is very persistent. Therefore, an intruder cannot exit a house where there is a pet

dog or cat without coming in contact with and carrying a sample of the animal's biomaterial away with him (D'Andrea et al. 1998).

Given the high probability of discovering comingled human, dog, and cat forensic samples, it is important to know the species of origin of the sample before attempting downstream nuclear DNA analyses, including short tandem repeat (STR) profiling. The ability to accurately detect and quantify target DNA in mixed-species samples is crucial when target templates could be overwhelmed by non-target DNA. The use of a reliable species-determining test to robustly ascertain if the probative sample is of human, canine, or feline origin will help analysts determine a priori the species-specific DNA typing tests that would be needed in a case. Besides aiding the analysts in minimizing the consumption of limited DNA, an accurate species-specific DNA quantification method will also facilitate cost efficient analyses as analysts can now adjust the amount of reagents used according to the concentrations of sample DNA.

Designed to be conceptually straightforward and practical, our triplex and quadriplex assays use species-specific TaqMan® MGB hydrolysis probes to target the STR region of the human tyrosine hydroxylase (TH01), a locus that is widely used for human forensic identification (Timken et al. 2005) and the canine and feline Melanocortin 1 Receptor (MC1R), a nuclear gene that encodes for a seven-pass transmembrane G protein-coupled receptor protein involved in hair and fur coloring in all mammals. This MC1R gene sequence is conserved in dogs and cats as well as their closely related species (Evans et al. 2007) but has sufficient inter-specific variability that can be used for species identification of dog and cat DNA with high levels of taxonomic confidence. Since all three are nuclear markers and have unique subchromosomal locations, they make ideal and relevant target sites to estimate the amount of human, canine, and feline nuclear templates for DNA profiling. The combination of kinetic real-

time PCR technology and fluorescent probes provides a greater functionally effective quantitative range and does not require extensive attention or expertise on the part of the analysts especially for post-PCR processing, and data collection and analysis.

Livestock and poultry assays

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 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 simplifies data collection and analysis (Kanthaswamy et al. 2012). 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. 2012; 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 qPCR

techniques for mitochondrial (mt) DNA analysis have already been used for species testing in food safety management and for screening for fraudulent or 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 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. 2012). Nuclear DNA concentration is pertinent information for optimal genotyping that can be used for individual identity testing and for identifying or tracing the source of meat products throughout the entire supply chain (Alonso et al. 2004, Timken et al. 2005).

The livestock 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 and horse, the beta-actin (ACTB) gene in pig, and the T-cell surface glycoprotein CD4 gene in goat and sheep. 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 nuclear DNA profiling than mtDNA-based

approaches that analyze non-nuclear loci that occur in multiple copies in a cell (Walker et al. 2004).

Of the few poultry assays developed, most rely on mtDNA genes as markers for detection (Girish et al. 2007, Haunshi et al. 2009, Herman 2001, Soares et al. 2010). Like the livestock assay, our poultry multiplex qPCR assay uses species-specific hydrolysis probes and primers to target nuclear genes in common poultry animals including the T-cell surface glycoprotein CD4 gene in duck, the transforming growth factor beta 3 gene (TGFB3) in chicken, and the prolactin receptor gene (PRLR) in turkey. The TFGB3 and PRLR genes have previously been used in studies and have been determined to be species-specific (Köppel et al. 2009). While this assay is sensitive and specific to chicken, turkey, and duck nuclear DNA detection and quantification, respectively, in single source samples, it is also capable of simultaneously detecting and quantifying all three poultry species' nuclear DNA in multi-species mixtures without cross-reactivity.

C. sativa assay

Approximately 90% of 700,000 drug-related cases in the United States involved *C. sativa* possession (Pollan 2001). According to the National Forensic Laboratory Information Systems (NFLIS) 2009 Annual Report (U.S. DEA 2010), Cannabis or THC was the most frequently identified illicit drug by American crime laboratories, accounting for 33.96% of all drug cases in the United States. *C. sativa* plant material is typically identified morphologically using cystolithic hairs on the leaves or using chemical tests designed to identify the presence of cannabinoids, such as high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) (Coutts & Jones 1979, Debruyne et al. 2003, Miller Coyle et al. 2003). Seeds suspected to be *C. sativa* are germinated into a viable plant so that these morphological and chemical

tests can be performed. Seed germination, however, causes delays in analysis that could be reduced with a fast, reliable DNA-based assay. Recent studies have shown that the genus Cannabis can be distinguished from its closest relative, within the genus *Humulus*, using both nuclear (ITS1, ITS2) and chloroplast loci (trnL, trnF) genes to identify *C. sativa* (Tsai et al. 2006, Linacre & Thorpe 1998, Siniscalco 1998, Gigliano et al. 1997). These assays require between five (Tsai et al. 2006) and 20 (Linacre & Thorpe 1998) nanograms (ng) of initial copies of the target (or template) DNA in order to make a positive identification and, therefore, may not be feasible for detecting pg amounts of a sample. More recently, SNaPshot™ (minisequencing) techniques that employ several single nucleotide polymorphisms (SNPs) at a time have been used to differentiate *C. sativa*, however, these assays require considerable post-PCR processing and handling of amplified material (Rotherham & Harbison 2011). These methods are also limited to samples from which DNA templates of sufficient quality can be isolated. Therefore, challenges arise when the forensic sample consists of only a single seed, a few seeds, and too little or degraded particulate material. In addition, the previously described methods are more time-consuming and expensive.

To address simultaneously the problems of low target DNA concentration and low quality DNA, we have designed and developed a real-time qPCR method that uses the TaqMan® chemistry and targets chloroplast DNA (cpDNA). Multiple chloroplasts exist per plant cell, and there are multiple copies of the chloroplast genome in each chloroplast. Additionally, the chloroplast membrane protects the DNA from degradation (Taberlet et al. 2007), therefore, cpDNA was chosen as the target for this assay to increase the probability of success of testing limited or degraded *C. sativa* samples. To further address the problem of low starting sample quantity, qPCR provides both the

specificity and the sensitivity needed to identify forensic samples from all tissue sources (Kanthaswamy et al. 2012, Evans et al. 2007).

As demonstrated elsewhere, qPCR technology has already been used to identify unknown samples of animal origin in forensic samples (Kanthaswamy et al. 2012, Evans et al. 2007) and is already widely used and accepted in forensic settings (Vallone et al. 2008). This method is not limited to those samples from which DNA of large quantity or high quality can be prepared. Unlike Sanger sequencing (Mukherjee et al. 2008), STR (Howard et al. 2008), or SNaPshot™ (Rotherham & Harbison 2011) analysis of *C. sativa*, qPCR also requires no post-PCR processing, cutting down on both the cost and time needed to identify an unknown sample, both important factors in a forensic setting. Because qPCR technology allows for the simultaneous amplification, identification, and quantification of one or more specific DNA sequences in real-time, the qPCR protocols minimize the risk of exposure to contamination. Therefore, the creation of a qPCR test capable of specifically identifying pg amounts of *C. sativa* DNA would further the forensic science community's ability to serve its clients in an efficient and timely manner. The novel qPCR assay for species identification of suspected *C. sativa* particulate material described in this study aims to provide such a test, while utilizing instruments and reagents either already in forensic laboratories, or ones that can be easily obtained by forensic laboratories.

II. Methods:

Human-dog-cat assays

Human hair samples and buccal swabs were collected from various volunteers using university approved procedures (IRB Protocol Number 200816356.1). Dog and cat hair, fecal, urine, and buccal samples were obtained from household pets with their owners' permission. Human and non-human DNA was purified using the vendor's

protocol (QIAGEN, Inc., Valencia, CA). Pre-quantified high molecular weight human, dog and cat DNA extracts (GH 130F, GD 150F, and GC 180F) purchased from Zyagen (San Diego, CA) were used as qPCR control samples and quantification standards for all experiments. DNA from human blood and feces were purchased from Zyagen (San Diego, CA). For the population study, in addition to a set of mixed breed dog DNA samples (N = 24) from Kanthaswamy et al. (2009), samples of mixed breed cat DNA (N = 24) obtained ex gratia from the National Cancer Institute (NCI), Questgen Forensics (Davis, CA), and the UC Davis Veterinary Hospital (Animal Blood Bank) were also included.

The human TH01 primers and probes were the same as those designed, validated and published by Swango et al. (2007). Primers to prime the synthesis of the dog and cat MC1R sequences were designed based on the MC1R sequences using the Primer Express® v3.0 software (Applied Biosystems, Carlsbad, CA). The program was used to design several candidate primer pairs that amplified the target input template in silico. These primer sequences were then submitted to the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) Basic Local Alignment Search Tool (Altschul et al. 1990) to search against the NCBI nucleotide database. The search results were screened to avoid primer pairs that were not specific to the dog and cat input template. Because many segments within the dog and cat MC1R genes exhibited identical sequence homology (i.e., constant sequences) only primer annealing sites in these orthologs that exhibited identical sequences were targeted for primer design. This facilitated the development of a common dog-cat forward primer and a common dog-cat reverse primer (Table 1) that not only met our criteria for input template-specificity but also enabled the simultaneous amplification of both dog and cat MC1R fragments without any off-target effects. The common primers bind to a segment

of the dog MC1R gene (GenBank accession number: AF064455.2) from nucleotide position (np) 549 to np 757, and the cat MC1R ortholog (GenBank accession number: AY237395.1) between nps 212 and 420. While the human TH01 PCR reaction generated a 180 bp long human amplicon as reported in Swango et al. (2007), both the dog and cat reactions generated a 209 bp PCR product, respectively.

To further ensure their species-specificity and to identify and prevent inadvertent cross-taxa homologies between the dog-cat primer pair and regions of the non-target genomes, the dog-cat MC1R primer sequences were aligned with commonly-occurring non-canid and -felid mammalian MC1R orthologs from GenBank including sequences from several cattle, goats, horses, humans, mice, pigs, rats, and sheep using Sequencher v4.9 (GeneCodes Corp., Ann Arbor, MI). This primer pair was aligned against other dog (accession numbers: GU220378.1 and GU220379.1) and cat (accession numbers: AB100089.1, FM180571.3 and FM877776.1) genomic sequences downloaded from the GenBank database as well as laboratory-generated sequences from four different mixed-breed dogs and five different mixed-breed cats to ensure that they annealed to regions that were conserved among the different breeds within each species, but not to non-target sites (paralogous sites) within the same species. The laboratory-generated dog (Accession numbers JF501537 to 40) and cat (JF501541-45) MC1R sequences which were generated using Sanger-sequencing techniques are available in the GenBank database.

Evaluation of the dog-cat forward and reverse primers (Table 1) for sensitivity and efficiency were based on PCR amplifications of 10-20 ng of genomic DNA in a 12.5 µL reaction volume. Each reaction contained a 10.5 µL of PCR cocktail comprising 8.14 µL distilled deionized water, 0.25 µM dNTPs, 1.25 µL 10X PCR Buffer, 0.3 µL of 50 µM of $MgCl_2$, 0.25 µL of 10 µM forward and reverse primers and 0.06 µL of Taq

polymerase. Thermalcycling was conducted using the Eppendorf Mastercycler epgradient instrument (Eppendorf North America, Hauppage, NY) under the following conditions: 3 min at 95°C, 20 sec at 95°C, 45 cycles at 56°C for 10 sec, and 72°C for 42 sec, and final hold at 4°C. Presence and purity of the dog and cat MC1R PCR products were confirmed using 6% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), with the aid of size standards, to confirm the presence of amplified product of specific length. Electrophoresis was conducted in Bio-Rad Mini PROTEAN Tetra Cell, 1x SB Buffer and Sucrose loading dye (375 µL 30% Glycerol, 250 µL Bromophenol Blue, and 125 µL 1% Xylene Cyanol) at 160V for approximately 45 minutes. Gels were stained in a 2 µg/mL Ethidium Bromide (EtBr) solution (Amresco, Solon, OH) for 3 minutes and visualized on a UV transilluminator using the Alphaimager program (Alpha Innotech Corp., San Leandro, CA). The dog and cat MC1R fragments were then sequenced using the ABI 3130 Genetic Analyzer (Applied Biosystems) following the manufacturer's protocol and were aligned against each other using Sequencher to identify taxon-specific SNPs.

The dog and cat MC1R ABI TaqMan® MGB probes that targeted these SNP sites were also designed using the Primer Express® software. As with the MC1R primer pair, the dog and cat MC1R probe sequences were also evaluated for species-specificity and non-specific binding to avoid spurious reactivity. Unlike the MC1R primer pair, the canine and feline MC1R probes were specific to dogs and cats, respectively. The dog and cat MC1R and human TH01 probes were first tested in qPCR singleplexes to check for specificity and efficiency using default ABI qPCR Absolute Quantification conditions. The human TH01 hydrolysis probe is NED-labeled for detection while the dog and cat MC1R probes utilize FAM and VIC-fluorophores, respectively. A ROX-

labeled passive reference dye (present in the master mix) was employed to correct for well-to-well variations in background fluorescence.

The triplex qPCR assay (i.e., incorporating the human TH01 and canine and feline MC1R primers and probes sets) were assembled and optimized following the recommendations in the Real-Time PCR handbook by Invitrogen (2008). The instrument's cycling configuration for the triplex assay was an initial hold of 95°C for 10 min, 45 cycles at 95°C for 15s, 56°C for 30s, and 70°C for 27s. The cycle threshold (Ct) value for each singleplex reaction of the triplex assay was optimized to an approximate range of 40 cycles. The total per reaction (single well) volume of each triplex assay PCR cocktail solution was 32 µL with 1.06 µL human forward and reverse primers, 2.11 µL each dog-cat forward and reverse primers, 0.59 µL of human probe, 0.99 µL dog probe, 0.66 µL cat probe, 6 µL mixture DNA, and 18.4 µL ABI TaqMan® Universal Master Mix-II no UNG. An Applied Biosystems 7300 Real-Time PCR instrument and ABI Prism software v1.4 were used for designing and optimizing the triplex assay, and for collecting real-time PCR data.

Development validation studies on the triplex assay were performed to define its limitations in detecting and quantifying target specific DNA, and in yielding reliable, reproducible, and robust results. All studies performed used the optimized triplex conditions as previously described. A DNA mixture containing equal amounts of human, dog, and cat DNA was used. For the quantification study the initial concentration of DNA from each species was normalized at 40 ng/µL. For subsequent studies, the per reaction concentration of 6 ng of DNA from each species, referred to hereafter as the standard DNA concentration (Co), were used. All studies were run in triplicate.

Quantification of DNA samples from 24 mixed breed dogs, 24 mixed breed cats, and 10 samples from anonymous human donors were performed to normalize DNA

concentration across samples. Post-normalization, equivalent Ct values across samples indicated that probe and primer annealing sites did not contain mutations. The presence of individual-specific mutations could potentially interfere with the efficiency of PCR and thus, the ability of the triplex assay to accurately quantify DNA.

Analytical sensitivity refers to the minimum amount of DNA in a sample than can be quantified accurately with the assay (Bustin et al. 2009). Singleplex and triplex qPCR assays were run with samples serially diluted from 1:1 to 1:1000 to obtain standard curves. Standard curves were generated for both singleplex and triplex runs by plotting cycle threshold values for each species against the log of the initial concentration (log Co) of the samples. Standard curves produced a good fit to the linear regression having an R^2 value of 0.95, 0.95, and 0.97 for the triplex assay and 0.99, 0.97, and 1.0 for the singleplexes for human, dog and cat samples, respectively. Separate serial dilutions were also conducted with the addition of 1 µL TaqMan® master mix into each well to determine the effect of increased TaqMan® master mix to the triplex assay

The triplex assay was evaluated with sample DNA purified from different human, dog, and cat tissues and body fluids or stains including blood, bone, feces, hair, saliva, and urine derived from the same individual human, dog, and cat to determine if the triplex assay is capable of detecting DNA from different types of biological sources routinely recovered in crime scenes. All these studies were conducted including one mixed sample of human, dog, and cat DNA at standard concentration.

An important step in the triplex assay's validation is to determine if it is target specific (i.e., exclusively detects DNA of human, canid, and felid origins) and if non-target DNA would interfere with its efficiency in target DNA detection. Cross-species amplification can limit the functional detection range of the quantitative analyses. To evaluate the triplex assay's ability to exclusively detect appropriate target sequences

rather than other non-specific targets (and without any interference from the latter that might be also present in the crime scene sample) we simulated the effect of mixtures with non-specific sources by combining the standard human, dog, and cat DNA with 2 ng of template DNA from each of the following commonly encountered animals: pig, chicken, duck, goose, turkey, goat, cow, sheep, horse, hamster, mouse, fish, rabbit, and rat in 1:1 (6 ng/μL each) and in 100:1 (6 ng/μL non-target species: 0.6 ng/μL target species) DNA dilutions.

Inhibition studies were conducted with humic acid (Sigma Aldrich, St. Louis, MO). Wilson (1997) demonstrated that 1 μL of humic acid-like extract could successfully inhibit a 100 μL PCR reaction. A 1 mg/mL humic acid solution was prepared with 10 mM NaOH and serially diluted from one- to 10-fold. DNA of standard concentration was mixed with 1 μl of each fold dilution of humic acid solution and subjected to qPCR conditions.

The degradation study was performed with enzyme DNase (1 U/μL). The experimental set up with DNase was identical to Swango et al.'s (2006) degradation study. Degradation of DNA was basically conducted by subjecting it to varying time intervals of DNase activity and the addition of 2 μL of EDTA to inactivate DNase as in Swango et al. (2006). Separate studies were also conducted with the addition of 2 μL of EDTA in each well of the triplex study to determine the effect of EDTA on Ct values.

Previous assays developed in the forensic community have emphasized the use of IPCs in order to detect the presence of PCR inhibitors (Swango et al. 2006, Hudlow et al. 2008). The IPC reported in Swango et al's (2006) paper could not be integrated into our assay due to optimization issues. Therefore for our quadriplex assay, we used the TaqMan® Exogenous Internal Positive Control (ABI catalog number-4308335). The thermal cycling conditions are the same as previously published. A volume of 0.56 μL of

Exo-IPC DNA, 2.52 µl of Exo-IPC mix, and 0.4 µL of molecular grade water is integrated into the assay. The qPCR cocktail volume is now reduced to 28 µL and uses 16.0 µL of Roche FastStart Universal Probe Master (ROX). The dyes on the target probes are NED, FAM, Cy5, and VIC for human, dog, cat, and IPC, respectively. Similar studies to determine the sensitivity, specificity, and robustness of the quadriplex assay were conducted in the same manner as the above-cited paper.

Livestock and poultry assays

Livestock assay

Purified and quantified high molecular weight livestock 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).

The bovine and equine primers and probes (Table 2) 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.'s (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 $MgCl_2$, 0.25 µL 10 uM primers (Integrated DNA Technologies, San Diego, CA), and 0.06 µL Platinum® Taq Polymerase (Invitrogen, Carlsbad, CA).

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 (Ng et al. 2012a, b). 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.

The qPCR assay was designed and optimized for a qPCR cocktail volume of 15 µL and 5 µL of DNA (Ng et al. 2012a). 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) 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 for each target were generated using a 10^4-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 in the equation $E=10^{[-1/m]}-1$ (Bustin et al. 2009). The standard curves facilitated the measurements of DNA quantities of unknown samples (Ng et al. 2012a).

Dilution studies were conducted using 10 µg/µL DNA per species for a total concentration of 50 µg/µL (Ng et al. 2012a). 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 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 (Ng et al. 2012a). 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 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. (2012a) 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 (Ng et al. 2012a).

A sample size of N =10 for each species was obtained ex gratia from Scidera (Davis, California), from 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 (Ng et al. 2012a). The assay was run according to qPCR conditions above.

Poultry assay

Purified and quantified high molecular weight genomic reference DNA extracts (Mallard: GD-220, Chicken: GC-120F, Turkey: GT-150) were purchased from Zyagen (San Diego, CA) and used as controls and quantification standards for all experiments. The remaining samples were used for the population study. Purified and quantified chicken DNA extracts (N = 8) were obtained ex gratia from Scidera (Davis, CA). Duck feathers from ten individuals, each representing one of the following breeds: White Layer, Buff, Khaki Campgell, Welsh Harlequin, Cayuga, Black Runner, Mallard, Chocolate Runner, Blue Runner, and Black Swedish were obtained ex gratia from Metzer Farms (Gonzales, CA). These duck breeds were derived from the wild Mallard (*Anas platyrhynchos*) which is the most commonly consumed species (Hird et al. 2005). Buccal swabs from livestock in the Davis, CA area were collected with owners' consent. The basal tip of the calamus and the blood clot from the superior umbilicus of the feathers were isolated following procedures by Horváth et al. (2005) and extracted and purified together using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) according to the vendor's tissue extraction protocol. Buccal samples were extracted and purified using the same kit following the 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).

The chicken and turkey primers and probes were the same as those designed and published by Köppel et al. (2009). All duck nuclear genes and corresponding genes for chicken and turkey were downloaded from GenBank and aligned using Sequencher v.4.10.1 (GeneCodes, Corp., Ann Arbor, MI) to screen for duck specific sequences for in silico primer and probe design using Primer Express® v.3.0 (Applied Biosystems, Carlsbad, CA). Candidate primer and probe sequences were screened in silico and in

vitro following the same procedures as our previous work (Ng et al. 2012b) for species specificity.

The qPCR assay was designed and optimized for a qPCR cocktail volume of 17 µL and 3 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) and distilled deionized water for a total volume of 17 µL. Three microliters of DNA consisting of 1 µL each of 10 ng/µL DNA for each of the three target poultry species was used in the multiplex. The qPCR run condition was the same as previously published (Ng et al. 2012b).

Standard curves were generated and efficiencies calculated following the same procedures as previously published (Ng et al. 2012b).

Validation studies were conducted using the same procedures as published previously in Ng et al. (Ng et al. 2012b). The dilution and inhibition studies were performed with 10 ng/µL of DNA from each target species for a total DNA concentration of 30 ng/µL. The only modification was in the non-target DNA dilution study. The non-target DNA species used in equal amounts for diluting included Cow (Cat. # GB-110), Sheep (GS-190), Goat (GG-150), Pig (GP-160), Horse (GE-170), Cat (GC-130F), Dog (GD-150F), and Human (GH-180F) purchased from Zyagen (San Diego, CA). Since the chicken and turkey primers and probes were previously validated by Köppel et al. (2009) to be species-specific, a population study was not done for these two species. However, the duck primers and probe were designed in-house and were validated for specificity using the population study.

C. sativa assay

Non-viable *C. sativa* seeds that were estimated to be over 30 years old (J. Effenberger, pers. comm. 2011) were obtained ex gratia from the California Department of Food and Agriculture's Seed Herbarium specifically for the purpose of conducting this research (Johnson et al. 2013). *Humulus lupulus* seed and leaf material were obtained ex gratia from the UC Davis Department of Food Science and Technology. Single seeds were placed in a 1.5 mL microcentrifuge tube with 400 µL of nuclease-free ddH_2O water. The seeds were then heated at 55°C for one hour and then ground with a plastic 1.5 mL microtube pestle until the seed was completely disrupted. DNA from the ground seed material was extracted using REDExtract-N-Amp Seed PCR Kit (Sigma, St. Louis, MO) stopping before amplification. The DNA extract was then further purified to remove inhibitors using a QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) resulting in 200 µl of DNA suspended in AE buffer and stored at -20°C.

Extracted seed DNA samples were quantified using a Qubit 1.0 fluorometer (Invitrogen, Carlsbad, CA) with reagents designed for low concentration DNA samples; however, the DNA content of the sample was below the detection limit of the instrument (0.5 ng/µL). The DNA samples were amplified using the C, D, G, and H primers that target the intergenic spacer flanked by the trnL 3' exon and trnF gene in the *C. sativa*, as described in Linacre & Thorpe (1998). The samples were PCR amplified using Sigma REDExtract-N-Amp Seed PCR Kit (Sigma, St. Louis, MO) according to the manufacturer's directions. Ten microliters of REDExtract-N-Amp PCR reaction mix was added to 2 µL of 25 µM of each of the four primers along with 4 µL of seed extract for a total volume of 22 µL. The mixture was incubated for 10 minutes at 94°C, followed by 45 cycles of 30 seconds at 94°C, 30 seconds at 60°C, 30 seconds at 72°C, and 7 minutes at 72°C, using a PTC-100 thermocycler (BioRad, Hercules, CA). Five microliters of the

product was then electrophoresed on a 2% agarose gel for 10 minutes at 200V to confirm the presence and size of PCR product. The C and D primer pair generated a 348 bp fragment in all green plants whereas the G and H primers produced a 197 bp fragment specific to *C. sativa* in accordance with Linacre & Thorpe (1998).

The amplicons were then purified using ExoSAP IT (USB, Santa Clara, CA) following the manufacturer's protocol. Once purified, samples were amplified for sequencing using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. After amplification, excess cycle sequencing reagents were removed with BigDye XTerminator (Applied Biosystems, Foster City, CA) again following the manufacturer's protocol. The cleaned product was then electrophoresed on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the BigDye Xterminator run settings and POP7 polymer (Applied Biosystems, Foster City, CA). After cycle sequencing, the sequences were trimmed for quality and chromatograms for the two sets of forward and reverse sequences were visualized and aligned using Sequencher v4.9 (GeneCodes Corp., Ann Arbor, MI). The resulting consensus sequences were compared to the National Center for Biotechnology Information's (NCBI) GenBank database (Acc. No. AY958396.1) using the BLAST program (Altschul et al. 1997) to confirm the species identity of both products.

The *C. sativa* sequences generated in our laboratory using Linacre & Thorpe's (1998) CD and GH primer sets did not yield suitable priming and probe sites for the development of our qPCR assay. Their CD and GH primers also generated fragments that were too large (348 and 197 bp, respectively) for optimum qPCR analysis. Our green plant and *C. sativa*-specific primers are different from those published by Linacre & Thorpe (1998) as they target different amplification sites and produce shorter amplicons.

Therefore, our laboratory-generated *C. sativa*-specific and green plant sequences (Table 4, Accession number: JF900767) were aligned with sequences of both *C. sativa* and *H. lupulus* from GenBank, including the trnL and trnL 3' exon-trnF intragenic spacer regions (Accession numbers: AB033890.1 and AY958396.1) into a single, FASTA-formatted file. For the green plant qPCR assay, only conserved priming sites and probe target sites were chosen for primer and probe design. SNPs that distinguished *C. sativa* and *H. lupulus* sequences were identified from the sequence alignment file using Sequencher V4.9 (GeneCodes Corp., Ann Arbor, MI). Potential priming sites containing *C. sativa*-specific SNPs were considered highly suitable for *C. sativa*-specific probe design to distinguish material from this plant from those of others, including *H. lupulus*. Potential primers were then evaluated for high GC content and used as input for NCBI's Primer BLAST (Altschul et al. 1997) to measure Tm (time) and potential for self-compatibility.

The green plant- and *C. sativa*-specific probes were first tested in singleplex assays to determine the optimum qPCR reaction conditions. The probes were combined into a single qPCR duplex assay and optimized further. After optimization, the qPCR cocktail contained 20 µL of 1x TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA), 0.31 µL of 0.25 µM green plant-specific forward and reverse primers, 1.24 µL of 1.0 µM *C. sativa* specific forward and reverse primers, 0.25 µL of 0.08 µM Green plant specific probe, 1.40 µL of 0.45 *C. sativa* specific probe, and 1.26 µL of EDTA per sample. From this master mix 15 µL of sample was added to 16 µL of master mix to achieve a final reaction volume of 31 µL per well. An Applied Biosystems 7300 qPCR instrument and ABI Prism software v1.4 were used for optimization of the assay and for collecting real-time PCR data. The instrument's cycling configuration for the assay was an initial hold of 55°C for 7 minutes and then 95°C for

10 minutes, 45 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and 70°C for 30 seconds. The green plant specific amplicon is 101 bp in length and is labeled with 6FAM-labeled probe for detection. The *C. sativa* specific amplicon is 180 bp in length and is labeled with a VIC-labeled probe for detection. A ROX-labeled passive reference dye (contained in the master mix) was used to account for well-to-well variations in background fluorescence.

Positive amplification, as determined by using the Real-Time PCR handbook by Invitrogen (2008), occurred consistently between cycles 30 and 40. Extracted DNA samples were also serially diluted in triplicates of 1:1, 1:10, 1:100, and 1:1000 ratios representing approximately 500 pg/μL, 50 pg/μL, 5 pg/μL, and 0.5 pg/μL of DNA, respectively to determine the sensitivity of the assay.

III. Results

Human-dog cat assays

Evans et al. (2007) redesigned their original K9MC1R-F primer because one dog in their sample set of 182 exhibited a mutation within the primer annealing site that affected the Ct estimates. Based on our evaluation with multiple samples of humans, dogs, and cats, the triplex assay produced optimal Ct values with equal efficiency for each individual within each species (Fig 1). Therefore, it is conceivable that none of the target species exhibited any intraspecific variation within the primer and probe annealing sites that can affect the binding affinities of the primers and probes, and the sensitivity of this assay.

The triplex assay was run with serial dilutions of human, dog and cat DNA in order to determine the lowest detection limit for DNA concentrations. It was found that the triplex assay can detect to as low as 0.4 pg of DNA in humans and cats, respectively, and 4.0 pg in dogs; these detection limits are slightly lower than nuclear

DNA amounts in diploid cells (6 pg). The minimum detection limits of our triplex assay also slightly exceeded the minimum limits of the dog (5.0 pg) and human (5.0 pg) quantification assays that were developed by Evans et al. (2007) and by Swango et al. (2006), respectively.

The triplex assay was able to detect DNA extracted from blood, bone, feces, hair (both pulled and shed hair), saliva, and urine from all three target species. DNA derived from different source material from different individuals of the same species unequivocally matched in terms of species identity; no individual variation within each species sample sets was observed. While exhibiting both sensitivity and specificity to lower concentrations of target DNA, greater consistency in probe-target binding affinity for measuring template concentrations was observed in tests that involved more concentrated templates. Figure 2 shows the results obtained from the dog bone sample included in this study.

The triplex assay unambiguously detected the known values of each target species' DNA quantities in the mixed-species sample studies that contained DNA from chicken, cow, duck, goat, goose, horse, sheep, pig, mouse, rat, fish, hamster, and turkey. None of these tests showed any amplification with non-target species nor were there any background cross-species reactivity when mixed-species templates were tested. This clearly demonstrates that the triplex assay is highly species-specific and does not reflect any interference nor exhibit cross-species amplifications with DNA from other commonly occurring species.

Inhibition of the triplex assay with humic acid did not entirely prevent amplification of target DNA. The assay as a whole failed to detect DNA when the concentrations of humic acid were high. The dog and cat specific components of the assay performed less successfully when the ratio of humic acid dilutions was higher than 1:100. Human

probes produced Ct values at 1:10 to 1:1000 humic acid dilutions and dog and cat probes produced Ct values starting from 1:100 humic acid dilution. The ability of this triplex assay to detect target DNA even in the presence of a natural inhibitor like humic acid is indicative of its robustness. Figure 3 shows the triplex assay (with dilutions) in the presence of humic acid.

The presence of 1 µL of EDTA in each well significantly reduced the Ct values thus enhancing the real-time amplification in each run. The addition of EDTA as a reagent enhanced the results of the DNA detection and quantification analysis. Figure 4 illustrates the result of this experiment with and without the addition of EDTA.

We have designed and tested a sensitive and reliable TaqMan® qPCR triplex assay for species-specific (human, dog, and cat) identification and quantification of DNA from forensic evidence including degraded remains and mixed-species samples. The design of this qPCR protocol is appealing because it is simple to use and does not require specialized expertise. Unlike SYBR Green-based assays (Walker et al. 2004), dissociation curves are not required to distinguish target fragments in the TaqMan® protocol, therefore our method requires a short run time. The triplex assay's probes and primers target single-copy sites in the nuclear genome that are specific to humans, dogs, and cats including the human TH01 locus and the dog and cat MC1R loci. Our detection and DNA quantification technique which relies on single copy targets is more relevant to DNA profiling than assays that interrogate loci that occur in multiple copies throughout the genome (Walker et al. 2004). Moreover, because it targets nucleated cells for species determination and quantification analysis it provides a crucial advantage over assays that employ mtDNA (Melton & Holland 2007) that confer accurate species confirmation but without a nuclear DNA quantification feature.

The results of the population, inhibition, degradation, and dilution studies involving the human-dog-cat quadriplex assay with IPC (Fig. 5) remained unchanged. Dilution studies were conducted using 48 ng/ul as the starting DNA concentration (mixture of 16 ng/ul human, dog, and cat DNA). The quadriplex started dropping off at dilution 1:10,000. It detects DNA as low as 8 ng/ul, which is very close to the amount of DNA present in one diploid cell (approximately 6 pg).

Livestock assay

Table 2 lists the primers and probes used for each target livestock species. The multiplex assay was run containing serial dilution mixtures with equal amounts of cattle, goat, sheep, horse, and pig DNA to test the 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 6 shows the multi-species dilution plot of the livestock assay.

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. 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. 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.

Poultry assay

QPCR reactions have a maximum efficiency of 100% since the amount of DNA can only be doubled per cycle. However, efficiencies greater than 100% have been observed (Clark-Langone et al. 2010, da Silva Coelho et al. 2010), but are most likely due to pipetting errors and other reaction inefficiencies such as primer dimer. Figure 7 shows the species-specificity of the poultry assay.

Serial dilution mixtures with equal amounts of chicken, duck, and turkey DNA samples were run to test the 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). In the non-target dilution study, all species were detected at $1:10^2$ dilutions with duck and turkey samples consistently detected down to $1:10^3$ dilutions.

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 2.5 minute time interval. All species were detected by qPCR for the digestion time intervals 0 and 5 minutes and only the chicken DNA sample was detected after 10 minute's digestion.

Hematin completely inhibited the qPCR reactions for samples combined with 1 mM to 31.25 µM of inhibitor. Only the duck DNA sample was detected in 15.63 and 7.81 µM of hematin and all samples were detected at 3.91and 1.95 µM of hematin. Humic acid completely inhibited the qPCR reactions from 1000 ng/µL to 15.6 ng/µL of inhibitor. Only the duck sample was detected at 7.81 ng/µL of humic acid, and duck and chicken samples were consistently detected at 3.91 ng/µL. All species were detected at 1.95 ng/µL of humic acid.

The duck assay was the only assay subjected to the population study. Each duck sample was run in duplicate and was the only sample detected in each reaction. The

reactions did not show signs of inhibition for any of the individuals or any inconsistencies between individuals. Since all 10 individuals were reliably detected and correctly identified without any inter-individual differences, it is reasonable to postulate that the target primer and probe annealing sites do not have mutations that affect primer and probe binding and the target fragment does not have mutations that affect the efficiency of the reaction and species specificity.

Though few poultry assays have been developed for detecting food adulteration and fraudulence (Stamoulis et al. 2010), increasing consumer awareness of food adulteration has led to more assays involving poultry species. With poultry increasingly becoming the preferred meat of many individuals, poultry meats adulterated with other meats (Soares et al. 2010, Doosti et al. 2011, Stamoulis et al. 2010) have become a focus of study as opposed to poultry meats being the adulterant. In addition to consumer demands for food transparency, food fraudulence has severe economic effects on the food industry. The quick and accurate detection of poultry species with qPCR can reduce fraudulent replacement of higher quality meats with lesser quality meats and provide quantification data for subsequent individual identification and traceability testing.

In our study we analyzed pure DNA extracts of our three target poultry species. Since DNA is present in all tissues we collected samples that were the least invasive to the animals even though our assay will most likely be applied to meat samples. The detection of our targets is based on the amount and quality of DNA in the reaction and not from which the DNA was derived. The quantitative analysis of heterogeneous meat samples (i.e. spiked meat) was considered, but the detection and quantification of targets in mixed meats is dependent on the sampling method, DNA extraction method, and assay detection. The mixed meat analysis was not done because there is no

definitive method to determine if the assay is capable of accurately quantifying the DNA or if the variation in DNA profiles that are generated are caused by the differences in sampling or extraction methods.

***C. sativa* assay**

Taxonomic identification of *C. sativa* using molecular DNA techniques provides accurate species determination of trace and particulate materials which may otherwise remain undetermined. Seeds were used in this experiment as they pose a greater challenge for positive identification than other parts of the plant. *C. sativa* seeds as a DNA source tend to limit options for efficient DNA extraction techniques, therefore the lack of a quick and accurate species identification method for seeds suspected of being *C. sativa* has been a problem in law enforcement. Although Sanger sequencing-based methods have been used in the forensic identification of suspected *C. sativa* seeds (Tsai et al. 2006), these DNA sequence-based techniques are both time consuming and costly.

The DNA concentration of the *C. sativa* seed samples was below the detection threshold of the Qubit 1.0 fluorometer for accurate quantification. Despite this low concentration, the DNA samples were amplified using end-point PCR and were visualized on agarose gels to confirm the presence of DNA from the desired species, and not simply contamination. Two bands of appropriate size as described by Linacre & Thorpe (1998) could clearly be seen suggesting that sufficient template DNA was present for successful PCR, even though it was too dilute for quantification using the fluorometer.

A three hundred forty-eight base pair sequence was generated using Linacre & Thorpe's (1998) CD primers and a consensus sequence was formed using the sequence from multiple seeds. A BLAST search using this consensus sequence

showed 12 matches with 100% identity to *C. sativa* along with many other close matches (ranging from *C. sativa* to *Ficus ampelas*). This was not unexpected as the purpose of assaying this locus as the target for the green plant probe was to serve as a green plant positive control for positive amplification of DNA from all green plants including *C. sativa*. The Linacre & Thorpe's (1998) GH primers generated a 197 bp sequence whose BLAST results showed 100% identity to 47 *C. sativa* sequences in GenBank.

When the duplex assay comprising the green plant and *C. sativa* primers and probes was tested on DNA extracted from known *C. sativa* seeds, and with known *H. lupulus* leaf material, respectively, samples from the *C. sativa* seed exhibited the fluorogenic activities of both probes (Fig. 8), whereas only the green plant probe yielded any signals when the *H. lupulus* extract was tested. This result is consistent with the expectation that both the green plant probe (serving as an internal positive control) and the test (*C. sativa* specific) probe would amplify given a starting sample that was in fact *C. sativa*, while the amplification of a non-*C. sativa* starting samples, as in this case *H. lupulus*, would show the amplification of only the green plant probe. This result shows that the duplex assay can robustly and accurately distinguish between *C. sativa* and its closest relative *H. lupulus*.

C. sativa cpDNA samples diluted 10-fold (approximately 50 pg/µL) routinely could be identified correctly by qPCR, however, the identification of samples diluted 100-fold (approximately 5 pg/µL) varied in success probably based upon the starting quality of the extract. This duplex assay could be designed for the quantification of cpDNA, and for optimization of DNA concentrations for downstream applications such genotyping. This was not done in the present study due to the limitations of the quality and quantity of reference DNA derived from antiquated and non-viable *C. sativa* seeds used as

starting material. These seeds were the only samples available for this research, but were unsuitable as pristine reference samples. Reference samples that can be serially diluted for the creation of an optimal standard curve for the development of a reliable quantification assay were not available.

IV. Conclusions

Human-dog cat assays

Our qPCR triplex assay is highly specific and sensitive to detecting minute concentrations of DNA. The lowest DNA detection limit of this triplex assay is 0.4 pg in humans and cats, and 4.0 pg in dogs. This remarkably low amount of input template that can be detected by the triplex assay portends its role in the analysis of forensic evidence. Apart from its high sensitivity, mixture studies do not show non-specific amplification of DNA from animals other than the target species, i.e., either human, dog, or cat. The presence of a strong inhibitor like humic acid in samples often tend to hinder PCR reactions, and the fact that this potent inhibitor did not prevent the triplex assay from detecting and measuring sample DNA further confers an added benefit to the assay. The quadriplex assay has an advantage over the triplex assay due to the inclusion of an IPC, shorter run time, reduced master mix volume, and the use of the 7500 Fast Real time system. It is essential that forensic DNA laboratories understand the importance of the robustness and specificity of the assay and validate this method at their respective laboratories for mixed-species DNA analysis.

Livestock assay

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 and will help to protect 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.

Poultry assay

The poultry assay that we designed and developed is a simple to use, one reaction step TaqMan®-based multiplex qPCR assay for the identification and DNA quantification of mixed poultry samples including duck, chicken, and turkey. Our assay has a limit of detection of 5 pg of poultry DNA. In the presence of overwhelming non-target DNA, the LOD of the assay is 50 pg of DNA for chicken and 5 pg for duck and turkey DNA, respectively. In addition to its use in the food industry for safety and fraudulence management, the simplicity and sensitivity of this assay lends itself to be very useful in criminal or civil forensic investigations that involve poultry biological evidence. The assay was successful in the identification and quantification of the target species even in the presence of 3.91 µM of hematin and 1.95 ng/µL of humic acid, two PCR inhibitors known to be highly potent. Therefore, while our assay can contribute to the quality and safety assurance of our food supply through the facilitation of downstream STR- and SNP-based identity testing and traceability assays, our robust qPCR assay can similarly be applied to downstream analyses in the animal forensic science field such as evidentiary and reference sample comparisons.

C. sativa

The goal of our *C. sativa* assay was to design a quick and easy test to identify trace amounts of *C. sativa* cpDNA using technology, instruments, reagents, and other resources readily available in most crime laboratories. The results clearly show that our duplex qPCR assay is able to robustly and accurately identify minute amounts (5 to 50 pg/µL) of *C. sativa* cpDNA. This assay enables crime laboratories to identify suspected seed samples in a timely manner that could not be done in the past.

V. References:

Alonso, A., Martin, P., Albarran, C., Garcia, P., Garcia, O., de Simon, L., Garcia-Hirschfeld, J., Sancho, M., de La Rua, C., & Fernandez-Piqueras, J. (2004) Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Sci Int, 139*(2-3), 141-149.

Altschul, S., Gish, W., Miller, W., Myers, E., & Lipman, D. (1990) Basic local alignment search tool. *J Mol Biol*. 215, 403–410.

Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25, 3389–402.

Ballin, N. (2010) Authentication of meat and meat products. *Meat Sci. 86*(3), 577-587.

Bustin, S., Benes, V., Garson, J., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M., Shipley, G., Vandesompele, J., & Wittwer, C. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem. 55*(4), 611- 622.

Clark-Langone, K., Sangli, C., Krishnakumar, J., & Watson, D. (2010) Translating tumor biology into personalized treatment planning: analytical performance characteristics of the Oncotype DX(R) Colon Cancer Assay. *BMC Cancer*. 10(1), 691.

Coutts, R. & Jones, G. (1979) A comparative analysis of Cannabis material. *J Forensic Sci*. 24(2), 291–302.

D'Andrea, E., Fridez, F., & Coquoz, R. (1998) Preliminary experiments on the transfer of animal hair during simulated criminal behavior. *J. Forensic Sci*. 43, 1257–1258.

da Silva Coelho, I., de Queiroz, M., Costa, M., Kasuya, M., & de Araujo, E. (2010) Identification of differentially expressed genes of the fungus *Hydnangium* sp. during the pre-symbiotic phase of the ectomycorrhizal association with *Eucalyptus grandis*. *Mycorrhiza*. 20(8), 531-540.

Dalmasso, A., Fontanella, E., Piatti, P., Civera, T., Rosati, S., & Bottero, M. (2004) A multiplex PCR assay for the identification of animal species in feedstuffs. *Mol Cell Probes. 18*(2), 81-87.

Debruyne, D., Albessard, F., Bigot, M., & Moulin, M. (1994) Comparison of three advanced chromatographic techniques for cannabis identification. *Bull Narc.* 46(2), 109–21.

Dooley, J., Paine, K., Garrett, S., & Brown, H. (2004) Detection of meat species using TaqMan® real-time PCR assays. *Meat Sci. 68*(3), 431-438.

Doosti, A., Ghasemi Dehkordi, P., & Rahimi, E. (2011) Molecular assay to fraud identification of meat products. *J Food Sci & Tech*. doi:10.1007/s13197-011-0456-3.

Evans, J., Wictum, E., Penedo, M., & Kanthaswamy, S. (2007). Real-time polymerase chain reaction quantification of canine DNA. *J Forensic Sci*. 52, 93–96.

Fajardo, V., González, I., Rojas, M., García, T., & Martín, R. (2010) A review of current PCR-based methodologies for the authentication of meats from game animal species. *Trends in Food Sci & Tech. 21*(8), 408-421.

Ferris, S., Sage, R., Huang, C., Nielsen, J., Ritte, U., & Wilson, A. (1983) Flow of mitochondrial DNA across a species boundary. *Proc Natl Acad Sci U S A. 80*(8), 2290-2294.

Ghovvati, S., Nassiri, M., Mirhoseini, S., Moussavi, A., & Javadmanesh, A. (2009) Fraud identification in industrial meat products by multiplex PCR assay. *Food Cont. 20*(8), 696-699.

Gigliano, G., Caputo, P., & Cozzolino, S. (1997) Ribosomal DNA analysis as a tool for the identification of *Cannabis Sativa L.* specimens of forensic interest. *Sci Justice*. 37(3), 171–4.

Girish, P., Anjaneyulu, A., Viswas, K., Santhosh, F., Bhilegaonkar, K., Agarwal, R., Kondaiah, N., & Nagappa, K. (2007) Polymerase chain reaction-restriction fragment length polymorphism of mitochondrial 12S rRNA gene: a simple method for identification of poultry meat species. *Vet Res Commun*. 31 (4), 447-455.

Halverson, J. & Basten, C. (2005) Forensic DNA identification of animal-derived trace evidence: tools for linking victims and suspects. *Croat Med J.* 46, 598–605.

Hajibabaei, M., Singer, G., Hebert, P., & Hickey, D. (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends Genet*. 23(4), 167-172.15

Haunshi, S., Basumatary, R., Girish, P., Doley, S., Bardoloi, R., Kumar, A. (2009) Identification of chicken, duck, pigeon and pig meat by species-specific markers of mitochondrial origin. *Meat Sci*. 83, 454-459.

Herman, B. (2001) Determination of the animal origin of raw food by species-specific PCR. *J of Dairy Res*. 68(3), 429-436.

Heid, C., Stevens, J., Livak, K., & Williams, P. (1996) Real time quantitative PCR. *Genome Res. 6*(10), 986-994.

Hird, H., Chisholm, J., & Brown, J. (2005) The detection of commercial duck species in food using a single probe-multiple species-specific primer real-time PCR assay. *European Food Res & Tech.* 221(3), 559-563.

Horváth, M., Martínez-Cruz, B., Negro, J., Kalmár, L., & Godoy, J. (2005) An overlooked DNA source for non-invasive genetic analysis in birds. *J of Avian Bio*. 36(1), 84-88.

Howard, C., Gilmore, S., Robertson, J., & Peakall, R. (2008) Developmental validation of a *Cannabis sativa* STR multiplex system for forensic analysis. *J Forensic Sci*. 53(5), 1061–7.

Hudlow, W., Chong, M., Swango, K., Timken, M., & Buoncristiani, M. (2008) A quadruplex real-time qPCR assay for the simultaneous assessment of total human DNA, human male DNA, DNA degradation and the presence of PCR inhibitors in forensic samples: a diagnostic tool for STR typing. *Forensic Sci. Int. Genet.* 2, 108–125.

Invitrogen. (2008) Real-time PCR: From theory to practice. Carlsbad, Print. http://corelabs.cgrb.oregonstate.edu/sites/default/files/Real%20Time%20PCR.From%20Theory%20to%20Practice.pdf.

Kanthaswamy, S., Tom, B., Mattila, A., Johnston, E., Dayton, M., Kinaga, J., Erickson, B., Halverson, J., Fantin, D., DeNise, S., Kou, A., Malladi, V., Satoski, J., Budowle, B., Smith, D., & Koskinen, M. (2009) A canine STR kit and population database for use in forensic casework. *J Forensic Sci*. 54, 829–840.

Kanthaswamy, S., Premasuthan, A., Ng, J., Satkoski, J., Goyal, V. (2012) Quantitative real-time PCR (qPCR) assay for human-dog-cat species identification and nuclear DNA quantification. *Forensic Sci Int*. Genetics, 6(2), 290–5.

Köppel, R., Zimmerli, F., & Breitenmoser, A. (2009) Heptaplex real-time PCR for the identification and quantification of DNA from beef, pork, chicken, turkey, horse meat, sheep (mutton) and goat. *European Food Res &Tech. 230*(1), 125-133.

Linacre, A. & Thorpe, J. (1998) Detection and identification of cannabis by DNA. *Forensic Sci Int.* 91(1), 71–6.

Lindquist, C., Evans, J., & Wictum, E. (2011) Developmental validation of feline, bovine, equine, and cervid quantitative PCR assays. *J Forensic Sci.* 56, Suppl 1, S29-35.

Melton, T. & Holland, D. (2007) Routine forensic use of the mitochondrial 12S ribosomal RNA gene for species identification. *J Forensic Sci*. 52, 1305–1307.

Miller Coyle, H., Palmbach, T., Juliano, N., Ladd, C., & Lee, H. (2003) An overview of DNA methods for the identification and individualization of marijuana. *Croat Med J*. 44(3), 315–21.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K., Bonnet, J., Vidal, R., Cabrita, J., & Megraud, F. (1997) Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *J Clin Microbiol. 35*(4), 995-998.

Mukherjee, A., Roy, S., De Bera, S., Jiang, H-E., Li, X., Li, C-S., & Subir, B. (2008) Results of molecular analysis of an archaeological hemp (*Cannabis sativa L.*) DNA sample from North West China. *Genet Resour Crop Evol*. 55(4), 481–5.

Ng, J., Satkoski, J., Premasuthan, A., & Kanthaswamy, S. (2012a) A TaqMan® Multiplex Assay for Hoofed Livestock Species Identification and Nuclear DNA Quantification. *Int Food Res J.* 19(3), 1279-1285.

Ng, J., Satkoski, J., Premasuthan, A., & Kanthaswamy, S. (2012b). A Nuclear DNA-Based Species Determination and DNA Quantification Assay for Common Poultry Species. Journal of Food Science and Technology. doi 10.1007/s13197-012-0893-7.

Pollan, M. (2001) The botany of desire: a plant's eye view of the world. New York, NY: Random House.

Rotherham, D. & Harbison, S. (2011) Differentiation of drug and non-drug Cannabis using a single nucleotide polymorphism (SNP) assay. *Forensic Sci Int*. 207(1), 193–7.

Siniscalco Gigliano, G. (1998) Identification of *Cannabis sativa L.* (*Cannabaceae*) using restriction profiles of the Internal Transcribed Spacer II (ITS2). *Sci Justice*. 38(4), 225–30.

Smalling, B., Satkoski, J., Tom, B., Szeto, W-Y., Joy-Alise, B., Erickson, Spear, T., Smith, D., Budowle, B., Webb, K., Allard, M., & Kanthaswamy, S. (2010) The significance of regional and mixed breed canine mtDNA databases in forensic science. *Open Forensic Sci. J*. 3, 22–32.

Soares, S., Amaral, J., Mafra, I., Oliveira, M. (2010) Quantitative detection of poultry meat adulteration with pork by a duplex PCR assay. *Meat Sci*. 85(3), 531-536.

Stamoulis, P., Stamatis, C., Sarafidou, T., & Mamuris, Z. (2010) Development and application of molecular markers for poultry meat identification in food chain. *Food Cont*. 21(7), 1061-1065.

Swango, K., Timken, M., Chong, M., & Buoncristiani, M. (2006) A quantitative PCR assay for the assessment of DNA degradation in forensic samples. *Forensic Sci Int. 158*(1), 14-26.

Swango, K., Hudlow, W., Timken, M., & Buoncristiani, M. (2007) Developmental validation of a multiplex qPCR assay for assessing the quantity and quality of nuclear DNA in forensic samples. *Forensic Sci Int*. 170, 35–45.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C., & Willerslev, E. (2007) Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Res*. 35(3), e14.

Timken, M., Swango, K., Orrego, C., & Buoncristiani, M. (2005) A duplex real-time qPCR assay for the quantification of human nuclear and mitochondrial DNA in forensic samples: Implications for quantifying DNA in degraded samples. *J Forensic Sci*. 50, 1044–1060.

Timken, M., Swango, K., Orrego, C., Chong, M., & Buoncristiani, M. (2005) Quantitation of DNA for Forensic DNA Typing by qPCR (quantitative PCR): Singleplex and Multiple Modes for Nuclear and Mitochondrial Genomes, and the Y Chromosome. *National Criminal Justice Reference Service*.

Tsai, L-C., Hsieh, H-M., Huang, L-H., Wang, J-C., Linacre, A., Lee, JC-I. (2006) Cannabis seed identification by chloroplast and nuclear DNA. *Forensic Sci Int*. 158(2–3), 250–1.

U.S. Drug Enforcement Administration. (2010) National Forensic Laboratory I System: year 2009 annual report. Washington, DC: U.S. Drug Enforcement Administration.

Vallone, P., Buel, E., Kline, M., & Foran, D. (2008). qPCR: Human DNA Quantification using Real-Time PCR Assays. Full-day workshop at AAFS meeting. Monday, February 18, 2008, in Washington, D.C. Available at: http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_qPCRworkshop.htm.

Wilson, I. (1997) Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol*. 63, 3741–3751.

Walker, J., Hughes, D., Hedges, D., Anders, B., Laborde, M., Shewale, J., Sinha, S., & Batzer, M. (2004) Quantitative PCR for DNA identification based on genome-specific interspersed repetitive elements. *Genomics. 83*(3), 518-527.

Woolfe, M., & Primrose, S. (2004) Food forensics: using DNA technology to combat misdescription and fraud. *Trends Biotechnol. 22*(5), 222-226.

Zha, D., Xing, X., & Yang, F. (2010) A multiplex PCR assay for fraud identification of deer products. *Food Cont. 21*(10), 1402-1407.

VI. Dissemination of Research Findings:

Publications:

Ng, J., Satkoski, J., Premasuthan, A., & Kanthaswamy, S. (2013) A Nuclear DNA-Based Species Determination and DNA Quantification Assay for Common Poultry Species. *Food Res Int*.

Johnson, C., Trask, J., Premasuthan, A., & Kanthaswamy, S. (2013) Species Identification of *Cannabis sativa* seeds using Real-Time Quantitative PCR (qPCR). *J of Forensic Sci*. 58(2): 486-490. doi: 10.1111/1556-4029.12055.

Ng, J., Satkoski, J., Premasuthan, S., & Kanthaswamy, S. (2012) A TaqMan® Multiplex Assay for Hoofed Livestock Species Identification and Nuclear DNA Quantification. *Int Food Res J*. 19(3), 1279-1285.

Kanthaswamy, S. & Premasuthan, A. (2012) Quadriplex real-time PCR (qPCR) assay for human-canine-feline species identification and nuclear DNA quantification. *Forensic Sci Int*. Genetics 6(3), e97-e98.

Kanthaswamy, S., Premasuthan, A., Ng, J., Satkoski, J., & Goyal, V. (2012) Quantitative Real-Time PCR (qPCR) Assay for Human-Dog-Cat Species Identification and nuclear DNA Quantification. *Forensic Sci Int*. Genetics 6(2), 290-295.

Presentations:

Sreetharan Kanthaswamy. Animal Forensics. Standing Committee on Animal Forensics Workshop, 33rd Conference of the International Society of Animal Genetics in Cairns, Australia on July 15-20th, 2012.

Sreetharan Kanthaswamy, Jillian Ng, Amritha Premasuthan, Chris Johnson, Jessica Satkoski. Multi-species determination and DNA quantification assays. Poster presentation at the 13th Annual US National Institute of Justice (NIJ) Conference 2010, Arlington, VA, June 19th, 2012.

Sreetharan Kanthaswamy, Non-Human Forensic DNA Analysis Workshop. 2011 California Association of Criminalists (CAC) Falls Seminar. Double Tree Hotel, Sacramento, California, October 24, 2011.

Sreetharan Kanthaswamy (Guest Lecturer). Non-human Forensic DNA Analysis. Laboratory of Forensic Biology, Department of Forensic Medicine, University of Helsinki, Kytösuontie 11, 00300 Helsinki, Finland. August 22, 2011.

Amritha Premasuthan, Jillian Ng, Chris Johnson, Jessica Satkoski and Sreetharan Kanthaswamy. Multi-species determination and DNA quantification assays. Poster presentation by AP at the 12th Annual US National Institute of Justice (NIJ) Conference 2010, Arlington, VA, June 20th, 2011.

Sreetharan Kanthaswamy (Invited Speaker). Development of a real-time quantitative PCR (qPCR)-based Human-dog-cat Species Identification and DNA Quantification Assay. Poster Presentation, Seventh ISABS Conference in Forensic, Anthropologic and Medical Genetics and Mayo Clinic Lectures in Translational Medicine. Bol, Island of Brač, Croatia, June 20-24, 2011.

Sreetharan Kanthaswamy. Animal Forensics. Standing Committee on Animal Forensics Workshop, 32nd International Society for Animal Genetics (ISAG) Conference, Edinburgh, Scotland, UK, July 26-30, 2010.

Sreetharan Kanthaswamy, Amritha Premasuthan, Vivek Goyal, Jessica Satkoski. The Development and Validation of a Multispecies DNA Speciation and Quantification Reagent Kit for Use in Forensic Casework. Poster presentation by SK at the 11th Annual US National Institute of Justice (NIJ) Conference 2010, Arlington, Virginia, June 13 – 16, 2010.

Vivek Goyal, Jessica Satkoski and Sreetharan Kanthaswamy. The Development and Validation of a Multispecies DNA Speciation and Quantification Reagent Kit for Use in Forensic Casework. Poster presentation by SK at the 10th Annual US National Institute of Justice (NIJ) Conference 2009, Arlington, Virginia, June 14 – 17, 2009.

VII. Tables and Figures

Tables

Table 1 Human, dog, and cat specific TaqMan® primer and probe sequences used for developing the human-dog-cat triplex assay. The human, dog and cat target amplicons were 180 bp, 209 bp and 209 bp, respectively.

Name	Sequence
Human Forward	AGGGTATCTGGGCTCTGG
Human Reverse	GGCTGAAAAGCTCCCGATTAT
Common Forward	CGCCCATGTATTACTTCATCTGTTGCC
Common Reverse	CACGGCGATGGCGCCCAGGAA
Human Probe	ATTCCCATTGGCCTGTTCCTCCCTT
Dog Probe	GCCTTGGCTGCGCAGGCTGCTGTGGTGCAG
Cat Probe	GCCCTGGCCGGCCGGGCCGCCGTGGTGCAG

Table 2 The TaqMan® primer and probe sequences used for developing the livestock assay. Probe lengths for this assay are in bp.

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-**IBRQ**	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		

Table 3 The poultry TaqMan® probe and primer sequences.

Oligonucleotide	Final conc. (μM)	Sequence, 5' - 3'	Position	Length (bp)	GenBank accession no.
Duck-F	0.2	CCAGTCCCATCCCACCTAATGTC	1067 - 1089	80	AY738732
Duck-R	0.2	CTGTTTTGACTTCTTGCCGTCCAT	1123 - 1146		
Duck-probe	0.1	**VIC**-GGTCCCATTAACACGTTCCCA-**MGB-NFQ**	1099 - 1119		
Chicken-F*	0.2	CAGCTGGCCTGCCGG	3323 - 3337	76	X60091
Chicken -R*	0.2	CCCAGTGGAATGTGGTATTCA	3378 - 3398		
Chicken -probe*	0.1	**FAM-**TCTGCCACTCCTCTGCACCCAGT **-MGB-NFQ**	3350 - 3372		
Turkey-F*	0.8	CAAAGAAAGCAGGGAAAAGGA	1799 - 1819	83	L76587
Turkey-R*	0.8	TGCACTCTCGTTGTTAAAAAGGA	1859 - 1881		
Turkey-probe*	0.2	**NED-**CTGGGAAAGTTACTGTGTAGCCTCAGAACG**-MGB-NFQ**	1821 - 1850		

Table 4 The TaqMan® probe and primer sequences of the duplex *C. sativa* qPCR assay. The green plant and *C. sativa* target amplicons were 101 bp and 180 bp, respectively

Forward primer *C. sativa* specific	5'-GGG GGA TTA CAT TGT TAA TTA TTC C-3'
Reverse primer *C. sativa* specific	5'-ATC CTC TCA TTC CGT TAG TGG-3'
Forward primer Green Plant-Specific control	5'-AGA GTC CCA TTC CAC ATG TCA-3'
Reverse primer Green Plant-Specific control	5'-GGG ATA GAG GGA CTT GAA C-3'
C. sativa specific probe	5'-AAA GGG TCA TTC AGG TCC GGT TGT AAA-3'
Green Plant control probe	5'-CCG TCG ACT TTA AAA ATC GTG AGG-3'

Figures

Fig. 1 Amplification curves of human-dog-cat triplex assay showing species-specificity.

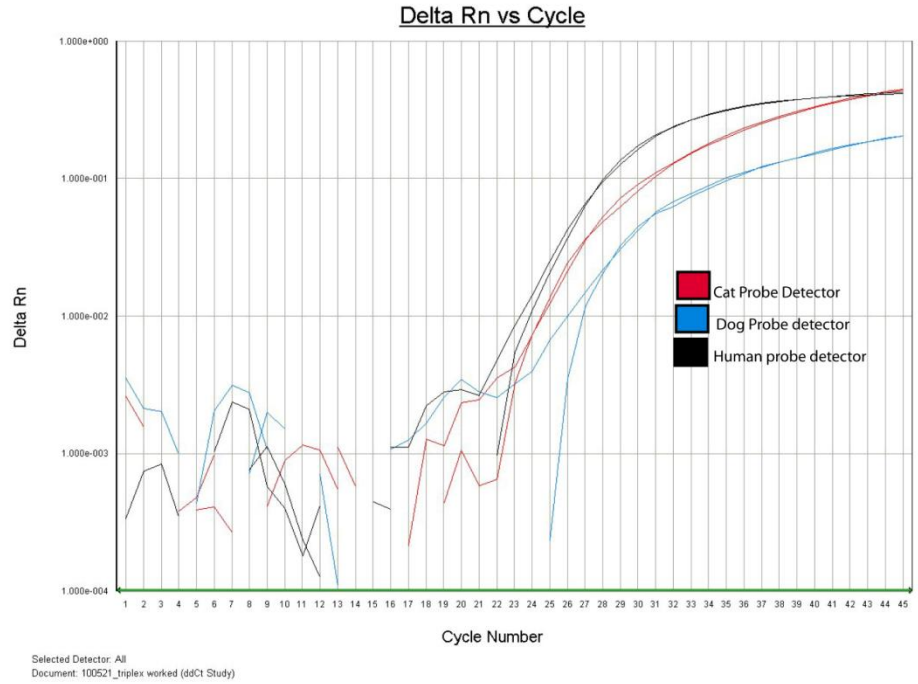

Fig 2. Amplification curve of the human-dog-cat triplex assay showing amplification of dog bone DNA.

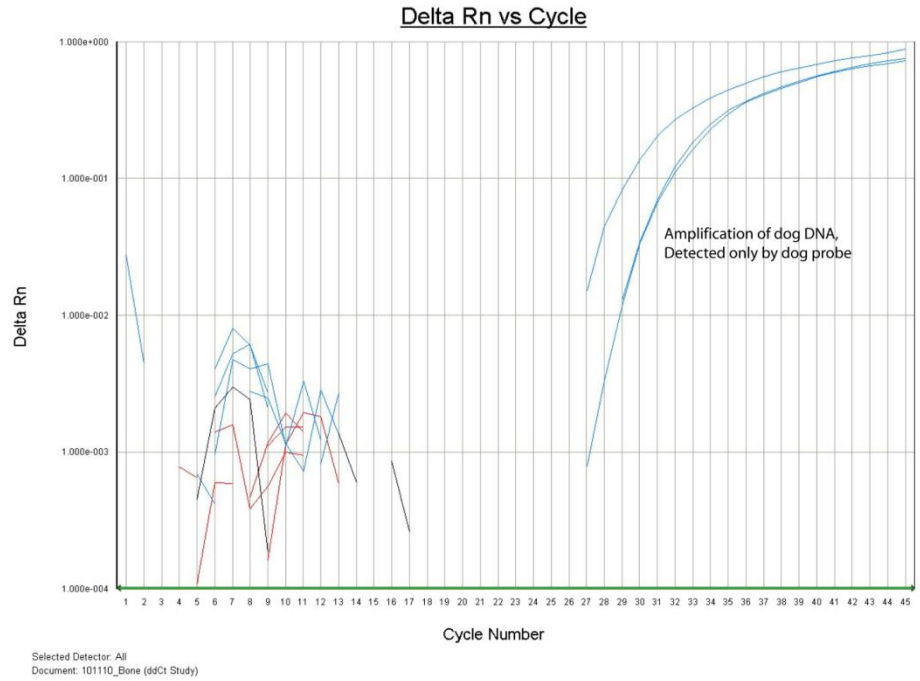

Fig 3. Amplification curves of the human-dog-cat triplex assay for inhibition study with humic acid.

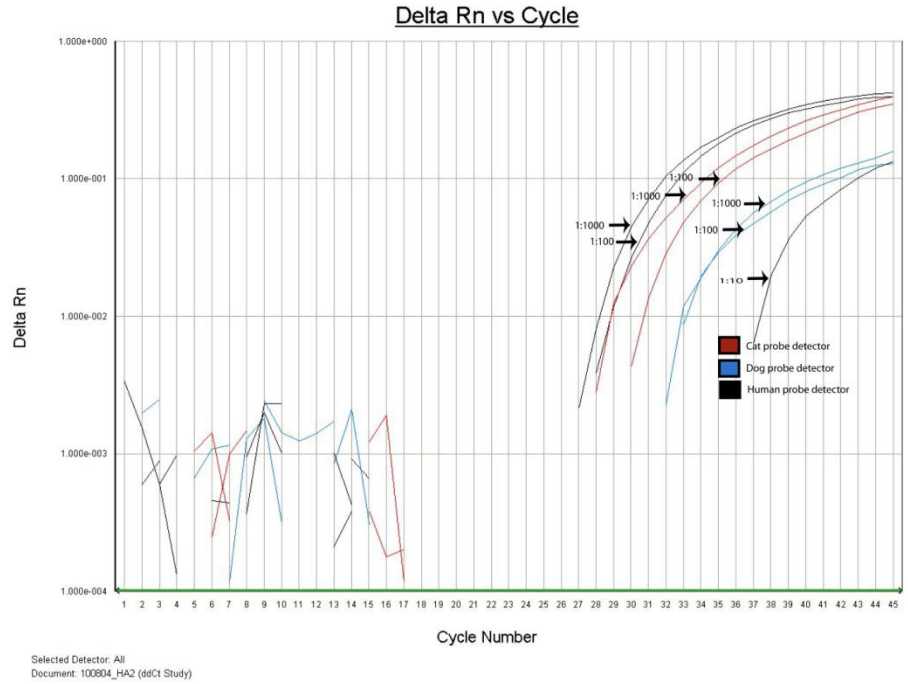

Fig. 4 Amplification curves of the human-dog-cat triplex assay showing higher Ct values in the presence of EDTA.

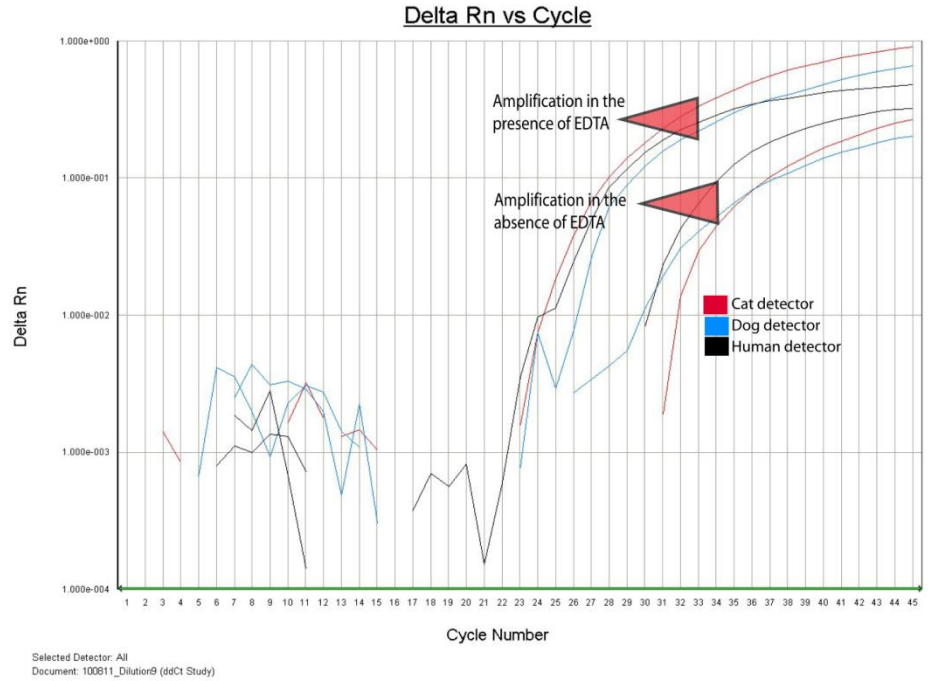

Fig 5. The human-dog-cat triplex quadriplex assay showing human, feline and canine targets with IPC amplification.

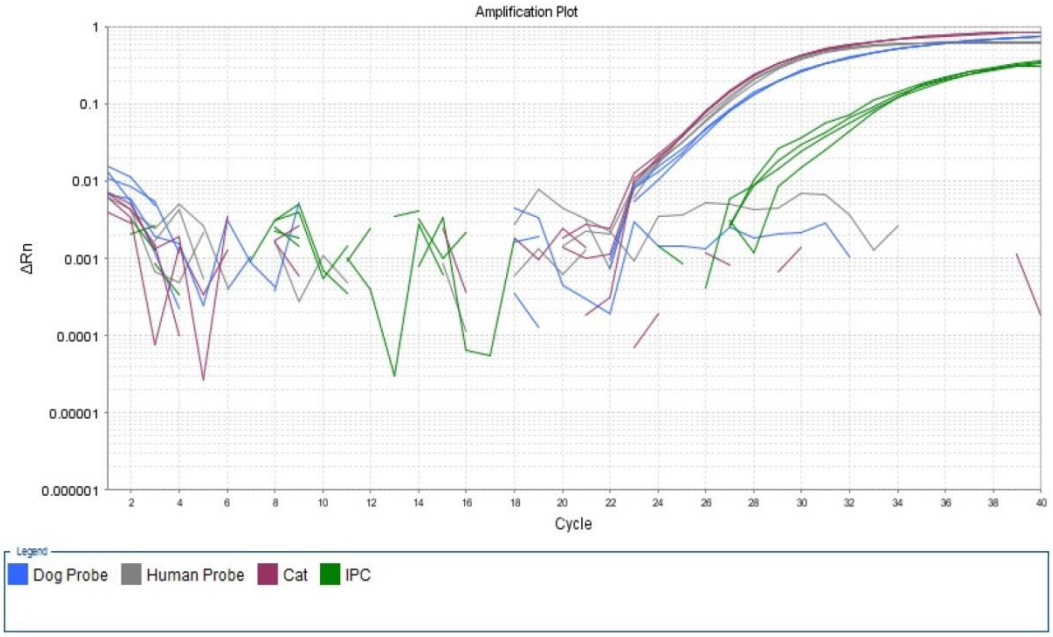

Fig. 6 Multiplex amplification plot showing livestock species-specificity

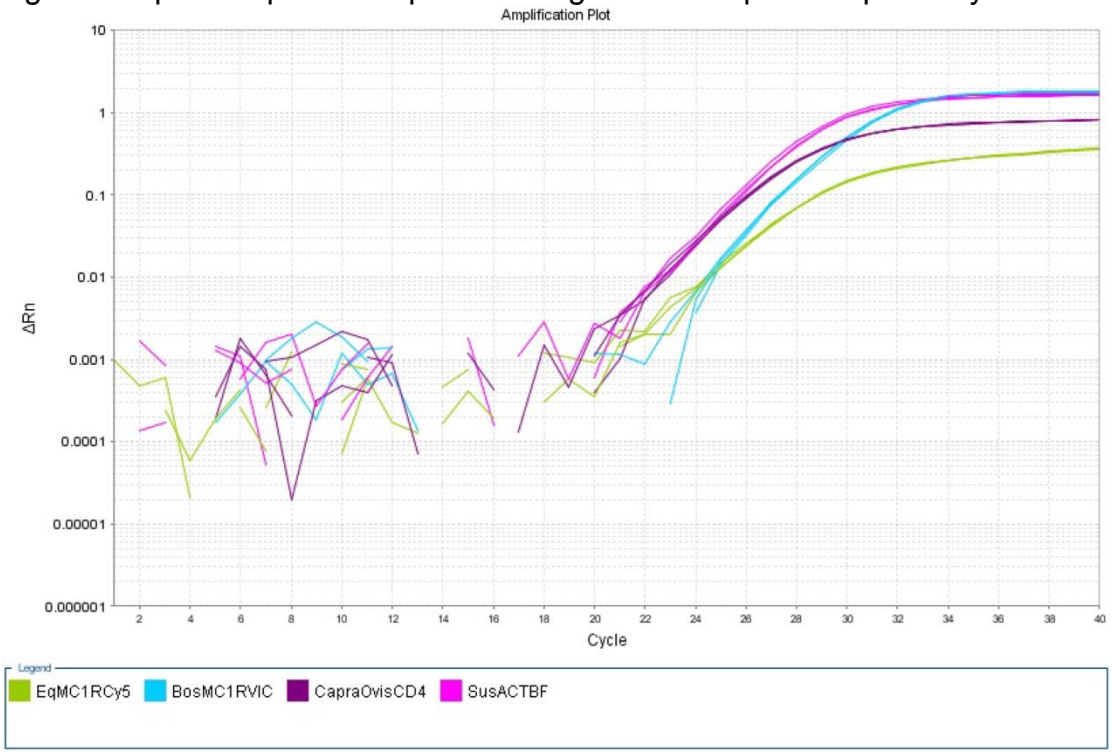

Fig. 7. The species-specificity of the poultry assay

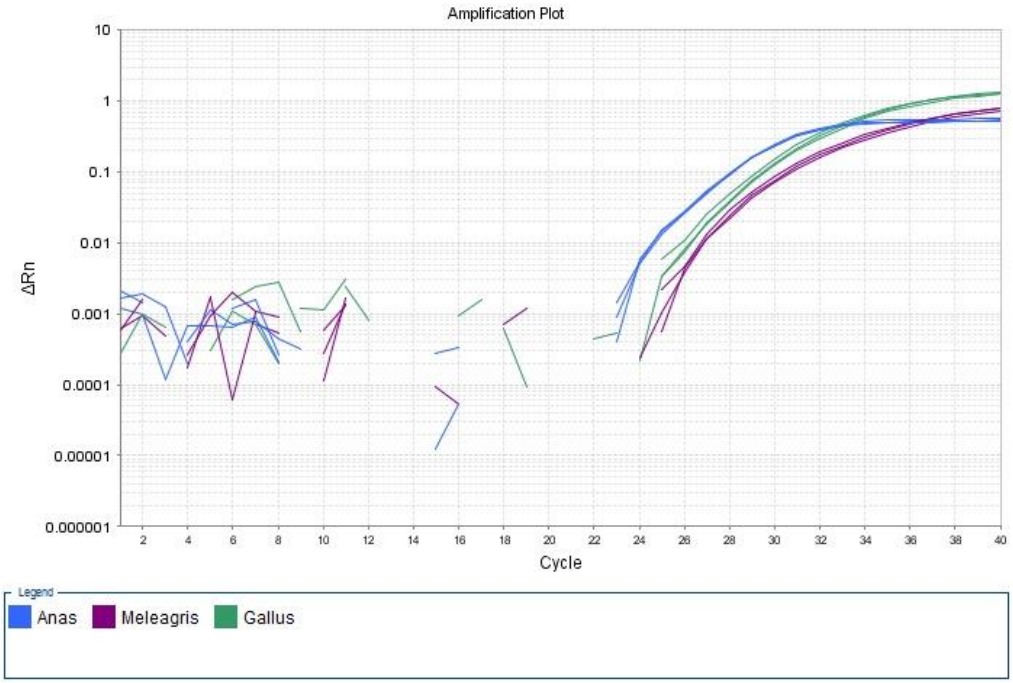

Fig. 8 The target site of the green plant primers and the green plant-specific probe that are used in the *C. sativa* assay.

Acknowledgements:

Many have contributed to this work in terms of sampling, research in the laboratory and the write up of research articles that have led to this final report. Some of the notable contributors are: David Glenn Smith, Cecilia von Beroldingen, Jillian Ng, Amritha Premasuthan, Christopher Johnson, Jessica Satkoski Trask, and Debra George. Without their contribution, this work would not have happened.

We wish to thank Mihn Nguyen for her steadfast support for this project.