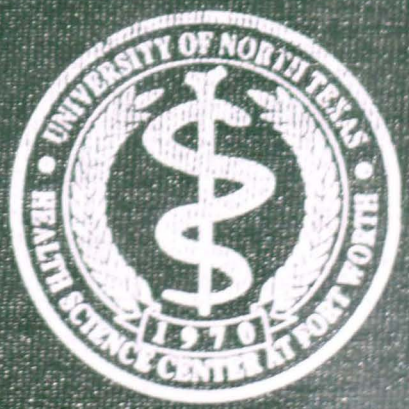
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EVALUATION OF UNLABELED PRIMERS FOR SEX DETERMINATION OF ANIMAL AND WILDLIFE SAMPLES

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EVALUATION OF UNLABELED PRIMERS FOR SEX DETERMINATION OF ANIMAL AND WILDLIFE SAMPLES

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CHAPTER I

INTRODUCTION

Background

Species identification is not only important in determining the origins of remains found in human forensic cases, but is also important in the growing field of non-human forensics. Animal forensics is an emerging discipline in the non-human forensics field. Animal forensics focuses on domestic animals, or animals that live in close contact with humans. These animals include dogs, cats, cattle, pigs, horses, sheep as well as others.

There are several different sub-disciplines within animal forensics. One is the involvement of domestic animals in human crime scene investigations. In 2003, The American Pet Products Manufacturers Association reported that approximately 50% of households in the United States owned at least one cat or dog. These domestic animals can provide important information to the investigator by the transfer of trace evidence [1, 2]. Animal hairs are often found in the homes and cars and on the clothing of animal owners and others who have been in contact with the animals. These hairs are easily transferred from one location to another and can be left at crime scenes by perpetrators as well as victims [3]. If an animal is a victim of a crime, the blood from the animal may be transferred to the perpetrator.

Another important sub-discipline of animal forensics is pedigree verification and breeding programs. Purebred horses are bred for speed and strength and domestic cattle are bred for increased milk production or a particular type of meat [4, 5]. Cats and dogs compete for best of breed in both national and international pet shows. The best of breed from all domestic animals are selectively bred for particular attributes and stud fees can reach great heights. The offspring of top animals can command high prices as well and purchasers are becoming more and more cautious about the product they are buying. Paternity tests of animals and testing of semen from studs are being done on a more regular basis to verify identity [4, 5].

A more recent use of animal forensics is lineage testing for animals infected with diseases such as Bovine Spongiform Encephalopathy (BSE). To determine age and location of the birth herd of the infected animal, paternity testing is done. If an animal was born after 1997, when North America banned using animal parts in feed, then the lineage testing is more important and can have great impacts on trade both domestically and internationally. It is necessary to ensure that correct testing is done as the repercussions both medically and economically can be disastrous.

Species identification is also important in the growing field of wildlife forensics. Wildlife forensics encompasses different areas, but some of the most important applications are for tissue identification, and for tracking down poachers of endangered and protected species [6]. As well as tracking poachers, wildlife forensics is used to

regulate the harvest of non-endangered species by determining the yearly hunting quotas. Tissue identification is not only employed in poaching cases, but also used in fraud cases, where a merchant may be selling common domestic animal tissue (beef, pork, chicken) at a higher price, representing it to be game tissue or an exotic tissue. Merchants can also sell exotic and endangered tissue, claiming it is domestic tissue, to escape the criminal charges of hunting protected animals [7]. There are many reasons why people hunt endangered species. Hunters collect trophies for their walls. Farmers shoot animals on their property and collectors of animal organs harvest them for supposed medicinal value (for example, bear gall bladders and tiger organs are used in traditional Chinese medicines) [6, 7].

Sex determination is important, because there are certain times of year where one of the sexes may be off-limits for hunting. Sex determination will aid in enforcing game laws and hindering poachers. Non-phenotypic sex determination is used when sex determination can no longer be achieved by visual methods, such as appearance or examination of genitalia. Sex determining regions of the DNA, such as the sex chromosomes, can be used in conjunction with other regions of DNA to identify or exclude individual animals, to identify their geographic origin and to lead to identifying the poacher or killer of a particular animal.

Methods

Quantification is required to determine the amount of deoxyribonucleic acid (DNA) present in a sample. The amount of DNA needs to be known to determine the amount of input DNA into polymerase chain reaction (PCR). There are many methods to quantify DNA. Given the many different species involved in this study, ultraviolet spectroscopy and SYBR Green I® quantitative plate reads were selected as they can be used effectively across different species.

Ultraviolet spectroscopy, or UV spectrophotometry, is a procedure that uses ultraviolet light to determine the amount of DNA, ribonucleic acid (RNA) and proteins in liquid samples. The procedure measures the absorbance of DNA at 260nm and 280nm and is effective for solutions with high concentrations of DNA. The procedure has been used in forensics, molecular biology and biology-related fields for years. A blank is used to set the baseline, against which all other samples are compared. This blank is generally the solution in which the DNA has been stored or diluted for the experiment. A major problem with UV spectrophotometry is that as well as giving a reading for DNA, the RNA and proteins are included at the same time and can cause the DNA reading to be higher than it really is. Another problem is that a high concentration of DNA solution must be used to obtain a good reading [8].

SYBR Green I® quantitative plate reads use the fluorescent dye SYBR Green I® (Molecular Probes, Eugene, OR) which is a minor-groove-binding nucleic acid stain

specific for double-stranded DNA [9]. The more DNA present in a sample, the more dye binds and the greater the fluorescence. The range for detection using SYBR Green I® is between 5 and 1000ng/ml (or about 250pg to 50ng of DNA in a 50μl reaction) [9]. This method is better than UV spectrophotometry because less DNA is needed to quantify the sample and it detects only the DNA in the sample.

The polymerase chain reaction (PCR) is a technique used to replicate DNA *in vitro*, or outside a living organism. The PCR method was described in 1986 by Kary Mullis, who went on to win the Nobel Prize in Chemistry in 1993 for the technique [10]. The uses a thermostable DNA polymerase, which functions the same way as the DNA polymerase in the human body to replicate the DNA, but which is not degraded from the repetitive heating cycles needed for the PCR technique [10]. This polymerase, *Taq* (from *Thermus aquaticus*), along with primers that define the area to be replicated are put into a reaction with other components that mimic the optimal conditions found in the cell nuclei. Optimized buffer conditions containing potassium chloride (KCl) and magnesium chloride ($MgCl_2$) are also needed for the reaction to occur efficiently. Dinucleotide triphosphates (dATP, dCTP, dGTP, dTTP) are needed to construct the newly replicated strands of DNA. The buffer solution also ensures proper conditions for all components by maintaining pH at levels needed for *Taq* polymerase. Finally, an optimal concentration of sample DNA is needed to allow the reaction to proceed effectively.

During the PCR process, the double-stranded DNA is first denatured at 94 -96°C for several minutes. Then, a cycling regime of three steps is repeated from 20 to 35 times as parts of the replication process. The PCR cycle begins with another denaturation step, followed by a primer annealing step at 45-60°C, where the primers bind to the now single-stranded DNA. Then there is an elongation step at 72-74°C where the dinucleotide triphosphates (dNTPs) are introduced to complete the newly copied strand to form double-stranded DNA again. After these three steps have been repeated, as many times as the protocol requires, there is a final elongation step at 72-74°C for 15-45 minutes to ensure all the strands of DNA have been completely replicated (see Figure 1).

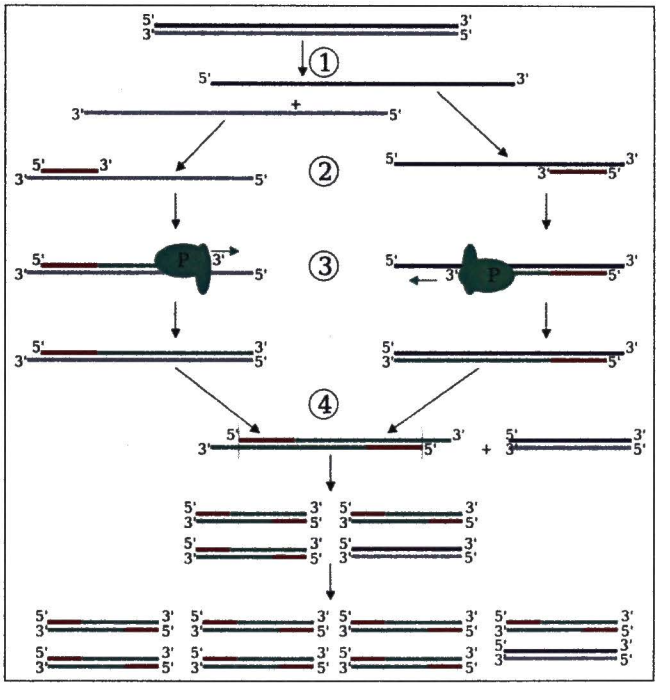


Figure 1. Schematic drawing of the PCR cycle. (1) Denaturing at 94-96°C. (2) Annealing at (*e.g.*) 56°C. (3) Elongation at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle [10].

Agarose gel electrophoresis can be used to separate the amplified DNA fragments by size. These fragments are then compared to a ladder which is made up of fragments of known size. The agarose is combined with a buffer - generally tris-acetate-EDTA (TAE), tris-borate-EDTA (TBE) or sodium boric acid (SB) [11] to provide the electrolytic quality necessary for electrophoresis. Ethidium bromide, a dye that intercalates between the bases of DNA causing fluorescence, is added to the gel to permit visualization under ultraviolet light. A gel comb is used to create little wells in the gel for the samples. This gel is placed horizontally into a gel box and covered in the same buffer used to make the gel. The samples are combined with glycerol to cause the sample to fall to the bottom of the well. Bromophenol blue, a dye that helps track movement through the gel towards the positive cathode, is pipetted into the wells. A negative charge is applied to the anode end of the gel, and the DNA is forced through the gel towards the positive, cathode end. The smaller the DNA fragment, the faster it will move through the gel. The bromophenol blue runs ahead of the DNA and is used to ensure that the smaller fragments are not allowed to run off the gel [11]. Once run, the gels are placed on a UV transilluminator, and visualized. The ethidium bromide intercalates between the bases, stretching the DNA molecule which then fluoresces under UV light. The different size fragments can then be seen and compared to the known ladder to determine their size.

CHAPTER II

RESEARCH DESIGN

Primers are short oligonucleotide sequences that hybridize to defined regions of interest on the DNA during PCR. Two sets of primers were provided for this assay. The first set is the ZFY/ZFX primers which hybridizes the zinc-finger protein region on the DNA. The zinc finger protein one of the most abundant DNA-binding motifs found [12]. It ranges from two copies of the protein in fruit flies (*Drosophila*) up to nine copies in the higher primates. The zinc finger protein is a finger shaped protein that allows it to interact with both DNA and RNA. The fold is created by the binding of specific amino acids in the protein to a zinc atom. Zinc finger proteins regulate the expression of genes as well as nucleic acid recognition, reverse transcription and virus assembly [13]. The zinc finger protein is being used as a region of interest in this assay due to the abundance in DNA and due to its presence in both males and females.

The second set of primers being used is the SRY gene primers. The SRY gene is the sex-determining region Y [14, 15, and 16]. This is the sex determining region on the Y chromosome and is responsible for "maleness" as it encodes the testis determining factor (TDF or SRY protein) [16]. The SRY protein binds to DNA and distorts it out of shape, enhancing other transcription factors. These transcription factors cause the chemical reactions that directly or indirectly cause the development of primary sex cords,

which later develop into the seminiferous tubules of the testes [15]. Without the development of the testes, secondary sexual characteristics do not develop (*i.e.* - facial hair, deeper voice and a prominent Adam's apple) [16]. Both genes that are targeted by the primer sets are abundant in DNA, the zinc finger protein gene in both males and females and the SRY gene in males only. Since the genes are found in many different species, from yeast (zinc finger protein) [12] to humans (both genes) they are termed universal. If both primer sets successfully bind and the regions of interest are amplified, we would expect to see two bands for the males (both zinc finger protein and SRY genes) and a single band for females (only have the zinc finger protein gene). The purpose of this assay is to evaluate these two primer sets, provided by DNA Solutions, Inc. in Oklahoma City, and to optimize the reaction conditions with regards to sensitivity and PCR cycle number. Another purpose of the assay is to look at the species specificity of the primers and determine if these specific primers can bind to all species that possess the two genes or only to a select few.

Objective 1

Sensitivity – Forensic samples are often low copy number so there may be little viable or non-degraded DNA to work with. The PCR reaction needs to be optimized to determine how little DNA you can start with and still obtain accurate results. The given protocol from DNA Solutions, Inc. (Appendix A) [17] suggests 20ng of input DNA. In practice, this level is rarely found in forensic cases, so this assay will begin at 5ng of

input DNA. The dilution series will be 5ng, 2ng, 1ng, 500pg, 250pg, 125pg, 62.5pg, 31.25pg and 15.63pg.

Objective 2

PCR cycle number – As a part of sensitivity, PCR cycle number is an important part of optimizing the reaction. Since agarose gels are being run to visualize results, increased cycle number can result in increased specificity. Cycle numbers being evaluated are 26 cycles [17], 28 cycles, 30 cycles, 32 cycles and 34 cycles.

Objective 3

Species Specificity – It must be determined if these universal primers do, in fact, bind to all species that possess the genes of interest. Samples from across the different animal classes will be evaluated to determine the specificity of the primers. The primers have already been tested on members of the deer family, with success, so a male deer sample has been sent to be used as a positive control as it shows both genes. Species of interest are mammals, birds and fish as there are many of each that are endangered and identifying the sex of the animal is an important step to identifying the individual animal.

CHAPTER III

MATERIALS AND METHODS

Samples

Tissue samples of pork (*Sus domesticus*), chicken (*Gallus gallus*), beef (*Bos taurus*), tuna (*Thunnus sp.*), salmon (*Salmo salar*), buffalo (*Bison bison*) and lamb (*Ovis sp.*) were obtained from local grocery stores. The white-tailed deer (*Odocoileus virginianus*) and black bear (*Ursus americanus*) tissue samples, dog (*Canis familiaris*) buccal samples and human blood samples were also collected. Unknown deer samples, and a known male deer sample to use as a positive control, were provided by DNA Solutions, Inc.

Primers

As explained in the research design, there are two regions of interest for sex determination in this assay. The first region is the Zinc Finger protein (ZFX/ZFY) genes in both males and females which is visualized as a 445bp fragment and uses the 3EZ primer (5'GCACTTCTTTGGTATCTGAGAAAGT 3') and the 5EZ primer (5'ATAATCACATGGAGAGCCACAAGCT 3'). The second region of interest is the SRY gene, which is found only in males and which is visualized as a 224bp fragment. The primers used for this region are 3D (5' ATTTAGCCTTCCGACGAGGTCGATA 3') and 3C (5' CCCATGAACGCATTCATTGTGTGG 3').

Organic Extraction

Approximately 0.02g of tissue or one half of a buccal or blood swab was used for the DNA extractions [18]. 300µl of Stain Extraction Buffer was added along with 5.0µl of Proteinase K (AMRESCO, Solon, OH) to each sample. The samples were incubated at 56°C (+/- 5°C) for 2 hours. The swab heads were removed using a spin-ease basket and centrifuged for 4 minutes at 12,000 rpm. 300µl of 25:24:1 Phenol-Chloroform/Isoamyl Alcohol (PCIA) (Invitrogen, Carlsbad, CA) was added to the digested cells, vortexed and centrifuged for 3 minutes at 12,000 rpm. Using a sterile pipette, the aqueous layer containing the DNA was added to a Microcon® concentrator filter (Millipore Corporation, Bedford, MA), with 100µl of dH_2O already wetting the filter. 300µl TE-4 was added to the top of the filter and the tube capped. The tubes were centrifuged at 4000 rpm for 20 minutes then the filtrate was discarded. The concentrator filters were inverted and 100µl of TE-4 was added and centrifuged at 9000 rpm for 3 minutes. The concentrator filters were then discarded and the retentate stored at -20°C [19].

Quantification

DNA samples were quantified by two different methods not only to study the efficacy of the methods, but to determine more precisely PCR input DNA amounts. First, UV spectrophotometry was used. A TE-4 blank was used to set the baseline for the spectrophotometer. Some of each sample was diluted to 1/50 of original concentration before running them on the UV spectrophotometer. 500µl of both the blank and the

samples were pipetted individually into a cuvette, placed in the reading chamber and analyzed at a wavelength of 260nm.

The second method used was a SYBR Green I® quantitative assay using the Stratagene Mx3000P (Stratagene, La Jolla, CA). A standard curve was set up using DNA template (Applied Biosystems, Foster City, CA) with a known concentration and performing serial dilutions (100pg/µl, 50pg/µl, 25pg/µl, 12.5pg/µl, 6.25pg/µl and a water blank). Some of each of the samples diluted to 1/50 and 1/100 of original concentration. 25µl of DNA sample and 25µl of SYBR Green I® master mix (Molecular Probes, Eugene, OR) - (1(10X PCR Buffer): 1(1:2500 SYBR Solution):3(dH_20)) were combined to give 50µl reaction volume. The quantitative plate read was set up according to Stratagene's protocol [9]. Once quantified, the DNA was diluted to the desired concentration (per 2µL) for sensitivity testing (5ng, 2ng, 1ng, 500pg, 250pg, 125pg, 62.5pg, 31.25pg and 15.625pg).

Polymerase Chain Reaction

A PCR master mix was prepared for each sample set containing all the reaction components as outlined in Table 1. From this master mix, 23µl was added to each PCR tube and 2µl of DNA template was added.

Table 1 – PCR Reaction Set-up (25μl final volume) [17]

Reaction Component	Amount per reaction
Taq Gold 10XPCR Buffer	2.5μl
dNTPs (10mM)	3.125μl
$MgCl_2$ (25mM)	2.6μl
Bovine Serum Albumin (2.5mg/ml)	1.6μl
Primer Mix (10μM)	1.0μl
Taq Gold Polymerase (5U/μl)	0.4μl
dH_20	11.775μl
DNA Template	2.0μl

PCR was evaluated at 26, 28, 30, 32 and 34 cycles following the DNA Solutions Inc. protocol [17]. The protocol consists of an 11 minute initial denaturation at 94°C followed by the selected amount of cycles of 45 seconds of denaturation at 94°C, 45 seconds of annealing at 60°C and 1 minute of extension at 73°C followed by a final extension at 72°C for 15 minutes.

Gel Electrophoresis

A 2% agarose gel was made using 4g of agarose (Fisher Scientific, Fair Lawn, NJ) and 200mL of 1X TAE (10X stock solution diluted to 1X with dH_2O - Invitrogen, Carlsbad, CA) buffer. After melting the agarose in a microwave and allowing it to cool slightly, 4μL of Ethidium Bromide, EtBr (10mg/mL, Invitrogen, Carlsbad, CA) was added to the gel. The solution was them poured into the gel plate in the gel apparatus.

Once set, the gel apparatus was filled with 1X TAE, so the gel was covered. Five microliters of 100bp ladder (Invitrogen, Carlsbad, CA) was added to the first well of each comb row. The gels were loaded with 7.5μl of PCR product, mixed with 2.5μl of loading dye. The amplified products were electrophoresed at 100V for one hour. Gels were visualized using a mid length wave UV transilluminator and photographed using a digital camera. Three small 8-well gels (0.5g agarose, 25mL 1X TAE and 0.5μL EtBr) were electrophoresed at 75V for 30 minutes due to their smaller size. Samples were run on the gels with 5 PCR cycle number samples grouped to compare the sensitivity of the primers, at both different cycle numbers and at different input concentrations.

CHAPTER IV

RESULTS

Quantification

A series of three extraction sets were used. Extraction set 1 did not include all of the species examined in the subsequent extraction sets. The dog, bear, lamb, and buffalo samples were added to the second and third extraction sets to increase the number of species evaluated by the assay. Both quantification methods gave comparable results for all samples (Tables 2 to 7). The average concentrations of DNA (ng/μl), for both UV spectrophotometry and the SYBR Green I® assay, were calculated for comparison purposes using the 1/50 dilution from the second and third extraction sets (Table 8).

A. UV Spectrophotometry

Extraction 1 was diluted to 1/50 before quantifying using the UV Spectrophotometer. Results (Table 2, below) indicated that the reagent blank did not contain any detectable DNA. Results also indicated that there was ample DNA in the samples to be able to obtain the desired input concentrations for testing. Both reagent blanks showed excessive absorbance at 260nm (Table 3, below). It is not known if this was truly DNA contamination, or if there may have been proteins or phenol (remnant from the organic extraction) present in the tube or on the blank swab that were being read by the UV spectrophotometer. The blank was run multiple times during the procedure to

ensure that contamination was not due to remnant DNA in the UV Spectrophotometer cuvette. The final blank showed less than a 0.00001ng/µl change in reading, so cross-sample contamination during the procedure was negligible. All samples, except the chicken, showed higher concentrations than the reagent blanks.

Table 2. UV Spectrophotometry Extraction 1

Sample	Total DNA (ng/µl)
Blank	0.0000
Deer	1.0435
Beef	0.8835
Pork	1.3730
Salmon	1.2960
Tuna	0.9795
Chicken	1.3730
PC – AC2695A	0.0835
RB	0.0000

PC - Positive Control, RB - Reagent Blank

Table 3. UV Spectrophotometry Extraction 2

Sample	Total DNA (ng/µl)
Blank	0.0000
Deer	1.3925
Salmon	1.6675
Beef	1.8875
Tuna	3.3925
Chicken	0.5425
Bear	1.8750
Pork	1.6450
Buffalo	1.1600
Lamb	2.2075
Dog M	1.5250
Dog F	1.2475
Human M	1.4025
Human F	1.0375
RB-Swab	0.8450
RB	0.9050

M - Male, F - Female, RB - Reagent Blank

Results for extraction 3 showed contamination of both reagent blanks once again. All samples did show higher concentrations than the reagent blanks, so there could have been a small amount of contamination in the tubes or on the blank swab, even though all tubes were sterilized using ultraviolet light, by crosslinking, before using them, to degrade the DNA, RNA and the proteins that may have been present (Table 4). As a result of the contamination present in all 4 of the reagent blanks in extractions 2 and 3, the dilutions made for UV spectrophotometry were not used for further analysis in the study.

Table 4. UV Spectrophotometry Extraction 3

Sample	Total DNA (ng/μl)
Blank	0.0000
Deer	1.1875
Salmon	1.2725
Beef	1.2000
Tuna	1.1850
Chicken	1.9175
Bear	1.7375
Pork	1.1075
Buffalo	2.2650
Lamb	1.0975
Dog M	1.9450
Dog F	1.0150
Human M	1.1575
Human F	1.4100
RB-Swab	0.9200
RB	0.8575

M - Male, F - Female, RB - Reagent Blank

B. SYBR Green I®

A single reagent blank was run with the extraction 1 samples and did show some contamination (Table 5). This contamination was lower than any of the samples, but the samples were not used for further analysis, due to the contamination. There may have been two reasons for the lower concentration in the human samples. First, the human samples in extraction 1 were the only samples that were taken from swabs as opposed to tissue samples. Secondly, the human blood samples used were old samples and may have been somewhat degraded. Fresh blood was drawn for extractions 2 and 3.

Table 5. SYBR Green I® Extraction 1

Sample	Dilution	Total DNA (ng/µl)	Dilution	Total DNA (ng/µl)
Deer	1 in 50	1.50540	1 in 100	1.50540
Salmon	1 in 50	1.50451	1 in 100	1.43816
Beef	1 in 50	1.50540	1 in 100	1.48860
Tuna	1 in 50	1.41213	1 in 100	1.11609
Chicken	1 in 50	1.50540	1 in 100	1.45011
Pork	1 in 50	1.48598	1 in 100	1.32311
Human M	1 in 50	0.10639	1 in 100	0.07757
Human F	1 in 50	0.10758	1 in 100	0.06516
RB	1 in 50	0.01491	1 in 100	0.00833

M - Male, F - Female, RB - Reagent Blank

Both reagent blanks showed no contamination in extraction 2 (Table 6). Some samples did show lower concentrations than seen in UV spectrophotometry and in the extraction 1 SYBR Green I® assay. The dog buccal swabs showed the lowest concentrations of DNA. This is to be expected, as there will not be as many cells in a

buccal swab as there will be in a piece of muscle. Samples from this extraction set were tested at 26 PCR cycles, 28 PCR cycles and 32 PCR cycles.

Table 6. SYBR Green I® Extraction 2

Sample	Dilution	Total DNA (ng/µl)	Dilution	Total DNA (ng/µl)
Deer	1 in 50	3.63869	1 in 100	2.09135
Salmon	1 in 50	1.09744	1 in 100	0.72853
Beef	1 in 50	3.41836	1 in 100	2.27215
Tuna	1 in 50	0.92352	1 in 100	0.39623
Chicken	1 in 50	3.67607	1 in 100	3.20841
Bear	1 in 50	3.45430	1 in 100	2.70971
Pork	1 in 50	1.14474	1 in 100	0.23394
Buffalo	1 in 50	3.34620	1 in 100	2.20194
Lamb	1 in 50	2.46032	1 in 100	1.64345
Dog M	1 in 50	0.10009	1 in 100	0.02740
Dog F	1 in 50	0.09329	1 in 100	0.03918
Human M	1 in 50	0.45279	1 in 100	0.22954
Human F	1 in 50	0.34082	1 in 100	0.21109
RB-Swab	1 in 50	0.00000	1 in 100	0.00000
RB	1 in 50	0.00000	1 in 100	0.00000

M - Male, F - Female, RB - Reagent Blank

There was a small amount of contamination in the swab reagent blank in the 1/50 dilution in extraction 3 (Table 7). This reading was much lower than any of the samples and could be due to several factors such as contamination of the tube itself or cross-sampling contamination. The swab samples were low again compared to the tissue samples. However, the lamb sample in the 1/50 dilution was lower than expected for a tissue sample based on the results obtained for other tissue samples being similar.

Samples from this extraction set were tested at 26 PCR cycles, 30 PCR cycles and 34 PCR cycles.

Table 7. SYBR Green I® Extraction 3

Sample	Dilution	Total DNA (ng/µl)	Dilution	Total DNA (ng/µl)
Deer	1 in 50	3.06695	1 in 100	0.53543
Salmon	1 in 50	3.10053	1 in 100	0.75479
Beef	1 in 50	3.50164	1 in 100	1.17269
Tuna	1 in 50	2.25790	1 in 100	0.35639
Chicken	1 in 50	3.76726	1 in 100	2.37542
Bear	1 in 50	2.78667	1 in 100	0.61241
Pork	1 in 50	1.68855	1 in 100	0.35003
Buffalo	1 in 50	2.62374	1 in 100	0.61224
Lamb	1 in 50	0.09278	1 in 100	0.21676
Dog M	1 in 50	0.06837	1 in 100	0.03058
Dog F	1 in 50	0.38040	1 in 100	0.05190
Human M	1 in 50	0.76077	1 in 100	0.72550
Human F	1 in 50	0.05375	1 in 100	0.38095
RB-Swab	1 in 50	0.00071	1 in 100	0.00000
RB	1 in 50	0.00000	1 in 100	0.00000

M - Male, F - Female, RB - Reagent Blank

As seen by the comparison in Table 8 the SYBR Green I® assay showed higher average concentrations of DNA in almost all sample. The swab samples - both dog samples, both human samples and both reagent blank samples, tuna and lamb - showed higher concentrations using UV spectrophotometry. The higher level in the swab samples could be due to the swabs themselves, which may have residual DNA, RNA or proteins on them from the manufacturing process, as the higher level was evident in the reagent blank as well. The tuna, lamb and non-swab reagent blank samples may have had more proteins in them, which may have increased the reading in spectrophotometry. Some of

the samples had very similar concentrations between the two methods which is what is sought. Since the SYBR Green I® quantitative plate read is DNA specific, and does not read proteins and RNA, as UV spectrophotometry does, the results from the SYBR Green I® were used to make dilutions for the sensitivity and specificity studies and for PCR.

Table 8. Average DNA concentrations (ng/µl) in UV Spectrophotometry versus SYBR Green I® Quantitative Plate Read (1/50 Dilution)

Sample	UV Spectrophotometry Average DNA Amount	SYBR Green I® Average DNA Amount
Deer	1.29000	3.35282
Salmon	1.47000	2.09899
Beef	1.54375	3.46000
Tuna	2.28875	1.59071
Chicken	1.23000	3.72167
Bear	1.80625	3.12049
Pork	1.37625	1.41665
Buffalo	1.71250	2.98497
Lamb	1.65250	1.27655
Dog M	1.73500	0.08423
Dog F	1.13125	0.23685
Human M	1.28000	0.60678
Human F	1.22375	0.19729
RB-Swab	0.88250	0.00036
RB	0.88125	0.00000

M - Male, F - Female, RB - Reagent Blank

Agarose Gel Electrophoresis

Five sensitivity gels were run, each with 51 wells, divided into three 17-well sections. Each gel was loaded with samples from extraction 2 and extraction 3 and was setup in decreasing concentrations with the 5 different PCR cycling regimes at each concentration next to each other. The first section had concentrations of 5ng, 2ng and

1ng. The second section had concentrations of 500pg, 250pg and 125pg. The final section had concentrations of 62.5pg, 31.25pg and 15.625pg. Each section had a 100bp ladder and one of three controls. The first section had the negative control, the second section had one of the two reagent blanks, and the third section had the positive control. Each concentration was started with the 26 PCR cycle sample and went up to the 34 PCR cycle sample. For species specificity, one medium sized, 14-well gel (see Figure 17, below) was run, and one small gel was run with two subsequent gels, to determine if an unexpected band that was seen in a sample could be replicated.

The first sensitivity gel comprised of deer samples from both extraction 2 and extraction 3. The second gel consisted of male dog samples from extraction 2 and male human samples from extraction 3. The third gel contained female human samples from extraction 2 and female dog samples from extraction 3. The fourth gel was made up of buffalo samples from extraction 2 and beef samples from extraction 3. The fifth sensitivity gel was composed of bear samples from extraction 2 and pork samples from extraction 3.

The first specificity gel (Figure 17), was run with 100bp ladder, chicken, salmon, tuna, deer, beef, pork and human samples as well as with a negative control and two reagent blanks. The first small gel (Figure 18) was run with a 100bp ladder, a lamb sample, a tuna sample, a salmon sample, a chicken sample a reagent blank and a positive control that had all been run at 34 cycles to ensure visibility of any results, and to

determine if there was contamination in the negative control and the reagent blank. The second and third small gels (Figures 19 and 20) were only run with the 100bp ladder, a chicken sample, and the three controls (positive, negative and a reagent blank) all from new amplifications of the original sample.

Sensitivity

For sensitivity, the five gels were split up into 3 parts, each comprised of 17 wells: deer samples (Figures 2, 3 and 4); male dog and male human samples (Figures 5, 6 and 7); female dog and female human samples (Figures 8, 9, and 10); buffalo and beef samples (Figures 11, 12 and 13); and bear and pork samples (Figures 14, 15 and 16).

Figure 2. Deer Samples. Lane 1: 100bp ladder, Lane 2: Negative Control, Lane 3: 5ng - 26 cycles, Lane 4: 5ng - 28 cycles, Lane 5: 5ng - 30cycles, Lane 6: 5ng - 32 cycles, Lane 7: 5ng - 34 cycles, Lane 8: 2ng - 26 cycles, Lane 9: 2ng - 28 cycles, Lane 10: 2ng - 30 cycles, Lane 11: 2ng - 32 cycles, Lane 12: 2ng - 34 cycles, Lane 13: 1ng – 26 cycles, Lane 14: 1ng – 28 cycles, Lane 15: 1ng - 30 cycles, Lane 16: 1ng - 32 cycles and Lane 17: 1ng - 34 cycles.

Figure *3*. Deer Samples. Lane 1: 100bp ladder, Lane 2: 500pg - 26 cycles, Lane 3: 500pg - 28 cycles, Lane 4: 500pg - 30cycles, Lane 5: 500pg - 32 cycles, Lane 6: 500pg - 34 cycles, Lane 7: 250pg - 26 cycles, Lane 8: 250pg - 28 cycles, Lane 9: 250pg - 30 cycles, Lane 10: 250pg - 32 cycles, Lane 11: 250pg - 34 cycles, Lane 12: 125pg – 26 cycles, Lane 13: 125pg – 28 cycles, Lane 14: 125pg - 30 cycles, Lane 15: 125pg - 32 cycles, Lane 16: 125pg - 34 cycles and Lane 17: Reagent Blank – 34 cycles.

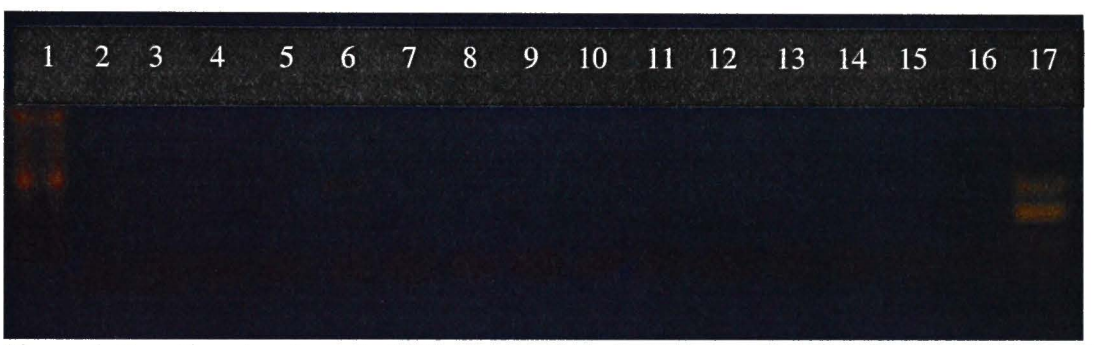


Figure *4*. Deer Samples. Lane 1: 100bp ladder, Lane 2: 62.5pg - 26 cycles, Lane 3: 62.5 pg - 28 cycles, Lane 4: 62.5pg - 30cycles, Lane 5: 62.5pg - 32 cycles, Lane 6: 62.5pg - 34 cycles, Lane 7: 31.25pg - 26 cycles, Lane 8: 31.25pg - 28 cycles, Lane 9: 31.25pg - 30 cycles, Lane 10: 31.25pg - 32 cycles, Lane 11: 31.25pg - 34 cycles, Lane 12: 15.125pg – 26 cycles, Lane 13: 15.125pg – 28 cycles, Lane 14: 15.125pg - 30 cycles, Lane 15: 15.125pg - 32 cycles, Lane 16: 15.125pg - 34 cycles and Lane 17: Positive Control 10ng– 30 cycles

As the cycle number increased, the bands became more intense, and more bands remained visible. The deer used in the study was male and the two bands are visible. As the concentrations reduced, the difference between 30, 32 and 34 PCR cycles was more apparent. In the low concentrations, 125pg and 62.5pg, the 34 cycle band was still visible

with only faint bands visible at 32 cycles. At 31.25pg and 15.625pg, a faint band could be seen at 34 cycles, but was not visible in the photograph (Figures 2 to 4).

Figure 5. Male Human (MH) and Male Dog Samples (MD). Lane 1: 100bp ladder, Lane 2: MD 5ng - 26 cycles, Lane 3: MD 5ng - 28 cycles, Lane 4: MH 5ng - 30cycles, Lane 5: MD 5ng - 32 cycles, Lane 6: MH 5ng - 34 cycles, Lane 7: MH 2ng - 26 cycles, Lane 8: MD 2ng - 28 cycles, Lane 9: MH 2ng - 30 cycles, Lane 10: MD 2ng - 32 cycles, Lane 11: MH 2ng - 34 cycles, Lane 12: MD 1ng – 26 cycles, Lane 13: MD 1ng – 28 cycles, Lane 14: MH 1ng - 30 cycles, Lane 15: MD 1ng - 32 cycles, Lane 16: MH 1ng - 34 cycles and Lane 17: Reagent Blank Swab (34 cycles).

Figure 6. Male Human (MH) and Male Dog Samples (MD). Lane 1: 100bp ladder, Lane 2: MH 500pg - 26 cycles, Lane 3: MD 500pg - 28 cycles, Lane 4: MH 500pg - 30cycles, Lane 5: MD 500pg - 32 cycles, Lane 6: MH 500pg - 34 cycles, Lane 7: MD 250pg - 26 cycles, Lane 8: MD 250pg - 28 cycles, Lane 9: MH 250pg - 30 cycles, Lane 10: MD 250pg - 32 cycles, Lane 11: MH 250pg - 34 cycles, Lane 12: MH 125pg – 26 cycles, Lane 13: MD 125pg – 28 cycles, Lane 14: MH 125pg - 30 cycles, Lane 15: MD 125pg - 32 cycles, Lane 16: MH 125pg - 34 cycles and Lane 17: Negative Control – 34 cycles.

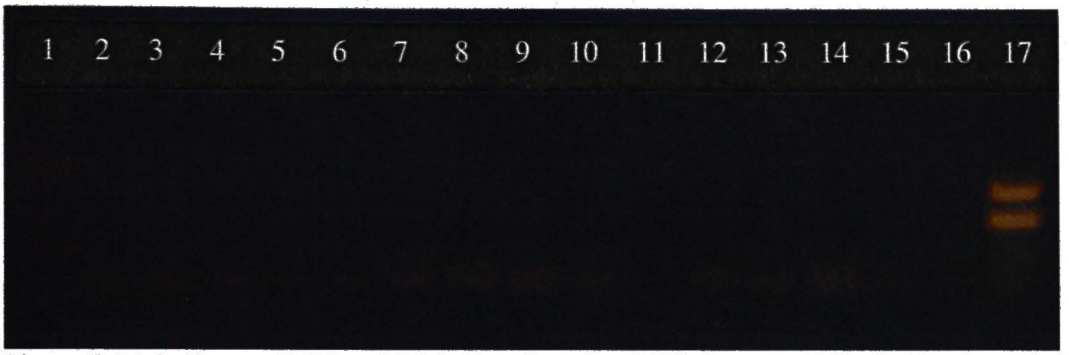


Figure 7. Male Human (MH) and Male Dog Samples (MD). Lane 1: 100bp ladder, Lane 2: MD 62.5pg - 26 cycles, Lane 3: MD 62.5 pg - 28 cycles, Lane 4: MH 62.5pg - 30cycles, Lane 5: MD 62.5pg - 32 cycles, Lane 6: MH 62.5pg - 34 cycles, Lane 7: MH 31.25pg - 26 cycles, Lane 8: MD 31.25pg - 28 cycles, Lane 9: MH 31.25pg - 30 cycles, Lane 10: MD 31.25pg - 32 cycles, Lane 11: MH 31.25pg - 34 cycles, Lane 12: MD 15.125pg – 26 cycles, Lane 13: MD 15.125pg – 28 cycles, Lane 14: MH 15.125pg - 30 cycles, Lane 15: MD 15.125pg - 32 cycles, Lane 16: MH 15.125pg - 34 cycles and Lane 17: Positive Control 10ng – 32 cycles.

The results with the male swab samples indicated the test was less sensitive than with the tissue samples. At 1ng and 500pg the bands of the swab were more difficult to see than the male tissue counterparts. However, the 30 and 32 cycle samples were fairly consistent with band intensity (Figures 5 and 6). At 62.5pg the final pair of bands in the sample set was visible at 34 cycles. No results, however, could be seen at 31.25pg and 15.625pg (Figure 7), even when viewing the gel directly (as opposed to viewing the photograph).

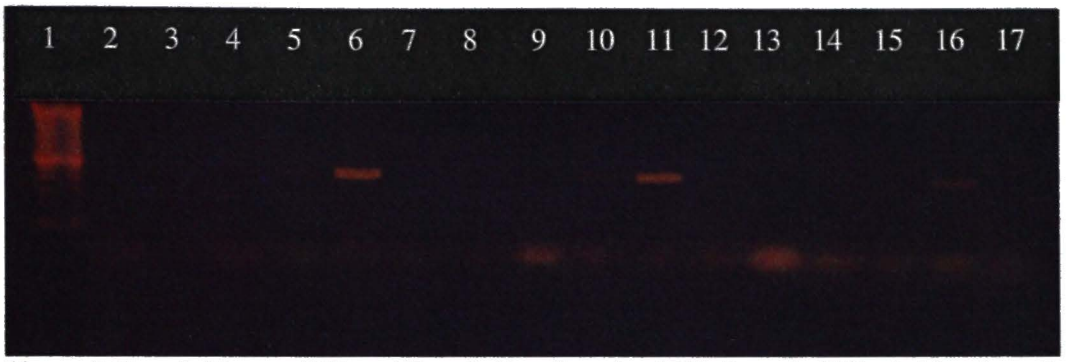


Figure *8*. Female Human (FH) and Female Dog Samples (FD). Lane 1: 100bp ladder, Lane 2: FH 5ng - 26 cycles, Lane 3: FH 5ng - 28 cycles, Lane 4: FD 5ng - 30cycles, Lane 5: FH 5ng - 32 cycles, Lane 6: FD 5ng - 34 cycles, Lane 7: FD 2ng - 26 cycles, Lane 8: FH 2ng - 28 cycles, Lane 9: FD 2ng - 30 cycles, Lane 10: FH 2ng - 32 cycles, Lane 11: FD 2ng - 34 cycles, Lane 12: FH 1ng – 26 cycles, Lane 13: FH 1ng – 28 cycles, Lane 14: FD 1ng - 30 cycles, Lane 15: FH 1ng - 32 cycles, Lane 16: FD 1ng - 34 cycles and Lane 17: Reagent Blank Swab (34 cycles).

Figure 9. Female Human (FH) and Female Dog Samples (FD). Lane 1: 100bp ladder, Lane 2: FD 500pg - 26 cycles, Lane 3: FH 500pg - 28 cycles, Lane 4: FD 500pg - 30cycles, Lane 5: FH 500pg - 32 cycles, Lane 6: FD 500pg - 34 cycles, Lane 7: FH 250pg - 26 cycles, Lane 8: FH 250pg - 28 cycles, Lane 9: FD 250pg - 30 cycles, Lane 10: FH 250pg - 32 cycles, Lane 11: FD 250pg - 34 cycles, Lane 12: FD 125pg – 26 cycles, Lane 13: FH 125pg – 28 cycles, Lane 14: FD 125pg - 30 cycles, Lane 15: FH 125pg - 32 cycles, Lane 16: FD 125pg - 34 cycles and Lane 17: Negative Control – 28 cycles.

Figure *10.* Female Human (FH) and Female Dog Samples (FD). Lane 1: 100bp ladder, Lane 2: FH 62.5pg - 26 cycles, Lane 3: FH 62.5 pg - 28 cycles, Lane 4: FD 62.5pg - 30cycles, Lane 5: FH 62.5pg - 32 cycles, Lane 6: FD 62.5pg - 34 cycles, Lane 7: FD 31.25pg - 26 cycles, Lane 8: FH 31.25pg - 28 cycles, Lane 9: FD 31.25pg - 30 cycles, Lane 10: FH 31.25pg - 32 cycles, Lane 11: FD 31.25pg - 34 cycles, Lane 12: FH 15.125pg – 26 cycles, Lane 13: FH 15.125pg – 28 cycles, Lane 14: FD 15.125pg - 30 cycles, Lane 15: FH 15.125pg - 32 cycles, Lane 16: FD 15.125pg - 34 cycles and Lane 17: Positive Control 10ng – 28 cycles.

The female swab samples did not show the same sensitivity as the tissue or male swab samples. Visible, definite bands at 30, 32 and 34 cycles were only present at 5ng and 2ng (Figure 8). Very faint bands at 30 and 32 were visible on the gel, but did not photograph well at 1ng. At 34 cycles however, the band was visible. A faint band at each of 250pg and 125pg were the last visible bands, and these bands were only present at 34 cycles (Figure 9). There were no bands visible below 125pg on either the photograph or the gel, making the female human/dog samples the least sensitive set of samples in the study (Figure 10).

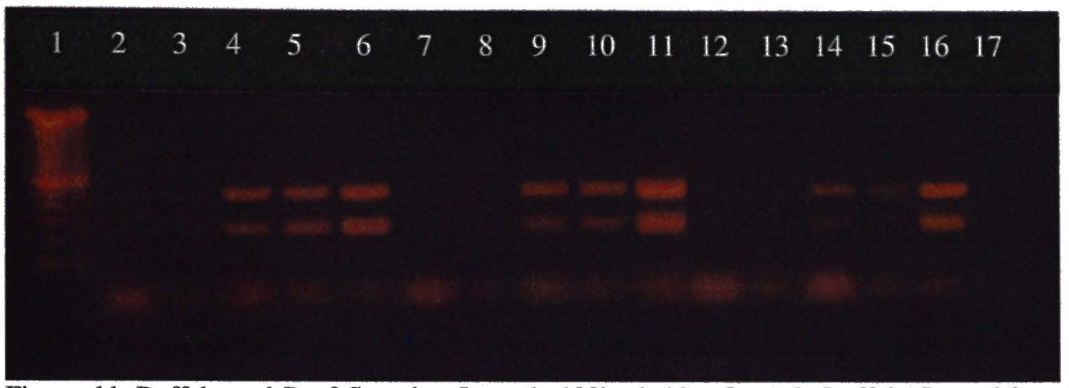


Figure *11*. Buffalo and Beef Samples. Lane 1: 100bp ladder, Lane 2: Buffalo 5ng - 26 cycles, Lane 3: Buffalo 5ng - 28 cycles, Lane 4: Beef 5ng - 30cycles, Lane 5: Buffalo 5ng - 32 cycles, Lane 6: Beef 5ng - 34 cycles, Lane 7: Beef 2ng - 26 cycles, Lane 8: Buffalo 2ng - 28 cycles, Lane 9: Beef 2ng - 30 cycles, Lane 10: Buffalo 2ng - 32 cycles, Lane 11: Beef 2ng - 34 cycles, Lane 12: Buffalo 1ng – 26 cycles, Lane 13: Buffalo 1ng – 28 cycles, Lane 14: Beef 1ng - 30 cycles, Lane 15: Buffalo 1ng - 32 cycles, Lane 16: Beef 1ng - 34 cycles and Lane 17: Reagent Blank (32 cycles).

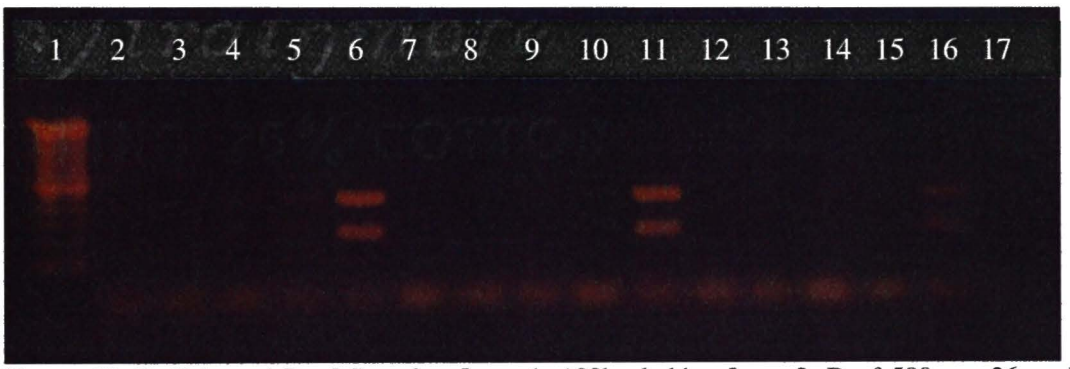


Figure *12*. Buffalo and Beef Samples. Lane 1: 100bp ladder, Lane 2: Beef 500pg - 26 cycles, Lane 3: Buffalo 500pg - 28 cycles, Lane 4: Beef 500pg - 30cycles, Lane 5: Buffalo 500pg - 32 cycles, Lane 6: Beef 500pg - 34 cycles, Lane 7: Buffalo 250pg - 26 cycles, Lane 8: Buffalo 250pg - 28 cycles, Lane 9: Beef 250pg - 30 cycles, Lane 10: Buffalo 250pg - 32 cycles, Lane 11: Beef 250pg - 34 cycles, Lane 12: Beef 125pg – 26 cycles, Lane 13: Buffalo 125pg – 28 cycles, Lane 14: Beef 125pg - 30 cycles, Lane 15: Buffalo 125pg - 32 cycles, Lane 16: Beef 125pg - 34 cycles and Lane 17: Negative Control – 32 cycles.

Figure *13*. Buffalo and Beef Samples. Lane 1: 100bp ladder, Lane 2: Buffalo 62.5pg - 26 cycles, Lane 3: Buffalo 62.5 pg - 28 cycles, Lane 4: Beef 62.5pg - 30cycles, Lane 5: Buffalo 62.5pg - 32 cycles, Lane 6: Beef 62.5pg - 34 cycles, Lane 7: Beef 31.25pg - 26 cycles, Lane 8: Buffalo 31.25pg - 28 cycles, Lane 9: Beef 31.25pg - 30 cycles, Lane 10: Buffalo 31.25pg - 32 cycles, Lane 11: Beef 31.25pg - 34 cycles, Lane 12: Buffalo 15.125pg – 26 cycles, Lane 13: Buffalo 15.125pg – 28 cycles, Lane 14: Beef 15.125pg - 30 cycles, Lane 15: Buffalo 15.125pg - 32 cycles, Lane 16: Beef 15.125pg - 34 cycles and Lane 17: Positive Control 10ng – 32 cycles.

Both the buffalo and beef samples were male, as can be seen from the double bands. The bands decreased in intensity as the concentration lowered. At 34 cycles, faint bands were visible as low at 15.625pg (Figure 13). It is possible that the bands seen at 15.623pg are as a result of the strong positive control being in the next lane. Bands were still intense down to 250pg with visible bands at 30, 32 and 34 cycles down to 62.5pg (Figure 11-13). At 5ng and 2ng, the bands were very bright and did not show much difference between 30, 32 and 34 cycles (Figure 11).

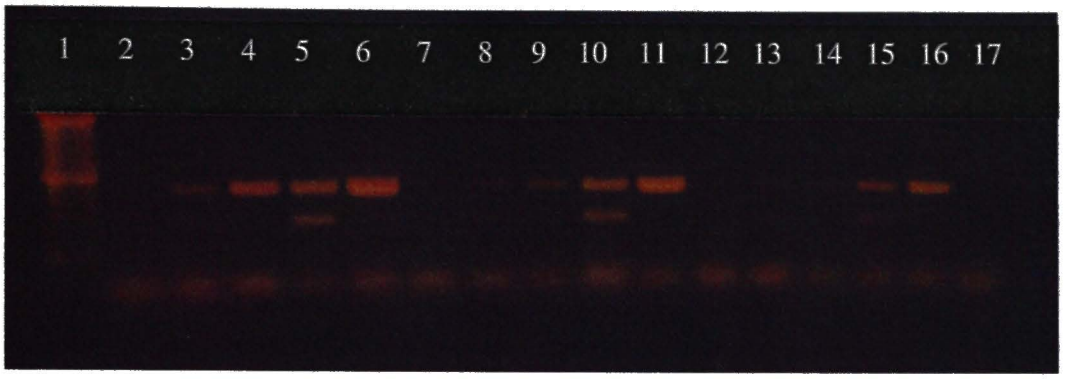


Figure *14*. Bear and Pork Samples. Lane 1: 100bp ladder, Lane 2: Bear 5ng - 26 cycles, Lane 3: Bear 5ng - 28 cycles, Lane 4: Pork 5ng - 30cycles, Lane 5: Bear 5ng - 32 cycles, Lane 6: Pork 5ng - 34 cycles, Lane 7: Pork 2ng - 26 cycles, Lane 8: Bear 2ng - 28 cycles, Lane 9: Pork 2ng - 30 cycles, Lane 10: Bear 2ng - 32 cycles, Lane 11: Pork 2ng - 34 cycles, Lane 12: Bear 1ng – 26 cycles, Lane 13: Bear 1ng – 28 cycles, Lane 14: Pork 1ng - 30 cycles, Lane 15: Bear 1ng - 32 cycles, Lane 16: Pork 1ng - 34 cycles and Lane 17: Reagent Blank (30 cycles).

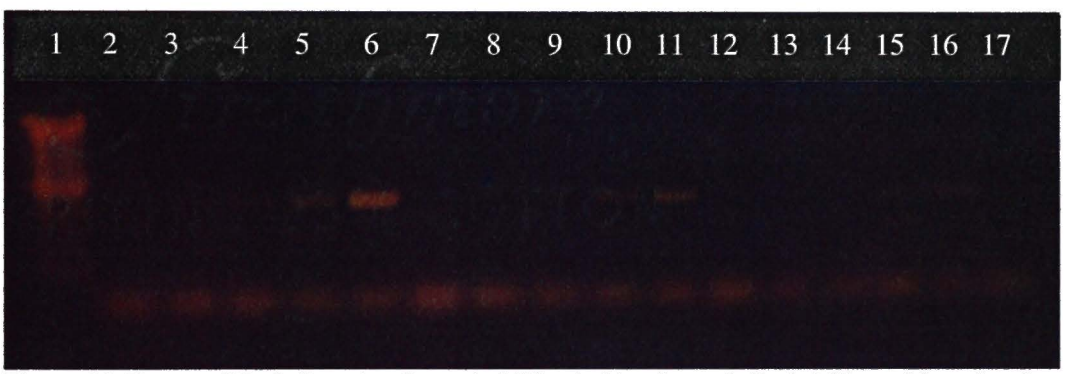


Figure *15*. Bear and Pork Samples. Lane 1: 100bp ladder, Lane 2: Pork 500pg - 26 cycles, Lane 3: Bear 500pg - 28 cycles, Lane 4: Pork 500pg - 30cycles, Lane 5: Bear 500pg - 32 cycles, Lane 6: Pork 500pg - 34 cycles, Lane 7: Bear 250pg - 26 cycles, Lane 8: Bear 250pg - 28 cycles, Lane 9: Pork 250pg - 30 cycles, Lane 10: Bear 250pg - 32 cycles, Lane 11: Pork 250pg - 34 cycles, Lane 12: Pork 125pg – 26 cycles, Lane 13: Bear 125pg – 28 cycles, Lane 14: Pork 125pg - 30 cycles, Lane 15: Bear 125pg - 32 cycles, Lane 16: Pork 125pg - 34 cycles and Lane 17: Negative Control – 30 cycles.

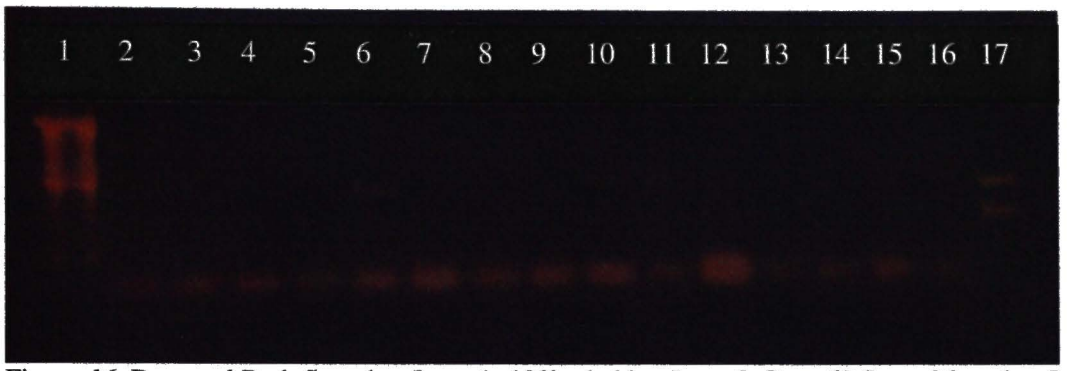


Figure *16*. Bear and Pork Samples. Lane 1: 100bp ladder, Lane 2: Bear 62.5pg - 26 cycles, Lane 3: Bear 62.5 pg - 28 cycles, Lane 4: Pork 62.5pg - 30cycles, Lane 5: Bear 62.5pg - 32 cycles, Lane 6: Pork 62.5pg - 34 cycles, Lane 7: Pork 31.25pg - 26 cycles, Lane 8: Bear 31.25pg - 28 cycles, Lane 9: Pork 31.25pg - 30 cycles, Lane 10: Bear 31.25pg - 32 cycles, Lane 11: Pork 31.25pg - 34 cycles, Lane 12: Bear 15.125pg – 26 cycles, Lane 13: Bear 15.125pg – 28 cycles, Lane 14: Pork 15.125pg - 30 cycles, Lane 15: Bear 15.125pg - 32 cycles, Lane 16: Pork 15.125pg - 34 cycles and Lane 17: Positive Control 10ng – 30 cycles

The bear sample was male, while the pork showed only the single female band. Once again, the tissue showed greater sensitivity than the swab samples, as bands were visible, in the bear sample down to 15.625pg (32 cycles), and down to 31.25pg (34 cycles) in the pork (Figure 16). Lane 12 showed a strong band at 26 cycles which should not be present. This was possibly due to a pipetting error. As expected, intensity decreased as the concentration decreased. The intensity of the bands at 5ng and 2ng was not overly varying between 28 and 34 cycles (Figure 14). At 1ng and 500pg, the bands increased in intensity as the cycle number increased (Figure 14-15).

Specificity

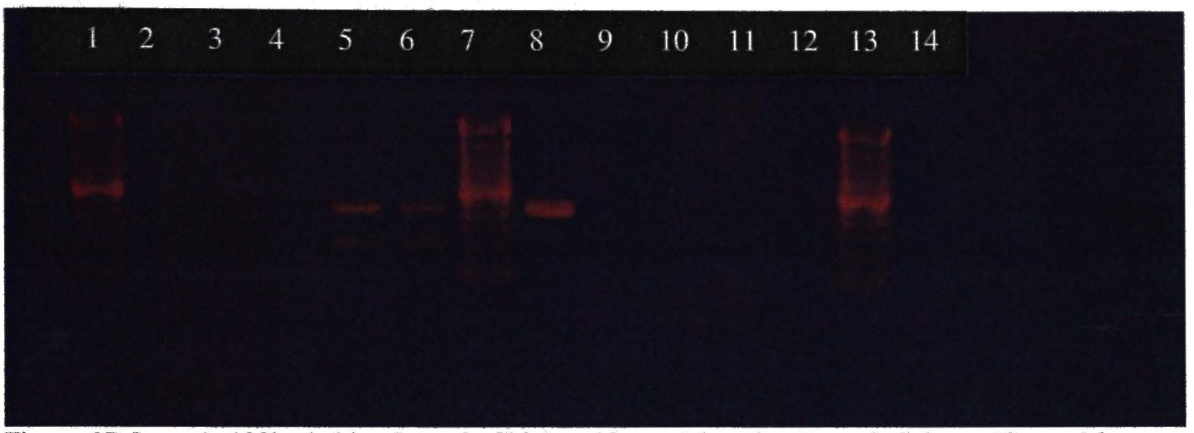


Figure *17*: Lane 1: 100bp ladder, Lane 2: Chicken 10ng – 26 cycles, Lane 3: Salmon 10ng – 26 cycles, Lane 4: Tuna 10ng – 26 cycles, Lane 5: Deer 10ng – 26 cycles, Lane 6: Beef 10ng – 26 cycles, Lane 7: 100bp ladder, Lane 8: Pork 10ng - 26 cycles, Lane 9: Reagent Blank - Swab , Lane 10: Male Human (Extraction 1) 10ng – 26 cycles, Lane 11: Female Human (Extraction 1) 10ng – 26 cycles, Lane 12: Negative Control, Lane 13: 100bp ladder, Lane 14: Reagent Blank.

As seen in figure 17 (above), there were no visible bands for chicken, salmon, tuna and both human samples. Since the primers were working on other mammals, new human blood samples were drawn, in case the lack of bands was due to old, degraded blood. As seen in previous gels (Figures 5-10), humans do exhibit bands, so the old blood must have been spoiled. The deer and beer showed two bands making them male, while the single band indicated a female pig.

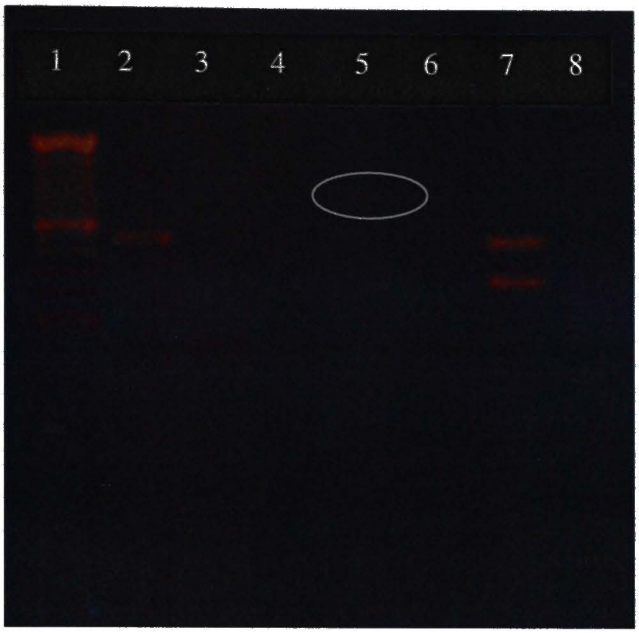


Figure *18*: Lane 1: 100bp ladder, Lane 2: Lamb 1ng – 34 cycles, Lane 3: Tuna 1ng – 34 cycles, Lane 4: Salmon 1ng- 34 cycles, Lane 5: Chicken 1ng – 34 cycles, Lane 6: Reagent Blank – 34 cycles, Lane 7: Positive Control 10ng – 34 cycles and Lane 8: Negative Control – 34 cycles. Lane 5 (chicken - circled) shows faint band at ~700bp

In Figure 18 lane 2 (above), the lamb exhibited a female band. Again, the tuna and salmon did not show any bands. The controls (positive, negative and reagent blank) showed the expected results. The chicken sample showed a single faint band at ~700bp (circled) which was not consistent with any expected bands in the study. A new chicken sample was amplified and re-run on a gel (Figure 19).

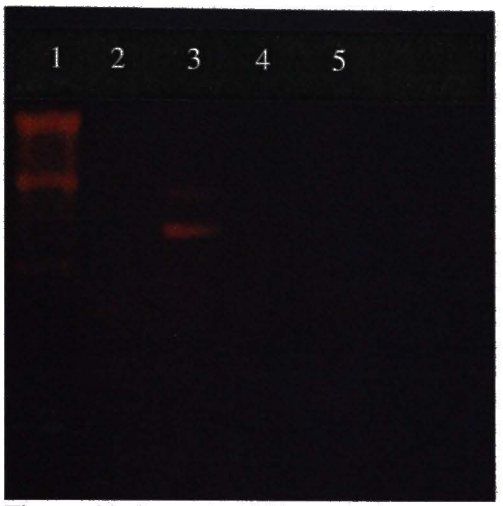


Figure *19*: Lane 1: 100bp Ladder, Lane 2: Chicken 1ng – 34 cycles, Lane 3: Positive Control 10ng – 34 cycles, Lane 4: Negative Control – 34 cycles and Lane 5: Reagent Blank – 34 cycles.

The chicken sample did not show the 700bp band on this gel, but, to ensure that it was a contamination or a spurious band in Figure 17, a third amplification was done, and another gel was run (Figure 20).

Figure *20*: Lane 1: 100bp Ladder, Lane 2: Chicken 1ng – 34 cycles, Lane 3: Positive Control 10ng – 34 cycles, Lane 4: Negative Control – 34 cycles and Lane 5: Reagent Blank – 34 cycles.

Once again, no band was seen at 700bp for the chicken, so it can be assumed that the band seen in Figure 17 was not related to the study, and that the primers are indeed mammal specific.

Database Search

A BLAST (Basic Local Alignment Search Tool) search was run on the National Center for Biotechnology Information (NCBI) website, using the reference SRY gene nucleotide sequence from black-tailed deer to search for other animals with DNA regions that are very similar to the reference sequence. The results showed that there are multiple species that show very similar SRY regions as the region of interest, including some that did not show bands in the study (see Table 9, below). The DNA could have differences in the primer region that do not allow for binding to the primers. If the primers do not bind, amplification cannot occur.

A BLAST search was also run on puffer fish (*Takifugu rubripes*) and on pipid frogs (*Xenopus tropicalis*) as representatives of fish and amphibians, and there was no similarity with these species. The puffer fish was chosen as it is a fully sequenced fish genome present in the NCBI database. Given the significant research into the SRY gene in fruit flies (*Drosophila melanogaster*), a search was conducted comparing the reference sequence examined above to the fruit fly genome. As a whole, the reference sequence did not have any similarities with the fruit fly SRY gene, but there were parts of that sequence that were very similar.

Table 9. SRY gene species homology

Species	Percent Homology
Bos Taurus	97%
Bison bison	97%
Ovis sp.	96-97%
Gallus gallus	90%
Canis familiaris	89%
Sus domesticus	87%
Homo sapiens	85%

BLAST searches on the individual primers turned up the following results. The 3EZ and 5EZ primers both had species that matched the sequence exactly. Some species only had partial sequence matches (Table 10). The 3D and 3C primers were not as specific, with none of the species searched matching at all 25 bases of the 3D primer, and only a few species matching at all 24 bases of the 3C primer (Table 11).

Table 10 – Zinc Finger protein primer homology

Species	3EZ primer	5EZ primer
Homo sapiens	100%	92%
Bos Taurus	100%	88%
Sus domesticus	100%	100%
Ovis sp.	100%	100%
Gallus gallus	76%	72%
Canis familiaris	96%	64%
Takifugu rubripes	52%	56%
Xenopus tropicalis	67%	64%
Equus caballus (Horse)	100%	100%
Mus musculus (Mouse)	-	100%
Eschrichtius robustus(Gray Whale)	100%	-

The zinc finger protein primers were not mammal specific, but did not bind well with non-mammalian species. The 3EZ primer showed great similarity to the sequence found in aquatic mammals, such as whales and porpoises, although it was similar to all of the mammals searched. The 5EZ primer showed similarities with the mammalian species. The dog sample showed low similarity which was surprising, as there was successful amplification of both dog samples during testing. The chicken, frog and puffer fish samples showed low levels of similarity with both primers. Some of mammalian species had lower values at 5EZ than the non-mammalian species. The lack of amplification for non-mammalian species could be attributed to the sequence of interest or to the efficiency of that part of the primer that can bind to the DNA. If the sequence of interest is very long, then it might not be able to replicate fully during PCR, or it may be too large to visualize on a 2% agarose gel. If only the middle or beginning of the primer binds, then amplification may not occur because the polymerase cannot bind.

Table 11 – SRY gene primer homology

Species	3C primer	3D primer
Homo sapiens	100%	80%
Bos Taurus	96%	64%
Sus domesticus	96%	52%
Ovis sp.	96%	80%
Gallus gallus	92%	56%
Canis familiaris	92%	68%
Takifugu rubripes	92% (not SRY gene)	48%
Xenopus tropicalis	92%	52%
Macaca mulatta (Monkey)	100%	80%
Mirounga leonina (Elephant Seal)	-	84%

The SRY gene primers were not as specific as the zinc finger protein primers. The 3C primer was universal and was similar in sequence to many different species. The primates showed the highest level of similarity in sequence. The puffer fish search showed 92% similarity with the sequence. However, the area where the primer bound was not the SRY gene, but transcription factor SOX17. The 3D primer was not very similar in sequence to any of the species searched. The highest level of similarity was observed in seals and walruses, and only 21 bases matched the primer sequence. The results were confusing, as some species, with low similarity in one or both of the Zinc Finger protein primer and the SRY gene primers, successfully amplified during testing with the primers provided by DNA Solutions, Inc., and species with higher similarity did not amplify. Again, this is likely due to the region of interest being too long to successfully amplify during PCR, or due to the primer not being able to bind enough for the polymerase to work successfully.

CHAPTER V

CONCLUSIONS

The protocol sent by DNA Solutions, Inc. stated that the primers were sensitive to 1ng at 26 cycles. From the testing done, no visible results could be obtained at 26 cycles for any concentrations, so the cycle number was increased. There was little visible improvement in sensitivity at 28 cycles with only the occasional faint bands being visible at the higher concentrations. At 30 cycles, the tissue samples were sensitive down to 125pg of DNA. At 32 cycles, the sensitivity was increased as far down as 62.5pg and 31.25pg in some cases with the tissue samples. At 34 cycles, the deer tissue sample showed a faint pair of bands as low as 15.625pg. The bands at 15.625pg may have been a result of the strong neighbouring positive control. Further tests should be done with the positive control kept separate from the samples. By increasing the cycle number the sensitivity of the system was increased to a point where it could be used for forensic samples which may not have a significant amount of DNA present.

However, when these samples were taken on swabs, the sensitivity was greatly reduced. The swabs also showed that the male samples amplified better with the primers than the female samples. The male human and male dog swabs showed bands as low as 62.5pg, but only at 34 cycles. At 5ng, there were results for 28 to 34 cycles. Very quickly though, the lower cycle numbers no longer had visible results and for 125pg and

62.5pg only the 34 cycle bands could be seen. As well, the male human samples showed better results than the male dog samples, which was expected, as whole blood was being tested instead of a buccal swab. The whole blood samples showed bands at 30 cycles down to 250pg, but bands were only visible at lower concentrations at 34 cycles. The female swab samples did not test as well. The female dog buccal samples were only visible down to 1ng where a faint band was present. The female human whole blood sample showed bands down to 1ng at 30 cycles and down to 125pg at 34 cycles. This shows that the male samples seem to amplify slightly better with the primers than the female samples. As well, the testing suggests that, although results are possible at low concentrations, when working with swabs, the protocol needs to be adjusted to increase the PCR cycle number that is being used to 34 cycles.

Species specificity testing suggests that the primers are mammal specific, although the BLAST search suggested there was some similarity to some non-mammalian species such as chicken. Neither the fish nor chicken samples showed any amplified product consistently at any cycle number. Further testing of non-mammalian species should be undertaken to ensure that the primers are indeed mammal specific. More samples from birds and fish should be studied, as well as representatives from the reptile and amphibian classes. Since the SRY gene is common, and is most notably studied in *Drosophila*, insects should be studied as well to ensure that their presence will not cross-react with the primers, especially in cases where the sample is old, and insects may be present. Obtaining some samples may also be an issue, as some potential

subjects are protected or are difficult to find. To be able to acquire exotic samples to test, documentation will be required under the *Convention on International Trade in Endangered Species of Wild Flora and Fauna* (CITES). These exotic samples, if they are available, will be perfect to test, as they are considered endangered and protected species. Identifying them could aid in catching poachers and in improving population conservation methods.

Once specificity has been determined, these primers can be added to animal or wildlife multiplexes to be used to identify individuals in the specific species. A multiplex contains a mixture of primers that can amplify multiple regions of DNA in a single reaction. These multiplexes increase the likelihood of individual identification by looking at all of these locations and comparing unknown samples to known samples to see if each of the DNA regions amplified are the same. For increased detection sensitivity, a Hitachi FMBIO image scanner can be employed with SYBR Green I® dye being used in the gels instead of ethidium bromide. This equipment will allow for visualization of bands that cannot be seen with the naked eye on an agarose/ethidium bromide gel. Labeled versions of these primers are also being studied, so that they can be placed in microsatellite multiplexes for use on a genetic analyzer.

For increased use in the animal and wildlife forensics field, the protocol was altered by changing the PCR cycle number. With the smaller amounts of DNA usually found at crime scenes, a more sensitive assay is needed. To achieve this, the PCR cycle

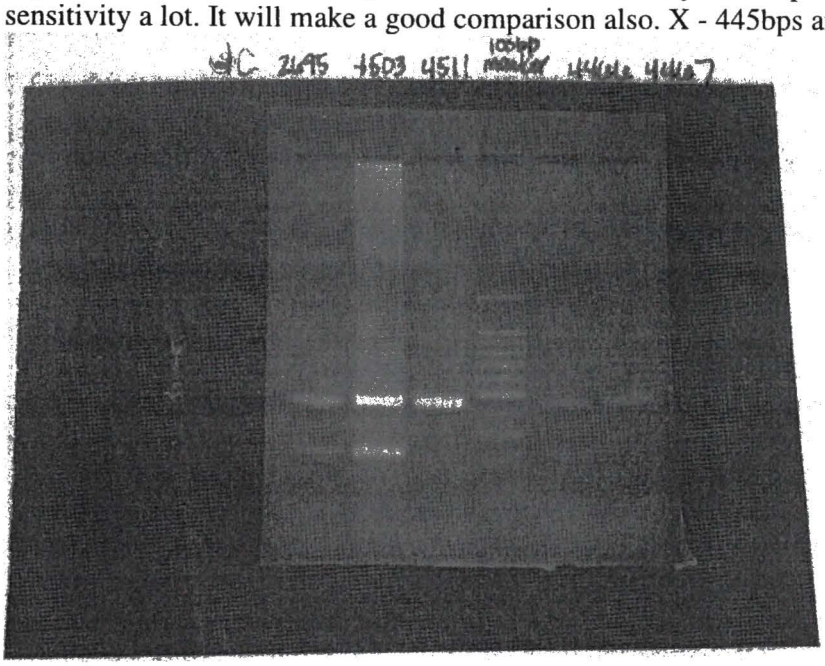
number should be increased to 34 cycles for low copy number cases and increased to at least 30 cycles for regular cases. If using swabs, then the cycle number should be increased to 34 cycles to ensure results at the lower concentrations. Since no visible results were obtained at 26 cycles, except early testing with over 20ng of input DNA, the 26 cycles should only be used when there is over 10ng of DNA present in the sample. If a large piece of tissue is recovered, this amount is possible, but for smaller samples, such as bone that has been crushed into powder and mixed with other substances, or small amounts of blood found at a scene, having over 10ng to work with is unlikely.

This protocol is excellent for those who do not have a genetic analyzer and need a less expensive and quick way to determine sex from a sample with no means of phenotypic identification. By increasing PCR cycle number, it can become a highly sensitive assay that is reproducible and accurate.

APPENDIX A

DNA SOLUTIONS, INC. PROTOCOL

Here is a picture of the gender analysis I currently use. Two bands is a male and one is a female. These are all deer samples. I have used about 20ngs of DNA so this assay is not all that sensitive. The STR system I use can get by with less than lng of DNA. I think switching this to a fluorescent assay will improve the sensitivity a lot. It will make a good comparison also. X - 445bps and Y - 224bps



Gel was loaded from left to right, with the wells oriented at the top, as follows: AC 2695, AC 4503, AC 4511, 100bp marker, AC 4466, and AC 4467

The system goes like this: 2 sets of primers
Made master mix of primers to 10µM Gender Mix:

Primer	Stock []	Amount Added to Mix
3D	100 M	10 1
3C	100 µM	10 µl
3 ez	100 µM	10 µl
5ez	100 µM	10 µl

Mixed with 60 µl DIH2O for a final concentration of 10 µM for each primer.

PCR setup:

Component	Concentration	Volume per rxn	Final [ ]	Total for 5 rxns
Taq gold buffer	10x	2.5 µl	1 x	12.5 µl
DNTP	2 mM	3.125 µl	250 µM	15.625 µl
MgC12	25 mM	2.6 µl	2.6 mM	13 µl
BSA	2.5 mg/mL	1.6 µl	160 ng/µl	8 µl
Primer Mix	10 µM	1 µl	400 nM	5 µl
Taq Gold	5 units/µl	.4 µl	2 units/µl	2 µl
H20		11.775 µl		58.875 µl
DNA Template		2 µl (- 20 ng)		
Final Volume		25 µl		115 µl

Added 23 µl of PCR mix to each tube.

Gender ID test will include Control AC 2695 Known Male AC 4503 Known Female AC 4511 100 bp marker
Strawser unknown sample AC 4466 Strawser unknown sample AC 4467

PCR Started at 8:55 am on 2400 thermocycler. Program found under BC/ Mammalsex60. Ends at approximately 11:00 am.

GEL:
Made 1 L of Ix TAE by mixing 20 mL SOx stock with 980 mL DI H20Mixed 100 mL lx TAE with 2g Agarose. Heated in microwave until boiling. Let cool to 60°C.
Poured in apparatus.
Loaded gel with 10 µl of PCR product mixed with 3 µl loading dye, with the exception of the marker which consisted of 3 µl DNA, 7 µl DI H2O, and 3 µl of loading dye.

Gel was loaded from left to right, with the wells oriented at the top, as follows: AC 2695, AC 4503, AC 4511, 100bp marker, AC 4466, and AC 4467

Gel was loaded, and run at 50 V. Start time was 11:26 am, and finishing time was 12:24pm.

Gel was stained with TAE + Ethidium Bromide, and destained with DI HzO. Poloroid photograph was taken.

The primer sequences are:

3D -5' ATTTAGCCTTCCGACGAGGTCGATA 3' 3C - 5' CCCATGAACGCATTCATTGTGTGG 3'

3EZ - 5' GCACTTCTTTGGTATCTGAGAAAGT 3' 5EZ - 5' ATAATCACATGGAGAGCCACAAGCT 3'

The PCR program I am currently running is:

94C/1 lmin (this is to activate the TAQ Gold enzyme. It is a hot start enzyme)
94C/45 sec 60C/45 sec 73C/1 min - 26cycles
72C/15 min
4C hold

I have a paper that describes this assay. California Fish and Game 84(4):159-169 (1998). I will make a copy of it and send it by mail. Along with primers and some known DNAs for you to work with. You should go ahead and isolate some DNA from other sources to run some tests. We still need to decide which primers to have labeled with fluorescent tags and what color these should be. I will look in to this and get something ordered.

Please think about this project and outline what you think would represent a possible work flow and time line. I think the first objective is to convert to a fluorescent assay, then test the sensitivity compared to the gel assay and look at the range of mammals that this assay would be good for. The National wildlife lab may be able to provide us some samples from the kind of mammals they run into on a regular basis. I think we should contact them once we have the assay working.

Let me know what you think.
Brandt

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