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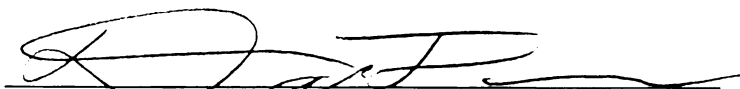
A More Sensitive Sex Determination Assay

presented by

Carrie Beth Jackson

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M.S. degree in Criminal Justice


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A MORE SENSITIVE SEX DETERMINATION ASSAY

By

Carrie Beth Jackson

A THESIS

**Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of**

MASTER OF SCIENCE

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ABSTRACT

A MORE SENSITIVE SEX DETERMINATION ASSAY

By

Carrie Beth Jackson

Forensic DNA analysis is a useful tool in the investigative process; however, analysis can be complicated by a lack of viable DNA. Aged skeletal material, shed hairs, nails, and skin cells often do not yield DNA amplification products when single copy nuclear loci are examined. If analysts encounter materials with low quantity or quality DNA, obtaining any information about the source is useful; however, sex determination is typically made using the single copy gene amelogenin, which is unlikely to amplify from degraded samples. In such instances, amplification may be successful using multicopy loci; these in theory could amplify with extremely low starting concentrations of DNA because many copies are present on the chromosome. This thesis describes the development of an assay that targets the Y chromosome multicopy locus, DYZ1, and positive control loci for sex determination. Optimizations of PCR were conducted in order to maintain specificity for male DNA with the DYZ1 primers and allow for amplification from forensic materials. Variation in magnesium concentration, annealing temperature and time, addition of DMSO, formamide, and BSA were tested. Semi-nested and SYBR Green real-time PCR techniques were attempted, however TaqMan® real-time PCR showed the most promise as a viable technique. Unlike the other techniques, TaqMan® real-time PCR reaction replicates had reproducible sex assessments. Overall, the ability to yield information using the high copy number markers on samples that could not otherwise be amplified will be a useful tool.

To my husband, Rich, for all your love and support.

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Images in this dissertation are presented in color

Introduction

State of the art forensic identification of an individual through DNA analysis involves amplification of short tandem repeats (STRs), which is routinely conducted in crime laboratories on biological evidence. Included in the kits used for obtaining DNA profiles is a locus for identifying the sex of the source. The quality or amount of DNA present in some items may be problematic for these standard analyses, limiting, either in part or completely, determination of the individualizing and class characteristics. In these instances, obtaining any information from biological evidence, however little, may aid the investigative process. Determining the sex of an individual can be of great importance to criminal and other investigations by limiting the suspect pool, aiding in the identification of missing persons, or in the analysis of ancient remains.

The X and Y chromosomes have obvious utility in assessing sex because it is these chromosomes that control male and female sex differentiation. Individuals typically possess a pair of sex chromosomes, females having two X chromosomes, while males have an X and a Y chromosome. The Y chromosome, the smallest of all human chromosomes, contains genes which affect male sexual development and differentiation (Rahman, 2004). During the evolutionary process, many of the genes initially located on the Y chromosome were lost or relocated to other chromosomes if they did not function in male sexual determination; as a result, the Y chromosome contains very few genes. Unlike other chromosome pairs, the majority of the X and Y chromosome do not recombine and exchange genetic material (Kunkel and Smith, 1982). Recombination between the two only occurs in the pseudoautosomal regions (PABY) 1 and 2, which constitute approximately five percent of the Y chromosome (Rahmann et al., 2004).

Differences between X and Y regions that no longer recombine can be exploited for sex determination; analysis can also target loci present only on the Y chromosome to distinguish males from females.

Sex Determination in the Forensic Laboratory

Anthropological Sex Determination

An advantage provided in the analysis of skeletal material is the ability to assess the sex of the individual using the sexually dimorphic characteristics of bones. Physical anthropologists are trained in the observation of these and other traits. When attempting to identify an individual based on skeletal remains, an important step is determination of sex because this affects other characteristics important in identification, such as assessment of stature and age.

According to Fairgrieve (1999) the completeness of skeletal remains can greatly affect the accuracy of sex determination; complete and intact skeletons provide the most precise estimation. When available, the pelvis and cranium are the two primary sources used to evaluate sex. Changes in size and shape arise between the male and female pelvis as biological development prepares females for childbirth. The features of the pubic region, such as the subpubic angle, ventral arc, ischio-pubic ramus, and sacrum show marked dimorphism between males and females.

Sexually dimorphic traits are present in the cranium as well, however these distinctive features are not the result of biological roles as in the pelvic region; rather they are attributed to differences in size or robusticity. Overall, males tend to have a more pronounced supraorbital ridge and sloping foreheads, while females tend to possess a

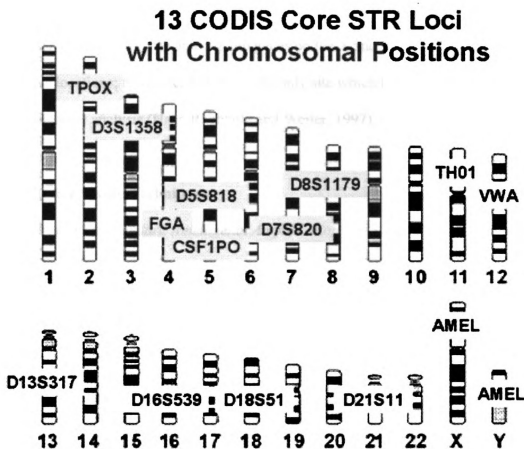
more vertical forehead and a more flattened supraorbital ridge. Differences also occur in the shape of the mandible and appearance of the mastoid process, among others (Fairgrieve, 1999). The overall robusticity and size of skeletal bones can also be used to evaluate sex. Application of these techniques is often critical in the identification of missing persons, and likewise can add to our understanding of previous cultures through evaluation of ancient remains.

Sexing by Molecular DNA Analysis

DNA analysis routinely completed in forensic laboratories involves the assessment of sex using the amelogenin locus included in standard STR kits (Figure 1); this allows for the generation of a DNA profile and assessment of sex simultaneously. Amelogenin is a single copy gene located on the X and Y chromosomes which encodes a protein found in tooth enamel (Sullivan et al., 1993). Recombination of the X and Y forms of amelogenin has ceased, which has allowed differences between the two loci to emerge (Huang et al., 1997); it is these unique features which are exploited for sex determination.

Figure 1: Thirteen Core CODIS Loci Included in STR Kits

The 13 STRs included in the Combined DNA Index System profile are identified below by boxes. Each STR locus is centered over the chromosome where it is located; for example TPOX is located on chromosome 2. Amelogenin (AMEL below) amplification is included in addition to the 13 STR loci for sex identification. The X and Y chromosomal forms of this gene have unique characteristics, which allow for distinction between males and females (Diagram from Butler and Reeder, 2006).



In order to perform STR analysis or sex assessment, the target DNA must be amplified. Amplification of DNA is completed using the polymerase chain reaction (PCR), a technique that targets a specific segment of DNA and increases its amount. The three steps of PCR include denaturation, primer annealing, and extension. In the initial step, the temperature is increased to separate the template DNA strands. Then primers, short segments of DNA that are complementary to the template DNA, bind (or anneal) to

either side of the target DNA. The polymerase extends the primers, forming a new DNA strand, and the process is repeated. With perfect amplification, after 30 cycles the target sequence or amplicon will be present in over a billion copies.

Primers that are typically used in forensic analysis of amelogenin target the first intron in the gene, which contains a six base pair deletion in the X chromosomal form (Sullivan et al., 1993). The amelogenin genes on the X and Y chromosomes differ in multiple locations; therefore, this is not the only site which has been exploited for sex determination analysis (Haas-Rochholz and Weiler, 1997).

Alternative Sexing Techniques

Modifications to Standard Amelogenin Amplification

The importance of accurate sex determination has led to the development of molecular DNA assays that approach it in different manners. In addition to the most commonly used primers which amplify across intron 1 of the amelogenin gene (Haas-Rochholz and Weiler, 1997), those which target alternative locations within the gene have been developed (Akane et al., 1991, 1992; Bailey et al., 1992; Faerman et al., 1995; Sullivan et al., 1993). In total, there are ten sites of sequence variation between the X and Y chromosomal forms of amelogenin that can be amplified (Haas-Rochholz and Weiler, 1997). Alternate priming sites may provide the benefit of producing smaller amplicons, which increase the likelihood of amplification from degraded samples as well as other benefits such as multiplexing with STRs. Modifications to the standard amelogenin protocol have also included the use of real-time PCR (see below) in place of traditional

amplification techniques. Such assays provide increased sensitivity by allowing for lower starting quantities of initial template DNA (Andréasson and Allen, 2003).

Targeting Different Loci for Sex Determination

Analysis of alternative loci for sex determination has been attempted in order to enhance sensitivity and to alleviate problems with inaccurate sex assessment, while other assays were developed prior to amelogenin assays. Instances where individuals lack all or portions of the Y chromosomal form of amelogenin, resulting in incorrect sex assessment, have been reported (Santos et al., 1998). Another locus that has been used is the Zinc Finger Protein gene, which is present on both the X and Y chromosomes. The primers for this assay produce amplicons of identical size from both chromosomes, however the intervening sequence of the two products are distinct (Stacks and Witte, 1996). Only the amplicon originating from the Y chromosome contains the specific sequence which can be cut by the restriction enzyme Hae III—it is digested into smaller fragments, while the X form remains whole—and these can be distinguished by gel electrophoresis. The sex-determining region Y (SRY) gene, which controls male sexual development in primates, has also been used in sex determination assays (Bartha et al., 2003). Additionally, alpha satellite DNA (see below), a repetitive, non-coding region located at the centromeres of chromosomes, has been targeted for sex assessment. The sequences of alpha satellite DNA are chromosome specific, and it is the differences between the X and Y chromosomal forms which has been targeted. Amplification of this highly repetitive DNA has allowed for sex determination from less than 1 ng of DNA (Nesser and Liechti-Gallati, 1995). Although modifications to protocols for sex

determination have increased amplification success in some cases, most still require the use of higher amounts of DNA than may be present in some samples.

Difficulties Encountered in Standard DNA Analysis

Sex determination through standard molecular techniques can be complicated by the fact that DNA does not always remain stable over time, and amplification by PCR can be inhibited. Additionally, some materials often contain little nuclear DNA. STR typing kits usually require between 0.5 and 1 nanograms of DNA (Andréasson and Allen, 2003), however hair shafts, aged skeletal material, nails, and shed skin cells typically contain little to no DNA (Chang et al., 2002). All of these factors can contribute to reduced amplification success and limit the information which can be obtained.

Concentrations and the quality of DNA can be diminished by degradation. As described by Burger et al. (1999), a major part of DNA degradation is the result of autolysis, which begins at the time of death. This process is the destruction of cellular material that results from the organism's own enzymes. DNA is further broken down over time by the processes of oxidation and hydrolysis of the nucleotides. Environmental factors, such as pH, humidity, temperature, microbial concentration, and UV light exposure also affect the degradation that occurs before and following extraction of the DNA from the biological sample. Low pH, high temperature, high concentrations of microorganisms, high humidity, and UV light can increase the rate of DNA degradation directly or by contributing to other factors, e.g. high humidity allows for increased microbial growth. Overall, these factors result in reduced amplification success, either

due to the fragmented nature of the DNA or by modifying nucleotides which diminish polymerase efficiency.

Inhibition of PCR amplification occurs in three primary ways: interference with the removal of DNA from the cell by ineffective lysis, cellular components may sequester or degrade DNA, or by prohibiting the action of polymerase. Some inhibitors commonly encountered include calcium ions, humic acid, heavy metals, and urea (Wilson, 1997). Inhibitors can originate from within the cell or from environmental sources extracted with the DNA. Prior to DNA extraction, humidity at the site of deposition has also been shown to affect the presence of inhibitors in a sample. Humid conditions allow humic acid as well as other organics from soil to enter skeletal materials at higher rates (Burger et al., 1999). Large concentrations of inhibitors can limit amplification even from rich DNA sources.

Overcoming Difficulties in Analyzing Low Quality/Quantity DNA Samples

Optimizing Traditional PCR Techniques

Optimizing PCR reaction conditions can increase the amplification efficiency from degraded and low quantities of DNA sources. Increasing the primer annealing temperature reduces the amount of non-specific products formed by requiring more precise complementarity between the template strand and primer (Henegariu et al., 1997). Magnesium ion concentration is critical to successful amplification of DNA samples; determining the optimal amount enhances product formation because magnesium leads to increased non-specific target formation at high concentrations while small amounts reduce polymerase activity (Ely et al., 1998). Targeting smaller amplicons is also useful

for analyzing samples which contain fragmentary DNA sequences (Coble and Butler, 2005). Additional modifications, such as those described below, can also be used to enhance the amplification success of standard PCR protocols.

Nested PCR

Nested PCR can help overcome situations where limited quantities of DNA are available. Traditional PCR involves a single round of amplification with one primer set and up to 40 amplification cycles; however this single round may not be sufficient to produce amplification products. Increased numbers of cycles with a single primer set can lead to non-target product formation because the primers can anneal in locations of non-complementarity. Nested PCR utilizes two different primer sets to alleviate this problem. The initial set is used to amplify the target DNA for several amplification cycles, then an aliquot of the first reaction is added to a reaction with fresh reagents and a second primer set. This secondary set is also specific for the target amplicon but lies inside the initial primers, amplifying a smaller fragment of DNA. The use of a second primer pair can reduce non-target product formation by maintaining specificity for the target amplicon (Strom and Rechitsky, 1998). Semi-nesting involves the addition of only one new primer to the second round of PCR; this is advantageous because there is less reduction in amplicon size while product specificity is maintained.

Addition of Adjuvants

The addition of a variety of chemicals to amplification reactions has been attempted in order to alleviate problems of inhibition, product yield, and specificity

(Henngariu, 1997). Additives can be effective in enhancing the amplification of difficult to analyze samples. Some of the adjuvants that have been tested in PCR include: dimethyl sulfoxide (DMSO), formamide, glycerol, bovine serum albumin (BSA), and non-ionic detergents (Cruickshank, 2004). DMSO and glycerol addition at 5 – 10% volume/volume have resulted in enhanced amplification efficiency and reduction in non-specific products; DMSO is believed to work by reducing formation of secondary structures (Henegariu et al., 1997). Formamide addition, at concentrations of 1 – 5% volume/volume, is particularly useful in enhancing amplification from templates with high guanine and cytosine content (Sarker et al., 1990). BSA addition at concentrations between 0.1 and 0.8 μ g/ μ L is effective in overcoming inhibition encountered in PCR, although the mechanism by which this occurs is not known (Wilson, 1997). It is critical to observe the effect of each chemical on individual primers and templates to determine the optimal adjuvant and concentration because poor selection can be detrimental to amplification (Henegariu et al., 1997).

Real-Time PCR Analysis

While traditional PCR amplicon detection involves measurement in the final phase or end point of a reaction, real-time PCR detects products during the early phases of the reaction as well. End-point detection is inconsistent due to differences in the rates at which reagents are depleted, leading to variability in the final amplicon concentration present in reactions with the same initial DNA quantity. Additionally, gel electrophoresis, used in traditional PCR detection, has limited resolution, and product concentrations cannot be easily quantified. Real-time PCR detects products before reagents become

limiting, and thus is more precise for quantifying DNA (Applied Biosystems). Because of the increased sensitivity, it can be applied to the analysis of low quantity DNA samples, and products can be quantified based on analysis of reference DNAs of known concentrations.

SYBR Green and TaqMan® Real-Time PCR

As detailed in the Applied Biosystems Reference Sheet, the two most commonly employed methods of real-time PCR analysis are by TaqMan® probes and SYBR Green chemistry. Detection of amplification products is based on changes in emitted fluorescence by the dye which is the result of laser excitation. The same equipment is used for detection in both methods.

The 5' exo-nuclease activity of the polymerase used in TaqMan® real-time PCR is essential to the function of this process. In the assay, a DNA probe is added to the reaction mix, which is complementary to the target DNA sequence and lies between the forward and reverse primers (Figure 2). The probe, like the primers, anneals to the template strand after denaturation of the DNA. At the 5' end of the TaqMan® probe, a reporter (high energy) dye is bound. The fluorescent emissions of the dye are suppressed when in the presence of the quencher, a low energy dye bound to the 3' end of the probe. The polymerase then begins to extend the primers; when the polymerase reaches the probe, the exo-nuclease activity begins to degrade it. The reporter and quencher dyes separate, and this leads to an increase in fluorescence from the reporter, which is detected by the instrument. The accumulation of fluorescence is proportional to the amount of product formed and is displayed as an amplification plot (Figure 3). The graph contains a

threshold line, which denotes the point when the fluorescent signal is more intense than the background fluorescence. The cycle threshold (Ct) is the cycle at which the fluorescent signal for a reaction reaches the threshold line (Applied Biosystems, 2006).

Figure 2: TaqMan® Real-Time PCR Primers and Probe Binding

Like traditional PCR, forward and reverse primers are used to amplify a target DNA sequence. Additionally, a TaqMan® probe, which is complementary to the sequence, binds internal to the primers. Dyes are bound at either end of the probe, a reporter at the 5' end and quencher at the 3' end. In the presence of the quencher, the fluorescent emission of the reporter is suppressed. When the probe is degraded and the quencher and reporter are separated, the reporter emits fluorescence.

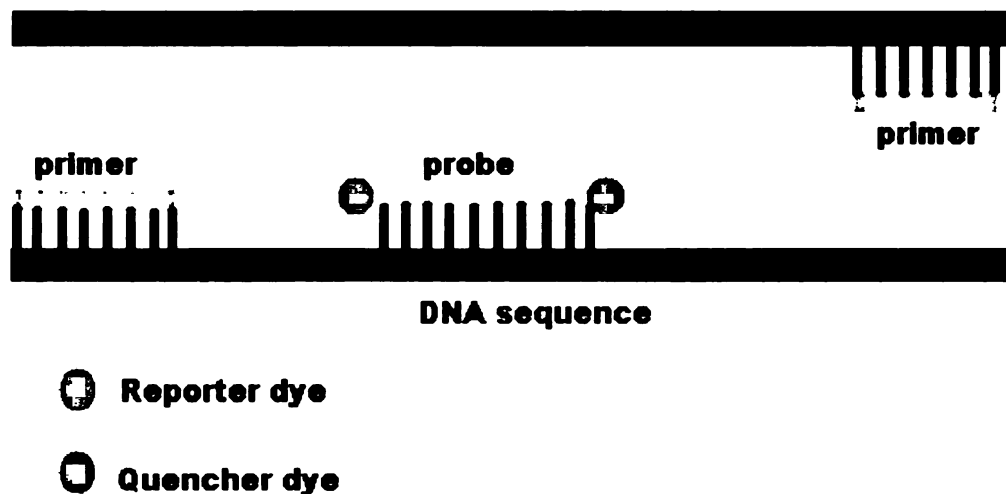
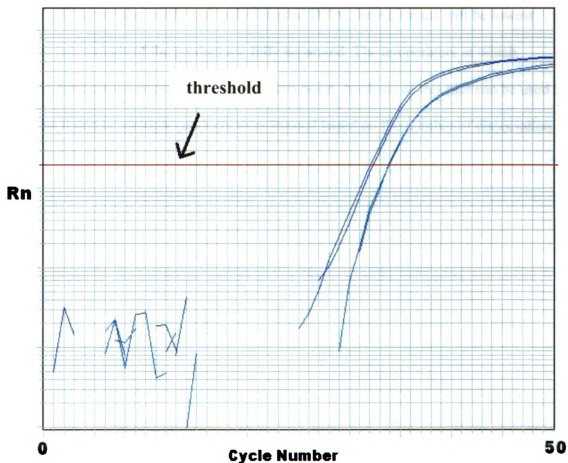


Figure 3: Real-Time PCR Data Output: An Example Amplification Plot

An amplification plot is a visual representation of what occurred in a reaction during amplification. The x-axis of the graph gives the cycle number, while the y-axis gives the R_n , which is the measure of the reporter signal. The red line is the threshold line or the point where the fluorescent emissions from the reporter is more intense than the background. Each blue curve represents a separate reaction; the cycle when this curve crosses threshold line is the C_t value for that reaction.

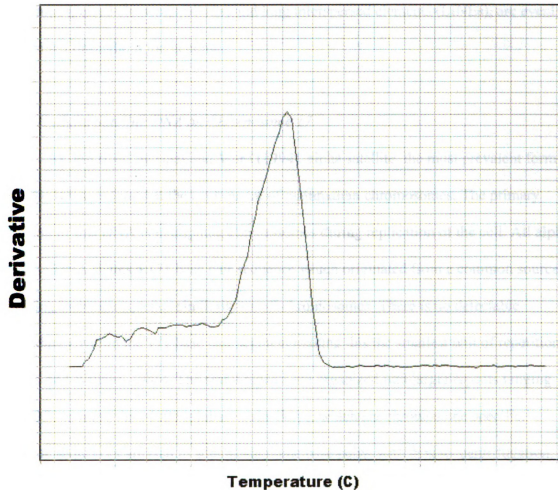


Unlike TaqMan® real-time PCR, there is no probe used in SYBR Green real-time PCR, but rather the SYBR Green dye is added to the reaction mix. When this dye binds to double stranded DNA, there is a rise in fluorescent intensity emitted. As the amount of product and therefore double stranded DNA increases, more fluorescence is detected by the instrument. This is displayed as an amplification plot as in TaqMan® real-time PCR

(Applied Biosystems, 2006). Additionally, a dissociation curve (Figure 4) can be produced from SYBR Green PCR, which gives information regarding the product or products formed. The data presented in the curve are collected in an additional heating-cooling-heating step, during which the instrument measures the fluorescence emitted. Since SYBR Green dye binds to double stranded DNA, the emissions will be lowest when the two strands of the product are fully separated. The temperature at which complete dissociation occurs is the dissociation point. Analysis of this plot can be used to determine if one or multiple products were formed based on the number of dissociation points.

Figure 4: SYBR Green Real-Time PCR Output: Example Dissociation Curve

The data presented are produced during a heating-cooling-heating step that follows the amplification cycles and yields information about the product(s) produced. The graph axes are temperature vs. derivative of the relative fluorescent units. The inflection point in the curve represents the point where the strands of the product dissociate.



Targeting Multicopy Loci for Sex Determination

In theory, loci which are present in a cell at multiple copies should allow for DNA analysis at lower quantities of total DNA. Primers which target repetitive sequences may enhance amplification success because multiple sites are available for annealing. Multiple primer binding sites are also advantageous in analysis of degraded DNAs as a result of their increased prevalence, and therefore opportunity for maintenance of the sites. The

two major classes of highly repetitive DNA found in humans include tandemly repeated DNA, such as satellite sequences and STRs, and interspersed elements. Satellite DNAs are typically repeated in long continuous stretches, while interspersed repeats, such as short interspersed elements (SINEs) and long interspersed elements (LINEs), are evenly distributed throughout the genome.

D17Z1, Alpha Satellite DNA on Chromosome 17

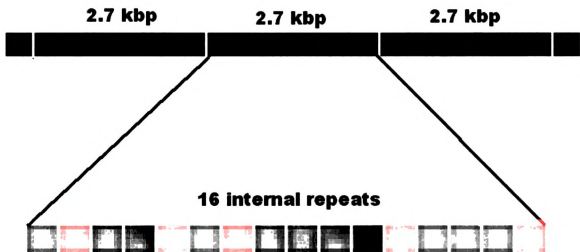
As described by Haaf and Ward (1994), alpha satellite, the most prevalent form of satellite DNA, is found at the centromere of mammalian chromosomes. The primary function of this DNA is in aligning chromosomes during replication of the cell. All alpha satellite DNA shares a 171 base pair sequence which originated from a common source, resulting in some sequence similarity on all chromosomes. There is no detectable redundancy within the 171 base pair segment making this the smallest unit of repetition (Waye and Willard, 1986). The small repeats are organized into larger repeats ranging from 250 to more than 4000 kilobase pairs (kbp), which are chromosome specific. Satellite sequences are rich in adenine-thymine base pairs; with density centrifugation, these sections segregate from other DNA sequences, forming a 'satellite' band separate from other DNAs because of its lower molecular weight (Haaf and Ward, 1994).

D17Z1, a repetitive sequence located on chromosome 17, is a member of the alpha satellite family. As described by Waye and Willard (1986), this repeat is approximately 2.7 kbp long and contains sixteen internal repeats of approximately 171 base pairs. The organization of the repeat is depicted in Figure 5; the arrangement of the internal repeats is represented by the smaller colored boxes. The internal sequences, when

compared directly, are sixty-six to ninety-four percent identical to each other; the boxes depicted in the same color represent those which have more sequence similarity. The larger 2.7 kbp repeats are maintained with higher integrity and have greater than ninety-nine percent similarity. Approximately 500 – 1000 copies of D17Z1 are present per chromosome 17, which represents between 1.5 to 3% of the chromosome's DNA.

Figure 5: Organization of D17Z1

The 2.7 kbp repeat represents the larger unit of repetition; internal to each of these are sixteen repeats of approximately 171 bp. The internal repeats share sequence similarity with all alpha satellite DNA. Internal repeats are represented by the colored squares; those which share the same color have more sequence similarity to each other than to repeats of other colors.



DYZ1, a Repetitive Locus on the Y Chromosome

Repetitive DNA is also prevalent on the Y chromosome. The DYZ1 locus, a tandemly arranged repeat, is located on the long arm of the Y chromosome. As reported by Rahmann et al. (2004), the repeat sequence is approximately 3.4 kilobase pairs in length and is present at 2000 – 4000 copies. Along with DYZ2, DYZ1 comprises

between fifty and seventy percent of the DNA on the Y chromosome. DYZ1, located in the non-recombining region, has no known function.

Alu Repetitive DNA

The most prevalent repeat in the human genome is the Alu family of SINES, which comprise approximately 10% of all human DNA. Alu sequences are believed to have originated from a 7SL RNA gene and replicate through retrotransposition (Batzer et al., 1995). Each repeat sequence is approximately 280 base pairs followed by a poly-A tail, which is variable in length. The biological function of Alu is unknown and movement of these repeats is associated with many human genetic diseases as a result of interruption of gene function. Differences in the consensus sequences have been used to break the larger Alu family into smaller related subfamilies which originated from a single source sequence (Price et al., 2006). The exact number of Alu subfamilies has yet to be determined.

Project Goal

The goal of this project was to develop an assay which could be used for sex determination of samples that are challenging based on standard forensic DNA analysis techniques. By targeting multicopy loci and small amplicons, samples which contain low quantity and quality DNA could potentially be amplified with more success. DNA from shed hairs, aged skeletal material, shed skin cells, or nails which have been analyzed with limited success by traditional nuclear DNA analysis could be analyzed with a multicopy assay. Additionally, skeletal remains of sub-adults and those containing fragmentary

skeletal remains, which are problematic using anthropological sex determination, can also be analyzed.

The Y chromosome locus, DYZ1, was targeted as a male identifier. Amplification of a positive control locus was necessary so that female samples and those samples which simply did not amplify could be differentiated. A locus that possessed similar copy number on the X chromosome was not identified, therefore an autosomal marker, D17Z1, was used as a positive control for the traditional PCR and SYBR Green real-time PCR assay, while Alu loci were amplified in TaqMan® real-time PCR. Targeting these multicopy loci should improve upon the sensitivity of existing methodologies allowing for information to be accessed, which would have been previously lost or unobtainable, providing a greater understanding of low quantity or degraded DNA sources.

Materials and Methods

DNA Samples Used to Develop an Assay for Sex Determination

Male and female control DNAs were used to develop an assay for sex determination. DNA previously extracted from skeletal remains from four different excavation sites was also examined (Table 1). The first set was from the 1959 excavation of Fort Michilimackinac, located in northern Michigan; these remains were approximately two hundred years old (Maxwell and Binford, 1961; Shunn, 2005; Thwaites, 1910). The second set was from remains interred in the Voegtly cemetery outside of Pittsburgh, Pennsylvania and were approximately one hundred and fifty years old (Misner, 2004; Mutolo, personal communication; Ubelaker et al., 2003). The third and fourth sets were from excavation sites located in Albania; three burials from a tumulus in Kamenica of approximately three thousand years old and an additional three burials from a site in Butrint, around two thousand years old, were analyzed (Murray, 2006 and personal communication) by TaqMan® real-time PCR. Mitochondrial analysis had been previously undertaken on these remains, and DNAs chosen for analysis had been successfully amplified; sex of the remains was assessed anthropologically. Skeletal material from a male individual who was deceased two weeks before discovery was also analyzed (Mutolo, personal communication).

Table 1: Skeletal Material Analyzed

The ethnicity and sex, which were estimated based on skeletal features, is given for the bone samples from each burial analyzed. The type of bone from which the DNAs were extracted are noted (MM – refers to burials from Fort Michilimackinac; VC – denotes burials from the Voegtly cemetery; A/ACDT – indicates burials from Albania).

Burial Number	Anthropological Sex	Ethnicity	Bone Type
MM			
10	male	Not Native American	femur
11	female	Not Native American	tibia
12	possibly female	Not Native American	femur
14	likely male	Not Native American	femur
16	male	European	femur
17	male	European	femur
18	possibly female	Not Native American	femur
19	subadult	Not Native American	femur
20	male	European	femur
21	male	European	femur
24	likely female	Not Native American	humerus
27	unknown	Not Native American	femur
29	likely male	Undetermined	tibia
34	subadult	Undetermined	femur
34B	unknown	Native American	femur
VC			
47	female	Swiss-German	femur
111	male	Swiss-German	femur
124	male	Swiss-German	femur
126	female	Swiss-German	femur
192	male	Swiss-German	femur
427	indeterminate	Swiss-German	femur
545	probably male	Swiss-German	femur
A/ACDT			
347	unknown	presumed European	petrous
358	likely male	presumed European	femur
391	subadult	presumed European	femur
CDT1	male	presumed European	femur
CDT2	male	presumed European	femur
CDT3	male	presumed European	femur

Hair shaft samples from two different studies were analyzed (Table 2); the DNA from these had been previously extracted (Graffy, 2003; Soltysiak, personal communication). During collection of the samples, volunteers filled out a questionnaire in which each participant was asked to state his or her sex. A 1 μ L sample of fresh blood, which was extracted as described in Barber (2005), was diluted and analyzed by TaqMan® real-time PCR.

Table 2: Hair Samples Analyzed

The sex declared and ethnicity given by each participant is recorded. Additionally the method of DNA extraction is given; standard organic indicates DNAs extracted according to the AFDIL protocol, while alkaline signifies DNAs extracted by the alkaline method described in Graffy and Foran (2005). (LG – refers to samples collected during the Graffy study (2003); SS – refers to DNAs collected during the Soltysiak study (personal communication))

Sample	Declared Sex	Ethnicity	Extraction Method
LG Hair			
8-2	female	African-American	alkaline
19-1	female	Caucasian	std. organic
19-2	female	Caucasian	alkaline
24-2	male	Caucasian	alkaline
27-1	male	Asian	std. organic
27-2	male	Asian	alkaline
28-1	female	Caucasian	std. organic
28-2	female	Caucasian	alkaline
SS Hair			
B101	female	Caucasian	std. organic
B102	female	Caucasian	std. organic
B105	female	Caucasian	std. organic
B106	female	Caucasian	std. organic
B107	male	Hispanic	std. organic
B111	male	Caucasian	std. organic
B143	male	Caucasian	std. organic
B146	male	Asian	std. organic
B152	male	Caucasian	std. organic
B155	female	Caucasian	std. organic
B157	male	Caucasian	std. organic
B163	female	Caucasian	std. organic
B170	female	Caucasian	std. organic
B173	female	Caucasian	std. organic

Development of the PCR-Based Sex Determination Assay

Steps Taken to Optimize Primers Targeting the DYZ1 Locus

Primers used for amplification of the Y chromosome locus, DYZ1, are listed in Table 3. New primer pairs were designed to eliminate non-target band formation from female control DNA; additionally, modifications were completed on two primer pairs, A-for forming A-for:mod, and D-rev forming D-rev:mod. A-for was modified by adding ten thymine bases to the 5' end of the primer in order to increase the size of the amplicon from 154 bp (A-for/A-rev) to 164 bp (A-for:mod/A-rev). Four nucleotides were added to the 3' end of D-rev to increase the specificity of the primer for the target sequence, and did not change the length of the amplicon. Primer concentrations were optimized to obtain the most favorable working concentration. All primers were tested using PCR master mixes: 1 μ L of Thermophilic DNA Polymerase Buffer (Promega), 1 μ L of 25 mM $MgCl_2$ (Promega), 1 μ L of 2 μ g/ μ L deoxynucleoside 5'-triphosphates (dNTPs), 1U Taq DNA Polymerase (Promega), 1 μ L of 20 ng/ μ L male control DNA, and sterile water, for a total volume of 8 μ L. One microliter each of forward and reverse primer was then added to reactions using concentrations of: 2000 μ M, 1000 μ M, 400 μ M, and 200 μ M. The following combinations of primers were tested with target amplicon sizes noted: A-for/A-rev (154 bp); A-for:mod/A-rev (164 bp); B-for/B-rev (129 bp); C-for/C-rev (118 bp); D-for/D-rev (102 bp); D-for/D-rev:mod (106 bp); A-for/D-rev (131 bp); D-for/A-rev (131 bp) (Figure 6).

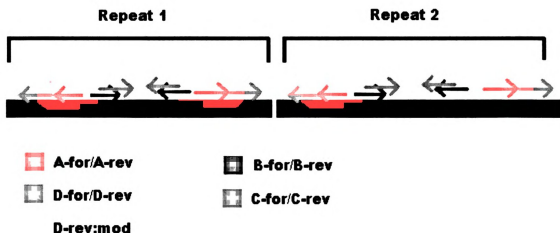
Table 3: Primers Used for Amplification of the DYZ1 Locus

Primer name and sequence are given. Primer set A was obtained from de la Torre et al. (2000); primer set D was obtained from Pfitzinger et al. (1993) (for = forward primer, rev = reverse primer, mod indicates primer derived from those with the same name; see text).

Primer Identifier	Sequence
A-for	5'-tccactttattccaggcctgtcc- 3'
A-rev	5' -ttgaatggaatgggaacgaatgg-3'
A-for:mod	5'-ttttttttttccactttattccaggcctgtcc- 3'
B-for	5'-acatcaattcctggcgatcc-3'
B-rev	5'-actgcaatggaaaggtctcg-3'
C-for	5'-tctctggcgattccattacat-3'
C-rev	5'-gcaatggaaaggtctcgaat-3'
D-for	5'-attacactacattccctcca-3'
D-rev	5'-agtgaattgtatgcagtagaagg-3'
D-rev:mod	5'-agtgaattgtatgcagtagaagg-3'

Figure 6: Primer Orientation for the DYZ1 Locus

Orientation of primer pairs used for amplification of the DYZ1 locus. The locus is a tandem repeat with two repeats shown; A-for/A-rev and D-for/D-rev are designed to amplify across repeats. (Not to scale.)



DNAs were amplified using the following PCR parameters: initial denaturation at 94°C for 5 minutes, followed by 35 – 40 cycles of 94°C for 30 seconds, primer annealing

at 60°C for 30 seconds, and primer extension at 72°C for 1 minute. The exception was primer pair D-for and D-rev for which annealing of 1 minute and extension of 30 seconds was used. In all reactions there was a final extension at 72°C for 5 minutes, after which samples were held at 4°C until placed in a -20°C freezer.

PCR products were electrophoresed on 2 – 4% agarose gels, followed by ethidium bromide staining and visualization under ultraviolet light. Primer dilutions that produced PCR products with the fewest non-specific targets (i.e., products produced when primers bind to template sequences that are not an exact match to primer sequences) and brightest bands were identified as the optimal concentration for subsequent reactions (Table 4).

Table 4: Optimal Primer Concentrations

Optimal primer concentrations for DYZ1 locus primers (optimal concentration = final concentration in the reaction)

Primer Identifier	Optimal Concentration
A-for	200 μ M
A-rev	200 μ M
A-for:mod	200 μ M
B-for	400 μ M
B-rev	400 μ M
C-for	400 μ M
C-rev	400 μ M
D-for	200 μ M
D-rev	200 μ M
D-rev:mod	200 μ M

Each DYZ1 primer set was tested in reactions containing female control DNAs. When bands of size similar to the target amplicon were seen, further optimization was performed, including modification of magnesium concentration, annealing temperature,

time and cycle number for annealing and extension steps, and DMSO or formamide addition.

Magnesium chloride concentrations were varied by adding between 0.5 – 2 μL of 25 mM MgCl_2 (in increments of 0.2 μL) to reactions. Based on findings (see Results), 2.5 mM MgCl_2 was the optimal concentration. Hot Master Taq Polymerase and 10 X Buffer (Eppendorf), which contains 25 mM MgCl_2 , was used in all subsequent optimizations.

Annealing temperature optimization was completed on a Mastercycler® gradient thermocycler (Eppendorf) for three primer pairs: A-for:mod/A-rev, C-for/C-rev, D-for/D-rev. This thermocycler creates a gradient of twelve temperatures between a given high and low. An annealing gradient from 52.6 – 65.2°C was used for primer pair D-for/D-rev and temperatures between 51.1 – 61.2°C were used to test A-for:mod/A-rev. Primer pair C-for/C-rev was tested using a gradient of temperatures between 57.6 – 67.7°C, with a modified annealing time of 45 seconds.

Variation of annealing time was conducted on primer pairs A-for/A-rev, D-for/A-rev, and D-for/D-rev. PCR parameters tested included annealing at 61°C for 5 – 20 seconds and extension for 20 – 30 seconds. Shortening of annealing and extension times had an adverse effect on product formation in reactions containing male control DNA; therefore, an annealing time of 30 seconds and extension of 45 seconds were used for subsequent reactions.

Addition of DMSO or formamide was tested with primer sets D-for/A-rev, D-for/D-rev, D-for/D-rev:mod. One, 5, and 10% DMSO or formamide were added to reactions with both male and female control DNAs.

DNA Sequencing of a High Molecular Weight Product from Female DNA

The DNA sequence of a high molecular amplicon formed from DYZ1 primer set D-for/A-rev with female controls DNAs, and occasionally with male controls, was determined. Reactions were prepared using 4 μ L of CEQ DTCS – Quick Start Kit (containing reaction buffer, dNTPs, labeled ddNTPs, and DNA polymerase (Beckman)), and 1 μ L each of forward or reverse primer and DNA template. The final volume was brought to 10 μ L with water. Sequencing parameters involved: DNA denaturation at 96°C for 20 seconds, annealing at 50°C for 20 seconds, and primer extension at 60°C for 4 minutes, which was repeated for 30 cycles.

Following amplification, a stop solution containing 1 μ L of 3 M NaOAc pH 5.2, 0.5 μ L glycogen, 0.2 μ L of 500 mM EDTA and 0.8 μ L of water was added to each reaction and mixed thoroughly. Thirty microliters of cold 95% ethanol was added and samples were vortexed, followed by centrifugation at 14,000 rpm for 15 minutes. The supernatant was removed without pellet disruption and 200 μ L of 70% ethanol was added and the samples were centrifuged at 14,000 rpm for 3 minutes. Again the supernatant was removed and the 70% ethanol wash was repeated, followed by ~ 30 minutes of vacuum drying. The pellet was resuspended in 40 μ L of Sample Loading Solution (Beckman) and allowed to resuspend for 1 – 5 minutes before it was vortexed and pipetted into the CEQ sample plate. Mineral oil was added to the top of each sample. The samples were analyzed on a Beckman CEQ 8000 Genetic Analyzer System, using the LFR 1 – 45 method which involved: capillary temperature at 50°C, denature at 90°C for 2 minutes, inject at 2.0kV for 15 seconds and separation at 4.2kV for 45 minutes. The forward and reverse sequences were aligned using BioEdit software (Hall, 1999), and Basic Local

Alignment Search Tool (BLAST) 2.0 web software (National Center for Biotechnology Information) was used to compare these sequences to human genome sequences.

DYZ1 Amplification with DNA from Bone and Hair

Limited amplification success was seen with only one round of cycling with DNA from hair and bone samples, therefore a second round of amplification was necessary. Nested or semi-nested PCR was carried out by transferring 1 μ L of product from the first round of PCR into a reaction containing fresh master mix. Different cycling combinations, including 25 – 45 cycles in the first round and 15 – 40 cycles in the second round were tested. Previous research with these samples had shown that the addition of BSA increased amplification success (Graffy, 2003; Shunn, 2006). One microliter of BSA was added in the initial round of amplification at 10 μ g/ μ L, 20 μ g/ μ L and 30 μ g/ μ L to determine an optimal concentration.

Primer Optimization for D17Z1, a Positive Control Locus

Primers designed for amplification of the positive control locus, D17Z1, are given in Table 5. The primers were diluted to determine the optimal concentration in the same manner as the DYZ1 locus primers; the optimal concentration was 200 μ M for both forward and reverse primers. Additional optimization steps were not necessary for these primers.

Table 5: Positive Control Primers

Primer pairs used for amplification of the D17Z1 locus. The primer name and sequence are given. (for = forward primer, rev = reverse primer)

Primer Identifier	Sequence
D17Z1-for	5'-gatcattgcactctttgaggag-3'
D17Z1-rev	5'-gtgtttctaaactgtacatcgc-3'

Multiplex Assay Development

Primers for D17Z1 and DYZ1 multiplexing included the standard D17Z1 primers and A-for/A-rev, D-for/A-rev and D-for/D-rev, using both single round and semi-nested PCR. Optimization of the reactions was completed by varying the number of cycles in the first and second round, examining combinations of cycles between 25 – 40 cycles. Additionally, the concentration of D17Z1 primers in the reactions was varied in order to establish the best conditions for equal amplicon formation using serially diluted male DNA.

Optimized PCR Assay Conditions

After optimization steps, the following PCR conditions were developed, which led to consistent identification of sex from control DNAs in multiplexed, semi-nested reactions. The master mix for both rounds of PCR contained 1 μ L Hot Master Taq Buffer (Eppendorf), 1 μ L dNTPs, 1U Hot Master Taq polymerase, 1 μ L each forward and reverse primer, and sterile water to a volume of 8 μ L. Primer pair D-for/A-rev was added in the first round at the optimized concentration; additionally, 1 μ L of BSA at 10 μ g/ μ L was incorporated in the mix. A second round of amplification was completed with D-for/D-rev:mod and contained 1 μ L of 10% formamide. Both rounds contained D17Z1-for/D17Z1-rev; these primers were added at 50 μ M in the first round and 100 μ M in the

second round. PCR parameters for the first round included an initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 45 seconds. This was followed by an additional round of amplification at an annealing temperature of 61°C for 40 cycles; all other conditions remained the same.

Real-Time PCR Assay Development

SYBR Green Real-Time PCR Assay

D-for/A-rev and D17Z1-for/D17Z1-rev were optimized for use in a SYBR Green real-time PCR assay. Combinations of forward and reverse primers at concentrations of 50 nM and 300 nM were tested in 15 µL reactions containing 7.5 µL of SYBR Green PCR Reaction Mix (Applied Biosystems), 1 µL of DNA and sterile water; 300 nM D-for, 50 nM A-rev, 50 nM D17Z1-for, and 500 nM D17Z1-rev were used in subsequent analysis. Reactions were run on an ABI 7900 HT Sequence Detection System (Applied Biosystems) under the following parameters: initial hold of 50°C for 2 minutes, followed by a hold at 94°C for 10 minutes—required for activation of AmpliTaq Gold polymerase (Applied Biosystems)—followed by 50 cycles of 95°C for 15 seconds, and extension at 60°C for 1 minute. Amplification of hair and bone samples required the addition of 1.5 µL of 0.67 µg/µL BSA.

TaqMan® Real-Time PCR Assay

A new primer set and probe were designed to target the DYZ1 locus for use in this assay by William R. Kiffmeyer, Applied Biosystems Field Applications Specialist,

because an acceptable probe was not identified by the Primer Express® software (Applied Biosystems). Additionally, a probe and primer for the Alu repeat designed by Nicklas and Buel (2005) were used as a positive control (Table 6). Optimization of DYZ1 primers was completed by adding combinations of forward and reverse primers at concentrations of 200 nM, 500 nM, and 900 nM, while the Alu primers were tested at concentrations of 50 nM, 500 nM and 900 nM. The optimal concentrations for multiplex reactions were 500 nM DYZ1-F, DYZ1-R, and Alu-F, while Alu-R was used at 900 nM; both probes were added to reactions at 250 nM. The Y-Probe was labeled with FAM reporter dye on the 5' end, while the Alu-Probe was labeled with Hex; both probes contained non-fluorescent quenchers, CMGBNFQ and DBH1, on the 3' end. Reactions contained 7.5 µL of TaqMan® Universal PCR Master Mix (Applied Biosystems), 1 µL of each primer and probe, and sterile water to 14 µL, to which 1 µL of DNA was added. DNAs were amplified on an ABI 7900HT using the same cycling parameters as the SYBR Green assay. Reactions containing DNA from hair and bone samples required the addition of 0.5 µL of BSA at 20 µg/µL.

Table 6: TaqMan® Primer and Probe Sequences

Primer/probe name and sequence are given. FAM and HEX are fluorescent markers on the 5' ends of the probe. CMGBNFQ and DBH1 are the quenchers on the 3' end of the probe, both of which are non-fluorescent.

Primer Identifier	Sequence
DYZ1-F	5'-ggcctgtccattacactacattcc-3'
DYZ1-R	5'-gaattgaatggaatggaacga-3'
Alu-F	5'-gagatcgagaccatcccggctaaa-3'
Alu-R	5'-ctcagcctccaagtagctg-3'
Probe Identifier	Sequence
Y-Probe	5'-6FAM-attccaatccattcctt-CMGBNFQ-3'
Alu-Probe	5'-DHEX-gggcgtagtggcggg-DBH1-3'

Following reaction amplification, the sex of the samples analyzed was determined based on the following criteria. If the Ct value for the Alu product was 38.0 cycles or fewer and the Ct for the DYZ1 product was not determined or was greater than 41.0 cycles, the sample was considered female. Male sex criteria included an Alu product Ct value of 38.0 cycles or fewer and a DYZ1 product Ct of 41.0 cycles or fewer. Reactions which did not meet the criteria for either sex were considered undetermined.

Results

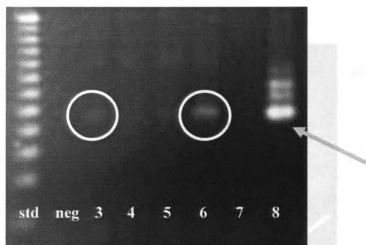
Development of PCR-based Assay

Optimization of Y Chromosome Primers

Y chromosome primer pairs were optimized by adding different concentrations of forward and reverse primers to PCR reactions (Table 3). Additional PCR optimizations were completed to eliminate undesirable amplification products seen with female DNA (circled in Figure 7). Variations in magnesium concentrations from 2.5 mM diminished amplification success (data not shown), therefore this concentration was used in successive reactions. Shortened annealing and extension times had an adverse effect on amplicon formation with male control DNA (data not shown). As a result, annealing times of 30 seconds and extension times of 45 seconds were used in subsequent reactions. When optimizations failed to eliminate these non-target bands new primer pairs were designed and optimized.

Figure 7: Amplification Products of D-for/D-rev

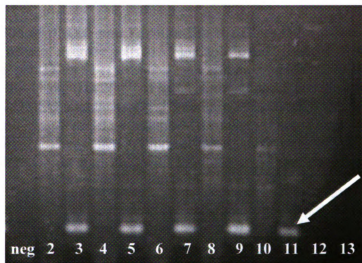
Four percent agarose gel of non-specific band formation with primer set D-for/D-rev. Lane 3: female control 1; lane 4: female control 2; lane 5: female control 3; lane 6: female control 4; lane 7: reagent blank; lane 8: male control. (std = 25 bp size standard; neg = negative control) The arrow is pointing to the target band in male DNA (102 bp), while the circles denote non-target bands of similar size in female DNA.



A gradient of annealing temperatures was tested using primer pairs A-for/mod/A-rev, C-for/C-rev, and D-for/D-rev, on male and female control DNAs. Reduced amounts of non-specific products were seen with increasing annealing temperatures (left to right in Figure 8); however temperatures above 63°C diminished production of the target amplicon (lane 11 compared to 9) up to 65.2°C, (lanes 12 and 13), when no amplification product was formed. Annealing temperatures of 61°C for D-for/D-rev and 60°C for C-for/C-rev and A-for-mod/A-rev were used in subsequent reactions.

Figure 8: Annealing Temperature Variation with D-for/D-rev

Four percent agarose gel showing amplification products of female (even lanes) and male (odd lanes) control DNAs. Lanes 2 – 3 = 55.1°C; lanes 4 – 5 = 56.2°C; lanes 6 – 7 = 58.4; lanes 8 – 9 = 61.1°C; lanes 10 – 11 = 63.6°C; lanes 12 – 13 = 65.2°C (neg = negative control). The arrow points to the target band. At temperatures of 63°C and above reduced target band formation resulted, along with decreased formation of non-target bands.

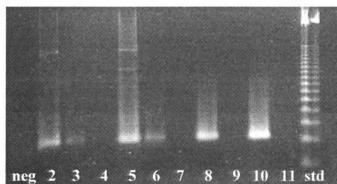


The use of DMSO or formamide to decrease non-specific primer binding was tested with primer pairs D-for/A-rev and D-for/D-rev. In reactions containing D-for/A-rev, addition of 1% DMSO increased production of a non-target amplicon rather than optimizing target amplicon formation (data not shown). Figure 9 contains the results of adding DMSO and formamide at different concentrations to reactions with male DNA. Concentrations of greater than 5% DMSO inhibited amplicon formation. Five percent formamide and ten percent DMSO eliminated product formation (lanes 4, 7, 9, and 11), while 5% DMSO noticeably reduced product formation (lanes 3 and 6). Although products were formed using 1% formamide or DMSO, the bands produced with

formamide were much brighter and only one product was present (lanes 8 and 10), unlike DMSO reactions (lanes 2 and 5) in which higher molecular weight products were formed. Therefore the addition of formamide at 1% was used in subsequent reactions to enhance specificity.

Figure 9: Addition of DMSO and Formamide at Different Concentrations

Three and a half percent agarose gel showing amplification products from male control DNA with primer pair D-for/D-rev. Lanes 2 – 4 and 8 – 9 contain 200 pg of DNA, while lanes 5 – 7 and 10 – 11 contain 20 ng of DNA. Lanes 2 and 5 contain 1% DMSO, lanes 3 and 6 contain 5% DMSO, and lanes 4 and 7 contain 10% DMSO. Lanes 8 and 10 contain 1% formamide and lanes 9 and 11 contain 5% formamide (neg = negative control; std = 50 bp size standard). Higher concentrations of DMSO and formamide reduced target amplicon formation. DNAs amplified with 1% formamide have the brightest bands.



Nested and Semi-nested PCR Reactions to Increase Amplicon Formation

DNA from aged bone and hair were not effectively amplified in a single round of PCR (40 cycles), thus nested and semi-nested PCR were attempted. Combinations of primers were tested on female control DNA to eliminate pairs that formed similar sized amplicons to the targets generated by the Y chromosome locus primers. A-for/D-rev (Figure 10) and C-for/C-rev (data not shown) were removed from further analysis as both pairs produced amplicons of similar size to the target. Lanes 7 – 10 of Figure 10 show the target amplicons formed from the primer pairs tested; amplification of female DNA with

A-for/D-rev (lane 3) produced a band similar in size to the amplicon produced with male DNA (lane 8) and thus was not considered in further analysis. Primer pair A-for/A-rev produced no visible products with female DNA (lane 2) while pairs D-for/D-rev (lane 4) and D-for/A-rev (lane 5) produced bands which were either weakly visible or outside of the target range, therefore these primer pairs were evaluated for further analysis. The higher molecular weight amplicon, of approximately 200 bp, seen in lanes 5 and 10 was only present when control DNAs were amplified, not with skeletal or hair samples. Table 7 contains the primer pairs used in further analysis. The frequency that amplicons were present in reactions which contained female control DNAs and DY1 primer sets was reduced when a semi-nested protocol was used.

Figure 10: Y Chromosome Locus, DY1, Primer Combinations

Four percent agarose gel showing amplification products from control DNAs with DY1 primers. Lanes 2 – 5 contain female control DNA and lanes 7 – 10 contain male control DNA. Lanes 2 and 7: A-for/A-rev; lanes 3 and 8: A-for/D-rev; lanes 4 and 9: D-for/D-rev; lanes 5 and 10: D-for/A-rev (neg = negative control; std = 50 bp size standard). The circle denotes the product formed from female DNA with primer pair A-for/D-rev, which is similar in size to the target amplicon produced with this pair (lane 8). The other pairs tested did not produce strong products of comparable size the target amplicons (lanes 7 – 10).

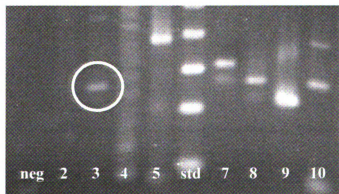


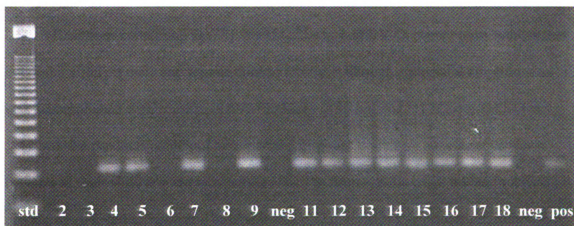
Table 7: Nested and Semi-nested Primer Pairs
Primer pairs used in nested and semi-nested PCR.

Nested Primers	
External Primers	Internal Primers
A-for/A-rev	D-for/D-rev
Semi-nested Primers	
External Primers	Internal Primers
D-for/A-rev	D-for/D-rev
D-for/A-rev	D-for/D-rev:mod

Experiments were completed to determine the optimal number of PCR cycles necessary for amplicon production from hair and bone samples. Reactions were shown to be quite sensitive to increases of as few as five cycles; equivalent increases could result in product formation in all samples analyzed. Figure 11 depicts samples initially amplified for 35 cycles with D-for/A-rev, then amplified for an additional 25 (lanes 2 – 10) or 30 cycles (lanes 11 – 20) with D-for/D-rev. Lanes 2, 3, 6, 8, 11, 12, 15 and 17 were female samples; all other samples were male. All female samples were negative after 25 semi-nested cycles; however all produced the target amplicon with an additional 5 amplification cycles.

Figure 11: Semi-nested PCR Reactions of Hair and Bone Samples

Four percent agarose gel showing amplification products after 25 (lanes 2 – 9) or 30 (lanes 10 – 20) cycles of semi-nested PCR with primers D-for/D-rev, which followed an initial 35 cycles of amplification with D-for/A-rev. Lanes 2, 3, 6, 8, 11, 12, 15, and 17 were females, while the other samples were males. Lanes 2 and 11: LG 8-1; lanes 3 and 12: LG 8-2; lanes 4 and 13: LG 24-1; lanes 5 and 14: LG 27-1; lanes 6 and 15: LG 19-1; lanes 7 and 16: MM B-20; lanes 8 and 17: MM B-12 (LG – DNA from hair extracted during the Graffy study (2003), MM – DNA from bone extracted during the Fort Michilimackinac study (Shunn, 2005), std = 50 bp size standard, neg = negative control, pos = positive control). The female samples LG 8-1 (lane 2), L.G. 8-2 (lane 3), LG 19-1 (lane 6), MM B-12 (lane 8) were negative after 25 cycles of semi-nested PCR, however these samples produced target bands when amplified for an additional 5 cycles (lanes 11, 12, 15 and 17). The negative control produced no amplicon after additional cycles. Male samples produced target amplicons under both cycling conditions.



DNA Sequencing of a High Molecular Weight Product

The amplicon formed from female control DNA with DYZ1 primer set D-for/A-rev was sequenced in order to identify a possible chromosomal source (Figure 10; lane 5). BLAST sequencing alignment indicated similarity between the amplicon sequence and regions of DNA on chromosomes 10 and 15. The primer sequences, however did not show similarity to DNA on either chromosome.

Optimization of Positive Control Primers, D17Z1 Locus

D17Z1-for and D17Z1-rev were optimized by adding different concentrations of forward and reverse primers to reactions with male and female control DNAs; optimal amounts were established at 200 nM for each primer. The primers required no further optimization because successful amplification was seen at all annealing temperatures tested and with DNA concentrations of approximately 2 fg.

Multiplex Assay Development

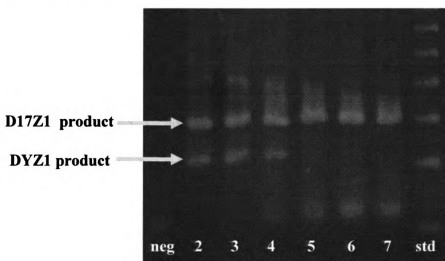
Reactions containing D17Z1-for/D17Z1-rev and DYZ1 primer pairs were tested in combination on male and female control DNAs. Although successful amplification was accomplished with primer pairs A-for/A-rev (154 bp) and D17Z1-for/D17Z1-rev (147 bp), the products could not be separated from each other by gel electrophoresis because of their similar size (data not shown). The ten thymine bases added to A-for, producing A-for:mod, appeared to hinder amplification with this primer set; although target amplicons were produced with 2 pg of male control DNA using A-for/A-rev, the modified forward A-for:mod and A-rev only produced products at concentrations of 200 pg of male DNA and higher.

Figure 12 shows male control DNA amplified with D-for/D-rev and D17Z1-for/D17Z1-rev simultaneously; reactions were more sensitive to amplification of D17Z1 than the Y chromosome locus. All samples analyzed contained male DNA, however amplification of the DYZ1 locus occurred only at concentrations of 200 pg and higher; at lower concentrations (lanes 5 – 7), no product was visible. These same reactions amplified with the D17Z1 locus primers (lane 7) amplified to 200 fg. In order to obtain

amplification at equal DNA concentrations, different cycling parameters and concentrations of primers were tested, which established optimal conditions (see Methods). Simultaneous amplification of both the D17Z1 locus and DYZ1 locus from male control DNA at concentrations of 2 pg was possible under the optimized conditions, while only the D17Z1 locus was amplified in female control samples.

Figure 12: Multiplex PCR of Serially Diluted Male Control DNA

Four percent agarose gel of PCR products from simultaneous amplification of D-for/D-rev and D17Z1-for/D17Z1-rev after 40 cycles. Lane 2: 20 ng of DNA; lane 3: 2 ng of DNA; lane 4: 200 pg of DNA; lane 5: 20 pg of DNA; lane 6: 2 pg of DNA; lane 7: 200 fg of DNA (neg = negative control; std = 50 bp size standard). No amplicons were present in reactions with D-for/D-rev:mod below 200 pg; however products were formed with D17Z1-for/D17Z1-rev to 200 fg of DNA.



Testing of Optimized PCR Assay on Hair and Bone Samples

DNA from hair was successfully amplified fifty-five percent of the time with the optimized PCR assay. Sixty percent of the samples that amplified once were amplified multiple times with replication; this limited the ability to confirm sex through multiple amplifications in many instances (Table 8). Results which did not conflict with previous

sex determinations by the assay were obtained sixty percent of the time. Analysis of DNA from hair samples showed some erratic PCR product formation. DNAs from female hair samples sometimes produced amplicons of similar size to the DY1 target while amplicons for D17Z1 were not visible (Figure 13, lane 7); this artifact was never present in control DNAs or male hair samples. Forty percent of the hair samples were inaccurately sexed by this analysis (i.e. the sex determined by the assay did not match the sex identified by the participant).

Figure 13: Optimized PCR Reactions of Hair Samples

Four percent agarose gel of DNA from hairs amplified with D-for/D-rev and D17Z1-for/D17Z1-rev in the second round of semi-nested PCR. Lane 1: LG 8-2; lane 2: LG 19-1; lane 3: LG 19-2; lane 4: LG 24-2; lane 5: LG 27-1; lane 6: LG 27-2; lane 7: LG 28-1; lane 8: male control DNA (LG – DNA extracted in Graffy study (2003), std = 50 bp size standard). Lanes 1, 2, 3, and 7 contain female DNA samples. All the male DNAs had both amplicons present. Sample 19-1, a female, has a strong D17Z1 sized amplicon, a very faint DY1 sized product is present. The circle indicates a female sample in which only a product of similar size to the DY1 target is present, no D17Z1-sized product is visible. This represents the unusual bands which were sometimes seen with analysis of DNA from female hairs. There are three bands in the control because these reactions were semi-nested and the product from the initial round of amplification is visible following the second round.

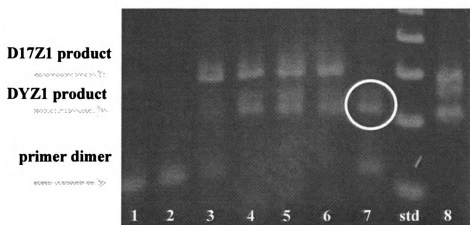


Table 8: Analysis of Reactions which Contained DNA from Hair

Results obtained from the hair samples which were analyzed in the optimized assay. The declared sex column lists the sex which was given by each participant. The female/male columns give the sex of the individual based on the assay; no amplification is the number of times the sample was analyzed and no amplification products were seen. The DYZ1 Sized Band Only column indicates the number of times that only a DYZ1 sized product was present and no D17Z1 amplicon was seen. (SS Hair – DNA extracted in the Soltysiak study (personal communication); LG Hair – DNA extracted in the Graffy study (2003))

Sample	Declared Sex	Female	Male	No Amplification	DYZ1 Sized Band Only
SS Hair					
B101	Female	1	0	2	
B102	Female	3	0	0	
B105	Female	1	0	2	
B106	Female	0	1	2	
B107	Male	1	1	1	
B111	Male	0	0	3	2
LG Hair					
8-2	Female	0	0	2	
19-1	Female	0	0	2	
19-2	Female	2	0	1	
24-2	Male	2	1	0	
27-1	Male	2	1	0	
27-2	Male	2	1	0	
28-1	Female	1	0	2	2

DNA from bone samples amplified fifty-six percent of the time through the optimized PCR assay, with 66.7% of those samples amplifying multiple times. Results from bone analysis had a higher reproducibility, with 82.4% of the samples yielding results which did not conflict with the sex assessed in previous reactions (Table 9). Unlike with hair samples, in analysis of bone samples there was only one instance where the DYZ1 sized product was present when there was no D17Z1 amplicon, this sample was estimated as male based on skeletal features. The percentage of inaccurate sex

assessment of bone samples cannot be directly stated as sex was estimated anthropologically and could be incorrect.

Table 9: Analysis of Reactions which Contained DNA from Bone

Results acquired from bone samples which were analyzed with the optimized assay. Anthropological sex indicates the sex which was estimated based on features of the skeletal remains. The female/male column is the sex determined based on the optimized molecular assay; no amplification is the number of times that no amplification products were present with that sample.

Sample	Anthropological Sex	Female	Male	No Amplification
Fort Michilimackinac (MM)				
B10	male	0	2	1
B11	female	1	2	0
B12	possibly female	1	0	1
B14	likely male	0	2	1
B16	male	0	0	1
B18	possibly female	0	2	1
B19	subadult	0	1	2
B20	male	1	0	0
B21	male	0	0	3
B24	likely female	0	0	3
B27	unknown	1	0	0
B29	likely male	0	1	2
B34	subadult	1	0	0
B34B	unknown	0	1	2
Voegtly Cemetery (VC)				
111	male	3	0	2
124	male	1	0	4
126	female	3	1	0
192	male	1	3	0
545	probably male	2	0	1
Fresh bone				
	male	0	1	0

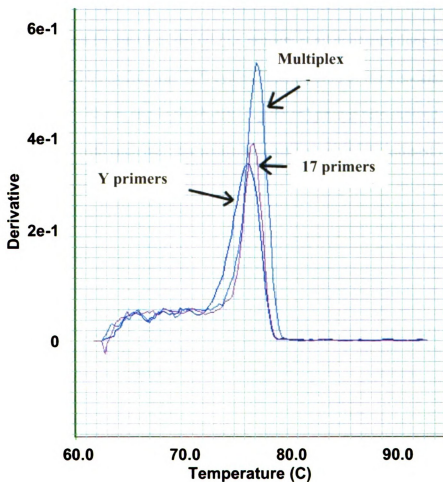
SYBR Green Real-Time PCR Assay Design and Development

Primer Optimization for SYBR Green Real-Time Assay

Primers, D-for/A-rev and D17Z1-for/D17Z1-rev, used in the standard PCR assay, were optimized for use in the SYBR Green real-time assay. Multiplex reaction optimizations, adding both DYZ1 and D17Z1 primers to a single reaction well, were attempted. With male control DNAs, the dissociation curves of the products produced from the two primer pairs could not be distinguished (Figure 14); i.e. only one peak was present in the dissociation curve even though two primer sets were present. Consequently reactions were not multiplexed, but rather primers were added to separate reaction wells. The optimal concentrations were found to be 300 nM D-for, 50 nM A-rev, 50 nM D17Z1-for, and 300 nM D17Z1-rev, which were used in all subsequent reactions.

Figure 14: Dissociation Curve of Male Control DNA Multiplexed Product

Dissociation curve from a reaction containing D17Z1-for/D17Z1-rev and D-for/A-rev overlain with curves from the reactions with each primer set added individually. Although two different products are formed from the primer sets, the melting points are too close to be reliably distinguished. The multiplex product has a dissociation curve which overlaps the individual curves and appears as though only one product was formed (Multiplex = reactions contains both D17Z1-for/D17Z1-rev and D-for/A-rev; Y primers = D-for/A-rev; 17 primers = D17Z1-for/D17Z1-rev).



Assay Optimization with Male and Female Control DNAs

Initial tests were conducted using serially diluted male and female control DNAs in duplicate reactions to determine whether the sexes could be differentiated from one

another. Male DNA was amplified at concentrations down to 2 fg, however at these concentrations allelic dropout was seen for both DYZ1 and D17Z1 in some replicate reactions. A trend observed in male controls was a large difference in the Ct value for the Y chromosome primers versus primers targeting chromosome 17, the cycle at which D-for/A-rev crossed the threshold was consistently lower than the Ct for D17Z1-for/D17Z1-rev (Figure 15). The average difference between Ct values, calculated by subtracting the Ct of the DYZ1 primers by the Ct of the D17Z1 primers at all concentrations, between 20 ng and 2 fg, of male DNA tested, was 5.99 cycles. Examination of the dissociation curves for the D17Z1-for/D17Z1-rev and D-for/A-rev (Figure 16) primer sets showed only one product being formed with each pair; the replicate reactions of serially diluted male DNA could be overlain

Figure 15: Amplification Plots of Male Control DNA

Male DNA had a consistent pattern of a lower Ct value for the DYZ1 amplicon than D17Z1 at all concentrations tested. The left curve presents duplicate reactions of each primer set, which contained 20 pg of DNA. A similar pattern of DYZ1 product having a lower Ct than the D17Z1 product was seen in diluted DNA. Dropout of reactions can be seen in the plot on the right. Although duplicate reactions were run for each primer set only one of the replicates successfully amplified with 2 fg concentrations of DNA.

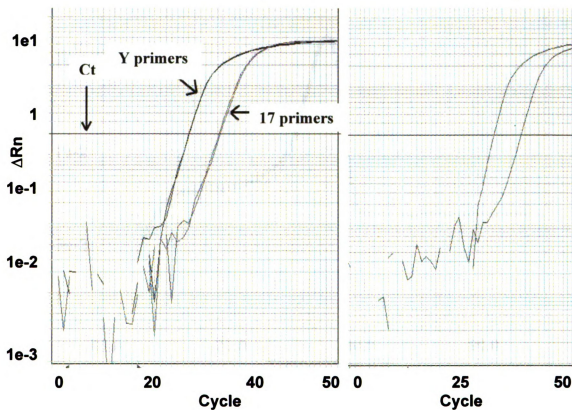
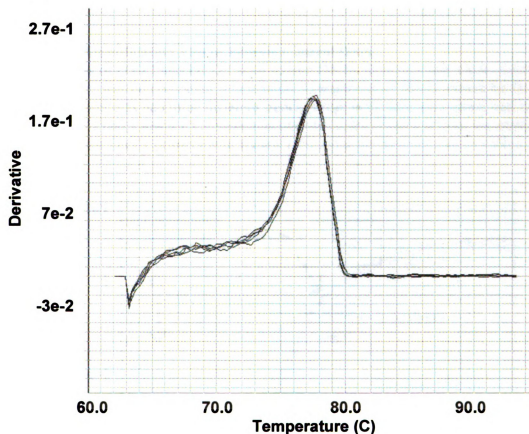


Figure 16: Dissociation Curves of Serially Diluted Male DNA Amplified with the DYZ1 Primers

The presence of only one peak in the graph, in which five separate reactions are overlaid, indicates that one product is formed with the D-for/A-rev primer pair.



Amplification products were formed with female DNA in the SYBR Green real-time assay; however there were some trends which distinguished male and female control samples. In particular, while the difference between the Ct values for DYZ1 and D17Z1 products were large in males, the Ct value for D17Z1 amplicons was often slightly lower or very close to that of the DYZ1 products with female DNAs (Figure 17); the average difference in Ct for all female controls and dilutions tested was 0.01. Comparison of the dissociation curves of the DYZ1 primers from male and female DNA showed that

different products were formed because the curves did not overlap one another (Figure 18); however the curves of the D17Z1 primers were indistinguishable, indicating a single product was formed (Figure 19), which was confirmed by gel electrophoresis (Figure 20).

Figure 17: Amplification Plot of Female Control DNA

The Ct values of DYZ1 and D17Z1 with female DNAs were typically close together; however there was no consistent relationship. Comparison of the Ct values for the DYZ1 and D17Z1 products had three different trends, either both were very close, had identical Ct's, or D17Z1 had a lower Ct. Unlike reaction replicates of male DNAs, the duplicate reactions of the DYZ1 primers with female DNAs did not have similar Ct values.

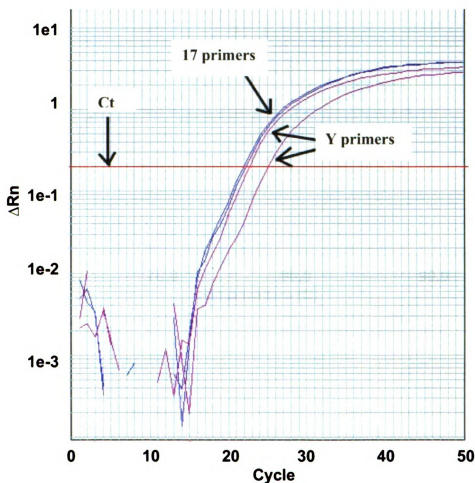


Figure 18: Dissociation Curve of Control DNAs Amplified using the DYZ1 Primers
Male and female control DNAs dissociation products for D-for/A-rev overlaid on one graph. The presence of distinct curves means different products were formed from the DNAs, leading to different melting points. Gel electrophoretic analysis of products showed that the amplicons were distinct in size (Figure 20).

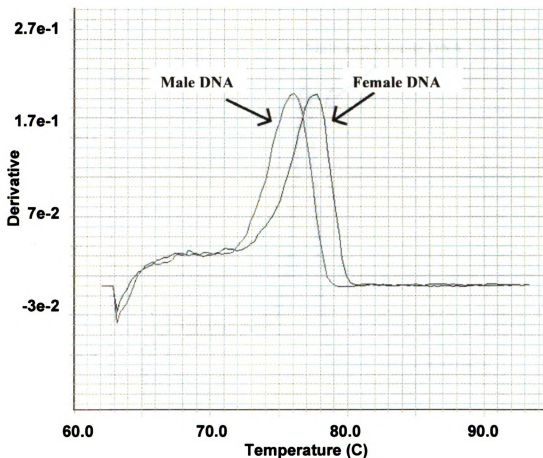
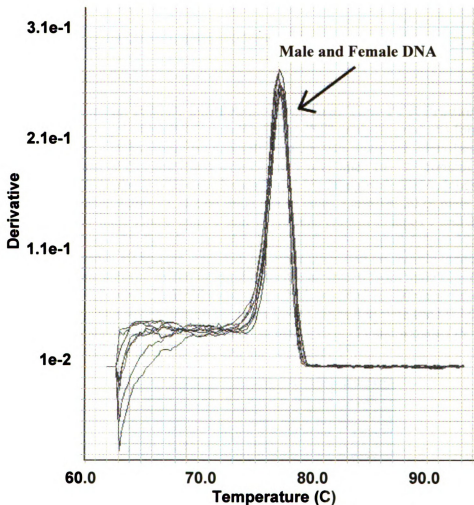


Figure 19: Dissociation Curve of Control DNAs Amplified Using the D17Z1 primers
Male and female dissociation curves for D17Z1-for/D17Z1-rev overlain on one graph. The curves peak at the same temperature indicating the same product was formed in both sexes, producing the same dissociation point. The amplicons from male and female DNAs were the same size when analyzed by gel electrophoresis (Figure 20).



Analysis of the products formed in SYBR Green real-time PCR by gel electrophoresis showed that different sized amplicons were formed from male and female DNA with the D-for/A-rev primer pair (Figure 20). Amplification of D-for/A-rev of male DNA produced a single product (upper half of gel), at approximately 130 bp; however,

amplification of female DNA produced multiple products at approximately 200 bp and 50 bp. All samples yielded the same sized product from D17Z1-for/D17Z1-rev (lower half of the gel). This corresponds with the data presented in the dissociation curves, which showed distinct melting points with DYZ1 amplicons and the same dissociation point for the D17Z1 primer products with male and female DNAs.

Figure 20: Agarose Gel of Male and Female DNA Real-Time PCR Products

Four percent agarose gel of SYBR Green Real-time PCR analysis products formed from analysis of controls. DNAs from four separate females was analyzed, identified as 1 – 4. Lanes 2 – 12 contain DNAs amplified with D-for/A-rev; lanes 14 – 19 contain DNAs amplified with D17Z1-for/D17Z1-rev. Lanes 2 and 13: 20 pg male DNA; lanes 3 and 14: 2 pg male DNA; lanes 4 and 15: 0.2 pg male DNA; lanes 5 and 16: 20 fg male DNA; lanes 6 and 17: 2 fg male DNA; lanes 8 and 19: female control 1; lanes 9 and 20: female control 2; lanes 10 and 21: female control 3; lane 11: female control 4; lane 12: 1:10 female control 4 (std = 50 bp size standard; all other lanes had no sample loaded). As can be seen in the upper section, different amplicons are produced from male (2 – 6) and female DNAs (8 – 12) with the DYZ1 primers, which resulted in different dissociation curves (Figure 18). The lower section contains DNAs amplified with the D17Z1 primers in which the same sized product was formed in male (13 – 17) and female (19 – 21), which resulted in an indistinct dissociation curve (Figure 19).



Overlaying the dissociation curves for the products formed from the DYZ1 and D17Z1 primers allowed for male and female DNAs to be differentiated. In reactions which contained male DNA, the DYZ1 product had a lower dissociation point than the D17Z1 amplicon (Figure 21), whereas with female DNAs where no distinct trend was present (Figures 22 and 23). The DYZ1 products shown in Figure 22 (female control 1) have two distinct melting points, indicating the formation of two products. In Figure 23 (female control 4) the D17Z1-for/D17Z1-rev primer product has a slightly lower dissociation point than that amplified by D-for/A-rev, which was opposite the trend seen in males.

Figure 21: Dissociation Curves for D-for/A-rev and D17Z1-for/D17Z1-rev: Male DNA

Dissociation curve of the products produced from amplification of male DNA with the DYZ1 primers and D17Z1 primers. The DYZ1 amplicon had a dissociation point that was slightly lower than the D17Z1 melting point. The same trend present at all male DNA concentrations tested, from 20 ng – 2 fg.

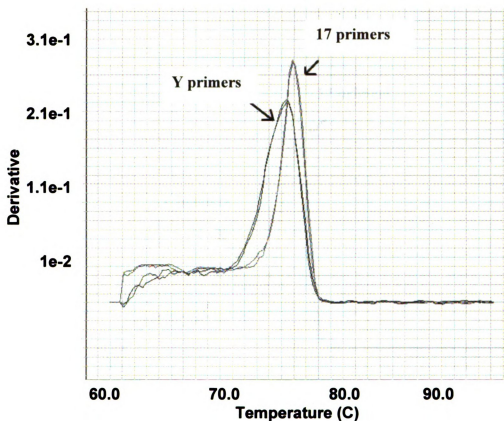


Figure 22: Dissociation Curves for D-for/A-rev and D17Z1-for/D17Z1-rev: Female Control 1

Dissociation curve of the products produced by amplifying female DNA with the D17Z1 and DYZ1 primers. In this sample, two products were formed with the DYZ1 primers (two distinct points in the pink curves). Unlike the trend seen the male dissociation curves (Figure 21), one amplicon had a melting point which was much lower, while the other had a very similar dissociation point to the D17Z1 product. There was no one single specific trend for females, as with male controls.

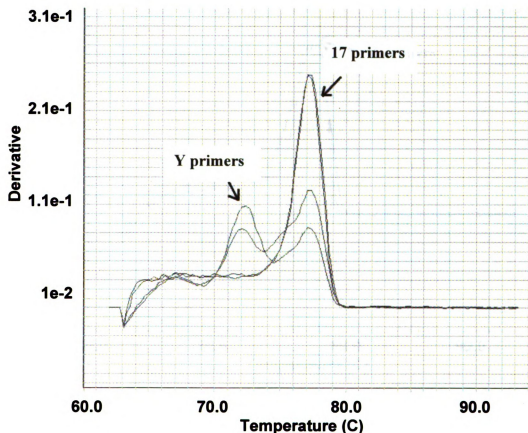
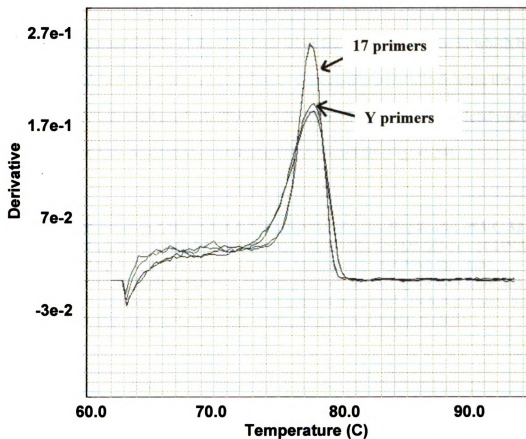


Figure 23: Dissociation Curves for D-for/A-rev and D17Z1-for/D17Z1-rev: Female Control 4

Dissociation curve of the products produced by amplifying female DNA with the D17Z1 and DYZ1 primers. In this sample, the dissociation curve for DYZ1 was directly under the curve for D17Z1.



Analysis of DNA from Hair and Bone Samples by SYBR Green Real-Time PCR

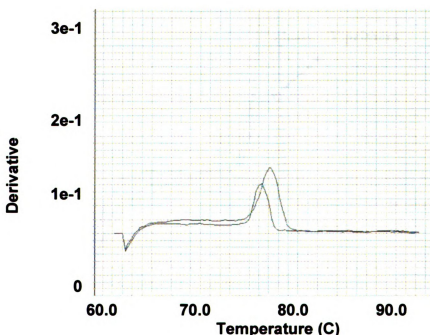
DNA from skeletal material did not effectively amplify without the addition of BSA; however BSA adversely affected amplification of the DYZ1 locus. Ct values of the products produced with the DYZ1 primers increased by approximately 2 cycles with its addition, particularly with male control DNA, however it did not appear to hinder amplification of the D17Z1 locus. This resulted in a decrease in the average difference in

Ct values between DYZ1 and D17Z1 from 5.99 without BSA addition to 2.50 with BSA when analyzing male controls, and from 0.01 to -0.43 with female DNA.

Successful amplification of the D17Z1 locus from DNA of bone or hair samples was limited; amplification of both replicate reactions occurred 38.5% of the time with DNA from bone and 22.2% of the time with DNA from hair. Reactions in which both replicates amplified successfully had different dissociation points, indicating the formation of two distinct products 29.6% of the time with DNA from skeletal material and 50% of the time in reactions containing DNA from hair (Figure 24).

Figure 24: Dissociation Curves for D17Z1-for/D17Z1-rev Replicates of VC 32

The dissociation curves for replicate reactions with D17Z1-for/D17Z1-rev of DNA from Voegtly Cemetery burial number 32. The two replicates do not have the same dissociation point, indicating that two distinct products are being formed from this one primer set. Different melting points were not present in control DNA replicates, but were seen approximately one-third of the time with DNA from skeletal material.



Consistent dissociation curve patterns, like those of control DNAs (Figures 21 – 23), were present in 28.6% of the bone samples and 18.2% of hair samples analyzed (Figure 25). A majority of the time the analyzed profiles had a pattern in which one replicate was consistent with a control male profile, while the other resembled patterns seen in female controls (Figure 26). Because most of the samples showed this type of pattern, no reliable determination of sex could be made from either bone or hair DNAs.

Figure 25: Dissociation Curves of DYZ1 and D17Z1 of Burial 34 from Fort Michilimackinac

The dissociation curves for the replicate reactions of the DYZ1 and D17Z1 primer sets have been overlain for this bone sample. This profile shows a pattern which was consistent with that seen in female control samples (Figure 23). Patterns which were similar to controls were rarely obtained when DNA from bone samples was analyzed.

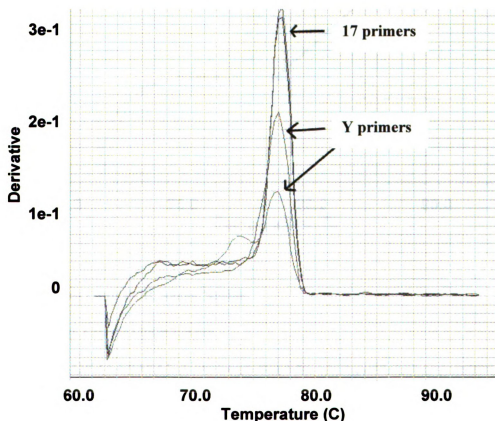
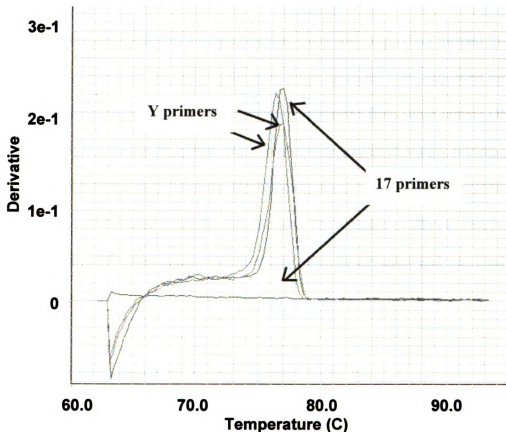


Figure 26: Dissociation Curves of DYZ1 and D17Z1 of Burial 10 from Fort Michilimackinac

The dissociation curves for the replicate reactions have been overlain, in which one D17Z1 replicate did not amplify. One replicate reaction with the D-for/A-rev primer set has a pattern consistent to that of male control DNA (Figure 21), in which the peak for the DYZ1 product overlaps the peak for the D17Z1 product on the left side of the curve. The other replicate has a pattern consistent with female controls (Figure 23), in which the DYZ1 product curve peaks under the curve for D17Z1. This inconsistent pattern made sex determination impossible.



TaqMan® Real-Time PCR Assay Design

TaqMan® Assay Primer Optimization

Primers DYZ1-F/DYZ1-R were optimized by adding different concentrations of forward and reverse primer; optimal quantities were determined to be 500 nM forward and either 900 nM or 500 nM reverse, at both concentrations the Ct value was the same.

Concentrations of DYZ1 primers were tested in reactions with different concentrations of the forward and reverse primers for the Alu loci. For multiplex reactions, the optimal concentration was established at 500 nM of DYZ1-F, DYZ1-R, and Alu-F, and 900 nM Alu-R; products for the two primer sets, DYZ1-F/R and Alu-F/R, had equivalent Ct values with 20 pg male control DNA at these amounts.

DYZ1-F/DYZ1-R had specificity for the Y chromosome when they and Alu-F/Alu-R primers were added to a single reaction; there was no product formed from female control DNA with the DYZ1 primer set when reactions were amplified for 40 cycles. With increased amplification, products were formed from some female DNAs analyzed at approximately 42 cycles. Male DNAs were amplified at concentrations down to 2 pg; after this point, dropout of the DYZ1 product was seen, although amplification products were still detected using the Alu primers. The addition of BSA to reactions did not affect the amplification success of either primer set in this assay and was used in subsequent reactions.

Analysis of a Fresh Blood Sample with TaqMan® Real-Time PCR

DNA from a 1 µl of fresh blood extracted in the Barber study (2005) was analyzed by TaqMan® real-time PCR. Serial dilutions between 1:1000 and 1: 10,000,000 of this male blood extraction were completed. The sample was correctly assessed to be male down to a one to one million dilution. After this point dropout of the DYZ1 locus began to appear, while the Alu loci still produced a detectable signal.

Analysis of Bone and Hair Samples with TaqMan® Real-Time PCR

DNA from bone samples amplified with limited success, 34% of the DNAs amplified using the TaqMan® primer set (Table 10). Replicate reactions were completed to test reproducibility, demonstrating that a majority of the bone samples which amplified once did so multiple times; only 8.3% of the samples analyzed amplified only once. Of the samples amplified, one sample produced a result which conflicted with previous analysis based on the established criteria (noted by the asterisk in Table 10; see Materials and Methods). However the amplification plot for this reaction contained a curve which did not reach the threshold line (Figure 27) indicating a product was formed with the DYZ1 primer set, and that this sample was male and in agreement with previous replicates.

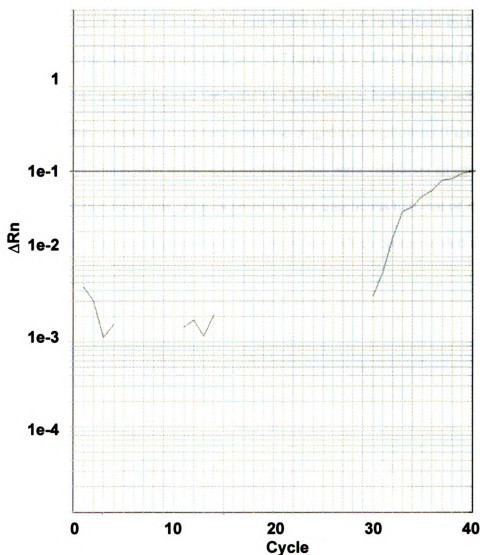
Table 10: The Results from TaqMan® Real-Time PCR Analysis of Bone Samples

Anthropological sex is the sex which was estimated based on anthropological features. The female/male column is the number of times a sample was determined to be that particular sex based on the assay criteria; not determinable signifies the number of times a sample was tested and not successfully amplified (MM – DNA extracted from remains in the Fort Michilimackinac study; VC – DNA extracted from remains at the Voegtly Cemetery; A – DNA extracted from remains from a tumulus in Kamenica, Albania; ACDT – DNA extracted from remains from Butrint, Albania; fresh bone – DNA extracted from remains two weeks of age). One replicate reaction for VC 192 (starred in the chart) amplified with the DYZ1 primers but did not cross the threshold; however a product was visible in the plot (Figure 27). As a result it was considered male.

Sample	Anthropological Sex	Female	Male	Not Determinable
Fort Michilimackinac (MM)				
B11	female		6	
B12	possibly female			4
B14	likely male		5	1
B16	male			2
B17	male		2	4
B19	subadult			2
B24	likely female		1	5
B27	unknown			4
B29	likely male	1		3
B34B	unknown			8
Voegtly Cemetery (VC)				
47	female			2
111	male			6
124	male	3		5
126	female			6
192	male		8*	
427	indeterminate	1		5
Albania (A/ACDT)				
347	unknown	3		1
358	likely male			4
391	subadult	4		2
CDT1	male			2
CDT2	male			2
CDT3	male			2
Fresh bone				
	male		2	

Figure 27: Amplification Plot of a Voegtly Cemetery Bone Sample

This plot is of the DYZ1 product for the Voegtly Cemetery burial 192. The curve did not reach the threshold line, however a product was present. Although this did not fit the set criterion, this result was interpreted as a male sample.



Eighty percent of the hair samples analyzed with the TaqMan® assay amplified (Table 11) and no conflicting results were produced through multiple amplifications from any of the samples. All of the DNA from hair samples analyzed were sexed as females based on the criteria set for sex determination (see Materials and Methods); the sex of the

hair samples analyzed were accurately assessed 62.5% of the time. However by observing the appearance of the amplification plots it was possible to accurately assess the sex of all the hair samples analyzed. In amplification plots of the DYZ1 product for male samples (Figure 28) curves were present which were not recognized by the software because each did not reach the threshold line. In Figure 29, a plot of female DNA, this same trend was not present; instead the baseline signal moved horizontally across the graph. The real-time reactions were then analyzed by gel electrophoresis (Figure 30) to determine if the products seen in the amplification plots were visible on an agarose gel. Analysis showed that male hair samples produced two amplicons like that of male controls, while in females only one product was present, which corresponded to the amplification plots.

Table 11: Results from TaqMan® Real-Time Analysis of DNA from Hair Samples

The declared sex column lists the sex which was given by each participant. The female (F1) and male (M1) columns denote the sex of the individual based on the assay criteria; not determinable (ND) indicates the number of times the sample was analyzed and not successfully amplified. The female (F2) and male (M2) columns indicate the sex assessed after observation of the amplification plots. Sex determination by this method accurately determined the sex of all DNAs which amplified (SS Hair – DNA extracted in the Soltysiak study (personal communication); LG Hair – DNA extracted in the Graffy study (2003)).

Sample	Declared Sex	F1	M1	ND	F2	M2
SS Hair						
B143	Male	2				2
B146	Male	1		1		1
B152	Male	2				2
B155	Female	2			2	
B157	Male	1		1		1
B163	Female	2			2	
B170	Female	2			2	
B173	Female	2			2	
LG Hair						
19-2	Female	2			2	
28-2	Female			2		

Figure 28: Amplification Plot of Male Hair Sample

The DYZ1 products formed from replicate reactions of sample B143 from Soltysiak hair extractions. Curves were present in each replicate that did not reach the threshold set for the assay. This trend was present in all male hair samples analyzed.

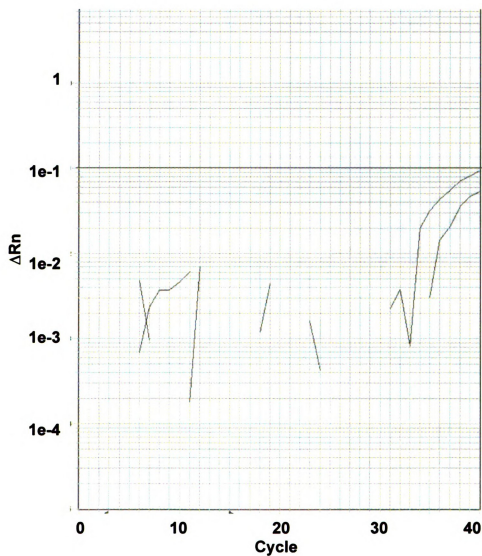


Figure 29: Amplification Plot of Female Hair Sample

Replicate plots of the DYZ1 product from sample B163 from the Soltysiak hair extractions. Only the background fluorescent signal was present. All reactions which contained DNA from female hair samples had a similar trend.

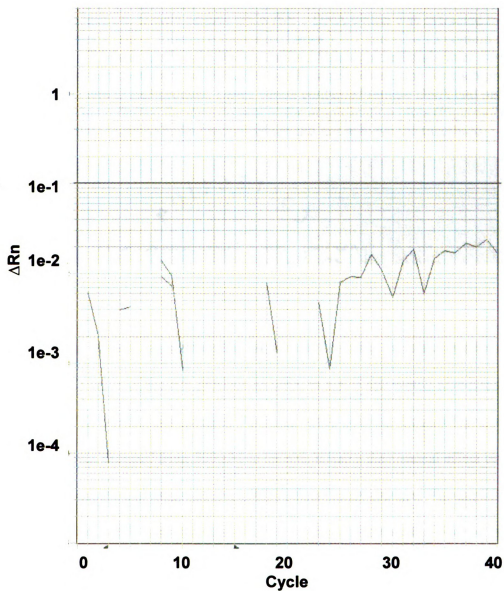
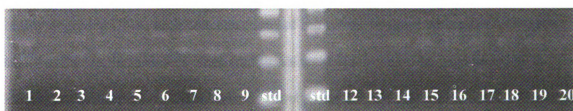


Figure 30: Agarose Gel of TaqMan® Real-Time PCR Products

Four percent agarose gel of DNA from hair samples and controls. All lanes contain reactions amplified with DYZ1-F/DYZ1-R and Alu-F/Alu-R primer sets. Lanes 1 – 7 and 12 – 13 contain male DNA, all others are females. Lane 1: male control DNA; lanes 2 – 3: B143 (SS); lanes 4 – 5: B146 (SS); lanes 6 – 7: B152 (SS); lanes 8 – 9: B155 (SS); lanes 12 – 13: B157 (SS); lanes 14 – 15: B163 (SS); lanes 16 – 17: B170 (SS); lanes 18 – 19: B173 (SS); lane 20: female control DNA (SS – DNA which was extracted in the Soltysiak hair study; std = 50bp size standard). Lanes 1 – 7 and 12 – 13 contain male DNA samples; all others are female DNA samples. Although the bands were very faint, in those lanes which contain male DNA two bands were present, one for each product; the Alu product and DYZ1 product. Lanes which contained female DNA had only one band present. This reflected the data present in the amplification plots (Figures 28 and 29), in which male samples had a product which did not achieve the threshold.



Previous primer optimization had shown that lowering the concentration of Alu-F increased the Ct value of the Alu product. In order to test if this would alleviate the problem of DYZ1 product not reaching threshold while Alu did, the Alu-F primer concentration was dropped from 500 nM to 50 nM. It had a detrimental effect on amplification success and assay accuracy; DNA from bones successfully amplified only 15.6% of the time, while amplification of DNA from hair dropped to 50% success, therefore subsequent reactions were run at the previously optimized concentrations.

Discussion

Analysis of biological materials in the crime laboratory involves amplification of STR loci to generate a DNA profile; however there are difficulties when attempting to analyze forensic samples containing low quality or quantities of DNA through either traditional or real-time PCR techniques. Often, these challenges can be overcome by protocol modifications although nuclear analysis of some materials may never be possible because of the inherent problems in amplifying those with little or no DNA, highly degraded DNA, high concentrations of inhibitors, or those where DNAs are not easily extracted. In instances when traditional techniques cannot be used to establish identity, determining any characteristics about the source the material is useful. Although establishing the sex cannot be used for identification it can assist in excluding possible origins.

Amplification of DNA from hair shafts and aged skeletal material may be made easier by targeting multicopy loci because multiple sites are available for primer binding and extension; however, there are difficulties in using these loci for analysis. Repetitive DNAs are generally present at high frequencies throughout the genome; they operate outside of the cellular controls that maintain the integrity of genome sequences. Some repeats, such as STRs, are produced as a result of slippage in the template strand by the replicating polymerase and are maintained within the same cellular location, while others replicate independently of the normal cellular machinery and insert into alternative locations. As a result, it is difficult to identify highly repetitive DNAs that are unique to a specific chromosome. The Y chromosome in particular has experienced dramatic changes in size over evolutionary time due to the loss of genes that were not important in male

sexual determination; portions of repeat sequences on the Y chromosome may have migrated with these genes. Movement of repeats can be problematic for developing a sex determination assay utilizing repetitive DNA; if any portion of the Y chromosome specific repeat is present on an autosome or the X chromosome, amplification may result from female DNAs. Migration of repeat sequences to alternative locations likely occurs in limited numbers, resulting in lower concentrations than are present at the repeat source. Over time mutations can change the sequences present at alternative and the original sites allowing differences to arise. Primers which target a Y chromosome repeat however may be able to lie down at other chromosomal sites if enough sequence similarity is present. When a large number of amplification cycles are used amplicons could result from another chromosomal sources.

Traditional PCR Analysis

Difficulty was encountered in developing a working sex determination assay utilizing traditional PCR techniques. The protocol needed to be stringent enough to not produce male-specific amplicons from female control DNAs, while allowing amplification of low quantity and quality DNAs, such as those originating from hair shafts and skeletal material. Because of the low concentrations of DNA in these samples, many amplification cycles were required; however, increases in cycles often led to product formation from female controls of similar size to the target. Optimization of PCR protocols for the male specific locus were attempted to balance these two factors.

Different primer sets for DYZ1 were designed in order to establish male-specific sites for primer binding. Primers were tested in alternative combinations to eliminate

pairs which were producing problematic non-target amplicons. Female DNAs amplified with primers A-for/D-rev consistently produced a product of similar size to the male target amplicon (Figure 10). It is possible that the sequence for these primers lies within a portion of the repeat which had migrated to another chromosome, since the amplicons produced were similar in size to the target. The higher molecular weight non-target amplicon was sequenced and compared to sequences generated in the Human Genome Project indicating that it originated from either chromosome 10 or 15. Although there was similarity in the intervening amplicon sequence, the primer sequences did not match the sequence of these chromosomes indicating that both were not annealing to their exact complement.

Occasionally other DYZ1 primer sets produced amplicons similar in size to the target. Determining the optimal annealing temperature (Figure 8) and addition of formamide (Figure 9) were often effective in eliminating product formation from female DNAs with DYZ1 primers. Although DMSO addition had been suggested to reduce non-target primer binding (Henegariu et al., 1997), its addition adversely affected amplicon formation with DYZ1 primer sets (Figure 9). Because the effects of these chemicals can vary it is recommended to test the addition of each chemical on primers and template DNAs. Reducing the annealing and extension times eliminated amplicon formation in all DNA samples tested. Reduced annealing times most likely diminished the amount of primer annealing which occurred, therefore reducing the amount of product.

Increasing the number of amplification cycles raised the amount of non-target amplicon formed from female DNAs, however a larger number of cycles was necessary to amplify DNAs from hair and bone samples. Extension of a primer does not require that

the entire primer be bound to the template DNA because the polymerase extends the primer from the 3' end. Binding of only a few bases allows the primer to be extended, typically with reduced efficiency, and the template DNA is amplified. Since the primer is incorporated into the newly amplified DNA, sites of exact complementarity between the primer and amplicon can accumulate; these non-target amplicons are then more efficiently amplified. Increasing the number of amplification cycles can therefore magnify this effect. Nesting and semi-nesting the PCR reactions helped to reduce this artifact, but did not totally eliminate non-target band formations. Variations in the number of cycles used in the second round demonstrated that increases of 5 cycles could alter the assessment of sex from hair and bone DNAs (Figure 11). The DNAs which did not produce the DYZ1 amplicon after 25 cycles yielded a visible product after 5 additional cycles. Because the negative control remained negative under both amplification conditions, this most likely resulted from non-target primer binding or the presence of the DYZ1 repeat on other chromosomes.

Addition of DYZ1 and D17Z1 primers in combination eliminated formation of the DYZ1 sized amplicon from female control DNAs (data not shown). Multiplexing may be beneficial because it supplies the polymerase with an alternate target for amplification and may reduce amplification from sites where the primers bound with less specificity. The D17Z1 primer binding sites are present in both male and female samples, while it is unknown whether females possess an exact complement to the DYZ1 primers, although none were found in a BLAST search of the human genome. In combination, the primers which bind more efficiently to the template DNA will be amplified preferentially. Multiplexing likely eliminated formation of the DYZ1 sized amplicon

because the D17Z1 primers out competed the DYZ1 primers for reagents due to their enhanced specificity for the template. Although this was beneficial for amplification of female DNAs, optimization of the protocol was necessary for reactions containing male DNA.

Even though the DYZ1 repeat is more prevalent than D17Z1, reactions that contained male DNA of the same concentration amplified the D17Z1 locus more successfully in separate (data not shown) and multiplex (Figure 12) reactions. It is possible that this results from the organization of the repetitive sequence (Figure 5). The D17Z1 locus, unlike DYZ1, contains sixteen repeats which are internal to the larger repeat. The primers designed to target D17Z1 lay within one of the internal repeats. Since the internal repeats share a similar sequence, it is possible that the primers annealed to multiple sites; effectively making the larger repeat more prevalent. In the multiplex reaction, the primer sets compete for the PCR reagents. If the D17Z1 primers are able to bind in more locations than the DYZ1 primers, they can out compete those primers for reagents, resulting in more D17Z1 product. Lowering the concentration of the D17Z1 primers added to the multiplex reactions allowed both loci to be amplified down to equivalent DNA concentrations.

Amplification of female control DNAs with D-for/A-rev produced an amplicon of approximately 200 bp; larger than the male target of 131 bp (Figure 10; lane 3). In rare instances this product was present in amplified male DNAs in addition to the target product. The absence of the higher molecular weight product in some male reactions indicates that the primers had higher specificity for the target amplicon, but the primer sequence had some similarity to the template at an additional site located either on the X

chromosome or an autosome. In female DNAs this product was amplified perhaps because of the high concentrations of primer available for binding. The higher molecular weight band was never present in shed hair or bone samples, possibly because of the limited quantities of high molecular weight DNA present in the samples.

Semi-nested PCR allowed approximately half of the hair and bone sample DNAs to amplify at least once. However, many of those that amplified multiple times yielded conflicting sex determinations. Irreproducibility in amplification of sample DNAs may have resulted from stochastic effects. On controls DNAs, the optimized multiplex assay demonstrated sensitivity down to 2 pg of DNA, which is less DNA than is present in a single cell. DNA sources, such as hair shafts and aged skeletal materials contain little quality nuclear DNA and are difficult to amplify by any PCR method. The low concentration of target DNA available affects the ability to successfully amplify because it is possible that the primer binding sites are not available in all replicate reactions. Since there are so few sites present, randomly sampling from the extracted DNA could result in replicates receiving a lot, a few or none at all. This could lead to inconsistencies in amplification success and as a result, the use of the optimized PCR methods for sex assessment of DNAs from samples such as these would cause inaccuracies.

SYBR Green Real-Time PCR Analysis

Real-time PCR, unlike the optimized PCR analysis, requires fewer amplification cycles in order to detect product formation. Sex determination of male and female control DNAs with SYBR Green real-time PCR showed promise for a viable assay. Although amplification products were formed with the DYZ1 primers when female DNA was

amplified, there were noticeable differences in amplification plots for male and female DNAs (Figures 16 and 17). The DYZ1 locus had a lower Ct values than D17Z1 when amplifying the same concentration of male DNA, however female DNAs often showed the reverse trend. Overlaying the dissociation curves demonstrated that the melting points for male (Figure 21) and female (Figures 22 and 23) products were distinct. Gel electrophoresis of the SYBR Green real-time products (Figure 20), showed that amplicons formed from female DNA with the DYZ1 primers, a product of approximately 200 bp, similar to the higher molecular weight product seen previously in the optimized PCR assay, and a primer dimer product. Differences in the products formed allowed male and female controls to be distinguished from one another. Addition of BSA to the reactions had an adverse effect on amplification of DNAs with the DYZ1 primer set, while no effect was seen in D17Z1 amplification. It is possible that BSA interacted with the DYZ1 primers inhibiting amplification success from this locus and not the other, which is why it is recommended to observe the effects of adjuvant addition with each primer set.

Analysis of DNA from hair and bone did not follow the trends present in the controls. Instead duplicate reactions for these samples often conflicted with one another (Figure 26), one replicate had trends which appeared male while the other did not. This is likely due to the quality and amount of target DNA present in these samples. Although the same volume of DNA was loaded into the reaction replicates, it is possible that the target DNA was available for primer binding in one and not the other, which could have resulted in inconsistency.

TaqMan® Real-Time PCR Analysis

Analysis using TaqMan® real-time PCR appeared to have the most potential for sex assessment. In male control DNAs both products, Alu and DYZ1, were formed, while female controls amplified only the Alu loci, with forty amplification cycles. At approximately 42 cycles, the DYZ1 amplicon began to reach the threshold with female controls; however dropout of the DYZ1 product was seen in male control DNAs, at concentrations of 20 and 2 fg, before this point. Because of this it was necessary to set the assay criteria (see Materials and Methods) at a lower cycle number to accurately assess the sex of the controls.

While optimized PCR or SYBR Green real-time analysis produced inconsistent sex assessments of hair and bone samples, TaqMan® real-time PCR produced only one result which conflicted with prior sex assessments by the assay—burial 192 from the Voegtly cemetery. Observation of the amplification plot (Figure 27) showed that a DYZ1 product was formed that did not reach the threshold indicating the sample was male as previous reactions had indicated. All hair samples analyzed were initially assessed as females based on the assay criteria. In each instance, the DYZ1 product for DNAs from male hair samples did not reach the threshold line (Figure 28), compared to female samples which moved across the baseline (Figure 29). Gel electrophoresis of the TaqMan® real-time reactions containing hair DNA confirmed that the DYZ1 target amplicon was present in male samples (Figure 30). Observation of the amplification plot allowed for accurate sex assessment of all the hair samples analyzed, unlike the other assays.

It is likely that the reduced amplification success of the DYZ1 amplicon compared to Alu was the result of the differences in the organization of the repeats. The continuous arrangement of the DYZ1 repeat could possibly make it more susceptible to stochastic effects. While both repeats are present in approximately the same number, DYZ1 is arranged in a continuous manner, while Alu is distributed throughout the genome. In samples such as these, where the DNA quantity is low, it is possible that none of the DYZ1 target is loaded into the reaction because all primer binding sites are in one stretch of DNA. Alu binding sites, present on multiple chromosomes are less susceptible to this effect. This could also explain why the Alu locus amplified in male controls which have higher quality DNAs, when DYZ1 did not amplify. Another possible reason for the increased amplification success of Alu is the fact that this amplicon was of smaller size than the DYZ1 amplicon. Although there were many samples which did not amplify with this assay, this is likely due to the condition of the DNA in the hair and bone. Overall the TaqMan® real-time PCR assay had enhanced success compared to SYBR Green real-time and optimized PCR assays.

Future Research

There is still work which can be done to further develop the TaqMan® real-time PCR assay and modify it so perhaps more samples will successfully amplify. Design of new probes, with alternative fluorescent markers, may help to alleviate the high background noise which was present. It was necessary to set the threshold line high for this assay because of the intensity of the background signal in the negative and female controls. Setting this threshold at a lower point may have allowed for those DNAs which

did not reach threshold, like the male hairs, to be accurately sexed without observation of the amplification plots. Increasing the number of amplification cycles would not alleviate this problem because reduced assay accuracy results; female controls begin to form products with the DYZ1 primer set at approximately 42 amplification cycles (data not shown).

Optimization of the primer concentrations for both primer sets may eliminate artifacts where the Alu loci product attains the threshold when the DYZ1 primer product does not, like was necessary for D17Z1 and DYZ1 in the optimized PCR assay. It may also be useful to design a D17Z1 primer and probe set for use as a positive control. Since the organization of this repeat is similar to DYZ1 it may relieve the problems of unequal amplification success at equivalent concentrations. Designing primer sets which amplify targets of equal size may also increase the amplification success. It might be useful to include a second probe with an additional fluorescent marker into the reaction which is specific to the DYZ1 target and internal to the primers. In addition to the initial probe it would require another level of specificity and possibly eliminate the signal seen in female controls after 42 amplification cycles. The absence of both fluorescent signals would indicate female DNA. Formamide addition to the real-time PCR reactions may also be helpful in enhancing specificity like it was in the optimized PCR assay.

Overall, high copy loci have proven beneficial for achieving amplification from samples which had not previously amplified with nuclear primers. Biological samples which contain very low quantities of nuclear DNA that cannot be analyzed by typical forensic DNA analysis techniques show promise for sex determination by TaqMan® real-time PCR.

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