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Infrared Fluorescent Detection of PCR Amplified Gender Identifying Alleles*

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ABSTRACT: An automated DNA sequencer utilizing high sensitivity infrared (IR) fluorescence technology together with Polymerase Chain Reaction (PCR) methodology was used to detect several sex differentiating loci on the X and Y chromosomes from various samples often encountered in forensic case work. Amplifications of the X-Y homologous amelogenin gene, the alpha-satellite (alphoid) repeat sequences and the X and Y chromosome zinc finger protein genes ZFX and ZFY (ZFX/ZFY) were performed. DNA extracted from various forensic specimens was amplified using either *Taq*, *Tth* or ThermoSequenase. Multiplexing using primers for all three loci in one reaction tube was achieved using *Tth* and ThermoSequenase.

Two IR labeling strategies for detection of PCR products were utilized. In the first strategy, one of the PCR primers contained a 19-base extension at its 5′ end identical to an IR-labeled universal M13 Forward (−29) primer which was included in the amplification reactions. During PCR the tailed primer generates sequence complementary to the M13 primer which subsequently primes the initial amplification products, thereby generating IR-labeled PCR products. In the second strategy, dATP labeled with an IR dye (IR-dATP) was included in the amplification reaction. During amplification IR-dATP was utilized by the polymerase and incorporated into the synthesized DNA, thus resulting in IR-labeled PCR products. X and Y specific bands were readily detected using both labeling methodologies.

Amplified products were electrophoretically resolved using denaturing Long-Ranger gels and detected with an automated detection system using IR laser irradiation. A separation distance of 15 cm allowed run times of less than 2 h from sample loading to detection. Because the gels could be run more than once, at least 120 samples (2 loads × 60 samples/load) can be typed using a single gel.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, multiplex amplification, automated DNA sequencer, infrared fluorescence, X-chromosome, Y-chromosome, amelogenin, ZFX, ZFY, α-satellite sequence

Advances in PCR methodology (1) have allowed amplification of samples that are degraded or of limited quantity thus yielding information about an individual's genetic identity. The genetic loci utilized for identification studies are usually small in size facilitating their amplification by PCR. Amplified alleles are separated by polyacrylamide gel electrophoresis which is capable of resolving amplification products varying in size by a single base. This allows for correct allele identification and sizing. DNA profiling most commonly utilizes short tandem repeat (STR) loci (2) which are also used for gene mapping, diagnostics, paternity testing, and forensic identification (3,4).

In forensic casework, it is sometimes necessary to determine the gender of biological material submitted as evidence. This is particularly useful for evidence such as a single hair or in sexual assault cases in which semen is donated by a vasectomized or aspermic male suspect. In cases in which no spermatozoa are present, it would be worthwhile subjecting a vaginal swab to direct gender identification. In Nebraska, numerous bloodstained items of evidence are submitted to the State Patrol Criminalistics laboratory for analysis in hopes of finding a stain deriving from the suspect. This is particularly true in homicide cases involving a struggle at the crime scene with bloodstains around the victim's body or in the residence. In the majority of these cases the suspects are male and the victims are female. During the initial phase of the investigation, it would be expeditious to analyze the gender of the bloodstains prior to analysis using other serological and DNA markers which are more expensive and time consuming. When the investigation is dependent on quick results, gender determination can give direction to investigators. Such analysis is particularly valuable in situations where the genders of the victim and suspect are different.

Gender determination can also be useful as an internal control and for body fluid mixture analysis as in sexual assault cases in which a female victim is subjected to oral sex. In Nebraska there are several possible homicide cases in which the women are missing. Should any of their remains be recovered in a suspect's residence or vehicle, gender determination from such evidence using DNA technology would be extremely useful. Gender determination of the female fraction during differential extraction of a seminal fluid stain can ensure that this fraction is devoid of any male DNA.

There are various reports in the literature on sex determination using DNA analysis (5–14). In this report we have utilized the amelogenin gene (15), the alpha-satellite (alphoid) repeat region (16) and polymorphic regions of the X-Y homologous zinc finger protein genes ZFX (17) and ZFY (18) for gender identification. These loci yield separate and distinct amplification products for both the X and the Y allele. For each locus, attempts were made to amplify both X and Y alleles simultaneously in one amplification reaction. Subsequently, we attempted multiplex amplifications using more than one locus in a single reaction tube. Where possible, two different labeling strategies were used and several thermostable DNA polymerases were evaluated. The feasibility of using an

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automated on-line system utilizing infrared (IR) laser irradiation to rapidly detect X and Y specific bands from various forensic specimens was tested. This would eliminate the need for radioactivity or gel staining required with other detection systems.

Materials and Methods

DNA Samples

Bloodstains, buccal swabs, and pulled hair samples were collected from various donors. Semen and vaginal secretions were collected on either cloth or paper from male and female volunteers. Semen from a vasectomized male was collected in a condom and stored frozen. 100 μL of this sample was used for DNA extraction. Bone samples were obtained from autopsy cases and DNA extracted following procedures described previously (19,20).

Other simulated forensic samples such as a toothbrush, etc. were supplied by volunteers. For the headband and cotton sock toe, approximately 0.25–0.50 $in.^2$ of material was carefully excised and used for extraction. Perspiration was collected using a sterilized cotton-tipped applicator from the forehead and underarm of a person who had been jogging. Skin wipes were obtained using a sterile cotton-tipped applicator moistened with water. Approximately 1 $in.^2$ inch of skin was wiped using pressure similar to an alcohol swab at a physician's office. One-half of each applicator was subsequently utilized for DNA extraction.

All samples (except vasectomized semen) were stored at room temperature until DNA was extracted, usually within one month. K562 DNA (Promega Corporation; Madison, WI) was used as a standard female control. One of the male volunteers served as a standard male control sample.

DNA Extraction

DNA was extracted from all samples using a Chelex extraction procedure (21,22). In some samples, such as a postcoital vaginal swab, a differential lysing procedure was used to differentiate between male and female fractions (22). Male and female samples were extracted and amplified at separate times to minimize the possibility of cross contamination.

Infrared Fluorescent Labeling Strategies

Two different IR labeling strategies were utilized. For the first strategy, one primer for a locus was synthesized with a 19-base extension at the 5′ end comprised of the sequence: 5′-CACGACGTTGTAAAACGAC-3′. This extension sequence is identical to an IR-labeled universal M13 Forward (−29) primer (23) which was also included in the amplification reaction. During amplification the tailed primer forms a complementary sequence to the M13 primer which subsequently primes the initial amplification products, thereby generating IR-labeled PCR products. This procedure has been described previously (24,25). This strategy was utilized with the forward primer for the lower molecular weight amelogenin product and the common primer (ZFXY) of the ZFX/ZFY loci.

The second strategy consisted of an IR-labeled dATP nucleotide (Fig. 1) which was included in the reaction mixture. During amplification, the polymerase incorporates a labeled molecule into the growing DNA chain thus producing PCR products internally labeled with IR fluorophore. All three of the loci listed above were amplified using this strategy, which has been described previously (26). This strategy is useful with commercially available kits, such as the Promega GenePrint™ Sex Determination System, because these primers are only available from a licensed manufacturer.

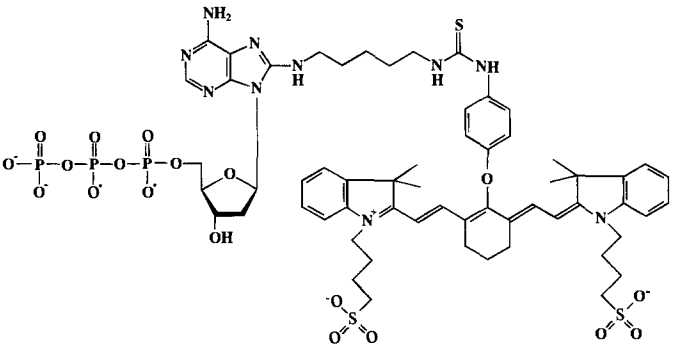

FIG. 1—*Structure of infrared dye IRD40 covalently attached to deoxyadenosine triphosphate (IR-dATP).*

Three different types of sizing standards were utilized in this research. The allelic ladders shown in some figures were generated by amplifying the CTT (CSF1PO, TPOX, and THO1) ladder (provided in the Promega CTT Multiplex Kit) in the presence of IR-dATP. In some cases, one lane of a standard DNA sequencing reaction was utilized. A molecular weight size marker produced internally at LI-COR was also used in some gels.

Amplification Primers

Where indicated the forward primers contained the 19-base universal M13 Forward (−29) sequence at their 5′ ends (underlined). The following gender loci were selected for evaluation: Locus name: Amelogenin (lower molecular weight products). Primer sequences were originally derived from a previous report (8). The 3′ end of the original reverse primer sequence was truncated to eliminate a stable hairpin loop structure.

Forward Tailed Primer:
5′-CACGACGTTGTAAAACGACCCCTGGGCTC-
TGTAAAGAATAGTG-3′
Reverse Primer: 5′-ATCAGAGCTTAAACTGGGAAG-3′

The Promega GenePrint™ Sex Determination System was used to generate higher molecular weight amelogenin amplification products. The primers for this locus are the Amelogenin 10X Primer Pair (Catalogue # DK628B) provided in this kit from Promega Corporation (Madison, WI). Locus name: Alpha-Satellite (Alphoid) Repeat Region. Primer sequences were derived from previous reports (6,12). Four primers were used for the amplification of this locus:

X3: 5′-TATTTGGACTCTCTCTGAGGA-3′
X4: 5′-TTCTACTACAAGGGTGTTGCA-3′
Y3: 5′-GTGTATTCACCTCCGGGAG-3′
Y4: 5′-ACAAAAGGTTCAATTCTGTGAG-3′

Locus name: Homologous Zinc Finger Protein Genes ZFX and ZFY (ZFX/ZFY). Primer sequences were obtained from a previous report (27).

ZFXY Forward Tailed Primer (common primer):
5′-CACGACGTTGTAAAACGACATTTGTTCTAAGTCG-
CCATATTCTCT-3′
ZFX Reverse: 5′-AGACACACTACTGAGCAAAATGTATA-3′
ZFY Reverse: 5′-CATCAGCTGAAGCTTGTAGACACACT-3′

Amelogenin primers (low molecular weight product) and alpha-satellite primers (X3, X4, Y3, and Y4) were synthesized by Genosys Biotechnologies (The Woodlands, TX). Primers for the ZFX/ZFY loci were obtained from Midland Certified Reagent Company (Midland, TX). The M13 Forward (−29) primer (23) labeled with

infrared dye IRD41 (28) was obtained from LI-COR, Inc. (Lincoln, NE).

Amplification Protocol

DNA extracted from various simulated forensic samples was amplified as described below. DNA was quantitated using a QuantiBlot Human DNA Quantitation Kit from Perkin Elmer (Norwalk, CT) and found to be 0.5–1.0 ng/μL in concentration. A DNA volume of 2.0 μL (1–2 ng) was used for amplification. This quantity has been determined empirically to produce acceptable amplification results in most cases. The volume of template DNA can be adjusted in subsequent amplifications for optimal results if necessary. A female control consisting of 1.0 ng of K562 DNA was utilized where indicated. DNA (1.0 ng) from a volunteer served as a standard male control.

All gender loci were amplified using PCR methodology (29). *Taq* and *Tth* polymerases together with their reaction buffers were obtained from Boehringer Mannheim (Indianapolis, IN). ThermoSequenase™ and its buffer were obtained from Amersham Life Science (Cleveland, Ohio). Where indicated, Promega GenePrint STR buffer (which includes dNTP's) from the Sex Determination kit was utilized. All synthesized primers were 5 pmol/μL in concentration. The concentration of Promega amelogenin primers was not disclosed. IR-labeled dATP (20 pmol/μL) and IR-labeled M13 Forward (−29) primer (1 pmol/μL) were obtained from LI-COR, Inc. (Lincoln, NE).

All amplification reactions were performed in both a Perkin Elmer 9600 Thermocycler (Perkin Elmer Corp.; Norwalk, CT) and an MJ Research MiniCycler (MJ Research, Inc.; Watertown, MA) for comparison. Reactions performed in the MJ MiniCycler were overlaid with mineral oil. Amplifications using the MJ MiniCycler contained programmed ramp times adjusted to simulate the Perkin-Elmer 9600 thermocycling profile.

I. Protocol using *Taq* or *Tth* polymerase with tailed primers:

A. Extracted DNA (1–2 ng)	2.0 μL
B. Appropriate PCR buffer	2.0 μL
C. Each PCR primer (5 pmol/μL)	0.5 μL
D. IR-labeled M13 Forward (−29) primer (1 pmol/μL)	0.7 μL
E. 2 mM dATP, dCTP, 7-deaza-dGTP, dTTP	1.0 μL
F. *Taq* (5 U/μL) or *Tth* polymerase (4 U/μL)	0.1 μL
G. Sterile distilled water to bring final volume to:	10 μL

These amplification reactions were cycled using Protocol No. 1 (Table 1).

II. Protocol using *Taq* with Promega GenePrint Sex Determination System:

A. Extracted DNA (1–2 ng)	2.0 μL
B. Promega STR buffer	2.5 μL
C. Amelogenin Primer Pair (10X)	2.5 μL
D. IR-labeled dATP (20 pmol/μL)	1.0 μL
E. *Taq* DNA polymerase (5 U/μL)	0.2 μL
F. Sterile distilled water to bring final volume to:	25.0 μL

Thermocycling was carried out using Protocol No. 2 (Table 1).

III. Protocol using *Tth* or ThermoSequenase with Promega GenePrint Sex Determination primers:

TABLE 1—*Protocols.*

Protocol Number	1	2	3
Initial Incubation	93°C for 5 min	96°C for 2 min	93°C for 5 min
Denaturation	94°C for 20 s	94°C for 1 min	94°C for 20 s
Annealing	67°C for 60 s	64°C for 1 min	67°C for 60 s
Extension	*	70°C for 1.5 min	*
Number of Cycles	10	10	10
Denaturation	94°C for 20 s	90°C for 1 min	94°C for 20 s
Annealing	62°C for 60 s	64°C for 1 min	62°C for 60 s
Extension	*	70°C for 1.5 min	*
Number of Cycles	22	20	26
Final Incubation	none	60°C for 30 min	72°C for 10 min

*Combined annealing and extension in one step.

A. Extracted DNA (1–2 ng)	2.0 μL
B. *Tth* or ThermoSequenase buffer	2.0 μL or 1.0 μL
C. Amelogenin Primer Pair (10X)	2.0 μL
D. IR-labeled dATP (20 pmol/μL)	1.0 μL
E. *Tth* (4 U/μL) or ThermoSequenase (1 U/μL)	0.2 μL
F. 2 mM dATP, dCTP, 7-deaza-dGTP, dTTP	1.0 μL
G. Sterile distilled water to bring final volume to:	10.0 μL

Cycling was performed using Protocol No. 2 (Table 1).

IV. Protocol using ThermoSequenase with tailed primers or internal label:

A. Extracted DNA (1–2 ng)	2.0 μL
B. ThermoSequenase buffer	1.0 μL
C. Each primer (5 pmol/μL)	0.5 μL
D. IR-labeled dATP (20 pmol/μL) or IR-labeled M13 Forward (−29) primer (1 pmol/μL)	1.0 μL
E. 2 mM dATP, dCTP, 7-deaza-dGTP and dTTP	1.0 μL
F. ThermoSequenase (1 U/μL)	0.2 μL
G. Sterile distilled water to bring final volume to:	10.0 μL

The reactions were cycled as described in Protocol No. 1 (Table 1).

V. Protocol for amplifying alpha-satellite repeat sequences:

A. Extracted DNA (1–2 ng)	2.0 μL
B. *Tth* or *Taq* buffer, or ThermoSequenase buffer	2.0 μL 1.0 μL
C. X3 and X4 primers (5 pmol/μL)	0.5 μL (of each)
D. Y3 and Y4 primers (5 pmol/μL)	2.5 μL (of each)
E. IR-labeled dATP (20 pmol/μL)	1.0 μL
F. 2 mM dATP, dCTP, 7-deaza-dGTP, dTTP	1.0 μL
G. *Taq* (5 U/μL) or *Tth* polymerase (4 U/μL), or ThermoSequenase (1 U/μL)	0.2 μL 0.3 μL
H. Sterile distilled water to bring final volume to:	15.0 μL

Thermocycling was performed as described previously (Protocol No. 1).

VI. Protocol for multiplex amplification of ZFX/ZFY, amelogenin and alphoid repeats:

A. Extracted DNA (1–2 ng)	2.0 μL
B. *Tth* or ThermoSequenase buffer	2.0 μL or 1.0 μL
C. Alphoid Y3 and Y4 primers (5 pmol/μL)	2.5 μL (of each)
D. All other PCR primers (5 pmol/μL)	0.5 μL (of each)
E. IR-labeled dATP (20 pmol/μL)	1.0 μL
F. 2 mM dATP, dCTP, 7-deaza-dGTP, dTTP	1.0 μL
G. *Tth* (4 U/μL) or ThermoSequenase (1 U/μL)	0.5 μL
H. Sterilized distilled water to bring total volume to:	20.0 μL

Thermocycling was performed using Protocol No. 3 (Table 1).

Removal of Unincorporated IR-dATP

Amplification reactions utilizing IR-dATP labeling were placed on ice after thermal cycling was completed. Because IR-dATP and its fluorescent thermal decomposition products may co-migrate with the amplification products, these species needed to be removed by gel filtration, 800 μL of preswollen Sephadex G-50 Fine (Sigma Chemical; St. Louis, MO) was added to filter cups of Costar SPIN-X® filter (0.45 micron Cellulose Acetate) apparatus (Corning Costar Corp.; Cambridge, MA). Columns were packed by spinning at 5000 RPM in a Tomy MTX-150 microcentrifuge for 1 min. Filter cups containing Sephadex G-50 were removed and placed into sterile 1.5-mL microcentrifuge tubes, 10 to 20 μL of each amplified sample was carefully loaded onto the resin bed and spun at 5000 RPM for 1 min. Filter cups were discarded and tubes containing purified filtrate were capped and stored frozen until use. Sephadex G-50 was prepared by adding 1.0 g of resin per 15 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and swelling at least overnight.

Gel Loading Protocol

For the internal labeling protocol, 4.0 μL of loading buffer (Stop Solution) (Amersham Life Science; Arlington Heights, IL) was added to 1.0 μL of reaction product; 1.0 μL of this diluted sample was loaded onto a gel mounted in a LI-COR Model 4000 automated DNA sequencer and electrophoresed. With tailed primers, 10.0 μL of loading buffer was added to 1.0 μL reaction product and 1.0 μL of this diluted sample loaded onto the gel. All samples were denatured at 95°C for 2 min and quickly cooled on ice prior to loading.

Electrophoresis

The electrophoresis procedure used in this research has been described previously (24). Briefly, a LI-COR Model 4000 automated DNA sequencer (30) was used for gel electrophoresis and detection of X and Y allele amplification products. All reagents and chemicals used for electrophoresis were of molecular biology grade. Square-toothed combs were used for casting of denaturing Long Ranger (FMC Bioproducts; Rockland, ME) gels. For most gels 1.0 μL volumes were loaded onto 25 or 33 cm length gels and electrophoresed according to the protocol recommended by the manufacturer. The raw data depicting X and Y alleles, allelic ladders and size markers is displayed as an autoradiogram-like image on the computer screen and stored on the hard drive of the computer.

Results

The goal of this research was to detect X and Y alleles from various forensic samples using several gender discriminating loci. In addition, we sought to minimize the time necessary to detect amplification products by use of an automated DNA sequencer. X and Y alleles were amplified using Chelex extracted DNA from bloodstains, saliva, hair roots, semen, vaginal swabs, nasal secretions, as well as other types of evidence commonly encountered in forensic cases. We have previously used *Tth* polymerase to amplify STR alleles directly from bloodstains using only a high temperature incubation to extract DNA (24). Due to our experience with *Tth* polymerase and because *Taq* polymerase is the most commonly used PCR enzyme, we initially focused upon amplifying individual gender loci utilizing these two polymerases. In addition, another enzyme (ThermoSequenase) was investigated in attempts to minimize spurious banding and to multiplex all loci in one reaction tube.

It was possible to amplify and detect allelic bands from regions of the X and Y chromosomes for each individual locus using all three polymerases. The amelogenin locus (both low and high molecular weight products), centromeric alphoid repeat sequences, and ZFX/ZFY loci were amplified individually using the internal labeling strategy and readily detected on the LI-COR automated DNA sequencer. In addition, the amelogenin (low molecular weight products) and ZFX/ZFY loci were amplified using the tailed primer strategy yielding results comparable to the internal labeling procedure, except for the increased molecular weight due to the 19 base tail on the amplification product.

Amelogenin Locus

The X-Y homologous amelogenin gene contains a six basepair deletion in the X chromosome thereby providing a means for differentiating between the two alleles. Figure 2A displays the low molecular weight amelogenin patterns of the X (125 bases) and

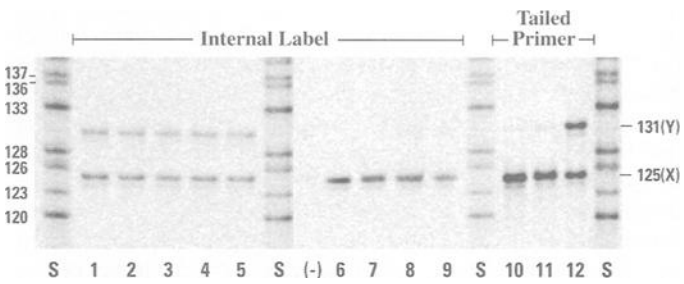

FIG. 2A—*Amplification of simulated forensic samples at the gender discriminating amelogenin locus (lower molecular weight products) using* Tth *polymerase with internal labeling (lanes 1–9) or tailed primer labeling (lanes 10–12) resolved on a 25-cm gel. Lanes 1–4 are blood, saliva, hair, and semen (containing spermatozoa), respectively, from a male. Lane 5 is the male fraction from a postcoital vaginal swab after differential extraction. Lanes 6–9 are blood, saliva, hair, and nasal secretion, respectively, from a female. Lane 10 is K562 DNA. Lane 11 is the female fraction of a postcoital swab after differential extraction. Lane 12 is semen from a vasectomized male. A negative control (−) and molecular weight size standards (S) (of 120–137 bases) are also displayed.*

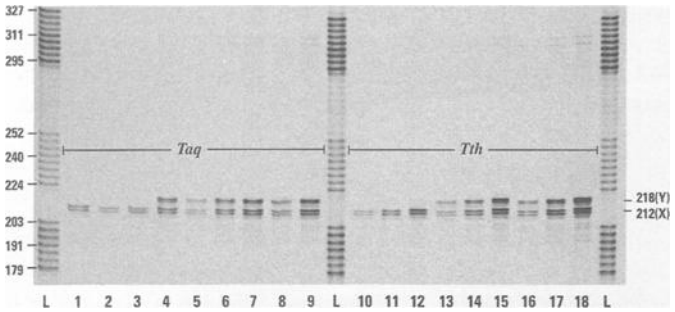

FIG. 2B—*Amplification of simulated forensic samples at the gender discriminating amelogenin locus (higher molecular weight products) using the Promega GenePrint System. Samples were amplified using either* Taq *(lanes 1–9) or* Tth *(lanes 10–18) together with the internal labeling protocol and resolved on a 25-cm gel. Lanes 1, 2, 3, and 11 are blood, saliva, vaginal swab, and hair, respectively, from a female. Lane 10 is a toothbrush used by a female. Lane 12 is K562 DNA supplied in the GenePrint kit. Lanes 4, 5, 6, and 7 are blood, saliva, semen (containing spermatozoa), and hair, respectively, from a male. Lanes 8, 9, 13, and 14 are toothbrush, cigarette butt, headband and skin wipe, respectively, collected from a male. Lanes 15, 16, 17 and 18 are semen (vasectomized), sunburned skin, toe of a cotton sock and blood, respectively, from a male. L represents the Promega GenePrint CTT allelic ladder internally labeled using IR-dATP in the amplification mixture. Lanes 7–9 and 15–18 contained 3.0 μL of sample.*

Y (131 bases) alleles amplified using either the internal labeling strategy (lanes 1–9) or the tailed primer strategy (lanes 10–12) and *Tth* polymerase. Because the primers in all of these reactions are identical (the forward primer in both cases contains an M13 tail), products of both amplification strategies are of identical size. Tailed primers can be utilized for internal labeling amplifications simply by including IR-dATP in the reaction in place of labeled universal M13 primer.

The amelogenin locus amplified using Promega GenePrint primers with the internal labeling protocol is shown in Fig. 2B. Similar results were obtained using either *Taq* (lanes 1–9) or *Tth* (lanes 10–18) polymerase. This amplification yielded higher molecular weight products (X allele at 212 bases and Y allele at 218 bases) than in Fig. 2A due to alternate placement of the amplification primers flanking the six basepair deletion in the amelogenin gene.

Inspection of Fig. 2B reveals the presence of dual amplification bands using the Promega amelogenin primers and internal labeling with both *Taq* and *Tth* polymerase. Similar dual bands have been observed using *Taq* polymerase (but not with *Tth* polymerase) for amplification of the lower molecular weight amelogenin product using both tailed primer and internal label strategies (data not shown). This phenomenon is absent in Fig. 2A where *Tth* polymerase was used with both labeling strategies. We hypothesize that dual bands are produced by differences in base composition of the two amplified strands which are both labeled by IR-dATP incorporation. Similar duality of bands has been observed when restriction fragments are labeled with IR-dATP via fill-in reactions (26). Some labeled restriction fragments show a greater degree of duality, probably due to larger base composition differences. Another possibility is that the terminal transferase activity of the polymerase generates the duality by adding an extension to a portion of the amplified fragments under these experimental conditions.

Alphoid Repeat Regions

The centromeric region of human chromosomes contains repetitive DNA known as the alpha satellite (alphoid) repeat region. A number of different alphoid repeat subfamilies exist which contain minor sequence differences between one another (16). Amplification of subfamilies specific to the X and Y chromosomes provides a means of gender discrimination. Simultaneous amplification of X and Y specific alphoid sequences required the use of four primers (X3, X4, Y3, and Y4). Amplification using only X3 and X4 primers produced the X allele (157 bases) alone for both male and female samples. Y3 and Y4 primers alone produced no allelic bands in female samples but yielded the Y allele band (196 bases) in male samples (data not shown). All four primers were necessary to amplify both X and Y allelic bands in male samples, yet produced only the X allele in female samples as shown in Fig. 3.

It was necessary to use at least five times more Y3 + Y4 primer as X3 + X4 primer for detection of the higher molecular weight Y allele when all four primers were present in a single reaction mixture. This may reflect the relative numbers of repeat units being amplified. For the alphoid repeat locus, amplification was only possible using the internal label protocol. This locus could not be amplified using the tailed primer strategy with X3 tailed forward and X4 reverse primers. Attempts to amplify the Y allele alone with Y3 tailed forward and Y4 reverse primers also failed. Tailed X3 and Y3 forward primers together with X4 and Y4 reverse primers in one reaction mixture also failed to amplify. It is possible that secondary structure problems arise when using these tailed primers making them less amenable to amplification.

Figure 3 also demonstrates the dual banding activity of *Taq* polymerase in amplification of the alphoid alleles. In this gel, male samples were amplified using *Tth* polymerase and female samples with *Taq* polymerase. Again, as with the amelogenin primers, dual bands were visible in the reactions using *Taq* polymerase but not with *Tth* polymerase. All female samples show dual bands whereas

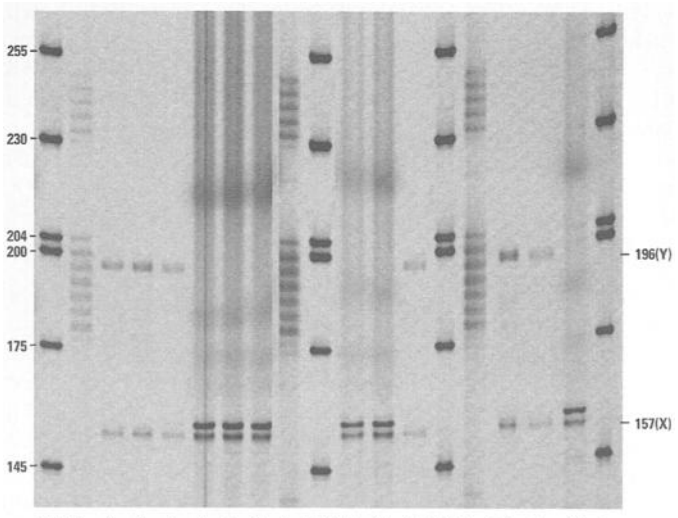

FIG. 3—*Amplification of simulated forensic samples at the gender discriminating alphoid repeat sequence locus using the internal labeling protocol resolved on a 33-cm gel. X3, X4, Y3, and Y4 primers were present in each amplification except where noted. Lanes 1–3 are blood, saliva and hair, respectively, from a male. Lanes 9–11 are semen (containing spermatozoa), male fraction after differential extraction of a postcoital vaginal swab and semen from a vasectomized male, respectively. Lanes 4–8 are blood, saliva, hair, vaginal secretion, and postcoital female fraction from a vaginal swab, respectively. Lane 12 is K562 DNA amplified using only X3 and X4 primers. L represents IR-labeled allelic ladder from the Promega CTT kit. MW is a molecular weight size marker. All female samples including K562 were amplified with* Taq *polymerase and all male samples with* Tth *polymerase.*

the male samples show none. The nature of this dual banding phenomenon is still under investigation.

ZFX + ZFY Loci

Similar to the case of the amelogenin gene, an X-Y homologous zinc finger gene exists on both the X chromosome (ZFX) and Y chromosome (ZFY). There is sufficient sequence variation between the two to allow for allele specific amplification which can be exploited for gender identification (13). Three primers were used for the ZFX/ZFY loci, including a common forward primer (ZFXY) containing an M13 tail which was utilized for amplification of both alleles. This tailed primer was used with both labeling strategies resulting in products of identical size. For the two loci discussed earlier, amplified X alleles were of smaller molecular weight than Y alleles. For the ZFX/ZFY loci the amplified X allele (500 bases) was larger than the Y allele (355 bases). Several reactions were performed to determine the amplification characteristics of the three ZFX/ZFY loci primers in male and female samples. Male samples were amplified using the following primer combinations in separate reaction mixtures:

1. ZFXY Forward and ZFX reverse primers,
2. ZFXY Forward and ZFY reverse primers,
3. ZFXY Forward, ZFX reverse and ZFY reverse primers.

As shown in Fig. 4, the first reaction mixture yielded only the X allele (lane 4), whereas the second reaction produced only the Y allele (lanes 1–3, 6, 17, 21). Only use of all three primers produced both X and Y allelic products (lanes 7, 9–16). Male gender is indicated by the presence of the Y allele band alone (because females normally lack a Y chromosome) or in combination with the X band (Fig. 4).

Similar amplifications were performed using female samples and the three different primer combinations. Figure 4 demonstrates that the first reaction yielded the X allele (lanes 5 and 19) whereas the second produced no allelic bands (lanes 8 and 20), as expected. Amplification with all three primers produced only the X allele (lane 18). Female samples require the ZFXY forward primer together with the ZFX reverse primer for amplification of the X allele. A female sample (K562) amplified using ZFXY forward and ZFY reverse primers yielded no detectable bands (lane 8). The presence of the Y allele alone is always indicative of a male, however, the additional amplification of the X allele serves as an internal positive control. All of the primers mentioned above have also been used to amplify primate bloodstains and the results will be reported elsewhere.

Figure 4 also demonstrates that these electrophoresis gels could be run multiple times. The X and Y allelic bands displayed in this figure were electrophoretically resolved and detectable even after reactions had been loaded in the lanes and electrophoresed twice previously. Although there is some degradation in gel quality with subsequent loads, this does not seem to cause inaccurate results

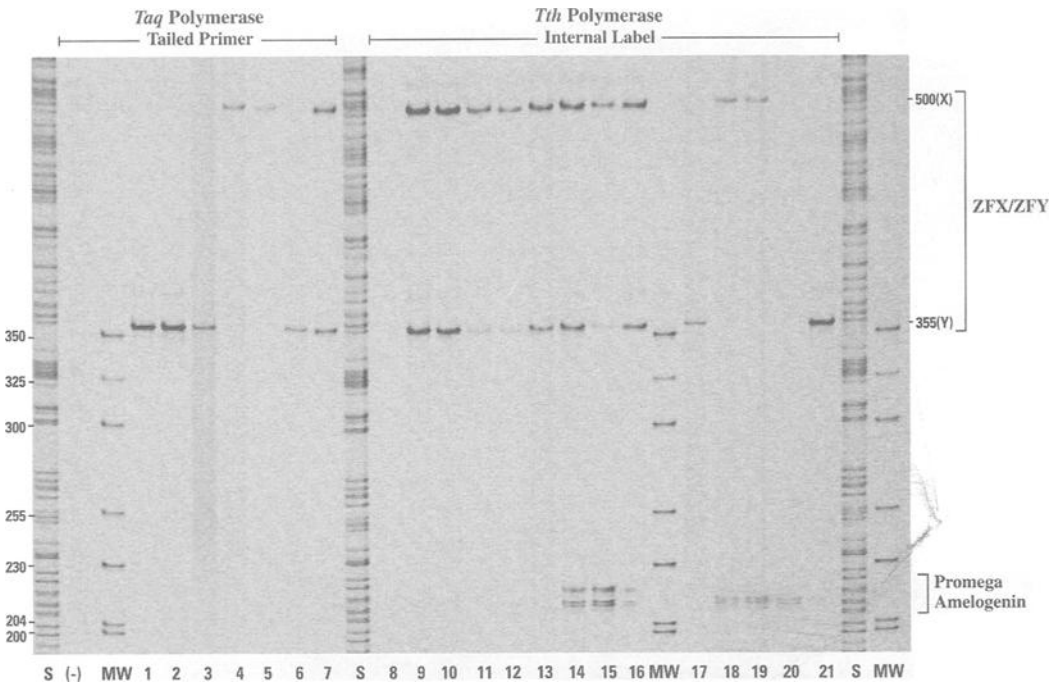

FIG. 4—*Amplification of simulated forensic samples at the gender discriminating ZFX and ZFY loci using different combinations of the three ZFX/ZFY primers resolved on a 25-cm gel. Lanes 1–7 utilized tailed primer labeling methodology and* Taq *polymerase, whereas lanes 8–21 utilized internal labeling with* Tth *polymerase. Lanes 1–3 and 6 are blood, saliva, hair, and semen, respectively, from a male amplified with ZFXY forward + ZFY reverse primers. Lanes 4 and 5 are blood from a male and female, respectively, amplified with ZFXY forward + ZFX reverse primers. Lanes 7 and 9–16 are male blood, saliva, hair, nasal secretion, semen (containing spermatozoa), perspiration (collected from males), semen (vasectomized), male fraction from a postcoital vaginal swab, and toothbrush used by a male, respectively, amplified with all three primers. Lane 8 is K562 DNA amplified with ZFXY forward + ZFY reverse primers. Lane 17 is the male fraction from a postcoital vaginal swab amplified with ZFXY forward + ZFY reverse primers. Lane 18 is a control vaginal swab (without semen) amplified using all three primers. Lanes 19 and 20 are bone from a female amplified with ZFXY forward + ZFX reverse and ZFXY forward + ZFY reverse, respectively. Lane 21 is a toothbrush used by a male amplified with ZFXY forward + ZFY reverse primers. Lanes 14–16 and lanes 18–20 also contained Promega amelogenin primers in the reaction. S is a size standard consisting of one lane of a standard sequencing reaction, MW are molecular weight markers and (−) is a negative control. This gel had been previously run twice prior to the loading of these reactions.*

for gender identification. With multiple sample loads the speed of electrophoresis and band resolution decreases slightly during each successive run, therefore we do not run gels more than three times. However, for applications requiring single base resolution, i.e., DNA sequencing, multiple loading is not recommended due to the decreased level of accuracy caused by the gel degradation.

Multiplex Amplifications

Because the three loci described above could be used individually for gender discrimination, we explored the feasibility of multiplexing them together. Figure 5A shows amplification results of the alphoid locus multiplexed with the amelogenin locus (low molecular weight product) using ThermoSequenase with IR-dATP labeling. All three samples from the male donor displayed both X and Y alleles of the amelogenin locus as well as the alphoid locus. This particular male displayed an interesting three-banded alphoid region. In addition to the normally occurring X (157 bases) and Y (196 bases) bands present in all other male samples analyzed, a third band (approximately 184 bases) was also visible. The amelogenin locus of the same donor male in Fig. 5A appears as only two bands of approximately equal intensity. The ZFX/ZFY and higher molecular weight amelogenin amplifications also yielded the expected bands (data not shown). In amplification reactions using only the four alphoid primers, the three-banded pattern was distinctly visible in all samples from this individual. Identical results were obtained regardless of which of the three polymerases was utilized. When reactions contained only Y3 and Y4 primers, two bands were visible at approximately 196 and 184 bases, with no band for the X allele (157 bases) (data not shown). Because manifestation of the three bands occurred only with the alphoid locus and was generated using only Y specific primers, we hypothesize that this phenomenon is due to a deletion in a portion of the alphoid repeat sequences in some of the Y chromosomes (mosaicism), or to a partial duplication of the Y alphoid region. The presence of a second Y chromosome containing altered alphoid repeats is not indicated because the amelogenin locus (containing one copy of the gene on both X and Y chromosomes) does not show a higher band intensity for the amplified Y allele (10).

Multiplexing was also possible using three primer sets: Alpha satellite, Promega amelogenin and ZFX/ZFY. Figure 5B displays both X and Y alleles for all three loci of male samples when *Tth* polymerase was used with the internal labeling strategy (lanes 3–7). Figures 5A and 5B show multiplexing of samples from male donors only. Female samples amplified using the three primer sets yielded only X allelic bands (data not shown).

Although we have demonstrated multiplex amplification of gender loci in Fig. 5, it was difficult to achieve in many situations

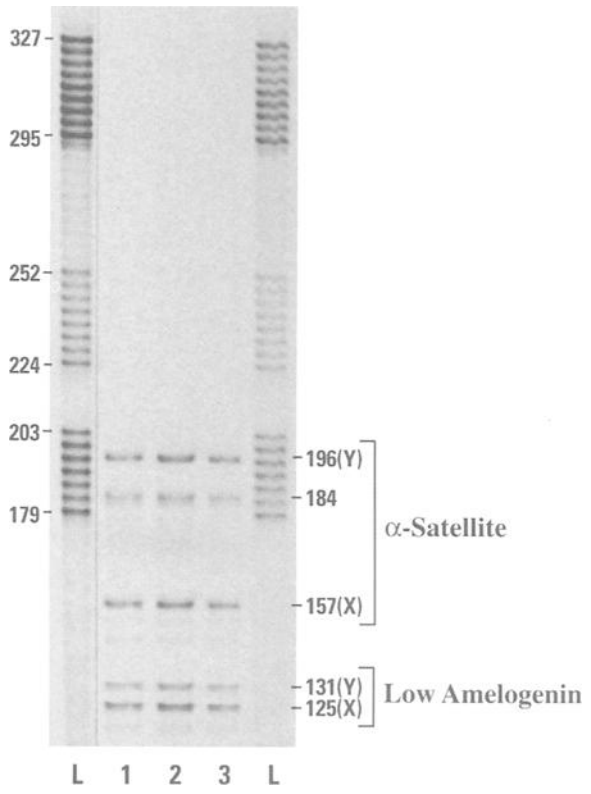

FIG. 5A—*Multiplex amplification using alpha-satellite and low molecular weight product amelogenin primers in one reaction resolved on a 33-cm gel. Lanes 1, 2, and 3 are blood, semen (containing spermatozoa) and hair, respectively, from a male. All reactions contained alpha-satellite primers (X3, X4, Y3, and Y4) together with amelogenin primers (low molecular weight product) and were amplified using ThermoSequenase and IR-dATP. L is a molecular weight size standard (Promega CTT allelic ladder).*

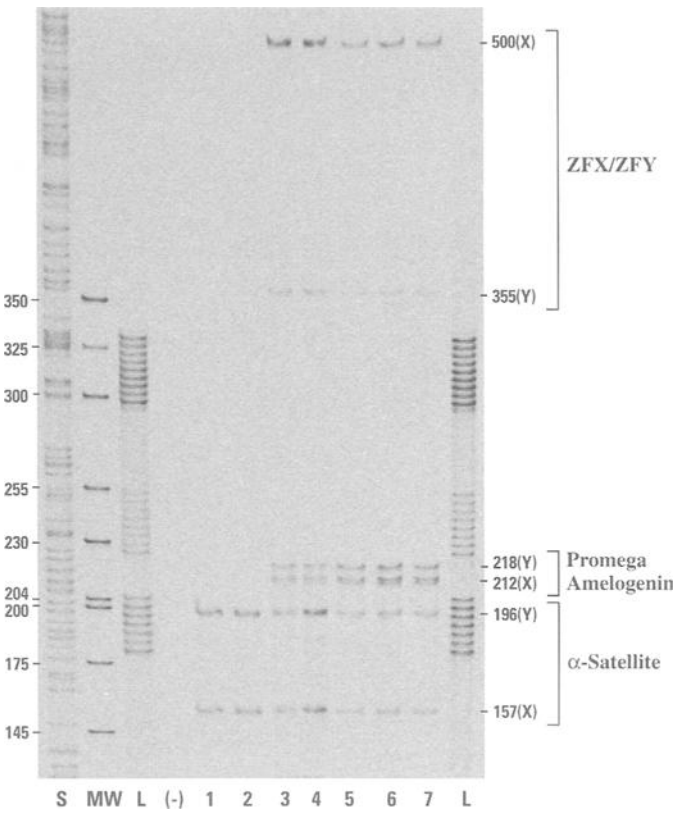

FIG. 5B—*Multiplex amplification using amelogenin, alpha-satellite and ZFX/ZFY primers in one reaction resolved on a 33-cm gel. All amplifications were performed with male samples using* Tth *polymerase and IR-dATP. Lanes 1 and 2 are bone and semen (vasectomized) from two males, respectively, amplified with alphoid primers. Lanes 3–7 are semen (vasectomized), blood, hair, toothbrush (used by a male) and a male toenail, respectively, amplified with alphoid primers, Promega amelogenin primers and ZFX/ZFY primers in one reaction tube. L designates IR-labeled Promega CTT allelic ladder, MW is a molecular weight size standard, and S is a size standard consisting of one lane of a standard sequencing reaction.*

and yielded unreliable results. The tailed primer labeling method failed to amplify all loci in the multiplex reactions. Multiplexing was only possible using *Tth* polymerase or ThermoSequenase with the internal labeling strategy. *Taq* polymerase failed to amplify all alleles from all of the loci in multiplexing reactions. Although we were able to detect all loci using either *Tth* polymerase or ThermoSequenase, various artifactual bands were often observed in multiplexing reactions. Artifactual bands such as the one observed in Fig. 5A below the alphoid X allele were quite common. A phenomenon similar to "allelic dropout" was also observed for some loci and was most commonly observed when *Taq* polymerase was used for multiplexing. Hence, a bloodstain originating from a male could be misinterpreted as coming from a female donor when only the X allele amplified. In some reactions in which both alleles of the amelogenin locus amplified, other loci such as the alphoid or ZFX/ZFY failed to do so. The reason for this is unknown at present.

Discussion

These gender differentiating loci were detected using a LI-COR Model 4000 infrared automated DNA sequencer. This system combines high sensitivity infrared fluorescence chemistry and laser technology which eliminates the necessity of using radioactivity, restriction digestion, probe hybridization, or the gel handling required with silver staining and fluor detection systems. Infrared fluorescence of solvents and biomolecules is extremely low, thus producing very low background signal and high sensitivity (31). Determination of detection limits for infrared fluorescent primers versus visible fluorescent primers reveals that infrared technology is approximately seven times more sensitive than visible fluorescent detection (26), allowing forensic analysis of diminutive quantities of template DNA. This on-line, real-time detection system permits immediate visualization of the raw data. X and Y specific allelic bands are displayed as familiar autoradiogram-like images that can be analyzed by software included in the computer.

Gender discriminating alleles could be amplified and detected using both internal labeling and tailed primer labeling strategies. The use of tailed primers seemed to generate more spurious bands compared to the internal labeling strategy (Fig. 2A). The visual impact of such artifacts can be minimized by dilution of the amplified products and/or loading less volume onto the gel (Fig. 2B). Artifacts are not uncommon among PCR amplification products and were reported earlier by the authors using the tailed primer method (24). Comparison of singleplex reactions using the M13 tail to identical reactions generated with IR-dATP indicates that internal labeling of the alleles eliminates many of these artifacts.

The nature of the dual banding phenomenon is still under investigation. We observed dual banding with *Taq* polymerase using both tailed primer and internal labeling strategies with the low molecular weight amelogenin products. Dual bands were also observed using all three polymerases with Promega amelogenin primers. Alphoid sequences produced dual bands when using *Taq* polymerase but not with *Tth* polymerase or ThermoSequenase. The X and the Y alleles of the ZFX/ZFY locus did not display dual bands using any of the three polymerases. A 30 min 60°C incubation subsequent to thermocycling did not eliminate dual bands from any loci amplified with *Taq* polymerase, implying that the cause was not due to incomplete polymerization. With Promega amelogenin primers, dual bands could not be eliminated in our system even when *Tth* polymerase or ThermoSequenase were used with an extension time subsequent to thermocycling. Because this primer set required use of the internal labeling strategy, dual banding could be exclusively due to base composition differences between the two strands.

Two different thermocyclers were utilized for amplifications. The Perkin Elmer 9600 is commonly found in many forensic laboratories whereas the MJ MiniCycler is not as widespread. However, gender discriminating alleles could be amplified with both thermocyclers yielding identical results. The only modification necessary was to utilize programmed ramp times in the MiniCycler to simulate the slower temperature ramping of the 9600. Gels could be run multiple times and throughput can be increased by use of 64-well combs. By the use of two loadings it is possible to analyze at least 120 samples within approximately 4 h using a single gel.

Conclusions

We have explored several means of amplifying X and Y specific sequences which can be utilized for gender identification. Although a multiplex system could perform several simultaneous gender determinations, this is not necessary in the vast majority of cases and was difficult to accomplish reliably in our hands. The amelogenin locus performed quite well in our experience yielding accurate gender discrimination. It could be amplified using only two primers with both IR labeling strategies. Primers are also commercially available for this locus. The six base difference between the X and Y alleles was easily resolvable within the 120–220 base size range and PCR products of this size are easily synthesized. In situations where the amelogenin gender results are called into question, the ZFX/ZFY locus can be utilized as a backup. It has also performed reliably using both labeling strategies, but requires the use of three primers and generates somewhat larger amplification products.

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References

1. Erlich HA, Gelfand D, Sninsky JJ. Recent advances in the polymerase chain reaction. Science 1991;252:1643–51.
2. Weber JL, May PE. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Gen 1989;44:388–96.
3. Sullivan KM, Pope S, Gill P, Robertson JM. Automated DNA profiling by fluorescent labeling of PCR products. PCR Meth Applic 1992;2:34–40.
4. Urquharat A, Oldroyd NJ, Kimpton CP, Gill P. Highly discriminating heptaplex short tandem repeat PCR system for forensic identification. BioTechniques 1995;18:116–21.
5. Witt M, Erickson RP. A rapid method for detection of Y-chromosomal DNA from dried blood specimens by the polymerase chain reaction. Hum Gen 1989;82:271–4.
6. Gaensslen RE, Berka KM, Grosso DA, Ruano G, Pagliaro EM, Messina D, et al. A polymerase chain reaction (PCR) method for sex and species determination with novel controls for deoxyribonucleic acid (DNA) template length. J Forensic Sci 1992;37:6–20.
7. Akane A, Seki S, Shioni H, Nakamura H, Hasegawa M, Kagawa M, et al. Sex determination of forensic samples by dual PCR amplification of an X-Y homologous gene. Forensic Sci Int 1992;52:143–8.
8. Sullivan KM, Mannucci A, Kimpton CP, Gill P. A rapid and quantitative DNA sex test: Fluorescent-based PCR analysis of X-Y homologous gene amelogenin. BioTechniques 1993;15:636–41.

9. Fattorini P, Caccio S, Gustincih S, Florian F, Altamura BM, Graziosi G. Sex identification by polymerase chain reaction of alpha-satellite in aged tissue samples. Electrophoresis 1993;14:23–6.
10. Pfitzinger H, Ludes B, Mangin P. Sex determination of forensic samples: Co-amplification and simultaneous detection of a Y-specific and an X-specific DNA sequence. Int J Leg Med 1993; 105:213–6.
11. Neto ED, Santos FR, Pena SDJ, Simpson AJ. Sex determination by low stringency PCR (LS-PCR). Nucl Acids Res 1993; 21:763–4.
12. Neeser D, Liechti-Gallati S. Sex determination of forensic samples by simultaneous PCR amplification of alpha-satellite DNA from both the X and Y chromosomes. J Forensic Sci 1995; 40:239–41.
13. Reynolds R, Varlaro J. Gender determination of forensic samples using PCR amplification of ZFX/ZFY gene sequences. J Forensic Sci 1996;41:279–86.
14. Stacks B, Witte MM. Sex determination of dried blood stains using the polymerase chain reaction (PCR) with homologous X-Y primers of the zinc finger protein gene. J Forensic Sci 1996; 41:287–90.
15. Nakahori Y, Takenaka O, Nakagome Y. A human X-Y homologous region encodes 'amelogenin'. Genomics 1991;9:264–9.
16. Choo KH, Vissel B, Nagy A, Earle E, Kalitsis P. A survey of the genomic distribution of alpha satellite DNA on all the human chromosomes, and derivation of a new consensus sequence. Nucl Acids Res 1991;19:1179–82.
17. Schneider-Gaedicke A, Brown LG, Nussbaum R. ZFX has a gene structure similar to ZFY, the putative human sex determinant, and escapes X inactivation. Cell 1989;57:1247–58.
18. Page DC, Mosher R, Simpson EM, Fisher EMC, Mardon G, Pollack J, et al. The sex-determining region of the human Y chromosome encodes a finger protein. Cell 1987;51:1091–104.
19. Lee H, Pagliaro E, Berka K, Folk N, Anderson D, Gualberto R, et al. Genetic markers in human bone: I. deoxyribonucleic acid (DNA) analysis. J Forensic Sci 1991;36:320–30.
20. Hochmeister M, Budowle B, Borer U, Eggman U, Comey C, Dirnhofer R. Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. J Forensic Sci 1991;36: 1649–61.
21. AmpliType® User Guide, Section 3 (Sample Preparation). Cetus Corporation, Emeryville, CA. 1990.
22. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 1991;10:506–13.
23. Steffens DL, Sutter SL, Roemer SC. An alternate universal forward primer for improved automated sequencing of M13. BioTechniques 1993;15:580–2.
24. Roy R, Steffens DL, Gartside B, Jang GY, Brumbaugh JA. Producing STR locus patterns from bloodstains and other forensic samples using an infrared fluorescent automated DNA sequencer. J Forensic Sci 1996;41:418–424.
25. Oetting WS, Lee HK, Flanders DJ, Wiesner GL, Sellers TA, King RA. Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. Genomics 1995;30:450–8.
26. Steffens DL, Jang GY, Sutter SL, Brumbaugh JA, Middendorf LR, Mühlegger K, et al. An infrared fluorescent dATP for labeling DNA. Genome Res 1995;5:393–9.
27. Strain L, Warner JP, Johnston T, Bonthron DT. A human parthenogenetic chimaera. Nature Gen 1995;11:164–9.
28. Narayanan N, Patonay G. A new method for the synthesis of heptamethine cyanine dyes: Synthesis of new near-infrared fluorescent labels. J Org Chem 1995;60:2391–5.
29. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487–91.
30. Middendorf LR, Bruce JC, Bruce RC, Eckles RD, Grone DL, Roemer SC, et al. Continuous, on-line DNA sequencing using a versatile infrared laser scanner/electrophoresis apparatus. Electrophoresis 1992;13:487–94.
31. Patonay G, Antoine MD. Near-infrared fluorogenic labels: New approach to an old problem. Anal Chem 1991;63:321A–327A.

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