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PERFORMANCE EFFICACY USING A COMPARISON OF COMMERCIAL AND IN-HOUSE Y-STR MULTIPLEX SYSTEMS FOR OPERATIONAL USE

by

KATHLEEN ANN MAYNTZ PRESS
B.S. University of Central Florida, 2004

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Chemistry
in the College of Arts and Science
at the University of Central Florida
Orlando, Florida

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2006

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ABSTRACT

It is routine for the forensic scientist to obtain a genetic profile of an individual from DNA recovered from a biological stain deposited at a crime scene. In contrast, only a limited number of laboratories in the United States have the capability of performing Y-STR analysis in casework. In order to aid in facilitating the transfer of Y-STR technology to the crime laboratory community for operational use, a comparison between commercial products from three main vendors (Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit, Promega PowerPlex® – Y System, Reliagene Y-PLEX™ 12) and two in-house Y-STR multiplexes (MPI and MPB) commenced. The main intention for this comparison was to ascertain whether commercial Y-STR kits are able to obtain a male profile from difficult samples which have been accomplished with our in-house Y-STR multiplexes; such as mixtures, post coital specimens, and environmental insults.

To aid the crime laboratory community an in depth comparison of the three main commercial Y-STR kits began in hopes to glean information in circumstances where Y chromosome polymorphisms may need to be employed. For example, the ability to provide investigators with the numbers of semen donors in multiple rape cases, identification of the genetic profile of the male component in a male/female mixture, and identification of the genetic profile of the male component in an extended interval post-coital sample.

The capability of typing Y-STR loci by the crime laboratory community could dramatically affect the admissibility of Y-STR evidence. Therefore, the comparison of commercially available kits is an imperative process by which the scientific community acquires the necessary information to assess the ability of a procedure to obtain reliable results, determine

the conditions under which such results can be obtained and define the limitations of the procedure. Thus the information for the study could lend itself to a standard being established amongst Y-STR kits for operational use and/or the production of a new Y-STR kit.

One example of how the comparison of the three main commercial Y-STR kits could directly impact a new standard being established is by examining post-coital samples and their extreme limits (>48 hrs) for each kit in which a full male genetic profile was observed and comparing it to other commercial Y-STR kit and in-house Y-STR multiplexes. This would help establish the types of cases where specific Y-STR kits would be most useful, and the parameters in which each kit is able to perform. Thus leading to the development of a highly sensitive Y-STR kit that would be more sufficient to perform with the variety of samples an operational crime laboratory would routinely analyze.

The capability of typing Y-STR loci by the crime laboratory community could dramatically affect the admissibility of Y-STR evidence. Therefore, the comparison of commercially available kits is an imperative process in order to inform the forensic community of different Y-STR kits available and their performance through direct comparison using modified SWGDAM validation guidelines.

For my Family

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LIST OF ACRONYMS/ABBREVIATIONS

BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HPLC	High Performance Liquid Chromatography
KCL	Potassium Chloride
MPB	Multiplex B
MPI	Multiplex I
NaCl	Sodium Chloride
NRY	Nonrecombining Region
PARs	Pseudoautosomal Regions
PCR	Polymerase Chain Reaction
PPLEX Y	Promega PowerPlex® Y-System
RFU	Relative Fluorescence Units
SDS	Sodium Dodecyl Sulfate
STR	Short tandem repeat
SWGDAM	Scientific Working Group on DNA Analysis Methods
TE^{-4}	Tris Base with Ethylenediaminetetraacetic acid
Tris-HCl	Tris base with hydrocholic acid

Y-filer	Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit
Y-PLEX 12	Reliagene Y-PLEX™ 12
Y-STR	Y chromosomal short tandem repeat

CHAPTER ONE: INTRODUCTION

Tandemly repeated DNA sequences, which are widespread throughout the human genome, are polymorphic in nature, making them important genetic markers for mapping studies, disease diagnosis, and human identity testing (1 and 2). Short tandem repeats (STRs) contain repetitive sequence elements that are 2–7 bp in length and can be readily amplified with the polymerase chain reaction (PCR) (3-9). More than a hundred and fifty STR loci have been described on the Y chromosome, a smaller number however are useful to forensic studies (10-13). Y –STR loci comprise of di-, tri-, tetra-, and penta-nucleotide repeats with the di-nucleotide repeat possessing the most polymorphism in addition to high amount of stutter artifacts (14). STRs in general have become popular in forensic laboratories because low amounts of DNA, even in a degraded form, can be successfully typed. In addition, sample mixtures can be more readily resolved with STR results than with previously used DNA typing technologies (1 and 2).

Autosomal STRs are currently used in forensic laboratories around the world to discriminate between unrelated individuals. However, given studies which indicate males commit the majority of all crime in the United States, committing 80% of all violent crimes and 95% of all sexual offenses there are several circumstances such as paternity testing, missing persons, multiple assailant sexual assault, and sexual assaults in which Y-STR polymorphisms are a useful addition to autosomal STR polymorphisms (15 and 16).

The use of Y chromosome STR polymorphisms has become commonplace in forensic laboratories. The application includes its use in deficiency paternity testing cases (where the father is unavailable for testing and inferences are made by references from male relatives in the

male lineage) and especially to discriminate stains in forensic investigation when a male suspect is involved. In addition, Y chromosome STR analysis can be useful to detect the male fraction from sexual assault stains that comprise of male-female mixtures, where the former is at lower concentration (17-24). The ability for Y-STRs to aid in discrimination of a male individual is due largely to the specificity of the human Y chromosome.

The human Y chromosome dictates male gender by the presence of the Y chromosome, an evolutionary relic of the X chromosome which contains a number of functional genes, some of which appear to be critical in male development (25). The Y chromosome exists in three functionally different forms including the pseudoautosomal regions (PARs), the euchromatin and the heterochromatin (26). The latter two forms of the Y chromosome are of interest to forensic science because they form the nonrecombining region (NRY) of the Y chromosome (26). The majority of the Y chromosome (95%) is the NRY, which does not undergo recombination during meiosis and therefore is always in the haploid state (27) Therefore, the NRY region of the Y chromosome is inherited as a functional block in a patrilineal manner in which a haplotype of physically linked genetic markers are transmitted unchanged, barring the occasional rare mutation estimated at 0.2% per generation, from father to son (28 and 29). In human identification there are main advantages and disadvantage for using the distinct qualities of Y-STRs instead of autosomal STRs (30-36).

The determination of the gender from a source which contains forensic biological materials can be of investigative and evidentiary value (37). Accordingly, the chief advantage which Y-STRs offer over the use of autosomal STRs is the ability to obtain a male profile in the presence of a much larger quantity of female DNA. The traditional method of extracting male DNA from an admixed sample involves performing a lengthy two-step differential extraction

that occasionally produces a mixed autosomal STR profile as seen in analysis of azoospermic semen samples, estimating the number of contributors in a multiple assailant rape case and oral sodomy. In which the female DNA masks the DNA profile of the male suspect making interpretation difficult (35, 38 and 39). Therefore, a sample left at a crime scene will be informative when using Y-STRs. In particular, Y-STRs will aid in the inclusion or exclusion of suspects when male and female DNA is mixed, as seen in rape cases (40).

On the contrary Y-STR haplotypes are less likely to differentiate between two unrelated individuals, whereas autosomal STR genotypes can differentiate between two individuals with the exception of identical twins (41). To increase the potential discrimination capacity of a particular Y-STR haplotype two approaches can be explored. The first approach would be to analyze as many Y-STRs as possible. Given that this approach is extremely labor intensive and largely unfeasible a second approach which is more practical is required.

The second approach would only use the most polymorphic markers and combine them into a single multiplex PCR assay, in which a number of Y chromosome STR loci can be examined. The single multiplex PCR assay would enlarge the discrimination capacity because the product rule cannot be used to increase the power of discrimination as seen with multiple autosomal marker assays (42). The discrimination capacity can be increased to aid in the differentiation between two unrelated males using the “minimal haplotype”, “extended haplotype” (minimal haplotype plus the addition of YCAII, a highly polymorphic dinucleotide repeat) or a combination thereof set forth by the European Y chromosome typing community (35, 10 and 43-45). The haplotypes produced using a combination of the minimal haplotype loci can distinguish 74-90% of male individuals in various local European populations (35). Due to the robust nature of these loci selected in the minimal haplotype it has been successfully

employed in casework analysis (46- 51). Moreover, in 2003 the Scientific Working Group on DNA Analysis Methods (SWGDAM) selected a set of core loci for Y-STR testing the United States termed the "U.S. haplotype" (41). This set of core loci contained the minimal haplotype loci plus two additional loci. Most forensic laboratories use commercially available and validated Y-STR multiplex systems instead of in-house multiplex systems. Therefore, to better aid the crime laboratory community in choosing or utilizing a multiplex system an in-depth parallel comparison commenced.

An in-depth parallel comparison of the three main commercial Y-STR multiplex systems (Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit, Promega PowerPlex® – Y System, Reliagene Y-PLEX™ 12) was launched to glean information from circumstances in which Y chromosome polymorphisms may need to be employed. All multiplexes compared had been validated before the end of this current comparison (52, 53 and 38). The multiplex systems reviewed were in large designed in a similar fashion to better differentiate a male profile.

Examples of the multiplex system's ability to differentiate a male profile providing investigators with the numbers of semen donors in multiple assailant rape case, identification of the genetic profile of the male component in a male/female mixture, and identification of the genetic profile of the male component in a biological stain. In light of the aforementioned example, the capability of typing Y-STR loci by the crime laboratory community could dramatically affect the admissibility of Y-STR evidence. Therefore, the comparison of commercially available kits is a needed process by which the scientific community acquires the necessary information to assess the ability of a procedure to obtain reliable results, determine the conditions under which such results can be obtained and define the limitations of the procedure.

Thus the information for the study could lend itself to a standard being established amongst Y-STR kits for operational use and/or the production of a new Y-STR kit.

One example of how the comparison of the three main commercial Y-STR kits could directly impact a new standard being established is by examining post-coital samples and their extreme limits (>48 hrs) for each kit in which a full male genetic profile was observed and comparing it to other commercial Y-STR kits and in-house Y-STR multiplexes. This would help establish the types of cases where specific Y-STR kits would be most useful, and the parameters in which each kit is able to perform. Consequently, leading to the development of a highly sensitive Y-STR kit that would be more sufficient to perform with the variety of samples an operational crime laboratory would routinely analyze.

CHAPTER TWO: MATERIALS AND METHODS

DNA Isolation and Purification

Blood was collected from human subjects by venepuncture. The blood stains obtained originated from four human male individuals (Y2, Y3, Y4 and Y5) and one human female individual (X2) in accordance with the procedures established by the University's Institutional Review Board. Fifty µL drops of blood were aliquotted onto a cotton cloth and dried overnight. Semen was collected from two human males (Y2 and Y3) whom deposited semen in a sterile plastic container. Fifty µL drops of semen were aliquotted onto a cotton cloth and dried overnight. Buccal swabs were collected by swabbing the subjects inside cheek with sterile swabs and allowing them to dry overnight. Body hair was also collected by plucking three to six hairs from various body regions. The buccal swabs obtained originated from six human male individuals (Y1, Y2, Y3, Y4, Y5 and Y6) and three human female individuals (X1, X2 and X3) in accordance with the procedures established by the University's Institutional Review Board. Body hairs collected from six human male individuals (Y1, Y2, Y3, Y4, Y5, and Y6) and one human female individual (X1) were done in accordance with the procedures established by the University's Institutional Review Board. DNA was extracted from the samples using a standard organic extraction protocol (54). Swab tips or hairs were placed in a Spin-Ease tube (Gibco-BRL, Grand Island, NY) and incubated overnight at 56˚C in 400 µl DNA extraction buffer for swabs or 200 µl extraction buffer for hairs (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K, for samples containing semen 39mM DTT). The

swab tip was removed from the tube and placed into a Spin-Ease basket and placed back into its original tube and centrifuged (5415C&D) at 13,200 rpm for 5 minutes to ensure all absorbed fluid was removed from the swab tip.

The extract was purified by adding either 400 µl of 25:24:1 phenol/ chloroform/ isoamyl alcohol (Fisher Scientific, Norcross, GA) to the Spin-Ease tube or 200 µl of 25:24:1 phenol/ chloroform/ isoamyl alcohol (Fisher Scientific, Norcross, GA) to the Spin-Ease tube for hair extractions. The upper organic layer was removed and added to a Microcon concentrator (Millipore, Bedford, MA) for purification according to the manufacturer's instructions. Samples were stored in TE^{-4} (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and kept at 4˚C until analysis.

Differential Cell Lysis for the Recovery of Sperm DNA

Sperm and nonsperm cells were separated using a standard differential lysis protocol, with minor modifications (55). Post-coital cervicovaginal swabs were incubated at 37˚C in 400µL of DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K). The swab tip was removed from the tube and placed into a Spin-Ease basket and placed back into its original tube and centrifuged (5415C&D) at 13,200 rpm for 5 minutes to ensure all absorbed fluid was removed from the swab tip. The resulting supernatant, containing the nonsperm DNA fraction, was removed into a separate tube for further analysis. The sperm pellet was resuspended in 400µL of DNA extraction buffer, and 0.1 mg/mL Proteinase K and 40µL of 0.39 M DTT and incubated overnight at 56˚C. DNA from both the sperm and nonsperm fractions was isolated and purified using the phenol: chloroform method described below.

The extract was purified by adding either 400 µl of 25:24:1 phenol/ chloroform/ isoamyl alcohol (Fisher Scientific, Norcross, GA) to the Spin-Ease tube or 200 µl of 25:24:1 phenol/ chloroform/ isoamyl alcohol (Fisher Scientific, Norcross, GA) to the Spin-Ease tube for hair extractions. The upper organic layer was removed and added to a Microcon concentrator (Millipore, Bedford, MA) for purification according to the manufacturer's instructions. Samples were stored in TE^{-4} (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and kept at 4˚C until analysis.

DNA Quantitation –Yield Gel

Extracted DNA was electrophoresed in a 1% agarose gel and stained using 1% ethidium bromide (Fisher Scientific, Norcross, GA). Samples were visualized using an Ultra-lum Omega 10. Quantitation was accomplished by comparing the intensities of the unknown bands to a set of known standards run concurrently with the samples (56).

DNA Quantitation - Real Time

DNA was quantitated using the Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Human Male DNA Quantification Kit (Applied BioSystems, Foster City, CA)(57). All quantitations were prepared in accordance with the manufacturer's instructions.

Standard PCR Conditions

Multiplex Reaction Components-The standard multiplex PCR reactions were as follows: *MPI*: 25µL total volume reaction containing 3 ng template DNA, 0.69 – 1.25 µM primers (16),

250 μM dNTP's, 10x PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3.25 mM $MgCl_2$, 10 μg nonacetlyated BSA (Sigma-Aldrich, St. Louis, MO), 2.5 units AmpliTaq Gold™ DNA Polymerase (Applied Biosystems, Foster City, CA). *MPB*: 25μL total volume reaction containing 1 ng template DNA, 0.0276 – 0.075 μM primers, 250 μM dNTP's, 10x PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3.25 mM $MgCl_2$, 10 μg nonacetlyated BSA (Sigma-Aldrich, St. Louis, MO) and 2.5 units AmpliTaq Gold™ DNA Polymerase (Applied Biosystems, Foster City, CA).

Commercial Kit Reaction Components- The standard commercial kit PCR reactions were as follows (58-60): *Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit*: 25μL total volume reaction containing 1 ng template DNA, *Applied BioSystems AmpFℓSTR® Y-Filer*™ Kit PCR Reaction Mix, *AmpFℓSTR® Y-Filer*™ Kit Primer Set and 5 units AmpliTaq™ Gold DNA Polymerase. The reaction was prepared in accordance with manufacturer's instructions. *Promega PowerPlex® – Y System*: 25μL total volume reaction containing 0.5- 1 ng template DNA, Gold STAR 10X Buffer, PowerPlex® Y 10X Primer Pair Mix, 2.75 units AmpliTaq Gold™ DNA Polymerase. The reaction was prepared in accordance with manufacturer's instructions. *Reliagene Y-PLEX™ 12*: 25μL total volume reaction containing 1-2 ng template DNA, 2.5X Y-PLEX™ 12 Primer Mix, 2.5 units AmpliTaq Gold™ DNA Polymerase. The reaction was prepared in accordance with manufacturer's instructions.

Primers-Either the forward or reverse primers of MPI and MPB were labeled with a fluorescent phosphoamidite dye (Invitrogen, Grand Island, NY) (tables 1 and 2). All commercial Y-STR kit primer concentrations are proprietary and provided by the manufacturer in the kit

(tables 1 and 3). The forward and reverse primer concentrations for the two multiplexes are as follows: *MPI*: DYS393- 0.055 μM, DYS392- 1.0 μM, DYS391-0.065 μM, DYS389-0.15 μM, DYS438- 0.15 μM, Y-GATA-A7.2-0.10 μM, and DYS385- 1.0 μM. *MPB*: DYS393- 0.0276 μM, DYS389- 0.075 μM, Y-GATA-H4- 0.05 μM, Y-GATA-A7.2- 0.045 μM.

Table 1- In-house multiplex systems locus specific information. Information provided includes the loci included in each system, the fluorescent dyes used, GenBank® Accession number, and repeat sequence.

Multiplex I

STR Locus	Label	GenBank® Accession Number	Repeat Sequence 5'→ 3'
DYS391	FAM	GO9613	TCTA
DYS392	FAM	GO9867	TAT
DYS393	FAM	G09601	AGAT
DYS389I/II	TET	AF140635	TCTA/TCTG
YGATA A7.2	HEX	G42671	TAGA
DYS438	HEX	AC002531	TTTTC
DYS385 a/b	HEX	Z93950	GAAA

Multiplex B

STR Locus	Label	GenBank® Accession Number	Repeat Sequence 5'→ 3'
DYS393	FAM	G09601	AGAT
DYS389I/II	TET	AF140635	TCTA/TCTG
YGATA A7.2	HEX	G42671	TAGA
YGATA H4	HEX	G42676	TAGA

Table 2- Y-STR multiplex systems characteristics. Information provided includes the loci included in each system, the fluorescent dyes used, optimum cycle number, recommended quantity of DNA added to a PCR reaction, and total PCR volume.

Commercial Y-STR Multiplexes	**Number of Loci**	**Labels**	**Optimum Number of PCR Cycles**	**Recommended (ng) of DNA added to PCR reaction**	**Total PCR Reaction (μL)**
Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit	(16) *DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385 a/b, DYS393, DYS391, DYS439, DYS635, DYS392, Y-GATA H4, DYS437, DYS438, DYS448*	6-FAM VIC NED PET	30	1	25
Promega Promega PowerPlex® – Y System	(11) *DYS391, DYS389I, DYS439, DYS389II, DYS438, DYS437, DYS19, DYS392, DYS 393, DYS390, DYS385a/b*	FL TMR JOE	32	2	25
Reliagene Y-PLEX™ 12	(11) *DYS392, DYS390, DYS385a/b, DYS393, DYS389I, DYS391, DYS389II, AMEL, DYS19, DYS439, DYS438*	6-FAM JOE NED	30	1-2	25
UCF Multiplexes	**Number of Loci**	**Labels**	**Optimum Number of PCR Cycles**	**Standard quantity of DNA added to PCR reaction (ng)**	**Total PCR Reaction (μL)**
Multiplex I (MPI)	(8) *DYS391, DYS392, DYS393, DYS389I, DYS389II, Y-GATA A7.2, DYS438, DYS385a/b*	6-FAM TET HEX	35	3	25
Multiplex B (MPB)	(5) *DYS393, DYS389I, DYS389II, Y-GATA A7.2, Y-GATA H4*	6-FAM TET HEX	45	1	25

Table 3- Commercial multiplex systems locus specific information. Information provided includes the loci included in each system, the fluorescent dyes used, GenBank® Accession number, and repeat sequence.

Applied BioSystems AmpFℓSTR®
Y-Filer™

STR Locus	Label	GenBank® Accession Number	Repeat Sequence 5'→ 3'
DYS456	FAM	AC010106	AGAT
DYS389I	FAM	AF140635	TCTA/TCTG
DYS390	FAM	AC011289	TCTG/TCTA
DYS389II	VIC	AF140635	TCTA/TCTG
DYS458	VIC	AC010902	GAAA
DYS19	VIC	X77751	TAGA
DYS385 a/b	NED	Z93950	GAAA
DYS393	NED	G09601	AGAT
DYS391	NED	G09613	TCTA
DYS439	NED	AC002992	GATA
DYS635	NED	AC004772 (r&c)	TSTA compound
DYS392	PET	G09867	TAT
Y GATA H4	PET	G42676	TAGA
DYS437	PET	AC002992	TCTA/TCTG
DYS438	PET	AC002531	TTTTC
DYS448	PET	AC025227	AGAGAT

Reliagene
Y-PLEX 12™

STR Locus	Label	GenBank® Accession Number	Repeat Sequence 5'→ 3'
DYS392	FAM	G09867	TAT
DYS390	FAM	G09611	TCTA/TCTG
DYS385 a/b	FAM	Z93950	GAAA
DYS393	JOE	G09601	AGAT
DYS389I	JOE	G09600	TCTG/TCTA
DYS391	JOE	G09613	TCTA
DYS389II	JOE	G09600	TCTG/TCTA
AMELOGENIN	NED	M55418 & M55419	--
DYS19	NED	X77751	TAGA
DYS439	NED	AC002992	GATA
DYS438	NED	AC002531	TTTTC

Promega PowerPlex®
Y-System

STR Locus	Label	GenBank® Accession Number	Repeat Sequence 5'→ 3'
DYS391	FL	G09613	TCTA
DYS389I/II	FL	AF140635	TCTG/TCTA
DYS439	FL	AC002992	GATA
DYS393	TMR	G09601	AGAT
DYS390	TMR	AC011289	TCTA/TCTG
DYS385 a/b	TMR	Z93950	GAAA
DYS438	JOE	AC002531	TTTTC
DYS437	JOE	AC002992	TCTA/TCTG
DYS19	JOE	X77751	TAGA
DYS392	JOE	G09867	TAT

Cycling Conditions- The cycling conditions were: *MPI*: 95˚C 11 minute hot start; 2 cycles: 96˚C 30 s, 62˚C 1 minute, 72˚C 1 minute; 2 cycles 96˚C 30 s, 60˚C 1 minute, 72˚C 1 minute; 31 cycles: 96˚C 30 s, 58˚C 1 minute, 72˚C 1 minute; final extension 72˚C 45 minutes. *MPB*: 95˚C 11 minute hot start; 2 cycles: 96˚C 30 s, 62˚C 1.5 minute, 72˚C 1 minute; 2 cycles 96˚C 30 s, 60˚C 1.5 minute, 72˚C 1 minute; 41 cycles: 96˚C 30 s, 58˚C 1.5 minute, 72˚C 1 minute; final extension 72˚C 45 minute. *Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit (52 and 58):* 95˚C 11 minute hot start; 30 cycles: 94˚C 1 minute, 61˚C 1 minute, 72˚C 1 minute; final extension 60˚C 80 minute. *Promega PowerPlex® – Y System (53 and 59)*: 95˚C 11 minute hot start; 96˚C 1 minute, 10 cycles: ramp 100% 94˚C 30 s, ramp 29% 60˚C 30 s, ramp 23% 70˚C 45 s; 18-22 cycles: 100% 90˚C 30 s, ramp 29% 58˚C 30 s, ramp 23% 70˚C 45 s; final extension 60˚C 30 minute. *Reliagene Y-PLEX™ 12 (38 and 60)*: 95˚C 01 minute hot start; 30 cycles: 94˚C 1 minute, 58˚C 1 minute, 70˚C 1 minute; final extension 60˚C 60 minute. All multiplex systems included a final extension time of at least 30 minutes to the amplification protocol to ensure full terminal nucleotide addition (61). Terminal nucleotide addition occurs when *Taq* DNA polymerase adds a nucleotide, generally an adenine, to the 3' ends of amplified DNA fragments in a template-independent manner (62 and 63). Generally, the artifact band is one base shorter than expected causing a split peak to appear on the electropherogram.

Sample Electrophoresis and Data Analysis – The amplified product was detected using a Macintosh based ABI Prism 310 capillary electrophoresis system (Applied Biosystems, Foster City, CA). *MPI*: 1 µl of PCR product was added to 24 µl Hi-Di formamide (Applied Biosystems, Foster City, CA) and 1 µl of the GeneScan 500 TAMRA internal lane standard (Applied

Biosystems, Foster City, CA). *MPB*: 1 µl of PCR product was added to 24 µl Hi-Di formamide (Applied Biosystems, Foster City, CA) and 1 µl of the GeneScan 500 TAMRA internal lane standard (Applied Biosystems, Foster City, CA). *Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit*: 1.5 µl of PCR product was added to 24.5 µl Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.5 µl of the GeneScan 500 LIZ internal lane standard (Applied Biosystems, Foster City, CA). *Promega PowerPlex® – Y System*: 1 µl of PCR product was added to 24 µl Hi-Di formamide (Applied Biosystems, Foster City, CA) and 1 µl of the ILS 600 internal lane standard (Promega). *Reliagene Y-PLEX™ 12*: 1.5 µl of PCR product was added to 24.5 µl Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.5 µl of the GeneScan 500 ROX internal lane standard (Applied Biosystems, Foster City, CA). Samples analyzed with MPI and MPB were injected using Module C (5 s injection, 15 kV, 60˚C, Filter set C). Samples analyzed with *Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit* were injected using Module G5v2 (5 s injection, 15kV, 60˚C, Filter set G5v2). Samples analyzed with *Promega PowerPlex® – Y System* were injected using Module A (5 s injection, 15kV, 60˚C, Filter Set A). Samples analyzed with *Reliagene Y-PLEX™ 12* were injected using Module F (5 s injection, 15kV, 60˚C, Filter Set F). All samples were heated at 95˚C for 3 minute and snap-cooled for no less than 3 minute. In addition, all samples were subjected to capillary electrophoresis, detected by laser-induced fluorescence, and analyzed by GeneScan Analysis 3.1.2 software (Applied Biosystems, Foster City, CA).

PCR Parameters

Thermocycling Conditions- The number of cycles for all kits with 35 cycles or less were increased in order to potentially improve the kits. The various cycle numbers added to the final set of cycles were 34, 35, and 40, since the preliminary determined standard cycling conditions were 30 (Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit), 35 (Multiplex 1), 32 (Promega PowerPlex® – Y System), and 30 (Reliagene Y-PLEX™ 12).

Sensitivity Studies

DNA from three males (Y1, Y2, and Y3) and three females (X1, X2, and X3) were tested using the two in-house multiplexes and the three commercially available kits. The quantities of male DNA used were 10 pg, 20 pg, 30 pg, 45 pg, 50 pg, 60 pg, 100 pg, 200 pg, 500 pg, 1 ng, 3 ng, 30 ng, 300 ng. The quantities of female DNA tested were 3 ng, 30 ng, 300 ng, and 1µg. The GeneScan Analysis threshold for these studies was set at 100 RFU.

Mixture Studies

Male/Female- In the first set of mixture experiments the male component was held constant at 1 ng while the female component was added in varying quantities and the entire sample amplified. The admixed male/female samples comprised of two differing males and two differing females; *Mixture 1*: (Y3 & X2); *Mixture 2*: (Y2 & X3). Male/female ratios tested were 1:1 (1 ng male DNA/1 ng female DNA), 1:10 (1 ng male DNA/ 10 ng female DNA), 1:100 (1 ng male DNA/ 100 ng female DNA), 1:500 (1 ng male DNA/ 500 ng female DNA), 1:600 (1 ng male DNA/ 600 ng female DNA), 1:800 (1 ng male DNA/ 800 ng female DNA), 1:1000 (1 ng male DNA/ 1000 ng female DNA), 1:2000 (1 ng male DNA/ 2000 ng female DNA), 1:3000 (1

ng male DNA/ 3000 ng female DNA), 1:4000 (1 ng male DNA/ 4000 ng female DNA), 1:5000 (1 ng male DNA/ 5000 ng female DNA), and 1:6000 (1 ng male DNA/ 6000 ng female DNA).

In the second set of mixture experiments the total input DNA was held constant at 300 ng and the entire sample amplified. The admixed male/female samples comprised of two differing males and two differing females; *Mixture 1*: (Y1 & X1); *Mixture 2*: (Y2 & X3), *Mixture 3*: (Y3 & X2). Male/female ratios tested were 1:1 (150 ng male DNA/150 ng female DNA), 1:10 (27.2 ng male DNA/ 272.8 ng female DNA), 1:100 (2.97 ng male DNA/ 297 ng female DNA), 1:500 (0.6 ng male DNA/ 299.4 ng female DNA), 1:600 (0.5 ng male DNA/ 299.5 ng female DNA), 1:800 (0.4 ng male DNA/ 299.63 ng female DNA), 1:1000 (0.3 ng male DNA/ 299.7 ng female DNA), 1:2000 (0.15 ng male DNA/ 299.8 ng female DNA), 1:3000 (0.10 ng male DNA/ 299.9 ng female DNA), 1:4000 (0.07 ng male DNA/ 299.9 ng female DNA), 1:5000 (0.06 ng male DNA/ 299.9 ng female DNA), 1:6000 (0.05 ng male DNA/ 299.9 ng female DNA), 1:8000 (0.04 ng male DNA/ 299.9 ng female DNA), 1:10,000 (0.03 ng male DNA/ 299.9 ng female DNA), 1:15,000 (0.02 ng male DNA/ 299.9 ng female DNA), 1:20000 (0.015 ng male DNA/ 299.9 ng female DNA), 1:21,000 (0.01 ng male DNA/ 299.9 ng female DNA), 1:22,000 (0.01 ng male DNA/ 299.9 ng female DNA), 1:25,000 (0.01 ng male DNA/ 299.9 ng female DNA), 1:28,000 (0.01 ng male DNA/ 299.99 ng female DNA), 1:30,000 (0.01 ng male DNA/ 299.99 ng female DNA) and 1:32,000 (0.01 ng male DNA/ 299.9 ng female DNA).

Male/Male- For mixture studies, DNA from four males were combined to make three male/male mixtures; *Mixture 1*: (Y3 & Y2); *Mixture 2*: (Y4 & Y6) and *Mixture 3*: (Y5 & Y1). A total of 1 ng of DNA for the minor component with an increasing quantity of DNA for the major component were added to standard MPI, MPB, Applied Biosystems AmpFℓSTR® Yfiler™ PCR

Amplification Kit, Promega PowerPlex® – Y System, and Reliagene Y-PLEX™ 12 reactions. Ratios tested were 1/1(1ng/1ng), 1:5(1ng/5ng), 1:10(1ng/10ng) and 1:20(1ng/20ng).

Female DNA Cross-Reactivity

To determine the amount of female DNA cross-reactivity, all five multiplexes were tested using increasing quantities of female DNA (3ng, 30ng, 300ng, 1µg) according to their respective standard conditions.

Species Specificity

Thirteen different species were tested during this study; all the species blood was donated by various sources. Non-primate blood was received from a number of sources: Tuscawilla Oaks Animal Hospital, Oviedo, FL (male cat and male dog); HemoStat Laboratories, Dixen, CA (male cow, male horse, and male sheep); Charles R. Daniels, DeLand, FL (male deer); West End Animal Hospital, Gainesville, FL (male ferret). Non-human primate blood was acquired from Coriell Cell Repository, Camden, NJ (male and female gorilla, male and female orangutan, male and female chimpanzee, male macaque, female pygmy chimpanzee and male spider monkey). The DNA was extracted using a standard organic extraction as by DNA extraction protocol. For amplification 1-3 ng of male DNA or 300 ng female DNA was added to the reaction respective to individual kits.

Stability Studies

Consistency of Y-STR Haplotypes in Different Tissues from the Same Individual- The consistency of Y-STR typing within different tissues of the same individual was confirmed by amplifying DNA extracted from blood, saliva, hair and vaginal secretions or semen collected from the same individual. Samples were obtained from four male individuals and one female individual.

Environmental Effects on Stability- Samples for the degradation studies were prepared by pipetting 50µL of liquid blood or semen onto a sterile cotton swatch, and allowed to dry overnight. The samples were exposed to the environment on July 11, 2005, in a small, fenced in, metropolitan area in Orlando, Florida. Samples were exposed for 1 day, 2 days, 1 week, 3 weeks and 6 weeks then collected and stored at -20˚C until analysis. Samples were extracted using a standard organic extraction as described in extraction protocol. The quantity of DNA added to each reaction varied depending on the condition of the sample.

Post-Coital Cervicovaginal Comparison

A post-coital cervicovaginal swab was obtained by two female volunteers who recovered the samples after coitus at the time points desired. To insure that the post-coital swabs were void of any previous male DNA, a pre-coital cervicovaginal swab was also obtained before coitus commenced but after a celebratory period of at least five days. The time points collected were: 0 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 144 hours, and 168 hours.

DNA was isolated using both a standard non-differential extraction, resulting in an extract containing both male and female DNA in an unknown ratio; and a differential extraction procedure, thus resulting in a separate male and female extracts. For nondifferentially extracted

samples 300ng of extract was added to standard PCR reactions for all multiplexes. In differentially extracted samples, only the male portion was added to the standard PCR reaction using 1ng of input DNA.

CHAPTER THREE: RESULTS AND DISCUSSION

PCR Parameters

The cycling conditions for all kits with 35 cycles or less (MPI, Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit and Reliagene Y-PLEX™ 12 systems) were increased in order to potentially improve the kits. The various cycle numbers used were applied to predetermined sensitivity and specificity limits using 5 pg to 300 ng template DNA and with a male/female DNA ratio ranging from 1:100 to 1:20,000. After increasing the cycling conditions for all aforementioned Y-STR systems a trend began to materialize. When the cycles were increased in MPI, Reliagene Y-PLEX™ 12, and Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit from the standard number of cycles to 40 cycles a significant amount of stutter above 15% of the parent allele was observed. Stutter products or repeat slippage, artifacts associated with *Taq* DNA polymerase, is due to the loss of a repeat unit during DNA amplification, somatic variation with in the DNA in sample material, or both (64 and 65). This effect caused full profiles to become partial profiles or the loss of the profile altogether as seen in the Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit and Reliagene Y-PLEX™ 12 systems. In addition, the Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit and Reliagene Y-PLEX™ 12 systems were also tested using 34 and 35 cycles. The results obtained showed similar results as when the cycles were increased to 40 cycles. Overall, the amount of stutter increased as the number of alleles involved decreased (Fig. 1).

In light of the results procured from the increase in cycle number for all multiplexes except MPB using the already predetermined sensitivity and specificity limits to determine if

there was an increase in alleles called the cycle number was not increased beyond the manufacturers preferred cycling conditions. For the reason that none of the multiplexes were able to consistently show an increase in allele call with the samples tested. As the numbers of cycles were increased the stutter also increased to greater than 15% of the parent allele in Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit and Reliagene Y-PLEX™ 12 systems. Promega PowerPlex® – Y System gave the same result of increased stutter at 35 cycles, as MPI did at 40 cycles.

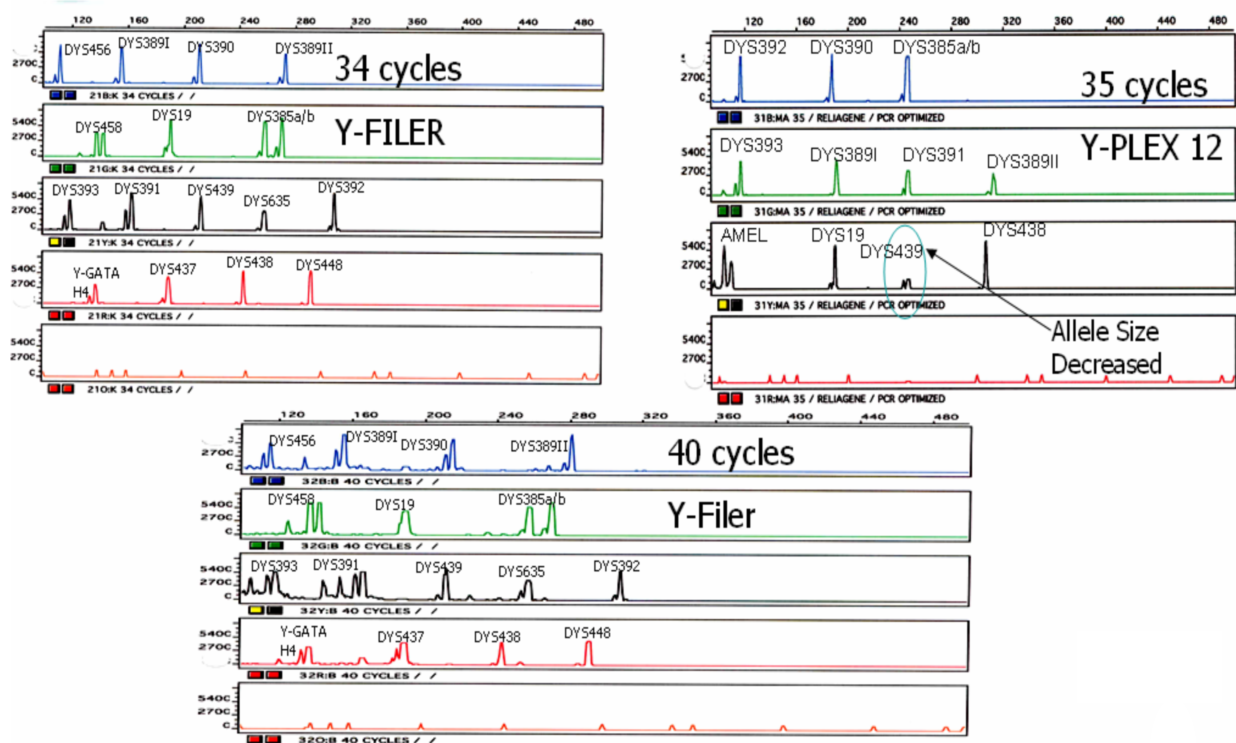


Figure 1- PCR Conditions Profile with Increased Cycle Number

Sensitivity

The sensitivity for all Y-STR systems was tested using three different male DNA samples and one female DNA sample as a control. Comparable results were obtained for the three male samples. While the optimum quantity of DNA added to each reaction ranged from 1-3ng, all Y-STR systems proved effective over a wider range of input template DNA (Table 4). The lower limit of template DNA necessary for a full seventeen-locus Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit profile was 0.045ng (Figure 2a). A partial profile was obtained using 0.040 ng, 0.035 ng and 0.020 ng of template DNA to obtain a thirteen-, ten-, and nine-locus Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit profile respectively. The profile was completely lost when 0.010ng of template DNA was added to the reaction. Similarly, the lower limit of template DNA necessary for a full nine-locus MPI profile was 0.050 ng (Figure 2d). A partial profile was obtained using 0.040 ng, 0.030 ng, 0.020 ng of template DNA to obtain a six-, five-, and two- locus MPI profile respectively. The profile was completely lost when 0.010 ng of template DNA was added to the reaction. The average lower limit of template DNA necessary for a full twelve-locus Promega PowerPlex® – Y System profile was 0.030 ng (Figure 2b). A partial profile was obtained using 0.015 ng of template DNA to obtain an eight-locus Promega PowerPlex® – Y System profile. The profile was completely lost when 0.005ng of template DNA was added to the reaction. Correspondingly, the average lower limit of template DNA necessary for a full five-locus MPB profile was 0.030ng (Figure 2e). A partial profile was obtained using 0.010ng to obtain a three-locus MPB profile. The profile was completely lost when 0.004ng of template DNA was added to the reaction. In the same way, the lower limit of template DNA necessary for a full twelve-locus Reliagene Y-PLEX™ 12 profile was 0.100 ng (Figure 2c). A partial profile was obtained using 0.050 ng of template DNA to

obtain an eleven-locus Reliagene Y-PLEX™ 12 profile. The profile was completely lost when 0.005ng of template DNA was added to the reaction.

The study of sensitivities ranging from 5 pg to 300ng template DNA illustrated that Promega PowerPlex® – Y System and MPB are the most sensitive Y-STR kits with full profile sensitivities of 20 pg in unique samples. However, these kits differ when comparing the partial profiles, MPB was able to achieve a partial profile at 10pg where as Promega PowerPlex® – Y System achieved a partial profile at 15 pg. In addition, the partial profiles of Promega PowerPlex® – Y System had five common core loci drop out; MPB on the other hand only had one common allele drop out. Both kits were unable to detect profiles at 5 pg. All other multiplexes reviewed did not render as sensitive results.

Table 4- Sensitivity Full/Partial/Lost Profile Details

Y-STR Multiplex	Full Profile (ng)	Partial Profile (ng)	Lost Profile (ng)	Reported Limits (ng)
Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit	0.045	0.04	0.01	0.05-0.1
Promega PowerPlex® – Y System	0.03	0.015	0.005	<0.25
Reliagene Y-PLEX™ 12	0.1	0.05	0.005	0.1
Multiplex I (MPI)	0.05	0.04	0.01	0.20
Multiplex B (MPB)	0.03	0.01	0.004	NA

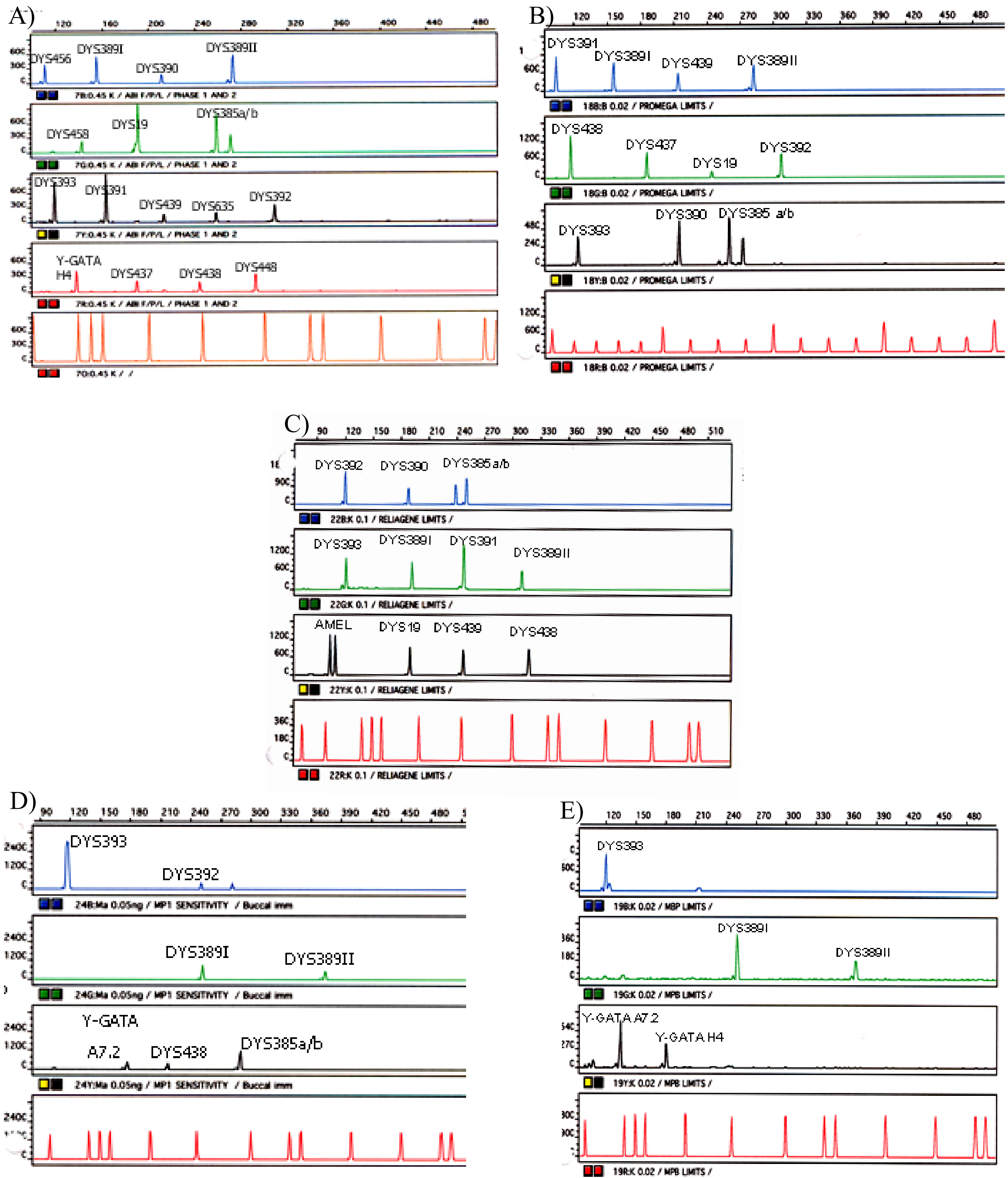


Figure 2- Multiplex System Profiles for Sensitivity

Female DNA Cross-Reactivity

Due to the evolutionary history of the Y-chromosome, Y-STR kits are able to determine the genetic profile of a male donor in the presence of excess female DNA. This ability is possible due to sequence homology that is different from the X-chromosome. However, the Y-chromosome still retains a considerable degree of sequence homology to the X-chromosome (26). Consequently, primers for Y-STR kits are designed to recognize specific Y-STR loci; nevertheless they still may also possess homology sequences on the X-chromosome. The degree of homology will determine the extent of X-chromosome artifacts detected when using DNA isolated from a male (XY) versus a female (XX).

Using all five multiplexes and increasing quantities of female DNA ranging from 3ng-1μg aided in identification of female DNA products in the ranges of Y-STR loci (Table 5). After the analysis of all Y-STR systems only Promega PowerPlex® – Y System, MPI, and MPB displayed female products in the ranges of Y-STR loci. The two remaining kits Reliagene Y-PLEX™ 12 and Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit proved to be highly specific for the Y-chromosome because no significant female DNA products were observed.

Two female products were observed using Promega PowerPlex® – Y System, one product was observed in the blue channel within the allele range of DYS391 once 300ng of female DNA was added to the system. The second product was observed located in the green channel was found in the range of DYS392 when 1μg of female DNA was added. Alternatively, in the blue channel MPI and MPB both displayed female DNA products in the DYS393 allele

range using a variety of female DNA quantities; this was counteracted in later studies by using HPLC purified primers to remove the female product in this range (Table 5)(22, 24 and 66). In addition, MPI had a female product in the yellow channel located in the DYS385a allele range which was only observable when 30ng of female DNA was added to the system. Female DNA artifacts can be explained by the binding of certain Y-STR primers to homologous, but not identical, sequences on the X-chromosome (16).

In light of the results the Y-STR loci effected by an abundant amount of Female DNA, Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit displayed zero artifacts from any of the titrations; Reliagene Y-PLEX™ 12 displayed a constant artifact at 104 in all windows which is a direct result of Amelogenin in the yellow channel. On the other hand, MPI, MPB, and Promega PowerPlex® – Y System all displayed at least one artifact in an allele calling region. In addition to determining that there are no female artifacts, we also determined that Applied BioSystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit is able to produce reliable human male genetic profile while in the presence of overwhelming quantities of Female DNA, a valuable advantage when used with forensic casework involving admixed samples procured from a female environment, such as a sexual assault sample.

Table 5- Female Titration 3, 30, 300, 1000 ng of Female DNA inputted into various Y-STR systems and female artifacts found in Y allele ranges recorded.

Multiplex System	Channel	Loci	Quantity of Female DNA added
MPI	Blue Yellow	DYS393 DYS385 a	3 ng-1 µg 30 ng
MPB	Blue	DYS393	3 ng-1 µg
Promega PowerPlex® – Y System	Blue Green	DYS391 DYS392	300 ng 1µg

Mixture Studies

Male/Female Mixtures- The aim of this study was to correctly determine the genetic profile of the male donor in the presence of large quantities of female DNA using the Y-STR multiplex systems. This study which is more akin to bona fide forensic casework a series of samples were prepared in which the total input of DNA was 300ng an admixture of male and female DNA in varying quantities. In addition, a second male/female mixture study was also preformed in which 1 ng of male DNA was added to an increasing amount of female DNA (Table 6). The ability for the Y-STR systems to dissect out a male profile from a male/female admixture is a unique advantage which allows for the inclusion of a greater quantity of total DNA added to a system than accommodated by autosomal systems. A full seventeen-locus profile was easily obtained using the Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit and 1ng of male DNA admixed with 3μg of female DNA (Figure 3ai) and a full profile with the male component comprising of 1/4000th of the total admixture (0.08ng/299.9ng) (Figure3bi). Furthermore, a full five-locus profile was easily obtained using the MPB and 1ng of male DNA admixed with 1μg of female DNA (Figure 3aii) and a full profile with the male component comprising on average of 1/16000th of the total admixture (0.02ng/299.9ng) (Figure3bii). In addition, a full twelve-locus profile as easily obtained using the Promega PowerPlex® – Y System and 1ng of male DNA admixed with 1μg of female DNA (Figure 3aiii) and a full profile with the male component comprising on average of 1/10000th of the total admixture (0.03ng/299.9ng) (Figure3biii). Notably, partial and lost profiles were also obtained upon further dilution of the male component with an increase in the female component

and continuing to decrease the male component all the while continuing to add a total of 300ng of DNA to the system. The number of loci also continued to decrease upon further dilution of the male component. For example, a three- and two locus profile was obtained using the Promega PowerPlex® – Y System at a dilution of 1/150000 and 1/20000 respectively and an eight- and five locus profile at a dilution of 1/2000 and 1/3000 respectively (data not shown). Lost profiles were obtained by continuing to increase the female contribution, further diluting the male contribution.

The specificity using male female mixtures yielded results for Promega PowerPlex® – Y System establishing a full profile using 300ng of input DNA in a male/female 1/ 10,000 dilution. On the other hand, MPB is able to give a full profile at a male/female at 1/20,000 using 300ng of input DNA. These results obtained from Promega PowerPlex® – Y System and MPB is an outstanding level of specificity. The specificities from these two multiplexes lend itself to forensic studies because only a few male cells are needed to obtain a full genetic haplotype in the presence of overwhelming female cells. All other multiplexes did not perform as well as these two multiplexes using the 300 ng input of male/female mixture. The least specific multiplex was Reliagene Y-PLEX™ 12, with all remaining multiplexes producing specificity results between Reliagene Y-PLEX™ 12 and MPB.

Shifting to the second set of specificity conditions, Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit has shown to have a specificity of 1:3000 using 1 ng Male DNA input and 3,000 ng Female DNA input. As a result Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit is the most specific kit when using 1ng of Male DNA and overwhelming quantities of Female DNA. MPI, MPB and Promega PowerPlex® – Y System have relatively the some specificity when using 1 ng of male DNA in a mixture with an overwhelming amount of

female DNA. The least specific multiplex when using these conditions was Reliagene Y-PLEX™ 12 with 1:100.

Table 6- Specificity 1ng male DNA with increasing female DNA added or 300 ng total DNA added as a male/female admixture.

Y-STR Multiplex	Full Profile	Partial Profile	Lost Profile	Manufacturer's Reported Limits
AmpFℓSTR® Yfiler™				
1 ng Male with increasing Female DNA added	1:3000	1:4000	1:8000	NA
300 ng total DNA added	1:4000	1:5000	1:15,000	1:2000
Promega PowerPlex® – Y System				
1 ng Male with increasing Female DNA added	1:1000	1:2000	1:5000	NA
300 ng total DNA added	1:10,000	1:15,000	1:22,000	
Reliagene Y-PLEX™ 12				
1 ng Male with increasing Female DNA added	1:100	1:300	1:1000	NA
300 ng total DNA added	1:100	1:300	1:2000	1:600-1:800
Multiplex I (MPI)				
1 ng Male with increasing Female DNA added	1:1000	1:2000	1:5000	NA
300 ng total DNA added	1:100	1:500	1:5000	1:2000
Multiplex B (MPB)				
1 ng Male with increasing Female DNA added	1:1000	1:2000	1:8000	NA
300 ng total DNA added	1:16,000	1:20,000	1:30,000	

Figure 3 a - Specificity limits for Y-STR multiplexes which had highest limit threshold using 1ng Male DNA with increasing quantity of Female DNA added. i) Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit 1:3000; ii) Multiplex B 1:1000; iii) Promega PowerPlex® – Y System 1:1000

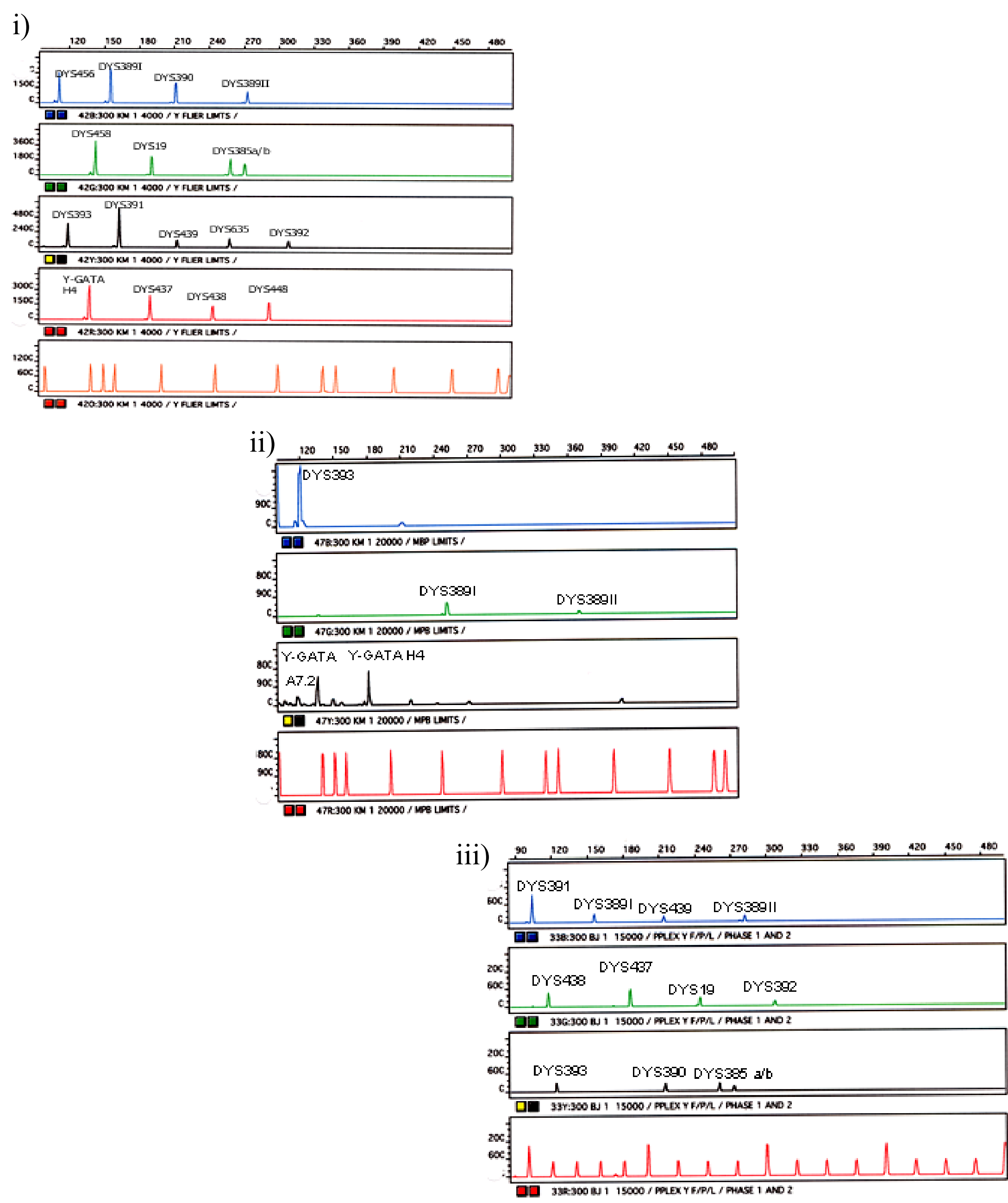


Figure 3b - Specificity limits for Y-STR multiplexes which had highest limit threshold using 300ng total DNA in ratios of male: female DNA. i) Applied Biosystems AmpFℓSTR® Yfiler™ PCR Amplification Kit 1:4000; ii) Multiplex B 1:20,000; iii) Promega PowerPlex® – Y System 1:15,000

Male/Male Mixtures- The number of male contributors should be easily obtained by the hemizygous state of the Y-chromosome (12 and 79). Therefore, the presence of a single allele from each contributor will ascertain the number of donors. To determine the limit at which two males in a mixed sample could be correctly detected and typed, DNA from six male individuals were arranged into three mixtures in various ratios (1:1, 1:5, 1:10, and 1:20). All Y-STR systems were amplified with two, six, eleven, and twenty-one ng of male DNA respectively (Table 7). The presence of two male individuals was determined by the presence of two allelic signals at a single locus except DYS385. The mixtures were chosen because each individual male component differed from the other at two or more loci.

The minor component was easily discernable at the ratios 1:1, 1:5 with all multiplexes compared. Conversely, the Promega PowerPlex® – Y System, MPB and Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit the presence of two males in each mixture could be established additional at a 1:10 mixture and only at a few loci with the 1:20 mixture. Furthermore, when the mixture was in excess of 1:10 the drop-out of the unshared minor contributor's alleles became significant and the major contributor's alleles were more discernable in some cases causing the minor alleles to become mixed with stutter products from the major component (Figure 4). Therefore, all multiplex systems performed adequately when male DNA was in a mixture with a second male contributor. This phase of the comparison represents an important part of forensic casework. The ability to discern the number of contributors as well as the relative ratios in which they exist aids in the determination of multiple assailant rape cases and consensual coital activities followed by a forced act from an assailant, as seen in some rape cases.

Table 7- Male/Male Mixtures Profile of Minor Component. Results illustrate drop-out of unshared minor contributor alleles at specific dilutions.

Y-STR Multiplex	**1:1**	**1:5**	**1:10**	**1:20**
AmpFℓSTR® Yfiler™				
Mixture 1	10/10	7/10	4/10	4/10
Mixture 2	13/13	11/13	6/13	6/13
Mixture 3	12/12	7/12	5/12	3/12
Promega PowerPlex® – Y System				
Mixture 1	6/6	4/6	5/6	3/6
Mixture 2	8/8	8/8	4/8	2/8
Mixture 3	9/9	7/9	5/9	4/9
Reliagene Y-PLEX™ 12				
Mixture 1	5/5	5/5	4/5	3/5
Mixture 2	6/6	5/6	3/6	1/6
Mixture 3	9/9	6/9	4/9	3/9
Multiplex I (MPI)				
Mixture 1	6/6	6/6	4/6	2/6
Mixture 2	4/4	3/4	1/4	1/4
Mixture 3	6/6	6/6	2/6	1/6
Multiplex B (MPB)				
Mixture 1	4/4	4/4	3/4	1/4
Mixture 2	2/2	2/2	2/2	2/2
Mixture 3	3/3	2/3	2/3	2/3

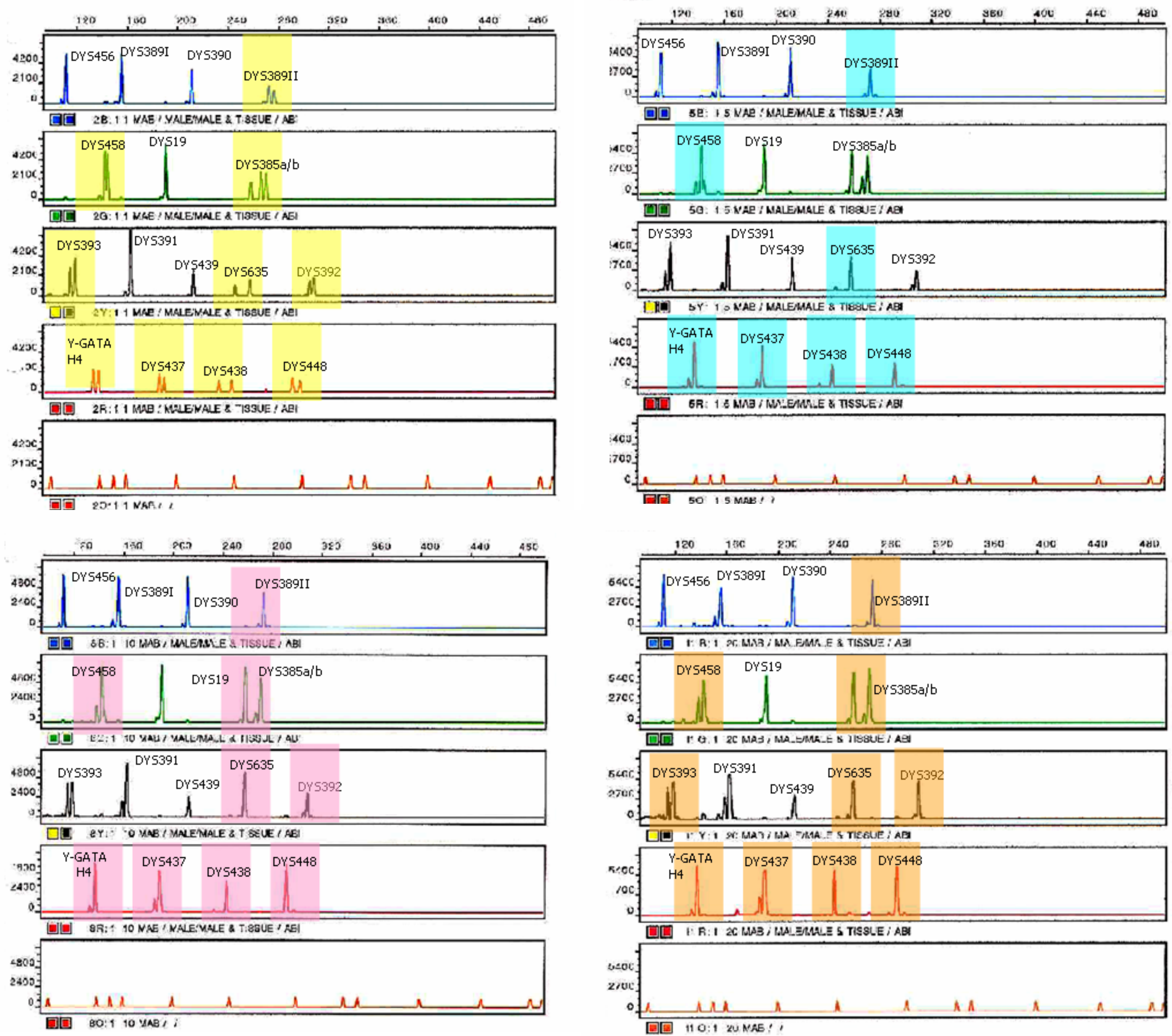


Figure 4- Male/Male Mixture Electropherograms. Minor contributor's allele dropout highlighted in blue, pink and orange. Mixed loci highlighted in yellow.

Stability Studies

Consistency of Y-STR Haplotypes in Different Tissues from the Same Individual- The ability to consistently identify a Y-STR haplotype by typing different tissues from the same individual has a momentous place in forensic science; because of the ability to correctly correlate a sample of one tissue type to an individual of interest, victim, or scene using a more common or easier tissue to be collected. All Y-STR multiplexes were typed using blood, semen, saliva and hair from four male individuals and blood, vaginal secretions, saliva and hair from one female (Table 8). Full identical haplotypes were obtained using the variety of tissue samples for all male individuals with all Y-STR systems. The female sample was used as a control and no tissue sample provided was able to be typed, except for the Reliagene Y-PLEX™ 12 system where in the amelogenin range a product with a size of 104.0 bp in the yellow channel was detected, consistent with a female sample (data not shown).

The ability to obtain full profiles from multiple sources is important to forensic casework and all multiplexes performed relatively equal. Some problems do exist however when comparing different tissues. The ability to obtain a full profile from hair samples can be inconsistent because of the nature of hair itself and the sampling technique. This must be carefully observed and should be used only as a comparative tissue when all others are not available. Meanwhile, in this current study all multiplexes were able to consistently provide an identical full haplotype for all male samples.

Table 8- Somatic Stability using 1ng Male DNA or 300 ng Female DNA. Results display total number of alleles correctly analyzed and called.

Y-STR Multiplex	Saliva	Blood	Semen	Hair	Vaginal Secretions
AmpFℓSTR® Yfiler™					
Male 1	16/16	16/16	16/16	16/16	NA
Male 2	16/16	16/16	16/16	16/16	NA
Male 3	16/16	16/16	16/16	16/16	NA
Male 4	16/16	16/16	16/16	16/16	NA
Female 1	0/16	0/16	NA	0/16	0/16
PowerPlex® – Y System					
Male 1	11/11	11/11	11/11	11/11	NA
Male 2	11/11	11/11	11/11	11/11	NA
Male 3	11/11	11/11	11/11	11/11	NA
Male 4	11/11	11/11	11/11	11/11	NA
Female 1	0/11	0/11	NA	0/11	0/11
Reliagene Y-PLEX™ 12					
Male 1	11/11	11/11	11/11	11/11	NA
Male 2	11/11	11/11	11/11	11/11	NA
Male 3	11/11	11/11	11/11	11/11	NA
Male 4	11/11	11/11	11/11	11/11	NA
Female 1	1/11	1/11	NA	1/11	1/11
Multiplex I (MPI)					
Male 1	8/8	8/8	8/8	8/8	NA
Male 2	8/8	8/8	8/8	8/8	NA
Male 3	8/8	8/8	8/8	8/8	NA
Male 4	8/8	8/8	8/8	8/8	NA
Female 1	0/8	0/8	NA	0/8	0/8
Multiplex B (MPB)					
Male 1	5/5	5/5	5/5	5/5	NA
Male 2	5/5	5/5	5/5	5/5	NA
Male 3	5/5	5/5	5/5	5/5	NA
Male 4	5/5	5/5	5/5	5/5	NA
Female 1	0/5	0/5	NA	0/5	0/5

Environmental Effects on Stability- The ability to obtain a Y-STR profile that has been exposed to harsh environmental conditions is significant to the science of forensics. DNA can be damaged in a variety of ways, but the most commonly encountered DNA damage in forensics is due to environmental insults, particularly sunlight (heat), humidity and precipitation (including precipitation in the form of drenching rain). Therefore, three conditions where insults could be introduced one at a time were implemented. The three types of conditions are heat/humidity (HH), heat/humidity and precipitation (HHR), and heat/humidity, sunlight and precipitation (HHSR). Two male individuals, whose haplotypes were already established, contributed semen and liquid blood which were subjected to the environmental insults using 50µL stains of each body fluid. A female also contributed blood as a control for this study. The samples were placed in an outdoor, metropolitan area in Central Florida and fully subjected to the elements. Exposure of the samples continued from July to September of 2005, with temperatures ranging from 79-95˚F. Humidity (relative) and precipitation for this period averaged 57% and a total of 10.5 in. (4.2 cm) was deposited on the samples respectively (Table 9). Samples were removed after 1 day, 2 days, 1 week, 3 weeks and six weeks and ultimately subjected to Y-STR analysis (Figures 5-9). The results are summarized in Table 10, which indicates the presence of a full, partial or lost profile at each exposure time.

The results confirm that semen persists longer than blood on a cotton cloth when exposed to environmental insults. This could be because of the nature of the sperm itself, the sperm has a sticky nature and interacts with the cotton weave which allows for it to remain on the cloth for a longer period of time despite the precipitation and heat (67). The multiplex system which performed the best in the presence of environmental insults and semen was Promega PowerPlex® – Y System by producing a full human male profile at two days, and a partial profile at one week

with the greatest number of loci when all other multiplexes were unable to obtain the same. Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit, Reliagene Y-PLEX™ 12 and MPB were the multiplexes the closest to obtaining a human male profile with semen in the presence of environmental insults. On the other hand, blood stains were unable to produce any full profile results at two days and beyond with all five multiplex systems, perhaps due to excessive precipitation, and sunlight.

Table 9- Environmental Conditions for months of exposure for environmentally insulted samples.

MONTH	AVERAGE TEMPERATURE	AVERAGE HUMIDITY	HEAT INDEX	TOTAL PRECIPITATION
JULY	90.85 ºF	58.45%	99.55 ºF	1.75 in
AUGUST	90.81 ºF	56.90%	99.29 ºF	5.35 in
SEPTEMBER	87.26 ºF	55.70%	91.20 ºF	3.37 in

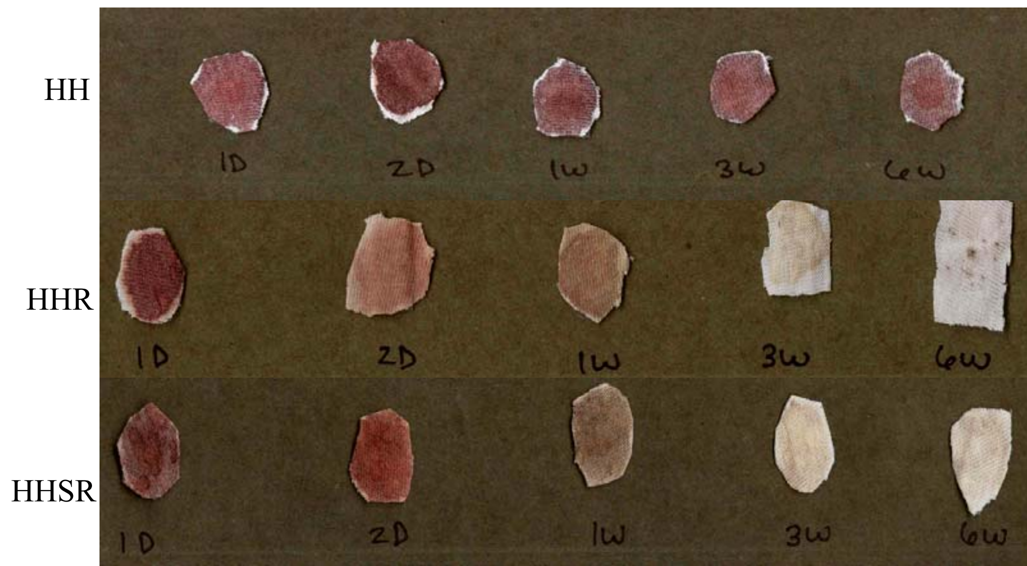


Figure 5- Stain cut-outs after environmental exposure

Table 10- Y-STR multiplex systems typing of environmentally insulted blood and semen stains. The number of loci present when not a full profile is indicated with a (+) #. The conditions tested are: Heat and Humidity (H); Heat, Humidity and Rain (R); Heat, Humidity, Sunlight and Rain (S).

Multiplex Systems	1 day			2 days			1 week			3 weeks			6 weeks		
	H	R	S	H	R	S	H	R	S	H	R	S	H	R	S
AmpFℓSTR® Yfiler™															
-Blood	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
-Semen	+	+	+	+	(+)13	(+)13	+	(+)4	(+)4	+	-	-	+	-	-
Multiplex I															
-Blood	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
-Semen	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-
Multiplex B															
-Blood	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
-Semen	+	+	+	+	(+)4	(+)4	+	(+)4	(+)3	+	-	-	+	-	-
PowerPlex® – Y System															
-Blood	+	+	+	+	(+)2	-	+	(+)2	(+)2	+	-	-	+	-	-
-Semen	+	+	+	+	+	+	+	(+)8	(+)6	+	-	-	+	-	-
Reliagene Y-PLEX™ 12															
-Blood	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
-Semen	+	+	+	+	(+)12	(+)12	+	-	(+)3	+	-	-	+	-	-

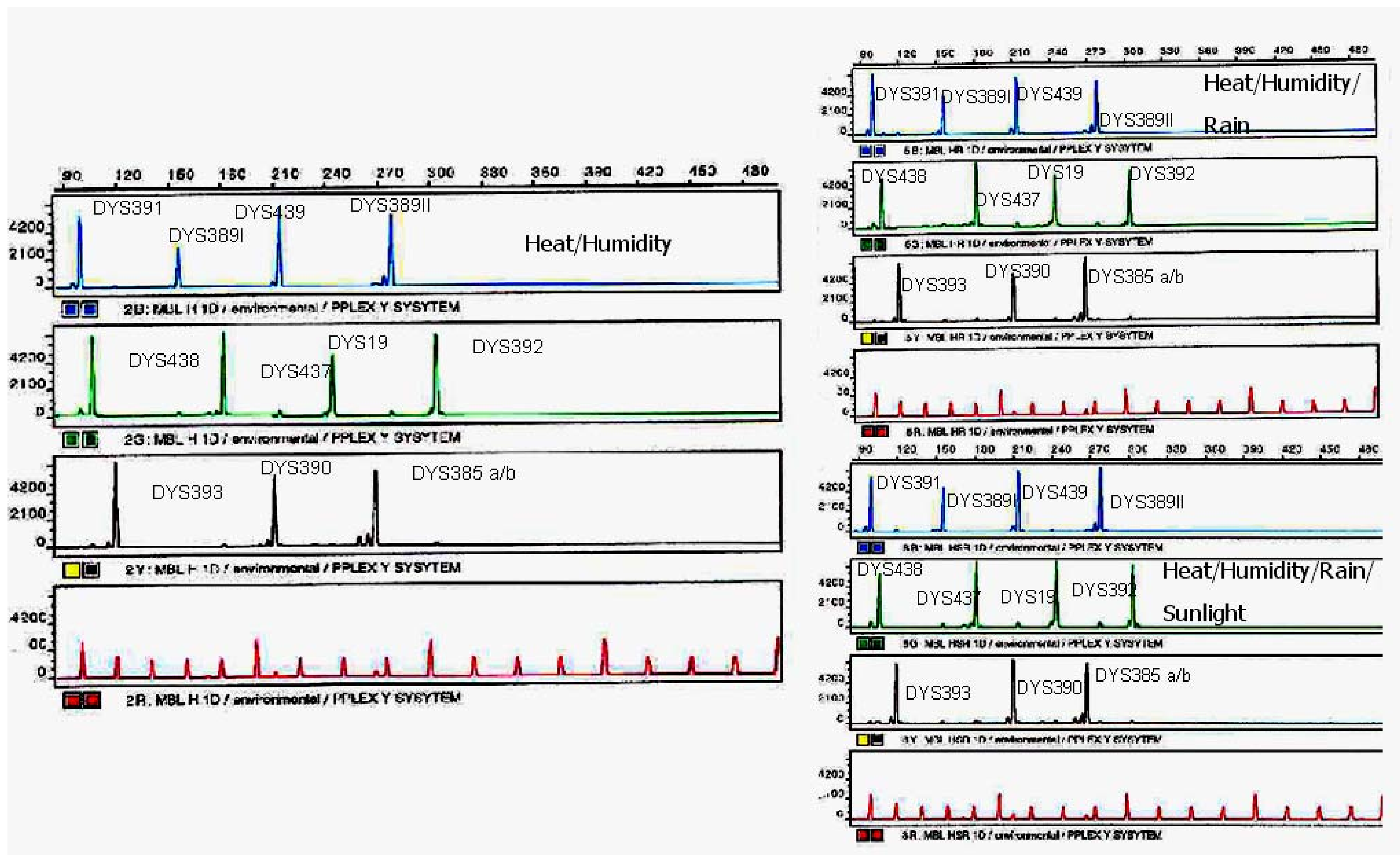
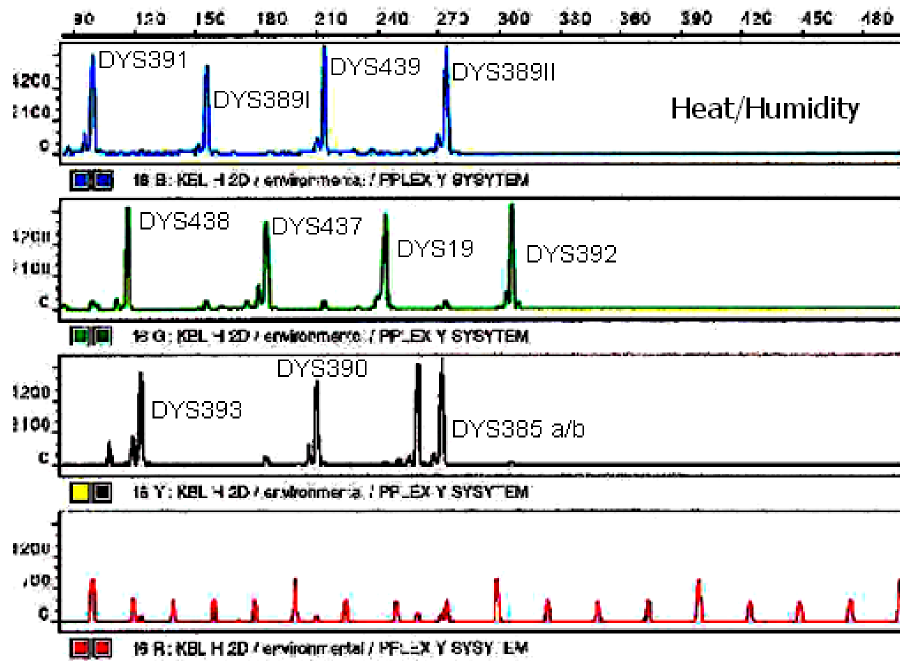
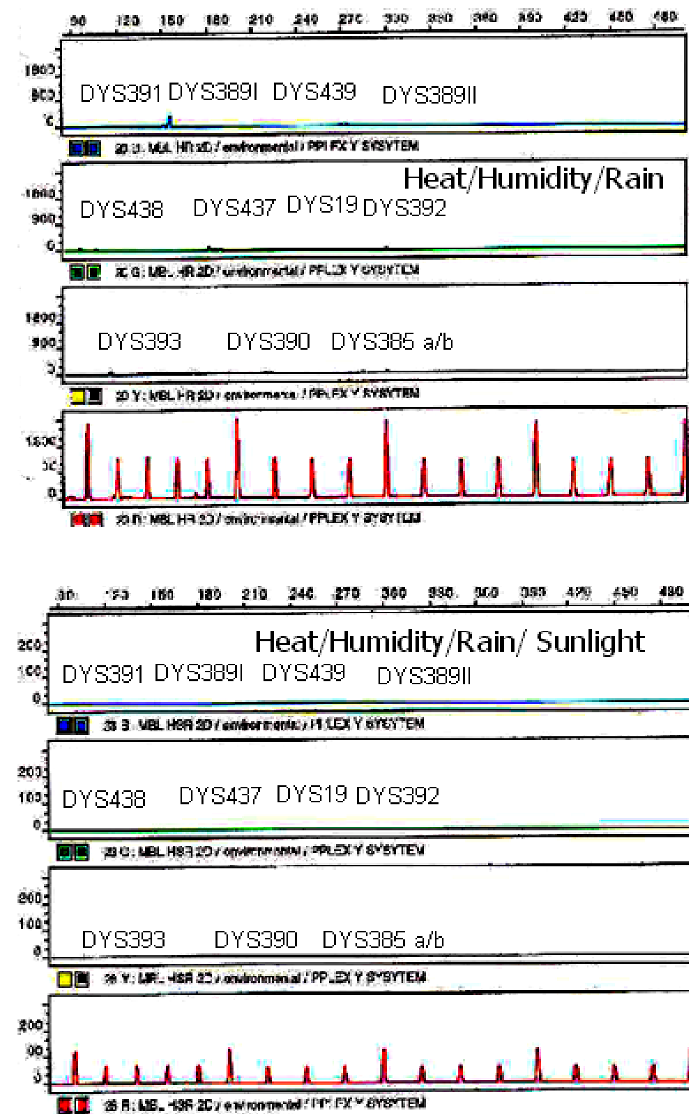


Figure 6- Electropherograms of blood stains exposed to environmental insults for 1 day; analyzed with Promega PowerPlex® Y-System





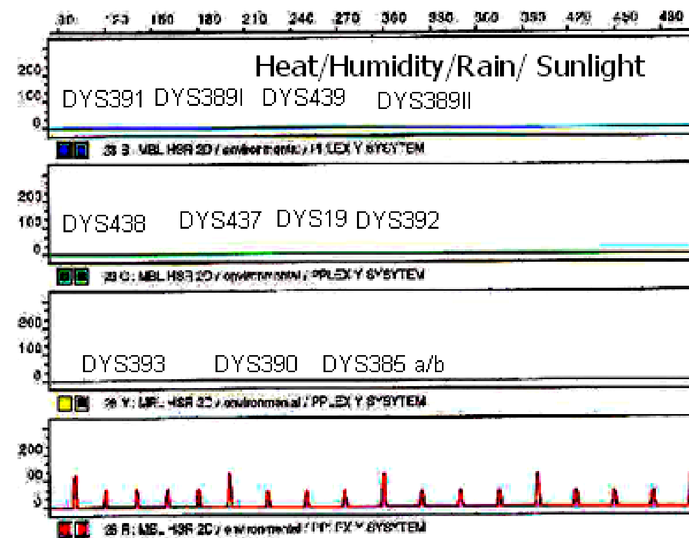


Figure 7- Electropherograms of blood stains exposed to environmental insults for 2 days; analyzed with Promega PowerPlex® Y-System

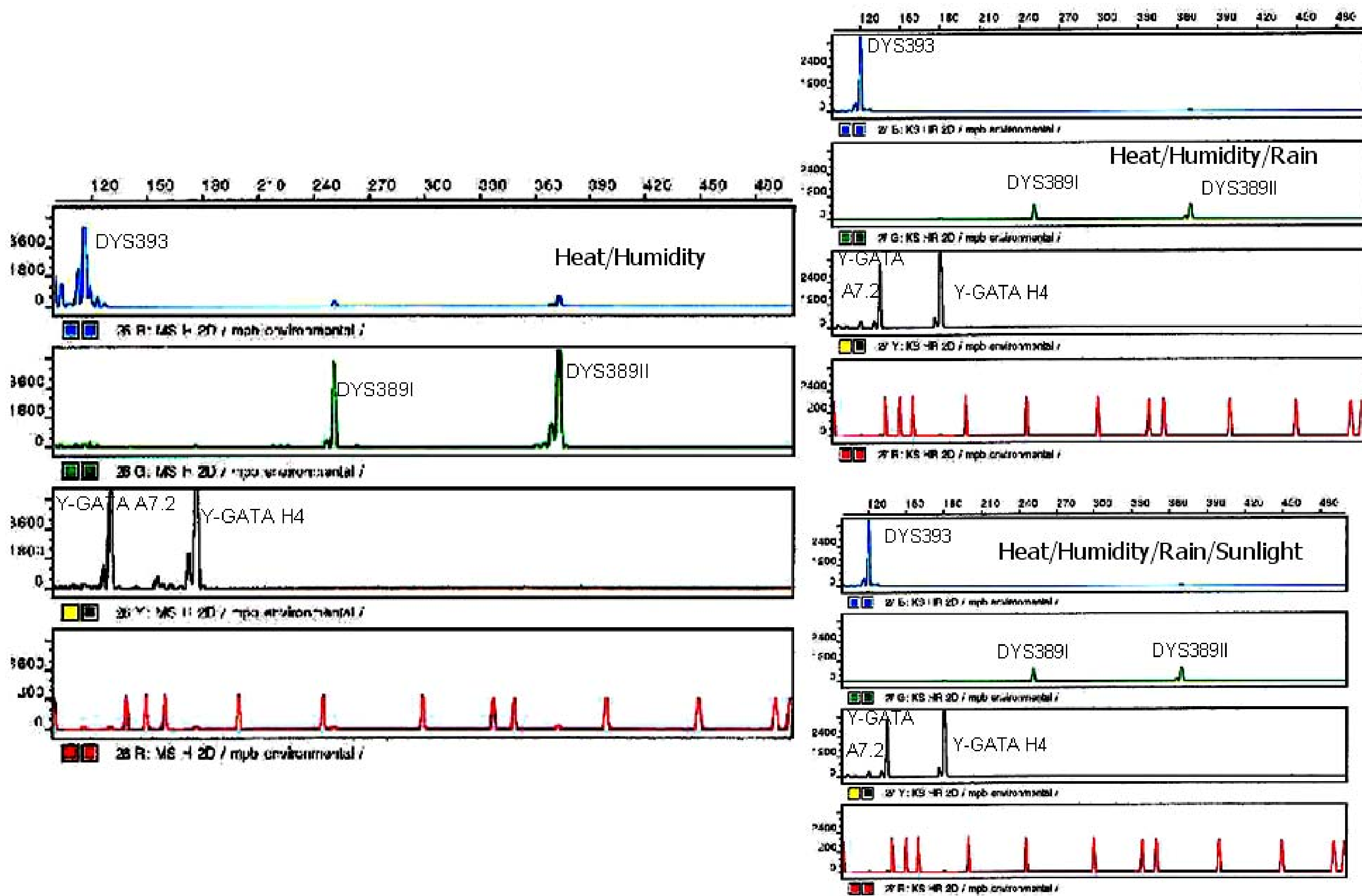
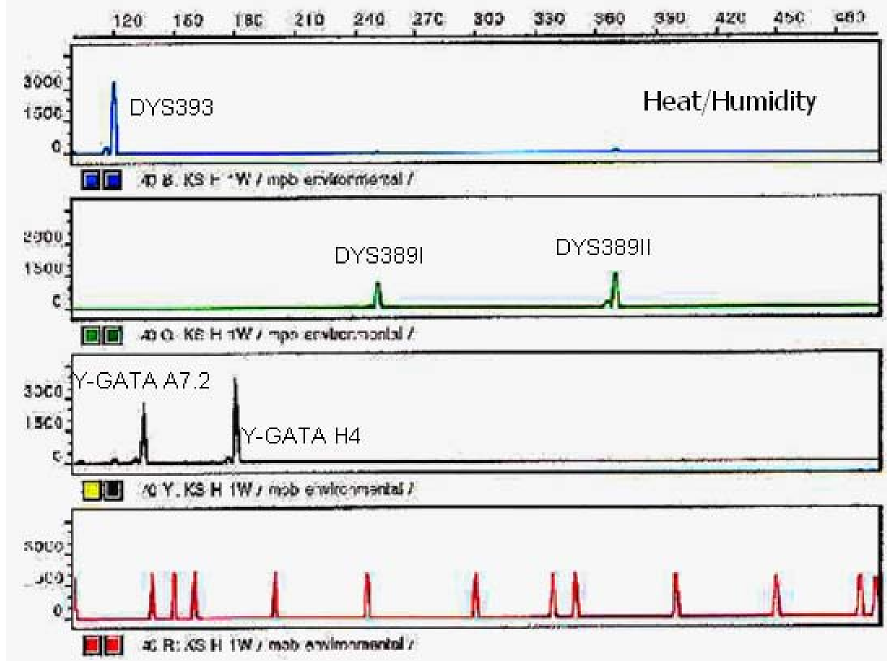


Figure 8- Electropherograms of semen stains exposed to environmental insults for 2 days; analyzed with MPB



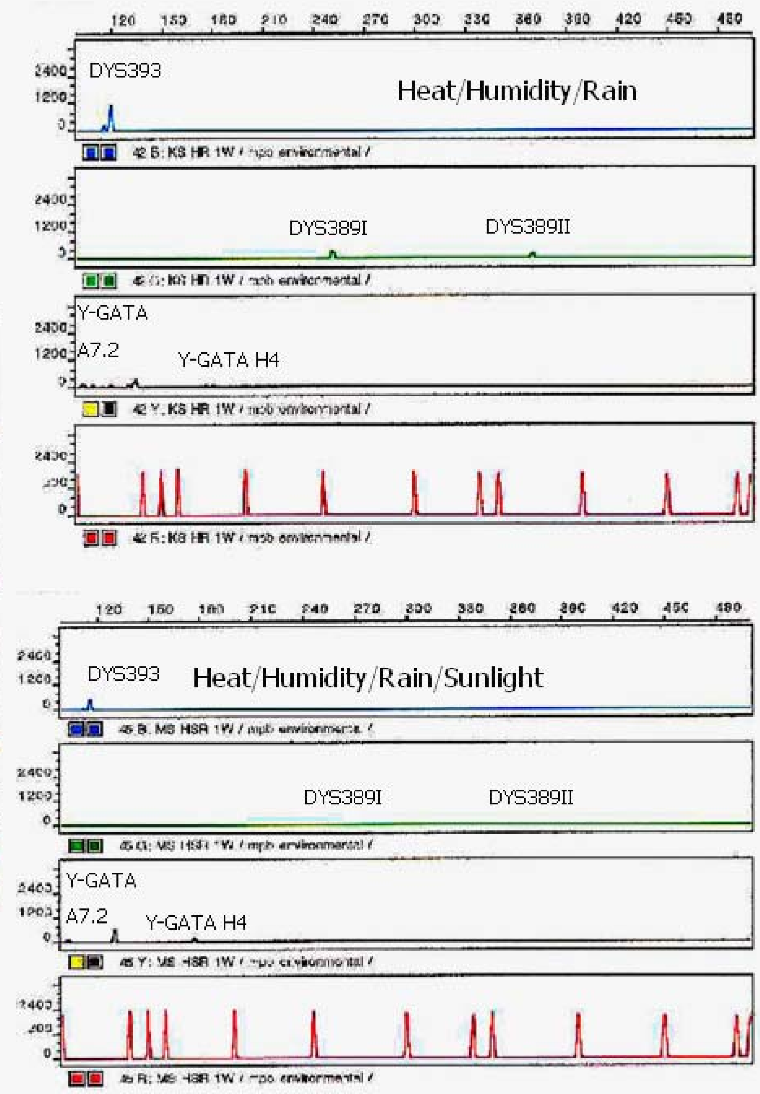


Figure 9- Electropherograms of semen stains exposed to environmental insults for 1 week; analyzed with MPB

Species Specificity

Species specificity was determined by testing all five Y-STR systems using DNA isolated from various common mammals and a number of nonhuman primates. Common mammals were tested in order to test their homology to the human Y chromosome. After amplification with MPI, MPB, Reliagene Y-PLEX™ 12, Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit, and Promega PowerPlex® – Y System; common mammals demonstrated homology to the Y-chromosome by producing detectable products in the alleles ranges of DYS391, DYS391 and DYS437 and DYS393, DYS439 and DYS389II (Horse, Cow, Sheep, and Deer respectively) for the Promega PowerPlex® – Y System; Reliagene Y-PLEX™ 12 was able to detect Cow, Cat, Dog, Sheep, and Deer products sized at 101 bp in the yellow channel; Ferret, Dog and Ferret, Ferret were observed at DYS456, DYS393, and Y-GATA 4 respectively using the Applied Biosystems AmpFℓSTR® Y-Filer system PCR Amplification Kit (data not shown).

A high level of sequence homology exists between humans and non-human primates. We tested one nanogram of DNA from five male non-human primates (gorilla, chimpanzee, orangutan, spider monkey, and macaque). Similarly, four female non-human primates were also tested (gorilla, chimpanzee, pygmy chimpanzee, and orangutan) using 300ng of DNA. MPI displayed many non-human primate products that were detected in the Blue Channel: Female Chimpanzee and Female Pygmy Chimpanzee (DYS393), Male Macaque (DYS392); Green Channel: Male chimpanzee (DYS389I); Yellow Channel: Male Macaque (DYS438 and DYS385b). MPB exhibited no non-human primate homology (Figure 10).

Conversely, Promega PowerPlex® – Y System detected in the Blue Channel: Female Orangutan (DYS391 and DYS389I), Female Chimpanzee (DYS389I and DYS439), Male

Macaque (DYS389I and DYS439), Female Pygmy Chimpanzee (DYS389I and DYS439);Green Channel: Female Orangutan (DYS437), Male Chimpanzee (DYS437), Male Macaque (DYS438 and DYS19);Yellow Channel: Female Chimpanzee (DYS393 and DYS390), Male Macaque (DYS393), and Female Pygmy Chimpanzee (DYS390) (Figure 11).

Furthermore, the Reliagene Y-PLEX™ 12 system detected in the Blue Channel: Male Gorilla (DYS385), Male Chimpanzee (DYS392); Green Channel: Male Gorilla (DYS389II), Male Chimpanzee (DYS393 and DYS389I), Female Chimpanzee (DYS393), Male Macaque (DYS391), Female Pygmy Chimpanzee (DYS391); Yellow Channel: Spider Monkey, Male and Female Orangutan (product at 101 bp in the range of amelogenin), Male Gorilla (amelogenin and DYS19), Female Gorilla (amelogenin), Male Chimpanzee (amelogenin and DYS439), Female Chimpanzee (amelogenin and DYS439), Male Macaque (amelogenin), Female Pygmy Chimpanzee (amelogenin) (Figure 12).

Moreover, the Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit detected in the Blue Channel: Male Chimpanzee (DYS389I); Yellow Channel: Female Orangutan (DYS393), Female Chimpanzee (DYS393 and DYS439); Red Channel: Male Macaque (DYS448) (Figure 13).

The species specificity portion was included to determine if there was cross reactivity between non-human primates as well as other animals that might be encountered while analyzing a biological stain. All multiplex systems except MPB produced non-human primate results in Y-STR allele ranges. MPB did not produce any cross reactivity because the primers and conditions used were chosen specifically during the multiplex development to remove all species cross reactivity. The high degree of homology that exists between non-human primates and humans caused multiple peaks to appear with the remaining four multiplexes. Reliagene Y-PLEX™ 12

exhibited the greatest amount of cross reactivity with the non-human primates this was due in large to the addition of amelogenin in the multiplex. The addition of amelogenin just provides the ability to determine sex; therefore there almost all samples tested will produce a peak in that range. Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit, MPI and Promega PowerPlex® – Y System all produced multiple peaks in allele ranges; however for the number of loci included in a multiplex Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit had the least amount of cross reactivity.

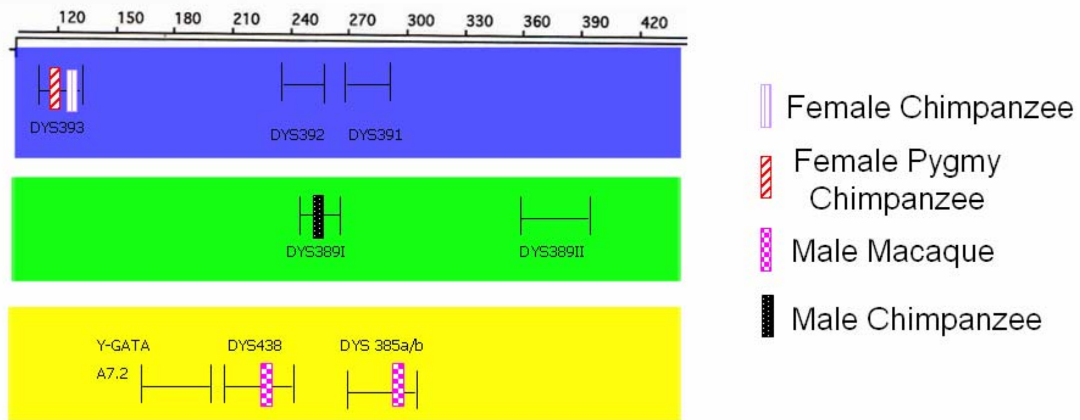


Figure 10- Non-Human primate artifact location for MPI

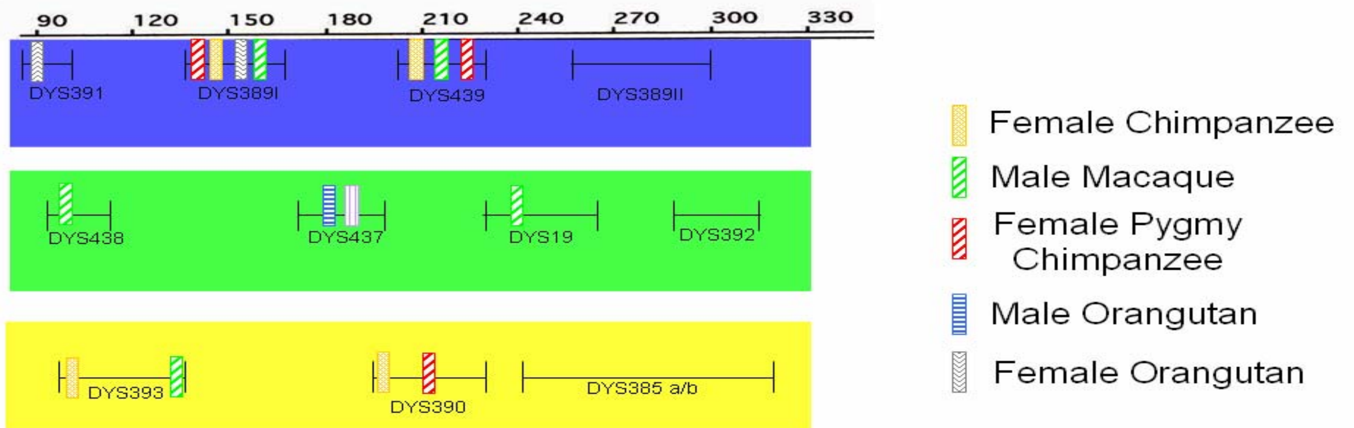


Figure 11- Non-Human primate artifact location for Promega PowerPlex® -Y System

Figure 12- Non-Human primate artifact location for Reliagene Y-PLEX™ 12

Figure 13- Non-Human primate artifact location for Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit

Post-Coital Cervicovaginal Studies

Nondifferentially extracted Post-Coital Cervicovaginal Comparison- A post-coital cervicovaginal swab was recovered independently by two females at specified intervals after coitus (0 hours, 12 hours, 24 hours, 48 hours, and 72 hours). In addition, a pre-coital swab was also recovered and analyzed to ensure the female environment was void of male DNA. The DNA was isolated using a standard organic extraction and analysis was conducted by adding 300ng of DNA to each system for all time points (Table 11).

Full profiles were able to be obtained from all kits from both mixtures up to 12 hours. However, Reliagene Y-PLEX™ 12 produced a partial profile at 24 hours, while all other kits still produced a full profile. Finally, after 48 hours MPI and Reliagene Y-PLEX™ 12 detected a partial profile. The profile remained partial after 72 (Table 12) (Figure 14). The importance of post-coital samples being collected $\geq$ 48 hours is that the sample is more likely to contain fewer sperm and many of the remaining sperm could be in a structurally fragile state.

Table 11 – Post-Coital Cervicovaginal Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Human Male DNA Quantification Kit results from 0 hours, 12 hours, 24 hours, 48 hours and 72 hours.

Mixture 1	**0 hours**	**12 hours**	**24 hours**	**48 hours**	**72 hours**
Non- Differential Quantity of Male DNA	2442 ng	146 ng	166 ng	50 ng	145 ng
Non- Differential Quantity of Female DNA	5959 ng	8755 ng	27035 ng	30650 ng	28556 ng
Differential Quantity of Male DNA	97 ng	21 ng	59 ng	12 ng	59 ng
Differential Quantity of Female DNA	3780 ng	5850 ng	15150 ng	15700 ng	26500 ng
Quantity of Male DNA in Female Cell Fraction	4630 ng	96 ng	37 ng	7 ng	47 ng
Mixture 2	**0 hours**	**12 hours**	**24 hours**	**48 hours**	**72 hours**
Non- Differential Quantity of Male DNA	1905 ng	190 ng	258 ng	56 ng	12 ng
Non- Differential Quantity of Female DNA	9795 ng	8010 ng	11243 ng	12994 ng	16438 ng
Differential Quantity of Male DNA	69 ng	184 ng	5 ng	4 ng	12 ng
Differential Quantity of Female DNA	4875 ng	11950 ng	8100 ng	6750 ng	0 ng
Quantity of Male DNA in Female Cell Fraction	84 ng	42 ng	72 ng	7 ng	50 ng

Table 12- Post-Coital profile results from 0 hours, 12 hours, 24 hours, 48 hours and 72 hours. + Full Profile, - Lost Profile, (+) Partial Profile

Mixture 1	**0 hours**	**12 hours**	**24 hours**	**48 hours**	**72 hours**
Profile Obtained Non-Differential Extraction 300 ng					
AmpFℓSTR® Yfiler™	+	+	+	+	+
Multiplex I	+	+	+	(+)	-
Multiplex B	+	+	+	+	+
Promega PowerPlex® – Y System	+	+	+	+	+
Reliagene Y-PLEX™ 12	+	+	(+)	(+)	(+)
Profile Obtained Differential Extraction 1 ng Male					
AmpFℓSTR® Yfiler™	+	+	+	+	+
Multiplex I	+	+	+	+	+
Multiplex B	+	+	+	+	+
Promega PowerPlex® – Y System	+	+	+	+	+
Reliagene Y-PLEX™ 12	+	+	+	+	(+)
Mixture 2	**0 hours**	**12 hours**	**24 hours**	**48 hours**	**72 hours**
Profile Obtained Non-Differential Extraction 300 ng					
AmpFℓSTR® Yfiler™	+	+	+	+	+
Multiplex I	+	+	+	(+)	(+)
Multiplex B	+	+	+	+	+
Promega PowerPlex® – Y System	+	+	+	+	+
Reliagene Y-PLEX™ 12	+	+	(+)	(+)	(+)
Profile Obtained Differential Extraction 1ng Male					
AmpFℓSTR® Yfiler™	+	+	+	+	+
Multiplex I	+	+	+	+	+
Multiplex B	+	+	+	+	+
Promega PowerPlex® – Y System	+	+	+	+	+
Reliagene Y-PLEX™ 12	+	+	+	(+)	(+)

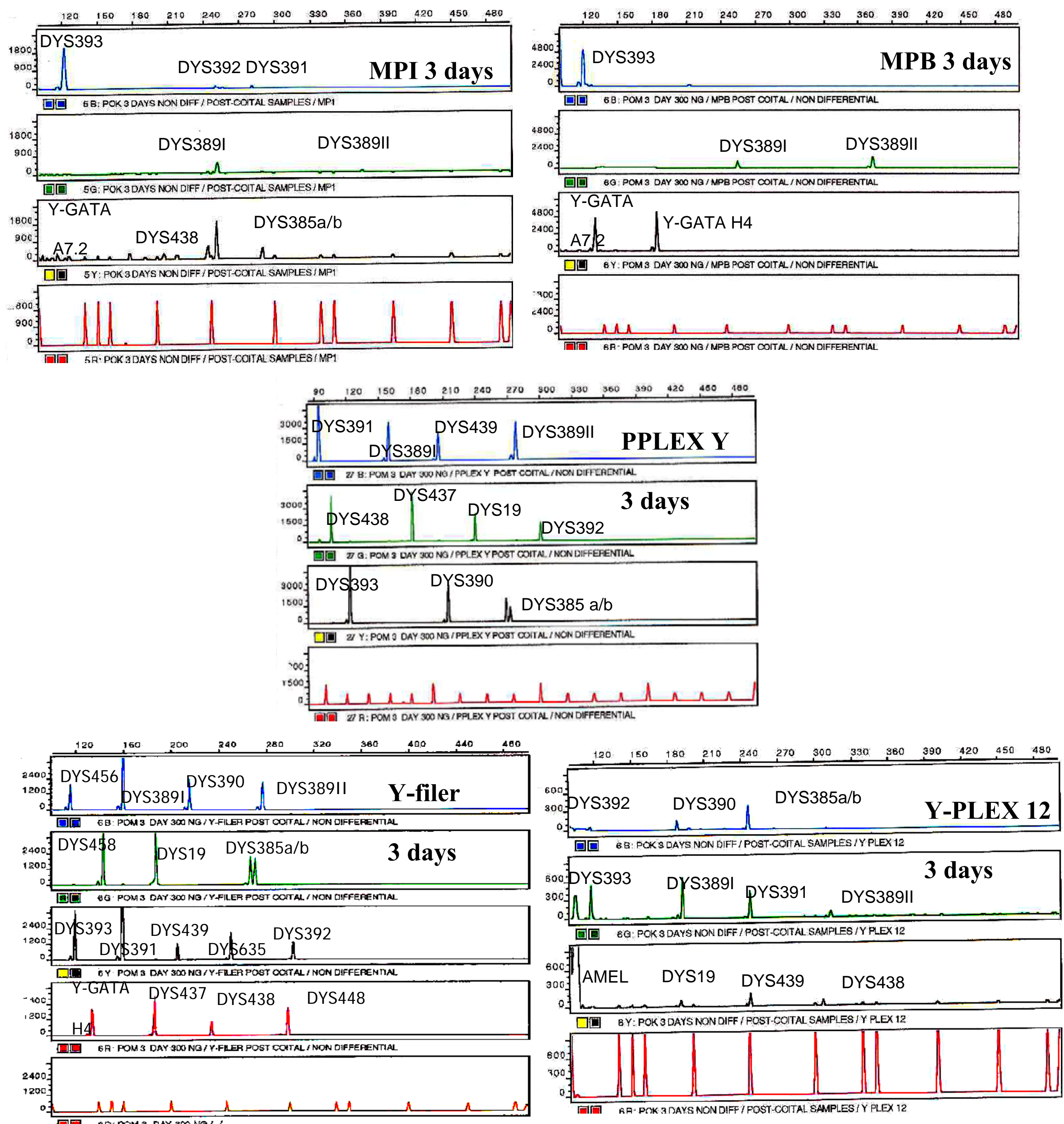


Figure 14- Post-Coital profiles after 3 days using non-differential extraction methods

Differentially extracted Post-Coital Cervicovaginal Comparison- The differentially extracted post-coital cervicovaginal comparison was included due to the theory that the differential extraction process would lyse the sperm prematurely and causes it to remain in the non-sperm fraction; overall causing a loss of male DNA to be tested in the sperm fraction. Therefore, a post-coital cervicovaginal swab was recovered independently by two females at specified intervals after coitus (0 hours, 12 hours, 24 hours, 48 hours, and 72 hours). In addition, a pre-coital swab was also recovered and analyzed to ensure the female environment was void of male DNA. The DNA was isolated using a standard differential extraction and analysis was conducted by adding one ng of male DNA to each system for all time points. The male DNA was quantitated using the Quantifiler™ Human Male DNA Quantification Kit which is specific for what is responsible for the male phenotype or the testis-determining SRY gene (68 and 69) which remains the most distinguishing characteristic of this chromosome. (Table 11).

After analysis of two independent mixtures from 0 hours to 72 hours, the data supports the contrary. Instead of a loss in profile or drop-out of alleles which might occur due to a low amount of male DNA remaining after the differential extraction, there is an increase with all multiplex systems in the ability to obtain a full profile till the 72 hour time point, except Reliagene Y-PLEX™ 12. Reliagene Y-PLEX™ 12 produced a partial profile at the 48 hour time point, which is expected given that Reliagene Y-PLEX™ 12 is known to be the least sensitive and specific. In addition, no profiles were lost as a result of this method of extraction as was the case in the non-differentially extracted samples (Table 12) (Figure 15). One plausible justification for this outcome is that the method employed for quantitation (Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Human Male DNA Quantification Kit (Applied BioSystems, Foster City, CA)) produced a more reliable quantity of DNA to add to the system.

Also, there is no overwhelming amount of female DNA present which would interfere in amplification and detection due to titrating out reagents in PCR.

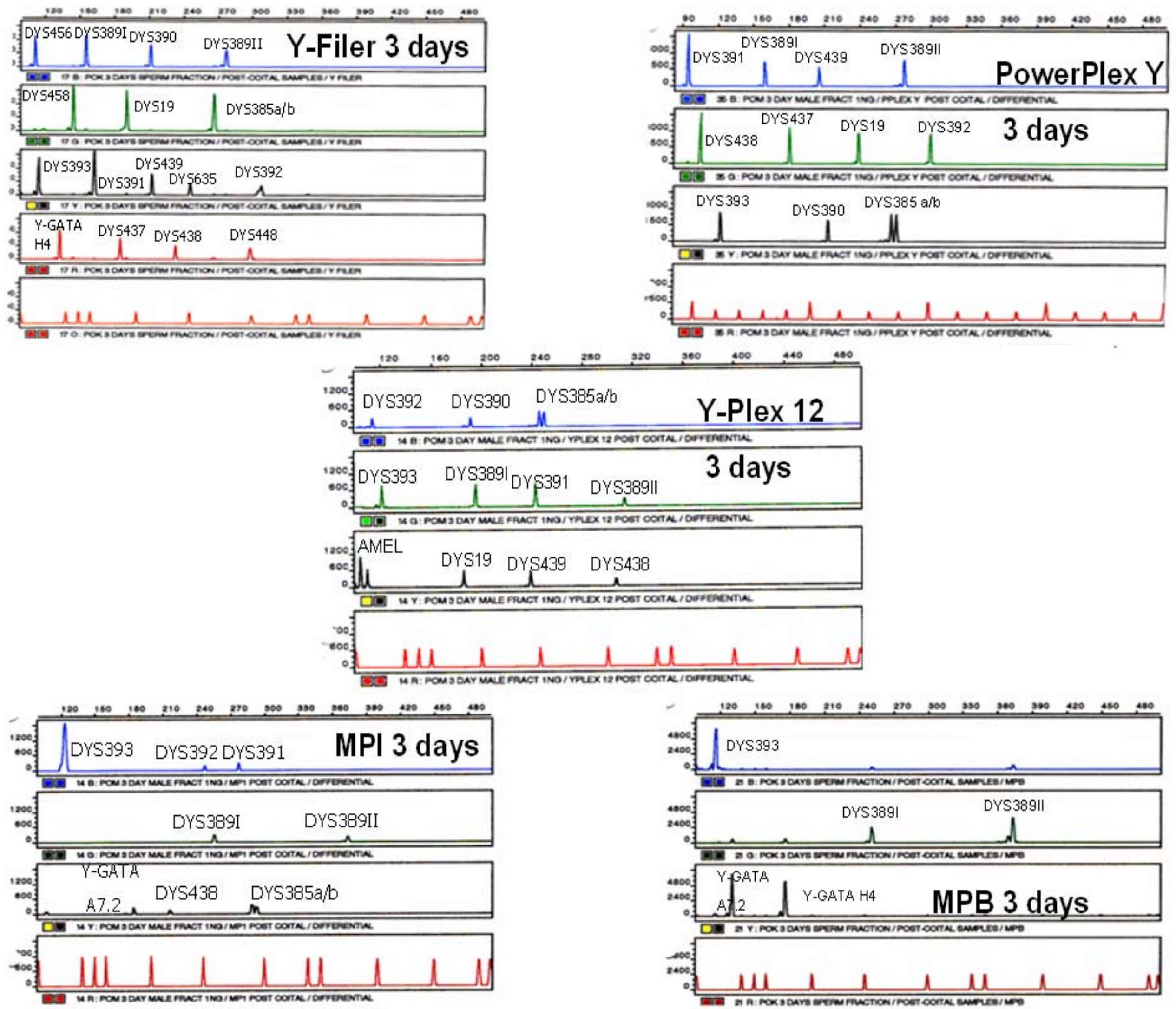


Figure 15- Post-Coital profiles after 3 days using differential extraction methods

Extended Interval Post-Coital Cervicovaginal Comparison Nondifferentially extracted - A post-coital cervicovaginal swab was recovered independently by two females at specified intervals after coitus (96 hours, 120 hours, 144 hours, 168 hours, and 192 hours). In addition, a pre-coital swab was also recovered and analyzed to ensure the female environment was void of male DNA. The DNA was isolated using a standard organic extraction and analysis was conducted by adding 300ng of DNA to each system for all time points (Table 13).

Full profiles were unable to be obtained from four out of the five kits after 72 hours using this extraction technique. However, MPB produced a full profile at 96 hours, while all other kits produced a partial profile (Figure 16). Finally, any attempt to obtain after 120 hours resulted in a loss of profile (Table 14). The importance of post-coital samples being collected ≥ 72 hours is that the sample is more representative of rape cases that occur with children or from date rape (70). In addition, detecting a male profile past the 24 hour time point is of value to forensic casework because, it is unlikely in most instances to obtain an autosomal STR profile of the semen donor from vaginal samples taken 24-36 hours after intercourse (71 and 72).

Table 13 – Extended Interval Post-Coital Cervicovaginal Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Human Male DNA Quantification Kit results from 96 hours, 120 hours, 144 hours, and168 hours.

Mixture 1	**96 hours**	**120 hours**	**144 hours**	**168 hours**
Non- Differential Quantity of Male/ Female DNA	42950 ng	20400 ng	11000 ng	10950 ng
Differential Quantity of Male DNA	0.8 ng	4 ng	2 ng	0.2ng
Differential Quantity of Female DNA	16950 ng	28550 ng	13900 ng	7700 ng
Mixture 2	**96 hours**	**120 hours**	**144 hours**	**168 hours**
Non- Differential Quantity of Male/Female DNA	7650 ng	30100 ng	14550 ng	18550 ng
Differential Quantity of Male DNA	125 ng	4 ng	1ng	0.9ng
Differential Quantity of Female DNA	7800 ng	9150 ng	14950 ng	16400 ng

Table 14- Post-Coital profile results from 96 hours, 120 hours, 144 hours, and 168 hours. + Full Profile, - Lost Profile, (+) Partial Profile

Mixture 1	96 hours	120 hours	144 hours	168 hours
Profile Obtained Non-Differential Extraction 300 ng				
Applied BioSystems Y-filer	(+)3	(+)5	-	-
Multiplex I	(+)1	-	-	-
Multiplex B	+	(+)4	-	(+)2
Promega PowerPlex Y	(+)9	(+)5	-	-
Reliagene Y-Plex 12	-	-	-	-
Profile Obtained Differential Extraction 1 ng Male or 300 ng Female DNA				
Applied Biosystem Y-filer	+	-	-	-
Multiplex I	+	+	-	-
Multiplex B	+	+	-	-
Promega PowerPlex Y	+	+	-	-
Reliagene Y-Plex 12	+	-	-	-
Mixture 2	**96 hours**	**120 hours**	**144 hours**	**168 hours**
Profile Obtained Non-Differential Extraction 300 ng				
Applied Biosystem Y-filer	(+)2	(+)10	(+)1	-
Multiplex I	-	-	-	-
Multiplex B	(+)1	(+)3	-	-
Promega PowerPlex Y	(+)5	(+)10	(+)3	-
Reliagene Y-Plex 12	-	-	-	-
Profile Obtained Differential Extraction 1ng Male or 300 ng Female DNA				
Applied Biosystem Y-filer	(+)11	-	-	-
Multiplex I	+	+	-	-
Multiplex B	+	+	-	-
Promega PowerPlex Y	+	+	-	-
Reliagene Y-Plex 12	-	(+)4	-	-

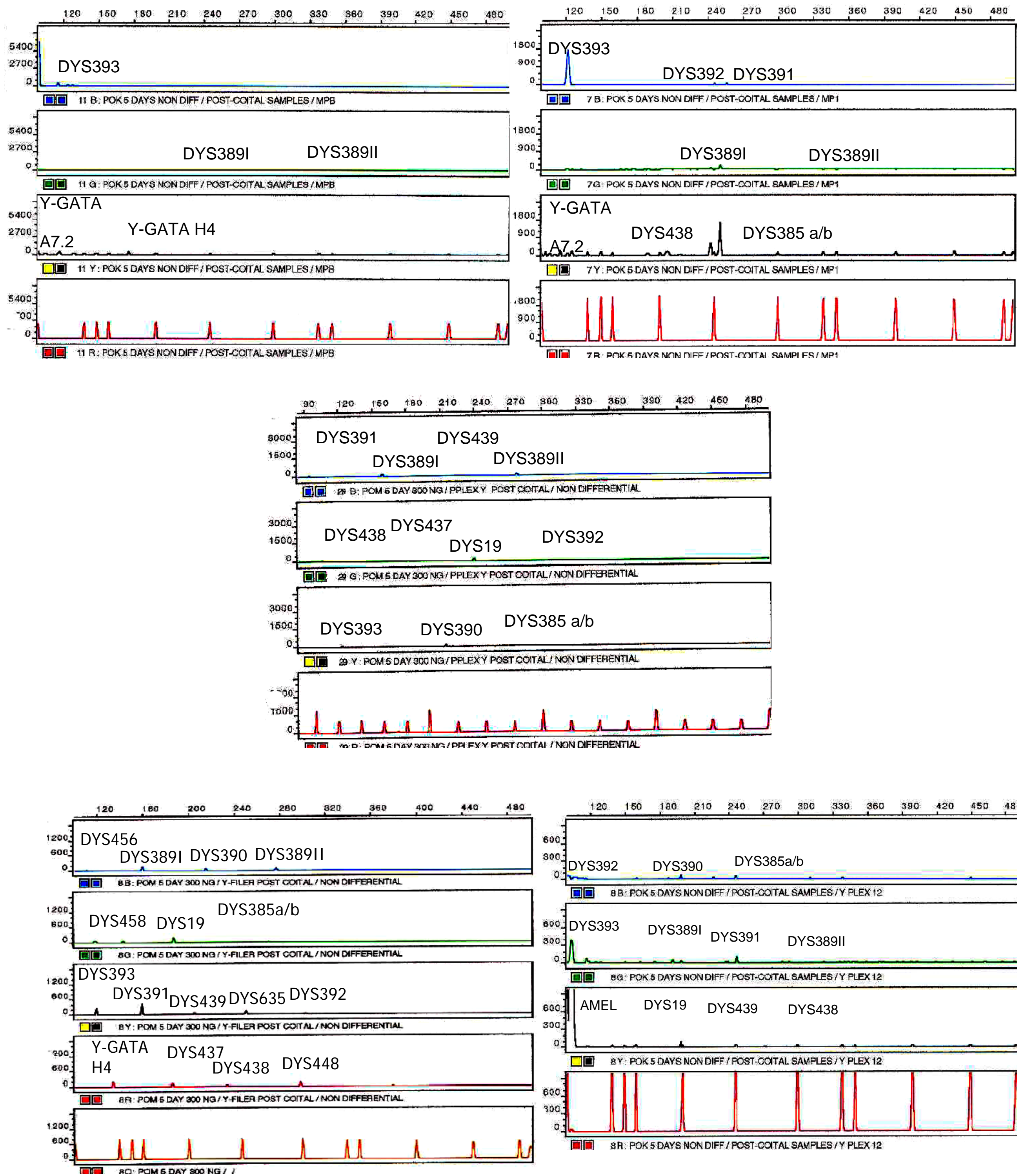


Figure 16- Post-Coital profiles after 5 days using non-differential extraction method

Extended Interval Post-Coital Cervicovaginal Comparison Differentially Extracted - The differentially extracted post-coital cervicovaginal comparison was included due to the theory that the differential extraction process would lyse the sperm prematurely and causes it to remain in the non-sperm fraction; overall causing a loss of male DNA to be tested in the sperm fraction. Moreover, classical serology studies have shown that spermatozoa can persist consistently in the vaginal canal up to three days after intercourse (73-75). A post-coital cervicovaginal swab was recovered independently by two females at specified intervals after coitus (96 hours, 120 hours, 144 hours, 168 hours, and 192 hours). In addition, a pre-coital swab was also recovered and analyzed to ensure the female environment was void of male DNA. The DNA was isolated using a standard differential extraction and analysis was conducted by adding 1ng of male DNA to each system for all time points if there was enough male DNA recovered to constitute 1 ng of DNA (Table 13). In the event that there was not enough male DNA to constitute 1 ng of DNA 300 ng of the female cell fraction was added to the multiplex system. The addition of the 300 ng of the female cell fraction to the multiplex only occurred for the six and seven day time point.

After analysis of two independent mixtures from 96 hours to 168 hours, there is data which supports the contrary. Instead of a loss in profile or drop-out of alleles which might occur due to a low amount of male DNA remaining after the differential extraction, there appears to be an increase in the ability to obtain a full profile beyond the 72 hour time point. In addition, no profiles were lost as a result of this method of extraction as was the case in the non-differentially extracted samples (Table 14). A magnificent ability to obtain a human male profile at 120 hours has never been done in other laboratories or by manufacturer's (70). A justification for this outcome is that the method employed for quantitation (Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Human Male DNA Quantification Kit (Applied

BioSystems, Foster City, CA)) produced a more reliable quantity of DNA to add to the system. Also, there is no overwhelming amount of female DNA present which would interfere in amplification and detection. An additional rationale for three multiplex systems (MPI, MPB and Promega PowerPlex® Y-System) achieving a full profile at the 120 hour time point would be the sample collection (Figure 17). The cervicovaginal swabs obtained were from the cervix which was brushed multiple times at the specific time point. Conversely, the reproductive biology literature is replete with reports demonstrating the persistence of several spermatozoa in the human cervix up to seven days post coitus, which is consistent with the concept of the cervix as a sperm repository prior to fertilization (71 and 76-78). Therefore, the current human male Y-STR detection methods are not sensitive enough if they routinely fail to detect these male cells found at seven days. It can then be suggested that future improvements of Y-STR multiplex systems could be able to type these extended post-coital samples.

Figure 17- Post-Coital profiles after 5 days using differential extraction methods

CHAPTER FOUR: CONCLUSION

A performance comparison of commercial and in-house Y-STR multiplex systems was performed in order to better establish the limits of each system, compare its capabilities using difficult samples in a parallel manner with the other Y-STR multiplexes commercially available. This data would then provide insight based on research as to where some systems are deficient. Presently, all five multiplex systems perform adequately for basic forensic use. However, some systems proved to be more efficient at differentiating a male profile under certain conditions.

When comparing the results procured from the increase in cycle number for all multiplexes except MPB only Promega PowerPlex® – Y System gave the same result at the manufacturers 32 cycle numbers as the increase to 34 cycle. Thus allowing for Promega PowerPlex® – Y System to increase the cycle number to 34 cycles. However, with the increase in cycle number no increases in sensitivity or specificity occurred. Therefore, all multiplexes at their recommended cycling conditions is adequate, but doing so precludes low copy number or ultra high sensitivity studies, as were possible with MPB.

The sensitivity study illustrated that Promega PowerPlex® – Y System and MPB are the most sensitive Y-STR multiplex systems with full profile sensitivities of 20 pg in unique samples. However, these systems differ when comparing the partial profiles, MPB was able to achieve a partial profile at 10pg where as Promega PowerPlex® – Y System achieved a partial profile at 15 pg. In addition, the partial profiles of Promega PowerPlex® – Y System had five common core loci drop out; MPB on the other hand only had one common allele drop out. Both kits were unable to detect profiles at 5 pg. All other multiplexes reviewed did not render as sensitive results. Therefore, MPB and Promega PowerPlex® – Y System can easily be

incorporated into the most sensitive forensic studies with low number of sample and still produce reliably sensitive results.

In addition, Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit displayed zero artifacts from any of the titrations; while Reliagene Y-PLEX™ 12, MPI, MPB, and Promega PowerPlex® – Y System all displayed at least one artifact in an allele calling region. In addition, we also determined that Applied BioSystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit is able to produce reliable human male genetic profile while in the presence of overwhelming quantities of female DNA, a valuable advantage when used with forensic casework involving admixed samples procured from a female environment, such as a sexual assault sample.

Moreover, a large number of samples encountered in forensic casework are mixtures, so the specificity of each multiplex was crucial in order to aid the crime laboratory community. The specificity using male/female mixtures yielded results for Promega PowerPlex® – Y System establishing a full profile using 300ng of input DNA in a male/female 1/ 10,000 dilution. On the other hand, MPB was able to give a full profile at a male/female at 1/20,000 using 300ng of input DNA. These results obtained from Promega PowerPlex® – Y System and MPB is an outstanding level of specificity. The specificities from these two multiplexes lends itself to forensic studies because only a few male cells are needed to obtain a full genetic haplotype in the presence of overwhelming female cells. All other multiplexes did not perform as well as these two multiplexes using the 300 ng input of male/female mixture. The least specific multiplex was Reliagene Y-PLEX™ 12, with all remaining multiplexes producing specificity results between Reliagene Y-PLEX™ 12 and MPB.

Applying a second set of specificity conditions, Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit was shown to have a specificity of 1:3000 using 1 ng Male DNA input and 3,000 ng Female DNA input. As a result Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit is the most specific kit when using 1ng of Male DNA and overwhelming quantities of Female DNA. MPI, MPB and Promega PowerPlex® – Y System have relatively the some specificity when using 1 ng of male DNA in a mixture with an overwhelming amount of female DNA. The least specific multiplex when using these conditions again was Reliagene Y-PLEX™ 12 with a specificity of 1:100 male to female DNA.

The ability to discern the number of contributors as well as the relative ratios in which they exist aids in the determination of multiple assailant rape cases and consensual coital activities followed by a forced act from an assailant, as seen in some rape cases. The minor component was easily discernable at the ratios 1:1, 1:5 with all multiplexes compared. Conversely, using the Promega PowerPlex® – Y System, MPB and Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit the presence of two males in each mixture could be established additional at a 1:10 mixture and only at a few loci with the 1:20 mixture. Therefore, all multiplex systems performed adequately when male DNA was in a mixture with a second male contributor, however Promega PowerPlex® – Y System, MPB and Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit out performed MPI and Reliagene Y-PLEX™ 12.

The ability to obtain full profiles from multiple sources is important to forensic casework and all multiplexes performed relatively equal. Some problems do exist however when comparing different tissues. The ability to obtain a full profile from hair samples can be inconsistent because of the nature of hair itself and the sampling technique. This must be

carefully observed and should be used only as a comparative tissue when all others are not available. Meanwhile, in this current study all multiplexes were able to consistently provide an identical full haplotype for all male samples.

Environmental insults and species cross reactivity are common problems associated with crime scene samples and were included in this comparison to better understand how each multiplex system would perform. The results from the environmental studies confirm that semen persists longer than blood on a cotton cloth when exposed to environmental insults. This could be because of the nature of the sperm itself. Overall the multiplex system which performed the best in the presence of environmental insults and semen was Promega PowerPlex® – Y System by producing a full human male profile at two days, and a partial profile at one week with the greatest number of loci when all other multiplexes were unable to obtain the same. Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit, Reliagene Y-PLEX™ 12 and MPB were the multiplexes the closest to obtaining a human male profile with semen in the presence of environmental insults. On the other hand, blood stains were unable to produce any full profile results at two days and beyond with all five multiplex systems, perhaps due to excessive precipitation, and sunlight.

The species specificity portion was included to determine if there was cross reactivity between non-human primates as well as other animals that might be encountered while analyzing a biological stain. All multiplex systems except MPB produced non-human primate results in Y-STR allele ranges. MPB did not produce any cross reactivity because the primers and conditions used were chosen specifically during the multiplex development to remove all species cross reactivity. The high degree of homology that exists between non-human primates and humans caused multiple peaks to appear with the remaining four multiplexes. Reliagene Y-PLEX™ 12

exhibited the greatest amount of cross reactivity with the non-human primates this was due in large to the addition of amelogenin in the multiplex. The addition of amelogenin just provides the ability to determine sex; therefore there almost all samples tested will produce a peak in that range. Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit, MPI and Promega PowerPlex® – Y System all produced multiple peaks in allele ranges; however for the number of loci included in a multiplex Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit had the least amount of cross reactivity.

Additionally, post-coital cervicovaginal samples were introduced into the study to replicate bona fide casework. Using a small set of time points that are more common to forensic laboratories full profiles were able to be obtained from all kits from both mixtures up to 12 hours. However, Reliagene Y-PLEX™ 12 produced a partial profile at 24 hours, while all other kits still produced a full profile. Finally, after 48 hours MPI and Reliagene Y-PLEX™ 12 detected a partial profile. The importance of post-coital samples being collected $\geq$ 48 hours is that the sample is more likely to contain fewer sperm and many of the remaining sperm could be in a structurally fragile state.

The second condition tested was not only how long post-coital results could be obtained but what method of DNA isolation was the best. It was originally believed that a loss in profile or drop-out of alleles might occur due to a low amount of male DNA remaining after the differential extraction. The results obtained in this comparison are contrary to that theory. The results show an increase with all multiplex systems in the ability to obtain a full profile till the 72 hour time point, except Reliagene Y-PLEX™ 12. Reliagene Y-PLEX™ 12 produced a partial profile at the 48 hour time point, which is expected given that Reliagene Y-PLEX™ 12 is known to be the least sensitive and specific. In addition, no profiles were lost as a result of this method

of extraction as was the case in the non-differentially extracted samples. One plausible justification for this outcome is that the method employed for quantitation (Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Human Male DNA Quantification Kit (Applied BioSystems, Foster City, CA)) produced a more reliable quantity of DNA to add to the system. Also, there is no overwhelming amount of female DNA present which would interfere in amplification and detection due to titrating out reagents in PCR.

In forensic casework some victims of sexual assault wait to provide vaginal samples more than 36 hours after the incidence. In these cases the ability to obtain sperm diminishes as the post-coital interval is extended. Therefore, we extended the range of time points to include 96 hours through 168 hours. The results from the extended interval post-coital study indicate the full profiles were unable to be obtained from four out of the five kits after 72 hours using the standard organic extraction technique. In addition, MPB produced a full profile at 96 hours, while all other kits produced a partial profile. Finally, any attempt to obtain after 120 hours resulted in a loss of profile. The importance of post-coital samples being collected $\geq$ 72 hours is that the sample is more representative of rape cases that occur with children or from date rape. In addition, detecting a male profile past the 24 hour time point is of value to forensic casework because, it is unlikely in most instances to obtain an autosomal STR profile of the semen donor from vaginal samples taken 24-36 hours after intercourse.

Moreover using the differential extraction method a human male profile at 120 hours was able to be obtained, which has never been reported in the scientific literature. A possible reason for this outcome is that the method employed for quantitation (Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Human Male DNA Quantification Kit (Applied BioSystems, Foster City, CA)) produced a more reliable quantity of DNA to add to the system.

Also, there is no overwhelming amount of female DNA present which would interfere in amplification and detection. An additional rationale for three multiplex systems (MPI, MPB and Promega PowerPlex® Y-System) achieving a full profile at the 120 hour time point would be the sample collection. The cervicovaginal swabs obtained were from the cervix which was brushed multiple times at the specific time point. Conversely, the reproductive biology literature demonstrates the persistence of several spermatozoa in the human cervix up to seven days post coitus (76-78). Therefore, the current human male Y-STR detection methods are not sensitive enough if they routinely fail to detect these male cells found at seven days.

Overall all multiplex systems produced results that were adequate for forensic crime laboratories. Nevertheless, three multiplex systems had unique abilities that should be considered with certain cases. For example, Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit being able to differentiate a male profile in a over abundance of female DNA has uses in mixture analysis, and multiple donor sexual assaults. Promega PowerPlex® Y-System and MPB provide additional benefits with post-coital samples, sensitivity and specificity using 300 ng total input of DNA However, there is a large gap between the outcomes that Promega PowerPlex® Y-System and MPB and those from MPI and Reliagene Y-PLEX™ 12. The first generation Y-STR systems namely MPI and Reliagene Y-PLEX™ 12 are inferior to Promega PowerPlex® Y-System, Applied Biosystems AmpFℓSTR® Y-Filer™ PCR Amplification Kit and MPB. In conclusion, forensic crime laboratories need to choose carefully the correct Y-STR multiplex system which would satisfy there own needs which reflect the nature of the cases submitted.

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