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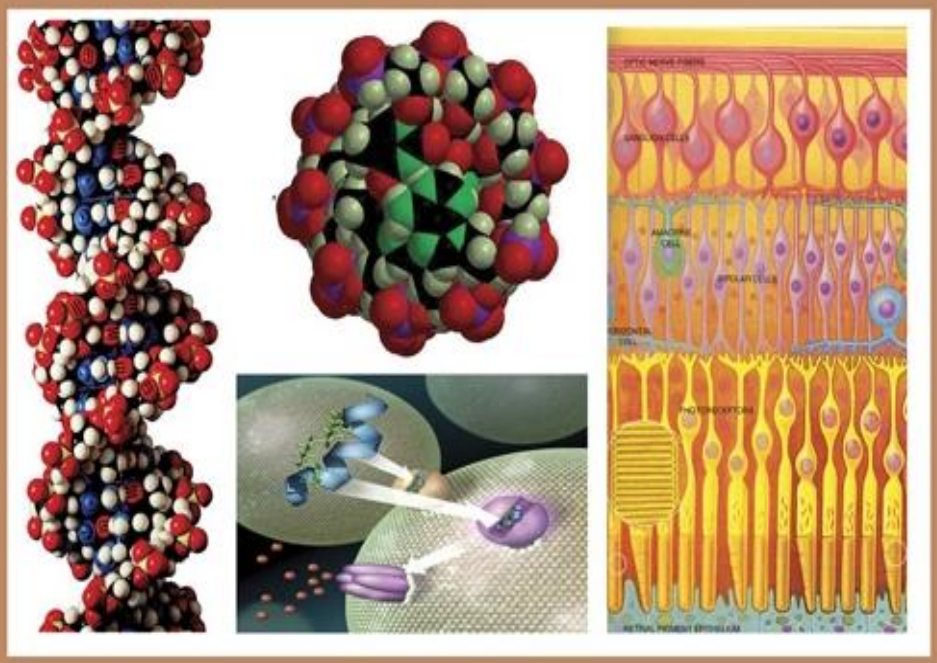

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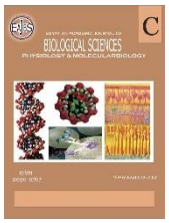

Study for the DNA Profiling of Mixed Samples of Multiple Contributors and Suggested Procedures to Overcome Mixture Troubleshooting in Forensic Investigation

Sobhy El-Sayed Hassab El-Nabi[1], Eman H. S. Khater[2], Ahmed E. Abd El-Salam Ahmed[3]and Mohammed Hussein Awwad[2]

1- Faculty of Science, Menoufia University, Shebin El-Koum, Menoufia, P. O. Box 32511
2- Faculty of Science, Benha University, Benha, Qalyubia, Egypt, P.O. Box. 13518
3- General Authority of Forensic Evidence, Ministry of Interior, Cairo, P.O. Box. 11517
E.Mail: **ahmedebrahim.aeae@gmail.com**

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ABSTRACT

In modern forensic genetics, the analysis of DNA mixtures is a challenge because of the obstacles found in defining the number of contributors, especially when they exceed two. The goal of the present study was to find the most reliable method of detection for profiling the two-male and female-male mixtures obtained. Bloodstains, saliva, and body tissues from various crime scenes were obtained as samples. All samples for DNA profiling have been subjected to DNA extraction, amplification, separation, and detection. Results revealed that DNA profiling showed two-male and female-male mixtures. Results also showed the presence of a major and a minor male contributor in each genetic locus according to peak heights. Differential extraction provided a reliable method for separating female and male contributors from each other in the case of the female-male mixture. The profiling of the male donor in the female-male mixture was furtherly verified using the Y-STR profiling process. Eventually, we can infer that a good approach to identifying the two-male mixture is autosomal STR interpretation centered on major and minor differentiation. In addition, differential separation and Y-STR profiling proved to be the best processes for profiling mixed samples comprising female-male contributors as only the male profile was identified.

INTRODUCTION

Samples recovered for DNA analysis in a forensic crime scene often consist of mixtures of cells that may belong to the suspect, victim, and an infinite number of additional persons. In modern forensic genetics, the analysis of complex DNA mixtures, especially when the number of contributors exceeds two, poses a challenge. As a result, laboratory methods, mathematical models, and software have recently been developed to enhance these studies (Kumar *et al*., 2013; Hu *et al*., 2014; Bieber *et al*., 2016 and Butler and Willis, 2020). Biological evidence that could be acquired from two or more granters is attributed to DNA mixtures and is established after a DNA profile is formed. Mixtures can be collected in a crime scene either from the evidence of sexual assault, fingernail cuttings taken by police or at autopsy, from conception items, and other related materials (Bieber *et al*., 2016). Two contributors are typically included in the mixtures obtained; one of them is predominantly identified and the remaining part of the DNA mixture profile is intended to belong to a second person (i.e. a potential person of interest or a foreign contributor).

Therefore, as the amount of DNA is abundant, the analysis of such evidence is relatively uncomplicated and as a result, the attribution of each individual in the collected sample can be easily determined and the allelic contributions to the DNA evidence of the known individual can be easily extracted from the profile of the DNA mixture (Bieber *et al*., 2016 and Semaan *et al*., 2020). In addition, the presence of three or more allelic peaks that are greater than a given heterozygote peak height ratio at two or more genetic loci or peak height differences are indications that multiple donors contributed to the particular DNA sample examined. A complex DNA mixture may contain more than two donors, a low amount of DNA template may have been contributed by one or more donors, or the sample may be somewhat degraded. During DNA amplification, low levels of input DNA can have stochastic effects on STR test results, which in turn can lead to failure to detect any or all of a true donor's alleles (i.e., drop-out of the allele) (Benschop *et al*., 2011; Bieber *et al*., 2016 and Semaan *et al*., 2020). Although the interpretation of a mixture prior to the statistical measurement involves the direct use of peak heights, such consideration is not needed for the assumed number of contributors, the genotype of identified contributors, or the genotype of persons of interest (Bieber *et al*., 2016). However, no unifying procedure for the application to test forensic DNA mixtures has been published so far, leading to ambiguity among forensic practitioners about the proper DNA mixture analysis methodology. In addition, samples are often enriched with imbalanced mixtures of epithelial cells and sperm in sexual assault cases, with an excess of the victim's content, resulting in an unfavorable male to female DNA ratio. According to several studies, it is not possible to detect the male autosomal DNA portion of the mixture beyond the 1:10 to 1:20 ratio of male: female DNA (Vuichard *et al*., 2011). This is basically due to rivalry during PCR amplification for the primers, which contributes to the main component of the mixture being preferentially amplified. In such cases, the use of Y-chromosome genetic markers, such as short tandem repeats (STRs), may allow small amounts of male DNA to be amplified independently of the DNA background of the victim (Vuichard *et al*., 2011 and Roewer, 2019). A Y-STR profile, however, is not as insightful as an autosomal STR profile. For complex mixtures for which the contributor profiles could not be deconvoluted and for mixtures for which an anticipated male component was not detected by autosomal analysis, Y-STR analysis was particularly useful (Purps *et al*., 2015 and Ramos González *et al*., 2020). Differential DNA extraction, which relies on differential tolerance of spermatozoa and epithelial cells to chemicals, is a solution to this issue (Gill *et al*., 1985 and Yoshida *et al*., 1995). Differential DNA extraction, which does not require expensive equipment and is quickly achieved, is used by most forensic laboratories. In brief, this method involves a mild step of cell lysis that allows an epithelial cell fraction enriched with DNA from the epithelial cells and leukocytes of the female to be recovered. To break the spermatozoa membrane and recover their DNA in the sperm fraction, stronger cell lysis is then used (Gill *et al*., 1985).

The purpose of this study is therefore to distinguish between samples collected from crime scenes whether they contain a mixture of DNA profiles and to study the contents of this genetic mixture and how genetic information can be extracted from it by separating profiles.

MATERAILS AND METHODS

Sample Collection:

From various crime scenes (sexual assault, murder, robbery), 44 saliva swabs, and 26 swabs of different body tissues were collected using a buccal swab (Silver Crest Mines Inc). Depending on color change, only samples collected from sexual assault crim scene were subjected to a presumptive and confirmatory semen test (Greenfield and

Sloan, 2002). In case of positive results, specimens underwent a differential extraction method according to McKiernan and Danielson, (2017) to separate the male sperm cells from the sample.

Sample Processing:

1-Differential Extraction of DNA:

Additional cell separation methods were used to differentiate sperm and female epithelial cells from each other in samples that showed positive semen tests (Yoshida *et al*., 1995 and Chen *et al*., 1998). 190 µl of Buffer G2 and 10 µl of Proteinase K were added to the samples. Samples were thoroughly vortexed for 10 s (MIX, FALC Instruments, Italy) and incubated with a thermomixer for 1-2 hours at 56 °C. The samples were centrifuged at 15,000 x g for 5 min after incubation (CF-10, DAIHAN Scientific Co., Ltd., Korea). The precipitated sperm cells were washed away in 500 µl Buffer G2 by resuspending the pellet. The tube was centrifuged for 5 min at 15,000 x g and the supernatant was discarded. This step has been repeated on two or three occasions. 160 µl of Buffer G2, 10 µl of Proteinase 11, then. K and 40 µl 1 M DTT were added to the pellet, vortexed for 10 s, and the pellet was resuspended. The samples were recently incubated in a thermomixer at 70 °C for 10 min at 850 rpm. Samples were vigorously vortexed for 10s following incubation. The specimens were finally centrifuged, and they were ready for further analysis.

2-DNA Extraction:

According to Montpetit *et al*. (2005), genomic DNA was obtained using the Biorobot EZ1 and Qiagen Kits EZ1 DNA Investigator Kit (cat. no. 952034). The extraction step was carried out using the Biorobot EZ1 equipment and the Qiagen EZ1 DNA Investigator Kit (cat. no. 952034) to obtain highly purified genomic DNA in accordance with the Biorobot EZ1 technical manual.

3-Amplification via PCR:

GlobalFiler™ and Yfiler™ Plus kits (Thermo Fisher Scientific) were used for autosomal STR and Y-STR amplification using the Gene Amp PCR System 9700 thermal cycler (AB/LT/Thermo) as per the GlobalFiler™ and Yfiler™ Plus user guide kits. Standard condition amplification reactions were 25 µl in total volume, consisting of 7.5 µl of Master Mix, 2.5 µl of Primer Set, and 15 µl of sample input. Using the Applied Biosystems GeneAmp™ PCR system 9700 (Thermo Fisher Scientific), samples were amplified. The standard parameters for thermal cycling were as follows: enzyme activation at 95 °C for 1 min; 29 denaturation cycles at 94 °C for 10 s and annealing/extension cycles at 59 °C for 90 s; followed by the final extension stage for 10 min at 60 C.

4-Sample Electrophoresis and Data Analysis:

Applied Biosystems ABI 3500 (Applied Biosystems, USA) and the appropriate software (GeneMapper™ID-X, version 1.4; Thermo Fisher Scientific, USA) were analyzed with capillary electrophoresis (CE) of amplification products using standard procedures. All peaks with a height exceeding 50 relative fluorescence units (RFU) on the electropherogram have been considered for interpretation.

Ethical Approval and Consent to Participate:

All sampling procedures were in compliance with the Ethics Regulations provided by Resolution No 238/2003 of the Egyptian Minister of Health, Articles 52-61 and Recommendations of the 4th session of the EC International Bioethics Dialogue (European Ethics Committee on Science and Emerging Technologies to the European Commission, Copenhagen, Denmark, 19 June 2012).

RESULTS AND DISCUSSION

Interpretation of Two-Males Mixture:

In most of the genetic loci, the DNA profiling of certain samples obtained expressed more than two peaks, confirming the existence in each sample of two contributors. Some collected samples of autosomal STR analysis revealed that the contributors were two males. DNA profiling of the case studied as autosomal STR profiling and peak heights were identified as

1st male, mixture, and 2nd male in Table (1). As a matter of fact, in DNA profiling of one contributor, autosomal STR genetic loci often showed two alleles in any genetic loci. A sign of blending is the identification of more than two alleles. Consequently, as the amount of DNA is abundant, the analysis of such evidence is relatively uncomplicated and as a result, the attribution of each individual in the collected sample can be easily determined and the allelic contributions to the DNA evidence of the known individual can be easily extracted from the profile of the DNA mixture (Bieber *et al*., 2016; Bille *et al*., 2019; and Semaan *et al*., 2020).

Excluding the following genetic loci; Yindel., Amel., and DYS391, results revealed that the 1st male was the major contributor, according to the major and minor method for interpretation, as 16 genetic loci displayed the highest peak heights. However, the 2nd male showed minor peak heights at the same genetic loci (Fig. 1). Owing to the unequal quantity of samples belonging to both authors, the occurrence of major and minor peaks may be due (Ballantyne *et al*., 2014; Lynch and Cotton, 2018; Tan *et al*., 2018).

Table 1: DNA profiling of two-males mixtures obtained from collected samples in accordance with major loci (red color) and minor loci (blue color) contributors**.**

Genetic Loci	Studied cases															
	Autosomal STR								Peak height							
	1st male		Mixture				2nd male		1st male		Mixture				2nd male	
D3S1358	15	18	15	16	17	18	16	17	4000	3500	6000	2500	2500	6000	6000	5000
vWA	15	15	15	16	17		16	17	6000	5500	6000	2500	2500		8000	7000
D16S539	11	13	11	13			11	13	6000	5750	5000	5000			7000	6000
CSF1PO	12	12	7	10	12		7	10	5000	5000	1500	1500	4000		5000	4000
TPOX	8	8	8	9			9	9	6000	6000	4000	1500			5000	5000
Yindel	2		2				2		15000		21000				25000	
Amel.	X	Y	X	Y			X	Y	5000	5000	5000	5000			5000	5000
D8S1179	15	15	13	14	15		13	14	20000	19000	15000	15000	7000		27000	25000
D21S11	27	29	27	29	31	31.2	31	31.2	10000	10000	9000	9000	2500	2500	10000	10000
D18S51	16	16	12	14	16		12	14	5000	5000	1500	1500	6000		5000	5000
DYS391	10		10				10		5000		5000				10000	
D2S441	11.3	14	11	11.3	13	14	11	13	15000	14000	8000	12000	8000	12000	15000	15000
D19S433	13	14	13	14	17		14	17	18000	17000	8000	14000	6000		20000	16000
TH01	6	7	6	7			6	7	15000	15000	12000	12000			20000	20000
FGA	19	22	19	22	24		24	24	20000	18000	9000	9000	12000		30000	30000
D22S1045	15	15	15	17			17	17	6000	5500	11000	4000			16000	16000
D5S818	12	12	11	12	13		11	13	3000	3000	1500	3000	1500		8000	8000
D13S317	12	13	12	13			12	13	5000	5000	4000	4000			6000	5500
D7S820	10	10	8	10	11		8	11	10000	10000	2000	4000	2000		5500	5500
SE33	16	33.2	16	19	20	33.2	19	20	5000	4000	3000	1000	1000	3000	4000	4000
D10S1248	15	15	13	15	17		13	17	1000	1000	500	1000	500		2000	2000
D1S1656	16	16	13	16	17.3		13	17.3	4000	4000	1000	2000	1000		3000	2500
D12S391	15	17	15	17	19	24	19	24	2000	2000	9000	9000	3000	3000	3000	2500
D2S1338	17	17	17	20	21		20	21	6000	6000	9000	4000	4000		9000	8000

Major loci (red). Minor loci (blue)

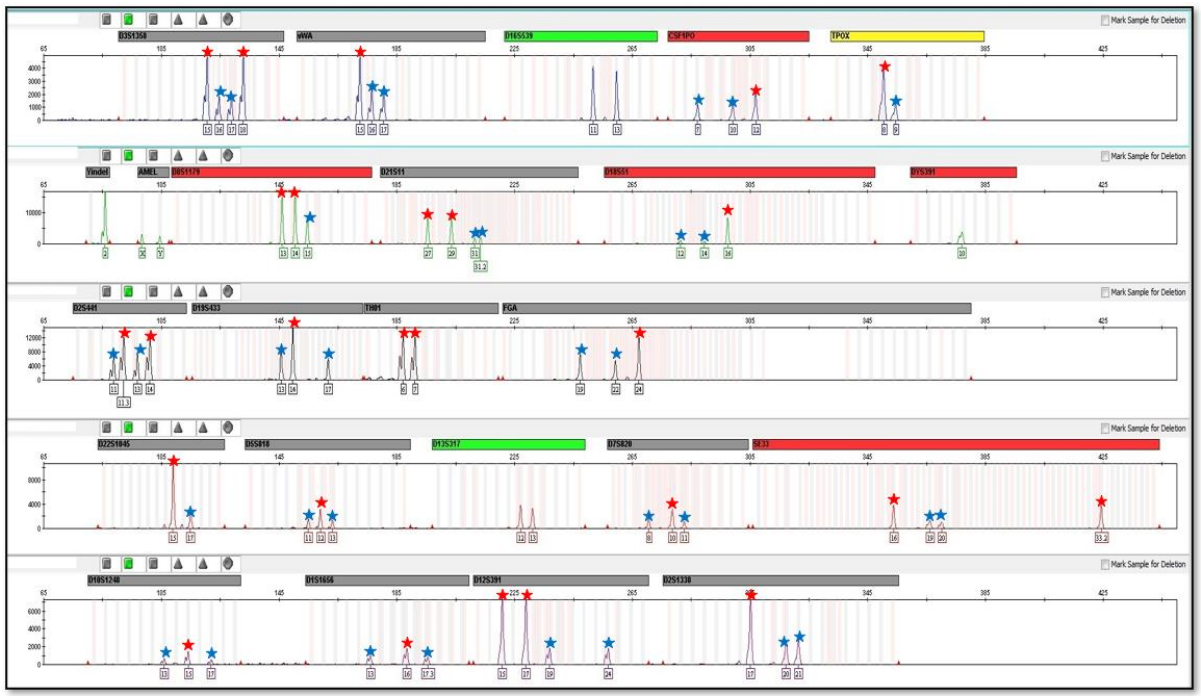

Fig. 1: Electropherogram showing the DNA profiling of all genetic loci from two-males mixture samples showing the major loci (red stars) and minor loci (blue stars).

Interpretation of Female-Male Mixture:

1-Interpretation According To Major And Minor Contributors:

Autosomal STR analysis of collected samples revealed that when more than two alleles were illustrated, there were two contributors. DNA profiling of the examined samples showed that as detected from Amel., Yindel., and DYS391 genetic loci, the mixtures belonged to female and male contributors. DNA profiling of the studied case as autosomal STR profiling and peak heights were mentioned in Table (2) as female, mixture, and male. By excluding Yindel, Amel., and DYS391 genetic loci, obtained results revealed that the female was the main contributor as 16 genetic loci displayed high peak heights, while the male contributor was the minor contributor at the same genetic loci. In addition, results revealed that both female and male contributors have three genetic loci in common; they were D19S433, D7S820, and D12S391). Moreover, the male contributor show majority in two genetic loci; CSFIPO and D10S1248, but the female was minor at the same genetic loci (Fig. 2). Using this method, the complete male profiling of the mixture proves its suitability for the identification of male donors apart from female contributors. As the collected samples contain only epithelial cells, autosomal STR is a potential procedure for male detection. However, if the detected common genetic loci are the majority of male and female contributors, other procedures such as Y-STR profiling should be considered (Berger *et al*., 2003 and Penn, 2019).

Table 2: DNA profiling of female-male mixtures obtained from collected samples in accordance with major loci (red color) and minor loci (blue color) contributors.

Genetic Loci	Studied cases														
	Autosomal STR							Peak height							
	Female		Mixture			Male		Female		Mixture			Male		
D3S1358	15	15	15	17	18	17	18	9800	6200	16000	4000	4000	15000	15000	
vWA	16	17	16	17	19	19	19	13000	10500	12000	12000	4000	14000	12000	
D16S539	12	12	10	12	13	10	13	13500	10000	4000	8000	4000	10000	9500	
CSF1PO	10	12	10	11	12	11	11	4000	3800	3000	6000	3000	9000	9000	
TPOX	8	8	8	9	11	9	11	6200	6000	6000	4500	4500	15000	15000	
Yindel			2			2				25000			25000		
Amel.	X	X	X		Y	X	Y	30000	30000	23000		10000	15000	13000	
D8S1179	12	13	10	12	13	14	10	14	23000	25000	8000	12000	12000	8000	27000	25000
D21S11	29	29	29	30	31.2	30	31.2	20000	17000	11000	6000	6000	16000	15000	
D18S51	15	15	15		17	17	17	4200	4000	9000		2000	7000	7000	
DYS391			10			10				4000			4000	4000	
D2S441	12	14	11	12	14	16	11	16	20000	12000	6000	8000	8000	6000	12000	12000
D19S433	13	14	13		14	13	14	15000	15200	12000		12000	20000	17000	
TH01	7	10	7	9	10	9	9	17000	18000	13000	5000	13000	14000	12000	
FGA	21	23	20	21	22	23	20	22	12000	10000	6000	12000	6000	12000	11000	10000
D22S1045	14	14	11	14	16	11	16	25000	19000	8000	12000	8000	16000	14000	
D5S818	9	11	9	11	12	13	12	13	12000	13000	6000	6000	2500	2500	12000	11000
D13S317	9	9	9	12	13	12	13	8000	7000	10000	3500	3500	18000	18000	
D7S820	10	11	10		11	10	11	6500	6000	8000		8000	12000	12000	
SE33	17	17	14	17	18	14	18	8000	8000	4000	6000	4000	4000	3700	
D10S1248	13	14	13	14	15	15	15	6200	6000	4000	4000	12000	12000	12000	
D1S1656	11	11	11	16	17.3	16	17.3	8000	8300	14000	4000	4000	18000	18000	
D12S391	15	18	15	18	20	22	20	22	5000	4000	2000	2000	2000	2000	3000	3000
D2S1338	18	18	18	19	23	19	23	8000	9000	10000	5000	5000	8000	7500	

Major (red). Minor (blue)

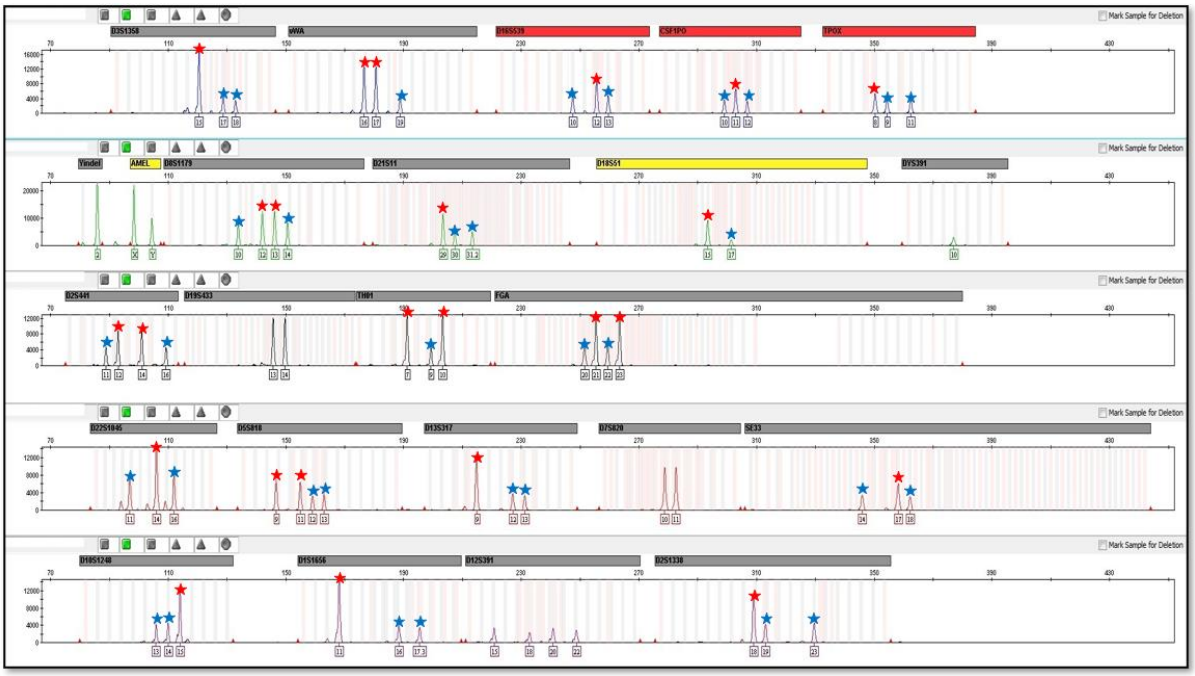

Fig. 2: Electropherogram showing the DNA profiling of all genetic loci from female-male mixture sample showing the major loci (red stars) and minor loci (blue stars).

2-Interpretation according to Y-STR:

Female-male mixture autosomal STR profiling obtained from collected samples shows high peak heights were displayed by both female and male contributors as shown in table (3) and figure (3). As a result, the individualization of the male profile was employed by Y-STR DNA profiling as shown in table (4) and figure (4).

The autosomal STR profiling of the female-male mixture showed that both contributors have the same high peak heights at about (17) genetic loci (Fig. 3). Therefore, using the Y-STR process can solve this

problem. When detecting small quantities of male DNA in a female/male mixture, Y-STRs are useful because Y-STRs are only present on the Y chromosome, so it does not matter how much female DNA is present in the sample. (Malsom *et al*., 2009; Kamodyová *et al*., 2013; Isir *et al*., 2015; and Penn, 2019). If the males are not from the same lineage, they can also distinguish between multiple male donors. The Y-chromosome is passed down from generation to generation, so all males in that lineage will have the same Y-STR profiles, with the possible exception of rapidly mutating Y-STRs that have been recently reported. (Prinz *et al*., 2001; and Purps *et al*., 2015). While Y-STRs cannot provide the same level of discrimination as autosomal STRs, Y-STRs should be used in those instances where it is not possible to recover an autosomal STR profile, as they can still identify the male contributor (Prinz *et al*., 2001; Cerri *et al*., 2003; and Penn, 2019).

Table 3: Autosomal STR profiling of female-male mixtures obtained from collected samples showing no distinguished major and minor peak heights at most genetic loci.

Genetic loci	Peak heights							
	Peak heights Of Mixture				Autosomal STR Of Mixture			
D3S1358	1900	1900	1800		15	16	17	
vWA	2400				17			
D16S539	500	1400	1200		9	10	11	
CSF1PO	3700				12			
TPOX	700	800	800		6	8	10	
Yindel	200				2			
Amel.	3600		3600		X		Y	
D8S1179	200	2300	200	2200	9	13	14	16
D21S11	1500		1700		30		31.2	
D18S51	1600		1500		13		15	
DYS391	600				10			
D2S441	3500		3500		11		14	
D19S433	4600		4500		14		15.2	
TH01	1400		7900		7		9	
FGA	3800	3600	3700	3600	19	20	24	25
D22S1045	1800		2100		15		16	
D5S818	2200		2100		11		12	
D13S317	800	200	1400		8	11	13	
D7S820	1800	1800	1700		10	11	13	
SE33	2400		2100		16		23.2	
D10S1248	1600	1700	1700		12	14	16	
D1S1656	1900		1600		14		15.3	
D12S391	1100	1000	1100		18	19	20	
D2S1338	3600	3600	3700	3600	18	20	22	23

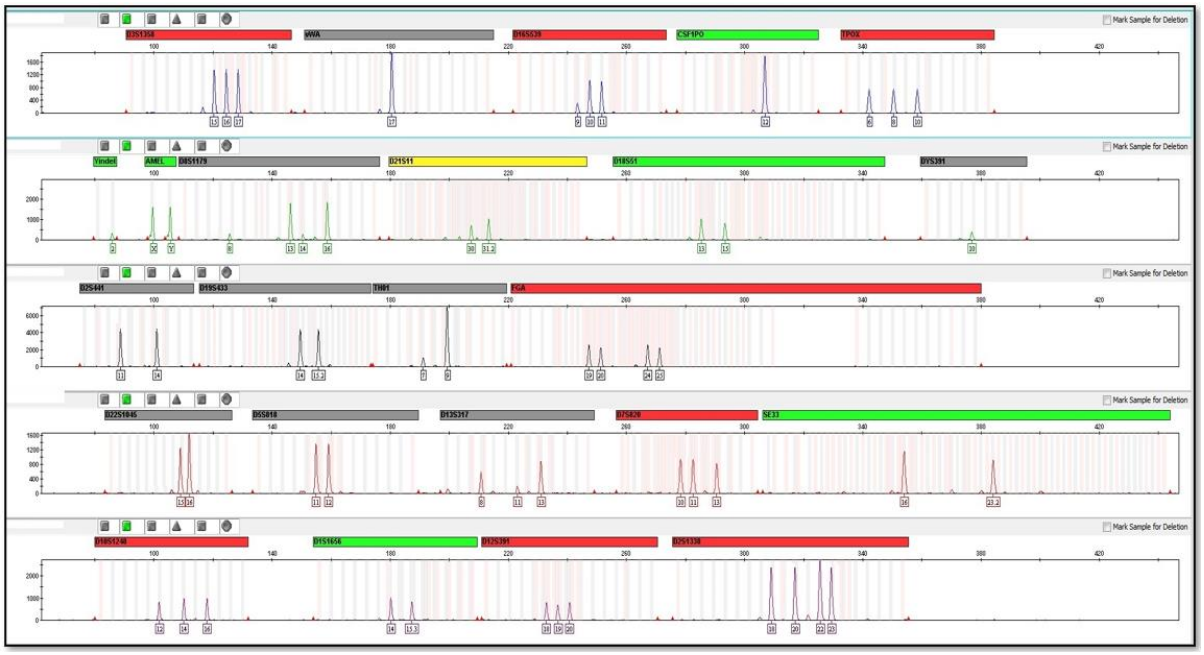

Fig. 3: Electropherogram of autosomal STR profiling of female-male mixture obtained from collected samples showing no distinguished major and minor peak heights at most genetic loci**.**

Table 4: Y-STR profiling of female-male mixtures obtained from collected samples in accordance with obtained alleles.

Y-STR loci	Alleles
DYS576	20
DYS3891	12
DYS635	19
DYS38911	29
DYS627	17
DYS460	10
DYS458	17
DYS19	14
YGATA H4	12
DYS448	20
DYS391	10
DYS456	17
DYS390	24
DYS438	10
DYS392	11
DYS518	39
DYS570	18
DYS437	14
DYS385a	17
DYS385b	19
DYS449	33
DYS393	13
DYS439	13
DYS481	23
DYS387S1a	36
DYS387S1b	36
DYS533	12

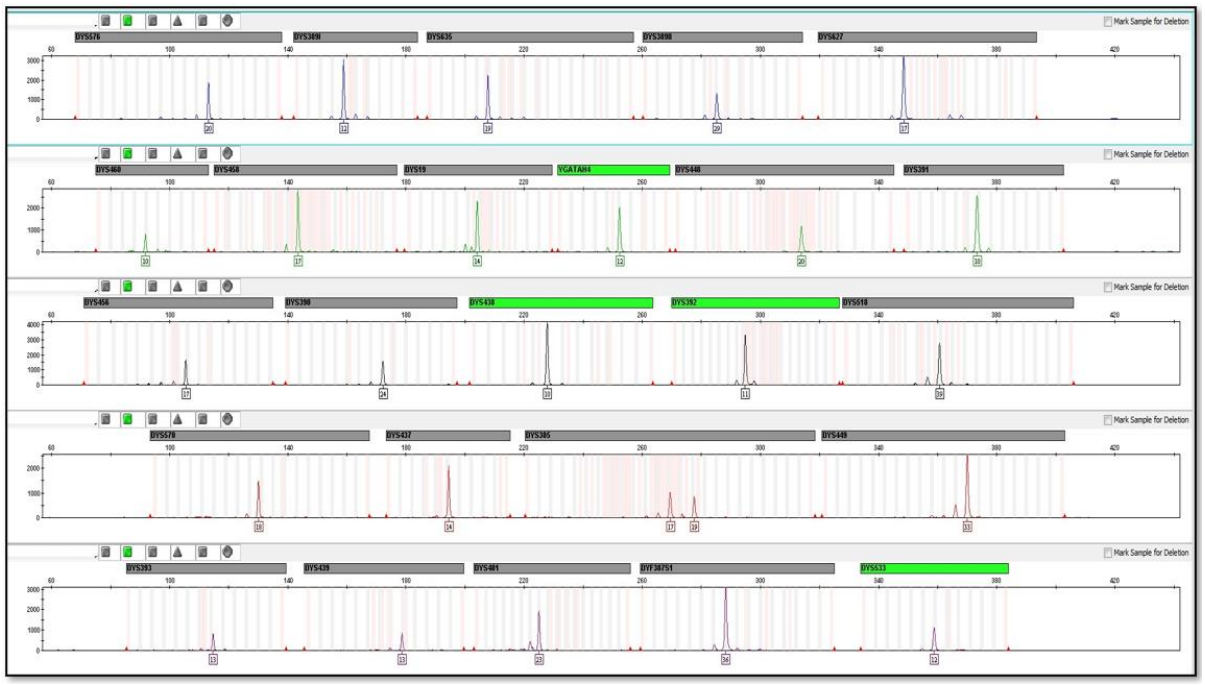

Fig. 4: Electropherogram of Y-STR profiling of female-male mixture obtained from collected samples showing that only the male Y-STR profile can be detected

3-Interpretation According To Differential Extraction:

The autosomal STR profiling of the female-male mixture before and after differential extraction was performed on the collected samples was shown by electropherograms in figures (5 and 6) and table (5). The results revealed that only the male profile was observed by applying differential extraction to mixed samples. The sperm and epithelial cell DNA are selectively lysed and isolated from a female-male mixture during the differential extraction. By increasing the probability of obtaining a clear male DNA profile from the sperm fraction, this extraction method can simplify the interpretation of DNA profiling results (Hudlow and Buoncristiani, 2012; McKiernan and Danielson, 2017; Schwerdtner *et al*., 2017 and Goldstein *et al*., 2020).

For male detection, autosomal STR is a potential technique as the amount of sperm is higher than the female epithelial cells (Berger *et al*., 2003; and Penn, 2019). However, in some cases, the quantity of the female's cells is larger than the male's cells. This represents an obstacle to the detection and isolation of male's cells especially when both cells are mixed together (Penn, 2019).

Eventually, we can conclude that autosomal STR interpretation for mixed samples depending on major and minor differentiation represents a good approach to define the two-males mixture. Furthermore, differential separation and Y-STR profiling proved to be the best procedures for profiling mixed samples contain female-male contributors as only the male profile was detected.

Table 5: Autosomal STR profiling of female-male mixture obtained from collected samples before and after differential extraction

Genetic loci	Mixture before differential extraction				Mixture after differential extraction	
D3S1358	16	17	18		17	17
vWA	15	17	18	19	15	19
D16S539	11		12		11	12
CSF1PO	10		11		10	11
TPOX	8	9	11		8	9
Yindel	2				2	
Amel.	X		Y		X	Y
D8S1179	11	13	15		11	15
D21S11	29		30		30	30
D18S51	13		18		13	15
DYS391	10				10	
D2S441	11		12		12	12
D19S433	11	13	15	16	11	16
TH01	6	7	8	9	6	7
FGA	21	23	26		21	26
D22S1045	15		16		15	16
D5S818	11	12	13		10	11
D13S317	10		12		12	13
D7S820	10	11	12		10	11
SE33	14		24.2		15	28.2
D10S1248	14		16		11	13
D1S1656	14	15	16.3	17.3	15	16.3
D12S391	18	22	23		16	22
D2S1338	17	21	24	25	21	24

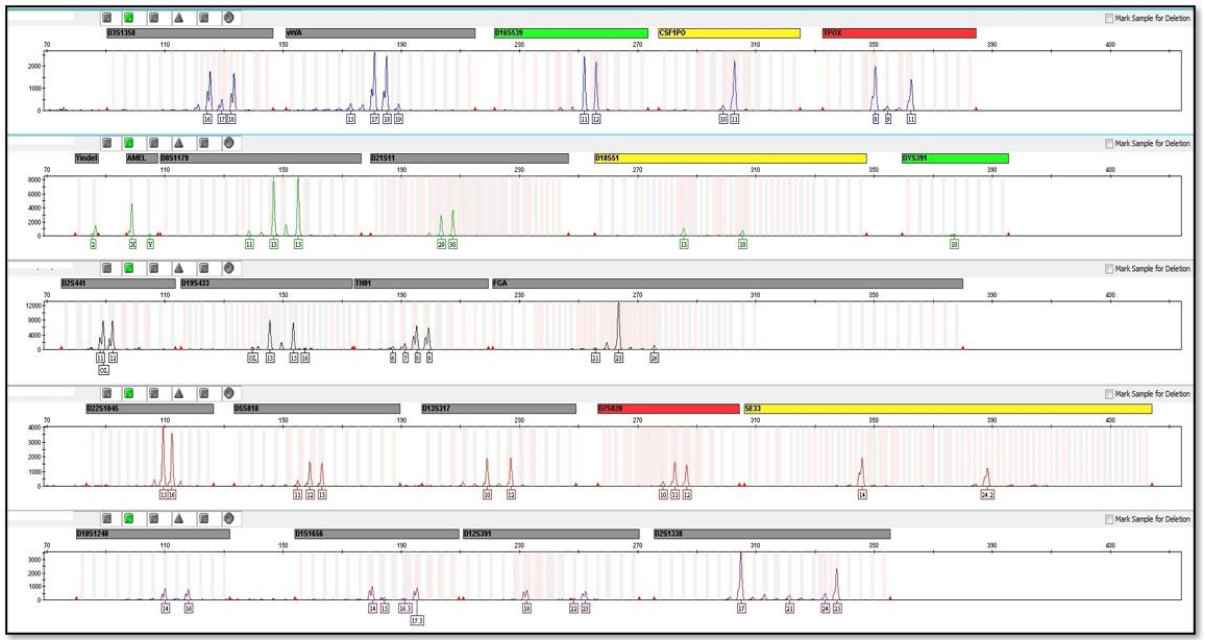

Fig. 5: Electropherogram of autosomal STR profiling of female-male mixture before differential extraction**.**

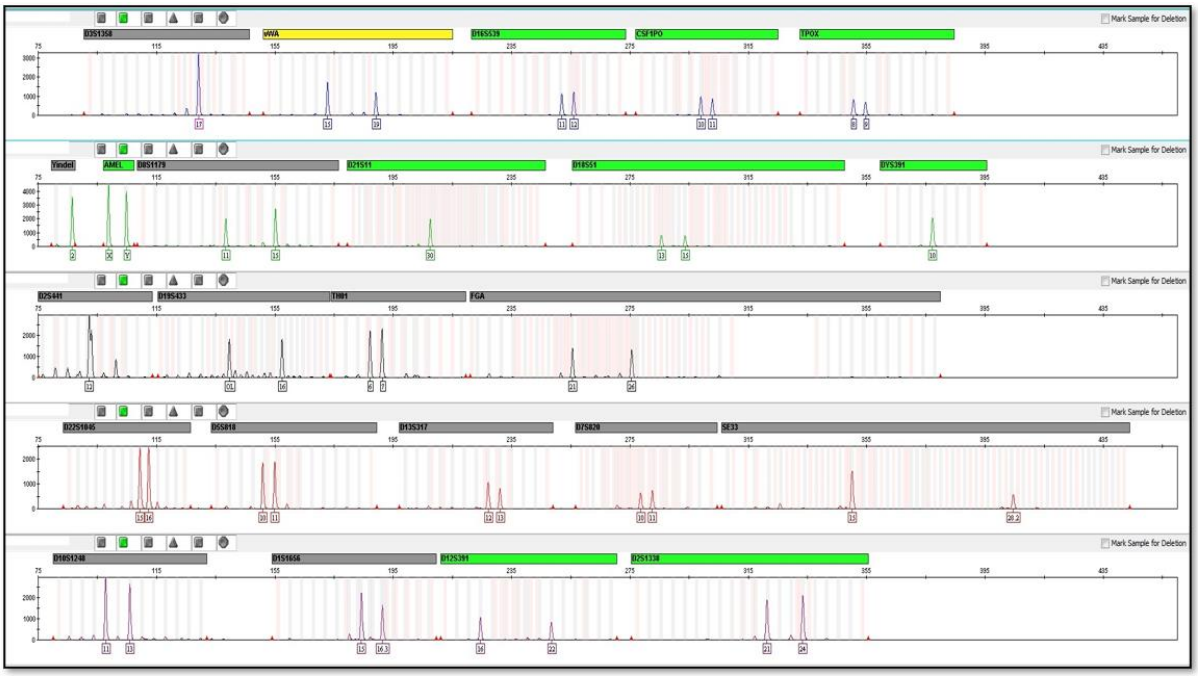

Fig. 6: Electropherogram of autosomal STR profiling of female-male mixture showing the male profiling involved in the mixture after separation by differential extraction

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ARABIC SUMMARY

دراسة توصيف الحمض النووي لعينات مختلطة لمساهمين متعددين وإجراءات مقترحة للتغلب على مشاكل الخليط في التحقيقات الجنائية

صبحي السيد حسب النبي[1]، إيمان حامد شاكر خاطر[2]، أحمد إبراهيم عبد السلام أحمد[3] ومحمد حسين عواد[2]

1 كلية العلوم، جامعة المنوفية، شبين الكوم، المنوفية، مصر

2 كلية العلوم، جامعة بنها، بنها، القليوبية، مصر

3الإدارة العامة لتحقيق الأدلة الجنائية، وزارة الداخلية، العباسية، القاهرة، مصر

يمثل الخليط من التصنيفات الجينية لأكثر من شخص أحد أصعب التحديات التي تواجه خبراء البصمة الوراثية في التحقيقات الجنائية، وأحد أهدافهم هو إيجاد تفسير وتحليل لهذا الخليط وذلك لإيجاد الطرق المناسبة للفصل وبخاصة عندما يزيد الخليط عن شخصين. وتهدف تلك الدراسة إلى إيجاد أفضل الطرق التي يمكن الاعتماد عليها لفصل الخليط لهذه التصنيفات الجينية من البصمة الوراثية، والكشف عنها من خلال النمط الجيني الناتج من الخليط بين ذكرين، وكذلك الخليط بين ذكر وأنثى. تم جمع عدد من العينات المختلفة (عينات دماء، لعاب، وأنسجة) من عدة مسارح للجريمة في قضايا جنائية مختلفة.

تم تعريض جميع العينات لعدة خطوات للحصول على البصمة الوراثية منها بداية باستخلاص الحمض النووي، مرورا بتكبير المواقع الجينية الخاصة بالبصمة الوراثية، وفصل المواقع الجينية على جهاز التحليل الوراثي للحصول في النهاية على النمط الجيني للبصمة الوراثية لتلك العينات. حيث أظهرت النتائج وجود خليط من التصنيفات الجينية لأكثر من شخص يفترض أن يكون بين ذكرين أو ذكر وأنثى. وبتحليل النتائج الخاصة بأطوال الأليلات في المواقع الجينية، أظهرت وجود اختلاف في الأطوال فيما بينها مما يشير إلى وجود أليلات أحد الأشخاص في الخليط أطول من أليلات الشخص الآخر. وفي حالات أخرى تبين تقارب أطوال الأليلات في المواقع الجينية للخليط مما استدعى استخدام كروموسوم الذكورة لتحديد التصنيفات الجينية الخاصة بكروموسوم الذكورة للذكر المشارك في الخليط وهي طريقة فعالة في حالة الخليط بين ذكر وأنثى. كما أن استخدام طريقة الاستخلاص التفاضلي في حالات الخليط بين الذكر والأنثى طريقة مميزة لفصل الذكر عن ذلك الخليط. مما سبق نستنتج أن هناك أكثر من طريقة لفصل الخبيط من أهمها طريقة أطوال الأليلات للأشخاص المشاركة في الخليط، تحديد كروموسوم الذكورة في الخليط بين الذكر والأنثى، ثم طريقة الفصل التفاضلي في حالات الخليط بين ذكر وأنثى في حالة وجود خلايا منوية مختلطة مع خلايا جسدية.