COMPARISON OF THE STATISTICAL RELIABILITY OF DIFFERENT STR MARKERS IN KINSHIP ANALYSIS

FINAL DEGREE PROJECT

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This section is written in Catalan, since it is my mother tongue, in order to convey my gratitude as sincerely and precisely as possible to those it is addressed to.

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ABBREVIATIONS

CODIS	Combined DNA Index System
DNA	Deoxyribonucleic Acid
H_0	Null hypothesis
H_1	Alternative hypothesis
ILS	Internal Lane Standard
ISS	Internal Size Standard
LINEs	Long Interspersed Nuclear Elements
LR	Likelihood Ratio
PCR	Polymerase Chain Reaction
SINEs	Short Interspersed Nuclear Elements
STR	Short Tandem Repeats
VNTR	Variable Number Tandem Repeats
W	Probability of Kinship

SUMMARY

This study evaluates the statistical reliability of autosomal, X-chromosome, and Y-chromosome STR markers in kinship analysis using real casework. Buccal swab samples were processed with commercial kits (VeriFiler™ Express, Argus X-12, YFiler™ Plus), and data were analyzed using Families, FamLinkX, and the YHRD database. Results showed autosomal STRs are highly effective in direct relationships (e.g., paternity), while X-STRs enhanced reliability in sibling analysis involving females. Y-STRs indicated paternal-line links in male cases but lacked resolution for direct brotherhood. The findings highlight the need for a marker-specific approach, tailored to relationship type, to ensure accuracy and robustness in forensic genetics.

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1. INTRODUCTION

1.1. FORENSIC SCIENCES

In its broadest sense, forensic science describes the usefulness of science in relation to legal matters, including many disciplines, such as chemistry, biology, pathology, anthropology, toxicology and engineering, among others. “Forense” comes from the Latin word forum, the central place of the city where debates and disputes were made public to be resolved, thus defining the law of the city. Forensic, in general, means ‘relative or applied to the law’).

Forensic science reconstructs past criminal events through the analysis of the physical remains of these activities (the evidence); the results of these analyses and their expert interpretation establish relationships between people, places and objects relevant to these events. This occurs through logical inferences, induction, abduction and deduction, all within the hypothetical-deductive method, in which investigative heuristics also play a role. (Schneider, 2007).

1.2. DNA

1.2.1. STRUCTURE AND COMPOSITION

Deoxyribonucleic acid (DNA) is a molecule present inside the cells of living beings that contains the genetic information necessary for their development and growth. The structure of this molecule is composed of two interlaced chains in a double helix structure (Figure 1).

Each chain is constructed by a sequence of sugar and phosphate bound to four nitrogenous bases: adenine (A), thymine (T), guanine (G) and cytosine (C). These bases are specifically paired (A with T and G with C) to form chemical bonds that maintain structure integrity (Alberts et al., 2017; Travers & Muskhelishvili, 2015).

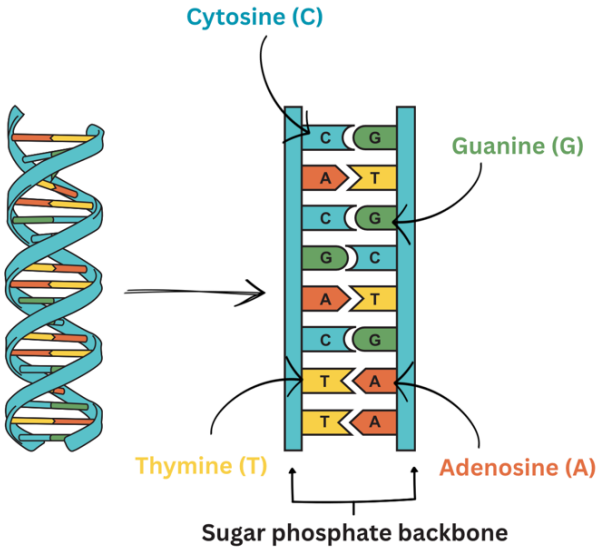

Figure 1 DNA molecular structure (Author's own data).

1.2.2. GENOME ORGANIZATION

1.2.2.1. DNA TYPES IN THE CELL

Cell DNA is presented in two forms: Nuclear DNA and mitochondrial DNA. In humans, the complete set of DNA within a cell is called the human genome and is organized into 23 pairs of chromosomes. This genome is diploid[1] except in sex cells, which are haploid[2], and in red blood cells, which have no nucleus or nuclear DNA, since they are anucleated.

1.2.2.2. INHERITANCE OF NUCLEAR AND MITOCHONDRIAL DNA

Regarding nuclear DNA, each individual inherits a copy of the genome from their progenitors, which are transmitted through the gametes. The mitochondrial DNA, on

[1] Diploid is a term that refers to the presence of two complete sets of chromosomes in an organism's cells, with each parent contributing a chromosome to each pair.

[2] Haploid refers to the presence of a single set of chromosomes in an organism's cells.

the other hand, is transmitted exclusively through the mother, because the mitochondria of the sperm are not preserved in fertilization.

1.2.2.3. CODING DNA

The DNA of the human genome is divided into coding and non-coding DNA. On the one hand, coding DNA contains information in the form of genes that determine protein synthesis. These proteins are essential for proper cell function and the expression of inheritable characteristics.

Coding DNA has a low level of variability, since any mutation in these regions can affect the function of proteins essential for cell functioning.

1.2.2.4. NON-CODING DNA

Non-coding DNA constitutes most of the genome and includes sequences that do not encode by proteins, but that have regulatory functions of gene and structural expression in chromosomes.

These regions show a high genetic variability, since they are repetitive regions prone to mutations during DNA replication without affecting their function, in this way there is a great diversity without significant selective pressure. This fact is what makes them especially useful in forensic genetics for the identification of individuals without showing phenotypic characteristics.

This non-coding DNA can be presented as single copy DNA or repetitive DNA. Within non-coding DNA, repetitive regions are classified into interspersed repetitions and tandem repetitions.

1.2.2.4.1. REPETITIVE REGIONS OF NON-CODING DNA

On the one hand, interspersed repetitions are repetitive sequences distributed throughout the genome, among which the Long Interspersed Nuclear Elements (LINEs) and the Short Interspersed Nuclear Elements (SINEs) stand out, which are mainly differentiated by their size, that is, by the number of base pairs they contain. These sequences play a role in genomic reorganization and can influence gene expression.

On the other hand, tandem repetitions are repetitive sequences grouped into blocks and can be classified into satellites, minisatellites and microsatellites.

Satellites are sequences of 5 to several hundred nucleotides repeated thousands of times that are located mainly in centromeres and telomeres, contributing to chromosomal stability.

Minisatellites, also known as Variable Number Tandem Repeats (VNTR), are repetitive units of 6-25 nucleotides that generate regions of 100 to 20.000 base pairs. Due to their high variability, they are used in studies of affiliation and forensic identification.

Microsatellites, also known as Short Tandem Repeats (STR), are repetitive units of 2-4 nucleotides with less than 150 base pairs. These are especially valuable in forensic genetics for individual identification, as they present great variability between individuals.

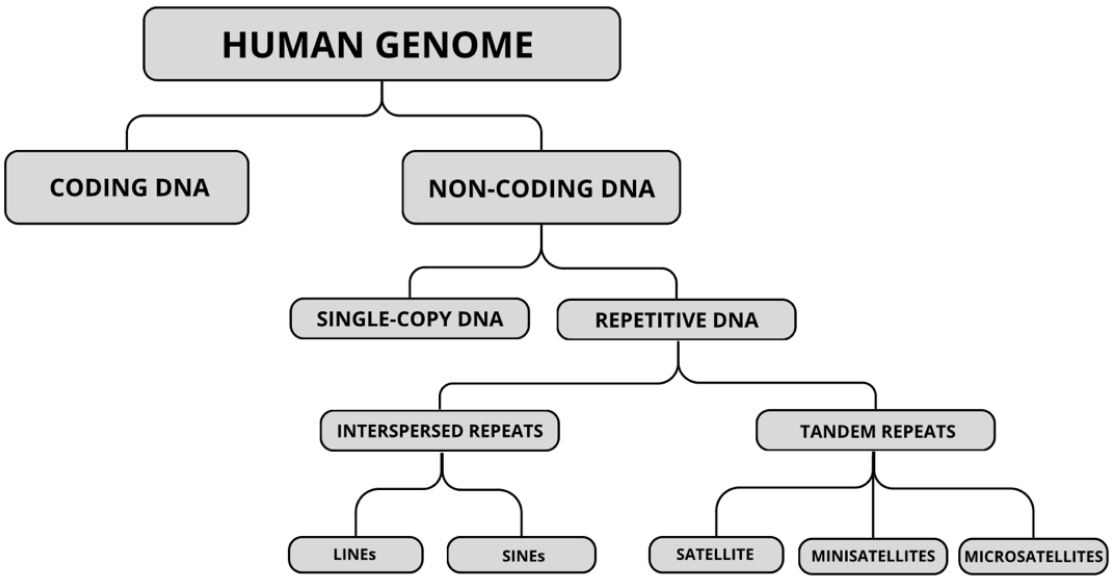

Figure 2 Organizational diagram of the human genome (Author's own data).

1.3. MARKERS OR LOCI STRs

The markers or STRs loci consist of short repetitive sequences of three to five base pairs of length. These are distributed throughout the human genome and represent a rich source of highly polymorphic[3] markers, which can be amplified using the Polymerase Chain Reaction (PCR).

The alleles of the STRs loci are differentiated by the number of copies of the sequence repeated in the amplified region. This means that, in the same loci, different individuals can have a variable number of repetitions, which makes these markers very useful for genetic analysis, since their high rate of polymorphism makes them essential tools for individual discrimination in genetic profiles.

Another aspect that makes them relatively interesting for forensic genetics is that, although they have a high variability rate, they have a relatively low mutation rate and are not expressed, that is, they do not give information neither encoding nor phenotypic (Wyner et al., 2020).

1.4. GENETIC MARKERS USED

STR markers are tandem-repeated nucleotide sequences found in the human genome that do not encode proteins. They are highly useful in molecular diagnosis, population studies, kinship relationship analysis and identity testing, as well as their applications in criminalistics, paternity testing and kinship studies.

Its short size, polymorphic nature, ease of amplification, development of multiplex systems and rapid analysis have increased the usefulness of STR markers, making this technology an essential part of most DNA analysis laboratories. However, degradation,

[3] Polymorphism, as related to genomics, refers to the presence of two or more variant forms of a specific DNA sequence that can occur among different individuals or populations. The most common type of polymorphism involves variation at a single nucleotide (also called a single-nucleotide polymorphism, or SNP).

low number of copies and mixed sources represent a major challenge for forensic DNA analysis using STR markers.

As for forensic genetics, a specific set of internationally standardized STRs loci is used, which are used to compare genetic profiles in criminal databases, such as the Combined DNA Index System (CODIS). This methodology is key to the identification of individuals and to be able to establish a kinship relationship in legal contexts. In addition, in medical and evolutionary genetics, the study of STRs allows analyzing genetic variability between individuals and populations, as well as reconstructing genealogical trees and establishing biological relationship.

Although current kits have facilitated the analysis of complex samples, only a basic set of STR markers is included for DNA analysis in various populations around the world. To obtain valid and understandable information, as well as for the generation of databases, a basic set of STR markers has been proposed for analysis in forensic and paternity applications (Caplinskiene et al., 2011; Dash et al., 2021; Sampaio et al., 2022; Udogadi et al., 2020; Wyner et al., 2020).

1.4.1. AUTOSOMAL STRs

1.4.1.1. LOCATION AND INHERITANCE OF AUTOSOMAL STRs

Autosomal STRs are those that are located in autosomal chromosomes, that is, in any of the 22 pairs of non-sexual chromosomes. These are inherited biparentally; therefore, the offspring inherits a copy of each parent (Figure 3). They are used in forensic biochemistry for genetic identification, paternity tests and population studies (Dash et al., 2021).

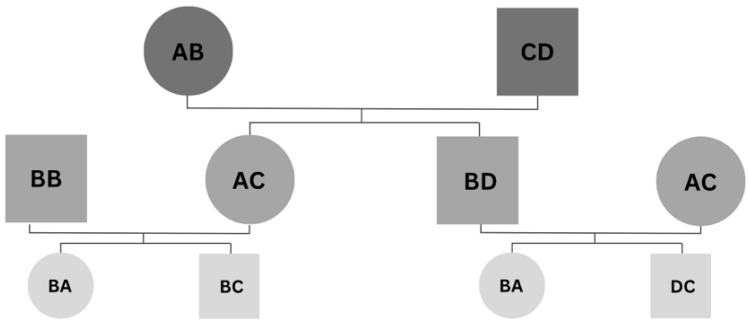

Figure 3 Inheritance pattern of autosomal STRs (Author's own data).

1.4.1.2. AUTOSOMAL STRs ANALYSIS

The VeriFiler™ Express PCR Amplification Kit is a PCR amplification kit developed by Thermo Fisher Scientific, designed for the direct amplification of STR loci in reference samples of human origin.

This kit allows the simultaneous amplification and detection of 25 loci, 23 loci STR (D3S1358, vWA, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, Penta E, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, D6S1043, D10S1248, D1S1656, D12S391, D2S1338 and Penta E), the Amelogenin[4] marker and a polymorphic marker for insertion/deletion located on the Y chromosome (Kurniawan et al., 2023).

1.4.1.3. AUTOSOMAL STRs STATISTICS

To perform the statistical calculations, the Families program is used. This computer program analyzes affiliation relationships using autosomal STR markers by comparing two hypotheses: that there is a biological relationship between individuals (e.g. siblings) or that they are not related. This program uses the calculation of the likelihood ratio (LR) to determine which of the two hypotheses is most likely. (Drábek, 2009; Egeland et al., 2000).

1.4.2. X CHROMOSOME STRs

1.4.2.1. LOCATION AND INHERITANCE OF X CHROMOSOME STRs

As for nuclear DNA, each individual inherits a copy of the genome from their progenitors, which is transmitted through gametes. In meiosis, genetic recombination occurs during the formation of eggs and sperm, especially in autosomal chromosomes and in women's X chromosome. This recombination makes the X chromosome inherited by female

[4] Amelogenin is a protein and a gene, both found in tooth enamel, and is used in forensic science to determine the sex of a DNA sample. The gene is located on both the X and Y chromosomes, but there are size differences between the two, which can be used to distinguish between male and female DNA.

offspring a combination of fragments of the mother's two X chromosomes, generating more genetic variability.

X chromosome STRs are located exclusively on this chromosome, so their inheritance varies by sex. Men have only one copy of the X chromosome, inherited from the mother, while women have two, one inherited from the father and one from the mother (Figure 4). This makes X chromosome STR analysis especially useful in maternal line kinship studies and in cases of complex paternity, such as when the supposed father is a close relative of the biological father.

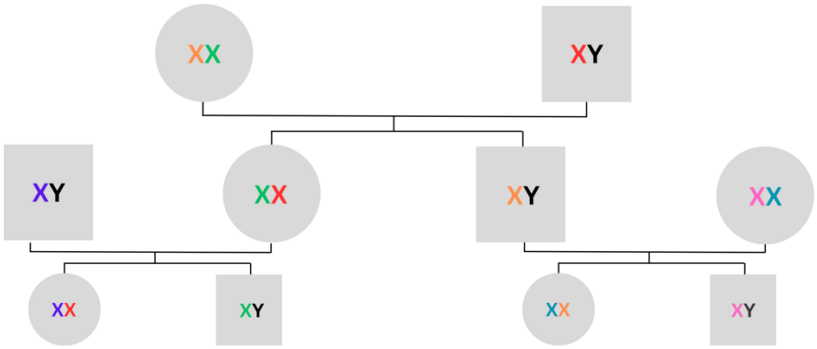

Figure 4 Inheritance pattern of X chromosome STRs (Author's own data).

1.4.2.2. X CHROMOSOME STRs ANALYSIS

The Argus X-12 commercial kit, developed by Qiagen, is a commercial kit for forensic analysis of the X chromosome STR. This kit includes a total of 12 specific STR markers for the X chromosome, and this allows us to obtain useful genetic profiles in kinship studies, especially in cases of maternal brotherhood or in parent absence filiation studies, where autosomal markers or Y chromosome markers are not enough.

This kit allows the simultaneous amplification and detection of 12 STR loci of the X chromosome, which are: DXS10135, DXS8378, DXS10148, DXS10159, DXS10162, DXS10164, DXS10165, DXS101, DXS6789, DXS7423, DXS7132 and HPRTB (Scherer et al., 2015).

1.4.2.3. X CHROMOSOME STRS STATISTICS

The statistical calculation is carried out through the computer program FamLinkX, a particularly useful tool to analyze filiation relationships from the X chromosome STR. This program allows to evaluate kinship hypotheses and calculates the LR, a value that allows to determine the probability of a biological relationship between the analyzed individuals (Kling et al., 2015).

1.4.3. Y CHROMOSOME STRs

1.4.3.1. LOCATION AND INHERITANCE OF Y CHROMOSOME STRs

Y chromosome STRs are regions of DNA that are located exclusively on the Y chromosome. These genetic markers are useful in forensic analysis because they are only transmitted by the paternal line, without recombination except in cases where there is a mutation (Figure 5). This allows establishing kinship relationships through the father and identifying male genetic profiles in mixed samples, such as in cases of sexual assault (Kayser, 2017).

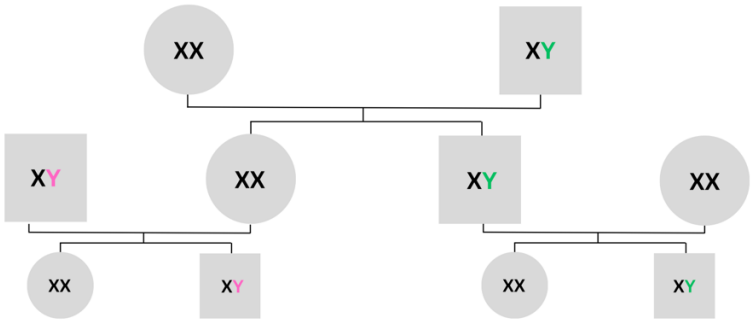

Figure 5 Inheritance pattern of Y chromosome STRs (Author's own data).

1.4.3.2. Y CHROMOSOME STR ANALYSIS

The YFiler Plus commercial kit, developed by Thermo Fischer Scientific, is a commercial kit for forensic analysis of Y chromosome STR. This kit includes 25 specific STR markers

of the Y chromosome, which allows obtaining haplotypes[5] that can be compared with databases to identify genetic relationships or exclude suspects in criminal investigations (Gopinath et al., 2016).

This kit allows the detection and simultaneous amplification of 25 STR loci of the Y chromosome, which are: DYS576, DYS289-I, DYS635, DYS389-II, DYS627, DYS460, DYS458, DYS19, YGATA H4, DYS448, DYS391, DYS456, DYS390, DYS438, DYS392, DYS518, DYS570, DYS437, DYS385a/b, DYS449, DYS393, DYS439, DYS481, DYF387 S1 a/b and DYS533.

1.4.3.3. Y CHROMOSOME STRs STATISTICS

The frequency of Y chromosome haplotypes varies according to the study population, since different ethnic groups and geographical regions present specific genetic distributions. Through the YHRD.org website, it is possible to compare a genetic profile with specific population databases, such as European, Asian or African populations, and determine their relative frequency to support the kinship hypothesis by paternal line.

In this case, the European population database has been used and the YFiler Plus Kit has been selected to perform statistical calculations.

[5] A haplotype is a group of alleles in an organism that are inherited together from a single parent. Many organisms contain genetic material which is inherited from two parents. Normally these organisms have their DNA organized in two sets of pairwise similar chromosomes.

2. HYPOTHESIS

Although autosomal STRs, thanks to their high power of discrimination, are the reference markers in the resolution of biological identification cases, they are not always suitable for all cases.

3. OBJECTIVES

The main objective of this work is to evaluate and compare statistical reliability in different cases of kinship using several STR markers.

In addition to the main purpose, this work pursues several specific objectives:

(1) Become familiar with different STR amplification kits, acquiring knowledge about their characteristics, applications and limitations.
(2) Learn to select the most appropriate genetic method for each case, considering factors such as the nature of the biological relationship and the complexity of the case, beyond the quality or quantity of DNA available in the sample, since it works with reference samples.
(3) To master the use of statistical programs for the interpretation of results.
(4) Apply the knowledge acquired in real practical cases to consolidate the understanding of the methods and tools used in forensic genetics.

4. MATERIALS AND METHODS

4.1. SAMPLE COLLECTION

Samples are obtained from the buccal epithelium using a dry oral swab, a simple and non-invasive method. This technique does not require sample stabilization, as the genetic material is adequately preserved without the need for specific additives or reagents. Furthermore, samples can be processed immediately or stored at room temperature until laboratory analysis, provided that optimal conditions are maintained to prevent genetic material degradation or contamination.

Both the samples collected for the study of a possible sibling relationship and those corresponding to the paternity test were treated in the same way, and the same analytical procedure was applied throughout the entire process.

4.2. EXTRACTION

DNA extraction is performed using the PrepFiler™ Automated Forensic DNA Extraction Kit by Thermo Fisher Scientific, a semi-automated system designed for various applications, including forensic samples. The process is conducted in batches of 24 samples, including a negative control to ensure the detection of potential contamination. All steps outlined in the manufacturer's protocol are strictly followed to guarantee efficient extraction and maximum DNA purity.

The extraction is carried out using the KingFisher™ instrument, which uses magnetic bead-based technology. This automated system enables DNA to bind to magnetic beads, which are subjected to a series of wash steps to remove impurities. Finally, the DNA is eluted into a clean, contaminant-free solution.

4.3. QUANTIFICATION

Once the DNA extract has been obtained, a quantification step is carried out to measure the DNA concentration, expressed in ng/µL, for each sample. For this purpose, the NanoDrop™ One spectrophotometer is used, which determines nucleic acid concentration based on optical absorbance.

Based on the concentration data obtained, sample dilutions are prepared to optimize the performance of the amplification reaction. The samples are diluted to achieve a final concentration of 2 ng/µL in a total volume of 25 µL, in accordance with the laboratory's internal validation protocol.

4.4. AMPLIFICATION

Once the DNA samples have been diluted to the optimal concentration, they are processed using PCR. Depending on the type of genetic marker to be analyzed, the appropriate kit is selected: VeriFiler™ Express, ArgusX-12, or Yfiler™ Plus (Section 1.4). These kits are designed for multiplex PCR amplification of genetic markers and follow specific protocols involving multiple cycles of denaturation, annealing, and extension of DNA.

4.5. SEQUENCING

Following amplification, the resulting DNA fragments are detected using the Applied Biosystems™ ABI 3500 Genetic Analyzer, which employs capillary electrophoresis to separate the fragments based on their size. This technique is based on the differential migration of DNA fragments through a polymer gel-filled capillary under the influence of an electric field. The separation is performed with high resolution, and the fragments are detected by means of specific fluorochromes attached to the amplification probes, which emit a signal as they pass through a laser detection system.

The result of this process is an electropherogram[6], where the fragments are represented as peaks according to their migration time and fluorescence intensity. The size of each

[6] An electropherogram is a graphical representation of the results from capillary electrophoresis. It is a plot that shows how DNA fragments (or other molecules) move through a capillary over time, helping scientists determine the size and quantity of those fragments.

fragment is determined through interpolation between an internal size standard[7] and an allelic ladder mix[8].

The analysis and interpretation of the resulting genetic profiles are performed using the specialized software GeneMapper™ ID-X v1.5. This software automates the sizing and preliminary allele designation, but the final interpretation and validation of the profiles are the responsibility of the analyst. A manual review is required to verify signal quality and to ensure consistency between the analyzed fragments and the corresponding allelic ladder. This process guarantees the accurate assignment of alleles for each marker and ensures the reliability of the obtained genetic profiles (McCord & Buel, 2013).

4.6. STATISTICAL ANALYSIS

As the final stage of the process, a statistical study is conducted with the aim of drawing conclusions based on probabilities derived from hypotheses, which are mutually independent and formulated for each case under analysis.

4.6.1. LIKELIHOOD RATIO (LR)

To perform the statistical analysis of kinship studies, the paternity index or sibling index is used, generally known as the likelihood ratio (LR) (Collins & Morton, 1994).

$$LR = \frac{P(H_0/E)}{P(H_1/E)} = \frac{\frac{P(E/H_0) \times P(H_0)}{P(E)}}{\frac{P(E/H_1) \times P(H_1)}{P(E)}} = \frac{P(E/H_0) \times P(H_0)}{P(E/H_1) \times P(H_1)}$$

[7] An internal size standard (ISS), also known as an internal lane standard (ILS), is a set of known DNA fragments of specific sizes that are used in conjunction with DNA samples during electrophoresis to accurately determine the size of other DNA fragments in the sample.

[8] Allelic Ladder Mix (Ladder): is a scale of the alleles of the most frequent STR markers or loci that serve as an internal reference to assign the size of a DNA fragment to a specific allele of each marker.

This index compares, based on genetic data, how many times more likely the proposed relationship (null hypothesis, H_0) is, in comparison to the alternative hypothesis (H_1), which assumes that the relationship is with a randomly selected individual from the reference population (Baksh et al., 2006; “The Evaluation of Forensic DNA Evidence,” 1997).

Bayes' theorem is used to calculate the LR, which allows the probabilities of two alternative genetic hypotheses, such as relatedness versus non-relatedness, to be compared.

In addition, it is assumed that the population is in Hardy-Weinberg equilibrium, a fundamental principle of population genetics that states that, in the absence of evolutionary factors such as mutation, natural selection, or migration, allele and genotype frequencies remain constant from one generation to the next. This assumption is essential for accurately calculating genetic probabilities in filiation studies (Abramovs et al., 2020; van de Schoot et al., 2017).

Once the value of LR has been calculated, it must be properly interpreted in order to assess the strength of the genetic evidence in support of, or against, a potential biological relationship. This value reflects the extent to which the observed genetic data are more probable under the hypothesis of biological relatedness (H_o) than under the alternative hypothesis (H_1), which assumes no biological relationship.

LR values are interpreted based on qualitative thresholds that are widely accepted in the field of forensic genetics, as summarized in Table 1.

Table 1 Criteria for Interpreting LR in kinship analysis.

LR VALUE	INTERPRETATION
LR<1	The tested individual is unlikely to have the proposed biological relationship (the smaller the value, the less likely).
LR=1	The genetic evidence is inconclusive (equal probability for both hypotheses).
LR>1	The tested individual could have the proposed biological relationship (the higher the LR, the stronger the supporting evidence).
LR >1000	The tested individual is very likely to have the proposed biological relationship (commonly accepted thresholds for confirmation).

Thus, the LR not only enables the quantification of the relative probability of a genetic relationship, but also provides an objective, graded, and practically interpretable criterion for the evaluation of biological kinship within the Bayesian probabilistic framework.

4.6.2. POSTERIOR PROBABILITY OF KINSHIP (W)

Based on this value, the probability of kinship (W) can be calculated, representing the level of confidence with which a biological relationship between individuals can be either accepted or excluded (Fung & Hu, 2008).

$$W = \frac{LR}{LR + 1}$$

In this type of analysis, it is common to assume a prior probability of 0.5 for each hypothesis, which implies that the analyzed samples have an equal probability of fulfilling hypothesis H_1 (a biological relationship exists) or H_0 (there is no relation). This assumption means that both hypotheses are considered equally probable before observing the data. However, the two hypotheses are mutually exclusive.

The higher the LR, the greater the support for the hypothesis of kinship. In this context, it is important to consider Hummel's guidelines, which discourage the use of verbal predicates, as they may be interpreted subjectively by the various parties involved in the judicial process. This highlights the importance of using objective, data-based language in expert conclusions.

5. RESULTS

5.1. SISTERHOOD CASE

Two sample donors arrived at the laboratory with the suspicion that they might be biological half-sisters through their paternal line. In order to determine whether this genetic relationship existed, a comparative analysis of their genetic profiles was conducted (Figure A 1, Figure A 2, Figure A 3 and Figure A 4) using buccal swab samples from both donors.

This study aimed to establish whether they shared the same biological father and, consequently, to confirm or rule out a paternal half-sibling relationship.

The two hypotheses that were considered, and upon which the subsequent statistical calculations were based, were:

H_0: The donors share the same biological father.	H_1: The sample donors are not genetically related.

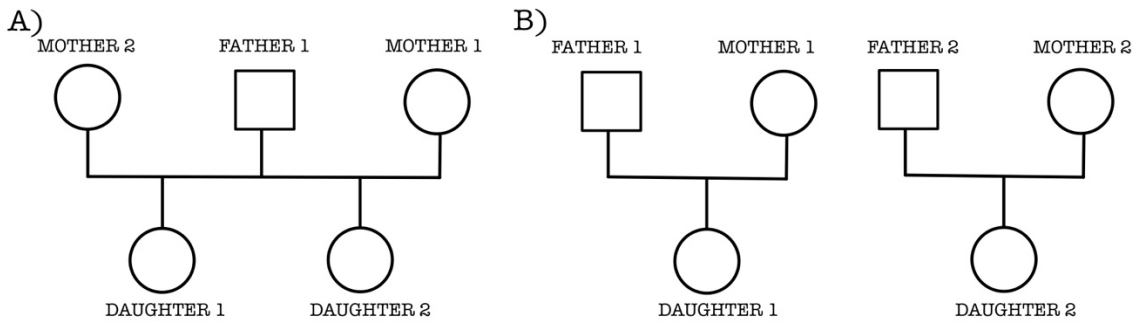

Figure 6 Genealogical tree of the individuals involved in Hypothesis 0 (A) and Hypothesis 1 (B) of the case of sisterhood (Author's own data).

Once the hypotheses were established, DNA was extracted from both samples, and an analysis of autosomal STRs and X chromosome STRs was performed. The results of these analyses are presented in Table 2 and Table 3, respectively.

Table 2 Genotype of autosomal STRs in the case of sisterhood.

MARKER	DAUGHTER 1		DAUGHTER 2		MATCHING ALLELES
D3S1358	16	18	15	16	16
vWA	18	20	18	20	18-20
D16S539	11	11	10	10	-
CSF1PO	10	11	11	12	11
TPOX	8	8	8	8	8
D8S1179	13	14	13	13	13
D21S11	30.2	31.2	28	32.2	-
D18S51	14	15	12	13	-
Penta E	7	12	12	12	12
D2S441	11	11.3	11.3	14	11.3
D19S433	13	13	13	14.2	13
TH01	7	9.3	6	9.3	9.3
FGA	21	25	21	25	21
D22S1045	15	15	12	15	15
D5S818	11	12	12	12	12
D13S317	8	11	9	11	11
D7S820	10	12	10	12	10-12
D6S1043	12	14	11	11	-
D10S1248	16	16	13	16	16
D1S1656	12	15	15	15.3	15
D12S391	18	20	20	22	20
D2S1338	17	20	20	20	20
Penta D	9	13	9	13	9-13
Y-Indel	-		-		---
Amelogenin	X	X	X	X	---

Table 3 Genotype of X chromosome STRs in the case of sisterhood.

MARKER	DAUGHTER 1		DAUGHTER 2		MATCHING ALLELES
DXS10103	18	19	19	19	19
DXS8378	11	12	11	12	11-12
DXS10101	30.2	32	30.2	31.2	30.2
DXS10134	34	36	34	34	34
DXS10074	16	18	16	17	16
DXS7132	14	15	13	14	14
DXS10135	21	21.2	21.2	23.1	21.2
DXS7423	14	16	14	14	14
DXS10146	28	30	26	30	30
DXS10079	16	19	16	19	16-19
HPRTB	11	12	11	12	11-12
DXS10148	13.3	18	13.3	24	13.3
D21S11	30.2	31.2	28	32.2	-

Based on the STR profiles obtained from the autosomal markers (Table 2) and the X chromosome markers (Table 3) a statistical analysis was conducted using the LR between the two samples, taking into account the previously formulated hypotheses (Figure 6). The results of this analysis are presented in Table 4.

Table 4 Summary table of LR and W results for autosomal and X chromosome STRs in the case of sisterhood.

STRs	LR	W
AUTOSOMAL	430,9506	99,7685%
X CHROMOSOME	4,664 x10^7	99,9999%

5.2. BROTHERHOOD CASE

Two sample donors arrived at the laboratory with the suspicion that they might be biological brothers. In order to resolve this uncertainty, a comparative analysis of their genetic profiles was performed (Figure A 6, Figure A 7, Figure A 8 and Figure A 9). This study aimed to determine whether they shared a common biological parent and, consequently, to confirm or rule out a sibling relationship.

The two hypotheses that were considered, and upon which the subsequent statistical calculations were based, were:

H_0: The sample donors are biological brothers.	H_1: The sample donors are not genetically related.

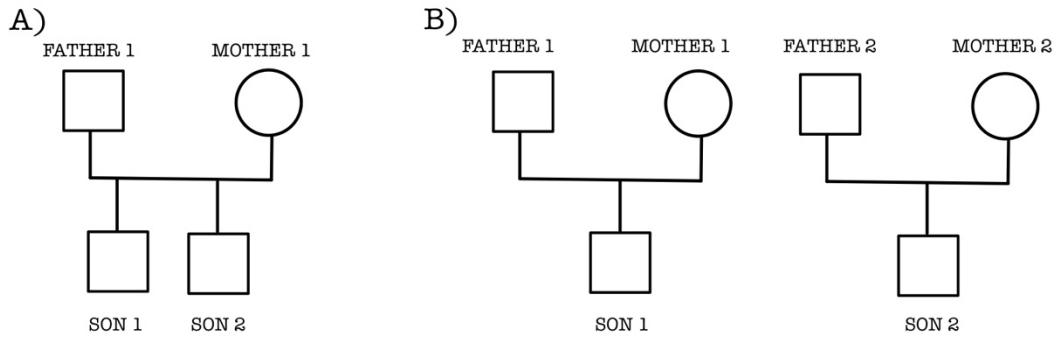

Figure 7 Genealogical tree of the individuals involved in Hypothesis 0 (A) and Hypothesis 1 (B) of the case of brotherhood (Author's own data).

Once the hypotheses were established, DNA was extracted from both samples, and an analysis of autosomal STRs and Y chromosome STRs was performed. The results obtained are presented in Table 5 and Table 6.

Table 5 Genotype of autosomal STRs in the case of brotherhood.

MARKER	SON 1		SON 2		MATCHING ALLELES
D3S1358	15	18	18	18	18
vWA	15	16	16	17	16
D16S539	9	12	12	13	12
CSF1PO	10	12	12	12	12
TPOX	8	11	11	11	11
D8S1179	12	13	12	14	12
D21S11	27	29	28	31.2	-
D18S51	15	16	16	17	16
Penta E	13	13	7	12	-
D2S441	11	11	11	14	11
D19S433	13	14	13	14	13-14
TH01	6	7	6	9	6
FGA	24	26	21	25	-
D22S1045	15	17	13	15	15
D5S818	8	13	11	12	-
D13S317	8	12	10	12	12
D7S820	11	12	11	12	11-12
D6S1043	11	11	15	19	-
D10S1248	15	17	14	16	-
D1S1656	15	18.3	15	18.3	15-18.3
D12S391	19.3	20	20	22	20
D2S1338	17	18	17	19	17
Penta D	9	11	9	12	9
Y-Indel	-		-		---
Amelogenin	X	Y	X	Y	---

Table 6 Genotype of Y chromosome STRs in the case of brotherhood.

MARKER	SON 1	SON 2	MATCHING ALLELES
DYS576	18	18	18
DYS389-I	13	13	13
DYS635	21	21	21
DYS389-II	31	31	31
DYS627	21	21	21
DYS460	10	10	10
DYS458	18.2	18.2	18.2
DYS19	13.1	13.1	13.1
YGATA H4	11	11	11
DYS448	20	20	20
DYS391	10	10	10
DYS456	14	14	14
DYS390	23	23	23
DYS438	10	10	10
DYS392	11	11	11
DYS518	41	41	41
DYS570	18	18	18
DYS437	14	14	14
DYS385 a/b	13-19	13-19	13-19
DYS449	24	24	24
DYS393	12	12	12
DYS439	11	11	11
DYS481	27	27	27
DYF387F1 a/b	36-37	36-37	36-37
DYS533	11	11	11

Based on the STR profiles obtained from the autosomal markers (Table 5) and Y chromosome markers (Table 6), a statistical analysis was conducted using the LR between the samples, taking into account the previously formulated hypotheses (Figure 7). The results of this analysis are presented in Table 7.

Table 7 Summary table of LR and W results for autosomal and Y chromosome STRs in the case of brotherhood.

STRs	LR	W
AUTOSOMAL	0,0176	1,7263%
Y CHROMOSOME	0,4999	33,33%

5.3. PATERNITY CASE

A man arrived at the laboratory requesting a paternity test based on X chromosome analysis to determine a possible biological relationship between himself and an alleged daughter. Additionally, it was decided to complement the study by performing an autosomal STR analysis as well.

Both genetic profiles (Figure A 10, Figure A 11, Figure A 12 and Figure A 13), from the father and the alleged daughter, were compared in order to reliably confirm or rule out the biological paternity relationship between the two sample donors.

The two hypotheses that were considered, and upon which the subsequent statistical calculations were based, were:

H_0: The donor of sample 1 (father) is the biological father of the donor of sample 2 (alleged daughter).	H_1: The biological father of the donor of sample 2 (alleged daughter) is a randomly selected individual from the population, who is not genetically related to either the donor of sample 2 or to the donor of sample 1.

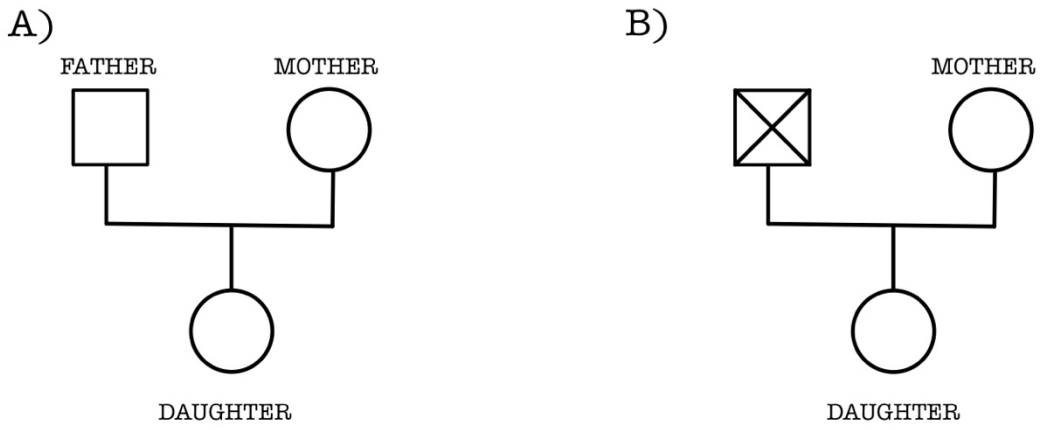

Figure 8 Genealogical tree of the individuals involved in Hypothesis 0 (A) and Hypothesis 1 (B) of the case of brotherhood (Author's own data).

Once the hypotheses were established, DNA was extracted from both samples, and an analysis of autosomal STRs and X chromosome STRs was performed. The results of these analyses are presented in Table 8 and Table 9, respectively.

Table 8 Genotype of autosomal STRs in the paternity case.

MARKER	FATHER		DAUGHTER		MATCHING ALLELES
D3S1358	15	15	15	15	15-15
vWA	14	16	14	14	14
D16S539	9	12	9	12	9-12
CSF1PO	7	10	10	11	11
TPOX	9	11	9	10	9
D8S1179	12	12	12	14	12
D21S11	28	30	28	30	28-30
D18S51	13	17	13	17	13-17
Penta E	16	18	7	16	16
D2S441	12	14	12	12	12
D19S433	13	13	12	13	13
TH01	6	9	6	8	6
FGA	23	24	22	23	23
D22S1045	11	15	11	11	11
D5S818	10	13	12	13	13
D13S317	11	11	11	12	11
D7S820	8	12	9	12	12
D6S1043	18	23	18	20	18
D10S1248	13	14	13	14	13-14
D1S1656	12	15	14	15	15
D12S391	16	19	19	21	19
D2S1338	17	21	19	21	19
Penta D	2.2	10	2.2	2.2	2.2
Y-Indel	-		-		---
Amelogenin	X	Y	X	X	---

Table 9 Genotype of STRs on the X chromosome in the paternity case.

MARKER	FATHER		DAUGHTER		MATCHING ALLELES
DXS10103	18		13.3	18	18
DXS8378	22		20	22	22
DXS10101	12		12	12	12
DXS10134	13		13	15	13
DXS10074	18		17	18	18
DXS7132	14		14	15	14
DXS10135	19		19	19	19
DXS7423	12		12	12	12
DXS10146	31		31	31	31
DXS10079	32		31	32	32
HPRTB	32		32	36	32
DXS10148	13		13	13	13
D21S11	28	30	28	30	28-30

Based on the STR profiles obtained from the autosomal markers (Table 8) and the X-chromosome markers (Table 9), a statistical analysis was conducted using the LR between the samples, taking into account the previously formulated hypotheses (Figure 8). The results of this analysis are presented in Table 10.

Table 10 Summary table of LR and W results for autosomal and X chromosome STRs in the paternity case.

STRs	LR	W
AUTOSOMAL	3,194 $x10^{11}$	>99,9999%
X CHROMOSOME	3,2687$x10^{11}$	>99,9999%

6. DISCUSSION

This section presents a critical and comparative analysis of the results obtained in the three cases studied: two sibling tests (one between two girls and another between two boys) and a paternity test between a father and an alleged daughter. Each case has been analyzed using autosomal STR markers and, depending on the familiar relationship studied or the request of the person seeking the test, specific markers from the X chromosome (in the case of the sisters and the paternity test) or the Y chromosome (in the case of the brothers) have also been used.

According to the literature, the most suitable and widely used markers for establishing biological relationships are autosomal markers, as they offer high resolution and greater discriminatory power in most cases (Udogadi et al., 2020; Wyner et al., 2020). However, this study discusses the extent to which this statement is true and whether these markers are always the most appropriate. The aim is to interpret the results from a genetic and statistical perspective and to assess their probative strength in establishing or excluding biological relationships.

In addition to the individual results, it is essential to consider the inherent limitations of forensic genetic studies. First, the discriminatory power of the results can be affected by the number and variety of markers used, as well as by their distribution within the population of origin. Secondly, statistical inference is based on the comparison between two hypotheses (H_0 and H_1), which are simplified theoretical models of reality. These limitations are particularly relevant when attempting to determine an indirect kinship relationship (such as siblinghood), as natural genetic variability can make it difficult to draw clear conclusions (Hasan et al., 2016; Henry et al., 2015; Szibor et al., 2003).

In the sisterhood case, the analysis of autosomal STRs offers a moderately high probability in favor of hypothesis H_0, although this evidence is limited by the nature of these markers. In a sibling relationship, the probability that two individuals share the same allele at a given locus is 25% if they share only one parent, and 50% if they share both, which may result in insufficient allelic matching to conclusively establish a biological relationship. However, the analysis of X chromosome STRs substantially improves the discriminatory power in this case (Hatsch et al., 2007; Zhang et al., 2021). Since biological sisters through the paternal line inherit exactly the same single copy of

the X chromosome from their father, allelic matching is expected at these loci. In the case under analysis, this matching clearly strengthens hypothesis H_0 and reduces the probability of H_1 to nearly zero, providing clear, robust, and conclusive genetic evidence of a biological sibling relationship through the paternal line between the two donors.

In the brotherhood case, the results obtained point in the opposite direction. The analysis of autosomal STRs shows very limited allelic matching between the samples, with a probability of 98.27% in favor of the hypothesis H_1, which indicates a clear absence of genetic relationship according to the statistical criteria applied. In addition, the study of STRs of the Y chromosome, which is transmitted from father to son without recombination, reinforces this conclusion (Henry et al., 2015; Kayser, 2017). In this case, the probability that the two individuals share the same paternal lineage is only 33.33%, a value too low to confirm direct siblingship. Although the Y chromosome match could suggest some paternal-line relationship (such as cousins or more distant relatives), it is not sufficient to affirm with certainty a sibling relationship. Therefore, the combination of both genetic analyses provides consistent support for hypothesis H_1 and does not allow for confirmation of a biological sibling relationship between the two individuals.

Finally, in the paternity case, both the results obtained with the autosomal STRs and the STRs of the X chromosome provide extremely solid and conclusive genetic evidence in favor of the biological relationship between the two individuals analyzed (H_0). The autosomal markers and the X chromosome STRs yield very high LR values, although with some differences between them. This slight difference is explained by the fact that, in a father-daughter relationship, the father transmits his only X chromosome in its entirety, which translates into a complete genetic coincidence in these markers, provided that no mutations occur. Therefore, the combination of both analyses allows for a scientifically certain conclusion of the paternity relationship between the two individuals (Hatsch et al., 2007; Wyner et al., 2020).

Therefore, while autosomal STRs are generally the most commonly used and appropriate markers for establishing biological relationships, especially in paternity testing, their effectiveness is not universal or sufficient in all contexts.

In the paternity case, autosomal STRs offer very high statistical power, and are sufficient to reach practically definitive conclusions. However, when it comes to indirect kinship

relationships, such as siblingship, these markers may be insufficient to provide clear evidence, especially when the familiar relationship does not involve both parents. In these cases, the complementary use of specific markers, such as X or Y chromosome STRs, becomes essential. For example, the analysis of X chromosome markers has been decisive in confirming the sibling relationship between two sisters, while Y chromosome markers have reinforced the absence of biological links between two supposed brothers (Gomes et al., 2012).

The Table 11 in the annexes is included to facilitate the comparison between the different types of STR markers mentioned above, highlighting their main advantages, limitations and contexts of use.

7. CONCLUSION

With the completion of this work, the main objective of the work has been achieved: to compare the statistical reliability of the different markers in various types of kinship relationships.

- X chromosome STRs have demonstrated very high statistical reliability in cases of female siblingship, especially when the paternal line is shared.
- Y chromosome STRs, in the case of male siblingship, have presented significant limitations, since haplotype matches may also occur among other paternal-line relatives who are not siblings.
- It is necessary to complement genetic results with other sources of information when there is no clear coincidence of alleles, especially in cases of indirect kinship relationships.
- In paternity testing, both autosomal STRs and X chromosome STRs have offered a clear and conclusive result, with high discriminatory power.

In addition, this study also successfully achieved the following specific objectives:

- Familiarization with the different amplification kits.
- Critical selection of the most appropriate genetic methods according to the case.
- Mastery of statistical programs such as Families and FamLink, as well as mastery of websites such as YHRD.org.

The results obtained confirm that there is no single optimal marker for all contexts, and that a critical and integrative approach is needed to select the most appropriate genetic tools. Despite their robustness in many scenarios, autosomal STRs are not always the most suitable markers when used exclusively. The choice of the most suitable type of marker should depend on the type of relationship being studied and the specific biological context.

In this sense, an adapted strategy and careful interpretation are key to guaranteeing reliable and scientifically sound conclusions in forensic genetics. This integrative approach allows maximizing the reliability of the results and adapting the genetic tools to the needs of each case.

8. REFERENCES

8.1. SISTERHOOD CASE

8.1.1. AUTOSOMAL STR ELECTROPHEROGRAMS

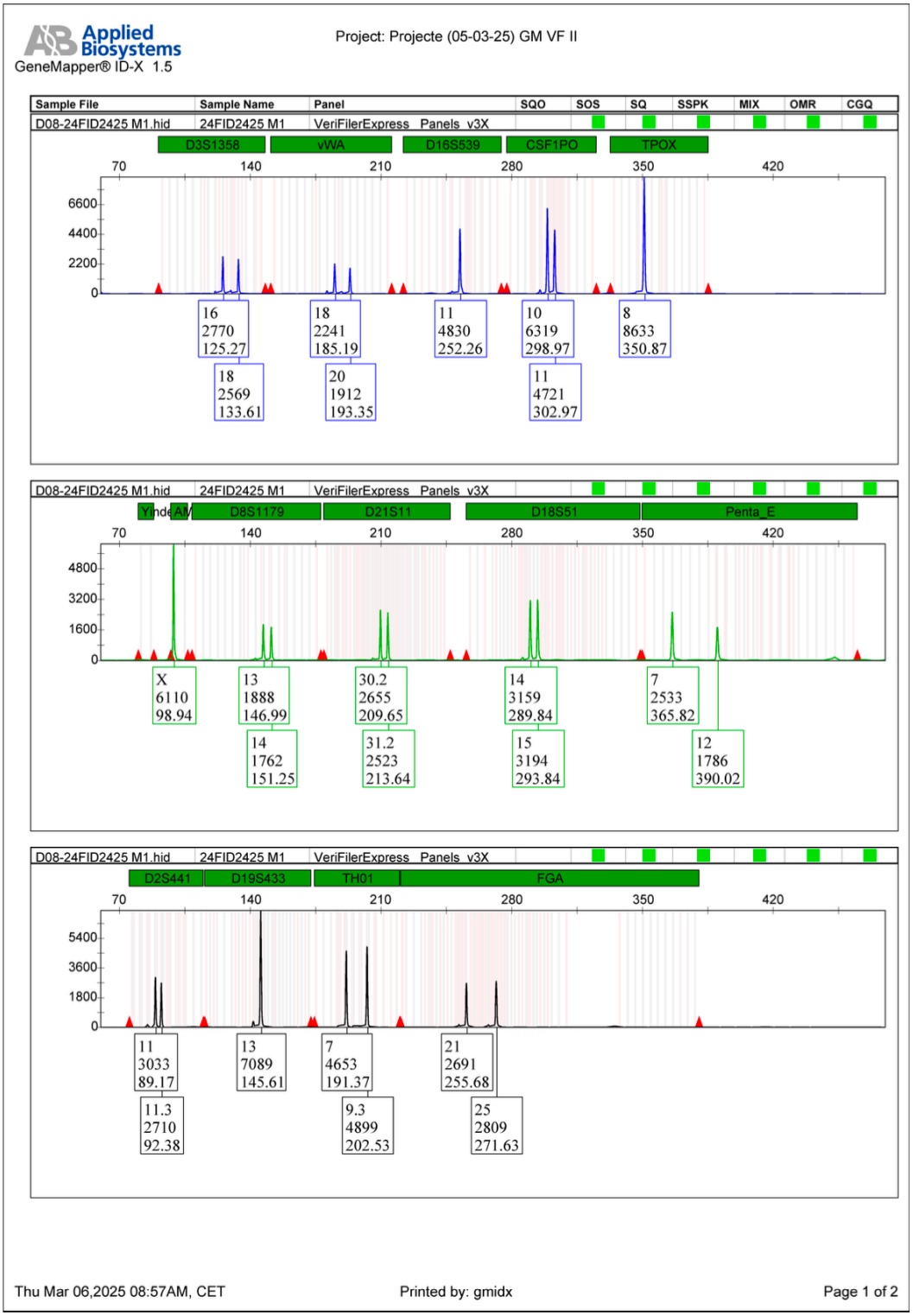

Figure A 1 Electropherogram corresponding to Sample 1 (M1) analyzed using autosomal STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M2).

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GeneMapper® ID-X 1.5

Project: Projecte (05-03-25) GM VF II 2

Sample File	Sample Name	Panel	SQO	SOS	SQ	SSPK	MIX	OMR	CGQ
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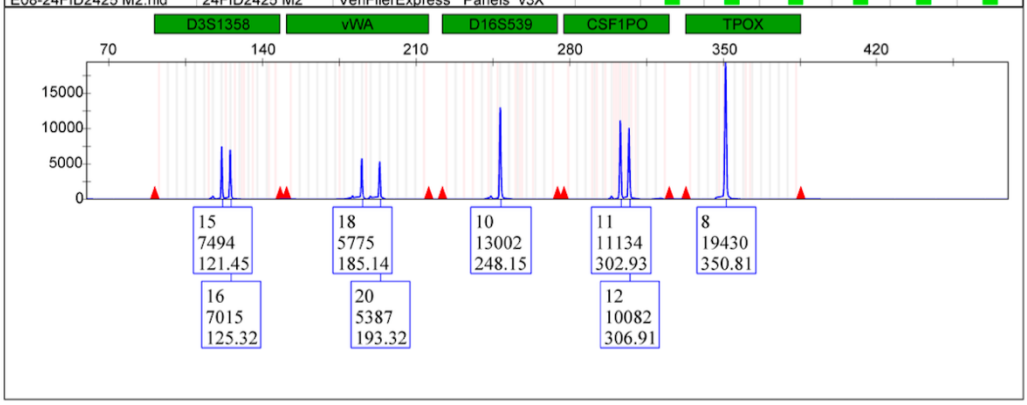
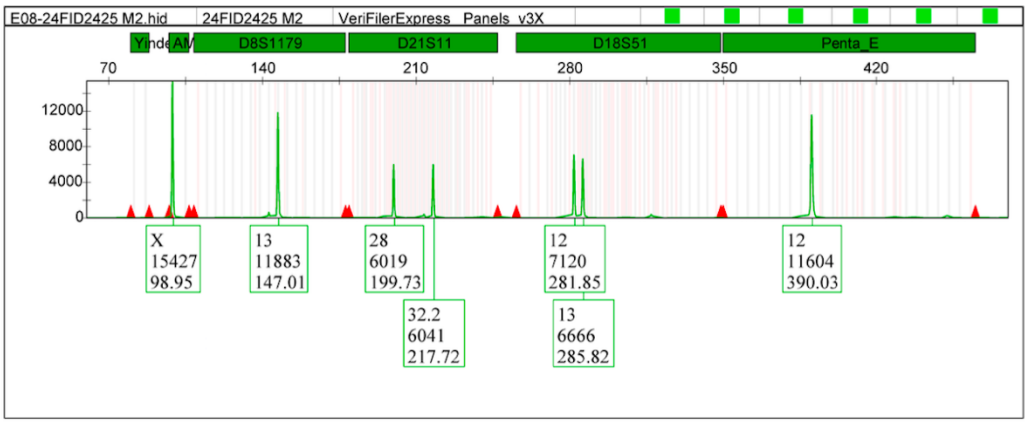




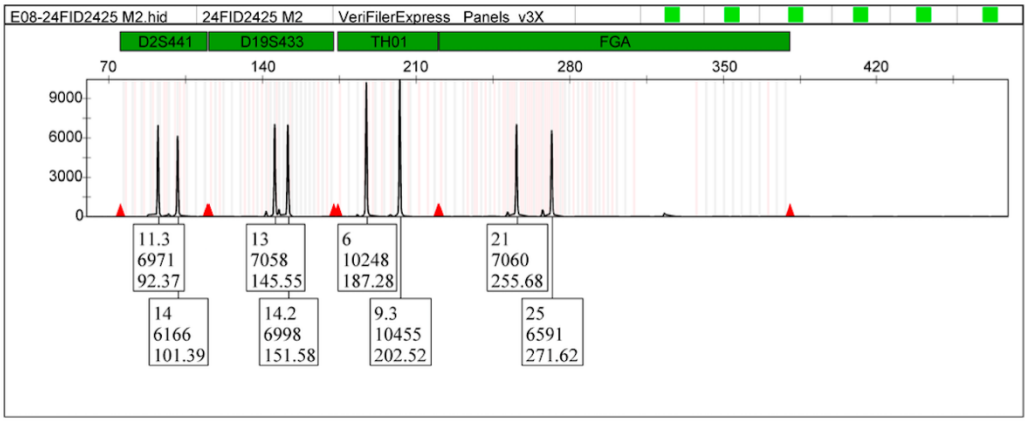
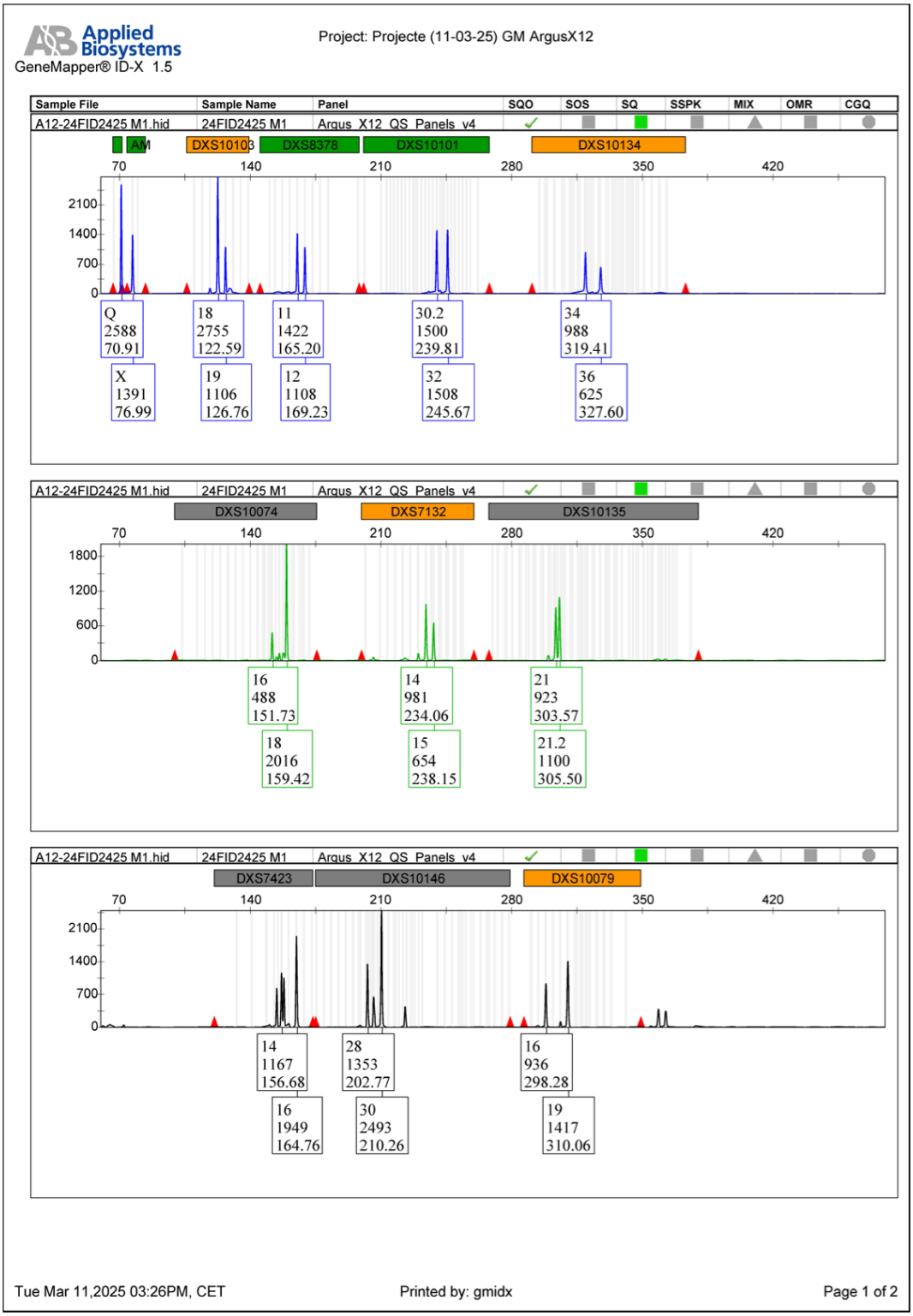


Figure A 2 Electropherogram corresponding to Sample 2 (M2) analyzed using autosomal STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M1).

8.1.2. X CHROMOSOME STR ELECTROPHEROGRAMS



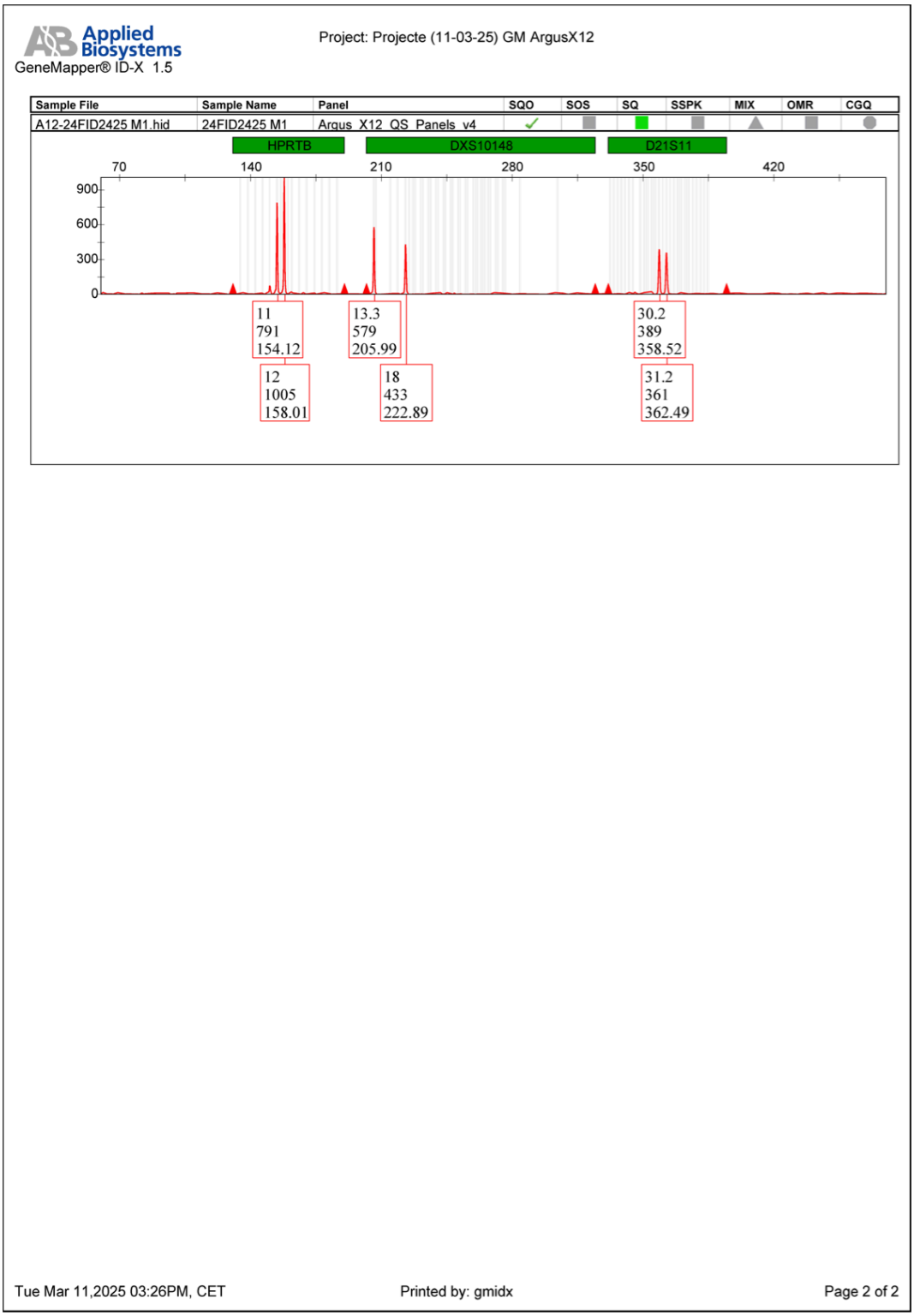


Figure A 3 Electropherogram corresponding to Sample 1 (M1) analyzed using X chromosome STR markers. The peaks represent the alleles detected at each X STR locus, shown by fragment size (in base pairs) and signal intensity (relative fluorescence units). In female individuals, two alleles may be observed per locus (heterozygous), or a single peak if homozygous. The marker labeled "Q" represents a quality control indicator used to assess the reliability and consistency of the electrophoretic run.

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Project: Projecte (24-03-25) GM Argus X-12

Sample File	Sample Name	Panel	SQO	SOS	SQ	SSPK	MIX	OMR	CGQ
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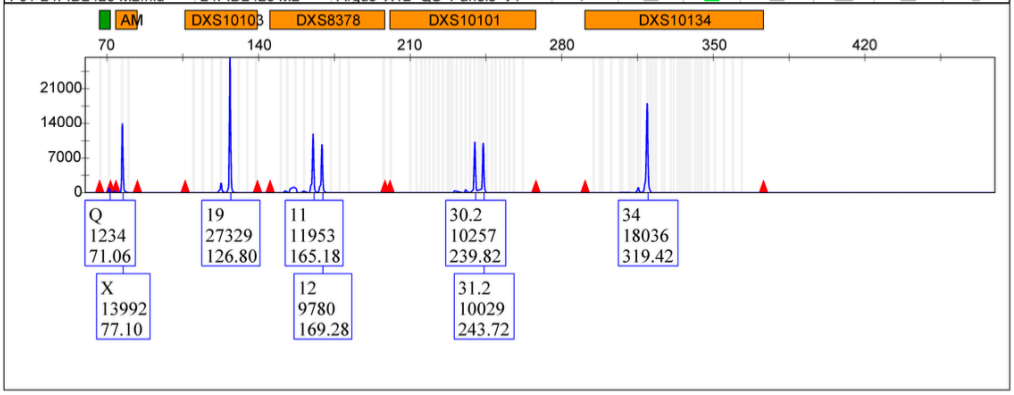
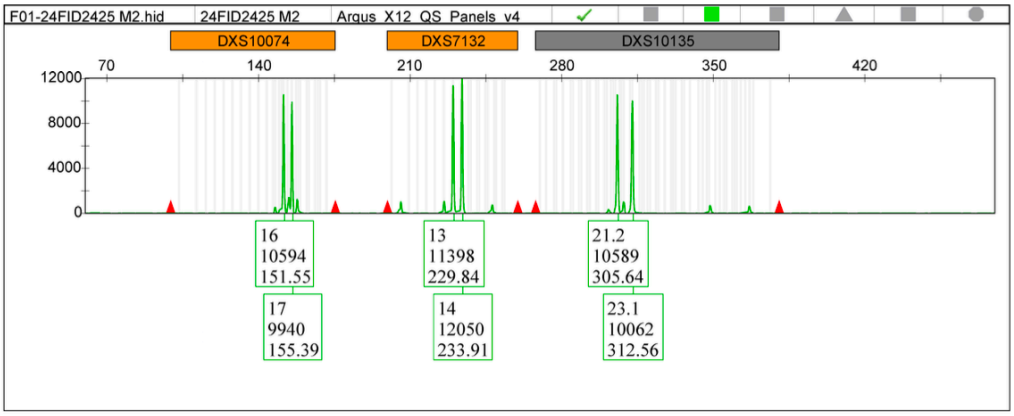
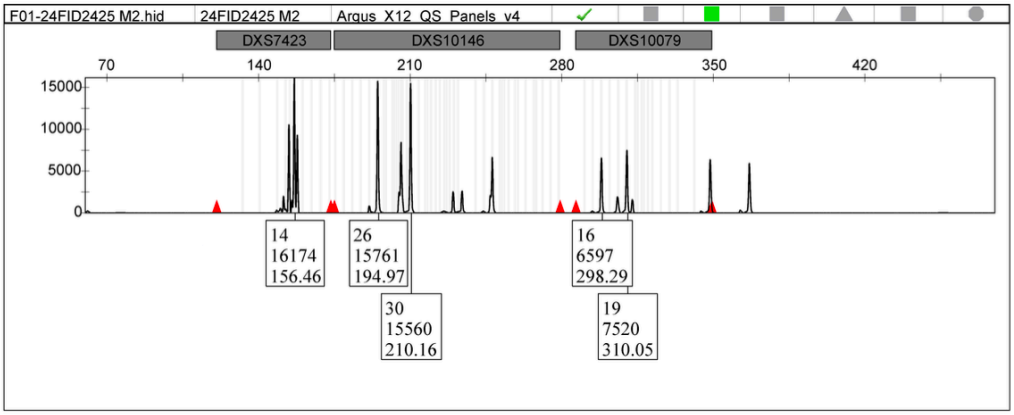


F01-24FID2425 M2.hid | 24FID2425 M2 | Argus_X12_QS_Panels_v4 | ✓



F01-24FID2425 M2.hid | 24FID2425 M2 | Argus_X12_QS_Panels_v4 | ✓



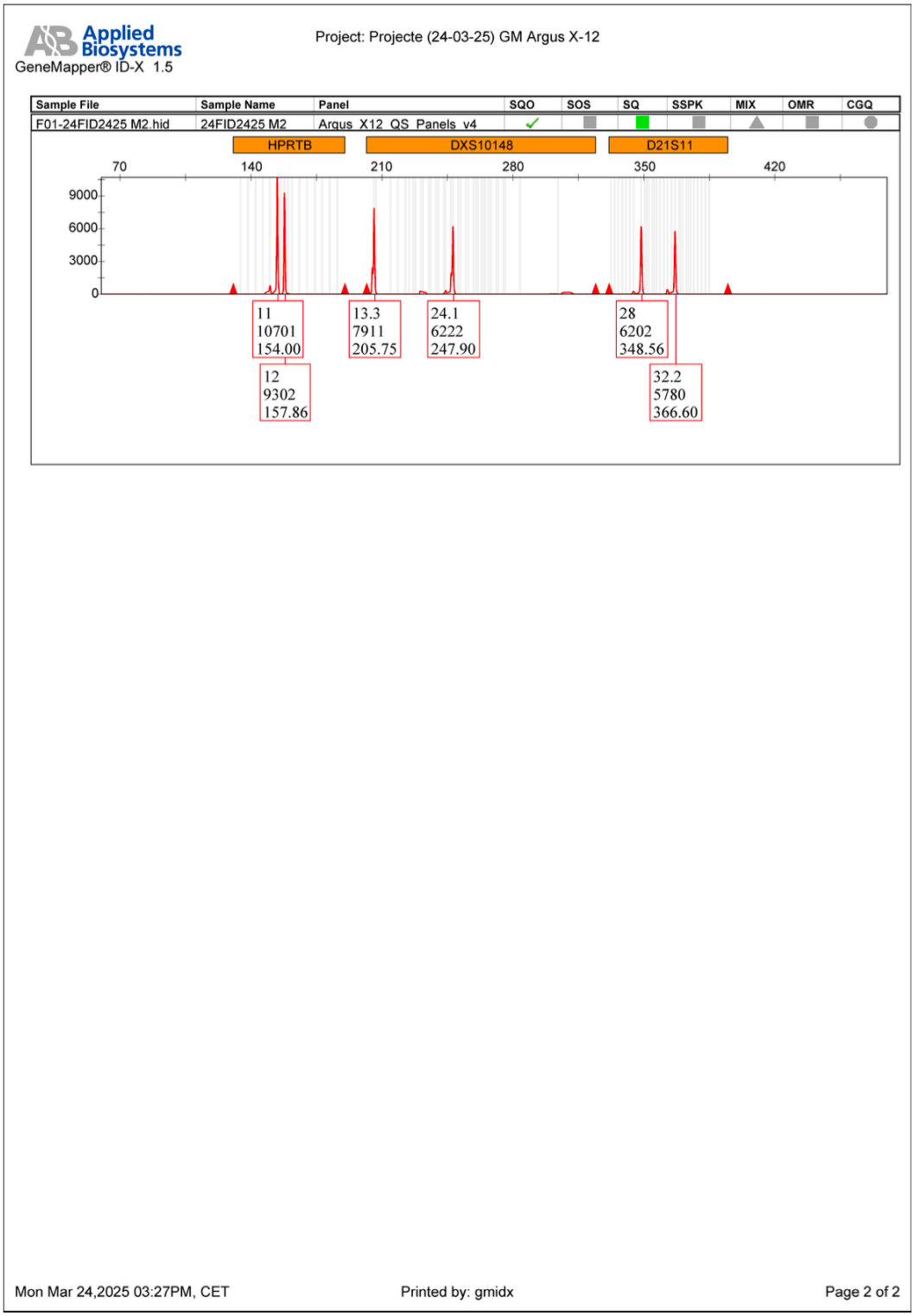
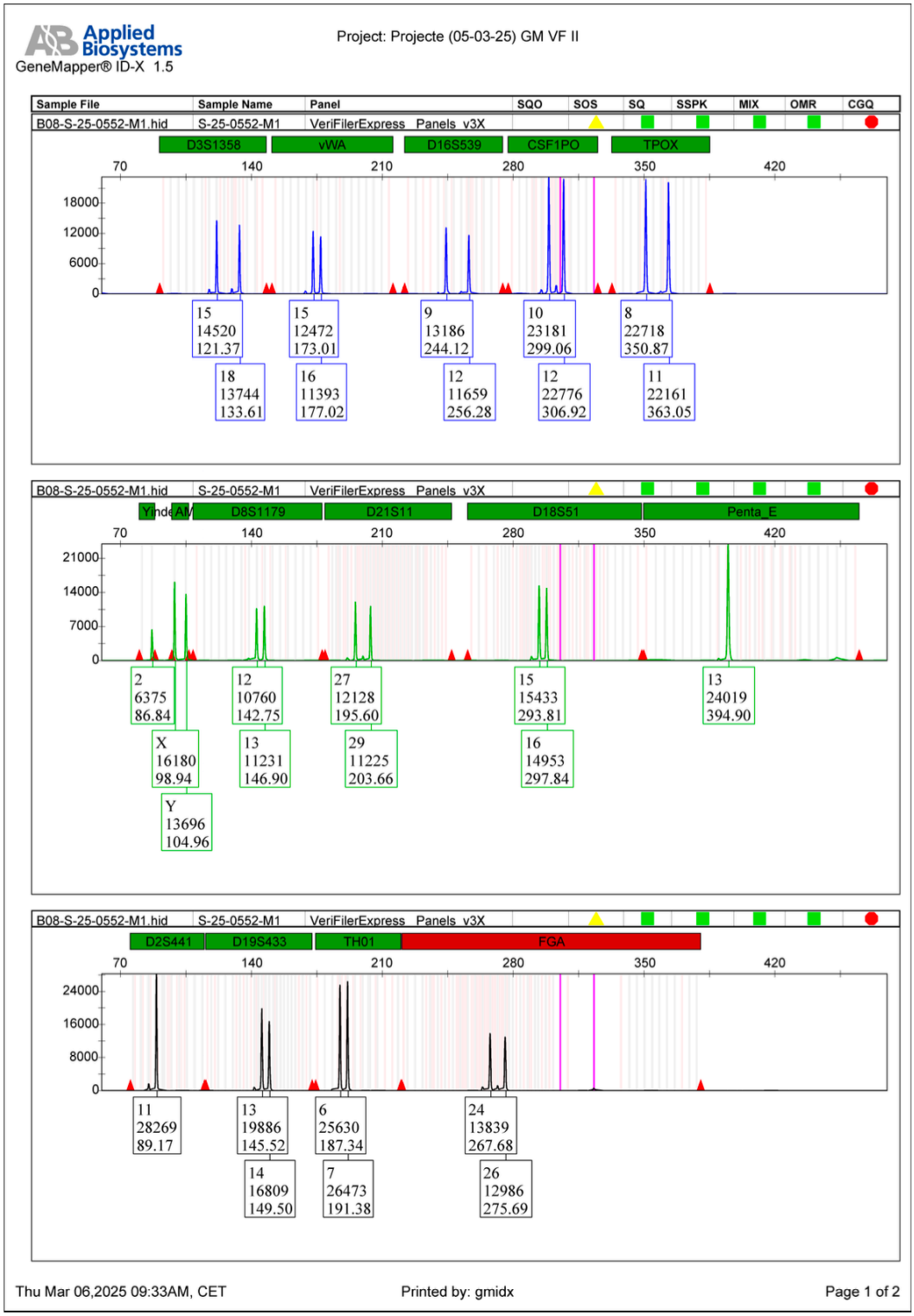


Figure A 4 Electropherogram corresponding to Sample 2 (M2) analyzed using X chromosome STR markers. The peaks represent the alleles detected at each X STR locus, shown by fragment size (in base pairs) and signal intensity (relative fluorescence units). In female individuals, two alleles may be observed per locus (heterozygous), or a single peak if homozygous. The marker labeled "Q" represents a quality control indicator used to assess the reliability and consistency of the electrophoretic run.

8.2. BROTHERHOOD CASE

8.2.1. AUTOSOMAL STR ELECTROPHEROGRAMS



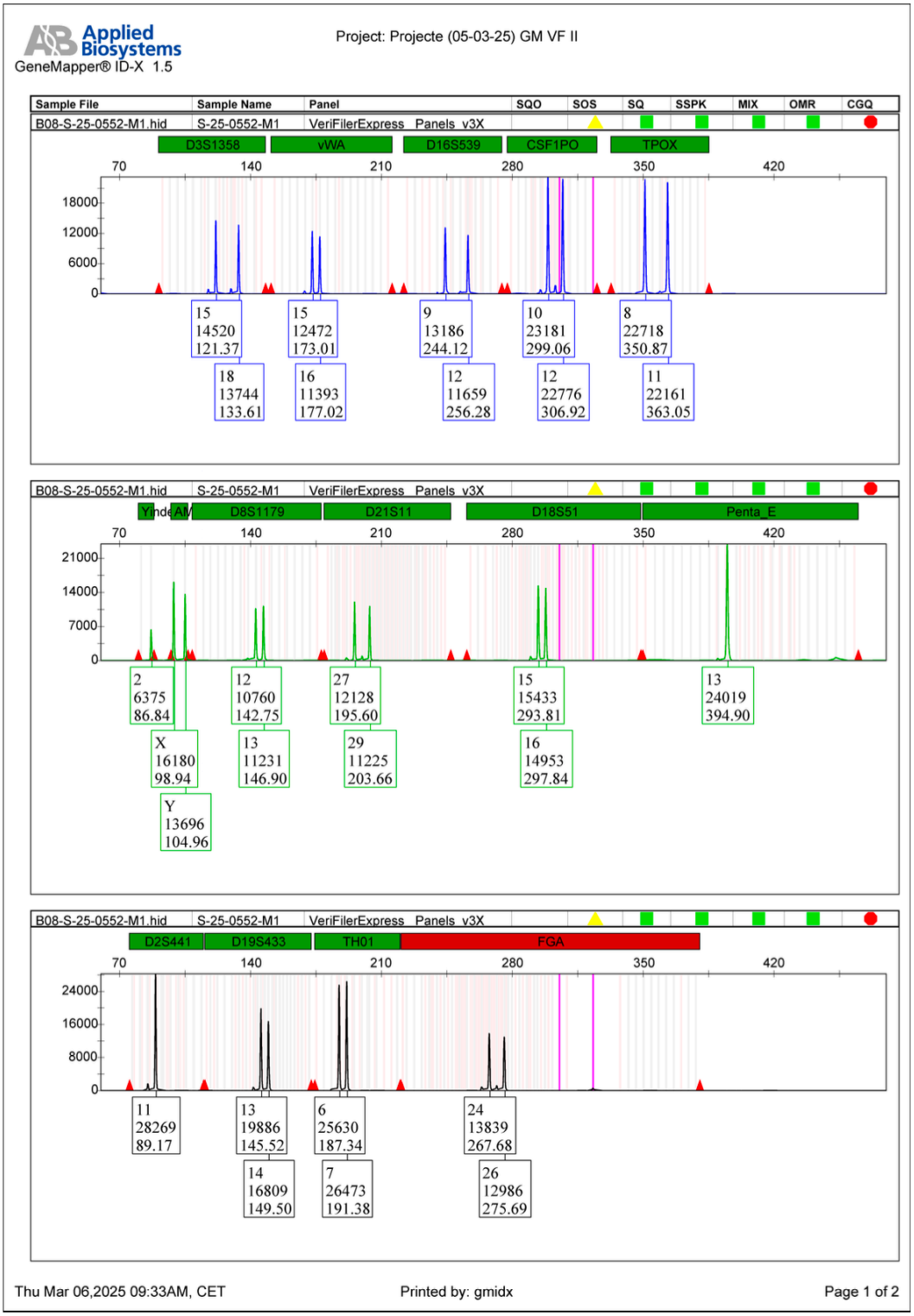


Figure A 5 Electropherogram corresponding to Sample 1 (M1) analyzed using autosomal STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M2).

The pink lines observed in the electropherogram are caused by a pull-up[9] artifact generated at markers D2S1338 and D6S1043. In this case, a sample re-injection with reduced injection time was performed, as the high DNA concentration prevented accurate interpretation of the results.

[9] A pull up is a deviation in an electrophorogram caused by an excess of DNA in the sample. This phenomenon manifests itself in an excessive height of the alleles causing aberrant alleles in the remaining fluorochromes

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GeneMapper® ID-X 1.5

Project: Projecte (12-03-25) GM VF II

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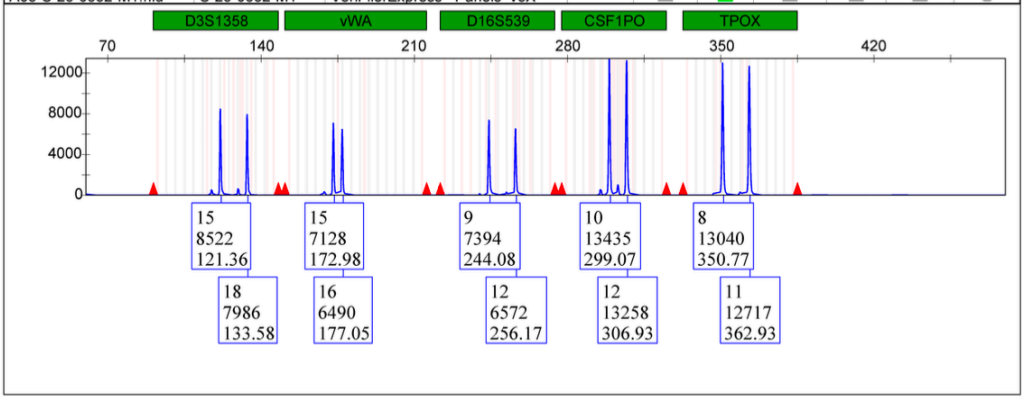

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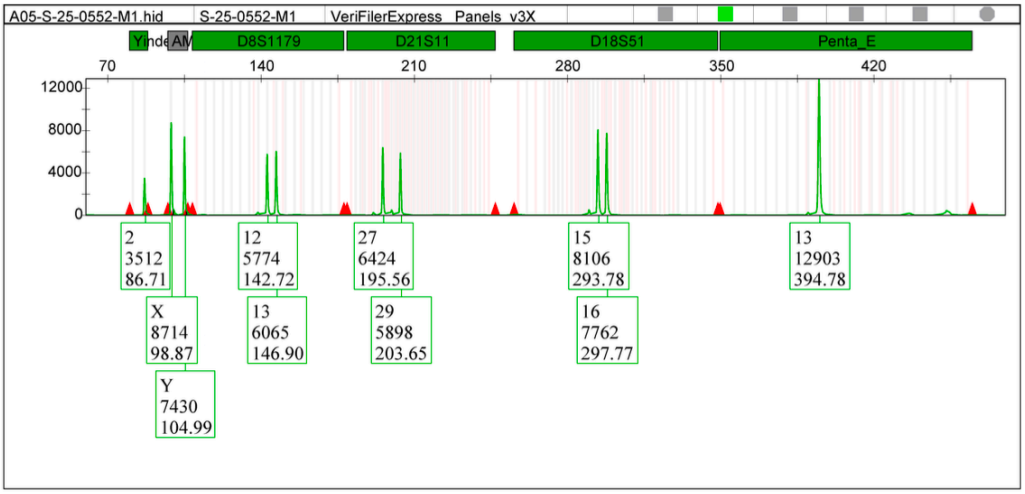

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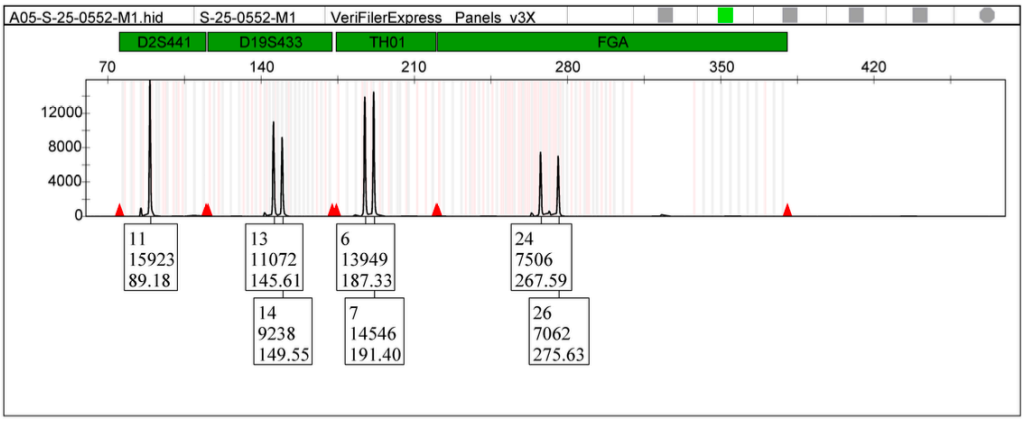

Thu Mar 13,2025 09:04AM, CET | Printed by: gmidx | Page 1 of 2

Figure A 6 Electropherogram corresponding to the re-injection of Sample 1 (M1) analyzed using autosomal STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M2).

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GeneMapper® ID-X 1.5

Project: Projecte (05-03-25) GM VF II 2

Sample File	Sample Name	Panel	SQO	SOS	SQ	SSPK	MIX	OMR	CGQ
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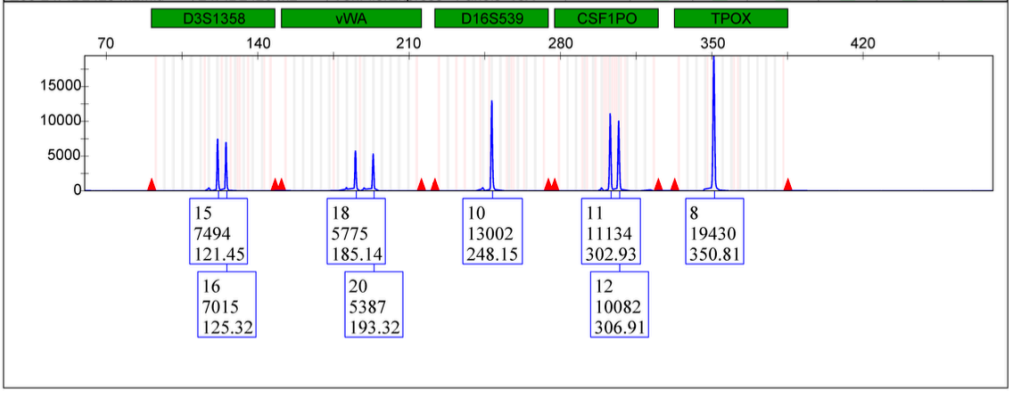
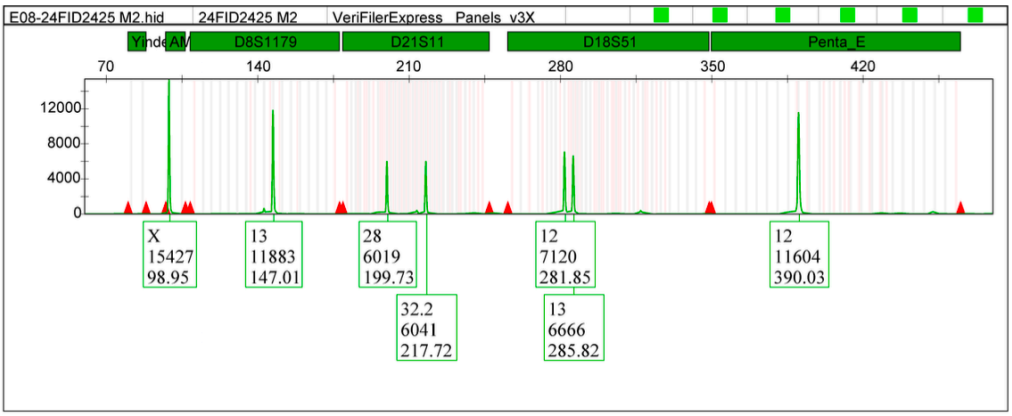


E08-24FID2425 M2.hid | 24FID2425 M2 | VeriFilerExpress_Panels_v3X



E08-24FID2425 M2.hid | 24FID2425 M2 | VeriFilerExpress_Panels_v3X

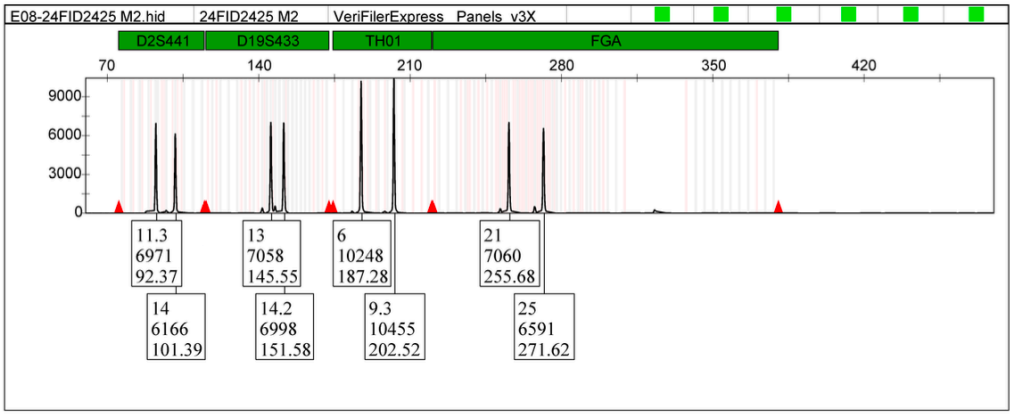
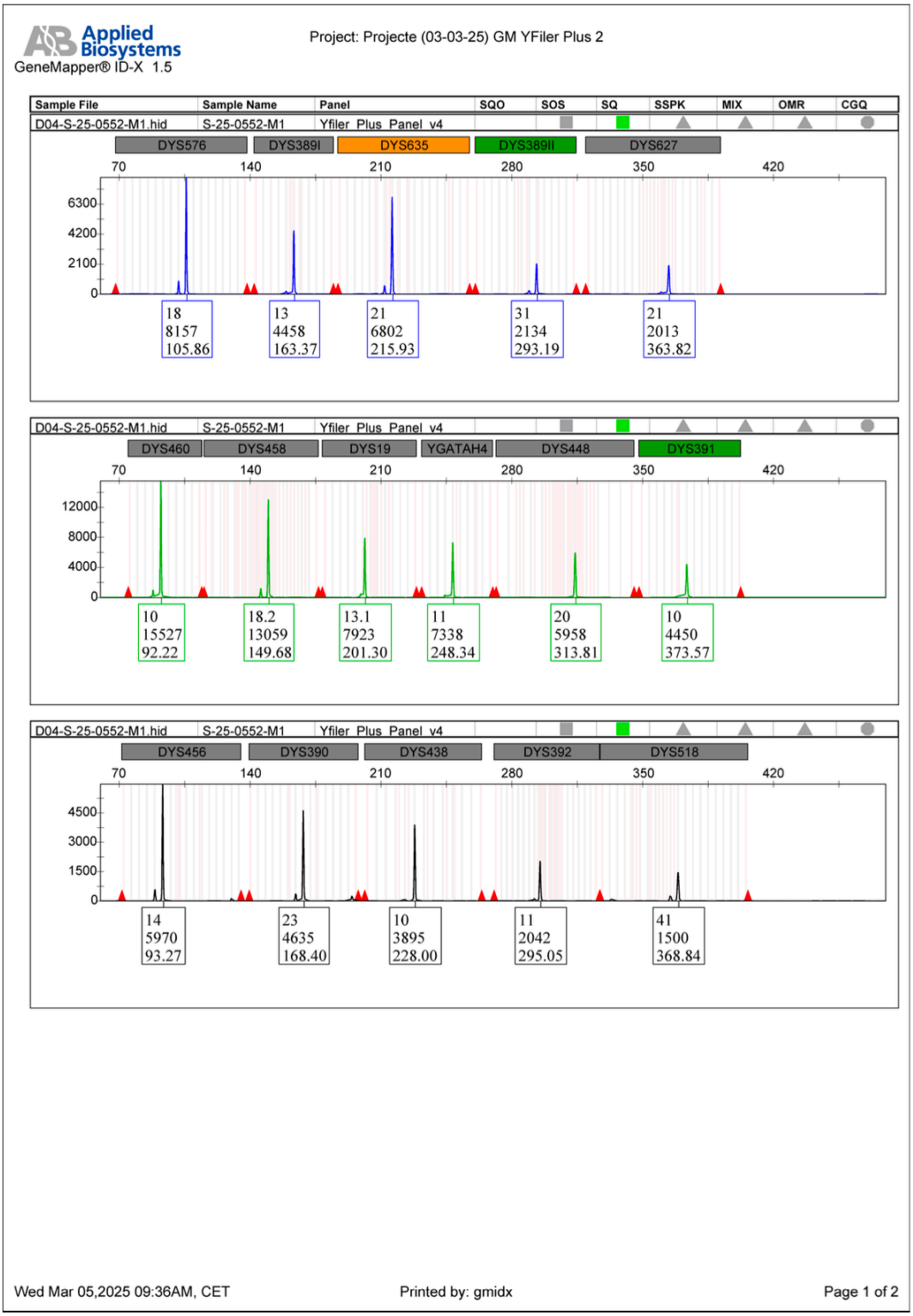


Figure A 7 Electropherogram corresponding to Sample 2 (M2) analyzed using autosomal STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M1).

8.2.2. Y CHROMOSOME STR ELECTROPHEROGRAMS



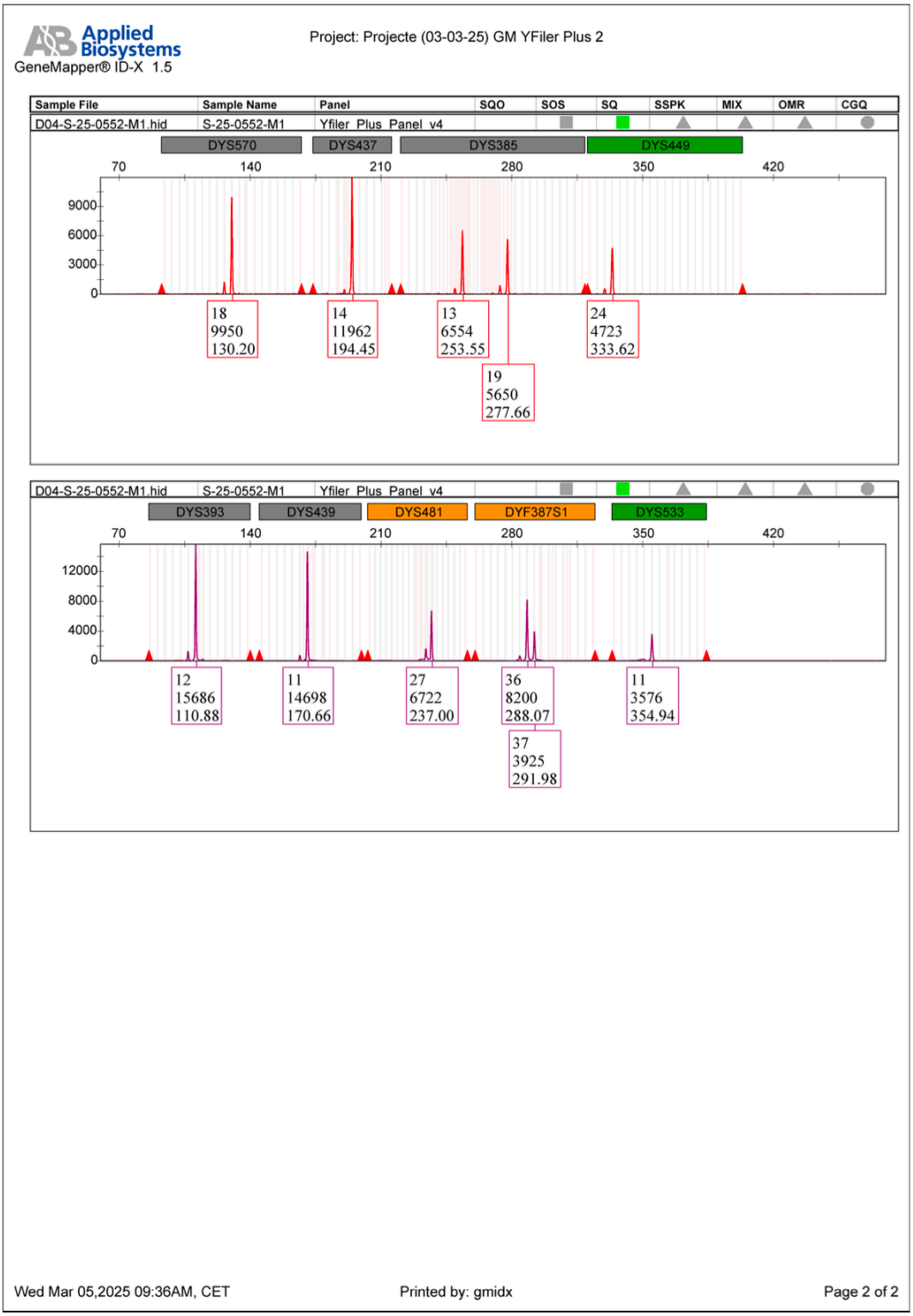


Figure A 8 Electropherogram corresponding to the analysis of Sample 1 (M1) using Y chromosome STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). In male individuals, only one allele will be observed per locus, as the Y chromosome is inherited exclusively from the father without recombination. This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M2).

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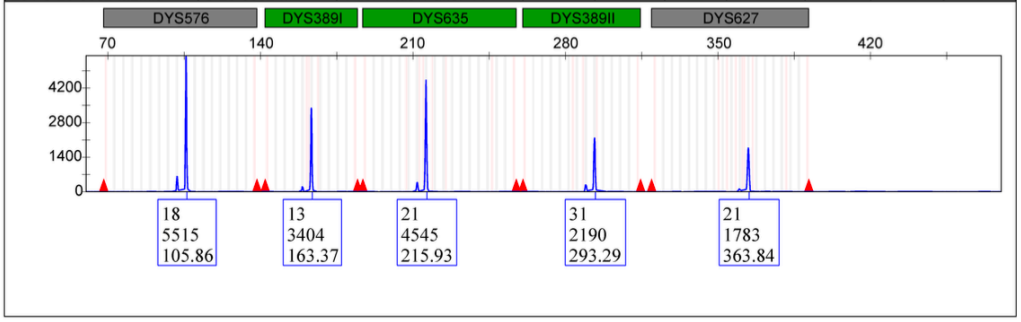
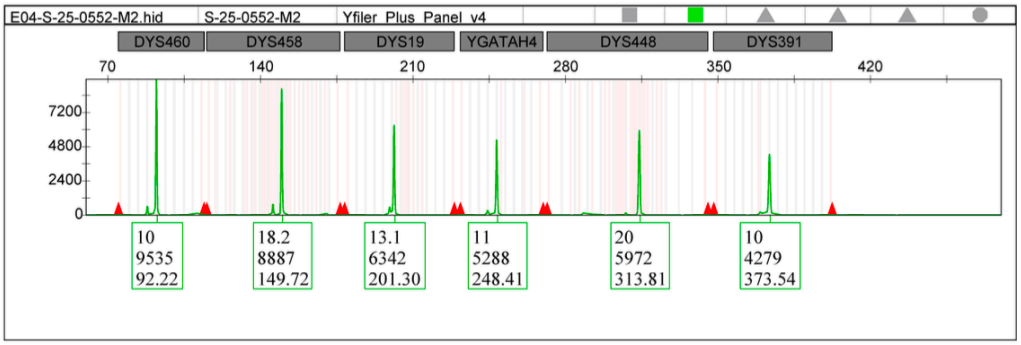
GeneMapper® ID-X 1.5

Project: Projecte (03-03-25) GM YFiler Plus 2

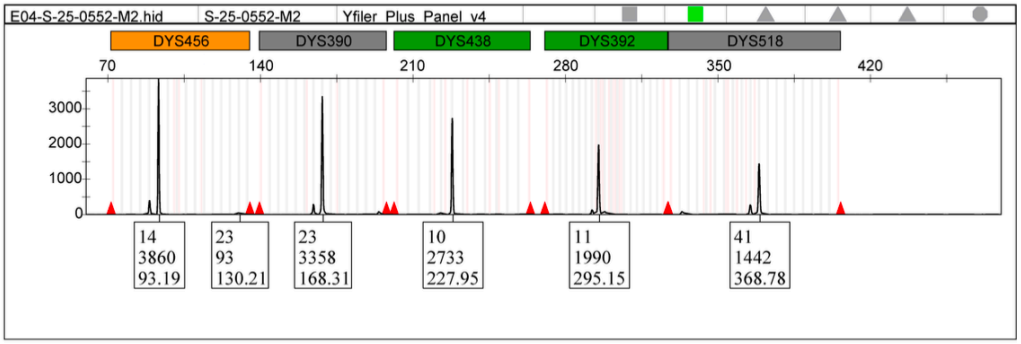
Sample File	Sample Name	Panel	SQO	SOS	SQ	SSPK	MIX	OMR	CGQ
E04-S-25-0552-M2.hid	S-25-0552-M2	Yfiler_Plus_Panel_v4							



E04-S-25-0552-M2.hid S-25-0552-M2 Yfiler_Plus_Panel_v4



E04-S-25-0552-M2.hid S-25-0552-M2 Yfiler_Plus_Panel_v4



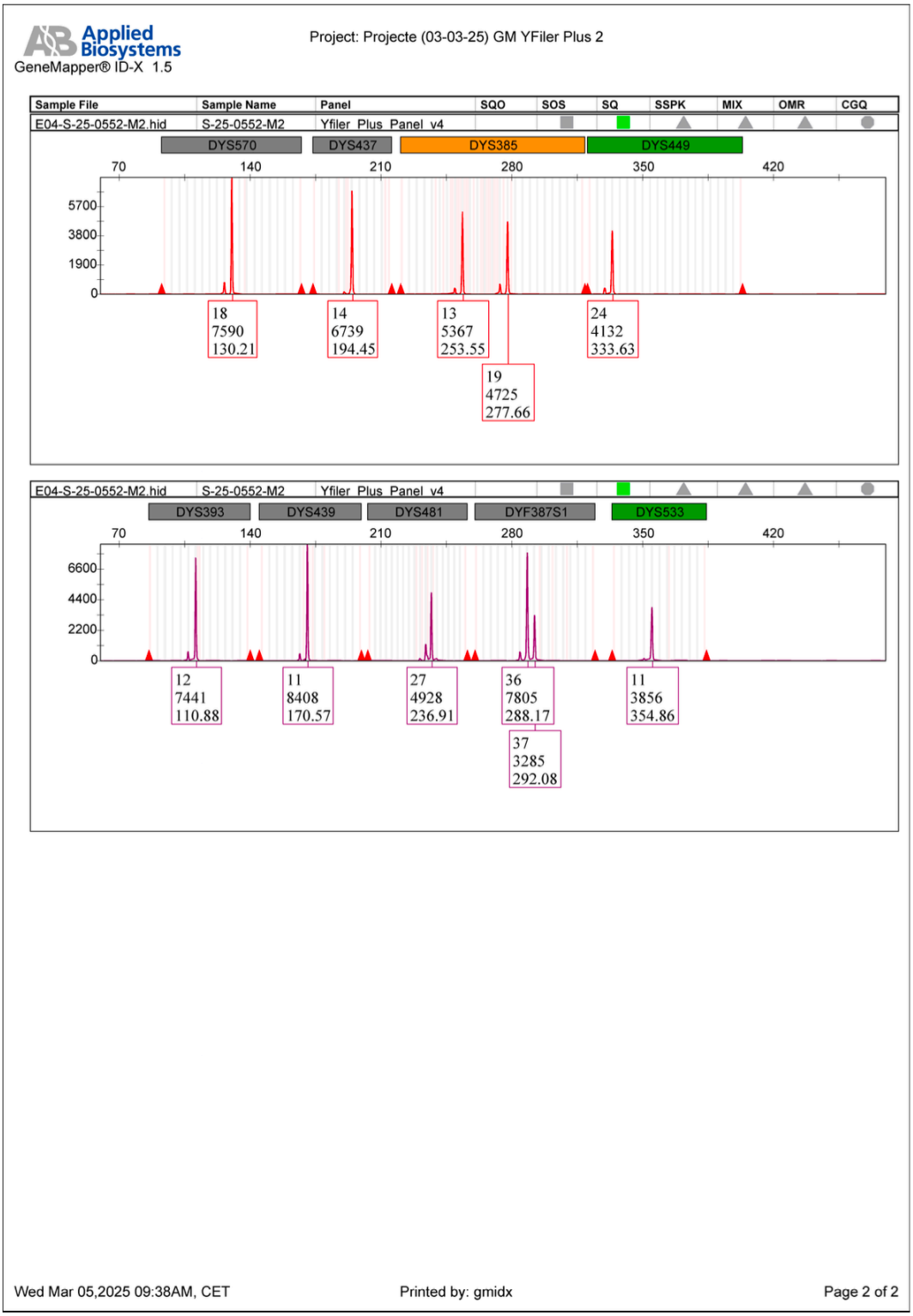


Figure A 9 Electropherogram corresponding to the analysis of Sample 2 (M2) using Y chromosome STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). In male individuals, only one allele will be observed per locus, as the Y chromosome is inherited exclusively from the father without recombination. This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M1).

8.3. PATERNITY CASE

8.3.1. AUTOSOMAL STR ELECTROPHEROGRAMS

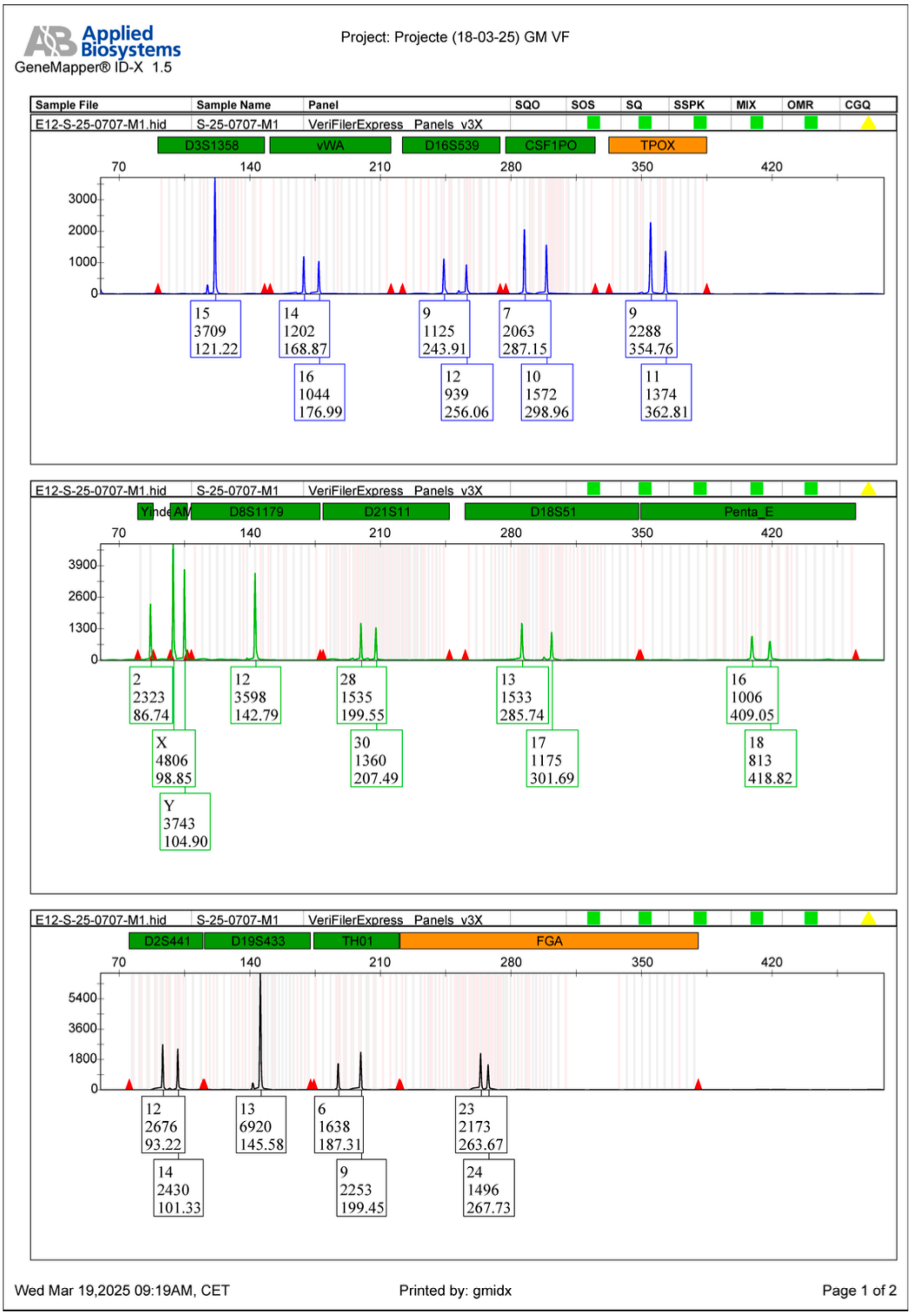
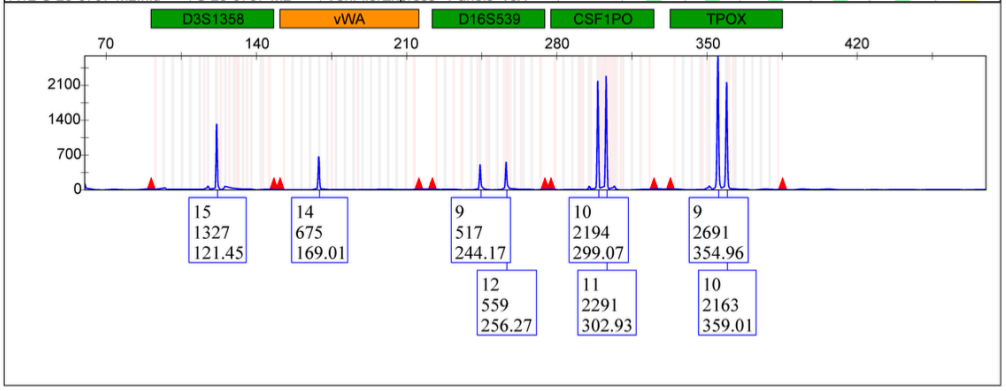


Figure A 10 Electropherogram corresponding to Sample 1 (M1) analyzed using autosomal STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M2).

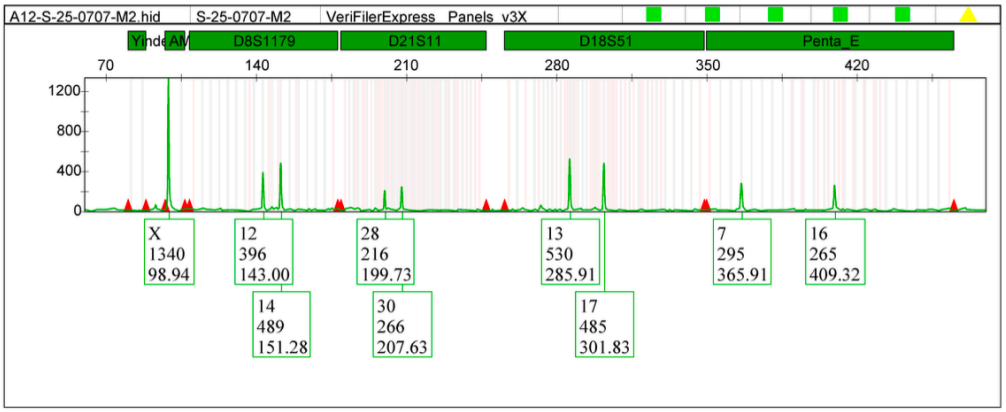
Applied Biosystems
GeneMapper® ID-X 1.5

Project: Projecte (19-03-25) GM VF II

Sample File	Sample Name	Panel	SQO	SOS	SQ	SSPK	MIX	OMR	CGQ
A12-S-25-0707-M2.hid	S-25-0707-M2	VeriFilerExpress_Panels_v3X							



A12-S-25-0707-M2.hid | S-25-0707-M2 | VeriFilerExpress_Panels_v3X



A12-S-25-0707-M2.hid | S-25-0707-M2 | VeriFilerExpress_Panels_v3X

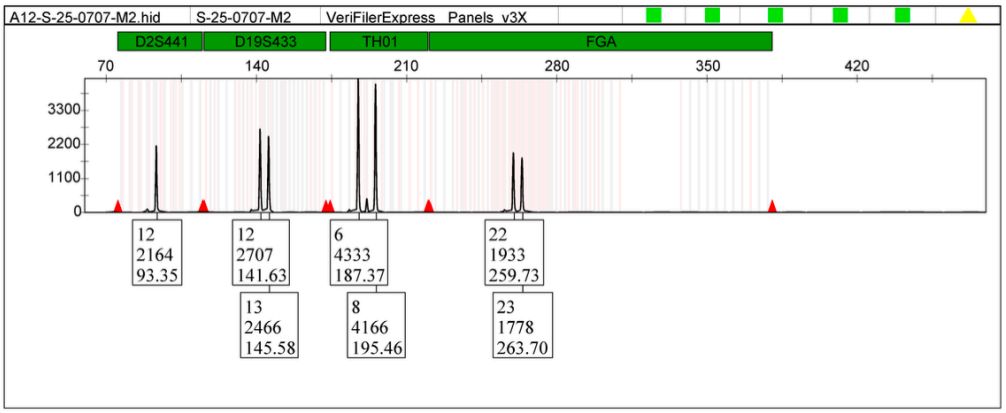


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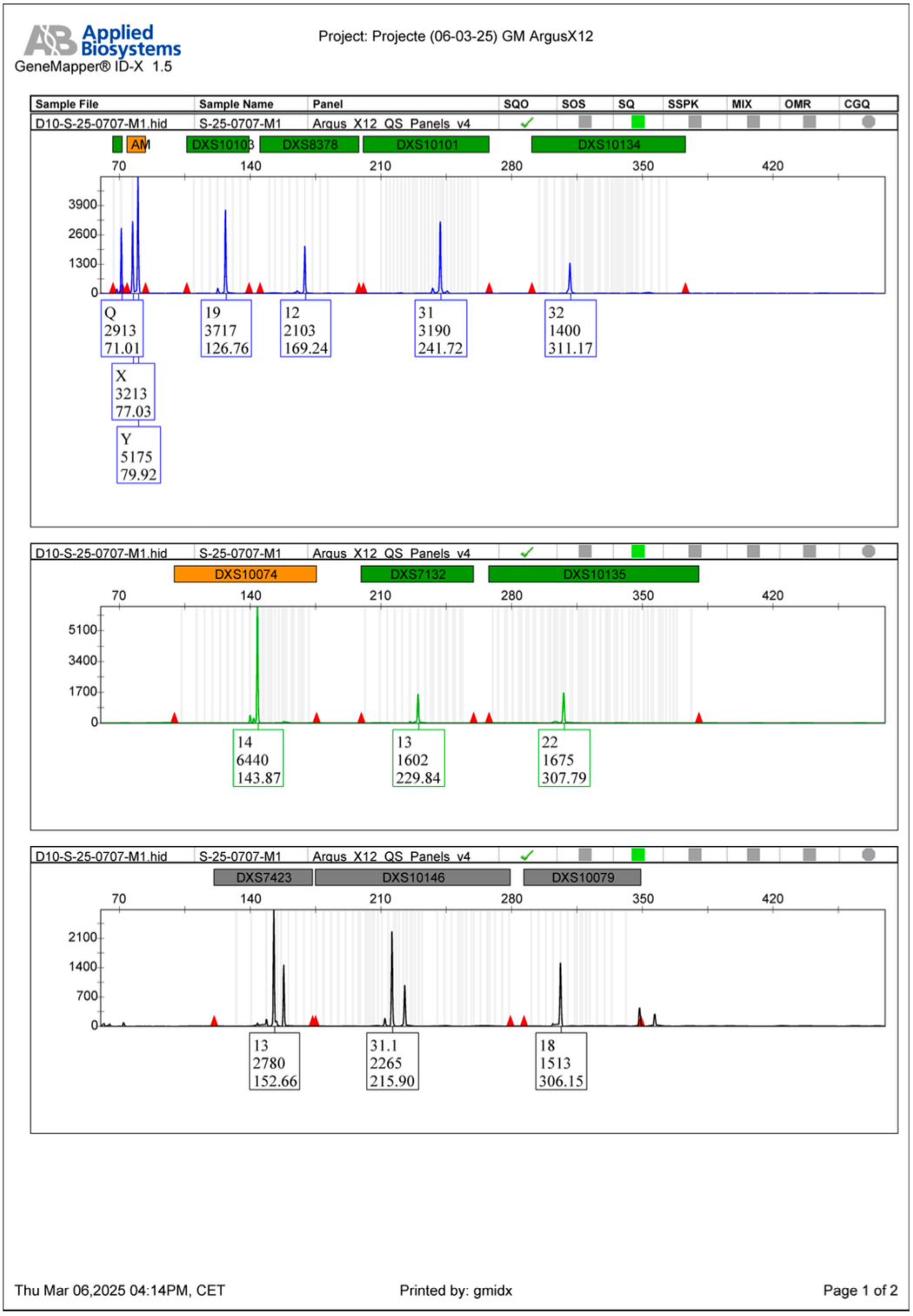
Figure A 11 Electropherogram corresponding to Sample 2 (M2) analyzed using autosomal STR markers. The image displays the peaks corresponding to the alleles detected at each locus, represented by their size (in base pairs) and signal intensity (relative fluorescence units). This graphical output illustrates the individual genetic profile of the sample and serves as the basis for comparative analysis with other samples (M1).

8.3.2. X CHROMOSOME STR ELECTROPHEROGRAMS



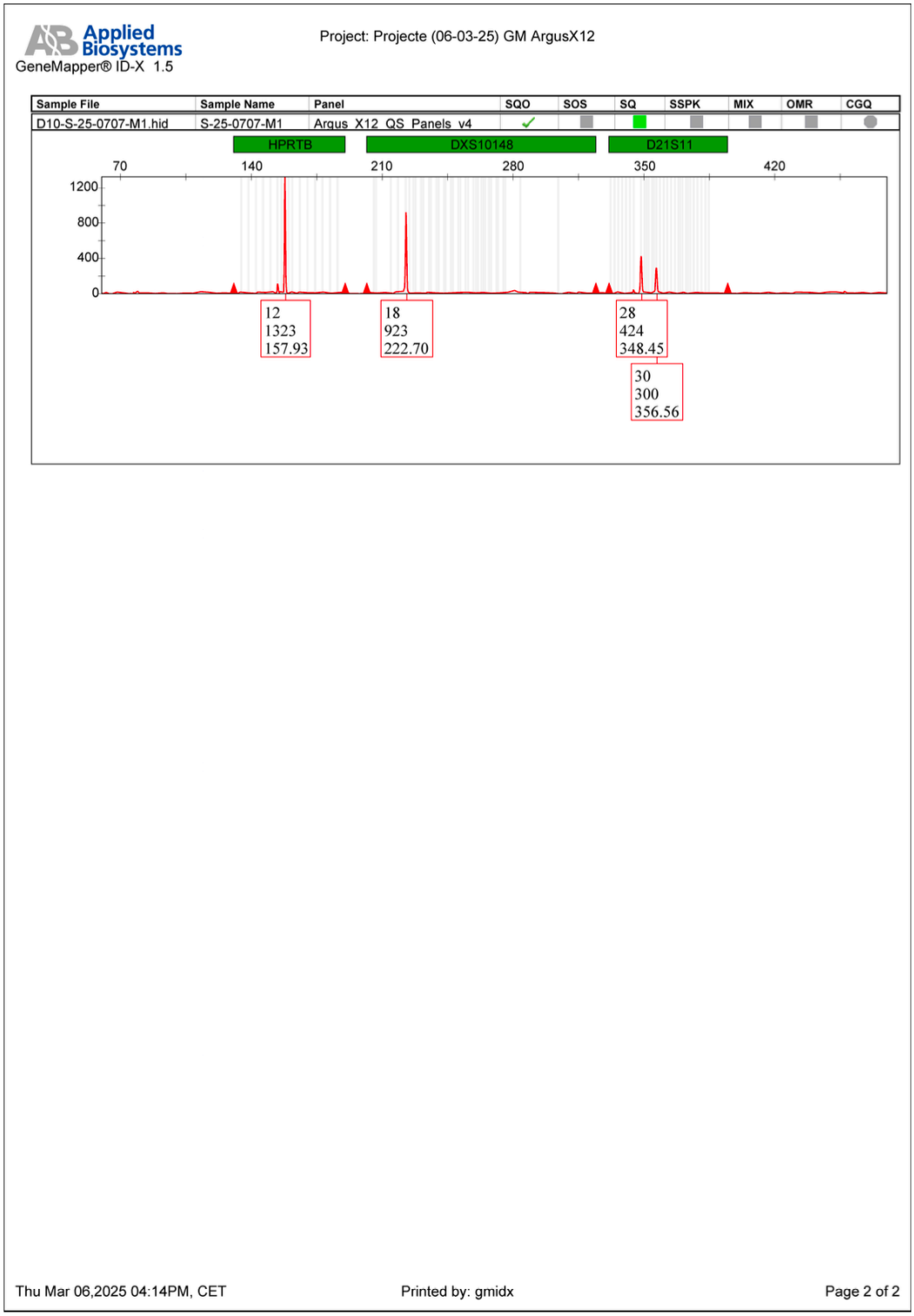
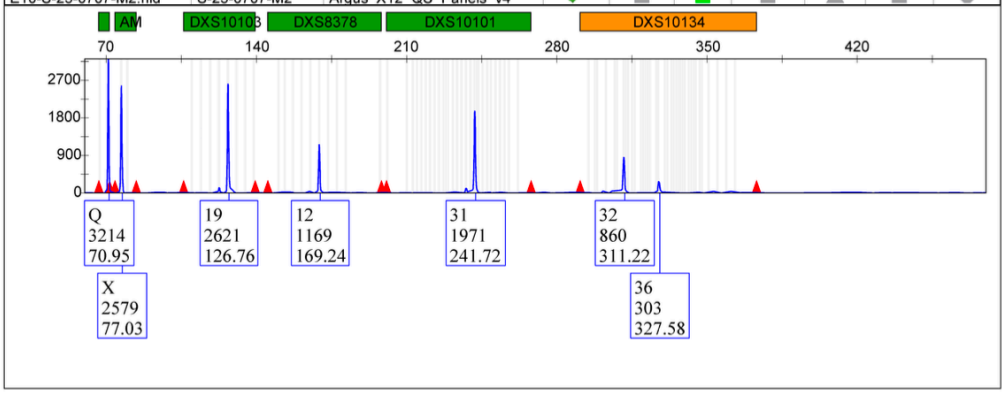


Figure A 12 Electropherogram corresponding to Sample 1 (M1) analyzed using X chromosome STR markers. The peaks represent the alleles detected at each X STR locus, shown by fragment size (in base pairs) and signal intensity (relative fluorescence units). In female individuals, two alleles may be observed per locus (heterozygous), or a single peak if homozygous. The marker labeled "Q" represents a quality control indicator used to assess the reliability and consistency of the electrophoretic run.

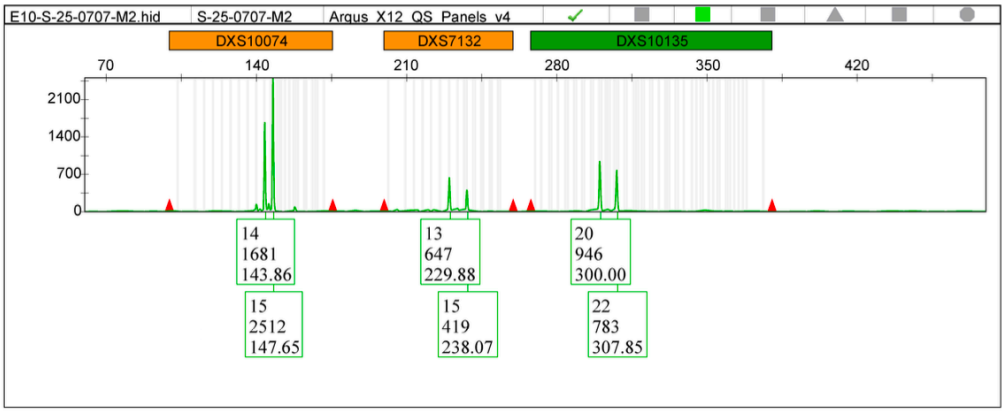
Applied Biosystems
GeneMapper® ID-X 1.5

Project: Projecte (06-03-25) GM ArgusX12

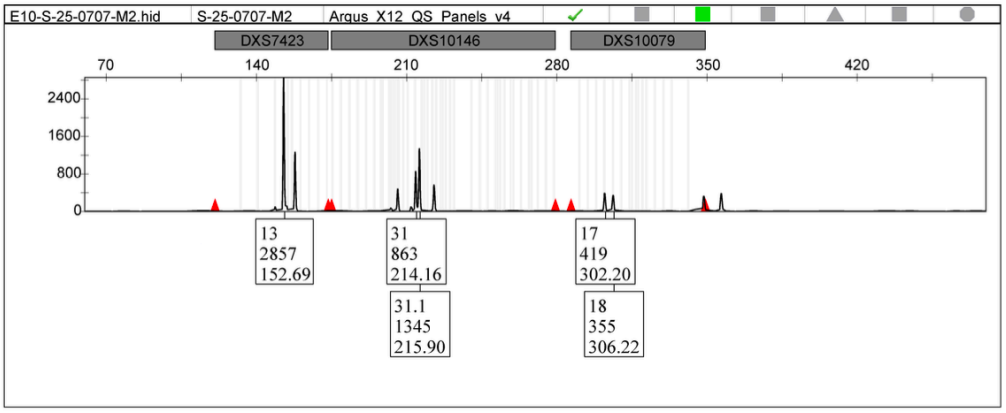
Sample File	Sample Name	Panel	SQO	SOS	SQ	SSPK	MIX	OMR	CGQ
E10-S-25-0707-M2.hid	S-25-0707-M2	Argus_X12_QS_Panels_v4	✓						



E10-S-25-0707-M2.hid | S-25-0707-M2 | Argus_X12_QS_Panels_v4 | ✓



E10-S-25-0707-M2.hid | S-25-0707-M2 | Argus_X12_QS_Panels_v4 | ✓



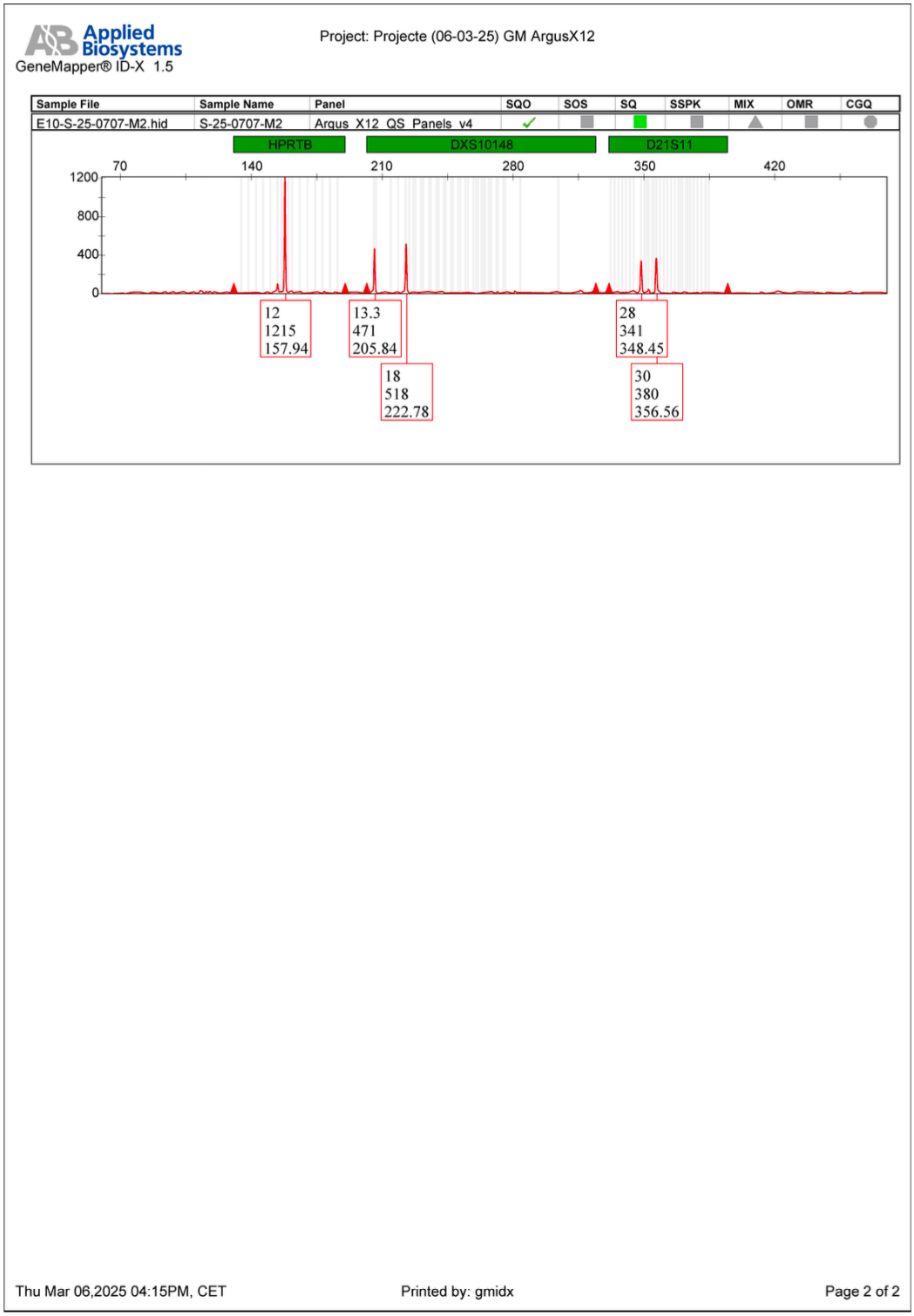


Figure A 13 Electropherogram corresponding to Sample 2 (M2) analyzed using X chromosome STR markers. The peaks represent the alleles detected at each X STR locus, shown by fragment size (in base pairs) and signal intensity (relative fluorescence units). In female individuals, two alleles may be observed per locus (heterozygous), or a single peak if homozygous. The marker labeled "Q" represents a quality control indicator used to assess the reliability and consistency of the electrophoretic run

Table 11 Comparative Table of STR Markers by Context, Advantages, and Limitations

MARKERS	ADVANTAGES	LIMITATIONS	MOST SUITABLE CONTEXTS
AUTOSOMAL STRs	High discriminatory power in most cases.	May lack resolution in indirect kinship (e.g., half-siblings).	Paternity testing.
	Widely used and validated in paternity testing.	Requires both individuals to share sufficient alleles.	General kinship analysis when both parents are potentially contributors.
	Biparental inheritance.		
X CHROMOSOME STRs	Highly informative in female sibling cases sharing the same father.	Limited informativeness in male–male comparisons.	Female-female sibling testing (Shared paternal lineage).
	Useful in father-daughter relationships.	Interpretation can be complex due to different inheritance patterns between sexes.	Paternity (father-daughter).
	Inherited with less recombination.		
Y CHROMOSOME STRs	Transmitted unchanged from father to son.	Cannot distinguish between close male relatives (e.g., brothers, paternal cousins).	Male-male kinship analysis (e.g., alleged brothers).
	Effective for tracing paternal lineages.		
	Useful in exclusion of male relationships.	Not useful in female lineage analysis.	Confirming or excluding paternal line consistency.

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