Genetisti Forensi Italiani (Ge.F.I.)

Ge.F.I. RECOMMENDATIONS FOR PERSONAL IDENTIFICATION ANALYSIS BY FORENSIC LABORATORIES

The present recommendations are the result of the work of experts who are part of the Ge.F.I working group and include: forensic genetics laboratories from Italian universities, Forensic Science Laboratories of the Carabinieri Force (Raggruppamento Carabinieri Investigazioni Scientifiche - Ra.C.I.S.), the Police Force and members of the Italian Society of Human Genetics (Società Italiana di Genetica Umana – SIGU).

The *Ge.F.I.* (Genetisti Forensi Italiani – Italian Forensic Geneticists) represents the Italian Speaking Working Group of the International Society for Forensic Genetics (*ISFG*) and, by the statute, is required to elaborate and regularly update recommendations for the adoption of guidelines on human identification analysis.
These guidelines have general validity and must facilitate the creation of a consensus regarding core operational strategies and utilized methods.
In 2015, the *Ge.F.I.* decided to take up the challenge of attempting to solve some of the emerging issues in the forensic field related to laboratory organization, complexity of human investigations for identification purposes and interpretation of analytical results. Thus, it undertook a course of action aimed at establishing guidelines to harmonize the work of Italian forensic laboratories and in compliance with the needs of the Italian Judicial Authorities.
The present *Ge.F.I* guidelines are the result of the work of several experts in the field affiliated to forensic genetics laboratories of Italian university institutions, Forensic Science Laboratories of the Carabinieri Force (*Raggruppamento Carabinieri Investigazioni Scientifiche - Ra.C.I.S.*), Forensic Police, and members of the Italian Society of Human Genetics (*Società Italiana di Genetica Umana – SIGU*).
Based on the *ISFG, SWGDAM,* and *ENFSI* recommendations recently published*, the herein work has been divided into three sections, each led by a working group of experts. The first section describes the minimum requirements for forensic laboratories and for personal identification analysis. This covers different topics including laboratory organization, quality of certifications and accreditations, laboratory reports, staff qualifications, and adequate/shared/recognized training programs. The second section provides a comprehensive overview of the different types of genetic biomarker, methods and technologies used in a forensic laboratory and it also includes a compendium on measures for prevention of DNA contamination.
The third section is focused on the assessment of the interpretative criteria applied in autosomal STR profiling and it was undoubtedly, and predictably, the most time consuming. It provides an introductory assessment and definition of the analytical criteria, evaluation of the conformity of analytical controls , description and detection of artefacts, and comprehensive procedures for data interpretation. This section is the result of numerous scientific meetings and discussions. Of particular importance is the statistical evaluation of the weight-of-evidence since it is common agreement that "*a conclusion of compatibility that is not supported by a statistical evaluation is not valid for identification purposes*".

* *International Society for Forensic Genetics* (ISFG):
https://www.isfg.org/Publications/DNA+CommissionScientific
Working Group on DNA Analysis Methods (SWGDAM):
https://www.swgdam.org/publications
European Network of Forensic Science Institutes (ENFSI) DNA working group:
http://enfsi.eu/about-enfsi/structure/working- groups/dna/

INDEX

SECTION 1- MINIMUM LABORATORY REQUIREMENTS

1. Laboratory organisation

1.1 Introduction

The work of a forensic genetics laboratory is to be organised through the definition of operative procedures and testing methods. Each laboratory must, where possible, make use of testing procedures defined by regulations, technical guidelines or official methods according to the ruling law. All methods, procedures, norms and regulations, reference manuals, and equipment instructions relative to the functioning of the laboratory must be kept up to date and made available to laboratory personnel. Prevention of contamination represents the most important requirement for a forensic genetics laboratory and must be ensured throughout all processes.

1.2 General recommendations

- Forensic genetics laboratories should guarantee physical separation of the main working areas including those reserved for item inspection and trace sampling, DNA extraction, and pre- and post-PCR process; fitting rooms for laboratory personnel must also be included.
- Each laboratory must use reagents and consumables for molecular biology; DNA-free, consumables and disposable items are preferable.
- Each laboratory must ensure the traceability and the identification of the reference samples, crime scene items and casework traces analysed.
- Each laboratory must ensure the traceability of all analytical operations carried out and maintain relevant supporting technical documentation.
- Each analytical method used in the laboratory must be defined, documented, validated, approved by internal quality control and made available to laboratory personnel.
- The internally validated method must be made available to the Judicial Authorities and consultants/expert witnesses upon motivated request.
- Expired reagents should not be used
- Each laboratory must create an *elimination database* to exclude any possible sample cross-contamination. This should include profiles of laboratory personnel, visitors and, where possible, external technicians, cleaning staff, expert witnesses, DNA examiners and analysts, police, personnel of the Judicial Authorities, emergency medical personnel who for different reasons come into contact with forensic items and biological samples. In case of refusal to provide a reference sample from external individuals, their access must be recorded. To ensure anonymity of database DNA profiles, an alpha-numerical code will be assigned to the biological sample of interest, following a pseudoanonymisation cirteria An authorized person is in charge of securing the anonymous data and it is the only person who can access such information in case this is relevant for the investigation or identification of a possible contamination The procedure must be fully documented and must include: methods of profiles management, the personnel in charge of genetic data handling, retention times and cancellation methods; these must appear on the informed consent forms of sample collection.
- All technical reports (e.g. photographic documentation of presumptive tests, reports on qualitative-quantitative DNA analyses, complete electropherograms with allele peak height and size, sequencer raw data, and all other instrumental reports) must be made available to consultants/experts upon request.
- In the context of implementation of judicial assignments, the data analysed and the resulting reports may be inspected by expert witnesses, to whom, however, they may only be made available upon authorisation granted by the Judicial Authorities.

- Laboratories performing forensic tests must have ISO 9001-2008 certification and also UNI EN ISO/IEC 17025 accreditation should they wish to submit DNA profiles to the national DNA database.

1.3 Recommendations on personnel

- Ttraining and skills of the personnel is a fundamental starting point for every forensic genetics laboratory.
- *Laboratory DNA analysts* in forensic genetics must possess adequate and recognised qualifications, according to the standard requirements of the UNI EN ISO / IEC 17025 regulation standard.
- *A laboratory manager* is required to:
  -evaluate the qualifications and acquired knowledge of the laboratory personnel by checking the relevant documentation;
  -train analysts on specific working tasks relative to the field of forensic DNA investigations conducted in crime DNA laboratories.

1.4 Recommendation on training

- Training should preferably be based upon the network of partnerships including universities, police/law enforcement, private companies, national and international forensic genetic societies (e.g. Ge.F.I., ISFG, ENFSI), and the European EUROFORGEN-NoE platform.

1.5 Recommendation on DNA analysis

- In a criminal investigation, relevant reference samples must always be analysed after casework trace samples or in separate designated area. Working spaces must be decontaminated according to procedures before and after the analysis of the reference samples and forensic items/traces.
- Daily plan of laboratory working activities must be made .
- Both positive and negative controls must be included at each step of the analytical DNA process.
- A paper- or electronic-based system aimed to document and ensure the traceability of operators, instruments, reagents, samples and testing methods and working environment conditions must be implemented to identify possible source of contaminations, sample or data mix-ups, or other errors.
- Genetic results from evidence samples must always be compared to the elimination database before drafting a final report.

2. Quality Assurance

Conformity of DNA analysis to internal and external quality control activities must be documented and ensured in a forensic genetic laboratory processing casework DNA samples. This is because DNA contamination issues may arise when analysing critical forensic traces (degraded DNA, low template DNA, etc).

2.1 Internal quality control

Internal quality control check must be applied to each analytical session.
-Corrective measures must be taken in cases where quality controls reveal lack of compliance with standards.
-Records of analytical quality control must be kept and periodically analysed to evaluate the performance of the laboratory and, if necessary, apply corrective measures to internal methods.

2.2 External inter-laboratory quality control

Laboratories are required to assess their skills in performing analytical tests and interpreting the results of forensic genetic investigations through external quality control in the form of inter-laboratory *exercises*. The term indicating the organisation, execution and evaluation of tests on identical or similar materials carried out by two or more laboratories on the basis of pre-set conditions.

They include:

- *Proficiency Tests (PT)* aim to evaluate the proficiency of forensic genetic laboratories in conducting analysis on biological traces, paternity/kinship/mixture testing; they must periodically be taken by all participating laboratories;
- *inter-laboratory comparisons* aim to demonstrate the reproducibility of a specific method and its forensic validity

The PTs require the analyses of biological traces (blood, saliva, sperm and/or other biological fluids, hair, etc), theoretical and applied statistics exercises and evaluations of the accuracy and precision of results obtained. A certification is issued upon test completion.

One of the aims is also to evaluate inter-laboratory variability and data concordance as well as the identification of number and type of errors made by the participating laboratories with the goal of reaching high performance standards drafted in specific recommendations. Proficiency testing is regulated by the ISO/IEC 17025, which requires at least one proficiency test to be performed per year. Experience gained through collaborative validation studies and proficiency testing clearly demonstrates that error reduction is the result of improved standardisation of procedures, not only of the methods and technologies employed, but also and above all of the interpretation of analysis results.

Here below different DNA projects organised worldwide are listed:

-GHEP Intercomparison Program “Analysis of DNA polymorphisms in bloodstains and other biological samples” is organised by the Spanish and Portuguese Speaking Working Group of the ISFG (GHEP-ISFG). It includes two difficulty levels (basic and advanced) and two training modules (kinship tests or forensic tests);

-GEDNAP “German DNA Profiling” is coordinated by the German Stain Commission. This organises two proficiency testing schemes per year with different forensic casework modules.

The “Ge.F.I. DNA Proficiency Test” represents the only external forensic genetics analysis evaluation programme that public and private laboratories can participate in at national level. This is organised by a technical and steering committee, which consist of three main experts in forensic genetics one of them of non-Italian nationality. The Ge.F.I PT requires all participating laboratories to genotype both reference samples and casework-like DNA traces, choosing from the following listed modules:

-identification of the biological nature of fluids
-autosomal STRs
-Y-STRs
-mtDNA
-biostatistical calculation
-theoretical kinship investigation.

A certification is released.

3. Accreditation of forensic genetic labs producing DNA profiles for DNA database

The European EN ISO/IEC 17025 regulation on "General requirements for the competence of testing and calibration laboratories" defines the criteria that laboratories must satisfy to demonstrate their technical competence and adherence to an accredited quality control system. , allowing them to achieve professionally qualified test and calibration results.

One of the requirements of the EN ISO/IEC 17025 system is the validation of the methods used. The EN ISO/IEC 17025 system represents one of the reference standards for laboratories that develop and accredit an internal method, while more detailed indications may be supplied by experts in the field.
Forensic genetic laboratories must establish minimum validation criteria for their procedures (internal validation). Just as the range and accuracy of the values obtainable through validated methods (for example the uncertainty of results, the analytical limits of detection, method selectivity, linearity, limitations in repeatability and or reproducibility, robustness in the face of external influences and/or cross-sensitivity with respect to interference coming from the sample matrix/object to be tested) are evaluated for their intended use, so too must they correspond to the demands of forensic application. For this purpose, the ENFSI, SWGDAM, EA, and ILAC guidelines represent useful tools of reference on methods validation.
All modifications/variations of a validated normalised or internal testing method must be the object of internal validation. The criteria applied for the validation of new methods must reflect the ones reported on the ENFSI documents (L. 85/2009 art. 11 c.1). All new validated test must conform to international standards and ensure concordance of genetic profiles from different laboratories.

Forensic genetics laboratories are required to set the primary goal of achieving ISO/IEC 17025:2005 accreditation. Failure to obtain such accreditation will unable a laboratory to provide data to the National DNA Database (L 85/2009).

'Accredia' is the only Italian national organisation authorised to grant accreditation and market surveillance activities (Reg. (CE) 765/2008 e DM 22.12.2009).

3.1 General indications

- Any modification of procedures that may influence results must be internally validated. It is essential to demonstrate that profiles obtained using the new procedure are, in terms of quality, better than or equivalent to those obtained using previous procedure.
- Environmental conditions must be controlled.
- Personnel variability must be examined in the validation protocol of each new choice of methods.
- At least five samples (negative control excluded) must be analysed for the approval of a specific parameter (repeatability, etc).

4. Laboratory reports

Different types of report exist:
1) Technical consultations, expert statements or technical investigations for the Judiciary Police: a full and comprehensive technical reports produced upon receiving an assignment from the Judiciary Authorities or upon private request.
The report should contain:
- the query/postulate (if formulated);
- circumstantial information and/or documentation on the case being examined if available and necessary to understand the investigation strategy and results obtained;
- the description of the items, chain of custody (preservation, transport, container, seals and state of the seals, signature, delivery receipt, acceptance receipt);
- list of items received for examination and relative internal laboratory codes and reference and if present, other previously used codes linked to the items (e.g. codes used in the police seizure receipts and previous investigations, etc);
- unambiguous identification of substances, materials or items sampled;

- original location of the sampled items accompanied with documented photographic record with appropriate metric references;
- requests made by the authorised parties;
- strategies of the analyses conducted, if necessary;
- materials and analytical methods used for analysis;
- all instrumental reports for the analyses performed (*e.g.,* photographic records of the presumptive tests, reports of the qualitative/quantitative DNA analysis, complete electropherograms with allele peak heights and sizes, and biostatistical reports)
- the results obtained and conclusions drawn by the expert.

2) The test report

The test report is defined by the ISO/IEC 17025 regulation (paragraphs 5.10.2 and 5.10.3.1) and the ACCREDIA documentation, and documents the final outcome of a forensic analysis with statement of genetic profile(s) obtained and accredited test method used in compliance with the relevant regulations.

3) Technical investigations report or preliminary report for the Judiciary Police:

This representa a concise communication to inform the Judiciary Authorities about new information on different aspects of an investigation. Such communication does not require a detailed description of all technical aspects.

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SECTION 2- LABORATORY METHODS

1. Detection of biological traces

1.1 Measures for prevention and detection of contamination

1.1.1 Introductions and definitions

For the purposes of the present guidelines, contamination is defined as: "the introduction of DNA, or biological material containing DNA to an item or biological sample when or after a monitored forensic process starts". This definition is distinct from "adventitious transfer", which refers to the transfer of biological materials to an item before it is recovered or before initial forensic activities and investigations, which start soon after the crime scene has been secured.

The main sources of DNA contamination are:

1) any type of professionals (crime scene manager and officer, evidence collector, etc.) handling an item or biological sample/fluid;

2) contaminated reagents and consumables (*e.g.,* swabs, test tubes) to the item or DNA sample;

3) from item to item or from DNA sample to DNA sample (cross-contamination) during collection, transport, storage or sampling of traces from an item.

Contamination may occur:

1) directly (e.g., deposition of saliva droplets on items);

2) indirectly (e.g., biological material present on the outer part of an evidence bag may be transferred to gloves of handlers who can subsequently transfer the contaminant if they fail to change gloves when opening the evidence bag and handling the item).

Contamination may sporadically occur as a single event affecting only one DNA sample per sample batch or 'widespread' event affecting simultaneously all DNA samples per batch at a given analytical stage (e.g., DNA extraction, amplification, etc.).

1.1.2 Measures for prevention of crime scene contamination

Restriction of crime scene access to authorised personnel only

All personnel authorized to access a crime scene, including individuals with no specifically assigned technical inspection activities, first responder and medical personnel, lawyers, public prosecutors and so on, must receive adequate education and training on measures to prevent and counter contamination events.

Wearing of personal protective equipment to minimize the possibility of direct contamination

All personnel accessing a 'complex' crime scene must wear, in the following sequential order, Personal Protective Equipment (PPE):

a) face mask;

b) mob cap or hair net;

c) first pair of gloves;

d) disposable over suit;

e) disposable shoe covers: these must be taken off or changed when leaving the environment or entering a separate area of interest within the crime scene itself);

f) second pair of gloves: these gloves must be regularly changed in a designated place, which must be separated from the area under examination, and always after handling any type of evidence items of forensic DNA relevance .

For 'simple' crime scenes, the following must be worn as minimum requirement PPE: face masks and one pair of gloves and also a second pair of gloves in case of collection of biological traces. In addition, individual access to crime scene areas should be constantly monitored to avoid cross-contamination across different secured areas.

In relation to the complexity of a single crime scene, it is strongly recommended to change PPE and decontaminate equipment before accessing different areas of the same crime scene. To avoid possible contamination during item packaging, each evidence item should be separately packaged in appropriate containers/evidence bags (e.g. paper bag for fresh traces) and carefully sealed. It is furthermore recommended to quickly identify the appropriate environmental conditions (room temperature, 4°C, -20°C, etc.) for items preservation, depending on their conditions (e.g., dry, wet, perishable, etc.). To guarantee and maintain the chain of custody, all packaged items should be unequivocally labelled.

Cleaning of equipment used at crime scenes
Any non-disposable equipment utilised to process crime scenes must be carefully decontaminated (*e.g.,* UV irradiation and/or treatment with an appropriate decontaminating fluid) before being re-utilized. Particular caution must be paid when the same equipment is used to process a place, environment or personal item from an alleged offender(s) or person under investigation that may potentially be linked to the same crime event.

Use of disposable equipment, materials and consumables
Equipment, materials and consumables used for collection, preservation and analysis of materials for forensic inquiries must be, wherever possible, disposable and DNA-free (ISO 17025:2005).

The general guidelines described above should be extended to the activities preceding the actual DNA analysis (extraction, quantification, amplification, etc.) in laboratory, which include item reception and inspection, and sampling of relevant biological traces.

1.1.3 Measures for prevention and detection of laboratory contamination
Refer to *Minimum Laboratory Requirements* – 1.2.

1.2 Detection of latent traces
The specialised personnel inspecting the crime scene not only proceeds to search for visible traces but also latent traces both using protective equipment and presumptive tests suitable for each type of trace. Initial non-invasive screening aiming to detect potential latent biological traces requires the use of ultra-violet lamps and visible light lamps (so-called forensic lights) to enhance initial observations of evidentiary items, produce photographic documentation, and also sample traces that are invisible to the naked eye. The use of forensic lights is required for initial screening of traces; however, it is insufficient for an accurate diagnose of the nature of traces. General criteria for the detection and diagnosis of the nature of substances and latent blood traces by chemical and enzymatic assays (e.g., Luminol) are described at point 2.1.2.

1.3 Collection methods of biological samples from and on bodies
If a corpse is found at the crime scene, biological samples and traces should be collected from the body for comparison purposes in accordance to the Recommendation No. R (99) 3 of the Council of Europe on the harmonisation of medico-legal autopsy rules. The International Society for Forensic Genetics has created specific recommendations concerning the type of tissue from which it is preferable to take samples for identification purposes from, also in relation to the conditions and conservation status of the corpse.
The sampling of traces from alive victims is specifically covered in the Guidelines for the collection of biological traces for forensic genetic analysis of sexually assaulted and/or abused victims (http://www.gefi-isfg.org/).

1.4 Chain of custody

The chain of custody is a written chronological record of all persons who maintained unbroken control over the items of evidence up to its eventual consumption/destruction. It ensures that the items of evidence collected at the scene of crime is the same evidence that is presented in a court of law. The chain of custody, when correctly established, represents an uninterrupted sequence of actions aimed to thoroughly document events of collection, custody, control, transfer, and storage of items. Samples derived from primary evidence items, such as DNA extracts, must have their own chain of custody maintained to the same standards as the original item. The chain of custody must be guaranteed through paper- or electronic-based documentation system or by any combination of the two. Manual record systems must, in any case, enable tracking the transfer of evidence items from person to person. The chain of custody records must include at least the following elements:

- description of the evidence item/object
- unique identification number (e.g., case number)
- location of evidence item
- storage room of evidence item
- list of persons in charge of the evidence items and their duities
- record of documented activities of each evidence item (e.g., analyses or re-packaging)
- record of dates and times activities were performed.

The chain of custody records must be stored for the period of time ruled by the judicial authority in charge of the investigation. Access to storage place must be limited exclusively to authorised personnel.

2. Laboratory activities

2.1 Identification of the nature of biological stains

The determination of the nature of stains allows to preliminary screen evidence items (discriminating biological and non-biological material, human and non-human material, etc) and integrate the genetic data obtained afterwards with circumstantial information useful for crime reconstruction (biological nature of human traces).

Since pre-screening tests of traces may lead to the destruction or dilution of the biological stain, this initial step may be omitted when in presence of latent or microscopic traces or when the determination of the biological nature of the sample is less relevant than the genetic identification of the donor. This must be stated in the technical report and made in agreement with the expert witnesses.

The determination of biological fluids is mandatory for traces collected from sexual victims or related evidence items allegedly containing sperm cells. The exclusion or positive confirmation of the presence of sperm is required in order to proceed further with the downstream differential extraction process.

2.1.1 Microscopic examinations

Microscopic examinations are based on the direct observation of cell-specific biological fluids and include, among others, optical and fluorescent evaluation (i.e., fluorescent monoclonal antibodies specific of a tissue antigen). For instance, specific staining protocols can be used to search for spermatozoa, identify different components of interest (e.g., head, tail) and estimate their amount in relation to the vaginal epithelial component in intimate swabs collected. A negative microscopic outcome for spermatozoa does not necessarily prove the absence of seminal fluid (e.g., azoospermic/vasectomised individuals or scarce/degraded samples) in the trace of interest. Additional immunological/biomolecular tests must be performed to confirm the presence/absence of non-cellular sperm components.

Moreover, the optical microscopic examination of cell morphology along with dedicated sources and reference databases is a pre-screening method, which can assist in the preliminary differentiation between natural and synthetic fibres as well as between human and animal hair. The microscopic examination of human hair morphology allows to determine the hair-specific development stage, a fundamental piece of information for following analytical tests.

2.1.2 Chemical and enzymatic assays

These tests exploit the capacity of a substrate to change colour or emit luminescence (Luminol) in the presence of a chemical compound or enzyme present in the target tissue. These commercial available tests largely documented in the literature are useful for the discrimination of biological fluids and relative chemical/enzymatic targets and include blood (heme group of haemoglobin), saliva (alpha-amylase), and sperm (prostatic acid phosphatase). These are 'presumptive' tests that produce high rate of false positives (low specificity limit) and low rate of false negative (high sensitivity limit). Overall they are useful for preliminary screening test of the biological nature of samples and can be performed either in laboratory or on-crime scene site; however, they are not considered confirmatory tests.

2.1.3 Immunological tests

These are methods involving the interaction between monoclonal antibodies produced in the laboratory and tissue specific antigen. Commercial systems (and relative antigenic targets) used for clinical testing (e.g., human faecal occult blood test) or forensic purposes are currently available for the following tissues: blood (haemoglobin, glycophorin A); saliva (alpha-amylase); sperm (prostate-specific antigen, semenogelin). A test for urine identification based on polyclonal antibodies that can bind the human Tamm-Horsfall protein has also been recently made commercial available. These are high specific immunological or 'confirmatory' tests; nevertheless, cross-reactivity should not be ruled out because the antigen used to identify a given target tissue is often present (though in lower concentrations) also in other human tissues. Lastly, low sensitivity of these tests does not allow excluding false negatives in minimal traces or traces previously subjected to washing.

2.1.4 Biomolecular tests

In recent years, biomolecular approaches have also been proposed as alternative or complementary methods to immunological tests for body fluid identification. The high level of sensitivity represents the main advantage of such methods and enables the whole trace to be processed for nucleic acids extraction. Although these are promising methods and some of them have already been validated through global inter-laboratory studies, a consensus marker panel and standardised/commercial available protocols have not been developed yet nor a universally accepted approach to data interpretation. For these reasons, caution must be taken and most up-to-date scientific literature used in the evaluation of results, which may be affected by individual-specific physio pathological conditions or environmental factors.

2.1.5 Coordinated interpretation of tests for determining the nature of traces and of genetic tests

For correct interpretation of results from presumptive, confirmatory, chemical/enzymatic, immunological and bimolecular tests, trained personnel must include the following information in the technical reports:

- commercial reagents used or description of custom-made preparation of reagents and reference works on their forensic validation;

- a brief description of the method: any deviation from protocols recommended by the manufacturer (e.g., incubation times for chemical and enzymatic assays or immunological tests) must be supported by scientific literature;
- a brief but clear description of the sensitivity and specificity limits of the type of test reported on manual of commercial products and/or in scientific literature.

If a diagnostic test gave a negative result, analysts are free to proceed with extraction, quantification, amplification and typing of DNA samples due to the high sensitivity level of genetic tests for human identification. If a positive result for a low specific chemical/enzymatic assay cannot be corroborated by a positive result from a 'confirmatory' test (i.e., microscopic examination for spermatozoa search, immunological test or biomolecular test), the resulting genetic profile reported in the technical report may not be referred to as certainly derived from a specific biological fluid. In such cases, any assumption concerning the nature of the trace should be made in relation to the relevant circumstantial information provided.

2.2 DNA extraction

2.2.1 Foreword

The choice of extraction protocol made by the laboratory is influenced by different factors including the type of equipment, personnel experience, type and amount of sample available for analysis and so forth. Precaution measures must be taken to prevent and identify possible contamination sources, irrespective of the method selected (refer to *Minimum laboratory requirements* – 1.5 Recommendations on DNA analysis). In the context of individual cases, reference samples and casework traces must be analysed in separate areas and/or at different times to avoid possible sample cross-contamination (after getting the genetic profiles from the evidence, where it is possible). A technical report must include the manual or automated sample processing technique, type of instrument and/or commercial kit used; any modifications from recommended protocols must be clearly stated and justified. Where a custom-made laboratory procedure is used, the internal validation should be avaible for consultation.

2.2.2 Extraction techniques in forensics

The most commonly used extraction techniques in forensic DNA laboratories include: phenol-chloroform, chelating resin-based DNA lysis, solid phase extraction with spin column and silica membrane- or magnetic bead-based process. DNA purification techniques that use magnetic beads and silica-membranes have made possible the commercialization of automated and high-throughput extraction process platforms. For reference samples such as saliva deposited on purpose-specific carrier material treated with DNA stabilisation reagents, direct PCR amplification protocols, which by-pass the extraction step, can be utilized. For bone tissue and dental material/teeth, a pre-cleaning of the surface of samples must be performed before proceeding further with the extraction process. For compact bone fragments, pulverisation of the bone material followed by decalcification in EDTA for varying lengths of time depending on the quantity of start material is also required. For dental material, tooth break and dissection is required to enable access of reagents to dental pulp. In addition, a pre-cleaning step is recommended before extraction of DNA from hair.

2.2.3 Differential extraction

Differential extraction protocol should be followed for the investigation of mixed traces allegedly containing sperm cells produced following an ejaculation event in sexual assault cases. Low-concentration proteinase K and a lysis buffer are added to isolate the 'epithelial fraction' (containing DNA from non-spermatozoa cells), which is then separated from the lysis-resistant spermatozoa fraction by centrifugation. The subsequent treatment of the spermatozoa fraction with dithiothreitol (DTT) extraction buffer enables the DNA extraction from spermatozoa.

Preferential DNA lysis can also be applied for the analysis of hair shafts embedded in biological fluids (e.g.; blood, saliva, vaginal fluid) where the first fraction will contain DNA from the biological fluid while the second fraction DNA from the hair shaft.

2.3 DNA quantification

The extreme sensitivity of STR amplification techniques requires that the preliminary quantification method must be at least equally sensitive.

The reliable quantification of human genomic DNA isolated from biological traces is an important analytical check-point to allow optimal input DNA volume in the PCR reaction and minimise a reaction failure due to sample inhibition or degradation. In the final report, the commercial kit and real time-PCR instrument used, results obtained, presence of any inhibitors and, if possible, degraded DNA material, and amount of male and female DNA must be indicated. Each laboratory may establish specific threshold values below which no useful genotyping results are expected, and also decide to proceed with STR typing should a negative quantification result be produced. Due to the high quantity of genomic DNA from reference samples collected from living individuals or from bodies during autopsies, quantification may either be disregarded or performed using less sensitive methods including spectrophotometry or fluorometry.

Alternatively, at the end of standardised extraction protocols, where such are in use, a standardised concentration of genomic DNA is to be obtained from the reference sample, so that a separate quantification step may be omitted.

2.4 Analysis of genetic markers

2.4.1 STR polymorphisms (STRs)

To meet the technical DNA standards set by the international forensic genetic community, commercial available PCR amplification STR kits must be utilized. Full list of kits used and relevant references must be reported and any changes in the amplification protocol (e.g., final PCR volume, number of PCR cycles) recommended by the manufacturer, must be accurately documented and supported by relevant scientific literature works or internal validation studies; if the analysis of additional non-commercial STR markers is required, internal/external validation procedures must also be documented (e.g., calibration of the comparative allelic ladder according to allelic nomenclature conforming to the ISFG guidelines; participation in collaborative exercises including quality controls or proficiency tests, etc.). For additional STRs, also, adequate population allele frequency databases must be available.

2.4.2 Autosomal STRs

Human identification is normally reached by the analysis of standard STR polymorphisms located on autosomes (i.e., non-sex determining chromosomes). The list of markers required by the European Standard Set (ESS) and Combined DNA Index System (CODIS) is reported in the table below.

Extended ESS	CODIS	Expanded CODIS
D3S1358	D3S1358	D3S1358
vWA	vWA	vWA
D8S1179	D8S1179	D8S1179
D21S11	D21S11	D21S11
D18S51	D18S51	D18S51
TH01	TH01	TH01
FGA	FGA	FGA
D1S1656	-	D1S1656
D2S441	-	D2S441
D10S1248	-	D10S1248
D12S391	-	D12S391

D22S10145	-	D22S10145
-	CSF1PO	CSF1PO
-	TPOX	TPOX
-	D5S818	D5S818
-	D7S820	D7S820
-	D13S317	D13S317
-	D16S539	D16S539
-	-	D19S433
-	-	D2S1328

2.4.3 Y-chromosome STRs

Male-specific STR loci located on the Y chromosome (Y-STRs) can be analysed together with or in alternative to autosomal STRs in cases of sexual assault (mixed male-female traces), kinship testing or missing person identification. The combination of alleles at multiple Y-STR loci generates a 'haplotype'. The minimal haplotype to investigate has, for many years, been identified as the core set. Today commercial multiplex PCR systems include up to 27 Y-STRs and also, in some cases, 'rapidly mutating' (RM) Y-STR loci characterized by a high mutation rate, which enhance the differentiation of males from the same paternal lineage. The table below lists the minimum number of 17 Y-STRs recommended for criminal DNA investigations.

Y-STR	**Indication**	**Comment**	**Y-STR**	**Indication**	**Comment**
DYS19	Recommended	Minimal haplotype	DYS449	Optional	RM Y-STR
DYS385	Recommended	Minimal haplotype	DYS460	Optional	
DYS389I	Recommended	Minimal haplotype	DYS481	Optional	
DYS389II	Recommended	Minimal haplotype	DYS518	Optional	RM Y-STR
DYS390	Recommended	Minimal haplotype	DYS533	Optional	
DYS391	Recommended	Minimal haplotype	DYS549	Optional	
DYS392	Recommended	Minimal haplotype	DYS570	Optional	RM Y-STR
DYS393	Recommended	Minimal haplotype	DYS576	Optional	RM Y-STR
DYS437	Recommended		DYS627	Optional	RM Y-STR
DYS438	Recommended		DYS643	Optional	
DYS439	Recommended		DYF387S1	Optional	RM Y-STR
DYS448	Recommended				
DYS456	Recommended				
DYS458	Recommended				
DYS635	Recommended				
YGATAH4	Recommended				

2.4.4 X-chromosome STRs

The X-STRs are most commonly applied to complement autosomal and Y-chromosomal STR analysis in complex kinship cases such as deficiency paternity tests involving female children, incest, identification of missing persons or victims of mass disasters. Since these *loci* are located on the same chromosome (*i.e.,* 'linkage'), they are not inherited independently and often display 'linkage disequilibrium' (LD) or non-random association of alleles in a haplotype.

The *loci* on the X chromosome are generally subdivided into 4 linkage groups, each of which is characterized by a specific haplotype data set and one to three loci:

- Linkage group 1 (Xp22): DXS10148, DXS10135 and DXS8378;
- Linkage group 2 (Xp12): DXS7132, DXS10079, DXS10074 and DXS10075;
- Linkage group 3 (Xp26): DXS10103, HPRTB and DXS10101;

- Linkage group 4 (Xp28): DXS8377, DXS10146, DXS10147, DXS10134 and DXS7423.
The haplotypes are established by commercial available kits or custom-made multiple panels containing 4 to 12 X chromosomal STR markers. For biostatistical evaluation of X-STR data, adequate haplotype frequencies of each linkage group must be used. The ISFG DNA Commission has recently established guidelines on the use of X chromosome microsatellites in parental studies and recommended its use as a supplement tool to standard autosomal marker analysis when an inconclusive result is obtained.

2.4.5 Gender identification markers

In general, conventional autosomal STR and X-STR typing systems include one or more sex-determining markers. The most widely used marker is the amelogenin, which is located in the pseudo-autosomal region of sex chromosomes and features an insertion/deletion polymorphism on intron 1 (6 bp deletion on X chromosome). A large deletion in the corresponding Y chromosomal segment is relatively frequent (2-8%) in males from Indian subcontinent and results in 'female' profile; in such cases additional supplemental Y- STR markers must therefore be tested. Although the lack of amplification of a specific Y-fragment with amelogenin test is a rare event, this is not completely negligible (1:5000) when tested in European populations.

2.4.6 Mitochondrial DNA (mtDNA) polymorphisms

The analysis of mtDNA is generally applied to the investigation of traces containing highly degraded nuclear DNA or made up of anucleated cells (*e.g.,* hairs in the telogen phase or bulb-free). Historically, Sanger sequencing was the prevalent technology for the forensic analysis of mtDNA haplotypes; however, this will be completely replaced by massively parallel sequencing in the near future (refer to section 2.4.8). Current forensic protocols require the sequencing of specific hypervariable regions (HVS-I, 16024-16365; HVS-II, 73-340 and HVS-III, 340-576) located within the mtDNA control region. To prevent the high risk of contamination in mtDNA analysis, each laboratory is required to observe and adhere to good laboratory practice and recommendations found in the guidelines established by the ISFG, published in 2000 and revised in 2014. Forensic laboratories performing mtDNA analysis are strictly required to include negative and positive control samples during the entire analytical process.
Lack of commercial mtDNA kits and automated analytical and data reporting process, high risk of contamination and possible sequencing of artefacts influence the quality of forensic mtDNA analysis. Careful revision of data on mtDNA haplotypes is crucial and recommended by published guidelines. Laboratories performing mtDNA analysis are also required to fully document internally and externally validated procedures followed (*e.g.,* PCR primers, control region, etc.) in their technical reports. To maintain high quality analytical standard, active and regular participation to proficiency testing programmes is recommended.

2.4.6.1 Mitochondrial haplotype nomenclature

MtDNA sequences must be aligned and the nucleotide variations relative to the *revised Cambridge Reference Sequence* (rCRS, NC001807) annotated, according to the nomenclature recommendations. Consistent nomenclature criteria must be adopted by each laboratory and documented in the analytical report. Adoption of a mitochondrial phylogeny-based nomenclature system is highly recommended, as specified in the ISFG guidelines established in 2000 and 2014. The nomenclature criteria used for haplotypes notation in casework samples must be the same of the population database used for estimation of haplotype frequencies; in alternative, a database allowing alignment-free queries such as EDNAP Mitochondrial DNA Population Database (EMPOP, www.empop.org) can be used.

2.4.7 SNPs and Indels

SNPs for human identification purposes can be useful for typing degraded DNA samples and they are included in manufacturing panels analysed by using MPS technology (see 2.4.8)
Indels (insertion/deletion polymorphisms) can be used as complementary markers for deficiency paternity testing cases.

2.4.8 Massively parallel sequencing (MPS)

The term massively parallel sequencing (MPS) indicates a high-throughput method used to determine a portion of the nucleotide sequence of an individual's genome. This technique utilizes DNA sequencing technologies capable of processing multiple DNA sequences in parallel. In general, MPS methods are based on initial enrichment of the target DNA sequence by PCR or DNA capture probes and followed by preparation of DNA libraries , which are then subjected to clonal amplification and sequencing. Commercial sequence-based STR and SNP kits have been developed for forensic applications including human identification, prediction of biogeographic ancestry and DNA phenotyping (i.e., determination of physical features such as eye, hair and skin colour) and target or whole sequencing of mtDNA. Requirements for the implementation and use of sequencing forensic kits include comprehensive developmental validation by manufacture, internal validation by laboratory, and documented scientific publications.

2.4.9 Interpretation of genetic typing of haploid markers

Y-STRs

Estimation of haplotype frequency in human identification and kinship testing.

To express the weight-of-evidence of full haplotype match between samples of interest frequency of haplotypes must be estimated. Since Y-STRs are linked on the same chromosome and not subjected to meiotic recombination, the 'Product Rule' cannot be applied to estimate the haplotype frequency. Extensive publicly available reference databases that record the frequencies of haplotypes and not the individual alleles must be used. The YHRD (www.yhrd.org) contains the highest number of populations (Release 62 at the time the recommendations were drafted) and more than 307,169 minimal haplotypes. The database includes 5275 Y chromosome haplotype data from Italy, making it one of the best represented countries in the database. In addition, automated calculation of haplotype frequencies (and specification of confidence intervals when applicable) using various approaches described in literature is supported.
A technical report must include the statistical approaches used to calculate the resulting haplotype frequency and whether the estimated value refers to the entire database or an individual 'meta populations' (groups of populations closely linked to one another by genetic, cultural or geographic factors) into which it is subdivided. For instance, the European population samples are grouped into east, southeast, and west meta populations or into 'national' database of population of interest (defined by political borders of the reference country).
The given number of database release (e.g., R62) and full list of investigated Y-STR markers (e.g., minimal haplotype, Yfiler, etc) must also be included in the technical report to allow repeatability and reproducibility of data search by third parties. Lastly, a statement on Y-STR haplotype sharing by all male individuals from the same paternal lineage must also be included.
It is possible, using RM Y-STR markers, to discriminate subjects belonging to the same paternal lineage.

X-STRs

With reference to the recently published guidelines on evaluation of X-STR haplotype results, it should be noted that the biostatistical calculation of the likelihood ratio is based on the two alternative hypotheses of kinship or non-kinship. Marker linkage and possible LD must also be considered in the analysis. However, effect of LD can only be evaluated using haplotype data

reported on digital reference databases (www.chrx-str.org) or in a limited number of scientific publications. When referring to the Italian population, the current largest database gather haplotype data of 200 individuals. Computational approaches relying on mathematical algorithms are available for the genetic analysis of markers in linkage (and also in LD) to produce probative LR values. Calculation approach (evaluation of linkage or LD alone), software used, allele/haplotype frequency database selected, recombination and mutation rate must be indicated in the final report.

mtDNA

The mtDNA haplotype obtained from a forensic specimen can be directly compared to the haplotype from a reference sample or to haplotypes from maternally related donors in case of corpse identification. The comparison of mtDNA haplotypes can lead to three different results: exclusion, non-exclusion or inconclusive result based on the 2000, 2014 ISFG's guidelines on interpretation of mtDNA results. In case of non-exclusion, the weight-of-evidence is expressed as frequency estimate of the study mitochondrial haplotype in the population database of interest.

The database search must be performed by taking into account all available sequences relative to the sequencing range considered. In this regard, length heteroplasmic position at homopolimer sequence should be excluded from the search while heteroplasmic positions should be inserted during the search in order not to exclude any possible heteroplasmic variant.

Public databases of mitochondrial haplotype frequencies exist. In particular, EMPOP is the largest database that ISFG recommends to use in light of the extensive number of population samples included (> 40.000 haplotypes grouped into 'meta populations' and 398 of which from Italian population, based on the most recent Release R13) and stringent quality controls of submitted sequence data. The choice of database and statistical approach used must be explained when reporting the results.

All analytical reports including estimates of haplotype frequencies from EMPOP must also indicate the database version, nucleotide range, type of match ("pattern" including all possible nucleotides that determine a heteroplasmic position, or "literal"), positions affected by length polymorphisms excluded from the search, subpopulation within which the estimate was made and the result of the estimation of the haplotype frequency. Lastly, a final statement on mitochondrial haplotype sharing among all individuals descending from the same maternal lineage. The likelihood ratio for mtDNA is normally calculated as illustrated in the ISFG Guidelines (2014).

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SECTION 3- EVALUATION CRITERIA FOR THE INTERPRETATION OF AUTOSOMAL STR PROFILES

1. Preparatory analytical and evaluation criteria for the interpretation of genetic typing results

For forensic genetic inquiries (personal identification, kinship testing and disaster victims identification (DVI) it is required that all laboratories preliminarily define analytical and evaluation criteria to be applied to DNA electropherograms (raw data, or .fsa or .hid files). The following phases should therefore be followed for the interpretation of genetic profiles:

A) Definition of threshold values for interpretation of STR profiles enables the detection of analytical signals specific to the samples/biological traces in the electropherograms and exclusion of artefact signals. The threshold values are defined following internal laboratory validation and are applied to the raw data. The validation method must be acknowledged by the international scientific community and also documented in the laboratory procedures.

- The **analytical threshold (AT), also known as limit of detection (LoD),** is the value in RFU, which allows an analytical signal (allele) to be distinguished from background noise. The specific level of confidence with which the threshold value was determined must be specified in the laboratory procedure. Only peaks equal to, or greater than the AT are assigned an allele call by the typing software used. A unique AT value across all spectral bands or a spectral-specific band AT value can be assigned.
- The **limit of linearity (LoL)** of the analytical system used is the value, in RFU, that corresponds to the saturation point of the detection system of the instrument (sequencer) beyond which the instrument no longer produces a linear signal when the LoL is exceeded. As a result, alleles beyond the LoL appear as double, or flat top peaks, the background noise increases, and non-specific signals that may complicate data interpretation can be generated. A lower input amount of DNA in the PCR reaction must be used or dilution of the amplified products must be performed.
- The **stutter threshold** may be defined as the limit value, expressed as a ratio (or percentage) relative to the highest peak, below which (backward stutter -1 or forward stutter +1) the analytical signals may be considered PCR by-products. The stutter value is defined per each specific locus as the ratio between the height (or area) of the analytical signal in the stutter position and that of the parent allele signal. Same stutter threshold cut-off value across all loci or locus-specific values can be set.
- The **stochastic threshold (ST)** is a quality indicator of allele signal that informs the DNA analyst about the possibility of not observing all expected genetic information in the sample. The ST is particularly relevant in cases of limited quantity and/or significantly degraded DNA. In such conditions, stochastic events may lead to allele imbalance in heterozygous loci, allele drop-out in heterozygous and/or homozygous loci, higher stutters, and allele drop-in events (refer to the corresponding entries in the glossary). The stochastic threshold is, therefore, a RFU value above which it is reasonable to assume – specifying the relative confidence level – that an allele drop out event has not occurred. The stochastic threshold must by empirically defined by each laboratory through internal validation and must be conducted on each multiplex-PCR system and typing procedure used in the laboratory.

B) Definition of parameters and criteria for interpretation of STR profiles

The electropherograms must be interpreted in relation to:

- **number of alleles detected at each locus** by the genotyping software, and in particular the presence of more than two allele signals at two or more loci indicates that at least two

individuals have contributed to the sample (mixed profile). The mixture ratio (M_x) of allele signals can be calculated and evaluated at each specific locus or across all STR profile following the procedure described in the glossary available in the SWGDAM Interpretation Guidelines 2017.

- **heterozygote balance (H_b)** or **Peak Height Ratio (PHR)** is an indication of the degree of imbalance between the sister alleles at a heterozygous locus. At optimal condition, the value of H_b at a locus should ideally be of ≥ 0.6 (60%) in biological trace or reference samples. The related method of H_b calculation is described in the glossary available in the SWGDAM Interpretation Guidelines 2017.

2. Evaluation of analysis results conformity

Once the analytical and evaluation criteria have been defined, the process continues with the evaluation and assignment of peaks in the *internal standard*, of electropherograms obtained from the *allelic ladder,* from *positive* and negative *control samples (of extraxction and amplification)* and with the verification of typing results of biological traces in the exclusion database.

2.1 *Internal lane standard* and *allelic ladder*

a) The internal lane standard. Correct fragment separation and allele size call of all fragments covered in the user's manual of the multiplex-PCR system in use must be verified.

b) The allelic ladder. Allelic ladder is required in each electrophoretic run and multiplex-PCR system used. More than one allelic ladder must be added when running a large number of samples. Use of allelic ladders from previous runs in not allowed and the correct electrophoretic separation and allele calls must be verified. If off-ladder alleles appear, internal standard must be used as a control reference to verify the correct allele call. If the problem persists, a new electrophoretic run must be performed.

2.2 Amplification of positive control

With the goal of evaluating and validating DNA profiles obtained from casework stains or reference samples, at least one *positive control sample* must be co-amplified with the batch of samples run. Genotype calls at all loci must be checked. If the observed genotype differs from the expected genotype or no genotypes are produced, even after a new electrophoretic run is performed, re-amplification of the positive control sample and of all samples in the same batch must be performed.

2.3 Extraction and amplification of negative controls

To reliably assess the typing results, two mandatory negative controls must be co-amplified with the sample batch: *negative extraction control* and *negative amplification control*.

- ***Negative extraction control:*** it is a sample containing all reagents used in the extraction process but the biological sample during each extraction session of stains or reference samples. This extraction blank sample must co-follow the analytical workflow of evidence traces under investigation from quantification/amplification to capillary electrophoresis.
    1. Where the negative extraction control does not yield allelic signals above the AT, the analysis may be considered valid.
    2. Where the extraction blank yields signals corresponding to alleles, the laboratory must establish and document the evaluation criteria by which the extraction of the biological traces obtained from stains and of the reference samples was considered valid or otherwise. The validity evaluation must bear in mind the possibility that such analytical results may have had a significant effect on the outcome of the analysis conducted on all the ***samples of that session.***

- ***Negative amplification control***. In each amplification session, two *negative control samples* consisting of PCR reagents and water must be amplified. Such samples must preferably be amplified and injected either at the beginning or end of electrophoretic run.
    1. If the two blanks do not yield allelic signals above the AT, the analysis is considered valid.
    2. If the two blanks yield consolidated allelic signals above the AT (the same allele is present in both negative controls) it is necessary to verify the presence of these alleles in the trace samples in the same analitycal batch, evaluating whether this analytical result (contamination) may have had a significant impact on the outcome of the analyses conducted on all the biological samples from that session.
    3. If the two blanks yield non-consolidated allelic signals above the AT (the same allele is not observed in both negative controls) it is necessary to verify the risk that this analytical result may have had a significant effect on the outcome of the analyses conducted on all the biological samples from that session.

For the hypotheses considered at points 2 and 3, a technical statement documenting the validity/non-validity of analytical results obtained must be produced.

One negative control may be amplified should the amplification session include $\leqslant$ 5 samples; no allele signals above the AT must appear in the negative control.

2.4 The elimination database

Once the typing results of positive and negative control samples and allelic ladder have passed all quality control check-points, DNA profile(s) generated from questioned traces or reference samples must be compared against the genetic profiles archived in the *elimination database* (refer to 'Minimum laboratory requirements – 1.2 General recommendations') and also those produced by the laboratory within a specific timeframe. This allows to exclude any possible cross-contamination from previously analysed casework traces or reference samples.

3. Amplification and fragment size separation-related artefacts

It is the DNA analyst's task to both identify PCR amplification- and capillary electrophoresis-related artefacts in the resulting electropherograms and determine the effects on the interpretation of the outcomes. This is a list of such products whose definition is available from the SWGDAM Interpretation Guidelines 2017

a. *Stutters*
b. *Spikes*
c. *Pull-ups*
d. *Dye-blobs*
e. *Split peaks.*

4. Interpretation of casework and reference DNA profiles

4.1 *Evaluation of electropherogram features*

The interpretation guidelines of DNA profiles is based on so-called *expert opinions*, which may involve several expert DNA analysts in accordance with internationally peer-reviewed scientific journals. This is based on the interpretation process that must be founded upon the most objective evaluation possible of analytical data and circumstantial information of the trace of interest even though human subjectivity may influence the decision-making process. The **objective** data to be evaluated by the DNA analyst are the following:

-qualitative features refer to the presence or absence of alleles at each locus relative to the AT and stutter threshold, the presence or absence of more than two alleles at each locus and the presence or absence of artefacts and stochastic phenomena;

-quantitative features refer to signal intensity (height) with reference to the AT, the ST and the stutter threshold; they also denote heterozygote balance, number of alleles per locus with peak height equal to or greater than the AT and ST, and finally, mixture ratio/mixture proportion when more than two alleles are detected at a locus.
DNA analysts must rely on both the evaluative indicators in the genotyping software and on their own 'expert opinion'.
The evaluation of electropherogram features will further allow to establish:
A. whether the genetic profile comes from a single DNA contributor (**single-source genetic profile**)
B. whether the genetic profile comes from at least two DNA contributors (**mixed-source genetic profile**). The number of DNA donors contributing to a mixed sample is mainly estimated by means of the maximum allele count (MAC) even though further methods have been described in the literature;
C. whether the **genetic profile is complex** it must meet at least one of the following features:

- alleles with a height lower than the ST at one or more loci;
- 4 alleles at one or more loci;
- partial genetic profiles of a lower number of correctly typed vs expected loci (2012 ISFG recommendations).

The interpretation of a DNA profile becomes more difficult when the number of alleles detected at each locus increases. In such a case, the consensus DNA typing approach from different profile replicates is recommended in order to verify the repeatability of genetic data produced. This approach requires the repeated amplification of the same DNA extract with multiplex-PCR systems using the same PCR conditions (same input DNA amount and number of cycles) and typing system. The change of PCR conditions including the input amount of DNA and/or the number of PCR cycles and/or the multiplex-PCR system is, however, permitted in the attempt to provide additional information to the trace profile if such modifications have been prior validated. If so, the amplification of the same number of replicates must be performed and the choice of multiplex-PCR system as well as the DNA quantity and PCR conditions documented.
The genetic profiles obtained in the various amplification replicas can be used to generate a "consensus" genetic profile , in which the alleles more frequently observed in the greatest number of the amplification replicas are reported, and/or a "compositus" profile, in which all the alleles obtained in the different amplifications are recorded.
D. whether the genetic profile is **suitable or not for personal identification**: if no alleles or only few of them above the AT are found, the genetic profile is considered unsuitable **for personal identification purposes**. Every different situation must be evaluated according to an expert opinion and must be accurately documented.
In general:
1. single-contributor genetic profiles of at least 10 loci typed in a reliable (consolidated) manner should be considered suitable for comparison even if the profiles are complex,
2. single-contributor genetic profiles of less than 10 loci typed in a reliable (consolidated) manner; in such cases, profiles should be considered potentially suitable for comparison;
3. multi-contributor genetic profiles of at least 10 loci typed in a reliable (consolidated) manner should be considered potentially suitable for comparison even if complex and/or with a high number of alleles per locus;
4. multi-contributor genetic profiles of less than 10 loci typed in a reliable (consolidated) manner, complex and/or with a high number of alleles per locus must be evaluated with extreme caution and should not be used for comparison purposes in any case,.

For cases 3 and 4, detailed documentation of criteria and internal laboratory procedures followed to evaluate genetic profiles with such characteristics suitable for comparison and reach an expert opinion must be provided.

4.2 Procedures for the interpretation of genetic profiles

4.2.1. DNA profile interpretation from reference and single-source samples

Single-contributor genetic profiles (refer to paragraph 4.1)

-reference sample must yield a single-contributor genetic profile; if this is not the case a new sample must be obtained from the individual. Single-contributor genotypes featuring three alleles at a single locus may occur and are generally associated with genetic anomalies (e.g., trisomy, segmental duplication or somatic mutation) or with bone marrow transplantation where a mixed recipient-and-donor profile can be detected. In all such cases, adequate clinical documentation and record should be provided to the laboratory.

-biological stain obtained from a crime scene item may results in a single-contributor genetic profile when at most one or two alleles per locus are observed with the exception of the genetic abnormalities aforementioned. With the aim of reaching a consensus DNA profiles, the generation of additional PCR replicates of the biological trace of interest from a stain is at the discretion of the DNA analyst even though reliable results have been obtained in the first round of amplification. The analysis of biological stain may lead to a complex single-contributor genetic profile. The interpretation of such genetic profiles should be performed following the ISFG recommendations

4.2.2. Profiles comparison procedures of single-contributor genetic profiles

Once a genetic profile from either a single stain or several stains is obtained, this must be compared to the biological reference sample. In general, the comparison may lead to one of the three conclusions listed hereafter:

a. exclusion: the person of interest is excluded as a possible contributor

b. inclusion: the person of interest cannot be excluded as a possible contributor to the genetic profile

c. inconclusive: DNA typing results are considered inadequate to draw a match or non-match conclusion

a. **Exclusion (discordance, non-match).** If the differences between the genetic profile obtained from the stain and the reference sample are such that they cannot reasonably be explained by stochastic phenomena, nor by degradation phenomenon, nor by sequence alteration (e.g., SNPs or indels), the individual can be possibly excluded as trace donor. If an individual discordance is detected in a profile with at least 10 loci, and if both the stain and the reference sample have been analysed with different STR typing kits, it is recommended to verify the result using the same commercial kit. If the genetic profile of the trace is complex, it is advisable to verify the possible exclusion result by calculating the likelihood ratio (LR) using a probabilistic genotyping semi continuous and/or continuous software.

b. Inclusion (**concordance, match).** If genotype concordance between the genetic profile obtained from the stain and the reference sample is found, this finding supports the hypothesis of identification of the person of interest as trace donor. To evaluate the evidence of a match, the calculation of the LR using binary and/or semi continuous and/or continuous computational/statistical approaches is recommended.

The greatest evidence in favour of the hypotheses of the prosecution consists of LR values above the level of 10^6 (one million), which is associated to the following two verbal equivalents: "*...provide extremely strong support for the first proposition (Prosecution Hypothesis-Hp) rather than the alternative (Defence Hypothesis-Hd)...*" and "*...are exceedingly more probable given… proposition...than proposition...*" . The numerical

reference values of LR and related verbal equivalents in support of this value is listed in the table published by the ENFSI.

c. **Inconclusive.** This result describes the situation in which, from the comparison of the genetic profile obtained from the questioned stain and the reference sample, identification or exclusion of an individual is not possible. The DNA analyst may, in any case, establish further evaluation criteria concerning the inconclusiveness of the result and provide detailed motivations for the opinion reported in the final report.

4.2.3. Interpretation of mixed profiles from biological stains collected on items

Mixed genetic profiles (refer to paragraph 4.1)

Mixed profiles arise when two or more individuals contribute with same or different biological fluids to the trace detected and collected on an evidence item.

Mixed genetic profiles may be affected by degradation and stochastic phenomena. However, the degree of complexity is greater than that of single-contributor profiles because the genetic contributions to the mixed profile may be affected in a different (and, often, undetectable) manner by such phenomena. The interpretation of mixed genetic profiles may be conducted following the guidelines proposed by the ISFG. Here below is a short summary.

a. Mixed genetic profiles with a major contribution from a single individual

It is sometimes possible to extrapolate a major contribution from a single individual in mixed stains in which many loci show more than two alleles, when one or two alleles at each locus are in a ratio of peak height ≥ 3:1 relative to the other alleles of the same locus. In these cases, the major profile thus extrapolated **may be treated like a single-contributor profile** (see par. 4.2.1) regarding comparison with a reference sample, and the analyst may proceed with the statistical calculation (restricted combinatorial approach). It is, in any case, possible to proceed with the calculation without extrapolation of the major contributor ("unrestricted approach", SWGDAM, 2017)

b. Mixed profiles without major contributor and stochastic phenomena

In the case of mixed profiles where a major contributor is not evident and cannot be extrapolated, it is necessary to proceed with the estimation of the number of mixture contributors. If all loci of a complete profile feature no more than 4 alleles, it may be assumed that the number of contributors is at least two. If the number of contributors is greater than two (several loci feature more than four alleles) it is best to consider the diverse scenarios in the statistical calculation relative to the number of known and unknown contributors, if possible, to be agreed upon by both the scientific consultant of the prosecutor and the defence. The opposing hypotheses for which the LR value is calculated must be scientifically consistent with the composition of the stain.

c. **Mixed profiles without major contributor and featuring stochastic phenomena**

Such profiles pose more difficulties than mixed profiles where stochastic effects are absent. In the verification of the reproducibility of the data with typing replicates (refer to paragraph 4.1), consolidation assumes fundamental importance. Genotypes can be evaluated following the "consensus" and/or "compositus" interpretation method.

4.2.4 **Profiles comparison procedures of mixed genetic profiles**

Comparison with a consolidated mixed genetic profile from a single stain may lead to one of the three conclusions: incompatibility, compatibility, or inconclusiveness.

a. Incompatibility (**exclusion**): if the alleles in the genetic profile obtained from the mixed stain do not match the alleles of the reference sample and the mismatch cannot be reasonably explained by neither stochastic phenomena nor degradation phenomena nor polymorphisms in the nucleotide sequence, this incompatibility is biologically coherent with the 'hypothesis of exclusion of the person of interest as contributor of the mixed trace. Since, in this case, the

genetic profile of the stain is complex, it is advisable to verify the exclusion by means of a probabilistic evaluation in terms of LR with semi-continuous and/or continuous models.

b. Compatibility (no exclusion): If alleles of the reference profile match the alleles of the genetic profile obtained from the biological stain and the possibility of a failure to find some alleles may reasonably be justified by stochastic and/or by degradation phenomena, this compatibility is biologically coherent with the hypothesis of inclusion of the person of interest as contributor of the mixed trace. Alleles from the reference profile not detected in the mixed profile must be reported in the report and it is mandatory to further support the compatibility using a probabilistic evaluation in terms of LR with semi-continuous and/or continuous models.

c. Inconclusive: this result describes situations in which, after a probabilistic evaluation, it is not possible to reach identification or exclusion. The DNA analyst may, in any case, establish further evaluation criteria concerning the inconclusiveness of the result, providing detailed justifications for this decision in the final report.

4.3 Probabilistic evaluation of the weight-of-DNA-evidence

The probabilistic evaluation of DNA results is performed to define the statistical significance of probability of inclusion or exclusion and requires the description of certain parameters needed for the statistical calculation. This includes:

- **allele frequencies of the reference population.** The reference population is defined as the population in which the crime was committed. It is also possible to use allele frequencies related to the population of the victim/suspect/person of interest (POI) if circumstantial information on ancestry of these individuals is available. The frequency database used in the case of interest must always be clearly indicated. In regard to the Italian population, the *GeFI* has made available the database of Italian frequencies, resulting from collaborative projects, for most of the loci included in commonly used commercial kits. If the reference population of interest is the Italian population, the use of the GeFI STR allele frequency database does not require any specific giustifications; the use of other population databases must, however, be stated. In cases where the POI belongs to a well-defined population, which does not correspond to the Italian population, the use of the allele frequency database specific to that population published in peer-reviewed journals is recommended.
- **FST value or θ (theta) parameter.** It is a measure of the population differentiation due to genetic structure and measures the difference in the allele frequency between two populations. This generates an increase in the frequency of level of homozygosity relative to the one predicted by the Hardy-Weinberg equilibrium, and is measured by a single parameter, which varies from zero (no layering) to 1 (a value that has never been measured). If the reference population is Italian, it is recommended to use FST values equal to 0 or 0.01 (1%) in the statistical calculations; the choice of a higher value must be explained. In the presence of a well-defined reference population distinct from the Italian population and in absence of specific allele frequency databases, the use of continental or sub-continental allele frequencies is recommended. In this case, the use of an FST value of 0.03 (3%) and up to 0.05 (5%) is recommended; however, the choice must be explained.
- **Drop-in and drop-out probabilities.** Amplification failure of expected alleles (drop-out) and amplification of unexpected additional alleles (drop-in) can be monitored in degraded DNA samples. The probability of such drop-in and out events can be set for all loci or per each locus of interest investigated. It is not reasonable to consider more than one or two drop-in events per profile if a drop-in probability of 5% is selected unless specified. Drop-out probability values must be empirically determined based on the criteria defined

in the test method and/or internal procedures and/or by means of mathematical simulations.

4.4 Principles and methods for probabilistic evaluation of DNA evidence

Likelihood Ratio (LR). The calculation of LR is the recommended method for the evaluation of evidence of a match. In line with the international scientific literature, calculated LR values are associated with 'verbal equivalents': descriptive phrases which express their meaning, using terms that are always more restrictive than the statistical significance of the support offered by the data to the Hp or the Hd. The reference is the table present in the ENFSI document.

In addition, it is possible, to use additional statistical approaches to the calculation of the LR:

- **Combined Probability of Exclusion (CPE) and Combined Probability of Inclusion (CPI) or Random Man Not Excluded (RMNE)**. These approaches provide an estimate of the fraction of a reference population, which cannot be excluded as possible contributors to the mixture.
- **Random Match Probability (RMP).** RMP calculation may be used for single-source and also mixed-source DNA profiles when all profiles of mixture donors can be clearly distinguished (RMP is calculated on each identified contributor).

4.5 Probabilistic genotyping (PG) software

The ISFG has made available on its website page (http://www.isfg.org/Software) a list of different internationally validated open-source and user friendly semi-continuous and continuous software packages for forensic statistical analyses. The listed semi-continuous software models includes the drop-in and drop-out probabilities in the LR calculation. Their use is also recommended in all cases where stochastic phenomena do not occur. The continuous-based software models instead take also into account the peak height or peak area, degradation and stutter effects in the calculation.

The decision-making process followed in reaching the conclusions given must always result from the analysis of the data, regardless of the type of PG software used. The presentation of results generated by the software is accepted if an adequate explanation of the software choice made is provided. In case of DNA profiles generated from repeated amplifications (replicates), the statistic evaluation of all replicates simultaneously is recommended. Alternatively, consensus or composite profile may be used for statistical evaluation.

When reaching the conclusion of DNA profile compatibility between the questioned and unknown samples, this can only be considered acceptable for the purpose of identifying a suspect if it is properly supported by adequate statistical analysis

The laboratory must, in any case, document in detail the procedures followed in the interpretation of mixed DNA profiles.

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