WORKING PROCEDURES MANUAL
FORENSIC DNA TESTING-2019

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CENTRAL FORENSIC SCIENCE LABORATORY

DIRECTORATE OF FORENSIC SCIENCE SERVICES
MINISTRY OF HOME AFFAIRS, GOVT.OF INDIA
DAKSHIN MARG, SECTOR 36-A
CHANDIGARH-160 036
Tel.: 91-172-2615068, 91-172-2605933
Fax: 91-172-2605923
Website: cfslchandigarh.gov.in, Email: cfsl-chd@nic.in

PUNYA SALILA SRIVASTAVA, IAS
JOINT SECRETARY

सत्यमेव जयते

भारत सरकार
GOVERNMENT OF INDIA
गृह मंत्रालय
MINISTRY OF HOME AFFAIRS
NORTH BLOCK
NEW DELHI-110001

FOREWORD

Forensic sciences have a critical role to play in criminal justice delivery system. Successful prosecution of offenders hinges on the quality of test reports. Exhibits in crime cases need to be examined timely, precisely and accurately as forensic examination report forms the basis for trial of the accused. In order to bring uniformity in the forensic analysis and the test reports of crime cases in forensic laboratories across the country, Directorate of Forensic Science Services (DFSS), Ministry of Home Affairs has taken the initiative to prepare Working Procedure Manuals (WPM) for various disciplines.

Due to advancement of technology and its global access, crime is continuously evolving and adapting. New crime trends are emerging with people committing crimes in cyberspace, trafficking occurring in new psychoactive substances and drugs etc. Therefore, incorporation of new technologies in the WPMs to meet the ever increasing challenges for solving crime is the need of the hour, which is systematically and comprehensively reflected in these WPMs.

A long-felt need for such uniform WPMs has been fulfilled and hope that these will be of immense use to forensic professionals of India. I congratulate the DFSS team for developing these manuals and urge them to keep on updating these at regular intervals.

Punya Salila

Punya Salila Srivastava, IAS
Joint Secretary (Women Safety)
Ministry of Home Affairs, Govt. of India

डॉ. एस. के. जैन
निदेशक–सह–मुख्य न्यायालयिक वैज्ञानिक

Dr. S.K. Jain, M.Sc Ph.D
Director-cum-Chief Forensic Scientist

सत्यमेव जयते

न्यायालयिक विज्ञान सेवा निदेशालय,
गृह मंत्रालय, भारत सरकार
ब्लॉक–9, तल नं. 8 , केन्द्रीय कार्यालय परिसर
लोधी रोड, नई दिल्ली–110 003

Directorate of Forensic Science Services,
Ministry of Home Affairs, Govt. of India
Block No. 9, 8th Floor, C.G.O. Complex
Lodhi Road, New Delhi-110 003 (India)
Tel. : 011-24362676 Fax : 011-24362819
E-mail : cfs-dfss@nic.in

PREFACE

The Directorate of Forensic Science Services (DFSS) is the nodal organization of Ministry of Home Affairs, Govt. of India to propagate and carry out high quality and credible forensic science practices in the country to serve the cause of criminal justice delivery system. In wake of its motto, DFSS has taken initiative and prepared a systematic and comprehensive Working Procedure Manual (WPM) for Forensic DNA testing to bring uniformity in the analytical procedures for routine analysis of biological samples.

The information in this WPM has been designed to provide methods to enable forensic biologist/DNA scientist/DNA Analyst to do the routine analysis of biological samples in a wide variety of situation. The manual consists of fourteen chapters including isolation and purification of DNA, quantification, amplification and capillary electrophoresis for STR analysis of forensic samples and essential equipments required to carry out the examination. Protocols for performing statistical calculations and report writing are also incorporated. As sexual assault, paternity and maternity disputes, human identification in mass disasters, rape and murder, child swapping cases are increasing day by day in our country and in most of the cases, court decisions are pending for DNA reports.Therefore, to meet the requirement in present scenario,efforts have been made to incorporate advanced automated techniques of Forensic DNA profiling to reduce turnaround time of the cases.

I am pleased to mention that Directorate of Forensic Science Services has now come out with this WPM in order to help the forensic laboratories to continue to follow standard and latest updated procedures in the examination of clue material as well as bring uniformity in the analysis. We understand that there is always a scope of improvement and perfection can be achieved with collective efforts, therefore, stakeholders are welcome to give their feedback.

17/12/15

Dr. S. K. Jain

AMENDMENT RECORD

Change No.	Section No.	Amendment Page/Clause No.	Amendment Date	Amendment Designator	Approved By

APPROVAL AND ISSUE

The Central Forensic Science Laboratory, Chandigarh towards its concern to quality in the Forensic laboratory examination of physical evidence items of crime cases, operates a quality system - such as to confirm to the ISO/IEC 17025: 2005 'General requirements for the competence of testing and calibration laboratories' issued by International Organization for Standardization/International Electro technical Commission, NABL 113: 2008 'Specific guidelines for Forensic science laboratories' issued by National Accreditation Board for Testing Laboratories and NABL 113/113A: 2008 'Specific guidelines for Forensic DNA Profiling laboratories' issued by National Accreditation Board for Testing Laboratories for the service.

This document, Working Procedures Manual of Forensic DNA Profiling, describes the test procedures for DNA profiling of test items like blood, semen, bone etc. It has to be read together with the other documents mentioned in it and amendments to them, if any. The manual is a controlled document, controlled according to the document control procedure described in it and any amendments to them, if any.

This manual is a controlled document, controlled according to the document control procedure describe in it and any amendments thereto.

The authorized holders of the manual are responsible for keeping the manual regularly updated with the amendments. They are to ensure its ready accessibility for use by their co-workers in the laboratory for the proper operation of the quality system.

Holders of the controlled copies of the document are to return the same to the Quality Manager when there is no further requirement of the copy or when the holder ceases to be an employee of the laboratory.

This issue viz. issue no.04 of the manual is approved and issued for use by the Laboratory.

20/8/19

Director
Central Forensic Science Laboratory, Chandigarh.

LIST OF AUTHORIZED HOLDERS

The following are the authorized holders of the Working procedure Manual of Forensic DNA Profiling, Doc. No.0-CFSL,CHD/WPM/DNA

Sr. No.	Authorized holder	Issue No.	Copy No.
1.	DIRECTOR ,CFSL, CHANDIGARH	04	00
2.	HOD DNA DIVISION	04	01

Note: The controlled copy no.00 is the master copy mentioned by the Director with the concerned activity desk.

INTRODUCTION

DNA (deoxyribonucleic acid) is the building block of human body, virtually every cell contains DNA. The DNA in people's blood is same as the DNA in their saliva, skin, tissue, hair and bone etc. Importantly DNA does not change throughout a person's life. Genetic marker typing was limited to the analysis of blood group markers and polymorphic protein markers. Because the number of suitable markers expressed in particular fluids and tissues is relatively small, and because mixtures of fluids cannot be separated for conventional genetic marker typing, a suspect frequently cannot be included or excluded as a fluid donor in a case.

An important development in the field of Forensic science was the introduction of DNA typing systems for the analysis of biological samples. All chromosomes in human genome have variety of unique repeat regions that serve as a powerful investigative tool. Except identical twins, no two persons have the same DNA; therefore DNA evidence collected from the crime scene can be linked to suspect or can eliminate the suspect from suspicion.

Most of the crime laboratories have acquired vast experience in performing human DNA identification tests on various biological samples and taking these results into the courtroom for presentation to judges and juries. Advancement in development of methods to isolate DNA from nearly all biological specimens has greatly expanded the potential for individual identification. With advances and modifications to the basic PCR method and Multiplex PCR based Short Tandem Repeat (STRs) analysis of specific regions of DNA has emerged as a powerful tool for the criminal justice system. Various Multiplex PCR based kits utilize the polymerase chain reaction (PCR) to amplify regions of DNA known as short tandem repeats (STRs) in order to characterize DNA extracted from Forensic specimens. These Multiplex kits allow the simultaneous amplification of numerous STR loci as well as a portion of the Amelogenin gene located within the X and Y chromosomes. The amplified fragments are separated according to size by Capillary gel electrophoresis using the Genetic Analyzers, detected by laser excitation and the subsequent emission spectra are captured by a charged coupled device (CCD) camera. The resulting data is graphically displayed as colored peaks in relative fluorescent units (RFUs) and scan number. This display is called an electropherogram.

Forensic DNA fragment analysis has allowed investigators to successfully analyze evidence samples of limited quality and quantity. This enables the laboratory to generate the DNA profile which can be compared with the DNA profile from the suspect. A statistical calculation is applied to reflect how often one would expect to find this particular DNA profile in the general population. While the current core loci are sufficient for general Forensic matching of evidence to suspect, additional autosomal or Y Chromosomal STRs loci can be beneficial in variety of other human identity/relationship testing questions. In casework, additional information can be obtained from the degraded DNA sample using Mini STRs.

With its remarkable sensitivity and power of discrimination, DNA analysis has become a key figure in the field of Forensic Science, for identity and relationship testing such as missing person, mass disaster, complex paternity analysis, parentage testing with only one available parent, sexual assault, homicide, etc.

CHAPTER 1

DNA QUALITY CONTROL AND ASSURANCE

1.1 PERSONNEL QUALIFICATIONS AND TRAINING

All persons involved in the actual recovery, evaluation, analysis, and interpretation of DNA evidence shall have a background and training appropriate to the duties assigned.

1.2 EDUCATION AND GENERAL REQUIREMENTS

All Forensic Scientists in the DNA Division, Central/State Forensic Science Laboratories must possess minimum Master's degree in Zoology, Botany, Anthropology, human Biology, Forensic Science, Biotechnology, Microbiology or Biochemistry. The DNA Technical Manager must have minimum Master's degree in biology-related area and successfully completed Master's course work covering any of the following subject areas: biochemistry, genetics, molecular biology, and statistics or population genetics, and a minimum of two to three years' experience as a Forensic DNA Analyst. The DNA Technical Manager should have successfully completed NABL auditor training.

- The DNA Manager shall have, at a minimum, the general requirements listed above and shall have a minimum of three years' experience as a Forensic DNA analyst along with court testimony. It is also desirable that the DNA Manager have a working knowledge of computers, networks, and computer database management.
- A DNA analyst shall have a minimum, the general requirements listed above and shall have successfully completed course work (postgraduate level) covering the following subject areas: biochemistry, genetics, molecular biology; and course work and/or training in statistics and/or population genetics as it applies to Forensic DNA analysis.
- A DNA supervisor (Supervising Forensic Scientist) SSO (Scientist-B)/AD (Scientist-C) must meet the Forensic Laboratory requirements for a Forensic Scientist and have a minimum of three years of experience in Forensic Biology as a Forensic Scientist. Junior technical staff i.e. JSO/SSA must have three years full-time paid technical experience in a Forensic laboratory which includes two years performing analyses of physical evidence.

1.3 TRAINING AND QUALIFICATIONS

All Forensic Scientists should have completed training in the fundamentals of

Forensic science, including training in handling biological evidences. The chemical Analysis Training Program and STR Training Program must be accomplished in-house or with a combination of in-house work and outsourcing to the manufacturers. The Forensic Scientist must complete well-documented sets of serology screening training.

In addition, a Forensic Scientist is qualified to examine and identify biological evidence for DNA casework, the scientist must have demonstrated, either through successful completion of formal course work or through in-house training, knowledge in the following areas:

- Documentation and reporting procedures.
- Safe laboratory practices.
- Serology screening methodologies for various body fluids and mixtures of body fluids.
- DNA Quality Control and Quality Assurance systems and methods.
- Equipment operation, calibration and maintenance.
- Quantitative and qualitative evaluation of DNA test results.
- DNA case acceptance policies.
- Interpretation of electropherograms.
- Mixture interpretation training.
- Statistical interpretation of STR results.
- DNA trouble-shooting.
- Courtroom demeanor and court testimony (prior to testifying in court).

When an experienced Forensic Scientist is hired, the DNA Technical Manager/Adviser shall be responsible for assessing their previous training and ensuring it is adequate and documented. Modification(s) to the training program may be appropriate and shall be documented by the DNA Technical Manager/Adviser.

A laboratory technician is qualified to prepare reagents, and perform QC checks on reagents, instruments and DNA kits, the technician must complete training and demonstrate knowledge and competence in the following areas:

- Safe laboratory practices
- Documentation procedures
- DNA Quality Control and Quality Assurance systems and methods
- Equipment operation, calibration and maintenance

Upon completion of an approved training program, the Laboratory Manager or DNA Technical Manager will authorize the Lab technician to perform reagent

preparation and QC checks on reagents, instruments and DNA kits. The DNA Laboratory Technician will not process evidence or perform casework, and is designated as Laboratory Support Staff for the purposes of the DNA Quality Assurance Standards.

1.4 TRAINING RECORDS

Supervisors have the responsibility of routing documentation and maintaining of all trainings received for each analyst. The QA/QC Manager will forward the record to the Employee training records.

1.5 EXPERIENCE

The DNA Analyst must have a minimum of two year of Forensic DNA laboratory experience. Prior to any independent serology screening and reporting on casework samples, the Analyst must have a minimum of 6 months of Forensic Serology laboratory experience.

The casework DNA analyst or Serology Screener shall complete the analysis of a range of samples routinely encountered in Forensic casework prior to independent work using DNA technology and shall successfully complete a competency test before beginning independent analysis.

1.6 CONTINUING EDUCATION

DNA analysts, SA/SSA/JSO must be aware of developments within the field by reading current scientific literature and by attending seminars/workshops, professional meetings, or documented training sessions/classes in relevant subject areas at least once each calendar year. A document pertaining to training must be submitted by the trainee and should be reviewed by the Technical Manager/Adviser. Continuing education is required annually and shall be documented. Management must provide analysts with an opportunity to comply with the above.

1.7 FACILITIES

DNA laboratory should be secured and have access only to limited personnel. Each laboratory will have an evidence storage vault, locked refrigerators and/or freezers, and personal evidence lockers for the secure storage.

The DNA section of laboratory should have a room separate from the examination area in which evidence examinations, DNA extractions and PCR setup, amplified DNA product (including real-time PCR) shall occur in separate spaces, or at separate times. When robotic workstations are used to carry out DNA extraction, quantification, PCR setup, and/or amplification, a single room may be used provided that appropriate validation has been performed and documented. If the robot performs analysis through amplification, the robot shall be housed in a separate room from that used for initial evidence examinations.

Each DNA laboratory shall have and follow written procedures for cleaning and decontaminating facilities and equipment. The procedures of cleaning record shall be maintained in a DNA Lab Cleaning Logbook for the particular laboratory. The procedures for equipment maintenance including cleaning shall also be maintained.

1.8 CASEWORK EVIDENCE AND SAMPLE CONTROL

1.8.1 Receipt, Storage, and Handling of Evidence Submitted to The Laboratory

Evidence items shall be received, stored and handled in such a manner to ensure integrity by protecting from loss, cross contamination or deleterious changes.

1.8.2 Sample Labeling and Documentation

Each DNA test sample must be labeled with a unique identifier (i.e. laboratory number and if appropriate, an item number and/or item description) and should be documented along with photographs/videography.

1.8.3 Evidence and Test Sample Handling

Evidence items and test samples shall be handled in a manner to prevent loss, alteration, contamination, or mixing. Analysts must wear gloves while handling evidence and test samples both to preserve the integrity of the evidence and for personal protection. When working with limited evidence or low-level DNA samples (such as handler/touch/cellular cases), disposable sleeves and a mask or plexiglass shield must be employed.

Analysts must use disposable plugged pipette tips, discard tips after each use involving possible contact with DNA sample or controls and use quality-controlled reagents when necessary. All items prior to coming into contact with sample must be sufficiently cleaned to prevent DNA cross contamination. For example, sample-handling tools (such as scissors or scalpels) must be cleaned between the preparations of each sample. Evidence samples shall be prepared separately (time and/or space) from the known reference samples.

DNA samples should be stored under controlled conditions to maintain shelf-life and reduce or prevent the degradation of DNA (refrigeration or freezing for liquid extracts). Analyst should follow the guidelines regarding inadvertent deleterious changes (loss, mixing, contamination, spillage or dropped etc.) to DNA samples at any step and must record/document the corrective steps taken in the case file and the Laboratory Quality Log book. The DNA technical Manager should be notified as soon as possible after the time of incident.

1.8.4 Consumption of DNA Evidence and Limited Samples

Forensic Scientists should make every reasonable attempt to conserve sufficient DNA evidence or sample for retesting, but may need to use it all in their analysis depending on the amount of biological material available. For evidence items that cannot be visually assessed for quantity of biological material (i.e. cells left on a surface from touching or other contact) or insufficient biological sample, DNA extracts can be stored as per the guidelines for retesting. Each sample should be labeled with a unique identifier (i.e., laboratory number).

1.8.5 Destruction of DNA

All extracted and amplified convicted offender DNA should be destroyed upon completion of typing.

1.9 ANALYTICAL PROCEDURES

Standard operating procedures are followed for reagent preparation, processing of samples, extraction and analysis, controls and equipment maintenance.

1.9.1 Definitions

Chemicals – substances with a distinct molecular composition (e.g. ethanol, sodium chloride).

Reagents – substances prepared from chemicals or other reagents, or commercially available kits used in laboratory analysis that might not be considered standalone chemicals (e.g. TE, amplification kits).

1.9.2 Preparation of Reagents

Reagents will be prepared according to the instructions in the appropriate STR Analysis Procedure Manuals. The quantities and lot numbers of the materials used to prepare the reagents shall be recorded in the laboratory's reagent preparation log book. Each batch of reagents prepared will be uniquely identified and recorded in the laboratory's reagent log book.

1.9.3 Labeling of Reagents Used for DNA Testing

All in-house prepared reagents shall be labeled with the following:

- The identity of the reagent.
- The date of preparation.
- The batch of the reagent.
- The expiration date.
- The initials of the individual preparing the reagent.

At a minimum, solutions on the bench, referred to as "working solutions," need to have the reagent name, the batch identifier, and expiration date on the label.

Commercially prepared reagents shall be labeled with the following:

- The identity of the reagent.
- The expiration date, if one is provided by the manufacturer or determined by the laboratory.
- Critical reagents shall be evaluated prior to the use.

1.9.4 Standards and Controls

All controls, internal lane standards and allelic ladders required for a given procedure will be run and checked to verify that the expected results are obtained.

1.9.5 Comparison to Known Standards

Once a year, each laboratory conducting DNA testing shall type a minimum of a DNA standard reference material, or standards traceable to a standard, using the DNA typing technology and methodology in current use. The results of the testing will be documented and reported to the DNA Technical Manager. The DNA Technical Manager should check the genotypes for concordance. Any discrepancy between the laboratory's results and the published standards or traceable standards profiles should be investigated and a root cause will be determined. If a serious finding in the DNA analysis is found, all typing should be stopped in that laboratory until the problem is corrected. All previous records will be examined back to ascertain that the error was not occurring. Appropriate actions regarding non-conforming work will be followed.

If a new method is introduced or the existing method modified substantially, it shall not be utilized for casework testing until it has been tested using a DNA standard or traceable standard and the appropriate values are obtained.

1.9.6 Storage, Disposition and Destruction of Evidence and Work Products

Any item submitted for analysis to the Forensic laboratory by a law enforcement agency is considered evidence. Forensic analysis of evidence items will result in work products. This may include microscope slides, DNA extracts, DNA amplicons, bloodstained cards and stain cutting etc. Work products as a result of crime lab analysis are not considered evidence.

All evidence items for DNA testing will be stored in secure areas (personal lockers, locked refrigerators/freezers) until returned to the submitting agency. Historical work product such as DNA extracts, bloodstained cards (e.g. stains made from reference samples), stain cuttings, microscope slides etc. may be retained for possible cold cases and post-conviction DNA investigations. To maintain the integrity of some DNA analysis work products such as bloodstained cards and stain cuttings, it may be necessary to use the laboratory's evidence vault for storage due to suitable space limitations. Cuttings with no probative serology result and that are not required for DNA extraction may be dis-

carded.

DNA extract work product will be stored as specified in the STR Analysis Procedures manual. Prior to approval of the procedure for returning extracted DNA, any extracted DNA not utilized in analysis will typically be retained (stored frozen) by the laboratory after the analysis was conducted. If not, the disposition will be recorded in case notes. Extracted DNA from reference items shall be disposed, and the disposition will be recorded in the case notes. Reagent blanks from extractions where there is no remaining DNA may be discarded. All amplified DNA shall be discarded.

The case notes and report will specify which item(s) contain returned DNA extracts and/or other work products. Additionally, where possible, evidence packaging will be labeled (i.e. stickers or other method) to indicate that DNA work product is enclosed.

1.10 GUIDELINES FOR THE PROPER RECORDING OF ALL ANALYTICAL DATA

The following information will be recorded in the permanent case file of every case submitted for DNA analysis:

- Request for Laboratory Examination (RFLE) Form for casework samples.
- Documentation of examination for casework.
- Worksheets filled out appropriately to document the flow of the sample, including a sample description, extraction, quantification, amplification, and capillary electrophoresis (CE) analysis. The appropriate reagent information for all reagents used will also be included, such as testing dates, lot numbers, etc. to allow for traceability of materials used in analysis.
- Results of the estimation of the quantity of human DNA recovered.
- Analytical data (stored electronically) which includes as appropriate, digital photographs of evidence, any robot templates, 7500 SDS file(s), GeneMapper®ID files and projects, the raw data generated, appropriate matrices, size standard(s), allele table(s) (if desired).
- Additionally, it will be verified and recorded that all controls run with the samples gave expected results.
- Any population frequency calculations and/or data sheets.
- A draft of the laboratory report bearing the technical reviewer's initials and the date of review on each page.
- The final laboratory report.

All case files shall be reviewed by at least one other DNA analyst. An approved

peer review checklist will be used to facilitate the review process. This must be retained in the case file. Following the checklist will ensure adequate review of results at every step of analysis.

1.11 DATA HANDLING, STORAGE, AND RETRIEVAL

All casework documentation shall be maintained according to laboratory policy.

Electronic data will be stored as follows:

- All original instrument data (run folders generated by the CEs or SDS files generated by the 7500) should be backed up from the appropriate instrument computer hard drives to a Data Repository (DR) hard drive. At least once per quarter, the instrument data which has been backed up to the DR will be copied to removable media (e.g. CDR, DVDR or equivalent technology) and transferred to a separate secure location.
- Completed casework files shall be placed in a Completed Cases (or equivalent) folder on the DR. Casework in this folder will be backed up at least quarterly (or more often depending on the number and size of the completed cases) using the same methods as outlined above.
- When possible, the backing up of data from instruments and casework files should be completed using the highest quality removable media available, and should be stored in a manner to prevent data loss from that removable media.

Electronic file records, proficiencies, and QC records shall be backed up at least annually and stored at a separate secure location. DNA data should be entered into the DNA Data system from reviewed case files and stored in the system according to procedures and protocols as soon as conveniently possible.

DNA Laboratory validation studies, performance testing results and population study work results and write-ups shall be retained in the DNA section of each laboratory conducting DNA typing. After the initial creation of the work results and write-ups, it will be the responsibility of the DNA Technical Manager to decide what is added to or deleted from these files. The DNA supervisor or designee will have the responsibility of ensuring that the material is added to or removed from all the files in their laboratory.

1.12 EQUIPMENT CALIBRATION AND MAINTENANCE

An inventory of all critical equipment used in DNA testing shall be maintained according to laboratory policy. Instruments or equipment that requires calibration or performance testing prior to use and periodically thereafter are critical as they may affect DNA testing.

New analytical instruments, or instruments which have undergone repair/maintenance, will be performance tested before use in casework sample analysis.

Any time a piece of equipment requires service or maintenance, that fact will be documented on the appropriate service form. Instruments which are on routine service contracts with the manufacturer will have routine service calls documented. All documentation of service, routine or otherwise, will be maintained in the appropriate instrument maintenance log book.

For equipment requiring calibration (such as pipettes, CEs, thermal cyclers etc.), the calibration should be done using appropriate certified standards and will be documented in the appropriate equipment/instrument log book. A written procedure or set of instructions should be available for calibration of more complex instruments.

Some instruments will be calibrated routinely by certified external agencies (e.g., NIST traceable thermometers, balances, pipettes).The equipment/instrument maintenance procedure will clearly define the calibration source to be used and the frequency of calibration.

Each DNA section should establish acceptable temperature ranges for their temperature-controlled equipment. Temperature checks should be done on each working day as specified in the Calibration of Instruments. The acceptable temperature range for passing the checks can be established by reviewing the history of temperature ranges where the functional performance of the equipment was acceptable. These ranges should be stored in the same binder as the Temperature Control Record Monthly Log form.

1.13 REPORTS

Any analytical results released from the laboratory shall be contained in a formal written report, and these reports will be prepared in accordance with WPM of DNA Testing. Prior to issuance of the report, the DNA analyst assigned to the case must have all data and conclusions independently reviewed by a second DNA analyst. This technical review will be documented in the case file.

The following information shall be included in all reports:

- Case identifier
- Description of evidence examined
- A statement addressing non-examined items
- A description of methodology
- Loci tested (where appropriate)
- Results and conclusions

- Quantitative or qualitative interpretation statement
- Disposition of evidence
- Date issued
- A signature and title of the person(s) accepting responsibility for the content of the report.

1.14 REVIEW

In addition to the Review procedures outlined in the Quality Manual, a qualified person performing a DNA Casework review (typically at the administrative review level) shall also review the chain of custody of item(s) for that case. The reviewer shall access the analysis program to verify that the scientist had possession of the item(s). The chain of custody review shall be documented on the review checklist within the case file.

1.15 PROFICIENCY TESTING

Participation in a proficiency testing program is a major element of the Forensic Science Laboratory Quality Program. Regular proficiency testing is a critical element in the DNA Analysis QA Program. It is used to demonstrate the quality performance of the DNA functional area (FA) and its staff, as well as assuring that the testing procedure is working properly.

1.15.1 Open Proficiency Testing

The DNA Forensic Analyst participates in open proficiency testing programs with samples provided by outside organizations (such as Collaborative Testing Services).

1.15.2 Personnel

All staff members in the DNA FA who are involved in the serology testing and DNA typing of samples from criminal cases, are required to complete proficiency testing on a semi-annual (or 2x) basis each year. Newly qualified Forensic scientists shall enter the external proficiency testing program within six months of the date of their qualification. Other personnel designated by the DNA technical Manager to technically review outsourced DNA cases are also required to participate in the external proficiency test programs.

1.15.3 Frequency

Each DNA analyst and other personnel designated by the DNA technical Manager shall undergo external proficiency testing twice a year, one between January 1stto June 30th and the other between July 1stto December 31st, with the interval between the two being 4 to 8 months. The designated date for proficiency test tracking for each lab is as follows:

• Received date

- Submitted date (results faxed to manufacturer)
- Due date

1.15.4 Technology

The typing of all STR core loci is required for each proficiency test. The proficiency testing for YSTRs must be done on a semi-annual basis. Technical reviewers outsourced for DNA cases must be qualified in the technology, platform and typing amplification test kit used to generate the data.

1.15.5 Methodology

Forensic scientists qualified in both automated and manual methods at minimum will perform alternate analysis of proficiency tests between both analytical processes.

1.15.6 Serology Screening

For those individuals involved in Serology Screening for biological evidence and preparation of cell pellets for DNA analysis, the proficiency test will involve teaming with DNA analysts twice a year. Individuals involved in Serology Screening for biological evidence only shall undergo annual external proficiency testing.

1.15.7 Specimens

For those individuals responsible for DNA typing, the proficiency samples will consist of dried bloodstains or buccal samples. For those individuals involved in DNA analysis and/or Serology Screening on criminal casework, the proficiency samples will consist of dried specimens of blood and/or other physiological fluids either singly or in mixtures. For other individuals designated only for outsourced case technical review, the proficiency samples will consist of electronic data of analysis results from dried specimens of blood and/or other physiological fluids either singly or in mixtures. These proficiency samples will normally be presented as a case situation with specific questions to be answered.

1.15.8 Sample Preparation, Storage, and Distribution

All internally developed proficiency test specimens should be uniformly prepared using materials and methods that ensure their integrity and identity. Each set and specimen must be labeled with a unique identifier.

Each proficiency test shall be treated as a laboratory case and as such, have an associated request for Laboratory Examination (RFLE) and assigned a unique laboratory number. Samples should be stored in an appropriate manner for pending analysis. Proficiency tests shall be assigned to individuals in the DNA section by the section supervisor. Proficiency tests shall be assigned to others designated only for outsourced case technical review by the DNA technical

Manager/reporting officer.

1.15.9 Documentation of Proficiency Test Results

The analyst shall, without assistance and as appropriate to their role

- Conduct analysis.
- Interpret analytical data.
- Form conclusions (as appropriate).
- Write a report/fill in the proficiency forms and answer questions on the proficiency forms.

If an examiner has questions or requires assistance, the immediate supervisor should be contacted. The proficiency test case file should include the same information as is included with a casework file including electronic data.

The proficiency test results shall be added to the proficiency test case file. Records of discrepancies between the test results as well as corrective actions taken shall be documented and maintained in the proficiency test case file. The completed proficiency test case file will be maintained in each lab.

1.15.10 Review and Reporting of Proficiency Test Results

The case file shall undergo a technical peer review by a qualified analyst. An administrative review will also be completed by someone other than the individual conducting tests. Upon completion of a proficiency test, the case file shall be returned to the appropriate supervisor, who will ensure that all necessary reporting forms have been completed. A copy of the required reporting forms should be sent to the organization providing the test materials.

When test results are available from the manufacturer, the Quality Process Manager shall review all test materials, determine whether the results reported by the analyst are correct and send out a report. The DNA Technical Manager or designee also reviews the Manufacturer's Information Report relative to the results reported by the DNA analyst to assist the Quality Manager. The Quality Manager will promptly notify the employee taking the test of the results and a written summary of the results should be included in the file.

1.16 NON-CONFORMING WORK

Non-conforming work is defined by the DNA Quality Manual. There are two levels of non-conforming work: substantive (as defined in the DNA QM) and quality variance.

Quality variance incidents in the DNA functional area include but are not limited to: contamination (confirmed or unconfirmed), sample switching, inadvertent sample loss (due to dropping or spilling), and sample mixing. The analyst shall document the incident in the case file(s) and notify their section supervisor. Quality variance incidents will be documented. If identified during a testimony evaluation, the testimony evaluation form can be used and the DNA

Quality Manual policy followed. Quality variances identified otherwise will be documented using the Quality Variance Incident form and maintained in a Quality Variance Log for each DNA lab. Log entries shall include the case number(s), date of filling of the form, the type of incident, if contamination, the deduced source of the DNA or the interpretable allele calls, description of circumstances, whether noted in the report or not, action(s) taken, and the analyst and supervisor's initials. A copy of the form then is to be sent to the DNA Technical Manager/reporting officer for system monitoring.

The DNA Technical Manager/Reporting officer, or designee, shall review all quality variance log forms and assess the severity. For those events determined to be substantive non-conformances, the DNA QM policy regarding the Corrective Action Process for Non-Conforming work should be followed. All other events will be treated as minor variances and monitored, as appropriate. Should multiple minor events occur for a single individual or multiple similar minor events occur for a laboratory or across a laboratory system, the group of instances may rise to the level of a substantive non-conformance. Supervisors, or their designee, shall review their lab's quality variances quarterly and report the result to their staff either via email or at a unit meeting. The DNA Technical Manager /Reporting officer shall also be notified of the quarterly variances summary.

If an event appears potentially substantive, where a formal Corrective Action would be required, the DNA Technical Manager /SSO/AD shall be notified as soon as practically possible.

If no results can be reported due to a quality variance incident, the final laboratory report shall indicate the reason(s) why.

Unconfirmed contamination based on data below the allele reporting threshold shall be assessed by the DNA analyst, peer reviewer and supervisor on a case-by-case basis for possibility of trace DNA contamination, potential for impact on DNA profile interpretation and as to whether it is sufficiently significant to be noted in the report.

Corrective actions involving DNA analysis shall not be implemented without the documented approval of the DNA Technical Manager. The policies and procedures related to non-conforming work and corrective actions are outlined in the Quality Manual.

1.17 AUDITS

Scientific audits are an important aspect of the DNA reporting officer's QA Program. These are the independent reviews conducted to compare the DNA Forensic Analyst's performance with a standard for that performance.

The audit shall be conducted in accordance with the NABL, DNA QUALITY

ASSURANCE SYSTEM. The internal annual audits will be mandatory every calendar year. External audits will be conducted by National Accreditation Authority once in two years to re-issue the accreditation certification.

1.17.1 Objectives

There are two primary objectives of an audit:

- To provide management with an evaluation of the DNA LABORATORIES performance in meeting its quality assurance policies and objectives.
- To identify the areas in which the DNA LABORATORIES performance may be improved.

1.17.2 Types of DNA Functional Area Audits

Audits of the DNA FORENSIC ANALYST/REPORTING OFFICER/TECHNICAL MANAGER: The DNA Technical Manager/reporting officer conducts and documents an annual review of the DNA Quality Assurance Program.On a biennial basis, each DNA laboratory undergoes an external audit designed to determine the extent to which the section meets the DNA Quality Assurance Audit document.

The audit is conducted by a team comprised of at least two individuals from an external agency one of whom must have successfully completed the DNA Auditor Workshop and has been qualified in the specific DNA technology used in the DNA laboratory to be audited.

A record (including a summary report prepared by the DNA Technical Manager) of each external audit will be maintained and include the date of the inspection, the names of the members of the inspection team, the findings and problems identified by the team and remedial actions taken to resolve existing problems. Any findings of non-conforming work will be addressed through corrective action processes.

Each DNA laboratory undergoes an internal audit annually designed to determine the extent to which the section meets the DNA Quality Assurance Audit document.

The audit is conducted under the direction of the DNA Technical Manager by at least one qualified auditor and one qualified (or previous qualified) DNA Analyst from another DNA laboratory and/or the DNA Technical Manager.

A record (including a summary report prepared by the DNA Technical Manager) of each internal audit will be maintained and include the date of the inspection, the names of the members of the inspection team, the findings and problems identified by the team, and remedial action taken to resolve existing problems. Any findings of non-conforming work will be addressed through

corrective action processes.

The annual DNA audits (both internal and external) will be incorporated in the DNA quality internal audits and Management System Reviews.

1.17.3 Management System Review and Internal Quality Audits

The annual internal quality audit and Management System Review (MSR) are reviews of the laboratory's management and quality systems and testing activities to ensure continuing suitability and effectiveness. These reviews will be used as tools to introduce necessary changes or improvements by management.

The procedures for the annual internal quality audit and MSR are outlined in the DNA Quality Manual.

1.17.4 NABL Inspection

Each laboratory of the Crime Laboratory Division is accredited by the NABL Board/Laboratory Accreditation Board (NABL). As part of the accreditation process, all sections of each laboratory are inspected every two years to ensure that current NABL guidelines are being met. An NABL inspection fulfills the biennial external audit requirement.

CHAPTER 2

GENERAL GUIDELINES FOR DNA CASEWORK

2.1 LABORATORY ORGANIZATION

- To minimize the possibility for carry-over contamination, the laboratory should be organized in unidirectional manner so that the areas for DNA extraction, PCR set-up and handling amplified DNA are physically isolated from each other. These work areas should be in separate rooms.
- Each room or work area must have dedicated micro centrifuges, tube racks, refrigerators, storage areas, pipettes etc.
- Sterilization and bleaching is required in every work area in stipulated time period.
- Dedicated equipment should not leave their designated areas. Only the samples in designated racks should move between areas.
- Analysts in each work area must wear appropriate personal protective equipment (PPE). Also, contamination preventive equipment (CPE) must be worn where available. Signs may be posted to designate appropriate personal protective equipment (PPE) in certain areas.
- Exhibit storage & case opening areas should be separated from the analysis area.

2.2 WORK PLACE PREPARATION

- Apply 10% bleach and/or 70% Ethanol to the entire work surface, cap opener, pipettes (when appropriate).
- Obtain clean racks and cap openers, and irradiated micro centrifuge tubes, and irradiated water from storage. Arrange work place to minimize crossover contamination or to reduce the aerosols.
- Change gloves frequently and whenever gloves may have become contaminated. Discard gloves when leaving a work area, except when transporting samples or reagents. Place gloves nearby in order to facilitate frequent glove changes

2.3 LABORATORY WASTE DISPOSAL MANAGEMENT

- **Human blood, other potential infectious body fluids** and contaminated materials must be decontaminated prior to disposal solid waste refuse or as per the guidelines.

- **Laboratory waste from infectious agents** must be autoclaved prior to disposal. Chemical disinfection is generally suitable for contaminated and non-disposal equipment and consumables (pipettes, glass etc.).
- **Nucleic acid (natural & synthetic) containing material** must be decontaminated or inactivated before disposal. Chemical treatment with an appropriate disinfectant is an acceptable method.
- **Sharps** including glass, razor blades, needles and other objects that can penetrate the skin and potentially get contaminated with blood borne pathogens or others bio-hazardous materials must be accumulated in rigid, leak-proof, labeled, red color containers specifically designed for the purpose.
- **Pipette tips** contaminated with blood borne pathogens or others bio-hazardous materials must be decontaminated before disposal. Decontamination can be achieved by autoclaving or chemical treatment with the appropriate disinfectant.
- Universal biohazard symbol should be visible on each container.

2.4 MICRO CENTRIFUGE TUBES AND PIPETTE HANDLING

- Pre-sterilized, polypropylene, DNase and RNase free, autoclavable, micro centrifuge tubes, Microcon collection tubes must be used.
- Avoid splashes and aerosols. Centrifuge all liquid to the bottom of a closed micro centrifuge tube before opening it.
- Avoid touching the inside surface of the tube caps with pipettes, gloves or lab coat sleeves.
- Use the correct pipette for the volume to be pipetted. For pipettes with a maximum volume of 20µL or over, the range begins at 10% of its maximum volume (i.e. a 100µL pipette can be used for volumes of 10-100µL). For pipettes with a maximum volume of 10µL or under, the range begins at 5% of its maximum volume (i.e., a 10µL pipette can be used for volumes of 0.5-10µL).
- Filter pipette tips must be used when pipetting DNA and for other reagents. Use the appropriate size filter tips for the different pipettes. The tip of the pipette should never touch the filter. Always change pipette tips between handling each sample.
- Never “blow out” the last bit of sample from a pipette. Blowing out increases the potential for aerosols, this may contaminate a sample with DNA from other samples. The accuracy of liquid volume delivered is not critical enough to justify blowing out.
- Discard pipette tips if they accidentally touch the bench paper or any other surface.

- Wipe the outside of the pipette with 70% ethanol solution if the barrel goes inside a tube.

2.5 SAMPLE HANDLING

- Samples that have not yet been amplified should never come in contact with equipment in the amplified DNA work area. Samples that have been amplified should never come in contact with equipment in the unamplified work area.
- The DNA extraction and PCR setup of evidence samples should be performed at a separate time from the DNA extraction and PCR setup of reference samples. This precaution helps to prevent potential cross-contamination between evidence samples and reference samples.
- Use disposable bench paper to prevent the accumulation of human DNA on permanent work surfaces. 10% bleach followed by 70% ethanol should always be used to decontaminate all work surfaces before and after each procedure.
- Limit the quantity of samples handled in a single run to a manageable number. This precaution will reduce the risk of sample mix-up and the potential for sample-to-sample contamination.
- Change gloves frequently to avoid sample-to-sample contamination. Change them whenever they might have been contaminated with DNA and whenever exiting a sample handling area.
- Make sure the necessary documentation is completely filled out, and that the analyst's ID is properly associated with the documents.

2.6 BODY FLUID IDENTIFICATION

- The general laboratory policy is to identify the stain type (i.e., blood, semen, or saliva) before individualization is attempted on serious cases such as sexual assaults, homicides, robberies, and assaults.
- Maximum and minimum detection range of body fluids for preliminary examination and confirmatory examination should be known.

2.7 DNA EXTRACTION GUIDELINES

Slightly different extraction procedures may be required for each type of specimen. Due to the varied nature of evidence samples, the user may need to modify procedures.

- All tube set-ups must be witnessed/ confirmed prior to starting the extraction (**NOTE:** For differential extractions, the tube set-up should be witnessed after the incubation step.)
- Use Kim wipes or a tube opener to open tubes containing samples,

only one tube should be uncapped at a time.

- When pouring or pipetting Chelex solutions, the resin beads must be distributed evenly in solution. This can be achieved by shaking or vortexing the tubes containing the Chelex stock solution before aliquoting.
- For pipetting Chelex, the pipette tip used must have a relatively large bore i.e. 1 mL pipette tips are adequate.
- Be aware of small particles of fabric, which may cling to the outside of tubes.
- For DNA examination, extraction negative control must be included with each batch of extractions to demonstrate extraction integrity.
- The extraction negative control contains all solutions used in the extraction process but no biological fluid or sample. For samples that will be amplified, the associated extraction negative controls should be re-quantified to confirm any quantification value of 0.2 pg/μL or greater.
- Sensitivity tests for PCR kits and instruments should be performed in the laboratory as per SWGDAM guidelines.
- After extraction, the tubes containing the unamplified DNA should be transferred to a box and stored in the appropriate refrigerator or freezer. The tubes should not be stored in the extraction racks.
- All tubes must have complete case number, sample identifier and Analyst's initials on the side of the tube. This includes aliquots submitted for quantification.

2.8 CONTROLS FOR PCR ANALYSIS

The following controls must be processed alongside the sample analysis:

- A positive control is a DNA sample where the STR alleles for the relevant STR loci are known. The positive control tests the success and the specificity of the amplification, and during the detection and analysis stage the correct allele calling by the software.
- An extraction negative control consists of all reagents used in the extraction process and is necessary to detect DNA contamination of these reagents.

  Note: Since the Y STR system only detects male DNA, one cannot infer the absence of female DNA from a clean Y STR extraction negative control. Therefore, an extraction negative control originally typed in Y STRs must be retested if the samples are to be amplified for autosomal STRs.
- Samples that were extracted together should all be amplified together, so that every sample must run parallel to its associated ex-

traction negative control.

- An amplification negative control consists of only amplification reagents without the addition of DNA, and is used to detect DNA contamination of the amplification reagents.

Failure of any of the controls does not automatically invalidate the test. Under certain circumstances it is acceptable to retest negative and positive controls. **See STR Results Interpretation Procedure for rules on retesting of control samples.**

2.9 CONCORDANT ANALYSES AND "DUPLICATE RULE"

The general laboratory policy is to confirm DNA results either by having concordant DNA results within a case by duplicating the DNA results with a separate aliquot, amplification, and electrophoresis plate. The most common situations are confirmation of a match or exclusion within a case and repeating DNA testing when a low amount of DNA is amplified. Concordant and duplicate analyses are also used to detect sample mix-up and confirm the presence of DNA mixtures.

2.9.1 For evidence samples, the following guidelines apply:

- Identical DNA profiles between at least two items-two evidence samples or one evidence sample plus a reference within a case are considered internally concordant results ("duplicate rule").
- If a sample does not match with any other sample in the case, it must be duplicated by a second amplification. If result was obtained using Y-STRs only, this must be duplicated in the Y system.
- If after the first DNA analysis, there is an indication that the sample consists of a mixture of DNA, several scenarios must be considered. Further analysis steps have to be decided based on the nature of each case. Consult with supervisor if encounter a situation that is not represented in the following examples:
    - If all alleles in a mixture are consistent with coming from any of the known or unknown samples in the case, e.g. a victim and a semen source, no further concordance testing is needed.
    - If two or more mixtures in a case are consistent with each other and display the same allele combinations, they are considered duplicated.
    - If one or more alleles cannot be accounted for by other contributors in the case, the presence of the foreign component must be confirmed by a second amplification.
    - If there is only one sample in a case and this happens to be a mixed sample, the results need to be confirmed by a second

amplification.

- Inconclusive samples (as defined in the STR Results Interpretation Procedure) that cannot be used for comparison do not require duplication.
- Another method to satisfy this policy is if two different kits with overlapping loci are used. At least two (2) autosomal loci must be duplicated to confirm results. (For example, using Identifiler/ Mini-Filer on the same evidence sample).

2.9.2 For reference samples, duplication is designed to rule out false exclusions based on sample mix-up, and also to streamline testing. Duplication must start with a second independent extraction, with the reference submitted for extraction at a different time. The two resulting extracts must be aliquoted for amplification separately at different times and aliquoted for electrophoresis separately and run on separate plates. If there is no additional reference material available for extraction, the duplication may begin at the amplification stage. To streamline testing, all suspect and victim references may be duplicated.

2.9.3 The following guidelines apply for required duplications:

- If the DNA profile of a victim's reference does not match any of the DNA profiles of evidence samples in the case, including mixtures, the victim's reference must be duplicated to eliminate the possibility of a reference mix-up. This is because it is highly likely that a reference mix-up would generate a false exclusion.
- Duplication of a victim's DNA profile is not necessary in a negative case (no alleles detected in evidence samples).
- Since duplicate reference analyses are performed to confirm the exclusion, a partial DNA profile (at least one complete locus) that demonstrates exclusion is sufficient.
- If the DNA profile of a victim's reference matches any of the DNA profiles of evidence in the case, or is present in a mixture, the reference does not need to be duplicated. This is because it is highly unlikely that a sample mix-up would generate a false inclusion.
- Non-victim elimination references (such as consensual partners, homeowners, business employees) will not be routinely duplicated. Duplication may be performed for specific cases, if necessary.
- If the DNA profile of a suspect's reference does not match any of the DNA profiles in the case, the reference does not have to be duplicated.
- If the DNA profile of a suspect's reference matches any of the DNA profiles in the case, or in the local database, the suspect's ref-

erence has to be duplicated to eliminate the possibility of a reference mix-up. This is meant to streamline the process.

- Pseudo references do not have to be duplicated, regardless if the DNA profile matches any of the DNA profiles in the case.

2.9.4 For evidence samples or reference samples analyzed in DNA systems containing overlapping loci, the DNA results for the overlapping loci must be consistent. If no or partial results were obtained for some of the overlapping loci, this amplification is still valid if consistent results were obtained for at least one overlapping locus (Amelogenin is not considered an overlapping locus in this context). If the partial amplification confirms match or exclusion of a reference or another evidence sample, it does not have to be repeated.

2.9.5 Partial profiles can satisfy the duplication policy. Consistent DNA typing results from at least one overlapping locus in a different amplification is considered a concordant analysis.

2.9.6 For Y-STR testing, the sample does not have to be re amplified if the internal duplication rule applies or if the Y-STR results are concordant with the autosomal results i.e. confirming an exclusion or inclusion, confirming the presence of male DNA. Based on the case scenario it might be necessary to re amplify in order to confirm the exact Y-STR allele calls if there might not be sufficient autosomal data to establish concordance.

2.10 EXOGENOUS DNA POLICY

Exogenous DNA is defined as the addition of DNA/biological fluid to evidence or controls subsequent to the crime. Sources of exogenous DNA could be first responders, police personnel, crime scene technicians, Medical Technician or laboratory personnel.

- Decontamination of materials is the first priority. Steps should be taken to minimize exogenous DNA as much as possible.

- The source of any exogenous DNA should be identified so that samples can be properly interpreted. It may be possible to identify the source by:
    - Examining other samples from the same batch for similar occurrences.
    - Examining samples from different batches, handled or processed at approximately the same time for possible similar occurrences (such as from dirty equipment or surfaces).
    - Processing elimination samples to look for exogenous DNA

occurring in the field or by laboratory personnel.

- Samples should be routinely compared to case specific elimination samples and personnel databases for possible matches. Mixtures may have to be manually compared. If a negative or positive control contains exogenous DNA, all the associated samples are deemed inconclusive and their alleles should not listed in the report. The samples should be re-extracted or re-amplified, if possible.

- If clean results cannot be obtained or the sample cannot be repeated then the summary section of the reports should state **"The following sample(s) cannot be used for comparison due to quality control reasons."**
- Once exogenous DNA has been discovered, the first step is to try to find an alternate sample.
- As appropriate, a new extraction, amplification, or electrophoresis of the same sample can serve as an alternate for the affected sample. For this type of alternate sample, the discovery of exogenous DNA is not noted in the report. However, all case notes related to the discovery of exogenous DNA are retained in the case file for review by the quality assurance group, forensic biology staff and experts. A form is created that identifies the source of the exogenous DNA by Lab ID Number, if known, and stating which samples were affected.
- If there are other samples from the crime scene which would serve the same purpose, they could be used as an alternate sample. For example, in a blood trail or a blood spatter, another sample from the same source should be used. Another swab or underwear cutting should be used for a sexual assault or If a sample has a single source of DNA and this DNA appears to be exogenous DNA or If a sample contains a mixture of DNA and all of the alleles from the source of the exogenous DNA appear in the mixture In this scenario, the sample containing the exogenous DNA should be listed in the summary section of the report as follows: "**The [sample] cannot be used for comparison because it appears to contain DNA consistent with a police personnel, crime scene technicians, Medical Technician or laboratory personnel. Instead please see [alternate sample] for comparison".** Names for the possible source(s) of the exogenous DNA should not be listed in the report. All case notes related to the event should be retained in the case file for review by attorneys and their experts. A form is created that identifies the source of the exogenous DNA by Lab Type ID Num-

ber, if known, and stating which samples were affected.

2.11 UNRESOLVED DISCREPANCIES

Valid differences of opinions or disputes concerning the interpretation of results may occur. If differences of opinion cannot be resolved by the analyst and/or supervisor, then the Technical Manager will be the final arbitrator. The "Unresolved Discrepancies Documentation" form shall be filled out and placed in the relevant associated case files.

2.12 DNA STORAGE

- Store evidence and unamplified DNA in a separate refrigerator or freezer from the amplified DNA.
- During analysis, all evidence, unamplified DNA, and amplified DNA should be store refrigerated or frozen. Freezing is generally better for long term storage.
- Amplified DNA is discarded after the Genotyper analysis is completed.
- DNA extracts should be retained refrigerated for a period of time, and then frozen for long-term storage.

CHAPTER 3

STERILIZATION AND SAMPLE PROCESSING

3.1 INTRODUCTION

All Forensic Biologists and DNA analysts must follow contamination prevention guidelines. The purpose of this is to prevent unwanted DNA from entering a sample. The supervisor through the laboratory director is responsible for ensuring this policy is followed. There are many sources of contamination such as: aerosols, liquids or dry flakes/dust, unclean tools, unclean gloves, and contaminating materials on lab coats. There are also laboratory areas that are more at risk for the introduction of unwanted DNA such as evidence examination areas and extraction areas. The laboratory should be designed to minimize the risk of contamination. Proper precautions must be taken to reduce the risk of contamination.

3.2 SAFETY CONSIDERATION

3.2.1 Reducing the Risk by Design

In general terms, segregation between activities is the single most effective measure for avoidance of contamination. For the DNA processes these include the following processes:

- For processing of casework and reference samples, dedicated areas, equipment should be assigned.
- For pre-PCR and post-PCR, dedicated laboratories and equipment should be used.
- Extracted, amplified DNA should be stored separately from reagents and kits in separate refrigerators and Freezers.
- Entry to DNA laboratory area should be restricted.

Warning: Treat all reagents/samples as potential biohazards.

3.2.2 Personal Protective Equipment (PPE)

Gloves must be worn whenever an individual is handling equipment or instruments that are used for casework within the Forensic DNA laboratory. Forensic DNA Analysts must wear a mask, gloves and disposable lab coat or reusable lab coat with disposable sleeve covers while examining all items of evidence. The gloves must either be sterile or after putting the gloves on, they must be bleached with 10% bleach and dried. Gloves must be changed or bleached between exhibits. Gloves must also be changed after handling non-evidence items prior to returning to casework. These non-evidence items may include but are not limited to refrigerators/freezers, biohazard waste bins, equipment, computers, and telephones. Gloves should be changed often.

3.2.3 Personal Protective Equipment (PPE) in POST-PCR

Disposable or designated lab coats must be worn in the post-PCR rooms and these lab coats should be worn only in the post-PCR rooms. Non-disposable lab coats should be removed from the laboratory for cleaning in a closed container.

3.2.4 Preparation of Work surfaces

Prepare10% Bleach Solution and use Laboratory disinfectant to clean the work surfaces.

3.3 PROCEDURES OR ANALYSIS

3.3.1 General Information

- Analysts should avoid taking phone calls when working in the laboratory. Conversations between laboratory personnel should be kept at a minimum when an analyst is working with evidence samples.
- Tube openers should be used to uncap tubes (micro centrifuge tubes, Microcons, etc.). The tubes must be centrifuged prior to opening. Open only one tube at a time. Tube openers should be cleaned in a 10% bleach solution after each use.
- Each analyst must use individual mini-stocks or aliquots of each reagent. These mini-stocks may not be shared between analysts. Mini-stocks may be replenished from the large stock solution.
- Do not pipette from a stock reagent bottle. Reagents must be poured from the stock reagent bottle into a disposable beaker. When finished, discard the beaker and its contents. All stock reagents must be closed when processing stains for extraction.
- Exhibits will be processed one at a time. Only one exhibit will be opened at a time.
- Unknown samples must be processed first and separately from standards throughout the Screening and DNA processes.

3.3.2 Decontamination

- Decontaminate the surface on which samples are to be processed with a 10% bleach solution. Ensure the surface is dry before examining evidence. Make a new bleach solution daily.
- All the equipment/tools which will be used to process forensic samples (e.g., forceps, scissors, centrifuge rotors, bone cutting equipment and pipettes) must be decontaminated by autoclaving or rinsing with a 10% bleach solution. In addition, these items may be placed under an ultraviolet (UV) light source for at least 15 minutes.
- Use a 10% bleach solution to rinse or wipe tools between samples.

Tools may be rinsed with distilled water. After rinsing with a 10% bleach solution, use a disposable cloth/tissue paper to dry.

- Fresh paper must be used for each exhibit throughout the screening process. The work area must be bleached with a 10% bleach solution between each exhibit. The only exception to this procedure is during the examination of exhibits from a Sexual Assault (SA) kit.

3.3.3 Sample Processing - DNA Extraction

- The process of examining DNA evidence, cutting samples for DNA extraction and adding reagents shall take place in bio-hood, if available. The bio-hood provides a clean environment for DNA extraction and must have a UV light. It must be cleaned thoroughly with a 10% bleach solution. If bio-hood is not available, the analyst may process samples on a freshly bleached bench top.
- A separate extraction process can be accomplished as follows:
    - Extraction of standards and unknown samples can be conducted on different days or at different times.
    - Extraction of standards and unknown samples can be conducted in different bio-hoods.

  If samples are to be processed on the same day using the same hood, the analyst must keep the unknown samples and their blank(s) in their own rack and process them first. The standard samples and their blank(s) will be kept in their own rack and processed after the unknowns throughout the analysis, when applicable.
- The order in which the samples are extracted and processed must be clearly documented in the case notes. This documentation must show the separate handling of the unknown(s) and their blank(s) from the standards and their blank(s). This can be accomplished by listing the samples in the order of extraction in the case notes along with the date on which the extraction started.
- For each extraction protocol followed, all the reagents without any sample must be processed as a reagent blank. Reagent blank must be processed with each set of unknown samples and each set of standard samples (separate blanks). Unknowns from different cases may be batched together with one reagent blank for each extraction protocol used. Standards from different cases may also be batched together with one reagent blank.
- Exhibits will be processed one at a time. Only one exhibit will be opened at a time. Unknowns must be processed first and separately

from standards throughout the DNA analysis. Unknowns will be cut, placed into tubes and placed into their own samples rack. A reagent blank for the unknown samples should be added to the unknown samples batch. After the unknowns have been processed, the hood will be thoroughly bleached. The standards can then be cut, placed into tubes and placed into their own samples rack. A reagent blank for the known samples will then be added to the standard samples batch.

3.2.4 Amplification Set-Up

- All amplifications must be set up in designated PCR work station. If the face shield on the hood does not shield the analyst's face, the analyst must wear a face mask during amplification set up.
- Pipettes dedicated for amplification set-up must be used.
- The PCR set-up work station must be cleaned thoroughly with a 10% bleach solution or absolute ethanol. Any tubes that will be used in amplification set-up must be exposed to 30 minutes of UV light prior to their use or sterilized tubes should be used.
- One master mix of amplification chemicals can be used for both unknown and standard samples. The amplification chemicals can be added to tubes in the rack of unknown samples and the tubes in the rack of standard samples. Close all tubes. Unknown samples and their blank(s) will continue to be placed within their own rack and processed prior to the standard samples and their blank(s). The positive and negative amplification controls will be setup in the last.
- All samples can be amplified at the same time using the same thermal cycler. Do not set the tube rack down in the post-amplification room while transferring samples to the thermal cycler. If the rack is set down, it must be bleached with a 10% bleach solution before being used in the main laboratory again.

3.2.5 Post-Amplification

- After working with amplified DNA, an analyst must not work with any other biology or non-amplified evidence.
- The door to the post-amplification room must remain closed.
- The sample preparation hood and pipettes must be cleaned with a 10% bleach solution or absolute ethanol before and after the samples are prepared.
- The unknown samples and their blank(s) should be prepared for injection first and separately from the standards and their blank(s), when applicable.

3.2.6 Sample Control

The laboratory shall have and follow a documented sample inventory control system to ensure the integrity of known samples. This system shall ensure that:

- Known and casework reference samples shall be marked with a unique identifier or the laboratory shall have and follow a method to distinguish each sample throughout the processing (such as plate or rack mapping) that may not require the assignment of unique identifiers.
- Documentation of sample identity, collection, receipt, storage, and disposition shall be maintained.
- If the laboratory is processing known or casework reference sample(s) as evidence, a chain of custody shall be documented and maintained in hard or electronic format. The chain of custody shall include the signature, initials or electronic equivalent of each individual receiving or transferring the known or casework reference sample(s), the corresponding date for each transfer and the known or casework reference sample(s) transferred.
- The laboratory shall have and follow documented procedures designed to minimize loss, contamination, and/or deleterious change of samples and work product in progress.
- The laboratory shall have secure areas for sample storage including environmental control systems consistent with the form or nature of the sample.

Note: Wherever possible, the laboratory shall retain the isolated DNA sample for re-testing for quality assurance and sample confirmation purposes.

3.3 MATERIALS AND METHODS

3.3.1 Bloodstained fabrics

Bloodstains are generally found on fabrics which may be wet or dry depending on the time at which they were obtained. To analyze these stains in the laboratory without disturbing its originality, certain steps are followed: -

- The exhibits along with the tag should be photographed with scale.
- The exhibits must be opened serially based on their labels.
- The stain on the fabric will then be marked and labeled with the case number and the date of opening the exhibit.
- The stained area should then be cut into piece/ pieces (depending on the area of stain) of 1-2 cm with a sterilized scissor.
- The cuttings should be packed into paper envelopes and labeled with the case number and the date on which the case was opened.

3.3.2 Liquid Blood

- Photograph of the vial containing blood should be taken with the tag of information and also after opening of vial. Condition of the blood should be noted and verified with authentication form.
- Blood from the vial will be taken with the help of the syringe in micro centrifuge tube and also on the gauge kept in labeled petriplate and then allowed to dry for further examination purpose.
- A small cutting of the gauge can then be taken for analysis.

3.3.3 Hair

Hair samples should be handled carefully as these are thin and their size and shape vary depending upon the part of the body they are obtained from. Also, hair samples may or may not have roots. It must be ensured that while handling such samples the roots do not get removed as they are of much importance. Hair samples are generally handled with forceps. The method followed is that: -

- The exhibits along with the tag should be photographed.
- Any foreign material such as blood found on the hair sample should be first removed.
- The hair sample should then be observed for the presence of roots and cut into pieces depending upon its length.
- If roots are present, these should be cut along with the shaft.
- The cut pieces should be packed into paper envelopes and labeled with the case number and the date on which the case was opened.

3.3.4 Cigarette and Cigar butts

Cigarettes obtained as an exhibit may be either fully smoked or half smoked. The method followed is: -

- The exhibits along with the tag should be photographed.
- Any foreign material present on the sample should be removed and secured separately.
- The area to be cut is marked and labeled.
- The area that may have saliva should be cut and separated from the rest of the portion.
- The cuttings should be packed into paper envelopes and labeled with the case number and the date on which the case was opened.
- Number of individual butts should be sampled separately.

3.3.5 Swabs

Swabs of cotton may be a part of exhibit with stains of blood, semen or vaginal secretions on it.

- The swab should be held in one hand with sterilized forceps and the

required portion is then cut into small pieces with a sterilized scissor.

- Individual swabs should be sampled separately.
- The cuttings should be packed into different paper envelopes for different tests and these packets should be labeled with the case number and the date on which the case was opened.

3.3.6 Bones

Bones may be wet or dry and may have lots of foreign materials on them. Also, the method to be followed depends on the kind of bone as the bone can be a long bone or a short/soft bone.

3.3.6.1 **For Long bones**- femur, spinal cord etc.

- The bones should first be treated with 10% NaOH solution for few minutes.
- It is then cleaned properly with bleaching solution.
- The area of consideration is then marked and labeled with a marker.
- The sample is then wrapped in aluminum foil paper to remove its contact with air which is then labeled with the case number and the date on which the case was opened.
- The area under consideration or the exposed portion is drilled and powdered form of a bone is obtained.

3.3.6.2 **For short/soft bones**- carpel, meta-carpel, ribs etc.

- The bones should be treated with 10% NaOH solution and then bleached with bleaching solution.
- The area under consideration is marked and labeled.
- In case of soft bones, the bones are cut with sterilized scissor into small pieces.
- The pieces are then put into the paper envelopes which are labeled with the case number and the date on which the case was opened.

3.3.7 Tooth

Single tooth or number of teeth may be obtained as an exhibit to be examined. Generally molar teeth should be preferred over others for the examination. Tooth should not be examined directly but should first be drilled into small pieces. For such purpose either drill machine or modern techniques like tissue lysers are used.

- The tooth sample is washed off with the washing buffer for half an hour to remove any foreign particles like food material in case of fresh tooth and other particles like dust in case of dry and old sam-

ples.

- The samples are then air dried for few minutes and packed in the paper envelopes which are labeled with the case number and the date on which the case was opened.
- The samples are now ready to be lysed. The sample is either drilled with the drill machine into the powder form or can be processed in automatic tissue lyser machine.
- The powdered form is collected on the aluminum foil and is properly packed to remove its contact with the air and labeled with the case number and the date on which the case was opened.

3.3.8 Bricks or stones

At times bricks and stones are received as an exhibit with blood stains on it that may be either dry or wet.

3.3.8.1 If the brick has a wet stain:

- The stained area should be marked and labeled with the case number and the date of case opening along with the sample number.
- Swab the stained surface of the brick with a clean and sterilized cotton bud.
- The cotton bud is then preserved carefully.

3.3.8.2 If the brick has a dry stain:

- The stained area should be marked and labeled with the case number and the date of opening the case along with the sample number.
- Swab the stained surface of the brick with a clean, sterilized and slightly wet cotton bud.
- The cotton bud is then air dried and preserved carefully.

3.3.9 Nail clippings

Nail clippings are important source of evidence as they may contain the skin scrapings of the accused which may be transferred due to retaliation by the victim. They may also have other biological samples such as blood, hair, dust etc. which may be necessary to relate a criminal to the crime as well as victim. Nail clippings are generally useful in foreign DNA detection.

- The nail scrapings should be first photographed properly.
- These are then cut into small pieces of 1-2mm and packed into different envelopes for different examinations.
- The packets are then labeled with the case number and the date on which the case was opened.

3.3.10 Weapons

In case a weapon is received as an exhibit, then

- Photograph of the exhibit should be taken with the tag.
- The blood-stained area is swabbed by moistening the sample collecting device with Milli-Q water.
- Photograph the weapon after swabbing also.
- The swab must be air dried and then placed in its case.

3.3.11 Tissue samples

3.3.11.1 Tissues with Formalin: Sometimes tissues are preserved in formalin and stored at lower temperature so that its originality is not disturbed. Such a sample follows a different method.

- The sample is washed several times with distilled water before the processing.
- To remove the fixative, tissue sample is then washed twice with a PBS solution for few minutes.
- The sample is then immersed in 70% alcohol followed by absolute alcohol.
- The sample is then air dried.
- Dried sample is cut into small pieces for performing different tests.

3.3.11.2 Tissues embedded with Paraffin:

Tissues with paraffin are washed with distilled water.

3.3.11.3 Normal Tissue

- At the time of processing, small cutting of the sample (10mg) should be taken in the petriplate, cleaned and taken into microcentrifuge tube for further processing.
- All steps should be performed in a bio-safety cabinet.

CHAPTER 4
PRESUMPTIVE AND CONFIRMATORY TESTS

4.1 INITIAL EVIDENCE EXAMINATION

In cases where the stains are not readily visible, the evidence samples should be examined by exposing to an alternate light source in order to locate biological stains. When body fluid stains are identified and preserved, a substrate control sample should be taken from an apparently unstained portion of the item, when appropriate.

4.2 BIOLOGICAL FLUID EXAMINATION

Biological fluid identification is accomplished by using presumptive and confirmatory tests. Presumptive testing is used to indicate possible presence of biological fluids such as blood, semen, saliva, etc. in stains and swabs. Testing is not limited to preliminary analysis only. In cases where positive results for preliminary tests are obtained, identification of stains is confirmed using microscopic examination.

4.3 DETECTION OF BLOOD

4.3.1 Tetramethylbenzidine (TMB) Test

Introduction

The tetramethylbenzidine test (TMB) is a presumptive chemical test for identification of blood and is based on the peroxidase like activity of heme portion of hemoglobin. This test is very sensitive, but not specific. If the test is positive, confirmatory testing can be performed for conclusive identification.

REAGENT 1

TMB Reagent:

3,3,5,5'- tetramethylbenzidine	0.25 gm
Glacial Acetic Acid	25 ml

Store the reagent in a dark colored bottle in refrigerator.

REAGENT 2

3% Hydrogen peroxide (H_2O_2)

30% H_2O_2 (SIGMA)	1 ml
Distilled water	9 ml

Mix H_2O_2 with water and store the reagent in the refrigerator.

4.3.2 Quality Control:

- Before using the TMB reagent and H_2O_2 on evidence samples, test the reagents with a known blood sample (positive control) and a clean piece of filter paper (negative control). If the known blood sample gives a positive result and piece of filter paper gives a negative result, the TMB and H_2O_2 may

be used.

- If either control does not give the expected result, do not proceed with testing evidence samples until the problem has been resolved. Document the results of the control in the lab worksheet.

4.3.3 Procedure:

- Moisten the swab with distilled water or phosphate buffer saline and rub on the stain. For small samples, place few fibers on the filter paper.
- Add few drops of TMB reagent to the swab or filter paper. Wait for a few seconds while carefully observing to ensure that no color change develops at this time.
- Add a few drops of 3% H_2O_2 and wait for up to 15 sec for a color change.

4.3.4 Interpretation of results:

- An almost immediate appearance of greenish blue color on the addition of H_2O_2 indicates a presumptive positive result for the presence of blood. If swab or paper does not turn greenish blue within 15 sec, it is a negative result.
- Positive reaction - immediate blue green color
- Negative reaction - no color change
- Inconclusive reaction - development of color combinations other than those specified for a positive reaction, including one test positive and other negative.

4.3.5 Reporting results:

- Report positive test result as "blood was indicated…."
- Report negative test result as "No blood was indicated…"
- Report inconclusive test result as "tests for blood are inconclusive…"

4.4 DETECTION OF SEMEN

4.4.1 Acid Phosphatase Test

Introduction

Acid phosphatase is an enzyme secreted by the prostate gland that is present in large amounts in seminal fluid. It is not unique to the prostate and can be found in other biological fluids including vaginal secretions. It is therefore considered a presumptive chemical test for the presence of semen and it must be confirmed by other means (sperm detection under microscope, PSA or DNA extraction).

Reagents

Acid Phosphatase (AP) buffer

Glacial Acetic Acid	1.0 ml
Sodium acetate (anhydrous)	2.0 gm
Distilled water	100 ml

Mix the reagents thoroughly until dissolved.

Reagent I (Sodium α naphthyl acid Phosphatase solution)

Sodium α naphthyl acid phosphatase	126 mg
Acid phosphatase buffer	50 ml

Mix the reagents thoroughly until dissolved.

Reagent II (Dye solution)

Ortho- dianisidine or napthanildiazo blue B	250 mg
Acid Phosphatase buffer	50 ml

Add in an appropriate labeled test tube or bottle until thoroughly dissolved.

NOTE: Protect from light by covering tube with foil or use a brown bottle.

4.4.2 Quality Control:

- Before using AP reagents on evidence samples, test the reagents with a positive control (known semen stain) and a negative control (a clean piece of filter paper) to ensure that the reagents are working properly. The results of this testing must be documented in a case file.
- If either control does not give the expected results, do not proceed with testing evidence samples until the problem has been resolved.

4.4.3 Procedure

- Place a small piece of the stain/swab on the filter paper.
- Add 1-2 drops of AP Reagent I.
- After 15 seconds, add 1-2 drops of AP Reagent II.
- The development of a pink/purple color within 10-15 seconds is indicative of semen.

NOTE: The presence of semen in all samples exhibiting an inconclusive result or a positive result may be confirmed by identifying spermatozoa or by p30.

4.4.4 Interpretation

- Positive reaction= pink/purple color with dye solution within 10-15 seconds.
- Negative reaction= no color development, slight/slow color development.
- Inconclusive result= slow moderate to strong development of color.

4.4.5 Reporting results

Report the results of semen testing as follows:

- Positive findings "seminal fluid was detected".
- Negative findings "no seminal fluid was indicated".
- Inconclusive findings "tests for seminal fluid were inconclusive".

4.5 IDENTIFICATION OF SEMEN-SPECIFIC PROTEIN P30 BY AB-ACARD@PSA (P30) TEST

p30 is a 30,000 Dalton semen glycoprotein of prostatic origin that is also known as PSA or Prostate Specific Antigen. PSA can be readily detected in seminal fluid and has been shown to be absent in other biological fluids commonly encountered in forensic situations. Although in certain cases such as azoospermia, vasectomy and various diseases can affect the spermatozoa production in males. The presence of PSA allows the forensic analyst to identify human semen even in the absence of spermatozoa.

The range of PSA is 200,000 to 5.5 million ng/ml of semen. The sensitivity of the ABAcard@PSA test is 4ng/ml and therefore seminal fluid diluted up to 1 in a million should be detectable.

The determination of the presence of semen can be done by using the ABAcard@PSA (p30) test. In this test, 200µl of sample is added to the sample well 'S' and allowed to soak in. If PSA is present in the specimen, it will react with the mobile monoclonal antihuman PSA antibodies and a mobile antigen-antibody complex is thus formed. This mobile antibody-antigen complex migrates through the absorbent device towards the test area 'T'. In the test area 'T', polyclonal antihuman PSA antibodies are immobilized. These immobilized antibodies capture the above antibody-antigen complex so that an antibody-antigen-antibody sandwich is formed. The conjugated pink dye particles will form a pink colored band in the test area 'T' indicating a positive test result. As an internal positive control, PSA antibody-dye conjugates cannot bind to the antibody in the test area 'T', but are captured by an immobilized anti immunoglobulin antibody present in the control area 'C' forming a complex. The captured pink dye particles will thus form a band in the control area 'C', indicating that the test worked properly. Presence of two-colored lines, one in

the test area 'T' and one in the control area 'C' indicates a positive result that identifies the presence of semen.

Note: Each new lot of kit must be validated using a positive control before using in casework.

4.5.1 Preparation

- If the sample has been refrigerated, bring it to room temperature.
- Cut a small section of the swab or stain, (more may be required if the stain is diffused), and extract with 3 to 4 drops (approx. 200µl) of deionized water at room temperature for a few minutes to one hour.
- Label ABAcard$^{@}$ with case and item/ exhibit numbers, date, and initials.
- Add 200µl of sample to the sample well 'S' of the test device.
- Read result after 10 minutes. Positive results can be seen as early as in 1 minute, depending on the p30 concentration. For negative results, one must wait for full 10 minutes.

4.5.2 Conclusions

Positive: If there are two pink lines, one each in the test area 'T' and in the control area 'C', the test result is positive and indicates that the PSA level is 4ng/ ml or above.

Negative: If there is only one pink line, (in the control area 'C'), the test result is negative. This may indicate that PSA is not present above 4 ng/ ml concentration or the presence of "High Dose Hook Effect". Presence of "High Dose Hook Effect" may give false negative results due to the presence of high concentration of PSA in the sample, for example in undiluted seminal fluid. In such cases the sample may be retested using a 10 to 10,000 fold dilution.

Invalid: If there is no pink line visible in the control area' C', the test is said to be inconclusive. Repeat the test and re-examine the test procedure carefully.

Limitation

Positive results may be obtained with male urine sample. Use of another appropriate test is recommended when male urine sample is in question.

High Dose Effect: When the PSA concentration is too high, it overwhelms the sensitivity of the test. The mechanism behind the high dose effect is that huge amounts of human PSA binds to the antibody to form an antigen-antibody

complex. But, both the complex and also free PSA migrates towards the test area 'T' and the antibodies in the test area 'T' are blocked by this free PSA. Therefore, the mobile antigen-antibody complex cannot bind to the antibodies in the Test region 'T' to give the pink color. This results in a false negative result.

Sensitivity Tests

Neat semen is not testable due to its extremely viscous nature. One Step ABAcard@PSA (P30) test gives positive result at dilutions of 1:100 and 1:1000. Additionally, the One Step ABAcard@PSA (P30) test shows a positive result at the 1:10 dilution.

Aspermatic Semen Sensitivity Tests

The One Step ABAcard@PSA (P30) test gives positive results at the dilutions: 1:1, 1:10: 1:100, 1:1,000, 1:10,000, 1:100,000.

Miscellaneous Fabrics with Seminal Stains

All fabrics tested positive for the presence of semen with the One Step ABAcard@PSA (p30) test.

4.6 IDENTIFICATION OF SPERMATOZOA

Additionally, individual sperm heads can accurately be identified based on their morphological characteristics using a microscope of 40-100x magnification. An ideal, mature spermatozoan has an oval shaped head with a regular contour (4.0-5.0 mm long and 2.5-3.5 mm wide) and a pale interior part (acrosome: 40-70% of the head area) and a darker posterior region. The sperm tail is attached in a symmetrically situated fossa at the base of the head. The base of the head is broad. Only one tail is attached (about 45 mm long), not coiled or bent over it-self. Immediately behind the head, the first part of the tail i.e. the mid piece is somewhat thicker (maximum width= 1µm) and about 7-8mm long.

4.6.1 Extraction of spermatozoa from a substrate

4.6.1.1 Equipment and materials:

- Microscope (with approximately 40x-100x magnification)
- Appropriate mounting medium.
- Cover slips
- Rotator, vortex or centrifuge (depending on extraction method used).
- Scissors
- Tweezers
- Dissecting needle (optional)

- Microscopic slides
- Test tubes

4.6.1.2 Reagents

- Distilled water
- Proteinase K (20mg/ml)

4.6.2 Extraction method

- Cut a portion of the stain into small pieces (size based upon the substrate) and place it into a labeled micro centrifuge tube.
- Add 250 µl distilled water and 5µl proteinase K (depending upon the amount of stain in the sample).
- Incubate at 37^0C for a minimum of 2 hours.
- Place the cuttings into a spin basket. Centrifuge for 5 minutes at 10,000 rpm to recover the liquid.
- Remove the supernatant and place it into a newly labeled micro centrifuge tube. The remaining extract and the pellet can be used for the sperm detection. Wash the pellet twice with distilled water.

4.6.3 Aniline blue staining

- Aniline blue (aqueous)
- Fixative (alcohol: ether)
- Distilled water
- DPX

4.6.4 Procedure

- Prepare a thin smear of the extract (pellet) from suspected semen stain sample and allow it to air dry. Fix the smear with a fixative (alcohol: ether), or by placing it on a heat block for 15 minutes.
- Add sufficient amount (2-5 drops) of aniline blue reagent to cover the smeared portion of the microscopic slide. Let it dry completely.
- Remove the excess stain by placing it in water. Dry the slide at room temperature.
- Mount a cover slip over the smear using DPX.
- Examine the slide at 100x magnification with oil immersion for the possible presence of spermatozoa.

4.6.5 Interpretation

A sperm cell can be identified by the presence of a head and an acrosome. Spermatozoa appear as differentially stained purple bodies, oval shaped with clearly distinguishable pale (almost colorless) acrosomal caps. Epithelial cells

and most bacteria stain green with some of the nuclei pink/red. However, these are shaped differently than spermatozoa. Yeast cells can also take on the same color as spermatozoa, but are shaped differently.

4.6.6 Reporting results

Report the results of microscopic examination of slides as follows:

- **Positive findings:** "Spermatozoa were identified". Document the approximate number of spermatozoa or spermatozoa heads on the smear, per length of slide, or per slide as appropriate (approximately 100X magnifications).

  OR

  A spermatozoon was identified. If only 1 spermatozoon is observed, there must be documented confirmation of its presence by a second qualified examiner.

- **Negative findings:** No spermatozoa were identified**.**

CHAPTER 5

DNA EXTRACTION

The DNA isolation procedure varies according to the type of biological evidence present e.g. liquid blood, bloodstains, sperm cells, buccal cells, hair, tissue, bone, tooth etc. Slightly different extraction procedures are required for each type of specimen for the purpose of STR analysis and therefore are outlined in this chapter. It is important to handle all samples aseptically to prevent contamination by extraneous DNA. It is also important to isolate evidence samples at a separate time and/ or space from reference samples to prevent possible cross contamination. **All extraction steps must be performed in a Non- amplified DNA Laboratory area.**

NOTE: Process a reagent blank and internal control along with each set of samples. The extraction method used must be recorded on the DNA extraction worksheet.

5.1 ISOLATION OF DNA FROM BLOOD/ SALIVA/ OTHER BODY FLUIDS ARCHIVED ON FTA CARDS

Whatman FTA products facilitate collection, transportation, purification and long-term room temperature storage of nucleic acids, all on a single device. FTA technology has the ability to lyse cells, denature protein, removal of contaminants, and protects DNA from degradation. On FTA Cards small area is encircled for spotting of blood/body fluids and this method is used to obtain DNA from the following types of samples:

- Peripheral blood
- Buccal cells
- Other body Fluids

5.1.1 Reagents:

- FTA Purification Reagent
- TE Buffer

5.1.2 Procedure:

- Spot peripheral blood directly on the FTA card.
- Allow drying for 1 hour to overnight before processing further.
- Using a clean 1.2mm punch, remove a sample from the middle of the stain and place it into an amplification tube (0.2µl volume).
- Add 150µl of FTA purification reagent to each tube. Cap each tube and vortex 1 to 2 seconds at low speed.
- Allow the tubes to sit for 5 minutes at room temperature with a second brief vortex halfway through the incubation.
- After 5-minute incubation, vortex for a third time and then carefully remove as much reagent as possible.
- Repeat steps 4 through 6 an additional 2 times for a total of three washes with the FTA purification reagent.

- After FTA purification reagent has been removed for the third time, add 150µl of TE (10mM Tris-HCl, pH 8.0, 0.1mM EDTA). Cap each tube and vortex 1 to 2 seconds at low speed.
- Allow the tubes to sit for 5-minutes at room temperature with a brief vortex half way through the incubation.
- Pour off the TE and replace with an additional 150µl of TE. Cap each tube and vortex 1 to 2 seconds.
- Allow the tubes to sit for 5-minutes at room temperature with a brief vortex half way through the incubation.
- Pour off the TE and allow the FTA paper punch to completely air dry for 1 hour at room temperature or alternatively by placing the tube at 37^0C for 30 minutes. The complete PCR amplification mix is directly added to the punch containing the purified immobilized DNA.

5.2 ORGANIC EXTRACTION

In this protocol the cell lysate is mixed with phenol, chloroform and isoamyl alcohol for the separation of nucleic acids from proteins. This method gives a high yield, but traces of organic solvents often contaminate the sample. Furthermore, the uses of these substances toxic to health make it necessary to carry out the entire process in a chemical fume hood.

- Add 500µl phenol: chloroform: isoamyl alcohol (PCI) to the sample. Shake the tube vigorously with hand or mix to achieve a milky emulsion in the tube. Spin the tube at high speed for a minimum of 3 minutes.
- After cell lysis and removal of proteins from the sample, nucleic acids are precipitated by adding isopropanol or ethanol. This method allows obtaining high purity DNA.

OR

Microcon/silica based column method is used.

5.2.1 Microcon dialysis and concentration of samples

Procedure:

- Add 100µl of TE^{-4} to the upper chamber of the Microcon device to pre-wet it.
- Transfer aqueous layer (from PCI) to the upper chamber of Microcon device.
- Place a spin cap on the concentrator and spin in a micro centrifuge at 500xg for 10 minutes.
- Remove the spin cap and add 200µl of TE^{-4} to the concentrator. Replace the spin cap and spin the assembly in a micro centrifuge at 500xg for 10 minutes.

- Remove the spin cap and add a measured volume of TE^{-4} (40µl to 75µl) to the concentrator.
- Remove the concentrator from the filtrate tube and carefully invert the concentrator onto a labeled retentate tube. Discard the filtrate tube.
- Spin the assembly in the micro centrifuge at 500xg for 5 minutes.
- Discard the concentrator and cap the retentate tube.

OR

5.2.2 Additional/Alternate Extraction Procedure using QIAamp® Micro Kit

Procedure:

- Briefly centrifuge the tube to force the condensation into the tube (after cell lysis).
- Carefully transfer the aqueous layer (from PCI) to the upper chamber of the QIAamp MiniElute Column(in a 2 ml Collection tube) without wetting the rim, close the cap and centrifuge at 6000Xg (8000rpm) for 1 minute. Place the QIAamp MiniElute Column in a clean 2 ml collection tube and discard the collection tube containing the filtrate.
- Carefully open the QIAamp MiniElute Column and add 500µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000xg (8000rpm) for 1minute. Place the QIAamp MiniElute Column in a clean 2 ml collection tube and discard the collection tube containing the flow-through.
- Carefully open the QIAamp MiniElute Column and add 500µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000Xg; 14,000rpm) for 3 minutes to dry the membrane completely.
- Place the QIAamp Mini Elute column in a clean 1.5 ml micro centrifuge tube and discard the collection tube containing the filtrate. Carefully open the lid of the QIAamp MinElute column and apply 20-50µl of Buffer AE to the center of the membrane.
- Close the lid and incubate at room temperature (15-25^{o}C) for 5-10 minutes. Centrifuge at full speed (20,000xg or 14,000rpm) for 1 minute.

The DNA template is now ready for PCR amplification or the sample can be

stored for long term at -20^{o}C.

5.3 INORGANIC EXTRACTION

5.3.1 Adsorption on a Silica Column

The principle of this type of extraction method is based on the capacity of nucleic acids to be adsorbed on a silica column in the presence of high concentrations of chaotropic salts. Salts/Contaminants of the sample are removed with subsequent washes of the column to remove unbound particles, and finally the DNA is eluted with H_20 or a low ionic strength resuspension buffer at neutral or slightly alkaline pH.

This method can be used to obtain DNA from the following types of samples:

- **Peripheral blood:** Blood volumes ranging from 0.02 ml to 10 ml can be processed.
- **Cells:** Not more than 1x10^{7} cells.
- **Saliva:** Samples collected by swab or in a container with Oragene® preservative.
- **Tissues:** Fresh frozen tissues not to exceed 25 mg of sample per column.

5.4 ISOLATION OF DNA FROM LIQUID BLOOD USING QIAamp®DNeasy MINI KIT

Liquid blood specimens should be collected in an EDTA vacutainer tube. Mix well before removing aliquots.

5.4.1 Reagents

- Proteinase K Solution:Proteinase K (100 mg):Dissolve in 5ml sterile Milli-Q Grade water (final concentration 20mg/ml). Divide into 1ml aliquots and store at –20^{o}C.
- 96-100% Ethanol
- Sterile Milli-Q Grade water

QIAamp Kit components: QIAamp spin column, Buffers ATL, AL, AE, AW1 and AW2.

5.4.2 Procedure:

- Pipette 20 µl Proteinase K into the bottom of a 1.5 ml micro centrifuge tube.
- Add 200µl of blood to the micro centrifuge tube.
- Add 200µl of buffer AL to the sample. Mix by pulse vortexing for 15 seconds to yield a homogeneous solution.
- Incubate at 56^{o}C for 10 minutes.
- Briefly centrifuge the micro centrifuge tube to force the condensation from lid into the tube.

- Add 200µl ethanol (96-100%) to the sample, and mix again by pulse –vortexing for 15 sec. briefly centrifuge the tube.
- Carefully apply the mixture from above to the QIAamp spin column (in a 2 ml Collection tube) without wetting the rim, close the cap and centrifuge at 6000Xg (8000rpm) for 1 minute. Place the QIAamp spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
- Open the QIAamp spin column and add 500 µl of buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000Xg (8000 rpm) for 1 minute. Place the QIAamp spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate.
- Open the QIAamp spin column and add 500 µl of buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000Xg; 14,000rpm) for 3 minutes.
- Place the QIAamp spin column in a clean 1.5 ml micro centrifuge tube and discard the tube containing the filtrate. Open the QIAamp spin column and add 200 µl of buffer AE. Incubate at room temperature for 1 minute and then centrifuge at 6000Xg (8000rpm) for 1 minute.

The sample is now ready for PCR amplification or can be stored for long term at -20^{o}C.

5.5 ISOLATION OF DNA FROM DRIED BODY FLUID STAINS USING QIAamp® DNeasy MINI KIT

Cut an approximate 3 mm bloodstain/buccal sample and if a stain is smeared over a large area of the fabric, cut the stain into small pieces and place the pieces into an extraction tube.

5.5.1 Reagents

- Proteinase K Solution:
- 96-100% Ethanol
- Sterile Milli-Q Grade water
- Kit components: QIAamp spin column,
- Buffers ATL, AL, AE, AW1 and AW2.
- Proteinase K (100 mg): Dissolve in 5ml sterile Milli-Q Grade water (final concentration 20mg/ml) Divide into 1ml aliquots and store at –20^{o}C.

5.5.2 Procedure:

- Place small cuttings from a dried stain into a 1.5 ml micro centrifuge tube and add 180 µl of Buffer ATL.

- Add 20µl of Proteinase K solution, mix by vortexing and incubate at 56 ^{0}C overnight.
- Centrifuge the tube for 3 minutes.
- Remove the cutting pieces and retain extracted substrates if desired.
- Add 200µl of buffer AL to the sample. Mix by pulse vortexing for 15 seconds to yield a homogeneous solution.
- Incubate at 56^{o}C for 10minutes**.**
- Briefly centrifuge the microcentrifuge tube to force the condensation from lid into the tube.
- Add 200µl ethanol (96-100%) to the sample, and mix again by pulse –vortexing for 15 sec. briefly centrifuge the tube.
- Carefully apply the mixture from step 2.6 to the QIAamp spin column (in a 2 ml Collection tube) without wetting the rim, close the cap and centrifuge at 6000Xg(8000rpm) for 1 minute. Place the QIAamp spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
- Open the QIAamp spin column and add 500 µl of buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000Xg (8000 rpm) for 1 minute. Place the QIAamp spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate.
- Open the QIAamp spin column and add 500 µl of buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000Xg; 14,000rpm) for 3 minutes.
- Place the QIAamp spin column in a clean 1.5 ml micro centrifuge tube and discard the tube containing the filtrate. Open the QIAamp spin column and add 200 µl of buffer AE.
- Incubate at room temperature for 1 minute and then centrifuge at 6000Xg (8000rpm) for 1 minute.
- The sample is now ready for PCR amplification. The sample can be stored at -20^{o}C for long term.

5.6 DNA ISOLATION FROM FRESH AND FROZEN TISSUES USING QIAamp® DNeasy KIT

Tissues should be stored at -20^{0}C until used.

5.6.1 Reagents

- Proteinase K Solution: Proteinase K (100 mg): Dissolve in 5ml sterile Milli-Q Grade water (final concentration 20mg/ml). Divide into 1ml aliquots and store at –20^{o}C.
- 96-100% Ethanol

- SterileMilli-Q Grade water
- QIAamp Kit components: QIAamp spin column, Buffers ATL, AL, AE, AW1 and AW2.

5.6.2 Procedure:

- Cut up to 25 mg of tissue into small pieces, place in a 1.5 ml micro centrifuge tube and add 180 µl of buffer ATL.
- Add 20 µl of proteinase K, mix by vortexing and incubate at 55^{o}C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or a rocking platform.
- Add 200µl of buffer AL to the sample. Mix by pulse vortexing for 15 seconds to yield a homogeneous solution.
- Incubate at 70^{o}C for 10minutes.
- Briefly centrifuge the micro centrifuge tube to force the condensation from lid into the tube.
- Add 200µl ethanol (96-100%) to the sample, and mix thoroughly by vortexing for 5 sec. briefly centrifuge the tube.
- Carefully apply the mixture from above step to the DNeasy spin column (placed in a 2 ml Collection tube) without wetting the rim and centrifuge at 6000Xg (8000rpm) for 1 minute. Discard flow-through and collection tube.
- Place the DNeasy spin column in a 2 ml collection tube, add 500µl buffer AW1 and centrifuge at 6000Xg (8000 rpm) for 1 minute and discard the collection tube containing the filtrate.
- Place the DNeasy spin column in a 2 ml collection tube, add 500µl buffer AW2 and centrifuge for 3 minute at full speed (20,000Xg; 14,000rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
- Place the DNeasy spin column in a clean 1.5 ml or 2 ml micro centrifuge tube and pipette 200µl buffer AE directly onto the DNeasy membrane.
- Incubate at room temperature for 1 minute, and then centrifuge for 1 minute at 6000Xg (8000rpm) to elute.
- The sample is now ready for PCR amplification. Depending upon the concentration, use 2 to 10 µl of sample in amplification procedure. The remaining sample may be stored for long term at -20^{o}C.

5.7 DNA ISOLATION FROM NAIL CLIPPINGS USING QIAamp® DNA MICRO KIT

To remove extraneous DNA from nail clippings, treat the nail clippings with

wash buffer.

5.7.1 Wash Buffer for surface cleaning:

For 1ml wash Buffer

- 1% SDS 100µl (10%SDS)
- 25mM EDTA 50µl
- 1µl Proteinase K (20mg/ml) 5µl
- Sterile Milli-Q Grade water 845 µl
- Transfer the nail clippings to a 1.5 ml micro centrifuge tube. Add wash buffer in proportions to saturate the sample for 30 minutes.
- Centrifuge the tube for 2 minutes. Remove the supernatant. Add sterile water to wash the nail clippings. Again centrifuge the tube for 2 minutes. Remove the supernatant. Repeat this step two times.

5.7.2 Reagents

- Proteinase K Solution /Proteinase K (100 mg): Dissolve in 5ml sterile Milli-Q Grade water (final concentration 20mg/ml). Divide into 1ml aliquots and store at –20^{o}C.
- 96-100% Ethanol
- Sterile Milli-Q Grade water
- 1M DTT (Dithiothreitol) - 20µl
- QIAamp Kit components: QIAamp spin column, Buffers ATL, AL, AE, AW1 and AW2.

5.7.3 Procedure:

- Transfer the nail clippings to a 1.5 ml micro centrifuge tube.
- Add 300µl of Buffer ATL, 20µl of Proteinase K and 20µl of 1M DTT, close the cap and mix by pulse vortexing for 10 seconds.
- Place the tube in a thermo mixer and incubate at 56^{0} C with shaking at 300 rpm overnight. Briefly centrifuge the tube to force condensation into the bottom of the tube.
- Add 300µl Buffer AL, close the lid and mix by pulse-vortexing for 10 seconds.
- Place the tube in a thermo mixer and incubate at 70^{o}C with shaking at 300 rpm for 10 minutes. Centrifuge the tube at full speed (20,000xg; 14,000rpm) for 1 minute.
- Carefully transfer the supernatant from step 2.5 to the QIAamp MinElute Column without wetting the rim.
- Close the lid and centrifuge at 6000xg (8000rpm) for 1 minute. Place the QIAamp MinElute Column in a clean 2 ml collection tube and

discard the collection tube containing the filtrate.

- Carefully open the QIAamp MinElute Column and add 500µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000xg (8000rpm) for 1 minute. Place the QIAamp MinElute Column in a clean 2 ml collection tube and discard the collection tube containing the filtrate.
- Carefully open the QIAamp MinElute Column and add 500µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at 6000xg (8000rpm) for 1 minute. Place the QIAamp MinElute Column in a clean 2.0 ml collection tube and discard the collection tube containing the filtrate.
- Centrifuge at full speed (20,000xg; 14,000 rpm) for 3 minutes to dry the membrane completely.
- Place the QIAamp MinElute column in a clean 1.5 ml micro centrifuge tube and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply 20-50µl of Buffer AE to the center of the membrane.
- Close the cap and incubate at room temperature (15-25^{o}C) for 1 minute. Centrifuge at full speed (20,000xg; 14,000rpm) for 1 minute.

OR

- Microcon method can be used for purification and concentration of samples as mentioned above.

  The sample is now ready for PCR amplification. The remaining sample may be stored for long term at -20^{o}C.

5.8 ISOLATION OF DNA FROM PARAFFIN EMBEDDED TISSUES

5.8.1 Pretreatment for Paraffin Embedded Tissues:

- Place a small section of paraffin embedded tissue in a 2ml micro centrifuge tube.
- Add 1200µl xylene. Vortex vigorously. Centrifuge at full speed for 5 minutes.
- Remove supernatant by pipetting. Do not remove any of the pellets.
- Add 1200 µl absolute ethanol to the pellet to remove residual xylene, and mix gently by vortexing.
- Centrifuge in a micro centrifuge at full speed for 5 minutes. Remove ethanol by pipetting. Do not remove any of the pellets.
- Incubate the open micro centrifuge tube at 37^{o} C for 10-15 minutes until the ethanol has evaporated. Now sample is ready for further

processing.

Note: Proceed to protocol for DNA isolation from fresh and frozen tissues using QAamp® DNeasy KIT or Microcon purification and concentration.

5.9 ISOLATION OF DNA FROM FORMALIN FIXED TISSUES

5.9.1 Pretreatment for Formalin Fixed Tissues:

DNA was extracted by immersing approximately 50 mg of tissue in 0.5 ml of PCR buffer (50 mMKCl, 10 mM Tris-HC1, pH 8.3, 1.5 mM MgC1), 1 µl of 1 M DTT and 5 µl of Proteinase K (10 mg/ml). The biopsies were incubated at 56^{o}C for 24 hr, with a second addition of 5 µl of Proteinase K after 1.5 hr. The digest was extracted with phenol/chloroform/isoamyl alcohol protocol.

- Add 400µl phenol/chloroform/isoamyl alcohol to the extract.Vortex briefly to attain a milky emulsion. Spin the tube in a micro centrifuge for 3 minutes.
- Transfer aqueous phase (top layer) carefully to a new 1.5ml Eppendorf tube.

Note: Proceed to Microcon®Purification Procedure and concentration of samples.

5.10 ISOLATION OF DNA FROM FIXED AND PERMOUNTED SLIDES/SMEARS USING ORGANIC EXTRACTION METHOD (DIFFERENTIAL PROCEDURE)

5.10.1 Procedure

- Place the stained, fixed and permounted smear/slide into a clean glass Petri dish with a cover.
- Pour a sufficient volume of xylene over the smear/slide until completely submerged. Incubate the smear/slide overnight at room temperature. If the coverslip does not float off easily, continue to soak the smear/slide in xylene until the coverslip can be removed easily.

NOTE: Smears/slides that have been mounted with an excess amount of DPX may need to be incubated in the xylene solution of as much as 5 days.

- Once the cover slip floats off, remove the smear/slide from the Petri dish and air dry for a minimum of 5 minutes**.** Do not discard the cover slip.

- Scrape the cellular material off the smear/slide with a clean, unused scalpel or razor blade and place it into a labeled 1.5 mL microcentrifuge tube.
- Remove half the cotton from a sterile cotton tipped swab. Wet the remaining portion of the swab slightly with sterile water and then use the swab to scrape smear/slide to remove any remaining cellular material.
- Remove the cotton from the stick and place it into the 1.5 mL microcentrifuge tube with the scrapings from the smear/slide and place into the 1.5 mL tube.
- Retain the smear/slide along with the cover slip until it is certain that DNA has successfully been obtained. Cells can stick to both the slide and the cover slip.

NOTE: Proceed to protocol for Differential Extraction Procedure, Microcon® Purification and concentration of samples.

5.11 ISOLATION OF TOTAL DNA FROM TRACE EVIDENCE (CHEWING GUM)

This protocol is for isolation of total (genomic and mitochondrial) DNA from chewing gum.

5.11.1 Digestion & Precipitation:

- Cut up to 30 mg of chewing gum into small pieces and transfer them to a 1.5 ml microcentrifuge tube.
- Add 300 µl Buffer ATL and 20 µl proteinase K, and mix by vortexing for 10 s.
- Incubate at 56°C with shaking at 900 rpm for at least 3 h.
- Add 300 µl Buffer AL.
- Incubate at 70°C with shaking at 900 rpm for 1 hour.
- Centrifuge at 20,000 x g; 14,000 rpm for 1 min.

5.11.2 DNA Binding to Silica membrane:

- Carefully transfer the supernatant from step 9 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim.
- Centrifuge at 6000 x g (8000 rpm) for 1 min.
- Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

5.11.3 Buffer Wash:

- Add 500 µl Buffer AW1

- Centrifuge at 6000 x g (8000 rpm) for 1 min.
- Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
- Add 700 µl Buffer AW2 and centrifuge at 6000 x g (8000 rpm) for 1 min.
- Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
- Add 700 µl of ethanol (96–100%) and centrifuge at 6000 x g (8000 rpm) for 1 min.
- Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
- Centrifuge at 20,000 x g; 14,000 rpm for 3 min to dry the membrane completely.

5.11.4 Incubation & Elution:

- Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the flow-through.
- Incubate at room temperature (15–25°C) for 10 min or at 56°C for 3 min.
- Add 20–100 µl Buffer TE or distilled water to the center of the membrane.
- Incubate at room temperature for 10-30 min. Centrifuge at 20,000 x g; 14,000 rpm for 1 min.

5.12 ISOLATION OF TOTAL DNA FROM CIGARETTE BUTTS

This protocol is for isolation of total (genomic and mitochondrial) DNA from Cigarette Butts.

5.12.1 Digestion & Precipitation

- Cut out a 1 cm2 piece of outer paper from the end of the cigarette or filter. Cut this piece into 6 smaller pieces. Transfer the pieces to a 1.5 ml microcentrifuge tube.
- Add 300 µl Buffer ATL and 20 µl proteinase K
- Incubate at 56°C with shaking at 900 rpm for at least 1 h.
- Add 300 µl Buffer AL
- Incubate at 70°C with shaking at 900 rpm for 10 min.
- Add 150µl ethanol (96–100%).
- Centrifuge at 20,000 x g; 14,000 rpm for 1 min.

5.12.2 DNA Binding to Silica membrane:

- Carefully transfer the supernatant from step 9 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim,
- Centrifuge at 6000 x g (8000 rpm) for 1 min.
- Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

5.12.3 Buffer Wash:

- Add 500 µl Buffer AW1
- Centrifuge at 6000 x g (8000 rpm) for 1 min.
- Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
- Add 700 µl Buffer AW2 and centrifuge at 6000 x g (8000 rpm) for 1 min.
- Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
- Add 700 µl of ethanol (96–100%) and centrifuge at 6000 x g (8000 rpm) for 1 min.
- Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
- Centrifuge at 20,000 x g; 14,000 rpm for 3 min to dry the membrane completely

5.12.4 Incubation & Elution:

- Place the QIAamp MinElute column in a clean 1.5 ml microcentrifugetube and discard the collection tube containing the flow-through.
- Incubate at room temperature (15–25°C) for 10 min or at 56°C for 3 min.
- Add 20–100 µl Buffer TE or distilled water to the center of the membrane.
- Incubate at room temperature for 10-30 min. Centrifuge at 20,000 x g; 14,000 rpm for 1 min.

5.13 LIST OF PROTOCOLS BASED ON SILICA BINDING TECHNOLOGY

- QiAamp® DNA Mini and Blood Mini Handbook for DNA purification from whole blood, plasma, serum, buffy coat, lymphocytes, dried blood spots, body fluids, cultured cells, swabs, and tissue. Qiagen. (Hilden, Germany). www.qiagen.com.
- QIAamp® DNA FFPE Tissue Handbook for purification of genomic DNA from formalin-fixed, paraffin-embedded tissues.

Qiagen. (Hilden, Germany). www.qiagen.com.

5.14 MAGNETIC SEPARATION

This method utilizes magnetic beads coated with natural or synthetic polymers that have a high affinity for nucleic acids. The magnetic beads are added to the lysed sample, which allows them to bind to DNA molecules. A magnetic stand is placed against the wall of the tube, the beads with bound DNA stick to the tube wall and dissolved contaminants of the sample are removed by pipetting or decanting. After several re-suspension/washing cycles in which the beads are retained by the magnet, the DNA is eluted in a suitable buffer. After a series of washes for maximum purification of the genetic material, the beads are removed from the solution by a magnetic separator.

This method is used to obtain DNA from the following types of samples:

- Peripheral blood: 5 and 10 ml of peripheral blood can be processed.
- Amniotic fluid: 1 to 3 ml can be processed.
- Saliva: 2 ml of saliva mixed with 2 ml of Oragene® preservative can be processed.
- Fresh frozen tissue: Not to exceed 10 mg of sample per extraction.
- Bone or tooth powder: 50 mg

5.15 PREPFILER™ /BTA FORENSIC DNA EXTRACTION KITS

The standard protocol is appropriate for most forensic sample types, including body fluids, stains and swabs of body fluids, and small tissue samples. This is brief procedure for using the PrepFiler™ Forensic DNA Extraction Kit with the standard protocol.

5.15.1 Materials

- Proteinase K 20mg/ml
- DTT (Dithiothreitol) 1.0 M
- PrepFiler™LysisBufferOne bottle, 50 ml
- PrepFiler™Isopropanol One 60 mL bottle
- PrepFiler™ Magnetic Particles One tube, 1.5 mL
- PrepFiler™Wash Buffer Concentrates two 125-mL bottles,each containing 26 ml ofconcentrate (user adds 74 ml of ethanol to each bottle)
- PrepFiler™ Elution Buffer One bottle, 12.5 ml
- PrepFiler™ Filter Columns 100 filter columns
- PrepFiler™ Spin Tubes 300 spin tubes
- Magnetic Stand

5.15.2 For BTA Extraction

- BTA Lysis buffer

Note: Items 1 and 2 are not provided in the Kit

5.15.3 Sample types and inputs for the standard protocol are shown below:

- Liquid samples (blood, saliva) Up to 40 µl
- Blood (on FTA paper or fabric) Up to 25 mm^2 cutting or punch
- Body fluids (saliva, semen) on fabric Up to 25 mm^2 cutting or punch
- Body fluids on swabs (Buccal and other body fluids) Up to one swab

Tissue fragments (from a razor or other swabbed substrate, or from a fingernail scraping or swabbing).

5.15.4 Lysis

- Place sample in a PrepFiler™ Spin Tube or standard 1.5-mL micro-centrifuge tube.
- Add 300 µl PrepFiler™ Lysis Buffer, 3 µL (use 5 µL for samples containing semen) DTT (1.0 M) vortex it for 5 seconds and then centrifuge briefly.
- Place the tube in a thermal shaker and incubate it at 70 °C and 900 rpm for 20 minutes (for liquid body fluids), 40 minutes (for swabs or dried stains) or 90 minutes (for neat semen samples).
- If a sample substrate is present, remove it from the lysate before continuing with the extraction procedure.
- Centrifuge the sample tube for 2 seconds to collect the condensation from the tube cap. Insert a PrepFiler™ Filter Column into a new 1.5 mL PrepFiler™ Spin Tube, and then carefully transfer the sample tube contents into the filter column.
- Cap the filter column/spin tube, then centrifuge for 2 minutes at 12,000-14,000 rpm or for 5 minutes at 3,000-4,000 rpm.
- Check the volume of sample lysate collected in the spin tube. If the volume is less than 180 µL, then centrifuge the filter column/spin tube for an additional 5 minutes.
- Remove the filter column from the spin tube, then properly dispose of the filter column and allow the sample lysate to come to room temperature (approximately 5 minutes).

Note: The collected sample lysate will remain in the spin tube and process the lysate further for extraction.

Important: To avoid precipitation of lysis buffer components, do not chill the sample lysate.

5.15.5 Binding of genomic DNA to magnetic particles

- Vortex the PrepFiler™ Magnetic Particles tube approximately for 5 seconds, invert the tube to confirm that no visible pellet remains in the bottom of the tube, then centrifuge briefly.
  Note: If you are pipetting multiple samples, vortex the magnetic particles tube every 5 minutes.
- Pipette 15 µl of PrepFiler™ Magnetic Particles into the tube containing the sample lysate, vortex it at low speed (approximately 500-1,200 rpm) for 10 seconds, and then centrifuge it briefly.
  Important: This step is required before you add isopropanol.
- Add 180 µL of isopropanol to the sample lysate tube, cap the sample tube, vortex it at low speed for 5 seconds, and then centrifuge it briefly.
- Place the sample lysate tube in a shaker or on a vortexer (with adaptor), then mix at room temperature at 1000 rpm for 10 minutes.

5.15.6 Wash bound DNA

- Vortex the sample DNA tube at maximum speed for 10 seconds, then centrifuge briefly.
  Note: It is acceptable to have magnetic particle aggregates suspended in the solution or on the side of the tube below the meniscus.
- Confirm that the magnet in the magnetic stand is properly aligned.
- Place the sample DNA tube in the magnetic stand, then wait until the size of the pellet stops increasing (approximately 1 to 2 minutes).
- With the sample DNA tube remaining in the magnetic stand, use a pipette to carefully remove and discard all visible liquid phase.
  Important! When removing the liquid phase, do not aspirate magnetic particles or disturb the magnetic particle pellet. Perform steps 1 through 4 three times.
- Add 300 µL of prepared PrepFiler™ Wash Buffer to the sample DNA tube, cap the tube and remove the tube from the magnetic stand.
- Vortex the sample DNA tube at maximum speed (approximately 10,000 rpm) until there is no visible magnetic particle pellet on the side of the tube (approximately 5 seconds), then centrifuge briefly.
- Place the sample DNA tube in the magnetic stand for 30 to 60 seconds, with the sample DNA tube remaining in the magnetic stand, use a pipette to carefully remove and discard all visible liquid phase.
  Important! When removing the liquid phase, do not aspirate mag-

netic particles or disturb the magnetic particle pellet.

- With the sample DNA tube remaining in the magnetic stand, open the tube, and then allow the magnetic particle-bound DNA to air-dry for 7 to 10 minutes.
  Important! Air-drying for more than 10 minutes may reduce DNA yield. If the room temperature is >25 °C, reduce the drying time to 5 minutes.

5.15.7 Elute the DNA

- Add 50 µL of PrepFiler™ Elution Buffer to the sample DNA tube.
- Cap the tube, vortex it at maximum speed (approximately 10,000 rpm) until there is no visible magnetic particle pellet on the side of the tube (approximately 5 seconds), then centrifuge it briefly.
- Incubate the sample tube at 70°C and 900 rpm for 5 minutes.
- Vortex the sample DNA tube at maximum speed (approximately 10,000 rpm) until there is no visible magnetic particle pellet on the side of the tube (approximately 2 seconds), then centrifuge briefly.
- Place the sample DNA tube in the magnetic stand until the size of the pellet at the side of the tube stops increasing (at least 1 minute).
- Pipette the liquid in the sample tube (which contains the isolated genomic DNA) to 1.5-mL microcentrifuge tube for storage.
  Important! When removing the liquid phase, do not aspirate magnetic particles or disturb the magnetic particle pellet.
  Note: The isolated DNA may be stored at 4 °C for up to one week, or at −20 °C for longer storage.
- If the eluted DNA extract is turbid (for example, this may occur in tissue samples with a high fat content), centrifuge the tube for 5 to 7 minutes at maximum speed (approximately 10,000 rpm), then transfer the clear supernatant to a new 1.5-mL microcentrifuge tube.

NOTE: PREPFILER® BTA FORENSIC DNA EXTRACTION KIT is designed specifically for DNA extraction from bone and tooth samples. The procedure for using this Extraction Kit is as per the manufacturer's the standard protocol.

5.16 DIFFERENTIAL EXTRACTION OF DNA FROM VAGINAL SWABS

Resolution of sperm cell DNA and vaginal cell DNA (Differential Extraction).

5.16.1 Reagents

- Proteinase K Solution: Dissolve 100 mg of Proteinase K in 5ml sterile Milli-Q Grade water (final concentration 20mg/ml) Divide into 1ml aliquots and store at –20^{o}C

- 96-100% Ethanol
- QIAamp Kit components: QIAamp spin column, Buffers ATL, AL, AE, AW1 and AW2.
- Sterile Milli-Q Grade water
- 20% Sarcosyl
- TNE (Tris/EDTA/NaCl) buffer
- 0.39M DTT (Dithiothreitol)

5.16.2 Procedure:

- Remove the swab from applicator stick or cut stain and place the swab/cutting into an extraction tube with basket or other appropriate tube.
- Add:
  - 400 µl Tris/EDTA/NaCl (TNE)
  - 25µl 20% Sarkosyl
  - 75µl H_2O
  - 5µl Proteinase K - 20 mg/mL
- Mix the contents and place at 37^0C for at least 2 hours and spin the tube for a minimum of 5 minutes.
- Remove the supernatant fluid and place it into a new 1.5ml tube.

5.16.3 Separation of Female and Male components

- This is the fraction that contains female DNA. Save the swab/cutting as a second male fraction. Be careful not to disturb the pellet on the bottom of the tube.
- Place a new cap on the original tube if necessary. Wash the male fraction tube with approximately 1.0 ml of TNE, vortex and centrifuge the tube for a minimum of 3 minutes. Repeat this washing at least two times.
- A small portion of the male fraction maybe examined microscopically for the evaluation of spermatozoa.
- To the pellet in the original tube add:
  - 150 µl TNE
  - 50µl 20% Sarcosyl
  - 40µl 0.39M DTT
  - 150µl H_2O
  - 10µl Proteinase K - 20 mg/mL
- Mix tube contents and place at 56^oC for at least 2 hours. This step may be prolonged overnight.
- Extract both tubes (female as well as male fraction) with an equal volume of phenol: chloroform: isoamyl alcohol.

- Add 500µl phenol: chloroform: isoamyl alcohol. Shake the tube vigorously with hand or mix to achieve a milky emulsion in the tube.
- Centrifuge the tube at 14,000 rpm for 5-10 minutes and transfer the supernatant into the microcon /Silica column.

5.16.4 Microcon dialysis and concentration of samples

- Add 100µl of TE^{-4} to the upper chamber of the Microcon device to pre-wet it.
- Transfer aqueous layer to the upper chamber of microcon device.
- Place a spin cap on the concentrator and spin in a micro centrifuge at 500xg for 10 minutes.
- Remove the spin cap and add 200µl of TE^{-4} to the concentrator. Replace the spin cap and spin the assembly in a micro centrifuge at 500xg for 10 minutes.
- Remove the spin cap and add a measured volume of TE^{-4} (40µl to 75µl) to the concentrator.
- Remove the concentrator from the filtrate tube and carefully invert the concentrator onto a labeled retenate tube. Discard the filtrate tube.
- Spin the assembly in the micro centrifuge at 500xg for 5 minutes.
- Discard the concentrator and cap the retenate tube.

OR

5.16.5 Additional/ Alternate Extraction Procedure using QIAamp® Micro Kit

Procedure:

- Briefly centrifuge the tube to force the condensation into the tube (from 2.8).Add 200µl Buffer AL to both male and female fractions.
- Place the tube in a thermo mixer and incubate at $70^{o}C$ with shaking at 300 rpm for 10 minutes.
- Briefly centrifuge the micro centrifuge tube to force the condensation from lid into the tube.
- Add 200µl ethanol (96-100%) to the sample, and mix again by pulse vortexing for 15 sec. briefly centrifuge the tube.
- Carefully transfer the mixture from step 4.2 to the QIAamp MiniElute Column(in a 2 ml Collection tube) without wetting the rim, close the cap and centrifuge at 6000Xg (8000rpm) for 1 minute. Place the QIAamp MiniElute Column in a clean 2 ml collection tube and discard the collection tube containing the filtrate.
- Carefully open the QIAamp MiniElute Column and add 500µl Buf-

fer AW1 without wetting the rim. Close the lid and centrifuge at 6000xg (8000rpm) for 1minute. Place the QIAamp MinElute Column in a clean 2 ml collection tube and discard the collection tube containing the flow-through.

- Carefully open the QIAamp MiniElute Column and add 500µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000Xg; 14,000rpm) for 3 minutes to dry the membrane completely.
- Place the QIAamp Mini Elute column in a clean 1.5 ml micro centrifuge tube and discard the collection tube containing the filtrate. Carefully open the lid of the QIAamp MinElute column and apply 20-50µl of Buffer AE to the center of the membrane.
- Close the lid and incubate at room temperature (15-25^{o}C) for 1 minute. Centrifuge at full speed (20,000xg; 14,000rpm) for 1 minute.

The DNA template is ready for PCR amplification. The sample can be stored for long term at -20^{o}C.

5.17 ISOLATION OF DNA FROM HAIR ROOT

Preparation of Hair Root for DNA Analysis

- Locate root end of designated hair/or hair on microscope slide. Using a scribe, puncture coverslip around the root. Add drop of xylene to root area.
- Remove pieces of cover slip. Using a razor with a new blade, cut the root end from the hair.
- While holding the hair with sterile forceps, rinse root with xylene.
- Rinse root in 100% ethanol.
- Rinse root with a thorough rinse in distilled water.
- Place hair root end into a labeled micro centrifuge tube.
- Add 200 µl Buffer ATL and 20 µl proteinase K, and mix by vortexing for 10 s.
- Incubate at 56°C with shaking for at least 3 hours or overnight.
- The lysate was extracted with phenol/chloroform/isoamyl alcohol protocol.
- Add 200µl phenol/chloroform/isoamyl alcohol to the extract.
- Vortex briefly to attain a milky emulsion. Spin the tube in a micro centrifuge for 3 minutes.
- Transfer aqueous phase (top layer) carefully to a new 1.5ml Eppendorf tube.

NOTE: Microcon method can be used for purification and concentration of sample.

5.18 REMOVAL OF DYES, IMPURITIES ETC. FROM ISOLATED DNA

SEPHAROSE 6B Cleaning Treatment

- Weigh approximately 100mg of dry Sepharose 6B beads (Sigma T-8387) and transfer to a 1.5 ml Eppendorf tube.
- Hydrate Sepharose 6B beads by adding 1.0 ml sterile water, mix well and let stand at room temperature for 5 minutes.
- Spin Eppendorf tube at 14000 RPM for about 2 minutes and discard the supernatant.
- Repeat steps 2 and 3 twice.
- Add appropriate volume of sterile water to the tube to make slurry of the beads of such a consistency that the beads can be aliquot using 1.0 ml pipette tip.
- Aliquot 100µl of hydrated beads slurry in a new Eppendorf tube. If dye needs to be removed from small volume of DNA, dilute the DNA sample to 400µl with sterile water and add to the beads slurry. Otherwise mix aqueous layer from Phenol purification step with hydrated beads.
- Gently agitate the tube for 15 minutes at room temperature.
- Spin at 14000 RPM for 5 min. and transfer supernatant to an appropriately labeled new tube. The supernatant has DNA in diluted form.
- Volume of the supernatant can be reduced by passing through Microcon.
- Concentrated DNA can be used for PCR after quantification.

CHAPTER 6

BONE PROCESSING AND DNA ISOLATION FROM TOOTH & BONE

In some cases, bone samples are the only biological evidence available for analysis and identification of a deceased victim. Anthropological approaches were widely used before the advent of DNA technology and its application to identify the human remains. However, those approaches were limited in identifying the human remains in specific cases such as in severely damaged remains. Therefore, DNA technology for genetic identification is more beneficial.

6.1 FACTORS AFFECTING DNA ANALYSIS FROM BONE:

- **Morphology of bone:** Whether spongy, brittle, non-compact bones are contributing in the overall weight of the bone for further DNA analysis.
- **Quality of bone:** Whether the bone is degraded, damaged, burnt, charred, exhumed, fragmented bone remains etc.
- **Age of bone:** Whether the bone is fresh, old or archived.

6.2 CLEANING AND DECONTAMINATION OF BONE SURFACE:

- Before extraction, the bone or tooth specimen should be cleaned entirely of any soft tissue and dirt using a range of methods, such as scraping, brushing, and rinsing with water or sonication. A combination of sterile scalpels, sterile toothbrushes and running water should be used to clean the specimen.
- Clean bone surfaces with 10% bleach using either Kim wipes or a bleach-sterilized toothbrush to clean the outer surface of the specimen. Repeat this step 2 –3 times to destroy any dirt or surface contaminants.

Or

Use wash buffer for surface cleaning as follows:

- **WASH BUFFER (For 1ml wash Buffer)**

1% SDS	100 µl
25mM EDTA	50 µl
1µl Proteinase K (20mg/ml)	5µl
Sterile Milli-Q Grade water	845 µl

- Clean the surfaces with UV sterilized molecular grade water/ethanol.
- Remove internal surface in powdered form using an electric drill and an engraving cutter bit in a safety hood.

- Store bone powder in a sterile 2 ml polypropylene tube.

6.3 QUANTITY OF BONE SAMPLE

- **For bones up to 1.0g (approximately):** Bone will be consumed and must be documented in DNA extraction worksheet.
- **For bones 1.0g to 1.50g (approximately):** Consumption will be determined by the nature of the bone and whether significant weight will be lost during the processing steps. If the nature of the bone will make the weight drop below the availability to be re-tested (at least 0.50g) then, the bone should be consumed and noted in DNA extraction worksheet.

6.4 SAMPLING METHODS FOR BONE

Prior to sampling, document the description/appearance, weight after cleaning and measurements of the bone/tooth. Initials of the analyst and date of examination/cutting performed should be documented.

6.4.1 Cutting/Sonication/Crushing

- **Cutting:** Protective eyewear, lab coats, cut resistant gloves, sleeve protectors, and HEPA filtered facial masks should be worn when cutting a bone. Avoid breathing bone dust. Cutting of bone must be done under a biosafety hood.
- **Sonication:** Bones that are too small to be cut should be sonicated.
- **Crushing/Grinding of Bone and Tooth**: There are two ways majorly used for crushing of bone and tooth sample, which helps in transforming a piece of bone or tooth into powdered form.
- **Mortar and Pestle:** The tooth or bone sample can be crushed to powdered form using the mortar and pestle by applying manual grinding. Applied force and continuous hitting on the tooth or bone changes the solid pieces into powder form. In case of tooth, the root part was often used to isolate the DNA. Similarly, bone shaft piece was used to isolate the ample amount of DNA.

6.4.2 Milling/Processing with Liquid Nitrogen

Some small bone fragments may not be suitable for milling. Consider going straight to extraction after cleaning if the fragment does not yield an appropriate amount of bone powder after milling.

Freezer Mill: It is a small cryogenic mill, which is used to grind tough as well as temperature sensitive samples. It brings the sample to cryogenic temperature and grinds/ pulverize it by impacting a magnetic based steel impactor in the back and forth pattern against two closed end plugs. It helps in avoiding the contami-

nation due to its enclosed structure. Liquid nitrogen provides optimum cryogenic temperature for the grinding of tough sample. Add liquid nitrogen slowly into the mill up to the FILL-LINE to avoid splashing and boiling over. Cryo gloves and eye protection are required and the liquid nitrogen must be at the fill mark.

- Program and use the freezer mill as per manufacturer's instruction.
- Transfer the bone dust to a labeled tube. Ensure complete bone powder transfer by tapping bottom of cylinder. Record the weight of the bone powder.

6.5 ISOLATION OF DNA FROM BONE (ORGANIC EXTRACTION)

6.5.1 Decalcification of bone

Bones or bone powder can be decalcified by using 0.5M EDTA solution.

Bone Lysis Buffer:

- Proteinase K (20mg/ml) 100µl
- QIAGEN® ATL buffer 1ml
- 1M DTT (Dithiothreitol) 50µl
- 1% SDS 40µl

Add Bone Lysis Buffer in proportional amounts to saturate the sample.

6.5.2 Procedure:

- Transfer bone powder (appx.200 mg) in a fresh 1.5 ml micro centrifuge tube.
- To the sample, add bone lysis buffer to saturate. Vortex for 5 seconds and briefly centrifuge. Parafilm the tube.
- Incubate overnight at 56^{o}C for digestion in a shaking water bath/with mild rocking.
- Next day, short spin the micro centrifuge to force the condensation into the bottom of the tube.
- Add 50µl Proteinase K and again vortex for 5 seconds and briefly centrifuge.
- Parafilm and again keep for overnight digestion at 56^{o}C in a shaking water bath/with mild rocking.
- Spin the sample tube for 5 minutes at 14,000 rpm and transfer the supernatant into a new 1.5 ml microcentrifuge tube.
- Add 500µl phenol/chloroform/iso-amyl alcohol to the extract. Vortex briefly to attain a milky emulsion. Spin the tube in a micro centrifuge for 3 minutes at high speed.
- Transfer the upper aqueous phase (top layer) carefully to a new 1.5ml microcentrifuge tube.

Proceed to Microcon Purification Procedure and concentration of samples.

6.6 ISOLATION OF DNA FROM TEETH (ORGANIC EXTRACTION METHOD)

Larger teeth with no restorations should be chosen over smaller or restored teeth. Thus, non-restored molars are the tooth of choice for DNA recovery.

6.6.1 Cleaning of Tooth Surface:

- Clean the outer surface of the tooth with an alcohol swab, then with 10% bleach using either Kim wipes or a bleach-sterilized toothbrush to clean the outer surface of the tooth. Repeat this step 2 –3 times to destroy any dirt or surface contaminants.
- Clean outer surface of the tooth with UV sterilized molecular grade water/ethanol.
- Using a cutter, cut the tooth vertically, if soft pulp tissue is available in the pulp chamber, extirpate the pulp tissue with a sterilized needle and place the extirpated tissue into a 1.5 ml micro centrifuge tube.
- If no soft tissue is available, remove internal surface in powdered form using an electric drill and an engraving cutter bit in a safety hood.
- Store tooth powder in a 1.5 ml microcentrifuge tube.

6.6.2 Reagents

- Proteinase K (20mg/ml)
- ATL buffer
- 1M DTT (Dithiothreitol)
- 1% SDS

Add reagents in proportional amounts to saturate the sample.

6.6.3 Procedure:

- Transfer tooth powder in a fresh 1.5ml micro centrifuge tube.
- To the sample add 200µl ATL, 20µl Proteinase K, 10µl SDS and 20µl DTT. Vortex for 5 seconds and briefly centrifuge to force the sample into the extraction fluid.
- Keep for overnight digestion at 56^{o}C in a shaking water bath/with mild rocking.
- Spin the sample tube for 5 minutes at 14,000 rpm and transfer supernatant fluid into a new 1.5 ml microcentrifuge tube.
- Add 400µl phenol/chloroform/isoamyl alcohol to the extract. Vortex briefly to attain a milky emulsion. Spin the tube in a micro

centrifuge for 3 minutes.

- Transfer aqueous phase (top layer) carefully to a new 1.5ml microcentrifuge tube.

Proceed to Microcon Purification Procedure and concentration of samples.

CHAPTER 7

AUTOMATED DNA EXTRACTION

Automated DNA Extraction system is a Robotic technology to isolate the DNA from various sources in an easiest way. We can process a number of samples simultaneously in less time as compared to manual procedures. The system comes with different technologies i.e. Silica based and magnetic beads-based technology.

EZ1 instruments and the EZ1 DNA Investigator Kit reproducibly automate purification of genomic DNA from 1–6 samples (EZ1 Advanced and BioRobot EZ1) or 1–14 samples (EZ1 Advanced XL) encountered in Forensic, human identity, and biosecurity applications. Magnetic-particle technology provides high-quality DNA that is suitable for direct use in downstream applications, such as STR analysis. AutoMate Express is also based on the separation of DNA using the silica-coated magnetic beads. These methods also remove PCR inhibitors and even the smallest amount of DNA can be recovered from various samples. These processes consume less time as compared to manual procedures. Before processing of the samples, pretreatment protocols are required to be followed as per the guidelines mentioned in the following Table.

7.1 EZ1® DNA INVESTIGATOR®

7.1.1 Principle

Magnetic-particle technology combines the speed and efficiency of silica-based DNA purification with the magnetic particles. DNA is isolated from lysates through its binding to the silica surface of the particles in the presence of a chaotropic salt. The particles are separated from the lysates using a magnet. The DNA is then washed and eluted either in water or TE buffer. The user can choose elution volumes of 40 µl (EZ1 Advanced XL only), 50 µl, 100 µl, or 200 µl.

7.1.2 Protocol

- **Pretreatment protocols**
  Since the type of samples that can be processed using the EZ1 DNA Investigator Kit can vary greatly, there is also a variety of different pretreatments optimized for specific sample types.

- **DNA purification protocols**
  There are 3 DNA purification protocols, which can be used in conjunction with the pretreatment protocols. Within each protocol, the user can specify elution in water or TE buffer, with elution volumes of 40 µl (EZ1 Advanced XL only), 50 µl, 100 µl, or 200 µl.
  - **Trace Protocol**is a standard protocol that can be used with all

sample types.

- **Tip Dance Protocol**, in this the filter-tip moves back-and-forth relative to the worktable platform while pipetting. This enables processing of solid materials, such as swabs, fabrics, blood discs, or cigarette butts directly in the sample tube. However, when processing fluffy sample material such as cotton wool, it is recommended to remove solid material, if the sample material is precious.
- **Large-Volume Protocol** enables fully automated processing of starting volumes up to 500 µl. This allows efficient DNA purification from dilute samples with low concentrations of DNA, such as diffuse stains, as well as purification from samples that require larger volumes for thorough lysis.

7.1.3 Working with EZ1 Instrument

7.1.3.1 The Main Features of the EZ1 Instrument Include:

- Purification of high-quality nucleic acids from 1–6 or 1–14 samples per run.
- Small footprint to save laboratory space.
- Preprogrammed EZ1 Cards containing ready-to-use protocols for nucleic acid purification.
- Prefilled, sealed reagent cartridges for easy, safe, and fast setup of EZ1 instruments.
- Complete automation of nucleic acid purification, from opening of reagent cartridges to elution of nucleic acids, with no manual centrifugation steps.

7.1.3.2 Additional Features of the EZ1 Advanced and EZ1 Advanced XL include:

- Bar code reading and sample tracking.
- Kit data tracking with the Q-Card provided in the kit.
- UV lamp to help eliminate sample carryover from run-to-run and to allow pathogen decontamination on the worktable surfaces.

7.1.3.3 EZ1 Cards, EZ1 Advanced Cards, and EZ1 Advanced XL Cards

Protocols for nucleic acid purification are stored on pre-programed EZ1 Cards (integrated circuit cards).

7.1.3.4 Reagent Cartridges

Reagents for the purification of nucleic acids from a single sample are contained in a single reagent cartridge. Each well of the cartridge contains a particular reagent, such as magnetic particles, lysis buffer, wash buffer, or elution buffer.

7.1.3.5 Worktable

The worktable of EZ1 instruments is where the user loads samples and the components of the EZ1 DNA Investigator Kit.

- **First row**: Elution tubes (1.5 ml) are loaded here.
- **Second row**: Tip holders containing filter-tips are loaded here.
- **Third row**: Tip holders containing filter-tips are loaded here.
- **Fourth row**: Sample tubes (2 ml) are loaded here.
- Reagent cartridges are loaded into the cartridge rack.
- Heating block with 2 ml tubes in the reagent cartridges for lysis.

7.1.4 Workflow for EZI Operation

- Insert EZ1 Card into the EZ1 Card slot.
- Switch on the EZ1 instrument.
- Follow on-screen messages for data tracking.
- Follow on-screen messages for worktable setup.
- Start the protocol.
- Collect purified nucleic acids.
- UV decontamination.

(* EZ1 Advanced and EZ1 Advanced XL only)

SAMPLE TYPE	PRETREATMENT PROTOCOLS	PURIFICATION PRO-TOCOLS	SAMPLE AMOUNT	BUFFER G2	PROTEINASE K	DTT
Blood/ saliva	Protocol 1	Trace	Up to 50µl	140-190µl	30µl	No
FTA card	Protocol 1	Trace or tip dance	4 x 3mm punches	290µl	30 µl	No
Surface swabs	Protocol 1	Trace or tip dance	1 swab	290µl	25 µl	No
Cigarette butts	Protocol 1	Traceor Tip Dance	$1cm^2$	190µl	25µl	No
Chewing gums	Protocol 1	Trace or Tip dance	Up to 40mg	190µl	25µl	No
Nail scrapping	Protocol 1	Trace	Up to 40mg	190µl	25µl	No
Nail clip-pings	Protocol 1	Trace	1	160µl	35µl	20µl
Hair	Protocol 1	Trace	0.5-1 cm	160µl	35µl	20µl
Tissues	Protocol 1	Trace	Up to10mg	190µl	35 µl	No
Blood or saliva stains	Protocol 1	Trace or tip dance	$0.5cm^2$	290µl	25 µl	No

Semen stains	Protocol 1	Trace or tip dance	0.5cm^2	270µl	40µl	30µl
Sexual assault cases	Protocol 2	Trace	Varies	Up to 2.5 ml	40µl	40µl
Bones or teeth	Protocol 3	Trace	150-200 mg	0.5 M EDTA	40µl	No
Soil	Protocol 4	Trace or Tip Dance	Up to 0.5 g	100µl	No	No
Paper or	Protocol 1	Trace or Tip Dance	0.5 cm^2 to 2.5 cm^2	190µl	10µl	No

7.1.5 Pre-Treatment Protocols for Various Samples

7.1.5.1 Protocol 1

Pre-treatment for Various Casework and Reference Samples

- Place sample in 2 ml sample tube.
- Add proteinase K according to table mentioned above.
- Incubate at 56°C for 15 min or overnight in a thermomixer at 900 rpm.
- Continue with protocol: DNA purification using either Trace Protocol (samples do not contain solid samples) volume of lysate should be approx. 400ul.

7.1.5.2 Protocol 2

Pre-treatment for Epithelial Cells Mixed with Sperm Cells (differential)

- Place sample in 2 ml sample tube.
- Add 190µl buffer G2.
- Add proteinase K according to table mentioned above and mix thoroughly by vortexing for 10s.
- Incubate at 56°C for 1-2 h (do not exceed 2 hour).
- Vortex the tube once or twice during the incubation.
- Centrifuge the tube briefly.
- Remove any solid material from tube.
- Centrifuge at 13,000 rpm for 5 min and carefully transfer pellet in fresh tube
  (DNA from epithelial cells can be purified from this fresh tube using trace protocol).

For Male Fraction

- Wash the sperm cell pellet by resuspending the pellet in 500 µl buffer G2.
- Centrifuge at 13000 rpm for 5 min and discard the supernatant. Repeat the steps for three to five times.

- Add 160µl buffer G2 and resuspend the pellet.
- Add 40µl proteinase K and 40ul 1M DDT, mix thoroughly by vortexing.
- Incubate at 70°C for 10 min at 850 rpm in a shaker incubator. Centrifuge briefly.
- Continue with protocol DNA purification (Trace DNA protocol).

7.1.5.3 **Protocol 3**

Pre-treatment for Bones or Teeth

- Remove or discard bone or teeth surface. Grind remaining bone or teeth using tissue lyser.
- Place 150mg to 200mg of powdered bone into a 2ml microcentrifuge tube.
- Add 600µl -700µl of 0.5 M EDTA, incubate for 24 to 48 hours at 37°C.
- Now add 40µl proteinase K and incubate at 56° C.
- Centrifuge at 6000 rpm for 4 min and transfer 200µl in sample tube if proceeding through Trace protocol and 500ul of sample if large volume protocol is used.

7.1.5.4 **Protocol 4**

Pre-treatment for Soil

- Place 0.5 g of soil sample in 2ml sample tube.
- Add 900ul of distilled water. Resuspend the soil by vortexing, and incubate at 95°C for 10 minutes.
- Centrifuge the tubes at 8000 rpm for 10 minutes. Transfer the supernatant to another 2ml sample tube and add 190µl buffer G2.
- Mix by vortexing.
- Add 1 Inhibit X tablet and incubate at room temperature for 1 minute.

Mix by vortexing and centrifuge at 13,000 rpm for 2 minutes. Transfer 200µl of the supernatant to an EZI sample tube if proceeding with "Protocol: DNA Purification (Trace Protocol)" or transfer 500µl of the supernatant to sample tube if proceeding with "Protocol: DNA Purification (Large volume Protocol)".

7.1.6 **Purification Protocols**

7.1.6.1 **DNA Purification Protocol**

Procedure

- Insert EZ1 Advanced DNA Investigator Card completely into the

EZ1 Advanced Card slot, or the EZ1 Advanced XL DNA Investigator Card completely into the EZ1 Advanced XL Card slot.

- Switch on the EZ1 instrument.
- Press “START” to start protocol setup. Follow the on-screen instructions for data tracking.
- Press “1” for Trace Protocol, “2” for Tip dance protocol, “3” for large DNA protocol.
- Choose the elution buffer and volume, press “1” to elute in water or “2” to elute in TE buffer. Then press “1”, “2”, or “3”, (or “4”, EZ1 Advanced XL only) to select the elution volume.
- Press any key to proceed through the text shown on the display and star worktable setup. The text summarizes the following steps which describe loading of the worktable. Wear gloves when loading the required items on the worktable.
- Open the instrument door.
- Invert reagent cartridges twice to mix the magnetic particles. Then tap the cartridges to deposit the reagents at the bottom of their wells. Check that the magnetic particles are completely resuspended.
- Load the reagent cartridges into the cartridge rack. Note: After sliding a reagent cartridge into the cartridge rack, ensure that you press down on the cartridge until it clicks into place.
- Load opened elution tubes into the first row of the tip rack.
- Load tip holders containing filter-tips into the second row of the tip rack.
- Load opened sample tubes containing digested samples into the back row of the tip rack. Note: When using the data tracking option, ensure that the sample ID follows the same order as the samples on the worktable to avoid data mixup.
- Close the instrument door.
- Press “START” to start the purification procedure. The automated purification procedure takes 15–20 min.
- When the protocol ends, the display shows “Protocol finished”. Press “ENT” to generate the report file.
- The EZ1 Advanced and the EZ1 Advanced XL can store up to 10 report files.
- Open the instrument door.
- Retrieve the elution tubes containing the purified DNA. The DNA is ready to use.
- Discard the sample-preparation waste. If the purified DNA is to be analyzed by real-time PCR, tubes containing elute should first be applied to a suitable magnetic separator and transferred to a clean

tube in order to minimize the risk of magnetic particle carryover.

- Optional: Follow the on-screen instructions to perform UV decontamination of the worktable surfaces.
- To run another protocol, press "ESC", prepare samples as described in the relevant protocol, and follow the procedure from step 4 onward. Otherwise, press "STOP" twice to return to the first screen of the display, close the instrument door, and switch off the EZ1 instrument.
- Clean the EZ1 instrument.

7.2 AUTOMATE EXPRESS DNA ISOLATION SYSTEM

The Automate works on the chemistry of reagents. The reagents used for DNA extraction are:

- Prep Filer Express™ Forensic DNA Extraction Kit (for soft tissues)
- Prep Filer Express BTA™ Forensic DNA Extraction Kit (for hard tissues)

The Prep Filer Express™ Extraction kit is based on the property of magnetic particles which efficiently binds the DNA and a multi-component surface chemistry. During the washing steps the magnetic particles + DNA complex remains stable, at the same time it removes inhibitors and allows the efficient release of DNA during elution to recover a highest amount of pure DNA. Thirteen (13) samples can be isolated at one time.

7.2.1 Configuration

- Single IC card
- 20" (w) x 22" (d) x 22.5" (h)

7.2.2 DNA to be Extracted from Various Samples

- **Routine Samples:** Liquid Samples (Blood, Saliva & Semen), Body fluid stains, Swabs, Pulled hair roots etc.
- **Challenged Sample Types:** Bone & Teeth, Cigarette butts & Chewing gum, Toothpick & Mouth wash, Fingernails scrapings etc.

7.2.3 Instrumentation

- **13 Knobs:** In AutoMate Express, the 13-knob system is designed in a way to process 13 samples at a time.
- **Syringe Unit:** Syringe is mainly used to pick and drop the solution and reagents to mix them for the separation of DNA.
- **Magnetic Unit:** DNA extraction from the sample is based on the magnetic separation, where DNA gets bind to the magnetic particles and gets separated into this magnetic unit.

- **Piercing Unit:** It is incorporated to pierce the cartridges comprised of reagents which are sealed to avoid spill.
- **Heating Unit:** It provides the optimal temperature for the DNA isolation. It heats at 95°C and provides elution at 70°C.
- **Tube & Tube Rack:** It holds the sample tube, elution tube, tip &tube. It comprises of four sections:
    - **S**- Sample Tube
    - **T_2**- Tip & Tube hold
    - **T_3**- Empty
    - **E**- Elution
- **Cartridge Rack:** It holds the cartridges which consist of the chemicals and reagents for DNA extraction.
- **Cartridge:** It is the section filled with various chemicals and reagents which helps our DNA to be extracted from the sample. There are 7 compartments filled i.e.;
    - Lysis buffer
    - Magnetic particles
    - Isopropanol
    - Wash buffer 1
    - Wash buffer 2
    - Wash buffer 3
    - Elution buffer
    - Other than these 7 compartments, 8, 9 &10, 11 are empty.
    - 12 is Heating chamber for Elution

7.2.4 Workflow for Extracting DNA

The sample was prepared for the lysis by adding the Prep Filer Lysis Buffer in the sample and incubated for 36 hours at 56°C.

- Prep Filer reagents from the specific kits according to the type of sample were added (Prep Filer Express and Prep Filer Express BTA).
- Prepare the instrument.
- Start the automated extraction run. Store the extracted DNA for further use.

7.2.5 Protocol for Various Samples

7.2.5.1 Body Fluids Protocol

For processing the various body fluids such as: Liquid samples (blood, saliva), Blood on FTA paper or fabric, saliva or semen on any kind of fabric, Buccal swabs, etc., the materials required for the lysis are as mentioned in Chapter 5 DNA Extraction. The body fluids such as blood sample on FTA or buccal swab

samples don't need the overnight incubation. They set for PCR directly, skipped the automate run step.

7.2.5.2 Body Fluid Stains

- A piece of fiber containing body fluid stain was placed in the LySep™ column proceeded by the addition of prepared PrepFiler™ Lysis buffer along with DTT.
- Place the sample tube combined with LySep column (LySep™ column assembly) on thermo mixer and incubated at 70°C overnight.
- The sample tubes were taken out carefully by detaching them from LySep™ column.
- The lysate was then transferred to a new sample tube and put directly for the automate run to isolate the DNA.

7.2.5.3 Bone and Tooth Protocol

Sampling of Bone and Tooth: as mentioned in Chapter 6 of DNA Isolation from Bone and Tooth.

7.2.6 Preparation of Lysate

- 50 gm of crushed powder was taken in a fresh new Prep Filer™ Bone and Tooth screw cap lysate tube.
- To the crushed powder, 3µl DTT solution, 7µl Proteinase K and 220µl Prep Filer™ BTA lysis buffer was added.
- The tube was then vortexed and centrifuged.
- Incubate at 56°C for 36 hours on Thermo mixer.

7.2.7 Automate Run

- Prep Filer™ BTA screw cap lysate tube taken out from the incubation and centrifuge for 3 min at 1000 rpm.
- Separate out the clear lysate to the new Prep Filer™ Sample Tube.
- Prepare the instrument for automate run.
- Start the AutoMate Express DNA Isolation System.

7.2.8 Trace Evidence Protocol

Substances which are used for the DNA isolation could be chewing gum, cigarette butts and adhesive tapes containing saliva or blood samples. The protocol followed for the adhesives is same as for the bone and tooth.

CHAPTER 8

DNA QUANTIFICATION

The quantification of total amount of human DNA isolated from a forensic evidence item is crucial for DNA normalization prior to the Short Tandem Repeat DNA analysis and a quality assurance standard requirement. The quantitative PCR is a very sensitive method and is routinely used to measure the concentration of total human and human male DNA and to assess the DNA degradation/inhibitors in forensic samples.

8.1 QUANTIFICATION BY AGAROSE GEL ELECTROPHORESIS

There are many types of electrophoresis methods but Horizontal gel electrophoresis is most commonly used method for checking the quality and quantity of DNA on 1.0 % Agarose gel. The separation occurs because the smaller molecules pass through the pours of the gel more easily than the larger molecules i.e. gel is sensitive to physical size of the molecule. The mobility of molecules during electrophoresis also influenced by the gel concentration and the volume of agarose gel electrophoresis depend upon the casting tray. The gel is viewed under U.V. Illuminator and the fluorescence was checked.

8.2 ABSOLUTE QUANTIFICATION OF HUMAN DNA THROUGH REAL TIME PCR SYSTEMS (ABI 7500 SDS)

Real-Time PCR 7500 system uses fluorescent based PCR chemistries to provide quantitative detection of nucleic acid sequences using real time analysis and quantitative detection of nucleic acid sequences using end point and dissociation curve analysis. For absolute quantification, Human Quantifiler, Y- Quantifiler, Quantifiler® Duo and Quantifiler™ Trio Kits are used on the Real-Time PCR 7500 system (Applied Biosystem, Foster City, CA) platform as per recommended protocol.

These systems are based on sets of oligonucleotide PCR primers specific for a target DNA sequence, plus a TaqMan Probe, labeled with a 5' fluorescent reporter dye and a 3' non-fluorescent quencher, that is homologous to the amplicon region between the PCR primers. At the start of PCR thermal cycling, all TaqMan probes are intact and in the close proximity of the reporter dye and quencher suppress the fluorescence of the dye molecules. During the course of synthesizing new DNA strands from the target Template, the Taq Polymerase enzyme encounters the Taqman probe annealing to the template and hydrolyzes it with its 5' to 3' exonuclease activity. The hydrolysis of the probe separates the reporter dye and quencher which allows the dye molecules to fluoresce more intensely. This hydrolysis occurs in proportion to the amplification of the target sequence and therefore the fluorescence signal. Eventually the fluorescence signal crosses a specific threshold defined as "Threshold Cycle", or CT. It is at this

point that the fluorescent signal has accumulated to the point where it can be detected over background fluorescence. There is an inverse mathematical relationship between the starting copy number of target sequence molecules and the resulting CT, which allows samples to be quantified reliably.

8.2.1 Technical Notes

- Monitor the accumulation of PCR product while amplification is occurring.
- Analyze cycle to cycle changes in fluorescent signal generated during the three phases of PCR.

8.2.2 Three phases of PCR

- **Geometric phase:** This is an exponential phase where doubling of product occurs at every cycle.
- **Linear phase:** One or more of the reaction components have become limiting and product accumulates arithmetically.
- **Plateau (End point of reaction):** The reaction has stopped. No more products are being made.

8.2.3 Regression Coefficients:

- **Slope:** Indicates the PCR amplification efficiency for the assay. A slope of -3.3 indicates 100% amplification efficiency.
- **Y-intercept:** Indicates the expected C_T value for a sample with Qty =1 (for example, 1 ng/µl).

8.2.4 Quantifiler® Duo DNA QUANTIFICATION

In a single real-time PCR reaction, the Quantifiler® Duo kit enables simultaneous quantification of human DNA and human male DNA (sex determining region Y target), as well as detection of PCR inhibitors using a synthetic Oligonucleotide sequence as an internal PCR control. The Quantifiler® Duo DNA Quantification Kit contains all the necessary reagents for the amplification, detection, and quantification of a human-specific DNA target and a human male-specific DNA target. The reagents are designed and optimized for use with the Applied Biosystems 7500 Real-Time PCR System and SDS Software.

8.2.4.1 Equipment, Materials, and Reagents

- Quantifiler® Duo DNA Quantification Kit
- Vortex
- TE Buffer
- Computer with HID Real Time PCR software v.1.0 7500 Sequence Detection System
- 96-well Optical Reaction Plates
- Optical Adhesive Covers

- Centrifuge with plate adaptor
- MicroAmp Splash Free Support Base

8.2.4.2 Preparation of the Standards for Quantifiler® Duo Kit

Human DNA standards will be prepared using a serial dilution of the standard DNA (200ng/µl) provided in the Quantifiler kits and TE buffer (Sterile water or dilution buffer from the Quantifiler Duo Kit are appropriate options). At least one quantification blank consisting of 23 µl of master mix and 2 µl Dilution Buffer shall be run in one well of each plate as a negative quantification control. Fresh standards should be made approximately every week. These standards will range from 50ng/ µl to 23pg/ µl and will be prepared as follows:

Table 1. Standard Dilution Series for Duo Kit.

Standards	Concentration (ng/µl)	Serial dilutionswith TE/Dilution Buffer	Dilution Factor
STD.1	50.0	10 µl (200ng/µl stock) + 30µl TE/Dil. Buffer	4x
STD.2	16.7	10 µl (Std.1) + 20 µl TE/Dil. Buffer	3x
STD.3	5.56	10 µl (Std.2) + 20 µl TE/ Dil. Buffer	3x
STD.4	1.85	10 µl (Std.3) + 20 µl TE/ Dil. Buffer	3x
STD.5	0.62	10 µl (Std.4) + 20 µl TE/ Dil. Buffer	3x
STD.6	0.21	10 µl (Std.5) + 20 µl TE Dil. Buffer	3x
STD.7	0.068	10 µl (Std.6) + 20 µl TE/ Dil. Buffer	3x
STD.8	0.023	10 µl (Std.7) + 20 µl TE/ Dil. Buffer	3x

- Label eight tubes 1-8 to prepare standards 1-8.
- Dilutions of standard DNA provided in the kit are prepared fresh for each set of experiment.
- Any mistake or inaccuracies in making the dilutions affect the quality of results.
- Mix the dilutions thoroughly.
- Standards 2 to 8 should be prepared after vortexing properly and should be mixed thoroughly before transfer to next tube.

8.2.4.3 Preparation of the Reaction Mix

Calculate the volume of each component needed to prepare the reaction, using the table below:

Table 2. Preparation of the Reaction mix

Reagents	Reaction volume (µl)
Quantifiler Reaction Mix	12.5 µl
Quantifiler Primer Mix	10.5 µl
Template DNA (Test sample)	2.0 µl
Final volume	25.0 µl

8.2.4.4 Preparation of Reaction Plate

- Prepare the reagents.
- Thaw the primer mix completely, then vortex 3 to 5 seconds and centrifuge briefly before opening the tube.
- Swirl the Quantifiler PCR Reaction Mix gently before using.
- Vortex the PCR Mix 3 to 5 seconds, then centrifuge briefly.
- Dispense the 23 µl of the PCR mix into each reaction well.
- Add 2 µl of sample, standards, positive control and negative control to the appropriate wells.
- Seal the reaction plate with the Optical Adhesive Cover.
- Centrifuge the plate at 2000 rpm for 5 minutes to remove any bubbles.
- Prepare the data sheet in Real-Time PCR System.
- Set all the data in Real-Time PCR according to the reaction plate.
- Start the Real-Time PCR machine and allow it to run as per manufacturer's recommended protocols.

8.2.4.5 Cycling Conditions for Real-Time PCR

The thermal conditions are as follows:

- 50°C for 2 minutes
- 95°C for 10 minutes
- 40 cycles of
- 95°C for 15 seconds
- 60°C for 1 minute

8.2.4.6 Analyzing the Run

- After the run is complete, turn off the instrument.
- Analyze the run by selecting the green "Analyze" icon at the upper right hand corner of the "Analysis" tab. Be sure the default analysis settings are used (i.e. Threshold: 0.2, Baseline Start Cycle:3, Baseline End:15)
- Verify the three quality indicators as specified below:

- **Slope** – this quality indicator indicates the amplification efficiency of the standard reactions. The slope should range from -2.9 to -3.6 (-3.3 indicate 100% amplification efficiency). If the slope falls outside this range the run may need to be repeated.
- **R2**- the co-relation coefficient indicates the statistical significance of the standard curve. A passing run should have an R2 value greater than 0.98. If the correlation coefficient falls below this value, the run may need to be repeated.
- **Y-intercept**- this value indicates the expected CT value for a sample with quantity of 1. A running list of Y-intercept values is kept in logs adjacent to each instrument. The Y-intercept for a given run should be similar to those from previous runs. This list can be used as a tool to troubleshoot potential quantification issues.

- Up to two non-concordant points may be removed from the standard curve in order to make minor adjustments should the slope and /or R2 fall out of range. The two points removed should be of different concentrations. Any standards removed should be noted on the Quantifiler Worksheet in the comments section.

8.2.4.7 Exporting the Results

- Following data analysis, the results should be exported to the Quantifiler Worksheet, which is located on the network: Q:\7500 Runs\7500 Templates and Forms\HID v1 templates.
- Select "Export" from the application toolbar.
- On the "Export Properties tab" specify the "Export File Name," set the file type to "*.xls" and designate the Export file Location on the network. The default settings (i.e., "Results in the field "Select Data to Export," "One File" in the field "Select One File or Separate Files") should not be altered.
- On the customize Export Tab, make sure "Well, Sample Name, target name, Task, CT, Quantity and M:F ratio" in the "Select Result Content" are checked, and appear in that order in the "Results Export" table. These are the default settings and should not need alteration.
- Select "Start Export". Open the "*.xls" export file from the network and select the complete set of cells or rows to be copied to the Quantifiler Worksheet.
- Open the Quantifiler Worksheet template, select the tab titled "Put SDS Data Here", and paste the data from the export file beginning in row A16.
- Select the tab titled "Quantification" to view the results.

- The fields at the top of the Quantifiler worksheet need to be manually entered. This information includes the quality indicator values, lot numbers, case number, analyst, date run name, comments section and instrument name.
- Print the results for inclusion in the case notes. It is only necessary that this record include the applicable case information.

8.2.4.8 Limitations/Notes

- Preparation of the DNA quantification standards is crucial for accurate analysis of run data. Any mistakes or inaccuracies in making the dilutions directly affect the quality of results. The quality of pipettes and tips and the care taken in measuring and mixing dilutions affect accuracy.
- Samples with a quantification value less than 200 ng/µL may be used to estimate target volumes for amplification. Samples with values greater than 200 ng/ µL should be diluted and re-quantified.
- All the samples that are potentially degraded and /or inhibited should be amplified regardless of the quantification value.
- All the samples selected for Y STR testing will be amplified regardless of the quantification value.
- Samples with a male quantification value but a zero human quantification should still be amplified for autosomal STRs.
- It may be appropriate in certain circumstances to terminate analysis when the human and male quantification values are zero. When making this decision, the analyst should carefully consider the sample type, expected levels of DNA, probative nature of the sample and the results of other samples in the case. The decision to terminate analysis must be approved by a supervisor in the unit. The supervisor will document this approval on the quantification worksheet. Justification for terminating the analysis will also be documented.
- Samples with IPC CT values of 31 or greater will be automatically highlighted on Quantifiler worksheet to indicate that the extract may be inhibited. Samples with high concentrations may also have IPC CT values of 31 or greater and should not be confused with an inhibited sample. If the sample volume is not a limiting factor, the inhibited sample should be diluted and re-quantified. If re-quantification will limit application opportunities the inhibition should be carefully considered during the amplification step. It should be noted that inhibition seen during real time PCR is not absolute indicator that there will be inhibition seen in downstream amplifications.
- The Quantifiler Duo kit simultaneously quantitates male and female

DNA in DNA mixtures. The male: female ratio can be used to dictate case approach.

8.2.5 Quantifiler™ Trio DNA Quantification

Quantifiler™ Trio DNA quantification kit quantifies the total amount of amplifiable human (and higher primate) DNA and human male DNA in a sample using Real Time PCR 7500 Sequence Detection System. The DNA quantification assay combines three assays. Each amplification well contains small human DNA, large human DNA, and human male DNA target-specific assays and an internal PCR control (IPC) assay. The target-specific assays consist of two primers for amplifying the small human DNA (80 bases), large human DNA (214 bases), or human male DNA (75 bases) and one TaqMan (MGB or QSY quencher) probe labelled with VIC dye (small human DNA), ABY dye (large human DNA) or FAM dye (human male DNA) for detecting the amplified sequence. The IPC assay consists of IPC template DNA (a 130 base synthetic sequence not found in nature), two primers for amplifying the IPC template DNA, and one TaqMan QSY quencher probe labelled with JUN dye for detecting the amplified IPC DNA. Successful amplification of the PCR control and the target DNA indicates that amplifiable DNA was detected. A scenario where the PCR control amplifies, but the sample does not, could indicate that sufficient amplifiable DNA is not present.

8.2.5.1 Equipment, Materials, and Reagents

- Quantifiler Trio Kit
- 96-well reaction plate
- Adhesive sealing supplies
- Computer with HID Real Time PCR software v.1.0 7500 Sequence Detection System
- Vortex
- Microcentrifuge tubes
- Tabletop centrifuge with 96-well plate adapters
- Pipettes and pipette tips
- MicroAmp Splash Free Support Base

8.2.5.2 Preparation of the Standards for Quantifiler™ Trio Kit

Human DNA standards will be prepared using a serial dilution of the standard DNA (100ng/µl stock) and dilution buffer from the Kit are appropriate. At least one quantification blank consisting of 23µl of master mix and 2µl Dilution Buffer shall be run in one well of each plate as a negative quantification control. Fresh standards should be made approximately every week. These standards will range from 50ng/ µl to 0.005ng/µl and will be prepared as follows:

- Label five microcentrifuge tubes (e.g., STD.1, STD.2 STD.3, etc)

- Dispense the required amount of diluents to each tube.
- Prepare STD.1.
- Vortex the Quantifiler Human DNA Standard for 3 to 5seconds.
- Add the calculated amount of the standard to the tube for STD.1.
- Mix the dilution thoroughly.
- Prepare Standards 2 through 5.
- Add the calculated amount of the prepared standard to the tube for the next standard.
- Mix the standard thoroughly and repeat the above steps until the dilution series is complete.

Table 3. Standard Dilution Series for Trio Kit.

Standard	Concentration (ng/µl)	Volume of 100ng/µL stock and dilution buffer	Dilution Factor
STD. 1	50	10 µl stock + 10 µl dilution buffer	2x
STD. 2	5	10 µl std 1 + 90 µl dilution buffer	10x
STD. 3	0.5	10 µl std 2 + 90 µl dilution buffer	10x
STD. 4	0.05	10 µl std 3 + 90 µl dilution buffer	10x
STD. 5	0.005	10 µl std 4 + 90 µl dilution buffer	10x

8.2.5.3 Preparing the Reactions/Loading the Instrument:

- Vortex the Quantifiler® Trio Primer Mix and then centrifuge. Add the volume as provided in the Setup Worksheet to a sterile 2ml tube.
- Gently vortex the Quantifiler® Trio THP Reaction Mix. Add the volume as provided in the Setup Worksheet to the 2ml tube already containing the Primer Mix. This is now the Master Mix.
- Assemble a 96-well optical plate into a Support base. Do not place the optical plate directly onto the bench topin order to protect it from scratches and other particulate matter.
- Vortex the Master Mix and then centrifuge. Dispense 18 µl of Master Mix into each well of the optical plate as per the Setup Worksheet.
- Add 2 µl of each Standard (in duplicate), control and sample to the appropriate wells.
- Seal the plate with an optical adhesive cover. Centrifuge the plate at 2000 rpm for approximately 5 minutes.
- To prepare the master mix (primer plus reaction mix) calculate the volume of each component needed to prepare the reactions using the table below. Include additional reactions to provide excess volume for the loss during reagent transfers.

Table 4. Preparation of the Reaction mix

Component	Volume per Reaction (µl)
Quantifiler Trio Primer Mix	8.0
Quantifiler THP Reaction Mix	10.0

- To load the plate, push in the button on the tray at the front of the instrument. The tray will move out to accept the reaction plate. Ensure the plate is oriented correctly in the upper left handcorner. Close the tray by pushing it.
- Now start the Real-Time PCR machine and allow it to run as per manufacturer's recommended protocols.

8.2.5.4 Analysis of Quantifiler Trio Data

Once the assay has finished, click on the green Analyze button in the top right corner of the screen.

8.2.5.5 Exporting Data

- In the Experiment Menu, click Analysis, then click either View Plate Layout or View Well Table.
- Select the wells to export.
- Complete the Export dialog box and export the data.
- In the toolbar, click Export.
- Select Results as the type of data to export.
- Select Separate Files or One File from the list.
- Enter a file name and export location.
- Click Start Export to export the data to the selected file(s).

8.2.5.6 Assessing the Standard Curve:

- The standard curve is a graph of the CT of quantification standard reactions plotted against the starting quantity of the standards.
- The range of the standard curve shall be between the following:

Targets	**Slope range**
Small Autosomal (SA):	-3.0 to -3.6
Large Autosomal (LA):	-3.1 to -3.7
Y Target (Y):	-3.0 to -3.6

The R^2 shall be ≥ 0.99.

- The standard curve for the Quantifiler™ Trio Kits is linear from 5 pg/µL to 100 ng/µL.

8.2.5.7 Assessing the Small Autosomal Target

- In the Experiment Menu, go to Analysis and QC Summary.
- This page provides a snapshot of the data and automatically flags possible sample issues.
- In the Analysis Summary tab, any such samples are hyperlinked to the View Plate Layout on the right hand side of the screen.
- Within the View Plate Layout, flagged wells have a yellow triangle with an exclamation point.
- Highlight the well(s) of interest in the View Plate Layout tab, and then select the View Well Table tab. The wells will be highlighted a darker blue in this tab (scroll through).
- Assess the quality of the data of the samples and controls by evaluating the IPC and Trio (Large and Small Autosomal Target), and Trio Male Target results.

Trio. Human S.A. Target (VIC® Dye) **Trio Human L.A. Target (ABY Dye)** **Trio Human Male Target (FAM™ dye)**	**Trio IPC (JUN™ Dye)**	**Interpretation**
No amplification	Amplification	Negative result (no human DNA detected)
No amplification	No amplification	Invalid result may be caused by severe PCR inhibition, improper formulation of reagents, or failure of critical assay components
Amplification, Sample quantity >5ng/ul	Amplification appears reduced relative to average IPC CT value for standards	High sample concentration may suppress IPC amplification that leads to inconclusive IPC results.
Amplification (high CT and low delta Rn), more pronounced in T. Large Autosomal targets.	No amplification or amplification appears slightly reduced relative to the average IPC Ct values for quantification Standards.	PCR inhibitor present

8.2.5.8 NTC (Negative Template Control)

Due to the sensitivity of the real-time PCR method, extremely low levels of DNA (levels that do not affect downstream applications such as STR analysis) can be detected. It is possible to detect CT values <40 for an extraction blank and negative control samples while performing a real-time PCR reaction. If the

other negative controls within the same assay show CT values greater than 40, the Forensic Scientist may proceed with the amplification of the samples. Positive amplification occurs when the Ct value for the target is <40.

8.2.5.9 Negative Controls

If the CT value of a negative extraction control (for either the Trio Human or Trio Male detectors) is less than 40, the processing of samples shall continue, realizing that DNA may be observed in those controls when amplified.

8.2.5.10 Samples

Samples shall be evaluated by taking the IPC results into consideration.

8.2.5.11 Invalid IPC

If both the IPC and the autosomal/male targets fail to amplify (undetermined), then it is not possible to distinguish between the absence of DNA, PCR reaction failure, or inhibition. Such samples shall be re-quantified with dilutions (unless a dilution of the sample has been quantified and valid IPC results were obtained).

8.2.5.12 Inconclusive IPC

If extremely high concentrations of DNA are present in a sample, competition between the human/male targets and the IPC for PCR reagents may suppress the IPC, which results in a low CT for the human/male targets and a CT higher or undetermined for the IPC. (Note: this situation is not necessarily inhibition; therefore, no notations shall be made as such.) Alternatively, if the CT is high for the human/male target as well as the IPC, then inhibition may be present. Inhibition may also be indicated when the CT is undetermined for the IPC and the CT is high for the human/male targets.

8.2.5.13 Negative Result

If the CT for the IPC is within an acceptable range in comparison to the CT for the IPC observed for the DNA Standards within the run and the autosomal/male targets are undetected, then this is a valid negative result (i.e., no DNA is detected).

8.2.5.14 PCR inhibition

No amplification or weak amplification of the IPC may indicate PCR inhibition (partial or complete) in the sample. In addition, suppressed amplification (high CT value and low Rn value) of the human and/or male-specific targets can occur due to PCR inhibition. This is typically more pronounced in the large autosomal target than the small autosomal target since the large autosomal target is more susceptible to inhibitory effects.

8.2.5.15 Analysis of Quantifiler Trio Data in Microsoft Excel

- Open the exported results in Excel.
- Open the DNA workbook, Dilution Calculation Tab (QI Agility or Manual) within the DNA Excel Worksheet. Copy the data into the Raw Data tab of the Dilution Worksheet.
- In the Dilution Worksheet tab, the data is now present in the upper portion of the worksheet. Copy the samples (highlighting the sample names only) and paste them into the Dilution Calculation Worksheet (Human) or (Male) portion.
- The Dilution Worksheet automatically calculates the amount of DNA to 0.5ng to 1ng/µl. For samples, the samples which require dilution due to large quantities, the Dilution Worksheet provides the amounts necessary to create a 1ng/µl or 0.5ng/ul dilution. Note: the Dilution Worksheet allows the Analyst to adjust the raw extract volume to be used in making the dilution, but is set to default with 5 µl.
- Samples that have a male:female mixture ratio of greater than 1:500 and meet the criteria set forth in the Procedure for Analysis and Interpretation of Y-STR Amplification shall be amplified with appropriate Y-STR kit. Samples with a male:female ratio greater than 1:2000 shall not be amplified, unless the male quant value is above 0.005 ng/ul.
- Samples below the quantification value of 0.005ng/µl shall not be amplified.
- Print the Dilution Worksheet as a PDF and add it to the PDF file(s) generated with the Setup Worksheet, standard curve, and results. The Dilution Worksheet provides the information necessary to perform further amplifications using various kits. This PDF, along with the instrument file, shall be added to the cases repository for documentation.

CHAPTER 9

AMPLIFICATION (POLYMERASE CHAIN REACTION)

Highly degraded biological samples may be encountered in cases of mass disasters, missing persons and other forensic casework. As biological tissues degrade, DNA becomes progressively more fragmented and a decrease in the ability to gain an accurate DNA profile result. The successful typing of highly degraded DNA further complicated by having chemical modifications (i.e. DNA damage). Such highly degraded samples often produce incomplete or no STR profiles. To analyze low copy number of DNA samples in forensic practice, alternate genotyping strategies such as mini-STRs have proven to be more successful in profiling such difficult samples. For STRs markers the repeat unit length range is from 3 to 7 bases.

9.1 5-DYE CHEMISTRY KITS:

Table-1. 5-dye Chemistry Kits

S.NO.	NAME OF KIT	NUMBER OF LOCI	NAME OF LOCI
1.	**AmpF*l* STR® Identifiler®**	16 loci	D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Amelogenin, D2S1338, D19S433, CSF1PO, D16S539, D7S820, D13S317, and D5S818.
2.	**AmpF*l* STR® Identifiler® Plus**	16loci	D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, Amelogenin, D5S818, FGA.
3.	**AmpF*l* STR® Y-Filer™**	17 loci	DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385, DYS393, DYS391, DYS439, DYS635, DYS392, YGATAH4, DYS437, DYS438, DYS448.
4.	**AmpF*l* STR® Minifiler™**	8 loci	D13S317, D7S820, Amelogenin, D2S1338, D16S539, D18S51, CSF1PO and FGA.
5.	**PowerPlex® Y23**	23 loci	DYS576, DYS389I/II, DYS448, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385a/b, DYS456, Y-GATA-H4.

6.	**Investigator Argus X-12 QS**	12 loci	QS1, Amelogenin, DXS10103, DXS8378, DXS10101, DXS10134, DXS10074, DXS7132, DXS10135, DXS7423, DXS10146, DXS10079, DXSHPRTB, DXS10148, D21S11.
7.	**PowerPlex® 21**	21 loci	D1S1656, D2S1338, D3S1358, D5S818, D6S1043, D7S820, D8S1179, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, Amelogenin, CSF1PO, FGA, Penta D, Penta E, TH01, TPOX and vWA.
8.	**PowerPlex® Fusion**	24 loci	D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01 ,vWA , D21S11 , D7S820 ,D5S818 ,TPOX , DYS391, D8S1179 , D12S391 , D19S433 , FGA , D22S1045.

9.2 6-DYE CHEMISTRY KITS:

Table-2. 6-dye Chemistry Kits

S.NO.	NAME OF KIT	NUMBER OF LOCI	NAME OF LOCI
1.	**GlobalFiler™**	24 loci	D3S1358, vWA, D16S539, CSF1PO, TPOX, Yindel, Amelogenin, D8S1179, D21S11, D18S51, DYS391, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D12S391, D2S1338.
2.	**Yfiler™ Plus**	27 loci	DYS576, DYS389I, DYS635, DYS389II, DYS627, DYS460, DYS458, DYS19, YGATAH4, DYS448, DYS391, DYS456, DYS390, DYS438, DYS392, DYS518, DYS570, DYS437, DYS385 a/b, DYS449, DYS393, DYS439, DYS481, DYF387S1, DYS533.
3.	**PowerPlex® Fusion 6C**	27 loci	Amelogenin, D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11,D7S820, D5S818, TPOX , D8S1179, D19S433, D12S391, SE33, D22S1045, DYS391, FGA,

			DYS576, DYS570.
4.	**Investigator®24plex QS**	24 loci	Amelogenin, TH01, D3S1358, vWA, D21S11, TPOX, DYS391, D1S1656, D12S391, SE33, D10S1248, D22S1045, D19S433, D8S1179, D2S1338, D2S441, D18S51, FGA, QS1, D16S539, CSF1PO, D13S317, D5S818, D7S820, QS2.

NOTE: A dedicated area, such as a biological hood or a separate room, should be used for preparing PCR amplification reactions. All equipments and supplies used to prepare amplification reactions should be kept in this dedicated "clean" area at all times. Do not use these items to handle amplified DNA or other potential sources of contaminating DNA. Trace amounts of amplified DNA, if carried over into other samples before amplification, can lead to results that are not interpretable. **Do Not** bring amplified DNA, or equipment and supplies used to handle amplified DNA into the designated Pre- PCR area.

Wear clean disposable laboratory gloves while preparing samples for PCR amplification. Change gloves frequently or whenever there is a chance that these have been contaminated with DNA. The positive amplification Control DNA (9947A and 2800M) for autosomal STRs, 007 for Y-STRs and Mini-STRs and negative amplification control must be amplified with each set of amplifications.

9.3 EQUIPMENT & MATERIALS

- 96 well PCR System 9700 (Veriti)
- Pipettes - 2 µL, 10 µL, 20 µL, 100 µL and/or 200 µL
- Micro centrifuges
- 0.2 ml thin walled Micro Amp® micro centrifuge tubes
- Aerosol resistant pipette tips
- Biological Safety Hood
- Microcentrifuge tube racks
- 1.5 mL microcentfuge tubes
- Kim wipes
- Gloves
- Calculator

9.4 REAGENTS

- Multiplex primers
- Reaction Buffers
- Amplification Grade Water

- DNA Control 9947A
- DNA Control 007
- DNA Control 2800M
- AmpliTaq Gold® DNA Polymerase

After quantification, dilute all the samples in the range of about 0.1-0.2ng/µl of DNA for each sample. Record all the samples that need to be amplified in the different Amplification Sheets as required**.**

9.5 AMPLIFICATION RECIPES

9.5.1 Prepare master mix for **AmpFl STR® Identifiler® Plus** as follows:

Table-3. Master mix for AmpFl STR® Identifiler® Plus

PCR Master Mix Component	**Volume per sample**
AmpF*l*STRIdentifiler® PCR reaction mix	10 µl
AmpF*l*STRIdentifiler® Primer set	5 µl
Total Master Mix volume	15 µl
Master Mix Volume to be added	15 µl
Template DNA to be added	10 µl
Total Reaction Volume	25ul

9.5.2 Prepare master mix for **GlobalFiler™** as follows:

Table-4.Master mix for GlobalFiler™

PCR Master Mix Component	**Volume per sample**
GlobalFiler™PCR reaction mix	7.5 µl
GlobalFiler™ Primer set	2.5 µl
Amplification Grade water	5 µl
Total Master Mix volume	15 µl
Master Mix Volume to be added	15 µl
Template DNA to be added	10 µl
Total Reaction Volume	25 µl

9.5.3 Prepare master mix for **AmpF*l*STR® Y-Filer™ Plus** as follows:

Table-5.Master mix for AmpF*l*STR® Y-Filer™ Plus

PCR Master Mix Component	Volume per sample
AmpF*l*STR® Y-Filer™ Plus PCR reaction mix	10 µl
AmpF*l*STR® Y-Filer™ Plus Primer set	5 µl
Total Master Mix volume	15 µl
Master Mix Volume to be added	15 µl
Template DNA to be added	10 µl
Total Reaction Volume	25 µl

9.5.4 Prepare master mix for **AmpF*l*STR®Minifiler™** as follows:

Table-6. Master mix for AmpFlSTR® Minifiler™

PCR Master Mix Component	Volume per sample
AmpF*l*STR®Minifiler™ PCR reaction mix	10 µl
AmpF*l*STR®Minifiler™ Primer set	5 µl
AmpliTaq Gold® DNA polymerase (5U/µl)	0.5 µl
Total Master Mix volume	15.5 µl
Master Mix Volume to be added	15 µl
Template DNA to be added	10 µl
TotalReaction Volume	25 µl

9.5.5 Prepare master mix for**PowerPlex® Fusion 6C** as follows:

Table-7. Master mix for PowerPlex® Fusion 6C

PCR Master Mix Component	Volume per sample
PowerPlex® Fusion 6C PCR reaction mix	5 µl
PowerPlex® Fusion 6C Primer set	5 µl
Amplification Grade water	10 µl
Total Master Mix volume	20 µl
Master Mix Volume to be added	20 µl
Template DNA to be added	5 µl
Total Reaction Volume	25ul

9.5.6 Prepare master mix for **Investigator Argus X-12 Kit** as follows:

Table-8. Master mix for Investigator Argus X-12 Kit

PCR Master Mix Component	Volume per sample
Reaction mix A	5 µl
Primer mix	2.5 µl
Taq DNA Polymerase	0.6 µl
Total Master Mix volume	8.1 µl
Master Mix Volume to be added	8 µl
Template DNA to be added	Variable
Amplification Grade water	Variable
Total Reaction Volume	25ul

Prepare the master mix according to the number of samples to be amplified. Calculate the required volume of each PCR amplification component to prepare a master mix by multiplying the volume by the number of samples. This should include reagent blanks, positive and negative amplification controls.

9.6 PROCEDURE:

- Determine the number of reactions to be set up, then place the required number of sterile 0.2ml Micro Amp reaction tubes in a rack in the pre-PCR hood. Label these tubes appropriately including positive and negative controls.
- Briefly vortex the required PCR reaction mix and primer mix for 5 to 10 seconds.
- Calculate the required amount of each component of the PCR master mix (Multiply the volume) per sample by the total number of reactions to obtain the final volume and two to three additional reaction volumes (depending on the number of samples being amplified) to compensate for any pipetting variation.
- As listed in above tables add the final volume of each reagent into a sterile, 1.5 ml/0.5ml tube and vortex gently and quick spin.
- Above tables show the component volumes per sample when using a DNA template volume of 5 or 10µl in 25µl of reaction volume.
- Aliquot required PCR master mix into each labeled reaction tube. Cap each tube and ensure that the solution is at the bottom of the tube by gently tapping each tube.
- Add the template DNA (0.5-1ng) of each sample into the respective tube containing the PCR master mix.

For controls

- For positive controls, use AmpF*l*STR Control DNA 9947A, Control DNA 2800M and AmpF*l*STR Control DNA 007 for autosomal and

Y-STRs.

- For negative amplification control, pipette amplification Grade water into the labeled reaction tubes. Close all the tubes firmly.

NOTE: For FTA extracted samples, aliquot required PCR master mix into each labeled reaction tube containing sample punch and add 10µl of amplification Grade water into each reaction tube.

9.7 THERMAL CYCLING PROTOCOLS FOR 96-WELL PCR SYSTEM 9700 (VERITI)

9.7.1 Parameters for AmpFlSTR® Identifiler® Plus Amplification

Table-9.AmpFlSTR® Identifiler® Plus Amplification

Initial Incubation	Denature	Anneal/Extend	Final Extension	Final Step
HOLD	28 /29 CYCLES		HOLD	HOLD
95°C 11 minutes	94°C 20 seconds	59°C 3 minutes	60°C 10 minutes	4°C ∞

NOTE: For FTA extracted samples, 25 cycles are recommended but thermal cycling parameters are same as mentioned in above table.

9.7.2 Parameters for GlobalFiler™ Amplification

Table-10.GlobalFiler™ Amplification

Initial Incubation	Denature	Anneal/Extend	Final Extension	Final Step
HOLD	30 CYCLES		HOLD	HOLD
95°C 1minute	94°C 10 seconds	59°C 90 seconds	60°C 10 minutes	4°C ∞

9.7.3 Parameters for AmpF*l*STR® Y-Filer™ Plus Amplification

Table-11.AmpF*l*STR® Y-Filer™ PlusAmplification

Initial Incubation	Denature	Anneal/Extend	Final Extension	Final Step
HOLD	30 CYCLES		HOLD	HOLD
95°C 1minute	94°C 4 seconds	61.5°C 1 minute	60°C 22 minutes	4°C ∞

9.7.4 Parameters for AmpF*l*STR® Mini filer™ Amplification

Table-12.AmpFl STR® Mini Filer™amplification

Initial Incubation	Denature	Anneal	Extend	Final Extension	Final Step
HOLD	30 CYCLES			HOLD	HOLD
95°C 11 minutes	94°C 20 seconds	59°C 2 minute	72°C 1 minute	60°C 45 minutes	4°C ∞

9.7.5 Parameters for PowerPlex® Fusion 6CAmplification

Table-13.PowerPlex® Fusion 6Camplification

Initial Incubation	Denature	Anneal/ Extend	Final Extension	Final Step
HOLD	29 CYCLES		HOLD	HOLD
96°C 1 minute	96°C 5 seconds	60°C 60 seconds	60°C 10 minutes	4-25°C ∞

9.7.6 Parameters for Investigator Argus X-12 Amplification

Table-14.Investigator Argus X-12 Amplification

InitialIncubation	5 CYCLES			25/27 CYCLES			Final Extension	Final Step
HOLD							HOLD	HOLD
94°C 4 minutes	96°C 30s	63°C 120s	72°C 75s	94°C 30s	60°C 120s	72°C 75s	68°C 60 minutes	10°C ∞

9.8 THERMAL CYCLING PROCESS

Place the MicroAmp ® tubes into the thermal cycler. Push the tubes down completely into the block. Close the heated cover and tighten.

- Start the thermal cycler.
- Select and run the recommended thermal cycling protocol.
- When the thermal cycler prompts the user for the sample volume, choose the required volume.
- Start the amplification protocol and verify the cycling parameters by monitoring the first cycle.
- After amplification process, remove the tubes from the thermal cycler and the samples should be stored in the dark at -20 ° C.

NOTE: Always store amplified DNA samples separate from pre-amplification reagents and extracted DNA samples.

9.9 ALLELIC LADDERS FOR DIFFERENT KITS

Table-15. Allelic ladder for AmpFlSTR® Identifiler® Plus kit

Locus	Alleles included in ladder	Dye	Control DNA 9947A
D8S1179	8,9,10,11,12,13,14,15,16,17,18,19	6-FAM™	13
D21S11	24,24.2,25,26,27,28,28.2,29,29.2,30,30.2,31,31.2,32,32.2, 33,33.2,34,34.2,35,35.2,36, 37,38		30
D7S820	6,7,8,9,10,11,12,13,14,15		10,11
CSF1PO	6,7,8,9,10,11,12,13,14,15		10,12
D3S1358	12,13,14,15,16,17,18,19	VIC®	14,15
TH01	4,5, 6,7,8,9,9.3,10,11,13.3		8,9.3
D13S317	8,9,10,11,12,13,14,15		11
D16S539	5, 8,9,10,11,12,13,14,15		11,12
D2S1338	15,16,17,18,19,20,21,22,23,24,25,26,27,28		19,23
D19S433	9,10,11,12,12.2,13,13.2,14,14.2,15, 15.2,16,16.2,17,17.2	NED™	14,15
vWA	11,12,13,14,15,16,17,18,19,20,21,22,23,24		17,18
TPOX	6,7,8,9,10,11,12,13		8±±
D18S51	7,9,10,10.2,11,12,13,13.2,14,14.2,15,16,17,18,19,20,21,22, 23,24,25,26,27		15,19
Amelogenin	X,Y	PET®	X
D5S818	7,8,9,10,11,12,13,14,15,16		11§§
FGA	17,18,19,20,21,22,23,24,25,26,26.2,27,28,29,30,30.2,31.2, 32.2,33.2,42.2, 43.2,44.2,45.2,46.2,47.2,48.2,50.2,51.2		23,24

Table-16. Allelic ladder for GlobalFiler™ kit

Locus	Alleles included in ladder	Dye	Control DNA 007
D3S1358	9,10,11,12,13,14,15,16,17,18,19,20	6-FAM™	15,16
vWa	11,12,13,14,15,16,17,18,19,20,21,22,23,24		14,16
D16S539	5,8,9,10,11,12,13,14,15		9,10
CSF1PO	6,7,8,9,10,11,12,13,14,15		11,12
TPOX	5,6,7,8,9,10,11,12,13,14,15		8,8
Y indel	1,2	VIC™	2
Amelogenin	X,Y		X,Y
D8S1179	5,6,7,8,9,10,11,12,13,14,15,16,17,18,19		12,13
D21S11	24,24.2,25,26,27,28,28.2,29,29.2,30,30.2,31, 31.2,32,32.2,33,33.2,34,34.2,35,35.2,36,37,38		28,31

D18S51	7,9,10,10.2,11,12,13,13.2,14,14.2,15,16,17,18, 19,20,21,22,23,24,25,26,27		12,15
DYS391	7,8,9,10,11,12,13		11
D2S441	8,9,10,11,11.3,12,13,14,15,16,17	NED™	14,15
D19S433	6,7,8,9,10,11,12,12.2,13,13.2,14,14.2,15,15.2, 16,16.2,17,17.2,18.2,19.2		14,15
TH01	4,5,6,7,8,8,9.3,10,11,13.3		7,9.3
FGA	13,14,15,16,17,18,19,20,21,22,23,24,25,26,26.2, 27,28,29,30,30.2,31.2,32.2,33.2,42.2,43.2,44.2, 45.2,46.2,47.2,48.2,50.2,51.2		24,26
D22S1045	8,9,10,11,12,13,14,15,16,17,18,19	TAZ™	11,16
D5S818	7,8,9,10,11,12,13,14,15,16,17,18		11,11
D13S317	5,6,7,8,9,10,11,12,13,14,15,16		11,11
D7S820	6,7,8,9,10,11,12,13,14,15		7,12
SE33	4.2,6.3,8,9,11,12,13,14,15,16,17,18,19,20,20.2, 21,21.2,22.2,23.2,24.2,25.2,26.2,27.2,28.2,29.2, 30.2,31.2,32.2,33.2,34.2,35,35.2,36,37		17,25.2
D10S1248	8,9,10,11,12,13,14,15,16,17,18,19	SID™	12,15
D1S1656	9,10,11,12,13,14,14.3,15,15.3,16,16.3,17,17.3, 18.3,19.3,20.3		13,16
D12S391	14,15,16,17,18,19,19.3,20,21,22,23,24,25,26,27		18,19
D2S1338	11,12,13,14,15,16,17,18,19,20,21,22,23,24,25, 26,27,28		20,23

Table-17. Allelic ladder for AmpF*l* STR® Y-Filer™ Plus kit

Locus	**Alleles included in ladder**	**Dye**	**Control DNA 007**
DYS576	10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25	6-FAM™	19
DYS389I	9,1011,12,13,14,15,16,17		13
DYS635	15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30		24
DYS389II	24,25,26,27,28,29,30,31,32,33,34,35		29
DYS627	11,12,13,14,15,16,17,18,19,20,21,22,23, 24,25,26,27		21
DYS460	7,8,9,10,11,12,13,14	VIC™	11
DYS458	11,12,13,14,15,16,17,18,19,20,21,22,23,24		17
DYS19	9,10,11,12,13,14,15,1617,18,19		15

YGATAH4	8,9,10,11,12,13,14,15		13
DYS448	14,15,16,17,18,19,20,21,22,23,24		19
DYS391	5,6,7,8,9,10,11,12,13,14,15,16		11
DYS456	10,11,12,13,14,15,16,17,18,19,20,21,22,23,24	NED™	15
DYS390	17,18,19,20,21,22,23,24,25,26,27,28,29		24
DYS438	6,7,8,9,10,11,12,13,14,15,16		12
DYS392	4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20		13
DYS518	32,33,34,35,36,7,38,39,40,41,42,43,44,45,46,47,48,49		37
DYS570	10,11,12,13,14,15,16,17,18,19,20,21,22, 23,24,25,26	TAZ™	17
DYS437	10,11,12,13,14,15,16,17,18		15
DYS385 a/b	6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24, 25,26,27,28		11,14
DYS449	22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40		30
DYS393	7,8,9,10,11,12,13,14,15,16,17,18	SID™	13
DYS439	6,7,8,9,10,11,12,13,14,15,16,17		12
DYS481	17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32		22
DYS387S1	30,31,32,33,34,35,36,37,38,39,40,41,42,43,44		35,37
DYS53	7,8,9,10,11,12,13,14,15,16,17		13

Table-18. Allelic ladder for AmpF*l*STR®Minifiler™ kit

Locus	Alleles included in ladder	Dye	Control DNA 007
D13S317	8,9,10,11,12,13,14,15	6FAM™	11
D7S820	6,7,8,9,10,11,12,13,14,15		7,12
Amelogenin	X,Y	VIC®	X,Y
D2S1338	15,16,17,18,19,20,21,22,23,24,25, 26,27,28		20,23
D21S11	24,24.2,25,26,27,28,28.2,29,29.2, 30,30.2,31,31.2,32,32.2,33,33.2,34, 34.2,35,35.2,36,37,38		28,31
D16S539	5, 8,9,10,11,12,13,14,15		9,10

		NED™	
D18S51	7,9,10,10.2,11,12,13,13.2,14,14.2, 15,16,17,18,19,20,21,22,23,24,25, 26,27		12,15
CSF1PO	6,7,8,9,10,11,12,13,14,15	PET®	11,12
FGA	17,18,19,20,21,22,23,24,25,26,26.2 27,28,29,30,30.2,31.2,32.2,33.2, 42.2,43.2,44.2,45.2,46.2,47.2,48.2,50.2,51.2		24,26

Table-19. Allelic ladder for PowerPlex® Fusion 6C kit

Locus	Alleles included in ladder	Dye	Control DNA 007
AMEL	X, Y	FL-6C	X,Y
D3S1358	9,10,11,12,13,14,15,16,17,18,19,20		17,18
D1S1656	9,10,11,12,13,14,15,15.3,16,16.3,17,17.3,18,18.3, 19,19.3,20.3		12,13
D2S441	8,9,10,11,11.3,12,13,14,15,16,17		10,14
D10S1248	8,9,10,11,12,13,14,15,16,17,18,19		13,15
D13S317	5,6,7,8,9,10,11,12,13,14,15,16,17		9,11
Penta E	5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25		7,14
D16S539	4,5,6,7,8,9,10,11,12,13,14,15,16	JOE- 6C	9,13
D18S51	7,8,9,10,10.2,11,12,13,13.2,14,15,16,17,18,19,20,21,22,23,24,25,26,27		16,18
D2S1338	10,12,14,15,16,17,18,19,20,21,22,23,24, 25,26,27,28		22,25
CSF1PO	5,6,7,8,9,10,11,12,13,14,15,16		12
Penta D	2.2,3.2,5,6,7,8,9,10,11,12,13,14,15,16,17		12,13
TH01	3,4,5,6,7,8,9,9.3,10,11,12,13,13.3	TMR- 6C	6,9.3
vWA	10,11,12,13,14,15,16,17,18,19,20,21,22,23,24		16,19
D21S11	24,24.2,25,25.2,26,27,28,28.2,29,29.2,30,30.2,31,31.2,32, 32.2,33,33.2,34,34.2,35,35.2,36,37,38		29,31.2
D7S820	5,6,7,8,9,10,11,12,13,14,15,16		8,11
D5S818	6,7,8,9,10,11,12,13,14,15,16,17,18		12
TPOX	4,5,6,7,8,9,10,11,12,13,14,15,16		11

D8S1179	7,8,9,10,11,12,13,14,15,16,17,18,19	CXR- 6C	14,15
D12S391	14,15,16,17,17.3,18,18.3,19,20,21,22,23,24,25,26,27		18,23
D19S433	5.2,6.2,8,9,10,11,12,12.2,13,13.2,14,14.2,15,15.2,16,16.2, 17,17.2,18,18.2		13,14
SE33	4.2,6.3,8,9,10,11,12,13,14,15,16,17,18,19,20,20.2,21.2,23.2,24.2,25.2,26.2,27.2,28.2,29.2,30.2,31.2,32.2,33.2,34.2,35,36,37,39		15,16
D22S1045	7,8,9,10,11,12,13,14,15,6,17,18,19,20		16
DYS391	5,6,7,8,9,10,11,12,13,14,15,16	TOM- 6C	10
FGA	14,15,16,17,18,18.2,19,19.2,20,20.2,21,21.2,22,22.2,23,23.2,24,24.2,25,25.2,26,27,28,29,30,31.2,32.2,33.2,42.2,43.2,44.2,45.2,46.2,48.2,50.2		20,23
DYS576	11,12,13,14,15,16,17,18,19,20,21,22,23		18
DYS570	10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25		17

Table-20. Allelic ladder for Investigator Argus X-12 Kit

Locus Designation	Alleles included in ladder	Dye	Control DNA 9947A
Amelogenin	X, Y	6-FAM	X, X
DXS10103	15, 16,17, 18, 19, 20, 21, 22		17,17
DXS8378	8, 9, 10, 11, 12, 13, 14, 15		10,11
DXS10101	21, 24, 24.2, 25, 25.2, 26, 26.2, 27, 27.2, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35		30,31
DXS10134	28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 38.3, 39.3, 40.3, 41.3, 42.3, 43.3, 44.3		35,36
DXS10074	4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 16.2, 17, 18, 19, 20, 21	BTG	16,19
DXS7132	10, 11, 12, 13, 14, 15, 16, 17		12,12
DXS10135	12.1, 13, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37.2, 39.2		12.1,27
DXS7423	12, 13, 14, 15, 16, 17, 18	BTY	14,15
DXS10146	21, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 32.2, 33, 33.2, 34, 34.2, 35.2, 39.2, 40.2, 41.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2		28,28
DXS10079	14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 27		20,23

HPRTB	8, 9, 10, 11, 12, 13, 14, 15, 16, 17	BTR	14,14
DXS10148	13.3, , 16, 17, 18, 19, 20, 21, 22.1, 23.1, 24.1, 25.1, 26.1, 27.1, 28.1, 29.1, 30.1, 31, 33.1, 38.1		22.1,23.1
D21S11	24, 24.2, 25, 26, 26.2, 27, 28, 28.2, 29, 29.2, 30, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 36, 36.2, 37		30,30

CHAPTER 10

DETECTION OF AMPLIFIED PRODUCTS USING THE GENETIC ANALYZERS

Fragment analysis determines the DNA fragments of different sizes in a sample. Amplified samples can be analyzed according to size by the capillary gel electrophoresis on the Genetic Analyzers. An internal lane size standard is loaded with each sample to allow for automatic sizing of the PCR products. The amplified DNA product is composed of a mixture of differently sized DNA fragments, each containing a fluorescent dye-labeled primer. These primers are specifically designed to differentiate the amplified loci. As the DNA fragments migrate through the capillary via electrophoresis, a laser beam excites the attached fluorescent dye generating an emission of light that is detected and converted to an electrical signal by a charge-coupled device (CCD) camera. The intensity of the resulting signals are converted to relative fluorescence units (RFU) and plotted against the measured time span for detection. The collected data corresponding to the amplified DNA fragments is ultimately represented as peaks called an electropherogram. The Genemapper ID-X software is then used for automatic analysis and genotyping of alleles in the collected data.

10.1 CAPILLARY ELECTROPHORESIS BY 3500 GENETIC ANALYZER

The ABI 3500 Genetic Analyzer is a multi-capillary electrophoresis instrument designed to separate amplified DNA product based on size and record the resulting data in a computerized data file capable of analysis using specialized software.

10.2 REAGENTS, MATERIALS AND EQUIPMENTS

- Hi-Di Formamide
- POP 4 Polymer
- Conditioning Reagent
- GeneScan™ 600 LIZ Size Standard
- GeneScan™ 500 LIZ Size Standard
- Amplified DNA products
- Anode and Cathode Buffer
- Capillary Array
- STR Ladders
- 96-well optical reaction plate
- 96-well septa and plate retainer
- 3500 Genetic Analyzer
- Micropipettes
- Aerosol resistant pipette tips
- Heat block

- 9700 ABI Thermal cycler

10.3 PROCEDURE

10.3.1 Starting the Computer and Instrument

- Power on the computer and monitor, but do not log in to the Windows® operating system.
- Once the computer and monitor are powered on, ensure that the instrument door is closed and power on the instrument by pressing the power on/off button on the front. Wait for the green status light.
- Once status light is green, log on to the Windows® operating system.

10.3.2 Launching the Application

- The Server Monitor should launch automatically, if not then go to: Start → Programs →Applied Biosystems→ 3500 →Server Monitor.
- Once all green checkmarks are displayed on the Server Monitor, click on the 3500 icon on the desktop.
- The 3500 Series Data Collection Software screen appears and will require a log in.
- Enter the User Name and password, click OK.
- Check System Status in the Dashboard.

10.3.3 Checking Consumable Status and Replenishing Consumables

- Perform appropriate maintenance tasks.
- Check Consumables Status.
- Click Refresh to update consumable status on the screen.
- Check the consumables gauges for the number of injections, samples, or days remaining for each. If consumables have expired or if buffer fill level is too low, replenish as directed.

10.3.4 Preparation of the Instrument

- Before conducting DNA fragment size analysis, it is necessary to perform a spectral calibration using the 6-Dye Matrix Standard Kit (J6 Dye Set).
- Before the spectral calibration process, ensure that the spatial calibration has been performed. This process is described in detail in the *Applied Biosystems 3500/3500xL Genetic Analyzers User Guide*.

10.3.5 Changing the Polymer

- Remove the polymer pouch from the refrigerator and allow to equilibrate to room temperature before use.
- Enter the appropriate information.

- Go to Dashboard, click Wizards, then click **Replenish Polymer** and follow the prompts.
- Or go to Maintenance Wizard, remove Conditioning pouch and Install Polymer pouch, Fill Array with fresh polymer.
- Remove bubbles from polymer pump path before each run.

10.3.6 Changing the Anode Buffer Container (ABC)

- Remove the ABC from the refrigerator and allow to come to room temperature.
- Document the appropriate information.
- Verify that the seal is intact. Do not use if the buffer level is too low or the seal has been compromised.
- Invert the ABC, and then tilt it slightly to move most of the buffer to the larger side of the container. The smaller side of the container should contain <1 mL of the buffer.
- Verify that the buffer is at the fill line.
- Peel off the seal at the top of the ABC and with the radio frequency identification (RFID) label pointed toward the instrument, place the ABC into the anode-end of the instrument below the pump. Position the anode in the large chamber of the ABC, then push the ABC up and back to install.
- Close the instrument door to re-initialize.
- In the Dashboard, click **Refresh**, then check the Quick View section for updated status.

10.3.7 Changing the Cathode Buffer Container (CBC)

- Remove the CBC from the refrigerator and allow it to come to room temperature.
- Document the appropriate information.
- Wipe away condensation on the CBC exterior with a Kimwipe.
- Check that seal is intact. Do not use if buffer level is too low or seal has been compromised.
- Tilt the CBC back and forth gently and carefully to ensure the buffer is evenly distributed across the top of the baffles.
- When ready to install CBC, place the container on a flat surface and peel off the seal.
- Wipe off any buffer on top of the CBC with a Kimwipe.
- Place the appropriate septum on each side of the CBC.
- Ensure the instrument door is closed, then click the Tray button to move the auto-sampler to the front position.
- With the tab facing you and the RFID tag to the right, install the

CBC on the autosampler.

- Close the instrument door to retract the autosampler.
- In the Dashboard, click Refresh, then check the Quick View section for updated status.

10.3.8 Conditioning reagent

The conditioning reagent for 3500 Series Genetic Analyzers is provided in a ready-to-use pouch. It is used for priming the polymer pump, washing the polymer pump between polymer type changes, and during instrument shut down. It has adequate volume for a one-time use. Maintenance wizards in the software indicate when to install conditioning reagent.

10.3.9 Capillary Array

Capillary Array enables the separation of the fluorescent-labeled DNA fragments by electrophoresis. It is a replaceable unit composed of 8 capillaries (50 cm and 36 cm length).

Note: The 36 cm capillary is for HID applications only.

10.4 PREPARING A RUN

10.4.1 Creating a Setup Sheet

- Create a set-up sheet. It is recommended to include one ladder per 1 injection for the 8- capillary instrument.
- The amount of each component needed in the Formamide/LIZ mixture will be calculated.
- The worksheet will calculate the appropriate volume of each component.
- # of Samples x 9.5 µl Hi-Di Formamide
- # of Samples x 0.5 µl GS 600/GS 500 LIZ Standard
- The number of reactions will include enough of the Formamide/LIZ mixture to complete an injection/run (8 wells). Alternatively, Formamide may be used to fill the remaining wells in an injection. An empty well may cause damage to the capillary.
- Extra reactions can be added to the calculations in order to account for any volume lost during pipetting.

10.4.2 Pre-heating the Oven

- This is an optional but recommended step. If this step is not done, the instrument run will not begin until the oven is 60°C.

- Set the oven temperature and then click Start Pre-heat**.**

10.4.3 Sample Preparation

- Retrieve Hi-Di Formamide, Size Standard and Allelic Ladder. Document the appropriate information.
- Vortex and pulse spin all the reagents.
- Retrieve a 1.5ml or 2.0ml tube and label.
- Add the required amount of each component to the tube.
- Vortex and pulse spin the Formamide/Size Standard mixture if prepared in a tube.
- Allow the amplified product to equilibrate to room temperature. Spin all of the tubes/plates.
- Label a 96-well plate with the plate name, date, and initials. Additional markings can be made on the plate to indicate rows and columns at the discretion of the analyst.
- Aliquot 10 µl of the Formamide/Size Standard mixture into each sample well. Be sure to fill all of the wells associated with the injection/run.
- Following the 3500 Setup Sheet, aliquot 1µl of Allelic Ladder, an amplification positive control, an amplification negative control or 1µl amplified product to the appropriate wells.
- Take a 96-well septum and be sure that all septum holes are open. Seal the plate by laying the septum flat on the plate, aligning the wells, and pressing down. Be certain that the septum fits securely and completely on the plate.
- Centrifuge the plate at 2000 rpm for approximately 30 seconds to 1 minute to ensure all liquid is concentrated at the bottom of each well and no bubbles are present.
- Denature the amplified DNA in the thermal cycler or a heat block at 95^{o}C for 3 minutes.
- After denaturation, keep the plate at 4^{o}C in the thermal cycler or place on an ice block for approximately 3 minutes until it is ready for further processing.

10.4.4 Performing the Run

- Prepare the instrument for the run as per the recommended instructions by the manufacturer.
- Assign the Assay type to the samples e.g. 3500 injection: 1.2kV for 15 seconds.
- To create a new plate record:
    - Click on the **Create New Plate** icon on the **Dashboard**.

- Fill out the **Plate Details** screen.
  - Enter the name of the plate.
  - The Number of Wells shall be set to 96.
  - The Plate Type shall be HID.
  - The Capillary Length shall be 36 cm.
  - The Polymer Type shall be POP-4.
  - The initials of the individual running the plate.
- Sample Types shall be assigned as follows:
  - Ladder: Allelic Ladder
  - Reagent Blank: Negative Control
  - Negative Amplification Control: Negative Control
  - Positive Amplification Control: Positive Control
  - Sample: Sample
- Click the "**Assign Plate Contents**" icon. Plate Contents may be entered in **Plate View** or **Table View**. **NOTE:** When completing a column, fill down by highlighting the desired cells and selecting "Ctrl" + "D" on the keyboard.
- When the plate layout is complete, the plate may be immediately run or saved to run at a later time.

10.4.5 Linking Plate for Run

- Access the Load Plates for Run in the Run Instrument menu.
- Place the 96-well plate that is to be processed onto the plate base and snap the plate retainer over the top. Ensure the holes of the plate retainer and the septa are aligned.
- Place the plate onto the 3500 autosampler, positioned correctly with the notch in the lower right corner. Close the instrument door.
- If a plate record is open in the Assign Plate Contents screen, click Link Plate for Run.
- When opening a previously saved or imported plate, select Open Plate and choose the option to Edit Existing Plate. Select the appropriate plate from the list and choose Open. After verifying the plate setup run and parameters, select Link Plate for Run.
- By default, plate A position is selected.
- A pop-up will give notification of the loaded plate's status. Click OK.
- Access the Load Plates for Run view of the Run Instrument menu.
- Repeat steps above to link a second plate if applicable.
- Verify plates are correctly linked. Click Create Injection List to view the run schedule. The screen will update to the Preview Run screen of the Run Instrument menu. Verify the plate setup and run parame-

ters.

- Select the appropriate plate from the Library, click Load Plate.

10.4.6 Starting the Run

- Click Start Run.
- A dialog box appears. Do not leave run unattended until the box has disappeared and the run has begun. The dialog box may bring up actions to complete or questions to answer. When a run is in progress, the injection will be listed as active and the corresponding plate map will have that injection highlighted in green.
- The monitor run screen is automatically displayed.

10.4.7 Exporting Data

- Once the run has been completed, select go to DATA (D :) > "Applied Biosystems" > "3500" > "DATA" or alternatively, click on the data collection shortcut.
- Locate the project folder, copy and paste the project to the designated storage location (i.e. share drive or thumb drive).

CHAPTER 11

STR DATA ANALYSIS WITH GENEMAPPER™ ID-X

GeneMapper® ID-X (GMID-X) is an automated genotyping software program for Forensic data analysis that supports the analysis of electropherogram data obtained from the Genetic Analyzers. The STR amplification kits are multiplex polymerase chain reaction systems that facilitate the identification and interpretation of specific short tandem repeat regions in human DNA. A multiplex amplification procedure is followed by size separation of the individual amplified fragments via capillary electrophoresis (CE). An internal size standard is included within the mixture that is used to prepare the amplicons for electrophoresis. This internal size standard in conjunction with the simultaneous electrophoresis of allelic ladders which denote the more common alleles occurring at each of the loci, allows for reliable genotyping of the controls and Forensic samples by GMID-X.

This chapter outlines the procedure for analyzing raw data generated from the 3500 Genetic Analyzer Data collection software. All project files, analysis methods, size standards, panels, bins, table settings, and plot settings will be assigned to or saved to the GMIDX security group.

11.1 AUTOSOMAL STR DATA ANALYSIS

11.1.1 Procedure

11.1.1.1 Logging in GeneMapper™ ID-X V1.6software

- Open the GeneMapper™ ID-X software.
- Select the User Name and Database Host from the drop-down list and enter the appropriate password.
- Click OK.
- The main project window will open.

11.1.1.2 Creating a Project

- Project Window is displayed.
- To add samples from the collection software, go to the edit menu and select **Add Samples to Project**.
- Select the raw data folder to be imported or select individual samples and click “Add to list” then “Add”.
- Select the appropriate Sample Type, Analysis Method, Panel and Size Standard for each sample and click the green arrow on the tool bar to analyze the samples.

- If changes need to be made to the panel, sample type, size standard or analysis method, select the necessary cells and choose the appropriate settings from the drop-down menus. Verify that the information is correct and analyze the project or re-analyze the affected sample(s).
- The settings for the Analysis Methods are viewed by selecting GeneMapper ID-X Manager under the Tools drop-down menu, then clicking on the Analysis Methods tab then double clicking to select a particular Analysis Method. These methods shall not be modified. New methods shall only be created or modified with permission from the DNA Technical Manager.
- Check the size standard results by examining the flags in the SQ (sizing quality) column. A green square indicates that a sample has passed the sizing criteria. For any samples that do not pass the sizing criteria, indicated by a yellow triangle or red octagon, assess the size standard by highlighting the sample(s) and click on the icon: or select Tools>Size Match Editor. Check that all expected size standard peaks are detected and labelled correctly. Spikes or other artifacts may be mislabelled by the software resulting in poor sizing.
- The Analytical Threshold of peak detector for all casework analysis is 175RFU.
- Ensure that all selections are properly designated.
- Name the project when prompted and save to the appropriate Security Group (GeneMapper ID-X Security Group). Projects are typically named with the Batch name, and the run date. Click OK.
- When the green arrows in the Status column on the left are gone, analysis is complete, and an Analysis Summary Screen appears.

11.1.1.3 Viewing the Data

- The ILS is viewed by highlighting all samples and clicking on the Size Match Editor, the icon with the red peaks on the toolbar.
- To view each sample, highlight the sample and click on the icon with the colored peaks to display plots. (This can also be done by View > display plots or Ctrl + L).
- Electropherograms can be printed from the sample's plot window. Various plots have been created for different sample types. Commonly used plot displays include:
    - Casework
    - Blank
    - Artefact View
- To view information on a sample's injection time and other run information, highlight the sample of interest and click **View** on the

toolbar > Sample Info (Ctrl + F1).

- To view raw data for a sample, click **View > Raw Data.**
- To view allele calls by sample and locus click the Genotypes tab from the main project window.

11.1.2 Reviewing the Data

11.1.2.1 Analyzing the Internal Lane Standard

- Select all of the appropriate sample(s) in the appropriate project files.
- Press the Display Plots button or select the "Analysis" tab. Select "Display Plots".
- Visually inspect each ILS peak within each sample to ensure it is labeled correctly.
- **GeneScan™-500 LIZ® Internal Lane Size Standard (ILS):** This standard contains DNA fragments of known sizes (35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500) used in-lane to estimate sizes (base pairs) of five dye STR products (Identifiler, Identifiler Plus, Minifiler, and Y-Filer). Each sample tested must have the correct peak sizes assigned.

  NOTE: The 250bp peak is sensitive to small temperature variations during electrophoresis and migrates at approximately 246bp. Accordingly, the 250 base fragment is used only as an indicator of precision within an injection and a run.
- **GeneScan™ 600 LIZ™ Size Standard (ILS):** This contains DNA fragments of known sizes (20, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580 and 600) to estimate sizes (base pairs) of 6-dye STR products (GlobalFiler, Y-filer Plus, Verifiler, Verifiler Plus).
- **WEN ILS 500 Size Standard** should be labeled as 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases used to estimate sizes (base pairs) of 6 dye based PowerPlex Fusion 6C kit and 5 dye based PowerPlex® Fusion &PowerPlex® 21 kit.
- **WEN ILS 500 Y23:** This size standard contains DNA fragments of known sizes (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases) for 6 dye based PowerPlex® Y23 kit.
- **Size Standard 550 (BTO):** It should be labeled as 60, 80, 90, 100, 120, 140, 160, 180, 200, 220, 240, 250, 260, 280, 300, 320, 340, 360, 380, 400, 425, 450, 475, 500, 525, and 550 bp These are used to

estimate sizes of 5 dye based Investigator® Argus X-12 kit and Investigator 24plex Kits®.

- Each sample tested must have the correct peak sizes assigned for its in-lane size standard before proceeding further with interpretation. Samples requiring interpretation with failed ILS/insufficient ILS peak heights must be re injected or re-run to obtain a suitable result. Documentation of re-injection or re-run will be made on the appropriate pages in the case file. When the ILS in a corresponding reagent blank or negative control meets requirements, the reagent blank or negative does not have to be re-injected or re-run.

11.1.2.2 Analyzing the Allelic Ladder Data

- The allelic ladder provided in the kit is used to ascertain the genotypes of the samples. While the ladder consists of the common alleles in the population for each locus, additional atypical alleles may also be included. Sample profiles require at least one valid/passing allelic ladder in a run to be considered for interpretation.
- Allelic ladders missing any alleles, having off-ladder alleles, insufficient peak heights or are consistent with a poor injection will be marked as failed/invalid.
- In the event an allelic ladder is determined to have failed, the injection may be reanalyzed using another/other ladder(s) within the plate. In the event all ladders in the run fail or no ladder in the run meets the migration criteria, all samples must be re-injected or re-run. Documentation of re-injection or re-run will be made on the appropriate page(s) in the case file as mentioned in Allelic tables of different commercial kits in chapter 9.

11.1.2.3 Analyzing the Negative Controls

- **Reagent Blank control/negative amplification control:** The reagent blank/ negative amplification control is a check for possible contamination of the extraction/amplification reagents by human DNA or by amplified STR product. The reagent blank is created by carrying out the DNA extraction in a tube containing no sample and the negative amplification control is a check for contamination during set up of the PCR amplification reaction. These negative controls are then processed in the same manner as the corresponding sample(s) through PCR amplification and run along with the test samples.
- The appearance of typeable test results in the reagent blank/negative amplification control may indicate that a sample preparation reagent(s)/PCR reaction setup has/have been contaminated, cross-

contamination between samples occurred during testing, or human DNA or STR product got into a sample(s) from some other source.

- When a reagent blank/ negative amplification control performs appropriately and passes analysis but a corresponding sample(s) needs to be re-injected or re-run, the reagent blank/ negative amplification control does not have to be re-injected or re-run.
- If a sample needs re-amplification, the reagent blank does not need to be re-amplified unless the prior amplification was amplified at a lower concentration.
- When a negative amplification control performs appropriately and passes analysis but a corresponding sample(s) needs to be re-injected or re-run, the negative amplification control does not have to be re-injected or re-run.
- Any type able peak (a reproducible peak above the analytical threshold) appearing in negative control samples (reagent blank or negative amplification control) in the defined analysis range of 60 – 460bp that is not attributable to an artifact, will be resolved.
- The analyst will ensure that the Technical Manager has been apprised of the contamination event as soon as practicable and initiate an evaluation to determine the source of the contamination.
- The analyst will ensure the Technical Manager remains apprised of the status of the contamination evaluation until at a time when the matter has been resolved by taking some corrective and preventive action and these actions should be documented.
- Re-examine the test samples to ensure the reproducibility and uniformity of the results before final reporting.
- When re-injection, re-running, and/or re-amplification have not resolved the situation, and it is not possible to re-extract a sample associated with a contaminated reagent blank or negative amplification control, consult with the Technical Leader. The raw data for all negative controls, including those with no signs of type able peaks, must be examined for the presence of primer peaks to ensure the samples were properly amplified.

11.1.2.4 Analyzing Positive Controls

- The positive control(s) should be checked to ensure that injection and electrophoresis performance was satisfactory and that the expected types have been called.
- If the positive control was injected poorly, it can be re-injected/re-run. The original sample injections can be analyzed if the correct types for the positive control are documented upon the re-injection/re-run.

- For samples with no typing results, the raw data may be viewed to verify the samples were, in fact, loaded onto the instrument, if indicated (for example, the quantification values indicating results should have been obtained).
- Artifacts such as pull-up, raised baseline, spikes, etc., may occasionally occur and can be documented.
- If pull-up peaks are due to poor color separation and not excessive peak height of peaks in other channels, then a new spectral calibration may need to be performed.
- Artifact peaks may be deleted and real alleles may be renamed, if necessary, as follows:
    - To delete an artifact, first highlight the peak by left clicking it. Then, right click on the artifact peak and select "Delete Label(s)". Enter the reason for the change when prompted (PU for Pull-up, ST for Stutter, etc.) and click OK. The label will now have a strike-through and the comment entered will appear at the bottom of the label.
    - To edit an existing peak label, first highlight the peak by left clicking it. Then, right click the peak and select "Rename Allele Label">"Custom Allele Label". Type in the allele name (e.g., <7) and click OK. Enter the reason for the change (OL for off-ladder, MV for microvariant, etc.) and click OK. The allele will now be re-named with the label showing both the new name and the reason for the change.

11.1.2.5 Analyzing Samples

- Select appropriate sample.
- Press the "Display Plots" button.
- In the plot settings drop down menu, choose the appropriate plot setting.
- For samples with no typing results, the raw data may be viewed to verify the samples were, in fact, loaded onto the instrument.
- Artifacts such as pull-up, raised baseline, spikes, etc., may occasionally occur and can be documented.
- Artifact peaks may be deleted and real alleles may be renamed, if necessary, as follows:
    - To delete an artifact, first highlight the peak by left clicking it. Then, right click on the artifact peak and select "Delete Label(s)". Enter the reason for the change when prompted (PU for Pull-up, ST for Stutter, etc.) and click OK. The label will now have a strike-through and the comment entered will appear at the bottom of the label.

- To edit an existing peak label, first highlight the peak by left clicking it. Then, right click the peak and select "Rename Allele Label">"Custom Allele Label". Type in the allele name (e.g., <7) and click OK. Enter the reason for the change (OL for off-ladder, MV for microvariant, etc.) and click OK. The allele will now be re-named with the label showing both the new name and the reason for the change.

NOTE: If a peak is right-clicked prior to highlighting by left-clicking, the option "Delete All Labels for Sample" will appear. Click elsewhere on the plot to remove this option.

- Print the appropriate electropherogram data by selecting File>Print in the Display Plots window.

NOTE: The plot settings for each amplification kit have been predefined in the software. Therefore, if using the plot settings saved for the amplification kit associated with the data, the x-axis plot region should default to the correct settings.

11.1.2.6 Display Settings

- To check Display Settings, from the GeneMapper ID-X manager select plot settings. A window will appear, click the "Labels" tab.
- Label 1 must be "allele call"
- Label 2 must be "height"
- Label 3 must be "size"
- Font size should be 8.
- For the Display Settings click the "Display Settings" tab. Under the display settings, the number of panels can be adjusted, show/hide off scale data, overlay/separate channels, and select the number of dyes, etc. The blue, green, yellow, red and orange dyes should be selected. Select the allele changes box from the show field. This will display deleted artefact and allele calls on the electropherogram. Click OK.

11.1.2.7 Archiving Run Folders

- Once the analyst has analyzed their project on the desktop, and the project file has been reviewed, export the project file folder (.ser) to a secure limited access network.
- Once the case file has been reviewed and the project file exported, the analyst must delete the project file folder from the GeneMapper ID-X database as follows:
  - Select GeneMapper Manager under the tools menu or the

GeneMapper® ID-X Manager icon.
    - Under the Project tab, select project name and select delete.
- Once the run folder has been removed from the GeneMapper® ID-X database, the project can be re-imported as follows:
    - Select GeneMapper® ID-X Manager under the tools menu or the GeneMapper® ID-X Manager icon.
    - Under the Project tab, select import.
    - Select appropriate file, select import and select done.
    - Under file menu, choose open project and select appropriate project.

11.2 Y-STR DATA ANALYSIS

This procedure specifies the steps for performing analysis on DNA samples amplified for Y-filer and Y-filer Plus using the GeneMapper® ID-X V 1.6 software and to provide guidelines for the interpretation of Y-STR DNA results. Y-STR testing is an additional tool used in cases where recovery of the male DNA profile in male: female mixtures is necessary.

The pattern of Y-STR peaks obtained during this testing is defined as the Y-STR haplotype of the individual. Due to this linked inheritance, the product rule cannot be applied to estimate the rarity of the haplotype and a counting method must be applied.

11.2.1 Initial Setup of Genemapper® ID-X V1.6

After installation of the **Genemapper®**ID-X software, customizations must be made. These include defining panels and bin sets, the size standard, plot settings, and table settings.

11.2.2 Creation of New Run Modules

- Instrument procedures are controlled by the module manager. The control of operations such as injection time, oven temperature, voltage during electrophoresis and data collection interval can be modified. Modules must be created or modified from the templates provided.
- To create a new module, navigate to Module Manager Window Click on "New" and a Run Module Editor window appears. A previously made module can be exported from another computer and imported into the Module Manager rather than creating a new module, if desired.

11.2.3 Module Manager

- Fill in the name and select the type of module (Regular) as well as a template (HIDFragmentAnalysis36_POP4) from the drop-down list. Make modifications to the run conditions as necessary to satisfy the

run requirements.

- After the settings have been verified, save the new module by clicking "OK".

11.2.4 Creation of New Protocols

- The instrument protocol is used to tell the instrument all of the necessary run conditions for the sample, including the type of run, the run module, and the dye set. Typically, once a protocol is defined for a particular multiplex, it will be used repeatedly and it will not be necessary to create a new one.
- If a new protocol needs to be generated, proceed for directions.
- In the 3500 Protocol Manager, click "New" in the Protocols section to open the Protocol Editor and create a new protocol.
- A previously-made instrument protocol can be exported from another computer and imported into the Protocol Manager, if desired.
- Type a name for the protocol. Typically, this names the amplification kit or dye set utilized for the samples of interest.
- Select the run module for your run.
- Typically: HID Fragment Analysis 36_POP4_1, as this is the default run module for Yfiler™.
- A custom run module may be selected.
- Select the Dye Set for your samples. Typically, this will be 'G5', which is the dye set for Yfiler or 'J6' for Y-filer Plus. Click OK.

11.2.5 Analysis Method Editor

- In the GeneMapper Manager, select the "Analysis Methods" tab and click "New". In the GeneMapper™ ID-X software, select Analysis>Analysis Method Editor.
- Click on the "Peak Detector" tab.
- Ensure that all selections are properly designated. The size calling method used for the AmpFℓSTR®Yfiler™kit and Y-filer Plus is the Local Southern method or as per the instructions given in commercially available Y-STR kits (Promega&Qiagen).
- It is important to note that ONLY the orange dye (ILS 600 size standard) may be adjusted.
- The Bin Set is defined as "CODISAmpFLSTR_Yfiler_Binset_v2".
- The default settings for the Peak Quality and Quality Flags tabs and "Allele" tab are used.

11.2.6 Panels and Bins

- Panels and bins must be created or imported for allele calling of Yfiler™data. In the GeneMapper®ID-X software, navigate to

Tools>Panel Manager.

- Choose File>Import Panels and browse to the file entitled CODISY-filer_v2 which has been created and named.
- Navigate to "CODIS_Yfiler_v2", choose File > Import Bin Set and browse to the bins file"CODISAmpFLSTR_Yfiler_Binset_v2".

11.2.7 Plot Settings

- In GeneMapper®ID-X, navigate to Tools >GeneMapper Manager. Choose Plot Settings to create custom plot settings.
- Create a new plot setting by clicking New and type a name for the plot settings in the Plot Settings Editor.
- In the Labels tab in the Plot Settings Editor, the appropriate settings should be used for samples. The ladders, if viewed, may be labeled differently (example:allele call only), Click OK.

11.2.8 Adding/Deleting samples

- If a Forensic Scientist/Analyst adds additional sample(s) to a current project, then newly added sample shall be analyzed before the data can be viewed.
- If the sample is from a different injection (e.g., run folder), the associated Allelic ladder shall also be imported in order for the sample data to be sized properly.
- If a Forensic Scientist/Analyst removes a sample(s) that has been added to the project (e.g., a sample from an unrelated case), highlight the sample to be removed, select Edit and Delete from Project.
- Samples from the same case that are present in a project, but are not used (e.g., redundancy, sizing data issues or similar), shall not be deleted.

11.2.9 Y-filer Plus Data Analysis

11.2.9.1 Procedure

- Y-filer Plus settings:
- Analysis Method = Yfiler Plus
- Panel = Yfiler_Plus_Panel_v3
- Bin = Yfiler_Plus_Bins_v3
- Stutter ratio = marker specific (values must be loaded into the Panel Manager).
- Review raw data to troubleshoot sizing difficulties. Check Analysis Method Editor to verify that the sizing range (3000-6000) brackets the set of GS600 LIZ size standards, all peaks are correctly labeled

and no artifacts are labeled.

- The LIZ 600 internal size standard must have the expected size results for the 60-460 base pair fragments.
- The peaks must be sizable but do not necessarily have to be above 100 RFU.
- To edit a particular size standard peak, right-click the peak.
- Examine the allelic ladders to determine that the software has assigned all allele designations correctly and the peaks are at least 100 RFU.
- Verify that positive controls give the correct type. Rarely, if the positive control fails to amplify, a single-profile case sample can be re-run with a working positive control and then used as asecondary positive control.
- Check for peaks in negative controls. If a non-artifact peak above threshold is detected in a reagent blank or negative control, investigate as per procedure.
- Review samples and mark artifacts: spikes, pull-up, raised base line, dye blobs and nonspecific female DNA amplification.
- Print the electropherogram.
- Base pair range for X-axis = 60-460.
- Y-axis = scale to top of highest peak for each dye color.
- Negative sample Y-axis = 75.
- Print allele call and bp only.
- Consider printing a second zoomed-in view where a very minor profile is present.

11.2.9.2 Assess Overall Quality of Data

11.2.9.2.1 Artifacts

The PCR process produces artifacts that are known and well characterized. If any peaks are to be removed from consideration as true alleles due to their presence as a known artifact (e.g., pull-up, dye-blob, stutter), highlight the sizing box associated with the peak and right-click. Select “Delete Label(s)” (multiple peaks can be deleted simultaneously by selecting them at the same time-when the warning dialog box appears, select “OK”). When prompted, type in artifact as the Reason(s) for Change.

11.2.9.2.2 Stutter

Results shall not be considered inconclusive if stutter peaks are present. Marker specific stutter percentages are incorporated into the GMID-X analysis. Additional stutter positions should be considered during interpretation. Peaks in stutter positions with ratios greater than the stutter guidelines should be interpreted

with caution. Excessive peak heights can cause stutter peaks in excess of the validated percentages.

11.2.9.2.3 OMR

GMID-X software flags peaks that fall outside the range of known alleles for a locus with the label "OMR" (Outside Marker Range). All instances of OMR should be assessed to ensure that a true allele was not mislabeled by the software. If the OMR call is an artifact, the Forensic Scientist/Analyst shall leave the peak labeled as OMR.

11.2.9.2.4 Spike

GMID-X software provides automatic labeling of artifacts known as "spikes" based upon peak morphology and typical presence in multiple dye channels. Peaks automatically labeled by the software as "spikes" can also include spectral pull-up. All instances of "spike" should be assessed to ensure that a true allele was not mislabeled by the software. If the "spike" call is an artifact, the Forensic Scientist/Analyst shall leave the peak labeled as "spike."

11.2.9.2.5 Off-ladder Alleles

Alleles which are not assigned a numeric designation in the standard ladder will be designated as off-ladder (OL) alleles. Peaks that are labeled as OL (off ladder) at a particular locus have a base size that is different from any of the alleles in the ladder and any of the virtual alleles. These peaks may be true alleles but additional analysis is required to verify this. Any allele designations as off-ladder (OL) peaks must be evaluated to determine if the peak is a true OL allele, an artifact, or a result of electrophoretic shifting.

11.2.9.3 OL Allele within locus range

- Microvariants may be observed and designated "Z.X", where the Z is the lower rung of the allelic ladder bracketing the microvariant. The "X" identifies the allele as having a size between alleles Z and Z+1. Using the nearest allelic ladder peak will enable the microvariant to be defined accurately. The microvariant will be designated as Z.1, Z.2, Z.3, Z.4, or Z.5 as applicable.
- Verification of OL: For single instance OL, the sample having alleles falling outside of allelic ladder bins will be reinjected in the same run as an allelic ladder for verification whether the allele is a true microvariant or the original designation was incorrect due to migration issues during electrophoresis. Multi-occurrence OLs do not require reinjection. Note: Questioned and known samples shall not be used for verification of OL alleles.

11.2.9.4 OL Allele located between two loci

- If the DNA profile has data at one locus bracketing the off-ladder allele, and no data at the other bracketing locus, then the off-ladder allele will be assigned to the locus with no data.
- Alleles which are located outside the range of the ladder or bin set (above or below) shall be described as "<" or ">" the largest or smallest allele for that locus with a set of ( ) placed around the off ladder allele. For example, if a band is located above the largest allele for the DYS19 locus, it would be designated as "(>19)". This should be clear when used and can be verified with a locus review.
- If the DNA profile has data at both bracketing loci, or if the DNA pattern is a mixture, then the locus to which the off-ladder allele belongs may not be determined. The off-ladder allele will not be given a locus designation. Both bracketing loci will be designated as inconclusive.
- It is possible for alleles to fall outside of the range of their locus and to be detected in the size range of a neighboring locus (e.g. an allele 20 in locus DYS391 would be detected in the range of locus DYS481). Such possibilities should be considered when a locus has a bi-allelic pattern and is adjacent to a locus with no data.
- If the bi-allelic pattern has one allele within a bin, and one allele designated as "off-ladder", the "off-ladder" allele will be designated as belonging to the locus showing no other data.
- If the bi-allelic pattern has both alleles within allelic bins, then it is not possible to determine which allele belongs to the locus showing no data. Both loci in question will be designated as inconclusive.
- Verification of OL: For single instance OL, the sample having alleles falling outside of allelic ladder bins will be reinjected in the same run as an allelic ladder for verification of whether the allele is a true microvariant or if the original designation was incorrect due to migration issues during electrophoresis. Multi-occurrence OLs do not require reinjection. Note: Questioned and known samples shall not be used for verification of OL.
- Duplication occurs when multiple alleles are present at a locus/loci. Instances of multiple duplications within a sample have been documented as well. Most duplications have a one repeat difference; however, two, three, and four repeat unit differences has been observed.
- A deletion occurs when there is a lack of a peak at single locus/loci. A deletion has been reported at the DYS448 locus. Samples with this deletion will show two peaks (duplication) in DYS576 and a null al-

lele in DYS448.

11.2.9.5 Off-scale Samples

Off-scale samples (consistently >15000 RFUs) shall not be used for comparison. Such samples shall be re-amplified with a lower concentration.

CHAPTER 12

EVALUATION AND INTERPRETATION OF STR DATA

Detailed and comprehensive assessment protocols are needed to ensure reliable and consistent interpretation and comparison of STR data. Specific requirements for laboratory's procedures for the interpretation and comparison of DNA data are provided. However, not every situation can or should be covered by specific rules. These guidelines establish a framework to ensure that interpretation is made objectively and consistently between analysts, reporting conclusions that are supported by analytical data.

12.1 AUTOSOMAL STR DATA INTERPRETATION GUIDELINES

STR alleles are small in size, generally less than 500 bp and contain repeat units ranging from 3 to 7 bases. If an allele contains an incomplete repeat, the allele is considered a microvariant and is designated by the number of complete repeats present followed by a decimal point, then the number of bases of the incomplete repeat. For example, the FGA 22.2 allele contains 22 tetrameric repeats plus 2 bases. Because of a deletion of two bases the FGA 22.2 allele is two bases shorter than the FGA 23 allele. The size of each allele is determined in the Gene Mapper ID software analysis by comparison to the internal size standard.

12.1.1 Allele Calling Criteria

12.1.1.1 Designation of Alleles

- Results are interpreted by observing the occurrence of electropherogram peaks for the loci that are amplified simultaneously. The identification of a peak as an allele is determined through comparison to the allelic ladder. An allele is characterized by the labeling color of the locus specific primers and the length of the amplified fragment.
- For each locus an individual can be either homozygous and show one allele, or heterozygous and show two alleles. In order to eliminate possible background and stutter peaks, only peaks that display intensity above the minimum threshold based on validation data i.e. 175 RFU (Relative Fluorescent Units) are labeled as alleles.
- Examine the batch controls (internal control specimen (ICS), positive and negative amplification controls, and extraction reagent blanks, Internal Lane Standard (ILS), allelic ladder(s) to ensure that the extraction, amplification and genetic analysis processes are functioning correctly.

NOTE: Repeat all or part of an extraction, amplification and/or run depending on the results of the batch controls. Any issues raised by the performance of a batch control must be addressed prior to the release of affected casework samples.

12.1.1.2 Computer Program/Processing Steps for Raw Data

- Recalculating fluorescence peaks using the instrument-specific spectral file in order to correct for the overlapping spectra of the fluorescent dyes.
- Calculating the fragment length for the detected peaks using the known in lane standard fragments.
- Comparing and adjusting the allele categories to the sizing of the co-electrophoresed allelic ladder by calculating the off sets (the difference between the first allele in a category and the first allele in the allelic ladder at each locus).
- Labeling of all sized fragments that are above threshold and fall within the locus specific size range. Removing the labels from minor peaks (background and stutter) according to the filter functions.

12.1.1.3 Internal Lane Standard (ILS)

- All samples run with Internal Lane Standard (ILS).
- Verify that all peaks from 60-460 base pairs are present and labelled.
- The peak heights for the ILS peaks must be at least 175 RFU (relative fluorescence units) to be identified and labelled by the GeneMapper™ ID-X software with a casework analysis method.

12.1.1.4 Allelic Ladder

- The allelic ladder contains the most common alleles determined for each of the loci in multiplex system. Verify that all peaks from the allelic ladder(s) are present and labelled.
- The GeneMapper™ ID-X software uses the allele calls of the ladder to assign allele calls to all the other samples in the project.
- In addition, alleles not labelled in the allelic ladder (virtual alleles), may be detected and labelled in some of the samples analyzed.
- If a spurious peak (not a true allelic peak) is labeled as a true allele (i.e. it falls within an allele size range and is designated and labeled as an allele) at any particular locus, the remaining allele designations /labels at the locus shift and will impact the correct allele call/ labelling of the samples in the project.
- Allelic ladders with such artifacts cannot be used to analyze data in the project. In such instances, re-injection is usually required.

12.1.1.5 Internal Control Specimen (ICS)

- An internal control specimen (ICS) is a designated, predetermined quality control sample whose expected profile is already known. The ICS is processed with an extraction batch and carried through at

least quantification concurrently with the other samples.

- While reagent blanks are designed to assess the possibility of contamination in extraction reagents, the purpose of the ICS is to demonstrate that all analytical processes prior to quantification are working correctly to produce an extract with amplifiable DNA.
- At least one ICS shall be created with each set of casework extractions. All ICS samples are quantified to ensure that they contain sufficient amplifiable DNA, and that the presence or absence of male DNA, is consistent with expected results based on known sample type. Each ICS which meets these criteria is considered as passing and does not require amplification.
- A partial profile for an ICS does not necessarily invalidate the batch.
- If a minimum of fifteen complete core loci are detected for the ICS, with no inconsistent allele calls, the ICS is acceptable.
- The Technical Manager, or other designated individual, shall determine whether or not to approve the ICS when fewer than fifteen complete core loci are detected for the ICS.
- The data quality of other positive control samples in the batch and/or the quality of other concurrently extracted samples will be reviewed to determine the approval process.
- If an incorrect STR profile is obtained for the ICS in a casework batch, the analyst will attempt to determine the cause of the discrepancy. The discrepancy will be documented and corrective and future preventive actions may be taken as deemed necessary by the Technical Manager.
- ICS extracts may also need to be amplified in batches where troubleshooting is required, e.g. assessing the possibility of sample switching.
- ICS extracts are discarded following completion of the DNA technical review of the batch.

12.1.1.6 Positive Amplification Control

- A positive control is defined as a single source sample whose genetic profile was previously determined and from which a full profile was developed, it is used to evaluate the performance of the amplification and typing procedures.
- Use suitable DNA Control (9947A, 007) as a positive control for amplification.
- A minimum of one positive amplification control is routinely amplified and processed concurrently with casework known amplification.

- The presence of artifacts in a positive control does not constitute a failed positive control, so long as they are not consistent with contamination.
- Artifacts are struck in the GMID-X project and checked during the electronic review of batch controls.
- It may be necessary to re-prepare and/or re-inject the positive control if the cause of failure appears to be a failed electrophoretic injection.
- As long as the re-prepared and/or re-injected positive control types successfully, the positive control is considered successful.
- Alternatively, other samples in an amplification may serve as a positive control (e.g. staff sample, casework known sample with a verified profile, or a buccal swab obtained from laboratory personnel whose genetic profile(s) was/were previously determined and documented.
- Use of an alternate positive control must have documented approval of the Technical Manager.
- All casework samples co-amplified will be re-analyzed if no positive control was successfully typed for that amplification.
- The laboratory will routinely include more than one positive control with every casework amplification reaction that includes questioned samples, in order to prevent reanalysis of samples that are limited in DNA content.
- If at least one of these positive control samples produces acceptable and expected results, the batch will not be reanalyzed.
- If all interpretable results agree for the samples between the first and second amplification/run, and the positive amplification control yields a correct STR profile for the second amplification/run, then the STR data obtained from either (but not both) amplification/run may be used.

12.1.1.7 Negative Amplification Control and Extraction Reagent Blanks

- A reagent blank is carried through the entire analytical process as part of each extraction or type of extraction. It contains all the reagents except DNA template, used during extraction, amplification and typing. Only one of the blanks (either replicate -1 or the blank with the higher observed quantification reading) is rehydrated with 15 µL sterile water and amplified.
- The negative amplification control contains only the PCR master mix (reagents used to prepare the PCR amplification mixture) for each batch of samples and the TE^{-4} buffer used to dilute the DNA samples.

- The purpose of the negative controls is to detect systemic DNA contamination that might occur from the reagents, the laboratory environment, between the samples being processed, and/or due to improper handling of the samples by the analyst.
- The negative controls must be run at the most stringent set of conditions for the batch.
- Verification of the presence of amplicon in the negative controls is performed by viewing the presence of unincorporated primer peaks.
- The negative controls should not yield any true STR allelic peaks above the analytical threshold (AT). When peaks greater than or equal to the reporting threshold are present in the range between 60-460 base pairs, the analyst will determine if the peaks are artefacts (e.g. spike, pull-up) or true allele peaks.
- The presence of peaks above the AT will not invalidate the sample as long as the data is at levels too low for interpretation, or the peaks can be shown to be artefacts.
- Artefacts will be struck in the project and checked in the electronic review of the batch controls.
- The failure of a negative control may indicate a problem at the extraction or amplification level.
- When probable true allele peaks are detected above the reporting threshold at an interpretable level, the analyst will re-examine the sample using the STR kit troubleshooting analysis parameters.
- In some cases, the contamination may be attributable to a known source, such as an adjacent sample or a lab worker who was in close proximity to the extract.
- If the analyst can identify the source of the contamination, a Quality Review Form must be initiated to investigate the root cause of the contamination. The DNA Technical Manager will then be consulted to determine the appropriate course of action.

12.1.1.8 Distinguishing Alleles from Artefacts

- The initial assessment of a casework sample electropherogram separates interpretable alleles from artefacts. Artefacts can occur in data Peaks determined to be artefacts are struck on the full view electropherogram.
- When it is not possible to distinguish between an artifact and a true allele, this is indicated on the electropherogram as Artefact/True Allele (A/TA). Artefacts are often associated with an excess of template DNA.
- If the presence of many artefacts complicates data analysis, it may

be appropriate to re-amplify the sample with less template DNA.

- Artefacts in questioned samples which do not fall in bin as alleles and are called OL, OMR or spike, may be struck without a documented explanation and without an artefact view printout.

12.1.1.9 Stutter

- The PCR amplification of tetranucleotide STR loci typically produces a minor product peak four bases shorter (N-4), four base pairs larger (N+4) or two bases shorter (N-2, rarely observed), than the corresponding base peak. This is the result of slippage of the Taq polymerase and is referred to as the stutter peak or stutter product.
- The proportion of stutter product relative to the main allele peak is calculated by dividing the height of the stutter peak by the height of the main allele peak. The stutter percentage is fairly reproducible for a particular locus.
- The macro for each system has an automated stutter filter for each locus.
- In addition, for single source samples, potential stutter peaks may be removed if they are within 20% of the larger peak for Identifiler and Yfiler.
- If the main allele has an additional label prior to the main allele label (e.g. a shoulder peak, 1bp less in size) this peak will be used for stutter percentage calculation and the stutter might not have been automatically removed. In this case, the label on the stutter peak can also be removed for mixtures.

12.1.1.10 Assessment of Stutter/ True Allele

Striking a stutter peak is acceptable in the following situations:

- In questioned samples, when there is no indication of a mixture other than elevated stutter at four or fewer loci, the analyst will document the stutter as such on the electropherogram (including the calculated % and a notation that it was determined to be an elevated stutter peak).
- Reference samples are assumed to be single source. Therefore, any elevated stutter may be struck without further documentation.
- Noting a stutter peak as indistinguishable artifact or true allele (A/TA) in questioned samples is acceptable in the following situations:
    - When expected stutter contribution (either +4, -4, or +4 / -4) is subtracted from a peak in a stutter position and the resulting peak is below the analytical threshold (AT) of 175 RFU, the peak should be noted as A/TA.

- When possible, elevated stutter peaks are observed at five or more loci in an otherwise apparent single-source questioned profile, and no minor peaks (or one minor peak) are observed outside of stutter positions, the elevated stutter peaks are documented as A/TA.

12.1.1.11 Baseline Noise

The analytical threshold (AT) for analysis of casework samples is 175 RFU. Data below the AT is considered indistinguishable from baseline noise and not suitable for interpretation. If baseline noise is difficult to distinguish from possible low-level DNA, it may be more appropriate to re-amplify the sample with less template DNA. Sometimes, an elevated baseline may occur adjacent to a shoulder peak and do not resemble distinct peaks.

12.1.1.12 Amplification and Injection Artefacts

The following are examples of artefacts which may be struck and initialled on electropherograms.

12.1.1.13 Pull-Up

- Pull-up is the result of incomplete separation of the emission spectra and is typically observed as a non-allelic peak at the same base size as a peak in another dye also.
- The label in the other color will have a base pair size very close to the real allele in the other color. The peak that is considered an artifact or "pull up" will always be shorter than the original, true peak.
- Documentation of a peak as pull-up includes a close-up view of the locus in question as well as the corresponding color with the peak causing the pull-up, with base pairs included on the zoomed view electropherogram.

12.1.1.14 Spikes

- A Spike is an electrophoresis artefact that is usually present in all dyes. Spikes are non-allele peaks that may arise due to air bubbles, urea crystals or voltage spikes.
- Spikes are typically sharp and easy to distinguish from a true allele. Spikes usually appear in more than one dye at the same base size and are not reproducible by re-injection.

12.1.1.15 Dye Blobs

Dye blobs occur when fluorescent dyes come off their respective primers and migrate independently through the capillary. Dye blob morphology is generally

characteristically broad, and not very "peak-like".

12.1.1.16 Incomplete Nucleotide Addition +A/-A (or split peaks)

- PCR amplification results in the addition of a single "A" nucleotide at the 3' end of double stranded PCR products, resulting in a product that is one bp longer than the actual target DNA sequence. PCR reactions have been optimized to favour this "A" addition, but incomplete "A" addition may occur when excessive amount of target DNA is present, or in other conditions less than optimal for the PCR reaction. Incomplete "A" addition, or "minus A" appears as a peak one bp shorter, and typically at a smaller peak height, than the true allele.
- Split peaks resulting from over amplification will be addressed on a case by case basis. Split peaks may be resolved by re-amplification with a lower amount of template DNA, if available. If the split peak is not resolved then the locus will be deemed inconclusive.

12.1.1.17 Off-Ladder (OL) Alleles

- The allelic ladder contains the most commonly observed alleles for the STR loci. Alleles which are not assigned a numeric designation in the standard ladder will be designated as off-ladder (OL) alleles.
- Peaks that are labeled as OL (off ladder) at a particular locus have a base size that is different from any of the alleles in the ladder and any of the virtual alleles. These peaks may be true alleles but additional analysis is required to verify this.
- Before determining whether an OL is a true allele or an artefact, the analyst must consider all other possible causes of OL occurrence.
- When a true OL allele lies within the range of alleles for a particular locus, the allele will be reported as a variant of the integer (i.e. X.1, X.2, etc.).
- The appropriate allele call is determined by simultaneously examining the base sizes for the sample allele peak and the associated allelic ladder peaks in the relevant locus.
- When the OL allele lies either above or below the largest or smallest allele in the ladder, the OL allele will be designated as either greater than (>) or less than (<) the allele from the ladder that is closest in proximity to the OL allele.
- OL alleles considered to be true alleles may require re-amplification to confirm, particularly when they are only observed in a mixture. If the OL allele is observed in multiple amplifications from a single item (such as two stains from the same item of clothing, or the sperm and epithelial fractions from a vaginal swab), further amplifications are not necessary to verify the OL allele.

- Documentation of the OL allele must include an electropherogram with both the sample and the associated allelic ladder for the locus, showing the OL allele(s) in the locus, with the bins and base pair sizes.

12.1.1.18 Off Ladder (microvariant) Alleles

The following guidelines should be followed to resolve an off-ladder allele:

- Off ladder alleles that fall outside of a bin or above or below the allelic ladder or virtual bin may be extrapolated from a single injection.
- An OL may either be a nominal or a microvariant STR. An OL may be reported following these guidelines:
  - In GeneMapper ID-X, analyse the evidentiary sample and ladder simultaneously.
  - Select plot setting on the label tab.
  - Set the parameters so that each allele is labelled with the allele call, height, and base pair size.
  - If the OL fall within a loci compare the base pair size of the OL allele to the two closest peaks of the ladder.
- For tetranucleotides, the OL allele must be 4 base pair or less. For pentanucleotides, the OL allele must be 5 base pairs or less, etc.
- An example of a Penta OL: OL = 385.06 base pairs, Allele 5 sizes at 380.05 and Allele 7 sizes at 389.85. The OL is 5 base pairs apart from both Alleles 5 and 7, therefore the OL is a 6.
- A rare microvariant may be called using the nominal allele designation followed by an ".x" or the allele may be extrapolated. For example, an allele that migrates between the 10 and 11 bin of CSF may be recorded as a "10.2."
- An allele that migrates above or below the allelic ladder or virtual bin may be extrapolated with caution from the base pair size. If the OL falls above or below the last allele in the loci the OL may be extrapolated with caution using the closest peak of the ladder or nearest virtual bin. If the OL is not extrapolated the OL may be electronically designated as < or > first or the last allele in the locus in the GMID-X allele call box.
- Alleles that migrate above or below the allelic ladder for a locus may not be flagged by GMID-X. In the event that an allele is not flagged by GMID-X the allele should be interpreted with caution and be recorded as outlined in the DNA Profile Interpretation Records Protocol.

12.1.1.19 Contamination

- The verification of reagents prior to use in casework is conducted in part to detect contamination which permeates an entire lot of a given reagent or set of reagents. However, such checks will not necessarily detect contamination arising from a single event (for example, cross contamination between two adjacent samples).
- For casework samples, whenever data suitable for comparison is not attributable to any reference samples associated with its case, the possibility that the profile was introduced via contamination must be considered.
- As a part of the review process, both the analyst and the technical reviewer must rule out laboratory staff working in direct proximity to the open evidence as well as cross-contamination within the batch of samples.
- This comparison may be done manually or via the Profile Comparison tool in GeneMapper ID-X. The same principle applies to control samples where no DNA should be detected.

12.1.1.20 Tri-alleles

If three alleles are present at one locus, the sample is not a mixture and the allele is not an artefact, or the allele is not off ladder then the sample may contain a tri-allelic profile:

- Verify that the allele is not from another locus by observing the allele pattern upstream and downstream of the locus in question.
- If the allele pattern is within the allelic ladder of the locus a tri-allelic pattern may be called.
- If the allele pattern falls between two allelic ladders it may not be called a tri-allele.
- If the allele pattern falls outside of the allelic range, for a dye labeled panel the allele will be designated as a >/< the appropriate locus.

12.1.1.21 No Interpretable Data (NID)

When no signal above baseline or a signal that is above the baseline but below the analytical threshold is generated in a profile, the data will be considered uninterpretable and will not be utilized for comparative analysis.

12.1.1.22 Designation of Amelogenin Fragments

To indicate the gender of the contributor of a particular biological sample the Amelogenin locus may be used. Amelogenin is typed by comparing fragments to the ladder. A male specimen consists of X and Y Amelogenin fragments, whereas a female specimen exhibits only the X fragments. A biological sample exhibiting a single band at X allele will generally be considered to have originated

from a female individual. A biological sample exhibiting a band at X allele and a band at Y allele will generally be considered to have originated from a male individual. If only a Y allele is obtained or only an X allele is obtained from a known male sample, the results at the Amelogenin locus will be reported as inconclusive.

12.1.1.23 Overall Interpretation of Specimens

- The analytical controls are evaluated and confirmed.
- Artifacts inherent to the PCR process as well as those generated by the detection instrumentation are isolated and eliminated.
- The overall profile is evaluated to assess its quality.
- The overall profile is defined as a single source or mixture.
- The potential number of contributors is determined.
- Profiles may be deconvoluted and/or compared to the known profiles submitted in connection with the case in order to make a forensic conclusion regarding the potential exclusion or inclusion of each individual as a contributor(s) of/to each sample.

12.1.1.24 Profile Assessment

- **Peak Evaluation**

  As a profile is evaluated, each peak identified by the GMID-X software must be checked to confirm it is a valid allele call. Typical characteristics and requirements related to the shape, size and location of a true allele are listed below along with a variety of recognizable artifacts.

- **Alleles**

  A reportable, true allele peak is defined as a distinct, triangular section of an electropherogram that is equal to or higher than the analytical threshold (AT). The analytical threshold is the minimum signal at which a peak can reliably be distinguished from noise. Based on internal validation studies, an AT of 175 RFU has been established for samples amplified with GlobalFiler™ (injected for 15 seconds at 1.2kV on the 3500 instrument).

  Based on an initial review of results, when appropriate and quantity of sample allows, the analyst may opt to concentrate the extract and re-amplify, re-amplify with more and/or less template DNA, and/or re-extract. When a sample is analyzed more than one time, the first page of the electropherogram used for reporting purposes will be clearly marked. If replicate amplifications will be reported for a sample, the first page of both electropherograms will be marked.

- **Overall Profile Quality**

  The overall quality of each profile, as well as the potential number

of contributors and any relevant source information, will be assessed prior to any in-depth determinations about the data observed. The presence of apparent degradation, inhibition, or significant stochastic effects in a profile will be considered during the interpretation process and may influence the assessments made at each locus. In addition, the number of potential contributors to a sample will affect how the sample is interpreted. It is also possible that knowledge regarding a potential contributor(s) to a mixture sample may affect sample interpretation (e.g., an intimate swab with semen positive results). These observations will be documented in the case file.

- **Degradation**
  DNA degradation is a process in which DNA molecules randomly break down into smaller pieces. This becomes problematic for forensic DNA typing when it occurs within the target DNA sequences of PCR. If the DNA has been degraded or damaged at these locations, sister peak height imbalance, abnormally high stutter product(s), allele dropout, and/or locus dropout may be observed. Typically, degraded profiles will display a downward sloping or "ski-slope" pattern from left to right for each dye. This pattern is due to the larger/longer loci being more likely to contain a break due to degradation than the smaller loci. It is important to note that it is possible for the profile from one or more donors in a multiple contributor profile to exhibit degradation in the absence of observed profile degradation of the other donor(s).
- **Inhibition**
  DNA samples may contain one or more of the PCR inhibitors commonly encountered in forensic casework. The presence of inhibitors may manifest itself by the failure to produce results at some or all loci and may mimic results seen when degradation is present. Samples containing inhibitors often produce partial profile results in which the smaller loci drop out before the larger loci.
- **Types of Profiles**
  A number of different types of profiles may be obtained from evidence samples. The comparisons with references that can be made are determined by the type of profile.
    - **No profile**: No DNA results obtained at all. No comparison can be made to reference samples.
    - **Uninterpretable**: Data obtained, but insufficient for comparison. This may be due to data at too few loci, or when the overall quality of the data is questionable. Indistinguishable mixtures that contain excessive contributors may also be uninterpretable. No comparison can be made to reference samples.

- **Single Source Profile**
  - Typically, each locus is characterized by one or two labeled peaks or alleles. If two peaks/alleles (heterozygous locus) are detected at a locus, they should ideally be of equal intensity that is, the peak height ratio (PHR) is approximately 1:1.
  - Peak height ratios are calculated by dividing the peak height (in RFUs) of the lower RFU allele by the peak height of the higher RFU allele, and then multiplying by 100 to express the PHR as a percentage.
  - Based on validation studies, the minimum expected PHR for single-source samples, where there is no indication of a mixture and 1 ng of template DNA is used, is 60%. This ratio, however, may be lower with lower amounts of DNA.
  - Peak-height-ratio imbalance may also result from degraded DNA or the presence of PCR inhibitors.
  - Allelic dropout may occur in degraded/inhibited samples and the possibility of a second allele should be considered.
  - In rare instances, an individual may exhibit a tri-allelic pattern at a locus. When a tri-allelic pattern is noted and not confirmed by multiple observations in a case, the sample will typically be re-amplified to confirm its presence.
  - The sample may be re-injected, re-amplified, or re-extracted at the analyst's discretion.

- **Partial profiles**

  Partial profiles exhibit allelic or locus dropout in some loci tested and can result from insufficient, degraded, or inhibited DNA. Typically, smaller loci amplify better under these conditions and larger loci tend to drop out. Extremely low levels of template DNA may also lead to stochastic effects which may under-represent one allele of a heterozygous locus. Again, care must be taken to ensure that non-matches are not a result of drop-out.

- **Mixtures**

  Evidence samples may contain DNA from more than one individual. A mixture can consist of full and/or partial profiles from multiple individuals. One or more of the following may indicate the presence

of a mixture:

- Greater than two alleles at a locus.
- A peak at the stutter position of significantly greater RFUs than is typically observed.
- Significantly unbalanced alleles at a heterozygous locus.
- Clear evidence of additional allelic activity below analytical threshold.
- Caution must be used if only a single example of a mixture is observed.
- Minor and major contributor peaks may be assigned if the peak height ratios of heterozygote types are within expected values.
- For purposes of mixture interpretation, peaks in the stutter position that exceed the expected percent stutter at a particular locus may be designated as a true allele.

- **Number of Contributors**
  - Generally, counting of alleles at the locus that exhibits the greatest number of allelic peaks can provide guidance towards determining the minimum number of contributors. For example, if at most five alleles are observed at a locus, then the DNA results are consistent with having arisen from at least three individuals. Proceed with caution when only one allele in the entire sample would lead to an increased number of possible contributors, as stutter, imbalance, and the potential for a tri-allele can complicate mixture interpretation. Loci more likely to exhibit a tri-allele pattern are D18S51, D21S11, FGA, TPOX, and vWA.
  - While counting allele peaks is very useful in determining a minimum number of contributors, the analyst must also consider allele sharing between individuals and allelic dropout may result in an underestimate of the actual number of contributors.
  - Mixtures may be reported as being from "at least" the fewest number of individuals possible, given the maximum number of alleles detected at a locus.
  - Determination of a finite number of contributors may be possible based on analysis of the data. Number of alleles per locus and peak height balance must be considered.
  - Using a known reference to account for alleles in an intimate sample can aid in determining the number of contributors.
  - Alleles that do not meet the stochastic threshold may be used to determine the number of contributors.

- If the number of contributors equals the number of individuals that cannot be excluded but there is activity foreign to these individuals, the sample interpretation must denote the foreign allelic activity.
- Indistinguishable mixtures of three or more individuals (complex mixtures), or the minor component of the distinguishable three or more person mixtures, must be interpreted with extreme caution. Allele sharing of alleles due to sharing creates more uncertainty.
- Two types of mixtures may be observed

- **Mixture with Major/Minor Contributors**
  - Some mixed source profiles may be clearly differentiated into major and minor components. A major component may consist of a single contributor or may be comprised of a mixture of two or more contributors. Major components of a single contributor should abide by the guidelines of a single source profile, including those for inclusion.
  - A major component can be identified by several means. The ratio of major/minor components can be considered and it should be ~4:1 or greater. Various combinations of homozygous and heterozygous genotypes of the major and minor components, along with allelic sharing between contributors, may influence the balance such that 4:1 parameter may be exceeded or insufficient to truly identify a major component.
  - It is permissible that a major component may not be identified at all loci. If a major component is not identified at all loci then caution must be used when deciding to consider the profile a major/minor mixture versus an indistinguishable mixture. This is especially true as the number of contributors increases. In general, a mixture of at least two contributors has a major component at least for half of the loci.
  - A mixture of three or more must have a major component(single or multiple contributors) at all loci.
  - Not all alleles of a minor component to a mixture may be determined since minor component alleles may be masked.
  - Consideration must be given to the possibility of the "swapping" of the major and minor contributors should one of the components exhibit evidence of degradation. If the major component is degraded, what appears to be from the major contributor at the loci of shorter fragments could appear to be from the minor component at the loci of longer fragments.

- **Mixtures with indistinguishable contributors (unresolved mixture)**

  A mixture must be considered indistinguishable when the major and minor components cannot be distinguished because of signal intensities. Individuals may still be included or excluded as possible contributors to the mixture. All interpretable loci are used in evaluating whether a person is included or excluded as a possible contributor. When evaluating whether a person should be excluded as a possible contributor, if an allele is not present at a locus, care must be taken to consider whether the allele may be missing due to drop-out. This determination can be difficult to make and consultation with a more experienced analyst or supervisor may be helpful.

- **Mixture Deconvolution**
  - Mixture deconvolution allows for the identification of obligate unknown alleles in a mixture given that a known contributor is present. This deduced profile is then eligible for comparison and statistical analysis as a single source sample.
  - It may not be possible to determine a complete genotype for the unknown contributor.
  - When a heterozygous genotype cannot be deduced and only a single obligate allele may be determined for the unknown contributor, then consideration must be given to any possible sister alleles. Three common reasons for a single allele may be:
  - The unknown contributor is homozygous.
  - A heterozygous sister allele is masked by the known contributor.
  - A heterozygous sister allele has dropped out.
  - Suitable mixtures are two person mixtures from intimate items with a finite number of contributors with one of the contributors known.
  - The major component of a three or more-person mixture may be eligible if a finite number of two contributors are identified in the major component and one contributor is known.

- **Steps for deconvolution**
  - Identify the alleles in the mixture that are attributed to the known individual.
  - Identify the remaining obligate alleles. At this step a full genotype of the unknown contributor has been identified for loci

where two obligate alleles remain. Document the deduced genotype for these loci.

- Identify the approximate ratio of the two contributors where a distinct genotype has been established for the unknown contributor.
- Examine the remaining loci with one obligate allele above stochastic threshold. It may be possible to determine a distinct genotype for the unknown contributor. Examine the RFU values for the alleles of the known contributor to determine if sharing with the foreign contributor could be occurring. Caution must be taken as natural heterozygous imbalance could lead to false deconvolution.
- Document the final genotype of the unknown contributor. Use a dash (--) with any single allele if the unknown is not being identified as homozygous.

12.2 Y-STR INTERPRETATION GUIDELINES

Y-Chromosome STR typing is an additional tool that can be used in concert with autosomal typing for the recovery of male DNA profiles in male:female mixtures and mixtures with an abundance of female DNA, such as with a vasectomized male or a product of conception. A Y-STR haplotype is shared by males from the same paternal lineage. Given Y chromosome markers are passed down from generation to generation without changing (except for mutational events), a match between an evidentiary item and a known sample only suggests that the individual could have contributed to the evidentiary sample, along with any relative from his paternal lineage. Y-STRs may also aid in determining the number of male contributors in a complex mixture.

Results and conclusions from DNA analysis must be scientifically supported by the analytical data with appropriate standards and controls. Whenever possible, evidentiary samples are be completely evaluated prior to the evaluation of any reference samples that are to be compared. The decision to attempt to develop a profile further through re-injection or re-amplification is be made without knowledge of the known profile for comparison, other than those of assumed contributors.

12.2.1 Interpretation of Results

The interpretation guidelines should determine if an observed peak is a true allele. The laboratory should define conditions in which the data would lead to the conclusion that the source of the male DNA is from either an apparent single male or two or more males of different paternal lineages. This may be accomplished by an examination of the number of alleles at each locus and the peak-height (or band-intensity) ratios at those loci that exhibit locus duplication such

as DYS385.

12.2.2 Apparent Single Male Contributor

A sample may be considered to represent a single male haplotype when the observed number of alleles at each locus is one and the signal intensity ratio of alleles at a duplicated locus is consistent with a profile from a single contributor. All loci should be evaluated in making this determination. It should be noted that individuals have been typed who exhibit multiple locus duplications at loci other than DYS385.

12.2.3 Mixtures with Major/Minor Male Contributors

A sample may be considered to consist of a mixture of major and minor male contributors if a distinct contrast in signal intensity exists among the alleles. All loci should be evaluated in making this determination.

12.2.4 Mixtures with a Known Male Contributor(s)

In some cases, when one of the male contributors (e.g., the victim) is known, the genetic profile of the unknown male contributor may be inferred. Depending on the profiles in the specific instance, this can be accomplished by subtracting the contribution of the known male donor from the mixed profile.

12.2.5 Mixtures with Indistinguishable Male Contributors

- When major or minor male contributors cannot be distinguished because of similarity in signal intensities or the presence of shared or masked alleles, individual males may still be included or excluded as possible contributors.
- The laboratory should have guidelines for the interpretation of partial profiles (i.e., profiles with fewer loci than tested) that may arise from degraded or limited-quantity DNA or from the presence of polymerase chain reaction (PCR) inhibitors. Occasionally, deletion of a portion of the Y-chromosome or a primer-binding site mutation can result in the failure to detect one or more Y-STR loci.

NOTE: The interpretation of inclusion or exclusion for Y STR profiles is the same as for autosomal STR profiles.

CHAPTER 13

STATISTICAL ANALYSIS OF STR DATA

13.1 ESTIMATING RARITY OF STR PROFILES

13.1.1 Introduction

- The presence of DNA from a victim, consent partner, or elimination standard on a sample which are reasonably expected and non-probative does not require a statistic for positive association. This includes DNA from a victim on intimate samples, i.e., samples from the body and clothing items in contact with the body do not require a statistic for positive association. A qualitative statement will be issued to express the significance of the association.
- Statistical analyses will be performed when the genetic typing results fail to exclude an individual(s) as a source of the DNA in a questioned sample and there is a positive association to a probative reference sample. The statistics is calculated from the questioned profile and includes the statistics-eligible loci which are completely detected and eligible for population statistics.

  Note: In the rare case that a known sample does not yield full profile at all statistics-eligible loci, then the questioned profile loci which could not be compared to the reference sample are not included in the statistics.
- To interpret the significance of a match between genetically typed samples, it is necessary to know the population distribution of alleles at the loci that were typed.
- If the STR alleles of the relevant evidence sample are different from the alleles of a subject's reference sample, then the subject is "excluded," and cannot be the donor of the biological evidence being tested. Exclusion is independent of the frequency of the alleles in the population.
- Qualitative statement similar to the following must be used if a statistics is not provided.

  Victim – included – expected contributor

  SUSPECT – included – consent partner

  SUSPECT – included – owner of [Item]

  Calculate statistics for each evidence profile where a victim, elimination, or suspect is included and considered probative.

  Examples: A vaginal swab match to a suspect is calculated.
- Five blood stains on the victim's clothing match the suspect, five stains

from the window at the scene match the suspect and five blood stains on the suspect's clothing match the victim. Calculations for all inclusions are needed: each profile from the window, each from profile victim's clothing and each profile from the suspect's clothing.

- In situations where the presence of a victim or elimination standard DNA profile would be reasonably expected, that profile may be subtracted out and the remaining profile calculated as a single source profile. The statistical statement in the report must describe what assumption is being made. Examples of items where the victim or elimination standard's profile would reasonably be expected are all intimate items, vehicle's owner, items in home, etc.
- If calculating a single source using the major contributor, every locus may be used where the major contributor is identified, even if the subject and victim have the same type at that locus, so long as there is not a reasonable chance that the target profile is dropping out.
- In rare instances, a single source minor profile can be calculated where the minor type is heterozygous and overlap can be ruled out. In less clearly defined circumstances, use a mixture calculation. Also use mixture calculation if there is no clear major and minor profile difference.

13.1.2 Single Source Profile Calculations

- If an evidence profile is different from that of a reference standard, then that individual is excluded as the source of the biological specimen. Exclusion is independent of the frequency of the profile in the population.
- If an evidence profile is the same as that of a reference standard, then that individual is included as a possible source of the biological specimen. The allele frequencies of Indian/Asian population may be used to calculate the profile frequency. A frequency may be calculated at one or more loci for a single source profile.

The following Popstats equations are recommended by the National Research Council (NRC II):

- **For Heterozygotes: 2pq** where p and q are the individual allele frequencies.
- **For Homozygotes: $p^2 + p(1-p)\theta$** where theta = 0.01

The product rule is used to calculate the combined profile frequency.

13.1.3 Mixed Source Calculations

- If a questioned sample contains DNA from more than one individual and clear major and minor contributors exist, the profile frequency of the major contributor can be calculated as a single source. The minor profile can be calculated as single source only for loci where there is no allele overlap.

- Multiple-donor profiles that cannot be resolved into major and minor contributors or for which it is desired to determine the frequency of the minor profile should be calculated as forensic mixtures. The frequency of the population that CANNOT be excluded as potential contributors to the mixture are then determined. For the frequency of each locus, Probability of Inclusion (PI) = $(P_1 + P_2 + P_3 + \ldots P_N)^2$ where P is the frequency of the allele detected for each allele 1 to N and N is the number of alleles detected at the locus, hence to calculate the frequency for each locus of the mixture. The product rule is then used to determine the overall combined probability of inclusion (CPI).

- CPE (combined probability of exclusion) = 1 – CPI

13.2 PROCEDURE

Statistical analysis is performed either using a statistics program or manually.

13.2.1 Random Match Probability for Autosomal STRs

The random match probability (RMP) is an estimate of the rarity of the observed DNA profile of a single individual, or the chance that a randomly selected individual from a population has an identical STR profile or combination of genotypes at the DNA markers used. The random match probability (RMP) will be used for the following samples:

13.2.2 Single Source Profile

- Deconvoluted major and/or minor profiles
    - The resultant values may be truncated for reporting, but should never be rounded up (i.e. 34,675,000 may be reported as 34 million or 34.6 million, but not 34.7 million).
    - Population frequencies can be estimated separately for each Indian caste population.
    - Profile frequency estimates are calculated according to the National Research Council report entitled *The Evaluation of Forensic DNA Evidence* (National Academy Press 1996, pp. 4-36 to 4-37).
    - For each population, the overall profile frequency estimate under the standard scenario of $\theta = 0.03$ unless there is reason to suspect that the "evidence DNA and subject are from the same subgroup" or a relative of the subject left the biological sample.
    - The overall profile frequency estimate is calculated by multiplying the individual locus genotype frequency estimates together.
    - In the standard scenario, homozygote genotype frequencies are estimated for each population using the formula $p^2+p(1-p)\theta$ for

$\theta = 0.03$ and heterozygote genotype frequencies are estimated using the formula 2pq.

- Calculations and allele frequencies are retained in the case file for referral at a later date if necessary.

- Instances where an individual's DNA on an item is reasonably expected may not require a statistic when making a positive association. In those instances, the positive association may be reported using a qualitative statement. Examples include:
  - Victim on intimate samples that originate directly from the individual's body: body cavity swabs, swabbing from any skin surface, or samples from fingernails.
  - Elimination/victim profile on their own clothing (single-source or mixtures).
  - Elimination homeowner on any item from their house (single-source or mixtures).
  - Person on any mixture on an item on which that person has already been demonstrated to be present elsewhere on that same item (Male Donor A on a mixture from cuffs scrapings of a shirt where Male Donor A was single-source or deduced major from the collar scrapings on the same shirt).
  - Person on any mixture from an item where that person has already been demonstrated to be present from a different item at the same location.
  - Mixtures on sexual assault items/swabs/fractions where Male Donor A was already identified on one of the items/swabs/fractions.

13.2.3 Combined Probability of Inclusion (CPI) for Mixtures

- The combined probability of inclusion (CPI) is defined as the probability that a randomly selected individual would be included as a contributor to a DNA mixture. In other words, it is the expected frequency of individuals who could be included as potential contributors to the mixture because all of their alleles are labeled in the evidence profile. CPI can only be used if all of the following circumstances are met:
  - When the evidence sample contains a non-deducible mixture.
  - When the alleles of the associated known sample are labeled at all of the conclusive loci in the evidence sample.
- A conclusive locus is a locus with concordant or repeating alleles. If an evidentiary sample is amplified more than once, loci with concordant alleles or repeating alleles are determined. Loci that are designated as "NEG" (for negative) or "INC" (for inconclusive) are not used in the CPI calculation. To avoid the possibility of bias, the determination to deem a locus inconclusive in the evidence profile must be made prior to

viewing the comparison sample profile.

- Although CPI can be calculated at any point, for efficiency and workflow, CPI is calculated (if necessary) after the DNA profile of the comparison sample(s) is determined to be included in the evidence sample. The CPI is calculated for informative samples. If RMP values have been generated, the CPI may not need to be calculated.
- Combined Probability of Inclusion is the expected frequency of individuals who are carrying only alleles that are labeled in the mixture in question, and if tested could potentially be included as contributors to this mixture. It is the expected frequency of individuals who could be included as potential contributors to the mixture because they do not carry any alleles that are not labeled in the evidence profile.

13.2.4 Parentage & Kinship Casework Procedures

The CFSLs perform parentage and relationship testing in criminal and civil cases of the following types:

- One-parent paternity where one biological parent is known and the other (often the father) is in question. Reference samples are analyzed for all three individuals in this scenario.
- Zero-parent paternity – where one biological parent is in question and the other is not available. Reference samples are collected from the two available individuals in this scenario.
- Reverse parentage – where a reference from an unidentified person or remains is compared against a pair of alleged biological parents. Reference samples for all three individuals are analyzed in this scenario.
- Caseswhich fall outside of the above, particularly those involving close biological relatives (incest), may have laboratory analysis performed at the CFSL.

13.2.5 Parentage Testing Terminology

- **Parentage**
  Refers to either paternity or maternity, paternity and parentage are often used interchangeably in genetic testing terminology.
- **Reverse parentage**
  A scenario in which a missing person's reference sample is compared to samples from a pair of alleged biological parents.
- **Likelihood Ratio**
  The ratio of two probabilities of the same event under different hypotheses. For example, in the case of one-parent paternity testing, the likelihood ratio compares the support of the genetic evidence for the hypothesis that the alleged man is the true biological father, against the support of the biological evidence that a random and unrelated man is the true biological father.

- **Paternity Index (PI)**
  A likelihood ratio at a single genetic locus that compares the probability of the observed genotypes (DNA profiles) if the tested man is the true biological father, to the probability of the observed genotypes (DNA profiles) if a random untested man is the true biological father.
- **Combined Paternity Index (CPI)**
  Because the genetic information at each of the loci is inherited independently, paternity indexes can be multiplied together to get a combined paternity index. The CPI is a measure of the strength of the genetic information from several loci.
- **Combined Paternity Index Ranges**
  In theory, for a CPI less than one, the genetic evidence is more consistent with non-paternity than paternity, for a CPI greater than one, the genetic evidence supports the hypothesis that the tested man is the father. "It is XXX times more likely that the tested man was the true biological father than if an untested random man was the father."
- **Probability of Paternity**
  A calculation based on both the paternity index and the prior odds. This probability should, in theory, include all evidence in the case, including both the non-genetic information and the genetic information from the DNA paternity test, as such, it is a measure of the weight of all the evidence. In practice, it is common to assume a prior probability of 0.5, or 1:1, when calculating and reporting probability of paternity.
- **Mean power of exclusion**
  The average probability that a random person would have a pattern of genetic information inconsistent with paternity at a particular locus. It indicates how often, on an average, one expects a random person in the population, who is unrelated to the child whose parentage is in question, to be correctly excluded as a biological parent.
- **One-parent Paternity**
  A scenario that includes biological reference samples from a child, one known parent and one alleged parent.
- **Zero-parent Paternity**
  A scenario that includes biological reference samples from a child and one alleged parent (with no reference from a known biological parent).
- **Exclusion**
  The obligate parental alleles in the child do not match the alleles in the alleged father in at least four loci. The alleged father is excluded from being the biological father of the child being tested.
- **Inclusion** (Cannot Exclude)
  The obligate parental alleles in the child match the alleles in the al-

leged father at all (or all but one) of the loci. The alleged father cannot be excluded from (or is consistent with being) the biological father of the child being tested.

13.3 FOR CASEWORK, CALCULATIONS WILL BE PERFORMED AND REPORTED FOR THE FOLLOWING POPULATION:

- Pooled samples (500) from Indian Caste Populations.
- Manual Calculations are performed using formulae.
- The resultant values may be truncated for reporting, but should never be rounded up.

13.3.1 Paternity Index (PI) Calculations

- Capital letters refer to the allele(s) present in each individual tested and the small letters refer to the frequency of the allele(s).
- After PI is calculated for each locus, the Combined Paternity Index (CPI) is calculated by multiplying together all the individual PIs.
- Assuming a prior probability of 0.5, probability of paternity (expressed as a percentage) is calculated as (CPI/(CPI+1)) x 100. Probability of paternity is reported to three decimal places (XX.xxx %). These values will be truncated, not rounded, for reporting purposes.
- The analysis of parentage cases is based on probability calculations.

13.3.2 Kinship Examinations: The purpose of a kinship investigation is to determine or exclude an assumed family relation. The calculations are based on the genotypes of the examined persons.

13.3.3 Paternity and Maternity Tests: Paternity tests aim to include or exclude a man (alleged father -AF) as biological father of a child (C). Analogously, one can include or exclude a woman (alleged mother - AM) from biological maternity by a maternity test.

- The values of paternity and maternity tests are determined for all investigated markers as:

  - **Paternity Index (PI)** is calculated for one examined marker according to BUTLER (2005):

    PI= X/Y

    where **X** is the probability of the null hypothesis and
    Y is the probability of the alternative hypothesis
    X and Y are mutually exclusive and assume non-relatedness.

- The paternity indexes are multiplied resulting in the **combined paternity index CPI**:

  - **Combined Paternity Index (CPI)**

    $$CPI=PI_1.PI_2.....PI_n$$

where **n** is the number of examined markers and
PI1…n is the paternity index of the markers 1 to n.

- **Likelihood Ratio (LR)**
  As a reciprocal of the paternity index the likelihood ratio LR indicates how much more likely is the alternative hypothesis than the null hypothesis. The likelihood ratio for one examined marker is calculated according to BUTLER (2005):

  LR= Y/X

  Where**X**is the probability of the null hypothesis.
  Y is the probability of the alternative hypothesis.
  n is the number of examined markers and
  LR1…n is the likelihood ratio of the markers 1 to n.
  The **combined likelihood ratio CLR** is calculated as the product of the LRs of the single markers:

  $$\mathbf{CLR=LR_1.LR_2....LR_n}$$

  where **n** is the number of examined markers and
  LR1…n is the likelihood ratio of the markers 1 to n.

- Testing paternity or maternity, one compares different genetic markers (loci) of the child with the markers of the examined parents. In an ideal situation one can conclude which of child's allele must originate from mother and which comes from the father. For instance, a child carrying the alleles 12 and 13 with its mother carrying the alleles 13 and 14 must have a biological father carrying allele 12 at least once.

- In order to make a statement about a putative paternity and maternity, one needs to compare the alleles of several genetic markers. The significance of the statement increases with the number of markers examined. At the present day, one usually compares at least 12 markers. These must be unlinked, that is they must be inherited independently. Furthermore, they have to be located on 10 different chromosomes at least (HOPPE, 2002).

- There are two possible scenarios for paternity and maternity tests:

  - **Trio case:** Genetic information is available about all three persons (child (C), mother (M) and alleged father (AF).
  - **Duo case:** Genetic information is only available for the child (C) and the alleged father (AF) or alleged mother (AM). The information about the second parent (mother (M) or father (F)) is missing.

- The investigation of trio cases presumes that:

- The mother is not related to the alleged father or other possible fathers, and that
- Different alleged fathers are not related to each other (maternity tests analogously).

Note: Trio maternity tests presume that the tested father is the biological father of the child. However, duo tests, in case of inclusion, are less significant than trio tests.

Table: 1 Calculation of a Trio Case

MOTHER	CHILD	FATHER	PATERNITY INDEX
AA	**AA**	**AB**	0.5/p(a)
AA	**AB**	**AB**	0.5/p(b)
AA	**AB**	**BC**	0.5/p(b)
AB	**AA**	**AB**	0.5/p(a)
AB	**AA**	**AC**	0.5/p(a)
BC	**AB**	**AB**	0.5/p(a)
BC	**AB**	**AC**	0.5/p(a)
BD	**AB**	**AC**	0.5/p(a)
AA	**AA**	**AA**	1/p(a)
AB	**AA**	**AA**	1/p(a)
BB	**AB**	**AA**	1/p(a)
BC	**AB**	**AA**	1/p(a)
AB	**AB**	**AC**	1/p(a)+p(b)
AB	**AB**	**AA**	1/p(a)+p(b)
AB	**AB**	**AB**	1/p(a)+p(b)

Table: 2 Calculation of a Duo Case

The calculation of duo cases is analogous to trio cases. Due to the missing parent more possible combinations of genotypes are feasible. The table for calculation of paternity indexes for all possible duo constellations is the following:

ALLEGED PARENT	CHILD	PATERNITY/MATERNITY INDEX
AC	**AB**	0.25/p(a)
AB	**AB**	p(a)+p(b)/4**.** p(a)**.**(b)
AA	**AB**	0.5/p(a)
AC	**AA**	0.5/p(a)
AA	**AA**	1/ p(a)

13.3.4 Paternity and Maternity Tests in Cases of Incest

- If the (alleged) parents of a child are related to each other, the probability of paternity might be affected by this. In the case of mother and alleged father (or father and alleged mother) being cousins, uncle and niece or aunt and nephew, there is only less influence. The probabilities of paternity or maternity determined when regarding the relationship hardly deviate from the results obtained without consideration of such. Thus, trio cases of this type are evaluated like normal trio cases (HUMMEL, 1997).
- However, if mother and alleged father (or father and alleged mother) are parent and child or siblings, one needs to use other formulas for the calculation of the PI because the genotypes of the parents are not independent from each other. The formulas used are based on the three-allele system formulas by MINATAKA (1996). The formulas have been adapted for multi-allele systems.

Table: 3 calculation of the paternity index in cases of incest

CHILD	FATHER	MOTHER	FATHER-DAUGHTER INCEST/ MOTHER-SON INCEST	BROTHER-SISTER INCEST
AA	**AA**	**AA**	1/(0.5a+0.5)	1/(0.5a+0.5)
AA	**AA**	**AB**	1/(a+0.5)	1/(a+0.5)
AA	**AB**	**AA**	1/(a+1)	1/(a+1)
AA	**AB**	**AB**	1/(a+0.5)	1/(a+0.5)
AA	**AC**	**AB**	0.5/a	0.5/a
AB	**AA**	**AB**	1	1
AB	**AA**	**BB**	exclusion	1/(0.5a)
AB	**AA**	**BC**	exclusion	1/(a)
AB	**AB**	**AA**	1/(b)	1/(b)
AB	**AB**	**AB**	1	1
AB	**AB**	**BC**	0.5/a	0.5/a
AB	**AC**	**AB**	0.5/(a+b)	0.5/(a+b)
AB	**AC**	**BC**	0.5/a	0.5/a
AB	**AC**	**BD**	exclusion	0.5/a
AB	**BC**	**AA**	exclusion	0.5/b

- A, B, C and D are different alleles of a multi-locus system. Their allele frequencies are a, b, c and d.
- The probability of paternity determined considering the incest is often lower than the one determined without consideration (HUMMEL, 1997). In addition, there are a higher number of incompatible constellations (at least for parent-child incest). For instance, if there is a mother with the genotype aa, it is not possible that the alleged father has genotype bb, at least not if he was

the true biological father of the mother.

13.3.5 Reverse Parentage Testing

Table: 4 Formulae for calculation of the Reverse Parentage Index

Parent -1	Child	Parent -2	INDEX
BD	AB	AC	1/8ab
BC	AB	AC	1/8ab
BC	AB	AB	1/8ab
BC	AB	A	1/4ab
B	AB	AC	1/4ab
B	AB	AB	1/4ab
B	AB	A	1/2ab
AB	AB	AC	1/8ab
AB	AB	AB	1/4ab
AB	AB	A	1/4ab
AB	A	AC	$1/4a^2$
AB	A	AB	$1/4a^2$
AB	A	A	$1/2a^2$
A	A	AC	$1/2a^2$
A	A	A	$1/a^2$

13.4 HAPLOTYPE FREQUENCY FOR Y STR

13.4.1 Introduction

Once a questioned sample is compared to a known reference standard and an inclusion is made, the frequency estimation of the haplotype must be determined. No calculations are required for known standards that are included on an intimate sample. The basis for haplotype frequency estimation is the counting method. This frequency estimation would be the same for all paternally related males as to the standard that is included.

13.4.2 Procedure

- Statistical calculations shall be performed for single source and single major contributors with a minimum of 10 loci.

- A Y-STR haplotype frequency estimation is calculated from the observed haplotype frequency by attaching a confidence interval (generally 95% or greater) to the haplotype frequency estimate to capture the effect of the database size. The sampling variance decreases as the database size increases.
- The frequency for a Y STR haplotype is estimated by counting the number of times the haplotype occurs in each of the population databases and dividing by the total number of individuals in the database.
- Haplotype Frequency (p) = x/N where x is the number of times the haplotype is observed in a database containing N number of haplotypes.
- If the haplotype has not been previously observed in the database, the formula for calculating the upper 95% confident limit is: 1 – (0.05)1/n where n = database size.
- The consolidated Indian YSTR Database shall be utilized for haplotype frequencies. Y-Chromosome Haplotype Reference Database (YHRD) may be used (https://yhrd.org). A list of other Y-STR haplotype databases can be found at http://cstl.nist.gov/biotech/strbase/y_strs.htm and utilized as necessary.
- The statistics which provides the most genetic information and/or the highest discrimination potential must be reported. This "either/or" scenario comes about due to databases containing different population sizes for different loci, as they are comprised of data from different multiplexes (for example, more population data exists for loci shared between the Applied Biosystems AmpF*l*STR® Yfiler® amplification kit and the Promega PowerPlex® Y23 than for the loci contained exclusively within the PowerPlex® Y23 kits). (As recommended by SWGDAM YSTR Committee_2014).
- The statistical calculations must be reported to the same significant figure as displayed on the results sheet.
- A haplotype that has not been previously observed in the Indian database, which includes 200 individuals, would be reported as "less than 1 in 200 Indians".
- A haplotype that has been observed once in the Asian database would be reported as "1 in 200 Indians".
- A haplotype that has been observed 5 times in the Indian database is reported as "1 in 40 Indians" (5 in 200 is equal to 1 in 40).

CHAPTER 14

REPORT WRITING

This document is a guideline intended to help achieve uniform contents in technical reports. Because all possible case scenarios cannot be addressed individually, different approaches from the statements mentioned here will at times be necessary to accommodate specific case circumstances. This document is intended to describe and explain terminology related to analysis. It shall be attached to or incorporated by reference in laboratory reports or included in the case file.

Examiners are expected to prepare reports and provide testimony consistent with the directives of this document. However, examiners are not required to provide a complete or precise recitation of the definitions or bases set forth in this document. This is supplemental information that is intended to clarify the meaning of and foundation for the approved conclusions.

14.1 PROCEDURE FOR CASE REPORT WRITING (AUTOSOMAL STR)

The purpose of this document is to provide casework report writing guidelines for autosomal DNA results using various multiplex amplification kits.

In accordance with the NABL's "Quality Assurance Standards for Forensic DNA Testing Laboratories", all Forensic DNA Reports (Certificates of Analysis) in which DNA analysis was conducted will contain at a minimum the following information:

- Case identifier
- Description of evidence examined
- A description of the methodology
- Polymorphic loci examined or amplification system
- A quantitative or qualitative interpretative statement
- Statistical frequencies of inclusion
- Date issued
- Disposition of evidence
- A signature and title, or equivalent identification of the person accepting responsibility for the content of the report.

14.2 GENERAL FORMAT OF REPORTING

The Forensic DNA Report will contain a METHODS section (as appropriate) and RESULTS section and may contain a CONCLUSIONS section. The METHODS section will be placed prior to the RESULTS section in the Forensic DNA Report and will include the description of methodology, the PCR kit used, and polymorphic loci tested.

14.3 METHODS SECTION

14.3.1 Methods of Sampling

The methods used to collect a sample for DNA is considered part of the examina-

tion process and will not be reported in the Forensic DNA Report. For example,

- If a bottle is submitted and a swab is used by the examiner to collect DNA from the mouth area of the bottle, the Report will refer to a DNA profile developed from the mouth area of the bottle, rather than a swab of the mouth area of the bottle.
- If it is clearly inferred from the report wording that a sample was combined, there is no need to use the word combined in the Report.

14.3.2 Methods for Body Fluid Testing (choose and use as appropriate based upon body fluid tested)

The indication of blood or seminal fluid based on preliminary testing of body fluids is not confirmatory. The indication of body fluid is conducted through visual, serological & immunological testing. The identification of spermatozoa is conducted through microscopic examinations and is confirmatory.

NOTE: The statement given in analysis report regarding identification of spermatozoa need only be included if a microscopic examination for spermatozoa is conducted.

14.3.3 DNA Analysis Methods

- **DNA Extraction** method should be mentioned in analysis method. In cases for which a Differential Extraction Procedure was conducted, the terms sperm fraction (male fraction) and non-sperm fraction (Epithelial fraction) are used to denote two samples for analysis produced from one parent sample during the DNA extraction process. DNA analysis of a sperm fraction does not necessarily imply the presence of spermatozoa.
- **The real-time PCR** amplification kit for DNA Quantification used, should be mentioned. It includes both total human DNA and male DNA as samples tested or mentioned in the report.
- The method of deoxyribonucleic acid (DNA) analysis used is the **Polymerase Chain Reaction (PCR)** which includes total number of autosomal loci/alleles tested or listed including Amelogenin for gender determination. Y-Chromosome PCR amplification kit includes loci/alleles tested used to generate the male DNA profile.
- **Instruments and Techniques:** Instruments and software used for the generation of genetic profiles in Forensic DNA Report should be mentioned.

14.4 RESULTS SECTION

The Results section will contain the results of all analyses including screening results, DNA results, comparisons/conclusions, result Data and statistics. The results section may list results in a bulleted format or a narrative format. All items sub-

mitted for Forensic Biology & DNA examinations will be addressed in the Report. Items not examined will be addressed in the Report as not having been examined. Results of tested alleles of examined samples should be in tabular form in the Report. Based on results of examination, observations or interpretations should be drawn.

14.5 CONCLUSIONS SECTION

Conclusion will follow the RESULTS section. This section is reserved for reports containing multiple items tested and hence more results to be reported. It may be incorporated into the report to assist in streamlining results, thus creating a more reader friendly report. When incorporated, it will contain results of comparisons, result data, and statistics. A CONCLUSIONS section is incorporated into the report as follows:

EXAMPLE: A reconstruction case involving 2 victims and 2 suspects and 21 items were submitted for analysis. DNA profiles were developed from 17 of the 21 items; the results of comparison to the known samples may be summarized in a CONCLUSIONS section in the Report. The RESULTS section would address whether DNA profiles were developed or not.

14.6 DEFINITIONS AND TERMINOLOGY USED IN CONCLUSIONS

- **Combined Probability of Exclusion (CPE)**: The probability that a randomly chosen, unrelated person from a given population would be excluded as a potential contributor to the observed DNA mixture.
- **Combined Probability of Inclusion (CPI):** The probability that a randomly chosen, unrelated person from a given population would be included as a potential contributor to the observed DNA mixture.
- **Intimate Sample:** A biological sample from an evidence item that is obtained directly from an individual's body, it is not unexpected to detect that individual's allele(s) in the DNA typing results.
- **Random Match Probability (RMP):** The chance that a randomly selected unrelated individual from a population will have an identical STR profile to that obtained from a forensic sample.
- **Reference Sample/Standard:** Biological material for which the identity of the donor is established and used for comparison purposes; also referred to as a known standard. These include victim, suspect, elimination and/or witness standards.

14.7 GENERAL PRINCIPLES

- If a profile is determined to be partial (whether a single source, mixture, evidentiary or reference), the word partial shall be used to qualify the result. NOTE: comparisons between partial reference profiles and evidentiary profiles can be made only for the loci at which results exist in the partial reference profile.

- If a differential extraction is performed, non-sperm and sperm fractions shall be used to qualify the results and both fractions shall be reported.
- If a single source or single predominant profile (unknown) is obtained and a Y is present at Amelogenin, such profiles shall be qualified as male in the report.
- If a Y is present at Amelogenin in a mixture and no inclusionary statement to a male reference sample has been made, the overall mixture shall be qualified as having a male contributor. This mixture contains at least one male contributor.
- If multiple unknown profiles are present within a case, they may be qualified numerically. For example: first unknown (male), second unknown (male), etc.

14.8 SINGLE SOURCE PROFILES

- Matches and Non-matches: A DNA profile was obtained from _____ (Item__) that **MATCHES** the DNA profile obtained from _____ (Item__) and **DOES NOT MATCH** the DNA profile(s) obtained from _____ (Item(s)__).
- An assumed contributor (i.e., victim or elimination reference profile) matches an intimate item. The DNA profile obtained from the _____ (Item__) is not different from that of the victim/subject (Item__) and **DOES NOT MATCH** the DNA profile obtained from _____ (Item__).
- Matches and Consistent With: if our population exceeds the world's population at the time the statistics are generated, the word **MATCHES** shall be used. If the population group is less than the world population at the time the statistics are generated, the phrase **IS CONSISTENT WITH** shall be used.

Note: If the phrase **IS CONSISTENT WITH** is used, add the following statement: (The term is consistent with means that the result of the population data statistics did not exceed the world's population; therefore, the term match was not used).

14.9 MIXTURES

Note: if the number of contributors can be determined, a statement indicating such shall be added to the result statement(s): "*…is CONSISTENT WITH A MIXTURE of at least (#) contributors*." If a determination of the number of contributors cannot be made, see indistinguishable mixture.

14.9.1 Mixtures with a Single Predominant Profile

14.9.2 Match to Known Reference Sample(s): *The DNA profile obtained from* ____

*(Item__) is **COSISTENT WITH A MIXTURE** of (# of contributors). The predominant profile **MATCHES** the DNA profile obtained from _____ (Item__).*
Non-match to Known Reference Sample(s) (i.e., unknown profile): *The DNA profile obtained from ____ (Item__) is **CONSISTENT WITH A MIXTURE** of (# of contributors). The predominant DNA profile **DOES NOT MATCH** the DNA profile(s) obtained from _____ (Item(s) __) and is from an unknown individual.*

14.9.3 Contribution to Overall Mixture (for 2 or 3 contributor mixtures)

- **Cannot exclude/excluded from the mixture:** *The DNA profile(s) obtained from _____ (Item(s)____) cannot be excluded/ is/are excluded as a contributor/as contributors to the mixture.*

14.9.4 Contribution to Overall Mixture (for mixtures of more than 3 contributors): (See Indistinguishable Mixture)

14.9.5 Mixtures with Multiple Major Profile:

- **Excluded from the multiple major profile but not excluded from the minor component:** The DNA profile from ____ (Item__) is excluded as a contributor to the multiple major profile of the mixture. However, the DNA profile from _____ (Item __) cannot be excluded as a contributor to the minor component of the mixture.
- **Excluded from the overall mixture:** The DNA profile(s) from _____ (Item __) is/are excluded as a contributor/contributors to the mixture.
- **Primary statement:** The DNA profile obtained from ____ (Item__) is **CONSISTENT WITH A MIXTURE** of (two or three) contributors/of at least four contributors which contains multiple major profiles.
- **Cannot exclude from the multiple major profile:** The DNA profile(s) obtained from _____ (Item_) cannot be excluded as a contributor/as contributors to the multiple major profile of this mixture.

14.9.6 Mixtures with No Predominance (Indistinguishable Mixture):

- **Primary statement:** *The DNA profile obtained from ____ (Item__) is **CONSISTENT WITH A MIXTURE** of (# of contributors).*
- **Cannot exclude from mixture:** *The DNA profile(s) obtained from _____ (Item__) cannot be excluded as a contributor to/as contributors to the mixture.*
- **Excluded from the mixture:** *The DNA profile(s) obtained from _____ (Item__) is/are excluded as a contributor to/are excluded as contributors to the mixture.*

14.9.7 Un-interpretable Profiles

- The following statement shall be used when the overall DNA profile is insufficient for comparison purposes. This statement shall also be used when results are only obtained at Amelogenin: No interpretable DNA profile was obtained from _____ (Item__). This DNA profile is not of sufficient quantity and/or quality for comparison purposes.
- The following statement shall be used for minor components of a mixture: No interpretable DNA profile was obtained from the minor component of the mixture from _____ (Item__). This DNA profile is not of sufficient quantity and/or quality for comparison purposes.

14.9.8 No Conclusion (for individual contribution)

In an overall mixture/to the minor component of a mixture: No conclusion can be rendered as to the contribution of the DNA profile(s) from _____ (Item __) to the mixture/to the minor component of the mixture due to insufficient quality and/or quantity.

14.10 LIMITED DNA PROFILE

- **Primary statement:** Examination of _____ (Item__) revealed the presence of _#_ alleles and is of insufficient quality and/or quantity for inclusionary purposes.

  Note: Amelogenin results shall not be considered when determining the total number of alleles present.
- **Exclusions:** The DNA profile(s) obtained from _____ (Item __) is/are excluded from this profile.
- **No conclusion:** No conclusion can be rendered as to the contribution of the DNA profile(s) from _____ (Item___) to this profile.
- **No DNA Profile:** When no alleles are detected above the analytical threshold: *No DNA profile was obtained from _____ (Item__).*
- **Additional Alleles:** When additional alleles are present in an interpretable mixture that cannot be attributed to any of the known reference standards provided: *Additional DNA was present which cannot be accounted for by the standard(s) submitted.*

14.11 STATISTICS

Note: Qualifiers such as partial, predominant, partially predominant, non-sperm fraction or sperm fraction shall be used as appropriate in the statistical statements. If no statistical data is generated, the following statement shall be used: *No population frequency data were generated for the contribution of _____ (Item) to this Item.*

14.11.1 Random Match Probability

- When Random Match Probability statistics have been generated for a profile in which database utilized is above 1 trillion, the following statement shall be used: *The probability of randomly selecting an unrelated individual with a DNA profile that matches the DNA profile obtained from the ___ is 1 in greater than 1 trillion (which is more than the world's population) in the Indian/Asian populations.*
- When Random Match Probability statistics have been generated for a profile in which the database utilized is below 1 trillion, the following statement shall be used: *The probability of randomly selecting an unrelated individual with a DNA profile that matches/is consistent with the DNA profile obtained from the ____ is approximately 1 in _____ in the Indian/Asian population.*

14.11.2 Combined Probability of Inclusion

If a known reference sample cannot be excluded from a mixture, the following statement shall be used: *The chance of randomly selecting an unrelated individual who would be expected to be included for the DNA mixture obtained from _____ (Item __) is approximately: 1 in ___.*

All inclusionary statements when compared to a reference sample shall be accompanied by the appropriate statistics (i.e., RMP or CPI/CPE). An exception to this requirement is when an assumed contributor is present in an intimate item (i.e., victim on a vaginal swab).

14.12 PROCEDURE FOR CASEWORK REPORT WRITING (Y-STR)

14.12.1 Y-STR

The following wording shall be added as applicable to the report.

Y-STR DNA testing was performed on the extracts from Items____ or/ DNA extractions were performed on Items _____ as well as on the extract(s) from the known DNA standard from _____ (Item _____). These extracts were previously quantitated, and then amplified, and tested with Y-STR DNA genetic markers DYS576, DYS389I, DYS448, DYS389II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385a/b, DYS456 and Y-GATA-H4.

14.12.2 Single Source Profiles

- **Match** - If the Y-STR DNA profile from a known sample is included in the single source questioned sample, then the results shall be reported as follows:
  - The Y-STR DNA profile obtained from ___ (Item __) matches the Y-STR DNA profile obtained from ___ (Item ___).
- If the sample is a partial profile, then the qualifying word partial shall be added to the above statement and matches will be reported

as consistent with.

- **Exclusion** – If the Y-STR DNA profile from a known sample is excluded as being a contributor, then the results shall be reported as:
    - The Y-STR DNA profile(s) obtained from ___ (Item __) is/are excluded as contributors to the Y-STR DNA profile obtained from _____ (Item __).

14.12.3 Mixture Profiles

- Primary Statement – For profiles containing a mixture of multiple males, the following statement shall be reported.
  The Y-STR DNA profile obtained from ___ (Item __) is consistent with a mixture from # male contributor.
- Major/minor – When a major/minor contributor can be ascertained from a mixture, the following statement shall be reported.
  The Y-STR DNA profile obtained from ___ (Item __) matches the major Y-STR DNA profile/the minor profile of this mixture.
- If the owner of the intimate sample is used to derive the remaining contributor of the mixture, the following statement shall be reported.
  Assuming the Y-STR DNA profile obtained from ____ (Item ___) as a contributor to the mixture obtained from ____ (Item __), the remaining component of the mixture matches the Y-STR DNA profile obtained from ____ (Item __).

14.12.4 Inconclusive

For Y-STR DNA profiles (or components of the profile) obtained whose results are not suitable for comparisons, the following statement shall be reported:
The Y-STR DNA profile obtained from ___ (Item __) is inconclusive due to insufficient quality and/or quantity.

14.12.5 No DNA result

If no Y-STR DNA was obtained, the result shall be reported as:

- No Y-STR DNA profile was obtained from ___ (Item __).

14.13 REPORT WRITING PROCEDURES

14.13.1 Forensic Relationship Comparison Methods

Forensic relationship comparisons may be requested as part of some criminal investigation cases or civil cases. Types of relationship comparisons that can be performed include paternity, maternity, reverse paternity, sibling relationship etc. These comparisons may establish potential relationships among individuals and /or aid in the identification of human remains and missing persons. The genetic profiles from standards/samples are compared to evaluate relationship.

14.13.2 Conclusions to be Reported

- **Parentage Relationship cases**
  - The genetic results strongly support the hypothesis of the alleged relationship; therefore the alleged individual cannot be excluded from the relationship.
  - The genetic results do not strongly support the hypothesis of the alleged relationship nor does it indicate no relationship; therefore it is deemed inconclusive. An inconclusive result may also be derived if a genetic profile cannot be interpreted or is of poor quality [100>combined parentage Index>1].
  - The genetic results support the hypothesis of no relationship; therefore the alleged individual can be excluded from the relationship [Combined Parentage Index<1].
- **Non-Parentage Relationship cases**
  - The genetic results support the hypothesis of the alleged relationship [combined Relationship Index CRI>10.
  - The genetic results do not strongly support the hypothesis of the alleged relationship nor does it indicate no relationship; therefore it is deemed inconclusive (10>CRI>0.05).
  - The genetic results support the hypothesis of no relationship (CRI<0.05).
- **Inconsistent Profiles in Parentage relationship Cases**
  - If the alleged father's profile is inconsistent with the child's profile at three or more loci then no relationship/exclusion shall be concluded.
  - If the alleged father's profile is inconsistent with the child's profile at fewer than three loci then other alternatives may be evaluated for these inconsistencies. Other alternatives may include possible mutation, null allele, first degree relative or true exclusion. If it is determined that a possible mutation could account for the inconsistencies than the mutation shall be included into the statistical calculations.
- **Mixtures**
  - With the product of conception/fetal samples it is necessary to have a standard from the mother. Only loci where obligate paternal alleles can be determined shall be used for statistical purposes.
  - With personal effects believed to belong to a missing person the degree of the mixture shall be evaluated to determine whether conclusive results can be obtained. Additional standards may be requested to aid in interpretation.

14.14 GENERAL GUIDELINES FOR REPORT WRITING

- The DNA profiles shall be reported in table format in the results area of the report.
- The combined paternity index and paternity index shall be reported as calculated.
- All reports shall include the introductory statement and retention statement.
- The paternity index for each locus shall be included in the table. Combined paternity Index shall be reported in the table.
- Any software calculation of the combined paternity Index with a decimal value shall be rounded down to the nearest whole number.

14.14.1 Example Table for Paternity Case Report

STR LOCUS	SAMPLE SOURCE			PATERNITY INDEX
	Alleged Father (Item/Exhibit 1)	Child (Item/Exhibit 2)	Mother (Item/Exhibit 3)	
D8S1179				
D7S820				
CSF1PO				
vWA				
FGA				
AMELOGEN -IN				
COMBINED PATERNITY INDEX=				

14.14.2 Sample Wording of Relationship Comparison Cases

- **Sample : A parentage case where Alleged father cannot be excluded**

  [NAME](Item#), the alleged father, cannot be excluded as the biological father of [NAME] (Item #) the child in focus.

  The genetic results are {CPI value} time more likely if [NAME] is the biological father of the child [NAME] than a random untested unrelated man in the Indian Population

  OR

  Statement of Results: Alleged relationship is not excluded.

  Under each of the 15 STR systems viz: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 and FGA, an obligate pa-

ternal allele present in the child [NAME] (ITEM#) is included in the 15 STR genotype of the alleged father [NAME] (ITEM#).

- **Opinion:**
  From the DNA profiling results of the above individuals and from the interpretations thereof:
  [NAME] (represented by ITEM#) cannot be excluded as the biological father of this male/female child in focus, [NAME] (ITEM#) of [MOTHER'S NAME] (ITEM#).

- **Sample: A parentage case where Alleged individual can be excluded**
  The lack of genetic markers that must be contributed to the child by the biological father in the genetic results listed in the table above, the Alleged father [NAME](Item#) can be excluded as the biological father of the child in focus[NAME](Item #).

  OR

  Statement of Results: Alleged Relationship is excluded.
  Based on the DNA analysis results, the Alleged Father, [NAME] is excluded as the biological father of the child [NAME] because they do not share sufficient genetic markers. Of the Genetic Identity System tested ___ of ___ do not match and the Combined Index is 0.
- **Sample: A parentage case with inconclusive results**
  The genetic results listed above do not strongly favour the hypothesis of [NAME] (Item#), the alleged father, being the biological father of [NAME] (the child in focus)(Item#). The combined Paternity index of [CPI] is low and does not meet the laboratory standards in order to establish paternity, therefore these results are inconclusive. The genetic results are based only on the current data.
- **Sample: A reverse parentage case where putative individuals cannot be excluded**
  The putative father [NAME](Item#) and the putative mother[NAME](Item #) cannot be excluded as biological parents of child [NAME](Item#). The genetic results are [Combined parentage Index] times more likely that [NAME] and [NAME] are biological parents of the child [NAME] than random untested unrelated couples in the Indian population.

  OR

  Statement of Results: Putative relationship is not excluded.

  Under each of the 15 STR systems viz: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338,

D19S433, vWA, TPOX, D18S51, D5S818 and FGA, Putative father[NAME] and Putative Mother[NAME] possess the necessary alleles to produce an offspring with STR types obtained from the bone [ITEM#] and/or blood stain on the shirt (ITEM#).

- **Opinion:**
  From the DNA profiling results of the above individuals and from the interpretations thereof:
  [NAME] (Represented by Exhibit-A) and [NAME] (Represented by Exhibit-B) qualify to be the possible biological parents of the donor of evidentiary samples (ITEM# and ITEM#). Furthermore the reverse parentage analysis (Combined Parentage Index) did not exclude the donor of the evidentiary samples as [NAME] and [NAME] child.

- **Sample: A Sibship Comparison with evidence of relationship**
  The genetic results listed above support the conclusion that [NAME](Item #) and [NAME](Item#) are related as full siblings. These genetic results are (CRI) times more likely if [NAME] is a full sibling of [NAME] than if they are unrelated.

  OR

  Biological specimens corresponding to [NAME](Alleged Sibling 1) and [NAME] (Alleged sibling 2) were submitted for genetic testing to help determine whether they are full siblings (share both parents). Based upon the genetic data the combined sibship index is XX (corresponding to a probability of sibship of YY%, assuming prior probability=0.5) and indicates that these data are XX times more likely if [NAME] and [NAME] are full siblings than if they are unrelated. Sibship indices greater than Z are generally regarded by the genetic testing community as comprising evidence in favour of the disputed biological relationship(in this case full sibship) However only those indices in excess of Z are regarded as conclusive evidence. Therefore the genetic data in this case is strictly speaking inconclusive but comprise evidence in favour of sibship. These results do not supersede any testing involving the biological parents of these individuals. The genetic data are inconclusive but support a sibship relationship involving [NAME] and [NAME].
- **Sample: A Sibship Comparison with no evidence of relationship**
  The genetic results listed above support the hypothesis that [NAME](Item#) and [NAME](Item#) have different biological parents over the hypothesis that [NAME] and [NAME] share biological parents. These genetic results are (CRI) times more likely if

[NAME] is unrelated to [NAME] than if they are full siblings. The genetic results are based only on the current data and do not supersede any additional genetic testing.

- **Sample: A Sibship Comparison with inconclusive Results**
  The genetic results listed above do not strongly support the hypothesis that [NAME](Item#) and [NAME](Item#) share the same biological parents nor does it indicate that [NAME] and [NAME] have different biological parents therefore it is deemed inconclusive. The combined relationship index for full siblings is low and does not meet the laboratory standards necessary to establish a relationship. The genetic results are based only on the current data and additional genetic testing is recommended.
- **Cases with an observed mutation**
  A single genetic inconsistency between the child and the alleged father was observed at locus (__). This has been incorporated into the calculation as a mutation.

APPENDICES

APPENDIX- A
ALLELE FREQUENCIES (INDIAN)

Allele	CSF 1PO	D10 S1248	D1 S3	D13 S317	D16 S539	D18 S51	D19 S433	D1 S1656	D21 S11	D22 S1045	D2 S1338	D2 S441	D3 S1358	D5 S818	D6 S1043	D7 S820	D8 S1179	F13 A01	F13 B	FE SFPS	FGA	LPL	Penta _C	Penta _D	Pen- _E	SE3	TH01	TPOX	vWA
2.2																													
3.2																		0.30											
4.0																		0.12											
4.2																													
5.0																		0.06					0.025		0.072				
6.0																		0.48	0.01					0.010			0.1701		
6.3																													
7.0	0.020													0.015		0.00		0.01	0.00			0.00	0.005				0.2680		
8.0				0.216								0.005		0.005		0.13			0.04				0.067	0.005	0.005		0.0722	0.5464	
8.1																													
9.0	0.067			0.144	0.355									0.097		0.04			0.19	0.015		0.00	0.355	0.314	0.025		0.4433	0.0825	
9.1												0.005																	
9.3																											0.0412		
10.0	0.201	0.0052		0.103	0.164							0.268		0.226	0.030	0.26	0.123		0.73	0.092		0.64	0.067	0.185	0.061		0.0052	0.0309	
10.1																													
10.2																													
10.3																				0.005									

11.0	0.216			0.268	0.190			0.030		0.2010		0.350		0.273	0.149	0.35	0.118		0.00	0.360		0.11	0.314	0.180	0.159			0.2938	
11.2																														
11.3												0.030																		
12.0	0.386	0.0876		0.211	0.175	0.036	0.036	0.046		0.0052		0.216		0.206	0.123	0.17	0.118			0.309		0.21	0.144	0.180	0.092			0.0464	
12.2							0.025																							
12.3												0.005			0.005															
13.0	0.087	0.3196		0.056	0.097	0.216	0.283	0.134				0.025		0.159	0.123	0.02	0.201			0.195		0.01	0.015	0.103	0.051				
13.2							0.020																							
13.3																														
13.4																														
14.0	0.015	0.2526			0.015	0.237	0.299	0.061		0.0103		0.082	0.025	0.015	0.154		0.201			0.020			0.005	0.010	0.051				0.1959
14.2							0.103																							
14.3																														
15.0	0.005	0.2062	0.0			0.180	0.061	0.278		0.3093		0.010	0.366		0.036		0.128							0.010	0.113	0.01			0.0206	
15.2							0.123																							
15.3																														
15.4																														
16.0		0.0979	0.0			0.128	0.010	0.201		0.2268	0.020		0.329		0.005		0.092								0.056	0.03			0.1392	
16.2							0.030																							
16.3								0.015																						
17.0		0.0309	0.0			0.067		0.072		0.2216	0.056		0.201		0.072		0.010				0.01				0.082	0.03			0.3144	
17.1																														
17.2							0.005																							
17.3								0.087																						
18.0			0.2			0.030		0.015		0.0206	0.134		0.067		0.159		0.005				0.02				0.077	0.06			0.2062	

18.1																														
18.2																														
18.3								0.051																						
19.0			0.1			0.041				0.0052	0.180		0.010		0.092						0.05				0.041	0.06			0.1082
19.1																														
19.2																														
19.3			0.0					0.005																						
19.4																									0.010					
20.0			0.1			0.025					0.159				0.041						0.08				0.020	0.09			0.0155
20.1																														
20.2																										0.01				
20.3																														
21.0			0.0			0.010					0.015				0.005						0.10				0.020	0.05				
21.2																										0.02				
21.3																														
22.0			0.0			0.015					0.051										0.24				0.030	0.01				
22.2																										0.04				
22.3																														
23.0			0.0			0.005					0.164										0.20				0.020	0.01				
23.2																										0.04				
23.3																														
24.0			0.0								0.128										0.14									

24.2																					0.00					0.06			
24.3			0.0																										
25.0			0.0								0.046										0.07				0.005				
25.2																										0.05			
26.0			0.0								0.036										0.03								
26.2																										0.07			
27.0			0.0								0.005										0.00								
27.2																										0.10			
27.3																													
28.0						0.005			0.056																				
28.2									0.005																	0.07			
28.3																													
29.0									0.201																				
29.2																										0.06			
29.3																													
30.0									0.329																				
30.2									0.005																	0.03			
30.3									0.010																				
31.0									0.123																				
31.2									0.036																	0.02			
32.0									0.056																				
32.2									0.113																	0.01			
33.0									0.015																				

33.1																													
33.2									0.046																				
34.0																													
34.2																													
35.0																													
36.0																													
37.0																													
38.0																													
39.0																													
43.2																					0.00								
H_{obs}	0.783	0.7526	0.8	0.804	0.742	0.876	0.783	0.835	0.773	0.7010	0.907	0.814	0.742	0.814	0.835	0.68	0.907	0.67	0.37	0.824	0.87	0.56	0.721	0.742	0.917	0.92	0.6804	0.6186	0.8144
P_I	0.104	0.0919	0.0	0.075	0.092	0.050	0.072	0.048	0.056	0.1030	0.042	0.118	0.148	0.079	0.032	0.09	0.049	0.19	0.38	0.147	0.05	0.28	0.101	0.076	0.019	0.01	0.1361	0.2122	0.0885
PPE	0.535	0.5619	0.6	0.606	0.558	0.680	0.613	0.691	0.644	0.5350	0.738	0.525	0.461	0.595	0.756	0.53	0.697	0.38	0.21	0.485	0.69	0.29	0.523	0.590	0.834	0.87	0.4535	0.3525	0.5872
HWE	0.339	0.6250	0.8	0.215	0.569	0.935	0.333	0.615	0.855	0.0256	0.167	0.444	0.491	0.520	0.438	0.34	0.551	0.16	0.06	0.095	0.07	0.21	0.395	0.551	0.795	0.00	0.8084	0.9791	0.0934

APPENDIX- B

LABORATORY FORMS

The various forms required for the acceptance of Forensic DNA cases are:

- Evidence Submission Form
- Chain of Custody Form
- Blood Sample Authentication Form
- Sexual Assault Victim Information Form
- Autopsy Specimen (s) Submission Form
- Aborted Foetus Identification Sheet (DNA Paternity Testing)

A. Forms required for Paternity Cases:

- Blood Sample Authentication Form
- Evidence Submission Form

B. Forms required for Sexual Assault Cases:

- Evidence Submission Form
- Chain of Custody
- Blood Sample Authentication Forms
- Sexual Assault Victim Information Form
- Autopsy Specimen (s) Submission Form
- Aborted foetus identification sheet.

C. Forms required for Human Identification/Homicide Cases:

- Evidence Submission Form
- Blood Sample Authentication Form
- Autopsy Specimen (s) Submission Form

CENTRAL FORENSIC SCIENCE LABORATORY
DNA Division,
Ministry of Home Affairs, Govt. of India,
CFIs Complex, DakshinMarg, Sector 36-A, Chandigarh – 160036

EVIDENCE SUBMISSION FORM

Government/Law Enforcement Agency Submitting the case

This form MUST be completed before processing can begin on this case

Case Information **Date:**

FIR_____U/S_____P.S_____

Full Address of Submitting Agency:

Telephone # **Fax #**

Delivering Officer_____Designation:_____P.S._____

Phone No._____ Email Address:_____

Signature

Type Of Case

Disputed Paternity/Disputed Maternity/ Criminal Paternity / Sexual Assault/ Homicide/Human identification

☐ ☐ ☐ ☐ ☐ ☐

Examination Required	Brief Description of Items Submitted	Brief Case History (Attach extra sheet if required)	Seal Impression (s)	No. Of Seals

Information to be provided in sexual assault cases

Please Answer these Questions For Requested Laboratory Services (**Serology/DNA Analysis**)

Who was bleeding? Suspect ☐ Victim ☐

Has victim had sexual relations within 3 days?

Did perpetrator use a condom?

Did ejaculation occur outside the body?

How much time elapsed between the sexual assault and medical examination?

Statement of Authorization

I authorize CFSL, Chandigarh to perform DNA analysis on the specimens submitted regarding the aforementioned case.

Name: Signature:

Date

CFSL/CHD/FM/106/01.09.2010/II

CENTRAL FORENSIC SCIENCE LABORATORY

DNA Division,
Ministry of Home Affairs, Govt. of India,
CFIs Complex, Dakshin Marg, Sector 36-A, Chandigarh – 160036.

BLOOD SAMPLE AUTHENTICATION FORM

(To be completed by the Authorized Medical officer collecting the samples. Identity of person from whom blood sample is being collected)

Name of person: _____ Father's/Guardian/Husband name: _____

Gender: Male [ ] Female [ ] Age _____ Caste/ Origin of State: _____

Address: _____ PIN_____

FIR/Crime Case No: _____

Collection Center Name _____

Sample Collected By _____Sample Collection Date _____

Collection Center Address_____

Storage conditions used _____

Attested Photograph by Medical officer

_____	_____	_____
Name of the person Collecting the blood sample	Date &Time	Signature
_____	_____	_____
Name of Investigating Officer/ Representative	Date &Time	Signature
_____	_____	_____
Name of Witness	Date &Time	Signature
_____ _	_____	_____
Name of Witness	Date &Time	Signature

Imp: A person from the opposite party in Paternity disputes and Sexual assault cases.

Chain of Custody

Blood samples sealed and released by: _____Blood samples released to: _____

Mode of release: Hand delivery [ ] or Mail [ ] Date sent to CFSL, Chandigarh:_____

SUBJECT'S STATEMENT OF VOLUNTARY CONSENT & RELEASE

I _____Son/Daughter/Wife/Guardian of Kum/Master_____hereby certify that the information provided above is true and accurate. I willingly consent to the collection of the blood sample from myself for the purpose of DNA analysis.

_____ _____
Signature/thumb impression of the donor Date &Time

For Office use only

CFSL File No: _____ Sample received on: _____

Laboratory Reference No: _____ Examined By: _____

Laboratory Exhibit Code No : _____

Signature of Authorized Medical Officer

CENTRAL FORENSIC SCIENCE LABORATORY

DNA Division,
Ministry of Home Affairs, Govt. of India,
CFIs Complex, DakshinMarg, Sector 36-A, Chandigarh – 160036

CHAIN OF CUSTODY

(FOR INVESTIGATING OFFICERS)

REFERRING INVESTING AGENCY: _____

FIR/DDR/CRIME CASE #:_____

NAME OF THE INVESTIGATING OFFICER: _____DESIGNATION_____

PARCEL #	# OF SEALS	SEAL IMPRESSION	DESCRIPTION OF ARTICLES (Indicate place, time and date of collection and the name of the Investigating Officer collecting /receiving the exhibits)

PARCELS/ EXHIBITS	TIME & DATE	EVIDENCE RECEIVED FROM (Name & Signature)	EVIDENCE RECEIVED BY

Signature of Investigating Officer

CENTRAL FORENSIC SCIENCE LABORATORY

DNA Division,
Ministry of Home Affairs, Govt. of India,
CFIs Complex, DakshinMarg, Sector 36-A, Chandigarh – 160036

AUTOPSY SPECIMEN (S) SUBMISSION FORM

(To be completed by the Authorized Medical Officer who conducted the Postmortem)

1. Identity of person from whom samples are being collected:

Name: _____ Religion/Caste _____.

Date of Death _____ Hospital Patient # (If any) _____.

2. Cause of Death_____

3. Has the individual received a blood transfusion or bone marrow transplant in the last three months?

4. Legal Contact: _____ Phone_____.

5. Specimen Collection:

Collection Centre Name: _____.

Collection Centre Address: _____.

Sample Collected by: _____. Sample collection date: _____.

6. Description of Samples Collected:

Sample	Storage conditions	Other remarks

Specimen Disposal: (Please check either option 1 or 2 or 3)
Note: If the disposal or return of the sample is not authorized, a Specimen(s) may be destroyed of in 1 year.

7. Chain of Custody

Specimen(s) sealed and released by_____.

Specimen(s) released to: _____.

Mode of release: Hand delivery_____. Mail_____.
Date sent to CFSL, Chandigarh_____.

Authorized Medical Officer Signature: _____Date_____

DNA Division,
Ministry of Home Affairs, Govt. of India,
CFIs Complex, DakshinMarg, Sector 36-A, Chandigarh – 160036

SEXUAL ASSAULT VICTIM INFORMATION FORM

(To be completed by the Authorized Medical Officer who conducted the Medical examination)

Attested Photograph by Medical Officer

1. VictimName: _____MLR/PMR Number _____

Address _____ Age _____ Sex _____

Date & Time of assault _____District & State of Incident: _____

Date of Examination:_____

Number of Assailants_____Age _____Sex _____

Sexual Assault Examiner: _____

Hospital Name: _____Hospital Telephone No.: _____

2. DETAILS OF ASSAULT: (e.g., oral, rectal, vaginal penetration/contact: perpetrator penetration of victim with fingers or with foreign object; oral contact by perpetrator; oral contact by victim; ejaculation, if known by victim, other injuries).

_____

_____

3. Pregnancy test to determine pre-existing pregnancy only.Yes/ No/Don't know_____

4. PRIOR TO EVIDENCE COLLECTION, VICTIM HAS:

1. Bathed / Urinated /Defecated /Vomited/Had Food or Drink/ /Brushed Teeth or Used Mouthwash _____ None of the above _____
2. Whether Clothes changed: **Yes/ No/Don't know_____**

4. For "Rape Drug" Test Blood and/or Urine Sample taken: **Yes/ No/Don't know_____**

5. AT TIME OF ASSAULT WAS:

1. Contraceptives / Spermicide / Lubricant/ Condom present /used? **Yes/ No/Don't know _____**

2. Victim menstruating? **Yes/ No/Don't know_____**

6. AT TIME OF EXAM WAS: Victim menstruating: **Yes/ No/Don't know_____**

7. RECENT CONSENSUAL COITUS:

Has Victim had consensual coitus within last 5 days? **Yes/ No/Don't know_____**

If yes, was birth control used? **Yes/ No/Don't know_____**

What method of birth control was used? _____

Over leaf

Brief Description of Evidence Submitted (One item per line.)

Parcel No.	No. of Seals	Description

Chain of Custody

Parcel Description	Evidence received From	Evidence delivered To	Date	Comments

Examinations Requested

9. Person authorizing release of

Information is (check one): Victim_____ Victim's parent _____Victim's guardian _____ Other (Specify) _____

If reporting anonymously, I have been informed that all evidence, including my clothing will be disposed of, if I do not report the crime within 3 months after the medical examination.

Date:

Signature:
VICTIM/PARENT/GUARDIAN SIGNATURE

Place:

Date:

Signature with stamp:
Sexual Assault Examiner

Place:

ABORTUS (Aborted Foetus) IDENTIFICATION SHEET

(DNA PATERNITY TESTING)

To be completed by the Authorized Medical Officer who conducted the Medical/Postmortem Examination

1. Identity of person from whom abortus sample is being collected

Name of person: _____

Address: _____

Whether the individual is juvenile or deceased? _____

2. Specimen Collection (See instructions)

Hospital Name: _____Hospital Telephone No.: _____

Medical Examiner_____ Date_____

3. Type of Specimen(s) Collected (Please specify the portion of Abortus)

i

ii.

iii.

4. Weeks Gestation_____Storage conditions used _____

5. Chain of Custody

Specimen sealed and released by: _____

Specimen released to: _____

Mode of release: Hand delivery_____ Mail_____

Date sent to CFSL, Chandigarh:

Signature of Authority Medical officer

ABORTUS SPECIMEN COLLECTION INSTRUCTIONS

Abortus collection	**1** **2** **3** **4**	**Wear gloves while collecting samples** **Tissue from an abortus shall be selected by the physician and approximately 2 cm^2 portion must be placed into a sterile plastic tube.** **Print the mother's name and the date of collection on the label.** **Physician should put his/her initials on the label.**
Mother's sample	**5**	**A blood sample needs to be collected and the appropriate form completed (DNA Paternity Test/Chain of custody form).**
Storage	**2** **3** **4**	**Do not preserve the tissue in formalin.** **Freeze the tissue and transport it on ice.** **Blood sample should be collected in sterile EDTA tubes. Do not freeze the blood sample.**
Forms	**5** **6**	**Complete the forms, documenting all the required information.** **Sign the form where indicated to verify collecting the biological samples.**
Packing	**A.**	**Package each sample separately and affix with a tamper proof seal.**

APPENDIX-C

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