WORKING PROCEDURES MANUAL
FORENSIC BIOLOGY-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 Biology 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 to do the routine analysis of biological samples in a wide variety of situation. This manual consists of five chapters including examination of blood & blood stains, semen & seminal stains, vaginal fluid, saliva, vomit, urine stains and Faecal stains. This manual also includes Forensic identification of hair & fibre, Diatom examination and Human skeleton examination. Therefore, to meet the requirement in present scenario, efforts have been made to incorporate advanced automated techniques of Forensic Biology 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.

17/11/19

(**Dr. S. K. Jain**)

AMENDMENT PAGE					
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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: 1999 'General requirements for the competence of testing and calibration laboratories' issued by International Organization for Standardization/International Electro technical Commission, NABL 113: 1998 'Specific guidelines for forensic science laboratories' issued by National Accreditation Board for Testing Laboratories and NABL 113: 2000 'Specific guidelines for Forensic Biology ' issued by National Accreditation Board for Testing Laboratories - for the service.

This document, Working Procedures Manual of Forensic Biology, serology and anthropology describes the test procedures for body fluids detection and anthropological test items like blood, semen, saliva, hair, bone, skeleton remains, skull 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 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.03 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 Biology, Doc. No.0- CFSL/CHD/WPM/BIO

Sr. No.	Authorized holder	Issue No.	Copy No.
1.	DIRECTOR ,CFSL, CHANDIGARH	03	00
2.	HOD DNA DIVISION	03	01

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

GENERAL INFORMATION

1. About manual

The purpose of this manual is to provide the Forensic Serologists with a set of procedures for examination of biological and serological evidence. Due to the wide variety of evidence received by the Forensic Serologists, a great deal of ingenuity is required in the analytical approach. The manual is a presentation of methods, which have been found to be workable for practicing Forensic Serologist.

1.1 Policy of examination

"No crime can be committed without leaving a clue". This statement forms the basis for our work in Forensic Serology; hence it is required for the examiner.

1. To observe or detect the clue.
2. To recognize the potential value of the clue.
3. To record the clue.
4. And try to individualize the clue material

General examination of evidence exhibit for the biological material encompasses all the steps taken from the time the evidence is first submitted to the laboratory until a specific examination is carried out.

Interim storage of evidence

The decision must be made whether to work on the evidence immediately or to store the evidence until it is possible to do the examination. If it is not possible to work on the evidence right away, it is necessary to preserve certain items of perishable evidence and liquid control samples for later examination. Here, the analyst must decide that how preservation can be accomplished using refrigeration or freezing, as the case may be. When the items to be preserved have been dealt with, the rest of the evidence should be sealed in appropriate evidence containers and placed in a storage vault, which provides physical security for the evidence.

GENERAL GUIDELINES

In general, screening tests and/or confirmatory tests are used to identify biological fluids such as blood, semen, and saliva prior to further analysis.
All reagents are quality control checked, where ever possible. Make own reagents or use supplies that have been quality control checked.

1.1 ASSIGNMENT OF CASE NUMBERS

This task should be performed by the supervisor.

1. Compile all appropriate documentation. The case worksheets are available in the laboratory.
2. Compare each case documentation with the manifest and the specimens received to ensure that all of the specimens designated for Forensic Biology have been received.
3. Screen all the documentation for potential Forensic Biology (FB) cases. Any case in which a Forensic Biology test is requested should be through court order or investigating agency.

Before the actual examination, certain preparations should be made:

1. Review all the provided information to determine what questions the investigator needs to be answered. If any case is complicated, discussion should be done with a colleague or supervisor to get his/her viewpoint.

2. Plan your approach to the case. Certain items may have greater potential value for information than others. The serologist may like to examine those items first. This concept also applies to items, which may have potential value to "investigative information".

3. Prepare the work area. The bench may be clean. If large items such as clothing or bedding are to be examined, a large table should be available for this purpose. The work area should be covered with white paper to prevent the loss of small particles of evidence and to prevent the cross-transfer of materials from one item to another. The necessary tools and reagents for the examination should be conveniently placed. Adequate lighting should be provided to allow close visual inspection

of evidence. If room lighting is not sufficient, supplemental lighting may be used. Keep noting pad or working sheets at hand to record observations. One low power magnification device may also be kept ready on working table.

4. Wear a lab coat to protect your own clothing from contamination.

5. Spread the object out and examine it carefully for presence of biological material, stains on exhibits.

   Record your observations on a work sheet. It is generally helpful to draw a sketch of the object to describe the location and size of various stains. At this time, stains can be given a number or letter designation to distinguish multiple stains. Area of unstained controls should also be marked and recorded. Detailed drawings on the sketch will be useful later to interpret results. Work sheets with printed diagrams of assorted clothing articles can also be used for the above.

6. The stains on the object may be marked to aid in locating at a later time. Care should be taken when examining objects, which are non-absorbent and bear crusty stains. These crusts may flake off if handled roughly. It is advisable to remove the crusty portion of the stain with a scalpel and preserve it in a paper packet. Label the paper packet. Photograph before collecting, if stain pattern is significant.

7. The general examination should continue until the object has been thoroughly search for traces of evidential material. At this point, the materials of evidential value may be samples for analysis. Following this, the object can be repacked for storage.

8. As a rule, all the parcels and the exhibits should be photographed or video-graphic recording may be done.

Precautions:

Having decided upon an analytical approach, it is time to examine the evidence.

1. Be certain that the previous item has been packed and put away before opening another item on the work surface.
2. Remove packing with case, remembering that materials of evidential value may be adhering to the item. Opening the evidence over the white surface paper will prevent the loss of these materials.
3. Mark the evidence for future identification with your initials; the laboratory case number, the date, and the exhibit number.

Weapons are submitted frequently for bloodstain, tissue, or hair examination. The forensic serologist must be aware of the possibility that latent prints may be present on the weapon. The possibility should be discussed with the investigator.

1.2 PROCESSING OF EVIDENCE SPECIMENS

This task should be performed reasonably soon after a batch of samples arrives in the Laboratory.

1. Specimens are delivered to the laboratory in sealed containers/parcels. After checking and receiving the case parcels, chain of custody is ensured.
2. To inventory the contents proceed with the following:
   - Inventory each container separately. **(Check for completeness and record discrepancies.)**
   - Compare the plastic tags with serial numbers to the serial numbers written on the chain of custody.
   - The person on the rotation must record the chain of custody.
3. Sealed examined parcels should be stored in a dedicated storage area.

Personal safety:

Note: Wear gloves, mask and head cover all through examination of evidence exhibit.

In some instances, mask may be helpful when dealing with foul smelling evidence. Following the examination of biological material, whether dried or liquid, wash your hands with disinfectant to prevent the possibility of infection.

Items infested with vermin:

Fleas, lice and insect larvae may be discovered while examining various objects. If these occur, the following steps should be taken.

1. Examine the item carefully on an isolated bench if possible.
2. If the pests are still living, dry the exhibit and take samples of evidence material.
3. Remnants should be packed in paper packets.

Precautions for Contaminated Evidence

In the event contaminated evidence is submitted to the laboratory, the following steps should be taken:

1. Ascertain the nature of the contamination from the submitting agency, if possible.
2. Exercise care in handling the evidence. Avoid excessive movement of clothing articles to prevent possible air borne spread of contaminants.
3. When examination is complete, discards all paper table coverings, soiled gloves, and contaminated disposable glassware in plastic bags, sealed and marked as hazardous or contaminated.

Biological & Serological Examination of Body Fluids

CHAPTER 1
EXAMINATION OF BLOOD & BLOOD STAINS

1.1 **Physical Examination:** In natural light examination of exhibits for brown, reddish brown stains, powder or crystals of reddish brown colour, these areas should be demarcated. In case of absence of clear and visible stains, washed stains should be examined under 230-269 nm frequency UV light.

1.2 **Presumptive Test:** These suspected bloodstains; contaminated materials should be tested for blood.

1.2.1 **Tetramethyl Benzidine (TMB) Test:**

NOTE: TMB is carcinogenic. Use of gloves is required.

Reagent preparation:

Acetate Buffer

Sodium acetate anhydrous	10 g
Glacial acetic acid	5 ml
Distilled water	500 ml

Working Solution 1:

TMB	0.25 g
Acetate Buffer	25 ml

Working solution 2:

3% H_2O_2	10 ml

Mix, filter and store in brown coloured bottle in refrigerator.

Procedure:

1. Place a cutting or swabbing of the stain on filter paper or spot test paper.
2. A drop of TMB Solution is placed on the stain, followed by a drop of 3% Hydrogen Peroxide.
3. An immediate blue-green colour is a positive test for peroxidase activity, indicative of hemoglobin. This is not a confirmatory test for blood.

Standards and Controls: A known bloodstain and unstained control must be tested.

1.2.2 Phenolphthalein Test (Kastle-Meyer Test):

Reagent Preparation:
Stock Solution:

Phenolphthalein	2.0 g
Potassium Hydroxide	20.0g
Distilled Water	100 ml
Zinc Dust	20.0 g

Mix, add a few boiling chips and boil under reflux 2-3 hours or until the solution has lost its pink colour. Cool and decant into a bottle containing some zinc to keep in the reduced form.

Working Solution:

Solution # 1:	Ethanol	10 ml
Solution # 2:	Phenolphthalein Stock	2 ml
	Distilled Water	10 ml
	Ethanol	2 ml
Solution # 3:	3% Hydrogen Peroxide	10 ml

Procedure:

1. A small cutting, swabbing or extract of the suspected bloodstain is placed on filter paper or spot test paper.
2. Two or three drops of Ethanol are placed on the stain.
3. Two drops of working phenolphthalein solution are added to the stain.
4. After waiting to insure that no colour develops at this stage, two or three drops of 3% Hydrogen Peroxide are added.
5. An intense pink colour is a positive test for peroxidase activity, indicative of hemoglobin. This is not a confirmatory test for blood.

Standards and Controls: A known bloodstain and unstained control must be tested.

NOTE:
Zinc powder or dust in contact with water or damp air evolves Hydrogen. The heat of the reaction is sufficient that the Hydrogen may ignite. Therefore, the Zinc should not be discarded in the wastebasket. The following procedure

should be followed for less than 20 g of Zinc:

1. Follow standard laboratory procedures of wearing gloves and safety aprons. Add dilute Hydrochloric acid to the Zinc dust in a beaker. Allow the bubbles to form and slowly add more of acid till no more bubbles are seen.
2. When all the Zinc has dissolved, add Sodium Carbonate solution to the mixture. Bubbles will be formed. Keep on adding the Sodium Carbonate small quantity at a time till the Zinc precipitates as Zinc Carbonate.
3. The Zinc Carbonate can be now filtered and disposed off as it is non-toxic.

1.3 **Confirmatory Test:** Stains positive for the presumptive test should be further examined by the following tests:

1.3.1 **Takayama Test:**

Reagent Preparation:

Standard Glucose Solution (100g/100ml)	3 ml
10% Sodium Hydroxide	3 ml
Pyridine	3 ml
Distilled Water	7 ml

Reagents should be made fresh daily.

Procedure:

1. Place material to be tested on a microscopic slide and cover with a cover slip.
2. Add a drop of Takayama reagent and allow to flow under the cover slip.
3. Warm slide gently on a hot plate at 65^{o}C for 10-20 seconds
4. Allow to cool and observe under microscope at 100X.
5. The appearance of pink needle shaped crystals of Pyridine Hemochromogen (Pyridine ferroprotoprophyrin) is positive reaction for heme.

1.3.2 Teichmann's Test:

Reagent Preparation:

Potassium Chloride or	0.1 g
Potassium Bromide	0.1 g
Potassium Iodine	0.1 g
Glacial Acetic Acid	100 ml

Mix and store in a stoppered bottle.

Procedure:

1. Place material to be tested on a microscopic slide and cover with a cover slip.
2. Let the Reagent flow under the cover slip.
3. Warm slide gently on a hot plate at 65^{o}C for 10-20 seconds.
4. Allow to cool and observe under microscope at 100X.
5. The appearance of brown rhombohedron shaped crystals of Ferroprotoprophyrin Chloride is a positive reaction for heme.

1.3.3 Spectrophotometric Estimation:

Reagent Preparation:

Solution # 1: 0.2% Sodium Lauryl Sulphate in water

Solution # 2: 0.2% Mercaptoethanol in 1% NH_3 solution

These reagents will keep approximately 4 days.

Procedure:

1. To a 1 cm long stained thread, add 10 ml of Solution # 1.
2. Incubate at 37 ^{o}C for 15-20 minutes.
3. Add 10 ml of Solution # 2 and mix.
4. Transfer liquid to a microcappillary cuvette.
5. On a Spectrophotometer, monitor the reaction at 560 mm against a reaction blank until absorption reaches maximum.
6. When the reaction is complete, after 5-10 minutes, scan the sample between 600 and 500 nm. Two peaks, which are clearly defined at 558 nm and 529 nm, indicate the presence of haemoglobin derivatives.

Standards and Controls: Known bloodstains of various ages must be tested, Oxyhaemoglobin exhibits absorption peak at 576 and 538 nm. The apparent shift is thought to be due to the formation of reduced haemoglobin derivatives.

1.4 Origin of Blood

1.4.1 Cross-over Electrophoresis

Principle:

Cross over electrophoresis is used to determine the species origin of bloodstains and tissues.

In this variation of the precipitin method, antigen and precipitating antisera are brought together electrophoretically.

- The antigens in the blood or tissue extract are serum albumin and α and β globulins which, under the test conditions, move toward the anode.
- The antibodies in the antisera are γ globulins and are moved by electroendosmosis toward the cathode.

- Reacting antibody and antigen form a precipitate that can be visualized with a protein stain.

Reagents:

Agar	1 g
Sodium barbiturate	7 g
Diethyl barbituric acid	1.1 g
Calcium lactate	1 g
Distilled water	1 litre

Standards and Controls:

- known blood or serum (human or other species, depending upon the species being tested for)
- Extractant blank (deionized water)

Sample preparation:

Extract an approximately 1mm x 1 mm cutting of stained material in 50 µl deionized water, or place the cutting directly into the gel. The extracts should be a light straw colour.

Procedure:

1. Prepare veronal buffer by mixing sodium barbiturate, Diethyl barbituric acid and Calcium lactate in 1 litre of distilled water.
2. Dissolve 1 gm of agar in 100 ml of veronal buffer, pH 8.6.
3. Heat the mixture to 100 ^{o}C until the solution is clear.
4. Centrifuge at 3000 rpm for two minutes to remove undissolved particles.
5. Pipette 7 ml of hot agar onto clean slides (3 in. x 2 in.).
6. After the agar has solidified, punch small wells in the gel approximately 1.5 mm apart.
7. Place diluted bloodstain extract (approx. 1:1000), known human blood (approx. 1:1000), blank extract and other controls into the right hand wells.
8. Fill the left hand wells with antisera.
9. Place the slide into the electrophoresis chamber. The stain extracts should be near the cathode and the antisera on the anode side.

10. Connect the gel to buffer chambers by means of four pieces of filter paper.
11. Carry out electrophoresis at 150 Volts for fifteen minutes. A fine white precipitin line between two holes of a pair represents a positive reaction.
12. Record the result photographically and stain the slide with amido black or other suitable protein stains.

Staining procedure:

1. Place the slide in 1 molar saline overnight at room temperature. This is to wash away any unreacted proteins.
2. Wash for 1 hour in distilled water at room temperature to remove any saline.
3. Remove from water, cover with a piece of damp filter paper (Whatman or comparable) and place in the oven to dry.
4. Remove the filter paper when dry and wash plate under running tap water, gently rubbing gel surface to remove fragments of filter paper.
5. Place in Amido Black stain for 10 minutes.
6. Transfer to destain solution, examine periodically.
7. Remove when background is clear and precipitin bands are stained a deep blue/black.
8. Allow to dry and double check your results.

Interpretation:

The following information must be considered when interpreting the results of this analysis:

1. Examine the area between the opposite wells. The presence of a stained precipitin between two opposing wells is a positive (+) reaction.
2. Multiple precipitin bands may occur if the amounts of antigen and antibody are not balanced. Multiple bands do not negate a positive finding.
3. The absence of a precipitin band indicates a negative (-) reaction.
4. Cross-reactions with closely related species may occur.

1.4.2 ABAcard®

The ABAcard® HemaTrace® is a testing device used to aid in the possible identification of human blood by detecting the presence of human hemoglobin. If human hemoglobin is present in the sample, its antigens will react with the mobile monoclonal antihuman Hb antibodies in the area marked "S". This will form a mobile antibody-antigen complex which migrates through the device toward the area marked "T". The "T" area contains stationary polyclonal antihuman antibodies which capture the mobile antibody-antigen complex. This forms an antibody-antigen antibody sandwich. The antibodies are labeled with a pink dye and upon aggregation of these antibodies, a pink line forms in the "T" zone indicating the presence of human hemoglobin in the sample. This test device also contains an internal positive control. In the area marked "C" there are stationary anti-immunoglobulin antibodies which bind excess antihuman Hb monoclonal antibodies that do not bind to the antibodies in area "T". The captured pink dye particles will form a pink line in area "C" indicating the test worked properly. The presence of two pink lines, one in area "T" and one in area "C" indicate a positive result. The presence of only one pink line in area "C" indicates a negative result. If there is no pink line in area "C" then the test is invalid. This test has shown that false positives may occur with ferret blood.

Standards and Controls:

- Positive control (known blood)
- Negative control – extraction buffer (included in kit)

Procedure:

1. Place approximately 3 mm^2 cutting or 1 /4 of a swab into a sterile 1.5 ml microcentrifuge tube. The size of the cutting may be adjusted based upon the amount of biological material.
2. Pipette 300 µl of extraction buffer from the commercial kit into the microcentrifuge tube. Vortex briefly.
3. Incubate for 1-5 minutes at room temperature.
4. Remove the ABAcard ® HemaTrace® kit device from the sealed pouch
5. Label the test device appropriately
6. Add 150 µl (or 4 drops with the enclosed dropper) of the sample to the sample well "S" on the test device.
7. Read the result after 10 minutes.
8. The presence of two pink lines, one in the "T" area and one in the "C" area indicate a positive result.

The presence of only 1 pink line in the "C" area indicates a negative result. A

negative result indicates there is no hemoglobin antigen present or is below the limit of detection of the test. If there is no pink line in the "C" area, the test is invalid.
The ABAcard® test has been extensively validated and shown to be sensitive, specific and rapid.

1.5 GROUPING OF BLOODSTAINS

Reactions between the RBCs and serum were related to the presence of markers (antigens) on the RBCs and antibodies in the serum. Agglutination occurred when the RBC antigens were bound by the antibodies in the serum. The antigens A and B, and depending upon which antigen the RBC expressed, blood either belonged to blood group A or blood group B. A third blood group contained RBCs that reacted as if they lacked the properties of A and B, and this group was later called "O" after the German word "Ohne", which means "without". The following year the fourth blood group, AB, was added to the ABO blood group system. These RBCs expressed both A and B antigens.

The ABO blood group antigens are encoded by one genetic locus, the ABO locus, which has three alternative (allelic) forms—A, B, and O. A child receives one of the three alleles from each parent, giving rise to six possible genotypes and four possible blood types (phenotypes). A and B antigens were inherited codominantly over O

Blood Type			
Type	**Possible Genotype**	**Antigen present on cell**	**Antibodies present in serum**
A	AA or AO	A antigen	Anti B
B	BB or BO	B antigen	Anti A
AB	AB	A and B antigen	No Antibodies
O	OO	No antigen	Anti A and Anti B

ABO Grouping is conducted routinely in forensic laboratories to individualize or to find a correlation between the suspect and the scene of crime using clothes stained with blood, semen, saliva etc. as exhibits recovered from the scene of crime.

Techniques used for determination of blood groups:

1.5.1 Absorption elution method:

Reagents:

Anti-A Sera
Anti-B Sera
Anti-H
0.5% suspensions of A, B & O indicator cells in isotonic saline.

Procedure:

1. Prepare 0.5% of A, B & O cell suspensions in buffered saline.
2. Take 3 test tubes for each crime case exhibit to be tested and place small cuttings of the exhibit to be tested in each of these tubes.
3. Add two drops of anti-sera Anti-A, Anti-B & Anti-H to each of these tubes.
4. Allow this to be absorbed overnight at 4^{o}C.
5. Pipette off the anti-sera the next day and wash the sample 5-6 times in ice-cold saline.
6. Add 2 drops of saline to each sample and elute the absorbed anti-serum at 56℃ for 15-20 min.
7. Remove the cloth material from the test tube and add one drop of an appropriate 0.5% indicator cell suspension to the test tubes.
8. Shake mechanically for 10 minutes.
9. Read results microscopically.

Biospecimen	Agglutination of Indicator cells in the zone marked			Inference (Regarding grouping)
	Anti-A	Anti-B	Anti-H	
Exhibit-1	+	-	+	A
Exhibit-2	-	+	+	B
Exhibit-3	+	+	+	AB
Exhibit-4	-	-	+	O
Negative Control	-	-	-	-

+Denotes agglutination

-Denotes lack of agglutination

Interpretation of Results:

The stains containing A antigens absorb Anti-A antiserum only and react with known group 'A' indicator cells. The stains containing B antigens absorb anti-B antiserum and consequently react with known group 'B' indicator cells only. AB stains will absorb both anti-A and anti-B sera and react with both known 'A' & 'B' group indicator cells. 'O' stains will absorb neither anti-A nor anti-B serum (it will absorb anti-H lectins) & react with known 'O' group indicator cells only.

This method is quite sensitive.

Note: Absorption Elution technique is another technique of directly testing the absorbed antibody. Agglutination indicates the presence of corresponding antibody in the elute i.e. the stained material contains the same blood group antigen as that of the indicator cells.

1.5.2 Absorption elution-Ammonia method:

Ammonia elution is especially useful when typing bloodstains on substrates, which do not lend themselves to Howard-Martin absorption elution typing.

1. Old, insoluble bloodstains.
2. Stains on material to which bloodstains are not readily fixed (e.g. nylon fibers).

Reagent preparation:

5% ammonia solution. The solution should be made fresh at least monthly.
Anti-A, Anti-B and Anti-H lectin.
0.5% suspensions of A, B and O indicator red blood cells in saline

Procedure:

1. Extract a 5 mm x 5 mm stain with six drops of 5% ammonia solution. Extract should be straw colored. (In this method, a lighter extract works considerably better than a darker extract).
2. Place one drop of extract in each of three wells of a serological ring slide.
3. Heat-fix the extract for a minimum of 3 hours at 37 º C or a minimum of one hour at 56 ºC.
4. Add one drop of appropriate antisera to each well and allow to absorb for five minutes in a moisture box at room temperature.
5. Quickly rinse off the antisera and place in an agitated saline bath at 4 º C for 10 minutes (Saline in the agitated bath must be changed frequently. Prolonged use of the same saline wash solution can cause inadequate washing to occur).
6. Carefully pat dry each well.
7. Add one drop of appropriate 0.5% cell suspension to each well.
8. Place slides in a warm moisture chamber at 37 ºC for 15 minutes for the elution process.
9. Transfer the slide to a room temperature moisture box and rotate for ten minutes.
10. Read results microscopically.

Titration of the antisera:

Reagents:

Anti-A antisera
Anti-B antisera
Anti-H
0.25% suspension of indicator A, B & O cells.

Procedure:

1. Prepare serial dilutions of anti-A, anti-B and anti-H (purified lectin, high titer) by diluting the antisera with isotonic saline. The dilution series is a function of (1/2) n, from n=1 to n=8 (i.e. ½, ¼, 1/8……1/256).
2. Prepare standard 0.25% indicator cell suspensions of A cells, B cells and O cells in isotonic saline.
3. Transfer one drop from each tube of diluted antisera to serological ring slides.
4. Add one drop of appropriate indicator cell suspension to the diluted antisera on the ring slides.
5. Place ring slides in moisture boxes and rotate at low speed for 10 minutes.
6. Allow cells to settle for 10-20 minutes (bringing total elapsed time, from the addition of the indicator cells to 20-30 minutes).
7. Read and grade the agglutination results

1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
4	4	4	4	4	2	1	neg.

8. Select the proper titer, that is, the greatest dilution that gives 4 plus agglutination.

Reagents:

Anti-A antisera
Anti-B antisera
Anti-H
0.5% suspension of A, B & O indicator cells in isotonic saline.

Procedure:

1. Prepare 0.5% of A, B & O cell suspension in saline solution.
2. Take 3 test tubes for each crime case exhibit to be tested and place small cutting of exhibit in each of these test tubes marked A, B & H along with exhibit and case details.
3. Add 3 drops of titer-adjusted antisera Anti-A, Anti-B & Anti-H to each of these tubes.

4. Incubate the tubes at room temperature for 2 hours (time of incubation may be increased for better results).
5. Add 3 drops of corresponding indicator cells in the respective tubes.
6. Gently but thoroughly mix and leave the tubes undisturbed for 2 hours at room temperature.
7. Transfer the contents (of each tube separately) to clean microscopic slide and observe under microscope.

1.5.3 **Absorption inhibition technique:**

The absorption inhibition method is a classical indirect way of demonstrating the presence of agglutinogen. This method involves the addition of titered antiserum to the stain. The titer of the antiserum is adjusted so that most the antibodies will be bound by the quantity of the stain employed in the test if homologous antigen is present. If the corresponding antigen is present in the stain, it will react with antibody and decrease the titer of antiserum, so that it is no longer available for agglutination of known test cells. Thus the absence of agglutination of known test cells is indicative of the presence of the agglutinate in the stain.

Table: Results of absorption – inhibition test employed for ABO grouping of body fluid stain.

Saliva	Agglutination of Indicator cells In the tube marked			Inference (Regarding grouping)
	Anti-A	Anti-B	Anti-H	
Exhibit-1	+	+	+	Nonsecretor
Exhibit-2	-	+	-	Secretor ‘A’
Exhibit-3	+	-	-	Secretor ‘B’
Exhibit-4	-	-	-	Secretor ‘AB’
Exhibit-5	+	+	-	Secretor ‘O’

+Denotes agglutination

-Denotes lack of agglutination

Interpretation of Results:

Group ‘A’ bloodstain contains ‘A’ agglutinates, which will absorb anti-A serum

and alter anti-A serum titer. Group 'B' bloodstain contains B agglutinates, which will absorb anti-B serum and alter anti-B titer so that it will not be available for binding with group 'B' indicator cells. Hence, absence of agglutination in this test tube is interpretated as positive indication of the presence of group 'B' agglutinates in the questioned exhibit. Group AB bloodstain contains both A & B antigen and hence reduces the titer of both anti-A & anti-B sera. Group 'O' bloodstain contains neither A nor B agglutinate so the serum titer would remain unaltered.

Note: This method is suitable for ABO typing of stains of body fluids, such as semen, sweat, saliva etc. The antigens of the ABO system are not confined exclusively to the red blood cells. Approximately 80% of individuals are classified as secretors, which means that their blood type antigens are found in high concentration in most body fluids (e.g. saliva, semen, vaginal secretions, sweat etc.). In fact saliva and semen have a higher concentration of A&B antigens than does blood.

AN ALTERNATIVE TECHNIQUE FOR GROUPING OF BLOODSTAINS

Mixed agglutination technique

1.5.4 In this technique, the known antiserum is added to the blood stain which has been fixed to a surface such as the thread of a fiber. After a period of absorption, the excess antiserum is washed away with cold saline so that only the antibody, which had reacted with the agglutinate, remains in the stain. Known indicator cells are then added. Homologous cells will attach themselves to the free ends of the bound antibody. A positive reaction is indicated by the presence of cells, which appear to be attached to the stain.

Reagents:

Anti-A sera
Anti-B sera
Anti-H sera
0.5% suspension of A, B & O indicator cells in isotonic saline.

Procedure:

1. Prepare 0.5% suspensions of A, B & O cells in saline solution.
2. Tease out the stained fabric under a microscope and cut individual threads to about 2 mm long.

3. Affix individual threads to three wells of a slide.
4. Add two drops of appropriate antiserum to each well.
5. Incubate overnight at 4 ºC
6. Pipette off antiserum, wash six times with ice-cold saline.
7. Add one drop of appropriate indicator cell suspension.
8. Place the slide in a moisture chamber at 50 ºC for ten minutes.
9. Remove the slide, allow it to cool, let sit at room temperature for two hours and examine under a microscope.

Table: Results of a mixed agglutination test employed for ABO grouping

Biospecimen	**Agglutination of Indicator cells In the zone marked**			**Inference (Regarding grouping)**
	Anti-A	**Anti-B**	**Anti-H**	
Exhibit-1	+	-	+	A
Exhibit-2	-	+	+	B
Exhibit-3	+	+	+	AB
Exhibit-4	-	-	+	O

+Denotes agglutination

-Denotes lack of agglutination

Interpretation of results:
Presence of agglutinate in zone (s) of particular antiserum/lectin directly denotes the presence of the corresponding (homologous) antigen(s) in the tissue/bloodstain sample tested.

Note: Using saline can modify the sensitivity – albumin cells or papain treated cells. This technique besides being used in tissue grouping may also be used for stained clothes and cotton swabs.
ABO groupings of the stains could be done by any one of the three given procedure.

1.6 Examination of Menstrual Blood and its Stains:

It may be relevant to establish that a bloodstain is of menstrual origin in cases of assault when grouping alone cannot differentiate between individuals or when as assailant claims that a bloodstain is menstrual.

When looking for menstrual blood there are certain factors that are to be borne in mind.

1.6.1 Physical Appearance:

The discharge is in the form of a trickling descent, it does not splash. Therefore, the nature and distribution of the suspect stain must be taken in account when making the examination.

1.6.2 Microscopic Examination:

Vaginal epithelium and residual cells will usually be present in menstrual bloodstains, along with endometrial cells.

1.6.3 Identification by Fibrin Degradation Product:

Menstrual blood is discharged from the uterus in a fluid state; it contains no fibrinogen and cannot be made to clot. This situation is a result of a fibrinolysis process during which the blood clots and subsequently reliquifies prior to menstrual period. The reliquifaction process is known as fibrinolysis and defined as enzymatic digestion of fibrin clot to soluble Fibrin Degradation Products (fdp). The test is carried out in two parts:

1. Assay of fdp in stain extract
2. The Assay of total soluble protein in the extract.

Stain Extraction:

Extract a small piece of bloodstain (0.5 sq.cm.) on 0.3 ml of saline for one hour at room temperature. Remove the stain, centrifuge at full speed for 10 minutes. Transfer the supernatant to a small clean tube and use for fdp and total soluble protein assay.

Assay for Fibrinogen Degradation Products:

Fibrinogen Degradation Products may be assayed by a haemagglutination inhibition method. In this technique they are quantitated by their ability to inhibit the agglutination of sheep red blood cells coated with human fibrinogen by anti- fibrinogen serum.

Materials:

Fibrinogen Standards:

Human Fibrinogen Standards may be purchased and diluted to a concentration of 10 µg/ml and stored at –20 ^{o}C and should be thawed twice only.

Phosphate Citrate Buffer pH 6.4
1.86 g DiSodium hydrogen phosphate
1.32 g Potassium dihydrogen phosphate
8.95 g triSodium citrate
0.050 g Sodium azide
500 ml distilled water
Use citric acid to bring pH to 6.4
1.15 ml 30% Bovine albumin solution
Store at 4 ^{o}C.

Antiserum:
Fibrinogen specific antisera are commercially available. Each vial of freeze-dried antiserum is dissolved in 1 ml water. The specificity of the antisera is to be checked with a wide variety of human serum dilution. Dilute the antisera with Phosphate Citrate Buffer to produce a working strength solution for the haemagglutination inhibition assay. This is usually between 1/2000 and 1/4000 dilutions of the stock antiserum depending on the batch of sensitized cells used.

Sensitized Cells:
Sheep red blood cells sensitized with human fibrinogen are obtained commercially. The cells supplied usually in 50 ml aliquots of 2.5% suspension. Prior to each assay, the cells are centrifuged and resuspended in Phosphate Citrate Buffer.

Microtitre Plates:
The assays are performed in Perspex Microtitre Plates. Each plate contains eight rows (A-H) with twelve V-cup shaped wells in each row.

Method:
1. Using Pasteur pipette, place one drop of buffer in wells 2-10 and two drops in well 11 row A.
2. Place one drop of buffer in wells 2-9 and well 12 of all subsequent rows – B, C, D, ……i.e., one row for each assay.
3. Place one drop of standard fibrinogen solution in wells 1, 2 of row A. Wash pipette in water.
4. Place one drop of stain extract in wells 1, 2 and 12 of row B. The

next sample should be placed in wells 1, 2 and 12 of row C. Wash pipette in water before application of each sample.

5. Prepare doubling dilution of the fibrinogen standard and samples. Take one drop of solution from well 2 and add it to well 3. Return any remaining solution to well 2.Take one drop of solution from well 3 and add it to well 4. Return any remaining solution to well 3 etc. Discard the final drop from well 9. Wash the pipette in distilled water after dilution of each sample.
6. Using a clean Pasteur pipette, place one drop of diluted antisera in wells 1-10 of row A and well 1-9 of all subsequent row. Mix contents of the wells by gently tapping the sides of the plates, cover and incubate at 4 ^{o}C for an hour.
7. Remove the plates from the refrigerator and add one drop of freshly suspended sensitized cells to wells1-11 of row A and well 1-9 and 12 of subsequent rows. Mix contents of the wells by gently tapping the sides of the plates, cover the plates and incubate at 4 ^{o}C for two and half hours. The results can be read after that.

Calculation and interpretation of Results:

The end point of the fibrinogen standard and the samples is taken as the last well showing complete no agglutination. This appears as a compact 'button' at the bottom of the appropriate well. Partial or complete agglutination appears as a curtain. If the controls are different from those described then the results must be discarded.

CHAPTER 2

EXAMINATION OF OTHER BODY FLUIDS AND THEIR STAINS

2.1 Examination of Semen & Seminal Stains

2.1.1 Physical Examination:

Colour: Thick, yellowish white, glairy, opalescent, secretion having a characteristic odor known as seminal odor.

Texture: On touch, seminal stains are starchy.

Appearance: Garments sent for forensic examination are usually dirty having variety of stains, in natural light some stains are reddish coloured, while others are brown, yellow or faint grey in colour. These are often mixed with stains of blood vaginal discharge, urine and semen, so as to restrict the investigation to seminal stains only, preliminary examination is done under filtered UV light. The fluorescence of the seminal stains is of a bluish white colour and such stains should be selected for further examination.

2.1.2 Presumptive Test:

2.1.2.1 Acid Phosphate Test: Sodium alpha-naphthyl phosphate method

Reagent Preparations:

Buffer

Glacial Acetic acid	1 ml
Sodium acetate anhydrous	2 g
Distilled water	100 ml

Step 1 Reagent

Buffer	50 ml
Sodium alpha-naphthyl Phosphate, 0.25% (w/v)	126mg

Step 2 Reagent

Buffer	50 ml
Naphthanil diazo blue B, 0.5% (w/v)	250mg

Step 1 Reagent and Step 2 Reagent can be made up in bulk and aliquoted into test tubes and frozen. When needed, one tube of each reagent can be thawed under warm running water for use.

Procedure:

1. Place a small piece (2 x 2 mm) of suspected seminar stain material on Whatman filter paper or other suitable test paper. Use proper standards and controls including positive, negative and unstained controls; see below.
2. Add 1-2 drops of Step 1 Reagent and allow to react for 30 seconds. (No colour should develop at this stage).
3. Add 1drop of Step 2 Reagent. Record the result after 10 seconds.
4. A positive reaction is recorded upon rapid development of a purple colour, which is indicative of semen. This is not a confirmatory test for semen.

2.1.3 Confirmatory Test: Microscopic Examination

Upon obtaining a positive preliminary test for acid phosphates, the suspected stain can be extracted as follows:

1. Cut a small portion (1 cm^2 maximum) of the stain and place in a test tube.
2. Add a few drops of acidulated water to cover the stain.
3. Agitate the stain on a vortex, or in an ultrasonic cleaner bath or manually, by flicking the tube. This will aid in freeing the spermatozoa from the dried stain.
4. When the solution is cloudy, withdraw the liquid with a pipette and place into a disposable 400ul plastic centrifuge tube and centrifuge in a microfuge for 30 seconds.
5. Carefully withdraw the supernatant, which contains soluble group-specific substances, enzymes and other solutes from seminal plasma.
6. Collect the button of cellular and other insoluble components from the tube and place on a clean-labeled microscope slide.
7. Fix in dilute H_2SO_4 acid and dry. It is now ready for staining.

2.1.3.1 Gram Staining

Reagent Preparations:

Reagent # 1: Ammonium oxalate crystal violet solution.

A: Add 0.2 g of crystal violet dye to 20 ml of 95% ethanol.

B: Add 0.8 g of Ammonium oxalate to 80 ml of distilled water.

Mix Solution A and Solution B to form Reagent # 1

Reagent #2: Gram's iodine

Iodine	1 g
Potassium Iodine	2 g
Distilled water	300 ml

Reagent # 3: Decolorizer – 95% ethanol.

Reagent # 4: Safranin solution.

Safranin (2.5% in 95% ethanol)	10 ml
Distilled water	300 ml

Procedure:

1. Fix the smear or stain extract to the slide by gentle heating or chemical fixative.
2. Add Crystal violet Solution (Reagent # 1) to slide for 1 minute.
3. Rinse with tap water (some of the material on the slide will remain as violet colour).
4. Add Gram's iodine Solution (Reagent # 2) to the slide for 1 minute (This fixes the crystal violet to the tissue). Rinse with tap water.
5. Add decolorizer (Reagent # 3) and tilt the slide back and forth, watching most of the violet colour wash away. This takes about 10 seconds.
6. Rinse with tap water. Add Safranin Solution (Reagent # 4) for 1 minute.
7. Allow to air dry. Examine with oil immersion at 1000X.

Gram positive protein will stain violet, gram-negative protein will stain red. Spermatozoa will appear as differentially stained purple bodies, somewhat oval in shape with a clearly discernible acrosomal cap.

2.1.3.2 Grading of Spermatozoa on Smear

1. Few; difficult to locate
2. Some in some fields
3. Some in many fields; easy to locate
4. Many in most fields

2.1.3.3 CROSS-OVER ELECTROPHORESIS

Seminal Material can be identified by demonstrating the presence of P30, a semen specific protein. One method of doing this is Cross-Over Electrophoresis.

An extract of the suspected stain is placed in the cathode well of a gel plate and anti P30 is placed in the anode well. Electrophoresis is commenced for 20 minutes at 200 volts, forcing two components together. When the antigen (P30) meets the antibody (anti-P30), a precipitin band is formed. The presence of a precipitin band within an extract of an unknown stain proves that the stain contains seminal material.

Plate Preparation:

1. Clean 1" x 3" or 2" x 3" microscope slides with detergent. Rinse and polish dry.

2. With a diamond tipped scribe number, slides should be consecutively numbered in the upper right-hand corner.

Pre-coating Plates: (Note: Omit this step if gel bond ® is used instead of glass slides.

(In order for final 1% agarose to adhere to the glass slide, it is necessary to pre-coat the slide with a thin layer of 0.2% agarose).

1. Add 1 gm of 0.2% stock agarose to 10 ml distilled water and boil until dissolved.
2. Paint a thin coating of this solution onto the pre-numbered glass slides with a soft brush.
3. Wipe the edges of the slides with tissue paper or Kimwipe to remove any overflow.
4. Allow to air dry.
5. Store pre-coated slides in a numbered slide box for later use. They will keep indefinitely.

Coating Plate with Gel:

1. On a level surface, place a pre-coated slide onto a small inverted beaker, allowing the slide edges to be suspended.

2. Place one or two tubes containing 3.5 ml each of 1% agarose gel (see data sheet) in a water bath to liquefy.

1 – 3.5 ml tube for a 1" x 3" slide
2 – 3.5 ml tube for a 2" x 3" slide

3. Pour the liquefied 1% agarose onto the slide. Surface tension and pre-coating of slide will prevent the liquid from overflowing the edges. A smooth level surface is formed. Allow to cool and harden.

Punching The Sample Wells:

1. Center the coated slide under the multi hole punch using the lines on the punch as a guide.
2. Depress the plunger once and release.
3. Remove the plugs of agarose from the slide with a pipette connected to a vacuum aspirator.

Sample Preparation:

1. Extract a small portion of the stain to be tested with a drop of saline at 4 ^{o}C for 1 hour.
2. Centrifuge and collect the cell-free extract.
3. Prepare sample extracts: neat, 1:1 saline.

Electrophoresis Tank Preparation:

1. Remove the cooling plate.
2. Fill tank with tank buffer.
3. Cut 3 mm filter paper bridges and arrange onto the support panels in the tank. Support panels must be close enough together to support a microscope slide i.e. slightly less than 3 inches apart.

Sample Application and Electrophoresis:

1. Using a double drawn pipette, fill the right-hand sample well of the pair with stain extract. (Volume is approximately 2 ul per well).
2. Fill the left hand well of the pair with the proper antiserum. See diagram for examples:

ANTI-P30 O O SUSPECTED SEMEN EXTRACT

ANTI-P30 O O SUSPECTED SEMEN EXTRACT 1:1 SALINE

ANTI-P30 O O SUSPECTED SEMEN EXTRACT 1:3 SALINE

3. Invert the slide onto the filter paper bridges of the electrophoresis tank. The inverted number on the slide should now appear in the lower right-hand corner of the plate with agarose gel in contact with the filter paper bridges. The stain extract should be nearest to the cathode, the antiserum nearest to the anode. If inverted in wrong

way, the same components will be driven apart, not together.

4. Electrophorese for 30 minutes at 120 volts. Record conditions.
5. Following Electrophoresis, switch off the power supply and remove the plate.
6. Observe the plate with the aid of a lamp. A fine white line of precipitate between a pair of wells is a positive reaction.
7. Record result in Electrophoresis Book. Recheck after staining.

Staining Procedure:

1. Place the slide in 1 molar saline overnight at room temperature to wash away any un-reacted proteins.
2. Wash for one hour in distilled water at room temperature to remove all saline.
3. Remove the water, cover with a piece of damp filter paper cover with a dozen thickness of dry paper towel followed by a glass plate. Place a weight on top of the glass to press the liquid from the gel. After 30 minutes, retrieve the slide with the pressed gel and place in the oven to dry.
4. Place in Amido Black stain for 10 minutes.
5. Transfer to distain solution, examining periodically.
6. Remove when background is clear and precipitin bands are trained a deep blue/black.
7. Allow to dry and double check your results.

NOTE: It should be noted that the results of crossover electrophoresis are only as accurate as the mono specificity of the antiserum used. Any precipitin bands formed by cross-reaction could be interpreted as a false positive reaction.

TANK BUFFER (pH 9.1)	Tris EDTA (free acid) Boric acid Distilled water	0.208 M 0.0086 M 0.0307 M	25.2 g. 2.5 g. 1.9 g. 1 L
GEL BUFFER (pH 9.1)	Same as tank buffer		
SUPPORT MEDIUM	1% AGAROSE, mr = 0.25, E25 or Sigma Type II.		
AMOUNT REQUIRED	3.5 ml for a 1" x 3" slide / 7 ml for a 2" x 3" slide		
2% STOCK AGAROSE	10 grams of agarose is added to 500 ml distilled water in a large flask. Heat to boiling on a constant stirring hot plate until all the agarose is dissolved. Pour into a plastic sandwich box and store upside down in the refrigerator. Blocks of 2% stock will be weighed out for later use.		
1% AGAROSE	50 grams of 2% stock agarose 50 ml of gel buffer	Melt stock agarose and gel buffer together and pour 3.5 ml quantities into 10 x 75 test tubes. Allow to cool, cork and store at 4^{o}C.	
TEMPERATURE & CONDITIONS	Room Temperature.		
VOLTAGE AND DURATION	**DAY:** 120V for 30 minutes (approximately 30 mA for 1x 3" slide)		
STAIN (0.1% AMIDO BLACK)	Naphthalene Black 10B Methanol Glacial acetic acid Distilled water	2.2 g 1 L 200 ml 1 L	
DESTAIN SOLUTION	Methanol Glacial acetic acid Distilled water	1 L 200 ml 1 L	
CONTROLS	Liquid semen diluted 1:50, 1:100, 1:200 with saline. Extract of unstained control (whenever possible to obtain). Saline black run against anti-P30. Cell free extract of questioned stain run against saline instead of anti-P30.		

2.2 Examination of Vaginal Fluid & Stains of Vaginal Secretions

2.2.1 Physical Examination

Vaginal Secretions, appear stiff on feeling when these are on clothing, under UV light examination, these show fluorescence.

2.2.2 SAP/VAP Electrophoresis

Although SAP/VAP electrophoresis is used only to identify the presence of seminal material, it can be used to indicate the origin of other acid phosphatase activity. A suspected stain should be extracted in a minimum of water for 10 minutes and placed in the gel. When used in conjunction with known samples of vaginal acid phosphatase, as per the Standards and Controls, the analyst can determine if the source of the activity in the unknown could be from vaginal secretions. This test is not Confirmatory for Vaginal Acid Phosphatase. The activity of the enzyme acid phosphatase in vaginal secretions is comparatively low. But this examination coupled with microscopic examination of the vaginal secretion can give conclusive proof regarding the vaginal origin of the stain.

TANK BUFFER (pH 8.5)

Sodium barbital (0.021M)	4.35 g
Barbital (0.013M)	2.39 g
Distilled water	1 litre

Adjust the pH with 0.1N NaOH to 8.5

GEL BUFFER (pH 8.5)

Use tank buffer

SUPPORT MEDIUM

1% Agarose

AMOUNT REQUIRED

Lysate Stain:

A small thread of the stain is moistened with a minimum of water. Allow to soak for 10 minutes. Place in sample slot on gel plate.

TEMPERATURE & CONDITIONS

Cooling plate at 4 ^{o}C

VOLTAGE AND DURATION

250V for 2 hours

REACTION BUFFER (pH 3.0)

Citric acid (0.05M)	10.5 g
Distilled water	1 Liter

Adjust with 0.1N NaOH to pH 3.0

REACTION MIXTURE

4-Methylumbelliferyl phosphate (4-MUP)	4 mg
Reaction Buffer	10 ml

Soak onto 3 mm filter paper and overlay onto gel from origin to anode. Place in moisture box at 37 ^{o}C for 30-60 minutes. Read under long wave UV.

CAUTION: Always wear safety eyeglasses which filter out UV radiation whenever viewing UV sensitive reactions.

2.2.3 LUGOL'S Stain

Reagent Preparation:

Iodine	1g
Potassium iodide	2 g
Distilled Water	200 ml

Dissolve the Potassium iodide in the water then add iodine.

Procedure:

1. Make a thin smear of the questioned material on a glass microscope slide.
2. Fix the smear by gentle heating or methanol fixation.
3. Cover the smear with Lugol's iodine solution. Add an equal volume of water to the smear.
4. Place a cover slip on the stain area.
5. Allow to stand 3-5 minutes at room temperature,
6. Observe microscopically at 200-400x.
7. Squamous epithelial cells with high glycogen content (notably vaginal and penile urethral epithelial cells) will exhibit a chocolate-brown or tan colour. Other epithelial cells will exhibit a yellow or gold colour.

2.3 Examination of Saliva and Saliva Stains

The saliva identification in Forensic Investigation is usually done on cigarettes or bidi ends, apparels and in sexual offenses to determine the secretor status.

2.3.1 Starch- Iodine Test

Reagent Preparation:

1. 0.5% soluble starch solution (50 mg soluble starch / 10 ml H_2O).
2. Lugol's iodine solution.

Procedure:

1. Place 3 tubes in a rack and add the following:
    a. In the first tube, place a 5 mm x 5 mm piece of sample to be tested.
    b. In the second tube, place a similar sized unstained control piece.
    c. In the third tube, place a 5 mm x 5 mm piece of a known saliva stain.
2. Add 3 drops of soluble starch solution to each tube.
3. Mix, cork and incubate the tubes for 1 hour at 37 ºC.
4. Add 2 drops of Lugol's iodine and note the colour formed.
5. A dark blue starch-iodine complex should be observed in the second and fourth tubes. The absence of the dark blue colour indicates that the starch has been hydrolyzed and is no longer available for complexing. Therefore, the lack of blue colour is a positive result for amylase activity, indicative of the presence of saliva. This is not a confirmatory test for saliva.

Notes: To dissolve the starch, heat the solution to a boil. Allow solution to cool to room temperature. Always use soluble starch. Do not use hydrolyzed starch. Starch solution must be prepared fresh daily. Never use starch solution older than 24 hours.

2.3.2 Radial Diffusion Test for Amylase

Reagent Preparation:

1. 0.1M Phosphate buffer, pH 6.9 prepared as follows:

NaH_2PO_4 anhydrous	2.7 g
Na_2HPO_4 anhydrous	3.9 g
NaCl	0.2 g
Distilled water	500 ml
Gel test plates (2% agarose, 0.1% soluble starch)	
Buffer, pH 6.9	10.0 ml
Agarose	0.2 g
Soluble starch	0.01 g

2. Heat to boiling and continue stirring constantly until all the agarose is dissolved. Divide gel solution and pour into 302" disposable plastic petri dishes. Allow to polymerize completely. Store gels inverted (to retard dehydration) at 4ºC.
3. Iodine development solution

KI	1.65 g
I_2	2.54 g
Distilled water	30 ml

Dissolve by stirring for 5 minutes at 65 ^{o}C. Store saturated I_2 solution in dark stoppered bottle.
Working solution in 1/50 dilution with distilled water.

Sample preparation:
Extract approximately 3 mm^2 piece of stained material with 50 µl of distilled water. Prepare an extract of unstained material (when available) in the same manner.

Procedure:
1. Punch holes in gel plate with a vacuum pipette, leaving 1.5 cm between the sample wells.
2. Place samples to be tested in the sample wells using a precision pipettor. Each well holds approximately 4 µl of liquid.
3. Cover the petri dish and place in an incubator at 30 ºC for 6 hours or overnight.
4. Stain the plate by pouring a 1:50 dilution of saturated iodine solution onto the surface. Rinse with H_2O.
5. Clear circles around the wells indicate areas of amylase activity. The diameter of the clear circle is proportional to the square root of the concentration of amylase.

1. Positive Controls:
   a. Known dilutions of fresh liquid saliva (1/200, 1/500 in H_2O)
   b. Known dried saliva stain extracted with H_2O (3 mm^2 extracted in 50 µl H_2O)
2. Negative Control: distilled H_2O.
3. Unstained Control: 3 mm^2 unstained material extracted in 50 µl H_2O).

2.4 Examination of Vomit

For the examination of vomit, presence of the following materials are to be taken

into account:

1. Presence of Mucus
2. Free HCl
3. Endothelial cells from gastric mucosa
4. Undigested and semi digested food material

2.4.1 Test for Mucus:

To the extract add 33% acetic acid drop by drop. Opalescence appears which may be due to mucus or lipoid substance or both. If on addition of more acetic acid opalescent does not disappear, presence of mucus is confirmed, because with excess of acetic acid lipoid globulins dissolve but not the mucus.

2.4.2 Test for Free HCl (Gunzberg's Test):

The reagent is prepared by 6 drops of 10% phloroglucinol in alcohol with 3 drops 10% vanillin in alcohol.

In a porcelain evaporating dish one drop of suspected extract is placed and 1-2 drops of Gunzberg's reagent is mixed at once. The contents are allowed to dry completely. A brilliant red colour indicates free HCl.

2.4.3 Endothelial Cells:

After centrifuging the extract for 10 minutes a thin film is made on a slide. The Endothelial Cells are observed under microscope.

2.5 Examination of Urine Stains

2.5.1 Physical Examination:

A suspected urine stain may fluoresce pale yellow or pale blue when viewed under long and short wave UV light. Safety eyeglasses, which absorb ultraviolet radiation, must be worn when viewing material for fluorescence.

2.5.2 Odour Test:

The characteristic odor of urine may be detected by placing a small sample of the stain in a test tube and heating it gently over flame. Avoid scorching the test material.

2.5.3 Urea Nitrate Crystal Test

An aqueous extract of stain is made and a thin film made on a microscopic slide. Add one drop of conc. Nitric acid and cover. In the presence of urea, hexagonal stacked crystals of Urea nitrate are formed.

2.5.4 Creatinine Test

To a drop of stain extract on filter paper, add one drop of picric acid followed by

one drop of 5% Sodium hydroxide.
Brown/orange colour shows presence of creatinine.

2.6 Examination of Faecal Matter and Faecal Stain

Faecal Matter is excretory product of animal metabolism. It consists of undigested vegetable refuge, bilirubin, muscle fibres and cellular material. Its identification may be sometimes be of great importance especially in cases of sodomy. For identification following examinations may be carried out.

2.6.1 Physical Appearance:

Faecal Matter is generally brown in colour due to urobilinogen, in infants it is yellow due to unchanged bilirubin and milk diet. Odour: Characteristic.

2.6.2 Microscopic Examination:

Suspected stains are softened with distilled water, for about half an hour. A small amount of scraping from the stain is transferred on to a microscopic glass slide and a drop of Lugol's iodine is added to it. The material is then covered with a cover slip and examined under microscope for the detection of undigested food particles, vegetable residues and muscle fibres.

2.6.3 Urobilinogen Test:

Urobilinogen is formed in the intestine by reduction of bilirubin. Urobilinogen is oxidized to Urobilin, which is soluble in alcohol. This test relies on the formation of a green fluorescent zinc-urobilin complex formed in the presence of neutral alcohol zinc salt.

Reagent preparation:

Solution 1:	40% Alcoholic Mercuric chloride solution	
	Mercuric chloride	4 g
	Methanol Mix and store in stoppered bottle.	10 ml
Solution 2:	40% Alcoholic Zinc chloride solution	
	Zinc chloride	4 g
	Methanol Mix and store in stoppered bottle.	10 ml
Solution 3:	Amyl alcohol.	

Standards and controls:
A known fecal stain stained and unstained control should be tested each time the testing is performed. Use distilled water as a negative control.

Note: The species of origin of fecal matter however is not detected normally, unless it is contaminated with blood or mucus.

CHAPTER 3

FORENSIC IDENTIFICATION OF HAIR & FIBRE

3.1 Sampling

1. Spread the exhibit on a clean white surface under proper illumination.
2. With hand magnifier, carefully locate any loose hair/fibre and collect.
3. If any hair or fibre is found adhering to the exhibit, it can be either picked up using forceps or may be transferred to adhesive tape.
4. If the exhibit happens to be a container or an object with crevices, vacuum sweeper with appropriate filter can be used to collect the hair/fibre.
5. Samples collected in the above manner should be individually packed in cellophane or paper folders and labeled and proper noting should be made on the worksheet for their exact location on the exhibit.
6. Each sample should be preliminary examined under microscope to note colour, texture and determine whether it is a hair, fibre or indistinguishable at that magnification.
7. Care should be taken to note the presence of root bulb or sheath of cells in the hair samples. They should be properly preserved for determination of sex or serological/DNA examination.

3.2 Hair Examination

Examination of hair can help in the determination of species or origin, sex, site (part of the body), genetic markers & source by comparison. Different morphological and histological characteristics of hair can be examined under microscope/stereomicroscope by temporary or permanent mount, scale casting, cross-sectioning and micrometric analysis.

3.2.1 Temporary Mount

1. Make a temporary mount of the hair sample on a clean slide with the distilled H_2O or glycerin. Cover with cover slip.
2. Examine under microscope from one end of hair to other for general appearance, length, colour, treatment-dye or bleached, presence or absence of root, tip and shaft characteristic and

contamination, if any.

3.2.2 Scale Casting

3.2.2.1 Nail Polish/Cellulose Acetate Method

1. Each hair sample should be cleaned before examination with suitable detergent to remove dust and debris and blot dried. If the hair is with root, care should be taken not to disturb the root in the above process.
2. On a clean microscope slide, place a thin layer of nail polish (cutex) or Cellulose Acetate paste with low viscosity.
3. With fine forceps, place hair onto the nail polish (cutex) or Cellulose Acetate paste and press with another clean slide.
4. Allow it to dry for 2-5 minutes.
5. With fine forceps, life the hair from root end and gently peel the hair from the slide and observe the scale impressions left on the cast microscopically.

3.2.2.2 Polaroid Coater Method

1. Place the hair on a clean microscope slide, securing the ends with cellophane tape.
2. Using a Polaroid film coater, make 2 or 3 passes along the length of the hair, wetting the slide and hair thoroughly.
3. Allow the coating to dry for 2-3 hours.
4. After removing the cellophane tape, gently peel the hair from the slide.
5. With a sharp scalpel, slice away the excess coating protruding above the flat surface of the scale cast. Be careful not to slice too deeply into the coating itself.
6. Observe the scale impressions microscopically

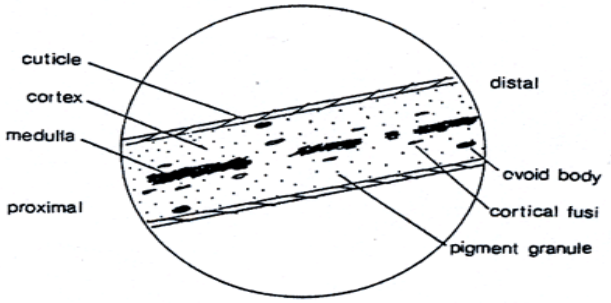
3.2.3 Permanent Mount

If the item is a hair, it may also be cleaned in xylene and mounted on a microscopic slide as follows:

1. Place hair on slide in a drop of xylene and add permanent mounting medium.
2. Place a cover slip on the hair allowing the medium to spread under cover slip-encasing hair.

3. Label the slide appropriately and allow it to dry for 48 hours.

Following permanent mounting of the hair, it can be examined for different morphological characteristics and micrometry.



3.2.4 Cross Sectioning

Cross sections are studied under microscope for their contour, thickness of cuticle, pigment distribution and for micrometry etc.

1. Clean hair in ether: ethanol (1:1) mixture.
2. Bundle the samples and dip in a block of molten wax (preferably with melting point 52 ºC) and allow to cool. Keep in refrigerator for 2-3 hours.
3. Cross sections can be taken either with a sharp blade or with a microtone to a thickness of 5-10 microns.
4. Place sections on a clean slide and dissolve wax with a drop of xylene.
5. Prepare permanent mount of the sections and examine under the microscope.

3.2.5 Micrometry

With the help of micrometer, measure following distances and calculate different indices:

1. Maximum diameter of the shaft.
2. Scale Count: Number of scales per unit length (100u)
3. Scale Count Index: Diameter of hair in microns/scale count
4. Medullary Index: Maximum diameter of medulla/ Maximum diameter of hair shaft
5. Hair Index: Minimum diameter of shaft/ Maximum diameter of the shaft X 100

3.2.6 Sex Determination from Hair Root Sheath

1. Mcllvaine's Buffer (pH-4.6):

I. 0.1M Citrate Buffer – Dissolve 10.5 g of Citric acid in 500 ml of distilled water.
II. 0.2M DiSodium hydrogen phosphate - Dissolve 35.91 g of Di-Sodium hydrogen phosphate in 500 ml of distilled water.
III. Mix 267 ml of solution I and 233 ml of solution II and store at 4 ^{o}C.

2. 1% Quinacrine dihydrochloride: Dissolve 0.5 g of Quinacrine dihydrochloride in 500 ml of Mcllvaine's Buffer (pH-4.6) II and store at 4 ºC. Keep for at least 2 weeks.
3. 2mM Magnesium Chloride: Dissolve 200 mg of Magnesium Chloride in 500 ml of distilled water.
4. Fixative: Glacial Acetic acid (3:1). Make fresh.

Procedure:

1. Place hair root sheath on a clean microscope slide, add one drop of $MgCl_2$ solution and finely tease the tissue.
2. Make a smear of the tissue on the slide and allow it to dry. Add two drops of fixative and leave for 10 minutes. Remove fixative and dry.
3. Cover the smear with Quinacrine dihydrochloride solution and stain for 15 minutes.
4. Wash the slide with $MgCl_2$ solution for 10 minutes, add a drop of glycerol solution and place a cover slip over.
5. Examine for Y-body under UV light in Fluorescent Microscope with appropriate filters at magnification 500-600X.
6. Examine at least 100 nuclei and score the percentage of nuclei with fluorescent Y-body. The present of Y-body in the case of males is generally more than 25% of nuclei.

3.2.7 Standards and Controls:

Animal Hairs: Sufficient samples of hairs from different species should be maintained in a laboratory for reference and comparison purposes.

Human Hairs: Hairs of unknown origin may be compared with hairs of known origin to determine the possibility of a common source. For comparison purposes, adequate number of standards (at least 10 strands collected at

random) should be examined.

3.2.8 General Differences between Human Hair and Animal Hair

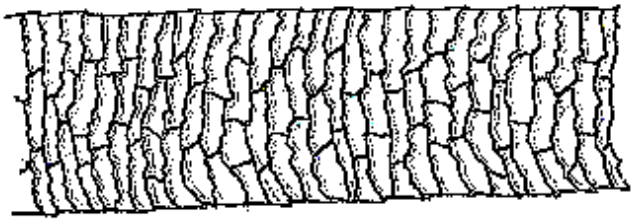
Feature	Human Hair	Animal Hair
Colour	Relatively consistent along shaft	Often showing profound colour changes and banding
Cortex	Occupying most of the width of shaft greater than medulla	Usually less than width of medulla
Distribution of pigment	Even, slightly more towards cuticle	Central or denser towards medulla
Medulla	Less than one-third width of shaft. Amorphous, mostly not continuous when present	Greater than one-third width of shaft. Continuous, often varying in appearance along shaft, defined structure
Scales	Imbricate, similar along shaft from root to tip	Often showing variation in structure along shaft from root to tip

3.2.9 Morphological Characteristics of Human Hair for Racial Determination

Race	Diameter	Cross-section	Pigmentation	Cuticle	Undulation
Negroid	60 – 90 um	Flat	Dense & clumped	-	Prevalent
Caucasoid	70 – 100 um	Oval	Evenly distributed	Medium	Uncommon
Mongoloid	90 – 120 um	Round	Dense auburn	Thick	Never

3.2.10 General characteristics of Human Hair from Different Sites

Scalp	Head hair, 100 – 1000 mm long, 25-125 um diameter, 0.4 mm/day growth; small root, tapered tip, little diameter variation, various medullation, often with cut tips, may artificially treated.
Pubic	Pudental, 10 – 60 mm long, coarse diameter and prominent diameter, variation and buckling, broad medulla, follicular tags common, asymmetrical cross section twisted and constricted, may be straight, curved or spirally tufted.
Vulvar	Secondary pubic hair, finer and shorter than pubic hair, may be abraided.
Chest	Pectoral, moderate to considerable diameter variation, long fine arch-like tip, usually longer than pubic hair.
Beard	Facial hair, very coarse, 50-300 mm long, large root, irregular structure, often triangular cross section, complex medullation, blunted or razor cut tips, grows 0.4 mm/day.
Axillary	Arm pit, 10-50 mm long, grows 0.3 mm/day, coarse, blunt tip, abraided or frayed, usually straighter than pubic hair, many cortical fusi, sometimes yellowed and bleached.
Eyebrow	Superciliary, 1 cm long, 0.16 mm/day growth, curved, relatively coarse for length, smooth curve with punctate tip and large medulla.
Eyelash	Ciliary, less than 1 cm long, short curved pointed hair.
Limb	Leg and arm hair, 3-6 mm long, fine tips, irregularly medullated, often indistinctly, slightly pigmented.
Ear	Tragi, pinnae, down
Buttocks	Anal hair, short blunted and abraided hair.
Nose	Similar to facial hair.



Human Hair Scales

3.2.11 General characteristics of Human Hair

1	**Colour:**	White, blonde, light brown, gray brown, dark brown, gray, black, auburn, red.
2	**Reflectivity:**	Opaque, gray, translucent, transparent, auburn, clear.
3	**Length:**	Fragment, 1", 1-3", 3-5", 5-8", 8-12", 12-18", 18-30", segment.
4	**Diameter:**	20-30 um, 30-40 um, 40-50 um, 50-60 um, 60-70 um, 70-80 um, 80-90 um, 90-100 um, 100-110 um.
5	**Spatial Configuration:**	Undulating, kinky, curly, wavy, curved, straight, sinuous
6	**Tip:**	Singed, uncut, tapered, rounded, sharp cut, cut at angle, frayed, split, crushed, broken.
7	**Base:**	Cut, damaged, pigmented, clear, enlarged, tapering, broken.
8	**Root:**	Stretched, absent, bulbous, sheathed, atrophied, follicular, wrenched.
9	**Cross-section:**	Polygonal, oval, round oval, undulating, round.
10	**Pigment:**	Absent, non-granular, granular, multicolour, chain, massive (clumped), dense, streaked, opaque.
11	**Medulla:**	Absent, sparse, scanty, fractional, broken, globular continuous, irregular, double, cellular.
12	**Cortical fusi:**	Absent, few, abundant, bunched, linear, central, periphery, roots.

13	**Cortical cells:**	Brittle, damaged, fibrous, cellular, invisible, fusiform, ovoid bodies.
14	**Cosmetic Treatment:**	Bleached, rinsed, natural, dyed, damaged.
15	**Cuticle:**	Ragged, serrated, looped, narrow, layered, wide, cracked, absent, clear, dyed.
16	**Scales:**	Flattened, smooth, level, arched, prominent, and serrated.

3.2.11 Report Writing

Species of Origin:	On the basis of morphological examination, the exhibit was found to be human hair. On the basis of morphological examination, the exhibit was found to be animal hair belonging to (name of family/species).
Sex:	On the basis of microscopic examination, the exhibit was found to be human hair of male/female.
Site:	On the basis of morphological examination, the exhibit was found to be (naturally fallen/plucked/cut) human (scalp/pubic/axillary etc) hair.
Comparison:	On the basis of morphological examination, the exhibit was found to be human (scalp/pubic/axillary etc) hair similar/dissimilar in characteristics with the control sample.

3.3 Fibre Examination:

Identification of common commercial textile fibres is done by microscopical examination, staining tests, solubility tests and physical methods of analysis.

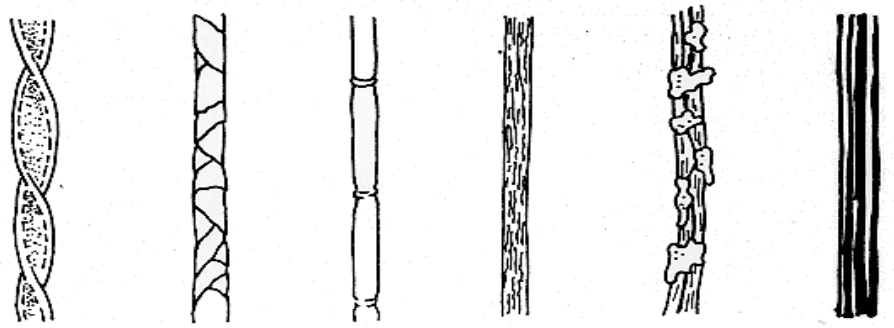
3.3.1 Microscopic Examination:

3.3.1.1 Temporary Mount:

Microscopy of fibres is carried out using temporary preparations of the fibers in distilled water or glycerine or chloral hydrate.

1. Place small quantity of fibre on a slide.
2. Add a drop of distilled water or glycerine or chloral hydrate and place a cover slip. In case of chloral hydrate, pass the slide over a micro burner flame until the liquid boils.

3. Examine under the microscope. A study of cell wall and lumen, crystals, fibre ends, dislocations and cross markings, cells from tissues other than sclerenchyma will help in the identification of plant fibres.



Cotton Wool Linen Nylon Silk Rayon

3.3.1.2 Maceration of Plant Fibres:

Maceration of plant fibres breaks the fibre bundle down into individual ultimates. The technique should be used whenever chloral hydrate preparation of the fibre bundle is not adequate for identification.

Procedure:

1. Prepare a solution of equal parts of Hydrogen peroxide and glacial acetic acid.
2. Place fibre bundles in the solution in a flask and close it with a stopper.
3. Heat over a boiling water bath for about eight hours.
4. When the fibre ultimates are seen to be separating, remove the fibre bundle and place then into a flask containing water.
5. Shake the flask to disperse the ultimates and mount a sample in glycerine.

3.3.2 Cross Sectioning:

Cross sections of fibres can be performed as given in hair section.

3.3.3 Physical Methods:

A number of physical methods of analyses like twist on drying, floatation test, examination of fibre ash and burning test are conducted for fibre identification.

3.3.3.1 Twist on Drying:

1. One end of a single fibre is held between the fingers and the free

end is directed towards the observer.

2. The free end is moistened and the direction of twist (clockwise or counter clockwise) during drying is noted.

3.3.3.2 Floatation Test:

Floatation of fibres in different chemicals like alcohol, Carbon tetrachloride, Chloroform, methyl salicylate, ortho-dichlor benzene etc. is observed.

1. Degrease a small sample of the fibre with petroleum ether.
2. Place fibre in the test liquid and push below surface by means of a glass rod. The liquid should be illuminated transversely and viewed against a black background to observe whether the sample floats to the surface or sinks.

3.3.3.3 Burning Test:

1. A small tuff of fibres in the raw untreated state is held by forceps in the flame of a micro-burner for about 10 seconds and then removed.
2. Note whether the tuff burns or not; whether it forms any bead or whether the ash skeleton is retained; the type of smell emitted during burning. The test is carried out in daylight.

3.3.4 Standards and Controls:

Sufficient known standards of the common fibres should be maintained in the laboratory for reference and comparison purposes.

3.3.5 Cotton (Gossypium sp.):

Cotton is distinguished from all other important textile fibres that under microscope it appears bright in all orientations. Fibres appear beaded, twisted, and thick walled, form the alternation of bright and dark portions.

Burning Test:	Burns with smell of burnt paper leaves delicate black or grayish ash skeleton.	
Twist on Drying:	Variable.	
Floatation Test:	**In alcohol**	Sinks
	In Carbon tetrachloride	Floats

3.3.6 Akund (Calotropis sp.):

Fibres are fine, soft and lustrous but very weak. Under microscope they appear to be similar to Kapok but do not show net like thickenings. Average length 30-

40 mm, average diameter is 20 u.

Burning Test:	As in cotton.
Twist on Drying:	No twist.
Floatation Test:	**In alcohol** Floats

3.3.7 Kapok (Bambax malabaricum):

Unicellular, lingo-cellulose hairs, smooth, cylindrical, hollow, thin-walled and frequently bent, twisted thin walls over on itself, it tapers to a point at one end and other end forms a bulbous base with annular or reticulate markings.

Burning Test:	As in cotton.
Twist on Drying:	Variable.
Floatation Test:	**In alcohol** Floats

3.3.8 Jute (Corchorus capsularis)

Fibres when viewed (cross section) under microscope show 6-2 u or upto 50u thick walled polygonal cells (ultimates), with broad lumen, natural ends of fibres blunt, lingo-cellulosic

Fibre Bundle:	Less than 30 ultimates in each bundle and show longitudinal striations on fibres.
Length of ultimate fibre	Less than 3.7 mm

Burning Test:	As in cotton.
Twist on Drying:	Counter clockwise.

3.3.9 True Hemp (Cannabis sativa)

Fibres show joints when seen under microscope, natural ends blunt.

Cross section:	Rounded polygons.	
Burning Test:	Burns with smell of burnt paper leaves delicate white ash skeleton.	
Twist on Drying:	Counter clockwise.	
Floatation Test:	**In alcohol** **In Carbon tetrachloride**	Sinks Floats

3.3.10 Sisal (Agave sislana)

Fibre Bundle:	Approx. 100 ultimates in each fibre bundle.	
Length of ultimate fibre	Less than 4 mm	
Burning Test:	Burns with smell of burnt paper leaves delicate white ash skeleton.	
Twist on Drying:	Counter clockwise.	
Floatation Test:	**In methyl salicylate** **In ortho-dihlor-benzene**	Sinks Floats

3.3.11 Abaca (Manila) (Musa textiles)

Fibre Bundle:	Approx. 100 ultimates in each fibre bundle.
Length of ultimate fibre	Less than 4 mm
Burning Test:	Burns with smell of burnt grass leaves delicate grayish skeleton.
Twist on Drying:	Counter clockwise.
Floatation Test:	**In methyl salicylate** Floats **In ethyl alcohol** Sinks

3.3.12 Coir (Cocosnucifera)

Length of ultimate fibre	Less than 1.5 mm
Burning Test:	Burns with smell of burnt paper, no bead formation or ash skeleton.
Twist on Drying:	Clockwise.
Floatation Test:	**In methyl salicylate** Floats **In ethyl alcohol** Sinks

3.3.13 Wool

Irregular diameter and prominent scale margins. Medulla present in some medium and coarse fibres. Medulla may be fragmental, interrupted or continuous. In cross section – Oval to circular, variable in diameter. Medulla if present is concentric and variable in size.

Burning Test:	Burns with smell of burnt hair, forms black bead.

3.3.14 **Silk (Bombyxmori)**

In longitudinal view, fibres are fine without any markings, cemented in pairs by silk gum. In cross section fibres are triangular with rounded corners in pair. Silk occurs as two filaments of proteins, fibroin coated and cemented together by a second protein, and sericin, termed as silk gum.

Burning Test:	Burns with smell of burnt hair, forms black bead.

3.3.15 **Asbestos**

Fibers composed of inorganic matter, not destroyed by fire, diameter of single fibre less than 0.001 mm, always in bundles, white, gray, greenish or slightly brown, smooth.

Asbestos can be corded and spun in pure form, but for commercial yarns other fibres are generally blended with it. The important varieties are:

Chrysolite	A crystalline form of serpentine and is a hydrated silicate of Magnesium having formula $Mg_3Si_3O_5(OH)_4$.
Crocidolite	A complex silicate of iron and sodium with a characteristic blue colour. It is resistant to acid attack.
Aniosite	A Ferrous silicate with a proportion of Iron replaced by Magnesium. It is gray to brown in colour.

3.3.16 **Cellulose Acetate Rayon**

Burning Test:	Melts and burns with smell of acetic acid, forma a black bead.	
Solubility Test:	**Phenol**	**Dissolves**
	Acetone	Dissolves

3.3.17 **Cuprammonium Rayon**

Burning Test:	Burns with smell of burnt paper leaves fine gray or black ash.	
Twist on Drying:	No twist.	
Solubility Test:	**80% Sulphuric acid**	**Dissolve**
	Cuprammonium hydroxide	Dissolve

3.3.18 **Viscose Rayon**

Burning Test:	Burns with smell of burnt paper leaves fine gray or black ash.
Twist on Drying:	No twist.

3.3.19 **Nylon**

Burning Test:	Shrinks from flame, melts and forms round hard and transparent bead turns black on further heating and gives pungent smell.

3.4 **Fabrics and Cordage**

Because of their general availability, fabric and cordage are often encountered by forensic scientists, who must compare these types of evidence in order to determine if the two pieces could have originated from the same source. Structural details such as design, construction and composition can provide information that may assist the examiner in reaching a conclusion. The construction, composition and colour of textiles involved in crimes are useful comparison characteristics for forensic examinations. Textiles appear in a variety of weaves, knits and non-woven constructions, and a combination of fabric types can occur in any one textile. The range of colours in which textiles are offered in the market place is enormous. Therefore, the construction, composition and colour of a textile can aid the examiner in including or excluding a textile for consideration in a forensic examination.

3.4.1 Terminology

Core:	A fibre or fibres running lengthwise through the center of a cordage.
Course:	The row of loops or stitches running across a knit fabric, corresponding to the filling in woven fabrics.
Crown:	The raised portion of a strand in a twisted cordage.
Knit Fabric:	A structure produced by interloping one or more ends of yarn or comparable material.
Pitch:	The number of crowns per inch of the same strand.
Ply:	The number of single yarns twisted together to form a plied cord. An individual yarn in a plied yarn or cord.
Selvage:	The narrow edge of woven fabric that runs parallel to the wrap. It is made with stronger yarns in a tighter construction than the body of the fabric to prevent raveling.
Strand:	A single fibre, filament or monofilament.
Twist (Lay):	The direction of twist in yarns is indicated by the capital letters S and Z. Yarn has an S-twist if when it is held vertically the spirals around its central axis slope in the same direction as the middle portion of the letter Z.
Wale:	A column of loops lying lengthwise in a knit fabric.
Warp:	The set of yarn in all woven fabrics that runs lengthwise and parallel to the selvage. It is interwoven with the filling.
Weft (Filling):	In a woven fabric, the yarn running from selvage to selvage at right angles to the wrap.
Woven Fabric	Generally used to refer to fabric composed of two sets of yarns, wrap and weft (filling), that is formed by weaving, which is the interlacing of these sets of yarns.

3.4.2 Sample Handling

Photograph the item prior to conducting any analyses in order to provide documentation of original condition. Document and remove other evidence (e.g. hair, blood and paint) that may require additional analysis. Document and record descriptions of any physical damage (e.g., worn, cut, broken and frayed). The following general macroscopic characteristics should be observed and documented.

1. Severed ends for possible physical matches;
2. Knots, ligatures or both;

3. Dimensions: size, length, diameter etc.
4. Components: number, type and twist.
5. Colour;
6. Dyes; and
7. Natural.

Do not bring a questioned specimen (e.g., piece of fabric, yarn and tuft of fibres) in contact with the known fabric from which it is suspected to have originated until you have performed a preliminary examination of the questioned specimen.

Do not alter the condition of a questioned specimen (e.g.. shape, position, layers, or relation of one yarn to another) before a preliminary examination and before receiving a known sample for comparison.

Do not cut a sample to be used for comparison testing from ends of yarn or edges of fabric if there is a possibility of physically matching a questioned specimen to a known specimen. Take the known sample away from the existing edge or edges and mark the location as known taken.

Fabric and cordage may be a source of other types of physical evidence (hairs, fibres, blood, etc.). In addition, cuts, tears, knots and severed ends may be of forensic value. Therefore, fabric and cordage evidence should be examined in a manner that preserves these types of evidence.
All pertinent data collected on questioned and standard samples should be placed into or referenced within the specific case file.

3.4.3 Analysis

Preliminary examination of fibres composing a fabric or cordage, with any adhering matter, should include its general appearance under a low power microscope before a sample is mounted on a slide. Any adhesives or other material used in bonding fabrics, carpet backings, and so forth should also be noted.

Physical matching should always be considered if two pieces of fabric or cordage need to be compared. If the ends have been cut or torn, a physical match may be possible. A physical match must be documented by photography. Additionally, describing the condition of corresponding threads and their relative positions in the damaged area on the questioned and known pieces (so-called longs and shorts) provides a detailed corroborative description.

If a physical match is not possible, comparison of the parameters determined in the checklist will assist the examiner in determining if the two pieces could have originated from the same source.

3.4.4 Fabric

Fabric examinations are primarily a process of deconstructing the fabric by dissecting its constituents elements. Each of these elements can have a number of sub-elements, all of which must be characterized to complete the examination. These elements include the following:

1. Construction (woven, knit, non-woven);
2. Threads per inch in wrap and weft direction;
3. Staple or continuous fibres in yarns;
4. Yarn twist;
5. Number of piles;
6. Direction of twist of piles;
7. Number of filaments in each ply;
8. Composition of yarn;
9. All fibre types composing the fabric;
10. Colours and design;
11. Blend of two or more types of fibres within each ply; and
12. Sewing threads, buttons, decorations, and so forth as detailed previously.

3.4.5 Cordage

The initial step in the identification of rope and cordage is to determine its construction and assembly. The checklist should include, but is not limited to the following characteristics:

1. Diameter;
2. Staple or filament fibres;
3. Twisted, braided or non-twisted;
4. Twist;
5. Crowns or turns per inch;
6. Number of plies or braids;
7. Twist of each ply or braid;
8. Crowns or turns per inch; and
9. Filaments in each ply or braid, which are evaluated for the following characteristics:
10. Core if any;
11. Twist;
12. Crowns or turns per inch;

13. Number of filaments;
14. Colour or colours;
15. Coatings, if any;
16. Tracers, if any, and
17. Coatings.

After the construction has been established, then the constituent fibres should be analyzed with the appropriate microscopic and instrumental techniques.

3.4.6 Report Writing

On the basis of morphological examination, the exhibit was found to be (name) fibre. On examination, the test item is found to be similar / dissimilar with the control sample.

Comparison: On the basis of morphological examination, the exhibit was found to be (name) fibre similar / dissimilar in characteristics with the control sample. (For comparison purposes, at least 10 fibre strands should be examined).

On the basis of fibre, texture and weaving pattern characteristics, the exhibit was found to be similar / dissimilar in characteristics with the control sample and has (not) originated from the same source.

CHAPTER 4

DIATOM EXAMINATION

4.1 **Importance:** In drowning deaths, whether death was due to suicide on homicide observation of diatoms is significant proof of drowning. In the postmortems carried out on bodies of drowning victims detection of diatoms in the autopsy signifies the cause of death. If the drowning had taken place in the fresh water, i.e. ponds, lakes or rivers then fresh water diatoms are found in the autopsied body. Presence of diatoms in the body is not a conclusive proof of drowning death. However, in many instances, when postmortem was conducted in accident deaths, diatoms were found in the autopsied bodies. Hence presence of diatoms is not a conclusive proof of drowning.

The nature of diatom found in the autopsied body indicates the site of occurrence of death i.e. if fresh water diatoms are found in the body, drowning had taken place in fresh waters, i.e. ponds, lakes, rivers. If marine diatoms are recovered in postmortem then body was drowned in ocean.

4.2 **METHOD:**

Before diatoms can be examined, they have to be cleaned. This involves the removal of cell contents, pigments, sand, mud or other material likely to interfere with microscope examination. A sample of material may conveniently be obtained from femoral or sternal bone marrow. Care should be taken to use dry and clean instruments to avoid any contamination from extraneous material. After removing the sternum, a small window is cut into it, and marrow exposed. A small portion of the marrow is scooped out into a test tube. Acid digestion of the marrow is then effected by mixing it with strong nitric acid, and heating it till a clear fluid is obtained. This is cooled, then centrifuged and the sediment is examined under the microscope. A remarkable collection of most delicate and beautiful skeletons of diatoms is seen in cases of death from drowning. In favorable circumstances, even the site of drowning can be determined since the fresh water and the sea water diatoms are different and the sea water plants even vary from place to place along the coast.

The diatom test is valid if it can be shown that:

1. The deceased did not drink this water immediately before submersion.
2. The species recovered from the specimen are all present in the sample from the site of drowning, and

The various species are present (if sufficient are found) in the same order of

dominance for the admissible size range and in approximately the same proportions.

Since diatoms resist putrefaction, the diatom test is particularly valuable. Where decomposition is advanced. Diatom test is negative in dead bodies thrown in water and in dry drowning.

4.3 Standards and Controls:

In diatom examination invariably the control water samples must be used for comparison purpose.

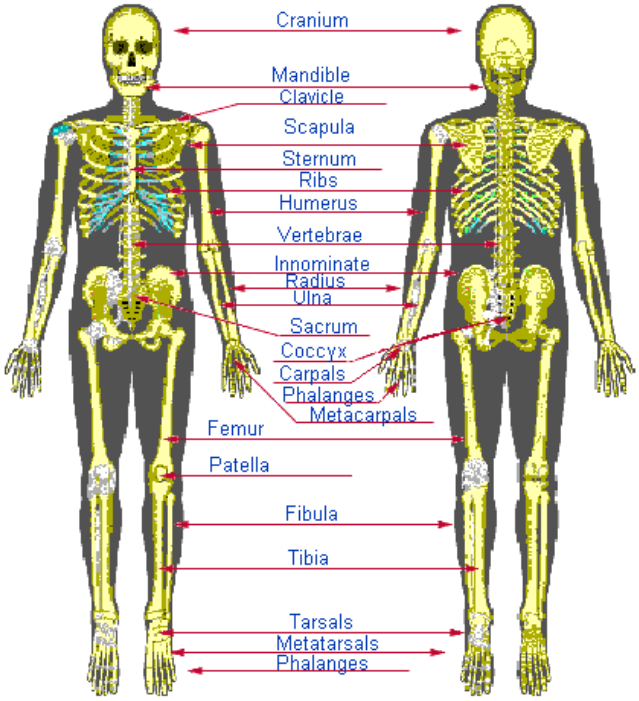
Standard diatom samples can be preserved on slides and can be used as standards for comparison purpose.

4.4 Diatom Report Format:

Diatoms were detected / not detected in the Exhibit (s) and were comparable/ not comparable with the control sample Exhibit (s)

Chapter 5

HUMAN SKELETON EXAMINATION



5.1 Human skeleton remains are invariably encountered in the forensic examination. Skeleton is made up of calcified tissues with dense matrix in which osteocytes are embedded. The matrix is composed of collagen fibers crystals of calcium phosphate complex and a ground substance or cement containing monosaccharide. There are 206 bones in adult human skeleton. Different bones in human skeleton are as follows (nos. in parentheses refers to total nos., right and left).

Skull (29):	
1.	**Cranium (8)** Frontal (1) Parietal (2) Occipital (1) Temporal (2) Sphenoid (1) Ethmoid (1)

2.	**Face (14)** Nasal (2) Vomer (1) InferiorNasal Concha (2) Lacrimal (2) Zygoma (2) Palatine (2) Maxilla (2) Mandible (1)
3.	**Ear Ossicles (6)** Malleus (2) Incus (2) Stapes (2)
4.	Hyoid (1)
Vertebral Column (26):	
1.	Cervical vertebrae (7)
2.	Thoracic vertebrae (12)
3.	Lumbar vertebrae (5)
4.	Sacrum (1, representing five fused sacral vertebrae)
5	Coccyx (1, representing three to five fused coccygeal vertebrae)
Thoracic Cage (25):	
1.	Ribs (24)
2.	Sternum (1, representing manubrium plus body, the latter of which represents the fusion of four elements.)
Arm (64):	
1.	**Shoulder girdle (4)** Scapula (2) Clavicle (2)
2.	**Upper arm (2)** Humerus (2)
3.	**Forearm (4)** Radius (2) Ulna (2)
4.	**Hand (54)** **<u>Carpals (16)</u>** a. Scaphoid (2) b. Lunate (2) c. Triquetral (2) d. Pisiform (2) e. Multangulum major or trapezium (2) f. Multangulum minor or trapezoid (2) g. Capitate (2)

	h. Hamate (2) **<u>Metacarpals (10)</u>** **<u>Phalanges (28)</u>**
Leg (62):	
1.	Pelvis (2, each pelvic bone representing the union of three bones, ilium, ischium, pubis)
2.	**Thigh and knee (4)** Femur (2) Patella (2)
3.	**Leg (4)** Tibia (2) Fibula (2)
4.	**Foot (52)** **<u>Tarsals (14)</u>** a. Talus (2) b. Calcaneus (2) c. Cuboid (2) d. Navicular (2) e. Cuneiform I (2) f. Cuneiform II (2) g. Cuneiform III (2) **<u>Metatarsals (10)</u>** **<u>Phalanges (28)</u>**

5.2 General Comparison

The following factors are to be taken into consideration to ascertain whether the bone is of human origin or otherwise.

1. Shape of the bone
2. Size of the bone
3. Features of the bone
4. Internal structure of the bones

Origin from small fragments of bone can also be determined by microscopic

examination, serological techniques or by DNA analysis.

5.3 SEX DETERMINATION FROM SKELETON:

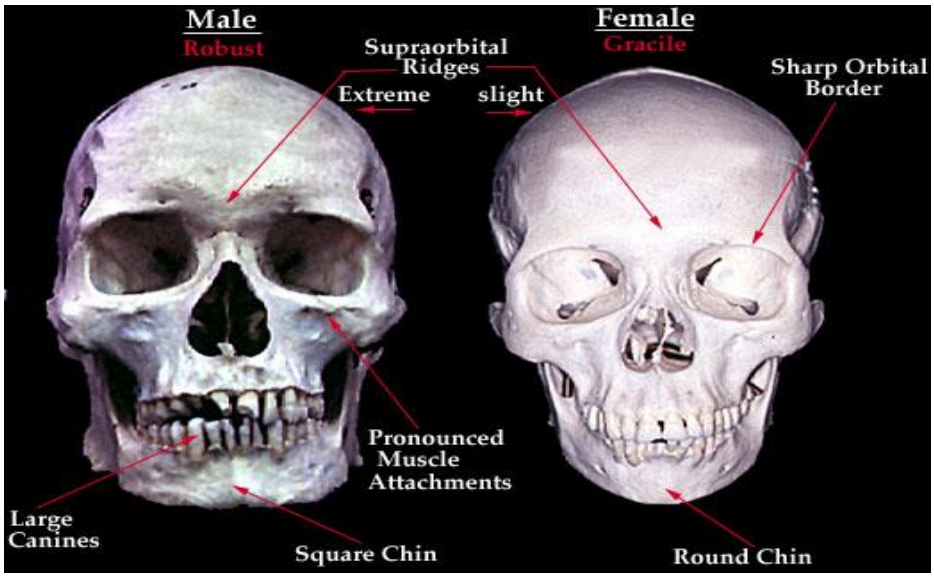
Sex differences do not appear until puberty. Up to that time the skeletons of two sexes differ only in size. Sex is determination is reliable if the essential parts of the skeleton are present and in good condition. Most of the criteria for determining sex pertain to the pelvis and the skull, although the round of ball joints also provides very reliable means of determining sex.

5.3.1 General Characteristic of the male and female skeleton

	Male	Female
1.	Skeleton comparatively bigger and stouter.	Skeleton comparatively smaller and slender.
2.	Muscular ridges, depression and process are more prominent.	Muscular ridges, depression and process are less prominent.
3.	Shaft of the long bones relatively rough and the articular surfaces and ends larger.	Shaft of the long bones relatively smooth and the articular surfaces and ends small.

5.3.2 Skull:

The female skull (adult) is usually lighter and smaller, 10% less capacious than that of the males. It is very difficult to determine sex of skull below the age of adolescence. The differentiating features between the two sexes are as follows:



		Male	Female
1.	Size	Bigger, heavier, contour is more rough and rugged.	Lighter, smaller, contour smoother and less rugged.
2.	Capacity	Cranial Capacity is approx. 10% more than that of females (approx. 200 c.c. more)	Less capacious, capacity 10% less than that of males.
3.	Glabella etc.	Glabella, Supraorbital ridges, zygomatic arches, mastoid processes, occipital protuberances, occipital condyles of adult male skulls are well marked and prominent. Condylar facets are long, large and slender.	All these are more delicate, small smooth and less prominent. Condylar facets are broad and short.
4.	Areas of muscular attachments	Muscular attachments and impression over occipital region of skull are well marked and prominent. Occipital surface is rough and protuberant more so in those who carry heavy weight on head, irrespective of sex. Digastrics grooves are deeper.	These are less prominent and marked in females. Occipital boney surface is relatively smooth, Digastric grooves are less deep.
5.	Base of skull	It is more rough and rugged. Foramen magnum large.	It is less rough and rugged, more smooth and plain. Foramen magnum small.
6.	Frontal Sinuses	These are more well developed. Frontal and parietal eminences are small. Frontal Sinuses may get atrophied in makes in Kartagener's Syndrome, which is characterized by Oh. Sinusitis dextrocardia & bronchiactesis.	Frontal Sinuses are less developed. Frontal Sinuses may get unduly developed in hyperpituitarism in females.
7.	Orbital Cavities	These are comparatively smaller square and placed low down with round upper edges.	These are bigger nearly circular and placed higher up with sharp upper edges, sizes being larger

			relative to the face as a whole.
8.	Forehead	It is steeper and less round with no tendency to bulge.	It is more round with full of tendency of bulging.
9.	Front nasal Junction	There are distinct angulations at the junction.	No such; instead there is smooth curve from forehead to upper part of bridge of nose.
10.	Facial bones	These are massive, rough and heavier and more laterally arched.	These are less massive, rather smaller and delicate and more compressed sideways.
11.	Palate	The breadth of palate is larger, wider and broader. Tends to be like "U" shape, due to relative length of check-tooth-jaw.	Usually smaller and narrower, is parabola shaped due to relative length of check-tooth-jaw.
12.	Chin	It is more prominent and square in male.	Chin is less prominent; it is "U" shaped.
13.	Cervical Vertebrae	Larger in males.	Smaller in females
14.	Teeth	Teeth are larger in males than the females. Lower 1st Molars are often 5 cusped.	Teeth are smaller in size comparatively. Molars are more often 4 cusped.
15.	Size	Bigger, heavier, contour is more rough and rugged.	Lighter, smaller, contour smoother and less rugged.
16.	Nasal apertures	These are usually placed high up and are narrower in breadth.	Nasal apertures in females are low, down and broader.

5.3.3 Mandible

		Male	Female
1.	Size	It is usually larger, thicker and heavier.	It is smaller, thinner and lighter.
2.	Mandibular symphysis	The height of symphysis menti is greater and higher up.	It is smaller and shorter.
3.	Ramus	The ascending ramus has greater breadth.	It has rather smaller breadth.
4.	Shape of Chin	Chin is square shaped.	Chin is pointed or rounded (U shaped).
5.	Anatomical angle	The anatomical angle is the angle between the body and the ramus on outer aspect will be less obtuse, under 125^0 more prominent and everted.	The anatomical angle will be more obtuse, less prominent and inverted.
6.	Condyles	These will be bigger and larger.	Condyles will be smaller.
7.	Impressions	Impressions for the muscular attachments are prominent.	These are less prominent.

5.3.4 Thorax & Ribs

	Male	Female
1.	The thoracic cage is longer and narrower.	It is shorter and wider.
2.	Ribs are thicker and comparatively massive in texture.	Ribs are thinner and delicate in texture.
3.	Ribs are disposed not so obliquely.	Ribs are disposed more obliquely.
4.	Costal arches are not larger.	Costal arches are larger.
5.	Ribs have lesser curvatures.	Ribs have greater curvatures.

5.3.5 Sternum

Sl. No.	Traits	Male	Female
1.	Body	The body of the sternum is bigger and is at least twice or more the length of the manubrium.	The body of the sternum is shorter and is less than twice the length of the manubrium.
2.	Level	The upper border of the sternum is usually at the level of lower part of the body of the 2nd Thoracic Vertebrae.	The upper border of the sternum is usually at the level of lower part of the body of the 3rd Thoracic Vertebrae.
3.	Manubrium	Manubrium is somewhat smaller.	It is somewhat bigger.

5.3.6 Pelvis

Sl. No.	Trait	Male	Female
1.	Pelvis as a whole	Massive, rugged, marked muscular sites	Less massive, gracile, smother
2.	Symphysis	Higher	Lower
3.	Sub-pubic angle	V-shaped, sharp angle	U-shaped, rounded, broader, divergent, obtuse angle
4.	Obturator foramen	Large, often ovoid	Small, triangular
5.	Acetablulum	Large, tends to be directed laterally	Small, tends to be directed antero-laterally
6.	Greater sciatic notch	Small, close, deep	Large, wider shallower.
7.	Ischipubic rami	Slightly everted	Strongly everted
8.	Sacro-iliac articulation	Large	Small, oblique
9.	Preauricular sulcus	Not frequent	More frequent, better developed
10.	Ilium	High, tends to be vertical	Lower, laterally divergent

11.	Sacrum	Longer, narrower with evenly distributed curvature, often 5+ segments	Shorter, broader with tendency to curve
12.	Pelvic brim	Heart shaped	Circular or elliptical
13.	True Pelvis	Relatively smaller	Oblique, shallow, spacious.

Examination of pelvis alone allows sex to be determined with fair accuracy in sex determination from isolated skeletal parts, showing data as follows:

1.	Pelvis alone	-	95%
2.	Skull alone	-	92%
3.	Pelvis and Skull	-	98%
4.	Long bones alone	-	80% - 85%
5.	Long bones and Skull	-	96%
6.	Long bones and Pelvis	-	85%

5.3.7 Sacrum

	Male	Female
1.	Long and Narrow	Short and wide
2.	Uniform curvature along its whole length	Sharply curved forward in its lower half
3.	The articular surfaces extend over two and half to three elongated bodies	The articular surfaces extend over two to two and a half segment

5.4 Age Estimation

5.4.1 IN INFANCY:

(i) The ramus of the mandible is short, oblique and forms and obtuse angle with the body.
(ii) The coronoid process projects about the level of the condyloid process.
(iii) The mental formation remains near the lower margin of the jaw.
(iv) The body is shallow.

5.4.2 BETWEEN 6 MONTHS – 2 YEARS:

(i) Eruption of all the temporary teeth.
(ii) Appearance of ossification centers in the heads of humerus, femur, ulna, tarsal and carpal bones.

(iii) Closure of the anterior fontanelle at about 3/2 years.

5.4.3 BETWEEN 2 - 6 YEARS:

(i) Appearance of centers of ossification in the epiphysis of long bones.
(ii) Closure of metopic suture.
(iii) Fusion of condylar part of occipital bone with the squama and basi-occiput.
(iv) Fusion of greater and lesser tubercles to the head of humerus.

5.4.4 BETWEEN 6 - 12 YEARS:

(i) Fusion of rami of ischium with pubis at about 7-9 years.
(ii) Ossification of pisiform bones 9 to 12 years in females.
(iii) Fusion of lateral epicondyles of the humerus with the shaft.
(iv) Shedding of all temporary teeth ad eruption of all permanent teeth except the 3rd molars around 12 years (12-14 years).
(v) Appearance of secondary sexual characteristics around 12 years.

5.4.5 BETWEEN 12 – 18 YEARS:

(i) Fusion of upper end of Radius with shaft.
(ii) Fusion of Olecranon to the ulna.
(iii) Eruption of 3rd molar teeth with calcification of roots of all the previously erupted teeth.
(iv) Union of lower ends of Radius and Ulna with the respective shafts.
(v) Union of head, greater and lesser trochanters of femur with the shaft so also of the upper and lower ends of tibia and fibula with the respective shafts.

5.4.6 BETWEEN 18 - 25 YEARS:

(i) Union of epyphysis of most of long bones with their respective shafts.
(ii) Union of iliac crest with ilieum, ischial tuberosity with ischium.
(iii) Union of coracoid and acromion with their respective bodies.
(iv) The union of the epiphysis at sternal ends of the clavicles; the secondary centers in the pelvis and articular facets of ribs usually get completed in between 20-25 years.
(v) Bony replacement of the cartilage between the basiocciput and the basisphenoid commences at about 17th year in both sexes; union is completed by 22 years in females and 24 years in males usually.
(vi) Four pieces of the body of the sternum fuse with one another from below upwards between 14-25 years. Union of all sacral vertebrae with one another occurs from below upwards to constitute the sacrum as a single piece of bone.

5.4.7 BETWEEN 24 - 35 YEARS:

(i) The closure of the lambdoid suture is usually not complete before 50 years, though coronal sutures get closed by 40 years approximately. The changes in S. pubis are also characteristics.

(ii) Around 40 years, the articular surfaces of lumbodorsal vertebrae show lipping, loss of joint space, presence of punched out areas of osteoporosis etc. under X-ray examination. Such changes are noticed first in the glenoid fossa of the scapulae; lipping becomes well advanced by 45-50 years but does not get complete before 60 years.

5.4.8 ABOVE 50 YEARS:

(i) In old age, long bones become more lighter and brittle owing to the increase in the inorganic constituents; skull becomes thinner and lighter from absorption and hence is more liable to fracture from even slight violence. In some cases, skull bones may become thicker and heavier due to hypertrophy of the inner table or may also change from an ivory like to granular appearance and feel with advancing years.

(ii) The closure of mastoid, occipital, squamous and parietomastoid sutures will occur in very old age parietal suture may not even close throughout life.

(iii) After 60-65 years, the angle of lower jaw opens out and the medico legal angles become obtuse.

(iv) Manubrium and body of sternum may fuse above 60 years.

(v) The calcification of the laryngeal and costal cartilages becomes more apparent.

5.4.9 SYMPHYSIS PUBIS:

S. Pubis helps greatly in determination of age from second to fifth decades. Below 20 years, the symphyseal surface has an even appearance with a layer of compact bone over its surface; between 20-25 years it looks markedly ridged and irregular – the "ridges or billowing" run transversely and irregularly across the articular surface. In X-ray films, the billowing appears ads undulating lines. Between 25-35 years – the billowing gradually disappears and the articular surface in macerated bone presents granular appearance with its anterior and posterior margins sharply defined.

5.4.10 Skull:

(i) The anterior fontanalle closes at 3/2 years and two halves of mandible usually unite at 2nd year. The metopic suture closes

between 2-4 years usually.

(ii) The condylar portion of the occipital bone gets united with its squamous part by 3rd year and with the basi-occipital portion by the 5th year. The cartilage between the basi-sphenoid and basi-occiput gets ossified in case of females by 19-22 years and in case of males by 19-24 years. It is claimed that bony replacement of the cartilage starts as early as 17-19 years in both the sexes.

(iii) Closure of the sutures of the skull has some bearing with milestone of life. Absence of any sign of closure of the sill sutures points to a strong possibility of the age having not exceeded thirty years.

(iv) Sutures commence closing first in the endocranial surface, then on the ectocranial one. As a rule, inner surface closes several years before the outer.

(v) The SAGITTAL SUTURE is the first to start closing endocranially at about 25 years at its back portion – para lambdica close to the parietal foramina. The fusion becomes completed both endo and ectocranially by 32-35 years – the last part to unite is pars bregmatica – the portion near the bregma; parts vertica & parts Obelica – the 2nd and 3rd part of sagittal suture unite in between.

(vi) The CORONAL SUTURE is the next to unite – it starts closing endocranially at about 25-30 years in its lowest part – pars pterica, close to the junction with the sphenoid but at times may close at parts bregmatica; the closure gets completed by 35-40 years; parts complicata & parts Stephanica - the 2nd and 3rd part of coronal suture unite in between.

(vii) The LAMDOID SUTURE starts closing endocranially near the Lambda, at parts lambdica at about 35 years (35-40 years) and the union at parts intermedia and parts asterica, the 2nd and 3rd part of lambdoid suture getting united in between usually by 45-50 years.

(viii) The spheno-temporal, occipito-mastoid, parieto-mastoid, or spheno-parietal sutures may not start closing before 50 years and the closure may not get completed as late as 60 to 70 years; spheno – parietal may close at about 80 years app.

Suture closure in skull occurs earlier in males than in females.

Coronal Suture:

The coronal sutures for age estimation purpose have been divided into four parts between the bregma* and pterion** bregmatic, fronto-bregmatic, stephanion*** and pteric i.e. C1, C2, C3 and C4.

- Point of junction of the sagittal and coronal sutures*
- Junction of the frontal, parietal, temporal and sphenoid bones**

- Point of junction of the coronal sutures and the temporal bones***

Synosteosis (age)			
Part	**Beginning**	**Development**	**Ending**
C1	38	41	46
C2	44	50	55
C3	35	38	45
C4	32	35	40

This table shows that the synosteosis begins in part C4 followed by parts C3, C1 and C2.

Sagittal Suture:
The sagittal sutures for age estimation purpose have been divided into four parts between the bregma* and the lambda** bregmatic, mesobregmatic, obliac*** and inial i.e. S1, S2, S3 and S4.

- Point of junction of the sagittal and coronal sutures*
- Junction of the sagittal and lambdoial sutures **
- Area corresponding to the almost straight part of the sagittal suture found at the level of S1***

Synosteosis (age)			
Part	**Beginning**	**Development**	**Ending**
S1	32	40	44
S2	28	36	41
S3	22	35	38
S4	29	40	42

This table shows that the synosteosis begins in part S3 followed by parts S2, S4 & S1.

Lambdoidal Suture:

The lambdoidal sutures for age estimation purpose have been divided into three parts between the lambda* and the asterion**. Initial inial, mesoinial and teloinial i.e. L1, L2 and L3.

- Point of junction of the sagittal and lambdoidal sutures*
- Point of junction of the occipital and parietal bones with the mastoid area of the temporal bones**

Synosteosis (age)			
Part	**Beginning**	**Development**	**Ending**
L1	38	44	49
L2	42	50	53
L3	48	58	64

N.B. estimation age from a study of the path of the synosteosis of cranial sutures is a method of long standing. However, the various research workers who have studied the subject have not agreed on any results except in rare cases (for example, the date when synosteosis begins. The age limit is generally accepted as being 30.

5.4.11 Estimation of Age from Teeth

The estimation of age from teeth, with some degree of accuracy can only be possible up to 17-25 years of age; beyond that age, it would be rather rough approximation. Children of same age from different countries have practically more or less the same eruption times. Eruption is not always symmetrical in both halves of the jaw. There may be differences in eruption time between the upper and lower jaws.

Factors like heredity, environment, climate, nutrition and endocrine factors may have some influence upon eruption of teeth. There may be some correlation between dental and skeletal age especially in case of females.

The teeth, under natural conditions of decomposition are for all practical purpose indestructible. Hence, they offer an excellent means of identification for an almost limited time.

The tooth may be temporary or permanent. In infancy and childhood, they are

fairly regular in their appearance and thus afford very useful criteria for age estimation.

5.4.12 Development & Eruption of Teeth

The alveolar cavities for tooth buds are formed at about the 4th or 5th month of intrauterine life. Tooth development begins with formation of cellular tooth-germ within the alveolar cavities of the jaw in the shape of the crown. Within this tooth-germ, apposition and calcification of enamel and dentin take place and before a change of position of tooth occurs the crown gets formed and calcified. At birth, the rudiments of all temporary teeth and 1st permanent molars are found in the jaws. After completion of the crown, root formation begins, with the roots getting longer; the crown erupts through the soft tissues of the gum and protrudes out inside the oral cavity. The roots get completed sometime after the teeth are in full functional occlusion.
As the permanent tooth erupts, the overlying root of its temporary predecessor undergoes simultaneous resorption, until only the crown remains. The unsupported crown then fall out.
X-ray examination will reveal the stage of development unerupted teeth.

5.4.13 Temporary/Deciduous Teeth

They are twenty in number, ten in each jaw comprising of four incisor, two canines and four molars. The first temporary tooth cuts usually in between 6-7 months after birth, eruption of all temporary teeth gets completed at about 2nd year.

Dentition is delayed in weak, rickety, debilitated children; whereas in case of syphilitic children, early dentition is the feature – when the child may even be born with teeth. In case of congenital syphilis, the permanent upper central incisors get notched and stunted – they are known as "HUTCHINSON'S TEETH".

ERUPTION OF TEMPORARY TEETH

CENTRAL INCISORS	(Lower) erupt	at 6 months	(6-8 months)	Root calcification
- DO -	(Upper) erupt	at 7 months	(7-9 months)	Completed
LATERAL - DO -	(Upper) erupt	at 9 months	(9-10 months)	½ years
LATERAL	(Lower)	at 10	(10-12	½ years

- DO -	erupt	months	months)	
FIRST TEMPORARY MOLAR	Erupt	at 12 months	(12-14 months)	2 - 2½ years
CANINES	Erupt	at 18 months	(17-18 months)	2½ - 3 years
SECOND TEMPORARY MOLAR	Erupt	at 24 months	(20-30 months)	3 years

Calcification of roots of milk teeth are complete by the end of 3rd / 4th year, the incisors and the second molars being the last to calcify. Resorption of the roots of the incisors starts at about the fourth year to be followed by molars at 6-7th year and canines at 8th year of age.

Resorption of milk teeth starts in this order:

Central Incisor- 4th year
Lateral Incisor- 5th year
1st Molar- 6th year
2nd Molar- 7th year
Canine- 8th year

The temporary teeth start shedding round about 6-7th year after the eruption of the 1st permanent molar. Hence in a child of 6-7th years of age, we will find 24 teeth in all – 20 temporary and four permanent. The period of mixed dentition is the age interval between 6-11 years, may persist until 12-13 years of age.

5.4.14 Differences in Temporary & Permanent Teeth

Sl. No.	***Temporary Tooth***	***Permanent Tooth***
1.	Smaller in size usually, except in case of temporary molars, which are larger than the permanent bicuspids replacing them.	Longer and larger than temporary teeth except permanent bicuspids replacing temporary molars.
2.	They are lighter and more delicate than the permanent ones except molars.	More strong, broad and heavy; temporary molars are bigger and longer than premolars replacing them.

3.	The anteriorly placed milk teeth are vertical.	Permanent teeth are more or less inclined forwards. Have small irregularities on its cutting edges.
4.	Neck is more constricted.	It is less constricted.
5.	Crowns are china white in colour.	Crowns are usually ivory white in colour.
6.	A ridge or thick edge is present at the junction of the crown and the fangs.	No such ridge is noticed.
7.	Temporary molars have their cusps flat, roots smaller and more divergent.	Bicuspids replacing temporary molars have bigger and less divergent roots with permanent cusps.
8.	Presence of tooth germ beneath the tooth if seen in X-ray will suggest that the tooth is temporary.	No such thing is visible in X-ray in case of permanent tooth.

5.4.15 Permanent Teeth

They are 16 in each jaw, 32 number in total, comprising of 4 incisors, 2 canines, 4 bicuspids or premolars and 6 molars, in each jaw.

5.4.16 Morphology of Permanent Teeth

Each tooth has a root, neck and crown the root is embedded in the jawbone and the crown is the visible part of the tooth. The tooth is composed of dentin, which is covered by enamel on the crown portion and on the root by cemented which is attached to the alveolar process of jaw by periodontal membrane. Neck of the tooth is the constricted portion between the root and crown being surrounded by gum.

INCISORS

The crown portion of the tooth is chisel shaped being convex on its labial surface and concave on its lingual surface, except near its neck, where the surface is convex. The neck of the incisor is slightly constricted and the root is single.

CANINE

It is peg shaped – its masticatory edge tapers to a blunt point; projects slightly beyond the level of other teeth. Its crown is large, has more or less concave

surface both on its lingual and labial surfaces. It has got a single conical root, which is larger and thicker than that of an incisor.

PREMOLAR OR BICUSPID

It is smaller and shorter than the canine. The crown of the premolar is more or less circular on cross section. The masticatory surface has got two cusps. It has got a single root usually. Cusps are the raised and prominent area on the masticatory surface of the tooth.

MOLARS

The molars are the biggest and largest amongst all other teeth, having broad crown, which are convex on the labial and lingual surfaces and flattened on its medial and distal surfaces. The cusps of the molars vary from three to five. Each upper molar has got three roots, while the lower has two usually.

5.4.17 Eruption of Permanent Teeth

Eruption of teeth is very good indication of age but there may be abnormalities in definition. The first permanent molars erupt fairly regularly at 6 years of age and second permanent molars do erupt round about 13 years (12-14 years). The premolars are most irregular to erupt and hence they are of little value in fixing the age. But 1^{st} and 2^{nd} molars are more or less constant as to their age of appearance.

All permanent molars are "SUPERADDED PERMANENT TEETH" as they do not have temporary predecessors, "SUCCESSIONAL PERMANENT TEETH" – 10 in each jaw are those, which erupt in the place of temporary teeth. All permanent teeth except the molars belong to this group.

5.4.18 Spacing of Jaw

After eruption of 2^{nd} molar – the ramus of the jaw grows behind, when the body of the jaw increases in length to make room for the eruption of the 3^{rd} molar teeth. Hence, while examining the teeth the space behind the 2^{nd} permanent molars is to be felt; if space is present, to see if it is hard in feel or not.

Usually the permanent tooth erupts first in the lower jaw, then after a short interval, in the upper jaw but this may not be always regular. Usually permanent teeth appear a few months earlier in girls than in boys.

The eruption of 3^{rd} molar is very irregular. They usually erupt by 17-25 years. Hence presence of all the four third molars indicate that the subject is over 18 years of age, but their absence gives no certain idea about age. If the X-ray shows no calcification of the roots of 3^{rd} molars – it is to be presumed that the

age is below 25 years. If calcification is found to be complete, then the age can be presumed to be at least 25 years. In general, complete calcification of the roots of the teeth takes place within 3-4 years of their eruption.

AVERAGE PERIOD OF ERUPTION OF PERMANENT TEETH

FIRST MOLAR	6 YEARS	6-7 YEARS
CENTRAL INCISOR	7 YEARS	7-8 YEARS
LATERALINCISOR	8 YEARS	8-9 YEARS
FIRST PREMOLARS	9 YEARS	9-11 YEARS
SECOND PREMOLAR	10 YEARS	10-13 YEARS
CANINES	11 YEARS	11-13 YEARS
SECOND MOLAR	12 YEARS	12-14 YEARS
THIRD MOLAR	17-18 YEARS	17-25 YEARS

NUMBER OF TEETH AT DIFFERENT AGES

Sl. No.	***Age***	***No. of teeth***	***Temporary / Permanent***	***Name of teeth***
a.	12 months	8/12 teeth	Temporary	C.I., L.I., 1st Molar
b.	15 months	12 teeth	- do -	C.I., L.I., 1st Molar
c.	18 months	16 teeth	- do -	C.I., L.I., 1st Molar, Canine
d.	24 months	20 teeth	- do -	C.I., L.I., 1st Molar, Canine, 2nd Molar
e.	6 years	24 (Temp 20 + Perm 4)	Permanent	All temporary teeth + 1st permanent Molar
f.	7 years	24 (Temp 16 + Perm 8)	- do -	1st permanent Molar & Canine
g.	8 years	24 (Temp 12 + Perm 12)	- do -	1st permanent Molar & Canine
h.	9 years	24 (Temp 8 + Perm 16)	- do -	Per IM, C.I., L.I., 1st
i.	10 years	24 (Temp 4 + Perm	- do -	Per IM, C.I., L.I.,

		20)		1st
j.	11 years	24 (Temp Nil + Perm 24)	- do -	Per IM, C.I., L.I., 1st

5.5. Estimation of Height from Long Bones

An idea about the height of the deceased from the skeletal remains is obtained by multiplying the length (in inches) of a long bone with a suitable multiplication factor. The following factors are useful:

Long bone	**Multiplication factor**
Humerus	5.2
Radius	6.6
Ulna	6.0
Femur	3.7
Tibia	4.3
Fibula	4.4

The exact multiplication factor varies for males, females and children and for various groups of population and should be used from proper database.

5.6 Identification of a person by Superimposition Technique

Identification of a person is not very difficult if the individual has been made to death or has committed suicide where not much of decomposition of the body or deliberate mutilation of the body has taken place. It becomes difficult if the skin or the fleshy portions of the body have disappeared and only the skeletal remains are left. It becomes still worse if some parts of the skeleton or even a single bone are left. On the basis of the stature and development of the bone, it is possible to determine the sex, age, race, height and massiveness of the individual. However, the skull is an exception because the bones of the face provide a clue to the physical features of the individual. Identification of a human dead body through has been made possible if the recent facial photograph of the individual is available for comparison.

Human Identification from the skull is generally done by (1) Reconstruction Method and by (2) Comparative Method. Reconstruction method suggests only possibilities and probabilities without certainty of as many criteria of individualization from the skull, viz., race, age, sex, stature etc. as possible.

Comparative Method gives a high degree of reliability and accuracy where the photographs and radiographs are used for comparison with the skull. Comparative method is being done either by 1) Metric measurements or by 2) Superimposition.
Evidential value of Superimposition Technique in the court of law is corroborative. Rare individual characteristics such as protuberance of the teeth or forehead etc. further help in arriving at a definite conclusion.

5.6.1 Metric Measurement

On the basis of the anthropometrical measurements on the photographs, it has been possible to judge whether the photograph of skull could be of the same individual whose identification photograph is available. There are some anthropometrical landmarks, which are marked on both the photographs, the distances measured and certain ratios calculated, assuming that there is least possibility of physical variation.

The following distances are recorded:

1. Nasion-subnasale distance
2. Nasion-prosthion distance
3. Nasion-gnathion distance
4. Glabella-subnasale distance
5. Glabella-prosthion distance
6. Glabella-gnathion distance
7. Zygion-zygion distance
8. Gonion-gonion distance
9. Alare-alare distance

Ratios of measurements are calculated for all possible combinations (i.e. vertical / horizontal measurements). Average variation of all the ratios along with the individual ratio variation are taken for reaching a conclusion. If the variation of ratio is below 0.06 it is taken towards similarity. This method is also useful in making a decision whether to proceed with the superimposition of skull or not.

5.6.2 Superimposition Technique

Conventional Method: Negatives of identification photograph and the skull are prepared. These two negatives when closely together gives superimposed positive prints. The technique is applied to see whether or not the skull of the individual in question belong to the same person whose identification photograph is available. For this purpose it is necessary to have a recent photograph with a front view and in certain exceptional cases where a slight

laterally tilted view have been taken up. If the negative of the identification photograph is not available it is first prepared and kept under the ground glass of the camera. The outlines of the face along with landmarks are marked on the ground glass. Then the skull is fixed on the tripod (stand) and placed in front of the camera and positioned so as to align it as accurately as possible with the outlines of the ground glass giving due allowance to the soft tissues covering the bones. In this position a negative of the skull is prepared. These two negatives of the skull and identification photograph are superimposed by taking full precautions that the points in one are coinciding with the other.

Following anthropometrical landmarks lines are marked all over comparison:

(1) Nasion,
(2) Prosthion,
(3) Gnathion,
(4) Gonion,
(5) Zygion,
(6) Vertical line (Mid sagittal plane),
(7) Horizontal line (line joining the two supraorbital ridges)

The two superimposed negatives are then photographed and a positive print is prepared. The superimposed photograph brings out the similarity or dissimilarity between skull and the photograph. The superimposition is correct, if the contour and the size of the skull accurately correspond to the fact of photograph giving full margins to the soft tissues of the face.

5.6.3 Interpupillary Distance

The magnification factor is obtained by measuring the interpupillary distance on the life size skull photograph, since the distance is constant for the normal adult individual (i.e. non squint persons) because both the pupils move simultaneously so that their interpupillary distance remains constant. The middle point of the orbit is determined by deciding both the orbits geometrically. The interpupillary distance can be adjusted and made to life size in questioned photographs and then superimposed.

5.6.4 Magnification Factor

It is possible to convert the questioned photograph into life size photograph also, if certain articles such as wearing apparel of the deceased or the chair or any article visible in the photograph which could be obtained from the

house/studio etc. If the shirt or saree is having stripped designs, the same can be measured for getting the required magnification. The distance between the two buttons or buttonholes or any such characteristics can help in reaching the life size magnification of the questioned photograph. After making the life size skull photograph, it is superimposed with the life size questioned photograph.

5.6.5 Video Superimposition Method

The video superimposition of the skull and face images is attempted only after the following two preparatory exercises are achieved satisfactorily.

(i) Preparation of the life size photograph of the suspected individual
(ii) Setting the skull in the same orientation of the head as seen in the photograph

It is possible to work out the life size face photograph from the articles such as shirt, spectacles, chair, sari, etc. seen along with the individual in the photograph. If the face of the dead person has to be taken out from a group photograph, measurement of the other living persons photographed along with dead person will be useful to work out the life size face photograph.

The life-size photograph thus prepared is fixed on the video stand and the skull is fixed on the specially made skull stand. If the skull has to be fixed in the same posture as the individual has posed, all the three factors viz., Flexion – extension, rotation and lateral flexion of the head as seen in the photograph are to be evaluated from the life size two-dimensional photograph.

Among the three factors, Flexion – extension is the major one for achieving success in employment of the superimposition techniques. A simple "tilt-measuring device" is also used for positioning the skull. Similarly the rotation and lateral flexion factors can be evaluated from the life-size photograph. The skull is then set in a specially made stand, which is capable of permitting the skull, all movements of the head.

The image of the skull along with the measuring scale is brought on to the monitor by a video camera and care is taken to see that the skull image in the monitor is exactly of 1:1 size. Similarly the life-size image of the face photograph is brought on to the same monitor by another video camera. Both the images are then superimposed one over the other with the help of the video vision mixer and the superimposed image is scrutinized for agreement of the several anthropometrical points and cephalofacial contours. To achieve superimposition it may be necessary to move the camera vertically or horizontally but at this stage the cameras are never to be adjusted to alter the size or angle of the skull and face images. In matching the cephalofacial

contours, modeling clay is applied on the skull at certain anthropometric points to indicate the flesh thickness that has to be considered.

The use of video vision mixer, which is an electronic special effects generator, makes it possible to scrutinize the facial view of skull and face juxtapositioned so as to have the skull on one half of the image and face on the other half. In fact it is possible to have any fractional combination of skull and face images.

The ESID not only helps to reduce the time taken for comparison but also eliminates the errors that could arise of the comparison is done manually using an X-ray reviewing illuminator. The technique can therefore be utilized for positive identification and exclusion as well.

ESID consists of two television cameras, a video vision mixer, a synchronizer, a videocassette recorder, a mixing monitor, two scrutiny monitors, a special skull stand and a photo stand.