DNA yield from Blood, hair, buccal cells, saliva and semen: comparison of methods for forensic analysis

ABSTRACT

In forensic research, blood and buccal swabs provide rich quantities of DNA. Present article presents the most probable biological samples as a source of evidence in forensic analyses. Here we present saliva, hair and semen samples, in addition, as a biological evidence too. Blood cells are composed of RBCs, WBCs and Platelets. Out of both corpuscles, WBCs are the one and only source of DNA as RBCs don't have DNA. From buccal swabs, scientists get adequate amount of DNA. Epithelial cells are the source in that case. Saliva has low cellular content and thus very low DNA but it has advantage of easy and simple collection from crime site. Hair follicle is another source of DNA which is the most probable type of evidence to be found at site and has the advantage of resistance to decay after death. Sperm in semen sample are the source of DNA, and culprit ID in cases sexual assaults. Hence semen samples can also be used for DNA isolation

INTRODUCTION

The introduction of forensics into the world of crime scene investigation has remarkably changed the concept of investigation. A wide variety of biological, chemical, physical and trace evidence are present on crime scene which can aid in conviction (Bandyopadhyay and Basu, 2015). Biological evidence at a crime scene is not confined to blood only rather it contains saliva, semen, sweat and hair as well. The diversity of biological evidence along with its power to make direct link with offender makes it potentially important evidence at crime scene (Norén *et al.*, 2013). Interestingly, there is another unique source of DNA available at crime scene i.e. the epithelial cells which are continuously shed from human body. Any evidence that has been collected from crime scene usually contains epithelial cells (Barash *et al.*, 2010).

Advancement in molecular biology has made it significantly easier to isolate DNA from any biological source. The adoption of various molecular biology techniques by forensic experts have enabled detection of minute quantities of DNA from evidence collected at crime scene (Budowle and Van Daal, 2009). The availability of DNA databases especially in USA has aided in early arrests of many offenders based on DNA evidence from crime scene (Budowle *et al.*, 2005).

The recent forensic approach to deal with DNA evidence involves initial detection of biological material at crime scene followed with packaging, sealing, labelling and transport to laboratory for further processing. Once in lab, the DNA is extracted, quantified and profile is generated by genotyping STR (short tandem repeats) and sex typing marker (Frumkin *et al.*, 2010).

Blood is one of the most frequently encountered sample or evidence at crime scenes. In the field of forensic investigation bloodstains at crime scene is a good source of DNA and is used for DNA typing to identify the culprit among suspects (Bremmer *et al.*, 2011).

BLOOD

A connective tissue in human body that has two major functions.

1. Transport
2. Defense

Respiration in which oxygen to cells and carbon dioxide to lungs is transported is main function performed by blood. It also transport amino acids, glucose and lipids of food to the cells and act as medium for the transportation of hormones that endocrine glands secrete into blood. Blood also has its role in waste removal through liver and kidney.

Role of a blood in defense of body is another important function performed by blood. White blood cells are the blood cells which have role in defense system and are produced in lymphatic tissues and bone marrow. Whenever there is an infection WBCs circulate in the whole body through blood stream and fight against that infection.

The liquid portion of blood is **Plasma** in which blood cells and cell fragments are suspended. If the whole blood sample is centrifuged, the red blood cells would settle at the tube bottom, on the top of layer of RBCs there would be **buffy coat** which consist of white blood cells and small layer of platelets. Above this would be liquid part of blood, plasma. **(Figure 1)** (Stuart H. James, 2005)

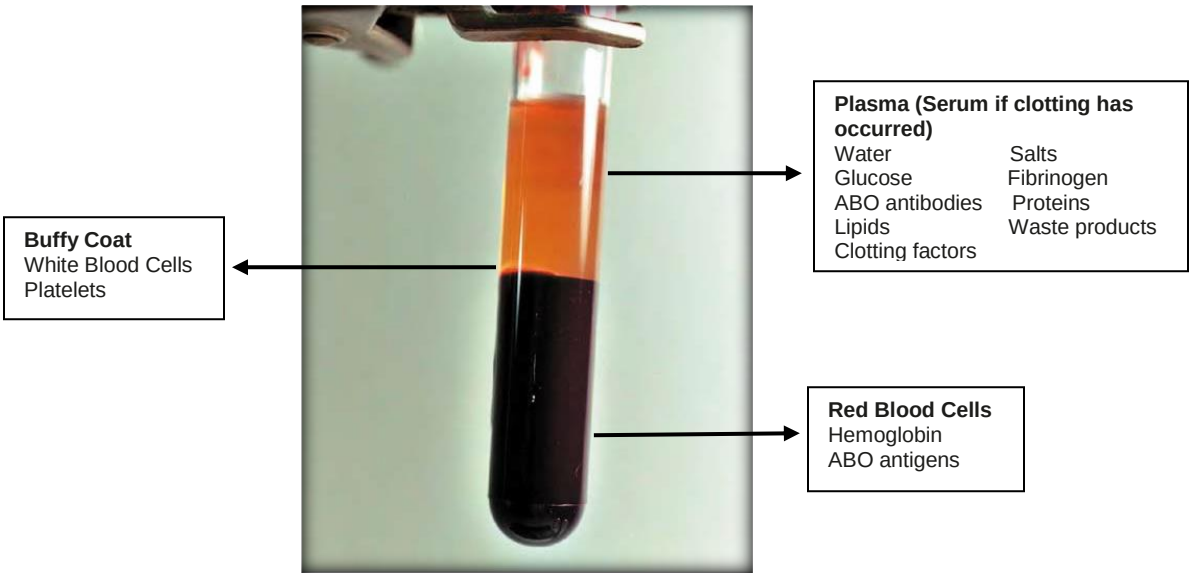


Figure 1: components of whole blood

There are three types of blood cells suspended in liquid plasma.

1. White blood cells (leukocytes)
2. Red blood cells (erythrocytes)
3. Platelets (thrombocytes)

Red blood cells and white blood cells are whole cells while platelets are cell fragments.

Erythrocytes:

Red blood cells are the most abundant among the major three blood cells. They are synthesized in bone marrow and their normal amount is **4.8-5.4 million** RBCs per µl of blood which makes 1 billion RBCs in 2-3 drops of blood (Stuart H. James, 2005). They are the donut shaped cells having thick rim and thin depressed center. They have diameter of about **7.5µm** and thickness of **2µm** at rim **(Figure 2).** During their development they lose all organelles so they are devoid of nucleus and have no DNA (Saladin, 2008).

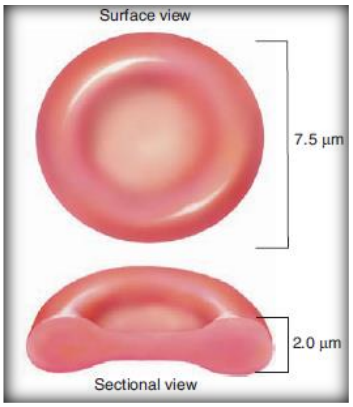


Figure 2: Shape of an erythrocyte

Hemoglobin, an oxygen carrying protein containing iron, is a major component of RBCs. The function of hemoglobin is to transport oxygen and carbon dioxide between tissue and cells and it makes up 97% of dry content of blood (Bremmer *et al.*, 2012).

Leukocytes:

White blood cells (leukocytes) are the cells found in smallest amount in blood approximately **5,000-10,000 WBCs** per µl of blood (Saladin, 2008). WBCs with different plasma proteins constitute the immune system. Immune system is a body defense system which recognize any particle that is foreign to body and destroy it. WBCs are colorless as they lack hemoglobin.

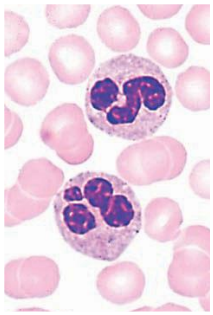
There are **5** different types of WBCs each having specific structure and function **(Table 1):**

- Lymphocytes
- Neutrophils
- Basophils
- Monocytes
- Eosinophils

On the basis of granules in the cytoplasm and shape of their nuclei these five types are divided into two classes:

- Polymorph nuclear granulocytes which have nucleus with many lobes and have granules in cytoplasm include Neutrophils, basophils and eosinophils.
- Mononuclear a granulocytes which have single and non-segmented nucleus with very few granules in cytoplasm include monocytes and lymphocytes (Sherwood).

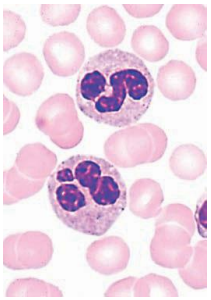
Table 1: Types of white blood cells and their properties

Types of WBCs and their properties
Basophils
Percent of WBCs Less than 0.5%–1% **Appearance*** large and irregular shaped nucleus with abundant and dark violet granules in cytoplasm (figure 3) **Functions** Release histamine, a vasodilator, which increases blood flow to a tissue Release heparin, an anticoagulant , which prevent clotting    *Basophils*

Neutrophils

Percent of WBCs

60%–70%



Neutrophils

Appearance*

Nucleus with 3–5 lobes

Small violet granules in cytoplasm (figure 4)

Functions

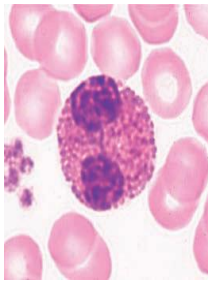
Bacterial phagocytosis

Secrete antimicrobial chemicals

Eosinophils

Percent of WBCs

2%–4%



Eosinophils

Appearance*

Nucleus with two large lobes linked by thin strand

Cytoplasm with large orange pink granules (figure 5)

Functions

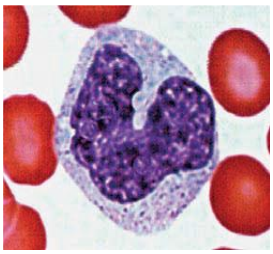
Phagocytosis of allergens, inflammatory chemicals and antigen–antibody complexes

Release enzymes that destroy parasites like worms

Monocytes

Percent of WBCs

3%–8%



Monocytes

Appearance*

Ovoid and kidney-shaped nucleus

Light violet cytoplasm with scarce, fine granules (figure 6)

Functions

Differentiate into macrophages when enter in tissue

Phagocytize debris of dead cells, dead neutrophils, and pathogens

Antigen presentation to trigger other immune cells

Lymphocytes

Percent of WBCs

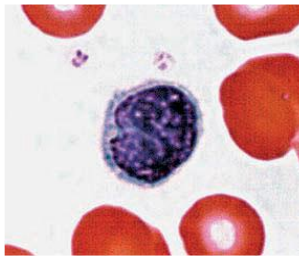
25%–33%

Appearance*[1]

Nucleus of dark violet color, ovoid and round

In small lymphocytes, nucleus occupy almost all of the cell and with small rim of light blue cytoplasm

In larger lymphocytes, cytoplasm is more abundant (figure 7)



Lymphocytes

Functions

Destroy cancer cells, cells infected with viruses, and foreign cells

Antigen presentation to activate other immune cells

Antibodies secretion

Immune memory response

Thrombocytes:

Platelets are third type of blood cells which have function in blood clotting. Actually platelets are not cells rather they are fragments of cells called megakaryocytes. Whenever there is a damage in

[1] Appearance refer to blood films dyed with Wright's stain.

blood vessel platelets form tangled web of insoluble fibrin molecules. Within a seconds after damage or bleeding this process of clotting starts (Bremmer *et al.*, 2012). After RBCs platelets are second most abundant blood cells and normal amount of platelets in blood is approx. **130,000-400,000 per µl** (Saladin, 2008).

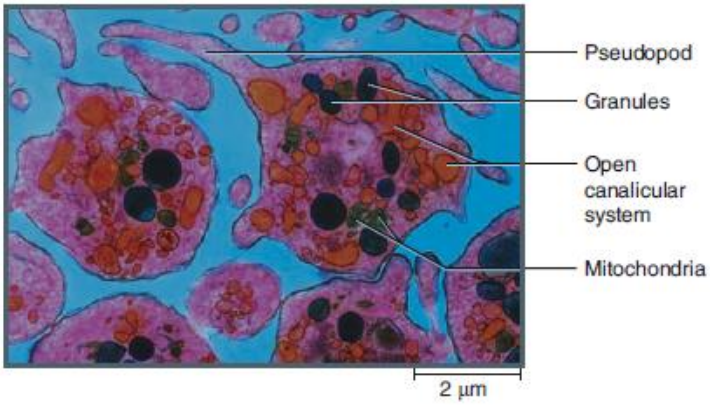


Figure 3 structure of platelets (TEM)

Plasma:

Liquid portion of blood which constitute **90%** of water and **10%** solutes including lipids, salts, glucose and proteins is Plasma. It is a medium of transport for hormones, electrolytes and nutrients to the cells and waste products out of the cells. In the absence of plasma blood cells would be unable to move throughout the body to carry out their function Components and composition of plasma is described in **Table 2 (Stuart H. James, 2005)**

Table 2: Composition of plasma

Composotion of plasma	
Water	90%
Solutes	10%
Plasma proteins	Approx. 7% ▪ Albumin ▪ Globulins ▪ Fibrinogen
Nutrients	Approx. 2% ▪ Lipids ▪ Glucose ▪ Vitamins ▪ Steroid hormones

	▪ Amino acids
Inorganic salts	Approx. 1% ▪ Sodium ▪ Potassium ▪ Calcium salt

The blood sample collected from crime scene is mostly in dry condition in the form of blood stains and there is a impact of different environmental conditions on it like humidity and temprature. These conditions along with small quantity of dry blood can reduce the DNA yeild in blood so the aim of this study is to find the DNA extraction method with best and maximum yeild. Below is a comparison of different protocols of DNA isolation from blood.

BUCCAL SWAB:

Among the various samples used in forensic analysis one is buccal swab that can be obtained rapidly and painlessly. With reference to storage, handling and transportation and when subjects are not willing to give blood buccal swabs can be preferred on blood samples. Moreover they are cost effective or economically feasible in terms of sampling, storage and transportation as no refrigeration is required for their transport.(Van Wieren-De Wijer *et al.*, 2009)

Buccal cell collection can be done by either mouthwash collection or by buccal swab. For buccal swab collection buccal swab is rubbed on cheeks of subject to collect buccal cells and buccal swabs are then allowed to dry and store. (Butler, 2011). For the collection of mouthwash mouth is vigorously rinsed with mouthwash or any solution with rubbing of tongue on teeth and oral mucosa and resulting mouthwash is collected in tube. (Aidar and Line, 2007)

Source of DNA in buccal swab are epithelial cells of cheeks. Oral mucosa is composed of 2 layers upper oral epithelium with underneath connective tissue called lamina propria. Both these layers are separated by basal membrane. In some regions of mouth oral mucosa is further attached to underneath structure by connective tissue called sub mucosa. (Moharamzadeh *et al.*, 2007) Lamina propria provides support and nourishment to the epithelium while the epithelium act as an obstacle for pathogens and foreign substances.

The epithelium of oral cavity is squamous epithelium which can be:

1. Keratinized epithelium
2. Non-keratinized epithelium

Regions of mouth which are associated with mechanical forces during mastication contain keratinized epithelium such as hard palate and gingiva. It has layer of dead cells at its surface. While the mouth floor with buccal area which need flexibility to allow swallowing, chewing and speech is composed of non-keratinized epithelium and it has nucleated and living cells at the surface. (Liu *et al.*, 2010)

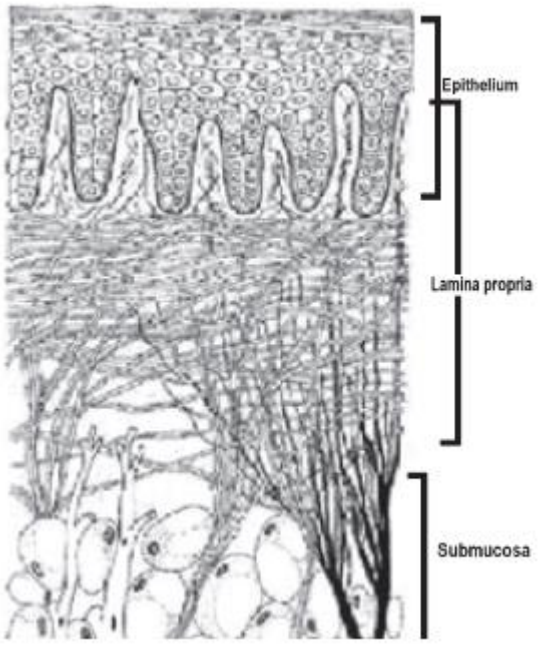


Figure 4 Structure of oral mucosa

Below is the comparison of different protocols for DNA isolation from buccal swabs and mouthwash samples.

SALIVA:

The clear biological fluid with slightly acidic pH (6-7) (Cuevas-Córdoba and Santiago-Garcia, 2014) in mouth is an exocrine solution which is 99% water and 1% different electrolytes and proteins, (De Almeida *et al.,* 2008) and coats our oral cavity is known as **SALIVA**. It is an aqueous mixture of lipids, proteins, mucins and other bioactive molecules. (Haward *et al.*, 2011) A normal human adult secrete 500-1500ml saliva per day, customary. (Cuevas-Córdoba and Santiago-Garcia, 2014) (Navazesh, 1993) Saliva has its role in maintenance of health of soft and hard tissues of mouth and supervising human oral microbial flora. Its proper flow in mouth is very crucial for many reasons. Reduced flow develops oral infections and dental caries.(Dodds *et al.*, 2015) There are organic and inorganic components of saliva. Inorganic ones are Calcium, magnesium, sodium, chloride, potassium, phosphate, ammonia, bicarbonate, while several immunoglobulins, several by-products of body secretions (uric acid, urea, creatinine), enzymes (mainly amylase), putrefaction products (cadaverine, lipids such as cholesterol and fatty acids and putrescine), albumin, lysozyme, mucins, lactoferrin, amylase, statherins, IgA, peroxidase, histatins, lipoproteins and glycoproteins (Cuevas-Córdoba and Santiago-Garcia, 2014) and more than 400 types of other such proteins are the key organic elements of saliva. (Lima *et al.*, 2010), (Hofman, 2001) Many of these protein components are proline rich (35-40%).

Other noticeable proteinaceous components including amazing hormones like thyroxine, catecholamine, growth hormones, cortisol, testosterone, triiodothyronine, progesterone, melatonin and prolactin. Also some cytokines for example IL-8 and enzymes and their inhibitors too. (Marcantoni, 1999) They all connected to the crucial oral functions attributed to saliva.(Humphrey and Williamson, 2001) (Buzalaf *et al.*, 2012) Salivary secretions may be mucous (rich in mucins), serous (rich in enzymes and ions) or mixed. (De Almeida *et al.*, 2008) **Figure 5** summarizes the salivary constituents.

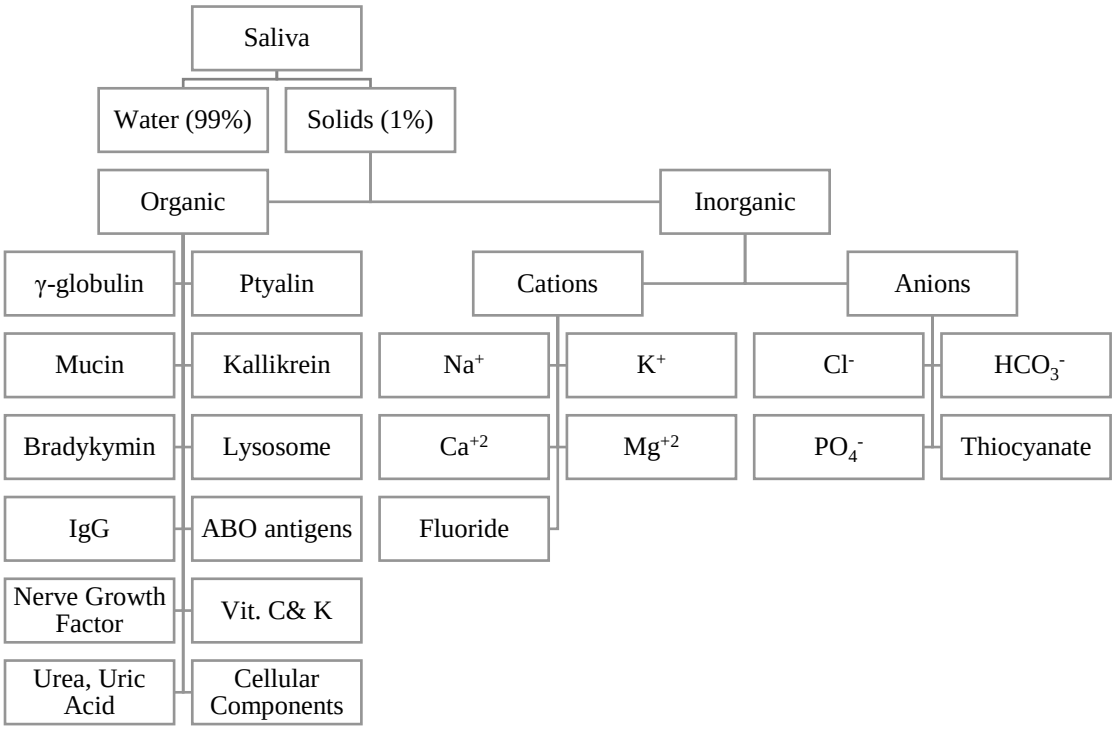


Figure 5 Summary of Salivary Constituent

Going in detailed study, three major salivary glands primarily (~90%) secretes the saliva. The submandibular gland (~65%), the parotid gland (~20%) and the sublingual gland (5-7%). The autonomic nervous system controls these glands. Distributed around the oral cavity are some minor salivary glands (lingual, buccal, labial and palatine), which produce the remaining saliva (<10%). (De Almeida *et al.*, 2008), (Spielmann and Wong, 2011), (Chicharro *et al.*, 1998) (Dodds *et al.*, 2015) (Buzalaf *et al.*, 2012). In addition to the organic and inorganic components of saliva, other components too are the part of it coming from upper airway, oropharynx, gastro intestinal reflux, food deposits, microorganisms, gingival sulcus fluid and blood-derived compounds. (Lima *et al.*, 2010), (Ouglas, 2002), (Dodds *et al.*, 2005), (Cuevas-Córdoba and Santiago-Garcia, 2014) In addition to all these, some components from organs too enters the saliva. These plasma components destines for salivary glands. Density range of saliva is from 1002 to 1012, with optimum pH 6.6.4 but variable depending upon blood carbon dioxide (CO_2) level. And can be in the range of 5.3 (low flow) to 7.8 (peak flow). (Chicharro *et al.*, 1998; Humphrey and Williamson, 2001).

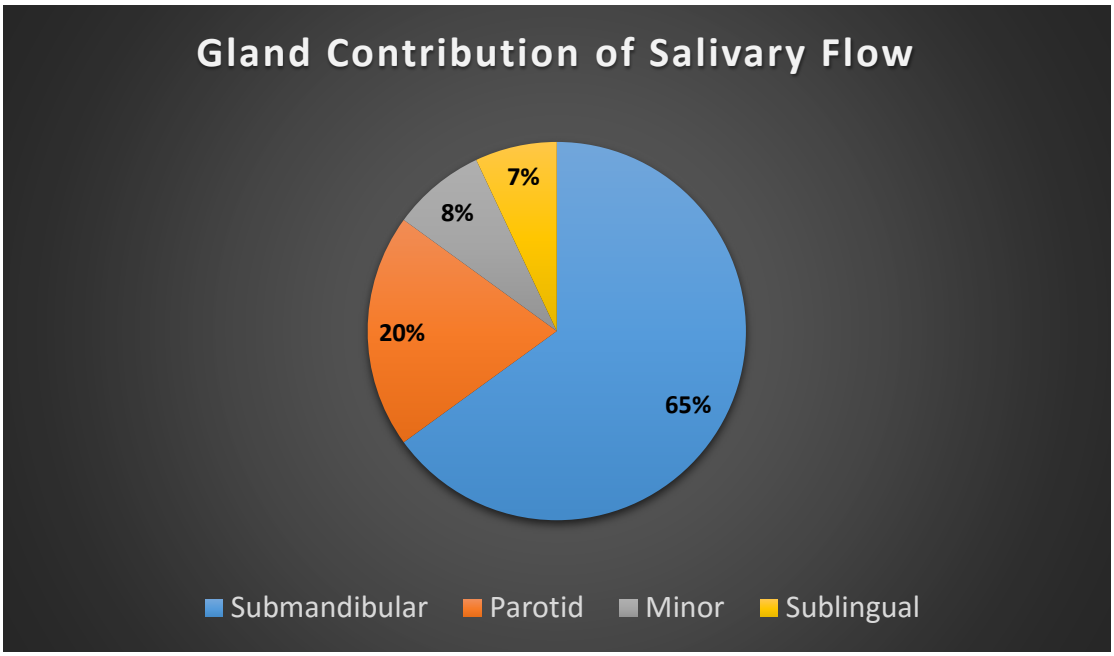


Figure 6 Gland Contribution of Salivary Flow *(De Almeida et al., 2008)*

The key functions of saliva are: (Dodds *et al.*, 2015), (Spielmann and Wong, 2011), (Lima *et al.*, 2010)

1. Oral tissues protection
2. Lubrication
3. Antimicrobial performance by degrading their cell wall using its enzymatic activity
4. It neutralizes acidity of plaque using bicarbonates preset in saliva
5. Phosphate and calcium remineralization on enamel. (Li *et al.*, 2014)
6. Facilitate eating, by helping in chewing and swallowing.
7. Breakdown the food in mouth by enzymes
8. Facilitates speech by lubricating oral tissues while they move.
9. Mastication
10. Antifungal and antiviral properties. **Figure 7**

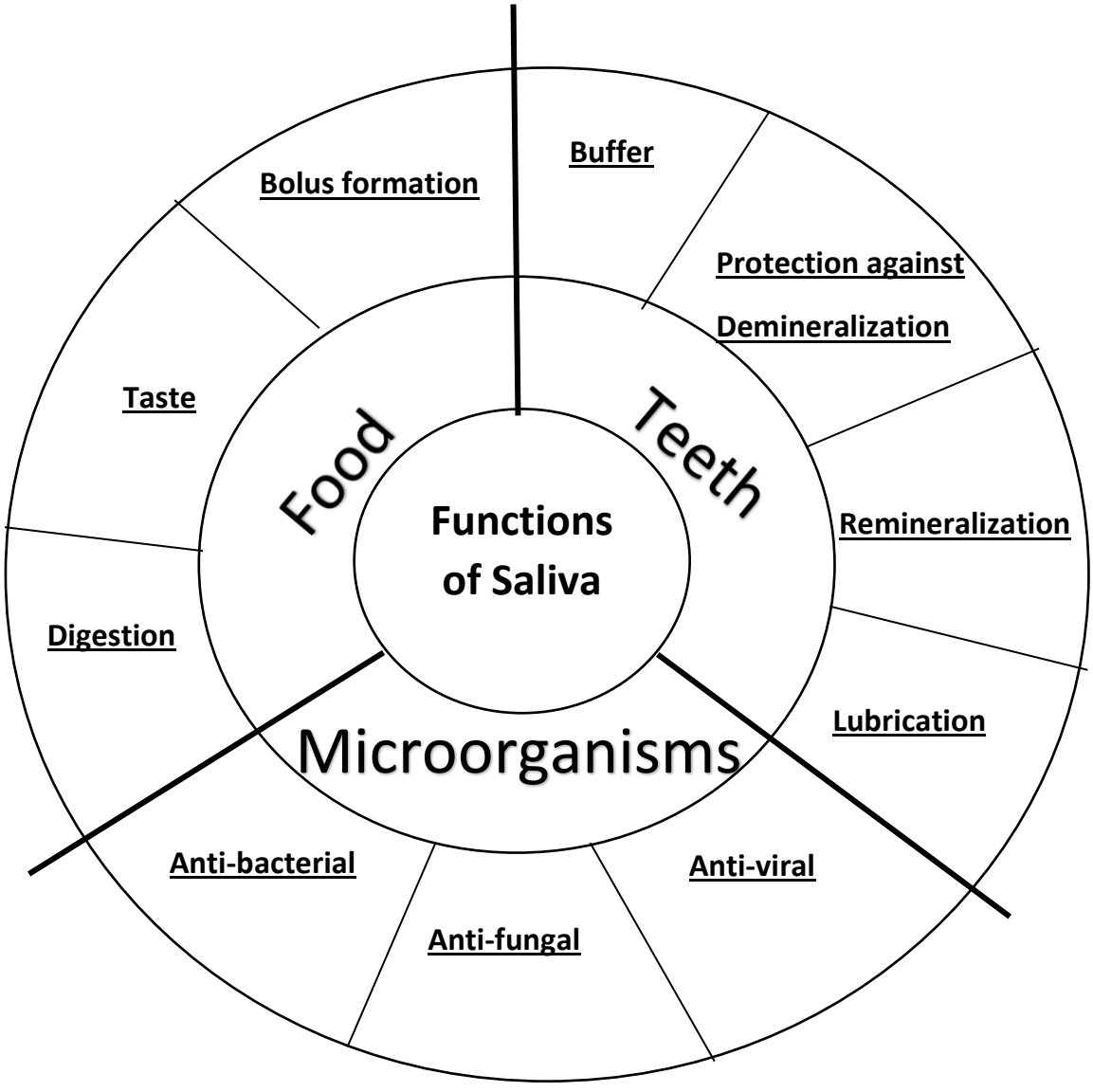


Figure 7 Essential Functions of Saliva (Amerongen and Veerman, 2002)

With reference to forensics investigation department and mainly to the area of its DNA serology unit, saliva has many advantages over other biological evidences found from crime scene, which includes its easy and simple collection and its convenient and cost-effective storage methods. (Lima *et al.*, 2010) It can be found at floors drooling down from a mouth of victim, and most probably from bite marks on objects like cigarettes, glass/cup rims, envelopes or from victim's body during fighting cases. In all such cases saliva traces can be recuperate easily. (Lima *et al.*, 2010)

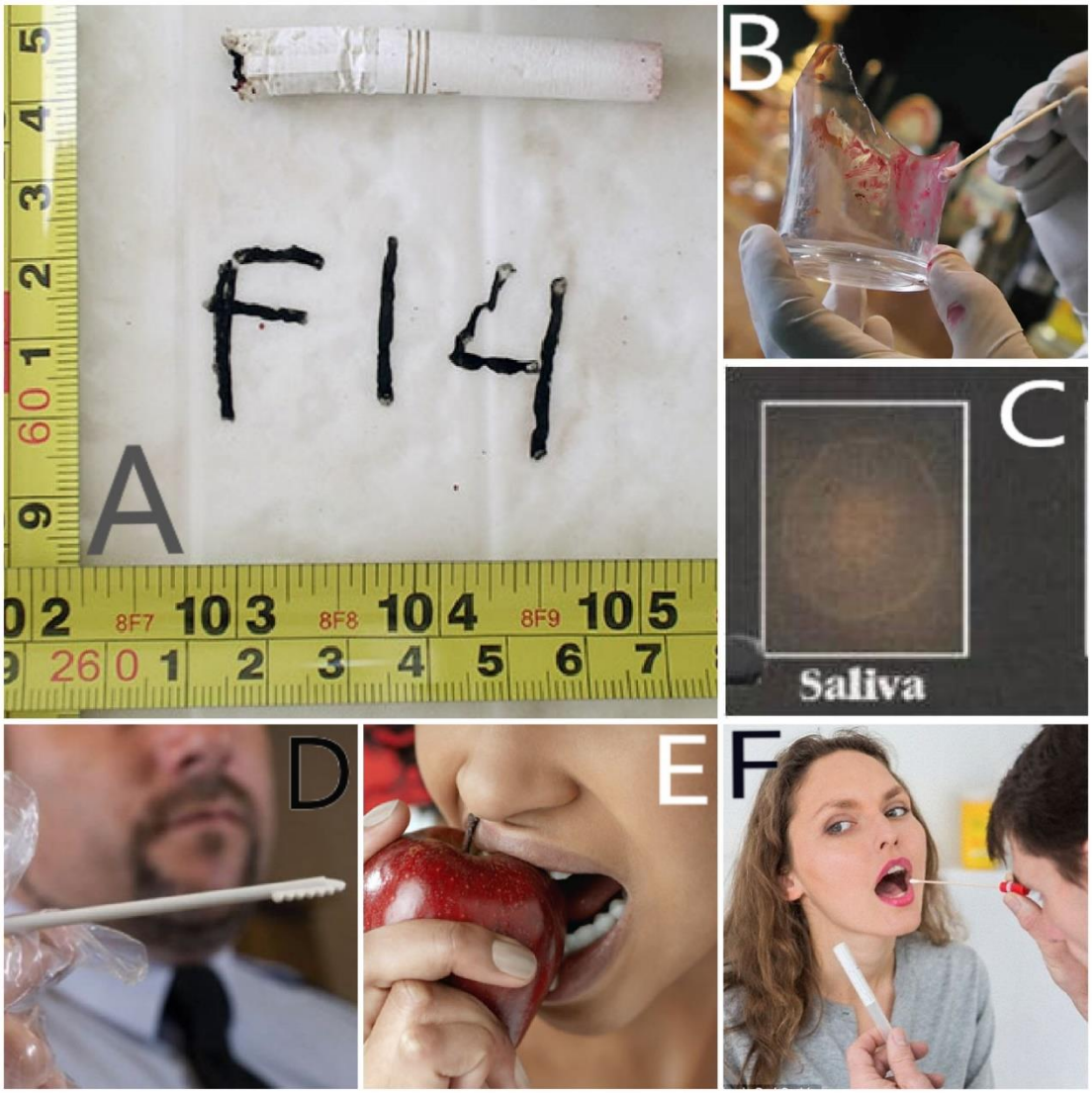


Figure 8 A) Saliva sample from used cigarette B) Lipstick and Saliva collection from broken glass C) Saliva sample from fabric D) A used toothbrush as a saliva sample E) Saliva on bitten apple (fruit) F) Saliva sampling from buccal cavity

Standardize DNA protocol serves the purpose usually and gives the high yield, but as at crime scenes saliva is in the dried conditions and has to be scraped off. Sometimes saliva is exposed to high or low temperatures and/or to different humidity levels. Some other times it is mixed with dirt or present on ground or on grass, if present on dirt it is contaminated with microbial DNA which can interrupt the interpretation. These conditions can low the yield of DNA from saliva and thus our aim is to compare the already documented and practiced standard DNA isolation protocols from saliva and compute the best one. And then artificially provide it the possible crime scene conditions, and check the yield from the chosen procedure. And then optimize to maximize the yield. DNA source from saliva can be T and B lymphocytes, PMNs, macrophages. And the saliva sample can have buccal epithelial cells in it too as a DNA source.

HAIR

Unique feature found only on mammals are, **hair** which is the derivative of epidermis. There are two defined and separate structural entities of hair: **Hair Shaft** and **Hair Follicle**. (Buffoli *et al.*, 2014) Three main layers which collectively make up hair shaft are: the outermost **cuticle**, which is made up of keratin, the central core **medulla,** made of shrunk and keratinized cell groups, and the inner sheath **cortex,** which is made up of macrofibrils, pigment granules and nuclear remnants**.** (**Figure 9, Figure 10**). Hair cuticle pattern can be easily seen through SEM (**Figure 11**). From the first single-layered epithelium of the scalp, originates the hair cuticle, which is surrounded by **epicuticle**. Epicuticle forms the outers covering of the hair and is semi permeable. But the major component of the hair is cortex. Cell composition of cortex is longitudinally arranged strongly keratinized cells. The pigment granules are found scattered all over the inner sheath and central core. (Lory *et al.*, 2011).

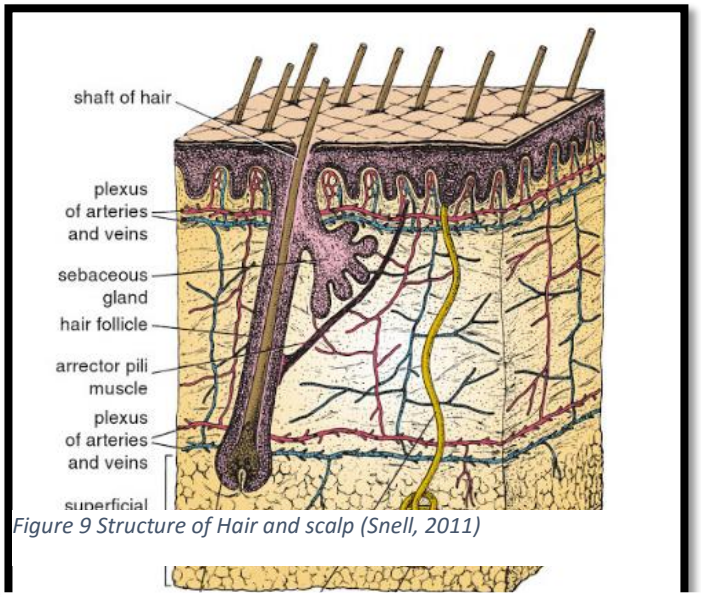


Figure 9 Structure of Hair and scalp (Snell, 2011)

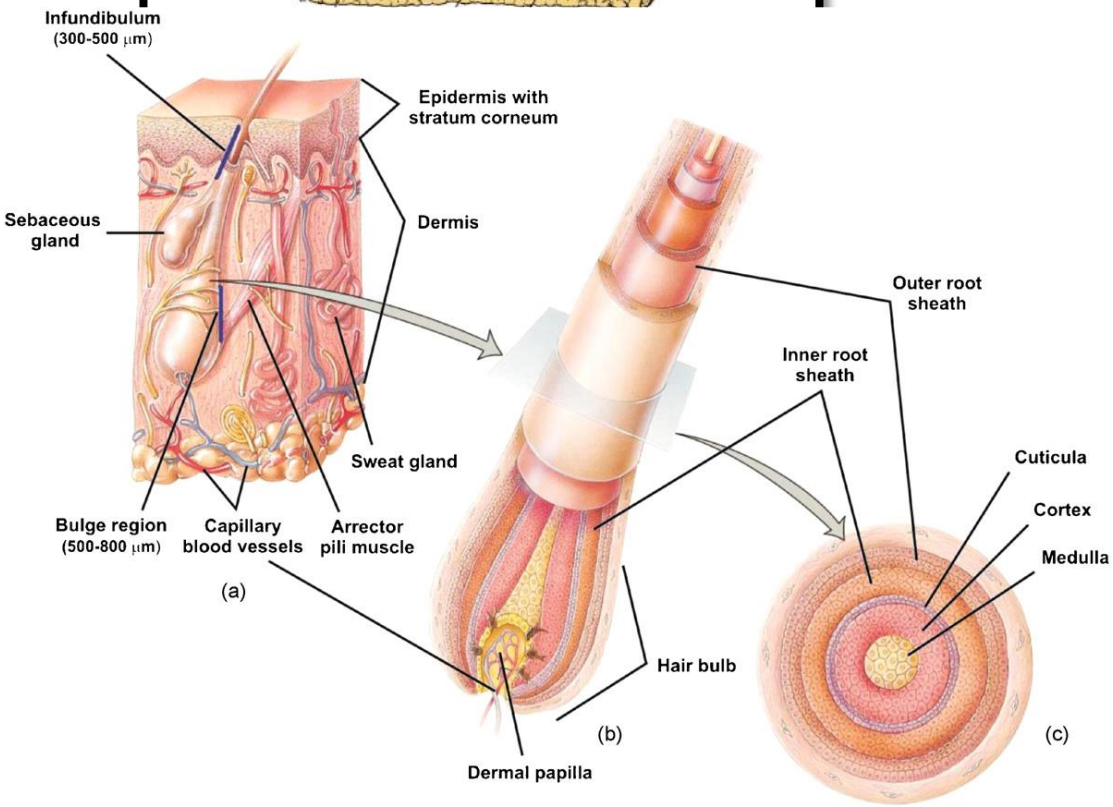


Figure 10 (a) Structure of the skin. (b) Structure of the hair follicle. (c) Cross-section of the hair (Wosicka and Cal, 2010) OR (Buffoli et al., 2014)

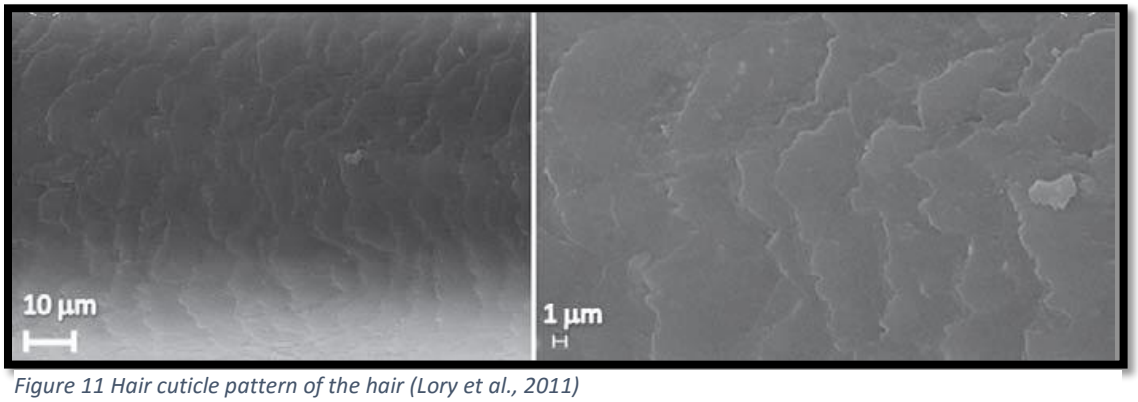


Figure 11 Hair cuticle pattern of the hair (Lory et al., 2011)

A category of hair shaft is called, **terminal hair** shaft. Either the hair on androgen-dependent regions (scalp, chest, beard, pubic region, axilla) or on androgen-independent regions (lashes, eyebrows) the hair shafts are thick (diameter = >60µm), long (>2cm), medullated and pigmented. (Wosicka and Cal, 2010), (Knorr *et al.*, 2009), (Vogt *et al.*, 2007). And these hair shafts protrude 3mm into the hypodermis. Another category of hair is called **vellus-hair**, the ones present on rest of the adult's body. These hair type are thinner (<30µm), shorter (diameter = <2cm) and usually unpigmented. They protrudes just 1mm into the dermis. (Wosicka and Cal, 2010), (Knorr *et al.*, 2009), (Meidan *et al.*, 2005), (Patzelt *et al.*, 2008), (Krause and Foitzik, 2006). The regions of skin which are denuded of hair include foot sole, palm, portions of genitalia and lips. (Wosicka and Cal, 2010), (Tobin, 2006), (Kertész *et al.*, 2007).

Hair follicle comes next. Hair follicles are actually a unit of 1 or 2 vellus-hair and 1 to 4 terminal hair. (Wosicka and Cal, 2010), (Meidan *et al.*, 2005), (Patzelt *et al.*, 2008). They are divide in to 4 portions: (**Figure 10**)

- Infundibulum (the part opening to the hair canal situated between skin surface and sebaceous gland)
- Isthmus (part between sebaceous gland and bulge area)
- Suprabulbar zone
- Hair bulb connected to the blood capillaries (**Figure 12**)

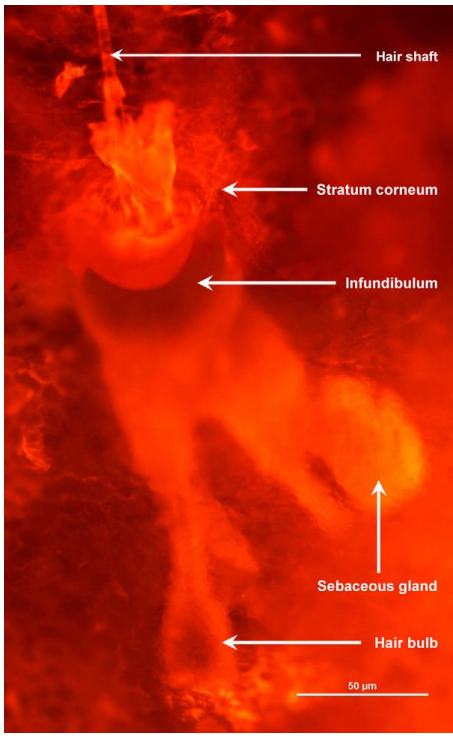


Figure 12 Fluorescence imaging of hair follicle

The bulge region is the attractive zone, which basically reconstitutes the hair follicle. It contains multipotent and highly proliferating stem cells. This bulb region controls pigmentation and hair growth. (Wosicka and Cal, 2010), (Knorr *et al.*, 2009), (Meidan *et al.*, 2005), (Kertész *et al.*, 2007). The hair cycle consists upon 5 phases out of which first 3 are major and 2 are recently described: (Meidan *et al.*, 2005), (Hordinsky, 2008)

- Anagen (growth phase – cells rapidly divides) (**Duration** 2-6 years)
- Catagen (involution – mitosis stops and cell dies) (**Duration** 2 weeks)
- Telogen (resting phase – just before hair sheds) (**Duration** 2-4 months)
- Exogen (telogen fibers start releasing)
- Kenogen (lag time between previous exogen and upcoming anogen)

So from outer side thin, flexible dead looking tubes, are a part of complete assembly, starting from follicle, down growing epithelia into dermis, and then to under-skin fat and finally enlarging into a hair bulb which encircles dermal papilla. It has some major functions for mammal's body. These includes, protection from mechanical injury and to social and sexual communication, protects scalp from cold and scorching weather effects, guards head and neck from hurting, eyebrows and

eye lashes prevent unwanted entries to eyes, aids thermoregulation and also has a sensory function. (Buffoli *et al.*, 2014)

Hair is preferred biological type of evidence at crime scene. This is due to two major reasons: firstly it is considered easily and commonly found sample at crime scene (Hue *et al.*, 2012) and secondly, due to its resistance from decay after death, towards frozen or dry environmental conditions, (Lory *et al.*, 2011). But in forensics investigation we need living cells for DNA isolation so it is important to preserve hair follicle of the hair recovered from crime scene. This is such because, hair follicle has self-regeneration ability as stem cells reside there due to which hair once shed, regrows. (Hordinsky, 2008). In forensics we found only 1 or 2 hair from crime scene and it is often difficult to extract good DNA yield from such a low number. Here is the comparison of different DNA isolation protocols from hair.

Semen

Semen is a fluid like material which is ejaculated during male sexual act. It is composed of two components:

1. Spermatozoa
2. Seminal plasma

Sperms or spermatozoa are formed in testes by the process of spermatogenesis. Sperm consist of tail and head with total length 5µ. Head contains the nucleus while tail is involved in movement of sperm(Harel *et al.*, 2015). Sperm constitute a small portion of the semen i-e from 1% to 5% of the total semen volume (Mortimer and Menkveld, 2001).Motility and fertility are the functions associated with spermatozoa. Spermatozoa contains many enzymes, co enzymes and intracellular nutrient reserve but also depends on extracellular nutrient reserve i-e seminal plasma which provide the natural medium for sperm cells(Mann, 1951).

Seminal plasma is a mixture of fluids which is produced by different glands and male sex organs including fluid from vas deferens (10%), 60% of fluid from seminal vesicle, 30% from prostate glands and small amounts from bulbourethral glands (Guyton, 1961). Besides providing medium for sperm this fluid provide protection from acidic environment of vagina to sperm (Organization, 1987)The fluid from different organs has different composition. Secretions from prostate is composed of citric acid, zinc, acid phosphatase, calcium, prostaglandins, inositol, magnesium and prostate specific antigen (p30 protein)(Fisher *et al.*, 2009). Contributions from seminal vesicle

consists of ascorbic acid and fructose while the source of of L-carnitine, and neutral alpha-glucosidase is epididymides (Owen and Katz, 2005). Seminal plasma is different from other body fluids due to the presence of free amino acids like glutamic acid (Mann and Lutwak-Mann, 1981). Other amino acids which are present in fluid are glycine and arginine (Brown-Woodman and White, 1974). Moreover seminal fluid is comprise of wide range of proteolytic and nucleolytic enzymes including nucleases, nucleosidases, nucleotide pyrophosphatases. Phosphatases includes acid 5'-nucleotidase and phosphatase while glycosides includes alpha-manosidase, beta-glucuronidase and beta-glycosidase (Mann and Lutwak-Mann, 1981).

Semen is among the important biological or physical evidence at crime scene use in cases like rape, sexual murders and sodomy etc. to confirm the testimony of victim. This evidence can be collected from clothes like bed sheets, pillow cover and carpet. Different body parts can also be a source of evidence including thigh, perineum, pubic hair and vaginal swabs (Harel *et al.*, 2015). Acid phosphatases and prostate specific antigen (p30) in a semen are used as a screening test for semen detection. Acid phosphatases are detected by change in their color after hydrolyzing the organic phosphates while p30 is detected through immunological assay (Tilstone *et al.*, 2006) Woods lamp is another technique for the detection of semen as it emits UV light which cause the semen to fluoresce (Santucci *et al.*, 1999).

But all of these are confirmatory tests which cannot recognize criminal for which DNA profiling is needed. After DNA extraction of sample collected at crime scene these profiles are then compared with profiles of suspects or search in databases to identify the culprit(Briody, 2002)

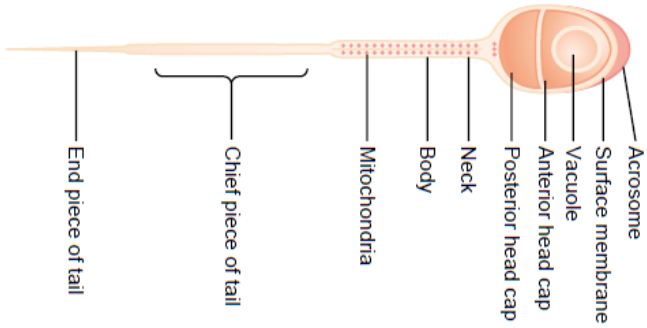


Figure 13 Structure of sperm(Guyton, 1961)

Comparison of different isolation protocols

DNA isolation from blood:

The **first** protocol was performed by **Debomoy K. Lahiri et al**. They use the salting out method of DNA extraction using sodium chloride solution. They also exclude the use of proteinase K which need prolong incubation. The protocol involve the collection of 5ml of blood in 15ml falcon tube then addition of 5ml of low salt TKM1 solution **(Table 3)**. Then add 125µl of nonidet P-40 (NP-40) for cell lysis. Mix the mixture by inverting the tube numerous time. Centrifuge the solution at 2200 RPM at room temprature for 10 minutes. After centrifugation decant the supernatent saving the pellet. Once again rinse the pellet with 5ml of TKM1 buffer and repeat the centrifugation step. After washing resuspend the pellet in 0.8ml high salt buffer TKM2 **(Table 4)**. Then add 50µl of SDS (10%) and mix the suspension using pipette and leave at 55˚C for 10 minutes. Now add 0.3ml of NaCl (6M) in the solution and mix it. The next step is centrifugation for 5 minutes at 12000 RPM. After centrifugation save the supernatent in a new tube and dispose of the pellet. Then add 2 volume of 100% ethanol in a supernatent (at room temperature). Mix the solution several time by inverting the tube to allow DNA to precipitate. Collect the precipitated DNA strands from mixture and transfer it into tube containing 1ml of 70% ethanol (ice cold). Then centrifuge at 12000 RPM for 5 minutes at 4˚C. Decant the supernatent and dry the pellet. After drying resuspend the pellet in 0.5ml of 10mM tris-HCl and 1mM EDTA (pH 8) and leave it at 65˚C for 15 minutes.

5ml of blood using this protocol give the yeild ranges from 130-160µg. This is a rapid method which give high yield in less time. This protocol give uncut or undegraded, high molecular weight and slow migrating DNA in agarose gel stained with ethidium bromide. The method give same results with the samples stored at 4˚C and even those at -70˚C. It exclude the use of many toxic reagents like phenol, isoamylalcohol and chloroform (Debomoy K.Lahiri and John I.Nurnberger, 1991).

Table 3: TKM1 recipe

Components	Molarity
Tris-HCl (pH 7.6)	10mM
KCl	10mM
$MgCl_2$	10mM
EDTA	2mM

Table 4: TKM2 recipe

Components	Molarity
Tris-HCl(pH 7.6)	10mM
KCl	10mM
$MgCl_2$	10mM
NaCl	0.4 M
EDTA	2mM

Souvik Ghatak et al. use different samples for DNA isolation including urine, buccal swab, hair and blood. They employ the modified conventional phenol chloroform method for DNA extraction from blood. The first step is lyses of RBCs using hypotonic buffer which contains ammonium chloride and ammonium bicarbonate. Add 3 volumes of RBCs lysis buffer in 50µl of blood. Invert and vortex the tube of 5 minutes and then centrifuge for 10 minutes at 2000 g. Now discard the supernatant after centrifugation and repeat the same step 2-3 times until supernatant get clear and pellet become clear white. After final washing completely decan the supernatant and suspend the pellet in 500µl of PBS, 10 µl of proteinase K (10mg/ml) and 400µl of cell lysis buffer **(Table 6).** Dissolve the pellet by vortexing and incubate at 56˚C (water bath) for 2 hours. Add equal volume of equilibrated phenol (pH 6), invert mix for 1 minute and centrifuge for 10 minutes at 10,000g at 4˚C. After centrifugation move the upper layer to a fresh tube and add 10µl of 10mg/ml RNase A followed by incubation at 37˚C for 30 minutes. Now add equal volume (1:1) of phenol and chloroform: isoamyl alcohol (24:1). Then invert mix the tube for 1 minute and

centrifuge at 10,000g for 10 minutes at 4˚C. Again transferr the supernatant to a new tube and add 2 volumes of absolute alcohol. Invert mix the tube and chill at -20˚C. Centrifuge at 10,000 g for 20 minutes at 4˚C. Decant the supernatant followed by addition of 250µl of 70% ethanol. Gently pat the pellet and centrifuge for 10 minutes at 10,000 RPM. Remove the supernatant and dry the pellet. Now suspend the pellet in 1X TE buffer or 50µl nuclease free water and keep the samples at −80°C or −20°C for storage.

The yield of the protocol calculated using gel electrophoresis and double-beam UV visible spectrophotometer was 57–94 ng/µl of blood and there was no effect of freezing temperature (-20˚C or -80˚C) on integrity of DNA **(Table 5)** (Ghatak *et al.*, 2013)

Table 5: Total DNA yield and yield after storage

Total DNA Yield and Quality by Immediate or Storage Processing of the Biological Samples

	Spectrophotometry																			
Biological sample	**Immediate processing**										**Storage processing**									
	Total DNA yield (ng/µl)					**A_{260}:A_{280}**					**Total DNA yield (ng/µl)**					**A_{260}:A_{280}**				
	R1	**R2**	**R3**	**R4**	**R5**	**R1**	**R2**	**R3**	**R4**	**R5**	**R1**	**R2**	**R3**	**R4**	**R5**	**R1**	**R2**	**R3**	**R4**	**R5**
Blood sample	86	74	92	57	94	1.82	1.86	1.77	1.82	1.76	79	91	83	82	71	1.89	1.65	1.81	1.77	1.81
Buccal swab sample	83	62	85	60	65	1.57	1.62	1.54	1.67	1.62	55	51	58	42	47	1.62	1.67	1.62	1.83	1.75
Hair sample	49	62	68	57	72	1.72	1.82	1.87	1.74	1.97	58	52	58	61	57	1.82	1.82	1.83	1.84	1.82
Urine sample	31	25	37	42	39	1.48	1.42	1.58	1.52	1.58	23	36	36	20	23	1.48	1.46	1.48	1.55	1.53

Table 6: cell Lysis buffer recipe (pH 7.5)

Components	**Molarity**
Tris-HCl	10mM
EDTA	10mM
NaCl	50mM
SDS	10%

The **third** protocol we studied was from **Higuchi, R.** 65-100µl of blood sample was dispensed in a tube having 20µl 10mM EDTA. 200µl of lysis buffer **(Table 7)** was added to each tube and was vortexed. Tubes were then centrifuged at 16000 g for 25 seconds. Supernatant was discarded then and same step was repeated until pellet become white. Pellet was again suspended in 100µl of PBND **(Table 8)** containing 60µg/ml proteinase K and incubated at 55˚C for 60 minutes. In the last to inactivate proteinase K samples were incubated at 97˚C for 10 minutes.(Higuchi, 1989)

Table 7: Cell lysis buffer recipe

Components	Molarity/Quantity
Sucrose	0.32M
Tris-HCl	10mM (pH 7.5)
$MgCl_2$	5mM
Triton X-100	1% v/v

Table 8: PBND (PCR buffer with non-ionic detergent) recipe

Components	Molarity/Quantity
KCl	50mM
Tris-HCl	10mM
$MgCl_2$	2.5mM
Gelatin	0.1mg/ml
Nonidet P-40	0.45%
Tween 20	0.45%
Proteinase K*	60µg/ml

*Add proteinase K just prior to use

The **fourth** protocol was proposed by **Laura Lee Boodram.** This is simple method which utilize the salting out method to precipitate proteins and there is no use of any organic solvent. To start with the protocol take a volume of blood and add 2 volumes of sterile and cold distilled water and 1 volume of buffer A **(Table 9)**. Now mix the solution by vortexing or inverting tube 6 to 8 times and leave it on ice for 2 to 3 minutes. Then centrifuge for 15 minutes at 3500 rpm at 4˚C. Dispose of supernatant and add 6ml of water and 2 ml of buffer A to resuspend pellet. Again centrifuge for 15 minutes at 3500 rpm at 4˚C. Repeat this step with every time discarding supernatant until pellet is white in color. After washing resuspend the pellet in 500µl of 10% SDS and 5 ml of Buffer B **(Table 10)**. Vortex for 30 to 60 seconds. Now add 50µl of Proteinase K (20mg/ml). Incubate at 55˚C for 2 hours in water bath. After 2 hours let the tube cool at room temperature or on ice for 2 to 3 minutes. Now add 4ml of NaCl solution (5.3M) and vortex for 15 seconds. Centrifuge for 15 to 20 minutes at 4500 rpm (4˚C). After centrifugation transfer supernatant to new tube and add equal volume of isopropanol (stored at 20˚C). Now invert tube few times to allow DNA to precipitate. Take out DNA with the help of wide bore tip from the solution and transfer to new tube. Then wash the DNA with 70% ethanol. After washing step dry the DNA by leaving it at 37˚C for 15-30 minutes. After drying add 300 to 400µl of Tris HCl (pH 8.5) and store DNA in refrigerated.

This method can give the yield upto 100-200ug of DNA from 4 to 8ml of frozen or fresh blood (Helms, 2002)

*Table 9: Buffer A (RBCs lysis buffer) recipe**

Components	**Molarity/Quantity**
Sucrose	0.32M
Tris-HCl	10mM
$MgCl_2$	5mM
Triton X-100	0.75 %

*pH 7.6

*Table 10: Buffer B (proteinase K buffer) recipe**

Components	Molarity/Quantity
Tris-HCl	20mM
Na_2EDTA	4mM
NaCl	100mM

*pH 7.4

All solutions should be sterilized. Buffer A should be sterilized before the addition of Triton X-100.

The **fifth** protocol is proposed by **Maxim V.Myakishev et al.** It is a method of DNA extraction using silica gel. The time they claim for the protocol is 15-20 minutes and it uses the strong reagent guanidine thiocyanate to lyse the cells followed by adsorption of DNA to glass based sorbents. To perform the protocol take 500µl of blood in an Eppendorf and add 1ml of **bind mix (Table 12)**. Now incubate the tube at room temperature for 3 minutes. Now centrifuge at 5000 g for 3 seconds to collect silica gel. Decant the supernatant and suspend the pellet in 1ml of **guanidine solution (Table 11)** and vortex. Again centrifuge at 5000 g to collect sorbent. Repeat this step of washing of silica gel using guanidine solution. Then use **propanol wash (Table 13)** for washing silica gel (twice) and once use absolute alcohol. Now remove ethanol and dry silica gel by heating under vacuum. Add 100µl of TE buffer to resuspend the silica gel and elute the DNA for 3 minutes at 65˚C. Again resuspend in TE buffer, spin for 10 seconds and collect the supernatant.
They claim the yield 40µg/ml of blood and size of DNA upto50kb.

Table 11: Guanidine solution recipe

Components	Quantity/molarity
GuSCN	6M
EDTA	20mM
Tris-HCl (pH 6.5)	10mM
Triton X-100	40g/l
DTT	10g/l

Table 12: Bind mix composition

Components	Quantity
Silica gel	4g
Guanidine solution	100ml

Table 13: Propanol wash composition

Components	Quantity/ Molarity
Isopropanol	25%
Ethanol	25%
NaCl	100mM
Tris-HCl (pH 8)	10mM

The **sixth** protocol was proposed by V. Iranpur M. and A. K. Esmailizadeh. In this protocol 500µl of blood was poured in eppendorf and 1000µl of RBCs lysis buffer **(Table 14)** was added. After shaking mixture was centrifuged at 7000rpm for 2 minutes. After centrifugation supernatant was removed and same step was performed two to three times to clean the pellet by removing hemoglobin. Then 400µl of cell lysis buffer **(Table 15)** was added to tube and pellet was dissolved in it by pipetting. After that 100µl of 5M NaCl and 600µl of chloroform was added. After vortexing mixture was centrifuged at 7000rpm for 2 minutes. 400µl of supernatant was transferred to new tube and 800µl of ethanol (cold) was added. After shaking mixture was vortexed. Then it was centrifuged at 12000rpm for 1 minute. Supernatant was removed and pellet was dried at room temperature. In the last 50µl of TE was added and DNA was stored at -20˚C or 4˚C.

According to authors the method give suitable yield of DNA from samples stored at 4˚C or -20˚C. The yield was 1-3µg from 500µl of blood that was stored at 4˚C for one year **(Figure 13)**.

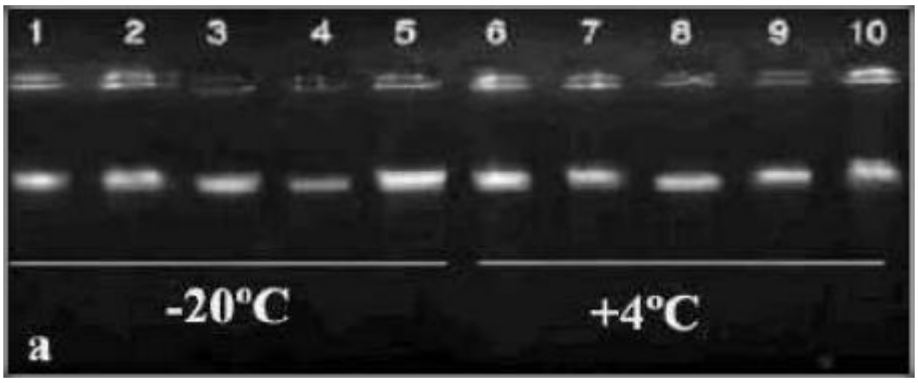


Figure 14 Genomic DNA extracted from sheep whole blood (lane 1-10), DNA extracted from blood stored at -20˚C and 4˚C for one year

Table 14: Red cell lysis buffer recipe

Components	**Molarity/quantity**
Tris-HCl (pH 7.6)	0.01M
Sucrose	320mM
$MgCl_2$	5mM
Triton X-100	1%

Table 15: Cell lysis buffer recipe

Components	**Quantity/Molarity**
Tris-HCl	0.01M
Sodium citrate	11.4mM
EDTA	1mM
SDS	1%

DNA isolation from Buccal Swabs:

First method of buccal swab DNA isolation we studied is proposed by **Marisi AIDAR and Sergio Roberto Peres LINE.** The protocol use salting out of proteins using ammonium acetate (8M) without use of any organic solvent. It is easy to perform and fast method with use of cheap chemicals. Talking about the procedures the first step is sample collection. For sample collection rinse your mouth with 5ml of sucrose solution 1 hour after brushing your teeth. Then rub your tongue on teeth and oral mucosa and collect mouthwash in 15ml tube. Now add 3ml of TNE solution (diluted in 66% of ethanol) **(Table 16).** Divide the mouthwash in four tubes and use only one tube for DNA extraction while leaving other tubes at room temperature for 2,15 and 30 days to check the integrity of DNA with the passage of time. For DNA isolation centrifuge the tube at room temperature at 3000rpm for 10 minutes and discard the supernatant. Now resuspend the cells in 1ml of TNE solution and centrifuge for 5 minutes at 2000rpm followed by supernatant decanting. Vortex the pellet for 5 seconds and add 1.3ml of lysis solution **(Table 17)** and 10µl of 20mg/ml proteinase K solution. Vortex the mixture at medium speed for 5 seconds and incubate for overnight at 55˚C. Take out 1.4ml of mixture after incubation and transfer it in 2ml micro centrifuge tube and add 500µl of solution containing 1mM EDTA and 8M ammonium acetate. Now vortex for 5 seconds followed by centrifugation for 10 minutes at 17000 g. transfer 900µl of supernatant in two separate tubes and add 540µl of isopropanol. Mix both tubes by gently inverting 20 times followed by centrifugation for 5 minutes at 17000 g. Decant supernatant and place the tubes on absorbent paper in inverted position for few minutes. Now add 2ml of 70% ethanol and invert the tubes to wash the pellet and centrifuge for 5 minutes at 17000 g. Pour off the supernatant and keep the tubes on absorbent paper. Then allow the pellet to dry for 45-60 minutes. Now to store the DNA resuspend the pellet in 100µl of TE buffer.

The yield of the protocol ranges from 5-93µg. Figure is showing the PCR products of DNA extracted using above procedure of all the samples including immediately processed sample and sample left for 2,15 and 30 days. (Aidar and Line, 2007)

Table 16: TNE solution recipe

Components	Molarity/ Quantity
Tris-HCl (pH 8)	17mM
NaCl	50mM
EDTA	7mM

Table 17: Lysis solution recipe

Components	Molarity/quantity
Tris (pH8)	10mM
SDS	0.5%
EDTA	5mM

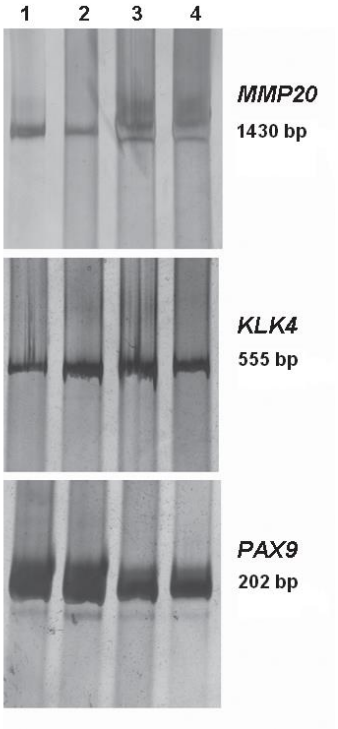


Figure 15 Gel electrophoresis displaying PCR products of DNA obtained from mouthwash. 1) DNA isolated immediately after sampling. 2) DNA isolated 2 days after sampling. 3) DNA isolated 15 days after sampling. 4) DNA isolated 30 days after sampling

The **second** protocol we studied was from **B. Freeman et al**. the protocol provide the enough DNA for several thousand PCR reactions. Concentration of DNA was effective even in samples stored for more than 4 years. First of all for sample collection scrap the cotton swab inside your mouth and place infused end of cotton swab in 15ml of tube and add 2.5ml of collection buffer **(Table 18).** Then centrifuge the tubes containing cotton swabs at 300 g for 4 minutes to collect the liquid at the base. Now incubate the tubes for 2 hours at 65˚C in water bath followed by centrifugation for 4 minutes at 300 g. Remove the liquid from 15ml tube and transfer in new 50ml tube and again centtrifuge at 300 g for 4 minutes. Remove and discard the cotton buds and transfer the supernatant to fresh tube followed by addition of 300µl of organic deproteinization reagent (ODPR). Shake the tube vigorously for 30 seconds and centrifuge at 5000g for 25 minutes. Again transfer the supernatant to a fresh tube and add 300µl of ODPR. Repeat the mixing and centrifugation step and transfer supernatant to new tube. Add 1.2ml of isopropyl-alcohol at room temperature. Mix gently for few minutes and centrifuge for 25 minutes at 5000g. Remove the supernatant and resuspend the pellet in 2ml of ice cold 70% ethanol for 10 minutes. Then centrifuge for 10 minutes at 5000g. Discard the supernatant and leave the tubes for 30 minutes for drying at room temperature. Then resuspend the DNA in 400µl of TE buffer (10mM Tris-HCl ,1mM EDTA, pH 8.0)(Freeman *et al.*, 2003)

Table 18: Collection buffer recipe

Components	Quantity/ Molarity
NaCl	100mM
Tris-HCl (pH 8)	10mM
EDTA	10mM
Proteinase K	0.2mg/ml
SDS	0.5% w/v

The **third** protocol we studied was used by **Amy H. Walker et al**. for DNA isolation from buccal swabs. Sampling was done by rubbing the buccal swab inside the mouth. After sample collection remove the handle of buccal swab and place the brush of swab in 1.5ml tube. Then add 600µl of 50mM NaOH and vortex for 10 minutes. Now heat the tube for 10 minutes at 95˚C. Next step is addition of 120µl of 1M Tris (pH 8) followed by the removal of brush from tube and store the resulting solution containing DNA at 4˚C.

They reported the mean DNA concentration as 3.7ng/µl and 2.7µg per buccal swab with the total volume of sample 720µl.(Walker *et al.*, 1999)

The **fourth** protocol is proposed by **Ellen M. Heath et al.** they used the mouth wash sample as a source of buccal epithelial cells. Rather than using conventional boiling lysis method and phenol-chloroform extraction they modify or optimize the PUREGENE DNA isolation kit with the addition of proteinase K and glycogen solution. The first step in the protocol is sample collection for which they ask donors to vigorously swish 10 ml of mouthwash 20 times and then spit the mouthwash back in a 50ml tube. Now for DNA extraction centrifuge the tube for 10 minutes at 2000g. Decant the supernatant leaving 100µl of liquid behind. To re suspend the pellet in a residual liquid vortex for 5 seconds. Then add 3ml of cell lysis solution and vortex for 5 seconds. Now add 15µl of 20mg/ml of proteinase K and incubate at 55˚C for 1 hour. After incubation period add 15µl of RNase A solution and invert the tube 25 times for mixing. Now leave the tube at 37˚C for 15 minutes. After 15 minutes allow the sample to cool by leaving it at room temperature and add 1ml of protein precipitation solution. To mix the solution vortex for 20 seconds at high speed and keep the tube on ice for next 10 minutes. Next step is centrifugation for 10 minutes at 2000g. At this step tight green pellet should be formed. Remove the supernatant in a new tube and add 3ml of 100% isopropanol and 5µl of 20mg/ml glycogen solution. Gently invert the tube 50 times for mixing and leave the tube for 5 minutes at room temperature followed by centrifugation for 10 minutes at 2000g. Discard the supernatant and leave the tube on clean absorbent paper for few minutes. Now to wash the DNA pellet add 3ml of 70% ethanol and centrifuge for 3 minutes at 2000g. Remove the supernatant and place the tube on absorbent paper for 10-15 minutes to dry the pellet. Now add DNA hydration solution (100 µl) and incubate for 1 hour at 65˚C or at room temperature for overnight. After incubation briefly vortex and centrifuge the sample to store the DNA at 2˚C to 8˚C.

The results of their experiment show the increase in DNA yield with the addition of proteinase K and glycogen solution. **Figure 15** is showing the effect of these additions on DNA yield. They claim high quality DNA yield of 12 to 16 µg per donor using this optimized protocol.(Heath *et al.*, 2001)

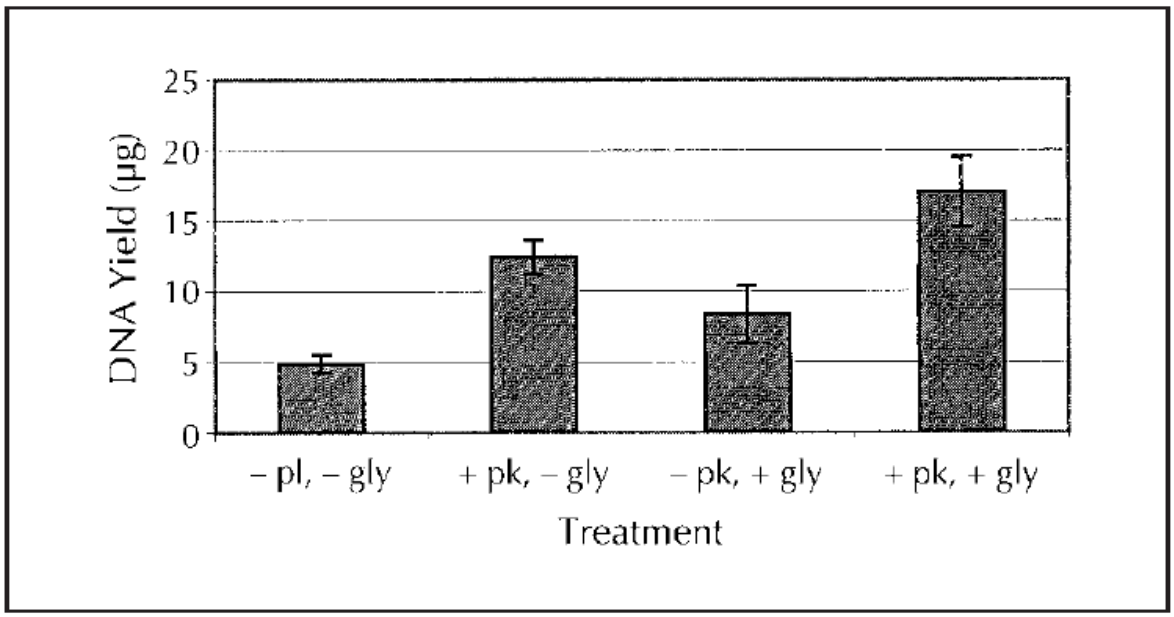


Figure 16 Graph showing effect of proteinase K and glycogen solution treatment on DNA yield

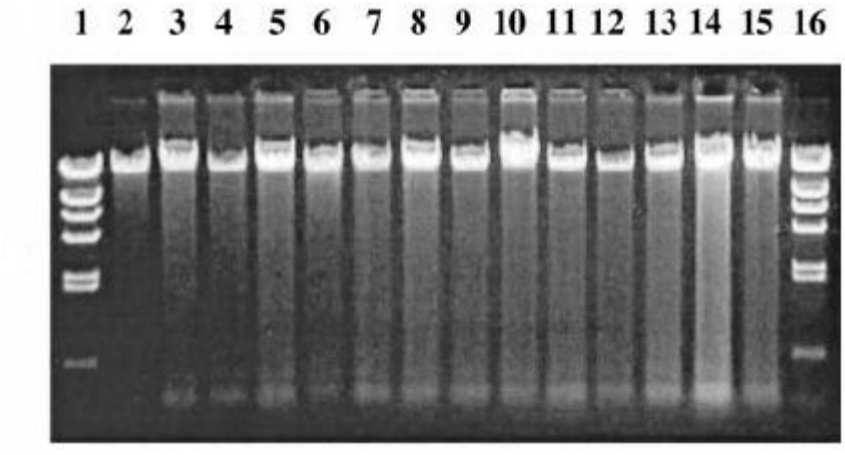


Figure 17 Gel electrophoresis showing the DNA bands of 14 mouthwash samples, Lane 1 and 16 are DNA markers while lane 2-15 are sample DNA

The **fifth** protocol is proposed by **D. B. M. A. van Wieren-de Wijer et al.** They send three buccal swabs with three 15 ml tubes to the donors. The tubes were containing 2ml of buffer (1880µl STE **(Table 19)**, 100µl of SDS (10%) and 20µl of proteinase K (10mg/ml). Donors were asked to rub their cheek with swab in morning, afternoon and evening. The swabs were collected in each of 3 tubes containing buffer. After arrival of samples in laboratory the concentration of proteinase K in samples was increased to 0.2mg/ml followed by incubation at 65˚C for 2 hours. After lysis buccal swabs were centrifuged for 60 sec at 1000rpm by placing them in syringe cover inside 50ml tube. Remaining buffer from original tube was also poured in new 50ml tube. Then 0.2 volumes of pottasium acetate was added and samples were incubated on ice for 15 minutes. Then choloroform isoamyle alcohol (24:1) was added and mixed for 30 minutes. The tubes were then centrifuged at 3000rpm for 15 minutes. After centrifugation aqueous phase was trnasferred to new 50ml tube. Then 2 volumes of absolute alcohol was added followed by centrifugation at 3000rpm for 10 minutes. Samples were then washed with 70% ethanol twise and stored in 200µl of TE at -30˚C.

They claim the total DNA yield of 0.08 to 1078.0 µg using above protocol. There was a difference of yield from male and females. Yield from male samples was 58.7µg (median) and 44.2µg from that of females.(Van Wieren-De Wijer *et al.*, 2009)

Table 19: STE recipe

Componenets	Quantity
NaCl	100mM
EDTA	10mM
Tris	10mM

DNA isolation from saliva:

The **first** protocol we followed was, from **'NC DNA Day'** (Day) It used the everyday life ingredients to get the saliva DNA out in 5 minutes. It starts with 1 ml of saliva and uses couple drops of dish washing soap as a detergent to lyse the cell membrane. Then add few drops of contact lens solution to break down the protein contaminants and separate them from the DNA. Then add a pinch of soapy saliva and invert mix the mixture, gently. This aggregates the dissolve DNA out of the solution. Then add 5-6ml of isopropyl alcohol and gently mix the tube. This will help DNA precipitate. Transfer your DNA using a stick or centrifuge it and resuspend the obtained DNA in nuclease free water. At this point, DNA is ready to be further analyzed by a forensics scientist. This protocol claims the enough DNA yield (Day).

The **second** protocol was proposed by (Akbar *et al.*, 2015) in journal of life sciences which is modified protocol for DNA isolation by (Ralser *et al.*, 2006). Properly rinse mouth for one and a half minutes, and then brush the teeth. Then wash the mouth with 3-5ml of 5% sucrose for about 3 minutes and then expectorate into falcon tube and can be stored at -20°C if to be processed later. Only 1ml of saliva is processed in an Eppendorf, to which add 100µl of Lysis Solution (**Table 20**) and mix well. Incubate the sample for one and a half hour at 56°C. Add 600µl of (1:1) phenol: chloroform and leave at room temperature for 5 minutes. Now centrifuge for 10 minutes at 10,000 rpm and carefully transfer the upper layer to fresh Eppendorf. Add equal amount of isopropanol and incubate at 20°C for 20 minutes. Repeat the centrifugation step at 10,000 rpm for 15 minutes and discard the resulting supernatent, followed by washing the pellet with 70% ethanol. Re-centrifuge the Eppendorf for 5 more mins at 8,000 rpm and ethanol in the supernatent was discarded. Air-dry the pellet and add 30µl of double distilled water and incubate at 56°C for 10 minutes. The yield of DNA was analyzed by Gel Electrophoresis. This protocol claims the excellent quality yield, without degradation suitable for PCR and downstream processing. (**Figure 17**)

Table 20 Recipe of Lysis Solution

Components	**Amount**
Lysis Buffer	2ml
Proteinase K	10µl
Marcaptoethanol	3µl

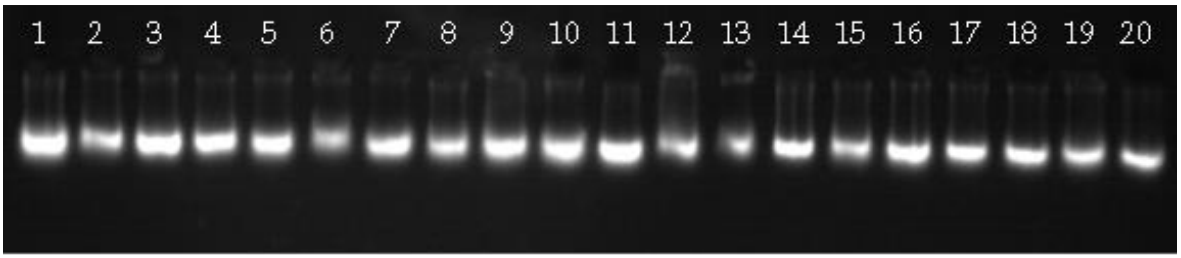


Figure 18 Gel documentation image of genomic DNA extracted from Human saliva. (Akbar et al., 2015)

The **third** protocol was performed by (Anzai-Kanto *et al.*, 2005) from the saliva deposited on skin due to the bite marks or some other reasons, including assault or homicide. This method was very helpful in forensics identification. Saliva was collected with the distance of half an hour to eating or drinking into a sterile plastic container and 1.5ml of it was stored in cryotubes at -20°C for further use. It is estimated that a bite mark leaves approx. 300µl of saliva, but to create artificial crime scene, 250µl of saliva was deposited on the researchers' arm and then after waiting for 10 minutes dried saliva was recovered using double swab technique. After extracting DNA from both the sources, DNA profiles were compared. Initial preparatory steps were different for both the types of samples. 1.5ml stored saliva samples were centrifuged for 10 minutes at 3,000 rpm and discard the supernatent and at the other side, the cotton part of swabs were disposed in 1.5ml propylene tube and hydrated with sterile water. Now both the samples were digested with 700µl of Lysis buffer (**Table 21**) with 35µl of (20mg/ml) Proteinase K. Phenol-chloroform method was

then used for DNA isolation which was precipitated and washed with ethanol. DNA isolated from both saliva and skin deposition were finally resuspended in 100µl and 30µl TE (**Table 22**), respectively. This protocol claims average DNA yield from saliva sample between 58.9 ± 43.2µg and average DNA yield from skin deposited saliva between 4.2 ± 0.9µg. (**Table 23**)

Table 21 Recipe of Lysis Buffer

Components	**Amount**
Tris	10mM (pH 8)
EDTA	10mM (pH 8)
NaCl	0.1M
SDS	2%

Table 23 Recipe of Low TE

Components	**Amount**
Tris-HCl	10mM (pH 8)
EDTA	1mM (pH 8)

Table 22 DNA yield for saliva samples (1-20) and skin-deposited saliva samples (A-E)

Samples / STR	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	A	B	C	D	E	Cont.
D3S1358	16	15	-	16	15	17	-	18	-	16	15	14	16	15	16	16	17	17	16	15	-	17	-	-	-	15
	17	17	-	18	16	18	-	18	-	18	15	16	18	16	17	18	17	18	17	15	-	18	-	-	-	16
D5S818	8	9	-	10	10	11	9	10	9	10	11	9	9	8	9	7	8	7	8	8	-	-	-	-	-	9
	9	10	-	11	11	12	10	10	11	11	11	10	9	10	10	8	10	8	9	10	-	-	-	-	-	10
D7S820	10	10	10	10	10	10	-	9	10	8	10	11	10	9	6	11	10	10	11	11	-	-	-	-	-	10
	11	11	13	11	12	11	-	12	11	10	11	11	12	10	9	12	12	10	12	12	-	-	-	-	-	12
D13S317	9	13	10	10	13	14	15	14	14	11	12	10	12	12	10	15	10	14	11	11	-	-	-	-	-	13
	10	13	10	11	15	14	15	15	14	13	12	12	14	15	14	15	14	14	13	13	-	-	-	-	-	15
D16S539	8	11	10	9	11	11	-	11	5	11	9	9	11	9	9	11	10	12	10	11	-	11	-	-	-	11
	12	13	11	12	12	13	-	13	11	11	13	11	12	12	10	13	13	12	11	11	-	13	-	-	-	11
D18S51	10,2	16	9	10,2	9	13	10	10	18	9	12	-	12	9	12	11	14	9	10,2	11	-	-	-	-	-	10
	15	23	9	15	14	23	24	23	22	11	12	-	12	9	12	11	14	11	14	11	-	-	-	-	-	17
D19S253	21	21	23	23	21	17	9	23	9	9	9	9	9	21	9	21	11	13	9	21	-	17	9	-	-	21
	21	21	23	23	21	19	21	23	21	19	23	9	17	21	23	21	25	19	23	23	-	19	21	-	-	21
D21S11	31	31	29	29	30	29	28	28	30	28	29	28	28	30	28	29	28	29	28	28	-	-	-	-	-	27
	33,2	31	31	30	30	30	31	32,2	30	30	30	31	28	30	30	30	28	29	32	32	-	-	-	-	-	30
F13A01	7	5	4	4	3	7	6	4	-	5	-	-	-	6	8	4	6	6	7	7	-	-	-	-	-	6
	7	7	4	4	7	7	6	4	-	5	-	-	-	6	8	4	6	6	7	7	-	-	-	-	-	6
F13B	10	8	9	9	8	9	9	6	9	8	8	6	8	9	9	9	8	9	8	8	-	-	-	-	-	9
	10	9	9	10	10	9	9	10	9	9	10	9	10	9	9	9	10	9	8	10	-	-	-	-	-	9
FES/ FPS	11	11	12	12	11	10	12	11	-	11	11	10	12	11	11	-	11	10	11	10	-	10	-	-	-	10
	13	12	13	12	11	10	13	12	-	11	11	13	12	11	13	-	11	11	11	12	-	10	-	-	-	13
FGA	23	20	20	18	20	20	19	20	22	19	20	23	22	19	19	20	19	19	21	22	-	-	-	-	-	22
	24	24	22	22	24	21	19	24	23	24	22	26	23	25	27	22	19	19	22	25	-	-	-	-	-	25
SE – 33	A14	A12	-	A12	A19	A15	A14	A19	A19	A17	A16	A13	A21	A9	A12	A17	A16	-	A18	A8	A19	A15	A14	-	-	A15
	A7	A5	-	A9	A8	A12	A8	A18	A12	A8	A4	A4	A9	A9	A12	A8	A5	-	A17	A8	A8	A12	A8	-	-	A10
TH01	6	-	6	8	8	6	6	7	8	9	8	6	6	9	9	8	6	6	8	6	-	6	6	-	6	5
	6	-	7	9,3	9	8	9	9	9	9	9	8	7	9	9	8	8	6	9	7	-	8	9	-	7	6
vWA	16	15	13	17	15	13	16	16	16	15	15	15	13	14	-	14	15	14	13	13	-	-	-	-	-	13
	16	15	18	17	16	15	16	16	17	16	18	18	16	16	-	15	15	16	16	15	-	-	-	-	-	13
Quant/DNA (µg)	25.7	27.8	31.4	67.9	40.3	156	12.8	39.6	16.0	16.1	90.8	93.5	151	91.7	83.9	26.7	25.9	56.2	95.2	29.9	3.0	4.8	3.4	5.4	4.2	63.3

(-) allele not found. Quant: quantification.

The **fourth** protocol was proposed by, Biometrica, THE BIOSTABILITY COMPANY, using organic extraction method. 350µl of saliva aliquot was processed in this protocol. The first step was **cell lysis and protein digestion** for which 163µl of Lysis Buffer (**Table 21**) and 8.2µl of Proteinase K was added to the sample. Incubate at 56°C in a water bath for 10 minutes and give it a short spin. For the second step of **organic extraction**, an equal volume of buffered phenol:chloroform:isoamylalcohol was added and sample was vortexed for few seconds and then centrifuged at 12,000 rpm for 2 minutes (at 4°C). The upper aqueous layer was transferred to fresh tube and equal volume of chloroform was added to it. Vortex and centrifugation step was repeated and upper layer was again transferred to a fresh tube, followed by the third step of **DNA precipitation.** 35µl 3M Sodium Acetate and 700µl of 1bsolute ethanol was added to the transferred layer. The sample was now vortexed briefly and then left at -80°C for an hour or at -20°C for overnight, to precipitate the DNA. Centrifugation was done at 12,000 rpm at 4°C for 20 minutes and the supernatent was very carefully decanted, avoiding the loss of DNA pellet. **DNA washing** was done in subsequent step, for which 1ml of 70% ethanol serves the purpose. Then sample was centrifuged again at 12,000 rpm at 4°C for 10 minutes and very carefully aspirate the

supernatent and dry the pellet for 10 minutes at room temperature. The last step was **DNA hydration,** for which 100µl of TE buffer was added and dried DNA pellet was dissolved.

The **fifth** protocol was DNA isolation method from saliva present on envelope. (Withrow *et al.*, 2003). The envelopes were opened with the help of steam and sterile forceps and the epithelial cells which were attached to glue was transferred to a sterile, moistened swab. Swab was continuously kept rubbing till all the visible glue was absorbed to the swab. Repeat the same procedure with a new swab on the opposite face of envelope to ensure the complete collection. The cotton part of the swab were inserted into a sterile microcentrifuge tube and they were incubated in 300µl of stain extraction buffer (**Table 24**) and 4µl of Proteinase K (10mM/ml) at 56°C or 24 hours. Add 300µl of Phenol:Chloroform:Isoamylalcohol. Now the extracted DNA was poured in 10 concentrator unit and its washing was done by adding 200µl of TE^{-4} Buffer (**Table 25**) and centrifuging it at 2500 X g for 10 minutes. The extract obtain contained purified DNA which was eluted from the membrane within the concentrator by adding 30µl of TE^{-4} Buffer. Invert the tube in microcentrifuge tube by up and down movements and centrifuging that tube at 2500 X g for 5 minutes. This protocol claims the DNA yield of 3.6 ng ± 90 ng.

Table 24 Recipe of Stained Extraction Buffer

Components	**Amount**
Tris	10mM
NaCl	100mM
DTT	39mM
EDTA	10mM
SDS	2%

Table 25 Recipe of TE Buffer

Components	**Amount**
Tris-HCl	10mM
EDTA	0.1mM

The **sixth** protocol we studied was by (Lucky and Baig, 2013) where person first rinsed their oral cavity for a minute with distilled water and then expectorated in 50ml tube. 10ml of saliva was used for DNA extraction which was first centrifuged at 4,000 rpm at room temperature for 10 minutes. Remove the supernatent carefully so that pellet do not get dislodged (leave 200µl behind if needed). 500µl of Lysis Buffer (**Table 26**) was added to the remaining pellet. The sample was then vortexed for 30 seconds and then given an incubation at 60°C for 30 minutes in water bath. Now sonicated the tubes and then given a short spin centrifugation. Then 500µl of isopropanol and 200µl of 3M Sodium Acetate were added and 2 minutes incubation at room temperature was given. Vortexed the tubes for 30 more seconds and then spun at 12,500 rpm for 15 minutes. Supernatent was decanted and for washing, 1ml of 70% ethanol was added to the pellet. Vortex well for 30 seconds and then spun again at 12,500 rpm for 5 minutes. Discard the supernatent carefully leaving behind the pellet, undisturbed. Place the tubes upside down on filter paper till the pellet got dried and then finally eluted in 50µl of TE buffer. According to this procedure, average DNA yield from oral rinse/saliva was 15.684 µg/ml ± 10.50 µg/ml (mean concentration = 14.5 µg/ml). 250 samples were processed where highest yield obtained was 145 µg/ml and lowest yield obtained was 4.72 µg/ml. (**Figure 18**)

Table 26 Recipe of Lysis Buffer

Components	**Amount**
SDS	50g
NaCl	8.8g
Sodium Citrate	4.4g
EDTA	0.3g
Final volume of 1 litre	

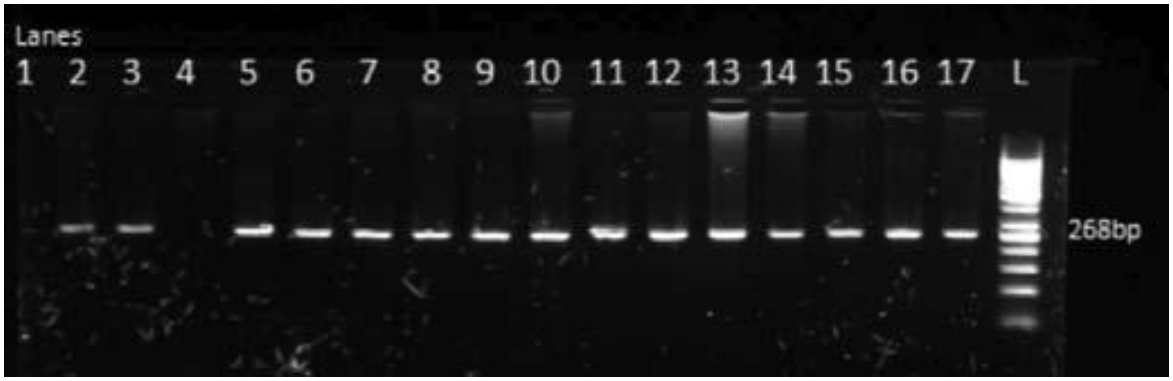


Figure 19 Lane 2-3: shows beta-globin amplified product (268 bp) of delayed oral rinse extraction, Lane 5-15: shows beta-globin amplified product (268 bp) of fresh sample.

DNA isolation from Hair:

The **first** protocol is the simplest one claimed by (Campbell *et al.*, 1997) which says to pluck a hair from roots and cut off its shaft up to maximum possible length leaving behind the follicle (approx.. 5mm). Now incubate it at 55°C for 60 minutes in the presence of 100µl of extraction buffer (**Table 27**) which contains 0.6µl (10mg/ml) of Proteinase K (6µg of Proteinase K) and then incubate at 95°C for 10 more minutes. Finally cool them at room temperature. This protocol claims 100 ng per 15 µl (6.67 ng per µl) of the extract obtained.

Table 27 Recipe of Extraction Buffer

Components	**Amount**	**Concentration**
KCl	1.86g	50mM
Tris	0.61g	10mM
$MgCl_2 \cdot 6H_2O$	0.25g	2.5mM
Gelatin	0.05g	0.1mg/ml
NP-40	2.25ml	0.45%
Tween 20	2.25ml	0.45%
pH 8.3. Water to **500ml** and autoclave. Store at **4°C**		

The **second** protocol was cited from (Ghatak *et al.*, 2013) in which hair sample was taken in an eppendorf, to which 500µl of digestion buffer (**Table 28**), 40µl of 1M DTT, 240mM of Sodium Acetate (pH 5.2) and 15µl of Proteinase K (10mg/ml) was added. After vortexing and incubating eppendorf at 56°C for 2 hours, the tubes were vortexed again and 40µl of 1M DTT and 15µl of Proteinase K (10mg/ml) was again added to it. Gently mixed and incubated at 60°C for 2 more hours until hair dissolves completely. Now equal volume of phenol:chloroform:isoamylalcohol was added and gently inverted the tubes for a few minutes to mix well. Then centrifugation at 10,000 g at 4°C was done for 10 minutes. 10µl of (10mg/ml) Rnase A was then added to a new tube in which upper layer of previous step is transferred and then incubated at 37°C for half an hour. Equal volume of (24:1) chloroform:isoamylalcohol was then added and centrifugation step was repeated with same parameters. Supernatent was then discarded and pellet was washed using

250µl of 70% ethanol. Pellet was tapped gently and then centrifuged at 10,000 rpm for 10 minutes. Finally, supernatent was decanted and when the pellet was dried in a laminar flow, it was dissolved in 50µl of 1X TE and stored at -20°C or -80°C.

Table 28 Recipe of Digestion Buffer

Components	**Amount**
Tris-HCl	10mM
EDTA	10mM
NaCl	50mM
SDS	20%
pH 7.5	

The **third** protocol referred her was proposed by (Matsuda *et al.*, 1997) in which 6cm long scalp hair were plucked and were washed with 1% tween 20, distilled water and ethanol in a sequence. Now cut the hair shaft leaving 1cm length from root area and both hair shaft and root were processed for DNA extraction. **Hair shafts** of 5cm length were fragmentize and kept in an eppendorf. 320µl of TNE buffer (**Table 29**), 40µl of 0.4M DTT, 40µl of 10% SDS and 15µl of Proteinase K (10mg/ml) was added to the hair fragments and incubated for 8 hours at 55°C. After, add 20µl of 0.4M DTT, 20µl of 10% SDS, and 5µl of Proteinase K (10mg/ml) and then incubated again for 3hours under same conditions. DNA extraction was performed thrice by TE saturated phenol (pH 8), once with phenol:chloroform (1:1), once with chloroform and finally with ether. Microcon 100 ultrafiltration was used for purification and concentration of the DNA from upper aqueous layer obtained in above step. Same protocol was followed by 3mm long **hair root.** Add TE buffer to the DNA solution obtained to make up the volume up to 50µl.

Table 29 Recipe of TNE buffer

Components	**Amount**
Tris-HCl	10mM (pH 8)
EDTA	10mM (pH 8)
NaCl	100mM

The **fourth** protocol was reported by (Nozawa *et al.*, 1999) where hair were plucked and washed in sterile water and then rinsed with absolute ethanol. 1cm from root portion was cut and the left over 40m portion of shaft was used for DNA isolation. This hair was divided into 4 equal parts, each was fragmentized and kept in an eppendorf. 1.22 ml digestion buffer (**Table 30**) was added and the tubes were left for digestion at 37°C overnight. And then DNA was extracted once with TE saturated phenol, twice with phenol:chloroform, once with chloroform. Purification of DNA extracted was proceed using CTAB method where ultrafiltration was done by Centricon 30 to desalt the above obtained aqueous phase and then precipitation was done with 0.02M NaCl and 0.2% CTAB. The precipitated DNA was re-dissolved in 300µl of 1.2M NaCl 9mix with 10µg of yeast tRNA and re-precipitated with 2.5 volumes of ethanol. Finally 10µl of TE buffer was use for dissolution and storage of precipitated DNA. According to this protocol 2ng/µl of DNA is obtained.

Table 30 Recipe of Digestion Buffer

Components	**Amount**
Tris-HCl	8mM (pH 8)
EDTA	1.6mM (pH 8)
NaCl	8mM
SDS	1%
DTT	50mM
Proteinase K	0.8mg/ml

Fifth cited protocol is by (Goossens *et al.*, 1998) in which one, three and 10 hair were proceeded together. Cut the 1cm portion of hair from root hair end place it in 400µl of 5% chelex-100 suspension and incubated for 5-6 hours at 56°C and then further incubated in water boiling bath for 8 minutes. Protocol claims presence of DNA in this extract.

DNA Isolation from Semen

First protocol was proposed by (Hossain *et al.*, 1997) in which, sperm pellet of every 30 X 10^6-1.2 X 10^7 spermatozoa count, was lysed in 5ml of lysis buffer (**Table 31**) for 3-4 hours at 55°C. Then 2x isopropanol was added to the lysate and tube was gently inverted. The DNA pellet which

was separated as a ball was taken out and was washed in 70% ethanol and then was dissolved in TE buffer. This protocol gives 189±23 mg of DNA with 260/280 ratio of 1.58±0.09.

Table 31 Recipe of Lysis buffer

Components	**Amount**
Guanidinium	6mM
Sodium Citrate	30mM (pH 7)
B-Marcaptoethanol	0.3mM
Sarkosyl	0.5%
Proteinase K	0.20mg/ml

Second protocol was proposed in the book **Spermatogenesis Methods and Protocols** according to which frozen semen was washed in 10 volumes of sperm wash buffer (**Table 32)** in a falcon tube, centrifuged at 750 X g for 10 minutes and discard the supernatent. 10ml of sperm wash buffer was added again and pellet was vortexed for thorough mixing and the steps were repeated. Careful about disturbing the pellet. Pellet was vortexed and 3m of extraction buffer (**Table 33**) was added and tube was inverted gently to mix the constituents. Tube was incubated at 56°C in water bath for 2 hours with frequent inversion mixing while incubation for up to 3 times. After getting on room temperature, 2.4ml of isopropanol was added and mixed until DNA strands were seen DNA was spooled out into a 2ml eppendorf containing 2ml of 0.1M sodium citrate dissolved in 10% ethanol. Tube was left at room temperature for ½ hour with periodic tube inversions. Buffer was carefully aspirated and washing was repeated once more. DNA pellet was twice washed in 1ml of 70% ethanol by invert mixing the tubes several times. Tube was inverted to drain all the ethanol and tube was dries for 10 minutes or until the tube is completely dried. Resuspend the DNA into 400-600µl of 10mM Tris-Cl. Tube was kept in water bath for 65°C for about an hour until all the DNA is dispersed in the solution form by tapping the tube 2-3 times (Carrell and Aston, 2013). This protocol promise 80% of DNA yield.

Table 32 Recipe of Sperm Wash Buffer

Components	Amount
EDTA	10mM (pH 8)
NaCl	150mM

Table 33 Recipe of Extraction Buffer

Components	Amount
Guanidine Thiocyanate	4.24mM (pH 8)
NaCl	100mM
Sarkosyl	1%
DTT	150mM
Proteinase K	20mg/ml
H_2O	240µl

Third protocol was proposed by (Alvarez *et al.*, 2004) according to which swab or stain was place into the tube and 500µl of extraction buffer (**Table 34**) was added and incubated at 56°C for 60 minutes. The swab or stained cloth was separated from the extraction buffer and was placed in the bucket which was then placed inside the same tube containing the extract. The tube was centrifuged at 16,000 X g for 5 minutes and bucket and cloth or swab remnants were dispose of. Then 50µl of 2M sodium citrate and 600µl of, 5:1, phenol chloroform was added, tubes were vortexed and placed at 4°C to separate aqueous phase from organic phase. Tubes were again centrifuged at same speed for 20 minutes. The aqueous phase was transferred to a new tube and 500µl of chilled absolute ethanol was added to precipitate DNA. Tube was kept for 1 hour or overnight and then spun at same speed for 15 minutes. The supernatent was carefully decanted and invisible pellet was washed with 1ml 70% ethanol by centrifuging at 16,000 X g for 5 minutes. Tube was properly dried and pellet was rehydrated in 50-100µl of TE buffer at 56°C for 45 minutes and stored. Approximately 1100ng/50µl is the yield.

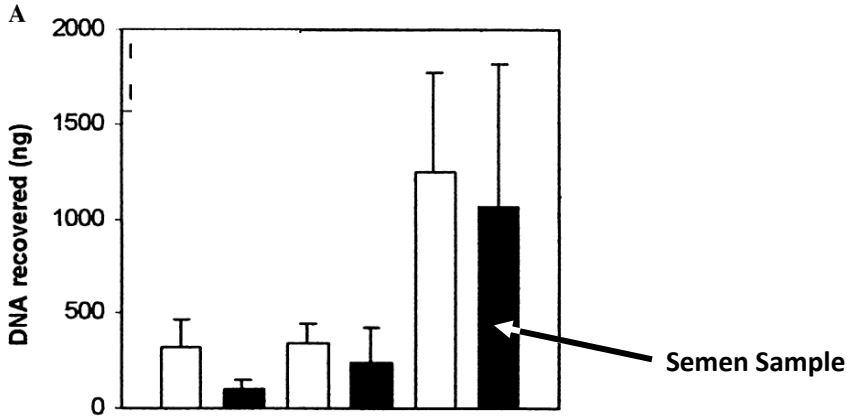


Figure 19 DNA recovery from semen

Table 34 Recipe of Extraction Buffer

Components	**Amount**
Tris-HCl	10mM (pH 8)
NaCl	0.1 M
EDTA	10mM (pH 8.0)
SDS	70mM
DTT	40mM
Proteinase K	0.65mg/ml

Fourth protocol was proposed by (Alvarez *et al.*, 2004) where semen sample was lysed overnight in lysis buffer (**Table 35**). Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added to the lysate and mixture was centrifuged at 16,000 X g for 15 minutes for phase separation. The aqueous phase was transferred to a separate tube and same procedure was followed as was done for third protocol. The yield is approximately 1250ng/50µl DNA.

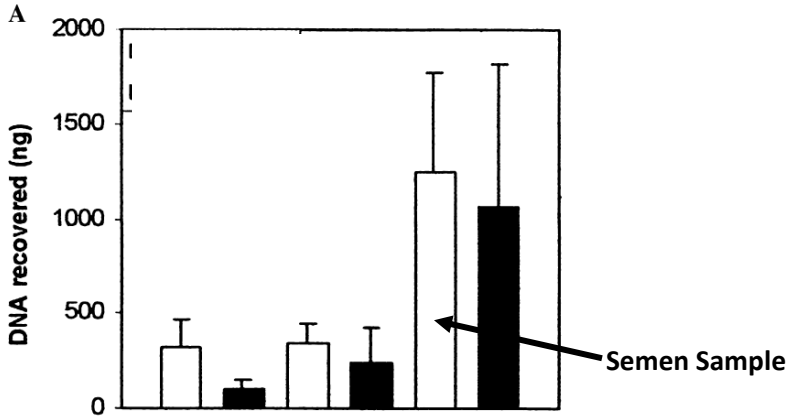


Figure 20 Recovery of DNA from semen sample

Table 35 Recipe of Lysis Buffer

Components	**Amount**
Tris-HCl	10mM (pH 8)
NaCl	0.1 M
EDTA	25mM (pH 8.0)
SDS	20mM
DTT	0.39 M
Proteinase K	0.5mg/ml

Fifth protocol is proposed by (Montjean *et al.*, 2013) where pellet was washed with 1XPBS twice and then incubated at 56°C for 1h with 10µl of 20mg/ml Proteinase K and 10µl of 1M DTT. Then 400µl of digestion buffer (**Table 36**) was added and continued incubation overnight. Finally the DNA was precipitated with 100% ethanol and washed with 70% ethanol, two times. Pellet was dried and stored in TE.

Table 36 recipe of Digestion buffer

Components	Amount
Tris-HCl	10mM (pH 8)
Sarkosyl	0.5%
EDTA	1mM (pH 8.0)
SDS	0.1%

Conclusion

Studies show that blood is the most encountered sample as evidence in forensic research and swabs are the second most. Main focus of DNA isolation for profile generation are these typical samples, since the beginning of field of forensic analysis. And this has also been a successful way out since then. But now molecular research has extended up to micro level. Finger prints, lip prints, foot print etc. based specific patterns are now generated from the crime scene and matched with that of victims. If suspect has some special microbial disease like TB or shed some specific uncommon microorganism, then air sample can too be taken to match the microbial flora with him. Chemical analyses of the victim is also the important field of forensic analysis.

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