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Comparative study of DNA yield from various blood samples under different conditions

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ABSTRACT: *Forensic/DNA analysis is completely based on the extraction of DNA from the biological samples available at crime scenes. Blood being one of the commonly available sample sources from crime scenes, is generally used for DNA extractions. DNA profiling and sequencingtechniques depend on the quantity of DNA below which the analysis becomes practically not possible. Thus, extraction methods used for sample processing must be accurate and specific based on the type of sample obtained to yield maximum DNA from available samples. Current work involves analysis of different types of blood samples that can be possibly obtained from crime scenes which include 1) blood-stained cloth, blood dried on a glasssurface, blood absorbed on cotton fiber, blood spread on a floor, and fresh blood obtained from the suspect. All the samples were subjected to DNA extraction using different protocols fortesting the yield. DNA extracted was subjected to purification, agarose gel run, and quantification. Based on the results obtained it would be possible to suggest a specific method for a given type of sample for maximum extraction yield of DNA.*

Keywords; *DNA Extraction, DNA analysis, Quantitative estimation, Agarose gel electrophoresis, Quantification, Purification*

1. INTRODUCTION:

1. DNA

DNA is a wide molecule that contains hereditary information. Cell regulation, metabolism, and reproduction all rely on the presence of DNA. The nucleus contains a significant amount of compressed DNA. Both the cell nucleus and the mitochondria contain genes. DNA can exist as single-stranded or double-stranded. The nucleus is composed of deoxyribose sugar, phosphates, and nitrogenous bases. DNA consists of four nitrogenous bases, forming a ladder-like structure made of deoxyribose and phosphates. The third and fifth carbon atoms bond each sugar molecule to a phosphate molecule.

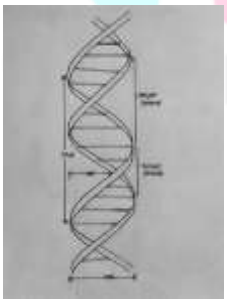


Fig 1: Structure of DNA

1.1 Discovery/History of DNA

1860's: Swiss Chemist Friedrich Miescher was the one person who recognized DNA in the early 1860's. After the Miescher discovery, further scientists Phoebus Levene and Erwin Chargaff gave additional details about the DNA.

1953: It was the year the two scientists discovered the structure of DNA, American biologist James Watson and British physicist Francis Crick. Their discovery was based on several scientist works: Rosalind Franklin (1952), who established the first X-ray picture of DNA. He is also known as a specialist in X-ray crystallography. He also gave an understanding of A and B forms of DNA. Maurice Wilkins was a British biophysicist, who discovered the DNA structure through X-ray diffraction. Linus Pauling established the triple helix structure of DNA with the bases on the inside and the nucleotides facing towards the outside, which later wants and crick prove false his proposal.

1.2 How DNA differs from Person to Person

The complete human DNA is mostly similar in its sequence or the base pair positioning. However, the dissimilarity that occurs in humans is due to genetic discrepancy, which createsdissimilarity in different people.

There are two major reasons for the difference in DNA genetic material.

1. Firstly, the parents with unique DNA and genetic material exchange through meiosis during reproduction. Both DNA combines to form the offspring's DNA, and during thiscombination, the genetic material is condensed and transferred to the offspring, hence bringing differences in human offspring with few similar characteristics.
2. Secondly, mutations can also lead to differences in DNA. In mutations, the DNA withany of its base pairs in the sequence is damaged, changed, or deleted, creating genetic variations even if it is only a single base pair or sequence.

DNA analysis is done to recognize a person's identity as it is unique in each human with moredissimilarities and similarities. In most cases, identical twins have identical DNA, but some scientists still do not agree with these identical characters of DNA. So, this is how the DNA isdifferent for different humans

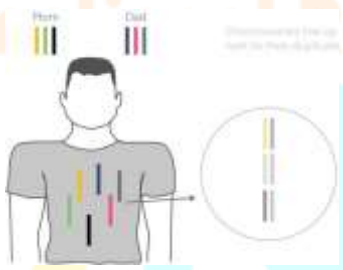


FIG 2: DNA DIFFERS FROM PERSON TO PERSON

1.3 Where DNA is found?

DNA or deoxyribonucleic acid, is found inside a peculiar region of the cell called the nucleus. Each DNA molecule is tightly packed, this pack of DNA is called a chromosome (23 pairs in humans). It is located in a peculiar region, which is the nucleus because of the tiny cell and also because organisms in each cell have multiple DNA molecules. DNA is also found in the nucleus of a cell, which is known as Nuclear DNA. The absolute set of DNA of an organism is also known as the Genome. Furthermore, humans and other sophisticated organisms also have miniature quantities of DNA in cellular networks called mitochondria. Mitochondria is the powerhouse of the cell; it can produce energy for the cells which need to function properly. Nuclear and Mitochondrial DNA are inherited from both parents whereas all the mitochondrial DNA transfers to offspring from the female parent, in sexual reproduction. Generally, a human cell holds around six Picograms (pg) of DNA and also it has 3.1 (3.1Gb) billion base pairs of DNA.

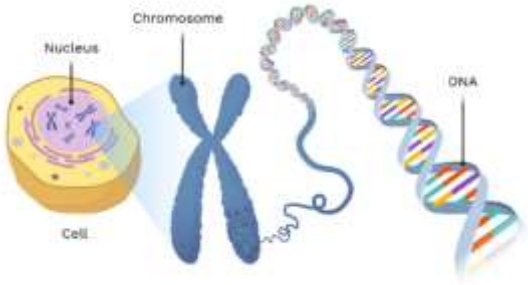


Fig 3: DNA Location

1.4 EXTRACTION AND ISOLATION OF DNA

1.4.1 Overview of DNA extraction

DNA extraction- It is the method/ technique used to purify the DNA by exploiting physical/chemical methods that help the sample separate DNA from Proteins, cell membranes, and other components.

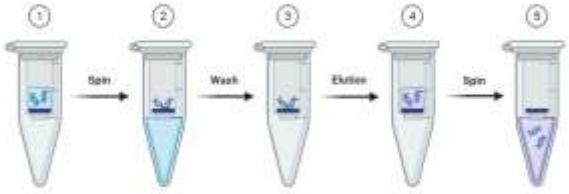


Fig 4: Overview of DNA extraction

1.4.2 History of Extraction of DNA

DNA was isolated by the first scientist Fredrich Miescher in 1869 while examining leucocyte composition (white blood cells). He could isolate unknown mediums that act dissimilarly to the proteins in the solution.

1.5 Blood DNA Extraction from Human

Blood is a fluid connective tissue that transports oxygen and nutrients and carries carbon dioxide from various body parts. A moderate human has 5 litres of blood.

1.5.1 Composition of Blood-

- Plasma
- White blood cells/Leucocytes
- RBC's
- Platelets

1.5.2 Importance of Blood in Forensic Science

1. Blood is the most common biological evidence usually found at the crime scene. It plays an essential forensic tool because it contains very significant information.
2. It links with the suspect
3. It also helps in the crime scene reconstruction.
4. Inspecting/investigating various states of bloodstains/spots can assist in clarifying the situation under which crimes have taken place. This crucial information signifies the suspect/criminal investigation is the right path to solve the crime.
5. It serves as evidence in the court of law.

1.6 Forensic Significance-

At the crime scene, various pieces of evidence play a very crucial role in identifying the suspect and unknown victim. The common evidence which usually found in the crime is biological evidence such as bite marks in sexual assault cases, hair, blood (menstrual blood, fresh, dry blood), bones, saliva, semen, vitreous, and many shreds of evidence. For further analysis, the samples/evidence were later sent to forensic laboratories and this evidence gives significant information about the suspect, victim, and the incident/crime. The analysed evidence gives information about the incident and the significance of DNA extraction are follows:

1. It links the crime scene with the Suspect
2. It helps to reconstruct the Crime scene
3. It helps in the determining or evaluating DNA profiling and RNA profiling
4. DNA analysis is the most important method which evaluates the samples and gives accurate results of the evidence. The common evaluation methods of DNA are DNA fingerprinting, UV spectrophotometer, etc.
5. In Forensic analysis, DNA typing compares the DNA that was extracted from the crime scene with suspect DNA extraction.
6. It also helps to provide information about the biological relationship between a mother and a child using prenatal testing screening/maternal testing
7. Forensic/DNA analysis
8. It also helps to find out Missing Persons/unknown deceased information.

2. OBJECTIVES

Aim for the Research

The research aims to compare DNA detection from various samples and conduct blood stainsin different scenarios.

Objectives for the research study:

1. To conduct a comparative study using three different protocols to predict thebloodstains collected from different conditions.
2. To find the DNA analysis results for five different blood conditions using two analyticalmethods.
3. To understand five different conditions of blood when aid with three different protocols
4. To explore the relationship between dried and fresh bloodstains.

3. METHODOLOGY

3.1 Materials:

Reagents and solution

Protocol 1

Solution A [100ml] = 1M Tris HCL = 1ml, Sucrose=10.9, Magnesium Chloride=0.047mg

Dissolved all the components in 50ml of distilled water adjust the pH to 8 makeup the volume to 100ml.

Calculation:

Solution B [100ml] = 1M tris HCL=10uL, 0.5m EDTA= 12uL, Sodium Chloride= 0.876g

Dissolved all the components in 50ml of distilled water adjust the pH to 8 makeup the volume to 100ml.

Protocol2

CTAB buffer, 300ul of 150mM of NH4cl, 1mM KHCO3 and 0.01mM EDTA solution. 200uL phenol buffer, 200uL chloroform and isoamyl alcohol (24:1)

Protocol 3

Lysis Buffer 1 [15ml] = 20Mm Tris HCL + 10Mm EDTA + {[0.1%] v/v SDS

Lysis Buffer 2 [10ml] = 20Mm Tris HCL + 5Mm EDTA + 10Mm Sodium chloride

Fig 5: Lysis buffer 1

Fig 6: Lysis buffer 2

TE Buffer [10ml] = 1M Tris + 0.5 EDTA

Fig 9: TE buffer

Colorimetry/Spectrophotometer

Standard DNA solution, DNA samples, Diphenylamine reagent, Glacial acetic acid, Conc. H2SO4.

Agarose gel electrophoresis

Ethidium bromide, DNA samples, DNA staining dye, buffer solution, Agar.

3.2 Equipment

Centrifuge, Rotating Mixer, Water Bath, Vortex, UV transilluminator, Colorimetry/Spectrophotometer, Test tubes, Electrophoresis equipment, & Micropipettes

3.3 Procedure

1. Collection of blood samples

Blood samples were collected in vacuum tubes containing EDTA from a nearby clinic/diagnostic facility with their approval. The samples were taken with consent. Various fresh blood samples were collected as needed for the study/research.

Fig 8: Sample

Sample Preparation/Preservation:

Fresh/liquid Blood: To preserve the blood, it is necessary to add some anticoagulants such as EDTA, heparin, and sodium nitrate. EDTA is often used to preserve the DNA.

- Fresh blood was directly added to the solution.

Dried Blood: I conducted a research study where I collected blood in five different situations: on fabric, cotton, surfaces, fresh blood, and glass. I then introduced dry blood stains from cotton, cloth, and glass to distilled water.

- The cotton was immediately dipped in the distilled water.
- The dried blood stains on the cloth were cut and dipped in the distilled water.
- The dried blood stains on the glass or glassware (generally in a petri dish) were immediately added to the distilled water.
- The dried blood stains on the surface were scraped and introduced to the solution.

2. ***Extracting/purification of blood samples.***

   In this study, five different conditions of blood samples were collected and processed using three different protocols to extract and purify the DNA. Generally, the chemical method is used for DNA extraction.

3. ***Performing DNA analysis using: Agarose gel electrophoresis/band identification & Quantitative estimation of DNA Using the DPA method.***

All the samples were subjected to DNA extraction using different protocols fortesting the yield. DNA extracted was subjected to purification, agarose gel run, and quantification. Based on the results obtained it would be possible to suggest a specific method for a given type of sample for maximum extraction yield of DNA.

4. ***Comparison of three protocol results***.

The study aims to compare DNA detection from various samples and conduct bloodstains in different scenarios.

1. Fresh Blood

2. Surface

3. Glassware

4. Cloth

5. Cotton

Fig:9 - Different conditions of blood

3.4 METHODS

DNA Extraction Procedure

For my Research study which is on blood and were extracted/collected from different conditions of blood: Fresh, Glassware, Surface, Cotton & Cloth. Three different DNA-extracting protocols used for the research:

Method-1 (2days process)

Day1

1. Take 1ml of Anti-coagulant blood (EDTA) and add 3ml of solution A
2. Shake it slowly for10mins
3. Incubate at 37 degrees Celsius for 5mins
4. Centrifuge at 5,000rpm for 5mins
5. Add 650uL of ice-cold chloroform [decolorizing]
6. Centrifuge at 4,000rpm for 10mins
7. Take the supernatant & add an equal amount of ice-cold Isopropanol/70% Ethanol
8. Incubate at 4 degrees Celsius overnight

Day2

9. Centrifuge at 12,000rpm for 5mins
10. Wash the pellet in 70% Ethanol (1ml)
11. Centrifuge at 12,000rpm for 5mins
12. Discard the supernatant, air dry the pellet
13. Dissolve the DNA pellet in 50-100uL TE buffer

Method-2 (2days process)

DAY-1

1. Take 1ml of blood and add 300uL of 150mM of NH4cl, 1mM KHCO3 and 0.01mM EDTA solution
2. Then mix the solution and incubate at room temperature for 10 mins

3. Centrifuge and discard the supernatant part
4. Add 160uL of distilled water and 200uL CTAB buffer
5. Vortex for 20 seconds, incubate at 56 degrees Celsius/hr
6. Vortex every 15 minutes and add 200uL phenol buffer, 200uL chloroform, and isoamyl alcohol (24:1) vortex.
7. Centrifuge at 10,000 rpm for 4mins.
8. Remove the supernatant part transfer it to a fresh tube and add 70% chilled ethanol/Isopropanol.
9. Incubate at 4 degrees Celsius overnight.

DAY-2

10. Centrifuge at 10,000 rpm for 4mins.
11. Take the pellet & wash it with 70% chilled ethanol.
12. Centrifuge at 10,000 rpm for 4 minutes and completely air dry the pellet.
13. Dissolve the DNA pellet in 50-100uL TE buffer

Method-3 (3 days process)

DAY-1

1. Take 1ml of blood + lysis buffer 2 [3ml], and mix properly.
2. Incubate at room temperature for 10 minutes.
3. Centrifuge at 10,000rpm for 5min.
4. Discard the supernatant and add ice-cold lysis buffer 2 (2ml), mix properly.
5. Add 30uL pf SDS (10%) and incubate at 56 degrees Celsius, overnight in the water bath (minimum 16hrs)

DAY-2

6. Bring the solution to room temperature
7. Add 2ml of potassium acetate solution
8. Mix properly, centrifuge at 10,000rpm for 5mins
9. Transfer the supernatant to another fresh tube and discard the pellet
10. Add isopropanol and incubate at 4 degrees Celsius overnight.

DAY-3

11. Centrifuge at 5,000 rpm for 5mins.
12. Take the pellet & wash it with 70% chilled ethanol.
13. Centrifuge at 10,000 rpm for 4 minutes and completely air dry the pellet.
14. Dissolve the DNA pellet in 50-100uL TE buffer

3.5 DNA ANALYSIS

The common methods to evaluate DNA extraction are Agarose gel electrophoresis and Estimation of DNA using Diphenylamine methods

Estimation of DNA using the Diphenylamine

Principle: In DNA deoxyribose reacts with diphenylamine to produce a blue colour in the presence of acids which forms β-hydroxylevulinaldehyde, with a sharp absorption maximum at 595nm. In DNA, only the deoxyribose sugar of purine nucleotides reacts, so the results obtained represent half of all deoxyribose sugars present.

DPA REAGENT:

1gm of DPA in 100ml glacial acetic acid and 2.5ml of concentrated H2SO4 (must be addedfreshly)

Quantitative Estimation of DNA Using DPA (Diphenylamine) method

1. Take Blank,0.2,0.4,0.6,0.8 and 1ml of standard working into the succession of labelled test tubes
2. Pipette out 0.5ml unknown samples into the 5 tubes (for three protocols 15 test tubes) T1, T2, T3, T4, & T5

3. The volume in all the samples made up to 1.5ml with distilled water and 2ml of distilled water in Blank which was served separately.
4. 5ml of diphenylamine reagent was added in all the test tubes along with a blank
5. Incubate all the test tubes for 15 minutes at 100°C in a water bath.
6. After incubation, the samples show a blue colour, which indicates the presence of DNA except for blank.
7. A standard graph is drawn taking the concentration of DNA on the x-axis and absorption of the y-axis. From the standard graph, the amount of DNA present in the unknown solution is calculated.

Agarose gel Electrophoresis

Principle: Agarose gel electrophoresis is the technique that is used to separate DNA/RNA molecules by size. This is achieved by twitching negatively nucleic acid molecules through an agarose matrix with an electrophoresis (electronic field). Shorter particles move and travel faster than longer ones.

MATERIALS REQUIRED:

Ethidium bromide, loading dye, DNA marker, Tri's borate, EDTA buffer, DNA samples & Agarose

TAE buffer

- Tri's base – 40mM
- Acetic acid – 20mM
- EDTA – 1mM

Dissolve each component in 2/3rd of the final volume of distilled water and adjust the PH of the solution to around 8.0 using HCl or NaOH if necessary.Make the volume to 300ml with distilled water.

Procedure

1. Wipe the electrophoresis tray and chamber with spirit
2. Seal the agarose gel electrophoresis tray with cellophane tape and test for leakage.
3. Prepare 0.7% agarose in TAE buffer by melting it in an oven
4. Cool it to 45℃ and add 5-10ul of ethidium bromide.
5. Mix it and pour it into the agarose gel tray.
6. Insert the comb and leave it to set
7. After setting remove the comb carefully without punching the walls. Remove the seal and place the gel tray in the electrophoresis chamber
8. Cover the gel with TAE buffer
9. Prepare the DNA samples for loading into wells as mentioned (10ul of DNA sample +3ul of loading dye)

Loading Gel:

1. Load 10-15ul of the sample carefully and note down the order of loading
2. Switch on the power supply and run the gel at 50V till the bands reach the 3/4th of the gel.
3. Turn off the power remove the lid of the electrophoresis chamber and carefully remove the tray and gel using a glove
4. Observe the gel in a UV transilluminator for visualization of DNA.

4. RESULTS & DISCUSSION

Agarose gel electrophoresis

Here, gel electrophoresis is used to confirm the presence of DNA in each sample and to obtain sufficient amounts of DNA from blood stains for band verification. It can also be used to assess the quality of DNA. The DNA samples are typically stained with a fluorescent dye such as ethidium bromide to visualize DNA bands on the gel. When exposed to UV light, DNA bands make them

visible for labelling and analysis. What is observed here is not a variation, but a DNA stain on the gel. This is because the DNA samples contained fragments of various sizes. This indicates the presence and quality of DNA with a range of fragment sizes suitable for band verification.

Fig 10: Band verification under UV transilluminator

Estimation of DNA using Diphenylamine

Sl. No	Samples	Vol. of standard (200 µg/ml) DNA (ml)	Vol. of distilled water (ml)	Conc. of DNA (µg)	Vol. of DPA reagent (ml)	Incubate in boiling water bath for 15 Min & Cool	OD at 595nm				
							Standard values	Unknown samples			
1	Blank	0 ml	2 ml	0 µg	5ml	15mins	0	Blank	0	0	0
2	S1	0.2 ml	1.8ml	200 µg	5ml	15mins	0.182	T1 (Fresh)	0.028	0.027	0.021
3	S2	0.4 ml	1.6ml	400 µg	5ml	15mins	0.296	T2 (Cloth)	0.174	0.023	0.092
4	S3	0.6 ml	1.4ml	600 µg	5ml	15mins	0.574	T3 (Glass)	0.029	0.079	0.085
5	S4	0.8 ml	1.2ml	800 µg	5ml	15mins	0.689	T4 (Cotton)	0.725	0.070	0.096
6	S5	1.0ml	1.0ml	1000 µg	5ml	15mins	0.844	T5 (Surface)	0.736	0.069	0.023

Table 1: Final table of Quantitative Estimation of DNA Using the DPA method

Calculation:

Protocol-1 unknown samples	**T1 (Fresh)**	**T2 (Cloth)**	**T3 (Glass)**	**T4 (Cotton)**	**T5 (Surface)**
Calculation	=170 µg/0.5ml =1ml=? **=340 µg/ml**	=340 µg/0.5ml =1ml=? **=680 µg/ml**	=500 µg/0.5ml =1ml=? **=1000 µg/ml**	=670 µg/0.5ml =1ml=? **=1340µg/ml**	=840 µg/0.5ml =1ml=? **=1680 µg/ml**

Table 2: Unknown sample calculation table of Protocol-1

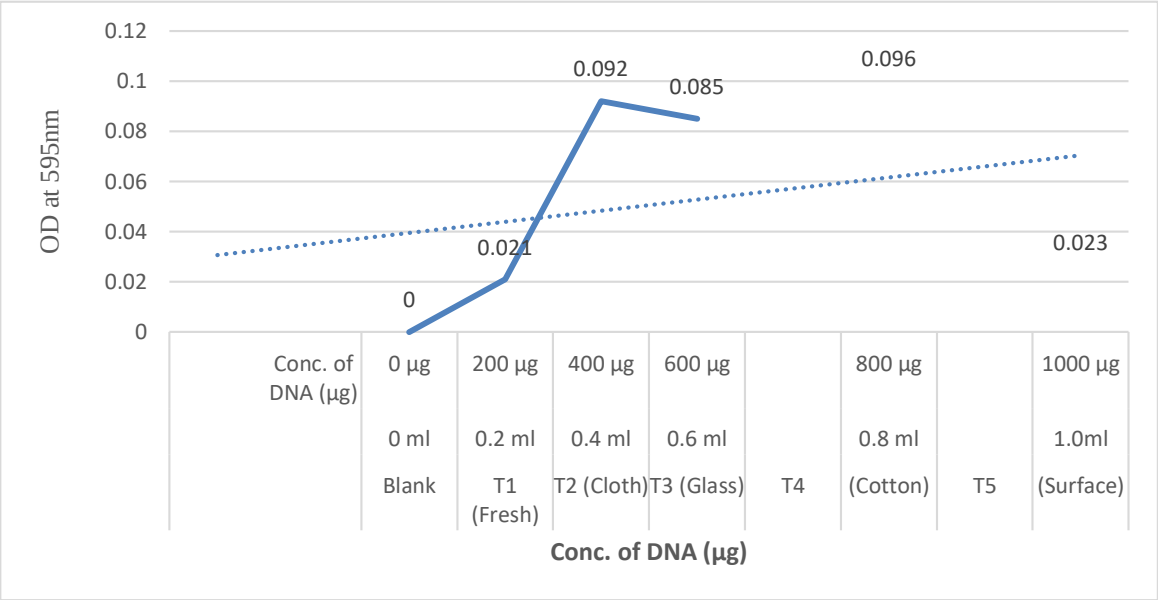
Protocol-2 unknown samples	**T1 (Fresh)**	**T2 (Cloth)**	**T3 (Glass)**	**T4 (Cotton)**	**T5 (Surface)**
Calculation	=170 µg/0.5ml =1ml=? **=340 µg/ml**	=340 µg/0.5ml =1ml=? **=680 µg/ml**	=500 µg/0.5ml =1ml=? **=1000 µg/ml**	=670 µg/0.5ml =1ml=? **=1340µg/ml**	=840 µg/0.5ml =1ml=? **=1680 µg/ml**

Table 3: Unknown sample calculation table of Protocol-2

Protocol-3 unknown samples	**T1 (Fresh)**	**T2 (Cloth)**	**T3 (Glass)**	**T4 (Cotton)**	**T5 (Surface)**
Calculation	=180 µg/0.5ml =1ml=? **=360 µg/ml**	=340 µg/0.5ml =1ml=? **=680 µg/ml**	=510 µg/0.5ml =1ml=? **=1020µg/ml**	=680 µg/0.5ml =1ml=? **=1360µg/ml**	=840 µg/0.5ml =1ml=? **=1680 µg/ml**

Table-4: Unknown sample calculation table of Protocol-2

PROTOCOL-1



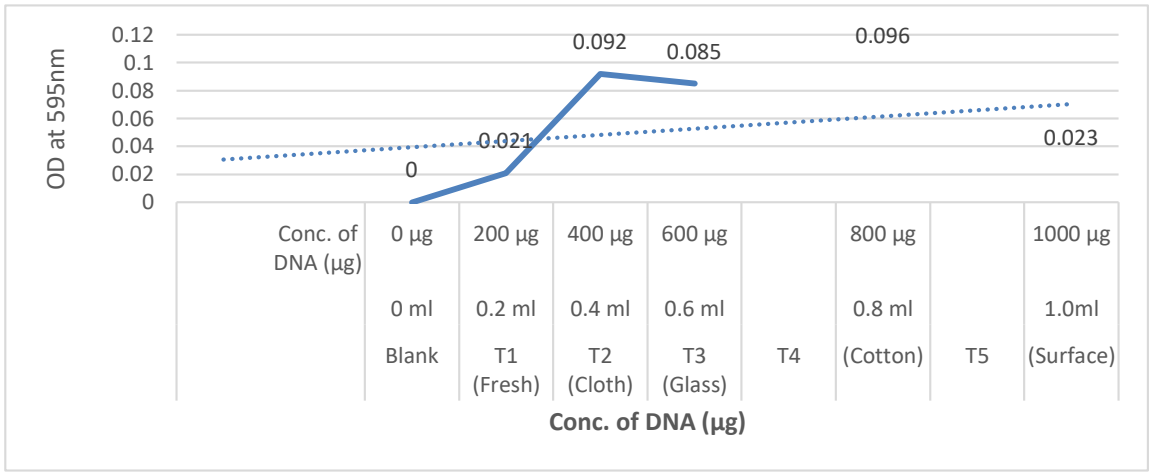
GRAPH 2: PROTOCOL 1

SL. No	**Sample**	**OD at 595nm**
1	T2 (Cloth)	0.174
2	T4 (Cotton)	0.725
3	T5 (Surface)	0.736

Table 6: Result analysis of Protocol-2

In protocol 1, of all 5 different blood conditions T2 (cloth), T4(Cotton), and T5(Surface)show high amounts of DNA.

PROTOCOL-2



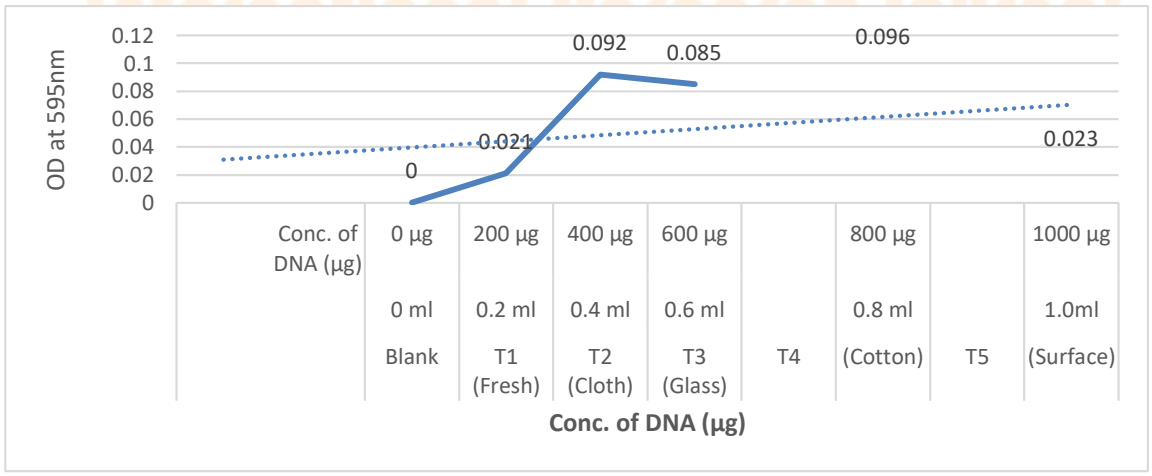
GRAPH 3: PROTOCOL 2

Table 6: Result analysis of Protocol-2

SL. No	Sample	OD at 595nm
1	T3 (Glass)	0.079
2	T4 (Cotton)	0.070
3	T5 (Surface)	0.069

In protocol 2, of all 5 different blood conditions T3 (Glass), T4(Cotton), and T5(Surface)show high amounts of DNA.

PROTOCOL-3



GRAPH 4: PROTOCOL

TABLE 7: RESULT ANALYSIS OF PROTOCOL-3

SL. No	Sample	OD at 595nm
1	T2 (Cloth)	0.092
2	T3 (Glass)	0.085
3	T4 (Cotton)	0.096

Protocol 1 T5 (surface) has a high amount of DNA, Protocol 2 T3 (glass) shows a high amount of DNA, and Protocol 3 T4 (cotton) shows a high amount of DNA.

In this research study, five different conditions of bloodstains (fresh, cloth, glass, cotton & surface) were analyzed using three different protocols. During the experiments, I observed the formation of white precipitation when 70% chilled ethanol/isopropanol was added, indicating the presence of DNA. Additionally, noticed the appearance of white thread-like structures.

Fig:11 White thread-like structure (DNA)

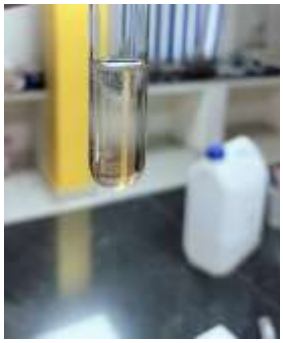


Fig:12 White Precipitation (DNA)

5. CONCLUSION

DNA extraction is a method used to understand the DNA of individuals and samples. It involvesspecial techniques to purify DNA. In forensic analysis, DNA extraction plays a crucial role because DNA contains significant genetic information. At a crime scene, various biological evidence such as hair, blood, nails, saliva, semen, vitreous, etc is collected and sent to forensic laboratories for analysis. These labs have various equipment, methods, and techniques available for DNA extraction. In recent years, new technologies have emerged to provide quicker results.

In this research study, five different conditions of bloodstains (fresh, cloth, glass, cotton & surface) using three different protocols, and we analyzed them using two analytical methods: estimation of DNA using DPA (Diphenylamine) and Agarose gel Electrophoresis. Research studies showed that each protocol has unique significance in providing accurate results, basedon the chemicals used, sample handling, and experimental procedures. Although the protocolsare universally the same, using different protocols to experiment with the same five conditionsof bloodstains yielded distinct values and results. During the experiments, I observed the formation of white precipitation when 70% chilled ethanol/isopropanol was added, indicatingthe presence of DNA. Additionally, noticed the appearance of white thread-like structures.

In conclusion, the comparative study using three different protocols provided valuable insightsinto predicting bloodstains collected from different conditions. The DNA analysis results for five different blood conditions using two analytical methods yielded significant findings. Understanding the five different conditions of blood with the aid of three different protocols proved to be informative. Additionally, exploring the relationship between dried and fresh bloodstains sheds light on their differences and similarities, contributing to a comprehensive understanding of bloodstain analysis.

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