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A Comparative Analysis of DNA Degradation Patterns in Forensic Samples Involved in Sexual Assault Cases

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Abstract- Sexual assault is a pressing global public health issue, with forensic experts recognising that the majority of such cases remain unreported. In India only, 31,677 rape cases are reported annually, averaging 86 daily, with only 3-5 solved each day. However, to identify suspected culprits, reported incidents require thorough investigations. DNA samples play a vital role in forensic investigations, particularly in cases of sexual assault, where they can provide critical evidence for identifying perpetrators. When collected from a crime scene or the victim's body, DNA evidence often serves as a vital source of clues, playing a pivotal role in the identification process and aiding in the resolution of cases. Extracting a genetic profile involves several stages: extraction, quantification, amplification, separation of STR fragments, and genotyping. While accurate profiling is possible when samples contain sufficient, high-quality genetic material, this ideal scenario is often challenging. Many cases involve samples with degraded or insufficient DNA from the perpetrator, complicating forensic analyses using current methodologies. DNA degradation, influenced by environmental factors, impacts samples from crime scenes, leading to varying degrees of integrity loss. By implementing proper methods and understanding degradation levels, we can prevent sample degradation, leading to faster and more reliable investigation results. Understanding the causes and mechanisms of DNA degradation is essential for improving analysis techniques. Degradation affects DNA universally, eroding its quality over time. Environmental factors such as light exposure, preservation techniques, and material type cause varying deterioration patterns in samples. This thesis investigates biological samples commonly encountered in sexual assault cases, analyzing factors that influence their condition and the rate of DNA degradation during the initial case days. It is a comparative study of degradation trends in forensic fluids, such as semen and vaginal fluid, under different environmental conditions. The study is intended to increase knowledge on DNA integrity to enhance forensic techniques and resolve challenges presented by degraded samples.

Keywords- DNA Degradation, Forensic Samples, Polymerase Chain Reaction, DNA Quantification, Seminal Fluid, Vaginal Fluid.

I. INTRODUCTION

Sexual violence is a pervasive public health issue that affects individuals across all ages and genders worldwide. While both genders are impacted, women are disproportionately affected, with statistics showing that in some countries, nearly 25% of women have experienced sexual violence, and up to 33% of adolescent girls were assaulted during their first sexual experience. Defined as any sexual act, attempt to obtain a sexual act, unwanted sexual comments or advances, or actions targeting a person's sexuality through coercion, sexual violence can occur in any setting, including homes and workplaces, regardless of the perpetrator's relationship to the victim. [1]

It includes anything from forceful penetration to verbal abuse. Coercion extends beyond physical force, encompassing psychological intimidation, social pressure, blackmail, and other forms of threats. Additionally, sexual assault includes situations where the victim is unable to provide consent due to circumstances such as intoxication, drug influence, unconsciousness, or an inability to comprehend the situation. This multi-faceted issue underscores the urgent need for global awareness and intervention to address its widespread impact. [2]

The term "sexual assault" refers to a broad variety of behaviours, such as unwelcome sexual approaches or harassment, rape by strangers or acquaintances, and rape inside partnerships. It also covers incidents of forced marriage or cohabitation, assaults of minors, and assaults of people with physical or mental impairments. Sexual violence also includes forced abortion and deprivation of the freedom to use contraception and other protections against sexually transmitted illnesses. Sexual assault also includes forced prostitution, human trafficking for sexual exploitation, and violent acts directed at an individual's sexual integrity. This wide range emphasizes how urgently awareness and action are needed to successfully address these infractions. [3]

Sexual assault has far-reaching consequences that affect victims physically, psychologically, and socially. It can lead to unintended pregnancies, complications during pregnancy, miscarriages, and induced abortions. Victims may also experience gynaecological issues, sexually transmitted infections, and a range of psychological effects, including depression, post-traumatic stress disorder (PTSD), and other mental health disorders. While physical force is not always involved in sexual assault, and physical injuries may not always be evident, the impact can still be severe. In some cases, deaths associated with rape have been reported, underscoring the devastating nature of such violence. These consequences highlight the urgent need for comprehensive support systems and preventive measures to address the multifaceted effects of sexual violence. Cases involving fatalities and a small proportion of forced intercourse—whether within relationships, through threats, or by strangers—are more likely to be reported to the police. Some additional cases of forced intercourse, under similar circumstances, are disclosed through surveys. However, a substantial number of incidents, including those related to sexual exploitation, remain unreported or undisclosed. For the reported cases, victims often require a range of specialised services. [4]

These include support for addressing physical and mental health consequences, assistance with recovery, access to social welfare programs, and legal aid to navigate the justice system. This highlights the critical need for comprehensive support systems to address the multifaceted impact of sexual violence. To identify the perpetrator and verify the circumstances surrounding the assault, an investigation must be conducted, requiring a thorough analysis of the collected physical and biological evidence. [5]

The primary purpose of collecting forensic evidence is to establish a connection between a suspect and the victim of a crime. To ensure the collection of appropriate evidence, medical practitioners must have a thorough understanding of the types of evidence typically present in sexual assault cases. Specimens can confirm contact between individuals or between an individual and a specific location, as such evidence is often generated at the time of the offence. Maintaining the integrity of the evidence depends on the timely collection of specimens. Conducting the collection promptly after the incident reduces the risk of loss or degradation, particularly of biological samples, ensuring their reliability for forensic analysis. This highlights the importance of swift and meticulous procedures in sexual assault investigations. [6]

Medical practitioners play a crucial dual role in supporting survivors of sexual assault. They provide essential medical care and psychological support while also assisting in medico-legal proceedings by collecting evidence and ensuring accurate documentation. A Registered Medical Practitioner (RMP) is required to do exams in rape cases. Under Section 27(2) of the POCSO Act, if the survivor is a girl under eighteen, the examination must be carried out by a female RMP employed in a government or local authority hospital. If unavailable, any RMP may conduct the examination (as per Section 164A of the CrPC) with the victim's or a competent person's consent within 24 hours of receiving information about the offence. Parental consent is mandatory for medico-legal examinations of minors. Importantly, survivors or their guardians have the right to refuse medico-legal procedures, but such refusal does not deny them access to necessary medical treatment. Medical practitioners must inform the individual being examined about the nature and purpose of the examination. In the case of a child, this information should be provided to the parent, guardian, or a trusted person. Evidence such as clothes, scalp hair, foreign objects, pubic hair, and samples from the mouth, rectum, anus, vagina, and blood are all gathered during medico-legal exams. Importantly, the "two-finger test" must not be conducted, and no comments should be made regarding the size of the vaginal introitus, elasticity, hymen, or past sexual experiences, as these have no relevance to cases of sexual violence.[7]

Practitioners should document injuries, bleeding, swelling, or tenderness reported by the survivor. While sexual violence is often perpetrated against females, it can also affect males, transgender individuals, and intersex individuals. The forensic evidence collected depends on the nature of the violence, the time elapsed since the incident, and whether the survivor has bathed or washed. Clothes, condoms, sanitary pads or tampons, body fluids (semen, blood, vaginal fluid, saliva, etc.), head hair, pubic hair, vulval swabs, vaginal swabs, anal/rectal swabs, cervical swabs, oral swabs, penile swabs, and urethral swabs are among the important biological samples that are typically gathered by medical examiners. Nail clipping or scraping, urine sample, vaginal sample, smear slides (vaginal, cervical, anal, oral, etc.), and an aborted foetus. After collecting samples or evidence, they are transferred to the Forensic Science Laboratory (FSL). A requisition letter must accompany the transfer, addressed to the relevant authority, detailing the samples provided and the specific tests required. [8]

Beyond backlogs and delays, sexual assault cases in India present additional difficulties. Due to the postponement in sample collection and processing, alongside improper preservation methods, evidence often deteriorates. This degradation critically impacts forensic reliability and can compromise justice, which is central to the focus of this study. While timely collection and preservation within the mandated 24–72-hour window are emphasized, logistical inefficiencies often render these efforts ineffective. The vast backlog of more than 9,000 ongoing cases hampers resolution even when samples get to the Forensic Science Laboratory (FSL); only a small number are given emergency priority. This systemic inadequacy highlights the urgent need for reforms in inefficient case handling and evidence management. The degradation of biological samples is influenced by various factors that compromise their integrity and suitability for forensic analysis. Environmental conditions such as temperature, humidity, and exposure to sunlight play a crucial role in accelerating the breakdown of DNA and other biomolecules within the samples. High humidity levels or the presence of water encourage microbial growth, which leads to the release of nucleases, proteases, and other enzymes capable of fragmenting and modifying DNA. Similarly, prolonged exposure to high temperatures or ultraviolet radiation can cause chemical alterations such as oxidation and hydrolysis, further damaging the DNA structure. The presence of soil contaminants, with their high microbial load and nutrients, also poses a significant threat by fostering rapid degradation of evidence. [9]

Additionally, biological factors contribute to degradation, including endogenous enzymes released from cells upon death, which drive the decomposition process. Forensic case exhibits that contain mixtures of fluids, such as seminal and vaginal fluid, provide nutrient-rich environments that promote microbial proliferation and biofilm formation. Improper storage and handling techniques, such as delays in processing or exposure to fluctuating temperatures, exacerbate degradation risks. To minimize these impacts, proper drying, the use of stabilizing solutions, and immediate storage in controlled environments are essential for preserving biological samples effectively.[10]

A substantial amount of research has focused on the degradation of blood samples. However, there remains a gap in the study of the degradation of semen and vaginal fluid. Therefore, this study aims to examine DNA degradation patterns in forensic samples from sexual assault cases. Given the critical role of these forensic samples in investigations, the current work, titled 'A Comparative Analysis of DNA Degradation Patterns of Forensic Samples Involved in Sexual Assault Cases,' will address the collection of various biological samples encountered in sexual assault cases (semen, and vaginal fluid), the effects of various environmental factors on DNA integrity in biological samples at different time intervals and surfaces and a comparative analysis of the degradation patterns.

II. REVIEW OF LITERATURE

Researchers have thoroughly investigated blood as it is one of the most commonly found pieces of evidence at crime scenes. Its forensic significance extends to identification, paternity cases, and sexual assault investigations. Through blood sample analysis, researchers have focused on DNA degradation patterns, which are essential for determining how long genetic material remains viable for forensic analysis. Various environmental factors contribute to this degradation, including exposure to water, nucleases, sunlight, extreme temperatures, bacteria, and fungi. When a body is subjected to these elements for an extended duration before discovery, extensive damage occurs to its genetic material, posing challenges for forensic analysis. In situations of deadly sexual assault, when DNA evidence is crucial, this problem is very important. All targets and internal positive controls (IPCs) were successfully amplified using DNA extracted from clean blood and soil-blood mixed control samples; intact DNA was indicated by degradation indices less than 1. Samples stored at room temperature for two weeks retained amplifications, though microbial degradation reduced DNA concentration slightly, without significantly impacting the degradation index. After four weeks of storage, DNA extractions continued to show amplification across all targets and IPCs, though concentration declined further while degradation remained minimal. By six to twelve weeks, degradation indexes ranged between 1–10, suggesting greater degradation of large autosomal targets compared to smaller ones. This indicates moderate DNA degradation, driven primarily by microbial activity fragmenting DNA over time. Additionally, wet samples stored in plastic containers exhibited poor preservation, whereas dried samples kept in brown paper envelopes maintained higher absorbance levels.[11]

For successful amplification using Polymerase Chain Reaction (PCR), the DNA template must remain intact at key binding sites where primers attach, as well as in the regions between primers to facilitate proper extension. Less whole DNA molecules are accessible for PCR amplification, the more a sample deteriorates. Research has demonstrated an inverse relationship between the length of a DNA locus and the probability of successful PCR amplification, with shorter fragments being more likely to amplify compared to larger ones under degradation. Capillary electrophoresis is widely used to analyse degraded DNA samples; however, due to environmental exposure, the results frequently yield only partial DNA profiles, complicating forensic investigations. Understanding these degradation patterns enables forensic experts to refine preservation techniques and optimize DNA recovery methods, ensuring that even compromised samples remain valuable in criminal cases.[12]

Other biological evidence, though harder to locate, plays a vital role in DNA identification. Seminal fluid, vaginal secretions, saliva, touch DNA, hair, and bone are crucial in forensic investigations, especially in sexual assault cases. These biological traces provide valuable genetic material, helping forensic experts establish links between victims and perpetrators. Although blood is frequently utilized, these substitute DNA sources are crucial in situations when blood is inaccessible or deteriorated. Advanced forensic techniques now allow DNA extraction from minimal biological samples, improving investigative accuracy. Proper collection and preservation of such evidence significantly enhance the chances of obtaining reliable DNA profiles for criminal cases. Researchers had evaluated the impact of the Quantifiler1 Trio Kit, and researchers analyzed 181 forensic samples, including oral swabs, blood, touch DNA, bones, human remains, sperm, and hair, selecting cases with single DNA profiles or mixtures with a distinguishable major contributor. The quantification data from the kit uses big amplicon signals to evaluate degradation levels and tiny amplicon signals to determine DNA concentration. The Degradation Index (DI) is determined by the ratio of the two signals because bigger DNA fragments are more susceptible to degradation. This study recorded DI values ranging from 0.47 to 158.73, confirming that higher DI values indicate greater DNA deterioration over time.

The impact of temperature on DNA degradation has also been extensively analyzed. Samples stored at 4°C for two weeks showed sharp declines in concentration, with degradation indexes between 1–10, affirming microbial activity as the main factor. By eight to twelve weeks at 4°C, DNA concentrations fell to zero, signifying substantial degradation. Conversely, extractions from samples stored at -20°C consistently showed amplification across all targets, with degradation indexes below 1, indicating minimal degradation. Notably, forensic DNA profiling of soil-blood mixed samples revealed that storage at -20°C and room temperature (25°C) was more effective for DNA preservation compared to storage at 4°C. This contradicts previous studies, such as Cushwa & Medrano's research on whole blood, which reported that both 4°C and -20°C storage conditions were optimal for DNA preservation over four weeks. Such discrepancies may arise from differences in sample storage methods, including tube storage versus dry-state preservation.[13]

Further analysis of short tandem repeat (STR) profiles demonstrated that DNA degradation significantly impacts STR typing after prolonged storage. While samples stored at room temperature and -20°C yielded complete STR profiles after twelve weeks, those stored at 4°C for two weeks retained intact profiles. However, by the fourth week, allele dropouts occurred, indicating substantial degradation. By six weeks and beyond, STR profiling became impossible due to severe degradation, reducing amplicon sizes beyond detection limits. These findings support the conclusion that storing forensic samples at -20°C is the most effective strategy for preserving DNA integrity, whereas storage at 4°C results in extensive degradation.

The study identified certain forensic samples as particularly challenging using standard protocols, requiring adjustments in PCR input and multiple amplifications to obtain viable DNA profiles. The Quantifiler1 Trio Kit's Degradation Index (DI) effectively characterizes degraded samples, offering valuable insights into DNA deterioration. This tool helps forensic experts predict profile quality and optimize processing methods for maximum allele recovery, enhancing efficiency while reducing time and costs in forensic casework.[14]

Operating guidelines were established to integrate Quantifiler1 Trio with the GlobalFiler Kit for Short Tandem Repeat (STR) analysis. For samples with minimal degradation (DI < 4), DNA concentration becomes the limiting factor; concentrated samples meeting the 0.5 ng input threshold produce full profiles, while lower-concentration samples require maximum amplification volume. For samples with a DI > 4, degradation limits profile completeness. Increasing DNA input may improve genotyping, but low-concentration samples may yield partial or null profiles. These findings can refine forensic protocols across different STR kits. [15]

Epidemiology of Sexual Assault Cases

Sexual assault remains a deeply troubling and pervasive issue in India, with tens of thousands of cases reported annually. Between 2005 and 2022, (As shown in Figure 1) official records indicate an average of approximately 30,000 rape cases registered each year. However, this alarming statistic likely represents only a small portion of the actual incidents, as many cases go unreported. Survivors often face significant barriers to coming forward, including societal stigma, fear of retaliation, and a lack of confidence in the justice system. These challenges contribute to the underreporting of such crimes, leaving countless survivors without the support and justice they deserve. The data underscores the urgent need for comprehensive reforms to address this critical societal problem. Strengthening the justice system, fostering public awareness, and creating robust support mechanisms for survivors are essential steps toward meaningful change. Efforts must focus on dismantling the stigma surrounding sexual assault, ensuring survivors feel safe and empowered to report incidents, and holding perpetrators accountable. By addressing these systemic issues, society can strive to create a safer and more equitable environment for all individuals.[16]

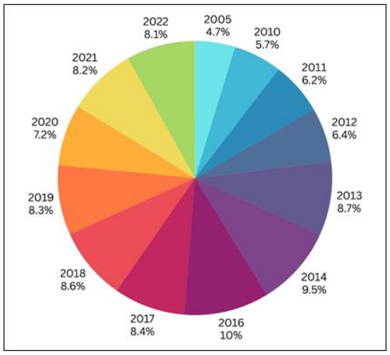

Figure-1- Total Number of rape cases reported in India from 2005-2022.

The lack of proper management, combined with equipment shortages and a high pendency rate, severely impacts the efficiency of handling sexual assault cases in India. Mishandling of evidence further exacerbates the issue, as biological samples—critical for forensic analysis—often degrade due to improper preservation and delays in processing. Such deterioration compromises the quality of results, weakening cases and hindering the pursuit of justice.

This systemic inefficiency not only prolongs the trauma experienced by survivors but also undermines their trust in the justice system.

Addressing these challenges requires immediate reforms in resource allocation, evidence management protocols, and streamlined case prioritization. According to the Open Government Data Portal, the State/UT-wise number of pending cases(refer to figure 2.1) under the Rape and POCSO Act as of October 31, 2024, highlights the significant backlog in the judicial system.

This data underscores the urgent need for reforms to expedite case resolution and ensure timely justice for survivors.[17]

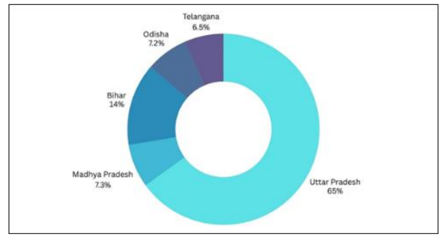

Figure 2: This data shows the high rate of pendency in major cities in India till 31st October 2024

In India, rape is one of the most common violent crimes against women, with an increasing number of recorded incidents. Vaginal swabs, which frequently include sperm and, thus, the offender's DNA, are among the most important pieces of forensic evidence in these types of instances. Crime labs seek to isolate pure male DNA from the sperm and the victim's epithelial cells to identify the perpetrator. Because all males of the same paternal lineage share the same Y profile, Y chromosome STR markers can be examined in mixed DNA samples, but they cannot be used to definitively identify the rapist. Differential lysis, a widely used method, selectively lyses non-sperm cells using detergent and Proteinase K, leaving sperm cells intact due to their highly disulfide cross-linked proteins. The sperm cells are then separated by centrifugation, washed to remove residual DNA, and treated with reducing agents to release pure male DNA. However, this process is time-consuming, difficult to automate, and may result in sperm loss. Alternative methods like filtration, flow cytometry, and laser dissection have been explored but are not yet standard practice.[18]

DNA analysis is pivotal in human identification, especially in cases involving sexual assaults, paternity disputes, mass disasters, and terrorist attacks. Degraded biological materials often complicate forensic investigations. DNA degradation is accelerated by full-blown cell death, which activates enzymes like lipases, nucleases, and proteases. Lysosomal proteases remove histones, facilitating DNA cleavage by endonucleases. Environmental factors such as hydrolysis, oxidation, and microbial activity further exacerbate degradation. Custom quantitative-PCR (Q-PCR) strategies can quantify damaged DNA fragments, aiding in minimizing amplification failures and improving forensic outcomes.

Biological evidence, such as bloodstains, is often exposed to environmental contaminants like soil, which harbors billions of microbes per gram. These bacteria quickly break down DNA, which compromises the precision of profiling. Timely processing of crime scene evidence is crucial to prevent degradation. However, delays due to backlogs, limited staff, or insufficient reagents highlight the need for effective storage protocols. Proper preservation methods, such as drying or using saline solutions, can mitigate degradation risks. Sexual assault evidence constitutes a significant portion of forensic DNA analyses. Sperm cells, rich in DNA, are crucial for profiling even from minute seminal stains. However, the time elapsed between the assault and sample collection is critical, as semen can persist for up to 72 hours post-coitus. Seminal fluid, with its nutrient-rich composition, fosters microbial growth, accelerating degradation. Vaginal fluid, composed of proteins and cervical mucus, further promotes microbial activity. Proper storage and rapid examination are essential to preserve evidence integrity and ensure reliable forensic analysis.[19]

III. MATERIALS AND METHODS

Material Required

DNA Extraction Using Qiagen QIAamp DNA Investigator Kit Sample (Cotton fabric with dried semen or vaginal fluid stains), QIAamp DNA Investigator Kit reagents which includes Buffer ATL, Buffer AL, Buffer AW1, Buffer AW2, Buffer ATE (elution buffer), Proteinase K Carrier RNA, Dithiothreitol (DTT) – not included (required for semen samples as it is essential for semen due to protamine-rich DNA packaging), Ethanol (96–100%), Microcentrifuge tubes (1.5 mL and 2 mL, DNase-free), Vortex mixer, Incubator or water bath (56°C and 70°C), Microcentrifuge, Sterile scissors or biopsy punch, Pipettes with aerosol-resistant tips.

Sample Collection

Biological samples were obtained from two donors: a 30-year-old male and a 23-year-old female. Semen and vaginal fluid samples were deposited onto clean cotton underwear under controlled conditions. Ethical approval was secured from the relevant authorities, and informed consent was obtained from both individuals. This study focuses on the early-stage DNA degradation patterns using techniques such as quantitative PCR and STR profiling. The semen sample was collected on March 25, 2025, and the vaginal fluid sample on March 28, 2025. Before packaging, both samples were exposed to ambient indoor light under standard laboratory conditions. They were then stored at room temperature in paper bags within a dark, dry environment until further analysis. Conducted at MRD Lifesciences Laboratory in Lucknow, DNA isolation and extraction were performed using the Qiagen QIAamp kit, commonly used in forensic investigations. Initially collected in vials, samples were applied to cotton underwear, dried, and later cut into smaller pieces for examination. The dried samples were stored in brown envelopes to replicate forensic laboratory settings, ensuring they underwent similar treatment to real crime scene samples before reaching forensic labs.

The QIAamp DNA Investigator Kit employs a silica-based membrane for selective DNA binding and offers flexible elution volumes between 20 µL and 100 µL. Additionally, the kit adheres to ISO 18385 standards, ensuring compliance with forensic quality control measures. The meticulous handling of these samples ensures their integrity for further forensic analysis, emphasizing the significance of controlled conditions when studying DNA degradation in biological materials.

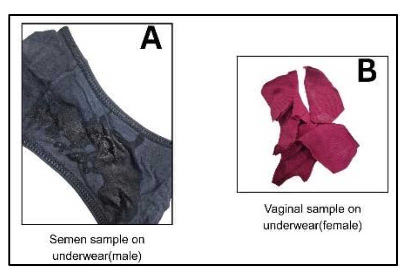

Figure 1.Samples before packaging

Table 3.1 Protocol Steps used to extract DNA

SNO.	STEPS	PROTOCOL
1	***Sample Preparation***	a) Cut ~1–2 cm² of the stained fabric (preferably from the visibly stained area). b) Place into a 1.5 mL microcentrifuge tube.
2	***Lysis and Digestion***	a) Add: 360 µL Buffer ATL b) 40 µL Proteinase K c) 20 µL DTT (for semen samples) d) Optional: Add 5 µL Carrier RNA (especially for low DNA samples). e) Vortex briefly. f) Incubate at 56°C for 3 hours, or overnight for highly stained or aged samples. g) Vortex occasionally or shake gently during incubation. *Standardly, we use 180 µL ATL, 20 µL Proteinase K, and 20 µL DTT (for semen). Optionally, add 5 µL Carrier RNA and Incubate at 56°C for 1–3 hours (depending on sample age), but above we have modified the protocol accordingly. The incubation is done before adding Buffer AL.*
3	***DNA Binding***	a) After digestion, add 400 µL Buffer AL. b) Mix thoroughly by vortexing. c) Add 400 µL ethanol (96–100%), mix again by vortexing. d) Transfer the entire lysate to the QIAamp MinElute column (in a 2 mL collection tube).

		e) Centrifuge at 8000 rpm for 1 min. f) Discard the flow-through and reuse the collection tube. *If total lysate volume exceeds column capacity (~800 µL), load in multiple spins.*
4	***Washing Steps***	a) Add 500 µL Buffer AW1, centrifuge at 8000 rpm for 1 min, discard flow-through. b) Add 700 µL Buffer AW2, centrifuge at 14,000 rpm for 3 min, discard flow-through. c) Perform dry spin at 14,000 rpm (or 20,000 × g) for 1 minute to ensure ethanol removal before elution.
5	***Elution***	a) Transfer contents to a clean 1.5 mL microcentrifuge tube. b) Add 50 µL Buffer ATE or nuclease-free water directly to the centre of the membrane. c) Incubate at room temperature for 5 minutes. d) Centrifuge at 14,000 rpm for 1 min to elute DNA. *Place the QIAamp column in a clean 1.5 mL microcentrifuge tube for elution. If low yield is expected, for higher DNA concentration, re-elute with the same 50 µL volume after the first spin or use a smaller volume (e.g., 30 µL).”*
6	***Storage***	Store DNA at 4°C and at -80°C for long-term use.

7	***DNA Quantification and Storage***	Quantify DNA using a fluorometric method (e.g., Qubit) or a spectrophotometer (e.g., Nanodrop), depending on concentration.

Ageing Studies Across Various Environmental Conditions and Substrates

The spectrophotometric analysis revealed notable variations in DNA integrity across different exposure periods—7, 15, and 25 days—demonstrating progressive degradation over time. Although the selected intervals of 7, 15, and 25 days do not necessarily reflect standard forensic evidence processing timelines, they provide insight into short-term degradation patterns. Previous research has examined DNA stability over months and years, demonstrating that environmental factors influence long-term degradation. Comparative studies can help refine preservation techniques for forensic applications. The A260/A280 ratio indicated that DNA purity remained relatively high in early stages but declined with extended exposure, suggesting increased fragmentation and contamination. Shed temperature (between 25°C and 28°C), humidity, and exposure to light (both fluorescent and sunshine). It was found to accelerate DNA breakdown, reinforcing the challenges of preserving forensic evidence in uncontrolled environmental conditions.

For this study, the unstressed controls could be preserved under optimal conditions, such as storage at -20°C without exposure to light, humidity, or microbial activity. By comparing these control samples with the experimental ones exposed to ambient conditions, it can accurately assess degradation trends and validate the extent to which preservation influences DNA integrity. Despite degradation, some samples retained sufficient quality for further forensic analyses, including PCR, sequencing, and genetic profiling. These findings emphasise the importance of optimised storage and handling protocols to ensure DNA viability and reliability in forensic investigations.

DNA Isolation and Extraction

After the designated periods, samples have undergone DNA isolation using the phenol-chloroform method. Phenol and chloroform are highly hazardous chemicals that pose health risks due to their toxicity and volatility. Their use requires proper safety precautions, including handling in a fume hood to prevent exposure to harmful fumes. Due to these risks, most forensic DNA extraction processes favor safer alternatives, such as silica column-based methods. Cotton underwear is the most commonly worn type in India, making it a suitable choice for forensic analysis. Cotton fabric has a high absorbency rate, allowing biological samples to be retained effectively. While other clothing items may also contain traces of biological evidence, depending on individual preferences, cotton underwear was preferred for this study due to its widespread use. Since a majority of people opt for cotton-based garments, it provided a reliable medium for sample collection and analysis, ensuring consistency in the research process.

This method effectively separates DNA from proteins and other contaminants, ensuring high-quality extraction suitable for downstream analysis. To begin the DNA isolation process, the cotton underwear sample must first be prepared by cutting out the stained or relevant portion using sterile scissors. The fabric is then placed in a microcentrifuge tube or a sterile container, and a suitable lysis buffer (commonly containing Tris-HCl, EDTA, SDS, and NaCl) is added to break open cells and release DNA. If bodily fluids are present, Proteinase K is included to digest proteins and enhance DNA extraction efficiency. The sample is incubated at 55°C for about an hour to ensure thorough lysis.(as shown in figure 3.2) If RNA removal is required, RNase can be added during this step. Once cell lysis is complete, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) is added to the lysate. This organic solvent mixture facilitates phase separation, allowing DNA to remain in the aqueous layer while proteins and lipids migrate to the organic phase. To guarantee adequate emulsification, the mixture is vortexed rapidly for 10–15 seconds. It is then centrifuged for 10 minutes at 4°C at 12,000–14,000 rpm. Three separate layers emerge during centrifugation: Lower organic phase with phenol and impurities; middle interface with denatured proteins; and upper aqueous phase with DNA.

The aqueous phase is carefully transferred to a fresh tube while avoiding the middle interface to prevent protein contamination. If further purification is needed, another round of phenol-chloroform extraction may be performed. For DNA precipitation, chilled isopropanol is added in a ratio of 2.5–3 volumes to the aqueous solution, mixed gently, and incubated at -20°C for 30 minutes to enhance DNA aggregation. The sample is then centrifuged at 12,000 rpm for 10 minutes to form a DNA pellet.

The supernatant is discarded, and the pellet is washed with 70% ethanol to remove residual phenol and impurities. After a brief air-drying step, the purified DNA pellet is resuspended in TE buffer or nuclease-free water for long-term storage (The nuclease-free water may be preferred if DNA will be used in PCR). Finally, a spectrophotometer analyses the extracted DNA for concentration and purity.

The A260/A280 ratio is used to assess sample integrity, with values around 1.8–2.0 indicating high purity. To improve purity, RNase treatment may be applied before phenol-chloroform extraction if RNA contamination is suspected. The spectrophotometry (Nanodrop) only gives a rough estimate and should ideally be supplemented by fluorometric assays (e.g., Qubit) for accurate quantification. DNA can then be used for further forensic analyses, including PCR, sequencing, or genetic profiling.

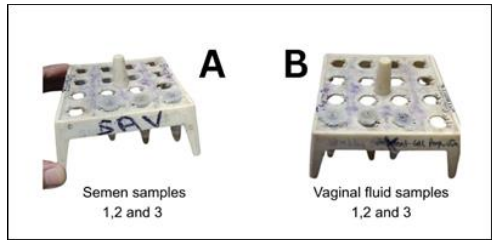

Figure 2.Seminal fluid and Vaginal fluid Samples for DNA Extraction

DNA Purification from Agarose Gel

The Agarose Gel Extraction Method is a widely used technique to isolate and purify specific DNA fragments after electrophoresis.(Refer Figure 3.3) It is a technique used after PCR or for cloning purposes, rather than being directly applicable to the extraction of forensic samples. This method is particularly useful in molecular biology and forensic applications where intact, high-quality DNA is required for downstream analyses such as cloning, sequencing, or PCR amplification. This method relies on separating DNA fragments by size, followed by excision from the gel and purification.

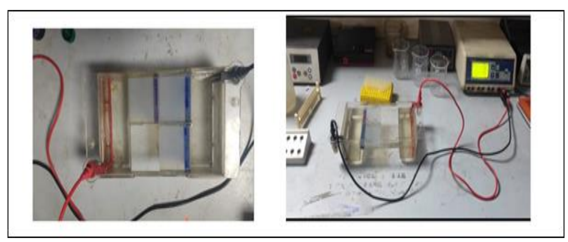

Figure 3.DNA Extraction using the Agarose Gel Extraction Method

To begin, the agarose gel is prepared by dissolving an appropriate percentage of agarose in Tris-acetate-EDTA (TAE) buffer or Tris-borate-EDTA (TBE) buffer, depending on the experimental requirements. The agarose solution is heated until fully dissolved, poured into a gel casting tray, and allowed to solidify. After setting, the gel is put in an electrophoresis chamber containing buffer, and the wells are carefully loaded with DNA samples that have been combined with a loading dye. A DNA ladder is also included as a molecular weight reference. Electrophoresis is conducted at a suitable voltage, typically between 80–120V, allowing DNA fragments to migrate towards the positive electrode. As DNA migrates through the gel, smaller fragments travel faster than larger ones, effectively separating them. To visualise DNA bands, ethidium bromide (EtBr) staining or non-toxic alternatives like SYBR Safe are used, and the gel is exposed to UV light in a gel documentation system. To minimize UV-induced DNA damage, limit exposure time and consider using blue light transilluminators or SYBR Safe dyes. The desired DNA fragment is identified and excised using a sterile scalpel or razor blade. Care is taken to remove excess gel material, ensuring minimal contamination. The gel slice containing the target DNA is transferred to a microcentrifuge tube, and gel solubilization buffer (typically containing salts such as guanidine hydrochloride) is added to dissolve the gel matrix. The sample is incubated at 50–60°C for 10–15 minutes, occasionally vortexing to ensure complete dissolution. Once the agarose has liquefied, the solution is subjected to silica-based column purification or organic extraction to remove contaminants. For silica-based purification, the dissolved gel solution is loaded onto a spin column containing silica membranes, which selectively bind DNA while impurities are washed away. Silica column-based methods are the gold standard for forensic DNA extraction, ensuring efficient and reliable purification. Phenol-chloroform extraction is rarely required unless purity is compromised, as it involves hazardous chemicals and complex procedures. Forensic laboratories prioritize silica-based techniques due to their safety, effectiveness, and consistency in preserving DNA integrity.

Successive washing steps using ethanol-based buffer ensure the removal of unwanted components. TE buffer or nuclease-free water are subsequently used to elute the purified DNA. In ethanol precipitation, proteins are removed using an equivalent proportion of phenol-chloroform-isoamyl alcohol. Absolute ethanol and sodium acetate are then added to precipitate DNA. The DNA pellet is centrifuged, cleaned with 70% ethanol, allowed to air dry, and then reconstituted in buffer. To ensure high purity and concentration, the final amount of purified DNA may be measured using spectrophotometers or calculated using gel documentation software by comparing band intensity to a DNA ladder. For extended usage, the sample is kept at either -20°C or -80°C. The A260/A280 ratio, which shows RNA and protein contamination, was used to assess the purity of the extracted DNA.Typically, a ratio of 1.8–2.0 suggests high-quality DNA suitable for molecular applications.

In the DNA extracted using the Agarose Gel Method, the purity ratios ranged from 1.75 to 1.92, signifying acceptable integrity for downstream analyses. This suggests that while minor protein contamination may be present, the DNA quality is sufficient for applications such as PCR, sequencing, and forensic identification.

IV. RESULTS

DNA concentration and absorbance at 260 nm (A260) are closely linked, as A260 is used to calculate concentration via the Beer-Lambert law, with 1 A260 equating to 50 ng/µL for double-stranded DNA (dsDNA). This establishes that DNA concentration is derived from absorbance rather than being independently measured. The data demonstrates a gradual decline in both DNA concentration and purity in semen and vaginal samples(Refer Figure 4.1) over time. As the preservation period lengthens, A260 values and DNA concentration progressively decrease, indicating ongoing genetic material degradation.

Figure 1. Extracted DNA Sample

Over 25 days, DNA concentration in semen samples reduced from 64 ng/µL at day 7 to 36 ng/µL, while vaginal samples followed a similar pattern, declining from 55 ng/µL to 30.5 ng/µL. The purity, assessed by the A260/A280 ratio, exhibited a slight reduction, with semen samples ranging from 1.76 to 1.68 and vaginal samples from 1.74 to 1.65. Despite this deterioration, purity values remained within acceptable limits for DNA analysis, ensuring sample integrity was largely preserved. However, the observed reduction at 25 days may affect highly sensitive applications such as STR profiling and qPCR, potentially impacting amplification efficiency and result accuracy. Optimizing preservation strategies is essential to maintaining DNA integrity, and improved storage conditions could mitigate degradation while enhancing the reliability of DNA analysis.

Since the values presented originate from a single trial, variations may exist, necessitating replicates for statistical accuracy. Although a decline in DNA concentration and purity suggests degradation, further verification via gel electrophoresis or qPCR is required to confirm DNA integrity loss. To ensure that observed intensity accurately reflects degradation and not variations in sample loading, equal amounts of DNA (ng) were used for analysis. These findings validate that prolonged preservation leads to measurable DNA loss, though samples still maintain reasonable quality for forensic and molecular studies. To ensure better long-term DNA stability, future studies might look at enhanced preservation methods that reduce degradation (as shown in Table 4.1).

Table 4.1: Time-Dependent Variation in DNA Yield and Quality in semen and vaginal samples

S.No	Sample	Period of Preservation	Absorbance at 260 nm (A260)	DNA Concentration (ng/µL)*	Purity (A260/A280)**
1	Semen (S1)	7 Days	0.128	64	1.76
2	Semen (S2)	15 Days	0.095	47.5	1.72
3	Semen (S3)	25 Days	0.072	36	1.68
4	Vaginal (V1)	7 Days	0.110	55	1.74
5	Vaginal (V2)	15 Days	0.084	42	1.70
6	Vaginal (V3)	25 Days	0.061	30.5	1.65

Each of these samples has its DNA extracted and amplified, as shown in Figure 4.2. The variance among each group is seen by the bands between the semen and vaginal samples. The intensity of the bands decreases with increasing preservation time, indicating the degree of sample deterioration.

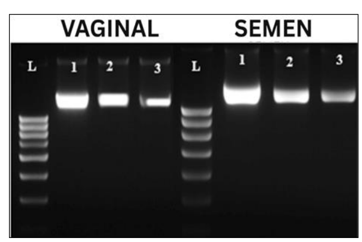

Figure 2. Separation of DNA fragments using Agarose Gel Electrophoresis

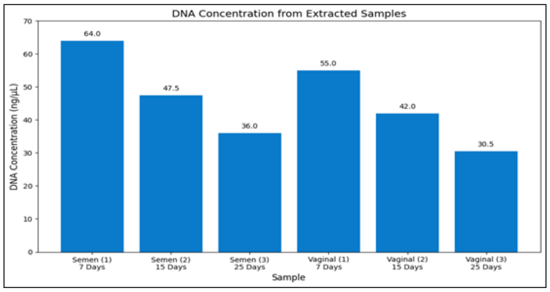

Figure 3. Graph showing DNA concentration from Extracted Samples

V. DISCUSSIONS

The impact of preservation time on DNA integrity in semen and vaginal samples is a critical concern in forensic science and molecular biology, as sample quality directly influences analysis accuracy. Over time, a steady decline in DNA concentration and purity has been observed, necessitating the development of optimized storage methods to preserve genetic material for forensic investigations. The data highlights a consistent reduction in DNA yield and purity due to prolonged storage, reinforcing the need for timely sample processing. The decrease in absorbance at 260 nm (A260) aligns with the reduction in DNA concentration, suggesting the inevitable molecular degradation that occurs when biological samples are not stored under ideal conditions. The decline from 64 ng/μL to 36 ng/μL in semen samples and 55 ng/μL to 30.5 ng/μL in vaginal samples over a span of 25 days exemplifies the gradual breakdown of nucleic acids, which can significantly impact forensic DNA profiling. Various environmental factors, enzymatic activity, and possible microbial contamination contribute to this degradation, making it imperative to establish preservation protocols that minimize such effects. Despite efforts to maintain DNA stability, natural deterioration remains unavoidable, and forensic scientists must account for these changes to ensure accurate genetic analysis.[20]

Several mechanisms contribute to DNA degradation in stored biological samples. Oxidative damage, a common molecular phenomenon, alters nucleotide structures, leading to fragmentation over time. Hydrolytic degradation, which occurs due to prolonged exposure to moisture, disrupts the phosphodiester bonds that maintain DNA's molecular integrity. Additionally, microbial contamination, particularly from bacteria and fungi, accelerates the breakdown of DNA through enzymatic activity. The presence of nucleases in biological fluids enhances degradation rates, further diminishing DNA concentration over time. Given these factors, forensic laboratories prioritize preservation techniques that mitigate these effects, employing cryogenic storage, chemical stabilization, and controlled environmental conditions to extend DNA viability. Nonetheless, even under optimized storage settings, degradation persists, reinforcing the urgency of rapid sample processing to prevent excessive loss of genetic material.[21]

DNA purity is another essential factor in forensic analysis, as contaminants can compromise the reliability of genetic profiling. The A260/A280 ratio serves as a standard measure of DNA purity, indicating potential protein contamination or degradation products. In this study, semen samples- maintained purity values between 1.76 and 1.68, while vaginal samples ranged from 1.74 to 1.65 over 25 days of storage. Although these values remain within acceptable limits for molecular analysis, their gradual decline indicates minor contamination. Protein residues, RNA fragments, and degraded nucleic acid byproducts can contribute to this reduction, influencing DNA usability in forensic investigations. While minor contamination does not necessarily render samples unusable, forensic applications often require highly pure DNA extracts for accurate profiling. In cases involving complex genetic analysis, such as kinship testing or degraded sample evaluation, forensic scientists must account for purity variations to ensure precise results. Despite the observed decline in DNA purity, the values remain sufficient for forensic purposes within the examined timeframe, reinforcing the feasibility of using stored samples for genetic analysis within reasonable preservation periods.[22]

Forensic science relies on efficient preservation methods to maintain DNA yield and integrity in biological samples. Cryogenic storage at ultra-low temperatures (-80°C or lower) minimizes enzymatic activity and oxidative damage, effectively slowing molecular degradation. Chemical stabilization, involving specialized buffer solutions and ethanol-based preservatives, enhances DNA stability by preventing hydrolytic breakdown and microbial contamination. Optimized storage environments, including humidity and temperature control, significantly reduce DNA degradation rates, contributing to reliable forensic analysis. Additionally, rapid sample processing ensures that DNA is extracted and analyzed before degradation reaches critical levels. Some forensic laboratories implement desiccation techniques to remove moisture from biological samples, improving DNA stability in settings where refrigeration is unavailable. By integrating these strategies, forensic investigators mitigate the effects of prolonged storage on DNA integrity, enhancing sample usability for genetic profiling and criminal investigations.[23]

Advanced preservation techniques hold promise for extending DNA viability beyond conventional methods. Cryogenic storage is among the most effective solutions, yet its accessibility and cost limitations prompt researchers to explore alternative stabilization approaches. Nanotechnology-based preservation, employing nanoparticle coatings to shield DNA from environmental damage, is emerging as a potential innovation in forensic science. Artificially engineered stabilization buffers designed to withstand prolonged storage conditions may further improve DNA longevity. [24]

Additionally, advancements in enzyme inhibition could significantly reduce nucleic acid breakdown by neutralizing DNA-degrading enzymes within biological samples. Future research into these preservation technologies could enhance forensic and clinical applications, allowing investigators to maintain reliable genetic profiles even in degraded samples. By exploring new methods for extending DNA stability, forensic scientists aim to improve sample usability, ensuring accurate results even from aged biological evidence.[24]

The findings of this study emphasize the progressive nature of DNA degradation and the necessity of strategic preservation approaches in forensic investigations. Despite the observable reductions in DNA concentration and purity, the analyzed samples retained sufficient quality for forensic purposes within a 25-day timeframe. These results highlight the importance of timely sample processing and optimized storage conditions to minimize degradation risks. Forensic scientists must employ specialized preservation protocols that maximize DNA yield while maintaining purity levels appropriate for genetic profiling. Implementing cryogenic storage, chemical stabilization, and rapid sample processing ensures that biological evidence remains viable for forensic applications. Further research into alternative preservation techniques may improve forensic sample management, enabling investigators to enhance DNA stability for extended periods. As forensic science continues to advance, the development of innovative storage solutions and stabilization methods will play a crucial role in optimizing DNA yield and purity in biological evidence.[25]

DNA degradation remains a fundamental challenge in forensic sample management, influencing the reliability of genetic analysis. While degradation over time is inevitable, forensic laboratories can adopt advanced preservation techniques to mitigate its effects. By maintaining optimal storage environments and implementing rapid processing protocols, forensic scientists ensure that DNA remains viable for criminal investigations. The study underscores the importance of efficient sample handling, reinforcing the need for strategic preservation methods that sustain DNA integrity in forensic and clinical applications. As research into DNA stability progresses, new preservation technologies may emerge, enabling forensic professionals to extend sample usability beyond current limitations. These advancements will contribute to the refinement of forensic methodologies, improving the accuracy and reliability of genetic profiling in criminal investigations. The continuous development of preservation techniques ensures that DNA degradation does not hinder forensic analysis, supporting the long-term usability of biological evidence in forensic science.[26]

Limitations and Challenges

Despite the advancements in forensic DNA preservation techniques, several limitations and challenges persist in ensuring sample integrity over extended periods. One of the primary limitations is the inevitable degradation of DNA due to environmental factors, such as temperature fluctuations, humidity, and exposure to microbial contaminants. Even with optimized storage conditions, nucleic acids deteriorate over time, affecting DNA yield and purity. Hydrolytic and oxidative degradation, in particular, remain significant concerns, as they disrupt the molecular structure of DNA, leading to fragmentation and reduced usability for forensic analysis. Another challenge is the variability in DNA preservation methods. While cryogenic storage at ultra-low temperatures is practical, it requires specialized facilities and resources, making it inaccessible to forensic laboratories with limited infrastructure. Chemical stabilization techniques, though useful, may introduce potential contaminants that affect downstream applications. The balance between preserving DNA while minimizing external influences remains a complex issue in forensic science.[27]

Additionally, sample contamination remains a persistent challenge, especially in biological fluids such as semen and vaginal samples. The presence of proteins, enzymes, and microbial agents can accelerate DNA degradation, requiring rigorous purification steps. Standard forensic protocols aim to minimize contamination, but external factors such as improper sample collection, handling errors, and suboptimal storage conditions continue to pose risks to DNA integrity.[28]

Long-term preservation attempts are also hampered by technological constraints. Innovations that prolong the survivability of stored forensic materials beyond traditional timeframes are required, as current preservation technologies mainly concentrate on short-term DNA stability. Cryogenic preservation slows down deterioration, but it is not a long-term fix, which highlights the need for ongoing research into new preservation techniques. Another level of complexity is introduced by ethical and legal issues. In forensic investigations, chain-of-custody requirements mandate strict sample handling protocols to ensure admissibility in court proceedings. The risk of degradation over time could lead to compromised forensic evidence, impacting the credibility of genetic profiling in legal cases. These limitations and challenges necessitate a multidimensional approach to DNA preservation, incorporating technological advancements, resource accessibility, and forensic best practices to maintain sample viability for genetic analysis.[29]

Future Directions

As forensic science advances, several promising directions are emerging in DNA preservation. One of the most critical areas of research is the development of enhanced stabilisation techniques that extend the longevity of DNA beyond conventional storage methods. Innovations in molecular preservation, such as nanoparticle-based encapsulation, could revolutionise DNA storage by protecting nucleic acids from environmental damage while ensuring long-term stability. Cryogenic conservation will continue to play a crucial role in forensic sample management, but researchers are exploring alternative low-temperature methods that require less infrastructure and cost. Freeze-drying techniques, for example, have shown the potential to preserve DNA for extended periods without requiring deep freeze storage. Further research into desiccation and vacuum-sealing methods could yield efficient alternatives for forensic laboratories with limited resources.[30]

Artificially engineered buffer solutions represent another avenue for improving DNA stability. Developing advanced chemical preservatives tailored to different biological sample types may provide more effective stabilization, ensuring minimal degradation over time. These solutions could be optimized to prevent enzymatic activity while maintaining DNA integrity for forensic profiling. The role of AI and computational modeling is also expanding in forensic applications. Machine learning algorithms can help predict DNA degradation rates, enabling forensic scientists to establish preservation protocols that maximize sample usability. AI-driven simulations can assess environmental impact factors, leading to improved preservation strategies and enhanced sample quality predictions.[31]

Ethical considerations in forensic DNA storage will likely evolve alongside technological advancements. With growing concerns about genetic privacy and data protection, researchers must establish standardized guidelines to ensure ethical DNA preservation practices. Future forensic methodologies must incorporate legal compliance and technological efficiency, balancing accuracy with ethical transparency.[32]

Integrating interdisciplinary approaches—including biotechnology, chemistry, AI, and forensic genetics—will shape the next generation of DNA preservation techniques. Continued research into molecular stability mechanisms and innovative storage solutions will enhance forensic capabilities, ensuring reliable genetic profiling in criminal investigations.

VI. CONCLUSION

DNA preservation plays a crucial role in forensic science, as the integrity of biological samples directly impacts the accuracy of genetic analysis. This study highlights the challenges associated with DNA degradation over time and underscores the importance of strategic preservation approaches to maintain sample viability. While conventional storage methods such as cryogenic freezing and chemical stabilization provide effective short-term solutions, forensic scientists must continually adapt to evolving preservation technologies to minimize DNA loss. The observed decline in DNA concentration and purity over 25 days demonstrates the inevitable molecular degradation of stored samples. Environmental exposure, enzymatic activity, and contamination contribute to DNA breakdown, reinforcing the necessity of optimized storage protocols. Preservation techniques, including cryogenic storage, specialized buffer solutions, and rapid sample processing, mitigate degradation risks while maximizing DNA yield for forensic investigations.[32]

As forensic science advances, future research must focus on extending DNA stability beyond conventional methods. Nanotechnology, AI-driven predictive modelling, and engineered stabilization solutions represent promising directions for improving forensic DNA preservation. The interdisciplinary integration of biotechnology, chemistry, and forensic genetics will further refine sample management practices, ensuring that DNA remains viable for reliable genetic profiling. Ultimately, forensic sample management must balance technological advancements with ethical considerations, ensuring DNA preservation aligns with legal standards and privacy regulations. The continuous development of innovative preservation techniques will strengthen forensic capabilities, enhancing the accuracy and reliability of genetic evidence in criminal investigations. By implementing strategic storage solutions and fostering research into molecular preservation, forensic scientists can sustain DNA integrity over extended periods, contributing to the progress of forensic and molecular biology applications.[33]

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