EVALUATION OF THE STABILITY OF DNA METHYLATION MARKERS IN BIOLOGICAL STAINS AND ITS IMPACT ON FORENSIC INVESTIGATIONS

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ABSTRACT

Background: Study of epigenetic modifications such as DNA methylation has become an important tool in forensic investigations due to its reliability and specificity. DNA methylation is highly dynamic and sensitive to several environmental and lifestyle factors. DNA samples collected from crime scenes can be tested according to their methylation patterns to help identify different types of biological pieces of evidence, including hair, blood, semen, and saliva found at the crime scene. Furthermore, it can help in the identification of sex, age and shed light on the overall identity of the suspect or victim. **Objectives:** This study aims to validate the use of DNA methylation-specific markers in identifying peripheral blood, menstrual blood, and saliva and investigate the stability of these markers. Additionally, this research assesses the effect of exposure of blood and saliva to different environmental conditions on detecting DNA methylation-specific markers. **Methodology:** The samples used in this study are peripheral blood, saliva, and menstrual blood. DNA has been extracted from all samples, and its quality has been detected on gel electrophoresis. Then bisulfite conversion and real-time PCR were applied using BLM1 primer to detect peripheral blood samples, MENS1 primer to detect menstrual blood samples, and SPEI1 to detect saliva samples. Dried Stains from the saliva, menstrual blood, and peripheral blood samples have been collected and exposed to different environmental conditions. **Results:** The results of real-time PCR and statistical analysis of BLM1 and MENS1 primers showed better results than SPEI1 primers in identifying fresh body fluids and those exposed to different environmental conditions of degradation. **Conclusion:** DNA methylation is highly specific to the person's tissue type, age, and sex. This unique characteristic of DNA methylation is exploited in the identification of victims or culprits during a forensic investigation. The amount and the integrity of DNA used for analysis are often the determining factors in the success of methylation studies. Various factors such as exposure to UV radiation, high temperature, PH, and salt concentration can affect DNA stability.

Keywords: DNA methylation, body fluids identification, environmental conditions, DNA extraction, real-time PCR.

INTRODUCTION

Body fluid identification is of immense importance in the forensic investigation as it can help reconstruct the crime scene. Several genome-wide analysis studies have been performed on tissue samples to identify tissue-specific methylation markers. This marker data is publicly available and can be used for forensic investigations **(Forat et al., 2016)**. If preliminary screening methods such as external morphology or physical examination can be narrowed down to a set of sample tissue, DNA methylation can effectively confirm the type of body fluid. This is very useful in the forensic

investigation if the sample recovered is very small in amount **(Vidaki et al., 2016)**.

Each body fluid has a specific origin, composition, and function. The cells present in each fluid receive different developmental signals that ultimately lead to terminal differentiation. Epigenetic signals are also important components of this specific tissue development. Each body tissue shows a particular pattern of methylation, which is a unique feature to be utilized to identify an unknown fluid found at the crime scene. Blood, saliva, and semen are the major body fluids utilized for forensic analysis in criminal investigations **(Gomes, Kohlmeier, and Schneider, 2011).** Genome sequencing and methylation data aid in the identification of the tissue evidence as well as the individual identity. Another distinguishing feature of DNA methylation is that it can also differentiate between normal and menstrual blood. Menstrual blood contains several proteins and other components of the endometrium wall. So, DNA methylation analysis can also confirm the presence of these components.

Epigenetics is the study of phenotypic changes caused by alterations in gene expression but not in the genome sequence itself. Histone modifications and DNA methylation are the two major epigenetic mechanisms. Due to its importance in gene regulation, mutations in epigenetic machinery components often result in embryonic lethality or pleiotropic effects. Altered epigenetic regulation has been reported to be responsible for several imprinting disorders and cancer in humans. Especially aberrant DNA methylation is a common feature in several human disorders **(Godman, 2006)**. They alter the overall morphology of the DNA structure, thereby affecting the accessibility of DNA for regulatory proteins. Alleles do not show a change in the sequence but show different expressions, termed epialleles. These epialleles are stably inherited to the successive generations. The expression of the other allele in the zygote is suppressed by epigenetic mechanisms **(Leme, 2012).**

When developmental signals trigger morphogenesis and differentiation, the epigenetic changes are modified accordingly for the proper growth and development of the organism. One important aspect of epigenetics is the environmental effect. The behavioural and the environmental impact on the parent generation are fit into the epigenetic code. Epigenetic code can also change according to the food habits of an individual. So, it is a highly dynamic and sensitive mechanism to fine-tune the expression of key regulators in metabolism. Epigenetic changes are also subject to evolution and natural selection. For example, humans share most of their genome with chimpanzees, yet both appear different. So, the genome sequence is not the only determinant of an organism's phenotype and its evolutionary fitness **(Alegría et al., 2011).** These modifications are known as epigenetic modifications, and they help the cell to regulate the extent of transcription **(Grummt and Längst, 2013).** Epigenetic modifications are major signals that respond to developmental and extrinsic signals, so there is a strong correlation between the types of DNA methylation at specific loci and a particular tissue. This uniqueness of tissue-specific Methylation has been successfully employed to identify the type of body fluid recovered from a crime scene **(Khader and ghai, 2015).**

DNA methylation is a reversible process. Several genes in humans are shown to be methylated in their genomic loci. DNA methylation is established early in the embryonic stages and is maintained throughout the life of an organism. Housekeeping genes in a cell are constitutively expressed, whereas few developmentally important or metabolic enzymes are expressed only in response to a signal. So, the expression of such genes is regulated at the DNA methylation level. Due to this intricate mechanism, each tissue type shows a characteristic DNA methylation pattern **(Zilberman and Henikoff, 2007).** Even monozygotic twins who share identical DNA sequences show different DNA methylation patterns **(Tillo**

et al., 2016). The most important advantage of DNA methylation analysis is that it is a stable mark. DNA is quite stable, unlike protein or RNA samples, and methylation markers do not show much variation.

Epigenetic DNA methylation is a good molecular marker that strongly correlates with age. Since it is a sensitive and highly reliable molecular method, it has become a convenient and popular method for age prediction in forensic studies **(Wu, H et al., 2014).** However, certain factors such as physical activity, diet, and exposure to mutagens affect the success rate of this prediction. Age prediction becomes difficult in the case of divergent populations as they show huge variations in methylation patterns. The sex of the individual also influences DNA methylation. Sex-specific CpG islands have been shown to be differentially methylated between male and female samples. So, gender differences must be considered while predicting age **(Nagase and Ghosh, 2008).**

Several methylation analysis techniques have been developed to provide accurate, reliable details that can be used as a supporting factor in judicial inquiry **(Farzeen Kader et al., 2019).**

Each of these methods has its advantages and disadvantages **(Shin et al., 2016).** They include Methylation-specific PCR **(Herman et al., 1996)**, MethyLight **(Eads, 2000)**, Next-generation sequencing, Methylation sensitive high-resolution melting (MS-HRM), Sanger sequencing of bisulfite-treated DNA, Mass array Details, Pyrosequencing **(Tost and Gut, 2007)**, and SMART-MSP. These methods provide highly accurate molecular data that can serve as conclusive evidence—the basic detection method of DNA methylation in bisulfite sequencing.

The epigenome is highly sensitive and reacts to changing external conditions. DNA methylation changes have been associated with cancer, immunologic disorders, infertility, and neurodegenerative pathologies. Several reports have shown that exposure to various chemicals during the prenatal or adult stage can result in DNA methylation changes in germ cells **(Feil and Fraga, 2012)**. These changes are stably transmitted across generations with phenotypic consequences. Changes in DNA methylation patterns in response to varying environmental conditions have been reported in human blood leukocytes **(Pacchierotti and Spanò, 2015).** It is known that environmental toxic chemicals such as As, Hg, and Pb have been associated with human pathologies such as cardiovascular diseases and autoimmune diseases. It has been found that exposure to these harmful chemicals leads to epigenomic changes in the DNA that result in pathological conditions. Exposure to air pollution due to urbanization and industrialization also has a similar effect on DNA methylation patterns. Several chemicals such as bisphenol and perfluoroalkyl substances cause hormonal imbalances in humans. Analysis of methylation patterns in these individuals revealed that indeed these chemicals cause alterations in the epigenome **(Seisenberger et al., 2012).** The amount and the integrity of DNA used for analysis are often the determining factors in the success of methylation studies. Various factors such as exposure to UV radiation, high temperature, pH, and salt concentration can affect DNA stability. Often contamination with nucleases, physical shearing, and repeated freeze-thawing also results in DNA sample degradation (Srinivasan et al., 2002). Under dry conditions, complete thermal degradation of DNA occurs at 190oC. At high temperatures, DNA gets denatured, and ssDNA is more prone to strand breakage. Direct DNA damage can occur when DNA is exposed to UV light. UV exposure results in the bond formation between successive thymidine. It leads to pyrimidine dimers, thereby causing a strand breakage **(Karni et al., 2013).** Temperature and relative humidity also affect DNA methylation status. DNA methylation at ICAM-1, TLR-2, CRAT, and IFN-γ has shown a strong correlation with increased temperature. A 10% increase in relative humidity showed a 5% decrease in DNA

methylation at ICAM-1 **(Bind et al., 2014).** UV radiation has been a major threat in recent years as the ozone layer is being depleted due to pollution. Human T-cells exposed to UV-B radiation show significant global hypomethylation. Upon exposure to UV-B radiation, a significant decrease in the transcript levels of DNA methyltransferase one was observed **(Zhu et al., 2013)**. This research aims were:

-To validate the use of DNA methylation body fluid specific markers in identifying peripheral blood, menstrual blood, and saliva.

-To investigate the stability of these DNA methylation markers in blood and salivary stains.

-To assess the effect of exposure of blood and saliva to different environmental conditions on detecting DNA methylation-specific markers.

MATERIALS & METHODS

The research was conducted after the approval of the council ethical committee of the Biotechnology college in a university in Dubai. Written consent was given to 30 participants. It was read and fully understood by the volunteers and signed. The peripheral blood was collected using a Venepuncture needle attached to it 1.5 ml tube containing an anticoagulant. For menstrual blood, the volunteers did a self-swab using a sterile cotton-tipped applicator and gloves, then they placed the swab in Eppendorf sterile tube. For saliva, the volunteers were asked to avoid foods with high sugar or acidity or high caffeine content immediately before sample collection since they may compromise the assay by lowering saliva pH and increasing bacterial growth. Then they were asked to rinse their mouth with water to remove food residue and wait at least 10 minutes after rinsing to avoid sample dilution before collecting saliva. After these instructions were given to allow saliva to pool in the mouth, with head tilted forward, volunteers should drool through the Saliva Collection Aid (SCA) to collect saliva in the cryovial. This step was repeated until a sufficient sample was collected. Then the samples were reserved in air space in the vial to accommodate the expansion of saliva during freezing at -20C.

According to the manufacturer's instructions, DNA was extracted from the blood, menstrual blood, and saliva using the QIAamp DNA Investigator Kit (Qiagen). Then the verification of successful DNA extraction was done using gel electrophoresis. DNA samples were treated with sodium bisulfite, which converts unmethylated cytosines into uracil, while the methylated ones remain unchanged using EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's protocol. Real-time PCR was used to amplify DNA methylated specific markers. It was conducted using real-time PCR CFX manager software. The markers used were Cg13763232 located on chromosome 2 for peripheral blood, Cg09696411 located on chromosome 12 for menstrual blood, and Cg21597595 located on chromosome 2 for saliva. The primers used are described in the table below.

Table (1): Primers used

Marker	Primer	Sequence (5′–3′)
cg13763232 **(Peripheral blood)**	BLM1-F	TAGTTGATATTGGT TTGGTA
	BLM1-R	CAAATAACTCAAT TTCTCTAC
cg09696411 **(Menstrual blood)**	MENS1-F	GAT TAG GTT TAG GGA AGT TTT TAT
	MENS1-R	ACC CTC TAA AAC TTA TAC TCC C
cg21597595 **(Saliva)**	Spei1-F	CTA CAA AAA TAA ATA TAA ATA TAA AA
	Spei1-R	TTT TGG TGG TTT GGG GTT TA

- Dried Stains from the saliva, menstrual blood, and peripheral blood samples were collected and exposed to different environmental conditions:

1) Freshly stained samples at room temperature:

- Peripheral blood (10 samples)
- Menstrual blood (10 samples)

- Saliva (10 samples)

2) Temperature at 40°C (2 weeks):

- Peripheral blood (10 samples)
- Menstrual blood (10 samples)
- Saliva (10 samples)

3) Exposure to UV light (10-15 minutes).

- Peripheral blood (6 samples)
- Menstrual blood (6 samples)
- Saliva (6 samples)

RESULTS

DNA extraction results:

DNA was extracted successfully from 10 fresh peripheral blood, saliva, and menstrual blood samples, as shown in fig. 1, 2, and 3, respectively.

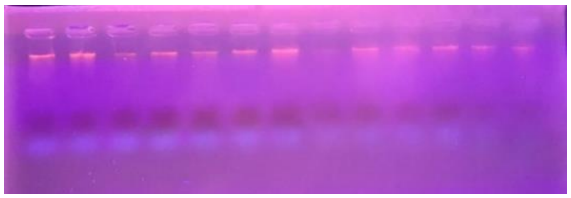


Figure (1): DNA of peripheral blood samples on gel electrophoresis

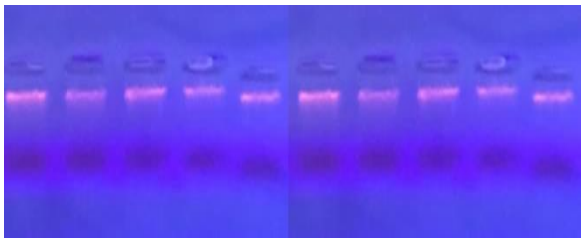


Figure (2): DNA of saliva samples on gel electrophoresis

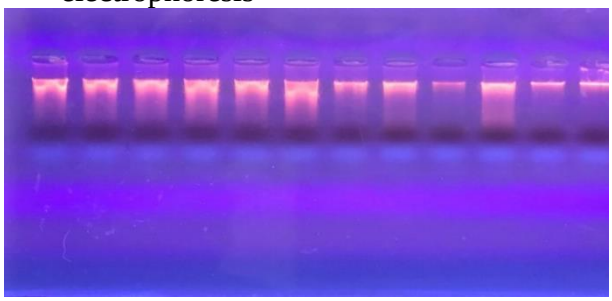


Figure (3): DNA of menstrual blood samples on gel electrophoresis

DNA was extracted successfully from 2 samples that were randomly selected from each of the peripheral blood, saliva, and menstrual blood after being exposed to 40°C temperature for two weeks.

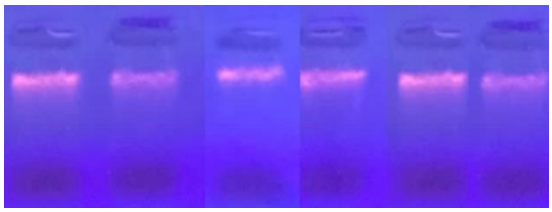


Figure (4): DNA samples exposed to 40°C temperature on gel electrophoresis

DNA was extracted successfully from 2 samples that were randomly selected from peripheral blood, saliva, and menstrual blood after being exposed to UV light for 15 minutes.

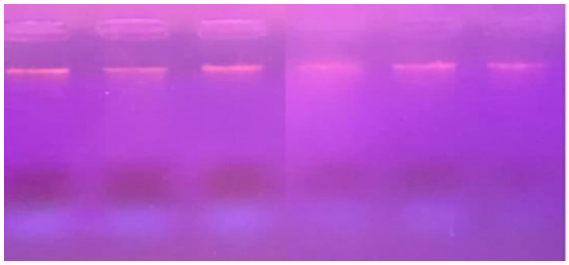


Figure (5): DNA samples that were exposed to UV light on gel electrophoresis

Real-time PCR results:

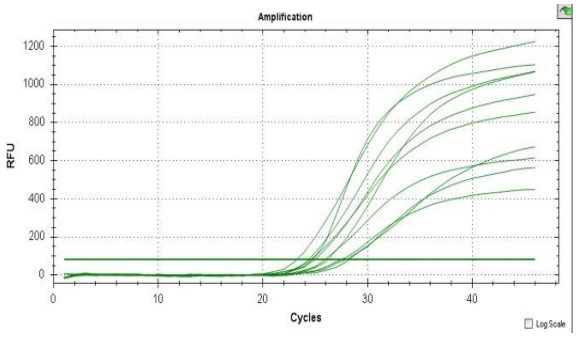


Figure (6): Amplification of peripheral blood samples using BLM1 primer.

Table (2): Peripheral blood samples and their Cq values

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	B1	28.22
SYBR		Unkn	B2	25.90
SYBR		Unkn	B3	27.44
SYBR		Unkn	B4	24.68
SYBR		Unkn	B5	27.91
SYBR		Unkn	B6	24.33
SYBR		Unkn	B7	23.21
SYBR		Unkn	B8	24.53
SYBR		Unkn	B9	26.19
SYBR		Unkn	B10	24.93

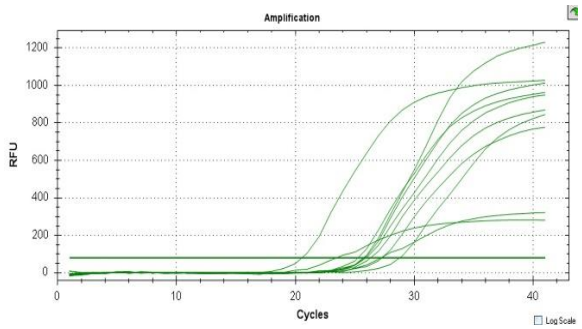


Figure (7): Amplification of menstrual blood samples using MENS1 primer. (The first peak to be amplified started at 18.5 cycles, while the eight other peaks started to amplify at 24 cycles and the lowest peak started to amplify at 20 cycles)

Table (3): Menstrual blood samples and their Cq values

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	MENS1	23.48
SYBR		Unkn	MENS2	25.75
SYBR		Unkn	MENS3	27.22
SYBR		Unkn	MENS4	25.28
SYBR		Unkn	MENS5	20.56
SYBR		Unkn	MENS6	25.84
SYBR		Unkn	MENS7	27.29
SYBR		Unkn	MENS8	26.33
SYBR		Unkn	MENS9	28.79
SYBR		Unkn	MENS10	25.99

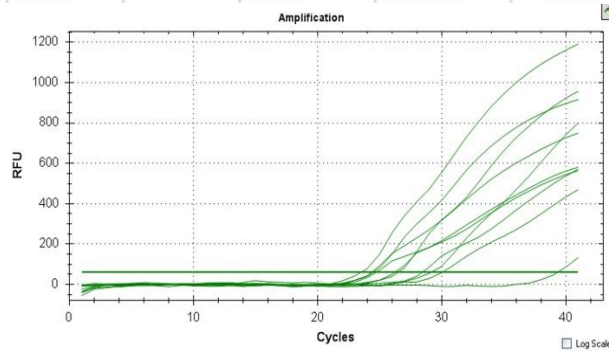


Figure (8): Amplification of saliva samples using SPEI1 primer. (9 peaks started to amplify at 22 cycles and the lowest peak started to amplify at 14 cycles)

Table 4: Saliva samples and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	SF1	28.48
SYBR		Unkn	SF2	30.11
SYBR		Unkn	SF3	29.21
SYBR		Unkn	SF4	39.40
SYBR		Unkn	SF5	24.78
SYBR		Unkn	SF6	24.52
SYBR		Unkn	SF7	26.27
SYBR		Unkn	SF8	24.38
SYBR		Unkn	SF9	26.54
SYBR		Unkn	SF10	23.55

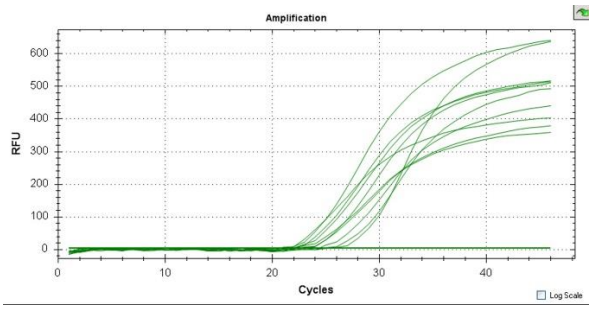


Figure (9): Amplification of freshly stained peripheral blood samples using BLM1 primer. (The peaks started from 22 cycles till 24.5 cycles)

Table (5): Stained blood samples and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	BS1	24.40
SYBR		Unkn	BS2	22.58
SYBR		Unkn	BS3	20.81
SYBR		Unkn	BS4	22.12
SYBR		Unkn	BS5	22.58
SYBR		Unkn	BS6	21.87
SYBR		Unkn	BS7	25.57
SYBR		Unkn	BS8	24.87
SYBR		Unkn	BS12	21.58
SYBR		Unkn	BS14	22.01

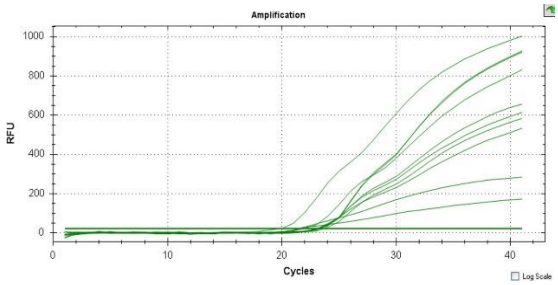


Figure (10): Amplification of freshly stained saliva samples using SPEI1 primer. (The highest peak started to amplify at 18 cycles and the other 9 peaks started to amplify at 20 cycles)

Table (6): Freshly stained saliva samples and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	SSF1	21.40
SYBR		Unkn	SSF2	21.49
SYBR		Unkn	SSF3	23.57
SYBR		Unkn	SSF4	23.05
SYBR		Unkn	SSF5	23.14
SYBR		Unkn	SSF6	23.05
SYBR		Unkn	SSF7	19.70
SYBR		Unkn	SSF8	22.99
SYBR		Unkn	SSF9	21.76
SYBR		Unkn	SSF10	23.26

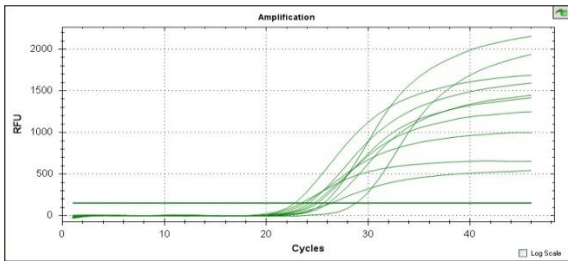


Figure (11): Amplification of 10 peripheral degraded blood samples at 40° C using BLM1 primer. (They all started to amplify at 20 cycles)

Table (7): Peripheral blood samples degraded at 40o C and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	BSD1	26.31
SYBR		Unkn	BSD2	24.64
SYBR		Unkn	BSD3	23.76
SYBR		Unkn	BSD4	23.39
SYBR		Unkn	BSD6	22.74
SYBR		Unkn	BSD7	26.06
SYBR		Unkn	BSD8	25.03
SYBR		Unkn	BSD9	25.58
SYBR		Unkn	BSD10	28.76
SYBR		Unkn	BSD12	24.19

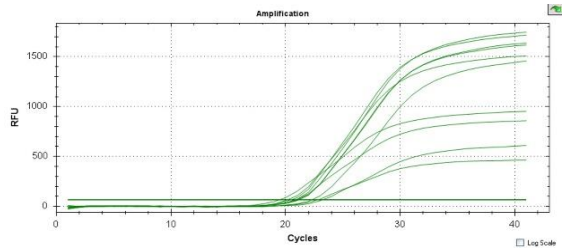


Figure (12): Amplification of 10 menstrual degraded blood samples at 40° C using MENS1 primer. (All started to amplify at 18 cycles)

Table (8): Menstrual blood samples degraded at 40° C and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	MSD1	22.74
SYBR		Unkn	MSD2	20.86
SYBR		Unkn	MSD3	23.18
SYBR		Unkn	MSD4	22.24
SYBR		Unkn	MSD5	19.40
SYBR		Unkn	MSD6	21.02
SYBR		Unkn	MSD7	20.11
SYBR		Unkn	MSD8	21.11
SYBR		Unkn	MSD9	20.78
SYBR		Unkn	MSD10	20.42

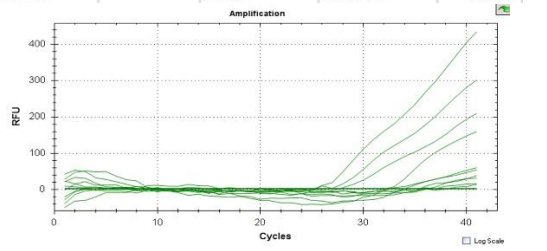


Figure (13): Unsuccessful amplification of all 10 saliva degraded samples at 40° C using SPEI1 primer.

Table (9): Saliva degraded samples at 40° C and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	s1	38.14
SYBR		Unkn	s2	36.26
SYBR		Unkn	s3	37.02
SYBR		Unkn	s4	35.48
SYBR		Unkn	s5	27.65
SYBR		Unkn	s6	32.59
SYBR		Unkn	s7	33.96
SYBR		Unkn	s8	25.44
SYBR		Unkn	s9	25.24
SYBR		Unkn	s10	32.12

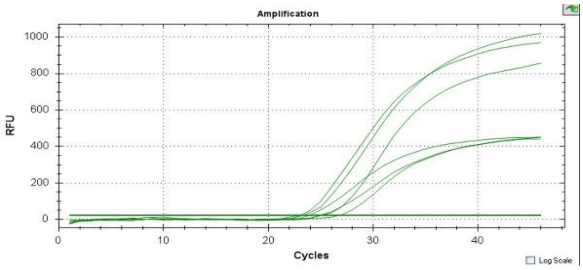


Figure (14): UV light degraded peripheral blood samples using BLM1 primer. (All started to amplify at 22 cycles)

Table (10): UV light degraded peripheral blood samples and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	BSUV1	24.87
SYBR		Unkn	BSUV2	26.72
SYBR		Unkn	BSUV3	23.86
SYBR		Unkn	BSUV4	23.24
SYBR		Unkn	BSUV5	25.04
SYBR		Unkn	BSUV6	23.04

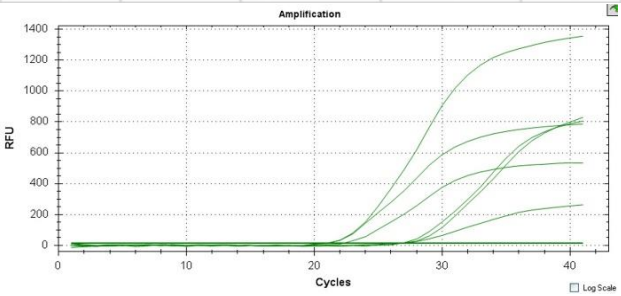


Figure (15): UV light degraded menstrual blood samples using MENS1 primer. (3 of the peaks started to amplify at 20 cycles and the other 3 peaks started to amplify at 28 cycles)

Table (11): UV light degraded menstrual blood samples and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	MUV1	22.24
SYBR		Unkn	MUV2	26.45
SYBR		Unkn	MUV3	26.74
SYBR		Unkn	MUV4	20.85
SYBR		Unkn	MUV5	21.06
SYBR		Unkn	MUV6	26.63

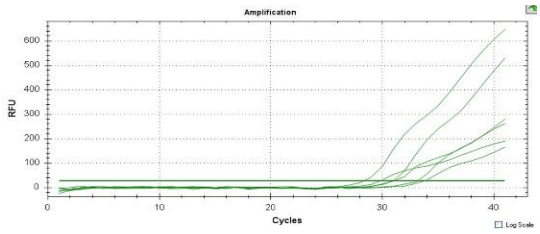


Figure (16): UV light degraded saliva samples using SPEI1 primer. (The highest peak started to amplify at 26 cycles and the other 5 peaks started to amplify at 28 cycles)

Table (12): UV light degraded saliva samples and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	SSUV1	33.80
SYBR		Unkn	SSUV2	29.83
SYBR		Unkn	SSUV3	31.07
SYBR		Unkn	SSUV4	33.17
SYBR		Unkn	SSUV5	28.36
SYBR		Unkn	SSUV6	31.00

Mixed conditions of the samples on real-time PCR:

All samples of peripheral and menstrual blood that were exposed to different environmental conditions were exposed to UV light and were amplified in real-time PCR together to assess the sensitivity of the primers and to compare between the different conditions of each sample.

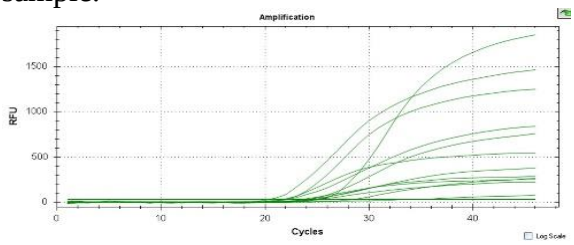


Figure (17): 12 peripheral blood samples exposed to different environmental conditions using BLM1 primer. (The highest peak started to amplify at 26 cycles and the other 5 peaks started to amplify at 28 cycles)

Table (13): Samples of peripheral blood exposed to different environmental conditions and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	FB1	23.73
SYBR		Unkn	FB2	24.94
SYBR		Unkn	FB3	25.06
SYBR		Unkn	SB1	28.61
SYBR		Unkn	SB2	35.58
SYBR		Unkn	SB3	24.39
SYBR		Unkn	SBDT1	22.31
SYBR		Unkn	SBDT2	24.34
SYBR		Unkn	SBDT3	20.50
SYBR		Unkn	SBDUV1	21.78
SYBR		Unkn	SBDUV2	25.31
SYBR		Unkn	SBDUV3	22.13

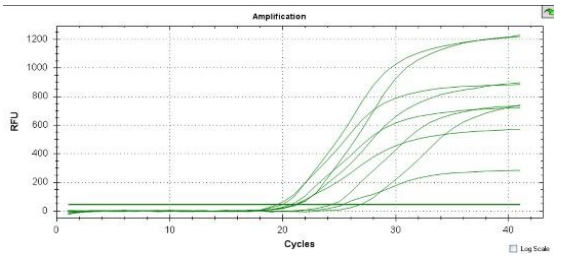
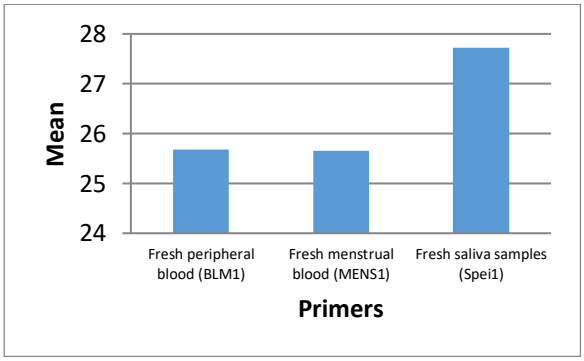


Figure (18): 9 menstrual blood samples exposed to different environmental conditions using MENS1 primer. (6 peaks was amplified at 18 cycles, while 3 peaks were amplified at 22 cycles)

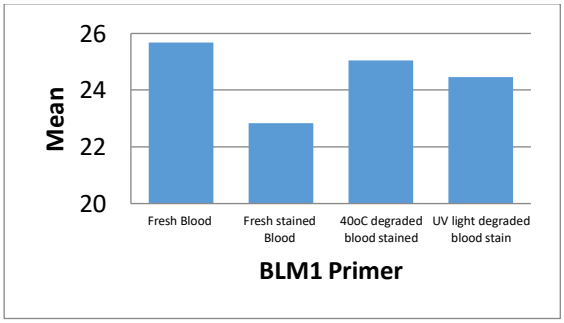
Table (14): Samples of menstrual blood exposed to different environmental conditions and their Cq values.

Fluor	Target	Content	Sample	Cq
SYBR		Unkn	MF1	25.30
SYBR		Unkn	MF2	24.26
SYBR		Unkn	MF3	19.43
SYBR		Unkn	MD1	20.63
SYBR		Unkn	MD2	21.07
SYBR		Unkn	MD3	19.96
SYBR		Unkn	MUV1	21.06
SYBR		Unkn	MUV2	21.27
SYBR		Unkn	MUV3	26.82

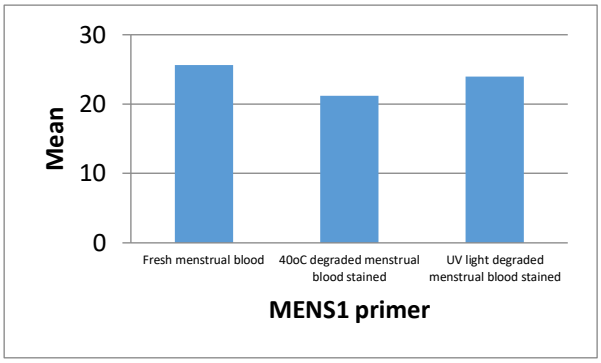
STATISTICAL ANALYSIS:



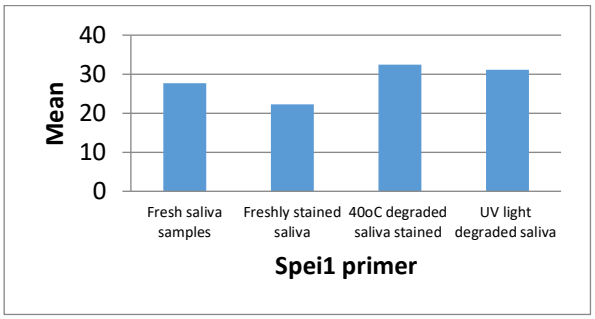
Graph (1): Comparison of different body fluid primers using mean value.



Graph (2): Comparison of different environmental factors of peripheral blood samples using BLM1 primer.



Graph (3): Comparison of different environmental factors of menstrual blood samples using MENS1 primer.



Graph (4): Comparison between different environmental factors of saliva samples using Spei1 primer.

DISCUSSION

DNA methylation is sensitive to environmental changes, nutritional status, age, and lifestyle habits. Each tissue exhibits specific methylation signatures at particular loci. These unique signature loci can be used as markers to identify the nature of an unknown biological sample. Several biological samples such as blood, saliva, and semen can be recovered from a crime scene. The methylation pattern analysis confirms the body fluid type and helps the investigation agency reconstruct the crime scene. Several genome-wide association studies have established DNA methylation signatures for several tissues. Analysis of these signature sequences can help in the identification of the tissue type, age, and sex of the tissue. Due to its immense importance in medicine and forensic study, various methods have been developed to accurately determine DNA methylation patterns at the whole genome level. DNA methylation patterns can respond to harmful chemicals, air pollution, and hormonal status. So, all this data must be considered while determining the future course of events **(Eades, 2005)**. Each tissue undergoes a specific developmental program that ultimately decides its final differentiation status. Changes in temperature and humidity can affect methylation status. The epigenome is very sensitive and shows high plasticity.

The results of real-time PCR in this research showed that fresh and stained samples of peripheral blood, menstrual blood, and saliva using BLM1, MENS1, and SPEI1 primers, respectively, were successfully detected. Also, the degraded samples of peripheral and menstrual blood at 40oC showed successful detection. While SPEI1 primer had mostly moderate to weak results in detecting the saliva DNA degraded at 40oC because the Cq results ranged from 38 to 33 in 7 samples and the other three saliva samples had positive results. In the samples exposed to the UV light, BLM1 and MENS1 showed successful detection, but the SPEI1 primer again showed moderate results for five saliva samples. In peripheral blood samples exposed to different environmental conditions, BLM1 primer was successfully detected under all conditions. Also, in menstrual blood samples exposed to different environmental conditions, MENS1 primer was successfully detected under all conditions.

Coincidently with this study, it was reported by Vidaki et al., 2016 that BLM1 primer is highly detective of blood tissues while it was weak in detecting any other tissues such as saliva or skin samples. Similarly, Forat et al., 2016 reported that SPEI1 and MENS1 are methylated in their target fluid and are hypomethylated in the others. This type of marker indicates the presence of the target fluid also in unknown mixtures with the other body fluids. Also, they reported that Mens1 is exclusively methylated in menstrual blood and unmethylated in other body fluids. SPEI1 shows no overlap with any other body fluid. It identifies saliva in any mixture containing more than 20% saliva.

Fresh peripheral blood (BLM1) and fresh menstrual blood (MENS1) markers had the same least mean value, 25.6, which indicates that it's the strongest. At the same time, fresh saliva samples (SPEI1) had a mean value of 27.7. When the variance of fresh saliva samples (SPEI1) was compared to fresh peripheral blood (BLM1) and menstrual blood (MENS1), the variance value was (*p < 13.63).

The study compared peripheral blood samples exposed to different environmental conditions using BLM1 primer. All the samples have almost the same mean value, but fresh stained blood had the best mean value, 22.8, while the highest mean value was the fresh blood which had a result of 25.6. The mean values show that the BLM1 primer could be used to detect the peripheral blood that has been exposed to different environmental factors because all the mean values are less than 37-40. Variance analysis of fresh blood value was compared individually to all the samples that were exposed to environmental conditions, and the results were, stained blood showed a difference in variance with value of (* p < 0.3), 40oC degraded bloodstains showed difference in variance with a value of ((* p < 0.06) and UV light degraded bloodstains showed a difference in variance with a value of ((* p < 1.08). This means that the variance compassion between fresh blood and bloodstains that have been exposed to different environmental conditions didn't show a lot of difference.

Also, the study has compared menstrual blood samples exposed to different environmental conditions using the MENS1 primer. 40oC degraded menstrual blood stained shows the best mean value, 21. The UV light degraded menstrual blood stained showed a mean value of 23.9. In comparison, the highest mean value was fresh menstrual blood, as it had a value of 25.6. The results concluded that MENS1 primer could detect menstrual blood degraded stains and the fresh samples as all the mean samples didn't exceed 37-40, but the stained samples had better values than the fresh samples. Variance analysis of fresh menstrual blood value was compared individually to all the samples that had been exposed to environmental conditions, and the results were, 40oC degraded menstrual blood stains showed a difference in variance with a value of ((* p < 3.74) and UV light degraded bloodstains showed a difference in variance with value of ((* p < 3.26). This means that the variance compassion between fresh and stained menstrual blood that has been exposed to different environmental conditions showed a high difference.

Upon comparing saliva samples exposed to different environmental conditions using the SPEI1 primer, freshly stained saliva had the best mean value, which was 22.3. And the fresh saliva samples had a mean value of 27.7, while both the 40oC degraded saliva stains and UV light degraded saliva samples had almost close results of mean value, 32.3 and 31.2. The study results concluded that SPEI1 had the highest mean values amongst the other two primers BLM1 and MENS1. Also, it showed that SPEI1 primer is the best to detect freshly stained saliva. Variance analysis of fresh saliva samples value was compared individually to all the samples exposed to environmental conditions. The results were, freshly stained saliva showed a difference in variance with a value of (* p < 0.53), 40oC degraded bloodstains showed a difference in variance with a value of ((* p < 0.06), and UV light degraded saliva stains showed a difference in variance with a value of ((* p < 1.08). This means that the variance compassion between fresh and stained saliva samples that have been exposed to different environmental conditions didn't show a lot of difference. Also, there was a study conducted by Maha et al. in 2016 which discussed the effect of different human muscle tissue preservatives on the quality and quantity of DNA.

CONCLUSION

Body fluid identification is an important component in forensic investigation as it helps to establish the sequence of events and determine an individual's identity. DNA methylation is highly specific to the person's tissue type, age, and sex. This unique characteristic of DNA methylation is exploited to identify victims or culprits during a forensic investigation. Several molecular markers have been identified to recognize the origin of a tissue sample identified at the crime scene. The amount and the integrity of DNA used for analysis are often the determining factors in the success of methylation studies. Various factors such as exposure to UV radiation, high temperature, pH, and salt concentration can affect DNA stability.

RECOMMENDATIONS

-Sequencing is recommended in the future as a further study of this research project.

-Different DNA methylation markers could be used to validate their effectiveness in body fluid identification.

REFERENCES

Alegría-Torres, J., Baccarelli, A. and Bollati, V. (2011). Epigenetics and lifestyle. Epigenomics, 3(3), pp.267-277.

Bind, M., Zanobetti, A., Gasparrini, A., Peters, A., Coull, B., Baccarelli, A., Tarantini, L., Koutrakis, P., Vokonas, P. and Schwartz, J. (2014). Effects of Temperature and Relative Humidity on DNA Methylation. Epidemiology, 25(4), pp.561-569.

Eads, C. (2000). MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Research, 28(8), pp.32e-0.

Farzeen Kader,Meenu Ghai, Ademola O. Olaniran (2019). Characterization of DNA methylation-based markers for human body fluid identification in forensics: a critical review. International Journal of Legal Medicine, pp. 1–20 https://doi.org/10.1007/s00414-019-02181-3.

Feil, R. and Fraga, M. (2012). Epigenetics and the environment: emerging patterns and implications. Nature Reviews Genetics.

Forat, S., Huettel, B., Reinhardt, R., Fimmers, R., Haidl, G., Denschlag, D. and Olek, K. (2016). Methylation Markers for the Identification of Body Fluids and Tissues from Forensic Trace Evidence. PLOS ONE, 11(2), p.e0147973.

Gomes, I., Kohlmeier, F. and Schneider, P. (2011). Genetic markers for body fluid and tissue identification in forensics. Forensic Science International: Genetics Supplement Series, 3(1), pp.e469-e470.

Goodman, J. (2006). Altered DNA methylation: An epigenetic mechanism underlying carcinogenesis. Toxicology Letters, 164, p.S19.

Grummt, I. and Längst, G. (2013). Epigenetic control of RNA polymerase I transcription in mammalian cells. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1829(3-4), pp.393-404.

Herman, J., Graff, J., Myohanen, S., Nelkin, B. and Baylin, S. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proceedings of the National Academy of Sciences, 93(18), pp.9821-9826.

Kader, F. and Ghai, M. (2015). DNA methylation and application in forensic sciences. Forensic Science International, 249, pp.255-265.

Karni, M., Zidon, D., Polak, P., Zalevsky, Z., & Shefi, O. (2013). Thermal Degradation of DNA. DNA and Cell Biology; 32(6), 298-301. doi:10.1089/dna..2056

Leme, D. H., & Maher, E. R. (2012) DNA methylation: a form of epigenetic control of gene expression. The Obstetrician & Gynaecologist, 12(1), 37-42.

doi:10.1576/toag.12.1.037.27556.

Maha I. Ali, Rehab I. Abdel-Karim, Ranya M. Mahmoud and Abeer M. Hagres (2016). Effect of different human muscle tissue preservatives on quality and quantity of dna: medico legal aspect. Egypt J. Forensic Sci. Appli. Toxicol. Article 14, Volume 16, Issue 1, Page 179-191

Shin, K Lee, H. Y., Lee, S. D. (2016); Forensic DNA methylation profiling from evidence material for investigative leads. BMB Reports, 49(7), 359-369. doi:10.5483/bmbrep.2016.49.7.070.

Nagase, H. and Ghosh, S. (2008). Epigenetics: differential DNA methylation in mammalian somatic tissues. FEBS Journal, 275(8), pp.1617-1623.

Pacchierotti, F. and Spanò, M. (2015). Environmental Impact on DNA Methylation in the Germline: State of the Art and Gaps of Knowledge. BioMed Research International, 2015, pp.1-23.

Seisenberger, S., Peat, J., Hore, T., Santos, F., Dean, W. and Reik, W. (2012). Reprogramming DNA methylation in the mammalian life cycle: building and breaking epigenetic barriers. Philosophical Transactions of the Royal Society B: Biological Sciences, 368(1609), pp.20110330-20110330.

Srinivasan, M., Sedmak, D., & Jewell, S. (2002). Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids. The American Journal of Pathology, 161(6), 1961-1971. doi:10.1016/s0002-9440(10)64472-0

Tillo, D., Mukherjee, S. and Vinson, C. (2016). Inheritance of Cytosine Methylation. Journal of Cellular Physiology, 231(11), pp.2346-2352.

Tost, J. and Gut, I. (2007). DNA methylation analysis by pyrosequencing. Nature Protocols, 2(9), pp.2265-2275.

Vidaki, A., Giangasparo, F. and Syndercombe Court, D. (2016). Discovery of potential DNA methylation markers for forensic tissue identification using bisulphite pyrosequencing.ELECTROPHORESIS, 37(21), pp.2767-2779.

Wu, H., Wang, Q., Chung, W. K., Andrulis, I. L., Daly, M. B., John, E. M., Terry, M. B. (2014); Correlation of DNA methylation levels in blood and saliva DNA in young girls of the LEGACY Girls study. Epigenetics, 9(7), 929-933. doi:10.4161/epi.28902

Zhu, J. et al, (2013). Effects of ultraviolet B exposure on DNA methylation in patients with systemic lupus erythematosus. Experimental and Therapeutic Medicine.

Zilberman, D. and Henikoff, S. (2007). Genome-wide analysis of DNA methylation patterns. Development, 134(22), pp.3959-3965.

الملخص العربي

تقييم استقرار علامات ميثيل الحمض النووي في البقع البيولوجية وتأثيرها على تحقيقات الطب الشرعي

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ج الطب الشرعي و التكنولوجيا الحيوية ، جامعة العلوم الحديثة ، دبي ، الإمارات العربية المتحدة

الخلفية: أصبحت دراسة التعديلات اللاجينية مثل مثيلة الحمض النووي أداة مهمة في تحقيقات الطب الشرعي نظرًا لموثوقيتها وخصوصياتها. مثيلة الحمض النووي ديناميكية للغاية بطبيعتها وحساسة للعديد من العوامل البيئية ونمط الحياة. يمكن اختبار عينات الحمض النووي التي تم جمعها من مسرح الجريمة وفقًا لأنماط المثيلة للمساعدة في تحديد أنواع مختلفة من الأدلة البيولوجية بما في ذلك الشعر والدم والسائل المنوي واللعاب الموجودة في مسرح الجريمة. علاوة على ذلك ، يمكن أن يساعد في تحديد الجنس والعمر وإلقاء الضوء على الهوية العامة للمشتبه به أو الضحية..

الأهداف: هدفت هذه الدراسة إلى التحقق من صحة استخدام الواسمات النوعية لمثيلة الحمض النووي في تحديد الدم المحيطي ودم الحيض واللعاب ، والتحقق من ثبات هذه الواسمات. بالإضافة إلى ذلك ، قيم هذا البحث تأثير تعرض الدم واللعاب لظروف بيئية مختلفة على اكتشاف علامات محددة لمثيلات الحمض النووي..

المنهجية: العينات المستخدمة في هذه الدراسة هي الدم المحيطي واللعاب ودم الحيض. تم استخلاص الحمض النووي من جميع العينات وتم الكشف عن جودته على الفصل الكهربائي للهلام. ثم تم تطبيق تحويل ثنائي كبريتيت و PCR في الوقت الحقيقي باستخدام تمهيدي BLM1 للكشف عن عينات الدم المحيطية ، وتمهيدي MENS1 للكشف عن عينات دم الحيض و SPEI1 للكشف عن عينات اللعاب. تم جمع البقع الجافة من اللعاب ودم الحيض والدم المحيطي وتعريضها لظروف بيئية مختلفة.

النتائج: أظهرت نتائج PCR في الوقت الفعلي والتحليل الإحصائي للبادئات ان BLM1 و MENS1 قد حصلا علي نتائج أفضل من SPEI1 التمهيدي في تحديد سوائل الجسم الطازجة وكذلك تلك المعرضة لظروف بيئية مختلفة من التدهور.

الخلاصة: إن مثيلة الحمض النووي خاصة جدًا بنوع الأنسجة وعمر وجنس الشخص. يتم استغلال هذه الخاصية الفريدة لمثيلة الحمض النووي في التعرف على الضحايا أو الجناة أثناء تحقيقات الطب الشرعي. غالبًا ما يكون مقدار وسلامة الحمض النووي المستخدم في التحليل هو العامل المحدد في نجاح دراسات المثيلة. يمكن أن تؤثر عوامل مختلفة مثل التعرض للأشعة فوق البنفسجية وارتفاع درجة الحرارة ودرجة الحموضة وتركيز الملح على استقرار الحمض النووي.

الكلمات الأساسية: مثيلة الحمض النووي ، تحديد سوائل الجسم ، الظروف البيئية ، استخراج الحمض النووي ، تفاعل البوليميراز المتسلسل