Wasit Journal for Pure Science

Journal Homepage: https://wjps.uowasit.edu.iq/index.php/wjps/index
e-ISSN: 2790-5241 p-ISSN: 2790-5233

A Successful Approach to Extracted Human mtDNA and Amplify some Genes from FFPE Samples

Nisreen Abdulkadhim Hanoush[1]*, Ahmed E. Dhamad[2]

[1]Department of Nursing Techniques, Technical Institute Kut, Middle Technical University, IRAQ

[2]Department of Biology, College of Science, Wasit University, IRAQ

*Corresponding Author: Nisreen Abdulkadhim

DOI: https://doi.org/10.31185/wjps.551
Received 10 September 2024; Accepted 21 October 2024; Available online 30 December 2024

ABSTRACT: Tissues that have been paraffin-embedded and fixed in formalin (FFPE) are a significant diagnostic resource for molecular and pathophysiological methods of cancer and numerous other illnesses. The most difficult obstacle is obtaining high-quality of DNA from FFPE tissues, especially mitochondrial DNA (mtDNA), as formalin fixation significantly compromises the integrity of DNA. To extract mtDNA, the Geneaid gSYNCTM DNA kit (cat#GS100) was used with fifty breast cancer tissues (block). To check the mtDNA quality, specific primers for the following genes, ATP6 gene (8527-9024) and D-Loop region (256-16277), were used to run a PCR reaction. Our data showed that the mtDNA was successfully extracted with high quantity and quality. The two genes were amplified via PCR with very high rate (97% average). In conclusion, the method described here has many possibilities for applications in various molecular research projects involving FFPET substances. It can be used to obtain a high quantity and quality of mtDNA from FFPE samples for different molecular diagnostic and research methods such as PCR, qPCR, Sanger sequencing, and whole genome sequencing.

Keywords: form FFPE sample, mtDNA, Conventional PCR, Breast cancer,

1. INTRODUCTION

Worldwide, pathology laboratories routinely process patient samples using formalin fixation and paraffin embedding (FFPE). FFPE allows for immunohistochemistry investigation for clinical diagnosis while maintaining tissue morphology [1, 2]. Several research investigations have documented noteworthy correlations between mtDNA and other illnesses, such as various forms of cancer [3]. Numerous researches comparing various deparaffinization and DNA extraction methods in different carcinomas, including cervical cancer, breast cancer lymphoma, hepatocellular carcinoma, stomach adenocarcinoma, and colonic tumor, have been conducted in the past [4-7].

Breast cancer is the most frequently identified malignant growth in women. Most of these cancers begin in the lobules' epithelial cells and the breast's nonepithelial tissue. However, on rare occasions, mixed carcinomas and sarcomas may also occur. Breast cancer is the most common cancer in women worldwide [8, 9].

Tissues that have been formalin-fixed and paraffin-embedded (FFPE) are a remarkable source of preserved and morphologically characterized disease-specific biological substance that allows molecular results to be correlated, treatment, and clinical results [10, 11]. The quantity of DNA obtainable from biological specimens may restrict the applicability of molecular research as interest in the genetic causes of diseases grows [12, 13]. They are frequently used in Real-Time PCR experiments and other PCR-based DNA amplification-based clinical and research molecular analyses [14]. Because of its many benefits, formalin is the most frequently employed fixative in histopathology. However, crosslinking tissue proteins destroys tissue nucleic acids, causing severe DNA and RNA fragmentation [15].

The amplification of longer DNA targets and lower PCR yields are known to be connected with fixation intervals and the difficulty of PCR when using DNA recovered from fixed tissues [1]. Before extraction, techniques for paraffin removal from fixed tissues (such as the use of ethanol and xylene washes) are thought to be crucial to maximizing the quality and quantity of extracted nucleic acid; failing to do so results in low-quality samples and inhibiting subsequent PCR reactions [16]. Recently, a new method for removing formaldehyde from samples has been introduced. This technique involves using a series of ethanol washes with varying concentrations [17]. When utilizing DNA extraction techniques, factors such as the extraction buffer's composition (including the presence of proteinase-K) and the temperature and duration of the digesting stage should be considered [18, 19].

The significance of mitochondria in disease development, particularly in aspects like oxidative phosphorylation (OXPHOS), chiefly involves their function in initiating apoptosis, generating free radicals, and producing ATP [20]. Mitochondria are organelles in the cell that produce ATP, a kind of energy that the cell can utilize [21, 22]. Margit and Sylvan Nass first discovered and isolated mitochondrial DNA (mtDNA) in 1963 while studying some mitochondrial fibers that appeared to be associated with DNA due to their fixation, stabilization, and staining characteristics [23]. The mtDNA found in mammals is circular and double-stranded; each mammalian cell possesses multiple copies [24, 25]. It is an intron-less and consists of ~ 16.5 kilobases in size. It consists of 37 genes, 13 protein subunits for the oxidative phosphorylation (OXPHOS) system, two ribosomal ribonucleic acids (12S and 16S) and all 22 transfer ribonucleic acids required to synthesize the mitochondrial proteins (Figure 1) [25-28].

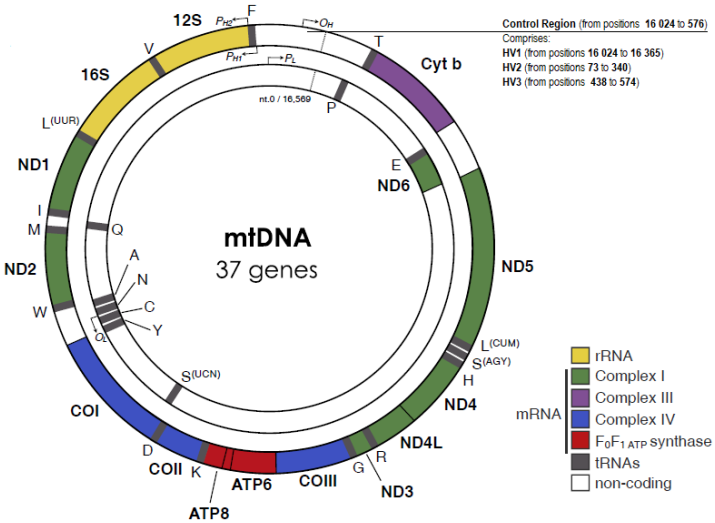

Figure 1: The human mtDNA with genes and control regions marked. Adapted from [29]

2. MATERIALS AND METHODS

2.1 Samples collection

Fifty blocks (FFPE) of breast tissues were prepared and stored for one year were used for mtDNA extraction and ATP6 and Dloop genes amplification. This research was conducted according to manufacturers of gSYNCTM DNA extraction kit (cat#GS100). Each block of tissue was cut into three sections with 8mm thickness.

2.2 MtDNA extraction protocol:

To extract human mtDNA from FFPE samples, the commercial gSYNC DNA kit (Geneaid, cat.GS100/ Taiwan) was used according to the manufacturer's guidelines. The tissues underwent dewaxing using xylene, then centrifuge for 3 min to remove the supernatant, then followed the extraction steps in the kit, which included: Add 200 µl of the GST buffer &20 µl of a proteinase K. Then shake thoroughly and incubate in a waterbath at 60°C for overnight, or until the sample clears, Cell Lysis (move the supernatant to a new1.5ml microcentrifuge tube and add 200 µl of GSB Buffer), Binding DNA (add 200 µl of pure ethanol), Add 400 µl of W1 Buffer to the GS Column to wash and finally steps DNA Elution (add 50 µl of pre-heated elution buffer into the center of the column then, centrifuge for 3sec to elute purified DNA).

2.3 PCR Amplification:

Extracted mtDNA was used to run a PCR experiment with specific primer sets to amplify mtDNA genes (ATP6 and D-Loop). The primer sequences for different genes are illustrated in Table 1. The amplification process involved 50 ml PCR reaction volume, which included the reaction master mix of 25µl Taq polymerase, 19 µl nuclease-free water, 2µl Forward primer,2µl reverse primer, and 2µl DNA template. The PCR program consisted of the first denaturation at 95 ºC for 4min, followed by 40 cycles. Every cycle consisted of denaturation at 95ºC for 30sec, annealing at 58ºC for 30

sec, extention1 at 72ºC for 1min, and final extenion at 72ºC for 5min. The analysis of PCR products was analyzed by electrophoresing with three microliters of the PCR product on 0.5g of agarose gel.

Table 1: PCR primers.

	DNA primers	Source	Gene Length	PCR amplicon size
ATP6	Forward 5`-ATGAACGAAAATCTGTTCGC-3` Reverse 5`-CCTGCAGTAATGTTAGCGGT-3`	NCBI/ NC_012920.1	8527-9024	500bp
D-Loop	Forward 5´-AGTGGCTGTGCAGACATTCA-3´ Reverse 5´-ACCAACAAACCTACCCACCC -3´		256-16277	558bp

3. RESULTS AND DISCUSSION

The amount of mtDNA yielded using the gSYNCTM DNA extraction kit was sufficient and can be used to run many molecular and genetic experiments, such as conventional PCR reactions. To test the quality of the extracted mtDNA, two mitochondrial genes were examined. Specific primers for the following genes ATP6 (8527-9024) and D-loop (256-16277) were designed and used. The results showed that the ATP6 gene was successfully amplified with a 98% percentage (figure 1). While the success rate of D-loop amplification was 96% (figure 2). The average failure to amplify the genes was 3%, which could be explained by the block preparation, poor storage, or an error during the technique. These results suggested that the extracted mtDNA was of high quality and could be used to do many molecular studies.

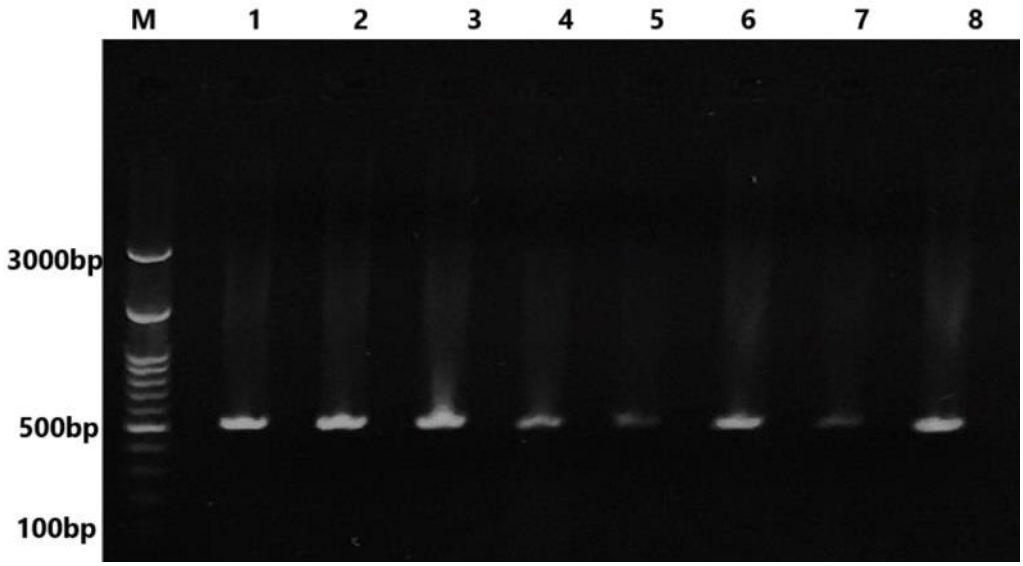

Figure 1: Agarose gel electrophoresis: 3 µl of PCR product were mixed with loading dye and loaded in the gel wells. M: 100 bp DNA marker (Geneaid#Cat. No. isDL007). Lanes 1 to 8 were ATP6 (positive) samples.

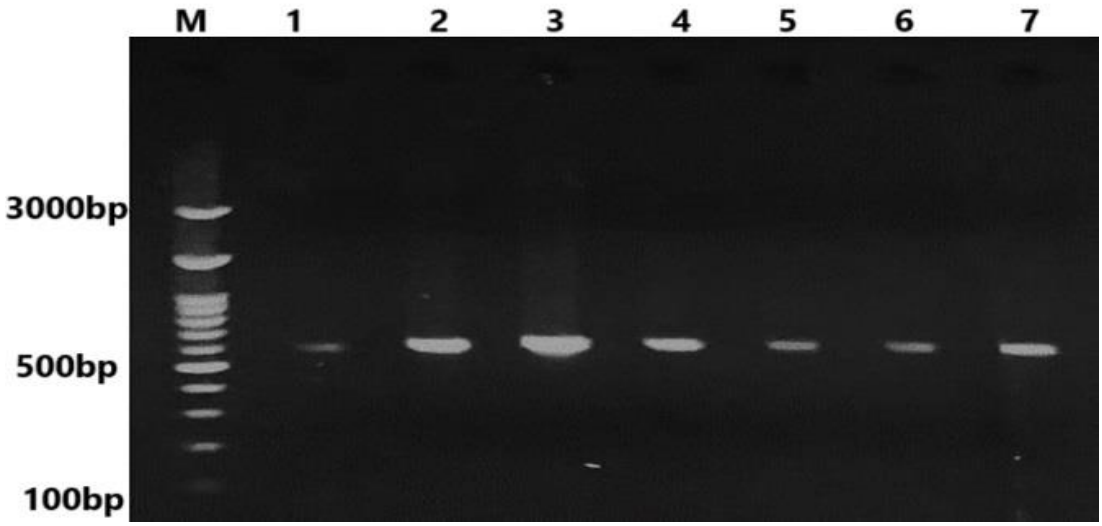

Figure 2: Agarose gel electrophoresis: 3 µl of PCR product were mixed with loading dye and loaded in the gel wells.M: 100 bp DNA marker (Geneaid/Cat. No. isDL007). lanes, 1-to 7 were D-Loop samples (positive).

Evaluating different susceptibility and tumor indicators in tissue samples preserved by FFPEsamples is becoming increasingly important in molecular epidemiology research. PCR is a technique that is particularly useful for examining tissues from FFPE since it is easily accessible. However, several factors, including the type of fixative chosen, the length of fixation, the technique for extracting DNA, the size of PCR amplimers, the quantity of template DNA, and the fine-tuning of PCR conditions, all affect PCR's effectiveness. These factors could explain why very small percentage (3%) of the samples were not be able to amplified [14, 30].

4. CONCLUSION

The present study's purpose was to extract mtDNA from FFPE samples, which were stored for a long time (one year) with high quantity and quality. FFPE samples are a valuable source in molecular, genetic, biomedicine, and biopharmaceutical studies. Our result showed that mtDNA was extracted with high amount. The high rate of genes' amplification (98% of ATP6 gene and 96% of D-Loop) confirmed the high quality of the extracted mtDNA. The technique described here has much potential for use in various molecular and genetic research, such as PCR, northern blot, and DNA sequencing. with FFPET material

5. ACKNOWLEDGEMENT

I want to express my genuine gratitude and thanks to Wasit Health Department for granting a letter facilitating the mission to enter the tissue laboratory at Al-Karamah Hospital and to Dr. Faris Lutfi at the National Oncology Laboratory/ Wasit Governorate for providing me breast tissue blocks of women who had cancer.

REFERENCES

[1] Q. Guo, E. Lakatos, I. A. Bakir, K. Curtius, T. A. Graham, and V. Mustonen, "The mutational signatures of formalin fixation on the human genome," Nature Communications, vol. 13, no. 1, p. 4487, 2022.

[2] W. Mathieson and G. Thomas, "Using FFPE tissue in genomic analyses: advantages, disadvantages and the role of biospecimen science," Current Pathobiology Reports, vol. 7, pp. 35-40, 2019.

[3] K. Zhou et al., "A novel next-generation sequencing–based approach for concurrent detection of mitochondrial DNA copy number and mutation," The Journal of Molecular Diagnostics, vol. 22, no. 12, pp. 1408-1418, 2020.

[4] M. Mirmomeni, S. Sajjadi Majd, S. Sisakhtnezhad, and F. Doranegard, "Comparison of the three methods for

DNA extraction from paraffin-embedded tissues," Journal of Biological Sciences, vol. 10, no. 3, pp. 261-266, 2010.

[5] K. Vitošević, M. Todorović, Ž. Slović, T. Varljen, S. Matić, and D. Todorović, "DNA isolated from formalin-fixed paraffin-embedded healthy tissue after 30 years of storage can be used for forensic studies," Forensic Science, Medicine and Pathology, vol. 17, pp. 47-57, 2021.

[6] P. Decruyenaere, K. Verniers, F. Poma-Soto, J. Van Dorpe, F. Offner, and J. Vandesompele, "RNA extraction method impacts quality metrics and sequencing results in formalin-fixed, paraffin-embedded tissue samples," Laboratory Investigation, vol. 103, no. 2, p. 100027, 2023.

[7] B. P. Bass, K. B. Engel, S. R. Greytak, and H. M. Moore, "A review of preanalytical factors affecting molecular, protein, and morphological analysis of formalin-fixed, paraffin-embedded (FFPE) tissue: how well do you know your FFPE specimen?" Archives of pathology and laboratory medicine, vol. 138, no. 11, pp. 1520-1530, 2014.

[8] L. Li, L. Chen, J. Li, W. Zhang, Y. Liao, J. Chen, and Z. Sun, "Correlational study on mitochondrial DNA mutations as potential risk factors in breast cancer," Oncotarget, vol. 7, no. 21, p. 31270, 2016.

[9] A. Talei, M. Akrami, M. Mokhtari, and S. Tahmasebi, "Surgical and clinical pathology of breast diseases," Histopathology-Reviews and Recent Advances, 2012.

[10] X. H. Gao et al., "Comparison of fresh frozen tissue with formalin-fixed paraffin-embedded tissue for mutation analysis using a multi-gene panel in patients with colorectal cancer," Frontiers in oncology, vol. 10, p. 310, 2020.

[11] S. M. Hickey et al., "Fluorescence microscopy—an outline of hardware, biological handling, and fluorophore considerations," Cells, vol. 11, no. 1, p. 35, 2021.

[12] S. Aviel-Ronen, C. Qi Zhu, B. P. Coe, N. Liu, S. K. Watson, W. L. Lam, and M. S. Tsao, "Large fragment Bst DNA polymerase for whole genome amplification of DNA from formalin-fixed paraffin-embedded tissues," BMC genomics, vol. 7, pp. 1-10, 2006.

[13] I. Oscorbin and M. Filipenko, "Bst polymerase—a humble relative of Taq polymerase," Computational and Structural Biotechnology Journal, 2023.

[14] P. Dedhia, S. Tarale, G. Dhongde, R. Khadapkar, and B. Das, "Evaluation of DNA extraction methods and real time PCR optimization on formalin-fixed paraffin-embedded tissues," Asian Pacific Journal of Cancer Prevention, vol. 8, no. 1, p. 55, 2007.

[15] S. Bonin, F. Petrera, B. Niccolini, and G. Stanta, "PCR analysis in archival postmortem tissues," Molecular Pathology, vol. 56, no. 3, p. 184, 2003.

[16] S. S. Khan et al., "Evaluation and Comparison of Genomic DNA Extraction Methods and PCR Optimization on Archival Formalin-Fixed and Paraffin-Embedded Tissues of Oral Squamous Cell Carcinoma," Diagnostics, vol. 12, no. 5, p. 1219, 2022.

[17] M. T. P. Gilbert et al., "The isolation of nucleic acids from fixed, paraffin-embedded tissues–which methods are useful when?" PloS one, vol. 2, no. 6, p. e537, 2007.

[18] S. Bonin and G. Stanta, "Nucleic acid extraction methods from fixed and paraffin-embedded tissues in cancer diagnostics," Expert Review of Molecular Diagnostics, vol. 13, no. 3, pp. 271-282, 2013.

[19] P. G. Patel et al., "Preparation of formalin-fixed paraffin-embedded tissue cores for both RNA and DNA extraction," JoVE (Journal of Visualized Experiments), no. 114, p. e54299, 2016.

[20] L. Grzybowska-Szatkowska, B. Ślaska, J. Rzymowska, A. Brzozowska, and B. Floriańczyk, "Novel mitochondrial mutations in the ATP6 and ATP8 genes in patients with breast cancer," Molecular medicine reports, vol. 10, no. 4, pp. 1772-1778, 2014.

[21] Y.-h. Jang and K.-i. Lim, "Recent advances in mitochondria-targeted gene delivery," Molecules, vol. 23, no. 9, p. 2316, 2018.

[22] P. Kozakiewicz et al., "Mitochondrial DNA changes in respiratory complex I genes in brain gliomas," Biomedicines, vol. 11, no. 4, p. 1183, 2023.

[23] M. M. Nass and S. Nass, "Intramitochondrial fibers with DNA characteristics: I. Fixation and electron staining reactions," The Journal of cell biology, vol. 19, no. 3, pp. 593-611, 1963.

[24] N. A. Bonekamp and N.-G. Larsson, "SnapShot: mitochondrial nucleoid," Cell, vol. 172, no. 1, pp. 388-388. e1, 2018.

[25] R. Filograna, M. Mennuni, D. Alsina, and N. G. Larsson, "Mitochondrial DNA copy number in human disease: the more the better?," FEBS letters, vol. 595, no. 8, pp. 976-1002, 2021.

[26] C. M. Gustafsson, M. Falkenberg, and N.-G. Larsson, "Maintenance and expression of mammalian mitochondrial DNA," Annual review of biochemistry, vol. 85, no. 1, pp. 133-160, 2016.

[27] A. L. Smith, J. C. Whitehall, and L. C. Greaves, "Mitochondrial DNA mutations in ageing and cancer," Molecular Oncology, vol. 16, no. 18, pp. 3276-3294, 2022.

[28] P. Kowalczyk et al., "Mitochondrial oxidative stress—a causative factor and therapeutic target in many diseases," International Journal of Molecular Sciences, vol. 22, no. 24, p. 13384, 2021.

[29] A. Amorim, T. Fernandes, and N. Taveira, "Mitochondrial DNA in human identification: a review," PeerJ, vol. 7, p. e7314, 2019.

[30] S. Santos, D. Sá, E. Bastos, H. Guedes-Pinto, I. Gut, F. Gärtner, and R. Chaves, "An efficient protocol for genomic DNA extraction from formalin-fixed paraffin-embedded tissues," Research in veterinary science, vol. 86, no. 3, pp. 421-426, 2009.