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RESEARCH ARTICLE

EFFICIENT AND COST-EFFECTIVE METHOD OF HIGH QUALITY DNA EXTRACTION FOR BISULFITE CONVERSION BASED METHYLATION ANALYSIS OF FORMALIN-FIXED PARAFFIN- EMBEDDED ARCHIVAL BREAST TISSUES

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Abstract

Robust standardized methods to extract DNA from formalin-fixed paraffin-embedded tissues are required for accurate bisulfite conversion based methylation analysis. Despite many different protocols are published, extraction of sufficient quantity of intact DNA from formalin-fixed paraffin-embedded (FFPE) tissues is still challenging. We compared two DNA extraction methods (Phenol-Chloroform and Qiagen mini kit) in terms of DNA yield, concentration, purity and devised a modified protocol of Qiagen minikit. DNA fragment size and amplification rate were assessed with *GAPDH* 499 bp and *BRCA1* non-methylated 86 bp primers. Efficiency of bisulfite conversion and subsequent methylation-specific PCR were assessed with *BRCA1* methylated 75 bp primers. The modified protocol of Qiagen minikit yielded a sufficient quantity of purified DNA for methylation analysis than phenol chloroform protocol. Each modified parameter excluding age of tumor blocks had significant ($P < 0.05$) effect on extracted DNA. Amplification rate was high with short amplicons (86 bp) than large amplicons (499 bp). 100% amplification rate of touchdown methylation-specific PCR (MSPCR) was found with *BRCA1* 75 bp primers. The devised modified protocol of Qiagen minikit coupled with touchdown MSPCR are suitable and cost-effective methods for wide-range of downstream molecular and epigenetic applications.

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Introduction:-

Epigenetics is an emerging field focuses on the regulation of many cellular and molecular processes including chromosome stability, chromatin structure, DNA repair in cell cycle and transcription. DNA methylation, the addition of a methyl group to cytosine bases, is the most widely studied epigenetic modification. Genome-wide methylation analysis has enabled in-depth investigation of complex disorders such as cancer, discovery of new disease biomarkers and drug targets. However, successful DNA extraction from formalin-fixed paraffin-embedded tissues and bisulfite conversion are key requirements for methylation analysis of targeted diseases (Balic and Pichler, 2009).

Formalin-fixed paraffin-embedded (FFPE) tissue samples are the only available material for many cases and using of this tissue is especially valuable for retrospective studies. Besides, there is growing need to use FFPE samples in

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routine molecular studies and sample type and quality are important factors that affect the performance of transcriptomic, genomics and epigenomics studies. Formalin is the most commonly used fixative for the preservation of a tumour and other tissues in histopathology (Sengüven et. al., 2014). However, the isolation of sufficient quantity of intact DNA from FFPE tissue samples includes several potential problems such as DNA-protein cross-linking, DNA fragmentation and the presence of PCR inhibitors. Bisulfite conversion involves converting cytosine to uracil while leaving intact 5-methylcytosine. Cross-linking of methyl group with formalin further degrades DNA and therefore methylation analysis based on bisulfite conversion presents an additional challenge (Gagnon et.al., 2010). Thus, the protocol for DNA purification from FFPE tissues must efficiently extract high quality DNA and reverse cross-linking caused by formalin fixation.

To overcome these limitations the objective of this research was to devise the method of high-quality DNA extraction whilst improving the performance and efficiency of bisulfite conversion and subsequent methylation-specific PCR (MSPCR) using template DNA from FFPE breast tissues.

Materials And Methods:-

Sample Collection

This study was approved by the Ethical Review Committee at Forman Christian College University (ERC-42-2018). 40 FFPE breast tissue samples were randomly selected from the bio-repository of Chughtais Lahore Lab. Six to eight sections (10 microns) of each tumour block were used for DNA extraction.

Deparaffinization

The sections were deparaffinized and incubated twice with 300ul of 0.1M NaOH for 20 minutes at 70⁰C. 1 ml of xylene was added and centrifuged for 2 minutes at 15000 rpm. The supernatant was removed and 1 ml of absolute ethanol was added to the tissue pellet. Centrifugation was done for 2 minutes at 15000 rpm and the ethanol step was repeated. The supernatant was removed and the pellet was dried at room temperature. Samples were incubated for 10 minutes at 37⁰C until all residual ethanol was evaporated.

Extraction of Genomic DNA from FFPE Breast Tissues:

The two DNA extraction protocols were Phenol-Chloroform method (Gilbert et.al., 2007) and Qiagen Qiamap DNA extraction mini kit with several modifications.

Genomic DNA Extraction using Qiagen mini kit:

DNA was extracted from FFPE tissues using Qiagen mini kit according to manufacturer instructions. Several parameters including age of tumour blocks, incubation time in lysis step, hot alkali method that used NaOH to remove formalin and paraffin from FFPE tissues, elution volume, carrier RNA addition, treatment with proteinase K and Rnase A were tested on original kit method to increase the yield of extracted DNA.

Modification 1:

Incubation time and the temperature were reduced from 2 hours to 1 hour 30 minutes and 90⁰C to 75⁰C in the lysis step. Samples were eluted one time in 100ul Buffer AE, then 2 times in 200ul buffer.

Modification 2:

Using an incubation time of 1 hour 30 minutes at a temperature of 75⁰C, 30ul of proteinase K (20 mg/ml) was added instead of 20ul (as in a kit method) to avoid protein contamination. 10ul carrier RNA was also added at DNA purification step. Samples were eluted in 70ul of elution buffer.

Modification 3:

Samples were incubated with 300ul of 0.1M NaOH for 20 minutes at 70⁰C in deparaffinization step prior to xylene treatment. Lysis and DNA purification steps were the same as in the modification 2. Samples were eluted in 50ul of elution buffer.

Modification 4:

Using modification 3, 4ul of RNase A was added after incubation in lysis step.

DNA Quantification

The absorbance (A260: A280) and concentration (ng/ul) of DNA were quantified by absorbance measurement using a Nano-drop Spectrophotometer.

Bisulfite Conversion of FFPE DNA

Bisulfite conversion was done using EPItect Bisulfite conversion kit, Qiagen according to manufacturer's instruction.

Assessment of Extracted DNA from FFPE Breast Tissues

DNA fragmentation was assessed with *GAPDH* 499 bp and *BRCA1* 86 bp primers. The integrity of bisulfite converted DNA and performance of MSPCR were assessed with *BRCA1* 75 bp methylated primers. PCR inhibition was minimized with touchdown PCR, hot start Taq polymerase, PCR elongation time and MgCl₂. *GAPDH* and *BRCA1* PCR products were analysed on 1% and 2.5% agarose gel respectively.

Statistical Analysis:

Effect of each parameter on FFPE extracted DNA was analysed with Pearson chi-square test using SPSS software. A P value <0.05 was considered significant.

Results and Discussion:-

FFPE tissues processed with Qiagen modified protocol here in described yield high quality and high quantity genomic DNA than phenol-chloroform protocol. Comparative analysis was done for each method and parameter. Following groups of sections were examined:

- A: Phenol-Chloroform method
- B: Qiagen mini kit manufacturer's instruction
- C: Qiagen mini kit with modified protocol 1
- D: Qiagen mini kit with modified protocol 2
- E: Qiagen mini kit with modified protocol 3
- F: Qiagen mini kit with modified protocol 4

DNA extraction methods (A, B, C, D, E and F) were compared in terms of DNA yield, concentration and purity. Highest DNA yield was obtained with method F. Extracted DNA from 40 FFPE breast tissue samples using protocol F was assessed in terms of DNA purity, fragmentation and amplification rate. The nano-drop (A260/A280) ratio between 1.8-2.0 was considered normal. Purity of extracted DNA was measured before and after bisulfite conversion. Table 1 shows the assessment of extracted DNA with different factors. In the recently published paper on methylation analysis, the authors suggested the use of Qiagen minikit for good quality DNA extraction from FFPE tissues but due to DNA degradation, yield quality was less than fresh frozen tissues (Ludgate et.al., 2017).

Table 1:- Assessment of FFPE Extracted DNA with Different Factors.

Assessment of FFPE Extracted DNA with Different Factors								
DNA Yield (ug)	DNA Purity (A260/A280)		GAPDH 499 bp Touch Down PCR		BRCA1 86 bp Touch Down PCR		BRCA1 75 bp Touch Down MSPCR	
	After DNA Extraction	After Bisulfite Conversion	Amplification Rate	DNA Fragmentation	Amplification Rate	DNA Fragmentation	Amplification Rate	DNA Fragmentation
0.1-1	50%	50%	5.8%	Highly Fragmented	100%	No Fragmentation	100%	No Fragmentation
1.1-2.9	60%	59%	38.8%	Highly Fragmented	100%	No Fragmentation	100%	No Fragmentation
6-8.9	100%	95%	100%	Less Fragmented	100%	No Fragmentation	100%	No Fragmentation
9-12	100%	98%	100%	Less Fragmented	100%	No Fragmentation	100%	No Fragmentation

Chi-square analysis showed significant association (P -value < 0.05) of each modified parameter including incubation time in lysis step, elution volume, incubation with an alkaline solution (NaOH), carrier RNA addition, treatment with proteinase K and Rnase A excluding age of tumour block with DNA yield. Few other studies have also reported that heating FFPE samples with an alkaline solution, use of proteinase K, short fixation period improve the quality of DNA extraction (Sengüven et al., 2014). It is noteworthy that another recent study has reported the robust PCR result with Rnase treatment (Wieczorek et al., 2014).

We have performed two-fold assessment of extracted DNA with *GAPDH*, a housekeeping gene and *BRCA1* methylated and non-methylated reference genes because highly fragmented DNA is usually extracted from FFPE tissues. Housekeeping genes are universally expressed in all the cells and thus important for regulating cell metabolism and its expression varies under different experimental conditions. *GAPDH* is the most commonly used housekeeping gene due to its stable gene expression under different experimental conditions (Wieczorek et al., 2014). We found the overall 25% amplification rate for 499 bp *GAPDH* gene and the 100% amplification rate for 86 bp *BRCA1* gene with touchdown PCR. Figure 1 shows the representative images of agarose gel electrophoresis. The authors of previous study also reported the efficient result with short (100bp) amplicons than large amplicons (300bp) (Balic et al., 2009; Gagnon et al., 2010).

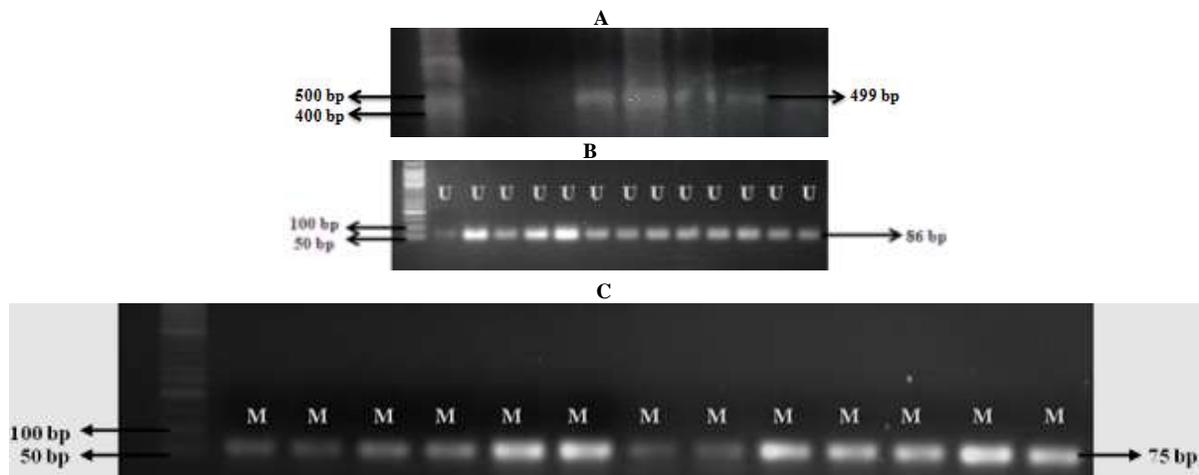


Figure 1:- Representative touchdown PCR results. A: *GAPDH* 499 bp touchdown PCR results. B: *BRCA1* 86 bp touchdown PCR results. C: *BRCA1* 75 bp touchdown MSPCR results.

FFPE DNA extraction methods for bisulfite conversion based methylation analysis are not well established yet. However, few studies are published but with non-consistent results (Killian et al., 2009; Gagnon et al., 2010; Shen et al., 2007). We found 100% amplification rate of touchdown methylation-specific PCR with *BRCA1* 75 bp methylated primers. Touchdown PCR is a simple and rapid means to increase the efficiency, specificity, and sensitivity without the need for lengthy optimization (Dietrich et al., 2013). Our study suggest that touchdown PCR with hot-start Taq polymerase is highly efficient and cost-effective method for methylation analysis of bisulfite converted FFPE fragmented DNA samples. The modified DNA extraction protocol of Qiagen mini kit coupled with touchdown methylation-specific PCR will have utility in detailed molecular studies especially for the screening of epigenetic changes in oncogenes, which require archival formalin-fixed paraffin-embedded tissues. However, methylation screening with more set of primers could be a limiting factor. Testing the FFPE extracted DNA by PCR amplification is a first valid approach to assessing the DNAs capacity for responding to PCR based assessment but it would be more informative to have continued to test the DNA on high-density platforms to ascertain the capacity of the FFPE DNA to be used on these modern platforms including methylation analysis.

References:-

1. Balic, M., Pichler, M., Strutz, J., Heitzer, E., Ausch, C., Samonigg, H., Cote, R.J. and Dandachi, N. 2009. High quality assessment of DNA methylation in archival tissues from colorectal cancer patients using quantitative high-resolution melting analysis. *The Journal of Molecular Diagnostics*, **11**(2), pp.102-108.

2. Dietrich, D., Uhl, B., Sailer, V., Holmes, E.E., Jung, M., Meller, S. and Kristiansen, G. 2013. Improved PCR performance using template DNA from formalin-fixed and paraffin-embedded tissues by overcoming PCR inhibition. *PLoS One*, **8**(10).
3. Gagnon, J.F., Sanschagrin, F., Jacob, S., Tremblay, A.A., Provencher, L., Robert, J., Morin, C. and Diorio, C. 2010. Quantitative DNA methylation analysis of laser capture microdissected formalin-fixed and paraffin-embedded tissues. *Experimental and molecular pathology*, **88**(1), pp.184-189.
4. Gilbert, M.T.P., Haselkorn, T., Bunce, M., Sanchez, J.J., Lucas, S.B., Jewell, L.D., Van Marck, E. and Worobey, M. 2007. The isolation of nucleic acids from fixed, paraffin-embedded tissues—which methods are useful when?. *PloS one*, **2**(6).
5. Killian, J.K., Bilke, S., Davis, S., Walker, R.L., Killian, M.S., Jaeger, E.B., Chen, Y., Hipp, J., Pittaluga, S., Raffeld, M. and Cornelison, R. 2009. Large-scale profiling of archival lymph nodes reveals pervasive remodeling of the follicular lymphoma methylome. *Cancer research*, **69**(3), pp.758-764.
6. Ludgate, J.L., Wright, J., Stockwell, P.A., Morison, I.M., Eccles, M.R. and Chatterjee, A. 2017. A streamlined method for analysing genome-wide DNA methylation patterns from low amounts of FFPE DNA. *BMC medical genomics*, **10**(1), p.54.
7. Sengüven, B., Baris, E., Oygur, T. and Berktaş, M. 2014. Comparison of methods for the extraction of DNA from formalin-fixed, paraffin-embedded archival tissues. *International journal of medical sciences*, **11**(5), p.494.
8. Shen, L., Guo, Y., Chen, X., Ahmed, S. and Issa, J.P.J. 2007. Optimizing annealing temperature overcomes bias in bisulfite PCR methylation analysis. *Biotechniques*, **42**(1), pp.48-58.
9. Wiczorek, D., Schagat, T. and Vincent, E. 2014. Overcoming Challenges of DNA Purification from FFPE Samples. The ReliaPrep™ FFPE gDNA Miniprep System. <https://worldwide.promega.com/resources/pubhub/overcoming-challenges-of-dna-purification-from-ffpe-samples/>.