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Rescue of highly degraded DNA by primerless PCR

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ABSTRACT. Next-generation sequencing (NGS) platforms are now implemented as routine analysis for K-RAS mutation in colon cancer patients before therapy. The DNA used in NGS platforms is extracted from colon cancer formalin-fixed paraffin-embedded (FFPE) blocks. In this study, we utilized 20 FFPE colon cancer blocks. In general, a good quality DNA sample includes compact high molecular weight DNA. The quality of the extracted DNA is checked by agarose gel electrophoresis. Some samples are found to be highly degraded due to natural mechanisms like autolysis and spontaneous depurination, or bacterial contamination and extracted DNA is then routinely fragmented by sonication. In this study, PCR was performed to reconstruct larger DNA fragments rather than to amplify DNA fragments. Primerless PCR relies on the natural power of two segments of the PCR cycle to reconstruct fragmented PCR by: the capability of denatured DNA to anneal randomly to its complementary sequence (annealing), and Taq polymerase to extend the DNA at the 3' end (extension). By repeating for 150 cycles a larger DNA fragment is generated instead of amplifying the DNA. Fragmented DNA was reconstructed by primerless PCR for 150 cycles. However, 1U of Taq polymerase was added to the PCR reaction every 50 cycles. The samples selected for this study were highly degraded. The degree of degradation of samples was

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visualized by running the samples on a 1% agarose gel. After running primerless PCR for 50, 100, and 150 cycles, larger fragments of highly intact DNA appeared as sharp, compact bands whereas degraded DNA was a diffuse smear. In conclusion, we have demonstrated that primerless PCR can authentically generate DNA fragments that are larger than the initial template, and the DNA polymerization of self-primed DNA fragments can increase the likelihood of successful regeneration ostensibly by reconstructing the template. Primerless PCR can help in regenerating larger DNA fragments from highly deteriorated samples, such as forensic, and ancient samples.

Key words: Primerless PCR**;** Degraded DNA; NGS; DNA rescue; DNA fragmentation

INTRODUCTION

The diagnostic potential of the use formalin-fixed paraffin-embedded (FFPE) tissue, as abundant resource, has unlocked the development of commercial reagents intended specifically for molecular testing. It is the perfect resource for molecular testing because both archival FFPE tissue and tissue from current patients are available. The study of DNA and RNA as biomolecules faces a variety of technological difficulties due to FFPE's stability and capacity to store morphological data. Cross-linking and processing, in particular, cause issues with the extraction and isolation of DNA, RNA, and protein and have an impact on how well these molecules work in subsequent analyses. FFPE malignant tissue samples are generally collected for routine histopathological procedures. When the diagnosis is confirmed, the blocks could be sent to the molecular diagnostics lab for DNA sequencing. However, the quality of DNA extracted from FFPE blocks mostly differs when compared with fresh frozen samples. DNA is usually fragmented by sonication into 150 to 300 base pairs (bp) to prepare the DNA library to target specific DNA targets of interest using next-generation sequencing (NGS) platforms. However, the rate of fragmentation depends heavily on the quality of the tissue delivered to the lab. Almost all samples received were colon cancer FFPE blocks (Lindahl and Nyberg, 1972; Bär et al., 1988). Problems with DNA degradation are well-known in both recently prepared and most archival FFPE blocks. Natural mechanisms of DNA degradation include autolysis, spontaneous depurination, and contaminating bacterial restriction enzymes (Lindahl and Nyberg, 1972; Bär et al., 1988). It has been observed that when the FFPE block is initially extensively degraded, the DNA fragments were smaller than required size and are not perfect for DNA sequencing (Akane et al., 1993).

The quality of the DNA extracted from the blocks must be high enough to allow for accurate analysis. The size of the biopsy or tissue sample, the quality of the formalin employed, the length of time the sample is fixed, and the storage conditions all have an impact on the DNA quality of the resulting blocks (Quach et al., 2004; Srinivasan et al., 2010; Williams et al., 2010; Van Allen et al., 2014; Do and Dobrovic, 2015).

When compared to FFPE blocks, the DNA quality of fresh frozen (FF) tissue is superior. Biological and immunohistochemical studies, especially those examining DNA

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integrity, work best in FF tissue. But FF tissue needs a solid setup and rigorous sample processing techniques (Oh et al., 2015). Inappropriate treatment decisions may be made due to a high rate of false positive calls caused by the use of low-quality DNA in next-generation sequencing technologies (Ye et al., 2013).

Mechanical methods, such as nebulization and sonication, or enzyme digestion are used to shear pure DNA material into small pieces in preparation for NGS library construction. Using the Roche Junior sequencer for library preparation, it was found that all three methods produced comparable results (Golenberg et al., 1996; Knierim et al., 2011). The quality of the resulting sequence is profoundly impacted by the quantity of the library used. As a result of DNA fragmentation's imprecise size, bioinformatics is unable to produce accurate data assembly. However, reliable scaling improves assembly and sequencing throughput. Because of the short-read sequences they generate, NGS technologies like Illumina TM MiSeq TM requires 150 and 300 bp DNA fragments. Because of this, smaller pieces need to be removed as well. The size of DNA fragments was determined via electrophoresis on a gel. Which is suitable for low-throughput labs. The size of the required DNA fragment can be identified on the gen and then eluted. For high-throughput laboratories, however, automated DNA sizing methods including bead-based systems (Duhaime et al., 2012; Larose et al., 2021) and disposable gel cassette platforms (Hoeijmakers et al., 2011) are already available. Even though the latter method has been demonstrated to be more precise in terms of size (Quail et al., 2012), the bead-based method has been shown to be superior in terms of cleanliness, reducing the likelihood of cross-contamination between samples, and increasing output (Duhaime et al., 2012; Larose et al., 2021).

While the aforementioned methods have shown moderate success when dealing with large amounts of DNA, the question of whether methodology is ideal for extremely low yield samples remains open. For NGS, DNA fragmentation was required to get the library to the right size for the platform. While most NGS systems require 50-500 ng of DNA, the DNA yield from FFPE biopsies is often only a few pg to a few ng. A wide variety of methods are available to boost the yield of low-yield samples. Target DNA is enriched in these processes. To do so successfully, you must have access to the target's amplified and sequenced DNA. Another technique requiring only 3-40 ng is linear amplified deep sequencing (LADS) (Hoeijmakers et al., 2011).

The PCR amplification capability is not tested in this article, but another function of the PCR will be added to its capability. PCR is a well-known method for amplifying a specific fragment flanked between two primers. The PCR capability intended to be investigated here is the capability of the PCR to regenerate larger DNA fragments. The PCR master mix was prepared in the absence of primers (primerless PCR) and highly degraded DNA was added and the tubes were loaded on the thermal cycler. In a primerless PCR cycle, the natural ability of denatured DNA fragments to randomly anneal to their complementary sequence (annealing segment) after denaturation segment of the PCR cycle, then was followed by Taq polymerase extension of the DNA at the 3' end (extension). The fragments obtained are utilized in primerless PCR, wherein denatured fragments spontaneously and randomly bind to one other, acting as both primer and template in a mutually beneficial manner (Meyer et al., 2014). The whole concept is illustrated in Figure 1. The process is repeated in each subsequent cycle to generate larger DNA fragments.

MATERIAL AND METHODS

FFPE blocks

Ethical approval for this investigation was obtained from the Deanship of Research, King Abdulaziz University, Jeddah, KSA. Twenty colon cancer FFPE blocks were utilized in this investigation that would have been thrown away otherwise. Since it is suggested that all patients' blocks should be kept for at least 10 years before discarding them, this batch has been marked for destruction.

DNA extraction

Following the manufacturer's instructions, DNA was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen, Germantown MD, USA) (Cat. # 56404). Using spectrophotometry with a NanoDrop 2000, the yield the DNA quantity and quality was determined (Thermo ScientificTM, Wilmington DE, USA). The 260/280 absorbance ratio of 1.22–1.62 was indicative of high-quality DNA in the samples. When measuring DNA degradation, first 100 ng of DNA was loaded onto a 1% agarose gel and run through an electrophoresis cycle. The DNA concentration was then normalized to 50 ng/mL.

PCR amplification

PCR master mix was prepared without adding primers (Table 1). the master mix was dispensed in tubes, then 10 ng of fragmented DNA was added to the master mix and the required volume was adjusted by adding DNase free ddH2O. The polymerase chain reaction (PCR) was performed using a Perkin Elmer thermal cycler set to 50 cycles (hold at 94°C for 30 sec., hold at 56°C for 30 sec., hold at 72°C for 30 sec.). Three tubes, labelled 50, 100, and 150 cycles, were kept at 4°C after each set of PCR reactions. After adding one unit of Taq polymerase to the remaining PCR mixture, the remaining 50 cycles were conducted. Then, another pair of tubes, designated 100 cycles, was kept at 4°C. Taq polymerase (1 unit) was added to the final two tubes, and 50 PCR cycles. PCR products were run on 1% agarose gel (Figure 2-a). For the gel quantification plot, MATLAB 2017b was used for bar diagram with standard deviations (Figure 2-b).

Finally, I've outlined the general strategy used for this work, which made use of a 200-microliter tube with a primerless PCR master mix and fragmented DNA (Figure 1).

Table 1. PCR master mix.

Reagent	Final concentration	Volume	
10x buffer	1x	10 µL	
$MgCl_2$ (25mM)	2.5 mM	4 µL	
dNTPs	0.2 mM	10 µL	
Taq polymerase (1.25 U/□L)	5 U	4 µL	
Sample DNA	10 ng	Variable	72 µL
DNase free H_2O		Variable	
Total Volume		**100** µL	

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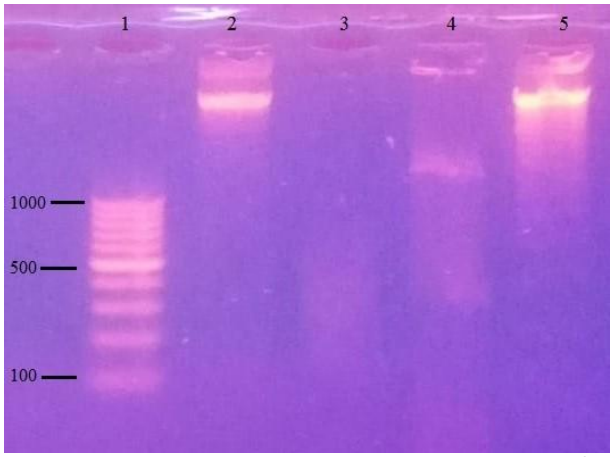

Figure 1: Two hundred microliter tube containing primerless PCR master mix and fragmented DNA. In each primerless PCR cycle, the fragmented DNA is dissociated during the denaturation segment into ssDNA. Denatured fragmented ssDNA anneals randomly to complementary segments during the annealing segment and then extends during the extension segment of the PCR cycle. By the end of the cycle DNA fragments become larger. After each cycle, the DNA fragments become larger and larger. The process of denaturation, random fragment DNA annealing, and DNA regeneration will reconstruct larger amplicon after each cycle.

RESULTS AND DISCUSSION

DNA extracted from colon cancer FFPE blocks is usually fragmented by sonication. Degraded DNA is then run on 1% agarose gel to determine the size of the DNA fragment. deterioration had occurred in the samples. The varying lane quality seen in Figure 2 represents the quality of the DNA samples. Lane 2 displays high-quality DNA. The DNA in this sample is extremely compact for its molecular weight. The degradation of DNA fragments between 100 and 500 base pairs in size is clearly visible in lane 3, which is the recommended size. The FFPE sample in lane 4 contains larger degraded DNA fragments can be seen as a result. The FFPE sample in lane 5 has been degraded less noticeably and contains a larger DNA fragment.

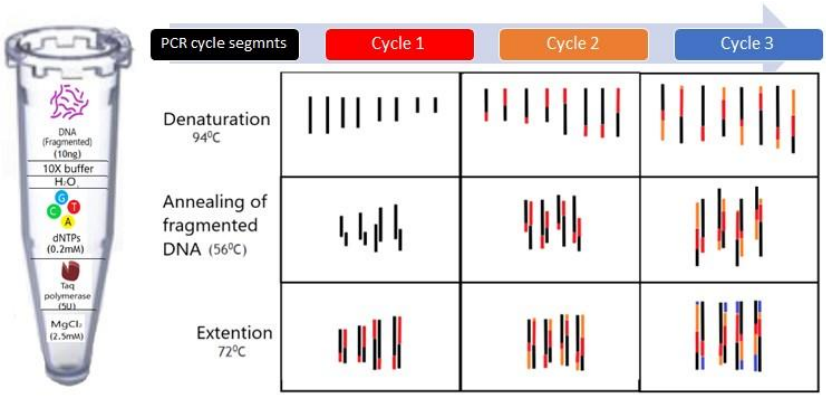

Figure 2. DNA Fragmentation. (a) Lane 1: 100bp ladder, Lane 2: DNA sample, Lane 3: Sample degraded by sonication, lanes 4: highly degraded sample, and Lane 5: moderately degraded sample and (b) respective quantification.

In this experiment, highly degraded DNA was added to the primerless PCR master mix. Then PCR was run for 50 cycles, 100 and 150 cycles. The PCR products were run on 1% agarose gel. Primerless PCR was able to reconstruct fragmented DNA generating a larger size DNA fragment. Highly intact DNA appears as comets ranging in size from 1500 bp to less than 100 bp for the first 50 cycles, allowing the sample's integrity to be visualized after it has been reassembled, as can be seen in lanes 3 and 4; Figure 3. After being subjected to a further 50 cycles (for a grand total of 100 cycles), DNA fragments ranging in size ranges from 300--5000 bp were reassembled, as can be seen in lanes 5 and 6; Figure 3. Finally, after being subjected to 150 cycles, fragmented DNA was assembled into fragments longer than 5000 base pairs, as can be seen in lanes 7; Figure 3.

Furthermore, Figure 1 was shown to summarize the overall approach applied for this study where two hundred microliter tube containing primerless PCR master mix and fragmented DNA were used (Figure 1). In each primerless PCR cycle, the fragmented DNA is dissociated during the denaturation segment into ssDNA. Denatured fragmented ssDNA anneals randomly to complementary segments during the annealing segment and then extends during the extension segment of the PCR cycle. By the end of the cycle DNA fragments become larger. After each cycle, the DNA fragments become larger and larger. The process of denaturation, random fragment DNA annealing, and DNA regeneration will reconstruct larger amplicon after each cycle.

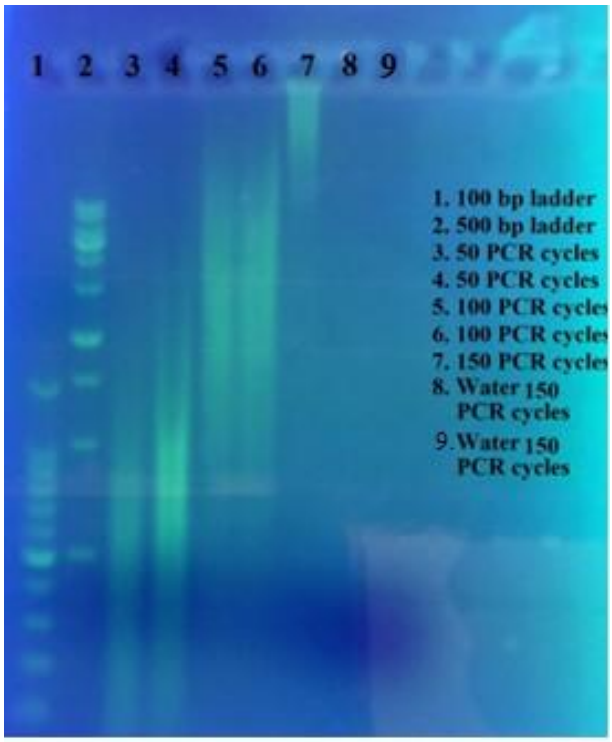

Figure 3. (a) Lane 1: 100 bp ladder, Lane 2: 500bp ladder, lanes 3 and 4: 50 PCR cycles of degraded DNA, Lanes 5 and 6: 100 PCR cycles of degraded DNA, lane 7: 50 PCR cycles of degraded DNA, and lanes 8 and 9: negative control (150 PCR cycles of water) and (b) the respective quantification.

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Generation of longer DNA strands is required for DNA isolated from old peripheral blood samples, forensic, ancient and deteriorated samples due to autolysis, bacterial degradation, and background spontaneous depurination, DNA extracted from old or deteriorated tissue is frequently extremely fragmented (Lindahl and Nyberg, 1972; Permenter, 2015). Highly fragmented DNA has a major impact on PCR efficiency, which has a direct effect on the sequencing results (Akane et al., 1993; Fisher et al., 1993; Permenter et al., 2015; Larose et al., 2021). Indeed, both the anticipation that aged DNA will decay and the expectation that degraded DNA will be resistant to PCR analysis are so widely acknowledged that the PCR's behaviour has been employed as a criterion for the template DNA's legitimacy (Pääbo, 1989; Fisher et al., 1993).

However, highly degraded DNA extracted from defective FFPE tissue blocks, and possibly ancient or forensic materials, can be rescued by exposing the DNA to 5-10 cycles of PCR before loading the DNA on the NGS platform (Nikkilä et al., 2002; Maradeo and Cairns, 2011; Zhu et al., 2020; Cobain et al., 2021; Garg et al., 2021). To reduce the need for unnecessary sample repetition, different researchers could try out this technique to salvage fragmented samples and also recommend applying the concept to the low yield tiny environmental virus samples might be received.

From studies of PCRs with highly fragmented DNA as template, three major conclusions may be derived. As a first point, the maximum lengths of the template molecules used in the procedure are not always a limiting factor for the amplified areas. DNA polymerase anneals and extends overlapping genomic regions, which might lead to template rebuilding (Golenberg et al., 1996; Burrell et al., 2013; Dunn et al., 2018). There may be a conflict between this data with the common perception that using amplified damaged DNA is challenging and, at best, severely limited in target size. The two primers must completely cover the target area, and there must be enough enzyme and substrate dNTP available for amplification to take place (Fisher et al., 1993).

In order to find therapeutically relevant mutations in cancer patient samples, next-generation deep sequencing of gene panels is being used as a diagnostic tool. Clinical samples, such as formalin-fixed, paraffin-embedded specimens, on the other hand, usually offer small amounts of damaged, subpar DNA. Many sequencing techniques use extensive PCR amplification, which results in a build-up of bias and artefacts, to get around these problems. Therefore, a focused sequencing assay is required that works well with DNA of low quality and quantity without heavily relying on PCR amplification (Martinez et al., 2013; Rehm, 2013; So et al., 2018; Dunn et al., 2018).

A targeted sequencing assay based on oligonucleotide selective sequencing has already had its effectiveness assessed. This assay enables the enrichment of genes and regions of interest as well as the discovery of sequence variants from small quantities of DNA damage. This test makes use of an adaptor ligation to single-stranded DNA, a repair process tailored to clinical FFPE materials, and a primer-based capture method. Their method produces sequence libraries of high quality while reducing the need for lengthy PCR amplification, which makes it easier to detect single nucleotide variants and insertion/deletion events and to accurately estimate copy number variations (Muellner et al., 2011; Picotti et al., 2013; Zhou et al., 2020).

Now, diagnostic clonality investigations and the discovery of PCR targets useful for the diagnosis of minimal residual illness can both be conducted using BIOMED-2 multiplex tubes (van Dongen et al., 2003). Previous research has provided evidence that, when DNA

concentration is reduced relative to inhibitors, a reagent-rich PCR technique, where all reagents are raised in same relative proportion, can boost amplification success. For the 112 extracts utilized in the study, the reagent-rich PCR technique, known as rescue PCR, enhanced amplification success by 51%. In particular for old, deteriorated, and low copy number DNA samples, rescue PCR is a straightforward and effective addition to the range of techniques already available to work with DNA in the presence of inhibition (Johnson and Kemp, 2017).

Technical issues associated with primerless PCR

Patients with colon cancer are now routinely screened for K-RAS mutations using Illumina- MiSeqDx platforms at the Molecular Diagnostics Laboratory-King Abdulaziz University Hospital (MDx lab-KAUH). In order to acquire the optimal DNA fragment size of 300bp for sequencing, DNA is degraded by sonication. Biopsies sent from the pathology lab are routinely processed for DNA extraction at MDx lab-KAUH-KAU. Thus, most samples come from biopsies that have been stored in FFPE (Formalin fixed paraffin embedded) blocks. There were no quality assurance (QA) issues with the DNA sequencing data for most of the samples. Some samples, however, do not make it through the rigorous QA threshold, therefore the sequencing findings are not shared. Excessive degradation of the original DNA used for sequencing before sonication is mostly to blame.

This research aimed to produce longer (150--300bp) DNA fragments from the highly damaged DNA that would still fit the 150 bp reads used for sequencing. To compensate for the low DNA amount, the library preparation procedure was performed using sonication. The approach was modified to maintain a consistent DNA fragment size between 100 and 500bp, hence preventing excessive degradation. Highly degraded DNA results in a large proportion of erroneous calls that may be misinterpreted as actual mutations, resulting in inconsistent DNA sequence calling.

CONCLUSIONS

In conclusion, this project has demonstrated that primerless PCR can authentically regenerate larger DNA fragments than the initial template. This experiment demonstrates how we can rely on natural power of PCR to regenerate fragmented PCR by both: the capability of denatured DNA to anneal randomly to its complementary sequence, and the Taq polymerase to extend the DNA at the 3’ end. And by repeating the cycle larger fragments are generated. In other words, self-primed DNA fragments can increase the likelihood of successful reconstructing larger DNA fragments. This can be a solution to overcome the over degradation of the DNA samples.

AUTHOR CONTRIBUTIONS

Conceptualization, I.A.; methodology, I.A.; software, I.A.; validation, I.A.; formal analysis, I.A.; investigation, I.A.; resources, I.A.; data curation, I.A.; writing—original draft preparation, I.A.; writing—review and editing, I.A.; visualization, I.A.; supervision, I.A.; project administration, I.A.; funding acquisition, I.A. I have read and agreed to the published version of the manuscript.

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INSTITUTIONAL REVIEW BOARD STATEMENT

This study was approved by the Ethically approved by the Deanship of Research, King Abdulaziz University. This study adhered to the principles of the declaration of 1964 Helsinki declaration and its later amendments or comparable ethical standards.

INFORMED CONSENT STATEMENT

Informed consent was not essential to be sought from any participants because there was no data generated in this study except the natural behaviour of DNA replication.

DATA AVAILABILITY STATEMENT

All the related data and information are included in this work.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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