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Comparison of two commercial kits for the efficiency of DNA extraction from FFPE tissue

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ABSTRACT
Commercial kits that can extract DNA from Formalin-fixed paraffin-embedded (FFPE) tissue from the center stage serves the primary prerequisite in the laboratories that deal with genomic assessments. For the molecular assessments, the kit that can extract DNA of larger quantity with greater purity is favored. In this era of capitalization, many local companies have developed kits that can serve such purpose where few but not all kits benchmark such DNA extraction. Therefore, in this study, we compared one such Indian local-made Alexgen DNA extraction kit (Alexius Biosciences, India) with globally renounced QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) for DNA extraction from breast cancer FPPE tissue. For the quality assessment of extracted DNA, we made use of three methods, where first the UV absorption assessments at A_{260}/A_{280} along with A_{260}/A_{230} using NanoPhotometer was performed, then as second assessment fluorometric measurement using Qubit was performed which quantified the DNA with providing profile of its integrity. Lastly, PCR was performed for KRAS gene to see how efficient amplicons are generated. On performing all this study, we found that Alexgen DNA extraction kit yielded the quantity and quality of DNA at par with globally renounced QIAamp DNA FFPE Tissue Kit with statistical austerity.
Keywords: DNA extraction; formalin-fix paraffin-embedded tissue; genetic analysis; breast cancer; QIAamp DNA FFPE Tissue kit; Alexgen DNA extraction kit

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INTRODUCTION

Genomic DNA (gDNA) is the crucial starting material for most molecular assessment. The quality and yield of gDNA serve as the decisive factor for getting appropriate results in experiments dealing with gene amplification using Polymerase Chain Reaction (PCR) [1]. gDNA can be isolated from various sources like blood, tissue, saliva, hairs, etc., and for isolating gDNA from each of these sources, kits are marketed commercially [2]. However, for molecular pathology assessment the most accepted methodology is to convert sample tissue to Formalin-fixed paraffin-embedded (FFPE) tissue, and kits that can isolate gDNA from FFPE are widely used [3]. Such kits that can isolate gDNA from FFPE should be carefully evaluated for quantity and quality as these factors will determine the overall outcome of molecular assessment which form the fundamental agenda for devising this manuscript.

Molecular pathology has advanced tremendously in the recent decade, and molecular methods are fast shifting from research to regular usage in diagnostic pathology [4]. At present, molecular diagnostics is already included in the pathogen identification, genetic mutations, microsatellite instability, and mRNA up- and down-regulation [5]. However, to perform various molecular and histopathological analyses, long-term

preservation of tissue samples would always be essential in research and diagnosis. FFPE tissue sources are usually kept for decades and proved as an enormous source of morphologically well-defined tissues that now allow retrospective investigations to link molecular discoveries with therapeutic and diagnostic outcome [6, 7].

Thus far, the adoption of modern DNA-based technologies to FFPE tissues is extremely complicated. Formalin, the most widely used fixative in histopathology, has a variety of advantages, namely ease of tissue processing, long-term stability, outstanding histological quality, and huge amounts at a cheap price [8]. Conversely, the fixation of tissue with the formalin leads to cross-links between DNA and tissue proteins, which can hinder the amplification of isolated DNA [9]. Additionally, due to specimen age or the pH of fixative might fragment the nucleic acid present in formalin-fixed tissues [10]. Consequently, all these variables have an adverse influence on the quality of the isolated gDNA. However, numerous strategies can be used to overcome these constraints in utilizing FFPE tissue derived DNA samples. For example, heat can be used to revert and disintegrate DNA protein crosslinks [11].

Several approaches have been employed for the isolation of DNA from FFPE tissue blocks with the good quality and quantity and developed it at commercial level [12]. There are variety of commercially available kits for extracting gDNA from FFPE tissues, which may be used for both research and diagnostic reasons. In current study we have analyzed two kits, (i) QIAamp DNA FFPE Tissue Kit (Qiagen, Duesseldorf, Germany) and (ii) Alexgen DNA extraction kit (Alexius Biosciences, Ahmedabad, India) for the quantity and quality of isolated gDNA from FFPE tissue using spectrophotometric as well as fluorometric approach. Further, we analyzed how specific gene amplification using PCR yields amplicons which we used as criteria to compare and evaluate both kits under study.

MATERIALS AND METHODS

Sample selection and preparation

In present study, total 50 FFPE blocks were prepared from the leftover breast cancer tissue specimens, which were provided by Unipath Specialty Laboratory Ltd., Ahmedabad, India. The specimens utilized in this investigation were obtained as part of routine disease diagnostic procedures. All patient records were anonymized, and no information about an individual's identification was revealed.

All 50 tissue specimens were formalin-fixed and paraffin-embedded in accordance with standard methods [6]. Briefly, obtained tissue specimens were first fixed by treating it with 10% neutral buffered formalin for 4 to 12 h and then allow to dehydrate using alcohol. For the dehydration procedure, formalin fixed tissues were incubated with 90% and 95% ethanol and then with 100% alcohol for thrice for 4 h each. The dehydrated tissues were then immersed in xylene for 180 minutes and then three times in paraffin at 56–58°C for a total of 180 minutes before being embedded in blocks. The study was approved by Gujarat University's Ethics Committee (approval no. GUJIEC/22/2019).

Sectioning of FFPE tissue blocks

For the genomic DNA extraction, prepared FFPE tissue blocks (n=50) were trimmed to remove exceed paraffin wax using a scalpel and then five to eight serial sections (8-10 mm thickness) were taken for all samples using a rotary microtome (HM 325 Rotary Microtome, Thermo Scientific). The first few sections were discarded because they had been exposed to air. Then, equal quantities of sections were dissected using nucleic acid free scalpel and pooled in 2 ml sterile microcentrifuge tubes and kept at 4°C for later use. For each tissue sample, two sets of samples were produced, for DNA extraction using two different DNA isolation kits. Furthermore, all the chemicals utilized in this research study were of molecular biology grade, and all slides were macro dissected by one individual to avoid the variance associated with misinterpretation of the chosen tissue sections.

DNA extraction

We compared the effectiveness of Alexgen FFPE DNA Extraction Kit (Product ID: AG-FT50) developed by Alexius Biosciences, Ahmedabad, India with the QIAamp DNA FFPE Tissue Kit (Product ID: 56404) from the Qiagen, Duesseldorf, Germany which is being widely used for extraction of DNA from FFPE tissues across the world. DNA isolation was performed according to the manufacturer's instructions. Both kits involve solid phase DNA extraction, which is divided into six stages. The first step is to dissolve paraffin in xylene. In the second phase, the sample is lysed with proteinase K under denaturing conditions, followed by the reversal of formalin crosslinking at 90°C in the third step. The very next step is to link DNA to the membrane in the spin column. The fifth step is repeated several times to wash out any remaining contaminants. Pure and concentrated DNA is eluted from the membrane in the last stage. Outline of the DNA isolation procedures for QIAamp and Alexgen FFPE DNA extraction kits are presented in Fig. 1.

DNA quantity and quality evaluation

Isolated DNA from both kits for each sample were quantified using two different instruments, the NanoPhotometer (NanoPhotometer P-300, Implen, Germany) and the Qubit fluorometer (Qubit 2.0, Invitrogen, USA). The former quantifies DNA spectrophotometrically based on absorbance at 260 nm wavelength, whereas the latter quantifies DNA fluorometrically based on binding of dsDNA-selective fluorescent dyes [13].

The quality of each extracted DNA sample was assessed using an absorbance ratio of 260 nm to 280 nm (A_{260}/A_{280}) and an absorbance ratio of 260 nm to 230 nm (A_{260}/A_{230}). Generally, DNA samples with an A_{260}/A_{280} ratio of 1.8–2.0 were considered "pure". A decreased absorbance ratio implies that the sample is contaminated by proteins, phenol, and other chemicals having an absorbance near to 280 nm [14]. Additionally, the A_{260}/A_{230} ratio widely used as a supplement to the A_{260}/A_{280} ratio as a measure of DNA purity [15]. Consequently, a DNA sample with an A_{260}/A_{230} ratio of 2.0-2.2 was regarded "pure" in most cases.

Downstream molecular analyses

After assessing the quantity and quality of extracted DNA, its amplification ability was investigated using Polymerase Chain Reaction (PCR), which is one of several downstream molecular analyses. For that, conventional PCR was performed using Premix Ex Taq master mix (Takara, India) along with 5`-GTATTAAAAGGTACTGGTGGAG-3` as forward primer and 5`- GTATCAAAGAATGGTCCTGC-3`as reverse primer, which amplifies a fragment of 268 bp of KRAS gene present in the extracted DNA samples [16]. The PCR process involved fourteen DNA samples, seven of which were obtained using QIAamp and the other seven using the Alexgen kit from the same FFPE tissue blocks. The PCR was carried out with a final volume of 25 µl. Each reaction was made up of 12.5 µl of Premix PCR master mix, 3 µl of extracted template DNA, 1 µl of each primer and 7.5 µl nuclease free water. Instead of template DNA, a PCR mixture with water was used as a negative control. The PCR conditions for the amplification of KRAS gene from isolated DNA are described in Table 1.

Statistical analysis

The statistical analyses were performed using Microsoft Excel. The DNA concentrations and quality (absorbance ratios of A_{260}/A_{280} and A_{260}/A_{230}) of two techniques were compared using Student's t-tests.

Results

Quantification of gDNA by spectrophotometric and fluorimetric method

In total, 50 breast cancer tissue samples were utilized in preparing 50 FFPE tissue blocks, and DNA was extracted from each block using the QIAamp DNA FFPE Tissue kit and the Alexgen gDNA extraction kit. We compared the quantity and quality of DNA samples (n=50) extracted with DNA isolation kits from two different manufactures. Along with Spectrophotometric measurements, the Qubit 2.0 Fluorometer was also used to evaluate the quantity of each DNA sample. Table 2 summarizes the statistical analysis for the amount and purity of the isolated DNA samples.

The spectrophotometric analysis showed 44.45 ng/µl average concentration of DNA for QIAamp DNA FFPE Tissue kit and 42.36 ng/µl for the Alexgen gDNA extraction kit ($p > 0.5$). Noticeably, the fluorimetric analysis with the Qubit method for evaluated DNA samples revealed similar variations in the results of the tested DNA isolation procedures. However, the average concentrations measured with fluorimetric approach were substantially lower with 8.14 ng/µl and 7.61 ng/µl, for the QIAamp and Alexgen kit, respectively. The Box and Whisker plot for the quantification of each isolated DNA sample is displayed in Fig. 2. Furthermore, we also investigated correlation between the results of DNA quantitative analysis using spectrophotometric and fluorimetric methods. The correlation was considerable, with the correlation coefficients for QIAamp and Alexgen kit being 0.8146 and 0.7987, respectively **(Fig. 3).** However, as previously demonstrated, the spectrophotometric approach yielded greater values of DNA concentration in all evaluated samples than the fluorimetric method.

Quality assessment of isolated gDNA

The spectrophotometric ratio of absorbance at 260 nm and 280 nm along with the ratio of absorbance at 260 nm and 230 nm was used to determine the quality of the extracted DNA samples using both the kits. For both kits, 25 of 50 isolated DNA samples (50%) achieved the purity threshold, with A_{260}/A_{280} ratios ranging from 1.8 to 2.0, with mean values of 1.94 and 1.96 for QIAamp and Alexgen, respectively. However, Student's t-test revealed a P value of 0.36 ($p>0.05$), indicating that the mean values of A_{260}/A_{280} ratios obtained with two separate kits are not significantly different. Additionally, the A_{260}/A_{230} ratio for QIAamp samples varies from 1.32 to 3.55 and for Alexgen from 1.67 to 3.15, with mean values of 1.98 and 2.01, respectively. When A_{260}/A_{230} was compared samples isolated using QIAamp and Alexgen, there was no significant difference ($p>0.05$). The Box and Whisker plot for the A_{260}/A_{280} and A_{260}/A_{230} of each isolated

DNA sample is displayed in **Fig. 4.** These results showed that DNA samples extracted with the Alexgen gDNA extraction kit are as reliable as isolated with QIAamp DNA FFPE Tissue kit.

Evaluation for amplification ability of the gDNA

We performed conventional PCR using a set of primers that amplify a region of 268 bp of *KRAS* to compare the amplification capacity of DNA samples acquired using Alexgen kit with those extracted using QIAamp kit. For comparison, seven DNA samples isolated using both the kits (S7 to S13 for QIAamp and A7 to A13 for Alexgen) were assessed for the amplification of *KRAS* gene, and PCR products were separated on 2% agarose gel to detect amplified *KRAS* gene product. In **Fig. 5**, the amplified product of the *KRAS* gene (268 bp) is clearly visible for all samples included in the PCR experiment, regardless of the DNA isolation kits used. These findings confirmed that DNA samples acquired with the Alexgen kit can amplify DNA fragments in adequate quantities for downstream applications.

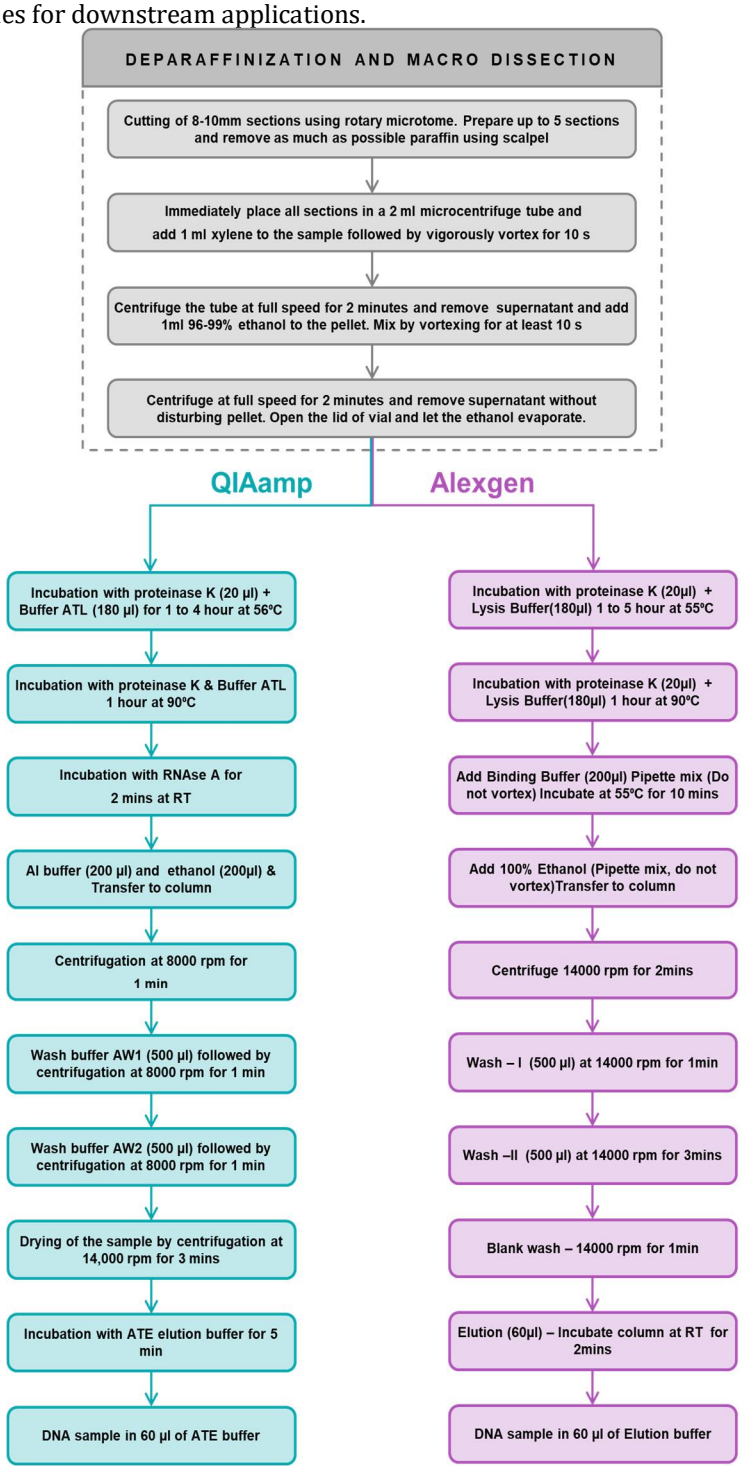

Fig.1. Outline of the DNA isolation procedure for QIAamp and Alexgen FFPE DNA extraction kits.

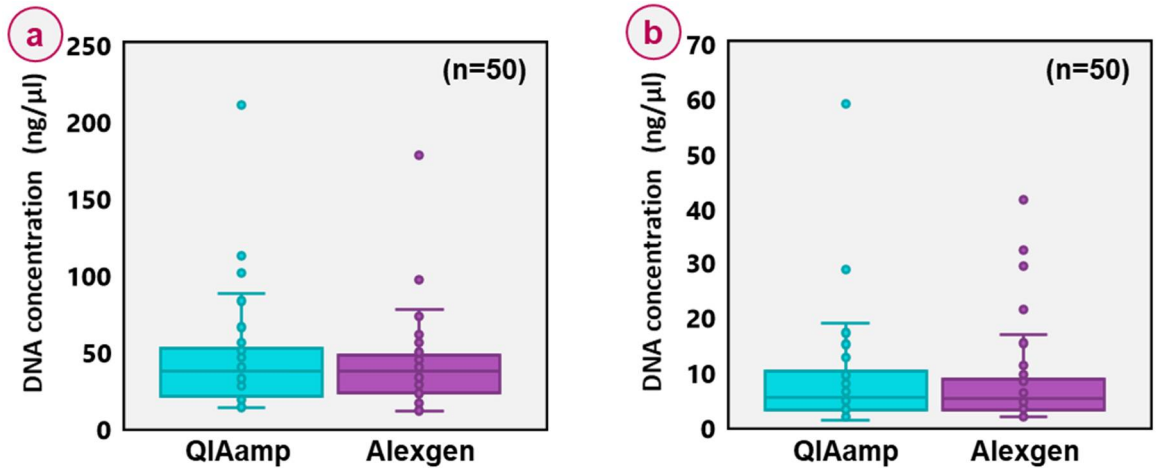

Fig. 2. Quantitative analysis of gDNA isolated from FFPE tissue specimens (a) Spectrophotometric analysis using NanoPhotometer, (b) Fluorimetric analysis with Qubit method.

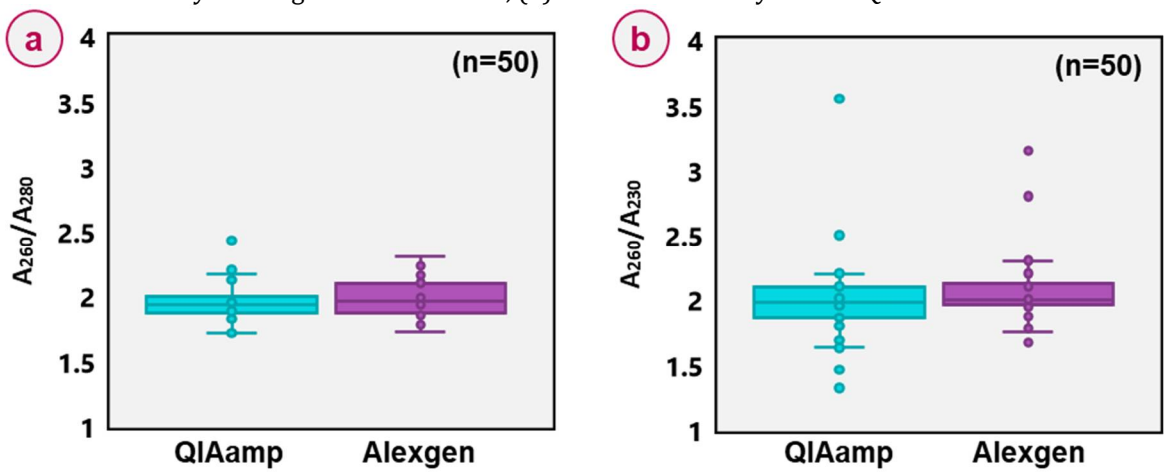

Fig. 3. Qualitative analysis of gDNA isolated from FFPE tissue specimens via spectrophotometric analysis with NanoPhotometer, expressed as (a) an absorbance ratio of 260 nm to 280 nm (A_{260}/A_{280}) and, (b) an absorbance ratio of 260 nm to 230 nm (A_{260}/A_{230}).

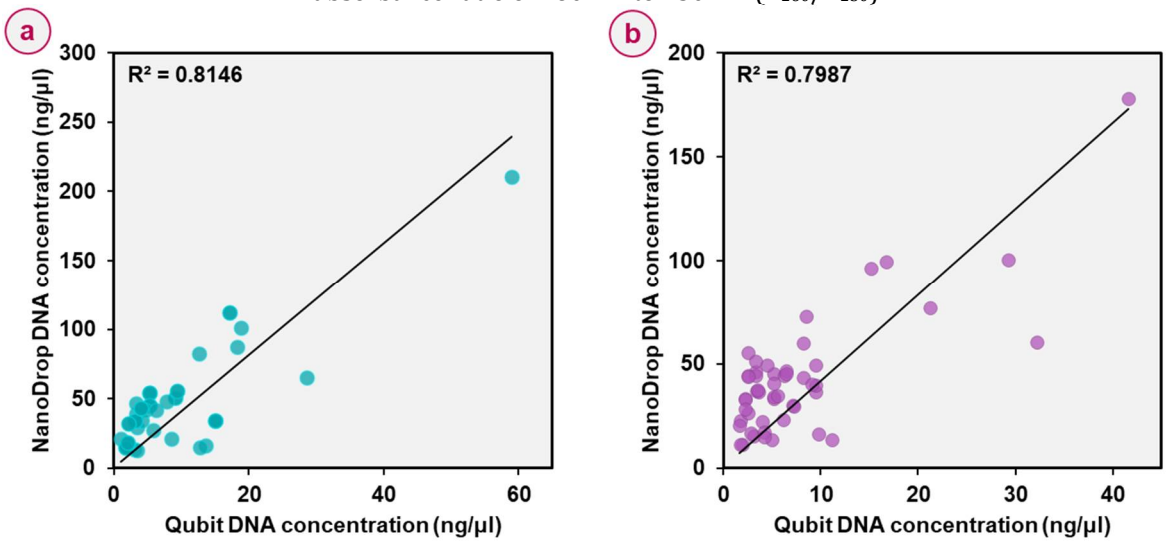

Fig. 4. Correlation of DNA quantification using the spectrophotometric NanoPhotometer method and the fluorimetric Qubit method (a) QIAamp kit (b) Alexgen kit.

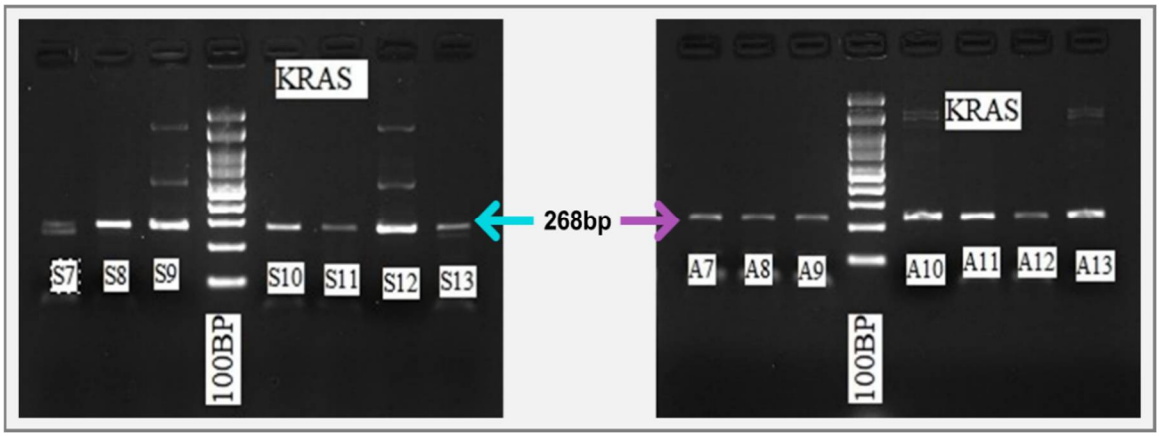

Fig. 5. Amplification of KRAS gene (268bp) in random samples (n=7) of isolated DNA using FFPE DNA extraction kits- QIAamp (Left panel-S7 to S13) and Alexgen (Right panel-A7 to A13).

Table 1 The PCR conditions for the amplification of KRAS gene from isolated DNA samples

PCR Steps	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Rest period
Temperature	95 °C	95 °C	55 °C	72 °C	72 °C	4 °C
Time	10 min	30 sec	40 sec	1 min	10 min	∞
Cycles		30-35 cycles				

Table 2 Results of statistical analysis of DNA samples extracted using QIAamp and Alexgen FFPE DNA extraction kits.

Measuring Device	Parameter	Kit	Mean	Median	Range	*p*-value
Nanophotometer	Quantity (ng/µl)	QIAamp	44.45	36.95	13.2 - 210.5	0.736
		Alexgen	42.36	37.15	11.2 - 178.1	
	Purity (A_{260}/A_{280})	QIAamp	1.94	1.94	1.71 - 2.43	0.3634
		Alexgen	1.96	1.96	1.72 - 2.30	
	Purity (A_{260}/A_{230})	QIAamp	1.98	1.98	1.32 - 3.55	0.1235
		Alexgen	2.01	2.0	1.67 - 3.15	
Qubit	Quantity (ng/µl)	QIAamp	8.14	5.3	1.07 - 59.0	0.7606
		Alexgen	7.61	5.2	1.7 - 41.6	

DISCUSSION

The extraction of DNA is pivotal to biotechnology. It is the starting point for numerous applications, ranging from fundamental research to routine diagnostic and therapeutic decision-making [17]. Extraction and purification are also essential to determining the unique characteristics of DNA, including its size, shape and function [18]. It is also essential for carrying out forensic science, sequencing genomes, detecting bacteria and viruses in the environment and for determining paternity. For quite a long time, numerous human tissue banks have been laid out to preserve FFPE specimens as a significant source of tissue sample for molecular assessments. The goal of preserving such FFPE samples is to protect tissue structure for histological examination as well as nucleic acids for diagnostic and prognostic examinations. The first successful DNA isolation was done in the year 1869 by Swiss Scientist, Friedrich Miescher, and from that point forward propels have been made to the techniques for removing and sanitizing DNA [19]. From the 1950s routine lab extraction of DNA became dependent on the utilization of density gradient centrifuges. Until as of late most strategies for extricating DNA remained tedious which required escalated amounts of work. They additionally gave just little amounts of DNA [19]. Today there are many particular extraction strategies which make use of solution based or column dependent principles [20]. The extraction of DNA has become a lot simpler with the development of commercial kits and automated mechanization. Such changes have both accelerated creation and expand the yield of DNA. The simplest, widely successful commercial kits deploy solid phase silica column-based extraction of DNA from FFPE tissue [21], whereby the QIAamp DNA isolation Kit from the company Qiagen, Duesseldorf, Germany is globally renowned.
However, the limitation of using such globally renowned kits is that they are expensive to the economically restrained researchers in the developing countries. To overcome such limitations, local companies of such developing countries have launched DNA isolation kits calming to extract DNA at par with kits by global

giants. Such a market is filled with genuine local products and products developed to scam researchers. Therefore, it is of importance to gauge the quality locally available kits, and for this the efficacy of DNA isolation and quality obtained from FFPE samples by using one such local kit, Alexgen DNA extraction kit from Alexius Biosciences, Ahmedabad, India is compared with QIAamp DNA isolation Kit from Qiagen. For such assessment we deployed DNA isolation with both the kits from the FFPE sample of breast cancer tissue, then the Quantity of total DNA extracted was assessed using NanoPhotometer spectrophotometry and Qubit fluorometry. Further, the purity of extracted DNA was studied using spectrophotometric absorbance values at A_{260}/A_{280} and A_{260}/A_{230}. Integrity of isolated DNA was examined by performing the PCR reaction for *KRAS* gene, this provided an account how intact the isolated DNA was and served as the parameter of DNA quality.

NanoPhotometer is an instrument which is the most widely spectrophotometer that can determine the absorbance in micro-volumes and is considered as the global reference spectrophotometer for analyzing the DNA absorbances [22]. Under current study we made use of NanoPhotometer P-300 Spectrophotometers from Implen, Germany. The NanoPhotometer results were used to compare the amount and quality of isolated DNA. Because of the aromatic structure of the purine and pyrimidine bases, DNA absorbs UV light at a wavelength of 260nm and can thus be used to determine DNA concentration. Several aspects must be considered while selecting an optimum extraction process. Any molecule which may hinder the downstream application of DNA should be present as minimum as possible. Protein, RNA, and organic substances such as phenol and Trizol are the most common contaminants found in extracted DNA and must be tested for their presence [17]. Protein absorbs at 280nm, hence A_{260}/A_{280} values in the range of 1.8-2.0 for DNA are used for protein-free DNA samples. If the protein is present in the extracted DNA sample, the A280 value will be high and the A_{260}/A_{280} ratio will be less than 1.8 [14, 23]. Similarly, phenol absorbs about 270-280nm and reduces the A_{260}/A_{280} ratio if it is present in the DNA sample [23]. However, because the DNA extraction in this investigation was done with a solid phase extraction kit, phenol contamination was not a concern. For both kits, our DNA purity values were optimum, and in most cases, they were inside the acceptable limit.

As mentioned earlier, quantity of DNA was also assessed using Qubit 2.0 fluorometer. The Qubit's sensitivity may be as low as 10 pg/mL, greatly exceeding that of the NanoPhotometer. As a result, Qubit may be used to analyze samples with extremely low concentrations. The Qubit also has a unique advantage over the NanoPhotometer in terms of specificity. The fluorescent dye used in the Qubit fluorometer selectively binds to the dsDNA and fluorescence intensity gives the concentration of only intact DNA present in sample [24]. In spectrophotometric analysis, however, both intact and fragmented DNA absorb UV light at 260nm, and so, despite the larger concentration reported, downstream application of DNA does not yield the best results owing to the lesser amount of intact DNA [25]. The Qubit values for FFPE-DNA were proportionate to the dilution ratio but were lower than that of the NanoPhotometer readings for each DNA sample collected in this investigation, indicating that fragmented DNA from the FFPE tissue was present. Further to check the quality of extracted DNA samples, PCR reaction was also performed for the amplification of *KRAS* gene, and the amplification ability of all tested DNA samples was observed.

Conclusively, For the quantitative, qualitative, and amplification ability measurements, the statistical non-significant difference between the DNA extracted using the QIAamp and Alexgen kits implies that the Alexgen kit is on par with the widely renowned QIAamp kit for FFPE tissue DNA extraction. The QIAamp kit costs roughly 300 INR each response for DNA extraction, which is much more than the Alexgen kit, which costs around 200 INR per reaction. Alexgen kit cost is around 33% less than QIAamp kit cost. As a result, using a locally accessible kit would be highly cost efficient for researchers in developing nations who are on a tight budget, and it would aid their research.

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Competing Interests
The authors have declared that no competing interest exists.

Availability of Data and material
All data generated or analyzed during this study are included in this published article**.**

Ethics approval
The study was approved by Gujarat University's Ethics Committee (approval no. GUJIEC/22/2019).

Author Contribution Statement
S.B. and U.D. conceptualized the idea; S.B., E.J., and D.M. performed the experiments, analyzed the data, and drafted the manuscript; U.D., and R.R. reviewed the manuscript and supervised the work.

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