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An Effective and Viable DNA Extraction Protocol for FFPE Tissues and its Effect on Downstream Molecular Application

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Abstract: Archival Formalin-Fixed Paraffin-Embedded (FFPE) tissues are a readily available source of DNA for retrospective studies including genetic predisposition. FFPE tissues present significant challenges for extraction of pure genomic DNA in adequate amounts due to the harsh fixation conditions and long-term storage associated with samples. The aim of the study is to propose a simple, in-expensive, viable protocol for the extraction of DNA from FFPE tissues and to optimize the same for the detection of 3'UTR miRNA binding site polymorphism in *ADAMTS1* gene in colorectal cancer tissues and normal colonic mucosa archival tissues using Sanger sequencing and TaqMan SNP genotyping assay. Five different DNA extraction methods were compared and analyzed. NanoDrop quantification demonstrated that out of the various methods tested, highest quantity and pure DNA was obtained from Modified non heating extraction. When we further applied this method for colorectal cancer tissues and normal colonic mucosa archival tissues the NanoDrop readout indicated that the DNA was intact and pure. Intra samples comparison over a time period of 6 months revealed that the DNA was intact. The samples isolated with Modified non heating extraction were found suitable for downstream processes such as Sanger sequencing and TaqMan SNP genotyping assay. This cost effective and non-arduous protocol can successfully extract even minute quantities of DNA from FFPE tissues and facilitate the downstream molecular analysis of a large number of archival specimens for retrospective studies.

Keywords: FFPE Tissues, Sanger Sequencing, Colorectal Cancer, Modified Non Heating Extraction, TaqMan SNP Genotyping Assay

Introduction

Worldwide FFPE tissues are archived in hospitals and tissue banks to serve as an important source of knowledge on genetic and pathological events involved in various aspects of clinical conditions (Blow, 2007). In the last decade there have been exceptional advancements in the field of molecular pathology, providing avenues to deduce molecular mechanisms; screen and evaluate biomarkers pertaining to a variety of diseases. Today these samples present both an incredible opportunity and an immense challenge to researchers. FFPE tissues have been extensively annotated and well preserved, allowing an in depth study of the development of disease such as Mycobacterium tuberculosis and tumor progression. Formalin fixation and paraffin embedding have been the clinical gold standard for preserving these precious samples (Lehmann and Kreipe, 2001; Gnanapragasam, 2010). FFPE tissues have numerous advantages compared to fresh or frozen tissues; they can be effortlessly handled and are inexpensive long-term storage materials which are valuable in various aspects (Ludyga *et al*., 2012). They are stable at ambient temperature, can be easily stored and do not require specialized amenities. FFPE tissue samples can be used for retrospective studies; these sections exhibit various histological features of cancer, including precancerous lesions and facilitate

evaluation of the genetic events correlated to the observed histological changes (Einaga *et al*., 2017).

Of late FFPE tissue specimens have shown compatibility with diverse analytical tools, such as isotope labeling, affinity enrichment and Laser Capture Micro dissection (LCM), increasing the viability of utilizing these tissues as a substitute to frozen tissues for retrospective and prospective protein biomarker discovery (Xiao *et al*., 2010; Toews *et al*., 2008; Azimzadeh *et al*., 2010; Wisniewski *et al*., 2011). In contrast, frozen tissues require specialized facilities for storage, which makes handling outside the research setting challenging, predominantly due to the possibility of rapid decaying. Despite the fact that FFPE tissue is often considered as one of the best choices for clinical molecular applications, isolation of DNA from FFPE tissues is a tedious task. Different protocols have been followed to extract DNA from FFPE tissues for molecular analysis. It is well known that PCR is very difficult to perform with DNA extracted from fixed tissues and fixation intervals are the main reason for decreased PCR yields and incapability to amplify longer DNA targets (Quach *et al*., 2004). It has been previously reported that only up to 300 bp of DNA was amplifiable from FFPE postmortem tissues and more often amplicon size up to 100 bp had given consistent PCR results (Bonin *et al*., 2003). Many studies have been done to optimize the extraction of DNA from FFPE tissues. Among the existing protocols, use of mineral oil for deparaffinization (Lin *et al*., 2009) and the use of high-temperatures with 0.1 M NaOH have shown to increase the efficiency of DNA extraction by manual methods (Pikor *et al*., 2011; Shi *et al*., 2004). Besides, a variety of commercial kits (Huijsmans *et al*., 2010) and have likewise been utilized for DNA extraction.

FFPE tissues have been used for downstream molecular analysis, such as real-time quantitative PCR, Next generation sequencing, Whole genome sequencing, Sanger sequencing and TaqMan SNP genotyping assay. Amongst these, TaqMan SNP genotyping assay is an ideal option for the analysis of cancer markers which can be performed on small copy targets and on degraded DNA due to its increased sensitivity and specificity for the DNA extracted from FFPE tissues. Sanger sequencing has been considered the gold standard for identifying single nucleotide polymorphisms in FFPE tissues for a long time because of its low false-positive rate and high specificity.

A Disintegrin And Metalloproteinase (ADAM) represents a protein family possessing both metalloproteinase and disintegrin domains. ADAMTS-1 is a member of the ADAM protein family which is involved in proteolytic modification of cell-surface proteins and extracellular matrices (Shindo *et al*., 2000). The unique structure of ADAMTS-1, characterized by the presence of thrombospondin type I motifs and is shared by other newly identified proteins in mammals and in *C. elegans*, which comprise the ADAMTS subfamily that may perform well-conserved biological functions. ADAMTS-1 was originally identified by differential display analysis as a gene highly expressed in the murine colon 26 cachexigenic tumors (Kuno *et al*., 1997). In vivo expression of the gene induced in the kidney and heart of mice treated with lipopolysaccharide, suggested a potential role of ADAMTS-1 in the inflammatory reactions (Kuno *et al*., 1997).

This study aims to (1) develop an efficient method for DNA isolation to obtain optimum DNA concentration and purity from FFPE tissues. (2) To optimize a protocol for the detection of 3'UTR miRNA binding site polymorphism in *ADAMTS1* gene in colorectal cancer tissues normal colonic mucosa archival tissues using Sanger sequencing and TaqMan SNP genotyping assay. Different isolation methods that were compared include Modified non heating extraction, Spin column extraction, Non heating extraction, Salting-out method and Phenol chloroform extraction.

Materials and Methods

Tissue Selection and Processing

Formalin-fixed paraffin-embedded colon cancer and normal colonic mucosa tissues (non cancerous tissues) were obtained from the pathology archives of Sri Ramachandra Medical College & Research Institute after due Institutional Ethical clearance (REF:IEC-NI/15/APR/46/26). A total of 50 FFPE tissues were utilized for the study. The tissues fixed in 10 % buffered formalin at ambient conditions were embedded and used. Twenty micron sections were cut utilizing a standard microtome (Shi *et al*., 2002) and the slices were taken into a sterile 1.5 mL micro centrifuge tube directly.

Deparaffinization and Tissue Digestion

The tissues were heated at 55-58°C for five min. Subsequently the sections were deparaffinized with 100% xylene twice and incubated at 56°C for five min, vortexed for 2 min and centrifuged at 13,000 rpm for ten min at ambient conditions. This was followed by subjecting it to 100%, 75% and 50% ethanol washes and centrifugation at 13,000 rpm (Pikor *et al*., 2011; Goelz *et al*., 1985). The tissue pellets were dried free of ethanol at 37°C.

DNA Isolation

DNA was extracted from FFPE tissues using Modified non heating extraction, Spin column extraction, Non heating extraction, Salting-out method and phenol chloroform extraction. A brief outline of the DNA extraction protocols is shown in Table 1. A pictorial representation of the various methods involved in the study is shown in Fig. 1.

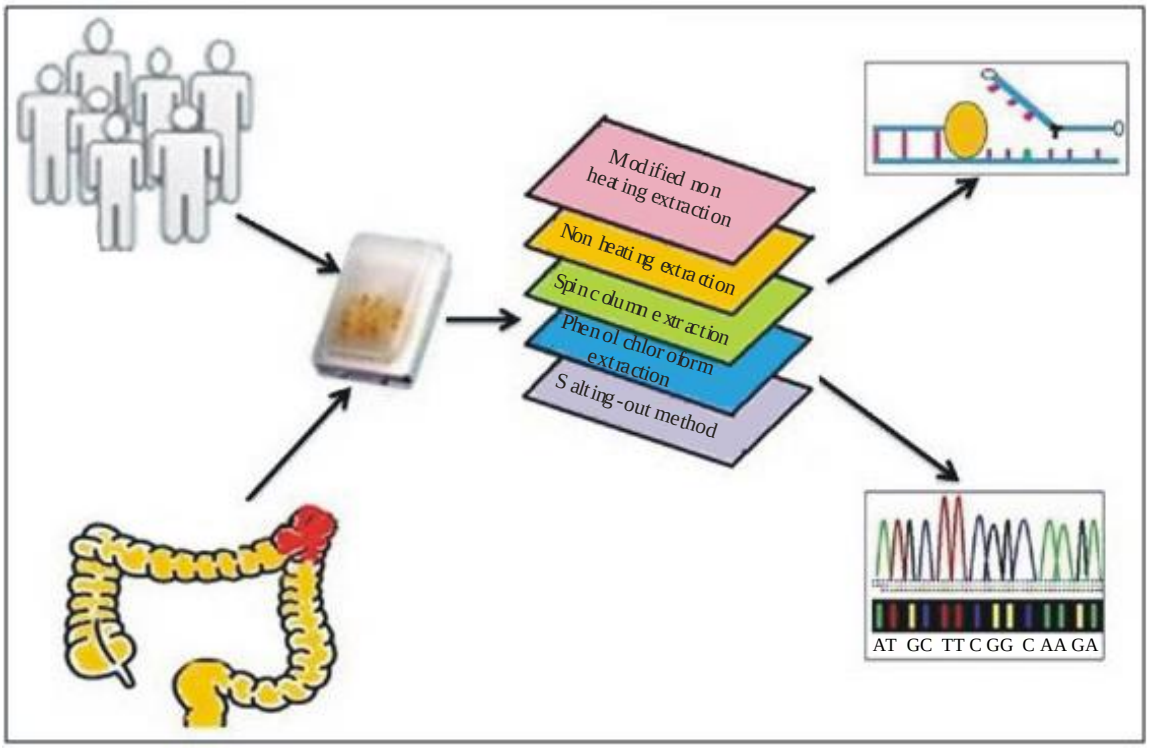

Fig. 1: Overview of the various extraction methods using FFPE tissues

Table 1: Outline of the DNA extraction protocols

Extraction method	Modified non heating extraction	Spin column extraction	Non heating extraction	Salting-out method	Phenol chloroform method
Column based	No	Yes	No	No	No
Trimming excess paraffin	Yes	No	No	No	No
Deparaffinization	Ethanol and xylene	Ethanol and xylene	Ethanol and xylene	Ethanol and xylene	Ethanol and xylene
Tissue Digestion	Digestion buffer Proteinase K	ATL buffer Proteinase K	Lysis buffer Proteinase K	Lysis buffer Proteinase K	Digestion buffer Proteinase K
Digestion conditions	56°C over night	56°C 1 h	52°C over night	55°C over night	55°C over night
Extraction	PCI	GB buffer	PCI	PCI	PCI
Precipitation	Sodium acetate and ethanol	-	Sodium acetate and isopropanol	Absolute alcohol	Isopropanol
Elution buffer	TE Buffer	ATE buffer	Distilled water	70% ethanol	70% ethanol
Final elution volume	40 µL	100 µL	50 µL	30 µL TE buffer	40 µL MilliQ water
Special conditions	Pre heat elution buffer at 95°C for 10 min, heat the samples at 65°C after eluting in TE buffer	-	-	-	-

TE, Tris EDTA, PCI, Phenol chloroform isoamyl alcohol

Modified Non Heating Extraction

Excess paraffin from the tissues was trimmed and the tissues were deparaffinized as mentioned above and PBS was added in two changes after which 500 µL of lysis buffer (proteinase K 20 mg/ml 50 µL, 1 M Tris-HCl solution 10 µL, 0.5 M EDTA 2 µL, 10% SDS 100 µL and distilled water 838 µL) was added and incubated at 56°C overnight until all tissue fragments were dissolved completely. Extraction and purification were performed by adding 500 µL of 25:24:1 phenol: Chloroform: Isoamyl alcohol to the dewaxed tissue, followed by vortexing (cyclo mixer) and centrifugation at RT, 12,000 g for 10 min. The

upper aqueous supernatant was transferred to a new micro centrifuge tube and one volume of chloroform was added, vortexed and was centrifuged at 12,000 g for 10 min. The upper aqueous supernatant was transferred into a new micro centrifuge tube, 0.1 volume of 3 M sodium acetate was added, vortexed after which one volume of ethanol was added and incubated at-20°C overnight. The precipitated DNA was centrifuged at 12,000 g at 4°C for 10 min. The supernatant was discarded and the precipitate was washed twice with 75% ethanol (Shi *et al*., 2004). The pellet was dried free of ethanol and was dissolved in 40 µL of preheated (heated at 95°C for 10 min) TE buffer. And the eluted DNA was again heated at 65°C for five min.

Spin Column Extraction

The commercial spin column DNA extraction was performed according to the manufacturer's instructions. The tissues were deparaffinized with xylene, followed by two washes with 100% ethanol to remove remaining xylene. After deparaffinization, tissues were digested at 56°C with 180 µL of ATL buffer and 20 µL proteinase K. The completely digested tissues were incubated at 90°C for 1 h. Subsequent to digestion, 200 µL of AL buffer was added, followed by ethanol precipitation. The solution was transferred into a new spin column and washed with wash buffers AW1 and AW2 provided in the kit. The DNA was eluted in 40 µL ATE buffer.

Non Heating Extraction Method

Tissues were deparaffinized as mentioned above and PBS was added in two changes after which 500 µL of lysis buffer (proteinase K 20 mg/ml, 50 µL, 1 M Tris-HCl solution 10 µL, 0.5 M EDTA 2 µL, 10% SDS 100 µL and distilled water 838 µL) was added and incubated at 52°C overnight until all tissue fragments were dissolved completely. Extraction and purification was performed by adding 500 µL of 25:24:1 phenol: Chloroform: Isopropanol alcohol to the dewaxed tissue, followed by vortexing (cyclo mixer) and centrifugation at 12,000 g for 10 min. The upper aqueous supernatant was transferred to a new micro centrifuge tube after which one volume of chloroform was added, vortexed and was centrifuged at 12,000 g for 10 min. The upper aqueous supernatant was transferred into a new micro centrifuge tube, 0.1 volume of 3 M sodium acetate was added vortexed after which one volume of isopropanol was added and incubated at – 20°C overnight. The precipitated DNA was centrifuged at 12,000 g at 4°C for 10 min. The supernatant was discarded and the precipitate was washed twice with 75% ethanol (Shi *et al*., 2004). The pellet was dried free of ethanol and was dissolved in 50 µL double distilled water.

Salting-Out Method

Tissues were deparaffinized as mentioned above. One mL of lysis buffer solution was added along with 50 µL of 10% SDS and inverted for 10 min. To this mixture, 6 M NaCl was added and incubated overnight. This was followed by centrifugation at 3000 rpm for 20 min. Supernatant was collected; to this double the volume of chilled 100% ethanol was added. The DNA was transferred into a new micro centrifuge tube and centrifuged at 12,000 rpm for 10 min at ambient conditions (Miller *et al*., 1988). Supernatant was disposed and 1 mL of 70% ethanol was added and centrifuged at 12,000 rpm at 4°C. The pellet was dried free of ethanol, after which 30 µL of TE buffer was added to dissolve the pellet.

Phenol Chloroform Extraction

After deparaffinization as described above the pellet was dried at 37°C. Dried tissue was dissolved in 300 µL of digestion buffer containing 50 mM Tris, 5 mM EDTA, pH 8 and 10 µg/ml proteinase K. Subsequent to overnight incubation at 56°C, the digested tissue was centrifuged with phenol chloroform. The upper aqueous layer was taken and the DNA was precipitated overnight with sodium acetate and ethanol. Centrifugation was done for 30 min at 4°C, the pellet formed was washed with 1 mL of 70% ethanol and was air dried at ambient conditions (Dedhia *et al*., 2007). Later the DNA was eluted in 40 µL of miliQ water.

Quality Assessment of DNA

After the completion of DNA isolation, 3 µL DNA was subjected to 0.8% agarose gel electrophoresis to check the presence of intact DNA. Comparison of the quality of FFPE tissue DNA isolated using five different isolation methods is shown in Fig. 2A. The yield was compared between five Different isolation methods by using NanoDropTM 1000 spectrophotometer. This was measured according to the standard protocol recommended by the manufacturer. Further FFPE tissues from the year 2011-2017 was subjected to Modified non heating extraction and checked for purity and stability of the DNA. Also NanoDrop quantification was done in 3 months and 6 months interval to follow up the DNA stability. The quantified DNA was subjected to Sanger sequencing and TaqMan SNP genotyping assay.

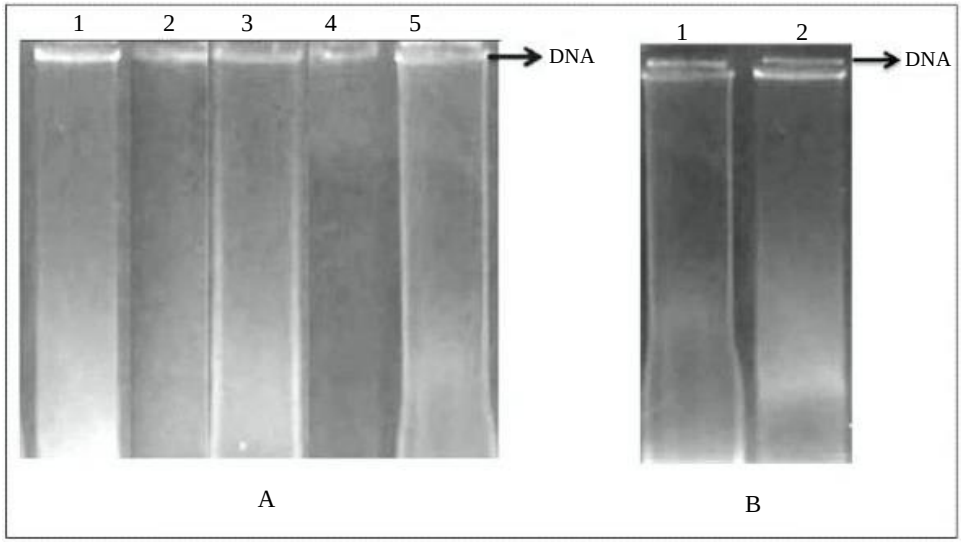

Fig. 2: An Agarose gel electrophoresis of DNA isolated from FFPE tissues using five different methods. Lane 1: Modified non heating extraction method, Lane 2: Spin column extraction, Lane 3: Non heating extraction method, Lane 4: Salting-out method, Lane 5

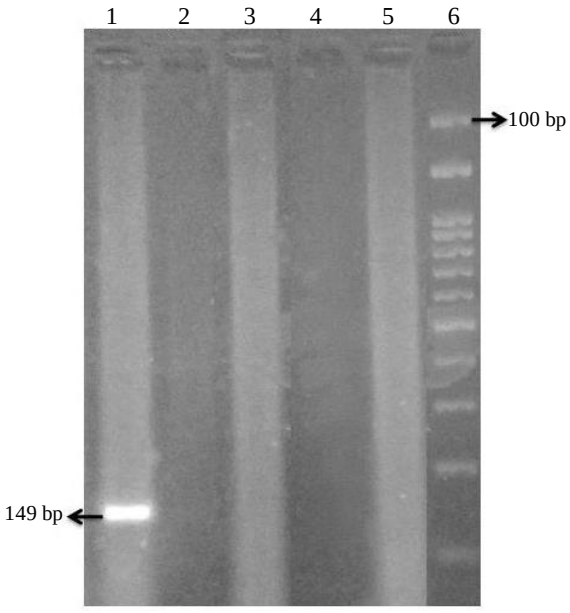

Fig. 3: Agarose gel electrophoresis of PCR product obtained from FFPE tissue DNA extracted by different methods. Lane 1: Modified non heating extraction method, Lane 2: Spin column extraction, Lane 3: Non heating extraction method, Lane: Salting -out metho

PCR Amplification

DNA extracted from five different extraction methods was used for performing PCR. PCR amplification was observed from the DNA isolated from Modified non heating extraction (Fig. 3). Further, this DNA was used for Sanger sequencing. 2 µL of 50 ng DNA template was added to PCR master mix containing 5 U Taq polymerase, 2 mM dNTPs, 5 pmol forward and reverse primer (Table 2), 10X buffer and double distilled water. The PCR reaction was carried out with a 4 min initial denaturation at 94°C, denaturation for 30 sec at 94°C, annealing for 30 sec at 56.4°C, extension for 30 sec at 72°C and final extension for 30 sec at 72°C for 35 cycles, followed by storage at 4°C. 10 µL of PCR products were resolved on a 2% agarose gel.

PCR Optimization

FFPE tissue DNA extracted from colon cancer tissue and normal colonic mucosa using Modified non heating extraction method was utilized for PCR optimization. ADAMTS1 gene was amplified using forward and reverse primer (Table 2). Annealing temperature, primer concentration and template quantity were standardized. Annealing temperatures tried were 54.6°C, 54.8°C, 55.4°C, 56.4°C, 58.0°C, 59.1°C and 59.7°C. Two different primer concentrations of 10 pmol, 5 pmol were tried, avoiding primer dimer formation was considered as a primary factor during optimization of primer concentration. Template concentrations of 100 ng, 50 ng, 250 ng and undiluted DNA were used. The reaction volume was 20 µL, which consisted of master mix, template DNA, forward and reverse primer. No template control was also included for the reaction.

Table 2: Primer details

Name of the gene	Tm	Product size	Sequence (5'-3')
ADAMTS1–Forward primer	56.4°C	218 bp	GTATCATGGGGGTTGGGAA
ADAMTS1–Reverse primer	56.4°C	218 bp	CGTTGCTGAGCCTTTCTCTC

Tm, primer melting Temperature, *bp*, base pair

Sanger Sequencing

The amplified products were subjected to Sanger sequencing using Big Dye Terminator V.3.1. (Applied Biosystems, Foster City, CA) in ABI Prism™ 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were evaluated with SeqScape analysis software V2.5.

Allelic Discrimination by TaqMan SNP Genotyping Assay

The allelic discrimination by TaqMan SNP genotyping assay was carried out in Fast 7900 HT Real Time PCR machine using DNA extracted from Modified non heating extraction. PCR was performed in a 5 µL reaction mixture containing 1 µL of 50 ng DNA, 2.5 µL TaqMan Genotyping Master mix, 0.1 µL TaqMan SNP genotyping assay Mix and 1.4 µL of water. The PCR conditions used were: Stage I: 50°C for 2 min Stage II: 95°C for 10 min Stage III: 95°C for 15 sec and 60°C for 1 min for forty cycles. Genotype identification was performed using Sequence Detection Software 2.0 (SDS).

Optimization of Allelic Discrimination by TaqMan SNP Genotyping Assay

TaqMan SNP genotyping assay was performed on DNA isolated from colon cancer FFPE tissues and normal colonic mucosa samples using the Modified non heating extraction. The concentration of DNA and quantity of master mix was standardized. Template concentrations of 50 ng, undiluted DNA, 2.5 µL, 2.75 µL and 3.0 µL of master mix were used for standardization. The reaction volume was 5µL, which consisted of Taqman Genotyping Master Mix, TaqMan SNP genotyping assay Mix, water and template DNA. No template control was also included.

Statistical Analysis

The statistical analysis was performed using SPSS software version 20.0. Student's t-test was done to compare the difference between concentrations of DNA obtained from five different extraction methods. P values below 0.05 were acknowledged as significant.

Results

FFPE Tissue DNA Extraction

Four diverse DNA extraction protocols along with one modified DNA extraction protocol were carried out in parallel to assess the yield and quality of DNA isolated from FFPE tissue samples. Maximum yield was observed in the range of 500-2500 µg which was obtained with Modified non heating extraction and minimum yield was seen in the range of 7 µg-20 µg which was obtained by Spin column extraction method as shown in Table 3. The maximum purity of DNA obtained at OD 260/280 was 1.9 which was observed in Modified non heating extraction. The quantity of DNA obtained using Modified non heating extraction protocol was good enough for optimization of Sanger sequencing and TaqMan SNP genotyping assay. Among the five different methods, significant results in terms of concentration, were obtained from the samples treated with the Modified non heating extraction (p = 0.0002) compared to the samples that were isolated with the Spin column extraction. Furthermore, the samples isolated with Modified non heating treatment produced remarkably significantly pure DNA compared to Salting-out and Phenol chloroform method (Table 3).

Student's *t*-test was performed to compare the DNA concentrations between the five different isolation methods, where Spin column extraction method was considered as the reference method. The results were significant where p value was less than 0.05. Further FFPE tissues from the year 2011-2017 was subjected to Modified non heating extraction and checked for purity and stability of the DNA. Also, NanoDrop quantification was done in 3 months and 6 months intervals to follow up the DNA stability most importantly; year-wise comparison of the NanoDrop reading also suggested that this method results in good quality and quantity of DNA. FFPE tissues collected from 2011 archive yielded 1091 ng of DNA and the highest of 2480 ng was obtained from FFPE tissues belonging to 2017 archive (Table 4 and Fig. 4). When intra sample NanoDrop comparison was done after three months and six months respectively, the DNA had minimal degradation and was intact to perform downstream molecular applications (Table 4 and Fig. 5). The NanoDrop reading for the colorectal cancer tissues and normal colonic mucosa tissues (non cancerous tissues) indicate that good quantity of intact DNA can be isolated by using Modified non heating extraction method (Table 4). However the quantity of DNA obtained from normal colonic mucosa tissues was lesser compared to colorectal cancer tissues. The primary reason for this was the size of the biopsy. But it was observed that the DNA obtained from normal colonic mucosa was stable, pure and was suitable for performing Sanger sequencing and TaqMan SNP genotyping assay.

Table 3: Quantity and quality of DNA extracted from formalin fixed paraffin embedded tissues

Extraction method	Mean ± SD (Concentration ng/µL)	P value	A260/280
Modified non heating extraction	1619.51±1012.97	0.0002	1.8-1.9
Spin column extraction	101.5±69.39	(reference)	1.8-1.9
Non heating extraction	1078.16±704.69	0.0004	1.8-1.9
Salting-out method	38.53±28.55	0.0161	1.4-1.7
Phenol chloroform method	152.1±158.99	0.3687	1.4-1.9

Bold values are significant, SD, Standard deviation; Student's *t*-test was done to compare the DNA concentrations between the five different isolation methods. The results are considered significant where P value was less than 0.05

Table 4: Comparison of DNA quality and quantity post 3 and 6 months of DNA isolation among samples from different time period

Sample No	Tissue Thickness	Year	A260/280	Initial values ng/µL	values after 3 months ng/µL	values after 6 months ng/µL
CA-18/17	60 micron	2017	1.9	2480	2440	2400
CON-15/17	60 micron	2017	1.9	220	210	195
CA-37/17	60 micron	2017	1.8	974	960	945
CA-12/16	60 micron	2016	1.9	2376	2356	2340
CON-41/16	60 micron	2016	1.9	352	340	332
CA-58/16	60 micron	2016	1.9	1663	1640	1630
CA-33/15	60 micron	2015	1.9	1312	1280	1240
CON-64/15	60 micron	2015	1.9	253	240	225
CA-25/15	60 micron	2015	1.8	2340	2320	2300
CA-17/14	60 micron	2014	1.8	1604	1580	1560
CON-60/14	60 micron	2014	1.8	476	460	440
CA-30/14	60 micron	2014	1.9	1841	1820	1800
CA-48/13	60 micron	2013	1.8	1091	1050	1000
CON-33/13	60 micron	2013	1.9	374	360	345
CA-77/13	60 micron	2013	1.8	1897	1870	1855
CA-11/12	60 micron	2012	1.8	1755	1740	1700
CON-16/12	60 micron	2012	1.8	481	465	440
CA-30/12	60 micron	2012	1.9	1841	1820	1800
CA-19/11	60 micron	2011	1.8	1091	1050	1000
CON-35/11	60 micron	2011	1.8	351	333	315
CA-84/11	60 micron	2011	1.8	2688	2650	2632

ng, nanogram, CA, Colorectal cancer tissues, CON, Normal colonic mucosa tissues

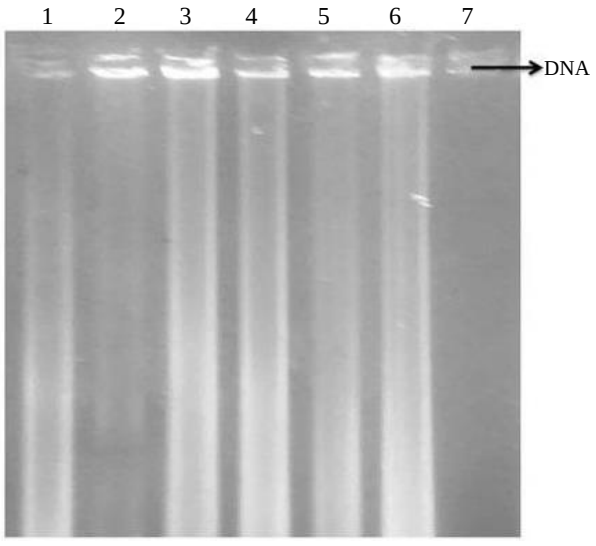

Fig. 4: Agarose gel electrophoresis of DNA isolated from FFPE tissues using Modified non heating extraction. Lane 1: 2011, Lane 2: 2012, Lane 3: 2013, Lane 4: 2014, Lane 5: 2015, Lane 6: 2016, Lane 7: 2017

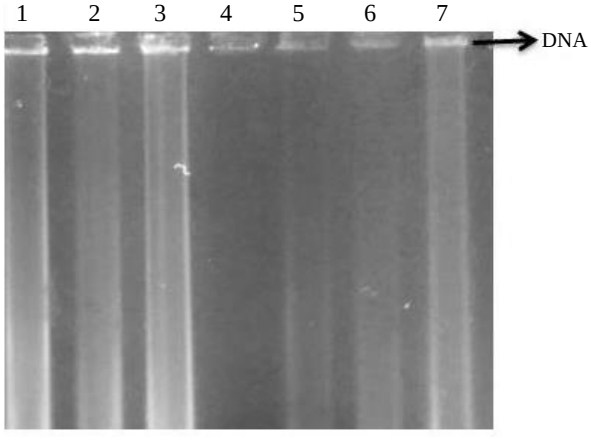

Fig. 5: Agarose gel electrophoresis of FFPE tissue DNA to compare stability of DNA after 6 months of isolation and storage. Lane 1: 2011, Lane 2: 2012, Lane 3: 2013, Lane 4: 2014, Lane 5: 2015, Lane 6: 2016, Lane 7: 2017

PCR Amplification and Optimization

Among the five different DNA extraction protocols, DNA extracted through Modified non heating extraction

method showed proper PCR amplification compared to other four methods (Fig. 3). PCR results indicated a prominent band in the range of 218 bp (Fig. 6). In case of *ADAMTS1* gene amplification, commendable results were acquired at an annealing temperature of 56.4°C. Primer concentration of 5 pmol was used for better results and primer dimmers were not observed. PCR amplification was visible only with 50 ng of DNA. While 100 ng, 250 ng and undiluted DNA samples failed to demonstrate any amplification.

Allelic Discrimination by TaqMan SNP Genotyping Assay

Template concentration of 50 ng and 2.5 µL master mix showed good genotyping results. Automatic calling was performed to analyze the results obtained from the Sequence detection systems software. The results of the allelic discrimination assay data were plotted as Allele 1 (VIC™ dye) versus Allele 2 (FAM™ dye).

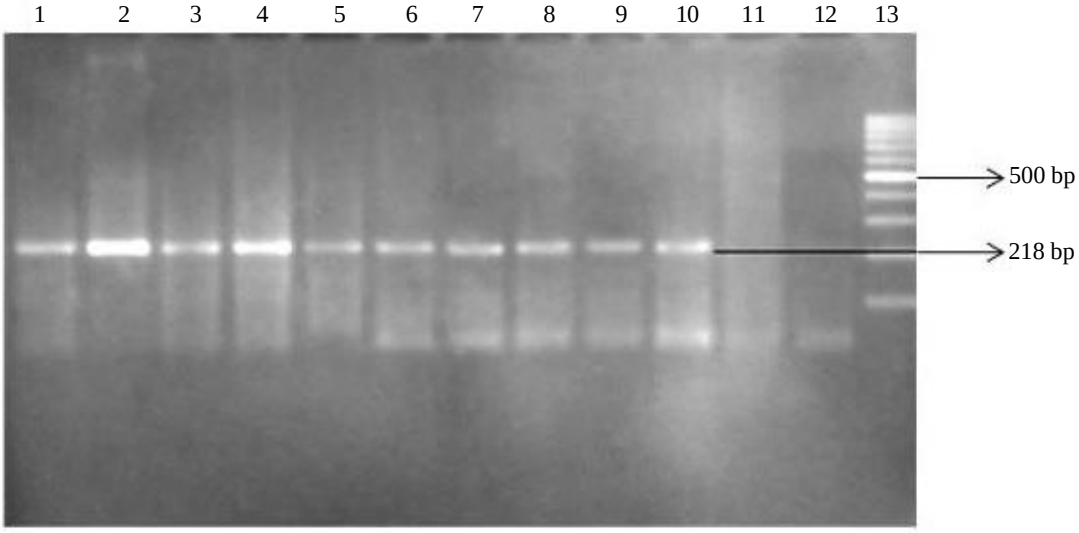

Fig. 6: Agarose gel electrophoresis of PCR products obtained from ADAMTS1 gene amplification using FFPE tissue DNA isolated from Modified non heating extraction (218 bp). Lane 1-6: Genomic DNA isolated from representative colorectal cancer tissues. Lane 7

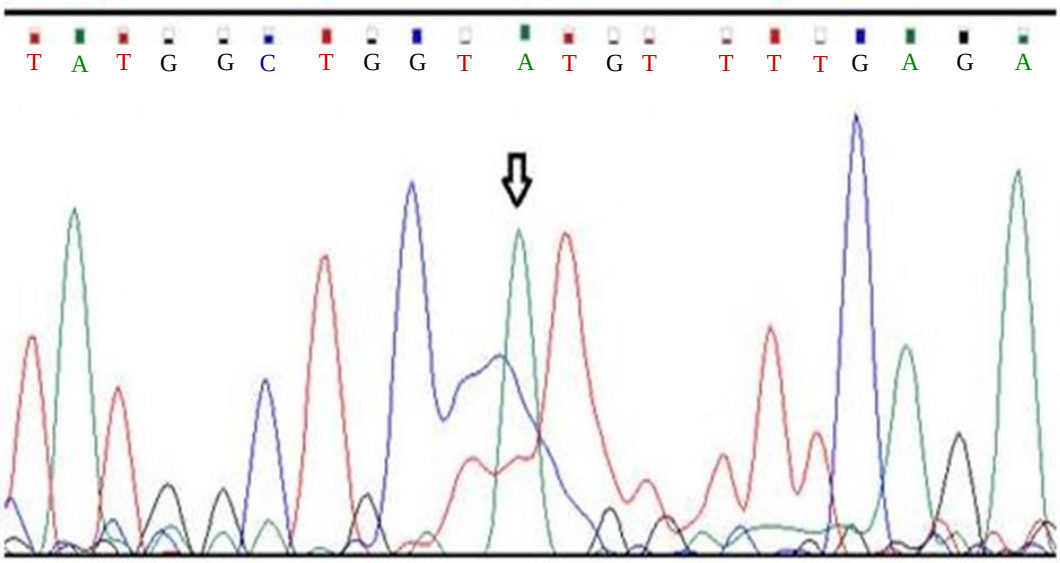

Fig. 7: Electropherogram showing Sanger Sequencing of ADAMTS1 gene. The arrow indicates the location of the Single nucleotide polymorphism

Discussion

Currently, FFPE tissues are progressively used for molecular analyses both in clinical and research laboratories. The expulsion of the paraffin wax encasing the thin layer of tissue and isolation of adequate intact DNA are major impediments to work with these samples. Lately, the methods and protocols for the isolation of DNA from FFPE tissues have been considerably improved (Regan *et al*., 2012; Okello *et al*., 2010; Huijsmans *et al*., 2010). In this study, we illustrate a dynamic protocol for the isolation of DNA from FFPE tissue samples. Our study demonstrated that amongst the five DNA extraction protocols assessed, Modified non heating extraction protocol was found to be the most reliable method for the clinical diagnosis in terms of quantity, quality (Table 3; Fig. 2A) and cost effectiveness. On the other hand, Phenol chloroform extraction was found to be quite laborious, involving a large number of steps, prone to cross contamination and has had environmental effects. Salting-out method was likewise prone to salt contamination which might result in PCR inhibition. In the case of Spin column extraction, the yield of DNA obtained was exceptionally low and comparatively very expensive. Non heating extraction method has shown effective results but it was found that Modified non heating extraction resulted in better quality and quantity of DNA which can be preserved for a longer time without rapid degradation (Fig. 2B).

The changes implemented for Modified non heating extraction include (a) Over night digestion using proteinase K at 56°C. Proteinase K gets activated at 56°C, an interesting characteristic of proteinase K is that it retains its activity in the presence of Sodium Dodecyl Sulphate (SDS). In turn increasing the temperature of the reaction from 37°C to 50°-60°C and thereby increases its activity by several folds. Another striking feature of proteinase K is its ability to digest native proteins at 56°C, thereby inactivating enzymes such as DNase and RNase without recourse to a denaturation process. (b) Elution of FFPE tissue DNA using TE buffer, where TE is derived from its components Tris and EDTA, a molecule that chelates cations like Mg^{2+} and solubilizes DNA or RNA. The pH of TE buffer is slightly basic which allows DNA to dissolve faster and EDTA helps to protect it from degradation. (c) Preheating TE buffer at 95°C is done to eliminate DNase contamination, heating at higher temperatures allows better hydration of DNA hence allowing a better absorption of DNA. (d) Heating the eluted DNA at 65°C for five min removes the ethanol traces and helps to dissolve the pellet quickly, thereby increases DNA yield.

Earlier studies on DNA extraction protocols have revealed that heating tissues at high temperature may lead to the extraction of good quality DNA (Frank *et al*., 1996; Faulkner and Leigh, 1998). Other studies have reported that higher temperature heating under an alkaline condition provided the most acceptable results (Shi *et al*., 2002). Sam *et al*. (2012) reported that Qiagen EZ1 DNA Tissue Kit requires less hands-on-time to yield adequate DNA concentration and quality and is also found to be a proficient method for purifying DNA from FFPE tissues. However, this method in all aspects seems to be quite expensive (Sam *et al*., 2012). In spite of the fact that there are some evidences that the simple heating method can yield adequate quantity of DNA and also be utilized for detection of genomic changes in DNA extracted from FFPE tissue sections, this approach has not been widely applied. One possible reason may be that the quantity of DNA extracted from FFPE tissue by heating alone is lower than that extracted by an enzyme-based protocol (Sam *et al*., 2012).

The enzymatic digestion step is without doubt a standout amongst most imperative steps in the Modified non heating extraction procedure. The enzyme volume plays a critical role in tissue digestion. One h and overnight digestions have been utilized in various studies earlier (Huijsmans *et al*., 2010; Santos *et al*., 2009; Oh *et al*., 2013) and researchers have also repeatedly acknowledge the fact that prolonged protein digestion time enhances DNA yield (Pikor *et al*., 2011; Isola *et al*., 1994; Wood *et al*., 2010). Among the two modifications in our Modified non heating extraction, overnight incubation of tissue fragment at 56°C is one of the key factors in increasing the DNA yield. Proteinase K is a member of the subtilisin family; the enzyme is a long polypeptide protein which is comprised of 278 amino acids. This enzyme acts on proteins by cleaving the peptide bonds adjacent to the carboxyl group of hydrophobic amino acid residues (aliphatic and aromatic). The optimal temperature for proteinase K is known to be between 50- 60°C and the specific activity of proteinase K is enhanced by increasing the temperature from 52°C to 56°C (Snow *et al*., 2014). This will unfold the proteins, exposing the hydrophobic amino acid residues that would be normally encrypted inside the hydrophobic core of the protein making it easier for enzyme action. Moreover, at higher temperatures most nucleases that would degrade DNA are denatured thus making the increase in temperature favorable for efficient DNA precipitation. The same was evident in our study

Further, the assumption underlying the extension of enzymatic (proteinase K) digestion is that the persistent protein-DNA cross-links are reversed by heat and that this reversal can be improved either by increasing the incubation temperature or time (Huijsmans *et al*., 2010; Shi *et al*., 2002; Isola *et al*., 1994; Einaga *et al*., 2017). Removal of the protein-DNA cross-links by maximizing the protease digestion, will ultimately lead to precipitation of relatively intact DNA.

Hence, in Modified non heating extraction we have used 56°C as the incubation temperature and overnight incubation time, which facilitates proper digestion of the tissues. Kumar *et al*. (2016) have reported that quantification of DNA influences the PCR results obtained from FFPE tissue DNA. They have demonstrated that considerable differences exist between DNA quantification protocols that might cause researchers to under/overestimate the quantity of DNA in their samples.

It has also been reported that the issue of quantification of DNA from human diagnostic specimens, either fresh-frozen or FFPE, is also not adequately addressed in the literature (Kumar *et al*., 2016). We noticed that the age and origin of the FFPE tissue sample influenced the DNA isolation efficiency (Fig. 4 and 5). However, our NanoDrop results suggest that the DNA yield from 7-year-old FFPE tissue material was equally good in comparison to a two-year-old block (Table 4). In fact the DNA showed long term stability and was found to be intact for the next 6 months (Table 4). Besides, the tissue samples vary with regard to tissue compositions that include inflammatory cells and parenchymal cells. These cells bring about differences in cell density and therefore different DNA quantities are observed in a given tissue surface area. Our study revealed that Spin column extraction, Salting-out method and Phenol chloroform method brought about low quality and yield of DNA which wasn't useful for downstream molecular studies, whereas Modified non heating extraction method lead to successful amplification of the *ADAMTS1* gene with an amplicon size of 218 base pairs (Fig. 6). Numerous studies have reported that fragmented DNA is obtained due to fixation and embedding processes, only allowing PCR analysis on short target gene sequences (Liu *et al*., 1993; Pavelić *et al*., 1996; Bonin *et al*., 2005; Shi *et al*., 2002).

In our study PCR amplification was visible only with 50 ng of DNA isolated by using Modified Non heating extraction method and not with 100 ng and 250 ng, it was observed that the input of higher concentrations of DNA can lead to inhibition of PCR amplification. The primary reason being the fragmented nature of the DNA, which not only influences the target sequence but also randomly generates short DNA debris. This debris might act directly as an inhibitor of the DNA polymerase. The DNA debris might bind to the polymerase and decrease its activity resulting in a decreased PCR reaction speed (Dietrich *et al*., 2013). However, the other four methods generated low quality of DNA and showed no PCR amplification (Fig. 3), due to the DNA being impure and extremely fragmented.

Gall *et al*. (1993) and Sato *et al*. (2001) have suggested that avoiding one or more of the following steps such as centrifugation, dewaxing, digestion with proteinase K, purification may improve PCR efficiency, (Gall *et al*., 1993; Sato *et al*., 2001; Coates *et al*., 1991; Shi *et al*., 2002) but due to this DNA purity may be reduced. Our results were obtained with modifications in conventional extraction procedures, by combining: A. Pre heating of tissue at 56°C; b. Deparaffinization with xylene; c. Digestion over night with concentrated proteinase K (20 mg/mL); d. Purification by phenol chloroform extraction and e. increase in the number of PCR amplification cycles up to 35. The most critical step affecting DNA integrity is the fixation process. Specifically, the degree of damage in DNA may depend on the kind of fixative used and the extent of fixation. However, formaldehyde, a principal active component of formalin causes degradation of the nucleic acids under extremely acidic environment which negatively influences the downstream applications (Blow, 2007; Gilbert *et al*., 2007; Turashvili *et al*., 2012). It decreases the efficiency of PCR due to the presence of chemical cross-linking between RNA, DNA and protein. In addition, monomethylol groups are also added to nucleotide base pairs (Specht *et al*., 2001; Kokkat *et al*., 2013). These processes increase the vulnerability of nucleotide sequences to shear, fragment and degrade. The age of the fixed tissue, the dimensions and conditions of storage, have been implicated as factors that can influence the process of cross linking and molecular degradation (Cukier *et al*., 2009).

In our study we have shown that DNA extracted from Modified non heating extraction can be used for downstream molecular analysis like Sanger sequencing and TaqMan SNP genotyping assay. We were able to successfully genotype 3'UTR miRNA binding site polymorphism in *ADAMTS1* gene. The genotyping results suggested that the most of the samples genotyped showed homozygous normal condition (C/C) (Fig. 7). For genotyping we utilized FFPE tissue DNA isolated from colorectal cancer tissues and normal colonic mucosa tissues. Even though the normal colonic mucosa archival tissue is a smaller biopsy compared to colorectal cancer archival tissue we were able to isolate good quality and quantity of DNA. Snow *et al*. (2014) have reported that extraction of DNA from cellular tumors with a solid growth pattern and less contaminating non-neoplastic tissue is comparatively easy. In contrast to small biopsies which usually contain minute nests of tumor cells surrounded by non-tumor tissue creating a challenge in obtaining adequate quantity of DNA material (Snow *et al*., 2014). Therefore our newly modified DNA extraction method enables us to acquire adequate quantity of DNA from diminutive foci of the tumor and can be utilized for both cancer tissues as well as non neoplastic tissues.

It was observed that our study showed concordance to Betge *et al*. (2015) who has tried to compare sequencing in fresh and frozen tissue samples and have

reported that convincing results were obtained with FFPE tissues (Betge *et al*., 2015). Past studies have reported that NGS (Gall *et al*., 1993), Genome-wide massively parallel sequencing (Schweiger *et al*., 2009), Whole genome (Van Allen *et al*., 2014) and Whole exome sequencing (Astolfi *et al*., 2015; Bonfiglio *et al*., 2016) have been performed on FFPE tissues. But all these methods are very expensive compared to Sanger sequencing and TaqMan SNP genotyping assay. One reason behind the slightly higher amplification rate for TaqMan SNP genotyping assay could be that this technique combines both amplification and hybridization with specific fluorescent probes, therefore making it more sensitive than amplification alone at an affordable price, which may be time consuming. In summary, the Modified non heating extraction results in greater yield, optimum DNA concentration and quality and it is found to be an efficient method for extracting DNA from FFPE tissues. This is a critical factor for consideration where DNA isolation is a routine in clinical laboratories for various genetic analyses, as molecular techniques are moving rapidly from bench to bed use in diagnostic pathology.

Conclusion

Modified non heating extraction is a cost effective and non-laborious protocol which can successfully extract DNA from FFPE tissues and facilitate the downstream molecular analysis of a large number of archival tissues in retrospective studies. Among the five methods tested for its effectiveness, Modified non heating extraction showed efficient DNA extraction for TaqMan SNP genotyping assay and Sanger sequencing used for SNP detection.

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Authors Contributions

Charles Emmanuel Jebaraj Walter and Thanka Johnson: Conceived and designed the experiments.

Sai Sushmitha Kontham: Carried out recruitment of subjects, experiments and drafted the manuscript.

Manjula Raju: Helped in carrying out Sanger sequencing.

Chinambedu Dandapani Mohanapriya: Analyzed the data.

Nirmala Karuppasamy and Zioni Sangeetha Shankaran: Provided their critical suggestions in completion of the experiments.

Charles Emmanuel Jebaraj Walter and Thanka Johnson: Reviewed and finalized the manuscript.

Ethics

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and ICMR ethical guidelines for biomedical research. For this type of study formal consent is not required. Institutional Ethical clearance (REF: IEC-NI/15/APR/46/26) was obtained from Sri Ramachandra Institute of Higher Education and Research.

Conflict of Interest

The authors declare that they have no competing interests. The corresponding author affirms that all of the authors have read and approved the manuscript.

References

Astolfi, A., M. Urbini, V. Indio, M. Nannini and C.G. Genovese *et al*., 2015. Whole Exome Sequencing (WES) on Formalin-Fixed, |Paraffin-Embedded (FFPE) tumor tissue in Gastrointestinal Stromal Tumors (GIST). BMC Genom., 16: 892-892. DOI: 10.1186/s12864-015-1982-6

Azimzadeh, O., Z. Barjaktarovic, M. Aubele, J. Calzada-Wack and H. Sarioglu *et al*., 2010. Formalin-Fixed Paraffin-Embedded (FFPE) proteome analysis using gel-free and gel-based proteomics. J. Proteome Res., 9: 4710-4720. DOI: 10.1021/pr1004168

Betge, J., G. Kerr, T. Miersch, S. Leible and G. Erdmann *et al*., 2015. Amplicon sequencing of colorectal cancer: Variant calling in frozen and formalin-fixed samples. PLoS One, 10: e0127146-e0127146. DOI: 10.1371/journal.pone.0127146

Blow, N., 2007. Tissue preparation: Tissue issues. Nature, 448: 959-963. DOI: 10.1038/448959a

Bonfiglio, S., I. Vanni, V. Rossella, A. Truini and D. Lazarevic *et al*., 2016. Performance comparison of two commercial human whole-exome capture systems on formalin-fixed paraffin-embedded lung adenocarcinoma samples. BMC Cancer, 16: 692-692. DOI: 10.1186/s12885-016-2720-4

Bonin, S., F. Petrera, J. Rosai and G. Stanta, 2003. PCR analysis in archival postmortem tissues. Mol. Pathol., 56: 184-186. DOI: 10.1136/mp.56.3.184

Bonin, S., F. Petrera, J. Rosai and G. Stanta, 2005. DNA and RNA obtained from Bouin's fixed tissues. J. Clin. Pathol., 58: 313-316. DOI: 10.1136/jcp.2004.016477

Coates, P.J., A.J. d'Ardenne, G. Khan, H.O. Kangro and G. Slavin, 1991. Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. J. Clin. Pathol., 44: 115-118. DOI: 10.1136/jcp.44.2.115

Cukier, H.N., M.A. Pericak-Vance, J.R. Gilbert and D.J. Hedges, 2009. Sample degradation leads to false-positive copy number variation calls in multiplex real-time polymerase chain reaction assays. Anal. Biochem., 386: 288-290. DOI: 10.1016/j.ab.2008.11.040

Dedhia, P., S. Tarale, G. Dhongde, R. Khadapkar and B. Das, 2007. Evaluation of DNA extraction methods and real time PCR optimization on formalin-fixed paraffin-embedded tissues. Asian. Pac. J. Cancer Prev., 8: 17477772-17477772. PMID: 17477772

Dietrich, D., B. Uhl, V. Sailer, E.E. Holmes and M. Jung *et al*., 2013. Improved PCR performance using template DNA from formalin-fixed and paraffin-embedded tissues by overcoming PCR inhibition. PLoS One. DOI: 10.1371/journal.pone.0077771

Einaga, N., A. Yoshida, H. Noda, M. Suemitsu and Y. Nakayama *et al*., 2017. Assessment of the quality of DNA from various Formalin-Fixed Paraffin-Embedded (FFPE) tissues and the use of this DNA for Next-Generation Sequencing (NGS) with no artifactual mutation. PLoS One, 12: e0176280-e0176280. DOI: 10.1371/journal.pone.0176280

Faulkner, S.W. and D.A. Leigh, 1998. Universal amplification of DNA isolated from small regions of paraffin-embedded, formalin-fixed tissue. Biotechniques, 24: 47-50. DOI: 10.2144/98241bm08.

Frank, T.S., S.M. Svoboda-Newman and E.D. Hsi, 1996. Comparison of methods for extracting DNA from formalin-fixed paraffin sections for non isotopic PCR. Diagn. Mol. Pathol., 5: 220-224. DOI: 10.1097/00019606-199609000-00012

Gall, K., J. Pavelić, D. Jadro-Santel, M. Poljak and K. Pavelić, 1993. DNA amplification by polymerase chain reaction from brain tissues embedded in paraffin. Int. J. Exp. Pathol., 74: 333-337. PMID: 8398805

Gilbert, M.T.P., T. Haselkorn, M. Bunce, J.J. Sanchez and S.B. Lucas *et al*., 2007. The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when? PloS One, 2: e537-e537. DOI: 10.1371/journal.pone.0000537

Gnanapragasam, V.J., 2010. Unlocking the molecular archive: The emerging use of formalin-fixed paraffin-embedded tissue for biomarker research in urological cancer. BJU Int., 105: 274-278. DOI: 10.1111/j.1464-410X.2009.08665.x

Goelz, S.E., S.R. Hamilton and B. Vogelstein, 1985. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem. Biophys. Res. Commun., 130: 118-126. DOI: 10.1016/0006-291x(85)90390-0

Huijsmans, C.J., J. Damen, J.C. van der Linden, P.H. Savelkoul and M.H. Hermans, 2010. Comparative analysis of four methods to extract DNA from paraffin-embedded tissues: Effect on downstream molecular applications. BMC Res. Notes, 3: 239-239. DOI: 10.1186/1756-0500-3-239

Isola, J., S. DeVries, L. Chu, S. Ghazvini and F. Waldman, 1994. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples. Am. J. Pathol., 145: 1301-1308. PMID: 7992835

Kokkat, T.J., M.S. Patel, D. Mc Garvey, V.A. LiVolsi and Z.W. Baloch, 2013. Archived Formalin-Fixed Paraffin-Embedded (FFPE) blocks: A valuable underexploited resource for extraction of DNA, RNA and protein. Biopreserv. Biobank, 11: 101-106. DOI: 10.1089/bio.2012.0052

Kumar, D., M.K. Panigrahi, M. Suryavanshi, A. Mehta and K.K. Saikia, 2016. Quantification of DNA extracted from formalin fixed paraffin-embedded tissue comparison of three techniques: Effect on PCR efficiency. J. Clin. Diagn. Res., 10: BC01-BC03. DOI: 10.7860/JCDR/2016/19383.8407

Kuno, K., N. Kanada, E. Nakashima, F. Fujiki and F. Ichimura *et al*., 1997. Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene. J. Biol. Chem., 272: 556-562. DOI: 10.1074/jbc.272.1.556

Lehmann, U. and H. Kreipe, 2001. Real-time PCR analysis of DNA and RNA extracted from formalin fixed and paraffin embedded biopsies. Methods, 25: 409-418. DOI: 10.1006/meth.2001.1263

Lin, J., S.H. Kennedy, T. Svarovsky, J. Rogers and J.W. Kemnitz *et al*., 2009. High- quality genomic DNA extraction from formalin-fixed and paraffin-embedded samples deparaffinized using mineral oil. Anal. Biochem., 395: 265-267. DOI: 10.1016/j.ab.2009.08.016

Liu, J., R.M. Johnson and S.T. Traweek, 1993. Rearrangement of the BCL-2 gene in follicular lymphoma. Diagn. Mol. Pathol., 2: 241-247. PMID: 8118601

Ludyga, N., B. Grünwald, O. Azimzadeh, S. Englert and H. Höfler *et al*., 2012. Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses. Virchows Arch, 460: 131-140. DOI: 10.1007/s00428-011-1184-9

Miller, S.A., D.D. Dykes and H.F.R.N. Polesky, 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res., 16: 1215-1215. DOI: 10.1093/nar/16.3.1215

Oh, S.Y., W.Y. Kim, T.S. Hwang, H.S. Han and S.D. Lim *et al*., 2013. Development of an ammonium sulfate DNA extraction method for obtaining amplifiable DNA in a small number of cells and its application to clinical specimens. Biomed. Res. Int.

Okello, J.B., J. Zurek, A.M. Devault, M. Kuch and A.L. Okwi *et al*., 2010. Comparison of methods in the recovery of nucleic acids from archival formalin-fixed paraffin-embedded autopsy tissues. Anal. Biochem., 400: 110-117. DOI: 10.1016/j.ab.2010.01.014

Pavelić, J., K. Gall-Troselj, M.H. Bosnar, M.M. Kardum and K. Pavelić, 1996. PCR amplification of DNA from archival specimens. A methodological approach. Neoplasma, 43: 75-81.

Pikor, L.A., K.S. Enfield, H. Cameron and W.L. Lam, 2011. DNA extraction from paraffin embedded material for genetic and epigenetic analyses. J. Vis. Exp.

Quach, N., M.F. Goodman and D. Shibata, 2004. *In vitro* mutation artifacts after formalin fixation and error prone translesion synthesis during PCR. BMC Clin. Pathol., 4: 1-1. DOI: 10.1186/1472-6890-4-1

Regan, J.F., M.R. Furtado, M.G. Brevnov and J.A. Jordan, 2012. A sample extraction method for faster, more sensitive PCR-based detection of pathogens in blood culture. J. Mol. Diagn., 14: 120-129. DOI: 10.1016/j.jmoldx.2011.10.001

Sam, S.S., K.A. Lebel, C.L. Bissaillon, C.L. Tafe and G.J. Tsongalis *et al*., 2012. Automation of genomic DNA isolation from formalin-fixed, paraffin-embedded tissues. Pathol. Res. Pract., 208: 705-707. DOI: 10.1016/j.prp.2012.08.008

Santos, S., D. Sá, E. Bastos, H. Guedes-Pinto and I. Gut *et al*., 2009. An efficient protocol for genomic DNA extraction from formalin-fixed paraffin-embedded tissues. Res. Vet. Sci., 86: 421-426. DOI: 10.1016/j.rvsc.2008.08.007

Sato, Y., R. Sugie, B. Tsuchiya, T. Kameya and T. Natori *et al*., 2001. Comparison of the DNA extraction methods for polymerase chain reaction amplification from formalin-fixed and paraffin-embedded tissues. Diagn. Mol. Pathol., 10: 265-271. DOI: 10.1097/00019606-200112000-00009

Schweiger, M.R., M. Kerick, B. Timmermann, M.W. Albrecht and T. Borodina, 2009. Genome-wide massively parallel sequencing of Formaldehyde Fixed-Paraffin Embedded (FFPE) tumor tissues for copy-number-and mutation-analysis. PLoS One, 4: e5548-e5548. DOI: 10.1371/journal.pone.0005548

Shi, S.R., R. Datar, C. Liu, L. Wu and Z. Zhang, *et al*., 2004. DNA extraction from archival formalin-fixed, paraffin-embedded tissues: Heat-induced retrieval in alkaline solution. Histochem Cell Biol., 122: 211-218. DOI: 10.1007/s00418-004-0693-x

Shi, S.R., R.J. Cote, L. Wu, C. Liu and R. Datar *et al*., 2002. DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: Heating under the influence of pH. J. Histochem. Cytochem., 50: 1005-1011. DOI: 10.1007/s00418-004-0693-x

Shindo, T., H. Kurihara, K. Kuno, H. Yokoyama and T. Wada *et al*., 2000. ADAMTS-1: A metalloproteinase-disintegrin essential for normal growth, fertility and organ morphology and function. J. Clin. Invest., 105: 1345-1352. DOI: 10.1172/JCI8635

Snow, A.N., A.A. Stence, J.A. Pruessner, A.D. Bossler and D. Ma, 2014. A simple and cost-effective method of DNA extraction from small formalin-fixed paraffin-embedded tissue for molecular oncologic testing. BMC Clin. Pathol., 14: 30-30. DOI: 10.1186/1472-6890-14-30

Specht, K., T. Richter, U. Müller, A. Walch and M. Werner, *et al*., 2001. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am. J. Pathol., 158: 419-429. DOI: 10.1016/S0002-9440(10)63985-5

Toews, J., J.C. Rogalski, T.J. Clark and J. Kast, 2008. Mass spectrometric identification of formaldehyde-induced peptide modifications under *in vivo* protein cross-linking conditions. Anal. Chim. Acta, 618: 168-83. DOI: 10.1016/j.aca.2008.04.049

Turashvili, G., W. Yang, S. McKinney, S. Kalloger and N. Gale *et al*., 2012. Nucleic acid quantity and quality from paraffin blocks: Defining optimal fixation, processing and DNA/RNA extraction techniques. Exp. Mol. Pathol., 92: 33-43. DOI: 10.1016/j.yexmp.2011.09.013

Van Allen, E.M., N. Wagle, P. Stojanov, D.L. Perrin and K. Cibulskis *et al*., 2014. Whole-exome sequencing and clinical interpretation of formalin-fixed, paraffin-embedded tumor samples to guide precision cancer medicine. Nat. Med., 20: 682-688. DOI: 10.1038/nm.3559

Wisniewski, J.R., P. Ostasiewicz and M. Mann, 2011. High recovery FASP applied to the proteomic analysis of microdissected formalin fixed paraffin embedded cancer tissues retrieves known colon cancer markers. J. Proteome Res., 10: 3040-3049. DOI: 10.1021/pr200019m

Wood, H.M., O. Belvedere, C. Conway, C. Daly and R. Chalkley *et al*., 2010. Using next-generation sequencing for high resolution multiplex analysis of copy number variation from nanogram quantities of DNA from formalin-fixed paraffin-embedded specimens. Nucleic Acids Res., 38: e151-e151. DOI: 10.1093/nar/gkq510

Xiao, Z., G. Li, Y. Chen, M. Li and F. Peng *et al*., 2010. Quantitative proteomic analysis of formalin-fixed and paraffin-embedded nasopharyngeal carcinoma using iTRAQ labeling, two-dimensional liquid chromatography and tandem mass spectrometry. J. Histochem. Cytochem., 58: 517-527. DOI: 10.1369/jhc.2010.955526