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RESEARCH ARTICLE

Extraction of Genomic DNA from Formalin Fixed - Paraffin Embedded Tissue for Forensic DNA Analysis: Influence of Proteinase K

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Abstract

A huge part of tissues preserved throughout the world used in diagnostic aim is formalin-fixed and paraffin embedded tissue (FFPET) which are a highly worthy supply for retrospective (genetic) application contain screening of mutations in cancer-critical genes in addition to detection of pathogens. The purpose of present study was to estimate the best method in the extraction of DNA from FFPET for utilizing in the forensic analysis by comparing three various extraction methods with and without proteinase K digestion and estimated the influence of these procedures of extraction in DNA quality, quantity and polymerase chain reaction (PCR) amplicon. In this study, the comparison was between three DNA extraction methods from five identically processed FFPET. The extraction methods, including DNA extraction kit, phenol: chloroform: isoamyl alcohol with and without proteinase K methods. Amplifiable DNA fragment size was assessed by amplification of exon 7 of *CYP1A1* gene and the size of the fragment was (312 bp). Extractions by different methods used in this study were found suitable for amplification of fragment (312 bp) from all tissues used. The best amplification was after extraction without proteinase K and DNA extraction kit. In conclusion, amplification by PCR shows extremely affected by the method of extraction utilized. Pre-treatment of proteinase K was not a prerequisite for extractions of DNA from FFPET and the suitable extraction method was phenol: chloroform: isoamyl alcohol method without proteinase K as it was less time, less cost, yielding a good DNA quantity and quality.

Keywords: *Formalin Fixed-Paraffin Embedded Tissue, Forensic Analysis, Proteinase K.*

Introduction

Since archival samples represented by FFPET became a quite common source of DNA for a variety of applications [1]. The genetic materials extraction from kept tissue or museum samples is an essential constituent of the abundant area of research, containing the phylogenetic studies containing extinct species, forensic investigations, biological studies, and cancer research utilizing FFPET [2, 3].

An abundant source of DNA for forensic applications as well as for disease investigations, especially for genomic studies on human cancer represented by FFPET [4, 5]. Previous findings displayed needing to a further effective procedure for extraction of DNA from archival FFPET and supply an improved understanding of changes which take place throughout modification of nucleic acids induced by formalin [6]. In particular circumstances, the only resource of DNA obtainable for forensic DNA analysis are tumor tissue samples.

However, though FFPET depicts an important source for such investigation, the DNA quality get from this sample may be an important limit. The utilize of FFPET as the biological source was estimated for the limitation of genetic analyses in the forensic area, emphasize the potential to make a comparison between the same tissue frozen

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and FFPET, many years after storage. Also, when compared between the genetic profiles of FFPET and frozen tumor tissues; genetic modification was identical in both types of samples [7].

In addition, quality controls of DNA from FFPET prostate biopsy for forensic analysis were recorded. The quality control is essential for the guarantee of the methods used in the laboratories and established standardization of the forensic laboratories. The STR (Short Tandem Repeat) can be helpful to solve suspicions of FFPET biopsy mix-up in pathology diagnostic.

Some parameters have to be well known, like the quantity of the DNA and the efficiency of thermal cycling used in PCR [8]. With the preface of the PCR method into forensic applications, the utility of DNA from FFPET as a biological source has been re-assessed. The previous investigation assesses the possibility of utilizing DNA from FFPET for PCR system [9].

Also, comparability of techniques in the retrieval of nucleic acids from archival FFPET autopsy was carried out by [10] who reported that successfully utilize these tissues in genomic applications, high nucleic acid purity, and yields.

Despite the growing number of FFPET kits useable, earlier investigations have examined their suitability in recovering nucleic acids of high-quality from archived samples of the human autopsy. DNA typing of biological material possesses most powerful equipment for personal identification in a criminal investigation and forensic medicine [11, 12].

Profiling of DNA was primarily improved as a technique of paternity determination; in which specimens captured below clinical states were tested for hereditary evidence which susceptible to bind a child to parent [13].

DNA extracted from FFPET can as well supply alternate sources of genetic material for forensic casework, like solving paternity cases or identifying unknown embalmed human remains [14]. In case of sexual assault victims, DNA can be extracted from FFPET sections obtained from victim's vaginal epithelium, aiming to detect assaulter profile.

However, this often results in mixed or partial profiles, because of the low abundance of the male component and its dilution in the more abundant female DNA [14, 15].

The rape or sexual assault cases sometimes culminate in not desirable gestation. In nearly all these conditions the fetus is aborted. A laboratory of forensic analyses may extradite the placenta, the fetus, or abortion material in paraffin-embedded blocks for paternity examining.

Acquiring a fetal DNA profile from a placenta or fetus may not be efficacious as a result of the tissue age or state. Besides, maternal pollution of material from placenta will cause a mixed DNA profile. Anyway, the utilize of well-screened paraffin blocks of abortion material will cause in acquiring a fetal DNA profile.

Additionally, paraffin blocks of fixed fetal tissue do not need particular states for storage and submission as demanded to keep fresh placental or fetal tissue; such specimens seem the favored choice for testing of paternity [16]. More than a billion tissue specimens archived in tissue banks and hospitals worldwide and the vast majority of these are FFPET samples [17].

During the gene expression microarray era, FFPET specimens had been extensively used for expression profiling purposes [18, 20]. Candidate biomarkers identification through different strategies utilizing FFPET, involving Single Reaction Monitoring for detection of Epidermal Growth Factor Receptor (EGFR) [21], and laser microdissection [22].

Previous studies have exhibited a high unison in single nucleotide polymorphism (SNP) calls between formalin fixed (FF) and FFPE samples [23]. Furthermore, genotyping arrangements can also be used to assess DNA copy number variance (CNV) but CNV assessment from DNA obtained from FFPE specimens can be challenging, as DNA usually degraded and fragmented [24].

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Different methods for isolation of RNA/DNA and following examination have been progressing for FFPET of different origin and preservation time.

In spite of a lot of analyses of nucleic acids (NA) that used for extraction from FFPET has enlarged, the typical operating protocols for extraction of NA from blocks of old tissue still require to be enhanced [25]. Epigenetic studies of DNA were carried out with FFPET extracted from 30-year-old have been provided [26].

Moreover, FFPET used in advanced genomic technologies such as gene expression profiling and Next-Generation-Sequencing (NGS) which are vital for the improvement of personalized medicines, as they are suitable for identification molecular disease[27].

Some pioneering studies using matched FF and FFPET samples have already been performed to evaluate the usefulness of FFPET sample for miRNA-seq technology [28, 29].

This study designed to evaluate a more efficient protocol for DNA extraction from FFPET for PCR amplification. Also, estimated the utilize of FFPET as a biological source for the identification of genetic profiles in the forensic analyses especially forensic pathology.

Materials and Methods

Collection of Sample

The FFPET of 5 randomly chosen blocks from histopathology laboratory was used for DNA was extracted and took 4 replication for each block (Table 1) and the design of the study was explained in Figure 1.

Table 1: Collection of Sample (FFPET) used for DNA Extraction

No.	Tissue Name	No. of Replications	Age of tissue
1-	Lung Tuberculosis	4	2011
2-	Uterus Fibroid	4	2011
3-	Uterus Adenocarcinoma	4	2011
4-	Abdominal wall mass (Endometriosis)	4	2011
5-	Thigh Mass Rhabdomyosarcoma	4	2011

The carrying out of DNA extraction was by employing three methods. First: DNA extracted by g SYNC™ DNA Extraction Kit according to Manufacture Company. Second: Phenol: Chloroform: Isoamyl Alcohol Method [8] with some modification. Third: Phenol: Chloroform: Isoamyl Alcohol Method applied according to [30] with some modification.

For each block, 4 sections were taken for extraction (5 blocks multiply by 4= 20 extractions for each method). Gel electrophoresis was carried out according to [31]. Visualization of agarose gel performed in a UV transilluminator provided with gel documentation unit.

Preparation of Solutions

Extraction of Genomic DNA

TEB buffer (0.5 X) Buffer

TEB buffer (10X) buffer was diluted to 0.5X with distilled water [31].

Ethidium Bromide Solution (10mg/ml)

One gram (1g) of Ethidium bromide was melted in 100ml of distilled water by using magnetic stirrer several hours until the dye completely dissolved and kept in dark vial at room temperature [31].

Sample Loading Buffer

About 0.25gm of Bromophenol blue with 40gm sucrose was solved in 10 ml of distilled water [31].

Agarose 1%

One gram of agarose was dissolved in 100ml 0.5X TBE buffer and heating by microwave oven until the solution became clear. After the solution cool, add 0.5µl of ethidium bromide and mix well [31].

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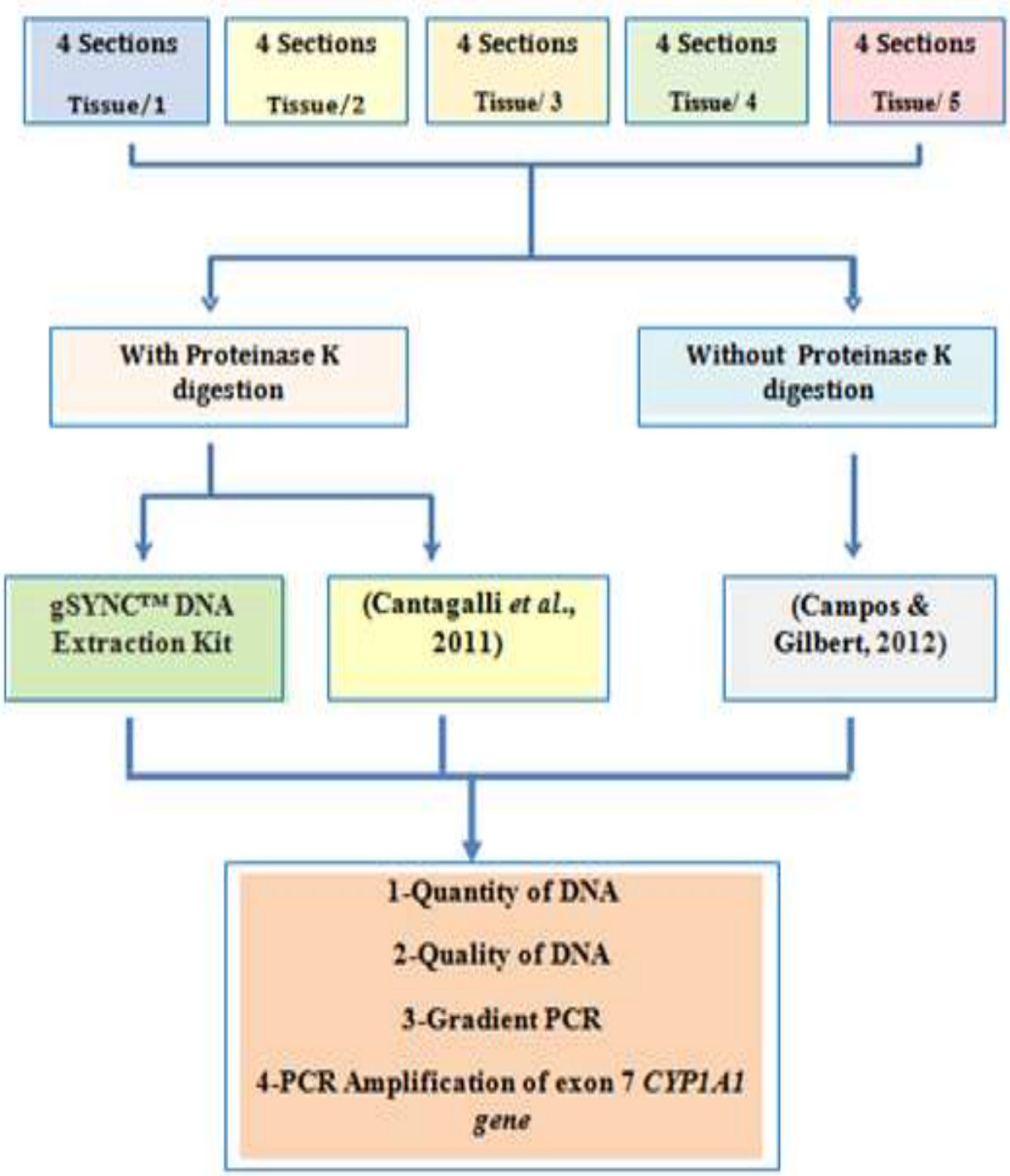

Figure 1: The study design explained comparative study of three methods in the following axes: 1-Quantity of DNA, 2-Qualityof DNA, 3-Gradient PCR, 4-PCR Amplification of exon 7 of CYP1A1 gene

DNA Purity and Concentration

DNA concentration and purity were estimated by using Biodrop spectrophotometer (Nanodrop).

Amplification of Exon 7 of *CYP1A1* gene by Conventional PCR and Gradient PCR

To amplify of exon 7 of *CYP1A1* gene (312bp) amplicon. Many temperatures (50°C, 55°C, 60°C, 65°C).The best temperature was 60°C which selected for PCR to amplification of Exon 7 *CYP1A1*gene (312bp) amplicon. The primers that used in this study were selected according to [32] and showed in Table (2).

Table 2: Sequences of the primers to detect Exon 7 of CYP1A1 gene

Primer Name	Primer Sequence5′-3′	Product Size/bp
Exon7-*CYP1A1*	Forward: GAACTGCCACTTCAGCTGTCT Reverse: CAGCTGCATTTGGAAGTGCTC	312 pb

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Primer Dilution

The primer was synthesized at Bioneer Company which was re-dissolved with nuclease-free water according to Manufacture Company, each 10 µl dissolve in 90 µl re-dissolved nuclease-free water.

PCR Master Mix Kit

PCR Pre Mix (20 µl reaction-Bioneer- Accu Power), which composed of the following components:

Table 3: Component of PCR Master Mix Kit

Component	20µl Reaction	Storage
DNA polymerase	1 U	-20°C
Each: dNTP (dATP, dGTP, dCTP, dTTP)	250 µM	
Tris –Hcl	10 mM	
KCl	30 mM	
$MgCl_2$	1.5 mM	
Stabilizer and tracking dye		

Prepare Solution for PCR

The solution was prepared as displayed in the Table (4). The exon -7 of *CYP1A1* gene was detected by PCR program as mentioned by [33] (Table 5).The detection of amplified PCR product by agarose gel electrophoresis with bromide ethidium dye.

Lastly, the gel was photographed using gel documentation system. According to ladder base pairs of DNA, bands were measured if the DNA band was equal to target product size the result that means a positive result.

Table 4: Prepare Solution for PCR

Stock Solution	20 µl Reaction
Mater mix	10µl
Foreword Primer	1µl
Reverse Primer	1µl
DNA Template	2 µl
ddH_2O	6µl

Table 5: Program of PCR

Stage	Temperature °C	Time (min./sec.)	No. of Cycles
Initial Denaturation	94	5 minutes	35
Denaturation	94	30 second	
Annealing	60	30 second	
Extension	72	30 second	
Final Extension	72	5 minutes	
Hold		5 minutes	

Statistical Analysis

The comparison of the DNA quantities (ng), qualities (purity) and amplification percentage which resulted from various extraction methods from FFPET of five blocks per method. All data is offered as the average and standard deviations (SDs) and the version16 of Statistical Package for the Social Sciences (SPSS), one way ANOVA and Duncan Test was performed. P values ≤0.05 was acceptable as significant.

Results

Gel Electrophoresis

Gel Electrophoresis used for detecting the integrity of DNA extraction from FFPET by three methods (agarose 1%, 15 minutes, at 100 volts) as shown in the Figures 2.

Estimation of the Quantities of DNA

The data of the current investigation recorded that the yields of DNA from FFPET altered rely on the method of extraction as shown in Table (6). Method 3 (phenol: chloroform: isoamyl alcohol without proteinase K) gave the highest yield and quantity. The results of the method 1 (gSYNC™ DNA Extraction Kit) and method 2 (phenol: chloroform: isoamyl alcohol with proteinase K) showed a significant decrease (P values ≤0.05) compared with of yields and quantity of method 3 while there was nonsignificant

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difference (P >0.05) when comparing method1 and method 2.

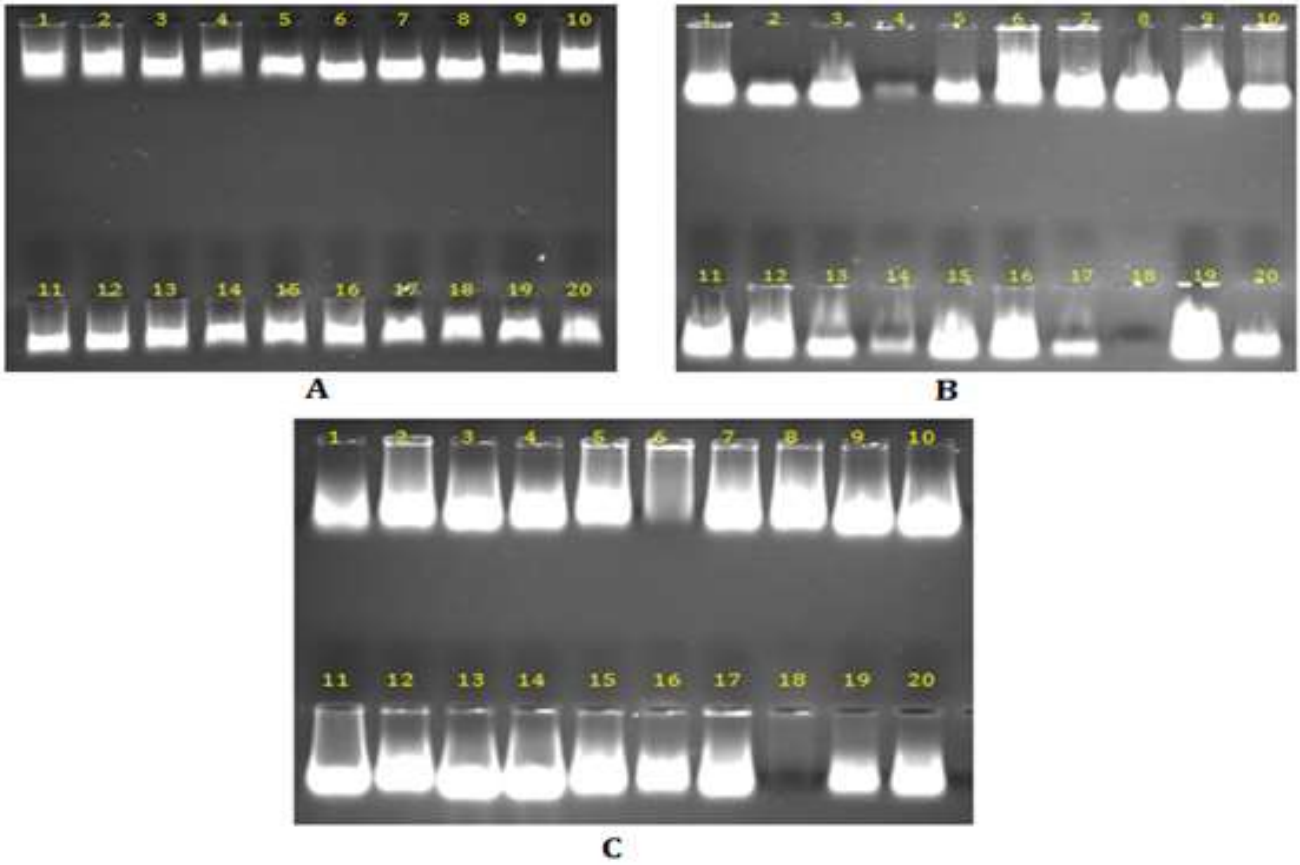

Figure 2: Gel electrophoresis of DNA extracted from FFPET (agarose 1%, 15 minutes at 100 volts). Lane1-4: Sample 1, Lane 5-8: Sample 2, Lane 9-12: Sample 3, Lane 13-16: Sample 4, Lane 16-20: Sample 5). A-gSYNC™ DNA Extraction Kit. B and C: Extraction by phenol: chloroform: isoamyl alcohol with and without proteinase K respectively

Estimation of the Quality of the Extracted DNA (A260/280) and (A260/230)

The estimation of DNA purity according to the proportion of the absorbance at 260 nm and 280 nm (A260/280) as recorded in Table 7. A260/280 ratios are commonly performed to estimation the nucleic acids purity from contamination of protein solutions. A260/280 ratios between 1.8 and 2 exhibit approximately DNA free from protein.

Also, the purity of the DNA extracted was estimated as the proportion of the absorbance at 260 nm and 230 nm (A260/230) as recorded in Table 8. A260/230 ratios between 1.8 and 2 exhibit approximately salt carryover-free DNA. When comparing the three methods depending on quantity, all specimens isolated with phenol-chloroform methods with or without proteinase K were of lower purity at (A260/280) and (A260/230) than those that were extracted by DNA extraction kit but method without proteinase K was significantly greater ($P \leq 0.05$) than the method with proteinase K. When comparing the three methods depending on ratios A260/280 and A260/230, there are significant differences **(**$P \leq 0.05$) between three extraction methods from FFPET.

The highest quality was for g SYNC™ DNA extraction kit followed by phenol: chloroform: isoamyl alcohol without proteinase K which was significantly higher **(**$P \leq 0.05$) than phenol: chloroform: isoamyl alcohol with proteinase K.

Table 6: Quantity of Extracted DNA

*Method	Number	Mean ± S.D
Method 1	**20**	**8.37±3.20 a**
Method 2	**20**	**14.96±7.87 a**
Method 3	**20**	**62.63±49.93 b**

P≤0.05

* Method 1: g SYNC™ DNA Extraction Kit, Method 2 and 3**:** phenol: chloroform: isoamyl alcohol with and without proteinase K respectively

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Table 7: Quality of extracted DNA (260/280)

*Method	Number	Mean±S. D
Method 1	**20**	**1.91±0.06 a**
Method 2	**20**	**1.44±0.1 b**
Method 3	**20**	**1.8±0.04 c**

$P \leq 0.05$

* Method 1: g SYNC™ DNA Extraction Kit, Method 2 and 3: phenol: chloroform: isoamyl alcohol with and without proteinase K respectively

Table 8: Quality of extracted DNA (A260/230)

*Method	Number	Mean ± S. D
Method 1	**20**	**2.10±0.08 a**
Method 2	**20**	**1.25±0.18 b**
Method 3	**20**	**1.90±0.25 c**

$P \leq 0.05$

* Method 1: g SYNC™ DNA Extraction Kit, Method 2 and 3: phenol: chloroform: isoamyl alcohol with and without proteinase K respectively

Gradient PCR

To amplify exon 7 of *CYP1A1* gene (312 bp) amplicon, gradient PCR (50°C- 65°C) was used. The best temperature was 60°C which selected for PCR amplification of Exon 7 of *CYP1A1* gene and the amplicon 312 bp was detected.

PCR Detection

To amplify the DNA from the samples that extracted from FFPET by using three methods, amplification of Exon 7 gene *CYP1A1* were employed, the size of the fragment was 312 bp. Electrophoresis of PCR product shown in Figure (6) revealed that method 1.

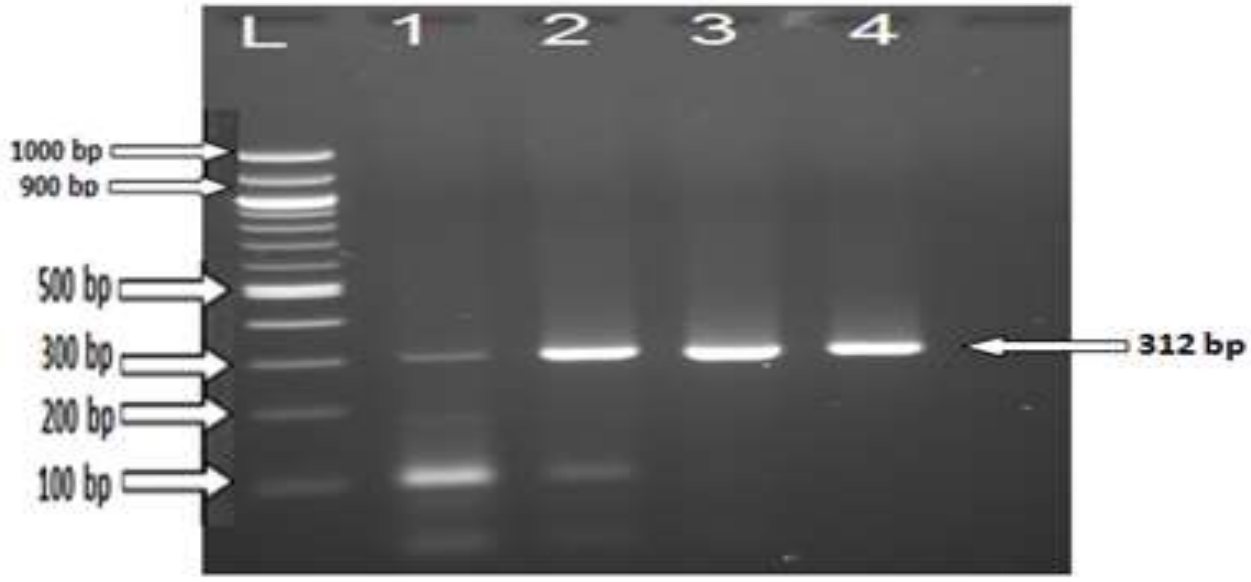

Figure 5: Gradient PCR to amplification of exon 7 of *CYP1A1* gene (312 bp) amplicon. (L:100 bp ladder, 1: 50°C, 2: 55°C, 3:60°C,4:65 °C)

(g SYNC™ DNA Extraction Kit) samples show 62 % specific band appeared in lanes labeled 1, while method 2 (phenol: chloroform: isoamyl alcohol with proteinase K) samples show 50 % specific band appeared in lanes labeled 2. Also, method 3 (phenol: chloroform: isoamyl alcohol without proteinase K) samples show 87 % specific band appeared in lanes labeled 3.

Discussion

In FFPET, cross-linking not merely make difficult extraction of nucleic acid but as well establish polymerase "blocks" through PCR. A great different method performed for the retrieval of RNA and DNA from FFPET, the previous study has undertaken wide-scale quantitative comparison [4]. Quantity and quality of DNA were detected by DNA concentration and purity [34].

In most DNA extraction protocols, enzymatic digestions, such as those with proteinase K, are used for cell and tissue disruption. The disruption process can also be carried out by boiling and by using alkali treatment. After lysis, cytoplasmic constituents, such as

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proteins and liquids that interfere with DNA extraction, are removed. Proteins and lipids are usually removed by one or more tours of extraction with organic solvents like phenol-chloroform mixtures.

Another strategy to eliminate cytoplasmic constituents is to utilize the reversible bound of DNA to a solid material such as silica, which selectively binds DNA in chaotropic salt solutions. The proteins and cytoplasmic constituents can then be removed through washing steps [35].

The present study shows the high quantity of DNA that extract by phenol: chloroform: isoamyl alcohol method without proteinase K **(**62.63±49.93**)** had a higher mean quantity of DNA than that extracted by utilizing phenol: chloroform: isoamyl alcohol method with proteinase K (14.96±7.87) and g SYNC™ DNA extraction kit (8.37±3.20). The key steps of successful phenol: chloroform: isoamyl alcohol method without proteinase K are those that act through causing a break of the DNA–protein cross-links using either heat or alkali treatment, or both [30].

In respect to purity, nucleic acid specimens usually had contamination with another molecule (i.e. organic compounds, proteins, other). The mainly usual technique to find out DNA purity and yield is an estimation of absorbance. Absorbance estimation is simple and needs usually available laboratory tools as the Nanodrop spectrophotometer. Whatever, DNA is not merely molecule that could absorb UV light at260 nm because RNA.

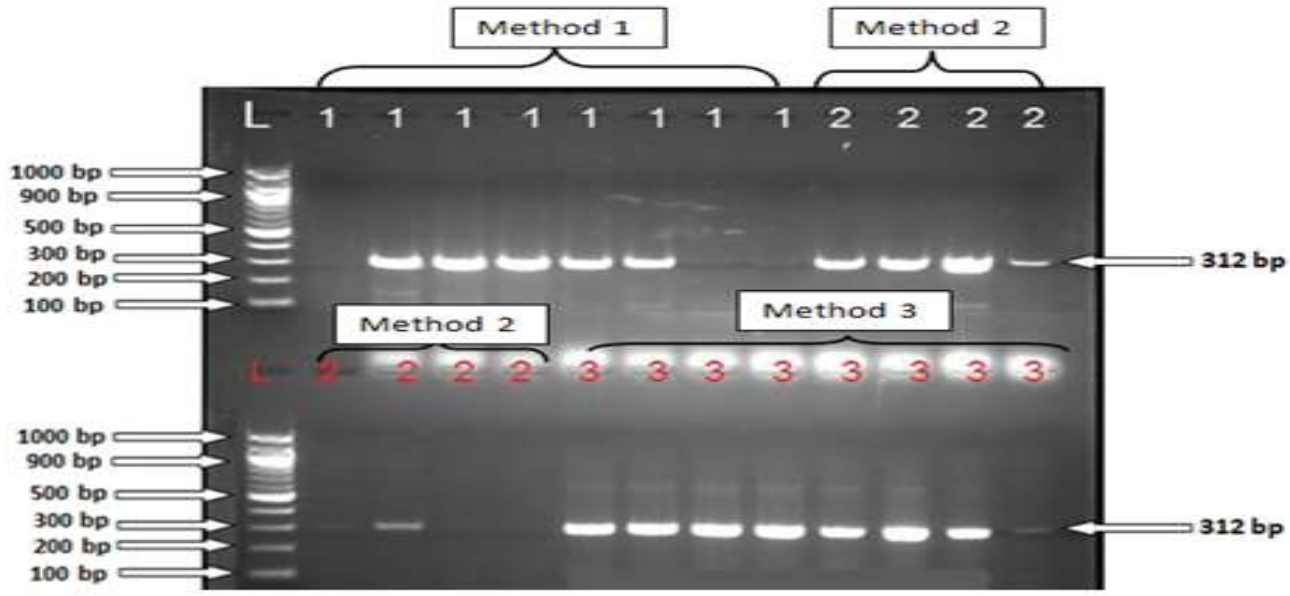

Figure 6: PCR amplification of Exon 7 *CYP1A1* gene (312 bp) amplicon. L: 100bp ladder, Method 1: **gSYNC™ DNA Extraction Kit**, **Method 2 and 3**: **phenol: chloroform: isoamyl alcohol with and without proteinase K respectively**

As well possesses a large absorbance at 260 nm. The absorption of aromatic amino acids found in protein also at 280 nm, both pollution, if found in the DNA solution, had a contribution to the total measurement at 260 nm. So that the ratio of absorbance at 260 vs 280 nm (A260/280) is commonly performed to estimate the nucleic acids purity from the pollution of protein solutions, for pure DNA, A260/280 is ~1.8 ± 0.2 [36].

The higher quality (260/280) of DNA that extracted by g SYNC™ DNA extraction kit (1.91±0.06) followed by the mean quality of DNA that extracted by used phenol: chloroform: isoamyl alcohol method without proteinase K (1.8±0.04) and with proteinase K (1.44±0.1). A ratio of 260 nm to 230 nm may assist in the estimation of the salt carryover level in the purified DNA. Powerful absorbance around 230 nm may exhibit that organic compounds or chemotropic salts are found in the purified DNA. So the diminish in the proportion, the larger the amount of thiocyanate salt is found, for example as a guideline, the A260/A230 is better if greater than 1.5 [31].

The higher quality (260/230) of DNA was for that extracted by g SYNC™ DNA extraction

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kit (2.10±0.08) followed by the quality of DNA that extracts by used phenol: chloroform: isoamyl alcohol method without proteinase K (1.90±0.25) and with proteinase K (1.25±0.18). These results of quality of g SYNC™ DNA extraction kit may due to a precise amount of solution, buffers and optimum temperature of reaction which improve the inactivation of nuclease and releasing of DNA from target cells.

The results of amplification show that 87 % specific band for samples that extracted by phenol: chloroform: isoamyl alcohol method without proteinase K and 62 % specific band for DNA samples that extracted by use g SYNC™ DNA extraction kit while the results of amplification shows 50 % specific band for samples that extracted by phenol: chloroform: isoamyl alcohol method with proteinase K. In conclusion, the samples isolated by a method without proteinase K, Gene aid kit gave a suitable result for PCR amplification and method without proteinase K was less cost and less time with a suitable quantity and quality.

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