

Evaluation Of DNA Extraction From Archieved Formalin –Fixed Paraffin Tissues (FFPT) By B-Globin Gene PCR.

تقييم الدنا المستخلص من قوالب الأنسجة الشمعية مخزونة باستخدام تفاعل أنزيم البلمرة لجين بيتا كلوبين

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الخلاصة

خلفية البحث : إن معرفة الحالة التي يكون عليها الحامض النووي قبل الشروع في الكشف الجزيئي له أهمية كبيرة في إجراء الحسابات الرياضية والتي من خلالها يتم معرفة كمية ونوعية الحامض النووي المستخدم.

الهدف : إن الطرق التقليدية لاستخلاص الدنا فيها من المحددات ما يمنع معرفة كفاءة ونوعية الدنا وخصوصا من العينات الحاوية على خلايا قليلة لهذا فان نوعية وكفاءة الدنا يمكن تحديدها من خلال فحص البيتا كلوبين وجهاز النانودروب على التوالي سيما إن استخلاص الدنا من الأنسجة المثبتة بالفورمالين ضروريا للأبحاث الجزيئية وخصوصا مع تقدم العلوم التقنية الجزيئية.

المنهجية: الدراسة الحالية اختبرت نماذج الأنسجة المثبتة بالفورمالين لسرطان الثدي لنساء عراقيات، والتي جمعت من مستشفى الصدر التعليمي في وحدة النسيج المرضي ووحدة الأورام، وعدة مختبرات خاصة في محافظة النجف، النماذج كانت مخزونة للسنوات (2008,2009,2010 و 2011). النماذج تضمنت، 54 نموذج لسرطان الثدي، 21 نموذج التليف الغدي، 18 نموذج لالتهاب ثدي، 19 نموذج لحالات توسع القنوات الثديية.

النتائج: كان متوسط كمية DNA (61.68 ng/ul) بينما نوعية DNA التي ظهرت إيجابية في تفاعل أنزيم البلمرة للبيتا كلوبين كانت 71.42 % (80 من 112)، فكانت نماذج سرطان الثدي 70.37 % (38 من 54)، ونماذج التليف الغدي 85.71 % (18 من 21)، ونماذج التهاب الثدي 77.77 % (14 من 18) و نموذج التهاب خلية البلاستولية الخارجية 52.63 % (10 من 19)، حيث كان هناك اختلاف معنوي ($p < 0.5$). كما اظهر تفاعل أنزيم البلمرة لجين البيتا كلوبين نتائج مختلفة من الحزم تبعا لحالة الدنا المستخلص.

الاستنتاجات: يعتبر تقييم الدنا المستخلص بالنانودروب والبيتا كلوبين من أسهل وأدق الطرق بالمقارنة مع نتائج باحثين آخرين عملوا على طرق تقييم أخرى .

التوصيات: توصي الدراسة باعتماد تقييم البيتا كلوبين لتجنب الحصول لنتائج سلبية كاذبة بسبب تكسر الدنا.

Abstract

Background : the knowledge of the nucleic acid state before start to molecular detection have a great importance to make a math measurements by which the nucleic acid quantity and quality will be knows.

Objective: Conventional methods have limitations for extracting amplifiable DNA from specimens containing a small number of cells. DNA quality and quantity was evaluated by β -globin amplification and NanoDrop instrument respectively. DNA extraction from FFPT has become essential for molecular research specially with advances in molecular technology.

Methods: The current study tested breast cancer FFPT specimens of Iraqi women which collected from AL-Sader teaching hospital histopathology and neoplasm units , and several privet histopathology labs in AL-Najaf province, the specimens were stored for the years (2008,2009,2010 and 2011). The specimens were included of BC 54 , fibroadenoma 21, mastitis 18, and ductectesia 19 samples.

Results: Specific primers were used to detect the activity of beta-globin gene by PCR technique and the results appeared that The mean of DNA quantity was (61.68 ng/ul) while the DNA extract quality which appeared positive by β -globine PCR was 71.42% (80 of 112), BC 70.37% (38 of 54), fibroadenoma 85.71% (18 of 21), mastitis 77.77% (14 of 18) and ductectesia 52.63 % (10 of 19), there was a significant difference ($p < 0.5$). The beta-globin PCR appeared different results depending on the DNA stats.

Conclusions: the evaluation of the extracted DNA by NanoDrop system and beta-globin gene is the easiest and definite method compared with other researchers who worked of other evaluation methods.

Recommendations : This study recommends that every study performed on DNA should be started with beta-globin testing to avoid false negative results caused by degraded DNA.

Keyword: FFPT: formalin –fixed paraffin tissues, BC: breast cancer.

INTRODUCTION

Archival fixed paraffin-embedded tissue (FFPT) is a valuable source for molecular genetic studies but the extraction of high quality DNA is still a problematic issue. The polymerase chain reaction (PCR) is an in vitro amplification method that depends, for successful results, on the adequate storage of samples, and a good protocol for DNA extraction. Formalin fixation paraffin embedding Tissues (FFPT) is a standard method for long-term preservation of most archived pathological specimens. Such samples provide an invaluable resource for subsequent molecular studies of clinical phenotypes, especially genetic studies in which DNA is not available from fresh or frozen tissue(s) because subjects are no longer alive. FFPT is an excellent source of DNA, but its extraction remains a challenge. Formaldehyde, the effective component of formalin, leads to the generation of cross-linking between nucleic acids and proteins, and causes nucleic acids to fragment because of fixation process conditions, e.g., the extremely low pH (<1). Cross-linking not only causes problems in DNA extraction but blocks polymerase chain reaction (PCR) amplification⁽¹⁾. This makes it very difficult to amplify high molecular weight DNA⁽²⁾. Despite these problems, FFPT samples are widely used as their DNA content, even in shorter fragments, is often invaluable for studying genetic diseases and is suitable for PCR requiring relatively short DNA fragments. Methods for DNA extraction from fresh tissue and cytological preparation have been described and adapted for use in archival specimens. The most common human archival specimens are formalin-fixed, paraffin-embedded tissues (FFPT). DNA can be extracted from (FFPT), but archival material may be unsuitable for most molecular techniques which require high molecular weight genomic DNA, as slow degradation of DNA occurs with time. However, short DNA segments are also useful as a substrate for PCR. Some authors have shown that PCR can be performed successfully on nucleic acids (NA) that are partially degraded over time⁽³⁾.

Some researcher said that the (FFPT) are the most invaluable source of diagnostic material for studying pathogenesis of cancer and a variety of other diseases. Unfortunately, DNA extracted from formalin fixed tissues is highly degraded due to cross-linking between nucleic acid strands. Extracted DNA was amplified with different primer sets that gave amplifiers of different size. Optimization of Real Time PCR for EMSY (an oncogenic interacting partner of BRCA2 that functions as a transcriptional repressor), cyclin D1 and beta-globin genes was carried out on DNA obtained using heat treatment protocol for annealing temperature, primer concentration and template concentration⁽⁴⁾. The presence of adequate DNA was confirmed by amplifying the human beta-globin gene⁽⁵⁾.

Polly Etkind suggest that To determine the quality of the isolated DNA from both paraffin blocks of tumor tissue and fresh normal breast tissue, globin primers were used in PCR and the resulting amplified products were electrophoresis in 1.8 % agarose gels⁽⁶⁾. On the same idea, Dr.Stella Melana suggest that the best methods to qualify the DNA fragments that extracted from FFPT was Beta-globin PCR by GH20 and PCO4 primers⁽⁷⁾.

Study aims

1. estimate the amount of DNA which was recoverable by conventional methods.
2. evaluate the DNA by beta-globin PCR to use this extracts for molecular detection of different items.

MATERIALS AND METHODS

Extraction of human DNA genome.

It is the first step in the PCR protocol. The DNA extracted according to the Invitrogen company (pureLink™, Genomic DNA mini kit, ca: k182001/USA).

DNA extraction protocol:

- 1 ml of xylene was added to the tissues.
- Centrifuged at maximum speed for 3 minutes at room temperature to pellet the tissue. Carefully removed the supernatant without disturbing the pellet.
- **Note :(Steps No.1 and No.2 were repeated, three times according to the modified protocol of Tisch cancer institute /Hematology & oncology department/ MSSM/NY/USA) .**
- 1 ml 96-100% ethanol was added and vortexed to re-suspend the tissue pellet.
- Centrifuged at maximum speed for 3 minutes at room temperature to pellet the tissue. Carefully removed the supernatant without disturbing the pellet.
- **Note :(Steps No.1 and No.2 were repeated, three times according to the modified protocol of Tisch cancer institute /Hematology & oncology department/ MSSM/NY/USA) .**
- The tubes with lid open were incubated at 37°C overnight (modified protocol of Tisch cancer institute /Hematology & oncology department/ MSSM/NY/USA) , to evaporate any residual ethanol.
- 180 µl PureLink™ Genomic Digestion Buffer and 20 µl Proteinase K (supplied with the kit) were added. Mix well by brief vortex.
- The tubes were incubated at 37°C -50°C for 3 hours to overnight respectively.
- Centrifuged the lysate at maximum speed for 3 minutes at room temperature to remove any particulate materials, the lysate was transferred to a new, sterile microcentrifuge tube.
- 20 µl RNase A (supplied in the kit) was added to the lysate, mixed well by brief vortex, and incubated at room temperature for 2 minutes.
- 200 µl PureLink™ Genomic Lysis/Binding Buffer was added and mixed well by brief vortex.
- 200 µl 96-100% ethanol was added to the lysate. Mixed well by vortex for 5 seconds to yield a homogenous solution.
- Removed PureLink™ Spin Column in a Collection tube from the package.
- the lysate (~640 µl) prepared with PureLink™ Genomic Lysis/Binding Buffer and ethanol were added to the PureLink™ Spin Column.
- Centrifuged the column at 10,000 × g (13000 rpm) for 1 minute at room temperature.
- Discarded the collection tube and placed the spin column into a clean PureLink™ Collection tube supplied with the kit.
- 500 µl (Wash Buffer 1) prepared with ethanol was added to the column.
- Centrifuged the column at room temperature at 10,000 × g (13000 rpm) for 1 minute.
- Discarded the collection tube and placed the spin column into a clean PureLink™ collection tube supplied with the kit.
- 500 µl (Wash Buffer 2) prepared with ethanol was added to the column.
- Centrifuged the column at maximum speed for 3 minutes at room Temperature, then discarded collection tube.
- the spin column was placed in a sterile 1.5-ml microcentrifuge tube.
- 50 µl of PureLink™ Genomic Elution Buffer was added to the column.
- Incubated at room temperature for 1 minute and centrifuged the column at maximum speed for 1 minute at room temperature.
- Step No.22 and No. 23 were repeated (modified protocol of Tisch cancer institute /Hematology & oncology department/ MSSM/NY/USA) . to make 100 µl total volume of DNA extracts

- while the tube contained purified DNA, the column was removed and discarded.
- The purified DNA was stored at -20°C or use DNA for immediately.
- **(Note: to avoid repeated freezing and thawing of DNA, store the purified DNA at 4°C for immediate use or aliquot the DNA and stored at -20°C for long-term storage).**

Evaluation of nucleic acid by quantity and quality:

DNA quantity:

The important steps following DNA extraction was the measuring of DNA quantity. The DNA concentration measured by NanoDrop instrument (Image 3)

DNA quantity protocol:

1. Open the program of NanoDrop icon.
2. From the system options, click on ((**single sample**)), and click on ((**nucleic acid**)), then the program started automatically.
3. For each reagent, 1ul was the standard volume that used with NanoDrop instrument
4. The sample site was cleaning by Kim white papers.
5. 1ul of water was added to exclude the contaminations.
6. The sample site was cleaning by Kim white papers.
7. 1ul of blank control (elution buffer) was added.
8. The sample site was cleaning by Kim white papers.
9. 1ul of the positive control (DNA template control) was added.
10. The sample site was cleaning by Kim white papers.
11. 1ul of each sample was added subsequently.
12. The DNA concentration and further information of each sample recorded automatically.

DNA quality

Because the FFPT samples were stored between 1-3 years, the DNA quality was subsequently tested by amplification of a 260-bp fragment of the β -globin gene by using (GH20 & PCO4) primers (**GH20 (GAAGAGCCAAGGACAGGTAC) PCO4 (CAACTTCATCCACGTTTACC)**). The amplified products were visualized on 2% agarose gel. To applied the DNA quality , β -globin PCR was used by Microsoft excel program to calculate the optimal amount of each sample depending on DNA concentration , as shown in the following example:

Example to explain the calculation process of PCR samples

A	B	C	D	E	F	G
sample NO.	modified NO.	ng/ul conc.	250 ng/ul	ul/used	master mix	H2O
1	BC	50	250	5	2	18

A. Sample NO.

B. Modified NO.(the number on the FFPT).

C. ng/ul conc.(DNA concentration which was read by NanoDrop).

D. 250 ng/ul (the standard volume of the nucleic acid in any optimal PCR technique).

- E. ul/used (the sample's amount should be used in the PCR according to the following equation, $=d2/c2----- =250/50----5$)
- F. master mix (the primer's amount was always 1ul for each primer ,forward and backward = 2 ul)
- G. H₂O (the water amount that used to complete the standard PCR volume (25ul) by used the following equation, $=25-(f2+e2)---- =25-(2+5)--- 18$).

β-globin PCR protocol:

- After doing the calculations above.
- PCR technique was done by used of , PCR beads (**GH healthcare,illustra[™] puretaq[™] ready to go[™] PCR beads, 0.2ml tubes/plates/UK, ca.27955702**) for samples, positive and three negative tubes.
- 2ul of master mix of 260-bp sequence of the β-globin primers (GH20 &PCO4) (**Appendix 13**) was added.
- Positive control tube made by added 2ul of primers, 0.5 ul of positive control, and 22ul water.
- The negative control made by added primers plus water (2ul primers + 23 ul water).
- The samples volume was added.
- The total volume of 25 ul was completed by water.
- Mix the PCR tubes by brief shaking.
- Spin down the materials by used of micro- centrifuge.

Thermal cycler instrument was used to applied PCR condition of β-glubine as the following: 95°C (5 minutes), 95°C (15 seconds), 55°C (30 seconds), 72°C (30 seconds), Go to 2 repeat 35 cycles, 72°C (10 minutes).

- After completed PCR run , gel electrophoresis instrument was done.
- Materials of gel electrophoresis was TAE (tris acetate EDTA), and agarose .
- 2% gel was prepared for medium cast gel (20 wells) (**Appendix 15**).
- After completed of cast gel, PCR results was loaded in the well's gel by the following procedure:
 - a. Filled the gel electrophoresis chamber with TAE.
 - b. 20ul of ladder was added.
 - c. 20ul of each sample , positive , and negative control were mixed with 4ul of loading dye on the parafilm paper, then loaded in the wells.
 - d. Turn on the gel electrophoresis at 95-100 volt for around 45 minutes.
- The results was shown by gel documentation system (Image 3).
- Only the samples were shown to be positive were suitable for PCR analysis.

Results of DNA extraction from FFPT:

Our study started by extraction of DNA from FFPT in MSMC successfully by used of decontaminated conditions inside the hood .

Results of DNA quantity:

After completion of the DNA extraction , the DNA concentration was determined by NanoDrop system (DNA quantity). The concentration of the DNA appeared in different reading according to the amount of DNA extract. Descriptive data showed that the mean of DNA concentration was 61.68 ng/ul with standard deviation of 51.35. The NanoDrop system applied automated sheet including several information related to tested samples

Results of DNA quality (β-glubine PCR)

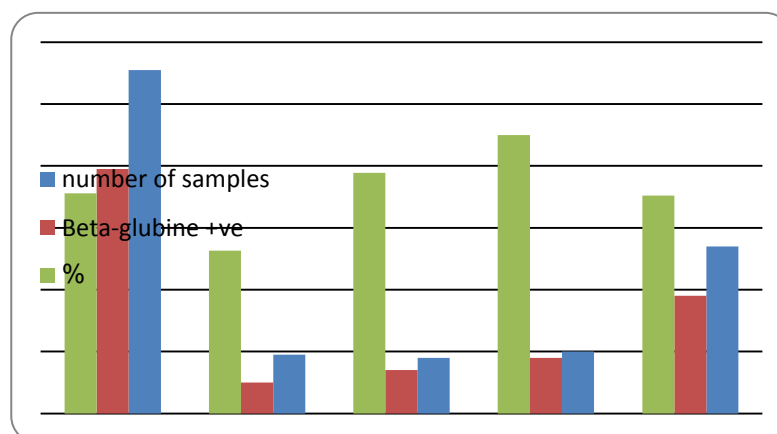
Each 12 samples (except final group, 4 samples) was processed to β -globine PCR twice times to confirm the results by using GH20 and PCO4 primers . The positive samples means it have good DNA and ready to the nested PCR protocol.

Table (1) Results of DNA quality by β -globin PCR.

Samples	No. of samples	β -globine negative	β -globine positive	%
BC	54	16	38	70.37
Fibroadenoma	21	3	18	85.71
Mastitis	18	4	14	77.77
Ductectesia	19	9	10	52.63
Total	112	32	80	71.42

($X^2=5.775$, $p<0.5=0.1231$, $df=3$)

The total positive **β -globine PCR** results were (80 of 112) samples (71.42%) as the following (BC %70.37, 38 of 54), fibroadenoma (%85.71, 18 of 21), mastitis (%77.77, 14 of 18) and ductectesia (%52.63, **10 of 19**), there was a significant differences $p<0.5$ (**Table 1**) & (**Histogram 1**).



Histogram (1) Results of DNA quality (β -globine PCR).

Table (2) β -globin PCR samples results.

Samples group	Positive samples in the 1 st trail	Negative samples in the 2 nd trail	Total	%
1-12	11	1,4,5	4	33.33
13-24	16,17,19,20,21,22	14,15,18	9	75
25-36	25,27,28,29,31,32,33,34,35, 36	26,30	12	100
37-48	37,39,40,41,44,46,47	43	8	66.66
49-60	53,58,60	49,50,51,52,57	8	66.66
61-72	61,62,63,65,66,67,68,69,70,71	64,72	12	100
73-84	73,75,78,80,81	74,76,77,79	9	75
85-96	85,86,87,88,89,90,92,93,94,95,96	0	11	91.66
97-108	100,104,105,107,108	0	5	41.66
109-112	0	109,110	2	50
Total			80	71.42

β -globin 260bp was amplified by 12 samples for each run (1st and 2nd trail), i.e, the negative β -globin in the 1st trail would be repeated in a second trail. The numbers of β -globin positive and negative in both trials are in the (Table 2) (Figures 1-4).

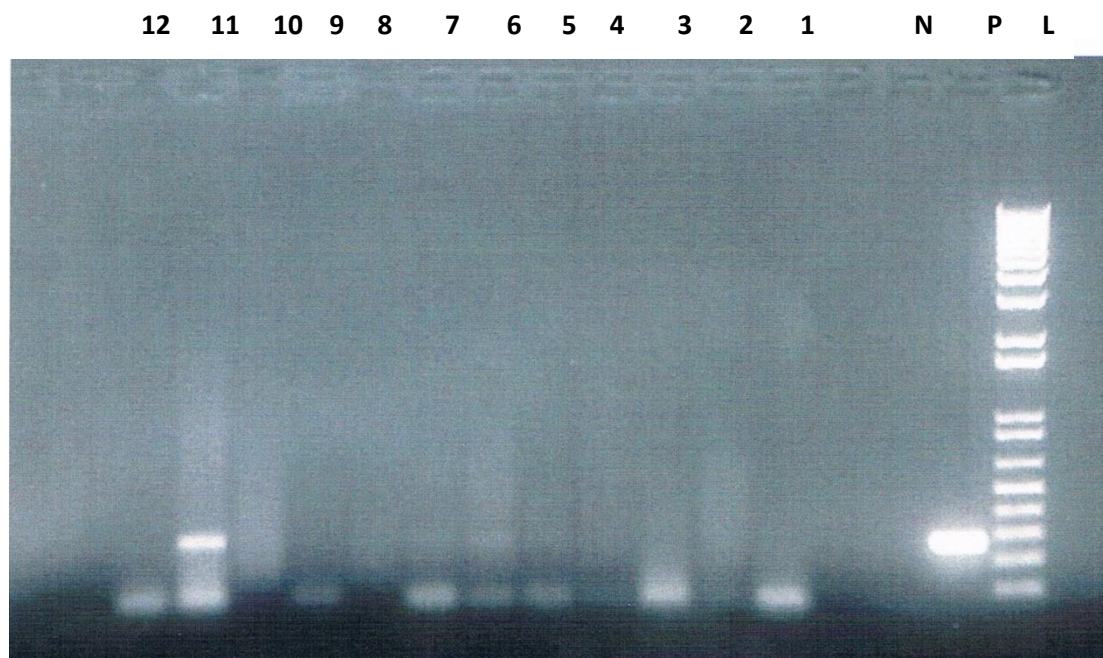


Figure (1) group 1-12 (1st trail) β -glubine PCR bands (No.11 positive) N: negative control, P: positive control, L: ladder

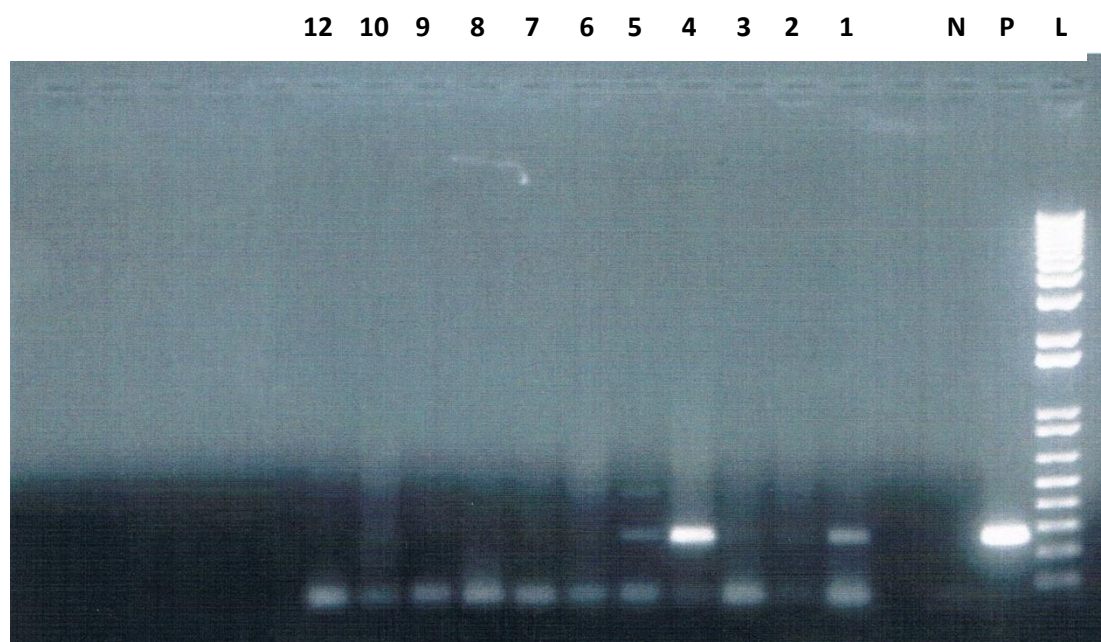


Figure (2) group 1-12 (2nd trail) β -glubine PCR bands(No. 1,4,5 positive) N: negative control, P: positive control, L: ladder

24 23 22 21 20 19 18 17 16 15 14 13 N P L

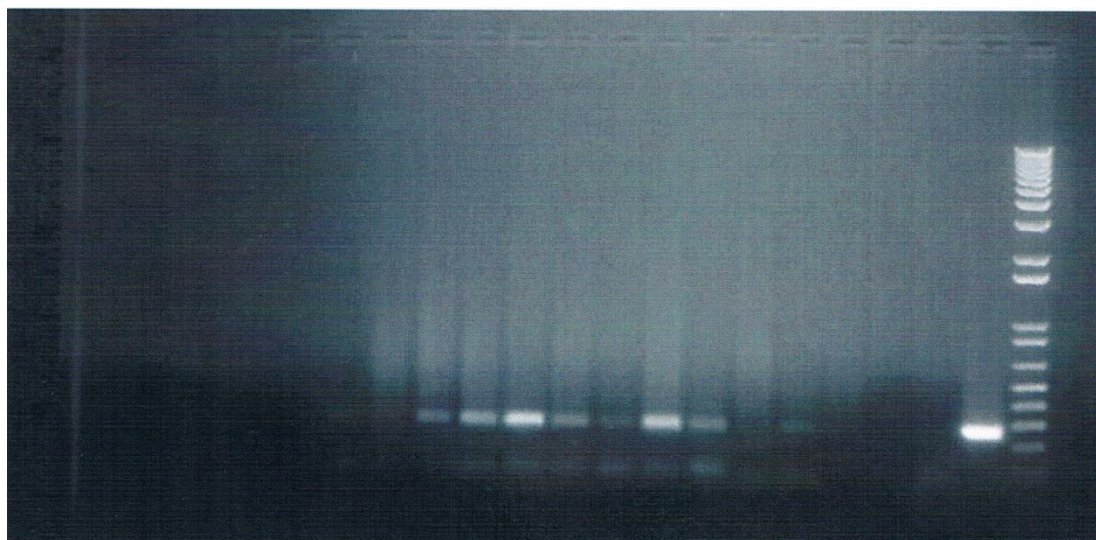


Figure (3) group 13-24 (1st trail) β -glubine PCR bands(No. 16,17,19,20,21,22 positive) N: negative control, P: positive control, L: ladder

24 23 19 18 15 14 13 N P L



Figure (4) group 13-24 (2nd trail) β -glubine PCR bands(No. 14,15,18 positive) N: negative control, P: positive control, L: ladder

DISCUSSION

The current study started the molecular protocol by the first step , which was DNA extraction by ethanol, phenol method including Protienase K, there were successful extraction from all FFPT but with a different concentration. Cornelis J. observed that Protienase K digestion is required for optimal purification of paraffin-embedded DNA ⁽⁸⁾. Merkelbach & Lehmann mentioned that there

are several reasons for the failure of the techniques using DNA isolated from paraffin-embedded tissues :

(1) the degradation of target DNA, which may occur due to long time lapses between surgical tissue removal and fixation, the type of fixative used, and the duration of the fixation.

(2) the fragmentation of nucleic acids due to formalin fixation

Therefore high quality DNA extraction from paraffin-embedded tissue specimens was problematic ⁽⁹⁾. Gilbert M.*et al* suggest that the Formalin fixation induces DNA-protein cross-links and it can interfere with the amplification ability of the DNA in the tissue specimen. The DNA damage may be induced by such as pH of the fixative and the duration of fixation ⁽¹⁰⁾. A number of intrinsic attributes of the tumor specimen, such as tissue area, degree of necrosis and endogenous inhibitors such as melanin and small specimens, can also contribute to the variability in yields of amplifiable DNA. A few studies comparing the recovery of nucleic acid from FFPT specimens by various DNA extraction kits have reported data from a limited number of tumor types using specimens with relatively large amounts of tissue ⁽¹¹⁾. Dedhia P., and Williams C. , and other researchers said that Although formalin is an excellent preservative for maintaining the integrity of tissues, the time since death, and the time to fixation, the paraffin embedding process and subsequent storage lead to nucleic acid degradation and extensive modification that may affect the yield and quality of nucleic acids (DNA and RNA) ⁽¹²⁾. Regarding to the DNA extraction which is a routine step in many biological studies including molecular identification, phylogenetic inference, genetics, genomics, medical examinations, clinical diagnostics, and forensic investigations. Therefore, a variety of methods have been established to isolate DNA molecules from biological materials ⁽¹³⁾.

DNA quantity

This study used the archived FFPT that stored for about 1-3 years ago, so, used of NanoDrop system to have assurance that the tissues have a good DNA concentration. The results of NanoDrop readings appeared that, from 112 FFPT samples, the mean concentration reading was 61.68 (ng/ul) , regarding to the minimum reading which was 3.86 (ng/ul) and the maximum reading which was 194.4 (ng/ul). The diversity of the DNA readings depended on the DNA extraction process. In the current study the DNA extraction procedure was done according to the modified processes of MSMC. Indeed, tissues that removed from the human body and used for diagnostic purposes can be fixed, paraffin wax embedded and stored for several years. These specimens are valuable sources of material for research ⁽¹⁴⁾. The use of a Thermo Scientific NanoDrop spectrophotometer to quality control of nucleic acid samples can result in significant savings in time and money. The micro volume capability of NanoDrop™ spectrophotometers allow the researcher to quickly and easily run quality control checks of nucleic acid and protein samples. In addition, the instrument's short measurement cycle and general ease of use greatly increases the rate at which samples can be processed, making it possible to implement multiple quality control checks throughout a procedure or process, on the other hand, several studies suggest that the paraffin-embedded tissues have higher quality and quantity of DNA than tissues that were not embedded after liquid fixation. While the DNA that kept for long time in an aqueous or liquid media of time did not yield usable DNA. Many factors can influence the results of obtaining high quality DNA from fixed and paraffin embedded tissue such as chemical composition of the fixative, impact of tissue hypoxia and autolysis, size of the tissue specimen and the length of storage time ⁽¹⁵⁾.

DNA quality by β -globin

In this study, to check the DNA quality, the specimens were amplified with beta-globin primers PC04 and GH20. The results was 80 positive samples(71.42%) from total of 112 samples, there was

a significant differences $p < 0.5$ (Table 1). Some of samples (28.5%) appeared negative because either the oldest FFPT with bad storage or there were a little tissues slices after cutting the FFPT by microtome system at pathology lab. Thus it is a routine protocol in mostly all molecular studies , Saiki, 1988 suggest that a segment β -globin was amplified using probes GH20 and PCO4 .The positivity of this segment guaranteed the viability of the DNA obtained ⁽¹⁶⁾. Regarding of FFPT, the Iraqi conditions like variety of temperatures & bad storage may consider a cardinal causes of DNA degradations. The current study agreed with several studies depends on the β -globin sequences to checking the DNA viability like Shikova E. *et al.* amplified β -globin sequences with beta-globin primers PC04 and GH20 during their study on Human Papilloma Virus ⁽¹⁷⁾. Yih-Lin Chung Amplified the b-globin gene fragment was also performed by use of PCO4 and GH20 primers as a control for the target DNA integrity ⁽¹⁸⁾. Specific primer sequences were used in other studies for amplification and sequencing of the β -globin gene like D-1,2,3(R,R,F respectively). Other studies used different method to checking the DNA quality by amplification of a 268-bp fragment of the β -globin gene using HotStarTaq DNA polymerase (Qiagen) and primers G073 (5'-GAAGAGCCAAGGACAGGTAC-3') and G074 (5'CAACTTCATCCACGTTTACC-3'). The cycling conditions were 95°C, 15minutes; followed by 35 cycles of 95°C, 30 seconds; 55°C, 30 seconds; 72°C, 1 minute; and a final extension at 72°C, 10 minutes. The amplified products were visualized on 2% agarose gel. All the samples were shown to be suitable for PCR analysis ⁽¹⁹⁾.

CONCLUSION:

1. For best DNA extraction, modified manual method by using of xylene and ethanol method is preferable.
2. The NanoDrop thermo-scientific 8000 was the best instrument for DNA quantitation.
3. Using β -globin gene PCR amplification was the specific and basic PCR protocol for evaluation of the ability of DNA replication

RECOMMENDATION:

1. FFPT used in genomic testing should be stored in suitable conditions to avoid DNA degradation.
2. This study recommends that every study performed on DNA should be started with beta-globin testing to avoid false negative results caused by degraded DNA.

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