RESEARCH COMMUNICATION

Evaluation of DNA Extraction Methods and Real Time PCR Optimization on Formalin-fixed Paraffin-embedded Tissues

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Abstract

Formalin-fixed, paraffin-embedded (FFPE) tissues 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. Real Time PCR has become the standard for gene copy as well as RNA transcript determination. Thus, optimum standardization of Real Time PCR is crucial for obtaining accurate quantification for both research as well as for clinical diagnosis. However there are various factors which have negative impact . The aim of our study was to establish a simpler method of extraction and Real Time PCR Optimization for FFPE extracted DNA. Five breast cancer tissues that were formalin fixed and paraffin embedded were used for DNA extraction with four different methods. Extracted DNA was amplified with different primer sets that gave amplimers of different size. Optimization of Real Time PCR for EMSY, cyclin D1 and β-globin genes was carried out on DNA obtained using heat treatment protocol for annealing temperature, primer concentration and template concentration. Highest quantity of DNA was obtained without the use of expensive reagents and in short time frame. PCR positivity was observed in case of shorter amplimer up to 250 bp in length. Amplimers of higher length failed to amplify with paraffin extracted DNA. Optimum annealing temperature for EMSY, Cyclin D1 and β-globin genes were 60°C, 60°C and 61°C respectively. Good results were seen with a primer concentration of 300 nM and 5 ng of template DNA. This study indicates that DNA obtained from formalin fixed paraffin embedded tissue is highly fragmented and can be used for successful amplification of shorter amplification products up to 250 bp in length. Optimization of real time PCR is important, especially while using SYBR green dye chemistry.

Key words: Real time PCR - protocol optimization - heat treatment

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Introduction

Formalin fixed and paraffin embedded (FFPE) tissues represent an extraordinary source of archived and morphologically defined disease-specific biological material enabling the correlation of molecular findings, therapy and clinical outcome (Lehmann, 2001). They are commonly used for both research and clinical molecular analysis based on DNA amplification by PCR assays and currently used Real Time PCR assays. DNA extracted from paraffin embedded tissues is also used for diagnosis of various infectious agents such as EBV, HPV, CMV, HSV and Mycobacterial tuberculosis (Takasaka et al., 1996, Yang et al., 2004, Mahaisavariya et al., 2005). Formalin is the most widely used fixative in histopathology due to several advantages, but it damages tissue nucleic acids by crosslinking it to tissue proteins, which subsequently results in extensive DNA and RNA fragmentation (Bonin et al., 2003). It is very well known that PCR is very difficult with DNA extracted from fixed tissues and fixation intervals are associated with decreased PCR yields and inability to amplify longer DNA targets (Quach et al., 2004). It has been reported previously that DNA amplifications only up to 300 bp are obtained from FFPE postmortem tissues and very often amplimers only up to 100 bp are obtained (Bonin et al., 2003).

Real Time PCR is a favorable option for quantitative analysis of cancer markers and can be performed on low copy targets and on degraded DNA due to increased sensitivity and is best suited for DNA extracted from paraffin embedded tissues (Lyon et al., 2001). Real Time PCR machines combine thermal cycling with florescence acquisition (Bernard et al., 2002) and the fluorescence of DNA dyes or probes is monitored at each cycle during PCR. Ct (threshold cycle) is the fractional cycle number at which the fluorescence passes the fixed threshold. SYBR Green is widely used florescent dye due to its low cost and ease of use. However, it has the inherent drawback of binding to the non-specific PCR products, primer dimers and the variable amount of DNA in the sample. The

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accumulation of non-specific products and primer dimers can be assessed from the dissociation curve since every product in a reaction gives a different peak at its Tm. This drawback can be taken care of by optimum primer design, primer concentration, Mg^{++} concentration, annealing temperature and template DNA concentration (Ponchel et al., 2003, Bustina et al., 2004).

The aim of our study was to extract good quantity and quality of DNA in a cost-effective manner from FFPE tissues and to standardize Real time PCR on paraffin extracted DNA using SYBR Green 1 assay. DNA was extracted from FFPE Breast Cancer tissues using four different extraction protocols: 1) Qiagen spin column Extraction, 2) Phenol/ Chloroform Extraction (Chan et al., 2001), 3) Extraction protocol by Wickham et al. (Wickham et al., 2000) and 4) Heat Treatment Protocol. This article focuses on our experience with FFPE tissue DNA extraction and Real time PCR standardization.

Materials and Methods

Five blocks of formalin fixed, paraffin wax embedded (FFPE) breast cancer tissues stored for two to three months were used for DNA extraction. The study was conducted in compliance with the Helsinki document. For each sample 50μM thick section were cut and one 50μM section was used per extraction protocol. Four different DNA extraction protocols were followed for each sample.

Qiagen Spin column extraction:

The commercial QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. The tissues were dewaxed with xylene, followed by two washes with 100% ethanol twice to remove residual xylene. After dewaxing, tissues were digested overnight at 56°C with 180μl of ATL buffer and 20μl proteinase K. After digestion, 200 μl of AL buffer was added and incubated at 70°C for 10 minutes, followed by ethanol precipitation. The solution was transferred into a spin column, and washed with the wash buffers provided in the kit. DNA was eluted with 200 μl of AE buffer.

Phenol/ Chloroform Extraction:

Tissues were deparaffinzed in the similar manner as described above. They were dried at 37ºC for two hours. Dried tissues were suspended in 300 μl of digestion buffer containing 50mM Tris, 5 mM EDTA, pH 8, and 300 μg/ml proteinase K. After overnight incubation at 56ºC, the digested tissue mixture was centrifuged with phenol/ chloroform. DNA was precipitated overnight with sodium acetate and Ethanol. The DNA pellet was collected by centrifugation for 30 minutes at 4ºC, followed by a wash with 70% ethanol and air dried at room temperature. DNA was resuspended in 50μl of H_2O.

Protocol by Wickham etal

This is modified Qiagen protocol. The 50μM tissue was deparffinized. The DNA was then precipitated with 1ml of absolute alcohol. The DNA pellet was then dried at 55ºC for 10 minutes. The tissue was then lysed using ATL (360 μl) and proteinase K (40 μl) and incubated at 55ºC for 72 hrs. The solution was transferred into a spin column, centrifuged for one minute, and washed with AW1 and AW2 buffers. DNA was eluted in 200 μl of AE buffer preheated to 70°C.

Heat Treatment Protocol

The 50μM tissue section was deparaffinized in the similar manner. The tissue pellet was dried at 56°C. 200μl of digestion buffer (50mM Tris HCL, pH 8.5, 1mM EDTA, 0.5% Tween) and 4μl of 20 mg/ml proteinase K was added to the pellet and incubate at 56°C for 2hr on a shaker. After incubation the tissue dissolves completely and then it is incubated at 95°C for 10 minutes and centrifuged at 13,000 rpm for 5 minutes. DNA is present in the supernatant.

Assessment of Extracted DNA Purity and Yield

The quality (OD_{260}/OD_{280}) and quantity (OD_{260}) of DNA obtained was measured spectrophotometrically using the standard method (Sambrook, 1989).

PCR Amplification

Extracted DNA preparations with heat treatment method were subjected to DNA amplification using different primer sets described earlier (Rodriguez et al., 2004, Sharma et al., 2005, Old et al., 2001). Primer sequences of different genes are summarized in Table 1. Amplification was set up in a 50 ml reaction mixture containing 1X PCR buffer, 1.5 mM MgCl2, 200 μM dNTPs, 10 pm of primers and 1.5 units Taq. Positive and negative controls were included in the assay (Data not shown). The PCR conditions were as follows: Initial denaturation at 95ºC for 3 minutes, followed by 35 cycles of amplification at 94ºC for 30 seconds, annealing for 1 min at the respective annealing temperature and extension at 72ºC for 1 min followed by an additional extension for 5 minutes at 72ºC. Ten microlitre of PCR products were electrophoresed on a 2% Agarose gel.

Optimization of Real Time PCR

Real Time PCR was performed on breast cancer tissue DNA extracted using the Heat treatment protocol. EMSY, Cyclin D1 and β-globin genes were amplified using QuantiTect™ SYBR® Green PCR, Qiagen on Stratagene Mx3000P® Real Time Machine (Table 2). The assay was optimized for annealing temperature, primer concentration and template quantity. For optimization of annealing temperature and primer concentration two factors were considered: a) Ct value obtained should be as low as possible and b) There should be no primer dimer formation. For EMSY PCR, annealing temperatures tried were 59°C, 60°C and 61°C. Cyclin D1 and β-globin PCRs were carried out at 60°C, 61°C and 62°C for optimization of annealing temperature. Three different primer concentrations of 100 nM, 300 nM and 500nM were tried for optimization of primer concentration. Template concentration of 100 ng, 5 ng and 2 ng were tried for all the three genes. A heat dissociation experiment was included at the end of the PCR to check for the presence of single product. The assay was set up as mentioned in the QuantiTect™ SYBR® Green PCR product literature. The reaction volume was 25 μl, which consisted of 1X Master Mix, forward and reverse primer and template DNA. No template control was

Table 1. PCR Primers

Oligo-	˚C*	bp#	Sequence (5'-3')
EMSY-F	55.0	105	AAGTTCCAAAGGCCGTTGTT
EMSY-R	55.0	105	GTGGTAAGGAGTTGGCAATGCT
Mito 1F	48.0	476	AAAGTCTTTAACTCCACC
Mito 1R	48.0	476	GCACTCTTGTGCGGGATATTG
β-glo HbS-F	64.5	245	GGCAGTAACGGCAGACTTCTCCT
β-glo HbS-R	64.5	245	CAGTGCCAGAAGAGCCAA
β-glo prim A	63.0	653	CCCCTTCCTATGACATGAACTTAA
β-glo prim B	63.0	653	ACCTCACCCTGTGGAGCCAC

*Annealing temperature #Product size

Table 2. Real Time Primers

Oligonucleotide	Tm	Sequence (5'-3')
EMSY-F	64.5	AAGTTCCAAAGGCCGTTGTT
EMSY-R	66.1	GTGGTAAGGAGTTGGCAATGCT
CCND1-F	65.2	GGGCAGTTTTCTAATGGAATGG
CCND1-R	67.5	CACCACAGTGGCCCACACT
β-globin-F	66.7	GGCAACCCTAAGGTGAAGGC
β-globin-R	67.4	GGTGAGCCAGGCCATCACTA

Table 3. Spectrophotometric Analysis of FFPE DNA

DNA Extraction Protocol	260:280	Yield
Qiagen DNA Extraction	1.2 – 1.7	13.5 – 17.5 μg
Phenol/ Chloroform extraction	1.4	9.0 – 13 μg
Wickham et al	1.3 – 1.7	12.5 – 19.5 μg
Heat Treatment Protocol	1.1 – 1.3	160 – 211 μg

included in every assay and all the reactions were carried out in duplicates.

Results

FFPE DNA Extraction

Four different DNA extraction protocols were carried out to evaluate the yield and quality of DNA formalin fixed paraffin embedded tissue samples. Maximum yield in the range of 160 - 211 μg was obtained with heat treatment protocol and minimum yield in the range of 9 μg – 13 μg was obtained with phenol/ chloroform extracted DNA (Table 3). However, the quality of DNA obtained with Wickham et al protocol was of maximum purity with $OD_{260:280}$ of 1.3 – 1.7. DNA obtained using heat treatment protocol gave $OD_{260:280}$ of not more than 1.3. The quantity of DNA obtained using heat treatment protocol was good enough to carry out optimization of Real Time PCR and further to analyze multiple cancer markers.

PCR Amplification

DNA obtained using heat treatment protocol was further subjected to PCR using different sets of primers that gave amplimers in the range of 105 bp to 653 bp. Amplification was seen with EMSY primers (105 bp) and β-globin primers (245 bp) (Figures 1 and 2). No amplification was obtained with mitochondria gene primers (476 bp) and with β-globin primers that gave higher amplimer of 653 bp.

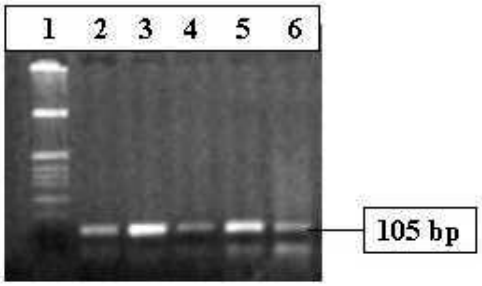

Figure 1. EMSY Gene Amplification. EMSY gene amplification on FFPE Extracted DNA (105 bp).Lane 1: pBR322 HinfI Marker (1632 bp, 504 bp, 396 bp, 344 bp, 298 bp, 220 bp, 154 bp); Lane 2, 3, 4, 5, 6: FFPE extracted breast cancer tissues.

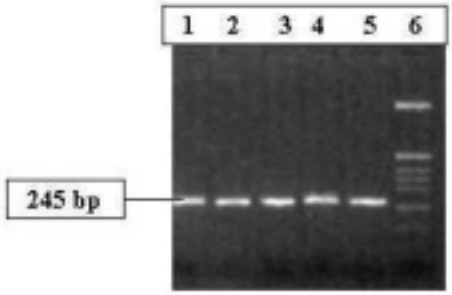

Figure 2. β-globin Gene Amplification. β-globin gene amplification on FFPE Extracted DNA (245 bp) Lane 1, 2, 3, 4, 5: FFPE extracted Breast Cancer tissues; Lane 6: pBR322 HinfI Marker (1632 bp, 504 bp, 396 bp, 344 bp, 298 bp, 220 bp, 154 bp)

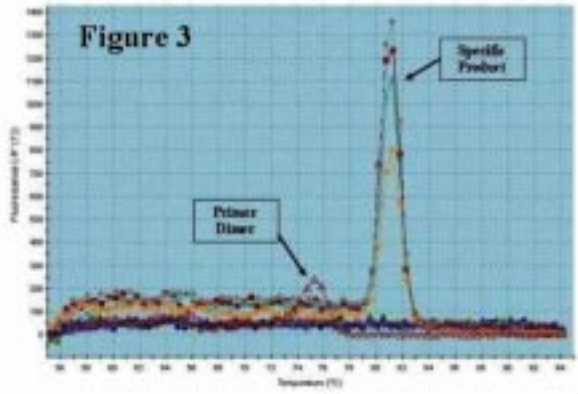

Figure 3. EMSY Gene Dissociation Curve - 59˚C Annealing Temperature. EMSY products show a specific peak at Tm of 81°C and primer dimer is observed at 75°C

Optimization of Real Time PCR

In case of EMSY gene amplification best results were obtained at 60°C with lower Ct value and no dimer formation (Table 4). At 59°C, primer dimer formation was observed (Figure 3). In case of Cyclin D1 and β-globin

Table 4. Optimization of Annealing Temperature for Real Time Analysis of FFPE Tissue DNA

	EMSY			Cyclin D1			β-globin		
Temperature	59°C	60°C	61°C	60°C	61°C	62°C	60°C	61°C	62°C
Ct values	29.24	29.92	30.38	29.2	32.28	33.0	28.0	27.4	26.89
Dimer Formation	Present	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

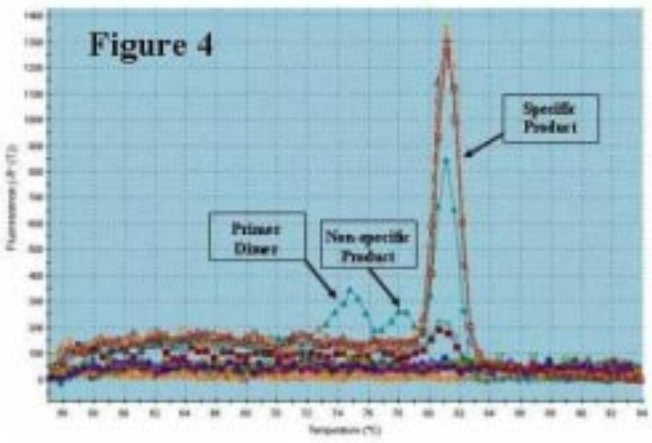

Figure 4. EMSY Gene Dissociation Curve – 500 nM Primer Concentration. EMSY products show a specific peak at Tm of 81°C and a primer dimer is observed at 75°C and a non-specific product at 78°C

best results were obtained at an annealing temperature of 60°C and 61°C respectively. Primer concentration of 100 nM, 300 nM and 500 nM were tried for all the three genes and best results were obtained with Primer concentration 300 nM. EMSY gene did not amplify at all with 100 nM primers. At 500 nM primer concentration, EMSY PCR showed formation of primer dimer and a non-specific product (Figure 4).

Template concentration of 100 ng and 10 ng were tried for EMSY, Cyclin D1 and β-globin PCR and good amplification curve was observed with 10 ng of Template DNA. With 2 ng of DNA some of the samples did not show amplification.

Discussion

There is a rapidly increasing need to evaluate a range of susceptibility and tumor markers in formalin-fixed, paraffin-embedded tissue specimens in molecular epidemiological studies. Polymerase chain reaction is one of the easiest molecular biology techniques that can be performed on FFPE tissues. However, the efficiency of PCR is influenced by a number of factors including the type of fixative and the fixation time, the DNA extraction procedure, PCR amplimer size, the concentration of template DNA and optimization of PCR (Mahaisavariya et al., 2005). Amongst the four DNA extraction protocols evaluated, Heat treatment protocol was found to be the most reliable for clinical diagnosis in terms of quantity of DNA obtained, cost effectiveness and short extraction time of 2-3 hours. Phenol/chloroform extraction is quite laborious, involves a number ofsteps and is prone to cross-contamination.

In case of the other two extraction protocols that use expensive Qiagen kit, the yield of DNA obtained is very low and is not suitable for studies that involve the use of multiple gene markers. Successful amplification was observed with low amplimer size products i.e. EMSY (105 bp) and _-globin (245 bp). Fragments of higher sizes, 476 bp Mitochondria fragment and 653 bp β-globin failed to amplify and the results were in compliance with the previous reports (Chan et al., 2001). DNA obtained using Heat treatment protocol is sufficiently intact to amplify a region of at least 250 base pairs and the quantity obtained is good enough to analyze multiple markers. In case of Real Time quantitation, sensitivity of detection with SYBR-Green I is compromised by the formation of primer-dimers, lack of specificity of the primers, primer concentration, annealing temperature, template concentration and the formation of secondary structures in the PCR product. Non-specific product and primer dimers were observed with increase in primer concentration and decrease in annealing temperature in case of EMSY PCR.

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