

Optimization of Real Time PCR for Precise Measurement of HER2 Overexpression in Breast Cancer Specimens

Samira Sadeghi¹, Hossein Tabatabaeian¹, Zohreh Hojati^{1*}

Genetics Division, Biology Department, Faculty of Sciences, University of Isfahan, Isfahan, Iran.

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Corresponding Author:

Zohreh Hojati.

Associate Prof. in Human Molecular Genetics. Genetics Division, Biology Department, Faculty of Sciences, University of Isfahan, Isfahan, Iran. Phone: +983117932478

E-mail: z.hojati@sci.ui.ac.ir

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Abstract

Background: Breast cancer is one of the most prevalent malignancies among women in various countries. HER2 overexpression, which is due to different reasons, occurs in 20-30% of breast cancers. HER2 gene encodes 185 kDa transmembrane glycoprotein with 1255 amino acids. This active product triggers downstream intracellular signaling pathways inducing cell proliferation and cell survival. These activities could be done in an uncontrolled manner when HER2 expression undergoes up-regulation. The aim of this study was optimization of Real Time PCR condition.

Materials and Methods: RNA purification, cDNA synthesis and then optimization of Real Time PCR method performed respectively. In this study, total RNA was extracted from fresh tissue samples, first strand of total cDNA was synthesized and in the following steps, Real Time PCR was performed to be optimized.

Results: Although altering the protocol, annealing temperature and concentration of MgCl₂ did not make any improvement and beneficial effects on reactions, changing the concentration of primers to 0.24 pm/μl was influential to eliminate primer dimers of Real Time PCR reactions. It demonstrated that the copy number of GAPDH transcripts is more than HER2 transcripts in normal breast tissues. Therefore, deviation in 2.5 differences between the Ct value of HER2 and GAPDH indicated that the copy number of HER2 transcripts was increased; therefore, HER2 underwent overexpression in these cases.

Conclusion: Under these optimized conditions, this technique can be applied as a powerful method in clinical laboratories.

Keywords: HER2 gene; cDNA, Purification; Real Time PCR

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Introduction

Breast cancer is the most prevalent cancer among women and HER2 is one of the most prominent agents involved in breast tumorigenesis. Human Epidermal growth factor Receptor 2 (HER2) is overexpressed because of miscellaneous reasons and this phenomenon generally occurs in 20 to 30 percent of breast malignancies. HER2 gene, which is called by some other names including HER2/neu, c-ErbB2 and P185HER2, is located on 17q21 position of human genome (1-3). HER2 gene encodes a 185 kDa transmembrane glycoprotein with 1255 amino acids. HER2 product, along with other members of HER - HER1, HER3 and HER4- family, form active tyrosine

kinase receptors on the surface of cells (4). All members of HER family have similar structure, an extracellular domain which is ligand binding region, a lipophilic transmembrane domain and an intracellular domain which have tyrosine kinase activity. As exceptions, HER3 and HER2 lack an active tyrosine kinase and ligand binding domains, respectively. In fact, these tyrosine kinase domains are activated by binding of Epidermal Growth Factor-Like Growth Factors (EGFs), while there is no particular ligand with ability of binding to HER2 receptors (5). However, homo or heterodimerization of these receptors occurs via ligand binding and then

autophosphorylation of tyrosine kinase domains launches. HER2 protein prefers to form heterodimers with other members of HER family, instead of homodimerization (6). Some intracellular signal transduction pathways are triggered by HER-2 activation, resulting in cell proliferation and cell survival (7, 8). HER2 amplification occurs in 20 to 25 percent of breast cancers and causes poor prognosis (9, 10). In principle, HER2 amplification changes normal epithelial tissue into an invasive carcinoma. Estrogen Receptor (ER) and HER2 signaling pathway are the major reasons of proliferation and immortality in 85 percent of breast cancers; therefore, studies on HER2 and ER targeting have developed therapeutic approaches in HER2 and ER positive patients (11). HER2 overexpression, which is happened due to amplification or other reasons, causes propagation of HER2 proteins on the cell surface. In this situation, HER2 proteins can form plenty of heterodimers and make each other active. It then causes transphosphorylation of intracellular domains with tyrosine kinase activity. These phosphorylated receptors are becoming anchors for other proteins which play role as secondary messengers in signal transduction pathways (12-16). Transcription factors activated by HER2 signaling pathway involve in regulation of genes responsible for various cellular activities, such as: cell proliferation, cell survival, cell differentiation, angiogenesis, invasion and metastasis (6, 12, 16, 17). Normal epithelial cells produce a few amounts of HER2 protein, but in breast carcinoma this restricted amounts of HER2 protein can increase to 40-100 folds more than standard level; consequently presence of about two million HER2 proteins is observed on the surface of HER2 positive tumor cells (18). These two different levels of HER2 receptor in normal and tumor cells form the basis of disease diagnosis and selection of appropriate therapeutic method. Real Time PCR is one of the suitable methods used for assessment of HER2 status in breast tumor cells. Thus, optimization of this technique can improve preciseness of HER2 evaluation for distinguishing HER2 positive tumors.

Materials and methods

Patients and Samples

All patients were provided standardized written consent. It was performed in fifty samples including 40 malignant and 10 normal specimens collected between 2010 and 2012 from the Breast Cancer Research Center in Seyed-Al-Shohada Hospital in Isfahan. Immediately after excision, fresh tumor samples were frozen in liquid nitrogen and stored at -80 °C, whereas a portion of tumor samples was transferred to the pathology laboratory for further

confirmation of breast cancer diagnosis.

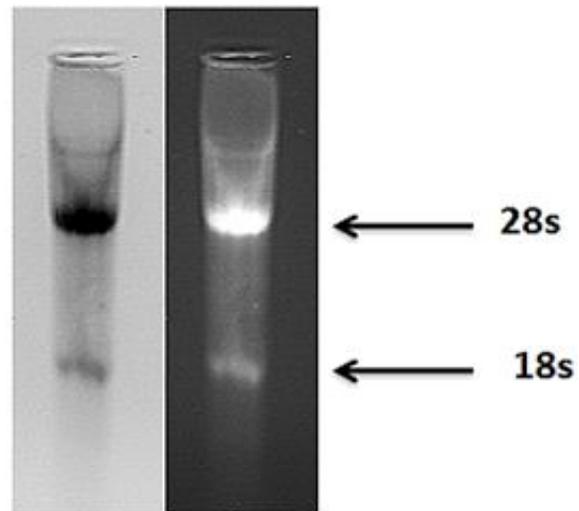


Figure 1. Integrity and purity of extracted RNAs. Extracted RNAs were loaded in agarose gel along with ethidium bromide staining followed by U.V detection. Two expected bands were seen in a condition that 28S band is sharper than 18S, which is a standard situation for appropriate purification when 10 mM Tris.Cl, pH=7.5 is used as dilutor.

RNA Purification and cDNA Synthesis

Twenty to thirty milligram fresh frozen specimens were collected from each patient for RNA isolation. Mortar and pestle with liquid nitrogen were recruited for disruption of samples. It was followed by homogenization using a syringe and needle. RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for extraction of total RNA from fresh frozen specimens.



Figure 2. Temperature gradient for amplification of HER2 cDNA. Amplification of HER2 cDNA in 3 different temperatures (Lane 1, 54 °C; lane 2, 56 °C; and lane 3, 58 °C). In 58 °C the sharpest band was observed. 103bp band shows the expected product.

The isolation was performed according to the manufacturer's instruction. RNA was then stored in RNase-free water at -70 °C. The quantity and quality of purified RNAs were measured by spectrophotometer and electrophoresis in a 2% agarose gel with ethidium bromide staining, respectively. Total RNA was then transcribed into total cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The manufacturer's instruction of this kit was used for cDNA synthesis. Synthesized cDNA was stored at -70 °C.

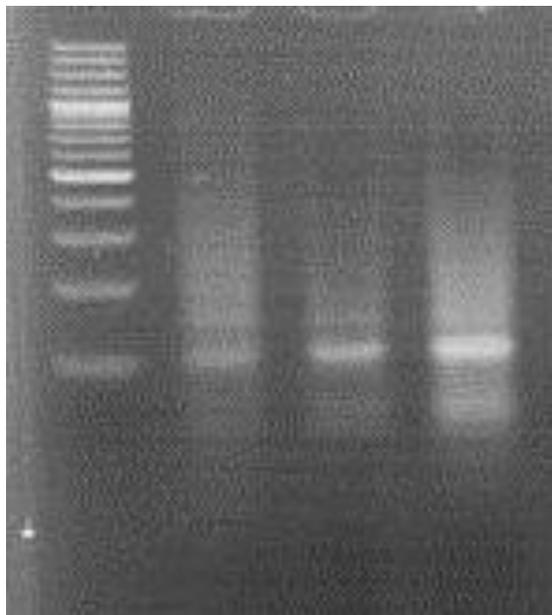


Figure 3. Temperature gradient for amplification of GAPDH cDNA. Amplification of GAPDH cDNA performed in 3 different temperatures. (Lane 1, 54 °C; lane 2, 56 °C; and lane 3, 58 °C). The sharpest band was observed in 58 °C. 115bp band shows the expected product.

Analysis of Real Time PCR

Primers

GAPDH was selected as the housekeeping gene. Primers were designed by AlleleID version 7.7 and Oligo version 7 software. They have been synthesized by Bioneer (Korea). The length of amplified products of HER2 and GAPDH is 103bp and 115bp, respectively.

Real Time PCR

Chromo4 (Bio-Rad) device and BioEasy SYBR Green I Real Time PCR kit were used for performing Real Time PCR. Amplification reactions were carried out in a volume of 25 µl with 4U Taq DNA polymerase, 0.4 pmol/µl of each primer, and 40 ng/µl cDNA. 2x SYBR Mix included PCR buffer, Mg²⁺, dNTP mixture and SYBR Green®. The thermal cycling conditions included an initial denaturation for 5 minutes at 94 °C and 40 cycles consisted of a

denaturation step at 94 °C for 30 seconds, an annealing step at 68 °C for 30 seconds, and an extension step for 40 seconds at 72 °C. Fluorescence measurements were performed after each extension step. Each sample was evaluated in duplicate manner. Average Ct values were utilized for further analysis. The same amount of cDNA was amplified in all experiments, in single measurement fluorescence.

Standard curve

The 2-ΔΔCt method could be used in a condition that the amplification efficiency of control and target templates is approximately identical and reliable. The efficiency of primers was computed from the threshold cycles attained by 10-fold serial dilution (1000 ng/µl to 1 ng/µl) of HER2 and GAPDH cDNAs. Calculating the Ct values, HER2 and GAPDH standard curves were plotted.

Ethics Statement

This study was approved by the institutional ethics committee of University of Isfahan.

Results

Purity and integrity of extracted RNAs

Spectrophotometer, used to check extracted RNAs, showed 200 to 10000 Nano gram amount of RNA per milliliter. The ratios of OD260/OD280 in studied specimens were 1.9 to 2.1 using 10 mM Tris.Cl, pH7.5 as dilutor. The purity of RNAs extracted in this study was confirmed by this ratio. Agarose gel and ethidium bromide staining were used for determining the integrity of purified RNAs. Two expected bands, related to 28S and 18S rRNAs, were detected successfully as illustrated in figure 1. These bands confirmed the integrity of extracted RNAs and dearth of excessive and irrelevant substances in final volume of extraction.

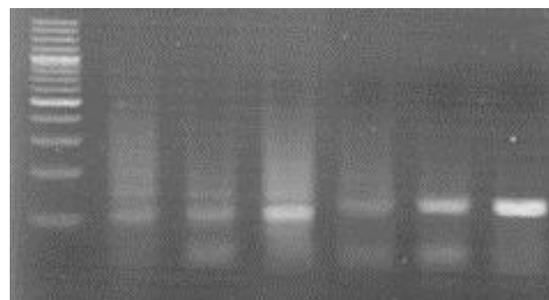


Figure 4. Primer concentration gradient for amplification of HER2 and GAPDH genes in 58°C. Lanes 1 to 3 show the concentration of HER2 primers 0.16, 0.24 and 0.32 pm/µl, respectively. Lanes 4 to 6 show the concentration of GAPDH primers 0.16, 0.24 and 0.32 pm/µl, respectively. Lanes 3 and 6 show that the best condition for eliminating primer dimer bands is 0.32 pm/µl concentration.

cDNA amplification of HER2 and GAPDH genes

In this study, PCR technique was used for obtaining the best condition for Real Time PCR. The main

variables including temperature, MgCl₂ concentration and primer concentration were evaluated by PCR.

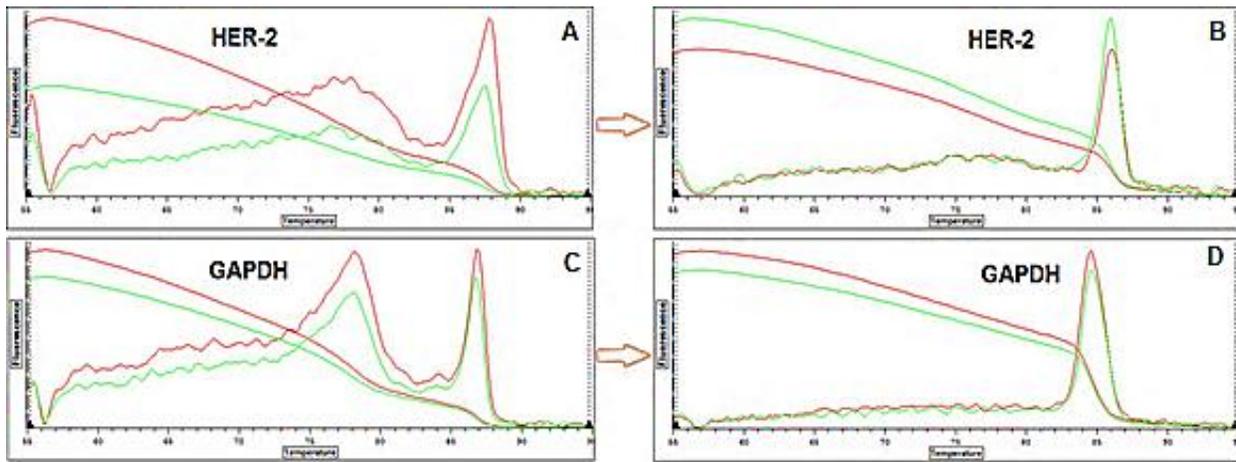


Figure 5. Optimization of Real Time PCR reaction. A and C. The dearth of typical peak in HER2 and GAPDH amplification melting curves. B and D. using 0.24 pm/μl concentration of specific primers to achieve almost standard melting curve with only one typical peak.

determination of appropriate annealing temperature for simultaneous amplification of HER2 and GAPDH cDNAs

In the Real Time PCR, the housekeeping gene (reference gene) and the main gene (HER2) should be amplified simultaneously at the same temperature.

Temperature gradient between 54°C to 58 °C (54 °C, 56 °C and 58 °C) was used for choosing the best annealing temperature. As it is displayed in figures 2 and 3, the best temperature for both HER2 and GAPDH cDNAs was 58 °C.

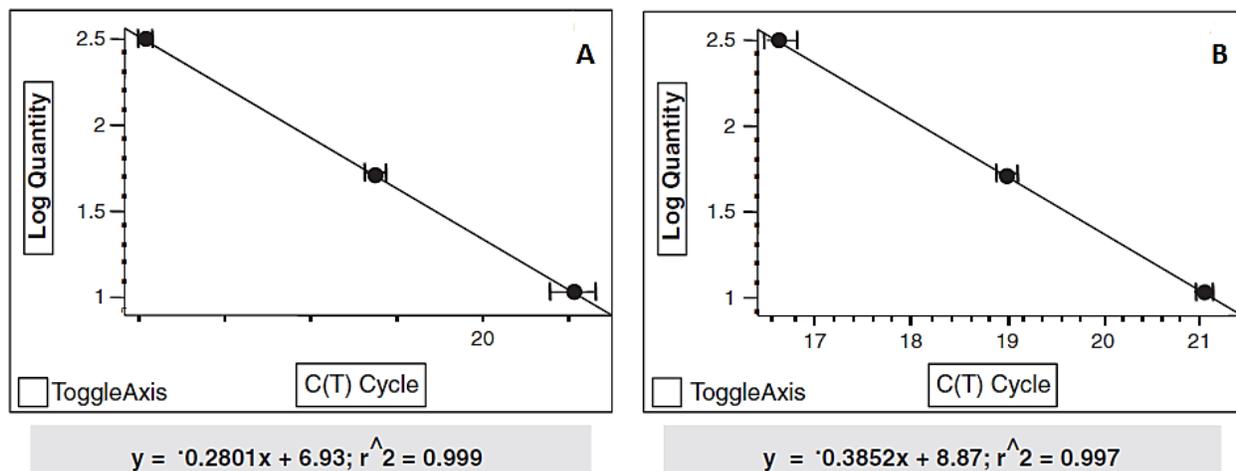


Figure 6. The standard curve of HER2 (A) and GAPDH (B) genes. The r² value of HER2 and GAPDH gene was approximately similar, near 1. The efficiency of amplification for both HER2 and GAPDH genes were perfect; therefore, it confirms using 2-ΔΔC_t method.

Appropriate primer concentration for amplification of HER2 and GAPDH cDNAs

As primer dimer and other excessive bands may cause false positive results, it is necessary to obviate them in Real Time PCR method. Therefore, the concentrations of specific primers were reduced to eliminate primer dimer bands. Thus, a gradient of different concentrations for specific primers (0.16, 0.24 and 0.32 pm/μl) in 58 °C were used to determine the best condition. As it is shown in figure 4, 0.32

pm/μl was the best concentration of HER2 and GAPDH primers for performance of Real Time PCR using SYBR Green dye.

Real Time PCR

The first results of the reaction of Real Time PCR under the optimized PCR conditions (annealing temperature=58 °C and primer concentration=0.32 pm/μl) revealed that it was not a good protocol for propagation of HER2 and GAPDH cDNAs by Real

Time PCR method, probably due to changes in device and amplification buffer. Although altering the protocol, annealing temperature and concentration of MgCl₂ did not make any improvement and

beneficial effects on reactions, changing the concentration of primers to 0.24 pm/μl was influential to eliminate primer dimers of Real Time PCR reactions, as illustrated in Figure 5.

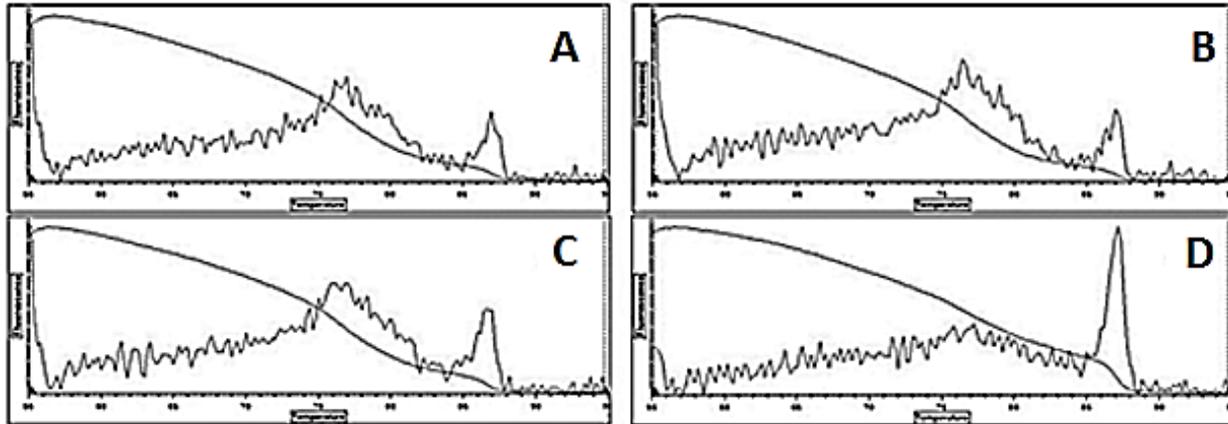


Figure 7. HER2 melting curve. The melting curve of HER2 propagation by Real Time PCR method in 1, 10, 100 and 1000 ng/μl is shown in parts A to D, respectively. The best condition is for 1000 ng/μl which only exhibits one typical peak.

Validation of the standard curve

The standard curve was drawn from the synthesized cDNAs of normal breast samples that were serially diluted tenfold (four points: 1, 10, 100 and 1000). The plotted curves of HER2 and GAPDH genes were within standard conditions. The r² values of HER2

and GAPDH genes were approximately similar. The efficiency of amplification for both HER2 and GAPDH genes were perfect and appropriate, thus the 2-ΔΔCt method was found valid and accurate (Figure 6).

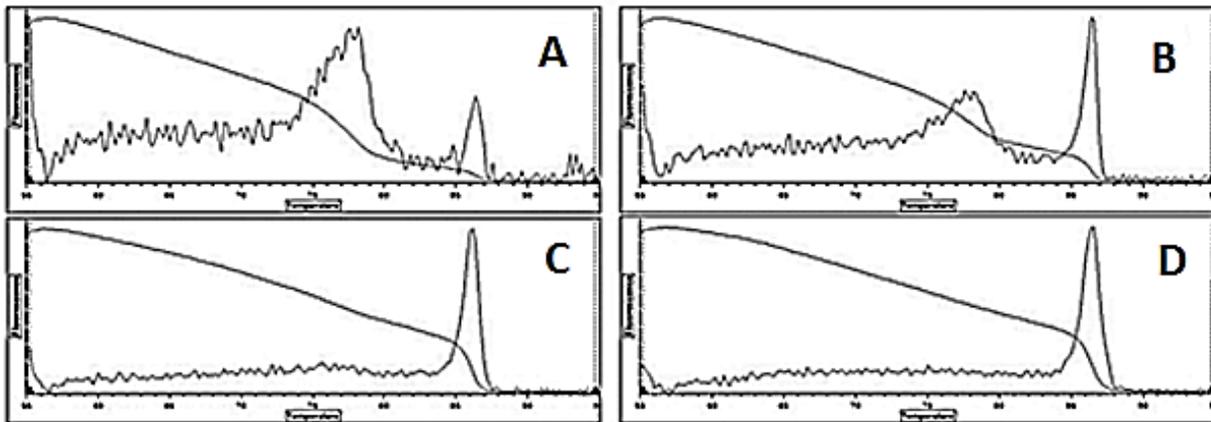


Figure 8. GAPDH melting curve. The melting curve of GAPDH propagation by Real Time PCR method in 1, 10, 100 and 1000 ng/μl is shown in parts A to D, respectively. The best conditions are for 100 and 1000 ng/μl concentrations which exhibit only one typical peak. Synchronization was required between HER2 and GAPDH amplifications, therefore, 1000 ng/μl concentration was selected for Real Time PCR reaction.

Determination of appropriate cDNA concentration and melting curve plotting

In analysis of the melting curves of four different cDNA concentrations (1, 10, 100 and 1000 ng/μl), 1000 ng/μl was identified as the best concentration for cDNA propagation in Real Time PCR (Figures 7 and 8). Although the best conditions for GAPDH propagations were obtained in 100 and 1000 ng/μl

concentrations, due to compulsory synchronization between the amplification of HER2 and GAPDH, 1000 ng/μl concentration was selected for performing the Real Time PCR reaction.

Amplification curve

As it is illustrated in figure 9, in normal specimens the Ct value difference between amplification of HER2

and GAPDH cDNAs was approximately 2.5 units. We found that the copy number of GAPDH transcripts is more than HER2 transcripts in normal breast tissues. Although this rule was observed in the majority of malignant specimens, not only Ct value of HER2 came close to Ct value of GAPDH in some

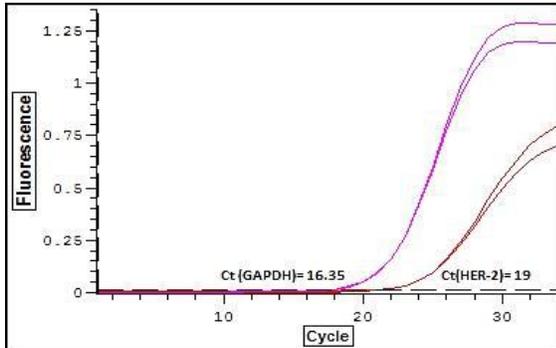
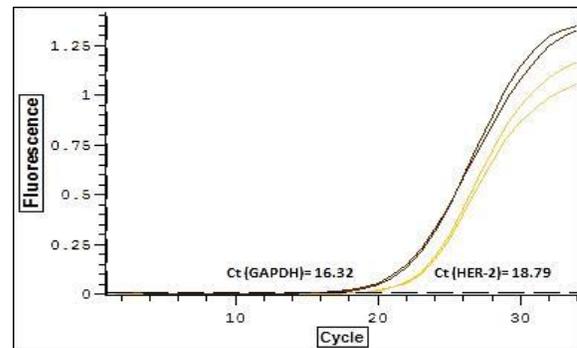


Figure 9. Amplification curve of HER2 and GAPDH in normal breast tissue. The difference between C_t value of HER2 and GAPDH was approximately 2.5 units.

cases, but also it was even less than C_t value of GAPDH. This deviation in 2.5 differences between the C_t value of HER2 and GAPDH indicated that the copy number of HER2 transcripts increased; therefore, in these cases HER2 underwent overexpression (Figure 10).



Discussion

The main aim of this study was to optimize a Real Time PCR method for assessment of HER-2 expression in breast cancer specimens. Real Time PCR was chosen because of two main reasons; first, it is one of

the most dependable methods for quantitative measurement of mRNA and second, quantifying of HER2 expression has been used to determine prognosis in breast cancer (19).

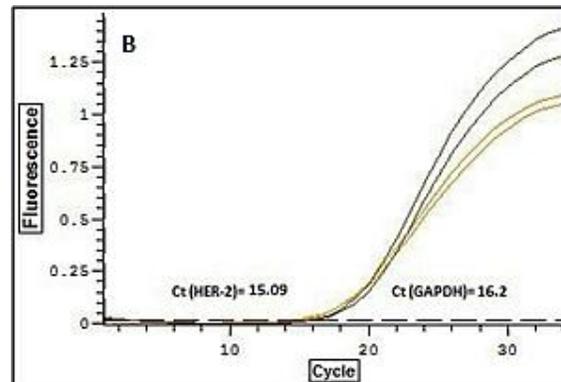
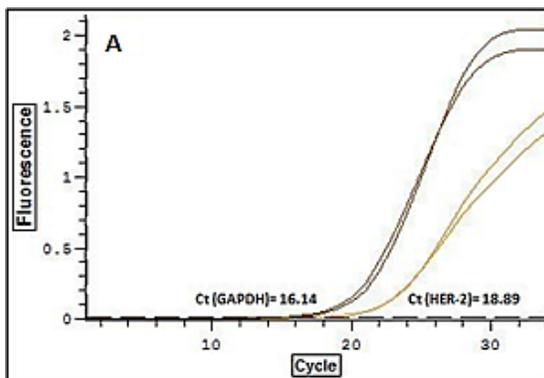


Figure 10. Amplification curve of HER2 and GAPDH in malignant breast tissue. A. The difference of C_t value between HER2 and GAPDH expression was approximately 2.5 units indicating that this sample has normal expression of HER2 gene B. C_t value of HER2 is less than C_t value of GAPDH indicating that HER2 has undergone overexpression in this case.

In this study, Real Time PCR technique was used for measurement of the level of HER2 mRNA transcribed in normal and malignant cells. Since benefiting from trastuzumab therapy (a monoclonal antibody against HER2) along with chemotherapy is only useful and practical for treatment of HER-2 positive types of breast cancer (20, 21), and on the other hand, using trastuzumab, as well as its toxicity, impose a huge cost on the patients (22), deciding whether it has to be used or not is mandatory and crucial; therefore, this diagnosis inevitably requires a reliable and accurate method. Initial diagnosis of HER2 overexpression also allows conservation of

breast tissue in patients who initially require a mastectomy operation.

Fifty fresh frozen tissue samples including 40 malignant and 10 normal specimens underwent RNA extraction. Quantity, integrity and purity of extracted RNAs were proved, according to the spectrophotometer and agarose gel outcomes. Initial optimization of Real Time PCR method was achieved by PCR technique using synthesized cDNAs, because it is more cost effective. But it was shown that the optimized PCR conditions did not work precisely in Real Time PCR. Thus, the next level of optimization was performed by Real Time PCR. A slight change in

concentration of primers provided the appropriate condition for Real Time PCR performance. Validate standard and melting curves were consequences of optimized Real Time PCR reaction. Except for HER2 gene amplification which accounts for 92–95% of HER2 overexpression cases (23), other factors such as chromosome 17 polysomy and mutations in the HER2 or upstream regulator genes are of the reasons of HER2 overexpression (24-26).

Therefore, it seems logical to assess transcripts of HER2 gene to encompass other various reasons that cause HER2 overexpression. Real time PCR can be a sensitive, credible and cost effective method provided that it is performed in a perfectly optimized manner. Under this circumstance, this technique can be applied as a reliable and powerful method in clinical laboratories.

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Author Contributions

Study concept and design: Zohreh Hojati. Analysis and interpretation of data: Samira Sadeghi, Hossein Tabatabaieian. Drafting of the manuscript: Samira Sadeghi, Hossein Tabatabaieian. Critical revision of the manuscript for important intellectual content: Zohreh Hojati.

Conflict of interest

The authors declare no conflict of interest.

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