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Research Article

Determination of Cut-off Value for HER-2 Scoring based on qPCR Technique using Frozen and Archival, Formalin-fixed, Paraffin-embedded Tissues of Breast Cancer

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Abstract: This study aimed at determining cut off value for HER-2 scoring based on qPCR technique using frozen and FFPE of breast cancer tissues samples. Total numbers of samples were 23 for FFPE and 72 for frozen tissues, obtained from breast cancer patients in several hospitals in Padang, West Sumatera Province, Indonesia. Plasmid containing HER-2 gene fragment insert and Plasmid containing WHN gene fragment were prepared for standard curve. An evaluation for qPCR optimal quality was determined by CV value, coefficient of determination (R^2) > 0.980, and value of efficiency percentage (% E; within the range of 90 to 105), which was then confirmed with the melt peak and agarose analysis. Determination of cut-off score was conducted with quartile calculation. The standard curve of this method showing the good performance of HER-2 scoring as shown by R^2, and efficiency value of 0.9977 (98 %) and 0.9984 (97 %) for HER-2 and WHN, respectively. In addition, both the standard curves showed a slope value of -3.3 with CV value less than 10 %. On the basis of further confirmation using melt peak and agarose analysis, a single band and a single peak, especially for WHN, were proven; however, in HER-2 a small unspecific melt peak was appeared. The cuts off obtained from qPCR technique as borderline for scoring the HER-2 gene status were 2.34 to 4.79 for FFPE tissues, and were 2.61 to 4.19 for frozen samples. Thus, qPCR method may be considered as complementary technique to IHC test for confirming equivocal IHC results to improve the outcomes and healthcare diagnosis for the sub type HER-2 breast cancer patients.

Keywords: Breast cancer, cut off, HER-2 scoring, qPCR technique

1. INTRODUCTION

HER-2 (human epidermal growth factor receptor 2) is a proto-oncogene (185 kDa), consisting of a protein receptor, transmembrane and tyrosine kinase. HER-2 is classified as the epidermal growth factor receptor (EGFR) which is also known as HER family encoded by *her-2* genes located on chromosome 17q21. Under normal conditions, these proteins along with other HER proteins form homodimers or heterodimers serving to regulate a variety of cellular functions, including cell proliferation, apoptosis, differentiation, development, immune response and so forth. HER-2 protein is one of the important biomarkers in breast cancer. In certain condition, HER-2 overexpression will cause HER-2 type breast cancer. HER-2 overexpression occurred in (20 to 30) % of overall breast cancer cases [1, 2].

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The examination of HER-2 status in breast cancer patient will determine the prognosis, the malignancy of cancer and the treatment. Patient with HER-2 overexpression breast cancer correlate with low prognosis and low life expectancy. For therapeutic treatment, patients with HER-2 breast cancer have been reported to be resistant to endocrine therapy and more responsive, while to improve patients'survival the monoclonal antibody trastuzumab may be applied, either in form of single use or in combination with other chemotherapeutic agents. Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) methods have been recommended for HER-2 determination status. Both of these methods have been approved by the Food and Drug Administration (FDA), where IHC is the most widely used method. The disadvantage of the IHC method is high subjectivity and low reproducibility. On the other hand, the FISH method is a gold standard for HER-2 scoring as it is more accurate although it is costly and requires sophisticated equipment. According to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines, the HER-2 scores that fall in borderline obtained by IHC should be followed by FISH test. In fact, most are not obeyed this procedure. Especially for developing countries such as Indonesia, the examination using the FISH is rarely done. A mistaken determination of HER-2 status will result improper treatments for the patients. This further causes low survival of breast cancer patients and then lead to the high medical cost [3–7].

In this recent year, a real-time PCR method has been studied for HER-2 scoring purpose. This method can be quantified, controlled and more importantly it has a high reproducibility. According to Mendoza et al. [8], HER-2 scoring using the real-time PCR method showed high concordance with the FISH, with low concordance with IHC, (20 to 40) %. Compare to FISH and IHC, the scoring method developed by this real-time PCR technique is easier, faster, and more sensitive and it can be applied for routine clinical purposes [8]. Due to its high accuracy and high reproducibility, Konigshoff et al. [3] and Rosa et al. [9] suggest that Real-time PCR can be a routine method for HER-2 status determination. Here in this report, we determined cut off value for HER-2 scoring in breast cancer patients using archival, formalin fixed, paraffin embedded (FFPE) tissue samples in comparison with frozen tissue samples. Both of those samples have previously been determined for the IHC status.

2. MATERIAL AND METHODS

2.1 Samples

This study has received an ethical clearance issued by the Ministry of Health of Indonesia. Frozen and FFPE tissues of breast cancer sample were obtained from several hospitals in Padang, West Sumatra Province, Indonesia. The sample has been examined with IHC method and identified to contain more than 50 % breast cancer cells. FFPE tissue examination was conducted by an anatomical pathologist from Dr. Sardjito Hospital Yogyakarta, and then confirmed by anatomical pathologist from M. Djamil hospitals in West Sumatera Province. The results of the examination from each hospital showed 100 % conformity which was identical with all the examined samples.

2.2 Preparation Plasmid Recombinant Containing HER-2 or WHN Gene Fragment Insert for qPCR Standard Curve Development

The extraction process of pGEM-T easy HER-2 and pGEM-T easy WHN was conducted following High-Speed Plasmid Mini Kit procedures (Geneaid #PDH300, Australia). The extraction results were confirmed by agarose gel electrophoresis and measured for its DNA concentration with spectrophotometer at a wavelength 260 nm. The extracted DNA plasmid was prepared to develop a qPCR standard curve.

2.3 Genome Extraction

The extraction of breast cancer DNA genome was performed using Invitrogen Purelink Genomic DNA kit (#LSinvC421-K182001, USA). Meanwhile, the DNA genome extraction from FFPE tissue was performed using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE from Ambion (#AM1975, TX, USA). The genomic DNA was further confirmed for its concentration with spectrophotometer at a wavelength of 260 nm.

2.4 Quantitative Real-Time PCR (qPCR)

qPCR was performed with CFX96TM qPCR using SYBR Fast KAPA Kit (#KK4600, USA). The reaction is conducted in a separate tube for HER-2 gene and WHN, with a total mixture reaction of 10 µL. The composition was 3 µL of TE buffer pH 8. 5 µL of 2 × KAPA SYBR fast kit, 0.5 µL for each forward and reverse primer and 2.5ng of DNA template. qPCR condition was 95 °C for 3 min, followed by 35× cycles of denaturation, annealing and elongation of each 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s. Each sample was run in three replicates to avoid false-positive results and the average value was used for further calculations. Non-template control was also included in each reaction to ensure no contamination to the reaction mixture. Each amplification reaction was further examined in 3 % agarose gel electrophoresis.

2.5 Standard Curve of qPCR

Standard curve was constructed by plotting the log value of the copy number of the value Ct generated from serial dilutions of plasmid DNA. Copy number value is obtained from the general formula (1) [8]

$$\frac{6.02\times10^{23}(\text{copy mol}^{-1})\times\text{plasmid concentration (g mL}^{-1})}{\text{weight of molecule (g mol}^{-1})} \quad (1)$$

Evaluation of qPCR optimal quality is determined by the value of coefficient of determination (R2) > 0.980, the value of efficiency percentage (% E) in the range of (90 to 105) % and it is intended for the CV value.

2.6 qPCR Ratio Calculations

Calculation of the relative copy number of HER-2 gene was done as the ratio of the average value of Ct HER-2/WHN each sample with the Equation (2)

$$\text{The ratio of R} = 2^{\Delta CT} \quad (2)$$

Where ΔCT = Ct WHN - Ct HER-2, assuming that the number of copies of both genes in normal cells is similar [6].

2.7 The Determination of the Cut-Off Value

The determination of the cut-off value to categorize each sample as the negative, borderline positive and HER-2 positive was calculated statistically using the quartiles. Value of quartile is assumed to be equivalent with the score of IHC to classify the status of HER-2.

3. RESULTS

3.1 Standard Curve of HER-2 and WHN for Relative qPCR Determination of HER-2 Scoring

The OD260 of template sample for both pGEMT containing HER-2 gene fragment insert and pGEMT containing WHN gene fragment insert was between 1.7 and 2.0, indicating the both of those plasmids were ready to be applied for the standard curve preparation. To generate a standard curve, 2-fold dilution was prepared. In terms of the integrity of the data, R2 and efficiency were 0.9977; 98 % and 0.9984; 97 %, respectively for HER-2 and WHN. Both of the standard curves showed a slope value of -3.3, while the CV value was below 10 % (data calculation is not shown). Based on the melting peak shape (both with DNA plasmid and DNA genome as template) followed confirmation with the agarose electrophoresis, it was shown as single band for HER-2 but with additional small non-specific peak below the threshold line, while WHN was shown as single band and single sharp peak. Non Template Control (NTC) for HER-2 standard curve was below the threshold line. NTC for WHN standard curve consistently showed Ct value at 30 to 31 which was eight cycles different from lowest DNA plasmid dilution (Fig.1 and Fig. 2).

3.2 Determination Cut-off Value

In this study, HER-2 gene quantification was determined by calculating the ratio between HER-2/WHN genes. The cut off for HER-2 scoring using qPCR was analyzed by quartile calculation. Quartile value was assumed to be equivalent with score used in classifying the HER-2 status by IHC method. By using frozen tissue as template in qPCR, the following cut off values were obtained: negative < 2.61; borderline 2.61 to 4.19; positive > 4.19, while for FFPE tissue, cut off value were: negative < 2.34; borderline 2.34 to 4.79; positive > 4.79. Both frozen tissues and FFPE tissue were identical with and similar to the cut off value.

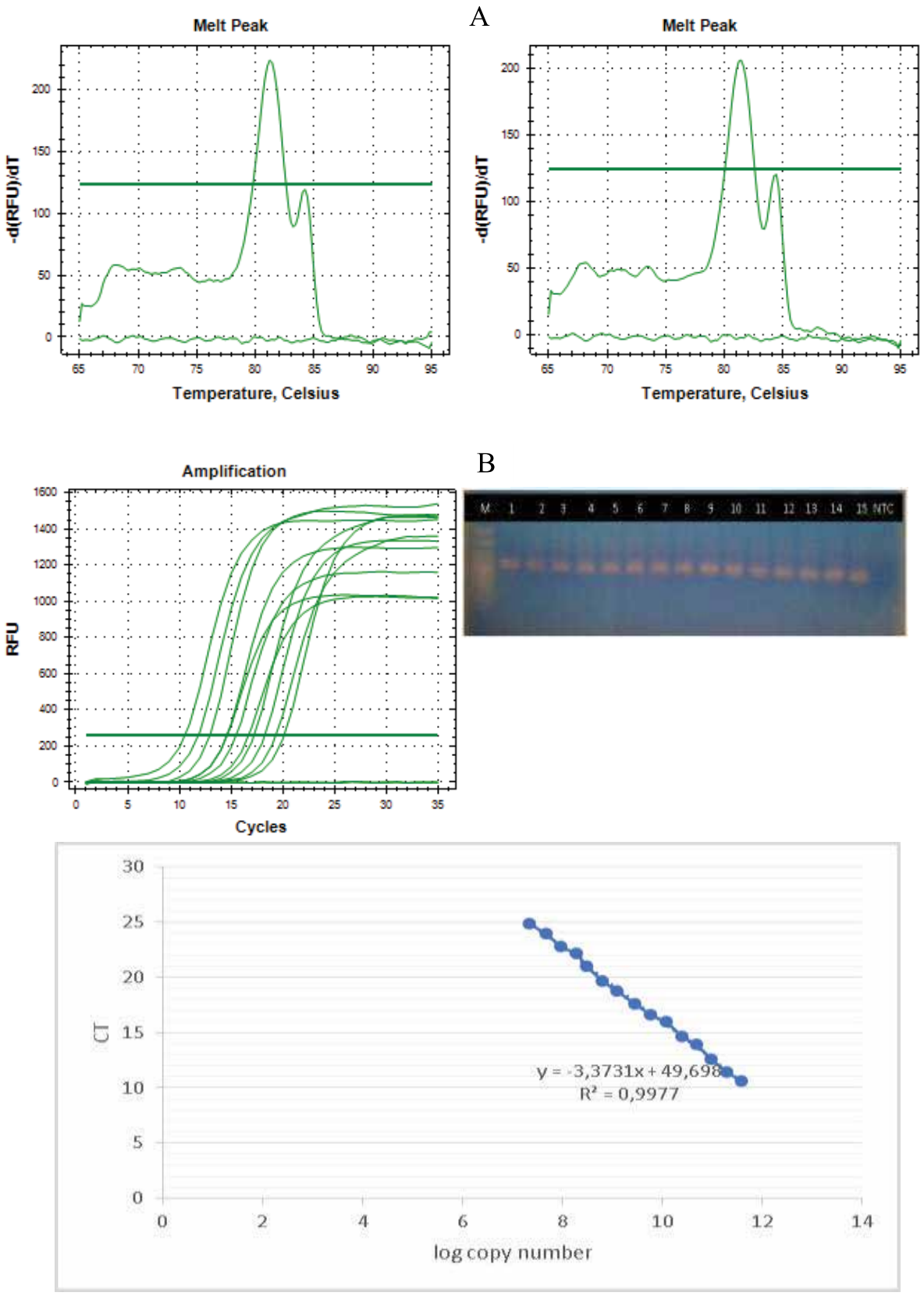

Fig. 1. A. Melting peak of HER2 gene amplification product: pGEM-T easy HER-2 (left) and genomic DNA as template respectively (right); **B.** Serial dilution of standard curve pGEM-T easy HER-2 and agarose confirmation.

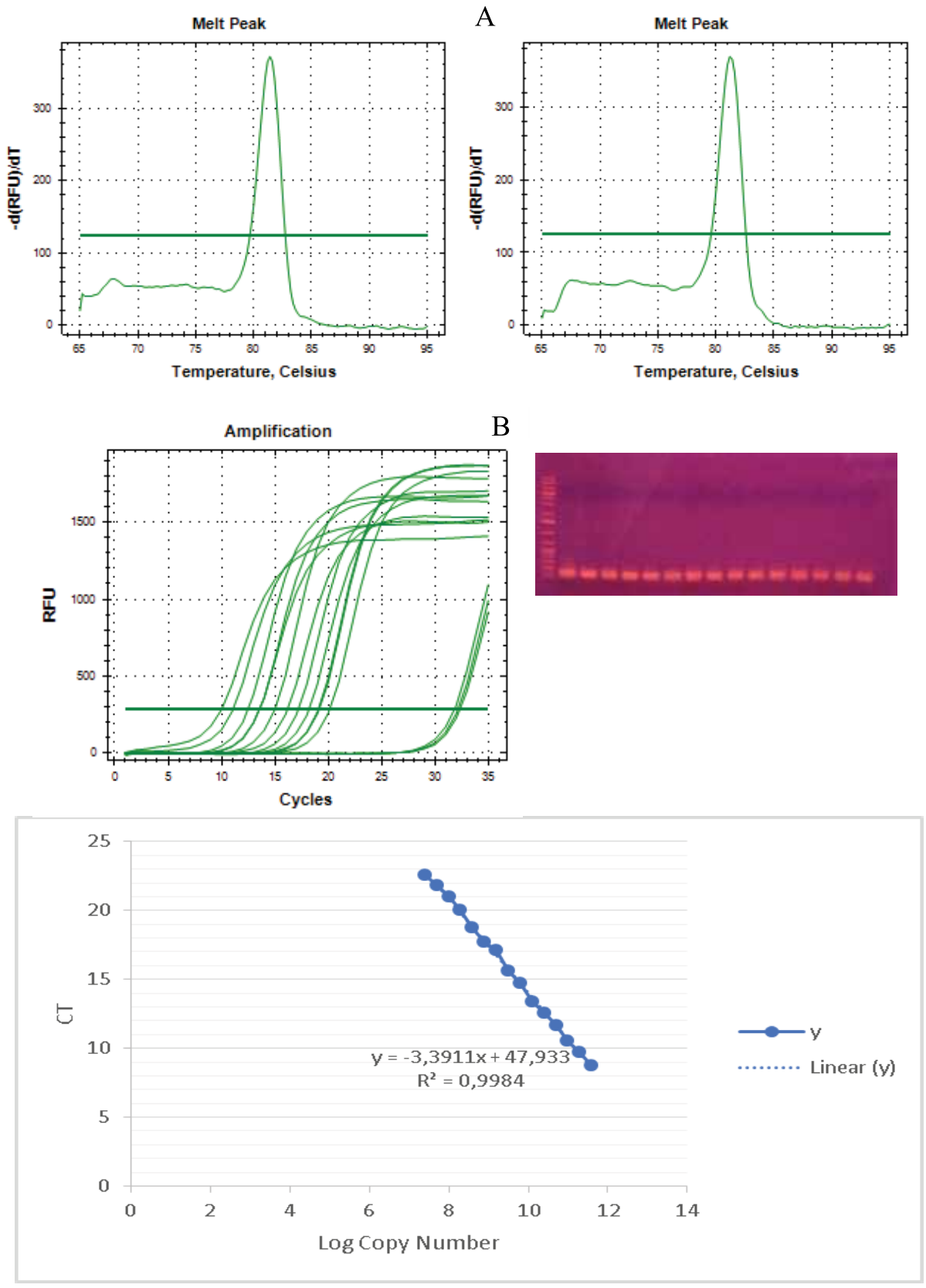

Fig. 2. A. Melting peak of WHN gene amplification product: pGEM-T easy WHN (left) and genomic DNA as template respectively (right); **B.** Serial dilution of standard curve pGEM-T easy WHN and agarose confirmation.

3.3 Comparison between IHC and Quantitative Real-time PCR method

All samples, frozen or FFPE tissues have been evaluated by IHC and scored for their HER-2 immunoreactivity. In frozen tissues, all of samples were scored as +3 by IHC which were also scored as +3 by qPCR, giving 100 % concordance. Eight samples showed discrepancies and most of which were scored +3 by qPCR and exhibited negative result by IHC (data was not shown). While in FFPE tissues, seven samples showed same scoring result between IHC and qPCR, (63 %). Four samples showed discrepancies between IHC and qPCR. Most of samples showed scores at borderline by IHC or conversely. Only one sample showed negative value in IHC results, yet it was +3 in qPCR test (Table 1).

Table 1. HER-2 scoring analysis based on IHC method compared to qPCR method by using FFPE tissue.

No.	ID sample	HER-2 status	
		IHC status	qPCR status
1	376-15	+2	borderline
2	025-15	+2	+3
3	078-14	(-)	(-)
4	808-14	(-)	(-)
5	1042-14	(-)	(-)
6	731-15	+3	+3
7	1292-14	+3	+3
8	0424-15	+3	borderline
9	0934-15	+3	+3
10	451-15	+2	(-)
11	458-15	+3	borderline
12	0045-15	+3	+3
13	0027-14	+1	borderline
14	0007-14	+1	borderline
15	1835-14	+3	+3
16	0392-15	+3	+3
17	1042-14	(-)	+3
18	0938-14	+3	+3
19	0423-14	(-)	borderline
20	1043-14	(-)	borderline
21	0416-14	(-)	borderline
22	0949-14	+1	borderline
23	0456-15	+1	borderline

4. DISCUSSION

In standard curve preparation, the plasmid was prepared as circular form, instead of as the linear one. According to Dhanasekaran et al. [10] circular plasmid was more stable during storage, in contrast with linear plasmid which was easily degraded, affecting the various copy number in qPCR assay. Standardized quality and also method stability were very important to be routinely tested using qPCR [10]. The prepared standard curve serves as the quality control for qPCR reaction. Regarding the curve standard using HER-2 gene fragment insert, its agarose analysis showed the formation of single band, yet an additional non-specific product below the threshold line appeared as shown by the melting peak analysis. This non-specific peak may be caused by the GC content in amplicon. According to Abathi et al. [11], asymmetric GC distribution contributed to the shape of melt peaks. In another hand, WHN curve standard showed single melting peak and sharper. Furthermore, single band of WHN amplicon was confirmed by agarose, confirming an ideal for standard curve in qPCR. The PCR reaction, accuracy and precision of pipetting in this study could be claimed to perform well as proven by standard curve using plasmid as indicated by the R2, efficiency, CV, melting peak shape and agarose confirmation. All of those parameters were important for judging and claiming an ideal qPCR reaction [12]. NTC below the threshold line or consistently showed in the same cycle means that no contamination was found in this standard curve preparation.

IHC is the most widely used method for HER-2 scoring, since it is low in cost, easy and less time consuming. However, IHC method has been reported to have high subjectivity affecting some misevaluation of HER-2 scoring due to variability of standard operating procedure among laboratories. False positive or false negative results for HER-2 scoring determination will increase the

change of cardiotoxicity, will incur high cost and denial from targeted breast cancer patients with trastuzumab therapy [4]. FISH, a gold standard method for HER-2 scoring is expensive and time consuming. In the developing country such Indonesia, FISH is rare to be applied. According to Mendoza et al. [8], qPCR could be as an alternative method for HER-2 scoring since it is shown to be less expensive, less time consuming than FISH and to be more accurate than IHC. By using WHN as the calibrator and FFPE tissue as HER-2 target for quantification, they suggested that qPCR ratios between 2.5 to 5.0 be considered as borderline. In this study, we referred to Mendoza et al. [8], using WHN as the calibrator. As the sample source, we used FFPE tissues and we added frozen sample as another tissue target. The borderline for scoring the HER-2 status in qPCR by using both FFPE tissue and frozen sample were as follows: 2.34 to 4.79 and 2.61 to 4.19, respectively. In the present study, similar and identical results as Mendoza et al. [8] were obtained. This result confirming and validating WHN as a calibrator in determining the cut off value based on our developed qPCR methods for HER-2 scoring. The discrepancy of IHC result with qPCR could be caused by the IHC subjectivity problem affecting the misevaluation scoring. Moreover, an isolation of tumoral cells by microdissection will further improve the qPCR methods in FFPE tissue as the DNA source. While frozen sample, due to heterogeneity problems in frozen tissue, dilution with non-tumoral cells will affect the PCR signals. Furthermore, simplicity and convenience offered by frozen tissue as source of DNA genome give some advantage than frozen tissue as the target for HER-2 scoring determination. This qPCR method could be considered as complement method of IHC test or as additional methods for confirming the equivocal result from IHC test, that suppose improve the outcomes and medical practices.

5. CONCLUSIONS

Standard curve of the developed methods, based on qPCR technique for HER-2 status scoring exhibited a good performance, as indicated by R2 value, efficiency, slope and CV value. It was further confirmed by the melting peak and agarose analysis which mostly exhibited a single band and single peak especially for WHN samples; however, in HER-2 it showed a small unspecific melting peak. Furthermore, the cut off values of qPCR techniques for scoring the HER-2 status suggest that the borderline using FFPE tissue and frozen samples was in the range of 2.34 to 4.79 and 2.61 to 4.19, respectively. The qPCR method could be considered as a complementary test along with IHC for HER-2 status scoring.

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