**BIODIVERSITAS**
Volume 18, Number 3, July 2017
Pages: 1041-1046

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d180323

# Housekeeping gene as a source of calibrator candidate for *HER-2* scoring in frozen tissue breast cancer study based on qPCR

**RISMAYA[1], AZAMRIS[2], SRI BUDIARTI[1], DESRIANI[3,♥]**

[1]Department of Biotechnology, Institut Pertanian Bogor. Jl. Raya Darmaga, Kampus IPB, Bogor 16680, West Java, Indonesia.
[2]Division of Surgical Oncology, Medical School of M. Djamil Hospital, Universitas Andalas. Jl Perintis Kemerdekaan No. 94, Padang 25171, West Sumatra, Indonesia
[3]Research Center for Biotechnology, Indonesian Institute of Sciences. Jl. Raya Jakarta-BogorKm 46, Cibinong, Bogor 16911, West Java, Indonesia. Tel.: +62-21-87907604/87907636, Fax.: +62-21-87907612, ♥email: desr002@lipi.go.id, desrianilipi@gmail.com

Manuscript received: 9 May 2017. Revision accepted: 11 June 2017.

**Abstract.** *Rismaya, Azamris, Budiarti S, Desriani. 2017. Housekeeping gene as a source of calibrator candidate for* HER-2 *scoring in frozen tissue breast cancer study based on qPCR. Biodiversitas 18: 1041-1046. HER-2* amplification in breast cancer gives an implication in therapy and prognosis. FDA have been approved Fluorescence In situ hybridization (FISH) for *HER-2* amplification and immunohistochemistry (IHC) for protein expression measurement. In this recently times, quantitative PCR techniques have been reported as an alternative method for *HER-2* amplification determination. Here in this report, we investigated new reference gene coming from housekeeping gene as an alternative calibrator for *HER-2* amplification scoring based on qPCR methods. We compared two recommended housekeeping gene, *18S rRNA* and β-*Actin*, as a candidate for *a HER-2* calibrator. For methods development, optimization and comparison study between *18S rRNA* and β-*Actin*, we used non-invasive samples (DNA buccal cells). Selected candidate reference further was tested to eighteen breast cancer sample, validated with qPCR methods refer to Mendoza et al. 2013. β-*Actin* is shown as the best candidate for *HER-2* scoring status determination. qPCR concordance result with other qPCR methods refers to Mendoza et al. 2013 shown 94.4%. The qPCR efficiency and % CV value shown as a requirement as a theory. qPCR efficiency was 103.8-105.3% for β-*Actin* and *HER-2* respectively while CV value was around 1.2%. Our result showed a significant correlation with reported methods, which could potentially complement with FDA approved methods.

**Keywords:** Breast cancer, *HER-2*, ERBB2, qPCR, FISH, IHC, housekeeping gene

## INTRODUCTION

Human epidermal growth factor receptor-2 (*HER-2,* official symbol *ERBB2)* is a proto-oncogene. Amplification of *HER-2* has been identified in 20-30% of breast cancer and endometrial cancer, which associated with malignancies, poor clinical outcome, and low survival rate. Status of *HER-2* could predict sensitivity to anthracycline-based chemotherapy regimens, cyclophosphamide-based regimens, tamoxifen therapies, and the most important is to targeted therapy such as trastuzumab and lapatinib in *HER-2* subtype carcinomas (Alaoui-Jamali MA et al. 2015). *HER-2* status could be detected in DNA, mRNA or the protein area. American Society for Clinical Oncology (ASCO) and National Comprehensive Cancer Network (NCCN) guidelines suggest *HER-2* testing for all of the breast cancer patients (Wolf et al. 2014). In this recently times, IHC was reported as the most used methods for *HER-2* scoring status, which FISH as the gold standard detection of *HER-2* amplification. FISH and IHC have been approved by the US Food and Drug Administration (FDA). IHC showed an easy, low-cost methods but shown high subjectivity. Inter-observer variation contributed to the high subjectivity problems in IHC. While FISH reported as sensitive, specific and accurate methods but with high cost and need sophisticated equipment. According to Gjerdrum et al. (2003), in carcinomas, there was a correlation between *HER-2* DNA amplification with *HER-2* protein expression detected respectively by FISH and IHC. Treating patients with breast cancer is expensive and have cardiotoxic risk which observed around 1% to 4% of patients. Accurate identification to determine *HER-2* status in breast cancer is needed, to avoid misinterpretation and mistreatment with trastuzumab. Recently times, quantitative PCR techniques have been reported as an alternative method for *HER-2* determination. In real-time PCR, selection of the reference genes should consider carefully to avoid from incorrect conclusions. Avoiding reference gene located at the chromosome with high frequently altered, specifically because of amplified or deleted problem should consider. According to Kytola et al. (2000), chromosomes 1, 8, 16, 17 and 20 were the most frequently involved in chromosomal alterations. Further, polysomy occurred in chromosome 17 where *HER-2* was located. In contrast with *HER-2* gene amplification, polysomy 17 were no related with high tumor grade or low survival rate. According to Bemt et al. (2008), polysomy 17 were more similar to *HER-2* negative tumors. Polysomy occurred at 13% to 46% in breast cancer. Since there was a polysomic problem in chromosome 17 that potentially contributed to misinterpretation of *HER-2* scoring status, He et al. (2016) recommended reference gene for *HER-2*

scoring calibrator located from another chromosome. In contrast with the statement above, Roche applied science used calibrator for *HER-2* quantification from the same chromosome as *HER-2,* localized on chromosome 17. They have commercialized LightCycler-HER2/neu DNA Quantification Kit since 2001.The concordance result between Roche product and FISH for *HER-2* quantification were in between 92%-100% (Beyser et al. 2001, Benohr et al. 2005; Murad et al. 2013). Further Mendoza et al. (2013), standardized qPCR analysis by using *whn* that localized also on chromosome 17. They reported that qPCR technique shown complementary with IHC analysis and similar to FISH, with concordance value were 60% with IHC methods, and 80-90% with FISH. The *HER-2*/whn ratio will show the value of 1,0 in a polysomic condition.

Liu et al. (2014), have been selected 13 housekeeping genes. Among those 13 housekeeping genes they conclude two genes potentially as best reference genes for normalization of RT-qPCR expression studies in human breast cancer, *18S rRNA* and β-*Actin*. β-*Actin* were located 7p22.1, while *18S rRNA* located 22p12 chromosome. Principally, scoring of *HER-2* based on real-time PCR was imitated FISH methods. *HER-2* scoring in FISH was determined based on the ratio of *HER-2* signals divided with a number of signals second probe to the centromeric portion of chromosome 17 (CEP17). According to ASCO-CAP *HER-2* test guidelines, the ratio of dual probe *HER-2*/*CEP17* for *HER-2* positive tumors is ≥2.0. Roche Molecular Biochemicals (Germany), applied the same cut-off value for *HER-2* scoring based on real-time methods. As already mention above, they choose *gastrin* localized on chromosome 17 as the calibrator (Hillig et al. 2012; Murad et al. 2013). Further, beta actin as a calibrator for *HER-2* copy number has been reported by Andolfo et al (2011), as DNA source were isolated from esophageal carcinoma patient plasma. Here in this activity, we tested and compared housekeeping gene,*18S rRNA* and β-*Actin* gene, potentially as calibrator candidates for *HER-2* scoring in breast cancer frozen tissue. Different from Andolfo et al (2011), we targeted to breast cancer frozen tissue and we considering the amplicon size, maintain the GC content 50-60% refer to Dorak (2006). For reliability data, we refer from MIQE (the Minimum Information for Publication of Quantitative real time PCR) guidelines for methods validations. The cut off for *HER-2* scoring was used the same value as FISH and Roche *HER-2* scoring based qPCR methods. We used DNA buccal cells for method development. Buccal cells reported as a non-invasive sample and acceptable as DNA source to be used in research and clinical applications (Heath et al. 2001).

## MATERIALS AND METHODS

### Samples

DNA samples were obtained from frozen tissue of West Sumatera breast cancer patient. While Buccal cells are isolated from normal women. This research had been approved for the ethical clearance from Health Ministry of Indonesia

### DNA extraction

Total tumor DNA and buccal cell DNA were extracted using the Purelink DNA Genome Kit from Invitrogen. The DNA concentration further was measured at 260/280nm ratio. Purity was confirmed above of 1.7-2.0 value. The DNA samples were separated on 1% agarose for confirming the result.

### Real-time PCR for candidate calibrator of *HER-2* scoring

We used Biorad CFX96 real-time PCR. The master mix of real-time PCR was done following protocol as manufactured determined from TOYOBO SYBR Green real-time PCR master mix #QPK-201. For methods validation, we refer to reported qPCR developed by Mendoza et al. (2013). For optimal and selection of reference candidate, we optimized the annealing temperature as below: denaturation steps 95$^{o}$C for three mins, followed by 35 cycles of initial temperature at 95$^{o}$C for 30 secs, annealing at 57-62$^{o}$C for 30 secs, and melt curve analysis from 65$^{o}$C-95$^{o}$C. The best annealing temperature then is chosen for further annealing temperature. Primer of 18S sRNA (RNA18S5) Fw are 5'-CTCTAGATAACCTCGGGCCG-3', 18S sRNA rev is 5'-TTT CTC AGG CTC CCT CTC CGG A-3'while for beta-actin and *HER-2* primers could contact the corresponding author for further information.

### Data analysis

The calculation of *HER-2* scoring was done followed below equation: Ratio R= $2^{\Delta Ct}$ (Hillig et al. 2012). The Δ is the difference between Ct calibrator gene and *HER-2* gene. The cut-off value was referred to ASCO-CAP guidelines. The reproducibility was tested by calculating the coefficient of variance (CV %).

## RESULTS AND DISCUSSION

### Quality and integrity of DNA sample

At the beginning of research activity, we prepared DNA genome from breast cancer frozen tissue samples and DNA buccal cells (Figure 1). The purity of the DNA samples with absorbance ratios 260/280nm was in between 1,7-2,0, both for DNA genome sample and DNA from buccal cells mean no protein contamination nor RNA. Further, 1% agarose strengthened above result for both DNA extraction result. The 1% agarose showed clearly no RNA in the bottom of agarose area. These indicated that the samples had high quality and suitable for clinically diagnostic development. Instead of FFPE tissue, frozen tissue will produce more high quality of DNA genome and more time-saving preparation compare using FFPE tissue.

For method development, we used non-invasive target buccal cell instead of blood. Utilization of buccal cells as non-invasive sample gives more benefit against blood. The problematic for blood sampling could is probably painful compared with buccal cells sampling. Buccal cells could be obtained as DNA source using mouthwashes, swabs, of whole saliva collection. (Kuchler et al. 2010).

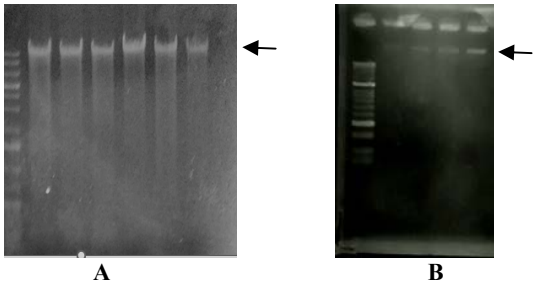



**Figure 1.** DNA extraction running at 1% agarose, (Marker: 1Kb DNA Ladders). A. DNA genome breast cancer sample, B. DNA Buccal cells.

**Amplification specificity and comparison study of two candidate reference genes**

Based on MIQE guideline, specificity of qPCR must be checked to ensure the reliable of developed methods for *HER-2* scoring based on qPCR (Bustin et al. 2009). Melting curve analysis of the PCR products following running to the gel of that product will allow confirming the specificity of the methods. In this activity, we optimize the annealing temperature for the calibrator candidate and also for the *HER-2* gene, in order to get the specific target for each (Figure 2). As already mentioned above, DNA buccal cell was prepared to generate optimal methods. Here in this activity, we can see very nice and sharp melt curve as expected for both β-*Actin* gene and 18S rRNA in melt curve analysis, *HER-2* indicated with blue lines, β-*Actin* gene with red lines, 18S rRNA with black line and NTC with green lines. All of the qPCR product shown as one single band running in 1% agarose (Figure 3). Primer specificity is one of important thing avoiding from qPCR misinterpretation result. There was no primer dimer exist in the qPCR result. The appearance of primer dimer will show in melt curve analysis around 70°C melting temperature. This indicated the developed method already have high specificity. Based on annealing temperature optimization we chosen 60,2°C with have most specific melt curve compared with other annealing temperature. In Figure 2, β-*Actin* gene (*ACTB*) with located on chromosome number 7 (p22.1 region) shown overlapping Ct valued with the *HER-2* gene as predicted since we used normal DNA sample, while 18SrRNA was shown distance Ct value. Principally, *HER-2* scoring in this developed method is determined based on Ct ratio between *HER-2* and calibrator gene. We targeted, no distant Ct value between *HER-2* and candidate calibrator for normal sample determination, and Ct distant exist in breast cancer positive *HER-2*. With this strategy will facilitate *HER-2* scoring be easier and simply. Since there is existence of distant between *HER-2* gene Ct value with 18S rRNA in a normal sample, this showing us that 18S rRNA was not suitable as a reference gene for *HER-2* scoring. Further, we used β-*Actin* as a calibrator for *HER-2* scoring with 60,2°C annealing temperature

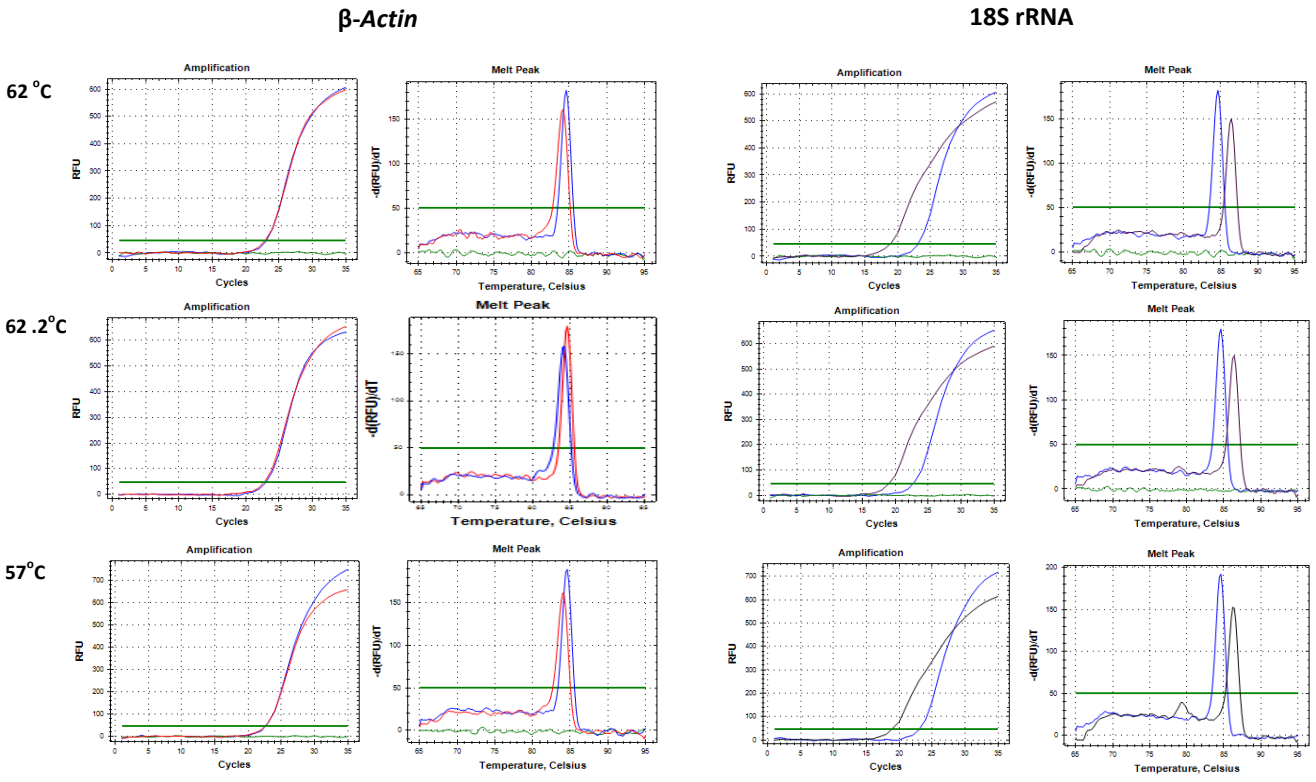



**Figure 2.** Optimization of annealing temperature for β-*Actin* and 18S rRNA as a candidate for calibrator gene of *HER-2* scoring analyzed based on Ct value and melt curve analysis. (*HER-2* indicated with blue lines, β-*Actin* gene with red lines, 18S rRNA with black line and NTC with green lines)

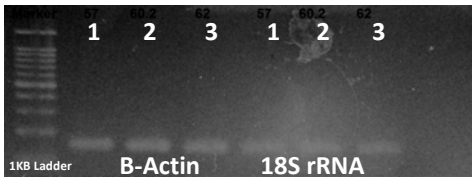



**Figure 3.** qPCR optimization of annealing temperature confirming in 1% agarose. 1. 62°C; 2. 60,2°C; 3. 57°C

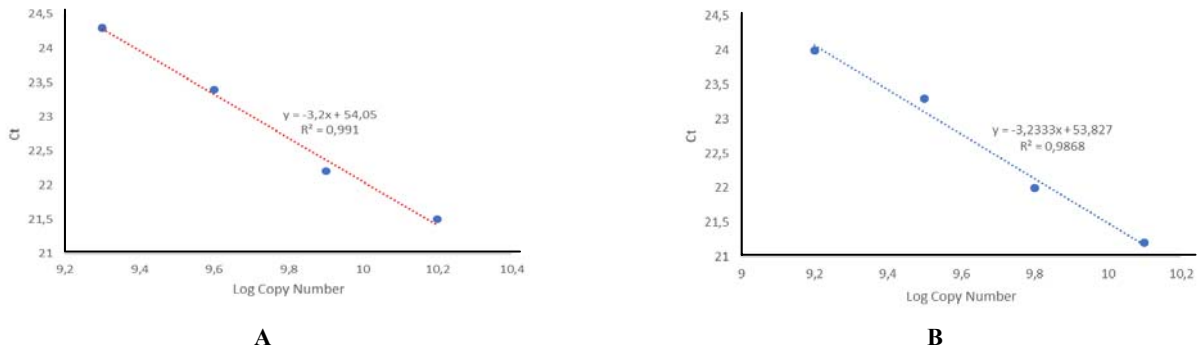
For the efficiency test for both *HER-2* and Beta-actin primers, pairs fulfilled the requirement amplification efficiencies (within the range of 90-110%). qPCR efficiency was 103.8-105.3% for β-*Actin* and *HER-2* respectively (Figure 4). The CV value were around 1.2%, which below 10% as the requirement mean no pipetting error and contaminations. This demonstrated that the developed methods here shown high efficiencies and specificities. (Dorak 2006; Bustin et al. 2009).

**Beta-actin as candidate of reference gene for *HER-2* scoring**

Based on above optimization result, we test β-*Actin* as the reference gene for *HER-2* scoring. In this activity, for methods validation, we refer to Mendoza et al. (2013), who developed qPCR for *HER-2* scoring based on *HER-2*/whn ratio. According to Mendoza et al. (2013), FISH and qPCR techniques shown an equivalence of 80-90%, while IHC and qPCR showed concordance around 60%. Further, discrepancies between IHC with FISH and qPCR were reported around 20-40% (Konigshoff et al. 2003; Millson et al. 2003; Ntoulia et al. 2006). Interestingly, Pu et al. 2015 reported that qPCR perform superior to FISH.

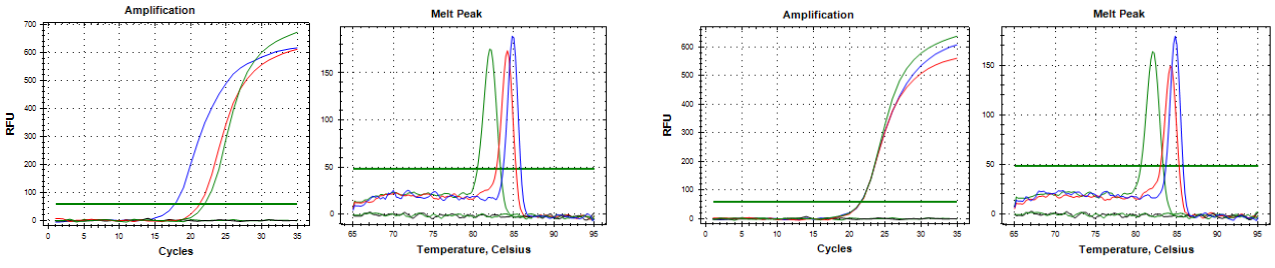
In our previous activity, we used whn gene as a calibrator for *HER-2* scoring refer to Mendoza et al. 2013. We got a similar trend of concordance value with IHC as Mendoza et al. (2013 did, 60%. We also have the similar cut-off value as Mendoza for negative, borderline and positive *HER-2*. This confirmed and shown us the high reproducibility of developed method by Mendoza at al..2013. Here In this activity, due to high cost of FISH and high subjectivity of IHC analysis, we validated our novel calibrator candidate with qPCR whn/*HER-2* refer to Mendoza et al. 2013. From eighteen sample, three sample shown as *HER-2* positive

In Figure 5 we can see Ct value for amplified and no amplified *HER-2* gene both using β-actin and whn as calibrator gene. Based on melt curve analysis, shown as one single peak. Confirming with 1% agarose also shown as a single band, whereas the band for NTC was not existed (Figure 6).




**A** **B**

**Figure 4.** Standard curve for Beta-actin (A) and *HER-2* (B)




**A** **B**

**Figure 5.** Amplification plots of *HER-2*. A. Non-amplification of *HER-2*, B. Amplification of *HER-2*. (*HER-2* indicated with blue lines, β-*Actin* gene with red lines, 18S rRNA with black line and NTC with green lines)

For the cut-off determination were referred to ASCO-CAP, ratio ≥2 determined as positive *HER-2*, while <2 as negative *HER-2* (Wolf et al. 2014). The ratio of *HER-2*/ beta-actin in this activity was ranged between 0,47-9,25 (Figure 7), the ratio of *HER-2*/whn was ranged between 0,53-14,22. With this qPCR approach, 17-22% showed as *HER-2* positive breast cancer. This percentage is similar as reported *HER-2* subtype breast cancer incidence in between 10-30%. (Kim et al. 2002).

Among eighteen samples, four sample detected as positive *HER-2* with qPCR *HER-2*/ β-*Actin* approach (Sample A, B, D, I), and three samples as positive *HER-2* with qPCR *HER-2*/ whn approach (sample A, D, I). It means the agreement qPCR *HER-2*/ β-*Actin* and qPCR HER2/whn is 94%. We repeat sample B measurement one more time to confirm the result. As a result, the sample shown consistency as positive with *β-Actin* as a calibrator, negative with *whn* as calibrator (Figure 8).

For next work activity, the dubious sample needs to be confirmed with FISH. Furthermore, for more accurate and reliable analysis, more samples are needed to be tested and compared with IHC and FISH. Not only applied in esophageal carcinoma patient plasma (Andolfo et al. (2011), in this activity *β-Actin* also confirmed potentially applied to breast cancer frozen tissue for *HER-2* scoring methods. The assessment of *HER-2* status is critical for disease management, therefore, accurate and reliable methods are needed. As conclusions, due of high concordance among the two qPCR methods, its mean *β-Actin* potentially used as candidate calibrator for *HER-2* scoring. The qPCR technique, shown faster than IHC, massive, less expensive and promising as an alternative for *HER-2* scoring methods.

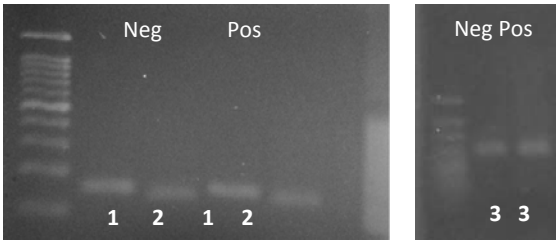



**Figure 6.** Confirmation of qPCR result from non-amplification (-) and amplification *HER-2* )+) on 1% agarose. (1. Beta actin: 156bp, 2. *HER-2*: 142bp, 3 whn : 93bp)

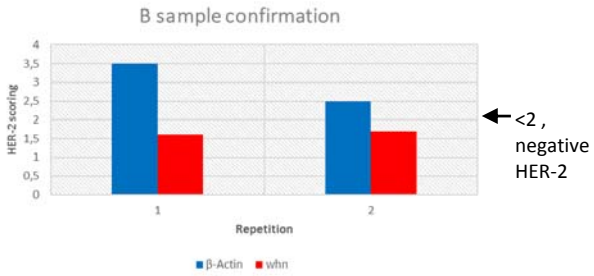



**Figure 8.** Repeating B sample to confirm its *HER-2* scoring status

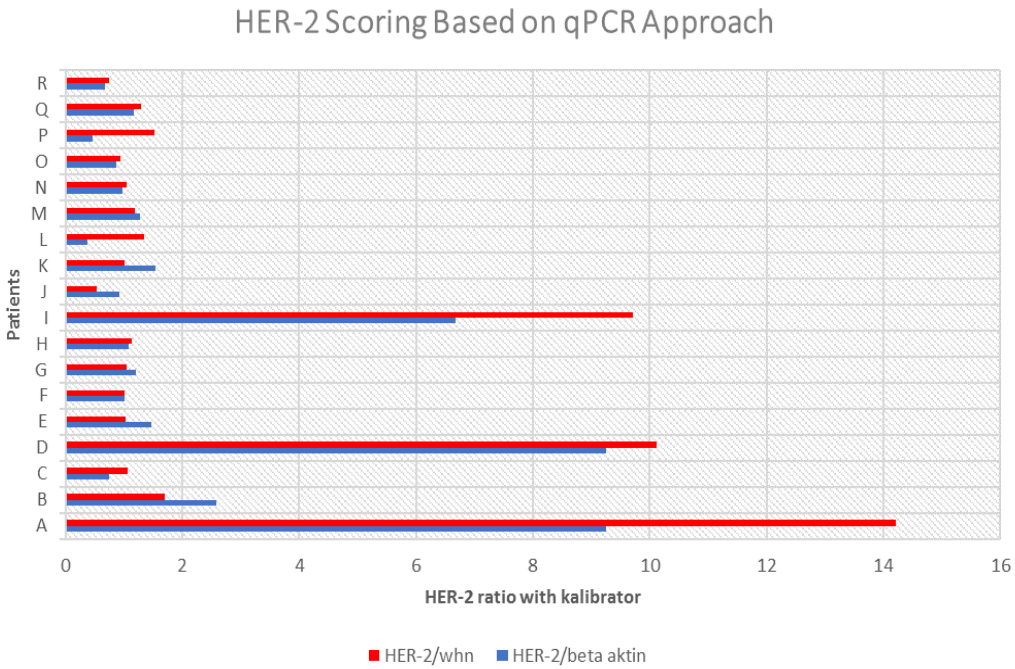



**Figure 7.** Scoring *HER-2* with recently developed methods often breast sample that already checked for their IHC status

## ACKNOWLEDGEMENTS

We thanks to the research funding Insinas of the Indonesian Ministry of Research, Technology and Higher Education to Dr. Eng. Desriani (P.I.). We would also like to thanks, Budi Ratno Budiarto and M. Ali Warisman.

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