การศึกษาเปรียบเทียบการแสดงออกของเฮอร์ทูอิมมูโนฮิสโตเคมีในมะเร็งเต้านมโดยการตรวจวิเคราะห์ด้วยภาพดิจิตอล และการตรวจประเมินโดยดูจากกล้องจุลทรรศน์

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Comparison between digital image analysis and visual assessment of immunohistochemical HER2 expression in breast cancer

นายมอร์เท็น ราเก็น ยาคอบเซ็น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเวชศาสตร์คลินิก
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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Thesis Title	การศึกษาเปรียบเทียบการแสดงออกของเฮอร์ทูอิมมูโนฮิสโตเคมีในมะเร็งเต้านมโดยการตรวจวิเคราะห์ด้วยภาพดิจิตอล และการตรวจประเมินโดยดูจากกล้องจุลทรรศน์
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มอร์เท็น ราเก็น ยาคอบเซ็น : Comparison between digital image analysis and visual assessment of immunohistochemical HER2 expression in breast cancer (การศึกษาเปรียบเทียบการแสดงออกของเฮอร์ทูอิมมูโนฮิสโตเคมีในมะเร็งเต้านมโดยการตรวจวิเคราะห์ด้วยภาพดิจิตอล และการตรวจประเมินโดยดูจากกล้องจุลทรรศน์) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สมบูรณ์ คีลาวัฒน์, 105 หน้า.

ภูมิหลัง: การตรวจหาความผิดปกติของยีน HER2 ในมะเร็งเต้านมเป็นวิธีการมาตรฐานในทางพยาธิวิทยาที่ใช้ในการพยากรณ์โรคและการวางแผนการรักษา การตรวจความผิดปกติของยีนนี้มีขั้นตอนหลักๆอยู่สองขั้นตอน คือ 1. การตรวจ ด้วยวิธี immunohistochemistry ซึ่งเป็นวิธีในการตรวจหา HER2 protein และ 2. การตรวจด้วยเทคนิคทาง in-situ hybridization เพื่อดูว่าเนื้องอกมี HER2 amplification หรือไม่ ซึ่งกรณีหลังนี้จะตรวจก็ต่อเมื่อการตรวจด้วย immunohistochemistry ให้ผลไม่ชัดเจน (equivocal result).

วิธีการ: ผู้วิจัยได้นำชิ้นเนื้อของมะเร็งเต้านมซึ่งเป็นชิ้นเนื้อของคนไข้ที่มารักษาในโรงพยาบาลจุฬาลงกรณ์ จำนวน 109 ราย จากภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ซึ่งทุกรายจะมีผลรายงานทางพยาธิวิทยา และผลการอ่านระดับการแสดงออกของยีน HER2 โดยพยาธิแพทย์ผู้รับผิดชอบ มาศึกษาเปรียบเทียบผลการอ่านด้วยวิธีการต่างๆ 3 วิธี ได้แก่ 1. การอ่านโดยพยาธิแพทย์ 1 ท่าน (ใช้ผลเดิมที่อยู่ในรายงาน) 2. การอ่านด้วยวิธี digital image analysis (DIA) โดยใช้ software ที่ชื่อว่า Aperio Imagescope เป็นเครื่องมือในการวิเคราะห์ และ 3. การอ่านโดยพยาธิแพทย์ 3 ท่านซึ่งผลการอ่านต้องได้รับความเห็นพ้องของพยาธิแพทย์สองในสามเสียงเป็นอย่างน้อย ผลการอ่านทั้งสามวิธีจะมีการนำไปเปรียบเทียบกับผลการตรวจทางโมเลกุลด้วย คือ dual in-situ hybridization (DISH).

ผลการศึกษา: การอ่านทั้ง 3 วิธี ให้ผลค่อนข้างสอดคล้องไปในทิศทางเดียวกัน โดยผลการอ่านด้วยพยาธิแพทย์ 1 ท่าน และพยาธิแพทย์ 3 ท่านได้ค่า weighted kappa coefficients อยู่ที่ 0.79 ส่วนการอ่านโดยพยาธิแพทย์ 1 ท่านและการอ่านด้วยวิธี digital image analysis (DIA) ได้ค่า weighted kappa coefficients อยู่ที่ 0.71 เมื่อนำวิธีการทั้งสามนี้ไปเปรียบเทียบผลกับ DISH จะได้ค่า weighted kappa coefficients ดังนี้ คือ 0.56 (เปรียบเทียบการอ่านโดยพยาธิแพทย์ 1 ท่าน และ DISH), 0.59 (เปรียบเทียบการอ่านโดยพยาธิแพทย์ 3 ท่าน และ DISH) และ 0.78 (เปรียบเทียบการอ่านโดยวิธี DIA และ DISH) ไม่พบว่ามีผลลบเทียมเลยจากการอ่านด้วยวิธีการทั้ง 3 แบบนี้ ส่วนผลบวกเทียมพบได้แต่ไม่สูง คือแค่ร้อยละ 0.9-2.8 เท่านั้น สัดส่วนของผลการอ่านที่ไม่ชัดเจน (equivocal หรือ 2+) ของการอ่านทั้งสามชนิด มีดังนี้ 1. การอ่านโดยพยาธิแพทย์ 1 ท่านมีสัดส่วนถึงร้อยละ 44 และ 2. การอ่านโดยพยาธิแพทย์ 3 ท่านมีสัดส่วนร้อยละ 33.3 และ 3. การอ่านโดย วิธี DIA มีสัดส่วนของ equivocal cases แค่ร้อยละ 14.7 เท่านั้น หรืออาจกล่าวได้ว่าการใช้ DIA ช่วยอ่านผล HER2 ช่วยลดจำนวน equivocal cases ลงไปถึงร้อยละ 67 ทีเดียว โดยจำนวนผลลบเทียมไม่ได้เพิ่มขึ้นแต่อย่างใด

สรุป: การอ่าน HER2 immunohistochemistry โดยวิธี DIA จะช่วยลดจำนวนผลการอ่านที่เป็น equivocal ลงได้ โดยไม่มีผลกระทบใดๆต่อความไวของการอ่าน

สาขาวิชา เวชศาสตร์คลินิก ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5974656130 : MAJOR CLINICAL SCIENCES

KEYWORDS: BREAST CANCER / HER-2 / DIGITAL IMAGE ANALYSIS / DUAL IN SITU HYBRIDIZATION

MORTEN RAGN JAKOBSEN: การศึกษาเปรียบเทียบการแสดงออกของเฮอร์ทูอิมมูโนฮิสโตเคมีในมะเร็งเต้านมโดยการตรวจวิเคราะห์ด้วยภาพดิจิตอล และการตรวจประเมินโดยดูจากกล้องจุลทรรศน์. ADVISOR: ASSOC. PROF. SOMBOON KEELAWAT, 105 pp.

Background: Assessment of HER2 status is considered standard of care in the histopathologic workup of breast cancer and conveys prognostic and predictive information used to guide treatment decisions. The assessment is often carried out in a two-step approach where immunohistochemical expression of HER2 protein is first evaluated by conventional microscopy and equivocal cases are further analyzed by in-situ hybridization techniques to assess gene amplification status.

Methods: In this study we compared conventional manual assessment of immunohistochemical HER2 expression with digital image analysis (DIA) and consensus manual assessment by a panel of three pathologists. From our archive we retrieved sections of 109 breast carcinomas stained for HER2 with corresponding HER2 score from the original pathology report. The glass slides were assessed by three pathologists to reach a consensus score. Next, the slides were scanned into whole slide images and DIA was performed using Aperio Imagescope. The scoring results were then compared with gene amplification status evaluated by dual in-situ hybridization (DISH).

Results: Comparing manual assessment with consensus assessment and DIA, good agreement was obtained with weighted kappa coefficients of 0.79 (manual vs. consensus) and 0.71 (manual vs. DIA). When compared with gene status assessment by DISH, agreement analysis yielded weighted kappa coefficients of 0.56 (manual vs. DISH), 0.59 (consensus vs. DISH) and 0.78 (DIA vs. DISH). There were no false negatives by any of the three methods and false positives ranging from 0.9 - 2.8%. The proportion of equivocal cases by each method was 44% (manual), 33.3% (consensus) and 14.7% (DIA). Application of DIA reduced the number of equivocal cases by 67% without increasing the proportion of false negatives.

Conclusion: We conclude that DIA is an accurate method to reduce the number of HER2 equivocal cases without affecting the sensitivity of the HER2 assessment.

Field of Study: Clinical Sciences Student's Signature .....

Academic Year: 2017 Advisor's Signature .....

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Abbreviations

ASCO/CAP	American Society of Clinical Oncology / College of American Pathologists
Consensus assessment	Here: The consensus visual assessment of immunohistochemical HER2 expression by a panel of three pathologists using conventional light microscopy
DIA	Digital image analysis
DISH	Dual in-situ hybridization
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in-situ hybridization
HER2	The HER2/*neu* gene or protein
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry / immunohistochemical
ILC	Invasive lobular carcinoma
KCMH	King Chulalongkorn Memorial Hospital
Manual assessment	Here: Visual assessment of immunohistochemical HER2 expression by a pathologist using conventional light microscopy
NPV	Negative predictive value
PPV	Positive predictive value
ROI	Region of interest
WSI	Whole slide image

1 Introduction

1.1 Background

Assessment of hormone receptor status, Ki67 labeling index and HER2 status is considered standard of care in the histopathological diagnostic workup of breast cancer [1]. These individual biomarkers, and their combination, reflect biological properties of individual breast carcinomas and convey important prognostic and predictive information used to guide decisions concerning adjuvant treatment. This is becoming increasingly important with the advent of stratified medicine and targeted therapy modalities. More recently, these markers also serve as surrogate biomarkers in molecular subtyping of breast cancers.

The HER2 gene is a proto-oncogene located on the long arm of chromosome 17 (17q12). It belongs to the epidermal growth factor receptor family whose gene product is a membrane-bound tyrosine kinase receptor involved in cellular growth signaling and proliferation. HER2 gene amplification is closely linked to overexpression of the HER2 protein, which is detected in approximately 15-20% of invasive breast cancers. These “HER2-positive” cancers are traditionally associated with aggressive biological behavior and poor prognosis, but the introduction of targeted anti-HER2 therapy (eg. Trastuzumab) as adjuvant or neoadjuvant treatment has mitigated this deleterious effect. However, the treatment is costly and carries a risk of cardiotoxicity among other adverse effects. An accurate and reproducible

assessment of HER2 status in breast cancer is therefore of paramount importance because of the substantial clinical, economic and safety implications of anti-HER2 therapy [2].

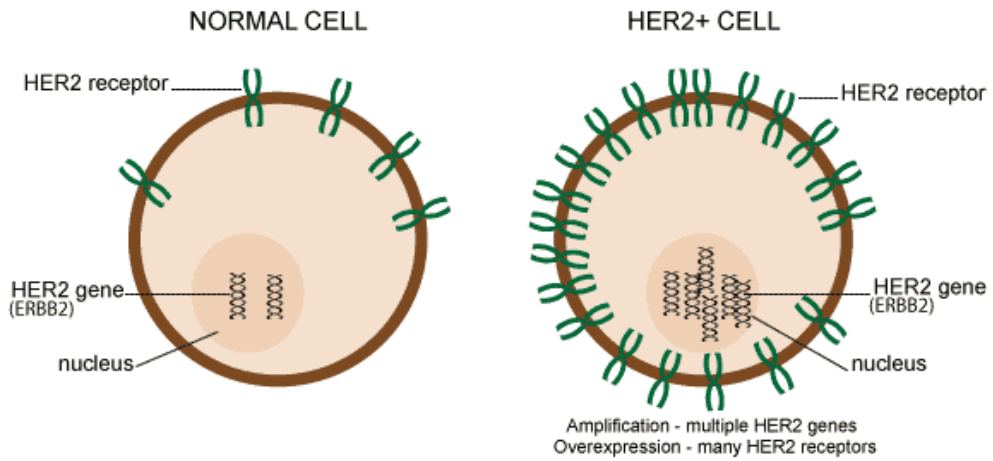

Figure 1: HER2 expression in normal and cancer cells (3)

HER2 status in breast cancer can be determined by assessing either HER2 protein expression by immunohistochemisty (IHC), or by quantification of the nuclear HER2 genes in cancer cells by in-situ hybridization techniques. Fluorescence in-situ hybridization (FISH) is considered the gold standard, but compared to IHC, the FISH technique has a higher failure rate, longer testing time, longer interpretation time and significantly higher costs [2, 3]. A two-step approach is therefore adopted in many centers (and recommended by ASCO/CAP) in which the HER2 status is first evaluated by IHC, and equivocal cases resolved by reflex in-situ hybridization techniques [1, 4]. As seen in the commonly utilized test algorithm below, a patient with a HER2 negative breast cancer (IHC result 0/1+) will not be considered for anti-HER2 treatment, whereas patients with HER2 positive cancers (IHC results 3+) will be

offered anti-HER2 treatment. Equivocal cases (2+) will undergo further testing with other test modalities before a definitive HER2 status is concluded.

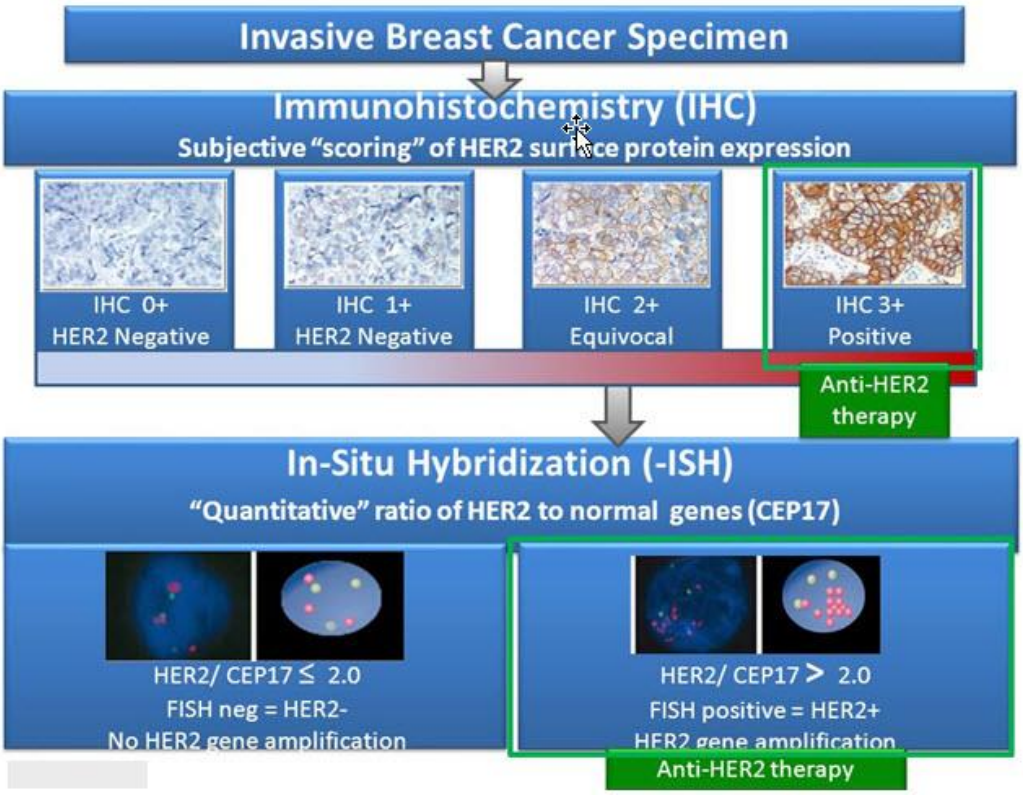

Figure 2: HER2 evaluation algorithm

Due to the significant clinical effect of anti-HER2 treatment in HER2 positive patients, methods that could decrease the number of false negatives would confer substantial therapeutic gains. On a similar note, increased accuracy with reduction of the proportion of IHC borderline cases (2+) could potentially reduce the need for expensive ISH testing. One method to reduce intra- and interobserver variability is by using digital image analysis (DIA), which is now recommended in current ASCO/CAP guidelines [5]. The potential advantages include improved accuracy, objectivity and

reproducibility. According to a 2015 CAP survey, approximately 33% of laboratories in the United States routinely use digital image analysis for HER2 IHC assessment [6].

1.2 Rationale

In our center (Department of pathology, King Chulalongkorn Memorial Hospital), HER2 status in breast cancer is evaluated in a similar two-step approach, using dual in-situ hybridization (DISH) to resolve equivocal cases. Patients with equivocal IHC HER2 expression are offered additional dual in-situ hybridization (DISH) analysis at a cost of 10,100 THB. Due to local health insurance policies (conditions for reimbursement), patients with HER2 IHC 3+ also require confirmatory DISH analysis performed in our center.

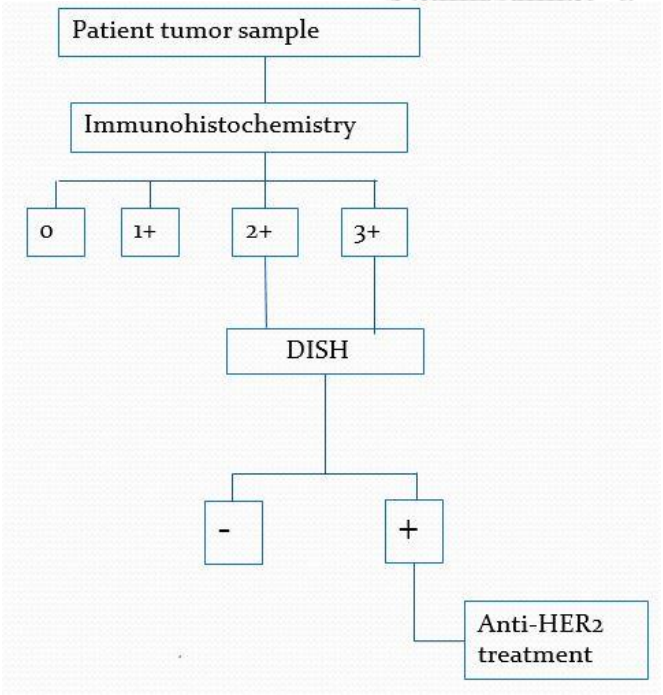

Figure 3: Current HER2 assessment algorithm at KCMH

A quick search in the hospital database (KCMH) for the period 1/1 - 30/4 2018 revealed 108 breast cancer cases with corresponding results of immunohistochemical HER2 study. Of 108 cases, approximately 44% were IHC

negative, 27% were IHC equivocal and 29% were IHC positive. Of the equivocal cases subjected to supplementary DISH, approximately 90% came out negative for amplification.

As shown by these numbers, there is a high proportion of equivocal cases, most of which are HER2 non-amplified. A method capable of reducing the proportion of equivocal cases would carry significant cost savings, potentially reduce the work load and possibly shorten the average turnaround time per case. To this end, we introduce two new modalities for HER2 evaluation in this study: DIA and consensus manual assessment by a panel of three pathologists. Based on published results from other centers (cf. Literature review), we hypothesize that DIA can improve the accuracy of IHC HER2 expression assessment in breast cancer, when compared to conventional assessment by one pathologist. We also seek to explore whether consensus manual assessment by three pathologists - in contrast to the conventional assessment by one pathologist - can improve the accuracy of IHC HER2 expression assessment.

This study is designed as a retrospective analysis in an unselected cohort of archived breast carcinomas in order to make the results applicable in our routine breast pathology practice. In the first part of the study, we compare conventional manual microscopy with consensus manual microscopy and DIA of IHC HER2 expression in breast cancer to determine the agreement between these three methods. In the following part, we correlate each of the three methods with results obtained by dual in-situ hybridization (DISH), which is chosen as our gold standard.

2 Objective

This study has two primary objectives:

- To determine the concordance between assessment of IHC HER2 expression in breast cancer by manual microscopy, manual consensus microscopy and DIA.
- To determine the concordance between assessment of HER2 status by DISH and assessment by manual microscopy, manual consensus microscopy and DIA.

It is the intention to contribute to the validation of DIA in the routine assessment of HER2 status in breast cancer in our department and thereby pave the way for implementation into our daily routine workflow. By doing so, we might be able to reduce the turnaround time and cut costs related to resolving immunohistochemically equivocal HER2 cases.

3 Literature review

3.1 Reproducibility and accuracy of manual HER2 assessment

HER2 status assessment by conventional immunohistochemical methods implies visual semi-quantitiative assessment of chromogenic IHC expression by a pathologist. This assessment is inherently subjective and prone to error, even when carried out by experienced pathologists, which negatively affects the accuracy and reproducibility of the method. Layfield et al. demonstrated an absolute interobserver agreement (of manual microscopy) ranging from 69% to 85% and agreement with

FISH ranging from 35%-54% explaining this with differences in experience, training and the antibody clone used [7]. Differences between laboratories, including pre-analytical, analytical and post-analytical factors have in several studies been shown to be substantial. Reports on IHC HER2 test performance in the years following introduction of anti-HER2 treatment demonstrated poor reproducibility between laboratories [8, 9] and false positive rates up to 18% when compared with FISH [10]. However, significant advances in standardization of pre-analytical factors, scoring and interpretation seem to have improved the test performance with time [11]. One recent study reported false positive rates at 1.3% and false negative rates at 0.7% when retesting with TMA centrally [12]. Another large study found a false positive rate of approximately 7% and a false negative rate of approximately 1% in recent years [6].

The proportion of equivocal cases reported typically range from 14-35% of cases [6, 13, 14]. It should be noted that the 2007 ASCO/CAP guidelines for HER2 assessment were revised in 2013, yielding a broader equivocal group and a larger positive group, which accounts for some of the historical changes in test performance [15].

3.2 HER2 assessment by DIA

The challenges with interrater and intermodality agreement have been mentioned in the previous section. As a means to overcome these, several studies have investigated the feasibility of applying DIA in the field of breast pathology. This

method offers the theoretical advantage of fully quantitative analysis in contrast to the semi-quantitative analysis performed by most pathologists (“eye-balling”). It is also safe to assume that compared to the human eye, DIA is better at discerning subtle differences in staining intensity. But even though DIA is now being widely implemented world-wide, no international guidelines exist concerning standardization and validation of digital techniques. Technical standardization remains an issue, and one fundamental concern to be addressed is the validity and reproducibility compared to conventional microscopy which is still considered the gold standard for assessment of IHC HER2 expression. For these reasons, assessment of HER2 status by DIA has been studied extensively, and many studies have shown results consistent with visual scoring [16-20] and FISH [13, 19, 20]. A number of studies [13, 14, 16, 21] conclude that digital image analysis reduces the need for reflex FISH analysis by lowering the number of equivocal cases (2+). In one recent study assessing the concordance with PAM50 gene expression assays, DIA was found to outperform manual microscopy of biomarkers in breast cancer [22].

Nazzar et al. conducted a study (n=180) comparing DIA (Aperio IA system) with manual microscopy in tumor sections stained with two different antibodies. They concluded that DIA was substantially equivalent to manual microscopy and that DIA improved the interpathologist agreement [23].

Holten-Rossing et al. compared manual reading with DIA of HER2 expression in tissue microarrays (TMA) of an unselected population of breast cancer using the HER2-

CONNECT algorithm (Visiopharm, Denmark). The results were compared with the HER2 gene amplification status obtained by FISH and showed a 68% reduction in equivocal cases with only 0.4% false negatives and 1.5% false positives [13].

In a study of 750 breast carcinomas, Helin et al. compared manual microscopy with DIA of HER2 expression in an unselected population of breast cancers using the web-based ImmunoMembrane (Institute of Biomedical Technology, University of Tampere, Finland). When compared with manual microscopy, the DIA resulted in a 70.3% reduction of equivocal cases with only 0.8% false negatives and 0.8 % false positives [14].

Dobson et al. also compared manual microscopy with DIA using SlidePath Tissue IA (Leica Microsystems) and found a moderate reduction in equivocal cases (21.7%) and equivalent proportions of false negatives and false positives [20]. Table 1 summarizes these three studies.

Table 1: Summary of relevant studies

		Manual			**DIA**			Reduction of equivocals
Study	Cases	False +	False -	% equivocal	False +	False -	% equivocal	
HO Helin et al. (2015) Immunomembrane (University of Tampere)	750	0	0	34.0	0.8	0.8	10.1	70.3%

H Holten-Rossing et al. (2015) HER2-CONNECT (Visiopharm)	904			14.0	1.5	0.3	4.5	67.9%
L Dobson et al. (2010) SlidePath (Leica Microsystems)	136	0.7%	5.1 %	23.5%	0	4.4 %	18.4%	21.7%

Even though automated DIA may intuitively be expected to produce more consistent and reproducible results than a pathologist performing conventional manual assessment, the DIA entails several sources of error. Some of these are pre-analytical errors relating to the immunostaining (time of fixation, staining protocol, antibodies, chemicals, etc.) affecting both manual assessment and DIA alike. Other sources of error relate specifically to DIA and include any factor affecting image properties or algorithm performance. The former includes parameters relating to image acquisition (eg. image scanner, scanning resolution, objective and illumination). Some algorithms (eg. Aperio Imagescope) are adjustable, which allows for optimization of performance, but also potentially reduces the interlaboratory reproducibility. This was the topic of investigation in a study by T Keay et al., who compared HER2 scores obtained by using different WSI systems and algorithms with a panel of expert pathologists. Different combinations of scanner and algorithms were shown to significantly impact the HER2 score results [24].

3.2.1 Aperio Imagescope

Several digital image analysis algorithms have been developed to assess HER2 status in breast cancer in both research and clinical settings. One of these is Aperio Imagescope, which is a digital image analysis platform developed to perform quantitative analysis of digital slides. In this project we use the Aperio Membrane v9 (version 9.1) algorithm developed specifically for digital quantification of membrane staining. This algorithm has received FDA clearance on diagnostic use together with DAKO HercepTest assay, but not with the Ventana Pathway HER2 (4B5) assay used in our center.

The Aperio membrane algorithm detects membrane staining of individual cells within manually selected regions of a virtual slide. Both intensity and completeness of the immunohistochemical membrane staining is quantified, and each cell within the selected area is categorized as 0, 1+, 2+ or 3+ in accordance with IHC HER2 scoring guidelines. Based on the proportion of each cell score category, a resultant slide score of 0, 1+, 2+ or 3+ is calculated (33). The Aperio membrane algorithm is tunable, which allows adjustment to local staining and image acquisition characteristics.

3.3 Dual in-situ hybridization (DISH)

In our center (and in this study) we use dual in-situ hybridization (DISH) as the gold standard to resolve equivocal cases. The HER2 DISH assay is a molecular technique

to assess HER2 amplification status in cancer cells by quantifying the average number of HER2 gene copies and centromere 17 (CEN17) per cell.

The DISH technique has been validated in several studies showing good agreement between DISH and FISH analysis of HER2 gene status, making it a reasonable alternative to FISH [25-27]. DISH testing was abstained from in cases consistently assessed as negative by all modalities (manual, all three consensus pathologists, DIA). This approach is supported by several studies reporting very low frequencies of false negatives by IHC. Thus, Helin et al. reported 0.5% false negatives in a set of 750 cases [14], and the study of Dekker et al. demonstrated 0.7% false negatives in a series of 1008 cases [12].

4 Method

The Chulalongkorn University Institutional Review Board has approved the study (IRB No. 112/61).

4.1 Study design

The study was designed as a retrospective method agreement analysis (case-control study design) and a diagnostic test evaluation. For the method agreement analysis, three datasets were collected (manual score from original pathology report, consensus manual assessment and digital image analysis). These three methods were

all based on analysis of the same original HER2 stained slides, as depicted in the conceptual framework below.

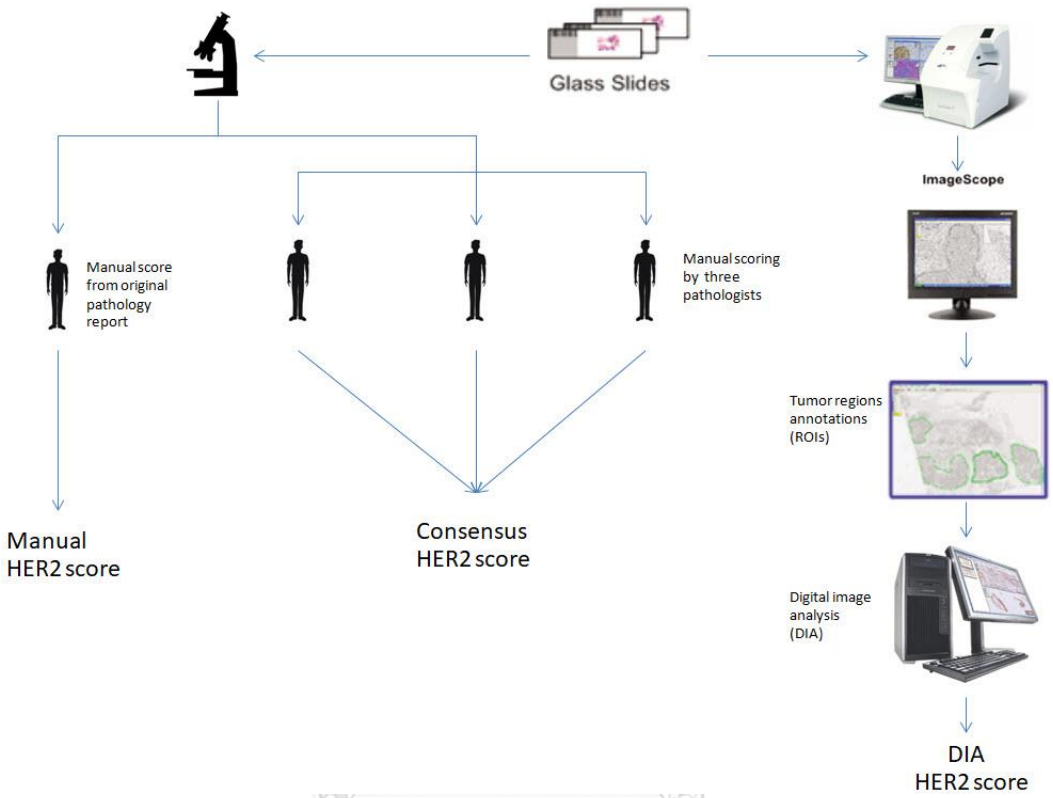

Figure 4: Study design

The diagnostic test evaluation featured HER2 dual in-situ hybridization as the gold standard with which results from the other test modalities were compared.

110 specimens were included in the study (see sample size calculation in next section). For the selection of IHC equivocal and positive cases, these were randomly chosen from a department registry list of specimens previously subjected to HER2 DISH. HER2 negative cases (0/1+) were randomly chosen from the hospital database, consecutively from 1/11-2017 and backwards irrespective of the numeric value of the scoring (0 vs. 1+). The calculated minimum sample size was enriched with

additional 24 IHC HER2 equivocal cases (2+), randomly chosen from a department registry list of specimens previously subjected to HER2 DISH.

These were the sequential steps of the main research activities:

1. Specimens were selected according to original HER2 score and adequacy of technical slide quality.
2. Relevant patient demographic data, tumor data and original manual microscopy scoring of IHC HER2 expression were obtained from the hospital pathology database.
3. The H&E and HER2 IHC stained glass slides of each patient were retrieved from the department archive.
4. All glass slides were scanned into whole slide images creating virtual slides.
5. The researcher performed digital image analysis of IHC HER2 expression of all cases.
6. The three consensus panel pathologists individually scored each HER2 stained glass slide by conventional microscopy.
7. Cases of total disagreement between all three consensus panel pathologists were rescored individually to reach consensus.
8. Data analysis and statistical calculations were performed. Outliers were scrutinized and analyzed.

4.2 Sample size calculation

A total of 110 patients were included in the study.

The sample size was calculated based on the “diagnostic test evaluation” part (comparison of DIA with DISH) focusing on specificity with the following parameters and assumptions:

- 95% confidence interval
- 25% of the study population is HER2 positive
- Precision = 5%

- Specificity = 96% (based on literature)

$$Sample\ size\ (n) = \frac{\sum^2_{1-\alpha/2}\ x\ specificity\ x\ (1-specificity)}{e^2\ x\ (1-prevalence)} = \frac{1.96^2\ x\ 0.96\ x\ 0.04}{0.05^2\ x\ (1-0.25)} \approx 79$$

Since the study focused particularly on the equivocal (2+) group, the population was enriched with additional 30% HER2 2+ patients to increase statistical power:

1.3 x 79 ≈ 103 patients (rounded up to 110 patients to allow for inadvertent exclusion of cases)

4.3 Patient population

The only inclusion criterion was presence of invasive breast cancer. The included slides were reviewed for technical quality issues, and tumor sections which were severely insufficiently fixated were excluded (2 cases from the preliminary selection). Slides with previously stained tumor sections from 110 invasive breast carcinomas diagnosed from August 2016 – November 2017 were collected from the archives at the department of pathology, King Chulalongkorn Memorial Hospital.

Table 2: Specimen characteristics based on original pathology report (n=109)

	n (%)
Patient gender	
Male	0 (0%)
Female	109 (100%)
Patient age (years)	29 - 82 (mean = 53)
Histological type	
IDC NOS	96 (88%)

	ILC	3 (3%)
	Others	10 (9%)
Tumor size		0,2 - 6 cm
Tumor grade		
	1	14 (13%)
	2	46 (42%)
	3	28 (26%)
	NA	21 (19%)
ER		
	Positive	81 (74%)
	Negative	28 (26%)
HER2 expression		
	0/1+	35 (32%)
	2+	49 (45%)
	3+	25 (23%)
	Others: including combinations	

All specimen types (biopsies, wide excisions, mastectomies) as well as primary, recurrent and metastatic breast carcinomas were included to reflect the actual patient population. Cases were chosen according to the original IHC HER2 score, so as to reflect the distribution of HER2 categories in the general population (46% negative, 30% equivocal and 24% positive). The calculated sample size was then enriched with additional 30% HER2 equivocal cases (24 cases) and 7 additional cases were randomly added to reach 110.

In the actual analysis, one case was excluded from the study due to lack of invasive carcinoma (only in-situ carcinoma was present) resulting in a total of 109 slides included in the study. In all 109 cases, the original H&E section and HER2 IHC stained section were used.

Tumor characteristics and background information on each patient were retrieved from the hospital pathology database, including patient gender, age, histological

tumor type, tumor size, tumor grade and tumor estrogen receptor status. The specimen characteristics are summarized in table 2.

4.4 H&E sections

An H&E section of each tumor was included in the study set. These slides were retrieved from the archive and had been constructed from 3 mcm sections from the FFPE tumor sections stained with hematoxylin (DAKO) and eosin (DAKO) in the automated DAKO CoverStainer according to the manufacturer's recommended protocol (see appendix for full protocol).

4.5 Immunohistochemistry

The HER2 stained slides of all included cases were retrieved from the archive. These slides had been constructed from 3 mcm sections of the original formalin-fixed paraffin-embedded tumor sections stained in an automated immunostainer (Ventana, Benchmark XT) using the PATHWAY anti-HER2/neu (4B5) rabbit monoclonal primary antibody (Ventana) according to the manufacturer's recommended protocol. In summary, slides were deparaffinized and submitted to heat-induced epitope retrieval by cell conditioning (Cell conditioning 1), followed by incubation with the primary antibody (HER2 clone 4B5 RTU Ventana) at 37 °C for 32 minutes. After washing in buffer (ultraWash), the antibody was visualized with UltraView DAB (Ventana) and developed with DAB (Ventana) followed by counterstaining with Hematoxylin II (Ventana) and a bluing agent.

4.6 Image acquisition

Whole slide images of both H&E slides and HER2 IHC slides were acquired by scanning of the conventional glass slides. A department technician performed the image acquisition using the Aperio CS2 whole slide scanner (Leica Biosystems, Germany) with a 40x lens and one focus layer without Z-stacking (ie. several focus planes). The default autofocus mode was used, but in a few cases the scanning needed manual focus to optimize the sharpness of the picture. The image files (.svs format) were stored on a Windows-based computer running the Aperio ScanScope software.

One HER2 stained tumor section and the corresponding H&E section were scanned for each case.

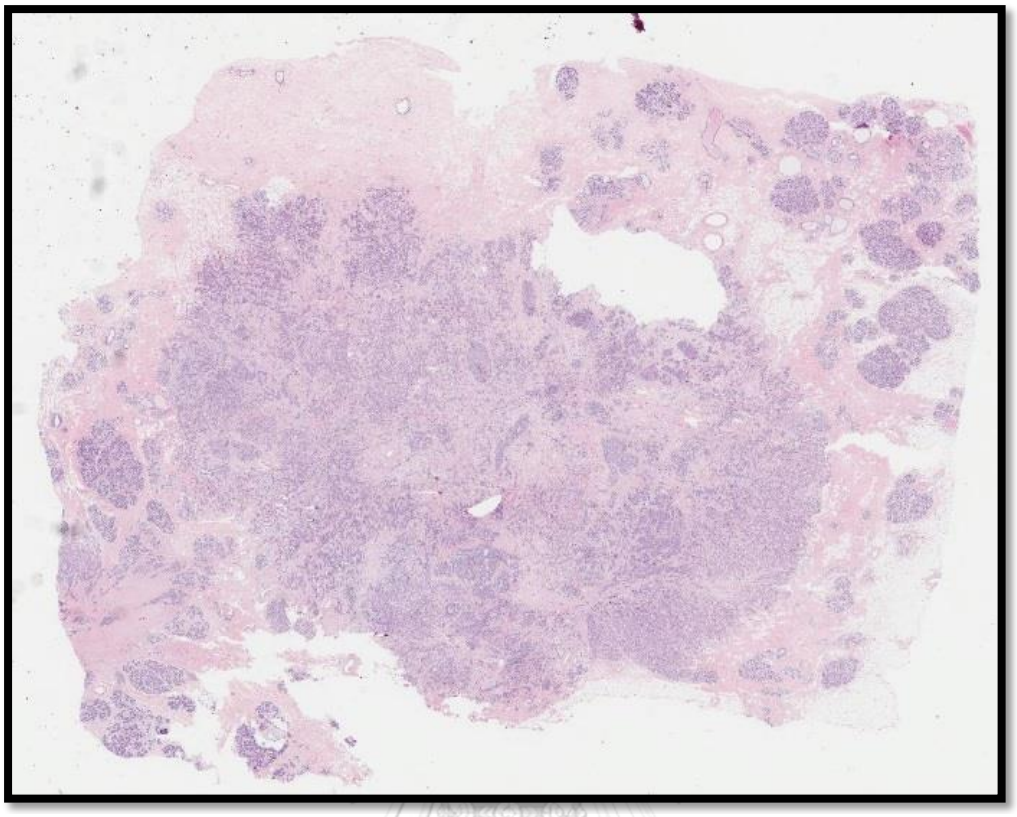

Figure 5: WSI of H&E stained tumor section

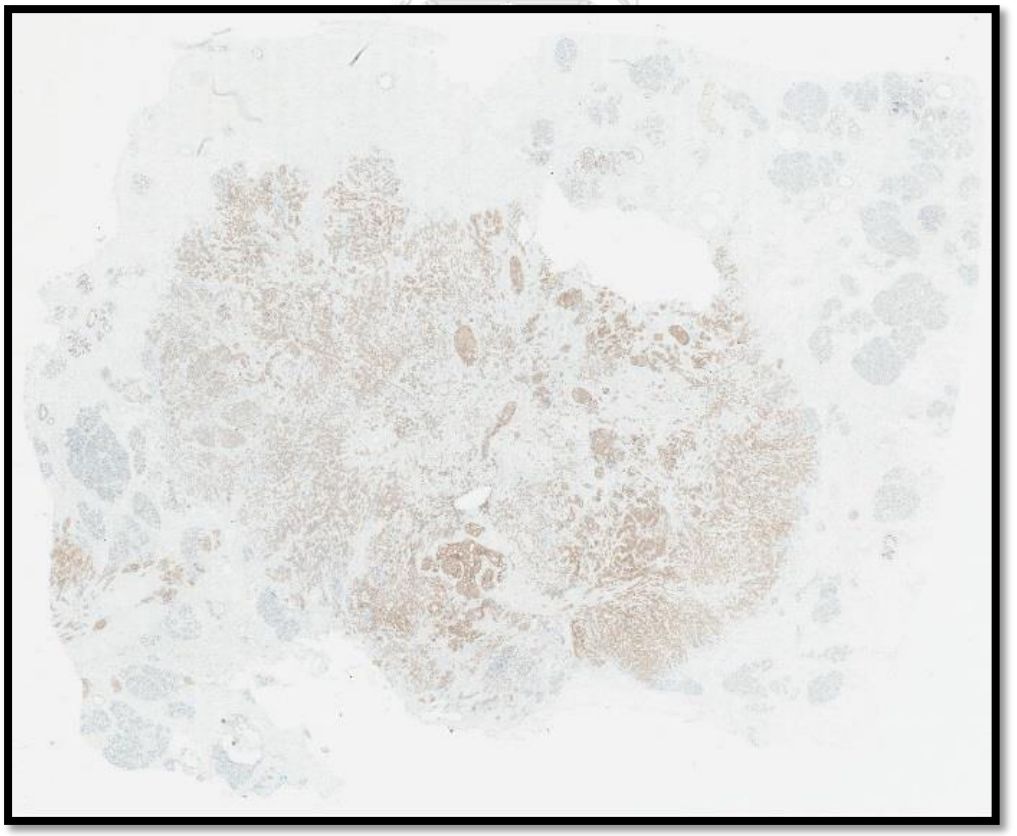

Figure 6: WSI of HER2 stained tumor section

4.7 Dual in-situ hybridization

All cases originally scored as IHC HER2 equivocal or positive (2+ or 3+) had previously been subjected to HER2 DISH analysis. It was ensured that the IHC result was signed off before the DISH result in each case in order to avoid any bias. Cases discordantly HER2 scored in the study, and for which a HER2 DISH result was not already available, were subjected to supplementary HER2 DISH. The DISH slides were scored according to the manufacturer's instructions [28] and interpreted in accordance with ASCO/CAP guidelines. Cases which were consistently scored as HER2 negative by all modalities (manual, consensus manual and DIA) were considered truly negative and not subjected to supplemental DISH (cf. Literature review, section 3.3).

For the DISH analysis, we used the Ventana INFORM HER2 Dual ISH DNA Probe Cocktail. The in situ hybridization was carried out according to the manufacturer's guidelines by placing the slides with formalin-fixed paraffin-embedded tumor sections in the automated BenchMark XT autostainer. After deparaffinization, the slides underwent heat-induced epitope retrieval (Cell conditioning CC2, Ventana) followed by proteolytic treatment in ISH-protease 3 (Ventana). The DNA probes (HER2 DNP-labeled and CEN17 DIG-labeled) were applied and incubated to hybridize for 6 hours, followed by stringency wash to reduce non-specific DNA hybridization. The DNP labeled probe was visualized by sequential incubation with rabbit anti-DNP antibody and goat anti-rabbit antibody followed by the addition of three sequential silver reagents.

The DIG-labeled probe was visualized by sequential incubation with mouse anti-DIG antibody and goat anti-rabbit antibody and developed with Ventana fast red reagent. By this method, silver precipitation is deposited in the nuclei, and single copies of *the HER2 gene* are visualized as single black dots while single copies of chromosome 17 are seen as red dots on the same slide.

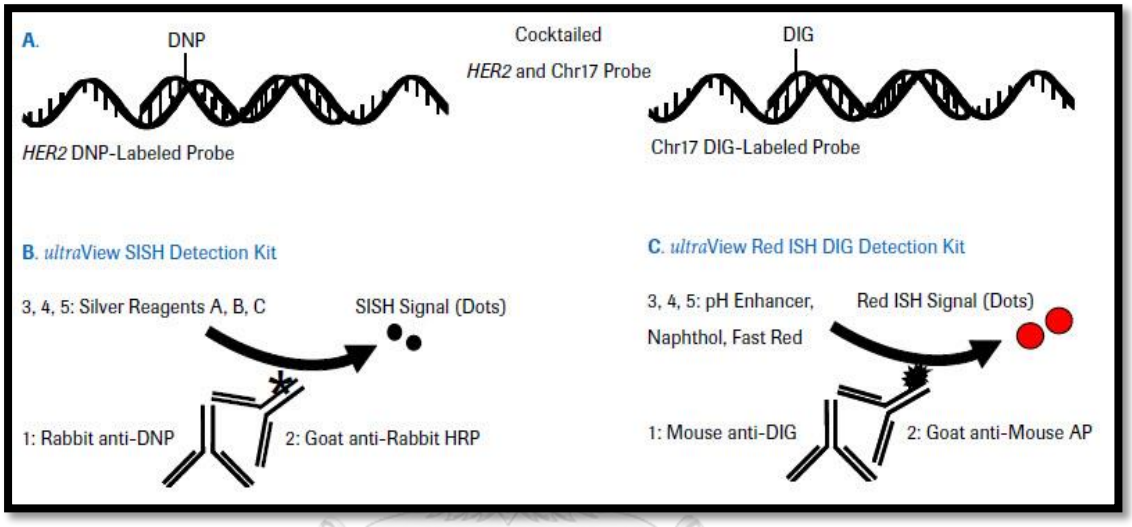

Figure 7: Dual in situ hybridization - detection of HER2 and CEN17 copies [28]

The HER2 gene status is reported as the ratio of the average number of HER2 gene copies per cell to the average number of CEN17 copies in nuclei of cells within the invasive part of the breast carcinoma.

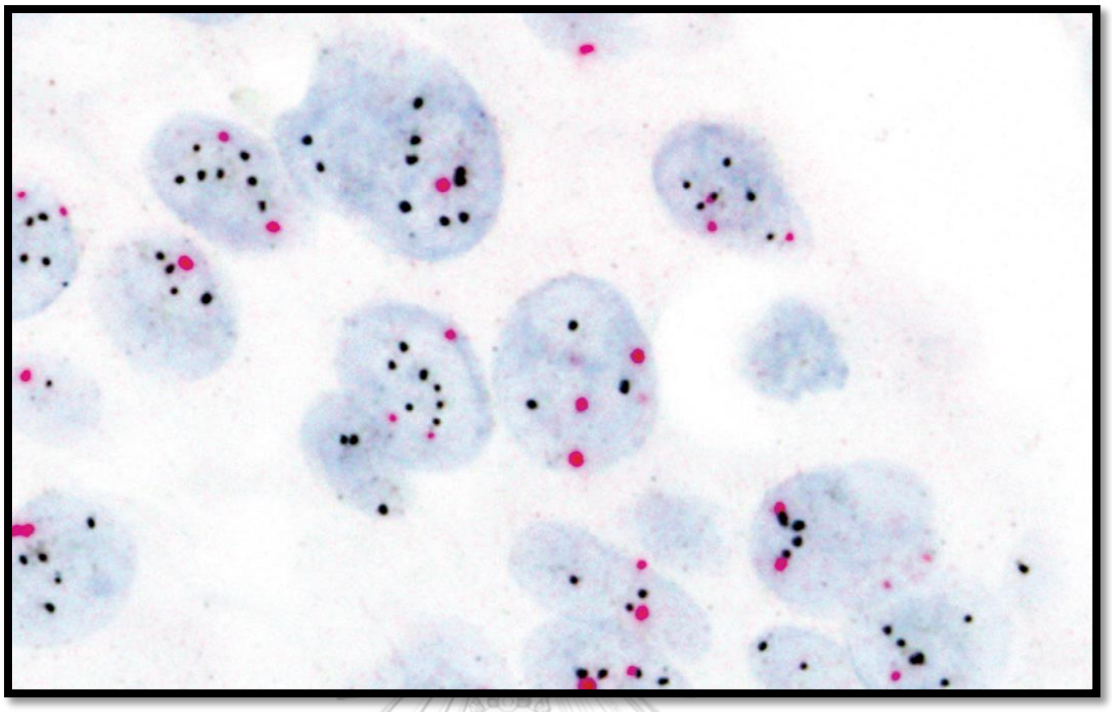

Figure 8: DISH (black=HER2 gene, red = centromere17) [28]

DISH is performed on a formalin-fixed paraffin-embedded tumor section and evaluated in a bright-field microscope. As per the ASCO/CAP 2013 interpretation guidelines, the number of HER2 gene and centromere17 signals are counted in 20 tumor cells and the HER2/CEN17 ratio is calculated. The results are interpreted as follows [1]:

Negative:

Dual-probe HER2/CEN17 ratio < 2.0 with an average HER2 copy number < 4.0 signals/cells

Equivocal

Dual-probe HER2/CEN17 ratio < 2.0 with an average HER2 copy number ≥ 4.0 and < 6.0 signals/cell

Positive:

Dual-probe HER2/CEN17 ratio ≥ 2.0 with an average HER2 copy number ≥ 4.0 signals/cell

Dual-probe HER2/CEN17 ratio ≥ 2.0 with an average HER2 copy number < 4.0 signals/cell

Dual-probe HER2/CEN17 ratio < 2.0 with an average HER2 copy number ≥ 6.0 signals/cell

4.8 IHC HER2 scoring

Both pathologists and DIA assessed the HER2 expression in accordance with the 2013 ASCO/CAP guidelines [1]. An updated guideline was “early online released” a week before the finalization of this thesis. It includes a small revision of the IHC score 2+ criteria, but the major changes pertain to the interpretation of ISH analysis in a quest to reduce the number of equivocal cases obtained with in-situ techniques [4]. This thesis is based upon the 2013 guidelines:

IHC 0 is defined as no staining observed or membrane staining that is incomplete and is faint/barely perceptible.

IHC 1+ is defined as incomplete membrane staining that is faint/barely perceptible and within >10% of the invasive tumor cells. (Together, category 0 and 1+ are considered negative for IHC HER2 expression.)

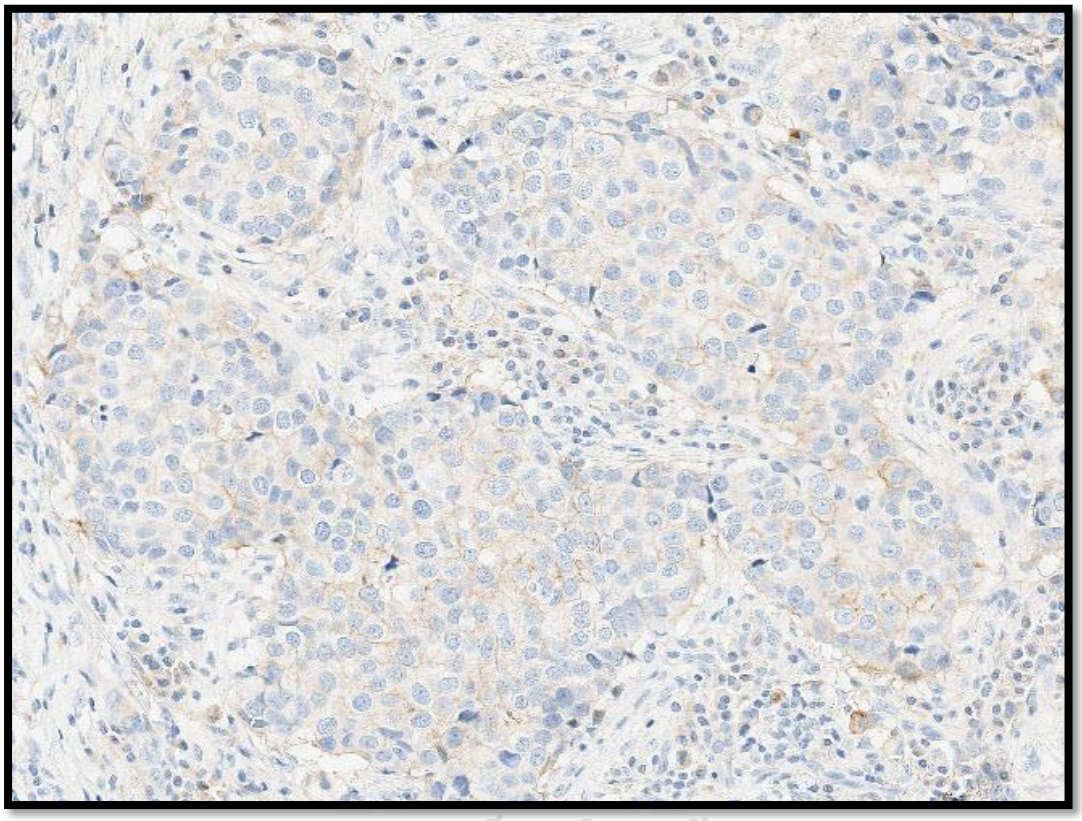

Figure 9: HER2 score 1+

IHC 2+ is equivocal, and defined as circumferential membrane staining that is incomplete and/or weak/moderate and within >10% of the invasive tumor cells; *or* complete and circumferential membrane staining that is intense and within ≤10% of the invasive tumor cells.

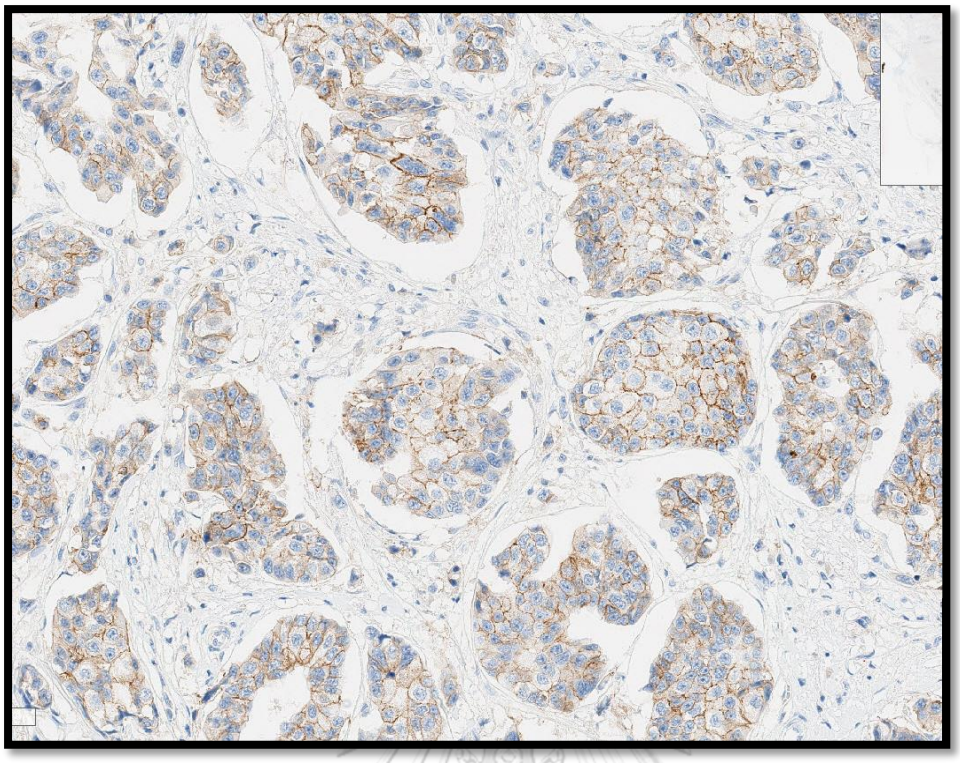

Figure 10: HER2 score 2+

IHC 3+ is considered positive, and defined as more than 10% of tumor cells showing homogeneous, dark circumferential (chicken wire) pattern.

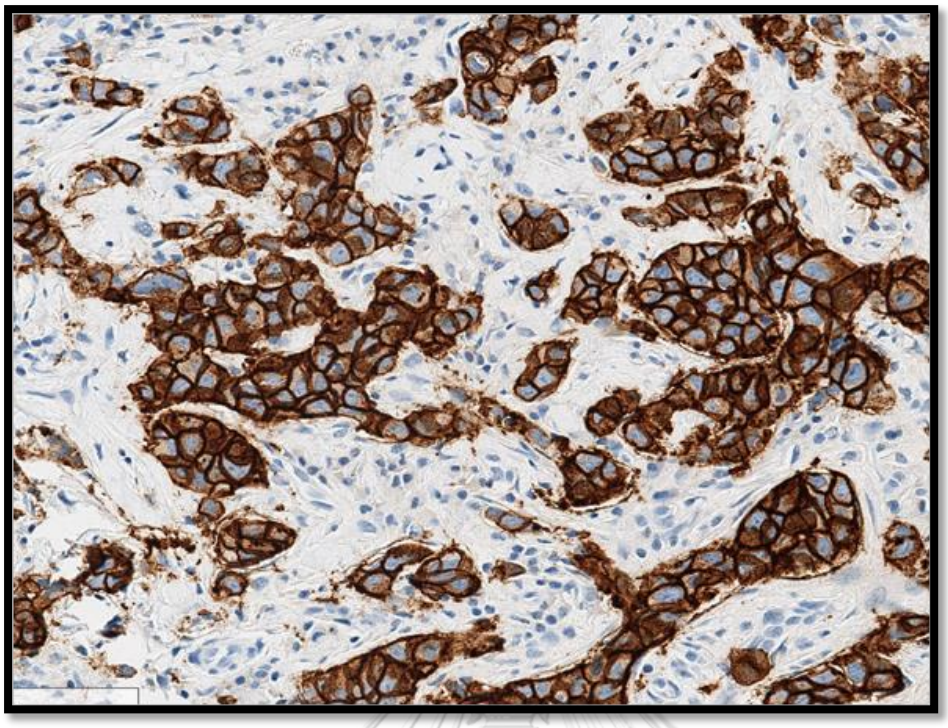

Figure 11: HER2 score 3+

Below is an overview of the 2013 ASCO/CAP HER2 IHC and DISH scoring criteria [1]:

Table 3: ASCO/CAP 2013 HER2 scoring criteria

	IHC	**DISH**
0 (negative)	IHC 0 is defined as no staining observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤10% of the invasive tumor cells.	HER2/CEN17 < 2.0
1+ (negative)	Incomplete membrane staining that is faint/barely perceptible and within >10% of the invasive tumor cells.	

2+ (equivocal)	Circumferential membrane staining that is incomplete and/or weak/moderate and within >10% of the invasive tumor cells; *or* complete and circumferential membrane staining that is intense and within ≤10% of the invasive tumor cells.	*
3+ (positive	More than 10% of tumor cells showing homogeneous, dark circumferential (chicken wire) pattern.	HER2/CEN17 ≥ 2.0

* HER2/CEN17 < 2.0 with an average HER2 copy number ≥ 4 and < 6 is considered equivocal.

4.9 Manual assessment of IHC HER2 expression

Results of the manual assessment of HER2 status were obtained from the original pathology report in the hospital pathology database (scores 0, 1+, 2+, 3+ or negative, equivocal and positive). These cases had previously been scored by experienced pathologists at our department.

4.10 Consensus manual assessment of IHC HER2 expression

The validation set consisted of an H&E tumor section with corresponding HER2 stained section for each case. No histopathological or clinical information was

provided. All 109 cases were manually scored individually by two senior pathologists and the researcher (resident pathologist) in a blinded manner, assigning each case a standard score from 0 - 3+. The consensus score was determined as the score assigned by the majority (2 of 3) or all of the three pathologists. In case of total disagreement (cases scored differently by all three pathologists), a renewed individual scoring was undertaken to reach a consensus.

4.11 Digital image analysis of IHC HER2 expression

The digital image analysis was performed on whole slide images (WSI) of the HER2-stained tumor sections using Aperio ImageScope (v. 12.1.0.5029) running the Membrane algorithm (v9.1) with standard settings slightly modified for optimization (see appendix). These adjustments were based on a preliminary testrun (16 HER2 stained WSI), where different settings were compared with manual counting of cells. The adjusted settings (minimal nuclear size increased from 10 to 25 mcm and minimal cell size increased from 25 to 50 mcm) did not in any case affect the final result (HER2 score), but increased the accuracy of the cell count by improving cell separation (Cf. Appendix 7.2). The adjusted algorithm settings were saved in a file ("macro") and used in the analysis of all images.

Whenever the algorithm returned a result within ±2% points of a significant cut-off (eg. 8% 3+ cells), the number of ROI was doubled and the analysis was repeated in order to reduce sampling bias and thereby increase the accuracy of the result. The DIA was performed by the author in a blinded manner more than four weeks after selection of the cases to avoid any bias. DIA results were saved and stored on file together with the digital images.

The main steps of the DIA process are outlined below.

1. The H&E WSI is reviewed to get an impresssion of tumor morphology, invasiveness and technical quality of the tissue (eg. adequacy of fixation).

2. The HER2 WSI is reviewed with regard to quality of the tissue, quality of the staining and any tumor heterogeneity.

3. ROI are manually annotated to include only tumor cells and exclude stroma or inflammatory cells.

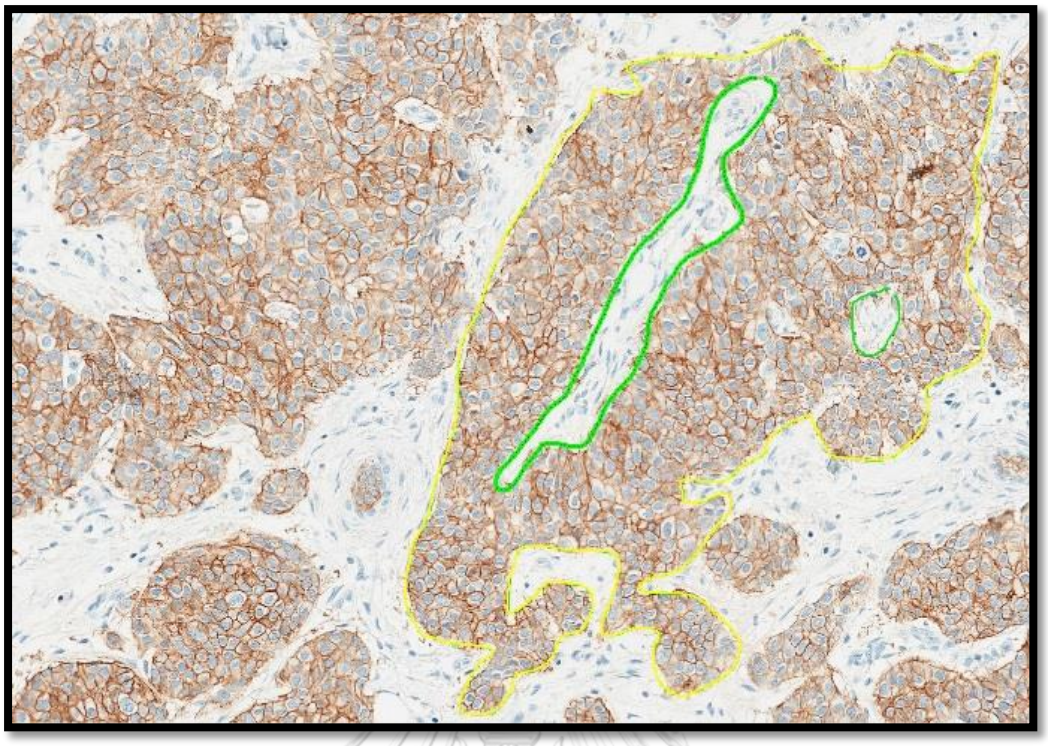

Figure 12: Close-up of ROI (yellow outline), areas outlined with green are excluded from analysis

4. ROI are annotated according to the manufacturer's recommendations (15-20 regions and at least 1000 tumor cells) – whenever possible - to appropriately represent any heterogeneity of the tumor [29]. Poorly fixated areas are avoided.

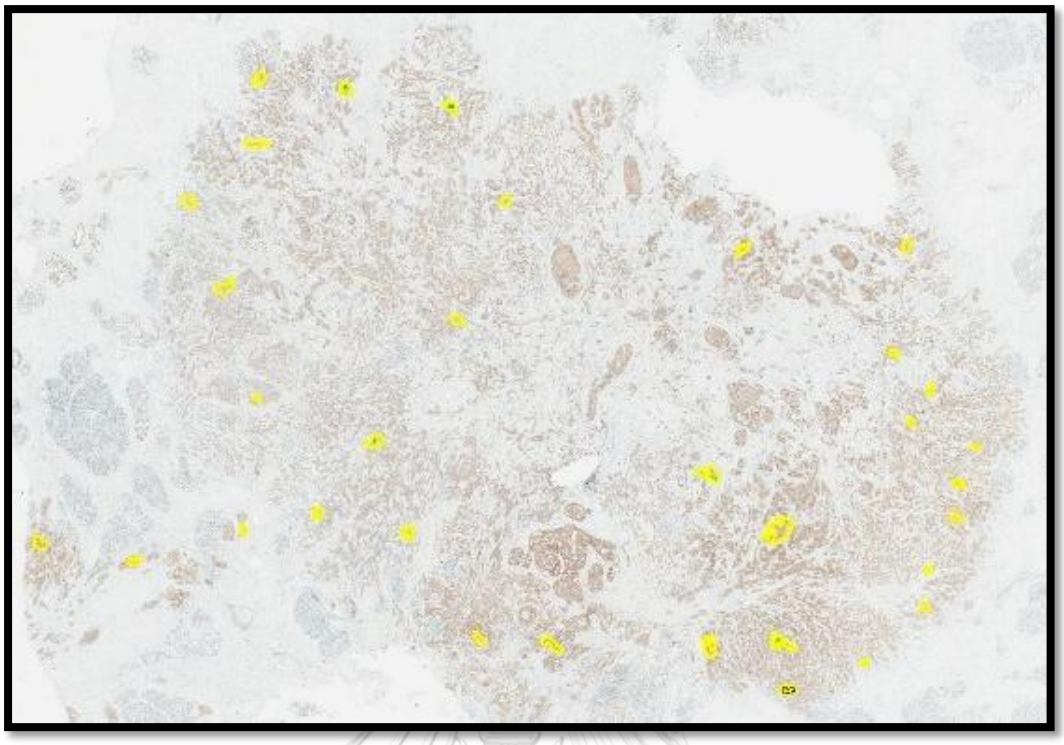

Figure 13: Representative ROI annotated (yellow outline)

5. The membrane algorithm (v9.1) is selected and the customized settings (”macro”) are loaded. The algorithm is started.

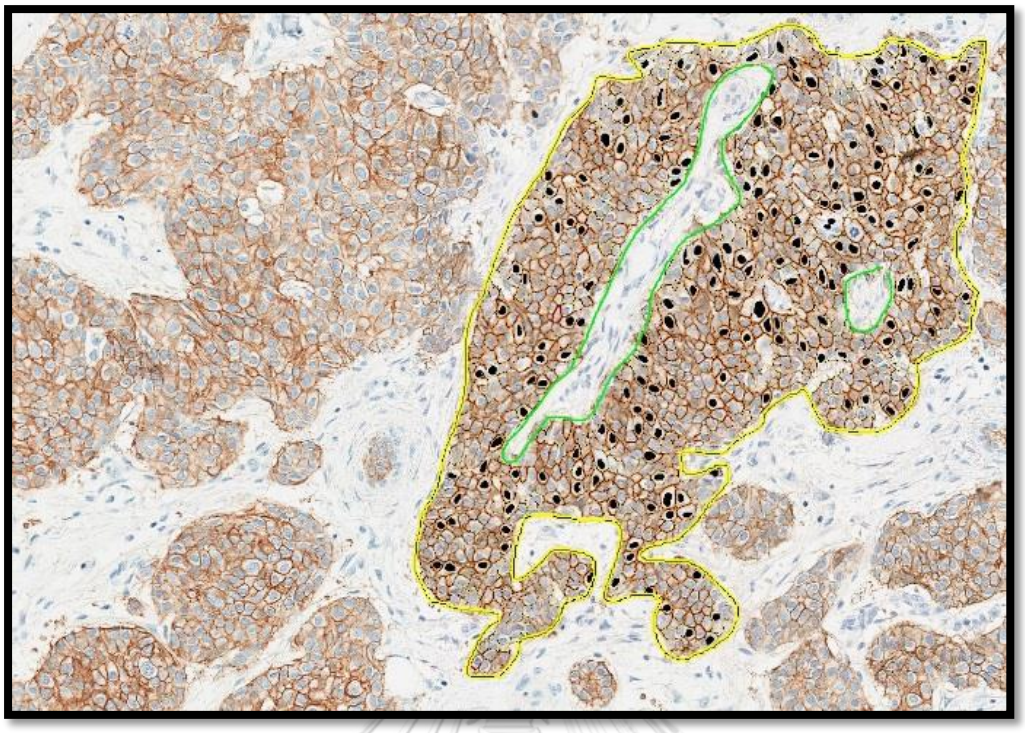

Figure 14: ROI after running the algorithm

6. The algorithm analyses each ROI individually and displays a markup with color codes signifying the HER2 score of each individual cell.

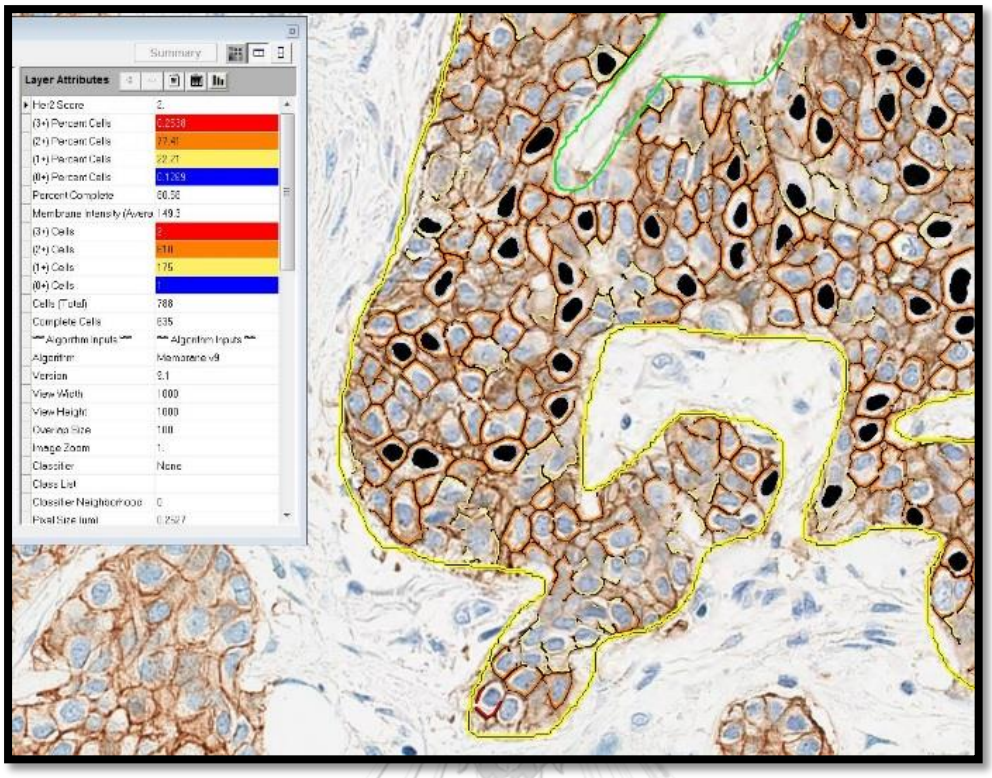

Figure 15: Close-up of ROI after running the algorithm (orange membranes: 2+, yellow membranes: 1+)

7. A summarized final score is displayed.

Her2 Score	2.
(3+) Percent Cells	0.2538
(2+) Percent Cells	77.41
(1+) Percent Cells	22.21
(0+) Percent Cells	0.1269
Percent Complete	80.58
Membrane Intensity (Avera	149.3
(3+) Cells	2
(2+) Cells	610
(1+) Cells	175
(0+) Cells	1
Cells (Total)	788
Complete Cells	635

Figure 16: Final DIA result

4.12 HER2 DISH scoring

HER2 DISH results for all cases previously scored as 2+ or 3+ were retrieved from the hospital pathology database. Those additional cases which needed DISH analysis due to IHC study score disagreement (n=4) were scored by the principal investigator according to ASCO/CAP guidelines.

4.13 Statistics

Comparing the three assessment modalities (manual, consensus manual and DIA), intermodality agreement was calculated using percentage agreement and weighted kappa with 95% confidence intervals. Significance of the differences in frequency distribution was evaluated by calculation of p-values with χ^2 test for independence (GraphPad InStat, v3.05).

Next, agreement between the three modalities and DISH was calculated. Diagnostic test parameters (sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV)) were determined with 95% confidence intervals using DISH as gold standard. It should be noted, however, that the NPV and PPV calculations do not include the equivocal cases. Bayes' theorem was used for the calculation of positive predictive value (PPV) and negative predictive value (NPV), since the study sample did not accurately reflect the prevalence of IHC HER2 scores in the population (the cohort was enriched with equivocal cases).

5 Results

Of the 110 cases included in the study, one was excluded due to lack of invasive carcinoma (only DCIS present). In the consensus manual assessment, one case was excluded as one of the pathologists found the tumor section unsuitable for evaluation due to insufficient fixation. There was only one case of total disagreement in the consensus reading; this was resolved after renewed individual reading by the three pathologists. The raw data can be found in appendix 9.4 - 9.7.

Scanning of the slides was performed by a department technician and took approximately 20-25 minutes per slide after which the images were saved on a portable hard disk. Once the algorithm had been tuned, the DIA process performed by the researcher took approximately 8-10 minutes per case. The DIA results were saved in a file together with the original images.

In the DIA, ROI were manually outlined (range: 4 - 97, mean 30, equal to 148 - 19929 cells, mean 2761) and the analysis was performed. Six cases were analyzed twice due to the first result falling within the "grey zone" of ±2%, and by doing so, one case was re-categorized after the second analysis: Four cases remained 1+ after the second scoring, one case remained 3+ after the second scoring and one case was reclassified from 2+ to 3+ after the second scoring (10.0% vs. 10.1 % 3+ cells).

Manual scoring (ie. original score from the pathology report, n=109) yielded 36 negative (33.0%), 48 equivocal (44.0%) and 25 positive cases (22.9%).

When scored manually by the **consensus** group (n=108[1]), 43 cases (39.8%) were categorized as negative, 36 (33.3%) as equivocal and 29 (26.9%) as positive. Scoring by **DIA** (n=109) yielded 65 (59.6%) negative, 16 (14.7%) equivocal and 28 (25.7%) positive cases.

Table 4: HER2 score by each modality

	Negative	Equivocal	Positive
Manual (n=109)	36 (33%)	48 (44%)	25 (22.9%)
Consensus (n=108[1])	43 (39.8%)	36 (33.3%)	29 (26,9%)
DIA (n=109)	65 (59.6%)	16 (14.7%)	28 (25.7%)

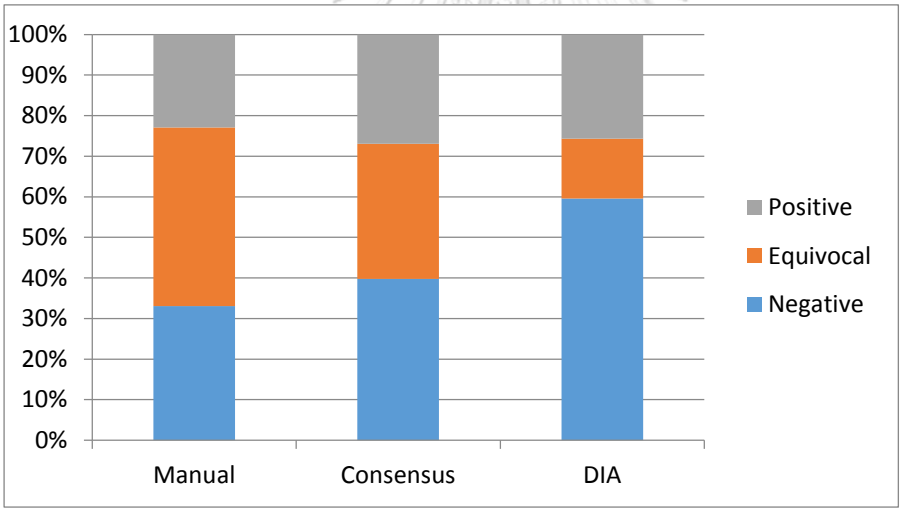

Figure 17: HER2 score by each modality

[1] One case (serial no. 48) was excluded in the consensus assessment because one of the panel pathologists found it inadequate for evaluation due to poor fixation. Hence, the total case number in the consensus analysis is 108 (vs. 109 in manual and DIA).

5.1 Intermodality IHC scoring concordance

Scoring of the 109 cases by the three different methods showed overall substantial agreement.

Comparing **manual vs. consensus scoring**, good agreement was obtained with percentage agreement 85.6% and weighted kappa 0.79 [0.70-0.88]. A lower proportion of equivocal cases was seen in the consensus scoring (33.3% vs. 44%), although not significant at 0.05 level (p = 0.31 : χ^2 test).

Table 5: Cross tabulation: Manual vs. Consensus score

		Consensus			
		Neg	Equi	Pos	Total
Manual	Neg	32	4	0	36
	Equi	11	32	4	47
	Pos	0	0	25	25
	Total	43	36	29	108[2]
Percentage agreement				85.6%	
Weighted kappa				0.79 [0.70-0.88]	

For **consensus scoring vs. DIA**, equally good agreement was obtained with percentage agreement 79.4% and weighted kappa 0.77 [0.68-0.86]. The DIA had significantly fewer equivocal cases (14.7% vs. 33.3%) and more negative cases (59.6% vs. 39.8%) (p = 0.001 : χ^2 test)

[2] One case (serial no. 48) was excluded in the consensus assessment because one of the panel pathologists found it inadequate for evaluation due to poor fixation. Hence, the total case number in the consensus analysis is 108 (vs. 109 in manual and DIA).

Table 6: Cross tabulation: Consensus vs. DIA

		DIA			
		Neg	Equi	Pos	Total
Consensus	Neg	43	0	0	43
	Equi	22	14	0	36
	Pos	0	1	28	29
	Total	65	15	28	108[2]
Percentage agreement			79.4%		
Weighted kappa			0.77 [0.68-0.86]		

For **manual scoring vs. DIA**, a slightly lower but yet substantial agreement was obtained with percentage agreement 70.6% and weighted kappa 0.67 [0.58-0.77]. The DIA returned significantly fewer equivocal cases (14.7% vs. 44%) and a larger proportion of negative cases (59.6% vs. 33%). ($p < 0.00001$: χ^2 test)

Table 7: Cross tabulation: Manual vs. DIA

		DIA			
		Neg	Equi	Pos	Total
Manual	Neg	36	0	0	36
	Equi	29	16	3	48
	Pos	0	0	25	25
	Total	65	16	28	109
Percentage agreement			70.6%		
Weighted kappa			0.67 [0.58-0.77]		

5.2 Concordance with HER2 DISH

HER2 DISH was performed on 78 of the included cases, either as part of the primary diagnostic work-up (n=74) or supplementary as part of the study (n=4). The 31 cases which were not subjected to DISH analysis were unanimously assessed as negative by all scoring modalities (original report, digital image analysis and all three consensus score pathologists) and thus presumed to be truly negative.

When comparing **manual scoring with DISH**, a moderate agreement was obtained with percentage agreement 55.6% and weighted kappa 0.52 [0.41-0.63]. There were no false negatives (IHC negative/DISH positive) and only one false positive (IHC positive/DISH negative).

Consensus scoring vs. DISH obtained a slightly better agreement with percentage agreement 65.7% and weighted kappa 0.59 [0.47-0.70]. There were no false negative and three false positives.

DIA compared with DISH obtained a high level of agreement with percentage agreement 85.0% and weighted kappa 0.78 [0.68-0.88]. There were no false negatives and two false positives.

Table 8: Manual, consensus and DIA vs. DISH

		HER2 DISH			
		Neg	Equi	Pos	Total
Manual	Neg	36 (33.0%)	0	0	36 (33.0%)
	Equi	43 (39.4%)	0	5 (4.6%)	48 (44.0%)
	Pos	1 (0.9%)	0	24 (22.0%)	25 (22.9%)
	Total	80 (73.4%)	0	29 (26.6%)	109 (100%)
Percentage agreement				55.6%	
Weighted kappa				0.52 [0.41-0.63]	
		Neg	Equi	Pos	Total
Consensus	Neg	43 (39.8%)	0	0	43 (39.8%)
	Equi	34 (31.5%)	0	2 (1.9%)	36 (33.3%)
	Pos	3 (2.8%)	0	26 (24.1%)	29 (26.9%)
	Total	80 (74.1%)	0	28 (25.9%)	108[3] (100%)
Percentage agreement				65.7%	
Weighted kappa				0.59 [0.47-0.70]	
		Neg	Equi	Pos	Total
DIA	Neg	65 (59.6%)	0	0	65 (59.6%)
	Equi	13 (11.9%)	0	3 (2.8%)	16 (14.7%)
	Pos	2 (1.8%)	0	26 (23.9%)	28 (25.7%)
	Total	80 (73.4%)	0	29 (26.6%)	109 (100%)
Percentage agreement				85.0%	
Weighted kappa				0.78 [0.68-0.88]	

Figure 19 offers a graphic overview of the test performance results of each of the three modalities.

[3] One case (serial no. 48) was excluded in the consensus assessment because one of the panel pathologists found it inadequate for evaluation due to poor fixation. Hence, the total case number in the consensus analysis is 108 (vs. 109 in manual and DIA).

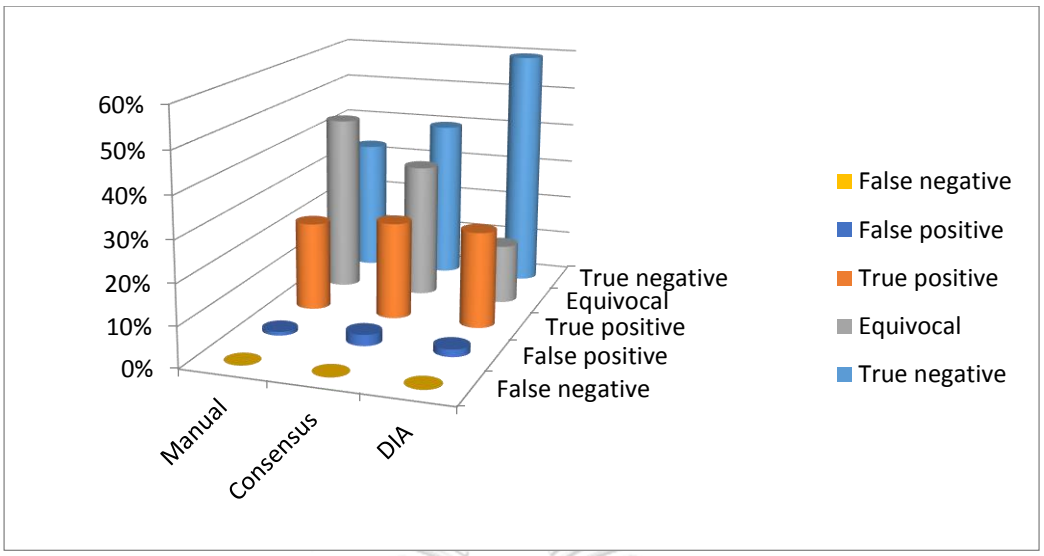

Figure 18: Test performance of the three IHC test modalities (manual, consensus and DIA) in the test population

The diagnostic test parameters calculated for the three methods, using DISH as the gold standard, are shown in the table below. The equivocal cases are not included in the calculation, since in the clinical reality an equivocal result would trigger reflex HER2 DISH and thereby prevent a false positive or false negative result.

Table 9: Diagnostic test parameters for the three modalities

DISH vs.	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Equivocal HER2 (%)
Manual	100	97.7	94.6	100	44.0
Consensus	100	93.5	86.1	100	33.3
DIA	100	97.0	93.1	100	14.7

Comparing DIA with manual microscopy, the number of equivocal cases was reduced approximately 67% with no false negatives. Importantly, a sensitivity of 100% was seen in all modalities (ie. no false negatives).

6 Discussion

Establishment of HER2 status is considered standard of care in the diagnostic workup of breast cancer and has high predictive value by identifying patients who might benefit from anti-HER2 treatment. The evaluation of HER2 status has traditionally been performed by manual assessment of immunohistochemical expression of HER2 with reflex to HER2 in-situ hybridization in equivocal cases. The semi-quantitative assessment of immunohistochemical HER2 expression performed by a pathologist depends on skills and experience, and is inherently subjective and prone to observer error and interobserver variance. In an average population of breast cancers, a significant number of cases are categorized as IHC equivocal (in our department approx. 25-30%). With a view to minimize the analytical variance and reduce the proportion of equivocal cases, digital image analysis has been shown to offer a standardized and highly reproducible method for assessment of immunohistochemical HER2 expression.

In our study we compared the IHC assessment by manual microscopy, consensus manual microscopy and digital image analysis, demonstrating substantial agreement across the three methods. When comparing the three methods with DISH, a very high

sensitivity and specificity were found for all three methods, but with a substantial 2/3 reduction of equivocal cases when evaluated by DIA ($p<0.00001$). Thus, 32 cases originally classified as equivocal by manual microscopy were reclassified as negative (n=29) or positive (=3) by DIA. Consensus reading by a panel of three pathologists saw a 24% reduction in equivocal cases compared to manual reading by a single pathologist, although this result was not significant at 0.05 level.

There are several possible explanations for the different proportions of equivocal cases seen in the three scoring modalities. Manual scoring by one pathologist places the entire responsibility on one person, who in any case of doubt might want to hedge himself by rendering an equivocal score and refer the case for supplementary in-situ analysis. Any factor that might affect the confidence of the assessing pathologist (experience, skills, fatigue, risk profile, etc.) could therefore potentially affect the individual scoring practice.

When the HER2 score is determined as a consensus by a panel of pathologists, the feeling of shared responsibility may reduce each pathologist's need for hedging and risk reduction. In our study, the decreased number of equivocal cases compared to single manual reading may also be partly due to awareness of the fact that the scoring result would have no clinical consequences (ie. no personal risk). Finally, DIA has the advantage of performing de-facto quantitative analysis (each separate cell is analyzed), in contrast to most pathologists who tend to use semi-quantitative methods or "eye-balling" rather than rigorously counting 1000 cells.

6.1 Analysis of “outliers”

There were three cases (case no. 20, 47 and 75) of potentially clinically significant discrepant assessment by IHC and DISH with positive ÍHC reading and negative DISH result (ie. false positives). Two of these (no. 20 and 47) had borderline IHC results and heterogeneous IHC staining pattern, while one case (no. 75) showed unanimous strong membranous staining.

Case no. 75 case was scored as equivocal in the original pathology report, but scored positive by consensus and DIA, despite being non –amplified by DISH analysis. When the HER2 slide without any additional information was shown to three breast pathologists who were not directly involved in this study (including the original evaluator), it was consistently re-scored as positive. However, when additional information was supplied (ER 100%, PR 100%, Ki67 10%), the pathologists re-scored the slide to 2+ in light of the additional information.

The HER2 stained section showed complete, intense circumferential staining in the majority of cells, as seen in figure 20. Lack of myoepithelial cells was confirmed by negative staining for SMA, p63 and smooth muscle myosin heavy chain.

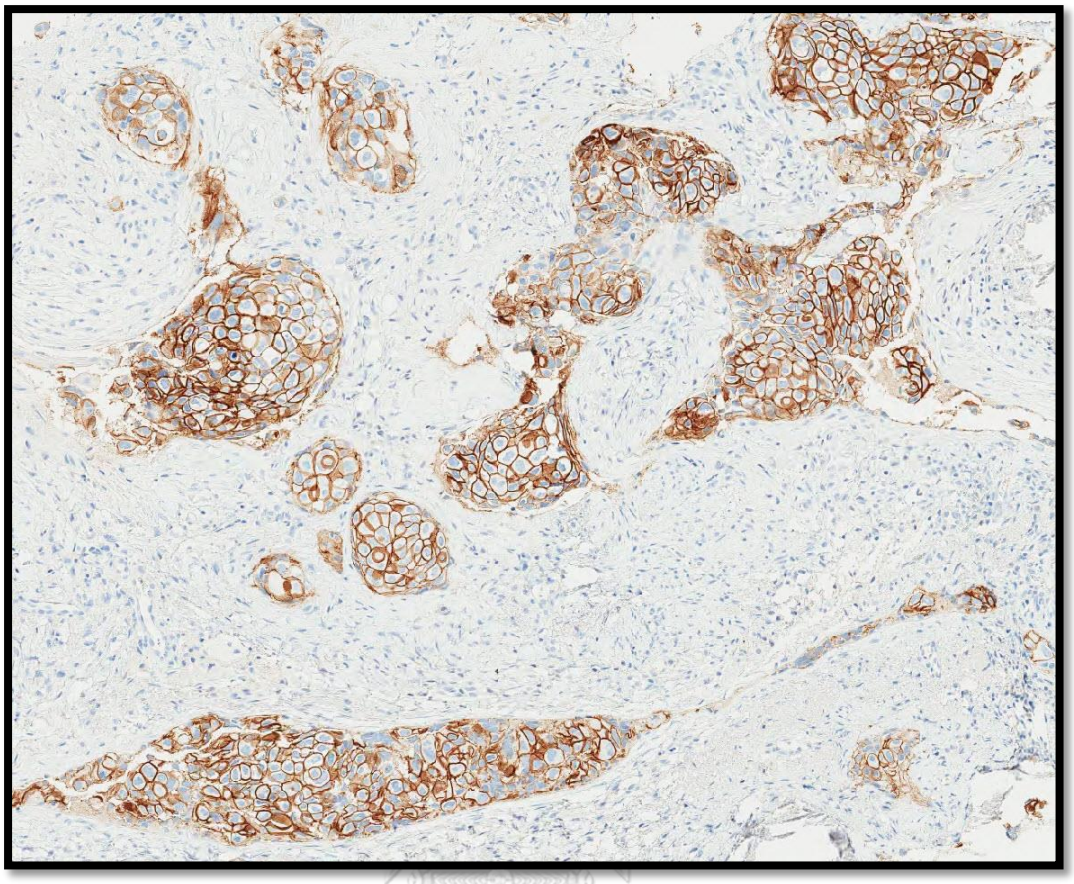

Figure 19: Case no.75 - Homogenous, strong membranous staining

The DISH result revealed a HER2/CEN17-ratio of 0.95:1 with an average of 3.3 CEN17/nucleus. Elevated centromere 17 count ("polysomy", by some defined as ≥ 3 CEN17 copies per cell [30] has in some reports been associated with positive IHC staining (3+) in HER2 non-amplified cases [30]. The clinical significance of this finding is still unclear, in particular the potential effect of adjuvant anti-HER2 treatment of IHC positive, HER2 non-amplified polysomal tumors. However, some studies suggest that this (small) patient group may benefit from anti-HER2 treatment (ibid).

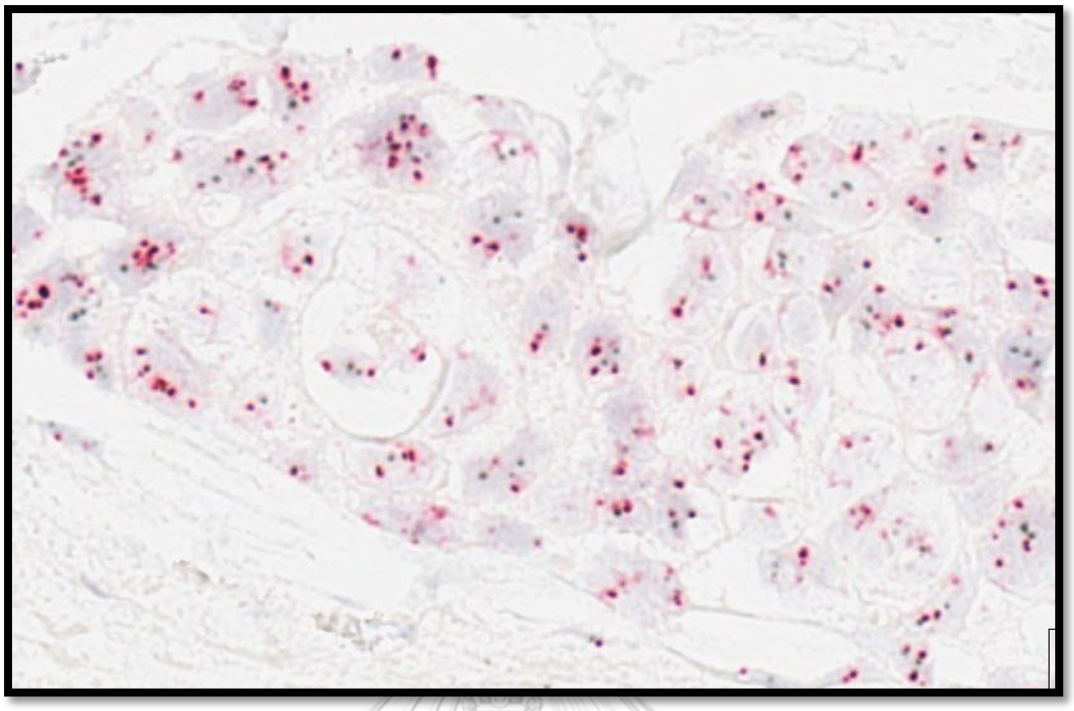

Figure 20: Case no. 75 - HER2 DISH with CEN17 copy gains ("polysomy") [red dots = CEN17, black dots = HER2 gene]

Case no. 20 was consistently IHC scored as positive by all modalities (original score from pathology report, consensus score and DIA) despite being non-amplified by DISH analysis. The HER2 stained sections revealed some areas of intense and complete membranous staining (3+), while other areas exhibited weak to moderate membranous staining (2+).

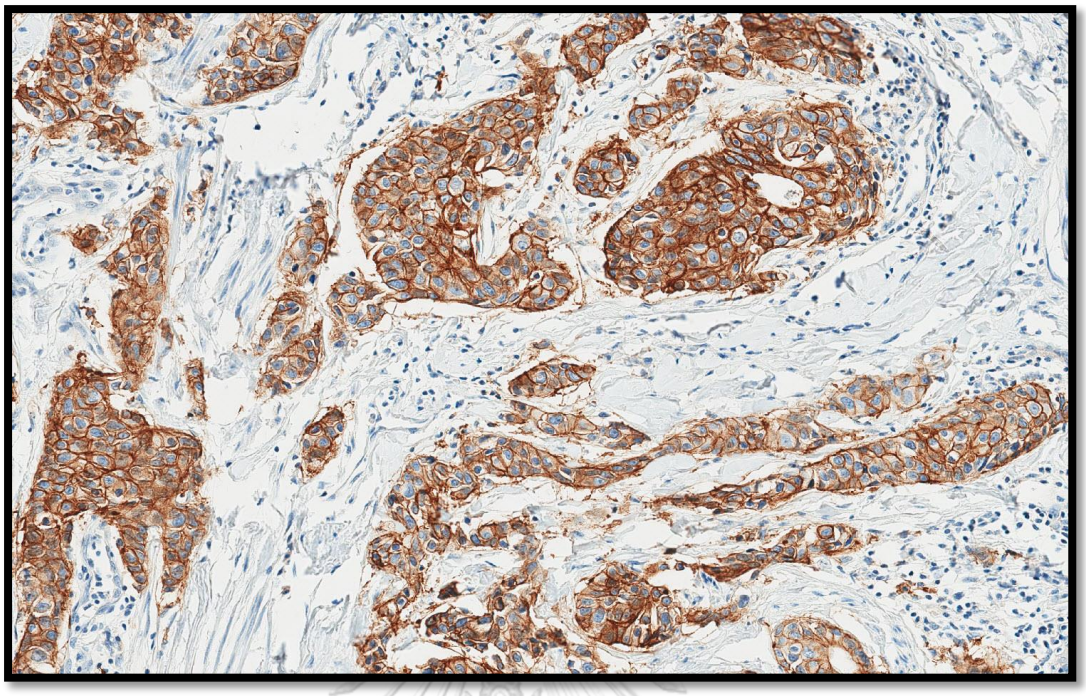

Figure 21: Case no. 20 - Areas with intense circumferential membranous staining (3+)

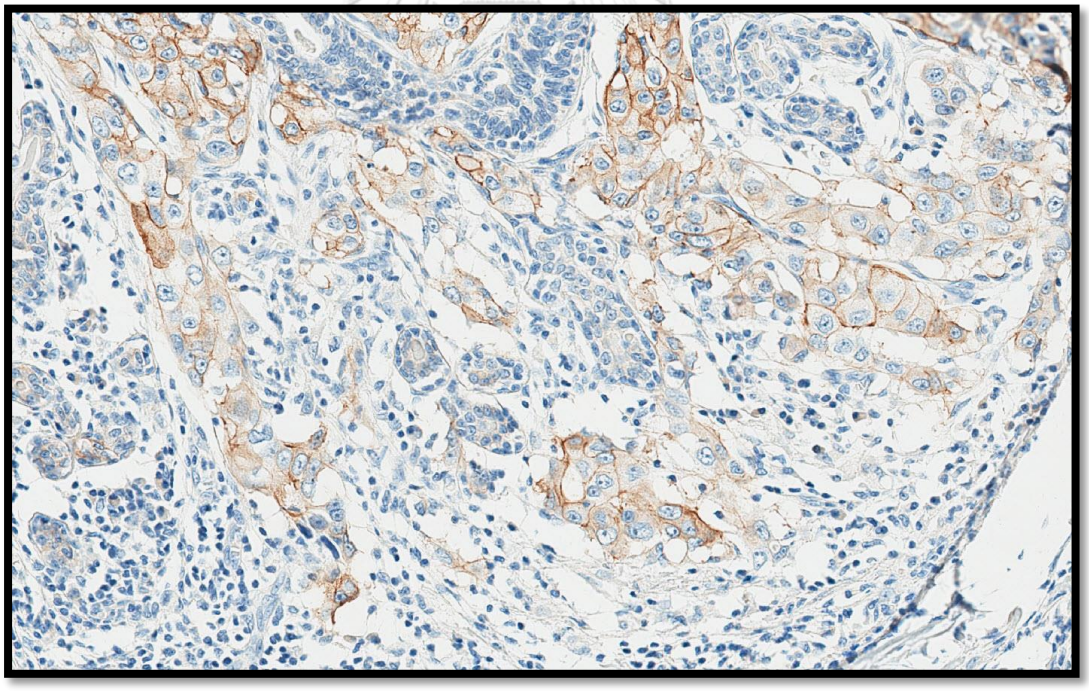

Figure 22: Case no. 20 - Areas with weak to moderate membranous staining (2+)

The DISH analysis revealed a HER2/CEN17 ratio of 1.24:1 with 2.25 CEN17/nucleus. Review of the DISH slide showed remarkable intratumoral variation in the number of HER2 copies per cell. Some tumor cells had a HER2/CEN17-ratio higher than 2.0, while most tumor cells retained at ratio of < 2. This intratumoral genotypic variability could possibly explain the discrepantly assessed HER2 status, the result of which would depend on the tumor region/tumor cells chosen for analysis [30].

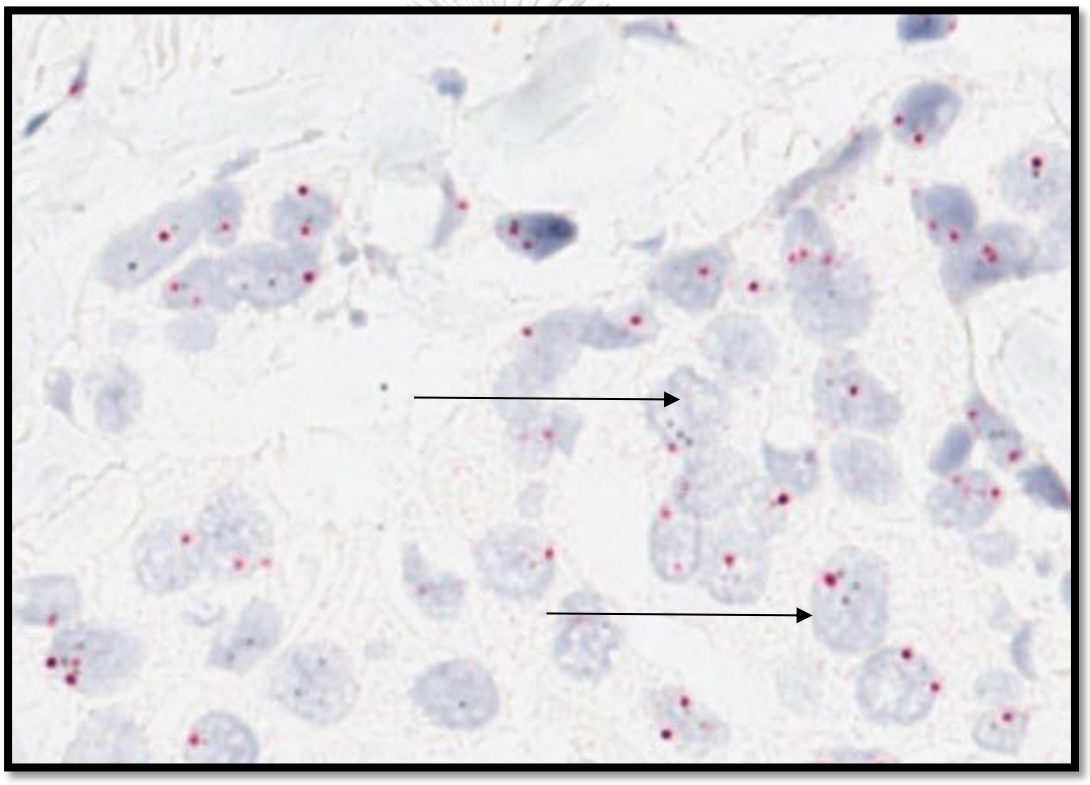

Figure 23: Case no. 20 - DISH shows HER2 copy number gain (>2) in some tumor cells

Case no. 47 case was scored as positive by consensus, but negative by DIA and conventional manual reading. Review of the HER2 stained slide demonstrated heterogeneous staining pattern with some tumor areas displaying intense and

complete membranous staining, while other tumor areas showed weak and incomplete staining.

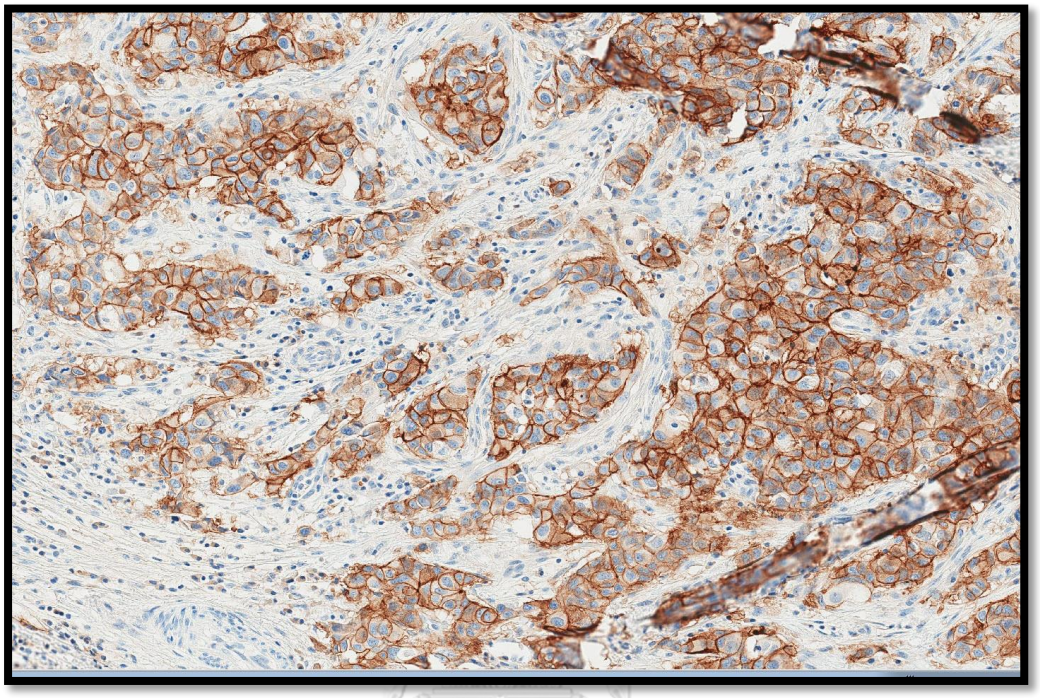

Figure 24: Case no. 47 - Areas with intense, complete membranous staining

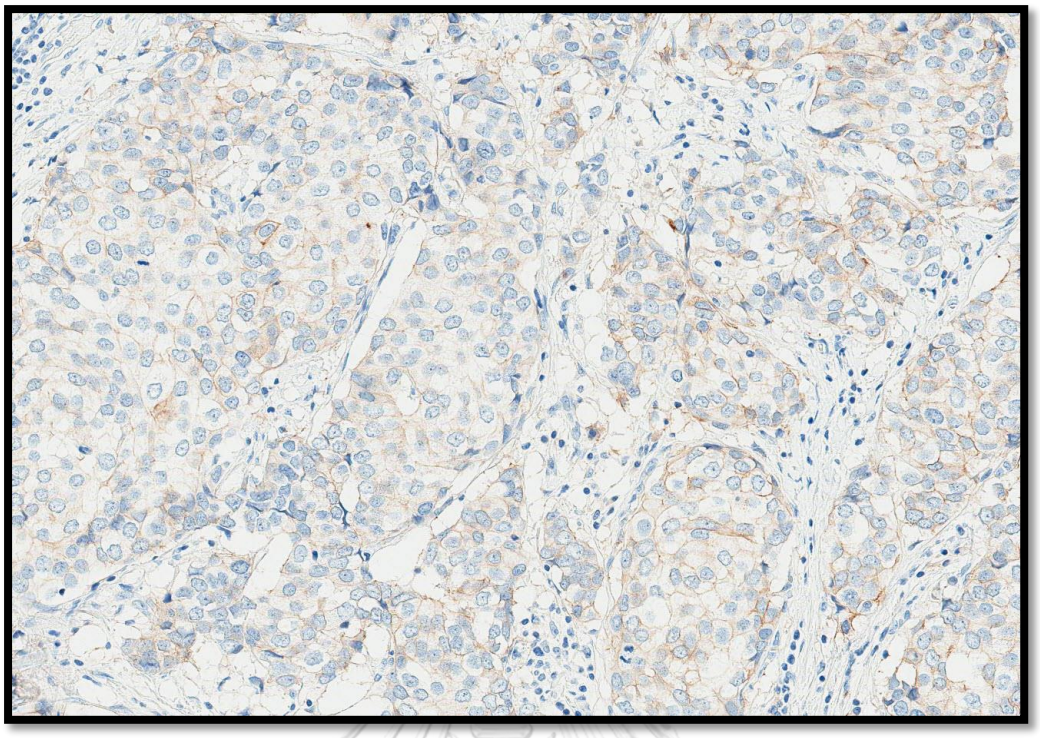

Figure 25: Case no. 47 - Areas with weak and incomplete staining

DISH analysis revealed a HER2/CEN17-ratio of 1.54:1 with an average of 1.75 CEN/nucleus.

These two cases (case no. 20 and 47) both showed heterogeneous staining with areas of 3+ cells while the predominant areas of the tumor were negative or equivocal. The clinical relevance of this has been investigated, and some studies indicate a poorer disease-free survival compared to patients with tumors exhibiting homogeneous HER2 amplification [30]. From a clinical perspective, however, a recent study found no clinical benefit of Trastuzumab treatment in patients with low levels of HER2 expression [31]. Other authors advocate considering a tumor HER2 amplified even if gene amplification is detected in only one area [32].

Particularly case no. 20, but also case no. 47 had areas of poor fixation, which is generally considered a significant source of pre-analytical error. According to the manufacturer, less than 6 hours fixation may cause nuclear digestion and loss of signals [28, 33]. In our situation, this could lead to concerns whether these cases were truly non-amplified or suffering from pre-analytical errors.

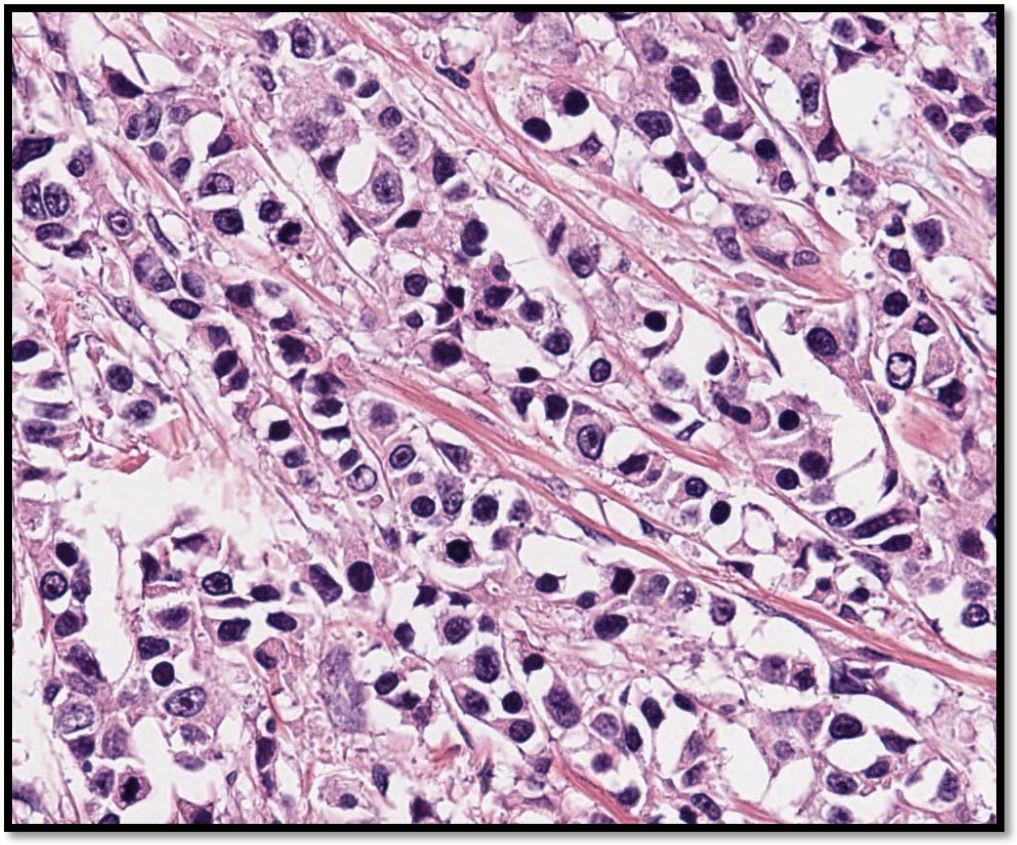

Figure 26: Case no. 20 - Area with poor fixation

The three clinically significant discordant “outliers” are summarized in table 10.

Table 10: Summary of "outliers"

Serial no.	IHC		DIA (% 3+ cells)	DISH	Conclusion
	Manual	Consensus			
20	Pos	Pos	Pos (13.4%)	Non-amp	Borderline case, heterogeneous IHC staining, areas with poor fixation
47	Equi	Pos	Equi (7.2%)	Non-amp	Borderline case, heterogeneous IHC staining, areas with poor fixation
75	Equi	Pos	Pos (52.4%)	Non-amp	Definite overexpression, CEN17 gain (polysomy)

It should be noted that the false positives is our study population would not have affected patient treatment, as all HER2 IHC positive cases are subjected to confirmatory DISH testing according to local guidelines.

Cases which by DIA fell within a grey zone of ±2% were reanalyzed after doubling of the ROI in order to reduce selection bias. Out of 109 cases, six cases had a second round of DIA which in 5 of 6 cases returned the same HER2 score. In one case (no. 56), the DIA reanalysis increased the final HER2 score from equivocal to positive in a HER2 amplified tumor. While these small numbers are hardly statistically significant, this method may theoretically reduce selection bias in cases with heterogeneous staining.

The results obtained in this study are in line with those of Holten-Rossing et al., who found a 68% decrease in numbers of equivocal cases when evaluated by DIA (HER2-CONNECT) instead of manual microscopy [13]. A comparable conclusion was reached by Helin et al., who in a series of 750 cases saw the proportion of equivocal cases reduced from 34% to 10.1 % (a reduction of 70%) by applying DIA [14].

While effectively offering a more accurate method of IHC HER2 assessment, digital image analysis still has its shortcomings. In the application used in this study, regions of interest still need to be manually delineated, which introduces some degree of subjectivity in the analysis. Newer algorithms have been developed which are capable of automated tumor detection (eg. Aperio GENIE), but this feature is not yet available at our department.

The imaging chain of a WSI acquisition system comprises several components each of which may affect the result: Light source, optics, sensor, image compression, color correction, etc. Also different algorithms used for DIA may obtain different results, and even reproducibility using the same algorithm may be affected whenever the algorithm is adjustable [24]. Adding to this are all the parameters relating to the IHC staining quality of each section. The immunostained sections remain the cornerstone on which the HER2 detection is based, hence the issues pertaining to this (incl. fixation time, protocol differences) also apply for the DIA.

Manual assessment of IHC HER2 expression has the advantage of including other factors in the evaluation, eg. histological grade, Ki67 and hormone receptor status,

which in some cases might aid the pathologist in the decision-making. These factors are not part of the DIA algorithm, and therefore all DIA HER2 score results should be reviewed by a pathologist before being signed off.

6.2 Impact of new ASCO/CAP HER2 scoring guidelines

An updated guideline on HER2 testing from ASCO/CAP was "early online released" a week before the finalization of this thesis. It includes revised recommendations concerning ISH scoring particularly relating to "borderline" situations, as well as rephrasing of the IHC 2+ score criteria.

According to the 2013 guideline, IHC HER2 score 2+ was defined as:

> *''...circumferential membrane staining that is incomplete and/or weak/moderate and within > 10% of tumor cells or complete and circumferential membrane staining that is intense and within ≤ 10% of tumor cells."(4)*

This definition is rephrased in the updated 2018 guideline as:

> *"...weak to moderate complete membrane staining observed in > 10% of tumor cells." [4]*

With a footnote stating that:

> *"Unusual staining patterns of HER2 by IHC can be encountered that are not covered by these definitions. In practice, these patterns are rare and if encountered should be considered IHC 2+ equivocal. As one example, some specific subtypes of breast cancers can show IHC staining that is moderate to intense but incomplete (basolateral or lateral) and can be found to be HER2 amplified. Another example is circumferential membrane IHC staining that is intense but within < 10% of tumor cells (heterogeneous but very limited in extent)." (ibid.)*

The new definition thus seems to include the same staining patterns in a more concise wording, while the more unusual equivocal 2+ staining patterns are mentioned in the footnote. It remains to be seen if or how this update will affect the scoring practice, but the impact is likely to be minimal, as it is mainly a linguistic update rather that a definitional.

The updated 2018 guideline also clarifies how to interpret certain ISH results previously deemed equivocal or contentious. A complete interpretation of the new guideline is beyond the scope of this thesis, but the situations in question deserve brief mentioning. These three (rare) scenarios were previously interpreted as positive (A and B) or equivocal (C), but the revised algorithmic approaches to interpret such cases could potentially change the final HER2 category:

A. Invasive cancers with an HER2/CEP17 ratio of ≥ 2.0 but an average HER2 copy number of < 4.0 signals per cell
B. Invasive cancers with an average HER2 copy number of ≥ 6.0 signals per cell but a HER2/CEP17 ratio of < 2.0
C. Invasive cancers with an average HER2 copy number of ≥ 4.0 but < 6.0 signals per cell and an HER2/CEP17 ratio of < 2.0 (ibid.)

The DISH results of all included cases were reviewed in light of the new guideline to get an impression of the potential impact. Out of 79 cases with an available DISH report, only one case fell within one of the revised categories (case no. 28). This case was assessed as equivocal (2+) by all IHC modalities and deemed amplified by DISH (HER2/CEP17-ratio = 2.59 with an average HER2 copy number of 3.75 signals per cell)[4]. Figure 28 displays the recommended revised approach to resolve such cases.

[4] The apple of discord lies in data from the early trastuzumab trials showing that patients in this subgroup who were assigned to the trastuzumab arm did not seem to derive any improvement in disease-free or overall survival despite being “HER2 amplified” according to previous definitions.

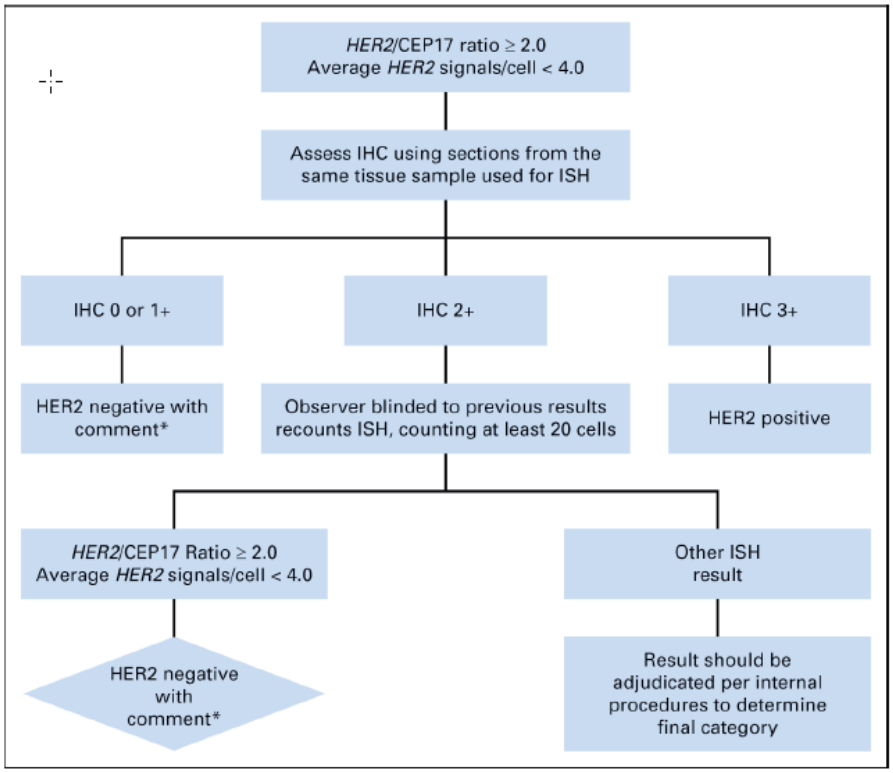

Figure 27: Revised 2018 DISH algorithm (ibid.)

Our case was IHC 2+ and would - according to the algorithm - require renewed DISH scoring by an observer blinded to the previous result to reach a final HER2 category. No matter the outcome, this case was IHC scored as equivocal by all modalities, so any revision of the final HER2 category would affect the DISH concordance calculations for all modalities equally. Sensitivity, specificity and clinical test parameter calculations would remain unchanged as they do not include the IHC equivocal category anyway. The revised guidelines are therefore high unlikely to significantly affect the conclusions of this study.

6.3 Potential benefit of implementing DIA in the routine pathology

Based on the results obtained in this study, the potential benefits of implementing DIA into the routine breast pathology in our department at King Chulalongkorn Memorial Hospital should be pondered. As previously discussed, the main outcome of this study is the reduction of IHC equivocal cases by approximately 67% without significantly affecting sensitivity or specificity. Assuming 600 cases per year with 27% equivocal results by IHC and a price tag of 10,100 baht/case, the yearly cost saving can be calculated:

Approximated yearly cost savings = 600 cases/year x 0.27 x 0.67 x 10,100 baht/case ≈ 1,096,000 baht/year

The implementation would not require any financial investments, but only a change in working procedures. A saving of this magnitude – be it private or public health care spending – is substantial and should constitute an incentive for implementation. A further advantage would be the possibility of reducing the average turnaround time for a final breast cancer pathology report, owing to the fact that DIA of equivocal cases could possibly be accomplished within 1-2 working days (depending on the workflow), whereas DISH analysis in our department has an average turnaround time of 4-5 days.

6.4 Considerations about DIA implementation

While DIA is widely incorporated into medical research, and increasingly into clinical use, official recommendations and guidelines are still lacking. CAP is allegedly working on a guideline, but as of yet (May 2018) nothing has been officially published.

Taking into account the significant clinical implications of HER2 assessment in breast cancer, the implementation of a new method should be carefully considered to ensure robustness of the system and appropriate quality control and assurance. Before implementing the new method into the routine pathology, results of the new method should be compared to an alternative, validated method serving as gold standard. - It was the intention with this study to contribute to this validation. Furthermore, reproducibility should be assured by comparing results of different batches (eg. immunostains) and different operators (eg. the personnel selecting ROI for analysis). The latter could be accomplished by making blinded double or triple analysis of the samples submitted for DIA in the introductory period following implementation. Reproducibility across different batches of immunostains and people involved in the immunostaining process has been validated by this study. After implementation, processes should be in place to ensure that any changes to the DIA system which might affect the clinical result are tested and validated. Designated staff should be chosen to oversee the DIA process, workflow and standard procedures and continuously monitor and document the performance of

the system. There should be standard operating procedures in place to ensure the necessary qualifications and the required training of any personnel involved in the DIA chain. As with other complex procedures, ensuring a certain volume of cases for each person involved is desirable.

A formal DIA scoring report should be created to include pertinent parameters which would add value to the clinical decision making (eg. percentage scores to highlight borderline cases and tumor heterogeneity). This DIA report should be integrated into the standard pathology report, and the final DIA result verified by a pathologist before signing off the report. All DIA results should be stored in easily accessible electronic files and should be subjected to internal auditing and external inspections as part of continuous quality assurance and quality control.

7 Conclusion

In this study we have compared the assessment of HER2 expression in 109 breast cancers performed by manual microscopy by a single pathologist (standard method), manual evaluation by a consensus panel and assessment by digital image analysis (Aperio Imagescope). Substantial agreement was found between the three different methods. In our sample population, the fraction of IHC equivocal cases ranged from 44.0% (original manual score), to 33.3% (consensus score) and 14.7% (DIA).

We then compared the results obtained by the same three methods with the results of dual in-situ hybridization, and excellent sensitivity and specificity was obtained for

all methods. None of the methods had any false negatives, and the false positives ranged from 0.9% (original manual score) to 1.8% (DIA) and 2.8% (consensus score). Possible explanations for these false positives include intratumoral heterogeneity, poor fixation and centromere 17 gains. The false positives would not have had any clinical impact in our setting, since all HER2 IHC positive cases are confirmed by ISH according to current guidelines and legislation in Thailand. This effectively prevents any “false positive” patients from receiving unnecessary anti-HER2 treatment.

The results of our study suggest that integration of DIA into the diagnostic workflow could significantly reduce the number of equivocal cases while maintaining a very high level of test sensitivity. These findings are integrated into a proposed new test algorithm for HER2 status evaluation of breast cancer in our department, as shown below in comparison with the current algorithm. The main difference lies in the group of cases initially assessed as equivocal by the case owner pathologist. These cases are then subjected to DIA, and only cases which are assessed as equivocal (2+) or positive (3+) by DIA will be referred for confirmatory DISH. If this approach was adopted in the evaluation of the study population, only 14.7% would have needed additional HER DISH (versus 44% according to the original algorithm), which equals a reduction of approximately 67%. Furthermore, following the proposed new algorithm, no patients would receive unnecessary anti-HER2 treatment and - importantly - no patients would mistakenly be deprived of relevant treatment with anti-HER2 medications due to a false negative test results.

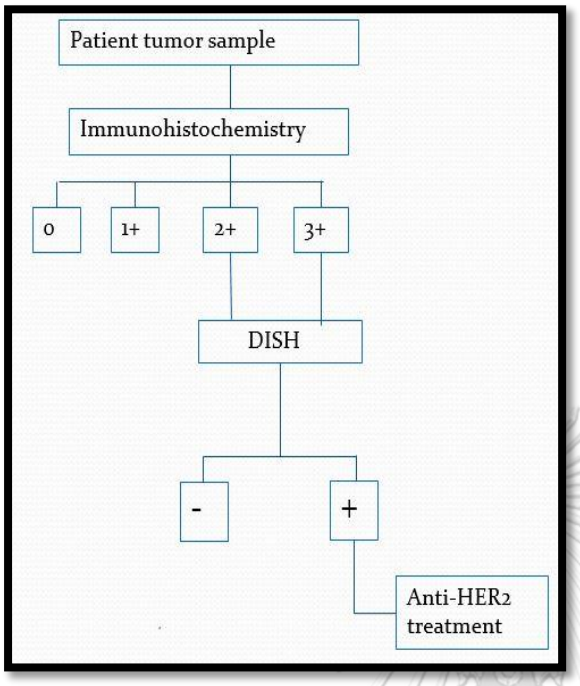

Figure 28: Current algorithm

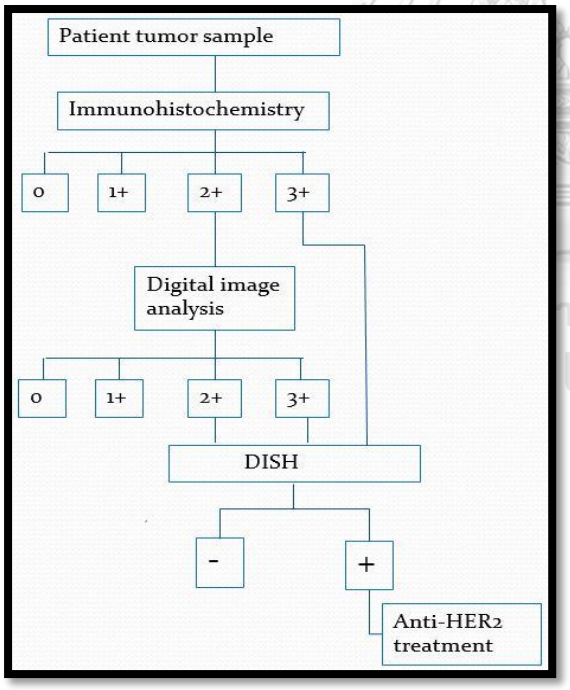

Figure 29: Proposed new algorithm with DIA

For institutes where DIA is not available, consensus manual assessment may be considered as an alternative to conventional manual assessment performed by a single pathologist. As demonstrated in this study, consensus assessment may reduce the proportion of equivocal cases, although not as significantly as with DIA. Figure 30 depicts a HER2 assessment algorithm featuring consensus assessment of equivocal cases.

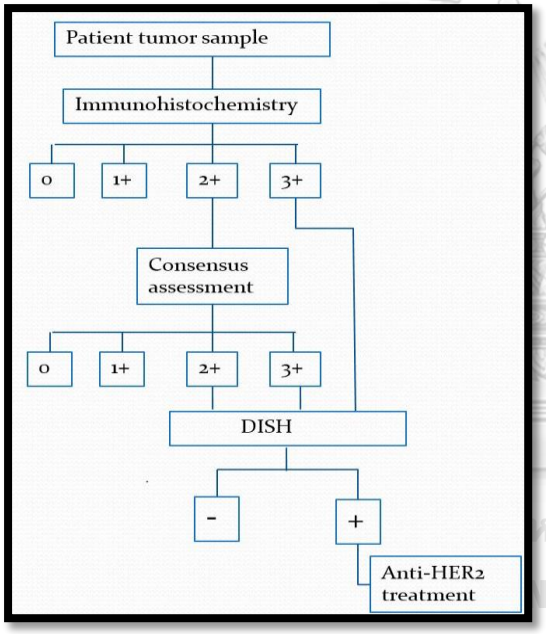

Figure 30: Proposed new algorithm with consensus assessment

In conclusion, quantitative digital image analysis is highly sensitive and specific when compared to DISH in detecting IHC HER2 overexpression. It is an accurate and objective method which can serve as a diagnostic aid in the assessment of HER2 expression in breast cancer, and the reduced need for reflex DISH testing would confer substantial economic savings. Prior to implementation of DIA into the routine pathology, a robust system must be in place to ascertain quality control and quality assurance.

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APPENDIX

7.1 H&E staining protocol

- Xylene 1 3.30 min
- Xylene 2 3.30 min
- Absolute alcohol 2 min
- 95% alcohol 2 min
- 95% alcohol 2 min
- Tap water 1 min
- Hematoxylin 5 min
- Deionized water 1 min
- Bluing buffer 1 min
- Tap water 3 min
- 95% alcohol 1 min
- Eosin 5 min
- 95% alcohol 1 min
- Absolute 1 min
- Absolute 1 min
- Xylene 1 min

Reagents:

Dako Hematoxylin (ready-to-use)

Dako Eosin (ready-to-use)

Dako Bluing Buffer (ready-to-use)

7.2 Imagescope parameter settings

'Membrane v9' Parameters

View Width	1000
View Height	1000
Overlap Size	100
Image Zoom	1
Markup Compression Type	Same as processed image
Compression Quality	30
Classifier Neighborhood	0
Classifier	None
Class List	
Averaging Radius (um)	1
Blue Curvature Threshold	1
Threshold Type	Edge Threshold Method
Lower Blue Threshold	0
Upper Blue Threshold	220
Min Nuclear Size (um^2)	25
Max Nuclear Size (um^2)	500
Min Nuclear Roundness	.1
Min Nuclear Compactness	0
Min Nuclear Elongation	.1
Cytoplasmic Correction	Yes
Cell/Nucleus Requirement	All Cells
Max Cell Radius (um)	5
Min Cell Size (um^2)	50
Max Cell Size (um^2)	2000
Min Cell Roundness	.1
Min Cell Compactness	.1
Min Cell Elongation	.1
Background Threshold	240
Weak(1+) Threshold	200
Moderate(2+) Threshold	170
Strong(3+) Threshold	105

Completeness Threshold	50
Use Mode	Analysis/Tuning
Mark-up Image Type	Analysis
Classifier Type	IHCMembrane
Classifier Definition File	IHCMembraneTraining
Display Plots	No

7.3 DIA algorithm test set

No.	SP no.	Algorithm setting	HER2 score	Cells %				Total cells	Pathology report	
				0	1	2	3		IHC	DISH
1	60-114	Default	2	6	68	26	0	6097	2+	Neg
		Adjusted	2	5	66	29	0	4130		
2	60-351	Default	2	7	65	27	0	4248	2+	Amp
		Adjusted	2	6	67	27	0	2964		
3	60-443	Default	1	31	66	3	0	5977	2+	Neg
		Adjusted	1	24	73	3	0	3357		
4	60-613	Default	3	1	35	17	47	6972	3+	Amp
		Adjusted	3	0	31	20	50	4570		
5	60-1094	Default	2	15	67	17	0	918	2+	Neg
		Adjusted	2	12	71	16	0	513		
6	60-1552	Default	1	8	85	6	0	509	2+	Neg
		Adjusted	1	7	84	9	0	357		
7	60-2644	Default	1	42	53	4	0	549	2+	Neg
		Adjusted	1	39	57	4	0	379		
8	60-3665	Default	3	0	23	8	69	12921	3+	Amp
		Adjusted	3	0	21	9	70	7183		
9	60-3683	Default	3	0	12	22	65	2265	3+	Amp
		Adjusted	3	0	10	24	66	1350		
10	60-3776	Default	1	71	29	0	0	4836	2+	Neg
		Adjusted	1	69	31	0	0	3136		
11	60-4035	Default	2	0	48	51	2	12210	2+	Amp
		Adjusted	2	6	58	35	2	7597		
12	60-4655	Default	2	1	41	53	6	6508	2+	Neg
		Adjusted	2	1	38	56	5	4190		
13	60-4866	Default	2	2	51	38	9	8939	3+	neg
		Adjusted	2	1	49	42	8	4936		
14	60-4881	Default	2	1	54	44	1	1121	2+	neg
		Adjusted	2	0	50	49	0	684		
15	59-309526	Default	1	60	37	3	0	1303	2+	neg
		Adjusted	1	59	38	3	0	902		
16	59-310983	Default	1	46	53	1	0	5470	2+	neg
		Adjusted	1	36	63	1	0	2030		

7.4 Raw data: Manual and DISH

Serial number	SP-number	DISH HER2 status	Manual IHC (pathology report)
1	60-1987	Amp	3+
2	60-1643		0
3	60-1094	Neg	2+
4	60-11245		0
5	60-2809		0
6	60-114	Neg	2+
7	60-2605	Neg	2+
8	60-13500	Neg	1+
9	60-3613 B	Neg	2+
10	60-1983		0
11	60-5646	Amp	3+
12	60-2249	Neg	2+
13	59-312129	Amp	3+
14	60-2621	Neg	2+
15	60-369		1+
16	59-312678	Amp	3+
17	60-209	Neg	2+
18	59-312932	Amp	2+
19	60-2375	Neg	2+
20	60-4866	Neg	3+
21	60-3577	Amp	2+
22	60-4881	Neg	2+
23	60-1419	Neg	2+
24	59-310806	Neg	2+
25	60-2644	Neg	2+
26	60-3232	Amp	3+
27	59-310078		0
28	60-5039	Amp	2+
29	60-1552	Neg	2+
30	60-3665	Amp	3+
31	60-4707	Neg	2+
32	60-6472		1+
33	60-2677	Neg	2+
34	60-6627		0
35	60-810	Neg	2+
36	60-1049	Amp	3+
37	60-2263		0
38	60-2678		1+
39	60-3442	Neg	2+
40	60-5886		0
41	60-6516	Neg	1+
42	60-1595	Amp	3+
43	60-3289	Neg	2+
44	60-6302	EXCLUDED (only DCIS)	
45	60-4062	Neg	2+
46	60-443	Neg	2+
47	60-4655	Neg	2+
48	60-4035	Amp	2+

49	60-1793	Neg	2+
50	59-312785	Neg	2+
51	60-1984	Neg	2+
52	60-5414		0
53	60-3614	Neg	2+
54	60-788	Amp	3+
55	60-1222	Neg	2+
56	60-4481	Amp	3+
57	59-310925		0
58	60-365	Neg	2+
59	60-7638		1+
60	60-3987	Neg	2+
61	60-4010		1+
62	59-311103		1+
63	60-2763	Amp	3+
64	60-3005	Neg	2+
65	60-4517	Neg	2+
66	60-5536		1+
67	59-310356		0
68	60-600	Neg	2+
69	60-613	Amp	3+
70	60-7037	Neg	1+
71	60-5633	Amp	3+
72	60-8131		0
73	60-3683	Amp	3+
74	59-312360	Amp	3+
75	60-414	Neg	2+
76	59-311356		1+
77	59-312201		1+
78	60-3101	Neg	2+
79	60-3167	Amp	3+
80	60-2290	Neg	2+
81	60-1986	Neg	1+
82	59-310470	Amp	3+
83	60-3776	Neg	2+
84	60-5223	Amp	3+
85	59-311827		0
86	60-1803	Amp	3+
87	60-5608	Amp	3+
88	59-308715	Neg	2+
89	60-4601		1+
90	60-3810	Amp	3+
91	59-312025		0
92	60-2374	Neg	2+
93	60-4039		0
94	60-13629		0
95	60-3515	Neg	2+
96	59-310983	Neg	2+
97	60-4905		1+
98	59-304688	Amp	3+

99	59-311777		0
100	60-1847	Neg	2+
101	60-351	Amp	2+
102	60-3995		1+
103	60-364	Neg	2+
104	59-309754	Amp	3+
105	60-3965		1+
106	60-1456	Neg	1+
107	60-4401	Neg	2+
108	60-2264	Neg	2+
109	59-310296		0
110	59-306780	Amp	3+

7.5 Raw data: Consensus score

Serial number	SP-number	Consensus	SK	ShS	MRJ
1	60-1987	3	3	3	3
2	60-1643	0	0	0	0
3	60-1094	2	2	2	2
4	60-11245	0	0	0	0
5	60-2809	0	0	0	0
6	60-114	2	2	2	2
7	60-2605	0/1	0	1	2
8	60-13500	2	2	2	2
9	60-3613 B	2	2	2	2
10	60-1983	0	0	0	0
11	60-5646	3	3	3	3
12	60-2249	2	3	2	2
13	59-312129	3	3	3	3
14	60-2621	2	2	2	2
15	60-369	0	0	1	0
16	59-312678	3	3	3	3
17	60-209	2	2	2	2
18	59-312932	3	3	3	3
19	60-2375	1	1	1	2
20	60-4866	3	3	3	2
21	60-3577	3	3	3	2
22	60-4881	2	2	2	2
23	60-1419	2	2	2	2
24	59-310806	2	2	2	1
25	60-2644	0/1	2	0	1
26	60-3232	3	3	3	3
27	59-310078	0	0	0	0
28	60-5039	2	2	2	2
29	60-1552		3	2	1
	2nd scoring	2	1	2	2
30	60-3665	3	3	3	3
31	60-4707	2	2	2	2
32	60-6472	1	1	0	1
33	60-2677	2	2	1	2
34	60-6627	0	0	0	0
35	60-810	2	1	2	2
36	60-1049	3	3	3	3
37	60-2263	0	0	0	0
38	60-2678	0	0	0	0
39	60-3442	2	2	2	2
40	60-5886	0	0	0	0
41	60-6516	2	1	2	2
42	60-1595	3	3	3	3
43	60-3289	2	2	1	2
44	60-6302		EXCLUDED (only DCIS)		
45	60-4062	1	1	1	1
46	60-443	1	1	1	2
47	60-4655	3	3	3	3
48	60-4035		EXCLUDED	1	2

49	60-1793	2	2	1	2
50	59-312785	2	2	2	2
51	60-1984	2	2	2	2
52	60-5414	1	1	1	0
53	60-3614	2	2	2	2
54	60-788	3	3	3	3
55	60-1222	1	1	1	1
56	60-4481	3	3	3	2
57	59-310925	0	0	0	0
58	60-365	2	3	2	2
59	60-7638	1	1	1	1
60	60-3987	2	2	2	2
61	60-4010	0	0	0	0
62	59-311103	0	0	0	0
63	60-2763	3	3	3	3
64	60-3005	2	2	2	2
65	60-4517	2	2	2	2
66	60-5536	0	1	0	0
67	59-310356	1	1	0	1
68	60-600	2	2	1	2
69	60-613	3	3	3	3
70	60-7037	2	1	2	2
71	60-5633	3	3	3	3
72	60-8131	0	0	0	0
73	60-3683	3	3	3	3
74	59-312360	3	3	3	3
75	60-414	3	3	3	3
76	59-311356	1	1	1	1
77	59-312201	1	1	1	1
78	60-3101	2	2	2	2
79	60-3167	3	3	3	3
80	60-2290	1	1	0	1
81	60-1986	1	1	1	1
82	59-310470	3	3	3	3
83	60-3776	1	1	1	1
84	60-5223	3	3	3	3
85	59-311827	0	0	0	1
86	60-1803	3	3	3	3
87	60-5608	3	3	3	3
88	59-308715	2	3	2	2
89	60-4601	0	0	0	0
90	60-3810	3	3	3	3
91	59-312025	0	0	0	1
92	60-2374	2	2	2	2
93	60-4039	0	0	1	0
94	60-13629	0	0	1	0
95	60-3515	1	1	2	1
96	59-310983	1	1	1	2
97	60-4905	1	0	1	1
98	59-304688	3	3	3	3

99	59-311777	1	0	1	1
100	60-1847	2	2	1	2
101	60-351	2	2	2	2
102	60-3995	0	0	1	0
103	60-364	1	0	1	1
104	59-309754	3	3	3	3
105	60-3965	0	0	0	0
106	60-1456	2	1	2	2
107	60-4401	2	2	2	2
108	60-2264	2	2	2	2
109	59-310296	0	0	0	0
110	59-306780	3	3	3	3

Ad no. 29: The slide was reevaluated due to total disagreement.
Ad no. 44: Excluded due to lack of invasive component.
Ad no. 48: Excluded because one pathologist found it inadequate for evaluation due to poor fixation.

7.6 Raw data: DIA

			DIA first run							DIA second run						
				%							%					
Serial number	SP-number	Final DIA	Score	3+	2+	1+	0,0	ROI	Total cells	Score	3+	2+	1+	0	ROI	Total cells
1	60-1987	3	3	10,3	62,5	27,2	0,0	28	7964	3	10,5	60,1	29,2	0,2	74	12491
2	60-1643	1	1	0,0	0,0	10,5	89,5	15	1268							
3	60-1094	2	2	0,2	16,5	73,3	10,0	54	502							
4	60-11245	1	1	0,0	0,3	19,5	80,3	30	4142							
5	60-2809	1	1	0,0	3,3	17,2	79,5	30	2223							
6	60-114	2	2	0,0	30,7	66,7	2,6	36	8383							
7	60-2605	1	1	0,0	5,7	74,2	20,1	24	5603							
8	60-13500	1	1	0,0	2,3	65,3	32,5	32	977							
9	60-3613 B	1	1	0,0	7,3	82,2	10,5	35	9206							
10	60-1983	0	0	0,0	0,0	0,0	100,0	24	256							
11	60-5646	3	3	83,9	0,5	15,0	0,5	25	7266							
12	60-2249	1	1	0,0	1,6	66,0	32,4	30	1160							
13	59-312129	3	3	84,5	4,1	11,4	0,0	32	12206							
14	60-2621	1	1	0,0	7,9	50,9	41,2	48	19929							
15	60-369	1	1	0,0	0,2	48,6	51,2	39	8854							
16	59-312678	3	3	80,4	0,8	18,9	0,0	38	3659							
17	60-209	1	1	0,0	9,3	79,9	10,8	48	2610	1	0	8,6	76,9	14,4	103	4546
18	59-312932	3	3	77,6	4,2	18,2	0,0	18	956							
19	60-2375	1	1	0,0	0,2	21,0	78,7	49	4733							
20	60-4866	3	3	13,4	49,0	37,0	0,7	53	4133							
21	60-3577	3	3	13,0	57,9	29,1	0,0	39	3609							
22	60-4881	2	2	0,9	38,2	59,2	1,7	32	1290							
23	60-1419	2	2	0,0	16,2	73,5	10,3	92	7046							
24	59-310806	1	1	0,0	3,4	79,6	17,1	28	5045							
25	60-2644	1	1	0,0	7,8	60,6	31,6	26	718							
26	60-3232	3	3	81,9	2,1	16,0	0,0	31	7919							
27	59-310078	0	0	0,0	0,0	0,4	99,6	31	558							
28	60-5039	2	2	0,2	25,9	68,3	5,6	50	4252							
29	60-1552	1	1	0,0	9,3	83,0	7,7	33	364	1	0	7,2	82,5	10,3	99	526
30	60-3665	3	3	66,5	12,3	21,1	0,1	97	8416							

31	60-4707	1	1	0,0	6,5	76,8	16,7	58	3444								
32	60-6472	1	1	0,0	0,0	16,2	83,8	38	1305								
33	60-2677	1	1	0,0	4,0	73,8	22,1	37	1338								
34	60-6627	0	0	0,0	0,0	3,9	96,1	22	1959								
35	60-810	1	1	0,0	4,0	90,1	5,9	35	2200								
36	60-1049	3	3	12,9	39,2	44,4	3,6	65	5231								
37	60-2263	0	0	0,0	0,0	1,4	98,6	18	1534								
38	60-2678	1	1	0,0	0,4	51,2	48,4	18	3748								
39	60-3442	1	1	0,0	4,5	74,3	21,2	19	9167								
40	60-5886	0	0	0,0	0,0	4,3	95,7	16	886								
41	60-6516	1	1	0,0	5,4	75,6	19,0	33	1174								
42	60-1595	3	3	73,9	6,4	19,7	0,0	17	5198								
43	60-3289	1	1	0,0	0,3	25,0	74,7	29	657								
44	60-6302	EXCLUDED (only DCIS)															
45	60-4062	1	1	0,0	0,3	17,6	82,2	26	2670								
46	60-443	1	1	0,0	2,1	68,7	29,2	34	2572								
47	60-4655	2	2	7,2	56,6	35,8	0,4	44	6268								
48	60-4035	2	2	1,5	52,9	45,6	0,0	16	6546								
49	60-1793	2	2	0,0	13,8	70,8	15,3	45	2197								
50	59-312785	2	2	0,1	26,2	65,0	8,7	37	4846								
51	60-1984	1	1	0,0	6,1	60,7	33,1	39	1648								
52	60-5414	1	1	0,0	0,1	16,4	83,5	20	3790								
53	60-3614	2	2	0,0	25,2	71,0	3,7	33	7644								
54	60-788	3	3	69,3	5,9	24,7	0,0	20	16300								
55	60-1222	1	1	0,0	0,6	37,8	61,7	23	2490								
56	60-4481	3+	2	10,0	52,8	36,5	0,8	34	7934	3	10.1	53.1	36.0	0.8	63	13346	
57	59-310925	0	0	0,0	0,0	0,7	99,3	16	440								
58	60-365	2	2	1,9	58,8	38,5	0,9	34	6299								
59	60-7638	1	1	0,0	0,0	33,8	66,2	17	1944								
60	60-3987	2	2	0,0	21,2	68,9	9,9	45	3597								
61	60-4010	0	0	0,0	0,0	1,1	98,9	17	1175								
62	59-311103	0	0	0,0	0,0	3,2	96,8	17	653								
63	60-2763	3	3	77,8	1,2	20,9	0,0	20	1285								

64	60-3005	1	1	0,0	6,0	75,5	18,5	27	3939								
65	60-4517	2	2	0,0	12,5	69,7	17,8	40	3178								
66	60-5536	1	1	0,0	0,2	53,5	46,4	22	666								
67	59-310356	1	1	0,0	0,3	39,0	60,7	27	2621								
68	60-600	1	1	0,0	9,2	62,5	28,3	52	18008	1	0	7,7	62	30,3	111	34827	
69	60-613	3	3	50,9	21,0	28,0	0,2	43	7506								
70	60-7037	1	1	0,0	0,2	62,0	37,8	40	7292								
71	60-5633	3	3	71,7	12,2	16,1	0,0	28	9109								
72	60-8131	0	0	0,0	0,0	9,7	90,3	48	780								
73	60-3683	3	3	75,5	15,4	9,1	0,0	24,0	3440,0								
74	59-312360	3	3	83,4	0,2	16,4	0,0	21	5183								
75	60-414	3	3	52,4	24,4	23,1	0,0	30	7168								
76	59-311356	1	1	0,0	0,5	45,4	54,1	39	1403								
77	59-312201	1	1	0,0	0,6	59,5	40,0	42	2646								
78	60-3101	1	1	0,0	7,0	83,8	9,2	52	4153								
79	60-3167	3	3	79,2	0,2	20,7	0,0	34	1453								
80	60-2290	1	1	0,0	0,1	47,8	52,1	28,0	11253,0								
81	60-1986	1	1	0,0	0,9	45,6	53,4	43	1271								
82	59-310470	3	3	64,3	16,5	19,1	0,0	29,0	6295,0								
83	60-3776	1	1	0,0	0,4	39,5	60,1	37,0	8834,0								
84	60-5223	3	3	83,2	0,0	16,7	0,0	12,0	1068,0								
85	59-311827	0	0	0,0	0,0	0,0	100,0	21,0	3732,0								
86	60-1803	3	3	82,1	0,5	17,4	0,0	25,0	5846,0								
87	60-5608	3	3	76,8	0,8	22,4	0,0	16,0	1555,0								
88	59-308715	2	2	6,9	49,3	41,9	1,9	48,0	1572,0								
89	60-4601	0	0	0,0	0,0	0,7	99,3	23,0	2761,0								
90	60-3810	3	3	78,1	0,0	21,9	0,0	16,0	894,0								
91	59-312025	1	1	0,0	0,5	31,2	68,3	31,0	948,0								
92	60-2374	1	1	0,0	3,6	70,4	26,0	36,0	713,0								
93	60-4039	0	0	0,0	0,0	6,8	93,2	31,0	732,0								
94	60-13629	0	0	0,0	0,0	1,4	98,6	17,0	348,0								
95	60-3515	1	1	0,0	5,5	68,3	26,2	38,0	634,0								
96	59-310983	1	1	0,0	1,4	70,8	27,8	19,0	1693,0								
97	60-4905	1	1	0,0	0,7	32,4	66,9	37,0	148,0								
98	59-304688	3	3	85,6	1,0	13,4	0,0	24,0	3326,0								
99	59-311777	1	1	0,0	0,3	18,9	80,7	31,0	1262,0								
100	60-1847	1	1	0,0	6,5	66,5	26,9	87,0	4818,0								
101	60-351	2	2	0,3	30,8	60,3	8,6	15	2420								
102	60-3995	1	1	0,0	0,4	47,9	51,8	19	1509								
103	60-364	1	1	0,0	0,8	46,2	52,9	21	2608								
104	59-309754	3	3	84,4	1,8	13,8	0,0	17	2144								
105	60-3965	1	1	0,0	0,5	36,2	63,3	17	3057								
106	60-1456	1	1	0,0	6,3	83,4	10,3	22	2015								
107	60-4401	1	1	0,0	7,2	61,2	31,5	60,0	5590,0								
108	60-2264	2	2	0,4	16,1	76,3	7,1	20	11831								
109	59-310296	0	0	0,0	0,0	0,6	99,4	4	11105								
110	59-306780	3	3	85,5	0,8	13,7	0,0	17	7235								

7.7 Score forms

HER2 score form			BOX 1 Morten
Number	SP-number	HER2 score	Comment
1	60-1987	3+	
2	60-1643	0	
3	60-1094	2+	
4	60-11245	0	
5	60-2809	0	
6	60-114	2+	
7	60-2605	2+	
8	60-13500	2+	
9	60-3613 B	2+	
10	60-1983	0	
11	60-5646	3+	
12	60-2249	2+	
13	59-312129	3+	
14	60-2621	2+	
15	60-369	0	
16	59-312678	3+	
17	60-209	2+	
18	59-312932	3+	
19	60-2375	2+	
20	60-4866	2+	
21	60-3577	2+	
22	60-4881	2+	
23	60-1419	2+	
24	59-310806	1+	
25	60-2644	1+	
26	60-3232	3+	
27	59-310078	0	
28	60-5039	2+	
29	60-1552	1+	
30	60-3665	3+	Micropap component
31	60-4707	2+	
32	60-6472	1+	
33	60-2677	2+	
34	60-6627	0	
35	60-810	2+	
36	60-1049	3+	
37	60-2263	0	
38	60-2678	0	
39	60-3442	2+	
40	60-5886	0	
41	60-6516	2+	
42	60-1595	3+	
43	60-3289	2+	
44	60-6302	0	Excluded: only DCIS
45	60-4062	1+	
46	60-443	2+	
47	60-4655	3+	
48	60-4035	2+	
49	60-1793	2+	
50	59-312785	2+	

HER2 score form			BOX 2 Morten
Number	SP-number	HER2 score	Comment
1	60-1984	2+	
2	60-5414	0	
3	60-3614	2+	
4	60-788	3+	
5	60-1222	1+	
6	60-4481	2+	
7	59-310925	0	
8	60-365	2+	
9	60-7638	1+	
10	60-3987	2+	
11	60-4010	0	
12	59-311103	0	
13	60-2763	3+	
14	60-3005	2+	
15	60-4517	2+	
16	60-5536	0	
17	59-310356	1+	~~Medullary~~ Medullary fea!
18	60-600	2+	
19	60-613	3+	
20	60-7037	2+	
21	60-5633	3+	
22	60-8131	0	
23	60-3683	3+	3683: 3+
24	59-312360	3+	
25	60-414	3+	
26	59-311356	1+	
27	59-312201	1+	
28	60-3101	2+	
29	60-3167	3+	
30	60-2290	1+	~~Solid~~ Solid pap
31	60-1986	1+	
32	59-310470	3+	
33	60-3776	1+	
34	60-5223	3+	
35	59-311827	1+	
36	60-1803	3+	
37	60-5608	3+	
38	59-308715	2+	
39	60-4601	0	
40	60-3810	3+	
41	59-312025	1+	
42	60-2374	2+	
43	60-4039	0	
44	60-13629	0	
45	60-3515	1+	
46	59-310983	2+	
47	60-4905	1+	
48	59-304688	3+	
49	59-311777	1+	
50	60-1847	2+	

HER2 score form			BOX 3 Morten
Number	SP-number	HER2 score	Comment
1	60-351	2+	
2	60-3995	0	
3	60-364	1+	
4	59-309754	3+	
5	60-3965	0	Mucinous ca.
6	60-1456	2+	
7	60-4401	2+	
8	60-2264	2+	
9	59-310296	0	
10	59-306780	3+	

HER2 score form			BOX 1 Sharop
Number	SP-number	HER2 score	Comment
1	60-1987	3	Poor control
2	60-1643	0	
3	60-1094	2	
4	60-11245	0	
5	60-2809	0	
6	60-114	2	
7	60-2605	1	
8	60-13500	2	
9	60-3613 B	2	
10	60-1983	0	
11	60-5646	3	
12	60-2249	2	
13	59-312129	3	
14	60-2621	2	
15	60-369	1	
16	59-312678	3	
17	60-209	2	
18	59-312932	3	
19	60-2375	1	
20	60-4866	3	
21	60-3577	3	
22	60-4881	2	
23	60-1419	2	
24	59-310806	2	
25	60-2644	0	
26	60-3232	3	
27	59-310078	0	
28	60-5039	2	
29	60-1552	2	
30	60-3665	3	
31	60-4707	2	
32	60-6472	0	
33	60-2677	1	
34	60-6627	0	
35	60-810	2	
36	60-1049	3	
37	60-2263	0	
38	60-2678	0	
39	60-3442	2	
40	60-5886	0	
41	60-6516	2	
42	60-1595	3	
43	60-3289	1	
~~44~~	~~60-6302~~	~~x~~	Excluded: Only DCIS
45	60-4062	1	
46	60-443	1	
47	60-4655	3	
48	60-4035	1	most cell stained, but membrane < 10 → DCIS??
49	60-1793	1	
50	59-312785	2	

HER2 score form			BOX 2 Sharop
Number	SP-number	HER2 score	Comment
1	60-1984	2	Intense Stain < 10%
2	60-5414	1	
3	60-3614	2	
4	60-788	3	
5	60-1222	1	
6	60-4481	3	
7	59-310925	0	
8	60-365	2	
9	60-7638	1	
10	60-3987	2	
11	60-4010	0	
12	59-311103	0	
13	60-2763	3	
14	60-3005	2	
15	60-4517	2	
16	60-5536	0	No PV control
17	59-310356	0	
18	60-600	1	No PV control
19	60-613	3	
20	60-7037	2	
21	60-5633	3	
22	60-8131	0	
23	60-3683	3	
24	59-312360	3	
25	60-414	3	
26	59-311356	1	
27	59-312201	1	
28	60-3101	2	
29	60-3167	3	poor control
30	60-2290	0	Solid papillary ca, score the encapsulated part
31	60-1986	1	
32	59-310470	3	
33	60-3776	1	
34	60-5223	3	
35	59-311827	0	
36	60-1803	3	
37	60-5608	3	
38	59-308715	2	
39	60-4601	0	
40	60-3810	3	
41	59-312025	0	
42	60-2374	2	
43	60-4039	1	
44	60-13629	1	
45	60-3515	2	
46	59-310983	1	
47	60-4905	1	
48	59-304688	3	
49	59-311777	1	
50	60-1847	1	

HER2 score form			BOX 3 Shamap
Number	SP-number	HER2 score	Comment
1	60-351	2	
2	60-3995	1	
3	60-364	1	The slide has two tumors; score only the one marked with t
4	59-309754	3	
5	60-3965	0	
6	60-1456	2	
7	60-4401	2	
8	60-2264	2	
9	59-310296	0	
10	59-306780	3	

HER2 score form			BOX 1 Somboon
Number	SP-number	HER2 score	Comment
1	60-1987	3+	
2	60-1643	0	
3	60-1094	2+	
4	60-11245	0	
5	60-2809	0	
6	60-114	2+	
7	60-2605	0	
8	60-13500	2+	
9	60-3613 B	2+	
10	60-1983	0	
11	60-5646	3+	
12	60-2249	3+	
13	59-312129	3+	
14	60-2621	2+	
15	60-369	0	
16	59-312678	3+	
17	60-209	2+	
18	59-312932	3+	
19	60-2375	1+	
20	60-4866	3+	
21	60-3577	3+	
22	60-4881	2+	
23	60-1419	2+	
24	59-310806	2+	
25	60-2644	2+	
26	60-3232	3+	
27	59-310078	0	
28	60-5039	2+	
29	60-1552	3+	
30	60-3665	3+	
31	60-4707	2+	
32	60-6472	1+	
33	60-2677	2+	
34	60-6627	0	
35	60-810	1+	
36	60-1049	3+	
37	60-2263	0	
38	60-2678	0	
39	60-3442	2+	
40	60-5886	0	
41	60-6516	1+	
42	60-1595	3+	
43	60-3289	2+	
~~44~~	~~60-6302~~		Excluded: Only DCIS
45	60-4062	1+	
46	60-443	1+	
47	60-4655	3+	
48	60-4035		* Can't be evaluated due to unpreserved cells
49	60-1793	2+	
50	59-312785	2+	

HER2 score form			BOX 2 Somboon
Number	SP-number	HER2 score	Comment
1	60-1984	2+	
2	60-5414	1+	
3	60-3614	2+	
4	60-788	3+	
5	60-1222	1+	
6	60-4481	3+	
7	59-310925	0	
8	60-365	3+	
9	60-7638	1+	
10	60-3987	2+	
11	60-4010	0	
12	59-311103	0	
13	60-2763	3+	
14	60-3005	2+	
15	60-4517	2+	
16	60-5536	1+	
17	59-310356	1+	
18	60-600	2+	
19	60-613	3+	
20	60-7037	1+	
21	60-5633	3+	
22	60-8131	0	
23	60-3683	3+	
24	59-312360	3+	
25	60-414	3+	
26	59-311356	1+	
27	59-312201	1+	
28	60-3101	2+	
29	60-3167	3+	
30	60-2290	1+	Solid papillary ca; ~~score~~ the encapsulated part
31	60-1986	1+	
32	59-310470	3+	
33	60-3776	1+	
34	60-5223	3+	
35	59-311827	0	
36	60-1803	3+	
37	60-5608	3+	
38	59-308715	3+	
39	60-4601	0	
40	60-3810	3+	
41	59-312025	0	
42	60-2374	2+	
43	60-4039	0	
44	60-13629	0	
45	60-3515	1+	
46	59-310983	1+	
47	60-4905	0	
48	59-304688	3+	
49	59-311777	0	
50	60-1847	2+	

HER2 score form			BOX 3 Somboon
Number	SP-number	HER2 score	Comment
1	60-351	2+	
2	60-3995	0	
3	60-364	0	The slide has two tumors; score only the one marked with blue
4	59-309754	3+	
5	60-3965	0	
6	60-1456	1+	
7	60-4401	2+	
8	60-2264	2+	
9	59-310296	0	
10	59-306780	3+	

VITA