Developing a routine lab test for absolute quantification of Her2 in Formalin Fixed Paraffin Embedded (FFPE) breast cancer tissues using Quantitative Dot Blot (QDB) method

Short title: Absolute quantification of biomarkers in FFPE samples with QDB method

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Abstract

While the transition from current prevailing method of relative and semi-quantitative assessment of solid tumor biomarkers to their absolute quantitation promises improved objectivity, consistency and accuracy in daily clinical practice, this transition is hindered by lacking of suitable technique, especially for Formalin Fixed Paraffin Embedded (FFPE) tissue samples. In this retrospective study, Quantitative Dot Blot (QDB) method was adopted to measure Her2 levels absolutely and quantitatively at protein level using 2X15 μm FFPE breast cancer tissue slices collected sequentially and non-selectively from local hospital. Her2 levels measured from 332 samples using two diagnostic antibodies respectively were strongly correlated (r=0.963, p=0.0000). When the result was evaluated with results from IHC or FISH analysis using Receiving Operating Characteristics (ROC) analysis, we were able to achieve 100% sensitivity (95% CI: 95.6% to 100%) and 99.3% specificity (95% CI: 96.14% to 99.98%) with results from IHC analysis using 0.267 nmole/g as cutoff, and 93.02% sensitivity (95%CI: 80.94% to 98.54%) and 93.68% specificity (95% CI: 86.76% to 97.65%) with results from FISH analysis using 0.261 nmole/g as cutoff. Thus, QDB method is demonstrated to provide objective and consistent assessment of Her2 levels in FFPE samples with comparable results from both IHC and FISH analyses. It is also the first method to achieve absolute quantitation of tissue biomarkers in FFPE samples to meet daily clinical need.

Keywords: Biomarker; Her2; breast cancer; QDB; quantitative; continuous; high throughput; absolute.

Introduction

The accurate assessment of protein biomarkers for diagnosis, prediction and prognosis is essential to the future of precision medicine with direct impact on the targeted therapies for cancer treatment. However, although Immunohistochemistry (IHC) is the prevailing method in biomarker assessment for solid tumors, it is far from satisfactory to meet this goal. The inherent problems with IHC, including its lack of consistency and objectivity, prevent the usage of biomarkers in clinical diagnostics to their full potentials. The overall situation is aggravated by the fact that IHC provides a discrete result, thus unable to reflect the wide range of protein biomarker levels among patients.

There are several ongoing efforts to develop alternative methods to circumvent these limitations. Considering that the majority of clinical samples are preserved as Formalin Fixed Paraffin Embedded (FFPE) block in pathological practice, one more pre-requisite for any method to be adopted in routine clinical practice is that this method must be compatible with FFPE samples.

Selected Reaction Monitoring Mass Spectrometry (SRM-MS) may be the closest one to achieve this goal[1–3]. This method is objective, consistent, sensitive and compatible with FFPE samples. It also measures biomarker in FFPE samples as absolute and continuous variables. However, the complicated analytical processes and high costs limit its usage in routine clinical practice, let alone in the local clinical laboratories and clinical labs in developing countries. In addition, not all the FFPE samples were suitable for SRM-MS analysis[1].

Recently, we have developed independently a Quantitative Dot Blot (QDB) method to measure the content of a protein molecule at tissue level as absolute and continuous variables[4,5]. It is a standardized assay, requiring minimum training and equipment to provide objective and consistent results in regular clinical laboratory. What is more, this method is designed for high throughput analysis.

We hypothesize that the large number of existing diagnostic antibodies clinically validated for IHC analysis (IHC antibodies) should be able to be adopted directly in QDB analysis. These antibodies are subjected to strict governmental regulations to be classified as either IVD or ASR antibodies. In a proof of concept (POC) study, we used frozen breast cancer tissues provided from local hospital to demonstrate that QDB method can measure several biomarkers including Her2, Estrogen receptor (ER), Progesterone receptor (PR) and Ki67 in breast cancer tissues simply, objectively, consistently and in high throughput format using these IHC antibodies (submitted for publication).

In this study, we extended QDB method to measure protein levels of Her2 (Her2/Neu or ERBB2) in 332 FFPE samples using the same two IHC antibodies, EP3 and 4B5 in our POC study. Her2 is one of the most used protein biomarkers among breast cancer patients[6]. Overexpression of this protein has been found among 20 ~30% invasive breast patients[7]. Targeted therapies against Her2, represented by Trastuzumab (Herceptin), have found success in treating patients testing Her2 positive (Her2+), but not with those testing negative (Her2-)[8].

Currently, Her2 level is assessed mainly through IHC. Under this system, the Her2 level is scored as 0, 1+, 2+ and 3+, with those scoring 0 and 1+ being defined as Her2-,

and those 3+ as Her2+. Samples scored as 2+ are defined as equivocal, requiring further Fluorescence in situ hybridization (FISH) analysis to differentiate Her2- from Her2+ samples. So far, the absolute quantification of Her2 levels in FFPE samples were only achievable using SRM-MS technique[1,3,9].

We validated our results by examining the consistency of results measured with EP3 and 4B5 antibodies respectively, and compared our results with those from both IHC and FISH analyses using Receiving Operative Characteristics (ROC) analysis. Finally, we re-evaluate the correlation between Her2 as absolute and continuous variables and other clinicopathologic parameters including age, tumor size, histological grades and metastasis statues of the tumor.

Materials and methods

Human subjects and human cell lines

A total of 332 of Formalin Fixed Paraffin Embedded (FFPE) breast cancer tissue specimens in 2X15 μm slices were provided sequentially and non-selectively by Yuhuangding Hospital, Yantai, P. R. China from Jan. 2015 to Aug. 2017. All the samples were obtained in accordance with the Declaration of Helsinki, and approved by the Medical Ethics Committee of Yuhuangding Hospital. The clinicopathological characteristics of these patients were listed in Table 1.

MCF-7 and BT474 cell lysates were used as controls. Both of the cell lines were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and maintained according to the provider's instruction.

General reagents

All of the chemicals were purchased from Sinopharm Chemicals (Beijing, P. R. China). Recombinant human HER2/ErbB2 protein was purchased from Sino Biological Inc. (Beijing, China). QDB plate was provided by Quanticision Diagnostics, Inc (RTP, USA). Ventana anti-HER2/neu (4B5) rabbit monoclonal primary antibody was purchased from Roche Diagnostics GmbH. Rabbit anti-HER2 antibody (clone EP3) was purchased from ZSGB-BIO (www.zsbio.com, Beijing, China). HRP labeled Donkey Anti-Rabbit IgG secondary antibody was purchased from Jackson Immunoresearch lab (West Grove, PA, USA). BCA total protein quantification kit was purchased from Thermo Fisher Scientific Inc (Calsband, CA, USA).

Preparation of FFPE tissue and cell lysates

Two FFPE tissue slices at 15μm each (2X15μm) were put into 1.5ml Eppendorf tubes, and deparaffinized before they were solubilized using lysis buffer (50mM HEPES, 137mM NaCl, 5mM EDTA, 1mM $MgCl_2$, 10mM $Na_2P_2O_7$, 1%TritonX-100, 10% glycerol) with protease inhibitors (2μg/ml Leupeptin, 2μg/ml Aprotinin, 1μg/ml pepstatin, 2mM PMSF, 2mM NaF). MCF-7 and BT474 cells were also lysed in the same lysis buffer with protease inhibitors. The supernatants were collected after centrifugation and the total amount of proteins was measured using BCA protein assay kit by following manufacturer's instructions.

QDB analysis

FFPE tissue lysates were adjusted to 0.5 μg/μl according to the BCA assay. Sample pool were prepared by mixing tissue lysates from four FFPE tissue specimens with an

IHC score of 3+, and was serially diluted side by side with the recombinant HER2 protein for defining the standard curve of QDB analysis.

The QDB process was described elsewhere with minor modifications[4,5]. In brief, the final concentration of the FFPE tissue lysates was adjusted to 0.25 μg/μl, and 2 μl/unit was used for QDB analysis in triplicate. The QDB plate was then dried for two hour at RT, soaked in transfer buffer for 10s, rinsed once with TBST, and then blocked in 4% non-fat milk for an hour. Next, it was put into a 96-well microplate with 100μl primary antibody (for clone EP3, 1:1500 in blocking buffer; for clone 4B5, 1:10 in PBS), and incubated overnight at 4℃. Afterward, the plate was rinsed twice with TBST and washed 3X10 mins. The plate was then incubated with a donkey anti-rabbit secondary antibody for 4 hours at RT, rinsed twice with TBST, and washed 4X10 mins. Finally, the QDB plate was inserted into a white 96-well plate pre-filled with 100μl/well ECL working solution for 3 mins. The chemiluminescence signals of the combined plate were quantified by using the Tecan Infiniti 200pro Microplate reader with the option “plate with cover”.

The consistency of the experiments was ensured by including BT474 and MCF-7 cell lysates with pre-documented Her2 level in all the experiments. The result was considered valid when the calculated Her2 level of BT474 was within 10% of documented Her2 level. The absolute her2 level was determined based on the dose curve of protein standard, with samples with chemiluminescence reading less than 2 fold over blank being defined as non-detectable, and enter 0 for data analysis. For those samples with the chemiluminescence reading less than that of 30 pg Her2

recombinant protein, the narrow range (0-125pg) linear regression formula was used to calculate low her2 level.

FISH analysis:

Total of 16 samples were submitted to ZSGB-Bio, Inc (www.zsbio.com) at Beijing, China for FISH analysis. The detailed reports are available upon request.

Statistical analysis

All the data were presented as Mean±SD. The difference between individual groups was calculated using unpaired two-tailed Student's t tests. P value <0.05 was considered statistically significant. The correlation analysis was performed using either Pearson's correlation coefficient analysis or Spearman's rank correlation analysis as indicated in the figure. The specificity, sensitivity of QDB method in comparison with either IHC or FISH was analyzed using receiver operating characteristic (ROC) analysis. All statistics were performed using the GraphPad Prism software version 7.0 (GraphPad Software Inc., USA).

Results:

Assay Development and Her2 measurement in FFPE samples

The linear ranges of QDB analysis were first defined using FFPE specimens and recombinant Her2 protein with EP3 and 4B5 antibodies respectively. Slices of 2X15μm FFPE specimens were used to extract total protein by de-paraffinization and solubilization with Triton-X 100 lysis buffer. As a routine practice, total protein extracted from three or four specimens determined as Her2+ either through IHC analysis or FISH analysis were pooled together to define the linear range of the QDB analysis (Fig. 1).

The FFPE slices were provided sequentially and non-selectively by local hospital with clinicopathological characteristics listed in Table 1. The absolute Her2 levels in 332 samples were measured with EP3 and 4B5 respectively. The flow diagram was shown in Fig. 2. The correlation between these results were analyzed using Pearson's correlation coefficient analysis with r=0.963, p=0.0000, n=332 (Fig. 3). For simplification of description, we limited our analysis in this study with results measured with EP3 clone only.

The distributions of Her2 levels among these samples were shown in Fig. 4A. Consistent with what we have observed with frozen tissues, the absolute Her2 level was distributed from non-detectable (we define the detectable signal below two fold of background as non-detectable, and enter 0 as final result) to as much as 31.31 nmole/g. The group average was **1.953±0.254** nmole/g with the 75 percentile at 0.987 nmole/g.

The samples were grouped further into 4 groups based on IHC scores (0, 1+, 2+, and 3+) in Fig. 4B. The average Her2 level was 0.045±0.006 (n=77), 0.049±0.008 (n=65), 0.537±0.122 (n=108), and 7.12±0.773 (n=82) nmole/g for group 0, 1+, 2+ and 3+ respectively. The differences with statistical significance were observed between each individual groups ($p<0.005$) when analyzed using unpaired two-tailed Student's t-test, except group 0 vs group 1+, where no statistical significant difference was observed.

Validation of QDB method

While the only method for absolute quantification of Her2 levels was SRM-MS, which was still in developmental stage[1,3,9], we had to rely on the results from both IHC and FISH analyses to validate our results indirectly, as the results from QDB measurement

were absolute and continuous variables, while those from FISH and IHC analyses were relative and discrete variables. Therefore, we evaluated the specificity and sensitivity of QDB method with both IHC and FISH analyses using receiver operating characteristic (ROC) analysis.

To compare QDB method with IHC analysis, we followed recommendations from both American Society of Clinical Oncology and College of American Pathologists [10,11] to group samples with IHC scores of 0 and 1+ as Her2-, and IHC score of 3+ as Her2+. IHC score of 2+ was excluded in the analysis. When the absolute Her2 levels from these two groups were used in ROC analysis, we achieved area under the ROC curve (AUC) at 0.9998±0.0001, 95% CI at 0.9994 to 1, with $p<0.0001$ ($n=224$) (Fig. 5A). Using 0.267 nmole/g as cutoff, we were able to achieve 100% sensitivity (95% CI: 95.6% to 100%) and 99.3% specificity (95% CI: 96.14% to 99.98%) with IHC results.

FISH analysis was recommended for samples with IHC score at 2+. There were 144 samples provided with FISH results, including 6 equivocal cases. Among the rest of 138 samples, 101 samples were with IHC score at 2+, and were excluded from ROC analysis of QDB and IHC analysis. Therefore we considered results from FISH analysis an independent validation of the developed cutoff value from ROC analysis of IHC results. Using 0.267 nmole/g as cutoff, we were able to achieve concordance rate with FISH analysis at 88.6% (the 6 equivocal cases were excluded in the analysis), and $\kappa=0.732$ with Cohen's kappa analysis).

We identified 16 samples (11.11%) in disagreement with provided FISH results using 0.267 nmole/g as cutoff. To rule out potential misdiagnosis, these samples were submitted to a third party for independent FISH analysis (Table 2). For 9 Her2- samples

198 from initial FISH reports, 4 samples were affirmed Her2-, 4 were reported Her2+ and 1
199 was reported as equivocal case. Among 7 Her2+ samples from initial FISH reports, 4
200 were affirmed Her2+, 2 was reported Her2-, and 1 was determined equivocal. When
201 these information was incorporated into the revised FISH results, concordance rate was
202 increased to 94.2% between FISH analysis and QDB method (κ=0.865 with Cohen's
203 Kappa analysis).

204 We also evaluated QDB results with FISH analysis using ROC analysis independently
205 using the revised FISH results. As shown in Fig. 5B, we were able to achieve area
206 under the ROC curve (AUC) at 0.978±0.0112, with 95% CI at 0.9561 to 0.9999,
207 $p<0.0001$ (n=138). Using Her2 level at 0.261 nmole/g as cutoff, we were able to
208 achieve 93.02% sensitivity (95% CI: 80.94% to 98.54%) and 93.68% specificity (95%
209 CI: 86.76% to 97.65%). At this value, we were able to achieve 99.6% concordance rate
210 between QDB and IHC analyses. The cutoff values developed from ROC analyses of
211 IHC and FISH analyses respectively were shown in a log scale chart in Fig. 5C.

212 Having evaluated QDB method with both FISH and IHC analyses, we analyzed next the
213 correlation between Her2 copy numbers from FISH analysis, reflected by the ratio of
214 Her2 number over chromosome 17 number (Her2/CEP17), with Her2 protein level as
215 continuous variables in Fig. 6. We found a strong correlation between DNA
216 amplification level and Her2 protein level, with r=0.75 when Pearson's correlation
217 coefficient analysis was performed (n=122).

218 *Exploration of the correlation between clinicopathologic factors and Her2 as absolute*
219 *and continuous variables*

The quantitated Her2 levels in FFPE samples allows us to investigate the correlation between Her2 levels as absolute and continuous variables with other clinicopathologic factors including age, histological grade by Nottingham grading system, tumor size and metastasis status (Table 3). Her2 levels were found to be associated significantly with histological grade based on Nottingham grading system using Spearman's rank correlation analysis (ρ=0.195, p=0.001), a conclusion consistent with previous studies based on IHC analysis[12,13]. In the same study, we found age was significantly associated with Her2 based on IHC analysis (ρ=-0.117, p<0.05). However, this conclusion was not supported when the absolute and quantitative levels of Her2 were used in the analysis.

Her2 distribution by histological grade was further analyzed in Fig. 7. We observed the average of these samples by Grades at 0.791±0.555, 1.554±0.330, 3.271±0.535 nmole/g for Grade I, Grade II, and Grade III respectively. There were statistical significance between Grade I vs Grade III (p< 0.05) and grade II vs Grade III (p=0.005) using unpaired two-tailed Student's t-test. We also calculated the percentage of Her2+ in each grade with 8.3% for Grade I, 29.7% for Grade II and 47.1% for Grade III. Thus, the possibility of Her2+ for Grade III patient was 5.7 fold over that of Grade I patient.

Discussion

In this study, we demonstrated the feasibility of QDB method to measure Her2 levels in FFPE samples as absolute and continuous variables. The method can be easily standardized to measure the protein level objectively, consistently and in high throughput format. It also requires minimum training to be adopted in any clinical lab

with the access of a microplate reader. Thus, we presented a method with clear advantage over the prevailing methods of IHC and FISH for assessment of Her2 levels to meet the routine clinical need. It is also the first method, to our knowledge, for absolute quantitation of tissue biomarkers in FFPE samples suitable for routine clinical practice. Since there is at least one clinically validated IHC antibody (either as IVD or ASR antibodies) for each biomarker in clinical diagnosis and prognosis, the adoption of QDB method promises assessment of most, if not all of these biomarkers as absolute and continuous variables in the near future.

Even for the assessment of Her2 levels *per se*, the minimum requirements in equipment and personnel training ensure the consistency, objectivity and accuracy of the results over both FISH and IHC analyses. The inherent validation steps in the analytical process, including cell lysates with pre-determined Her2 content, would significantly reduce the inter-laboratory variations. It is perceivable the adoption of this method would significantly improve the accuracy of Her2 testing in clinical practice, especially for local laboratories, and clinical laboratories in developing countries.

QDB method and IHC analysis were both based on antigen-antibody interaction. In addition, we on purposely used those antibodies already validated for IHC analysis in our assay (either as IVD or ASR antibodies). Therefore, this method can be considered a tradeoff of morphology for accuracy. Theoretically, results from QDB analysis should match very well with those from IHC analysis when IHC analysis was performed properly as dichotomous variables.

However, QDB method is fundamentally different from FISH analysis, as one analyzed at protein level (QDB), while the other one analyzed at DNA level (FISH). It is well

recognized the FISH analysis has the inherent drawback of being unable to reflect faithfully changes at protein level[14]. Therefore, we do not expect our QDB results to match perfectly with those from FISH analysis. In this study, even after third party verification, there were still 8 samples with their QDB results different from FISH reports, leading to a concordance rate between QDB and FISH at 94.2%. Nonetheless, considering one major usage of Her2 assessment is for antibody-based targeted therapy, we believe QDB results are more clinical relevant than those from FISH analysis.

Our results were also comparable with those from SRM-MS. The distribution of Her2 protein in this study was from non-detectable to 31.31 nmole/g (n=332), in comparison with 0.16 to 17.45 nmole/g (the unit was converted from amole/μg for comparison purpose) in one of the SRM-MS studies (n=270). When groups by IHC scores of 0, 1+, 2+, and 3+, the averages for SRM-MS were 0.19, 0.26, 0.41 and 4.21 nmole/g, compared with 0.04, 0.05, 0.54 and 7.12 nmole/g in QDB measurements. However, the suggested cutoff from SRM-MS method was at 0.74 nmole/g, in contrast to the suggested either 0.261 or 0.267 nmole/g in this study. We do not have a definite answer to this discrepancy yet.

One possible explanation might be that QDB methods highly epitope dependent. In fact, we consistently obtained different results with EP3 from those with 4B5 when analyzed side by side using the same lysate prepared from either frozen tissue or FFPE specimens. It is possible that these two antibodies recognize different fraction of total Her2 protein with different conformational epitopes. For this reason, it is absolute necessary to specify which antibody was used when reporting Her2 levels using QDB

method. In contrast, in SRM-MS analysis, total Her2 protein is measured after all forms of Her2 protein are eventually broke down with the aid of trypsin digestion.

Results from both QDB and SRM-MS showed that even among those samples currently classified as Her2+, there still existed significant differences among the samples. The highest level we measured in QDB analysis was over 100 fold over the proposed cutoff value with both antibodies. This wide distribution of Her2 levels among individual samples were also reported in several other studies including Nuciforo et al study[1,15–17]. Studies based on SRM-MS method showed that patients with higher level of Her2 responded better to Herceptin treatment in two types of cancer (gastric cancer and breast cancer), which further emphasized the necessity to quantify accurately the Her2 levels among cancer patients[1,17].

In conclusion, QDB method was demonstrated in this study to measure her2 level in FFPE specimens as absolute and continuous variables, with clear advantage of being simple, objective, consistent and in high throughput format. This method provides a platform to develop assays systematically for absolute quantitation of tissue biomarkers in FFPE specimen for routine clinical use. The fully implementation of this method will also allow us measure systematically tissue biomarkers as absolute and continuous variables over a huge number of FFPE specimens collected over the years. The future perspective in this area is very encouraging, as it may lead a new direction in proteomic research where bioinformatical tools will be used to further explore these biomarkers at population level.

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Author contributions: FT, WZ, and YZ performed all the experiments, collected data

311 and performed data analysis, GY provided clinical samples and performed data analysis,
312 JL participated in data collection, GT, FT & JZ participated in data interpretation, FT
313 supervised all the experiments, JZ designed and supervised the overall study and drafted
314 the manuscript.

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388 **Tables**

389 **Table 1: The clinicopathological characteristics of the patients.**

Variable		No. of patients	Average±SEM	Percentage
Age (y)	Total	332	53.3±0.6	
	<50	122		36.7%
	≥50	209		63.0%
	Unknown	1		0.35
Histological Grade	I	36		10.8%
	II	145		43.7%
	III	119		35.8%
	Unknown	32		9.6%
Tumor Size (mm)	Total	332	2.3±0.6	
	≤20	173		52.1%
	20~50	151		45.5%
	>50	5		1.5%
	Unknown	3		0.9%
Histological Type	Ductal	298		89.85
	Lobular	9		2.7%
	Other	23		6.9%
	Unknown	2		0.6%
Nodal Status	Negative	220		66.3%
	Positive	112		33.7%

Her2 (IHC)	0	77		23.2%
	1+	65		19.6%
	2+	108		32.5%
	3+	82		24.7%
Her2 (FISH)	Negative	95		28.6%
	Equivocal	6		1.8%
	Positive	43		13.0%
	Unknown	188		56.6%

390

391 **Table 2: Verification by the third party of 16 samples with disagreed QDB results**

392 **from locally provided FISH results.**

Sample NO.	FISH *(local hospital)*	QDB *(nmole/g)*	FISH *(Third party)*
290	negative	**0.547**	Equivocal
79		**0.459**	Negative
119		**0.686**	Negative
141		**0.365**	Negative
298		**0.681**	Negative
149		**0.531**	**Positive**
293		**0.520**	**Positive**
294		**0.849**	**Positive**
159		**0.967**	**Positive**
22	**positive**	0.068	Equivocal
96		0	Negative
286		0.050	Negative
64		0.102	**Positive**
288		0.212	**Positive**
218		0.213	**Positive**
301		0.262	**Positive**

393

394 The Her2 levels measured with QDB method were used to separate samples into Her2+
395 and Her2- using suggested cutoff value at 0.267 nmole/g. There were16 samples
396 identified with disagreed QDB and FISH results. These samples were sent to a third
397 party to rule out potential misdiagnoses, and the results were shown at the right column.
398 All the positive results, either from FISH or QDB analyses, were in bold. The
399 concordance rate would improve to 94.2% (κ=0.865 using Cohen's Kappa analysis)
400 when the results from third party were incorporated in the analysis.

Table 3: Assessment of relationships between clinicopathologic features and Her2 levels by QDB method and IHC analysis respectively.

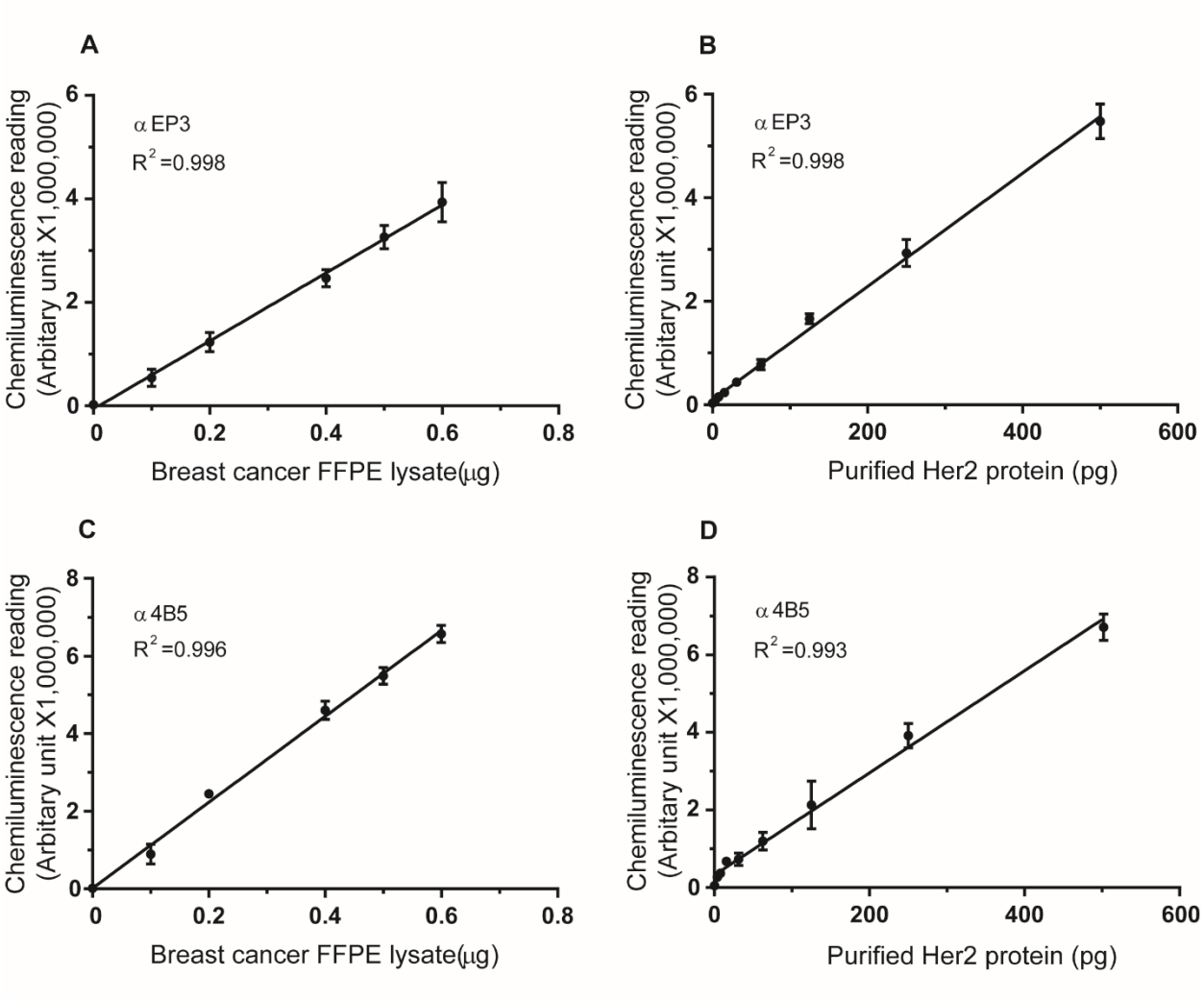
Variable	Age	Histological Grade	Tumor size	Nodal status
QDB	-0.084	0.195**	0.039	-0.041
IHC	-0.117*	0.204***	0.087	-0.019

*** p<0.05; ** p<0.001; *** p<0.0005.**

Her2 levels, assessed either by QDB method as absolute and continuous variables, or IHC analysis as relative and discrete variables, were used to explore the putative association with clinicopathological features using Spearman's rank correlation analysis using Graphpad 7.0. The statistical significant associations were indicated in the figure. The histological grades based on Nottingham grading system were found to be associated with Her2 levels assessed either by IHC (ρ=0.195, p<0.001) or QDB measurement (ρ=0.204, p<0.0005) with statistical significance.

Figures

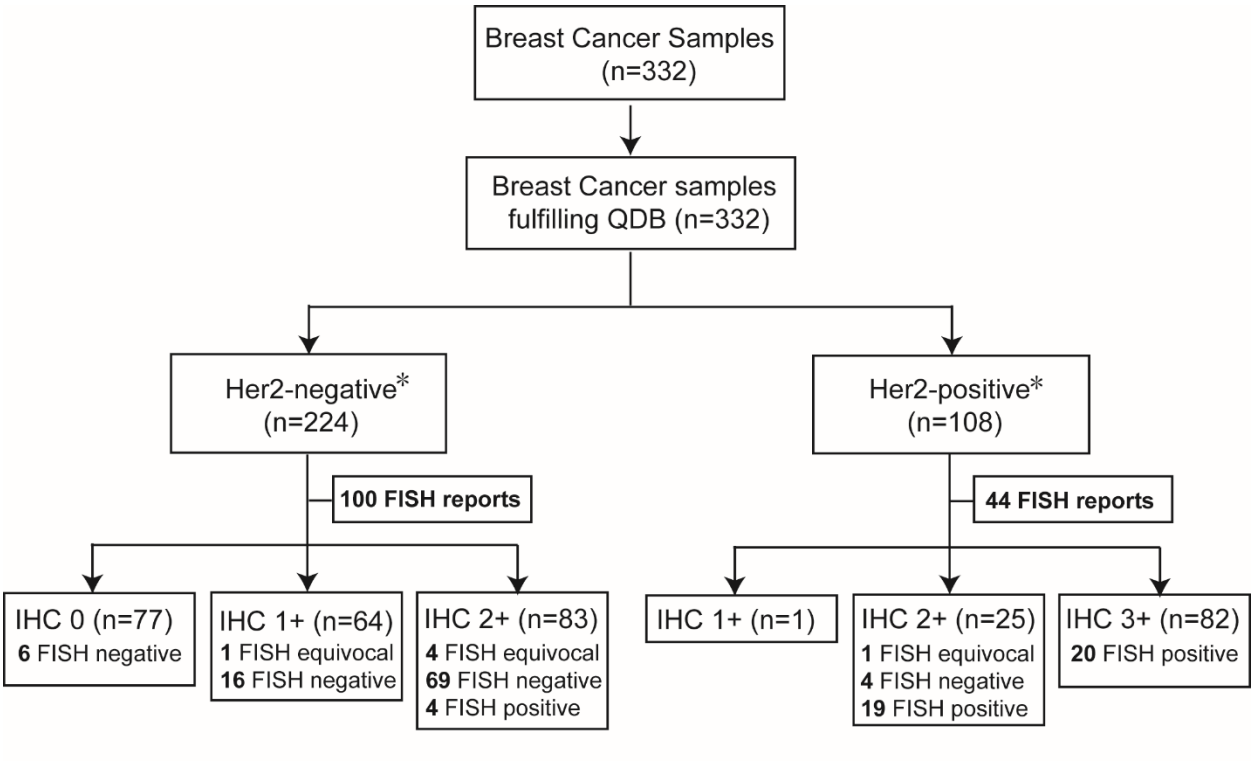
Fig.1: Defining the linear range of QDB measurements with two anti-Her2 antibodies, EP3 and 4B5.



(A, C) Defining the linear range of QDB method for analysis of breast cancer FFPE tissue lysates. Human breast cancer FFPE tissue blocks in two 15 μm slices (2X15 μm) were obtained from a local hospital, and the sample lysates were prepared as described in Materials and Methods. Breast cancer FFPE tissue lysates prepared from 4 samples

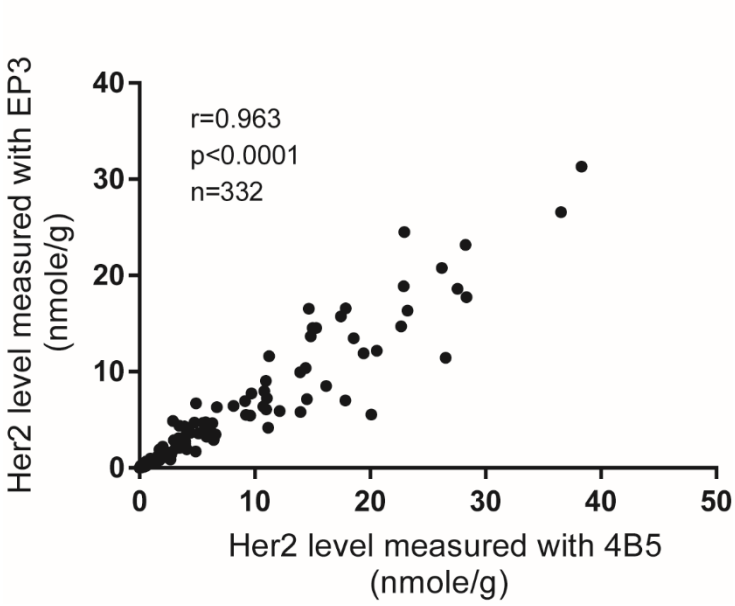
with an IHC score of 3+ were mixed in equal amounts based on the BCA assay. The sample pools were serially diluted, as indicated in the figure, supplemented with 0.5 μg/μl IgG-free BSA solution to ensure equal loading of the samples. The lysates were then applied onto the QDB plate at 1 μg/unit in triplicate for QDB analysis using two anti-Her2 antibodies, EP3 and 4B5 respectively. (B, D) Defining the linear range of QDB method for analysis of purified Her2 recombinant protein. The Her2 recombinant protein was serially diluted supplemented with 0.5 μg/μl IgG-free BSA solution. The diluted solution was then used for QDB analysis at 1 μg /unit in triplicate for measurement with EP3 and 4B5 antibodies respectively.

Fig. 2: The flow diagram of participants.



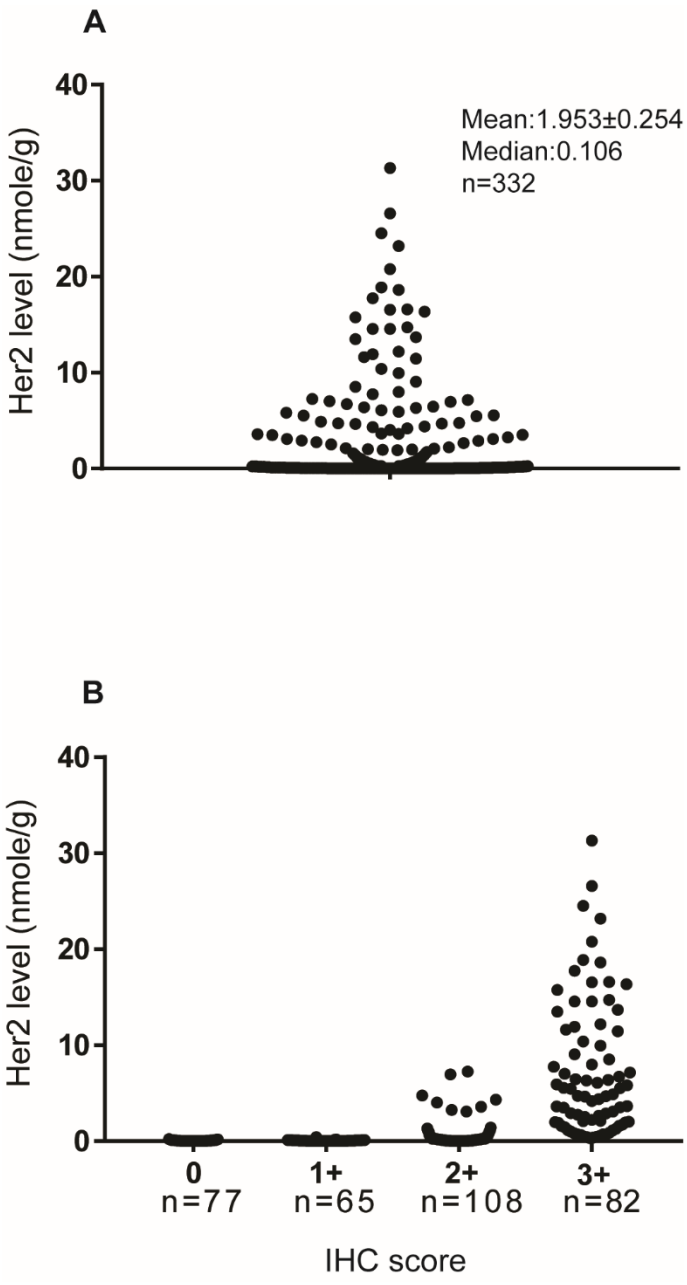
∗ based on cutoff value at 0.267 nmole/g derived from ROC analysis with results from IHC analysis when analyzed with EP3 antibody

Fig. 3: Correlation of Her2 levels measured with 4B5 and EP3 respectively.



A total of 332 breast cancer FFPE tissues in 2x15 μm slices were provided by a local hospital. MCF-7 and BT474 cell lysates were used as internal controls. FFPE tissue lysates (about 0.5 μg /unit) and cell lysates (about 0.3 μg/unit) were applied onto the QDB plates at 2 μl/unit in triplicate for the QDB measurements with clone EP3 and 4B5 respectively. A set of serially diluted Her2 recombinant protein were included in each plate to develop plate-specific standard curve. All results were averaged from three independent experiments, with each sample in triplicate. The correlation of Her2 levels measured with 4B5 and EP3 was analyzed with Pearson's correlation coefficient analysis using Graphpad software, r=0.963, p<0.0001.

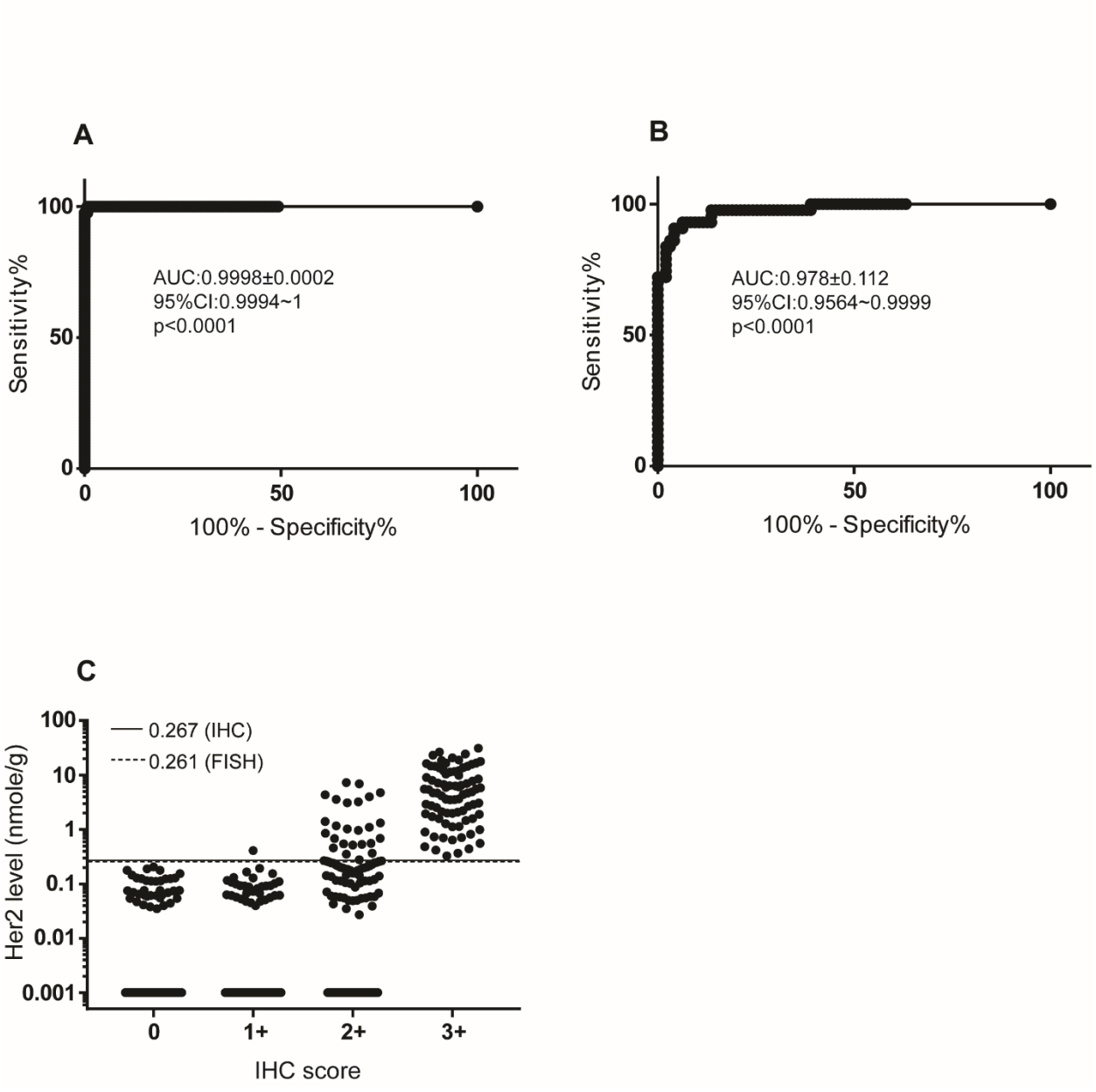
Fig. 4: Distribution of all 332 samples.



Her2 levels in all 332 breast cancer FFPE sample lysates were measured with QDB method using EP3 antibody. The lysates were diluted to about 0.25 μg/μl, and then 2 μl lysate was used for each sample. (A) the distribution of Her2 levels among 332 samples. Her2 levels were ranging from 0 (chemiluminescence readings less than two times the background) to 31.31 nmole/g, with the mean at 1.953±0.254 nmole/g. The

448 25th percentile was at 0 nmole/g and the 75th percentile was at 0.987 nmole/g. (B) All
449 samples were grouped by their IHC scores provided by local hospital. The distributions
450 of Her2 levels in each IHC group were recorded as following: **0**, 0~0.205 nmole/g,
451 mean=0.045±0.006 nmole/g, n=77; **1+**, 0~0.41 nmole/g, mean=0.049±0.008 nmole/g,
452 n=65; **2+**, 0~7.25 nmole/g, mean=0.537±0.122 nmole/g, n=108; and **3+**, 0.329~31.31
453 nmole/g, mean= 7.12±0.773 nmole/g, n=82. The intra- and inter-CV were 8.98% and
454 9.89% respectively.

455 **Fig. 5: Evaluation of QDB method with IHC and FISH analyses using Receiver**
456 **Operating Characteristic (ROC) analysis.**

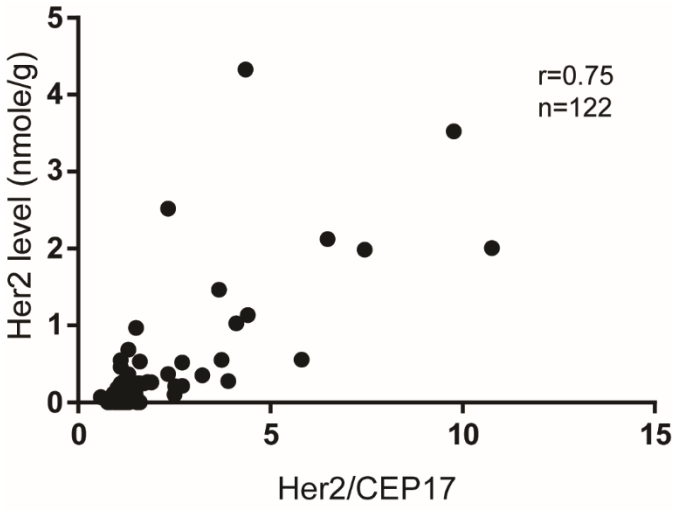


457

458 Samples were separated into negative (her2-) and positive (Her2+) groups based on the
459 recommendations from ASCO/CAP. In (A), samples were grouped based on their IHC
460 scores, with 142 samples in the negative group (IHC 0 and 1+), and 82 samples in the
461 positive group (IHC 3+). Absolute Her2 levels from these samples were used for ROC
462 analysis with Graphpad Prism7.0 software. The ROC curve of QDB analysis was
463 obtained with area under the Curve (AUC) at 0.9998± 0.0002; 95% CI: 0.9994~1;

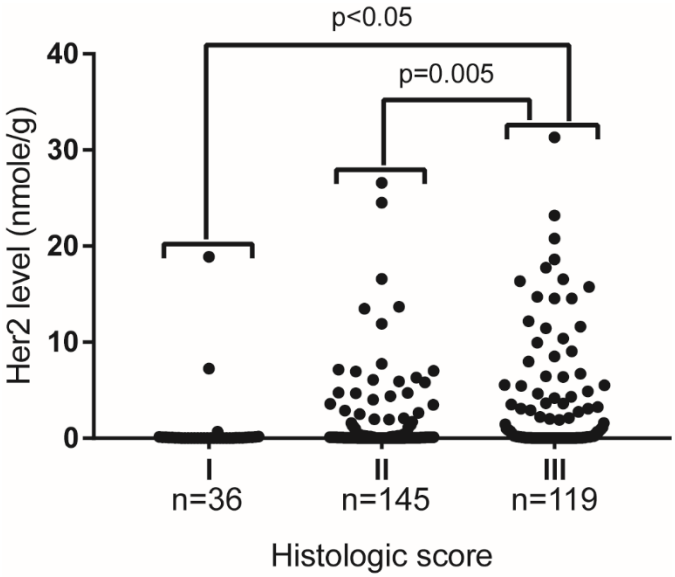
P<0.0001. In (B), samples were grouped based on FISH results, with 95 samples as negative (her2-) group and 43 samples as positive (Her2+) group. 6 equivocal cases were excluded in the analysis. Absolute Her2 levels from these samples were used for ROC analysis with Graphpad Prism7.0 software. The area under the curve (AUC) was at 0.978±0.0112, with 95% CI at 0.9561~0.9999; P<0.0001. (C) The samples were grouped by IHC scores, and the suggested cutoff values from ROC analyses in (A) at 0.267 nmole/g (solid line), and in (B) at 0.261 nmole/g (dashed line) were shown to demonstrate the effectiveness of these cutoff values to separate samples from Her2+ to Her2- groups. Her2 levels were plotted in log scale to better demonstrate the distribution of QDB results among these samples. For those samples with undetectable Her2 level, a value of 0.001 nmole/g was arbitrarily entered to avoid omitting any sample in the log scale graph.

Fig. 6: Assessment of correlation between Her2 gene copy numbers (Her2/CEP17) and their protein levels measured by QDB method.



The correlation was assessed with Pearson correlation coefficient analysis using Her2 protein levels from QDB analysis and Her2/CEP17 ratio from FISH analysis. r= 0.75, p<0.0001. The statistical analysis was performed using Graphpad 7.0.

Fig. 7: Assessing Her2 levels by histologic grade determined by Nottingham histologic scores.



The FFPE specimens (300 out of 332) were grouped according to their Nottingham histologic scores into grades I, II, and III. The Her2 levels of each grade were used for column statistics analysis with Graphpad Prism7.0 software. The mean±SD of the Her2 levels were 0.791±0.555 nmole/g for grade I (n=36), 1.554±0.330 nmole/g for grade II (n=145), and 3.271±0.535 nmole/g for grade III (n=119). The statistical difference was assessed with an unpaired two-tailed Student's t-test, with p<0.05 between grades I and III, and p=0.005 between grades II and III. There was no statistical difference between grade I and grade II samples.