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Development of a highly specific HER2 monoclonal antibody for immunohistochemistry using protein microarray chips

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ABSTRACT

HER2 is an orphan receptor tyrosine kinase of the EGFR families and is considered to be a key tumor driver gene [1]. Breast cancer and gastric cancer with HER2 amplification can be effectively treated by its neutralizing antibody, Herceptin. In clinic, Immunohistochemistry (IHC) was used as the primary screening method to diagnose HER2 amplification [2]. However, recent evidence suggested that the frequently used rabbit HER2 antibody 4B5 cross reacted with another family member HER4 [3]. IHC staining with 4B5 also indicated that there was strong non-specific cytoplasmic and nuclear signals in normal gastric mucosal cells and some gastric cancer samples. Using a protein lysate array which covers 85% of the human proteome, we have confirmed that the 4B5 bound to HER4 and a nuclear protein ZSCAN18 besides HER2. The non-specific binding accounts for the unexpected cytoplasmic and nuclear staining of 4B5 of normal gastric epithelium. Finally, we have developed a novel mouse HER2 monoclonal antibody UMAB36 with similar sensitivity to 4B5 but only reacted to HER2 across the 17,000 proteins on the protein chip. In 129 breast cancer and 158 gastric cancer samples, UMAB36 showed 100% sensitivity and specificity comparing to the HER2 FISH reference results with no unspecific staining in the gastric mucosa layer. Therefore, UMAB36 could provide as an alternative highly specific IHC reagent for testing HER2 amplification in gastric cancer populations.

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1. Introduction

Human epidermal growth factor receptor 2 (HER2) is an orphan receptor tyrosine kinase of the EGFR families. The amplification of HER2 has been observed in several types of malignant tumors [1,4]. Overexpression of the HER2 protein activates multiple signaling pathways including the Ras/MAPK and PI3K/AKT pathways which drive cell proliferation, invasion and survival. As a result, HER2

became one of the first targets for targeted therapy for cancer. HER2 targeting agents such as Herceptin (Trastuzumab) have been approved to treat HER2 amplified cancers [1,4].

Diagnosis of HER2 is crucial for identifying patients that are eligible for HER2-targeted therapy. For breast and gastric cancer, in which about 20% of the patients are HER2-positive, immunohistochemistry (IHC) and (fluorescence) in situ hybridization (FISH) are the most frequently used diagnostic methods [2]. Because of low cost and low technique barrel, IHC with a carefully designed score system now becomes a popular screening methodology for HER2 diagnosis. The sensitivity and specificity of the primary anti-HER2 antibody are crucial for the accuracy of the test.

In this work, we have developed a high density protein lysate array covering about 85% of the human proteome. Using the protein microarray, we evaluated the specificity of a widely used rabbit

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anti-HER2 primary antibody 4B5 [5] and found it recognized multiple protein targets including HER2, HER4 and a nuclear protein ZSCAN18. These interactions were subsequently confirmed by western blotting. Furthermore, we found that the non-specific interaction between HER4/ZSCAN18 and 4B5 antibody may be responsible for the unexpected high level of cytoplasmic/nuclear staining in the normal gastric mucosa. By incorporating the protein microarray as one of the screening criteria, we have obtained a highly specific mouse anti-HER2 antibody UMAB36.

2. Materials and methods

2.1. Protein array and assays

Lysate protein chips were printed as described previously [6] and stored at -80° prior to use. Protein arrays were incubated at room temperature (RT) with 0.5% PVA for 15 min and then blocked with 1X StartingBlock T20 (Thermo) for 1 h. Each protein array was applied with 1 ml primary antibody (5 μ g/ml in blocking buffer) and incubated at 37° for 1 h. The chip was then rinsed for 5 min, 3 times in 1X TBS at RT. DyLight 649-conjugated secondary antibody (Jackson Labs, 1:500 in blocking buffer) was used to probe the primary antibody. After 30 min incubation, the secondary antibody was washed with 1X TBS and rinsed briefly in ddH₂O. The arrays were finally air-dried and scanned with a Genepix 4100A scanner (Molecular Devices).

Protein array data were annotated and processed with GenePix Pro software (Molecular Devices). The exported gpr files were subsequently analyzed with R (<http://www.r-project.org/>). A positive signal was defined if the value was 2 standard deviations higher than the maximal background value.

2.2. Antibody generation

Antibodies to HER2 were generated by immunizing C57BL/6 mice with a purified cytoplasmic domain of the HER2 protein. Enriched B cells from immunized animals were fused to myeloma SP2/0 myeloma cells (ATCC) to generate hybridomas using standard techniques. Single clones were produced by limiting dilution assays through 3 rounds of screening and subcloning. Purified HER2 antibodies were screened using HER2 2+ breast cancer tissues and the specificities of the final candidates were confirmed using the 10k protein lysate chips.

Antibodies to ZSCAN18 were generated similarly using full length ZSCAN18 as an antigen. The specificity was also confirmed by 10k protein chips (Supplementary Fig. 1).

2.3. Immunohistochemistry and histology

Immunohistochemical staining was carried out manually on 4- μ m thick paraffin tissue sections. Briefly, deparaffinized slides were treated in 1 mM EDTA (pH 8) antigen retrieval solution at 120° for 3 min in a high pressure cooker. Slides were incubated with primary antibodies for 90 min at room temperature without serum blocking. Ready to use rabbit anti-HER2 (4B5) was purchased from Ventana. Mouse anti-HER2 (UMAB36) was used at 1:400; mouse anti-ZSCAN18 (UMAB114) was used at 1:200. Rabbit anti-HER4 (E200) was purchased from Epitomics and used at 1:400. DAKO's EnVision Plus and ZSBO's 2-step polymer detection system PV9000 were used in manual IHC assays.

2.4. Patient samples and HER2 assay comparison

Clinical histories were retrieved from the electronic medical record following approval by the Beijing Cancer Hospital

institutional review boards. Patient information was de-identified and replaced with case number prior to analysis. The HER2 statuses of all the breast and stomach cancer samples were previously diagnosed with 4B5 or PATHVYSION HER-2 DNA probe kit. For the comparison, tissues were retrieved from tissue bank and made into tissue arrays. Each tissue array contains maximal 60 samples (duplicated) and 8 HER2 reference tissues (2 for each score). IHC staining of HER2 by 4B5 and UMAB36 were performed using Ventana's BenchMark automatic staining station. HER2 FISH tests were performed using PATHVYSION HER-2 DNA probe kit (Abbott). HER2 IHC statuses (0+–3+) were scored according to published guidelines. Each antibody was scored without knowledge of the results obtained using other antibody or FISH results.

The samples were not included in the study if either antibody staining or FISH staining result could not be obtained due to technique problems. HER2 FISH scores were treated as the reference for "True" HER2 positives and the sensitivity and specificity was calculated as following:

Sensitivity = (number of true positives) / (number of true positives + number of false negatives);

Specificity = (number of true negatives) / (number of true negatives + number of false positives)

2.5. Epitope mapping

The binding sequences of 4B5 on HER2 (NP_001005862) and HER4 (NP_001036064) were reported by Brunner et al. [3]. Alignment of the corresponding sequences from EGFR (NP_005219), HER2, HER3 (NP_001973) and HER4 was first performed. A 10 amino acids (AA) sequence (TAENPEYLGL, AA 1212–1221 for HER2) was selected to search for similar sequences in ZSCAN18 (NP_001139014). The candidates were subsequently synthesized and linked onto bovine serum albumin (BSA). For ELISA validation, 1 μ g peptide-BSA was coated on each well of 96 well-plate and 0.5 μ g/ml 4B5 antibody was used as primary antibodies. HRP-conjugated anti-mouse antibody (Jackson Immuno, 1:5000) was used as secondary antibody.

2.6. Immunoblotting

HeLa, A549 and MCF7 cells were purchased from ATCC. HEK293T cells were transfected with EGFR, HER2, HER3, HER4, ZSCAN18 constructs for 48 h and then lysed on ice for 20 min in Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors. Lysates were fractionated by electrophoresis in SDS denaturing polyacrylamide gels and proteins were then transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% non-fat dried milk in 0.05% Tris-buffered saline (TBST) for 1 h, and then probed in primary antibody overnight at 4° C. The following antibodies were used: rabbit anti-HER2 (4B5, Ventana, 1:10), mouse anti-HER2 (UMAB36, OriGene Technologies, 1:2000), mouse anti-DDK (4C5, OriGene Technologies, 1:2000). After three 10 min washes with TBST, the membranes were incubated with corresponding secondary antibodies that were diluted at 1:5000.

3. Results

3.1. Rabbit anti-HER2 antibody 4B5 recognizes multiple antigens

In order to evaluate antibody specificity, we have developed a high density lysate array, on which lysates of HEK293T cells transfected with plasmids coding for various full length human

proteins were spotted (Fig. 1A). Our first generation protein array contains 11,000 unique full length human proteins (10k chip). In a pilot study to evaluate the specificity of popular primary antibodies currently used in pathology IHC tests, we found that the rabbit anti-HER2 antibody 4B5 bound to HER4 and ZSCAN18 besides HER2 (Fig. 1B). The same binding pattern was also observed using another protein chip containing 17,000 full length proteins (17k chip, data not shown).

We then utilized 4B5 antibody to probe HEK293T lysates

expressing various EGFR family members or ZSCAN18 by western blotting. Consistent with the protein array data, 4B5 can detect HER2, HER4 and ZSCAN18 expression from the cell lysates (Fig. 1C and D).

It was reported that the epitope of 4B5 was located between amino acids (AA) 1212–1221 of the HER2 C-terminal region. We scanned ZSCAN18 for similar peptide and discovered that AA 275–284 of ZSCAN18 shared high homology with the 4B5 epitope (Fig. 1E), which was further confirmed experimentally by enzyme-

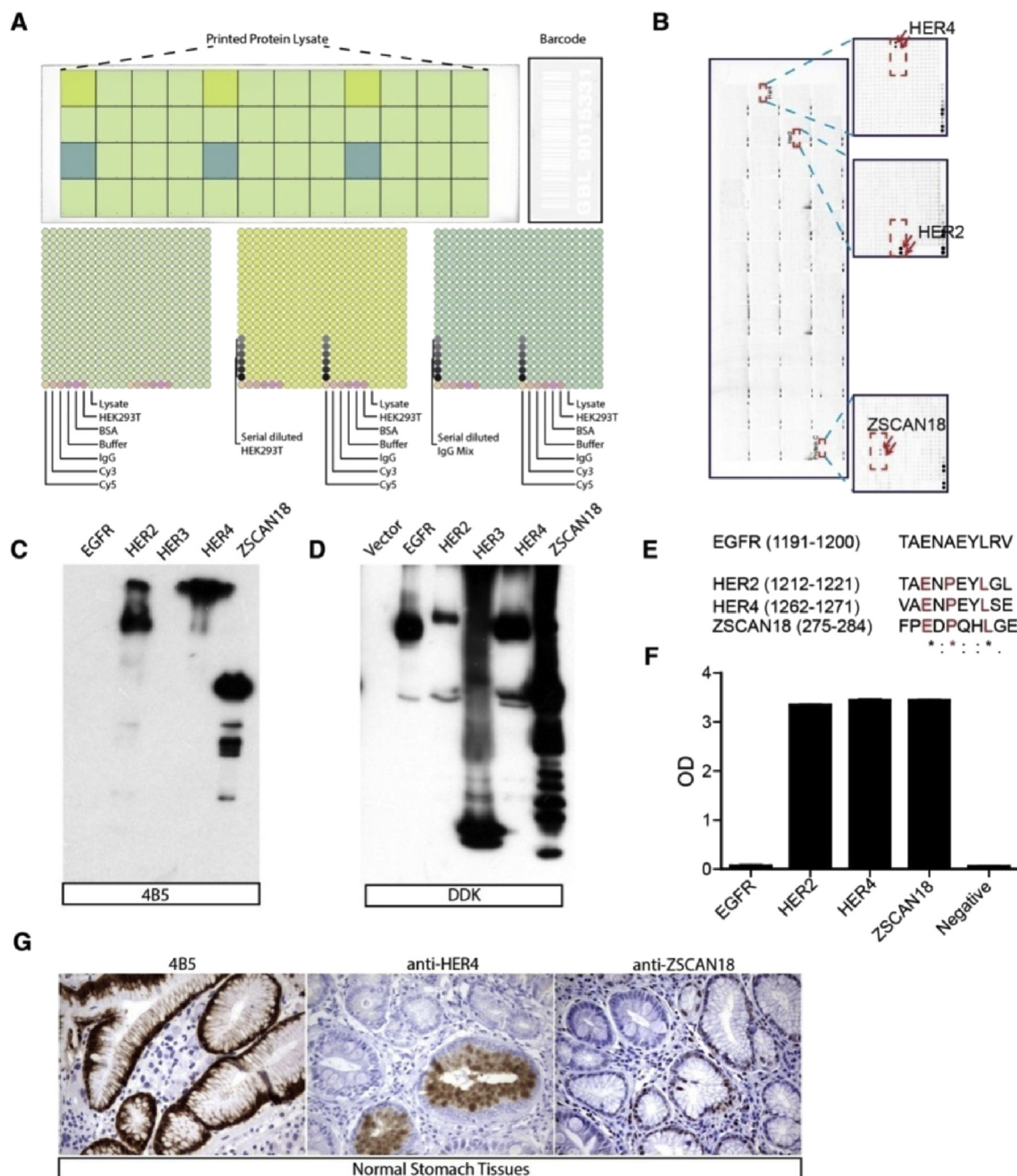


Fig. 1. Anti-HER2 antibody 4B5 non-specifically binds to HER4 and ZSCAN18. (A) Layout of the 10k protein lysate array. The protein chip is consistent of 48 blocks, with multiple controls on each block. Each block contains 21 × 22 spotted protein lysates and controls. (B) 4B5 binding results on the 10K protein lysate chip. The blocks with positive signals were enlarged and the positive signals were pointed by red arrows. (C & D) Western blot analysis of HEK293T cell lysates expressing different DDK tagged EGFR family members or ZSCAN18 with 4B5 (C) or anti-DDK (D) antibody. (E) CLUSTALW alignment of potential targeting sequences of 4B5 in HER2, HER4 and ZSCAN18. The crucial amino acids for 4B5 binding were marked in red. The corresponding region of EGFR was also displayed. (F) ELISA results of 4B5 binding to the 10 amino acid peptides shown in E. Peptides were synthesized and linked to BSA. (G) IHC staining of normal stomach tissues with 4B5 (left), rabbit anti-HER4 antibody (middle), or mouse anti-ZSCAN18 antibody (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

linked immunosorbent assay (ELISA, Fig. 1F). We aligned the multiple 4B5 targeting sequences from HER2, HER4, ZSCAN18 and found that the proline in the middle of 4B5 epitope is crucial for its binding affinity. A proline to alanine change in the corresponding EGFR C-terminal region has totally abolished 4B5 binding capacity (Fig. 1E).

Non-specific cytoplasmic and nuclear staining by 4B5 has been reported from IHC staining of gastric tissues. We also observed that 4B5 stained heavily in both the cytoplasm and nucleus of normal gastric foveolar epithelial cells although high level of HER2 expression was usually observed on the membrane of HER2 amplified gastric tumor cells (Fig. 1G, left, Fig. 2E, up right).

Interestingly, cytoplasmic and nuclear staining in the normal gastric mucosa cells were observed using rabbit anti-HER4 and mouse anti-ZSCAN18 antibodies, respectively, which suggests that the non-specific staining of 4B5 could be the result of the non-specific interactions with HER4 and ZSCAN18 (Fig. 1G, middle and right).

3.2. Development of a highly specific mouse monoclonal antibody (mAb) for IHC diagnosis of HER2 in breast and gastric cancers

With the high density protein array as a tool to evaluate antibody specificity, we set out to develop a specific HER2 antibody

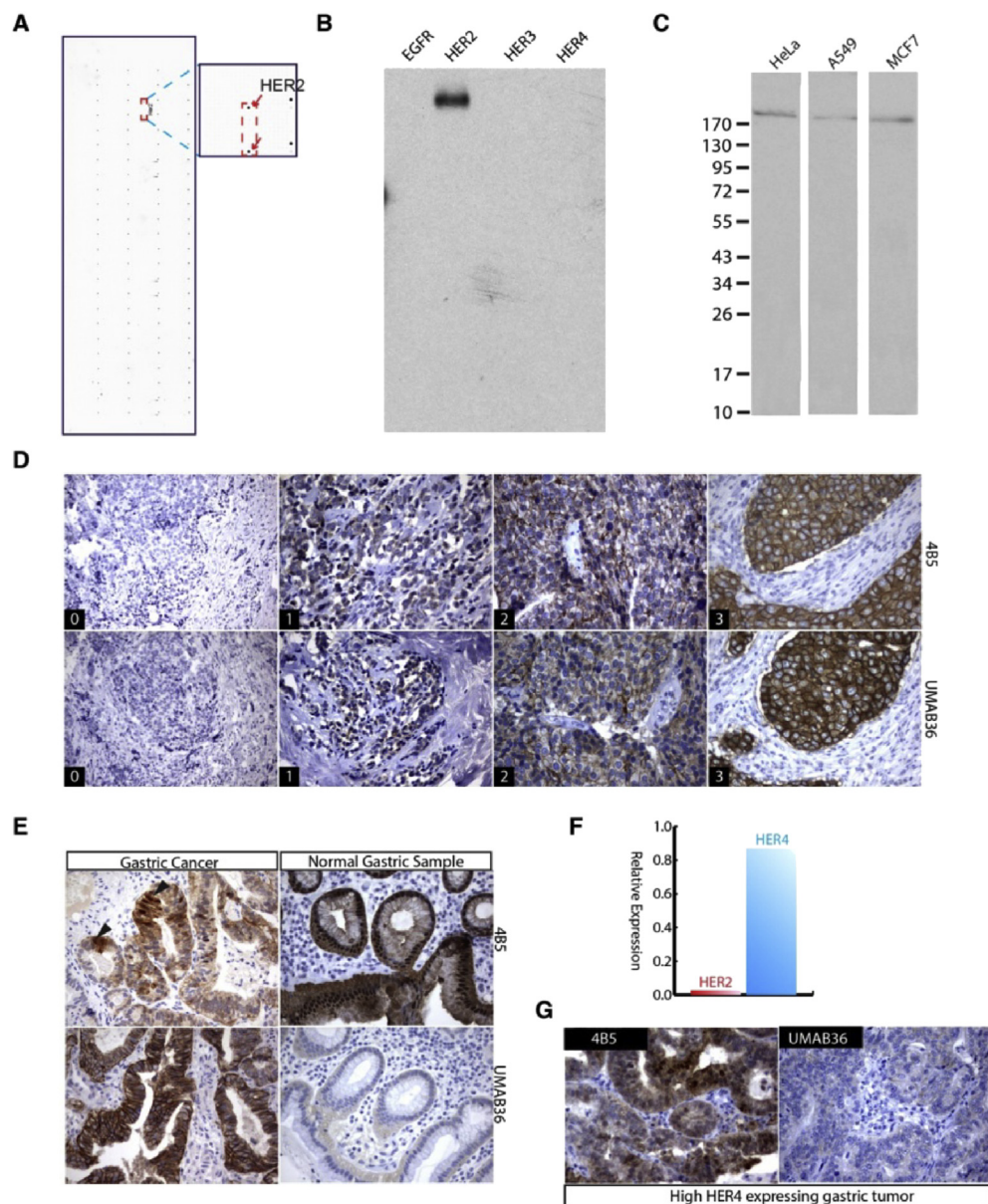


Fig. 2. A highly specific anti-HER2 antibody UMAB36 was capable of determining HER2 status in breast cancer and gastric cancer. (A) UMAB36 binding results on the 10K protein lysate chip. The block with HER2 positive signals was enlarged and the positive signals were pointed by red arrows. (B) Western blot analysis of HEK293T cell lysates expressing different DKK tagged EGFR family members with UMAB36. (C) Western blot analysis of endogenous HER2 with UMAB36 in different cancer cell lines. (D) IHC staining of breast cancer tissues with different HER2 scores using 4B5 (upper panel) or UMAB36 (bottom panel). (E) IHC staining of gastric cancer tissues (left) or normal gastric tissues (right) with 4B5 (upper) or UMAB36 (bottom). Cytoplasmic and nuclear 4B5 staining were pointed by arrowheads. (F) Relative HER4 and HER2 expression level of a representative gastric cancer tissue from OriGene's biorepository with high levels of HER4 but low levels of HER2 using qPCR analysis. (G) IHC staining by 4B5 (left) or UMAB36 (right) of the representative gastric cancer tissues in (F) which expressed high levels of HER4 but low level of HER2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with comparable sensitivity as 4B5 for IHC test of HER2. After several rounds of screening, we obtained a mouse mAb clone UMA36, which only recognized HER2 on our 10K (Fig. 2A) and 17K protein chips (data not shown). We confirmed that the antibody did not cross react with other EGFR family members by western blotting (Fig. 2B) and the antibody is sensitive enough for detecting endogenous HER2 expression in various cancer cell lines (Fig. 2C).

In order to evaluate the IHC performance of the antibody, we retrieved breast cancer samples with different HER2 scores from OriGene's biorepository and stained with either 4B5 or UMA36. Both antibodies stained similarly and the HER2 scores were in line with previously documented diagnoses (Fig. 2D). However, on gastric cancer samples, we observed greater heterogeneous staining with 4B5 as it showed obvious nuclear and cytoplasmic staining in some regions of the tumors (Fig. 2E up left). On the other hand, UMA36 mainly stained cell membrane of HER2 positive gastric tumor cells (Fig. 2E down left) and did not exhibit obvious staining on normal gastric epithelium cells (Fig. 2E down right).

It has been documented that HER4 is highly expressed in some gastric cancer cases. We speculated that there could be some cases with high levels of HER4 expression while no HER2 amplification. Using qPCR, we examined HER2 and HER4 expression levels from 48 gastric cancer samples in OriGene's biorepository and found 2 such cases with paraffin tissues available at the same time (Fig. 2F, Supplementary Table 1). Consistent with our protein array and western blotting data, 4B5 showed intense staining on the 2 tissue samples while the staining with UMA36 was negligible (Fig. 2G).

3.3. Performance of 4B5 and UMA36 in a larger number of breast and gastric cancer samples

Since HER2 targeting agent Herceptin has been approved for treating both breast cancer and gastric cancer, we sought to determine the performance of UMA36 and 4B5 in a larger cohort of patient samples. We examined HER2 status by IHC on tissue microarrays with breast cancer and gastric cancer samples using either UMA36 or 4B5 and compared the results with FISH results using a specific HER2 probe. A total of 129 breast cancer samples and 158 gastric cancer samples were used for the analysis. As expected, IHC results from 4B5 staining displayed excellent correlation with the FISH results in the cohorts of breast cancer samples, which is consistent with the finding that HER4 expression in breast cancer samples was normally low (Table 1). Interestingly, although 4B5 was shown to non-specifically interact with HER4 and ZSCAN18 in the gastric tissues (Fig. 1A–E, Fig. 3B), we did not observe the non-specific binding which might interfere with the specificity of the HER2 diagnosis (Table 2). Meanwhile, IHC results from UMA36 staining exhibited 100% concordance with the FISH results (Tables 1 and 2).

To further confirm the specificity of 4B5 and UMA36, we utilized another tissue microarray including 12 samples with each for four different tumor tissues. HER2 amplification was not reported from 4B5 or UMA36 staining score of pancreas, thyroid and colon tumors samples. Interestingly, one ovary sample showed a “2+” IHC score by both HER2 antibodies and subsequently diagnosed as HER2 amplification by FISH. IHC results from both 4B5 and

UMA36 staining were in full agreement with HER2 FISH results in these small groups of control samples (Table 3). Similar to the stomach, 4B5 stained heavily in the cytoplasmic regions of colon mucosa cells (Fig. 3B).

4. Discussion

Unlike assays which utilize antibody pairs to improve specificity, the accuracy of the IHC tests mostly depends on the specificity of the primary antibody only. While multiple measurement methods have been set up to evaluate the affinity of an antibody, the specificity of an antibody is still mainly reported by the trial and error methods. Taking advantage of the large cDNA collection, we have generated a protein lysate array including the largest collection of full length human proteins to our knowledge. The lysate protein array enabled us to evaluate the interaction of antibodies to 85% of the proteins in the human proteome. Using this technique, we had successfully identified the non-specific binding partner PCYT1A of the ERCC1 antibody 8F1 [6]. The expression of the DNA endonuclease ERCC1 was hypothesized to correlate with the resistance to cisplatin treatment in Non-small cell lung cancer [7]. However, non-specific interaction of 8F1 to PCYT1A, which is also a nuclear protein, compromised the ERCC1 diagnosis and subsequent clinical trial [8].

Despite the fact that non-specific interaction between HER2 antibody 4B5 to its family member HER4 has been reported earlier [3], non-specific staining of 4B5 in the breast cancer was not observed [9]. One possible explanation is that HER4 is not expressed in normal breast tissue and HER4 overexpression/amplification is very rare in breast carcinoma. In contrast, cytoplasmic HER2 staining in the stomach has been documented in the 4B5 interpretation manual [10]. Nuclear and cytoplasmic HER2 non-specific staining has also been reported by Methorst et al. and Polydorides et al. in gastric cancer cases [11,12]. Using the protein lysate array, we confirmed the interaction of 4B5 with HER4 and revealed binding of 4B5 to another nuclear protein, ZSCAN18. Interestingly, we also found that cells of the normal stomach mucosa expressed a high level of HER4 in the cytoplasm and ZSCAN18 in the nucleus (Fig. 1G), which correlated well with the non-specific staining pattern of 4B5 on the stomach tissues.

Interestingly and a little bit surprisingly, 4B5 staining on a relatively large cohort of breast and gastric cancer samples exhibited pretty good correlation with FISH results. One of the reasons could be that all the samples were chosen from biopsies that have been diagnosed by 4B5 and FISH. The HER2 statuses of these samples were clearly defined so that any potentially problematic tissues or staining had been re-sampled or repeated. More importantly, the robust HER2 interpretation guideline, according to which only membrane signals were counted for the scoring system, played a big role for the accurate diagnosis [13]. Furthermore, the non-specific staining of 4B5 were mostly observed in normal gastric tissues, few cytoplasmic/nuclear non-specific staining were found in our tests. However, intensive cytoplasmic/nuclear 4B5 staining were reported in gastric cancer previously [12]. Unlike breast cancer, where HER4 is rarely expressed, HER4 was weakly expressed in normal gastric mucosal. In addition, the expression of HER4 was reported to be elevated in gastric cancer and gastric cancer cell lines comparing to normal tissues [14]. In another independent study, HER4 overexpression was observed in the membranes in 22% and in the cytoplasm in 84% of the gastric tumor cells [15]. The non-specific nature of 4B5 could still interfere with the accurate HER2 scoring and diagnosis.

Specificity of IHC antibodies were normally determined by the rule of “innocence inference”, according to which specificity means no unexpected staining patterns in a panel of reference tissues. The

Table 1
Sensitivity and specificity of 4B5 and UMA36 for breast cancer and stomach cancer.

Tissue type	Indicator	UMA36	4B5
Breast	Sensitivity	100.0%	97.3%
	Specificity	100.0%	100.0%
Stomach	Sensitivity	100.0%	91.7%
	Specificity	100.0%	100.0%

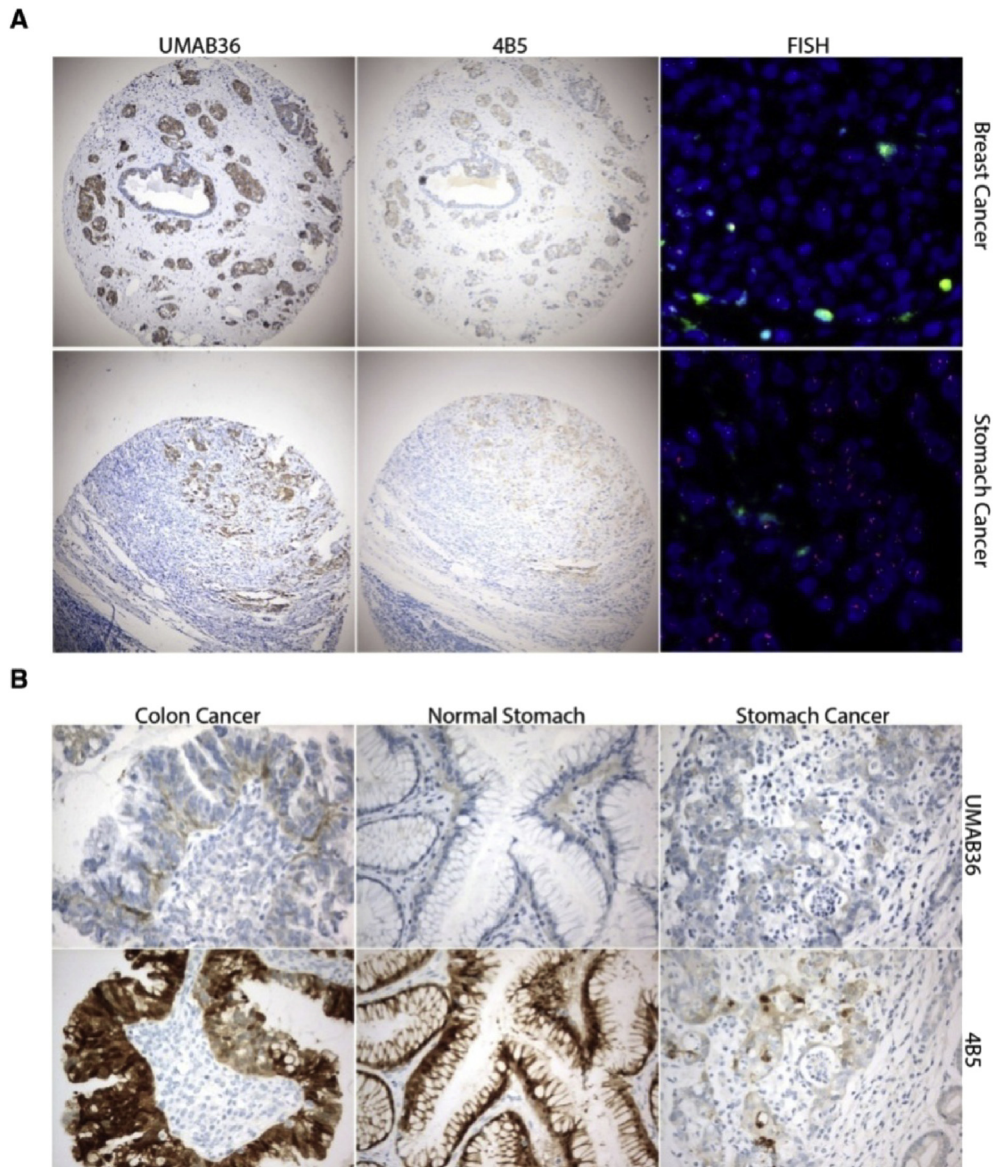


Fig. 3. 4B5 and UMAB36 staining in large collections of tumor tissues. (A) Representative IHC and FISH imaging of the breast cancer (upper panel) and stomach cancer (lower panel) tissues where 4B5 (1+) and UMAB36 (2+) showed discrepant results. (B) IHC results of 4B5 and UMAB36 in normal colon (left), normal stomach (middle) and stomach cancer (right) tissues.

Table 2

Summary of HER2 scores diagnosed by UMAB36, 4B5 and FISH in breast cancer and stomach cancer cases.

Tissue type	FISH result	Cases	UMAB36				4B5			
			IHC score							
			0	1	2	3	0	1	2	3
Breast	FISH NEG	93	29	38	26	0	51	29	13	0
	FISH POS	36	0	0	4	32	0	1	4	31
Stomach	FISH NEG	103	73	21	9	0	95	5	3	0
	FISH POS	55	0	0	10	45	0	5	5	45

Table 3

Summary of HER2 scores by UMAB36, 4B5 and FISH in pancreas, thyroid, colon and ovarian cancer samples.

Tissue type	FISH result	Cases	UMAB36				4B5			
			IHC score							
			0	1	2	3	0	1	2	3
Pancreas	FISH NEG	12	11	1	0	0	10	2	0	0
	FISH POS	0	0	0	0	0	0	0	0	0
Thyroid	FISH NEG	12	12	0	0	0	11	1	0	0
	FISH POS	0	0	0	0	0	0	0	0	0
Colon	FISH NEG	12	12	0	0	0	11	1	0	0
	FISH POS	0	0	0	0	0	0	0	0	0
Ovary	FISH NEG	11	6	4	1	0	9	2	0	0
	FISH POS	1	0	0	1	0	0	0	1	0

fact that the number of proteins expressed on these tissues was unknown makes it difficult to know how comprehensive such method is. The specificity of an antibody becomes difficult to evaluate even for antibody like 4B5 which was cleared by the FDA for clinic diagnosis. However, the high throughput protein lysate

array provides us the exact coverage of the specificity tests among the human proteome for the first time. The technique could be very

useful in determining the antibody specificity and developing ultra-specific antibodies for clinical application. Our previous effect of developing highly specific ERCC1 antibody 4F9 showed better clinical prediction value than the non-specific 8F1 [16]. Here in this article, we have presented a highly specific HER2 antibody developed by similar strategies. The antibody only recognized HER2 among 17,000 full length human proteins spotted on the chip. In addition, the antibody displayed good IHC performance, which correlated excellently with HER2 FISH results. The UMAB36 HER2 antibody thus would be a good candidate for HER2 IHC diagnosis alternative to 4B5, especially for gastric cancers.

Conflict of interest

The authors declare that they have no conflict of interests.

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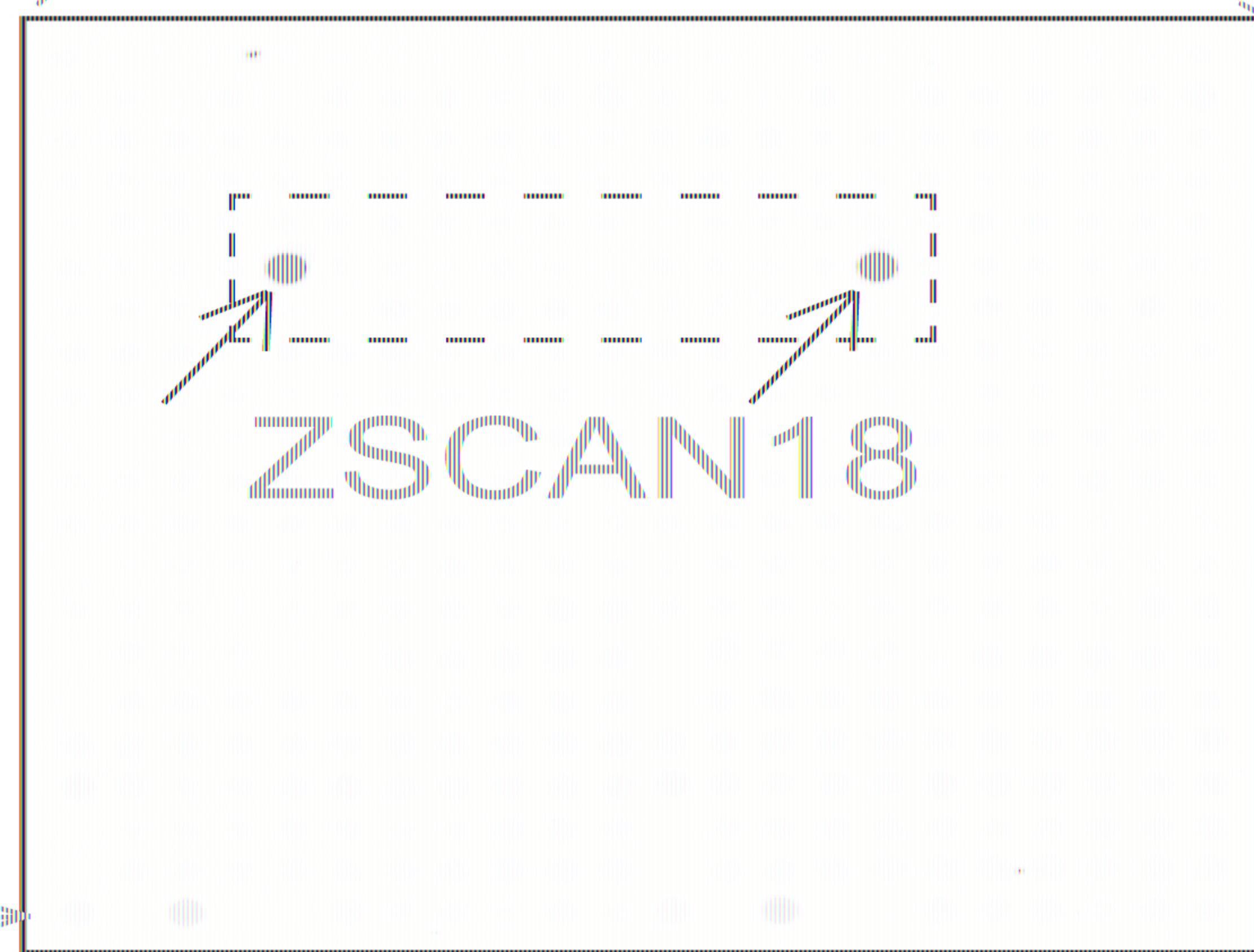
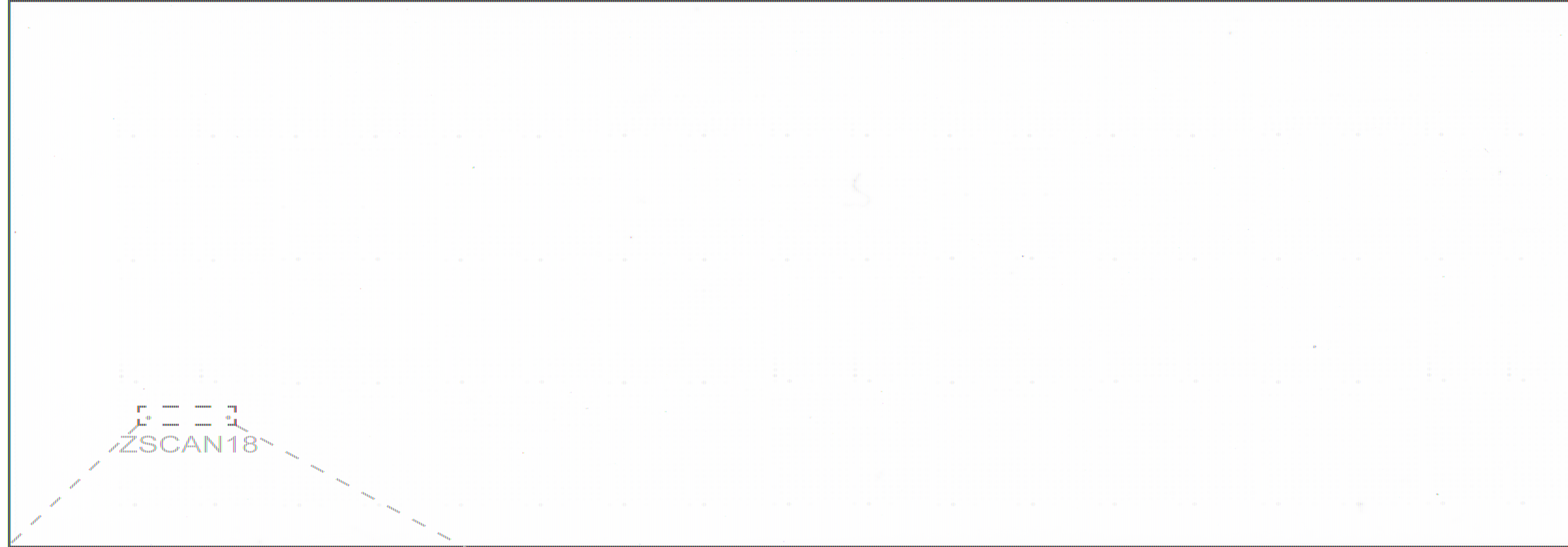
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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2017.01.086>.

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Cy5 orientation marker →

IgG mix