Accepted Article

TECHNICAL BRIEF

Multiplex Epitope Detection: A New Method Overcomes Limitations of Antibody Arrays

Hui Cen[1], Sarah L. Windler[1], Lyle S. Rice[1], Amy Zhang[2] and Hua Zhou[2]

[1] LEAP Biosciences Corporation, 502 Lowell Avenue, Palo Alto, CA 94301

[2] Epitomics, Inc., 863 Mitten Road, Suite 103, Burlingame, CA 94010

Correspondence: Dr. Hui Cen, LEAP Biosciences Corporation, 502 Lowell Avenue, Palo Alto, CA 94301, USA

Email: dcen@leapbio.com

Phone: +1-650-965-3616

Fax: +1-650-965-3618

Keywords: biomarker / multiplex detection / IHC / phosphoprotein / protein array

Total number of words: 3115

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/pmic.201200381

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Received: August 22, 2012; Revised: February 27, 2013; Accepted: February 28, 2013

Accepted Article

ABBREVIATIONS

NFκB - Nuclear Factor-KappaB

IkB – Inhibitor of Kappa B

STAT3 - Signal transducer and activator of transcription 3

p38 – p38 Mitogen activated protein kinase

EGFR – Epidermal Growth Factor Receptor

ErbB2 - Epidermal Growth Factor Receptor 2

PDGFRβ-Platelet-derived growth factor receptor beta

TfR – Transferrin Receptor

PE - Phycoerythrin

TNFα - Tumor Necrosis Factor Alpha

IFNα - Interferon alpha

ABSTRACT

Antibody arrays have been used as an effective method for simultaneously detecting multiple proteins such as cytokines. However, their use in quantifying a large number of cellular proteins has the following limitations: 1) unsuitable for simultaneously detecting proteins that may form complexes with each other; 2) incapable of quantitatively detecting more than one epitope of each protein such as phospho- and non-phospho epitopes; and 3) incapable of simultaneously detecting multiple biomarkers on solid surfaces such as formalin-fixed tissue sections. Using a novel multiple epitope detection (MED) technique, we have overcome these limitations and have improved upon currently available antibody-based protein detection technologies. The MED technique employs primary antibody detection of epitopes within fixed cells or tissue, followed by elution of bound antibodies, and subsequent quantification of the eluted antibodies by an epitope array. Using the MED method, we demonstrate accurate detection of individual proteins even in complex with each other,

simultaneous quantitative detection of phospho- and non-phospho epitopes on a protein, and sensitive detection of multiple biomarkers on formalin-fixed tissue sections.

Accepted Article

Antibody arrays are used as effective tools for simultaneously detecting multiple secreted proteins such as cytokines [1]. However, their application in detecting non-secreted cellular signaling proteins has been limited. One reason is that cytokines are primarily present as monomers while cellular signaling proteins often form complexes with each other [2, 3]. An antibody array is designed to detect a unique target protein per spot or bead, but the immobilized antibody on each spot/bead can also indirectly capture other proteins that form complexes with the target protein [4, 5]. The signal of other proteins can potentially generate a spurious signal on the target spot/bead by the presence of the detection antibody cocktail (Fig. 1A, upper panel). Thus, the antibody array is not suitable for simultaneously detecting multiple proteins such as signaling proteins that may form complexes with each other.

An additional limitation of the antibody array is the inability to quantitatively detect more than one epitope of each protein within a single assay unless each epitope level is in large excess over its antibody. That is because the epitope-specific antibodies on multiple spots/beads of the array will compete for the binding of the same protein. An example is shown in the lower panel of Fig. 1A. The same amount of protein A is present in two samples: sample #1 is free of phospho-epitope and sample #2 carries phospho-epitope (half green circle). If we assume that spots with antibodies against the total protein A epitope (orange) and protein A's phospho-epitope (green) share the same binding capacity, the sample #2 array will generate up to 50% less signal in comparison to sample #1 array for the total protein A amount, leading to the incorrect conclusion that total protein A levels are different between the two samples. Therefore, when trying to quantitatively detect multiple proteins for both the total amount of each protein and the amount of the protein that is phosphorylated, the non-phospho epitopes and the phospho-epitopes are normally detected in separate assays [6, 7].

Accepted Article

Separate assays not only demand a greater volume of valuable samples and higher cost, but also increase assay variation. Finally, the antibody array method is limited to detecting soluble proteins and is incapable of detecting biomarkers in solid phase such as formalin fixed paraffin embedded (FFPE) tissue sections. Most clinical tissue samples to date are prepared by the FFPE method and solubilization of FFPE tissue for the analysis of protein expression and modification remains highly challenging [8].

To overcome the aforementioned limitations of antibody arrays, we have developed the MED method whereby multiple protein epitopes are detected *in situ* (e.g. in fixed cells or tissue) by antibodies that can then be eluted and quantified simultaneously (Fig. 1B). In the MED technique, target cells or tissue sections are first incubated with an antibody cocktail containing antibodies specific to each epitope of interest. After incubation, unbound antibodies are washed away and bound antibodies are recovered by elution from the fixed cells or tissue sections through partial denaturation followed by renaturation back to the physiological condition. The recovered antibodies are then quantified by an epitope array that can be planar or bead based. Each spot or bead in an epitope array contains a specific epitope to which its corresponding antibody in the recovered sample binds and is subsequently detected with a labeled secondary antibody. By using antibodies that pair, for example antibodies that simultaneously detect both phospho- and non-phospho epitopes of a protein (shown in half orange and half green circle on cells in Fig. 1B), multiple epitopes of the same protein can be detected at the same time. The MED technique can also allow for accurate quantitative detection of proteins even if they are in complex with each other (shown in red and blue circles on cells in Fig. 1B). The MED technique is expected to detect any antibody accessible epitope in cells and tissue sections.

The feasibility of the MED technique was demonstrated based on the following three aspects: 1) epitope bound antibodies can be effectively eluted from their epitopes; 2) the antibodies partially denatured during the elution step can be renatured and detected by their cognate epitopes on epitope arrays; 3) the results obtained by the MED technique are correlated with

Accepted Article

the results obtained by Western blotting (Fig. S1 in Supporting Information). We have confirmed that each antibody remains specific to its own epitope after denaturing and renaturing in the multiplex assay format though epitope binding efficiency varies greatly among antibodies (Table S1 in Supporting Information). Additionally, MED assays are found to be reproducible (Table S2 and S3 in Supporting Information).

First, we used the complex-forming proteins NFκB and IκB to demonstrate that the MED method can accurately detect individual proteins even in complex with each other while the conventional antibody array method cannot. Untreated HeLa cells were fixed, permeabilized, blocked and subjected to antibody binding with the cocktail containing either single antibody (IκB or NFκB) (S-plex for single-plex) or both antibodies (IκB and NFκB) (M-plex for multiplex). The bound antibodies were then eluted, renatured and measured by a Luminex bead-based antibody assay (see Supporting Information). As shown in Fig. 2A, we detected similar amount of IκB or NFκB in both single-plex and multiplex formats using the MED method. In contrast, when using the antibody array based Milliplex assay (Millipore), the multiplex format detected dramatically different levels of IκB or NFκB from the single-plex format (Fig. 2B). In the single-plex format of the Milliplex assay, NFκB (or IκB) was detected using NFκB (or IκB) capturing beads and the NFκB (or IκB) detecting antibody. In the multiplex format of the Milliplex assay, NFκB and IκB were simultaneously detected in the presence of both NFκB and IκB capturing beads and both NFκB and IκB detecting antibodies. To determine if the non-IκB signal in the multiplex format came from NFκB and the non-NFκB signal in the multiplex format came from IκB due to the formation of the NFκB and IκB complex, we performed matching and mismatching assays using IκB or NFκB capturing bead and IκB or NFκB detecting antibody. We indeed detected the presence of NFκB on IκB beads and IκB on NFκB beads (Fig. 2C). This is highly suggestive that the non-IκB signal detected in the multiplex format came from NFκB and the non-NFκB signal detected in the multiplex format came from IκB. This result demonstrates that antibody

Accepted Article

arrays are unsuitable for accurately detecting multiple proteins that may form complexes with each other.

NFκB and STAT3 were used to demonstrate that the MED method can simultaneously detect two epitopes of the same protein (Fig. 2D). For NFκB, we used an antibody that recognizes a non-phospho epitope to reflect the total amount of NFκB ("NFκB" in Fig. 2D) and a phospho-specific antibody recognizing the phospho epitope surrounding Serine 536 to reflect the NFκB activation state ("p-NFκB" in Fig. 2D). Similarly for STAT3, we chose an antibody against a non-phospho epitope "STAT3" and a phospho-specific antibody for the phospho epitope "p-STAT3" for the assay. The MED method showed a consistent level of NFκB and p-NFκB, STAT3 and p-STAT3 between single-plex and multiplex formats (Fig. 2D) either in starved or stimulation conditions. This result shows that unlike antibody array (Fig. 1A lower panel), the MED method is capable of simultaneously detecting multiple epitopes of the same protein within a single assay.

Next, we used four antibody pairs to measure protein phosphorylation as well as the total protein expression of four signaling proteins simultaneously by the MED method. Each antibody pair consists of one antibody that detects a phospho epitope to determine the protein activation level and another antibody that detects a non-phospho epitope to determine the total protein amount (total epitope). Fig. 2E is an example of an 8-plex MED assay detecting both phospho epitopes and total epitopes of the inflammation related signaling proteins NFκB, IκB, STAT3 and p38 in HeLa cells. HeLa cells were either untreated or stimulated with 20 ng/ml TNFα, 100 ng/ml IFNα and 200 ng/ml Anisomycin and then subjected to the MED assay using 8 epitope-specific antibodies simultaneously. The 8-plex assay revealed a significant increase of all four phospho epitopes: p-NFκB, p-IκB, p-STAT3 and p-p38 and no change of all four total epitopes: NFκB, IκB, STAT3 and p38 (Fig. 2E). This indicates that the increased activation level of these four inflammation related proteins came from increased phosphorylation of their phospho epitopes and not from

Accepted Article

increased expression of the four inflammation related proteins. This demonstrates the ability of the MED method to detect expression and modification of multiple signaling proteins simultaneously.

We wished to explore the application of the MED technique for biomarker detection in FFPE tissue sections since conventional immunohistochemistry (IHC) suffers from the following limitations: 1) single-plex: limited to one biomarker per tissue section; 2) subjective scoring: scored by visual inspection of the pathologist; and 3) narrow scoring range: from 0 for negative to 3 for strong positive [9, 10]. We used comparably sized human breast cancer tissue and human placental tissue to detect 9 biomarkers, EGFR, ErbB2 PDGFRβ, TfR, NFκB, p38, p-p38, p-S6, and p-STAT3, by the MED method and compared the results to the conventional IHC method. For the MED method, these 9 markers were detected simultaneously with a single section slide of each tissue. Each deparaffinized tissue section was stained with the same marker-specific antibodies as for IHC, at the same antibody dilution, except in an antibody cocktail containing all 9 antibodies. After removing unbound antibodies from each tissue section, the bound antibodies were then eluted, renatured and quantified by the Luminex antibody assay (see Supporting Information). The mean fluorescence intensity (MFI) of each marker-specific antibody was subsequently converted into antibody concentration based on its standard curve (Fig. S2 in Supporting Information). IHC detection and scoring were carried out at Pantomics independently. As shown in Table 1, we detected all 9 marker-specific antibodies from each tissue section ranging from 10 to 15173 in MFI or from 20 to 9766 pg/ml in antibody concentration by the MED method. This suggests a wide range of expression levels of these markers between tissues and among markers, which IHC is unable to report. In general, we found good correlation of the MED level and IHC score. Higher IHC scores correlated with higher MED levels for each marker between two tissues. However, the MED levels provide a much wider dynamic range. For example, the IHC scores of top four biomarkers were 3 to 3.5 while MED levels were 2007 to 9766 pg/ml. Furthermore, the MED method provided more sensitivity than IHC since MED

Accepted Article

detected about 6 fold difference in signal for ErbB2 and more than 2 fold difference in signal for p-STAT3 between breast cancer and placenta tissues which were both negative according to IHC score. Although the baseline of the MED assay for each biomarker cannot currently be assessed without biomarker knockout tissue or the equivalent, the relative amount of each biomarker among different tissue specimen can be assessed either by MFI or antibody concentration (Table 1). This comparison study demonstrates that the MED method can be used to detect multiple markers in a single tissue section. The MED method is more sensitive than IHC while also providing a much wider dynamic range. However, the MED method is not able to provide the percentage and distribution of positive cells within a tissue as provided by the IHC method. The values determined by using the MED method in this application reflect the average signal of the whole tissue section. Therefore, caution is needed when interpreting data from tissues with mixed cell populations.

For further developing the MED method into a true accurate and quantitative biomarker detection technology, we believe following efforts need to be made: 1) Select and optimize biomarker-specific antibodies for effective and similar epitope binding and recovery to achieve minimal variation among assays and to allow comparison among different biomarkers; 2) Determine baseline of each biomarker perhaps through epitope blocking; 3) Develop a cell number or total protein amount normalizing biomarker for normalization such as tissues in different sizes; 4) For tissue with mixed cell populations, use laser capture microdissection technique to extract target cells if necessary for the most accurate quantification.

In conclusion, our results indicate that the MED method overcomes limitations of conventional antibody arrays and can accurately and simultaneously detect multiple epitopes of a single protein and multiple proteins even when they are in complex with each other. The MED method can be applied to detect a large panel of signaling proteins for their post-translational modification and expression level at the same time. Lastly, the MED method

provides a sensitive and quantitative method for detecting multiple biomarkers in FFPE tissue sections.

ACKNOWLEDGEMENTS

This study was supported by LEAP Biosciences Corp. and Epitomics, Inc.

CONFLICT OF INTEREST STATEMENT

Hui Cen and Lyle Rice own significant shares of LEAP Biosciences Corp. The remaining authors are former or current employees of either LEAP Biosciences Corp. or Epitomics, Inc.

REFERENCES

1. Knickerbocker, T., MacBeath, G., Detecting and quantifying multiple proteins in clinical samples in high-throughput using antibody microarrays. *Methods Mol Biol.* 2011, *723*, 3-13.
2. Heilker, R., Freuler, F., Pulfer, R., Di Padova, F. et al., All three IkappaB isoforms and most Rel family members are stably associated with the IkappaB kinase 1/2 complex. *Eur J Biochem.* 1999, *259*, 253-61.
3. Vermeer, P. D., Bell, M., Lee, K., Vermeer D. W. et al., ErbB2, EphrinB1, Src Kinase and PTPN13 Signaling Complex Regulates MAP Kinase Signaling in Human Cancers. *PLoS One.* 2012, *7*, e30447.
4. MacBeath, G., Protein microarrays and proteomics. *Nat Genet*. 2002, *32*, Suppl: 526-32
5. Han, M. K., Hong, M. Y., Lee, D., Lee, D. E. et al., Expression profiling of proteins in L-threonine biosynthetic pathway of Escherichia coli by using antibody microarray. *Proteomics*, 2006, *6*, 5929-40.

Accepted Article

Accepted Article

6. Stamper, B.S., Bammler, T. K., Beyer, R. P., Farin, F. M. et al., Differential Regulation of Mitogen-Activated Protein Kinase Pathways by Acetaminophen and Its Nonhepatotoxic Regioisomer 3'-Hydroxyacetanilide in TAMH Cells. *Toxicol. Sci.,* 2010, *116*, 164 - 173.

7. Khan, I. H., Mendoza, S., Rhyne, P., Ziman, M. et al., Multiplex analysis of intracellular signaling pathways in lymphoid cells by microbead suspension arrays. *Mol Cell Proteomics*, 2006, *5*, 758-68.

8. Berg, D., Hipp, S., Malinowsky, K., Böllner, C. et al., Molecular profiling of signalling pathways in formalin-fixed and paraffin-embedded cancer tissues. *Eur J Cancer*, 2010, *46*, 47-55.

9. Idikio, H. A., Immunohistochemistry in diagnostic surgical pathology: contributions of protein lifecycle, use of evidence-based methods and data normalization on interpretation of immunohistochemical stains. *Int J Clin Exp Pathol.* 2009, *3*, 169-76.

10. Leong, A.S., Zhuang, Z., The changing role of pathology in breast cancer diagnosis and treatment. *Pathobiology*, 2011, *78*, 99-114.

Accepted Article

Fig. 1. Antibody array method can produce falsely elevated signal when detecting multiple proteins which form complexes with each other (A, Upper panel). Without complex formation (left), only detecting antibody X binds to the array spot for protein X. With complex formation (right), detecting antibody Y also binds to the array spot for protein X besides detecting antibody X and produces falsely elevated signal. The antibody array method can produce a false conclusion when detecting multiple epitopes of the same molecule within an array (A, Lower panel). Orange circle: Protein A without phosphorylation; Half orange and half green circle: Protein A with phosphorylation. The orange antibody site detects the epitope for total protein A level while the green antibody site detects a phospho-epitope for protein A phosphorylation level. The MED method (B) elutes antibodies bound to their target proteins within the cells or tissues and quantifies them with an epitope array. The formation of protein complexes does not interfere with the accuracy of MED's protein-specific detection since each eluted antibody exists as a separate molecule. The MED method can be used to detect multiple epitopes of the same protein (shown in orange or green half circle), proteins in a complex (shown in red and light blue) as well as other distinct proteins (shown in purple, yellow and dark blue) within the cells and tissues.

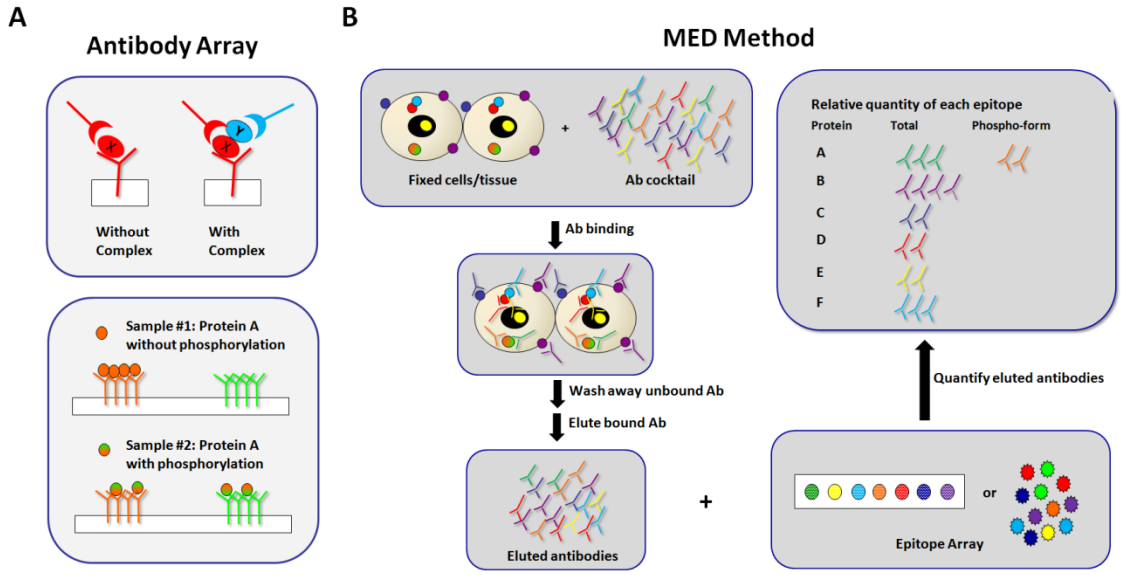

Fig. 1

Fig. 2. Detection of IκB and NFκB either individually (S-plex) or together (M-plex) by the MED method (A). MFI represents mean fluorescence intensity of duplicates. Detection of IκB and NFκB either individually (S-plex) or together (M-plex) using antibody array based Milliplex MAP kits (B). Matching and Mismatching single-plex assays: IκB beads with IκB detection antibody (IκB/ IκB); IκB beads with NFκB detection antibody (IκB/ NFκB), NFκB beads with NFκB detection antibody (NFκB/ NFκB) and NFκB beads/ IκB detection antibody (NFκB/IκB) (C)**.** Detection of NFκB and p-NFκB , STAT3 and p-STAT3 either individually (S-plex) or together (M-plex) by the MED method (D). Simultaneous detection of NFκB, p-NFκB, IκB, p-IκB, STAT3, p-STAT3, p38 and p-p38 by the MED method (E). The fluorescence value of eluted antibody for each target obtained from "untreated" sample is normalized to "1".

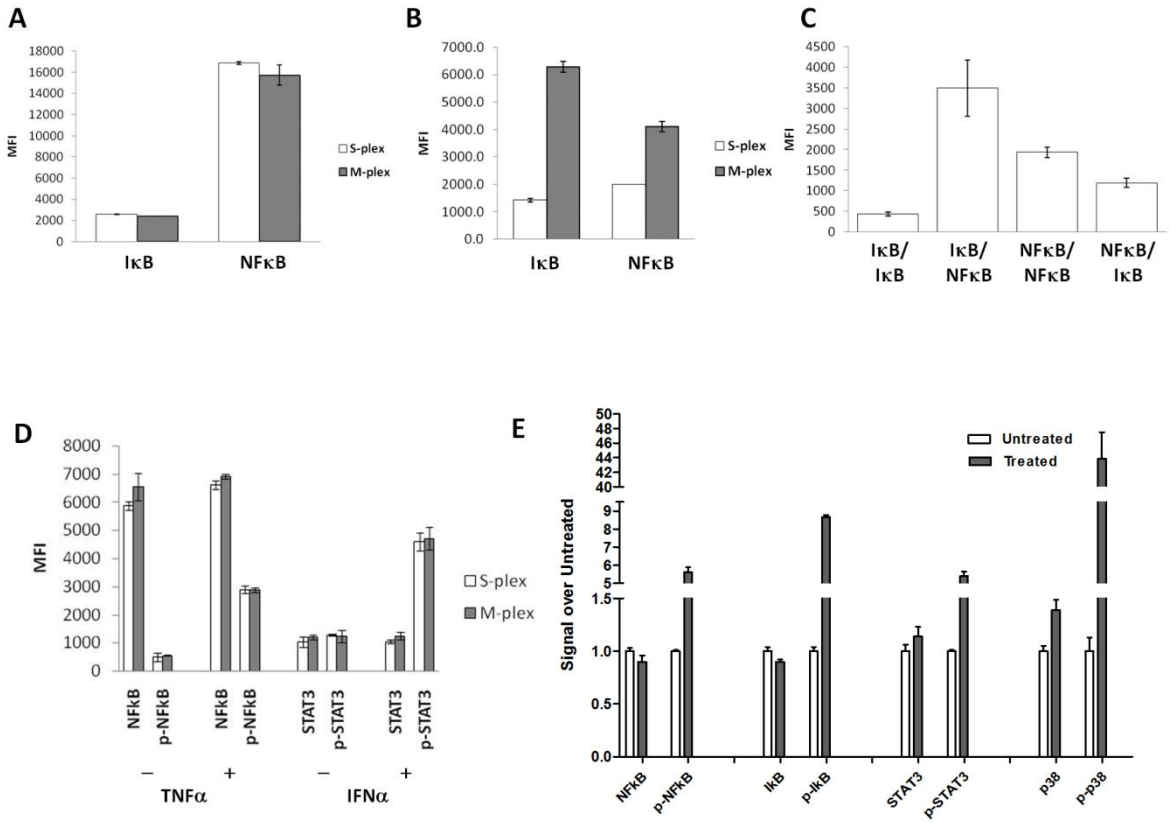

Fig. 2

Accepted Article

Accepted Article

Table 1: Comparison of Biomarker Detection between IHC and MED

Marker	Tissue	IHC		MED			
		Score	Positive Cells %	MFI		Ab (pg/mL)	
				Mean	SD	Mean	SD
EGFR	Breast Cancer	0		587.3	23.0	246.53	9.79
	Placenta	3	>90%	4022.5	108.2	2006.95	65.86
ErbB2	Breast Cancer	0		318.3	6.0	1827.43	36.63
	Placenta	0		58.5	0.7	333.74	4.07
PDGFRβ	Breast Cancer	0		10.0	0.0	211.2	0
	Placenta	2	40%	60.5	0.7	1393.92	17.28
TfR	Breast Cancer	0		120.5	4.9	209.43	8.95
	Placenta	2.5	90%	599.8	11.0	1038.2	19.69
NFκB	Breast Cancer	3.5	>90%	11583.5	307.6	5654.4	262.26
	Placenta	3.5	>90%	15173.8	642.4	9765.93	983.01
p38	Breast Cancer	1	5%	108.0	8.5	81.48	6.72
	Placenta	1.5	30%	453.3	15.2	359.52	12.6
p-p38	Breast Cancer	0		229.5	7.8	19.6	0.98
	Placenta	0		299.5	7.8	26.46	0.98
p-S6	Breast Cancer	1.5	20%	3476.3	216.0	2489.72	158.12
	Placenta	3.5	80%	5455.8	13.8	3994.54	10.72
p-STAT3	Breast Cancer	0		585.8	36.4	45.6	2.85
	Placenta	0		1391.5	23.3	115.9	1.9

Tissue section thickness: 4 mm. Tissue size: ~200 mm^2. Mean: average of duplicates. Antibody concentration is derived from its standard curve and adjustment factor shown in Fig. S2 of Supporting Information.