ANTIGEN DISCOVERY INC

WHITE PAPER

Qualification of Antibody- Cross-Reactivity Using HuProt™ Protein Microarray Version 3.0

Cynthia French, Krista Trappl-Kimmons, Arlo Randall, Gary Hermanson and Tim Le

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Introduction

The application of antibodies in research, diagnostics, and therapeutics has relied on reagents that have been characterized by a wide variety of methods without consistent or standardized validation criteria. The lack of standard guidelines for determining high quality, high-affinity, and target specific antibodies has resulted in variation and inconsistent reproducibility in research and clinical studies. Antibodies sold by the same company have been shown to demonstrate lot-to-lot variability; binding to one target in a series of experiments but performing dramatically different with a different lot of the same reagent. Unfortunately, with more rigorous characterization, these results have become more frequent than obtaining an antibody that performs identically across multiple lots. (1,2)

With the explosive increase of monoclonal and bi-specific antibody reagent use in therapeutic immune-oncology applications, there is an even more critical need for antibody characterization and validation of off-target cross-reactivity. Insuring that therapeutic antibody clinical candidates are highly- specific with no off-target effects and are calibrated to perform successfully is vital to patient safety and clinical efficacy.

Recently, an ad hoc "International Working Group for Antibody Validation" was convened to formulate standardized guidelines for antibody use and validation that may be used in an application-specific manner.(3) This report describes specific recommendations for antibody validation methods, reproducibility criteria, and orthogonal strategies that may serve as a road map for defining experimental approaches of measuring antibody performance and improving research and clinical reproducibility of these essential reagents.

Antigen Discovery Incorporated (ADI) a leading provider of protein microarray services has recently developed qualification procedures and methods to provide clients with antibody cross-reactivity screening services utilizing the HuProt™ human proteome microarray chip platform. The HuProt™ microarray contains the largest number of eukaryotic expressed human proteins individually printed on a single array slide; allowing thousands of protein-antibody interactions to be profiled in a high-throughput manner. The protein microchip is manufactured by CDI NextGen Proteomics, Mayaguez, PR, USA.

Antibody Profiling and Cross-Reactive Study Objectives

In this qualification, we aim to address the objectives below and to develop a protocol for future antibody and serum profiling.

- Quantify target-specific binding of individual monoclonal antibodies (mAbs) to specific protein targets on the microarrays using commercial mAbs
- Develop statistical analysis and target ranking methods for evaluation of mAbs and antiserum performance
- Develop standard operating procedures (SOPS) for probing HuProt™ human protein microarray using reference control reagents, mAbs, and human serum samples.

Materials and Methods

Materials

The HuProt™version3.0 microarray contains >19,000 unique proteins representing 75% of the human proteome. This content encompasses 15,581 unique human genes and 124 unique mouse control proteins. Recombinant proteins are expressed in the yeast *S. cerevisiae*, purified, and printed on glass slides in duplicate. In addition to these proteins, the following are printed as controls: H1 - Histone H1, H2 (A+B) - Histone H2A and H2B mixture, H3 - Histone H3, H4 - Histone H4 (the histones are non-specific binding proteins used as positive controls in a variety of assays), Alexa Fluor 488/594-labeled IgG, Alexa Fluor 555/647-labeled IgG (positive control and landmarks for fluorescent detection in 555/647 channels), glutathione S-transferase (GST) at 10 ng/µl, GST at 50 ng/µl, GST at 100 ng/µl, GST at 200 ng/µl, mouse anti-biotin IgG, rabbit anti-

biotin IgG, biotinylated BSA, BSA (negative control), buffer (printing buffer only, negative control), and mouse IgM. (4)

These expressed recombinant proteins are N-terminal GST and RGS-His6-tagged, and the quality of each microarray batch is determined by GST immunoblotting (98% of all proteins show GST signals significantly higher than negative controls).

Experiments described in this qualification have been performed from June 2016 to February 2017.

Monoclonal Antibody Cross-Reactivity Specificity Assay

HuProt™ microarrays are shipped in closed plastic slide holders on dry ice, or with gel coolant sheets. Upon arrival, microarrays should immediately be stored at -80°C. Probing procedures were performed according to the CDI Human Proteome Microarray v3.0 User Guide instructions. To ensure optimum performance of the HuProt array the following instructions were followed:

- Do not touch the active surface of the microarray (the surface where the bar code label is attached) with hands, with pipette tips or with tweezers. The active surface should always face up.
- Handle microarrays only along the edge near the barcode, using tweezers.
- Do not let the HuProt™ microarray dry out at any time during the assay.
- When conducting low volume assays, be very careful when applying the glass cover slips to the active surface of the microarray (used to minimize evaporation). Likewise, when the assay is completed, be careful when removing the cover slips from the microarrays prior to the washing steps. If the microarray surface is scratched, proteins printed on the glass may be smudged or removed. One alternative is to immerse the covered microarray in a large volume of wash buffer and allow the cover slip to float off.

HuProt™ protein microarrays are blocked for 5 minutes with 4.0 ml of blocking solution (5% BSA/TBS-T). The blocking solution is removed, 4.0 ml of fresh blocking solution is added, and incubation continued at room temperature for 1.5–2 hours with gentle shaking. Primary antibodies are diluted in blocking solution (5% BSA/TBS-T) and applied to the microarray chambers. Arrays are incubated with gentle shaking on an orbital shaker at room temperature for 1 hour. After incubation, microarrays are briefly rinsed three times (~3-5 minutes) with 4.0 ml of TBS-T. After the rinse, microarrays are incubated at room temperature in 4.0 ml of TBS-T with gentle shaking for 10 minutes and buffer removed by aspiration. This wash is repeated 3 times. Secondary antibodies are then diluted to manufacturer's recommendations; 3.0 ml of diluted secondary antibody is added and incubation continued at room temperature under aluminum foil cover for 1 hour with gentle shaking. Arrays are washed 3 times with 4.0 ml TBS-T and gentle shaking for 10 minutes at room temperature. After washing, arrays are briefly rinsed three times with ddH2O. The arrays are put into 50 ml conical tubes with wipes to cushion the slide. The 50 ml conical tubes with arrays are spun at 500 rpm for 3 minutes.

Microarray Data Output and Computational Analysis

Following antibody probing, the slide is analyzed with Mapix data acquisition and analysis software. (Innopsys, France)

Hits from the probing are identified by cross-referencing the array map and exact location of the reactive protein on the array.

We have chosen to quantify the affinity of the individual monoclonal antibodies to specific targets on the array by performing the statistical analysis scoring methods detailed in Jeong, JS et al 2012, and the CDI White Paper 2016. (5,6)

Mapix data points are analyzed in terms of signal strength and ranked by two scores: A-score that indicates the number of standard deviations above background seen for the mean signal bound by the target antigen; and the S-score which represents the difference between the A-score of the target antigen and the next best hit on the array.

S-scores greater than 3 standard deviations over the next listed target are deemed statistically significant and indicative of high specificity antibodies.

A-Score Calculation

The A-Score (Affinity Score) is the normalized signal intensity of spots on the HuProt™ Array. The A-score indicates the number of standard deviations above background seen for the mean signal bound by the target antigen.

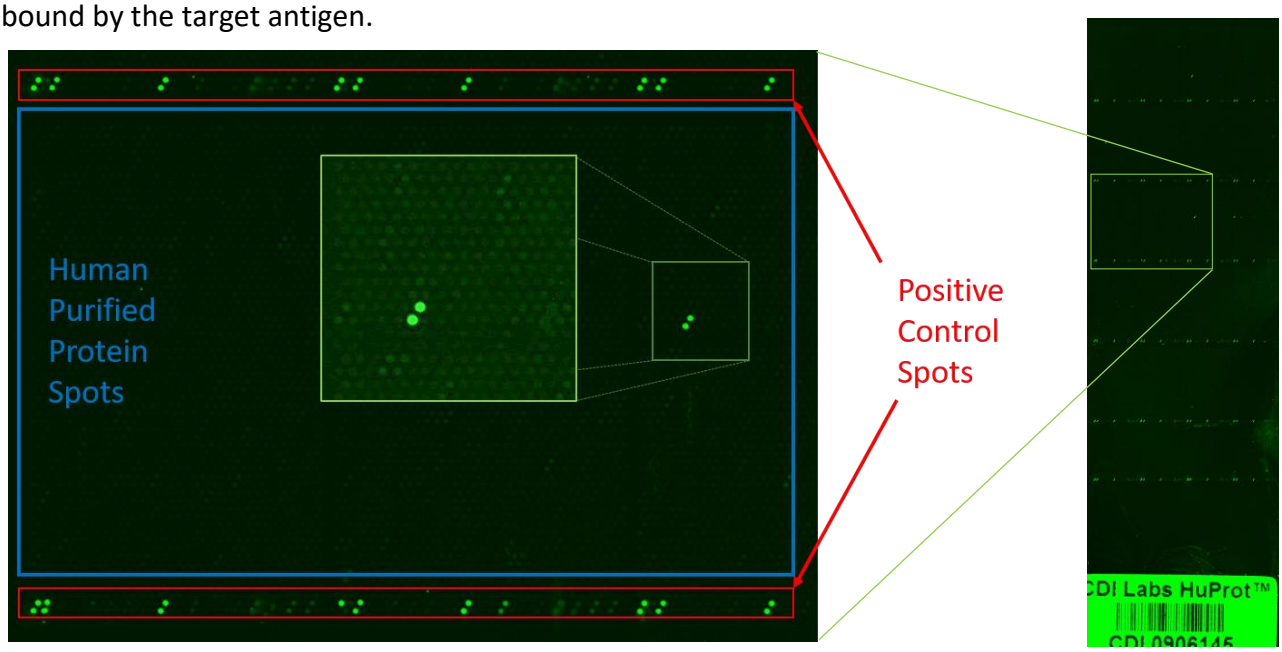


Figure 1: Magnified image of a monoclonal antibody -probed whole slide microarray showing localization of a mAb specific target antigen binding signal in duplicate. Positive controls are included in image.

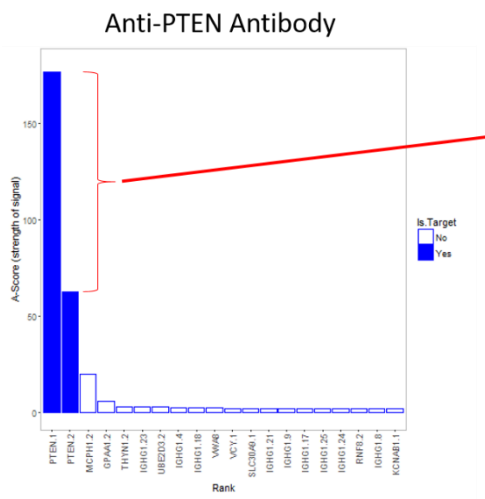
To quantify the affinity of an individual mAb to specific proteins on the array, the following A-score calculations are performed:

1. Calculate raw signal (x) of each spot as ratio of foreground signal to local background signal.
2. Calculate global median (g.m) and global standard deviation (g.sd) of all purified protein spots on the array.
3. Calculate A-Score as: (x – g.m)/g.sd.

NOTE: this is conservative since true positive spots are used in calculation of global standard deviation (g.sd).

S-Score Calculation

The S-score (Specificity Score) represents the difference between the A-score of the target antigen and the next best hit on the array. S-scores greater than 3 standard deviations over the next listed target are deemed statistically significant and indicate highly specific antibodies.



Rank	Name	Name.Unique	ID	A Score	S Score
1	PTEN	PTEN.1	JHU14142.P148D03	176.64	114.15
2	PTEN	PTEN.2	JHU15357.P161H12	62.49	42.69
3	MCPH1	MCPH1.2	JHU15340.P161G12	19.81	14.17
4	GPAA1	GPAA1.2	JHU14114.P148F03	5.64	2.78
5	THYN1	THYN1.2	JHU16333.P171E07	2.86	0.03
6	IGHG1	IGHG1.23	JHU15994.P179G07	2.83	0.08
7	UBE2D3	UBE2D3.2	JHU10732.P112H09	2.75	0.27
8	IGHG1	IGHG1.4	JHU01487.P016B05	2.48	0.02
9	IGHG1	IGHG1.18	JHU15224.P160D07	2.46	0.26
10	VWA8	VWA8	JHU19205.P195C03	2.21	0.16

Figure 2: Example of S-Score analysis. If the expected target is ranked #1 and the S-Score (the difference between Rank #1 and #2) is >3, then the antibody is considered monospecific.

Note: All protein spots are ranked by A-Score, then the S-Score is calculated as the difference between the A-Score of that protein and the next highest ranked protein (5).

Results

Five Commercially Available Monoclonal Antibodies Tested on HuProt™ Arrays.

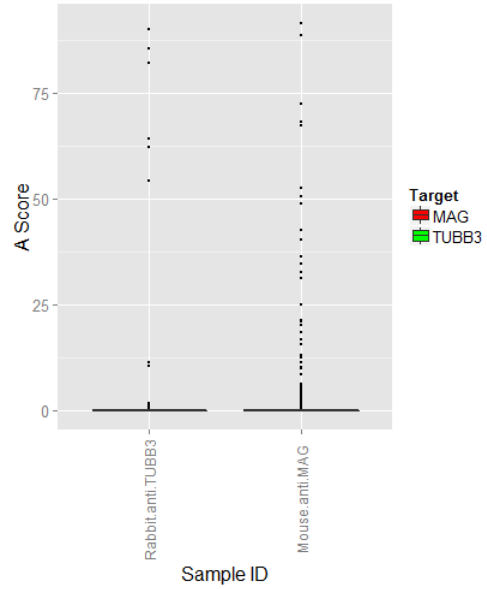
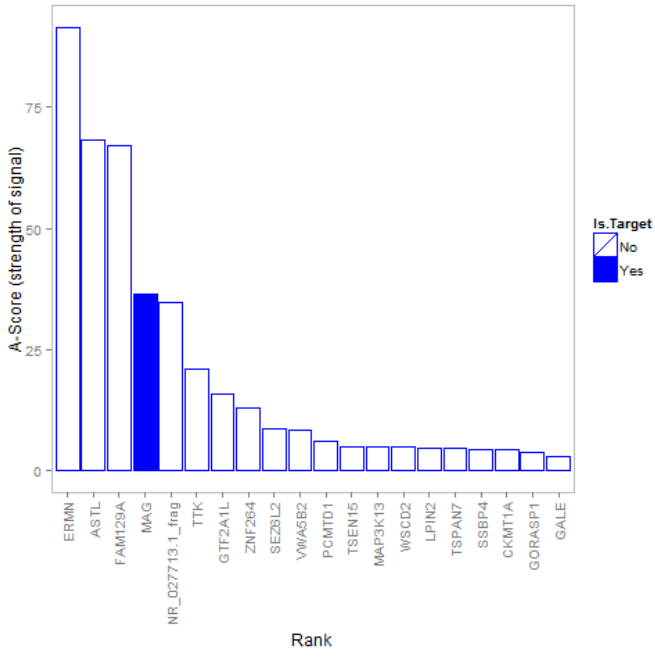


Figure 3: A-score distribution for three different antibodies targeting MAG and TUBB3

Anti-MAG Monoclonal Antibody



Rank	Name	ID	A Score	S Score
1	ERMN	JHU07443.P078C07	91.46	23.26
2	ASTL	JHU19261.P196B01	68.19	0.98
3	FAM129A	JHU12860.P135E12	67.21	30.90
4	MAG	JHU07056.P074G02	36.30	1.71
5	NR_027713.1_frag	JHU15602.P164E08	34.59	13.67
6	TTK	JHU08530.P089C10	20.93	5.26
7	GTF2A1L	JHU01736.P186C03	15.67	2.65
8	ZNF264	JHU19786.P183H02	13.02	4.52
9	SEZ6L2	JHU08510.P089B10	8.50	0.08
10	VWA5B2	JHU29124.P078B07	8.42	2.49

Figure 4: Bar plot and A-Score and S-Score analysis of top-10 hits for Anti-MAG monoclonal antibody.

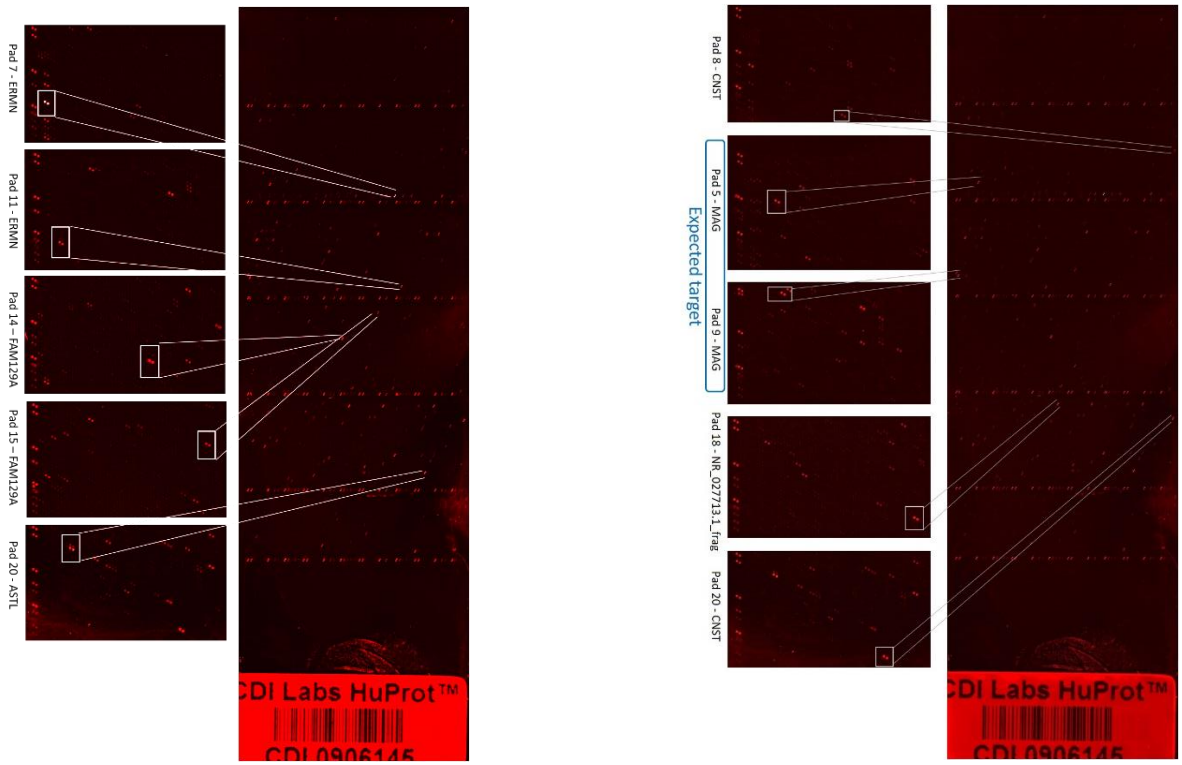
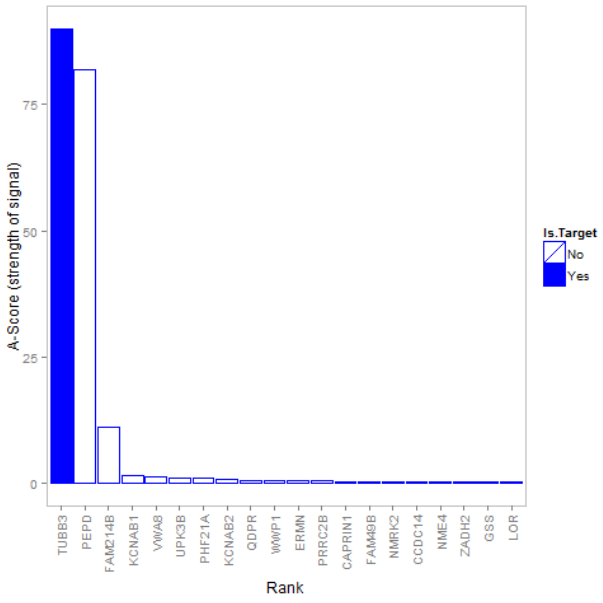


Figure 5: HuProt™ slide probed with Anti-MAG antibody, top hits have been magnified and highlighted on the left.

Anti-TUBB3 Monoclonal Antibody



Rank	Name	ID	A Score	S Score
1	TUBB3	JHU02583.P027C11	89.96	8.01
2	PEPD	JHU05526.P058H09	81.95	70.72
3	FAM214B	JHU09451.P099B01	11.23	9.69
4	KCNAB1	JHU05510.P058B02	1.54	0.14
5	VWA8	JHU19205.P195C03	1.39	0.37
6	UPK3B	JHU09497.P099A01	1.03	0.07
7	PHF21A	JHU01110.P012E12	0.96	0.15
8	KCNAB2	JHU07150.P075E02	0.81	0.23
9	QDPR	JHU13192.P138H11	0.59	0.07
10	WWP1	JHU12347.P129H10	0.51	0.05

Figure 6: Bar plot and A-Score and S-Score analysis of top-10 hits for Anti-TUBB3 monoclonal antibody.

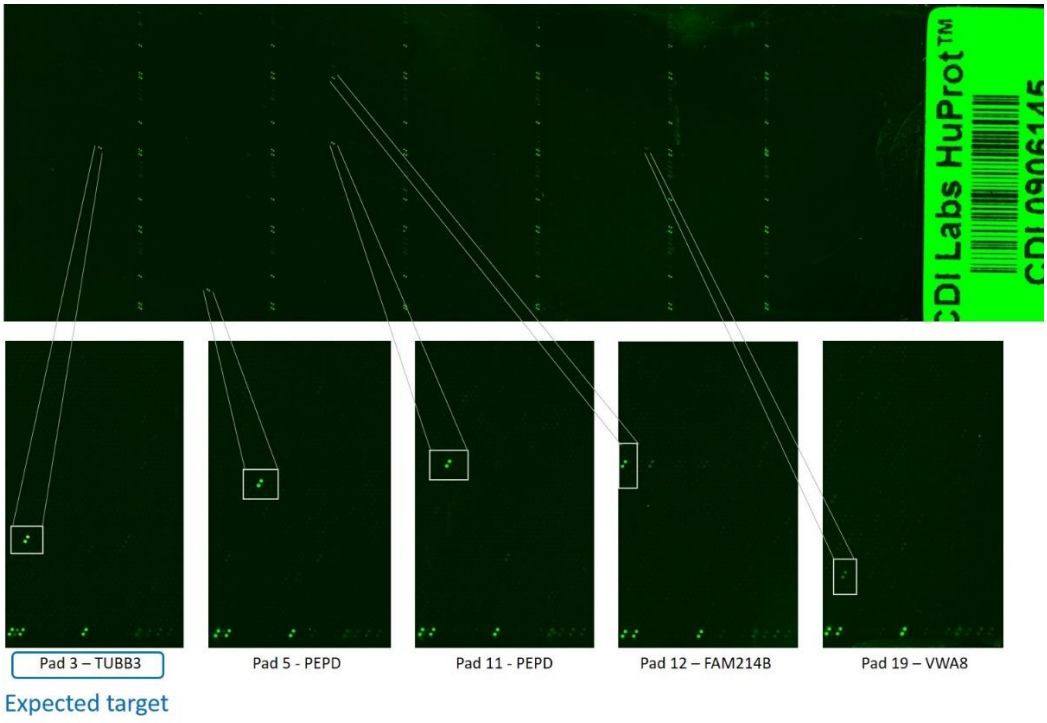


Figure 7: HuProt™ slide probed with Anti-TUBB3 antibody, top hits have been magnified and highlighted underneath.

Anti-PTEN Monoclonal Antibody Comparison

Three commercially available antibodies targeting the human PTEN protein are tested for specificity side-by-side.

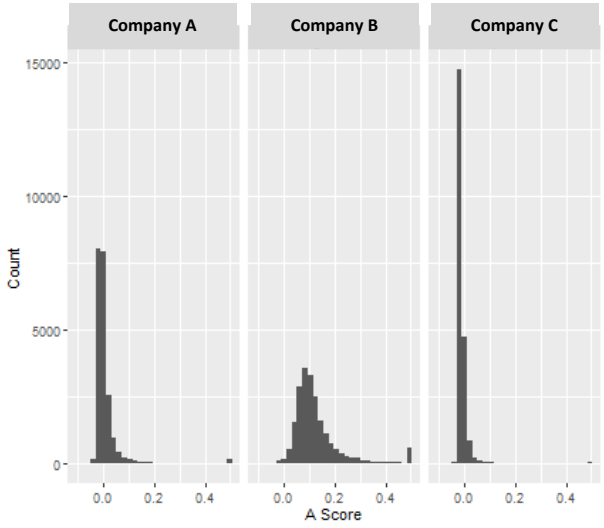
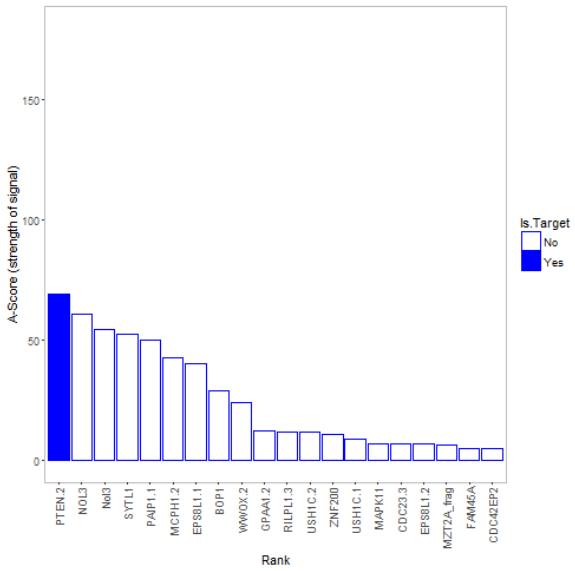
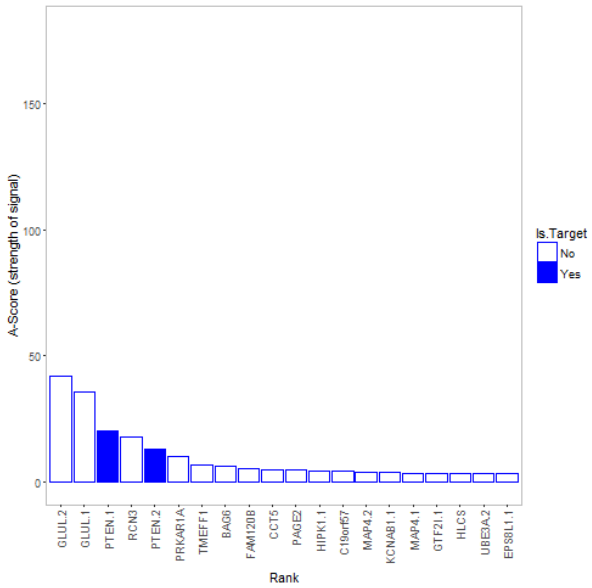


Figure 8: A-score distribution for three different antibodies targeting PTEN



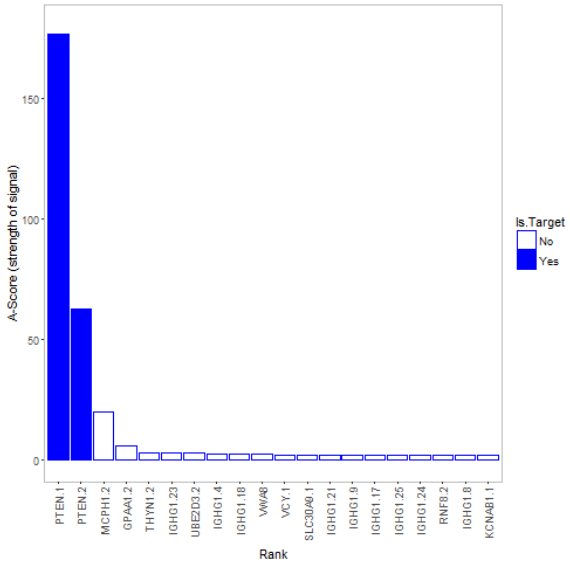
Rank	Name	Name.Unique	ID	A Score	S Score
1	PTEN	PTEN.2	JHU15357.P161H12	69.04	8.34
2	NOL3	NOL3	JHU09079.P095G02	60.70	6.30
3	Nol3	Nol3	JHU19589.P181C05	54.40	1.97
4	SYTL1	SYTL1	JHU08341.P087A03	52.43	2.50
5	PAIP1	PAIP1.1	JHU02075.P022C05	49.93	7.27
6	MCPH1	MCPH1.2	JHU15340.P161G12	42.66	2.57
7	EPS8L1	EPS8L1.1	JHU08289.P087C03	40.09	10.92
8	BOP1	BOP1	JHU08549.P090A11	29.17	5.28
9	WWOX	WWOX.2	JHU11317.P118B12	23.88	11.63
10	GPAA1	GPAA1.2	JHU14114.P148F03	12.25	0.46

Figure 9: Bar plot and A-Score and S-Score analysis of top-10 hits for Anti-PTEN (Company A) monoclonal antibody.



Rank	Name	Name.Unique	ID	A Score	S Score
1	GLUL	GLUL.2	JHU05602.P059F10	42.11	6.26
2	GLUL	GLUL.1	JHU01583.P017C04	35.85	15.81
3	PTEN	PTEN.1	JHU14142.P148D03	20.04	2.08
4	RCN3	RCN3	JHU18948.P226G11	17.96	4.98
5	PTEN	PTEN.2	JHU15357.P161H12	12.98	2.71
6	PRKAR1A	PRKAR1A	JHU05633.P059G11	10.27	3.34
7	TMEFF1	TMEFF1	JHU05647.P059F11	6.94	0.86
8	BAG6	BAG6	JHU03468.P037D02	6.08	0.98
9	FAM120B	FAM120B	JHU08667.P091A04	5.10	0.34
10	CCT5	CCT5	JHU08938.P094C04	4.76	0.20

Figure 10: Bar plot and A-Score and S-Score analysis of top-10 hits for Anti-PTEN (Company B) monoclonal antibody.



Rank	Name	Name.Unique	ID	A Score	S Score
1	PTEN	PTEN.1	JHU14142.P148D03	176.64	114.15
2	PTEN	PTEN.2	JHU15357.P161H12	62.49	42.69
3	MCPH1	MCPH1.2	JHU15340.P161G12	19.81	14.17
4	GPAA1	GPAA1.2	JHU14114.P148F03	5.64	2.78
5	THYN1	THYN1.2	JHU16333.P171E07	2.86	0.03
6	IGHG1	IGHG1.23	JHU15994.P179G07	2.83	0.08
7	UBE2D3	UBE2D3.2	JHU10732.P112H09	2.75	0.27
8	IGHG1	IGHG1.4	JHU01487.P016B05	2.48	0.02
9	IGHG1	IGHG1.18	JHU15224.P160D07	2.46	0.26
10	VWA8	VWA8	JHU19205.P195C03	2.21	0.16

Figure 11: Bar plot and A-Score and S-Score analysis of top-10 hits for Anti-PTEN (Company C) monoclonal antibody.

In summary, the three tested Anti-PTEN antibodies all bound to the intended target (targets) but varied in specificity and sensitivity. Company A antibody was sensitive to detection of intended target PTEN (highest A-Score), however it was not specific as many off-targets where bound with similar A-Scores. Antibody from Company B showed rather weak binding overall, the highest intensity target spot was not PTEN. In this test, the antibody from Company C performed the best in both, intensity and specificity; binding strongly to the intended target and very low binding to other non-target proteins.

Conclusion

We have utilized a comprehensive human purified protein microarray- HuProt™ 3.0 platform to determine the specificity and binding of protein epitopes by commercial monoclonal antibody reagents. Qualification of cross-reactivity and off-target binding effects using protein microarray technology is an efficient and cost-effective method that enables rapid identification of high quality mAbs as reproducible protein specific reagents for research and clinical studies.

In addition, we have demonstrated standardized microarray data analysis methods of mAb specificity scoring based on the work of CDI that enable comparison of results from mAb reagents obtained from commercial suppliers and other scientific laboratories.

All studies shown in this white paper were performed using Good Laboratory Practices (GLP) including SOP documentation and training of technologists, providing a high level of quality assurance for a successful outcome in the use of microarray based-analysis for the determination antibody specificity.

Our results of probing five different monoclonal antibodies against three separate targets showed the inconsistent quality of antibodies in both, spot intensity and specificity. Our results add to the literature illustrating that the HuProt™ microarray is a well-suited platform to qualify monoclonal antibodies.

By providing highly qualified cross-reactivity analysis services to the scientific community we can reduce research costs by identifying poor performing mAb reagents which will dramatically improve lab-to-lab reproducibility of results leading to greater research and clinical study progress.

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