Antibody Cross-Reactivity Testing Using the HuProt™ Human Proteome Microarray

Analysis of several commercially available antibodies against nearly 75% of the human proteome.

A **CDI Laboratories in partnership with Arrayjet Ltd.** White Paper

by Pedro Ramos, Andrew Leahy, Ignacio Pino, and Scott Paschke

April, 2016

CONCLUSION: The use of protein microarrays to evaluate antibodies is likely to set new quality standards to evaluate antibody cross-reactivity and will also address the need for new methods to identify antibodies that can be used in robust scientific investigations, grant proposals and for commercialization.

ARRAYJET
inkjet liquid handling

CDI
NEXTGEN PROTEOMICS

Table of Contents

Introduction

A number of recent articles and commentaries published in high impact journals detail the problems with antibody cross- reactivity, its impact on data relevancy and the amount of time and money wasted on the use of poor antibodies. In addition, there is growing demand from the NIH regarding the need for antibody standardization that would ensure that reagents used in publications are actually detecting the proteins of interest.

Antibodies are among the most commonly employed biological research reagents - tools used primarily to identify and/or isolate molecules of interest. It is becoming clear that they are also the cause of many problems regarding data interpretation and may hinder researcher's abilities to reach unambiguous conclusions. (Baker M. (2015) Reproducibility crisis: Blame it on the Antibodies. *Nature* **521**: 274-276.)

Scientists - along with non-scientists - contend that the complex system for ensuring the reproducibility of biomedical research is failing at certain levels and is in need of restructuring. (Bradbury Aand PlückthunA(2015) Reproducibility: Standardize antibodies used in research. *Nature* **518**: 27-29.)

Glenn Begley, chief scientific officer at TetraLogic Pharmaceuticals in Malvern, Pennsylvania, and coauthor of the controversial article "Drug development: Raise standards for preclinical cancer research" (Begley and Ellis, *Nature* **483:**531–533, 2012) suggested that "poorly characterized antibodies probably con- tribute more to the problem than any other laboratory tool". While antibodies are the undisputed workhorses of biological experimentation, they are also littering the field with false find- ings. A few crusaders are pushing for change.

This recent and growing concern over commercial antibody quality indicates that the day is coming when all commercially-available antibodies will need to undergo standardized testing to meet quality criteria. Only then will the data generated using these antibodies be accepted for journal publication or for inclusion in NIH grants.

As an example, some companies have begun using knockout cell lines to address antibody specificity issues. This is a good move forward but is still not a complete solution. A knockout cell line does give a snapshot of how a particular antibody behaves in a particular cell line under the given assay conditions. The knock- out does not, however, guarantee that antibody will behave similarly in a different cell line under different conditions, or in a different tissue type. This is because radically different expression levels of proteins can occur across cell lines, tissue types and treatments. In contrast, using high content protein microarrays produces a comprehensive picture of antibody cross-reactivity against a large part of the entire human proteome. Since the proteins spotted on the arrays are robustly expressed, array analysis can give a clear profile of cross-reactivity across proteins that are selectively expressed in a wide range of cell lines.

In addition, with tools such as the CDI Labs HuProt™ array, it is possible to measure antibody cross-reactivity to proteins in their native, folded conformation, (e.g., in an immunoprecipitation or immunofluorescence assay), or to denatured proteins, (as found on western blots, or in IHC) in which antibodies do not recognize native proteins and thus the tissues need to be processed by antigen retrieval methods. The HuProt™ arrays are ideal for both types of screening, as the proteins are initially spotted in native conformation, but the arrays can also be de- natured with 9M urea and 5mM DTT. See Hu, *et al.* "Proteins on HuProt™ Array Are Well Folded": A poster presentation i**ncluded in the Appendix, page 25.**

Continued on page 3.

Continued from page 2.

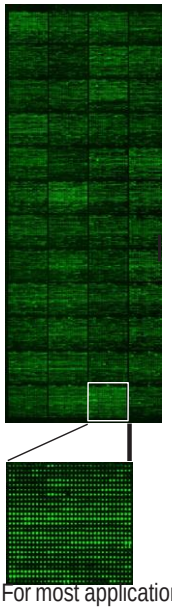
In a move aimed at improving the quality and usefulness of commercial antibodies, the NIH started the Protein Capture Reagents Program to produce validated and highly specific antibodies /protein detection reagents. CDI Laboratories is part of this program and uses a patented technique to produce mono- clonal antibodies with unprecedented specificity. All of the antibodies produced by CDI Labs are tested for monospecificity on the HuProt™ protein microarray, which is the world's highest- content protein microarray.

In order to demonstrate the usefulness of human proteome microarray analysis of research reagents, a number of commercial MAbs were screened on CDI's HuProt™ protein microarrays to look at possible cross reactivities.

In the pages that follow, CDI presents cross-reactivity data on a number of commercial antibodies, both as a function of working concentration and protein conformation (native vs denatured).

Methods and Materials

HuProt™ human proteome microarray



The HuProt™ human proteome microarray provides the largest number of unique hu- man proteins known to be included on a single slide, allowing thousands of interactions to be profiled in high-throughput.

The HuProt™ version 2.0 microarray contains thousands of purified human and ~100 mouse proteins. This content encompasses the encoded products of more than 15,000 unique human genes (~75% of the human proteome) and over 100 unique mouse gene symbols. Recombinant proteins are expressed in the yeast *S. cerevisiae*, individually purified using N-terminal GST or RGS-His6-tags, and printed on glass slides in duplicate. The quality of each microarray batch is determined by GST immunoblot- ting (98% of all proteins show GST signals significantly higher than negative controls).

For most applications, CDI prints the microarray on glass slides that are coated with 3D polymers that contain functional groups. This allows the protein samples to be immobilized on the glass by covalent bonding.

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/5l, GST at 50 ng/5l, GST at 100 ng/5l, GST at 200 ng/5l, mouse anti- biotin IgG, rabbit anti-biotin IgG, biotinylated BSA, BSA (nega- tive control), buffer (printing buffer only, negative control), mouse IgM.

Monoclonal antibody specificity determination assay

HuProt™ protein microarrays were blocked for 5 min with 3.0 ml of blocking solution (5% BSA/TBS-T). The blocking solution was removed, 3.0 ml of fresh blocking solution was added, and incubation was continued at room temperature for 1.5–2hrs with gentle shaking.

Primary antibodies were diluted in blocking solution (5% BSA/ TBS-T) to the concentrations detailed in the figures below. Arrays were incubated with gentle shaking on an orbital shaker at room temperature for 1 hr.

After incubation, microarrays were briefly rinsed with 4.0 ml of TBS-T. After the rinse, microarrays were incubated at room temperature in 4.0 ml of TBS-T with gentle shaking for 10 min and buffer was removed by aspiration. This wash was repeated 3 times.

Secondary antibodies were then diluted to manufacturer's recommendations. 3.0 ml of diluted secondary antibody was add- ed and incubation continued at room temperature for 1 hr with gentle shaking. Arrays were washed 3 times with 4.0 ml TBS-T and gentle shaking for 10 min at room temperature. After washing, arrays were briefly rinsed three times with ddH_2O.

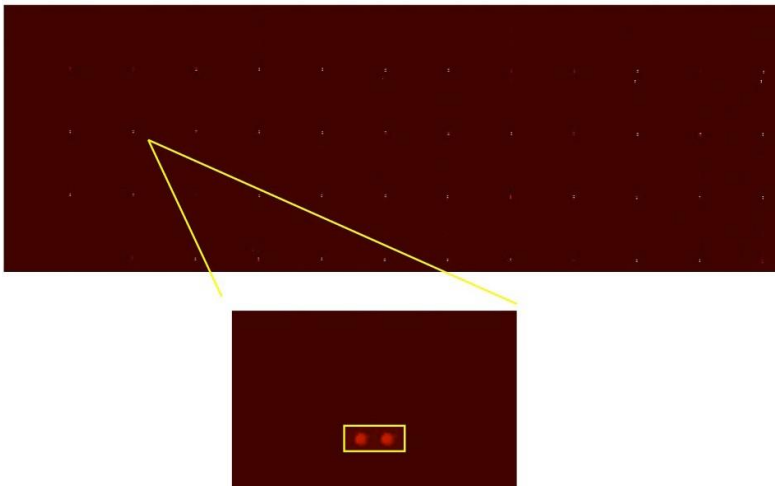
The arrays were put into 50 ml conical tubes that contained Kimwipes at the bottom. The 50 ml conical tubes with arrays were spun at 800 rpm for 3 minutes. Arrays were then immedi- ately analyzed by scanning with a Genepix scanner.

Native vs denatured arrays

To determine whether the antibodies bound to native and/or denatured forms of their target proteins, HuProt™ arrays were blocked as previously described. 3 ml of denaturation buffer (9 M urea with 5 mM DTT) was added and the arrays were incubated at room temperature WITHOUT shaking for 20 min. Arrays were then washed 3 times for 5 min with gentle shaking in TBS-T.

Arrays were probed with primary and secondary antibodies and then scanned as described above.

Microarray data output and statistical analysis

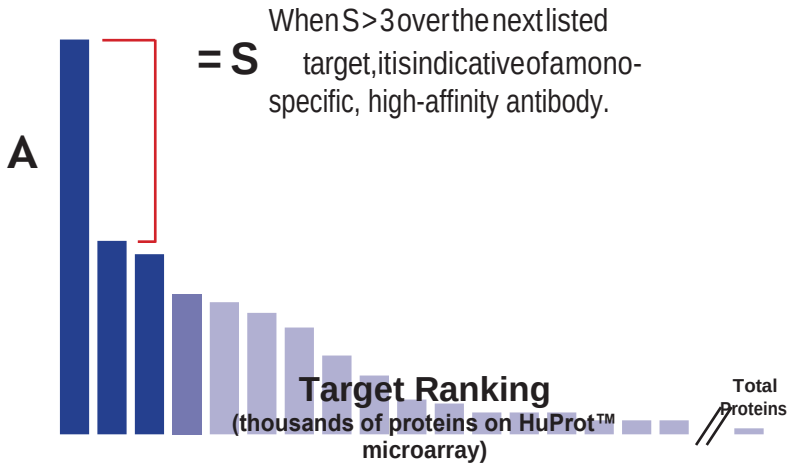


Microarray Analysis: Antibody specificity was evaluated using the CDI HuProt™ Human Proteome Microarray (~75% of the human proteome) and subsequently analyzed with GenePix Pro Image Acquisition and Analysis Software, the benchmark tool for the acquisition and analysis of microarray images. The top 3 "hits" are identified by cross-reference to the array map which stores the exact location of each protein. 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.

Statistical Analysis: Thousands of GenePix data points (from the microarray) are analyzed in terms of signal strength and ranked accordingly.

SUMMARY: The A-score indicates the number of standard deviations above background seen for the mean signal bound by the target antigen. The S-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.**



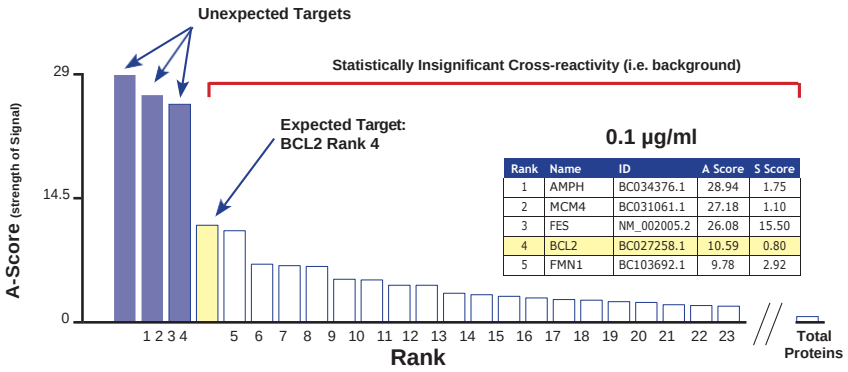
IN DEPTH: The following is excerpted from Jeong JS *et al.* (2012). Rapid identification of monospecific monoclonal antibodies using a hu- man proteome microarray. *Mol Cell Proteomics* **11**(6):O111.016253.

> "To quantify the affinity of individual mAbs to specific proteins on the array, we first calculated the mean and standard deviation of the signal intensity across all spots on the chip. We obtain normalized signal intensity for any pair of spots, which we define as ***A***, which is the mean ***z*** score for each duplicate pair of spotted proteins, where $A_n = (I_n - m)/\sigma$. Here, ***I*** is the ratio of median foreground and median background fluorescence for any given spot pair ***n***, ***m*** is the median value for ***I*** for all spots on the array, and σ is the standard deviation for ***I***. mAbs found in one unique row pool and one column pool that showed ***z*** scores greater than 2.8 for both duplicate spots of any given protein were then flagged for individual analysis.
>
> mAbs identified as potentially specific using this pooling strategy were then tested individually against the entire array, and ***A*** was measured for each spotted protein. We next quantitatively evaluated the specificity of any individual mAb identified as potentially specific by means of this analysis. To do this, we calculated a value for specificity that we define as ***S***, where $S = A_1 - A_2$. Here, A_1 represents the spot pair on the array that shows the highest value of ***A***, and A_2 represents the spot pair with the second highest value of ***A***."

The A-Score (Affinity Score) is the normalized signal intensity of spots on the HuProt™ Array. The S-Score (Specificity Score) is the difference between the A-score of a target ranking and the A-score of the target ranking that immediately follows. S-scores greater than 3 standard deviations over the next listed target are deemed statistically significant and indicate highly specific antibodies.

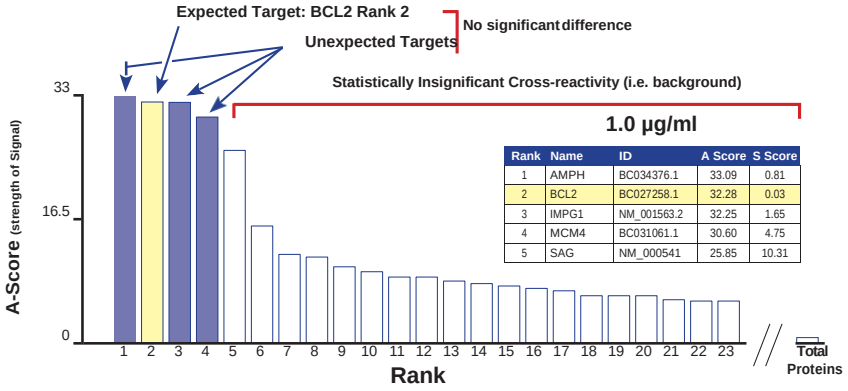
ELISA confirmation of cross reactivity: Company C Anti-BCL2 (2 working concentrations)

HuProt™ Microarray Analysis



0.1 µg/ml

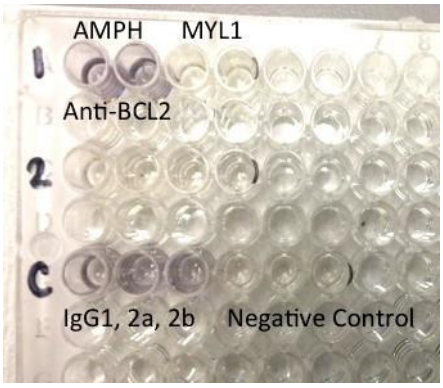
Rank	Name	ID	A Score	S Score
1	AMPH	BC034376.1	28.94	1.75
2	MCM4	BC031061.1	27.18	1.10
3	FES	NM_002005.2	26.08	15.50
4	BCL2	BC027258.1	10.59	0.80
5	FMN1	BC103692.1	9.78	2.92



1.0 µg/ml

Rank	Name	ID	A Score	S Score
1	AMPH	BC034376.1	33.09	0.81
2	BCL2	BC027258.1	32.28	0.03
3	IMPG1	NM_001563.2	32.25	1.65
4	MCM4	BC031061.1	30.60	4.75
5	SAG	NM_000541	25.85	10.31

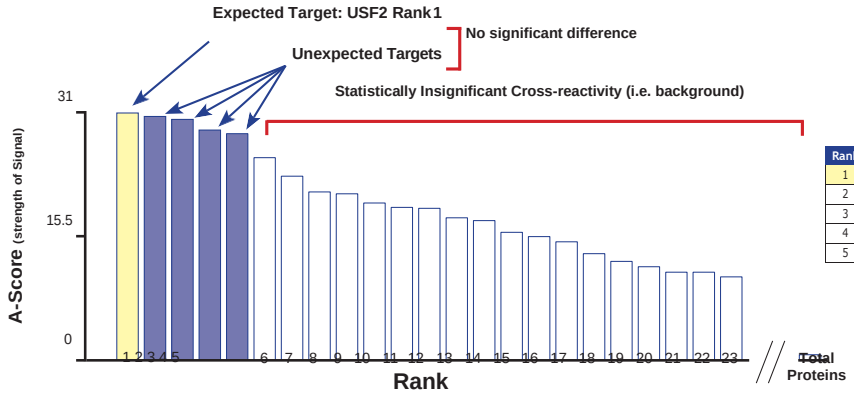
ELISA



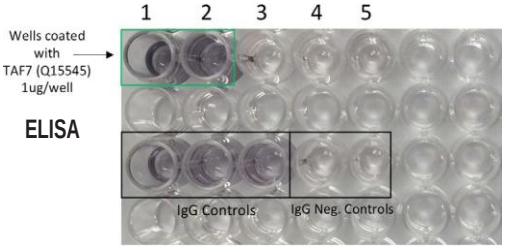
The BCL2 antibody from Company C exhibited cross-reactivity on the HuProt™ microarray and recognized AMPH as the top target at two different concentrations (0.1 and 1.0 µg/mL). Confirmation of cross-reactivity was done via an ELISA using a commercially available recombinant AMPH (NM_001635, Origene - TP306620) as the coating material. The plate was coated overnight at a concentration of 1 µg/well. Results from the ELISA clearly show that the Anti-BCL2 from Company C recognized AMPH. When commercially avail- able recombinant MYL1 (NM_079422, Origene - TP302750) was used to coat wells, the same antibody gave no reaction. These results correlate with the HuProt™ findings.

ELISA confirmation of cross reactivity: Company A Anti-USF2 (1.0 µg/ml)

HuProt™ Microarray Analysis



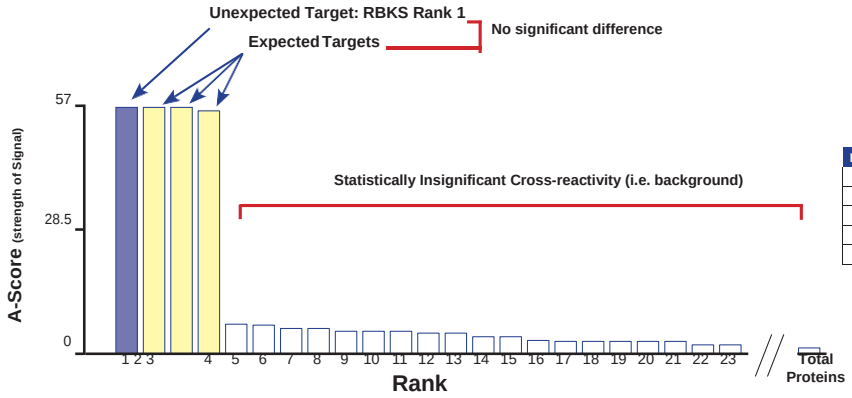
Rank	Name	ID	A Score	S Score
1	USF2	NM_003367.2	31.26	0.65
2	N4BP1	NM_153029	30.61	0.32
3	TAF7	NM_005642.2	30.29	1.51
4	SSH3	BC007709.1	28.78	0.26
5	RAB4B	NM_016154.3	28.51	2.10



We tested a commercial antibody (Company A) against USF2 at 1.0 µg/ml in native and denatured HuProt™ conditions. This mAb recognized the expected target (USF2) as the top target (Rank 1) with a S-score of 0.65. However, the mAb also showed cross reactivity with TAF7 in both conditions. To confirm that this cross reactive hit is real, we purchased the human recombinant protein TAF7 - Q15545 and coated a plate (wells 1-5) overnight at 1 µg/well. In the results above the Anti-USF2 from Company A recognized TAF7, therefore correlating with the HuProt™ results. When commercially available recombinant protein TFDP1 - Q14186 was used to coat wells, the same antibody gave no reaction.

ELISA confirmation of cross reactivity: Company G Anti-KCNP2 (1.0 µg/ml)

HuProt™ Microarray Analysis



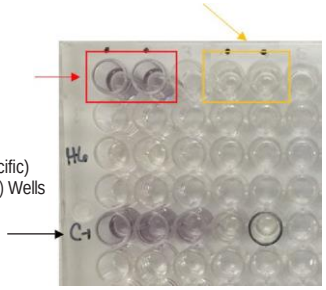
Rank	Name	ID	A Score	S Score
1	RBKS	IOH14265	56.58	0.00
1	KCNIP2	IOH29635	56.58	0.00
3	KCNIP2	IOH39571	56.58	0.57
4	KCNIP2	IOH39568	56.01	50.44
5	NSDHL	IOH4468	5.573	0.15

ELISA

Wells Coated: RBKS Rec. Protein
Primary MAb: N19_2.2 (DLG3) Neg. Control

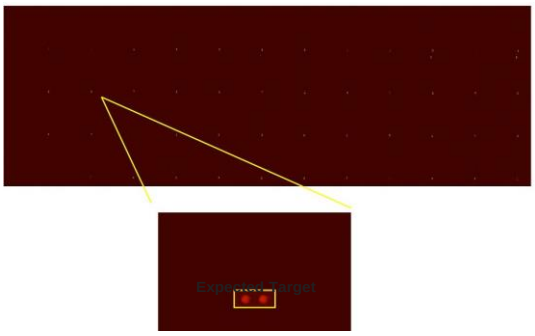
Wells Coated: RBKS Rec. Protein
Primary MAb: K60_73.1 (KCNP2)

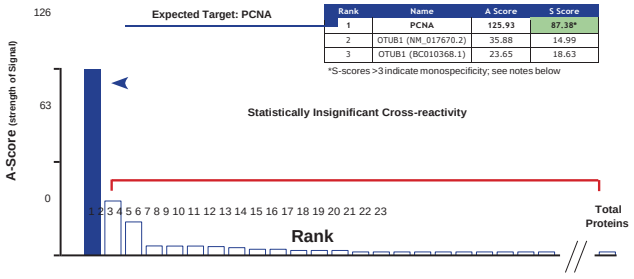
Wells Coated: Anti-Mouse IgG (Fc specific)
Primary MAb: Wells 1-3 (Anti-IgG1, -2a, -2b) Wells 4-5 (Neg. Control)



This antibody demonstrated cross-reactivity on HuProt and recognized RBKS as one of top targets. We proceeded to confirm the cross-reactivity by ELISA using LsBio human recombinant RBKS (Cat# LS-G1046). The ELISA plate was coated plate overnight (1 µg/well) with RBKS. The results indicate the mouse monoclonal Anti-KCNP2 recognized RBKS (red box). As expected, it correlated with the HuProt results. An additional mouse monoclonal antibody (Anti-DLG3) was tested as a negative control against RBKS and no reaction was observed (yellow box).

A monospecific, high-quality PCNA antibody

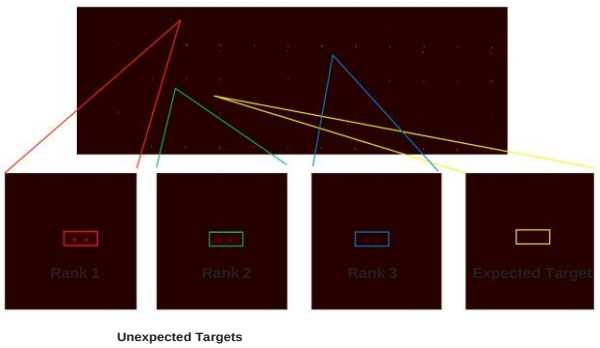




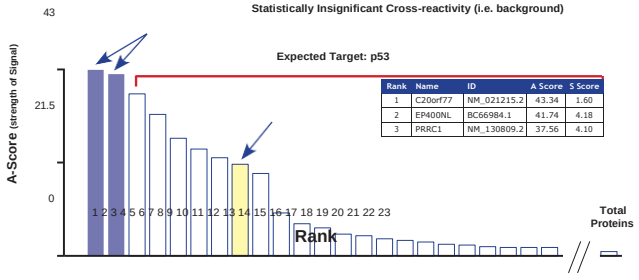
Rank	Name	A Score	S Score
1	PCNA	125.93	87.38*
2	OTUB1 (NM_017670.2)	35.88	14.99
3	OTUB1 (BC010368.1)	23.65	18.63

*S-scores >3 indicate monospecificity; see notes below

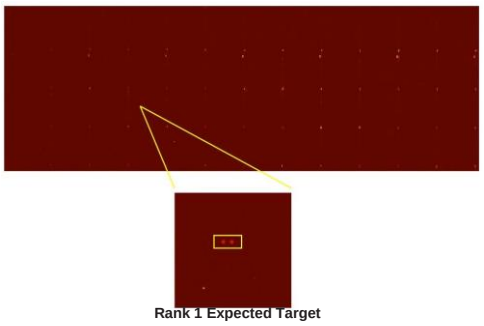
A cross-reactive, low-quality p53 antibody



Unexpected Targets



Rank	Name	ID	A Score	S Score
1	C20orf77	NM_021215.2	43.34	1.60
2	EP400NL	BC66984.1	41.74	4.18
3	PRRC1	NM_130809.2	37.56	4.10

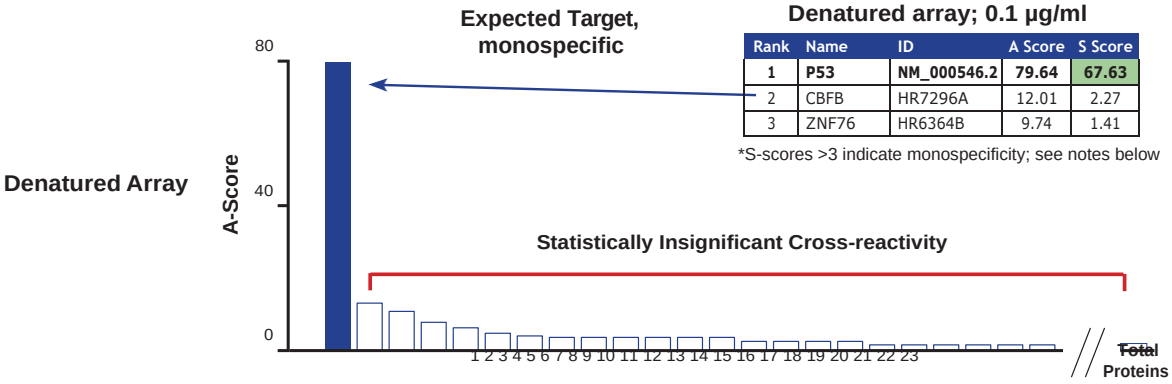


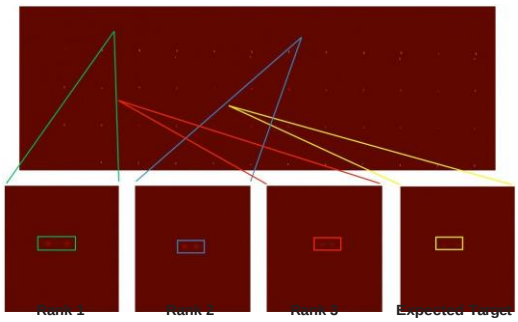
Ab Specificity:
native vs. denatured proteins (Anti-p53)

Denatured array; 0.1 µg/ml

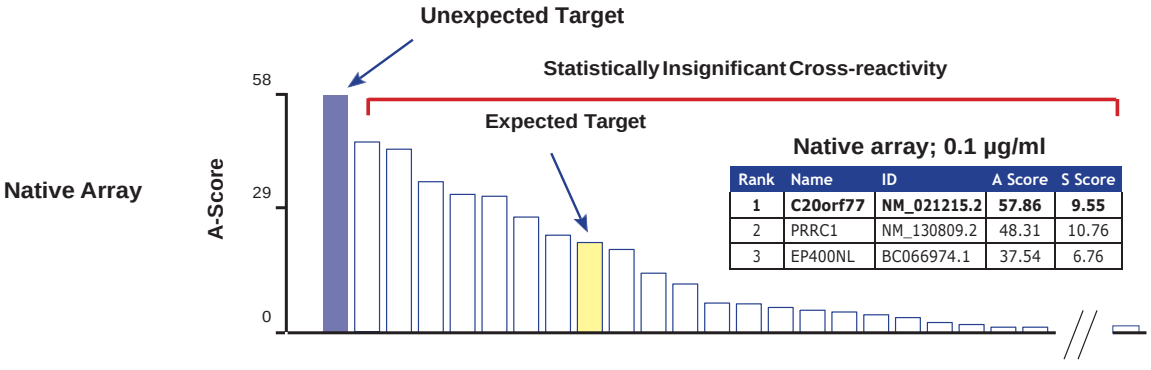
Rank	Name	ID	A Score	S Score
1	P53	NM_000546.2	79.64	67.63
2	CBFB	HR7296A	12.01	2.27
3	ZNF76	HR6364B	9.74	1.41

*S-scores >3 indicate monospecificity; see notes below





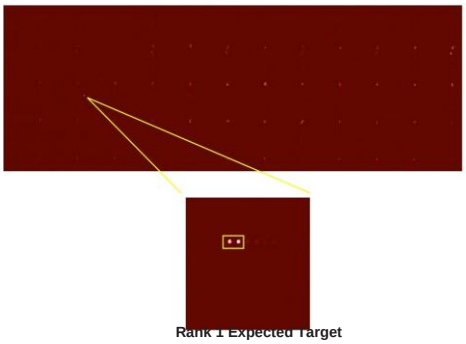
Rank



Native array; 0.1 µg/ml

Rank	Name	ID	A Score	S Score
1	C20orf77	NM_021215.2	57.86	9.55
2	PRRC1	NM_130809.2	48.31	10.76
3	EP400NL	BC066974.1	37.54	6.76

Ab Specificity:
and working concentration
(Anti-PCNA)

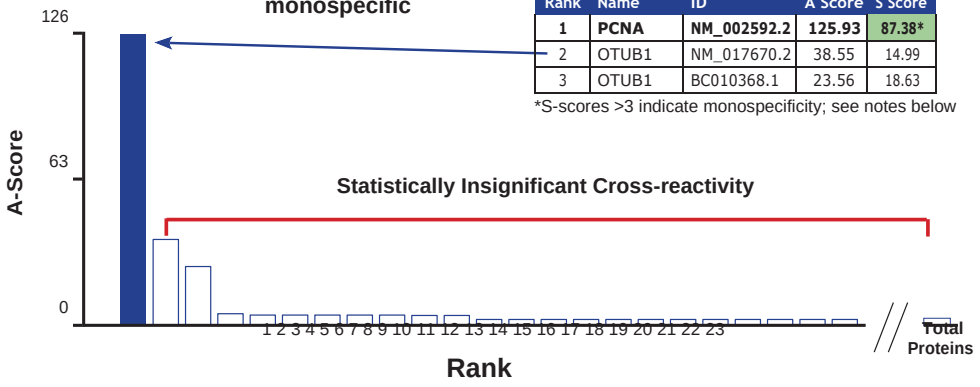


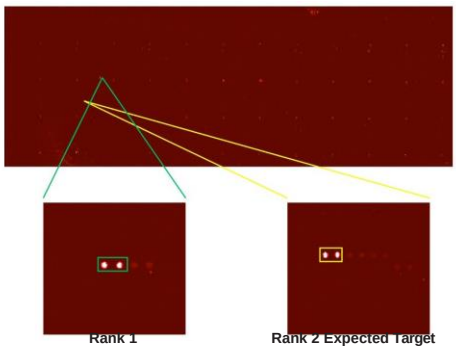
0.1 µg/ml

0.1 µg/ml

Rank	Name	ID	A Score	S Score
1	**PCNA**	**NM_002592.2**	**125.93**	**87.38***
2	OTUB1	NM_017670.2	38.55	14.99
3	OTUB1	BC010368.1	23.56	18.63

*S-scores >3 indicate monospecificity; see notes below

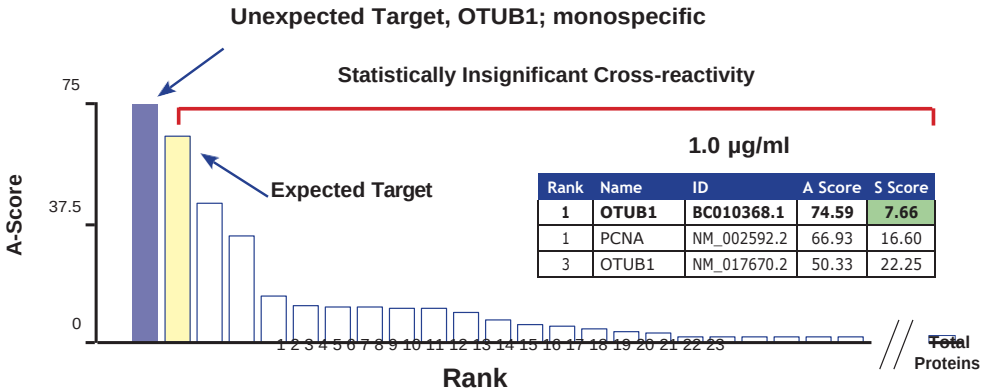




1.0 µg/ml

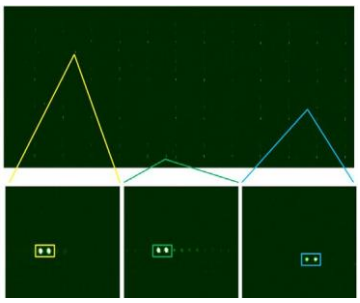
1.0 µg/ml

Rank	Name	ID	A Score	S Score
1	**OTUB1**	**BC010368.1**	**74.59**	**7.66**
1	PCNA	NM_002592.2	66.93	16.60
3	OTUB1	NM_017670.2	50.33	22.25



PTEN Antibody: Cross-reactive, low-quality

(commercially available; Company C; 1.0 µg/ml)



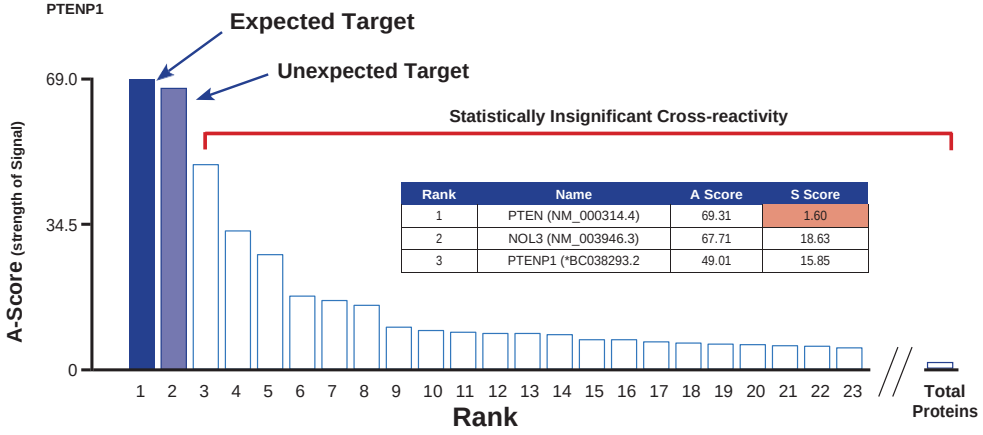
Summary:

- Recognizes the target protein
- Significant cross-reactivity with one other protein.

Top Hits:

1. PTEN (non-specific)
2. NOL3 (non-specific)
3. PTENP1 (statistically insignificant)

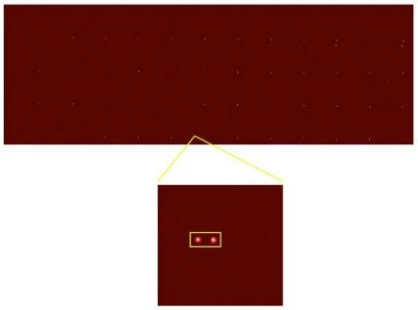
Native Array



Rank	Name	A Score	S Score
1	PTEN (NM_000314.4)	69.31	1.60
2	NOL3 (NM_003946.3)	67.71	18.63
3	PTENP1 (*BC038293.2	49.01	15.85

PTEN Antibody: Monospecific, high-quality

(commercially available; Company B; 1.0 µg/ml))



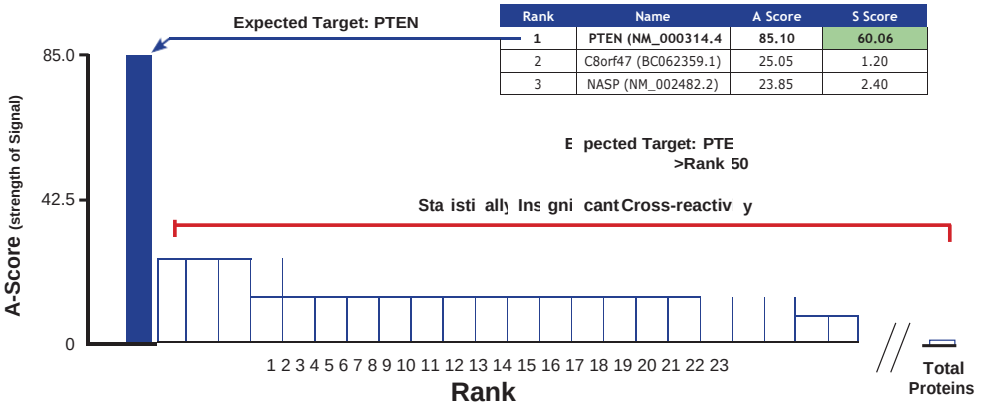
Summary:

- RECOGNIZES target protein and is monospecific
- Does not significantly cross-react with other proteins

Top Hits:

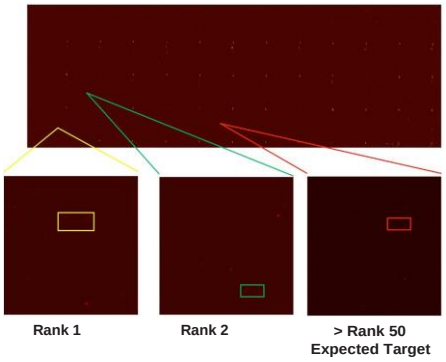
1. PTEN
2. C8orf47 (statistically insignificant)
3. NASP (statistically insignificant)

Native Array



Rank	Name	A Score	S Score
1	PTEN (NM_000314.4	85.10	60.06
2	C8orf47 (BC062359.1)	25.05	1.20
3	NASP (NM_002482.2)	23.85	2.40

PTEN Antibody: Non-specific, low-quality (commercially available; Company D; 0.1 µg ml)



Summary:

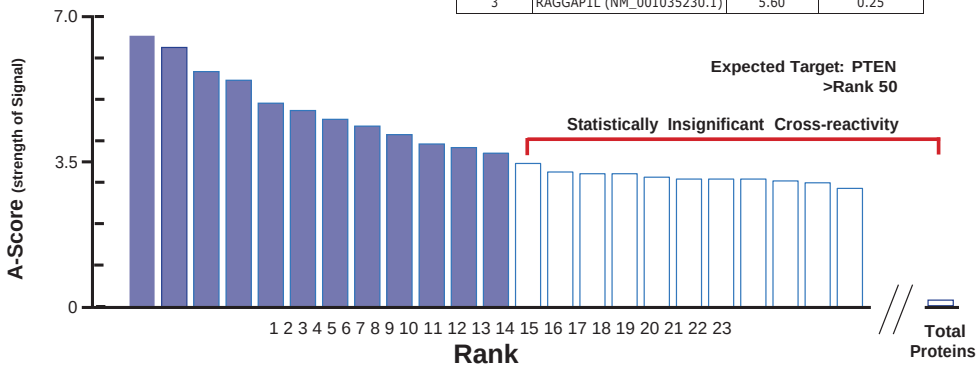
- Does not recognize the target protein
- Significant cross-reactivity with other proteins

Top Hits:

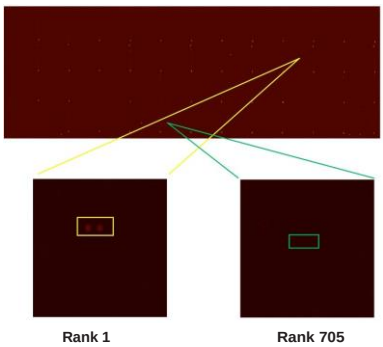
1. ZNF175 (non-specific)
2. KRTAP5-8 (non-specific)
3. RABGAP1L (non-specific)

Rank	Name	A Score	S Score
1	ZNF175 (NM_007147.2)	6.53	0.27
2	KRTAP5-8 (NM_021046)	6.25	0.66
3	RAGGAP1L (NM_001035230.1)	5.60	0.25

Native Array



PTEN Antibody: Monospecific, low-quality (commercially available; Company D; 1.0 µg ml)



Summary:

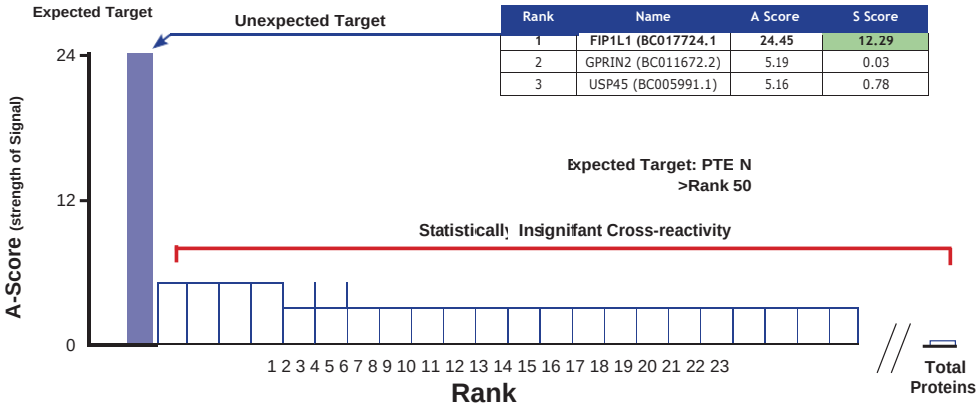
- DOES NOT RECOGNIZE target protein but is monospecific
- Does not significantly cross-react with other proteins

Top Hits:

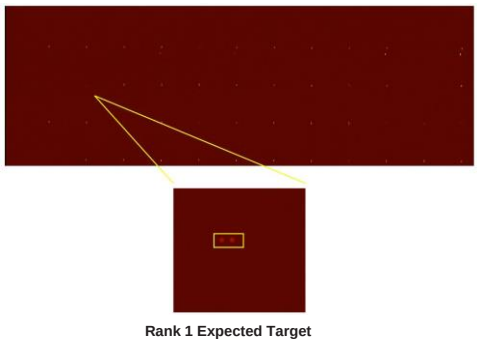
1. FIP1L1
2. GPRIN2 (statistically insignificant)
3. USP45 (statistically insignificant)

Rank	Name	A Score	S Score
1	FIP1L1 (BC017724.1	24.45	12.29
2	GPRIN2 (BC011672.2)	5.19	0.03
3	USP45 (BC005991.1)	5.16	0.78

Native Array



PCNA Antibody: Monospecific, high-quality (commercially available; Company E; 0.1 µg/ml)



Rank 1 Expected Target

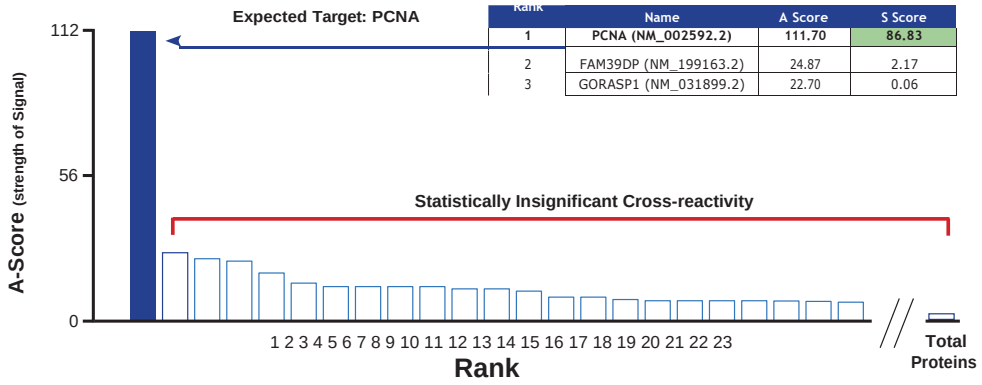
Summary:

- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

Top Hits:

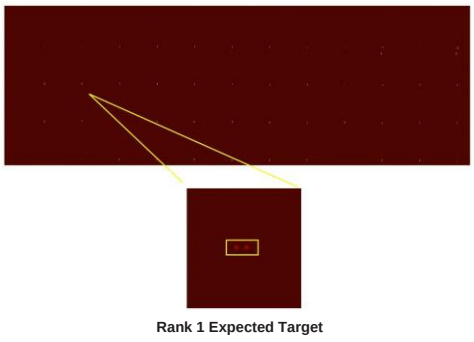
1. PCNA
2. FAM39DG (statistically insignificant)
3. GORASP1 (statistically insignificant)

Native Array



Rank	Name	A Score	S Score
1	PCNA (NM_002592.2)	111.70	86.83
2	FAM39DP (NM_199163.2)	24.87	2.17
3	GORASP1 (NM_031899.2)	22.70	0.06

PCNA Antibody: Monospecific, high-quality (commercially available; Company C; 0.1 µg/ml)



Rank 1 Expected Target

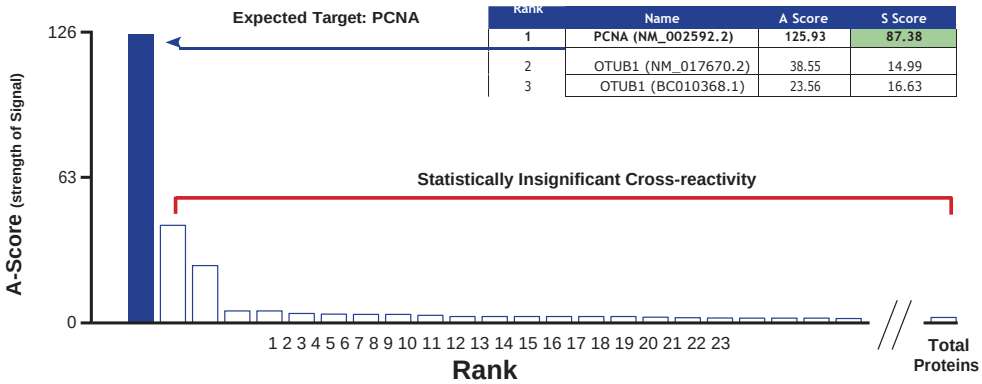
Summary:

- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

Top Hits:

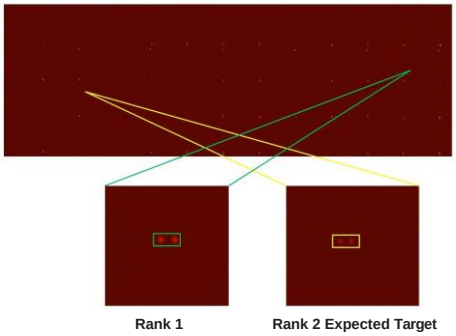
1. PCNA
2. OTUB1 (statistically insignificant)
3. OTUB1 (statistically insignificant)

Native Array



Rank	Name	A Score	S Score
1	PCNA (NM_002592.2)	125.93	87.38
2	OTUB1 (NM_017670.2)	38.55	14.99
3	OTUB1 (BC010368.1)	23.56	16.63

PCNA Antibody: monospecific, low-quality (commercially available; Company C, 1.0 µg/ml)



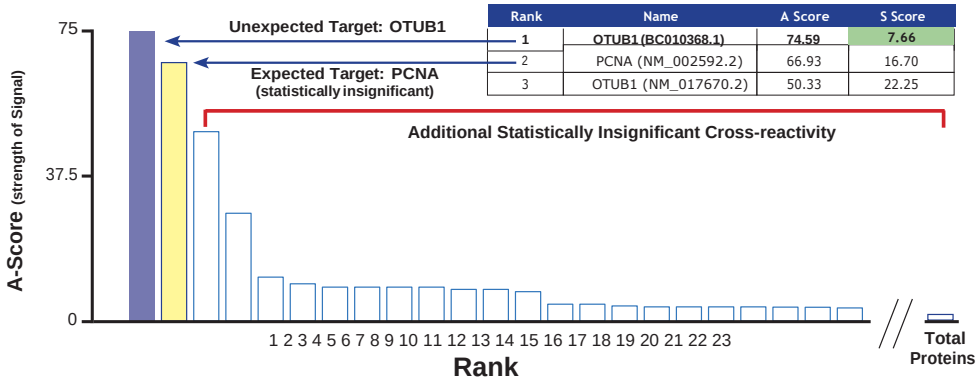
Summary:

- DOES NOT RECOGNIZE target protein but is monospecific
- No significant cross-reactivity with other proteins

Top Hits:

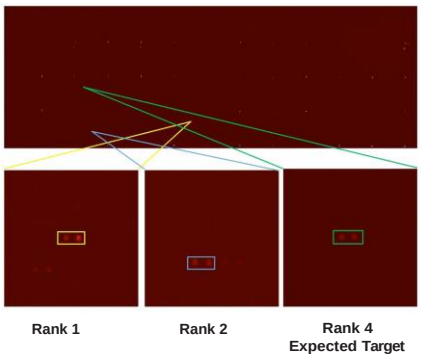
1. OTUB1
2. PCNA (statistically insignificant)
3. OTUB1 (statistically insignificant)

Native Array



Rank	Name	A Score	S Score
1	OTUB1 (BC010368.1)	74.59	7.66
2	PCNA (NM_002592.2)	66.93	16.70
3	OTUB1 (NM_017670.2)	50.33	22.25

PCNA Antibody: Monospecific, low-quality (commercially available; Company E, 1.0 µg/ml)



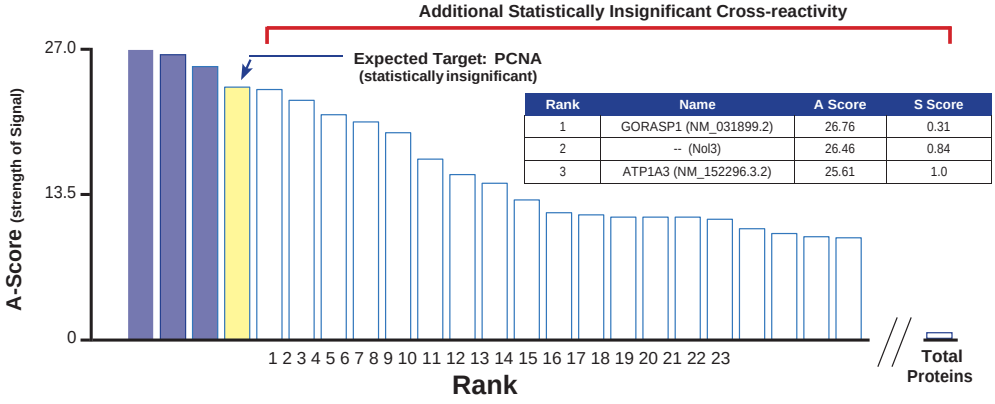
Summary:

- DOES NOT RECOGNIZE target protein
- No significant cross-reactivity with other proteins

Top Hits:

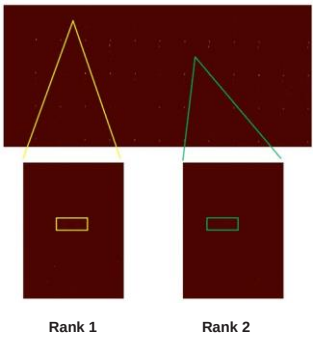
1. GORASP1
2. Nol3 (statistically insignificant)
3. ATP1A3 (statistically insignificant)

Native Array



Rank	Name	A Score	S Score
1	GORASP1 (NM_031899.2)	26.76	0.31
2	-- (Nol3)	26.46	0.84
3	ATP1A3 (NM_152296.3.2)	25.61	1.0

PCNA Antibody: Non-specific, low-quality (commercially available; Company D, 0.1 µg/ml)



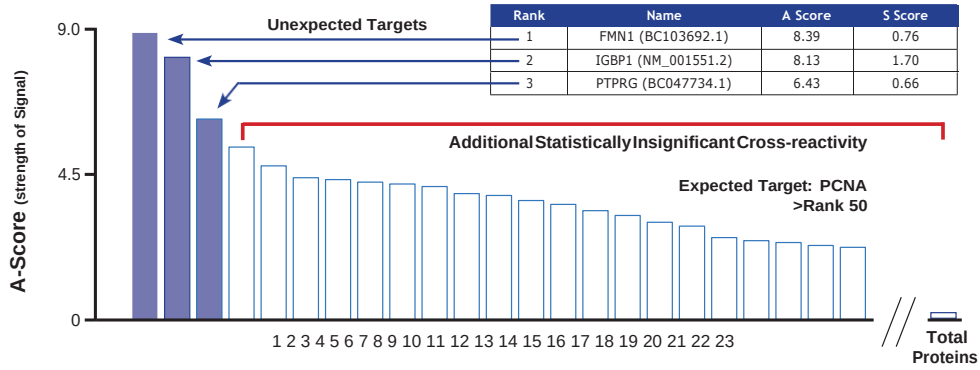
Summary:

- DOES NOT RECOGNIZE target protein
- Significant cross-reactivity with other proteins

Top Hits:

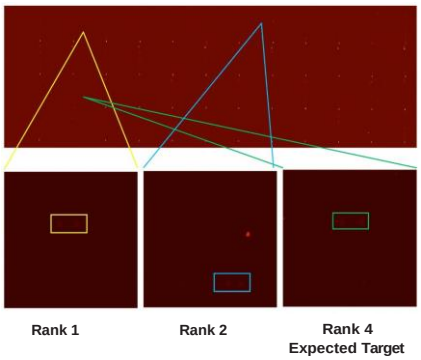
1. FMN1
2. IGBP1
3. PTPRG

Native Array



Rank	Name	A Score	S Score
1	FMN1 (BC103692.1)	8.39	0.76
2	IGBP1 (NM_001551.2)	8.13	1.70
3	PTPRG (BC047734.1)	6.43	0.66

PCNA Antibody: Non-specific, low-quality (commercially available; Company D, 1.0 µg/ml)



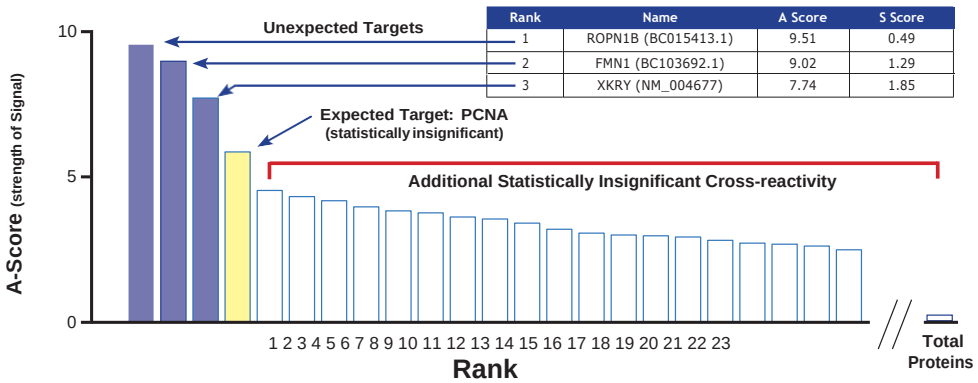
Summary:

- DOES NOT RECOGNIZE target protein
- Significant cross-reactivity with other proteins

Top Hits:

1. ROPN1B
2. FMN1
3. XKRY

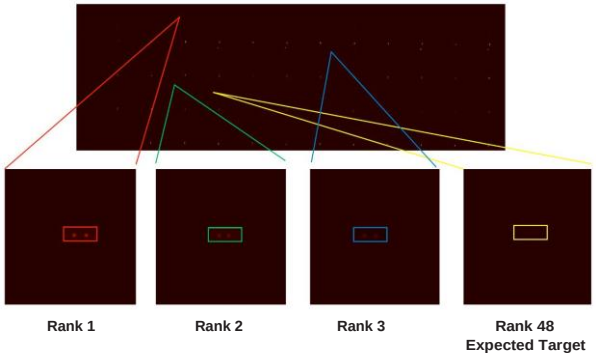
Native Array



Rank	Name	A Score	S Score
1	ROPN1B (BC015413.1)	9.51	0.49
2	FMN1 (BC103692.1)	9.02	1.29
3	XKRY (NM_004677)	7.74	1.85

TP53 Antibody: Non-specific, low-quality

(commercially available; Company C, 0.1 µg/ml)



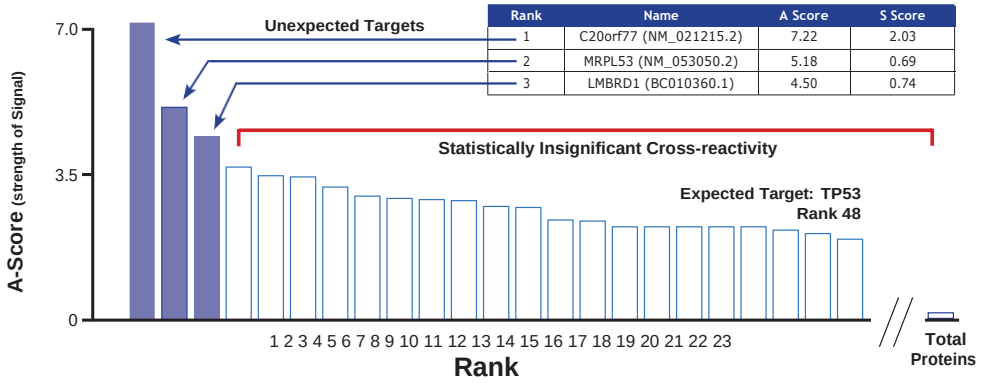
Summary:

- DOES NOT RECOGNIZE target protein
- Significant cross-reactivity with other proteins

Top Hits:

1. C20orf77
2. MRPL53
3. LMBRD1

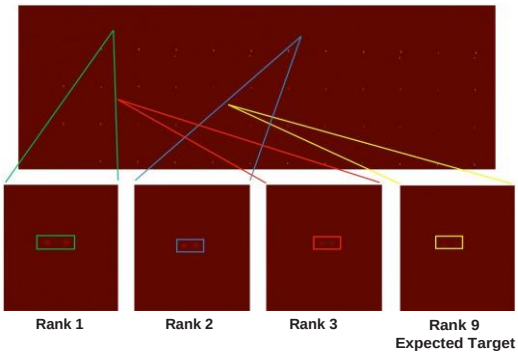
Native Array



Rank	Name	A Score	S Score
1	C20orf77 (NM_021215.2)	7.22	2.03
2	MRPL53 (NM_053050.2)	5.18	0.69
3	LMBRD1 (BC010360.1)	4.50	0.74

TP53 Antibody: Monospecific, low-quality

(commercially available; Company F, 0.1 µg/ml)



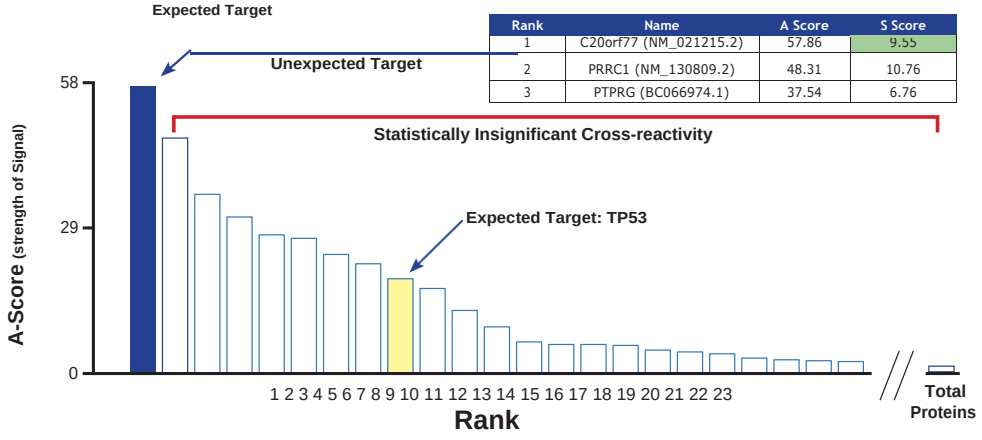
Summary:

- DOES NOT RECOGNIZE target protein but is monospecific
- Insignificant cross-reactivity with other proteins

Top Hits:

1. C20orf77
2. PRRC1 (statistically insignificant)
3. EP400NL (statistically insignificant)

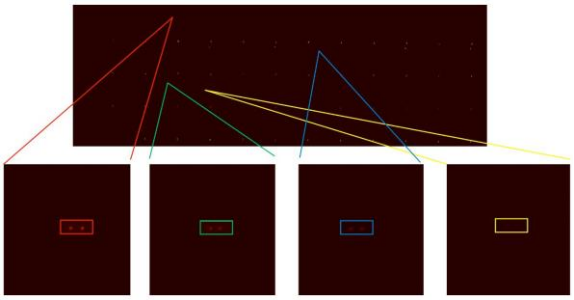
Native Array



Rank	Name	A Score	S Score
1	C20orf77 (NM_021215.2)	57.86	9.55
2	PRRC1 (NM_130809.2)	48.31	10.76
3	PTPRG (BC066974.1)	37.54	6.76

TP53 Antibody: Non-specific, low-quality

(commercially available; Company C, 1.0 µg/ml)



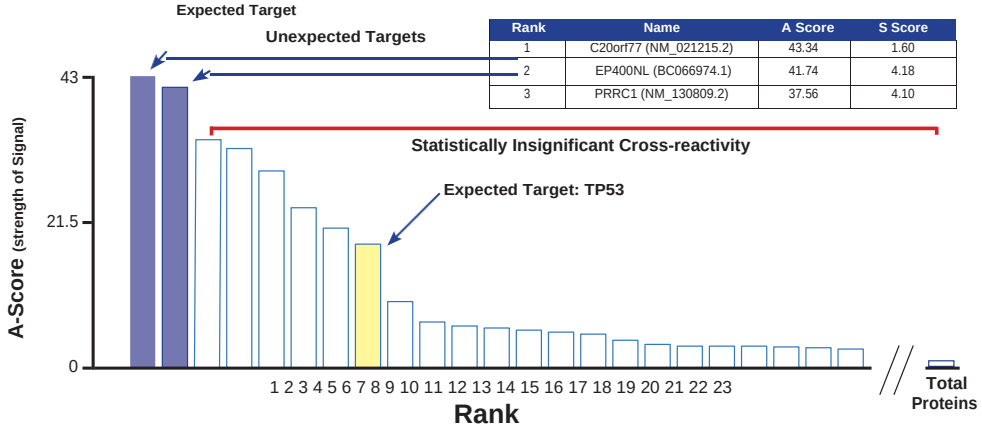
Summary:

- DOES NOT RECOGNIZE target protein
- Significant cross-reactivity with other proteins

Top Hits:

1. C20orf77
2. EP400NL
3. PRRC1

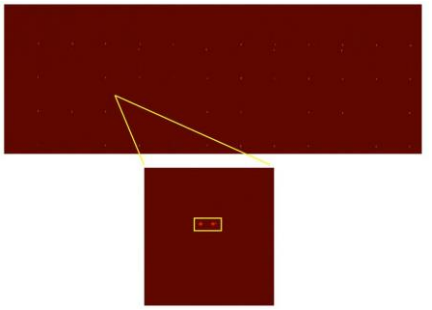
Native Array



Rank	Name	A Score	S Score
1	C20orf77 (NM_021215.2)	43.34	1.60
2	EP400NL (BC066974.1)	41.74	4.18
3	PRRC1 (NM_130809.2)	37.56	4.10

TP53 Antibody: Monospecific, high-quality

(commercially available; Company F, 1.0 µg/ml)



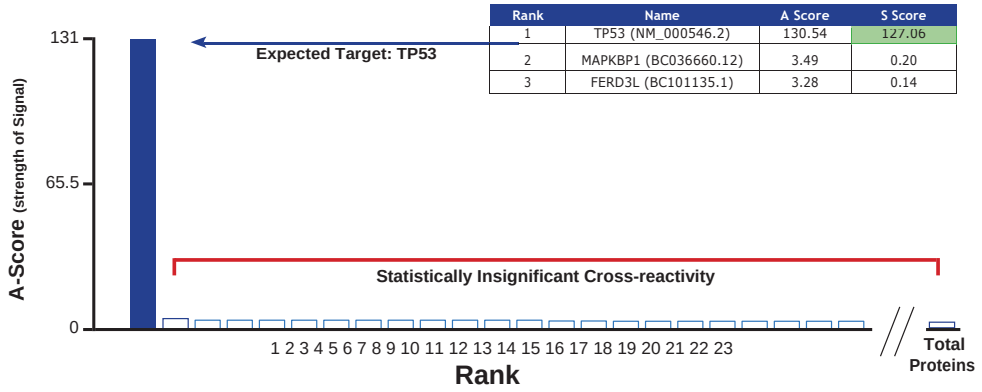
Summary:

- RECOGNIZES target protein; monospecific
- Insignificant cross-reactivity with other proteins

Top Hits:

1. TP53
2. MAPKBP1 (statistically insignificant)
3. FERD3L (statistically insignificant)

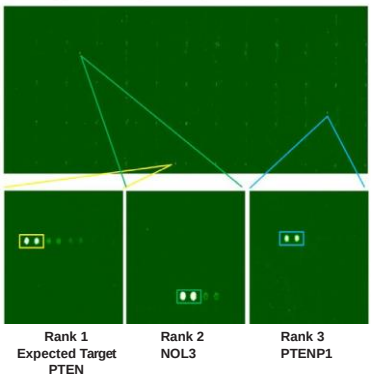
Native Array



Rank	Name	A Score	S Score
1	TP53 (NM_000546.2)	130.54	127.06
2	MAPKBP1 (BC036660.12)	3.49	0.20
3	FERD3L (BC101135.1)	3.28	0.14

PTEN Antibody: Non-specific, low-quality

(commercially available; Company C, 0.1 µg/ml)



Summary:

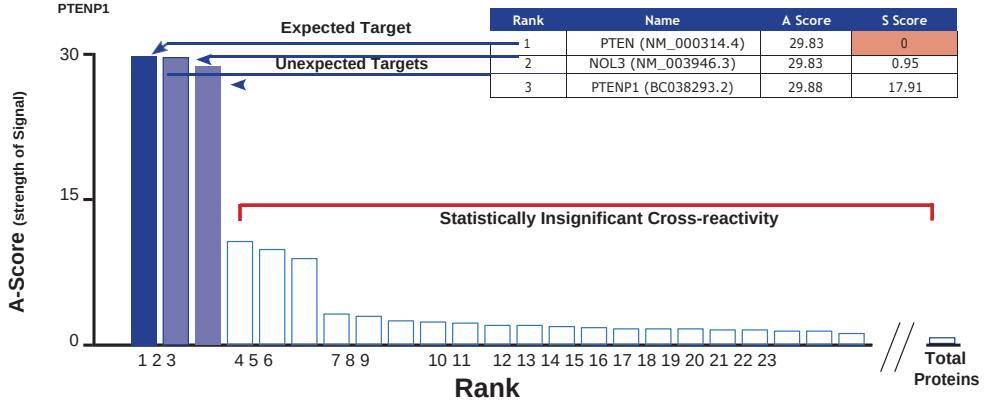
- RECOGNIZES target protein
- Significant cross-reactivity with other proteins

Top Hits:

1. PTEN
2. NOL3
3. PTENP1

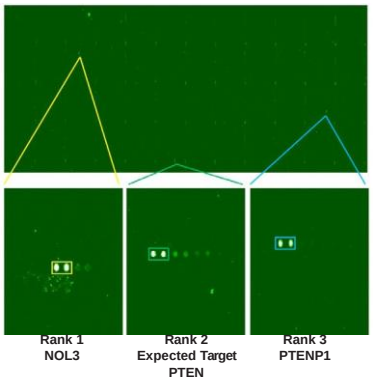
Rank	Name	A Score	S Score
1	PTEN (NM_000314.4)	29.83	0
2	NOL3 (NM_003946.3)	29.83	0.95
3	PTENP1 (BC038293.2)	29.88	17.91

Denatured Array



PTEN Antibody: Non-specific, low-quality

(commercially available; Company C, 1.0 µg/ml)



Summary:

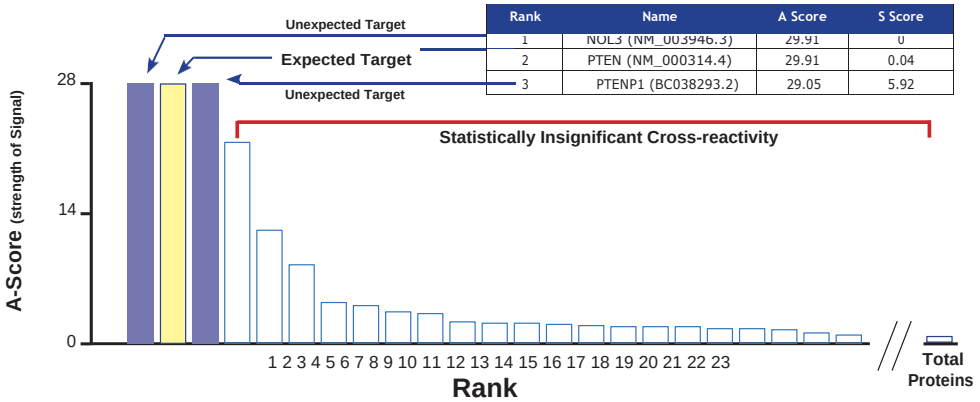
- RECOGNIZES target protein but is non-specific
- Significant cross-reactivity with other proteins

Top Hits:

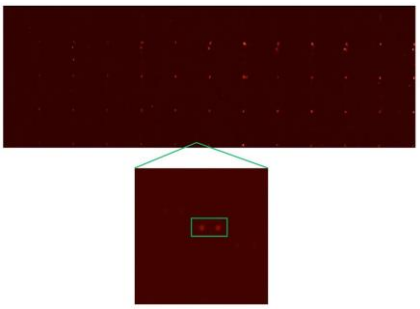
1. NOL3
2. PTEN
3. PTENP1

Rank	Name	A Score	S Score
1	NOL3 (NM_003946.3)	29.91	0
2	PTEN (NM_000314.4)	29.91	0.04
3	PTENP1 (BC038293.2)	29.05	5.92

Denatured Array



PTEN Antibody: Monospecific, high-quality (commercially available; Company B, 0.1 µg/ml)



Rank 1 (Expected Target)

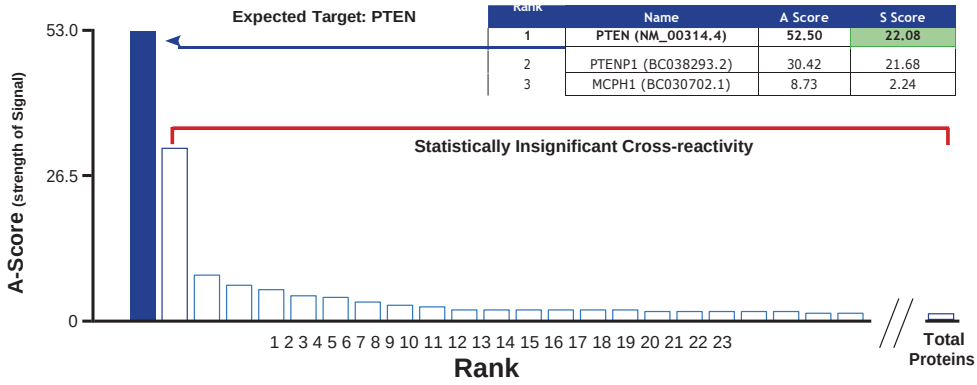
Summary:

- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

Top Hits:

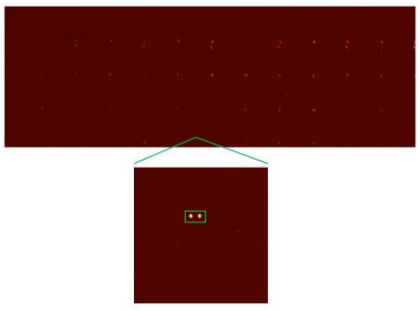
1. PCNA
2. PTENP1 (statistically insignificant)
3. MCPH1 (statistically insignificant)

Denatured Array



Rank	Name	A Score	S Score
1	PTEN (NM_00314.4)	52.50	22.08
2	PTENP1 (BC038293.2)	30.42	21.68
3	MCPH1 (BC030702.1)	8.73	2.24

PTEN Antibody: Monospecific, high-quality (commercially available; Company B,1.0 µg/ml)



Rank 1 (Expected Target)

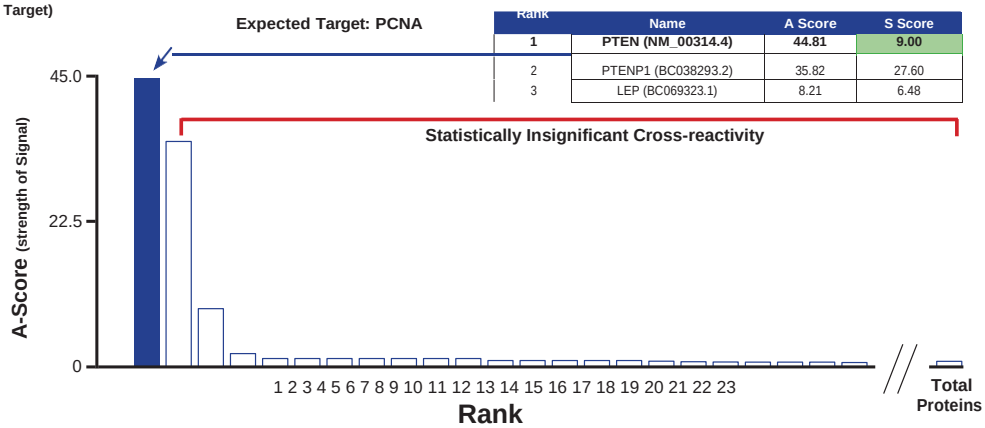
Summary:

- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

Top Hits:

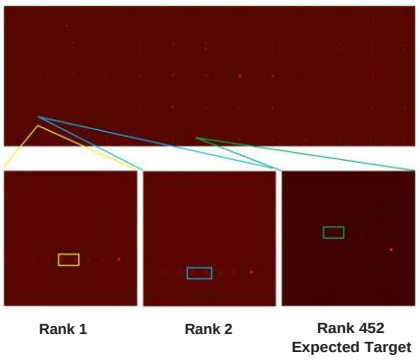
1. PCNA
2. PTENP1 (statistically insignificant)
3. LEP (statistically insignificant)

Denatured Array



Rank	Name	A Score	S Score
1	PTEN (NM_00314.4)	44.81	9.00
2	PTENP1 (BC038293.2)	35.82	27.60
3	LEP (BC069323.1)	8.21	6.48

PTEN Antibody: Non-specific, low-quality (commercially available; Company D; 0.1 µg/ml)



Summary:

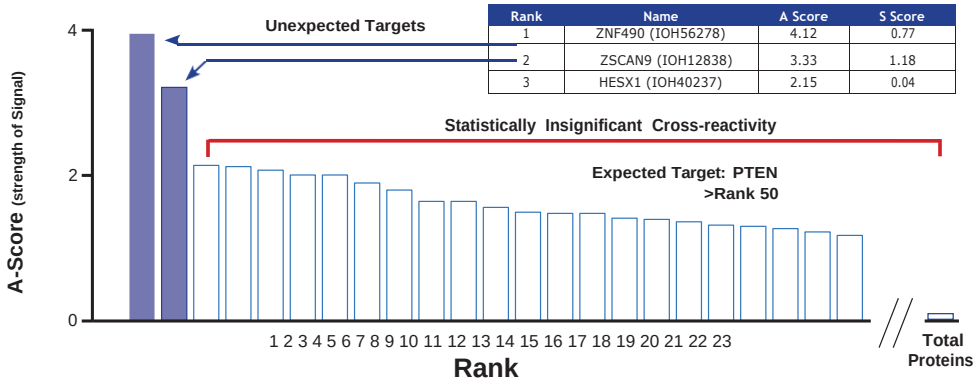
- DOES NOT RECOGNIZE target protein
- Significant cross-reactivity with one other protein

Top Hits:

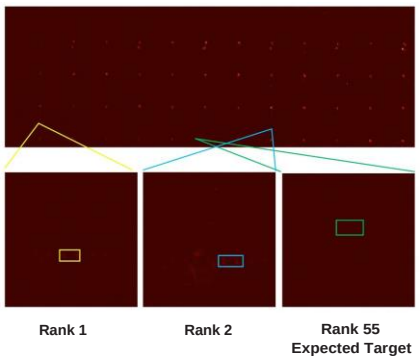
1. ZNF490
2. ZSCAN9
3. HESX1 (statistically insignificant)

Rank	Name	A Score	S Score
1	ZNF490 (IOH56278)	4.12	0.77
2	ZSCAN9 (IOH12838)	3.33	1.18
3	HESX1 (IOH40237)	2.15	0.04

Denatured Array



PTEN Antibody: Monospecific, low-quality (commercially available; Company D; 1.0 µg/ml)



Summary:

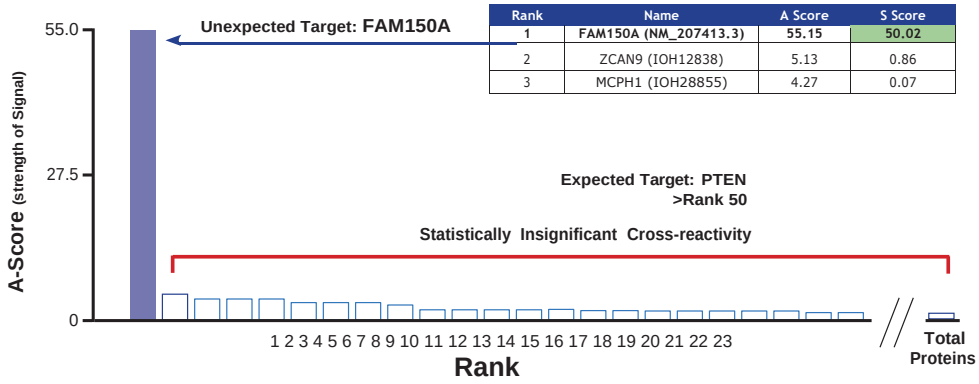
- DOES NOT RECOGNIZE target protein but is monospecific
- No significant cross-reactivity with other proteins

Top Hits:

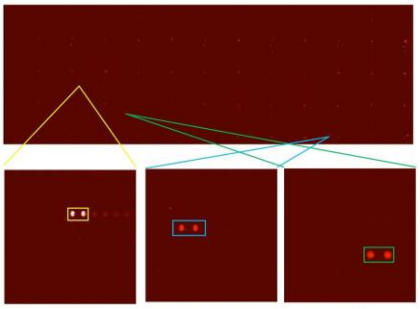
1. FAM150A
2. ZSCAN9 (statistically insignificant)
3. ZNF63 (statistically insignificant)

Rank	Name	A Score	S Score
1	FAM150A (NM_207413.3)	55.15	50.02
2	ZCAN9 (IOH12838)	5.13	0.86
3	MCPH1 (IOH28855)	4.27	0.07

Denatured Array



PCNA Antibody: Monospecific, high-quality (commercially available; Company C, 0.1 µg/ml)

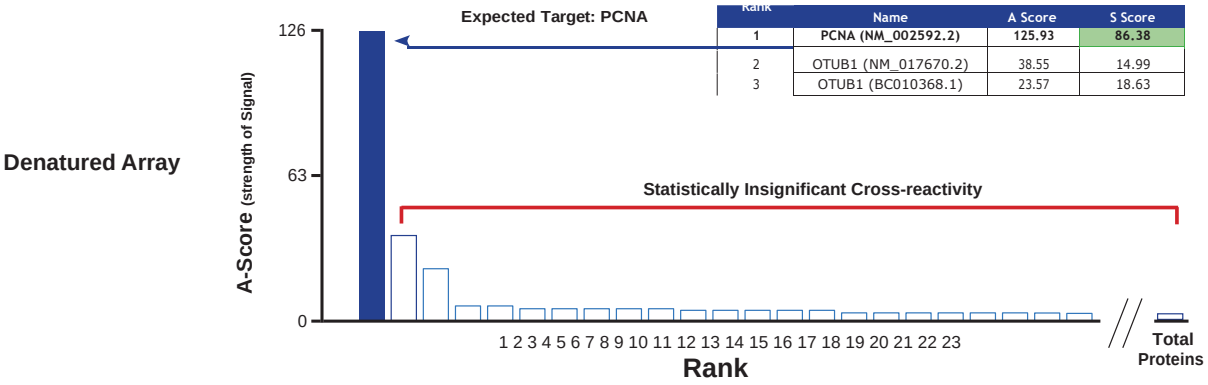


Summary:

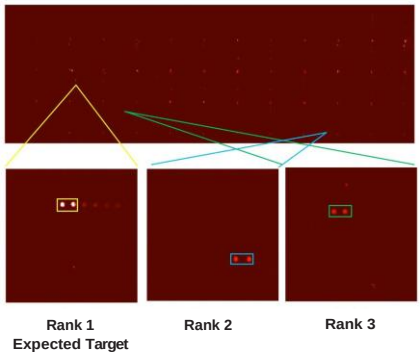
- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

Top Hits:

1. PCNA
2. OTUB1 (statistically insignificant)
3. OTUB1 (statistically insignificant)



PCNA Antibody: Monospecific, high-quality (commercially available; Company C, 1.0 µg/ml)

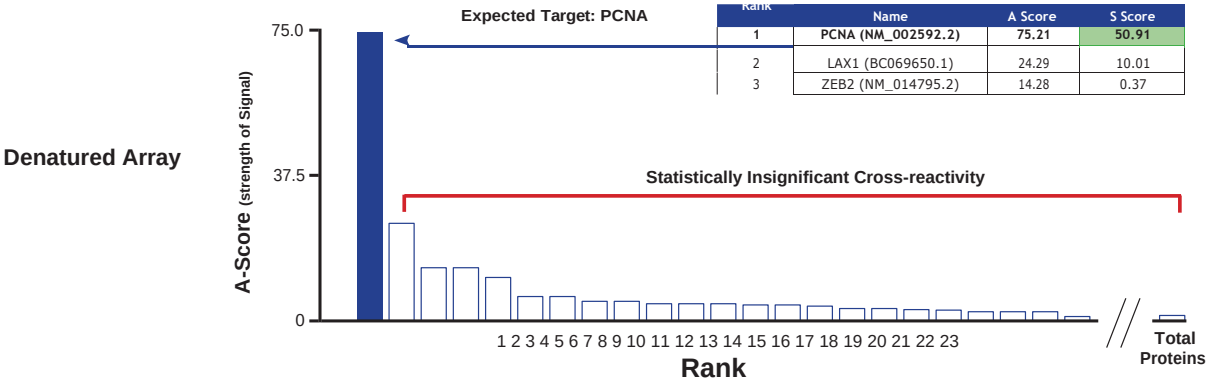


Summary:

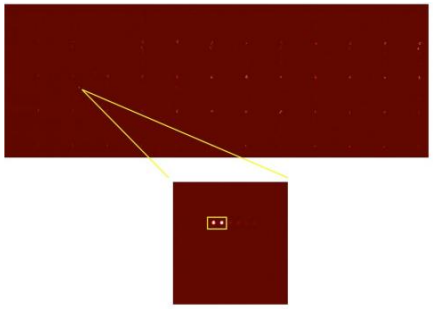
- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

Top Hits:

1. PCNA
2. LAX1 (statistically insignificant)
3. ZEB2 (statistically insignificant)



PCNA Antibody: Monospecific, high-quality (commercially available; Company E, 0.1 µg/ml)



Rank 1 Expected Target

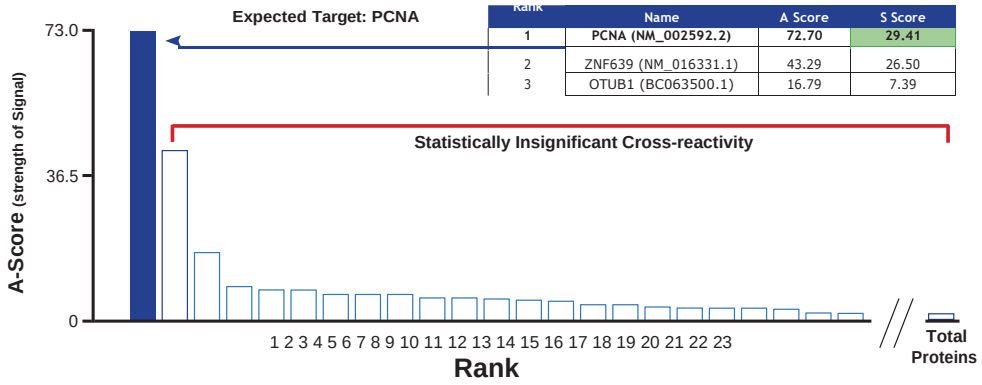
Summary:

- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

Top Hits:

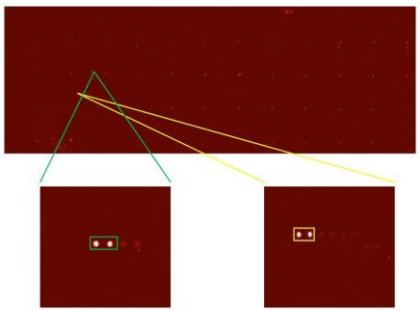
1. PCNA
2. ZNF639 (statistically insignificant)
3. TIGD1 (statistically insignificant)

Denatured Array



Rank	Name	A Score	S Score
1	PCNA (NM_002592.2)	72.70	29.41
2	ZNF639 (NM_016331.1)	43.29	26.50
3	OTUB1 (BC063500.1)	16.79	7.39

PCNA Antibody: Non-specific, low-quality (commercially available; Company E, 1.0 µg/ml)



Rank 1 Rank 2 Expected Target

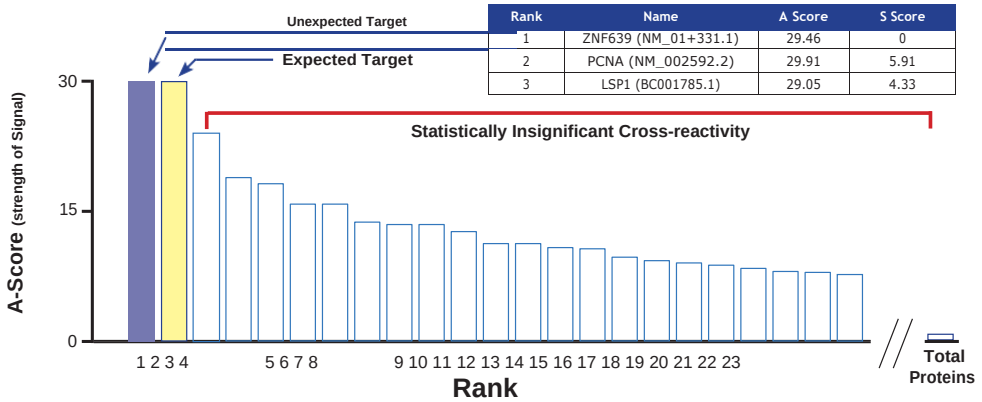
Summary:

- RECOGNIZES target protein but is non-specific
- Significant cross-reactivity with one other protein

Top Hits:

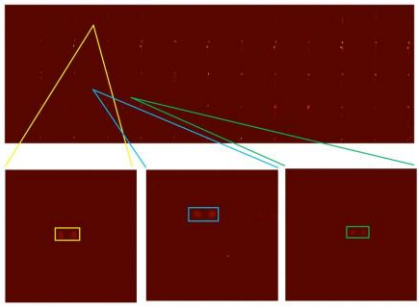
1. ZNF639
2. PCNA
3. LSP1 (statistically insignificant)

Denatured Array



Rank	Name	A Score	S Score
1	ZNF639 (NM_01+331.1)	29.46	0
2	PCNA (NM_002592.2)	29.91	5.91
3	LSP1 (BC001785.1)	29.05	4.33

P53 Antibody: Non-specific, low-quality (commercially available; Company C, 0.1 µg/ml)



Summary:

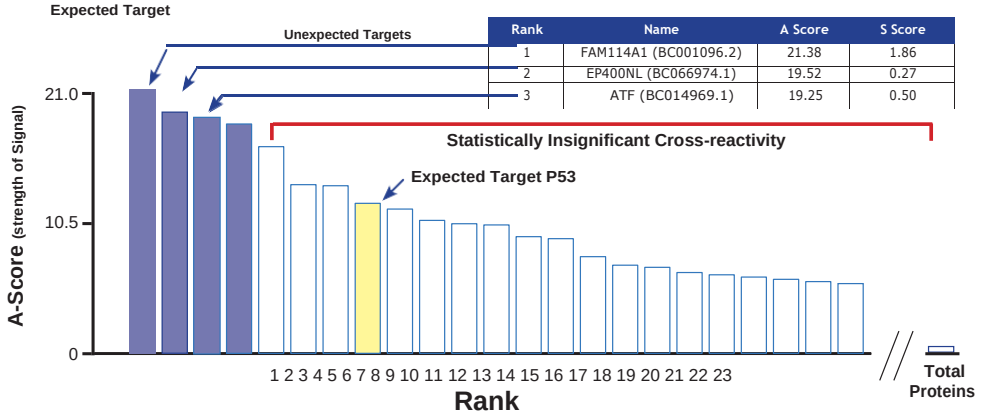
- RECOGNIZES target protein but is non-specific
- Significant cross-reactivity with other proteins

Top Hits:

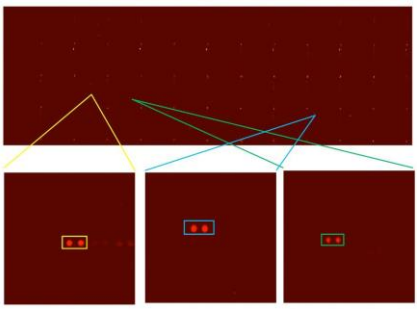
1. FAM114A1
2. EP400NL
3. ATF6

Rank	Name	A Score	S Score
1	FAM114A1 (BC001096.2)	21.38	1.86
2	EP400NL (BC066974.1)	19.52	0.27
3	ATF (BC014969.1)	19.25	0.50

Denatured Array



P53 Antibody: Non-specific, low-quality (commercially available; Company C, 1.0 µg/ml)



Summary:

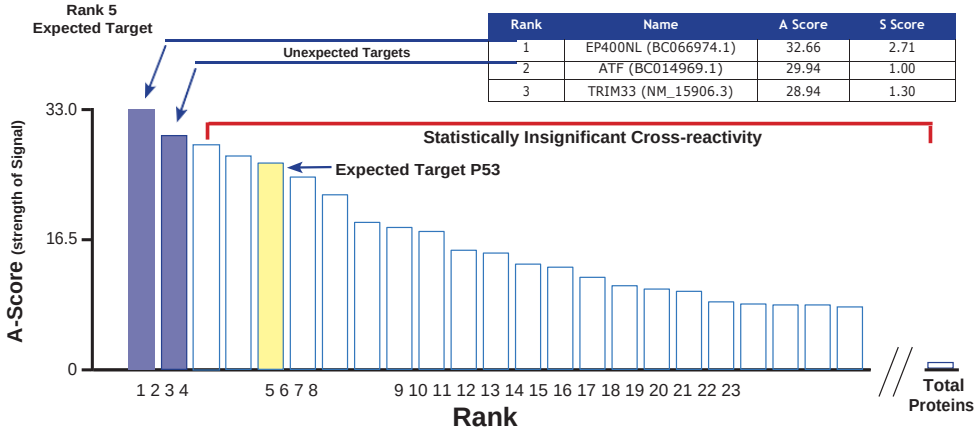
- RECOGNIZES target protein but is non-specific
- Significant cross-reactivity with other proteins

Top Hits:

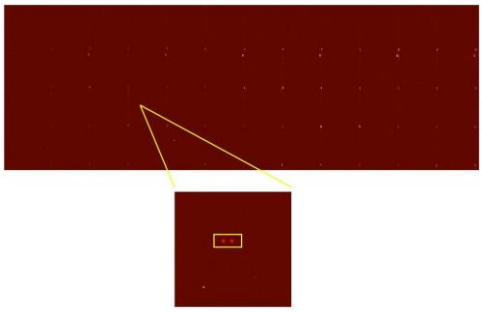
1. EP400NL
2. ATF6
3. TRIM33 (statistically insignificant)

Rank	Name	A Score	S Score
1	EP400NL (BC066974.1)	32.66	2.71
2	ATF (BC014969.1)	29.94	1.00
3	TRIM33 (NM_15906.3)	28.94	1.30

Denatured Array



TP53 Antibody: Monospecific, high-quality (commercially available; Company F, 0.1 µg/ml)



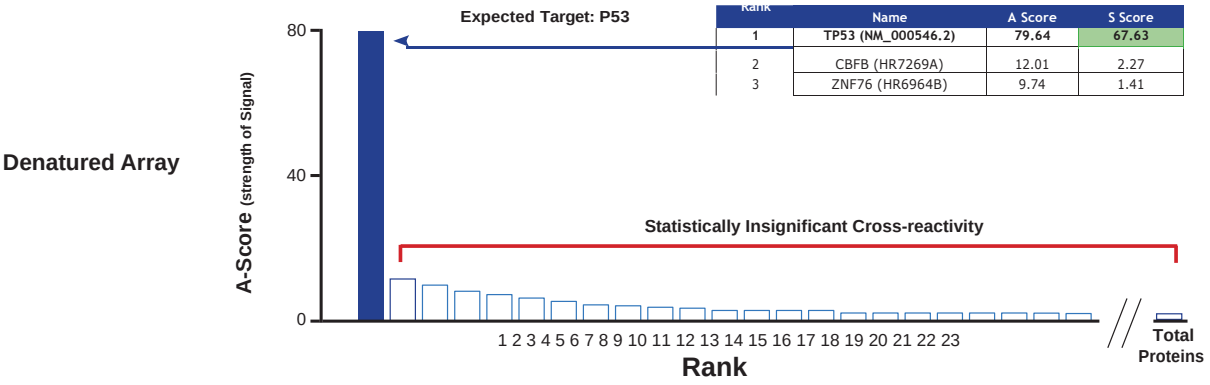
Rank 1 Expected Target

Summary:

- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

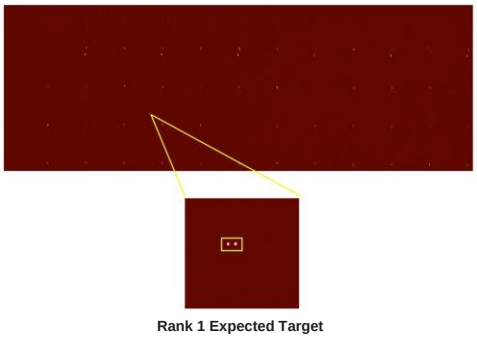
Top Hits:

1. TP53
2. CBFB (statistically insignificant)
3. ZNF76 (statistically insignificant)



Rank	Name	A Score	S Score
1	TP53 (NM_000546.2)	79.64	67.63
2	CBFB (HR7269A)	12.01	2.27
3	ZNF76 (HR6964B)	9.74	1.41

TP53 Antibody: Monospecific, high-quality (commercially available; Company F,1.0 µg/ml)



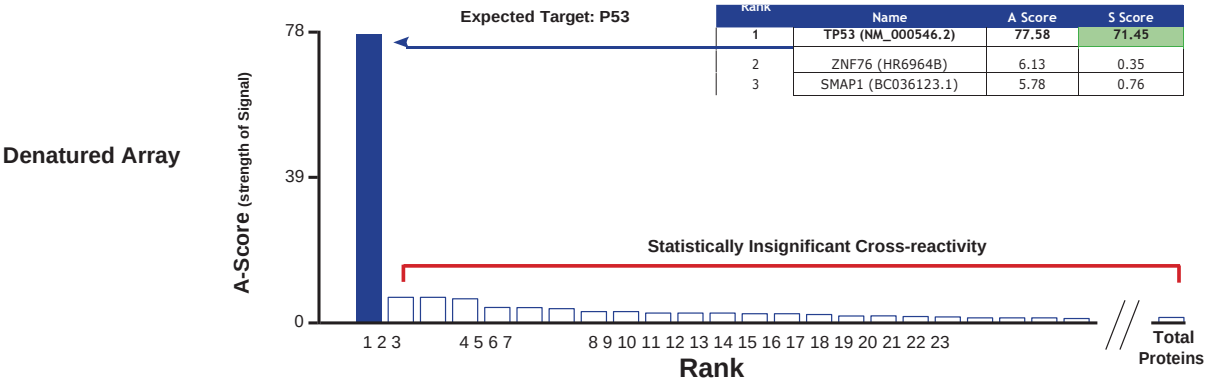
Rank 1 Expected Target

Summary:

- Recognizes the target protein; monospecific
- No significant cross-reactivity with other proteins

Top Hits:

1. TP53
2. ZNF76 (statistically insignificant)
3. SMAP1 (statistically insignificant)



Rank	Name	A Score	S Score
1	TP53 (NM_000546.2)	77.58	71.45
2	ZNF76 (HR6964B)	6.13	0.35
3	SMAP1 (BC036123.1)	5.78	0.76

Data Summary and Conclusion

Company	Intended Target (IT)	Dilution µg/ml	Top Target	AScore	S Score*	Specific for IT	Array+	Page	Notes
ELISA Confirmation									
Company C	BCL2	0.1	AMPH	28.94	1.75				
Company A	USF2	1.0	USF2	31.26	0.65	**NO**	N	p.5	ELISA confirmation of HuProt™ analysis; each antibody is non-specific for the intended target (IT)
Company G	KCNP2	1.0	RBKS	56.58	0.00			p.6	
Native Microarray									
Company C	PTEN	1.0	PTEN	69.31	1.60	**NO**	N	p. 9	head-to-head comparison; Company B PTEN Ab is also monospecific on denatured array (see below)
Company B				85.10	**60.06**	**YES**			
Company D	PTEN	0.1	ZNF175	6.53	0.27	**NO**	N	p. 10	**monospecific for unintended target** at higher concentration
		1.0	FIP1L1	24.45	**12.29**				
Company E	PCNA	0.1	PCNA	111.70	**86.83**	**YES**	N	p. 11-12	loss of monospecificity at higher concentra- tion; same results on denatured array (see below)
		1.0	GORASP1	26.76	0.31	**NO**			
Company C	PCNA	0.1	PCNA	125.93	**87.38**	**YES**	N	p. 11-12	**monospecific for unintended target** at higher concentration
		1.0	OTUB1	74.59	**7.66**	**NO**			
Company D	PCNA	0.1	FMN1	8.39	0.76	**NO**	N	p. 13	no specificity at either concentration
		1.0	ROPN1B	9.51	0.49				
Company C	TP53	0.1	C20orf77	7.22	2.03	**NO**	N	p. 14-15	no specificity at either concentration
		1.0		43.34	1.60				
Company F	TP53	0.1	C20orf77	57.86	**9.55**	**NO**	N	p. 14-15	**monospecific for unintended target** at lower concentration; monospecific for intended target at higher concentration
		1.0	TP53	130.54	**127.06**	**YES**			
Denatured Microarray									
Company C	PTEN	0.1	PTEN	29.83	0.00	**NO**	D	p. 16	no specificity on either array
		1.0	NOL3	29.91	0.00				
Company B	PTEN	0.1	PTEN	52.50	**22.08**	**YES**	D	p. 17	high quality antibody as demonstrated on both native and denatured arrays
		1.0		44.81	**9.00**				
Company D	PTEN	0.1	ZNF490	4.12	0.77	**NO**	D	p. 18	**monospecific for unintended target** at lower concentration
		1.0	FAM150A	55.15	**50.02**				
Company C	PCNA	0.1	PCNA	125.93	**86.38**	**YES**	D	p. 19	high quality antibody as demonstrated on a denatured array; use with **caution** on native proteins (see above)
		1.0		75.21	**50.91**				
Company E	PCNA	0.1	PCNA	72.70	**29.41**	**YES**	D	p. 20	loss of monospecificity at higher concentra- tion; same results on native array (see above)
		1.0	ZNF639	29.46	0.00	**NO**			
Company C	TP53	0.1	FAM114A1	21.38	1.86	**NO**	D	p. 21	no specificity on either array
		1.0	EP400NL	32.66	2.71				
Company F	TP53	0.1	TP53	79.64	**67.63**	**YES**	D	p. 22	high quality antibody as demonstrated on a denatured array; use with **caution** on native proteins (see above)
		1.0		77.58	**71.45**				

Data Summary and Conclusion ... continued from page 23.

Our analysis of monoclonal antibodies shows that while some high quality, monospecific antibodies are commercially available, there are also many poorly-performing antibodies on the market that are not specific to their intended targets. At present, there is a groundswell from within the research community that advocates higher quality standards for commercial antibodies. Funding agencies such as the NIH have also started to adopt higher standards for commercial antibodies that are to be selected for use in grant-funded research.

The new technical developments we described now make it possible to test monoclonal antibodies for cross-reactivity at an unprecedented level. We recommend that all commercial monoclonal antibodies made against human proteins be evaluated for binding specificity using human protein microarrays, such as the CDI HuProt™ Array.

As HuProt™ microarray analysis provides a global snapshot of monoclonal antibody cross-reactivity, this could serve as an authentication standard that is independent of the cell lines or tissues used.

For example, the recent NIH notice **NOT-OD-15-103** (Enhancing Reproducibility through Rigor and Transparency, in effect for grants submitted after January, 25 2016), states:

> ***Authentication of Key Biological and/or Chemical Resources***
>
> "The quality of the resources used to conduct research is critical to the ability to reproduce the results. NIH expects that key biological and/or chemical resources will be regularly authenticated to ensure their identity and validity for use in the proposed studies. Key biological and/or chemical resources may or may not be generated with NIH funds and: 1) may differ from laboratory to laboratory or over time; 2) may have qualities and/or qualifications that could influence the research data; and 3) are integral to the proposed research. These include, but are not limited to, cell lines, specialty chemicals, antibodies and other biologics.
>
> There are not clear consensus guidelines on authenticating different types of resources, and NIH encourages the research community to inform the development of such guidelines. In the absence of clear guidelines, researchers should transparently report on what they have done to authenticate key resources, so that consensus can emerge."

HuProt™ arrays have also been very useful in determining the best working concentrations for a given antibody, as antibodies sometimes show non-specific binding when used at sub-optimal concentrations. Some antibodies, when used at high concentrations, may bind in a non-specific fashion; such antibodies may need to be diluted in order to show specific binding to the target. In contrast, other antibodies may need to be used at a high concentration in order to show any binding at all; in such cases, monospecificity will be of paramount importance.

HuProt™ arrays are also very useful for analyzing the cross-reactivity of antibodies to both native and denatured forms of the target proteins. Antibodies that bind to folded proteins often function best for IP, ICC and IHC, as well as for ChIP and ChIP-seq applications. In contrast, antibodies that recognize linear epitopes (denatured protein) are usually best for western blot ap- plications. Experimental results obtained from many of the commercial monoclonal antibodies tested are simply not consistent with the manufacturers' recommendations. By evaluating antibodies on both denatured and native microarrays, it is possible to determine in advance the applications to which they are best suited prior to distribution.