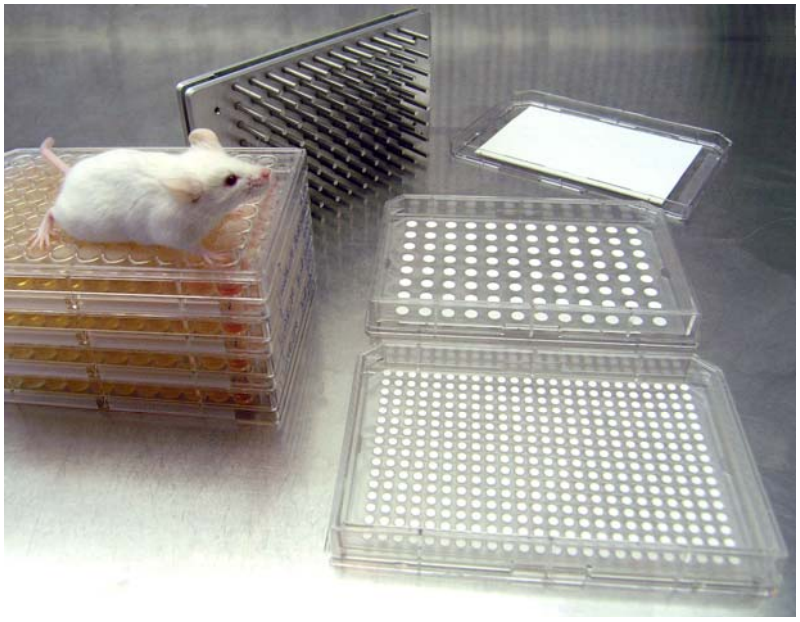
Testing hybridoma supernatants with the Spots On Dots™ Antibody Screening Kit

Application Note AN001



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Overview

Primorigen's Spots On Dots™ Antibody Screening Kit is a multiplexed direct ELISA platform that allows the researcher to screen monoclonal antibody hybridoma supernatants against multiple different antigens (target proteins, conjugated peptides, or cell lysates) in parallel. The Spots On Dots™ sheets have been configured with 96 or 384 nitrocellulose Dots conforming to SBS-standard microplate well positions, allowing simple integration into automated screening processes. The nitrocellulose Dots are hydrophobically isolated, eliminating the requirement for a frame or well superstructure to maintain sample isolation.

Immunogen and other antigens of interest can be printed on each of the 96 or 384 nitrocellulose Dots. During incubation with hybridoma supernatant samples, antigen-specific antibodies bind the targets of interest. The supernatants are removed, and the sheet is probed by incubation with a species-specific antibody conjugated to alkaline phosphatase. Bound antibodies are visualized by the addition of a precipitating alkaline phosphatase substrate to generate a colorimetric signal.

This Application Note contains a series of figures highlighting some of the hybridoma screening and antibody characterization applications for the kit. These include the rapid testing of antibody specificity and sensitivity, and the ability to simultaneously screen against antigens such as peptides, proteins, and cellular lysates. Very small amounts of antigens and samples are needed for multiplex analysis, meaning that large amounts of information can be gleaned at an early stage of hybridoma development. The resulting savings in cost, time, and labor offer significant advantages over traditional ELISA plate-based screening.

Detailed instructions on the use of the Spots On Dots Antibody Screening Kit can be found In the User Protocol, available online at www.primorigen.com.

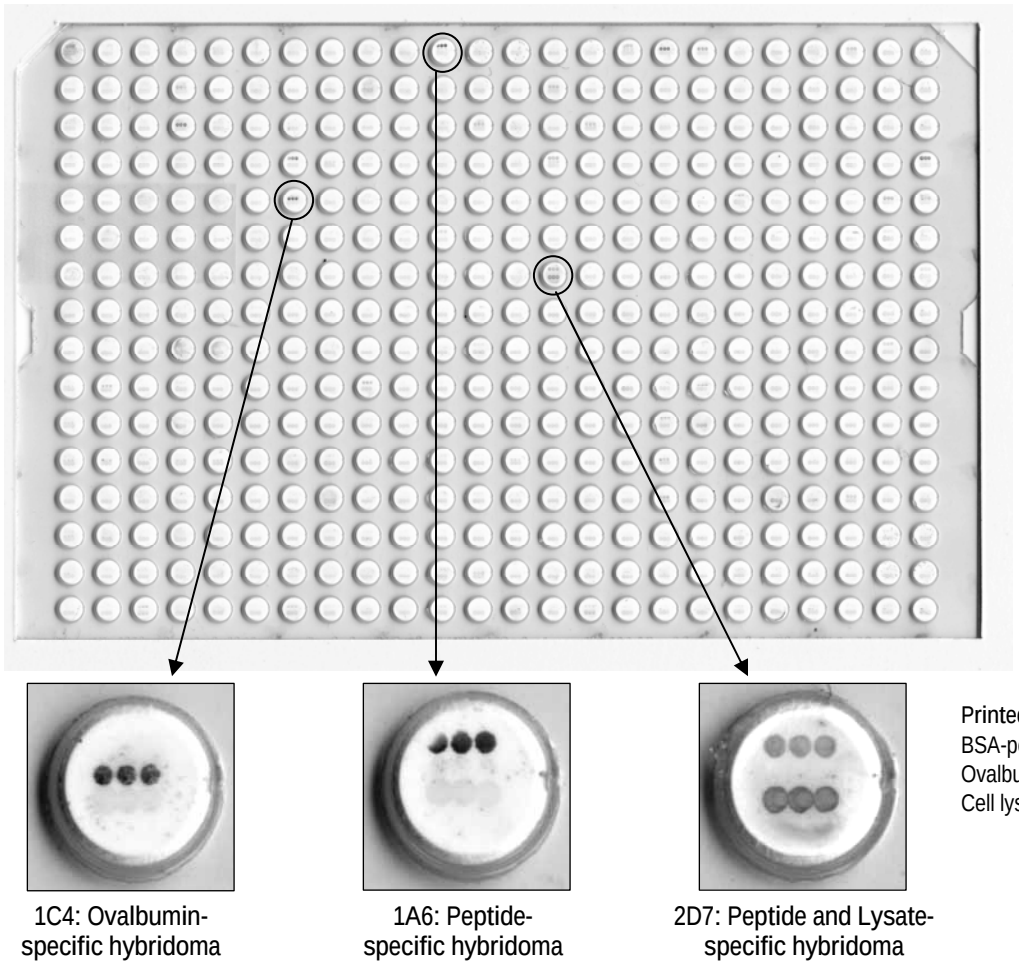


Figure 1. Screening of hybridomas raised against peptide antigens

BALB/c mice were immunized with a synthetic peptide coupled to ovalbumin (OVA), and splenocytes from a single spleen were fused to myeloma partner Sp2/0. The resulting hybridomas were plated out into four 96-well tissue culture plates (384 wells total). Following drug selection and hybridoma growth, each well was screened for monoclonal antibodies of interest by transferring 3 μl diluted supernatants to a Spots On Dots 384 sheet with a 96-well pin tool device, using methods described in the User Protocol. The Spots On Dots 384 sheet has been printed in triplicate with the synthetic peptide immunogen coupled to bovine serum albumin (BSA), OVA, and a cell lysate made from cells overexpressing the target protein.

Following the assay procedure, visual inspection of the developed 384 Dot sheet readily differentiates hybridoma clones secreting monoclonal antibodies (mAbs) specific for the immunogen carrier protein (OVA, left inset) from mAbs specific for the synthetic peptide (middle inset). In addition, some peptide-specific mAb clones are also able to bind to cell lysates expressing the target protein (from which the peptide was designed), thus identifying mAb clones that are likely to bind the target protein in the native, folded conformation (right inset).

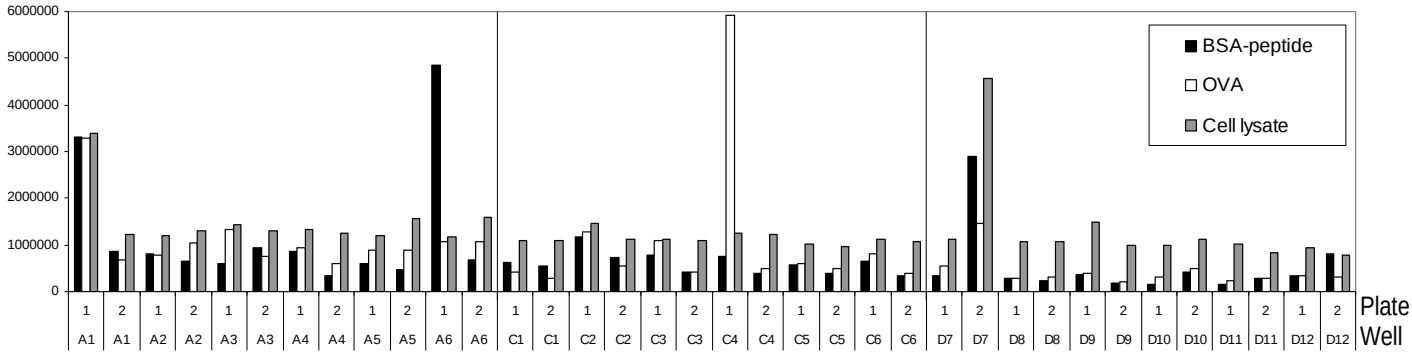


Figure 2. Scanning analysis of antibody screening

The Spots On Dots 384 sheet shown in Figure 1 was scanned on a flatbed scanner at 1200 dpi resolution, and integrated signal intensities for the printed Spots were derived from the image. Segments of the data have been graphed, illustrating that quantitative cut-offs can be assigned to identify clones with broad non-specific binding (e.g. 1A1), peptide-specific binding (e.g. 1A6), carrier protein binding (e.g. 1C4), or peptide/ lysate binding (e.g. 2D7).

Table 1. Comparison to ELISA: Ranking of positive clones

Well	Spots On Dots rank	ELISA rank
4B5	1	3
2A11	2	4
1A10	3	1
2E8	4	2
3G7	5	8
1E7	6	9
2H7	7	5
1F1	8	14
4E2	9	47
4G3	10	11

Cell supernatants from an anti-Lin28 hybridoma fusion (384 wells total) were screened in parallel using a single Spots On Dots sheet (384 Dot) and four 96-well ELISA plates, spotted or coated with antigen (respectively). Positive wells were then ranked according to signal strength, with “1” being the highest signal.

As shown, the top ten clones identified using Spots On Dots correlate very closely with the ranking of clones using ELISA. Similar results have been seen with all monoclonal hybridoma fusions tested, indicating that Spots On Dots can replace traditional methods for isolating the best antigen-specific hybridomas.

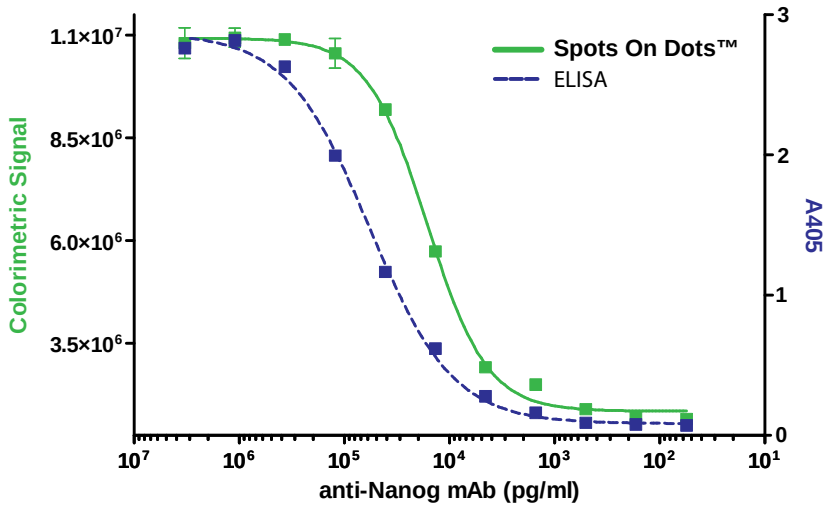
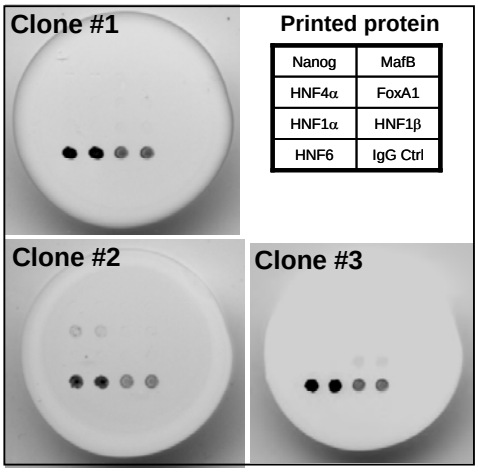


Figure 3. Comparison to ELISA: Sensitivity

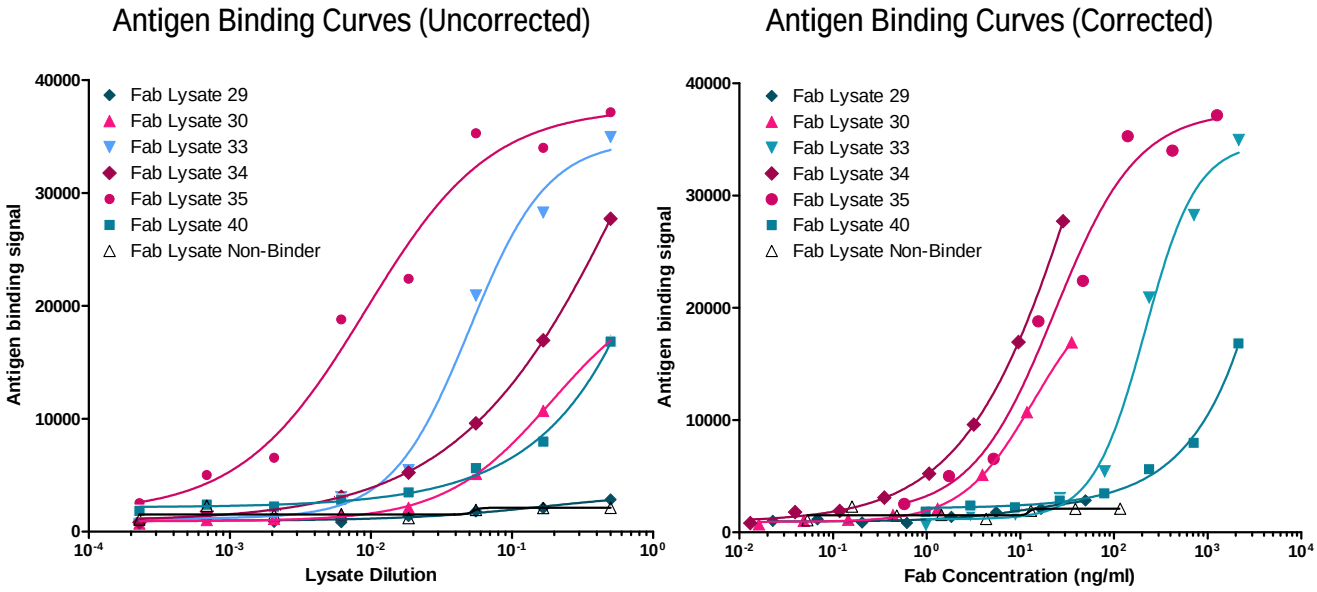
Titered monoclonal antibody was tested for the ability to detect recombinant antigen immobilized on Spots On Dots (solid line), or ELISA plates (dashed line). For the example shown, the lowest concentrations of antibody to detect cognate antigen were 0.05 ng/ml (Spots On Dots) or 0.2 ng/ml (ELISA). For all antibodies tested, the calculated lower limits of detection using Spots On Dots is equivalent to or greater than an optimized ELISA.

Figure 4. Specificity testing of monoclonal antibody clones



The Spots On Dots multiplex platform allows for the rapid testing of mAb specificity at a very early stage of hybridoma development. In this example, recombinant HNF6 and six related or unrelated proteins (plus IgG positive control) were printed in duplicate. Hybridomas were generated from a mouse immunized with HNF6, and supernatants were screened.

The results from three clones are shown here. Although all three exhibit strong binding to HNF6, Clone #2 and Clone #3 exhibit slight cross-reactivity to HNF4α and HNF1β, respectively. Clone #1 does not cross-react with any of the antigens tested. Equivalent ELISA testing would have required seven separate experiments and large amounts of the recombinant proteins.



Lysate	Affinity Rank (before correction)	Affinity Rank (after correction)	Biacore K_D (nM)	Fab Conc. (ng/ml)
Fab Lysate 34	3	1	0.4	57
Fab Lysate 35	1	2	0.9	2525
Fab Lysate 30	5	3	29	71
Fab Lysate 33	2	4	52	4298
Fab Lysate 40	4	5	97	4265
Fab Lysate 29	6	6	273	100
Non-binder	7	7	nd	233

Figure 5. Ranking the affinity of unpurified Fabs expressed from E. coli

Phage display of Fab (antibody fragment) libraries is a powerful technology for the *in vitro* selection of antigen binding molecules. However, it is difficult to quickly identify the highest-affinity binders following the screening process. Here, a Spots On Dots multiplex is used to rank the affinities of recombinant Fab molecules expressed in crude E. coli lysates at unknown concentrations. A titration of each lysate is tested for Fab binding to printed antigen (uncorrected graph). In parallel, a quantitative Fab assay is used to determine the concentration of Fab in each lysate (Table).

If the apparent affinities of the binders are ranked prior to correcting for the amount of Fab found in the expression lysates, the best binders are not identified, as shown in the table above. However, once the antigen binding curves are shifted based on Fab concentrations in the lysates (corrected graph), a more accurate ranking of affinities is obtained. The corrected ranking agrees perfectly with Biacore (surface plasmon resonance) affinity measurements of the purified Fab clones.

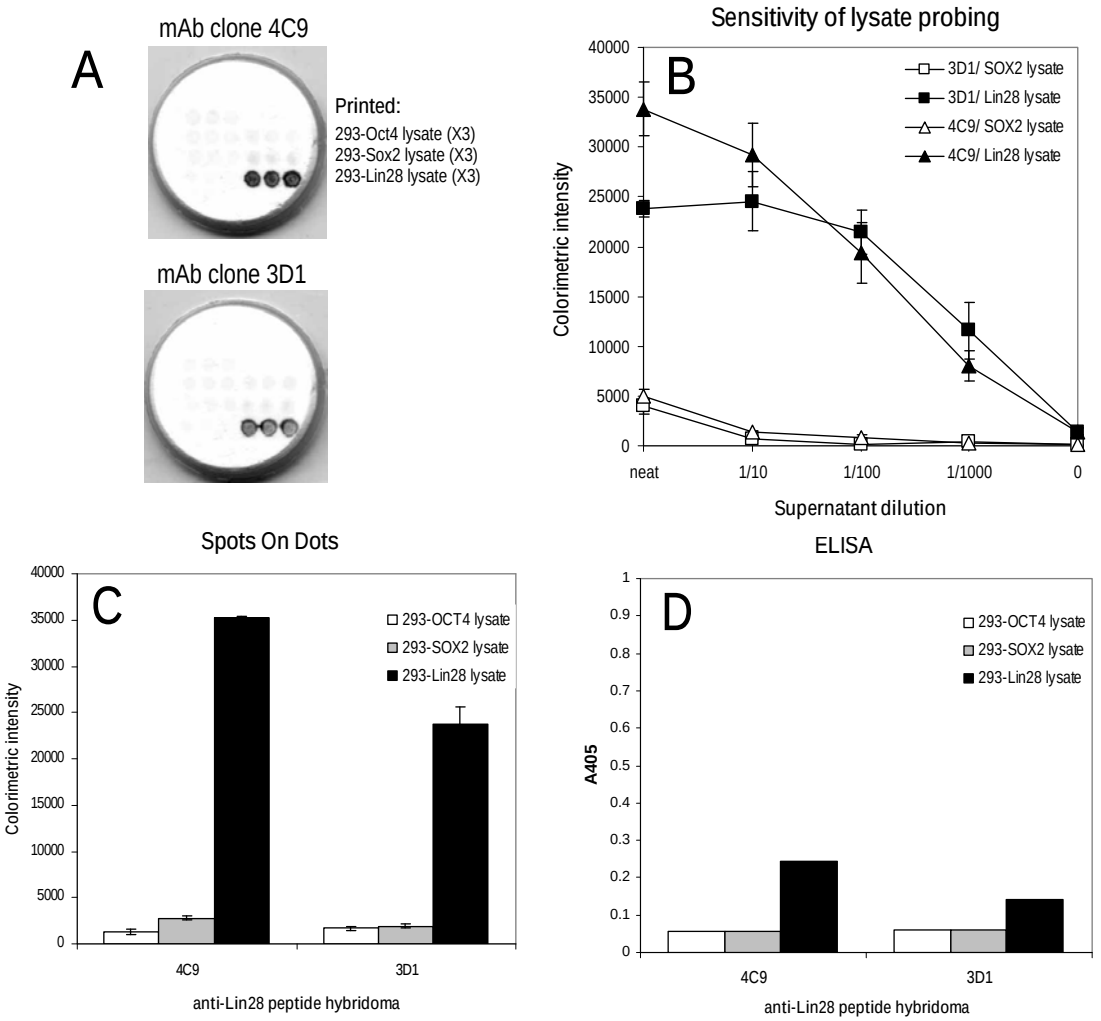


Figure 6. Identifying anti-peptide antibodies that recognize folded protein

Raising antibodies to synthetic peptides is a useful approach in cases where recombinant proteins are not available. Unfortunately, only a subset of antibody clones raised against a peptide sequence are also able to recognize the target protein as found in the cellular context. If antibodies are needed for ELISA, immunoprecipitation, immunostaining or flow cytometry applications, it is critical to identify those rare hybridomas able to bind native, folded protein.

Cell lines expressing high levels of the target protein provide a potential means for screening against folded protein, and lysates from transiently transfected cells can be made or purchased. However, purchased lysates are expensive, and ELISA plates bind lysates poorly due to the low binding capacity of plastic and blocking by detergents in the lysis buffer. The Spots On Dots platform overcomes these obstacles; very little lysate is needed for printing, and the thick Spots On Dots nitrocellulose membrane has an extremely high protein binding capacity.

(A) Using Spots On Dots screening, monoclonal antibodies (4C9 and 3D1) raised against two different Lin28 peptide sequences were identified that also have the ability to specifically bind lysates from HEK-293 cells over-expressing full-length Lin28. (B) The detection of Lin28 in lysate is sensitive enough that significant signals are seen even when the hybridoma supernatants are diluted 1000-fold. (C and D) The signal-to-background ratios of the Spots On Dots assay is much better than screening lysates using ELISA plates; ELISA signals are low even when lysates are coated at high concentration (20 μg/ml) and the alkaline phosphatase reaction is developed > 1 hr.

Ordering Information

Spots On Dots™ Antibody Screening Kit: Components

Qty		Component	Storage
8	ea.	Spots On Dots Sheets (96- or 384-Dot format)	RT desiccated
8	ea.	Incubation Trays	RT
8	ea	Humidification Pads	RT
1.2	ml	Printing Buffer (10X)	4 °C
100	ml	Blocking Buffer A	4 °C
200	ml	Wash Buffer A (10X)	4 °C
300	µl	Mouse IgG Control	4 °C
25	µl	anti-Mouse IgG Alkaline Phosphatase Conjugate*	-20 °C
80	ml	BCIP/NBT Substrate	4 °C

Products:

Kits:	Part Number
Spots On Dots™ Antibody Screening Kit (96-Dot format)	S2060-96
Spots On Dots Antibody Screening Kit (384-Dot format)	S2060-384

Hardware:	Part Number
Bel-Blotter™ 96-well Replicating Tool	S2067
PrimoriScan™ Flatbed Scanner	S2068

Consumables:	Part Number
Spots On Dots Sheet Set (4 pack, 96-Dot format)	S2063-4
Spots On Dots Sheet Set (8 pack, 96-Dot format)	S2063-8
Spots On Dots Sheet Set (4 pack, 384-Dot format)	S2064-4
Spots On Dots Sheet Set (8 pack, 384-Dot format)	S2064-8
Antibody Screening Buffer Set (Buffers, anti-Mo-AP, substrate)	S2059
Printing Buffer (10X)	S2056-1.2ML
Blocking Buffer A	S2039-100ML
Wash Buffer A (10X)	S2040-200ML
Mouse IgG Control	S2055-300UL
Anti-Mouse IgG Alkaline Phosphatase Conjugate	S2035-25UL
BCIP/NBT Substrate	S2038-80ML