

# Spots On Dots™ Antibody Screening Kit      User Protocol L1007

1. Overview.....	1
2. Kit Components .....	1
3. Storage and Handling Conditions .....	2
4. PROTOCOL.....	2
5. Abbreviated Protocol for Experienced Users .....	7
6. Related Products .....	7
7. Limited Use Label License.....	7
Appendix A: Sample Application Templates.....	8
Appendix B: General Recommendations for Robotics.....	9

## 1. Overview

The 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, antibodies bind the targets of interest. The supernatants are removed, and the sheet is probed by incubation with a mouse IgG-specific antibody conjugated to alkaline phosphatase. Bound antibodies are visualized by the addition of a precipitating alkaline phosphatase substrate to generate a colorimetric signal.

Images of the developed sheet can be captured with a standard flatbed scanner, eliminating the need for costly, dedicated readers. Quantification of signals can be rapidly obtained using dedicated software from Primorigen, free microarray analysis software, or other array analysis software.

## 2. Kit Components

Spots On Dots™ Antibody Screening Kit (96-Dot format)	Prod. No.S2060-96
Spots On Dots Antibody Screening Kit (384-Dot format)	Prod. No.S2060-384

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

\* Anti-Mouse IgG AP Conjugate is specific for mouse IgG (H+L), and also binds light chains on other mouse immunoglobulin isotypes. If secondary antibody conjugates specific for other species or isotypes are needed, please contact Primorigen technical service for recommendations.

Required or recommended components not included:

- Multichannel pipettor for small volumes (< 10 µl)
  - *or*
- Bel-Blotter™ 96-Well Replicating Tool\* (recommended for 96 Dot format)
  - *or*
- PrimaDotter™ 96-pin Transfer Device with Alignment Jig\* (recommended for 384 Dot format)
- Plate rocking platform or rotator (such as Labquake® or Belly Dancer®)
- Flatbed scanner with ≥1200 dpi optical resolution and 16-bit grayscale capability (such as the PrimoriScan™ scanner\*)
- Vacuum pump and vacuum chamber with indicating desiccant (recommended)
- Squirt bottle for wash buffer (optional)

\*See Section 6 (Related Products) for ordering information

### 3. Storage and Handling Conditions

Store buffers, BCIP/NBT, and Mouse IgG Control at 4 °C upon receipt. Store anti-Mouse IgG AP Conjugate at -20 °C; this reagent is formulated in 50% glycerol and does not need to be thawed before use. Store Spots On Dots™ sheets, incubation trays and humidification pads at room temperature. The packaging of Spots On Dots™ sheets contains a desiccant; reseal the packaging after opening, or store in a desiccator cabinet at room temperature. Gloves should be worn at all times when handling Spots On Dots sheets to avoid contaminating the nitrocellulose surface. Take care to avoid moisture and excessive dust.

Printing Buffer (10X), Blocking Buffer A, Wash Buffer A (10X), Mouse IgG Control and anti-Mouse IgG Alkaline Phosphatase Conjugate contain 0.09% sodium azide as a preservative. Use standard precautions for handling and disposal according to your local regulations.

## 4. PROTOCOL

### 4.1 Buffer Preparation:

Wash Buffer A (1X)	
Reagent	Volume
Wash Buffer A (10X)	200 ml
Add dH <sub>2</sub> O to:	2000 ml total

Store Wash Buffer A (1X) at 4 °C.

## 4.2 Printing:

1. Spots On Dots™ sheets are supplied with a pre-applied double-sided (high-tack/low-tack) adhesive that keeps the sheet flat and aligned during the printing step. Peel off the protective paper backing to expose the high-tack adhesive. Carefully align the top (notched) edge of the sheet with the inside upper edge of the incubation tray, then lay the sheet down into the tray. Place the blue separator paper on top of the Spots On Dots sheet and press gently to adhere the sheet evenly. All subsequent assay steps can be performed with the Spots On Dots sheet in the incubation tray.
  - ➡ *Note: If printing or performing the immunoassay without the adhesive layer is desired, the Spots On Dots sheet can be peeled away from the low-tack surface of the double-sided adhesive at any stage of the procedure.*
2. Dilute antigens in detergent-free and protein-free buffer (e.g. PBS or TBS) for printing. The Spots On Dots nitrocellulose will bind printed proteins up to 2 mg/ml. The following ranges are recommended for best assay performance:
  - a) **Purified protein:** Dilute to 100 – 500 µg/ml and add 1/10 volume of 10X Printing Buffer.
    - ➡ *Note: Printing Buffer contains octyl glucoside to improve spot morphology. Addition of the Printing Buffer is optional, depending on the performance characteristics of your printer/ microarrayer.*
  - b) **Synthetic peptide:** To improve binding to nitrocellulose, synthetic peptides should be conjugated to a carrier protein such as KLH, OVA or BSA (preferably not the carrier used for immunization). Dilute conjugate to 100 – 500 µg total protein/ml and add 1/10 volume of 10X Printing Buffer.
  - c) **Cell lysate:** Dilute cell lysates (1-2 mg total cell protein/ml) 5-fold to 10-fold to mitigate the blocking of protein binding by high detergent concentrations. Non-ionic detergents (Nonidet-P40, Tween-20, etc.) and ionic detergents (SDS, etc.) should be diluted to ≤ 0.2% and ≤ 0.05%, respectively. Do not add printing buffer.
3. (Optional) Mouse IgG Control can be used as a positive control for printing, and also verifies that the immunoassay procedures and reagents are working correctly. This control is 100 µg/ml mouse IgG in 1X Printing Buffer, and is ready to print.
4. Place samples in source plate according to printer/ microarrayer requirements.
5. Print samples according to printer/ microarrayer methods.

### Key Dimensions of the 96 and 384 format Spots On Dots sheets

Dimension	96 Dot	384 Dot
Dot diameter (mm)	5.59	2.75
Center to center Dot spacing (mm), horizontal and vertical	9	4.5
Dot surface height above bench (mm) when mounted in incubation tray	3.30	3.30
Dot A1 center inset (mm) from left outermost edge of incubation tray	14.14	11.87
Dot A1 center inset (mm) from top outermost edge of incubation tray	11.44	8.87
Array of printed Spots (375 mm diameter solid pins, 400 mm spacing)	7 x 7*	3 x 3*

\* Larger arrays can be achieved using smaller pins and/or tighter spot spacing.

6. [Recommended for lowest background:] After printing, place incubation tray into vacuum chamber with desiccant, pump to exhaust air (-26" Hg) and maintain vacuum for 1 hr minimum. For extended storage, keep printed sheets under vacuum or in desiccator at 4 °C. Stability of printed sheets is antigen-dependent, but is on the order of months.

### 4.3 Immunoassay:

#### Blocking

1. Peel protective sheet from humidification pad and attach the humidification pad to the underside of the incubation tray lid. Dampen with dH<sub>2</sub>O, and press out excess liquid.
  - ➡ *Note: Humidification is necessary to prevent evaporation when small volumes are incubated on individual Dot membranes. As an alternative to using the humidification pad, a sealed plastic food container containing a wet paper towel can be used as a humidification chamber.*
2. Block the printed Spots On Dots sheet. If you intend to add samples after blocking buffer has been removed from the Dots (damp or dry), follow the **Bulk Block** instructions. If you intend to dilute samples by adding undiluted samples to Dots pre-loaded with blocking buffer, follow the **Pre-Load** instructions:
  - a) **Bulk Block:** Add 10 ml Blocking Buffer A to incubation tray and incubate at least 30 min on rocking or rotating platform. Make sure that all Dot membranes are hydrated, and that the buffer contacts all Dots as the tray tips/ rotates.
 

*or*
  - b) **Pre-Load:** Apply Blocking Buffer A directly to each Dot (4 - 8 μl for 96-Dot format, 1 - 2 μl for 384-Dot format) and incubate at least 30 min on bench *with humidification*.

#### Sample Loading

3. (Optional) Prepare dilutions of antibody samples with Wash Buffer A in 96-well or 384-well dilution plates. Hybridoma supernatants can be added to the assay neat (undiluted). Alternatively, confluent hybridoma supernatants can typically be diluted 10-fold without a decrease in signal.
  - ➡ *Note: Samples can be diluted without preparing dilution plates by following the Pre-Load instructions.*
4. Prepare the Spots On Dots sheet for sample application:
  - a) **Bulk Block:** Dump Blocking Buffer from incubation tray and use a wadded Kimwipe® or lint-free lab wiper to gently blot the entire surface of the sheet, including the corners of the incubation tray. **Important:** Be careful to remove all visible liquid from between the Dots and from the surface of the Dots. Allow the sheet to air dry for 3-5 min (for damp membranes) or for ≥ 15 min (for dry membranes).
    - ➡ *Note: Blocked and dried Spots On Dots sheets can be stored for at least two weeks before use. Allow the membranes to air dry completely (≥ 1 hr) and store in a sealed bag with desiccant at 4 °C.*

*or*
  - b) **Pre-Load:** Do not remove blocking buffer. Proceed to the next step.
5. Apply samples according to the chosen method, using the volumes shown below. To aid in applying samples to the proper quadrants, set the incubation tray onto the attached Sample Application Template (Appendix A). **Important:** To prevent sample leakage, do not overload the Dots.

Dot membrane is:	Optimal sample volume per Dot	
	96-Dot format	384-Dot format
Dry	8 μl	2 μl
Damp	5.5 μl	1.2 μl
Pre-loaded with blocking buffer	Bring vol. to 8 μl total	Bring vol. to 2 μl total

- ➡ *Note: A range of volumes is acceptable (approximately +/- 50% of the volumes shown). It is not essential for a sample to form a droplet on the Dot. As long as each Dot is saturated (no visible dry areas), the assay will work.*

The following tools may be used for applying samples:

Tool	Vol./ Tip	# Samples	Sterile Transfer	Notes
PrimaDotter™ 96-pin Transfer Device and Alignment Jig*	1 – 2.2 µl	96	Flame, autoclave, or alcohol	Recommended for transfer to 384-Dot sheets (and pre-loaded 96-Dot sheets)
Bel-Blotter™ 96-Well Replicating Tool*	3 - 6 µl	96	Autoclave	Recommended for transfer to 96-Dot sheets
Multi-channel pipettor	1 - 20 µl	8 - 12	Disposable tips	To pipet, touch hanging drops to Dots
Single-channel pipettor	1 - 20 µl	1	Disposable tips	NOT RECOMMENDED due to sample evaporation during procedure

\* See Section 6 (Related Products) for ordering information

6. Incubate on bench for 1 hr *with humidification*.
7. Add ~10 ml Wash Buffer A to incubation tray and immediately discard, or rinse with wash buffer from squirt bottle.
8. Add ~10 ml Wash Buffer A to incubation tray and agitate 10 min. Dump buffer and repeat 10 min wash twice more.

➡ *Important: During wash steps, make sure the Spots On Dots sheet is fully adhered to the incubation tray. If not, press a forceps or P1000 pipet tip around the edge of the sheet (avoiding the Dots) to form a good seal. This prevents reagents from leaking behind the sheet and forming a substrate reaction on the plastic.*

### Secondary Antibody

9. Dilute 2 µl anti-Mouse IgG AP Conjugate in 10 ml Wash Buffer A (1:5000). Discard final wash and add diluted secondary antibody to incubation tray.
10. Incubate 1 hr with agitation.
11. Discard secondary antibody solution. Add ~10 ml Wash Buffer A to incubation tray and immediately discard, or rinse with Wash Buffer A from squirt bottle.
12. Add ~10 ml Wash Buffer A to incubation tray and agitate 10 min. Dump buffer and repeat 10 min wash twice more.


### Colorimetric Reaction

13. Discard final wash and add 10 ml BCIP/NBT Substrate to incubation tray. Incubate with occasional agitation, carefully monitoring the signal development.
14. Develop until positive signals are visualized as dark Spots, but before significant background develops on the nitrocellulose membranes. Signal should develop within 5 min at room temperature; however, weak signals may require 10-15 min incubation.
15. Rinse several times with tap water or dH<sub>2</sub>O to stop the colorimetric reaction.
16. Using forceps, peel the Spots On Dots sheet out of the incubation tray, leaving behind the adhesive layers. Blot any excess water from the sheet with a Kimwipe, and allow it to air dry completely.

#### 4.4 Image Capture:

The intensity of each spot can be determined from an image of the Spots On Dots sheet, captured using a standard flatbed scanner. For quantitative analysis by array analysis programs, the scanner must be capable of generating a high resolution image (1200 dpi or greater) with a 16-bit grayscale data file output. The PrimoriScan™ Flatbed Scanner is a low cost option (see Section 6: Related Products).

1. Place the Spots On Dots sheet face down on the scanner.

 *Important: The sheet must be aligned perfectly square to the scanner bed so that data analysis programs can find and assign the spots properly. Use of the PrimeAlign™ scanning template (bundled with the PrimoriScan scanner) will ensure accurate orientation.*

2. Open the software program for the scanner and select the following settings:

- Reflectance scanning
- 1200 dpi resolution
- 16-bit grayscale
- TIFF file format (uncompressed)
- Turn off automatic exposure adjustments. For example, select full input/ output ranges (0 – 255) and gamma = 1.0 under Histogram settings. These setting options differ by scanner software.

3. Scan the image and save the file.

#### 4.5 Data Analysis:

To obtain numerical values for each spot signal, use one of the following data analysis options:

- PrimeAlyzer™ analysis software has been custom designed by Primorigen Biosciences to extract and analyze data from scanned Spots On Dots sheets (96- or 384-well format) with minimal hands-on time. Go to [www.primorigen.com](http://www.primorigen.com) for more information and ordering.
- Spotfinder freeware is available from the TIGR website at [www.tm4.org/spotfinder.html](http://www.tm4.org/spotfinder.html). Templates for use with Spots On Dots sheets are available at [www.primorigen.com/software](http://www.primorigen.com/software).
- Other microarray image analysis programs such as GenePix®, ArrayVision™, and others can be used to extract data, but will require the creation of templates for the Spots on Dots layout.

 *Note: If using an analysis program intended for fluorescent microarrays, colorimetric images must be inverted to yield white spots on a dark background. Open the TIFF image file in Adobe® Photoshop®, type “Ctrl + I” to invert the image, and save.*

## 5. Abbreviated Protocol for Experienced Users

Step	Volume	Incubation time
Blocking Buffer (Pre-Load) <i>or</i> (Bulk Block)	4 - 8 $\mu$ l (96 Dot); 1 - 2 $\mu$ l (384 Dot) <i>or</i> 10 ml	Apply to each printed Dot, 30 min Add to tray and rock, 30 min
Remove Bulk Block		Dump, blot & air dry (5-15 min)
Add Samples	5.5 - 8 $\mu$ l total (96 Dot) 1 - 2 $\mu$ l total (384 Dot)	Apply to each Dot, 1 hr
Wash	4x 10 ml	Rinse once, 3 washes with rocking (10 min ea)
Secondary Antibody	10 ml	Dilute 1:5000, add to tray, 1 hr
Wash	4x 10 ml	Rinse once, 3 washes with rocking (10 min ea)
NBT/BCIP Substrate	10 ml	5-15 min or until desired intensity is obtained
Stop		Rinse 4x water and air dry
Scan Sheet		Capture data using flatbed scanner

## 6. Related Products

<b>Kits:</b>	<b>Prod. No.</b>
Spots On Dots™ Antibody Screening Kit (96-Dot format)	S2060-96
Spots On Dots Antibody Screening Kit (384-Dot format)	S2060-384
<b>Hardware:</b>	<b>Prod. No.</b>
PrimaDotter™ 96-pin Transfer Device	S2065
PrimaDotter Alignment Jig	S2066
Bel-Blotter™ 96-well Replicating Tool (4 pack)	S2067
PrimoriScan™ Flatbed Scanner	S2068
<b>Consumables:</b>	<b>Prod. No.</b>
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, control, substrate)	S2059

## 7. Limited Use Label License

Use of this product is covered by one or more patent applications. The purchase of this product includes a limited, non-transferable license for using only this amount of product for the purchaser's own internal research. This product is for research use only and may not be resold without written permission from Primorigen Biosciences.

### Trademark Information


The following trademarks and registered trademarks apply: Primorigen®, Spots On Dots™, PrimaDotter™, PrimeAlign™ and PrimoriScan™ (Primorigen Biosciences); Labquake® (Thermo Scientific); Belly Dancer® (Stovall Life Science Inc.); Bel-Blotter™ (Bel-Art Products); GenePix® (MDS Analytical Technologies); ArrayVision™ (GE Healthcare); Photoshop® (Adobe Systems); Kimwipe® (Kimberly-Clark)

## Appendix A: Sample Application Templates

8 x 12

	1	2	3	4	5	6	7	8	9	10	11	12	
A													A
B													B
C													C
D													D
E													E
F													F
G													G
H													H
	1	2	3	4	5	6	7	8	9	10	11	12	

16 x 24



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A																									A
B																									B
C																									C
D																									D
E																									E
F																									F
G																									G
H																									H
I																									I
J																									J
K																									K
L																									L
M																									M
N																									N
O																									O
P																									P
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	



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## Appendix B: General Recommendations for Robotics

### *Blocking*

- The key dimensions of the 96 and 384 format Spots On Dots sheets are shown in protocol Section 4.2 (Printing).
- Blocking Buffer A can be dispensed directly onto printed Dots using a multi-channel head with 20  $\mu$ l tips. Deliver 3-6  $\mu$ l per Dot (96-Dot format) or 1-2  $\mu$ l per Dot (384-Dot format).
- The blocking buffer can be placed in a single column of a 96-well source plate or in a bulk reagent reservoir.
- Humidity can be controlled during blocking and subsequent steps using the incubation tray lid with attached humidification pad, or by attaching the humidification pad to the bottom of the next incubation tray in a stack (no lids except top of stack).

### *Sample Loading*

- Samples can be delivered to blocked Dots using disposable tips or by using a multi-pin transfer tool.
- Removal of the blocking buffer is not necessary, but be careful not to exceed the maximum total volume per Dot of 6  $\mu$ l per Dot (96-Dot format) or 2  $\mu$ l per Dot (384-Dot format). See main Protocol for details.
- If desired, blocking buffer may be removed from individual Dots by aspiration (multi-channel wash tool in aspirate mode) or by manual blotting with lint-free lab wipes (e.g. Kimwipe).

### *Wash Steps*

- In the absence of agitation during washing, we recommend compensating by:
  - a) Increasing the volume of wash buffer (bulk dispense of ~25 ml per wash),
  - b) Increasing the number of washes after each assay step (~6 washes), and
  - c) Decreasing the length of each wash incubation (~2 min ea).
- Use TBST (Tris-buffered saline, pH 7.4 with 0.05% Tween-20) for extra wash buffer.

### *Secondary Antibody and Colorimetric Reaction*

- Bulk incubations (10 ml ea) can be performed on the automated system or as manual steps following sample incubation and washing.