Monoclonal antibody specificity validation using protein microarray, using purified mAbs

Introduction:
As described in the recent publication by Jeong et al. (Mol Cell Proteomics 11(6):O111.016253), use of a proteome microarray to validate antibodies leads to a high yield of MAbs that recognize full length human proteins in a cell line.

Overview steps:
1) A human proteome microarray is used as the primary validation tool.
2) The arrays are incubated with Protein G-purified MAb, and washed.
3) A dye labeled goat anti-mouse antibody is used to detect bound MAb
4) Array is scanned and analyzed

Materials:
Alexa Fluor 555 goat anti-rabbit IgG (H+L)*2mg/ml (Invitrogen)
Alexa Fluor® 647-AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories)
Aluminum foil
Anti-Glutathione-S-Transferase, S. japonicum form (EMD Millipore)
Automatic pipettes
BD Shoulder Cover Microscope Slide Box (Fisher Scientific)
Bovine Serum Albumin (IgG-Free, Protease-Free) (Jackson ImmunoResearch Laboratories)
Computer that meets minimum requirements (please see appendix 1)
Distilled water (ddH2O)
Filtration unit, 0.2 micron
Human proteome microarray (see below)
KCl
Microarray program (GenePix 6.1)
Microarray scanner (Molecular Devices GenePix 4000B)
Micropipettes
NaCl
Orbital shaker
Sterile disposable micropipette tips
Sterile serological pipettes
Thermo Scientific* Nunc* Dishes, Rectangular (4-Well)( Fisher Scientific)
Tris base
Tween-20
Tweezers
Vacuum system
Vortex
Reagents:

Blocking solution 2%BSA [w/v] in TBS-T (Recipe)
Dissolve 2g of (BSA) Bovine Serum Albumin (IgG-Free, Protease-Free) in 60ml of TBS-T.
Once dissolved, dilute to 100ml with TBS-T and sterile filter (0.2 micron filter) before use.
TBS-T (Recipe)
Dissolve the following in 800ml of distilled water:
0.2 KCl
8.8 NaCl
3g of Tris base
Add 0.5ml of Tween-20 and adjust pH to 7.5.
Complete with distilled water to 1L.
Sterilize by autoclave or filtration before use.

Array information. 17000+ individually purified human fusion proteins, expressed in yeast and
purified on glutathione beads. Overall structure of proteins is N-GST-His6-human ORF–C.
Proteins are randomly oriented on a Fullmoon slide surface (proprietary formula binds a variety
of side-chains). Proteins are arrayed as paired adjacent spots so 34,000 spots plus controls on
array. Nitrocellulose slides (FAST) have also been used successfully for antibody specificity
testing.

QC on the arrays is done with anti-GST. At a minimum, the first and last slide in each
batch are probed with anti-GST, and from this, the absolute quantity of fusion protein in
each spot can be calculated (by comparison to a standard curve of GST alone).

1) Maintain slides in -80 or dry ice until the moment they will be placed in blocking solution.
   (Warning: Avoid condensation or dryness at all times, this would ruin the chips).
2) Add 3ml of blocking solution (2% BSA/TBS-T) to 4-well plates prior to placing slides to
   plate.
3) Incubate the slides at room temperature for 1.5-2 hours with gentle shaking.
4) Use 3 ml blocking buffer for primary antibody preparation. Use a 20 ng/ml concentration
   for purified MAb to be validated. Also include rabbit anti-GST antibody to a final
   concentration of 1 micg/ml and vortex briefly.
5) Use vacuum system to remove blocking from 4-well plate.
6) Add the 3 ml of primary antibody previously prepared and leave incubating on orbital
   shaker at room temperature for 1 hour. (gentle shaking)
7) Once incubation period has passed, wash slides with 5 ml of TBS-T (Tris pH 7.5 and
   0.05% Tween-20) to each well three consecutive times.
8) After initial three washes add 3 ml of TBS-T and incubate 5-10 min at room temperature
   with gentle shaking.
9) Repeat the wash step two more times, removing completely the wash buffer after each
   wash step.
10) Add 3 ml of 2nd antibodies (goat anti-mouse IgG DyLight™649 conjugated – detects your antibody - and goat anti-rabbit IgG Alexa Fluor 555 conjugated – detects GFP) at a dilution of 1:800 in blocking solution. Cover the plates with aluminum foil and incubate for 1 hour with gentle shaking.

11) Wash three consecutive times with TBS-T.

12) After initial three washes add 3 ml of TBS-T and incubate 5-10min at room temperature with gentle shaking. (Warning: Incubations MUST be done covered with aluminum at ALL times.)

13) Repeat the wash step two more times, removing completely the wash buffer after each wash step.

14) Briefly rinse the slides three times with ddH₂O.

15) Once washes are complete remove chips from 4-well box and tap lightly on paper towel to remove excess water.

16) Place paper towel on the bottom of black microscope slide box and place slides in box.

17) Spin microscope slide box with chips in centrifuge at 2000 rpm for 3min to eliminate excess water.

18) Once centrifugation is complete, remove paper towel. Chips can be used for scanning immediately or be stored at 4°C to be used promptly.

GenePixpro 6.1 program and Array

Turn on the microarray instrument beforehand so it can warm up before use. Program will take a few minutes to start up as well.

Program features:
1) Place chip face down in the microarray apparatus with barcode towards the bottom.

2) Once chip is placed be sure to fasten in place and slide door shut.

3) Select ‘hardware’ to adjust wave lengths 635 (PMT Gain:600) and 532 (PMT Gain:500). Also adjust pixels; the lower you use the pixels the longer the run will take; however the image will be superior. The regular parameter is 10 pixels this will account for a 15 min run approximately.
4) Once proper wavelengths are established proceed to do a preview scan. The scan can be stopped or zoomed in at any time during this run.

5) Once preview is done verify chip for smudges or merged proteins. Select view scan area and outline the area that is to be scanned without including the barcode area.
6) Once scan is finished be sure to save photo as *.tif file and select the option “barcode prefix” that will save the name as the barcode number.
7) Select in the save menu open settings. This will accommodate the blocks on your scanned area. Each pair of spots is a different protein, it is crucial that the protein spot aligns with its respective circle, if not there will be false positives and negatives. 

*Note: If using the same chip batch it is simpler to use the same settings, the manipulation of the grid is less then starting from scratch.*

8) Align the entire grid with the specs using the block mode and overlapping both. At this point it’s possible to know the position and protein of each spec using the mouse arrow and placing it on the area of interest.
9) It is also possible to select a part of the block and modify a section instead of the block as a whole using feature mode.

*Currently there are two ways to identify positive hits: Manual (visual) and Program (z-score)

**Manual:**

10) Change wavelength to 532. Scan through each block to identify a positive hit (positive controls are on each block on the lower right corner.)
11) Once a proper hit has been identified, select feature mode and use the flag tool to mark your spots. Select the “Flag Good” good option.

12) Once finished flagging use the Analyze option. Save document as genpix. (.gps file)
13) Once document is saved open with Excel and identify your positive hits using the “sort by” tool on the flag column of the analysis. (Sort by highest number. Flagged areas will be 100.)

**Program (z-score):**

10) Once step 9 is complete, immediately use “analyze” option and save document as genpix (.gps file).

11) Use “z-Score” program to analyze data using the z-score values. This program will exclude positive control and previously identified non-specific array hits.

12) Finally results will appear with hit name, IOH number, z-score value.
**Appendix 1 Computer specs to drive GenePix**

**Minimum**
- IBM-compatible computer with a 1 GHz Pentium or faster
- Windows 2000 or XP operating system
- Memory requirements:
  - Full-size scan region at 5-µm resolution: 1 GB RAM for two colors; 2 GB for three or more colors
  - 10-µm scanning or partial-size scan regions at 5-µm resolution: 512 MB RAM for two colors; 768 MB for three or more colors
- 40 GB hard drive (for image storage)
- Available full-size PCI slot for SCSI adapter (*Note*: the provided PCI SCSI adapter will not fit in a low-profile or slimline case)
- CD-ROM drive
- 1024 × 768 display system with 65K colors
- Internet Explorer 5.0 or higher

**Recommended**
- IBM-compatible computer with a 2 GHz Pentium or faster
- Windows 2000 or XP operating system
- 2 GB RAM
- 80 GB hard drive (for image storage)
- Available full-size PCI slot for SCSI adapter (*Note*: the provided PCI SCSI adapter will not fit in a low-profile or slimline case)
- DVD-RW drive
- 1280 × 1024 display system with 16M colors
- Internet Explorer 6.0 SP1