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The “Reverse Capture” Autoantibody Microarray

An Innovative Approach to Profiling the Autoantibody Response to Tissue-Derived Native Antigens

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Summary

Recently, we reported the development and use of a “reverse capture” antibody microarray for the purpose of investigating antigen-autoantibody profiling. This platform was developed to allow researchers to characterize and compare the autoantibody profiles of normal and diseased patients. Our “reverse capture” protocol is based on the dual-antibody sandwich immunoassay of enzyme-linked immunosorbent assay (ELISA), and we have previously reported its use to detect autoimmunity to epitopes found on native antigens derived from tumor cell lines. In this protocol, we used ovarian cancer as a model system to adapt the “reverse capture” procedure for use with native antigens derived from frozen tissue samples. The use of this platform in studies of autoimmunity is valuable because it allows for the detection of autoantibody reactivity with epitopes found on the post-translational modifications (PTMs) of native antigens, a feature not present with other protein array platforms. In the first step in the “reverse capture” process, tissue-derived native antigens are immobilized onto the 500 monoclonal antibodies that are spotted in duplicate on the array surface. Using the captured antigens as “baits,” we then incubate the array with labeled IgG from test and control samples, and perform a two-slide dye-swap to account for any dye effects. Here, we present a detailed description of the “reverse capture” autoantibody microarray for use with tissue-derived native antigens.

Key Words: Autoantibodies; autoantigens; protein microarrays; biomarkers; tissues; ovarian cancer.

1. Introduction

Autoimmunity is a key feature of many disease states. Recently, the association of autoimmunity with cancer has been well established (1–3).

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01 In cancer, host antibodies react with a unique group of autologous intra-
 02 cellular proteins known as tumor-associated antigens (TAAs). Additionally,
 03 autoimmunity is consistently observed in a variety of well-known autoimmune
 04 diseases, including rheumatoid arthritis, multiple sclerosis, and insulin-
 05 dependent diabetes mellitus. The detection of autoimmunity is useful because
 06 autoantibodies can serve as biomarkers for disease, and their presence may help
 07 to elucidate the role of significant disease-related biochemical pathways.

08 Pervious studies of autoimmunity using microarray technology have relied
 09 on recombinant proteins and/or synthetic peptides as arrayed features (4–7).
 10 The major shortcoming of this approach is that these platforms may fail to
 11 detect the full range of autoimmunity present in a given disease state because
 12 of the absence of native protein conformations and relevant post-translational
 13 modifications (PTMs) of the arrayed features. Our laboratory recently reported
 14 on the development of a “reverse capture” autoantibody microarray (8,9). The
 15 “reverse capture” approach overcomes this significant shortcoming of other
 16 array platforms by detecting autoimmunity to proteins derived from a native
 17 source such as frozen tissue samples.

18 The “reverse capture” autoantibody microarray is based on the dual-antibody
 19 sandwich immunoassay of enzyme-linked immunosorbent assay (ELISA) (see
 20 Fig. 1). The basic platform consists of a glass microarray slide arrayed with
 21 1000 highly specific well-characterized monoclonal antibodies against 500
 22 unique antigens. These antibodies are used to immobilize native proteins.
 23 Because the reagents used in the procedure are non-denaturing, antigens are

40 Fig. 1. A schematic representation of the “reverse capture” autoantibody microarray
 41 platform.

01 immobilized on the array in their native conformations with all of their appropriate PTMs. The antigens captured on the slide then serve as “baits” for
02 differentially labeled patient IgG samples. By labeling test and control IgG
03 samples with different fluorescent dyes and scanning the “reverse capture”
04 slides, it is possible to identify the target antigens to which a test sample
05 differentially expresses autoantibodies, as compared with the control. We term
06 this array platform a “reverse capture” autoantibody microarray, because in
07 standard microarray nomenclature, “capture” refers to the immobilization and
08 detection of the analyte-antigen, not the antibody.
09

10 Additionally, the “reverse capture” protocol employs a two-slide dye-swap
11 method in which the dye/sample pairings used on one microarray slide are
12 reversed and applied to a second, parallel slide. This component of the protocol
13 facilitates the normalization of data, and controls for differences in the labeling
14 efficiencies of the different dyes, as well as differences in antibody-binding
15 efficiencies following the labeling reactions.

16 There are several key factors we have determined that are necessary to
17 account for in the analysis of “reverse capture” data: (a) variation in average spot
18 fluorescence intensity between dye channels, (b) outlying/aberrant data points,
19 and (c) average background intensity. Accordingly, we suggest eliminating far-
20 outlying data points and centering all data, through the method of ratio-based
21 normalization, before determining significant autoantibody expression using a
22 Student’s *t*-test and hierarchical clustering. There are numerous commercially
23 available computer programs that will assist with these types of analyses, as
24 well as with the construction of heat maps for graphical presentation of your
25 results. In this chapter, we describe a general normalization, transformation,
26 and analysis method that can be carried out using any high-quality software
27 package. We also introduce the recently developed Mixed Effect Model that
28 can be used to account for interdependence and correlation of results.

29 Additional information regarding our biostatistical methods and findings
30 using the “reverse capture” platform is available (8,10). In this chapter, we
31 used ovarian cancer as a model system to adapt the “reverse capture” platform
32 for use with native antigens derived from frozen tissue samples. This method,
33 however, may be used to study differential autoantibody expression in any
34 disease where autoimmunity is presumed to exist.

35 2. Materials

36 2.1. Purification of IgG

- 37 1. Serum samples acquired in accordance with IRB-approved protocol and stored
38 at -80°C .
- 39 2. Pipettes.

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- 01 3. Pipette tips.
- 02 4. Microcentrifuge.
- 03 5. Vortex.
- 04 6. Melon™ Gel Kit (Cat. No. 45206, Pierce Biotechnology) containing Melon™
- 05 Gel IgG Purification Support, Melon™ Gel Purification Buffer, and Handee™
- 06 Mini-Spin Columns and accessories.
- 07 7. End-over-end rotator.
- 08 8. BCA™ Protein Assay Reagent Kit (Cat. No. 23225, Pierce Biotechnology)
- 09 containing BCA™ Reagent A., BCA™ Reagent B, and Albumin standard
- 10 ampules (2 mg/ml).
- 11 9. 96-well plate or other size-appropriate plate for spectrometer.
- 12 10. Spectrometer capable of reading absorbance at 562 nm.

13 **2.2. Labeling of IgG with Fluorescent Dyes**

- 14 1. DyLight™ 547 and Dylight™ 647 Monoclonal Antibody Labeling Kits (Cat.
- 15 Nos. 53009 and 53015, Pierce Biotechnology) containing DyLight™ 547 and 647
- 16 NHS Esters, dimethylformamide (DMF), Borate buffer (0.67 M), and Zebra™
- 17 Desalt Spin Columns.
- 18 2. 0.5-ml microfuge tubes.
- 19 3. Pipettes.
- 20 4. Pipette tips.
- 21 5. Vortex.
- 22 6. Microcentrifuge.

23 **2.3. Removal of Unbound Dye**

- 24 1. Zebra™ Desalt Spin Columns (Cat. No. 89882, Pierce Biotechnology, also
- 25 supplied as a component of the Pierce Dylight™ Monoclonal Antibody Labeling
- 26 Kits).
- 27 2. Kimwipes.
- 28 3. Pipettes.
- 29 4. Pipette tips.
- 30 5. 2-ml microcentrifuge collection tubes.
- 31 6. Microcentrifuge.
- 32 7. Ultra-pure water.

33 **2.4. Native Protein Extraction from Tissue**

- 34 1. 50–100 mg of frozen tissue.
- 35 2. Mortar and pestle.
- 36 3. Alumina (Cat. No. A-2039, Sigma).
- 37 4. Ab Microarray Buffer Kit (Cat. No. 631792, Clontech) containing, Incubation
- 38 Tray, Extraction/Labeling Buffer, Stock Incubation Buffer, Background
- 39 Reducer, and Wash Buffers 1–7.
- 40
- 41

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- 01 5. Microcentrifuge.
- 02 6. 1.5- or 2-ml microcentrifuge tubes.
- 03 7. Pipettes.
- 04 8. Pipette tips.
- 05 9. BCA™ Protein Assay Reagent Kit (Cat. No. 23225, Pierce Biotechnology).
- 06 10. 96-well plate or other size-appropriate plate for spectrometer.
- 07 11. Spectrometer capable of reading absorbance at 562 nm.

09 **2.5. Antibody Array Incubation with Native Antigens**

- 10 1. Ab Microarrays (Cat. No. 631790, Clontech) containing 2 Ab Microarrays, and
- 11 one Storage Vial (empty).
- 12 2. Ab Microarray Buffer Kit (Cat. No. 631792, Clontech).
- 13 3. 50-ml conical centrifuge tubes.
- 14 4. Rocking platform.
- 15 5. Pipettes.
- 16 6. Pipette tips.
- 17 7. Phosphate-buffered saline (PBS) (pH 7.4).

19 **2.6. Antibody Microarray Incubation with Patient IgG**

- 20 1. Ab Microarray Buffer Kit (Cat. No. 631792, BD Clontech).
- 21 2. 15-ml conical centrifuge tubes.
- 22 3. Forceps.
- 23 4. Pipettes.
- 24 5. Pipette tips.
- 25 6. Rocking platform.
- 26 7. Kimwipes.
- 27 8. Swinging bucket centrifuge with adaptor for 50-ml tubes.

30 **2.7. Microarray Scanning and Statistical Analysis**

- 31 1. Microarray scanner compatible with 75 × 25 × 1-mm slides and capable of dual-
- 32 color analysis. The scanner must be capable of measuring fluorescence in the
- 33 ranges of the Cy3 and Cy5 fluorescent labels.
- 34 2. Microarray scanning software.
- 35 3. Microsoft Excel.
- 36 4. Microarray data analysis software.

38 **3. Methods**

40 The following protocol has been optimized to detect the presence of autoanti-
 41 bodies directed against native host antigens derived from frozen tissue samples.

01 This procedure should be carried out at room temperature wearing latex or
02 nitrile examination gloves, and all reagents used in the protocol should be
03 kept at 4 °C or on ice at all times, unless otherwise specified explicitly in the
04 protocol.

07 **3.1. Purification of IgG using Melon™ Gel Kit**

08 The following steps may be carried out several days before the rest of the
09 experiment. If you have not purified IgG before the day of your “reverse
10 capture” experiment, we recommend that you perform the purification and
11 labeling of patient IgG during the Incubation with Native Antigens (*see*
12 **Subheading 3.5.**); this will allow you to optimize the time spent performing
13 this procedure. Purified IgG can be stored for up to 1 week at 4 °C. If IgG is to
14 be stored for longer than 1 week, aliquots should be placed in a -20 °C freezer
15 for storage until use; avoid repeated freeze/thaw cycles.

- 17
18 1. Equilibrate the Melon™ Gel IgG Purification Support and Purification Buffer to
19 room temperature (about 30 min) and swirl the bottle containing the Purification
20 Support (do not vortex) to obtain an even suspension. To ensure proper gel
21 slurry dispensing, use a wide bore or cut pipette tip to dispense 500 µl of gel
22 slurry into a Handee™ Mini-Spin Column placed in a microcentrifuge tube. Swirl
23 the bottle of gel slurry before pipetting each sample to maintain an even gel
24 suspension.
- 25 2. Centrifuge the uncapped column/tube assemblies for 1 min at 3000 × g, then
26 remove the spin columns and discard the flow-through.
- 27 3. Add 300 µl of Purification Buffer to the column, pulse the centrifuge for 10 s,
28 and discard the flow-through. Repeat this wash twice. Place the bottom caps on
29 the columns.
- 30 4. Add 50 µl of each serum sample diluted 1:10 in 1× Melon™ Gel Purification
31 Buffer to a column. Cap the columns and incubate for 10 min at room temperature
32 with end-over-end rotation.
- 33 5. Remove the bottom caps from the columns, loosen the top cap, and insert the spin
34 columns into fresh 2-ml collection tubes. Then, centrifuge for 1 min at 3000 × g
35 to collect the purified antibody in the collection tubes.
- 36 6. Set up a new column corresponding to each sample that has been purified and
37 repeat **steps 2–5** in order to further purify the collected IgG using fresh Melon™
38 Gel (*see Note 1*).
- 39 7. Measure the concentration of IgG in each purified sample using Pierce’s BCA™
40 Protein Assay Reagent Kit.
- 41 8. Dilute each sample to 1 mg antibody/ml by adding the appropriate volume of 1×
Melon™ Gel Purification Buffer (*see Note 2*). The final amount of IgG should
be at least 200 µg per sample.

3.2. Labeling of IgG with Fluorescent Dyes

The following steps may be carried out several days before the rest of the experiment. However, **Subheadings 3.2** and **3.3** must be carried out together so that unbound dye is removed before storage of the labeled IgG. Labeled IgG can be stored for a maximum of 1 week at 4 °C protected from light. If labeled IgG is to be stored for longer than 1 week, aliquots should be protected from light in a -20 °C freezer until use. Repeated freeze/thaw cycles should be avoided.

Complete **steps 1–10** rapidly without interruption. Once the DyLight™ dyes are reconstituted, they must be used immediately (*see Note 3*).

1. Set up and label one 0.5-ml microfuge tube for each sample (four tubes total: A-DyLight™ 547, A-DyLight™ 647, B-DyLight™ 547, B-DyLight™ 647).
2. Transfer 100 µg of the appropriate purified antibody (1 mg/ml) from **Subheading 3.1** to the corresponding tube prepared in **step 1** and add 10 µl of Borate Buffer (0.67 M) to each tube.
3. Tap the bottom of the DyLight™ Reagent vials against a hard surface to ensure there is no dye in the caps and reconstitute one vial of DyLight™ 547 Reagent and one vial of DyLight™ 647 Reagent by adding 20 µl of DMF to each vial.
4. Vortex the two dye vials and briefly and centrifuge to collect the reconstituted dyes at the bottom.
5. Add 8 µl of DyLight™ 547 to each of the corresponding tubes from **step 2**.
6. Add 5 µl of DyLight™ 647 to each of the corresponding tubes from **step 2**.
7. Vortex the four microfuge tubes gently and briefly centrifuge the microfuge tubes to collect the samples at the bottom.
8. Incubate the tubes for 45 min at room temperature protected from light.
9. Proceed immediately with the removal of unbound dye.

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3.3. Removal of Unbound Dye with Desalting Columns

As long as you work quickly, desalting can be completed at room temperature. Otherwise, if you have access to a cold room, we suggest you complete the procedure at 4 °C.

1. Use two Zebra™ Desalt Spin Columns for each sample (8 columns in total) (*see Note 4*).
2. Twist off the bottom of each column and loosen the caps before placing each one in its collection tube and centrifuge each column at $1500 \times g$ for 1 min to remove the storage buffer. Note the side of each column where the compacted resin is slanted upwards, and be sure to place the columns in the centrifuge with this area of the column facing outward in all subsequent centrifugations.
3. Blot the bottom of the columns against a laboratory tissue to remove excess liquid.

- 01 4. Place each column into a fresh 2-ml collection tube and label two tubes for each
02 sample: A-DyLight™ 547, A-DyLight™ 647, B-DyLight™ 547, B-DyLight™
03 647 (8-column/tube assemblies in total).
- 04 5. Remove the caps from the columns and carefully apply half (~60 µl) of each
05 labeling reaction directly onto the center of the resin bed of its corresponding
06 column. Each labeling reaction tube should have two corresponding desalting
07 columns.
- 08 6. After the samples have been fully absorbed (~2 min protected from light), apply
09 15 µl of ultrapure water to the resin bed of each column and centrifuge the
10 columns for 2 min at 1500 × *g* to collect the desalted, labeled antibodies.
- 11 7. Combine the paired desalted samples so that each sample, A-DyLight™ 547,
12 A-DyLight™ 647, B-DyLight™ 547, B-DyLight™ 647, is consolidated into one
13 0.5-ml tube and has a volume of approximately 140 µl.
- 14 8. At this point, purified, labeled IgG samples can be stored for future use or used
15 in **Subheading 3.6** of a current experiment.

16 **3.4. Extraction of Native Antigen from Frozen Tissue**

17 This protocol employs the Clontech Ab Microarray 500; however, in a
18 different capacity from that set forth by the manufacturer. Nevertheless, in
19 order to use this microarray platform for autoantibody profiling, native antigens
20 should be extracted from a frozen tissue sample in the *exact* same method
21 set forth in Clontech's protocol. Please refer to and follow the protocol in
22 **Subheading 3.1.1** (*Extracting Protein from Tissue Samples*) of Chapter 9,
23 in order to extract native antigens from your frozen tissue sample. After
24 completing the antigen extraction, continue immediately to **Subheading 3.5**
25 (Chapter 10) of our "reverse capture" autoantibody microarray protocol (*do not*
26 *continue with the Clontech protocol*). Also, please note that we recommend
27 performing the native antigen extraction on the same day as the remaining
28 portions of the "reverse capture" protocol. If proteins are extracted at an earlier
29 time, phosphatase activity, proteolysis, and other proteolytic degradations may
30 occur that will alter the outcome of the experiment.

32 **3.5. Antibody Array Incubation with Native Antigens**

- 33 1. Set up the Incubation Tray provided. On the lid of the Incubation Tray, label the
34 four chambers as follows: Slide 1 Incubation, Slide 1 Wash, Slide 2 Incubation,
35 Slide 2 Wash (*see Fig. 2*).
- 36 2. Prepare 50 ml Incubation Buffer from 45 ml Stock Incubation Buffer and 5 ml
37 Background Reducer. Then, add 5 ml of Incubation Buffer to the incubation
38 chambers.
- 39 3. Transfer 200 µg of protein from **Subheading 3.4** to each of the incubation
40 chambers and incubate the tray at room temperature for 30 min with gentle
41 rocking.

Fig. 2. An illustration of the Incubation Tray showing incubation and wash chambers.

4. About 5 min before the incubation in **step 3** is complete, prepare the Ab Microarrays by washing the slides two times. Wash as follows:
 - a. Decant the Storage Buffer from the Storage Vial.
 - b. Add 30 ml of Stock Incubation Buffer.
 - c. Cap the Storage Vial and slowly invert the vial 10 times.
 - d. Decant the Stock Incubation Buffer from the Storage Vial.
 - e. Add 20 ml of Incubation Buffer and repeat **steps c** and **d**.
5. Record the lot number of the slides found on the label on the outside of the Storage Vial.
6. Carefully remove the slides from the Storage Vial and place each one, label side facing up, into one of the two designated incubation chambers containing the native antigen mix.
7. Incubate the slides at room temperature for 45 min with constant gentle rocking. Every 15 min, use a fresh pipette tip to gently lift each slide while rocking the incubation tray to allow the native antigen mix to come into contact with all sides of the slide.
8. Remove the buffer from the incubation chambers and discard.
9. Add 5 ml of PBS (pH 7.4) to the incubation chambers, taking care to pipette the liquid on the portion of the slide containing the manufacturer's label.
10. Incubate at room temperature for 5 min with gentle rocking.
11. Repeat **steps 9** and **10** two times.
12. Proceed immediately with the microarray incubation with patient IgG.

01 **3.6. Antibody Array Incubation with Patient IgG**

02 The labeled IgG samples should be incubated with the microarrays in the
 03 same incubation chambers used in **Subheading 3.5** for the incubation with
 04 native antigens. Some biologically relevant antigens that have not been captured
 05 by the arrays' monoclonal antibodies in **Subheading 3.5** will be found on the
 06 walls of the incubation chamber. The high affinity of certain IgG molecules
 07 for these antigens' epitopes limits non-specific binding of labeled IgG to the
 08 microarray slide.

- 09 1. Label one 15-ml conical tube "Mix 1" and label one 15-ml conical tube "Mix
 10 2"; then add 5 ml of Incubation Buffer to each of these tubes.
- 11 2. Transfer the entire sample of IgG A-DyLight™ 547 and IgG B-DyLight™ 647
 12 (200 µg total; from **Subheading 3.3**) to the Mix 1 tube from **step 1**.
- 13 3. Transfer the entire sample of IgG A-DyLight™ 647 and IgG B-DyLight™ 547
 14 (200 µg total; from **Subheading 3.3**) to the Mix 2 tube from **step 1**.
- 15 4. Add the contents of the tube labeled "Mix 1" to incubation chamber 1 and "Mix
 16 2" to incubation chamber 2.
- 17 5. Incubate the native protein array slide (prepared in **Subheading 3.5**) at room
 18 temperature for 45 min with gentle rocking. Every 15 min, use a pipette tip to
 19 lift one end of the slide while gently rocking the Incubation Tray.
- 20 6. Add 5 ml of Incubation Buffer (prepared in **Subheading 3.5**) to each wash
 21 chamber, transfer the slides to their respective wash chambers, and incubate at
 22 room temperature for 5 min with gentle rocking.
- 23 7. Remove the buffer from the wash chambers.
- 24 8. Add 5 ml of Wash Buffer 1 to each wash chamber.
- 25 9. Incubate at room temperature for 5 min with gentle rocking.
- 26 10. Repeat **steps 7–9** using Wash Buffer 2, then Wash Buffer 3, etc., until each
 27 slide has been washed with each of the Wash Buffers 1–7.
- 28 13. Dry the slides. It is important to remove as much moisture as possible from the
 29 surface of the slides before the liquid evaporates passively.
 - 30 a. Using scissors or a knife, puncture a small, round hole in the bottom of the
 31 Storage Vial provided. This will facilitate the removal of excess liquid from
 32 the slides during centrifugation.
 - 33 b. Using gloved hands and touching only the edges of the slides, hold the slides
 34 so that the excess liquid drips toward the bottom of the array slides (the area
 35 containing the manufacturer's label) and gently touch this edge to a clean
 36 Kimwipe several times.
 - 37 c. Carefully place the slides in the empty Storage Vial with the ends containing
 38 the manufacturer's label at the bottom of the vial. Do not touch the array
 39 surface.
 - 40 d. Cap the vial and centrifuge the slides at $1000 \times g$ for 25 min at room
 41 temperature.
14. Proceed immediately with microarray scanning (*see Subheading 3.7*).

3.7. Microarray Scanning and Quantitation

Antibody microarray slides should be scanned using a laser scanner, such as the Axon GenePix 4000B or the Perkin Elmer ScanArray 5000, according to the manufacturer's specifications. The scanner must be able to measure fluorescence in the ranges of the Cy3 and Cy5 fluorophores (*see Note 5*).

1. Turn on the scanner and allow the lasers to warm up. The lasers on the Perkin Elmer ScanArray 5000 require 15 min to warm-up before scanning your arrays.
2. Run a quick/preview scan of the entire slide in order to determine the area containing the arrayed features.
3. Create a scan protocol on the computer attached to the microarray scanner (*see Note 6*).
 - a. Set the protocol to scan for DyLight™ 547 and DyLight™ 647 fluorescence. It is acceptable to use the standard settings for Cy3 and Cy5 in order to scan for the DyLight™ dyes because these fluors have similar excitation/emission maxima (excitation; emission): DyLight™ 547: 557 nm; 570 nm Cy3: 550 nm; 570 nm DyLight™ 647: 652 nm; 673 nm Cy5: 649 nm; 670 nm
 - b. Determine the area containing the arrayed monoclonal antibodies and select this as the portion of the array to be scanned.
 - c. If possible, select an area on the array that contains no arrayed features to be scanned for background intensity.
 - d. Set the laser powers and PMT (photomultiplier) Gains so that the signal is high enough without being saturated (*see Note 7*). We suggest the following settings for scanning with the Perkin Elmer ScanArray 5000:
 Cy3/DyLight™ 547: PMT = 62%; laser power = 90%
 Cy5/DyLight™ 647: PMT=50%; laser power=90%
4. Insert the first slide, containing the Mix 1 autoantibodies, into the scanner. For the ScanArray 5000, the microarray surface is inserted face up.
5. Begin the scan and make sure that the signal is sufficiently high, but not saturated. You may need to readjust the laser and PMT Gain settings or rerun an Automatic Sensitivity Calibration if the image is saturated or the signal is too low.
6. Save the DyLight™ 547 and 647 images as separate TIFF files. The single-file TIFF format is the most useful for quantifying data from the images. Other file formats, such as Bitmap (BMP), may be appropriate for other tasks (*see Note 8*).
7. Scan the second microarray slide, containing the Mix 2 autoantibodies, using the same settings used to scan the first slide and save the images in the same manner.
8. Obtain the Axon Grid (GAL file) that corresponds to the lot number of the Clontech™ Ab 500 Microarray used. This can be found at <http://bioinfo.clontech.com/abinfo/array-list-action.do>

- 01 9. Using the GenePix Pro software, use the “Alt Y” command to open the GAL
02 file downloaded in **step 8** and open the TIFF files corresponding to your first
03 slide using the “Alt O” command.
- 04 10. Automatically align the Axon Grid with the array features using “F8.” Use the
05 Zoom-In feature to ensure the proper alignment of the grid with the features of
06 your array. You can adjust the fit of the Grid to the entire array or to individual
07 array features using the tools on the left-hand side of the screen.
- 08 11. Carry out an Automatic Analysis using the “Alt A” command (*see Note 9*) and
09 save your data as a GPR (GenePix Results) file using the “Alt U” command
10 (*see Note 10*).
- 11 12. Data can be exported to Excel using the “Ctrl A” command to select all data,
12 followed by the “Ctrl C” command to copy all data to the clipboard.
- 13 13. Data from your first slide can now be pasted into a blank Excel sheet with the
14 “Ctrl V” command.
- 15 14. Repeat **steps 9–13** using the TIFF images from your second slide (*see Note 11*).
16 Note, the same GAL file should be used to extract data from the second slide
17 because slides provided as a pair always have the same lot number.

18 **Fig. 3** shows the scan images from a “reverse capture” experiment where
19 the dye-swap method was employed. In this experiment, native antigens were
20 extracted from malignant ovarian tissue and hybridized with the two microarray
21 slides, as described. Test IgG was taken from the serum of an ovarian cancer
22 patient, whereas control IgG was prepared from the sera of group of 20 age-
23 matched normal females; IgGs were differentially labeled. The spots that appear
24 both green (DyLight™ 547) on one slide and red (DyLight™ 647) on the other,
25 represent candidate antigen-autoantibody reactivities. The significance of these
26 spots is investigated in **Subheading 3.8**.

27 3.8. Biostatistical Data Analysis

28 Biostatistical analyses are needed to appropriately normalize, analyze, and
29 interpret the vast amounts of data obtained from “reverse capture” studies.
30 There are many analysis tools to extract reliable information from microarray
31 data [commercial software packages as well as programs provided on the web
32 at no cost to investigators (*11*)]. Regardless of the tools that are employed,
33 “reverse capture” data must be normalized and transformed before reasonable
34 data comparisons can be made (*see Note 12*).

- 35 1. The raw array data acquired in GenePix Pro have to be pre-processed to separate
36 signals from noise, eliminate low-quality measurements, handle missing values,
37 and adjust the measured intensities to facilitate comparisons (*12,13*). Image
38 analysis tools reduce the background signal and minimize the impact of poor-
39 quality spots on the final array data (*11,12*).

Fig. 3. An illustration of results from the two-slide dye-swap. In Mix 1, control IgG is labeled with DyLight™ 647 dye (red) and ovarian cancer patient IgG is labeled with DyLight™ 547 dye (green). In Mix 2, the dyes are swapped, so control IgG fluoresces green, whereas ovarian cancer IgG fluoresces red. A portion of each array slide is enlarged to clearly illustrate that green spots denoting ovarian cancer autoantibody reactivity in Mix 1, fluoresce red in Mix 2.

2. The simplest method of normalization is a linear transformation of the data to correct for experimental variability because of differences in sample quantity, fluorescent labeling, hybridization efficiencies, and inter-array variations (14–16).
 - a. General linear modeling methods such as ANOVA (17) or a mixed model (18) protocol can be used to link the normalization process with downstream data analysis.
 - b. Non-linear normalization approaches, such as locally weighted regression, are useful for removing intensity-dependent effects on the fluorescence ratios (16,19,20).
3. Other data transformation procedures such as intensity-based filtering and replicate filtering can increase the reliability of measurements and assist with downstream clustering. For our dye-swapped replicate data, background-subtracted Log_2 -transformed intensity ratios of the dye-swapping slides can be plotted against each other; outliers with more than two standard deviations should then be excluded from clustering analysis.

- 01 4. Missing value estimation methods based on k -nearest neighbors (k -NN) or a
02 singular value decomposition (SVD approach) can be used to remedy the data
03 where multiple missing expression values exist (13,21).
- 04 5. Following normalization of the data, the underlying patterns of antigen-
05 autoantibody reactivity can be examined by clustering antigen signals into groups
06 with similar patterns (22,23).
 - 07 a. Agglomerative hierarchical clustering, k -means clustering, and self-organizing
08 maps (SOM) are widely used methods (23,24).
 - 09 b. Recently, mixture model-based clustering has become very popular for the
10 clustering analysis of microarray data (13,25,26), as it provides a sound mathe-
11 matical framework for clustering and a principled statistical approach to the
12 determination of the ideal number of clusters (13).
 - 13 c. For clustering correlated antigen-autoantibody reactivities from different kinds
14 of “reverse capture” experiments (such as time-course experiments, experi-
15 ments with repeated measurements, and cross-sectional experiments), a new
16 method has been proposed through the formulation of a linear mixed-effects
17 model (LMM) (27). This model reduces the possibility of misleading clusters
18 of microarray data by overlooking the presence of correlations between obser-
19 vations and the dependence between microarray data (28,29).
- 20 6. For a “supervised” approach to building a classifier suitable for diagnosis, as well
21 as drug discovery, support vector machine (SVM) methods based on a backward
22 selection procedure known as recursive feature elimination (RFE) have been
23 developed to select a small subset of significant data points from broad patterns of
24 data (30,31). Data points are ranked in relative importance as markers on the basis
25 of the absolute values of their fitted coefficients in the linear form of the classifier
26 for an SVM formed with linear kernel (30). In estimating the error rate of the
27 classifier based on a smaller set of significant parameters, care must be taken to
28 ensure that the selection bias associated with choosing the optimal of parameters
29 has been assessed and corrected for, using either an “external” cross-validation
30 or a bootstrap approach (32).
- 31 7. Another method for identifying biomarkers from a very large number of candidates
32 is to rank antigen-autoantibody reactivities using a supervised selection procedure
33 that makes use of known clinical data such as survival time, time to distant
34 metastases, or time to recurrence of tumor (33,34).

36 4. Notes

- 37 1. The IgG purification procedure is repeated in order to ensure exclusive isolation
38 of IgG from patient sera. We have found that with only one purification run,
39 high-abundance proteins other than IgG may be present in the eluted solution.
- 40 2. To label the purified antibodies with fluorescent dyes, the antibodies must be in a
41 buffer free of ammonium ions and primary amines. The Melon™ Gel Purification

- 01 Buffer used in **Subheading 3.1** is compatible with all of the DyLight™ labeling
02 reagents used in **Subheading 3.2**.
- 03 3. Pierce Biotechnology supplies the DyLight™ 547 and DyLight™ 647 dyes
04 as monofunctional *N*-hydroxysuccinimide (NHS)-esters in dried pre-measured
05 amounts. The NHS-ester is a functional group that cross-links to primary amines.
06 This reaction releases the NHS group and produces a covalent amide bond
07 that links the dye to the amine. Because of the unique affinity ratio of each
08 dye to the antibody that it is labeling, different volumes of the two dyes are
09 used in the labeling reactions. Once reconstituted in the DMF supplied in the
10 DyLight™ labeling kits, unused aliquots of the DyLight™ dyes cannot be saved
11 for future use. The binding efficiency is quickly diminished in this medium. If
12 you wish to save unused portions of the dyes for later use, they should initially
13 be reconstituted in anhydrous DMF (or DMSO) and frozen at -20 °C until use.
- 14 4. The Zebra™ Desalt Spin Columns that come with the DyLight™ Labeling
15 Kits remove free dye. These columns contain a desalting resin and molecular
16 weight cutoff. They perform well in desalting small sample volumes, providing
17 excellent protein recovery and $\geq 95\%$ retention of small molecules and salts
18 (<7 kDa).
- 19 5. The excitation and emission maxima of the more commonly used CyDye
20 fluorophores are very close to those of the DyLight™ dyes, where DyLight™
21 547 is analogous to Cy3 and DyLight™ 647 is analogous to Cy5. Thus, the
22 preprogrammed settings for the scanning of CyDyes may be used in scanning
23 arrays labeled with the Pierce DyLight™ dyes.
- 24 6. There are various software packages available for creating scan and quantitation
25 protocols. ScanArray Express by Packard Bioscience or GenePix Pro 6.0 by
26 Molecular Devices is recommended for the scanning of the microarray slides.
27 GenePix Pro is recommended for the quantitation of your results.
- 28 7. The microarray may need to be scanned several times before the ideal laser power
29 and PMT settings are ascertained. If your scanner has the option of running an
30 Automatic Sensitivity Calibration, this option may be perused. We recommend
31 that you also refer to our published "reverse capture" troubleshooting tips for
32 additional suggestions (9).
- 33 8. If the images will be displayed as graphics in a presentation or article, they
34 should be saved individually as BMP files and then readjusted and/or overlaid
35 in a photo-editing program, such as Adobe Photoshop™.
- 36 9. The tab labeled "Histogram" at the top of the screen allows you to check the
37 normalization of your data. When dye and scanning intensities are perfectly
38 normalized, the Count Ratio will be equal to 1.0. If your Count Ratio deviates
39 significantly from 1.0, you should attempt to adjust the PMT gains used for
40 scanning so that this value approximates 1.0. The "Scatter Plot" tab allows you
41 to view the distribution of your data to ensure that there are a minimal number
of outlying data points.
10. GPR files are used in some biostatistical analyses. For example, the microarray
data analysis software Acuity 4.0 by Molecular Devices uses this file format

in order to perform data manipulation and analyses, including ratio-based normalization, lowess normalization, hierarchical clustering, non-hierarchical clustering, and *t*-tests for statistical significance.

11. When opening the TIFF files from the second slide, it is necessary to check the box next to “Replace current images” in the “Open Images” dialogue box.
12. Normalization and transformation of data accomplish two aims. First, log-transformation of data scales the range of your data; as “reverse capture” data is acquired in ratio form, it is important that the scale allows for equal differential expression by the sample represented in both the numerator and the denominator of the ratio. Second, normalization centers data around an expected mean value; log-transformed data are centered around the expected median (or mean) log-ratio of 0.0 across all data points.

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Disclosure

International patent protection is currently pending for the “reverse capture” autoantibody microarray platform.

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