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Discovery and Customized Validation of Antibody Targets by Protein Arrays and Indirect ELISA

Tara K. Sigdel and **Minnie M. Sarwal**

Abstract

Because of our access to human genome data and ever improving genome sequencing and proteome analysis methods we are much better in terms of our understanding of biological processes. In addition to genomics, proteomics, and other “omics” methods, availability of more sophisticated molecular assaying methods have augmented our knowledge about immune processes towards autogeneic and allogeneic targets. High-density protein arrays are developed to analyze protein–small molecule interactions, enzyme–substrate profiling, protein–protein interaction, and immune monitoring by assessing antibodies in the serum.

Keywords

Antibodies; ELISA; Protoarray; Proteomics; Transplant; Urine; Biomarkers

1 Introduction

Organ transplantation is the optimal treatment for most end stage organ failure. However, due to lack of a specific and sensitive means to monitor graft injury long-term outcomes are not optimal [1]. Given the current status of organ shortage, advanced methods of organ monitoring and improving graft life expectancy is an unmet need in organ transplantation. Antibodies are known to be involved in acute and chronic rejection of transplanted organs, but their ability to predict graft injury events is poorly understood. Antibody’s role in causing graft injury in different injury types has not been studied. Recently, microarray technology has evolved beyond nucleic acids hybridization to serve as a platform for detecting protein-specific antibodies [2]. Protein-array technology that can be used to analyze protein–small molecule interactions for identification of small molecules that have specific affinity to human antigens, enzyme–substrate profiling to identify novel substrates for enzymes, and protein–protein interaction, has also been used to assess antibody level in the blood collected from transplant patients to identify reactive antigens activated in different graft injury phenotypes.

We have used the protein array platform to develop an immunogenic and anatomic roadmap of the most likely non-HLA antigens that result in serological responses in kidney transplantation [3]. We have also identified a number of antigens that are associated with kidney transplantation, end stage renal disease, and IgA nephropathy [4–7]. A successful

study requires well designed experiments and optimized methods, sophisticated data analysis and subsequent validation of findings made by high-throughput “omic” platform. This chapter provides two independent platforms for identification and validation of antibody levels in human sera. First, we provide a detailed method of identification of increased antibody response against auto/allo antigens by assaying antibodies against ~9,500 human antigens using high-density protein arrays (ProtoArray®, Life technologies, Carlsbad, CA). Then we present protocols for antibody ELISA methods to validate the discovery made by protein arrays. Protein array and MSD ELISA protocol (Meso Scale Discovery, Gaithersburg, MD) include methods that are based on our protocol adopted from corresponding manufacturer’s protocols.

2 Materials

2.1 Protein Arrays (ProtoArray®, Life Technologies, Carlsbad, CA)

1. Human serum or plasma sample (dilute the sample 1:150 in washing buffer, store on ice until use).
2. ProtoArray® Human Protein Microarray v5.0.
3. Alexa Fluor® 647 Goat Anti-Human IgG (Life Technologies, Cat. no. A21445).
4. Blocking buffer: either ProtoArray® Blocking Buffer Kit (Life Technologies, Cat. no. PA055) or 10× Synthetic Block (Life Technologies, Cat. no. PA017). *See* Table 1 for preparation using Synthetic Block.
5. Clean, 4-chamber incubation tray with cover (QuadriPERM® Culture Dish. 4-chamber polystyrene culture dish, VWR, San Francisco, CA), chilled on ice Forceps and deionized water Shaker (capable of circular shaking at 50 rpm, set the shaker at 4 °C).
6. Microarray slide holder and centrifuge equipped with a plate holder.
7. Fluorescence microarray scanner (e.g., GenePix 4000B Microarray Scanner or equivalent).
8. Microarray data acquisition software (e.g., GenePix® Pro from Molecular Devices).
9. Data analysis software (ProtoArray® Prospector recommended, available from www.invitrogen.com/protoarray, Significant Analysis of Microarray (SAM) software [8], GraphPad Prism (GraphPad Software, La Jolla, CA), or equivalent software for basic statistics and visualization and presentation).

2.2 ELISA Assays

Conventional ELISA

1. Coating buffer: 15 mM Na_2CO_3, 30 mM $NaHCO_3$, 0.02 % NaN_3.
2. Nonfat dry milk powder (BioRad, Blotting Grade Blocker Non Fat Dry Milk Cat. no. 170-6404XTU).
3. PBST buffer (1× PBS, 0.05 % tween 20).

4. Primary antibody (positive control) and secondary antibody (anti human IgG AP).
5. AP-PNPP liquid substrate (Sigma, Cat. # 7998).
6. The standard for UV-visible microplate reader absorbance (absorbance detection in the UV-visible wavelength range 190–1,000 nm).

MSD Platform

1. The SECTOR Imager 2400 reader or 6000 reader (Meso Scale Discovery, Gaithersburg, MD).
2. Standard and/or High Bind Plates (Meso Scale Discovery, Gaithersburg, MD).
3. Blocker A (Meso Scale Discovery, Gaithersburg, MD).
4. Read Buffer T (Meso Scale Discovery, Gaithersburg, MD).
5. SULFO-TAG labeled antibody (Meso Scale Discovery, Gaithersburg, MD).

3 Methods

3.1 Serum and Plasma Processing and Sample Preparation

3.1.1 Serum Processing

1. Collect a blood sample in a red top tube with blood using standard venipuncture technique. Let the tube stand in upright position for 20–30 min until clot forms.
2. Centrifuge blood at 800 × *g* for 10 min and aliquot serum and store at −80 °C until ready to use.

3.1.2 Plasma Processing

1. Collect blood sample into commercially available anticoagulant-treated tubes, e.g., EDTA-treated (lavender tops) or citrate-treated (light blue tops). Heparinized tubes (green tops) are indicated for some applications.
2. Centrifuge tubes for 10 min at 1,000–2,000 × *g* using a refrigerated centrifuge and aliquot the upper plasma portion and store at −80 °C until ready to use.

3.1.3 Sample Preparation (Serum or Plasma)

1. Prior to use, process the sample to remove any aggregates by centrifugation (12,000 × *g* for 30 s in a microcentrifuge).
2. Recommended dilution is 1:500 by the manufacturer but in our hands 1:150 dilution in washing buffer works the best. Users may have to optimize dilution based on their initial results.

3.2 ProtoArray

3.2.1 Blocking and Detecting—A summary of the probing method is presented in Fig. 1a.

1. Thaw the protein array slides by placing them at 4 °C for at least 15 min.

2. Place the protein array slides with barcoded side facing up into each well of a 4-chamber tray.
3. Pipet 5 ml blocking buffer (cooled to 4 °C) into each chamber, avoiding any direct pipetting onto the slides.
4. Incubate the slides for 1 h at 4 °C on a shaker set at 50 rpm (circular shaking preferred).
5. After the incubation step, aspirate blocking buffer using vacuum or a pipette.
6. Wash the slides with 5 ml washing buffer by incubating the tray for 5 min at 4 °C on a shaker set at 50 rpm (circular shaking).
7. Aspirate the buffer using vacuum or pipette.
8. Add 5 ml serum or plasma sample diluted (1:150 or 1:500 or project specific optimized dilution) in washing buffer without touching the slide surface.
9. Incubate the tray for 90 min at 4 °C on a shaker set at 50 rpm (circular shaking).
10. Aspirate the sample using vacuum or pipette.
11. Wash each array with 5 ml washing buffer with gentle shaking on a shaker set at 50 rpm for 5 min at 4 °C. Aspirate the washing buffer.
12. Repeat wash step four more times using fresh washing buffer each time to obtain a total of five washes.
13. Prepare detection antibody by mixing 2.5 μl Alexa Fluor® 647 goat anti-human IgG antibody with 5 ml washing buffer per array to obtain a final antibody concentration of 1 μg/ml. Store on ice until use.
14. Add 5 ml Alexa Fluor® 647 antibody solution to the incubation tray.
15. Incubate the tray for 90 min at 4 °C on a shaker set at 50 rpm (circular shaking).
16. Aspirate the antibody solution.
17. Wash each array with 5 ml washing buffer with gentle shaking on a shaker set at 50 rpm for 5 min at 4 °C. Aspirate the washing buffer and repeat wash four more times.

3.2.2 Drying of Slides and Scanning

1. Remove slides from the 4-chamber incubation tray by inserting the tip of the forceps into the indentation at the numbered end of the slides and gently pry the array upward pick up the array by holding the array by its edges only.
2. Insert the array into a slide holder and quickly rinse by dipping the slides into a large beaker filled with deionized water five times. It is important to properly place the slides in the slide holder to prevent damage to the array during centrifugation.

3. Immediately centrifuge the array in the slide holder or 50 ml conical tube at 200 × *g* for 1 min in a centrifuge (equipped with a plate rotor, if you are using the slide holder) at room temperature. Ensure the array is completely dry.
4. After drying, store the arrays vertically or horizontally in a slide box protected from light and avoid prolonged exposure to light. To obtain the best results, scan the array within 24 h of probing.
5. To scan the array, start the appropriate array acquisition and analysis software on the computer connected to the fluorescence microarray scanner (GenePix 4000B Microarray Scanner or equivalent).
6. Follow the instrument manufacturer's protocol to scan the slides with the following setting: Wavelength: 635 nm, Pixel Size: 10 μm, PMT Gain: 700, Lines to Average: 1.0 Laser Power: 100 %, Focus Position: 0 μm.
7. Save the image to a suitable location as "multi-image TIFF" file.

3.2.3 Data Generation

1. Use GenePix® Pro microarray data acquisition software or equivalent on the computer. Open the saved image (.tiff) and open the .GAL files downloaded from ProtoArray® Central (Life Technologies, Carlsbad, CA).
2. Adjust the subarray grid to ensure the grid is in proper location for each subarray. After the grid is properly adjusted and all features are aligned save .GPS file.
3. Once the gridding is completed acquire the pixel intensity data for each feature by clicking the Analyze button in GenePix® Pro, and save/export the results as a .GPR (GenePix® Results) file.

3.2.4 Data Analysis (Using Prospector Analyzer®, Life Technologies)

1. Install ProtoArray® Prospector (www.invitrogen.com/protoarray).
2. Start ProtoArray® Prospector from the desktop icon. Set the Application to Immune Response Profiling (for serum samples), or Immune Response Profiling with Plasma (for plasma samples).
3. Select the Load and Analyze button from the Tool Bar to normalize single protein arrays by selecting .GPR files with immune response profiling option selected from Application pull down menu.
4. Single array analysis generates a list of human proteins having significant IgG antibodies against them.
5. After single array analysis, group normalize group of array data by grouping the arrays from same sample type (i.e., disease group or control).
6. Once multiple arrays of the same phenotype/class are group normalized perform group comparison analysis. This allows identifying significantly increased antibodies in case of disease or injury.

7. Group normalization provides normalized data for further analysis.
8. Once the result is generated, users can see what antibodies are increased in how many samples per phenotype (% increase) and Chebyshev's Inequality (CI) *P* value.

3.2.5 Data Analysis Using Significant Analysis of Microarrays (SAM)

1. The SAM software is available at http://www-stat.stanford.edu/~tibs/SAM/
2. Normalized protein array data output from Prospector Analyzer (Life Technologies, Carlsbad, CA) can be used.
3. Data should be cube root transferred as the software does not handle a wide range of data such as that from ProtoArray very well.
4. SAM allows performance of a number of analyses such as two class unpaired (control and treatment groups with samples from different patients), two class paired (samples before and after treatment from the same patients), multiclass (more than two groups with each containing different experimental units), time course (each experimental units is measured at more than one time point; experimental units fall into a one or two class design), etc.

3.3 Conventional ELISA Method (A Summary of Indirect ELISA is Presented in Fig. 1b)

1. Coat the plates by pipetting 50 μl protein/coating buffer mixture onto each well of Nunc-Immuno™ Plates (Thermo Scientific, Cat. no. 449824). Seal the plate with clear sealing film.
2. Store the plates at 4 °C at least overnight.
3. Dump the protein and the coating buffer out of the plates by dabbing it on a pad of absorbant paper stack. Briefly blot on paper to remove excess protein on the surface.
4. Wash the plates five times with PBST (1× PBS, 0.05 % tween 20).
5. To block nonspecific protein binding, coat wells with 100 μl per well 2 % milk in PBST. Allow the plate to incubate at room temperature for at least 1 h.
6. Add primary antibody as a positive control and standard or diluted serum in 2 % dry milk in PBST and incubate at 4 °C overnight or 1 h at room temperature.
7. Wash the plates five times with PBST with 200 μl buffer per well.
8. Without letting the plate go dry add the secondary antibody, Anti-IgG-alkaline phosphatase(AP) diluted 1:1,000 in 2 % milk in PBST.
9. Incubate for 1 h at room temperature and wash the plates five times with PBST.
10. Prepare developing reagent with PNPP tablets using manufacturer's protocol.
11. Add 75 μl of developing reagent per well and incubate the plates for 30 min.
12. Read the plate using a standard UV-visible microplate reader at 405 nm.

3.4 MSD ELISA

Protocol overview of MSD antibody ELISA.

1. Coat Hi Bind microwell plate with target protein diluted in PBS. If it is spot coat, pipet 5 µl of target protein and dry on plate. If it is solution, coat the wells by pipetting 30 µl of target protein per well.
2. Block with 150 µl per well of Blocking solution (either 5 %(w/v) Blocker A or 3 % nonfat dry milk in PBS) 1 h at RT with shaking (300–600 rpm).
3. Wash the plate once with PBS or PBST.
4. Perform a titration for serum dilution optimization by comparing different fold dilutions (1:10, 1:100, 1:500 fold dilution in sample dilution buffer) e.g., 1 % Blocker A in PBS-T, Low Cross Buffer (Candor BioScience).
5. Seal the plate and shake for 1–2 h at RT.
6. Wash the plate three times with PBST.
7. Add 25 µl per well detection antibody+**(diluted in 1 % Blocker A, seal, and shake for 1 h at RT.

   +Concentration of detection antibody, e.g., 0.25 or 1 µg/ml.

   **Source of detection antibody, e.g., using a biotinylated version of the antibody you are using for ELISA in conjunction with an equal concentration of SULFO-TAG streptavidin or use the catalog MSD SULFO-TAG goat anti-human IgG).
8. Wash the plates three times with PBST.
9. Add 150 µl Read BufferT per well and read plate immediately using The SECTOR Imager 2400 reader or 6000 reader (Meso Scale Discovery, Gaithersburg, MD) following manufacturer's manual.

4 Notes

4.1 Protein Arrays

1. If multiple experiments are to be run for a project, it is very critical that all experiment conditions are maintained as consistent as possible. Especially, the incubation time, number and wash techniques and times, shaker speed, sample and antibody dilutions must be maintained consistently.
2. Proper laboratory precautions are critical such as wearing a laboratory coat, disposable gloves, and eye protection.
3. Temperature of the arrays and reagents should be kept at 2–8 °C throughout the experiment.
4. Never touch the surface of the array as it will scratch and damage surface of the array which will contribute to smears and scratches.
5. It is critical to keep the arrays wet all the time during the experiment. Make sure the slides are submerged in the appropriate reagent.

6. A clean dust free experiment location helps to reduce high background signals.
7. Arrays must be dried prior scanning and scanning must be performed immediately after the experiment is completed.
8. Protect the arrays from direct light after probing with the fluorescent detection reagent and before scanning.
9. *Probing other immunoglobulins such as IgA, IgM*: The Protoarry® design favors measurement of IgGs as the platform contains internal control IgGs that can be used for linear normalization. However, measurement of other immunoglobins using this platform is possible. The measurement of other immunoglobin requires, labeling of detection antibodies such as anti-human IgA, anti-human IgM by an Alexa Fluor tag. These antibodies and Alexa Fluor labeling kits are commercially available. Once the detection antibodies are labeled, the experiment described in this protocol are equally applicable to measure other antibodies other than IgG.
10. *Strengths*: ProtoArray enables us to assay antibodies against almost 10,000 human antigens in one single experiment. The method is robust, reproducible and has a potential to provide us with information that otherwise would have taken years by conventional low throughput methods. This sophistication has provided researchers a quick screening of reactive antigens and move on to validation steps which consists fewer number of antigen–antibody pairs.
11. *Issues*: The arrays and reagents involved are expensive. There is no way to determine specificity of antibody–antigen. Even though it includes almost 10,000 antigens it still doesn't provide information on reactivity of other remaining thousands of antigens.

4.2 ELISA

4.2.1 Conventional ELISA Method

1. It is important to determine the appropriate dilution of protein in coating buffer to achieve desired protein concentration.
2. Coated plates can be kept for a prolonged period up to 12 months at 4 °C. However, it is recommended to assess quality of the plate using a positive control.
3. It is important that once plates are coated the plates are never allowed to sit dry for more than a minute or two as this could disrupt binding of protein with the surface at the bottom of the well. If there is a possibility of either of the blocking buffer or the primary antibody remaining on the plates for more than a week, use 0.1 % sodium azide.
4. *Strength*: It is simple to set up and does not require specific equipment. Reagents and scanner are readily available.
5. *Issues*: Since protein arrays use fluorescence detection system, discovery made by protein array is difficult to reproduce using conventional chromophores AP or HRP. Since the detection is not sensitive it requires relatively concentrated sample which contributes to high background.

4.2.2 MSD ELISA Method

1. MSD method is preferred for validation because of its sensitivity to low abundance analytes and its electrochemiluminescence detection system.
2. While coating standard plates, the coating buffer is PBS + 0.03 % Triton X-100. No Triton should be added to the PBS coating buffer for spot coating standard plates or for solution coating high bind or standard plates.
3. The amount of protein to be tested for optimal amount is 10, 40, 160 ng protein per well.
4. *Strength*: Because of its electrochemiluminescence detection system it is sensitive and is compatible with ProtoArray detection system. MSD provides multiplexed ELISA option that enables users to perform ELISA on as many as ten antibodies on a single plate thereby saving time and reagents to be used in the assays.
5. *Issues*: It requires specific reagents compatible with electrochemiluminescence detection system. The scanner for this detection system is expensive and may not be available to many researchers readily.

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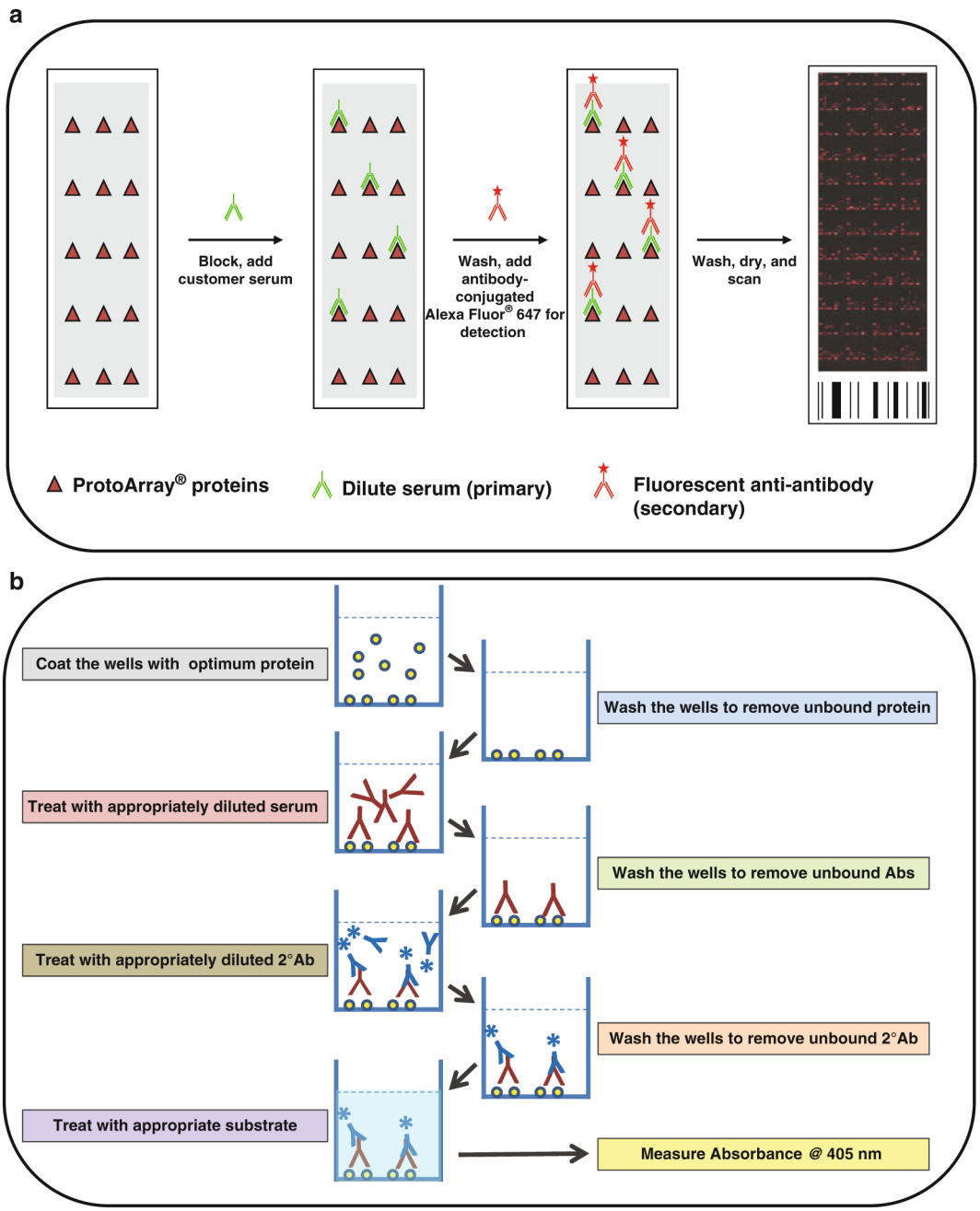

Fig 1.

Overview of ProtoArray methodology. A summary of the probing method is presented in (**a**). The indirect ELISA is shown in (**b**)

Table 1

Recipe to prepare blocking buffer and wash buffer using 10× Synthetic Block

Blocking Buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 0.08 % Triton ® X-100, 25 % Glycerol, 20 mM Reduced glutathione, 1× Synthetic Block, 1 mM DTT) *5 ml buffer required per microarray*.	*Washing Buffer* (1× PBS, 0.1 % Tween 20, 1× Synthetic Block) *60 ml buffer required per microarray*.
1 Prepare 50 ml Blocking Buffer fresh as follows: 1 M HEPES, pH 7.5, 2.5 ml 5 M NaCl, 2 ml 10 % Triton ® X-100, 0.4 ml 50 % Glycerol, 25 ml Reduced glutathione, 305 mg 10× Synthetic Block 5 ml Deionized water to 50 ml **2** Adjust pH to 7.5 with NaOH. **3** Mix reagents, chill to 4 °C, and add 50 µl of 1 M DTT prior to use. **4** Use buffer immediately. Store any remaining buffer at 4 °C for <24 h.	**1** Prepare 600 ml washing buffer fresh as follows: 10× PBS 60 ml 10 % Tween 20 6 ml 10× Synthetic Block 60 ml Deionized water to 600 ml **2** Mix reagents and cool to 4 °C. **3** Use buffer immediately. Store any remaining buffer at 4 °C for <24 h.