Chapter 15

High-Density Antigen Microarrays for the Assessment of Antibody Selectivity and Off-Target Binding

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Abstract

With the increasing availability of collections of antibodies, their evaluation in terms of binding selectivity becomes an important but challenging task. Planar antigen microarrays are very suitable tools to address this task and provide a powerful proteomics platform for the characterization of the binding selectivity of antibodies toward thousands of antigens in parallel. In this chapter, we describe our in-house developed procedures for the generation of high-density planar antigen microarrays with over 21,000 features. We also provide the details of the assay protocol, which we routinely use for the assessment of binding selectivity of the polyclonal antibodies generated within the Human Protein Atlas.

Key words Affinity proteomics, Protein microarrays, Antigen microarrays, Antibody selectivity

1 Introduction

Antibodies are utilized in a very broad spectrum of proteomics applications such as immunoassays, Western blotting, immunofluorescence, and immunohistochemistry analysis. The wide use and applicability of such affinity-based proteomics approaches is very much dependent on the availability of well-characterized affinity binders and antigens [1, 2]. Thus, in addition to the access to large collections of antibodies, availability of tools for the assessment of antibody-binding characteristics plays a very important role.

The Human Protein Atlas represents one of the large-scale efforts and resource, which produces antibodies on a proteomic scale toward all representative products of protein-coding human genes [3]. It uses recombinantly produced fragments of human proteins as antigens for the generation of affinity-purified rabbit polyclonal antibodies. These protein fragments are 50–150 amino acids in length and are designed in silico as unique representations of their corresponding proteins with lowest homology to the rest of

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the proteome [4]. They are sequence-verified and contain an N-terminal hexa-histidine albumin-binding protein tag (His_6ABP) for purification and solubility purposes [5].

All antibodies produced within the Human Protein Atlas are validated using multiple approaches to ensure that the antibodies bind to their intended target [6]. One of these approaches is based on the generation and use of protein fragment microarrays [7]. These routinely produced antigen microarrays host 384 protein fragments per subarray and allow for characterization of antibodies in parallel for their binding profile over 384 random antigens including the intended targets of the profiled antibodies. Besides, upon adjustment of assay conditions, their utility has been demonstrated for autoantibody profiling of body fluid samples in the context of autoimmune conditions such as multiple sclerosis [8].

In the following protocol, we describe the procedure for the generation and use of microarrays with a much higher antigen content, which host 21,120 features corresponding to 16,728 unique human protein fragments and representing 12,412 unique Ensembl Gene IDs [9]. Using these high-density protein fragment microarrays, we generated the binding profile of two polyclonal rabbit antibodies (Fig. 1). Here, the profile of Antibody-A revealed several off-target interactions whereas for Antibody-B only the binding to the intended target was observed. As demonstrated by these two examples, such high-content antigen microarrays offer a very valuable tool for a highly multiplex evaluation of the off-target binding characteristics of antibodies. Furthermore, by adjusting the described assay protocols, they can be easily adapted for the analysis of the autoantibody repertoire in body fluid samples.

2 Materials

2.1 Production of Planar Antigen Microarrays

1. Microarray printer: ArrayJet Marathon (ArrayJet Ltd.).
2. 384-well microplates: JetStar Microarray-Specific 384 Microplates (ArrayJet Ltd.).
3. Plate lids: JetGuard Probe Protector (ArrayJet Ltd.).
4. Microarray substrate: Epoxy activated glass slides (OPEpoxySlide, CapitalBio Corp.).
5. Antigens: His_6ABP-tagged human protein fragments stored in 0.1 M urea at a concentration of 0.8 mg/mL.
6. Printing buffer: 0.05 M carbonate-bicarbonate buffer, pH = 9.6 (Medicago AB) supplemented with 49.3% glycerol (Merck KGaA).
7. Blocking buffer: Phosphate-buffered saline buffer (1 × PBS), pH = 7.4, supplemented with 0.1% (v/v) Tween-20 (BDH

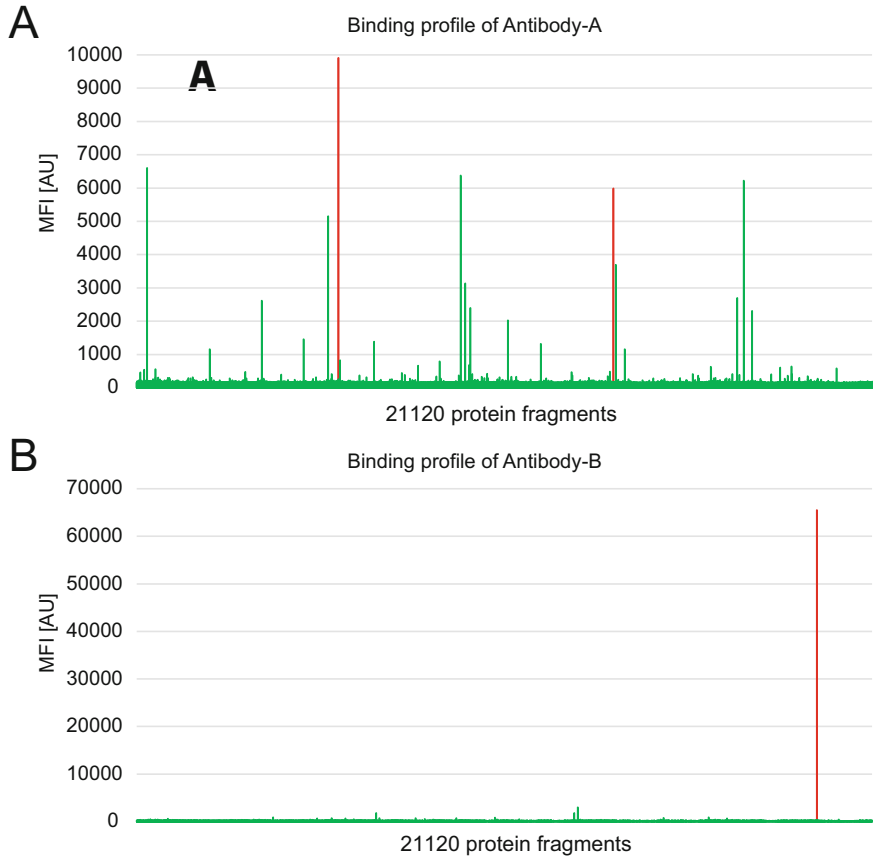

Fig. 1 Binding profile of two polyclonal rabbit antibodies on 21,120 protein fragments. (**a**) The intended target for Antibody-A is present as dual features in the array and is shown as red bars, while multiple off-target antigens are seen as green bars. The fluorescence intensities shown in this plot lie within the dynamic range of the scanner and the relative relationship between the on-target and off-target interactions can be inferred. (**b**) The intended target for Antibody-B is present as a single feature in the array and is shown as a red bar, while almost no off-target antigens can be seen. Here, the fluorescence intensity for the on-target binding reaches the saturation level of the scanner, however due to the lack of any off-target interactions no further adjustments are necessary for neither the antibody concentration nor the scanner settings

Prolabo) (1 × PBS-T), and 3% bovine serum albumin (BSA) (Saveen Werner AB).

8. Wash buffers: 1 × PBS and 1 × PBS-T.

2.2 Assay Procedure on Planar Antigen Microarrays

1. Slide coverslip: 85 μL LifterSlip® (Erie Scientific LLC) (*see* **Note 1**).
2. Orbital shaker (ELMI DOS-10 L).
3. Assay incubation buffer: 1 × PBS-T supplemented with hen anti-His_6ABP IgY (Agrisera) at a concentration of 312.5 ng/mL.
4. Assay wash buffers: 1×PBS and 1×PBS-T.

5. Secondary antibody solution: 1×PBS-T supplemented with goat anti-rabbit IgG Alexa Flour® 647 (Molecular Probes®) for the detection of the analyzed primary rabbit antibody and goat anti-hen IgY Alexa Flour® 555 (Molecular Probes®) for the detection of the hen anti-His_6ABP antibody, both at a concentration of 67 ng/mL.

2.3 Image Analysis of Planar Antigen Microarrays

1. Microarray scanner: Agilent G2505C (Agilent Technologies Inc.).
2. GenePix Pro 5.1 (Molecular Devices LLC).

3 Methods

3.1 Printing of Planar Antigen Microarrays

Printing of planar antigen microarrays can be performed using arraying robots that are based on different principles for the deposition of the antigens on the slides. The requirements for printing buffers and post-arraying procedures can therefore vary depending on the type of arrayer that is used. In the following, we describe the arraying procedure using an ArrayJet Marathon printer that deposits drops through the use of an inkjet printhead. This makes it suitable to use with glycerol-based buffers, which have higher viscosity than water. Please note that other arrayers might not be compatible with glycerol-based buffers.

1. Prepare the printing buffer by dissolving one carbonate-bicarbonate tablet in 42 mL MilliQ water before adding 58 mL of 85% glycerol (by first dissolving the carbonate-bicarbonate tablet in water, the dissolving process is sped up). Once the buffer is prepared it should be stored at 8 °C and preferably used within 1 week.
2. Dilute the antigens to 0.08 mg/mL in printing buffer into 384-well microplates and store the prepared plates at −20 °C until arraying.
3. Allow the plates to reach 20 °C prior to arraying procedure. Ensure that the diluted antigens in the plate wells are free from air bubbles by vortexing the plates and spinning them down in a plate centrifuge before placing the plates together with the glass slides in the arrayer (*see* **Note 2**).
4. Ensure that the temperature and humidity in the arrayer stabilize at 20 °C and 50%, respectively, before starting the printing process.
5. Array the antigens in a hexagonal lattice with a horizontal gap and pitch of approximately 0.2 mm. The arrayed antigens will produce features up to 0.1 mm in diameter. The extra space between the features minimizes merging and ensures a good separation between the features.

6. Remove the slides from the printer after arraying and allow the deposited drops to dry by placing the arrayed slides into a heat cabinet at 37 °C for 16 h. If the slides are not dried properly, tailing and bleed-off of the features can occur during the following blocking step.
7. Block the arrayed slides by gently submerging them in the blocking buffer for 1 h on an orbital shaker at 85 rpm.
8. Wash the blocked slides by submerging them in 1 × PBS-T for 5 min on an orbital shaker at 85 rpm, repeat this once. Wash with 1 × PBS to wash away any residual Tween-20 and finish the washing process by a quick rinse in deionized water to remove any residual salts. Spin-dry the slides before storing them in air-tight containers at 8 °C.

3.2 Assay Procedure

Here we describe an assay using a dual color detection system where one fluorophore channel is used for detection of the primary antibody that is being profiled, and the other channel is used for detection of the common tag for visualization of the features in the array.

1. Dilute the primary antibody in the assay incubation buffer (*see* **Note 3**).
2. Remove the slides from the 8 °C storage and allow them to reach room temperature.
3. Apply the coverslip to the slide so that the raised edge on the coverslip separates the coverslip from the glass slide. Also ensure that the coverslip is centered over the arrayed area on the slide.
4. Carefully deposit 85 μL of diluted primary antibody sample by placing individual drops of sample along the edge of the coverslip, so that it can be drawn under the coverslip by capillary forces (*see* **Note 4**). Once the entire volume has been applied, allow at least 1 h for the antibody to bind to the antigens (*see* **Note 5**).
5. Remove the coverslip from the slide (*see* **Note 6**). Wash the slide for 5 min by placing it in a small trough on an orbital shaker at 85 rpm and applying 15 mL of 1 × PBS-T, or enough to cover the entire slide. Discard the used 1 × PBS-T and repeat the wash once.
6. Submerge the entire slide in 15 mL of secondary antibody solution, similar to the previous wash, and incubate the slide under the dark for 1 h.
7. Wash the slide twice for 5 min in 1×PBS-T as in **step 5**. Remove any remaining Tween-20 by washing once in 1×PBS for 5 min, followed by a quick rinse in deionized water to remove any remaining salt. Spin-dry the slide before scanning.

8. Ensure that the slide is free from dust before placing it in the scanner. Care should be taken to only handle the slide by the edges to prevent any fingerprints to being transferred to the slide as it is placed in the slide holder in the scanner. Scan the slide with the maximum settings on the photomultiplier tube (and laser intensity if the scanner allows for this adjustment) and adjust as necessary to bring the recorded signals within the detectable range for the scanner. A suitable resolution for arrays with features of a diameter of 0.1 mm is 10 μm per pixel. This will result in approximately 80 measurements per feature as a basis for the reported values. Ensure that the setting "split/rotate" is set to "Yes" before starting the scan.

3.3 Image Analysis

1. Import the false-color images obtained from the scanner in to GenePix Pro 5.1 and assign a color to each image.
2. Load the array list and manually align it roughly to the array before using the auto-aligning function. Adjust individual features as needed before analyzing and saving the results (*see* **Note** 7).
3. Single result files can be opened, analyzed, and plotted in Microsoft Excel or similar software. However, if multiple files are to be handled simultaneously, or if the dataset is large, a statistical computing and graphics software, such as R-project, is recommended.
4. For the selectivity assessment of an antibody a simple bar plot, such as seen in Fig. 1, is often informative regarding the overall number and degree of on-target and off-target interactions.

4 Notes

1. The use of a coverslip is recommended if the array is spatially large enough to cover the entire slide. For smaller arrays, which can be arrayed in multiple sub-arrays, other masks and slide-holders allowing to compartmentalize the sub-arrays can be preferable.
2. Always take care to only handle the slides using gloves and/or tweezers and only by touching them by their edges or their barcode area, if the slides are barcoded. Also take care not to damage the barcode if tweezers are used as this might prevent the barcode from being read properly by the microarray scanner.
3. A final antibody concentration of at least 0.5 μg/mL is recommended, although the optimal concentration will vary depending on the properties of each antibody. If saturation of the fluorescence signal occurs during scanning, the signal

amplification by the photomultiplier tube (or the intensity of the lasers if the scanner allows for it) can be adjusted to bring the signals into the detection range of the scanner.

4. Before applying the coverslip to the slide, ensure that both the coverslip and the slide are free from dust and dirt. If dust or dirt is present, the sample will not be drawn in under the coverslip evenly and air might become trapped under the coverslip, preventing parts of the array to be exposed to the sample.
5. If the ambient humidity is low, or if a longer incubation time is used, the use of a humidity chamber is advisable as evaporation of the sample might adversely affect the results, or make it difficult to detach the coverslip from the slide.
6. This step can be performed by filling a 50 mL Falcon tube with the wash buffer and carefully dipping the slide in the wash buffer. The coverslip will start to slide off after a short while.
7. A GenePix Array List file (GAL-file) can be generated by either the arrayer, the GenePix software, or manually produced. Refer to the manual of either the arrayer or GenePix, or to instructions on Molecular Devices Knowledge Base (www.mdc.custhelp.com) for further information.

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