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Multiplexed Detection of Antibodies using Programmable Bead Arrays

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Summary

The detection of antibodies in sera has broad applications for detection and monitoring of infectious diseases, autoimmunity, and cancer. Proteomic methods of antigen detection, such as protein microarrays, are excellent clinical discovery tools, but due to both cost and specialization of manufacture, these are limited to screening small numbers of sera. Downstream assays for biomarker validation studies require rapid, reproducible, multiplexed assays for the simultaneous screening of fewer (<100) antigens with hundreds or thousands of sera. Traditional clinical ELISA assays use recombinant proteins, but these are limited by the ability to purify proteins free of cross-reacting contaminants and are limited to one antigen at a time. Here, we describe the application of coupled in vitro protein production with anti-tag capture onto bead arrays, for the rapid multiplexed detection of antibodies in sera. These assays can be readily adapted for detection of any protein-specific infectious, autoimmune, or cancer-specific antibodies.

Keywords

Bead array; Luminex; autoantibodies; biomarkers; ELISA

1. Introduction

The induction of antibodies in sera is a highly specific response to changes in protein structure and content. Antibodies to target antigens are well defined for infectious agents, but can be generated to autoantigens in the setting of clinical autoimmunity and cancer-derived proteins (reviewed in(1)). However, for most infectious and autoantigens, detection of the full breadth and specificity of the immune response has been limited by the ability to generate and purify hundreds, or thousands of protein assays for serologic detection. The recent development of proteomic technologies, including protein microarrays, phage display, and reverse-phase protein arrays, have the potential for proteome-wide immune monitoring. These assays have all been used to identify serum antibodies to infectious, autoimmune, and cancer antigens (2-5). However, these assays are generally limited to antigen discovery, as they are both labor-intensive and expensive for high-throughput clinical monitoring of immune responses.

When immunogenic antigens are identified using proteomic technologies, it is necessary to validate those select target antigens using larger serum (or plasma) sets. Currently, the standard ELISA assay is the primary assay for the confirmation of antibody biomarkers. ELISA requires recombinant protein expression and purification, and detects antibodies to one antigen at a time. For multiplexed analysis, recombinant protein antigens can be directly

or indirectly coupled onto spectrally-distinguishable fluorescent beads (Luminex)(6, 7) which can theoretically detect up to 100 antibodies per serum sample, although cross-talk and interference with multiplexed assays remains a concern. As with ELISA assays, these assays require antigen production and purification (8).

Here, we describe two related assays for the high-throughput detection of antigen-specific antibodies in patient sera, RAPID ELISA(9) and bead-array ELISA (10). Both assays use rabbit reticulocyte lysate for the in vitro transcription/translation of target antigens from plasmids and can be readily coupled to ORFeome-derived vector systems. Antigens are expressed in vitro and captured via anti-tag antibodies onto plates (RAPID ELISA) or fluorescent beads (Luminex platform). While RAPID ELISA can be used for the detection of one antigen-specific antibody at a time, the bead array allows for multiplexed detection of up to 100 individual antigens from one serum sample (see Note 1).

2. Materials

2.1. In vitro protein expression

1. DNA preparation systems (Qiagen, Valencia, CA or Nucleobond, Clontech, Mountain View, CA)
2. TNT T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI)
3. RNase Out (Invitrogen, Carlsbad, CA)

2.2. RAPID ELISA (96-well format)

1. PBS-T/milk blocking buffer: PBS, 5% fat-free dried milk, 0.2% Tween20, pH 7.4
2. Serum samples
3. SuperBlock blocking buffer (Pierce, Rockford, IL)
4. GST-coated 96-well detection module plates (GE Healthcare, Piscataway, NJ)
5. Mouse anti-GST (26H1) MAb (Cell Signaling Technology, Beverly, MA)
6. Goat anti-human IgG-HRP (Jackson ImmunoResearch, Westgrove, PA)
7. Sheep anti-mouse IgG-HRP (GE Healthcare, Piscataway, NJ)
8. SuperSignal ELISA Femto (Pierce, Rockford, IL)
9. Chemiluminescence Plate Reader (PerkinElmer Victor3, Waltham, MA)
10. Plate shaker (Eppendorf Thermomixer R, Westbury, NY)

2.3. Bead-based Multiplexed ELISA

1. 50 mM MES, pH 5.0
2. PBS-BN: PBS, 1% BSA, 0.05% sodium azide, pH 7.4
3. PBS-T: PBS, 0.05% Tween20, pH 7.4
4. PBS-1% BSA, pH 7.4
5. Sulfo-NHS

¹The bead-array ELISA(10) and the RAPID ELISA(9) have comparable sensitivities, specificities, and limits of detection as standard protein ELISA for the detection of antibodies in sera to the viral antigen EBNA-1 and the tumor antigen p53. The bead-array ELISA is comparable in sensitivity as direct covalent attachment of recombinant p53 protein to the microspheres.

6. EDC
7. Slide-a-Lyzer 10K Dialysis cassette
8. Goat anti-GST antisera (GE Healthcare, Piscataway, NJ)
9. Rabbit anti-FLAG antisera (Sigma-Aldrich, St. Louis, MO)
10. Goat anti-mouse IgG-PE antisera (Jackson ImmunoResearch, Westgrove, PA)
11. Mouse anti-GST MAb clone 26H1 (Cell-Signaling Technology, Beverly, MA)
12. Goat anti-human IgG-biotin (Jackson ImmunoResearch, Westgrove, PA)
13. Streptavidin-R-Phycoerythrin (Invitrogen, Carlsbad, CA)
14. Carboxylated SeroMap microspheres (see Note 2, Luminex, Austin, TX)
15. Copolymer microfuge tubes (USA Scientific, Ocala, FL)
16. 1.2 μ m MultiScreen HTS BV filter plate, (Fisher Scientific, Pittsburgh, PA)
17. Sonicator (Cole Parmer, Vernon Hills, IL)
18. Benchtop tube rotator (Fisher Scientific, Pittsburgh, PA)
19. Vacuum manifold (Millipore, Billerica, MA)
19. Luminex 200 machine (Luminex, Austin, TX)

3. Methods

Both assays described here, the plate-based RAPID ELISA, and the bead-array ELISA, involve the *in vitro* production of recombinant protein generated immediately prior to use (see Note 3). These proteins are produced from T7-based plasmid vectors using an optimized *in vitro* transcription/translation system, and captured onto anti-GST plates (RAPID ELISA, Figure 1A) or anti-GST-coupled fluorescent beads (bead-array ELISA, Figure 1B). Diluted sera are added, bound IgG is washed and secondary PE-labeled anti-IgG detection antibodies are added. Bound IgG is detected with a luminometer for RAPID ELISA, or with a Luminex 200 instrument for the bead-array ELISA.

Classical bead-based (Luminex) arrays rely on covalent crosslinking of antigen to reactive moieties on the bead surface. Each antigen is then ‘addressable’ based on bead color. In this bead-array ELISA, anti-tag antibodies (i.e. GST) are first directly coupled to the beads, and the same antibody is coupled to all the bead colors required for the assay. Both GST and FLAG-tagged proteins can be captured and detected on the beads with this approach. Confirmation of coupling efficiency is confirmed with anti-Ig secondary antibodies. Anti-GST coupled beads are prepared in advance, and are stable for at least one year. Proteins are captured onto individual beads, pooled for multiplexed detection, and aliquoted for serologic assays.

²There are different chemical compositions of Luminex microspheres. This assay uses the SeroMap microspheres, but also works with magnetic microspheres, with comparable sensitivities and specificities as the SeroMap microspheres.

³Protein expression with IVTT can vary from batch to batch. These studies have been optimized for the pCITE-c-terminal GST vector. FLAG-based vectors have also been tested, but not as extensively. Over 100 tumor and viral antigens have been expressed and captured using this assay on Luminex beads, with over 90% of the antigens expressed as determined by GST detection. Both high-quality DNA, and careful use of RNase-free pipets and filter tips are needed for efficient protein expression. Batches of rabbit reticulocyte lysate are tested and ordered in bulk.

3.1 In vitro transcription/translation of recombinant proteins

3.1.1. DNA preparation—Highly-purified plasmid DNA is required for effective in vitro transcription/translation (IVTT). For this protocol, a modified T7-based pCITE vector optimized for protein expression was used. This vector contains *att* sites for subcloning using the Invitrogen Gateway system and encodes a c-terminal GST fusion gene (plasmid available from <http://www.hip.harvard.edu>)(11). Creator-based vectors (Clontech) and FLAG-tagged vectors also have been tested (see Note 3). Standard DNA maxipreps (either Qiagen or Nucleobond), or high-throughput DNA preparations(12) were used.

3.1.2. DNA precipitation (if not already done)

1. For one well, transfer 300 μ l DNA (at least 2 μ g) to microfuge tube.
2. Add 30 μ l 3M sodium acetate (10% final).
3. Add 240 μ l isopropanol (80% final). Vortex.
4. Spin 5 min at 18,300 (18.3K) *g*. Decant.
5. Add 500 μ l 80% ethanol. Vortex, spin 5 min at 18.3K *g*, and decant.
6. Leave uncapped to dry.
7. Resuspend pellet with 100 μ l DEPC-treated water.
8. Spin briefly and quantify at OD₂₈₀ nm.

3.1.3. In vitro transcription/translation

1. The recombinant proteins are prepared fresh on the day of the ELISA assay.
2. Remove components of reticulocyte lysate kit and RNase Out from freezers. Thaw lysate, RNA Polymerase and RNase Out on ice. Thaw other components at room temperature (RT) and place on ice. (See Note 4).
3. Set water bath to 30°C.
4. For each well of the final RAPID ELISA assay, transfer 500 ng of DNA to microfuge tube. Bring all DNA samples to equal volumes in DEPC water.
5. Keep reaction on ice. For a master mix, add to 1 tube reticulocyte lysate (200 μ l), 16 μ l reaction buffer, 8 μ l polymerase, 4 μ l AA mix –Met, 4 μ l amino acid mix –Leu, 8 μ l RNase Out, and DEPC water for a total volume of 400 μ l when mixed with DNA.
6. Add reticulocyte lysate mix to each tube of DNA (25 μ l total/well, including DNA).
7. Incubate DNA/lysate at 30°C for 1.5 hrs.

3.2 RAPID ELISA (96-well format)

1. Block wells of GST-coated 96-well plates with PBS-T/milk overnight at 4°C. Blocking at RT for 4 hours is also effective. (See Note 5).

⁴The cost of RAPID ELISA and the multiplexed bead-array ELISA is approximately \$10/antigen, due to the high cost and volume required of IVTT reagent. Dilution of the IVTT greater than 1:2 lowers the sensitivity of detection, but could be used for high-titer antibody detection. Increasing the DNA concentration beyond 500-1000 ng/reaction had little effect.

⁵Some sera (<10%) have persistently high background in these assays. Multiple blocking reagents have been tested on the RAPID ELISA, including goat sera and rabbit sera, and commercial ELISA blocking reagents. Of these, addition of PBS-T/milk after protein expression and capture (prior to sera addition) overnight can decrease the background.

2. Prepare recombinant proteins from IVTT preparation (see 3.1.3). Add 1X PBS to each tube for a final volume of 50 μ l per well. Transfer 50 μ l to each well and seal plate. Each serum is tested in duplicate.
3. Incubate at RT for 2hrs while shaking at 800 rpm on an orbital shaker. The shaking is important and speeds the reaction.
4. Wash 5X with 200 μ l PBS-T/milk per well.
5. Add 100 μ l human serum (diluted 1:300 in PBS-T/milk). Optimal dilutions are established for each assay, usually 1:100-1:600.
6. To test protein expression of GST fusions, add mouse anti-GST MAb, 1:1000 in SuperBlock to separate wells.
7. Incubate 1hr RT while shaking at 800 rpm on an orbital shaker.
8. Wash 5X with PBS-T/milk.
9. Add 100 μ l secondary antibody (anti-human-IgG-HRP for wells with human sera, 1:1000 in PBS-T/milk or anti-mouse-IgG-HRP for wells with mouse anti-GST, 1:1000 in SuperBlock). Incubate for 1hr at RT while shaking.
10. Wash 5X with 1X PBS.
11. Mix equal volumes each of solutions 1 and 2 from SuperSignal ELISA Femto kit. Add 100 μ l mixed solution to each well. Mix 1 min with shaker.
12. Detect by chemiluminescence on Victor3 plate reader or equivalent.

3.3 Bead-array ELISA

To screen for antibodies using a bead-array (Luminex), anti-GST antisera is first coupled to the individual beads and stored. Antigenic proteins are produced with IVTT as with the RAPID ELISA assay (3.1.3) and captured onto the individual beads prior to mixing for multiplexed analysis (see Note 6).

3.3.1 Preparation of antibodies for bead conjugation—The commercial anti-GST antibodies contain sodium azide, which needs to be removed first with dialysis.

1. Pre-wet dialysis cassette membrane with cold PBS.
2. Dilute antibody in 1 ml PBS.
3. Inject diluted antibody into cassette. Remove air.
4. Dialyze against 3X with 1L cold PBS over 12-24 hrs total.
5. Remove antibody from dialysis cassette.
6. Determine concentration by absorbance ($\text{mg/ml} = \text{UV}_{280}/1.6$)
7. Aliquot and store in -20°C .

3.3.2. Antibody coupling to Luminex microspheres—This assay has been optimized for the anti-GST antibody/bead ratios shown below. For other anti-tag antibodies, the antibody/bead ratios should be titrated.

⁶Up to 8 antigens have been multiplexed at one time using the bead-array ELISA. Theoretically, this assay could be used to multiplex up to 100 antigens (limited only by the number of spectrally-distinguishable microspheres).

1. Volumes listed below are for small scale (2.5×10^6 beads) and large scale [in brackets, 12.5×10^6 beads (1 ml)].
2. Dilute Sulfo-NHS to 50 mg/ml in sterile water, immediately prior to use.
3. Dilute EDC to 50 mg/ml in sterile water, immediately prior to use.
4. Equilibrate microspheres to RT. Rotate 3 min on benchtop rotator.
5. Transfer 2.5×10^6 [12.5×10^6] microspheres to copolymer microfuge tubes.
6. Wash microspheres by centrifuging at $18.3K \ g$ for 2 min. Remove supernatant and add 100 μ l [200 μ l] sterile water. Resuspend microspheres by vortexing 10 sec, sonicating 20 sec, and vortexing 10 sec. Centrifuge and remove supernatant.
7. Activate microspheres by resuspending in 80 μ l 100 mM monobasic sodium phosphate, pH 6.2.
8. Add 10 μ l [50 μ l] 50 mg/ml sulfo-NHS to microspheres. Vortex. Add 10 μ l [50 μ l] 50 mg/ml EDC to microspheres. Vortex.
9. Incubate 20 min at RT. Vortex 10 sec every 10 min.
10. Wash microspheres 2X with 250 μ l [500 μ l] 50 mM MES, pH 5.0.
11. To couple the microspheres, resuspend in 100 μ l [200 μ l] 50 mM MES, pH 5.0.
12. Add 12.5 μ g [62.5 μ g] goat anti-GST antisera or rabbit anti-FLAG antibody.
13. Add 50 mM MES, pH 5.0 to final volume 500 μ l [1 ml].
14. Vortex 10 sec.
15. Incubate 2 hours, rotating at RT.
16. Wash microspheres with 500 μ l [1 ml] PBS-BN.
17. Incubate 30 min, rotating at RT.
18. Wash microspheres 2X with 1 ml PBS-T.
19. Resuspend in 250 μ l [1 ml] PBS-BN.
20. Count microsphere suspension with hemacytometer.
21. Store at 4°C wrapped in foil. Beads are stable for at least a year.

3.3.3. Confirmation of antibody coupling to microspheres—Prior to use in ELISA assays, the amount of anti-GST antibody that is covalently attached to the microspheres is confirmed using an independent anti-GST MAb. See Note 7 for technical notes on using the vacuum manifold to wash the microspheres and handling the filter plates. Beads of any fluorescent color can be used; colors in adjacent fluorescent spectra can be readily distinguished, and cross-reactivity was not observed.

1. Make 2-fold serial dilutions of donkey anti-goat IgG-PE detection antibody to total volume per reaction of 50 μ l.

⁷Using the vacuum to wash microspheres can be tricky. Turn on vacuum first before putting any plate on top. Set vacuum manifold to -6 in. Hg by placing solid bottom plate on top. Do not vacuum to dryness, otherwise beads will get stuck in filter (count to about 3 then turn off vacuum). For the filter plate, tape unused wells with 96-well plate sealing tape. Tape the filter plate on top of an upside down lid. Gently blot under drain of filter plate after last vacuum of each wash before adding or resuspending beads. Cover wells with a plate lid and wrap with foil to protect the beads from light when not adding reagents. Seal wells with tape if the incubation is overnight. Occasionally beads will get trapped in the filter plates (no bead counts during the assay). These can be rescued by resuspending the beads in the plates with vigorous shaking or vortexing.

2. Resuspend and aliquot coupled microspheres into a copolymer microfuge tube for 5000 microspheres/reaction. Dilute to 100 microspheres/ μ l in PBS-1% BSA (50 μ l/reaction).
3. Pre-wet filter plate with 100 μ l/well PBS-1% BSA. Vacuum.
4. Resuspend microspheres. Add 50 μ l coupled microspheres to appropriate wells in the filter plate.
5. Add 50 μ l diluted detection antibody to each well.
6. Incubate 30 min. at RT shaking at 750 rpm on an orbital shaker.
7. Wash wells 2X with 100 μ l PBS-1% BSA by vacuum.
8. Add 100 μ l PBS-1% BSA. Shake for 5 min at 750 rpm on an orbital shaker to resuspend beads.
9. Analyze with a Luminex 200 machine per manufacturer's instructions.

3.3.4. Bead-array ELISA, Day 1—The multiplexed bead-array ELISA is a two-day assay. Antigens are expressed with IVTT on the day of the reaction. For assay planning, sera are tested in duplicate, and protein capture onto the beads are tested in duplicate at the same time using separate wells, using anti-GST MABs. Controls include the vector control or any control GST fusion protein, including GST alone. Prior to use in ELISA assays, the amount of anti-GST antibody that is covalently attached to the microspheres is confirmed using an independent anti-GST MAB.

1. Express protein antigens by IVTT on the same day as the assay (section 3.1.3). Use 500 ng DNA per 25 μ l reaction.
2. Resuspend coupled microspheres and aliquot enough of the coupled microspheres into a copolymer microfuge tube for 5000 microspheres/reaction. Dilute to 100 microspheres/ μ l in PBS-1% BSA (should be 50 μ l/reaction).
3. Add 25 μ l antigen-expressed IVTT reaction to beads.
4. Mix IVTT protein and beads at RT for 2 hrs while rotating in dark.
5. Pre-wet filter plate with 100 μ l/well PBS-1% BSA. Vacuum and blot.
6. Spin tubes 2 min at 18.3K *g* to pellet the beads. Remove supernatant. Resuspend in 10 μ l PBS-1% BSA per reaction.
7. Mix beads in one copolymer microfuge tube to multiplex. Vortex 10 sec, sonicate 20 sec, vortex 10 sec. Add equal volumes of pooled beads to each well. There will be 5000 beads per antigen per well.
8. Wash 2X with 100 μ l PBS-1% BSA per well.
9. Resuspend in 50 μ l PBS-1% BSA per well. Shake 5 min at 750 rpm on an orbital shaker.
10. Dilute serum 1:80 in PBS-1% BSA and add 50 μ l serum to appropriate wells. Sera are tested in duplicate, and dilutions should be titered. More concentrated sera inhibits the reaction.
11. To test binding of GST protein to the beads, in separate wells add anti-GST MAB at 1:400 (5 μ g/ml).
12. Seal filter plate with tape, cover with lid, and wrap in foil. Incubate overnight at 4°C while shaking at 750 rpm on an orbital shaker.

3.3.5. Bead-array ELISA, Day 2

1. Shake plate at 750 rpm on an orbital shaker for 30 min at RT. Wash wells 2X with 100 μ l PBS-1% BSA.
2. Resuspend microspheres in 50 μ l PBS-1% BSA. Shake 5 min at 750 rpm on an orbital shaker.
3. For human serum samples, dilute biotin-labeled goat anti-human IgG antibody to 4 μ g/ml in PBS-1% BSA (1:375). Add 50 μ l to each well. Add 50 μ l PBS-1% BSA to wells for GST detection.
4. Incubate 30 min RT shaking. Wash 2X with 100 μ l PBS-1% BSA.
5. Resuspend microspheres in 50 μ l PBS-1% BSA. Shake 5 min at 750 rpm on an orbital shaker.
6. For human serum samples, dilute streptavidin-R-PE to 4 μ g/ml in PBS-1% BSA (1:250). For GST detection wells, add goat anti-mouse IgG-PE to 1:200. Add 50 μ l to each well. (See Note 8).
7. Incubate 30 min RT shaking. Wash 2X with 100 μ l PBS-1% BSA.
8. Resuspend microspheres in 100 μ l PBS-1% BSA. Shake until ready to analyze with Luminex, minimum of 5 min at 750 rpm on an orbital shaker.
9. Analyze with Luminex 200 instrument per manufacturer's instructions (Figure 2).

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⁸The assay presented here uses indirect labeling of bound human IgG with a biotinylated anti-human IgG secondary antibody and streptavidin-PE detection. Direct labeling of the secondary antibody with PE also works, but with decreased sensitivities. This can be overcome by overnight incubation of sera.

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Figure 1.

Schematic of RAPID ELISA and Bead-array ELISA for the detection of antibodies in serum. **A. RAPID ELISA.** cDNA encoding full-length antigens are expressed as tagged fusion proteins using rabbit reticulocyte lysate. The proteins are captured by anti-tag antibodies in individual wells. Human serum containing antigen-specific antibodies is added and detected with HRP-conjugated secondary anti-IgG antibodies. Reproduced with permission from Wiley Interscience(9). **B. Bead-array ELISA.** After expression with rabbit reticulocyte lysate, the recombinant proteins are captured onto anti-GST-coupled microspheres. The beads displaying different antigens are pooled and re-aliquoted prior to addition of human serum so that there are 5000 of each color/antigen bead per serum. Human IgG is detected with PE-conjugated secondary anti-IgG antibodies. Reproduced with permission from Elsevier (10).

Figure 2. Multiplexed detection of viral antibodies in human sera

Six individual GST-tagged protein antigens, p21, N-EBNA1, C-EBNA1, EBNA-3A, EBNA-3B, and LMP-2, were expressed, loaded onto individual anti-GST-coupled microspheres, and pooled. IgG-specific responses to the viral antigens were detected from six healthy donor sera (A-F). Differential patterns of IgG were readily detected with the bead array. Reproduced with permission from Elsevier (10).