Protein Microarrays for Parasite Antigen Discovery

Running title: Parasite Protein Microarrays

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Summary

The host serological profile to a parasitic infection, such as schistosomiasis, can be used to define potential vaccine and diagnostic targets. Determining the host antibody response using traditional approaches is hindered by the large number of putative antigens in any parasite proteome. Parasite protein microarrays offer the potential for a high throughput host antibody screen to simplify this task. In order to construct the array, parasite proteins are selected from available genomic sequence and protein databases using bioinformatic tools. Selected open reading frames are PCR amplified, incorporated into a vector for cell-free protein expression, and printed robotically onto glass slides. The protein microarrays can be probed with antisera from infected/immune animals or humans and the antibody reactivity measured with fluorophore labelled antibodies on a confocal laser microarray scanner to identify potential targets for diagnosis or therapeutic or prophylactic intervention.

Keywords Schistosomiasis; protein microarray; parasite; antibody/serum screening; vaccine and diagnostic discovery

1 Introduction

The serological profile of a parasitic disease, such as schistosomiasis, is the result of the interaction between the host's immune system and exposed parasite antigens. The recognition by and affinity of host antibodies for specific components of the parasite proteome indicates which antigens are accessible to the host immune response. When correlated with disease immunity or severity, these data can provide important information for vaccine and diagnostic target selection. Conventionally, antibody specificity and reactivity against native or recombinant antigens are measured using techniques such as ELISA or two-dimensional protein gels but these methods are difficult to adapt for high throughput screens (*1, 2*). Clearly, a protein microarray comprising hundreds to thousands of antigens which can be probed with antisera and individual antigen reactivity measured with a laser scanner is a highly efficient approach for quantifying the host antibody response (*3, 4*).

As with other pathogens, high throughput DNA sequencing and proteomics of schistosomes (*5, 6*) have provided rich sets of genomic, transcriptomic and protein data. These data, coupled with DNA microarray technologies and analysis methods, have enabled the development of schistosome and other parasite protein microarrays. However, compared with nucleic acid microarrays, there are available a wide and diverse range of protein microarray systems each with its own inherent strengths and weaknesses (*7*). The variables to be considered for construction of a protein microarray include: the source of protein used (e.g.

native extract, recombinant cellular or cell-free synthesis); whether the microarray is printed or is of *in situ* construction; the type of detection system; the microarray surface chemistry; whether an analytical, functional or reverse phase microarray is produced; and whether the microarray is manufactured commercially or in the laboratory.

Currently, we suggest researchers consider a purpose built microarray fabricated within the laboratory or with the assistance of a collaborator. The protein microarray can be made using standard 96-well format laboratory equipment, a commercial cell-free protein expression kit, nitrocellulose-coated glass slides and a microarray contact printer. We encourage researchers to seek specialist help when using a contact microarrayer although there are comprehensive reviews available (*8*). Here we present, as an example, details of our development and application of the first schistosome protein microarray (*2*). While the procedures described may require some modification dependent on, for example, the parasite species under consideration or the size of the protein microarray to be produced, the general methods we present are likely applicable for the construction of most parasite protein microarrays. The chapter describes the development of a parasite protein microarray comprising four steps: 1) Selection of target genes of interest for protein expression; 2) Production of the cDNA template; 3) Manufacture of the microarray; and 4) Scanning and probing of the microarray (**Figure 1**).

2 Materials

2.1 Protein selection, RNA purification and cDNA amplification

1. Data-mining software: e.g., TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), SignalP (http://www.cbs.dtu.dk/services/SignalP/), BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), interProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/)
2. Protein, genomic and transcriptomic datasets; e.g. NCBI (http://www.ncbi.nlm.nih.gov/genbank/), *Schistosoma japonicum* Genome Project (http://www.chgc.sh.cn/japonicum/), and SchistoDB (www.schistodb.net)
3. *Schistosoma japonicum* and *S. mansoni* lifecycle stages
4. Sterile homogeniser tips and cordless motor (Kimble 749521-1590)
5. TRIZOL (Invitrogen 15596-026)
6. RNeasy Minikit (Qiagen 74104)
7. Chloroform
8. Ethanol
9. 3M sodium acetate, pH 5.2
10. 1M Tris-HCl, pH 7.4
11. RNase-free water
12. RQ1 RNAse-free DNAse (Promega M6101) or equivalent
13. Bioanalyzer RNA Pico Kit (Agilent 5067-1513)
14. Bioanalyzer 2100 (Agilent)
15. QuantiTect Whole Genome Amplification kit (Qiagen 207043) or equivalent
16. Sensiscript Reverse Transcription kit (Qiagen 205211)

2.2 Polymerase chain reaction (PCR), recombination cloning, plasmid purification, cell-free protein expression and microarray printing

1. PCR primers specific for target sequences with adaptor sequences for homologous recombination in 96-well plate format (from a commercial supplier)

2. 96-well PCR plates
3. Reagents for PCR
4. 96-well PCR thermocycler
5. Reagents and equipment for agarose gel electrophoresis
6. Multichannel pipettes
7. Commercial or lab prepared chemically competent cells (e.g. DH5α or Top10)
8. Linearised vector for cell-free expression (e.g. pXT7, pXi, pIVEX or similar) (*see* **Note 1**)
9. Adhesive plastic sheet for 96-well plates
10. Super Optimal Catabolic (SOC) media (2% tryptone, 0.55% yeast extract, 10mM NaCl, 10mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, and 20mM glucose)
11. Luria-Bertani (LB) Media (1% tryptone, 1% NaCl, and 0.05% yeast extract)
12. 96-well flat bottom blocks for bacterial culture -2ml (19579 Qiagen)
13. Airpore Tape Sheets (19571 Qiagen)
14. QIAprep 96 Turbo Miniprep kit with vacuum manifold or equivalent
15. 96 well round-bottom plates
16. 384 well round-bottom plates
17. Cell-free protein expression kit (e.g. rapid translation system (RTS) 100 *E. coli* HY kits, 5 PRIME; or similar system such as Expressway, Invitrogen; EasyXpress, Qiagen)
18. Complete, mini, EDTA-free protease inhibitor cocktail tablets (Roche) or equivalent
19. Tween 20 (P1379 Sigma-Aldrich)
20. Microarray spotting robot including printing pins (e.g. OmniGrid family of microarrayers, Digilab)
21. 3, 8 or 16 pad nitrocellulose coated slides (Whatman or GraceBio Labs)

2.3 Protein microarray probing, scanning and data analysis

1. Array Blocking Buffer (Whatman 10485356)
2. *E. coli* lysate (from a commercial supplier)
3. FAST frame (Whatman 10486001)
4. FAST slide incubation chambers (Whatman 10486046)
5. TBST (20mM Tris HCl pH 7.5, 0.5M NaCl, 0.05% Tween 20) and TBS (20mM Tris HCl pH 7.5, 0.5M NaCl) washing buffers
6. EBNA1 recombinant protein or antibodies reactive against test sera for use as primary antibody positive control (from a commercial supplier)
7. Mixed species IgG for use as secondary antibody positive control (from a commercial supplier)
8. Biotin conjugated antibody reactive against antibodies or subtypes to be measured in test sera (from a commercial supplier)
9. Streptavidin conjugated Cy5 fluorophore (Surelight P3, Columbia Biosciences)
10. Laser microarray scanner (e.g Genepix 4300A, Molecular Devices or ScanArray, Perkin Elmer)
11. Scanner imaging software (e.g. Genepix Pro, Molecular Devices or ScanArray Express, Perkin Elmer)
12. Data analysis software (e.g. Excel, Microsoft; Bioconductor packages/R project for statistical computing, www.bioconductor.org/ www.r-project.org)

3 Methods

3.1 Protein selection, RNA purification and cDNA generation

It is a prerequisite that the researcher has access to genomic, transcriptomic or proteomic data for the parasite species under consideration from which to bioinformatically select a subset of protein-coding genes for microarray printing. A cDNA PCR template is prepared by isolating RNA from one or more parasite lifecycle stages, using standard techniques. The isolated RNA is affinity purified, treated with DNAse and the quality is assessed using a Bioanalyzer. Finally, cDNA is produced using the whole transcriptome amplification and reverse transcriptase kits. While there are many advantages to using cDNA as the PCR template, it is also possible to use genomic DNA (*see* **Note 2**) or phage libraries (*see* **Note 3**) with some modification of the methods we describe here.

3.1.1 Protein selection

The ideal protein microarray would have full proteome coverage; however, given the thousands of proteins expressed by most parasites, this is currently technologically and financially unfeasible. Therefore, genes must be selected using criteria suited to the particular aim of the project and the parasite species used. For vaccine discovery, as was the case with the schistosome protein microarray, genes were selected from available *Schistosoma japonicum* and *S. mansoni* gene and protein datasets on the basis of protein localisation, lifecycle stage expression, and sequence homology within schistosome and host species (*see* **Note 4**). Well established schistosome vaccine candidates and other characterised antigens were also included. Our final selection process was completed using standard bioinformatic tools and spreadsheet software; the details of this selection process, and other potential strategies, are beyond the scope of this chapter. The reader should refer to recent reviews on vaccine bioinformatics (*9*) for further information.

3.1.2 RNA purification

For specific details regarding parasite RNA isolation and purification, readers should refer to the excellent chapter in a previous volume of this series by Hoffmann and Fitzpatrick (*10*); note that a protocol that is best suited to individual need and the characteristics of the specific parasite should be selected. In brief, for schistosomes, we use a method combining guanidium thiocyanate-phenol-chloroform extraction (TRIZOL reagent) and affinity column purification (Qiagen RNeasy Minikit). The purified RNA is DNAse-treated (Promega) and then checked for quality by Bioanalyzer (Agilent). As many selected genes may not be universally expressed in male or female parasites or across all development stages, we recommend extracting and combining RNA from several parasite lifecycle stages of mixed sex to provide complete cDNA coverage for PCR amplification (*see* **Note 5)**.The basic procedure is as follows:

1. Mechanically homogenize pooled freshly collected parasites using a hand-held motor (Kimble) and disposable tips in TRIZOL as recommended by the manufacturer. It is important to ensure that there are sufficient parasites to provide a suitable yield of RNA for cDNA amplification (at least 10ng of total RNA).
2. Complete remaining steps for TRIZOL RNA isolation as per Hoffmann and Fitzpatrick (*10*) but with some modifications if necessary (*see* **Note 6**).

3. Remove the aqueous phase containing RNA and gently mix in a separate tube with an equal volume of 70% ethanol.
4. Transfer to a Qiagen affinity column. Centrifuge at room temperature for 15 seconds. Repeat for several aliquots if volume is larger than column capacity.
5. Complete remaining steps of RNA purification as described in Hoffmann and Fitzpatrick (*10*) and in the Qiagen kit instructions.
6. Resuspend RNA pellet in RNAse-free water following ethanol precipitation.
7. Check RNA concentration spectroscopically and store aliquots at -80˚C if not needed immediately.
8. Treat RNA samples with DNAse to remove all contaminating DNA as directed in the RQ1 RNAse-free DNAse protocol (Promega).
9. Mix 1-8 µL of RNA sample with 1µL RQ1 10x reaction buffer, RQ1 RNAse-free DNAse at 1unit/µg RNA, and RNAse-free water to a final volume of 10 µL. If sample contains less than 1µg RNA include only 1 unit of RQ1 DNAse.
10. Incubate at 37˚C for 30 minutes.
11. Add 1µL of RQ1 DNAse stop solution and incubate at 65˚C for 10 minutes.
12. Assess the quality of the isolated and purified RNA using a Bioanalyzer RNA Pico kit and 2100 Bioanalyzer (*see* **Note 7**). This method only requires 1µL of RNA sample.
13. Follow the detailed instructions for the RNA Pico kit carefully for consistent results, taking particular care to avoid bubbles when loading the Pico chip.
14. Ensure that all RNA samples have high RNA integrity number (RIN) values as determined by the Bioanalyzer software (*see* **Note 8**).

3.1.3 cDNA generation

If parasite RNA is limited or precious (e.g. in our case, RNA samples from schistosome miracidia, eggs or schistosomula lifecycle stages) a cDNA amplification step can be useful. Whole transcriptome amplification kits (QuantiTect WTA) use a combination of random and oligo-DT primers to amplify up to 40µg of cDNA from as little as 10ng of RNA (*see* **Note 9**). For higher yield or less precious RNA samples we used conventional reverse transcription kits (Sensiscript RT, Qiagen) with oligo-DT primers to generate cDNA (protocol not described here). Finally, equal concentrations of each cDNA source were mixed for the final PCR template. The summarized protocol for whole transcriptome amplification is as follows (see the QuantiTect manual for further details):

1. Prepare fresh RT mix as directed and add 5µL to ≥10ng RNA in 5µL nuclease-free water/TE buffer. Vortex and centrifuge.
2. Incubate at 37˚C for 30 minutes, stop reaction at 95˚C for 5 minutes, and cool to 22˚C.
3. Prepare fresh ligation mix as directed and add 10µL to RT reaction. Vortex and centrifuge.
4. Incubate at 22˚C for 2 hours.
5. Prepare fresh amplification mix as directed and add 30µL to the ligation reaction. Vortex and centrifuge.
6. Incubate at 30˚C for 8 hours (high yield reaction) and stop reaction at 95˚ for 5 minutes.
7. Quantify cDNA, diluting if necessary, and store at -20˚C.

3.2 PCR, recombination cloning, plasmid purification, cell-free protein expression and microarray printing

In contrast to the PCR amplification and plasmid purification methods that are standard, variations are possible for the cloning (*see* **Note 10**) and protein expression steps. While the DNA template for cell-free protein expression can be generated using other methods, cloning based on homologous recombination is efficient and suitable for high throughput workflow (*2*, *3*). In addition, as the highly efficient *E. coli* based cell-free protein expression system can potentially cause the loss of post-translational modifications and disulfide bonds important for epitope formation, disulfide kits and cell-free systems using wheat germ cells, rabbit reticulocytes and human cells are available for use. Readers are encouraged to examine the suitability of these other methods for construction of their particular parasite protein microarray.

3.2.1 PCR amplification

1. Design PCR primers for each of the selected parasite genes including 20 base pairs complementary to the expression vector of choice to allow for homologous recombination cloning.
2. Order primers from a commercial supplier in 96-well format.
3. Prepare the PCR template by diluting amplified cDNA to 50ng/µL and mixing equal volumes from each source.
4. Amplify using a standard PCR protocol in a 25µL volume (*see* **Note 11**).
5. Check 3µL of the PCR reaction by electrophoresis in a 1% (w/v) agarose gel.

3.2.2 Recombination cloning and plasmid purification

1. Combine 1µl PCR product with 4µl of linear vector in a new 96-well plate chilled on ice (*see* **Note 12**).
2. Add 10µl of thawed DH5α cells to each well with care to avoid contamination between wells.
3. Cover with adhesive plastic sheet and store on ice for 30 minutes.
4. Heat shock in a 42˚C water bath for 1 minute.
5. Chill on ice for 2 minutes.
6. Dispense 200µL SOC media to each well, cover plate with adhesive plastic sheet and incubate for 1 hour at 37˚C.
7. Add 1.1mL of LB media (with 50µg/ml kanomyocin) into flat-bottom well blocks and transfer transformation mixture.
8. Cover with Airpore Tape Strips and incubate overnight at 37˚C with 600rpm shaking.
9. Check for turbid media in cells – high turbidity indicates a successful transformation while slight turbidity suggests background from an empty vector. If necessary, repeat cloning steps for missing inserts with the option of picking single colonies.
10. Make glycerol stocks by mixing 80µL of cell culture with an equal volume of 50% (v/v) glycerol. Store at -80˚C in 96-well round bottom plates (*see* **Note 13**).
11. Pellet remaining cells by centrifuging at 3000rpm for 8 minutes.
12. Discard supernantant and proceed with QIAprep 96 turbo Minikit protocol (including optional purification step) using a vacuum manifold.
13. Elute plasmid in 100µL of EB buffer (supplied in kit).
14. Run agarose gel electrophoresis to check size of plasmids and inserts compared with empty vector. Quality control (QC)-PCR can be used to check for the presence of the insert (*see* **Note 14**).

15. It is recommended that all or a subset of the purified plasmids or previously stored glycerol stocks are sequenced prior to protein expression.

3.2.3 Cell-free protein expression

1. On the basis of the PCR and cloning gels, the QC-PCR and sequencing results select plasmids for cell-free protein expression and printing.
2. Prepare the RTS reaction mix as directed.
3. Transfer 10µL (>0.5µg DNA) of miniprep DNA into 96-well round bottom plates, add 40µL of the reaction mix, cover with an adhesive plastic sheet and briefly spin the plate (*see* **Note 15**).
4. Incubate for 5 hours at 30˚C and shake at 300rpm.
5. Stop the reaction with the addition of 16µL 4X stop solution (0.2% v/v Tween 20 and 5 complete, mini, EDTA-free protease inhibitor cocktail tablets (Roche) per 10ml) (*see* **Note 16**).
6. Cover plate and centrifuge at 3000rpm for 3 minutes and store on ice ready for printing.

3.2.4 Microarray printing

Load the cell-free protein solution onto printing plates including controls and recombinant proteins. We recommend including dilutions of recombinant cellularly-expressed parasite proteins with known antigenicity that are also printed as cell-free extracts (comparison control), secondary antibody positive controls (mixed species IgG), host-specific positive control (e.g. Epstein-Barr virus nuclear antigen 1 (EBNA1) or anti-host antibodies), parasite extract antigen (e.g. schistosome soluble worm antigen preparation (SWAP)), no plasmid DNA negative control (protein expression mix only), and buffer only negative control (*see* **Note 17**). Setup the microarrayer as directed by the manufacturer and load the gal file defining feature location. Print protein microarrays and allow to dry. Store at room temperature in a desiccator cabinet.

3.3 Protein microarray probing, scanning and data analysis

3.3.1 Protein microarray probing

Probe the protein microarrays with sera or with antibodies directed against protein expression tags. However, when probing with sera pre-absorption with *E. coli* lysate is required to reduce background signal from antibodies reactive with bacterial antigens present in the RTS protein extract. A quality control probe to check the print quality is also recommended. Select slides from the start, the middle and the end of the print run for probing with antibodies directed against the N- and C-terminal expression tags (in pXi/pXT7 the N- and C-terminal tags are 10xHis and HA, respectively). All incubation steps use a platform rocker.

1. Fit the incubation chambers over the slide and mount on the slide frame.
2. Hydrate each microarray chamber with blocking buffer (BB) and leave at room temperature for 30-60 minutes with gentle rocking.
3. Dilute sera in 1:100 with BB and 10% (w/v) *E. coli* lysate and incubate at room temperature for 30-60 minutes with gentle rocking.
4. For each microarray chamber, aspirate BB. Add pre-absorbed sera or QC antibodies diluted (1:500 typically) in BB alone. Take care not to let microarray pads dry out.
5. Incubate overnight in a humidified box at 4˚C with gentle rocking.
6. Aspirate and wash 3 times with TBST.

7. Add diluted (typically 1:1000 in BB) biotin-conjugated secondary antibody. Incubate at room temperature for 1 hour with rocking.
8. Aspirate and wash 5 times with TBST.
9. Add diluted streptavidin-conjugated Cy5 fluorophore (typically 1:200 in BB), and incubate for 1 hour at room temperature. Then aspirate and wash 3 times with TBST and 3 times with TBS.
10. Remove slides from incubation chambers and frame. Wash with purified water.
11. Centrifuge slide for 5 minutes at 500Xg. Store slide in the dark until scanned.

3.3.2 Scanning and data analysis

The image acquisition and basic data analysis techniques presented here, although still under development, are applicable to most projects; however, the reader is advised to consult the scanner and imaging software manuals as well as specialist references for further details of image acquisition and data analysis. Scan the probed slides using a confocal laser microarray scanner (e.g., Genepix 4300A). Adjust the laser and photomultiplier tube (PMT) values to maximise signal while not over-saturating features. Quantify the signals with image analysis software (e.g., Genepix Pro 7) and calculate the final feature intensity by subtracting the local background from the signal. Use a standard spreadsheet package (Excel) or more specialized software (R project, Bioconductor) for further data analysis. Transform the signal intensities and normalise between the microarrays using the No DNA negative controls (*11*). Positive features are defined as having a signal greater than the average of the No DNA negative controls plus 2-3 standard deviations. Confirm the probing protocol was successful and sample integrity was maintained by checking the printed control features within each protein microarray. Similarly, comparison controls should have comparable reactivity to the cell-free protein equivalent features. Ensure that over 90% of features have full-length protein expression in the quality control probed microarrays across the entire print run. Full-length proteins will have positive signal intensities for both the N- and C-terminal expression tags for each feature. When completing serological profile studies, positive antigens can be statistically compared between infection-resistant and -susceptible host groups for selection of vaccine targets. Diagnostic antigens are selected by comparing different cohorts of infected and uninfected sera samples.

4 Notes

1. Theoretically, any T7 vector capable of *in vitro* expression may be used. For the schistosome protein microarray the proprietary pXi vector, similar to the pXT7 vector (*3*), was used. Linear vector is generated by digesting the multiple cloning site in the circular vector with specific restriction enzymes and PCR amplifying further linear vector.

2. When using genomic DNA instead of cDNA, PCR primers must be designed to include coding regions only and it may be necessary to express and print several polypeptide fragments for a protein with introns. Similarly, due to the efficiency limitations of PCR coding sequences longer than 3000 base pairs must also be split into fragments. Open reading frames can be found using online ORF/gene finder or prediction programs.

3. In our hands, λ phage libraries were not as successful as cDNA when PCR amplifying large (>1kb) sequences

4. To allow for some losses during the PCR amplification, cloning and protein expression stages, select >20% more genes than required for protein microarray printing (*2*).

5. This may depend on the genes selected and the transcriptional differences within the parasite. In our case, numerous genes were only expressed in male or female schistosome worms or a particular lifecycle stage.
6. Our group routinely extracts RNA from schistosome parasites; however we have made several minor modifications in the Hoffmann and Fitzpatrick (*10*) protocol regarding homogenisation, incubation times and purification. Therefore, for optimal results, each laboratory is encouraged to empirically determine the optimal method.
7. RNA quality of samples can also be assessed using electrophoresis on a denaturing agarose gel.
8. The Bioanalyzer literature notes that a particular RIN is no guarantee of experimental success but it is a good indication of the quality of the RNA samples. For the schistosome protein microarray, we used samples with RIN values above 4-5 before attempting cDNA synthesis.
9. Due to random priming, the QuantiTect WTA kit cannot guarantee full-length sequences. However, in practise our template, consisting of cDNA prepared from multiple lifecycle stages and male and female worms using the WTA and conventional RT kits was successful in amplifying most sequences.
10. It is possible to use other methods adaptable for high throughput cloning including restriction site cloning, ligation independent cloning, or sequence and ligation independent cloning (*12*). The 5 Prime RTS manual also suggests using overlap extension PCR to generate linear DNA template.
11. Using 1µL of cDNA template, amplify in a typical cycling protocol: denature for 2 minutes at 94˚C; followed by 30 cycles of 94˚C for 30 seconds; 55˚C for 15 seconds and 68˚C for 1minute/kb; and a final extension for 10 minutes at 68˚C.
12. Cloning efficiency may be improved by varying PCR product volumes between 0.5-1µl.
13. It is useful to take glycerol stocks at this stage for repeat printing or sequencing checks.
14. A quality control PCR is also recommended. Perform a standard PCR reaction using previously designed primers and purified plasmids and check for presence of the correct insert on an agarose gel.
15. The volume required depends on the size of the microarray to be printed and the printing protocol used.
16. The final concentration of Tween 20 will determine the viscosity of the printing solution. Viscosity has a large impact on the quality of the final protein microarray and can cause spot bleeding or incomplete spot printing (*8*). Users are encouraged to empirically determine the best concentration, dependant on the printing pins used, the humidity of the microarrayer, and the print surface.
17. Prior to the final print, it is recommended that a range of dilutions of recombinant proteins and parasite antigen extracts is printed and probed to determine their optimal concentration.

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Figure 1: Parasite protein microarray workflow – construction of the array, its printing, probing with antibodies, scanning and analysis of the results