Chapter 14

Whole-Genome Analysis of *Plasmodium* spp. Utilizing a New Agilent Technologies DNA Microarray Platform

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Abstract

The application of DNA microarray technologies to malaria genomics has been widely used but has been limited by sample availability and technical variability. To address these issues, we present a microarray hybridization protocol that has been optimized for use with two new Agilent Technologies DNA microarrays for *Plasmodium falciparum* and *P. berghei*. Using the most recent genome sequences available for each species, we have designed ~14,000 oligonucleotide probes representing ~5,600 transcripts for each species. Included in each array design are numerous probes that allow for the identification of parasite developmental stages, common *Plasmodium* molecular markers used in genetic manipulation, and manufacturer probes that control for array consistency and quality. Overall, the Agilent *Plasmodium* spp. array designs and hybridization methodology provides a sensitive, easy-to-use, high-quality, cost-effective alternative to other currently available microarray platforms.

Key words: DNA microarray, *Plasmodium* genomics, Microarray hybridization, Transcription profiling, Gene expression, Comparative genomic hybridization, Agilent Technologies

1. Introduction

There are currently many DNA microarray designs that have been used to study transcriptional changes in various strains, species, and field isolates of *Plasmodium* during parasite development (see Chapter 13 by Z. Bozdech et al.). However, these technologies have not been broadly implemented in many laboratories for a number of reasons. First and foremost, the original spotted arrays were often of variable print quality and commercial arrays that attempted to overcome these issues have generally been costly. Furthermore, DNA microarrays traditionally required significant

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amounts of starting genetic material or have relied on amplification for sample preparation.

Here we present a microarray hybridization protocol optimized for two new commercially available DNA microarrays for *Plasmodium falciparum* and *P. berghei* that are manufactured by Agilent Technologies (1). Unlike spotted arrays, which directly place full-length oligonucleotides on glass slides, Agilent Technologies uses an ink jet-based technology to synthesize 60-mer oligonucleotides in situ (2). Agilent DNA microarrays provide excellent spot uniformity that results in a greater dynamic range of signal intensity, more signals scoring above background level, and a vast reduction in inter-array inconsistencies (3). Due to the high sensitivity of the Agilent platform, cRNA, cDNA, and gDNA (for comparative genomic hybridization (CGH)) can be hybridized using minimal quantities of labeled material (reviewed in ref. 4).

Using the most recent genome sequences, we designed ~14,000 oligonucleotide probes representing ~5,600 transcripts for each species using OligoRankPick (5). The *P. falciparum* custom Agilent array design is based on the PlasmoDB v7.1 3D7 genome, whereas the *P. berghei* design is based on the annotation from the Welcome Trust Sanger Institute (March 2011, with thanks to Dr. U. Boehme, The Wellcome Trust Sanger Institute). For practical purposes, additional probes were included in both Agilent microarray designs that allow for the recognition of common genetic markers frequently used in *Plasmodium* molecular biology (e.g., *egfp* and *hdhfr*). For microarray applications that seek to compare transcript levels during intraerythrocytic development, we have also randomly distributed repeats of oligonucleotides representing genes that have very high levels of expression at a single stage, thus allowing for the determination of intra-array variability as well as for an accurate measurement of the timing of developmental progression. As is standard with all Agilent DNA microarrays, this design also includes control probes to ensure manufacturing quality and reduce array-to-array variation. Finally, this platform allows for a single 1×3 in. glass microscope slide to contain eight individual microarrays, each having over 14,000 unique probes. We find that having eight microarrays on one slide reduces inter-array variability and is ideally suited for a detailed analysis of the full 48-h asexual developmental cycle of *P. falciparum* (every 6 h). Both array designs and details of the array oligonucleotides are available to the malaria research community at PlasmoDB.org and through Agilent Technologies (AMADID #037237 (*P. falciparum*) and AMADID #038059 (*P. berghei*) (further information is available at the Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/)). Since these custom arrays are not listed as catalog items, Agilent requires users to seek authorization from the designer, which we will happily provide for anyone expressing interest, no questions asked.

The following protocol has been optimized specifically for hybridizing 250 ng of cDNA on either the *P. falciparum* or *P. berghei* Agilent Technologies custom 8 × 15k microarray.

2. Materials

1. Agilent Technologies SurePrint Array AMADID #037237 (*P. falciparum*) or AMADID #038059 (*P. berghei*). Store at room temperature (RT).
2. Nitrogen purge box for slide storage.
3. Nuclease-free H_2O.
4. 10× GE blocking agent (Agilent, #5188-5281) (see Note 1).
5. 2× Hi-RPM hybridization buffer (Agilent, #5190-0403). Store at RT.
6. 20× SSPE: dissolve 175.3 g of NaCl, 27.6 g sodium phosphate monobasic, and 7.4 g of EDTA in 800 ml of deionized water. Adjust the pH to 7.4 with 10 N NaOH. Adjust the volume to 1 l with additional deionized water. Store at RT.
7. Wash buffer A: 700 ml deionized water, 300 ml 20× SSPE, 0.25 ml 20 % *N*-lauroylsarcosine. Store at RT.
8. Wash buffer B: 997 ml deionized water, 3 ml 20× SSPE, 0.25 ml 20 % *N*-lauroylsarcosine. Store a RT.
9. Acetonitrile (caution: extremely flammable, should be used in a fume hood).
10. Microcentrifuge.
11. Hybridization chamber gasket slides: eight microarrays/slide, (Agilent, #G2534-60014).
12. Stainless steel hybridization chamber with clamp assembly (Agilent #G2534A).
13. Hybridization oven with rotator for hybridization chambers; temperature set at 65°C and rotation at 10 rpm (Agilent, #G2545A and #G2530-60029).
14. Glass slide staining dish (×3) with removable slide rack.
15. Magnetic stir plate (×3).
16. Magnetic stir bars, 1 in. (×3).
17. Forceps for separating and handling glass slide.
18. Powder-free latex gloves.
19. Sterile pipette tips.
20. Sterile, nuclease-free 1.5-ml tubes.

3. Methods

1. Follow protocols for RNA isolation, cDNA synthesis, cDNA dye-coupling, CGH, and DNA labeling as detailed in Chapter 13 by Z. Bozdech et al. and/or the Llinás lab Web site (http://www.molbio1.princeton.edu/labs/llinas/protocols.html). Please note the following.
   (a) Starting amounts of RNA for cDNA generation can range from 500 ng to 10 µg. This input will result in sufficient quantity of cDNA for dye-coupling and hybridization on the Agilent microarray.
   (b) Dye-coupled/labeled cDNA/gDNA should be eluted into 10 µl of nuclease-free water for addition to the Agilent microarray hybridization mixture.
2. Determine cDNA quantity and efficiency of dye-coupling by measuring 1 µl of eluted sample using the microarray measurement tab on a NanoDrop spectrophotometer.
   (a) Record the following values: Cyanine-3 or Cyanine-5 dye concentration (pmol/µl), DNA absorbance ratio (260 nm/280 nm should be between 1.8 and 2.0), and cDNA concentration (ng/µl).
   (b) Determine the yield and specific activity of each reaction:

$$\text{cDNA}(\mu\text{g})\text{yield} = (\text{cDNA concentration (ng}/\mu\text{l}) \times 10\mu\text{l}) / 1{,}000.$$

$$\text{Specific activity}\left(\frac{\text{pmol dye}}{\mu\text{g cDNA}}\right) = \frac{\text{concentration of Cy}-3 \text{ or Cy}-5}{\text{concentration of cDNA}}.$$

   (c) Examine yield and specific activity results (see Note 2).
3. For each hybridization, combine equal amounts of dye-labeled reference (Cy3) and sample (Cy5) RNA/DNA in a 1.5-ml microcentrifuge tube so that each channel contains 250–1,000 ng and 2.5–5 pmol dye.
4. Adjust total volume to 22 µl with nuclease-free water.
5. To each hybridization mixture add 5.5 µl 10× GE blocking agent and 27.5 µl 2× Hi-RPM hybridization buffer (viscous), resulting in a total volume of 55 µl. Pipet gently to mix and remove bubbles by spinning for 30 s at maximum speed in a microcentrifuge.
6. Wearing powder-free latex gloves, remove a fresh 8-well gasket slide from protective sleeve and place into the hybridization chamber base being careful to align the numeric barcode in the rectangular end section.
7. Pipet hybridization mixture onto the center of the gasket that corresponds to the array being hybridized (for slide orientation

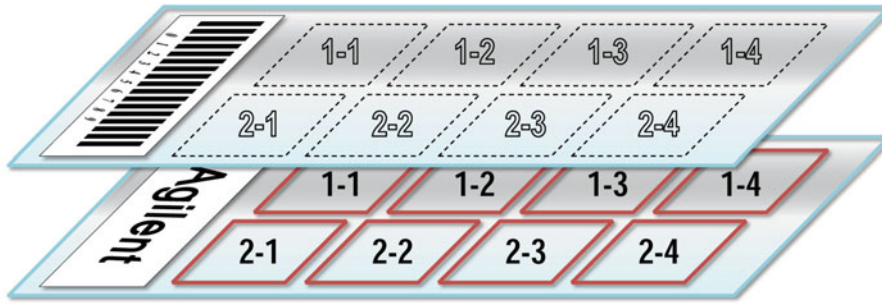

Fig. 1. Agilent Microarray Hybridization Setup. Orientation of the 8-well gasket slide (*bottom*) and microarray slide (*top*). Place gasket slide in metal array chamber (not shown), load hybridization mixture in the order noted in the text, and then carefully place microarray slide on top. Array numbers correspond to output files generated by Agilent Feature Extraction software.

and numbering of arrays, see Fig. 1) (see Notes 3 and 4). With the pipette tip, spread in a dragging motion without touching the silicone gasket. Do not expel the last 1–2 µl of hybridization mixture from the pipette tip to reduce the formation of small bubbles.

8. Remove Agilent microarray slide from desiccator and note the barcode number of the slide and the array number (see Fig. 1).
9. In one smooth motion, slowly lower the Agilent microarray slide (numeric barcode facing up, "Agilent" barcode facing down, as per Fig. 1) parallel on top of gasket slide.
10. Place the chamber cover on the chamber base, followed by the clamp assembly and hand-tighten the thumbscrew to create an airtight seal around each array.
11. Once sealed, rotate the entire chamber 2–3 times vertically, to wet the gasket seals and ensure proper bubble formation (see Note 5).
12. Place the hybridization chamber containing the microarray/gasket sandwich into the rotating hybridization oven and balance the rotating carousel.
13. Incubate at 65°C while rotating at 10 rpm for at least 17 h.
14. Following the incubation period, prepare washing buffers and apparatus.
    (a) Arrange three glass slide staining dishes, one removable slide rack, 3–1 in. stir bars, and three stir plates (see Fig. 2).
    (b) Fill 3/4 of glass dish 1 with Buffer A. Add stir bar and slide rack.
    (c) Fill 3/4 of glass dish 2 with Buffer B and stir bar.
    (d) Fill 3/4 of glass dish 3 with acetonitrile, stir bar, and cover until used.

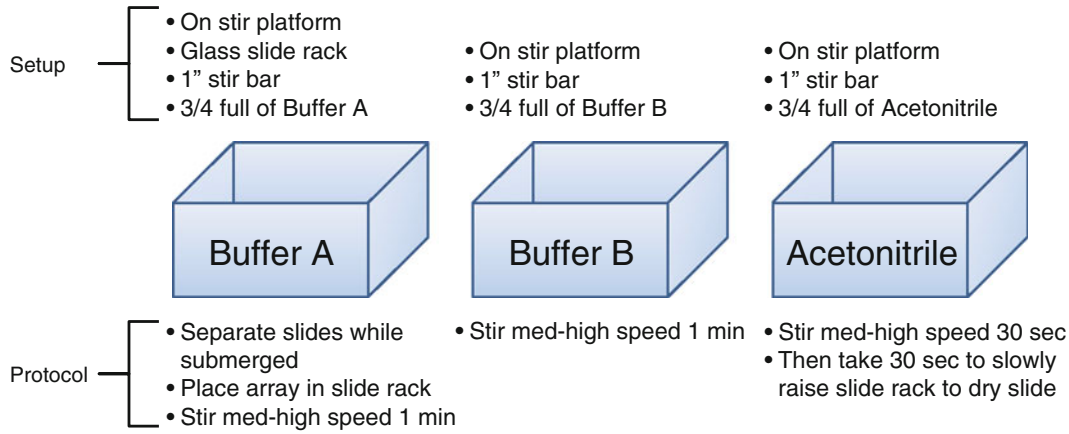

Fig. 2. Agilent Microarray Washing Apparatus and Protocol Setup. Position each of the three glass wash chambers on stir plates (not shown) with 1 in. stir bars. Fill with Buffer A, Buffer B, and Acetonitrile in the order shown. Add slide rack to Buffer A. Spin at med-high speeds for indicated length of time to wash microarray slide.

(e) Start chambers two and three stirring at medium to high speed (turbulence should be visible).

15. Wash Agilent microarray
    (a) Disassemble the hybridization chamber and remove the Agilent microarray/gasket sandwich. Separate the slides using forceps while submerged in wash buffer A and place microarray slide into rack. Wash slide for 1 min with stirring avoiding contact with the stir bar. Dispose of 8-well gasket appropriately.
    (b) Move slide rack with array slide to buffer B and wash for 1 min with stirring.
    (c) Move slide rack with array slide to the acetonitrile and submerge for 30 s while stirring. Slowly raise the slide rack out of the acetonitrile taking an additional 30 s (this will dry the washed slide). Remove any remaining liquid by blotting the edge of the slide on a lint-free tissue.
16. The microarray slide is now ready to be scanned (see Note 6). Slides should be scanned immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, slides can be stored in a nitrogen purge box, in the dark.

4. Notes

1. Resuspend 10× GE Blocking Agent lyophilized pellet in Nuclease-free H_2O and store at −20°C.
2. Using the NanoDrop readings taken at the end of the labeling protocol, calculate dye incorporation efficiency (pmol of dye

per μg cDNA). Target dye incorporation is 10–20 pmol/μg, but 5 pmol/μg is sufficient. It is not recommended to hybridize samples with inefficient dye incorporation.

3. Any number of arrays (1–8) can be hybridized simultaneously with unused arrays left empty. Unused arrays on a slide can be hybridized at a later date utilizing this same protocol.
4. If an Agilent scanner is being utilized, it is recommended that the arrays closest to the barcode be hybridized first and then in order moving away from barcode. This will assist in scanning accuracy.
5. Ideally, there should be one large moving air bubble per array. Stationary air bubbles can be dislodged by tapping the metal array chamber on a solid surface.
6. It is recommended to scan Agilent microarrays with an Agilent G2505B Microarray Scanner; however, *any* two-color array scanner can be utilized to obtain images for further analysis.

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