Protocol

Tips on Hybridizing, Washing, and Scanning Affymetrix Microarrays

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Starting in the late 1990s, Affymetrix, Inc. produced a commercial system for hybridizing, washing, and scanning microarrays that was designed to be easy to operate and reproducible. The system used arrays packaged in a plastic cassette or chamber in which the prefabricated array was mounted and could be filled with fluid through resealable membrane ports either by hand or by an automated “fluidics station” specially designed to handle the arrays. A special rotating hybridization oven and a specially designed scanner were also required. Primarily because of automation and standardization the Affymetrix system was and still remains popular. Here, we provide a skeleton protocol with the potential pitfalls identified. It is designed to augment the protocols provided by Affymetrix.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution’s Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

See Affymetrix protocols (<http://www.affymetrix.com>)

Target sample(s) of interest

Depending on the type of array being used, this target might be composed of cDNA, double-stranded cDNA, or “crRNA” made by amplification of cDNA derived from the original sample RNA of interest. This protocol assumes that the investigator has converted the RNA samples of interest into the appropriate target preparation. Several different kits are available for these various target preparation methods.

Equipment

Affymetrix fluidics station
Affymetrix hybridization oven
Affymetrix microarray chips
Affymetrix scanner
Computer
Heat blocks (set at 99°C, 65°C, and 50°C)
Microcentrifuge
Microcentrifuge tubes (1.5 mL)
Pipettors and tips
Racks for chips
Rotating oven (set at 50°C)

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Safe-Lock tubes
Tape or Tough-Spots

METHOD

There are three main parts to this process. First, the chips must be prehybridized briefly (~10 min) and then the biotinylated target sample prepared from the isolated RNA of interest is combined in a hybridization fluid and loaded into the chip. After hybridization overnight, the target is removed and the chip is filled with a high-salt (nonstringent) buffer and loaded into the fluidics station, along with tubes of stain (streptavidin–phycoerythrin fusion protein, SAPE) and a biotinylated antibody against streptavidin (available from Affymetrix). The fluidics station runs a program to wash the array with stringent buffer at the appropriate temperature, then applies a first staining step in which the SAPE binds to biotin moieties on the hybridized target. After washing off unbound SAPE, this signal is amplified by then binding a biotinylated antistreptavidin antibody to the SAPE left after the first staining. In a third staining step, a second binding of fresh SAPE is used, which binds to biotins on the antibody. Finally, the array is washed again and ready for scanning. About 1 h is needed for the hybridization setup. The next day, ~1 h is needed for the wash setup; the fluidics station then runs for ~1.5 h to stain and wash. It takes 5–10 min to scan each chip. For further information, see Schinke-Braun and Couget (2007).

1. Gather the reagents and set up the equipment.
 - i. Set the heat blocks to 99°C, 50°C, and 65°C.
 - ii. Set the Affymetrix rotating hybridization oven to 50°C.
 - iii. Take the chips to be hybridized out of storage at 4°C to equilibrate to room temperature on the bench.
 - iv. Allow the reagents stored at –20°C (e.g., bovine serum albumin [BSA], HS DNA, 20× hybridization controls, B2 oligo) to thaw.
 - v. Take 2× hybridization buffer (in 50-mL tube covered with foil) out of storage at 4°C and bring to room temperature.
2. Make the hybridization cocktail mix.
 - i. Follow the recipe in the appropriate Affymetrix protocol and prepare enough for 131 µL per chip.
 - ii. Make a little extra to account for pipetting variability (i.e., if making cocktail for four chips, multiply all amounts by 4.2).
 - iii. Vortex to mix and quick-spin down.
3. Add 131 µL of hybridization premix cocktail to each of the target sample tubes that have been prepared from RNA (~69 µL). Mix well and quick-spin down.

There should be ~200 µL of 1× hybridization solution with the target sample.
4. Make enough 1× hybridization buffer to prehybridize all chips (200 µL per chip).
5. Prehybridize the chips.
 - i. Take the chips out of their packets, being careful not to touch the glass surface, and place them on a clean bench with the clean laboratory mat face down.
 - ii. Carefully insert a pipette tip into the top septum (note that regular yellow tips, unbeveled, probably work best) to create an exit path for air in the chamber, which will be replaced by hybridization buffer.
 - iii. Take up 200 µL of 1× hybridization buffer in a pipettor.
 - iv. Holding the chip upright, pierce the bottom septum with the loaded pipette tip and slowly fill the chip while keeping the tip perpendicular to the chip surface, so as not to damage the septum. Wiggling or twisting the tip while in the septum may damage the septum.
 - v. Pull the pipette tip out of the septum while the plunger is depressed at the first stop, not the “blow out” stop—all of the buffer should be out.

- vi. Remove the other pipette tip, pulling straight out again, so as not to damage the septum.
 - vii. Rotate the chip manually while inspecting the glass side to ensure that the entire surface is wetted and that liquid is moving freely. The fluid can be seen in the chamber through the glass facing.
 - viii. When all of the chips are loaded with buffer, put them in racks (balance the racks by putting the chips in opposite positions on two racks; use an old empty chip to balance) and rotate the chips in the hybridization oven at 60 rpm for at least 10 min at 50°C.
6. Denature the samples (now in 1× hybridization mix) for 10 min at 99°C. Use Safe-Lock tubes or place a weight on top so that the tubes do not pop their tops at this higher than usual temperature. Transfer the tubes for 5 min at 50°C.
7. Spin the tubes at full speed in a microcentrifuge for 5 min to pellet denatured and aggregated material. Orient the tubes all in one direction to avoid the part where pelleted particulates will be.
8. Remove the prehybridized chips from the oven.
9. Remove the buffer from the chips, reversing the previous manipulations.
 - i. Pierce the top septum with a pipette tip.
 - ii. With the P200 set to 200 µL (and empty), depress the plunger to the first stop.
 - iii. Holding the chip vertically, pierce the bottom septum with the pipette tip and slowly release the plunger to suck out the prehybridization buffer.
 - iv. Pull the pipette tip out of the bottom septum and discard the fluid and the tip.
10. Fill the chips with denatured, pelleted target samples.
 - i. When pipetting the sample, take care to position the tip at the bottom of the tube on the side away from the pellet. Get all of the sample (~200 µL).
 - ii. Note which sample was applied to which chip. It is best to write the sample name on the chip label.

It is essential to mark the chips with the name of the target. Although each array has a bar code, in practice, only core or heavy-usage laboratories use bar-code readers to ensure that the right sample is associated with the right array.
11. Apply small pieces of tape or “Tough-Spots” over both the septa on each chip in case of leakage.
12. Hybridize in a rotating oven at 60 rpm for 16 h at 50°C. Before the end of the hybridization, start preparing for the wash and scan.
 - i. Ready the reagents for the stains (e.g., BSA removed from storage at –20°C and SAPE, antibodies, and 2× stain buffer [stored in a foil-wrapped tube] removed from storage at 4°C). Vortex and quick-spin each before use. See the Affymetrix standard protocol for more information.
 - ii. Turn on the fluidics station.
 - iii. Prepare buffers for the fluidics station. Fill each bottle with the appropriate buffer or water, checking for particulates, and empty the waste bottle. Nonstringent buffer is stored at room temperature. Stringent buffer is stored in foil at 4°C. Match bottles up to the labeled intake lines on the right of the fluidics machine.
 - iv. Log onto the computer and start up GCOS Affymetrix software that runs the fluidics station and the scanner.
 - v. Open the fluidics station window.
13. Prime the fluidics station. The fluidics station has four “modules,” each able to wash one chip.
 - i. For each module to be used, select <no probe array> and run the appropriate priming program (e.g., <PRIME_450>). Click Run to start the priming protocol for each module.

- ii. Follow the instructions on the module windows to start the protocol. The window commands are explained as follows:
 - Remove vials: This means lift up the lever for the needles (no need to actually remove vial except when loading the stains).
 - Load empty vials: This means gently push the lever back down.
 - Eject washblock: This means push down on the cartridge lever to open the module so that a chip can be inserted or retrieved.
 - Engage washblock: This means flip up the cartridge lever. Do not do this too gently, because a good amount of pressure is required to get the septa pierced appropriately so that the chip is engaged properly with the washblock.

When a protocol finishes, a notification will appear on the computer screen for each module.

14. For each chip, make an “experiment” entry.
 - i. Use templates for sample and experiment to record the lot numbers for the chips and notes for the samples.
 - ii. Choose the appropriate target sample type (cDNA, cRNA) and the proper array type. Select the appropriate analysis desired. This can be customized depending on the kind of analysis performed.
 - iii. Typically, the sample name and experiment name will be the same (but use whatever system that is both informative and systematic).
 - iv. Group samples together under project headings.
 - v. After saving an entry, edit it to save as a new experiment.

It is essential to double check the naming of the computer files and the association of the files with the array and with the sample. The computer believes what it is told, and thus it may be difficult to figure out why a sample that is named “brain” looks exactly like it came from liver if in fact the file name is incorrect.

15. After 16 h (or pick a consistent time to stop all hybridizations for a given project), remove the chips from the oven.
16. Remove the samples from the chip by removing the tape from the ports, piercing the upper port septum with a yellow 200- μ L pipettor tip and pulling the sample from the lower port. Save the samples because they can be hybridized to a second chip if necessary. Store them at -20°C .
17. Fill each chip completely ($\sim 250\ \mu\text{L}$) with nonstringent wash buffer.
 - i. Dial a 200- μ L pipettor to $\sim 135\ \mu\text{L}$ and fill with nonstringent wash buffer. Empty into the chip as described above. Reload the pipettor with buffer and insert into the bottom port septum again. Holding the chip upright, slowly fill the chip to ensure that there are no bubbles. The air space in the chip chamber should slowly disappear from the top center of the chip when the first stop on the plunger is reached.
 - ii. With the plunger depressed, remove the pipette tip. Check that the chip surface is completely filled (no new bubbles have appeared); remove the other pipette tip and check the chip surface again.
18. Allow the filled chips to sit at room temperature while the stains are prepared. Store hybridized chips (after replacing the hybridization fluid with nonstringent wash buffer) for a few hours at 4°C if necessary (e.g., eight chips hybridized but only four at a time on fluidics machine). Bring them to room temperature before putting them on the fluidics station.
19. Prepare the stain solutions.
 - i. Make one master mix in a 15-mL tube. Make up to 10% additional mix to account for pipetting variation.
 - ii. For each chip, aliquot $2 \times 600\ \mu\text{L}$ of the SAPE mix and $1 \times 600\ \mu\text{L}$ of the antibody mix into black 1.5-mL microcentrifuge tubes (this protects the samples from light).

- iii. Store the tubes to be used later at 4°C along with chips waiting to be stained.

Protect the SAPE and the stained arrays from light as much as possible because SAPE will bleach and signal will be lost. Keep the arrays face down on a soft paper or cloth in a dark drawer or in the refrigerator when not actually in the scanner.

20. Start washing the chips.

- i. In the fluidics station window on the computer, select an experiment from the pull-down list for each module (these were previously entered above).
- ii. Run a protocol that is appropriate to the chip and target sample of interest.
- iii. Click Run to start when ready and then follow the instructions on the fluidics station windows. Eject the washblock and make sure to load the chip matching the experiment selected for each module. When loading stain vials, make sure to load the antibody mix in position 2. To begin the washing protocol, first press the lever down to snap the needles into the stain vials. The washing takes ~1 h 15 min.
- iv. When the protocol is almost done, follow the prompt to remove the vials and the chip. Before engaging the washblock again, check the chip for bubbles—it should be completely full. If bubbles are present (that cover any part of the scan area), place the chip back into the washblock and engage the washblock. The fluidics station will drain and fill the array and again prompt to eject cartridge. Repeat as necessary, but after several tries, manual filling of the chip cartridge with nonstringent buffer is required.
- v. After vials and chip are removed, the module performs a 5-min purging procedure.

21. Cover septa with tape or “Tough-Spots” again to protect scanner from possible leaks.

22. Place the chips in a rack and put them in a drawer to protect them from the light until scanning. Store the chips at 4°C if it will be several hours before a scan.

23. Scan the chips. Turn on the scanner and let it warm up for 10 min.

- i. Open the scanner window in the software (still open from the wash protocol).
- ii. Select the experiment to be scanned from the pull-down menu.
- iii. Check the chip for streaks and dust. Blow off the dust carefully. If absolutely necessary, remove streaks by wiping from center to edge (glue at edges may smear even worse), using ethanol if desired.
- iv. Click the Start button. The sample door on the top of the scanner opens automatically.
- v. Load the chip (make sure to match the chip to the experiment selected) chamber window facing away, gently. It only fits one way so if there is a problem, do not force!
- vi. Click OK to close the door and start the scan.
- vii. The scan is automatically completed and the image is processed into a .cel file.
- viii. The sample door opens automatically at the end of the scan for chip removal. If it closes again before retrieval, simply click Load Sample in the scanner window to open the door.

24. Perform a quick quality check and initial analysis of images and .cel files.

- i. In the Expression assay tab, set scaling to user-defined (1), normalization to (1). This means no massaging of the data.
- ii. Right click on the .cel file and select Analyze. This generates a .chp file.

If a modified analysis method was selected when first inputting and saving the experiment, this may already be done.

- iii. Right click on the .chp file and select Report. This generates a .rpt file, which can be printed or simply noted for comparison between chips:

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- Noise
- Signals, 5'/3' ratios for some test gene probes, Present/Absent calls for housekeeping genes and spiked controls (meaning if intensity is enough to determine the presence of mRNA for the given gene)

25. Shut down and clean up.

- i. Place all fluidics station lines into the dH₂O bottle (fill again if necessary).
- ii. For all modules used, select <no probe array> and Shutdown_450.
- iii. Run the fluidics station shut down protocols, following the instructions on the computer.
- iv. When completed, the fluidics station can be turned off. Dump waste, remaining buffers, and rinse bottles.
- v. Turn scanner off (front button).
- vi. Log off computer.

REFERENCES

Schinke-Braun M, Couget JA. 2007. Expression profiling using Affymetrix GeneChip probe arrays. *Methods Mol Biol* 366: 13–40.

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