Chapter 10

DNA Microarray Analysis of Estrogen-Responsive Genes

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Abstract

DNA microarray is a powerful, non-biased discovery technology that allows the analysis of the expression of thousands of genes at a time. The technology can be used for the identification of differential gene expression, genetic mutations associated with diseases, DNA methylation, single-nucleotide polymorphisms, and microRNA expression, to name a few. This chapter describes microarray technology for the analysis of differential gene expression in response to estrogen treatment.

Key words DNA microarray, Gene expression, Estrogen

1 Introduction

DNA microarray is a powerful, non-biased discovery technology that has been adapted to many applications. DNA microarray allows the analysis of the expression of thousands of genes at a time. In addition to analysis of differential gene expression [1–3], microarray technology has been applied to the identification of genetic mutations associated with diseases [4–6], DNA methylation [7, 8], single-nucleotide polymorphisms [9, 10], and microRNA expression [11, 12], and others [13].

This chapter describes microarray technology for the analysis of differential gene expression in response to estrogen treatment. In this application, DNA sequences are spotted on treated microscope slides to form the arrays. Total RNA extracted from vehicle-treated control and from estrogen-treated cells or tissues is used as the starting material. The RNA is reverse transcribed to complementary DNA (cDNA) and the second strand of cDNA is synthesized. The double-stranded cDNA is purified and used as a template for the synthesis of antisense RNA (aRNA, also called complementary RNA, cRNA). The aRNA is labeled with the incorporation of biotin-UTP into the aRNA during aRNA synthesis. The aRNA is hybridized with the microarray slide overnight. Post-hybridization processing steps incorporate washing and incubation of the

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microarrays with streptavidin-Alexa fluor 647 to complete the labeling of the samples on the arrays. The washed microarrays are scanned and pixel intensity is translated to gene expression. Data are associated with gene names, and statistical comparisons are made between controls and treated samples.

2 Materials

1. RNAlater (Ambion) or similar RNA preservation reagent.
2. Four-week-old female rats.
3. Vehicle (2-hydroxypropyl-β-cyclodextrin) and estrogenic compounds (e.g., ethinylestradiol, 0.15 mg/kg) for gavage treatment.
4. Dissecting tools: Scissors, scalpels, forceps.
5. Tri reagent (Molecular Research Center).
6. 2 mL microfuge tubes.
7. Polytron homogenizer with 7 mm probe (Kinematica).
8. Bromochloropropane.
9. Sodium acetate, 3 M.
10. RNase Zap (Ambion) or RNase Away (Life Technologies).
11. Diethylpyrocarbonate (DEPC)-treated water: Mix 999 mL purified water (18.2 MΩ resistivity) with 1 mL DEPC. Mix on a stir plate overnight. Autoclave the water to destroy the toxicity of the DEPC. Store at room temperature.
12. Silica gel membrane RNA purification spin columns such as the RNeasy columns (Qiagen) or equivalent. The RNeasy Kit contains silica gel columns, binding and washing buffers, and nuclease-free water.
13. RLT buffer: Add 10 μL β-mercaptoethanol to 1 mL of the RNeasy lysis buffer/binding buffer RLT before use.
14. RPE buffer: Add 44 mL ethanol to 11 mL washing buffer RPE concentrate before use.
15. 100 % ethanol.
16. RNase-free DNase (Qiagen) stock solution: Add 550 μL nuclease-free water to the vial of lyophilized DNase and mix gently.
17. RNase-free DNase (Qiagen) working solution: Add 10 μL DNase stock solution to 70 μL RNase-free DNase dilution buffer for each sample and mix gently.
18. Agilent Bioanalyzer and Nano 6000 LabChip and reagents for RNA analysis.
19. Gel matrix for the Agilent RNA 6000 Nano LabChip: Mount a spin filter cartridge in a microfuge tube and add 550 μL of

the gel matrix to the cartridge. Centrifuge at 1500 × *g* for 10 min at room temperature. Store the gel in 65 μL aliquots at 4 °C for up to 1 month.

20. MessageAmp II-Biotin *Enhanced* Kit (Ambion): Contains oligo dT primer, ArrayScript reverse transcriptase, RNase inhibitor, 10× first-strand buffer, dNTP mix, 10× second-strand buffer, DNA polymerase, RNase H, T7 enzyme mix, T7 10× reaction buffer, biotin-NTP mix, nuclease-free water, cDNA filter cartridges, aRNA filter cartridges.
21. Reverse transcription master mix: For each sample/each microarray, mix 1 μL nuclease-free water, 1 μL T7 oligo dT primer, 2 μL 10× first-strand buffer, 4 μL dNTP mix, 1 μL RNase inhibitor, and 1 μL ArrayScript reverse transcriptase. Increase the volume by 5 % for pipetting overage.
22. Second-strand master mix: For each sample/each microarray, mix 63 μL nuclease-free water, 10 μL second strand buffer, 4 μL dNTP mix, 2 μL DNA polymerase, and 1 μL RNase H plus 5 % volume overage.
23. Wash buffer for cDNA and aRNA spin column purification: add 24 mL ethanol to the entire bottle of wash buffer before using.
24. In vitro transcription (IVT) master mix: For each sample/each microarray, mix 4 μL T7 10× reaction buffer, 4 μL T7 enzyme mix, and 12 μL Biotin-NTP mix, plus 5 % volume overage. (The Biotin-NTP mix contains ATP, GTP, CTP, and biotin-11-UTP.)
25. DNA microarrays spotted on treated microscope slides, such as those from Microarrays, Inc., Phalanx Biotech, or CodeLink.
26. Adhesive hybridization chamber (Microarrays, Inc.).
27. Prehybridization buffer: 5× SSC, 0.1 % SDS, 0.1 % bovine serum albumin (BSA).
28. Hybridization buffer: 40–70 % deionized formamide, 10× SSC, 0.2 % SDS, 0.02 % sheared salmon sperm DNA.
29. Post-hybridization wash buffer #1: 0.75 × TNT buffer (75mM Tris–HCl, pH 7.6, 112.5 mM NaCl, 0.0375 % Tween-20).
30. Streptavidin Alexa 647 fluor: for stock solution, dissolve 1 mg Streptavidin Alexa 647 in 1 mL 1 × PBS, pH 7.4. Aliquot the stock solution and freeze at –80 °C. For working solution, dilute 6.8 μL of stock solution in 3.393 mL of TNB buffer for each microarray. TNB buffer: 100 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.5 % NEN Blocking reagent, filtered through 0.88 micron filter.
31. Post-hybridization wash buffer #2: 1 × TNT buffer (100 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05 % Tween-20).
32. Post-hybridization wash buffer #3: 0.1× SSC, 0.05 % Tween-20 at room temperature.

3 Methods

3.1 Tissue Procurement

All experiments utilizing animals must have prior approval by the appropriate institutional ethics committee for animal usage, such as the Institutional Animal Care and Use Committee.

1. Ovariectomize 4-week-old female rats (e.g., Sprague Dawley or strain of choice); allow 2 weeks for recovery of the animals from surgery.
2. Treat the animals with vehicle or ethinylestradiol for 3 weeks (*see* **Note 1**).
3. Euthanize the animals and rapidly dissect the estrogen-responsive tissues of interest (e.g., uterus, mesenteric arteries, liver) (*see* **Note 2**).
4. Place the tissues in the RNA preservation reagent, RNAlater, and store at −80 °C until processed for RNA extraction, or proceed directly to RNA extraction.

3.2 RNA Hygiene and RNA Extraction

High-quality purified RNA is critical for applications such as DNA microarray or real-time RT-PCR. Therefore, care must be taken to prevent degradation of RNA by RNases and to remove contaminating substances such as genomic DNA from the RNA.

1. Clean the surfaces of all instruments, racks, bench tops, and any other materials that will be used in the extraction of RNA with an RNase degradation reagent such as RNase Zap. Spray surfaces with RNase Zap and rinse with DEPC-treated water.
2. Wear gloves at all times and periodically change gloves and/or clean gloves with RNase Zap.
3. Before using the Polytron homogenizer, soak the probe in RNase Zap and then run the probe in a graduated cylinder (that has been cleaned with RNase Zap) full of DEPC-treated water. Dry the probe before homogenizing each sample.
4. Use nuclease-free microfuge tubes and pipette tips.
5. To extract total RNA from tissue stored in RNAlater, blot the reagent from the surface of the tissue. Weigh the tissue; the capacity of the RNA purification columns is approximately 30 mg tissue weight.
6. Mince the tissue into fine pieces with two scalpels under Tri reagent.
7. Place 1 mL of Tri reagent and the minced tissue in a 2 mL microfuge tube or a 12 × 75 mm nuclease-free test tube (*see* **Notes 3** and **4**).
8. Homogenize the tissue using the 7 mm probe for 10 s three times with 30-s rest periods between each homogenization run (*see* **Notes 5–7**).

9. Clean the homogenizer probe carefully between each sample. Rinse the probe with DEPC-treated water then repeat **step 3** of Subheading 3.2.
10. When all of the samples have been homogenized, immediately clean the homogenizer probe very thoroughly. Tri reagent is corrosive and will destroy the probe if it is allowed to remain on the probe.
11. Add 200 μL bromochloropropane and 60 μL 3 M sodium acetate to each homogenized sample.
12. Shake the samples well to mix; the clear pink Tri reagent will turn milky. Incubate on ice for 15 min.
13. Centrifuge the samples for 5 min at 8000 × *g* to separate the phases.
14. Transfer the top (clear, aqueous) layer containing the RNA to a nuclease-free 12 × 75 test tube. Avoid the layer of genomic DNA at the interface between the two layers (*see* **Note 8**).
15. Add 1 mL RLT buffer to the aqueous phase containing the RNA (approximately 450 μL volume).
16. Add 1.2 mL 100 % ethanol to the sample and mix.
17. Transfer a 700 μL aliquot of the sample to a silica gel membrane spin column (RNeasy) that has been placed in a microfuge collection tube and centrifuge for 30 s at 10,000 × *g*. Discard the flow through and repeat until all of the sample has been passed through the column (*see* **Note 9**).
18. Wash the column with 350 μL of RW1 wash buffer from the extraction kit.
19. Perform an on-column DNase treatment: add 80 μL of RNase-free DNase working solution directly to the gel membrane of the column.
20. Incubate at room temperature for 15 min.
21. Add 350 μL of wash buffer RW1 to the column; centrifuge the column for 30 s at 10,000 × *g*. Discard the flow through.
22. Add 500 μL RPE buffer to the column and centrifuge the column for 30 s at 10,000 × *g*; discard the flow through.
23. Repeat the wash step with 500 μL RPE buffer.
24. Dry the membrane by centrifuging the column at 14,000 × *g* for 2 min without adding any buffer.
25. Move the column to a new, dry, well-labeled 1.5 mL microfuge collection tube.
26. Add 50 μL nuclease-free water (not DEPC-treated water) to the center of the membrane.
27. Incubate for 10 min at room temperature.

28. Elute the total RNA by centrifuging the column for 1 min at 10,000 × *g*. Save the flow-through; it contains the RNA.
29. Repeat **steps 26** and **27** of Subheading 3.2, eluting the second aliquot of RNA into the same collection tube (*see* **Note 10**).
30. Discard the column.
31. Store the purified RNA at −80 °C until used for DNA microarray.

3.3 Analysis and Quantitation of RNA

1. Assess the quality and quantity of the RNA using the Agilent RNA 6000 Nano LabChip (or the RNA 6000 Pico Chip if concentration is expected to be low) (*see* **Note 11**).
2. Remove kit reagents from storage at 4 °C 30 min before use and place them in an enclosed box to allow them to come to room temperature in the dark: dye concentrate, marker mixture, and one 65 μL aliquot of the gel matrix (from **item 18** of Subheading 2). Make sure that the water bath is turned on and set at 70 °C.
3. Mix the dye concentrate by vortexing, and then centrifuge for 5–10 s at 10,000 × *g*.
4. Mix 1 μL of dye concentrate with the aliquot of gel matrix and vortex well. Centrifuge for 10 min at 13,000 × *g*.
5. Incubate the RNA ladder and samples at 70 °C for 2 min to denature and chill on ice.
6. Pipet the gel matrix into the specified wells on the chip and pressurize. Pipet the marker mixture, RNA ladder, and samples according to the manufacturer's directions.
7. Use the vortex adaptor to vortex the chip at 2400 rpm for 1 min.
8. Read the chip in the Agilent Bioanalyzer and record the RNA concentration and RNA integrity number for each sample.

3.4 Synthesis and Labeling of aRNA

The following reactions will result in the synthesis of enough antisense RNA (aRNA, also called complementary or cRNA) for one microarray from each sample processed.

1. The manufacturer of your microarray platform will indicate the ideal starting RNA concentration for processing samples for their arrays. This is typically 0.2–2.0 μg total RNA. For vehicle- and estrogen-treated samples, calculate the volume containing the appropriate quantity of total RNA (*see* **Note 12**). If the volume is greater than 10 μL, reduce the volume to 10 μL using a vacuum concentrator (e.g., SpeedVac). Add nuclease-free water to raise the volume to 10 μL if the starting volume is less than 10 μL.
2. Add 10 μL reverse transcription master mix to each sample containing 10 μL of total RNA.

3. Place the samples in a heat block or thermocycler at 42 °C for 2 h. Remove from heat and place on ice. This reaction synthesizes the first strand of cDNA.
4. Add 80 μL of second-strand master mix to each reaction tube.
5. Incubate at 16 °C for 2 h. Use a thermocycler or heat block in which the temperature does not fluctuate.
6. Place the samples on ice. The sample now contains double-stranded cDNA.
7. Heat nuclease-free water to 55 °C (24 μL per sample) in preparation for cDNA purification.
8. To purify the double-stranded cDNA on the cDNA filter cartridges from the kit, add 250 μL cDNA binding buffer to each 100 μL sample of double-stranded cDNA.
9. Mix by pipetting, and then transfer each sample of cDNA onto a spin column filter cartridge.
10. Centrifuge at 10,000 × *g* for 1 min. Discard the flow-through.
11. Pipet 500 μL of wash buffer onto each filter cartridge and centrifuge at 10,000 × *g* for 1 min.
12. Discard the flow-through.
13. Centrifuge the filter cartridge without adding buffer at 10,000 × *g* for 1 min.
14. Transfer the cartridge to a new well-labeled collection tube.
15. Add 22 μL of 55 °C nuclease-free water (preheated in **step 7** of Subheading 3.4) to the center of each spin column.
16. Incubate at room temperature for 2 min.
17. Centrifuge the cartridge at 10,000 × *g* for 1 min. This step will deposit the purified double-stranded cDNA in the eluate.
18. Add 20 μL IVT Master Mix to the 20 μL of purified double-stranded cDNA from **step 17** of Subheading 3.4.
19. Incubate the IVT reaction for 14 h at 37 °C. This reaction synthesizes biotin-labeled aRNA (*see* **Note 13**).
20. Add 60 μL nuclease-free water to each sample to stop the aRNA synthesis reaction.
21. Heat nuclease-free water to 55 °C (200 μL per sample) in preparation for purification of the aRNA.
22. Add 350 μL of aRNA-binding buffer to the aRNA sample.
23. Add 250 μL of molecular grade 100 % ethanol to one sample. Mix by gently pipetting up and down. Do not vortex and do not centrifuge as this will precipitate the aRNA and reduce its recovery. Immediately transfer the entire volume of the sample to an aRNA filter cartridge that has been placed into a collection tube. Perform the entire step of adding ethanol, mixing,

and transfer to a filter cartridge of one sample before moving on to the next sample.

24. When all samples have been mixed with ethanol and transferred to their respective filter cartridges, centrifuge the samples at $10,000 \times g$ for 1 min.
25. Discard the flow-through.
26. Wash each filter cartridge with 650 μL Wash buffer; centrifuge at $10,000 \times g$ for 1 min.
27. Discard the flow-through and dry the cartridge by centrifuging at $10,000 \times g$ for 1 min without the addition of any buffer.
28. Add 200 μL 55 °C nuclease-free water to each cartridge filter. Incubate the samples in the 55 °C heating block for 10 min.
29. Elute the aRNA from the cartridge by centrifugation at $10,000 \times g$ for 1.5 min (*see* **Note 14**).
30. Calculate the concentration of the aRNA by reading the absorbance of the sample at 260 nm in a spectrophotometer. To calculate the aRNA concentration, use the equation

$$A_{260} \times \text{dilution factor} \times 40\ \mu\text{g} / \text{mL} \times 0.001\ \text{mL} / \mu\text{L} = \text{mg aRNA} / \mu\text{L}.$$

31. Store the purified aRNA in aliquots at −80 °C or proceed to hybridization.

3.5 Hybridization

1. Calculate the volume of aRNA containing 10 μg of biotinylated aRNA. If this volume is greater than 20 μL, reduce the volume using a vacuum concentrator. If the volume is less than 20 μL, raise the volume with nuclease-free water.
2. Incubate the 20 μL containing 10 μg of biotinylated aRNA with 5 μL of fragmentation buffer for 20 min at 94 °C (*see* **Note 15**).
3. Chill on ice for a minimum of 5 min.
4. Thoroughly mix the 25 μL of fragmented aRNA with 185 μL of hybridization buffer.
5. Denature the aRNA by incubating at 90 °C for 5 min.
6. Chill the samples on ice for 5 min before loading the microarray slides. Load all of the microarray slides within 30 min of completing the denaturation step.
7. Some microarrays require a prehybridization step. Prehybridize the microarrays for 30 min by incubating the slides with gentle rotation in prehybridization buffer that has been heated to 55 °C.
8. Rinse the slides with purified distilled water; to avoid allowing the warm prehybridization buffer from drying on the slide, place the slide holder in the sink and carefully pour the water over the slides.

9. Wash the slides four more times, 1 min each wash, with purified distilled water.
10. Dry the slides by centrifugation using a slide holder that fits into a 96-well plate rotor. Centrifuge for 3 min at $1000 \times g$. The prehybridization should be initiated so that the prehybridization steps and the denaturation of aRNA are completed at the same time.
11. Attach a flexible adhesive cover slip (called a sealed hybridization chamber) to the microarray slide. Line up the adhesive gasket with the edges of the slide and make sure that the gasket is adhered at all edges.
12. Load 200 μL of the hybridization solution containing labeled aRNA through one of the ports in the hybridization chamber.
13. Seal the ports with the seals that are supplied with the chambers.
14. Incubate the microarrays at 42 °C for 14–18 h in a shaking incubator set at 300 rpm or hybridization oven.
15. Place a container of 240 mL 0.75× TNT buffer in a 42 °C water bath during the hybridization incubation to ensure that it reaches temperature. This warmed buffer will be used for the first washing step after the hybridization.

3.6 Post-hybridization Processing

1. Prepare two containers of room temperature 0.75× TNT.
2. Remove the microarray slides from the incubator.
3. Submerge each slide in a container of 0.75× TNT and peel off the flexible cover slip.
4. Place the slide into a rack that is submerged in the second container of 0.75× TNT and continue until the cover slips have been removed from all microarrays.
5. Move the rack of microarray slides into the container of 42 °C 0.75× TNT and incubate at 42 °C for exactly 1 h. This step will remove non-hybridized aRNA.
6. Place the rack of microarray slides into a container containing streptavidin-Alexa fluor 647. Incubate at room temperature for 30 min in the dark. (Alexa 647 does not photobleach as rapidly as many other fluorescent dyes.)
7. Prepare four containers with room temperature 1× TNT buffer. Move the rack of microarray slides from the fluor into the first container and incubate for 5 min.
8. Move the rack from one container to the next, incubating for 5 min in each container of 1× TNT.
9. For a final wash, use 0.1× SSC/0.05 % Tween for 30 s. Gently agitate the rack of slides during this final wash step.
10. Place the rack of slides in a 96-well plate rotor and dry the slides by centrifugation for 3 min at $1000 \times g$.

11. Use an opaque slide box to store the slides until they can be scanned (preferably as soon as possible after completion of the washing and drying of the slides).
12. Use a GenePix Pro 4000B scanner or equivalent to scan the slides. Turn on the scanner and open the GenePix Pro software 15 min before use.
13. If the scans identify regions of high background on the slides, repeat the final washing step in 0.1× SSC/0.05 % Tween for 30 s, dry the slides by centrifugation, and scan the slides again.
14. Analyze the microarray data using a specialized microarray analysis software program such as GeneSpring (Figs. 1 and 2). Freeware programs are also available for microarray analysis. The software performs statistical analyses to identify differentially expressed genes (Fig. 3). Make sure to apply multiple testing correction statistical tests to account for the many comparisons that are performed in this type of experiment.
15. Perform confirmatory experiments using complementary technologies. Real time RT-PCR [14] or in situ hybridization [15, 16] can be used to confirm differential gene expression. Western blot [17, 18], enzyme-linked immunosorbent assay (ELISA) [19, 20], or immunohistochemistry [16, 21, 22] can be used to confirm differential expression of the cognate proteins of differentially expressed genes.
16. Deposit the raw data and the experimental details from each microarray experiment in a public database at the time of publication in compliance with the recommendations of the

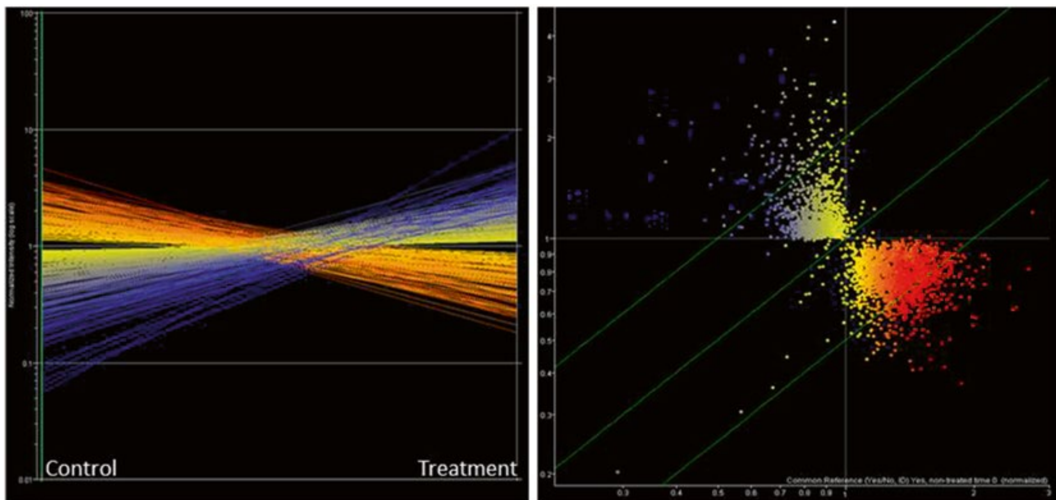

Fig. 1 DNA microarray data can be illustrated in a number of ways. In the *left panel*, each *line* represents one gene and compares expression in control versus treatment. In the *right panel*, each *dot* represents one gene. The location of each *dot* on the matrix is determined by the expression of the gene in control vs. treatment

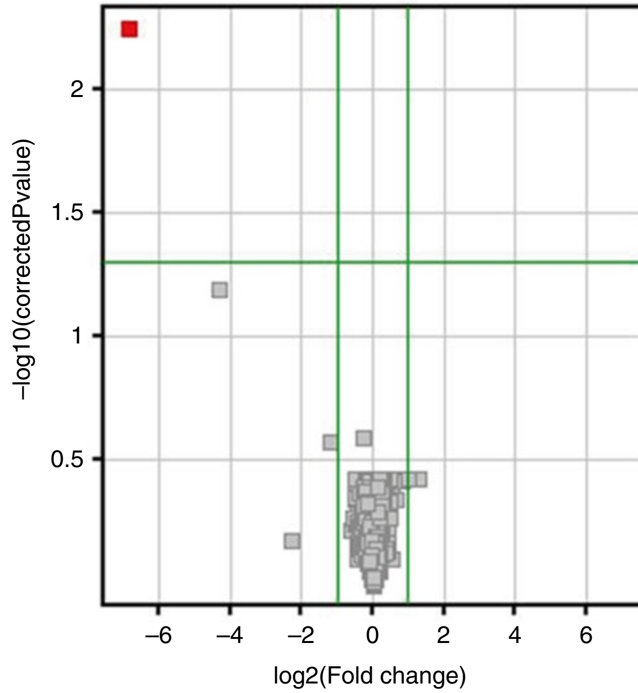

Fig. 2 DNA microarray data can also be represented by a volcano plot. In this data set, only one gene met the cutoff criteria of $p < 0.05$ and greater than two-fold change in expression

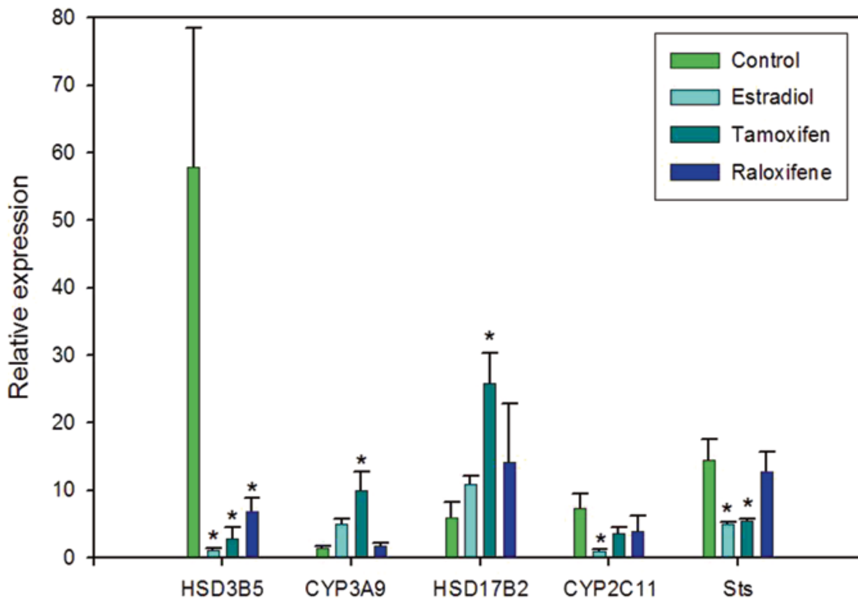

Fig. 3 These microarray data were taken from an experiment in which ovariectomized Sprague-Dawley rats were treated by gavage for 2 days with vehicle (2-hydroxypropyl-beta-cyclodextrin), ethinylestradiol (0.15 mg/kg), tamoxifen (3 mg/kg), or raloxifene (3 mg/kg). Total RNA was extracted from the liver and gene expression signatures were analyzed by DNA microarray. *Asterisks* denote significant difference from control (ANOVA). *HSD* hydroxysteroid dehydrogenase, *CYP* cytochrome P450, *Sts* steroid sulfatase

Minimum Information About a Microarray (MIAME) standards [23]. In the USA, the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo) maintains the Gene Expression Omnibus (GEO) database for deposition of microarray and related data for this purpose.

4 Notes

1. Treatment of the animals for 3 weeks is designed to mimic the type of long-term treatment that is administered to women.
2. Be careful not to place too large a piece of tissue into too small a volume of RNAlater as it is possible to overwhelm the RNA protection capacity of the reagent.
3. The 7 mm probe fits readily into these sizes of tubes but check your tubes before beginning. The bottom shape of some microfuge tubes may not be compatible with the probe. Also use caution as the 2 mL microfuge tubes are near capacity when the probe is introduced into 1 mL of volume.
4. If samples are especially small, homogenize the sample in 600 μL of Tri reagent, then rinse the probe in 400 μL of fresh Tri reagent and combine the two aliquots. The rinsing step rescues the drop of Tri reagent containing RNA that gets caught in the end of the probe and can substantially increase the recovery of RNA from small samples.
5. For soft tissue such as liver, three homogenization pulses will thoroughly disperse the tissue. For tissue with more muscle or connective tissue, more pulses of homogenization may be necessary to completely disperse the tissue. For especially difficult tissues, soak the minced tissue in Tri reagent for 1 h before beginning the homogenization step.
6. The RLT buffer in the RNeasy kits is a lysis buffer that can be used in place of Tri reagent for the homogenization step. Another alternative for sample homogenization is the QIAShredder column (Qiagen) which performs a homogenization step on the columns.
7. If using cultured cells for RNA extraction, remove the culture medium from the cells and rinse the cells with phosphate buffered saline. Add 1 mL Tri reagent to the cells in a T25 flask; the cells essentially “melt” in Tri reagent. If necessary, scrape the cells and transfer the Tri reagent containing the cell debris into vials. If pooling cells from several flasks, the milliliter of Tri reagent can be transferred from one flask to the next to keep the volume of Tri reagent at just 1 mL. Store the Tri reagent containing cellular material at −80 °C or proceed directly to RNA extraction.

8. The protein from the sample can be extracted from the bottom layer after this centrifugation step [24]. However, some proteins do not survive the protein extraction protocol well.
9. The advantage of using the RLT lysis buffer for the homogenization step (as in **Note 6** above) is that the sample can be placed directly onto the RNA purification column without the intervening centrifugation step. The disadvantage is that all of the genomic DNA and protein are still in the sample and go onto the column with the RNA. The columns are designed to bind the RNA while the DNA and protein pass through; however, the more genomic DNA that remains in the sample, the more likely it is that some will contaminate the RNA.
10. We observe better recovery of RNA if we elute twice with 50 μL volume each time than if we elute with a smaller volume. If the RNA is too dilute in this elution volume, it can be concentrated with a vacuum device such as the SpeedVac concentrator. We have found the silica gel membrane columns to yield more consistent RNA samples than the phenol/chloroform/isoamyl alcohol method [24]. However, not all of the silica gel columns are of equal quality and cheaper columns may compromise the quantity and quality of recovered RNA.
11. The quantity and quality of RNA can be assessed by spectrophotometry and agarose gel electrophoresis [24]. However, the Agilent Bioanalyzer has the advantage of producing both the quantity measurement and image of RNA bands while consuming only 1 μL of sample. At the same time, the Bioanalyzer provides an expression of the quality of the RNA sample called the RNA integrity number (RIN). Samples with an RIN number less than 8 should not be used for DNA microarray analysis.
12. If the quantity of total RNA available for the reaction is less than 0.2 μg or if you have reason to believe that a sample may not amplify efficiently, then carry out two rounds of aRNA amplification. Use the MessageAmp II kit to synthesize aRNA using the same reactions as described in Subheading 3.4 except do not incorporate biotin label into the aRNA in the final synthesis step. (Biotinylated aRNA cannot be used to synthesize cDNA.) Prime the single-stranded aRNA from the first reaction with random primers for synthesis of a second round of first-strand cDNA. Synthesize the second strand of cDNA using T7 oligo(dT) as the primer, and use the double-stranded cDNA for the synthesis of aRNA with the incorporation of biotin-11-UTP as described in Subheading 3.4.
13. In the initial reverse transcription reaction, the T7 oligo dT primer contains oligo (dT) sequence plus the T7 promoter sequence. The oligo (dT) sequence binds to the poly-A tail of messenger RNAs in the sample. During the reverse transcription

reaction, the T7 promoter sequence is added to each new cDNA transcript so it is present in the double-stranded cDNA when the second strand reaction is complete. The T7 enzyme in the IVT reaction is an RNA polymerase that will transcribe all transcripts that have the T7 promoter at its 5′ end, so it will transcribe the double-stranded cDNA that was synthesized in the reactions described here.

14. The synthesis of biotin-labeled aRNA from total RNA is a fairly long process. The process can be stopped and the samples frozen at −80 °C for continuation later after the second-strand synthesis reaction (**step 6** of Subheading 3.4), after purification of double-stranded cDNA (**step 17** of Subheading 3.4), or after purification of aRNA (**step 30** of Subheading 3.4).
15. The aRNA is fragmented before the hybridization step to reduce secondary and tertiary RNA structure which has been shown to interfere with RNA hybridization to probes on the microarray. The fragmentation is carried out by metal-induced hydrolysis.

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