

Microarray Technology for Use in Molecular Epidemiology

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Summary

Microarrays are a powerful laboratory tool for the simultaneous assessment of the activity of thousands genes. Remarkable advances in biological sample collection, preparation and automation of hybridization have enabled the application of microarray technology to large, population-based studies. Now, microarrays have the potential to serve as screening tools for the detection of altered gene expression activity that might contribute to diseases in human populations. Reproducible and reliable microarray results depend on multiple factors. In this chapter, biological sample parameters are introduced that should be considered for any microarray experiment. Then, the microarray technology that we have successfully applied to limited biological sample from all our molecular epidemiology studies is detailed. This reproducible and reliable approach for using microarrays should be applicable to any biological questions asked.

Key Words: Biological sample; epidemiology; microarray methods; microarray; molecular epidemiology; clinical specimen; gene expression; oligonucleotide array.

1. Introduction

Epidemiology is the distribution and determinants of diseases in human populations. Along with the development of powerful, high-throughput molecular technologies, the field of molecular epidemiology has evolved. As the name implies, molecular epidemiology focuses on the distribution and determinants of diseases at the molecular level. Now, studies of diseases in human populations can interrogate the contributions of genes, gene activity, proteins, and the environment.

Because population-based molecular epidemiology studies have the greatest potential to positively impact public health, the importance of the biological sample cannot be understated. The quality and standardization of the sample are as much a consequence of collection, handling, and storage as they are of isolation, extraction, and purification procedures (**Fig. 1**). The standardization of

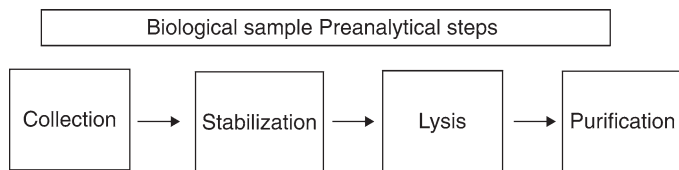


Fig. 1. The optimization and standardization of the preanalytic steps for the biological sample ensures reproducible and reliable results for any assay.

these first steps should be optimized to ensure representative and reproducible results. Each of these steps might vary depending on the sample type, what is analyzed (e.g., RNA, DNA, or protein) and the assay that is used and should be optimized prior to commencing any molecular epidemiology study.

The most logical and convenient biological sample for large, population-based molecular epidemiology studies is collected using noninvasive sampling methods and uniformly applied to a population. Examples of noninvasive samples commonly used in molecular epidemiology studies are peripheral blood, saliva, stool, urine, and exfoliated cells obtained by scraping or brushing. Peripheral blood is an attractive sample because it likely reflects ongoing systemic processes. Exfoliated cells, such as those collected for a Pap smear, might more closely reflect the disease but might have variable quality nucleic acids because of the dead and dying cells naturally present in this sample type (1,2). Different collection medium with various storage and extraction procedures should be attempted to extract the best possible quality RNA (Fig. 2).

The yield of nucleic acids from biologic samples is another important consideration for microarray technology. For example, exfoliated cervical cytology samples typically yield a low concentration of RNA so extracting total nucleic acid (RNA and DNA) to improve RNA recovery might be necessary (2). Our laboratory regularly uses microarray technology on peripheral blood samples and exfoliated cervical cytology. However, because exfoliated cervical cytology samples are more particular and are not as commonly used as peripheral blood for gene expression profiling, this chapter will focus on microarray technology as it applies to peripheral blood.

Peripheral blood serves as a representative sample of the systemic state allowing for evaluation and profiling of multiple pathological and physiological pathways (3,4). It is often desirable to use peripheral blood cell samples in multiple platforms, for example, total RNA in microarrays and live cells in functional assays. Typical venous collection provides live peripheral blood cells that are an ideal source of material for different assays. The ideal collection system would be blood tube that collects and separates the cells while maximizing cell viability, such as the BD Vacutainer® CPT™ (BD, Franklin Lakes, NJ) cell preparation

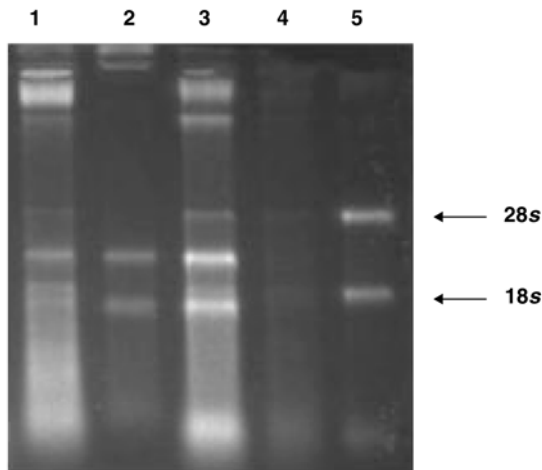


Fig. 2. RNA quality and quantity is variable depending on the sample source. **Lanes 1–3** have total RNA derived from exfoliated cervical cytology. **Lanes 4 and 5** have total RNA derived from peripheral blood cells.

tube. Because the cells are live following collection, steps should be taken to minimize the ex vivo cell stimulation to minimize alterations in gene expression (5). If only RNA from peripheral blood sample is necessary, then integrated blood collection systems such as the Paxgene[™] Blood RNA System (Qiagen Inc., Valencia, CA; PreAnalytix, <http://www.preanalytix.com/RNA.asp>) can be used. It should be noted that because this collection system is RNA specific, additional blood samples should be collected for other non-RNA assay types.

It is universally accepted that RNA quality and quantity are of paramount importance for assuring reliability of microarray results. Many different total RNA extraction methods are commercially available and each offers advantages. Given the different extraction chemistries involved, the single step phenol/guanidinium isothiocyanate methodology that is the basis to TRIzol[®] reagent (Invitrogen Corp., Calsbad, CA) and an RNA binding glass filter methodology for total RNA extraction was examined (6). Both methods produced similar quality microarray results but the single step method resulted in slightly greater total RNA yields and less DNA contamination. Use of a single method total RNA extraction method is one way to reduce experimental variability. RNA quality and quantity can be simultaneously assessed by evaluating 28S:18S rRNA using denaturing agarose gel electrophoresis. With appropriate standards and imaging software, this method is quantitative (Fig. 3) and has the advantage of indicating the presence of contaminating DNA. Recently, microfluidic-based platforms for the analysis of RNA have been developed (Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA). These “lab-on-a-chip” technologies are excellent

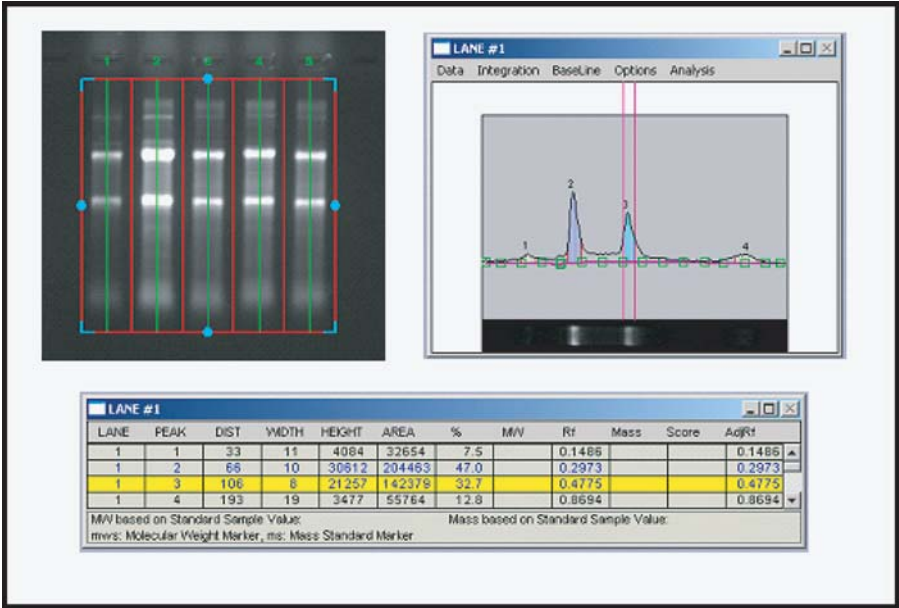


Fig. 3. Quantitation of ribosomal RNA (rRNA) bands by denaturing agarose gel electrophoresis using a control RNA of known concentration (**Lane 1**, 100 ng control RNA loaded). Using the Alpha Innotech gel imaging system (Alpha Innotech, San Leandro, CA), the area under the curve of the 28S and 18S rRNA bands are calculated, for the control RNA this area equates to 100 ng. The areas of each sample are then compared to the standard and the amount of RNA present in that sample determined. RNA concentration is determined by volume of RNA loaded onto the agarose gel. Intact total RNA run on a denaturing gel will have sharp, clear 28S and 18S rRNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band, a good indication that the RNA is completely intact. From clinical samples we rarely get such high ratios and have found that rRNA with a 28S:18S = 1 gives high quality microarray data.

alternatives to gel electrophoresis because they are sensitive, reproducible, and fast. The disadvantage is that residual DNA is not detected, which is an important consideration if the total RNA is subjected to any enzymatic amplification steps.

Although population-based studies offer the luxury of collecting hundreds to thousands of biological samples, the limitation is the quantity (e.g., volume) and the yield of the total RNA necessary for microarray applications. It is found that the average total RNA from an 8-mL blood collection tube is 5 μ g (3,6). One of the more common microarray methods uses RNA from two sources (reference and sample), each labeled with a different fluorophore that is hybridized to a microarray. This dual intensity method calculates the ratio of fluorescence signals

to determine the over- or underexpression of each gene. Although common, this method is not suited or feasible for population-based, high-throughput molecular epidemiology studies because 5–50 μg of total RNA is recommended for target synthesis (http://www.protocol-online.org/prot/Genetics___Genomics/Microarray/).

Therefore, in addition to optimizing sample collection and RNA extraction procedures to maximize yield, an alternative target-labeling technique and hybridization protocol (6) has been evaluated, adopted, and optimized. A biotin-labeled cDNA was generated from 2 μg of total RNA from each sample that is used as the target. Each target is hybridized to one microarray, generating a single intensity signal. The hybridized biotinylated cDNA is detected using the highly sensitive GeniconRLSTM (Resonance Light Scattering) system (Invitrogen Corp.). This employs gold particles for detection and has been demonstrated to have greater sensitivity than fluorescent detection systems (7). This higher sensitivity allows for the identification of low abundant mRNAs and is easily used with limited sample. Importantly, the signal is stable and does not photobleach, quench, or decay, thus hybridized microarray experiments can be archived indefinitely.

Automated hybridization of microarrays provides consistent hybridization conditions in between runs, low experimental variability and is high throughput. An automated platform such as the Ventana Discovery SystemTM (Ventana Medical Systems, Tucson, AZ) provides individual temperature-controlled slide pads, low hybridization volumes, and continuous mixing (Fig. 4). One disadvantage is the high cost; the hybridization stations are often expensive and not always feasible for a laboratory. In a comparison of manual with automated hybridization systems using the protocols described in this chapter, it is found that the reproducibility can be as good for manual hybridization as for automated hybridization if five times higher probe concentration is used in the manual protocol compared with the automated hybridization (5 μg compared with 2 μg , respectively).

Our molecular epidemiological studies using microarrays show low experimental variability and have proven that microarrays are powerful tools for screening for perturbations in multiple biological pathways (8,9). Great efforts have been given to optimize and standardize sample collection and microarray procedures (Figs. 5 and 6). All microarray experiments should adhere to the MIAME (Minimal Information About a Microarray Experiment) recommendations (10). In this chapter, the microarray experimental design used in all our molecular epidemiology studies has been provided in detail.

2. Materials

For all protocols use RNAase-free reagents, water, and plasticwares to minimize RNase contamination. Nonpowdered gloves are used for all manipulations and the best quality reagents are recommended for all manipulations.

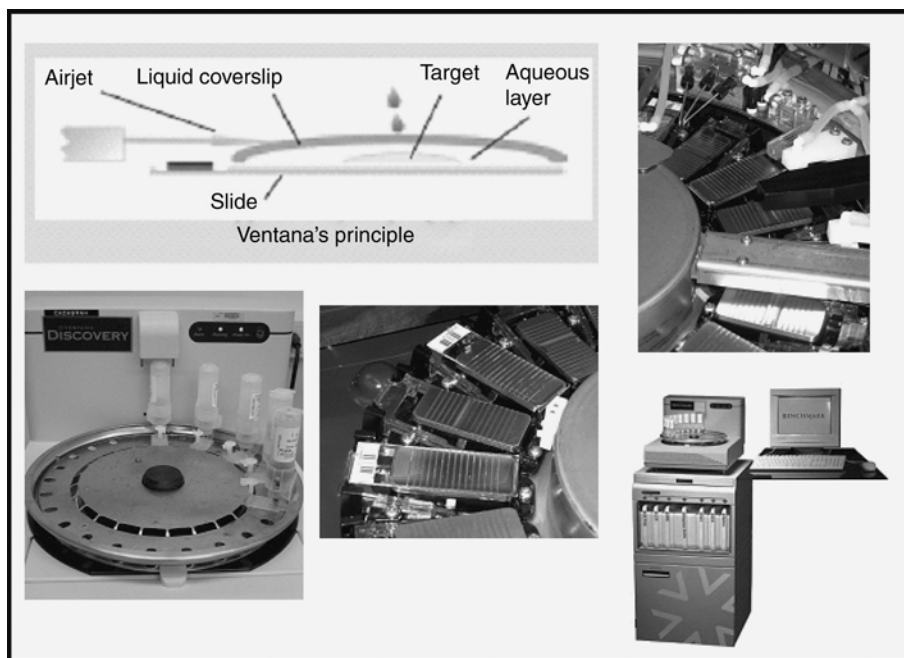


Fig. 4. Automated hybridization using the Ventana Discovery System.

2.1. RNA Extraction Using TRIzol

1. TRIzol Reagent (Invitrogen, cat. no. 15596-026).
2. Chloroform.
3. Isopropyl alcohol (2-propanol).
4. Ethanol (EtOH).

2.2. RNA Quantitation

1. An RNA sample of known concentration will work as the standard for the quantitation. Stratagene offers high quality, pure RNA from multiple sources.
2. UltraPure™ agarose (Invitrogen, cat. no. 15510-019).
3. Formaldehyde (Sigma, Aldrich, St. Louis, MO; cat. no. F8775).
4. Northern Max 10X MOPS (3-[N-morpholino] propane sulfonic acid) gel running buffer (Ambion Inc., Austin, TX cat. no. 8671).
5. RNA sample loading dye with ethidium bromide, (Sigma cat. no. R4268).

2.3. Direct Labeling of Target cDNA

1. Superscript II Reverse Transcriptase (Invitrogen, cat. no. 18064-014) supplied with 0.1 M DTT and 5X first strand buffer.
2. RNaseOut™ Recombinant Ribonuclease Inhibitor (Invitrogen, cat. no. 10777-019).
3. Random Primers, pd(N)₆ (Invitrogen, cat. no. 48190-011).
4. Oligo(dT)₁₂₋₁₈ (Invitrogen, cat. no. 18418-012).
5. Biotin-11-dUTP (Enzo Life Sciences, Farmingdale, NY; cat. no. 42806).

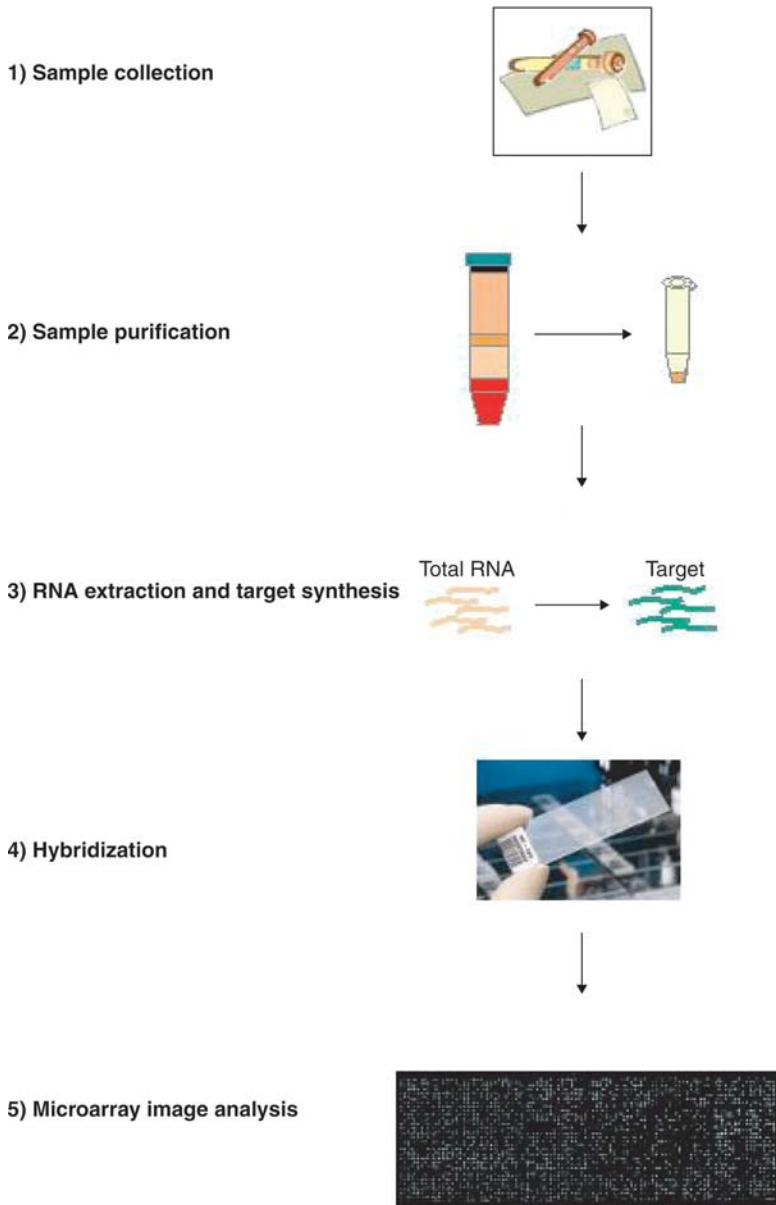


Fig. 5. Optimized and standardized procedures for sample collection and microarray methods results in reproducible and reliable results from limited biological sample.

6. dNTP Mix: 10 mM each of dGTP, dATP, and dCTP, and 2 mM dTTP. Aliquot and store in single use volumes. (Invitrogen, cat. nos. 10218014, 10216018, 10217016, and 18255018, respectively).

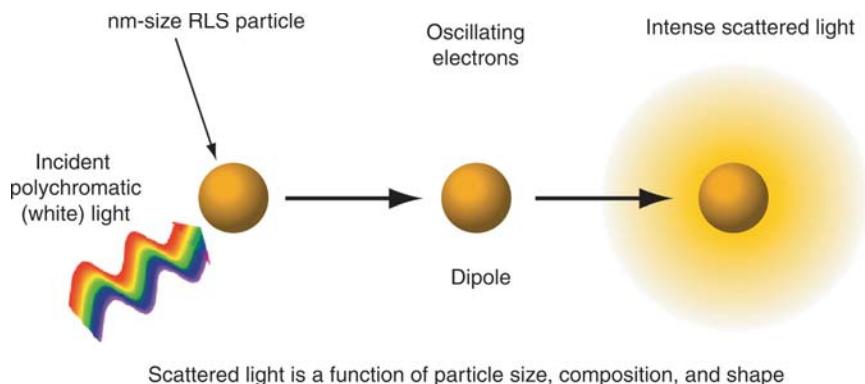


Fig. 6. GeniconRLS technology overview. When illuminated with configured white light, spherical gold and silver RLS Particles of uniform dimension (between 40 and 120 nm diameter) generate intense, scattered light signals. The signal obtained from an individual RLS Particle is up to 10^4 – 10^6 times greater than that obtained with the most sensitive fluorescent molecules. This improved signal intensity translates at the assay level to an approximate 10-fold greater sensitivity with RLS as compared with fluorescence. (Reproduced with kind permission of Invitrogen Corp.)

7. QiaQuick PCR purification kit (Qiagen, cat. no. 28106).
8. 2 M NaOH.
9. 1 M Tris-HCl, pH 7.5.
10. Ethanol.

2.4. Hybridization

1. Blocking buffer (400 mL): 4X saline sodium citrate buffer (SSC), 0.5% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin.
2. Lint-free paper towels.
3. Tissue-Tek® slide staining dishes (Sakura, Torrance, CA; cat. no. 4456).
4. A 24-slide capacity holder with a detachable handle for staining jars previously described (Sakura, cat. no. 4465A).
5. cDNA and oligonucleotide microarrays with compatible glass surface for hybridization and detection system (*see Note 1*).

2.4.1. Manual Hybridization

1. Water bath at 42°C.
2. Hybridization oven at 42°C.
3. Hybridization chamber (Rainin disposable pipet tip rack).
4. LifterSlips™ 25 × 60 mm² (Erie Scientific, Portsmouth, NH; cat. no. 25x60I-2-4789).
5. Hybridization cocktail: 50% formamide, 10X SSC, 0.2% SDS.
6. Wash buffer 1: 2X SSC, 0.1% SDS.

7. Wash buffer 2: 1X SSC.
8. Wash buffer 3: 0.1X SSC.
9. GeniconRLS Au particles and diluent (Invitrogen, *see Note 2*).

2.4.2. Automated Hybridization

1. Ventana Discovery System (Ventana Medical Systems Inc.).
2. ChipMap™ Kit: this contains optimized reagents for microarray applications on the Ventana Discovery System. It contains ChipPrep™ 1 reagent (Ventana Medical, ChipPrep™ 2 reagent Systems), ChipClean™ reagent, and ChipHybe™ reagent (Ventana Medical Systems, cat. no. 760-106).
3. Liquid cover slip (LCS) (Ventana Medical Systems, cat. no. 650-010).
4. RiboWash reagent (Ventana Medical Systems, cat. no. 760-105).
5. Reaction buffer (Ventana Medical Systems, cat. no. 950-300).
6. GeniconRLS Au particles and diluent (*see Note 2*).
7. Scanner: GSD-501™ (Invitrogen Corp.).
8. Pipet tips (Rainin Instrument, LCC, Woburn, MA).

2.5. Archiving

1. Archiving solution (Invitrogen, cat. no. 106225) (*see Note 2*).
2. 0.1% Tween-20.

3. Methods

3.1. RNA Extraction

1. Add 1 mL of TRIzol per 5×10^6 cells, gently resuspend the cells. Place the suspension in a microfuge tube and leave at room temperature for 5 min, insuring complete dissociation of protein complexes (*see Note 3*).
2. Following complete sample lysis add 200 μ L chloroform.
3. Cap tightly and vortex for 15 s.
4. Centrifuge at 12,000g for 15 min at 4°C.
5. Transfer the upper aqueous phase into a microfuge tube. Be careful not to incorporate any of the interphase.
6. Discard the tube with remaining organic phenol–chloroform phase into a hazardous waste container.
7. Precipitate the RNA from the aqueous phase by adding 0.5 mL isopropanol and mixing.
8. Incubate samples at room temperature for 10 min.
9. Centrifuge at 12,000g for 10 min at 4°C. The RNA forms a gel-like pellet on the side and bottom of the tube.
10. Remove the supernatant and wash the pellet by vortexing with 1 mL of 75% ethanol.
11. Collect the pellet by centrifugation at 7500g for 5 min at 4°C.
12. After removing the supernatant, air-dry the pellet and resuspend in 20 μ L RNase-free water.

3.2. Determining Quality and Quantity of Extracted RNA by Denaturing Agarose Gel Electrophoresis

These instructions assume the use of a $7 \times 10\text{-cm}^2$ agarose gel tray. They are easily adaptable to other formats.

3.2.1. Pouring the Agarose Gel (see **Note 4)**

1. Add 36 mL H_2O to 0.5 g agarose in 250-mL flask.
2. Microwave for 45 s until agarose is melted.
3. Place flask in a fume hood to cool down for 5 min.
4. Add 5 mL 10X MOPS buffer followed by 9 mL formaldehyde.
5. Mix and pour the gel into the tray ($7 \times 10\text{ cm}^2$).
6. Apply comb and let the gel harden for 30 min.

3.2.2. Preparing Samples to Load Gel

1. Prepare samples by placing 1 μL specimen RNA (or 100 ng control RNA), 4 μL H_2O , and 5 μL loading dye into a tube.
2. Heat at 70°C for 10 min. This will denature the RNA allowing it to migrate to its true molecular weight.
3. Spin.

3.2.3. Load and Run

1. Pour 1X MOPS buffer into the gel apparatus to cover the solidified gel.
2. Place the gel tray in the apparatus so samples migrate to the positive cathode.
3. Remove the comb.
4. Add 10 μL of sample per well.
5. Run at a constant 90 V with a 400 mA setting, for 60 min. Two running dyes are evident (xylene cyanol and bromophenol blue), the lower should be two-thirds of the way down the gel.
6. Visualize and quantitate RNA.
7. Examine the gel with ultraviolet (UV) illumination (see **Note 5**).
8. Densitometric measurements of band intensities of the 18S and 28S ribosomal RNA bands (as measured by the area under the curve) are used to determine amount of specimen RNA loaded. Calculated from the area under the curve of the known standard RNA (see **Fig. 1** and **Note 6**).

3.3. Preparation of Target cDNA

1. Adjust 2 μg of specimen total RNA to a final volume of 8 μL with RNase-free water (see **Note 7**).
2. To each 8 μL of RNA mix, add the following (see **Note 8**):
 - 1 μL Stock random primers (3 μg).
 - 1 μL dNTP mix.
 - 1 μL Oligo(dT) primers (0.5 μg).
 - 1 μL Biotin-11-dUTP (1 mM).

3. Incubate tube at 65°C for 5 min, spin down to remove condensation.
4. Add the following to the tube (*see Note 8*):
 - 4 μL 5X First strand buffer.
 - 2 μL 0.1 M DTT.
 - 1 μL RNaseOUT (40 U/ μL).
5. Mix gently and collect the contents of the tube by brief centrifugation.
6. Incubate at 42°C for 2 min.
7. Add 1 μL of SuperScriptII (200 U/ μL) to each tube.
8. Incubate at 42°C for 60 min. After cDNA synthesis is complete, immediately perform the following hydrolysis reaction to degrade the original RNA.
9. Add 2 μL 2 M NaOH to each reaction tube.
10. Incubate the tube at 70°C for 15 min.
11. Add 5 μL 1 M Tris-HCl, pH 7.5 to neutralize the pH and mix gently.
12. Cleanup reaction products using the QiaQuick PCR Purification kit. Add 135 μL buffer PB (Binding buffer, supplied with kit) to each sample (27 μL), and mix well (*see Note 9*).
13. Apply the whole volume of sample (162 μL) to the QiaQuick column preinserted into a collection tube, and centrifuge for 1 min at 3500g.
14. Discard the flowthrough and place the column back in the same collection tube.
15. Wash the column with 750 μL buffer PE (Wash buffer supplied with kit) and centrifuge for 1 min at 3500g.
16. Discard flowthrough, and place the column back in the same tube, and centrifuge for 1 min at 16,000g.
17. Place the column in a clean 1.5-mL microfuge tube.
18. To elute the DNA, add 30 μL buffer EB (Elution buffer supplied with kit) to the column, let stand 1 min and centrifuge for 1 min at 16,000g.
19. Add a further 30 μL of buffer EB to the column, incubate at room temperature for 1 min, and then centrifuge at 16,000g.
20. The combined eluate contains the purified labeled cDNA. This can be stored at -20°C until used for hybridization. Avoid freeze/thawing.

3.4. Hybridization

3.4.1. Manual Hybridization

3.4.1.1. BLOCKING OF ARRAYS

1. Block the array slides in preheated blocking buffer at 42°C for 45 min (*see Note 10*).
2. Rinse the arrays by dipping them in water five times.
3. Dry the arrays with filtered compressed air.

3.4.1.2. PROBE PREPARATION

1. For manual hybridizations the probe volume is reduced by vacuum centrifugation to 40 μL and an equal volume of hybridization cocktail added for a final volume of 80 μL .
2. Vortex the probe and heat at 95°C for 5 min to denature the probe. Centrifuge to collect condensation.

3. Place the blocked, dry array on a flat surface.
4. Clean a LifterSlip™ with 70% EtOH and lint-free wipes. Place the LifterSlip on the array covering the spotted area, such that the Teflon strips of the LifterSlip are on the long axis of the array, touching the glass (*see Note 11*).
5. Add the probe by placing the pipet tip along the open edge of the slip and slowly pipet the probe out of the tip, allowing capillary action to draw the probe mix under the slip. Be careful not to let air bubbles form.

3.4.1.3. HYBRIDIZATION

1. Prepare a humid hybridization chamber. Disposable pipet tip boxes that have a removable “shelf” that carries the pipet tips are used. This is removed and several layers of Whatmann filter paper (cut to fit) are saturated in 2X SSC and placed at the bottom of the box. This ensures that saturated filter paper does not come in contact with the array in such a way that the target would be absorbed by the filter paper. The shelf is then put in place over the filters (*see Note 12*).
2. Place the in a horizontal and level position and then put the humid chamber into a hybridization oven at 42°C and incubate overnight (16 h). Humidity is maintained in the hybridization oven with a container of nuclease-free water.

3.4.1.4. POSTHYBRIDIZATION WASHES

1. All wash buffers are prewarmed to 42°C in the staining jars.
2. Remove the hybridization chamber and dry the outside to prevent water dripping on the array. Keep the chamber setup for use in the detection of target on the array.
3. Place the array with LifterSlip into a jar of prewarmed wash 1. Angle the array so that the LifterSlip can fall to the bottom of the jar under its own weight. Place the array into the wash tank of wash 1 buffer at 42°C. (*see Note 13*).
4. After all slides are in the tank wash the arrays for 5 min. Agitate the arrays by lifting the slide carrier up and down.
5. Repeat this wash step in a second container of prewarmed wash buffer 1 at 42°C for 5 min.
6. Transfer the slide carrier to the prewarmed tank of wash buffer 2 at 42°C. Wash for 5 min with agitation.
7. Repeat **step 6** once.
8. The final stringency wash is in prewarmed wash buffer 3 at 42°C for 5 min.

3.4.1.5. DETECTION OF BIOTINYLATED TARGET

1. After the stringency washes slides are again blocked in blocking buffer at room temperature for 10 min.
2. To the hybridization chamber add a lint-free paper towel that has been prewetted with nuclease-free water to the rack on which the slides are placed. This is now used as a hydration chamber for the detection step.
3. Immediately before use prepare the GeniconRLS particles by mixing the suspension

with gentle vortexing.

4. In a microfuge tube add 60 μL of GeniconRLS Au particles to 30 μL of GeniconRLS particle diluent for each array on which a detection reaction needs to be performed. Mix gently to ensure dispersion.
5. Work on one slide at a time: remove the slide from the block solution, and drain the slide on a lint-free paper towel by dabbing the bottom edge of the slide on the towel.
6. Place the slide on the wetted paper towel in the hybridization chamber ensuring that the slide lies flat on the paper. The back of the slide needs to remain on the damp surface to prevent residue forming and drying.
7. Add 90 μL of the GeniconRLS particles to the bottom end of the slide.
8. Lower one edge of a LifterSlip onto the slide over the RLS particles and gently settle the other end of the LifterSlip onto the array, allowing the particles to wick over the full area.
9. Repeat **steps 6–8** until all arrays are set.
10. Seal the hybridization chambers and incubate at room temperature for 1 h.
11. Rinse the slide in wash buffer 3 and dry.

3.4.2. Automated Hybridization

The Ventana DiscoveryTM system is used for the automated hybridizations. This system offers the advantages of automated liquid-handling, individually controlled temperature pads for each microarray slide, and continuous mixing of hybridization solution under an oil-based LCS. This maximizes hybridization kinetics and allows for posthybridization stringency washes and target detection to be performed on the system.

3.4.2.1. METHODOLOGY FOLLOWS AS CLOSELY AS POSSIBLE THE CONDITIONS FOR MANUAL HYBRIDIZATION

1. The microarrays are blocked as for the manual hybridization (*see steps 1–3 in Subheading 3.4.1.1.*).
2. A bar-coded label is added to each array and the slides loaded onto the Ventana Discovery.
3. The ChipPrepTM1, ChipPrepTM2, and ChipCleanTM dispensers are loaded onto the reagent carousel.
4. The run is started.

3.4.2.2. AUTOMATED STEPS

1. Rinse slide with SSC solution
2. Apply ChipPrepTM1 and LCS. Incubate at 70°C for 10 min.
3. Rinse slides.
4. Apply ChipPrepTM 2 and LCS and incubate for 30 min.
5. Rinse slides.
6. Apply cover slip.

3.4.2.3. MANUAL STEPS

1. Add 200 μ L of ChipHybe™ to the 60 μ L of biotinylated target, denature at 100°C for 2 min, spin to collect and keep at room temperature until applied to the array.
2. Apply the targets to the appropriately labeled arrays, by pipetting just under the LCS. Do not allow the pipet tip to touch the surface of the array.

3.4.2.4. AUTOMATED STEPS

1. Warm the slide to 70°C and incubate for 6 min (denaturation step).
2. Warm the slide to 42°C and incubate for 8 h.
3. Stringency wash 1: 2X SSC at 42°C for 6 min.
4. Stringency wash 2: 1X SSC at 42°C for 6 min.
5. Stringency wash 3: 0.5X SSC at 42°C for 10 min.
6. Apply LCS.
7. Apply GeniconRLS Au particles and incubate for 30 min.
8. Rinse slide in reaction buffer.
9. Apply ChipClean™ and incubate for 2 min at 37°C.
10. Rinse slide in reaction buffer.

3.4.2.5. MANUAL STEPS

1. Lift the microarray slide carefully from the platform and wipe off residual oil from the back of the slide with lint-free paper. Rinse the slide with a squash-bottle filled with reaction buffer.
2. Place slides into the slide holder, bar-code down and wash with vigorous plunging in three changes of reaction buffer, each of about 1 min.
3. Rinse slides in two changes of 1X SSC, about 20 dips per change.
4. Dip slides in 0.1X SSC for 20 plunges.
5. Dry arrays with compressed air.

3.5. Archiving (see Note 14)

1. Aliquot 45 mL archiving solution into a 50-mL tube and let stand so air bubbles will dissipate. Bubbles might cause unevenness in the optical coating.
2. Dip each array into archiving solution up to the bar code or label.
3. Withdraw the array slowly. Ensure no air bubbles are trapped on the surface. If bubbles are evident redip. A smooth even coating is desired.
4. Blot the bottom edge of the array on a lint free wipe. Repeat this blotting several times.
5. Lean the array against a clean surface barcode edge up, ensuring that the wet surface does not come in contact with anything. Use a dust-free location this step is performed in a chemical cabinet. Allow to dry for 60 min.
6. Store in a well-sealed slide box.
7. To remove the archiving coating from the glass arrays. Immerse the array in a staining jar containing deionized water for 10 min. If the arrays are particularly dirty place them in 0.1% Tween-20 and agitate for 5 min. Briefly rinse in water and repeat the archiving procedure.

3.6. Scanning RLS™ Microarrays

1. The microarray images are captured using a GSD-501 scanner.
2. The slides are inserted face down with the slide label to the front.
3. In the image window mark the slide holders to be scanned and enter in file names.
4. In the preview window, select the particle type as gold and set exposure time in seconds (0.1–0.2 s is generally appropriate).
5. To determine the positioning of the array in the slide holder, set the area to be scanned by holding the mouse button and highlighting the space required.
6. Start the preview scan.
7. Open the “Brightness and Contrast” window select “mark saturated pixels.” Count the number of features that are saturated and set a final scan time that will give 1–2% of features saturation. This effectively scales the arrays from 0 to 65,000 pixels.
8. In the layout window, align the blue box to surround the area of the array for the final scan.
9. Start imaging.
10. Remove arrays from the scanner and place in a dirt-free container.

4. Notes

1. Slide surfaces that are compatible with the Ventana Discovery System include: aldehyde, amine, GAPS2, MWG epoxy, and UltraGAPS. Those that were found to be incompatible are from Agilent and the Erie epoxy (the Erie aldehyde and aminosilane are compatible).
2. These reagents are available as part of the GeniconRLS One-color Kit from Invitrogen.
3. An insufficient amount of TRIzol will result in contamination of the isolated RNA with DNA.
4. **Caution:** Formaldehyde is a suspected carcinogen and must be used and disposed of in accordance with federal, state, and local regulations. Always use formaldehyde in a fume hood.
5. Wear UV-protective safety glasses or a full safety mask to prevent UV damage to the face and skin.
6. A drawback to using denaturing agarose gels to assess integrity RNA is the amount of RNA required for visualization. For this protocol, at least 50 ng of RNA must be loaded onto the gel in order to be visualized with ethidium bromide. Some RNA preparations, such as those from exfoliated cervical cells, needle biopsies or from laser capture microdissected samples result in very low yields. In these cases, it might be impossible to spare 200 ng of RNA to assess integrity before proceeding with the expression profiling application. Currently we use an alternative to this methodology, the Agilent Technologies 2100, a microfluidic system that provides detailed information about the condition of RNA samples using as little as 1 μ L of 10 ng/ μ L per analysis.
7. RNA quantified by agarose gel electrophoresis or Agilent Bioanalyzer.
8. It is best to prepare master mixes when several reagents are to be added to each tube. The larger pipetting volumes increase accuracy and the reduced number of

steps saves time. When scaling calculations are made, generally an extra sample is added to the calculation to ensure adequate volumes are prepared.

9. Proceed with the purification of the labeled cDNA by removing any unincorporated nucleotides with the QiaQuick polymerase chain reaction purification kit. Before starting ensure ethanol has been added to the appropriate buffers in the kit.
10. It is found that blocking of glass arrays helped reduce the nonspecific background sometimes encountered with the RLS detection system. To ensure the blocking solution is clear and free from any precipitate filter it through a 0.40- μ m membrane before use. Make sure that all slides are adequately covered by the blocking solution.
11. Use of the LifterSlip greatly improves data quality by preventing nonuniform hybridization. These cover slips have a little edge of Teflon along two opposing outer edges (available from Erie Scientific). This creates a platform, which allows even distribution of the hybridization solution across the array.
12. If more than one array is being hybridized, use several chambers and do not overcrowd arrays. It is important to make sure nothing touches each array, so that the target does not wick out from the LifterSlip. Process one chamber at a time before and after hybridization.
13. Do not include more than eight arrays per wash station (the plastic holder can carry 24 slides). This insures proper washing.
14. The archiving process places an optical coating on the slides reducing the signal to background ratio during scanning. It also allows for prolonged storage of the arrays. The optical coating can be replaced if slides become scratched or dirty.

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