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Protocol for Gene Expression Profiling Using DNA Microarrays in *Neisseria gonorrhoeae*

Lydgia A. Jackson and David W. Dyer

Abstract

Gene expression profiling using DNA microarrays has become commonplace in current molecular biology practices, and has dramatically enhanced our understanding of the biology of *Neisseria* spp., and the interaction of these organisms with the host. With the choice of microarray platforms offered for gene expression profiling and commercially available arrays, investigators must ask several central questions to make decisions based on their research focus. Are arrays on hand for their organism and if not then would it be cost-effective to design custom arrays. Other important considerations; what types of specialized equipment for array hybridization and signal detection are required and is the specificity and sensitivity of the array adequate for your application. Here, we describe the use of a custom 12K CombiMatrix ElectraSense™ oligonucleotide microarray format for assessing global gene expression profiles in *Neisseria* spp.

Keywords

DNA microarray; Gene expression analysis; RNA purification; CombiMatrix; Electrochemical detection; *Neisseria* spp

1. Introduction

The last decade of study of the human pathogenic *Neisseria* spp. has been dramatically facilitated by the determination of the genome sequences of both *Neisseria gonorrhoeae* and *Neisseria meningitidis*. These achievements have supported the development of various microarray strategies that have been employed to perform gene expression profiling in *Neisseria* spp. (1–18), comparative genomics (19–26), multi-locus sequence typing (27), identification of potential vaccine antigens (28, 29), and the development of clinical assays (30–32) to identify these pathogens and their antibiotic resistance profiles. With the completion of the sequence of the human genome, microarrays also have been used to examine the host response to neisserial infection (33–39). Thus, it is no understatement that microarray technology has been instrumental in the rapid progress that has been made in the last decade in the study of *N. meningitidis* and *N. gonorrhoeae*. Our own experience with microarrays has employed spotted DNA arrays (13), Affymetrix arrays (1, 2), and most recently CombiMatrix ElectraSense arrays (manuscript in preparation) for gene expression profiling in the gonococcus. Each microarray format has advantages and disadvantages. Spotted microarrays offer low cost and ease of use, but often suffer from a lack of printing uniformity between microarrays. The Affymetrix oligonucleotide-based microarray is an extremely robust platform, but requires the availability of costly dedicated equipment and, in our experience with custom arrays, proprietary concerns can significantly hamper the easy

availability of this format. Affymetrix microarrays also are the most expensive of commercially available options. CombiMatrix oligonucleotide microarrays have many of the advantages of the Affymetrix format, but are less expensive and can be reused multiple times, cutting costs even further. The CombiMatrix 12K ElectraSense array is a sensor chip that is a specially modified semiconductor circuitry with over 12,000 individual microelectrodes (features). Following hybridization of the biotin-labeled target to the oligonucleotide probe, HRP-streptavidin conjugate binds to the biotin-labeled target. The resulting redox reaction occurring on each microelectrode that releases an electron is detected by the ElectraSense reader as a current proportional to the biotin-labeled target present, eliminating problems with spot uniformity present in other platforms and differences in array to array variability when using fluorescent detection. Using the ElectraSense process has additional advantages, including the elimination of the use of fluorescent dyes with concomitant problems including higher cost, photo bleaching, and the limited storage life of the labeled cDNA. Below, we describe the use of custom CombiMatrix ElectraSense microarrays for gene expression profiling in *N. gonorrhoeae*.

2. Materials

2.1. Gonococcal Growth Conditions

1. Complete defined medium (CDM); iron-free CDM-0 and CDM-10 (10 μ M Ferric nitrate) (¹, ²).
2. 10 mM Fe(NO₃) pH 1.0.
3. *N. gonorrhoeae* FA1090.
4. GC agar supplemented with 2 % Isovitalex (Becton Dickinson).
5. 37 °C incubator. 5 % CO₂.
6. 37 °C shaking incubator.
7. 500 mL side-arm flasks.

2.2. Gonococcal Total RNA Extraction “Hot Phenol Method” (See Note 1)

1. Water saturated phenol pH 7.9.
2. 100 % (v/v) Ethanol (store at –20 °C).
3. TE Buffer pH 8.0: 0.1 M EDTA, 10 mM Tris–HCl, pH 8.0.
4. 10 % SDS.
5. 1 M sodium acetate pH 5.5.
6. 3 M sodium acetate pH 5.5.

¹Isolation of RNA requires the researcher to take precautions to minimize the introduction of nucleases by treating the bench-top, pipettes, and any equipment with commercial products that eliminate RNases. We routinely use RNaseZap RNase Decontamination Solution. RNase-free buffers and reagents are available from Ambion. Gloves must always be worn to prevent contamination from nucleases present on the skin. Equally important is the use of barrier pipette tips. All dilutions of stock solutions listed in the Subheading 2 were done with Nuclease-free water.

7. 80 % (v/v) Ethanol.
8. Nuclease-free water.
9. 15 mL Falcon tubes.
10. RNaseZap RNase Decontamination Solution (Ambion/Applied Biosystems) (see Note 2).
11. Sterile 1.5 mL Eppendorf snap-cap microcentrifuge tubes (see Note 3).
12. 65 °C water bath.
13. NanoDrop™ ND1000 (NanoDrop Products, Fisher Thermo Scientific).
14. Microlite RF Centrifuge (Thermo IEC).
15. Freezers (−20 and −80 °C).
16. 100 % (v/v) Chloroform.

2.3. Assessment of RNA Quality

1. Agilent 2100 Bioanalyzer.
2. RNA 6000 Nano Chip Kit (Agilent).

2.4. DNase Treatment

1. RQ1 RNase-Free DNase (1 U/μL) with 10× Reaction Buffer (Promega).
2. Tri Reagent: phenol and guanidine thiocyanate in a mono-phase solution (Molecular Research, Inc.).
3. 100 % (v/v) Chloroform.
4. 100 % (v/v) Isopropanol.
5. Sterile 1.5 mL Eppendorf snap-cap microcentrifuge tubes (see Note 3).
6. Nuclease-free water (Ambion/Applied Biosystems).
7. 37 °C heat block.
8. 80 % (v/v) Ethanol (store at 4 °C).
9. Freezer (−80 °C).
10. NanoDrop™ ND1000 (NanoDrop Products, Fisher Thermo Scientific).
11. Microliter centrifuge.

2.5. Reverse Transcriptase and cDNA Labeling

1. High-Capacity cDNA Archive Kit (Applied Biosystems).

²Surfaces and equipment are decontaminated by spraying RNaseZap followed by rinsing with Nuclease-free water.

³Nuclease-free microcentrifuge tubes are available through Ambion. In our experience, sterilizing the microcentrifuge tubes by autoclaving is a cost-efficient alternative.

2. *Arabidopsis thaliana* mRNA positive controls (Stratagene):
 - SpotReport® mRNA Spike 1, Cab 10 ng/μL (store at –80 °C).
 - SpotReport® mRNA Spike 2, RCA 10 ng/μL (store at –80 °C).
 - SpotReport® mRNA Spike 3, RBCL 10 ng/μL (store at –80 °C).
3. LabelIT® μArray® Biotin Labeling Kit (Mirus).
4. 0.5 M EDTA, pH 8.0.
5. MicroAmp® Optical 96-well reaction plate (Applied Biosystems).
6. MicroAmp® 96-well clear adhesive film (Applied Biosystems).
7. QIAquick® PCR Purification Kit (Qiagen).
8. GeneAmp® 9700 thermal cycler (Applied Biosystems).
9. Nuclease-free water.
10. Sterile 1.5 mL Eppendorf snap-cap microcentrifuge tubes (see Note 3).
11. 37 °C heat block.
12. Centrifuge with 96-well plate carriers.

2.6. CombiMatrix Arrays: Hybridization and Data Collection

1. *N. gonorrhoeae* FA1090 CombiMatrix 12K ElectraSense™ Custom Array.
2. 12K ElectraSense™ Hybridization Chamber (CombiMatrix).
3. CustomArray™ holders for rotisserie (CombiMatrix).
4. Hybridization oven with rotisserie (e.g., Fisher Thermo Scientific).
5. Nuclease-free water.
6. Pre-Hybridization Buffer (50 μL): 5 μL 2× Hyb Solution Stock, 34 μL Nuclease-free water, 10 μL 50× Denhardt's Solution, 1 μL Sonicated Salmon sperm DNA (10 mg/mL). Before adding to the Pre-Hybridization Buffer, heat the Salmon sperm DNA for 5 min at 95 °C followed by 1 min on ice.
7. 2× Hyb Solution Stock (10 mL): 6 mL 20× SSPE, 100 μL 10 %/v/v Tween-20, 560 μL 0.5 M EDTA, 3.34 mL Nuclease-free water.
8. 50× Denhardt's Solution (Sigma-Aldrich).
9. Sonicated Salmon sperm DNA (Ambion/Applied Biosystems).
10. 10 % (v/v) Tween-20.
11. 0.5 M EDTA, pH 8.0.
12. 95 °C heat block.
13. Ice and ice bucket.
14. 20× SSPE Buffer: 3 M NaCl, 0.2 M Na₂PO₄, 0.020 M EDTA.

15. Hybridization Buffer (100 μ L): 50 μ L Hyb Solution Stock, 1 μ L Salmon sperm DNA heat denatured as listed for Pre-Hybridization Buffer, 4 μ L 1 % SDS, biotin-labeled cDNA up to 45 μ L, Nuclease-free water to bring the volume to 100 μ L.
16. Biotinylated cDNA (see Subheading 2.5).
17. 3 $\times$ SSPET Wash Solution (100 mL): 15 mL 20 $\times$ SSPE, 500 μ L 10 % Tween-20, 84.5 mL Nuclease-free water.
18. 0.5 $\times$ SSPET Wash Solution (100 mL): 2.5 mL 20 $\times$ SSPE, 500 μ L 10 % Tween-20, 97 mL Nuclease-free water.
19. PBST Wash Solution (100 mL): 20 mL 10 $\times$ PBS, 1 mL 10 % Tween-20, 79 mL Nuclease-free water.
20. 10 $\times$ Phosphate Buffered Saline (PBS): 1.37 M NaCl, 0.08 M Na₂PO₄, 0.02 M KH₂PO₄, 0.027 M KCl pH 7.4.
21. CombiMatrix ElectraSense Detection Kit™ (CombiMatrix).
22. CombiMatrix ElectraSense™ reader (CombiMatrix).

3. Methods

3.1. Gonococcal Growth Conditions

1. Growth in iron minus (CDM-0) and iron plus (CDM-10) medium was carried out as previously reported (¹, ²). *N. gonorrhoeae* strain FA1090 was grown overnight from glycerol stocks on GC agar supplemented with 2 % IsoVitalax at 37 °C and 5 % CO₂. Non-piliated organisms were selected and inoculated to another GC agar plate and incubated overnight for growth. Piliated and non-piliated colonies will grow as two distinct colony sizes with non-piliated colonies being larger than piliated colonies on GC agar. Growth of colonies from piliated *N. gonorrhoeae* causes significant clumping in broth making O.D. determinations difficult.
2. Non-piliated cells were inoculated into a 500 mL flask of CDM-0 to an absorbance of 0.100 at 600 nm. The flask was incubated at 37 °C (150 rpm) and the cells were allowed to double to an absorbance of 0.200 without the addition of ferric nitrate to deplete internal iron stores. Doubling time typically takes between 1.5 and 2 h.
3. The iron-depleted cells were then split and diluted twofold into 500 mL flasks containing either CDM-0 or CDM-10. 10 mM Fe(NO₃) pH 1.0 was added to the CDM-10 to a final concentration of 10 μ M.
4. The two flasks were returned to the 37 °C (150 rpm) incubator. Cells were collected at 3 h time points for RNA isolation.

3.2. Gonococcal Total RNA Extraction “Hot Phenol Method”

1. Add 5 mL of a 3 h culture (0.600 O.D.) into a 15 mL plastic conical Falcon tube.
2. Add 1:10 v/v of water saturated phenol pH 7.9 to ice cold 100 % ethanol to 5 mL of culture. (500 μ L water saturated phenol and 4.5 mL 100 % ethanol) (see Note 4).

3. Centrifuge for 10 min at $5,000 \times g$ and 4°C to pellet the cells.
4. Aspirate off the supernatant leaving as little medium behind as possible.
5. At this point, cell pellets may be snap frozen using dry ice or liquid nitrogen and stored at -80°C for up to a week.
6. Start the process of lysing the cells by adding 700 μL Buffer TE, pH 8.0, and transfer to a 1.5 mL sterile microcentrifuge tube.
7. Add 7 μL of 10 % SDS, invert several times, and put in a 65°C water bath for 1–2 min to lyse cells.
8. Add 77 μL of 1 M sodium acetate, pH 5.5, and 850 μL of hot (65°C) water saturated phenol and invert ten times (see Note 4).
9. Place the tubes in a 65°C water bath for 6 min. Invert tubes every 60 s.
10. Centrifuge 15 min at 4°C , $18,000 \times g$.
11. There will be two apparent phases after centrifugation. Aspirate off the top layer into a new sterile 1.5 mL microcentrifuge tube containing 850 μL 100 % chloroform and invert several times.
12. Centrifuge 15 min at 4°C , $18,000 \times g$.
13. Pull off the top aqueous layer being careful not to disturb the lower organic layer. Split the aqueous layer that carries the RNA into two sterile 1.5 mL microcentrifuge tubes (see Note 5).
14. Precipitate the RNA by adding 800 μL of cold ethanol (100 %) and 35 μL of 3 M sodium acetate to each tube.
15. Place the tubes at -80°C for at least 2 h (see Note 6).
16. Centrifuge tubes for 25 min at 4°C , $18,000 \times g$.
17. Decant the supernatant leaving the RNA pellet which should appear as white or translucent (see Note 7).
18. Wash the RNA pellet by adding 1 mL of cold 80 % ethanol and vortex gently.
19. Centrifuge for 15 min at 4°C , $18,000 \times g$.
20. Repeat ethanol wash steps 18 and 19.
21. Remove the last ethanol wash carefully and allow the RNA samples to air-dry for 10–15 min and then resuspend the pellets in Nuclease-free water (see Note 8). For our application, the desired RNA concentration is 1–2 $\mu\text{g}/\mu\text{L}$.

⁴Ensure that the water-saturated phenol is mixed well prior to use. The aqueous layer will separate out quickly.

⁵We find it advantageous to leave a small amount of the aqueous layer to prevent DNA and protein contamination, however; if the RNA quantity will be low, recover as much of the aqueous layer as possible.

⁶2 h is the minimum time to precipitate the RNA. We typically leave RNA samples overnight at -80°C to increase RNA yields.

⁷If the RNA pellet does not adhere to the side of the tube after centrifugation, aspirate off the supernatant being careful not to disturb the RNA pellet.

⁸Remove any residual ethanol from the sides of the tube with a pipette. We find it easier to remove small drops of ethanol using 10 μL barrier pipette tips. Do not overdry the RNA pellet as this makes the RNA less soluble.

22. Determine the RNA concentration of the samples using the NanoDrop 1000 (NanoDrop Products, Fisher Thermo Scientific) and store all samples at -80°C until DNase treatment.

3.3. Assessment of RNA Quality

Isolation of high quality RNA is essential for many downstream applications including generation of cDNA for microarrays and qRT-PCR, making it crucial to assess the RNA integrity prior to investing additional time and resources. A 260/280 ratio of 1.8 or greater indicates the purity of the RNA, but does not address the status of RNA degradation. We assess the quality of RNA samples using the Agilent Bioanalyzer. RNA samples were analyzed with the Agilent RNA 6000 Nano Chip Kit. The Bioanalyzer software generates an RNA Integrity Number (RIN) taking into account any degradation along the entire electropherogram and not just the 16S and 23S ribosomal RNA peaks. As seen in the electropherogram example in Fig. 1, the peaks for 16S and 23S ribosomal RNA are sharp with little degradation. The RIN score for this RNA sample was 9.2. An RIN of 10 is ideal indicating the least amount of degradation. Figure 2 shows an RNA sample with significant degradation. Note the 16S and 23S peaks are one broad peak and the increase in size of the 5S ribosomal RNA peak. The RIN was 4.2 for this sample, however, the 260/280 ratio was 2.0 (see Notes 9 and 10).

3.4. DNase Treatment

1. Add 1 U of Promega RQ1 RNase-Free DNase to 1 μg total RNA into a sterile 1.5 mL microcentrifuge tube.
2. Add 1/10 volume RQ1 RNase-Free DNase 10 $\times$ Reaction Buffer and Nuclease-free water to the appropriate final reaction volume taking into account the volume of the RNA and RQ1 RNase-free DNase (see Note 11).
3. Incubate the tube in a heat block at 37°C for 30 min.
4. Add 1 mL of TriReagent to the tube and proceed to RNA clean-up as detailed in step 5 below (see Note 12).
5. Add 200 μL chloroform, vortex, and centrifuge $12,000 \times g$ for 15 min, 4°C .
6. Carefully pull off the top aqueous layer containing the RNA into a new sterile 1.5 mL microcentrifuge tube.
7. Precipitate the RNA by adding 250 μL 100 % isopropanol, followed by a 15 min centrifugation at 4°C , $12,000 \times g$.

⁹An RIN number of 8.5 or greater generally indicates good quality RNA with modest degradation; however, this number will vary for different cells and/or tissues and different experimental conditions. Lower RIN values are acceptable if the downstream application is tested and validated by other methods.

¹⁰The 5S ribosomal peak will not be present if using Qiagen RNeasy columns to isolate the RNA. Nucleic acids smaller than 200 bp pass through the column. Many microarrays are now designed to detect small RNAs transcripts and may be missed using a column.

¹¹The following is an example of a 100 μL DNase treatment reaction using Promega DNase; 25 μL RNA at a concentration of 2.0 $\mu\text{g}/\mu\text{L}$, 10 μL RQ1 RNase-Free DNase 10 $\times$ Reaction Buffer, 50 μL RQ1 RNase-Free DNase (1 U/ μL), 15 μL Nuclease-free water.

¹²Total volume of the DNase reaction to which 1 mL of Tri Reagent is added should not exceed 200 μL .

8. Wash the RNA pellet by adding 1 mL cold 80 % ethanol. Centrifuge at 4 °C for 10 min, $12,000 \times g$.
9. Remove the supernatant and allow the pellet to air-dry for 10–15 min to remove any residual ethanol (see Note 8).
10. Resuspend all pellets in Nuclease-free water and determine the RNA concentration in the samples using the NanoDrop 1000 (NanoDrop Products, Fisher Thermo Scientific) and assess RNA quality as in Subheading 3.3. Store all samples at -80°C .

3.5. Reverse Transcription and cDNA Labeling

1. To generate the cDNA, reverse transcribe 10 µg of DNase-treated total RNA in a final volume of 100 µL using Applied Biosystems High-Capacity cDNA Archive Kit (see Note 13):
2. Prepare the MicroAmp 96-well reaction plate by adding 50 µL of a 2× Reverse Transcription (RT) master mix to the appropriate number of wells. Each 50 µL aliquot of 2× RT master mix contains, from the High-Capacity cDNA Archive Kit: 10 µL 10× RT Buffer, 4 µL 25× 100 µM dNTP, 10 µL Random Primer mix, 5 µL Multiscribe™ Reverse Transcriptase (50 U/µL), 21 µL Nuclease-free water.
3. Add 50 µL of RNA sample to each well containing the RT master mix and pipet up and down to mix. The 50 µL RNA sample will have 10 µg of DNase-treated RNA and 1 µL each of the three SpotReport mRNA positive control spikes diluted to 1 ng/µL. Add the appropriate amount of Nuclease-free water to bring the total volume to 50 µL (see Note 14).
4. Seal the plate with MicroAmp 96-well clear adhesive film. Briefly centrifuge the plate to spin down the contents and remove any air bubbles present.
5. Set the thermal cycler conditions: 25 °C for 10 min followed by 37 °C for 120 min and load the plate containing the RT reactions. The cDNA is now ready for labeling.
6. Following the Mirus cDNA labeling protocol, hydrolyze the RNA template by adding 0.3 volumes 0.5 M EDTA and 0.1 volume. Reagent D1 to the completed RT reaction in the 96-well plate.
7. Set the thermal cycler at 65 °C for 30 min and allow samples to cool to room temperature before continuing to the next step.
8. Neutralize samples by adding 0.125 volume (according to the original reaction volume) of Neutralization Buffer N1.

¹³The following is an example of a 100 µL RT reaction using the High-Capacity cDNA Archive Kit (Applied Biosystems) with an RNA sample at a concentration of 2.0 µg/µL; 10 µL 10× RT Buffer, 4 µL 25× dNTP, 10 µL Random Primers, 5 µL Multiscribe™ Reverse Transcriptase (50 U/µL), 5 µL DNase-treated RNA (2.0 µg/µL), 1 µL SpotReport® mRNA Spike 1 (1 ng/µL), 1 µL SpotReport® mRNA Spike 2 (1 ng/µL), 1 µL SpotReport® mRNA Spike 3 (1 ng/µL), 63 µL Nuclease-free water.

¹⁴All reagents and 96-well plates should be placed on ice when adding RT master mix and RNA samples.

9. Purify the cDNA using QIAquick PCR Purification method. Add 5 volumes of Buffer PB to 1 volume of the biotin-labeled cDNA.
10. Bind the cDNA to the column by applying the sample to a QIAquick column and spin for 30 s. Discard the flow through (see Note 15).
11. Wash the column by adding 750 μ L Buffer PE (see Note 16) and centrifuge for 30 s. Discard the flow through and centrifuge again for an additional 1 min to remove any residual ethanol.
12. Place the column in a new 1.5 mL microcentrifuge tube and elute by carefully adding 30 μ L of Nuclease-free water directly to the column membrane. Allow the column to set for 1–2 min. Centrifuge the column for 2 min.
13. Measure the cDNA concentration on the NanoDrop 1000, choosing ssDNA-33 as sample type.
14. Label 3 μ g of purified cDNA in a 100 μ L reaction volume according to the Mirus kit protocol: in a sterile 0.5 μ L micro-centrifuge tube, add the appropriate volume of cDNA sample up to 86 μ L, 10 μ L 10 $\times$ Labeling Buffer and Nuclease-free water to bring the volume to 96 μ L, then add 4 μ L *Labe/IT* μ Array Biotin Reagent (see Note 17).
15. Incubate the labeling mixture at 37 $^{\circ}$ C for 1 h in a heat block.
16. Add 0.1 volume Reagent D1 and incubate at room temperature for 5 min, followed by addition of 0.1 volume Neutralization Buffer N1, incubate on ice for 5 min.
17. Clean up the biotin-labeled cDNA using the QIAquick PCR Purification method as described previously in steps 9–13. The biotin-labeled cDNA is now ready to proceed to hybridization (see Note 18).

3.6. CombiMatrix Arrays: Hybridization and Data Collection

Our laboratory focuses on iron regulation in the human pathogen *N. gonorrhoeae*. We are currently interested in the role small RNAs play in the gonococcal iron regulon by assessing the global transcriptional profile in iron minus and iron plus growth conditions. Our custom 12K gene expression array was designed by CombiMatrix's bioinformatics team. The in situ technology uses an electrochemical process to synthesize probes/features (30–40-mers) directly onto the chip. Included in the custom array are factory-built negative controls and “no-synthesis” (quality controls) positioned in areas throughout the chip. *A. thaliana* genes were added as positive controls. We also included intergenic regions meeting bioinformatic criteria for small RNA expression. These intergenic features were designed for both the sense and antisense strands. If possible, all annotated genes had five to six features spanning the entire open reading frame. Figure 3 shows a typical gonococcal gene expression microarray. Each feature is visually represented as a square. Since the ElectraSense is an

¹⁵All steps in purification process of the biotinylated cDNA and centrifugation (14,000 $\times g$) are done at room temperature.

¹⁶Ensure that the appropriate amount of 100 % ethanol has been added to the Buffer PE.

¹⁷The micrograms of cDNA that are biotin-labeled should be empirically determined for each organism and/or sets of experimental conditions. We found that 3 μ g of biotin-labeled cDNA gave an optimal hybridization signal on our custom array.

¹⁸We have used biotin-labeled cDNA that was stored at -20° C for up to 2 days on our arrays without significant loss of signal.

electrochemical detection method, the read out will be a direct numeric quantification of the hybridization signal. Grid alignment or spot finding is not necessary.

1. Place the array in the 12K ElectraSense hybridization chamber, add Nuclease-free water, and incubate at 65 °C for 10 min. Unless otherwise stated, all arrays are rotated during incubations using the custom array holders for the rotisserie. After the 65 °C incubation step, bring the hybridization chamber to room temperature and refill with Pre-Hybridization Buffer. Incubate at 50 °C for 30 min.
2. Denature the hybridization solution containing the biotin-labeled cDNA by heating for 3 min at 95 °C and 1 min on ice.
3. Add the denatured biotin-labeled cDNA to the hybridization chamber and incubate for 16 h at 50 °C.
4. Rinse the array by filling the hybridization chamber with 3× SSPET pre-warmed to 50 °C. Pull off the rinse, add fresh 3× SSPET, and incubate for 5 min.
5. Rinse the array with 0.5× SSPET and again add fresh 0.5× SSPET and incubate at room temperature. All room temperature steps are done on the bench top.
6. Rinse the hybridization chamber with PBST Wash Solution and refill the chamber. Incubate at room temperature for 2 min. The array is now ready for post-hybridization labeling. Bring the ElectraSense Detection Kit™ to room temperature.
7. Remove the PBST Wash Solution and add the Blocking Buffer from the ElectraSense Detection Kit. Incubate at room temperature for 15 min.
8. Remove the Blocking Buffer and add the Biotin Labeling Solution to the hybridization chamber. Incubate at room temperature for 30 min.
9. Rinse the hybridization chamber with Biotin Wash Solution and refill with the Wash Solution. Incubate for 5 min at room temperature.
10. Repeat the Biotin Wash Solution step two more times.
11. Remove the Biotin Wash Solution and rinse with TMB Rinse Solution of the ElectraSense detection kit. Quickly remove the TMB Rinse Solution and add TMB Substrate Solution of the ElectraSense detection kit.
12. Place the hybridization chamber in the ElectraSense reader and scan the array within 1 min. Arrays can be rescanned by taking out the TMB Substrate Solution, rinsing first with Biotin Wash and then with TMB Rinse Solution before adding fresh TMB Substrate (see Note 19).

¹⁹A common scan error message is failure of some of the electrodes to read. This is usually the result of the electrical contact pads on the array slide not being clean. When this occurs, the contacts are cleaned and the hybridization chamber is washed first with Biotin Wash and then TMB Biotin Rinse, followed by fresh TMB substrate, and the slide rescanned. The ElectraSense arrays have the advantage of being able to be scanned multiple times, but in our hands most arrays were scanned at least twice and occasionally three to four times to get a good read.

4. Notes

¹Isolation of RNA requires the researcher to take precautions to minimize the introduction of nucleases by treating the bench-top, pipettes, and any equipment with commercial products that eliminate RNases. We routinely use RNaseZap RNase Decontamination Solution. RNase-free buffers and reagents are available from Ambion. Gloves must always be worn to prevent contamination from nucleases present on the skin. Equally important is the use of barrier pipette tips. All dilutions of stock solutions listed in the Subheading 2 were done with Nuclease-free water.

²Surfaces and equipment are decontaminated by spraying RNaseZap followed by rinsing with Nuclease-free water.

³Nuclease-free microcentrifuge tubes are available through Ambion. In our experience, sterilizing the microcentrifuge tubes by autoclaving is a cost-efficient alternative.

⁴Ensure that the water-saturated phenol is mixed well prior to use. The aqueous layer will separate out quickly.

⁵We find it advantageous to leave a small amount of the aqueous layer to prevent DNA and protein contamination, however; if the RNA quantity will be low, recover as much of the aqueous layer as possible.

⁶2 h is the minimum time to precipitate the RNA. We typically leave RNA samples overnight at -80°C to increase RNA yields.

⁷If the RNA pellet does not adhere to the side of the tube after centrifugation, aspirate off the supernatant being careful not to disturb the RNA pellet.

⁸Remove any residual ethanol from the sides of the tube with a pipette. We find it easier to remove small drops of ethanol using 10 μL barrier pipette tips. Do not overdry the RNA pellet as this makes the RNA less soluble.

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¹¹The following is an example of a 100 μL DNase treatment reaction using Promega DNase; 25 μL RNA at a concentration of 2.0 $\mu\text{g}/\mu\text{L}$, 10 μL RQ1 RNase-Free DNase 10 $\times$ Reaction Buffer, 50 μL RQ1 RNase-Free DNase (1 U/ μL), 15 μL Nuclease-free water.

¹²Total volume of the DNase reaction to which 1 mL of Tri Reagent is added should not exceed 200 μL .

¹³The following is an example of a 100 μL RT reaction using the High-Capacity cDNA Archive Kit (Applied Biosystems) with an RNA sample at a concentration of 2.0 $\mu\text{g}/\mu\text{L}$; 10 μL 10 $\times$ RT Buffer, 4 μL 25 $\times$ dNTP, 10 μL Random Primers, 5 μL MultiscribeTM Reverse Transcriptase (50 U/ μL), 5 μL DNase-treated RNA (2.0 $\mu\text{g}/\mu\text{L}$), 1 μL SpotReport[®] mRNA Spike 1 (1 ng/ μL), 1 μL SpotReport[®] mRNA Spike 2 (1 ng/ μL), 1 μL SpotReport[®] mRNA Spike 3 (1 ng/ μL), 63 μL Nuclease-free water.

¹⁴All reagents and 96-well plates should be placed on ice when adding RT master mix and RNA samples.

- ¹⁵All steps in purification process of the biotinylated cDNA and centrifugation (14,000×g) are done at room temperature.
- ¹⁶Ensure that the appropriate amount of 100 % ethanol has been added to the Buffer PE.
- ¹⁷The micrograms of cDNA that are biotin-labeled should be empirically determined for each organism and/or sets of experimental conditions. We found that 3 µg of biotin-labeled cDNA gave an optimal hybridization signal on our custom array.
- ¹⁸We have used biotin-labeled cDNA that was stored at –20 °C for up to 2 days on our arrays without significant loss of signal.
- ¹⁹A common scan error message is failure of some of the electrodes to read. This is usually the result of the electrical contact pads on the array slide not being clean. When this occurs, the contacts are cleaned and the hybridization chamber is washed first with Biotin Wash and then TMB Biotin Rinse, followed by fresh TMB substrate, and the slide rescanned. The ElectraSense arrays have the advantage of being able to be scanned multiple times, but in our hands most arrays were scanned at least twice and occasionally three to four times to get a good read.

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Fig. 1. Electropherogram of RNA isolated from *N. gonorrhoeae* FA1090 grown in CDM in iron minus conditions. The smallest peak is 5S ribosomal RNA. The peak at 25 nucleotides is the internal sizing standard. nt indicates nucleotide size. FU indicates Fluorescent Units (see Note 10).

Fig. 2. Electropherogram of RNA isolated from *N. gonorrhoeae* FA1090 grown in CDM in iron minus conditions showing significant RNA degradation. The peak at 25 nucleotides is the internal sizing standard. nt indicates nucleotide size. FU indicates Fluorescent Units.

Fig. 3. Biotinylated cDNA from *N. gonorrhoeae* FA1090 grown in CDM in iron minus conditions was hybridized to a 12K CombiMatrix ElectraSense™ CustomArray. The array was scanned and electrochemical detection read on the CombiMatrix ElectraSense™ reader. Signal intensity values and annotation information for each feature were exported for analysis in GeneSpring GX 11.