Preface

Finding a cure for melanoma will be dependent on a greater understanding of the complex molecular interactions that begin in the skin, prior to melanoma's ruthless spread and distribution at distant metastatic sites. Melanoma is one of the most virulent forms of cancer that is now at epidemic proportions worldwide. It is increasingly responsible for deaths and loss of livelihood for countless individuals in their second, third, or fourth decades. With the completion of the sequencing of the 3.1 billion nucleotides that comprise the Human Genome Project, it is now possible to intensify efforts at deciphering the biochemical cascade of events in melanoma, beginning with the relevant genes and the proteins they encode. One of the main challenges is not only to come to grips with the rapid pace of technological advances, but to integrate the new tools of modern genomics with a sound biological perspective that has clinical relevance.

The aim of *Melanoma Techniques and Protocols: Molecular Diagnosis, Treatment, and Monitoring* a volume in the *Methods in Molecular Medicine*™ series, is to provide a comprehensive and up-to-date summary of the most important advances in the field pertaining to melanoma. Each author was instructed to provide clear-cut experimental protocols (including detailed "Notes" on points that often are not spelled out in regular publications), to ensure that investigators outside the field could successfully use these techniques in their own laboratories. As can be appreciated by surveying the table of contents, a highly diverse group of authors were enlisted to provide expert reviews in their respective areas including perspectives in clinical medicine, molecular biology, tumor immunology, and pathology. Authors who could write from first-hand experience were selected to ensure that relevant expertise and direct knowledge of the technique and literature were presented for the reader.

This book is divided into four major categories including: Biology, Diagnosis, Treatment, and Monitoring of Patients with Melanoma. For each category, "cutting-edge" techniques are presented, and discussed within a biological context so that the complexities of melanoma may be better understood by each reader who completes the entire book. Though it is true that only modest clinical improvements have been made for melanoma patients, despite the exhilarating progress in the dissection of many molecular mysteries, it is hoped that this book will be seen as providing forward-thinking perspectives and experimental pro-

tocols designed to help ensure more rapid clinical breakthroughs. Coupling technology to biology at the molecular level should provide a sound basis for progress, and hope to both patients and investigators grappling with this disease.

I would like to express my gratitude to all of the authors who contributed chapters to this book, and to Professor John Walker for his editorial assistance. This book is dedicated to my wife, Debra, and our daughters, Megan and Kelly.

Brian J. Nickoloff, MD, PhD

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Isolation of Tumor Suppressor Genes in Melanoma by cDNA Microarray

Yan A. Su and Jeffrey M. Trent

1. Introduction

The multistep genetic alterations thought to involve both oncogenes and tumor suppressor genes that are causally related to melanocytic transformation remain largely undetermined ***(1)***. Mapping of alterations to chromosome 6 indicates that multiple genetic loci on 6q contribute causally to the development and progression of malignant melanoma ***(1)***. This notion is also supported by the introduction of chromosome 6 in malignant melanoma cell lines suppressing either their tumorigenicity ***(2)*** or metastasis ***(3,4)***. However, the suppressor genes involved have yet to be identified.

Human melanoma cell lines UACC903, UACC903(+6), and SRS3 were derived from two steps of genetic manipulation ***(2,5)***. Specifically, the parental malignant melanoma cell line UACC903 was derived from a primary melanoma specimen and displays anchorage-independent growth and rapid population doubling in plastic culture ***(2)***. The UACC903(+6) cell line was generated by introduction of a *neo*-tagged human chromosome 6 into the parental cell line via a microcell-mediated chromosome transfer ***(2)***. Phenotypically, the chromosome 6–mediated suppressed cell line UACC903(+6) is anchorage dependent and slower in growth than the parental cell line UACC903 ***(2)***. The SRS3 cell line was induced from the UACC903(+6) cell line by retroviral transduction ***(5)***. The phenotypic features of SRS3 are similar to those of its grandparental cell line UACC903. These three cell lines are genetically linked and phenotypically display readily distinguishable growth features. They provide us with the unique cellular resource for the successful identification of tumor suppressor genes by DNA microarrays ***(6)***.

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The DNA microarrays allow the simultaneous detection of RNA levels of thousands of genes ***(7,8)***. Briefly, cDNA templates for genes of interest are amplified from plasmid clones carrying human genes by polymerase chain reaction (PCR) using the vector sequence-specific primers. Following purification and quality control, aliquots of cDNA (1–16 ng) are printed on poly-lysine-coated glass microscope slides using a computer-controlled, high-speed robot. Total RNA from both the test and reference samples are labeled with either Cy3-dUTP or Cy5-dUTP using a single round of reverse transcription from oligo-(dT) primers. Equal amounts of the labeled DNA are combined and allowed to hybridize under stringent conditions to the probes on the array. Laser excitation of the incorporated fluorescence yields an emission with characteristic spectra, which are measured using a laser scanner. The scanned images are pseudo-colored and merged for comparison of a normalized ratio between the labeled Cy3-dUTP and Cy5-dUTP DNA hybridized to the clones on the array. Information about the clones, including gene name, clone identifier, intensity values, intensity ratios, normalization constant, and confidence intervals, is attached to each clone. The normalized intensity ratios from a single hybridization experiment are interpreted as follows. The significant deviations in the ratio from 1 (no change) are indicative of increased (>1) or decreased (<1) levels of gene expression relative to the reference sample. This technology has greatly facilitated studies of genomewide gene expression in various cancers ***(6,8–17)***. Applying this technology, we have measured the relative expression levels of thousands of genes among the cell lines UACC903, UACC903(+6), and SRS3 and have identified tumor suppressor genes ***(6)***. In this chapter, we describe this technology as a general method for isolation of tumor suppressor genes.

2. Materials

1. Tissue culture dish (60-mm) with grid (cat. no. 83.1801.001; Sarstedt, Newton, NC).
2. PCR plates (96-well) (cat. no. T-3031-21; ISC BioExpress, Kaysville, UT).
3. SeqPlaque low-melting agarose (cat. no. 50101; FMC BioProducts, Rockland, ME).
4. Bio-Spin 6 chromatography column (cat. no. 732-6002; Bio-Rad, Hercules, CA).
5. Cleaning solution: 400 mL of ddH_2O, 100 g of NaOH (cat. no. S-0899; Sigma, St. Louis, MO), 600 mL 95% ethanol (190 proof; Warner-Graham, Cockeysville, MD).
6. Cot1 DNA, 10 mg/mL (cat. no. 15279-011; Life Technologies, Rockville, MD).
7. Cover slips (22 × 55 mm) (cat. no. 125485E; Fisher, Pittsburgh, PA).
8. Cy3-dUTP (1 m*M*) (cat. no. NEL578; NEN, Boston, MA).
9. Cy5-dUTP (1 m*M*) (cat. no. NEL579; NEN).
10. ddH_2O: deionized water repurified by Barnstead E-pure System (Chesapeake Instruments, Columbia, MD).

11. Diethylpyrocarbonate (DEPC)-treated ddH_2O: 1 mL of diethylpyrocarbazole (cat. no. D-5758; Sigma) and 1 L of ddH_2O; mix well, leave at room temperature overnight, and autoclave for 20 min.
12. dNTP (100 m*M* each) (cat. no. 27-2035-02; Amersham Pharmacia Biotech, Piscataway, NJ).
13. dNTP with low dTTP (10X): contains 5 m*M* dATP, dGTP, and dCTP; 2 m*M* dTTP.
14. 0.1 *M* Dithiothreitol (cat. no. 18064-014; Life Technologies).
15. 0.5 *M* EDTA, pH 8.0 (cat. no. 360-500; Biofluids, Rockville, MD).
16. 100% Ethanol (200 proof; Warner-Graham).
17. Ethanol/acetate precipitation mixture: 247 mL of 100% ethanol and 13 mL of 3 *M* sodium acetate (pH 6.0).
18. 5X First-strand buffer (cat. no. 18064-014; Life Technologies).
19. Glass slide racks (cat. no. 900200; Wheaton Science, Millville, NJ).
20. Gold Seal slides (cat. no. 3011; Gold Seal Products, Portsmouth, NH).
21. Hybridization bottles (35 × 150 mm) (cat. no. 052-002; Biometra, Tampa, FL).
22. KH_2PO_4 (cat. no. P-0662; Sigma).
23. Microcon 100 (cat. no. 42412; Millipore, Bedford, MA).
24. Microcon 30 column (cat. no. 42409; Millipore).
25. MicroHyb hybridization solution (cat. no. HYB125.GF; Research Genetics, Huntsville, AL).
26. 1 *M* Na borate buffer: 61.83 g of boric acid (Sigma, Cat. No. B0394), 750 mL ddH_2O, adjust pH to 8.0 with 10 *N* NaOH (cat. no. S-0899; Sigma), add ddH_2O to 1 L, autoclave for 20 min, cool to room temperature, and filter through with a 0.22-mm filter.
27. Oligo dT primer (1 μg/μL of 10–20mer mixture) (cat. no. POLYT.GF; Research Genetics).
28. pcDNA3 (Invitrogen, Carlsbad, CA).
29. Poly dA (cat. no. POLYA.GF; Research Genetics).
30. Poly-L-lysine solution: 70 mL of poly-L-lysine (0.1% [w/v]) (cat. no. P8920; Sigma), 70 mL of tissue culture phosphate-buffered saline (PBS), and 560 mL ddH_2O.
31. Primers for PCR amplification of cDNA inserts (cat. no. GF200.primer; Research Genetics) including forward primer (5′-ctgcaaggcgattaagttgggtaac-3′) and reverse primer (5′-gtgagcggataacaatttcacacaggaaacagc-3′).
32. RNAsin (40 U/μL) (cat. no. 799025; Boehringer Mannheim, Indianapolis, IL).
33. RNeasy Midi Kit 50 (cat. no. 75144; Quiagen, Valencia, CA).
34. Sodium dodecyl sulfate (SDS) (cat. no. L5750; Sigma).
35. Sequence-verified human cDNA clones (cat. no. 042600; Research Genetics).
36. Succinic anhydride blocking solution: 6 g of succinic anhydride (cat. no. 23969-0; Aldrich, Milwaukee, WI), 325 mL of 1-methyl-2-pyrrolidinone (cat. no. 32863-4; Aldrich), and 25 mL of 1 *M* Na borate buffer (pH 8.0).
37. Superbroth (cat. no. 08-406-001; Advanced Biotech, Columbia, MD).
38. Superscript II reverse transcriptase (200 U/μL) (cat. no. 18064-014; Life Technologies).
39. *Taq* polymerase with PCR buffer and 50 m*M* Mg^{2+} (cat. no. 10342-020; Life Technologies).

40. TE buffer: 10 m*M* Tris and 1 m*M* EDTA, pH 7.5.
41. TE_L buffer: 10 m*M* Tris-HCl and 0.1 m*M* EDTA, pH 8.0.
42. AGTC kit (cat. no. 91528; Edge BioSystems, Gaithersburg, MD).
43. Tissue culture PBS: 8.00 g of NaCl (cat. no. S9625; Sigma), 0.20 g of KCl (cat. no. P-3911; Sigma), and 1.44 g Na_2HPO_4 (cat. no. S-0876; Sigma); add ddH_2O up to 1 L, autoclave for 20 min, cool to room temperature, and filter through a 0.22-µm filter (cat. no. 430517; Corning Costar, Corning, NY).
44. Tris base (cat. no. BP152-1; Fisher).
45. 1 *M* Tris-HCl, pH 7.5 (cat. no. 351-007-10; Quality Biological, Gaithersburg, MD).
46. Trizol reagent (cat. no. 15596-026; Life Technologies).
47. U-bottomed and 96-well plates (cat. no. 3799; Costar).
48. V-bottomed 96-well plates (cat. no. 3894; Costar).
49. Yeast tRNA (cat. no. R-8759; Sigma).
50. α^{33}P-dCTP (cat. no. AH9905; Amersham Pharmacia Biotech).
51. 2X RPMI medium (cat. no. 402G-777; Biofluids, Rockville, MD).
52. Fetal bovine serum (FBS) (cat. no. 10437-028; Life Technologies).

3. Methods

3.1. Extraction of Plasmid DNA from Cultured Bacteria

3.1.1. Day 1

1. Add 100 µL of Superbroth with 200 µg/mL of ampicillin in the wells of U-bottomed 96-well plates. Label the A1 corner.
2. Place the frozen 96-well plates holding the cDNA library at room temperature.
3. Spin the thawed plates at 1000 rpm for 2 min in a centrifuge (Sorvall Super T21).
4. Fill a container with 200 proof alcohol. Dip the 96-pin inoculation block in the alcohol. Flame the pins using a lit gas burner.
5. Allow the inoculation block to cool. Dip the pins in the library plate. Inoculate the LB plate (be sure to match the A1 corners of two plates). Reflame the inoculation block. After the flames are extinguished, return the inoculation block to the alcohol bath.
6. Repeat as necessary for each plate that you need to inoculate.
7. Reseal the library plates and return to –80°C.
8. Place the inoculated plates into a “zip-lock” bag containing a moistened paper towel. Inoculate the bag at 37°C overnight.

3.1.2. Day 2

1. Label the A1 corner of the 96-well AGTC culture blocks. Fill each well with 1 mL of Superbroth with 200 µg/mL of ampicillin.
2. Inoculate the culture blocks with the 96-pin inoculation block as in **Subheading 3.1.1., step 4**. Incubate the culture at 37°C and 200 rpm overnight in an Innova 4300 (New Brunswick Scientific, Edison, NJ).

3.1.3. Day 3 (Using the AGTC Kit)

1. Place the lysis buffer at 37°C. Fill the receiver plates with 350 μL of 100% ethanol. Label the receiver plates and place the filter plates on top.
2. Spin the 96-well AGTC culture blocks containing the bacteria at 3000 rpm for 7 min. Decant the supernatant immediately. Invert briefly and tap on a clean paper towel to remove the remaining droplets.
3. Add 100 μL of resuspension buffer with 1% RNase (v/v) to each well. Mix thoroughly using the Vortex Genie II Mixer (cat. no. 12-812; Fisher) with the 96-well plate insert (cat. no. 12-812D; Fisher). Add 100 μL of lysis buffer and mix well by tilting the block. Incubate at room temperature for 5 min.
4. Add 100 μL of precipitation buffer and then 100 μL of neutralization buffer. Seal the plates with the sealers from the AGTC kit. Vortex the plates.
5. Transfer the mixture immediately to the labeled filter/receiver plates prepared in **step 1**. Tape the stacks together without the lids.
6. Spin the stacked plates at 3000 rpm for 12 min in the centrifuge (Sorvall Super T21).
7. Remove the filter block. Decant the liquid in the receiver block. Touch off on clean paper.
8. Add 500 μL of 70% ETOH to each well. Decant immediately. Touch off excess drops on a clean paper towel.
9. Place the plates in a drawer with the lids off and cover with clean paper towels and allow to dry overnight.

3.1.4. Day 4

1. Add 200 μL of TE_L buffer to each well. Place the plates at 4°C overnight to allow the DNA to dissolve in the solution.
2. Randomly select 10 samples from each plate to measure the concentrations using a spectrophotometer (Beckman DU640). The concentrations are 100–300 ng/μL.

3.2. PCR Amplification of cDNA Inserts from Plasmid DNA

3.2.1. Day 1

1. Make up the PCR mixture (scale up the volume as necessary) as given in **Table 1**.
2. Add 79 μL of the PCR mixture to each well of 96-well PCR plates and then 1 μL of DNA templates (100–300 ng).
3. Carry out PCR cycles at 95°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s; 72°C for 10 min. Store at 4°C.
4. Fill each well of the V-bottomed 96-well plates with 160 μL of the alcohol/acetate mixture. Label each plate appropriately (library, plate number, date).
5. Transfer the PCR products from **step 3** to the equivalent wells containing the alcohol/acetate mixture.
6. Keep the plates overnight at –20°C.

Table 1
TK

Reagent	Stock solution	Final concentration	1 Reaction (μL)	100 Reactions (μL)
PCR buffer	10X	1X	8	800
dATP	100 m*M*	0.2 m*M*	0.16	16
dTTP	100 m*M*	0.2 m*M*	0.16	16
dGTP	100 m*M*	0.2 m*M*	0.16	16
dCTP	100 m*M*	0.2 m*M*	0.16	16
Forward primer	1 m*M*	0.4 μ*M*	0.032	3.2
Reverse primer	1 m*M*	0.4 μ*M*	0.032	3.2
Taq polymerase	5 U/μL	0.03 U/μl	0.5	50
ddH_2O			70.79	7079.6
Total			80	8000

3.2.2. Day 2

1. Keep the plates at room temperature for 5 min.
2. Spin the plates at 3200 rpm for 60 min. Decant the supernatant and add 70% ethanol.
3. Turn on the ImmunoWash 1575 (cat. no. 170-7009; Bio-Rad). Open the cover. Prime the system twice following the manufacturer's instructions. Select "Run" and then "Remove ETOH." Remove the ETOH and reprime. Select "Run" and then "Add ETOH" to add ETOH.
4. Spin the plates at 3200 rpm for 60 min. Remove ETOH with the ImmunoWash 1575.
5. Place the plates in a drawer, cover with clean paper towels, and allow the plates to dry overnight.

3.2.3. Day 3

1. Add 30 μL of 3X saline sodium citrate (SSC) to each well of the plates. Seal the plates with foil sealer. Keep the plates at 60°C for 2 h and room temperature for 2 h to dissolve the DNA. On average, the concentrations of these DNA samples are >200 ng/μL. Randomly select six clones from each plate to run 1% agarose gel to visualize the sizes of the clones.
2. Store the plates at –20°C.

3.3. Coating Slides with Poly-L-lysine

1. Place Gold Seal slides in a glass slide rack (10 slides/rack) in a glass tank.
2. Add 250 mL of cleaning solution. Shake the glass tank at room temperature for 2 h on an Environ Shaker (model 3527-5; Lab-Line, Melrose Park, IL).
3. Rinse the slides with ddH_2O five times, 2–5 min each time.

4. Transfer the slides into a new slide tank with 250 mL of poly-L-lysine solution.
5. Shake at room temperature for 1 h. Rinse the slides for 1 min with ddH_2O once.
6. Spin the slides at 1000 rpm for 2 min in the centrifuge (Sorvall Super T21).
7. Place the slides in the slide rack within a dust-free drawer at room temperature overnight.
8. Transfer the slides into a clean slide box. Age the slides for 2 wk at room temperature.

3.4. Printing cDNA Clones on Treated Slides

1. Turn on GMS 417 Arrayer (Affymetrix) and the PC controlling the arrayer. Run GMS 417 Arrayer software.
2. Click the "Setup" button. Select "Microplates Preference." Select 96-well plates. Enter "3" for microplates per group and "1" for hits per dot. Click the "OK" button.
3. Click the "Setup" button. Select "Slides Preference." Select the type of slides to print. Click the "OK" button.
4. Click the "Microplates" button. Select "Auto Generate Microplate" button. Click the "Array" button. Enter the number of 96-well plates with genes to be printed. As many as 72 plates (6912 genes) can be printed on a single slide by using pins with 150-μ diameters.
5. Prime the pumps of the GMS 417 Arrayer by clicking the "Prime Pumps" button.
6. If necessary, adjust the levels of the ring and the pin by following the manufacturer's instruction in the user handbook.
7. Wash the pin and ring by clicking the "Wash Pin Head" button.
8. Place 42 of the prepared glass slides on the slide holders in the GMS 417 Arrayer.
9. Transfer 25 μL of prepared DNA samples into V-bottomed 96-well plates.
10. Place three 96-well plates on the plate holders in the GMS 417 Arrayer and close the door.
11. Start to print by clicking the "START" button.
12. Change the 96-well plates when all three plates are printed. Repeat changing until all the plates of genes are printed.
13. Age the printed slides for 1 wk at room temperature.
14. Create one spreadsheet of 96 genes for each 96-well plate printed. Each spreadsheet contains Row (A–H), Column (1–12), Clone ID, and gene title. This spreadsheet will be used to generate the Array List file to analyze arrayed images later. *See* **Table 2** from example of 14 genes on Plate 1 printed.

3.5. Succinic Anhydride Blocking

1. Place the printed slides in the UV crosslinker (Life Technologies).
2. UV crosslink DNA on the slides at a dosage of 450 mJ.
3. Place the slides in a glass slide rack (10 slides/rack).
4. Place the slide rack in the glass tank with 250 mL of the succinic anhydride blocking solution. Shake at room temperature for 25 min on the Environ Shaker at 75 rpm.

Table 2
Example of 14 Genes on Plate 1

Row	Column	Clone ID	Title of Gene
A	1	136798	Fibronectin 1
A	2	34449	Expressed sequence tags
A	3	141953	CD36 antigen
A	4	271478	Max-interacting protein 1
A	5	24415	Tumor suppressor gene p53
A	6	131268	Growth factor receptor-bound protein 14
A	7	138345	Protein tyrosine phosphatase type IVA
A	8	140352	Colony-stimulating factor 2 receptor, alpha
A	9	155145	Matrix metalloproteinase 19
A	10	161172	Growth arrest–specific homeo box
A	11	49496	Programmed cell death 8
A	12	50893	Expressed sequence tags
B	1	172726	Neurexin II
B	2	259291	Integrin, beta 5

5. Transfer the slides immediately into boiling ddH_2O in a beaker on a stirrer/hot plate (cat. no. 43-2904-50; PGC Scientific). Turn off the heat, and incubate for 2 min.
6. Transfer the slide rack in the slide tank with 100% ethanol for 1 min.
7. Spin the slides at 1000 rpm for 2 min in a Sorvall Super T21 centrifuge.
8. Keep the slides at room temperature in a clean slide box overnight.

3.6. Purification of Total RNA from Cultured Cells

3.6.1 RNA Extraction (using Trizol Reagent)

1. Decant the medium in two culture flasks (175 cm^2) containing 90% confluent cells. Wash the cells with 15 mL of PBS once. Add 17.5 mL of Trizol reagent to each flask.
2. Transfer the cell lysate to a 50-mL Oak Ridge centrifuge tube (cat. no. 3119-0050; Nalge Nunc, Rochester, NY). Add 0.2 mL of chloroform/mL of Trizol reagent used. Mix well and incubate at room temperature for 5 min.
3. Spin the tubes at 12,000*g* for 15 min at 4°C in a centrifuge (Sorvall RC5B).
4. Transfer the aqueous phase solution to three fresh tubes (cat. no. 352059; Falcon, Becton Dickinson, Franklin Lakes, NJ). Add 0.9 mL of isopropyl alcohol to each milliliter of aqueous solution collected. Mix well and incubate at room temperature for 10 min.
5. Spin the tubes at 12,000*g* for 10 min at 4°C. Remove the supernatant. Wash the RNA pellet once with 75% ethanol. Air-dry the pellet at room temperature for 10 min.

6. Dissolve the pellet into 200 μL of DEPC-treated ddH_2O. Measure the concentration of RNA and adjust the concentration to 0.5 mg/mL.

3.6.2. RNA Purification (using RNeasy Midi Kit 50)

1. Mix 1 mL (500 μg) of the extracted RNA with 3.8 mL of Buffer RLT. Mix well.
2. Add 2.8 mL of 100% ethanol. Mix well.
3. Transfer the sample to one Rneasy Midi spin column. Spin at 5000*g* for 2 min.
4. Apply 2.5 mL of Buffer RPE. Spin at 5000*g* for 5 min.
5. Transfer the column to a new collection tube.
6. Add 250 μL of DEPC-treated ddH_2O. Spin at 5000*g* for 5 min. Repeat the elution once.
7. Transfer the samples to a Microcon 100 with collection tubes. Spin at 500*g* for 12 min.
8. Invert the Microcon 100 and place them into new collection tubes. Spin at 3000 rpm for 3 min to collect purified RNA samples.
9. Measure the concentrations of RNA. Adjust the concentration to 10 μg/μL with DEPC-treated ddH_2O.

3.7. Labeling First-Strand cDNA with Cy3- and Cy5-dUTP (using MicroMax Kit)

1. Mix the following in a tube: 5 μL of RNA (10 μg/μL), 2 μL of unlabeled control RNA, 2 μL of DNTP/primer mix, 4 μL of DEPC-treated ddH_2O.
2. Incubate for 10 min at 65°C. Cool for 5 min at room temperature.
3. Add 4 μL of Cy3-dUTP to one sample. Add 2 μL of Cy5-dUTP and 2 μL of DEPC-treated ddH_2O to the other sample. Warm to 42°C for 3 min.
4. Add 2.5 μL of 10X RT reaction buffer and 2 μL of AMV RT/Rnase inhibitor mix to each tube. Mix well, and quick spin.
5. Incubate for 1 h at 42°C. Cool to 4°C for 10 min.
6. Add 2.5 μL of 0.5 *M* EDTA and 2.5 μL of 1 *N* NaOH to each tube.
7. Incubate for 30 min at 65°C. Cool to 4°C for 5 min.
8. Add 6.2 μL of 1 *M* Tris-HCl (pH 7.5) to each tube.
9. Add 500 μL of 10 m*M* Tris-HCl (pH 7.5) to the Microcon 100. Spin for 10 min at 500*g*.
10. Add 200 μL of 10 m*M* Tris-HCl (pH 7.5) to the Microcon 100. Add Cy3-dUTP-labeled cDNA and Cy5-dUTP-labeled cDNA into the Microcon 100.
11. Spin at 500*g* for 4 min. Check the volume in the Microcon 100. Repeat the spin until the sample volume reaches about 25 μL.
12. Add 500 μL of 10 m*M* Tris (pH 7.5). Repeat the spin until the sample volume reaches about 25 μL.
13. Invert the Microcon 100 and place them into a new collection tube. Spin at 500*g* for 5 min to collect the labeled cDNA sample.
14. Dry the sample in a SpeedVac Concentrator (Savant).

15. Completely dissolve the sample in 20 μL of hybridization buffer by heating at 50°C for 10 min.

3.8. Microarray Hybridization

1. Overlay a cover slip onto a microarrayed glass slide.
2. Heat the labeled sample at 90°C for 2 min to denature the DNA. Cool for 5 min at room temperature. Quick spin.
3. Pipet all 20 μL of the sample onto the edge of the cover slip and allow the material to be drawn underneath the cover slip by capillary action.
4. Pipet 400 μL of 2X SSC onto a 10-cm^2 dust-free tissue (e.g., small KimWipe) and place in a 50-mL conical tube.
5. Place the microarrayed glass slide over the tissue and seal the cap tightly.
6. Ensure that the slide is level and stable in a 65°C incubator. Allow the hybridization to proceed overnight.
7. Wash the slide in 0.5X SSC and 0.01% SDS until the cover slip falls off.
8. Wash in 0.5X SSC and 0.01% SDS for 15 min.
9. Wash in 0.06X SSC and 0.01% SDS for 15 min.
10. Wash in 0.06X SSC for 15 min.
11. Spin the slide at 1000 rpm for 2 min in a centrifuge (Sorvall Super T21).

3.9. Scanning and Analyzing Microarray Images (using GenePix 4000A)

1. Turn on the GenePix 4000A Microarray Scanner (Axon Instruments, Foster City, CA). Turn on the computer of the scanner, and run the GenePix software.
2. Slide the door of the GenePix 4000A open. Lift the locking latch to unlock and open the door of the slide holder. Insert a microarray slide with the arrays facing down into the holder. Close the door and lock the latch. Slide the door of the GenePix 4000A closed.
3. Click the "Preview Scan" button to acquire rapidly a rough representation (40 μm/pixel) of the microarray.
4. Click the "View Scan Area" button to draw a region to be scanned. Select the "Zoom" button and zoom in the region.
5. Click the "Hardware Settings" button. By increasing or decreasing the current voltage, each of the photomultiplier tubes can be set such that only a few pixels are saturating in each image and the peak of the green histogram overlaps quite closely with the peak of the red histogram.
6. Click the "High Resolution Scan" button to acquire a high resolution (10 μm/pixel) of the selected microarray image with the selected photomultiplier tubes.
7. Click the "Save Images" button to save the images as 16-bit multiimage TIFF files with a file name you select.
8. Click the "New Blocks" button. For "Blocks," enter the row and column numbers for total blocks that are 2 and 2 for the GMS 417 Arrayer 2 × 2 pins. Specify the distance between the blocks. For "Features," enter the row and column num-

bers for total features (printed spots) within each block. Specify the distance between two spots and the size of the spots. For “Feature Layout,” select rectangular.

9. Align the features within a single block precisely on the image by zooming, moving, resizing and rotating the blocks on the computer screen.
10. Double-click the block that has been aligned to open the “Block Properties” dialog box. Select the “Apply to all” to align all blocks.
11. Click “Feature Mode.” Click a feature indicator that is not aligned precisely. Move it by clicking arrows. Resize it by pressing “Control Key” and clicking arrows.
12. Click “Save/Loading Settings” to save the image with the settings.
13. Click “Array List Generator.” Add each spreadsheet generated at **Subheading 3.4, step 14** in the same order as printing, i.e., from Plate 1, Plate 2, Plate 3, to the last plate printed.
14. Click “Create Array List.” Save the Array List file.
15. Click the “Load Array List” button. Open the Array List file.
16. Click the “Analyze” button. The results (gene title, clone ID, intensities, ratios, and others) analyzed and computed from the raw images of each printed gene are displayed and can be saved as a tab-delimited text file using the “Save As” button.
17. From these results, differentially expressed genes are readily identified. If necessary, one can perform Northern analysis to confirm the differential expression of genes interested.
18. If analyses such as clustering, construction of two-dimensional classification trees (dendogram), and principal components are needed, investigators should consult a statistician or a bioinformatic expert.

3.10. Database Search

1. Go to Netscape.
2. Go to www.nhgri.nih.gov/DIR/LCG/arraydb/cgi/query_clone_sets.cgi.
3. Type one of the items such as gene title, clone ID, accession number, plate number, or cluster number that you need to search. Click the “Submit” button.
4. Click on the gene title to find bioinformation under UniGene Cluster for ESTs or Gene Card, PubMed, GeneBank, Genomes, LocusLink, OMIM, Proteins, and Structures for a known gene.
5. The database search allows one to see the current bioinformation of genes interested.

3.11. Transfection (using Cell-Porator [cat. no. 71600-019, 11609-013; Life Technologies])

1. Clone a DNA fragment containing an interested coding region into mammalian expression vector pcDNA3.1 (cat. no. V790-20; Invitrogen) by using standard molecular cloning methods ***(18)***.
2. Harvest human cells cultured in an appropriate medium to 70% confluence.
3. Mix 5 μL of plasmid DNA (1 μg/μL) with 5×10^6 cells in 0.5-mL serum-free culture medium in a 0.4-cm gap electroporation chamber (cat. no. 11601-028; Life Technologies). Keep the chamber on ice for 10 min.

4. Add ice water between the safety chamber and the chamber rack of the Cell-Porator.
5. Place the electroporation chamber into the chamber rack.
6. Set up the conditions at 500 V/cm, low Ω, and 330 μF capacitance.
7. Start the electroporation by clicking the "Start" button.
8. Remove the chamber from the chamber rack and place on ice for 10 min.
9. Transfer the electorporated cells on 100-mm tissue culture dishes (2.5×10^6 cells/dish). Add 10 mL of culture medium. Incubate at 37°C with 5% CO_2 overnight.
10. Change the fresh medium with 600 μg/mL of G418 on the next day to select for the transfected cells.

3.12. Soft Agar Assay

1. Bottom SeqPlaque low-melting agarose: Mix 0.9 g of agarose with 34 mL of ddH_2O in a 125-mL bottle. Autoclave for 20 min. Keep the bottle in a 45°C water bath for 45 min.
2. Medium for bottom agarose. Mix 10 mL of FBS, 45 mL of 2X culture medium, 0.6 mL of G418 (100 mg/mL), and 10.4 mL of ddH_2O for a total of 66 mL. Filter through a 0.22-μ Sterile Filter System (cat. no. 430767; Corning Costar) and warm in a 45°C water bath for 45 min.
3. Mix the medium with the bottom agarose very well. Pool 5 mL to each 60-mm dish. Store the dishes at 4°C overnight.
4. Top SeqPlaque low-melting agarose: Mix 0.35 g of agarose with 20 mL of ddH_2O in a 125-mL bottle. Autoclave for 20 min. Keep the bottle in a 45°C water bath for 45 min.
5. Medium for top agarose: Mix 10 mL of FBS, 45 mL of 2X culture medium, 0.6 mL of G418 (100 mg/mL), and 24.4 mL of ddH_2O for a total of 80 mL. Filter through the 0.22-μ Sterile Filter System and warm in a 45°C water bath for 45 min.
6. Mix the top agarose with the medium very well. Keep the bottle in a 45°C water bath.
7. Harvest the cells from 90% confluent monolayer culture. Resuspend the cells in culture medium at a concentration of 10^5 cells/mL of medium.
8. Aliquot 8 mL of top agarose medium. Incubate in a 37°C water bath for 3–10 min.
9. Mix 100 μL of cell suspension with 1.9 mL of the top agarose medium. Pour the mixture on hardened bottom agarose medium in a 60-mm dish. Plate at least three dishes for each cell line.
10. Leave the dishes at 4°C for 30 min.
11. Place the dishes in a 37°C incubator with 5% CO_2. Count the colonies after 3 to 4 wk of culture.

4. Notes

The cDNA microarrays provide an unprecedented high throughput technology for detection of genomewide gene expression. Microarray, array hybrid-

ization, scanning image, and data analysis are essential components of this technology. The number of cDNA clones (or unigenes) has been increasing greatly owing to the advancement of the Human Genome Project. The easy access to cDNA clones, array robots, and image scanners has made this technology available widely. The techniques for robotic microarrays, array hybridization, and scanning image have become mature. In addition, many kits are available to facilitate microarray-related expression studies. There has been a dramatic increase in the use of cDNA microarrays.

We wish to address a few areas related to the microarrays. First, analysis of genomewide gene expression is a daunting task, especially on a large sample. How one can extract a nature law out of expression patterns is extremely challenging. Expertise combining biology, computer science, and statistics would be quite helpful in analyzing expression data. Second, microarrays frequently reveal hundreds of genes with the differential expression. To determine which genes play an important role in determining a given phenotypic feature remains to be solved. It is extremely important to design an experiment to ask and answer a precise question. We demonstrated that the comparison of expression profiles between multiple genetically close-linked and phenotypically distinguishable cell lines led to the identification of tumor suppressor genes ***(6)***. This rationale can be generalized because it allows the recognition of a small number of genes critical to the determination of phenotype. Third, if the aim of experiments is to find an unknown gene, that gene may not be included in a particular microarray. The methods such as cDNA subtraction ***(19)***, differential display ***(20)***, and representational difference analysis ***(21)*** may be considered to complement this limitation. Fourth, it is desirable to be able to compare expression profiles of the same cells between experiments, even if they are carried out under different experimental designs. The cross-comparison would expand our knowledge of expression changes of the same genes under the different conditions and decrease experimental costs. However, because of the lack of a "common" reference, gene expression levels of the same cells derived from different experiments or from different laboratories cannot be cross-compared currently. Finally, microarrays allow the detection of RNA levels. If RNA levels are different, the protein level may be different too. If RNA levels are the same, the protein levels may or may not be the same. The microarray detects only one of layers of gene expression. One should use other experiments to confirm or verify microarray data.

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