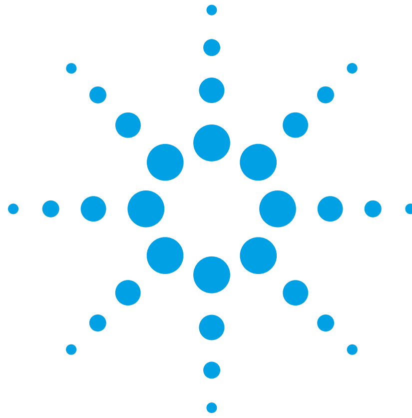


Two-Color Microarray-Based Prokaryote Analysis

FairPlay III Labeling

Before you begin, view hands-on videos of SurePrint procedures at http://www.agilent.com/genomics/protocolvideos.

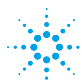
Protocol

For use with Agilent Gene Expression oligo microarrays

Version 2.0, August 2015

Microarrays manufactured with Agilent SurePrint Technology

For Research Use Only. Not for use in diagnostic procedures.



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Manual Part Number

G4813-90010

Edition

Version 2.0, August 2015

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Santa Clara, CA 95051 USA

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WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In this Guide...

This document describes the Agilent recommended procedures for the preparation and labeling of complex biological targets and hybridization, washing, scanning, and feature extraction of Agilent 60-mer oligonucleotide microarrays for microarray-based two-color gene expression analysis.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Procedures

This chapter describes the steps to prepare samples with the Fairplay III Microarray Labeling Kit; hybridize, wash and scan gene expression microarrays; and to extract data using the Agilent Feature Extraction Software.

3 Supplemental Procedures

This chapter contains instructions for quality assessment of template RNA and labeled cDNA, and steps to prevent ozone-related problems.

4 Reference

This chapter contains reference information related to the protocol.

What’s new in 2.0

- Expanded solvent wash details to prepare for microarray wash.
- Added list of supported microarrays.
- Added note to calibrate hybridization oven on a regular basis for accuracy of the collected data.
- Updated loading instructions for hybridization oven.
- Added reference to compatibility matrix for non-Agilent scanners.
- Expanded instructions to prepare hybridization assembly.
- Updated product labeling statement.

What’s new in 1.4

- Support for Agilent SureScan microarray scanner.
- Change of formula to quantify cDNA before hybridization.

What’s new in 1.3

- Addition of Agilent Two-Color Spike-In Kit.
- Support for the G2565CA Scanner.
- Support for the Agilent Ozone-Barrier Slide Cover Kit for ozone reduction in the wash step.
- Agilent Feature Extraction software requirement changed to version 10.7.1 or later.
- Agilent GeneSpring GX software requirement changed to version 11.0 or later.

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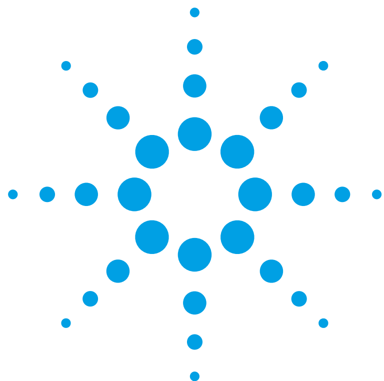

1
Before You Begin

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Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE Agilent cannot guarantee microarray performance and does not provide technical support to those who use non-Agilent protocols in processing Agilent microarrays.

Procedural Notes

- Determine the integrity of the input RNA for labeling and hybridization prior to use to increase the likelihood of a successful experiment.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Refer to the Fairplay III Microarray Labeling Kit User Guide for more procedural notes.

Safety Notes

CAUTION

- Inspect the Stabilization and Drying Solution bottle for chips or cracks prior to use. Failure to do so may result in bottle breakage.
- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

WARNING

- **Cyanine dye reagents are potential carcinogens. Avoid inhalation, swallowing, or contact with skin.**
- **LiCl is toxic and a potential teratogen. May cause harm to breastfed babies. Possible risk of impaired fertility. Harmful if inhaled, swallowed, or contacts skin. Target organ: central nervous system. Wear suitable PPE. LiCl is a component of the 2× Hi-RPM Hybridization Buffer.**
- **Lithium dodecyl sulfate (LDS) is harmful by inhalation and irritating to eyes, respiratory system, and skin. Wear suitable PPE. LDS is a component of the 2× Hi-RPM Hybridization Buffer.**
- **Triton is harmful if swallowed. Risk of serious damage to eyes. Wear suitable PPE. Triton is a component of the 2× Hi-RPM Hybridization Buffer and is an additive in wash buffers.**
- **Acetonitrile is a flammable liquid and vapor. Harmful if inhaled, swallowed, or contacts skin. Target organs: liver, kidneys, cardiovascular system, and CNS.**
- **Stabilization and Drying Solution is toxic and flammable and must be used in a suitable fume hood. This solution contains acetonitrile and must be disposed of in a manner consistent with disposal of like solvents. Gloves and eye/face protection should be used during every step of this protocol, especially when handling acetonitrile and the Stabilization and Drying Solution.**

Agilent Oligo Microarrays

For more information on microarray designs visit the following web site: http://www.chem.agilent.com

To get design files or create a custom design, go to the Agilent eArray web site at http://earray.chem.agilent.com.

NOTE Store entire kit at room temperature. After breaking foil on microarray pouch, store microarray slides at room temperature (in the dark) under a vacuum dessicator or nitrogen purge box. Do not store microarray slides in open air after breaking foil.

Eight microarrays printed on each 1-inch × 3-inch glass slide

Catalog SurePrint HD Microarray

Table 1 Catalog SurePrint HD Microarrays

Part Number	Description
G4813A-020097	E. coli Gene Expression Microarray, 8×15K (1 slide)

Custom Microarrays

Table 2 Custom SurePrint HD Microarrays

Part Number	Description
G2509F	Custom Gene Expression Microarray, 8×15K

Table 3 Custom SurePrint G3 Microarrays

Part Number	Description
G4102A	SurePrint G3 Custom Gene Expression Microarray, 8×60K

Required Equipment

Table 5 Required Equipment

Description	Vendor and part number
Agilent Microarray Scanner	Agilent p/n G4900DA, G2565CA or G2565BA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization gasket slides 8 microarrays/slide, 5 slides/box Go to www.agilent.com/genomics to see all available kit configurations.	Agilent p/n G2534-60014
Hybridization oven; temperature set at 65°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
nuclease-free 1.5 mL microfuge tube	Ambion p/n 12400 or equivalent
magnetic stir bar (×2)	Corning p/n 401435 or equivalent
magnetic stir plate (×2)	Corning p/n 6795-410 or equivalent
circulating water baths or heat blocks set to 37°C, 40°C, 60°C, 65°C, 70°C, and 80°C,	Corning p/n 6795-420 or equivalent
microcentrifuge	Eppendorf p/n 5417R or equivalent
sterile storage bottle	Nalgene 455-1000 or equivalent
spectrophotometer	NanoDrop p/n ND-1000 UV-VIS or equivalent
micropipettor	Pipetman P-10, P-20, P-200, P-1000 or equivalent
slide-staining dish, with slide rack (×3)	Thermo Shandon p/n 121 or equivalent
vacuum concentrator	Thermo Scientific p/n DNA 120-115 or equivalent
clean forceps	
ice bucket	
powder-free gloves	
sterile, nuclease-free aerosol barrier pipette tips	
vortex mixer	

Table 5 Required Equipment (continued)

Description	Vendor and part number
timer	
nitrogen purge box for slide storage	

Required Reagents

Table 6 Required Reagents

Description	Vendor and part or catalog number
RNA Spike-In Kit, Two-Color	Agilent p/n 5188-5279
Gene Expression Hybridization Kit	Agilent p/n 5188-5242
Gene Expression Wash Buffer Kit	Agilent p/n 5188-5327
FairPlay III Microarray Labeling Kit	
10 reactions	Agilent p/n 252009
30 reactions	Agilent p/n 252012
Cy3 Mono-Reactive Dye	GE Healthcare p/n PA23001
Cy5 Mono-Reactive Dye	GE Healthcare p/n PA25001
DNase/RNase-free distilled water	Invitrogen p/n 10977-015
RNeasy Mini Kit	Qiagen p/n 74104
Sulfolane	Sigma-Aldrich p/n T22209
ethanol (95% to 100% molecular biology grade)	Sigma-Aldrich p/n E7023-6×500ML
Milli-Q water or equivalent	
DNA size standard, useful range 400–1000 bp	
1 M NaOH	
1 M HCl	
10 mM Tris base, adjust pH to 8.5 with HCl	
3 M Sodium Acetate, pH 4.5	
isopropyl alcohol (molecular biology grade)	

Optional Equipment/Reagents

Table 7 Optional Equipment/Reagents

Description	Vendor and part number
2100 Bioanalyzer	Agilent p/n G2939AA
RNA 6000 Nano Assay Kit (RNA Series II Kit)	Agilent p/n 5067-1511
Stabilization and Drying Solution*	Agilent p/n 5185-5979
Ozone-Barrier Slide Cover*	Agilent p/n G2505-60550
slide box	Corning p/n 07201629
acetonitrile	Sigma p/n 271004-1L

* Recommended when processing microarrays in high ozone environment.

Required Hardware and Software

Table 8

Description
Feature Extraction software 10.7.1 or later
Agilent Scan Control software. Refer to Agilent Scanner user guide for specifications.
For system and supported Internet Explorer/Adobe Reader versions, please see the System Requirements for your Feature Extraction and Scan Control Software.

Optional Software

Table 9

Description
GeneSpring GX 11.5 or later

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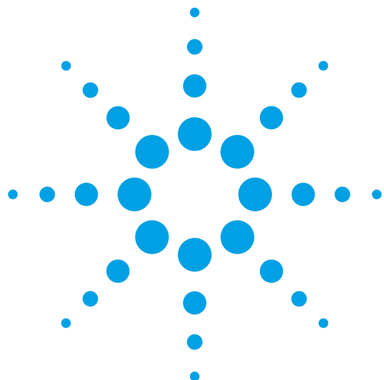

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The Agilent Two-Color Microarray-based Model Organism Analysis uses cyanine 3- and cyanine 5-labeled targets to measure gene expression in experimental and control samples. Figure 1 is a standard workflow for sample preparation and array hybridization design.

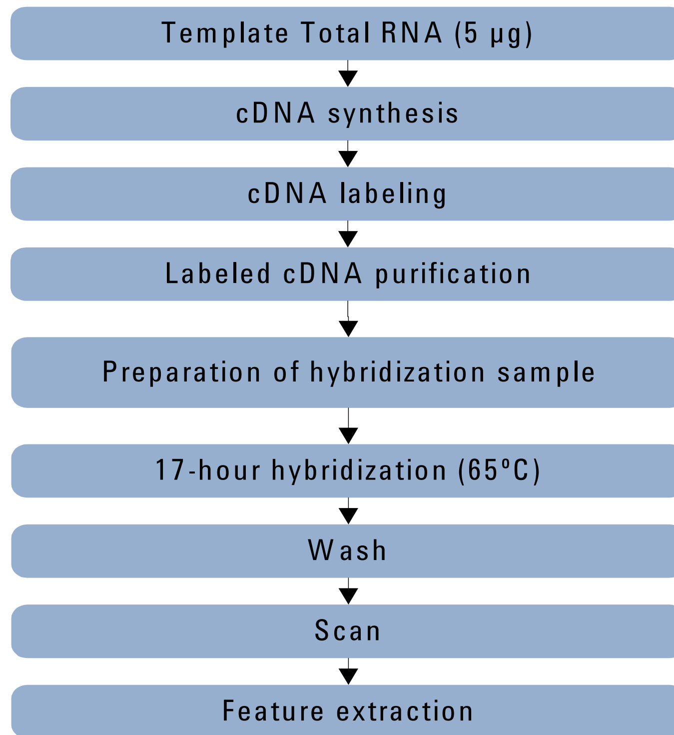


Figure 1 Workflow for sample preparation and array processing.

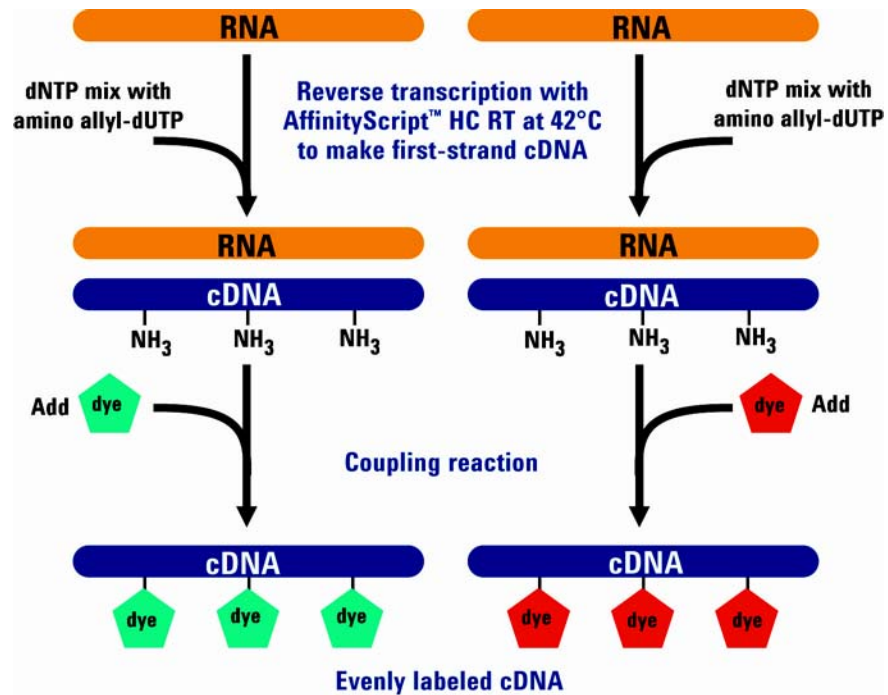


Figure 2 The FairPlay III Microarray Labeling Kit creates evenly labeled cDNA. The FairPlay kit labels sample with fluorescent dyes using a chemical coupling method. First strand synthesis incorporates an amino allyl dUTP. This reactive group is then coupled to a conjugated dye, resulting in evenly labeled cDNA.

Sample Preparation

Step 1. Dilute Spike-in Solution

1 Equilibrate a water bath to 37°C.
2 Thaw Spike A Mix concentrate and mix vigorously in a vortex mixer.
3 Heat at 37°C in a circulating water bath for 5 minutes.
4 Mix the spike mix vigorously on a vortex mixer.
5 Briefly spin in a centrifuge to spin contents to bottom of tube.
6 Add 5 µL of Spike A Mix to 15 µL of Dilution buffer.

   This is sufficient for up to 9 labeling reactions.
7 Use the Spike B Mix to prepare Spike B solution in the same way as Spike A solution.

Spike A Mix is to be added to Cy3 labeling reactions. RNA Spike B Mix is to be added to Cy5 reactions. Do not interchange the spike mixes with the dyes.

Step 2. Synthesize cDNA

Use reagents from:

- RNA Spike-In Kit, Two-Color
- FairPlay III Microarray Labeling Kit

NOTE Prepare separate labeling reactions for each fluorescent dye.

If you begin with 5 µg of RNA, you have enough cDNA for one 8×15K microarray.

CAUTION Do not interchange the diluted Spike A Mix with Spike B Mix.

1 Add 5 µg total RNA to a 1.5-mL microcentrifuge tube in a volume of 10 µL or less. If samples are concentrated, dilute with water until 5 µg of total RNA is added in at least 2 µL volume with a pipette to ensure the accuracy.
2 Add the diluted Spike Mix to Total RNA:
   - For the RNA being labeled with Cy3, add 2 µL of diluted Spike A Mix to 5 µg of Total RNA.
   - For the RNA being labeled with Cy5, add 2 µL of diluted Spike B Mix to 5 µg of Total RNA.

   If needed, bring the total volume of the Spike Mix/RNA solution to 12 µL with nuclease-free water.
3 Add 1 µL of Random Primers (pink cap) to the Spike-in/RNA solution. Gently mix on a vortex mixer and briefly spin the tube in a centrifuge.
4 Incubate the tube at 70°C for 10 minutes.
5 Put reagents on ice and incubate for 5 minutes.
6 Immediately prior to use, gently mix the components listed in Table 10 for the cDNA Master Mix by adding in the order indicated, and put on ice.

   Use a pipette to thoroughly mix the components. Make sure the enzymes are homogenous. Immediate after use, return components to -20°C.

Table 10 cDNA Master Mix

Component	Volume per reaction	Volume per 8 reactions (includes excess)
10× AffinityScript Reaction Buffer (clear cap)	2 µL	20 µL
20× dNTP Mix with amino allyl dUTP (green cap)	1 µL	10 µL
0.1 M DTT (green cap)	1.5 µL	15 µL
RNase Block (purple cap)	0.5 µL	5 µL

7. Add 5 uL of cDNA Master Mix to each sample tube. Draw the solution into a pipette and release to mix the solution.
8. Spin the AffinityScript HC Reverse Transcriptase (purple cap) right before use.
9. Add 3 µL of AffinityScript HC Reverse Transcriptase (purple cap) and incubate at 42°C for 60 minutes.
10. Add 10 µL of 1 M NaOH and incubate at 70°C for 10 minutes to hydrolyze RNA.
11. Let the sample cool to room temperature slowly. *Do not cool on ice.*
12. Spin tube briefly to collect contents.
13. Add 10 µL of 1 M HCl to neutralize the solution.

Step 3. Purify cDNA

The cDNA must be purified to remove unincorporated nucleotides, buffer components and hydrolyzed RNA. Incomplete removal of the Tris and EtOH will result in lower amino allyl-dye coupling efficiency. Care must be taken to ensure that the pellet is completely dry (at the end of step 6) indicating complete removal of the ethanol before proceeding to the dye coupling reaction.

1 Add 4 µL of 3 M Sodium Acetate, pH 4.5 to the reaction.
2 Add 1 µL of 20 mg/mL Glycogen (green cap) to the reaction.
3 Add 100 µL of ice-cold 95% ethanol.
4 Incubate at -20°C for at least 30 minutes. The reaction can be stored at this point for several days or up to 2 months.
5 Spin the reaction at 13,000 to 14,000 × g for 15 minutes at 4°C. Carefully remove the supernatant.
6 Wash the pellet with 0.5 mL ice cold 70% ethanol and spin at 13,000 to 14,000 × g for 15 minutes at 4°C. Carefully remove the supernatant and allow the pellet to air dry.

A vacuum dryer can be used but the pellet *must not* be excessively dried.

Step 4. Label the modified cDNA

NOTE Note that dye packs can be stored at 4°C until use, but make sure they are at room temperature before you open and add DMSO.

CAUTION Do not scale down the dye coupling instruction. If you do the reaction with reduced volumes, significantly reduced coupling efficiency can result.

1. Resuspend cDNA pellet in 5 µL of 2× Coupling Buffer (clear cap). Excessively dried pellets are difficult to get back into solution.
2. Gently heat at 37°C for 15 minutes to make sure that the precipitate is soluble before use.

   A visible precipitate may be seen in the 2× Coupling Buffer (clear cap).
3. If the tube of Cy3 Mono-Reactive Dye or Cy5 Mono-Reactive Dye is unopened:
   - Bring tube to room temperature before you open it.
   - Resuspend in 45 µL DMSO (green cap). Use the high-purity DMSO (green cap) provided in the kit. Do not substitute another DMSO.

   The unused dye can be stored in single-use aliquots and stored at -20°C in the dark for several months.
4. Mix gently to ensure that the pellet is completely soluble.

NOTE DMSO (green cap) is hygroscopic and will absorb moisture from the air. Water absorbed from the air will react with the NHS ester portion of the dye and significantly reduce or eliminate dye:cDNA-coupling efficiency. To reduce absorption, allow the dye to reach room temperature before opening and store the DMSO (green cap) at room temperature. Do not leave either the dye or DMSO (green cap) uncapped when not in use. During storage, tightly cap the resuspended dye and store at -20°C in the dark.

5. Add 5 µL of Cy3 Mono-Reactive Dye or Cy5 Mono-Reactive Dye to the cDNA. If the dye was stored at -20°C before use, allow the dye to reach room temperature before you open the container.
6. Mix by gently pipetting up and down.
7. Incubate for 30 minutes at room temperature in the dark.

Step 5. Purify the dye-coupled cDNA

Use the reagents in the FairPlay III Microarray Labeling Kit.

In summary:

- In the presence of a chaotropic salt (introduced by the DNA-binding solution, included in this kit), the dye-coupled cDNA binds to the silica-based fiber matrix seated inside the microspin cup.
- Washing steps remove buffer salts and uncoupled fluorescent dye from the bound cDNA.
- The cDNA is eluted from the matrix using a low-ionic strength solution. Do not use the DNA-binding solution and microspin cups provided in the FairPlay III Microarray Labeling Kit in conjunction with alternative purification protocols.

1. Prepare microspin cup elution buffer:
   a. Add 1 M HCl to 10 mM Tris base until the pH reaches 8.5.
   b. If the elution buffer is made by diluting a higher molarity Tris base, pH 8.5, to a final molarity of 10 mM, verify that the pH is still 8.5. If not, adjust the pH to 8.5 using either HCl or NaOH.
2. Prepare 80% Sulfolane:
   a. Incubate the 100% Sulfolane in a 37°C water bath until liquefied. 100% sulfolane is a solid at room temperature. 80% Sulfolane solution is a liquid at room temperature and can be stored at room temperature for at least a month.
   b. Add 1 mL of DNase/RNase-free distilled water to 4 mL of 100% Sulfolane to make 5 mL of 80% sulfolane.
3. Add 90 µL of DNase/RNase-free distilled water to the 10 µL labeled cDNA.
4. Combine 100 µL of DNA-binding solution and 100 µL of 80% Sulfolane mixture. Mix well on a vortex mixer.

   The two solutions must be well mixed before use.
5. Add the 200 µL of DNA-binding solution and 80% Sulfolane mixture to the labeled cDNA and mix on a vortex mixer.
6. Put a microspin cup into a 2 mL receptacle tube.

7 Use the pipette to transfer the mixture to the microspin cup that is seated in the 2 mL receptacle tube.

Do not touch the pipette to the matrix in the microspin cup as you transfer the mixture.

8 Snap the cap of the 2 mL receptacle tube onto the top of the microspin cup.

To ensure proper sample flow, use the 2 mL receptacle tube that is provided with the microspin cup. Do not substitute another tube.

9 Spin the 2 mL receptacle tube in a microcentrifuge at 11,000 to 13,000 rpm for 30 seconds.

The labeled cDNA is retained in the fiber matrix of the microspin cup.

10 Open the cap of the 2 mL receptacle tube, remove and retain the microspin cup, and discard the DNA-binding solution that contains the uncoupled dye.

11 Combine 100 µL of the DNA-binding solution and 100 µL of 80% Sulfolane mixture. Mix well on a vortex mixer. Make sure that the two solutions are well mixed before use.

12 Wash with DNA-binding solution and 80% Sulfolane mixture:

   a Add the 200 µL of DNA-binding solution and Sulfolane mixture to the microspin cup. Snap the cap of the 2 mL receptacle tube onto the top of the microspin cup.

   b Spin the 2 mL receptacle tube in a microcentrifuge at maximum speed for 30 seconds.

   c Open the cap of the 2 mL receptacle tube, remove and retain the microspin cup, and discard the DNA-binding /sulfolane mixture.

13 Wash with 75% ethanol:

   a Add 750 µL of 75% ethanol to the microspin cup. Snap the cap of the 2 mL receptacle tube onto the top of the microspin cup.

   b Spin the 2 mL receptacle tube in a microcentrifuge at 11,000 to 13,000 rpm for 30 seconds.

   c Open the cap of the 2 mL receptacle tube, remove and retain the microspin cup, and discard the wash buffer.

   d Repeat entire step.

14 Place the microspin cup back in the 2 mL receptacle tube and snap the cap of the 2 mL receptacle tube onto the microspin cup.

15 Spin the 2 mL receptacle tube in a microcentrifuge at 11,000 to 13,000 rpm for 30 seconds. When the tubes are removed from the centrifuge, make sure that all of the wash buffer is removed from the microspin cup.

16 Transfer the microspin cup to a fresh nuclease-free 1.5 mL microfuge tube and discard the 2 mL receptacle tube.

17 Incubate and spin with 10 mM Tris base:

- **a** Add 50 µL of 10 mM Tris base, pH 8.5 directly above, but not touching, the fiber matrix at the bottom of the microspin cup.
- **b** Incubate the tube at room temperature for 5 minutes.

NOTE Maximum recovery of the labeled cDNA from the microspin cup depends on the pH, ionic strength, and volume of the elution buffer added to the microspin cup; the placement of the elution buffer into the microspin cup; and the incubation time. Maximum recovery is obtained when the elution buffer is ≤10 mM in concentration with pH 7–9, when no less than 50 µL of elution buffer is added directly onto the fiber matrix at the bottom of the microspin cup, and when the tube is incubated for 5 minutes.

- **c** Snap the cap of the nuclease-free 1.5 mL microfuge tube onto the microspin cup and spin the tube in a microcentrifuge at maximum speed for 30 seconds.
- **d** Open the lid of the microcentrifuge tube and recover the flow through that contains the purified labeled cDNA.

18 Elute additional labeled cDNA:

- **a** Elute additional labeled cDNA by pipetting the flow through back onto the fiber matrix of the same microspin cup.
- **b** Re-seat the spin cup on the same 2 mL receptacle tube that contained the liquid from the first-pass elution.
- **c** Incubate the tube at room temperature for 5 minutes.
- **d** Snap the cap of the nuclease-free 1.5 mL microfuge tube onto the microspin cup and spin the tube in a microcentrifuge at maximum speed for 30 seconds.
- **e** Open the lid of the microcentrifuge tube and recover the flow-through containing the purified labeled cDNA.

19 Repeat step 18 to harvest one final elution from the microspin cup.

20 Remove a 2-µL sample of the labeled cDNA for analysis of dye incorporation using a small-volume spectrophotometer (such as a NanoDrop instrument). See "Step 6. Quantify the cDNA" on page 27.

21 If needed, reduce the volume of the labeled cDNA. For each hybridization, do not exceed 20 µL for the total volume of both labeled samples (see "Hybridization" on page 29). To reduce the volume, continue to spin the sample in a centrifuge and use a concentrator (such as a SpeedVac) to concentrate the sample.

Step 6. Quantify the cDNA

Quantify the cDNA using NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1.

1 Start the NanoDrop software.
2 Click the **Microarray Measurement** tab.
3 Before initializing the instrument as requested by the software, clean the sample loading area with nuclease-free water.
4 Load 1.0 to 2.0 µL of nuclease-free water to initialize. Then click **OK**.
5 Once the instrument has initialized, select **ssDNA-33** as the **Sample type** (use the drop down menu).
6 Make sure the **Recording** button is selected. If not, click **Recording** so that the readings can be recorded, saved, and printed.

CAUTION Failure to engage recording causes measurements to be overwritten, with no possibility of retrieval.

7 Transfer 1.0 to 2.0 µL of 10 mM Tris base, pH 8.5 with a pipette to the instrument sample loading area. Click **Blank**.
8 Clean the sample loading area with a laboratory wipe. Transfer 1.0 to 2.0 µL of the sample onto the instrument sample loading area. Type the sample name in the space provided. Click **Measure**.

   Be sure to clean the sample loading area between measurements and ensure that the baseline is always flat at 0, which is indicated by a thick black horizontal line. If the baseline deviates from 0 and is no longer a flat horizontal line, reblank the instrument with 10 mM Tris base, pH 8.5, then remeasure the sample.
9 Print the results. If printing the results is not possible, record the following values:
   - cyanine 3 or cyanine 5 dye concentration (pmol/µL)
   - DNA absorbance ratio (260 nm/280 nm)
   - cDNA concentration (ng/µL)

10 Determine the yield and specific activity of each reaction as follows:

a Use the concentration of cDNA (ng/µL) to determine the µg cDNA yield as follows:

$$\frac{(\text{Concentration of cDNA}) \times 50\ \mu\text{L (elution volume)}}{1000} = \mu\text{g of cDNA}$$

b Use the concentrations of cDNA (ng/µL) and cyanine 3 or cyanine 5 (pmol/µL) to determine the specific activity as follows:

$$\frac{\text{Concentration of Cy3 or Cy5}}{\text{Concentration of cDNA}} \times 1000 = \text{pmol Cy3 or Cy5 per }\mu\text{g cDNA}$$

11 Examine the yield and specific activity results.

CAUTION

If the yield is <650 ng (or 13 ng/µL) and the specific activity is <40 pmol of Cy3 or Cy5 per µg of cDNA, do not proceed to the hybridization step. Repeat cDNA preparation.

NOTE

If labeling results are poor, please refer to "Quality Assessment of Template RNA" on page 52 for general guidance and procedural recommendations on quality assessment of RNA.

Hybridization

An instructional video that shows hybridization and washing steps can be found at http://genomics.agilent.com. Search for "Running a microarray experiment".

If you are a first time user, practice the hybridization process before you begin. Use water instead of blocking mix, and use a clean microscope slide and a gasket slide. Make sure you mix and apply the hybridization solution with minimal bubbles. Practice the hyb assembly and the slide disassembly and wash.

CAUTION

You must calibrate the hybridization oven regularly for accuracy of the collected data. Refer to *Agilent G2545A Hybridization Calibration Procedure* (p/n G2545-90002, version A1 or higher) for more information.

Step 1. Prepare the 10× Blocking Agent

1 Add 500 µL of nuclease-free water to the vial containing lyophilized 10× Gene Expression Blocking Agent supplied with the Gene Expression Hybridization Kit, *or* add 1250 µL of nuclease-free water to the vial containing lyophilized large volume 10× Gene Expression Blocking Agent.

2 Gently mix on a vortex mixer. If the pellet does not go into solution completely, heat the mix for 4 to 5 minutes at 37°C.

3 Drive down any material that sticks to the tube walls or cap by spinning in a centrifuge for 5 to 10 seconds.

NOTE

Divide the 10× Gene Expression Blocking Agent into aliquots small enough to keep the freeze-thaw cycle to 5 times or less. Store at -20°C for up to two months. Before use, repeat step 2 and step 3.

Step 2. Prepare hybridization samples

1 For each microarray, add each of the components as indicated in Table 11 to a 1.5 mL nuclease-free microfuge tube:

2 Mix well but gently on a vortex mixer.

Table 11 Blocking Mix

Components	Volume/Mass
cyanine 3-labeled cDNA	300 ng
cyanine 5-labeled cDNA	300 ng
10× Gene Expression Blocking Agent	5 µL
Nuclease-free water	bring volume to 25 µL
Total Volume	25 µL

3 Add 2× Hi-RPM Hybridization Buffer. See Table 12.

Table 12 Hybridization mix

Components	Volumes per hybridization
cDNA from Blocking Mix	25 µL
2× Hi-RPM Hybridization Buffer	25 µL

4 Mix well by careful pipetting part way up and down. Do not introduce bubbles to the mix. The surfactant in the 2× Hi-RPM Hybridization Buffer easily forms bubbles. Do not mix on a vortex mixer; mixing on a vortex mixer introduces bubbles.

5 Spin briefly on a microfuge.

Use immediately. Do not store.

Refer to "Microarray Handling Tips" on page 65 for information on how to safely handle microarrays.

Step 3. Prepare the hybridization assembly

Refer to the *Agilent Microarray Hybridization Chamber User Guide* for more details to load slides, and to assemble and disassemble the chambers. This user guide is included with the Agilent Microarray Hybridization Chamber Kit (G2534A) and can also be downloaded from the Agilent web site at www.genomics.agilent.com. Search for **G2534A**.

1 Load a clean gasket slide into the Agilent SureHyb chamber base with the label facing up and aligned with the rectangular section of the chamber base. Make sure that the gasket slide is flush with the chamber base and is not ajar.

CAUTION

Do not let the pipette tip or the hybridization solution touch the gasket walls. Allowing liquid to touch the gasket wall greatly increases the likelihood of gasket leakage.

When you lower the microarray slide on top of the SureHyb gasket slide, make sure that the two slides are parallel at all times.

---

2 Slowly dispense 40 µL of hybridization sample onto the gasket well in a “drag and dispense” manner.
   - Position the slides so that the barcode label is to your left.
   - Load the samples left to right. For 8-pack slides, start with the first row. The output files will come out in that same order. Refer to “Array/Sample tracking microarray slides” on page 69 for guidelines on tracking sample position for multipack slide formats.
   - Avoid the introduction of air bubbles to the gasket wells. Air bubbles can affect the final sample volume and can cause leakage from the gasket well.
3 If any wells are unused:
   a Make a 1× solution of the 2× Hi-RPM Hybridization Buffer.
   b Add the volume of 1× Hybridization Buffer equal to the sample volume to each unused well.

   Make sure all wells contain sample or 1× Hybridization Buffer. Empty wells can cause failure in hybridization.
4 Grip the slide on either end and slowly put the slide “active side” down, parallel to the SureHyb gasket slide, so that the “Agilent”-labeled barcode is

facing down and the numeric barcode is facing up. Make sure that the sandwich-pair is properly aligned.

CAUTION Do not drop the array slide onto the gasket. Doing so increases the chances of samples mixing between gasket wells.

5. Put the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
6. Firmly hand-tighten the clamp onto the chamber.
7. Vertically rotate the assembled chamber to wet the gasket and assess the mobility of the bubbles. If necessary, tap the assembly on a hard surface to move stationary bubbles.

Step 4. Hybridize

1. Load each assembled chamber into the oven rotator rack. Start from the center of the rack (position 3 or 4 when counting from the left). Set your hybridization rotator to rotate at 10 rpm when using 2× Hi-RPM Hybridization Buffer.
2. Hybridize at 65°C for 17 hours.

CAUTION If you are not loading all the available positions on the hybridization rotator rack, be sure to balance the loaded hybridization chambers on the rack so that there are an equal number of empty positions on each of the four rows on the hybridization rack.

NOTE The Gene Expression Wash Buffer 2 needs to be warmed overnight. Make sure that you prepare the wash buffer the night before you plan to do the microarray wash. See “Step 2. Prewarm Gene Expression Wash Buffer 2”.

Microarray Wash

Step 1. Add Triton X-102 to Gene Expression wash buffers

This step is optional but highly recommended.

The addition of 0.005% Triton X-102 (10%) to the Gene Expression wash buffers reduces the possibility of array wash artifacts. Add Triton X-102 (10%) to Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2 when the cubitainer of wash buffer is first opened.

Do this step to *both* Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2 before use.

1. Open the cardboard box with the cubitainer of wash buffer and carefully remove the outer and inner caps from the cubitainer.
2. Use a pipette to add 2 mL of the provided Triton X-102 (10%) into the wash buffer in the cubitainer.
3. Replace the original inner and outer caps and mix the buffer carefully but thoroughly by inverting the container 5 to 6 times.
4. Carefully remove the outer and inner caps and install the spigot provided with the wash buffer.
5. Prominently label the wash buffer box to indicate that Triton X-102 (10%) has been added and indicate the date of addition.

Triton X-102 (10%) can be added to smaller volumes of wash buffer as long as the final dilution of the 10% Triton X-102 is 0.005% in the Gene Expression wash buffer solution.

Step 2. Prewarm Gene Expression Wash Buffer 2

Warm the Gene Expression Wash Buffer 2 to 37°C as follows:

1 Dispense 1000 mL of Gene Expression Wash Buffer 2 directly into a sterile storage bottle. Repeat until you have enough prewarmed Wash Buffer 2 solution for your experiment.
2 Tightly cap the sterile storage bottle and put in a 37°C water bath the night before washing arrays. Alternatively, remove the plastic cubitainer from the box and put it in a 37°C water bath the night before washing the arrays.

Step 3. Prepare the equipment

Always use clean equipment when doing the hybridization and wash steps. Designate and dedicate dishes to two-color experiments.

Solvent wash

Wash staining dishes, racks and stir bars with acetonitrile or isopropyl alcohol to avoid wash artifacts on your slides and images.

- Use acetonitrile for equipment that was exposed to Stabilization and Drying Solution.
- Use isopropyl alcohol for equipment that was not exposed to Stabilization and Drying Solution.

WARNING **Conduct solvent washes in a vented fume hood.**

1 Add the slide rack and stir bar to the staining dish.
2 Transfer the staining dish with the slide rack and stir bar to a magnetic stir plate.
3 Fill the staining dish with 100% acetonitrile or isopropyl alcohol.
4 Turn on the magnetic stir plate and adjust the speed to a setting of 4 (medium speed).
5 Wash for 5 minutes.
6 Discard the solvent as is appropriate for your site.

7 Repeat step 1 through step 6.
8 Air dry the staining dish in the vented fume hood.
9 Proceed to "Milli-Q water wash".

Milli-Q water wash

Wash all dishes, racks, and stir bars with Milli-Q water.

1 Run copious amounts of Milli-Q water through the staining dish.
2 Empty out the water collected in the dish.
3 Repeat step 1 and step 2 at least 5 times, as it is necessary to remove any traces of contaminating material.
4 Discard the Milli-Q water.

CAUTION Some detergents may leave fluorescent residue on the dishes. Do not use any detergent in the washing of the staining dishes. If detergent is used, all traces must be removed by copiously rinsing with Milli-Q water.

Step 4. Wash the microarray slides

NOTE The microarray wash procedure for the Agilent two-color platform must be done in environments where ozone levels are 5 ppb or less. For Scanner C and Scanner B, if ozone levels are between 5 to 10 ppb in your laboratory, use the Agilent Ozone Barrier Slide Cover (described in this topic). If ozone levels exceed 10 ppb, see "Preventing Ozone-Related Problems" on page 56 for more information.

NOTE When setting up the apparatus for the washes, be sure to do so near the water bath containing the pre-warmed Wash 2 solutions.

Table 13 lists the wash conditions for the wash procedure.

Table 13 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	1	Gene Expression Wash Buffer 1	Room temperature	
1st wash	2	Gene Expression Wash Buffer 1	Room temperature	1 minute
2nd wash	3	Gene Expression Wash Buffer 2	Elevated temperature*	1 minute

* The elevated temperature of the second wash step is usually around 31°C due to cooling by the room temperature dish and the rack of arrays.

1. Completely fill slide-staining dish #1 with Gene Expression Wash Buffer 1 at room temperature.
2. Put a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Gene Expression Wash Buffer 1 at room temperature to cover the slide rack. Put this dish on a magnetic stir plate.
3. Put the empty dish #3 on the stir plate and add a magnetic stir bar. Do not add the prewarmed (37°C) Gene Expression Wash Buffer 2 until the first wash step has begun.
4. Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.

5 Prepare the hybridization chamber disassembly.
   a Put the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counterclockwise.
   b Slide off the clamp assembly and remove the chamber cover.
   c With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.
   d Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Gene Expression Wash Buffer 1.
6 With the sandwich completely submerged in Gene Expression Wash Buffer 1, pry the sandwich open from the barcode end only:
   a Slip one of the blunt ends of the forceps between the slides.
   b Gently turn the forceps upwards or downwards to separate the slides.
   c Let the gasket slide drop to the bottom of the staining dish.
   d Grasp the top corner of the microarray slide, remove the slide, and then put it into the slide rack in the slide-staining dish #2 that contains Gene Expression Wash Buffer 1 at room temperature. Transfer the slide quickly so avoid premature drying of the slides. *Touch only the barcode portion of the microarray slide or its edges!*
7 Repeat step 4 through step 6 for up to seven additional slides in the group. For uniform washing, do up to a maximum of eight disassembly procedures yielding eight microarray slides.
8 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4 for 1 minute.
9 During this wash step, remove Gene Expression Wash Buffer 2 from the 37°C water bath and pour into the slide-staining dish #3.
10 Transfer slide rack to slide-staining dish #3 that contains Gene Expression Wash Buffer 2 at elevated temperature. Stir using setting 4, or a moderate speed setting, for 1 minute.
11 Slowly remove the slide rack minimizing droplets on the slides. It should take 5 to 10 seconds to remove the slide rack. If liquid remains on the bottom edge of the slide, dab it on a cleaning tissue.
12 Discard used Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2.

13 Repeat step 1 through step 12 for the next group of eight slides using fresh Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2 pre-warmed to 37°C.

14 Put the slides in a slide holder.

For SureScan microarray scanner

- Carefully put the end of the slide without the barcode label onto the slide ledge.
- Gently lower the microarray slide into the slide holder. Make sure that the active microarray surface (with “Agilent”-labeled barcode) faces up, toward the slide cover.
- Close the plastic slide cover, pushing on the tab end until you hear it click.
- For more detailed instruction, refer to the *Agilent G4900DA SureScan Microarray Scanner System User Guide*.

Figure 3 Slide in slide holder for SureScan microarray scanner

For Agilent Scanner B or C only:

- In environments in which the ozone level exceeds 5 ppb, immediately put the slides with active microarray surface (with “Agilent”-labeled barcode) facing up in a slide holder. Make sure that the slide is not caught up on any corner. Put an ozone-barrier slide cover on top of the array as shown in Figure 4. Refer to the *Agilent Ozone-Barrier Slide Cover User Guide* (p/n G2505-90550), included with the slide cover, for more information.

  As an alternative, use the Stabilization and Drying Solution. See “Preventing Ozone-Related Problems” on page 56.

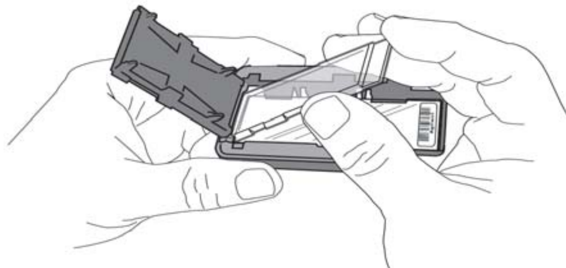


Figure 4 Inserting the ozone-barrier slide cover (shown for Scanner B and Scanner C)

- In environments in which the ozone level is below 5 ppb, put the slides with Agilent barcode facing up in a slide holder.

15 Scan slides immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a nitrogen purge box, in the dark.

Scanning and Feature Extraction

Step 1. Scan the slides

Agilent provides support for Agilent microarrays scanned on select non-Agilent scanners. Please see “Feature Extraction Compatibility Matrix for Non Agilent scanners” for scanner compatibility and settings (http://www.chem.agilent.com/Library/usermanuals/Public/G1662-90043_ScannerCompatibilityMatrix.pdf).

Agilent can guarantee the quality of data only if the data comes from Agilent microarrays scanned on Agilent scanners.

To get scanner profiles from Agilent:

- For Scan Control 9.1.3 or later, go to http://www.genomics.agilent.com/article.jsp?pageId=2610
- For Scan Control 8.x, go to http://www.genomics.agilent.com/article.jsp?pageId=2074

Agilent SureScan Microarray Scanner

1 Put assembled slide holders into the scanner cassette.
2 Select the protocol **AgilentHD_GX_2color.**
   - **AgilentHD_GX_2color** (for HD format)
3 Verify that the Scanner status in the main window says Scanner Ready.
4 Click **Start Scan**.

Agilent C Scanner Settings

1 Put assembled slide holders with or without the ozone-barrier slide cover into scanner carousel.
2 Select Start Slot *m* End Slot *n* where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.
3 Select **Profile AgilentHD_GX_2color**.
4 Verify scan settings for two-color scans. See Table 14.

CAUTION Do not scan G3 microarrays with HD format settings. The resolution of the resulting image will not be high enough for data analysis.

Table 14 C Scanner Scan Settings

	For HD Microarray Formats
Dye channel	R+G (*red and green*)
Scan region	Agilent HD (61 × 21.6 mm)
Scan resolution	5 µm
Tiff file dynamic range	20 bit
Red PMT gain	100%
Green PMT gain	100%

5 Verify that **Output Path Browse** is set for desired location.

6 Verify that the Scanner status in the main window says Scanner Ready.

7 Click **Scan Slot *m*-*n*** on the Scan Control main window where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.

Agilent B Scanner Settings

Agilent Scan Control software v7.0.01 is required.

1 Put slide into slide holder, with or without the ozone-barrier slide cover, with Agilent barcode facing up.

2 Put assembled slide holders into scanner carousel.

3 Verify scan settings for two-color scans. See Table 15.

For version 7.X, to change any settings, click **Settings > Modify Default Settings**. A window pops up from which you can change the settings.

Table 15 B Scanner Scan Settings

	For All Formats
Scan region	Scan Area (61 × 21.6 mm)
Scan resolution (µm)	5
5µm scanning mode	Single Pass
eXtended Dynamic range	(selected)
Dye channel	Red&Green
Red PMT	XDR Hi 100% XDR Lo 10%
Green PMT	XDR Hi 100% XDR Lo 10%

4 Select settings for the automatic file naming.
   - **Prefix1** is set to **Instrument Serial Number**.
   - **Prefix2** is set to **Array Barcode**.

5 Verify that the Scanner status in the main window says **Scanner Ready**.

6 Click **Scan Slot *m-n*** on the Scan Control main window where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.

Step 2. Extract data using Agilent Feature Extraction Software

Feature Extraction is the process by which information from probe features is extracted from microarray scan data, allowing researchers to measure gene expression in their experiments. To get the most recent Feature Extraction software for gene expression, go to the Agilent web site at www.agilent.com/chem/fe.

From the Feature Extraction online Help, you can find the Quick Start Guide, the detailed User Guide, and the Reference Guide. The Reference Guide includes descriptions of all feature output and the algorithms used.

Feature Extraction (FE) 10.7.1 and later support extraction of two-color .tif images of Agilent microarrays scanned on Agilent Scanner. Images from the Axon/Molecular Devices model 4000B can be analyzed with the use of Feature Extraction version 9.5 found at www.agilent.com/chem/fe.

After generating the microarray scan images, extract .tif images using the Feature Extraction software.

1 Open the Agilent Feature Extraction (FE) program.

   To get the most recent Feature Extraction protocols for gene expression, go to www.agilent.com/chem/feprotocols.

2 Add the images (.tif) to be extracted to the FE Project.

   a Click **Add New Extraction Set(s)** icon on the toolbar or right-click the **Project Explorer** and select **Add Extraction...**

   b Browse to the location of the .tif files, select the .tif file(s) and click **Open**. To select multiple files, use the Shift or Ctrl key when selecting.

   The FE program automatically assigns a default grid template and protocol for each extraction set, if the following conditions are met:

   - For auto assignment of the grid template, the image must be generated from a Agilent scanner and have an Agilent barcode.
   - For auto assignment of the Two-Color Gene Expression FE protocol, the default **Gene Expression** protocol must be specified in the FE Grid Template properties.

     To access the FE Grid Template properties, double-click on the grid template in the Grid Template Browser.

3 Set FE Project Properties.

a Select the **Project Properties** tab.

b In the **General** section, enter your name in the **Operator** text box.

c In the **Input** section, verify that at least the following default settings as shown in Figure 5 are selected.

d In the **Other** section, choose a QC Metric Set for the project. For Agilent two-color microarrays, select **GE2_QCMT_Sep09.**

For outputs that can be imported into Rosetta Resolver, select MAGE and JPEG.

Setting	Value
⊟ General	
Operator	Unknown
⊟ Input	
Number of Extraction Sets Included	0
⊟ Output and Data Transfer	
⊟ Outputs	
⊞ MAGE	None
JPEG	None
⊞ TEXT	Local file only
Visual Results	Local file only
Grid	Local file only
QC Report	Local file only
FTP Send Tiff File	False
⊟ Local File Folder	
Same As Image	True
Results Folder	
⊞ FTP Setting	
⊟ Automatic Protocol Assignment	
Highest Priority Default Protocol	Grid Template Default
Project Default Protocol	
⊟ Automatic Grid Template Assignment	
Use Grid file if available	True
⊟ Other	
QC Metric Set	
External DyeNorm List File	
Overwrite Previous Results	False

Figure 5 Default settings

4 Check the Extraction Set Configuration.

a Select the **Extraction Set Configuration** tab.

b Verify that the correct grid template is assigned to each extraction set in the **Grid Name** column. To assign a different grid template to an extraction set, select one from the pull down menu.

If a grid template is not available to select from the pull down menu, you must add it to the Grid Template Browser. To add, right-click inside the Grid Template Browser, select **Add**. Browse for the design file (.xml) and click **Open** to load grid template into the FE database.

To update to the latest grid templates via Online Update, right-click **Grid Template Browser** and select **Online Update**. You can also download the latest grid templates from Agilent web site at http://earray.chem.agilent.com. After downloading, you must add the grid templates to the Grid Template Browser.

After a new grid template is added to the Grid Template Browser, remember to specify the default protocol for the new grid template if you want the Feature Extraction program to automatically assign a FE protocol to an extraction set.

c Verify that the correct protocol is assigned to each extraction set in the **Protocol Name** column. To assign a different protocol to an extraction set, select from the pull down menu. The appropriate protocol begins with “GE2” for two-color analysis.

The protocols automatically distinguish the formats for processing the data.

If a protocol is not available to select from the pull down menu, you must import it to the FE Protocol Browser. To import, right-click **FE Protocol Browser**, select **Import**. Browse for the FE protocol (.xml) and click **Open** to load the protocol into the FE database. Visit the Agilent web site at www.agilent.com/chem/feprotocols to download the latest protocols.

NOTE These FE Protocols were optimized using data from Agilent catalog arrays, which have many replicated probes and validated Negative Control probes. If custom arrays without enough replicated probes are used, or arrays with custom probes designated as Negative Control probes are used, the default FE Protocols may not be optimal.

NOTE When the Agilent XDR scanned images are added to Feature Extraction software version 9.1 or later, the High and Low images are automatically combined for data extraction. Images are not combined with non-Agilent scanned images.

NOTE 20-bit single images from the C Scanner are equivalent to 16-bit XDR images from the B Scanner.

5 Save the FE Project (.fep) by selecting **File > Save As** and browse for desired location.

6 Verify that the icons for the image files in the FE Project Window no longer have a red X through them. A red X through the icon indicates that an extraction protocol was not selected. If needed, reselect the extraction protocol for that image file.

7 Select **Project > Start Extracting**.

8 After the extraction is completed successfully, view the QC report for each extraction set by double-clicking the QC Report link in the **Summary Report** tab. Determine whether the grid has been properly placed by inspecting Spot Finding at the Four Corners of the Array. See Figure 7.

If a QC Metric Set has been assigned to the FE Project, you can view the results of the metric evaluation in three ways:

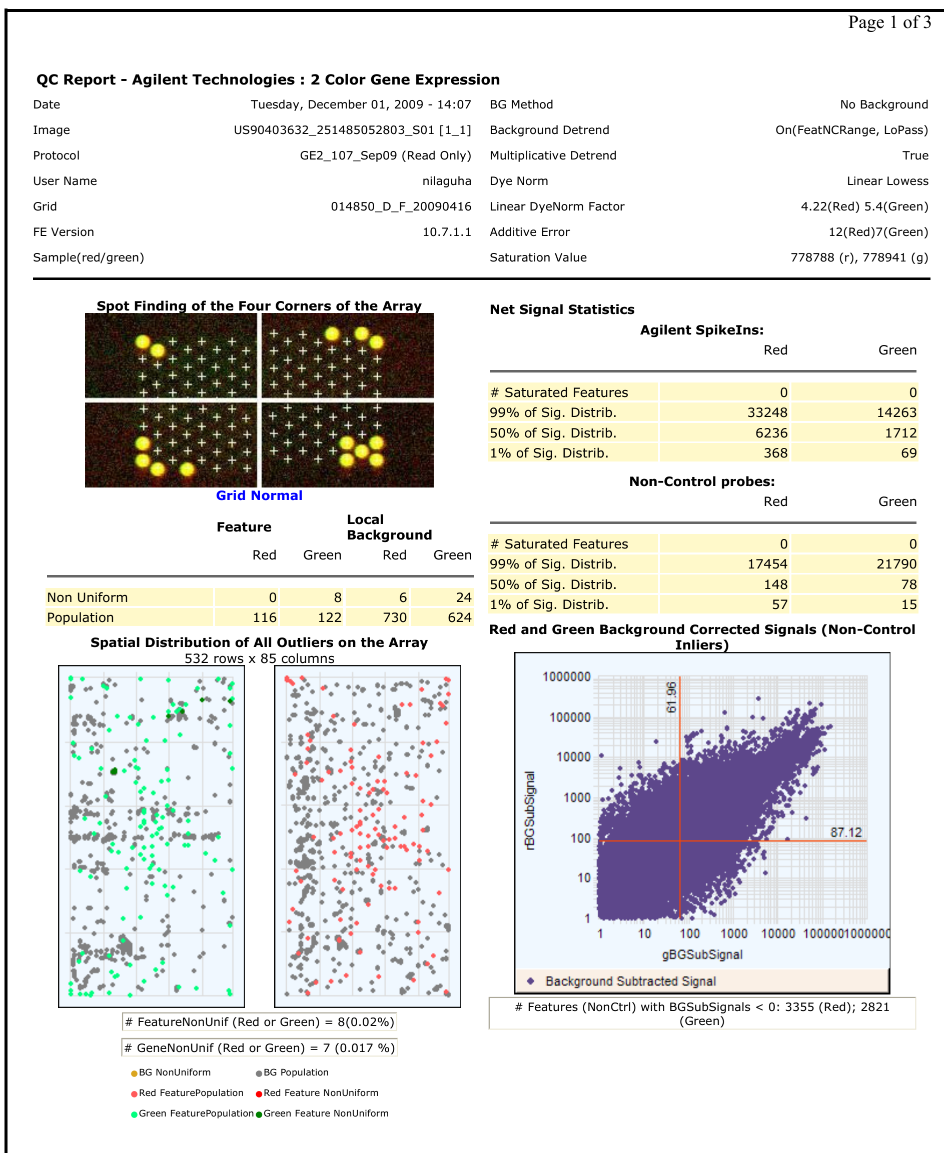
- Project Run Summary - includes a summary sentence.
- QC Report - includes both a summary on the header and a table of metric values.
- QC Chart - includes a view of the values of each metric compared across all extractions in FE Project.

Refer to the application note *Enhanced Quality Assessment Using Agilent Feature Extraction QC Metric Sets, Thresholds, and Charting Tools* (p/n 5989-5952EN) for more details on quality assessment and troubleshooting with the Feature Extraction QC Report. This technical note can be downloaded from the Agilent web site at www.agilent.com. Search for the part number 5989-5952EN.

Automatic Download from eArray

Feature Extraction version 10.7 or higher can automatically download Grid Templates, protocols and QC metrics (QCM or QCMT). To set this up, in the eArray Login Setting dialog box, under **Advanced Options**, click **Use eArray server during extraction**. See Figure 6.

Figure 6 eArray Login Setting



Page 1 of 3

QC Report - Agilent Technologies : 2 Color Gene Expression

Date	Tuesday, December 01, 2009 - 14:07	BG Method	No Background
Image	US90403632_251485052803_S01 [1_1]	Background Detrend	On(FeatNCRange, LoPass)
Protocol	GE2_107_Sep09 (Read Only)	Multiplicative Detrend	True
User Name	nilaguha	Dye Norm	Linear Lowess
Grid	014850_D_F_20090416	Linear DyeNorm Factor	4.22(Red) 5.4(Green)
FE Version	10.7.1.1	Additive Error	12(Red)7(Green)
Sample(red/green)		Saturation Value	778788 (r), 778941 (g)

Spot Finding of the Four Corners of the Array

Grid Normal

	Feature		Local Background	
	Red	Green	Red	Green
Non Uniform	0	8	6	24
Population	116	122	730	624

Spatial Distribution of All Outliers on the Array
532 rows x 85 columns

FeatureNonUnif (Red or Green) = 8(0.02%)

GeneNonUnif (Red or Green) = 7 (0.017 %)

Net Signal Statistics

Agilent SpikeIns:

	Red	Green
# Saturated Features	0	0
99% of Sig. Distrib.	33248	14263
50% of Sig. Distrib.	6236	1712
1% of Sig. Distrib.	368	69

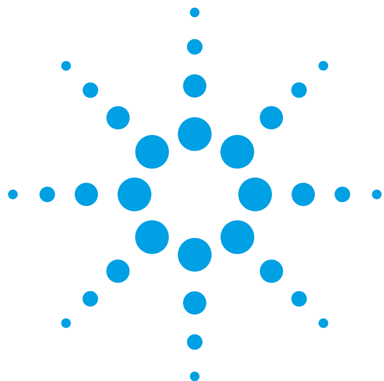
Non-Control probes:

	Red	Green
# Saturated Features	0	0
99% of Sig. Distrib.	17454	21790
50% of Sig. Distrib.	148	78
1% of Sig. Distrib.	57	15

Red and Green Background Corrected Signals (Non-Control Inliers)

Features (NonCtrl) with BGSubSignals < 0: 3355 (Red); 2821 (Green)

Figure 7 Example of the first page of a QC Report generated by Feature Extraction Software



3
Supplemental Procedures

Quality Assessment of Template RNA 52
Preventing Ozone-Related Problems 56

The procedures in this chapter are supplemental to the main protocol.

Quality Assessment of Template RNA

This section gives a general guideline for template RNA quality assessment before proceeding with amplification or hybridization. Although optional, this step is highly recommended.

High quality RNA have minimal residual protein, gDNA, or organic solvent from isolation. As such, A260/A280 and A260/230 ratios are above 2.0 or 1.8 respectfully. Genomic DNA is removed with a high quality DNase treatment.

Make sure you determine the integrity of the input template RNA before you label/amplify and hybridize respectively. Use the Agilent 2100 bioanalyzer. The RNA 6000 Nano LabChip kit can be used to analyze total RNA, mRNA, or cRNA with the appropriate assay at the assay specified concentration. For low concentration samples consider using the RNA 6000 Pico LabChip kit.

For the assessment of total RNA quality, the Agilent 2100 Expert Software automatically provides a RNA Integrity Number (RIN). RIN provides a quantitative value for RNA integrity that facilitates the standardization of quality interpretation. Users should define a minimum threshold RIN number based on correlative data in order to eliminate experimental bias due to poor RNA quality. Analysis of single stranded RNA provides information on size distribution and concentration. It allows relative quantification of fragments within a size range.

Step 1. Prepare for quality assessment

- Refer to Table 16 and Table 17 to make sure that you have the appropriate analyzer, kits, and compatible assays.

Table 16 Analyzer and Kits

Description	Vendor and part number
2100 Bioanalyzer	Agilent p/n G2938C or G2939A
RNA 6000 Nano LabChip Kit	Agilent p/n 5067-1511
RNA 6000 Pico LabChip Kit	Agilent p/n 5067-1513

Table 17 Compatible Assays

Description	Compatible Assay
RNA 6000 Nano LabChip Kit	Prokaryotic Total RNA Nano Assay Qualitative range 5 to 500 ng/µL
RNA 6000 Nano LabChip Kit	mRNA Nano Assay* Qualitative range 25 to 250 ng/µL
RNA 6000 Pico LabChip Kit	Prokaryotic Total RNA Pico Assay Qualitative range 50 to 5000 pg/µL in water
RNA 6000 Pico LabChip Kit	mRNA Pico Assay* Qualitative range 250 to 5000 pg/µL in water

* The mRNA assays are suitable for analysis of cDNA as well.

Step 2. Assess the quality using the Agilent 2100 Bioanalyzer

1. Choose the kit and assay according to your needs. Typically the RNA Nano 6000 kit and assay will be appropriate.
2. Ensure the 2100 bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
3. Start the Agilent 2100 Expert program (version B.02.06 or higher), turn on the 2100 bioanalyzer and check communication.
4. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
5. Load the prepared chip into the 2100 bioanalyzer and start the run within five minutes after preparation.
6. Within the instrument context, choose the appropriate assay from the drop down list.
7. Start the run. Enter sample names and comments in the Data and Assay context.
8. Verify the results.

Template RNA results (total RNA)

The resulting electropherogram should have at least two distinct peaks representing the prokaryotic 16S and 23S ribosomal RNA. Additional bands are the lower marker, and the potentially 5S RNA. Presence of 5S RNA depends on the purification method generally showing lower abundance in column purified total RNA (see Figure 8). Degradation of RNA samples can lead to compromised array results. Both the electropherogram and the RIN values can help determine the quality of the sample.

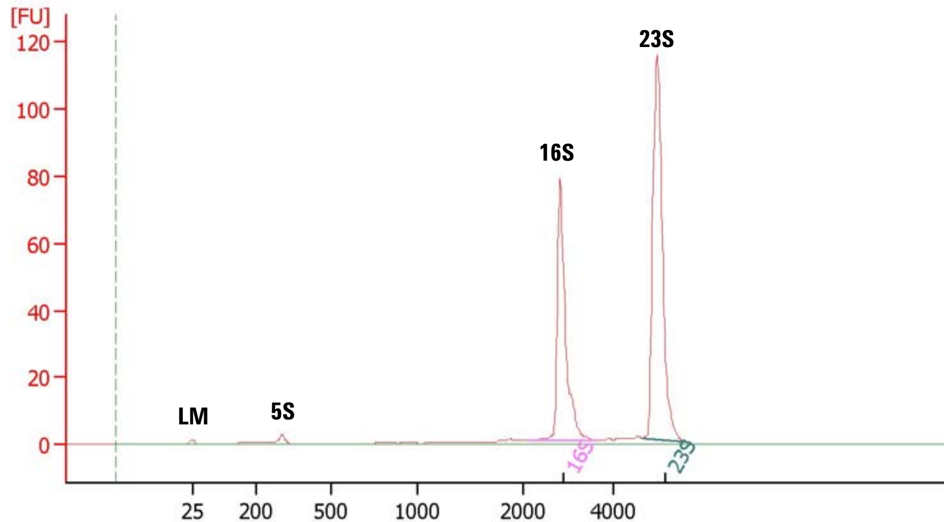


Figure 8 Analysis of *Escherichia coli* total RNA with the Prokaryote Total RNA Nano assay. Ribosomal RNA peaks are clearly defined, and indicated as 16S and 23S. This high quality RNA sample has a RIN value of 10.

For general assistance on evaluation of total RNA with emphasis on the RNA integrity number, see the corresponding application note: “RNA integrity number (RIN) - Standardization of RNA quality control”, 5989-1165EN.

To download application notes regarding the 2100 bioanalyzer visit Agilent web site at www.agilent.com/chem/labonachip.

Preventing Ozone-Related Problems

The Agilent two-color platform is robust in environments where the ozone level is 5 ppb (approximately 10 µg/m^3) or less. Beyond this level, ozone can significantly affect cyanine 5 signal and compromise microarray performance.

For Scanner C and Scanner B, the Agilent Ozone-Barrier Slide cover is designed to protect against ozone-induced degradation of cyanine dyes and is recommended when using Agilent oligo-based microarrays in high-ozone environments. See step 14 on page 39.

For the Agilent SureScan scanner, two built-in mechanisms minimize dye signal degradation by ozone and other dye oxidants:

- SureScan slide holder with an integrated ozone barrier in its lid.
- Catalytic ozone decomposition filtering system inside the scanner.

In addition to the ozone barriers, the Agilent Stabilization and Drying Solution, which is an organic solvent based wash, can reduce background variability produced by wash artifacts.

The use of the Agilent Stabilization and Drying Solution is described in this section. Before you begin, make sure that you follow the correct wash procedure:

Table 18 Wash procedure to follow

Ozone level in your lab	Wash Procedure	Ozone-Barrier Slide Cover
< 5 ppb	Wash Procedure *without* Stabilization and Drying Solution. "Step 4. Wash the microarray slides" on page 37	No
> 5 ppb and < 10 ppb	Wash Procedure *without* Stabilization and Drying Solution. "Step 4. Wash the microarray slides" on page 37	Yes
> 10 ppb	Wash Procedure *with* Stabilization and Drying Solution. See "Step 1. Prepare the Stabilization and Drying Solution" on page 57 and "Step 2. Wash with Stabilization and Drying Solution" on page 58.	Yes

For more information, visit www.agilent.com/chem/dnatechnicalnotes to download the technical note on *Improving Microarray Results by Preventing Ozone-Mediated Fluorescent Signal Degradation* (p/n 5989-0875EN).

Step 1. Prepare the Stabilization and Drying Solution

The Agilent Stabilization and Drying Solution contains an ozone scavenging compound dissolved in acetonitrile. The compound in solution is present in saturating amounts and may precipitate from the solution under normal storage conditions. If the solution shows visible precipitation, warming of the solution will be necessary to redissolve the compound. Washing slides using Stabilization and Drying Solution showing visible precipitation will have a profound adverse effect on microarray performance.

WARNING

The Agilent Stabilization and Drying Solution is a flammable liquid. Warming the solution will increase the generation of ignitable vapors.

Do not use an open flame or a microwave. Do not increase temperature rapidly. Warm and mix the material away from ignition sources.

Use gloves and eye/face protection in every step of the warming procedures.

WARNING

Failure to follow the outlined process will increase the potential for fire, explosion, and possible personal injury. Agilent assumes no liability or responsibility for damage or injury caused by individuals performing this process.

1 Warm the solution slowly in a water bath or a vented conventional oven at 40°C in a closed container with sufficient head space to allow for expansion.

NOTE

The original container can be used to warm the solution. Container volume is 700 mL and contains 500 mL of liquid. If a different container is used, maintain or exceed this headspace/liquid ratio. The time needed to completely redissolve the precipitate is dependent on the amount of precipitate present, and may require overnight warming if precipitation is heavy. DO NOT FILTER the Stabilization and Drying solution.

2 If needed, gently mix to obtain a homogeneous solution.

   Mix under a vented fume hood away from open flames, or other sources of ignition. Warm the solution only in a controlled and contained area that meets local fire code requirements.

3 After the precipitate is completely dissolved, let the covered solution stand at room temperature, allowing it to *equilibrate to room temperature and make sure that precipitation does not occur prior to use.*

Step 2. Wash with Stabilization and Drying Solution

NOTE Use fresh Gene Expression Wash Buffer for each wash group (up to eight slides). The acetonitrile and Stabilization and Drying Solution can be reused for washing of up to three groups of slides (for a total of 24 slides).

WARNING **The Stabilization and Drying Solution must be set-up in a fume hood. Wash 1 and Wash 2 set-up areas should be put close to, or preferably in, the same fume hood. Use gloves and eye/face protection in every step of the warming procedures.**

Table 19 lists the wash conditions for the wash procedure with Stabilization and Drying Solution.

Table 19 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	1	Gene Expression Wash Buffer 1	Room temperature	
1st wash	2	Gene Expression Wash Buffer 1	Room temperature	1 minute
2nd wash	3	Gene Expression Wash Buffer 2	Elevated temperature*	1 minute
Acetonitrile Wash	4	acetonitrile	Room temperature	10 seconds
3rd wash	5	Stabilization and Drying Solution	Room temperature	30 seconds

* The elevated temperature of the second wash step is usually around 31°C due to cooling by the room temperature dish and the rack of arrays.

1 Completely fill slide-staining dish #1 with Gene Expression Wash Buffer 1 at room temperature.
2 Put a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Gene Expression Wash Buffer 1 at room temperature to cover the slide rack. Put this dish on a magnetic stir plate.
3 Put the empty dish #3 on the stir plate and add a magnetic stir bar. Do not add the pre-warmed (37°C) Gene Expression Wash Buffer 2 until the first wash step has begun.

4 Fill slide-staining dish #4 approximately three-fourths full with acetonitrile. Add a magnetic stir bar and put this dish on a magnetic stir plate.

5 Fill slide-staining dish #5 approximately three-fourths full with Stabilization and Drying Solution. Add a magnetic stir bar and put this dish on a magnetic stir plate.

6 Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization, and if all bubbles are rotating freely.

7 Prepare the hybridization chamber disassembly.

   a Put the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.

   b Slide off the clamp assembly and remove the chamber cover.

   c With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

   d Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Gene Expression Wash Buffer 1.

8 With the sandwich completely submerged in Gene Expression Wash Buffer 1, pry the sandwich open from the barcode end only:

   a Slip one of the blunt ends of the forceps between the slides.

   b Gently turn the forceps upwards or downwards to separate the slides.

   c Let the gasket slide drop to the bottom of the staining dish.

   d Remove the microarray slide and put into slide rack in the slide-staining dish #2 containing Gene Expression Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

9 Repeat step 6 through step 8 for up to seven additional slides in the group. A maximum of eight disassembly procedures yielding eight microarray slides is advised at one time in order to facilitate uniform washing.

10 When all slides in the group are put into the slide rack in slide-staining dish #2, stir using setting 4 for 1 minute.

11 During this wash step, remove Gene Expression Wash Buffer 2 from the 37°C water bath and pour into the Wash 2 dish.

12 Transfer slide rack to slide-staining dish #3 containing Gene Expression Wash Buffer 2 at elevated temperature. Stir using setting 4 for 1 minute.

13 Remove the slide rack from Gene Expression Wash Buffer 2 and tilt the rack slightly to minimize wash buffer carry-over. Immediately transfer the slide rack to slide-staining dish #4 containing acetonitrile and stir using setting 4 for less than 10 seconds.

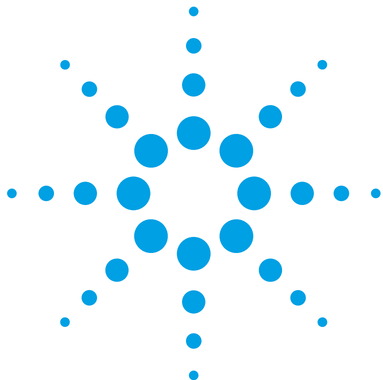
14 Transfer the slide rack to dish #5 filled with Stabilization and Drying Solution and stir using setting 4 for 30 seconds.

15 Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.

16 Discard used Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2.

17 Repeat steps 1 through 16 for the next group of eight slides using fresh Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2 pre-warmed to 37°C.

18 Scan slides immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a nitrogen purge box, in the dark.



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Reference

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Supplemental User Guides 64
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Array/Sample tracking microarray slides 69

This chapter contains reference information related to the protocol and Feature Extraction default parameter settings

Kit Contents

The content of the kits used in this protocol (required and optional) are listed here.

Table 20 RNA Spike-In Kit, Two-Color

Content
Spike A Mix
Spike B Mix
Dilution Buffer

Table 21 FairPlay III Microarray Labeling Kit

Content
10× AffinityScript Reaction Buffer (clear cap)
Oligo d(T) primer (pink cap)
0.1 M DTT (green cap)
DEPC water (green cap)
20× dNTP Mix with amino allyl dUTP (green cap)
RNase Block (purple cap)
2× Coupling Buffer (clear cap)
Glycogen (green cap)
DMSO (green cap)
AffinityScript HC Reverse Transcriptase (purple cap)
Random Primers (pink cap)
DNA-binding solution
Microspin cup
Receptacle tube (2 mL)

Table 22 Gene Expression Hybridization Kit

Content
10× Gene Expression Blocking Agent
25× Fragmentation Buffer
2× Hi-RPM Hybridization Buffer

Table 23 Gene Expression Wash Buffer Kit

Content
Gene Expression Wash Buffer 1
Gene Expression Wash Buffer 2
Triton X-102 (10%)

Table 24 RNeasy Mini Kit

Content
RNeasy Mini Spin Column (pink)
Collection Tube (1.5 ml)
Collection Tube (2 ml)
Buffer RLT
Buffer RW1
Buffer RPE
RNase-Free Water

Supplemental User Guides

First-time users of the Agilent oligo microarray system, please refer to the following user manuals for detailed descriptions and operation recommendations for each of the hardware and software components used in the two-color platform workflow. The user guides can be downloaded from the Agilent web site at www.agilent.com/chem/dnamanuals-protocols.

- Agilent Microarray Hybridization Chamber User Guide
- Hybridization Oven User Manual
- Microarray Scanner System User Guide
- G4900DA SureScan Microarray Scanner User Guide
- Feature Extraction Software Quick Start Guide
- Feature Extraction Software User Guide
- Feature Extraction Software Reference Guide

Microarray Handling Tips

Each microarray is printed on the side of the glass slide containing the "Agilent"-labeled barcode. This side is called the "active" side. The numeric barcode is on the inactive side of the slide.

CAUTION You must familiarize yourself with the assembly and disassembly instructions for use with the Agilent Microarray Hybridization Chamber (G2534A) and gasket slides. Practice slide kits are available.

In this "processing and hybridization" procedure, the hybridization mixture is applied directly to the gasket slide, and not to the active side of the oligo microarray. Instead, the active side of the oligo microarray is put on top of the gasket slide to form a "sandwich slide" pair.

To avoid damaging the microarray, always handle glass slides carefully by their edges. Wear powder-free gloves. Never touch the surfaces of the slides. If you do, you may cause irreparable damage to the microarray.

Never allow the microarray surface to dry out during the hybridization process and washing steps.

General Microarray Layout and Orientation

Agilent oligo microarray (1 microarray/slide format) as imaged on the Agilent microarray scanner

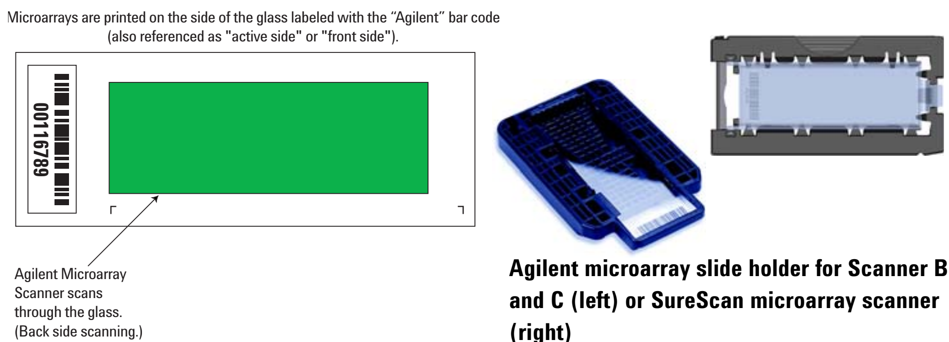

Figure 9 Agilent microarray slide and slide holder. The opposite or "non-active" numerically barcoded side is shown.

Agilent oligo microarray formats and the resulting "microarray design files" are based on how the Agilent microarray scanner images 1-inch × 3-inch glass slides. Agilent designed its microarray scanner to scan through the glass slide (back side scanning). The glass slide is securely placed in an Agilent microarray slide holder with the "Agilent" labeled barcode facing the opening of the slide holder (on SureScan Microarray Scanner G4900DA) or facing the inside of the slide holder (C scanner G2565CA). In this orientation, the "active side" containing the microarrays is protected from potential damage by fingerprints and other elements. Once securely placed, the numeric barcode, non-active side of the slide, is visible from the outside of the slide holder.

Figure 9 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the "microarray design files" that Agilent generates during the manufacturing process of its *in situ*-synthesized oligonucleotide microarrays. Thus, if you have a scanner that reads microarrays from the "front side" of the glass slide, the collection of microarray data points will be different in relation to the "microarray design files" supplied with the Agilent oligo microarray kit you purchased. Therefore, please take a moment to become familiar with the microarray layouts for each of the Agilent oligo microarrays and the layout information as it pertains to scanning using a "front side" scanner.

Non-Agilent front side microarray scanners

When imaging Agilent oligo microarray slides, you must determine:

- If the scanner images microarrays by reading them on the “front side” of the glass slide (“Agilent”-labeled barcode side of the slide) and
- If the image produced by the non-Agilent scanner is oriented in a “portrait” or “landscape” mode, “Agilent”-labeled barcode left-side, right-side, up or down, as viewed as an image in the imaging software (see Figure 10).

This changes the feature numbering and location as it relates to the “microarray design files” found on the CD in each Agilent oligo microarray kit. Microarray layout maps are available from Agilent. For more information, go to www.agilent.com/chem/dnamanuals-protocols and download *Agilent Microarray Formats Technical Drawings with Tolerance* (publication G4502-90001). This document contains visual references and guides that will help you determine the feature numbering as it pertains to your particular scanner configuration.

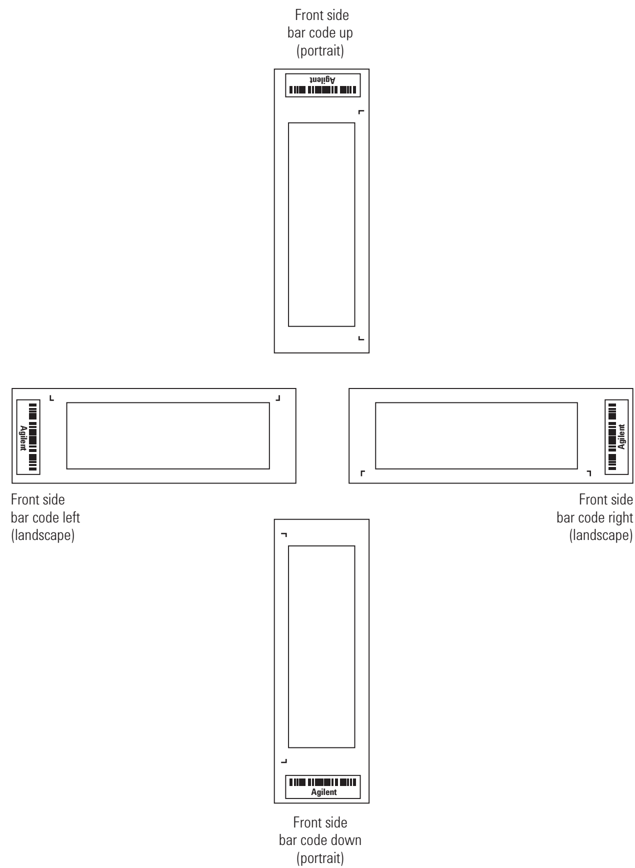

Figure 10 Microarray slide orientation

Array/Sample tracking microarray slides

Use the form below to make notes to track your samples on microarray slides.

Position the gasket slide in the SureHyb chamber base with the label to the left. Load the samples: top row, left to right, then lower row, left to right. The array suffix assignments from Feature Extraction will be in the order shown.

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
BARCODE	**Sample:**	**Sample:**	**Sample:**	**Sample:**
	Sample:	**Sample:**	**Sample:**	**Sample:**
	Array 2_1	Array 2_2	Array 2_3	Array 2_4

Barcode Number ____________________

Figure 11 8-pack microarray slide

www.agilent.com

In This Book

This guide contains information to run the Two-Color Microarray-Based Prokaryote Analysis protocol.

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Version 2.0, August 2015

G4813-90010
Revision B0

Agilent Technologies