Two-Color Microarray-Based Gene Expression Analysis
Low Input Quick Amp Labeling

Protocol
For use with Agilent Gene Expression oligo microarrays
Version 6.5, May 2010

Microarrays manufactured with Agilent SurePrint Technology

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Agilent Technologies
Two-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol

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In this Guide...

This document describes Agilent’s 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, 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 cRNA, and steps to prevent ozone-related problems.

4 Reference

This chapter contains reference information related to the protocol.
What’s New in Version 6.5?

- SurePrint G3 (1x1M, 2x400K, 4x180K, and 8x60K) microarray formats are supported.
- Stratagene Absolute RNA Nanoprep kit to purify labeled/amplified RNA is supported.
- The cRNA hybridization input has been updated for the 1-pack and 2-pack microarray format.
- The starting input requirement has been reduced to 10 ng for the 8-pack microarray format.

What’s New in Version 6.0?

- The Agilent Low Input Quick Amp Labeling Kit is supported in addition to the Agilent Quick Amp Labeling Kit.
- An optional thermocycler protocol has been added.
- Agilent Ozone-Barrier Slide Covers can be used to prevent ozone-related problems.
- Updated settings are provided for the Agilent Microarray Scanner.
- The hybridization procedure now includes the addition of an ice step.
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Before You Begin

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’s 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 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.
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 3-CTP and cyanine 5-CTP 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 Agilent’s 2X 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 Agilent’s 2X Hybridization Buffer.
- Triton is harmful if swallowed. Risk of serious damage to eyes. Wear suitable PPE. Triton is a component of Agilent’s 2X 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.
- Agilent 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.
1 Before You Begin
Agilent Oligo Microarray Kit Contents

Agilent Oligo Microarray Kit Contents

Check the Agilent Web site at www.agilent.com/chem/dualmode for the most up to date list of supported microarray designs.

Catalog microarray kits

- One to eight microarrays printed on each 1-inch × 3-inch glass slides, of a five slide kit.
- DVD containing microarray design files in various file formats

Custom microarray kits

- One to eight microarrays printed on each 1-inch × 3-inch glass slide
- Number of microarrays varies per kit and per order

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.
## Required Equipment

<table>
<thead>
<tr>
<th>Description</th>
<th>Vendor and part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent Microarray Scanner</td>
<td>Agilent p/n G2565BA or G2565CA</td>
</tr>
<tr>
<td>Hybridization Chamber, stainless</td>
<td>Agilent p/n G2534A</td>
</tr>
<tr>
<td>Hybridization Chamber gasket slides</td>
<td></td>
</tr>
<tr>
<td>1 microarray/slide, 5 slides/box</td>
<td>Agilent p/n G2534-60003</td>
</tr>
<tr>
<td>2 microarrays/slide, 5 slides/box</td>
<td>Agilent p/n G2534-60002</td>
</tr>
<tr>
<td>4 microarrays/slide, 5 slides/box</td>
<td>Agilent p/n G2534-60011</td>
</tr>
<tr>
<td>8 microarrays/slide, 5 slides/box</td>
<td>Agilent p/n G2534-60014</td>
</tr>
<tr>
<td>Hybridization oven; temperature set at 65°C</td>
<td>Agilent p/n G2545A</td>
</tr>
<tr>
<td>Hybridization oven rotator for Agilent Microarray Hybridization Chambers</td>
<td>Agilent p/n G2530-60029</td>
</tr>
<tr>
<td>Nuclease-free 1.5 mL microfuge tubes</td>
<td>Ambion p/n 12400 or equivalent</td>
</tr>
<tr>
<td>Magnetic stir bar (×2)</td>
<td>Corning p/n 401435 or equivalent</td>
</tr>
<tr>
<td>Magnetic stir plate (×2)</td>
<td>Corning p/n 6795-410 or equivalent</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>Eppendorf p/n 5417R or equivalent</td>
</tr>
<tr>
<td>NanoDrop ND-1000 UV-VIS spectrophotometer</td>
<td>NanoDrop p/n ND-1000 or equivalent</td>
</tr>
<tr>
<td>Slide-staining dish, with slide rack (×3)</td>
<td>Thermo Shandon p/n 121 or equivalent</td>
</tr>
<tr>
<td>Circulating water baths or heat blocks set to 37°C, 65°C, 80°C, 40°C, 70°C, and 60°C</td>
<td></td>
</tr>
<tr>
<td>Clean forceps</td>
<td></td>
</tr>
<tr>
<td>Ice bucket</td>
<td></td>
</tr>
<tr>
<td>Pipetman micropipettors, (P-10, P-20, P-200, P-1000) or equivalent</td>
<td></td>
</tr>
<tr>
<td>Powder-free gloves</td>
<td></td>
</tr>
<tr>
<td>Sterile, nuclease-free aerosol barrier pipette tips</td>
<td></td>
</tr>
<tr>
<td>Vortex mixer</td>
<td></td>
</tr>
<tr>
<td>Timer</td>
<td></td>
</tr>
<tr>
<td>Nitrogen purge box for slide storage</td>
<td></td>
</tr>
</tbody>
</table>

**Before You Begin**

Two-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol
1 Before You Begin

Required Reagents

<table>
<thead>
<tr>
<th>Description</th>
<th>Vendor and part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Input Quick Amp Labeling Kit, Two-Color</td>
<td>Agilent p/n 5190-2306</td>
</tr>
<tr>
<td>RNA Spike-In Kit, Two-Color</td>
<td>Agilent p/n 5188-5279</td>
</tr>
<tr>
<td>Gene Expression Hybridization Kit</td>
<td>Agilent p/n 5188-5242</td>
</tr>
<tr>
<td>Gene Expression Wash Buffer Kit</td>
<td>Agilent p/n 5188-5327</td>
</tr>
<tr>
<td>DNase/RNase-free distilled water</td>
<td>Invitrogen p/n 10977-015</td>
</tr>
<tr>
<td>Milli-Q water or equivalent</td>
<td></td>
</tr>
<tr>
<td>RNeasy Mini Kits (50 columns or 250 columns)</td>
<td>Qiagen p/n 74104 or 74106</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>Amresco p/n E193</td>
</tr>
</tbody>
</table>

Optional Equipment/Reagents

<table>
<thead>
<tr>
<th>Description</th>
<th>Vendor and part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>2100 Bioanalyzer</td>
<td>Agilent p/n G2938A</td>
</tr>
<tr>
<td>RNA 6000 Nano Assay Kit (RNA Series II Kit)</td>
<td>Agilent p/n 5067-1511</td>
</tr>
<tr>
<td>Slide box</td>
<td>Corning p/n 07201629</td>
</tr>
<tr>
<td>Stabilization and Drying Solution</td>
<td>Agilent p/n 5185-5979</td>
</tr>
<tr>
<td>Ozone-Barrier Slide Cover</td>
<td>Agilent p/n G2505-60550</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Sigma p/n 271004-1L</td>
</tr>
<tr>
<td>Absolutely RNA Nanoprep Kit</td>
<td>Stratagene p/n 4000753</td>
</tr>
<tr>
<td>Thermocycler</td>
<td></td>
</tr>
<tr>
<td>PCR 96-well plate or 0.2 mL PCR tubes</td>
<td></td>
</tr>
</tbody>
</table>
**Required Hardware and Software**

<table>
<thead>
<tr>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentium III 1.5 GHz or higher (Pentium IV 2.0 GHz or higher recommended)</td>
</tr>
<tr>
<td>Agilent’s Scan Control software, version A.7.0.1 for the B Scanner or A.8.4.1 for the C Scanner</td>
</tr>
<tr>
<td>2 GB RAM (4 GB recommended for 64-bit operating systems)</td>
</tr>
<tr>
<td>40 GB available disk space (if saving images and results files locally)</td>
</tr>
<tr>
<td>Windows 2000 with SP2 or later (fully tested on SP4), Windows XP SP2</td>
</tr>
<tr>
<td>Feature Extraction software 9.5 or later</td>
</tr>
<tr>
<td>Internet Explorer 6.0 or later</td>
</tr>
<tr>
<td>Adobe Acrobat Reader 4.0 or later</td>
</tr>
</tbody>
</table>

**Optional Software**

<table>
<thead>
<tr>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneSpring GX 10.0 or later</td>
</tr>
</tbody>
</table>

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Agilent's Two-Color Microarray-based Gene Expression 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.

* Samples can be stored frozen at -80°C after these steps, if needed.
Sample Preparation

Agilent's Low Input Quick Amp Labeling Kit generates fluorescent cRNA (complementary RNA) with a sample input RNA range between 10 ng and 200 ng of total RNA or a minimum of 5 ng of poly A⁺ RNA for two-color processing. The method uses T7 RNA polymerase, which simultaneously amplifies target material and incorporates cyanine 3- or cyanine 5-labeled CTP. Amplification is typically at least a 100-fold from total RNA to cRNA with the use of this kit.

NOTE
For optimal performance, use high quality, intact template total or poly A⁺ RNA. Please refer to "Quality Assessment of Template RNA and Labeled cRNA" on page 70 for general guidance and procedural recommendations on quality assessment of template RNA.
Figure 2  Schematic of amplified cRNA procedure. Generation of cRNA for a two-color microarray experiment is shown. When you generate targets for a one-color microarray experiment, only the Cy3-labeled “B” sample is produced and hybridized.
Step 1. Prepare Spike A Mix and Spike B Mix

(Time required: ~0.5 hours)

Refer to the protocol on Agilent RNA Spike-In Kit (publication 5188-5928) for in-depth instructions and troubleshooting advice on how to use two-color spike mixes. This protocol is available with the Two-Color RNA Spike-In Kit and can also be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols.

1. Equilibrate water baths to 37°C, 65°C, 80°C, 40°C and 70°C.
2. Vigorously mix the stock solutions on a vortex mixer.
3. Heat at 37°C for 5 minutes, and mix on a vortex mixer once more.
4. Briefly spin in a centrifuge to drive contents to the bottom of the tube prior to opening. Settlement of the solution on the sides or lid of the tubes may occur during shipment and storage.

Table 1 provides the dilutions of Spike A Mix and Spike B Mix for four different starting sample inputs of total RNA or 5 ng of poly A mRNA. Always label Spike A Mix with cyanine 3 and Spike B Mix with cyanine 5. The dilution scheme for Spike A Mix with cyanine 3 is the same as the dilution scheme for Spike B Mix with cyanine 5. The output of the Agilent SpikeIns LogRatio Statistics table in the QC report, generated when using Agilent Feature Extraction Software 9.5 or later, uses this orientation. It is built into the software code and cannot be set by the user. If the polarity is flipped, you will have to manually adjust the output.
2 Procedures

Step 1. Prepare Spike A Mix and Spike B Mix

For example, to prepare the Spike A Mix dilution appropriate for 25 ng of total RNA starting sample:

1 Create the First Dilution:
   a Label a new sterile 1.5 mL microcentrifuge tube “Spike A Mix First Dilution.”
   b Mix the thawed Spike A Mix vigorously on a vortex mixer.
   c Heat at 37°C in a circulating water bath for 5 minutes.
   d Mix the Spike A Mix tube vigorously again on a vortex mixer.
   e Spin briefly in a centrifuge to separate contents to the bottom of the tube.
   f Into the First Dilution tube, put 2 µL of Spike A Mix stock.
   g Add 38 µL of Dilution Buffer provided in the Spike-In kit (1:20).
   h Mix thoroughly on a vortex mixer and spin down quickly to collect all of the liquid at the bottom of the tube. This tube contains the First Dilution.

<table>
<thead>
<tr>
<th>Starting Amount of RNA</th>
<th>Serial Dilution</th>
<th>Spike Mix Volume to be used in each labeling reaction (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA (ng)</td>
<td>PolyA RNA (ng)</td>
<td>First</td>
</tr>
<tr>
<td>10</td>
<td>1:20</td>
<td>1.40</td>
</tr>
<tr>
<td>25</td>
<td>1:20</td>
<td>1.40</td>
</tr>
<tr>
<td>50</td>
<td>1:20</td>
<td>1.40</td>
</tr>
<tr>
<td>100</td>
<td>1:20</td>
<td>1.40</td>
</tr>
<tr>
<td>200</td>
<td>1:20</td>
<td>1.40</td>
</tr>
<tr>
<td>5</td>
<td>1:20</td>
<td>1.40</td>
</tr>
</tbody>
</table>

NOTE Use RNase-free microfuge tubes and tips. Make sure you dispense at least 2 µL with a pipette to ensure accuracy.
2 Create the Second Dilution:
   a Label a new sterile 1.5 mL microcentrifuge tube “Spike A Mix Second Dilution.”
   b Into the Second Dilution tube, put 2 µL of First Dilution.
   c Add 78 µL of Dilution Buffer (1:40).
   d Mix thoroughly on a vortex mixer and spin down quickly to collect all of the liquid at the bottom of the tube. This tube contains the Second Dilution.

3 Create the Third Dilution:
   a Label a new sterile 1.5 mL microcentrifuge tube “Spike A Mix Third Dilution.”
   b Into the Third Dilution tube, put 2 µL of Second Dilution.
   c Add 30 µL of Dilution Buffer (1:16).
   d Mix thoroughly on a vortex mixer and spin down quickly to collect all the liquid at the bottom of the tube. This tube contains the Third Dilution.

4 Create the Fourth Dilution:
   a Label a new sterile 1.5 mL microcentrifuge tube “Spike A Mix Fourth Dilution.”
   b Into the Fourth Dilution tube, add 4 µL of Third Dilution to 28 µL of Dilution Buffer for the Fourth Dilution (1:8).
   c Mix thoroughly on a vortex mixer and spin down quickly to collect all of the liquid at the bottom of the tube. This tube contains the Fourth Dilution (now at a 102,400-fold final dilution).

5 Add 2 µL of Fourth Dilution to 25 ng of sample total RNA as listed in Table 1 and continue with cyanine 3 labeling using the Agilent Low Input Quick Amp Kit protocol as described in “Step 2. Prepare labeling reaction” on page 23.
2 Procedures
Step 1. Prepare Spike A Mix and Spike B Mix

Storage of Spike Mix dilutions

Store the Agilent RNA Spike-In Kit, Two-Color at −70°C to −80°C in a non-defrosting freezer for up to 1 year from the date of receipt.

The first dilution of the Agilent Spike Mix (A or B) positive controls can be stored up to 2 months in a non-defrosting freezer at −70°C to −80°C and freeze/thawed up to eight times.

After use, discard the second, third and fourth dilution tubes.
Step 2. Prepare labeling reaction

(Time required: ~5.5 hours)

For each assay, make sure that the volume of the total RNA sample plus diluted RNA spike-in controls does not exceed 3.5 µL. Because the 1x reaction involves volumes of less than 1 µL, prepare components in a master mix and divide into the individual assay tubes in volumes >1 µL. When preparing 4 samples, use the 5x master mix. When preparing 8 samples, use the 10x master mix.

The starting input for the Low Input Quick Amp Labeling Kit ranges from 10 ng to 200 ng of total RNA. For best results, start with at least 25 ng of total RNA for the 4-pack and 8-pack formats, and 50 ng of total RNA for the 1-pack and 2-pack formats. For the 8-pack microarray format, as little as 10 ng of total RNA can be used to generate high quality data.

1 Add 10 to 200 ng of total RNA or 5 ng polyA RNA to a 1.5-mL microcentrifuge tube in a final volume of 1.5 µL. If samples are concentrated, dilute with water until 10 to 200 ng of total or 5 ng of polyA RNA is added in a 1.5 µL volume. Dilute the total RNA just prior to use and store the total RNA at concentrations over 100 ng/µL.

2 Prepare tubes for both Spike A Mix/cyanine 3-CTP and Spike B Mix/cyanine 5-CTP dyes.

3 Add 2 µL of diluted Spike Mix to each tube. Each tube now contains a total volume of 3.5 µL.
2 Procedures

Step 2. Prepare labeling reaction

4 Prepare and add T7 Promoter Primer:
   a Mix the T7 Promoter Primer and water to prepare the T7 Promoter Primer Master Mix as listed in Table 2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL) per reaction</th>
<th>Volume (µL) per 5 reaction</th>
<th>Volume (µL) per 10 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 Promoter Primer (green cap)</td>
<td>0.8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Nuclease-free water (white cap)</td>
<td>1</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>1.8</strong></td>
<td><strong>9</strong></td>
<td><strong>18</strong></td>
</tr>
</tbody>
</table>

b Add 1.8 µL of T7 Promoter Primer Mix to the tube that contains 3.5 µL of total RNA and diluted RNA spike-in controls. Each tube now contains a total volume of 5.3 µL.

c Denature the primer and the template by incubating the reaction at 65°C in a circulating water bath for 10 minutes.

d Place the reactions on ice and incubate for 5 minutes.

5 Prewarm the 5X first strand buffer at 80°C for 3 to 4 minutes to ensure adequate resuspensions of the buffer components. For optimal resuspension, briefly mix on a vortex mixer and spin the tube in a microcentrifuge to drive down the contents from the tube walls. Keep at room temperature until needed.

6 Prepare and add cDNA Master Mix:
   a Immediately prior to use, add the components for cDNA Master Mix listed in Table 3, use a pipette to gently mix, and keep at room temperature.

   The AffinityScript RNase Block mix is a blend of enzymes. Keep the AffinityScript RNase Block mix on ice and add to the cDNA Master Mix immediately prior to use.
Step 2. Prepare labeling reaction

b Briefly spin each sample tube in a microcentrifuge to drive down the contents from the tube walls and the lid.

c Add 4.7 µL of cDNA Master Mix to each sample tube and mix by pipetting up and down. Each tube now contains a total volume of 10 µL.

d Incubate samples at 40°C in a circulating water bath for 2 hours.

e Move samples to ice. Incubate for 5 minutes.

f Move samples to ice. Incubate for 5 minutes.

g Spin samples briefly in a microcentrifuge to drive down tube contents from the tube walls and lid.

Stopping Point

If you do not immediately continue to the next step, store the samples at –80°C.

7 Prepare and add Transcription Master Mix:

a Immediately prior to use, gently mix the components listed in Table 4 in the order indicated for the Transcription Master Mix by pipetting at room temperature.

The T7 RNA polymerase blend is a blend of enzymes. Keep the T7 RNA polymerase on ice and add to the Transcription Master Mix just before use.

**Table 3** cDNA Master Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL) per reaction</th>
<th>Volume (µL) per 5 reaction</th>
<th>Volume (µL) per 10 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X First Strand Buffer (green cap)</td>
<td>2</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>0.1 M DTT (white cap)</td>
<td>1</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>10 mM dNTP mix (green cap)</td>
<td>0.5</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>AffinityScript RNase Block Mix (violet cap)</td>
<td>1.2</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>4.7</strong></td>
<td><strong>23.5</strong></td>
<td><strong>47</strong></td>
</tr>
</tbody>
</table>

**NOTE** Incubation at 70°C inactivates the AffinityScript enzyme.
2 Procedures

Step 2. Prepare labeling reaction

Table 4 Transcription Master Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL) per reaction</th>
<th>Volume (µL) per 5 reaction</th>
<th>Volume (µL) per 10 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water (white cap)</td>
<td>0.75</td>
<td>3.75</td>
<td>7.5</td>
</tr>
<tr>
<td>5X Transcription Buffer (blue cap)</td>
<td>3.2</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>0.1 M DTT (white cap)</td>
<td>0.6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>NTP mix (blue cap)</td>
<td>1</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>T7 RNA Polymerase Blend (red cap)</td>
<td>0.21</td>
<td>1.05</td>
<td>2.1</td>
</tr>
<tr>
<td>Cyanine 3-CTP or cyanine 5-CTP</td>
<td>0.24</td>
<td>1.2</td>
<td>2.4</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>6</strong></td>
<td><strong>30</strong></td>
<td><strong>60</strong></td>
</tr>
</tbody>
</table>

b Add 6 µL of Transcription Master Mix to each sample tube. Gently mix by pipetting. Each tube now contains a total volume of 16 µL.

c Incubate samples in a circulating water bath at 40°C for 2 hours.

Stopping Point If you do not immediately continue to the next step, store the samples at −80°C.
Step 3. Purify the labeled/amplified RNA

(Time required: ~0.5 hours)

Qiagen’s RNeasy mini spin columns are recommended for purification of the amplified cRNA samples.

If sample concentration causes difficulty, you can use the Stratagene Absolutely RNA Nanoprep kit as an alternative. See “Absolute RNA Nanoprep Purification” on page 52.

NOTE

Make sure that ethanol was added to the RPE buffer as specified in the Qiagen manual before you continue.

1 Add 84 µL of nuclease-free water to your cRNA sample, for a total volume of 100 µL.
2 Add 350 µL of Buffer RLT and mix well by pipetting.
3 Add 250 µL of ethanol (96% to 100% purity) and mix thoroughly by pipetting. Do not centrifuge.
4 Transfer the 700 µL of the cRNA sample to an RNeasy mini column in a 2 mL collection tube. Centrifuge the sample at 4°C for 30 seconds at 13,000 rpm. Discard the flow-through and collection tube.
5 Transfer the RNeasy column to a new collection tube and add 500 µL of buffer RPE (containing ethanol) to the column. Centrifuge the sample at 4°C for 30 seconds at 13,000 rpm. Discard the flow-through. Re-use the collection tube.
6 Add another 500 µL of buffer RPE to the column. Centrifuge the sample at 4°C for 60 seconds at 13,000 rpm. Discard the flow-through and the collection tube.
7 If any buffer RPE remains on or near the frit of the column, transfer the RNeasy column to a new 1.5 mL collection tube and centrifuge the sample at 4°C for 30 seconds at 13,000 rpm to remove any remaining traces of buffer RPE. Discard this collection tube and use a fresh tube to elute the cleaned cRNA sample.

CAUTION

Do not discard the final flow-through in the next step. It contains the cRNA sample.
Step 3. Purify the labeled/amplified RNA

8 Elute the cleaned cRNA sample by transferring the RNeasy column to a new 1.5 mL collection tube. Add 30 μL RNase-free water directly onto the RNeasy filter membrane. Wait 60 seconds, then centrifuge at 4°C for 30 seconds at 13,000 rpm.

9 Maintain the cRNA sample-containing flow-through on ice. Discard the RNeasy column.
Step 4. Quantify the cRNA

Quantitate cRNA 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 RNA-40 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. Blank the instrument by pipetting 1.0 to 2.0 µL of nuclease-free water (this can be the same water used to initialize the instrument) and click Blank.
8. Clean the sample loading area with a laboratory wipe. Pipette 1.0 to 2.0 µL of the sample onto the instrument sample loading area. Type the sample name in the space provided and 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 nuclease-free water, 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)
   - RNA absorbance ratio (260 nm/280 nm)
   - cRNA concentration (ng/µL)
2 Procedures

Step 4. Quantify the cRNA

10 Determine the yield and specific activity of each reaction as follows:
   a Use the concentration of cRNA (ng/µL) to determine the µg cRNA yield as follows:
   \[
   \text{(Concentration of cRNA) } \times \frac{30 \, \mu\text{L (elution volume)}}{1000} = \mu\text{g of cRNA}
   \]
   b Use the concentrations of cRNA (ng/µL) and cyanine 3 or cyanine 5 (pmol/µL) to determine the specific activity as follows:
   \[
   \frac{\text{Concentration of Cy3 or Cy5}}{\text{Concentration of cRNA}} \times 1000 = \text{pmol Cy3 per } \mu\text{g cRNA}
   \]

11 Examine the yield and specific activity results. See Table 5 for the recommended cRNA yields and specific activities for hybridization.

<table>
<thead>
<tr>
<th>Microarray format</th>
<th>Yield (µg)</th>
<th>Specific Activity (pmol Cy3 or Cy5 per µg cRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-pack</td>
<td>2.5</td>
<td>6</td>
</tr>
<tr>
<td>2-pack</td>
<td>1.875</td>
<td>6</td>
</tr>
<tr>
<td>4-pack</td>
<td>0.825</td>
<td>6</td>
</tr>
<tr>
<td>8-pack</td>
<td>0.825</td>
<td>6</td>
</tr>
</tbody>
</table>

**NOTE** Please refer to “Quality Assessment of Template RNA and Labeled cRNA” on page 70 for general guidance and procedural recommendations on quality assessment of labeled cRNA.
Hybridization

Step 1. Prepare the 10X Blocking Agent

1. Add 500 µL of nuclease-free water to the vial containing lyophilized 10X Blocking Agent supplied with the Agilent Gene Expression Hybridization Kit, or add 1250 µL of nuclease-free water to the vial containing lyophilized large volume 10X Blocking Agent (Agilent p/n 5188-5281).

2. Mix by gently vortexing. 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 adhering to the tube walls or cap by centrifuging for 5 to 10 seconds.

**NOTE**

10X Blocking Agent can be prepared in advance and stored at ~20°C for up to 2 months. After thawing, repeat the vortexing and centrifugation procedures before use.
2 Procedures
Step 2. Prepare hybridization samples

Step 2. Prepare hybridization samples

1. Equilibrate water bath to 60°C.
2. For each microarray, add each of the components as indicated in the tables below to a 1.5 mL nuclease-free microfuge tube:
   - Table 6 for 1-pack or 2-pack microarray formats
   - Table 7 for 4-pack or 8-pack microarray formats
3. Mix well but gently on a vortex mixer.

**NOTE**
For 1-pack and 2-pack microarrays, if you did not generate enough labeled cRNA, add the amount of labeled cRNA to the fragmentation mix such that the same amount is used for each microarray within the same experiment (at least 1.65 µg).

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume/Mass 1-pack microarrays</th>
<th>Volume/Mass 2-pack microarrays</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyanine 3-labeled, linearly amplified cRNA</td>
<td>2.5 µg</td>
<td>1.875 µg</td>
</tr>
<tr>
<td>cyanine 5-labeled, linearly amplified cRNA</td>
<td>2.5 µg</td>
<td>1.875 µg</td>
</tr>
<tr>
<td>10X Blocking Agent</td>
<td>50 µL</td>
<td>25 µL</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>bring volume to 240 µL</td>
<td>bring volume to 120 µL</td>
</tr>
<tr>
<td>25X Fragmentation Buffer</td>
<td>10 µL</td>
<td>5 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>250 µL</strong></td>
<td><strong>125 µL</strong></td>
</tr>
</tbody>
</table>
Step 2. Prepare hybridization samples

4 Incubate at 60°C for exactly 30 minutes to fragment RNA.
5 Immediately cool on ice for one minute.
6 Add 2x GEx Hybridization Buffer HI-RPM to the 1-pack, 2-pack, 4-pack, and 8-pack microarray formats at the appropriate volume to stop the fragmentation reaction. See Table 8.

Table 7  Fragmentation mix for 4-pack or 8-pack microarray formats

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume/Mass 4-pack microarrays</th>
<th>Volume/Mass 8-pack microarrays</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyanine 3-labeled, linearly amplified cRNA</td>
<td>825 ng</td>
<td>300 ng</td>
</tr>
<tr>
<td>cyanine 5-labeled, linearly amplified cRNA</td>
<td>825 ng</td>
<td>300 ng</td>
</tr>
<tr>
<td>10X Blocking Agent</td>
<td>11 µL</td>
<td>5 µL</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>bring volume to 52.8 µL</td>
<td>bring volume to 24 µL</td>
</tr>
<tr>
<td>25X Fragmentation Buffer</td>
<td>2.2 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>55 µL</td>
<td>25 µL</td>
</tr>
</tbody>
</table>

**CAUTION**

Do not incubate sample in the next step for more than 30 minutes. Cooling on ice and adding the 2x Hybridization Buffer will stop the fragmentation reaction.

7 Mix well by careful pipetting. Take care to avoid introducing bubbles. Do not mix on a vortex mixer; mixing on a vortex mixer introduces bubbles.
8 Spin for 1 minute at room temperature at 13,000 rpm in a microcentrifuge to drive the sample off the walls and lid and to aid in bubble reduction. Use immediately. Do not store.
9 Place sample on ice and load onto the array as soon as possible.

Refer to “Microarray Handling Tips” on page 85 for information on how to safely handle microarrays.
2 Procedures
Step 3. Prepare the hybridization assembly

**Step 3. Prepare the hybridization assembly**

Refer to the *Agilent Microarray Hybridization Chamber User Guide* (G2534-90001) for in-depth instructions on how to load slides, assembly and disassembly of chambers, as well as other helpful tips. This user guide is available with the Agilent Microarray Hybridization Chamber Kit (G2534A) and can also be downloaded from the Agilent Web site at [www.agilent.com/chem/dnamanuals-protocols](http://www.agilent.com/chem/dnamanuals-protocols).

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. Ensure that the gasket slide is flush with the chamber base and is not ajar.

**NOTE**

Place unused gasket wells in the multi-pack array format at the far end opposite the barcode. In the unused wells, maintain the appropriate volume of 1x hybridization buffer for the corresponding format design.

**CAUTION**

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

2. Slowly dispense the volume of hybridization sample (see Table 9) onto the gasket well in a “drag and dispense” manner.

**Table 9** Hybridization Sample

<table>
<thead>
<tr>
<th>Components</th>
<th>1-pack</th>
<th>2-pack</th>
<th>4-pack</th>
<th>8-pack</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume Prepared</td>
<td>500 µL</td>
<td>250 µL</td>
<td>110 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Volume to Hybridize</td>
<td>490 µL</td>
<td>240 µL</td>
<td>100 µL</td>
<td>40 µL</td>
</tr>
</tbody>
</table>

**CAUTION**

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

3. Slowly place an array “active side” down onto the SureHyb gasket slide, so that the “Agilent”-labeled barcode is facing down and the numeric barcode is facing up. Make sure the sandwich-pair is properly aligned.
Do not drop the array slide onto the gasket. Doing so increases the chances of samples mixing between gasket wells.

4 Place the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
5 Hand-tighten the clamp onto the chamber.
6 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.
7 Place assembled slide chamber in rotisserie in a hybridization oven set to 65°C. Set your hybridization rotator to rotate at 10 rpm when using 2x GEx Hybridization Buffer HI-RPM.
8 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 to the Gene Expression wash buffers reduces the possibility of array wash artifacts. Add the Triton X-102 to Gene Expression wash buffer 1 and 2 when the cubitainer of wash buffer is first opened.

Do this step to both Gene Expression wash buffer 1 and 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 10% Triton X-102 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 has been added and indicate the date of addition.

Triton X-102 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 1000-mL bottle. Repeat until you have enough prewarmed Wash Buffer 2 solution for your experiment.

2. Tightly cap the 1000-mL bottle and place in a 37°C water bath the night before washing arrays. Alternatively, remove the plastic cubitainer from the box and place 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. The acetonitrile wash is only necessary if the staining dishes, racks and stir bars were used in previous experiments with the Agilent Stabilization and Drying Solution. Otherwise proceed to “Milli-Q water wash” on page 38.

Acetonitrile wash

Wash staining dishes, racks and stir bars that were used in previous experiments with the Agilent Stabilization and Drying Solution with acetonitrile to remove any remaining residue.

WARNING Conduct acetonitrile 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.
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 acetonitrile as is appropriate for your site.
2 Procedures

Step 3. Prepare the equipment

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” below.

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

The microarray wash procedure for Agilent’s two-color platform must be done in environments where ozone levels are 5 ppb or less. 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 76.

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 10 lists the wash conditions for the wash procedure.

<table>
<thead>
<tr>
<th>Dish</th>
<th>Wash Buffer</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disassembly</td>
<td>1 GE Wash Buffer 1</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>1st wash</td>
<td>2 GE Wash Buffer 1</td>
<td>Room temperature</td>
<td>1 minute</td>
</tr>
<tr>
<td>2nd wash</td>
<td>3 GE Wash Buffer 2</td>
<td>Elevated temperature</td>
<td>1 minute</td>
</tr>
</tbody>
</table>

1. Completely fill slide-staining dish #1 with Gene Expression Wash Buffer 1 at room temperature.
2. Place 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. Place this dish on a magnetic stir plate.
3. Place 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.
2 Procedures

Step 4. Wash the microarray slides

5 Prepare the hybridization chamber disassembly.
   a. Place 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. Remove the microarray slide and place 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!

More effort is needed to separate the 4x44K than the 1x44K sandwiched slides.

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.

**NOTE**
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.

10 Transfer slide rack to slide-staining dish #3 containing Gene Expression Wash Buffer 2 at elevated temperature. Stir using setting 4 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.
Step 4. Wash the microarray slides

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 so that the Agilent barcode faces up:
   - In environments in which the ozone level exceeds 5 ppb, immediately put the slides with Agilent barcode facing up in a slide holder with an ozone-barrier slide cover on top of the array as shown in Figure 3. Refer to the Agilent Ozone-Barrier Slide Cover User Guide (p/n G2505-90550), included with the slide cover, for more information.

![Figure 3: Inserting the ozone-barrier slide cover](image)

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

**NOTE**

Use fresh Gene Expression Wash Buffer 1 and 2 for each wash group (up to 8 slides).

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 C Scanner Settings

1. Put assembled slide holders with or without the ozone-barrier slide cover into the scanner carousel.
2. In the Scan Control main window, choose the slot number of the first slide for Start Slot and the slot number for the last slide for End Slot.
3. For 1x244K, 2x105K, 4x44K and 8x15K microarrays, select Profile AgilentHD_GX_2Color.
4. For 1x1M, 2x400K, 4x180K and 8x60K microarrays, select Profile AgilentG3_GX_2Color.

If you are unable to find the profile AgilentG3_GX_2Color from the Scan Control program, download the profile from https://www.genomics.agilent.com/GenericA.aspx?PageType=Custom&SubPageType=Custom&PageID=2074. To import the profile into the Scan Control program, click Tools > Profile Editor and select Import.
5. Verify scan settings for two-color scans. See Table 11 and Figure 4.

Table 11 C Scanner Scan Settings

<table>
<thead>
<tr>
<th>Dye channel</th>
<th>For 1x244K, 2x105K, 4x44K and 8x15K HD Microarray Formats</th>
<th>For 1x1M, 2x400K, 4x180K and 8x60K G3 Microarray Formats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan region</td>
<td>Scan Area (61 x 21.6 mm)</td>
<td>Scan Area (61 x 21.6 mm)</td>
</tr>
<tr>
<td>Scan resolution (µm)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Tiff</td>
<td>20 bit</td>
<td>20 bit</td>
</tr>
</tbody>
</table>
Procedures

Step 1. Scan the slides

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

2. In the Scan Control main window, click **Scan Slot m-n** where \( m \) is the slot of the first slide, and \( n \) is the slot for the last slide.

**Agilent B Scanner Settings**

The Agilent B Scanner does not support G3 microarrays. For G3 microarrays, use the Agilent C Scanner.

1. Put assembled slide holders with or without the ozone-barrier slide cover into the scanner carousel.

2. Verify the scanner settings in **Table 12**.
2 Procedures

Step 1. Scan the slides

To change any settings, click Settings > Modify Default Settings. A window pops up from which you can change the settings.

3 Select settings for the automatic file naming
   - **Prefix 1** is set to Instrument Serial Number
   - **Prefix 2** is set to Array Barcode

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

5 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.

---

### Table 12

<table>
<thead>
<tr>
<th></th>
<th>For 1x244K, 2x105K Formats</th>
<th>For 4x44K, 8x15K Formats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan region</td>
<td>Scan Area (61 x 21.6 mm)</td>
<td>Scan Area (61 x 21.6 mm)</td>
</tr>
<tr>
<td>Scan resolution (µm)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>5µm scanning mode</td>
<td>Single Pass</td>
<td>Single Pass</td>
</tr>
<tr>
<td>eXtended Dynamic range</td>
<td>(selected)</td>
<td></td>
</tr>
<tr>
<td>Dye channel</td>
<td>Red&amp;Green</td>
<td>Red&amp;Green</td>
</tr>
</tbody>
</table>
| Green PMT | 100% | XDR Hi 100%  
XDR Lo 10% |
| Red PMT | 100% | XDR Hi 100%  
XDR Lo 10% |
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.

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

1. Open the Agilent Feature Extraction (FE) software.
   To get the most recent Feature Extraction protocols for gene expression, go to the Agilent Web site at 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 an 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.

   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 below are selected.
   d. For FE 9.5, under Other, select GE2_QCM_Feb07. For FE 10.5 or higher, the metric sets are part of the protocol, so you do not need to set them.
2 Procedures

Step 2. Extract data using Agilent Feature Extraction Software

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

<table>
<thead>
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<td>JPEG</td>
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</tr>
<tr>
<td>Output Package</td>
<td>Local file only</td>
</tr>
<tr>
<td>Visual Results</td>
<td>Local file only</td>
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<td>Grid</td>
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</tr>
<tr>
<td>QC Report</td>
<td>Local PDF file only</td>
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<td>Same As Image</td>
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<td>Results Folder</td>
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<tr>
<td>User Grid File Available</td>
<td>False</td>
</tr>
<tr>
<td>External Database Use File</td>
<td>False</td>
</tr>
<tr>
<td>Overwrite Previous Results</td>
<td>True</td>
</tr>
</tbody>
</table>

Figure 5  Default settings in FE 10.5.

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 www.agilent.com/chem/downloaddesignfiles. 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 one from the pull down menu. For Agilent two-color microarrays, select **GE2-v5_95_Feb07** (in FE 9.5), **GE2_105_Dec08** (in FE 10.5) or **GE2-107_Sep09** (in FE 10.7).

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](http://www.agilent.com/chem/feprotocols) to download the latest protocols.

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.

If scans are done with an Agilent scanner in XDR mode, the High and Low images are automatically combined when imported into the Feature Extraction software version 9.1 or newer.

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**.
2 Procedures
Step 2. Extract data using Agilent Feature Extraction Software

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 on Use of Agilent Feature Extraction Software (v8.1) QC Report to Evaluate Microarray Performance (publication 5989-3056EN) 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/chem/dnaapplications.
Procedures

Step 2. Extract data using Agilent Feature Extraction Software

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
2 Procedures

Step 2. Extract data using Agilent Feature Extraction Software
3 Supplemental Procedures

Absolute RNA Nanoprep Purification  52
Thermocycler Protocol  55
Quick Amp Labeling Kit Sample Preparation  58
Quality Assessment of Template RNA and Labeled cRNA  70
Preventing Ozone-Related Problems  76

The procedures in this chapter are optional but recommended.
Absolute RNA Nanoprep Purification

As an alternative to the Qiagen RNeasy purification columns, the Absolutely RNA Nanoprep kit can be used to purify the amplified cRNA after “Step 2. Prepare labeling reaction” on page 23. Use the Absolutely RNA Nanoprep Kit when it is necessary to concentrate purified cRNA samples. The Absolutely RNA Nanoprep Kit uses an elution volume of 20 µL.

Step 1. Prepare the reagents

1 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 RNase-free water to 4 mL of 100% sulfolane to make 5 mL of 80% sulfolane.
      5 mL of 80% sulfolane is enough to process 50 RNA preparations (from up to 0.1 mL lysate each).

2 Prepare 1x high-salt wash buffer:
   a Add 16 mL of 100% ethanol to the bottle of 1.67X High-Salt Wash Buffer.
   b On the High-Salt Wash Buffer container, mark the check box for 1x (Ethanol Added).
   c Tighten the cap on the container of High-Salt Wash Buffer and store at room temperature.

3 Prepare the 1x low-salt wash buffer:
   a Add 68 mL of 100% ethanol to the bottle of 5X Low-Salt Wash Buffer.
   b On the Low-Salt Wash Buffer container, mark the check box for 1x (Ethanol Added).
   c Tighten the cap on the container of High-Salt Wash Buffer and store at room temperature.
Step 2. Purify the labeled/amplified RNA

1. Add 100 µL of the Lysis Buffer to each reaction tube for a total volume of 116 µL.

2. Mix on a vortex mixer, or pipette repeatedly until homogenized.

3. Add an equal volume (116 µL) of 80% sulfolane (room temperature) to the cell lysate. Mix thoroughly on a vortex mixer for 5 seconds.
   You must use equal volumes of 80% sulfolane and cRNA sample. Mix on a vortex mixer until the cRNA sample and sulfolane are thoroughly mixed.

4. Put an RNA-binding nano-spin cup into a 2-mL collection tube.

5. Transfer the 80% sulfolane and cRNA sample mixture to the RNA-binding nano-spin cup and snap the cap onto the top of the spin cup.

6. Spin the sample in a microcentrifuge at \( \geq 12,000 \times g \) for 60 seconds.

7. Remove and keep the spin cup. Discard the filtrate. Put the spin cup back into the same 2-mL collection tube.
   Up to this point, the RNA has been protected from RNases by the presence of guanidine thiocyanate.

8. Add 300 µL of 1x High-Salt Wash Buffer to the spin cup. Cap the spin cup, and spin the sample in a microcentrifuge at \( \geq 12,000 \times g \) for 60 seconds.

   **CAUTION**
   The High-Salt Wash Buffer contains the irritant guanidine thiocyanate.

9. Remove and keep the spin cup. Discard the filtrate. Put the spin cup back into the same 2-mL collection tube.

10. Add 300 µL of 1x Low-Salt Wash Buffer to the spin cup. Cap the spin cup, and spin the sample in a microcentrifuge at \( \geq 12,000 \times g \) for 60 seconds.

11. Repeat step 9 and step 10 for a second low-salt wash.

12. Remove and keep the spin cup. Discard the filtrate. Put the spin cup back into the same 2-mL collection tube.

13. Add 300 µL of 1x Low-Salt Wash Buffer to the spin cup. Cap the spin cup, and spin the sample in a microcentrifuge at \( \geq 12,000 \times g \) for 3 minutes to dry the fiber matrix.

14. Transfer the spin cup to a fresh 2-mL collection tube.
3 Supplemental Procedures

Step 2. Purify the labeled/amplified RNA

15 Add 20 µL of Elution Buffer directly onto the fiber matrix inside the spin cup. Cap the spin cup and incubate the sample at room temperature for 2 minutes.

NOTE
The Elution Buffer must be added directly onto the fiber matrix so that the buffer can permeate the entire fiber matrix.

To increase the RNA yield, warm the Elution Buffer to 60°C.

16 Spin the sample in a microcentrifuge at ≥12,000 x g for 5 minutes.

17 If needed, repeat the elution step (step 15 and step 16) to increase the yield of total RNA.

18 Transfer the eluate in the collection tube to a capped microcentrifuge tube to store the RNA.

The RNA can be stored at -20°C for up to one month, or at -80°C for long-term storage.
Thermocycler Protocol

The procedure in this section is an optional thermocycler protocol for the Low Input Quick Amp Labeling Kit.

Use a thermocycler to label reactions if you have a limited number of water baths. The use of a thermocycler can slightly lower the yield of cRNA when compared to the use of water baths.

Step 1. Program the thermocycler

- Store the following programs into your thermocycler:
  - Program 1: 65°C for 10 minutes, 4°C hold
  - Program 2: 40°C for 2 hours, 70°C for 15 minutes, 4°C hold
  - Program 3: 40°C for 2 hours, 4°C hold

Five minutes at 4°C is enough. Hold at that temperature if the reagents for the next step are not ready.

NOTE

Use a heated lid for optimal results.
**Step 2. Synthesize cDNA from Total RNA**

(Time required: ~3 hours)

1. Add 25 to 200 ng of total RNA to a 0.2 mL PCR tube or the well of a 96-well PCR plate in a volume of 1.5 µL. For optimal performance, use at least 25 ng of input total RNA.

2. Add 2 µL of the diluted Two-Color Spike Mix. Please refer to Table 1 on page 20 for detailed instructions on the preparation and use of Spike-in kits.

3. Immediately prior to use, use a pipette to gently mix the components in Table 2 on page 24.

4. Add 1.8 µL of T7 Promoter Primer Mix to the tube that contains 3.5 µL of total RNA and diluted RNA spike-in controls. Each tube now contains a total volume of 5.3 µL.

5. Put the tubes in the thermocycler and run Program 1 to denature the template and anneal the primer.

6. Keep the reaction tubes in the thermocycler at 4°C, or move to benchtop rack on ice.

7. Immediately prior to use, gently mix the components in Table 3 on page 25 in the order listed by pipetting, and keep at room temperature.

8. To each sample tube, add 4.7 µL of cDNA Master Mix for a total volume of 10 µL. Pipette up and down to mix.

9. Put reaction tubes in thermocycler and run Program 2 to synthesize double-stranded cDNA.

**NOTE**

Prewarm the 5X first strand buffer by incubating the vial in an 80°C water bath for 3 to 4 minutes to ensure adequate resuspension of the buffer components. For optimal resuspension, mix briefly on a vortex mixer and spin the tube briefly in a microcentrifuge to drive the contents off the tube walls. Keep at room temperature until use.

**NOTE**

Keep the AffinityScript RNase Block Mix on ice. Do not add the AffinityScript RNase Block Mix until just before you start the reactions.

**NOTE**

Incubation at 70°C inactivates the AffinityScript enzyme.
**Step 3. Synthesize Fluorescent cRNA Synthesis in vitro**

(Time required: ~2.5 hours)

1. Immediately before use, make Master Mix for each cyanine dye:
   a. Add the first four components listed in Table 4 on page 26 in the order shown to 1.5 mL nuclease-free microfuge tubes at room temperature.
   b. Mix thoroughly on a vortex mixer.
   c. Add the T7 RNA Polymerase Blend and cyanine dyes.
   d. Mix gently, but completely, by pipetting up and down without introducing bubbles.

   **NOTE**
   Do not add the T7 RNA Polymerase Blend to Transcription Master Mix until just before you do the reaction.

2. Keep the reaction tubes from step 9 above in the thermocycler at 4°C, or move to benchtop rack on ice.

3. To each sample tube, add 6 µL of Transcription Master Mix. Gently mix by pipetting up and down. The final volume of the reaction is now 16 µL.

4. Return the reaction tubes to the thermocycler and run Program 3 ("Step 1. Program the thermocycler" on page 55) to synthesize labeled cRNA.

5. Purify the labeled cRNA as described on "Step 3. Purify the labeled/amplified RNA" on page 27.
Quick Amp Labeling Kit Sample Preparation

The Low Input Quick Amp Labeling Kit replaces the Quick Amp Labeling Kit. If you have studies that are ongoing, continue to use the Quick Amp Labeling preparation steps described in this section. For new studies, use the Low Input Quick Amp Labeling Kit as described in Chapter 2, “Procedures”.

Agilent's Quick Amp Labeling Kit generates fluorescent cRNA (complimentary RNA) with a sample input RNA range between 50 ng and 5 µg of total RNA or a minimum of 10 ng of poly A+ RNA for two-color processing. The method uses T7 RNA polymerase, which simultaneously amplifies target material and incorporates cyanine 3- or cyanine 5-labeled CTP. There is routinely at least a 100-fold RNA amplification with use of this kit.

NOTE
For optimal performance, use high quality, intact template total or poly A+ RNA. Please refer to “Quality Assessment of Template RNA and Labeled cRNA” on page 70 for general guidance and procedural recommendations on quality assessment of template RNA.
**Figure 8**  Schematic of amplified cRNA procedure. Generation of cRNA for a two-color microarray experiment is shown. When you generate targets for a one-color microarray experiment, only the Cy3-labeled “B” sample is produced and hybridized.
**Step 1. Prepare Spike A Mix and Spike B Mix**

Refer to the protocol on Agilent RNA Spike-In Kit (publication 5188-5928) for in-depth instructions and troubleshooting advice on how to use two-color spike-ins. This protocol is available with the Two-Color RNA Spike-In Kit and can also be downloaded from the Agilent Web site at [www.agilent.com/chem/dnamanuals-protocols](http://www.agilent.com/chem/dnamanuals-protocols).

1. Equilibrate water baths to 37°C, 65°C, 40°C, and 80°C.
2. Mix the stock solutions vigorously on a vortex mixer.
3. Heat at 37°C for 5 minutes, and mix on a vortex mixer once more.
4. Briefly centrifuge to drive contents to the bottom of the tube prior to opening. Settlement of the solution on the sides or lid of the tubes may occur during shipment and storage.

Table 13 provides the dilutions of Spike A Mix and Spike B Mix for three different starting sample ranges of total RNA or 0.2 µg of poly A mRNA. Always label Spike A Mix with cyanine 3 and Spike B Mix with cyanine 5. The output of the Agilent SpikeIns LogRatio Statistics table in the QC report, generated when using Agilent Feature Extraction Software 9.5.3, uses this orientation. It is built into the software code and cannot be set by the user. If the polarity is flipped, you will have to manually adjust the output.

<table>
<thead>
<tr>
<th>Starting amount of RNA</th>
<th>Serial dilution</th>
<th>Spike A Mix or Spike B Mix volume to be used (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA (ng)</td>
<td>PolyA RNA (ng)</td>
<td>First</td>
</tr>
<tr>
<td>50-200</td>
<td></td>
<td>1:20</td>
</tr>
<tr>
<td>201-2000</td>
<td></td>
<td>1:20</td>
</tr>
<tr>
<td>&gt;2000</td>
<td>200</td>
<td>1:20</td>
</tr>
</tbody>
</table>
Supplemental Procedures
Step 1. Prepare Spike A Mix and Spike B Mix

For example, to prepare the Spike A Mix dilution appropriate for 200 ng of total RNA starting sample:

1 Make the First Dilution:
   a Mix the thawed Spike Mix vigorously on a vortex mixer.
   b Heat at 37°C in a circulating water bath for 5 minutes.
   c Mix the Spike Mix tube vigorously again on a vortex mixer.
   a Label a new sterile 1.5 mL microcentrifuge tube “Spike A Mix First Dilution.”
   b Into the First Dilution tube, put 2 µL of the concentrated Spike A Mix.
   c Add 38 µL of the Dilution Buffer, provided in the Spike-In kit (1:20).
   d Mix thoroughly on a vortex mixer and spin down quickly in a centrifuge to collect all of the liquid at the bottom of the tube. This tube contains the First Dilution.

2 Make the Second Dilution:
   a Label a new sterile 1.5 mL microcentrifuge tube “Spike A Mix Second Dilution.”
   b Into the Second Dilution tube, put 2 µL from the First Dilution tube.
   c Add 78 µL of the Dilution Buffer (1:40).
   d Mix thoroughly on a vortex mixer and spin down quickly in a centrifuge to collect all of the liquid at the bottom of the tube. This tube contains the Second Dilution.

3 Make the Third Dilution:
   a Label a new sterile 1.5 mL microcentrifuge tube “Spike A Mix Third Dilution.”
   b Into the Third Dilution tube, put 2 µL from the Second Dilution tube.
   c Add 30 µL of the Dilution Buffer (1:16).
   d Mix thoroughly on a vortex mixer and spin down quickly in a centrifuge to collect all of the liquid at the bottom of the tube. This tube contains the Third Dilution.

NOTE
Use RNase-free microfuge tubes and tips. Avoid pipetting volumes less than 2 µL to ensure accuracy.
3 Supplemental Procedures
Step 1. Prepare Spike A Mix and Spike B Mix

Storage of Spike Mix dilutions
Store the Agilent RNA Spike-In Kit, Two-Color at −70°C to −80°C in a non-defrosting freezer for up to 1 year from the date of receipt.

The first dilution of the Agilent Spike Mix (A or B) positive controls can be stored up to 2 months in a non-defrosting freezer at −70°C to −80°C and freeze/thawed up to eight times.

After use, discard the second and third dilution tubes.
Step 2. Prepare labeling reaction

1. Add 50 to 5000 ng of total or polyA RNA to a 1.5-mL microcentrifuge tube in an appropriate volume of 8.3 µL or less. Dilute samples so that at least 2 µL of sample is pipetted into the tube.

2. Prepare tubes for both Spike A Mix/cyanine 3-CTP and Spike B Mix/cyanine 5-CTP dyes.

3. Add 1.2 µL of T7 Promoter Primer (from the Agilent Quick Amp Kit, Two-Color). See Table 14.

4. Add 2 µL of diluted Spike Mix (A or B).

5. Use nuclease-free water to bring the total reaction volume to 11.5 µL.

6. Denature the primer and the template by incubating the reaction at 65°C in a circulating water bath for 10 minutes.

7. Place the reactions on ice and incubate for 5 minutes.

8. Immediately prior to use, gently mix the components listed in Table 15 for the cDNA Master Mix by adding in the order indicated, and keep at room temperature.

9. Prewarm the 5X first strand buffer at 80°C for 3 to 4 minutes to ensure adequate resuspensions of the buffer components. For optimal resuspension, briefly mix on a vortex mixer and spin the tube in a microcentrifuge to drive down the contents from the tube walls. Keep at room temperature until needed.

MMLV-RT and RNaseOUT are enzymes, which need to be kept on ice and are to be added to the cDNA Master Mix just before starting the reactions. Be sure to use the 10 mM dNTP mix tube from the kit.
3 Supplemental Procedures

Step 2. Prepare labeling reaction

Briefly spin each sample tube in a microcentrifuge to drive down the contents from the tube walls and the lid.

Add 8.5 µL of cDNA Master Mix to each sample tube and mix by pipetting up and down.

Incubate samples at 40°C in a circulating water bath for 2 hours.

Move samples to a 65°C circulating water bath and incubate for 15 minutes.

Move samples to ice. Incubate for 5 minutes.

Spin samples briefly in a microcentrifuge to drive down tube contents from the tube walls and lid.

Immediately prior to use, gently mix the components listed in Table 16 in the order indicated for the Transcription Master Mix by pipetting at room temperature.

Prewarm the 50% PEG solution at 40°C for 1 minute. For optimal resuspension, briefly mix on a vortex mixer and spin the tube in a microcentrifuge to drive down the contents from the tube walls. Careful pipetting is required to ensure accurate volume. Keep at room temperature until needed.

RNaseOUT, inorganic pyrophosphatase, and T7 RNA polymerase are enzymes, which need to be kept on ice and should be added to the Transcription Master Mix just before starting the reactions.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL) per reaction</th>
<th>Volume (µL) per 4.5 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X First Strand Buffer</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>MMLV-RT</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>RNaseOut</td>
<td>0.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Total Volume</td>
<td>8.5</td>
<td>38.3</td>
</tr>
</tbody>
</table>

Two-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol
**Supplemental Procedures**

**Step 2. Prepare labeling reaction**

**Table 16  Transcription Master Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL) per reaction</th>
<th>Volume (µL) per 4.5 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>15.3</td>
<td>68.9</td>
</tr>
<tr>
<td>4X Transcription Buffer</td>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>6</td>
<td>27</td>
</tr>
<tr>
<td>NTP mix</td>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td>50% PEG</td>
<td>6.4</td>
<td>28.8</td>
</tr>
<tr>
<td>RNaseOUT</td>
<td>0.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Inorganic pyrophosphatase</td>
<td>0.6</td>
<td>2.7</td>
</tr>
<tr>
<td>T7 RNA Polymerase</td>
<td>0.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Cyanine 3-CTP or cyanine 5-CTP</td>
<td>2.4</td>
<td>10.8</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>60</strong></td>
<td><strong>270</strong></td>
</tr>
</tbody>
</table>

18 Add 60 µL of Transcription Master Mix to each sample tube. Gently mix by pipetting.

19 Incubate samples in a circulating water bath at 40°C for 2 hours.
Step 3. Purify the labeled/amplified RNA

Qiagen’s RNeasy mini spin columns are recommended for purification of the amplified cRNA samples.

**NOTE**
Ensure that ethanol was added to the RPE buffer as specified in the Qiagen manual before proceeding.

1. Add 20 µL of nuclease-free water to your cRNA sample, for a total volume of 100 µL.
2. Add 350 µL of Buffer RLT and mix well by pipetting.
3. Add 250 µL of ethanol (96% to 100% purity) and mix thoroughly by pipetting. Do not centrifuge.
4. Transfer the 700 µL of the cRNA sample to an RNeasy mini column in a 2 mL collection tube. Centrifuge the sample at 4°C for 30 seconds at 13,000 rpm. Discard the flow-through and collection tube.
5. Transfer the RNeasy column to a new collection tube and add 500 µL of buffer RPE (containing ethanol) to the column. Centrifuge the sample at 4°C for 30 seconds at 13,000 rpm. Discard the flow-through. Re-use the collection tube.
6. Add another 500 µL of buffer RPE to the column. Centrifuge the sample at 4°C for 60 seconds at 13,000 rpm. Discard the flow-through and the collection tube.
7. If any buffer RPE remains on or near the frit of the column, transfer the RNeasy column to a new 1.5 mL collection tube and centrifuge the sample at 4°C for 30 seconds at 13,000 rpm to remove any remaining traces of buffer RPE. Discard this collection tube and use a fresh tube to elute the cleaned cRNA sample.

**CAUTION**
*Do not discard the final flow-through in the next step.* It contains the cRNA sample.
8 Elute the cleaned cRNA sample by transferring the RNeasy column to a new 1.5 mL collection tube. Add 30 µL RNase-free water directly onto the RNeasy filter membrane. Wait 60 seconds, then centrifuge at 4°C for 30 seconds at 13,000 rpm.

9 Maintain the cRNA sample-containing flow-through on ice. Discard the RNeasy column.
3 Supplemental Procedures

Step 4. Quantify the cRNA

Quantitate cRNA 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 RNA-40 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. Blank the instrument by pipetting 1.0 to 2.0 µL of nuclease-free water (this can be the same water used to initialize the instrument) and click Blank.
8. Clean the sample loading area with a laboratory wipe. Pipette 1.0 to 2.0 µL of the sample onto the instrument sample loading area. Type the sample name in the space provided and 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 nuclease-free water, 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)
   - RNA absorbance ratio (260 nm/280 nm)
   - cRNA concentration (ng/µL)
Step 4. Quantify the cRNA

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

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

\[
\text{Concentration of cRNA} \times \frac{30 \text{ µL (elution volume)}}{1000} = \mu g \text{ of cRNA}
\]

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

\[
\frac{\text{Concentration of Cy3 or Cy5}}{\text{Concentration of cRNA}} \times 1000 = \text{pmol Cy3 per µg cRNA}
\]

11 Examine the yield and specific activity results.

**CAUTION**
If the yield is <825 ng and the specific activity is <8.0 pmol Cy3 or Cy5 per µg cRNA do not proceed to the hybridization step. Repeat cRNA preparation.

**NOTE**
Please refer to “Quality Assessment of Template RNA and Labeled cRNA” on page 70 for general guidance and procedural recommendations on quality assessment of labeled cRNA.

12 Continue to the hybridization step at “Hybridization” on page 31.
Quality Assessment of Template RNA and Labeled cRNA

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

Make sure you determine the integrity of the input template RNA, as well as labeled cRNA, before you label/amplify and hybridize, respectively. Use the NanoDrop UV-VIS Spectrophotometer and 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, e.g. mRNA and cRNA, 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 17 and Table 18 to make sure that you have the appropriate analyzer, kits, and compatible assays.

**Table 17  Analyzer and Kits**

<table>
<thead>
<tr>
<th>Description</th>
<th>Vendor and part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent 2100 Bioanalyzer</td>
<td>Agilent p/n G2938C or G2939A</td>
</tr>
<tr>
<td>Agilent RNA 6000 Nano LabChip Kit</td>
<td>Agilent p/n 5067-1511</td>
</tr>
<tr>
<td>Agilent RNA 6000 Pico LabChip Kit</td>
<td>Agilent p/n 5067-1513</td>
</tr>
<tr>
<td>NanoDrop ND-1000 UV-Vis Spectrophotometer</td>
<td>NanoDrop p/n ND-1000 or equivalent</td>
</tr>
</tbody>
</table>

**Table 18  Compatible Assays**

<table>
<thead>
<tr>
<th>Description</th>
<th>Compatible Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent RNA 6000 Nano LabChip Kit</td>
<td>Eukaryote Total RNA Nano Assay Qualitative range 5 to 500 ng/µL</td>
</tr>
<tr>
<td>Agilent RNA 6000 Nano LabChip Kit</td>
<td>mRNA Nano Assay Qualitative range 25 to 250 ng/µL</td>
</tr>
<tr>
<td>Agilent RNA 6000 Pico LabChip Kit</td>
<td>Eukaryote Total RNA Pico Assay Qualitative range 50 to 5000 pg/µL in water</td>
</tr>
<tr>
<td>Agilent RNA 6000 Pico LabChip Kit</td>
<td>mRNA Pico Assay Qualitative range 250 to 5000 pg/µL in water</td>
</tr>
</tbody>
</table>

* The mRNA assays are suitable for analysis of cRNA as well.
Supplemental Procedures

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. Open the Agilent 2100 expert software (version B.02.02 or higher), switch 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 18S and 28S 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 9.
Step 2. Assess the quality using the Agilent 2100 Bioanalyzer Two-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol

Labeled cRNA

![Labeled cRNA electropherogram](image)

The resulting electropherogram should have a broad band. The majority of signal for amplified sample should fall into the size range from 200 to 2000 nucleotides. If there isn't a band in this range, and there are distinct bands less than 200 nucleotides in length, DO NOT proceed with that sample since it has likely been degraded and will not provide accurate results. See Figure 10.

**Figure 9** Analysis of (human) total RNA with the Eukaryote total RNA Nano assay using three different samples with decreasing integrity: Red, RIN 8.4; Blue, RIN 5.9; Green, RIN 3.6. Characteristic regions for ribosomal peaks and the lower marker (LM) are displayed.
Supplemental Procedures

Step 2. Assess the quality using the Agilent 2100 Bioanalyzer

Figure 10  Non-fragmented cRNA products from Agilent’s Low RNA Input Linear Amplification Kit PLUS. Concentration was 120 ng/µL according to NanoDrop, 1 µL was analyzed with the mRNA Nano assay. Red, Cy5 labeled cRNA; Blue, Cy3 labeled cRNA show the same size distribution. Since Cy5 is excited under the 2100 bioanalyzer assay conditions, the Cy5 label reagents generate a dominant peak at 120 nt. In comparison to Cy3 labeled cRNA, the Cy5 labeled cRNA yields additional fluorescence in the profile for the same reason.

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.

Additional information on mRNA can be found in the corresponding application notes: Interpreting mRNA electropherograms, publication 5988-3001EN, and Optimizing cRNA fragmentation for microarray experiments using the Agilent 2100 bioanalyzer, publication 5988-3119EN.

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

Two-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol
Step 3. Assess the quality using a NanoDrop Spectrophotometer

Accurate assessment of total RNA quantity and quality are crucial to the success of an Agilent Gene Expression experiment. High quality RNA should be free of contaminants such as carbohydrates, proteins, and traces of organic solvents, and should also be intact with minimal degradation.

Use the NanoDrop UV-VIS Spectrophotometer (or equivalent) to assess RNA concentration and purity.

UV-VIS Spectrophotometry

1. In the Nanodrop program menu, select Nucleic Acid Measurement, then select Sample Type to be RNA-40.
2. Use 1.5 µL of nuclease-free water to blank the instrument.
3. Use 1.5 µL of each total RNA sample to measure RNA concentration. Record the RNA concentration (ng/µL) for each sample.
4. Record the $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios.

High-quality total RNA samples have an $A_{260}/A_{280}$ ratio of 1.8 to 2.0, which indicates the absence of contaminating proteins. They also have an $A_{260}/A_{230}$ ratio of >2.0, which indicates the absence of other organic compounds, such as guanidinium isothiocyanate, alcohol and phenol as well as cellular contaminants such as carbohydrates.
Preventing Ozone-Related Problems

While cyanine 5 is sensitive to ozone degradation, the Agilent two-color platform is robust in environments where the ozone level is 5 ppb (approximately 10 µg/m³) or less. Beyond this level, ozone can affect cyanine 5 signal and compromise microarray performance. 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 41.

In addition to the Ozone-Barrier Slide cover, 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>
<thead>
<tr>
<th>Ozone level in your lab</th>
<th>Wash Procedure</th>
<th>Ozone-Barrier Slide Cover</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5 ppb</td>
<td>Wash Procedure without Stabilization and Drying Solution. “Step 4. Wash the microarray slides” on page 39</td>
<td>No</td>
</tr>
<tr>
<td>&gt; 5 ppb and &lt; 10 ppb</td>
<td>Wash Procedure without Stabilization and Drying Solution. “Step 4. Wash the microarray slides” on page 39</td>
<td>Yes</td>
</tr>
<tr>
<td>&gt; 10 ppb</td>
<td>Wash Procedure with Stabilization and Drying Solution. See “Step 1. Prepare the Stabilization and Drying Solution” on page 77 and “Step 2. Wash with Stabilization and Drying Solution” on page 78.</td>
<td>Yes</td>
</tr>
</tbody>
</table>
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. Gloves and eye/face protection should be used in every step of the warming procedures.

**WARNING**

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

**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. 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 homogenous 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 prior to use.
Step 2. Wash with Stabilization and Drying Solution

Cyanine 5 is susceptible to degradation by ozone. The following procedure is strongly recommended if the ozone levels exceed 10 ppb in your laboratory. 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 (publication 5989-0875EN).

NOTE

Fresh Gene Expression Wash Buffer 1 and 2 should be used for each wash group (up to eight slides). The acetonitrile and Stabilization and Drying Solution may be reused for washing of up to three groups of slides (that is, 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 placed close to, or preferably in, the same fume hood. Gloves and eye/face protection should be used in every step of the warming procedures.

Table 20 lists the wash conditions for the wash procedure with Stabilization and Drying Solution.

Table 20  Wash conditions

<table>
<thead>
<tr>
<th>Dish</th>
<th>Wash Buffer</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disassembly</td>
<td>GE Wash Buffer 1</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>1st wash</td>
<td>GE Wash Buffer 1</td>
<td>Room temperature</td>
<td>1 minute</td>
</tr>
<tr>
<td>2nd wash</td>
<td>GE Wash Buffer 2</td>
<td>Elevated temperature</td>
<td>1 minute</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Acetonitrile</td>
<td>Room temperature</td>
<td>10 seconds</td>
</tr>
<tr>
<td>3rd wash</td>
<td>Stabilization and Drying Solution</td>
<td>Room temperature</td>
<td>30 seconds</td>
</tr>
</tbody>
</table>
Supplemental Procedures

Step 2. Wash with Stabilization and Drying Solution

1. Completely fill slide-staining dish #1 with Gene Expression Wash Buffer 1 at room temperature.

2. Place 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. Place this dish on a magnetic stir plate.

3. Place 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 place 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 place 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. Place 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 place 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!
3 Supplemental Procedures

Step 2. Wash with Stabilization and Drying Solution

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 placed 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.

NOTE

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.

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 Immediately continue at step 14 on page 41.

CAUTION

Dispose of acetonitrile and Stabilization and Drying Solution as flammable solvents.
This chapter contains reference information related to the protocol and Feature Extraction default parameter settings.
Supplemental User Guides

First-time users of Agilent’s 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.

G2534-90001 Agilent Microarray Hybridization Chamber User Guide
G2545-80001 G2545A Hybridization Oven User Manual
G2566-90009 Agilent G2565AA and G2565BA Microarray Scanner System User Manual
Agilent G2567AA Feature Extraction Software Quick Start Guide
Agilent G2567AA Feature Extraction Software User Guide
Agilent G2567AA 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.

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 placed 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 (G2565BA)

Figure 11  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 inside of the slide holder. In this orientation, the “active side” containing the microarray 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 11 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 12).

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](http://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.
4 Reference

General Microarray Layout and Orientation

Figure 12  Microarray slide orientation
Array/Sample tracking on a 4-pack array slide

Use the form below to make notes to track your samples on a 4-pack array slide.

Arrays

<table>
<thead>
<tr>
<th>Barcode</th>
<th>Array 1_1</th>
<th>Array 1_2</th>
<th>Array 1_3</th>
<th>Array 1_4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

Barcode number _____________________________________________
Array/Sample tracking on a 8-pack array slide

Use the form below to make notes to track your samples on a 8-pack array slide.

<table>
<thead>
<tr>
<th>Arrays</th>
<th>Array 1_1</th>
<th>Array 1_2</th>
<th>Array 1_3</th>
<th>Array 1_4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample:</td>
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<tr>
<td>Barcode</td>
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<td></td>
</tr>
<tr>
<td>Array 2_1</td>
<td>Array 2_2</td>
<td>Array 2_3</td>
<td>Array 2_4</td>
<td></td>
</tr>
<tr>
<td>Barcode Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Related Microarray Reagents

<table>
<thead>
<tr>
<th>Description</th>
<th>Vendor and part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal Human Reference RNA</td>
<td>Stratagene p/n 740000</td>
</tr>
<tr>
<td>Universal Mouse Reference RNA</td>
<td>Stratagene p/n 740100</td>
</tr>
<tr>
<td>Universal Rat Reference RNA</td>
<td>Stratagene p/n 740200</td>
</tr>
</tbody>
</table>
4 Reference
Related Microarray Reagents

92 Two-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol
In This Book

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

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Revision A