

miRNA Microarray System with miRNA Complete Labeling and Hyb Kit

Protocol

Version 3.1.1, August 2015

Microarrays manufactured with Agilent SurePrint
Technology

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

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



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In this Guide...

This document describes the Agilent recommended procedures for the preparation and labeling of complex biological targets and hybridization, washing, scanning, and feature extraction of Agilent oligonucleotide microarrays for microarray-based miRNA systems.

1 Before You Begin

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

2 Procedures

This chapter describes the steps to prepare Spike-In solutions (optional), samples, hybridize, wash and scan miRNA microarrays, and to extract data using the Feature Extraction Software.

3 Characterization of Total RNA

This chapter contains instructions for characterization of total RNA, and steps to prevent ozone-related problems.

4 Reference

This chapter contains reference information related to the protocol and the limited use license.

What's New in 3.1

- Corrected CIP Master Mix table.
- Updated product labeling statement.

What's New in 3.0

- Added list of microarrays that are supported by this protocol.
- Added note to calibrate hybridization oven on a regular basis for accuracy of the collected data.
- Updated loading instructions for hybridization oven.
- Added reference to compatibility matrix for non-Agilent scanners.
- Added details to normalize Agilent one-color miRNA microarrays.

Content

1 Before You Begin

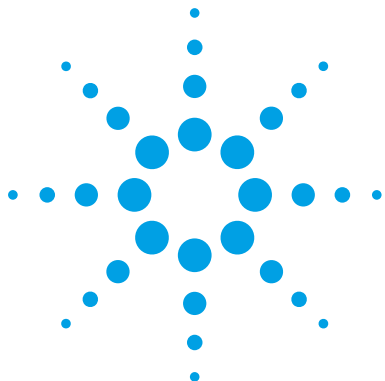
| | |
|--------------------------------|----|
| Procedural Notes | 8 |
| Safety Notes | 9 |
| Agilent Oligo Microarrays | 10 |
| Required Equipment | 12 |
| Required Reagents | 13 |
| Optional Equipment/Reagents | 14 |
| Required Hardware and Software | 15 |
| Optional Software | 15 |
| RNA Extraction Recommendation | 16 |

2 Procedures

| | |
|--|----|
| Sample Preparation and Labeling | 19 |
| Step 1. Prepare Spike-In solutions (optional) | 19 |
| Step 2. Dephosphorylate the sample | 21 |
| Step 3. Denature the sample | 23 |
| Step 4. Ligate the sample | 24 |
| Step 5. Purify the labeled RNA (optional) | 25 |
| Step 6. Dry the sample | 26 |
| Hybridization | 27 |
| Step 1. Prepare the 10× Blocking Agent | 27 |
| Step 2. Prepare hybridization samples | 28 |
| Step 3. Prepare the hybridization assembly | 30 |
| Step 4. Hybridize | 32 |
| Microarray Wash | 33 |
| Step 1. Add Triton X-102 to Gene Expression wash buffers | 33 |
| Step 2. Prewarm Gene Expression Wash Buffer 2 and slide-staining dish #3 | 34 |
| Step 3. Prepare the equipment | 34 |

Contents

| | |
|---|----|
| Step 4. Wash the microarray slides | 36 |
| Scanning and Feature Extraction | 40 |
| Step 1. Scan the slides | 41 |
| Step 2. Extract data using Agilent Feature Extraction Software | 44 |
| 3 Supplemental Procedures | |
| Normalizing Agilent One-Color Microarray Data | 52 |
| To do downstream analysis of Agilent microarray data | 52 |
| To use Feature Extraction | 53 |
| Characterization of Total RNA | 54 |
| Step 1. Prepare for characterization of total RNA | 56 |
| Step 2. Characterize the RNA samples using the Agilent 2100 Bioanalyzer | 57 |
| 4 Reference | |
| Kit Contents | 62 |
| Supplemental User Guides | 63 |
| Microarray Handling Tips | 64 |
| General Microarray Layout and Orientation | 65 |
| Array/Sample tracking microarray slides | 68 |



1 Before You Begin

| | |
|--------------------------------|----|
| Procedural Notes | 8 |
| Safety Notes | 9 |
| Agilent Oligo Microarrays | 10 |
| Required Equipment | 12 |
| Required Reagents | 13 |
| Optional Equipment/Reagents | 14 |
| Required Hardware and Software | 15 |
| Optional Software | 15 |
| RNA Extraction Recommendation | 16 |

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

NOTE

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



Procedural Notes

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

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.
-

WARNING

- **Cyanine dye reagents are potential carcinogens. Avoid inhalation, swallowing, or contact with skin.**
 - **Dimethyl Sulfoxide (DMSO) may be harmful by inhalation, ingestion or skin absorption. DMSO vapor or mist is irritating to the eyes, mucous membranes and upper respiratory tract. It can cause skin irritation and allergic respiratory and skin reactions. Overexposure may cause reproductive disorder(s) based on tests with laboratory animals.**
 - **LiCl is toxic and a potential teratogen. May cause harm to breastfed babies. Possible risk of impaired fertility. Harmful if inhaled, swallowed, or contacts skin. Target organ: central nervous system. Wear suitable PPE. LiCl is a component of the **2× Hi-RPM Hybridization Buffer**.**
 - **Lithium dodecyl sulfate (LDS) is harmful by inhalation and irritating to eyes, respiratory system, and skin. Wear suitable PPE. LDS is a component of the **2× Hi-RPM Hybridization Buffer**.**
 - **Triton is harmful if swallowed. Risk of serious damage to eyes. Wear suitable PPE. Triton is a component of the **2× Hi-RPM Hybridization Buffer** and is an additive in wash buffers.**
 - **Acetonitrile is a flammable liquid and vapor. Harmful if inhaled, swallowed, or contacts skin. Target organs: liver, kidneys, cardiovascular system, and CNS.**
-

Agilent Oligo Microarrays

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

To get design files or create a custom design for any species in the miRBASE, go to the Agilent eArray web site at <http://earray.chem.agilent.com>.

NOTE

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

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

Catalog SurePrint HD and G3 Microarrays and Microarray Kits

Table 1 Catalog SurePrint HD and G3 miRNA Microarrays - Human

| Part Number | Description | miRBase Release |
|---------------|--|-----------------|
| G4870C | G3 Human miRNA Microarray Kit, Release 21, 8×60K (3 slides) | 21.0 |
| G4872A-070156 | G3 Human miRNA Microarray, Release 21, 8×60K (1 slide) | 21.0 |
| G4872A-046064 | G3 Human miRNA Microarray, Release 19.0, 8×60K (1 slide) | 19.0 |
| G4870A | G3 Human miRNA Microarray Kit Release 16.0, 8×60K (3 slides) | 16.0 |
| G4872A-031181 | G3 Human miRNA Microarray, Release 16.0, 8×60K (1 slide) | 16.0 |
| G4471A-029297 | HD Human miRNA Microarray, Release 14.0, 8×15K (1 slide) | 14.0 |
| G4470C | HD Human miRNA Microarray Kit (V3), 8×15K (3 slides) | 12.0 |
| G4471A-021827 | HD Human miRNA Microarray (V3), 8×15K (1 slide) | 12.0 |
| G4470B | HD Human miRNA Microarray (V2) Kit, 8×15K (3 slides) | 10.1 |
| G4471A-019118 | HD Human miRNA Microarray (V2), 8×15K (1 slide) | 10.1 |
| G4470A | HD Human miRNA Microarray (V1) Kit, 8×15K (3 slides) | 9.1 |
| G4471A-016436 | HD Human miRNA Microarray (V1), 8×15K (1 slide) | 9.1 |

Table 2 Catalog SurePrint G3 and HD miRNA Microarrays - Mouse

| Part Number | Description | miRBase Release |
|---------------|---|-----------------|
| G4859C | G3 Mouse miRNA Microarray Kit, Release 21, 8×60K (3 slides) | 21.0 |
| G4872A-070155 | G3 Mouse miRNA Microarray, Release 21, 8×60K (1 slide) | 21.0 |
| G4872A-046065 | G3 Mouse miRNA Microarray, Release 19.0, 8×60K (1 slide) | 19.0 |
| G4872A-038112 | G3 Mouse miRNA Microarray, Release 18.0, 8×60K (1 slide) | 18.0 |
| G4859B | G3 Mouse miRNA Microarray Kit, Release 17.0, 8×60K (3 slides) | 17.0 |
| G4872A-035430 | G3 Mouse miRNA Microarray, Release 17.0, 8×60K (1 slide) | 17.0 |
| G4859A | G3 Mouse miRNA Microarray Kit Release 16.0, 8×60K (3 slides) | 16.0 |
| G4872A-031184 | G3 Mouse miRNA Microarray, Release 16.0, 8×60K (1 slide) | 16.0 |
| G4471A-029152 | HD Mouse miRNA Microarray, Release 15.0, 8×15K (1 slide) | 15.0 |
| G4471A-021828 | HD Mouse miRNA Microarray, Release 12.0, 8×15K (1 slide) | 12.0 |
| G4472B | HD Mouse miRNA Microarray Kit (V2), 8×15K (3 slides) | 12.0 |
| G4472A | HD Mouse miRNA Microarray Kit, 8×15K (3 slides) | 10.1 |
| G4471A-019119 | HD Mouse miRNA Microarray, 8×15K (1 slide) | 10.1 |

Table 3 Catalog SurePrint G3 and HD miRNA Microarrays - Rat

| Part Number | Description | miRase Release |
|---------------|--|----------------|
| G4473C | HD Rat miRNA Microarray Kit, Release 21, 8×15K (3 slides) | 21.0 |
| G4471A-070154 | HD Rat miRNA Microarray, Release 21, 8×15K (1 slide) | 21.0 |
| G4471A-046066 | HD Rat miRNA Microarray, Release 19.0, 8×15K (1 slide) | 19.0 |
| G4473B | HD Rat miRNA Microarray Kit Release 16.0, 8×15K (3 slides) | 16.0 |
| G4471A-031189 | HD Rat miRNA Microarray, Release 16.0, 8×15K (1 slide) | 16.0 |
| G4471A-029200 | HD Rat miRNA Microarray, Release 15.0, 8×15K (1 slide) | 15.0 |
| G4473A | HD Rat miRNA Microarray Kit V1, 8×15K (3 slides) | 10.1 |
| G4471A-019159 | HD Rat miRNA Microarray Slide V1, 8×15K (1 slide) | 10.1 |

1 Before You Begin

Required Equipment

Custom Microarrays

Table 4 Custom SurePrint Microarray

| Part Number | Description |
|-------------|-----------------------------------|
| G4871A | Custom G3 miRNA Microarray, 8×60K |
| G4474A | Custom HD miRNA Microarray, 8×15K |

Required Equipment

Table 5 Required Equipment

| Description | Vendor and part number |
|---|---|
| Agilent Microarray Scanner | Agilent p/n G4900DA, G2565CA or G2565BA |
| Hybridization Chamber, stainless | Agilent p/n G2534A |
| Hybridization gasket slides 8 microarrays/slide, 5 slides/box | Agilent p/n G2534-60014 |
| Go to www.agilent.com/genomics to see all available kit configurations. | |
| Hybridization oven; temperature set at 55°C | Agilent p/n G2545A |
| Hybridization oven rotator for Agilent Microarray Hybridization Chambers | Agilent p/n G2530-60029 |
| nuclease-free 1.5 mL microfuge tube | Ambion p/n 12400 or equivalent |
| magnetic stir bar (×2) | Corning p/n 401435 or equivalent |
| magnetic stir plate | Corning p/n 6795-410 or equivalent |
| magnetic stir plate with heating element | |
| circulating water baths or heat blocks set to 16°, 37°C, and 100°C, | Corning p/n 6795-420 or equivalent |
| microcentrifuge | Eppendorf p/n 5417R or equivalent |
| sterile storage bottle | Nalgene 455-1000 or equivalent |

Table 5 Required Equipment (continued)

| Description | Vendor and part number |
|---|--|
| micropipettor | Pipetman P-10, P-20, P-200, P-1000 or equivalent |
| 1.5L glass dish | Pyrex p/n 7211 or equivalent |
| slide-staining dish, with slide rack (×3) | Thermo Shandon p/n 121 or equivalent |
| vacuum concentrator with heater | Savant SpeedVac p/n SPD111V or equivalent |
| clean forceps | |
| ice bucket | |
| powder-free gloves | |
| sterile, nuclease-free aerosol barrier pipette tips | |
| vortex mixer | |
| timer | |
| nitrogen purge box for slide storage | |

Required Reagents

Table 6 Required Reagents

| Description | Vendor and part or catalog number |
|---|-----------------------------------|
| miRNA Complete Labeling and Hyb Kit | Agilent p/n 5190-0456 |
| Gene Expression Wash Buffer Kit | Agilent p/n 5188-5327 |
| DNase/RNase-free distilled water | Invitrogen p/n 10977-015 |
| Milli-Q water or equivalent | |
| isopropyl alcohol (molecular biology grade) | |

Optional Equipment/Reagents

Table 7 Optional Equipment/Reagents

| Description | Vendor and part number |
|---|-------------------------|
| Universal miRNA Reference Sample | Agilent p/n 750700 |
| 2100 Bioanalyzer | Agilent p/n G2939AA |
| RNA 6000 Nano Assay Kit (RNA Series II Kit) | Agilent p/n 5067-1511 |
| RNA 6000 Pico Kit | Agilent p/n 5067-1513 |
| Stabilization and Drying Solution* | Agilent p/n 5185-5979 |
| Ozone-Barrier Slide Cover* | Agilent p/n G2505-60550 |
| Small RNA Kit | Agilent p/n 5067-1548 |
| microRNA Spike-In Kit | Agilent p/n 5190-1934 |
| Micro Bio-Spin P-6 Gel Column | Bio-Rad p/n 732-6221 |
| slide box | Corning p/n 07201629 |
| PCR 96 plate | Eppendorf p/n 951020401 |
| acetonitrile | Sigma p/n 271004-1L |
| dye filter | Sigma p/n Z361569 |
| thermal cycler | |
| 1× tris EDTA pH 7.5 | |

* Recommended when processing microarrays in high ozone environment.

Required Hardware and Software

Table 8

| Description |
|---|
| Feature Extraction software 9.5 or later without Spike-In or Feature Extraction 10.7.3 with Spike-In |
| Agilent Scan Control software. Refer to Agilent Scanner user guide for specifications. |
| For system and supported Internet Explorer/Adobe Reader versions, please see the System Requirements for your Feature Extraction and Scan Control Software. |

Optional Software

Table 9

| Description |
|--------------------------|
| GeneSpring 10.0 or later |

RNA Extraction Recommendation

Total RNA extraction methods differ in numerous ways and may impact:

- yield
- the inclusion of small RNAs in the total RNA extraction
- the quantification of the total RNA

The Agilent miRNA Microarray system is for use with total RNA extracts. Do not use size fractionation or small RNA enrichment protocols. The following method has been validated on the Agilent miRNA Microarray system:

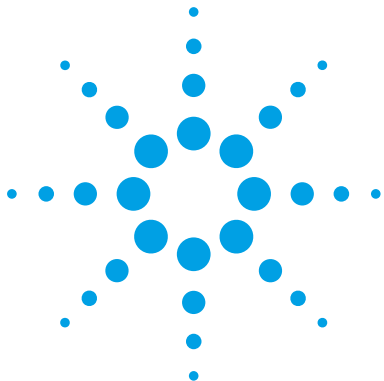
- Absolutely RNA miRNA Kit (Agilent p/n 400814)

To get consistent results for comparative experiments, use the same total RNA extraction method. Different total RNA extraction methods can result in slightly different miRNA profiles.

CAUTION

Do not use purified miRNA. This protocol supports the use of total RNA only.

Extraction methods that use organic solvents such as TRIZOL can result in inaccurate quantification, because organic solvent contamination from carry-over during the RNA extraction can compress the 260/230 ratio. The affected 260 measurement can result in inaccurate quantification of the total RNA. To ensure the removal of residual TRIZOL reagent, do one to two additional chloroform extractions after the final extraction step in the TRIZOL protocol, before you continue to the ethanol precipitation. See [“Characterization of Total RNA”](#) on page 51 for more information on RNA purity and quality assessment.



2 Procedures

Sample Preparation and Labeling 19

- Step 1. Prepare Spike-In solutions (optional) 19
- Step 2. Dephosphorylate the sample 21
- Step 3. Denature the sample 23
- Step 4. Ligate the sample 24
- Step 5. Purify the labeled RNA (optional) 25
- Step 6. Dry the sample 26

Hybridization 27

- Step 1. Prepare the 10× Blocking Agent 27
- Step 2. Prepare hybridization samples 28
- Step 3. Prepare the hybridization assembly 30
- Step 4. Hybridize 32

Microarray Wash 33

- Step 1. Add Triton X-102 to Gene Expression wash buffers 33
- Step 2. Prewarm Gene Expression Wash Buffer 2 and slide-staining dish #3 34
- Step 3. Prepare the equipment 34
- Step 4. Wash the microarray slides 36

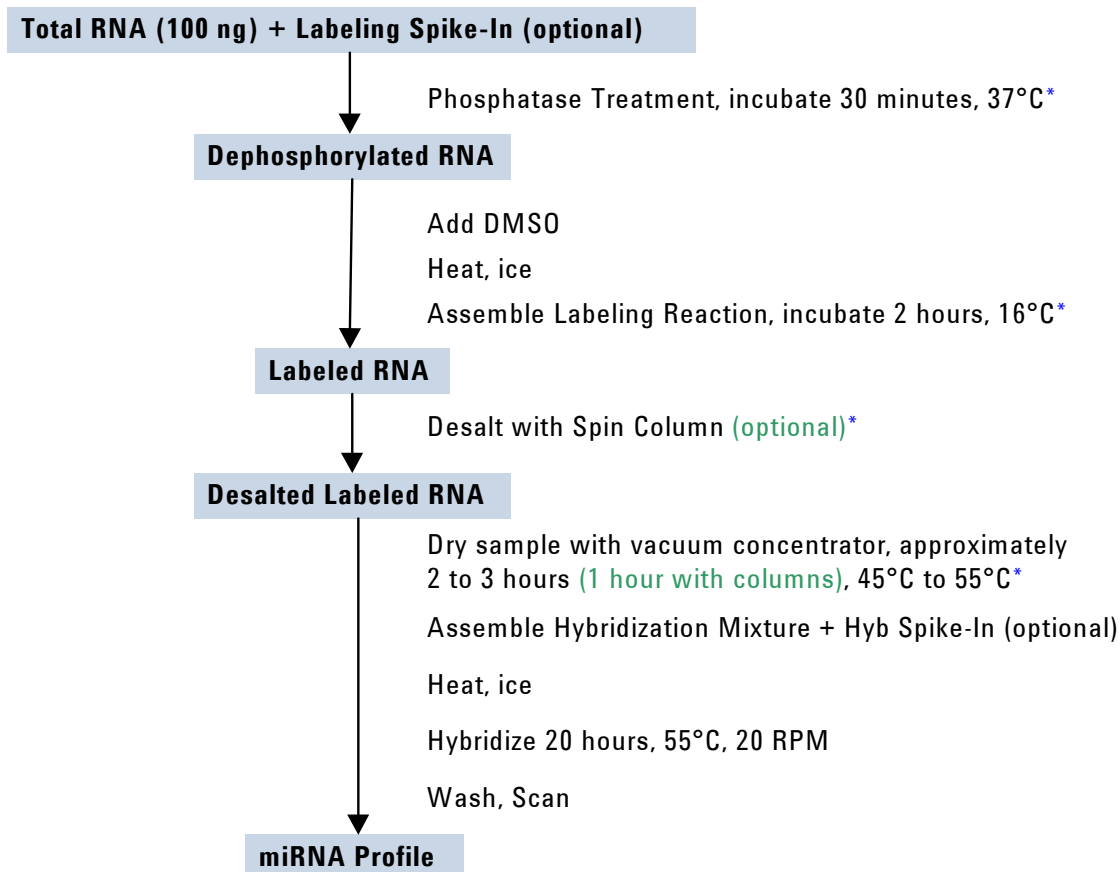
Scanning and Feature Extraction 40

- Step 1. Scan the slides 41
- Step 2. Extract data using Agilent Feature Extraction Software 44

The Agilent miRNA Microarray System uses cyanine 3-labeled targets to measure miRNA in experimental and control samples. [Figure 1](#) is a standard workflow for sample preparation and array hybridization.



2 Procedures



*The sample can be stored at -80°C after this step, if needed.

Figure 1 Workflow for sample preparation and array processing with the optional Bio-Rad columns and spike-ins.

Sample Preparation and Labeling

The [miRNA Complete Labeling and Hyb Kit](#) generates fluorescently-labeled miRNA with a sample input of 100 ng of total RNA. This method involves the ligation of one Cyanine 3-pCp molecule to the 3' end of a RNA molecule with greater than 90% efficiency. The [miRNA Complete Labeling and Hyb Kit](#) provides all the needed components for sample labeling and hybridization preparation. See [Table 24](#) on page 62.

NOTE

Please refer to “[Characterization of Total RNA](#)” on page 54 for procedural recommendations on total RNA characterization.

Read the protocol in its entirety before you continue.

This protocol is compatible with hybridization of labeled miRNA to either 8×15K or 8×60K microarray formats.

Step 1. Prepare Spike-In solutions (optional)

The [microRNA Spike-In Kit](#) consists of two microRNA Spike-In solutions for process control. Use the Spike-In solutions to help distinguish significant biological data from processing issues. The [Labeling Spike-In](#) solution is used in the labeling reaction while the [Hyb Spike-In](#) solution is used in the hybridization reaction.

CAUTION

Do not use spike-in solutions for normalization purposes.

Do not use with drosophila miRNA microarrays. Spike-in solutions are derived from drosophila sequences.

NOTE

Prepare the [2nd Dilution Spike-In solutions](#) and the [3rd Dilution Spike-In solutions](#) just prior to use. If [1st Dilution Spike-In solutions](#) is already prepared, start at step 7.

- 1 Thaw all components.
- 2 Briefly spin tubes.

2 Procedures

Step 1. Prepare Spike-In solutions (optional)

3 Label 10 reaction tubes:

- Label 5 tubes “1st Dilution Labeling Spike-In”
- Label 5 tubes “1st Dilution Hyb Spike-In”.

4 In each tube, add the components in [Table 10](#) according to label on the tube.

Table 10 1st Dilution Spike-In solutions

| 1st Dilution Labeling Spike-In solution | 1st Dilution Hyb Spike-In solution |
|---|--|
| 198 μ L of the Dilution Buffer | 198 μ L of the Dilution Buffer |
| 2 μ L of the Labeling Spike-In solution | 2 μ L of the Hyb Spike-In solution |

5 Mix well and briefly spin tubes.

6 (*Optional*) To increase the number of uses of this kit, and to prevent excess freeze/thaw cycles, store small aliquots of the unused [1st Dilution Spike-In solutions](#) in labeled tubes at -80°C .

7 Label one new tube each with these labels (total of 4 tubes):

- “2nd Dilution Labeling Spike-In”
- “3rd Dilution Labeling Spike-In”
- “2nd Dilution Hyb Spike-In”
- “3rd Dilution Hyb Spike-In”

8 Make the [2nd Dilution Spike-In solutions](#):

- Into each of the tubes that are labeled for **2nd Dilution**, add the contents in [Table 11](#) according to label on the tube.

Table 11 2nd Dilution Spike-In solutions

| 2nd Dilution Labeling Spike-In solution | 2nd Dilution Hyb Spike-In solution |
|--|---|
| 198 μ L of nuclease-free water. | 198 μ L of nuclease-free water. |
| 2 μ L of 1st Dilution Labeling Spike-In solution | 2 μ L of 1st Dilution Hyb Spike-In solution |

9 Mix well and briefly spin in a centrifuge.

Step 2. Dephosphorylate the sample

10 Make the 3rd Dilution Spike-In solutions:

- Into each of the tubes that are labeled for **3rd Dilution**, add the contents in [Table 12](#) according to the label on the tube.

Table 12 3rd Dilution Spike-In solutions

| 3rd Dilution Labeling Spike-In solution | 3rd Dilution Hyb Spike-In solution |
|--|---|
| 198 μ L of nuclease-free water. | 198 μ L of nuclease-free water. |
| 2 μ L of 2nd Dilution Labeling Spike-In solution | 2 μ L of 2nd Dilution Hyb Spike-In solution |

11 Mix well and briefly spin in a centrifuge.

This dilution is used in the labeling reaction or the hybridization reaction.

12 Discard the 2nd Dilution Spike-In solutions and 3rd Dilution Spike-In solutions after use.**13** Store 1st Dilution Spike-In solutions at -80°C .**CAUTION**

Do *not* freeze/thaw the original or 1st Dilution Spike-In solutions more than twice.

Step 2. Dephosphorylate the sample

- 1 Dilute total RNA sample to 50 ng/ μ L in $1\times$ tris EDTA pH 7.5 or DNase/RNase-free distilled water.
- 2 Add 2 μ L (100 ng) of the diluted total RNA to a nuclease-free 1.5 mL microfuge tube and maintain on ice.
- 3 Immediately prior to use, add the components in the order indicated in [Table 13](#) to create the CIP Master Mix. Maintain on ice.

If you did not prepare 3rd Dilution Labeling Spike-In solution, use nuclease-free water.

2 Procedures

Step 2. Dephosphorylate the sample

Table 13 CIP Master Mix

| Components | Volume (μL) per reaction | Volume (μL) per 9 reactions |
|--|---------------------------------------|--|
| 10 \times Calf Intestinal Phosphatase Buffer | 0.4 | 3.6 |
| 3rd Dilution Labeling Spike-In solution <i>or</i> nuclease-free water | 1.1 | 9.9 |
| Calf Intestinal Phosphatase | 0.5 | 4.5 |
| Total Volume | 2.0 | 18.0 |

- 4 Add 2 μL of the **CIP Master Mix** to each sample tube for a total reaction volume of 4 μL . Gently mix by pipetting.
- 5 Incubate the reaction at 37°C in a circulating water bath or heat block for 30 minutes to dephosphorylate the sample.

If needed, the samples can be stored at -80°C after incubation.

NOTE

A thermal cycler, in conjunction with PCR plates, can be used to process a large number of samples. Use a PCR cap to seal the plates. Do not use tape.

Step 3. Denature the sample

- 1 Add 2.8 μL of **DMSO** to each sample.
- 2 Incubate samples at 100°C in a circulating water bath or heat block for 5 to 10 minutes.

CAUTION

Incubate the sample for no less than 5 minutes and no more than 10 minutes, or the labeling efficiency of the sample will be affected.

- 3 Immediately transfer to ice-water bath.

CAUTION

You must use an ice-water bath and not just crushed ice to ensure that the samples remain properly denatured.

When using a thermal cycler, transfer the PCR plate to an ice-water bath immediately after incubating at 100°C to prevent the RNA from re-annealing. Thermocyclers do not cool as quickly as is needed.

- 4 Continue to the next step immediately.

2 Procedures

Step 4. Ligate the sample

Step 4. Ligate the sample

- 1 Warm the **10× T4 RNA Ligase Buffer** at 37°C and mix on a vortex mixer until all precipitate is dissolved.

CAUTION

Make sure that the **10× T4 RNA Ligase Buffer** has cooled to room temperature before you proceed. Failure to do so will affect the T4 RNA Ligase activity, and thus the labeling efficiency.

CAUTION

The **Cyanine 3-pCp** is light-sensitive. Please minimize any exposure to light.

- 2 Immediately prior to use, prepare the **Ligation Master Mix**. Gently mix the components listed in **Table 14** and maintain on ice.

Table 14 Ligation Master Mix

| Components | Volume (μL) per reaction | Volume (μL) per 9 reactions |
|--------------------------|--------------------------|-----------------------------|
| 10× T4 RNA Ligase Buffer | 1.0 | 9.0 |
| Cyanine 3-pCp | 3.0 | 27.0 |
| T4 RNA Ligase | 0.5 | 4.5 |
| Total Volume | 4.5 | 40.5 |

CAUTION

Be sure to use the **Ligation Master Mix** within 15 minutes of mixing all the components in **Table 14**. Failure to do so may affect the labeling efficiency.

- 3 Immediately add 4.5 μL of the **Ligation Master Mix** to each sample tube for a total reaction volume of 11.3 μL.
- 4 Gently mix by pipetting and gently spin down.
- 5 Incubate at 16°C in a circulating water bath or cool block for 2 hours.

The sample can be stored at -80°C, if needed.

Step 5. Purify the labeled RNA (optional)

If you are not using the columns for purification, continue at “Step 6. Dry the sample” on page 26.

This step removes DMSO and free Cyanine 3-pCp. The use of the column reduces the needed drying time in “Step 6. Dry the sample”, but it adds hands-on processing time.

Use the [Micro Bio-Spin P-6 Gel Column](#).

Preparation of [Micro Bio-Spin P-6 Gel Column](#)

- 1 Invert the [Micro Bio-Spin P-6 Gel Column](#) sharply several times to resuspend the settled gel and to remove any air bubbles.
- 2 Snap off the tip and place into a **2-mL collection tube** supplied with the [Micro Bio-Spin P-6 Gel Column](#).
- 3 Remove the green cap from the [Micro Bio-Spin P-6 Gel Column](#). If the buffer does not drip into the **2-mL collection tube**, press the green cap back onto the [Micro Bio-Spin P-6 Gel Column](#) and remove it again. Let the buffer drain for about 2 minutes.
- 4 Check to make sure that all columns are evenly drained.
- 5 Discard the drained buffer from the **2-mL collection tube** and then place the [Micro Bio-Spin P-6 Gel Column](#) back into the tube.
- 6 Spin the microcentrifuge tube containing the [Micro Bio-Spin P-6 Gel Column](#) for 2 minutes at $1000 \times g$ in a centrifuge.

CAUTION

The speed of the microcentrifuge must be accurately set (for example, $1000 \times g$, *not* 1000 rpm). If the spin speed is too low, sample can be lost. If the spin speed is too high, desalting may be ineffective and the column may break down.

- 7 Remove the [Micro Bio-Spin P-6 Gel Column](#) from the **2-mL collection tube** and place it into a clean nuclease-free 1.5 mL microfuge tube. Discard the **2-mL collection tube**.

The sample can be stored at -80°C , if needed.

2 Procedures

Step 6. Dry the sample

Sample purification

- 1 Add 38.7 μL of 1 \times tris EDTA pH 7.5 or DNase/RNase-free distilled water to the labeled sample for a total volume of 50 μL .
- 2 Without disturbing the gel bed, use a pipette to transfer the 50 μL sample onto the gel bed from [step 7](#) above.
- 3 Spin the microcentrifuge tubes that contain the columns for 4 minutes at 1000 \times g in a centrifuge to elute the purified sample.
- 4 Discard the columns and keep on ice the flow-through that contains miRNA sample.
- 5 Check that the final flow-through is translucent and slightly pink. The flow-through volume needs to be uniform across the samples and close to 50 μL .

CAUTION

Do not discard the final flow-through. It now contains the labeled miRNA sample.

The sample can be stored at -80°C , if needed.

Step 6. Dry the sample

- 1 After the 16°C labeling reaction or sample purification, completely dry the samples. Use a [vacuum concentrator with heater](#) at 45 to 55°C or on the medium-high heat setting. This step can take up to 1 hour after column purification and up to 3 hours without column purification.
- 2 Check the samples after 30 minutes. Continue vacuum concentration until they are dry.
To check for sample dryness, flick hard on the tube and make sure that the pellets do not move or spread.

CAUTION

The sample must be completely dried after labeling. Residual [DMSO](#) will adversely affect the hybridization results.

The sample can be stored at -80°C , if needed.

Hybridization

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

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

CAUTION

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

Step 1. Prepare the 10× Blocking Agent

- 1 Add 125 μL of nuclease-free water to the vial containing lyophilized 10× Gene Expression Blocking Agent supplied with the miRNA Complete Labeling and Hyb Kit.
- 2 Gently mix on a vortex mixer. If the pellet does not go into solution completely, heat the mix for 4 to 5 minutes at 37°C.

NOTE

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

2 Procedures

Step 2. Prepare hybridization samples

Step 2. Prepare hybridization samples

- 1 Equilibrate water bath or heat block to 100°C.
- 2 Resuspend the dried sample in 17 μL of nuclease-free water when the [Hyb Spike-In](#) solution is used and 18 μL when the [Hyb Spike-In](#) solution is not used.
- 3 For each microarray, add each of the components as indicated in [Table 15](#) or [Table 16](#) to a 1.5 mL nuclease-free microfuge tube:
- 4 Mix well but gently on a vortex mixer.

Table 15 Hybridization mix for miRNA microarrays with [Hyb Spike-In](#) solution

| Components | Volume (μL) |
|--|--------------------------|
| Labeled miRNA sample | 17 |
| 3rd Dilution Hyb Spike-In solution | 1 |
| 10 \times Gene Expression Blocking Agent | 4.5 |
| 2 \times Hi-RPM Hybridization Buffer | 22.5 |
| Total Volume | 45 |

Table 16 Hybridization mix for miRNA microarrays without [Hyb Spike-In](#) solution

| Components | Volume (μL) |
|--|--------------------------|
| Labeled miRNA sample | 18 |
| 10 \times Gene Expression Blocking Agent | 4.5 |
| 2 \times Hi-RPM Hybridization Buffer | 22.5 |
| Total Volume | 45 |

For best results, stagger the tubes in sets of eight such that eight arrays on one array slide can be loaded at once. Have the [SureHyb](#) chamber along with the gaskets and microarrays easily available to prepare the hybridization assembly before you continue to the next step.

- 5 Incubate at 100°C for 5 minutes.
- 6 Immediately transfer to an ice water bath for 5 minutes.

Step 2. Prepare hybridization samples

CAUTION

Do not leave samples in the ice water bath for more than 15 minutes. Longer incubations may adversely affect the hybridization results.

- 7 Quickly spin in a centrifuge to collect any condensation at the bottom of the tube.
- 8 Immediately proceed to “Step 3. Prepare the hybridization assembly” on page 30.

Step 3. Prepare the hybridization assembly

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

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

CAUTION

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

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

- 2 Slowly dispense all of the volume of hybridization sample onto the gasket well in a “drag and dispense” manner.
 - Position the slides so that the barcode label is to your left.
 - Load the samples left to right. For 8-pack slides, start with the first row. The output files will come out in that same order. Refer to “[Array/Sample tracking microarray slides](#)” on page 68 for guidelines on tracking sample position for multipack slide formats.
 - Avoid the introduction of air bubbles to the gasket wells. Air bubbles can affect the final sample volume and can cause leakage from the gasket well.
- 3 If any wells are unused:
 - a Make a 1× solution of the 2× [Hi-RPM Hybridization Buffer](#).
 - b Add the volume of 1× Hybridization Buffer equal to the sample volume to each unused well.

Make sure all wells contain sample or 1× Hybridization Buffer. Empty wells can cause failure in hybridization.

miRNA microarray slides processed with fewer than 8 samples can result in gridding errors in Feature Extraction, which can require manual gridding.

Step 3. Prepare the hybridization assembly

- 4 Grip the slide on either end and slowly put the slide “active side” down, parallel to the SureHyb gasket slide, so that the “Agilent”-labeled barcode is facing down and the numeric barcode is facing up. Make sure that the sandwich-pair is properly aligned.

CAUTION

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

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

Step 4. Hybridize

- 1 Load each assembled chamber into the oven rotator rack. Start from the center of the rack (position 3 or 4 when counting from the left). Set your hybridization rotator to rotate at 20 rpm.
- 2 Hybridize at 55°C for 20 hours.

CAUTION

Be sure that the arrays are hybridized for at least 20 hours. Hybridization can occur for longer than 20 hours but the actual hybridization time should be consistent if the results are to be compared. Failure to maintain consistent hybridization times may adversely affect your data.

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 and slide-staining dish #3](#)”.

Microarray Wash

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

This step is optional but highly recommended.

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

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

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

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

2 Procedures

Step 2. Prewarm Gene Expression Wash Buffer 2 and slide-staining dish #3

Step 2. Prewarm Gene Expression Wash Buffer 2 and slide-staining dish #3

Warm the [Gene Expression Wash Buffer 2](#) to 37°C as follows:

- 1 Dispense 1000 mL of [Gene Expression Wash Buffer 2](#) directly into a [sterile storage bottle](#). Repeat until you have enough prewarmed Wash Buffer 2 solution for your experiment.
- 2 Tightly cap the [sterile storage bottle](#) and put in a 37°C water bath the night before washing arrays. Alternatively, remove the plastic cubitainer from the box and put it in a 37°C water bath the night before washing the arrays.
- 3 Place slide-staining dish #3 (see [Table 17](#) on page 36) for numbering of the wash dishes) into a 1.5 L glass dish three-fourths filled with water. Warm to 37°C by storing overnight in an incubator set to 37°C.

Step 3. Prepare the equipment

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

Solvent wash

Wash staining dishes, racks and stir bars with [isopropyl alcohol](#) to avoid wash artifacts on your slides and images.

WARNING

Conduct solvent washes in a vented fume hood.

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

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](#)”.

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.

2 Procedures

Step 4. Wash the microarray slides

Step 4. Wash the microarray slides

NOTE

The microarray wash procedure must be done in environments where ozone levels are 50 ppb or less. For Scanner C and Scanner B, if ozone levels exceed 50 ppb in your laboratory, use the Agilent Ozone Barrier Slide Cover (described in this topic). SureScan microarray scanner uses a slide holder with a built-in ozone barrier.

NOTE

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

Table 17 lists the wash conditions for the wash procedure.

Table 17 Wash conditions

| | Dish | Wash Buffer | Temperature | Time |
|-------------|------|-------------------------------|------------------|-----------|
| Disassembly | 1 | Gene Expression Wash Buffer 1 | Room temperature | |
| 1st wash | 2 | Gene Expression Wash Buffer 1 | Room temperature | 5 minutes |
| 2nd wash | 3 | Gene Expression Wash Buffer 2 | 37°C | 5 minutes |

- 1 Completely fill slide-staining dish #1 with [Gene Expression Wash Buffer 1](#) at room temperature.
- 2 Put a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough [Gene Expression Wash Buffer 1](#) at room temperature to cover the slide rack. Put this dish on a magnetic stir plate.
- 3 Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with [Gene Expression Wash Buffer 2](#) (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of [Gene Expression Wash Buffer 2](#) at 37°C. Use a thermometer to check the temperature.
- 4 Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.

Step 4. Wash the microarray slides

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

CAUTION

You must maintain [Gene Expression Wash Buffer 2](#) at 37°C for the duration of the wash step. Failure to do so can result in alterations of stringency, which can reduce the consistency of experimental results.

2 Procedures

Step 4. Wash the microarray slides

- 10 Slowly remove the slide rack minimizing droplets on the slides. It should take 5 to 10 seconds to remove the slide rack. If liquid remains on the bottom edge of the slide, dab it on a cleaning tissue.
- 11 Discard used Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2.
- 12 Repeat step 1 through step 11 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.
- 13 Put the slides in a slide holder.

For SureScan microarray scanner

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



Figure 2 Slide in slide holder for SureScan microarray scanner

For Agilent Scanner C only:

- In environments in which the ozone level exceeds 50 ppb, immediately put the slides with active microarray surface (with “Agilent”-labeled barcode) facing up in a slide holder. Make sure that the slide is not caught up on any corner. Put an ozone-barrier slide cover on top of the array as shown in [Figure 3](#). Refer to the *Agilent Ozone-Barrier Slide Cover User Guide* (p/n G2505-90550), included with the slide cover, for more information.

Step 4. Wash the microarray slides

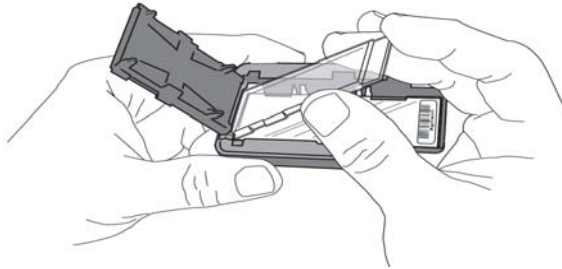


Figure 3 Inserting the ozone-barrier slide cover (shown for Scanner C)

- In environments in which the ozone level is below 50 ppb, put the slides with Agilent barcode facing up in a slide holder.

14 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

This section describes how to scan and extract data from miRNA microarrays. Refer to the Agilent Microarray Scanner *User Guide* for more information on how to use the scanner.

Table 18 Scanner to GeneSpring Workflow version compatibility

| Microarray Format | Scanner version | Scan Control version | Scan settings/profile | Spike-In | Feature Extraction version | GeneSpring version |
|-------------------|-----------------|----------------------|-------------------------|----------|----------------------------|--------------------|
| 8x60K | SureScan | 9.X | AgilentG3_miRNA | Yes | 10.7.3 or later | 11.5 or later |
| 8x60K | SureScan | 9.X | AgilentG3_miRNA | No | 10.5 or later | 10.0 or later |
| 8x15K | SureScan | 9.X | AgilentHD_miRNA | Yes | 10.7.3 or later | 11.5 or later |
| 8x15K | SureScan | 9.X | AgilentHD_miRNA | No | 10.5 or later | 10.0 or later |
| 8x60K | C | 8.X | AgilentG3_miRNA | Yes | 10.7.3 or later | 11.0 or later |
| 8x60K | C | 8.X | AgilentG3_miRNA | No | 10.5 or later | 10.0 or later |
| 8x15K | C | 8.X | AgilentHD_miRNA | Yes | 10.7.3 or later | 11.0 or later |
| 8x15K | C | 8.X | AgilentHD_miRNA | No | 10.5 or later | 10.0 or later |
| 8x15K | B | 7.X | See Table 20 on page 43 | Yes | 10.7.3 or later | 11.0 or later |
| 8x15K | B | 7.X | See Table 20 on page 43 | No | 9.5.3 or later | 10.0 or later |

Step 1. Scan the slides

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

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

A SureScan or Agilent C microarray scanner is required for SurePrint G3 formats.

To get scanner profiles from Agilent:

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

Agilent SureScan Microarray Scanner

- 1 Put assembled slide holders into the scanner cassette.
- 2 Select the appropriate scanner protocol:
 - **AgilentG3_miRNA** (for G3 format)
 - **AgilentHD_miRNA** (for HD format)
- 3 Verify that the Scanner status in the main window says Scanner Ready.
- 4 Click **Start Scan**.

Agilent C Scanner Settings

- 1 Put assembled slide holders with or without the ozone-barrier slide cover into scanner carousel.
- 2 Select Start Slot *m* End Slot *n* where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.
- 3 Select **AgilentG3_miRNA** (for SurePrint G3 formats) or **AgilentHD_miRNA** (for SurePrint HD formats).
- 4 Verify scan settings for -color scans. See Table 19.

2 Procedures

Step 1. Scan the slides

CAUTION

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

Table 19 C Scanner Scan Settings

| | For HD Microarray Formats | For G3 Microarray Formats |
|--------------------------------|---------------------------|---------------------------|
| Dye channel | G (<i>green</i>) | G (<i>green</i>) |
| Scan region | Agilent HD (61 × 21.6 mm) | Agilent HD (61 × 21.6 mm) |
| Scan resolution | 5 μm | 3 μm |
| Tiff file dynamic range | 20 bit | 20 bit |
| Green PMT gain | 100% | 100% |

- 5 Verify that **Output Path Browse** is set for desired location.
- 6 Verify that the Scanner status in the main window says Scanner Ready.
- 7 Click **Scan Slot *m-n*** on the Scan Control main window where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.

Agilent B Scanner Settings

Agilent Scan Control software v7.0. is recommended for 5 μm scans of SurePrint HD formats The Agilent B Scanner does not support G3 microarrays. For G3 microarrays, use the Agilent C Scanner or SureScan microarray scanner

- 1 Put slide into slide holder, with or without the ozone-barrier slide cover, with Agilent barcode facing up.
- 2 Put assembled slide holders into scanner carousel.
- 3 Change scan settings-color scans. See .

CAUTION

The scanner settings for miRNA microarray scanning are not the same as the default XDR settings for version 7.x.

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

Table 20 B Scanner Scan Settings

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

- 4** Select settings for the automatic file naming.
 - **Prefix1** is set to **Instrument Serial Number**.
 - **Prefix2** is set to **Array Barcode**.
- 5** Verify that the Scanner status in the main window says **Scanner Ready**.
- 6** Click **Scan Slot *m-n*** on the Scan Control main window where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.

Step 2. Extract data using Agilent Feature Extraction Software

Feature Extraction is the process by which information from probe features is extracted from microarray scan data, allowing researchers to measure miRNA 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.

FE version 9.5.3 or higher is required for extraction of .tif images of Agilent miRNA microarrays scanned on an Agilent Scanner. FE version 10.7 gives you the option to automatically download new grid templates, protocols and QC metrics (QCM or QCMT) via eArray, and supports analysis of the spike-ins miRNA. See “Automatic Download from eArray” on page 49 for configuration. Refer to Table 18 on page 40 to determine the version of Feature Extraction you need to use.

FE version 10.7 protocol uses surrogates in determining the gTotalGeneSignal which eliminates negative expression values. Earlier versions of FE and FE protocols do not use surrogates and may create negative gTotalGeneSignal values.

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

For more information about normalizing one-color microarray data, see “Normalizing Agilent One-Color Microarray Data” on page 52.

1 Open the Agilent Feature Extraction (FE) program.

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

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

- a Click **Add New Extraction Set(s)** icon on the toolbar or right-click the **Project Explorer** and select **Add Extraction...**
- b Browse to the location of the .tif files, select the .tif file(s) and click **Open**. To select multiple files, use the Shift or Ctrl key when selecting.

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

- For auto assignment of the grid template, the image must be generated from a Agilent scanner and have an Agilent barcode.

Step 2. Extract data using Agilent Feature Extraction Software

- For auto assignment of the -Color miRNA FE protocol, the default miRNA protocol must be specified in the FE Grid Template properties. To access the FE Grid Template properties, double-click on the grid template in the Grid Template Browser.

3 Set FE Project Properties.

- a** Select the **Project Properties** tab.
- b** In the **General** section, enter your name in the **Operator** text box.
- c** In the **Input** section, verify that at least the following default settings as shown in [Figure 4](#) are selected.
- d** For versions of FE earlier than 10.7.3, in the **Other** section, from the **QC Metric Set** drop-down list, select **miRNA_QCMT_Jan09** as shown in [Figure 4](#) on page 46.

If the QC Metric Set is not available to select from the pull down menu, you must add it to the QC Metric Set Browser. To add, right-click inside the QC Metric Set Browser, and click **Add**. Browse for the QC Metric Set file and click **Open** to load the QC Metric Set into the FE database.

If you don't have a local copy of the QC Metric Set or would like to download the latest QC Metric Set, you can do so from Agilent Web site at www.agilent.com/chem/feqcmetrics. After downloading, you must add the QC Metric Set to the QC Metric Set Browser.

After a new QC Metric Set is added to the QC Metric Set Browser, remember to specify the default protocol for the new QC Metric Set if you want the Feature Extraction program to automatically assign a FE QC Metric Set to an extraction set.

2 Procedures

Step 2. Extract data using Agilent Feature Extraction Software

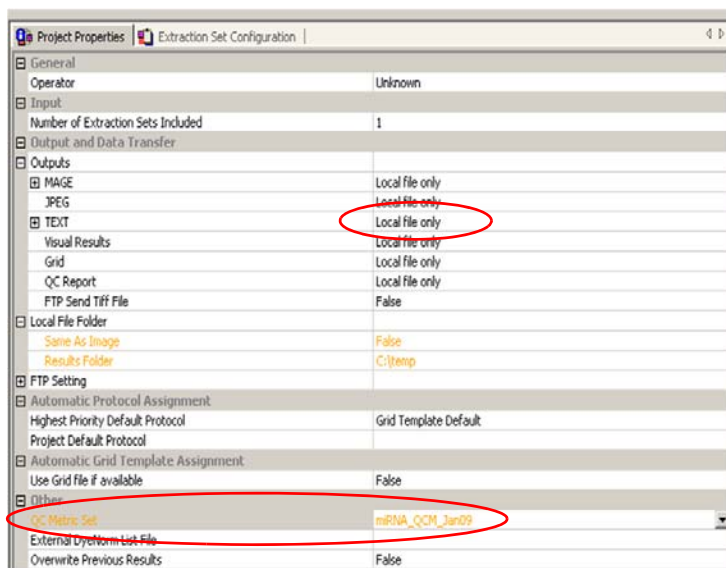


Figure 4 Feature Extraction version 9.5 dialog box

4 Check the Extraction Set Configuration.

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

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

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

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

Step 2. Extract data using Agilent Feature Extraction Software

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. For Agilent miRNA microarrays, select **miRNA_107_Sept09**.

The protocols automatically distinguish the formats for processing the data.

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

NOTE

When the Agilent XDR scanned images are added to Feature Extraction software version 9.5 or later, the High and Low images are automatically combined for data extraction.

NOTE

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

- 5 Save the FE Project (.fep) by selecting **File > Save As** and browse for desired location.
- 6 Verify that the icons for the image files in the FE Project Window no longer have a red X through them. A red X through the icon indicates that an extraction protocol was not selected. If needed, reselect the extraction protocol for that image file.
- 7 Select **Project > Start Extracting**.
- 8 After the extraction is completed successfully, view the QC report for each extraction set by double-clicking the QC Report link in the **Summary Report** tab. Determine whether the grid has been properly placed by inspecting Spot Finding at the Four Corners of the Array. See [Figure 6](#).

2 Procedures

Step 2. Extract data using Agilent Feature Extraction Software

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

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

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

Agilent Feature Extraction Result Files

Agilent Feature Extraction software generates two text files for each miRNA microarray. The text file that uses the name *ScannerNum_BarcodeNum_SO#_FEProtocol_Array_Num.txt* (for example, “US22502705_251911810634_S01_miRNA-v1_95_May07_1_1.txt”) includes all feature, probe and gene level data, as well as array level statistics and parameters. Use these files for analysis and to review the QC metrics in the GeneSpring GX version 10 or later.

The other text file, the “GeneView” file, uses the name *BarcodeNum_SO#_Array_Num_GeneView.txt* (for example, “251911810634_S01_1_1_GeneView.txt”). The GeneView file provides the summarized TotalGeneSignal, TotalGeneError, and a detection flag for each miRNA gene. This simple file is appropriate for any analyses and can be loaded into GeneSpring GX version 9.0, or similar software for analysis. However, it does not contain the QC metric data for review. The “gTotalGeneSignal” column has been background subtracted and outlier rejected and is appropriate for use as the measured intensity for each miRNA gene without further manipulation.

Please refer to the Agilent Feature Extraction Software *User Guide* or *Reference Guide* for more detail on the data summarization algorithms and for information on other columns in the feature extraction result files.

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 5.

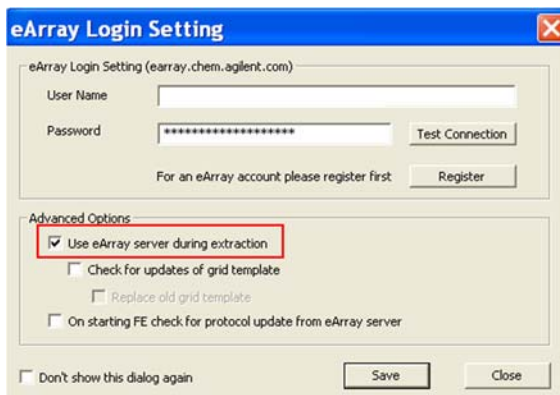


Figure 5 eArray Login Setting

2 Procedures

Step 2. Extract data using Agilent Feature Extraction Software

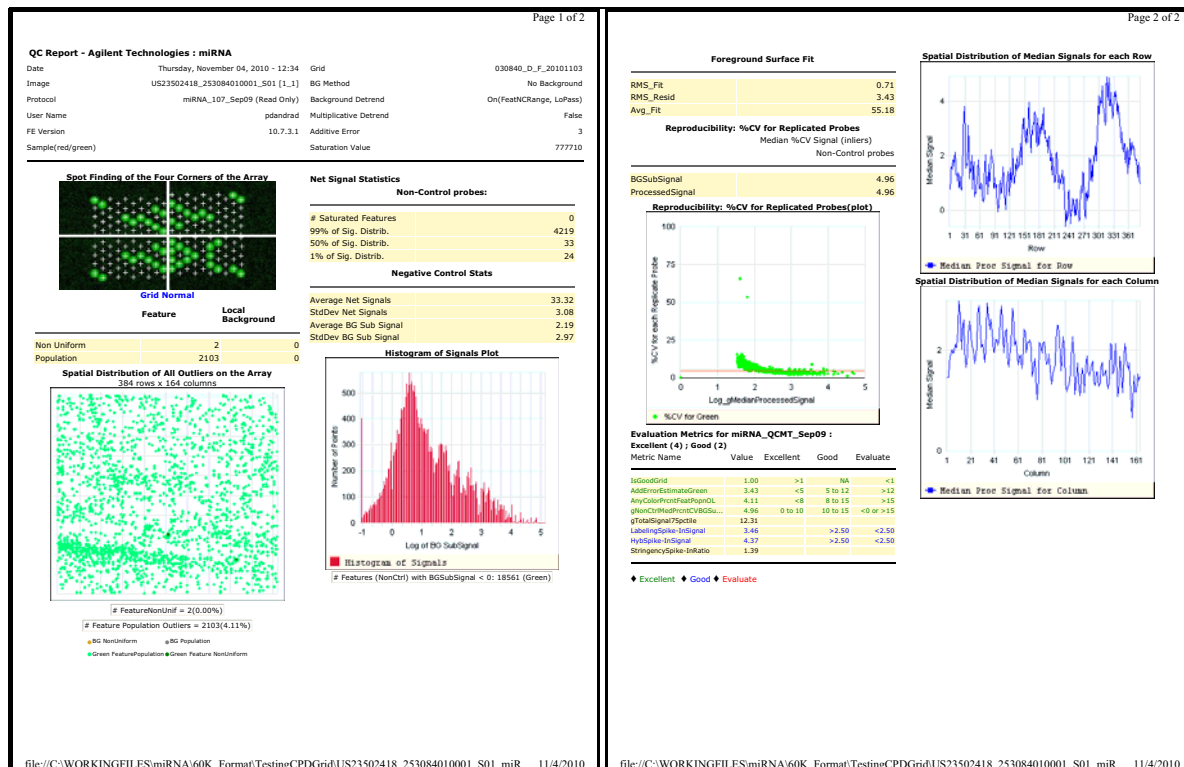
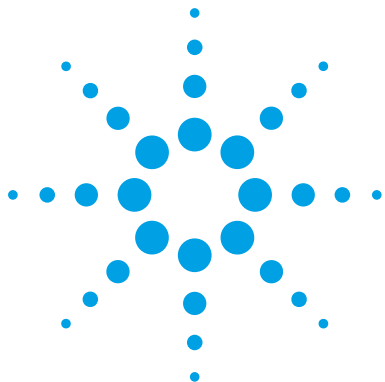


Figure 6 Example of a QC Report for 8x15K miRNA microarray, generated by Feature Extraction version 10.7.3 with the use of Spike-In miRNAs.



3 Supplemental Procedures

Normalizing Agilent One-Color Microarray Data 52
Characterization of Total RNA 54

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

Normalizing Agilent One-Color Microarray Data

When comparing data across a set of one-color microarrays, a simple linear scaling of the data is usually sufficient for most experimental applications.

The appropriate normalization method depends on the sample type and experimental design. Agilent recommends to use the signal value of the 90th percentile of all of non-control probes on the microarray as a start. Adjust this value as needed.

To do downstream analysis of Agilent microarray data

- Use GeneSpring GX 9.011.5 or later.

Note that the default normalization scheme for data in the GeneSpring GX 9.011.5 (or later) program is 90th percentile normalization.

For more information on the GeneSpring GX program, go to <http://www.agilent.com/chem/genespring>.

To use Feature Extraction

To normalize Agilent one-color microarray data without the GeneSpring program, use the 90th percentile value for each microarray assay in the Agilent Feature Extraction text file.

- 1 Generate a Feature Extraction text file.
- 2 Find the “STATS Table” in the middle section of the text file. This section describes the results from the array-wide statistical calculations.
- 3 Find the 90th percentile value of the non-control signals under the column with the heading **gPercentileIntensityProcessedSignal**.
- 4 Divide each of the green processed signals (**gProcessedSignal**) by the 90th percentile signal (**gPercentileIntensityProcessedSignal**) to generate the 90th percentile normalized microarray processed signals.

You can further scale the resulting 90th percentile-normalized signals by a constant, such as the average of the 90th percentile signals of the arrays in the experiment.

For more information on the output from the Agilent Feature Extraction program, please refer to the *Agilent G2567AA Feature Extraction Software Reference Guide*. You can download this guide from the Agilent web site at www.agilent.com/chem/dnamanuals-protocols.

Characterization of Total RNA

Although this step is optional in the miRNA microarray work-flow, Agilent strongly recommends that you characterize the total RNA for sample integrity and possible presence of contaminants. This chapter gives a general guideline for total RNA and small RNA characterization prior to proceeding with labeling and hybridization.

Depending on the method used for purification of the total RNA, your sample may have contaminants such as phenol, salts, and/or genomic DNA. You can check the absorption spectrum from 220 nm to about 300 nm to detect some of these possible contaminants. In the absorption spectra, you should see only one peak with an absorption maximum at 260 nm. You may also see a shoulder of an additional peak at < 220 nm that overlaps with and inflates the absorption at 260 nm. The level of inflation can be seen on the absorption spectrum. If the absorption spectrum is not available, calculate the ratio of absorbances at 260/230 nm. This ratio should be greater than 1.8. If the ratio is less than 1.8, then you need to purify the sample further by doing additional extractions with chloroform, followed by an ethanol precipitation. To ensure recovery of the RNA, do this purification with a large (microgram) quantity of total RNA.

Unfortunately, absorption spectra cannot differentiate between RNA and DNA. Contamination with DNA can result in overestimation of total RNA amounts, and may lead to decreased sensitivity on the miRNA assay due to the effective decrease in the amount of total RNA added.

Characterization of the input total RNA using the Agilent 2100 bioanalyzer provides information on the sample quality prior to labeling and hybridization. You can use the RNA 6000 Nano kit to analyze total RNA with the appropriate assay at the assay specified concentration. For low concentration samples, use the RNA 6000 Pico kit.

The small RNA assay characterizes the total RNA sample with an emphasis on the small RNA content of which a fraction is miRNA. This can be done using the Small RNA Kit along with the Small RNA assay on the Agilent 2100 bioanalyzer.

For 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.

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

To download application notes and for more information regarding Agilent’s 2100 bioanalyzer along with the various kits offered for analysis of both total RNA and small RNA, visit the Web site at www.agilent.com/chem/labonachip.

3 Supplemental Procedures

Step 1. Prepare for characterization of total RNA

Step 1. Prepare for characterization of total RNA

- Refer to [Table 21](#) and [Table 22](#) to make sure that you have the appropriate analyzer, kits, and compatible assays.

Table 21 Analyzer and Kits

| Description | Company and part no. |
|-------------------|------------------------------|
| 2100 Bioanalyzer | Agilent p/n G2938C or G2939A |
| RNA 6000 Nano Kit | Agilent p/n 5067-1511 |
| RNA 6000 Pico Kit | Agilent p/n 5067-1513 |
| Small RNA Kit | Agilent p/n 5067-1548 |

Table 22 Recommended Assays

| Description | Compatible Assay |
|-------------------|--|
| RNA 6000 Nano Kit | Agilent Eukaryote Total RNA Nano Assay Qualitative range 5 to 500 ng/ μ L |
| RNA 6000 Pico Kit | Agilent Eukaryote Total RNA Pico Assay Qualitative range 50 to 5000 pg/ μ L in water |
| Small RNA Kit | Agilent Small RNA Assay <ul style="list-style-type: none">• 1 to 100 ng/μL of total RNA• 100 to 2000 pg miRNA• 1 to 20 ng/μL of purified small RNA < 200 nt |

Step 2. Characterize the RNA samples using the Agilent 2100 Bioanalyzer

To measure the total RNA integrity, purity and concentration, use one of the Agilent RNA 6000 kits. Follow the instructions in the kit.

To measure the small RNA amount and profile, use the Agilent Small RNA kit. Follow the instructions in the kit.

- 1 Ensure the 2100 bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
- 2 Open the Agilent 2100 expert software (version B.02.04 or higher), switch on the 2100 bioanalyzer and check communication.
- 3 Prepare the chip, samples and ladder as instructed in the reagent kit guide.
- 4 Load the prepared chip into the 2100 bioanalyzer and start the run within five minutes after preparation.
- 5 Within the instrument context, choose the appropriate assay from the drop down list.
- 6 Start the run. Enter sample names and comments in the Data and Assay context.
- 7 Verify the results.

RNA 6000 - Total RNA

Total RNA characterization provides information on the quality of the total RNA, which may impact the overall RNA quantification and the microarray miRNA profile.

The resulting electropherogram typically has at least two distinct peaks representing the 18S and 28S ribosomal RNA. The peak representing the lower marker can also be seen as shown in [Figure 7](#). The presence of the 5S RNA peak, as shown in [Figure 7](#), depends on the purification method, and generally shows lower abundance in column purified total RNA. [Figure 7](#) demonstrates the differences in the peaks with varying degrees of total RNA integrity and provides RNA Integrity Number (RINs) as examples. Make sure you define a minimum threshold RIN number based on correlative data in order to eliminate experimental bias due to poor total RNA quality.

3 Supplemental Procedures

Step 2. Characterize the RNA samples using the Agilent 2100 Bioanalyzer

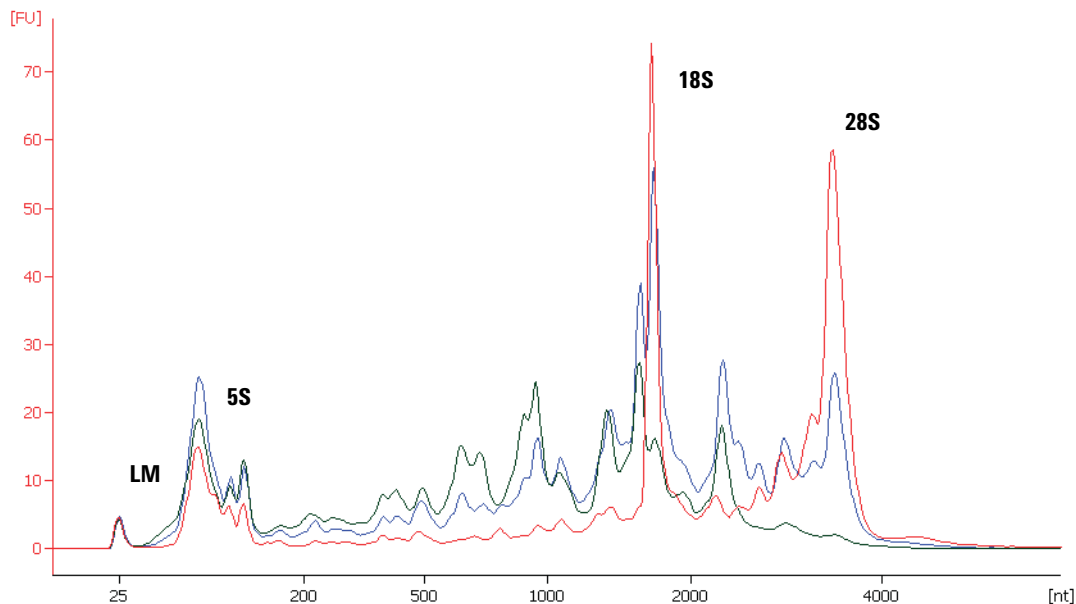


Figure 7 Analysis of (human) total RNA with the Eukaryote total RNA Nano assay using three different samples with decreasing integrity: Red, RIN 8.4; Blue, RIN 5.9; Green, RIN 3.6. Characteristic regions for ribosomal peaks and the lower marker (LM) are displayed.

Small RNA - miRNA in total RNA

The small RNA assay characterizes the total RNA sample with an emphasis on the small RNA content of which a fraction is miRNA.

The resulting electropherogram typically has a number of bands or peaks representing small RNAs ranging in size from 10 to 150 nt. The miRNA portion is represented by the band or peak ranging in size from 10 to 40 nt. These bands or peaks will vary in abundance depending on the total miRNA preparation. [Figure 8](#) shows an example of the resulting electropherogram of the small RNA assay for a total RNA sample.

You can record the results of the small RNA assay to correlate to your miRNA microarray data. However, the small RNA assay results may not necessarily be an indicator for the miRNA content in the sample, and the miRNA microarray assay is a more sensitive technique for detecting low levels of miRNAs.

Step 2. Characterize the RNA samples using the Agilent 2100 Bioanalyzer

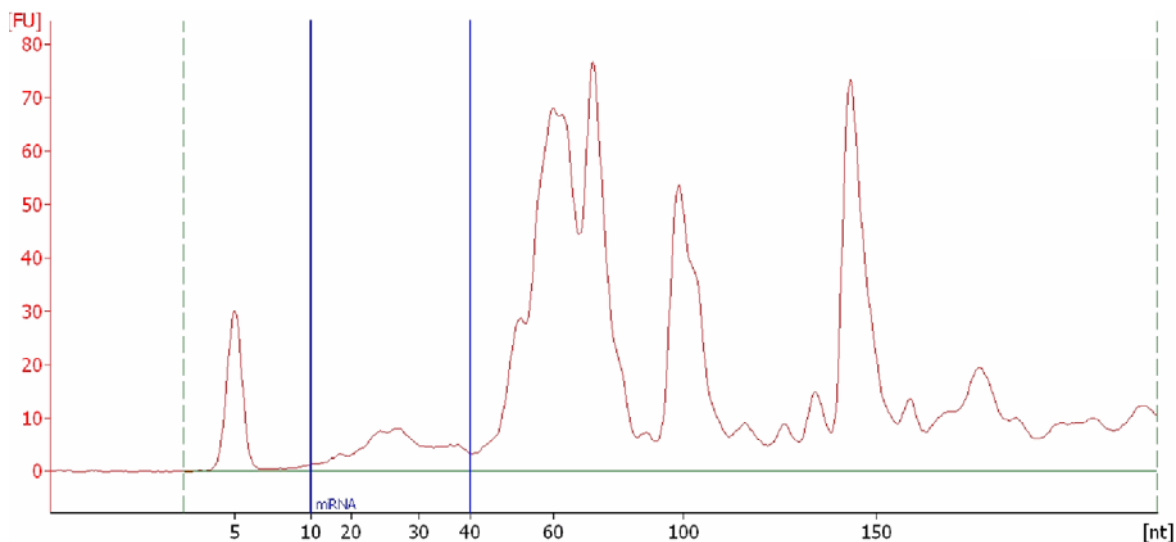
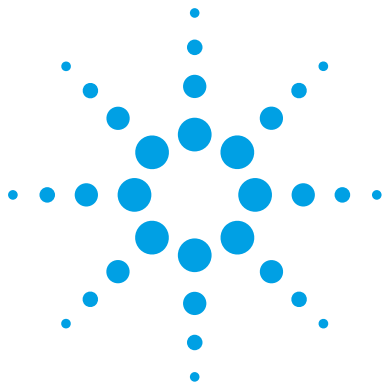


Figure 8 Characterization of small RNA within a total RNA sample. miRNA is typically found in the 10 to 40 nt region, which is a subset of the small RNA that range from 10 to 150 nt

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4 Reference

| | |
|---|----|
| Kit Contents | 62 |
| Supplemental User Guides | 63 |
| Microarray Handling Tips | 64 |
| General Microarray Layout and Orientation | 65 |
| Array/Sample tracking microarray slides | 68 |

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



Kit Contents

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

Table 23 microRNA Spike-In Kit

| Content |
|-------------------|
| Dilution Buffer |
| Labeling Spike-In |
| Hyb Spike-In |

Table 24 miRNA Complete Labeling and Hyb Kit

| Content |
|--|
| 10× Gene Expression Blocking Agent |
| 2× Hi-RPM Hybridization Buffer |
| T4 RNA Ligase |
| 10× T4 RNA Ligase Buffer |
| Calf Intestinal Phosphatase |
| 10× Calf Intestinal Phosphatase Buffer |
| DMSO |
| nuclease-free water |
| Cyanine 3-pCp |

Table 25 Gene Expression Wash Buffer Kit

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

Supplemental User Guides

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

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

Microarray Handling Tips

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

CAUTION

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

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

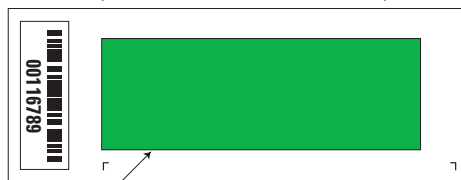
To avoid damaging the microarray, always handle glass slides carefully by their edges. Wear powder-free gloves. Never touch the surfaces of the slides. If you do, you may cause irreparable damage to the microarray.

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

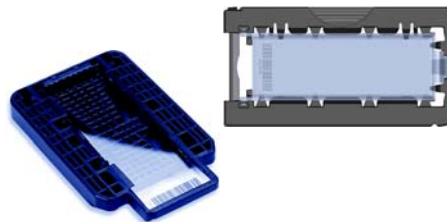
General Microarray Layout and Orientation

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

Microarrays are printed on the side of the glass labeled with the "Agilent" bar code (also referenced as "active side" or "front side").



Agilent Microarray Scanner scans through the glass. (Back side scanning.)



Agilent microarray slide holder for Scanner B and C (left) or SureScan microarray scanner (right)

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

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

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

Non-Agilent front side microarray scanners

When imaging Agilent oligo microarray slides, you must determine:

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

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

General Microarray Layout and Orientation

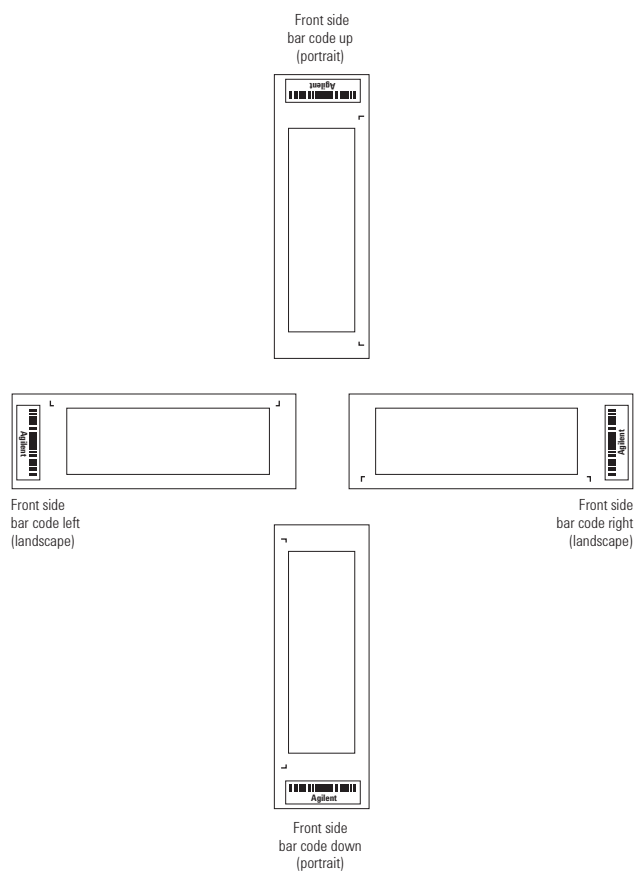


Figure 10 Microarray slide orientation

4 Reference

Array/Sample tracking microarray slides

Array/Sample tracking microarray slides

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

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

Arrays

| | Array 1_1 | Array 1_2 | Array 1_3 | Array 1_4 |
|---------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| B A R C O D E | Sample: <hr/> <hr/> <hr/> | Sample: <hr/> <hr/> <hr/> | Sample: <hr/> <hr/> <hr/> | Sample: <hr/> <hr/> <hr/> |
| | Sample: <hr/> <hr/> <hr/> | Sample: <hr/> <hr/> <hr/> | Sample: <hr/> <hr/> <hr/> | Sample: <hr/> <hr/> <hr/> |

Array 2_1

Array 2_2

Array 2_3

Array 2_4

Barcode Number _____

Figure 11 8-pack microarray slide

www.agilent.com

In This Book

This document describes the Agilent recommended procedures for the preparation and labeling of complex biological targets and hybridization, washing, scanning, and feature extraction of Agilent oligonucleotide microarrays for microarray-based miRNA systems.

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