

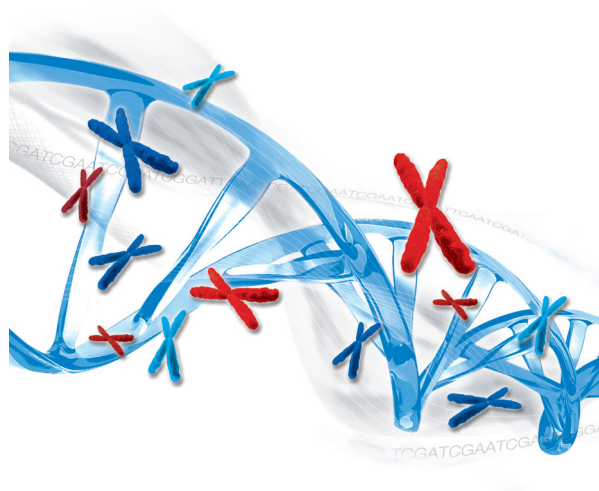
miRNA Microarray System with miRNA Complete Labeling and Hyb Kit

Protocol

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

Version 4.1, October 2021

Microarrays manufactured with Agilent SurePrint Technology



Notices

Manual Part Number

G4170-90011 Version 4.1
October 2021

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Safety Notices

CAUTION

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WARNING

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

In this Guide...

This document describes the Agilent recommended procedures for the preparation and labeling of complex biological targets and hybridization, washing, scanning, and feature extraction of Agilent 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 Supplemental Procedures

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 4.1

- Clarification of sample storage temperature on **page 32**.

What's new in 4.0

- Updated document look and feel.
- Instructions on handling the newly redesigned "secure fit" slide boxes in which the microarray slides are shipped. Before opening the box for the first time, see **"Secure Fit" Slide Box Opening Instructions** on page 68.
- Expanded instructions and new images in the **Procedures** chapter to help avoid common problems and optimize hybridization of your sample to the microarray.
- Updated web addresses for Agilent materials.
- Removed microarray scanning instructions for the Agilent B scanner.
- Replaced detailed **Safety Notes** with guidance on consulting the safety data sheets and product hazard labels.

What's New in 3.1

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

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

NOTE

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

Procedural Notes

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

Safety Notes

CAUTION

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

For reagent safety information, consult the safety data sheets and any product hazard labels. Agilent safety data sheets are available at www.agilent.com.

Agilent Oligo Microarrays

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

Table 1 Catalog SurePrint HD and G3 miRNA Microarrays - Human (continued)

Part Number	Description	miRBase Release
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

Before You Begin

Required Equipment

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

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 Chamber gasket slides 8 microarrays/slide, 5 slides/box	Agilent p/n G2534-60014
Go to www.agilent.com to see all available kit configurations.	
Hybridization oven; temperature set at 55°C	Agilent p/n G2545A

Before You Begin

Required Equipment

Table 5 Required Equipment (continued)

Description	Vendor and part number
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
nuclease-free 1.5 mL microfuge tube	Thermo Fisher Scientific p/n AM12400 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	Corning p/n 6795-420D or equivalent
microcentrifuge	Eppendorf p/n 5417R or equivalent
sterile storage bottle	Nalgene 455-1000 or equivalent
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 Fisher Scientific p/n 121 or equivalent
vacuum concentrator with heater	Savant SpeedVac p/n SPD111V or equivalent
circulating water baths or heat blocks set to 16°, 37°C, and 100°C,	
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	Thermo Fisher Scientific 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 Required Hardware and Software

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 Optional Software

Description
GeneSpring 10.0 or higher

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

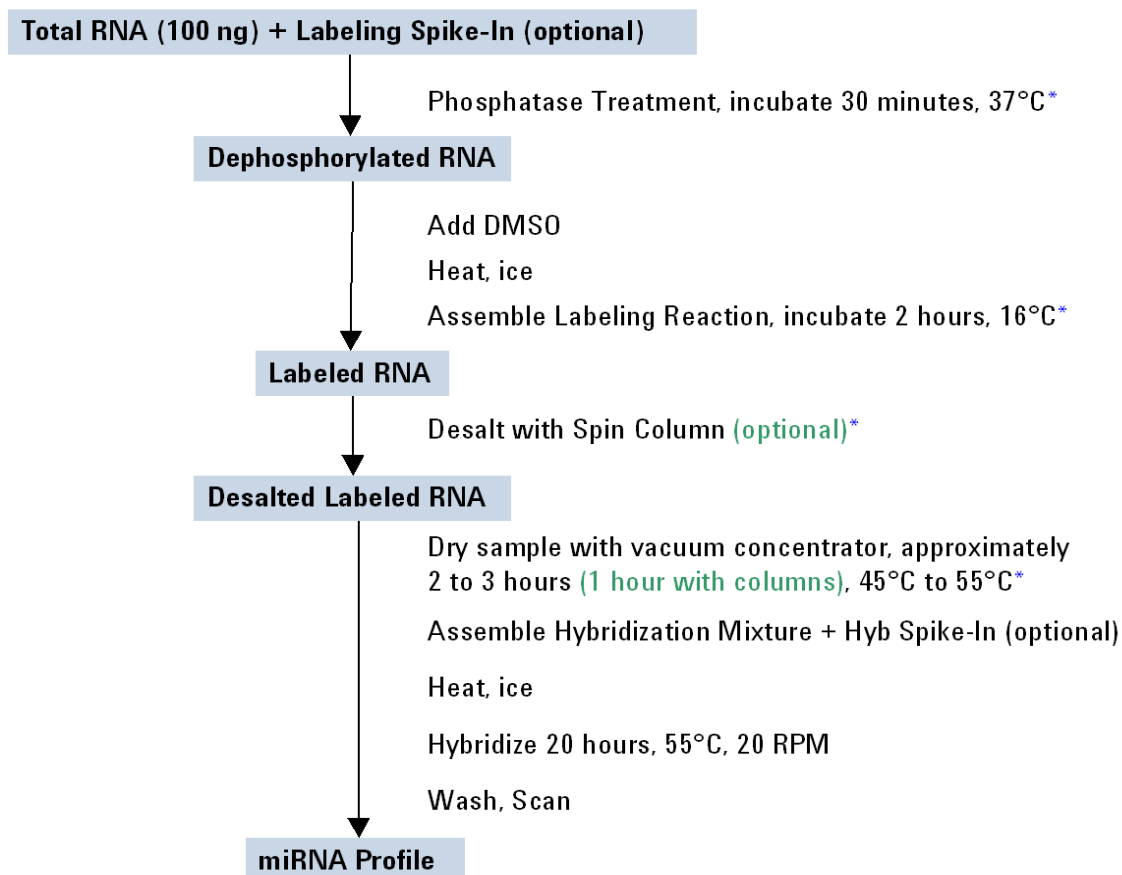
Do not use purified miRNA. This protocol supports the use of total RNA only. Extraction methods that use organic solvents such as TRIZOL can result in inaccurate quantification, because organic solvent contamination from carry-over during the RNA extraction can compress the 260/230 ratio. The affected 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 60 for more information on RNA purity and quality assessment.

2

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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.



*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** on page 66.

NOTE

Please refer to “**Characterization of Total RNA**” on page 60 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**.

Procedures
Step 1. Prepare Spike-In solutions (optional)

- 1 Thaw all components.
- 2 Briefly spin tubes.
- 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 µL of the Dilution Buffer	198 µL of the Dilution Buffer
2 µL of the Labeling Spike-In solution	2 µL 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 µL of nuclease-free water .	198 µL of nuclease-free water .
2 µL of 1st Dilution Labeling Spike-In solution	2 µL of 1st Dilution Hyb Spike-In solution

- 9 Mix well and briefly spin in a centrifuge.

Procedures
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 µL of nuclease-free water.	198 µL of nuclease-free water.
2 µL of 2nd Dilution Labeling Spike-In solution	2 µL 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/µL in **1× tris EDTA pH 7.5** or **DNase/RNase-free distilled water**.
 - 2 Add 2 µL (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**.

Table 13 CIP Master Mix

Components	Volume (μL) per reaction	Volume (μL) per 9 reactions
10× 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.

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 to “**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 x 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.

Sample purification

- 1 Add 38.7 µL of **1× 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 x 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.

Microarray Hybridization

Before you begin, make sure you read and understand **“Secure Fit” Slide Box Opening Instructions** on page 68 and **“Microarray Handling Tips”** on page 70.

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* (publication 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**.

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× Gene Expression Blocking Agent	4.5
2× 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× Gene Expression Blocking Agent	4.5
2× 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.

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 29.

Step 3. Prepare the hybridization assembly

Refer to the *Agilent Microarray Hybridization Chamber User Guide* (publication G2534-90004) for in-depth instructions on how to load samples, assemble and disassemble chambers, as well as other helpful tips. This user guide can be downloaded from the Agilent Web site at www.agilent.com.

Before you begin, make sure you read and understand **“Secure Fit” Slide Box Opening Instructions** on page 68 and **“Microarray Handling Tips”** on page 70.

Remove gasket slide from its packaging

NOTE

- Do not remove gasket slide from protective sleeve until ready for use.
 - Do not slice or cut open the gasket slide protective packaging.
 - Handle only the edges of the gasket slide.
 - Prior to use, inspect gasket slides for visible gaps or cuts through the gaskets or any debris within the hybridization areas as these are indications of instability. Do not use gasket slides that have these features.
-

Procedures

Step 3. Prepare the hybridization assembly

- 1 With tweezers, carefully lift up the corner of the clear plastic covering and slowly pull back the protective film.



Figure 2. Removal of clear plastic covering

- 2 With clean, powder-free gloved fingers, remove the gasket slide from its package. Handle the slide only on its edges.

To avoid any potential contamination from surrounding surface materials, immediately insert the gasket slide in the chamber base using the instructions below.

Insert the gasket slide into the chamber base

- 1 Hold the gasket slide so that the barcode label is facing towards you. This side of the slide is the gasket side.
- 2 Locate the four chamber base guideposts and rectangular barcode guide in the chamber base.
- 3 Position the gasket slide between the 4 chamber base guide posts with the barcode label resting over the base's rectangular barcode guide.

Figure 3 shows the proper positioning using a 4-well gasket slide. Note, however, that your gasket slide will contain 8 wells rather than 4.

Procedures

Step 3. Prepare the hybridization assembly

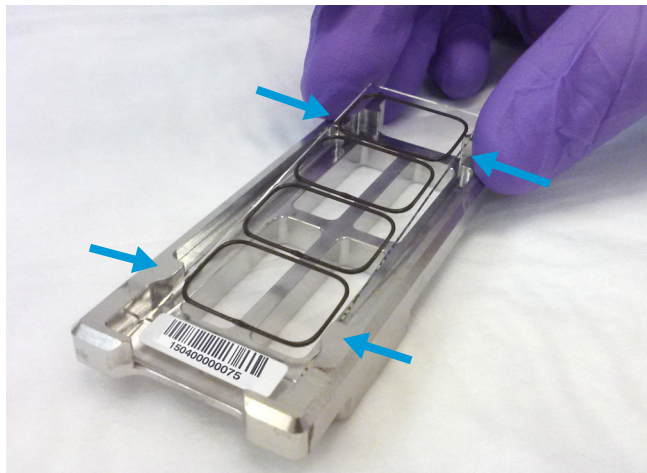


Figure 3. Chamber base, guide posts denoted with arrows

- 4 Gently place the gasket slide into the chamber base.
- 5 Make sure the gasket slide rests flush against the chamber base. Re-adjust to a flush position against the chamber base if needed.



Slide and gasket are flush

Figure 4. Correct positioning of gasket slide in chamber base

Load the sample

- 1 Slowly dispense the entire volume of a hybridization sample mixture onto a gasket well in a “drag and dispense” manner (described below). Load all 8 gasket wells before you add the microarray slide.
 - Load the samples left to right, starting with the first row. The output files will come out in that same order. Refer to **“Array/Sample tracking microarray slides”** on page 74 for guidelines on tracking sample position for multipack slide formats.

Procedures

Step 3. Prepare the hybridization assembly

The “drag and dispense” method helps to distribute the sample evenly across the surface of the well and avoids spillover of sample over the gasket edge. Start with the pipette tip near the top edge of the well. *Do not directly touch the gasket or the glass with the pipette tip.* Then, dispense the mixture while you move your pipette tip to the opposite end of the well so that the sample is distributed across the well space. Avoid creating large air bubbles as you dispense the mixture as they could lead to spillover.



Figure 5. Drag and dispense method – Start dispensing when the pipette tip is near the top of the well. Finish dispensing when the pipette tip is near the bottom of the well.

2 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.

Add the microarray slide

- 1 Remove a microarray slide from the slide storage box between your thumb and index finger, *numeric barcode side facing up and Agilent label facing down*.
- 2 Use the four chamber base guideposts and rectangular end of the base to position the microarray slide as you lower it to within 3 mm (1/8”) above the gasket slide, making sure the microarray slide is not tilted with respect to the gasket slide. Barcode ends of both the gasket slide and the microarray slide must line up at the corners of the chamber base. Once positioned, gently rest the microarray slide on the lower gasket slide.

Step 3. Prepare the hybridization assembly

Refer to **Figure 6** for proper technique on holding the microarray slide with both hands. Although the image uses a 4-well gasket slide and 4-pack microarray slide, the technique is the same for an 8-well gasket slide and 8-pack microarray slide.

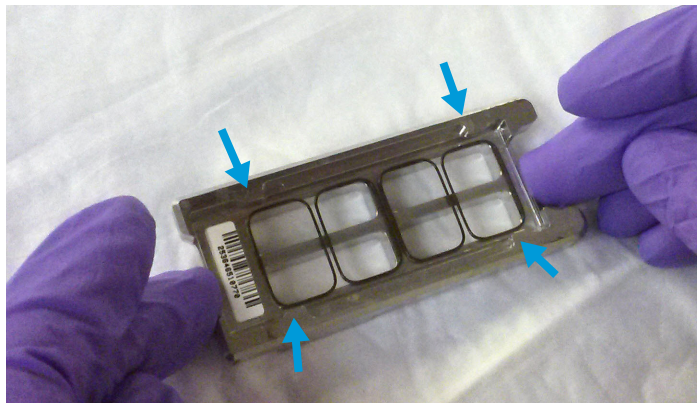


Figure 6. Chamber base with gasket and microarray slide applied, guide posts denoted with arrows

CAUTION

Do not drop the microarray slide onto the gasket slide as this increases the chances of sample mixing between gasket wells.

Once placed, do not attempt to move the chamber and sandwiched slides as this can cause leakage of the hybridization solution.

Assemble the chamber

- 1 Place the chamber cover, correct side facing up, onto the chamber base which contains the “sandwiched” slides.

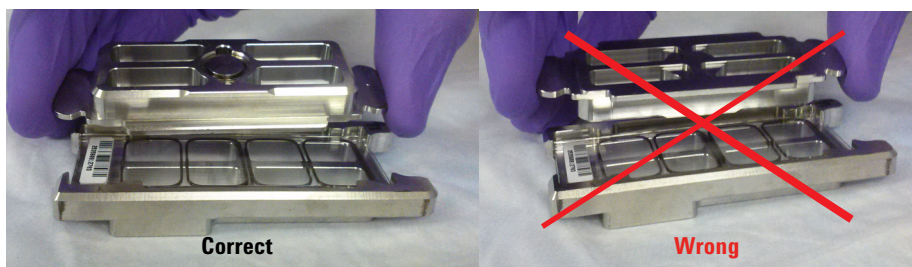


Figure 7. Chamber cover in correct (left) and incorrect (right) orientations

Procedures

Step 3. Prepare the hybridization assembly

- 2 From the rounded corner of the chamber base, slip the clamp onto the chamber base and cover until it stops firmly in place, resting at the center of the two pieces.

Keep the chamber assembly flat on the lab bench to avoid spilling the hybridization solution.

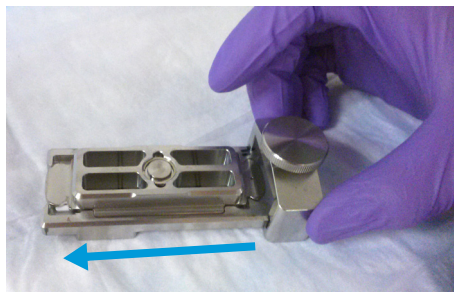


Figure 8. Slipping the clamp onto the chamber base

- 3 Firmly tighten the thumbscrew fully.
The slides will not be harmed by hand-tightening.

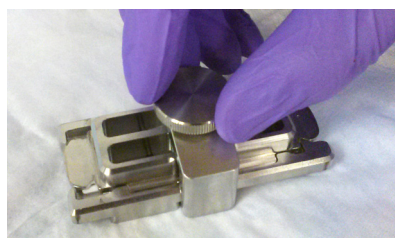


Figure 9. Tightening of the thumbscrew on the clamp

CAUTION

If you do not completely tighten the thumbscrew, hybridization solution can leak out during hybridization.

Do not use tools to tighten the thumbscrew. The use of pliers or other tools can damage the parts and will void the warranty.

- 4 Rotate the final assembled chamber in a *vertical orientation*, clockwise, 2 to 3 times to wet the gaskets (see **Figure 10**).

Rotation helps ensure that the hybridization solution will coat the entire surface of the microarray during the incubation process.

Procedures

Step 3. Prepare the hybridization assembly

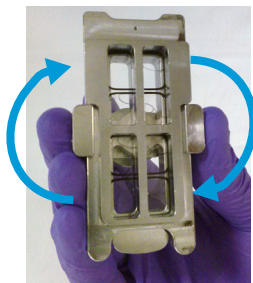


Figure 10. Rotation of the final assembled chamber

5 Inspect for good bubble formation.

- Hold the chamber vertically and inspect for stray or small bubbles that do not move as you rotate the chamber.
- Use the “large mixing bubble” to dislodge small stray or stationary bubbles.
- If the small stray or stationary bubbles persist, gently tap the assembled chamber on a firm surface. Rotate the chamber on its sides as you tap. Inspect again and repeat if needed until the small stray or stationary bubbles dissipate.

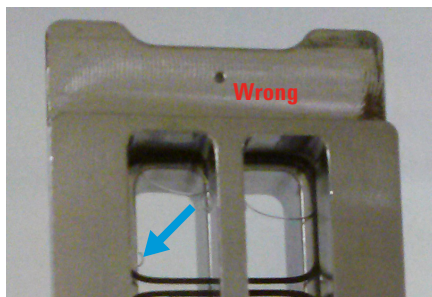


Figure 11. The slide on the left shows a stray, stationary bubble (denoted with arrow), which must be removed before hybridization. The slide on the right shows only large mixing bubbles, which move freely around the chamber when rotated. Bubbles are acceptable, as long as they move freely when you rotate the chamber.

Step 4. Hybridize

- 1 Load the assembled chamber into the oven rotator rack, starting from the center of the rack (position 3 or 4 when counting from left to right). Refer to the figure below for correct and incorrect orientations.

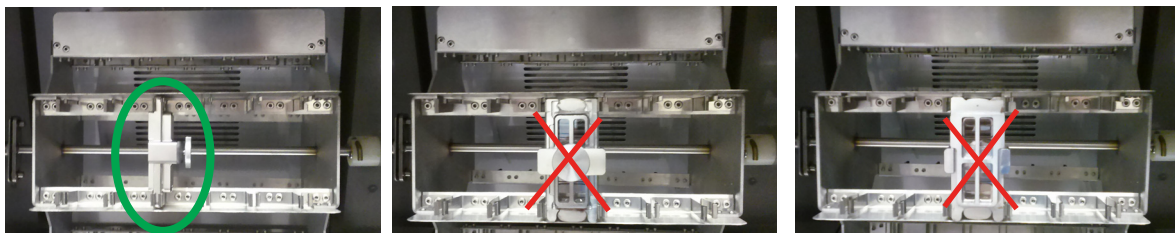


Figure 12. Assembled chambers in correct (left) and incorrect (middle and right) orientations

- 2 Close the door and set the rotator speed to 20 rpm.
- 3 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.

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 40) 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.

Step 4. Wash the microarray slides

- 6 Discard the solvent as is appropriate for your site.
- 7 Repeat **step 1** through **step 6**.
- 8 Air dry the staining dish in the vented fume hood.
- 9 Proceed to **“Milli-Q water wash”**.

Milli-Q water wash

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

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

CAUTION

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

Step 4. Wash the microarray slides**NOTE**

The microarray wash procedure 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.

Procedures
Step 4. Wash the microarray slides

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

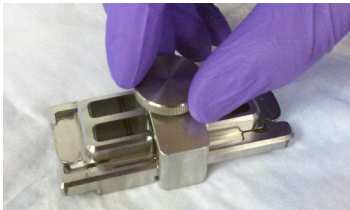


Figure 13. Loosening of the thumbscrew

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

Procedures

Step 4. Wash the microarray slides

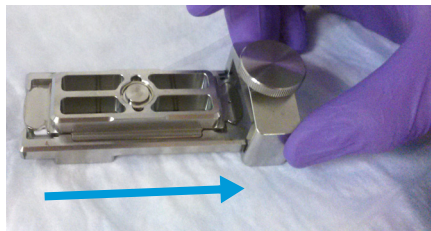


Figure 14. Removal of the clamp

- 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!*

Procedures

Step 4. Wash the microarray slides

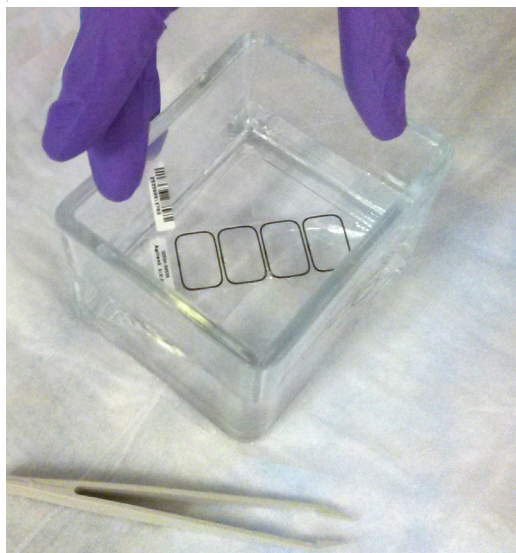


Figure 15. Removal of the microarray slide from the staining dish

- 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.

- 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.

Step 5. Put slides in a slide holder

For SureScan microarray scanner

- 1 Before you insert the slide into a slide holder, inspect the slide holder for any dust or fingerprints. If found, remove the dust or fingerprints with compressed air or a soft, dust-free cloth. If the slide holder is scratched, worn, or damaged, has a lid that does not close tightly, or has a hinge that does not move freely, discard the slide holder and select a different one.
- 2 Place the slide holder on a flat surface, with the clear cover facing up, and the tab on the right. This helps to ensure that you have the slide aligned properly when you insert it into the slide holder.
- 3 Gently push in and pull up on the tabbed end of the clear plastic cover to open the slide holder.

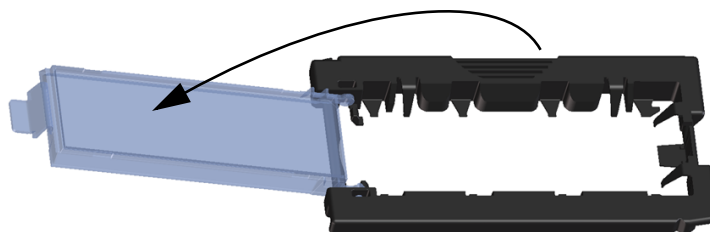


Figure 16. Opening the slide holder

- 4 Insert the slide into the holder.
 - a Hold the slide at the barcode end and position the slide over the open slide holder. Make sure that the active microarray surface faces up with the barcode on the left, as shown in **Figure 17**.
 - b Carefully place the end of the slide without the barcode label onto the slide ledge. See **Figure 17**.

Step 5. Put slides in a slide holder

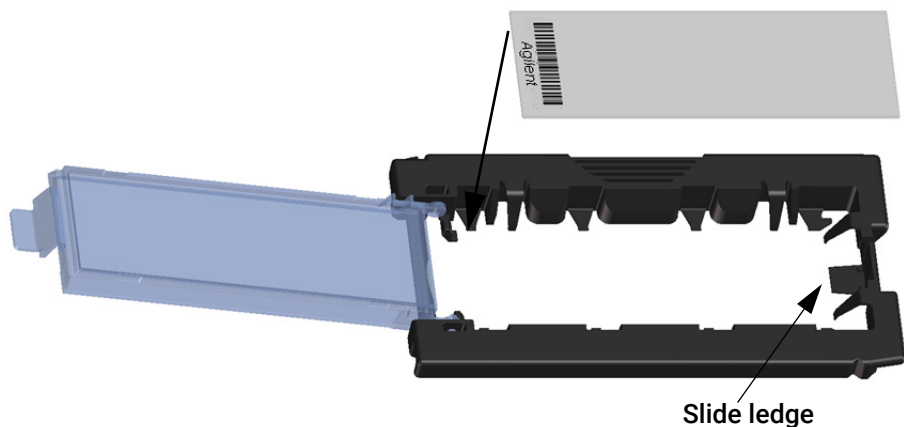


Figure 17. Inserting slide into the slide holder

- c** Gently lower the slide into the slide holder. See **Figure 18**.
- d** Close the plastic slide cover, pushing on the tab end until you hear it “click.” This moves the slide into position in the holder.

CAUTION

An improperly inserted slide can damage the scanner.

- e** Gently push in and pull up on the tabbed end of the clear plastic cover to open it again and verify that the slide is correctly positioned.
- f** Close the plastic slide cover, gently pushing on the tab end until you hear it “click”. See **Figure 19**. Make sure that the slide holder is completely closed.

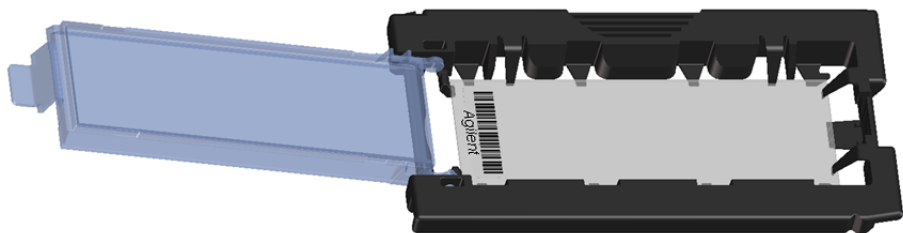


Figure 18. Slide inserted in slide holder – cover open

Procedures

Step 5. Put slides in a slide holder

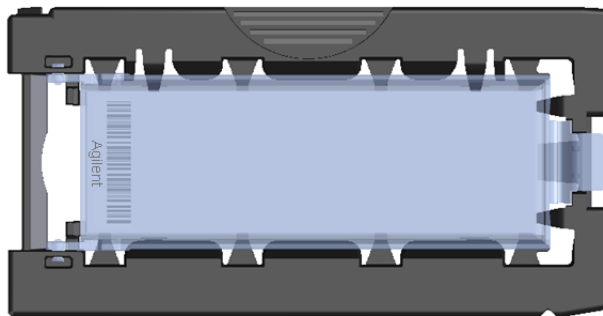


Figure 19. Slide inserted in slide holder – cover closed

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 20**. Refer to the *Agilent Ozone-Barrier Slide Cover User Guide* (publication G2505-90550), included with the slide cover, for more information.

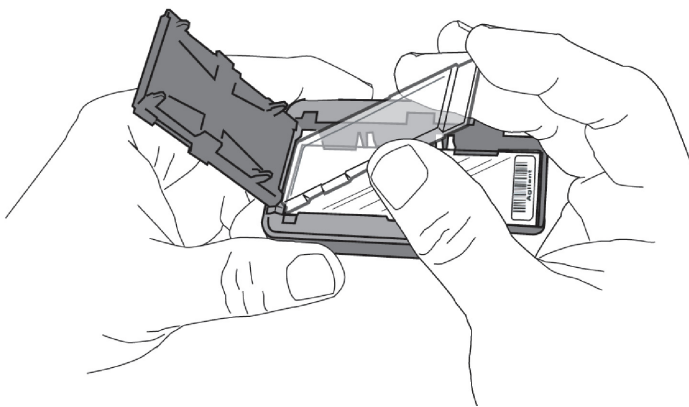


Figure 20. Inserting the ozone-barrier slide cover (shown for Scanner C)

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

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 (www.agilent.com/cs/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.

Step 1. Scan the slides

To get scanner profiles from Agilent:

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

Agilent SureScan Microarray Scanner

- 1 Put assembled slide holders into the scanner cassette. Refer to **Figure 21** and **Figure 22**.

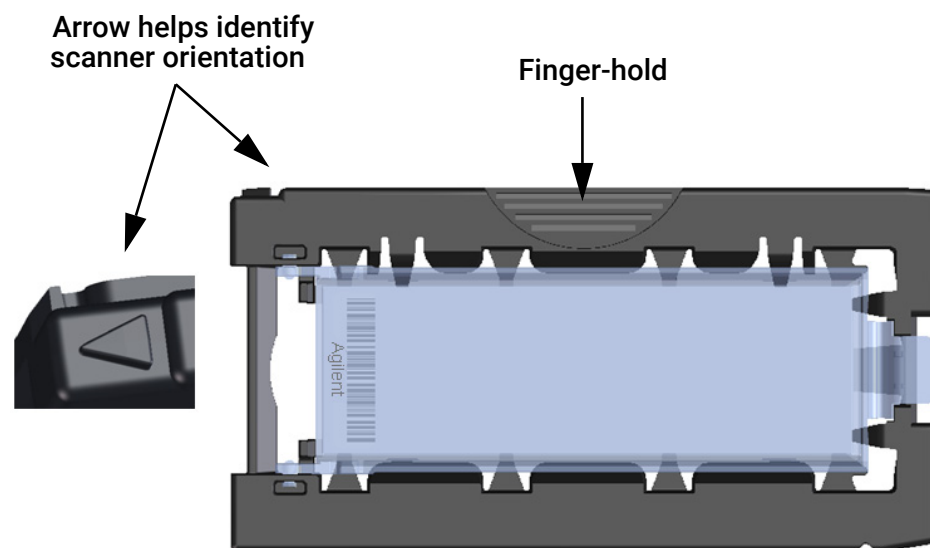


Figure 21. Slide holder helps you to insert slides correctly

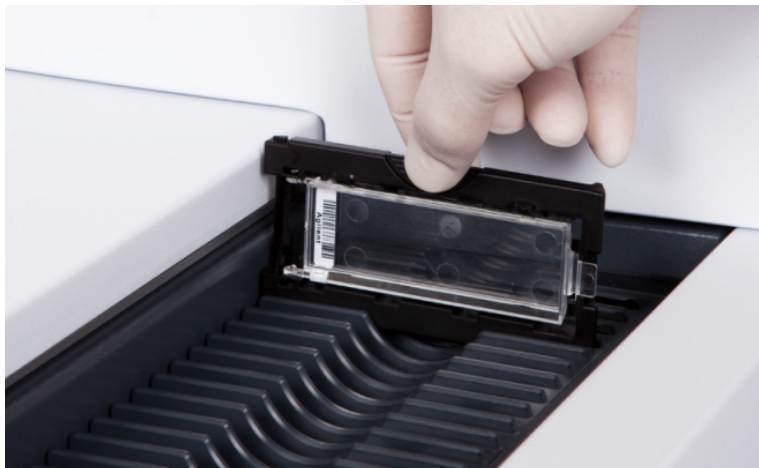


Figure 22. Inserting slide holder into cassette

- 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](#).

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 (green)	G (green)
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.

Step 2. Extract data using Agilent Feature Extraction Software

Feature Extraction (FE) 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.genomics.agilent.com/article.jsp?pagelId=2058.

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 54 for configuration. Refer to **Table 18** on page 46 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.

Step 2. Extract data using Agilent Feature Extraction Software

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 58.

- 1 Open the Agilent Feature Extraction (FE) program.

To get the most recent Feature Extraction protocols for gene expression, go to www.genomics.agilent.com/article.jsp?pagelId=2058.

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

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

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

- For auto assignment of the grid template, the image must be generated from a Agilent scanner and have an Agilent barcode.
- For auto assignment of the -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 23** 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 23** on page 51.

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.genomics.agilent.com/article.jsp?pagelId=2058. After downloading, you must add the QC Metric Set to the QC Metric Set Browser.

Step 2. Extract data using Agilent Feature Extraction Software

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.

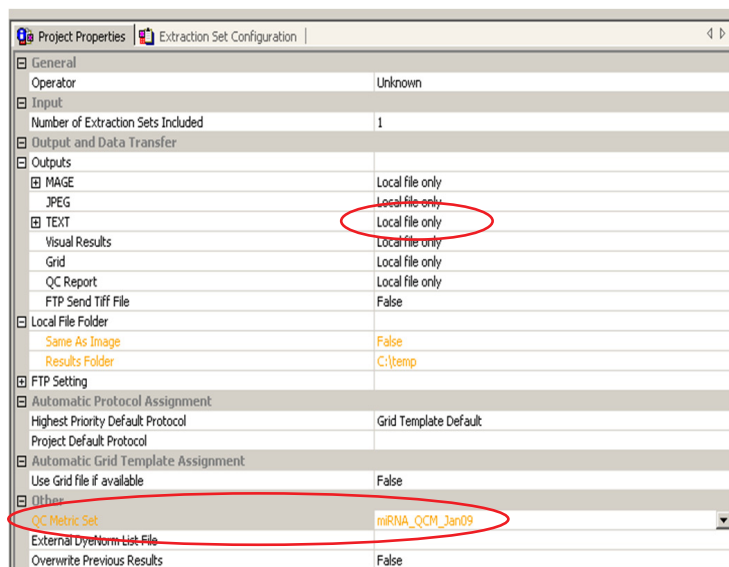


Figure 23. Feature Extraction version 9.5 dialog box

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.genomics.agilent.com/article.jsp?pagelId=2058 to download the latest protocols.

NOTE

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

NOTE

When the Agilent XDR scanned images are added to Feature Extraction software version 9.5 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**.

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 25**.

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.

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.

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 24**.

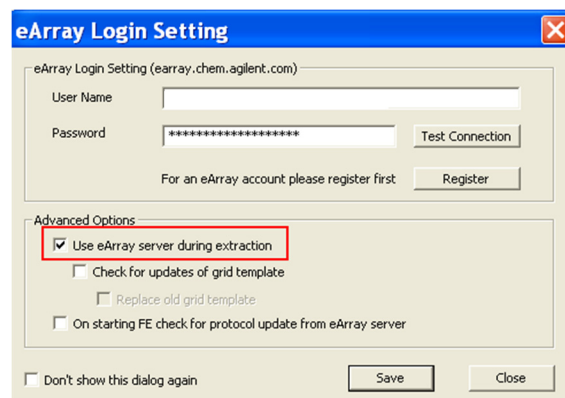


Figure 24. eArray Login Setting

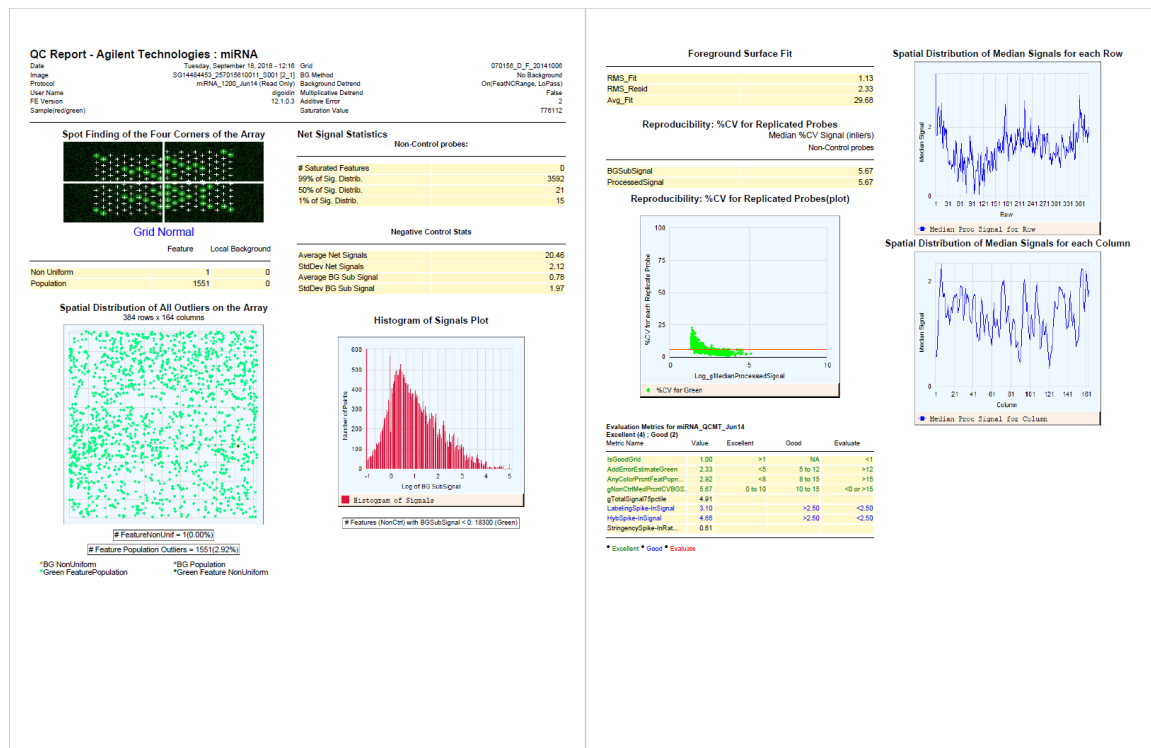


Figure 25. Example of a QC Report for an Agilent miRNA microarray, generated by Feature Extraction version 12.1 with the use of Spike-In miRNAs.



3

Supplemental Procedures

Normalizing Agilent One-Color Microarray Data 58

Characterization of Total RNA 60

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 11.5 or later.

Note that the default normalization scheme for data in the GeneSpring GX 11.5 (or later) program is 90th percentile scaling.

For more information on the GeneSpring GX program, go to 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 Feature Extraction Software Reference Guide*.

Characterization of Total RNA

Although this step is optional in the miRNA microarray workflow, 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.

Step 1. Prepare for characterization of total RNA

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.

Step 1. Prepare for characterization of total RNA

- Refer to **Table 20** and **Table 21** to make sure that you have the appropriate analyzer, kits, and compatible assays.

Table 20 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 21 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 26**. The presence of the 5S RNA peak, as shown in **Figure 26**, depends on the purification method, and generally shows lower abundance in column purified total RNA. **Figure 26** 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.

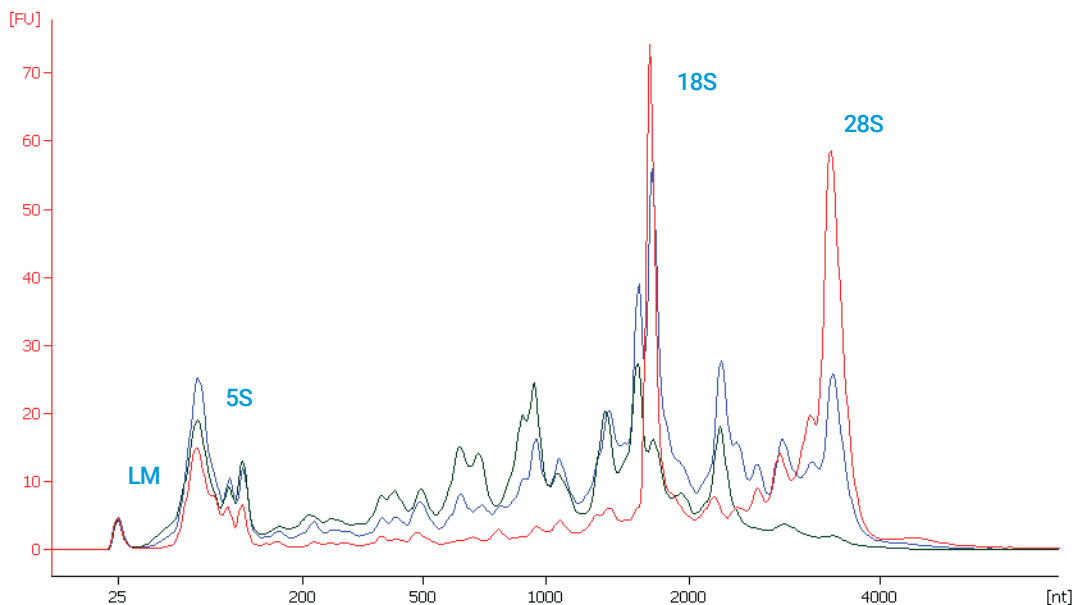


Figure 26. Analysis of (human) total RNA with the Eukaryote total RNA Nano assay using three different samples with decreasing integrity: Red, RIN 8.4; Blue, RIN 5.9; Green, RIN 3.6. Characteristic regions for ribosomal peaks and the lower marker (LM) are displayed.

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 27 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.

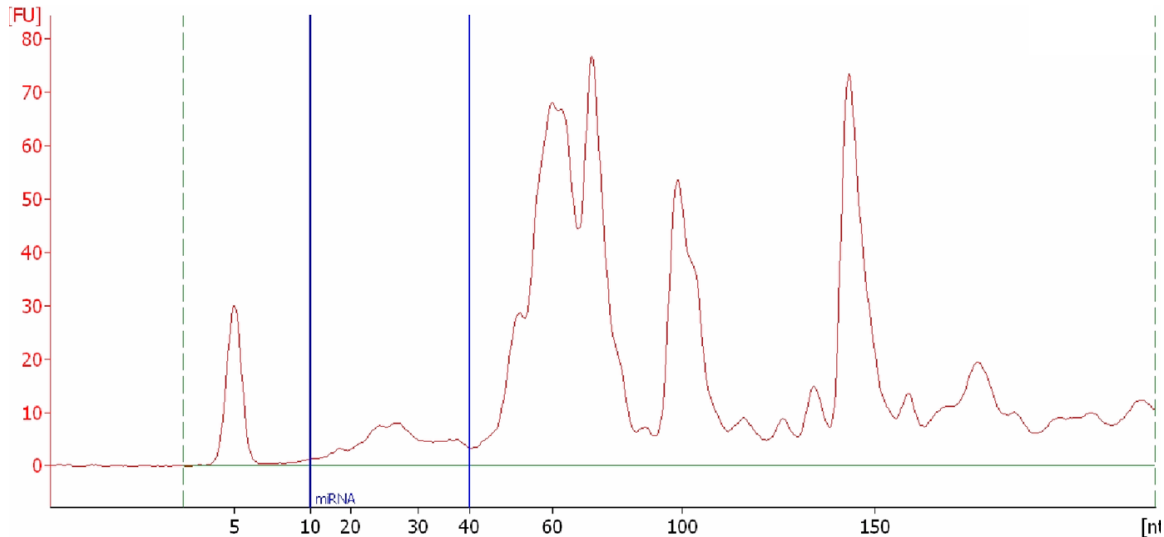


Figure 27. Characterization of small RNA within a total RNA sample. miRNA is typically found in the 10 to 40 nt region, which is a subset of the small RNA that range from 10 to 150 nt

4

Reference

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

Kit Contents

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

microRNA Spike-In Kit

Dilution Buffer
Labeling Spike-In
Hyb Spike-In

miRNA Complete Labeling and Hyb Kit

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

Gene Expression Wash Buffer Kit

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 platform workflow.

- 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

“Secure Fit” Slide Box Opening Instructions

Agilent now ships all microarray slides in a newly designed “secure fit” slide box. The instructions below describe how to remove the slide box from the shipping pouch, how to open the slide box, and how to properly remove a microarray slide.

- 1 Use scissors to cut below the seal and remove box from its foil pouch.

After breaking foil on microarray pouch, store microarray slides in the slide box at room temperature (in the dark) under a vacuum desiccator or nitrogen purge box.

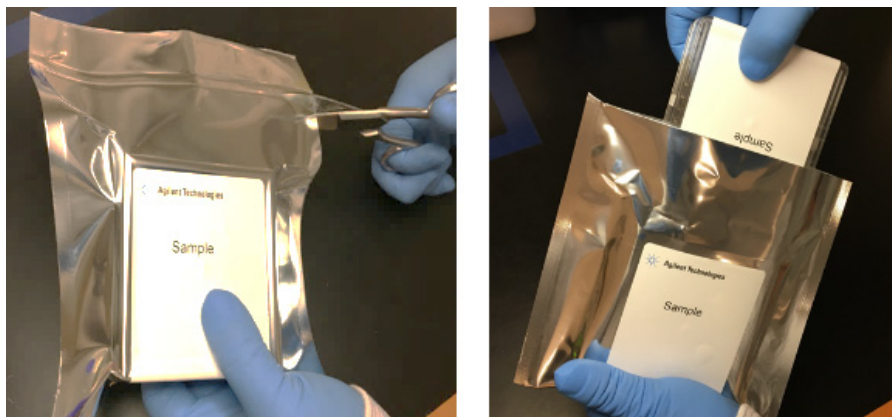


Figure 28. Opening foil pouch (left) and removing the slide box (right)

- 2 Place the slide box on a flat surface. While stabilizing the box from the top with one hand, use a sharp edge to cut the sealing tape on both sides of the box before opening.

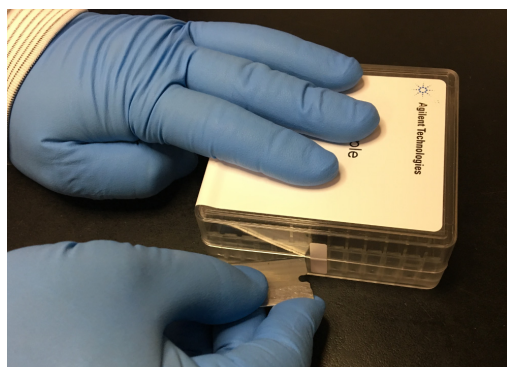


Figure 29. Cutting the sealing tape

Reference**“Secure Fit” Slide Box Opening Instructions**

- 3 With one hand, firmly hold the base of the box on the sides with the indentations (or dimples) for added grip.

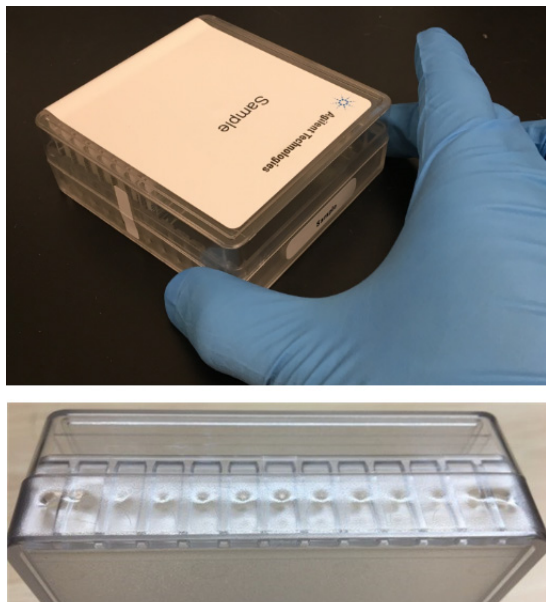


Figure 30. Gripping the base at the indentations (top) and close-up of the indentations (bottom)

- 4 Using your free hand, grasp the lid and gently lift it away from the base as if it is hinged. Set the lid aside.

Depending on your preference, you can reverse the hand positions so that the left hand holds the base while the right hand grasps the lid.

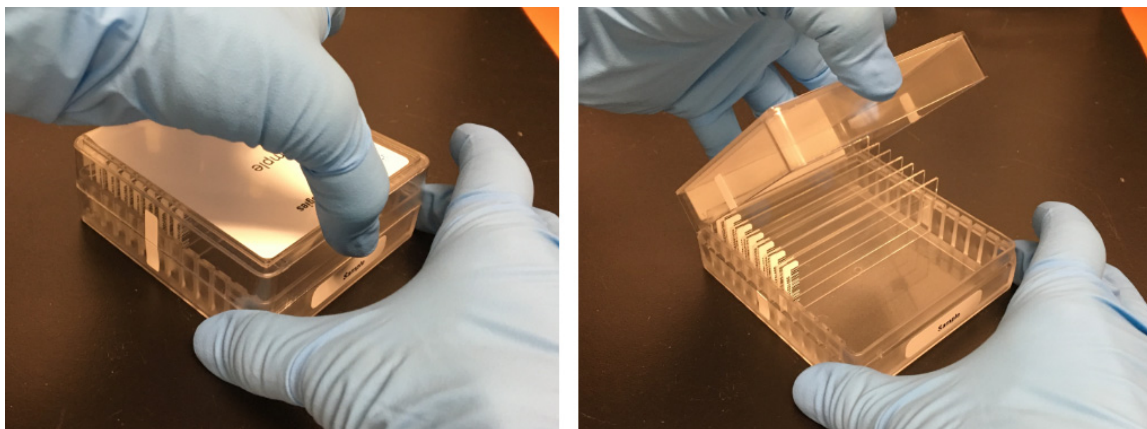


Figure 31. Grasping the lid (left) and lifting the lid from the base (right)

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.

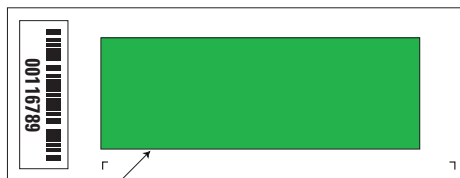
To avoid damaging the microarray, always handle glass slides carefully by their edges. Wear powder-free gloves. Never touch the surfaces of the slides. If you do, you may cause irreparable damage to the microarray.

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

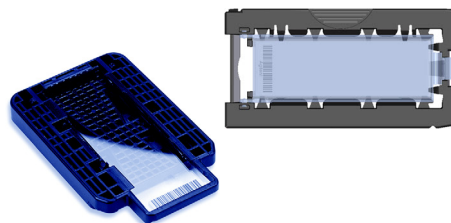
General Microarray Layout and Orientation

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 32. 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 32 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 33**).

This changes the feature numbering and location as it relates to the “microarray design files” For more information, go to

www.agilent.com/cs/library/technicaloverviews/public/G4502-90002_G3MicroarrayMap.pdf 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

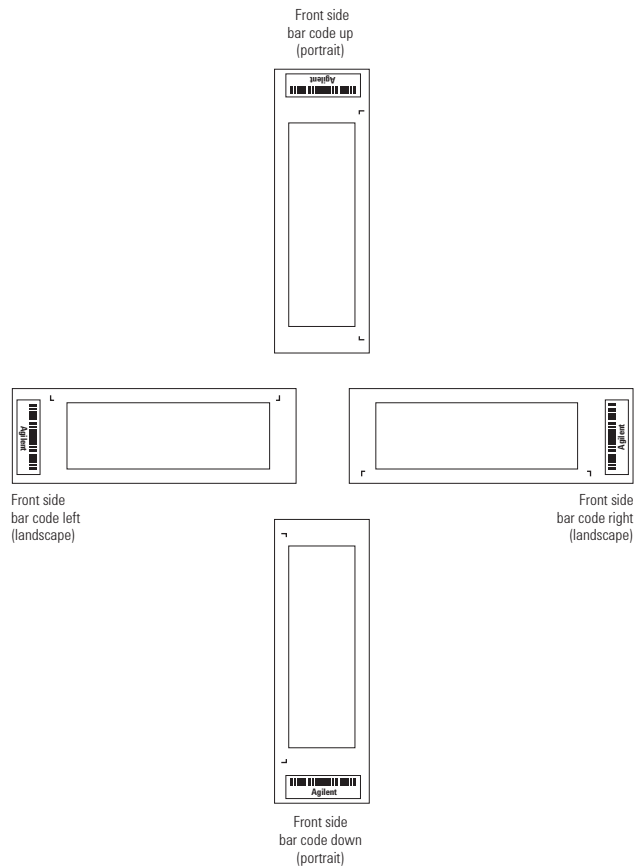


Figure 33. Microarray slide orientation

Array/Sample tracking microarray slides

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

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

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
B A R C O D E	Sample:	Sample:	Sample:	Sample:
	Sample:	Sample:	Sample:	Sample:
	Array 2_1	Array 2_2	Array 2_3	Array 2_4
Barcode Number _____				

Figure 34. 8-pack microarray slide

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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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