

miRCURY LNA™ microRNA Array kit

v.11.0 - human, mouse & rat

Instruction manual

for product # 208200-A, 208201-A, 208202-A
December 2009

Literature citations:

Please refer to miRCURY LNA™ microRNA Array when describing a procedure for publication using this product.

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GenePix® Array List (GAL) files can be found at www.exiqon.com/arrays.



Product summary

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miRCURY LNA™ microRNA Array content

Microarray slides

miRCURY LNA™ microRNA Arrays consist of control probes, mismatch probes, and 1769 capture probes, complementary to human, mouse, rat, and their related viral sequences from the v.11.0 release of miRBase. Arrays also contain a number of proprietary human miRPlus™ sequences not yet in miRBase.

Hybridization buffer (product # 208022)

Products with 3, 6 and 24 slides: 1 bottle x 5 mL

20x Salt buffer (product # 208023)

Products with 3 and 6 slides: 1 bottle x 125 mL

Products with 24 slides: 2 bottles x 125 mL

10% Detergent solution (product # 208024)

Products with 3 and 6 slides: 1 bottle x 15 mL

Products with 24 slides: 2 bottles x 15 mL

Spike-in microRNA kit (product# 208040)

10 synthetic unlabeled microRNAs, dried-down, 2x 24 reactions

Additional required material

miRCURY LNA™ microRNA Power Labeling Kit

Fluorescent labeling of microRNAs from total RNA samples ready for hybridization on arrays (product # 208030-A, 208031-A, 208032-A).

For manual hybridization

Microarray Hybridization Chamber - SureHyb (Agilent product# G2534A)

Hybridization Gasket Slide Kit (Agilent product# G2534-60003)

Hybridization oven with rotation.

Glass staining jar/dish or equivalent.



Product description

T_m -normalized capture probes

The miRCURY LNA™ microRNA Array slides contain capture probes complementary to mature microRNAs registered in miRBase. The capture probes are Locked Nucleic Acid (LNA™) enhanced oligonucleotides. By varying the LNA™ content and length, the capture probes are T_m -normalized to hybridise optimally under the conditions described in this protocol.

Coverage of probe set

The slides contains capture probes for all microRNAs in human, mouse, rat and their related viruses as annotated in miRBase Release 11.0. Please go to www.exiqon.com/array to see the coverage in respect to latest version of miRBase. In addition, a number of capture probes are available for detection of microRNAs not included in miRBase. These miRPlus™ probes give researchers access to information unavailable elsewhere.

Please go to our online microRNA resource at www.exiqon.com/miRCURY/array to:

- Download species-specific GenePix® Array Lists (GAL) files, consistent with the latest updates to miRBase. Please note the lot# on the array slide box and on the slide pouch. This number is needed to identify the GAL file.

Control capture probes

Fourtythree control capture probes are included in the probe set.

- Ten spike-in control probes to ensure optimal labeling and hybridization.
- Seven negative control capture probes.
- Twentysix capture probes complementary to small nuclear RNAs.

Please see table on next page for details.



Control probes

Probe ID	Positive controls	Validated positive control in these organisms
11278	U6-snrRNA-1	hsa, rno, mmu
11279	U6-sn-RNA-2	hsa, rno, mmu
19005	hsa_SNORD118	hsa
19603	hsa_SNORD13	hsa
19007	hsa_SNORD3@	hsa
19008	hsa_SNORD2	hsa
19604	hsa_SNORD4A	hsa
19605	hsa_SNORD6	hsa
19011	hsa_SNORD10	hsa
19606	hsa_SNORD12	hsa
19013	hsa_SNORD14B	hsa
19607	hsa_SNORD15A	hsa
46196	SNORD38B-3	hsa
46204	SNORD38B-5	hsa
46201	SNORD44-3	hsa
46206	SNORD44-5	hsa
46200	SNORD48-3	hsa
46205	SNORD48-5	hsa
46199	SNORD49A-3	hsa
46203	SNORD49A-5	hsa
46198	SNORD66-3	hsa
46197	SNORD66-5	hsa
Probe ID	Negative controls	Validated negative control in these organisms
14258	hsa_negative_control-1	hsa, mmu, rno
14259	hsa_negative_control-2	hsa
14260	hsa_negative_control-3	hsa, mmu, rno
14266	hsa_negative_control-4	hsa, mmu, rno
10901	hsa_negative_control-6	hsa, mmu, rno
10902	hsa_negative_control-7	hsa, mmu, rno
10903	hsa_negative_control-8	hsa, mmu, rno



Table content continues on next page



Probe ID	Spike-in controls	Validated spike-in microRNA control in these organisms
14261	spike_control_a	hsa, mmu
14263	spike_control_b	hsa, mmu
14264	spike_control_c	hsa, mmu, rno
10904	spike_control_d	hsa, mmu,
10906	spike_control_e	hsa, mmu, rno
14262	spike_control_f	hsa, mmu, rno
10905	spike_control_g	hsa, mmu, rno
10907	spike_control_h	hsa, mmu, rno
14257	spike_control_i	hsa, mmu,
10899	spike_control_j	hsa, mmu, rno

The different control capture probes were compared against the genomic sequence of hsa, mmu and rno, with the BLAST tools at www.ensembl.org.

Positive control probes with 100% match to genomic target is in this table. Negative control capture probes with less than 100% match to genomic target is in this table. Spike-in microRNA control capture probes with less than 100% match to genomic target is in this table.

Note

In the GAL-file, only capture probes relevant to the species in question are annotated with a name. Probes that do not have a name could be designed for another species, internal controls or obsolete probes no longer in use. Some of these may show signal although they are not annotated, but they should be ignored in the analysis.

Some capture probes have been optimized from previous versions of the miRCURY LNA™ Array. These will appear with a new probe ID on this array compared to earlier versions. For more details about comparisons to older versions of the arrays, please contact support@exiqon.com.

Spike-in microRNA controls

The miRCURY LNA™ microRNA Array Spike-in kit contains 10 different synthetic unlabeled microRNAs in different concentrations. The set can be spiked into an RNA sample prior to labeling and the synthetic Spike-in microRNA kit will hybridize to corresponding capture probes on the miRCURY LNA™ microRNA

See tip
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Array. The Spike-in microRNA kit has been designed and tested not to cross-react with endogenous microRNAs from human, mouse or rat, and is provided at concentrations compatible with endogenous microRNA expression levels. The Spike-in microRNA kit is supplied with different concentrations of synthetic spike-in microRNAs aimed at spanning the whole intensity range of microRNAs in most tissue samples. The corresponding capture probes have been printed once in every subgrid, thus 48 times each.

Note

.....
Please refer to the Instruction Manual for miRCURY LNA™ microRNA Power Labeling Kits, for further instructions on how to use the Spike-in microRNA kit during the labeling procedure.

When the spike-in microRNAs are added in equal amounts to labeling reactions before a dual-color array hybridization, the signals from the spike-in capture probes can be used:

- As a control of the labeling reaction and hybridization
- As a help in deciding scanner settings between channels
- As a control of the data normalization procedure
- To estimate the variance of replicated measurements within arrays
- To assess technical variability between different parts of the array

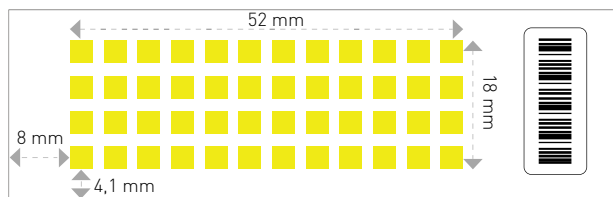
miRCURY LNA™ microRNA Array layout

The array is located on a standard size slide as illustrated in the drawing below (25.4 mm by 76.2 mm or 1 in by 3 in). The array carries the following specifications:

- Outer dimensions: 18 mm wide by 52 mm long
- Coordinates of first spot on slide = 4,1 mm, 8 mm
- 12 sub-arrays in 4 replicates
- Spot size: 90 µm
- Distance between spots: 250 µm



Figure 1



To assist in orientation of the array and positioning of the image analysis grid, Hy3™ fluorescent labeled “landing lights” are present in all 4 corners plus one extra in lower right corner of the 48 sub-arrays, 240 total. The slides are compatible with all major brands of microarray scanners, that take glass slides of the above described specifications (1 in x 3 in, not Affymetrix and Illumina scanners).

Storage

miRCURY LNA™ microRNA Arrays should be stored desiccated at room temperature and protected from light. When properly stored, arrays will remain hybridization competent for at least 12 months. The arrays are shipped in desiccated, re-sealable storage pouches that are ideal for this purpose. Both the storage pouches and slide storage boxes are manufactured from materials that minimize outgassing and effectively block exposure to foreign contaminants that can elevate background levels. When accessing arrays, remove only the arrays you plan to immediately use and return the remaining arrays to the pouch quickly, leaving the desiccant pack in place. If stored properly shelf life for the miRCURY LNA™ microRNA Array kit is 1 year.

Dissolve the miRCURY LNA™ Array spike-in microRNAs in 30 μ L of RNA free water (supplied) upon receipt. Vortex to thoroughly dissolve the lyophilized RNA, pulse briefly in a microfuge, and leave the suspension on ice for 30 min. to dissolve. Vortex and then spin to collect tube contents. Store the dissolved Spike-in microRNA at -20° C until use and avoid repeated cycles of freeze/thawing. You may wish to aliquot the dissolved spike-in microRNAs to avoid repeated freeze/thawing. For long-term storage, keep the vial at -80° C. If stored properly shelf life for the miRCURY LNA™ Array spike-in microRNAs is 1 year. In solution the shelf life for the spike-in microRNAs is 3 months.

Related products

Exiqon offers a range of tools designed for microRNA isolation, expression analysis, localization and functional analysis.

Figure 2



miRCURY™ RNA Isolation Kits

Get high quality total RNA suitable for miRCURY LNA™ microRNA Array analysis in as little as 20 minutes. Protocols are available for a large number of sample types and organisms.

miRCURY LNA™ microRNA Power Labeling Kit

For fluorescent labeling of microRNAs from total RNA samples ready for array hybridization (product # 208030-A, 208031-A, 208032-A).

miRCURY LNA™ microRNA Array, ready-to-spot probe set

Ready-to-spot oligo for direct printing of arrays, or coupling in bead-based applications (product # 208210-A).

miRCURY LNA™ microRNA Array, Spike-in microRNA kit

Ten different synthetic unlabeled microRNAs in different concentrations. The spike-in microRNA kit will hybridize to corresponding capture probes on the miRCURY LNA™ microRNA Array (product # 208040).



miRCURY LNA™ microRNA Array, Hybridization buffer

5 mL hybridization buffer optimal for microRNA hybridization to the miRCURY LNA™ microRNA Arrays (product # 208022).

miRCURY LNA™ microRNA Array, Wash buffer kit

125 mL salt buffer and 15 mL detergent optimal for wash of miRCURY LNA™ microRNA Arrays. (product # 208021).

miRCURY LNA™ microRNA Detection

For *in situ* hybridization and northern blotting of all annotated microRNAs.

miRCURY LNA™ microRNA Knockdown

microRNA Knockdown probes: Determine or confirm microRNA function.

miRCURY LNA™ microRNA Real-time PCR

Quickly and accurately determine microRNA expression levels using real-time PCR. Recommended for validation of miRCURY LNA™ microRNA Array data.



Protocol overview

miRCURY LNA™ microRNA Power Labeling Kit

CIP treatment

Mix: RNA sample, CIP buffer,
Spike-In microRNA and CIP enzyme



Labeling reaction

Mix: CIP treated RNA sample
Labeling buffer
Hy3™ or Hy5™
DMSO
Enzyme



miRCURY LNA™ microRNA Array Kit

Mix samples

Mix: Hy3™ labeled sample
Hy5™ labeled sample
Hybridization buffer
Denature sample



Hybridize

Hybridize at 56°C for 16 hours



Stringency wash

Wash 2 min. in buffer A at 56°C
Wash 2 min. in buffer B at 23°C
Wash 2 min. in buffer C at 23°C
Dry slides



Image acquisition

Scan slides (recommended scan at 10µm)
Download relevant GAL files from
www.exiqon.com



Protocol

Hybridization and washing using Tecan HS Pro™ hybridization stations

Before starting the experiment

Total RNA should be prepared using a method that preserves small RNA species. When using commercially available kits, please verify that the total RNA preparation contains small molecular weight RNAs. We recommend using the miRCURY™ RNA Isolation Kits for total RNA preparations. For labeling, we recommend that you use a miRCURY LNA™ microRNA Power Labeling Kit. Please visit www.exiqon.com to learn more about this product.

See tip
1

The amount of total RNA to be labeled for an array hybridization depends on the microRNA content of the cells or tissue being analyzed as this amount. Without prior knowledge of microRNA content in the sample, we would recommend to use between 250 ng and 1 µg of total RNA per labeling reaction.

Check the hybridization buffer for any precipitate. If necessary, warm the solution at 56° C and agitate to dissolve the precipitate completely.

Dissolve the Spike-in microRNA in 30 µl of RNase free water (supplied) upon receipt. Leave the suspension on ice for 30 minutes to dissolve. Vortex and then spin to collect tube contents. In order to avoid repeating freeze/thaw cycles we recommend to aliquote the dissolved spike-in microRNAs. Store the dissolved spike-in microRNA at -20° C until use.

Please refer to the instruction manual of your hybridization station for correct volume of buffers required to perform the hybridization.

The volumes in Table 1 applies to the hybridization of 4 slides in a Tecan HS400/HS4800 hybridization station.



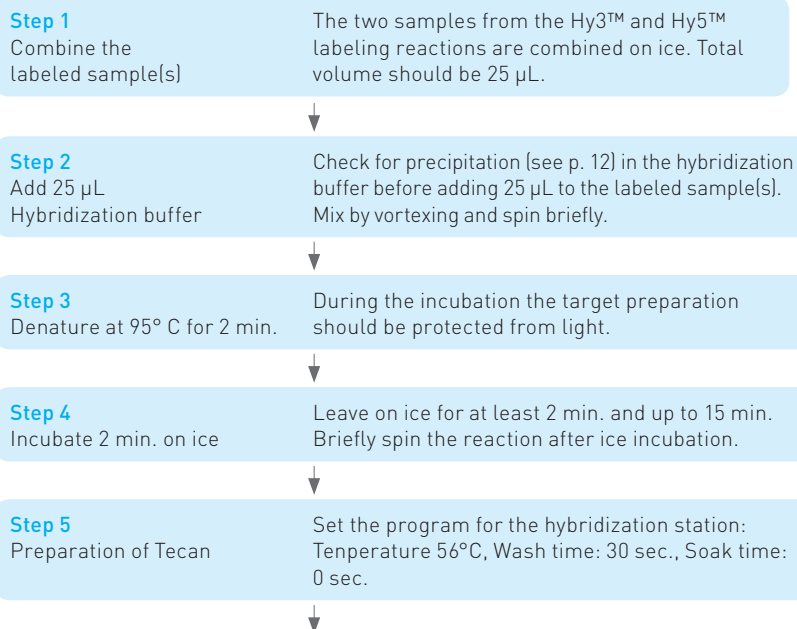
Table 1

Recipes for preparation of 200 mL Wash buffers

	Wash buffer A	Wash buffer B	Wash buffer C
20x Salt buffer	20 mL	10 mL	2 mL
10% Detergent solution	4 mL	-	
Nuclease-free water	176 mL	190 mL	198 mL

Protocol

Total handling time: 1 hour

See tip
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See tip
4**Step 6**

Flush hybrid chamber with
1x Hybridization buffer

The slide chamber in the hybridization station should be primed. Check the appropriate volume of the chamber in the suppliers manual and add 1x diluted Hybridization buffer. Dilute with water. (e.g. use 100 μ L for a Tecan HS400/HS4800).

Step 7

Inject reaction mixture

Inject the 50 μ L target preparation to the hybridization station. In order to flush injection inlet, it is recommended to inject 10 μ L 1x diluted Hybridization buffer after target injection.

Step 8

Incubate at 56° C
for 16 h.

Set the program for the hybridization station to 56° C and 16 h. incubation. Agitation should be set to medium, if possible.

Step 9

Two runs of wash at 56° C for
1 min. using Wash buffer A

Set the program for the hybridization station:
Temperature 56° C, Wash time: 1 min.,
Soak time: 1 min.

Step 10

Two runs of wash at 23° C for
1 min. using Wash buffer B

Set the program for the hybridization station:
Temperature 23° C, Wash time: 1 min.,
Soak time: 1 min.

Step 11

Two runs of wash at 23° C for
1 min. using Wash buffer C

Set the program for the hybridization station:
Temperature 23° C, Wash time: 1 min.,
Soak time: 1 min.

Step 12

Wash at 23° C for 30 sec.
using Wash buffer C

Set the program for the hybridization station:
Temperature 23° C, Wash time: 30 sec.,
Soak time: 0 sec.

Step 13

Dry slides

Set the program for the hybridization station:
Slide drying for 5 min.



Hybridization and washing using an Agilent hybridization SureHyb chamber kit and gasket slide kit

We recommend using an automatic hybridization station like the Tecan HS Pro hybridization stations for optimal quality (see procedure at page 12). If a hybridization station is not available manual hybridization can be carried out according to the protocol in this section using an Agilent hybridization SureHyb chamber kit and gasket slide kit. Please contact support@exiqon.com for an alternative protocol using cover slip.

We recommend that you use a miRCURY LNA™ microRNA Power labeling kit for labeling of your sample(s). Please visit www.exiqon.com to learn more about this product.

Additional required materials:

Hybridization Chamber Kit - SureHyb enabled, Agilent part # G2534A
Hybridization Gasket Slide Kit (5) - 1 microarray per slide format, Agilent part # G2534-60003

Hybridization oven with rotation (e.g. SciGene, # 400).

Ethanol 99%

Before starting the experiment, day 1

Total RNA should be prepared using a method that retains small RNA species. When using commercially available kits, please ensure that the total RNA preparation is guaranteed to contain microRNAs. The amount of total RNA to be labeled for an array hybridization depends on the microRNA content of the cells or tissue being analyzed as this amount is known to vary. Without prior knowledge of microRNA content in the sample to be analyzed we would recommend to use between 250 ng and 1 µg of total RNA per labeling reaction per slide hybridization.

We recommend that you use a miRCURY LNA™ microRNA Power labeling kit for labeling of your sample(s). Please visit www.exiqon.com to learn more about this product.

See tip
1



Check the hybridization buffer for any precipitate. If necessary, warm the solution at 56° C and agitate to dissolve the precipitate completely.

Before starting the experiment, day 2

Glass staining jar/dish and Wash buffer A should be placed at 56° C before starting the experiments at day 2.

If one or two miRCURY LNA™ microarrays are processed together in an experiment, the miRCURY LNA™ microarrays could be washed in a 50 mL screw-top tube (e.g. Falcon) by gently inverting the tube.

If three or more miRCURY LNA™ microarrays are processed in an experiment the miRCURY LNA™ microarrays could be placed in a slide rack and washed in a glass staining jar/dish. Use appropriate volume of washing buffer to cover the slides and shake gently. The volumes in Table 2 below are required for a large glass staining dish (8 slides, Sigma-Aldrich product # S-S6016 or similar). The following protocol is for hybridization of miRCURY LNA™ microRNA Arrays using a Agilent Hybridization chamber - SureHyb.

An instructional video on how to perform the hybridization using SureHyb Chambers can be found here:

www.exiqon.com/Is/Pages/man-hyb-high-res.htm

Table 2

The volumes in this table are required for glass staining jars of 200 mL.

Recipes for preparation of Wash buffers

	Wash buffer A	Wash buffer B	Wash buffer C
20x Salt buffer	60 mL	20 mL	2 mL
10% Detergent solution	12 mL	-	-
Nuclease-free water	528 mL	380 mL	198 mL



Protocol

Total handling time: 1 hour

Step 1
Prepare the labeled sample(s)

Combine the two samples from the Hy3™ and Hy5™ labeling reactions on ice and adjust the volume to 200 µL by adding nuclease free water to the labeled sample(s).

Step 2
Add 200 µL Hybridization buffer

If there is precipitation in the Hybridization buffer, then warm the solution at 56° C and agitate to dissolve. Add 200 µL to the labeled sample(s). Mix by vortexing and spin briefly.

Step 3
Denature at 95° C for 2 min.

During the incubation the target preparation should be protected from light.

Step 4
Incubate 2 min. on ice

Leave on ice for at least 2 min. and up to 15 min. Briefly spin the reaction after ice incubation.

Step 5
Add 400 µL to reservoir

Add 400 µL of the target sample mixture to the reservoir of backing gasket slides. Place the slide on top of the the backing gasket slides with the array side facing the target samples.

Step 6
Incubate at 56° C for 16 h.

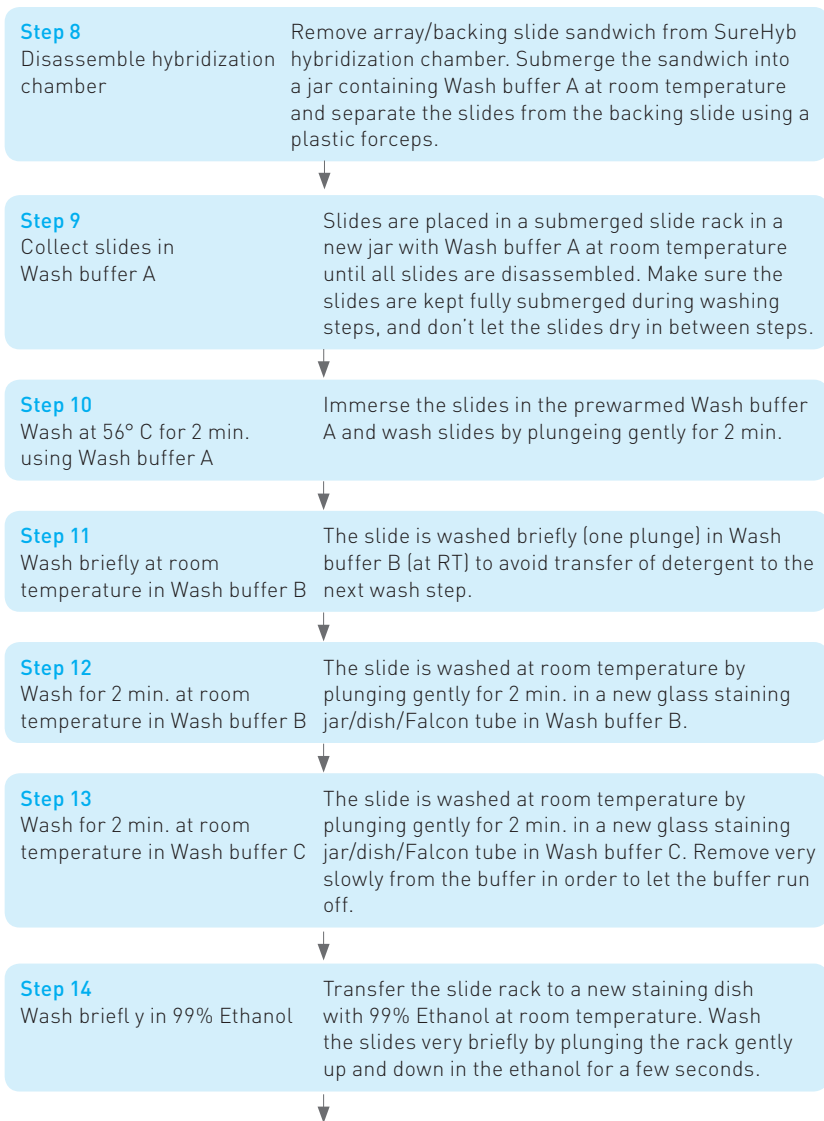
Clamp the array/backing slide sandwich into the SureHyb hybridization chambers and make sure all bubbles move freely. Incubate at 56° C for 16 h. in a hybridization oven with rotation (e.g. SciGene, #400).

Step 7
Place Wash buffer A at 56° C overnight

Pre-warm the glass staining jar/dish and Wash buffer A by placing them at 56° C.

See tip
3





Step 15

Dry the slide(s)

Dry the slides by centrifugation for 5 minutes at 1000 rpm. At this stage the fluorophores on the slides are very susceptible to degradation by ozone in atmospheric air. Keep ozone-induced photo bleaching to a minimum by working in an ozone free lab or keeping the slides under a controlled atmosphere. Scan slides immediately after drying.

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Hybridization and washing using MAUI® 4-Bay or 12-Bay hybridization stations.

This Protocol provides information for the use of Exiqon miRCURY LNA™ Arrays with the MAUI® Hybridization System using a MAUI® SC Mixer.

The MAUI® Hybridization System is comprised of two main components, the disposable MAUI® Mixer hybridization chambers and MAUI® instrument that powers the mixing bladders in the Mixer and maintains a constant incubation temperature. The MAUI® Mixer adheres to the microarray slide via an adhesive gasket forming a uniform, low volume, sealed hybridization chamber. Once attached, the Mixer-slide is clamped into one of the heated slide bays in the base unit, where hybridization takes place. For details about using the MAUI® Hybridization System please see the User's Guide that come with the MAUI® Hybridization System or is available from BioMicro Systems. This Protocol provides detailed information about performing hybridization of labeled RNA samples on Exiqon miRCURY LNA™ Arrays using the MAUI® Hybridization System. For details about preparing the samples and performing the RNA labeling reactions please see the Instruction Manual for the miRCURY LNA™ Power Labeling Kit available from Exiqon's website.

Additional required materials:

RNA samples (labeled with miRCURY LNA™ Power Labeling Kit)

MAUI® SC-mixers

MAUI® Humidity tray

MAUI® A/D jig

MAUI® Gasket brayer



Positive Displacement Pipette (optional)
 7 Rectangular Staining Dishes, 250 mL, w/slide washing racks
 (e.g. Wheaton # 900200 /VWR# 25461-003).
 Heating block set to 56° C.
 Oven set to 56° C.

Before starting the experiment, day 1

Total RNA should be prepared using a method that retains small RNA species. When using commercially available kits, please ensure that the total RNA preparation is guaranteed to contain microRNAs. The amount of total RNA to be labeled for an array hybridization depends on the microRNA content of the cells or tissue being analyzed as this amount is known to vary. Without prior knowledge of microRNA content in the sample to be analyzed we would recommend to use between 250 ng and 1 µg of total RNA per labeling reaction per slide hybridization.

See tip
1

We recommend that you use a miRCURY LNA™ microRNA Power Labeling Kit for labeling of your sample(s). Please visit www.exiqon.com to learn more about this product.

Check the hybridization buffer for any precipitate. If necessary, warm the solution at 56° C and agitate to dissolve the precipitate completely prepare the wash buffers and leave min. 500 mL Wash Buffer A in an oven at 56° C over night. Leave 2 Rectangular Staining Dishes in the oven at 56° C as well.

Table 2

The volumes in this table are required for glass staining jars of 200 mL.

Recipes for preparation of Wash buffers

	Wash buffer A	Wash buffer B	Wash buffer C
20x Salt buffer	60 mL	20 mL	2 mL
10% Detergent solution	12 mL	-	-
Nuclease-free water	528 mL	380 mL	198 mL



Protocol

Step 1

Attach the MAUI® SC-mixer to the miRCURY LNA™ Array

For details on how to assemble the mixer and the array slide please see the MAUI® User's Guide. Briefly:

- Remove the miRCURY LNA™ Array from the slide box and pre-heat it to 56° C by putting it on a heating block at 56° C for 5 min.
- Take out the SC-mixer from the packaging and remove the protective liner
- Insert the pre-heated array slide in the A/D jig with the barcode facing up and into the jig.
- Align the SC-mixer with the array slide in the A/D jig with the tab-end of the mixer facing away from the barcode on the slide. Carefully adhere the SC-mixer to the slide.
- Remove the mixer-slide assembly from the A/D jig and place the assembly with the mixer side up on the heating block at 56° C.
- Use the MAUI® Gasket brayer and moderate pressure to ensure good mixer to slide adhesion.
- Leave the mixer-slide assembly with the mixer side upon the heating block at 56° C. The sample must be loaded onto the 56° C-heated slide within 30 min of assembly.



Step 2

Prepare the labeled sample(s)

Combine the two samples from the Hy3™ and Hy5™ labeling reactions on ice. Each sample is 12.5 µL. Final volume 25 µL.



See tip
9**Step 3**

Add 25 μ L
2X Hybridization buffer

Add 25 μ L 2X Hybridization buffer to the labeled sample(s). Mix by vortexing and spin briefly. Final volume 50 μ L.

**Step 4**

Denature at 95° C for 2 min.

During the incubation the target preparation should be protected from light.

**Step 5**

Cool 2 min. on ice

Leave on ice for at least 2 min. and up to 15 min. Briefly spin the reaction after ice incubation. Heat the sample to 56° C for 2 min. prior to loading into the slide-mixer assembly.

**Step 6**

Load the sample into the pre-heated SC-mixer-slide assembly

Briefly spin the pre-heated sample prior to opening the tube. Aspirate 45 μ L of sample into the pipette by pipetting up and down a few times.



Step 6 (Continued)

Load the sample into the pre-heated SC-mixer-slide assembly.

- Insert the pipette tip into the fill port in the tab-end of the SC-mixer and carefully inject the sample into the chamber at a moderate speed until sample emerges from the vent port (if sample is injected too slowly, a bubble might form). The actual volume of the SC-mixer varies slightly from batch to batch, so do not be alarmed should some of the sample bubble up from the vent port.
- Keep the plunger depressed and remove the pipette tip from the fill port. Any excess sample from the fill and vent ports are wiped clean with a tissue.
- Using forceps, place adhesive port seals directly over both ports. Use a finger on each port seal and press down firmly on both seals simultaneously to seal the ports.

Step 7

Incubate at 56° C for 16 h.

Place the loaded slide-mixer assembly in one of the bays of a MAUI® mixer, cover the bays with a wet MAUI® humidity chamber, close the lid and incubate at 56° C for 16 h with mixing mode B.

Step 8

Preparation of washing procedure

In preparation of next day's washing procedure pre-heat min. 500 mL Wash Buffer A at 56° C overnight. Leave 2 Rectangular Staining Dishes in the oven at 56° C as well. Make sure the MAUI® A/D jig will fit in one of the Staining Dishes or find an alternative container, e.g. 1 mL pipette tip box lid.

Step 9

Prepare wash buffers

At room temperature prepare the following washing solutions, each in a separate staining dish. Add sufficient liquid to completely cover the slides when they are placed in a washing rack in the staining dish: Wash A, B and C buffer according to table 2. Ethanol, 99%.



Step 10

Disassemble A/D jig

Perform the following operations at 56° C by working in the door opening of an oven:

- A. Place the A/D jig in the heated staining dish and add sufficient pre-heated Wash Buffer A to cover the A/D jig.
- B. To the other pre-heated staining dish add sufficient Wash Buffer A to completely cover the slides when placed in the washing rack.
- C. Remove the slide-mixer assembly from the MAUI® unit and quickly insert it into the submerged A/D jig to avoid cooling of the slide. Hold the A/D jig firmly, grasp the top of the mixer and slowly peel the mixer off the slide. Discard the mixer.

**Step 11**

Wash for 2 min. at 56° C in Wash buffer A

- D. Quickly transfer the slide to the rack in the next staining dish with Wash Buffer A at 56° C.
- E. Wash the slide for 2 min at 56° C by gentle plunging of the slide rack.
- F. Repeat steps C to F for each slide in the MAUI® Hybridization Station, collecting the slides submerged in Wash Buffer B at room temperature.

**Step 12**

Wash for 2 min. at room temperature in Wash buffer B

When all slides have been collected in Wash Buffer B, wash the slides for additional 2 minutes by plunging the rack gently up and down in the buffer at room temperature. Make sure the slides are kept fully submerged during washing steps, and don't let the slides dry between steps.

**Step 13**

Wash for 2 min. at room temperature in Wash buffer C

Transfer the slide rack to a new staining dish with Wash Buffer C at room temperature. Wash the slides for 2 minutes by plunging the rack gently up and down in the buffer. Remove very slowly from the buffer in order to let the buffer run off.



Step 14

Wash briefly in 99% Ethanol

Transfer the slide rack to a new staining dish with 99% Ethanol at room temperature. Wash the slides very briefly by plunging the rack gently up and down in the ethanol for a few seconds.

**Step 15**

Dry the slide(s)

Dry the slides by centrifugation for 5 minutes at 1000 rpm. At this stage the fluorophores on the slides are very susceptible to degradation by ozone in atmospheric air. Keep ozone-induced photobleaching to a minimum by working in an ozone free lab or keeping the slides under a controlled atmosphere. Scan slides immediately after drying.

**See tip
5**

Recommendations based on our experience

As mentioned before it is possible to hybridize one sample (i.e. single color) or two samples (i.e. dual color) to one array. Since microarray expression profiling without appropriate standards cannot be used for absolute quantification, expression levels of a microRNA in a sample can only be determined in comparison to other samples. In single color experiments each sample is hybridized to a separate array; the comparison must then occur between arrays. So far there are no established or thoroughly tested control or housekeeping small ncRNAs or microRNAs that can be used as common factors for normalization. The only options for single-color experiments are the use of the characteristic signal distributions, assuming that the similarity between the samples is high enough to allow normalization, or the use of synthetic spiked-in microRNAs. A set of spiked-in control microRNAs could also be an option, but again, it has to be considered that the number of spots used is limited and may thus introduce bias.

We believe that the best way to enable optimal normalization across arrays is to use dual-color arrays with a common reference sample on all arrays in the study (see experimental design options above). Once intra-slide normalization has taken place, the log₂ ratios between sample and reference for each microRNA can be calculated allowing the immediate direct comparison of all log₂ ratios from all slides. The fact that all microRNA signals are expressed as a ratio to a reference, which should be the same on each slide, in essence removes technical variations from the comparison.

Protocol

Step 1 Scanning

We are using an Agilent G2505B Microarray Scanner System. The scanning is normally performed with 10 μm. The sensitivity should be adjusted to 100% PMT. To avoid ozone bleaching, we scan the microarrays in an ozone-free environment (less than 2 ppb ozone). Before starting any analysis, confirm that the tiff image is in the correct orientation (two landing lights in lower right corner). Depending on the scanner, the image may need to be flipped from upper left to lower right.



Step 2

Spot evaluation and background subtraction

In general, we recommend using local background subtraction. We subtract the local median background signal from each spot using Imagene (BioDiscovery). When using more advanced background subtraction, 'Normexp plus offset' convinced us with satisfying results. We are not using Feature Extraction software (Agilent) on a routine level. However, we provide a short protocol for customers who like to use this software (www.exiqon.com/micrna-microarray-analysis-micrna-array).

**Step 3**

Normalization

At a minimum, we recommend a lowest intra-slide normalization for the signal intensities of each channel. This eliminates the dye- and label-specific variances. In addition, it is then recommended to monitor inter-slide comparability based on the spike-ins and or signals derived from constantly expressed RNAs.

miRCURY LNA™ microRNA Arrays contain several control capture probes (e.g. detecting U6 snRNA and snoRNAs) and the signal obtained from these probes could theoretically be used in normalization after confirming the constant expression of these small RNAs under the given experimental conditions. However, we believe that normalization based on these very few probes alone is not optimal. Therefore, we recommend using these control capture probes to monitor the analyzed samples for uniformity than for normalization. In theory, it is possible to use signals from a set of spike-in synthetic microRNAs (added to each labeling reaction and for which control capture probes exist) to perform normalization. However, apart from being something synthetic added to the samples, the use of spike-ins for normalization focuses on a small number of data points, which is a problem if the differences between the samples are very large or if something in the samples themselves affect the synthetic microRNAs during labeling or hybridization. We found 10 spike-ins to be insufficient and found that a minimum of 30 different spike-ins is necessary to perform an efficient normalization. Therefore, the spike-ins supplied should be considered as a monitoring tool.



Step 3 (Continued)

For most cases, we recommend using an experimental design that uses a common reference sample on all arrays (see experimental design above). The design actually resembles the design for a set of single-color experiments, but allows for the direct comparison of all arrays within a study.

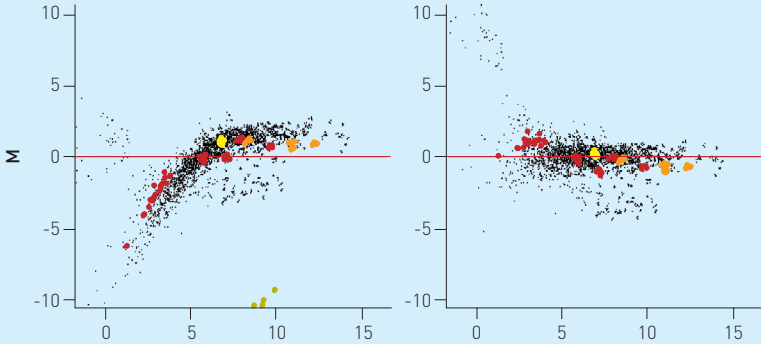
When two directly related samples are to be compared, we recommend a direct comparison. We obtain most reliable results when performing a technical replicate in form of a dyeswap for each analysis.

**Step 4**
Data analysis and
visualization

From each spot and each channel the median signal intensity obtained after image analysis should be measured and normalized (i.e. a global lowess normalization using the R software package after either local background subtraction or normexp plus offset background subtraction). The difference of a normalized and an unnormalized dataset can be seen in the MA plots below. For each of the normalized four replica datasets the ratio between the Hy3 and Hy5 channels is determined. We further recommend to use the median of the four ratios and to calculate the corresponding logratio. The data can be sorted for the relative differences between samples and can be used to generate the expression matrix and graphical visualizations.

The way the actual comparison is performed depends on the experimental setup. For direct comparison, the log ratios can be used directly. For common reference comparisons log₂ differences between sample are compared indirectly between the slides by using the common reference as normalizer added up to obtain the difference between the samples. Visualization of candidate signal intensity differences (e.g. by using heatmaps or clustering) can be performed by using software like dChip (www.dchip.org).



Step 4 (Continued)

Two color intra-slide MA-plots obtained before (left) and after (right) lowess intra-slide normalization. Colored spots represent spike-ins of different signal intensities.

**Step 5**

Data evaluation

We strongly advise users to evaluate the microRNA data for their cluster and family performance. MicroRNAs which cluster in close proximity are expected to react similarly in their expression pattern, due to common transcriptional activity. MicroRNA families can be interesting to analyze since they may react similarly due to their common target sequences or to understand how family members are tissue-specifically regulated. An analysis of how the data of families or clusters correlate can therefore provide relevant data in addition to the actual microRNA signal of initial interest. Additionally, a further analysis of potentially regulated mRNA targets will be useful. A short list of useful software and databases can be found below.



Software and databases

There are several software available for image analysis, as well as, for statistical analysis. In addition to the statistical software R (www.r-project.org/) and the bioconductor (www.bioconductor.org/) and limma (<http://bioinf.wehi.edu.au/limma/>) package for microarray analysis, other freeware programs are available. The selection below is not complete and we advise users to search the web for most appropriate solution for their projects. A far more comprehensive overview than the one below can be found on statweb (www.statsci.org/micrarra/index.html).

Image analysis software

The analysis requires gridding, spot classification and flagging of bad spots. Due the diversity of programs we cannot provide a detailed 'howto'. Image analysis can be done with the commercial programs Imagene or Genepix.

TM4 package TIGR Spotfinder

The program for Windows can be downloaded here (www.tm4.org/scgi-bin/getprogram.cgi?program=spotfinderwin, Linux and sourcecode are available at the website as well), the manual can be found here (<ftp://occams.dfc.harvard.edu/pub/bio/Spotfinder/Spotfinder311doc.pdf>).

ScanAlyze

The program can be downloaded here (http://rana.lbl.gov/downloads/ScanAlyze/ScanAlyze2_vers_2_51.exe) and the manual here (<http://rana.lbl.gov/manuals/ScanAlyzeDoc.pdf>).

Bzscan

Is a Java based platform the direct Java webstart is launched via this link here (http://web-tagc.univ-mrs.fr/bioinformatics/bzscan_files/BZScan.jnlp) and the manual is found here (http://tagc.univ-mrs.fr/bioinformatics/bzscan/bzscan_manual.php).



Further image analysis reading

Gonzalez, R.C., Woods, R.E. (2002) Digital Image Processing, Prentice-Hall, New Jersey

Zhang, W., Shmulevich, I., Astola, J. (2004) Microarray Quality Control, JohnWiley & Sons, New Jersey

Basic statistical analysis software

DChip is a Windows software package allowing sample comparison or hierarchical clustering. You can download the software here (http://biosun1.harvard.edu/~cli/dchip_2007_11.exe) and the manual here (http://biosun1.harvard.edu/complab/dchip/dchip_manual_oct05.pdf).

TM4 package Midas, the TIGR Microarray Data Analysis System is a Java-based microarray data quality filtering and normalization tool that allows raw experimental data to be processed through various data normalizations, filters, and transformations (e.g. lowess and total intensity normalization, low-intensity cutoff, intensity-dependent Z-score cutoff and replicate consistency trimming) by way of a user-designed analysis pipeline. The software can be downloaded here (www.tm4.org/scgi-bin/getprogram.cgi?program=midas) and the manual here (www.tm4.org/documentation/MIDAS2_19.pdf).



MicroRNA Software and Databases:

Annotation database:

Sanger<http://microrna.sanger.ac.uk/sequences/>
 MicroRNA viewer<http://cbio.mskcc.org/microRNAviewer/>
 miPlantBrowser.....<http://miplant.binf.ku.dk/main.pl>

Promoters:

miPromotor<http://people.binf.ku.dk/morten/services/miPromotor/>

production site:

microSite<http://www.microarray.fr/microRNA/microsite/index.php>
 ProMirII<http://cbit.snu.ac.uk/~ProMIR2/>
 miPrecursor.....<http://people.binf.ku.dk/morten/services/miPrecursor/>

Annot. Target database:

TarBase<http://www.diana.pcbi.upenn.edu/tarbase.html>

Target prediction:

Miranda.....<http://www.microrna.org/microrna/home.do>
 TargetScan<http://www.targetscan.org/>
 TargetScanS.....<http://genes.mit.edu/tscan/targetscanS2005.html>
 PicTar<http://pictar.bio.nyu.edu/>
 DIANA MicroT.....http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi/
 D. mel. Targets.....<http://www.russell.embl.de/microRNAs/>
 Argonaute2.....<http://www.ma.uni-heidelberg.de/apps/zmf/argonaute/>
 miRacle.....<http://miracle.igib.res.in/miracle/>
 miRTarget2.....<http://mirdb.org/miRDB/>
 miTarget<http://cbit.snu.ac.kr/~miTarget/>
 RNAhybrid<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid>
 miTargetFinder<http://people.binf.ku.dk/morten/services/miTargetFinder>



Meta Target prediction:

miRGator<http://genome.ewha.ac.kr/miRGator/>

Sequences alignments:

MirAlign<http://bioinfo.au.tsinghua.edu.cn/miralign/>

RNA relation:

CrossLink<http://www-ab.informatik.uni-tuebingen.de/software/crosslink/>

GenMiR++<http://www.psi.toronto.edu/genmir/>

Secondary structure:

miRScan<http://genes.mit.edu/mirscan/>

RNAFold<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>

MFold<http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.htm>

Pfold<http://www.daimi.au.dk/~compbio/rnafold/>

Sfold<http://www.bioinfo.rpi.edu/applications/sfold/srna.pl>

Meta database:

miRGen<http://www.diana.pcbi.upenn.edu/miRGen.html>

plant microRNA:

Micro harvester<http://www.ab.informatik.uni-tuebingen.de/>

miRU<http://bioinfo3.noble.org/miRU.htm>

miplant<http://miplant.binf.ku.dk/>



Tips and Trouble shooting

Experimental procedure

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Tip 1

Preparation of RNA sample

Total RNA should be prepared using a method that retains small RNA species. When using commercially available kits, please ensure that the total RNA preparation is guaranteed to contain microRNAs.

We recommend using our miRCURY™ RNA Isolation Kits for total RNA purification. Procedures that include acidic phenol chloroform extraction are generally also recognized as methods that preserve small RNAs. However, we recommend a column purification step following the phenol:chloroform extraction to remove any trace of these chemicals, as they could potentially inhibit the labeling reaction. Our miRCURY™ RNA Isolation Kits can be used for this procedure.

The purified total RNA should be dissolved in RNase-free water or TE buffer at a concentration of no more than 2 µg/µL. It is recommended to assess the integrity of the RNA isolated before proceeding with labeling. This may be performed either on the Agilent Bioanalyzer (RIN values should be above 7) or by denaturing gel electrophoresis. Degraded RNA is generally not suitable for labeling or for hybridization to microarrays, however RNA extracted from FFPE samples does give good results on miRCURY LNA arrays. The procedure miRCURY LNA™ microRNA Power Labeling Kit can be used for efficient labeling of both microRNA enriched and total RNA. However, microRNAs constitute only a small fraction (~0.01%) of the total RNA. Attempts to purify this small fraction can result in loss of microRNAs or co-purification of larger RNA species. For this reason, we recommend using total RNA for labeling and hybridization.

The amount of total RNA to be labeled for an array hybridization depends on the microRNA content of the cells or tissue being analyzed. Without prior knowledge of the microRNA content we recommend using between 250 ng and 1 µg of total RNA per labeling reaction.



Tip 2

Black spots

Ghost spots can be avoided by removing unincorporated dye. We recommend that the labeled RNA is purified using an exclusion column like mini quick spin OLIGO columns (Roche) or Micro spin columns (BioRad). Alternatively, perform the ethanol precipitation shown below.

- Mix the Hy3™ and Hy5™ labeling reactions before precipitation.
- Add 2.5µL RNase free Sodium acetate (3M, pH5.5) to the 25µL labeling reaction and 75µL 99.9% ethanol.
- Incubate the sample at -20° C for 30 minutes.
- Centrifuge 30 minutes at max speed in a cooled centrifuge.
- Remove supernatant and wash with 80% precooled ethanol, by centrifuging 5 min at max speed in a cooled centrifuge.
- Remove supernatant and if necessary speedvac for a few minutes to remove remainder of ethanol.
- Dissolve in a mixture of 4µL DMSO, 15µL water and 6µL labeling buffer.

Tip 3

Solid particles

If you are concerned about introducing microscopic solid particles onto your array, then filter the sample through a Millipore 0.22 micro spin column (product # UFC30GV0S): Wet the filter with 20 µL Nuclease-free water, spin 1 min. at 12,000 rpm and remove water. Add the target preparation and repeat the centrifugation. The flow-through contains the labeled sample(s).

Tip 4

Flushing the hybridization chamber

In order not to lose any target (to “waste”) when using automated hybridization stations, it is recommended that you inject a volume smaller than the total volume of the hybridization chamber. The mixing mechanism of the hybridization station will ensure that the injected sample will be distributed equally across the entire array. To ensure that the composition of the hybridization buffer is the same after mixing it is recommended to flush the hybridization chamber with 1X hybridization buffer immediately prior to sample injection.



Tip 5 **Dry slides**

If you are doing manual hybridization and have more than 2 slides in your experiment you can dry the slides in a centrifuge by placing the slides in a slide rack on a swinging plate tray (1,000 rpm for 5 minutes). Alternatively, place your slides back to back in a screw-top tube and spin at 1,000 rpm for 5 min. in a centrifuge.

Tip 6 **No signals**

Check that the Hy3™ labeled “landing lights” can be seen. They are located in all 4 corners plus one extra in the lower right corner of the 48 sub-arrays, 240 total. If they can be seen, then check that signals from the spike-in controls used in the labeling can be seen. If not the labeling procedure probably has failed. If the spike-in controls can be seen then check that your total RNA sample is of good quality by gel electrophoresis and optical density analysis. If the RNA quality is good, then increase the amount of RNA used in the labeling. If signals in the Hy5™ channel are unexpectedly low, it could be due to high ozone levels in the air. Ozone has a bleaching effect on the Hy5™. Exiqon recommends to perform labeling reaction, slide handling and scanning in an ozone free environment.

Tip 7 **High signals**

Due to high binding affinity of the LNA™-enriched miRCURY™ capture probes it is of utmost importance to use high stringency experimental settings, i.e. using the miRCURY LNA™ microRNA Array hybridization buffer and an overnight hybridization temperature of 56° C. Furthermore, use of ½-1 µg total RNA will in most cases result in optimal array signal intensities.

Tip 8 **High background**

Using a manual hybridization procedure with cover slip high background around the margins of the coverslip might be seen. This is usually caused by evaporation of the hybridization solution. To avoid uneven distribution of the hybridization solution, it is important to position the slide horizontally. To increase the humidity, we recommend using a water bath.



Tip 9

Concentration and purification of the labeling reaction

We recommend that you follow the standard instruction manual for the labeling reaction, which will yield 50 μL . To minimize the number of handling steps through which the concentration of microRNAs may be compromised, we recommend loading 45 μL of the sample into the MAUI[®] SC mixer without a concentration/purification step; However if high fluorescent background is repeatedly observed, you may purify the labeling reactions to remove unincorporated dyes. For purification and/or concentration of the sample we recommend either ethanol precipitation or the RNeasy Mini Kit into the MAUI[®] SC mixer chamber.

Due to the bleaching effect of ozone on Hy5[™] it is important to finish the concentration of the labeling reaction in the shortest amount of time possible.

Ethanol precipitation:

If the combined labeling reaction exceed 20 μL , then the volume could be concentrated to 20 μL by either ethanol precipitation as shown below, or as described in Tip 1:

- Add 1/10 vol RNase free sodium acetate (3M, PH 5.5) to the 25 μL labeling reaction + 3 vol 100% ethanol
- Incubate the sample at -20°C for 20 minutes
- Centrifuge 20 minutes at $> 12000g$ at 4°C
- Remove supernatant and wash the pellet with 200 μL 80% precooled ethanol (-20°C)
- Centrifuge 5 minutes at $> 12000g$ at 4°C
- Remove supernatant and let the pellet dry for 5 minutes
- Dissolve in a mixture of 20 μL : 3.2 μL DMSO, 12 μL H₂O and 4.8 μL labeling buffer

miRCURY[™] RNA Isolation Kits

- Please refer to the miRCURY[™] RNA Isolation Kit manual for an RNA concentration protocol.



Use of Spike-in microRNAs

Tip 10

Scanner settings

The 10 spike-in control capture probes are located on the diagonal of each subgrid, each in a total of 48 replicas. When scanning the images, some of the spike-in capture probes can be used to determine appropriate scanner settings. Spike_control_j and spike_control_i should appear saturated or close to saturation. They are marked in red in the figure below. Spike_control_d and spike_control_h should give quite high signal but not be saturated. They are marked in pink in the figure below. If spike-in mix was added in equal amounts to both RNA samples, the signal from the spike-in capture probes should be similar in both channels after scanning. First find a laser power setting that gives the expected signal range and then adjust PMT settings so that both channels give similar signal in the spike-in capture probes.

The landing lights (annotated as Hy3™ in the gal-file, probe ID 13138) should not be used for finding the proper scanner settings as these spots contain dye spotted directly on the arrays. The intensity of these spots may vary from batch to batch of slides. These spots are only included for gal-file orientation, and their corresponding data points should be removed prior to normalization of the dataset.

Figure 3

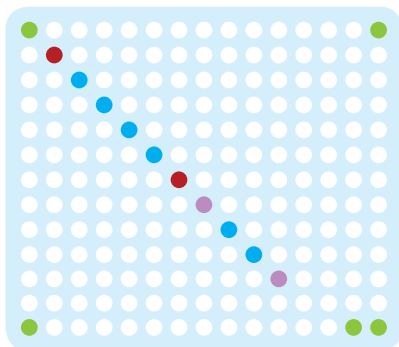


Figure 3. The location of spike-in capture probes in each subgrid on miRCURY LNA™ Arrays. The green spots are the Hy3 landing lights. The red spots are spike_control_i and spike_control_j that should have high signal close to saturation. The pink spots are spike_control_d and spike_control_h that should be well below saturation but have significant signal. The other spike-in capture probes are marked in blue. Some of these may give signal below detection limit, as they are present in very small amounts in the spike-in mix. More information on spike-in signal position is available in figure 4 on next page.

Tip 11

Spike-in microRNA signal distribution

Figure 4 below shows the distribution of the 10 spike-in microRNAs spiked into 1 μg of total RNA from human lung samples. The concentration of the various spike-in microRNAs are optimized so the signal intensities of these spike-in microRNAs are in the dynamic range of naturally expressed microRNAs in most tissues.

Figure 4 - Note

The position of signals from the spike-in microRNA set compared to signals from microRNAs will depend upon the microRNA expression level in the sample.

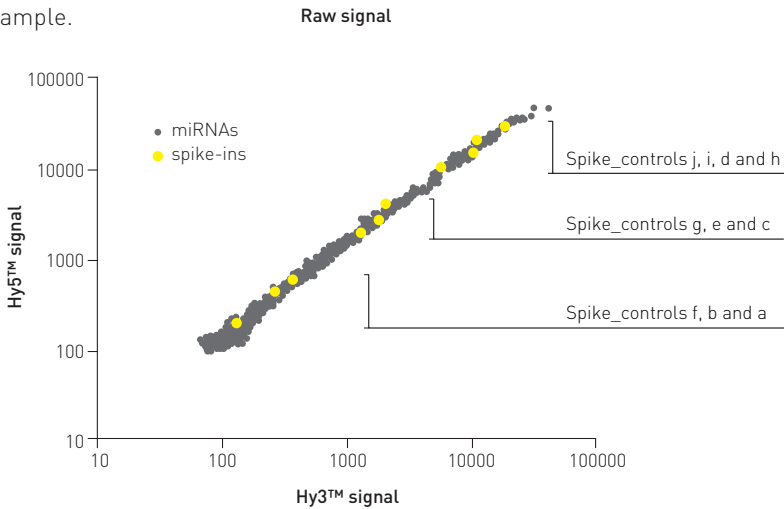


Figure 4. Scatter plot of a self-self hybridization with spike-in mix. One μL of the Spike-in microRNA kit was spiked into a sample of 1 μg total RNA from human lung labeled with Hy3™. Another 1 μL of spike-in microRNAs were spiked into 1 μg RNA from human lung and labeled with Hy5™. Labeling was performed using the miRCURY LNA™ microRNA Power Labeling Kit. Hybridization was performed using the Tecan HS4800™ Pro hybridization station.

Tip 12

Criteria for a good array run using spike-in microRNAs

The array contains specific capture probes for 10 Spike-in microRNAs. The Spike-in microRNAs cover the full signal range (High range; spike-in d, h, i and j, medium range; spike-in c, e and g; low range; a, b and f). Each spike-in microRNA has 48 replicates of capture probes on the array distributed from top to bottom.

- If the variation between replicates of each of the medium and high range spike-in controls exceed 20-25%, it could be an indication of insufficient agitation of the sample.
- Inter- as well as intra correlations between all spike-in microRNAs are normally within 0.950 and 0.999 (R2).



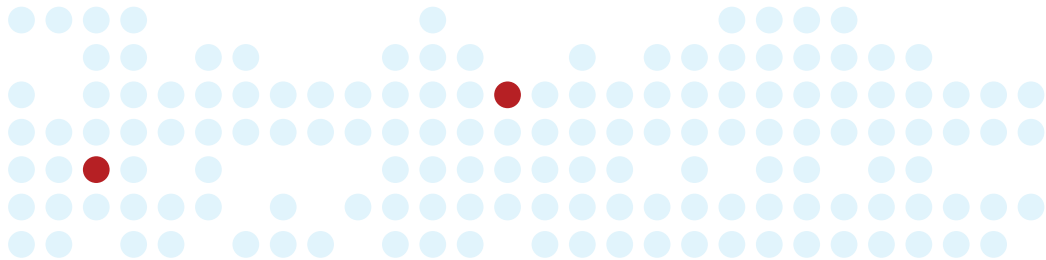
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- miRBase, Wellcome Trust Sanger Institute. <http://microrna.sanger.ac.uk>
- www.exiqon.com/miRCURY/array







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