

## miRCURY LNA™ microRNA Array

ready-to-spot probe set, 6<sup>th</sup> gen

- human, mouse & rat

### Instruction manual v2.0

for product # 208410

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Microplate layout can be found at [www.exiqon.com/miRCURY/array](http://www.exiqon.com/miRCURY/array)



# Product Summary

## 6<sup>th</sup> gen miRCURY LNA™ microRNA Array, ready-to-spot probe set content

### Microplates

The 9 x 384-well microplates contain 300 pmol of each of the capture probes dried down in individual wells.

### miRCURY LNA™ microRNA Array, Spike-in miRNA kit v2 (product # 208041)

One kit containing 52 synthetic unlabeled microRNAs, dried-down. The kit is sufficient for minimum 48 rxns.

### Hybridization buffer (product # 208022, 5 mL)

5 mL high stringency buffer optimized for hybridization of microRNAs to miRCURY LNA™ microRNA Array probes.

### Wash buffer (product # 208021)

20x Salt buffer (2 x 125 mL).  
10% Detergent solution (2 x 15 mL).

## Additional required material

### miRCURY LNA™ microRNA Array Hi-Power Labeling Kit

Fluorescent labeling of microRNAs from total RNA samples ready for hybridization to arrays (product # 208033, 208034, 208035).

### 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 for manual hybridization.



## Product description

Please visit [www.exiqon.com/array](http://www.exiqon.com/array) for download of a list of the capture probe IDs and their well locations in the microplates for use in creating e.g. GenePix® Array List (GAL) file.

Please note the lot# on the microplate and on the microplate pouch. This number is needed to identify the correct microplate layout file.

### $T_m$ -normalized capture probes

The miRCURY LNA™ microRNA Array ready-to-spot probe set contain capture probes complementary to mature microRNAs registered in miRBase Release v.16.0 Please go to [www.exiqon.com/array](http://www.exiqon.com/array) or contact [www.exiqon.com/contact](http://www.exiqon.com/contact) to see the coverage for individual organism in respect to latest miRBase release. The capture probes are Locked Nucleic Acid (LNA™) enhanced oligonucleotides. By varying the LNA™ content and the length of the capture probes the probes have been  $T_m$ -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 v.16.0 Please go to [www.exiqon.com/array](http://www.exiqon.com/array) to see the coverage of 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.



## Control capture probes

A number of control capture probes are included in the probe set. Details of the control capture probes can be downloaded at:

[www.exiqon.com/array-downloads](http://www.exiqon.com/array-downloads).

- Spike-in control probes to assure optimal labeling and hybridization.
- Negative capture probes.
- Capture probes are included that hybridize to small nuclear RNAs.

## Control probes

Probe ID	Positive controls	Aliases	Validated in these organisms
46202	5S_rRNA	-	hsa
145657	RNU1-1	U1; HSD1; RNU1; U1A1; HU1-1; RNU1A; RNU1A3; RNU1G4; Rnu1a1	hsa, mmu
145659	RNU5G	U5a; Rnu5a	hsa, mmu
11278	RNU6-1	U6; RNU6; RNU6A	hsa, mmu, rno
11279	RNU6-1/RNU6-2	U6; RNU6; RNU6A / U6; RNU6B	hsa, mmu, rno
19011	SNORD10	mgU6-77	hsa
145661	SNORD110	HBII-55	mmu
19005	SNORD118	U8	hsa
19606	SNORD12	HBII-99	hsa
19603	SNORD13	U13	hsa
19013	SNORD14B	U14; U14B; RNU14B	hsa
19607	SNORD15A	U15A; RNU15A	hsa
19008	SNORD2	R39B; SNR39B	hsa
19007	SNORD30	U3; U3A; U3B	hsa
46204	SNORD38B	U38B; RNU38B	hsa
46206	SNORD44	U44; RNU44	hsa
46205	SNORD48	U48; RNU48	hsa
46203	SNORD49A	U49; U49A; RNU49	hsa
19604	SNORD4A	Z17A; RNU101A; mgh18S-121	hsa
19605	SNORD6	mgh28S-2412	hsa
145663	SNORD65	HBII-135	mmu
46197	SNORA66	HBII-142	hsa
145666	SNORD68	HBII-202	mmu



Table content continues on next page



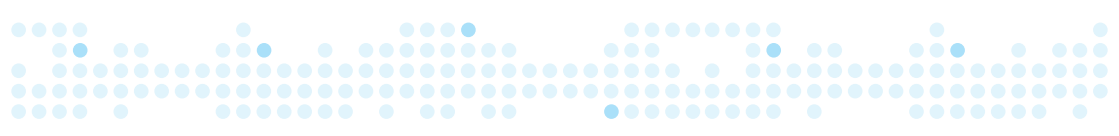
Probe ID	Negative controls	Validated negative control in these organisms
14258	hsa_negative_control-1	hsa, mmu, rno
14259	hsa_negative_control-2	hsa
14260	hsa_negative_control-3	hsa, mmu, rno
14266	hsa_negative_control-4	hsa, mmu, rno
10901	hsa_negative_control-6	hsa, mmu, rno
10902	hsa_negative_control-7	hsa, mmu, rno
10903	hsa_negative_control-8	hsa, mmu, rno

Probe	ID Name
1100	spike_control_v2_1
13186	spike_control_v2_2
13367	spike_control_v2_3
13371	spike_control_v2_4
13388	spike_control_v2_5
13389	spike_control_v2_6
13393	spike_control_v2_7
13417	spike_control_v2_8
13421	spike_control_v2_9
13430	spike_control_v2_10
24127	spike_control_v2_11
24136	spike_control_v2_12
24163	spike_control_v2_13
24199	spike_control_v2_14
24217	spike_control_v2_15
24226	spike_control_v2_16
25557	spike_control_v2_17
25593	spike_control_v2_18
25611	spike_control_v2_19
25728	spike_control_v2_20
26160	spike_control_v2_21
27291	spike_control_v2_22
27318	spike_control_v2_23
27350	spike_control_v2_24
27676	spike_control_v2_25
27821	spike_control_v2_26

Probe	ID Name
27833	spike_control_v2_27
27953	spike_control_v2_28
27968	spike_control_v2_29
28038	spike_control_v2_30
28098	spike_control_v2_31
28393	spike_control_v2_32
28444	spike_control_v2_33
28488	spike_control_v2_34
28568	spike_control_v2_35
28581	spike_control_v2_36
28684	spike_control_v2_37
28876	spike_control_v2_38
28929	spike_control_v2_39
29001	spike_control_v2_40
29056	spike_control_v2_41
29138	spike_control_v2_42
29146	spike_control_v2_43
29544	spike_control_v2_44
29564	spike_control_v2_45
29837	spike_control_v2_46
30147	spike_control_v2_47
30207	spike_control_v2_48
30293	spike_control_v2_49
30747	spike_control_v2_50
30756	spike_control_v2_51
32812	spike_control_v2_52

The different control capture probes were compared against the genomic sequence of hsa, mmu and rno, with the BLAST tools at [www.ensembl.org](http://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 in in this table. Spike-in microRNA control capture probes with less than 100% match to genomic target is in this table.



## Note

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In the microplate layout 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 [www.exiqon.com/contact](http://www.exiqon.com/contact).

## Spike-in microRNA Controls

The miRCURY LNA™ Array Spike in miRNA kit v2 contains 52 different synthetic unlabeled microRNAs in different concentrations. The set can be spiked into an RNA sample prior to labeling and the synthetic Spike-in miRNA kit v2 will hybridize to corresponding capture probes included in the miRCURY LNA™ microRNA Array ready-to-spot probe set.

The Spike-in miRNA kit v2 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 miRNA kit v2 is supplied with different concentrations of synthetic spike-in microRNAs aimed at spanning the whole intensity range of microRNAs in most tissue samples.

## Note

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Please refer to the instruction manual for miRCURY LNA™ microRNA Hi-Power Labeling Kits, for further instructions on how to use the Spike-in miRNA kit v2 during the labeling procedure.

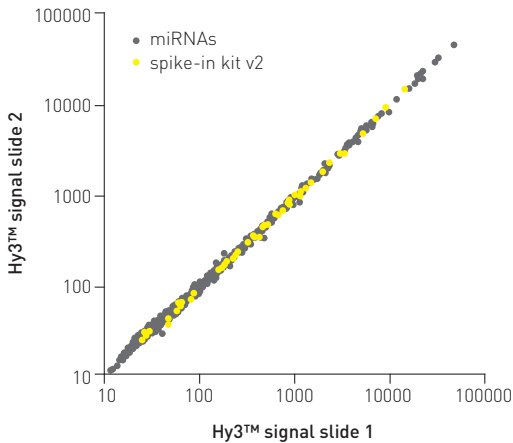


When the spike-in microRNAs are added in equal amounts to labeling reactions before a dual-color array hybridization, the signals from the spike-in capture probes can be used:

- 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 (if multiple replicates of the probes are printed scattered around the array)

### Guidelines for the spike-in microRNA signal distribution

The figure below shows the distribution of the 52 spike-in microRNAs spiked into 0.25µg universal reference RNA (Ambion, AM6000). 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.



**Figure1.** Scatter plot of two hybridizations with Spike-in miRNA kit v2 added. One µL of the Spike-in miRNA kit v2 was spiked into each sample of 0.25 µg total RNA from a mix of human tissues and labeled with Hy3™. Labeling was performed using the miRCURY LNA™ microRNA Power Labeling Kit. Hybridization was performed using the Tecan HS4800™ Pro hybridization station.





## Storage

The miRCURY LNA™ microRNA Array, ready-to-spot probe set should be stored desiccated at  $-20^{\circ}\text{C}$  and protected from light. When properly stored, the ready-to-spot probe set remains hybridization competent for at least 1 year. Exiqon ships the microplates at room temperature in sealable storage pouches that are ideal for long term storage at  $-20^{\circ}\text{C}$ .

Printed arrays should be stored according to the recommendations of the slide provider. If stored properly shelf life for the miRCURY LNA™ microRNA Array, ready-to-spot probe set is 12 months.

Dissolve the Spike-in miRNA kit v2 in 30  $\mu\text{L}$  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. Store the dissolved spike-in microRNA at  $-20^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$ . If stored properly shelf life for the miRCURY LNA™ Array Spike-in miRNA kit v2 is 12 months. In solution the shelf life for the spike-in microRNAs is 3 months.



# Protocol

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## Spotting recommendations

Spotting of the capture probes should be carried out according to the protocol recommended by the provider of the slide substrate.

The capture probes should be spotted onto amine reactive slide substrates for covalent attachment to the slide surface. The following slides have successfully been tested with the capture probes: Surmodics CodeLink™, SCHOTT Nexterion® and Corning® Epoxide. However, other amine-reactive substrates may function equally well. The capture probes are dried down in the wells of the microplates and need to be re-dissolved in spotting buffer according to the recommendations given by the provider of the slide substrate. Generally we have found that a phosphate buffer of 150-300 mM, pH 8.5 with 0.001% SDS is optimal for most amine reactive slides.

It is suggested to dissolve the capture probes in 15  $\mu\text{L}$  spotting buffer resulting in a final concentration of 20  $\mu\text{M}$  capture probe during spotting. Several other concentrations of capture probes can be successfully applied, but generally it is common to use 10-40  $\mu\text{M}$ , i.e. re-dissolve in 30-7.5  $\mu\text{L}$  spotting buffer.

Please go to [www.exiqon.com/array-downloads](http://www.exiqon.com/array-downloads) to download the microplate layout file.



# Hybridization Protocol

## Hybridization and washing using Tecan HS Pro™ hybridization stations

### Before starting the experiment

For labeling, we recommend that you use a miRCURY LNA™ microRNA Hi-Power Labeling Kit. Please visit [www.exiqon.com/array](http://www.exiqon.com/array) to learn more about this product.

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 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.



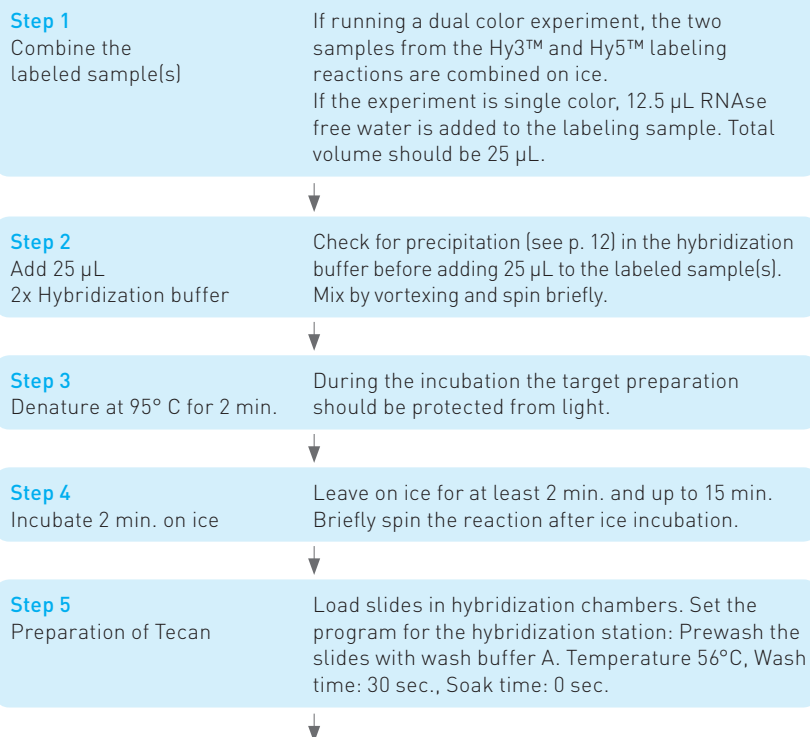
Table 1

## 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

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**Step 6**  
Flush hyb 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  $\mu$ L for a Tecan HS400/HS4800).

**Step 7**  
Inject reaction mixture

Inject the 50  $\mu$ L target preparation to the hybridization station. In order to flush injection inlet, it is recommended to inject 10 $\mu$ 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 station for optimal quality. 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 [www.exiqon.com/contact](http://www.exiqon.com/contact) for an alternative protocol using cover slip.

We recommend that you use a miRCURY LNA™ microRNA Hi-Power Labeling Kit for labeling of your sample(s). Please visit [www.exiqon.com](http://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 or Agilent).
- Ethanol 99%
- Hybridization chamber user guide (G2534-90002)

### **Before starting the experiment, day 1**

We recommend that you use a miRCURY LNA™ microRNA Hi-Power Labeling Kit for labeling of your sample(s). Please visit [www.exiqon.com](http://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.



## 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/e-talk](http://www.exiqon.com/e-talk)

**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)  
Adjust the volumes of the labeled sample to 200  $\mu\text{L}$  by adding nuclease free water to the labeled sample(s) (kept on ice). If running a dual color experiment, combine the two samples from the Hy3™ and Hy5™ labeling reactions before adjusting the volume.

**Step 2**  
Add 200  $\mu\text{L}$  2x hybridization buffer  
If there is precipitation in the Hybridization buffer, then warm the solution at 56° C and agitate to dissolve. Add 200  $\mu\text{L}$  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  $\mu\text{L}$  to reservoir  
Add 400  $\mu\text{L}$  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.

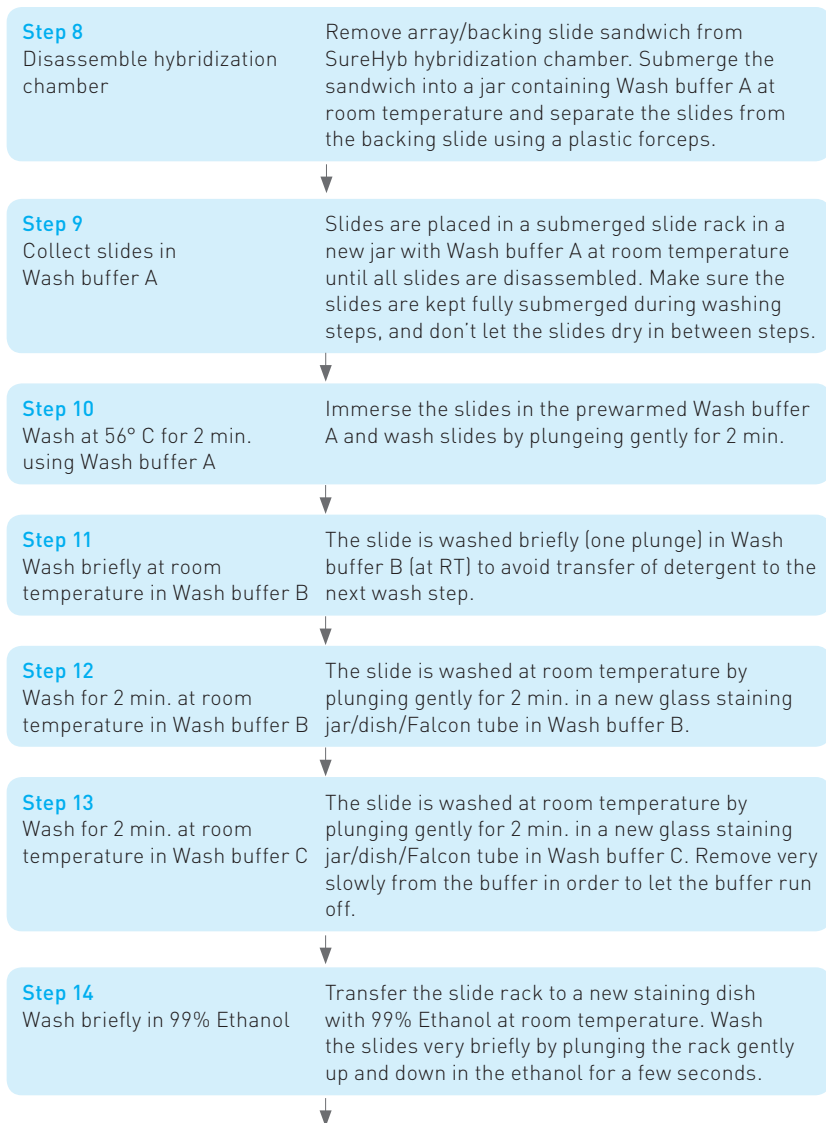
**Step 6**  
Incubate at 56° C for 16 h.  
Clamp the array/backing slide sandwich into the SureHyb hybridization chambers and make sure all bubbles move freely. Incubate at 56° C for 16 h. in a hybridization oven with rotation (e.g. SciGene, #400).

**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.

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**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 NimbleGen/ MAUI® 4-Bay or 12-Bay hybridization stations

This Protocol provides information for the use of Exiqon miRCURY LNA™ Arrays with the NimbleGen/ MAUI® Hybridization System using a NimbleGen HX1 mixer. The protocol is designed for the arrays that are printed on the opposite side of the slide compared to the barcode [prod. # 208420, 208421 and 208422].

The NimbleGen/ MAUI® Hybridization System is comprised of two main components, the disposable NimbleGen Mixer hybridization chambers and NimbleGen/ MAUI® instrument that powers the mixing bladders in the Mixer and maintains a constant incubation temperature. The NimbleGen 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 NimbleGen/ MAUI® Hybridization System please see the User's Guide that come with the Hybridization System.



**Additional required materials:**

RNA samples (labeled with miRCURY LNA™ Hi-Power Labeling Kit)  
 NimbleGen HX1-mixers  
 NimbleGen Precision mixer alignment tool (PMAT)  
 NimbleGen Mixer Disassembly Tool  
 NimbleGen Gasket brayer  
 Positive Displacement Pipette (optional, but highly recommended)  
 Wide pipette tips (see filling video at [www.exiqon.com/e-talk](http://www.exiqon.com/e-talk))  
 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**

We recommend that you use a miRCURY LNA™ microRNA Hi-Power Labeling Kit for labeling of your sample(s). Please visit [www.exiqon.com](http://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 NimbleGen HX1 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.
- Insert the array slide in the PMAT with the barcode facing down and away from the hinge.
- Place the HX1 mixer in the lid of the PMAT with the end of the mixer towards the hinge and the mixer's adhesive gasket facing outward. Remove the protective lining from the mixer using a forceps and close the lid of the PMAT and carefully press the mixer.
- Remove the mixer-slide assembly from the PMAT and place the assembly with the mixer side up on a heating block at 56° C for 5 min.
- Use the NimbleGen 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)

If running a dual color experiment, the two samples from the Hy3™ and Hy5™ labeling reactions are combined on ice. If the experiment is single color, 12.5 µL RNase free water is added to the labeled sample. Total volume should be 25 µL.



**Step 3**  
Add 25  $\mu\text{L}$   
2X Hybridization buffer

Add 25  $\mu\text{L}$  2X Hybridization buffer to the labeled sample(s). Mix by vortexing and spin briefly. Final volume 50  $\mu\text{L}$ .



**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 HX1-mixer-slide assembly

Briefly spin the pre-heated sample prior to opening the tube. Aspirate 45  $\mu\text{L}$  of sample into the pipette by pipetting up and down a few times (avoid bubbles).



**Step 6 (Continued)**

Load the sample into the pre-heated HX1-mixer-slide assembly.

- Insert the pipette tip into the fill port in the tab-end of the HX1-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 HX1-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 NimbleGen mixer, 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.

**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 HX1-mixer and slide

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

- A. Place the mixer/slide assembly in the Mixer Disassembly Tool in the heated staining dish and add sufficient pre-heated Wash Buffer A to cover it.
- 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 HX1-mixer from the slide and quickly insert it into the submerged washing rack to avoid cooling of the slide.

**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. Transfer the slide to the rack in Wash Buffer B
- F. Repeat steps C to F for each slide in the NimbleGen 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 for Experimental Set-up and Data Handling

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.

A way to enable optimal normalization across arrays is to use dual-color arrays with a common reference sample or a universal reference on all arrays in the study. Once intra-slide normalization has taken place, the  $\log_2$  ratios between sample and reference for each microRNA can be calculated allowing the immediate direct comparison of all  $\log_2$  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.

For help on designing array experiments, an overview can be downloaded from [www.exiqon.com/array:Guidelines\\_for\\_setting\\_up\\_microRNA\\_array\\_profiling\\_exp.pdf](http://www.exiqon.com/array:Guidelines_for_setting_up_microRNA_array_profiling_exp.pdf)

## Protocol

### Step 1 Scanning

We are using an Agilent G2505B Microarray Scanner System. The scanning is normally performed with 10  $\mu\text{m}$ . 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 the Exiqon-tailored ImaGene data analysis software (see [www.exiqon.com/mirna-array-software](http://www.exiqon.com/mirna-array-software)). 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/microna-microarray-analysis-microna-array](http://www.exiqon.com/microna-microarray-analysis-microna-array)).

**Step 3**

Normalization

As a minimum, we recommend a lowess intra-slide normalization for the signal intensities of each channel if running a dual color experiment. 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. If running single color experiments we recommend to normalize the data using the quantile normalization method, as we have found that this generates the most reliable data. Both of these normalization methods are supported in the Nexus software, supplied with ImaGene (the Exiqon array data analysis software package, for details see [www.exiqon.com/mirna-array-software](http://www.exiqon.com/mirna-array-software)). 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 and not for normalization.



### Step 3 (Continued)

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. Exiqon offers two different spike-in kits. The one supplied with the array (spike-in miRNA kit v2, 208041), contains 52 synthetic miRNAs and could in theory be used for normalization, although we recommend using all detection probes instead. The other (spike-in kit, 208040) only contains 10 different miRNAs and should not be used for normalization.



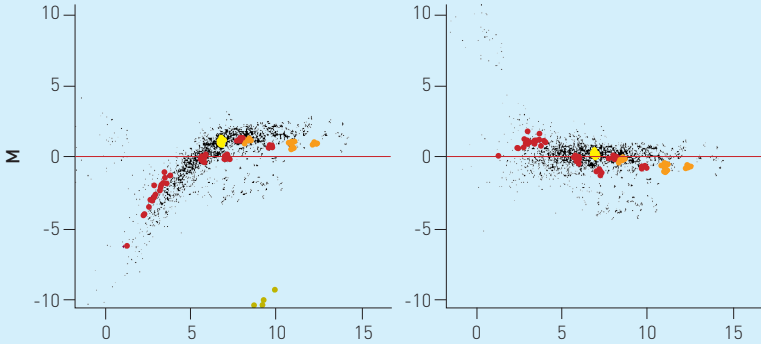
### Step 4 Data analysis and visualization

From each spot and each channel the median signal intensity obtained after image analysis should be measured and normalized (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.

The way the actual comparison is performed depends on the experimental setup. For direct comparison, the log ratios can be used directly. For common /universal reference comparisons log<sub>2</sub> 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. We use the special tailored Exiqon-offered Nexus software (see [www.exiqon.com/mirna-array-software](http://www.exiqon.com/mirna-array-software)), that calculates significant differentially expressed miRs across samples and visualizes this in heatmaps/cluster diagrams.



## 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 and help understanding 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

Exiqon offers a software package especially tailored to suit the needs of analysis of the miRCURY LNA™ microRNA Arrays. The easy to use software package includes the ImaGene®9 for image analysis and Nexus Expression™ 2 for array data analysis. Together with the Exiqon specific settings file and the Exiqon quick manual a successful array analysis can be obtained with a few clicks. Brief descriptions of the two software tools are given below. Further information is available at [www.exiqon.com/mirna-array-software](http://www.exiqon.com/mirna-array-software).

## ImaGene

The Exiqon-offered image analysis software ImaGene places a grid on top of the scanned array image, and identifies which probe is located in each spot. The software quantifies the signal intensity and the surrounding background. ImaGene can also be used for basic data normalization. ImaGene in our hands outperforms other software regarding the correct spot recognition and flagging.

## Nexus Expression

Nexus Expression is the miRCURY LNA™ microRNA Array supportive for statistical analysis of array profiling data. Nexus Expression reads ImaGene but also other common image analysis program output formats. Nexus Expression allows background subtraction, normalization and visualization of the array data. It can combine the replicated measures of each probe on the arrays into one output value per array and make statistical calculations of the differentially expressed data obtained by comparing the microRNA signal data of different array experiments. The active links of the probe-target information to miRBase allows the easy collection of further microRNA information as soon as it is needed. Additional information is found on the Exiqon website at [www.exiqon.com/mirna-array-software](http://www.exiqon.com/mirna-array-software).

Several other commercial and free software packages for microarray image and data analysis are also available. A selection of these is listed below. The list is not complete and a more appropriate solution may be available for certain projects. For a more comprehensive overview of software packages, it is advised to visit statweb at [www.statsci.org/micrarra/index.html](http://www.statsci.org/micrarra/index.html).



**Links to other array software:****image analysis:**

- Bzscan ..... <http://tagc.univ-mrs.fr/ComputationalBiology/bzscan/>  
ScanAlyze ..... <http://rana.lbl.gov/EisenSoftware.htm>  
Spotfinder ..... <http://www.tm4.org/spotfinder.html>

**statistical analysis:**

- Carmaweb ..... <https://carmaweb.genome.tugraz.at/>  
DChip ..... <https://sites.google.com/site/dchipsoft/downloading-dchip-software>  
Midas ..... <http://www.tm4.org/midas.html>  
R/bioconductor /  
limma ..... <http://www.r-project.org/>  
<http://www.bioconductor.org/> <http://bioinf.wehi.edu.au/limma/>

Links to additional array analysis software and microRNA software and databases in general are available at [www.exiqon.com/array](http://www.exiqon.com/array)



# Tips and Trouble Shooting

## Experimental procedure

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 miRCURY LNA™ microRNA Hi-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 miRCURY™ RNA Isolation Kit or 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 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™ dye, especially after the slide has been dried. 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 (procedure in Tip 13) 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.



### 34 Concentration and purification of the labeling reaction

Tip 9

We recommend that you follow the standard instruction manual for the labeling reaction, which will yield 50  $\mu$ L. To minimize the number of handling steps through which the concentration of microRNAs may be compromised, we recommend loading 45  $\mu$ L of the sample into the NimbleGen HX1 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 miRCURY™ RNA Isolation Kits, alternatively either ethanol precipitation or the RNeasy Mini Kit. 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:

The combined labeling reaction can be concentrated 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  $\mu$ L labeling reaction + 3 vol 100% ethanol
- Incubate the sample at  $-20^{\circ}$  C for 20 minutes
- Centrifuge 20 minutes at  $> 12000g$  at  $4^{\circ}$  C
- Remove supernatant and wash the pellet with 200  $\mu$ L 80% precooled ethanol ( $-20^{\circ}$  C)
- Centrifuge 5 minutes at  $> 12000g$  at  $4^{\circ}$  C
- Remove supernatant and let the pellet dry for 5 minutes
- Dissolve in a mixture of 20  $\mu$ L: 3.2  $\mu$ L DMSO, 12  $\mu$ L H<sub>2</sub>O and 4.8  $\mu$ L labeling buffer

#### miRCURY™ RNA Isolation Kits

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



## Ready-to-spot Probe Set

Tip 10

### Spotting buffer

Spotting of the capture probes should be carried out according to the protocol recommended by the provider of the slide substrate. We have found that a phosphate buffer of 150-300 mM, pH 8.5 with approx. 0.001% SDS works well with most substrates.

Tip 11

### Empty microplate wells

A number of wells in some of the plates are empty. The remaining wells may appear to be empty but each well contains 300 pmol of dried down capture probe according to the microplate layout file available at [www.exiqon.com/miRCURY/array](http://www.exiqon.com/miRCURY/array).

Tip 12

### Spot morphology

Use of the proper spotting conditions for your particular printing setup is essential for obtaining a satisfactory spot morphology. Several factors can influence the spot morphology, e.g. slide substrate, temperature and humidity during spotting. Of particular importance is the use of the correct spotting buffer with the right amount of detergent. It is generally recommended to follow the spotting protocols provided for the slide substrate.

Tip 13

### Storage and treatment of miRCURY LNA™ capture probes

The capture probes have physical and chemical properties identical to similar DNA capture probes and should be treated accordingly. The capture probes are short, amino-modified oligo-nucleotides with individual monomers substituted with LNA™. When dissolved the capture probes should not be subjected to repeated freeze-thaw cycles but kept at 4° C during periods of frequent use and stored at -20° C for long term storage. Do not expose the capture probes to light.

Tip 14

### Single color protocol

In order to be able to run single color experiments, the quality of all the components applied in the analysis is very important, including the arrays. The production of arrays should be very tightly monitored and QC in order to meet the strict requirements to a single color protocol. We therefore recommend that customers use preprinted Exiqon miRCURY LNA™ arrays, for running single color experiments.



# References

36

- The microRNA Registry.  
Griffiths-Jones S. Nucleic Acid Research, 2004, 32, Database Issue, D109-11
- miRBase, <http://www.mirbase.org/>
- [www.exiqon.com/miRCURY/array](http://www.exiqon.com/miRCURY/array)



# Related Products

Exiqon offers a tool kit enabling new discoveries concerning the expression, function, and spatial distribution of microRNAs:

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 Hi-Power Labeling Kits

For fluorescent labeling of microRNAs from total RNA samples ready for array hybridization (product # 208033, 208034, 208035).

## miRCURY LNA™ microRNA Array, Microarray Kit

Pre-printed miRCURY LNA™ microRNA Array microarray slides, available in pack sizes of 3, 6 and 24 (product # 208400, 208401, 208402).

## miRCURY LNA™ microRNA Array, Spike-in miRNA 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, Spike-in miRNA Kit v2

52 different synthetic unlabeled microRNAs in different concentrations. The spike-in microRNA set will hybridize to corresponding capture probes on the miRCURY LNA™ microRNA Array (product # 208041).



**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 ISH Optimization kit (FFPE)**

Complete kit with control probes and hybridization buffer for easy set up of microRNA in situ hybridization.

**miRCURY LNA™ microRNA Inhibitors and Power Inhibitors**

Unravel the function of microRNAs by microRNA inhibition. Sophisticated LNA™ design ensures potent inhibition of all microRNAs regardless of their GC content. Chemically modified, highly stable Power Inhibitors for unrivalled potency.

**miRCURY LNA™ microRNA Inhibitor Library**

For genome-wide high throughput screening of microRNA function.

**miRCURY LNA™ Universal RT microRNA PCR**

Exiqons microRNA qPCR system offers the best available combination of performance and ease-of-use on the microRNA real-time PCR market. The combination of a Universal RT reaction and LNA™-enhanced PCR primers results in unmatched sensitivity and specificity. The Ready-to-use microRNA PCR panels enable fast and easy microRNA expression profiling.



### Literature citations:

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

### Patents and Trademarks

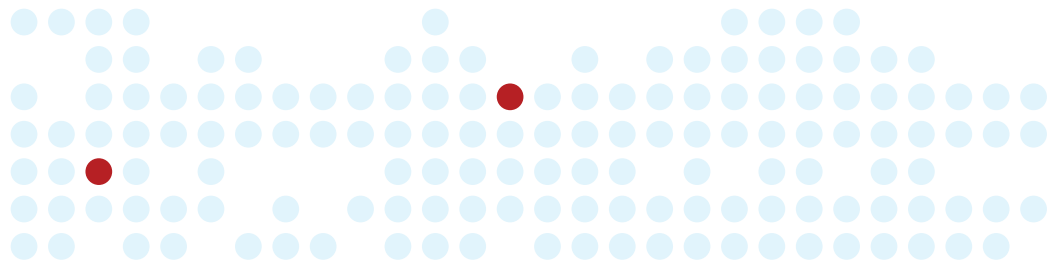
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