

# miRCURY™ LNA microRNA Array microarray kit

## Instruction manual

for product # 208000-A, 208001-A, 208002-A

### 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/miRCURY/arrays](http://www.exiqon.com/miRCURY/arrays) and Lot# of the miRCURY™ LNA microRNA Arrays can be found on the slide pouch and slide plastic box.



# Product summary

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## miRCURY™ LNA microRNA Array content

### Microarray slides

miRCURY™ LNA microRNA Arrays consist of control probes, mismatch probes, and 2056 capture probes, perfectly matched probes for all miRNAs in all organisms as annotated in miRBase Release 9.2. Arrays also contain a number of proprietary miRPlus™ sequences not 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 miRNA kit (product# 208040)

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

## Additional required material

### miRCURY™ LNA microRNA Power labeling kit

Fluorescent labeling of miRNAs 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<sub>m</sub>-normalized capture probes

The miRCURY™ LNA microRNA Array slides contain capture probes complementary to mature miRNAs registered in miRBase Release 9.2. Please go to [www.exiqon.com/array](http://www.exiqon.com/array) or contact [support@exiqon.com](mailto:support@exiqon.com) to see the coverage for individual organisms 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<sub>m</sub>-normalized to hybridise optimally under the conditions described in this protocol.

### Coverage of probe set

The slides contains capture probes for all miRNAs in all organisms as annotated in miRBase Release 9.2. Please go to [www.exiqon.com/array](http://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 miRNAs not included in miRBase. These miRPlus™ probes give researchers access to information unavailable elsewhere.

Please go to our online miRNA resource at [www.exiqon.com/miRCURY/array](http://www.exiqon.com/miRCURY/array) to:

- help to manage the complex relationships between the miRCURY™ LNA microRNA Array capture probes and their targets.
- 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

Thirty control capture probes are included the probe set.

Details of the control capture probes can be downloaded at [www.exiqon.com/miRCURY/array](http://www.exiqon.com/miRCURY/array)

- Ten spike-in control probes to assure optimal labeling and hybridization.
- Eight negative capture probes.
- Twelve capture probes are included that hybridize 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-snRNA-1	hsa, rno, mmu
11279	U6-sn-RNA-2	hsa, rno, mmu, dme, cel, ath
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

Probe ID	Negative controls	Validated negative control in these organisms
14258	hsa_negative_control-1	hsa, mmu, rno, dro, cel
14259	hsa_negative_control-2	hsa, dro, cel
14260	hsa_negative_control-3	hsa, mmu, rno, dro, cel
14266	hsa_negative_control-4	hsa, mmu, rno
10898	hsa_negative_control-5	hsa, mmu, rno, dro, cel
10901	hsa_negative_control-6	hsa, mmu, rno, cel
10902	hsa_negative_control-7	hsa, mmu, rno, dro, cel
10903	hsa_negative_control-8	hsa, mmu, rno, dro, cel

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

The different control capture probes were compared against the genomic sequence of hsa, mmu, rno, dre, dme, cel and ath with the BLAST tools at [www.ensembl.org](http://www.ensembl.org) and [www.arabidopsis.org](http://www.arabidopsis.org). Other organisms may also be valid.

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 miRNA control capture probes with less than 100% match to genomic target is in this table.

## Note

In the GAL-file, all capture probes which are designed for species other than the one in question, are annotated as e.g. "No\_known\_hsa\_target" – in the case of a human sample. However, many species show a high degree of conservation in the sequence of their miRNAs. Thus, it is entirely possible that a capture probe for one species can correspond to an as yet unidentified miRNA in another species. It is also possible that a target sequence differing by only one nucleotide located at either end of the sequence might bind to the capture probe producing a signal. Information on target sequences and miRNA interactions can be found at the Exiqon resource centre at [www.exiqon.com/miRCURY/array](http://www.exiqon.com/miRCURY/array). There are also a number of probes named "obsolete" present on the arrays. These are capture probes that were used in an earlier version of the arrays, and they can be ignored in the analysis as there is an optimized design of that capture probe present on these arrays. The optimized probe has another probe ID, and is annotated according to latest version of the miRBase.

## Spike-in miRNA controls

The miRCURY™ LNA microRNA Array Spike-in kit contains 10 different synthetic unlabeled miRNAs in different concentrations. The set can be spiked into an RNA sample prior to labeling and the synthetic Spike-in miRNA kit will hybridize to corresponding capture probes on the miRCURY™ LNA microRNA Array. The Spike-in miRNA kit has been designed and tested not to cross-react with endogenous miRNAs from human, mouse or rat, and is provided at concentrations compatible with endogenous miRNA expression levels. The Spike-in miRNA kit is supplied with different concentrations of synthetic spike-in miRNAs aimed at spanning the whole intensity range of miRNAs in most tissue samples. The corresponding capture probes have been printed once in every subgrid, thus 32 times each.

See tip  
11-12

## Note

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



When the spike-in miRNAs 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

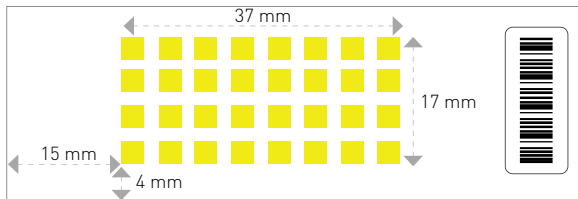
- as a control of the labeling reaction and hybridization
- as a help in deciding scanner settings between channels
- as a control of the data normalization procedure
- to estimate the variance of replicated measurements within arrays
- to assess technical variability between different parts of the array

## 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: 17 mm wide by 37 mm long
- Coordinates of first spot on slide = 4 mm, 15mm
- 8 sub-arrays in 4 replicates
- Spot size: 90  $\mu\text{m}$
- Distance between spots: 225  $\mu\text{m}$

**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 32 sub-arrays, 160 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 six 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.

Dissolve the miRCURY™ LNA Array spike-in miRNAs in 30  $\mu$ L of RNase free water (supplied) upon receipt. Leave the suspension on ice for 30 min. to dissolve. Vortex and then spin to collect tube contents. Store the dissolved Spike-in miRNA at  $-20^{\circ}$  C until use and avoid repeated cycles of freeze/thawing. You may wish to aliquot the dissolved spike-in miRNAs to avoid repeated freeze/thawing. For long-term storage, keep the vial at  $-80^{\circ}$  C.



## Related products

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

### **miRCURY™ LNA microRNA Power labeling kit**

For fluorescent labeling of miRNAs 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 # 208010-A).

### **miRCURY™ LNA microRNA Array, Spike-in miRNA kit**

Ten different synthetic unlabeled miRNAs in different concentrations. The spike-in miRNA 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 miRNA 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 miRNAs.

### **miRCURY™ LNA microRNA Knockdown**

miRNA knockdown probes: determine or confirm miRNA function.

### **miRCURY™ LNA microRNA Real-time PCR**

Determine miRNA expression using real-time PCR system. Available soon.



## Protocol overview

### miRCURY™ LNA microRNA Power Labeling Kit

CIP treatment

Mix: RNA sample  
Spike-In miRNA kit



Labeling reaction

Mix: CIP'ed 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 5µm)  
Download relevant GAL files from  
[www.exiqon.com](http://www.exiqon.com)



# Protocol

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## Hybridization and washing using a hybridization station

### Before starting the experiment

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

We recommend that you use a miRCURY™ LNA microRNA Power labeling kit for labeling of your sample(s). Please visit [www.exiqon.com](http://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 miRNA content of the cells or tissue being analyzed as this amount is known to vary. Without prior knowledge of miRNA 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.

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 miRNA 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 miRNAs. Store the dissolved spike-in miRNA 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.

Protocols for various automated hybridization stations are available at [www.exiqon.com](http://www.exiqon.com)

**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

### Step 1

Combine the labeled sample(s)

The two samples from the Hy3™ and Hy5™ labeling reactions are combined on ice. Total volume should be 25 µL.

See tip 2

### Step 2

Add 25 µL Hybridization buffer

Check for precipitation (see p. 12) in the Hybridization buffer before adding 25 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.

See tip 3

### 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

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 µL for a Tecan HS400/HS4800).

See tip 4

### Step 6

Inject reaction mixture

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

### Step 7

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 8**

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 9**

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 10**

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 11**

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 12**

Dry slides

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



## Hybridization and washing using a manual procedure

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. An alternative protocol using cover slip can be found in tip 13.

### 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)

### Before starting the experiment, day 1

Total RNA should be prepared using a method that preserves 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 miRNA content of the cells or tissue being analyzed as this amount is known to vary. Without prior knowledge of miRNA 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](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.

See tip  
1



## 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. The microarray kit instruction manual can be downloaded at [www.exiqon.com/array](http://www.exiqon.com/array)

**Table 2**

The volumes in this table are required for a glass staining jar 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).

See tip 2

**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.

See tip 3

**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.



- Step 8**  
Disassemble hybridization chamber
- Remove array/backing slide sandwich from SureHyb hybridization chamber. Submerge the sandwich into a jar containing Wash buffer A at room temperature and separate the slides from the backing slide using a plastic forceps.
- Step 9**  
Collect slides in Wash buffer A
- Slides are placed in a submerged slide rack in a new jar with Wash buffer A at room temperature until all slides are disassembled. Make sure the slides are kept fully submerged during washing steps, and don't let the slides dry in between steps.
- Step 10**  
Wash at 56° C for 2 min. using Wash buffer A
- Immerse the slides in the prewarmed Wash buffer A and wash slides by plunging gently for 2 min.
- Step 11**  
Wash briefly at room temperature in Wash buffer B
- The slide is washed briefly (one plunge) in Wash buffer B (at RT) to avoid transfer of detergent to the next wash step.
- Step 12**  
Wash for 2 min. at room temperature in Wash buffer B
- The slide is washed at room temperature by plunging gently for 2 min. in a new glass staining jar/dish/Falcon tube in Wash buffer B.
- Step 13**  
Wash for 2 min. at room temperature in Wash buffer C
- The slide is washed at room temperature by plunging gently for 2 min. in a new glass staining jar/dish/Falcon tube in Wash buffer C.
- Step 14**  
Wash briefly in water
- Briefly (~1 sec.) wash the slide(s) at room temperature in nuclease free water (one plunge).
- Step 15**  
Dry the slide(s)
- The slide is dried by centrifugation for 5 min. at 200 G (1000 rpm). Scan slides immediately after drying.

See tip 5

# Image acquisition and quantification

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Relevant GAL files can be found at <http://www.exiqon.com/miRCURY/array>

Please note the lot# on the slide box and slide pouch. This number is needed to identify the correct GAL file.

A wide variety of different scanning instruments are available, and a number of different image acquisition and quantification packages are associated with them. In general, selection of image quantification parameters (e.g. 'adaptive', 'fixed circle', 'spot distance') should be carefully assessed and decided for each project as a whole as this depends on the array design, slide type and spot morphology. It should be noted, that the image quantification method should be identical for all slides constituting a project, whereas image acquisition parameters, such as laser power and/or photo multiplier can be optimized from slide to slide. For optimal quantification and reproducibility, slides should be scanned at 5  $\mu$ M resolution.

The miRCURY™ LNA microRNA Power labeling kit (product # 208031-A, 208032-A) has the dyes Hy3™ and Hy5™ included. The two dyes are equivalent to the well-known Cy3™ and Cy5™, fluorophores having emissions of 556 nm and 656 nm, respectively.



# 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 preserves small RNA species. When using commercially available kits, please ensure that the total RNA preparation is guaranteed to contain microRNAs.

We recommend miRNeasy Mini Kit or the miRNeasy 96 kit (Qiagen) as a column purification procedure. Please use the protocol recommended by the manufacturer.

We strongly recommend to clean up a total RNA sample after RNA isolation with guanidinium solutions with or without phenol/chloroform extraction (Trizol (Molecular Research, Inc.), Qiazol™ (Qiagen GmbH), Tri reagent, etc.). The cleanup procedure must preserve miRNAs. Examples of column purification procedures which preserve small RNAs are the RNeasy MinElute Cleanup Kit (See tip 14) and miRNeasy Mini Kit from Qiagen (please use the protocol recommended by the manufacturer).

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 not suitable for labeling or for hybridization to microarrays.

The procedure used for labeling of miRNAs in the miRCURY™ LNA microRNA Power labeling kit uses total RNA or enriched small RNA. In general, enrichment for small RNAs is not necessary. Exiqon has carried out extensive comparisons of slides that were hybridized with labeled miRNA enriched samples versus total RNA samples. We do see some differences comparing miRNA enriched and non-enriched miRNA samples. Due to the risk of losing information we do not recommend miRNA enrichment of the total RNA samples.

The amount of total RNA to be labeled for an array hybridization depends on the miRNA content of the cells or tissue being analyzed as this amount is known to vary. Without prior knowledge of miRNA 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.



Tip 2

### Black spots

In case you experience ghost spots, it is possible to avoid it by removing unincorporated dye. We recommend to perform an ethanol precipitation. It is important to work fast due to the Hy5™ sensitivity to ozone.

- 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



Tip 5  
continued

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 32 sub-arrays, 160 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™ 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.



## Guidelines for microRNA profiling experiments

Tip 9

### Normalization

Normalization is performed on data from individual arrays (**intra-slide normalization**) and on data from a set of arrays (**inter-slide normalization**) and is used to remove system related variations (i.e. technical variations), such as dye labeling bias and differences in hybridization and scanning. The process involves normalizing the signal intensities from all the spots to a common factor. The common factor can be based on statistical parameters such as overall signal intensities or signal mean from the whole data set or on controls or “house-keeping” genes that are assumed to stay unchanged between different samples. Either of these types of constant parameter are easily applicable to microarrays with large numbers of spots or a host of unchanged signals (such as mRNA arrays). However, miRNA arrays have relatively few spots, microRNA expression levels can vary a lot between samples and there are no identified house-keeping miRNAs or unchanged controls.

In dual colour experiments, **intra-slide normalization** is performed to minimize intensity related differences between the colours (dye bias). We have found that the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm produces a good intra-slide normalization to minimize the intensity-dependent differences between the dyes in most cases. This (and other similar types of normalization) has to assume that most signals (i.e. miRNAs) are unchanged and equal between samples. It is also possible to use signals from a set of spiked-in synthetic miRNAs (added to each labeling reaction and for which control capture probes exist) to perform the Lowess normalization. However, apart from being something synthetic added to the samples, this reduces the number of data points used, and can be a problem if the differences between the samples are very large or if something in the samples themselves affect the synthetic miRNAs during labeling or hybridization.

**Inter-slide normalization** may be needed in order to remove technical variations from labeling, hybridization and scanning of a set of arrays with different samples and to compare data directly across arrays. Inter-slide normalization is dependent on a parameter which can be assumed to be constant between arrays. The miRCURY™ LNA microRNA Arrays contain



**Tip 9**  
continued

12 snRNA capture probes and the signal obtained from these probes could in some cases be used in normalization. Using the signals from a set of spike-in control miRNAs would also be an option but again is limited by the number of spots used. One way to enable optimal normalization across arrays is to use a common reference sample on all arrays in the study. Once intra-slide normalization has taken place, the  $\log_2$  ratios between sample and reference for each miRNA calculated allowing the immediate direct comparison of all  $\log_2$  ratios from all slides. The fact that all miRNA signals are expressed as a ratio to a reference, which should be the same on each slide, reduces technical variations from the comparison.



## Use of Spike-in miRNAs

**Tip 10**

### Scanner settings

The 10 spike-in control capture probes are located on the diagonal of each subgrid, each in a total of 32 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 should give quite high signal but not be saturated. It is marked 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.

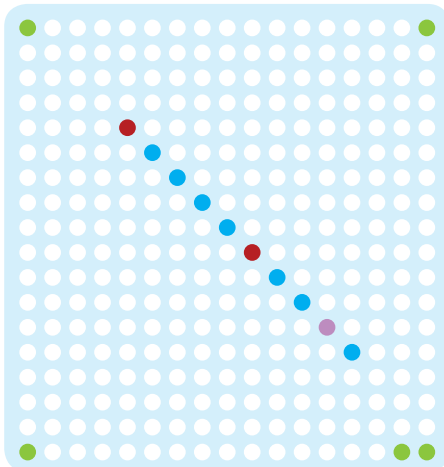


Figure 1. The location of spike-in capture probes in each subgrid on miRCURY™ LNA Arrays. The green spots are the Hy3 landing lights. The red spots are spike\_control\_i and spike\_control\_j that should have high signal close to saturation. The pink spot is spike\_control\_d that should be well below 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 2 on next page.

Tip 11

### Spike-in miRNA signal distribution

Figure 2 below shows the distribution of the 10 spike-in miRNAs spiked into 1  $\mu\text{g}$  of total RNA from human lung samples. The concentration of the various spike-in miRNAs are optimized so the signal intensities of these spike-in miRNAs are in the dynamic range of naturally expressed miRNAs in most tissues.

#### Note

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

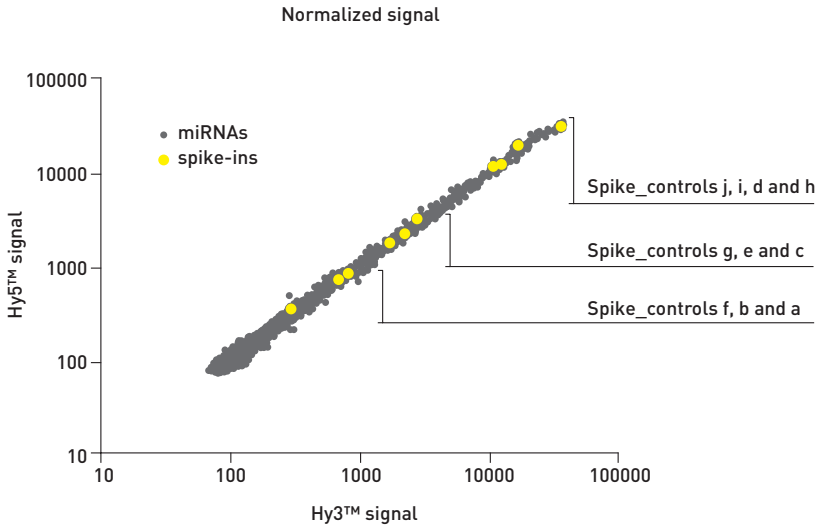


Figure 2. Scatter plot of a self-self hybridization with spike-in mix. One  $\mu\text{L}$  of the Spike-in miRNA kit was spiked into a sample of 1  $\mu\text{g}$  total RNA from human lung labeled with Hy3™. Another 1  $\mu\text{L}$  of spike-in miRNAs were spiked into 1  $\mu\text{g}$  RNA from human 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.

## 28 **Criteria for a good array run using spike-in miRNAs**

Tip 12

The array contains specific capture probes for 10 Spike-in miRNAs. The Spike-in miRNAs 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 miRNA has 32 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 miRNAs are normally within in 0.950 and 0.999 (R2).



Tip 13

## Alternative protocol for hybridization and washing using a manual procedure

### Additional required materials

LifterSlip™, for manual hybridization (e.g. Erie Scientific Company product # 22x50I-2-4711). Slide chambers for manual hybridization (e.g. Die-Tech).

### Before starting the experiment, day 1

Check the hybridization buffer for any precipitate. If necessary, warm the solution at 56° C and agitate to dissolve the precipitate completely. Heat a water bath to 56° C for overnight hybridization of the slides.

### 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™, BD Biosciences) 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 3 below are required for a large glass staining dish (8 slides, Sigma-Aldrich product # S-S6016 or similar)

Prepare for each slide wash buffers for the three washing steps.

**Table 3**

#### 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

### Day 1

#### Step 1

Combine the labeled sample(s)

The two samples from the Hy3™ and Hy5™ labeling reactions are combined on ice. Total volume must be 25 µL.

See tip 2



#### Step 2

Add 25 µL Hybridization buffer

Check for precipitation (see p. 14) in the Hybridization buffer before adding to the labeled sample(s). Mix by vortexing and spin briefly.

See tip 3



#### Step 3

Incubate 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 1x Salt buffer to the slide chamber

Prepare the slide chamber by putting 1x Salt buffer in both ends for humidity (e.g. recommended volume in a Die-Tech chamber is 2x30 µL). Place the microarray slide on top.



#### Step 6

Place slide in the slide chamber and add target preparation

Place the slide in the slide chamber and place a LifterSlip™ (Erie Scientific Company) over the spotted area and add the target preparation by pipetting into the gap between the slide and the LifterSlip™. The capillary effect draws the solution underneath the lifter slip. It is important that no air bubbles are introduced.



#### Step 7

Incubate at 56° C for 16-18 h

The slide chamber (Die-Tech or equivalent) is closed tightly and incubated 16-18 hours in a water bath at 56° C.



Total handling time: 0,5 hour

**Step 8**

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.

**Day 2****Step 9**

In Wash buffer A

The microarray slide is placed in a rack in Wash buffer A at room temperature until the LifterSlip™ falls off (Max. 30 sec., otherwise remove the LifterSlip™ manually). Repeat until all slides are disassembled.

**Step 10**

10. Wash at 56° C for 2 min. using Wash buffer A

Immerse the slides in the prewarmed buffer A and gently wash the slides by rotating the jar or moving the slides up and down in the jar.

**Step 11**

Wash briefly at room temperature in Wash buffer B

The slide is washed briefly (one plunge) in Wash buffer B (at RT) to avoid transfer of detergent to the next wash step.

**Step 12**

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

The slide is washed at room temperature by plunging gently for 2 min. in a new glass staining jar/dish/Falcon tube in Wash buffer B.

**Step 13**

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

The slide is washed at room temperature by plunging gently for 2 min. in a new glass staining jar/dish/Falcon tube in Wash buffer C.

**Step 14**

Wash briefly in water

Briefly (~1 sec.) wash the slide at room temperature in nuclease free water (one plunge).

**Step 15**

Dry the slide(s)

The slide is dried by centrifugation for 5 min. at 200 G (1000 rpm). Scan slides immediately after drying.

See tip 5



## Tip 14

**Sample concentration using RNeasy Mini Kit from Qiagen**

RNeasy Mini Kit from Qiagen (product # 74104). The following protocol has been validated and found to concentrate miRNAs with minimal depletion:

- 1 Add 350  $\mu\text{L}$  Buffer RLT to the sample, and disrupt and homogenize immediately (Vortex).
- 2 Add 3.5 volumes of 100% ethanol (1225  $\mu\text{L}$ ), and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 3.
- 3 Pipet 700  $\mu\text{L}$  of the sample, including any precipitate that may have formed, into an RNeasy Mini spin column placed in a 2 mL collection tube. Close the lid gently, and centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm). Discard the flow-through.

Repeat third step until the whole sample has been pipetted into the spin column. Discard the flow-through each time.

- 4 Place the RNeasy Mini spin column into a new 2 mL collection tube. Pipet 500  $\mu\text{L}$  Buffer RPE into the spin column. Close the lid gently, and centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash the spin column membrane. Discard the flow-through.

**Note**

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: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting" in the handbook supplied with the RNeasy Mini Kit).

- 5 Pipet another 500  $\mu\text{L}$  Buffer RPE into the RNeasy Mini spin column. Close the lid gently, and centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash the spin column membrane. Discard the flow-through and the collection tube.
- 6 Place the RNeasy Mini spin column into a new 2 mL collection tube. Centrifuge at full speed for 1 min.
- 7 Place the RNeasy Mini spin column into a 1.5 mL collection tube. Pipet 25  $\mu\text{L}$  RNase-free water directly onto the spin column membrane. Close the lid gently, and centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute the miRNA and total RNA.

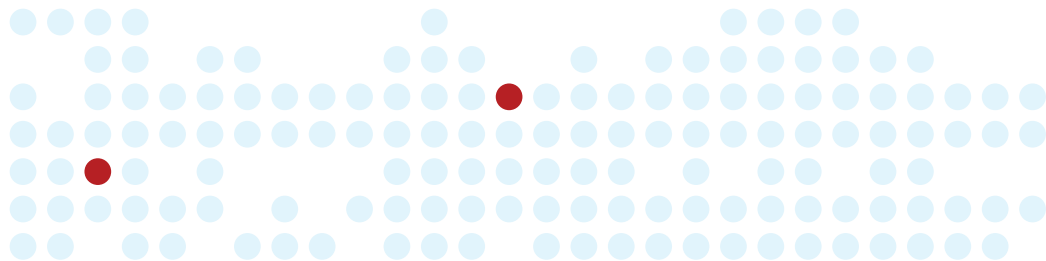
If the expected RNA yield is  $>30 \mu\text{g}$ , repeat step 7 with a second volume of RNase-free water. Elute into the same collection tube.



# References

- The microRNA Registry.  
Griffiths-Jones S. *Nucleic Acid Research*, 2004, 32, Database Issue, D109-11
- miRBase, Wellcome Trust Sanger Institute. <http://microrna.sanger.ac.uk>
- [www.exiqon.com/miRCURY/array](http://www.exiqon.com/miRCURY/array)





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