

miRCURY™ LNA microRNA Array

ready-to-spot probe set

Instruction manual
for product # 208010-A

Literature citations:

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

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Microplate layout can be found at www.exiqon.com/miRCURY/array



Product summary

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

Microplates

The 6 x 384-well microplates contain 300 pmol of each of the 2056 capture probes dried down in individual wells.

miRCURY™ LNA microRNA Array, Spike-in miRNA kit (product # 208040)

One kit containing 10 synthetic unlabeled miRNAs, 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.

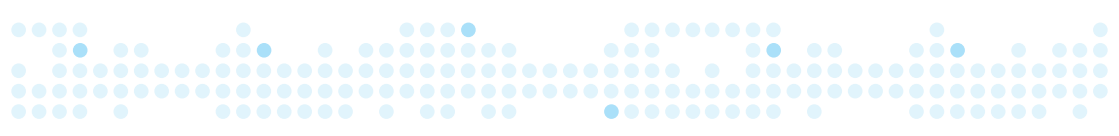
Wash buffer (product # 208021)

20x Salt buffer (2 x 125 mL).

10% Detergent solution (2 x 15 mL).

Spike-in miRNA kit (product# 208040)

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



Additional required material

miRCURY™ LNA microRNA Array Power labeling kit

Fluorescent labeling of miRNAs from total RNA samples ready for hybridization to 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 for manual hybridization.

Product description

Please visit <http://www.exiqon.com/miRCURY/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 miRNAs registered in miRBase Release 9.2. Please go to www.exiqon.com/array or contact support@exiqon.com 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 miRNAs in all organisms as annotated in miRBase Release 9.2. Please go to 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 miRs not included in miRBase. These miRPlus™ probes give researchers access to information unavailable



elsewhere.

Due to the high degree of cross-species miRNA sequence conservation, many of the capture probes specific for a miRNA in one organism can serve as mismatch controls for miRNA targets in another organism. Please go to our online miRNA resource at 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 in the probe set. Details of the control capture probes can be downloaded at 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 and 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 microplate layout 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 microRNA interactions can be found at the Exiqon resource centre at www.exiqon.com/miRCURY/array.

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 included in the miRCURY™ LNA microRNA Array ready-to-spot probe set. 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.

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

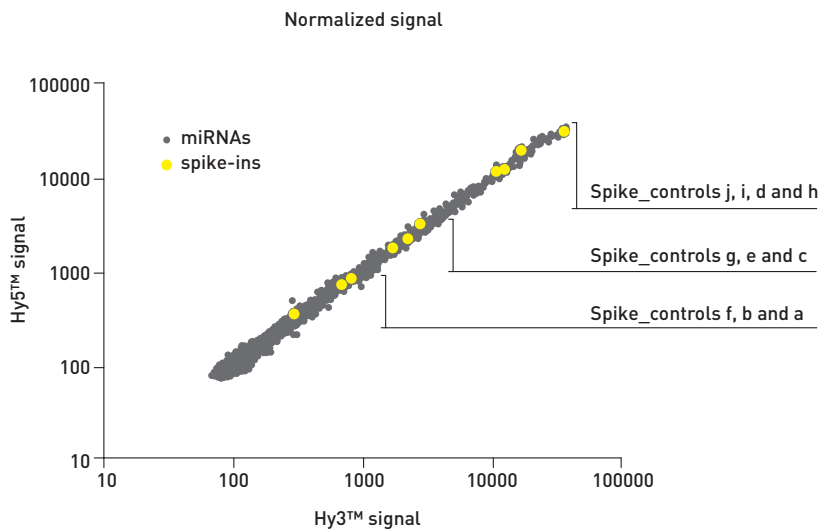


Guidelines for the spike-in miRNA signal distribution.

The figure below shows the distribution of the 10 spike-in miRNAs spiked into 1 μg 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 kit compared to signals from miRNAs will depend upon the miRNA expression level in the sample.



1 μL of the spike-in miRNAs were spiked into a sample of 1 μg total RNA from human lung labeled with Hy3™. Another, 1 μL of spike-in miRNAs were spiked into 1 μg RNA from human lung and labeled with Hy5™. Labeling was performed using the miRCURY™ LNA microRNA Array Power labeling kit. Hybridization was performed using a Tecan HS4800 hybridization station.

Storage

See tip
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The miRCURY™ LNA microRNA Array, ready-to-spot probe set should be stored dessicated at -20°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°C .

Printed arrays should be stored according to the recommendations of the slide provider.

Dissolve the spike-in miRNA 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 miRNA at -20°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°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 kits

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

miRCURY™ LNA microRNA Array, microarray kit

Pre-printed miRCURY™ LNA microRNA Array microarray slides, available in pack sizes of 3, 6 and 24 (product # 208000-A, 208001-A, 208002-A).

miRCURY™ LNA microRNA Array, Spike-in miRNA kit

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

Quickly and accurately determine miRNA expression using real-time PCR system. Available soon.



Protocol

12 Spotting recommendations

See tip
15-18

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: GE 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 μ L spotting buffer resulting in a final concentration of 20 μ M capture probe during spotting. Several other concentrations of capture probes can be successfully applied, but generally it is common to use 10-40 μ M, i.e. re-dissolve in 30-7.5 μ L spotting buffer.

Plate#3, well D23 contain a capture probe that is labeled with Hy3™. This is used as an internal control and can also be used as orientation of the spotted slides. You can remove the internal control probe by washing of the well 2 times with 3% peroxide and one time with water.

Please go to www.exiqon.com/mircury/array to download the microplate layout file.



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 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

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. 13) 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 Set the program for the hybridization station:
Two runs of wash at 56° C for 1 min. using Wash buffer A Temperature 56° C, Wash time: 1 min.,
Soak time: 1 min.



Step 9 Set the program for the hybridization station:
Two runs of wash at 23° C for 1 min. using Wash buffer B Temperature 23° C, Wash time: 1 min.,
Soak time: 1 min.



Step 10 Set the program for the hybridization station:
Two runs of wash at 23° C for 1 min. using Wash buffer C Temperature 23°C, Wash time: 1 min.,
Soak time: 1 min.



Step 11 Set the program for the hybridization station:
Wash at 23° C for 30 sec. using Wash buffer C Temperature 23° C, Wash time: 30 sec.,
Soak time: 0 sec.



Step 12 Set the program for the hybridization station:
Dry slides 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 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

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.



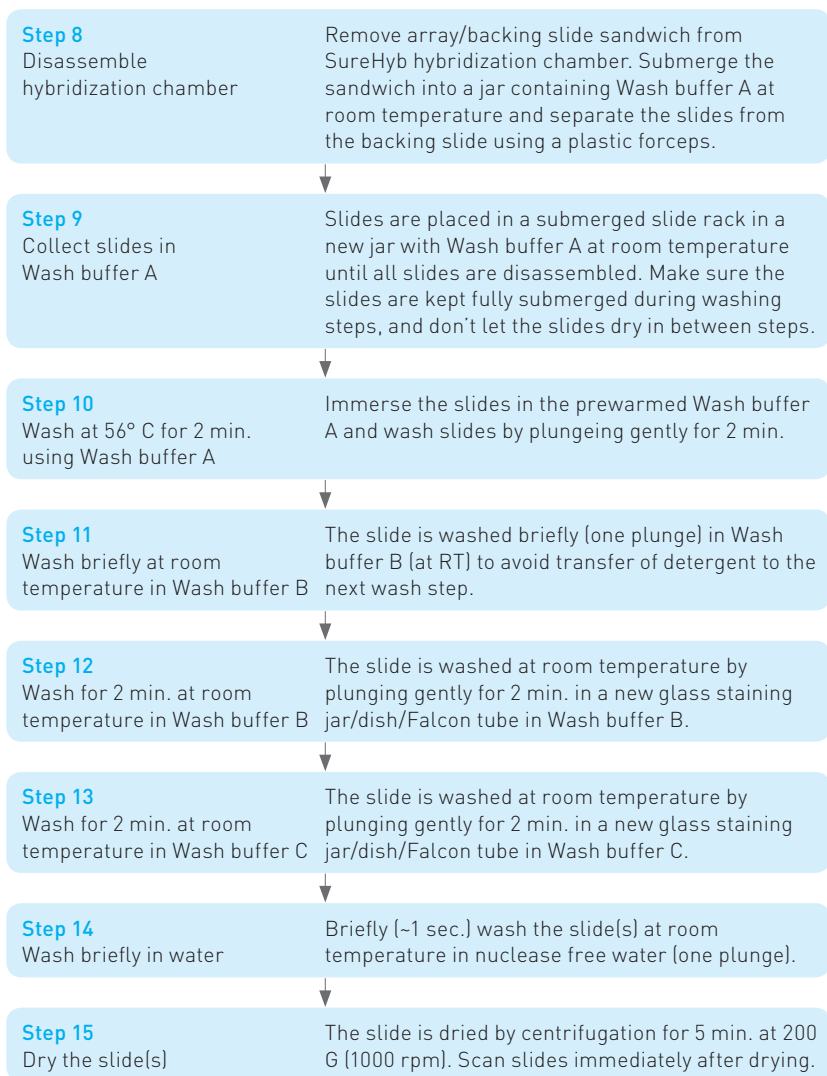


Image acquisition and quantification

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 μm 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

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Experimental procedure

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.

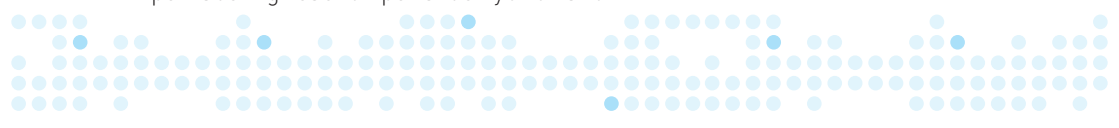
We strongly recommend to clean up a total RNA sample after RNA isolation with guanidinium solutions with or without phenol/chloroform extraction (Trizol (Molecular Research, Inc.), Qiazol™ (Qiagen GmbH), Tri reagent, etc.). The cleanup procedure must preserve miRNAs. Examples of column purification procedures 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 log₂ ratios between sample and reference for each miRNA calculated allowing the immediate direct comparison of all log₂ 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

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. Spike_control_d should give quite high signal but not be saturated. 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.



Tip 11

Spike-in miRNA signal distribution

Figure 2 below shows the distribution of the 10 spike-in miRNAs spiked into 1 μg 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.

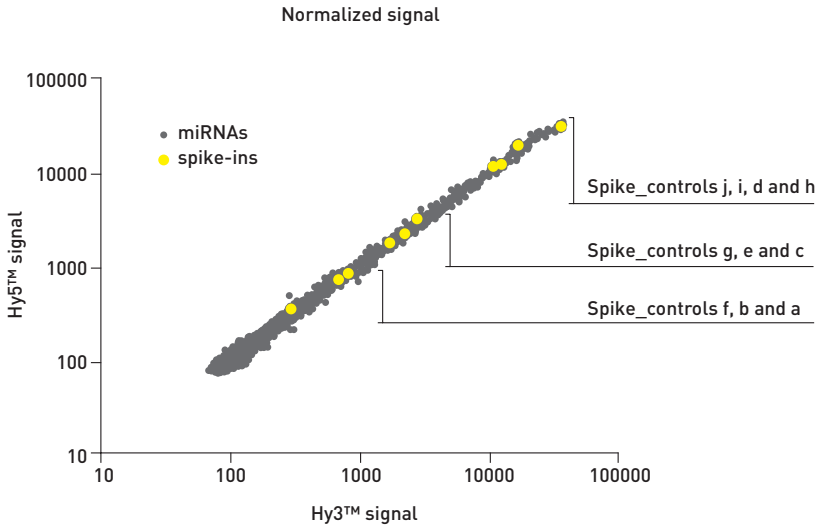


Figure 2. Scatter plot of a self-self hybridization with spike-in mix. One μL of the Spike-in miRNA kit was spiked into a sample of 1 μg total RNA from human lung labeled with Hy3™. Another 1 μL of spike-in miRNAs were spiked into 1 μg RNA from human lung and labeled with Hy5™. Labeling was performed using the miRCURY™ LNA microRNA Power labeling kit. Hybridization was performed using the Tecan HS4800™ Pro hybridization station.

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:

- Add 350 μL Buffer RLT to the sample, and disrupt and homogenize immediately (Vortex).
- Add 3.5 volumes of 100% ethanol (1225 μL), and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 3.
- Pipet 700 μL 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.

- Place the RNeasy Mini spin column into a new 2 mL collection tube. Pipet 500 μL 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).

- Pipet another 500 μL 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.
- Place the RNeasy Mini spin column into a new 2 mL collection tube. Centrifuge at full speed for 1 min.
- Place the RNeasy Mini spin column into a 1.5 mL collection tube. Pipet 25 μL 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.



Ready-to-spot probe set

Tip 15

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 16

Empty microplate wells

A number of wells in plate #4 are empty. These are D21, O21, P21, D22–P22 and all wells in column #23 +24. 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.

Tip 17

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 18

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.



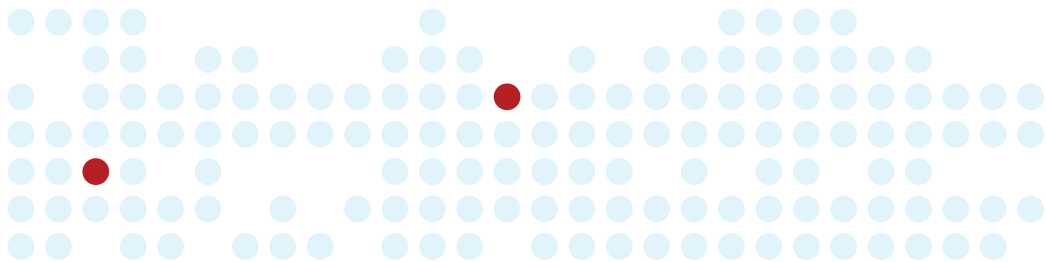
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