

miRCURY LNA™ microRNA Array

ready-to-spot probe set, v.11.0

- human, mouse & rat

Instruction manual

for product # 208210-A

Literature citations:

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

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Table of contents

Product summary	5
miRCURY LNA™ microRNA Array, ready-to-spot probe set content	5
Additional required material	5
Product description	6
Control probes	7
Storage	11
Related products	12
Protocol	13
Spotting recommendations	13
Protocol	14
Hybridization and washing using Tecan HS Pro™ hybridization stations	14
Hybridization and washing using an Agilent hybridization SureHyb chamber kit and gasket slide kit	18
Hybridization and washing using MAUI® 4-Bay or 12-Bay hybridization stations	22
Image acquisition and quantification	29
Guidelines for setting up miRNA profiling experiments	30
Single-color experiments	30
Dual-color experiments	30
Direct comparison	31
Dye swap	31
Experimental designs	32
Reference sample	35
Background subtraction	36
Local background	36
Kooperberg	36
Edwards	37
Normexp	37
Normexp plus offset	37
Normalization	38
Scaling	39
Quantile normalization	39
LOWESS	39
Cyclic LOWESS	40
Print-tip LOWESS	40
Global LOWESS	40



Variance stabilization and normalization	40
Cross correlation	41
Recommendations based on our experience	42
Software and databases	46
Tips and Trouble shooting	49
Experimental procedure	49
Guidelines for microRNA profiling experiments	52
Use of Spike-in microRNAs	53
Alternative protocol for hybridization and washing using a manual procedure	56
Ready-to-spot probe set	60
References	61

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



Product summary

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

Microplates

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

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

One kit containing 10 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].

Spike-in microRNA kit (product# 208040)

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

Additional required material

miRCURY LNA™ microRNA Array Power Labeling Kit

Fluorescent labeling of microRNAs 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 microRNAs registered in miRBase Release v.11.0. 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 microRNAs in human, mouse, rat and their related viruses as annotated in miRBase Release v.11.0. 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 microRNAs not included in miRBase. These miRPlus™ probes give researchers access to information unavailable elsewhere.

Due to the high degree of cross-species microRNA sequence conservation, many of the capture probes specific for a microRNA in one organism can serve as mismatch controls for microRNA targets in another organism. Please go to our online microRNA 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 microplate layout files, consistent with the latest updates to miRBase.



Control capture probes

Fortythree 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.
- Twentysix capture probes are included that hybridize to small nuclear RNAs.

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
19005	hsa_SNORD118	hsa
19603	hsa_SNORD13	hsa
19007	hsa_SNORD30	hsa
19008	hsa_SNORD2	hsa
19604	hsa_SNORD4A	hsa
19605	hsa_SNORD6	hsa
19011	hsa_SNORD10	hsa
19606	hsa_SNORD12	hsa
19013	hsa_SNORD14B	hsa
19607	hsa_SNORD15A	hsa
46196	SNORD38B-3	hsa
46204	SNORD38B-5	hsa
46201	SNORD44-3	hsa
46206	SNORD44-5	hsa
46200	SNORD48-3	hsa
46205	SNORD48-5	hsa
46199	SNORD49A-3	hsa
46203	SNORD49A-5	hsa
46198	SNORD66-3	hsa
46197	SNORD66-5	hsa
11278	U6-snRNA-1	hsa
11279	U6-snRNA-2	hsa



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

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

Note

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 LNATM array. These will appear with a new probe ID on this array compared to earlier versions. For more details about comparisons to older versions of the arrays, please contact support@dexiqon.com.



Spike-in microRNA Controls

The miRCURY LNA™ microRNA Array Spike in kit contains 10 different synthetic unlabeled microRNAs in different concentrations. The set can be spiked into an RNA sample prior to labeling and the synthetic Spike-in microRNA kit will hybridize to corresponding capture probes included in the miRCURY LNA™ microRNA Array ready-to-spot probe set. The Spike-in microRNA kit has been designed and tested not to cross-react with endogenous microRNAs from human, mouse or rat, and is provided at concentrations compatible with endogenous microRNA expression levels. The Spike-in microRNA kit is supplied with different concentrations of synthetic spike-in microRNAs aimed at spanning the whole intensity range of microRNAs in most tissue samples.

Note

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Please refer to the instruction manual for miRCURY LNA™ microRNA Power Labeling Kits, for further instructions on how to use the Spike-in microRNA kit during the labeling procedure. When the spike-in microRNAs are added in equal amounts to labeling reactions before a dual-color array hybridization, the signals from the spike-in capture probes can be used:

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



Guidelines for the spike-in microRNA signal distribution.

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

Note

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

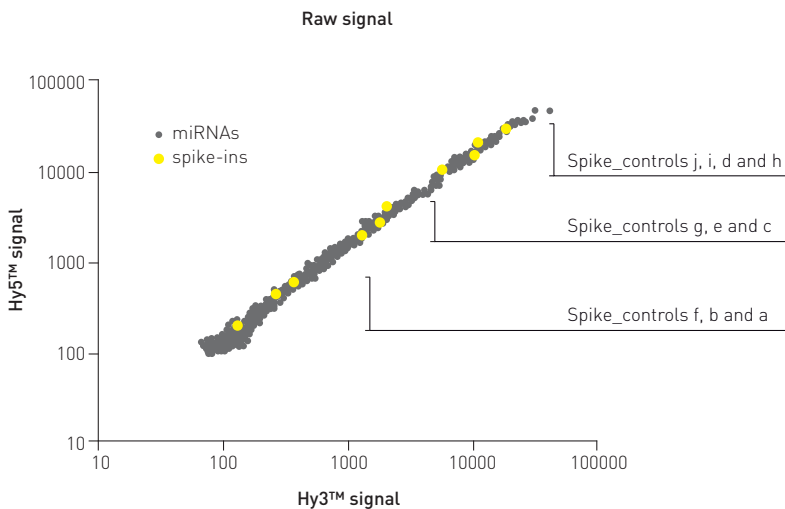


Figure 2. Scatter plot of a self-self hybridization with spike-in mix. One μL of the Spike-in microRNA kit was spiked into a sample of 1 μg total RNA from human lung labeled with Hy3™. Another 1 μL of spike-in microRNAs were spiked into 1 μg RNA from human lung and labeled with Hy5™. Labeling was performed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

Storage

See tip
18

The miRCURY LNA™ microRNA Array, ready-to-spot probe set should be stored desiccated 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. If stored properly shelf life for the miRCURY LNA™ microRNA Array, ready-to-spot probe set is 1 year.

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. Store the dissolved spike-in microRNA at -20° C until use and avoid repeated cycles of freeze/thawing. You may wish to aliquot the dissolved spike-in microRNAs to avoid repeated freeze/thawing. For long-term storage, keep the vial at -80° C If stored properly shelf life for the miRCURY LNA™ Array spike-in microRNAs is 1 year. In solution the shelf life for the spike-in microRNAs the is 3 months.



Related products

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

miRCURY LNA™ microRNA Power Labeling Kits

For fluorescent labeling of microRNAs 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, 208200-A, 208201-A, 208202-A).

miRCURY LNA™ microRNA Array, Spike-in microRNA kit

Ten 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 # 208040).

miRCURY LNA™ microRNA Array, Hybridization buffer

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

miRCURY LNA™ microRNA Array, Wash buffer kit

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

miRCURY LNA™ microRNA Detection

For in situ hybridization and northern blotting of all annotated microRNAs

miRCURY LNA™ microRNA Knockdown

microRNA knockdown probes: determine or confirm microRNA function

miRCURY LNA™ microRNA Real-time PCR

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



Protocol

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



Protocol

14 Hybridization and washing using Tecan HS Pro™ hybridization stations

Before starting the experiment

Total RNA should be prepared using a method that preserves small RNA species. When using commercially available kits, please verify that the total RNA preparation contains small molecular weight RNAs.

We recommend 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 microRNA content of the cells or tissue being analyzed as this amount is known to vary. Without prior knowledge of microRNA content in the sample to be analyzed we would recommend to use between 250 ng and 1 µg of total RNA per labeling reaction per slide hybridization.

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



Total handling time: 1 hour

Protocol

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 an Agilent hybridization SureHyb chamber kit and gasket slide kit

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

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

Additional required materials:

Hybridization Chamber Kit - SureHyb enabled, Agilent part # G2534A
Hybridization Gasket Slide Kit (5) - 1 microarray per slide format, Agilent part # G2534-60003
Hybridization oven with rotation (e.g. SciGene, # 400).
Ethanol 99%

Before starting the experiment, day 1

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

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

See tip
1



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

Before starting the experiment, day 2

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

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

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

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



Total handling time: 1 hour

Protocol

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].

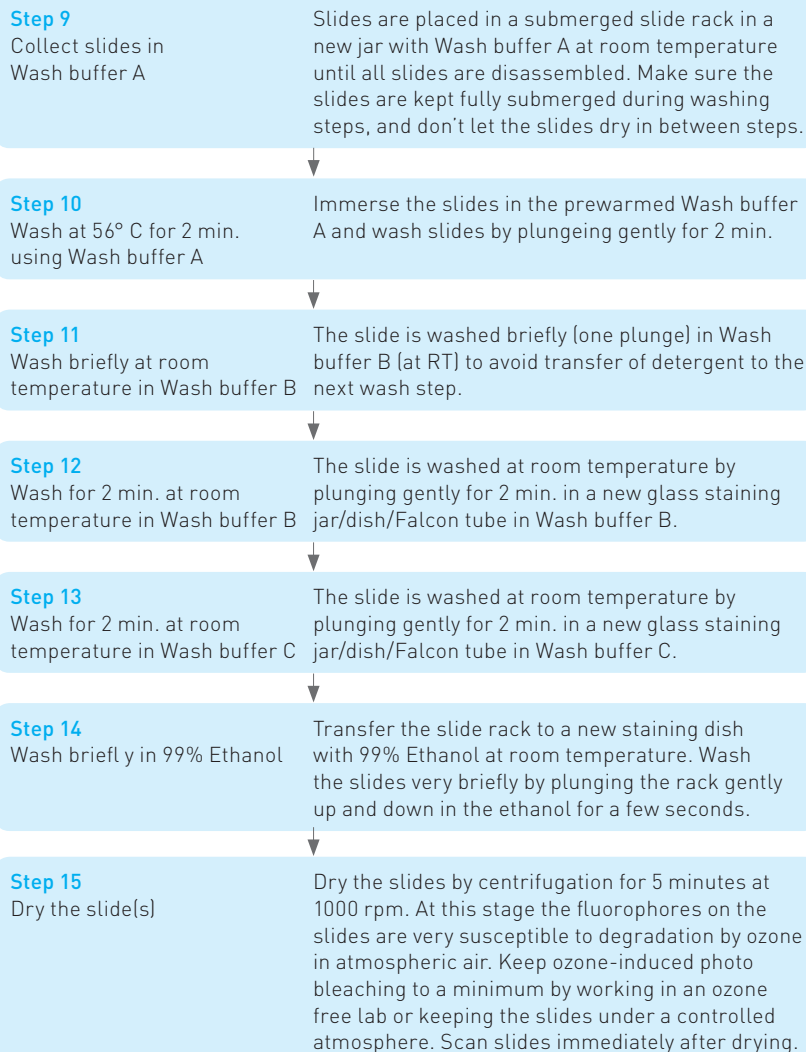
Step 7
Place Wash buffer A at 56° C overnight

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.



**See tip
5**

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

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

The MAUI® Hybridization System is comprised of two main components, the disposable MAUI® Mixer hybridization chambers and MAUI® instrument that powers the mixing bladders in the Mixer and maintains a constant incubation temperature. The MAUI® Mixer adheres to the microarray slide via an adhesive gasket forming a uniform, low volume, sealed hybridization chamber. Once attached, the Mixer-slide is clamped into one of the heated slide bays in the base unit, where hybridization takes place. For details about using the MAUI® Hybridization System please see the User's Guide that come with the MAUI® Hybridization System or is available from BioMicro Systems. This Protocol provides detailed information about performing a competitive hybridization of two Hy3/Hy5 labeled RNA samples on Exiqon miRCURY LNA™ Arrays using the MAUI® Hybridization System. For details about preparing the samples and performing the RNA labeling reactions please see the Instruction Manual for the miRCURY LNA™ Power Labeling Kit available from Exiqon's website.

Additional required materials:

- RNA samples (labeled with miRCURY LNA™ Power Labeling Kit)
- MAUI® SL-mixers
- MAUI® Humidity tray
- MAUI® A/D jig
- MAUI® Gasket brayer
- Positive Displacement Pipette (optional)
- 7 Rectangular Staining Dishes, 250 mL, w/slide washing racks (e.g. Wheaton # 900200 /VWR# 25461-003).
- Heating block set to 56° C.
- Oven set to 56° C.



Before starting the experiment, day 1

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

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

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

See tip
1

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

Step 1

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

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

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



Step 2

Prepare the labeled sample(s)

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

See tip
2



**See tip
9****Step 3**

Add 25 μ L
2X Hybridization buffer

Add 25 μ L 2X Hybridization buffer to the labeled sample(s). Mix by vortexing and spin briefly. Final volume 50 μ L. Prepare additional 25 μ L water + 25 μ L 2X Hybridization Buffer and leave it at 56° C for use in a later pipette pre-wetting step.

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 5 min. prior to loading into the slide-mixer assembly.

Step 6

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

- Wet the pipette tip by pipetting up and down in 1X Hybridization Buffer pre-heated to 56° C. Briefly spin the pre-heated sample prior to opening the tube. Aspirate 45 μ L of sample into the pipette being careful not to draw any air into the pipette. Should any bubbles appear within the loaded tip, dispense the solution back into the tube and draw again. Discard the remainder of the sample.



Step 6 (Continued)

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

- Insert the pipette tip into the fill port in the tab-end of the SL-mixer and carefully inject the sample into the chamber until sample emerges from the vent port. The actual volume of the SL-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 and wipe surface clean with a tissue.
- Using forceps, place adhesive port seals directly over both ports. Use a finger on each port seal and press down firmly on both seals simultaneously to seal the ports.

**Step 7**

Incubate at 56° C for 16 h.

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

**Step 8**

Preparation of washing procedure

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

**Step 9**

Prepare wash buffers

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



Step 10

Disassemble A/D jig

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

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

**Step 11**

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

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

**Step 12**

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

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

**Step 13**

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

Transfer the slide rack to a new staining dish with Wash Buffer C at room temperature. Wash the slides for 2 minutes by plunging the rack gently up and down in the buffer.



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**

Image acquisition and quantification

Relevant microplate layout 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 microplate layout 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.



Guidelines for setting up miRNA profiling experiments

30

In the following chapter we will briefly comment on various subjects that must be considered before setting up microRNA profiling experiments. Exiqon miRCURY LNA™ Arrays are designed for dual-color experiments using Hy3 and Hy5 as labeling dyes. Provided that normalization overrules interslide differences, experienced users may want to perform single-color experiments using the miRCURY LNA™ Array platform.

Single-color experiments

Either Hy3 or Hy5 can be used for single-color experiments; however, Hy3 is more commonly used due to its greater stability. While single-color experiments avoid dye association problems (e.g. ozone bleaching and dye bias), they do require inter-slide signal monitoring and adequate normalization.

Dual-color experiments

When performing dual-color array experiments the following must be considered:

The two dyes used must have a different dynamic range for their concentration-dependent light emission: For low intensity signals, green fluorescent dyes show a higher signal intensity compared to red fluorescent dyes when both are used for labeling the same amount of a specific sample. These intra-slide differences of dye-dependent signal intensities can be corrected using the LOWESS algorithm to normalize.

Furthermore, the labeling reaction itself is dependent on the dye used. This means that a specific microRNA can show different labeling efficiency with two different dyes due to their chemical/sterical characteristics. In a direct comparison of two samples labeled with two different dyes, this dye-specific difference may be misinterpreted as a biological effect unless it is ruled out by a dye-swap control experiment.

The two primary experimental design options when using dual-color array platforms are: A direct comparison of pairs of samples on individual arrays,



or a reference design in which each sample of the study is hybridized against a common reference sample.

Direct comparison

Ideally, the direct comparison of pairs of samples by hybridization on one slide lets you see the differences in microRNA expression between two samples. This can be useful in cases in which natural pairs exist which are not related to other samples (such as tumor and normal samples from the same patient). However, this design has several limitations, such as: The analysis is limited to looking directly at differences between the pairs. As each pair of samples is different, the normalization across arrays and comparison between samples on different slides is impaired (due to the lack of a common factor). The array results will reflect both technical and biological variations between the two samples. If dye-specific labeling differences exist, a dye swap experiment helps to discriminate between technical and biological signal intensity changes.

Dye swap

The dyes used to label the sample have different physical and chemical characteristics, which may cause some dye-specific labeling differences. In direct comparison experiments in which different samples are labeled with different dyes and compared with each other, labeling differences can be misinterpreted as biological differences. To avoid such a misinterpretation, a dye swap is recommended. This technical replicate with inversed labeling allows for discrimination between the biological differences and the dye-specific differences.



Experimental designs

Figure 2

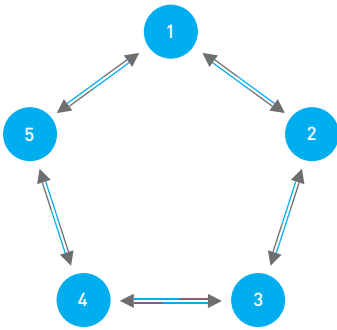


Figure 2. The loop design allows the pairwise comparison of each sample with its direct neighbor in a circular fashion. It is used for samples of equal importance. This design enables the detection of differences and reduces the variance per estimate because each sample occurs twice. This setup makes the evaluation of samples that are not directly compared more difficult.

Figure 3

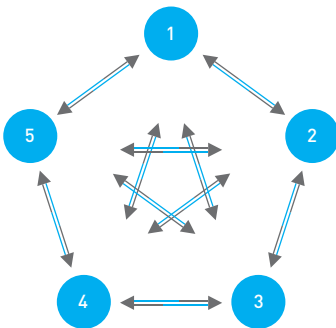


Figure 3. The all pairwise comparison design allows the comparison of each sample with all other samples in a balanced block design. It is used for samples of equal importance. This design is robust and redundant, giving a good base for statistical evaluation.



Figure 4

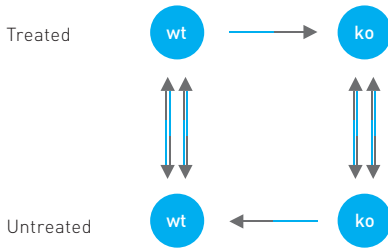


Figure 4. The multiple pairwise comparison design takes into account that the difference between two samples is more important and, therefore, is evaluated with more replicates than the comparison between different subsets.

Figure 5

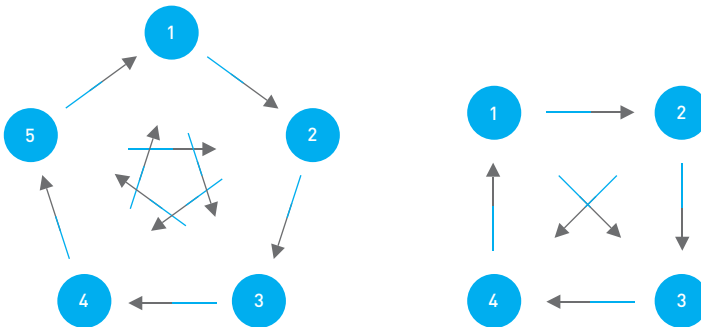
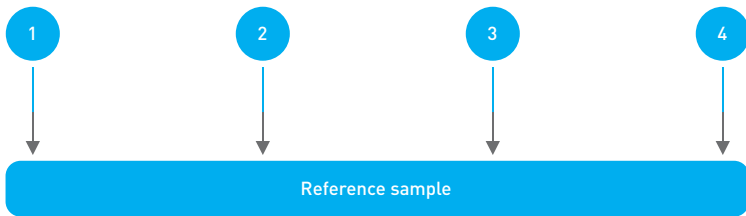


Figure 5. In a balanced experimental setup each sample is labeled with both dyes and hybridized in a manner that results in an equal number of data sets for each dye and each sample. Therefore, the resulting interwoven loop can be used to discriminate between dye-specific and biological differences. This type of setup can be difficult to analyze and interpret.

The common reference design is essentially equivalent to performing single-color hybridizations with each sample in the study, but uses a common reference sample in one of the channels that all other samples are hybridized against. The common reference can then be used as a common factor to which signals from all samples can be normalized, enabling direct comparison. The common reference design is recommended when the study contains more than two different types of sample to be compared. In addition, it also has advantages for typical paired sample studies in that it allows technical and biological variations to be separated. The common reference design provides information concerning variation between replicates and, importantly, can be used to identify outlying samples.

Figure 6



Reference sample

The ideal reference sample is always one that is as similar as possible to the actual samples and is likely to contain all microRNAs found in any of the samples in the study. Both of these conditions are fulfilled by making a reference sample from a pool of all the samples in the study. This requires that there is enough RNA available from each sample to contribute to both the pool and the sample to be hybridized. In addition, if the study might be extended at a later date with further samples, it is best to use the same reference for all studies.

The reference sample should always be either as closely related as possible to the samples in the study (but still containing all microRNAs likely to be found in the samples), or as complex as possible. A complex reference sample could be a pool of total RNA extracted from many different tissues and in which most microRNAs would likely be found.

Commercially available options include Ambion's FirstChoice® Human Total RNA Survey Panel consisting of 10µg each from 20 different tissues. The same type of panel exists for mouse, but only covers 10 tissues. This commercially available reference also enables the comparison of large-scale experiments run in several batches.

A universal reference sample, such as the one from Stratagene/BIOTAQ/ SuperArray, may be another solution. However, we have not tested those preparations.

Further reading on experimental design

Townsend, J.P. Multifactorial experimental design and the transitivity of ratios with spotted DNA microarrays. *BMC Genomics* 2003, 4: 41

Dobbin, K., *et al.* Statistical design of reverse dye microarrays. *Bioinformatics* 2003 May 1;19(7):803-10

Yang, C.W., *et al.* Evaluation of experimental designs for two-color cDNA microarrays. *J. Comput Biol.* 2005 Nov;12(9):1202-20



Background subtraction

Background subtraction is the first step in adjusting the raw data towards comparability. It has consistently been found to correct log ratios preventing underestimation of the observed changes in signal intensities. Different methods of background correction should be considered.

Local background

The easiest and best-known standard approach is to subtract the local background (mean or median) surrounding the spot from the spot signal. This results in unbiased intensities of the signal based solely on hybridization. As a consequence, this procedure gives highly variable low intensity values. In addition it may lead to loss of values due to negative, and thus unprocessable, signal intensities.

Several other methods using the local background have been established in order to circumvent the above-mentioned problems. These approaches are based on either subtracting values derived from mathematical models or nonlinear filters instead of the actual local background (TV-L and Morph) or they are based on signal intensity calculations that do not use subtraction to obtain a background corrected value (Koopberg, Edwards, Normexp and VSN).

Koopberg

The Koopberg background correction is a Bayesian model-based approach with convolution of normal distributions for spotwise background correction. This computationally intensive approach uses mean and standard deviation of foreground and background together with single pixel data of the same. The Koopberg background correction is channel separating; the background is obtained from the three to four nearest-neighbor spots.



Edwards

The Edwards background correction is based on a threshold and log-linear interpolation. The background subtraction is only performed on signals in which the difference between foreground and background is higher than the given threshold. For the remaining values, a monotonous smoothing function replaces subtraction.

Normexp

The Normexp background correction is based on the former RMA algorithm. It is a convolution model assuming that background signals are normally distributed while the sample signals are exponentially distributed. In contrast to the RMA algorithm the Normexp algorithm is channel separating and uses a maximum likelihood estimation simplified by way of a saddlepoint approximation.

Normexp plus offset

In addition to the Normexp algorithm, the background correction method Normexp plus offset uses a small correction factor to shift small values around zero to a value higher than zero which serves as variance stabilizing element.



Normalization

Normalization is a mathematical adjustment of data to eliminate systemic errors, such as dye bias and differences in labeling, hybridization and scanning, which can occur when performing an array experiment. The aim of the normalization is to make data of different sources comparable. However, each data modification may also affect the biological data the experimenter wants to preserve. Therefore, normalization should be kept minimal. Normalization is performed on data from individual arrays (intra-slide normalization) and on data from a set of arrays (inter-slide normalization). 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 the signal mean from the whole data set (global normalization) or on controls like housekeeping genes that are assumed to stay constant between different samples (internal normalization) or added spike-in controls (external normalization).

However, the normalization methods that are readily applicable to microarrays with large numbers of spots (such as mRNA arrays), and are thus based on a statistically solid foundation, have to be cautiously evaluated for microRNA arrays. This is due to the fact that microRNA arrays have relatively few spots and that unlike mRNA expression levels microRNA expression levels can vary significantly between samples. In addition, there are no thoroughly verified small RNA housekeeping genes or constant controls.

Spike-ins are artificial RNAs added to the sample used for monitoring the performance of the experiment. Depending on the time point of spiking in, the signals from a set of additional synthetic microRNAs (added prior to each labeling reaction and for which control capture probes exist) can be used as a monitoring control for RNA preparation, labeling or inter-array reproducibility. The signal of the labeled spike-in is recovered by hybridization to the appropriate capture probes present on the array. If the spike-ins provide sufficient data, they may even be used for normalization (see recommendations).

Inter-slide normalization may be necessary to remove technical variations from labeling, hybridization and scanning a set of arrays with different samples and to compare data directly across arrays. Inter-slide normalization is dependent on a parameter that can be assumed to be constant between arrays. For the different normalization methods the majority of signal



intensity remains unaffected, thus using statistics to separate biological relevant signal differences from the mass of unchanged signal. This approach is impaired when the differences between samples are big and the number of unaffected signal to normalize on is reduced. While this problem is rare in mRNA array experiments, it has to be considered when setting up microRNA analysis using microarrays.

Scaling

Scaling is performed on the linear scale not the log scale in a chipwise manner. It adjusts the overall mean or median signal intensity. The obtained scaling factor for each array is then used to multiply each of the signal values of the chip. Because microRNA arrays have low data density, scaling may not be the best choice to normalize these arrays.

Quantile normalization

Quantile normalization is based on the assumption that two sets of closely related data should sort themselves in a linear fashion forming a diagonal when plotted against each other. For example, quantile normalization can be used to adjust the data from two different arrays. It enforces an equal intensity distribution on the data.

LOWESS

LOWESS (Locally Weighted Scatterplot Smoothing) is the use of a locally established regression to smooth the M/A (log ratio/log mean-intensity) scatterplot toward a linear distribution. The LOWESS algorithm works under the assumption that the majority of the signals between samples do not differ and it enforces equal overall means on all signal intensities. Therefore, LOWESS allows the correction of systematic deviations in the MA plot giving an intensity-dependent adjustment of MA-data to a straight line. The different LOWESS normalizations differ in the subpopulations used in the algorithm.



Cyclic LOWESS

The cyclic LOWESS algorithm allows normalization of single-color experiments using the pairwise LOWESS comparison of the signal intensities from two array experiments at a time. For each comparison, a trend line for correction will be established, thus allowing each array to be compared against each other by repeated pairwise comparison of two arrays.

Print-tip LOWESS

The print-tip LOWESS algorithm uses the LOWESS algorithm for each print-tip group, thus allowing the normalization for print-tip specific differences.

Global LOWESS

Global LOWESS does not take into account spatial difference when using the LOWESS algorithm for normalization. It is a LOWESS algorithm that is based on the determination of the lowest variance of data from the probe sets from all slides in their signal intensity subgroups.

Variance stabilization and normalization

Variance stabilization and normalization (VSN) is an integrated normalization method using a background correction factor, as well as, additional additive and multiplicative error correction factors. The variance stabilizing transformation is used in order to reduce the dependence of variance and signal intensity. It is based on an arcsinh function that replaces the logarithmic transformation. The output of the formula is equivalent to a log-ratio for high signal intensities while it resembles a mere signal subtraction for low signal intensities.



Cross correlation

Cross correlation is another form of normalization that is based on a M/A plot pattern recognition algorithm. This algorithm is peak matching to the distribution of normalized log-ratios assuming the largest number of signals being invariant. It requires a template like the distribution of normalized log ratio of a self-hybridizing experiment. The calculation is done for intensity subsets to cope with nonlinearity when matching the distribution of the log ratio.

Further reading on background correction and normalization methods:

Ernst Wit, E., McClure, C.J.D. *Statistics for Microarrays: Design, Analysis and Inference.*

Ritchie, M.E., *et al.* A comparison of background correction methods for two colour microarrays. *Bioinformatics.* 2007 Oct 15;23(20):2700-7

Bolstad B.M., *et al.* Experimental design and low-level analysis of microarray data. *Int Rev Neurobiol.* 2004;60:25-58

Bolstad, B.M., *et al.* A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003 Jan 22;19(2):185-93

Chua, S.W., *et al.* A novel normalization method for effective removal of systematic variation in microarray data. *Nucleic Acids Res.* 2006 Mar 9;34(5)



Recommendations based on our experience

42

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

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

Protocol

Step 1 Scanning

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



Step 2

Spot evaluation and background subtraction

In general, we recommend using local background subtraction. We subtract the local median background signal from each spot using ImageJ. When using more advanced background subtraction, 'Normexp plus offset' convinced us with satisfying results. Since we like to perform the data analysis in a controlled manner, we are not using feature extraction software on a routine level. However, we provide a short protocol for customers who like to use this software (www.exiqon.com/12602).

**Step 3**

Normalization

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

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



Step 3 (Continued)

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

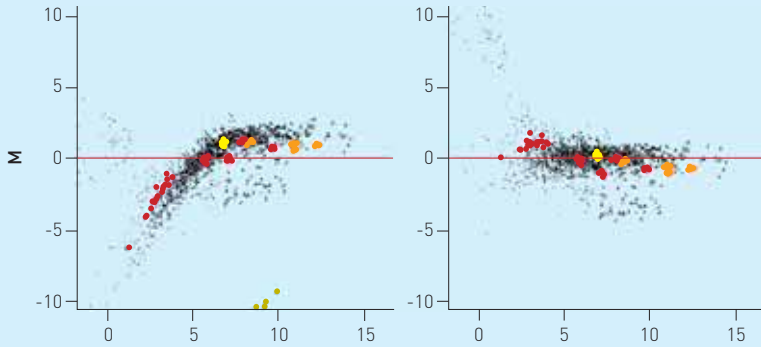
When two directly related samples are to be compared, we recommend a direct comparison. We obtain most reliable results when performing a technical replicate in form of a dyeswap for each analysis.

**Step 4**
Data analysis and
visualization

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

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



Step 4 (Continued)

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

Step 5
Data evaluation

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



Software and databases

46

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

Image analysis software

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

TM4 package TIGR Spotfinder

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

ScanAlyze

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

Bzscan

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



Further image analysis reading

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

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

Basic statistical analysis software

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

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



MicroRNA Software and Databases:

Annotation database:

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

Promotors:

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

production site:

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

Annot. Target database:

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

Target prediction:

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



Tips and Trouble shooting

Experimental procedure

49

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 microRNAs. 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 microRNAs 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 microRNA enriched samples versus total RNA samples. We do see some differences comparing microRNA enriched and non-enriched microRNA samples. Due to the risk of losing information we do not recommend microRNA enrichment of the total RNA samples.

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



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, microRNA arrays have relatively few spots, microRNA expression levels can vary a lot between samples and there are no identified house-keeping microRNAs 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. microRNAs) are unchanged and equal between samples. It is also possible to use signals from a set of spiked-in synthetic microRNAs (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 microRNAs 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 microRNAs 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 microRNA calculated allowing the immediate direct comparison of all log₂ ratios from all slides. The fact that all microRNA signals are expressed as a ratio to a reference, which should be the same on each slide, reduces technical variations from the comparison.

Use of Spike-in microRNAs

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 microRNA signal distribution

Figure 2 below shows the distribution of the 10 spike-in microRNAs spiked into 1 µg of total RNA from human lung samples. The concentration of the various spike-in microRNAs are optimized so the signal intensities of these spike-in microRNAs are in the dynamic range of naturally expressed microRNAs in most tissues.

Note

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

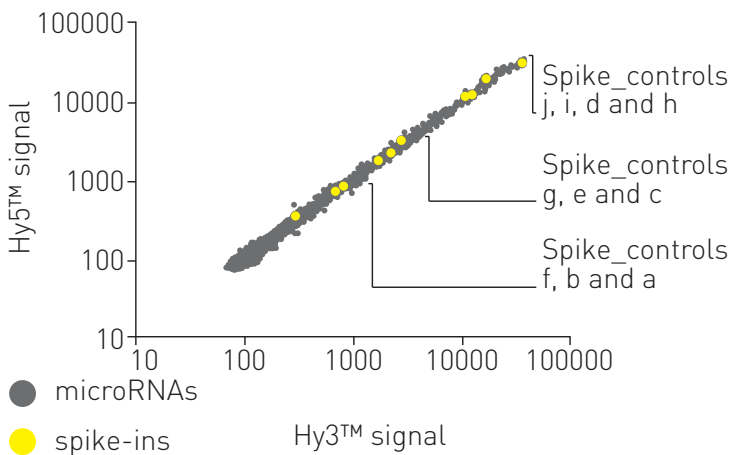


Figure 2. Scatter plot of a self-self hybridization with spike-in mix. One µL of the Spike-in microRNA kit was spiked into a sample of 1 µg total RNA from human lung labeled with Hy3™. Another 1 µL of spike-in microRNAs were spiked into 1 µg RNA from human lung and labeled with Hy5™. Labeling was performed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

Tip 12

Criteria for a good array run using spike-in microRNAs

The array contains specific capture probes for 10 Spike-in microRNAs. The Spike-in microRNAs cover the full signal range (High range; spike-in d, h, i and j, medium range; spike-in c, e and g; low range; a, b and f). Each spike-in microRNA has 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 microRNAs are normally within in 0.950 and 0.999 (R2).



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

Place Wash buffer A at 56° C overnight

Day 2

Step 9 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.)

In Wash buffer A

Step 10 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.

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

Step 11 The slide is washed briefly (one plunge) in Wash buffer B (at RT) to avoid transfer of detergent to the next wash step.

Wash briefly at room temperature in Wash buffer B

Step 12 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.

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

Step 13 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.

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

Step 14 Briefly (~1 sec.) wash the slide at room temperature in nuclease free water (one plunge).

Wash briefly in water

Step 15 The slide is dried by centrifugation for 5 min. at 200 G (1000 rpm). Scan slides immediately after drying.

Dry the slide(s)

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 microRNAs with minimal depletion:

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

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

- Pipet another 500 μ 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.
- 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 μ 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 microRNA and total RNA.

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



Ready-to-spot probe set

60

READY-TO-SPOT PROBE SET | Instruction Manual

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

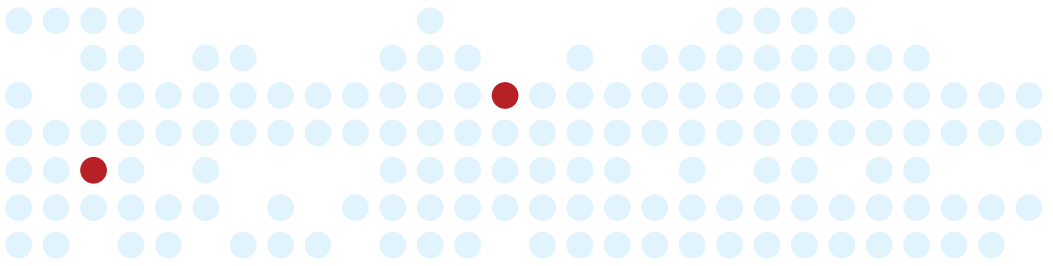
The capture probes have physical and chemical properties identical to similar DNA capture probes and should be treated accordingly. The capture probes are short, amino-modified oligo-nucleotides with individual monomers substituted with LNA™. When dissolved the capture probes should not be subjected 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.



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





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