# miRCURY LNA™ microRNA Array

## Power Labeling kit

**Instruction manual v3.0**

for product # 208030-A, 208031-A, 208032-A

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EXIQON

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# Product Summary

## miRCURY LNA™ microRNA Array, Power labeling kit content

The miRCURY LNA™ microRNA Array Power labeling kit consists of 8 vials as described in Table 1.

**Table 1**

| Kit Components | Amount supplied | Tube Label |
|---|---|---|
| Calf Intestine Phosphartase (CIP) | 20 µL | White |
| CIP buffer | 20 µL | Brown |
| Hy5™ fluorescent label | 12 or 24 reactions§ | Blue |
| Hy3™ fluorescent label | 12 or 24 reactions§ | Red |
| Labeling enzyme | 48 µL | Yellow |
| DMSO | 100 µL | Transparent |
| Labeling buffer | 150 µL | Orange |
| Nuclease-free water | 500 µL | Green |

§ Product # 208032-A for dual colour labeling contain one tube with the Hy5™ dye and one tube with the Hy3™ dye. The kits for single colour labeling (product # 208030-A and 208031-A) contains two tubes with the same dye. Each tube contains sufficient label for 12 labeling reactions.

## Additional required material

Total RNA containing the small RNAs.
RNase-free 0.5 mL microcentrifuge tubes.
Microcentrifuge.
Incubator or heating block.
Nuclease-free water.
miRCURY LNA™ microRNA Array kit.

# Product description

The miRCURY LNA™ microRNA Array Power labeling kit will label RNA molecules with a single fluorophore per molecule. All small RNAs are uniformly labeled. The miRCURY LNA™ microRNA Array labeling kit will label all microRNAs, including those from animals, plants as well as viruses. The labeled RNA molecules may be hybridized to the pre-spotted miRCURY LNA™ microRNA Arrays.
The miRCURY LNA™ microRNA Array Power labeling system follows a simple and fast 2-step protocol. The first step includes a Calf Intestinal Alkaline Phosphatase for removal of 5'-phosphates from terminal of the microRNAs. In the second step, a fluorescent label is attached enzymatically to the 3'-end of the microRNAs in the total RNA sample. This is followed by an enzyme inactivation step after which the sample is ready for hybridization.

## Spike-in miRNA Controls

The miRCURY LNA™ microRNA Array Spike-in miRNA kit v2 is included in the miRCURY LNA™ microRNA Array, microarray kits, the miRCURY LNA™ microRNA Array, ready-to-spot probe sets, (product# 208410) and available as a seperate product (product# 208041).
The miRCURY LNA™ microRNA Array Spike-in miRNA kit v2 contains 52 different synthetic unlabeled microRNAs in different concentrations. The set can be spiked into an RNA sample prior to labeling and the synthetic spike-in microRNAs will hybridize to corresponding capture probes on the miRCURY LNA™ microRNA Array.
For detailed description please see the instruction manual for the miRCURY LNA™ microRNA Array Kit.

## Storage

The miRCURY LNA™ microRNA Array Power labeling kit is shipped in plastic foam (polystyrene) containers with dry ice. Though it is recommended to store the kit at -20° C, exposure to higher temperatures (4 to 10° C) during shipping does not pose any risk to the enzymes.
The fluorescent labeling dyes are shipped dry for increased stability. Before use, add 30 µL of RNase-free water, vortex and spin to collect tube content. Before opening the tubes, briefly spin down all tubes to collect content. The fluorescent labeling dyes in the kit should not be subjected to repeated cycles of freeze/thawing. Instead it is recommended to aliquot the dyes and store at -20° C. For long-term storage of the dyes keep the vial(s) at –80° C.
All vials in the kit should be kept on ice during laboratory work and the dyes should always be kept in the dark protected from light.

## Protocol overview

### miRCURY LNA™ microRNA Power Labeling Kit

| | |
|---|---|
| CIP treatment | Mix: RNA sample, CIP buffer, Spike-In microRNA and CIP enzyme |
| ↓ | |
| Labeling reaction | Mix: CIP treated RNA sample<br>Labeling buffer<br>Hy3™ or Hy5™<br>DMSO<br>Enzyme |
| ↓ | |

### miRCURY LNA™ microRNA Array Kit

| | |
|---|---|
| Mix samples | Dual color mix:<br>Hy3™ labeled sample<br>Hy5™ labeled sample<br>Hybridization buffer<br>Denature sample<br><br>Single color mix:<br>Hy3™ labeled sample<br>Hybridization buffer<br>Denature sample |
| ↓ | |
| Hybridize | Hybridize at 56°C for 16 hours |
| ↓ | |
| Stringency wash | Wash 2 min. in buffer A at 56°C<br>Wash 2 min. in buffer B at 23°C<br>Wash 2 min. in buffer C at 23°C<br>Dry slides |
| ↓ | |
| Image aquisition | Scan slides (recommended scan at 5µm)<br>Download relevant GAL files from www.exiqon.com |

# Protocol



## Preparation of RNA sample

See tip 7

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 using our miRCURY™ RNA Isolation Kits for total RNA purification.

Procedures that include an acidic phenol chloroform extraction are generally recognized as methods that preserve small RNAs. Subsequent to any RNA extraction procedure using a guanidine isothiocyanate–phenol:chloroform extraction, we recommend a column purification procedure to remove traces of theses chemicals that potentially may inhibit labeling. Our miRCURY™ RNA Isolation Kits can be used for this procedure. The purified total RNA should be dissolved in RNAse-free water at a concentration of 0,5-1 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 microRNA in the miRCURY LNA™ microRNA Array labeling kit uses total RNA and 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 recommend using 0,25-1 µg of total RNA per labeling reaction per slide hybridization. Exiqon has compared microRNA expression pattern using a range of 0,25-5 µg labeled total RNA, obtaining same expression pattern in this range.

## Before starting the experiment

Prior to performing the labeling, dissolve the fluorescent dye(s) by adding 29 µL of nuclease-free water to the tube with the labeling dye, followed by vortexing and a brief centrifugation to collect the content of the tube.

**Important note:**

Minor lot variations of the labeling dyes may occur. For optimal performance of dual color hybidization with Hy3™ and Hy5™ labeled RNA, it is recommended to use matched pairs of Hy3™ and Hy5™ labels.
The miRCURY LNA™ microRNA Array, Power labeling kit (product # 208035) always contains matching pairs of dyes.

To assure optimal labeling and hybridization, 52 spike-in control probes (Spike-in miRNA kit v2) are supplied in the miRCURY LNA™ microRNA Array kit and in the Ready-to-spot probe set.
Dissolve the spike-in miRNAs 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 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.

* Unless labeled together with miRCURY LNA™ microRNA Array spike-in kit (product # 208040). Then se the manual for miRCURY LNA™ microRNA Array Kit.

# Protocol & Notes

Total handling time: 1 hour

## Protocol

**Step 1**
Thaw all kit components

Place all kit components on ice and thaw for 15-20 min. Mix thoroughly by vortexing followed by brief centrifugation. Do not thaw or vortex the enzymes. Flick these tubes followed by brief centrifugation.

See tip 1+2

**Step 2**
Combine reagents according to Table 2. Mix on ice

Reagents should be combined in an RNase-free microcentrifuge tube and should be mixed by pipetting up and down to ensure that all reagents are mixed thoroughly.

See tip 5+7

| Table 2 | Volume (µL) |
|---|---|
| Total RNA** | 2 |
| Spike-in miRNA kit v2 | 1 |
| CIP buffer | 0.5 |
| CIP enzyme | 0.5 |

** We recommend using between 0.25 - 1 µg, depending on the microRNA content in the sample.
Mastermix preparation: In order to minimize variation between slides it is recommended to prepare master mixes for both the CIP reaction (Table 2 ) and the labeling reaction (Table 3 ).

**Step 3**
Incubate 30 min. at 37° C

Incubate 30 min. at 37° C, using a PCR cycler with heated lid.

**Step 4**
Incubate 5 min. at 95° C

Stop the enzyme reaction and denaturate the RNA by incubation at 95° C followed by snap cooling on ice.

**Step 5**
Incubate 2 min.on ice.

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

**Step 6**
Combine reagents listed in Table 3. Mix on ice

Add the reagents listed in Table 3 to the 4 µL CIP reaction from step 5.

| Table 3 | Volume (µL) |
|---|---|
| CIP reaction from step 5 | 4 |
| Labeling buffer | 3 |
| Fluorescent label (Hy3™ or Hy5™) | 1.5 |
| DMSO | 2 |
| Labeling enzyme | 2 |

Mastermix preparation: In order to minimize variation between slides it is recommended to prepare master mixes for both the CIP reaction (Table 2) and the labeling reaction (Table 3).

**Step 7**
Mix and centrifuge the reagents briefly

Reagents should be mixed by gentle vortexing or by pipetting up and down to ensure that all reagents are mixed thoroughly.

**Step 8**
Incubate at 16° C for 1 hour

Incubate for 1 hour at 16° C, using a PCR cycler with heated lid. Protect the reaction from light.

**Step 9**
Incubate for 15 min. at 65° C

After stopping the labeling procedure, briefly spin the reaction and leave it at 4° C. The labeled sample is now ready for hybridization on the array. Hybridization should preferably occur within 1-2 h.

See tip 3+6

# Tips and Trouble Shooting



Tip 1

## RNA quality

Total RNA should be prepared using a method that preserves small RNA species. When using commercially available kits, make sure the total RNA preparation is guaranteed to contain microRNAs. An example of a column purification procedure which preserves small RNAs is the miRCURY™ RNA Isolation Kits for total RNA purification. Procedures that include an acidic phenol chloroform extraction are generally recognized as methods that preserve small RNAs. Subsequent to any RNA extraction procedure using a guanidine isothiocyanate–phenol:chloroform extraction, we recommend a column purification procedure to remove traces of these chemicals that potentially may inhibit labeling. Our miRCURY™ RNA Isolation Kits can be used for this purpose. It is recommended to assess the integrity of the RNA isolated before proceeding with labeling. This may be performed either on the Agilent Bioanalyzer or by denaturing gel electrophoresis. These methods do not reveal/show microRNAs, but yield an assessment of the overall RNA quality. Degraded RNA is generally not suitable for labeling or for hybridization to microarrays, however RNA extracted from FFPE samples does give good results on miRCURY LNA™ microRNA Arrays. The miRCURY LNA™ microRNA Power Labeling Kit can be used for efficient labeling of both microRNA enriched and total RNA.

Tip 2

## Enrichment for microRNA

The Power labeling kit will efficiently label RNA that has been enriched for microRNAs as well as miRNAs in total RNA preparations. It should be noted that microRNAs only constitute a minor fraction (~0.01%) of total RNA and attempts to purify this fraction often results in depletion of the microRNAs or a co-purification of large RNAs. We recommend to label and hybridize the total RNA.

Tip 3

## Weak hybridization signals

Several options to investigate the cause of weak signals have been build into the miRCURY LNA™ microRNA Array and labeling kit. Key components of this investigation involves checking the intensities on the landing lights and on the Spike-in miRNAs capture probes. Hy5™ label is sensitive to ozone, thus it is important to work fast during entire procedure. If possible handle labeling and especially washing and scanning of the slide in an ozone free environment.

Tip 4

## Landing lights

Landing lights are Hy3™ labeled capture probes spotted directly onto the array. Very weak signals from the landing lights could indicate that the PMT settings of the microarray scanner are too low. Consult the instruction manual for your microarray scanner to determine how to choose the optimal PMT settings and redo the scanning. Alternatively, the slide might have been placed incorrectly in the scanner. Scanning of the slide in an up-side-down or a backwards orientation will result in very little or no signal. Consult the miRCURY LNA™ microRNA Array manual to identify the side and area of the slide where the array has been spotted. See tip 5 & 6.

Tip 5

## Spike-in capture probe

The miRCURY LNA™ microRNA Array Power Labeling kit can be used with a spike-in kit, consisting of a mixture of synthetic microRNA. When spiked into the labeling reaction the synthetic microRNAs will co-label with the sample. Subsequently, the synthetic microRNAs will hybridize to control capture probes on the miRCURY LNA™ microRNA Array. If the signals of all spike-in microRNA capture probes are weak, this may indicate that the labeling reaction was performed under suboptimal conditions e.g. pipetting errors, incubation at too low temperatures, exposure of labeling dyes to excess light, loss of microRNA during concentration of the labeling reaction, presence of RNases or enzyme inhibiting compounds such as phenol or ethanol residues from the sample preparation. Alternatively, hybridization at too stringent conditions (low salt, high temperature) will also result in low signals. If the signal from the spike-in control capture probe is high but signal from most other capture probes are low, this may indicate low quality of the RNA sample (see above) or that the RNA concentration was lower than anticipated.

Tip 6

## Removal of unincorporated dye

In contrast to chemical labeling procedures, the labeling dye in the The miRCURY LNA™ microRNA Array Power Labeling kit will not produce high background on the array slide. Therefore, removal of unincorporated dye is normally not necessary. If elevated background is experienced (usually in the Hy5 channel) and removal of dye is desired, we recommend performing 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 (3 M, pH 5.5) to the 25µL labeling reaction + 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 H2O and 6µL labeling buffer.

If negative spots are experienced due to elevated background, it might be more feasible to use signal values from probes that are not expressed/ negative controls as background level instead of local background, which may lead to negative values.

Tip 7

### 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 microRNA 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 ≥8000 x g (≥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 ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

**Note**

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 ≥8000 x g (≥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 25µL RNase-free water directly onto the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the microRNA and total RNA.

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

# References


- The microRNA Registry.
  Griffiths-Jones S. NAR, 2004, 32, Database Issue, D109-D111

- miRBase, http://www.mirbase.org

- www.exiqon.com/array

# Related products

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

**Figure 1**


### miRCURY LNA™ microRNA Array, microarray kit

Pre-printed miRCURY LNA™ microRNA Array microarray slides, available in pack sizes of 3, 6 and 24 (product # 208400, 208401, 208402, 208212-A, 208213-A, 208214-A).

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

Ready-to-spot oligo set for direct printing of arrays, or coupling in bead-based applications (product # 208410, 208215-A).

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

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

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

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

### miRCURY LNA™ microRNA Array, Hybridization buffer

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

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

### miRCURY LNA™ microRNA Detection
For in situ hybridization and northern blotting of all annotated microRNAs.

### miRCURY LNA™ microRNA ISH Optimization kit (FFPE)
Complete kit with control probes and hybridization buffer for easy set up of microRNA in situ hybridization.

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

### miRCURY LNA™ microRNA Inhibitor Library
For genome-wide high throughput screening of microRNA function.

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

## Literature citations:

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

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