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miRXplore™ Microarray Kit User manual

miRXplore Microarray Kit, 4 130-093-254
miRXplore Microarray Kit, 8 130-093-272



For technical questions, please contact your local distributor or our Technical Support Team in Germany:
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Contents

1. Description	3
1.1 Components and size	3
1.2 miRNA analysis with miRXplore™ Microarrays	4
1.3 Kit capacities	8
1.4 Reagent and instrument requirements	8
1.5 Related products	8
2. Protocol for miRXplore Microarray hybridization	9
2.1 Sample preparation	9
2.2 Fluorescent sample labeling	9
2.3 Hybridization and wash	9
2.4 Data analysis	15
3. Tips & hints	16
4. Troubleshooting	16
5. Appendix	17
6. References	17

The cover photo shows a replica of the DNA model built in 1953 by James D. Watson and Francis Crick at the Cavendish Laboratory in Cambridge. This model is located at Heureka, the Finnish Science Centre. Photography by Alexander Budde; © Miltenyi Biotec GmbH, Germany. Detailed information on the history of the Watson-Crick model can be found in: de Chadarevian, S. (2003) Relics, replicas and commemorations. *Endeavour* 27: 75–79.

1. Description

1.1 Components and size

miRXplore™ Microarray Kit, 4 microarrays (# 130-093-254)
miRXplore Microarray Kit, 8 microarrays (# 130-093-272)

Kit for 4 or 8 microarrays includes:

1 or 2 (as ordered)	tray(s) with 4 miRXplore Microarrays
50 mL	1× Prehybridization Solution
50 mL	Wash Buffer 1 (25×)
50 mL	Wash Buffer 2 (25×)
1	PIQOR™ Navigator, miRXplore Annotation file (CD-ROM), and miRXplore Configuration overview

Box

3 RNA controls	miRControl 1, miRControl 2, and miRControl 3
3 × 1 mL	Hybridization Solution (2×)

Product format

miRControl 1, 2, and 3 are supplied as lyophilisates and are stable for at least two days at room temperature. Upon receipt, dissolve each miRControl: Add 20 µL of RNase-free water and heat to 65 °C for 5 minutes, mixing constantly by repeated up and down pipetting. Store at –20 °C.

All buffers included in the miRXplore Microarray Kit are evaluated for the absence of RNase activity.

Storage

miRXplore Microarrays should be stored at room temperature, dry, and protected from light. Avoid condensation on the miRXplore Microarray (e.g. caused by temperature changes).

Important: Keep miRXplore Microarray dry before use!

Store Wash Buffer 1 and Wash Buffer 2 at room temperature.

Store dissolved miRControls, Prehybridization and Hybridization Solution at –20 °C.

The expiration dates are indicated on the vial and microarray labels.

1.2 miRNA analysis with miRXplore™ Microarrays

1.2.1 miRNAs

MicroRNAs (miRNAs) are presumed to be key regulators of gene expression. They are derived from endogenous transcripts that contain complementary or near-complementary 20–50 base pair-inverted repeats. These transcripts fold back on themselves to form double-stranded RNA hairpins, which are processed into mature 21–28 nucleotide miRNAs.¹ Discovered in 1993 in *Caenorhabditis elegans*,^{2,3} more recent genetic screens as well as other experimental and computational approaches have led to the identification of hundreds of such small RNA coding genes, referred to as miRNAs.^{4–6} Animal miRNAs preferentially target mRNAs at partially complementary yet evolutionary conserved sites, which are predominantly located within the 3' untranslated region (UTR).^{7–9} Among diverse methods that can be used to identify and quantify expressed miRNAs, microarray technology—established as valuable tool in mRNA expression profiling—is increasingly used for the simultaneous detection of many or all known miRNAs.

1.2.2 miRXplore Microarrays

The miRXplore™ Microarray Kit offers a system for semiquantitative analysis of differential miRNA expression to be carried out in samples of interest. miRXplore Microarrays cover all human, mouse, rat, and viral miRNAs as deposited in the newest version of the smRNA Database^{10,11} and the miRBase Sequence Database^{12–15} (please inquire for updates).

Each microarray contains a set of different positive, negative, and calibration controls (see section 1.2.3, 1.2.4, and fig. 1); thereby, improving the quantification of differential expression patterns. All oligonucleotides are spotted in quadruplicates. After hybridization of one or more fluorescently labeled miRNA samples to the appropriate number of mi-

croarrays, fluorescent scanning generates data for the comparison of specific miRNAs expression levels between samples (see section 1.2.8 and 1.2.9).

1.2.3 miRNA oligonucleotides

miRXplore Microarray probes spotted on the microarray are DNA oligonucleotides whose sequences are reverse-complementary to the corresponding mature miRNAs. The number of miRNA is constantly updated on the miRXplore Microarray; in August 2007, a total of 5164 spots, encompassing 1194 distinct miRNAs and 72 controls, are present in quadruplicates on the microarray. Please inquire for updates.

The 1194 probes cover all currently known and sequence-verified miRNAs of the species human, mouse, and rat as well as viral miRNAs. In case a miRNA displays the same sequence in different species, only one probe is present on the microarray. If the sequence differs among the species, all distinct sequences are spotted on the microarray. Thereby, the 1194 miRNA probes cover all together 728 human, 584 mouse, 426 rat, and 122 viral miRNAs, which sums up to 1860 miRNAs. Please refer to the enclosed PIQOR™ Navigator software to track the position of each miRNA oligonucleotide on the miRXplore Microarray.

	Human	Mouse	Rat	Virus
Sequence no.	728	584	426	122

Table 1: Number of miRNA oligonucleotides present on the miRXplore™ Microarray (August 2007). Please inquire for updates.

1.2.4 Control oligonucleotides

Positive controls

Three positive controls miRControl 1, miRControl 2, and miRControl 3 are included in the kit. miRControl 1, 2, and 3 are RNA sequences, which are not present in any of the human, mouse, rat, or viral genome avoiding cross-hybridization.

miRControl 1 consists of a mixture of four RNA oligonucleotides at a concentration of 5 fmol/μL each. miRControl 2 is a single RNA oligonucleotide with a concentration of 100 fmol/μL. miRControl 3 consists of 18 RNA oligonucleotides (please inquire for updates) at a concentration of 5 fmol/μL each.

miRControl 1 and miRControl 3 are added to the total or enriched small RNA before labeling, to indicate the efficiency and sensitivity of the labeling reaction. The corresponding DNA oligonucleotides to miRControl 1 are positioned at the corners of each microarray (fig. 1). The miRControl 1 signals thus help the alignment of the grid provided by the imaging software.

The miRControl 3 signals are used for the normalization of the signal intensities of different arrays or differently labeled samples on the same microarray (see section 2.4).

miRControl 2 can be added to the homogenized tissue or to the total RNA before small RNA enrichment to assess efficiency of the enrichment reaction.

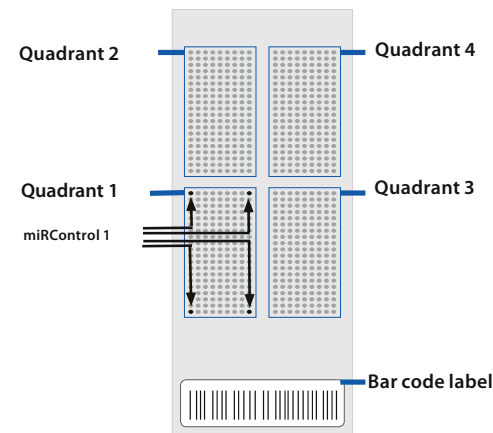


Figure 1: Position of control RNA miRControl 1 on each microarray.

In addition, each miRXplore™ Microarray contains further 36 internal positive small but non-miRNA control probes including probes for 5.1S RNA, tRNAs, and U6 RNAs. For a complete list please refer to the PIQOR™ Navigator on CD-ROM.

Negative controls

Each miRXplore™ Microarray contains 13 negative controls: Different mismatch probes are included for specific miRNAs or shuffled miRNA sequences. Furthermore, herring sperm DNA and buffer-only serve as background quality control. For a complete list please refer, for example, to the miRXplore Configuration overview file on the PIQOR navigator CD-ROM.

1.2.5 Normalization of miRXplore™ Microarrays

Normalization of microarray data

The normalization of microarray signals, detected from two independently labeled samples hybridized to one or more microarrays, is used to compensate for biased signal intensities. Certain experimental differences may influence signal intensities: varying amounts of sample material and label, inconsistent efficiency of dye incorporation, differing quantum yield of fluorochromes, setting of laser power and photo-multiplier, etc. To overcome these systematic variation, standard normalization procedures can be performed, for example, normalizing against the median of all single-spot ratios. These standard normalization methods only lead to reproducible results if a sufficient number of miRNA oligonucleotides show no difference in their respective expression level.

Normalization using spike-in controls

The field of miRNA microarray research is particularly challenging with respect to normalization, reproducibility, and quantification of results. The main reasons are i) the missing so-called housekeeping

genes, ii) limited number of expressed miRNAs, and iii) the observation that under certain experimental circumstances a general up- or down-regulation of many miRNAs may happen all at once. Thus, some conventional methods for normalizing signal intensities over multiple experiments may fail. To overcome these obstacles, a set of spike-in calibration controls is included in miRxplore™ Microarray Kits. The provided 18 control RNAs of miRControl 3 can be used as a spiked-in control. When using the miRControl 3, or additional internal positive controls, it is recommended to normalize all signal intensities of each two of the investigated samples using the median of the miRControl 3 signals (see section 2.4).

1.2.6 Production and quality control of miRxplore™ Microarrays

miRxplore Microarrays are high-quality DNA microarrays designed and accurately produced for valid and reliable miRNA expression profiling.

The proprietary miRxplore Microarray technology consists of continuously updated miDNA (DNA complementary to miRNA) collections, a high-through-put piezoelectric spotting device, and an optimized DNA immobilization procedure. For the miRxplore Microarrays, each oligonucleotide has been purified by HPLC prior to spotting. Each miRxplore Microarray is quality-controlled via an online camera system detecting and qualifying each single spot.

1.2.7 Performance of miRxplore Microarrays

Specificity

In general, miRNAs differing in at least one single nucleotide (nt) can be distinguished with the miRxplore Microarrays: By using perfect-match and corresponding mismatch probes—that have one or more mismatches at different positions—the miRNA probes were investigated extensively (please

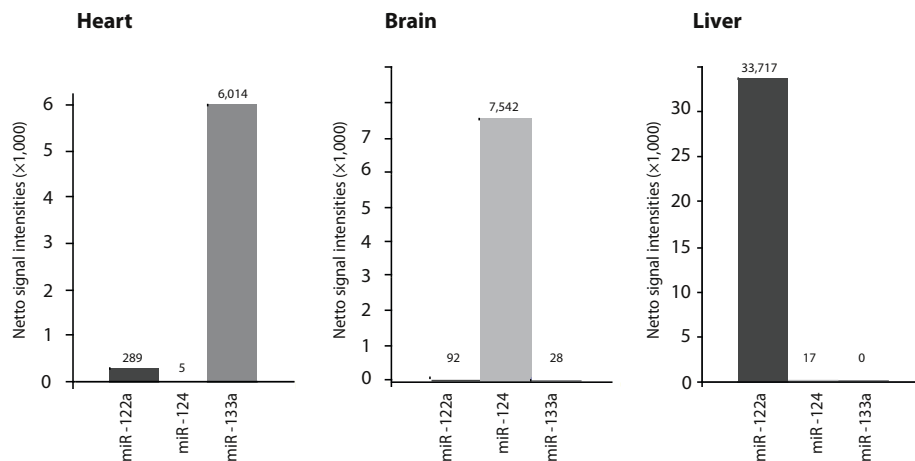


Figure 2: Examples of specifically expressed miRNAs are miR-133a in heart, miR-124 in brain, and miR-122a in liver (Lagos-Quintana *et al.*, 2002). 5 µg of total RNA of each tissue were hybridized on different miRxplore™ Microarrays. The net signal intensities (signal minus background) for miR-122a, miR-124 and miR-133a are shown for each tissue.

see data on website www.miltenyibiotec.com). Tissue-specific detection of miRNAs is shown below in figure 2.

Sensitivity

The detection limit depends on the melting temperature of the respective miRNAs and has been detected to be as low as 0.01 fmol.

Dynamic range

The dynamic range of miRNA detection is influenced by the signal-to-noise ratio of hybridized microarrays as well as by the dynamic range of the scanning device. Using conventional scanners, miRNAs quantities of 0.01–10 fmol can be detected linearly offering a dynamic range of 3 logs. However, by performing multiple scans the dynamic range can be enhanced to more than 3 logs.

Reproducibility

Intra-microarray variance: The mean coefficient of variation of the signals detected on replicate spots is usually below 10%. The coefficient of variation of the ratios derived from two or more labeled samples hybridized to a single microarray on replicate spots is typically 5%. The coefficient of variation depends on the signal intensity and therefore on the melting temperature and concentration of the respective miRNA.

Inter-microarray variance: The regression coefficients of expression profiles detected for samples hybridized independently on different microarrays is approximately 0.98. The correlation coefficient of repeated multiple color hybridizations is about 0.99.

1.2.8 Application procedures

A microarray experiment for gene expression profiling consists of RNA preparation and labeling, followed by hybridization of the labeled sample to the microarray. The starting material for detection of miRNAs can either be total RNA or size-fraction-

ated total RNA. Size fractionation can be carried out using denaturing polyacrylamide gel electrophoresis or other methods to enrich for the fraction of small RNAs (e.g. silica-based columns).

In most cases, enrichment of small RNA is not necessary. Depending on the isolation method, small RNA may get lost. Thus, we recommend to use total RNA.

▲ **Note:** RNA samples may contain different types of small RNA that vary in size.

miRxplore™ Microarrays carry DNA oligonucleotides with a reverse-complementary sequence of mature miRNAs. Therefore, RNA samples to be analysed need to be directly labeled. After fluorescent labeling of miRNAs, the sample is hybridized to the miRxplore Microarray. The fluorescence signals generated by the hybridization of miRNAs to arrayed complementary DNAs are detected and quantified using a scanner and appropriate software.

1.2.9 Handling of miRxplore™ Microarrays

For optimal performance, please pay attention to the following points when handling the microarrays:

- Always pick up or hold the microarrays at the bar code-end of the slide. Do not touch the spotted surface of miRxplore Microarrays located on the same surface as the bar code.
- Store microarrays in the trays provided so that they remain free from grease, dust, and other contaminants. Always wear gloves when handling them. If cleaning is necessary, use pressured gas from a clean supply.

1.2.10 Hybridization recommendations

miRxplore Microarrays have the standard slide format of 25×75 mm and can be hybridized manually or in an automated procedure using a hybridization machine.

Due to the short length of miRNAs (21–28 nt) the hybridization of miRNAs is sensitive to even slight changes of the hybridization conditions. A very pre-

cise control of hybridization conditions, like wash temperature and incubation times, is a prerequisite for reproducible results.

To reach optimal and robust results, we recommend to use an automated hybridization station.

The a-Hyb™ Hybridization Station combines tight temperature control and precise liquid handling. The unique active circulatory system evenly distributes liquids via micropumps over the surface of the slides and accelerates the usually diffusion-controlled hybridization process. In addition, the continuous sample flow minimizes non-specific hybridization. Altogether, the a-Hyb Station improves the specificity of miRNA hybridization and allows sensitive miRNA detection on miRXplore™ Microarrays.

1.3 Kit capacities

The kit is for hybridization of 4 (8) or more miRNAs samples with 4 (8) miRXplore™ Microarrays.

1.4 Reagents and instrument requirements

All additionally required equipment and reagents must be RNase-free.

- For homogenization and lysis of tissue: mortar, pestle, and/or rotor-stator homogenizer.
- For RNA isolation: It is very important to choose a method that preserves small RNAs. We recommend Tri Reagent (Sigma-Aldrich, # T9424) or Trizol (Invitrogen, # 15596-026) for isolation of total RNA. For purification of small RNA, Pure Link™ miRNA Isolation Kit (Invitrogen, # K1570-01) or mirVana™ miRNA isolation (Ambion, #1560) can be used.
- For fluorescent labeling: commercially available labeling kits for miRNAs, for example, miRCURY™ LNA Array Labeling Kit (Exiqon, #208032) or mirVana miRNA Labeling Kit (Ambion, #1562)
▲ Note: Please remember to use the spike-in RNA

controls provided with the miRXplore Kit to monitor the labeling process.

For the automated hybridization

- The a-Hyb Hybridization Station is recommended (#130-092-181).
- Wash container (Milian Laboratories supplies, # KART-922)
- Distilled water
- Water bath
- For scanning: microarray scanner for standard microscope glass slides
- RNase-free tubes and pipette tips
- Microcentrifuge suitable for 2 mL tubes
- Heat plate (70 °C)

For manual hybridization

- SecureSeal™ Hybridization Chambers SA600FL-6L (Grace Bio-Labs)

▲ Note: These chambers are designed for use with fluorescent labels.

- MACSmix™ Tube Rotator, (#130-090-753)

▲ Note: This instrument is strongly recommended for the manual hybridization protocol: It enables a continuous, tumbling rotation of the microarray solution as it is needed for hybridization in the SecureSeal chamber. The chamber content is moved constantly in circles over the spotted area. Thus, the labeled nucleic acids are distributed as evenly as possible across the hybridization area.

- Hybridization incubator

1.5 Related products

- a-Hyb Hybridization Station for automated hybridization of standard glass slide (#130-092-181)
- MACS® Products for gene expression profiling, see www.miltenyibiotec.com
- miRXplore™ Microarray Service and miRXplore Microarray Universal Reference Service, the latter uses a defined synthetic universal reference pool containing miRNAs that are spotted on the miRXplore Microarray (August 2007).
- *Coming soon:* miRXplore Universal Reference

2. Protocol for miRXplore™ microarray hybridization

All additionally required equipment must be RNase-free.

2.1 Sample preparation

To generate a fluorescently labeled target that allows a sensitive miRNA expression analysis, we recommend using 1–5 µg of total RNA. If the miRNA of interest is expressed at a very low level we recommend to increase the total RNA concentration.

▲ Note: In case less than 1 µg total RNA is available, do not hesitate to contact Technical Support for further information (macstec@miltenyibiotec.de). Any RNA extraction method which preserves small RNAs can be used. Please note that standard silica-based columns kits for total RNA isolation do not preserve small RNAs due to an exclusion of nucleic acid sized smaller than 100 nt.

▲ Note: Perform the total RNA isolation according to the manufacturer's protocol. miRControl 2 can be spiked either into the intact material before lysis or after lysis, for example, to the aqueous phase when performing phenol-based RNA isolation, or after RNA isolation.

2.2 Fluorescent sample labeling

▲ Fluorescent dyes and labeled samples are light-sensitive material. Whenever possible keep the reactions containing fluorescent dyes in the dark.

Before starting

Add 1 µL of miRControl 1 per 1–5 µg total RNA and 1 µL of miRControl 3 per 1–5 µg total RNA to the RNA sample.

For the comparison of RNA sample 1 with sample 2, miRControl 3 has to be added to each RNA sample. The RNA samples to be compared should have equal amounts of RNA. If this is not possible, adapt volume of miRControl 3 accordingly, e.g. 1 µL of miRControl 3 per 5 µg of sample RNA, or 0.6 µL of miRControl per 3 µg of sample RNA.

▲ Note: Pipetting of small volumes is error-prone and might lead to artefacts during the normalization. Transferring volumes less than 0.5 µl should be avoided.

▲ Note: Each miRControl has to be dissolved by adding 20 µL of RNase-free water and heating to 65 °C for 5 min, mixed constantly by repeated up and down pipetting. The labeling reaction has to be carried out according to the manufacturer's protocol (see section 1.4). We recommend to use 1–5 µg of total RNA or 1 µg of small RNA.

2.3 Hybridization and wash

miRXplore Microarrays use the standard slide format of 25×75 mm and can be hybridized manually or in an automated procedure using a hybridization machine.

2.3.1 Automated hybridization with the a-Hyb™ Hybridization Station

Before starting

- Please use the a-Hyb™ Hybridization Station setting recommendations for your protocol shown in table 1.
- Pre-warm 2× Hybridization Solution to 42 °C.
▲ Note: 2× Hybridization Solution may form a precipitate during storage. Warming to 42 °C allows the precipitate to dissolve and facilitates pipetting of the viscous solution. Mix the pre-warmed solution thoroughly before pipetting.
 Heat Prehybridization Solution at 98 °C for 2 minutes, centrifuge briefly, and cool to 42 °C. Dilute 25× Wash Buffer 1 and 25× Wash Buffer 2 in 0.5 L aliquots to a final concentration of 1× Wash Buffer.

▲ Note: Prehybridization Solution, 25× Wash Buffer 1, and 25× Wash Buffer 2 may form precipitates when stored. If necessary, redissolve by warming to 50 °C, then cool to room temperature. Make sure that no undissolved particles are present in the washing solutions as they may cause background fluorescence.

▲ Note: It is recommended to sterile filtrate (0.45 µm filter) all wash buffers prior to use.

Connect buffer bottles with the corresponding tube for automated medium supply:
 Medium tube 3: Prehybridization Buffer
 Medium tube 1: Wash Buffer 1
 Medium tube 2: Wash Buffer 2

▲ Note: For automatic buffer supply in the a-Hyb™ Hybridization Station, about 5 mL of buffer is required

per step and activated microarray position. If all four positions are selected, approximately 20 mL per step are necessary (20 mL of Prehybridization Solution and 40 mL per washing buffer for two wash cycles).

For manual sample application in the a-Hyb Station, use a volume of 200 µL of labeled sample per position.

See a-Hyb™ Hybridization Station user manual for further instructions.

1. Start the a-Hyb™ Hybridization Station: Switch power button on.
2. Switch to the hybridization screen designated by the blue area.
3. Select slide positions to be used.
4. Select the miRNA Protocol.
 - ▲ **Note:** Select View protocol to ensure that the protocol is unchanged and the buffer selection corresponds to the selected tube numbers. See table 1 for details of the miRNA protocols.
5. Switch to the hybridization screen and press **Start** to begin the hybridization process.
6. Load slides into a-Hyb™ Slide Carrier using slide loading device. Use blank slides for free positions. Place a-Hyb Sealing Plate on top of slide carrier.

Insert slide carrier together with sealing plate into drawer and press **Continue**.

▲ **Note:** See a-Hyb™ Hybridization Station user manual for further instructions.

7. Adjust your labeled RNA sample from section 2.2 to a volume of 100 µL with nuclease-free water (e.g. if you have a sample volume of 20 µL from section 2.2, add 80 µL of nuclease-free water).
8. Add 100 µL of 2× Hybridization Solution, pre-warmed to 42 °C (total sample volume: 200 µL).
9. Incubate 200 µL of labeled sample from the previous step at 70 °C for 3 minutes. Centrifuge briefly.
10. When the a-Hyb Station software prompts for manual application of sample, press **Continue** and add 200 µL of sample from step 9 into the reservoir, and again press **Continue**. Repeat for all selected positions when requested. All wash steps will be performed automatically.
11. Fill a dish with distilled water. When prompted to cool down, press **No**. Press **Continue** to open the drawer of the a-Hyb Station and remove Sealing Plate. Remove slide carrier from a-Hyb Station and immediately place slide carrier in slide loading device and take out microarrays.

Step	Medium	Temperature (°C)	Time (min)	Pump speed (mL/min)	Cycle no.	Comment
1. Incubation	3	42	5	1.0	–	Use Prehybridization Solution, pre-warmed to 42°C.
2. Incubation	M	42	840	1.0	–	Hybridization: Fill in 200 µL of sample solution.
3. Wash	1	10	1	1.0	2	Use Wash Buffer 1.
4. Wash	2	10	1	1.0	2	Use Wash Buffer 2.
5. End						

Table 1: Recommended protocol specifications for hybridization of miRXplore™ Microarrays in the a-Hyb™ Hybridization Station.

12. Dip microarray three times quickly into distilled water (at room temperature).

▲ **Note:** This final wash step ensures that no remaining salt residues are left on the slide.

13. Dry microarray by centrifugation at 500×g for 3 minutes, or with compressed, dry air (or inert gas like He, N₂) from a clean supply, and store in a dust-free hybridization cassette.

▲ **Note:** If using compressed gas, dry slide starting from the opposite side of the bar code label towards bar-code label.

2.3.2 Manual hybridization

We strongly recommend controlled sample mixing during hybridization. Active mixing minimizes non-specific hybridization and improves the quality of hybridization. Best results are achieved by automated hybridization.

For further information contact technical support macstec@miltenyibiotec.de or our website www.miltenyibiotec.com.

For satisfying manual hybridization, the sample solution should be moved or mixed continuously! Static hybridization generally results in low signal intensities with a higher degree of non-specific signals and increased variance. The manual hybridization procedure described below takes advantage of the MACSmix Tube Rotator for a continuous tumbling movement of the labeled sample solution over the hybridization area. Also, SecureSeal™ hybridization chambers are used. These peel-and-stick adhesive enclosures frame the hybridization area on the slide, providing barriers for the hybridization solution.

Before starting

- Pre-warm 2× Hybridization Solution to 42 °C.
- ▲ **Note:** 2× Hybridization Solution may form a precipitate during storage. Warming to 42 °C allows the precipitate to

dissolve and facilitates pipetting of the viscous solution. Mix the pre-warmed solution thoroughly before pipetting.

- Heat Prehybridization Solution at 98 °C for 2 minutes, centrifuge briefly, and cool to 42 °C. Dilute 25× Wash Buffer 1 and 25× Wash Buffer 2 in 0.5 L aliquots to a final concentration of 1× Wash Buffer.
 - ▲ **Note:** Prehybridization Solution, 25× Wash Buffer 1, and 25× Wash Buffer 2 may form precipitates when stored. If necessary, redissolve by warming to 50 °C, then cool to room temperature. Make sure that no undissolved particles are present in the washing solution as they may cause background fluorescence.
 - ▲ **Note:** It is recommended to sterile filtrate (0.45 µm filter) all wash buffers prior to use.

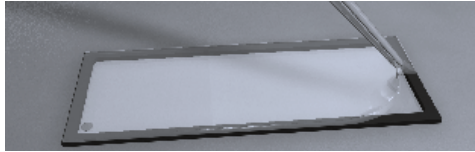
- The secure adhesion of the hybridization chamber on the glass slide is very important; thus, it is recommended to practice this technique with blank slides before using miRXplore™ Microarrays!
 - ▲ **Note:** Improper sealing of the hybridization chamber will result in leakage of the hybridization solution and high background due to dried areas. Please read also the Secure Seal™ instructions from GraceBio (www.grace-bio.com).

Sample preparation

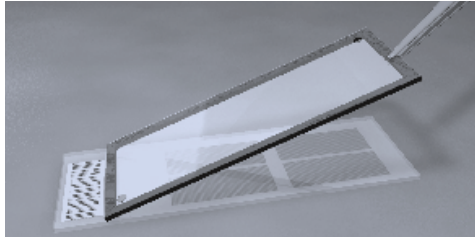
1. Adjust your labeled RNA sample from section 2.2 to a volume of 350 µL with nuclease-free water (e.g. to a sample volume of 20 µL from section 2.2, add 330 µL of nuclease-free water).
2. Add 350 µL of 2× Hybridization Solution, pre-warmed to 42 °C (total sample volume: 700 µL).
3. Incubate 700 µL of labeled sample from the previous step at 70 °C for 3 minutes. Centrifuge sample briefly.
4. Place your miRXplore™ Microarray on a flat surface. Be sure that the slide surface is dry and dust-free.

Setting-up of the hybridization chamber

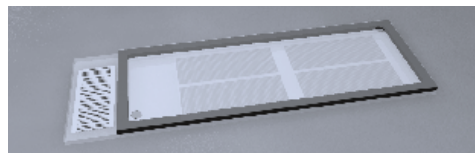
1. Take out a SecureSeal™ hybridization chamber. Peel off the thin adhesive foil on the gasket surface of SecureSeal chamber.




2. Place chamber on microarray, aligning the spotted area with the gasket interior.



3. Ensure a firm adherence of the hybridization chamber by pressing gently along the chamber edges with the provided wooden stick. Avoid high pressure that might lead to breakage of the slide.



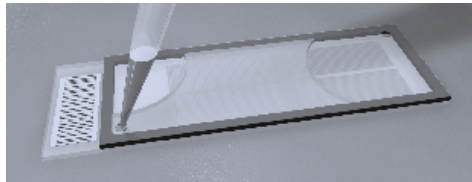
4.  **Warning:** Hot surface! Touching hot surfaces can result in body injury!

For a secure adhesion of chamber to the slide, place SecureSeal chamber upside-down for a at least 20 seconds on a hot surface (about 70 °C).

Filling of the chamber

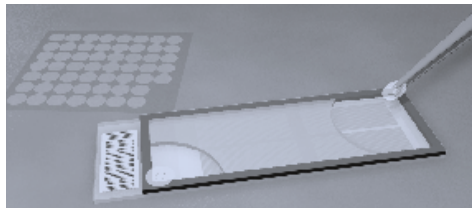
1. Pipet 700 µL of Prehybridization Solution through one port of the chamber while allowing air to escape through the other port. Make sure that the surface around both ports is dry.

▲ Note: Intentionally, the chamber will not be filled completely. The remaining air supports mixing of the labeled sample. Sufficient and correct mixing – over the slide edges – is essential for the continuous movement of the solution.

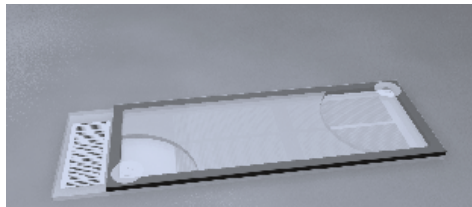


Sealing of the chamber and prehybridization

1. Using forceps, remove a seal tab from liner strip and place it carefully over each filling port.



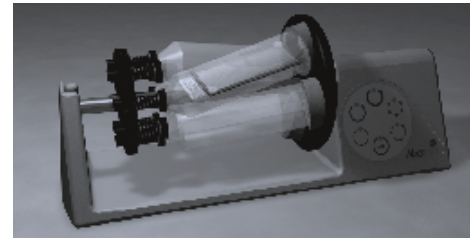
2. Press gently on both seal tabs simultaneously for at least 5 seconds to ensure a secure seal.



3. For constant agitation of the Prehybridization Solution over the slide surface, place miRXplore™ Microarray gasket with hybridization chamber in a 50 mL tube.

▲ Note: It is recommended to fix the microarray inside the tube with few laboratory tissues (e.g. Kim wipes). To ensure a humid atmosphere, pipet 4 mL of ddH₂O over the tissue.

4. Place the 50 mL tube into MACSmix™ Tube Rotator. Please see MACSmix User manual for further instructions.



5. Choose medium rotation velocity on the MACSmix Tube Rotator touch pad.

▲ Note: The MACSmix Rotator rack should be loaded symmetrically. For sufficient mixing, the tubes need to be placed in a sloping position into the rack. Overhead rotation (e.g. like in a hybridization oven) is not recommended as sample solution will just move up and down without sufficient mixing.

The MACSmix Tube Rotator runs on rechargeable batteries for at least 24 hours at room temperature. The device can be placed into a refrigerator or an incubator at temperatures between 2 °C and 42 °C. The operation time varies with temperature. Read the MACSmix™ User manual for details (MACSmix Tube Rotator #130-090-753).

6. Place the MACSmix Tube Rotator in an adequate laboratory oven for hybridization at 42 °C. Keep the chambers rotating at 42 °C at least for 10 minutes.

Sample hybridization

1. Take the MACSmix™ Rotator out of the laboratory oven and let it run until the chambers have reached room temperature.

2. Take the microarrays out of the 50 mL tubes.

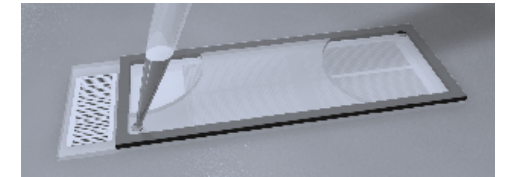
3. Remove seal tab from each filling port.

4. Remove 700 µL of Prehybridization Solution from one port using a pipette.

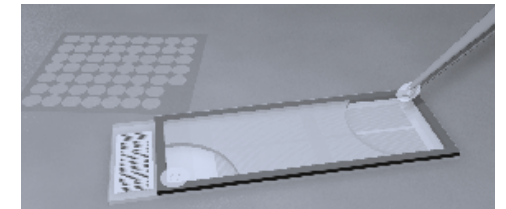
▲ Note: Residual amounts of Prehybridization Solution may stay in the chamber.

5. Pipet 700 µL of sample solution from Sample preparation, step 3 (p. 11) through one port of the chamber while allowing air to escape through the other port. Make sure that the surface around both ports is dry.

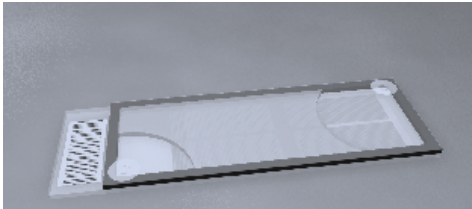
▲ Note: Intentionally, the chamber will not be filled completely. The remaining air supports mixing of the labeled sample. Sufficient and correct mixing – over the slide edges – is essential for the continuous movement of the solution.



6. Using forceps, place seal tabs centred over each filling port.



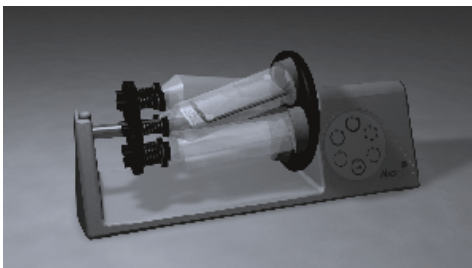
7. Press gently simultaneously for at least 5 seconds on both tabs.



8. Place miRXplore™ Microarray gasket with hybridization chamber in a 50 mL tube.

▲ **Note:** It is recommended to fix the microarray inside the tube with few laboratory tissues (e.g. Kim wipes). To ensure a humid atmosphere, pipet 4 mL of ddH₂O over the tissue.

9. Place the 50 mL tube into MACSmix™ Tube Rotator; please see MACSmix User manual for further instructions.



10. Choose slow rotation velocity on the MACSmix Rotator touch pad.

▲ **Note:** The rack should be loaded symmetrically. For sufficient mixing, the tubes need to be placed in a sloping position into the rack. Overhead rotation (e.g. like in a hybridization oven) is not recommended as sample will just go up and down without sufficient mixing.

The MACSmix Tube Rotator runs on rechargeable batteries for at least 24 hours at room temperature. The device can be placed into a refrigerator or an incubator at temperatures between 2 °C and 42 °C.

The operation time varies with temperature. Read the MACSmix™ User manual for details (MACSmix Tube Rotator #130-090-753).

11. Place MACSmix Rotator in an adequate laboratory oven for hybridization. The incubation should last minimal 14 hours at 42 °C.

▲ **Note:** With the continuous, tumbling rotation of the microarray chambers inside the MACSmix Rotator, the chamber content is moved constantly in circles over the spotted area. The inserted air helps distributing the labeled nucleic acids as evenly as possible across the hybridization area.

Postprocessing

1. Fill a wash container with fresh Wash Buffer 1.

▲ **Note:** In order to ensure a correct workflow we recommend to work with two wash containers.

2. Remove miRXplore™ Microarray from the hybridization oven.

3. Grasp the edge of chamber firmly and peel the SecureSeal away from the microarray.

▲ **Note:** Take care to minimize the formation of splashes or drop-lets, wear goggles as a precaution.

4. Place microarray immediately in the wash container filled with 50 mL of fresh 1× Wash Buffer 1 for 5 minutes at room temperature.

5. Repeat step 4 using fresh 1× Wash Buffer 1.

6. Wash miRXplore Microarray with 50 mL of fresh 1× Wash Buffer 2 for 5 minutes at room temperature.

7. Repeat step 6 using fresh 1× Wash Buffer 2.

8. Dip microarray three times quickly into ddH₂O (room temperature).

▲ **Note:** This final wash step ensures that no remaining salt residues are left on the slide.

9. Dry microarray by centrifugation at 500×g for 3 minutes, or with compressed, dry air (or inert gas like He, N₂) from a clean supply, and store it in a dust-free hybridization cassette.

▲ **Note:** If using compressed gas, dry slide starting from the opposite side of the barcode label towards barcode label.

2.3.3 Scanning

Scan the microarrays according to the instruction of your scanning instrument.

▲ **Note:** In case of repeated scanning of the same microarray, scanner-derived bleaching may occur, mainly in the Cy5 channel. This is also dependent on additional environmental factors.

2.4 Data analysis

miRXplore™ Microarray Kits include a software package to support the analysis of data: the miRXplore Microarray annotation list, miRXplore Configuration overview, and the PIQOR™ Navigator.

The **miRXplore Annotation file** links each gene on the microarray to the miRBase database¹⁵.

The **miRXplore Configuration overview** shows the exact number of the constantly updated miRNA oligonucleotides that are spotted on the current microarray. Also, any newly spotted miRNA sequences—as compared to previous miRXplore Microarray versions—are listed.

The **PIQOR Navigator** is a visualization tool that contains the gene information for every spot and enables the localization of the spot position of each miRNA sequence on the microarray. In addition, it enables the generation of gene ID lists required for microarray image analysis with software packages like ImaGene and GenePix. These software packages will extract the microarray raw data.

2.4.1 Normalization using miRControl 3

To compensate for experimental bias, it is recommended to normalize all signal intensities of two or

more investigated samples. miRControl 3 contains 18 different synthetic oligonucleotides called calibration oligos which can be used for normalization (August 2007, please inquire for updates). The normalization procedure using the median of the miRControl 3 signals is depicted below.

2.4.2 Calculation of normalization factor

1. Take the raw data set(s) of the two samples to be normalized.
2. From these, select all 18 calibration oligos named miRControl 3.
3. Exclude all data points from this selection showing only low signal intensities compared to background signal intensities.
4. Use this filtered subset to calculate the single spot ratios of the background-corrected signals. For example, divide the background-corrected signal intensity of each of the four spots of calibration oligo 5, derived from sample A, by the background-corrected signal intensity of the respective spot in sample B and so on.
5. Calculate the median of this single spot ratios. The median of the calibration oligos is a useful estimator for a global normalization factor

2.4.3 Normalization of detected signals

1. Calculate the single spot ratios for each miRNA and control by dividing the background-corrected signals.
2. Calculate the mean ratio for the four replicates of each miRNA or control.
3. Divide each miRNA mean ratio or control mean ratio by the normalization factor calculated above for the respective pair of samples.

4. Repeat transformation for each pair of samples.

▲ **Note:** Please inquire for further data analysis services offered by our bioinformatics specialists (e.g. cluster, pathway, or discriminatory gene analysis).

3. Tips & hints

Speedvac vacuum concentrator

If the volume of RNA is too large, it is recommended to concentrate the RNA sample by using a Speedvac vacuum concentrator at 45 °C. The time length depends on the volume of the sample.

▲ **Note:** Do not prolong Speedvac incubation when RNA is dried.

Quality control of RNA

Check RNA quality by spectrophotometric absorbance measurements: Calculate amount of nucleic acids with OD260 (40 µg/mL/OD260 RNA). The ratio of OD260/280 absorbance values should be 1.8–2.0; a lower value indicates protein contamination. Another tool for quality control of RNA are PAGE-Gels or the Agilent Bioanalyzer.

Photobleaching

Avoid direct exposure of the hybridized microarray and the solutions containing the fluorescent dyes to light.

4. Troubleshooting

No signals of sample but signal of miRControl 1

Signals at the corner position for miRControl 1 indicate efficient fluorescent sample labeling. If you did not obtain signals of miRNAs check your isolation method. Make sure that the isolated RNA contains the complete fraction of small RNA.

No or weak signals

Check your labeling method and washing conditions. Incorrect incubation temperatures during hybridization can lead to no or weak signals. Avoid photobleaching of the fluorescent sample (see chapter 3, Tips & hints).

No or weak signal of miRControl 2

A weak or no signal of miRControl 2 indicates loss of miRNAs during RNA isolation and/or labeling.

High non-specific background

• RNA sample quality

High background implies that impurities, such as cell debris including proteins, carbohydrates or salts, are binding to the probe on the microarray in a non-specific manner. If these substances show fluorescence signals at the scanning wavelength 550 or 650 nm, they give rise to background. High background creates an overall loss of sensitivity in the experiment due to a low signal-to-noise ratio.

• Wash conditions

The temperature of the wash solution can have a dramatic effect on the removal of non-bound labeled probes. Please use wash temperature as indicated in the protocol to ensure complete removal of non-hybridized probes. If slides are washed with low stringency or still have high background it is necessary to rewash and rescan the slide. The background can also be monitored via the negative controls buffer-only and herring sperm DNA, they should not give signals above background.

• Precipitates in Prehybridization/Hybridization Solution or Wash Buffers

Make sure that all buffers or reagents are free of particles after pre-warming. Generally, it is recommended to filter the solutions (e.g. 0.45 µm filters).

During manual hybridization

• Leaky SecureSeal chamber

Perform the heating step to adhere the chamber before applying the sample. After filling the chamber, the areas around the ports have to be dry to apply the sealing taps. The taps have to adhere tightly to seal the ports.

• Uneven hybridization or dried areas during manual hybridization

Uneven hybridization may be caused by insufficient mixing or by moving the sample just up and down. Make sure that the microarray is in a sloping position when agitated during hybridization.

If the hybridization volume is less than 700 µL, some areas of the microarray might not be covered by the hybridization sample during agitation.

It is very important to avoid drying of the slides during the handling steps. Quickly place the microarrays in the wash containers, exchange the wash buffers, dip slides in ddH₂O, and dry them immediately.

5. Appendix

Automated hybridization using the a-Hyb™ Hybridization Station

The a-Hyb™ Hybridization Station is a fully automated system for processing diverse microarrays: cDNA microarrays, oligo microarrays, protein microarrays, etc. The active circulatory system results in an even distribution of liquid over the surface of the slides and accelerates diffusion-controlled processes.

Combined with a tight temperature control, these features lead to greater reproducibility and reliability of the assays. Using the microtiterplate (MTP)-format slide carrier, four standard slides (25.65 mm ± 0.65 × 75.65 mm ± 0.65 × 1 mm ± 0.5 mm) can be handled in parallel.

The a-Hyb Hybridization Station can be linked with standard laboratory robot systems for integration into fully automated microarray processing. Each station can be used as a single instrument controlled via touchscreen, or up to 32 a-Hyb Stations can be controlled by a PC through RS485 serial ports.

The a-Hyb Software offers a highly flexible protocol structure. Users can choose from standard actions such as incubation, temperature change, washing, and drying and can combine up to 15 of these steps suiting their individual applications. In addition, temperature and media can be selected independently for each slide.

6. References

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