



miRXplore™ Universal Reference

miRXplore™ Universal Reference 25 version 1.0

Order no. 130-093-521

miRXplore™ Universal Reference 5 version 1.0

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1. Description

Components	Lyophilized pool of more than 950 synthetic, unmodified, HPLC-purified RNA oligonucleotides identical to the mature human, mouse, rat, and related virus microRNAs according to miRBase 9.2 ¹ . The RNA oligonucleotides each contain 5'-phosphate moiety as in wildtype microRNAs. 0.3 mL sterile, RNase-free water
Capacities	miRXplore™ Universal Reference 25: For 25 microarray applications; lyophilizate contains 125 fmol of each synthetic microRNA. miRXplore™ Universal Reference 5: For 5 microarray applications; lyophilizate contains 25 fmol of each synthetic microRNA.
Product format	Supplied as a lyophilizate.
Storage	Store lyophilized and dissolved aliquots at -70 °C. Dissolved aliquots are stable for three months at -70 °C. Dissolve as described below. miRXplore™ Universal Reference 25: Dissolve lyophilizate in 28 µL sterile, RNase-free water. With approximately 10% liquid loss due to adherence to vial wall, a remaining total volume of 25 µL can be pipetted (concentration per oligonucleotide: 5 fmol/µL). miRXplore™ Universal Reference 5: Dissolve lyophilizate in 5.6 µL sterile, RNase-free water. With approximately 10% liquid loss due to adherence to vial wall, a remaining total volume of 5 µL can be pipetted (concentration per oligonucleotide: 5 fmol/µL).

All RNA oligonucleotides have been controlled by mass spectroscopy and PAGE analysis. The concentration of each oligonucleotide was determined by at least two measurements before adding equimolar amounts (5 fmol/µL) to the pool.

A complete list of all synthetic miRNA oligonucleotides of the miRXplore™ Universal Reference is available upon request.

1.1 Principle of miRXplore™ Universal Reference for two-color microarray hybridization

In microarray experiments, the direct comparison of absolute signal intensities of different microarrays can be jeopardized due to different hybridization efficiencies. Therefore, two-color microarray hybridizations are frequently the method of choice: The experimental sample e.g. labeled with Cy5, and the control e.g. labeled with Cy3, are hybridized on one microarray. As the labeled molecules compete for the same probes on the microarray, the hybridization efficiency stays the same, and allows a comparison of sample versus control signals. Thus, the ratio of the signal intensities of the two dyes represents the proportion of sample to control. The two-color microarray experiments can achieve a high accuracy.

Still, if all samples of a collection have to be compared with each other, a high number of hybridizations is necessary since at least one microarray experiment has to be performed for each pair of samples.

Now, with the miRXplore™ Universal Reference (miRXplore Reference), multiple experimental samples can be compared without the need of pairwise hybridizations. Each sample—or control—is hybridized with the miRXplore Reference to one microarray. Then, signal ratios are calculated from the ratio of each sample(s) versus miRXplore Reference over the ratio of control versus miRXplore Reference—resulting in a so-called re-ratio, which reflects indirectly the ratio of the sample versus control (see fig. 1). Thereby, the results of multiple microarray experiments can be arithmetically linked without the need for pairwise hybridizations. For larger experimental series, the miRXplore Reference facilitates the comparison of multiple samples and helps to save sample material.

1.2 Background information

microRNAs 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. For the investigation and quantification of microRNA expression, microarray technology is frequently the method of choice. The miRXplore Reference was developed to provide microRNA researchers with a sample-independent reference tool to monitor microRNA experiments. The miRXplore Reference version 1.0 consists of equimolar amounts of all known microRNAs for human, mouse, rat, and related viruses according to miRBase version 9.2¹. The final concentration was optimized for robust signal measurement above background threshold.



1.3 Applications

- Microarray experiments: The miRXplore Reference is a comprehensive, synthetic microRNA pool and has been designed to give reproducible signals for most microRNA probes present on microRNA microarrays. When used in two-color microarray experiments, the miRXplore Reference enables the comparison of data derived from serial microarray experiments.

In addition, the miRXplore Reference can be used as a hybridization control and for the quality control of microRNA microarrays.

- Real-time PCR: The miRXplore Reference can also be applied as positive control for real-time PCR experiments. For absolute quantification of microRNAs, serial dilutions of the miRXplore Universal Reference can be used to generate standard curves for the microRNAs of interest. For instructions, please contact your real-time PCR vendor.
- Positive control for microRNA cloning: The miRXplore Reference is optimally suited as a positive control for small RNA cloning procedures². As the microRNAs are present in equimolar concentrations in the miRXplore Reference, the appearance frequency will—ideally—be equal for the microRNAs in the experimental sample. Thereby, a possible bias due to the cloning procedure can easily be monitored.

1.4 Reagent and instrument requirements

- RNase-free tubes and pipette tips
- Microcentrifuge suitable for 2 mL tubes

Further recommendations

- For microRNA labeling: e.g. miRCURY™ LNA microRNA Hy3™/Hy5™ Power labeling kit, Exiqon #08032-A) or mirVana miRNA Labeling Kit (Ambion, #1562)
- For manual hybridization: miRXplore Hyb Frames (# 130-094-454), MACSmix™ Tube Rotator (# 130-090-753)
- For automated processing of standard glass slides: a-Hyb™ Hybridization Station for active circulation of liquids (# 130-092-181), see miRXplore Microarray Kit user manual at www.miltenyibiotec.com.
- For scanning: standard microarray scanner

Related products

- miRXplore Microarray Kits and Services
- Bioinformatics Services
- MACS® Products for gene expression profiling, see www.miltenyibiotec.com

2. Protocol

2.1 Before starting

- ▲ All additionally required equipment must be RNase-free.
- ▲ Standard methods for microRNA labeling can be used for labeling the miRXplore Universal Reference. The experimental sample is usually labeled with Cy5, a widely-used fluorescent CyDye, and the miRXplore Reference is labeled with Cy3. Labeling of the miRXplore

Reference should be performed using the respective amount of experimental sample, total RNA or enriched small RNAs, in a standard labeling reaction.

- ▲ For microarray hybridization, the amount of miRXplore Reference used should be in the range of the sample RNA. In general, an amount of 5 fmol per RNA oligonucleotide of the miRXplore Reference is recommended for hybridization versus 5 µg of total RNA. In case less total RNA is used, the amount of miRXplore Reference has to be adapted accordingly, e.g. 1 fmol RNA oligonucleotides should be used in combination with 1 µg total RNA.

- ▲ Depending on the labeling efficiency, microarray platform, and detection sensitivity, lower amounts of the miRXplore Reference can be sufficient and should be used to avoid signal saturation.

- ▲ When using miRXplore Microarrays, miControl3 has to be spiked into the sample material (see manual at www.miltenyibiotec.com). This is also necessary when using the miRXplore Reference as the respective synthetic microRNAs are not included in the pool.

2.2 Reconstitution of lyophilizate

- ▲ RNA oligonucleotides are susceptible to degradation by exogenous ribonucleases introduced during handling. Wear gloves when working with the miRXplore Reference. In addition, RNase-free reagents, tubes, and barrier pipette tips should be used.

1. miRXplore Universal Reference 25: Dissolve the miRXplore Reference in 28 µL of RNase-free, distilled water. For dissolving leave tube on ice for 30 minutes. Vortex solution thoroughly.

miRXplore Universal Reference 5: Dissolve the miRXplore Reference in 5.6 µL of RNase-free, distilled water. For dissolving leave tube on ice for 30 minutes. Vortex solution thoroughly.

2. Briefly centrifuge to collect the content at the bottom of the tube.

▲ **Note:** The final concentration of the solution is set up to be 5 fmol/µL for each microRNA of the miRXplore Reference.

3. Prepare aliquots immediately after dissolving the miRXplore Reference thoroughly. The aliquots have to be stored at -70 °C to -80 °C. Do not subject aliquots to more than two freeze-thaw cycles.

2.3 Analysis and normalization of reference microarray data

Introduction to normalizing procedures

Normalization calculations are usually necessary to correct for dye bias such as inconsistent labeling efficiencies, varying quantum yields of the dyes, or different scanning parameters.

When performing reference hybridization, standard global normalizing procedures cannot always be applied: The number of detected signals and the dynamic range differ significantly between the reference and the sample. Therefore, the ratios of sample or control versus reference will not correspond to a normal distribution, an assumption on which global normalization procedures are based.

miControl 3—spike-in normalization control set provided with miRXplore™ Microarray Kits

Complementing standard normalization methods, spike-in normalization controls such as miControl 3 have been developed for the miRXplore™ Microarrays in order to give researchers the possibility to apply different normalization procedures.

Briefly, the artificial calibration oligonucleotides provided with the miRXplore Microarray Kits are spiked into all experimental and control samples in identical concentrations; thus, they can be used for normalization. The corresponding antisense sequences for the normalization oligonucleotides are spotted on the miRXplore Microarrays. As the amount of the normalization oligonucleotides is kept constant within all samples and the miRXplore Reference, the signals of the calibration control spots on the miRXplore Microarrays can be used for the correction of the dye bias.

Spike-in normalization is advantageous to normalize the primary ratios in case standard normalization methods cannot be used (e.g. for reference hybridizations).

Calculation of normalization factor

1. Take the raw data set(s) of the two samples to be normalized.
2. Select miRControl 3 probes (in total 18 probes).
3. Exclude all data points from this selection showing only low signal intensities as estimated by background signal intensities.
4. Use the 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 for the miRXplore Reference, etc.
5. Calculate the median of these single-spot ratios. The median of the normalization oligos is a useful estimator for a global normalization factor

Normalization of detected signals

1. Calculate the single-spot ratios for each microRNA and control by dividing the background-corrected signals gained by the hybridized sample and the miRXplore Universal Reference, respectively.
2. Calculate the mean ratio for the four replicates of each microRNA or control.
3. Divide each microRNA mean ratio or control mean ratio by the normalization factor calculated above for the hybridized sample and the miRXplore Reference.
4. Repeat transformation for each pair of samples.

Re-ratio calculation

To compare different samples, the primary ratios of sample A versus reference can be divided by the ratio of sample B versus reference, resulting in the so-called re-ratio of sample A versus sample B. Following this re-ratio calculation, standard normalization procedures can be performed as the results approximate the data of direct hybridizations.

$$\text{Re-ratio} = \frac{\frac{\text{Sample}}{\text{UR}}}{\frac{\text{Control}}{\text{UR}}} = \frac{\text{Sample}}{\text{Control}}$$

Figure 1: Principle of re-ratio.

3. Troubleshooting

No or weak signals due to RNase contamination

Although the miRXplore Reference itself is RNase-free, it is susceptible to degradation by exogenous ribonucleases introduced during handling. Wear gloves when working with the miRXplore Universal Reference. In addition, RNase-free reagents, tubes, and barrier pipette tips should be used.

Insufficient labeling or hybridization

If the sample and the miRXplore Reference give weak signals, check labeling method and washing conditions. Incorrect incubation temperatures during hybridization can lead to weak or no signals. Avoid photobleaching of the fluorescent sample.

Saturated signals

Depending on the labeling efficiency, microarray platform, and detection sensitivity, the recommended amount of 5 fmol per RNA oligonucleotide for the miRXplore Reference may give rise to saturated signals. In case of saturated signals, the amount of the miRXplore Reference should be adapted accordingly.

Broadly distributed or varying signal intensities

The signal intensities gained by microarray hybridization strongly depend on the labeling and hybridization efficiency of each individual microRNA. Differences in length, sequence, melting temperature, or secondary structure of the microRNAs may affect the labeling and hybridization efficiency.

As long as the signal intensities are reproducible, differences in the signal intensities from one microRNA to another are not critical. The miRXplore Reference can be used as a control tool to monitor the labeling and hybridization efficiency of miRNA oligonucleotides. Generally, 1 amol to 10 fmol will typically span the detection range for the miRXplore Reference on the miRXplore Microarrays.

4. References

1. miRBase sequence database version 9.2: <http://microrna.sanger.ac.uk/sequences/>
2. Landgraf, P. *et al.* (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 129: 1401–1414.
3. Pfeffer, S. *et al.* (2003) Cloning of small RNA molecules; in Ausubel, F. M. *et al.* (eds.): *Current Protocols in Molecular Biology*. New York, John Wiley & Sons.
4. Koenig, R. *et al.* (2004) Reliability of gene expression ratios for cDNA microarrays in multiconditional experiments with a reference design. *Nucleic Acids Res.* 32: e29.

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