

# PIQOR™ Microarray Technology

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# PIQOR™ Microarray Technology

## PIQOR™ Microarray Kit

Parallel Identification and Quantification of RNAs

User manual

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## 1. Description

### 1.1 Components and capacity

#### ■ Components

1 or 2 (as ordered)	tray(s) with 4 PIQOR™ Microarrays
50 mL	Wash Buffer 1 (25×)
50 mL	Wash Buffer 2 (25×)
1	PIQOR Navigator (CD-ROM)
1 box	Coverslips, 60×24 mm

#### Inner box

	Control RNA 1
	Control RNA 2
	Either 50 µL Primer Mix (s) with PIQOR Microarray, sense*, or 50 µL Primer Mix (as) with PIQOR Microarray, antisense
3 × 1.5 mL	Prehybridization Solution
2 × 1 mL	Hybridization Solution (2×)

#### ■ Capacity

For 4 or 8 microarray hybridizations as ordered.

#### ■ Product format

Control RNA 1 and Control RNA 2 are supplied as lyophilisates and are stable for at least two days on room temperature. **Upon receipt, dissolve Control RNA 1 and Control RNA 2: Add 50 µL RNase-free water and heat to 55 °C for 5 minutes with repeated up and down pipetting.**

#### ■ Storage

PIQOR Microarrays should be stored at room temperature, dry and protected from light. Avoid condensation on the PIQOR Microarray (e.g. caused by temperature changes). It is important, that the PIQOR Microarray remains dry before use. Store Wash Buffer 1, Wash Buffer 2, and coverslips at room temperature. Store inner box with dissolved Control RNA 1 and 2, Primer Mix, Prehybridization and Hybridization Solution at –20 °C.

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

\* Use Primer Mix (s) for labeling of samples for PIQOR Microarray, sense, when µMACS One-step cDNA Labeling Kit is not used.



## 1. Description

### 1.2 Reagent and instrument requirements

#### ■ (Optional) Sample stabilization

PrepProtect™ Stabilization Buffer is a non-toxic solution that stabilizes RNA in fresh or frozen cell or tissue samples for storage, shipment, or when handling of multiple samples is necessary.

PrepProtect Stabilization Buffer 10 mL  
(# 130-092-643)

PrepProtect Stabilization Buffer 100 mL  
(# 130-092-642)

#### ■ Sample preparation

##### (PIQOR™ Microarray, sense)

- (Optional) Total RNA isolation reagents and instruments
- μMACS™ One-step cDNA Labeling Kit (# 130-092-443) for up to 10 μg mRNA from 10<sup>7</sup> cells, 30 mg animal tissue, 100 mg plant tissue, 0.5 mL whole blood, or 200 μg total RNA (20 isolations)
- thermoMACS™ Separator (#130-091-136)
- MACS® MultiStand (# 130-042-303)
- **Alternatively**  
μMACS One-step cDNA Labeling Starting Kit (# 130-092-521) including 1 μMACS One-step cDNA Labeling Kit (20 isolations), 1 thermoMACS Separator, and 1 MACS MultiStand
- Fluorescent dye  
The sample labeling is optimized for usage with the following dyes: Cy3-dCTP (# PA53021); Cy5-dCTP (# PA55021), both 25 nmol, GE Healthcare, or radioactive nucleotides: alpha-32P-dCTP, 10 μCi/μL, e.g. 250 μCi; 9.25 MBq; 3,000 Ci/mmol; 25 μL,

# AA0005, GE Healthcare. Radioactively-labeled probes should have a high specific activity. Use only fresh (less than one week old) α32P-dCTP

For further requirements please refer to μMACS One-step cDNA Labeling Kit user manual.

#### ■ Sample preparation with amplification (PIQOR Microarray, antisense)

- (Optional) Total RNA isolation reagents and instruments
  - μMACS One-step T7 Template Kit (# 130-092-866)
  - thermoMACS Separator (# 130-091-136)
  - MACS MultiStand (# 130-042-303)
  - **Alternatively**  
μMACS One-step T7 Template Starting Kit (# 130-092-943) including 1 μMACS One-step T7 Template Kit (20 isolations), 1 thermoMACS Separator, and 1 MACS MultiStand
  - T7 *in vitro* transcription reagents, for example, Megascript™ T7 Kit, Ambion Inc.; Microarray RNA Target Synthesis Kit (T7), Roche Diagnostics GmbH
  - Kit for the purification of amplified RNA, for example, NucleoSpin® RNA Clean-up Kit (# 740948.10 or # 740948.50), Macherey&Nagel GmbH
- For further requirements please refer to μMACS One-step T7 Template Kit user manual.

### Fluorescent aRNA labeling and sample clean-up

- Superscript™II RNase H-Reverse Transcriptase (RT) including 5× First Strand Buffer and 0.1 M DTT (Invitrogen, # 18064-071)
- dNTP set for low C dNTP mix (see section 3.2.4), for example, peqGOLD dNTP-Set (# 20-2010), Peqlab Biotechnologie GmbH; 100 mM dNTP Set (Invitrogen, # 10297-018)
- Ribonuclease H (RNaseH, 2.2 U/μL), (Invitrogen, # 18021-014 )
- **Fluorescent dye**  
The sample labeling is optimized for use with the following dyes:  
Cy3-dCTP (# PA53021); Cy5-dCTP (# PA55021), both 25 nmol, GE Healthcare,
- CyScribe GFX Purification Kit (# 27-9606-01), GE Healthcare
- RNase-free water

### ■ Manual hybridization and washing

- Double-distilled water (ddH<sub>2</sub>O)
- 96% Ethanol (EtOH)
- Hybridization chamber (e.g. PIQOR™ HybChamb #130-091-726)
- Staining trough (e.g. Coplin, Carl Roth GmbH, # H548.1)
- Staining glass box for 60 slides in vertical position (e.g. Hauser box, Carl Roth GmbH, # T725.1)
- Lid (e.g. Carl Roth GmbH, # T726.1)
- Slide rack for 60 slides in vertical position (e.g. staining insert, Carl Roth GmbH, # T727.1)

- Water bath, optional: hybridization oven or suitable PCR Cycler
- Compressed, dry air/ inert gas like He, N<sub>2</sub>, from a clean supply or slide centrifuge

### ■ Automated hybridization and washing in the a-Hyb™ Hybridization Station

- a-Hyb™ Hybridization Station (#130-092-181)
- Double-distilled water (ddH<sub>2</sub>O)
- (Optional) PIQOR Prehybridization Solution (50 mL, # 130-091-776) for automated supply of prehybridization solution
- Isopropanol for automatic drying of slides, alternatively, staining dish for microscopic slides and compressed, dry air/inert gas or slide centrifuge for drying of slides outside of a-Hyb Station
- 50% formamide in ddH<sub>2</sub>O for automatic microarray pretreatment; alternatively, 96% ethanol (EtOH) and Hauser staining box for manual pretreatment
- (Optional) Empty glass slides when using less than four PIQOR Microarrays per experiment

### ■ Scanning

- Microarray scanner (for standard microscope glass slides)



## 1. Description

### 1.3 Safety warnings and precautions

PIQOR™ Microarrays are developed, designed, and sold for research applications only. They are not to be used for human diagnostic purposes. All due care and attention should be exercised in the handling of many of the materials described in this text. We recommend that the components of this product are handled only by personnel trained in laboratory techniques, and in accordance

with the principles of good laboratory practice. As all chemicals should be considered potentially hazardous, it is advisable to wear suitable protective clothing such as laboratory overalls, safety glasses and gloves when handling chemical reagents. If contact with skin or eyes does occur, wash immediately with water.

### 1.4 Introduction to microarray technology

Hybridization has been a powerful and widely-used tool for the characterization and quantification of nucleic acids since Edwin Southern introduced the Southern blot technique in 1975.<sup>1</sup> By immobilizing a limited number of electrophoretically separated, heterogeneous DNA samples on a solid support, such as a membrane, and hybridizing the separated DNA with a labeled, specific nucleic acid, the presence of this specific gene or DNA sequence could be analyzed in different samples.

For gene expression analysis on the RNA level, the analogous northern blotting technique was established and used as a standard for many years: Northern blotting uses electrophoretically separated RNA samples, immobilized by transfer to nylon membranes, and hybridized with a specific labeled nucleic acid sequence. Subsequently, dot blots were developed, in which larger numbers of DNA or RNA samples (no longer separated by gel electrophoresis) were immobilized and tested by hybridization in a single experiment.<sup>2</sup>

To increase expression profiling throughput, this technique has been reversed to develop DNA arrays: multiple homogeneous DNA sequences, often referred to as probes, are arrayed on a solid substrate, and are tested with a free, heterogeneous RNA mixture which is to be analyzed and is termed target or sample.<sup>3,4,5</sup> For this purpose, the sample is labeled, for example, with a fluorescent dye, and hybridized to the microarray. The fluorescence signal—generated by the hybridization of a complementary nucleic acid in the sample to an arrayed DNA—is detected and quantified using a scanner and appropriate software.

The resulting data enables the gene expression analysis of thousands of different, specific DNAs present on the microarray, in a complex mRNA population. Rather than characterizing a single gene, like in Northern blotting, RT-PCR, or RNase protection assays, microarrays can provide a complete gene expression profile from a cell or tissue.

## 1.5 Product application

Microarrays provide an effective method to screen very large numbers of genes rapidly in parallel. Consequently, they are becoming the tool of choice for generating gene expression data at the RNA level.

The PIQOR™ Microarray Kit, developed at Miltenyi Biotec, enables a semiquantitative description of differential gene expression to be carried out in samples of interest. PIQOR Microarrays use fragments of fully characterized human and murine cDNAs that are arrayed on glass slides in functional categories (e.g. signal transduction, cytokine signaling, leukocyte differentiation, apoptosis, receptors, oncogenes and tumour suppressors).

After hybridization of two or more fluorescently labeled samples to the appropriate number of microarrays (see fig. 1 and 2 as well as section 2.1 for details on general procedure), fluorescent scanning is used to generate data for comparison of amounts of specific RNAs between samples. Depending on the sources of the RNA samples, microarray experiments identify genes that are differentially expressed either in different tissues or cells, or in different biological states of the same tissue or cell type (e.g. activated/unactivated, diseased/healthy, treated/untreated).<sup>6</sup> Comparison of gene expression profiles in healthy and diseased states of the same tissue or cell types can, for example, lead to the identification of multiple drug targets. Likewise, the mechanism of drug action can be investigated by identifying marker genes, and by studying changes in their expression following administration of the drug.<sup>8,9,10,11</sup>

For research on stem cells and development, microarrays allow monitoring as well as optimization or quality control of differentiation protocols. Furthermore, microarrays enable researchers to analyse biological pathways or to classify associated genes by cluster analysis.

To enable high-quality gene expression profiling, MACSmolecular provides reagents and kits based on  $\mu$ MACS™ Technology for sample preparation optimized with PIQOR Microarrays.

### 1.6 Characteristics of PIQOR™ Microarray Technology

PIQOR™ Microarray Technology (Parallel Identification and Quantification of RNAs) has developed to produce premium microarrays for generation of high quality gene expression data. The proprietary technology consists of continuously updated human, mouse, and rat cDNA collections, a high throughput spotting device, an optimized DNA immobilization procedure (patent pending), a hybridization kit and a hybridization cassette for manual processing, the PIQOR HybChamb as well as the a-Hyb™ Hybridization Station for automated slide hybridization.

#### ■ PIQOR™ Collection

The cDNA collection used for PIQOR Microarrays consists of fully characterized human, mouse, and rat fragments. More fragments are permanently added to the library as a result of ongoing research. Fragments in the collection have been selected using the following criteria:<sup>12</sup>

- **The region is selected to contain no repetitive elements** (e.g. Alu, B1, MIRs, microsatellites) to avoid hybridization to unrelated RNAs.
- **The region selected has minimal homology (<85%) to other members of the same gene family** to prevent cross-hybridization.
- **PIQOR Fragments are 200 – 400 bp in length.** This is long enough for efficient hybridization, but short enough to avoid cross-hybridization and allow effective attachment to the coating of the glass slides. Furthermore, the homogeneous length of the fragments ensures that all the arrayed cDNAs have comparable hybridization kinetics.

- **Wherever possible, we generate fragments from the same sections of orthologous human, mouse, and rat cDNAs, enabling a direct interspecies comparison of microarray-based expression profiles.** This is, for example, valuable for the comparison of pre-clinical results such as from mouse or rat cells, with expression profiles of human cells.
- **The region selected is present in all known alternatively spliced variants of a gene, and if alternative polyadenylation signals are present, it is upstream of the first signal.**

#### ■ Spotting

The spotting device used to produce PIQOR™ Microarrays is instrumental to the quality and reproducibility of the microarray data. Miltenyi Biotec has developed a piezoelectric spotter for the production of microarrays on an industrial scale. This device allows selection of optimal spotting settings, which guarantees complete reproducibility of spot shape and width, and ensures that each spot contains the same quantity of DNA.

#### ■ Substrate

The coating of the glass slides used for microarrays is critically important. They must be coated uniformly with a substance that shows no autofluorescence, has a high DNA-binding capacity and produces minimal background signals after hybridization. Miltenyi Biotec uses a proprietary surface which covalently binds DNA molecules, allowing the attachment of DNA at an optimal density, at which an adequate amount of cDNA is present but charge and steric effects are minimal.

### ■ Intra-array and inter-array variance

Control experiments in which the same RNA sample is labeled with two fluorescent samples show that the intra-array variance (measured by comparing the expression ratio of two identical samples on a single microarray) in the signals is typically less than two-fold. Experiments in which the same RNA samples are applied to different microarrays with the same cDNA configuration show that inter-array variance (measured by comparing the expression ratio of two identical samples on different microarrays) is in average over all genes less than 10%.

### ■ Data mining and analysis with PIQOR™ Navigator

PIQOR Microarray Kits include a software package, PIQOR Navigator. The software is a visualization tool that shows the gene information for every cDNA spot and enables the localization of the spot position of a cDNA on the microarray. In addition, it enables the generation of gene ID lists required for microarray image analysis with software packages like ImaGene, GenePix, and ArrayWorx. Furthermore, the PIQOR Navigator links each gene on the microarray to external databases like Unigene and SwissProt.

Please inquire if any further support for data analysis using advanced bioinformatics tools is of interest (e.g. cluster or pathway analysis).

## 2. General considerations

### 2. General considerations

#### 2.1 RNA requirements

The amount and quality of the RNA sample is a critical factor for each microarray experiment. To generate a fluorescently labeled target that allows a sensitive gene expression analysis, we recommend using the  $\mu$ MACS™ One-step cDNA Labeling Kit (see section 3.1, Sample preparation for PIQOR Microarrays, sense).

If the amount of starting material is limited, such as from rare cells or biopsy material, or one sample is required for multiple analyses, we strongly recommend mRNA amplification before labeling. The  $\mu$ MACS One-step T7 Template Kit enables magnetic RNA isolation and T7 amplification in one column (see section 3.2, Sample preparation with amplification). RNA amplified in this way is complementary to mRNA, and termed antisense RNA (aRNA).

Please see table 1 below for sample requirements. If even less than  $5 \times 10^4$  cells or 250 ng total RNA is available, please contact Technical Support for further information.

The RNA purity is of critical importance for the labeling, especially when using fluorescent dyes. Cellular contaminants in the RNA sample, mainly carbohydrates (which aggregate with nucleic acids when dried or precipitated), but also proteins and lipids (combined with the highly hydrophobic fluorescent dyes), can cause problems such as dispersed, fine-grain noise over the entire microarray surface, and non-specific binding of fluorescent dye to the immobilized cDNAs on the slide.

Therefore, when using RNA extraction kits, it is essential to use the recommended volumes of lysis solution and extraction buffer, and to carefully follow instructions when carrying out washing steps. Extraction and labeling methods using ethanol precipitation should be avoided.

Amount of starting material	Sample preparation kit	Amplification required	PIQOR™ Microarray Kit
Up to $1 \times 10^7$ cells, 30 mg human and animal tissue, or up to 200 $\mu$ g total RNA	$\mu$ MACS™ One-step cDNA Labeling Kit	no	sense
$5 \times 10^4$ – $1 \times 10^6$ cells, up to 6 mg human and animal tissue, or 250 ng – 40 $\mu$ g total RNA	$\mu$ MACS One-step T7 Template Kit	yes	antisense

Table 1: Overview on required amounts of starting material and appropriate kits

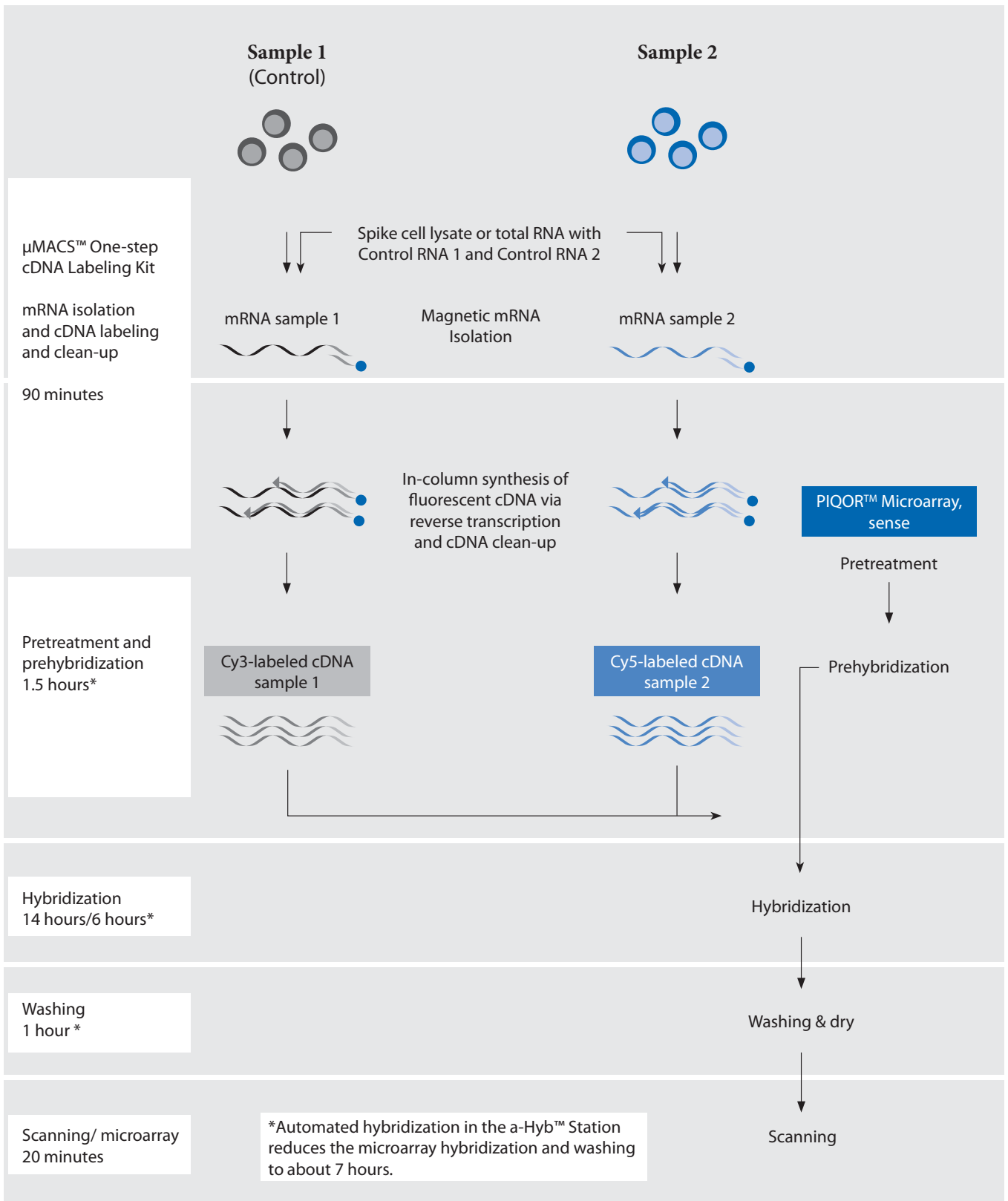


Figure 1: PIQOR™ Microarray procedure (PIQOR Microarray, sense)

## 2. General considerations

$\mu$ MACS™ Kits for sample preparation include poly(A)<sup>+</sup> RNA isolation to provide highly pure sample material for microarray experiments. A poly(A)<sup>+</sup> RNA sample is much less complex and thus can be more specifically labeled, compared to a total RNA sample. Additionally, the reverse transcriptase reaction works more efficiently with lower concentrations of substrate. Starting material for  $\mu$ MACS sample preparation can be cells or tissues (direct mRNA isolation) or total

RNA (two-step mRNA isolation). A two-step mRNA isolation from total RNA can improve the purity of RNA samples. Therefore, depending on tissue or cell material, Trizol-based or other RNA extraction kits can be used for total RNA preparation. For suitable tissues and cells, poly(A)<sup>+</sup> RNA can be isolated directly from cells or tissues, in a single-step procedure.

### 2.2 Procedure overview

A microarray experiment for gene expression profiling consists of RNA preparation and labeling, followed by hybridization of the labeled sample to the microarray. If only limited amount of sample material is available, a sample amplification step should be included. The  $\mu$ MACS One-step cDNA Kits enable the one-step mRNA purification with paramagnetic Oligo(dT) MicroBeads with a subsequent in-column cDNA synthesis.

For sufficient amount of starting material,  $\mu$ MACS One-step cDNA Labeling Kit combines mRNA isolation and synthesis of labeled cDNA. The direct sample labeling is performed by reverse transcription in the presence of labeled dNTPs. The obtained cDNA is complementary to mRNA and hybridized to **PIQOR™ Microarray, sense** (refer to protocol 3.1, Sample preparation for PIQOR Microarray, sense).

When starting material is limited, sample amplification following the protocol of the  $\mu$ MACS One-step T7 Template Kit is recommended. After magnetic mRNA isolation, double-strand cDNA with a T7 promoter is synthesized for a subsequent RNA amplification.

This linear amplification is performed using T7 polymerase in an *in vitro* transcription (IVT) reaction. After labeling and purification, this target can be hybridized to PIQOR Microarrays. Labeled cDNA obtained from amplified antisense RNA (aRNA) has the same orientation as mRNA and is hybridized to **PIQOR Microarray, antisense** (refer to protocol 3.2, Sample preparation for PIQOR Microarray, antisense). Since only one strand of the respective PIQOR Fragment is covalently attached to the surface of a PIQOR Microarray, it is important to use the appropriate PIQOR Microarray Kit.

In order to facilitate multiple comparisons of PIQOR Microarray data, it is recommended to consistently label the control sample with one CyDye dCTP (wild type, uninduced cells/tissue, time point 0, etc.), and the experimental sample with the other: Most investigators use Cy3 for the control, and Cy5 for the experimental sample. The fluorescence signal generated by the hybridization of a complementary nucleic acid sequence to an arrayed cDNA is detected and quantified using a scanner and appropriate software.

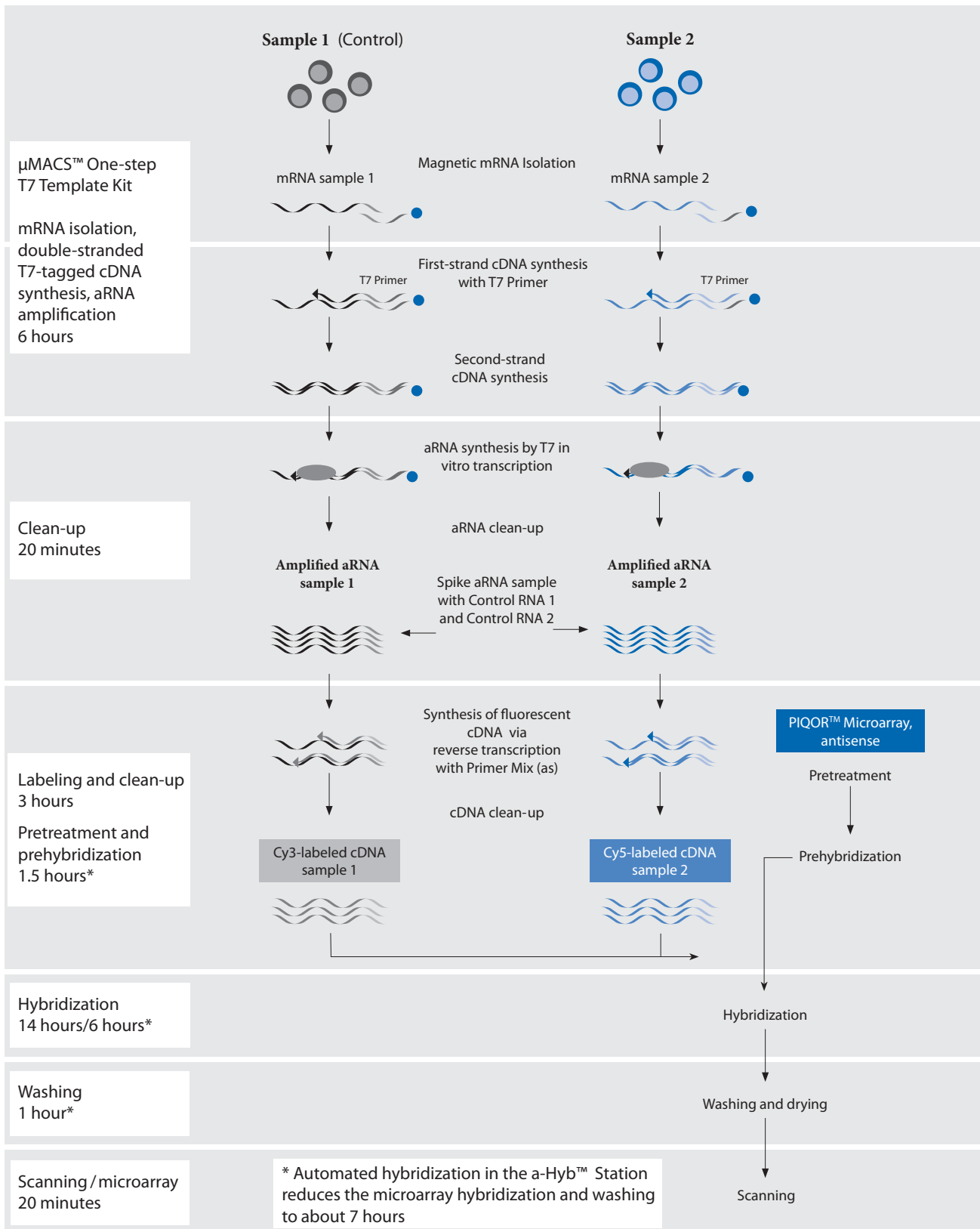


Figure 2: PIQOR™ Microarray procedure with RNA amplification (PIQOR Microarray, antisense)

## 2. General considerations

### 2.3 Handling of PIQOR™ Microarrays

For optimal performance, the following points should be observed when working with microarrays:

- Always handle the microarrays by holding the barcode-end of the slide. Do not touch the spotted surface of PIQOR™ Microarrays (located on the same side as the barcode) or an edge covered by coverslip (as this may cause loss of applied solutions through capillary conductance).
- Store microarrays and coverslips in the provided trays, 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.)
- When applying the coverslip, avoid trapping air bubbles by lowering the coverslip slowly on to the PIQOR Microarray from one side to the other.
  - ▲ Note: Small air bubbles trapped under the coverslip disappear after incubation at 65 °C for several minutes. If larger bubbles are trapped, they can be removed by gently pressing on the surface of the coverslip, thereby directing the air bubble to the nearest edge of the coverslip.
- If the coverslip is not in the right position (see figure 3 below), move it by touching its surface, not by touching the edges.

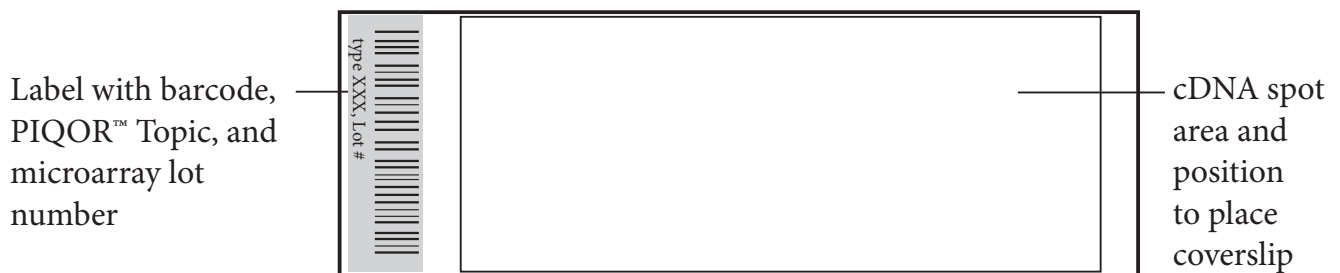


Figure 3: PIQOR™ Microarray with coverslip. The PIQOR™ Microarray type is given on the label with first 3 letters of name, first letter of species (h= human, m= mouse, r= rat), and orientation (s= sense, a= antisense); for example, PIQOR Immunology Microarray, human, sense is abbreviated to „Imm h s“.

## 2.4 Manual versus automated hybridization

PIQOR™ Microarrays use the standard 25×75 mm slide format and can be hybridized manually, or in an automated procedure using a hybridization machine. With an appropriate chamber, microarrays can be hybridized in a standard water bath or hybridization oven. However, when throughput is increased, automated hybridization reduces handling time and increases reproducibility and speed of microarray hybridization.

- **For manual hybridization**, care should be taken not to let the microarray get dry, as hybridization solutions can evaporate at the recommended hybridization temperature. Thus, the use of a humidified hybridization cassette, such as the PIQOR HybChamb is recommended. The PIQOR HybChamb is a small-sized, watertight chamber for four microarrays. It can be used for hybridization in a water bath or hybridization oven or on a MJ Research Thermocycler with a temperature-controlled lid. To humidify the chamber, water is applied in the respective cavities without risk of the hybridization solution being diluted or contaminated. Small racks in the PIQOR HybChamb allow easy insertion and removal of microarrays, spacers avoid cross-contamination between different microarrays.

- **For automated hybridization**, a fully automated hybridization/incubation station has been developed: the a-Hyb™ Hybridization Station. In this instrument, the sample solution is actively circulated over the spotted probe area in a closed system via micropumps. This unique active circulation 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. Additionally, the a-Hyb Station can be integrated into standard laboratory robot systems for fully automated slide processing. The microtiter plate-format slide carrier of PIQOR Microarrays facilitates the handling of up to four standard slides in parallel.

## 2. General considerations

### 2.5 PIQOR™ Microarray controls

#### ■ Positive controls

Two positive control RNAs, Control RNA 1 and Control RNA 2, are included in the kit. Control RNA 1 and Control RNA 2 are *in vitro* transcripts of *E. coli* genomic DNA fragments. Control RNA 1 consists of one transcript, CR1, at a concentration of 10 fmol/μL. Control RNA 2 is a mixture of three different transcripts, CR2<sub>A</sub>, CR2<sub>B</sub>, and CR2<sub>C</sub> with concentrations of 5 fmol/μL, 2.5 fmol/μL and 1 fmol/μL, respectively.

Control RNAs are added to the total RNA or cell lysate before poly(A)<sup>+</sup> enrichment to assess efficiency and sensitivity of the enrichment, labeling, and (optional) amplification reaction. The control RNA spots are positioned at the corners of each microarray. Thus, the Control RNA signals support the alignment of the grid provided by the imaging software. Brightest signals should be obtained for Control RNA 1 and Control RNA 2<sub>A</sub>, Control RNA 2<sub>B</sub> and in particular Control RNA 2<sub>C</sub> give lower signal intensities.

#### ■ Negative controls

As background quality controls, herring sperm DNA and buffer are spotted as negative controls.

#### ■ Housekeeping genes

Each PIQOR™ Microarray contains six housekeeping genes which are considered to be expressed in most tissues and cells and give strong signal intensities.

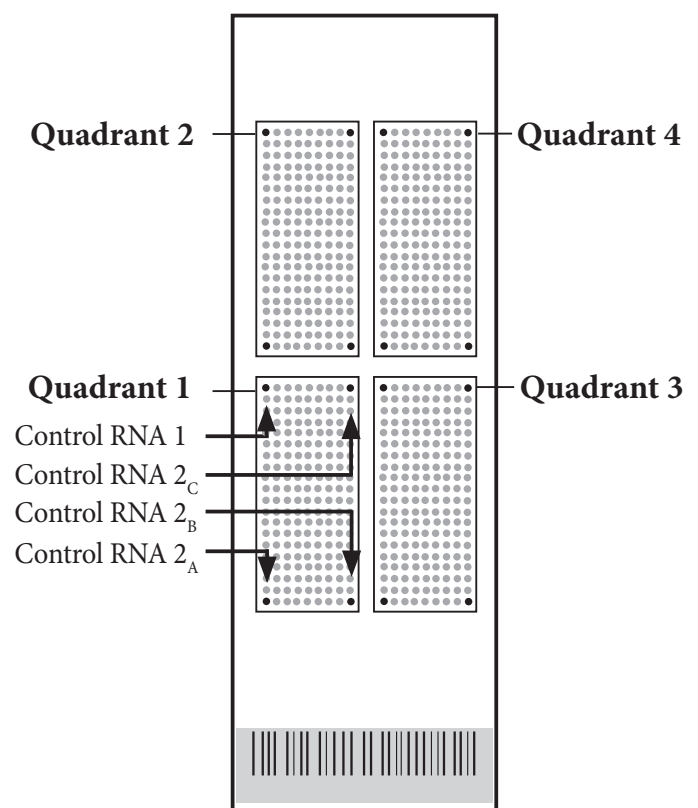


Figure 4: Position of control RNAs on PIQOR™ Microarray spotted with quadruplicates (quadrant 1–4)

### 3. Sample preparation

For **PIQOR™ Microarray, sense**, please refer to 3.1, Sample preparation for PIQOR Microarrays, sense. For **PIQOR Microarray, antisense** and limited starting material, please refer to 3.2, Sample preparation and amplification for PIQOR Microarrays, antisense.

#### 3.1 Sample preparation for PIQOR™ Microarrays, sense

##### ■ 3.1.1 Two-step RNA preparation

###### Extraction of total RNA, followed by poly(A)<sup>+</sup> RNA enrichment and cDNA labeling

1. Add 2 µL of Control RNA 1 and 2 µL of Control RNA 2 to the total RNA before poly(A)<sup>+</sup> RNA enrichment.
2. Carry out poly(A)<sup>+</sup> RNA enrichment and cDNA labeling according to the protocol supplied with µMACS™ One-step cDNA Labeling Kit.

##### ■ 3.1.2 Single-step RNA preparation

###### Direct isolation of poly(A)<sup>+</sup> RNA from samples and cDNA labeling

- ▲ Note: Single-step mRNA preparation is recommended for suitable tissues and cells only. If suitability of the individual tissue/cells is not tested, the two-step process described above is recommended for optimal results.
1. Prepare cell or tissue lysate according to µMACS One-step cDNA Labeling Kit User Manual.
  2. Add 2 µL of Control RNA 1 and 2 µL of Control RNA 2 to the lysed cell or tissue sample.
  3. Isolate poly(A)<sup>+</sup> RNA directly from the sample and label cDNA according to the protocol supplied with µMACS One-step cDNA Labeling Kit.



## 3. Sample preparation

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### 3.2 Sample preparation with amplification for PIQOR™ Microarrays, antisense

#### ■ 3.2.1 Two-step RNA preparation

##### Extraction of total RNA, followed by poly(A)<sup>+</sup> RNA enrichment and aRNA amplification

1. Isolate poly(A)<sup>+</sup> RNA, synthesize double-strand T7 promoter-tagged cDNA, and amplify aRNA according to the protocol supplied with μMACS™ One-step T7 Template Kit.

#### ■ 3.2.2 Single-step RNA preparation

##### Direct isolation of poly(A)<sup>+</sup> RNA from samples and aRNA amplification

- ▲ Note: Single-step mRNA preparation is recommended for suitable tissues and cells only. If suitability of the individual tissue/cells is not tested, the two-step process described above is recommended for optimal results.
1. Isolate poly(A)<sup>+</sup> RNA directly from the sample, synthesize double-strand T7 promoter-tagged cDNA, and amplify aRNA according to the protocol supplied with μMACS One-step T7 Template Kit.

### ■ 3.2.3 *In vitro* transcription reaction clean-up

The clean-up protocol for the *in vitro* transcription reactions below uses the NucleoSpin® RNA Clean-up kit (for further information refer to NucleoSpin RNA Clean-up kit user manual).

1. Fill up aRNA sample with RNase-free water to 100  $\mu$ L.
2. Prepare a RA1-ethanol premix:  
Mix 300  $\mu$ L of RA1 with 300  $\mu$ L of ethanol (96 – 100%).
3. Add 600  $\mu$ L of RA1-ethanol premix to 100  $\mu$ L aRNA sample. Mix by vortexing.
4. Transfer sample to NucleoSpin RNA Binding Column placed in a 2 mL centrifuge tube and centrifuge for 30 seconds at 8,000 $\times$ g. Discard collecting tube with flow-through and place the column in a new collecting tube.
5. Add 700  $\mu$ L Wash buffer RA3 to the column. Centrifuge for 30 seconds at 8,000 $\times$ g. Discard flowthrough and reuse collecting tube.
6. Add 350  $\mu$ L Wash buffer RA3 to the column. Centrifuge for 2 minutes at 8,000 $\times$ g. Discard flow-through and place the column into a fresh collection tube. Open the lid of the spin column and let it sit for 3 minutes.
7. Add 60  $\mu$ L RNase-free H<sub>2</sub>O to the membrane in the spin column. Centrifuge for 1 minute at 8,000 $\times$ g.
8. Take an aliquot of the purified aRNA, dilute 1:50 and measure OD 260 to determine nucleic acid concentration.
9. Store aRNA at –20 °C or continue with 3.2.4, Fluorescent sample labeling, to generate fluorescently labeled first strand cDNA. Use 1  $\mu$ g of aRNA for one labeling reaction and add 2  $\mu$ L of Control RNA 1 and 2  $\mu$ L of Control RNA 2 to the sample before labeling. Adjust sample volume to 20  $\mu$ L.

### 3. Sample preparation

#### ■ 3.2.4 Fluorescent sample labeling

The labeling reaction generates fluorescently labeled first-strand cDNA, using the isolated the amplified aRNA from Section 3.2.2 as a template.

Note: This protocol can also be used for labeling of not amplified samples for PIQOR™ Microarray, sense, when  $\mu$ MACS™ One-step cDNA Labeling Kit is not used.

**Before starting: Prepare low C dNTP mix (see below).**

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

1. For the labeling reaction, combine the following in a 1.5 mL RNase-free microcentrifuge tube:

8 $\mu$ L	5 $\times$ First Strand Buffer
2 $\mu$ L	Primer Mix (as)
2 $\mu$ L	Low C dNTPs (10 mM dATP, 10 mM dGTP, 10 mM dTTP, 4 mM dCTP)*
2 $\mu$ L	Cy3-dCTP (sample 1) or 5-dCTP (sample 2)
4 $\mu$ L	0.1 M DTT

Mix by vortexing.

- ▲ Note: Use Primer Mix (s) for labeling of samples for PIQOR Microarray, sense, when  $\mu$ MACS One-step cDNA Labeling Kit is not used.
2. Add 20  $\mu$ L RNA-Mix from section 3.2.2 (or section 3.1). Vortex again and centrifuge briefly.

3. Incubate at 70 °C for 3 minutes.
4. Cool sample to 42 °C.
5. Add 1  $\mu$ L RT (200 U) and mix thoroughly. Incubate reaction at 42 °C for 30 minutes.
6. Repeat step 5: Add 1  $\mu$ L RT (200 U) and mix thoroughly. Incubate reaction at 42 °C for 30 minutes.
7. Add 0.5  $\mu$ L RNaseH. Incubate at 37 °C for 20 minutes to hydrolyze RNA. Proceed to section 3.2.4, Fluorescent sample clean-up.

#### \*Preparation of low C dNTP mix

For effective incorporation of the fluorescently labeled dCTPs, low amounts of unlabeled dCTP are included in the dNTP mix used for section 3.3, Fluorescent sample labeling.

To prepare the required low C dNTP mix, combine the following in a 1.5 mL RNase-free microcentrifuge tube:

10 $\mu$ L	dATP (100 mM)
10 $\mu$ L	dGTP (100 mM)
10 $\mu$ L	dTTP (100 mM)
4 $\mu$ L	dCTP (100 mM)
66 $\mu$ L	RNase-free H <sub>2</sub> O

Aliquot and store at –20 °C.

### ■ 3.2.5 Fluorescent sample clean-up

The clean-up protocol for fluorescent samples below makes use of the CyScribe GFX purification kit (for further information refer to CyScribe GFX purification kit user manual).

**Before starting: Pre-warm 2× Hybridization Solution, included in PIQOR™ Microarray Kit, to 42 °C.**

1. Use one GFX column for each sample. Add 500 µL capture buffer on GFX column.
  - ▲ Note: Differently labeled cDNAs can be combined to one sample.
2. Pipette labeled cDNA sample 5 times up and down and transfer into GFX column.
3. Centrifuge column immediately for 30 seconds at 11,000xg.
4. Discard flow-through. Place the GFX column back into the same tube.
5. Add 600 µL wash buffer to column and centrifuge for 30 seconds at 11,000xg.
6. Discard flow-through and place the GFX column back into the same tube.
7. Repeat last wash steps (5 and 6) to a total of 3 washes. After the final wash, discard the liquid and place column back in the used collection tube.
8. Centrifuge column for an additional 10 seconds at 11,000xg to remove all wash buffer in the tip of the column. Discard the collection tube.
9. Transfer GFX column to a fresh 1.5 mL microcentrifuge tube and add 60 µL elution buffer to the top of the glass fiber matrix in each GFX column. Make sure that the elution buffer completely covers the membrane.
10. Incubate the GFX column at room temperature for 1–5 minutes. Centrifuge for 1 minute at 11,000xg to collect the purified labeled cDNA.
11. Quantify the frequency of CyDye incorporation by spectrophotometric absorption.
12. For manual hybridization only: Concentrate purified sample under vacuum at 45 °C until volume is 20 µL.
  - ▲ Note: Take care not to dry the sample.
13. For manual hybridization only: add 20 µL 2× Hybridization Solution, pre-warmed to 42 °C.
  - ▲ Note: 2× Hybridization Solution may form precipitates during storage. Pre-warming to 42 °C allows the precipitates to dissolve and facilitates pipetting of the viscous solution. Mix the pre-warmed solution thoroughly before pipetting. The total sample volume should be 40 µL.
14. Store sample at room temperature in the dark until pre-hybridization is finished.
  - ▲ Note: Labeled samples can be stored protected from light at –20 °C for at least six months without significant loss of signal intensity.

## 4. Manual hybridization and wash

### 4. Hybridization and wash

#### 4.1 Manual hybridization and wash

##### ■ 4.1.1 PIQOR™ Microarray pretreatment

Before starting: Heat water bath to 95 °C.

1. Heat PIQOR™ Microarray in distilled H<sub>2</sub>O in a water bath at 95 °C for 2 minutes.
  - ▲ Note: This can be performed as follows: Fill a cylindrical beaker (2 L, made of stainless steel) with distilled water. Use an old microarray placed in a slide rack in vertical position (with barcode label at top) to adjust water level: The water should cover the microarrays up to—but not touching—the barcode label. Place beaker into a 95 °C water bath. Place microarrays vertically into a slide rack and immerse it into the beaker filled with ddH<sub>2</sub>O preheated to 95 °C.
  - ▲ Note: This step denatures the double-stranded cDNA molecules on the glass slide, leaving only the strand complementary to the sample covalently bound to the slide surface.
2. Immediately transfer the PIQOR Microarray into a Hauser staining box filled with 96% EtOH for at least 30 seconds. Avoid contact of the barcode label with 96% EtOH.
3. Dry by centrifugation for 3 minutes at 500×g, or with pressured gas from a clean supply.
  - ▲ Note: If using pressured gas, dry slide starting from the opposite site of the bar code towards bar code label.
4. Place dry microarray in a dust-free slide box.

##### ■ 4.1.2 Prehybridization

For optimum manual hybridization, PIQOR Microarrays should be incubated using the PIQOR HybChamb. Prehybridization of PIQOR Microarrays significantly reduces the background fluorescence and is therefore strongly recommended.

- ▲ Note: Microarrays should be placed in horizontal, „face-up“ position during all prehybridization and hybridization steps.
1. Heat Prehybridization Solution at 98 °C for 2 minutes, centrifuge briefly and cool to 42 °C.
    - ▲ Note: Prehybridization solution may form a precipitate when stored. Redissolve the precipitate by heating the solution to 50 °C.
  2. Apply 40 µL of PIQOR Prehybridization Solution onto the microarray in the cDNA spot area (see section 2.3, Handling of microarrays). Place the coverslip over the PIQOR Microarray.
  3. Humidify hybridization cassette by pipetting 90 µL distilled H<sub>2</sub>O into each of the four cavities at the bottom of the PIQOR HybChamb.
  4. Place microarray in hybridization cassette. Seal the cassette and incubate in a water bath, hybridization oven, or PCR cycler at 65 °C, for at least 30 minutes.
    - ▲ Note: Make sure that the hybridization cassette is properly sealed to prevent the hybridization reaction from drying out. Seal PIQOR Hyb Chamb by tightly turning the screw of the fixing device downwards or by tightly closing the PCR cycler lid.

### ■ 4.1.3 Hybridization

1. Cool the hybridization cassette to 25 °C by leaving it at room temperature for at least 20 minutes.
2. Incubate 40 µL labeled sample from section 3.4 at 65 °C for 2 minutes.
3. Remove the PIQOR™ Microarray from the cassette and carefully remove the coverslip.
4. Centrifuge sample briefly and apply immediately to the spot area of the PIQOR Microarray.
  - ▲ Note: Simply apply the labeled sample without removing residual Prehybridization Solution. Do not wash the PIQOR Microarray, and do not allow it to dry.
5. Place fresh coverslip on to the PIQOR Microarray.
  - ▲ Note: Do not reuse the coverslip from the prehybridization step.
6. Place in a humidified hybridization cassette. Seal the cassette and incubate overnight (minimum 6 hour) in a hybridization oven, water bath or appropriate PCR cycler at 65 °C (see also Appendix, Handling of the PIQOR HybChamb).
  - ▲ Note: Make sure that the hybridization cassette is properly sealed to prevent the hybridization reaction mix from drying out.

### ■ 4.1.4 Wash

**Before starting:**

**Dilute 25× Wash Buffer 1 and 25× Wash Buffers 2 in 0.25 L aliquots to a final concentration of 1× Wash Buffer.**

- ▲ Note: 25× Wash Buffer 1 and 25× Wash Buffer 2 may form a precipitate when stored. If necessary, redissolve by warming to 50 °C. To prevent that undissolved particles cause background fluorescence 1× Wash Buffers should be filtered.

**Pre-heat Wash Buffer 1 and Wash Buffer 2 to 50 °C.**

Set a water bath to 50 °C. Coplin staining troughs filled with the Wash Buffers should be placed into this water bath to keep the temperature of the Wash Buffers constant during the washing process.

1. Remove PIQOR Microarray from the hybridization cassette and place it immediately in a Coplin staining trough filled with preheated 1× Wash Buffer 1. If the coverslip does not slide off the PIQOR Microarray upon placing it into the Wash Buffer, remove coverslip using forceps.
2. Remove the coverslip out off the Coplin staining trough and move the microarray several times up and down immediately. Wash the microarray for 5 minutes at 50 °C.
3. Repeat step 1: Wash PIQOR Microarray with fresh 1× Wash Buffer 1 for 5 minutes at 50 °C.
4. Wash PIQOR Microarray with 1× Wash Buffer 2 for 5 minutes at 50 °C.

## 4. Automated hybridization and wash

5. Repeat step 3: Wash PIQOR™ Microarray with 1× Wash Buffer 2 for 5 minutes at 50 °C.
6. Dip microarray three times quickly into distilled water filled in a staining trough at room temperature.
  - ▲ Note: This final wash step ensures that no remaining salt residues are left on the slide.
7. Dry microarray by centrifugation at 500×g for 3 minutes, or with compressed, dry air (or inert gas like He, N<sub>2</sub>) from a clean supply.
  - ▲ Note: If using compressed gas, hold up slide nearly vertically and start drying the slide from the upper opposite side of the barcode label down to the end with the barcode label.

Thus, the transfer of background material from the barcode label on to the spots is avoided.

8. Store slide in a dust-free slide box. The PIQOR Microarray is ready for scanning.

### 4.2 Automated hybridization and wash in the a-Hyb™ Hybridization Station

#### 4.2.1 Preparation of buffers

- ▲ Note: For automatic buffer supply, 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. For manual application, a volume of 200 µL is needed per step and slide.

If the wash buffers contain precipitates, heat the buffer to 50 °C for several minutes until the precipitate dissolves.

Depending on the given experimental design, it might be advisable to fill the volumes of prehybridization and wash buffers required for the specific experiment in appropriate containers (e.g. 50 mL Falcon™ Conical Centrifuge Tubes).

1. Heat Prehybridization Solution to 98 °C for 2 minutes, centrifuge briefly and cool down to 42 °C before usage.
2. Pre-heat water bath or heating block to 65 °C.

### ■ 4.2.2 Sample preparation

1. Dissolve labeled cDNA sample in a total volume of 200  $\mu\text{L}$  by adding the appropriate amount of molecular biology grade water and 100  $\mu\text{L}$  of hybridization buffer.
2. Heat sample to 65  $^{\circ}\text{C}$  for 2 minutes prior to sample application.

### ■ 4.2.3 Setting-up of instrument

Connect buffer bottles with the corresponding tube for automated medium supply.

Medium tube 3: Prehybridization Solution

Medium tube 1: Wash Buffer 1

Medium tube 2: Wash Buffer 2

▲ Note: Tube numbers might be different if the a-Hyb™ Station protocol is altered. Please make sure, that the usage of tubes corresponds to the selected a-Hyb Protocol, See section 6.1 for an overview of the a-Hyb Station protocol.

Medium tube 8: ddH<sub>2</sub>O

(Optional) For automated microarray pre-treatment: Medium tube 4 (50% formamide in ddH<sub>2</sub>O)

(Optional) For automated drying  
Medium tube 7: Isopropanol

### ■ 4.2.4 PIQOR™ Microarray pretreatment

The microarray pretreatment denatures the double-stranded cDNA molecules on the glass slide, leaving only the strand complementary to the sample covalently bound to the slide surface. For automated microarray pretreatment in the a-Hyb Hybridization Station, two extra steps have to be added to the hybridization protocol, see Appendix, section 6.2.

Alternatively, see section 4.1.1 and 4.1.2 for manual pretreatment.

1. Start process according to section 4.2.5, Hybridization, using special protocol as described in 6.2. Use additional Medium 4 (50% Formamide in ddH<sub>2</sub>O) and Medium 8 (ddH<sub>2</sub>O).

### ■ 4.2.5 Hybridization

Please refer to the a-Hyb Station user manual for detailed information on installation, setup, and usage of the a-Hyb Instrument.

1. Switch on power button of a-Hyb Station and choose the hybridization screen, designated by the blue area.
2. Select slide positions to be used for hybridization.
3. Select PIQOR™ Protocol.
  - ▲ Note: Select View protocol to check protocol and match of buffer and tube number selection. See section 6.1 and 6.2 for further information.
4. Switch to the hybridization screen and press Start to begin the hybridization process.
5. Open package with PIQOR Microarrays fixed in a slide carrier. Remove white bottom plate.

Alternatively, load PIQOR Microarrays in a-Hyb Slide Carrier, and place plain glass slides in unoccupied positions.



## 4. Automated hybridization and wash

6. Place a-Hyb™ Sealing Plate on top of a-Hyb Slide Carrier.
7. Place a-Hyb Slide Carrier with sealing plate into drawer and press Continue. The hybridization process will start.
8. Pre-heat cDNA samples to 65 °C for 2 min.
9. After 5 minutes of prehybridization, the software will ask to press continue before adding the sample: Press Continue when you are ready to add the sample.
10. The software will immediately ask for sample application: Pipette 200 µL of labeled cDNA sample in the reservoir and press Continue.
11. Apply further samples for all selected positions.

### ■ 4.2.6 Washing

All washing steps will be performed automatically.

End of process

#### Option 1: Manual drying

1. Fill Wash Buffer 2 or ddH<sub>2</sub>O into slide-staining box.
2. At the end of the process the software will ask whether it should cool down to 25 °C. Press No. Press Continue to open the instrument.
3. Immediately remove the sealing plate, place the slide carrier on the slide loading device to take out the slides.
4. Immediately transfer slides to the slide rack within the slide-staining dish containing Wash Buffer 2 or ddH<sub>2</sub>O.
5. Dip the slides twice in ddH<sub>2</sub>O.
6. Dry microarray by centrifugation at 500×g for 3 minutes, or with compressed, dry air (or inert gas like He, N<sub>2</sub>) from a clean supply.
  - ▲ Note: If using compressed gas, dry slide starting from the opposite side of the bar code label towards bar code label.

#### Option 2: Automatic drying

For automatic drying, an additional step has to be added as last step to the PIQOR™ Protocol. Drying will be done automatically at the end of the process.

Store slides in a dust-free hybridization cassette. The PIQOR Microarray is ready for scanning.

## 5. Troubleshooting

Please also refer to  $\mu$ MACS™ One-step cDNA Labeling Kit and  $\mu$ MACS One-step T7 Template Kit user manuals and a-Hyb™ Station instruction manual.

### ■ No or weak signals

#### RNA sample quality

High-quality RNA is a prerequisite for microarray analysis. However, RNA is chemically unstable and susceptible to ubiquitous RNases. Therefore, RNase-associated degradation during sample collection, storage, thawing of frozen samples, and RNA preparation should be prevented. The non-toxic **PrepProtect™ Stabilization Buffer** allows instant RNA stabilization in freshly harvested or frozen samples. Upon addition of PrepProtect Buffer, **freshly harvested samples** are stable for at least one day at 37 °C, one week at room temperature, one month at 4 °C, or unlimited below –20 °C. This facilitates sample collection and parallel processing of multiple samples. Additionally, PrepProtect Buffer seeps through previously frozen material, and protects RNA during thawing. **Frozen samples** incubated in PrepProtect Buffer are stable for up to one hour at room temperature or overnight at 4 °C. This enables researchers to prepare their samples, for example, to weigh or dissect tissues, before RNA isolation. PrepProtect Buffer compensates the hardness of frozen tissues by softening their structure; thereby, facilitates tissue lysis and homogenization without prior pulverization with mortar and pestle.

When performing two-step **mRNA isolation** check total RNA quality by spectrophotometric absorbance measurements: Calculate amount of nucleic acids with OD260 (40  $\mu$ g/mL/OD260 RNA). The ratio of OD260/280 absorbance values should be between 1.8–2.0, a lower value indicates protein contamination. Integrity of RNA samples

with regard to RNA sizes, potential degradation or genomic DNA contamination can be checked by gel electrophoresis. To obtain high-quality samples, poly(A)<sup>+</sup> RNA isolation is performed using  $\mu$ MACS One-step cDNA Labeling or T7 Template Kits (refer to section 2.1. and 2.2). Strong signals at the indicated corner position for labeled Control RNAs indicate efficient sample preparation (see fig. 3, section 2.5, PIQOR Microarray controls, or use the PIQOR Navigator for spot allocation).

#### Sample amplification for PIQOR™ Microarray, antisense, only

##### • Average transcript length of amplified RNA

For quality control of sample amplification, the transcript length of amplified aRNA can be monitored by formamide gel electrophoresis or with a Bioanalyzer system (Agilent Technologies). When starting with intact and pure total RNA, the average aRNA transcript length should be approximately 1,500 bases. If the length of the transcripts is significantly lower (e.g. 500 bp) the labeling efficiency (incorporated CyDyes per transcript) will decrease.

• **Reverse Transcriptases (RT)** are thermolabile enzymes. To avoid RT degradation and loss of function, RT should always be kept on ice and stored at –20 °C (refer to manufacturer's recommendations).

#### Sample labeling

Strong signals at respective positions on the PIQOR™ Microarray of labeled Control RNAs indicate efficient fluorescent sample labeling. If no or only weak signals for the Control RNAs were obtained, check DNA quantity and incorporation rate of Cy dyes.



## 5. Troubleshooting

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We recommend checking the quality of fluorescent sample labeling by OD measurements of concentration (37 µg/OD260) and determining the dye incorporation per nucleotide (see section 6.3, Calculation of fluorescent label incorporation rate). Furthermore, each PIOQR Microarray contains six housekeeping genes, which are considered to be expressed in most tissues/cells; therefore, they serve as additional positive controls. Fluorescent CyDyes are light sensitive. Dyes and labeled samples should be kept protected from light.

### Scanner-derived bleaching

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.<sup>13</sup>

### ■ High non-specific background

#### RNA sample quality

A 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 at the scanning wavelength 550 or 650 nm, they give rise to higher 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 targets. 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 (which can also be monitored via checking the negative controls “buffer only” and “herring sperm DNA” that should not give signals above background), it is necessary to rewash and rescan the slide.

### Unincorporated Cy dyes (for amplified samples only)

If after the clean-up step of the fluorescently labeled probes the cDNA solution in the tube is still strongly colored, it is recommended to repeat the fluorescent sample clean-up procedure (section 3.2.4) to remove unincorporated dyes that will cause a higher background.

### Precipitates in Prehybridization/Hybridization solution/Wash Buffers

Make sure that all used buffers or reagents are free of particles after prewarming. If particles are still present after warming, filter the solutions using 0.45 µm filter.

## 6. Appendix

### 6.1 Overview of a-Hyb™ Station protocol for PIQOR™ Microarrays

Step	Medium	Temperature (°C)	Time (min)	Pumpspeed (mL/min)	Cycles
Incubation	3	63	5	1.0	–
Incubation	M	63	360–960	1.0	–
Wash	1	50	1	1.0	2
Wash	2	35	1	1.0	2
End					

#### Automated buffer supply

Media 1: Wash Buffer 1 via tube 1

Media 2: Wash Buffer 2 via tube 2

Media 3: Prehybridization Solution via tube 3

#### Manual buffer supply

Media M: Labeled sample via sample reservoir

### 6.2 Automatic pretreatment of PIQOR™ Microarrays

Step	Medium	Temperature (°C)	Time (min)	Pumpspeed (mL/min)	Cycles
Incubation	4	75	2	1.5	–
Wash	8	75	2	1.5	2
Incubation	3	63	5	1.0	–
Incubation	M	63	360–960	1.0	–
Wash	1	50	1	1.0	2
Wash	2	35	1	1.0	2
End					

#### Protocol

For automatic denaturation, insert an extra incubation and wash step.

1. Incubate microarrays at 75 °C for two minutes with a pump speed of 1.5 mL/min, using a solution of 50% formamide in distilled water (medium bottle 4).
2. Wash slides with distilled water at 75 °C for 2 minutes with a pump speed of 1.5 mL/min

and two cycles should be performed to wash away the formamide and any unbound DNA molecules.

#### Buffer supply

As in section 4.2.3, plus Media 4 (50% formamide in ddH<sub>2</sub>O) and Media 8 (ddH<sub>2</sub>O).



### 6.3 Calculation of fluorescent label incorporation rate

Cy3 and Cy5 have absorbance maxima at 550 nm and 650 nm, respectively. The amounts of Cy3 and Cy5 incorporated into cDNA probes can be calculated from their respective extinction coefficients using the following equations:

#### Calculation of total DNA amount [ $\mu\text{g}$ ]

Total DNA [ $\mu\text{g}$ ] = OD 260 nm  $\times$  dilution factor  $\times$  37  $\times$  total sample volume [ $\mu\text{l}$ ] /1,000

#### Calculation of total dNTP amount [nmol]

According to molecular weight of dNTPs, 1  $\mu\text{g}$  DNA contains 3.1 nmol dNTPs.

total dNTP [nmol] = total DNA  $\times$  3.1

#### Calculation of content of fluorescent label [nmol]

Molar extinction coefficient (550) of Cy3 is 150,000  $\text{M}^{-1}\text{cm}^{-1}$ .

Cy3 content [nmol] = OD 550 nm  $\times$  dilution factor  $\times$  total sample volume [ $\mu\text{L}$ ]  $\times$  1,000/150,000

Molar extinction coefficient (650) of Cy5 is 250,000  $\text{M}^{-1}\text{cm}^{-1}$ .

Cy5 content [nmol] = OD 650 nm  $\times$  dilution factor  $\times$  total sample volume [ $\mu\text{L}$ ]  $\times$  1,000/250,000

#### Calculation of specific activity of labeled cDNA

##### Cy3-labeled cDNA

Cy3 [nmol]/total DNA [nmol]

##### Cy5-labeled cDNA

Cy5 [nmol]/total DNA [nmol]

A result of 0.01 corresponds to 1 Cy dye per 100 dNTPs. Typically, incorporation rates between 0.01 and 0.005 will be obtained. These values allow a qualitative measurement of the fluorescent labeled probe, they do not allow absolute quantification. Microarray hybridization of a labeled probe with incorporation rates  $> 0.01$  or  $< 0.005$  may not give optimal results.

Alternatively, the incorporation rate can be determined by simultaneous OD measurement at 260, 550 and 650 nm using the ND-1000 Spectrophotometer (NanoDrop Technologies).

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