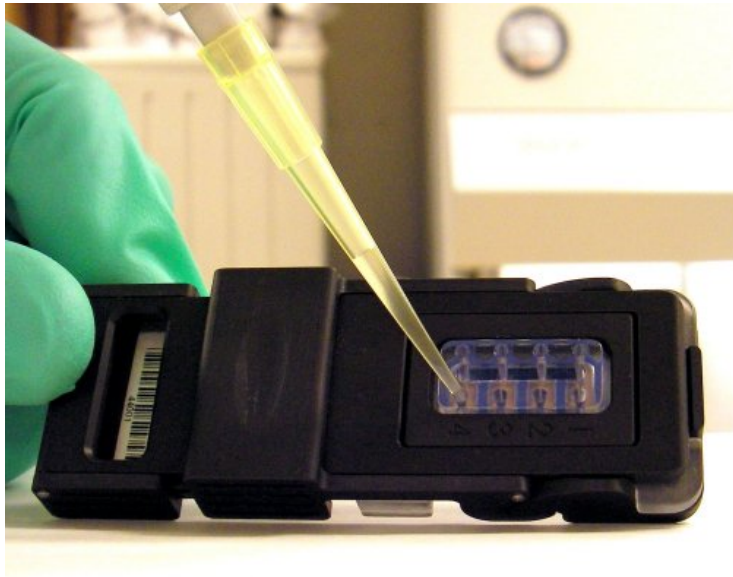


COMBIMATRIX

MicroRNA 4X2K Microarray: Hybridization and Imaging Protocol (PTL012)

(for Species-Specific and Content Choice MicroRNA Microarrays)



MicroRNA 4X2K Microarray: Hybridization and Imaging Protocol

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MicroRNA 4X2K Microarray: Hybridization and Imaging Protocol

Introduction

This manual describes how to hybridize labeled target samples to the CombiMatrix Species Specific or Content Choice MicroRNA 4X2K Microarray, and how to prepare the microarray for imaging. Preparation of labeled microRNA target samples is the user's responsibility. For best results, Cy5® fluorescent dye is recommended for microRNA labeling. The protocol "Preparation of Labeled MicroRNA Samples for Hybridization with 4X2K Microarrays" is available from CombiMatrix.

The CombiMatrix Species Specific or Content Choice MicroRNA 4X2K Microarrays carry anti-sense oligonucleotide probes with a median length of 22 nucleotides and an average T_m of 55 to 60°C. The hybridization conditions described in this manual have been optimized for these probes. Further optimization of hybridization conditions may be required if the user has added custom probes.

Each microarray has four identical array sectors that can be hybridized with different microRNA samples using the sectorized Hybridization Cap provided. All four sectors must be hybridized simultaneously. Hybridization is performed with 0.5 to 2 µg of a labeled target sample per array sector.

Imaging of the MicroRNA 4X2K microarray is performed using a high-resolution fluorescent scanner that is compatible with a 1"x3" slide format, with a minimum resolution of 5µm and an adjustable focus. All four sectors are scanned simultaneously.

The hybridized MicroRNA 4X2K microarray can be stripped of targets using the CombiMatrix CustomArray™ Stripping Kit (see Appendix A), and then re-used up to three times according to the hybridization protocol described in this manual.

Materials and Equipment Provided

IMPORTANT! Do not touch the semiconductor microarray surface. Wear gloves when handling. CustomArray 4X2K microarrays can be stored in a cool dry place for up to 4 months.

- ❑ Species Specific or Content Choice MicroRNA 4X2K Microarray
- ❑ Sectored Hybridization Cap
- ❑ LifterSlip™ coverslip for imaging
- ❑ Imaging Solution

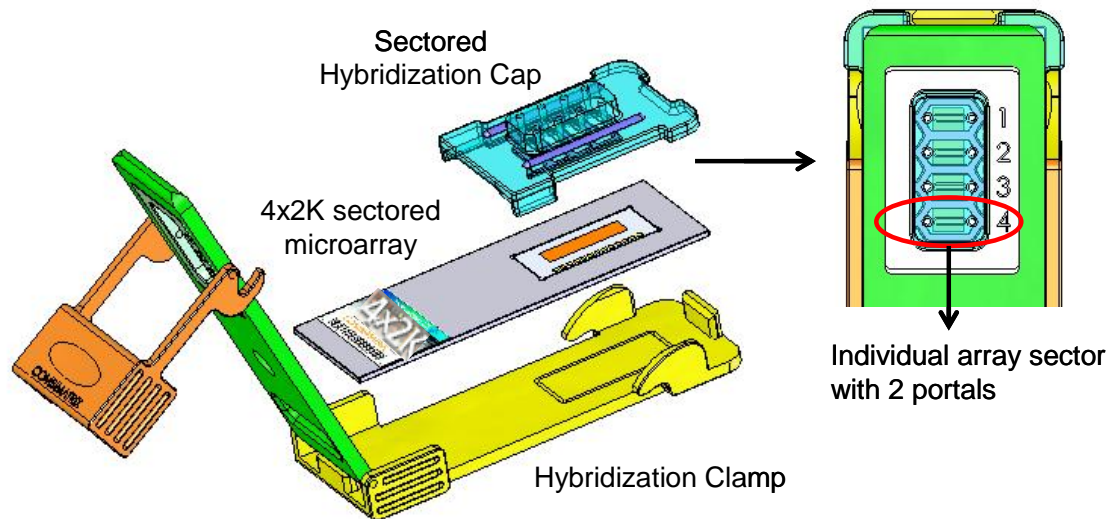


Figure 1. 4X2K Microarrays and accessory components. The colorization of the Hybridization Clamp is for illustrative purposes and does not reflect the real color (see Fig. 2).

Materials and Equipment Required (not provided)

- ❑ Fluorochrome-labeled target microRNA, such as small RNA enriched samples labeled with Cy5®
- ❑ CombiMatrix Hybridization Clamp for the CustomArray™ 4X2K microarray, Product Number 610009
- ❑ Rotisserie oven for array hybridization
- ❑ CombiMatrix CustomArray™ holder for rotisserie oven (see Appendix A)
- ❑ 95°C Heating Block
- ❑ Micropipettors and tips, sterile plastic ware, gloves (powder-free)
- ❑ Adhesive tape: Scotch® Magic transparent tape, or a PCR sealing tape (PN 276014, aluminum liner)
- ❑ Nuclease-free water
- ❑ 0.5 M EDTA (pH 8.0)
- ❑ 10% Tween-20
- ❑ 20X SSPE Buffer
- ❑ 10% SDS
- ❑ Deionized (DI) Formamide
- ❑ Bovine Serum Albumin, Ultrapure, 50 mg/ml (5% w/v) solution, Ambion, Cat. # 2616
- ❑ 1X PBS prepared from 10X Phosphate-buffered Saline (1.37M Sodium Chloride, 0.027M Potassium Chloride, 0.08M Sodium Phosphate dibasic, 0.02M Sodium Phosphate monobasic, pH 7.4; Ambion, Cat.# 9625)
- ❑ Standard, high-resolution fluorescent microarray scanner (CombiMatrix recommends the Axon Instruments Genepix® 4000B and 4200A microarray scanners, and the Perkin Elmer ScanArray® 4000, 5000, Lite, and Express microarray scanners).

4X2K Microarray Assembly

IMPORTANT! The Hybridization Cap is intended for single use only. Dispose of the cap upon completion of this protocol.

1. Open the Hybridization Clamp and place the 4X2K Microarray with the semiconductor array side up as shown in Figures 1 and 2.



Figure 2. Placement of the CombiMatrix 4X2K microarray in the Hybridization Clamp.

2. Align the Hybridization Cap over the slide so that the top edge of the slide is positioned against the stop on the Hybridization Cap, and the Cap is centered over the semiconductor array (see Figures 1 and 3). There is only one way to position the Hybridization Cap inside the clamp.

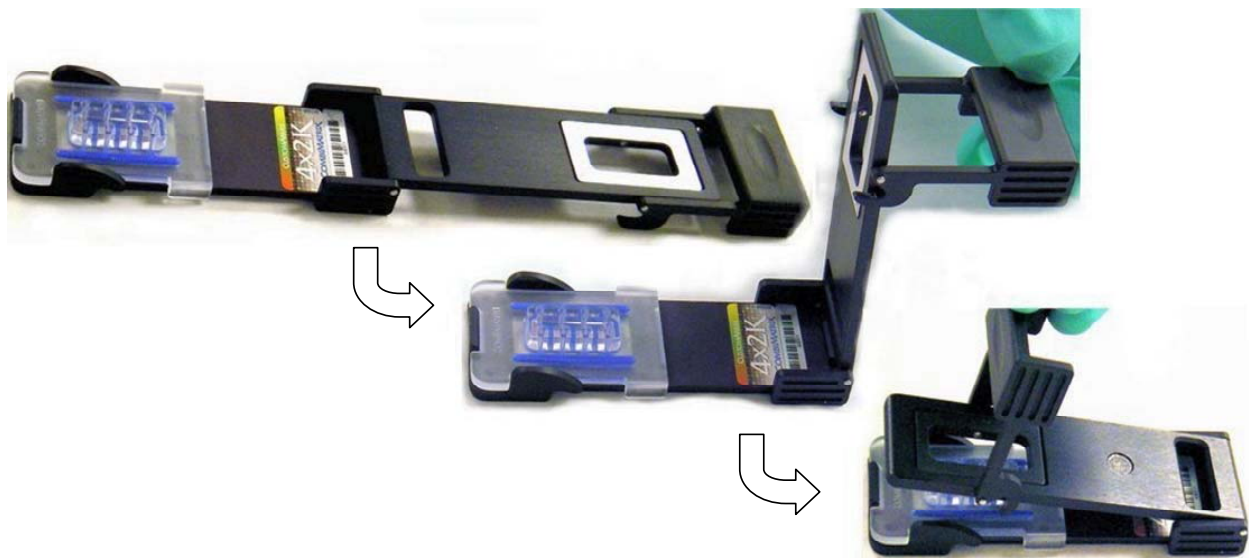


Figure 3. Placement of the Hybridization Cap on the top of the 4X2K microarray, and closing of the Hybridization Clamp.

3. Secure the 4X2K Microarray and Hybridization Cap by closing the Clamp (Fig. 3).

Handling of Assembled Microarrays

1. Wear gloves at all times when handling microarrays and reagents.
2. After a 4X2K microarray is assembled and re-hydrated, keep the Hybridization Cap in place during all hybridization and wash steps, and do not open it. Do NOT allow the semiconductor microarray surface to become dry at any step in the protocol. Proceed rapidly when changing solutions, and do not leave the hybridization chambers empty for any significant length of time.
3. Use a 200 μ l micropipettor with nuclease-free pipet tips to add or remove solutions through the solution portals of the Hybridization Cap. Hold the microarray with the assembled Hybridization Cap at a 45 degree angle, and add/remove solutions through the lower portals (Fig. 4).

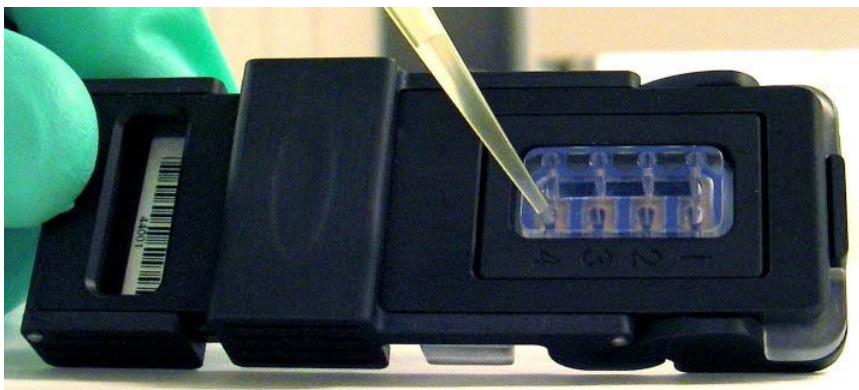


Figure 4. Ready to use 4X2K Microarray assembled with the Hybridization Cap in the Hybridization Clamp. To add/remove solutions, hold the assembled microarray at a 45 degree angle and pipet through the lower portals of hybridization chambers.

4. The volume of each chamber of the Hybridization Cap is 35 μ l. However, when filling a chamber with hybridization buffer containing labeled targets, add only 30 μ l per chamber to prevent overfilling and spilling of the labeled target solution to a neighboring chamber. The air bubbles that form in the chambers will improve mixing when arrays are rotated during hybridization incubation.

IMPORTANT! Do NOT overfill the chambers with hybridization buffer. Carefully wipe all spilled solution from the upper surface of the hybridization cap. This is critical to prevent cross contamination between array sectors that may be caused by solution leaking from one portal and being absorbed by capillary action into a neighboring portal.

5. Use of a rotisserie oven or a rotating incubator is recommended to ensure mixing during hybridization, and it is critical to improve performance of 4X2K microarrays. Special holders are available from CombiMatrix for attachment of 4X2K microarrays to standard rotisseries (see Appendix A).
6. For all incubations longer than 10 minutes, seal the solution portals of Hybridization Cap with non-permeable adhesive tape to prevent evaporation. Use Scotch® Brand Magic® Transparent Tape or a PCR sealing tape such as Nunc Brand PN 232702 (clear polyolefin liner) or PN 276014 (aluminum liner). Wipe the surface clean with a lint-free tissue before sealing.
7. If you plan to strip and re-use a hybridized 4X2K microarray with the CombiMatrix CustomArray™ Stripping Kit (see Appendix A), do not allow the semiconductor microarray surface to dry, as this will prevent any further use. Avoid prolonged storage of hybridized microarrays prior to stripping; instead, strip the microarrays first, and store them wet in Imaging Solution or 1X PBS at 4°C for a maximum of 2 weeks.

Preparation for Hybridization

The following solutions can be prepared beforehand. All reagents used should be RNase and DNase-free.

1. Prepare the **Pre-hybridization Solution** (see Table 1). The prepared Pre-hybridization Solution should be filter-sterilized and can be stored at room temperature up to 1 month. If the Pre-hybridization solution forms precipitate, heat it to 45°C.

Table 1. Pre-hybridization Solution		
<u>Reagent</u>	<u>Volume for 1 ml</u>	<u>Final Concentration</u>
20X SSPE	300 µl	6X
10% SDS	100 µl	1%
50 mg/ml BSA Solution	40 µl	0.2%
Nuclease-free water	560 µl	
Total Volume	1 ml	

2. Prepare the Hybridization Wash Solutions (see Table 2). The solutions should be filter-sterilized and can be stored at room temperature for up to 6 months.

Table 2. Hybridization Wash Solutions		
<u>Step</u>	<u>Solution</u>	<u>For 10 ml</u>
6X SSPET Wash	6X SSPE, 0.05% Tween-20	3 ml 20X SSPE 50 µl 10% Tween-20 6.95 ml Nuclease-free water
3X SSPET Wash	3X SSPE, 0.05% Tween-20	1.5 ml 20X SSPE 50 µl 10% Tween-20, 8.45 ml Nuclease-free water
0.5X SSPET Wash	0.5X SSPE, 0.05% Tween-20	250 µl 20X SSPE 50 µl 10% Tween-20 9.7 ml Nuclease-free water

Pre-Hybridization

NOTE: The 4X2K microarray must be re-hydrated prior to hybridization. A pre-hybridization step is then recommended to block non-specific binding of targets.

1. Pre-heat an incubator to 65°C.
2. Set a hybridization rotisserie oven to 37°C.

3. Fill the hybridization chambers with nuclease-free water. Avoid introducing air bubbles into the chambers. Cover the solution portals with adhesive tape to prevent evaporation.
4. Incubate at 65°C for 10 min.
5. Remove the microarray from the incubator and bring to room temperature. Remove the adhesive tape and aspirate the water out of the hybridization chambers.
6. Fill the hybridization chambers with the Pre-hybridization Solution. Mix gently by pipetting. A small air bubble can be introduced to improve the mixing process if the arrays are rotated. Wipe the surface clean with a lint-free tissue and cover the solution portals with adhesive tape.
7. Load the microarray onto the rotisserie in the hybridization oven and incubate at 37°C for 60 min with gentle rotation.

Hybridization

IMPORTANT! The following conditions are recommended for standard microRNA 4X2K microarrays. Customized arrays may require different hybridization conditions.

1. Prepare the **Hybridization Solution** (see Table 3), The volume of each chamber of the Hybridization Cap is 35 μ l. However, when filling a chamber with hybridization buffer containing labeled targets, add only 30 μ l per chamber to prevent overfilling and spilling of the labeled target solution to a neighboring chamber. We recommend using 0.5 to 2 μ g of labeled microRNA targets per one array sector.

Table 3. Hybridization Solution		
<u>Reagent</u>	<u>Volume for 30 μl</u>	<u>Final Concentration</u>
20X SSPE	9 μl	6X SSPE
50 mg/ml BSA Solution	4.8 μl	0.8% BSA
DI Formamide	3.6 μl	12%
Labeled microRNA targets	up to 5.1 μl	15-67 ng/μl
10% SDS	7.5	2.5%
Nuclease-free water	to 30 μl	
Total Volume	30 μl	

NOTE 1: The Hybridization Solution should be prepared fresh each time you hybridize arrays. Add 10% SDS after all other components are combined. If precipitation occurs, proceed with the denaturation step.

NOTE 2: You can dry the labeled target RNA sample using SpeedVac®, and then dissolve the pellet directly in 1X Hybridization Solution prepared as described in the Table 3.

2. Denature the Hybridization Solution at 95°C for 3 minutes, and then cool for 20 seconds on ice. Remove the Hybridization Solution from ice and keep it at room temperature to prevent SDS precipitation. If you observe precipitation, repeat the heating step.
3. Spin down the Hybridization Solution in a microcentrifuge for 5 seconds at maximum speed.
4. Remove the adhesive tape and pipet the Pre-hybridization Solution out of the hybridization chambers.

5. Fill the hybridization chambers with the Hybridization Solution (30 μ l per chamber) and mix gently with repeated pipetting. A small air bubble will form, and it will improve the mixing process if the arrays are rotated in the hybridization rotisserie oven.

IMPORTANT! Do NOT overfill the chambers with the Hybridization Solution. This is critical to prevent cross contamination between array sectors that may be caused by solution leaking from one portal and being absorbed by capillary action into a neighboring portal.

6. Carefully wipe excess solution from the surface of the Hybridization Cap with a lint-free tissue, and cover the solution portals with adhesive tape.
7. Load the microarray onto the rotisserie in the hybridization oven and incubate at 37°C for 2-4 hours with gentle rotation.

NOTE 1: To improve microarray performance, use of a rotisserie oven or a rotating incubator is recommended to ensure mixing during hybridization (see Appendix A).

NOTE 2: A short incubation time of 2 hours is sufficient for microRNA hybridization (due to the small target size), however the incubation can be extended to overnight if desired.

Hybridization Washing

IMPORTANT! Do not allow the array to become dry. Proceed rapidly when changing solutions. Do not leave the hybridization chamber empty for any significant length of time.

NOTE 1: For every wash step, we recommend rinsing the hybridization chambers with the corresponding Wash Solution prior to the wash incubation. Add the Wash Solution to the chamber, gently mix by pipetting. Remove the Wash Solution, and fill the chambers again with the same solution.

NOTE 2: Protect the array semiconductor surface from light by covering with aluminum foil during all incubations exceeding 5 minutes.

1. Remove the microarray from the hybridization oven. Remove the adhesive tape and pipet the Hybridization Solution out of the chambers.
2. Using the **6X SSPET Wash** solution, rinse the hybridization chambers, fill the chambers, and incubate the array for 3 minutes at room temperature. Remove the 6X SSPET Wash solution from the hybridization chambers.
3. Using the **3X SSPET Wash** solution, rinse the hybridization chambers, fill the chambers, and incubate the microarray at room temperature for 3 minutes. Remove the 3X SSPET Wash solution from the hybridization chambers.
4. Using the **0.5X SSPET Wash** solution, rinse the hybridization chambers, fill the chambers, and incubate the microarray at room temperature for 3 minutes. Remove the 0.5X SSPET Wash solution from the hybridization chambers.
5. Repeat step 4 one more time for a total of two washings with the 0.5X SSPET Wash solution. Retain the 0.5X SSPET Wash solution in the hybridization chambers until you are ready to proceed with the imaging step.

Imaging of the Hybridized 4X2K Microarray

IMPORTANT! The microRNA 4X2K microarray must be scanned wet using the Imaging Solution supplied. The LifterSlip™ coverslip provided with the 4X2K microarray has been specifically designed to retain the Imaging Solution without contacting the array surface.

NOTE: The Imaging Solution contains phosphate buffer, which may precipitate during shipping. If a precipitate is visible, heat the Imaging Solution at 60-70°C for about 5 minutes until it dissolves. Allow the solution to cool to room temperature before applying it to the microarray.

1. Remove the 0.5X SSPET Wash solution from the hybridization chambers.
2. Open the Hybridization Clamp and carefully lift the Hybridization Cap off the slide surface. Remove the microarray from the Clamp and place it horizontally.
3. Immediately cover the semiconductor microarray surface with the Imaging Solution.
4. Using thin-tipped forceps, pick up a fresh LifterSlip™ and hold it so that the raised edges face the microarray. The raised edges can be detected by gently rubbing an edge with the tip of the forceps – the raised edge will feel rougher than the glass surface.
5. Lay the LifterSlip™ at an angle onto the microarray so that it is centered over the semiconductor area (see Fig. 5). First touch the Imaging Solution with one side of the LifterSlip™, then slowly lower the slip down, taking care not to introduce air bubbles. If bubbles still form, lift one side of the LifterSlip™ with forceps (or a razor blade) to let the bubbles out, and lower it down again.

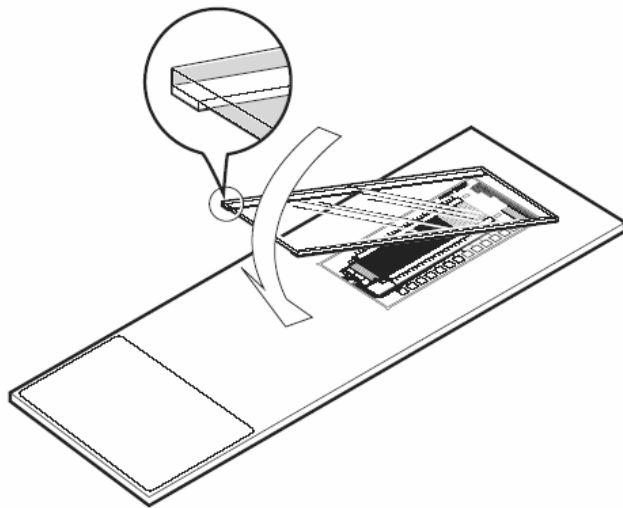


Figure 5. A CombiMatrix microarray with LifterSlip™ coverslip

6. Carefully remove any excess Imaging Solution from the edges of the LifterSlip™ using a lint-free tissue, until it is resting evenly over the microarray.
7. Load the 4X2K microarray into the scanner, taking care not to disturb the LifterSlip™ coverslip. Follow the manufacturer's recommendations for loading the slide into the scanner.

8. After you complete the scan, save the image as a .tiff image file. The data can be extracted from the image using the CombiMatrix Microarray Imager Software. Please refer to the Quick Start Guide or the Microarray Imager User's Manual on our web site (<https://webapps.combimatrix.com>).
9. After imaging is completed, you can proceed to stripping of the 4X2 microarray for subsequent re-hybridization using the CombiMatrix CustomArray™ Stripping Kit (see Appendix A). Do not allow the semiconductor microarray surface to dry; keep the microarray wet, either in a tube or slide-holder containing 1X PBS, or in the Imaging Solution with the LifterSlip™ attached. Avoid prolonged storage of hybridized microarrays prior to stripping; instead, first strip the microarray, then store wet in Imaging Solution or 1X PBS at 4°C for a maximum of 2 weeks.

Appendix A. Related Products Available from CombiMatrix

CombiMatrix Rotisserie Holders for 4X2K Microarrays

To improve microarray performance, use of a rotisserie oven or a rotating incubator is recommended to ensure mixing during hybridization. Microarrays can be attached to standard rotisseries using holders available from CombiMatrix. Please note that the rotisserie holders for CombiMatrix 4X2K microarrays are different from the 12K ones, and they are not interchangeable. The appropriate holder is necessary because the rotation axis is different for these two types of microarrays.

- **CombiMatrix 8 CustomArray™ Rotisserie for 4X2K:**
 - Product Number for 3/8" diameter shaft is 610020,
 - Product Number for 3/4" diameter shaft is 610014,
 - Product Number for 1/2" diameter shaft is 610021,
 - Product Number for 10 mm diameter shaft is 610022,
 - Product Number for 13 mm diameter shaft is 610023.

The 8 CustomArray™ Rotisserie for 4X2K allows the hybridization of 8 microarray slides (32 microarray sectors) at one time. This custom engineered product will mount on a shaft of an incubation oven with simple spring clasps. CombiMatrix have several models adapted for different oven shaft diameters. Please indicate the shaft diameter of your rotisserie oven when ordering.

CombiMatrix CustomArray™ Stripping Kit and Clamp

- **CombiMatrix CustomArray™ Stripping Clamp**, Product Number 610010.

The CombiMatrix CustomArray™ Stripping Clamp enables the microarrays to be stripped and re-hybridized up to three times. The Stripping Clamp is specially designed to withstand chemicals used for stripping and to provide appropriate pressure to prevent leakage from the stripping cap. The CustomArray™ Stripping Clamp is recommended for up to 100 stripping procedures.

- **CombiMatrix CustomArray™ Stripping Kit**, Product Number 610024.

The CustomArray™ Stripping Kit enables re-hybridization of a microarray three times, which makes the use of a single CombiMatrix microarray four times. Each kit contains reagents for 25 microarray stripping reactions, and accessories for microarray re-hybridizations.