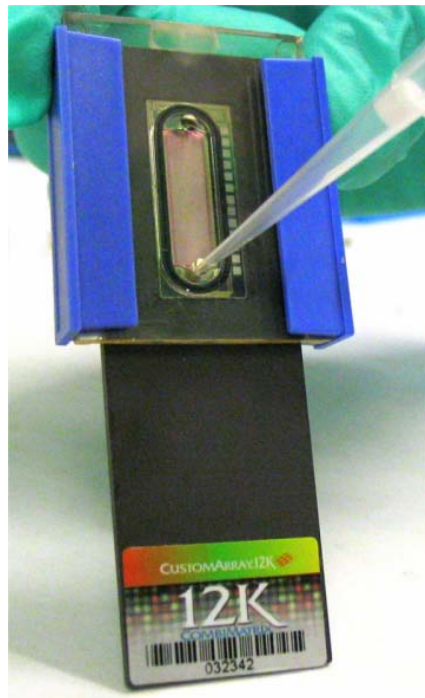
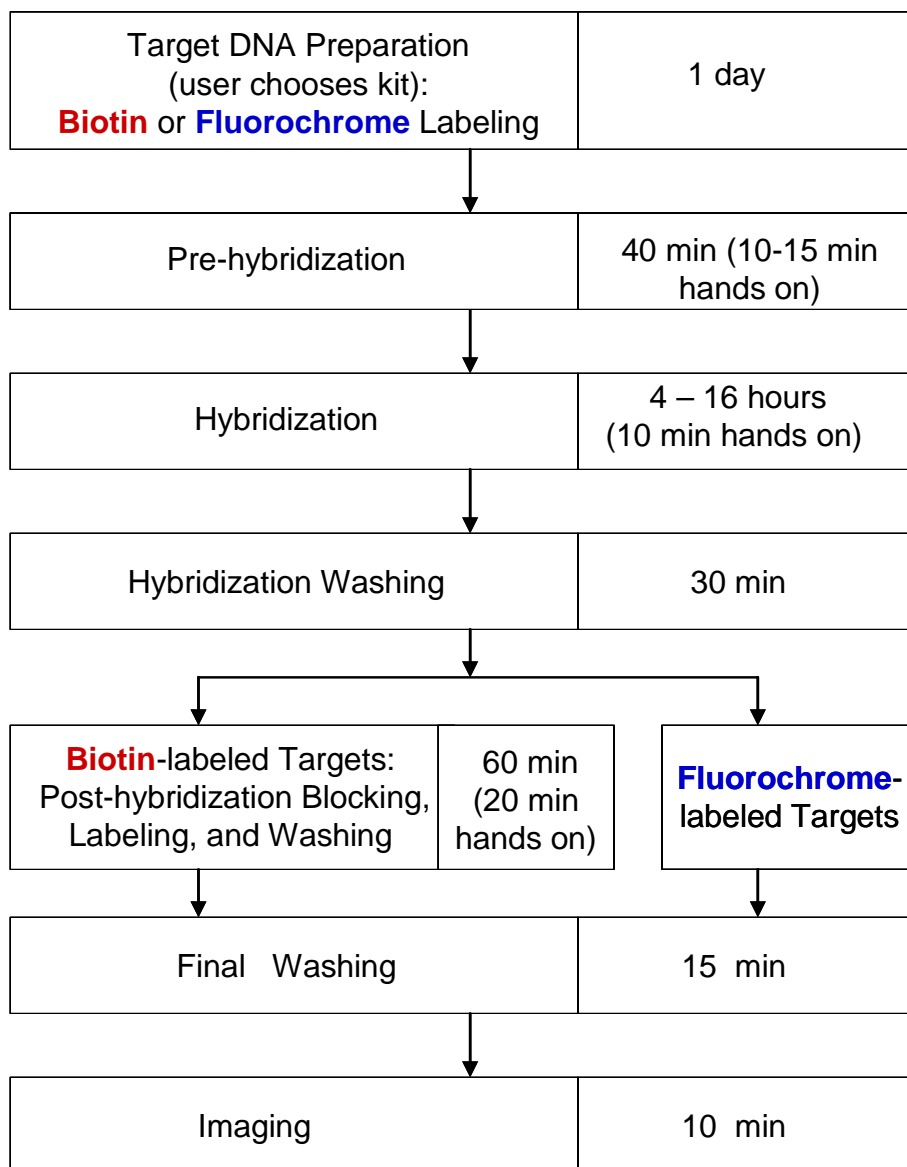


# COMBIMATRIX

## Comparative Genome Hybridization: Hybridization and Imaging of CustomArray™ 12K Microarrays (PTL004)



# Comparative Genome Hybridization: Hybridization and Imaging Workflow



# Comparative Genome Hybridization: Hybridization and Imaging of CustomArray™ 12K Microarrays

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# Comparative Genome Hybridization: Hybridization and Imaging of CustomArray™ 12K Microarrays

## Introduction

This manual describes how to use CustomArray™ 12K microarrays for comparative genome hybridization applications with labeled genomic DNA, and how to prepare the microarray for imaging. Genomic DNA samples can be labeled by incorporation of either **biotin** or a **fluorochrome** (Cy3®, Cy5®, or AlexaFluor® 555 and 647 fluorescent dyes). Preparation of labeled targets is the user's responsibility. Hybridization is performed with 1 to 4 µg of labeled DNA target sample per microarray. If two labeled samples are to be analyzed on the same array (for a dual-color experiment), use 1 to 4 µg of each sample (for a total of 2-8 µg). The hybridization conditions recommended in this protocol are designed for standard oligonucleotide probes of 35 to 40-mer length with a balanced melting temperature (T<sub>m</sub>) of approximately 72°C. If non-standard oligonucleotide lengths are used, conditions such as hybridization temperature and hybridization buffer composition must be optimized empirically.

Imaging of the CustomArray™ 12K must be 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.

The hybridized CustomArray™ 12K can be stripped of targets using the CombiMatrix Stripping Kit (see Appendix A), and then re-used according to the hybridization protocol described in this manual.

## Materials and Equipment Provided

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**IMPORTANT! Do not touch the semiconductor microarray surface. Wear gloves when handling. Keep in a dry cool place. Store up to 4 months.**

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- ❑ CustomArray™ 12K microarray
- ❑ Hybridization Cap (100 µl Volume)
- ❑ Clips for Hybridization Cap
- ❑ Gasket (O-ring)
- ❑ LifterSlip™ coverslip
- ❑ Imaging Solution

## Materials and Equipment Required (not provided)

- ❑ Target DNA or RNA samples labeled with **biotin** or **fluorochrome** dyes
- ❑ Rotisserie oven
- ❑ CombiMatrix CustomArray™ Holder for rotisserie oven (optional, see Appendix A)
- ❑ Standard, high-resolution fluorescent microarray scanner (CombiMatrix recommends the Axon Instruments Genepix® 4000B and 4200A microarray scanners, or the Perkin Elmer ScanArray® 4000, 5000, Lite and Express microarray scanners).
- ❑ 95°C Heating Block
- ❑ Micropipettors and nuclease-free pipette tips
- ❑ Sterile plastic ware
- ❑ Gloves (powder-free)

- ❑ Adhesive tape: Scotch® Brand Magic® Transparent Tape is suitable for hybridization temperatures of 50°C or less; for extended incubations at higher temperatures use a PCR sealing tape such as Nunc Brand PN 232702 (clear polyolefin liner) or PN 276014 (aluminum liner)
- ❑ Nuclease-free water
- ❑ 50X Denhardt's solution
- ❑ 10% and 1% Tween-20
- ❑ 20x SSPE Buffer
- ❑ 10 mg/ml Sonicated Salmon Sperm DNA (sheared), Ambion, Cat.#9680
- ❑ 1% SDS
- ❑ 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)
- ❑ Optional: Human Cot-1 DNA, Invitrogen Cat. # 15279-011 (recommended for working with human samples, use mouse Cot-1 DNA if working with murine material)

**For Biotin-labeled targets:**

- ❑ Acetylated Bovine Serum Albumin (BSA, 20 mg/ml solution), Ambion Cat.#2614, or 5X PBS-Casein Blocking Buffer, BioFX Laboratories, Cat.# PBSC-0100-01
- ❑ Biotin detection reagent: Fluorolink™ Cy5®-labeled streptavidin, GE Healthcare/Amersham Biosciences Cat. # PA45001

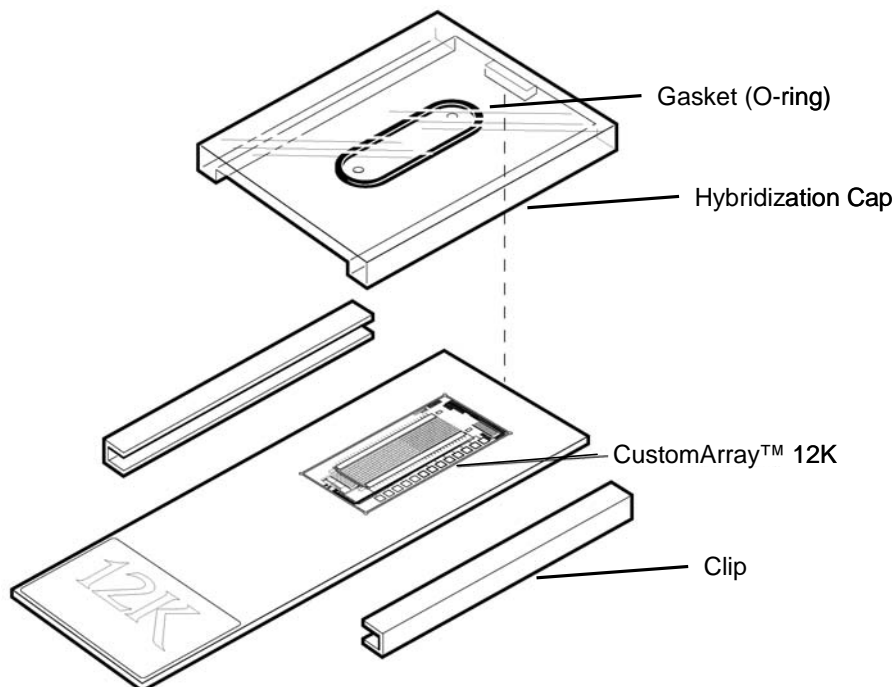
## CustomArray™ 12K Assembly

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**IMPORTANT! The Hybridization Cap, Clips and O-ring are intended for single use only.**

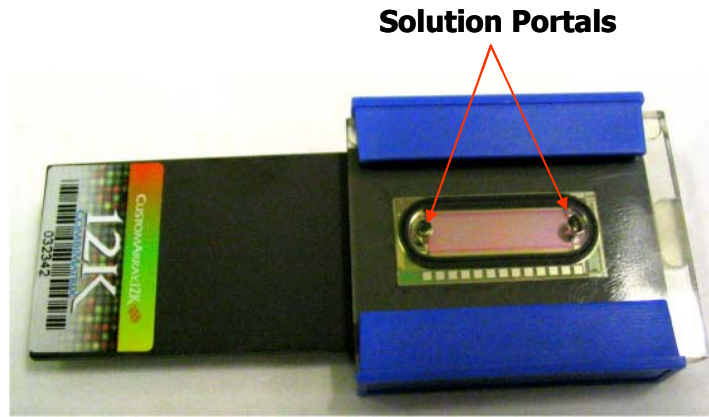
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1. Insert the provided Gasket (O-ring) in the groove of the Hybridization Cap.
2. Align the Hybridization Cap over the slide so that the top edge of the slide is flush against the stop on the Hybridization Cap, and the Cap is centered over the semiconductor area.
3. Secure the Hybridization Cap in place with the Clips provided (see Fig. 1 and 2). The Clips can be attached only if the Hybridization Cap is positioned with the top edge of the slide at the stop of the Cap.



**Figure 1.** CustomArray™ 12K microarray, Hybridization Cap and Clips.

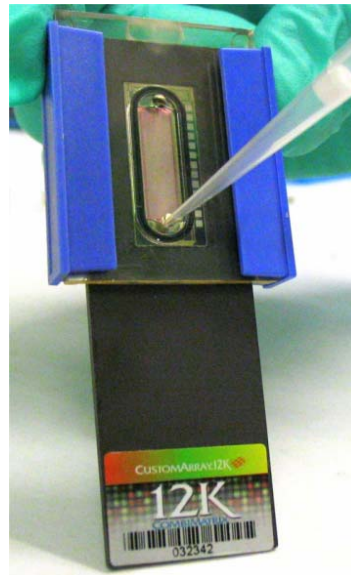
**Figure 2.** Assembled CustomArray™ 12K microarray with Hybridization Cap and Clips.



## Handling of Assembled Microarrays

1. Wear gloves at all times when handling microarrays and reagents.
2. After a CustomArray™ 12K microarray is assembled and re-hydrated, keep the Hybridization Cap in place during all hybridization and wash steps. Do NOT allow the semiconductor microarray surface to become dry at any step in the protocol.
3. To add or remove solutions, hold the microarray with the assembled Hybridization Cap at a 45 degree angle. Use a 200  $\mu$ l micropipettor with nuclease-free pipet tips to add or remove solutions through the lower solution portal of the Hybridization Cap (see Fig. 3).

**Figure 3.** Pipetting of solutions into the hybridization chamber of the assembled CustomArray™ 12K microarray.



4. Proceed rapidly when changing solutions during the pre-hybridization, hybridization, and washing steps. Do not leave the hybridization chamber empty for any significant length of time.
5. 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 for hybridization temperatures of 50°C or less; for higher temperatures use 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.
6. If you plan to strip and re-use a hybridized CustomArray™ 12K microarray (see Appendix A), do not allow the semiconductor surface to dry, as this will prevent any further use. Avoid prolonged storage of hybridized microarrays prior to stripping; instead, strip the microarrays, then 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.

### Solutions Required for All Targets

1. Prepare the **2X Hyb Solution Stock** (see Table 1). This stock will be used for preparation of the Pre-hybridization and Hybridization Solutions. The 2X Hyb Solution Stock should be filter-sterilized and can be stored at room temperature for up to 6 months.

<b>Table 1. 2X Hyb Solution Stock</b>		
<u>Reagent</u>	<u>Volume for 10 ml</u>	<u>Final Concentration</u>
<b>20X SSPE</b>	<b>6 ml</b>	<b>12X</b>
<b>10% Tween-20</b>	<b>100 µl</b>	<b>0.1%</b>
<b>0.5M EDTA</b>	<b>560 µl</b>	<b>40mM<sup>a</sup></b>
<b>Nuclease-free water</b>	<b>3.34 ml</b>	
<b>Total Volume</b>	<b>10 ml</b>	

<sup>a</sup>Final concentration includes the EDTA from the SSPE.

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

<b>Table 2. Wash Solutions</b>		
<u>Step</u>	<u>Solution</u>	<u>For 10 ml</u>
<b>6X SSPET Wash</b>	<b>6X SSPE, 0.05% Tween-20</b>	<b>3 ml 20X SSPE 50 µl 10% Tween-20 6.95 ml Nuclease-free water</b>
<b>3X SSPET Wash</b>	<b>3X SSPE, 0.05% Tween-20</b>	<b>1.5 ml 20X SSPE 50 µl 10% Tween-20, 8.45 ml Nuclease-free water</b>
<b>0.5X SSPET Wash</b>	<b>0.5X SSPE, 0.05% Tween-20</b>	<b>250 µl 20X SSPE 50 µl 10% Tween-20 9.7 ml Nuclease-free water</b>
<b>PBST Wash</b>	<b>2X PBS, 0.1% Tween-20</b>	<b>2 ml 10X PBS, 100 µl 10% Tween-20, 7.9 ml Nuclease-free water</b>
<b>PBS Wash</b>	<b>2X PBS</b>	<b>2 ml 10X PBS, 8 ml Nuclease-free water</b>

## Solutions Required Only for Biotin-labeled Targets

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NOTE: If working with **fluorochrome**-labeled targets, skip this step.

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1. Prepare the 1 mg/ml stock solution of the Fluorolink™ Cy5®-labeled streptavidin. This reagent is supplied as a lyophilized powder. Dissolve in 1.0 ml of nuclease-free water, dispense into 10-20 µl aliquots, freeze and store at -20°C. Avoid repeated freeze-thaw cycles.
2. Prepare the Biotin Blocking Solution (see Table 3). All reagents should be nuclease-free. The prepared Biotin Blocking Solution should be filter-sterilized and can be stored at 4°C for up to 1 month.

<b>Table 3. Biotin Blocking Solution</b>		
<b>Reagent</b>	<b>Volume for 1 ml</b>	<b>Final Concentration</b>
<b>10X PBS</b>	<b>200 µl</b>	<b>2X</b>
<b>10% Tween-20</b>	<b>10 µl</b>	<b>0.1%</b>
<b>BSA (20 mg/ml)</b>	<b>500 µl</b>	<b>1%</b>
<b>Nuclease-free water</b>	<b>290 µl</b>	
<b>Total Volume</b>	<b>1 ml</b>	

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NOTE: You can replace the Biotin Blocking Solution with the 5X PBS-Casein Blocking Buffer for both blocking and labeling steps.

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## Pre-Hybridization

NOTE 1: The CustomArray™ microarray must be re-hydrated prior to hybridization. A pre-hybridization step is then recommended to block non-specific binding of targets.

NOTE 2: The Pre-hybridization and Hybridization buffers listed in this protocol have been optimized for comparative genome hybridization with standard oligonucleotide probes of 35 to 40-mer length with a balanced melting temperature (T<sub>m</sub>) of approximately 72°C. If non-standard oligonucleotide lengths are used, conditions such as hybridization temperature and hybridization buffer composition must be optimized empirically.

1. Pre-heat an incubator to 65°C.
2. Set a hybridization rotisserie oven to 50°C.
3. Prepare fresh **Pre-hybridization Solution** (see Table 4).

<b>Reagent</b>	<b>For 100 µl Volume Cap</b>	<b>Final Concentration</b>
<b>2X Hyb Solution Stock</b>	<b>50 µl</b>	<b>6X SSPE, 0.05% Tween-20, 20mM EDTA</b>
<b>Nuclease-free water</b>	<b>34 µl</b>	
<b>50X Denhardt's solution</b>	<b>10 µl</b>	<b>5X</b>
<b>Salmon sperm DNA (10mg/ml)<sup>a</sup></b>	<b>1 µl</b>	<b>100 ng/µl</b>
<b>1% SDS</b>	<b>5 µl</b>	<b>0.05%</b>
<b>Total Volume</b>	<b>100 µl</b>	

<sup>a</sup> Heat the Salmon sperm DNA solution to 95°C for at least 5 minutes and then place on ice for at least 1 minute before use.

4. Fill the hybridization chamber with nuclease-free water. Avoid introducing air bubbles into the chamber. Cover the solution portals with adhesive tape to prevent evaporation. Incubate at 65°C for 10 minutes. Remove the microarray from the incubator and bring to room temperature. Remove the adhesive tape and aspirate the water out of the hybridization chamber.
5. Fill the hybridization chamber 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 during incubation. Cover the solution portals with adhesive tape.
6. Load the microarray onto the rotisserie in the hybridization oven and incubate at the desired hybridization temperature for 30 minutes with gentle rotation.

NOTE: To improve microarray performance, use of a rotisserie oven or a rotating incubator is recommended to ensure mixing during hybridization. The CustomArray™ 12K can be attached to standard rotisseries using holders available from CombiMatrix (see Appendix A).

# Hybridization

1. Prepare fresh **Hybridization Solution** (see Table 5).

<b>Table 5. Hybridization Solution</b>		
<b>Reagent</b>	<b>For 100 <math>\mu</math>l Volume Cap</b>	<b>Final Concentration</b>
<b>2X Hyb Solution Stock</b>	<b>50 <math>\mu</math>l</b>	<b>6X SSPE, 0.05% Tween-20, 20mM EDTA</b>
<b>Labeled DNA target sample</b>	<b>Varies (up to 45 <math>\mu</math>l)</b>	<b>10-40 ng/<math>\mu</math>l recommended</b>
<b>1% SDS</b>	<b>5 <math>\mu</math>l</b>	<b>0.05%</b>
<b>Nuclease-free water</b>	<b>to 100 <math>\mu</math>l</b>	
<b>Total Volume</b>	<b>100 <math>\mu</math>l</b>	

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OPTIONAL: You can add human Cot-1 DNA to the final concentration of 0.5 mg/ml if working with human DNA samples. Use mouse Cot-1 DNA if working with murine DNA samples. Cot-1 DNA should be heated to 95°C for at least 5 minutes and then placed on ice for at least 1 minute before use.

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2. Denature the Hybridization Solution at 95°C for 3 minutes, and then cool for 1 minute on ice.
3. Spin down the solution in a microcentrifuge for 5 seconds at maximum speed to collect condensate.
4. Remove the adhesive tape from the microarray and pipet the Pre-hybridization Solution out of the hybridization chamber.
5. Fill the hybridization chamber with the Hybridization Solution and mix gently with repeated pipetting. A small air bubble can be introduced to improve the mixing process if the microarray is rotated during hybridization.

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NOTE: Usually the volume of Hybridization Cap is slightly less than 100  $\mu$ l. You can adjust the final volume of Hybridization Solution to 95 instead of 100  $\mu$ l, if you want the whole amount to fit into the chamber.

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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 50°C for 4-16 hours with gentle rotation.

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NOTE: To improve microarray performance, use of a rotisserie oven or a rotating incubator is recommended to ensure mixing during hybridization. The CustomArray™ 12K can be attached to standard rotisseries using holders available from CombiMatrix (see Appendix A).

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## Hybridization Washing

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**IMPORTANT!** The hybridization washing conditions listed in this protocol have been optimized for comparative genome hybridization with standard oligonucleotide probes of 35 to 40-mer length with a balanced melting temperature ( $T_m$ ) of 72°C.

**If your CustomArray™ has been designed with longer probes (ie,  $\geq 50$ mers), we suggest increasing the stringency of the Hybridization Washing by performing the first wash step with Hybridization Wash 1 at a higher temperature. This temperature should be between 15-20°C below the average  $T_m$  of the probes on your CustomArray™ design (for example, we recommend washing at 60-65°C for 50mer probes with  $T_m$  of 75-80°C). Also, we recommend increasing the length of the first washing step from 15 minutes to 20-30 minutes.**

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

NOTE 2: If the microarray has been hybridized with **fluorochrome**-labeled targets, protect it from light by covering with aluminum foil during all incubations exceeding 5 minutes.

NOTE 3: With the exception of the first wash step, microarrays should be incubated in the Hybridization Wash solutions for a minimum of 1 minute. However, if processing multiple microarrays, you can extend the wash incubation time until you rinse and fill all hybridization chambers.

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1. Prior to starting the wash procedure, preheat the **6X SSPET Wash** Solution to the hybridization temperature (50°C for standard conditions). The stringency of the hybridization washing can be increased by performing the first wash step at a higher temperature (between 15-20 °C below the average  $T_m$  of the probes on your CustomArray™ design).
2. Remove the microarray from the hybridization oven. Remove the adhesive tape, and pipet the Hybridization Solution out of the chamber.
3. Using the pre-heated **6X SSPET Wash** solution, rinse the hybridization chamber, fill the chamber, cover the portals with adhesive tape, and return the microarray to the hybridization oven for 5 minutes (with gentle rotation). Remove the 6X SSPET Wash Solution from the hybridization chamber.
4. Using the **3X SSPET Wash** solution, rinse the hybridization chamber, fill the chamber, and incubate the microarray at room temperature for 1 minute (see NOTE 3). Remove the 3X SSPET Wash Solution from the hybridization chamber.
5. Using the **0.5X SSPET Wash** solution, rinse the hybridization chamber, fill the chamber, and incubate the microarray at room temperature for 1 minute. Remove the 0.5X SSPET Wash Solution from the hybridization chamber.
6. Using the **PBST Wash** solution, rinse the hybridization chamber, fill the chamber, and incubate the microarray at room temperature for 1 minute. Remove the PBST Wash Solution from the hybridization chamber.
7. If the microarray has been hybridized with **fluorochrome**-labeled target samples, proceed to the final wash step.

8. If the microarray has been hybridized with **biotin**-labeled target samples, proceed to the Post-hybridization Labeling Step. Retain the PBST Wash Solution in the hybridization chamber until you are ready to proceed to the Post-hybridization Blocking step.

## Post-hybridization Labeling for **Biotin**-labeled Samples

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NOTE: If working with **fluorochrome**-labeled targets, skip this step.

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1. Prepare the **Dye Labeling** Solution using the 1 mg/ml stock solution of Fluorolink™ Cy5®-labeled streptavidin. Thaw an aliquot, and make a 1:1000 dilution (v/v, 1 µl per 1 ml) in the Biotin Blocking Solution (or 5X PBS-Casein Blocking Buffer). We recommend that you discard any unused Cy5®-labeled streptavidin.

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NOTE: Prepare the Dye Labeling Solution fresh each time you hybridize microarrays.

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2. Remove the PBST Wash Solution from the hybridization chamber.
3. Add the **Biotin Blocking** Solution (or 5X PBS-Casein Blocking Buffer) to the hybridization chamber, mix gently by pipetting, and incubate the microarray at room temperature for 15 minutes. Remove the solution from the hybridization chamber.
4. Add the **Dye Labeling** Solution to the hybridization chamber, mix gently by pipetting, and cover the solution portals with adhesive tape. Incubate the microarray at room temperature for 30 minutes. Protect from light by covering with aluminum foil. Remove the Dye Labeling Solution from the hybridization chamber.
5. Using the **PBST** Wash solution, rinse the hybridization chamber, fill the chamber, and incubate the microarray at room temperature for 1 minute. Remove the PBST Wash Solution from the hybridization chamber.
6. Repeat Step 4.

## Final Washing

1. Remove the PBST Wash Solution from the hybridization chamber.
2. Using the **PBS Wash** solution, rinse the hybridization chamber, fill the chamber, and incubate the microarray at room temperature for 1 minute. Remove the PBS Wash Solution from the hybridization chamber. Repeat a second time.
3. Retain the PBS Wash Solution in the hybridization chamber until you are ready to proceed to the Imaging step.

# Imaging of CustomArray™ 12K

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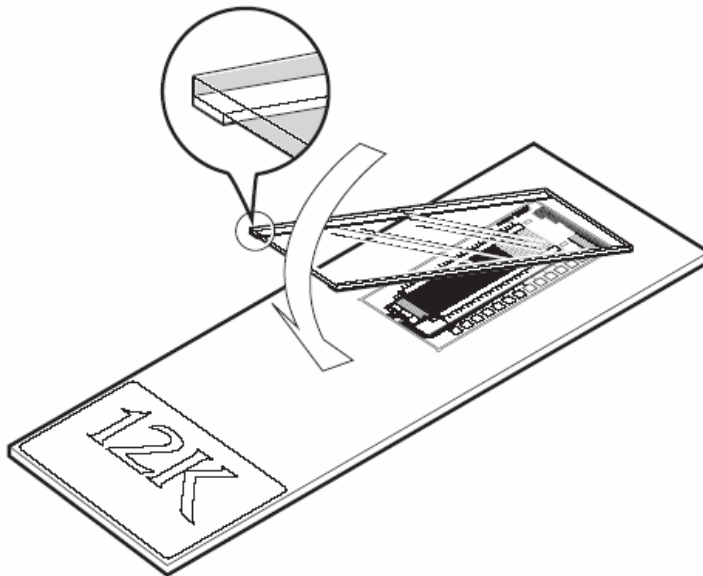
**IMPORTANT! The CustomArray™ 12K must be scanned wet using the Imaging Solution supplied. The LifterSlip™ coverslip provided with the CustomArray™ has been specifically designed to retain the Imaging Solution without contacting the microarray surface. Do not use a standard coverslip with CustomArray™ 12K.**

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**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.

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1. Remove the PBS Wash Solution from the hybridization chamber.
2. Carefully remove the hybridization chamber from the microarray by removing the clips and lifting the Hybridization Cap off the slide surface.
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. 4). 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 4.** CustomArray™ 12K with LifterSlip™ coverslip

6. Carefully remove any excess Imaging Solution from the edge of the LifterSlip™ using a lint-free tissue, until it is resting evenly over the microarray.
7. Load the CustomArray™ 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 CustomArray™ 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 CustomArray™ 12K Microarrays

To improve microarray performance, use of a rotisserie oven or a rotating incubator is recommended to ensure mixing during hybridization. CustomArray™ microarrays can be attached to standard rotisseries using holders available from CombiMatrix.

- **CombiMatrix 32 CustomArray™ Rotisserie for 12K**, Product Number 610004.

The 32 CustomArray™ Rotisserie for 12K allows the hybridization of 32 microarrays at a time. The unit has four plates, each with eight positions, using ball detent clamps to secure the microarray. The 32 CustomArray™ Rotisserie is supplied with a shaft that fits most Fisher Scientific Isotemp Hybridization Incubators. The Rotisserie can also be adapted to fit other incubation ovens.

- **CombiMatrix 8 CustomArray™ Rotisserie for 12K:**
  - Product Number for 3/8" diameter shaft is 610012,
  - Product Number for 3/4" diameter shaft is 610013.

The 8 CustomArray™ Rotisserie for 12K allows the hybridization of 8 microarrays at a time. The Rotisserie will mount on a 3/8" or 3/4" diameter shaft of an incubation oven with simple spring clasps. The model can also be adapted to fit other incubation ovens. Please indicate the oven shaft diameter when ordering.

- **CombiMatrix 3-Tray CustomArray™ for 12K**, Product Number 610008.

CustomArray™ 3-Tray for 12K is a three-position tray that fits in place of the standard Affymetrix tray/bucket in an Affymetrix oven. As many as eight trays can be loaded in one oven, allowing for 24 CustomArray™ 12K microarrays to be hybridized at a time.

### CombiMatrix Stripping Kit and Clamp

- **CombiMatrix CustomArray™ Stripping Kit**, Product Number 610011.

The Stripping Kit enables re-hybridization of a CustomArray™ microarray three times, thus allowing the use of a single CustomArray™ up to four times. Each kit contains reagents for 25 stripping reactions, plus accessories for subsequent re-hybridizations.

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

The CombiMatrix Stripping Clamp is designed to be used with the **CombiMatrix CustomArray™ Stripping Kit**. The CustomArray™ Stripping Clamp is recommended for up to 100 stripping procedures.