



SIGNAL + SAMPLE AMPLIFICATION PRODUCTS

| Table of Contents | Page |
|--|-------------|
| Characteristics of 3DNA Array 900MPX Kit Labeling | 2 |
| Kit Contents | 4 |
| Other Materials Required | 5 |
| Sample Preparation | 6 |
| Procedure for Use | |
| Preparation of Tagged DNA | |
| cDNA Synthesis (Reverse Transcription) | 7 |
| Purification of cDNA | 8 |
| Terminal Deoxynucleotidyl Transferase (TdT) Tailing Reaction | 9 |
| Ligation to 3DNA Capture Sequence (Preparation of “Tagged” DNA) | 9 |
| Purification of Tagged DNA | 9 |
| Array Hybridization | |
| Tagged DNA Hybridization and Wash | 10 |
| 3DNA Hybridization and Wash | 12 |
| Signal Detection | 13 |
| Troubleshooting | 14 |
| References | 15 |
| Appendix A: Concentration of DNA | 16 |
| Appendix B: Reducing Array Background | 17 |
| Appendix C: Reducing the Degradation of Cy TM 5 or Alexa Fluor [®] 647 | 18 |

Characteristics of 3DNA Array 900MPX Kit Labeling

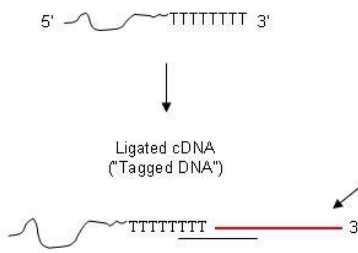
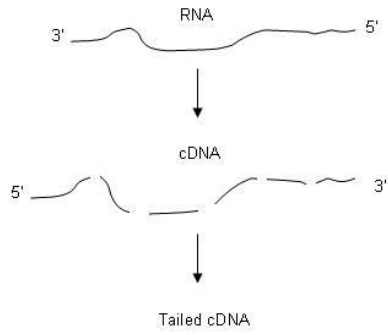
Genisphere continues to pioneer labeling technology for microarrays. The 3DNA Array 900MPX kit is designed to provide increased sensitivity on microarrays when using small quantities of RNA. Any RNA sample can be used, including intact or partially degraded samples, prokaryotic RNA, or amplified RNA .

The 3DNA Array 900MPX kit is also capable of labeling any type of DNA (genomic, PCR products, cDNA, oligonucleotides of 30 bases or more, random-primed DNA, etc.). The labeled DNA can be used in a variety of microarray assays, including chromosome immunoprecipitation (ChIP), single nucleotide polymorphism (SNP), and genomic hybridization experiments.

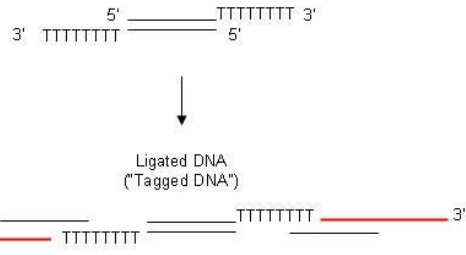
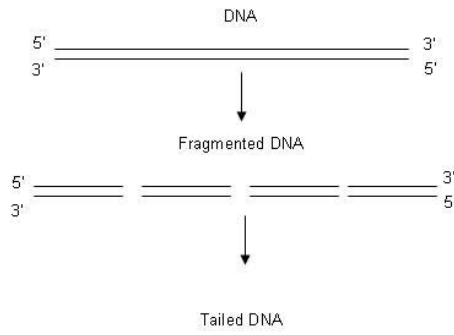
Note: This kit is not designed for use in hybridization volumes greater than 60 μ l (such as large hybridization chambers and hybridization stations). Please contact Genisphere Technical Support for recommendations for high hybridization volumes.

Caution: This kit is not designed for use with Agilent microarrays. Please contact Genisphere Technical Support for more information.

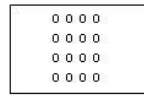
Array 900MPX Procedural Overview RNA Samples (for Gene Expression)



Array 900MPX Procedural Overview DNA Samples (for DNA Analysis)



Tagged DNA Array Hybridization

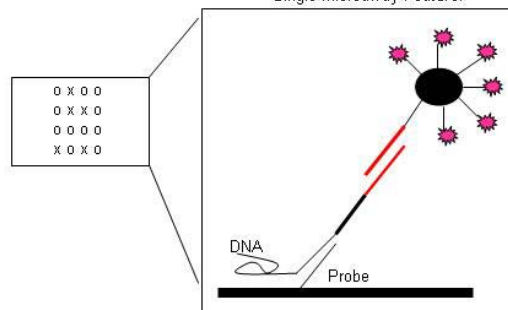


3DNA Array Hybridization

3DNA Capture Reagent:



Single Microarray Feature:



Kit Contents

| | |
|---------|---|
| Vial 1 | Cy3 / Alexa 546 (red cap) or Cy5 / Alexa 647 (blue cap) 3DNA Capture Reagent |
| Vial 2 | Random Primer (1 μ g/ μ l) |
| Vial 3 | Deoxynucleotide Triphosphate Mix (10mM each dATP, dCTP, dGTP, and dTTP) (packaged separately) |
| Vial 4 | Superase-In™ RNase Inhibitor |
| Vial 5 | 2X Enhanced DNA Hybridization Buffer |
| Vial 6 | 2X SDS-Based Hybridization Buffer |
| Vial 7 | 2X Formamide-Based Hybridization Buffer |
| Vial 8 | MPX dT Primer (15ng/ μ L) |
| Vial 9 | LNA™ dT Blocker |
| Vial 10 | Nuclease Free Water |
| Vial 11 | Cy3 / Alexa 546 (red cap) or Cy5 / Alexa 647 (blue cap) 6X Ligation Mix |

Ligation Mix Capture Sequence Information:

Cy3 / Alexa Fluor 546: 5' - (PO₄) TTC TCG TGT TCC GTT TGT ACT CTA AGG TGG A - 3'

Cy5 / Alexa Fluor 647: 5' - (PO₄) ATT GCC TTG TAA GCG ATG TGA TTC TAT TGG A - 3'

| | |
|---------|---|
| Vial 12 | T4 DNA Ligase |
| Vial 13 | 10X Tailing Buffer |
| Vial 14 | 10mM dTTP (packaged separately) |
| Vial 15 | Terminal Deoxynucleotidyl Transferase |

Store Vials 1-15 at -20°C in the dark.

Instructions for Handling Kit Contents:

Vial 1: Refer to page 12.

Vials 5, 6, 7: Refer to pages 10 and 12.

Vials 2, 8, 9, 10, and 13: Thaw at room temperature, vortex, and briefly microfuge.

Vials 3, 4, 12, 14, and 15: Thaw on ice, briefly microfuge if necessary, and **keep on ice at all times. Do not vortex.**

Vial 11: Thaw on ice, vortex, and briefly microfuge.

Other Materials Required

- Microarray
- Microarray reader equipped to read Cy3 / Alexa 546 and/or Cy5 / Alexa 647 fluorochromes
- **Qiagen® MinElute PCR Purification Kit (Catalog number 28004)**
- 10 mM Tris-HCl, pH 8.0 / 1 mM EDTA (1X TE Buffer)
- 0.5M EDTA
- Microcon® YM-30 Centrifugal Filter Device (Millipore Cat No. 42409) (optional: for use with Appendix A)
- Cot-1 DNA (optional, species specific, available from Invitrogen)
- Glass coverslips
- Reagent Grade Deionized Water for preparing wash buffers (Recommended: VWR Cat No. RC9150-5)
- 2X SSC, 0.2% SDS buffer
- 2X SSC buffer
- 0.2X SSC buffer
- Glass Coplin Jars (or equivalent)
- DyeSaver 2 (Genisphere Cat No. Q500500) (optional: use to preserve fluorescent signal and prevent photobleaching)

For RNA Samples (Gene Expression):

- 0.5-4 μ g total RNA sample or 1-3 μ g senseRNA sample in a volume of 6-9 μ L
- Reverse Transcriptase enzyme
 - SuperScript II (Invitrogen Cat No. 18064-014 – 10,000 Units @ 200U/ul)
 - Genisphere RT Enzyme (Genisphere Cat No. RT300320)
 - or other equivalent reverse transcriptase enzyme (Promega, BD Biosciences Clontech, Stratagene etc.)
- 0.5M NaOH, 50 mM EDTA (cDNA synthesis stop solution)
- 1M Tris-HCl, pH 7.5

For DNA Samples (DNA Analysis):

- 0.5-5 μ g purified DNA sample, 250bp fragments (Genomic DNA, PCR products, random primed DNA, etc.) in a volume of 16.5 μ L

Sample Preparation

RNA Samples (for Gene Expression)

- RNA samples can be isolated and purified by most standard or commercially available methods. Carriers composed of nucleic acid should not be used when purifying RNA samples for Array 900MPX labeling, since these carriers will also be labeled by Array 900MPX. Choose a carrier that does not reverse transcribe or have a functional 3' hydroxyl, like linear acrylamide.
- RNase inhibitor should be added to stored RNA samples suspected of being contaminated with RNases.
- If contaminating genomic DNA is present in the RNA sample, it should be degraded by using RNase-free DNase. If DNase is used, **it is essential that the DNase be inactivated completely** before proceeding with the cDNA synthesis procedure to prevent degradation of the RT Primer. Methods for inactivating DNase include phenol-chloroform extraction and use of the RNeasy[®] kits from Qiagen. Inactivation of the DNase by high temperature may not completely inactivate the enzyme.
- **For RNA samples, begin the protocol on page 7 (cDNA Synthesis).**

DNA Samples (for DNA analysis)

- DNA samples can be purified by most standard or commercially available methods. If necessary, samples may be concentrated by ethanol precipitation or other suitable methods.
- For efficient labeling, DNA fragments should be around 250bp. Restriction enzymes, DNase I, or other methods may be used to fragment the DNA to 250bp prior to labeling. Ensure that the fragmentation procedure creates DNA fragments with 3'OH ends.
- **For DNA samples, begin the protocol on Page 9 (TdT Tailing Reaction).**

Procedure For Use

Preparation of Tagged DNA

cDNA Synthesis (Reverse Transcription):

Since microarrays and RNA preparations vary in quality, the exact amount of RNA required for a given experiment will typically range from 0.5-2 μ g of animal total RNA or 1-4 μ g of plant total RNA. Larger or smaller amounts of RNA may be required to achieve optimal results. For new users, the following quantities of RNA are recommended as a starting point for cDNA synthesis:

- Animal total RNA: 2 μ g
- Plant total RNA: 4 μ g
- Poly(A) RNA: 100ng
- SenseRNA: 1-3 μ g

The reverse transcription reaction can be performed with Random Primer (Vial 2), MPX dT Primer (Vial 8), or both primers. The primers are designed to be used differently: the MPX dT Primer (Vial 8) should be used at 1 μ l per reaction, whereas the Random Primer (Vial 2) should be used at 2X by mass of RNA; for example, use 2 μ l of Random Primer per 1 μ g of RNA. When using small quantities of RNA, the Random Primer may be diluted in Nuclease Free Water (Vial 10) for ease of pipetting. For example, when using 100ng of poly(A) RNA, dilute the Random Primer 1:10 and use 2 μ l (200ng) of this dilution in the reaction.

1. In a microtube, prepare the **RNA-RT primer mix**:
 - 1-9 μ l RNA
 - 1-4 μ l Random Primer (Vial 2) USE RANDOM PRIMER AT 2X BY MASS OF RNA.
 - 1 μ l MPX dT Primer (Vial 8) MAY BE OMITTED WHEN PRIMING PROKARYOTIC SAMPLES
 - Add Nuclease Free Water (Vial 10) to a final volume of 11 μ l
2. Mix the **RNA-RT primer mix** and microfuge briefly to collect contents in the bottom of the tube.
3. Heat to 80°C for 10 minutes and immediately transfer to ice for 2-3 minutes.
4. In a separate microtube on ice, prepare a **reaction mix** for every RT reaction:
 - 4 μ l 5X SuperScript II First Strand Buffer (or equivalent reaction buffer supplied with enzyme)
 - 2 μ l 0.1M dithiothreitol (if supplied with enzyme; otherwise use 2 μ l Nuclease Free Water (Vial 10))
 - 1 μ l dNTP mix (Vial 3)
 - 1 μ l Superase-In RNase inhibitor (Vial 4)
 - 1 μ l Superscript II enzyme, 200 units (or equivalent reverse transcriptase)
5. Gently mix (do not vortex) the **reaction mix** and microfuge briefly to collect reaction mix contents in the bottom of the tube.
6. Add the 9 μ l of **reaction mix** from step 5 to the 11 μ l of **RNA-RT primer mix** from step 3 (20 μ l volume).
7. Gently mix (do not vortex) and incubate at 42°C for 2 hours.
8. Stop the reaction by adding 3.5 μ l of 0.5M NaOH/50mM EDTA.
9. Incubate at 65°C for 15 minutes to denature the DNA/RNA hybrids and degrade the RNA.
10. Neutralize the reaction with 5 μ l of 1M Tris-HCl, pH 7.5.
11. Add 21.5 μ l of 1X TE buffer for a total volume of 50 μ l.

Purification of cDNA:

Purify the 50 μ l of cDNA using the Qiagen MinElute PCR Purification Kit (Catalog # 28004) as follows:

1. Add 250 μ l Buffer PB to the 50 μ l cDNA sample and mix.
2. Apply the cDNA mixture from step "1" to the MinElute column and centrifuge for 1 minute at 10-14,000 x g (~13,000 rpm) in a conventional tabletop microcentrifuge.
3. Discard the flow-through. Place the MinElute column into the same collection tube.
4. Add 750 μ l Buffer PE to the MinElute column and centrifuge for 1 minute.
5. Discard the flow-through. Place the MinElute column back into the same collection tube and centrifuge for an additional 2 minutes to remove residual ethanol.
6. Place the MinElute column into a clean 1.5mL microfuge tube.
7. To elute cDNA, add 10 μ l Buffer EB to the center of the column membrane. Incubate at room temperature for 2 minutes. Centrifuge for 2 minutes. Discard column and save the 10 μ l eluted DNA. Proceed to the next page.

Terminal Deoxynucleotidyl Transferase (TdT) Tailing Reaction:

1. Add Nuclease-Free Water (Vial 10) to adjust the volume of DNA to 16.5 μ l.
2. Heat the 16.5 μ l of DNA to 95° for 10 minutes and immediately transfer to ice for 2 minutes.
3. Add the following components for a volume of 25 μ l:
 - 2.5 μ l 10X Tailing Buffer (Vial 13)
 - 4 μ l 10mM dTTP (Vial 14)
 - 2 μ l Terminal Deoxynucleotidyl Transferase (Vial 15)
4. Incubate at 37° for 30 minutes.
5. Proceed immediately to ligation, below.

Ligation to 3DNA Capture Sequence (Preparation of “Tagged” DNA):

1. Heat the 25 μ l of tailed DNA to 95° for 10 minutes and immediately transfer to ice for 2 minutes.
2. Add the following components for a volume of 32 μ l:
 - 5 μ l appropriate 6X Ligation Mix (Vial 11: Cy3 / Alexa 546 or Cy5 / Alexa 647)
 - 2 μ l T4 DNA Ligase (Vial 12)
3. Mix gently, and incubate at 18-25°C (approximately room temperature) for 30 minutes.
4. Add 3.5 μ l of 0.5M EDTA to stop the ligation reaction, and vortex thoroughly (5-10 seconds).
5. Add 14.5 μ l of 1X TE buffer for a total volume of 50 μ l.

Purification of Tagged DNA:

Purify the 50 μ l of Tagged DNA using the Qiagen MinElute PCR Purification Kit (Catalog # 28004) as follows:

1. Add 250 μ l Buffer PB to the 50 μ l Tagged cDNA sample and mix.
2. Apply the cDNA mixture from step “1” to the MinElute column and centrifuge for 1 minute at 10-14,000 x g (~13,000 rpm) in a conventional tabletop microcentrifuge.
3. Discard the flow-through. Place the MinElute column into the same collection tube.
4. Add 750 μ l Buffer PE to the MinElute column and centrifuge for 1 minute.
5. Discard the flow-through. Place the MinElute column back into the same collection tube and centrifuge for an additional 2 minutes to remove residual ethanol.
6. Place the MinElute column into a clean 1.5mL microfuge tube.
7. To elute cDNA, add 10 μ l Buffer EB to the center of the column membrane. Incubate at room temperature for 2 minutes. Centrifuge for 2 minutes. Discard column and save the 10 μ l eluted DNA.

For single color assays: add competitor DNA, if desired. If the volume of Tagged DNA exceeds the desired volume per the table on page 10, follow Appendix A, Concentration of DNA. Otherwise, proceed to **Array Hybridization** on page 10.

For dual color assays: combine the two 10 μ l purifications for a total of 20 μ l DNA. Then, add competitor DNA, if desired. If the volume of Tagged DNA exceeds the desired volume per the table on page 10, follow Appendix A, Concentration of DNA. Otherwise, proceed to **Array Hybridization** on page 10.

Array Hybridization

Tagged DNA Hybridization:

1. Thaw and resuspend the 2X Hybridization Buffer. Vial 5, Enhanced Hybridization Buffer, is recommended. Other 2X hybridization buffers, including Vial 6, or Vial 7, may be used. Heat the buffer to 65-70°C for at least 10 minutes or until completely resuspended. Vortex to ensure that the components are resuspended evenly. If necessary, repeat heating and vortexing until all the material has been resuspended. Microfuge for 1 minute.
2. For each array, prepare a **Hybridization Mix** according to the tables below. **Note:** 2X Enhanced Hybridization Buffer (Vial 5) requires higher hybridization volumes due to its increased viscosity.

Option 1 (Recommended): Use of Enhanced Hybridization Buffer (Vial 5)

| Glass Coverslip Size, mm | 24x30 | 24x40 | 24x50 | 24x60 |
|---|-------------|-------------|-------------|-------------|
| Final Hybridization Volume | 30µl | 38µl | 48µl | 58µl |
| Tagged DNA | 10µl | 10µl | 20µl | 20µl |
| LNA dT Blocker (Vial 9) | 2µl | 2µl | 2µl | 2µl |
| Nuclease Free Water (Vial 10) | 3µl | 7µl | 2µl | 7µl |
| 2X Enhanced Hybridization Buffer (Vial 5) | 15µl | 19µl | 24µl | 29µl |

Option 2: Use of Vial 6 or Vial 7 Hybridization Buffers

| Glass Coverslip Size, mm | 24x30 | 24x40 | 24x50 | 24x60 |
|---------------------------------------|-------------|-------------|-------------|-------------|
| Final Hybridization Volume | 26µl | 34µl | 43µl | 50µl |
| Tagged DNA | 10µl | 10µl | 20µl | 20µl |
| LNA dT Blocker (Vial 9) | 2µl | 2µl | 2µl | 2µl |
| Nuclease Free Water (Vial 10) | 1µl | 5µl | 0µl | 3µl |
| 2X Hybridization Buffer (Vial 6 or 7) | 13µl | 17µl | 21µl | 25µl |

3. Gently vortex and briefly microfuge the **Hybridization Mix**. Incubate the **Hybridization Mix** first at 75-80°C for 10 minutes, and then at the hybridization temperature until loading the array (see the table located below step 5 for recommended hybridization temperatures). Pre-warm the microarrays to the hybridization temperature.
4. Gently vortex and briefly microfuge the **Hybridization Mix**. Add the **Hybridization Mix** to a pre-warmed microarray, taking care to leave behind any precipitate at the bottom of the tube.
5. Apply a glass coverslip to the array. Incubate the array overnight in a dark humidified chamber at the appropriate hybridization temperature:

| Spotted DNA | Vial 5 or 6 Buffer | Vial 7 Buffer |
|--------------------|---------------------------|----------------------|
| Oligonucleotide | 55-62°C | 43-50°C |
| PCR Product (cDNA) | 60-65°C | 48-53°C |

The hybridization temperatures recommended in this protocol are intended as a starting point and should be used as a guide. It may be necessary to adjust the temperatures to meet the stringency requirements.

Array Washes:

1. Prewarm the 2X SSC, 0.2%SDS wash buffer to:
 - 60-65°C for PCR product (cDNA) arrays
 - 42°C for oligonucleotide spotted arrays
2. Remove the coverslip by washing the array in prewarmed 2X SSC, 0.2% SDS for 2-5 minutes or until the coverslip floats off.*
3. Wash for 15 minutes in prewarmed 2X SSC, 0.2%SDS.
4. Wash for 10-15 minutes in 2X SSC at room temperature.
5. Wash for 10-15 minutes in 0.2X SSC at room temperature.
6. Immediately transfer the array to a dry 50mL centrifuge tube. Do this quickly to avoid streaky background on the slide. Orient the slide so that any label is down in the tube. Centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide. Avoid contact with the array surface.

***Note:** If the coverslip is difficult to remove, this may be an indication of drying. To prevent this problem from recurring in future experiments, increase the total volume of the **Hybridization Mix** by adding equal volumes of Nuclease Free Water (Vial 10) and 2X Hybridization Buffer. In addition, ensure that the hybridization chamber is properly humidified and sealed.

3DNA Hybridization:

1. Prepare the 3DNA Array 900MPX Capture Reagent (Vial 1). It is necessary to break up aggregates that may form as a result of the freezing process.
 - a. Thaw the 3DNA Array 900MPX Capture Reagent (Vial 1) in the dark at room temperature for 20 minutes.
 - b. Vortex at the maximum setting for 3 seconds and microfuge briefly.
 - c. Incubate at 50-55°C for 10 minutes.
 - d. Vortex at the maximum setting for 3-5 seconds.
 - e. Microfuge the tube briefly to collect the contents at the bottom.

Be sure to check the sample for aggregates prior to use and repeat vortex mixing if necessary. Aggregates may appear as small air bubbles or flakes at the side of the tube below the surface of the solution. Repeat steps a-e if necessary.

2. Thaw and resuspend the 2X Hybridization Buffer. Vial 6, 2X SDS-based Hybridization buffer, is recommended. Heat to 70°C for at least 10 minutes or until completely resuspended. Vortex to ensure that the components are resuspended evenly. If necessary, repeat heating and vortexing until all the material has been resuspended. Microfuge for 1 minute. **Caution: Do not use the 2X Enhanced cDNA Hybridization Buffer (Vial 5) in the 3DNA Hybridization step.**
3. For each array, prepare a **3DNA Hybridization Mix** according to the table below.
Note: For single channel analysis, use 2.5µl of Nuclease Free Water (Vial 10) in place of the second 3DNA Capture Reagent.

| Glass Coverslip Size, mm | 24x30 | 24x40 | 24x50 | 24x60 |
|---------------------------------------|--------------|--------------|--------------|--------------|
| Final Hybridization Volume | 26µl | 34µl | 42µl | 50µl |
| 3DNA Capture Reagent #1 (Vial 1) | 2.5µl | 2.5µl | 2.5µl | 2.5µl |
| 3DNA Capture Reagent #2 (Vial 1) | 2.5µl | 2.5µl | 2.5µl | 2.5µl |
| Nuclease Free Water (Vial 10) | 8µl | 12µl | 16µl | 20µl |
| 2X Hybridization Buffer (Vial 6 or 7) | 13µl | 17µl | 21µl | 25µl |

4. Gently vortex and briefly microfuge the **3DNA Hybridization Mix**. Incubate the **3DNA Hybridization Mix** first at 75-80°C for 10 minutes, and then at the hybridization temperature until loading the array (see the table located below step 6 for recommended hybridization temperatures). Pre-warm the microarrays to the hybridization temperature.
5. Gently vortex and briefly microfuge the **3DNA Hybridization Mix**. Add the **3DNA Hybridization Mix** to a pre-warmed microarray, taking care to leave behind any precipitate at the bottom of the tube.
6. Apply a glass coverslip to the array. Incubate the array for 4 hours in a dark humidified chamber at the appropriate hybridization temperature:

Spotted DNA
Oligonucleotide
PCR Product (cDNA)

Vial 6 Buffer
55-65°C
60-65°C

Vial 7 Buffer
43-53°C
48-53°C

Array Washes:

Perform these washes **in the dark to avoid photobleaching and fading** of the fluorescent dyes.

1. Prewarm the 2X SSC, 0.2%SDS wash buffer to 60-65°C.
2. Remove the coverslip by washing the array in prewarmed 2X SSC, 0.2% SDS for 2-5 minutes or until the coverslip floats off.*
3. Wash for 15 minutes in prewarmed 2X SSC, 0.2%SDS.
4. Wash for 10-15 minutes in 2X SSC at room temperature.
5. Wash for 10-15 minutes in 0.2X SSC at room temperature.
6. Transfer the array to a dry 50mL centrifuge tube, orienting the slide so that any label is down in the tube. Immediately centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide (any delay in this step may result in high background). Avoid contact with the array surface.

Proceed to Signal Detection, or first apply DyeSaver 2 coating to preserve fluorescent signal (Genisphere Cat No. Q500500).

***Note:** If the coverslip is difficult to remove, this may be an indication of drying. To prevent this problem from recurring in future experiments, increase the total volume of the **3DNA Hybridization Mix** by adding equal volumes of Nuclease Free Water (Vial 10) and 2X Hybridization Buffer. In addition, ensure that the hybridization chamber is properly humidified and sealed.

Signal Detection:

Scan the microarray according to the manufacturer's recommendations.

Troubleshooting

All Genisphere reagents are carefully checked for quality before shipment to customers. Genisphere labeling kits are designed to achieve excellent microarray results. The sensitivity and performance of the kits is influenced by factors such as array quality, sample quality and user technique. Good microarray results can be achieved by considering the following tips:

- Properly store and handle kit components and other reagents. DO NOT use expired reagents.
- Carefully read and follow the recommended procedures outlined in the protocol. Any modifications may affect results.
- Check the purity, quality and quantity of RNA prior to each use. See Appendix A of the Troubleshooting Guide for 3DNA Array 350RP and Array 900MPX Kits for a procedure for running RNA gels (www.genisphere.com/array_detection_troubleshooting.html).
- Keep thawed RNA solutions on ice at all times.
- Contaminants and miscalculations of RNA input can adversely affect results. Always handle RNA with extreme care, avoiding any exposure to ribonucleases. Keep thawed RNA solutions on ice at all times.
- Check the purity and quantity of synthesized cDNA using gel electrophoresis or other quantitative methods. See Appendix B of the Troubleshooting Guide for 3DNA Array 350RP and Array 900MPX Kits for a procedure for running cDNA gels (www.genisphere.com/array_detection_troubleshooting.html).
- If using custom arrays, confirm viability prior to using Genisphere labeling kits.
- Scan arrays prior to hybridization to determine inherent background characteristics.
- Closely monitor all temperature sensitive procedures. Genisphere kits require a variety of temperature-controlled instruments (ovens, heat blocks, water baths etc.). Calibrated thermometers should be used routinely to verify all temperatures outlined in the protocols. Any fluctuation or divergence in temperature can adversely impact results.
- If using Agilent arrays, please call Genisphere Technical Support.
- The protocols are written for use of glass coverslips. Avoid using plastic coverslips. If using hybridization volumes greater than 60 μ L (such as large hybridization chambers or hybridization stations), please contact Genisphere Technical Support for recommendations.

In the event that a problem should arise, consult the Troubleshooting Guide for 3DNA Array 350RP and Array 900MPX Kits, which can be viewed or downloaded at www.genisphere.com/array_detection_troubleshooting.html. This guide is designed to help identify and resolve problems quickly. Each section pertains to a specific problem area: procedural, background, signal and data. Under these categories, from left to right, are listed symptoms, causes and resolutions to many of the most common microarray problems. If a particular problem is not listed in this guide, please contact Genisphere Technical Support:

Phone: (215) 996-3040 (Monday through Friday, 8:30am to 5pm EST)

Email: info@genisphere.com

References

1. Nilsen, T.W., Grayzel, J., and Prenskey, W. Dendritic Nucleic Acid Structures. *J. Theor. Biol.* (1997) 187: 273-284.
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Appendix A:

Concentration of DNA

DNA samples may be concentrated using Microcon[®] YM-30 Microconcentrators (30,000 molecular weight cutoff, Millipore catalog number 42409). These devices are capable of reducing the volume of cDNA to 3-10 μ l in as little as 8-10 minutes. The procedure below reiterates the manufacturer's directions with minor adaptations for Genisphere labeling kits.

Important: When using microconcentrators, evaluate centrifuge time and speed settings to yield final volumes of 3-10 μ l. A fixed angle rotor tabletop centrifuge capable of 10-14,000g should be used.

1. Adjust the volume of the DNA sample to 100 μ l with 1X TE buffer.
2. Place the Microcon[®] YM-30 sample reservoir into the 1.5mL collection tube.
3. Pre-wash the reservoir membrane by adding 100 μ l 1X TE buffer to the sample reservoir. Do not touch the membrane with the pipet tip.
4. Secure the tube cap and centrifuge for 3 minutes at 10-14,000g.
5. Add the 100 μ l DNA to the sample reservoir. Do not touch the membrane with the pipet tip.
6. Secure the tube cap and centrifuge for 8-10 minutes at 10-14,000g.
7. Carefully separate the sample reservoir from the collection tube. Discard the collection tube.
8. Add 5 μ l of 1X TE buffer to the sample reservoir membrane without touching the membrane. Gently tap the side of the reservoir to promote mixing of the concentrate with the 1X TE buffer.
9. Carefully place the sample reservoir upside down in a **new collection tube**.
10. Centrifuge for 2 minutes at top speed.
11. Separate the sample reservoir from the collection tube and discard the reservoir. Note the volume of DNA collected in the bottom of the tube (3-10 μ l total volume).
12. Proceed to Array Hybridization.

Appendix B

Reducing Array Background

To reduce array background, one or both of the procedures outlined below may be used.

Option 1: Array Prewashing Procedure

Prewashing the array can help to remove salts and other debris that can become immobilized during hybridization, resulting in high background.

1. Wash the microarray by the following conditions:
 - a. 2X SSC/0.2% SDS for 20 minutes at 55°C
 - b. 0.2X SSC for 5 minutes at room temperature
 - c. Deionized distilled water for 3 minutes at room temperature
2. Immediately transfer the array to a dry 50 mL centrifuge tube. Do this quickly to avoid streaky background on the slide. Orient the slide so that any label is down in the tube. Centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide.

The array is now ready for either prehybridization or hybridization with Tagged DNA.

Option 2: Array Prehybridization with BSA

Prehybridization of the array with BSA can help reduce some types of nonspecific binding, a common cause of high background. 2X Formamide-Based Hybridization Buffer (Vial 7) contains BSA and can be used to pre-coat the array.

1. Prewarm the microarray to 50°C for 10 minutes.
2. Thaw and resuspend the 2X Formamide-Based Hybridization Buffer (Vial 7) by heating to 70°C for at least 10 minutes or until completely resuspended. Vortex to ensure that the components are resuspended evenly. If necessary, repeat heating and vortexing until all the material has been resuspended. Microfuge for 1 minute.
3. Prepare the **Prehybridization Mix** as follows:
 - 25µl 2X Formamide-Based Hybridization Buffer (Vial 7)
 - 1µl Human Cot-1 DNA
 - 24µl Nuclease free water
4. Heat the **Prehybridization Mix** to 80°C for 10 minutes.
5. Apply the **Prehybridization Mix** to the prewarmed microarray and cover with a 24x60mm coverslip.
6. Incubate at 50°C for 1-2 hours.
7. Wash the array by the following conditions:
 - a. 2X SSC, 0.2% SDS for 15 min at 60-65°C.
 - b. 2X SSC for 10 min at room temperature.
 - c. 0.2X SSC for 10 min at room temperature.
8. Immediately transfer the array to a dry 50 mL centrifuge tube. Do this quickly to avoid streaky background on the slide. Orient the slide so that any label is down in the tube. Centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide. Avoid contact with the array surface.

The array is now ready for hybridization with Tagged DNA.

Appendix C

Reducing the Degradation of Cy5 or Alexa Fluor 647

Cy5/Alexa Fluor 647 dye performance may be affected by a variety of factors that are particularly prevalent during the summer months. Exposure of the Cy5/Alexa Fluor 647 dye solutions and the hybridized arrays to light and to oxidative environments may cause rapid fading of the dye, regardless of the labeling system used. Limiting or controlling the exposure of the arrays to these environments has been shown to significantly reduce Cy5/Alexa Fluor 647 fading. Below are recommendations for reducing the degradation of Cy5/Alexa Fluor 647 when performing microarray experiments:

1. Always keep solutions and arrays containing Cy5/Alexa Fluor 647 away from light, particularly sunlight. Cy5/Alexa Fluor 647 will photo-bleach when exposed to light, including normal fluorescent lighting.
2. Protect the hybridized, dried array from contact with air, particularly on hot and sunny days. We have found that ambient ozone levels resulting from summertime air pollution can cause oxidative degradation of Cy5/Alexa Fluor 647. Keeping the Cy5/Alexa Fluor 647-containing arrays in an inert atmosphere (nitrogen) in a small container (50mL tube) can significantly delay fading of the Cy5/Alexa Fluor 647. Some investigators also add small quantities of dithiothreitol (DTT) or beta mercapto-ethanol (BME) to the bottom of the tube to further promote a reducing micro-environment. Be certain to avoid contact of the array with these chemicals. If possible, do not place the arrays or the array scanner near high ozone-generating equipment, such as a freezer or laser printer.
3. In the preparation of wash buffers, avoid the use of water that may damage Cy5/Alexa Fluor 647. We recommend using deionized, reagent grade water from VWR (Cat. No. RC91505) to prepare wash buffers.
4. In the preparation of wash buffers, be certain that any DEPC treated solutions have had all of the DEPC fully removed, since DEPC is a potent oxidizer. We recommend using 10% SDS from Ambion (Cat. No. AM9823) and 20X SSC from Ambion (Cat. No. AM9763) to prepare wash buffers.
5. Add a small quantity of dithiothreitol (DTT) to the first two wash buffers used after the 3DNA hybridization. The DTT should be at a final concentration of 0.1-0.5mM. Be sure to work with fresh DTT, as old or poor quality DTT may cause an increase in background visible as a "haze" in the Cy3/Alexa Fluor 546 channel.
6. Use DyeSaver 2 (Genisphere Cat. No. Q500500) after the final washing and drying of the microarray. DyeSaver 2 is easy to use, compatible with most array surface chemistries, and protects Cy5/Alexa Fluor 647 from atmospheric oxidation for at least three weeks. DyeSaver 2 has also been shown to reduce Cy5/Alexa Fluor 647 damage due to photobleaching. For more information about DyeSaver 2, please refer to www.genisphere.com or call Genisphere Technical Support.