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CHARACTERISTICS OF 3DNA ARRAY 50 (Version 2) KIT LABELING

The 3DNA Array 50 (Version 2) kit is easy to use and is designed for use with arrays printed with oligonucleotides or PCR products (cDNA). First, reverse transcribe RNA using the included deoxynucleotide triphosphate mix and special RT dT primer. Then, hybridize the cDNA and the fluorescent 3DNA reagent to the microarray in succession. The fluorescent 3DNA reagent will hybridize to the cDNA because it includes a "capture sequence" that is complementary to a sequence on the 5' end of the RT primer.

The 3DNA Array 50 (Version 2) labeling system provides a more predictable and consistent signal than direct or indirect dye incorporation for two reasons. First, since the fluorescent dye is part of the 3DNA reagent, it does not have to be incorporated during the cDNA preparation. This avoids the inefficient hybridization of the cDNA to the array that results from the incorporation of fluorescent dye nucleotide conjugates into the reverse transcript. Second, because each 3DNA molecule contains an average of about 45 fluorescent dyes and each bound cDNA will be detected by a single 3DNA molecule, the signal generated from each message will be largely independent of base composition or length of the transcript. In contrast, the signal generated from each message labeled through dye incorporation will vary depending on the base composition or length of the message.

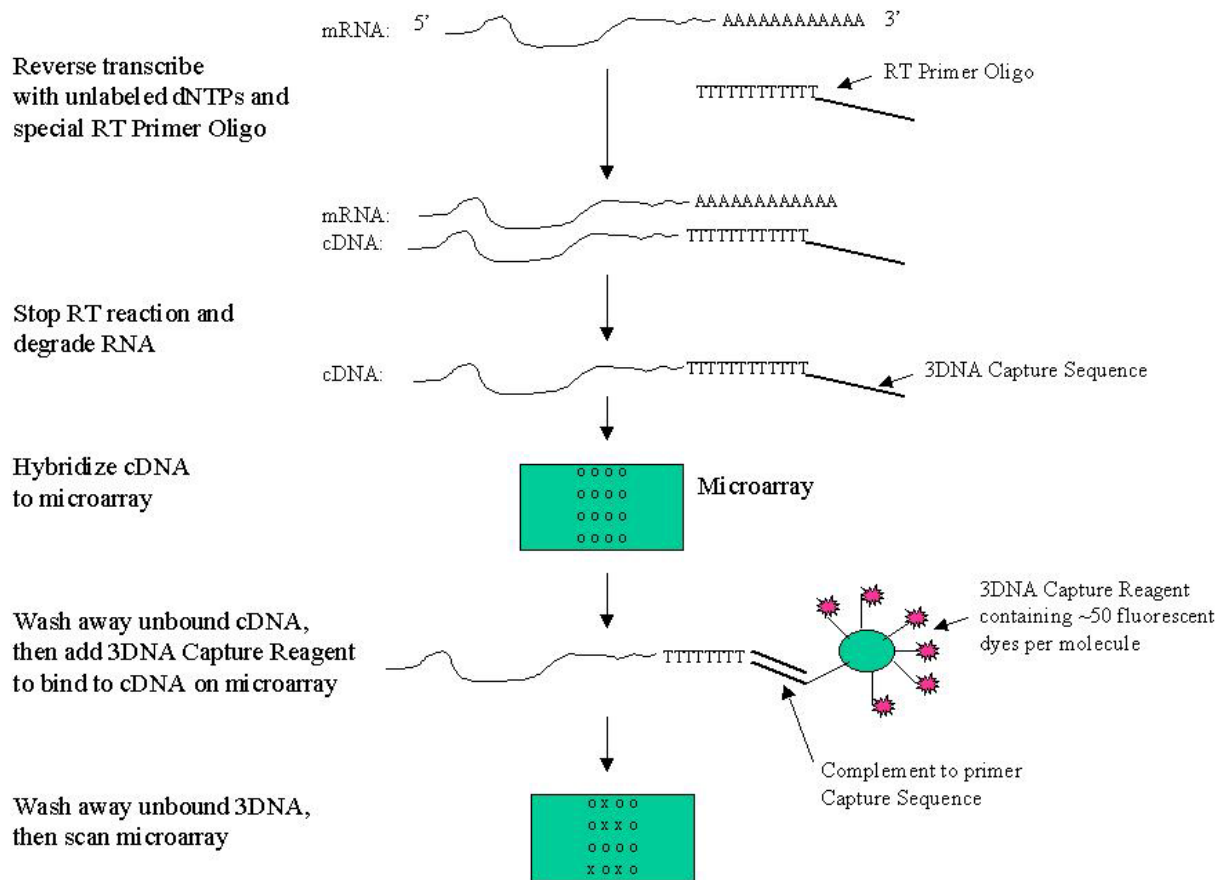
Please note that the array pattern produced by this kit may differ somewhat from the pattern produced by direct or indirect dye incorporation labeling methods when total RNA samples are used. The reason for this is that reverse transcriptase enzyme is known to label genomic DNA (without the need for a primer) as well as RNA. Dye incorporation labeling methods can therefore produce labeled genomic DNA. The labeled genomic DNA will bind to microarrays, resulting in false positives for negative genes and/or inappropriate and misleading fluorescent signals for array elements simultaneously bound with cDNA produced by reverse transcription of RNA. The 3DNA reverse transcription process utilizes unlabeled nucleotides that cannot incorporate any fluorescence into genomic DNA, thus eliminating the possibility of signal contribution from genomic DNA. Because 3DNA labeling differs from dye incorporation labeling in this way, the array pattern produced may vary depending on which labeling method is used. However, in a differential expression experiment, the expression differences between the two RNA samples should be the same regardless of the labeling method used as long as genomic DNA has been eliminated from samples labeled by dye incorporation.

3DNA Array 50 (Version 2) Kit expression array reagents are available with CyTM3, CyTM5, Alexa FluorTM 546, or Alexa FluorTM 647 dye attached to the 3DNA molecule, making possible either single or dual channel detection in array experiments. The diagram on the following page summarizes the Array 50 protocol.

Note: This protocol is not written for use in hybridization volumes greater than 60µl (such as large Lifter Slips, hybridization chambers and hybridization stations). Please contact Genisphere Technical Support for a specific protocol for hybridization volumes greater than 60µl.

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

Microarray Detection with 3DNA™ Reagents



KIT CONTENTS

- Vial 1 Cy3 / Alexa Fluor 546 (red cap) or Cy5 / Alexa Fluor 647 (blue cap) 3DNA Array 50 Capture Reagent
- Vial 2 1.0 pmole/ μ l RT Primer for Cy3 / Alexa Fluor 546 (red cap) or Cy5 / Alexa Fluor 647 (blue cap)
- Primer Sequence Information:
Cy3/Alexa Fluor 546: 5' - TTC TCG TGT TCC GTT TGT ACT CTA AGG TGG A –T(17)- 3'
Cy5/Alexa Fluor 647: 5' - ATT GCC TTG TAA GCG ATG TGA TTC TAT TGG A – T(17)-3'
- Vial 3 Deoxynucleotide Triphosphate Mix (10mM each dATP, dCTP, dGTP, and dTTP)
(packaged separately)
- Vial 4 Superase-In™ RNase Inhibitor
- Vial 5 Linear Acrylamide (co-precipitant)
- Vial 6 2X SDS-Based Hybridization Buffer
- Vial 7 2X Formamide-Based Hybridization Buffer
- Vial 8 Anti-Fade Reagent
- Vial 9 LNA™ dT Blocker
- Vial 10 Nuclease Free Water
- Vial 11 5.0 pmole/ μ l RT Primer for Cy3 / Alexa Fluor 546 (red cap) or Cy5 / Alexa Fluor 647 (blue cap)
(see above for sequence information)

Store Vials 1-11 at –20°C in the dark. Vial 1 may be kept at 4°C for short-term storage (~1 week).

Instructions for Handling Kit Contents

Vial 1: Refer to page 14.

Vials 6 and 7: Refer to pages 12 and 14.

Vials 2, 8, 9, 10 and 11: Thaw at room temperature, vortex, and briefly microfuge.

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

OTHER REQUIRED MATERIALS

Critical materials required for successful use of this kit include:

- Microarray: Commercial or in-house prepared from either oligonucleotides or PCR/cDNA products
- Microarray reader equipped to read Cy3 / Alexa Fluor 546 and/or Cy5 / Alexa Fluor 647 fluorochromes
- RNA sample
 - Total RNA sample greater than or equal to 900ng/ μ l
 - or Poly(A) RNA sample greater than or equal to 125ng/ μ l
 - or senseRNA sample greater than or equal to 375ng/ μ l
- Reverse Transcriptase enzyme
 - SuperScript II (Invitrogen Cat No. 18064-014 – 10,000 Units @ 200U/ μ l)
 - or Genisphere RT Enzyme (Genisphere Cat No. RT300320)
 - or other equivalent reverse transcriptase enzyme (Promega, etc).
- Reagent Grade Deionized Water (Recommended: VWR Cat No. RC9150-5)
- 0.5M NaOH/50 mM EDTA (cDNA synthesis stop solution)
- 1M Tris-HCl, pH 7.5
- 10 mM Tris-HCl, pH 8.0 / 1 mM EDTA (1X TE Buffer)
- Millipore Microcon[®] YM-30 Centrifugal Filter Device (30,000 molecular weight cutoff, Millipore Cat. No. 42409)
- Glass coverslips (Corning brand distributed by Fisher or VWR) or LifterSlips[™] (Erie Scientific)
- 2X SSC, 0.2% SDS buffer
- 2X SSC buffer
- 0.2X SSC buffer
- Glass Coplin Jars (or equivalent)

Optional reagents:

- Cot-1 DNA (species specific)
- Additional high-concentration RT primer (for use with Appendix B):
 - 5 pmole/ μ l RT Primer for Cy3/ Alexa Fluor 546: Genisphere Cat. No. CW31100A
 - 5 pmole/ μ l RT Primer for Cy5/ Alexa Fluor 647: Genisphere Cat. No. CW31100B
 - 30 pmole/ μ l RT Primer for Cy3/ Alexa Fluor 546: Genisphere Cat. No. CMR960UA
 - 30 pmole/ μ l RT Primer for Cy5/ Alexa Fluor 647: Genisphere Cat. No. CMR960UB
- 100% ethanol (for use with Ethanol Precipitation Procedure)
- 3M ammonium acetate or 5M NaCl (for use with Ethanol Precipitation Procedure)
- 70% ethanol in reagent grade water (v/v) (for use with Ethanol Precipitation Procedure)
- Isopropanol (for use with Appendix C)
- 0.2% SDS buffer (for use with Appendix C)
- 95% ethanol (for use with Appendix C)
- 2X Enhanced cDNA Hybridization buffer, Genisphere Cat. No. CW31200 (for use in the cDNA hybridization step only, if additional sensitivity is desired)
- DyeSaver 2, Genisphere Cat. No. Q500500 (use to preserve fluorescent signal and prevent photobleaching)

IMPORTANT INFORMATION BEFORE BEGINNING

RNA Preparation

Preparation and use of high-quality RNA is critical to the success of microarray experiments. Recommended protocols for RNA purification are available on the Genisphere website at www.genisphere.com/Array_Detection_protocols.html

- If degraded RNA is used, the RT reaction using dT primer will only generate short poly dT tails as opposed to full length cDNA, and little or no specific signal will be produced upon subsequent array hybridization. If degraded RNA samples must be used, good results can be obtained by labeling the samples with Genisphere's 3DNA Array 900MPX kit. Or, degraded RNA samples may be amplified with Genisphere's SenseAmp Plus kit and then labeled with the Array 50 kit.
- The use of an RNase inhibitor (Superase-In™, Vial 4) is strongly recommended. RNase inhibitor should be added to stored RNA samples suspected of being contaminated with RNases. Inhibitor should also be added during the reverse transcriptase reaction to avoid RNA degradation during cDNA synthesis. Please refer to the following references for more information regarding RNA degradation by RNases:

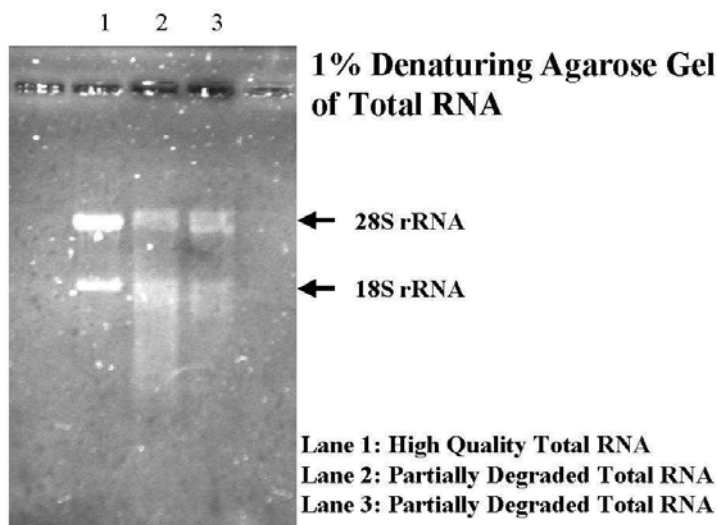
Sambrook, J., Fritsch, E.F., and Maniatis, T. *Molecular Cloning, A Laboratory Manual (Second Edition)* Cold Spring Harbor Laboratory Press, 1989.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., 1998.

- 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.

High-quality RNA will have the following characteristics:

1. OD 260/280 ratio will be between 1.9 and 2.1.
2. On an agarose gel, total plant and mammalian RNA will be represented as two sharp, bright bands. For mammalian RNA, the bands will be at ~ 4.5 kb and ~ 1.9 kb, representing the 28S and 18S ribosomal subunits, respectively.



Selection and Preparation of Microarrays

Quality control testing of all microarrays should be performed immediately after spotting (or receipt of arrays) and periodically thereafter, to establish non-specific background noise characteristics of the arrays as they age. Also, all solutions used in post-spotting array processing should be tested to assure consistency and minimal contribution to non-specific array background.

Genisphere recommends using amino-silane coated slides for spotting PCR products, particularly Clontech DNA-Ready Type II, Corning GAPS II and UltraGAPS, and Telechem SuperAmine slides. One of the protocols in Appendix C should be followed for processing the arrays after they are spotted. Arrays prepared on poly-L-lysine, aldehyde or amino-silane surfaces may require either a prewash or prehybridization step to reduce the background observed after hybridization. Please refer to Appendix D for these procedures.

Genisphere labeling kits are compatible with most array chemistries currently on the market. Most purchased arrays should be prepared as described by the manufacturer. Pre-spotted cDNA arrays manufactured by Takara do not require special treatment prior to use. Pre-spotted oligo arrays manufactured by MWG Biotech require prewashing as described in Appendix D for optimal results.

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

Hybridization Conditions

Because microarrays vary, it is important to determine the optimal hybridization buffer and temperature for each array type. The following buffers are provided in this kit:

- **2X SDS-Based Hybridization Buffer (Vial 6)**—for use in both the cDNA and 3DNA hybridization steps, this buffer may be used on arrays that tolerate higher temperatures.
- **2X Formamide-Based Hybridization Buffer (Vial 7)**—for use in both the cDNA and 3DNA hybridization steps, this buffer is designed for use at lower temperatures due to its higher stringency formulation.

Each hybridization buffer should be tested to determine which buffer is best for a particular array type. The optimal hybridization temperature should be empirically determined for each lot of microarrays. The ranges of hybridization temperatures included in this product manual are provided as a guide. If a lower temperature is required for a certain array type, please contact Genisphere Technical Support for instructions on adding formamide to a hybridization buffer.

For optimal data quality, the LNA dT Blocker (Vial 9) should be included in the cDNA hybridization step for both cDNA and oligo arrays. LNA dT Blocker is a high-performance poly T based blocking reagent designed by Genisphere (patent pending) to completely block all poly A sequences present in array features, including control spots containing only poly dA sequences. This blocking reagent contains Locked Nucleic Acid (LNA) nucleotides (a patented Exiqon™ technology) at key positions within the poly dT synthetic strand. The presence of these modified nucleotides stabilizes the hybridization between complementary strands of nucleic acids, thus improving the blocking capacity of the poly dT reagent. See reference 3 for additional information relating to LNA chemistry. Although average array signal intensity for a blocked array may be lower compared to a non-blocked array, specific signal from reversed transcribed cDNA binding to complementary array elements should not be adversely affected. While a volume of 2µl of the LNA dT Blocker (Vial 9) is recommended for each hybridization, some arrays may demonstrate better performance if additional LNA dT Blocker is used (3-4µl). Additional LNA blocker may be purchased (Cat. No. CW3910).

PROCEDURE FOR USE

Step 1: Preparation of cDNA from Total RNA (Reverse Transcription)

Since microarrays and RNA preparations vary in quality, the exact amount of RNA required for a given experiment will typically range from 10-20 μ g of animal total RNA or 25-50 μ g of plant total RNA. For new users, 20 μ g of animal total RNA or 50 μ g of plant total RNA is recommended as a starting point for cDNA synthesis. Larger or smaller amounts of RNA may be required to achieve optimal results, depending on the quality of the RNA sample and the array.

For use of higher quantities of RNA in large-scale cDNA synthesis, follow Appendix A.
For use of senseRNA or poly(A) RNA, follow Appendix B.

1. In a microtube, prepare the **RNA-RT primer mix**:
 - 1-22 μ l total RNA (10-20 μ g mammalian total RNA or 25-50 μ g plant total RNA)
 - 1 μ l RT primer (Vial 2, 1pmole/ μ l, either Cy3/Alexa Fluor 546 or Cy5/Alexa Fluor 647)
 - Add Nuclease Free Water (Vial 10) to a final volume of 23 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:
 - 8 μ l 5X SuperScript II First Strand Buffer (or equivalent reaction buffer supplied with enzyme)
 - 4 μ l 0.1M dithiothreitol (if supplied with enzyme; otherwise use Nuclease Free Water (Vial 10))
 - 2 μ l dNTP mix (Vial 3)
 - 1 μ l Superase-In™ RNase inhibitor (Vial 4)
 - 2 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. Keep on ice until used.
6. Add the 17 μ l of **reaction mix** from step 5 to the 23 μ l of **RNA-RT primer mix** from step 3 (40 μ l volume).
7. Gently mix (do not vortex) and incubate at 42°C for 2 hours.
8. Stop the reaction by adding 7 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 10 μ l of 1M Tris-HCl, pH 7.5.
11. For Single Channel Assays:
 - Add 73 μ l of 1X TE buffer to the reaction from step 10 (130 μ l volume).

For Dual Channel Assays:

- Combine the Cy3/Alexa Fluor 546 and Cy5/Alexa Fluor 647 reactions from step 10 into one tube.
- Rinse the empty tube with 16 μ l of 1X TE buffer.
- Combine the rinse with the reaction mixture (130 μ l volume).

Proceed to *Step 2: Concentration of cDNA*.

Step 2: Concentration of cDNA

Two methods are provided below for concentrating the cDNA, which must be done before the cDNA can be used in the hybridization mix. Although ethanol precipitation is a traditional method for nucleic acid concentration, this method may lead to variable results due to partial or complete loss of the pelleted cDNA or incomplete re-solubilization of the precipitated cDNA. Microcon[®] concentration is an alternative method that may offer better performance characteristics. Please compare both methods to determine which is suitable for your laboratory.

Note: Evaporative drying of the cDNA is NOT recommended, as a dried sample may result in lower signal and/or higher background on the array.

Option 1 (Recommended): Millipore Microcon[®] YM-30 Centrifugal Filter Devices

cDNA samples may be concentrated using the Millipore Microcon[®] YM-30 Centrifugal Filter Devices (30,000 molecular weight cutoff, Millipore cat. no. 42409). These devices are capable of reducing the volume of the cDNA synthesis reaction to 3-10 μ l in as little as 8-10 minutes. The procedure below reiterates the manufacturer's directions with minor adaptations for the 3DNA Array 50 Kit.

Important: When using the Microcon[®] YM-30 column, evaluate centrifuge time and speed to yield final volumes of 3-10 μ l. A fixed angle rotor tabletop centrifuge capable of 10-14,000g should be used.

1. Place the Microcon[®] YM-30 sample reservoir into the 1.5mL collection tube.
2. 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.
3. Secure the tube cap and centrifuge for 3 minutes at 10-14,000g.
4. Add the cDNA to the sample reservoir. Do not touch the membrane with the pipet tip.
5. Secure the tube cap and centrifuge for 8-10 minutes at 10-14,000g.
6. Carefully separate the sample reservoir from the collection tube. Discard the collection tube.
7. Add 5 μ l of 1X TE buffer to the sample reservoir without touching the membrane. Gently tap the side of the reservoir to distribute the TE across the membrane surface.
8. Carefully place the sample reservoir upside down in a **new collection tube**.
9. Centrifuge for 0.5-2 minutes at top speed in the same centrifuge.
10. Separate the sample reservoir from the collection tube and discard the reservoir. Note the volume collected in the bottom of the tube (3-10 μ l). The cDNA sample may be stored for later use.
11. Add Nuclease Free Water (Vial 10), if necessary, to a final volume of 10 μ l.

Proceed to *Step 3: cDNA Hybridization and Wash*.

Option 2: Ethanol Precipitation

The ethanol precipitation procedure (below) may lead to variable microarray results if not performed carefully because reverse transcription of small quantities of RNA produces a cDNA pellet that is very small and easily lost during processing or by adherence to the inside of pipet tips. Tracking the pellet through the addition of the linear acrylamide co-precipitant (Vial 5) is helpful; additional reagents (i.e. non-fluorescent Pellet Paint from Novagen) may also be used to help avoid loss of precipitated cDNA. If the cDNA pellet disappears or is lost, do NOT proceed with the array hybridization.

1. Thoroughly mix the linear acrylamide (Vial 5) by vortexing for several seconds.
2. Add 3 μ l of 5.0mg/ml linear acrylamide (Vial 5) to the combined cDNA mix.
3. Add 6 μ l of 5M NaCl or 250 μ l 3M Ammonium Acetate and mix.
4. Add 540 μ l of 100% ethanol if using NaCl or 875 μ l of 100% ethanol if using 3M Ammonium Acetate. Mix by moderate vortexing.
5. Incubate at -20°C for 30 minutes.
6. Centrifuge the sample at $>10,000\text{g}$ for 15 minutes.
7. Carefully aspirate the supernatant to avoid loss of the cDNA pellet. **Do not decant**, as decanting may dislodge the pellet and cause it to be lost.
8. Add 300 μ l of 70% ethanol to the cDNA pellet. Gently mix by tapping the side of the tube. Avoid over mixing, which may cause the cDNA pellet to break up.
9. Centrifuge at $>10,000\text{g}$ for 5 minutes and remove the supernatant. **Do not decant.**
10. Dry the cDNA pellet completely by heating for 10-30 minutes at 65°C . If the cDNA pellet is not completely dry, it will be difficult to resuspend, and incomplete resuspension may produce high speckled background on the microarray and/or weak results.
11. Resuspend pellet in 10 μ l of nuclease free water (heating and vortexing as necessary).

Proceed to *Step 3: cDNA Hybridization and Wash*.

Step 3: cDNA Hybridization and Wash

cDNA Hybridization

1. Thaw and resuspend the hybridization buffer (see *Hybridization Conditions*, pg. 7, for help in selecting the appropriate buffer) by heating to 65-70°C for at least 10 minutes or until completely resuspended. Vortex to ensure that the buffer is 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 **cDNA Hybridization Mix** according to the table below. **Optional:** 1.0µl Cot-1 DNA may be added to the cDNA Hybridization Mix. Cot-1 DNA (species specific) should be denatured at 95-100°C for 10 minutes prior to use.

Glass Coverslip Size, mm	24x30	24x40	24x50	24x60
Final Hybridization Volume	26µl	34µl	42µl	50µl
Concentrated cDNA	10µl	10µl	10µl	10µl
LNA dT Blocker (Vial 9)	2µl	2µl	2µl	2µl
Nuclease Free Water (Vial 10)	1µl	5µl	9µl	13µl
2X Hybridization Buffer (Vial 6 or 7)	13µl	17µl	21µl	25µl

3. Gently vortex and briefly microfuge the **cDNA Hybridization Mix**. Incubate the **cDNA 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 **cDNA Hybridization Mix**. Add the **cDNA 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:

<u>Spotted DNA Size</u>	<u>Vial 6 Buffer</u>	<u>Vial 7 Buffer</u>
30mer	42-47°C	30-35°C
50mer	55-60°C	43-48°C
70 mer	55-62°C	43-50°C
PCR Product (cDNA)	55-65°C	43-53°C

The hybridization temperatures recommended in this protocol are intended as a starting point and should be used as a guide for further optimizations. It may be necessary to adjust the temperatures to meet the stringency requirements dictated by the nature of the nucleic acids spotted on the array as well as the slide surface chemistry. In particular, increasing the hybridization temperature by 5°C may remove non-specific signal.

Post cDNA Hybridization Wash

1. Prewarm the 2X SSC, 0.2%SDS wash buffer to:
 - 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. Transfer the array to a dry 50mL centrifuge tube, orienting the slide so that any label is at the bottom of 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.

Further optimization of wash conditions may be required to achieve optimal array performance. If necessary to reduce background on the array, we recommend increasing the time of some or all of the washes to 15-20 minutes. Agitation during washing may also help to reduce background due to non-specific binding to the surface of the array.

Proceed to *Step 4: 3DNA Hybridization and Wash*.

***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 **cDNA Hybridization Mix** by adding equal volumes of Nuclease Free Water (Vial 10) and 2X Hybridization Buffer (Vial 6 or 7). In addition, ensure that the hybridization chamber is properly humidified and sealed.

Step 4: 3DNA Hybridization and Wash

3DNA Hybridization

1. Prepare the 3DNA 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 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 or 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. **Optional:** Prepare a stock solution of Anti-Fade Reagent (Vial 8) by combining 1 μ l of Anti-Fade with 100 μ l of either of the 2X Hybridization Buffers to be used in the 3DNA hybridization (Vial 6 or Vial 7). The Anti-Fade Reagent helps reduce fading of the fluorescent dyes both during and after the hybridization process. Store any unused hybridization buffer containing Anti-Fade Reagent at -20°C and use within two weeks of preparation. **Do not use Anti-Fade Reagent on arrays printed on aldehyde-coated glass**, as this may cause high background.
4. For each array, prepare a **3DNA Hybridization Mix** according to the table below. **Optional:** 1.0 μ l Cot-1 DNA may be added to the cDNA Hybridization Mix. Cot-1 DNA (species specific) should be denatured at 95-100°C for 10 minutes prior to use. **Note:** For single channel expression analysis, use 2.5 μ l 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)	2.5 μ l	2.5 μ l	2.5 μ l	2.5 μ l
3DNA Capture Reagent #2 (Vial 1)	2.5 μ l	2.5 μ l	2.5 μ l	2.5 μ l
Nuclease Free Water (Vial 10)	8 μ l	12 μ l	16 μ l	20 μ l
2X Hybridization Buffer (Vial 6 or 7), may contain Anti-Fade Reagent from step 3	13 μ l	17 μ l	21 μ l	25 μ l

5. 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 7 for recommended hybridization temperatures). Pre-warm the microarrays to the hybridization temperature.
6. 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.
7. Apply a glass coverslip to the array. Incubate the array for 2-3 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

Post 3DNA Hybridization Wash

Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes. Please refer to Appendix E for recommendations for reducing the degradation of Cy5 / Alexa Fluor 647.

1. Prewarm the 2X SSC, 0.2%SDS wash buffer as follows:
 - 65°C for PCR product (cDNA) arrays and oligonucleotide arrays greater than 50 nucleotides long
 - 42°C for oligonucleotide arrays less than 50 nucleotides long
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 at the bottom of 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.

Further optimization of wash conditions may be required to achieve optimal array performance. If necessary to reduce background on the array, increase the time of some or all of the washes to 15-20 minutes. Agitation during washing may also help to reduce background due to non-specific binding to the surface of the array.

Proceed to *Signal Detection*, or first apply DyeSaver 2 coating (Genisphere Cat No. Q500500) to preserve fluorescent signal and reduce photo-bleaching.

***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 (Vial 6 or 7). In addition, ensure that the hybridization chamber is properly humidified and sealed.

SIGNAL DETECTION

IMPORTANT: Store the array in the dark until scanned. The fluorescence of the 3DNA reagents, especially Cy5 and Alexa Fluor 647, can diminish rapidly even in ambient light because of oxidation. Please refer to Appendix E for recommendations for reducing the degradation of Cy5/Alexa Fluor 647 when performing microarray experiments.

Scan the microarray as described by the scanner's manufacturer. Avoid excess multiple scans as the dyes may photo-bleach from exposure to the scanner light source.

If using a Packard scanner, the recommendation is to start by setting the laser at 80% power and either use the "autobalance" feature or the table below to set up the initial scanning parameters for proper channel balance. Adjustment of your scanner laser power and photo-multiplier tube (PMT) voltage may be required to balance the various fluorophore channels. If the PMT setting is set too high, the background observed may be unacceptable. In these instances the PMT setting should be reduced and the laser power should be increased to optimize the signal-to-noise ratio. However, to prevent photo-bleaching the fluorescent dyes, especially Cy5/Alexa Fluor 647, after a single scan, avoid setting the laser too high (>90-95% power).

Note: Balancing the image by offsetting the laser or PMT may result in a non-linear distribution of the data between each channel. In these instances, a statistical normalization may be required. Please consult the instrument's user manual for further instructions.

Initial Scanner Setting for Packard ScanArray 5000 4 Channel Scanner

<u>Dye</u>	<u>Laser</u>	<u>PMT</u>
Cy3 / Alexa Fluor 546	80	70 +/-5
Cy5 / Alexa Fluor 647	80	65 +/-5

If using an Axon 4000 series scanner, the recommended PMT settings are as follows:

Initial Scanner Setting for Axon 4000B 2 Channel Scanner

<u>Dye</u>	<u>Laser</u>	<u>PMT</u>
Cy3 / Alexa Fluor 546	100	500-700 volts
Cy5 / Alexa Fluor 647	100	600-800 volts

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, as RNA may degrade between times of use. See Appendix A of the Troubleshooting Guide for 3DNA Array 900, Array 350 and Array 50 V2 Kits for a procedure for running RNA gels (www.genisphere.com/array_detection_troubleshooting.html).
- Keep thawed RNA solutions on ice at all times. Immediately return RNA to -80°C storage after use.
- Contaminants can cause miscalculations of RNA input, which can adversely affect results. Always handle RNA with extreme care, avoiding any exposure to ribonucleases.
- 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 900, Array 350 and Array 50 V2 Kits for a procedure for running cDNA gels (www.genisphere.com/array_detection_troubleshooting.html).
- If using custom arrays, confirm appropriate performance characteristics prior to using Genisphere labeling kits.
- Scan arrays prior to hybridization to determine inherent background characteristics. Array features often non-specifically fluoresce in the Cy3/Alexa 546 channel.
- 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 or Affymetrix arrays, please call Genisphere Technical Support.
- This protocols is written for use of glass coverslips. Do not use plastic coverslips. If using high volume equipment such as hybridization stations or chambers, contact Genisphere Tech Support for a specific protocol.

In the event that a problem should arise, consult the Troubleshooting Guide for 3DNA Array 900, Array 350 and Array 50 V2 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

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2. Stears, R.L., Getts, R.C., Gullans, S.R. A novel, sensitive detection system for high-density microarrays using dendrimer technology. *Physiol Genomics* 3: 93-99, 900.
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For additional references of Genisphere products, please visit the following link:

http://www.genisphere.com/array_detection_data_ref.html

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Appendix A: Scaled-Up cDNA Preparation

A scaled-up reverse transcription reaction can be performed (20-50 μ g mammalian total RNA or 50-125 μ g plant total RNA) to provide extra cDNA for additional array sensitivity, duplicate experiments, quantitation of the cDNA, or other parallel analysis. This procedure may also be used to avoid concentrating the cDNA prior to hybridization.

1. In a microtube, prepare the **RNA-RT primer mix**:
 - 1-22 μ l total RNA (20-50 μ g mammalian total RNA or 50-125 μ g plant total RNA)
 - 1 μ l RT primer (Vial 11, 5pmole/ μ l, either Cy3/Alexa Fluor 546 or Cy5/Alexa Fluor 647)
 - Add Nuclease Free Water (Vial 10) to a final volume of 23 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:
 - 8 μ l 5X SuperScript II First Strand Buffer (or equivalent reaction buffer supplied with enzyme)
 - 4 μ l 0.1M dithiothreitol (if supplied with enzyme; otherwise use 2 μ l Nuclease Free Water (Vial 10))
 - 2 μ l dNTP mix (Vial 3)
 - 1 μ l Suprase-In™ RNase inhibitor (Vial 4)
 - 2 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. Keep on ice until used.
6. Add the 17 μ l of **reaction mix** from step 5 to the 23 μ l of **RNA-RT primer mix** from step 3 (40 μ l volume).
7. Gently mix (do not vortex) and incubate at 42°C for 2 hours.
8. Stop the reaction by adding 7 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 10 μ l of 1M Tris-HCl, pH 7.5.
11. For dual channel assays, combine the appropriate amount of the Cy3/Alexa Fluor 546 and Cy5/Alexa Fluor 647 cDNA synthesis reactions. If concentration is required, proceed Step 2: *Concentration of cDNA*. Otherwise, proceed to Step 3: *cDNA Hybridization and Wash*. Extra cDNA may be stored at -20°C.

Appendix B: cDNA Preparation from senseRNA or poly (A) RNA

The procedure below summarizes the steps necessary to synthesize cDNA from senseRNA (produced by Genisphere SenseAmp Plus or RampUP Plus kits) or poly(A) RNA. Since microarrays and RNA preparations vary in quality, the exact amount of RNA required for a given experiment will range from 5-10 μ g of senseRNA or 0.5-1 μ g of poly(A) RNA. To obtain optimal array results, it is important to use sufficient amounts of primer. Generally, 1 μ g of senseRNA requires 7.5-10 picomoles of oligo(dT) primer for efficient reverse transcription. Usually, 0.1 μ g of poly(A) RNA requires 5 picomoles of oligo(dT) primer for efficient reverse transcription. To achieve reasonable reaction volumes for cDNA synthesis, high-concentration primers are available from Genisphere. 20 μ l of 5 pmole/ μ l RT Primer (Vial 11) is provided in the Array 50 kits. If necessary, additional primer can be ordered as follows:

- 5 pmole/ μ l RT Primer for Cy3/ Alexa Fluor 546: Genisphere Cat. No. CW31100A
- 5 pmole/ μ l RT Primer for Cy5/ Alexa Fluor 647: Genisphere Cat. No. CW31100B
- 30 pmole/ μ l RT Primer for Cy3/ Alexa Fluor 546: Genisphere Cat. No. CMR960UA
- 30 pmole/ μ l RT Primer for Cy5/ Alexa Fluor 647: Genisphere Cat. No. CMR960UB

cDNA Synthesis

1. In a 1.5mL RNase-free microcentrifuge tube on ice, prepare the **RNA-RT primer mix**:
 - 1-8 μ l senseRNA or poly(A) RNA
 - 1-3 μ l RT Primer (5 pmole/ μ l or 30 pmole/ μ l)
 - Add RNase free water 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. Continue with step 3 on page 8 (*Step 1: Reverse Transcription*).

Appendix C: Array Processing

Arrays should be processed, post manufacturing, according to the glass manufacturer's recommendations or by one of the procedures below. **Note:** Do not use succinic anhydride when processing cDNA arrays, as this reagent may interfere with 3DNA detection. Succinic anhydride may be used on oligo arrays.

Option 1 (cDNA arrays): Cross-link, isopropanol wash, and boil

1. Preheat 2 liters of reagent grade deionized distilled water (best quality water available) to 95°C-100°C (boiling) in a 4 liter beaker on a hot plate.
2. Transfer 250 mL of isopropanol into a glass rectangular staining dish and place a small stir bar into the dish. Place the dish on a magnetic stir plate and allow the bar to stir at a slow steady rate.
3. Retrieve the unprocessed arrays. Carefully, pick up one slide by the corner and hold it in the steam above the boiling water (from step 1) for five seconds. Make sure the arrays are facing up. Wave the slide in the air for three seconds and place onto a fiber free lab wipe, array side up. Repeat until eight slides have been hydrated and dried.
4. Transfer the eight slides, array side up, to a cross-linker set to 50-220 mJ.
5. After cross-linking, transfer the eight arrays into a glass/metal Wheaton staining slide holder with grooves (do not place slides on each end groove). Put the holder with the slides into the isopropanol (from step 2) and incubate for 15 minutes with stirring.
6. Transfer the slide holder to the boiling water (from step 1) and incubate for 8-10 minutes. Make sure the slides are under the water.
7. Remove the slide holder from the boiling water and place onto a lab wipe to remove excess liquid. The arrays are now ready for hybridization.

Option 2 (cDNA arrays): Cross-link, SDS wash, boil, and cold ethanol rinse

1. Prepare 2 liters of a 0.2% SDS solution in reagent grade deionized distilled water (best quality water). For example, mix 40 mL of 10% SDS and 1960 mL of water in a two-liter autoclaved glass bottle. Filter the solution to remove any precipitated SDS. Transfer 250 mL of this 0.2% SDS solution into a glass rectangular staining dish and place a small stir bar into the dish. Place the dish on a magnetic stir plate and allow the bar to stir at a slow steady rate.
2. Preheat 2 liters of reagent grade deionized distilled water (best quality water available) to 95°C-100°C (boiling) in a 4 liter beaker on a hot plate.
3. Transfer 2 liters of reagent grade deionized distilled water to a 4-liter beaker. Keep at room temperature.
4. Transfer 250 mL of ethanol into a glass rectangular staining dish. Place this dish into an ice bucket to set up an ice cold ethanol bath.
5. Retrieve the unprocessed arrays. Carefully, pick up one slide by the corner and hold it in the steam above the boiling water (from step 2) for five seconds. Make sure the arrays are facing up. Wave the slide in the air for three seconds and place onto a fiber free lab wipe, array side up. Repeat until eight slides have been hydrated and dried.
6. Transfer the eight slides, array side up, to a cross-linker set to 50-220 mJ.
7. After cross-linking, transfer the eight arrays into a glass/metal Wheaton staining slide holder with grooves (do not place slides on each end groove). Put the holder with the slides into the 0.2% SDS (from step 1) and incubate for 10 minutes with stirring.
8. Remove the slide holder and place onto a lab wipe to remove excess liquid. Then dunk the holder into the 2 liters of room temperature water (from step 3) five times.
9. Transfer the slide holder to the boiling water (from step 2) and incubate for 8-10 minutes. Make sure the slides are under the water.
10. Remove the slide holder from the boiling water and place onto a lab wipe to remove excess liquid. Transfer the slide holder into the ice cold ethanol (from step 4) and incubate for five minutes. Make sure the slides are under the ethanol.
11. Remove the slide holder and place onto a lab wipe to remove excess liquid. Transfer each slide into a 50 mL centrifuge tube. Centrifuge at 1000 rpm for 3 minutes to dry the slides. The arrays are now ready for hybridization.

Option 3 (oligo arrays): Cross-link, wash

1. Transfer eight slides, array side up, to a cross-linker set to 50-220 mJ.
2. After crosslinking, follow the Array Prewashing Procedure found in Appendix D.

Appendix D: Reducing Array Background

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

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 at the bottom of 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 either prehybridization or hybridization with cDNA.

Array Prehybridization Procedure

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 at the bottom of 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 cDNA.

Appendix E: Reducing Degradation of Cy5/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 Cy5/Alexa Fluor 647 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 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. Use the Anti-Fade Reagent (provided with the 3DNA kits) in the hybridization solution containing Cy5/Alexa Fluor 647 Capture Reagent. The Anti-Fade Reagent has anti-oxidant properties that will retard the oxidative process.
4. In the preparation of wash buffers, avoid the use of water that may damage Cy5/Alexa 647. As noted in the Internet List Serve, MilliQ[®] water has been shown to damage Cy5 (<http://groups.yahoo.com/group/microarray/messages/2867>). We recommend using deionized, reagent grade water from VWR (Cat. No. RC91505) to prepare wash buffers.
5. 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. 9823) and 20X SSC from Ambion (Cat. No. 9763) to prepare wash buffers.
6. 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 channel.
7. 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 photo-bleaching. For more information about DyeSaver 2, please refer to the Genisphere web site (www.genisphere.com) or call Genisphere Technical Support.