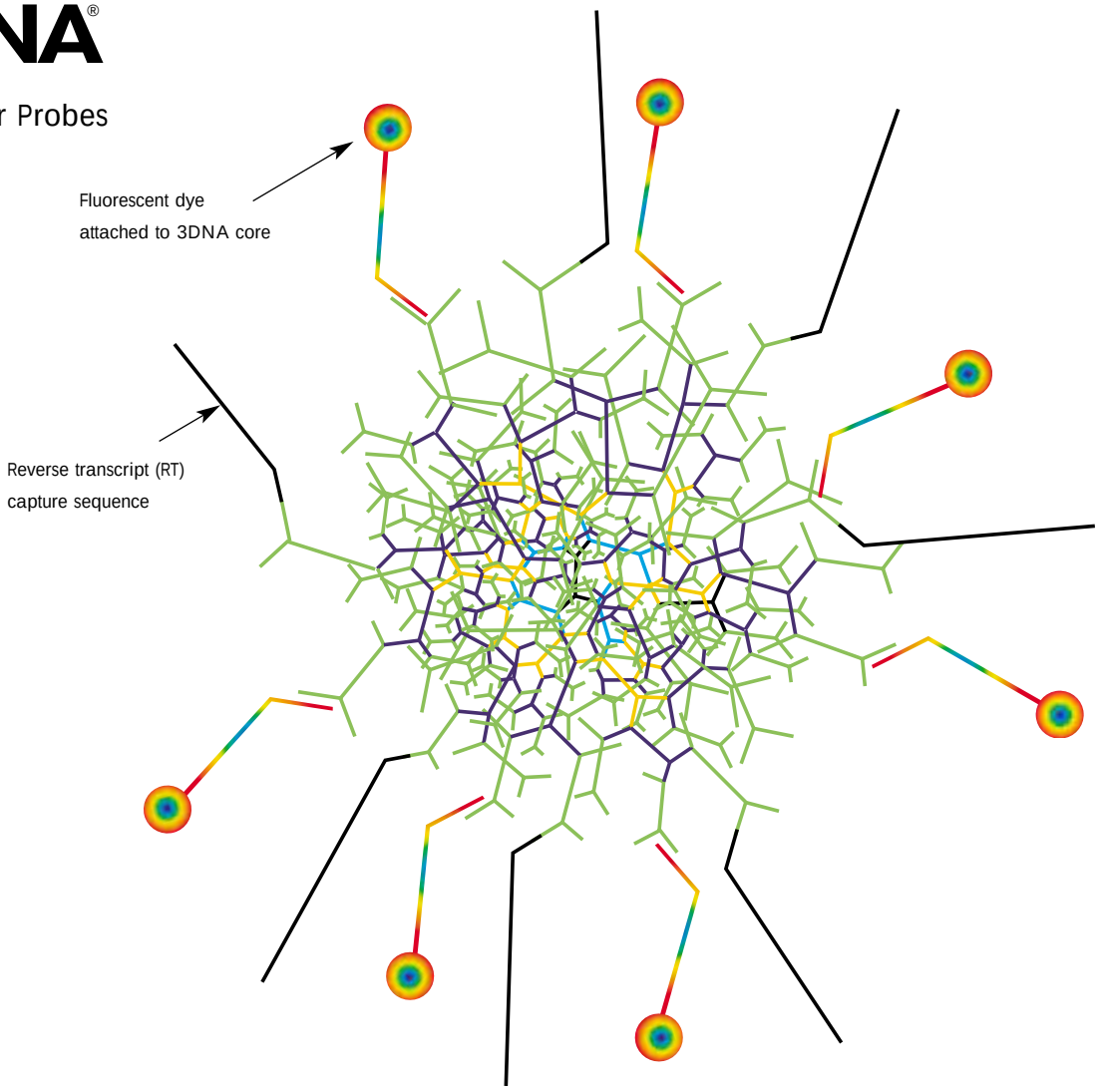
3DNA®

DNA Dendrimer Probes



3DNA® Array 350™

Expression Array Detection Kit for Microarrays

Alexa Fluor 546™ Kit	Cat No. W300100	20 Assays
Alexa Fluor 647™ Kit	Cat No. W300110	20 Assays
Cy3™ Kit	Cat No. W300130	20 Assays
Cy5™ Kit	Cat No. W300140	20 Assays
Cy3™/Cy5™ Kit	Cat No. W300180	5 Assays each
Alexa Fluor 546™ /Alexa Fluor 647™ Kit	Cat No. W300184	5 Assays each

Genisphere Inc.
2801 Sterling Drive
Hatfield, PA 19440
Tel: 877.888.3DNA
201.651.3100
Fax: 877.FAX.3DNA
215.996.3070
www.genisphere.com

Genisphere®

LABELING SYSTEMS + MICROARRAY SERVICE LABORATORY

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Characteristics of 3DNA Array 350 Kit Labeling

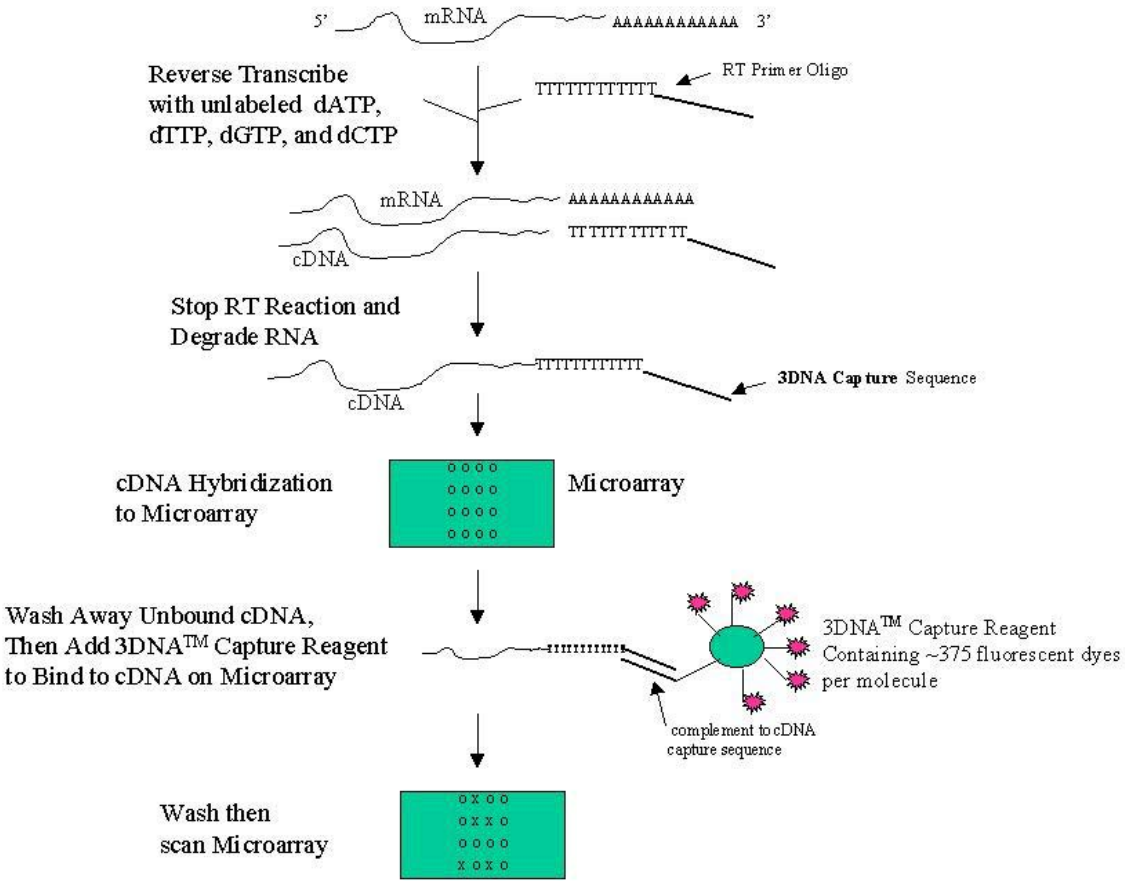
The 3DNA Array 350 kit is easy to use and is designed for use with arrays printed with oligonucleotides or PCR products (cDNA). First, reverse transcribe your RNA, total or Poly(A)$^+$, using the included deoxynucleotide triphosphate mix and special RT primer oligo. Then, hybridize your cDNA and the fluorescent 3DNA reagent to your microarray in succession. The fluorescent 3DNA reagent will hybridize to your cDNA because it includes a "capture sequence" that is complementary to a sequence on the 5' end of the RT primer.

The 3DNA Array 350 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 375 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 fluorescence levels 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 350 Kit expression array reagents are available with Cy™3, Cy™5, Alexa Fluor™ 546, or Alexa Fluor™ 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 350 protocol.

Microarray Detection with 3DNA™ Reagents - 2 Step Hybridization



Kit Contents

Warning: The contents of the 3DNA Array 350 kit, specifically Vials 1, 2 and 11, are unique to this product and are not compatible with any other Genisphere 3DNA microarray labeling kits.

Vial 1	Cy3 / Alexa Fluor 546 (red cap) or Cy5 / Alexa Fluor 647 (blue cap) 3DNA Array 350 Capture Reagent.
Vial 2	1.0 pmole/μl RT Primer for Cy3 /Alexa Fluor 546 (red cap) or Cy5 / Alexa Fluor 647 (blue cap).
Vial 3	Deoxynucleotide Triphosphate Mix (10mM each dATP, dCTP, dGTP, and dTTP).
Vial 4	Superase-In™ RNase inhibitor.
Vial 5	Linear Acrylamide (co-precipitant).
Vial 6	2X SDS-Based Hybridization Buffer (see Buffer Components section p. 6).
Vial 7	2X Formamide-Based Hybridization Buffer (see Buffer Components section p. 6).
Vial 8	Anti-Fade Reagent.
Vial 9	LNA™ dT Blocker (for use with PCR product (cDNA) micorarrays).
Vial 10	Nuclease Free Water.
Vial 11	5.0 pmole/μl RT Primer for Cy3 /Alexa Fluor 546 (red cap) or Cy5 / Alexa Fluor 647 (blue cap) (for use with Poly(A)$^+$ and Scaled-Up procedures).

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

Hybridization Buffer Components and Selection

2X SDS-Based Hybridization Buffer (Vial 6):

0.50M $NaPO_4$
1% SDS
2mM EDTA
2X SSC
4X Denhardt's Solution

2X Formamide-Based Hybridization Buffer (Vial 7):

50% Formamide
8X SSC
1% SDS
4X Denhardt's Solution

Because microarrays vary, it is important to determine the optimal hybridization conditions, including the optimal buffer selection, for each array type. It has been observed that the 2X SDS-Based Hybridization Buffer (Vial 6) has given stronger signals on some arrays relative to the 2X Formamide-Based Hybridization Buffer (Vial 7), and vice versa. We recommend testing the hybridization buffers to determine which is best for your array type. Additionally, the ranges of hybridization temperatures included in this product manual are provided as a guide. Genisphere recommends that the optimal hybridization temperature be empirically determined for each lot of microarrays.

On some arrays prepared on poly-L-lysine surfaces, the poly-L-lysine coating may begin to peel off at the hybridization temperature required for use of the Vial 6 buffer. Use the Vial 7 buffer as directed if you experience this problem.

Blocker and Competitor DNA Use

Because most PCR products (cDNAs) contain poly(dA/dT) sequences, we recommend the use of the LNA dT Blocker (Vial 9). The LNA dT Blocker is a high-performance poly T based blocking reagent designed by Genisphere (patent pending). It is designed to completely block **all** the poly A sequences present in array features, including control spots containing only poly dA sequences. This new 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.

While a volume of 2μl of the LNA dT Blocker (Vial 9) is recommended for each hybridization, some arrays may demostrate better performance if additional LNA dT Blocker is used (3-4μl). **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.**

Add additional competitor DNA as required (e.g. C0T-1 DNA (human, mouse, etc.) (Life Technologies)). Use competitor nucleic acid at 1/10 by mass of input total RNA (i.e. use 100ng of C0T-1 DNA for every microgram of total RNA). If too much competitor is used the signal may be reduced due to nonspecific interactions of the excess competitor with the limited cDNA in the hybridization. Denaturation of C0T-1 and other competitor nucleic acids is recommended (95-100°C for 5-10 minutes) prior to addition to hybridization mix.

Other Materials Required

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
- Total RNA sample (greater than or equal to 0.5μg/μl) or Poly(A)$^+$ RNA sample (greater than or equal to 25ng/μl)
- Reverse Transcriptase enzyme

  SuperScript II (Gibco Cat No. 18064-014 – 10,000 Units @ 200U/ul)

  Genisphere RT Enzyme (Genisphere Cat No. RT300320)

  or other equivalent reverse transcriptase enzyme (Promega, Clontech, Stratagene etc).
- C0T-1 DNA (optional, species specific, available from Gibco / Life Technologies)
- RNase-free deionized distilled water. Note: Cy5 dye may be damaged by contact with DI water produced by the MilliQ® purification system.
- 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 catalog number 42409).
- 100% ethanol (optional: for use with Ethanol Precipitation Procedure)
- 3M ammonium acetate or 5M NaCl (optional: for use with Ethanol Precipitation Procedure)
- 70% ethanol in reagent grade water (v/v) (optional: for use with Ethanol Precipitation Procedure)
- Glass coverslips (Corning brand distributed by Fisher or VWR) or Lifter Slips (Erie Scientific)
- 2X SSC, 0.2% SDS buffer
- 2X SSC buffer
- 0.2X SSC buffer
- 95% ethanol – Molecular Biology Grade

PROCEDURE FOR USE

This kit is intended for use with arrays made with spotted oligonucleotides or double stranded DNA generated from cloned genes via PCR amplification.

The protocol below summarizes the steps required to use the 3DNA Array 350 Kit for gene expression array detection.

Reducing Background through Proper Preparation and Selection of Microarrays:

Pre-spotted cDNA arrays manufactured by Genomic Solutions, Agilent and Takara do not require special treatment prior to use. With other purchased arrays, prepare or pre-treat the microarray as described by the manufacturer. For arrays made "in-house", we recommend using the protocols in Appendix C for pre-treating the arrays. These protocols do not require succinic anhydride treatment and on many array types have yielded stronger signal and lower background.

Pre-spotted oligo arrays manufactured by MWG Biotech require prewashing as described in Appendix D for optimal results.
Note: The Prewash procedure in Appendix D may also be used with other array types to help reduce background.

Genisphere recommends use of certain aminosilane coated slides for spotting PCR products (cDNAs), particularly Clontech DNA-Ready Type II, Corning GAPS II and UltraGAPS, and Telechem SuperAmine slides. These slides demonstrate good DNA binding when used with 3DNA Array 350 kits.

Arrays prepared on poly-L-lysine, aldehyde or certain aminosilane (e.g., Corning GAPS) surfaces may require either a prewash or prehybridization step to reduce the background observed after hybridization. Refer to Appendix D or E, respectively, for procedures to help reduce background on microarrays. Increasing the post-hybridization wash times by 5 minutes each may also reduce background.

As arrays age, they may exhibit lower specific signal and higher levels of background noise. In some cases, as an array ages the spotted probe demonstrates a "green" (Cy3) channel background. We have experienced this phenomenon with both commercial and "in-house" arrays on all substrate surfaces. Quality control testing of both commercial and "in-house" arrays should be performed immediately after spotting (or receipt of arrays) and periodically thereafter to establish non-specific background noise characteristics of the arrays and other materials 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.

RNA Preparation:

Preparation and use of high-quality RNA is critical to the success of microarray experiments.

- If degraded RNA is used, the RT reaction 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. As an alternative, Genisphere recommends the use of the 3DNA Array 350RP kit for use with degraded RNA.

- 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:

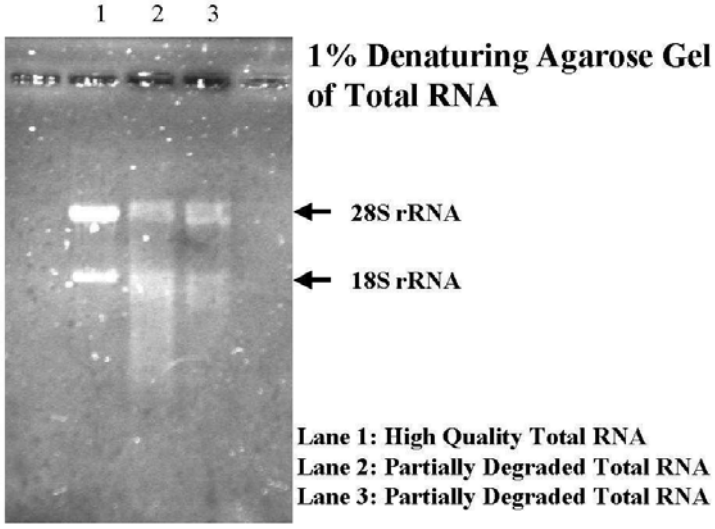
  Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning, A Laboratory Manual (Second Edition) Cold Spring Harbor Laboratory Press, 1989.
  Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., 1998.

- The 3DNA Array 350 labeling system will not label genomic DNA, so it is not essential to remove genomic DNA contamination. (In contrast, labeling methods that incorporate fluors into the cDNA will label genomic DNA, producing a false signal.) However, it is better to digest away genomic DNA so that the quantity and quality of the RNA present may be determined more accurately. Also, if the genomic DNA is allowed to remain in the sample, it may bind to some of the RT enzyme and make the enzyme unavailable for reverse transcription. RNase-free DNase is recommended for degrading contaminating genomic DNA.
  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 RNeasy® kits from Qiagen, following the DNase treatment. 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 sub-units, respectively. Please refer to the image below.



Recommended protocols for RNA purification are available on the Genisphere website at www.genisphere.com/Array_Detection_protocols.html

Preparation of cDNA (reverse transcription):

The procedure below summarizes the steps necessary to synthesize cDNA from total RNA**. If you are using poly(A)$^+$ RNA, follow the procedure described in Appendix B for cDNA synthesis.** Since microarrays and RNA preparations vary in quality, the exact amount of RNA required for a given experiment will range from 1-5μg of mammalian total RNA or 2.5-10μg of plant total RNA. For new users, 5μg of mammalian total RNA or 10μg of plant total RNA is recommended as a starting point for cDNA synthesis.

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 RNeasy® kits from Qiagen, following the DNase treatment. Inactivation of the DNase by high temperature may not completely inactivate the enzyme.

cDNA Synthesis from Total RNA:

1. In a microtube combine:

   1-10μl total RNA (1-5μg mammalian total RNA or 2.5-10μg plant total RNA)
   1μl RT primer (Vial 2, 1pmole/μl)
   Add Nuclease Free Water (Vial 10) to a final volume of 11μl
   This is the **RNA-RT primer mix**.

2. Mix and microfuge briefly to collect contents in the bottom of the tube.

3. Heat to 80°C for ten minutes and immediately transfer to ice for 2-3 minutes.

4. Add 1μl Superase-In (Vial 4) to bring the RNA-RT primer mix to a final volume of 12μl.

5. Reverse transcriptase enzyme and the enzyme's reaction buffer are not included in the kit and must be purchased separately. We recommend the use of SuperScript II Reverse Transcriptase Enzyme (Gibco Cat No. 18064-014 – 10,000 Units @ 200U/μl).

   In a separate microtube combine (on ice):

   4 μl 5X SuperScript II First Strand Buffer or equivalent reaction buffer (supplied with enzyme)
   1 μl dNTP mix (10mM each for dATP, dCTP, dGTP, dTTP) (Vial 3)
   2 μl 0.1M dithiotreitol (DTT) (supplied with enzyme)
   1 μl Superscript II enzyme, 200 units

   This is the **reaction mix.** The final volume should be 8μl. Keep on ice until used.

6. Gently mix (do not vortex) and microfuge briefly to collect reaction mix contents in the bottom of the tube.

7. Add the 8μl of **reaction mix** from step 6 to the 12μl of **RNA-RT primer mix** from step 4 (20μl final volume).

8. Gently mix (do not vortex) and incubate at 42°C for two hours.

9. Stop the reaction by adding 3.5μl of 0.5M NaOH/50mM EDTA.

10. Incubate at 65°C for ten minutes to denature the DNA/RNA hybrids and degrade the RNA.

11. Neutralize the reaction with 5μl of 1M Tris-HCl, pH 7.5.

12. For **dual** channel expression analysis proceed to step 13. For **single** channel assays add 101.5μl of 10mM Tris, pH 8.0, 1mM EDTA for a total volume of 130μl.

13. For **dual** channel expression analysis combine the cDNAs from step 11 into one tube. Rinse the original tubes with 73μl of 10mM Tris, pH 8.0, 1mM EDTA. Combine wash with the cDNA mixtures into one tube for a total volume of 130μl.

Concentration of the 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.

Concentration of cDNA with Millipore Microcon® YM-30 Centrifugal Filter Devices (recommended)

cDNA samples may be concentrated using the Millipore Microcon® YM-30 Centrifugal Filter Devices (30,000 molecular weight cutoff, Millipore catalog number 42409). These devices are capable of reducing the volume of the cDNA synthesis reaction from 130μl to 3-10μl in as little as 8-10 minutes. The procedure below reiterates the manufacturer's directions with minor adaptations for the 3DNA Array 350 Kit.

Important: Users of the MicroconYM-30's should evaluate their own centrifuge settings to determine the optimal time and speed settings to yield final volumes of 3-10μl.

1. Place the Microcon® YM-30 sample reservoir into the 1.5mL collection tube.
2. Pre-wash the reservoir membrane by adding 100μl TE pH 8.0 to the Microcon® YM-30 sample reservoir.
3. Place the tube/sample reservoir assembly into a fixed angle rotor tabletop centrifuge capable of 10-14,000g.
4. Spin for 3 minutes at 10-14,000g.
5. Add all 130μl from the cDNA reaction to the Microcon® YM-30 sample reservoir. Do not touch the membrane with the pipet tip.
6. Place the tube/sample reservoir assembly into a fixed angle rotor tabletop centrifuge capable of 10-14,000g.
7. Centrifuge for 8-10 minutes at 10-14,000g.
8. Remove the tube/sample reservoir assembly. Separate the collection tube from the sample reservoir with care, avoiding spilling any liquid in the sample reservoir.
9. Add 5μl of 1X TE buffer (10mM Tris-HCl, pH 8.0 / 1mM EDTA) to the sample reservoir membrane without touching the membrane. Gently tap the side of the concentrator to promote mixing of the concentrate with the 1X TE buffer.
10. Carefully place the sample reservoir upside down on a new collection tube. Centrifuge for 2 minutes at top speed in the same centrifuge.
11. Separate the sample reservoir from the collection tube and discard the reservoir. Note the volume collected in the bottom of the tube (3-10μl total volume). The cDNA sample may be stored in the collection tube for later use.

Proceed to "Successive Hybridization of cDNA and 3DNA to Microarray", p. 13.

Ethanol Precipitation of the cDNA

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 3µl of 5.0mg/ml linear acrylamide (Vial 5) to the combined cDNA mix.

3. Add 6µl of 5M NaCl or 250µl 3M Ammonium Acetate and mix.

4. Add 540µl of 100% ethanol if using NaCl or 875µl 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 >10,000g 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 300µl of 70% ethanol to the cDNA pellet. Gently mix by tapping the side of the tube. Avoid overmixing, which may cause the cDNA pellet to break up.

9. Centrifuge at >10,000g 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.

Proceed to “Successive Hybridization of cDNA and 3DNA to Microarray”, p. 13.

Successive Hybridization of cDNA and 3DNA to Microarray (2-Step Protocol)

The cDNA and 3DNA hybridization conditions are described below using the two Genisphere hybridization buffers (Vial 6 and Vial 7) provided in the kit. Prior to starting this step, please decide which buffer you prefer. The 2X SDS Based Buffer (Vial 6) may be used on arrays that tolerate higher temperatures (up to 65°C), which may exclude certain array surfaces (i.e. poly-L-lysine). The 2X Formamide Based Hybridization Buffer (Vial 7) is designed for use at lower temperatures due to its higher stringency formulation.

Caution: If you are using a type of slide or array for which a different buffer is specifically recommended, you should contact Genisphere Technical Support for specific recommendations. For example, Agilent cDNA microarrays contain a poly-L-lysine surface that requires the use of the Agilent Deposition Buffer for cDNA hybridization. The subsequent hybridization of the fluorescent 3DNA reagents is performed using the 2X SDS-Based Hybridization Buffer (Vial 6). Please contact Genisphere Technical Support for more detailed information regarding the use of Agilent arrays.

cDNA Hybridization:

1. (Optional) Wash the glass coverslips as follows: Briefly submerge the coverslip in 0.2% SDS followed by a brief rinse in reagent grade deionized distilled water. Let dry before use. Remove excess liquid with a fiber-free lab wipe if necessary.

<u>2X SDS-Based Hybridization Buffer (Vial 6)</u>

2. Thaw and resuspend the 2X SDS-Based Buffer (Vial 6) by heating to 65°C for at least 10 minutes or until completely resuspended. Mix by inversion to insure that the components are resuspended evenly. If necessary repeat heating and mixing until all the material has been resuspended. Microfuge for 1 minute.

<u>2X Formamide-Based Hybridization Buffer (Vial 7)</u>

2. Thaw and resuspend the 2X Formamide-Based Buffer (Vial 7) by heating to 55°C for at least 10 minutes or until completely resuspended. Mix by inversion to insure that the components are are resuspended evenly. If necessary repeat heating and mixing until all the material has been resuspended. Microfuge for 1 minute.

3a. If the cDNA sample was concentrated using the Millipore Microcon® YM-30, add Nuclease Free Water (Vial 10) to the cDNA preparation to achieve a total volume of 10µl.

3b. If the cDNA sample was concentrated using ethanol precipitation, add Nuclease Free Water (Vial 10) to the cDNA preparation to achieve a total volume of 10µl. Heat the mixture of cDNA and water for 10-15 minutes at 65°C. Resuspend the cDNA pellet by vortexing at maximum speed for about 5 seconds. **Do not pipet the pellet "up and down",** as this may cause the pellet to lodge in the pipet tip and be lost! Repeat the heating and mixing steps until the cDNA pellet is completely resuspended. If the cDNA is not completely resuspended, high background and low signal will be observed on the array.

4. Following the table below, add the appropriate volumes of additional reagents based upon the final desired volume:

<u>Desired Final Hybridizaton Mix Volume:</u>	<u>25µl</u>	<u>30µl</u>	<u>35µl</u>	<u>40µl</u>	<u>45µl</u>	<u>50µl</u>
Concentrated cDNA (from step 3a/3b)	10µl	10µl	10µl	10µl	10µl	10µl
2X Hybridization Buffer (Vial 6 or 7)	12.5µl	15µl	17.5µl	20µl	22.5µl	25µl
LNA dT Blocker (Vial 9)***	2µl	2µl	2µl	2µl	2µl	2µl
Nuclease Free Water (Vial 10)	0.5µl	3µl	5.5µl	8µl	10.5µl	13µl

******Oligo arrays may not require the addition of this reagent. Use 2µl water (Vial 10) in place of the LNA dT blocker (Vial 9).***

Optional: 1.0µl C0T-1 DNA may also be added if desired (must be denatured at 95-100°C for 10 minutes prior to use).

Gently vortex and briefly microfuge the Hybridization Mix after addition of all components.

Recommended Hybridization Mix Volume for various coverslip sizes:

Coverslip Dimensions	Total Volume Required	
22 x 22mm	25µl	If LifterSlips are used add approximately 25-50% additional volume.
22 x 30mm	25-30µl	
22 x 40mm	30-40µl	
24 x 50mm	35-45µl	
24 x 60mm	40-50µl	

5. 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 7 for recommended hybridization temperatures).

6. 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. (Pre-warming the microarray to the hybridization temperature may reduce background.)

7. Apply a glass coverslip to the array and incubate overnight in a dark humidified chamber at the appropriate hybridization temperature (see table below).

Note: If drying of the slide occurs then increase the hybridization volume by 10-15% using additional hybridization buffer/water (in a 50/50 mix).

Spotted DNA Size	**Vial 6 Buffer**	**Vial 7 Buffer**
30 mer	42-45°C	30-35°C
50 mer	55-60°C	42-48°C
70 mer	55-65°C	42-53°C
PCR Products (cDNA)	60-65°C	50-55°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 dictated by the nature of the nucleic acids spotted on the array. In particular, increasing the hybridization temperature by 5°C may remove non-specific signal that might otherwise be visible on a negative control spot.

Post cDNA Hybridization Wash:

After hybridization the slides are washed briefly several times to remove unbound cDNA molecules.

1. Prewarm the 2X SSC, 0.2%SDS wash buffer to the first wash temperature (see step 3, below).

2. Remove the coverslip by washing the array in 2X SSC, 0.2% SDS for 2-5 minutes at room temperature (or until coverslip floats off).

3. For PCR product (cDNA) arrays, wash for 10-15 minutes with 2X SSC, 0.2%SDS at 55-65°C. For oligonucleotide spotted arrays, wash for 10-15 minutes with 2X SSC, 0.2%SDS at 42°C.

4. Wash for 10-15 minutes at room temperature with 2X SSC.

5. Wash for 10-15 minutes at room temperature with 0.2X SSC.

6. Wash for 1-2 minutes at room temperature in 95% ethanol to fix the cDNA molecules to the probes. Note: this step can be omitted if 95% Ethanol is not available.

7. Immediately transfer the array to a dry 50mL centrifuge tube. Do this quickly to avoid streaky background on the slide and 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.

Further optimization of wash conditions may be necessary to achieve optimal array performance. If necessary to reduce background on the array, we recommend increasing the wash temperature of the first wash by 5-10°C and 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.

3DNA Hybridization:

1. (Optional) Wash the glass coverslips as follows: Briefly submerge the coverslip in 0.2% SDS followed by a brief rinse in reagent grade deionized distilled water. Let dry before use. Remove excess liquid with a fiber-free lab wipe if necessary.

2X SDS-Based Hybridization Buffer (Vial 6)

2. Thaw and resuspend the 2X SDS-Based Buffer (Vial 6) by heating to 65°C for at least 10 minutes or until completely resuspended. Mix by inversion to insure that the components are resuspended evenly. If necessary repeat heating and mixing until all the material has been resuspended. Microfuge for 1 minute.

2X Formamide-Based Hybridization Buffer (Vial 7)

2. Thaw and resuspend the 2X Formamide-Based Buffer (Vial 7) by heating to 55°C for at least 10 minutes or until completely resuspended. Mix by inversion to insure that the components are are resuspended evenly. If necessary repeat heating and mixing until all the material has been resuspended. Microfuge for 1 minute.

3. The Anti-Fade Reagent (Vial 8) reduces fading of the fluorescent dyes post hybridization. Prepare a stock solution by adding 1µl of Anti-Fade to 100µl of either 2X Hybridization Buffer (Vial 6 or Vial 7). Store any unused hybridization buffer at –20°C and use within two weeks. However, do not use the Anti-Fade Reagent if your arrays are printed on aldehyde-coated slides, as background haze may result**.** Refreeze the Anti-Fade Reagent after use.

4. Prepare the 3DNA Array 350 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 350 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 (1 second).
    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.
    f. 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 4a-4f if necessary.

5. Following the table below, add the appropriate volumes of the 3DNA Array 350 kit components to a fresh tube to make the Hybridization Mix based upon the final desired volume:

Desired Final Hybridizaton Mix Volume:	25µl	30µl	35µl	40µl	45µl	50µl
2X Hybridization Buffer (Vial 6 or 7)	12.5µl	15µl	17.5µl	20µl	22.5µl	25µl
3DNA Capture Reagent #1 (Vial 1, e.g. Cy3)	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl
3DNA Capture Reagent #2 (Vial 1, e.g. Cy5)	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl
Nuclease Free Water (Vial 10)	7.5µl	10µl	12.5µl	15µl	17.5µl	20µl

Optional: 1.0µl C0T-1 DNA may also be added if desired (must be denatured at 95-100°C for 10 minutes prior to use).

Note: For single channel expression analysis, use 2.5µl of Nuclease Free Water (Vial 10) in place of the second 3DNA Capture Reagent.

Gently vortex and briefly microfuge the Hybridization Mix after addition of all components.

Recommended Hybridization Mix Volume for various coverslip sizes:

Coverslip Dimensions	Total Volume Required	
22 x 22mm	25µl	If LifterSlips are used add approximately 25-50% additional volume.
22 x 30mm	25-30µl	
22 x 40mm	30-40µl	
24 x 50mm	35-45µl	
24 x 60mm	40-50µl	

6. 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 8 for recommended hybridization temperatures).

7. Gently vortex and briefly microfuge the Hybridizaton Mix. Add the Hybridization Mix to a pre-warmed microarray, taking care to leave behind any precipitate at the bottom of the tube. (Pre-warming the microarray to the hybridization temperature may reduce background.)

8. Apply a glass coverslip to the array and incubate 2.5-3 hours in a dark humidified chamber at the appropriate hybridization temperature (see table below).

Note: If drying of the slide occurs then increase the hybridization volume by 10-15% using additional hybridization buffer/water (each 50% of the solution).

Spotted DNA Size	**Vial 6 Buffer**	**Vial 7 Buffer**
30 mer	55-65°C	45-53°C
50 mer	55-65°C	45-53°C
70 mer	55-65°C	45-53°C
PCR Products (cDNA)	60-65°C	50-55°C

Post 3DNA Hybridization Wash:

After hybridization the slides are washed briefly several times to remove unbound 3DNA molecules. Perform these washes **in the dark to avoid photobleaching and fading** of the fluorescent dyes. To reduce fading of Cy5 post hybridization, it may also be beneficial to include DTT in the first two washing buffers at a final concentration of 0.5-1mM. 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. Please refer to Appendix F for recommendations for reducing the degradation of Cy5 when performing microarray experiments.

Caution: Be careful with the water you use for your post-hybridization wash buffers and other solutions. As noted in the Internet List Serve, MilliQ® water has been shown to damage Cy5 dye (http://groups.yahoo.com/group/microarray/messages/2867). Also, be certain that any DEPC treated solutions have had all of the DEPC completely removed (DEPC is a potent oxidizer). As an alternative, we recommend the use of non-DEPC treated nuclease free solutions. Commercially available solutions (water, buffers, etc.) from Ambion have been found to work well with Cy5 labeled microarrays.

1. Prewarm the 2X SSC, 0.2%SDS wash buffer to the first wash temperature (see step 3, below).

2. Remove the coverslip by washing the array in 2X SSC, 0.2% SDS for 2-5 minutes at room temperature (or until coverslip floats off).

3. For oligonucleotide arrays spotted with oligos which are less than 50 nucleotides long, wash for 10-15 minutes at 42°C with 2X SSC, 0.2%SDS. For all other array types, wash for 10-15 minutes at 60-65°C with 2X SSC, 0.2%SDS.

4. Wash for 10-15 minutes at room temperature with 2X SSC.

5. Wash for 10-15 minutes at room temperature with 0.2X SSC.

6. Immediately transfer the array to a dry 50mL centrifuge tube. Do this quickly to avoid streaky background on the slide and 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. Immediately transfer the array to a light-protective slide box, taking care not to touch the array surface.

Further optimization of wash conditions may be necessary to achieve optimal array performance. If necessary to reduce background on the array, we recommend increasing the wash temperature of the first wash by 5-10°C and 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 "Signal Detection" p.18.

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 F 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 photobleach from exposure to the scanner light source. If you are working with a Packard scanner, we suggest that you 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 photobleaching 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

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

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For Research Use Only

Genisphere Inc.
2801 Sterling Drive
Hatfield, PA 19440

To Order:

Tel: 877.888.3DNA
215.996.3002
Fax: 877.FAX.3DNA
215.996.3070

Web: www.genisphere.com

Technical Support:

Tel: 877.888.3DNA
215.996.3040
Fax: 215.996.3070

Appendix A

Scaled-Up cDNA Preparation/Concentration Protocol:

When your RNA is not limited to quantities less than 10-50μg of mammalian total RNA or 25-125μg of plant total RNA, a scaled up reverse transcription reaction can be performed. The resulting extra cDNA can be used for duplicate experiments, quantitation of the cDNA, or other parallel analysis.

cDNA Synthesis:

1. In a 1.5mL RNase-free microcentrifuge tube combine:
   1-10μl total RNA (10-50μg mammalian total RNA or 25-125μg plant total RNA)
   1μl RT Primer (Vial 11, 5 pmole/μl)
   Add RNase free water to a final volume of 11μl
   This is the **RNA-RT primer mix**.

3. Mix and microfuge briefly to collect contents in the bottom of the tube.

4. Heat to 80°C for ten minutes and immediately transfer to ice for 2-3 minutes.

5. Add 1μl Superase-In (Vial 4).

6. In a separate microtube combine (on ice):
   4μl 5X Superscript II First Strand Buffer or equivalent reaction buffer (supplied with enzyme)
   1μl dNTP mix (Vial 3)
   2μl 0.1M dithiotreitol (DTT) (supplied with the enzyme)
   1μl Superscript II enzyme, 200 Units
   This is the **reaction mix.** The final volume should be 8μl. Keep on ice until used.

7. Gently mix (do not vortex) and microfuge briefly to collect contents in the bottom of the tube.

8. Add the 8μl of **reaction mix** from step 7 to the 12μl of **RNA-RT primer mix** from step 5 (20μl final volume).

9. Gently mix (do not vortex) and incubate at 42^{o}C for two hours.

10. Stop the reaction by adding 3.5μl of 0.5M NaOH/50 mM EDTA.

11. Incubate at 65°C for ten minutes to denature the DNA/RNA hybrids and degrade RNA.

12. Neutralize the reaction with 5μl of 1M Tris-HCl, pH 7.5. **The resulting solution is your cDNA.**

13. For **dual** channel expression analysis proceed to step 14. For **single** channel assays add 28.5μl of RNase free water. The final volume of the cDNA solution should be 57μl. Proceed to step 15.

14. For **dual** channel expression analysis combine the cDNA preparations from step 12 into one tube. The final volume of the cDNA solution will be 57μl.

15. Transfer into a new microfuge tube up to 10μl (the equivalent of ~1-5μg mammalian total RNA or 2.5–12.5μg plant total RNA) of your cDNA solution. For example, if you start with a 20μg total RNA sample and use 6μl of your 57μl cDNA solution (in a dual channel analysis experiment), you will be using the equivalent of 2.1μg of total RNA in your hybridization. If necessary, add nuclease free water to a final volume of 10μl. Larger fractions of the cDNA mixture may be used if necessary to achieve greater signal. It may not be possible to hold hybridization volumes below 25-30μl in these circumstances.

Proceed to “Successive Hybridization of cDNA and 3DNA to Microarray”, p. 13.

Appendix B

cDNA Preparation from Poly(A)$^+$ RNA

The procedure below summarizes the steps necessary to synthesize cDNA from poly(A)$^+$ RNA. Since microarrays and RNA preparations vary in quality, the exact amount of RNA required for a given experiment will range from 50-250ng of poly(A)$^+$ RNA. For new users, 150ng of poly(A)$^+$ RNA is recommended as a starting point for cDNA synthesis.

cDNA Synthesis:

1. In a 1.5mL RNase-free microcentrifuge tube combine:
   1-9µl poly(A)$^+$ RNA (50-250ng)
   1µl RT Primer (Vial 11, 5 pmole/µl)
   Add RNase free water to a final volume of 11µl
   This is the **RNA-RT primer mix**.

3. Mix and microfuge briefly to collect contents in the bottom of the tube.

4. Heat to 80°C for ten minutes and immediately transfer to ice for 2-3 minutes.

5. Add 1µl Superase-In (Vial 4).

6. In a separate microtube combine (on ice):
   4µl 5X Superscript II First Strand Buffer or equivalent reaction buffer (supplied with enzyme)
   1µl dNTP mix (Vial 3)
   2µl 0.1M dithiotreitol (DTT) (supplied with the enzyme)
   1µl Superscript II enzyme, 200 Units
   This is the **reaction mix.** The final volume should be 8µl. Keep on ice until used.

7. Gently mix (do not vortex) and microfuge briefly to collect contents in the bottom of the tube.

8. Add the 8µl of **reaction mix** from step 7 to the 12µl of **RNA-RT primer mix** from step 5 (20µl final volume).

9. Gently mix (do not vortex) and incubate at 42^{o}C for two hours.

10. Stop the reaction by adding 3.5µl of 0.5M NaOH/50 mM EDTA.

11. Incubate at 65°C for ten minutes to denature the DNA/RNA hybrids and degrade RNA.

12. Neutralize the reaction with 5µl of 1M Tris-HCl, pH 7.5.

13. For **dual** channel expression analysis proceed to step 14. For **single** channel assays add 101.5µl of 10mM Tris, pH 8.0, 1mM EDTA for a total volume of 130µl.

14. For **dual** channel expression analysis combine the cDNAs from step 12 into one tube. Rinse the original tubes with 73µl of 10mM Tris, pH 8.0, 1mM EDTA. Combine wash with the cDNA mixtures into one tube for a total volume of 130µl.

Proceed to “Concentration of cDNA”, p. 11.

Appendix C

Array Processing Procedure (No Succinic Anhydride)

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

1. Preheat 1.0-2.0 liters of reagent grade water (best quality water available) to 95°C-100°C (boiling) in a beaker on a hot plate.
2. Pour 200-250 ml of isopropanol into a rectangular tray and place on magnetic stir plate.
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 damped 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 rectangular staining dish containing isopropanol (from step 3) on the magnetic stir plate for 15 minutes.
6. Transfer the holder with the slides to the boiling hot water (from step 1). Incubate the slides for 8-10 minutes. Make sure the slides are under the water.
7. Remove the slides from the hot water and remove the excess water by blotting onto a lab wipe. The arrays are now ready for hybridization.

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

1. Prepare 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.
2. Preheat 1.0-2.0 liters of reagent grade water to 90°C-100°C in a beaker on a hot plate or in a glass tray in a microwave.
3. Pour at least 700 ml of RGDD into a separate small clean one-liter glass beaker. Place the small beaker on a hot plate and boil on high. This will be used to rehydrate the array prior to cross-linking (step 7).
4. Add 2.0 liters of reagent grade water to a 4 liter beaker. This will be used to wash the slide (see step 10) after the SDS wash and prior to denaturing the spotted DNA.
5. Pour 250 ml of the 0.2% SDS solution (from step 1) into a glass rectangular staining dish and place a small stir bar into the rectangular dish. Place the rectangular dish on a magnetic stir plate and allow the bar to stir at a slow steady rate.
6. Set up an ice cold ethanol bath using a beaker or slide tray. This will be used in step 12, below.
7. Retrieve the unprocessed arrays. Carefully, pick up one slide by the corner and hold it above the boiling water in the steam (from step 3) 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 damped and dried.
8. Transfer the eight arrays to a cross-linker set to 50-220 mJ.
9. 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 rectangular dish on the magnetic stir plate (from step five) for ten minutes.
10. Take the holder with the slides out of the rectangular dish and place onto lab wipe to remove excess liquid. Then dunk the holder into the clean two liters of water (from step 4) five times.
11. Transfer the holder with the slides to the hot water (from step 2). Incubate the slides for 8-10 minutes. Make sure the slides are under the water.
12. Remove the slides from the hot water and remove the excess water by blotting onto a lab wipe. Place the slides into the cold ethanol on ice (from step 6) for five minutes. Again, make sure the slides are under the ethanol.
13. Place slide holder onto a lab wipe to remove the excess ethanol and place each side into one 50 ml centrifuge tube.
14. Transfer centrifuge tubes to centrifuge set at 1000 rpm for 2 ½ minutes. Once arrays are dry, place the slides into a slide box for storage.

Appendix D

Array Prewashing Procedure to Reduce Background

1. Place microarray slide in a Coplin jar with 2X SSC/0.2% SDS at 55^{o}C for 20 minutes.

2. Transfer the microarray array to dish or Coplin jar with 0.2X SSC at room temperature for 5 minutes.

3. Transfer the array to dish or Coplin jar with deionized distilled water at room temperature for 3 minutes.

4. Transfer to 50ml tube and centrifuge at 1000 rpm in clinical centrifuge for 2-3 minutes to dry. Do this quickly to avoid streaky background on the slide and orient the slide so that any label is down in the tube.

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

Appendix E

Array Prehybridization to Reduce Background:

Non-specific binding to the array surface is a common problem on many array types. The prehybridization protocol described below is recommended for reducing this non-specific binding, thereby reducing the background seen post-hybridization.

1. Prewarm the microarray to 50°C for 10 minutes.

2. Prepare Prehybridization Mixture as follows:

   25µl 2X Formamide-Based Hybridization Buffer (Vial 7)
   1µl Human C0T-1 DNA
   24µl Nuclease free water

3. Heat the Prehybridization Mixture to 80°C for 10 minutes.

4. Apply the Prehybridization Mixture to the prewarmed microarray and cover with a 24x60mm coverslip.

5. Incubate at 50°C for 1-2 hours.

6. Wash the array as follows:

   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.

7. Immediately transfer the array to a dry 50 mL centrifuge tube. Do this quickly to avoid streaky background on the slide and 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.

Appendix F

Recommendations for Reducing the Degradation of Cy5 or Alexa Fluor 647 When Performing Microarray Experiments

Cy5/Alexa Fluor647 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 Fluor647 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 photobleach 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 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).

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. Be careful with the water you use for your post-hybridization wash buffers and other solutions. As noted in the Internet List Serve, MilliQ® water has been shown to damage Cy5 dye (http://groups.yahoo.com/group/microarray/messages/2867). Also, be certain that any DEPC treated solutions have had all of the DEPC fully removed (DEPC is a potent oxidizer). Alternatively, we recommend the use of non-DEPC treated nuclease free solutions. Commercially available solutions (water, buffers, etc.) from Ambion have been found to work well with Cy5 labeled microarrays.

5. Add a small quantity of dithiothreotol (DTT) to the post hybridization wash buffers, i.e. 0.1mM final concentration. This potent reducing agent will protect the Cy5/Alexa Fluor 6477 on the array from attack by any oxidative agents in the wash buffers.

In addition, always be certain to mix your 3DNA Cy3 /Alexa Fluor 546 and Cy5/Alexa Fluor 647 Capture Reagents (Vial 1) to break up any aggregates that may form during storage:

1. Thaw the 3DNA Capture Reagent (Vial 1) in the dark at room temperature for 20 minutes.
2. Vortex at the maximum setting for 3 seconds and microfuge briefly (1 second).
3. Incubate at 50-55°C for 10 minutes.
4. Vortex at the maximum setting for 3-5 seconds.
5. 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 1-6 if necessary.