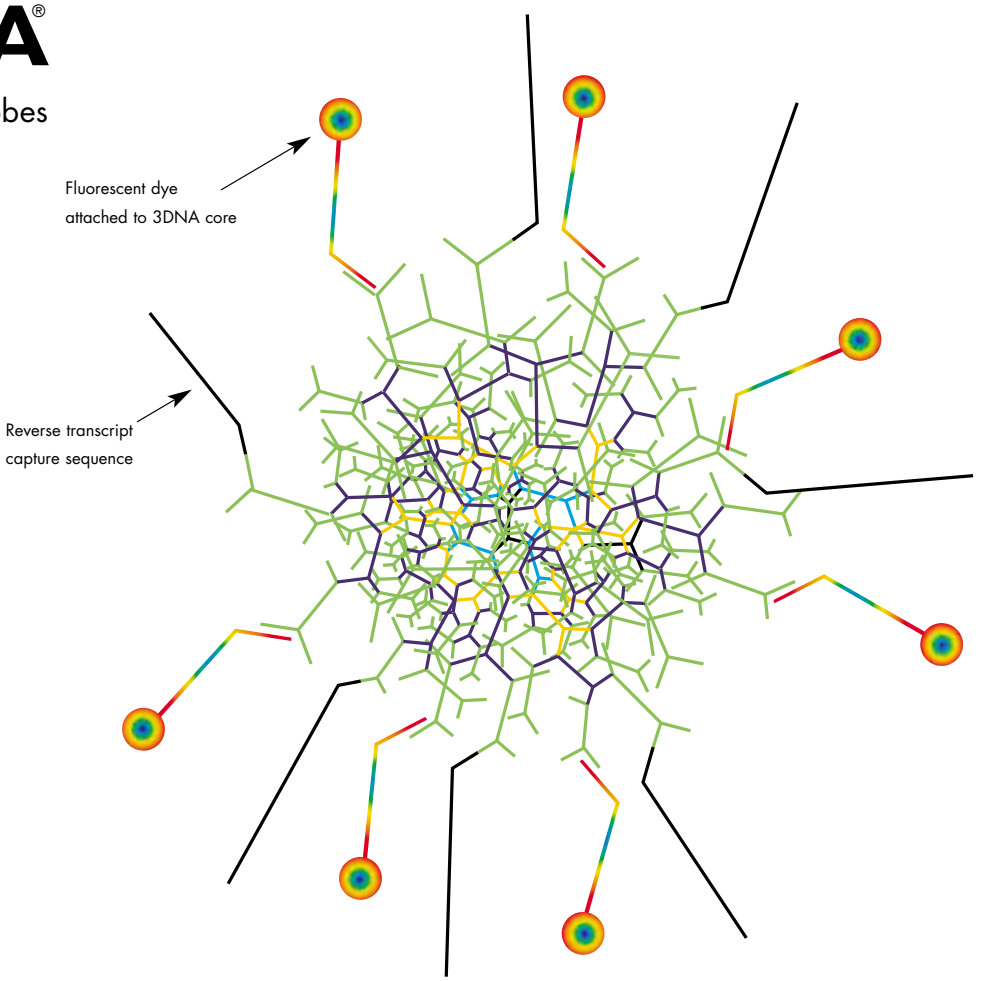
3DNA®

DNA Dendrimer Probes



3DNA™ Submicro® EX Expression Array Detection Kit

Product Manual for Use with New 2X Hybridization Buffers

Alexa Fluor 488™ Kits (Cat No. A100752/A100757)
Alexa Fluor 546™ Kits (Cat No. A100332/A100337)
Alexa Fluor 594™ Kits (Cat No. A100762/A100767)
Alexa Fluor 647™ Kits (Cat No. A100342/A100347)
Cy3™ Kits (Cat No. A100732/A100737)
Cy5™ Kits (Cat No. A100742/A100747)

Alexa Fluor 546™/Alexa Fluor 647™ Trial Kit (Cat No. A100382)
Alexa Fluor 488™/Alexa Fluor 594™ Trial Kit (Cat No. A100784)
Cy3™/Cy5™ Trial Kit (Cat No. A100782)

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

Genisphere®

LABELING SYSTEMS + MICROARRAY SERVICE LABORATORY

TABLE OF CONTENTS

Characteristics of Submicro Labeling

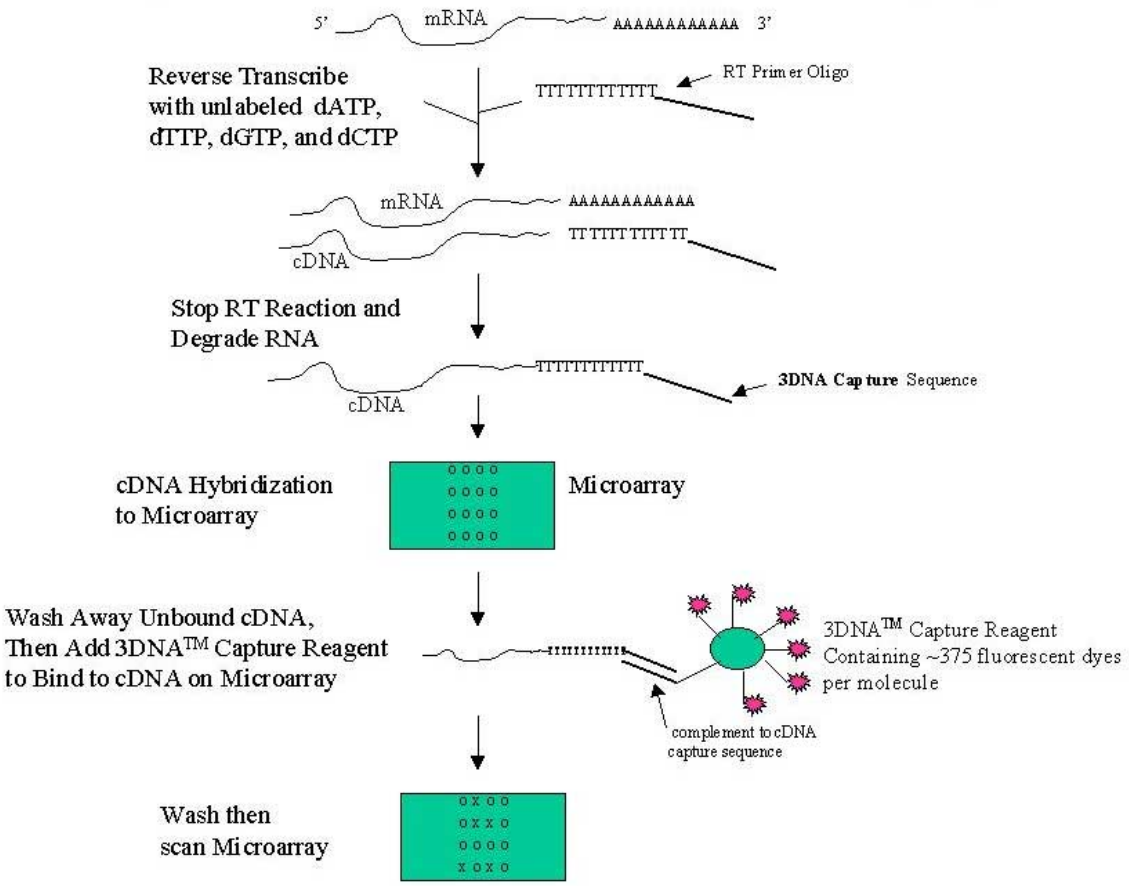
The 3DNA Submicro EX kit is easy to use. First, reverse transcribe your total or poly $(A)^+$ RNA using the included deoxynucleotide triphosphate mix and special RT primer oligo (or your custom RT primer oligo). Next, remove the excess unextended RT primer using a Genisphere SCL spin column (or follow the protocol outlined in Appendix B). Then, hybridize your cDNA and the fluorescent 3DNA reagent to the array in succession (the "2-step protocol). 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 Submicro labeling system provides a more intense, 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 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 expression array reagents are available with Alexa Fluor 488™, Alexa Fluor 546, Cy3™, Alexa Fluor 594™, Alexa Fluor 647 or Cy5™ dye attached to the 3DNA molecule, making possible either single or multiple channel detection in array experiments. The diagram on the following page summarizes the 2-step Submicro protocol.

Microarray Detection with 3DNA™ Reagents - 2 Step Hybridization



Note: Use of this kit requires Genisphere's SCL spin columns (sold separately) unless the Appendix B protocol is used.

Genisphere SCL Spin Columns: **10 pack: Cat No. A100620**
50 pack: Cat No. A100625

Kit Contents

Vial 1	Cy3™ /Alexa Fluor 546 (red cap), Cy5™ /Alexa Fluor 647 (blue cap), Alexa Fluor 594™ (purple cap) or Alexa Fluor 488 (green cap) 3DNA Capture Reagent.
Vial 2	RT Primer for Cy3™ /Alexa Fluor 546 (red cap), Cy5™ /Alexa Fluor 647 (blue cap), Alexa Fluor 594™ (purple cap) or Alexa Flour 488 (green cap) (5 pMole/µl).
Vial 3	Reverse Transcriptase Enzyme (200 Units/µl).
Vial 4	Deoxynucleotide Triphosphate mix (10 mM each dATP, dCTP, dGTP, and dTTP).
Vial 5	5X RT Reaction Buffer.
Vial 6	Hybridization Buffer (not required for this protocol, not included in the Submicro EX trial kit).
Vial 7	Alternate Hybridization Buffer (not required for this protocol, not included in the Submicro EX trial kit).
Vial 8	Anti-Fade Reagent.

Vial 9 Oligo dT Blocking Reagent (250 ng/ul) (not included in the Submicro EX trial kit).

Vial 9b LNA™ dT Blocker.

Vial 10 High-End Differential Enhancer.

Vial 11 Linear Acrylamide (co-precipitant).

Vial 12 Superase-In™ RNase Inhibitor.

Vial 13 2X SDS Based-Hybridization Buffer (see Buffer Components section below).

Vial 14 2X Formamide Based-Hybridization Buffer (see Buffer Components section below).

Store Vials 1-14 at -20°C in the dark. Store Vial 1 may be kept at 4°C for short-term storage (~1 week).

Note: The **LNA dT blocker** (Vial 9b) is a new, high-performance poly T based blocking reagent designed by Genisphere (patent pending). It is designed to completely block **all** poly A containing elements including spotted 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.

Buffer Components and Selection

This protocol is designed for use with the 2X Hybridization Buffers (Vials 13 & 14).

2X SDS-Based Hybridization Buffer (Vial 13):	**2X Formamide-Based Hybridization Buffer (Vial 14):**
0.50M NaPO4	50% Formamide
1% SDS	8X SSC
2mM EDTA	1% SDS
2X SSC	4X Denhardt's Solution
4X Denhardt's Solution	
60-65°C hybridization	50-55°C hybridization

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 13) has given stronger signals on some arrays relative to the 2X Formamide-Based Hybridization Buffer (Vial 14), and vice versa. We recommend testing the hybridization buffers to determine which is best for your array type.

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 13 buffer. Use the Vial 14 buffer as directed if you experience this problem.

Hybridization at the temperatures recommended by this protocol may cause the morphology of spots to change on certain arrays. If this occurs, lower the hybridization temperature by using additional formamide in a buffer similar to the 2X Formamide-based Hybridization Buffer (above).

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) or at 2-fold by mass of poly(A)$^+$ RNA (i.e. use 100ng of C0T-1 DNA for 50µg of poly(A)$^+$ 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 buffer.

To avoid nonspecific hybridization of 3DNA-labeled cDNA to elements on the array containing poly A sequences, 500-1000 ng (2-4µl) of the oligo dT blocking reagent (Vial 9) or 2µl of the LNA dT blocking reagent (Vial 9b) should be added to the hybridization mixture as directed prior to applying it to the microarray. **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.**

Other Materials Required

Critical materials required for successful use of this kit include:

- **Genisphere SCL Spin Columns (10 pack: Cat No. A100620, 50 pack: Cat No. A100625)**
- Microarray: Commercial or in-house
- Microarray reader equipped to read Cy3™ /Alexa Fluor 546, Cy5™ /Alexa Fluor 647, Alexa Fluor 488™ and/or Alexa Fluor 594™ fluorochrome
- Total RNA Sample (greater than or equal to 0.25µg/µl)
- C0T-1 DNA (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)
- 3M ammonium acetate or 5M NaCl
- 100% ethanol
- 70% ethanol in reagent grade water (v/v)
- 0.2% SDS (w/v) in reagent grade deionized distilled water
- 2X SSC, 0.2% SDS buffer
- 2X SSC buffer
- 0.2X SSC buffer
- Glass coverslips (Corning Brand distributed by Fisher or VWR) or Lifter Slips (Erie Scientific)
- RNase-free 1.5mL tubes with caps (Ambion Cat # 12450)
- Optional: Millipore Microcon® YM-30 Microconcentrators (30,000 molecular weight cutoff, Millipore catalog number 42409).

PROCEDURE FOR USE

The protocol below summarizes the steps required to use 3DNA fluorescent reagents 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.

Genisphere recommends use of certain aminosilane coated slides for spotting cDNAs, particularly Clontech DNA-Ready Type II, Corning GAPS II and Telechem SuperAmine slides. These slides demonstrate good cDNA binding and exhibit low background without BSA prehybridization (see below) when used with Submicro kits.

Arrays prepared on poly-L-lysine, aldehyde or certain aminosilane (e.g., Corning GAPS) surfaces may require a prehybridization in 5X SCC, 0.1% SDS, 1% BSA (fraction V) to reduce the background observed after hybridization. The prehybridization should be performed at 50°C for 30 minutes, followed by a 3-minute wash at room temperature in 2X SSC, and then a 3-minute wash at room temperature in 0.2X SSC. Finally, the array should be dried. The amount of BSA required will depend on the quality of the BSA. Some BSA sources may not be suitable for this application and may produce higher background. Ambion Ultra Pure BSA (Non-Acetylated), Cat. No. 2616, has been used successfully at a final concentration of 0.1%. Increasing the post-hybridization wash times from 10 minutes each to 15 minutes each (see below) 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.

If you are using arrays made with spotted oligonucleotides (from MWG Biotech, Mergen, Clontech, etc.) you should label your samples with Genisphere's 3DNA® Submicro™ Oligo labeling kits. Please call Genisphere Technical Support at 877.888.3DNA for more information.

RNA Preparation:

The Submicro EX kit typically performs best when used to label total RNA samples. If poly(A)$^+$ RNA samples are used, sensitivity may be reduced because oligo dT sequences, which are part of the binding media used to purify poly(A)$^+$ RNA, may be present as a contaminant in the purified poly(A)$^+$ material. These oligo dT sequences act as a primer in the subsequent RT reaction (in competition with the special "capture sequence" primer included in the kit) and produce cDNA molecules that do not contain capture sequence, cannot bind to fluorescent 3DNA molecules, and therefore cannot generate a signal. Although the kit has been designed to minimize this phenomenon by providing a high concentration capture sequence primer (Vial 2), it cannot be eliminated entirely.

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.

- The use of an RNase inhibitor (Superase-In™, Vial 12) 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.

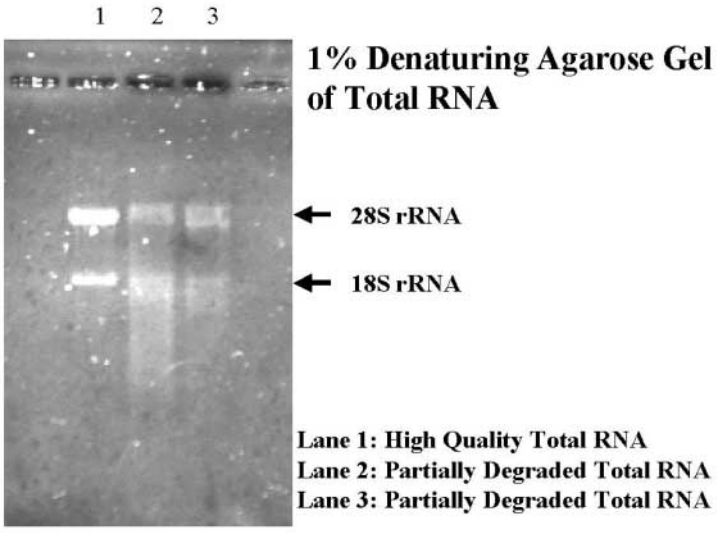
Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., 1998.

- The Submicro™ 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 remains 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 either total or poly(A)$^+$ RNA. Since microarrays and RNA preparations vary in quality, the exact amount of RNA required for a particular experiment will range from <1μg of mammalian total RNA or <2.5μg of plant total RNA (or <50ng of poly(A)$^+$ RNA) to 5μg of mammalian total RNA or 12.5μg of plant total RNA (or 250ng of poly(A)$^+$ RNA). If a very small sample does not produce an adequate signal with an overnight hybridization, the signal can be improved by increasing the hybridization time to 30-60 hours, which allows for better cDNA hybridization to the features on the array.

The cDNA synthesis procedure below recommends either a Millipore Microcon® YM-30 Microconcentrator or an ethanol precipitation step to concentrate the cDNA prior to hybridization. This cDNA concentration step can be avoided by starting with 20-25μg of total RNA or 1000-1250ng of poly(A)$^+$ RNA and by following one of the alternate protocols outlined in Appendix A and Appendix B. These alternate protocols maintain the ~1-5 μg of input total RNA or 50-250 ng of input poly(A)$^+$ RNA for the hybridization step by using only a portion of the cDNA generated during the reverse transcription step for the subsequent hybridization to the array (see Appendix A and/or Appendix B). In addition to avoiding the cDNA concentration step, the alternate protocol of Appendix B (for use only with total RNA samples) also avoids the spin column step for removing the excess RT primer after the cDNA synthesis. This is accomplished by using less RT primer for the reverse transcription reaction (see Appendix B).

Since some applications require the use of sequence specific primers, the "capture sequence" portions of the Cy3™ /Alexa Fluor 546, Cy5™ /Alexa Fluor 647, Alexa Fluor 594 ™ , and Alexa Fluor 488™ RT primers are provided below. When designing custom primers these sequences should be attached to the 5' end of the corresponding oligonucleotide primer. To use a custom primer with the reagents in this kit substitute the custom primer for the respective RT primer at a concentration of 5 pmole/μl and proceed according to the standard protocol. If more than one custom primer will be used, be sure that the final concentration of all primers (combined) does not exceed 5 pmole/μl. Also, take into account the level of expression (low, medium, or high) of each message when setting the final concentration of each primer in the mix. Since the protocol has been designed for use with the enclosed primer, some optimization may be required when substituting a custom primer.

Cy3™ /Alexa Fluor 546 RT primer capture sequence: 5'- ggC CgA CTC ACT gCg CgT CTT CTg TCC CgC C -3'

Cy5™ /Alexa Fluor 647 RT primer capture sequence: 5'- CCT gTT gCT CTA TTT CCC gTg CCg CTC Cgg T -3'

Alexa Fluor 594 ™ primer capture sequence: 5'- gAC gAC AgA TCg ggg ggC TAg TgC TTT CAT g -3'

Alexa Fluor 488™ primer capture sequence: 5'- TTC TCg TgT TCC gTT TgT ACT CTA Agg Tgg A -3'

cDNA Synthesis:

Note: The RT enzyme (Vial 3) included in the kit may not be suitable for some RNA samples - - particularly certain plant RNA preparations - - including arabidopsis, corn and drosophila. When labeling such samples, we recommend replacement of the kit RT enzyme with SuperScript II or equivalent RNase H minus mutant enzyme. Also, if you are already using an RT enzyme that is working well with your samples, you may wish to continue using that enzyme and purchase Submicro kits that do not contain enzyme.

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.

1. In a RNase-free 1.5 mL tube combine:

   1-9μl total RNA (<1-5μg mammalian total RNA or <2.5-12.5μg plant total RNA or <50-250ng of mammalian or plant poly(A)+ RNA).
   1μl RT primer (5 Pmole) (Vial 2).
   Add RNase free water to a final volume of 10μl.

   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.

4. Add 1µl Superase-In™ (Vial 12).

5a. In a separate microtube combine (on ice):
4µl 5X RT buffer (Vial 5).
1µl dNTP mix (Vial 4).
3µl RNase free water.
1µl (200 Units) reverse transcriptase enzyme (Vial 3). The reverse transcriptase enzyme should be kept on ice during use to avoid loss of activity.
This is the **reaction mix.** The final volume should be 9µl. Keep on ice until used.

5b. Alternatively, if you purchased a kit that does not contain Vials 3, 4 and 5 (enzyme, dNTPs and enzyme buffer) we recommend the use of SuperScript II Reverse Transcriptase Enzyme (Gibco Cat No. 18064-014 – 10,000 Units @ 200U/ul). The following reaction mix should be used:

4µl 5X SuperScript II RT buffer (supplied with enzyme).
1µl dNTP mix (10mM each for dATP, dCTP, dGTP, dTTP).
2µl 0.1M dithiotreitol (DTT) (supplied with enzyme).
1µl Superscript II enzyme, 200 units.
1µl RNase free water.

This is the **reaction mix.** The final volume should be 9µl. Keep on ice until used.

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

7. Add the 9µl of **reaction mix** from step 5 to the 11µl 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/50 mM EDTA.

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

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

12. For **dual**, **three or four** channel expression analysis proceed to step 13. For single channel assays add 71µl of 10 mM Tris, pH 8.0, 1 mM EDTA and proceed to "Removal of Excess RT Primer via the SCL Spin Column", p. 12.

13. For **dual** channel assays, rinse the one empty tube with 42µl of 1X TE Buffer Add the rinse to the combined cDNA samples. For **three** channel assays, rinse the two empty tubes with a total of 15µl of 1X TE Buffer by transferring rinse from one tube to the next. Add the rinse to the to the combined cDNA samples. For **four** channel assays, rinse the three empty tubes with a total of 16µl of 1X TE Buffer by transferring rinse from one tube to the next. Add the rinse to the combined cDNA samples. Proceed to "Removal of Excess RT Primer via the SCL Spin Column", p. 12.

cDNA Purification: Removal of Excess RT Primer via the SCL Spin Column

The media in the spin columns is a size exclusion resin. The resin pores are large enough to capture the RT primer, but the cDNA molecules will pass in the void volume. The columns are easy to use and will function properly if 1) a swinging bucket clinical centrifuge is used, 2) the pre-spin is done correctly, 3) the sample is loaded directly onto the top of the media, and 4) the recommended centrifugation times and g forces are used. **Do not** use a fixed angle rotor for this application.

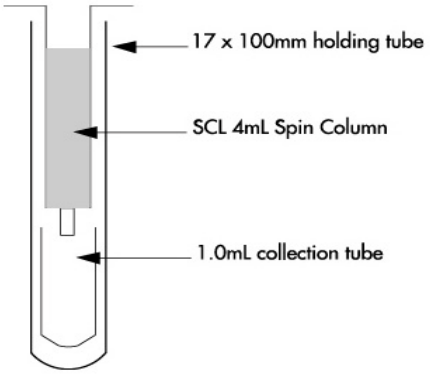
Prepare the spin column by using the following steps to remove the excess buffer:

1. Invert or gently vortex to completely re-suspend the media and create an even slurry in the column.
2. Remove the top cap and then the bottom cap.
3. Place the SCL spin column into the 17x100mm holding tube provided with the columns.
4. Centrifuge at 1,000g for 3.5 minutes.
5. Make sure the column is fully drained after centrifugation. The 17x100mm holding tube should contain about 2-2.5mLs of clear buffer voided from the spin column. The resin will appear nearly dry in the column barrel, well packed without distortions or cracks. Discard the column if the resin is cracked or disturbed in any way, or if the voided buffer volume is less or more than 2-2.5mLs (1.5-2.0cm from the bottom of the tube to the top of the buffer). Also discard the column if there is substantial leakage of the resin into the holding tube (>10% of the column resin).

Now use the spin column to remove the excess primer:

6. Remove the drained spin column and discard the voided buffer. Place the provided 1.0 mL collection tube into the 17x100mm holding tube. Place the drained spin column on top of the fresh collection tube (see schematic below).
7. Load all of the cDNA mixture (100µl) directly into the center of the column media.
8. Centrifuge at 1000g for 2.5 minutes.
9. Save the eluate by transferring the entire volume into a 1.5 ml microcentrifuge tube. The recovered volume should be 90-110µl. THIS IS YOUR cDNA PROBE!

If there is more than 110µl of eluate from the spin column, then your cDNA probe will contain excess buffer eluted from the spin column. This will not affect the quality of your cDNA probe; however, the amount of ammonium acetate and ethanol used for precipitation of the cDNA should be increased proportionally to compensate for the excess diluent buffer from the spin column. (Note: If you are following the **four** channel analysis protocol of Appendix A your final volume will range between **120-140µl**).



Concentration of cDNA:

Two methods are provided below for concentrating the cDNA. The cDNA must be concentrated before it 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. Microconcentration 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® microconcentrators

cDNA samples may be concentrated using the Millipore Microcon® YM-30 Microconcentrators (30,000 molecular weight cutoff, Millipore catalog number 42409). These devices are capable of reducing the volume of the cDNA synthesis reaction from 100-130µl to 3-10µl in as little as 8-10 minutes. The procedure below reiterates the manufacturer's directions with minor adaptations for the 3DNA Submicro Expression Array Detection Kit.

Important: users of the microconcentrators 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 sample reservoir into the 1.5mL collection tube.
2. Pre-wash the reservoir membrane by adding 100µl TE pH 8.0 to the microconcentrator 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 100-130µl from the cDNA reaction to the microconcentrator sample reservoir. Do not touch the membrane with the pipet tip.
6. Centrifuge for 8-10 minutes at 10-14,000g.
7. Remove the tube/sample reservoir assembly. Separate the collection tube from the sample reservoir with care, avoiding spilling any liquid in the sample reservoir.
8. Add 5µl of 1X TE buffer (10 mM Tris-HCl, pH 8.0 / 1 mM 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.
9. Carefully place the sample reservoir upside down on a new collection tube. Centrifuge for 30 seconds 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 (3-10µl 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 Using Vial 13 or Vial 14 Hybridization Buffers (2-Step Protocol)", p.15.

Ethanol Precipitation of the cDNA

The ethanol precipitation step (below) may lead to variable microarry results if not performed carefully because reverse transcription of microgram 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 11) 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 11) by vortexing for several seconds.
2. Add 3µl of 5.0mg/ml linear acrylamide (Vial 11) to the combined cDNA to act as a co-precipitant.
3. Add 250µl 3M ammonium acetate and mix (or 6µl 5M NaCl and mix).
4. Add 875µl of 100% ethanol (or if using 5M NaCl add 250µl of 100% Ethanol).
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.

Proceed to “Successive Hybridization of cDNA and 3DNA to Microarray Using Vial 13 or Vial 14 Hybridization Buffers (2-Step Protocol)”, p.15.

Successive Hybridization of cDNA and 3DNA to Microarray (2-Step Protocol) Using Vial 13 or Vial 14 Hybridization Buffers

The cDNA and 3DNA hybridization conditions using either the Genisphere 2X SDS-Based Hybridization Buffer (Vial 13) or the 2X Formamide-Based Hybridization Buffer (Vial 14) provided in the kit are described below. If you are using a type of slide or array for which a different buffer is specifically recommended, you can use it in place of those provided in the kit for the cDNA hybridization step. It may also be possible to use a different buffer in the 3DNA hybridization step, but we recommend that you first try a "negative" control experiment with no cDNA to confirm the compatibility of the buffer, as well as the appropriate temperature for its use, for this hybridization. Typically, when the same buffer is used for both the cDNA and 3DNA hybridization steps, the hybridization temperatures used in the two hybridizations should be about the same.

Note on Hybridization Temperatures:

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.

cDNA Hybridization:

1. 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 13)

2. Thaw and resuspend the Hybridization Buffer (Vial 13) by heating to 65°C for 10 minutes. Mix by inversion to insure that the components are resuspended evenly. If necessary repeat heating and mixing until all the material has been resuspended.

2X Formamide-Based Hybridization Buffer (Vial 14)

2. Thaw and resuspend the Alternate Hybridization Buffer (Vial 14) by heating to 55°C for 10 minutes. Mix by inversion to insure that the components are resuspended evenly. If necessary repeat heating and mixing until all the material has been resuspended.

3. Included in the kit are two reagents unique to the Submicro kit that will improve the quality of your cDNA hybridization. Thaw the reagents listed below and refreeze after use:

   - **Oligo dT Blocking Reagent (Vial 9)** blocks the nonspecific hybridization of labeled cDNA to elements containing poly dA sequences. **$PolyA_{40-80}$** should **not** be added as a blocker because it interacts with the 3DNA reagents and can result in the precipitation of the sample.

   - **LNA dT Blocker (Vial 9b)** is a new, high performance poly T based blocking reagent designed by Genisphere (patent pending). It is designed to completely block **all** poly A containing elements including spotted 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.

4a. If the cDNA sample was concentrated using the Millipore Microcon® YM-30 Microconcentrator, add RNase-free deionized distilled water to the cDNA preparation to achieve a total volume of 10µl.

4b. If the cDNA sample was concentrated using ethanol precipitation, add RNase-free deionized distilled water to the cDNA preparation to achieve a total volume of 10µl. However, for smaller hybridization volumes (~20µl), the cDNA should be resuspended directly into hybridization buffer, to avoid excessive dilution of the buffer.

a. Heat the mixture of cDNA and water or buffer for 5-10 minutes at 65°C.

b. 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!

c. Repeat the heating and mixing steps an additional two times to insure the cDNA pellet is completely resuspended. If the cDNA is not completely resuspended, high background and low signal will be observed on the array.

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

Desired Final Hybridizaton Mix Volume:	25µl	30µl	35µl	40µl	45µl	50µl
Concentrated cDNA (from step 4a/4b or Appendix A or B protocols)	10µl	10µl	10µl	10µl	10µl	10µl
2X Hybridization Buffer (Vial 13 or 14)	12.5µl	15µl	17.5µl	20µl	22.5µl	25µl
dT Blocking Reagent (Vial 9 or 9b)	2µl	2µl	2µl	2µl	2µl	2µl
Nuclease-free water	0.5µl	3µl	5.5µl	8µl	10.5µl	13µl

This is your Hybridization Mix.

Optional: 1.0µl COT-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% additional volume.
22 x 30mm	25-30µl	
22 x 40mm	30-40µl	
24 x 50mm	35-45µl	
24 x 60mm	40-50µl	

Hybridization volumes greater than 50µl may require additional cDNA and/or 3DNA reagents.

2X SDS-Based Hybridization Buffer (Vial 13)

6. Incubate the Hybridization Mix first at 75-80°C for 10 minutes, and then at 55-60°C for 15-20 minutes.

2X Formamide-Based Hybridization Buffer (Vial 14)

6. Incubate the Hybridization Mix first at 75-80°C for 10 minutes, and then at 45-50°C for 15-20 minutes.

7. Add the Hybridization Mix to a pre-warmed microarray. (Pre-warming the microarray to the hybridization temperature may reduce background.)

2X SDS-Based Hybridization Buffer (Vial 13)

8. Apply a washed glass coverslip (from Step 1) to the array and incubate overnight in a dark humidified chamber at 60-65°C.

2X Formamide-Based Hybridization Buffer (Vial 14)

8. Apply a washed glass coverslip (from Step 1) to the array and incubate overnight in a dark humidified chamber at 50-55°C.

Note: If drying of the slide occurs then increase the hybridization volume by 10-15% using additional hybridization buffer or switch to the 2X Formamide-Based Hybridization Buffer (Vial 14).

Post cDNA Hybridization Wash:

After hybridization the slides are washed briefly several times to remove unbound cDNA/3DNA molecules.

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

2. Wash for 10 minutes at 55-65°C with 2X SSC, 0.2%SDS.

3. Wash for 10 minutes at room temperature with 2X SSC.

4. Wash for 10 minutes at room temperature with 0.2X SSC.

5. Wash for 2 minutes at room temperature in 95% ethanol to fix the cDNA molecules to the probes.

6. 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.

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. 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 13)</u>

2. Thaw and resuspend the Hybridization Buffer (Vial 13) by heating to 65°C for 10 minutes. Mix by inversion to insure that the components are resuspended evenly. If necessary repeat heating and mixing until all the material has been resuspended.

<u>2X Formamide-Based Hybridization Buffer (Vial 14)</u>

2. Thaw and resuspend the Alternate Hybridization Buffer (Vial 14) by heating to 55°C for 10 minutes. Mix by inversion to insure that the components are resuspended evenly. If necessary repeat heating and mixing until all the material has been resuspended.

3. Included in the kit are two reagents unique to the Submicro kit that will improve your signal and differential. Thaw the reagents listed below and refreeze after use:

   - **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 Hybridization Buffer. 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.

   - **High-End Differential Enhancer (Vial 10)** increases the differential between labeled samples run on the same array, especially for differentials above 10-fold. It is not necessary to use this reagent when performing single-channel experiments.

4. 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 (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 "a-f" if necessary.

5. To a fresh tube, add together the following 3DNA Submicro Expression Array Detection kit components to make the Hybridization Mix. Please note the table below for determining the Final Hybridization Volume.

Desired Final Hybridizaton Mix Volume:	25µl	30µl	35µl	40µl	45µl	50µl
Hybridization Buffer (Vial 13 or 14)	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
High-End Differential Enhancer (Vial 10)	1µl	1µl	1µl	1µl	1µl	1µl
Nuclease Free Water	6.5µl	9µl	11.5µl	14µl	16.5µl	19µl

This is your Hybridization Mix.

Optional: 1.0µl COT-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 in place of the second 3DNA Capture Reagent. For three or four channel expression analysis, add 2.5µl of each additional 3DNA Capture Reagent as required and decrease the amount of Nuclease Free Water added accordingly.

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	

Hybridization volumes greater than 50µl may require additional cDNA and/or 3DNA reagents.

2X SDS-Based Hybridization Buffer (Vial 13)

6. Incubate the Hybridization Mix first at 75-80°C for 10 minutes, and then at 55-60°C for 15-20 minutes.

2X Formamide-Based Hybridization Buffer (Vial 14)

6. Incubate the Hybridization Mix first at 75-80°C for 10 minutes, and then at 45-50°C for 15-20 minutes.

7. Pre-warm the microarray to 55-65°C for 10-15 minutes.

8. Remove the microarray 1 minute prior to adding the 3DNA hybridization mixture.

9. Add the Hybridization Mix to the pre-warmed microarray.

2X SDS-Based Hybridization Buffer (Vial 13)

10. Apply a washed glass coverslip (from Step 1) to the array and incubate 2-3 hours in a dark humidified chamber at 60-65°C.

2X Formamide-Based Hybridization Buffer (Vial 14)

10. Apply a washed glass coverslip (from Step 1) to the array and incubate 2-3 hours in a dark humidified chamber at 50-55°C.

Note: If drying of the slide occurs then increase the hybridization volume by 10-15% using additional hybridization buffer or switch to the 2X Formamide-Based Hybridization Buffer (Vial 14).

Post 3DNA Hybridization Wash:

After hybridization the slides are washed briefly several times to remove unbound cDNA/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 0.5-1 mM. 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 shorter wavelength channels, Alexa Fluor 488 and Cy3/Alexa Fluor 546.

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

2. Wash for 10 minutes at 60-65°C with 2X SSC, 0.2%SDS.

3. Wash for 10 minutes at room temperature with 2X SSC.

4. Wash for 10 minutes at room temperature with 0.2X SSC.

5. 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. 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" protocol, p. 20.

Signal Detection:

IMPORTANT: Store the array in the dark until scanned. The fluorescence of the 3DNA reagents, especially Cy5, can diminish rapidly even in ambient light because of oxidation. Please refer to Appendix D for recommendations for reducing the degradation of Cy5 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, after a single scan, avoid setting the laser too high (>90-95% power). Please consult the instrument's user manual for further instructions. Alternatively, additional RNA may be used for cDNA synthesis to compensate for a weaker signal in any one channel.

Initial Scanner Setting for Packard ScanArray 5000

Dye	Laser	PMT
Alexa Fluor 488	80	75 +/-5
Cy3/Alexa Fluor 546	90	65 +/-5
Alexa Fluor 594	80	80 +/-5
Cy5/Alexa Fluor 647	80	74 +/-5

Note that Alexa Fluor 594 demonstrates about 3-6% "bleedover" into the Cy3/Alexa Fluor 546 channel when scanned at the settings listed above. This is a consequence of the chemical structure of the Alexa Fluor 594 dye and does not imply inappropriate performance of the product. It is important to compensate for this phenomenon when calculating the specific signal generated by Cy3-labeled cDNA on your arrays by subtracting the bleedover value of the Alexa Fluor 594 in the Cy3 channel from the total Cy3 signal measured for Cy3-labeled samples. The bleedover of the Alexa Fluor 594 dye into the Cy3 channel is proportional to the setting of the PMT on the Packard ScanArray 5000, with higher PMT settings generating higher bleedover artifact. We recommend testing the Alexa Fluor 594-labeled 3DNA, run by itself on an array, to determine the percentage bleedover in the Cy3 channel on your scanner; this will provide a baseline of bleedover percentage that may then be used for calculating the true Cy3 signal on your arrays.

REFERENCES

1. Nilsen, T.W., Grayzel, J., and Prensky, W. Dendritic Nucleic Acid Structures. J. Theor. Biol. (1997) 187: 273-284.
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, 2000.
3. Singh, S.K., Nielsen, P., Koshkin, A. A., and Wengel, J. LNA (Locked Nucleic Acids): Synthesis and high-affinity nucleic acid recognition. Chem. Commun., 455-456, 1998.

ACKNOWLEDGEMENT

Genisphere gratefully acknowledges the contributions of Robin L. Stears, Ph.D., and Steven R. Gullans, Ph.D., both of Brigham & Women's Hospital, who played a major role in the development of 3DNA Expression Array Detection reagents.

For Research Use Only

Genisphere Inc.
2801 Sterling Drive
Hatfield, PA 19440

To Order:

Tel: 877.888.3DNA
201.651.3100
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

Alternate cDNA Preparation/Concentration Protocol: Scale-up with SCL Spin Column

When your RNA is not limited to quantities less than 10-50µg of total RNA, 25-125µg plant total RNA or 500-2500ng of poly(A)$^+$, ethanol precipitation is not required because the cDNA is concentrated enough to perform the array hybridization. The excess material can be used for duplicate experiments, quantitation of the cDNA, or other parallel analysis.

cDNA Synthesis:

1. In a RNase-free 1.5 mL tube combine:

   1-9µl total RNA (10-50µg mammalian total RNA, 25-125µg plant total RNA or 500-2500ng poly(A)$^+$).
   1µl RT primer (5 pmole) (Vial 2).
   Add RNase free water to a final volume of 10µl.

   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.

4. Add 1µl of Superase-In™ (Vial 12).

5. In a separate microtube combine (on ice):
   4µl 5X RT buffer (Vial 5).
   1µl dNTP mix (Vial 4).
   3µl RNase free water.
   1µl (200 Units) reverse transcriptase enzyme (Vial 3). The reverse transcriptase enzyme should be kept on ice during use to avoid loss of activity.

   This is the **reaction mix.** The final volume should be 9µl. Keep on ice until used.

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

7. Add the 9µl of **reaction mix** from step 5 to the 11µl 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/50 mM EDTA.

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

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

12. For **dual**, **three** or **four** channel expression analysis proceed to step 13. For single channel assays add 71µl of 10 mM Tris, pH 8.0, 1 mM EDTA (~100µl final volume) and proceed to "Removal of Excess RT Primer via the SCL Spin Column", p.12. After following the SCL Spin Column procedure proceed to step 14, p.23.

13. For **dual** channel assays, combine the cDNAs in one tube, and rinse the one empty tube with 42µl of 1X TE Buffer. Add the rinse to the combined cDNA samples (~100µl final volume).

    For **three** channel assays, combine the cDNAs in one tube, and rinse the two empty tubes with a total of 15µl of 1X TE Buffer by transferring the rinse from one tube to the next. Add the rinse to the combined cDNA samples (~100µl final volume).

For **four** channel assays, combine the cDNAs in one tube, and rinse the three empty tubes with a total of 16µl of 1X TE Buffer by transferring the rinse from one tube to the next. Add the rinse to the combined cDNA samples (~130µl final volume).

Proceed to "Removal of Excess RT Primer via the SCL Spin Column", p.12. After following the SCL Spin Column procedure proceed to step 14, below.

14. Transfer into a new microfuge tube up to 10µl for single, dual or three channel analysis or up to 13µl for four channel analysis (the equivalent of ~1-5µg mammalian total RNA, 2.5-12.5µg plant total RNA, or 50-250ng of poly(A)$^+$ RNA) of the material eluted from the spin column, which is your cDNA. (For example, if you start with a 40µg total RNA sample and use 5µl of your 100µl cDNA, you will be using the equivalent of 2µg of total RNA in your hybridization.) For single, dual, or three channel analysis add nuclease free water to a final volume of 10µl if necessary. For four channel analysis add nuclease free water to a final volume of 13µl if necessary. (Note that a 10µl final volume is preferred.)

    Larger volumes of the cDNA mixture may be used if necessary or 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 Using Vial 13 or Vial 14 Hybridization Buffers (2-Step Protocol)", p.15.

Appendix B

Alternate cDNA Preparation/Concentration Protocol: Scale-up without SCL Spin Column

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. This scaled-up protocol eliminates the spin column procedure for the removal of excess primer as well as the ethanol precipitation procedure. The resulting extra cDNA can be used for duplicate experiments, quantitation of the cDNA, or other parallel analysis.

cDNA Synthesis:

1. Prepare a 1/5 dilution of the RT primer by combining 2µl of RT primer (Vial 2) with 8µl of RNase free reagent grade deionized distilled water. Mix.

2. In a 1.5mL RNase-free microcentrifuge tube combine:

   1-9µl total RNA (10-50µg mammalian total RNA or 25-125µg plant total RNA).
   1µl DILUTED RT primer (1 pmole) from step 1.
   Add RNase free water to a final volume of 10µl.

   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.

5. Add 1µl Superase-In™ (Vial 12).

6. In a separate microtube combine (on ice):
   4µl 5X RT buffer (Vial 5).
   1µl dNTP mix (Vial 4).
   3µl RNase free water.
   1µl (200 Units) reverse transcriptase enzyme (Vial 3). The reverse transcriptase enzyme should be kept on ice during use to avoid loss of activity.

   This is the **reaction mix.** The final volume should be 9µl. 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 9µl of **reaction mix** from step 5 to the 11µl 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.

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

13. For **dual**, **three or four** 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 58µl. Proceed to step 15, p. 25.

14. For **dual**, **three or four** channel expression analysis combine the cDNA preparations from step 12 into one tube. The final volume of the cDNA solution will be 59µl, 88.5µl or 118µl, respectively.

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 59µl cDNA solution (in a dual channel analysis experiment), you will be using the equivalent of 2µg of total RNA in your hybridization.) If necessary, add nuclease free water to a final volume of 10µl.

    Larger volumes of the cDNA mixture may be used if necessary or 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 Using Vial 13 or Vial 14 Hybridization Buffers (2-Step Protocol)", p.15.

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

Recommendations for Reducing the Degradation of Cy5 When Performing Microarray Experiments

Cy5 dye performance may be affected by a variety of factors that are particularly prevalent during the summer months. Exposure of the Cy5 dye solutions and the hybridized arrays to light and to oxidative environments may cause rapid fading of the Cy5 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 fading.

Below are recommendations for reducing the degradation of Cy5 when performing microarray experiments:

1. Always keep solutions and arrays containing Cy5 away from light, particularly sunlight! Cy5 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 of Cy5. Keeping the Cy5-containing arrays in an inert atmosphere (nitrogen) in a small container (50mL tube) can significantly delay fading of the Cy5. 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 any Cy5 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 on the array from attack by any oxidative agents in the wash buffers.

In addition, always be certain to mix your 3DNA Cy3 and Cy5 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.