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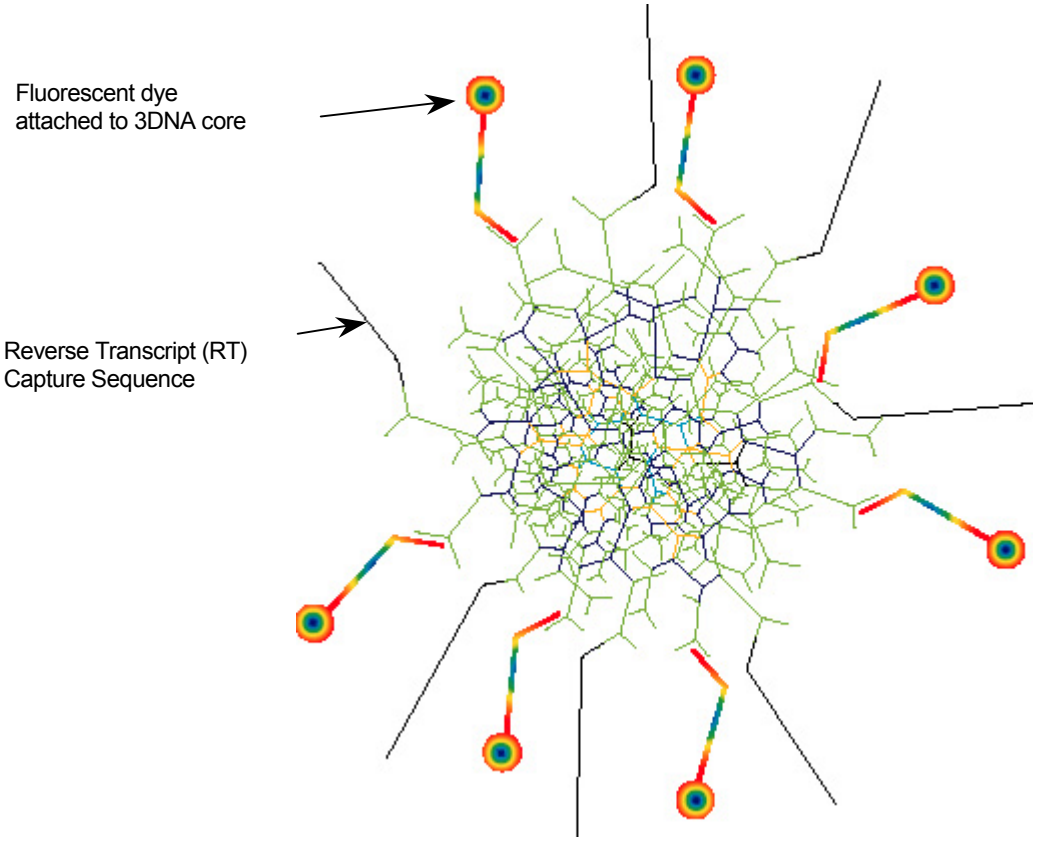

3DNA® Submicro™ Expression Array Detection Kit
Protocol for Agilent cDNA Arrays

3DNA DNA Dendrimer Probes

Genisphere Inc.
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Hatfield, PA 19440

Protocol Specific for Agilent cDNA Arrays

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 Fluor 488 (green cap) (0.067 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. Do not use for Agilent Arrays, see Buffer Components section (below).
Vial 7	Alternate Hybridization Buffer. Do not use for Agilent Arrays, see Buffer Components section (below).
Vial 8	Anti-Fade Reagent.
Vial 9	Oligo dT Blocking Reagent (250 ng/µl) (not included in the Submicro trial kits).
Vial 9b	LNA™ dT Blocker .
Vial 10	High-End Differential Enhancer.
Vial 11	Linear Acrylamide (co-precipitant).
Vial 12	Superase-In™ RNase inhibitor.

Store Vials 1-12 at –20°C in the dark. Vial 1 may be kept at 4ºC 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
(Contact Genisphere for the 2X Formamide Based Hybridization Buffer described below).

This protocol requires that the cDNA be hybridized to the array overnight, and that the fluorescent 3DNA reagent then be hybridized to the array in a second, 2-3 hour hybridization. The overnight cDNA hybridization should be performed using the Agilent 2X Deposition Hybridization Buffer only. The 2-3 hour 3DNA hybridization should be performed using the Genisphere 2X Formamide Based Buffer only (available free of charge from Genisphere Technical Support). **The Vial 6 and Vial 7 buffers included in the kit should not be used with Agilent arrays.**

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 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:

- Agilent Microarray and 2X Depositon Hybridization Buffer.
- 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, pH7.5
- 10 mM Tris-HCl, pH 8.0 / 1 mM EDTA (1X TE Buffer)
- 3M ammonium acetate
- 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)

PROCEDURE FOR USE

The protocol below summarizes the steps required to use 3DNA fluorescent reagents for gene expression array detection.

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.

- 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:

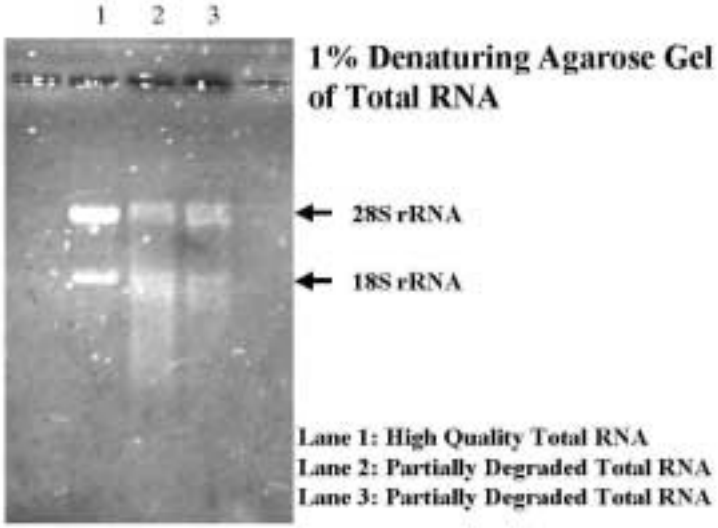
  Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning, A Laboratory Manual (Second Edition) Cold Spring Harbor Laboratory Press, 1989.
  Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. Current Protocols in Molecular Biology. John Wiley & Sons,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

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/RNApurification.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, we recommend that you do not use this kit; instead, use the 3DNA Submicro EX kit.) Since microarrays and RNA preparations vary in quality, the exact amount of RNA required for a given experiment will range from <1μg of mammalian total RNA or <2.5μg of plant total RNA to 5μg of mammalian total RNA or 12.5μg of plant total RNA

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 0.067 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 0.067pmole/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.

1. In a microtube combine:

   <1-5μg mammalian total RNA or <2.5-12.5μg plant total RNA (1-7μl).
   3μl RT primer (0.2pmole) (Vial 2).
   Add RNase free water to a final volume of 10μl.

   This is the **RNA-RT primer mix**.

   Note: When using the 2-step hybridization procedure additional primer may be used in the RT reaction because excess primer will be removed during the washing steps after the cDNA hybridization and before the 3DNA hybridization (see Hybridization Procedure below).

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™ RNase inhibitor (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 stored 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:

4 ul 5X SuperScript II RT buffer (supplied with enzyme)
1 ul dNTP mix (10mM each for dATP, dCTP, dGTP, dTTP)
2 ul 0.1M dithiotreitol (DTT) (supplied with enzyme)
1 ul 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 reaction mix 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 38.5µl of 10 mM Tris, pH 8.0, 1 mM EDTA and proceed to step 14.

13. For **dual**, **three, or four** channel expression analysis combine the cDNAs from step 10 into one tube. Rinse the original tubes with 10µl of 10 mM Tris, pH 8.0, 1 mM EDTA. Combine wash with the cDNA mixtures.

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.

Note: Care should be taken when using microconcentrators since cDNA may be lost due to adherence to the vessel. **Also, evaporative drying of the RT reaction product (synthesized cDNA) is NOT recommended, as a dried sample may result in lower signal and/or higher background on the array.**

A. Thoroughly mix the linear acrylamide (Vial 11) by vortexing for several seconds.

B. Add 3µl of 5.0mg/ml linear acrylamide (Vial 11) to the combined cDNA to act as a co-precipitant.

C. Add 250µl 3M ammonium acetate and mix.

D. Add 875µl of 100% ethanol.

E. Incubate at –20°C for 30 minutes.

F. Centrifuge the sample at >10,000g for 15 minutes.

G. 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.

H. 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.

I. Centrifuge at >10,000g for 5 minutes and remove the supernatant. **Do not decant.**

J. 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.

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

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.

Hybridization with 2X Agilent Deposition Hybridization Buffer

2. Thaw and resuspend the Agilent Deposition Hybridization Buffer 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.

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

   - **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.

4. Resuspend the cDNA in 5.5µl of reagent grade deionized distilled water.

   a. Heat the mixture of cDNA and water 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. Mix the cDNA hybridization components together:

5.5µl	resuspended cDNA in water
12.5µl	2X Agilent Deposition Hybridization Buffer
2.0µl	LNA dT Blocker (Vial 9b)
2.5µl	Water OR Deposition Control Targets (Operon)**
2.5µl	COT-1 DNA (must be denatured at 90-100°C for 10 minutes prior to use)
25µl	Total Volume **This is your Hybridization Mix.**

**** Use of the Deposition Control Targets (from Operon) may cause non-specific signal on the array.**

6. Incubate the Hybridization Mix at 98°C for 2 minutes. Allow to cool to room temperature.

7. Centrifuge for 5 minutes at maximum speed in a microfuge (~10,000Xg).

8. Clean a coverslip with a cannister air duster to eliminate any dust or debris.

9. Load 20uL of the Hybridization Mix to the array. Do not load any of the pellet from the bottom of the tube of the Hybridization Mix. Do not touch the array. Immediately place a 24x30mm coverslip on top of the array. Note that each slide contains two separate arrays. Using Agilents array template will allow correct placement of the coverslip over the array grid area.

10. Transfer to a dark humidified hybridization chamber and incubate at 65°C for no more than 17 hours.

11. Proceed to Post cDNA Hybridization Wash protocol below.

Post cDNA Hybridization Wash:

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

1. Remove coverslips by gently dipping the slide into 0.5X SSC, 0.01% SDS (Agilent Wash Solution 1). Do not pull off the coverslip; the coverslip should float off by itself with gentle agitation.

2. Transfer the slide into room temperature Wash Solution 1. Incubate with moderate stirring for 5 minutes.

3. Transfer to 0.06X SSC (Wash Solution 2) for 2 minutes at room temperature with moderate stirring.

4. Carry the immersed array to a large swinging bucket benchtop clinical centrifuge. Immediately transfer the array to a dry 50 mL centrifuge tube pre-loaded into the centrifuge. Centrifuge without the tube cap for 2 minutes at 1000 RPM to dry the slide. Do not touch the microarray surface.

5. Place array into a slide rack. Place the rack and array into a 53-55°C hybrization oven or incubator.

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.

2. Thaw Genisphere's 2X Formamide Based Hybridization Buffer at 65°C for 10 minutes. Vortex to mix.

3. Included in the kit is a reagent unique to the Submicro kit that will improve your signal and differential. Thaw the reagent listed below and refreeze after use:

   - **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. Mix the 3DNA hybridization components together:

12.5µl	Genisphere's 2X Formamide Based Hybridization buffer
2.5µl	of each 3DNA capture reagent (Vial 1) required (labeled with Cy3™/Alexa Fluor 546, Cy5™/Alexa Fluor 647, Alexa Fluor 488™, Alexa Fluor 594™).
1.0µl	High-End Differential Enhancer (Vial 10)
2.5µl	COT-1 DNA (must be denatured at 90-100°C for 10 minutes prior to use)
25µl	Total Volume **This is your Hybridization Mix.**

6. Incubate the Hybridization Mix at 55°C for 10 minutes.

7. Remove the microarray from the incubator just prior to adding the 3DNA hybridization mixture.

8. Add the Hybridization Mix to the pre-warmed microarray. Do not touch the microarray. Use the Agilent template for pipetting accuracy.

9. Incubate in a humidified dark hybridization chamber at 53-55°C for 3 hours.

10. Proceed to Post 3DNA Hybridization Wash protocol below.

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.

1. Remove coverslips by gently dipping the slide into 2.0X SSC, 0.01% SDS (Agilent Wash Solution 1). Do not pull off the coverslip; the coverslip should float off by itself with gentle agitation.

2. Wash for 15 minutes at 42°C with 2X SSC, 0.01%SDS. Do not agitate the slide.

3. Wash for 10 minutes at room temperature with 2X SSC. Do not agitate the slide.

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

5. Carry the immersed array to a large swinging bucket benchtop clinical centrifuge. Immediately transfer the array to a dry 50 mL centrifuge tube pre-loaded into the centrifuge. Centrifuge without the tube cap for 2 minutes at 1000 RPM to dry the slide. Do not touch the microarray surface.

Proceed to Signal Detection protocol below.

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.

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	80	83 +/-5
Alexa Fluor 594	90	65 +/-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

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