

FlashTag™

RNA Labeling Kit

Table of Contents	Page
Introduction	
Background Information	2
Procedure Overview	3
Components, Storage, and Handling	4
Other Required Materials	4
Microarrays	5
RNA Sample and Quantitation	5
Recommended: Enrichment and Concentration of LMW RNA Using YM-100 and YM-3 Columns	6
FlashTag RNA Labeling Procedure	
Poly (A) Tailing of RNA	7
FlashTag Ligation	7
Microarray Procedures	
NCode™ Multispecies miRNA Array (Invitrogen) or Non-commercially Printed Array	8
miRCURY® LNA Array (Exiqon)	9
CustomArray™ 4X2 MicroRNA Array (CombiMatrix)	10
References	12
Appendix A: Reducing Degradation of Oyster®-650	13

Introduction

Background Information

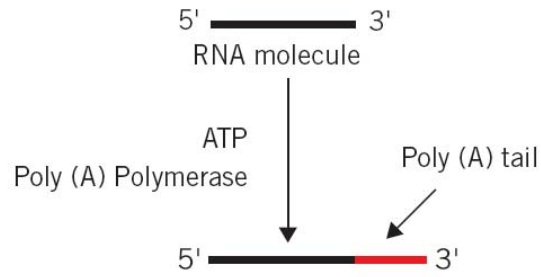
The FlashTag™ kit will label any RNA sample, including total RNA, senseRNA, severely degraded RNA, and low molecular weight RNA. This protocol describes labeling low molecular weight (LMW) RNA for microarray analysis. LMW RNA (snRNA, hnRNA, piRNA, miRNA, etc.) has recently been shown to be involved in important biological processes such as mRNA degradation, transcriptional gene silencing (TGS) and translational repression.¹⁻⁶ As a result, these newly discovered biomolecules are gaining the interest of the scientific community as possible new drug targets and for use in diagnostics. FlashTag provides the necessary tools to identify such targets.

FlashTag labeling is simple, accurate and highly sensitive. Starting with approximately 1µg of total RNA (or LMW RNA enriched from 1µg of total), the process begins with a brief tailing reaction followed by ligation of the signal molecule to the target RNA sample. Samples can be labeled with either Oyster®-550 (equivalent to Cy™3) or Oyster®-650 (equivalent to Cy™5). The labeling process is complete in less than one hour. For application to microarrays, the reactions are simply combined, brought up in hybridization buffer and applied to the array. FlashTag has been tested and is compatible with several commercially available array platforms (see “Microarrays” on page 5).

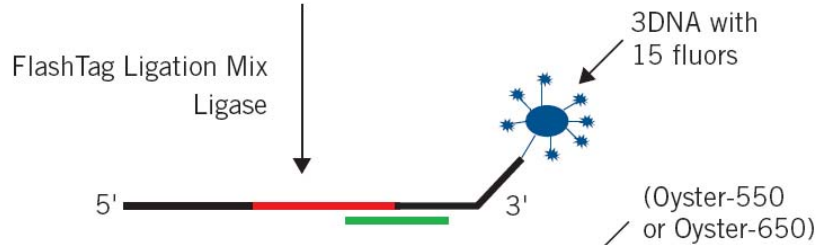
The high sensitivity of FlashTag is due to Genisphere's proprietary 3DNA dendrimer signal amplification technology. The 3DNA dendrimer is a branched structure of single and double stranded DNA conjugated with numerous fluorescent dyes.⁷⁻⁸ Whereas other labeling strategies typically target a single fluor to the sample, FlashTag's 3DNA molecule delivers approximately 15 fluors to the sample. Please refer to the procedural overview diagram on page 3.

FlashTag: Procedure Overview

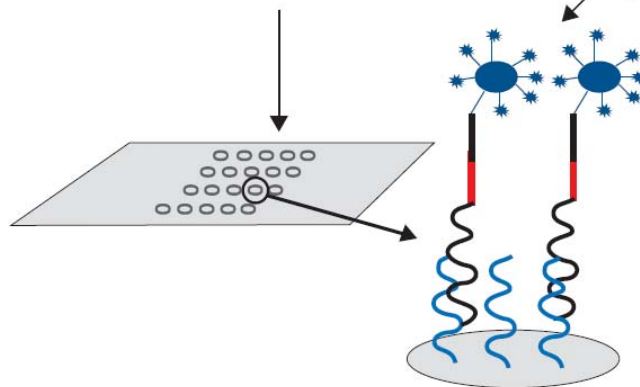
1 Poly (A) Tailing



2 Ligation



3 Array Hybridization



Components, Storage, and Handling

FlashTag RNA Labeling Kit: **Store all components at –20°C**

Vial 1	10X Reaction Buffer
Vial 2	25mM MnCl ₂
Vial 3	ATP Mix
Vial 4	PAP Enzyme
Vial 5 (red cap)	5X FlashTag Ligation Mix Oyster-550
OR	
Vial 5 (blue cap)	5X FlashTag Ligation Mix Oyster-650
Vial 6	T4 DNA Ligase
Vial 7	Stop Solution
Vial 8	2X Enhanced Hybridization Buffer
Vial 9	2X SDS-Based Hybridization Buffer
Vial 10	10% BSA

Handling Kit Contents

Vials 1, 2, and 7: Thaw at room temperature, vortex, and briefly microfuge.

Vials 3, 4, 6, and 10: Thaw on ice, microfuge if necessary, and keep on ice at all times. Do not vortex.

Vial 5: Thaw at room temperature in the dark, vortex, and briefly microfuge. **Protect from light.**

Vials 8 and 9: Heat to 70-80°C for 10 minutes, vortex, and repeat as necessary to resuspend the buffer. Microfuge for 1 minute.

Other Required Materials

- RNA sample containing low molecular weight (LMW) RNA (see “RNA Sample and Quantitation” on page 5)
- Optional: Microcon[®] YM-100 Centrifugal Filter Devices (Millipore[®] cat. no. 42413)
- Optional: Microcon[®] YM-3 Centrifugal Filter Devices (Millipore[®] cat. no. 42404)
- Optional: Quant-iT[™] RiboGreen[®] RNA Assay Kit (Invitrogen cat. no. R11490)
- Nuclease-Free water (Ambion cat. no. AM9934 or equivalent)
- 1mM Tris pH 8.0
- 10mM Tris pH 8.0
- miRNA Microarray: see “Microarrays” on page 5 for a list of suggested arrays and “Microarray Procedures” on pages 8-11 for additional required materials for these array types.
- Optional: NCode[™] Multi-Species miRNA Microarray Controls (Invitrogen cat. no. MIRAC-01)
- Optional: Spike-in miRNA kit for miRCURY[®] LNA Arrays (Exiqon cat. no. 208040)
- Glass coverslips or other hybridization chamber
- Array Wash Buffers: See “Microarray Procedures” on pages 8-11 for specific buffer formulations. Recommended solutions for wash buffer preparation: deionized, reagent grade water (VWR cat. no. RC91505), 20X SSC (Ambion cat. no. AM9763), and 10% SDS (Ambion cat. no. AM9823)
- Optional: DyeSaver[®] 2 protective coating (Genisphere cat. no. Q500500)

Microarrays

FlashTag has been tested to be compatible with the following commercially available miRNA microarrays:

- NCode Multispecies miRNA Array (Invitrogen)
- miRCURY LNA Array (Exiqon)
- CustomArray 4X2 MicroRNA Array (CombiMatrix)

FlashTag can also be used with non-commercially printed arrays. Please see the “Microarray Procedures” on pages 8-11 for specific hybridization recommendations.

RNA Sample and Quantitation

FlashTag can accommodate total RNA, enriched RNA, and senseRNA that has been amplified from LMW RNA using the SenseAMP™ Plus LMW RNA Amplification Kit.⁹ Some applications may require enrichment for optimal profiling. For example, to distinguish mature and precursor miRNAs, enrichment may be necessary. In addition, degraded total RNA samples should be enriched prior to FlashTag labeling. To accurately determine the concentration of the enriched miRNA sample, Genisphere recommends the use of the Quant-iT RiboGreen RNA Assay Kit (Invitrogen cat. no. R11490) or the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). If the enriched sample is not quantitated, use LMW RNA enriched from between 0.5 to 3µg of total RNA. Refer to the table below for RNA input recommendations for FlashTag labeling.

RNA Sample	Input for FlashTag Labeling
Total RNA containing LMW RNA	0.5 - 3µg
Enriched LMW RNA that is not quantitated	Enriched from 0.5 - 3µg total RNA
LMW RNA enriched by Microcon YM-100 Filter Unit Recommended, see page 6 (Millipore cat. no. 42413)	10 - 80ng
LMW RNA enriched by PureLink™ miRNA Isolation Kit (Invitrogen cat. no. K1570-01)	50 - 300ng
LMW RNA enriched by mirVana™ miRNA Isolation Kit (Ambion cat. no. 1560)	50 - 300ng
LMW RNA enriched by flashPAGE™ Fractionator (Ambion cat. no AM13100)	0.1 - 1ng
LMW RNA enriched by Qiagen RNA/DNA Midi Kit (Qiagen cat. no. 14142)	50 - 300ng
senseRNA* amplified by Genisphere's SenseAMP Plus LMW RNA Amplification Kit (cat. no. RAMP110MIR or RAMP120MIR)	125 - 750ng

*If labeling **senseRNA**, the “Poly (A) Tailing” reaction is not required. Adjust the volume to 15µl with Nuclease-Free water and proceed directly to “FlashTag Ligation” on page 7.

Recommended: Enrichment and Concentration of LMW RNA Using YM-100 and YM-3 Columns

Enrich the total RNA sample using a Microcon YM-100 column (Millipore cat. no. 42413) and a conventional tabletop microfuge.

1. Dilute the total RNA sample to 100 μ l with 10mM Tris pH 8.0.
2. Heat to 80°C for 3 minutes, then immediately cool on ice for 3 minutes.
3. While the sample is cooling on ice, add 50 μ l of 10mM Tris pH 8.0 to the Microcon column, and spin for 3 minutes at 13,000g.
4. Discard the flow-through and the collection tube. Place the column into a new collection tube.
5. Add the 100 μ l of RNA to the Microcon column, and centrifuge for 7 minutes at 13,000g.
6. Save the eluate (~95 μ l) in the collection tube. This is the enriched LMW RNA. The LMW RNA can be quantitated with the Quant-iT RiboGreen RNA Assay Kit or the NanoDrop ND-1000 Spectrophotometer. Proceed to Concentration, below, or "FlashTag RNA Labeling Procedure" on page 7.

Note: To collect the high molecular weight RNA, add 5 μ l of 10mM Tris pH 8.0 to the Microcon column and gently mix by tapping the side. Carefully place the sample reservoir **upside down** in a **new collection tube** and centrifuge for 3 minutes at 13,000g.

Concentration of LMW RNA with Microcon YM-3 column (Millipore cat. no. 42404)

1. Add the LMW RNA from step 6 above (~95 μ l flow-through of YM-100) to the YM-3 sample reservoir. Do not touch the membrane with the pipette tip. Secure the tube cap and centrifuge for 30 minutes at 13,000g.
2. Check the volume of the flow-through and continue the centrifugation if necessary. For adequate concentration, the flow-through volume should be equal to the loaded volume minus 5 μ l. For example, if 95 μ l was loaded, then the flow-through volume should measure 90 μ l.
3. Add 5 μ l of 10mM Tris-HCl, pH 8.0 to the sample reservoir and gently mix by tapping the side.
4. Carefully place the sample reservoir **upside down** in a **new collection tube**. Centrifuge for 3 minutes at 13,000g to collect the concentrated LMW RNA (~5-10 μ l). The LMW RNA can be quantitated with the Quant-iT RiboGreen RNA Assay Kit or the NanoDrop ND-1000 Spectrophotometer. Proceed to "FlashTag RNA Labeling Procedure" on page 7.

FlashTag RNA Labeling Procedure

FlashTag will label LMW RNA from total, enriched or amplified senseRNA. For RNA input requirements, see "RNA Sample and Quantitation" on page 5. Spike-in controls, such as those recommended by Invitrogen and Exiqon (see page 4 for catalog numbers), may also be added to the RNA sample prior to FlashTag labeling.

Note: If labeling **senseRNA**, the "Poly (A) Tailing" reaction is not required. Adjust the volume of senseRNA to 15µl with nuclease-free water and proceed directly to "FlashTag Ligation" below.

Poly (A) Tailing

1. Adjust the volume of RNA, including any spike-in controls, to 10µl with Nuclease-Free Water.
2. Dilute the ATP mix (Vial 3) in 1mM Tris (pH 8.0) as follows:

For **total RNA samples**, dilute the ATP Mix 1:500.

For **enriched, quantitated samples**, calculate the dilution factor according to the following formula:

$$5000 \div \text{ng input LMW RNA}$$

Example: If using 100ng of enriched LMW RNA, the dilution factor is $5000 \div 100 = 50$.
Dilute the ATP Mix 1:50.

For **enriched samples that are not quantitated**, calculate the dilution factor according to the following formula:

$$1000 \div \mu\text{g input total RNA}$$

Example: If the sample was enriched from 2µg total RNA, the dilution factor is $1000 \div 2 = 500$.
Dilute the ATP Mix 1:500.

3. Add the following components to the 10µl RNA, for a volume of 15µl:
 - 1.5µl 10X Reaction Buffer (Vial 1)
 - 1.5µl 25mM MnCl₂ (Vial 2)
 - 1µl diluted ATP Mix (Vial 3 dilution from step 2)
 - 1µl PAP Enzyme (Vial 4)
4. Mix gently (do not vortex) and microfuge.
5. Incubate in a 37°C heat block for 15 minutes. Discard any unused, diluted ATP Mix from step 2.

FlashTag Ligation

Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes.

1. Briefly microfuge the 15µl of tailed RNA and place on ice. If labeling **senseRNA**, adjust the volume to 15µl with nuclease-free water and proceed to step 2.
2. Add 4µl of the appropriate 5X FlashTag Ligation Mix (Vial 5, Oyster-550 or Oyster-650).
3. Add 2µl of T4 DNA Ligase (Vial 6).
4. Mix gently and microfuge.
5. Incubate at room temperature for 30 minutes. Protect the reactions from light.
6. Stop the reaction by adding 2.5µl Stop Solution (Vial 7). Mix and microfuge the 23.5µl of ligated sample. The samples are now ready for hybridization to microarrays.

Microarray Procedures

NCode MultiSpecies miRNA Array (Invitrogen) or Non-commercially Printed Arrays

Additional required materials:

- Glass coverslips (Corning, VWR or other manufacturer)
- Heat blocks set to 65°C and 70-80°C
- Wash buffers: 2X SSC/0.2% SDS, 2X SSC and 0.2X SSC
- Hybridization oven set to 52°C

Note: Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes.

Array Hybridization

1. Resuspend the 2X Hybridization Buffer. Vial 8, 2X Enhanced Hybridization Buffer, is recommended. Other hybridization buffers, including Vial 9, 2X SDS-based Hybridization Buffer, can be used. Heat the hybridization buffer to 70-80°C for 10 minutes, vortex, and repeat as necessary to resuspend the buffer. Microfuge for 1 minute.
2. For dual-color assays: combine the two ligation reactions and add 5µl of 10% BSA (Vial 10). Then add 52µl of hybridization buffer. The **Hybridization Mix** is 104µl.

For single-color assays: add 5µl of 10% BSA (Vial 10) and 28.5µl of hybridization buffer. The **Hybridization Mix** is 57µl. For larger volumes, add equal parts 2X hybridization buffer and water.

3. Heat the **Hybridization Mix** to 65°C for 10 minutes. Gently vortex and briefly microfuge.
4. Apply some or all of the **Hybridization Mix** to a microarray, and cover with a glass coverslip (see table below). Other hybridization chambers can also be used. **Note**: 2X Enhanced Hybridization Buffer (Vial 8) requires higher hybridization volumes due to increased viscosity.

	<u>24x40mm</u>	<u>24x50mm</u>	<u>24x60mm</u>
Vial 8, 2X Enhanced Buffer	43µl	53µl	63µl
Vial 9, 2X SDS Buffer	37µl	45µl	53µl

5. Incubate the array 6 hours to overnight (6-20 hours) in a dark, humidified chamber at 52°C.

Array Washing

1. Prewarm the 2X SSC/0.2%SDS wash buffer to 52°C.
2. Remove the coverslip by washing the array in 52°C 2X SSC/0.2% SDS for 2 minutes or until the coverslip floats off.
3. Wash for 15 minutes in 52°C 2X SSC/0.2%SDS.
4. Wash for 15 minutes in 2X SSC at room temperature.
5. Wash for 15 minutes in 0.2X SSC at room temperature.
6. Transfer the array to a dry 50mL centrifuge tube, orienting the slide so that any label is down in the tube. Immediately centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide (any delay in this step may result in high background). Avoid contact with the array surface.

Signal Detection

Scan the array according to the manufacturer's recommendations, or first apply DyeSaver 2 coating (Genisphere cat. no. Q500500) to preserve fluorescent signal.

miRCURY LNA Array (Exiqon)

Additional required materials:

- LifterSlips™ (Erie Scientific cat. no. 22x50I-2-47711)
- Heat blocks set to 60°C and 95°C
- 2x Hybridization buffer (Exiqon cat. no. 208020)
- Wash buffers: 2X SSC/0.2% SDS, 1X SSC and 0.1X SSC
- Hybridization oven set to 60°C

Note: Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes.

Array Hybridization

1. Resuspend the Exiqon 2x Hybridization buffer by heating to 60°C and agitating. Microfuge for 1 minute.
2. For dual-color assays: combine the two ligation reactions and add 47µl of the Exiqon 2x Hybridization buffer. The **Hybridization Mix** is 94µl.

For single-color assays: add 2.5µl nuclease-free water and 25µl of the Exiqon 2x Hybridization buffer. The **Hybridization Mix** is 50µl.

3. Heat the **Hybridization Mix** to 95°C for 10 minutes. Gently vortex and briefly microfuge.
4. Apply 50µl of the **Hybridization Mix** to a miRCURY array and cover with a 22x50mm LifterSlip.
5. Incubate the array 6 hours to overnight (6-20 hours) in a dark, humidified chamber at 60°C.

Array Washing

Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes.

1. Prewarm the 2X SSC/0.2%SDS wash buffer to 60°C.
2. Remove the coverslip by washing the array in 2X SSC/0.2% SDS at 60°C for 2 minutes or until the coverslip floats off. Incubate for an additional 2 minutes.
3. Wash for 5 seconds in 1X SSC at room temperature.
4. Wash for 2 minutes in a fresh solution of 1X SSC at room temperature.
5. Wash for 2 minutes in 0.1X SSC at room temperature.
6. Transfer the array to a dry 50mL centrifuge tube, orienting the slide so that any label is down in the tube. Immediately centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide (any delay in this step may result in high background). Avoid contact with the array surface.

Signal Detection

Scan the array according to the manufacturer's recommendations, or first apply DyeSaver 2 coating (Genisphere cat. no. Q500500) to preserve fluorescent signal.

CustomArray 4X2 MicroRNA Array (CombiMatrix)

Note: CombiMatrix arrays are designed for single color only (Oyster-650 channel).

Required materials:

- Sectored Hybridization Cap (CombiMatrix)
- CombiMatrix CustomArray Hyb Clamp for 4X2K (CombiMatrix cat. no. 610009)
- LifterSlip coverslip for imaging (Erie Scientific cat. no. 12X30I-2-5631)
- Imaging Solution (CombiMatrix)
- Hybridization oven with rotisserie set to 43°C
- Incubator set to 65°C
- Heat block set to 95°C
- Adhesive tape: Scotch® Magic™ tape or equivalent
- Nuclease-free water
- 20X SSPE
- 10% Tween-20
- Wash buffers: 6X SSPE/0.05% Tween-20 (6X SSPET), 3X SSPE/0.05% Tween-20 (3X SSPET), 0.5X SSPE/0.05% Tween-20 (0.5X SSPET)
- Deionized (DI) formamide
- Bovine Serum Albumin, Ultrapure (5% w/v) (Ambion cat. no. AM2616)
- 1X PBS prepared from 10X phosphate-buffered saline (Ambion cat. no. AM9625)

Note: For detailed instructions on assembly of the CombiMatrix Hybridization Clamp, please refer to the *CombiMatrix MicroRNA 4X2K Microarray: Hybridization and Imaging Protocol (PTL012)*.

Solution Preparation

Prehybridization Solution:

	Volume (μl)	Final Concentration
20X SSPE	300	6X
10% SDS	100	1%
BSA Solution (5% w/v)	40	0.2%
Nuclease-free water	560	
Total volume	1000	

Pre-Hybridization

1. Assemble the 4X2K Microarray using the Hybridization Cap and Clamp.
2. Fill the hybridization chambers with 30μl of nuclease-free water. Cover the ports with adhesive tape and incubate at 65°C for 10 minutes.
3. Remove the microarray from the incubator and bring to room temperature. Remove the adhesive tape and aspirate the water out of the chambers. Immediately fill the chambers with 30μl of *Prehybridization Solution*, introducing a small air bubble. Wipe the surface and apply adhesive tape over the ports.
4. Load the microarray onto the rotisserie in the hybridization oven and incubate at 43°C for 1 hour with slow rotation.

Note: Perform the following steps in the dark to avoid degradation and fading of the fluorescent dyes.

Hybridization

1. Prepare the hybridization solution in the order listed below. Note: Use 5.1 μ l of the labeled RNA for hybridization. Alternatively, use the entire sample by first concentrating to 5.1 μ l by evaporation (avoid complete drying).

	Volume (μl)	Final Concentration
20X SSPE	9	6X SSPE
BSA solution (5% w/v)	4.8	0.8% BSA
DI Formamide	3.6	12% Formamide
Labeled LMW RNA (Oyster-650)	5.1	
10% SDS	7.5	2.5%
Total Volume	30	

2. Denature the hybridization solution at 95°C for 3 minutes. Cool on ice for 20 seconds. Remove the samples from ice and keep at room temperature to avoid SDS precipitation. If precipitation occurs, repeat this step. Briefly spin down.
3. Remove the adhesive tape and pre-hybridization solution from each chamber. Fill the chambers with 30 μ l of hybridization solution and mix gently by pipetting up and down, introducing a small air bubble into the chamber. Wipe excess solution from the Hybridization Cap and apply adhesive tape.
4. Load the microarray onto the rotisserie in the hybridization oven and incubate at 43°C overnight (12-16 hours).

Array Washing

Note: For each wash step, rinse the chamber with the wash solution by pipetting up and down several times, discard and apply fresh solution for the incubation. **Do not allow the array to dry.**

1. Remove the adhesive tape and hybridization solution from each chamber.
2. Apply 6X SSPET wash solution and incubate at room temperature for 3 minutes. Remove the solution.
3. Apply 3X SSPET wash solution and incubate at room temperature for 3 minutes. Remove the solution.
4. Apply 0.5X SSPET wash solution and incubate at room temperature for 3 minutes. Remove the solution.
5. Repeat step 4. Retain the 0.5X SSPET solution in the chambers until ready to scan.

Signal Detection

1. Remove the 0.5X SSPET wash solution from the chambers and disassemble the Hybridization Clamp. Remove the microarray from the Clamp.
2. Immediately apply Imaging Solution to the semiconductor surface and cover with a 22x30mm LifterSlip. Avoid introducing air bubbles. Scan the array in the Oyster-650 channel only (Cy5 equivalent).
3. Proceed to stripping according the manufacturers recommendations.

Note: CombiMatrix arrays are not compatible with DyeSaver.

References

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Appendix A: Reducing Degradation of Oyster-650

Oyster-650 dye performance may be affected by a variety of factors that are particularly prevalent during the summer months. Exposure of the Oyster-650 dye solutions and the hybridized arrays to light and to oxidative environments may cause rapid fading of the dye, regardless of the labeling system used. Limiting or controlling the exposure of the arrays to these environments has been shown to significantly reduce Oyster-650 fading. Below are recommendations for reducing the degradation of Oyster-650 when performing microarray experiments:

1. Always keep solutions and arrays containing Oyster-650 away from light, particularly sunlight. Oyster-650 will photo-bleach when exposed to light, including normal fluorescent lighting.
2. Protect the hybridized, dried array from contact with air, particularly on hot and sunny days. We have found that ambient ozone levels resulting from summertime air pollution can cause oxidative degradation of Oyster-650. Keeping the Oyster-650-containing arrays in an inert atmosphere (nitrogen) in a small container (50mL tube) can significantly delay fading of the Oyster-650. Some investigators also add small quantities of dithiothreitol (DTT) or beta mercapto-ethanol (BME) to the bottom of the tube to further promote a reducing micro-environment. Be certain to avoid contact of the array with these chemicals. If possible, do not place the arrays or the array scanner near high ozone-generating equipment, such as a freezer or laser printer.
3. In the preparation of wash buffers, avoid the use of water that may damage Oyster-650. We recommend using deionized, reagent grade water from VWR (Cat. No. RC91505) to prepare wash buffers.
4. In the preparation of wash buffers, be certain that any DEPC treated solutions have had all of the DEPC fully removed, since DEPC is a potent oxidizer. We recommend using 10% SDS from Ambion (Cat. No. AM9823) and 20X SSC from Ambion (Cat. No. AM9763) to prepare wash buffers.
5. Add a small quantity of dithiothreitol (DTT) to the first two wash buffers used after the 3DNA hybridization. The DTT should be at a final concentration of 0.1-0.5mM. Be sure to work with fresh DTT, as old or poor quality DTT may cause an increase in background visible as a "haze" in the Oyster-550 channel.
6. Use DyeSaver 2 (Genisphere Cat. No. Q500500) after the final washing and drying of the microarray. DyeSaver 2 is easy to use, compatible with most array surface chemistries, and protects Oyster-650 from atmospheric oxidation for at least three weeks. DyeSaver 2 has also been shown to reduce Oyster-650 damage due to photobleaching. Note: DyeSaver 2 is not compatible with CombiMatrix arrays. For more information about DyeSaver 2, please refer to the Genisphere web site (www.genisphere.com) or call Genisphere Technical Support.