FlashTag™ Biotin HSR RNA Labeling Kit

For GeneChip™ miRNA Arrays

User Guide

Catalog Numbers 901910, 901911

Document Part Number 703095

Publication Number MAN0018109

Revision A.0

ThermoFisher SCIENTIFIC

Life Technologies Corporation
7335 Executive Way
Frederick, MD 21704 USA

The information in this guide is subject to change without notice.

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, THERMO FISHER SCIENTIFIC INC. AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. MAN0018109

Revision	Date	Description
A.0	18 December 2018	• Initial release in Thermo Fisher Scientific document control system. • Supersedes legacy Affymetrix publication number 703095. • Updated to the current document template, with associated updates to trademarks, logos, licensing, and warranty.

Important Licensing Information: These products may be covered by one or more Limited Use Label Licenses. By use of these products, you accept the terms and conditions of all applicable Limited Use Label Licenses.

TRADEMARKS: All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified.

Contents

Chapter 1 Introduction

Background Information

The FlashTag™ Biotin HSR Labeling Kit will label any RNA sample, including total RNA, severely degraded RNA, plant RNA, and low molecular weight RNA. This protocol describes labeling total RNA or low molecular weight (LMW) RNA for analysis by GeneChip™ miRNA Arrays and includes an in-process ELOSA QC Assay.

LMW RNA molecules (snRNA, hnRNA, piRNA, miRNA, etc.) have recently been shown to be involved in important biological processes such as mRNA degradation, transcriptional gene silencing (TGS) and translational repression.[1, 2, 3, 4, 5, 6, 7, 8] 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 Biotin HSR provides the necessary tools to identify such targets.

FlashTag Biotin HSR labeling is fast, simple, accurate, highly sensitive and reproducible. Starting with total RNA (see Table 1.2 for recommended input amounts), the process begins with a brief tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample (see Figure 1.1). The labeling process is complete in less than one hour.

The high sensitivity of FlashTag Biotin HSR is due to proprietary 3DNA™ dendrimer signal amplification technology. The 3DNA dendrimer is a branched structure of single and double stranded DNA conjugated with numerous labels.[9, 10] The 3DNA molecule in the FlashTag Biotin HSR Labeling Kit provides ultrasensitive biotin labeling.

Please review this manual before beginning experiments. Materials needed for GeneChip miRNA Arrays are listed. Materials needed for the ELOSA QC Assay are listed in Appendix A. Note that ELOSA wells must be coated with DNA Spotting Oligos and incubated overnight before the ELOSA assay may be run.

[1] Schembri et al. MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. PNAS 2009 vol. 106 no. 7, 2319-2324.

[2] Taylor and Gant. Emerging fundamental roles for non-coding RNA species in toxicology. Toxicology 2008 vol. 246 Issue 1, 34- 39.

[3] Ronemus, M. et al. MicroRNA-Targeted and Small Interfering RNA–Mediated mRNA Degradation Is Regulated by Argonaute, Dicer, and RNA-Dependent RNA Polymerase in Arabidopsis. The Plant Cell. 2006, 18(7):1559-1574.

[4] Morel, JB. et al. Hypomorphic ARGONAUTE (ago1) Mutants Impaired in Post-Transcriptional Gene Silencing and Virus Resistance. The Plant Cell. 2002, Vol. 14(3), 629-639.

[5] Krichevsky, AM. et al. A microRNA array reveals extensive regulation of microRNAs during brain development. RNA. 2003, 9(10):1274-1281.

[6] Schmittgen, TD. et al. A high-throughput method to monitor the expression of microRNA precursors. Nucleic Acids Res. 2004, 32(4):e43.

[7] Thomson, JM. et al. A Custom Microarray Platform for Analysis of MicroRNA Gene Expression. Nature Methods. 2004, 1(1) 47-53.

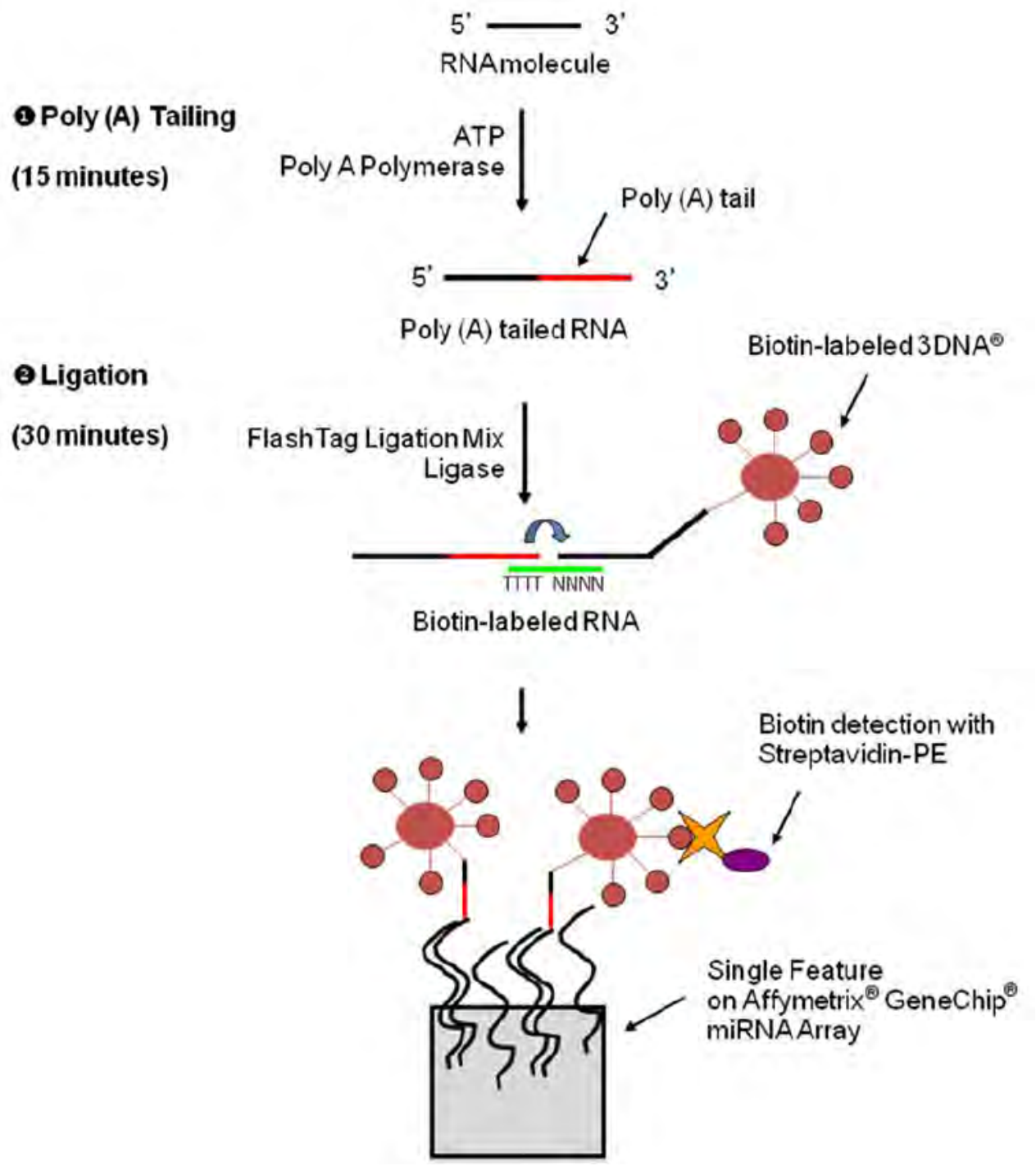
[8] Ambros, V. The functions of animal microRNAs. Nature. 2004, 431:350.

[9] Nilsen, TW. et al. Dendritic Nucleic Acid Structures. J. Theor. Biol. 1997, 187:273-284.

[10] Stears, RL. et al. A novel, sensitive detection system for high-density microarrays using dendrimer technology. Physiol. Genomics. 2000, 3:93-99.

Procedure Overview

Figure 1.1 **FlashTag™ HSR: Procedure Overview**



Materials Required

FlashTag™ Biotin HSR RNA Labeling Kit

FlashTag Biotin HSR RNA Labeling Kit, 10 rxn (P/N 901910) or 30 rxn (P/N 901911).

Table 1.1 FlashTag™ Biotin HSR RNA Labeling Kit Reagents and Storage Conditions

Vial	Component	Storage	Handling
1	10X Reaction Buffer	–20°C	Thaw at room temperature, vortex, and briefly microfuge.
2	25mM MnCl2	–20°C	Thaw at room temperature, vortex, and briefly microfuge.
3	ATP Mix	–20°C	Thaw on ice, microfuge if necessary, and keep on ice at all times.
4	PAP Enzyme	–20°C	Remove from freezer just prior to use, and briefly microfuge. Keep on ice at all times.Do not vortex.
5	5X FlashTag Biotin HSR Ligation Mix	–20°C	Thaw at room temperature, vortex, and briefly microfuge.
6	T4 DNA Ligase	–20°C	Remove from freezer just prior to use, and briefly microfuge. Keep on ice at all times.Do not vortex.
7	HSR Stop Solution	–20°C	Thaw at room temperature, vortex, and briefly microfuge.
8	RNA Spike Control Oligos	–20°C	Thaw on ice, microfuge if necessary, and keep on ice at all times.
9	ELOSA Spotting Oligos	–20°C	Thaw at room temperature, vortex, and briefly microfuge.
10	ELOSA Positive Control	–20°C	Thaw on ice, microfuge if necessary, and keep on ice at all times.
11	Nuclease-Free Water	–20°C	Thaw at room temperature, vortex, and briefly microfuge.
12	27.5% Formamide	–20°C	Thaw at room temperature, vortex, and briefly microfuge.

NOTE: The FlashTag Biotin HSR RNA Labeling Kit is recommended for no more than three freeze-thaw cycles.

Other Required Materials

Refer to Appendix C for example reagent preparation and storage.

IMPORTANT: All materials should be nuclease-free, and all reagents should be prepared with nuclease-free components.

- RNA sample containing low molecular weight (LMW) RNA (see *RNA Sample and Quantitation*)
- Nuclease-free water (Thermo Fisher P/N AM9932 or equivalent)
- 1mM Tris (Appendix C)
- Reagents and materials for analysis by GeneChip miRNA Array:
  - miRNA Array
  - Reagents for ELOSA QC Assay: Refer to Appendix A, *ELOSA QC Assay* and Appendix C, *Example Reagent Preparation and Storage*.
  - GeneChip™ Eukaryotic Hybridization Control Kit (P/N 900454)
  - GeneChip™ Hybridization, Wash and Stain Kit (P/N 900720)
  - Laser Tough-Spots™ 3/8" diameter (Diversified Biotech P/N SPOT-1000)
  - Laser Tough-Spots™ 1/2" diameter (Diversified Biotech P/N SPOT-2000)
  - GeneChip Command Console™ Software (AGCC)
  - Expression Console™ Software (EC) v1.2 or higher
  - GeneChip™ Fluidics Station 450 (P/N 00-0079)
  - GeneChip™ Hybridization Oven 645
  - GeneChip™ Scanner 3000 7G
- Reagents and materials for analysis by miRNA Plates:
  - miRNA Plate
  - Reagents for ELOSA QC Assay: Refer to Refer to Appendix A, *ELOSA QC Assay* and Appendix C, *Example Reagent Preparation and Storage*.
  - GeneChip™ Eukaryotic Hybridization Control Kit (P/N 900457)
  - GeneTitan™ Hybridization, Wash and Stain Kit for miRNA Array Plates (P/N 902276)
  - GeneChip™ Command Console™ Software (AGCC)
  - Expression Console™ Software (EC) v1.2 or higher
  - GeneTitan™ SC or MC Instrument

RNA Sample and Quantitation

Either Total RNA or LMW (Low Molecular Weight) RNA can be labeled with FlashTag Biotin HSR. Using total RNA can save time and money, and prevent sample loss.[1, 2]

1 http://www.genetics.pitt.edu/forms/flyers/miRNAextractionevaluation.pdf

2 Masotti et al. Quantification of Small Non-Coding RNAs Allows an Accurate Comparison of miRNA Expression Profiles. Journal of Biomedicine and Biotechnology 2009, Article ID 659028

RNA Isolation

Any kit for purification of total RNA or LMW RNA will be compatible with FlashTag Biotin HSR. Elute or resuspend the RNA in nuclease-free water. Ensure that the purification method retains low molecular weight species. Some commercial products that have been tested successfully with FlashTag Biotin HSR include:

- Thermo Fisher Scientific: mirVana™ miRNA Isolation Kit
- Thermo Fisher Scientific: RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE
- QIAGEN: miRNeasy Mini Kit
- Thermo Fisher Scientific: PureLink™ miRNA Isolation Kit
- Thermo Fisher Scientific: TRIzol™ reagent (total RNA only) with additional overnight –20°C precipitation step during isopropanol precipitation[1]

Quantitation

To accurately determine the concentration of the RNA sample, we recommend the use of the Quant-iT™ RiboGreen RNA Assay Kit (Thermo Fisher P/N R11490) or the NanoDrop™ ND-1000 Spectrophotometer (NanoDrop Technologies).

RNA Input for FlashTag™ Biotin HSR

Table 1.2 describes general recommendations for RNA input for FlashTag Biotin HSR labeling. To maintain comparability to previous generation arrays, a minimum of 130 ng input is recommended for 100 format arrays.

Table 1.2

RNA Sample	Input for FlashTag Biotin HSR Labeling for miRNA 400/169 Format Arrays (miRNA 1.0 and 2.0 Arrays)	Input for FlashTag Biotin HSR Labeling for miRNA 100 Format Arrays (miRNA 3.0 and later designs)
Total RNA containing LMW RNAT	100–1000 ng total RNA	130–1000 ng total RNA
Enriched LMW RNA, quantitated	100–400 ng LMW RNA	130–400 ng LMW RNA
Enriched LMW RNA, not quantitated	Enriched from 100–1000 ng total RNA	Enriched from 130–1000 ng total RNA

1 Wang et al. The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme. The Journal of Neuroscience, January 30, 2008, 28(5):1213-1223

Chapter 2 FlashTag™ Biotin HSR RNA Labeling Procedure

To confirm target labeling, we suggest running an ELOSA QC Assay prior to array hybridization. Refer to Appendix A, *ELOSA QC Assay*. Note that ELOSA wells must be coated with DNA Spotting Oligos and incubated overnight before the ELOSA assay may be run, and that Plate Washing and Blocking steps may be completed prior to or during the FlashTag Biotin HSR labeling procedure.

Poly (A) Tailing

1. Adjust the volume of RNA to 8 µL with Nuclease-Free Water (Vial 11).
2. Transfer the 8 µL RNA to ice. Add 2 µL RNA Spike Control Oligos (Vial 8) and return to ice.
3. Dilute the ATP mix (Vial 3) in 1 mM Tris 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 ÷ ng input LMW RNA

     Example: If using 100 ng of enriched LMW RNA, the dilution factor is 5000 ÷ 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 ÷ µg input total RNA

     Example: If the sample was enriched from 500 ng total RNA, the dilution factor is 1000 ÷ 0.5 = 2000. Dilute the ATP Mix 1:2000.
4. Assemble a Poly A Tailing Master Mix in a nuclease-free tube in the order listed in Table 2.1. Include 10% overage to cover pipetting errors.

Table 2.1 Poly A Tailing Master Mix

Component	Volume per miRNA Array	16-Array Plate*	24-Array Plate*	96-Array Plate*
10X Reaction Buffer (Vial 1)	1.5 µL	26.4 µL	39.6 µL	158.4 µL
25mM MnCl2 (Vial 2)	1.5 µL	26.4 µL	39.6 µL	158.4 µL
Diluted ATP Mix (Vial 3 dilution from Step 3)	1.0 µL	17.6 µL	26.4 µL	105.6 µL
PAP Enzyme (Vial 4)	1.0 µL	17.6 µL	26.4 µL	105.6 µL

**** Includes ~10% overage to cover pipetting error.***

5. Add 5 µL of Master Mix to the 10 µL RNA/Spike Control Oligos (Step 2 above), for a volume of 15 µL.

6. Mix gently (do not vortex) and microfuge.
7. Incubate in a 37°C heat block for 15 minutes. Discard any unused, diluted ATP mix from Step 3.

FlashTag™ Biotin HSR Ligation

1. Briefly microfuge the 15 µL of tailed RNA and place on ice.
2. Add 4 µL 5X FlashTag Biotin HSR Ligation Mix (Vial 5) to each sample.
3. After the Ligation Mix has been added, add 2 µL of T4 DNA Ligase (Vial 6) to each sample. Do not make a master mix at this step, as auto-ligation can occur.
4. Mix gently (do not vortex) and microfuge.
5. Incubate at 25°C (room temperature) for 30 minutes.
6. Stop the reaction by adding 2.5 µL HSR Stop Solution (Vial 7). Mix and microfuge the 23.5 µL of ligated sample.
7. Remove 2 µL of the biotin-labeled sample for use with the ELOSA QC Assay (Appendix A). It is acceptable to store the 2 µL of biotin-labeled sample on ice for up to 6 hours or at –20°C for up to 2 weeks, and run the ELOSA QC Assay at a convenient time. If the ELOSA QC Assay is not performed, it is recommended that 2 µL of biotin-labeled sample be saved until the array QC is complete. Retaining this sample will enable one the ability to troubleshoot possible target preparation issues, if needed.
8. The remaining 21.5 µL biotin-labeled sample may be stored on ice for up to 6 hours, or at –20°C for up to 2 weeks, prior to hybridization on GeneChip™ miRNA Arrays or miRNA Array Plates.

Chapter 3 GeneChip™ miRNA Array Procedure

Preparation of Ovens, Arrays, and Sample Registration Files

1. Download and install the miRNA Array library file package (if not performed previously) into GeneChip™ Command Console™ (AGCC) software using the Command Console Library File Importer tool. The files can be found on our web site.
2. Turn the Hybridization Oven 645 on and set the temperature to 48°C. Set the RPM to 60.

   Turn the rotation on and allow the oven to preheat for at least one hour.
3. Upload the sample and array information (sample names, barcode IDs, etc.) into AGCC.
4. Unwrap the arrays and place on the bench top. Allow the arrays to warm to room temperature (10-15 minutes). Mark each array with a meaningful designation.
5. Insert a 20 µL or 200 µL pipet tip (unfiltered type recommended) into the upper right septum to allow for proper venting when hybridization cocktail is injected.

For more information, refer to the *Command Console™ User Guide* (Pub. No. 702569).

Hybridization

1. Bring the reagents listed in Step 3, below, to room temperature.
2. Completely thaw and then heat the 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre* from GeneChip™ Eukaryotic Hybridization Control Kit) for 5 minutes at 65°C.
3. Add the following components in Table 3.1 to the 21.5 µL biotin-labeled sample in the order listed, to prepare the array hybridization cocktail:

Table 3.1 Hybridization Cocktail (for a single reaction)

Component	Volume for a 400/169 Format Array (miRNA 1.0 and 2.0 Arrays)	Volume for a 100 Format Array (miRNA 3.0 and later designs)	Final Concentration
2X Hybridization Mix	50 µL	66 µL	1X
27.5% Formamide (Vial 12)	15 µL	19.2 µL	4%
DMSO	10 µL	12.8 µL	9.7%
20X Hybridization Controls	5 µL	6.6 µL	1X
Control Oligo B2, 3nM	1.7 µL	2.2 µL	50 pM
Nuclease-free Water	N/A	3.7 µL	
Total Volume	**81.7 µL**	**110.5 µL**	

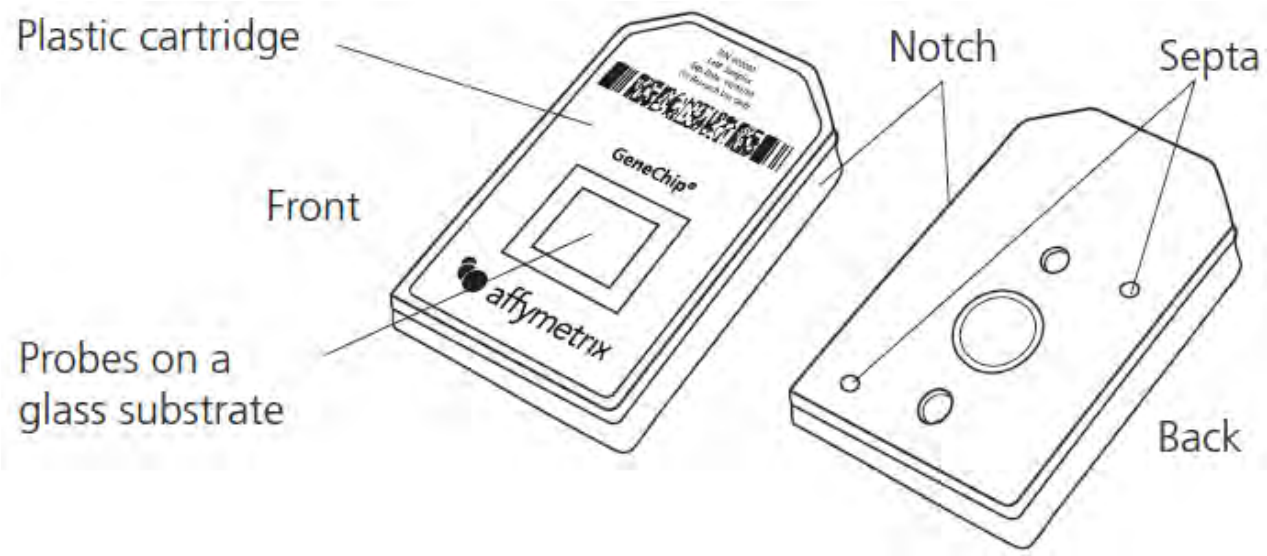
NOTE: If preparing multiple samples, a master mix of the hybridization cocktail components in Table 3.1 may be prepared by multiplying the volumes by the number of arrays plus 10% overage to cover pipetting error. Refer to Table 3.2 for appropriate Hybridization Master Mix volumes to add to the biotin-labeled sample

Table 3.2 Hybridization Cocktail Using a Hybridization Master Mix

Component	400/169 Format Array (miRNA 1.0 and 2.0 Arrays)	100 Format Array (miRNA 3.0 and later designs)
Biotin-labeled Sample	21.5 µL	21.5 µL
Hybridization Master Mix	81.7 µL	110.5 µL

4. Incubate at 99°C for 5 minutes, then 45°C for 5 minutes.
5. Aspirate 100 µL (400/169 format array) or 130 µL (100 format array) and inject into an array.

Figure 3.1 **GeneChip™ Cartridge Array**



6. Remove the pipet tip from the upper right septum of the array.
7. Cover both septa with 1/2" Tough-Spots™ to minimize evaporation and/or prevent leaks.
8. Place the arrays into hybridization oven trays.
9. Load the trays into the hybridization oven.
10. Incubate the arrays at 48°C and 60 rpm for 16 to 18 hours.

Washing and Staining

For additional information about washing, staining, and scanning, refer to the *Genechip™ Expression Wash, Stain, and Scan User Guide for Cartridge Arrays* (Pub. No. 702731) and the *Command Console™ User Guide* (Pub. No. 702569).

1. After 16 to 18 hours of hybridization, remove the arrays from the oven. Remove the Tough-Spots from the arrays.
2. Extract the hybridization cocktail from each array and transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail. Store on ice during the procedure, or at –80°C for long-term storage. Refer to Appendix B, *Array Rehybridization Procedure*, if necessary.
3. Fill each array completely with Array Holding Buffer.
4. Allow the arrays to equilibrate to room temperature before washing and staining.

NOTE: Arrays can be stored in the Array Holding Buffer at 4°C for up to 3 hours before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

5. Place vials into sample holders on the fluidics station:
   a. Place one (amber) vial containing 600 µL Stain Cocktail 1 in sample holder 1.
   b. Place one (clear) vial containing 600 µL Stain Cocktail 2 in sample holder 2.
   c. Place one (clear) vial containing 800 µL Array Holding Buffer in sample holder 3.
6. Wash and stain with Fluidics Station 450 using the appropriate fluidics script for the array format.

Table 3.3 Fluidics Protocols

	Fluidics Station 450 Protocol FS450_0002 (100 format array)	Fluidics Station 450 Protocol FS450_0003 (400/169 format array)
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	8 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
1st Stain	Stain the probe array for 5 minutes with Stain Cocktail 1 (Vial Position 1) at 35°C	Stain the probe array for 10 minutes with Stain Cocktail 1 (Vial Position 1) at 25°C
Post Stain Wash	Wash 10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C	Wash 10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C
2nd Stain	Stain the probe array for 5 minutes with Stain Cocktail 2 (Vial Position 2) at 35°C	Stain the probe array for 10 minutes with Stain Cocktail 2 (Vial Position 2) at 25°C
3rd Stain	Stain the probe array for 5 minutes with Stain Cocktail 1 (Vial Position 1) at 35°C	Stain the probe array for 10 minutes with Stain Cocktail 1 (Vial Position 1) at 25°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C
Array Holding Buffer	Fill the probe array with Array Holding Buffer (Vial Position 3)	Fill the probe array with Array Holding Buffer (Vial Position 3)

7. Check for air bubbles. If there are air bubbles, manually fill the array with Array Holding Buffer. If there are no air bubbles, cover both septa with 3/8" Tough-Spots. Inspect the array glass surface for dust and/or other particulates and, if necessary, carefully wipe the surface with a clean lab wipe before scanning.

Scanning

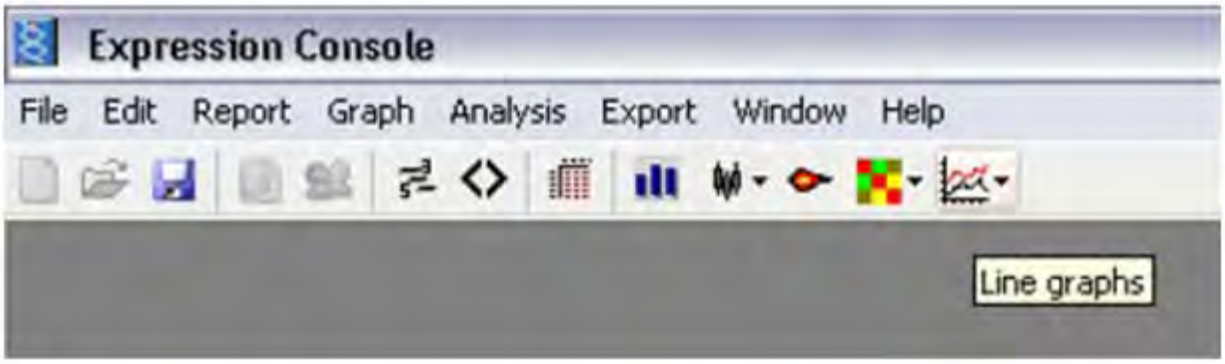
The instructions for using the scanner and scanning arrays can be found in the *Command Console™ User Guide* (Pub. No. 702569).

Analysis

Use Expression Console software for data summarization, normalization, and quality control (v. 1.2 and higher). Please refer to the website for Expression Console for instructions on miRNA Array analysis. We recommend using RMA + DABG for analysis.

To evaluate the success of the labeling protocol and array processing, open line graphs for the spike-in labeling control probe sets in Expression Console. Once the RMA+DABG analysis is complete, click on the icon for line graphs or select **Graph → Line Graph - Report Metrics** and select the check boxes for desired probe sets.

Figure 3.2



If the labeling protocol was successful, the following spike-in control probe sets (representing synthetic miRNAs present in vial 8) should have signal greater than or equal to 1000 (or 9.96 for log2 signal):

- spike_in-control-2_st
- spike_in-control-23_st
- spike_in-control-29_st
- spike_in-control-31_st
- spike_in-control-36_st.

Oligos 2, 23 and 29 are RNA, and confirm the poly(A) tailing and ligation. Oligo 31 is poly(A) RNA and confirms ligation. Oligo 36 is poly(dA) DNA and confirms ligation and the lack of RNases in the RNA sample.

If the array hybridization, wash, stain and scan procedure was successful, the arrays should be gridded successfully and cel and chp files should be generated. The hybridization control probe sets should have signal commensurate with concentration:

AFFX-r2-Ec-c1-BioB-3_at < AFFX-r2-Ec-c1-BioC-3_at < AFFX-r2-Ec-c1-BioD-3_at < AFFX-r2- Ec-c1-cre-3_at

Export the data into third party software for further analysis.

Chapter 4 miRNA Array Plates Procedure

This chapter includes hybridization cocktail preparation instructions for processing miRNA Array Plates using the GeneTitan™ Instrument and the FlashTag Biotin HSR RNA Labeling Assay. The hybridization mix described below was specifically formulated for use with the FlashTag Biotin HSR RNA Labeling Kit P/N 901910 (10 rxn) and P/N 901911 (30 rxn). For instructions on setting up hybridizations on cartridge arrays, please go to *Chapter 3, GeneChip™ miRNA Array Procedure*.

Reagents and Materials Required

- GeneTitan™ Hybridization, Wash and Stain Kit for miRNA Array Plates (P/N 902276, 96 Rxn) consists of:
  - GeneTitan™ Hybridization Module for miRNA Array Plates (P/N 902275)
  - GeneTitan™ Wash Buffers A & B Module (P/N 901583)
- GeneChip™ Hybridization Control Kit (P/N 900457, 150 rxns)

Prepare the Hybridization Cocktail Mix

1. Bring the reagents listed in Step 3, below, to room temperature.
2. Completely thaw and then heat the 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre* from GeneChip™ Eukaryotic Hybridization Control Kit) for 5 minutes at 65°C.
3. Prepare a Hybridization Cocktail Master mix according to Table 4.1 below:

Table 4.1 Hybridization Cocktail (for a single reaction and for multiple reactions)

Component	Volume per miRNA Array	16-Array Plate*	24-Array Plate*	96-Array Plate*	Final Concentration
2X Hybridization Mix	62.5 µL	1100 µL	1650 µL	6600 µL	1X
27.5% Formamide (Vial 12)	18.2 µL	320.3 µL	480.5 µL	1921.9 µL	4%
DMSO	12.1 µL	213 µL	319.4 µL	1277.8 µL	9.7%
20X Hybridization Controls	6.3 µL	110.9 µL	166.3 µL	665.3 µL	1X
Control Oligo B2, 3nM	2.1 µL	37 µL	55.4 µL	221.8 µL	50pM
Nuclease-free Water	2.3 µL	40.5 µL	60.7 µL	242.9 µL	
Total Volume	103.5 µL	1821.7 µL	2732.3 µL	10929.7 µL	

**** Includes ~10% overage to cover pipetting error.***

4. Add 103.5 µL cocktail mix to 21.5 µL of labeled sample for each array.
5. Denature the hybridization cocktail with target at 99°C for 5 minutes, followed by 45°C for 5 minutes.
6. After denaturation, centrifuge hybridization cocktail with target to remove any insoluble material from the hybridization mixture.
7. Carefully transfer 120 µL of the denatured and centrifuged supernatant hybridization target into the appropriate well of the HT Hybridization Tray.
8. Please follow the instructions provided in the *GeneTitan™ Instrument User Guide for Expression Array Plates* (Pub. No. 702933 rev. 1 or higher) to process miRNA array plates on the GeneTitan™ Instrument.

Appendix A
ELOSA QC Assay

The Enzyme Linked Oligosorbent Assay (ELOSA) is designed to provide confirmation that the FlashTag Biotin HSR Labeling Kit has performed appropriately as a biotin labeling process. Specifically, the ELOSA is designed to detect the RNA Spike Control Oligos (Vial 8) included in all FlashTag Biotin HSR labeling reactions. Only 2 µL of the labeling reaction is required for the ELOSA assay. Successful biotin labeling is verified via a simple colorimetric ELOSA assay through the hybridization of the biotin-labeled RNA Spike Control Oligos (Vial 8) to complementary ELOSA Spotting Oligos (Vial 9) immobilized onto microtiter plate wells. The ELOSA Positive Control (Vial 10) confirms the ELOSA assay is working properly.

We suggest that this assay be run prior to the use of any labeling reaction on microarrays to ensure the FlashTag Biotin HSR labeling process worked appropriately with known controls. As an alternative, the 2 µL aliquot from the labeling reaction may be stored at –20°C for up to two weeks and used for troubleshooting, if needed. Please note that this procedure does not assure the performance of any RNA sample on a microarray.

Additional Required Materials

Refer to Appendix C for example preparation and storage.

- Flat bottom Immobilizer™ Amino – 8 well strips

  Nunc P/N 436013 (30 plates)

  Do not use strips or plates from other manufacturers.
- Adhesive plate sealers (VWR P/N 62402-921) or equivalent
- Wash bottle (or washing instrument) for vigorous washing
- 1X PBS
- 1X PBS, 0.02% Tween-20
- 5X SSC, 0.05% SDS, 0.005% BSA (If a precipitate forms in this buffer, warm at 42°C to dissolve. Use at room temperature.)
- 5% BSA in 1X PBS
- 25% dextran sulfate – see Appendix C
- Streptavidin-HRP (Thermo Fisher P/N N100) or equivalent
- TMB Substrate Solution (Thermo Fisher P/N N301) or equivalent
- Optional: TMB Stop Reagent (Thermo Fisher P/N N600) or equivalent
- Optional: Plate reader or instrument capable of reading absorbance at 450 nm

Procedural Notes

- All materials should be nuclease-free, and all reagents should be prepared with nuclease-free components.
- 2 µL of each biotin labeling reaction, will be used in the ELOSA. It is acceptable to store the 2 µL of biotin-labeled sample on ice (up to 6 hours) or at –20°C (up to 2 weeks) and run the ELOSA at a convenient time.
- The ELOSA Positive Control (Vial 10) is already labeled with biotin and should be added to its own well each time the ELOSA assay is run.
- Bring all solutions to room temperature before using them in the ELOSA.
- During all incubation steps, cover the plate with an adhesive plate sealer.
- To blot dry, expel the liquid into a sink, and repeatedly tap the inverted plate on a stack of paper towels. Do not insert laboratory wipes into the ELOSA wells.
- A multichannel pipette (8 or 12 tip) is recommended, but not required.
- Do not touch pipette tips to the bottom of the ELOSA wells at any step of the procedure.
- Vigorous washing is required to minimize non-specific background signals in negative control wells. Vigorous manual washing of the ELOSA wells with a squirt bottle filled with washing buffer is a simple and inexpensive method that works well when performed over a sink; alternatively, an automated washing instrument capable of vigorous washing may be used.

Experimental Design Recommendations

To understand the validity of this ELOSA method, appropriate controls should be included in all ELOSA assays.

- Negative controls should include a FlashTag Biotin HSR labeling reaction that does not contain any RNA Spike Control Oligos (Vial 8). It is optional to include Total RNA in the negative control. This type of control should result in a negative reaction in the ELOSA assay and will define any baseline non-specific background signals. If a Negative control FlashTag Biotin HSR reaction is not run, another acceptable negative control is 50 µL 5X SSC, 0.05% SDS, 0.005% BSA + 2.5 µL 25% Dextran sulfate.
- Spike controls should include a FlashTag Biotin HSR labeling reaction containing both total RNA and the RNA Spike Control Oligos (Vial 8). Labeled samples that have previously demonstrated appropriate reactivity for the ELOSA assay should be used. Labeled samples that have shown appropriate performance on microarrays may also be of value.
- Positive controls should include the ELOSA Positive Control (Vial 10), an oligo which is already biotinylated and confirms the ELOSA is working properly.

Coating Wells with ELOSA Spotting Oligos

1. Dilute the ELOSA Spotting Oligos (Vial 9) 1:50 in 1X PBS according to the table below:

Table A.1

Number of Wells	Total Volume Required	ELOSA Spotting Oligos	1X PBS
3	225 µL	4.5 µL	220.5 µL
12	900 µL	18 µL	882 µL
24	1800 µL	36 µL	1764 µL

2. Add 75 µL of the diluted ELOSA Spotting Oligos to each well of the plate or strip. Avoid touching the bottom of the ELOSA wells with the pipette tip.
3. Cover with an adhesive plate sealer and incubate overnight at 2–8°C. The plates (or wells) may be stored at 2–8°C for up to 2 weeks if covered tightly with an adhesive plate sealer and no evaporation occurs, but for best results, incubate overnight.

Washing and Blocking

These steps may be completed prior to or during the FlashTag Biotin HSR labeling procedure.

1. Remove the ELOSA Spotting Oligos by expelling the liquid into a sink.
2. Wash 2 times with 1X PBS, 0.02% Tween-20, blot dry.
3. Add 150 µL of 5% BSA in 1X PBS to each well.
4. Cover the wells and incubate for 1 hour at room temperature.

Sample Hybridization

1. Make up a Hybridization Master Mix, by adding the following components to a tube and gently vortexing until the dextran sulfate is in solution. It is recommended that a larger master mix volume be made than is needed, as the dextran sulfate is difficult to pipette. Briefly microfuge.

Table A.2 Hybridization Master Mix

Component	Volume
5X SSC, 0.05% SDS, 0.005% BSA (Appendix C)	48.0 µL
25% Dextran sulfate (Appendix C)	2.5 µL

2. For the **positive control**, add 2 µL of vial 10 to 50.5 µL of Master Mix for a total volume of 52.5 µL.
3. For the **negative control**, add 2 µL of water to 50.5 µL of Master Mix for a total volume of 52.5 µL.
4. For each **sample**, add 2.0 µL of biotin labeling reaction to 50.5 µL of Master Mix for a total volume of 52.5 µL.
5. Remove the BSA blocking solution by expelling the liquid into a sink. Blot dry.
6. Add all 52.5 µL of hybridization solution to a designated well.
7. Cover the wells and incubate for 1 hour at room temperature.

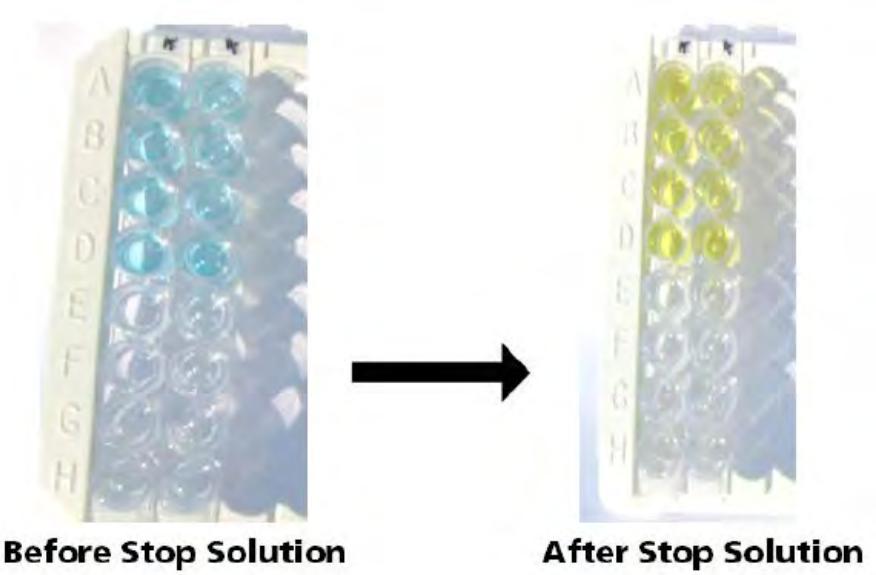
SA-HRP Binding

1. Dilute SA-HRP in 5% BSA in 1X PBS. If using Thermo Scientific SA-HRP, a dilution of 1:4000 to 1:8000 is recommended.
2. Remove the hybridization solution by expelling the liquid into a sink.
3. Wash 3–4 times with 1X PBS, 0.02% Tween 20, blot dry.
4. Add 75 µL of the diluted SA-HRP from Step 1 to each well.
5. Cover the wells and incubate for 30 minutes (up to 2 hours) at room temperature.

Signal Development

1. Remove the SA-HRP by expelling the liquid into a sink.
2. Wash 3 times with 1X PBS, 0.02% Tween-20, blot dry.
3. Add 100 µL of TMB Substrate to each well.
4. Cover the wells and incubate at room temperature for 5-30 minutes in the dark (or covered with aluminum foil).
5. The blue substrate color indicates a positive result and may be used as qualitative results (see Figure A.1).
6. Optional: For instrument quantitation, remove the adhesive plate sealer and add 100 µL Stop Reagent (or equivalent acidic TMB stop reagent) to each well. This will convert the blue substrate to a yellow color (see Figure A.1). Read the absorbance at 450 nm on a plate reader. Readings of greater than 0.10 OD (450 nm) over a negative control should be considered positive. Typically, this assay generates positive results of at least 0.15–1.00 OD when working appropriately.

Figure A.1



7. After a successful ELOSA QC Assay, proceed to *Chapter 3, GeneChip™ miRNA Array Procedure*.

Appendix B
Array Rehybridization Procedure

Follow the procedure below if it is necessary to rehybridize another GeneChip™ miRNA Array.

1. Record the volume of recovered hybridization cocktail from *Washing and Staining*, Step 2.
2. Prepare a 1X Hyb Mix:

Table B.1

Component	Volume for 400/169 Format Array (miRNA 1.0 and 2.0 Arrays):	Volume for 100 Format Array (miRNA 3.0 and later designs):
Nuclease-Free Water (Vial 11)	21.5 µL	25.2 µL
2X Hybridization Mix (from GeneChip™ Hyb, Wash and Stain Kit, P/N 900720)	50 µL	66 µL
27.5% Formamide (Vial 12)	15 µL	19.2 µL
DMSO (from GeneChip™ Hyb, Wash and Stain Kit, P/N 900720)	10 µL	12.8 µL
20X Eukaryotic Hybridization Controls *bioB, bioC, bioD, cre* (from GeneChip™ Eukaryotic Hybridization Control Kit, P/N 900454)	5 µL	6.6 µL
Control Oligonucleotide B2, 3nM (P/N 900301)	1.7 µL	2.2 µL

3. Adjust the volume of recovered hybridization cocktail (Step 1) to 103.2 µL (169 format array) or 132 µL (100 format array) with 1X Hyb Mix (Step 2, above).
4. Follow the hybridization instructions to complete the hybridization process.
5. Continue with *Washing and Staining*.

Appendix C
Example Reagent Preparation and Storage

For all of the reagents below, it is important to remove the amount that is needed for the day (or step of the protocol) by carefully pouring off or using a long pipette to avoid contamination of the stock buffer. All components should be nuclease-free and stored in nuclease-free tubes or bottles. Recommended suppliers and part numbers are listed. Equivalent suppliers may be used for reagents other than BSA. We strongly recommend BSA from Sigma (Sigma P/N A3294).

1mM Tris (50 mL)

- Transfer 50 mL nuclease-free water (P/N AM9932) to a 50 mL conical tube.
- Remove and discard 50 µL water.
- Add 50 µL of 1M Tris-HCl, pH 8 (P/N 22638).
- After this dilution is made, do not take a pH reading.
- Store at room temperature up to 3 months.

25% Dextran Sulfate (10 mL)

- Slowly pour 5 mL 50% dextran sulfate (Millipore P/N S4030) into a 15 mL conical tube.
- Add 5 mL nuclease-free water (P/N AM9932) and vortex thoroughly.
- Store at room temperature up to 3 months.

1X PBS (1L)

- 100 mL 10X PBS pH 7.4 (Thermo Fisher Scientific P/N AM9625)
- 900 mL nuclease-free water (P/N AM9932)
- Store at room temperature up to 3 months.

1X PBS, 0.02% Tween-20 (1L)

- 100 mL 10X PBS pH 7.4 (P/N AM9625)
- 0.2 mL Tween-20 (200 µL) (Sigma P/N P-9416)
- Add water to a final volume of 1L.
- Store at room temperature up to 3 months.

5% BSA in 1X PBS (40 mL)

- Transfer 2g of powdered BSA (Sigma P/N A3294) to a 50 mL conical tube.
- Slowly add 1XPBS to a final volume of 40 mL.
- Shake or vortex to mix.
- Make 8 aliquots of 5 mL.
- Store each aliquot at –20°C, up to 6 months. Do not freeze/thaw each 5 mL aliquot more than 4 times.
- Once thawed, store one aliquot at 4°C for 1 week.

5X SSC, 0.05% SDS, 0.005% BSA (10mL)

- 2.5 mL 20X SSC (P/N AM9763)
- 0.05 mL 10% SDS (50 µL) (P/N AM9823)
- 0.01 mL 5% BSA in 1XPBS (10 µL)
- Add water to a final volume of 10 mL.
- Make 10 aliquots of 1 mL.
- Store each aliquot at –20°C, up to 6 months. Do not freeze/thaw each 1 mL aliquot more than 4 times.
- Once thawed, store one aliquot at 4°C for 1 week.
- If a precipitate forms in this buffer, warm at 42°C to dissolve. Use at room temperature.

Documentation and support

Obtaining support

Technical support

For the latest services and support information for all locations, visit **www.thermofisher.com**.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**thermofisher.com/support)**
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **thermofisher.com/support**.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.