



User Manual

FlashTag™ Biotin HSR RNA Labeling Kit

For Affymetrix® GeneChip® miRNA Arrays

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Contents

Chapter1	Introduction	4
	Background Information	4
	Procedure Overview	5
	Materials Required	6
	FlashTag™ Biotin HSR RNA Labeling Kit	6
	Other Required Materials	7
	RNA Sample and Quantitation	7
	RNA Isolation	8
	Quantitation	8
	RNA Input for FlashTag™ Biotin HSR	8
Chapter2	FlashTag™ Biotin HSR RNA Labeling Procedure	9
	Poly (A) Tailing	9
	FlashTag™ Biotin HSR Ligation	10
Chapter3	Affymetrix® GeneChip® miRNA Array Procedure	11
	Preparation of Ovens, Arrays, and Sample Registration Files	11
	Hybridization	11
	Washing and Staining	12
	Scanning	13
	Analysis	13
Appendix A	ELOSA QC Assay	16
	Additional Required Materials	16
	Procedural Notes	16
	Experimental Design Recommendations	17
	Coating Wells with ELOSA Spotting Oligos	17
	Washing and Blocking	17
	Sample Hybridization	18
	SA-HRP Binding	18
	Signal Development	18
Appendix B	Array Rehybridization Procedure	20
Appendix C	Example Reagent Preparation and Storage	21

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 Affymetrix® 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 on page 5](#)). 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 Affymetrix GeneChip miRNA Arrays are listed [on page 6](#). 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.

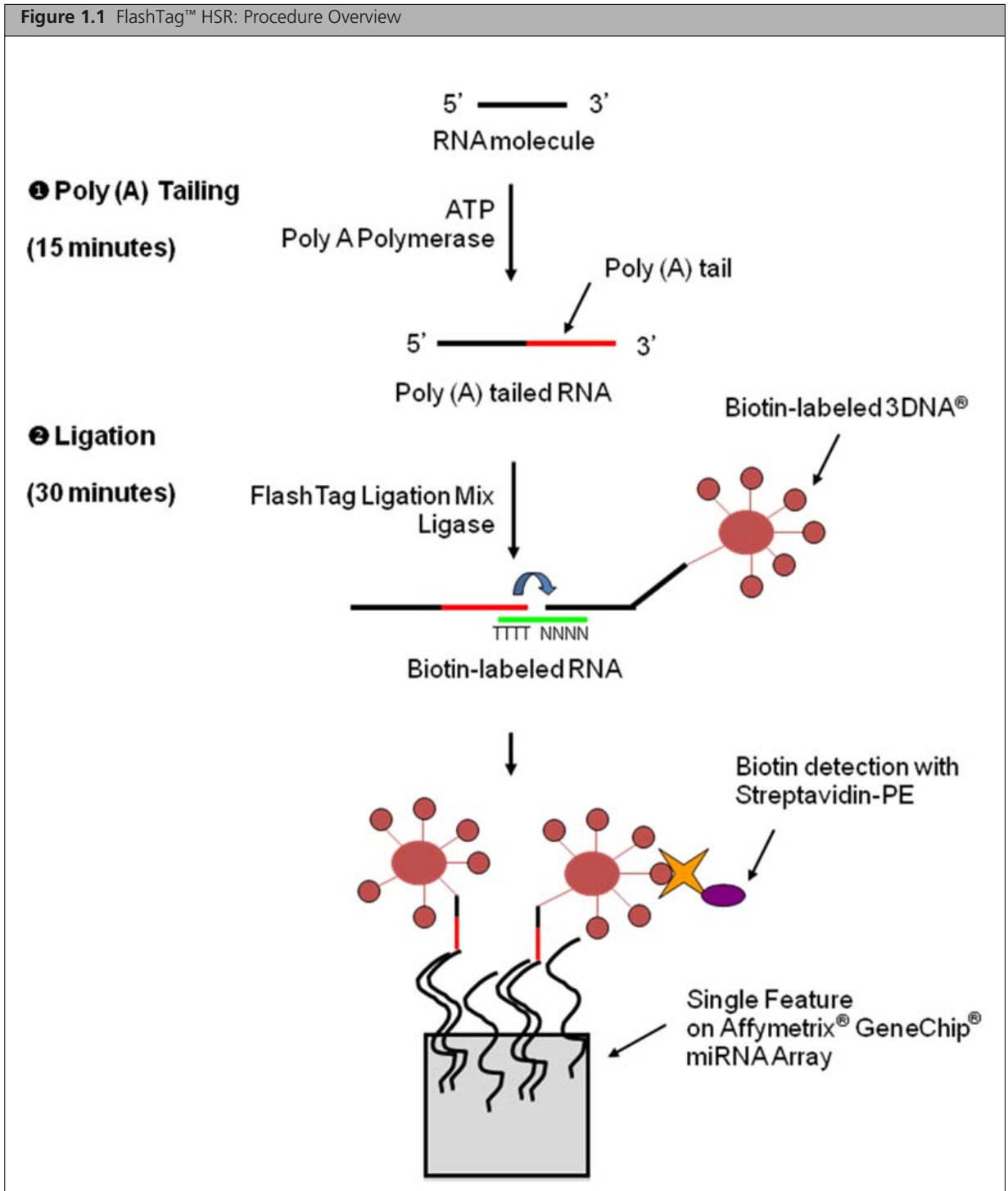
⁷ Thomson, JM. et al. *A Custom Microarray Platform for Analysis of MicroRNA Gene Expression*. *Nature Methods*. 2004, 1(1) 47-53.

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



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 MnCl ₂	-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 on page 7*)
- Nuclease-free water (Applied Biosystems P/N AM9932 or equivalent)
- 1mM Tris ([Appendix C](#))
- Reagents and materials for analysis by Affymetrix GeneChip miRNA Array:
 - Affymetrix miRNA Array:
 - GeneChip® miRNA Array (Affymetrix P/N 901324, P/N 901325, or P/N 901326)
 - GeneChip® miRNA 2.0 Array (Affymetrix P/N 901753, P/N 901754, or P/N 901755)
 - GeneChip® miRNA 3.0 Array (Affymetrix P/N 902017, P/N 902018, or P/N 902019)
 - Reagents for ELOSA QC Assay: Refer to [Appendix A](#), *ELOSA QC Assay on page 16* and [Appendix C](#), *Example Reagent Preparation and Storage on page 21*.
 - GeneChip® Eukaryotic Hybridization Control Kit (Affymetrix P/N 900454)
 - If necessary, Control Oligonucleotide B2, 3nM (included in Hybridization Control Kit) can be ordered separately (Affymetrix P/N 900301)
 - GeneChip® Hybridization, Wash and Stain Kit (Affymetrix P/N 900720)
 - If necessary, Wash Buffer A (included in Hybridization, Wash and Stain Kit) can be ordered separately (Affymetrix P/N 900721)
 - If necessary, Wash Buffer B (included in Hybridization, Wash and Stain Kit) can be ordered separately (Affymetrix P/N 900722)
 - Laser Tough-Spots® 3/8" diameter (Diversified Biotech P/N SPOT-1000)
 - Laser Tough-Spots® 1/2" diameter (Diversified Biotech P/N SPOT-2000)
 - Affymetrix® GeneChip Command Console® Software (AGCC)
 - miRNA QC Tools v 1.0.33.0 (miRNA 1.0 array) or v 1.1.1.0 (miRNA 2.0 array)
 - Affymetrix® Expression Console™ Software (EC) v1.2 or higher (miRNA 2.0 and 3.0 Arrays)
 - GeneChip® Fluidics Station 450 (Affymetrix P/N 00-0079)
 - GeneChip® Hybridization Oven 645
 - GeneChip® Scanner 3000 7G

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:

- Applied Biosystems: mirVana™ miRNA Isolation Kit
- Applied Biosystems: RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE
- QIAGEN: miRNeasy Mini Kit
- Invitrogen: PureLink™ miRNA Isolation Kit
- Invitrogen: TRIzol® reagent (total RNA only) with additional overnight –20°C precipitation step during isopropanol precipitation¹

Quantitation

To accurately determine the concentration of the RNA sample, Affymetrix recommends the use of the Quant-iT™ RiboGreen RNA Assay Kit (Invitrogen 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 Arrays)
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 β -Site Amyloid Precursor Protein-Cleaving Enzyme. *The Journal of Neuroscience*, January 30, 2008, 28(5):1213-1223

FlashTag™ Biotin HSR RNA Labeling Procedure

To confirm target labeling, Affymetrix® suggests running an ELOSA QC Assay prior to array hybridization. Refer to [Appendix A, ELOSA QC Assay on page 16](#). 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:

$$5000 \div \text{ng input LMW RNA}$$
 Example: If using 100 ng 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 500 ng total RNA, the dilution factor is $1000 \div 0.5 = 2000$. 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 (for a single reaction)

Component	Amount
10X Reaction Buffer (Vial 1)	1.5 µL
25mM MnCl ₂ (Vial 2)	1.5 µL
Diluted ATP Mix (Vial 3 dilution from Step 3)	1.0 µL
PAP Enzyme (Vial 4)	1.0 µL

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 μ L of tailed RNA and place on ice.
2. Add 4 μ L 5X FlashTag Biotin HSR Ligation Mix (Vial 5) to each sample.
3. After the Ligation Mix has been added, add 2 μ L 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 μ L HSR Stop Solution (Vial 7). Mix and microfuge the 23.5 μ L of ligated sample.
7. Remove 2 μ L of the biotin-labeled sample for use with the ELOSA QC Assay ([Appendix A](#)). It is acceptable to store the 2 μ L 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 μ L 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 μ L 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 Affymetrix GeneChip miRNA Arrays.

Affymetrix® 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 Affymetrix® GeneChip® Command Console® (AGCC) software using the Command Console Library File Importer tool (see Table 3.1). The files can be found at: www.affymetrix.com

Table 3.1

Array Type	Library File
GeneChip miRNA Array	miRNA 1_0_2Xgain
GeneChip miRNA 2.0 Array	miRNA 2_0
GeneChip miRNA 3.0 Array	miRNA 3_0

2. Turn Affymetrix® 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 *Affymetrix® Command Console® User Manual* (P/N 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 3.2 to the 21.5 µL biotin-labeled sample in the order listed, to prepare the array hybridization cocktail:

Table 3.2 Hybridization Cocktail (for a single reaction)

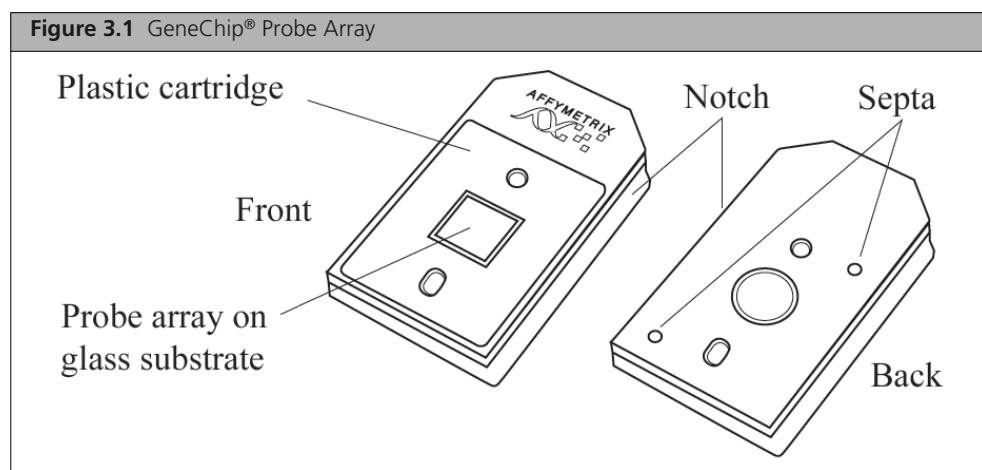
Component	Volume for a 400/169 Format Array (miRNA 1.0 and 2.0 Arrays)	Volume for a 100 Format Array (miRNA 3.0 Arrays)	Final Concentration
2X Hybridization Mix	50 µL	66 µL	1X
27.5% Formamide (Vial 12)	15 µL	19.2 µL	4%
DMSO	10 µL	12.8 µL	9.7%
20X Hybridization Controls	5 µL	6.6 µL	1X
Control Oligo B2, 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 3.2](#) may be prepared by multiplying the volumes by the number of arrays plus 10% overage to cover pipetting error. Refer to [Table 3.3](#) for appropriate Hybridization Master Mix volumes to add to the biotin-labeled sample

Table 3.3 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 Array)
Biotin-labeled Sample	21.5 μ L	21.5 μ L
Hybridization Master Mix	81.7 μ L	110.5 μ L

4. Incubate at 99°C for 5 minutes, then 45°C for 5 minutes.
5. Aspirate 100 μ L (400/169 format array) or 130 μ L (100 format array) and inject into an 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 Manual for Cartridge Arrays* (PN 702731) and the *Affymetrix® Command Console® User Manual* (P/N 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 on page 20](#), 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 3.4 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 *Affymetrix® Command Console® User Manual* (P/N 702569).

Analysis

Use the following free software for data summarization, normalization, and quality control. There are two software packages on the website. Please choose the correct software package and version depending on the array type:

Table 3.5

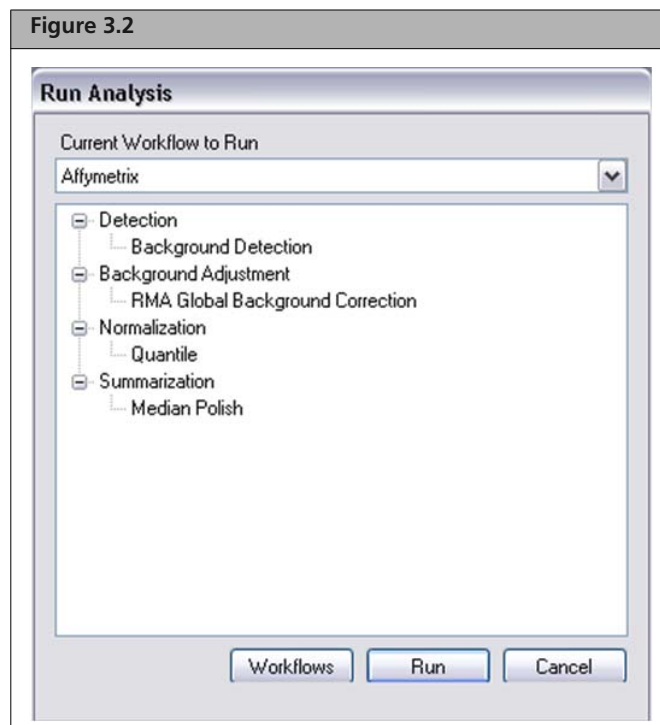
Array Type	Software
GeneChip® miRNA 1.0 Array	miRNA QC Tool Version 1.0.33.0
GeneChip® miRNA 2.0 Array	miRNA QC Tool Version 1.1.1.0/ Expression Console 1.2 or higher
GeneChip® miRNA 3.0 Array	Expression Console 1.2 or higher

■ **For miRNA QC Tool:**

If using miRNA QC Tool, both versions of the miRNA QC Tool cannot exist on the same computer. If you are planning on using both versions of the miRNA arrays, please install versions 1.0.33.0 and 1.1.10 on separate computers.

If you have previously installed miRNA QC Tool version 1.0.33.0 and are now running miRNA 2.0 arrays, please uninstall/remove the program before installing miRNA QC Tool version 1.1.10. After installation, reboot your computer before analyzing miRNA 2.0 array CEL files.

Affymetrix recommends the following workflow in miRNA QC Tool:



To edit the workflow:

1. Open QCTool software.
2. Click **Cancel** (do not load any CEL files).
3. Select **Tools** → **Workflow Editor**.
4. Select **File** → **New**.
5. Type a name for the new workflow.
6. Add the procedures (listed above) by selecting them on the left, and clicking the middle “arrow” button.
7. Select **File** → **Save**.

8. Click **OK**.
9. Click **Close** to close the editor.

Using miRNA QC Tool software, look for Vial 8, RNA Spike Control Oligos in the Quality Control Table (Select **Tables** → **Quality Control**). The Affymetrix library file lists the following names for these probe sets:

- 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

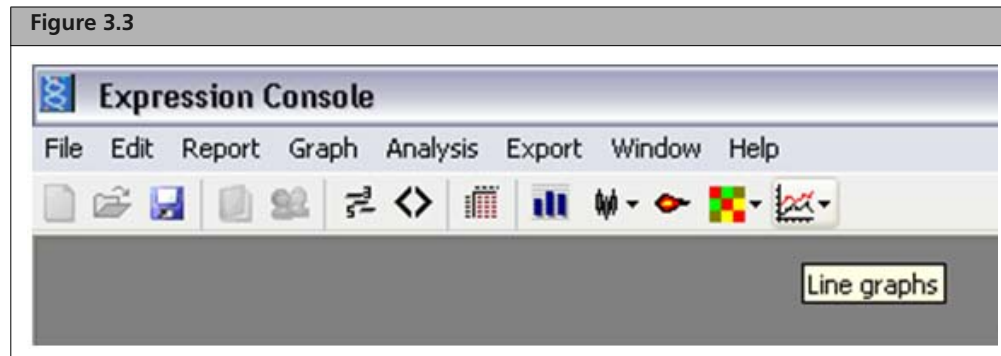
Each probe set should show >1000 units (signal-background).

Export the data into third party software for further analysis.

■ **For Expression Console:**

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. Affymetrix recommends 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.



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.

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 μ L 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.

Affymetrix suggests 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 μ L 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 Scientific / Pierce P/N N100) or equivalent
- TMB Substrate Solution (Thermo Scientific / Pierce P/N N301) or equivalent
- Optional: TMB Stop Reagent (Thermo Scientific / Pierce 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 μ L of each biotin labeling reaction ([Step 6 on page 10](#)), will be used in the ELOSA. It is acceptable to store the 2 μ L 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 μ L
25% Dextran sulfate (Appendix C)	2.5 μ L

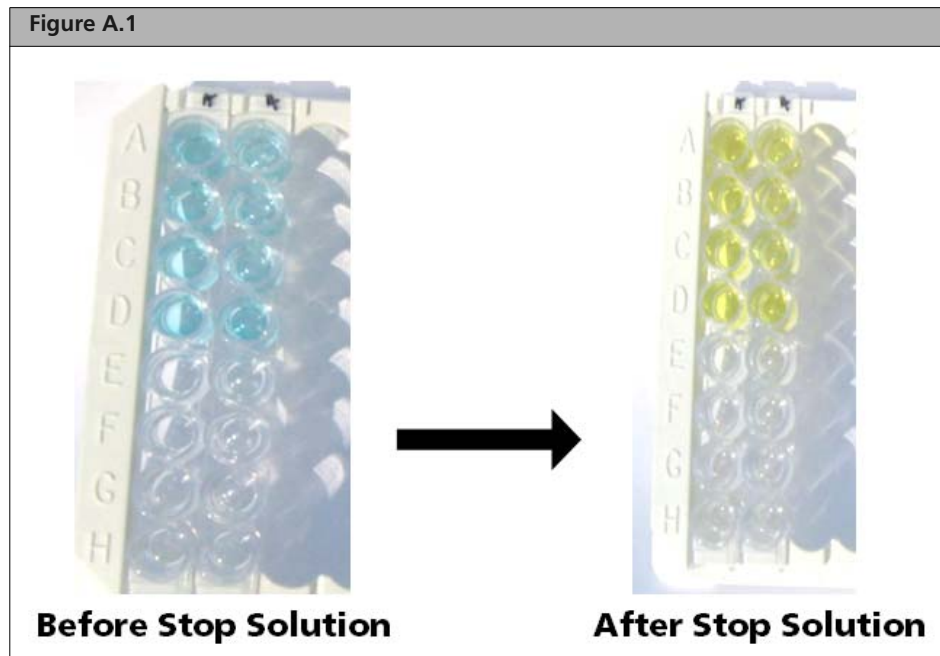
2. For the **positive control**, add 2 μ L of vial 10 to 50.5 μ L of Master Mix for a total volume of 52.5 μ L.
3. For the **negative control**, add 2 μ L of water to 50.5 μ L of Master Mix for a total volume of 52.5 μ L.
4. For each **sample**, add 2.0 μ L of biotin labeling reaction (Step 6 on page 10) to 50.5 μ L of Master Mix for a total volume of 52.5 μ L.
5. Remove the BSA blocking solution by expelling the liquid into a sink. Blot dry.
6. Add all 52.5 μ L 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 μ L 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 μ L 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 μ L 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.



7. After a successful ELOSA QC Assay, proceed to [Chapter 3, Affymetrix® GeneChip® miRNA Array Procedure](#) on page 11.

Array Rehybridization Procedure

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

1. Record the volume of recovered hybridization cocktail from [page 12, *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 Arrays):
Nuclease-Free Water (Vial 11)	21.5 µL	25.2 µL
2X Hybridization Mix (from GeneChip® Hyb, Wash and Stain Kit, Affymetrix 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, Affymetrix P/N 900720)	10 µL	12.8 µL
20X Eukaryotic Hybridization Controls <i>bioB</i> , <i>bioC</i> , <i>bioD</i> , <i>cre</i> (from GeneChip® Eukaryotic Hybridization Control Kit, Affymetrix P/N 900454)	5 µL	6.6 µL
Control Oligonucleotide B2, 3nM (Affymetrix 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 [on page 11](#) to complete the hybridization process.
5. Continue with [Washing and Staining on page 12](#).

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 (Applied Biosystems P/N AM9932) to a 50 mL conical tube.
- Remove and discard 50 μ L water.
- Add 50 μ L of 1M Tris-HCl, pH 8 (USB 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 (10mL)

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

1X PBS (1L)

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

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

- 100mL 10X PBS pH 7.4 (Applied Biosystems P/N AM9625)
- 0.2mL Tween-20 (200 μ L) (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 (40mL)

- 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 (Applied Biosystems P/N AM9763)
- 0.05mL 10% SDS (50 μ L) (Applied Biosystems P/N AM9823)
- 0.01mL 5% BSA in 1XPBS (10 μ L)
- 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.