applied biosystems

# FlashTag™ Biotin HSR RNA Labeling Kit

For miRNA Array Strips

User Manual



ThermoFisher SCIENTIFIC

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P/N 703112

# 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 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 1.7 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 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.

**NOTE: The GeneAtlas™ miRNA Expression Kit Assay is not compatible with array strips designed to focus on the 3' ends of transcripts or WT transcripts. For the 3' arrays, please use the GeneAtlas™ 3' IVT PLUS Kit and for WT array strips, please use The GeneAtlas™ WT PLUS *Kit.***

[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.*

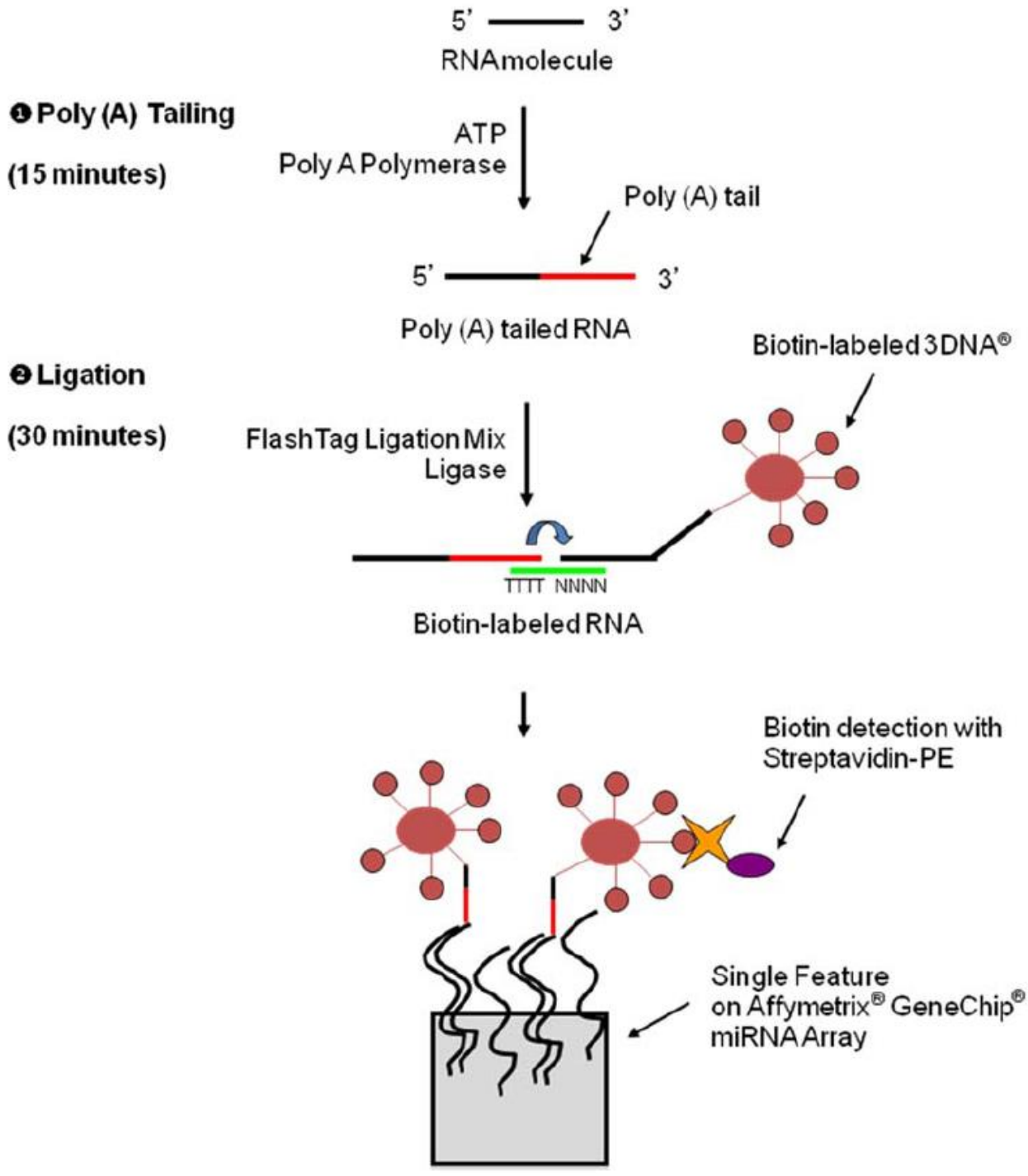
[8] *Ambros, V. The functions of animal microRNAs. Nature. 2004, 431:350.*

[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 $MnCl_2$ | –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.**

**Table 1.2** Other Required Materials

| Material | Source | P/N |
|---|---|---|
| RNA sample containing low molecular weight (LMW) RNA | (see *RNA Sample and Quantitation*) | |
| Nuclease-free water | Applied Biosystems | AM9932 or equivalent |
| 1mM Tris | See Appendix C | |
| 1M Tris-HCl, pH 8 | USB | 22638 |

## GeneAtlas™ Hybridization, Wash and Stain Kit for miRNA Array Strips

**Table 1.3** GeneAtlas™ Hybridization, Wash, and Stain Kit for miRNA Array Strips (P/N 902134)*

| Component | Storage |
|---|---|
| **GeneAtlas™ Hybridization and Stain Module for miRNA Array Strips (P/N 902135)** | |
| 2X Hybridization Mix | 2°C to 8°C |
| DMSO | 2°C to 8°C |
| Nuclease Free Water | 2°C to 8°C |
| Stain Cocktail 1 | 2°C to 8°C |
| Stain Cocktail 2 | 2°C to 8°C |
| Array Holding Buffer | 2°C to 8°C |
| **GeneAtlas™ Wash Buffers (P/N 901625)** | |
| Wash Buffer A (P/N 901544) | 2°C to 8°C |
| Wash Buffer B (P/N 901545) | 2°C to 8°C |

**The GeneAtlas Hybridization, Wash and Stain Kit for miRNA Array Strips (P/N 902134) consists of P/Ns 902135, 901544, and 901545. Part number 902135 is not available for purchase separately.*

**Table 1.4** Reagents and Materials for Analysis by miRNA Array Strips

| Material | Source | P/N |
|---|---|---|
| miRNA Array Strips* | Thermo Fisher | Various. See website. |
| GeneAtlas™ Hybridization, Wash and Stain Kit for miRNA Array Strips† | Thermo Fisher | 902134 |
| GeneChip™ Eukaryotic Hybridization Control Kit‡ | Thermo Fisher | 900454 |

**Do not use strips or plates from other manufacturers.*

*†If necessary, Gene Atlas Wash Buffers A and B Module (included in Hybridization, Wash and Stain Kit) can be ordered separately (P/N 901625; contains Wash Buffer A, P/N 901544 and Wash Buffer B, P/N 901545)*

*‡If necessary, Control Oligonucleotide B2, 3nM (included in Hybridization Control Kit) can be ordered separately (P/N 900301).*

**Table 1.5** Reagents for ELOSA QC Assay (Refer to Appendix A, *ELOSA QC Assay* and *Appendix C, Example Reagent Preparation and Storage*)

| Material | Source | P/N |
|---|---|---|
| Flat bottom Immobilizer™ Amino - 8 well strips | Nunc | 436013 (30 plates) |
| Adhesive plate sealers | VWR | 62402-921 or equivalent |
| Wash bottle (or washing instrument) | Various | |
| 1X PBS<br>1X PBS, 0.02% Tween-20<br>5X SSC, 0.05% SDS, 0.005% BSA<br>5% BSA in 1X PBS<br>25% dextran sulfate | Appendix C | |
| Streptavidin-HRP | Thermo Scientific / Pierce | N100 or equivalent |
| TMB Substrate Solution | Thermo Scientific / Pierce | N301 or equivalent |
| Optional: TMB Stop Reagent | Thermo Scientific / Pierce | N600 or equivalent |
| Optional: Plate reader or instrument capable of reading absorbance at 450 nm | | |

## Instruments and Software

**Table 1.6** Instruments and Software

| Instruments | Manufacturer | P/N |
|---|---|---|
| GeneAtlas™ Instrument Control Software v1.0.6 or higher | Thermo Fisher | |
| Expression Console™ Software (EC) v1.2 or higher | Thermo Fisher | |
| GeneAtlas™ Fluidics Station | Thermo Fisher | 00-0377 |
| GeneAtlas™ Imaging Station | Thermo Fisher | 00-0376 |
| GeneAtlas™ Hybridization Station | Thermo Fisher | 00-0380 (115VAC)<br>00-0381 (230VAC) |
| GeneAtlas™ Workstation | Thermo Fisher | 00-0894 |
| GeneAtlas™ Barcode Scanner | Thermo Fisher | 00-0379 |

# 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]

## 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 precipitation[3]

## Quantitation

To accurately determine the concentration of the RNA sample, we recommend 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 1.7 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.

**Table 1.7**

| RNA Sample | Input for FlashTag Biotin HSR Labeling for one miRNA Array |
|---|---|
| Total RNA containing LMW RNA | 130 - 1000 ng total RNA |
| Enriched LMW RNA, quantitated | 130 - 400 ng LMW RNA |
| Enriched LMW RNA, not quantitated | Enriched from 130 - 1000 ng total RNA |

[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*

[3] *Wang et al. The Expression of MicroRNA miR-107 Decreases Early in Alzheimer's Disease and May Accelerate Disease Progression through Regulation of ?-Site Am-yloid 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, suggests 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 (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 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 miRNA Arrays.

# Chapter 3 miRNA Array Strip Procedure

This chapter outlines the basic steps involved in hybridizing your array strip(s) on the GeneAtlas™ System. The three major steps involved in array strip hybridization are:

- *GeneAtlas™ Software Setup*
- *Array Strip Hybridization*
- Hybridization of Array Strips on the GeneAtlas™ System

**NOTE: If you are re-hybridizing previously made hybridization cocktail refer to Appendix B.**

**IMPORTANT: Before preparing hybridization ready samples, register samples as described in *Sample Registration.***

## GeneAtlas™ Software Setup

Prior to setting up the target hybridization and processing the Array Strips on the GeneAtlas™ System, each array strip must be registered and hybridizations setup in the GeneAtlas Software.

- **Sample Registration**: Sample registration enters array strip data into the GeneAtlas Software and saves and stores the Sample File on your computer. The array strip barcode is scanned, or entered, and a Sample Name is input for each of the four samples on the array strip. Additional information includes Probe Array Type and Probe Array position.
- **Hybridization Software Setup**: During the Hybridization Software Setup the array strip to be processed is scanned, and the GeneAtlas Hybridization Station is identified with hybridization time and temperature settings determined from installed library files.

### Sample Registration

The following information provides general instructions for registering Array Strips in the GeneAtlas Software. For detailed information on Sample Registration, importing data from Excel and information on the wash, stain and scan steps, please refer to the *GeneAtlas™ System User's Guide* (P/N 08-0246).

**NOTE: The Download Library Files function lets you download the latest library files from the NetAffx website.**

**You will need to create a NetAffx account if you have not already done so. To download libraries:**

**From the Utility Actions menu, select Download Library Files and download the respective miRNA library files before proceeding to sample registration**

1. Click **Start → Programs → Affymetrix→ GeneAtlas** to launch the GeneAtlas Software.
2. Click the Registration tab.

   Figure 3.1 appears.

**Figure 3.1** Registration Tab of GeneAtlas™ Software


3. Click the **+ Strip** button: 

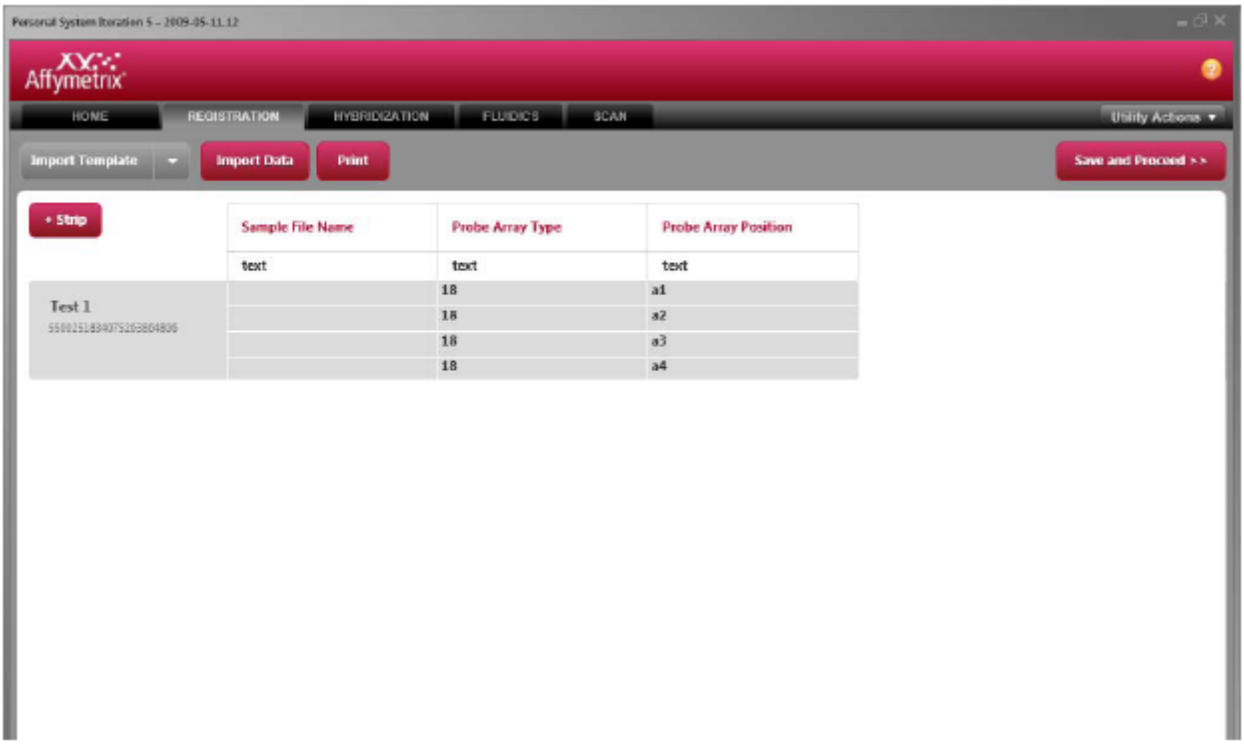
.

   The Add Strip Window appears (Figure 3.2).

**Figure 3.2** Add Strip Window


4. Enter or scan the array strip **Bar Code** and enter a **Strip Name**, then click **Add**. The array strip is added and appears in the Registration window (Figure 3.3)

**Figure 3.3** Array Strip added to Registration window




5. Under the **Sample File Name** column, click in the box and enter a sample name and press **Enter**. Enter a unique name for each of the four samples on the array strip.
6. When complete click the **Save and Proceed** button: 

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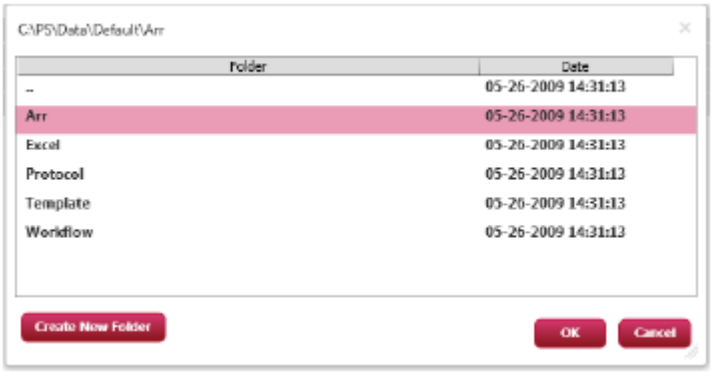
   The Save dialog box appears (Figure 3.4).

**Figure 3.4** Save Dialog




7. In the Save dialog box, click to select a folder in which to save your data. Click **OK**.

   Your files are saved to the selected folder and a confirmation message appears (Figure 3.5).

**Figure 3.5**


8. Click **OK** to register additional array strips, or click **Go to Hybridization**.

**NOTE: You may enter a total of four array strips during the registration process. To add additional strips please repeat Step 3 through Step 8.**

9. Proceed to *Hybridization Software Setup*.

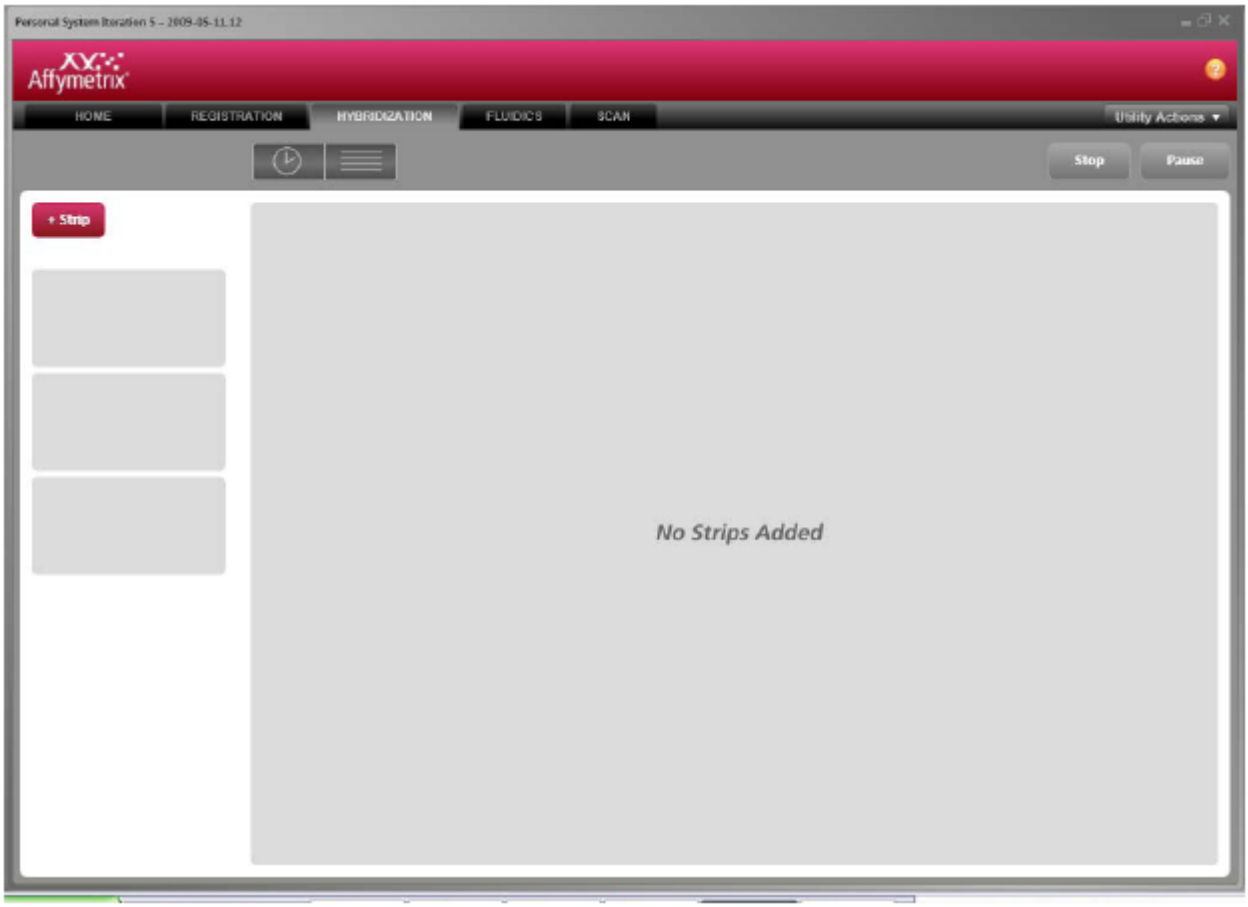
## Hybridization Software Setup

All Array Strips to be processed must first be registered prior to setting up the hybridizations in the GeneAtlas Software. Refer to *Sample Registration* for instruction on registering array strips.

**IMPORTANT: When hybridizing more than one array strip per day, it is recommended to keep the hybridization time consistent. Set up hybridizations for one array strip at a time, staggered by 2 hours so that washing and staining can occur immediately after completion of hybridization for each array strip the next day. Recommended hybridization time is 20 ± 1 hour.**

1. Navigate to the **Hybridization** tab on the GeneAtlas Software interface.

**Figure 3.6** Hybridization window

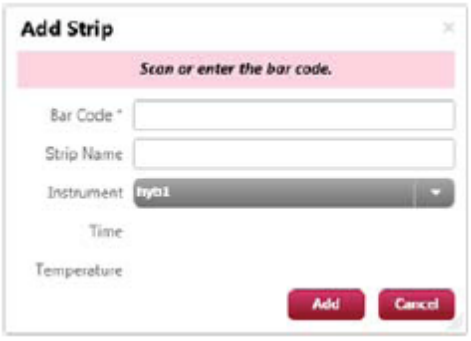



2. Click the **+ Strip** button: 

.

The Add Strip Window appears (Figure 3.7).

**Figure 3.7**



3. Scan or enter the **Bar Code** (required) of the array strip you registered.

   The **Strip Name** field is automatically populated.

4. Click the **Add** button in Figure 3.7.
5. **DO NOT click the Start button at this time**. Proceed to *Array Strip Hybridization*.

## Array Strip Hybridization

This section provides instruction for setting up array hybridizations using the GeneAtlas™ Hybridization, Wash, and Stain Kit for miRNA Array Strips, (60 rxns). For ordering information please refer to *GeneAtlas™ Hybridization, Wash and Stain Kit for miRNA Array Strips*.

1. In preparation of the hybridization step, prepare the following:
   1. Pull the array strip from storage at 4°C so that it can begin to equilibrate to room temperature.
   2. Gather one (1) hybridization tray per array strip.
   3. Set the temperature of the GeneAtlas Hybridization Station to 48°C. Press the Start button.
2. Bring the reagents listed in Step 4, below, to room temperature.
3. 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.
4. 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 one miRNA Array | Final Concentration |
|---|---|---|
| 2X Hybridization Mix | 62.5 µL | 1X |
| 27.5% Formamide (Vial 12) | 18.2 µL | 4% |
| DMSO | 12.1 µL | 9.7% |
| 20X Hybridization Controls | 6.3 µL | 1X |
| Control Oligo B2, 3nM | 2.1 µL | 50 pM |
| Nuclease-free Water | 2.3 µL | |
| **Total Volume** | **103.5 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**

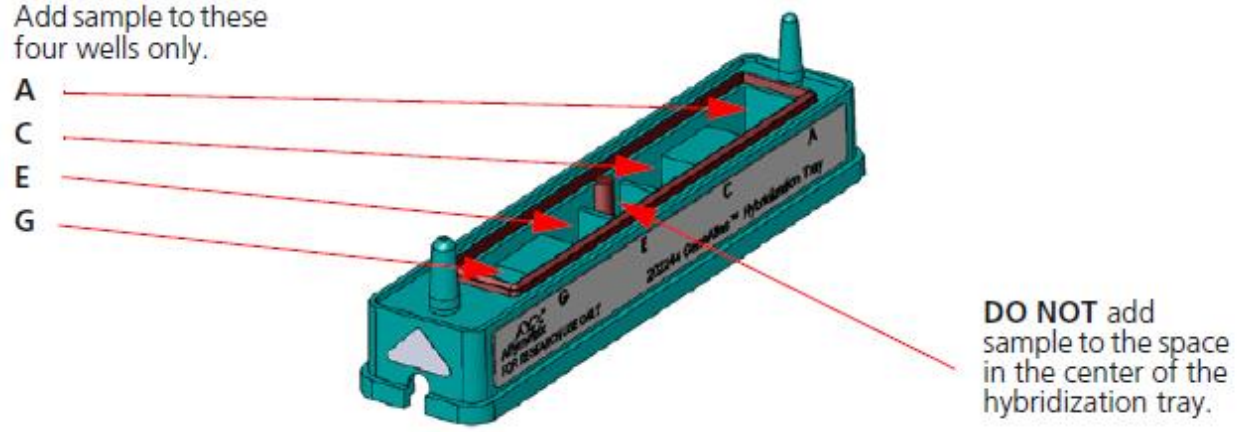
**Table 3.2** Hybridization Cocktail Using a Hybridization Master Mix

| Component | Volume |
|---|---|
| Biotin-labeled Sample | 21.5 µL |
| Hybridization Master Mix | 103.5 µL |

5. Denature the hybridization cocktail with target at 99°C (1.5 mL tubes) or 95°C (thermocycler plates) for 5 minutes, followed by 45°C for 5 minutes.
6. Array Strip Sample Hybridization.
    1. Apply 120 µL of hybridization cocktail to the middle of the appropriate wells of a new clean hybridization tray (see Figure 3.8).

**IMPORTANT:** **Do not add more than 120 µL of hybridization cocktail to the wells as that could result in cross-contamination of the samples.**

**Figure 3.8** Location of the sample wells on the hybridization tray




    2. Carefully remove the array strip and protective cover from its foil pouch and place on bench (Figure 3.9).

**IMPORTANT:** **Leave array strip in protective cover.**

**Figure 3.9** Array Strip in protective tray


3. Place the array strip into the hybridization tray containing the hybridization cocktail samples (Figure 3.10). Refer to Figure 3.11 for proper orientation of the array strip in the hybridization tray.

**Figure 3.10** Placing the array strip into the hybridization tray

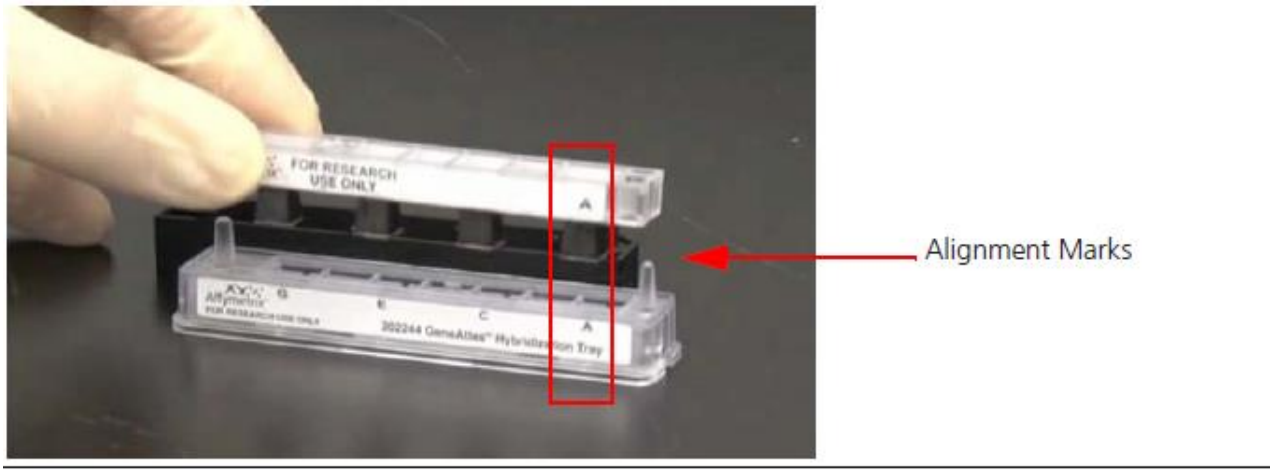
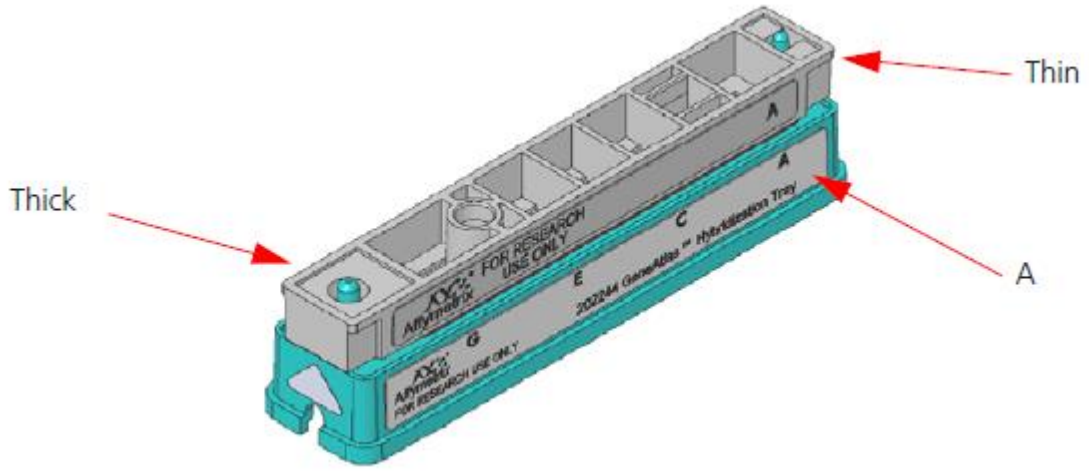


**Figure 3.11** Proper Orientation of the Array Strip in the Hybridization Tray




4. Optional: the remainder of the hybridization cocktail Master Mix can be stored at –20°C to supplement Hybridization Cocktail volume should a rehybridization be necessary.

**CAUTION:** **Be very careful not to scratch/damage the array surface.**

**TIP:** **To avoid any possible mix-ups, the hybridization tray and array strip should be labeled on the white label if more than 1 array strip is processed overnight.**

5. Bring the hybridization tray to just above eye level and look at the underside of the hybridization tray to check for bubbles.

**CAUTION:** **Be careful not to tip the hybridization tray to avoid spilling.**

**IMPORTANT:** **Insertion of the array strip and air bubble removal should be performed quickly to avoid drying of the array surface.**

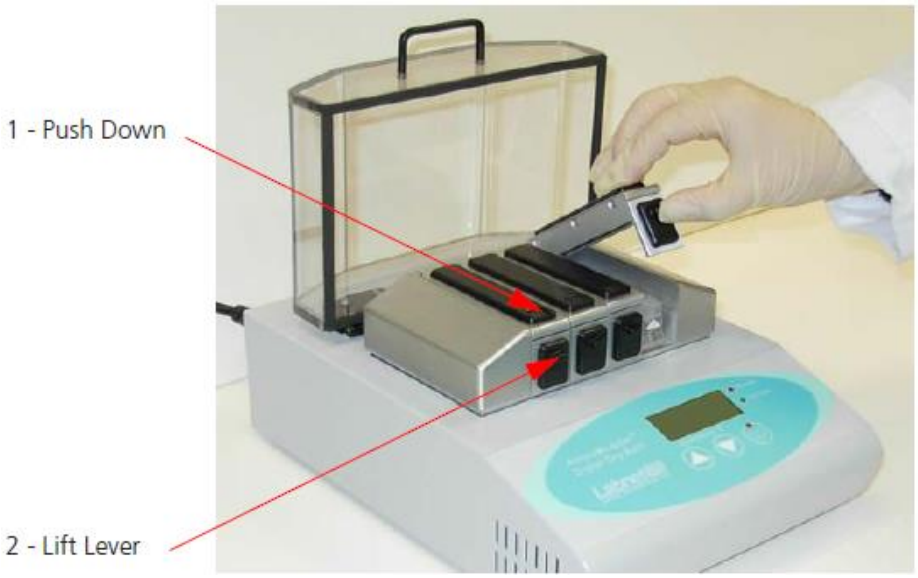
6. If an air bubble is observed, separate the array strip from the hybridization tray and remove air bubbles. Place array strip back into hybridization tray and recheck for air bubbles.

7. Open a Hybridization Station clamp by applying pressure to the top of the clamp while gently squeezing inward. While squeezing lift the clamp to open (Figure 3.12).

**WARNING:** **Do not force the GeneAtlas Hybridization clamps up. To open, press down on the top of the clamp and simultaneously slightly lift the protruding lever to unlock. The clamp should open effortlessly. Refer to Figure 3.12.**

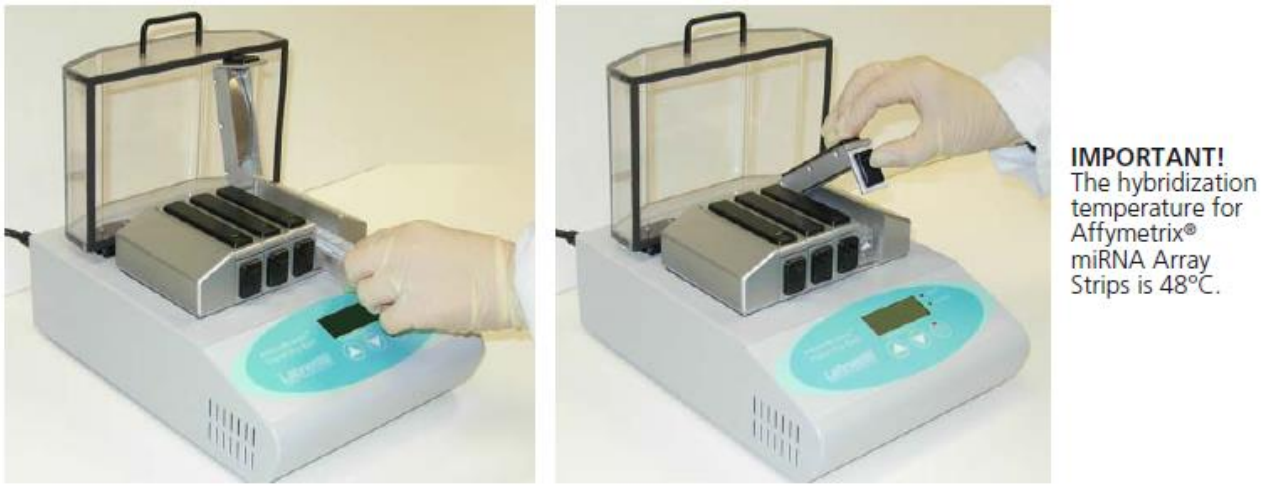
**IMPORTANT:** **The hybridization temperature for miRNA Array Strips is 48°C.**

**Figure 3.12** Opening the clamps on the GeneAtlas™Hybridization Station




8. Place the hybridization tray with the array strip into a clamp inside the Hybridization Station and close the clamp as shown in Figure 3.13.

**Figure 3.13** Laterally inserting an array strip and closing the clamp of the GeneAtlas™ Hybridization Station



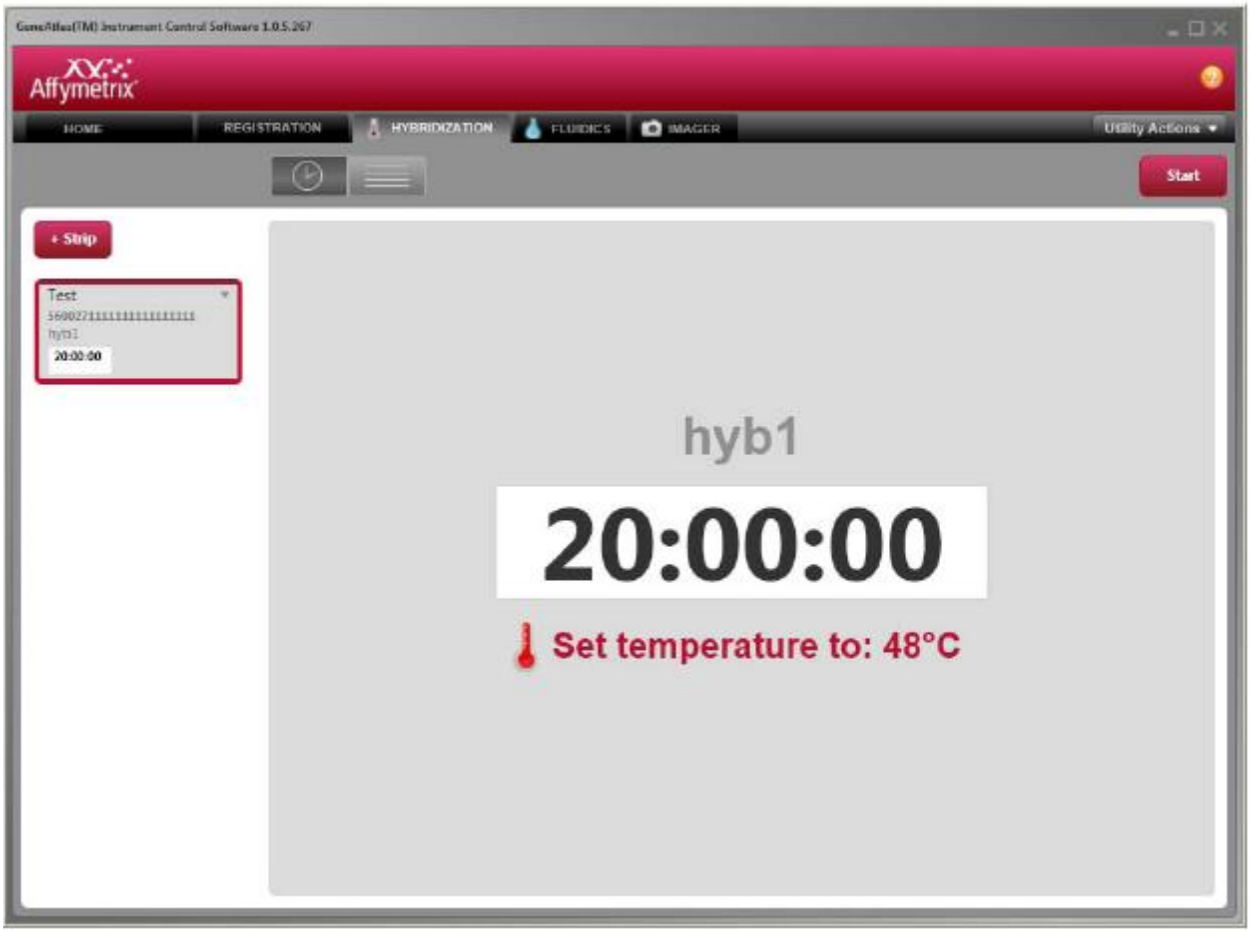

7. Proceed to *Hybridization of Array Strips on the GeneAtlas™ System*.

# Hybridization of Array Strips on the GeneAtlas™ System

After the array strip are registered in the GeneAtlas Software and the target hybridization steps are complete, processing on the GeneAtlas System may begin.

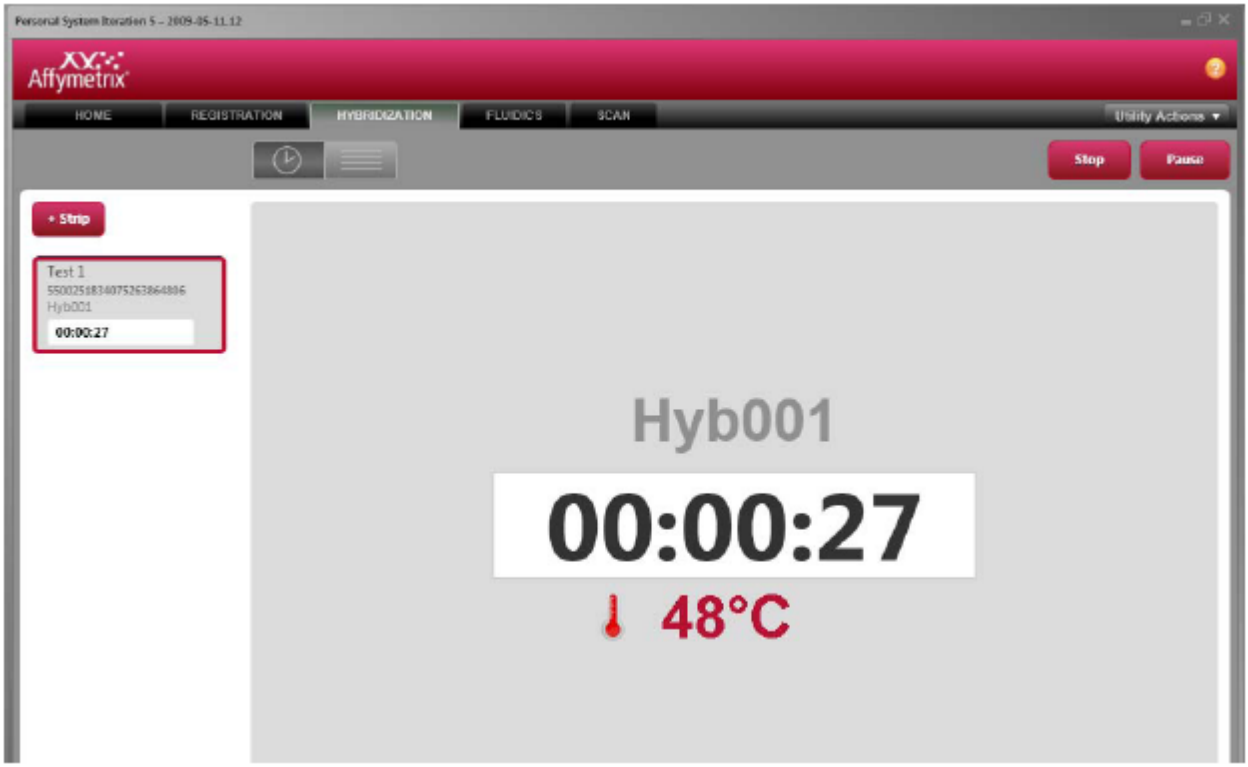
**1.** Return to the GeneAtlas Software interface. Figure 3.14 should be visible

**Figure 3.14**




**2.** With the hybridization tray and array strip already in the GeneAtlas Hybridization Station, click **Start** in Figure 3.14.

**Figure 3.15** Hybridization Countdown




**NOTE:** **The software displays the hybridization time countdown. This time is displayed with a white background (Figure 3.15). When the countdown has completed the display turns yellow and the time begins to count up.**

**Figure 3.16** Hybridization Count up

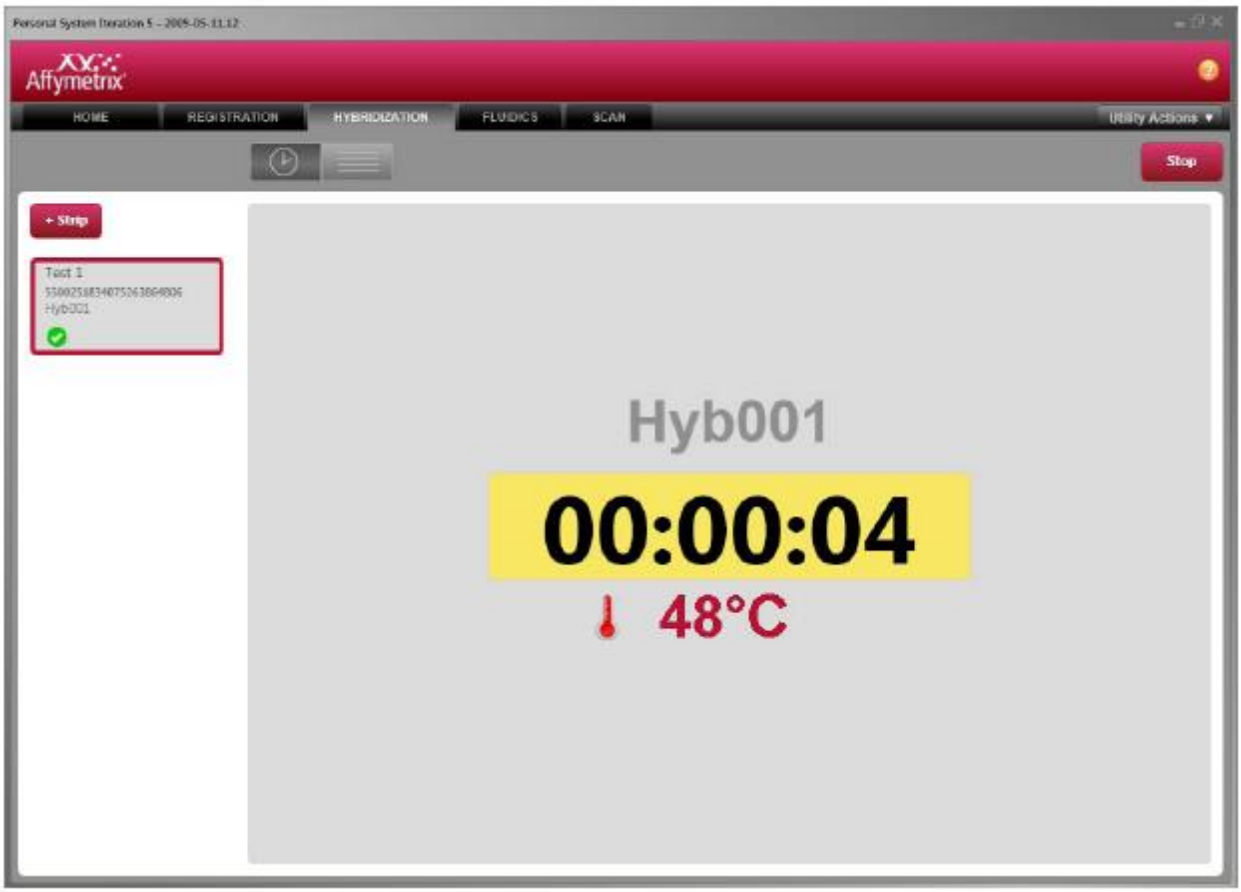



**Figure 3.17** Confirmation Message


3. Click **Yes** to complete hybridization.
4. It is important to remove the hybridization tray from the Hybridization Station after the timer has completed the countdown as the Hybridization Station does not shut down when the hybridization is complete.
5. Save the remaining hybridization cocktail in –20°C for future use.
6. Immediately proceed to the GeneAtlas Wash, Stain and Scan protocol. Please refer to *GeneAtlas™ System User's Guide* (P/N 08-0246) for further detail.

### Rehybridizing Used Cocktails

A used hybridization cocktail can be rehybridized to a new array if necessary. Collect the used hybridization cocktail immediately after the Fluidics run is completed, add to the remainder of the hybridization cocktail master mix from Step 6D and store at –20°C. For rehybridization see Appendix B, *Array Strip Rehybridization Procedure*.

**IMPORTANT:** Rehybridization of hybridization cocktails should only be necessary in case of serious array problems. The performance of rehybridized samples has not been thoroughly tested and is recommended only when absolutely necessary.

## 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 3.18**


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.

# 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 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 µL of each biotin labeling reaction (Step 6), 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 (Step 6) 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

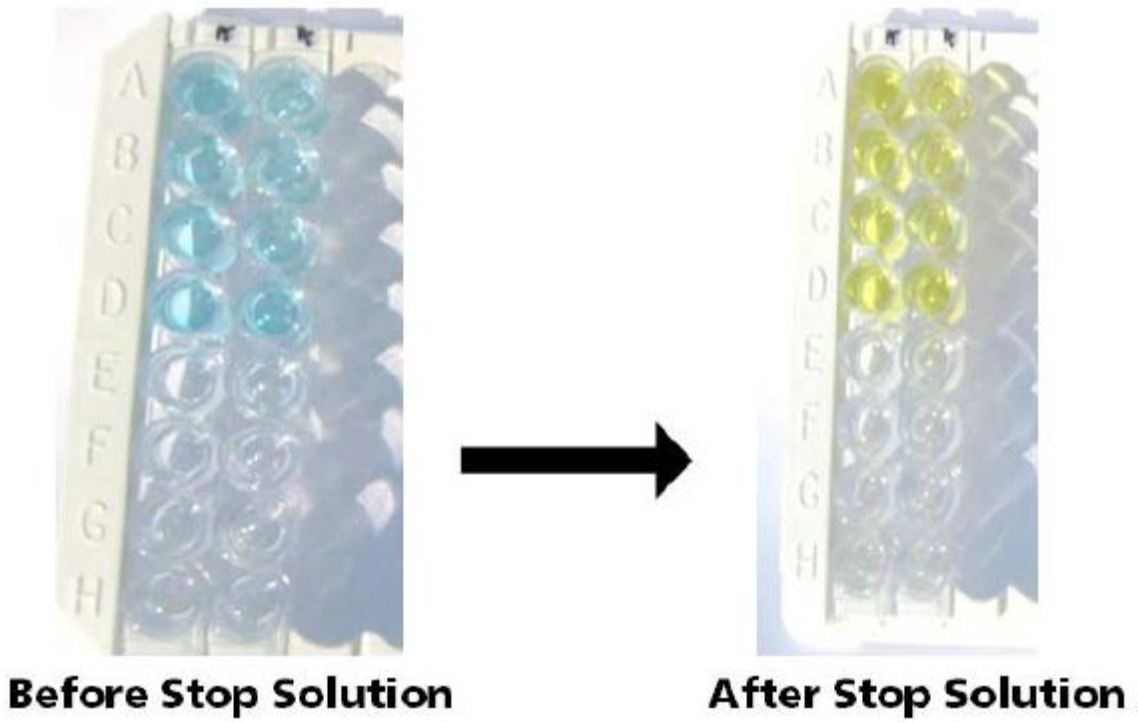
1. Dilute SA-HRP in 5% BSA in 1X PBS. If using Thermo Scientific SA-HRP, a dilution of 1:4000 to 1:8000 is recommended.
2. Remove the hybridization solution by expelling the liquid into a sink.
3. Wash 3-4 times with 1X PBS, 0.02% Tween 20, blot dry.
4. Add 75 µL of the diluted SA-HRP from Step 1 to 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, miRNA Array Strip Procedure*.

# Appendix B Array Strip Rehybridization Procedure

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

**1.** Record the volume of recovered hybridization cocktail from page 24, *Rehybridizing Used Cocktails*.

**2.** Prepare a 1X Hyb Mix:

**Table B.1**

| Component | Volume for one miRNA Array: |
|---|---|
| Nuclease-Free Water (Vial 11) | 23.8 µL |
| 2X Hybridization Mix (from GeneAtlas™ Hybridization, Wash, and Stain Kit for miRNA Array Strips (P/N 902134)) | 62.5 µL |
| 27.5% Formamide (Vial 12) | 18.2 µL |
| DMSO (from GeneAtlas™ Hybridization, Wash, and Stain Kit for miRNA Array Strips (P/N 902134)) | 12.1 µL |
| 20X Eukaryotic Hybridization Controls *bioB*, *bioC*, *bioD*, *cre* (from GeneChip™ Eukaryotic Hybridization Control Kit, P/N 900454) | 6.3 µL |
| Control Oligonucleotide B2, 3nM (P/N 900301) | 2.1 µL |

**3.** Adjust the volume of recovered hybridization cocktail (Step 1) to 125 µL with 1X Hyb Mix (Step 2, above).

**4.** Follow the hybridization instructions from Step 5 to complete the hybridization process.

# 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 (Applied Biosystems P/N AM9932) to a 50 mL conical tube.
- Remove and discard 50 µL water.
- Add 50 µL 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 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 (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 µL) (Applied Biosystems P/N AM9823)

- 0.01mL 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

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