Affymetrix®

User Manual

GeneChip®
HT 3' IVT Express Kit

P/N 702662 Rev. 6

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Overview

Introduction

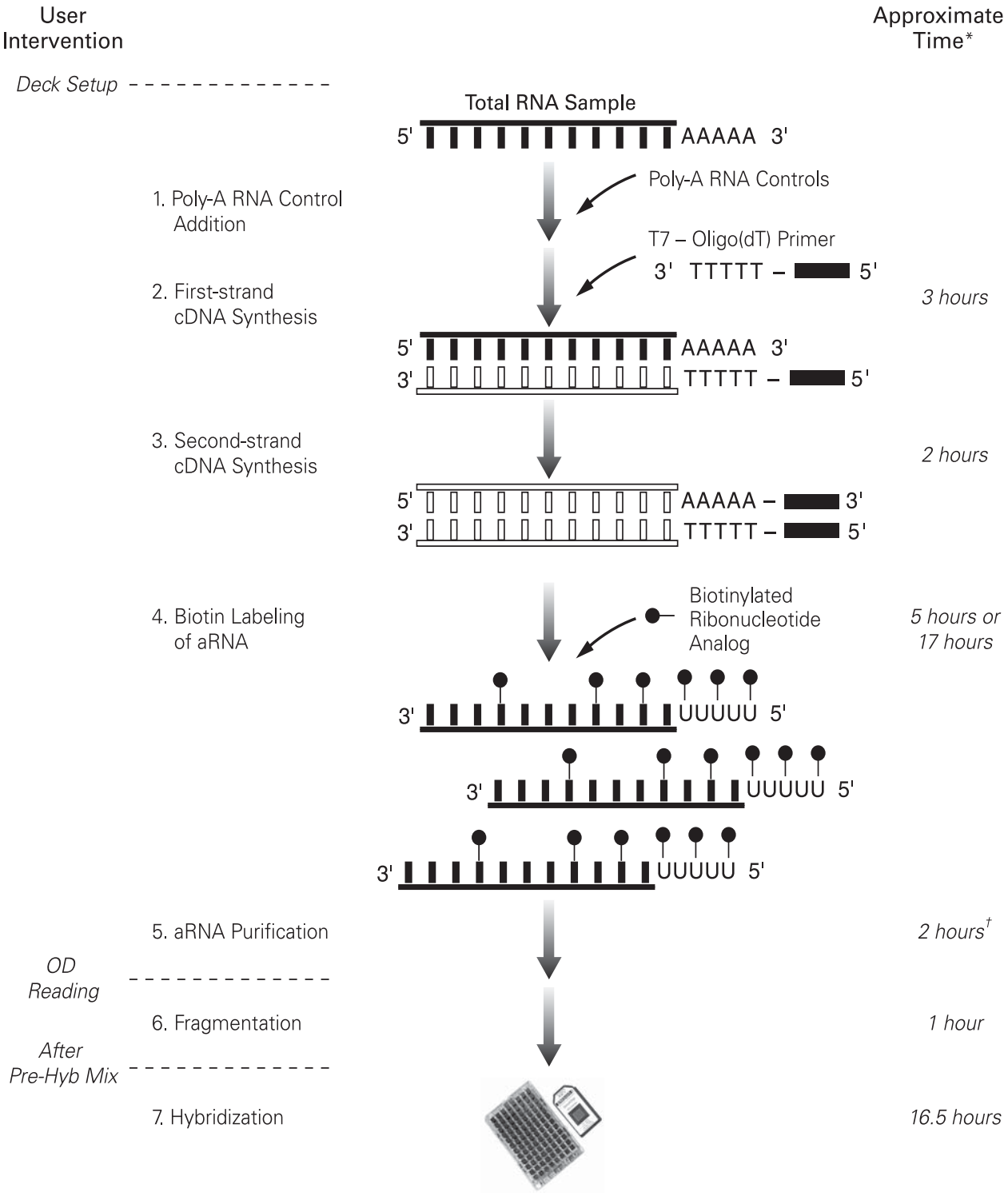
Welcome to the GeneChip® HT 3' IVT Express Kit User Manual developed for the Beckman Biomek® FXᵖ Target Prep Express (TPE) System. The Beckman Biomek FXᵖ Target prep express System makes use robotic technology to automate many of the labor intensive tasks required when preparing a eukaryotic mRNA sample for gene expression analysis. The Beckman Biomek FXᵖ TPE System also automates the hybridization of a target to a GeneChip HT Array Plate and prepares the stain and scan trays for insertion into the GeneTitan® Instrument.

This manual describes the assay procedures recommended for eukaryotic target labeling for expression analysis and subsequent HT Array Plate hybridization and processing using the Beckman Biomek® FXᵖ Target Prep Express (TPE) System. By following the protocols and using high-quality starting materials, a sufficient amount of biotin-labeled amplified RNA (aRNA) target can be obtained for hybridization. The reagents and protocols have been developed and optimized specifically for use with either automated system.

NOTE: Previous versions of the *GeneChip Expression Analysis Technical Manual* referred to complementary RNA (cRNA) rather than amplified RNA (aRNA). Complementary RNA is also known as amplified RNA or aRNA.

The GeneChip HT 3' IVT Express Labeling Assay outline is represented in Figure 1.1. Total RNA (50-500 ng) is first reverse transcribed using a T7-Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction. The cDNA is converted into double-stranded cDNA template during second strand cDNA synthesis. *In vitro* transcription synthesizes aRNA that is biotin incorporated and purified. Fragmentation of the biotin-labeled aRNA prepares the sample for hybridization onto GeneChip cartridge arrays.

Figure 1.1 Overview of the GeneChip HT 3′ IVT Express Labeling Assay



Beckman Biomek® FXP Target Prep Express System

The Biomek FXP TPE System utilizes task-specific hardware combined with easy to use software to process the procedures of the GeneChip HT 3' IVT Express Assay.

Summary of the Beckman Biomek® FXP Target Prep Express System Run

Target preparation on the Beckman Biomek FXP TPE System takes between ~10 hours and ~23 hours depending on selected parameters. At the end of target preparation, the sample is ready to be denatured and hybridized onto cartridge or array plates. Hybridization takes 16 hours. Additional time is required for scanning and is dependent on the number of samples processed and the array type. The typical workflow and chapter references are provided below.

1. Isolate high quality total RNA (refer to Chapter 2).
2. Perform Array Station system check, home all Axis and Purge air from lines of the Biomek FXP TPE System (refer to Chapter 4).
3. Day One procedures: IVT Synthesis and Labeling (refer to Chapter 4).
4. Day Two procedures: Purification, Quantitation, Normalization, Fragmentation, and Hybridization. Optional: Gene Titan Setup (refer to Chapter 4).
5. Denature sample and hybridize overnight
   - **GeneChip Cartridge Arrays** using the GeneChip® Hybridization, Wash and Stain Kit (refer to Chapter 5).
   - **GeneChip HT Array Plates** using the GeneChip® HT Hybridization, Wash and Stain Kit processed on the GeneTitan Instrument (refer to the *GeneTitan® Instrument User Guide for Expression Array Plates*, P/N 702933).

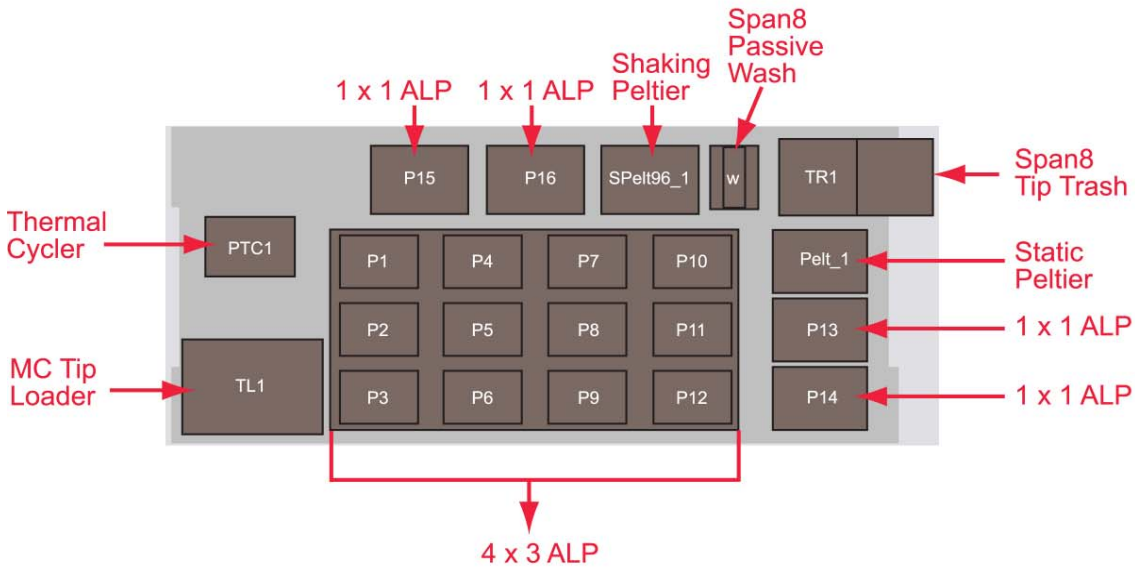
   NOTE: Affymetrix does not recommend the use of user-prepared reagents for the 3' IVT Express Assay. Please use the GeneTitan Hybridization, Wash and Stain kit for IVT Arrays (PN 901530) or the GeneChip® Hybridization, Wash and Stain Kit (cartridge arrays, P/N 900720) for all the reagents needed for the hybridization, wash and stain steps of this assay.
6. Wash, stain and scan cartridge arrays (refer *GeneChip® Expression Wash, Stain and Scan User Manual*, P/N 702731).
7. Scan HT Array Plates using the HT Scanner (refer *Affymetrix GeneChip® HT Scanner User's Guide*, P/N 701978).

Safety Information

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Caution: All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as lab coat, safety glasses and gloves. Care should be taken to avoid contact with skin and eyes. In case of contact with skin or eyes, wash immediately with water. See MSDS (Material Safety Data Sheet) for specific advice.

Figure 1.2 Biomek® FXᴾ TPE System and Deck / Pod Locations



RNA Preparation

Total RNA Isolation for the GeneChip 3' IVT Express Assay

This chapter describes the general requirements for RNA isolation methods and poly-A control preparation for spiking into your RNA sample.

IMPORTANT: **The quality of the RNA is essential to the overall success of the analysis. Since the most appropriate protocol for the isolation of RNA can be source dependent, we recommend using a protocol that has been established for the tissues or cells being used. In the absence of an established protocol, using one of the commercially available kits designed for RNA isolation is suggested.**

When using a commercial kit, follow the manufacturer's instructions for RNA isolation.

Materials List

Total RNA Isolation Reagents

- TRIzol® Reagent: Invitrogen Life Technologies, P/N 15596-018, or QIAzol™ Lysis Reagent: QIAGEN, P/N 79306
- RNeasy® Mini Kit: QIAGEN, P/N 74104

Miscellaneous Reagents

- 80% ethanol (stored at –20°C)
- Pellet Paint®: Novagen, P/N 69049-3 (optional)
- Glycogen: Ambion, P/N 9510 (optional)
- 3M Sodium Acetate (NaOAc): Sigma-Aldrich, P/N S7899

Isolation of RNA from Yeast

Total RNA

Quality total RNA has been isolated successfully from yeast cells using a hot phenol protocol described by Schmitt, *et al. Nucl Acids Res* **18**:3091-3092 (1990).

Isolation of RNA from Arabidopsis

Total RNA

TRIzol® Reagent from Invitrogen Life Technologies has been used to isolate total RNA from Arabidopsis. Follow the instructions provided by the supplier and, when necessary, use the steps outlined specifically for samples with high starch and/or high lipid content. QIAzol™ Lysis Reagent from QIAGEN can also be used.

Isolation of RNA from Mammalian Cells or Tissues

Total RNA

High-quality total RNA has been successfully isolated from mammalian cells (such as cultured cells and lymphocytes) using the RNeasy® Mini Kit from QIAGEN.

If mammalian tissue is used as the source of RNA, it is recommended to isolate total RNA with a commercial reagent, such as TRIzol® or QIAzol™ reagent.

IMPORTANT: If going directly from TRIzol- or QIAzol™-isolated total RNA to cDNA synthesis, it may be beneficial to perform a second cleanup on the total RNA before starting. After the ethanol precipitation step in the Lysis Reagent extraction procedure, perform a cleanup using the QIAGEN RNeasy Mini Kit. Much better yields of labeled aRNA are obtained from the *in vitro* transcription-labeling reaction when this second cleanup is performed.

Precipitation of RNA

Total RNA

It is not necessary to precipitate total RNA following isolation or cleanup with the RNeasy Mini Kit. Adjust elution volumes from the RNeasy column to prepare for cDNA synthesis based upon expected RNA yields from your experiment. Ethanol precipitation is required following TRIzol or QIAzol reagent isolation and hot phenol extraction methods; see methods on page 6 for details.

Precipitation Procedure

1. Add 1/10 volume 3M NaOAc, pH 5.2, and 2.5 volumes ethanol.*
2. Mix and incubate at –20°C for at least 1 hour.
3. Centrifuge at ≥ 12,000 x g in a microcentrifuge for 20 minutes at 4°C.
4. Wash pellet twice with 80% ethanol.
5. Air dry pellet. Check for dryness before proceeding.
6. Resuspend pellet in DEPC-treated H_2O. The appropriate volume for resuspension depends on the expected yield and the amount of RNA required for the cDNA synthesis. Please read ahead to the cDNA synthesis protocol in order to determine the appropriate resuspension volume at this step.

*Addition of Carrier to Ethanol Precipitations

Adding carrier material has been shown to improve the RNA yield of precipitation reactions.

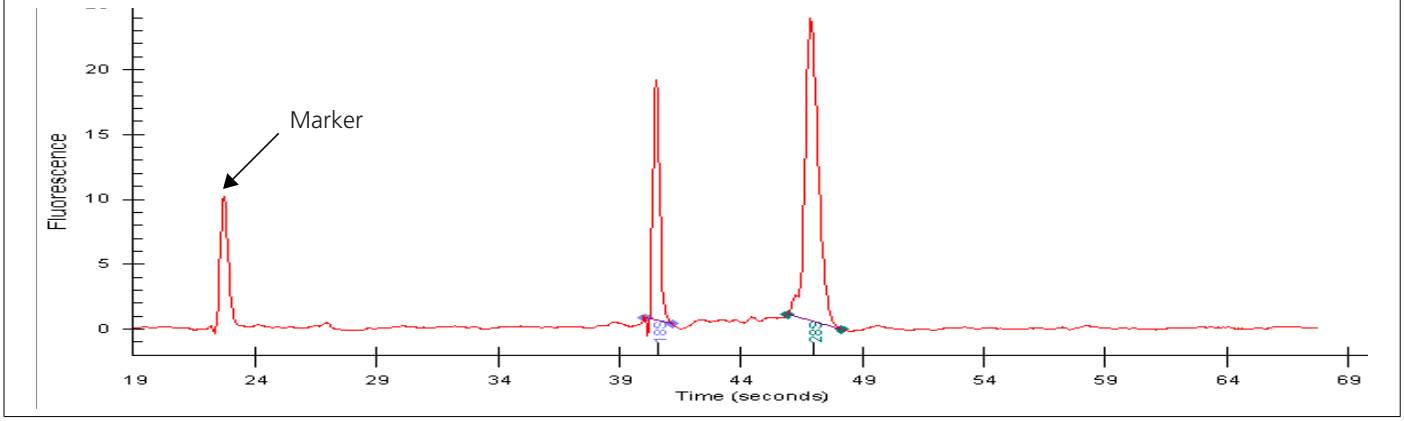
- **Pellet Paint®**
  Addition of 0.5 µL of Pellet Paint per tube to nucleic acid precipitations makes the nucleic acid pellet easier to visualize and helps reduce the chance of losing the pellet during washing steps. The pellet paint does not appear to affect the outcome of subsequent steps in this protocol; however, it can contribute to the absorbance at 260 nm when quantifying the total RNA.
- **Glycogen**
  Addition of 0.5 to 1 µL of glycogen (5 mg/mL) to nucleic acid precipitations aids in visualization of the pellet and may increase recovery. The glycogen does not appear to affect the outcome of subsequent steps in this protocol.

Quantitation of RNA

Quantify RNA yield by spectrophotometric analysis using the convention that 1 absorbance unit at 260 nm equals 40 µg/mL RNA.

- The absorbance should be checked at 260 and 280 nm for determination of sample concentration and purity.
- The A_{260}/A_{280} ratio should be close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).
- Integrity of total RNA samples can also be assessed qualitatively on an Agilent 2100 Bioanalyzer. Refer to Figure 2.1 for an example of good-quality total RNA sample.

Figure 2.1 Electropherogram (from the Agilent 2100 Bioanalyzer) for HeLa Total RNA. For a high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands.



Preparation of Poly-A RNA Controls

Reagents and Equipment

- GeneChip® HT 3' IVT Express Kit components: Affymetrix, P/N 901225 (4 x 24 rxn), or P/N 901253 (96 rxn)

  Components used in this step:

  - Eukaryotic Poly-A RNA Controls

Designed specifically to provide exogenous positive controls to monitor the entire eukaryotic target labeling process, a set of poly-A RNA controls is supplied in the GeneChip HT 3' IVT Express Kit.

Each eukaryotic GeneChip probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys*, *phe*, *thr*, and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are pre-mixed at staggered concentrations. The concentrated Poly-A Control Stock can be diluted with the Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final dilutions (relative to estimated copy number of total mRNA population) summarized in Table 2.1.

Table 2.1 Final Dilutions of Poly-A RNA Controls in Samples

Poly-A RNA Spike	Final Dilution (estimated ratio of copy number)
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:6,667

The controls are then amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples. Anticipated relative signal strength follows the order of *lys* < *phe* < *thr* < *dap*.

The **Poly-A RNA Control Stock** and **Poly-A Control Dil Buffer** are provided in the GeneChip HT IVT Express Kit to prepare the appropriate serial dilutions based on Table 2.2. This is a guideline when 100, 250 or 500 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

IMPORTANT: Use non-stick RNase-free microfuge tubes to prepare all of the dilutions (not included).

Table 2.2 Serial Dilution of Poly-A RNA Control Stock

Total RNA Input Amount	Serial Dilutions				Volume of 4th dilution to add to total RNA
	First Dilution	Second Dilution	Third Dilution	Fourth Dilution	
100 ng	1:20	1:50	1:50	1:10	2 µL
250 ng	1:20	1:50	1:50	1:4	2 µL
500 ng	1:20	1:50	1:50	1:2	2 µL

Recommendation: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.

For example, to prepare the Poly-A RNA dilutions for 1 µg of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 38 µL of Poly-A Control Dil Buffer for the First Dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the First Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the Second Dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 2 µL of the Second Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the Third Dilution (1:50).
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of this Third Dilution to 1 µg of total RNA sample directly. The final volume of the total RNA with the diluted poly-A controls should not exceed 4 µL.

NOTE: The first dilution of the Poly-A RNA controls can be stored up to six weeks in a frost-free freezer at –20°C and freeze-thawed up to eight times.

Table 2.3 Total RNA/Poly-A RNA Contral Mixture

Component	Volume
Total RNA Sample (50-500 ng)	Variable
Diluted Poly-A Controls (4th Dilution)	2 µL
Nuclease-free Water	Variable
Total Volume	**4 µL**

Preparation of Total RNA Plates for Processing on the Biomek® FXp Target Prep Express (TPE) System

The Target Preparation protocol starts with 4 µL of material in a Bio-Rad 96-Well Hard-Shell PCR Plate. This plate can either be prepared offline (manually) or on the Beckman Biomek® FXᵖ Target Prep Express (TPE) System, as described below.

User-prepared Plate Preparation

Preparing Samples with PolyA Controls

Pipet 2 µL of the total RNA sample (100 - 500 ng) and 2 µL of the appropriate PolyA spike control solution into the 96-well plate. Samples should be placed into the plate in a column-wise fashion starting from the left side of the plate. For example, if preparing 24 samples, pipet the samples into the sample wells for columns 1, 2, and 3.

Optional – Running without PolyA Controls

Affymetrix highly recommends that you utilize PolyA controls as described above. However, if you choose not to utilize these controls, the total RNA sample in the Bio-Rad 96-Well Hard-Shell PCR Plate must be adjusted to a final volume of 4 µL.

GeneChip® HT 3′ IVT Express Kit and Assay

This chapter presents information on the GeneChip® HT 3' IVT Express Kit and assay and is applicable for users of both the GeneChip Array Station (GCAS) as well as the Beckman Biomek® FXᵖ Target Prep Express (TPE) System. After reading this chapter, users processing on the GeneChip Array Station should proceed to Appendix C, *Reagent Preparation for the GeneChip Array Station* on page 101; and users processing on the Biomek® FXᵖ TPE System should proceed to Chapter 4, *Beckman® Biomek® FXᵖ Target Prep Express Setup and Target Preparation* on page 17.

GeneChip® HT 3′ IVT Express Kit

The GeneChip® HT 3' IVT Express Kit is the latest technology in RNA target preparation for microarray expression analysis. This kit features:

- Low RNA input requirements; from as little as 100 ng of total RNA for a single round of amplification
- Streamlined workflow, with the option to decrease target labeling time with appropriate inputs of total RNA
- Master mixes, consumables included and a simple protocol for ease of use, convenience and a high rate of success
- A complete kit that includes Poly-A RNA controls and hybridization controls
- Magnetic-bead aRNA purification for high recovery and ease of use.

The kit is based upon linear RNA amplification and employs T7 *in vitro* transcription technology. Also known as the Eberwine or reverse transcription-IVT (RT-IVT) method, this process is considered the gold standard for target preparation for gene expression analysis. RT-IVT was experimentally validated using TaqMan® RT-PCR (MAQC Consortium *et. al*, 2006).

In the GeneChip® HT 3' IVT Express Protocol total RNA undergoes reverse transcription to synthesize first-strand cDNA. This cDNA is converted to double-stranded cDNA template during second-strand cDNA synthesis. *In vitro* transcription synthesizes aRNA that gets biotin incorporated and purified (cRNA is also known as amplified RNA or aRNA). Fragmentation of the biotin-labeled aRNA prepares the sample for hybridization onto GeneChip 3' expression arrays.

Kit Contents and Storage Conditions

Table 3.1 GeneChip® HT 3' IVT Express Kit Components and Storage Conditions

Component	Vol/Qnty 4 x 24 rxn P/N 901225	Vol/Qnty 96 rxn P/N 901253	Storage
BOX 1 of 2			
aRNA Binding Buffer Concentrate	4 x 1.8 µL	7.2 mL	room temp
RNA Binding Beads	4 x 360 µL	1.44 mL	2-8 °C*
aRNA Wash Solution Concentrate (*Add 100% ethanol before use, as shown on the label)*	4 x 7.5 mL	25 mL	room temp
aRNA Elution Solution	4 x 5 mL	20 mL	room temp
5X Array Fragmentation Buffer	4 x 1.8 mL	1.8 mL	room temp
BOX 2 of 2			
First-Strand Enzyme Mix	4 x 49 µL	143 µL	–20 °C
First-Strand Buffer Mix	4 x 167 µL	541 µL	–20 °C
Second-Strand Enzyme Mix	4 x 76 µL	264 µL	–20 °C
Second-Strand Buffer Mix	4 x 176 µL	644 µL	–20 °C
IVT Enzyme Mix	4 x 209 µL	771 µL	–20 °C
IVT Labeling Buffer	4 x 661 µL	2 x 1,266 µL	–20 °C
IVT Biotin Label	4 x 143 µL	517 µL	
Control RNA (1 mg/mL HeLa total RNA)	10 µL	10 µL	–20 °C
Poly-A Control Stock	16 µL	16 µL	–20 °C
Poly-A Control Dilution Buffer	3.8 mL	3.8 mL	–20 °C
20X Hybridization Controls	2 x 1,125 µL	2 x 1,125 µL	–20 °C
Control Oligo B2	750 µL	750 µL	–20 °C

** Do not freeze.*

Assay Overview

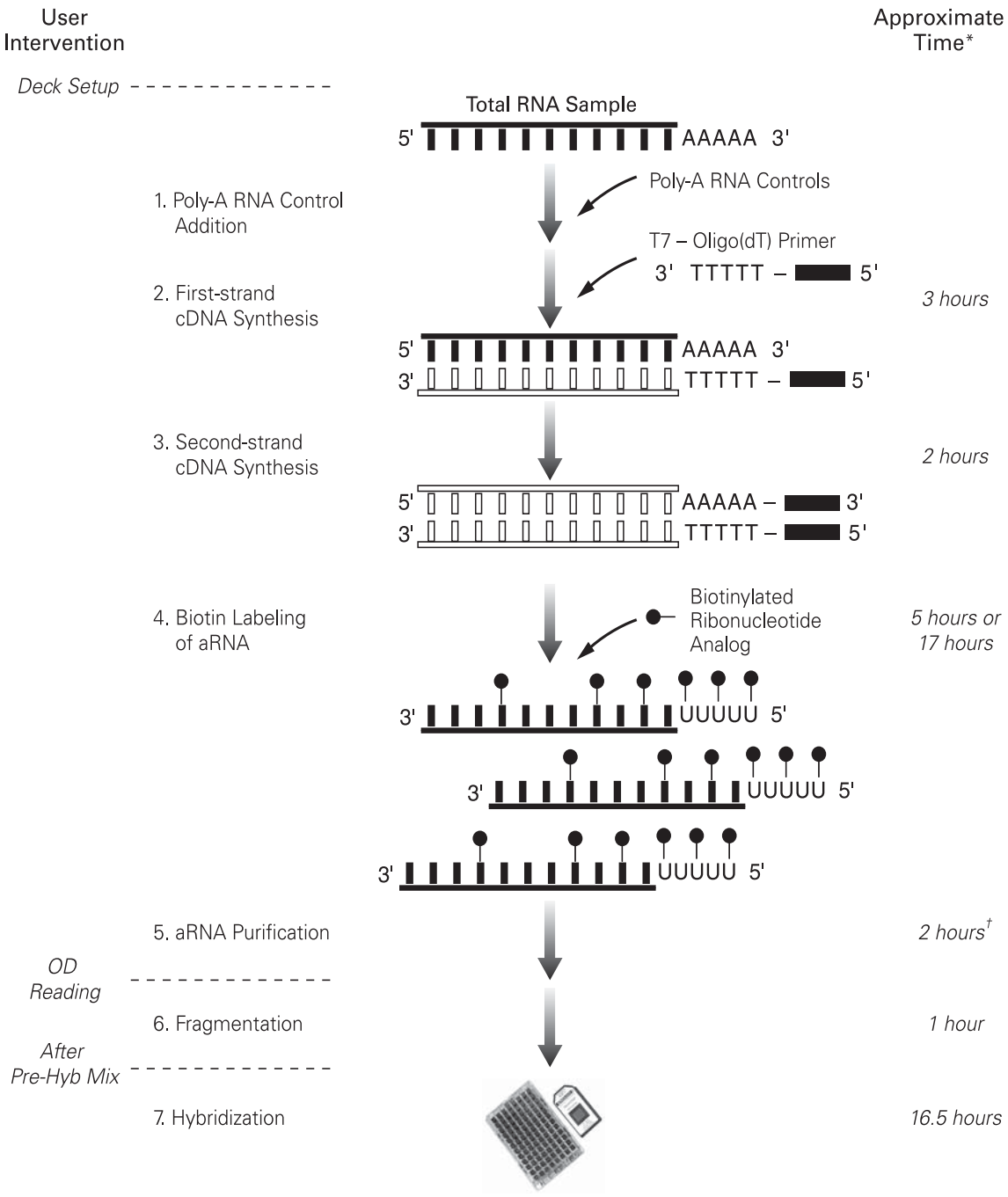
The GeneChip HT 3' IVT Express Kit aRNA amplification procedure is depicted in Figure 3.1 on page 13.

- **Reverse Transcription to Synthesize First-Strand cDNA** is primed with T7 oligo(dT) primer to synthesize cDNA containing a T7 promoter sequence.
- **Second-Strand cDNA Synthesis** converts the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription. The reaction employs DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA.
- ***In Vitro* Transcription to Synthesize Biotin-Modified aRNA with IVT Labeling Master Mix** generates multiple copies of biotin-modified aRNA from the double-stranded cDNA templates; this is the amplification step.
- **aRNA Purification** removes unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the biotin-modified aRNA.

Control RNA

Use the included Control RNA to familiarize yourself with the GeneChip HT 3' IVT Express Kit RNA Amplification procedure. Instructions for the positive control reaction are provided in Appendix F, *Troubleshooting* on page 171.

Figure 3.1 Overview of the GeneChip® HT 3' IVT Express Kit Labeling Assay



aRNA Amplification Protocol

Important Parameters for Successful Amplification

Input RNA Quantity and IVT Reaction Incubation Time

NOTE: The RNA volume must be = 4 µL.

Consider both the type and amount of sample RNA available and the amount of aRNA needed for your analysis when planning experiments using the GeneChip® HT 3’ IVT Express Kit. Because mRNA content varies significantly with tissue type, the optimal amount of total RNA input should be determined empirically for each experimental system. The recommended input RNA amounts listed in Table 3.2 are based on using total RNA from HeLa cells; use these recommendations as a starting point. Table 3.3 shows the corresponding recommended IVT incubation times.

Table 3.2 Input RNA Limits

Recommendations	Amount
Recommended	100 ng
Minimum	100 ng
Maximum	500 ng

Table 3.3 Recommended IVT Incubation Times

RNA Amount	IVT Incubation Time
100 ng	16 hours
100–250 ng	8 hours
250–500 ng	4 hours

RNA Purity

RNA quality is the single most important factor affecting how efficiently an RNA sample will be amplified using GeneChip® HT 3’ IVT Express Kit. RNA samples should be free of contaminating proteins, DNA, and other cellular material as well as phenol, ethanol, and salts associated with RNA isolation procedures. Impurities can lower the efficiency of reverse transcription and subsequently reduce the level of amplification. An effective measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The ratio of A_{260} to A_{280} values should fall in the range of 1.7–2.1. RNA must be suspended in high quality water, TE (10 mM Tris-HCl, 1 mM EDTA).

RNA Integrity

The integrity of the RNA sample, or the proportion that is full length, is another important component of RNA quality. Reverse transcribing partially degraded mRNAs will generate cDNAs that may lack portions of the coding region. RNA integrity can be evaluated by microfluidic analysis using the Agilent 2100 bioanalyzer with an RNA LabChip® Kit. Primarily full-length RNA will exhibit a ratio of 28S to 18S rRNA bands that approaches 2:1. Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity. The RIN, a metric developed by Agilent, includes information from both the rRNA bands and outside the rRNA peaks (potential degradation products) to provide a picture of RNA degradation states. Search for “RIN” at the following web site for further information: www.chem.agilent.com.

Denaturing agarose gel electrophoresis and nucleic acid staining can also be used to separate and visualize the major rRNA species. When the RNA resolves into discrete rRNA bands (i.e., no significant smearing below each band), with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band, then the mRNA in the sample is likely to be mostly full-length. The primary drawback to gel electrophoresis is that it requires microgram amounts of RNA.

Other Important Parameters

- **Keep reaction incubation times precise and consistent**:
  The incubation times for the enzymatic reactions in the protocol were optimized in conjunction with the kit reagents for maximum yield in each step—adhere to them closely.
- **Mix each kit component before use**:
  - Mix enzyme solutions by gently flicking the reagent tube a few times and centrifuge before inserting them into the reagent cold block.
  - Thaw frozen reagents completely at room temperature, then mix thoroughly by inverting tubes several times, spin down and place on ice.
- **Incubate reactions in a calibrated thermal cycler**:
  - We do not recommend using ordinary laboratory heat blocks, water baths, or hybridization ovens for any of the reaction incubations.
  - The procedure is very sensitive to temperature; therefore use a thermal cycler that has been calibrated according to the manufacturer's recommended schedule. Variable or inaccurate incubation temperatures can negatively impact aRNA synthesis.
  - Heated lids: It is important that condensation does not form in the tubes during any of the incubations, because it would change the reaction composition and can greatly reduce yield. If possible, set the lid temperature to match the block temperature. Otherwise, incubate all reactions with the heated lid on (~100 °C).
- **Maintain procedural consistency**:
  Procedural consistency is very important for amplification experiments. Consider implementing a detailed procedural plan that will be used by everyone in the lab to maintain consistency. This type of plan will minimize variation due to subtle procedural differences that can influence RNA amplification and may complicate gene expression studies. The plan should include basic information such as the method of RNA isolation, the amount of RNA to use in the procedure, and how long to incubate the IVT reaction. It should also address specifics that are not often included in protocols such as which tubes and thermal cycler to use for each step in the process. Finally, develop a consistent work flow. For example standardize stopping points in the method. The idea is to standardize all of the variables discussed in this section of the Instruction Manual and carefully follow all the protocol steps in order to maximize amplification consistency among samples.

After reading this chapter, users processing on the GeneChip Array Station should proceed to Appendix C, *Reagent Preparation for the GeneChip Array Station* on page 101; and users processing on the Biomek® FXᵖ TPE System should proceed to Chapter 4, *Beckman® Biomek® FXᵖ Target Prep Express Setup and Target Preparation* on page 17.

Beckman® Biomek® FXP Target Prep Express Setup and Target Preparation

This chapter describes a typical run using the Beckman Biomek® FXP Target Prep Express (TPE) System. A schematic of the automated target preparation protocol is provided at the beginning of this chapter to outline the steps in the procedure.

This chapter also describes the procedures for using the Biomek® Software, setting up the deck, and performing quantitation and normalization of aRNA.

Reagents and Materials Required

Table 4.1 Beckman Biomek FXP Target Prep Instrument

Product	Qnty	Source	P/N
Beckman Coulter Biomek FXP Target Prep Instrument	1	Beckman Coulter	A83103

Table 4.2 3′ IVT Express Reagents and Miscellaneous Reagents and Materials

Product	Source	P/N
GeneChip® HT 3′ IVT Express Kit Kit packaging options: ▪ 1 x 96 reaction Kit ▪ 4 x 24 reactions Kit	Affymetrix	 901253 901225
GeneTitan® Hybridization, Wash and Stain kit for IVT Arrays – (Sufficient for 1x96 or 3x24 reactions)	Affymetrix	901530
DNAZap	Ambion	9890
RNaseZap	Ambion	9786
100% ETOH	various	
Cold Reagent Block template: 24 or 96 samples	Affymetrix	15-0432
Affymetrix® GeneChip® HT 3′ IVT Reservoir Template	Affymetrix	90-0905
SPRIPlate 96R Super Magnet	Agencourt	A32782

Table 4.3 Biomek Consumables Kit from Affymetrix

Product	Quantity	Source	P/N
Affymetrix® Labware Kit for IVT Express Method on Beckman Biomek® FXP Target Prep Instrument* Consists of:		Affymetrix	901561
▪ Plate, 1.2 mL Square Well Storage, Low Profile	4		
▪ Plate, Bio-Rad Hardshell PCR	16		
▪ Plate, Costar Brand Serocluster U-bottom	8		
▪ Plate, UV Star Greiner	8		
▪ Plate, 96 Well, Conical, PP, 340 µL/well	4		
▪ Tube, Conical Bottom, 2.0mL, Screwcap, Sterile (Mixing Tube)	24		
▪ Lid, Autosealing Microplates, Arched	1		
▪ Pad, Microseal, P	1		

** Labware sufficient for 4x24 or 4x96 rxn runs.*

Table 4.4 Biomek® Tips and Universal labware

Product	Packaging	Total 24X	Total 96X	Source	P/N
P50 Barrier Tips (Span 8 Pipettor, magenta)	96 ea./rack- 10 rack/case	365 tips	576 tips	Beckman	A21586
P250 Barrier Tips Sterile (AP 96 Multichannel Pipettor, light blue)	96 ea./rack - 10 rack/case	480 tips *576 tips**	480 tips *576 tips**	Beckman	717253
P250 Barrier Tips Sterile (Span 8 Pipettor, green)	96 ea./rack - 10 rack/case	85 tips	59 tips	Beckman	379503
P1000 LLS Barrier Tips (Span 8 Pipettor, yellow)	96 ea./rack - 5 rack/case	47 tips *71 tips**	69 tips *93 tips**	Beckman	987925
Quarter Reservoir, Divided by Width	40 unit/case	3	3	Beckman	372792
Half Reservoir	24 unit/case	1	1	Beckman	372786
Quarter Reservoir	40 unit/case	8	8	Beckman	372790
Frame for Reservoirs	1 ea.	2	2	Beckman	372795

**Number required if running GeneTitan setup.*

Automated Target Preparation Schematic

The automatic target preparation schematic for the Beckman Biomek FXP TPE System is presented for both 24 and 96 sample runs.

Day One: 24-Sample Workflow

Figure 4.1 24-Sample, Day 1: Single Strand Synthesis

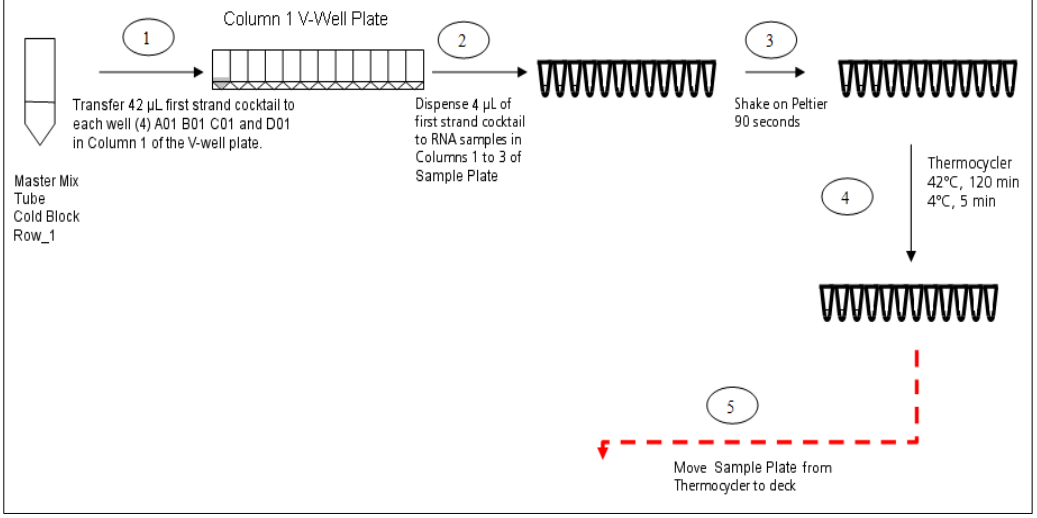


Figure 4.2 24-Sample, Day 1: Second Strand Synthesis

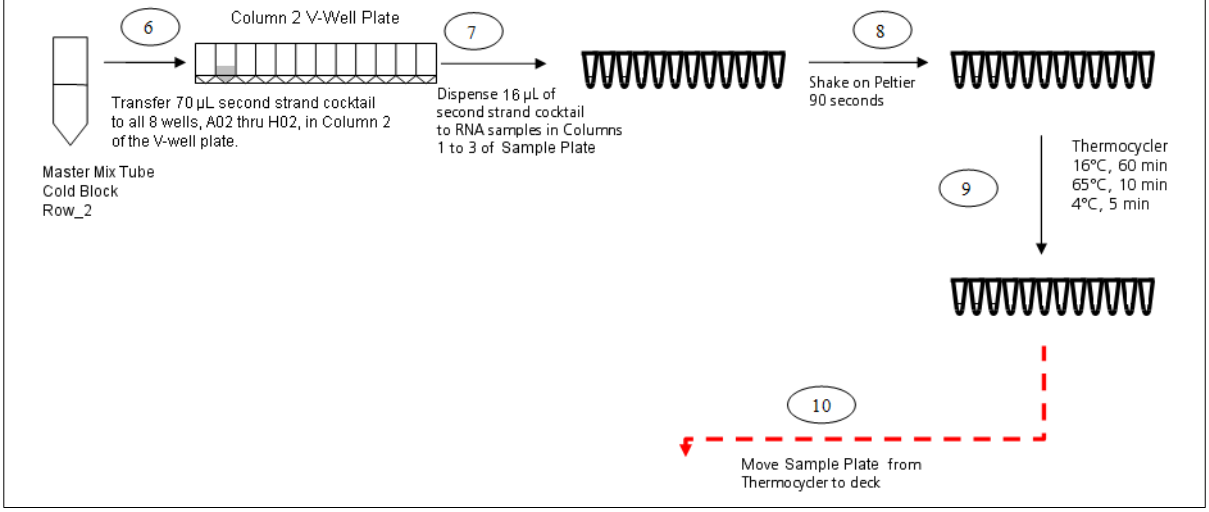
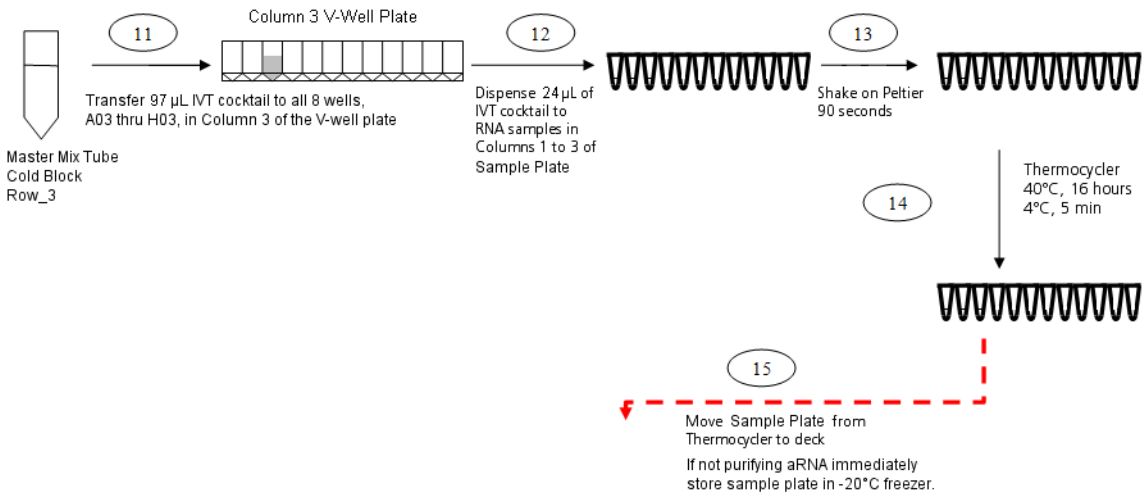


Figure 4.3 24-Sample, Day 1: IVT Labeling



Day One: 96-Sample Workflow

Figure 4.4 96-Sample, Day 1: Single Strand Synthesis

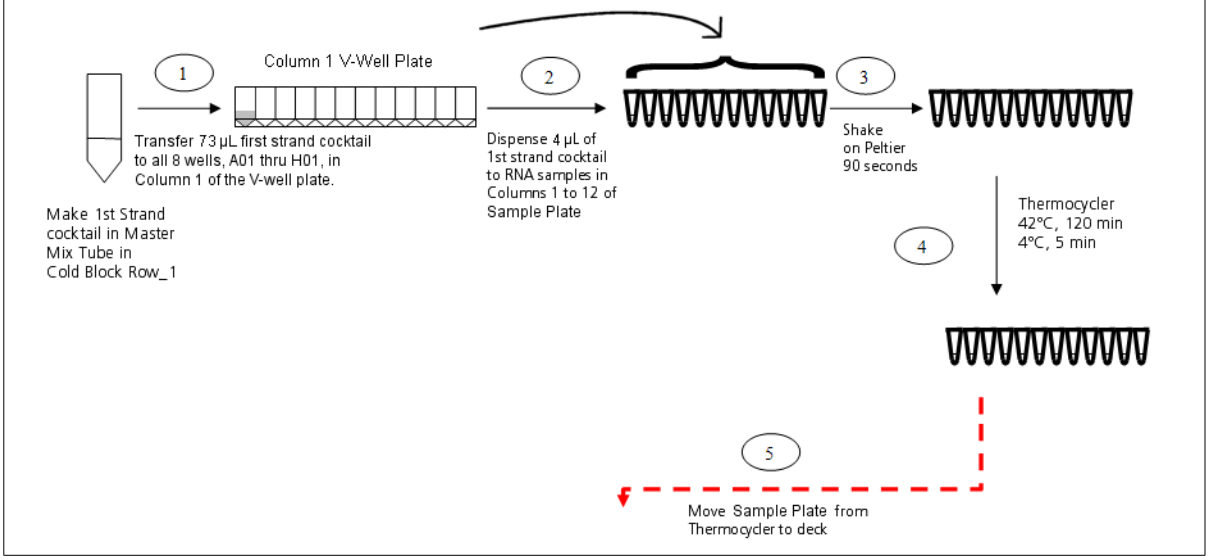


Figure 4.5 96-Sample, Day 1: Second Strand Synthesis

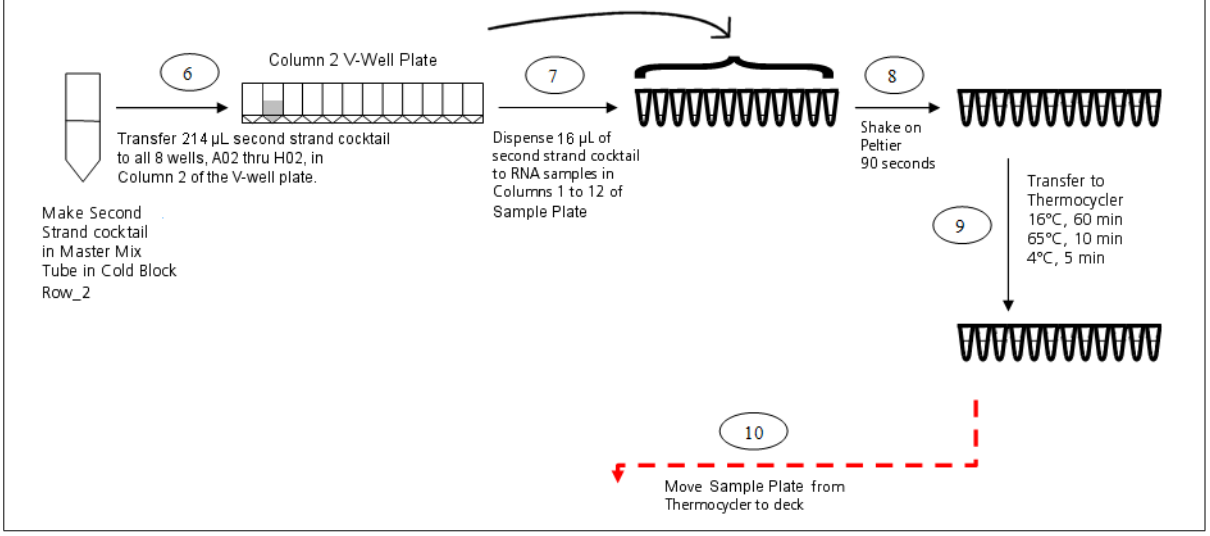
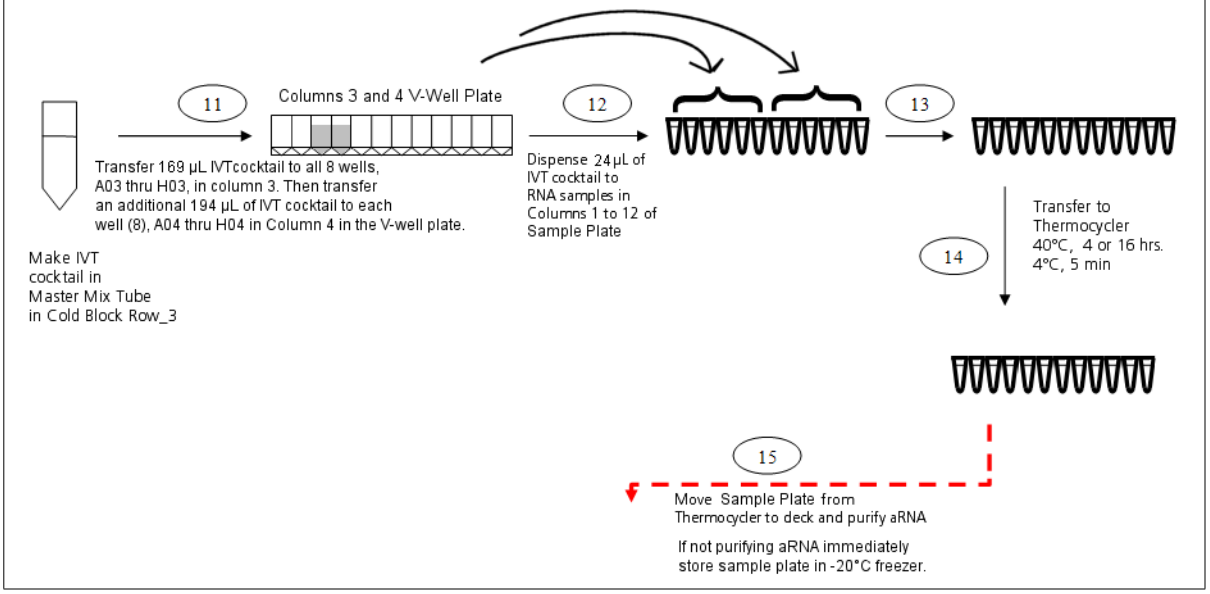


Figure 4.6 96-Sample, Day 1: IVT Labeling



Day Two: 24- and 96-Sample Workflow

Figure 4.7 24- and 96-Sample, Day 2: Purification

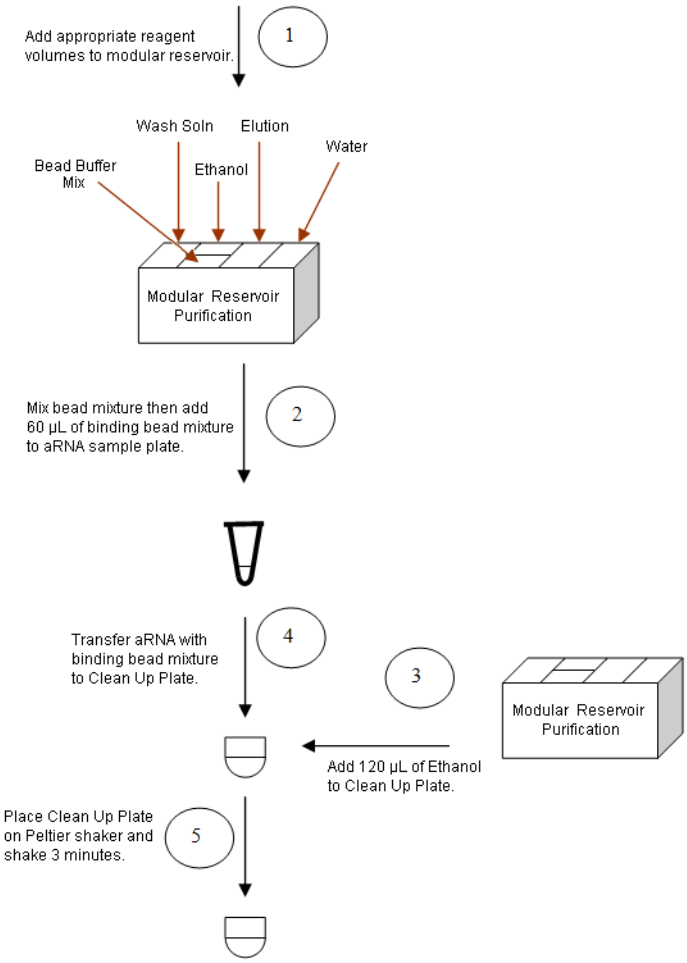


Figure 4.8 24- and 96-Sample, Day 2: Purification and Elution

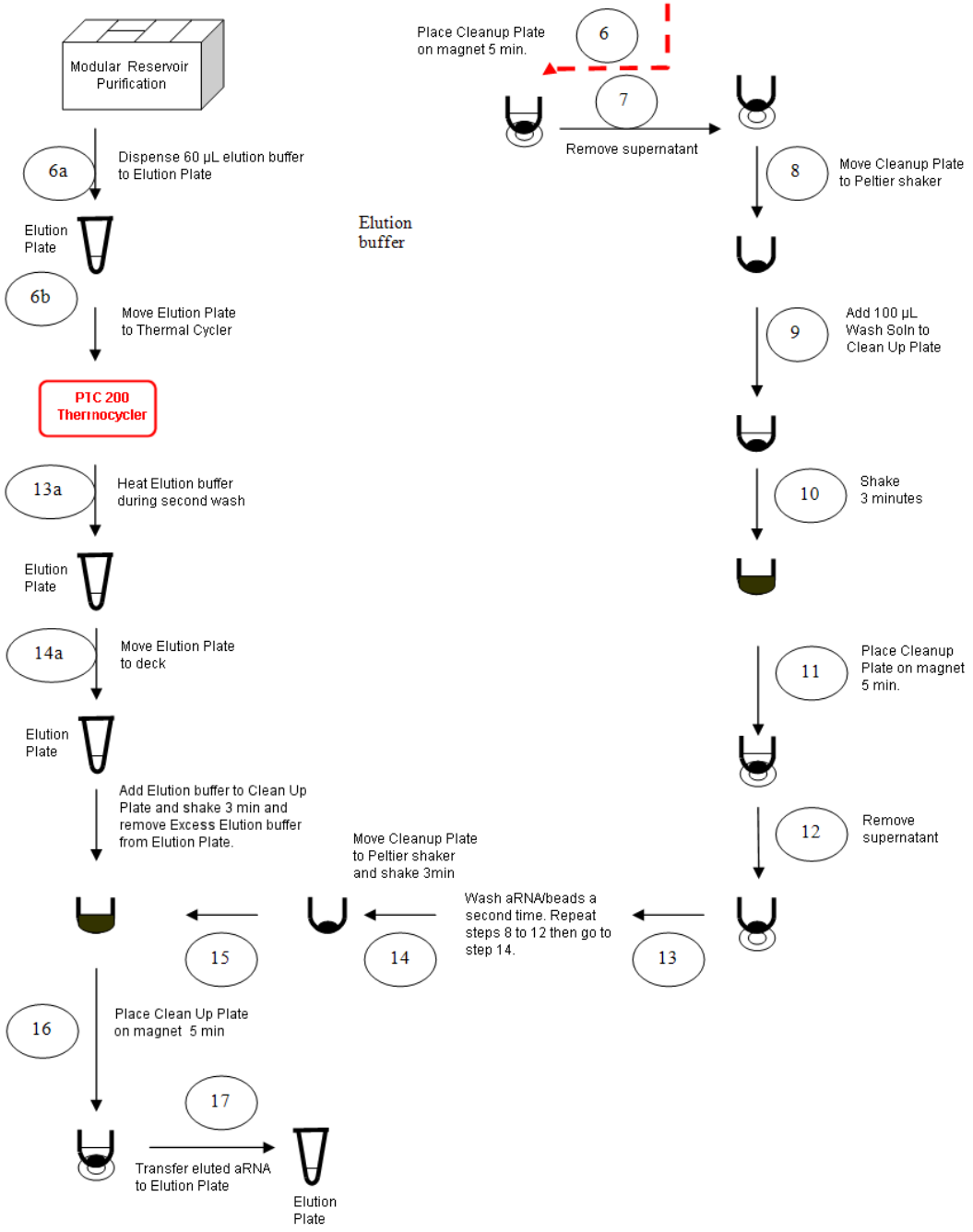


Figure 4.9 24- and 96-Sample, Day 2: Quantitation and Normalization

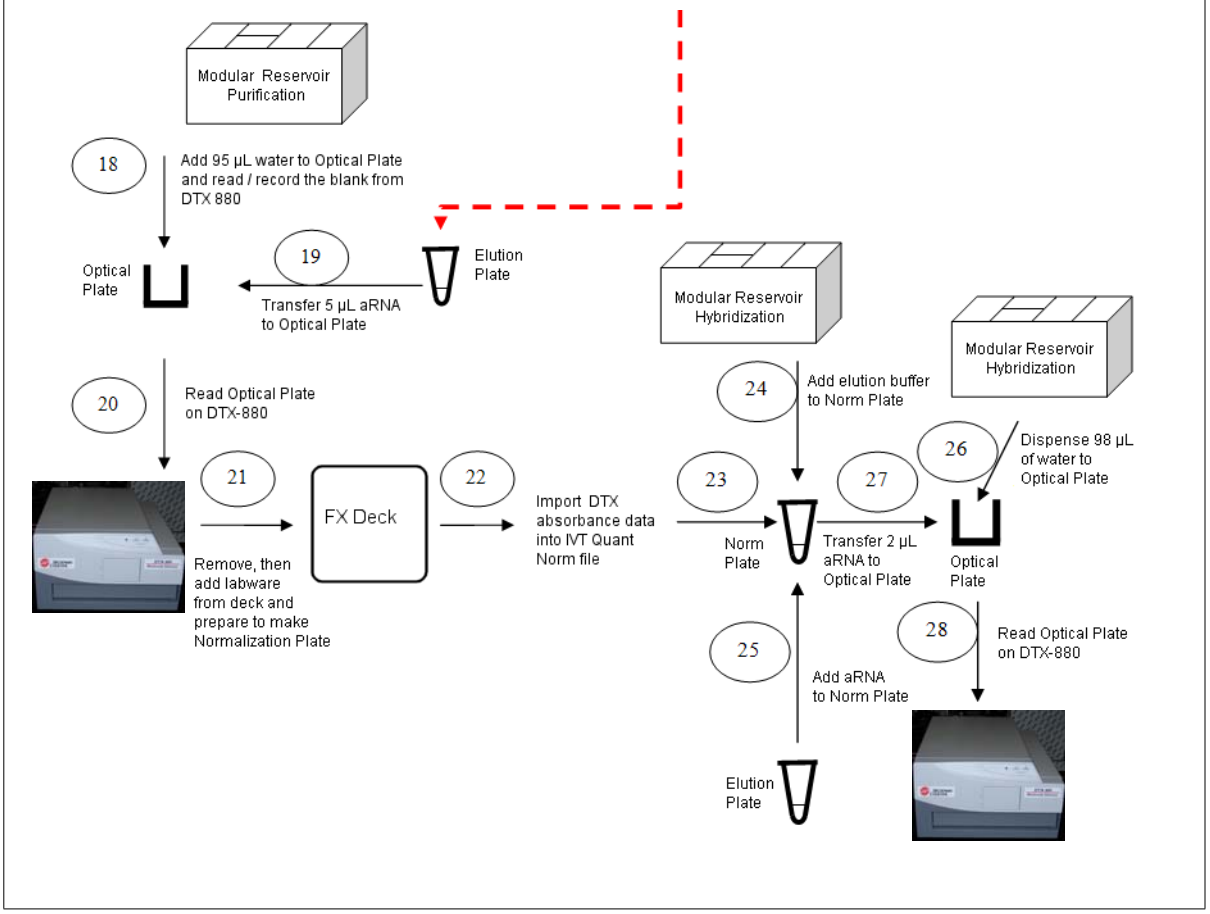
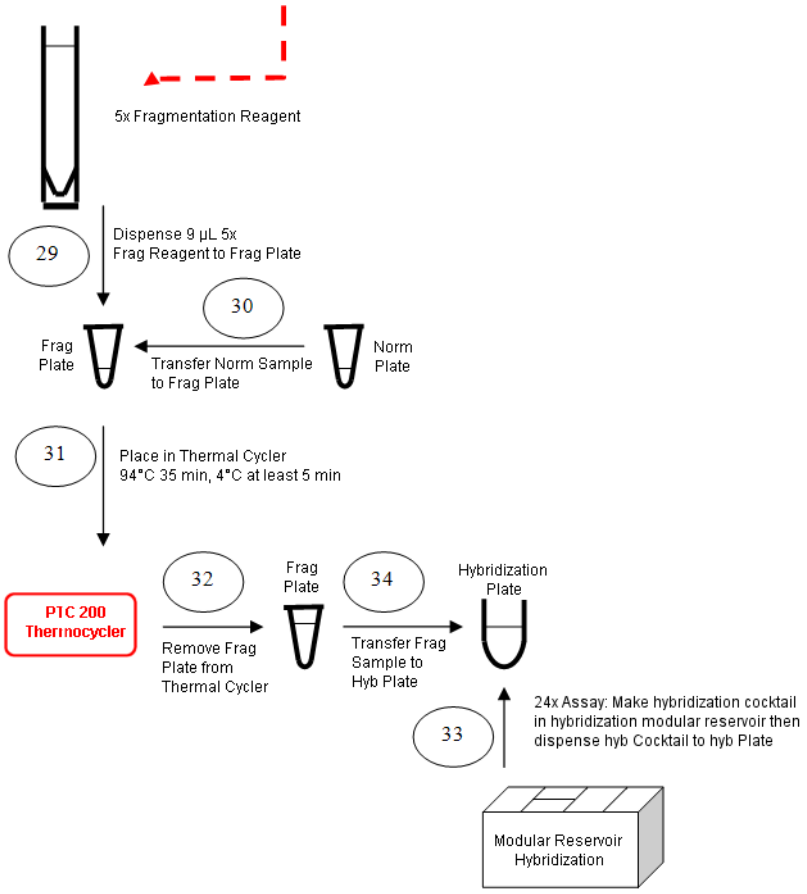


Figure 4.10 24- and 96-Sample, Day 2: Fragmentation and Hybridization



Check List Before a Run - Day 1

Materials Required - Day 1

Table 4.5 Materials Required for Day 1 Run

# 24X	# 96X	Description
1	1	GeneChip HT 3' IVT Express Kit Components needed: ▪ 1st Strand Enzyme Mix ▪ 1st Strand Buffer Mix ▪ 2nd Strand Enzyme Mix ▪ 2nd Strand Buffer Mix ▪ IVT Enzyme Mix ▪ IVT Labeling Buffer ▪ IVT Biotin Label
		Nuclease-free Water
		DNAZap
		RNaseZap
1	1	Cold Reagent Block, kept in 4°C storage
1	1	Cold Reagent Block template
4 tips	22 tips	P1000 LLS Barrier Tips (Span 8 Pipettor, yellow), Beckman P/N 987925
21 tips	19 tips	P250 Barrier Tips Sterile (Span 8 Pipettor, green), Beckman P/N 379503 (Note: The P250 Barrier Pipette Tips are loaded where the user interface shows P200.)
77 tips	288 tips	P50 Barrier Tips (Span 8 Pipettor, magenta), Beckman P/N A21586
4	6	Mixing Tube, Conical Bottom, 2.0mL, Screwcap Unskirted
1	1	Plate, Greiner V-well mixing plate, EK21201
		Bio-Rad Hardshell 96 sample/reaction plate
1	1	Lid, Autosealing Microplates, Arched
1	1	Pad, Microseal, P

Clean the Bio-Rad 96-Well Hard-Shell PCR Plate Lids

NOTE: The disposable pad under the arched lids should be cleaned before every run.

Material required

- Ambion DNAZap™
- Ambion RNaseZap® Wipes

Procedure

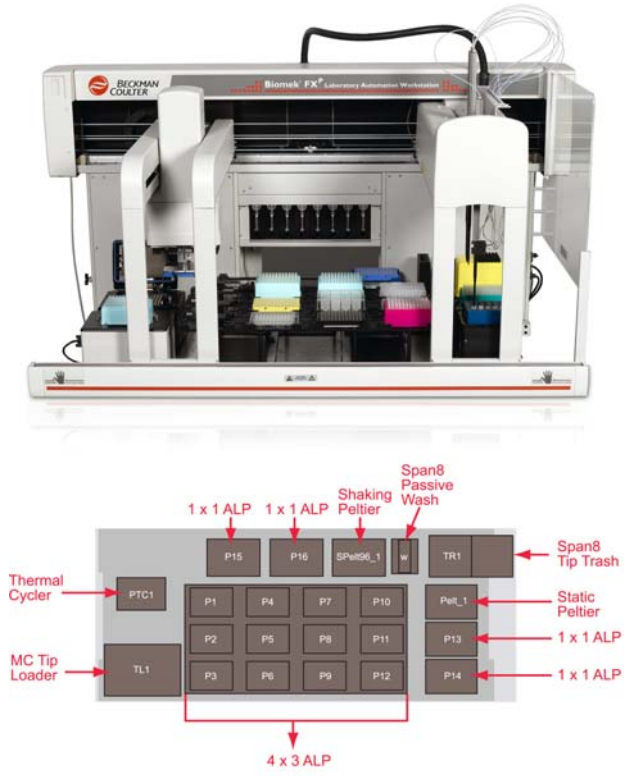
1. Rinse the pad with DI water.
2. Wipe the pad with RNaseZap.
3. Rinse the pad with DI water.
4. Wipe the pad with DNAZap.
5. Thoroughly rinse the pad with DI water.
6. Dry the pad with pressurized clean air or nitrogen.

NOTE: The disposable pad under the arched lids should be replaced every 25 runs.

Preparing the Biomek FXP TPE System

Before beginning a sample preparation run, you must make the following checks of the system.

Figure 4.11 Biomek® FXP TPE System with Deck / Pod Locations



Check the Water and Waste Containers

To check the system water and waste containers:

1. Ensure that the water supply connection and waste water drainage are properly installed.

1. If necessary, fill the system supply bottle must be filled with distilled or de-ionized water (ultra-pure water not required).
2. If necessary, empty the system waste bottle.
3. If necessary empty the tip waste container.

Turn on the Biomek FXP Target Prep Express

To turn on the workstation:

1. Power on the workstation.
2. Ensure that all of the peripherals are powered on (Switches are found on the back of the boxes.).
   - Watlow temperature controllers control the Static Peltier (Pelt_1) and the Shaking Peltier (SPelt_96); no additional power supply.
   - Thermal cycler:
     - BIO-RAD PTC-200 with DNA Engine, or
     - Whatman Biometra TRobot-96

CAUTION: If using a PTC-200 thermal cycler, before turning it on confirm that the Multichannel head (pod_1) is not positioned over the thermal cycler.

3. Launch the Biomek Software by double-clicking the **Biomek Software** icon on the desktop, or click **Start → Programs → Beckman Coulter→ Biomek Software** to launch the Biomek FXP TPE System software application.

NOTE: The instructions that follow pertain to the PTC-200 thermal cycler. If using a Biometra TRobot proceed to *Home All Axes* on page 31.

NOTE: For detailed instructions on how to use either the PTC-200 or the Biometra TRobot, please refer to the appropriate Beckman Coulter user manual for your specific thermal cycler.

Close the PTC-200 Thermal Cycler Lid

The PTC-200 lid may remain open upon startup. You must close the PTC-200 lid prior to homing the axes or starting a method.

Close the PTC-200 lid:

- **if it remains open after you have powered on the workstation.**
- **if the lid is up before you home the axes or before you begin a method.**

If not closed, the MC Pod may collide with the PTC-200 lid and damage the instrument.

Close the PTC-200 Thermal Cycler Lid

The PTC-200 lid may remain open upon startup. You must close the PTC-200 lid prior to homing the axes or starting a method.

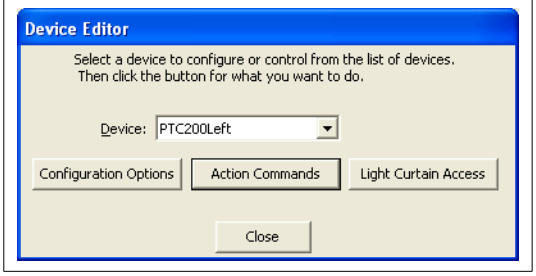
Close the PTC-200 lid:

- if it remains open after you have powered on the workstation.
- if the lid is up before you home the axes or before you begin a method.

If not closed, the MC Pod may collide with the PTC-200 lid and damage the instrument

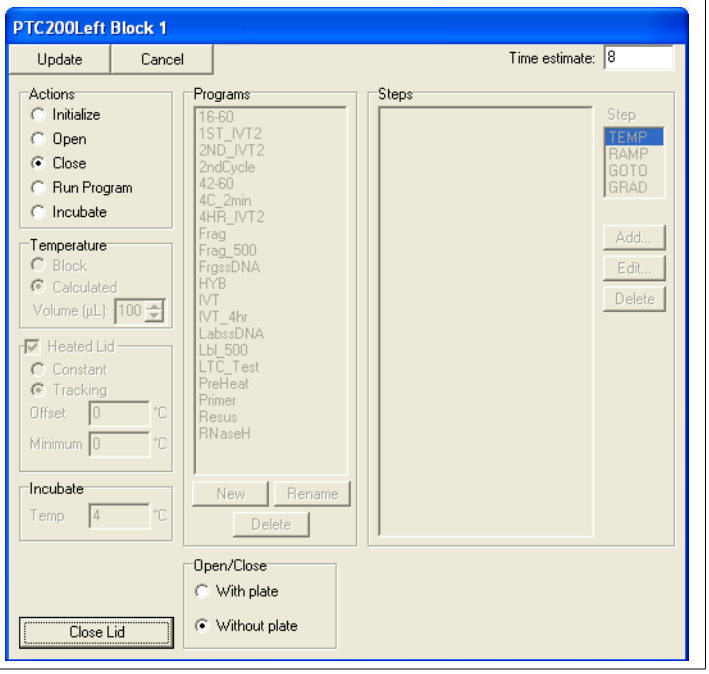
1. To close the thermal cycler lid, click **Instrument → Device Editor**.
   The Device Editor window appears (Figure 4.12)

Figure 4.12



2. Select the PTC thermal cycler from the Device drop-down list box.
3. Click the **Action Commands** button.
   The PTC Command window appears (Figure 4.13).

Figure 4.13

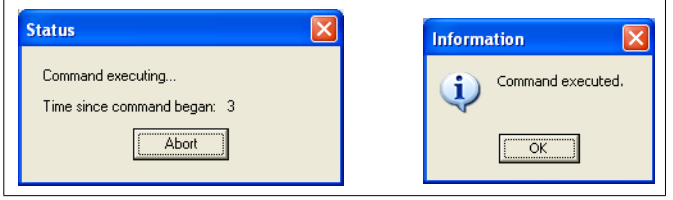


4. Select the following options:
   - **A.** In the *Actions* box, select **Close**.
   - **B.** In the *Open/Close* box, select **Without plate**.

! IMPORTANT: **It is critical that you select *Without plate* in the Open/Close box. If a plate is present, remove it before closing the lid.**

5. Click the **Close Lid** button.
   A Status box is followed by an Information box (Figure 4.14).

Figure 4.14



6. Click **OK** in the Information box. Click the **Cancel** button. Click the **Close** button in the Device Editor window.

> **WARNING:** Do not use the blue button on the on-deck thermal cycler to close the lid.

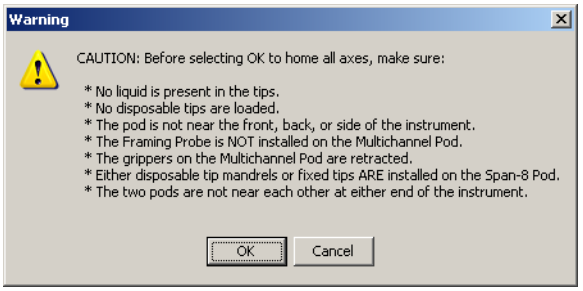
Home All Axes

This procedure will home all axes and prime the fluidics lines.

To home all axes:

1. Click **Instrument** → **Home All Axes**.

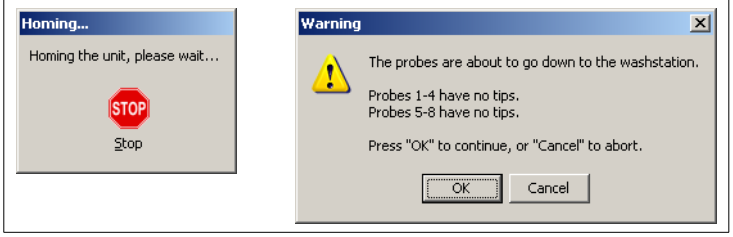
Figure 4.15 Warning message



2. Follow the instructions on the warning message (Figure 4.15). Verify the following:
   - No liquid is present in the tips.
   - No disposable tips are loaded.
   - The pod is not near the front, back, or side of the instrument.
   - The Framing Probe is NOT installed on the Multichannel Pod.
   - The grippers on the Multichannel Pod are retracted.
   - Either disposable tip mandrels or fixed tips ARE installed on the Span-8 Pod.
   - The two pods are not near each other at either end of the instrument.

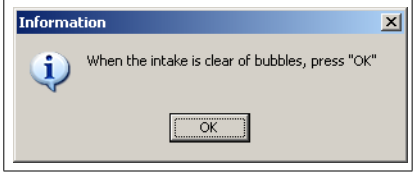
3. When ready click **OK**.

Figure 4.16



4. When the warning message appears as shown in Figure 4.16, click **OK**.
   The Biomek FXP TPE System starts purging the tubes of air.
5. When you no longer see bubbles in the lines (approximately 20 times), click **OK**, as shown in Figure 4.17.

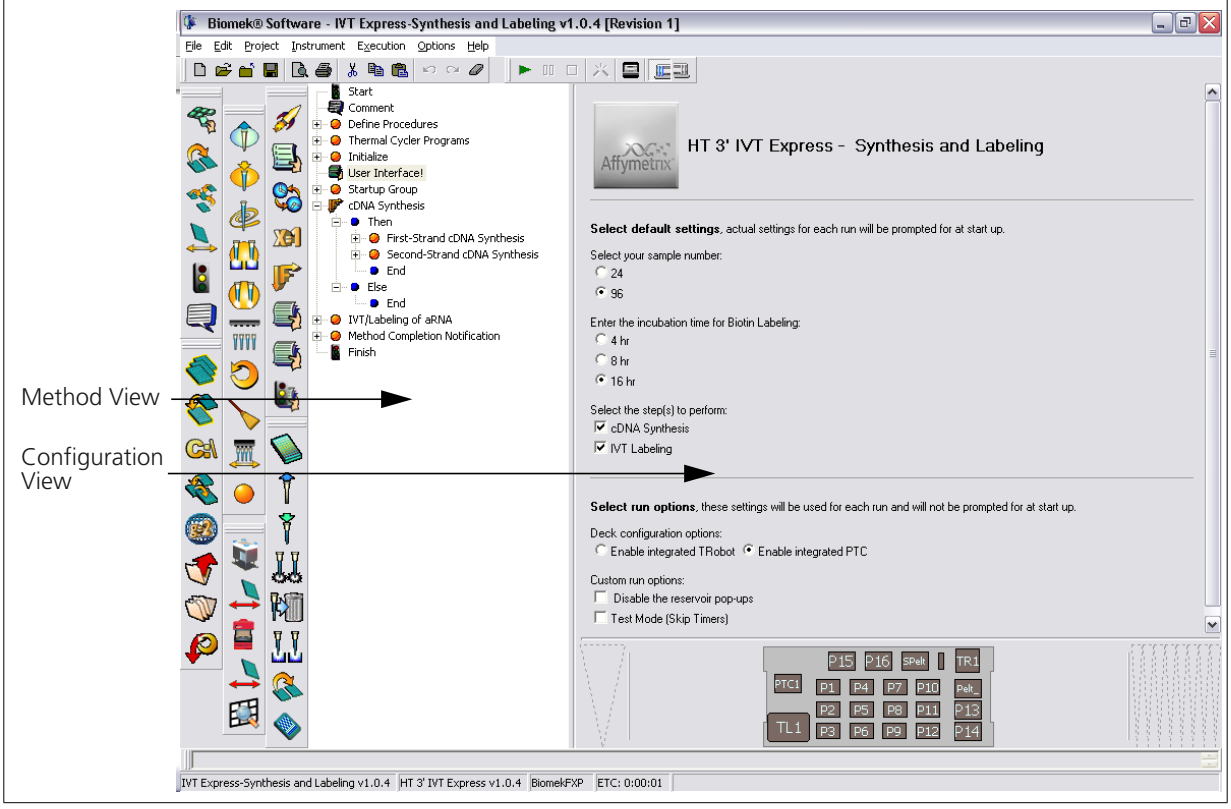
Figure 4.17



Selecting Run Options

1. From the Method View, click **User Interface!**
   The Configuration View is populated with information (Figure 4.18).

Figure 4.18



2. Select run options:

NOTE: The HT IVT Express Method on the Beckman requires an on-deck Thermo-cycler. Verify that your robot is the FXP Target Prep Express configuration with an on-deck thermo-cycler.

- **Disable the reservoir pop-ups**: Select this option if you do not need to see pop-up instructions for reservoir setup.
- In the Configuration View area, under **Device Configuration Option**, confirm that the correct integrated thermal cycler device is selected (Bio-Rad PTC-200 or Whatman Biometra TRobot).
- **Test Mode** (Skip Timers): DO NOT select Test Mode as it will disable timers.

The Biomek FXP TPE System is now ready for use.

Day One: IVT Synthesis and Labeling

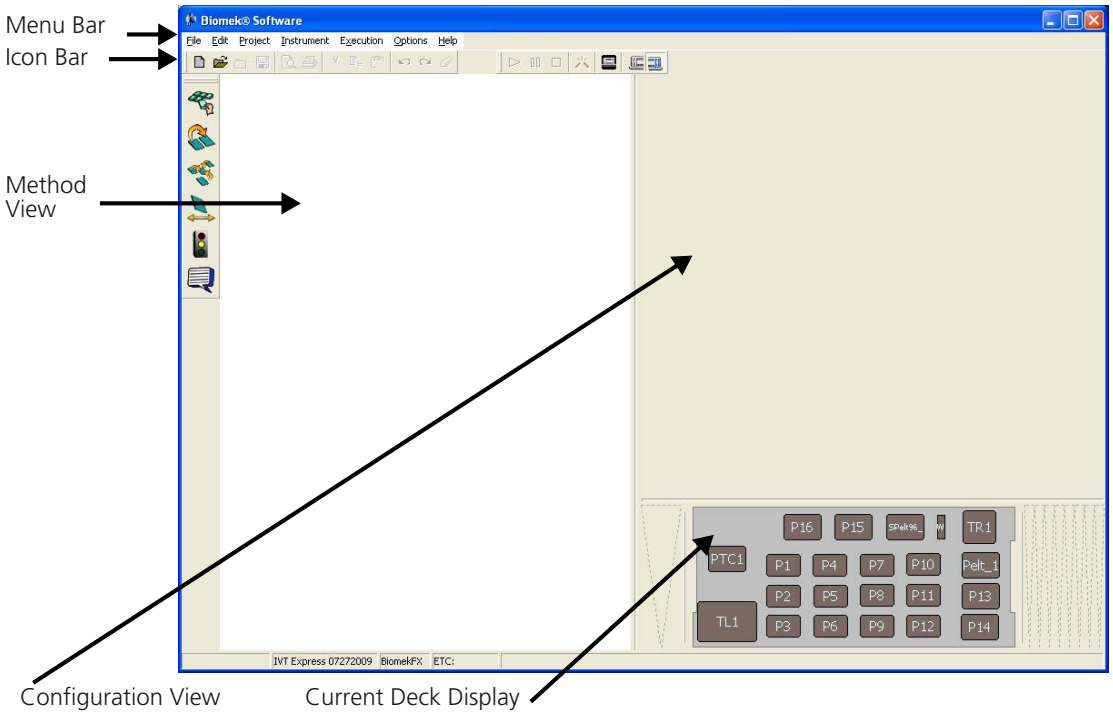
Obtain First-Strand, Second-Strand and IVT Reagents

1. From the GeneChip® HT 3’ IVT Express Kit, thaw the First Strand Buffer, Second Strand Buffer, IVT Buffer and Biotin reagent.
2. Store the reagent cold block in 4°C refrigerator.

Running the Biomek Software

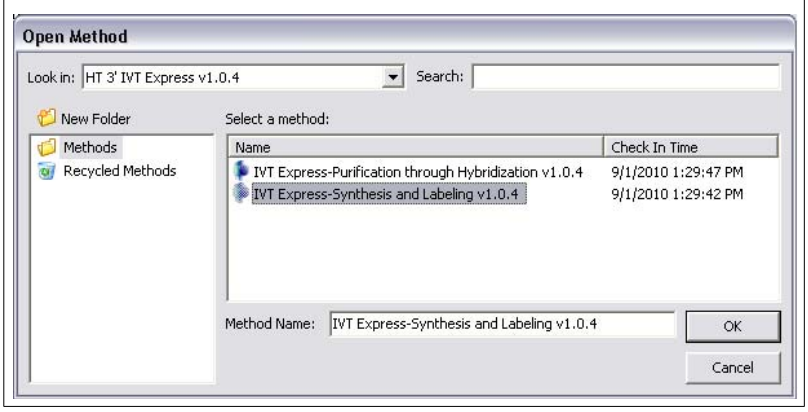
1. If necessary, click **Start** → **Programs** → **Beckman Coulter** → **Biomek Software** to launch the Biomek FXP TPE System software application (Figure 4.19).

Figure 4.19 Biomek software interface



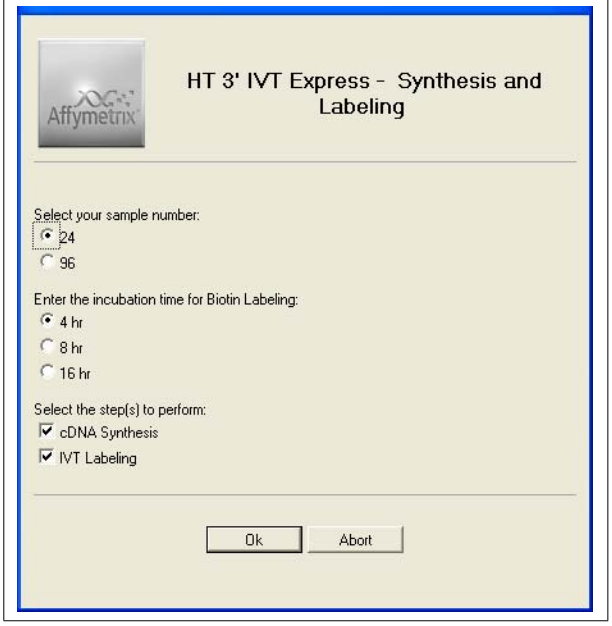
2. From the menubar, click **File** → **Open**.
The Open Method dialog box appears (Figure 4.20).

Figure 4.20 Open Methods dialog box



3. From the Open Method dialog box:
   A. Click the **Look in** drop down box and select the **HT 3' IVT Express** project name.
   B. In the **Select a method** list box, click to select **IVT Express Synthesis and Labeling**.
   C. Click **OK**.
4. Click the Run icon.
   A Run Settings window appears summarizing the options available for the current run (Figure 4.21).

Figure 4.21 Run Settings



5. In the Run Settings window, select from the options listed:
   A. **Select your Sample Number** for your run (24 or 96).
   B. **Enter The incubation time for Biotin Labeling** (4, 8 or 16 hours).
   Refer to Table 3.3 on page 14 for Recommended IVT Incubation Times.
   C. **Select the step(s) to perform**:
      - **cDNA Synthesis**: Runs methods: (1) Reverse Transcription to Synthesize First-Strand cDNA and (2) Second-strand cDNA Synthesis
      - **IVT Labeling**: Runs method: In Vitro Transcription to Synthesize Labeled aRNA

6. Confirm both static and shaker Peltier is set to 4°C (Figure 4.22).

Figure 4.22 Setting Peltier temperatures - Day One

7. When ready click **OK** in Figure 4.21.
   The deck layout window appears for your run, Figure 4.23 or Figure 4.24.

Figure 4.23 Deck layout for 24-samples

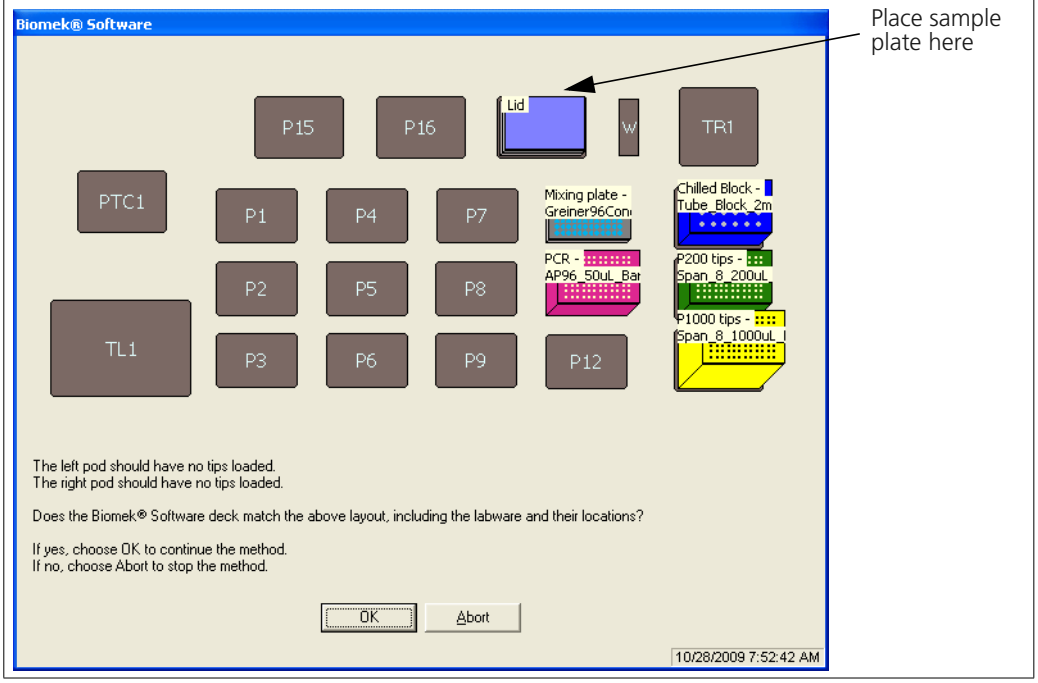
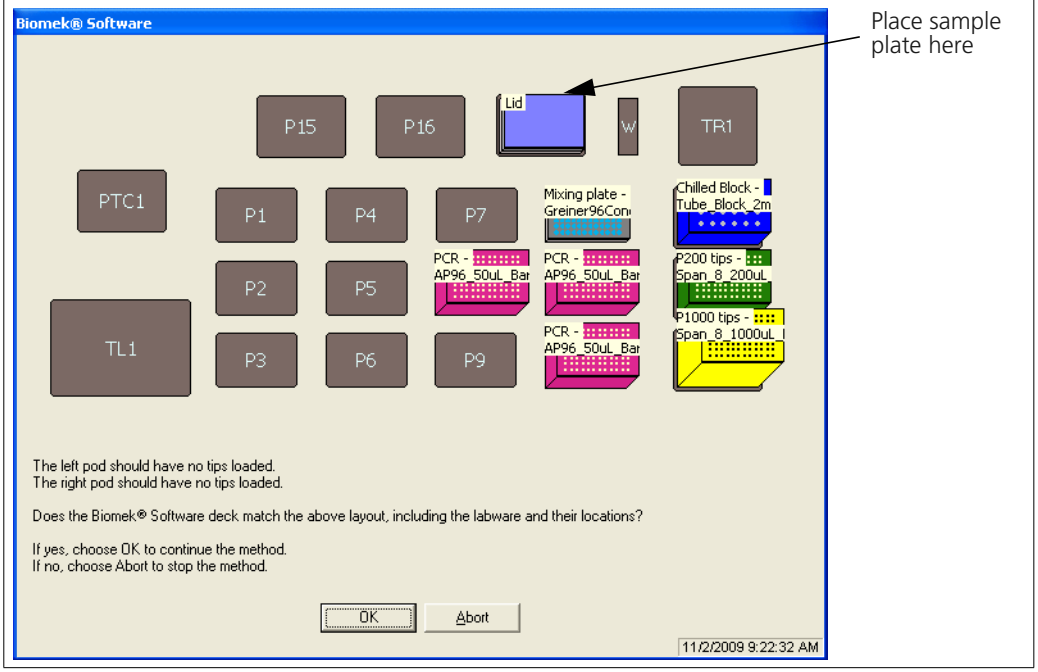


Figure 4.24 Deck layout for 96-samples



8. Set up the deck as illustrated in your deck layout window.
   - Remove the cold block from 4°C storage and place on the static Peltier. Verify that the correct side of the Reagent Cold block template is being used for your run.
   - Remove lids from tip boxes.
   - Place sample plate (see page 9) covered with an arched lid onto the shaking Peltier.

Table 4.6 Biomek® Tips and Universal labware

Product	P/N
P50 Barrier Tips (Span 8 Pipettor, magenta)	A21586
P250 Barrier Tips Sterile (Span 8 Pipettor, green)	379503
P1000 LLS Barrier Tips (Span 8 Pipettor, yellow)	987925

9. When ready click **OK** in Figure 4.23.
   Reagent Cold Block window appears (Figure 4.25).

Figure 4.25 Reagent Cold Block setup, 24 samples

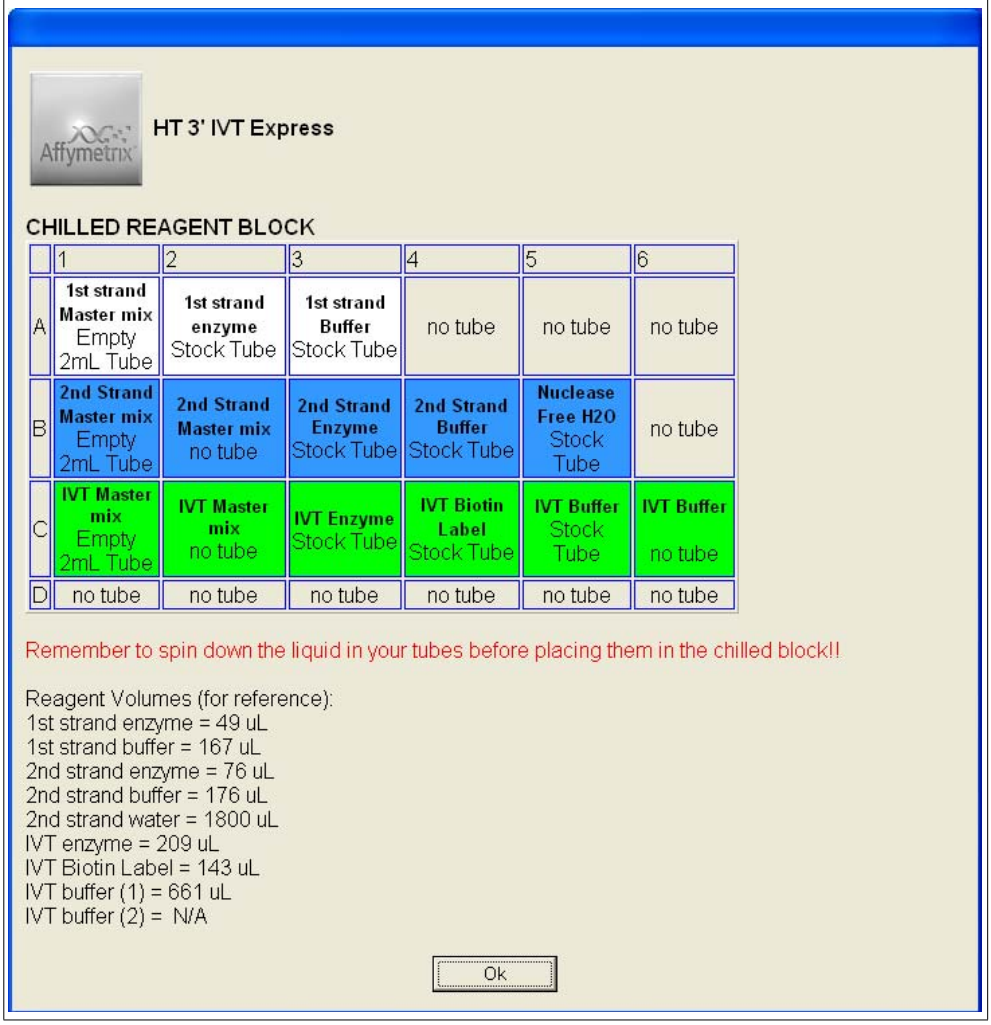
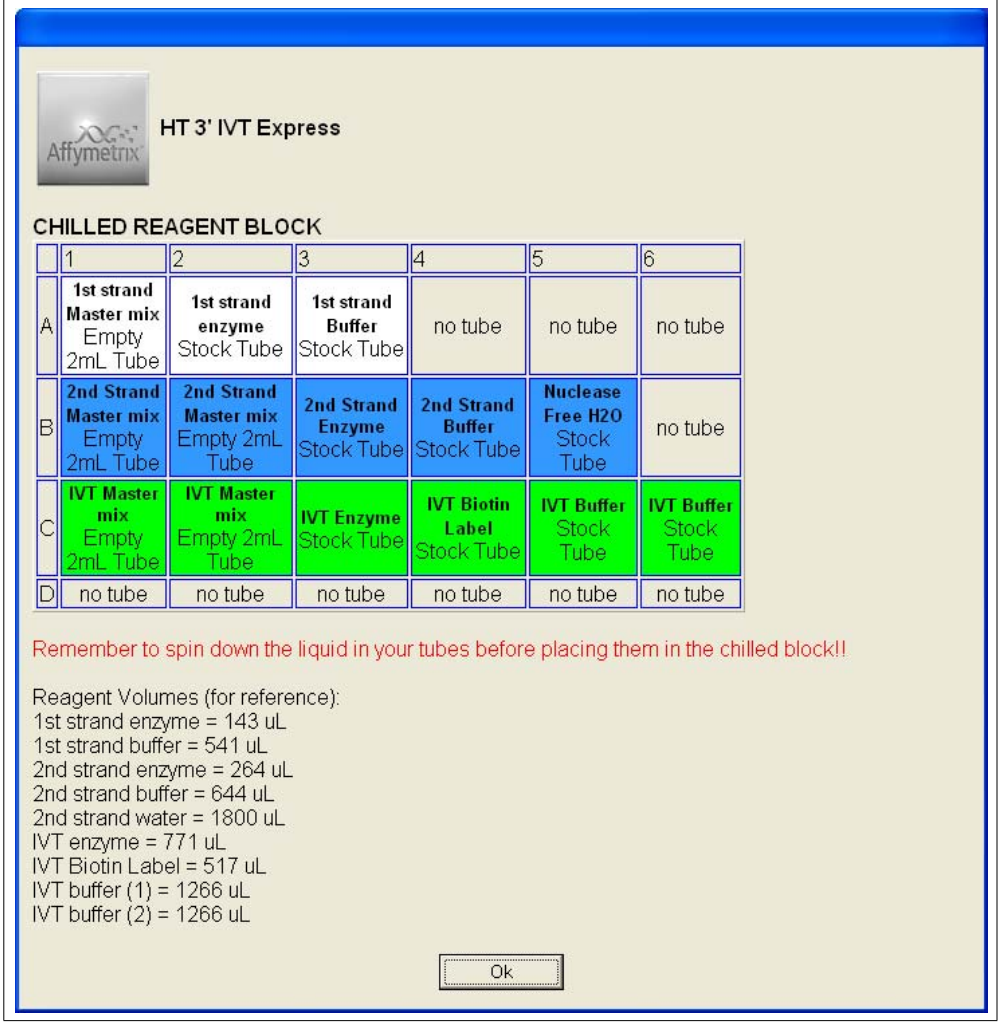


Figure 4.26 Reagent Cold Block setup, 96 samples



	1	2	3	4	5	6
A	1st strand Master mix Empty 2mL Tube	1st strand enzyme Stock Tube	1st strand Buffer Stock Tube	no tube	no tube	no tube
B	2nd Strand Master mix Empty 2mL Tube	2nd Strand Master mix Empty 2mL Tube	2nd Strand Enzyme Stock Tube	2nd Strand Buffer Stock Tube	Nuclease Free H2O Stock Tube	no tube
C	IVT Master mix Empty 2mL Tube	IVT Master mix Empty 2mL Tube	IVT Enzyme Stock Tube	IVT Biotin Label Stock Tube	IVT Buffer Stock Tube	IVT Buffer Stock Tube
D	no tube	no tube	no tube	no tube	no tube	no tube

! IMPORTANT: **Ensure Biotin reagent and all buffers are completely thawed prior to use. If a precipitant is present in buffer dissolve by placing in a heat block at 40°C 1 to 2 minutes, vortex, and return to heat block until dissolved. Keep all enzymes cold. Gently flick enzymes to mix. Centrifuge all reagent tubes prior to loading. Check no bubbles present.**

10. Setup the reagent cold block for your run. Refer to Figure 4.27 and Figure 4.28 for reagent cold block setup.

A. Place the reagent block template appropriate for your run onto the cooled (4°C) reagent block.

B. Uncap the First-Strand, Second-Strand and IVT reagent tubes and insert them into the cooled reagent block following the color codes on the template, or as shown in Figure 4.27 and Figure 4.28.

C. Fill a 2 mL mixing tube with 1800 µL nuclease-free water and insert it into the loaded reagent cold block.

D. Uncap all tubes and load the mixing tubes onto the assembled cold reagent block and adaptor following the color codes on the template, or as shown in Figure 4.27 and Figure 4.28.

Figure 4.27 Cold Block Reagent Layout for 24-Sample Plate - Day 1

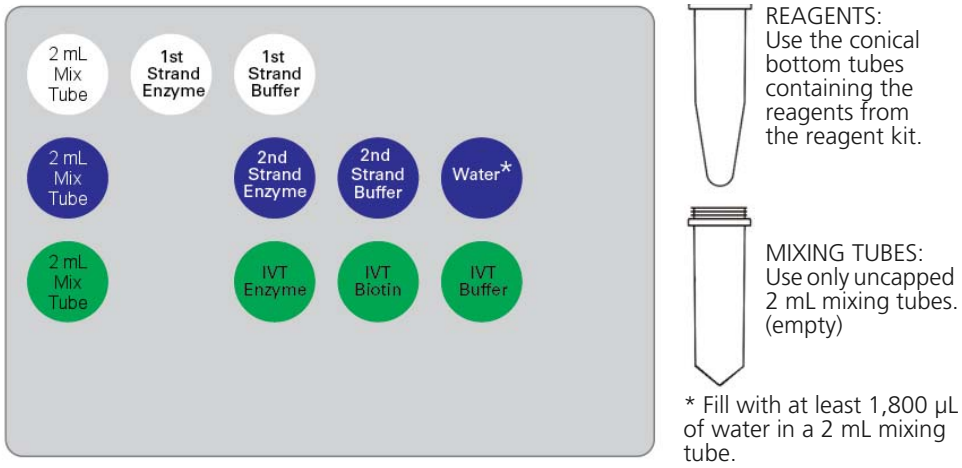
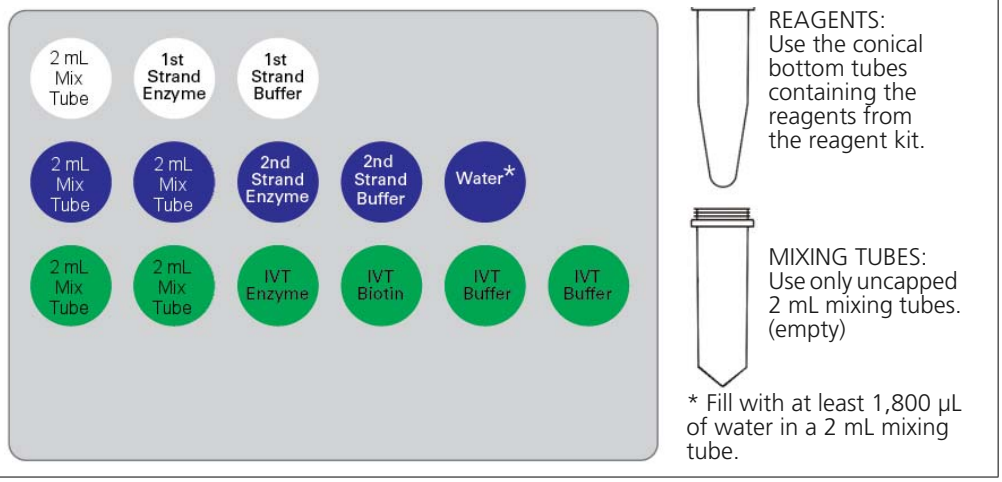


Figure 4.28 Cold Block Reagent Layout for 96-Sample Plate - Day 1



! IMPORTANT: All mixing tubes used should be empty, uncapped 2 mL tubes. For reagents, use the conical bottom tubes containing the reagents in the GeneChip® HT 3' IVT Express Kit. For the location of the reagents and mixing tubes in the cold block please refer to the cold block template or Figure 4.27 or Figure 4.28 on page 40.

11. When you have completed the Reagent Cold Block setup, click **OK** in Figure 4.25.

The robot proceeds to First-Strand Synthesis.

Reverse Transcription to Synthesize First-Strand cDNA

The Biomek FXP TPE System begins the First-Strand Synthesis step without user intervention. This step takes approximately 2.5 hours to complete and then proceeds to Second-strand cDNA Synthesis.

The composition of the First-Strand Master Mix is shown in Table 4.7.

Table 4.7 First-strand Master Mix/RNA Volumes

Component	Amount
First Strand Buffer Mix	3.2 µL
First Strand Enzyme Mix	0.8 µL
RNA	4 µL
Total Volume	**8 µL**

Second-strand cDNA Synthesis

After the First-strand Synthesis, the Biomek FXP TPE System continues to the Second-strand cDNA Synthesis step. This step takes approximately 1.75 hours to complete. The composition of the Second-strand Master Mix is shown in Table 4.8. The Biomek FXP TPE System proceeds to the next step without user intervention.

Table 4.8 Second-strand Master Mix (for a single reaction)

Component	Amount
Nuclease-free Water	10.4 µL
Second Strand Buffer Mix	4 µL
Second Strand Enzyme Mix	1.6 µL
Total Volume	**16 µL**

In Vitro Transcription to Synthesize Labeled aRNA

After the Second-strand Synthesis, the Biomek FXP TPE System continues to the IVT Synthesis step. This step requires approximately 20 minutes preparation and with a 16 hour incubation time. The composition of the IVT Master Mix is shown in Table 4.9.

The Biomek FXP TPE System assembles the **IVT Master Mix**, mixes the component reagents, then aliquots 24 µL IVT Master Mix to 24 µL sample in Bio-Rad hardshell PCR plate.

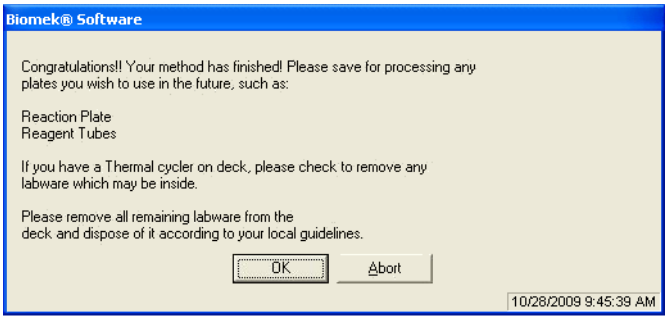
Table 4.9 IVT Master Mix (for a single reaction)

Component	Amount
IVT Biotin Label	3.2 µL
IVT Labeling Buffer	16 µL
IVT Enzyme Mix	4.8 µL
Total Volume	**24 µL**

After incubation is complete the reaction plate is held at 4°C.

After the IVT synthesis and labeling reaction has completed, a user message will appear (Figure 4.29).

Figure 4.29



12. Click **OK** in Figure 4.29.

TIP: STOPPING POINT: The aRNA can be stored at –20°C at this point, if desired.

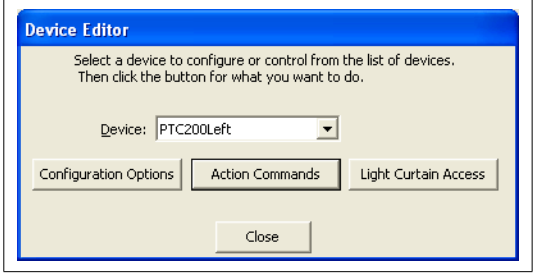
If you have selected the 8 or 16 hour incubation time the sample plate is held in the incubator overnight.

If you have selected a 4 hour incubation time, remove the sample plate from the thermal cycler. The sample may be placed at –20°C for storage or held a short time at 4°C until Purification. If proceeding to Purification immediately hold at room temperature.

To Remove Sample Plate from PTC

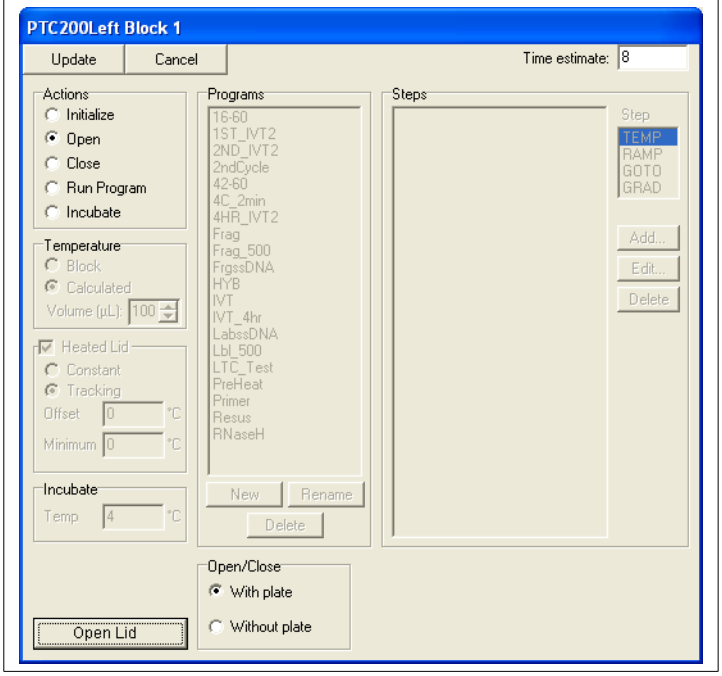
1. To open the thermal cycler lid, click **Instrument** → **Device Editor**.
   The Device Editor window appears (Figure 4.12)

Figure 4.30



2. Select the PTC thermal cycler from the Device drop-down list box.
3. Click the **Action Commands** button.
   The PTC Command window appears (Figure 4.13).

Figure 4.31



4. Select the following options: **Open**, and **With plate**.
5. Click the **Open Lid** button.
6. Remove sample plate from thermal cycler.
7. Select the following options: **Close**, and **Without plate**.
8. Click the **Close Lid** button. Click the **Cancel** button. Click the **Close** button.

Day Two: Purification

Materials Required - Day 2

Table 4.10 Materials Required for Day 2 Run

# 24X	# 96X	Description
		GeneChip HT 3’ IVT Express Kit Components needed: ▪ aRNA Wash Solution ▪ aRNA Elution Solution ▪ Beads and Buffer
		(cartridge arrays only) GeneChip® Hybridization, Wash, and Stain Kit, Affymetrix P/N 900720 Components needed: ▪ DMSO
		(array plates) If using Gene Titan: GeneTitan Hybridization, Wash, and Stain Kit for 3' IVT Arrays, Affymetrix P/N 901530
		100% Ethanol
		Nuclease-free water
		Sample plate and lid
480 tips *576 tips**	480 tips *576 tips**	P250 Barrier Tips Sterile (AP 96 Multichannel Pipettor, light blue), Beckman P/N 717253
64 tips	40 tips	P250 Barrier Tips Sterile (Span 8 Pipettor, green), Beckman P/N 379503
43 tips *67 tips**	47 tips *71 tips**	P1000 LLS Barrier Tips (Span 8 Pipettor, yellow), Beckman P/N 987925
288 tips	288 tips	P50 Barrier Tips (Span 8 Pipettor, magenta), Beckman P/N A21586
1	1	Cold Reagent Block
1	1	Cold Reagent Block template
2	2	Plate, Bio-Rad Hardshell PCR
2	2	Plate, UV Star Greiner
1	1	Plate, 1.2mL Square Well Storage, Low Profile
2	2	Plate, Costar Brand Serocluster U-bottom
		SPRIPlate 96R Super Magnet
3	3	Quarter Reservoir, Divided by Width, Beckman P/N 372792
8 *11**	8 *11**	Quarter Reservoir, Beckman P/N 372790
2	2	Frame for Reservoirs, Beckman P/N 372795
1	1	Affymetrix® GeneChip® HT 3’ IVT Reservoir Template

**Number required if running GeneTitan setup.*

Check List Before Proceeding with the Run

1. Check Peltiers for condensation. If the Peltiers have condensation blot if off with a Kimwipe.
2. If necessary, remove sample plate from storage.
3. Gather Day 2 Purification reagents and leave them at room temperature:

NOTE: All components and reagents should be at room temperature. Allow samples to sit at room temperature for at least 10 minutes. The aRNA Binding Buffer will precipitate if cold, therefore keep beads and binding buffer at room temperature.

- Beads
- Bead Buffer
- Wash Solution Concentrate
- aRNA Elution Solution
- Ethanol
- Water

4. Turn on Shaking Peltier and Static Peltier and make sure that both Peltiers are at room temperature.
5. If not using a Beckman DTX-880 Multimode Plate Reader perform the following:

   The quantitation portion of this protocol has been written to be used with a Beckman DTX 880 Multimode plate reader. If you are using a different OD plate reader, then please ensure your plate reader is capable of the following:

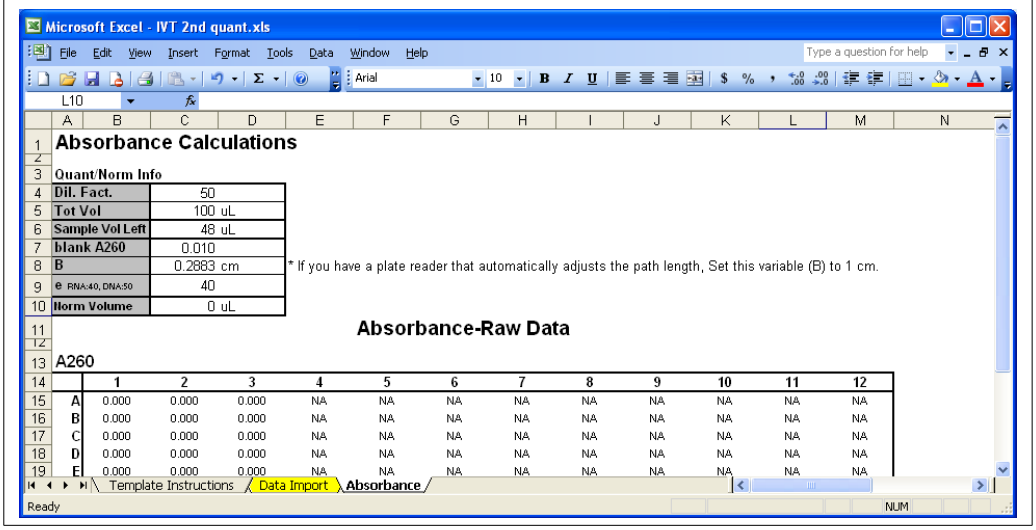
   - Instrument is capable of reading the absorbance at 260nm.
   - If spectrophotometer has pathlength correction to 1cm, then reset pathlength correction and resave the two templates. See directions below.
   - During the quantitation run you will need to manually enter (copy and paste) the reader data into the template.

   To Reset Pathlength Correction in Template Files for Non- DTX 880 Users:

   A. Locate the two template files on your computer. They can be found at: C:\Documents and Settings\All Users\Documents\IVT 2
      - **IVT Quant and Norm.xls** template
      - **IVT 2nd quant.xls** template

   B. Open a template file and click the **Absorbance** tab (Figure 4.32).

Figure 4.32 Reset Pathlength Correction in Template File



C. Set variable **B** in cell C8 to **1.0**.

D. Save and close the template file.

E. Repeat this procedure for the second template file.

6. Prepare Day 2 Purification Reagents:

A. Prepare Beads.

- Vortex beads and ensure beads are completely suspended. Beads tend to be stuck at the bottom of the tube so use a micropipettor if necessary to suspend the beads completely.

B. Check Bead Buffer.

- Make sure buffer has not precipitated out of solution. If precipitated warm tube to room temperature and gently swirl tube. Do not shake or introduce bubbles into the bead buffer.

C. Prepare aRNA Wash Solution Concentrate.

1) Add ethanol to the aRNA Wash Solution Concentrate bottle.

- For 24X: Add 6 mL 100% ethanol (ACS reagent grade or equivalent) to the bottle containing the aRNA Wash Solution Concentrate. Total volume = 7.5 mL.
- For 96X: Add 20 mL 100% ethanol (ACS reagent grade or equivalent) to the bottle containing the aRNA Wash Solution Concentrate. Total volume = 25 mL.

2) Mix well and mark the label to indicate that you have added the ethanol. Do not vortex. Refer to this solution as **aRNA Wash Solution** throughout this protocol. Store at room temperature.

Preparing the Biomek FX^P TPE System

NOTE: The instructions that follow pertain to the PTC-200 thermal cycler. If using a Biometra TRobot proceed to *Home All Axes* on page 48.

NOTE: For detailed instructions on how to use either the PTC-200 or the Biometra TRobot, please refer to the appropriate Beckman Coulter user manual for your specific thermal cycler.

Close the PTC-200 Thermal Cycler Lid

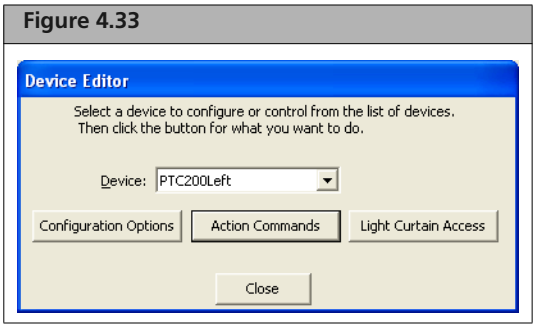
The PTC-200 lid may remain open upon startup. You must close the PTC-200 lid prior to homing the axes or starting a method.

1. Remove the sample plate from the PTC thermal cycler and close lid:

WARNING: Use device editor to remove the sample plate from the on-deck thermal cycler and close the lid to avoid collision of pod 1 with the automated lid of the PTC.

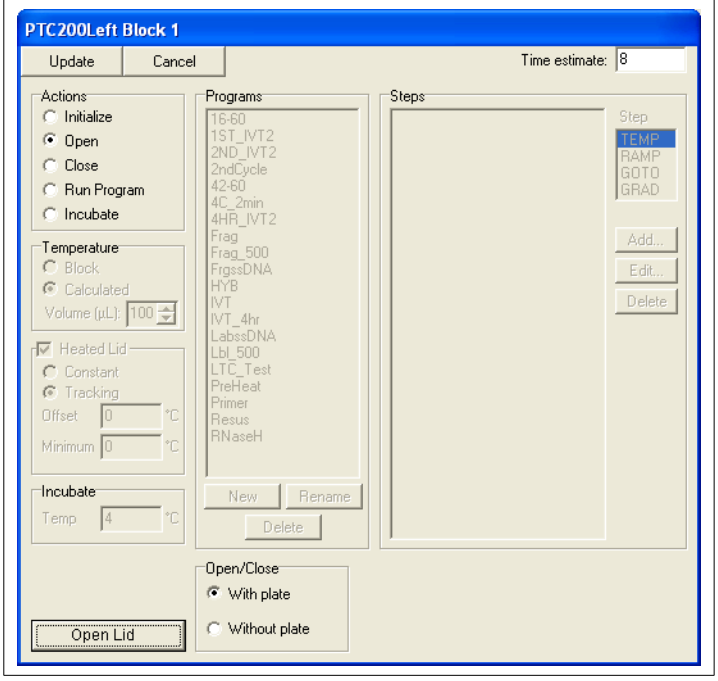
2. To open the thermal cycler lid, click **Instrument** → **Device Editor**.
The Device Editor window appears (Figure 4.33)

Figure 4.33



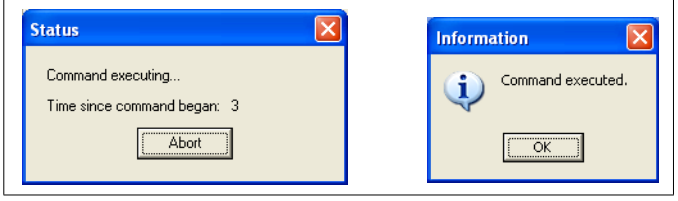
3. Select the PTC thermal cycler from the Device drop-down list box.
4. Click the **Action Commands** button.
   The PTC Command window appears (Figure 4.34).

Figure 4.34 PTC Command window



5. Select the following options: **Open**, and **With plate**.
6. Click the **Open Lid** button.
   A Status box is followed by an Information box (Figure 4.35).

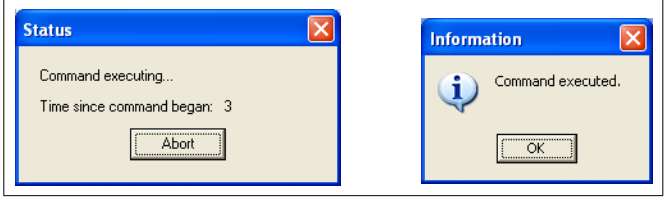
Figure 4.35



7. Click **OK** in the Information box.
8. Remove plate from thermal cycler and place on shaking Peltier at room temperature.
9. In the PTC Command window select the following options: **Close**, and **Without plate** to close the thermal cycler lid.

10. Click the **Close Lid** button.
A Status box is followed by an Information box (Figure 4.36).

Figure 4.36



11. Click the **Cancel** button. Click the **Close** button in the Device Editor window.

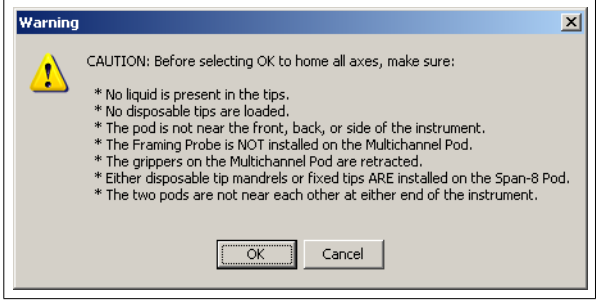
Home All Axes

This procedure will home all axes and prime the fluidics lines.

To home all axes:

1. From the Biomek FXP TPE System software application, click **Instrument → Home All Axes**.

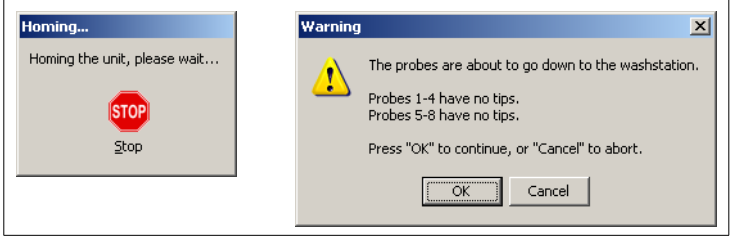
Figure 4.37 Warning message



2. Follow the instructions on the warning message (Figure 4.37). Verify the following:
- No liquid is present in the tips.
- No disposable tips are loaded.
- The pod is not near the front, back, or side of the instrument.
- The Framing Probe is NOT installed on the Multichannel Pod.
- The grippers on the Multichannel Pod are retracted.
- Either disposable tip mandrels or fixed tips ARE installed on the Span-8 Pod.
- The two pods are not near each other at either end of the instrument.

3. When ready click **OK**.

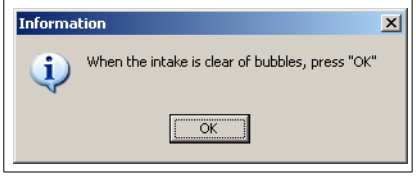
Figure 4.38



4. When the warning message appears as shown in Figure 4.38, click **OK**.
The Biomek FXP TPE System starts purging the tubes of air.

5. When you no longer see bubbles in the lines, click **OK**, as shown in Figure 4.39.

Figure 4.39



6. Verify that both static and shaker Peltier are at room temperature (Figure 4.40).

Figure 4.40 Setting Peltier temperatures - Day Two

7. Ensure that the receptacle that holds the waste tips is empty.
   The Biomek FXP TPE System is now ready for use.

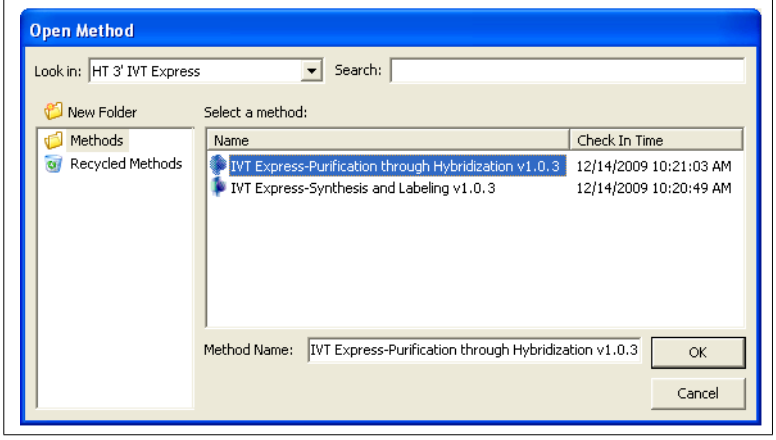
Continuing the Run - Day Two

1. Manually move the arched lid on and off the sample plate to ensure the robot can remove the lid.

> **NOTE:** **At this point the sample plate should have been removed from the thermal cycler and the lid of the thermal cycler closed. If not, please repeat the directions in *Preparing the Biomek FXᴾ TPE System* on page 46.**

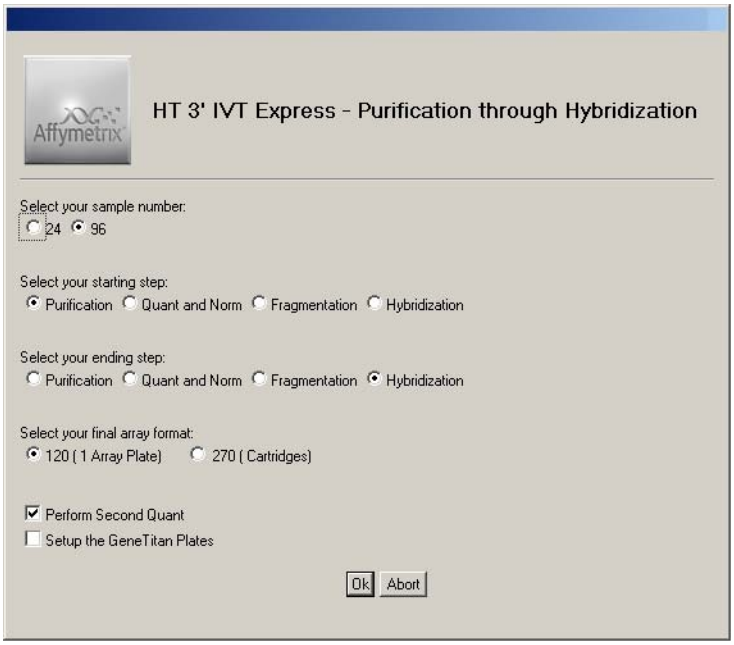
2. With the Biomek FXᴾ TPE System software application open, select **File** → **Open** from the menubar. The Open Method dialog box appears (Figure 4.41).

Figure 4.41



3. From the Open Method dialog box:
   A. Click the **Look in** drop down box and select the **HT 3’ IVT Express** project name.
   B. In the **Select a method** list box, click to select **IVT Express - Purification through Hybridization**.
   C. Click **OK**.
4. Click the Run icon.
   The Run Settings window is displayed (Figure 4.42).

Figure 4.42 Run Settings



5. Select the settings for your run:
   - **A.** **Select Sample Number** for your run: (24, or 96).
   - **B.** Select your starting step:
     - Purification
     - Quant and Norm
     - Fragmentation
     - Hybridization - If starting a run from the Hybridization step, please refer to *Starting a Run at Hybridization* on page 77.
   - **C.** Select your ending step:
     - Purification
     - Quant and Norm
     - Fragmentation
     - Hybridization
   - **D.** Select your final array format:
     - **120 (1 array plate)**: When preparing the hybridization cocktail for the 3' IVT PM only array plates, select the 120 (1 Array Plate) option. This option will produce 120 µL of hybridization cocktail per well.
     - **270 (cartridges)**: When preparing the hybridization cocktail for use on cartridges, select the 270 (Cartridge) option. This will create 270 µL of hybridization cocktail per well.
   - **E.** **Perform Second Quant -** Select this option if you wish to perform a second quantitation after normalization as a QC step.
   - **F.** **Setup the GeneTitan Plates -** Select this option to aliquot the hybridization cocktail, stains, and array holding buffer into the hybridization tray, stain trays and scan tray. This option is only available if plates are the array format.
6. When ready click **OK** to proceed with the run.
   The Deck Setup window appears (Figure 4.43). The deck layout for your run may appear different than the image displayed below, depending on the run option selected.

Figure 4.43 Deck Setup for 24 and 96 Sample Run

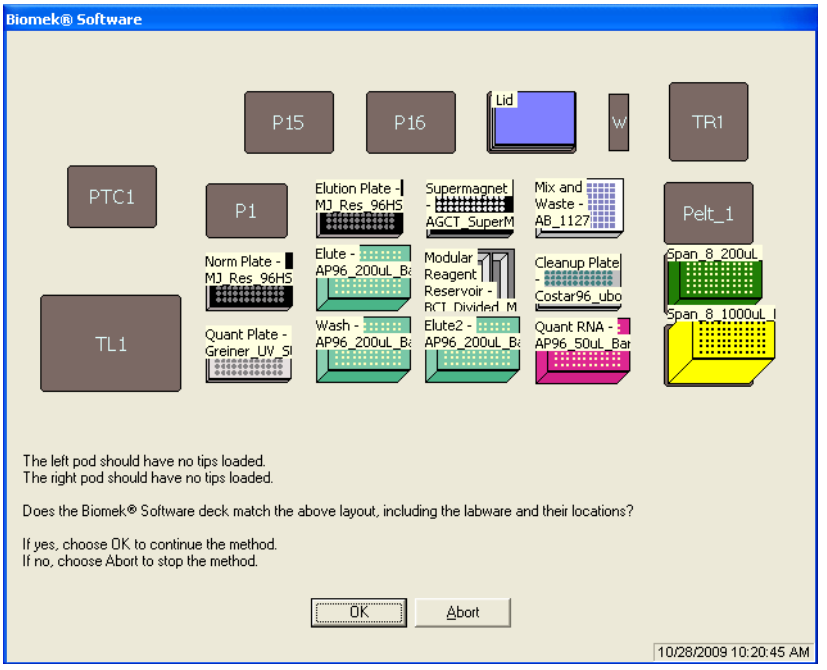


Table 4.11 Biomek® Tips and Universal labware

Product	P/N
P50 Barrier Tips (Span 8 Pipettor, magenta)	A21586
P250 Barrier Tips Sterile (AP 96 Multichannel Pipettor, lt. blue)	717253
P250 Barrier Tips Sterile (Span 8 Pipettor, green)	379503
P1000 LLS Barrier Tips (Span 8 Pipettor, yellow)	987925

7. Ensure that the deck is set up as displayed onscreen. When ready, click **OK**.
   The Modular Reservoir Setup window appears (Figure 4.44 for 24X and Figure 4.45 for 96X).

Figure 4.44 Modular Reservoir Setup, 24 sample

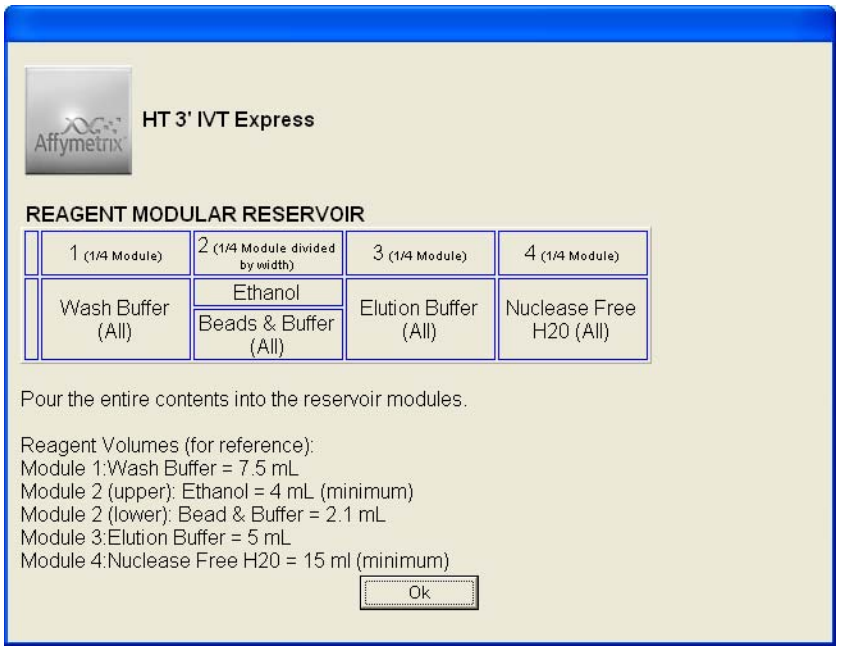
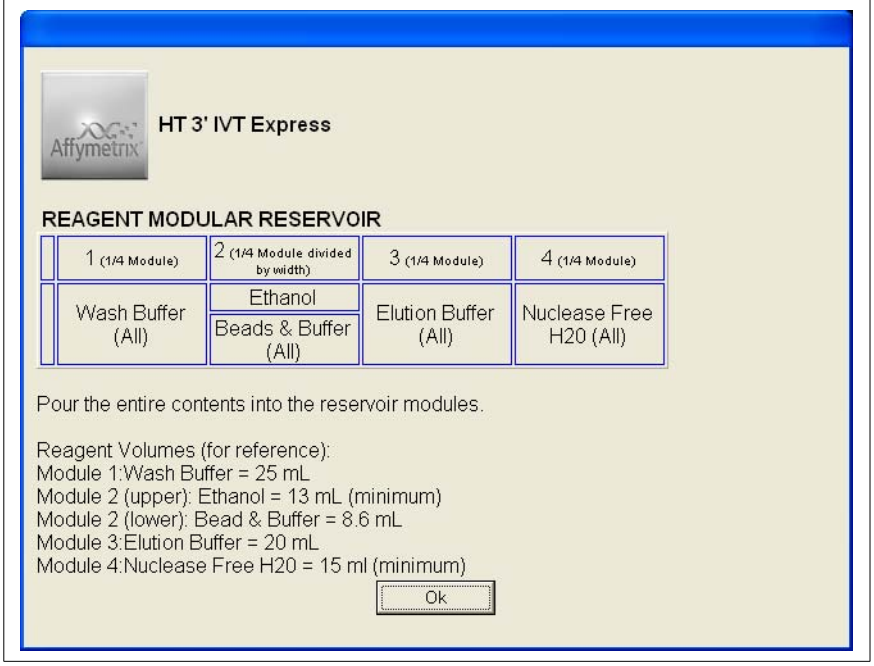


Figure 4.45 Modular Reservoir Setup, 96 Sample



8. Setup the Quarter Modular Reservoir Holder:
   - **A.** In columns 1, 3, and 4 of the Quarter Modular Reservoir Holder: insert a fresh quarter modular reservoir.
   - **B.** In column 2, insert a fresh quarter modular reservoir divided by width.
   - **C.** Fill the Quarter Modular Reservoir with reagents as instructed in Table 4.12.
     - **1)** Add aRNA Wash Solution to the quarter modular reservoir in Column 1.

2) Add Ethanol to the top half of quarter modular reservoir divided by width in Column 2.

3) Add entire contents of binding bead buffer to bottom half of quarter modular reservoir divided by width in Column 2.

NOTE: The aRNA Binding Buffer will precipitate if cold, therefore keep beads and binding buffer at room temperature.

Carefully pipet to prevent bubbles from forming in the quarter modular reservoir. Do not vortex the aRNA Binding Buffer. If bubbles are excessive, then remove bubbles from the quarter modular reservoir prior to proceeding.

- Vortex beads and add beads to binding buffer with a P1000 pipetor. Make sure all beads have been added to the binding buffer in the modular reservoir. Mix beads and binding buffer with a pipetor a few times be careful not to introduce bubbles.

4) Add a RNA Elution Solution to the quarter modular reservoir in Column 3.

5) Add Nuclease-free Water to the quarter modular reservoir in Column 4.

Table 4.12 Quarter Modular Reservoir Setup

Column 1	Column 2	Column 3	Column 4
a RNA Wash Solution and 100% Ethanol 24x = All (7.5mL) 96x = All (25 mL)	100% Ethanol 24x = 4 mL 96x = 13 mL	aRNA Elution Solution 24x = All (5 mL) 96x = All (20 mL)	Nuclease-free water 24x = Fill to the Top 96x = Fill to the Top
	Beads & Buffer 24x = All buffer and All bead solution (2.1 mL) 96x = All buffer and All bead solution (8.6 mL)		

D. Place filled Quarter Modular Reservoir Holder on the deck in position P8.

9. Click **OK** in Figure 4.44 when ready.

10. The Biomek FXP TPE System proceeds through Purification and Elution without user intervention.

aRNA Purification

After the IVT synthesis is complete, the aRNA is purified to remove enzymes, salts, and unincorporated nucleotides. The composition of the aRNA Binding Mix is shown in Table 4.13. At the completion of this step, the Biomek FXP TPE System proceeds to aRNA Quantitation and Normalization without user intervention.

Table 4.13 aRNA Binding Mix

Component	Amount
RNA Binding Beads*	10 µL
aRNA Binding Buffer Concentrate	50 µL

**Mix the RNA Binding Beads by vortexing before dispensing.*

aRNA Quantitation and Normalization

This section of the protocol details the user intervention steps necessary for calculating the yield of aRNA generated from the *in vitro* transcription reaction. The accurate calculation of the yield is necessary so that the correct amount of aRNA is added to the fragmentation reaction. Too much or too little aRNA added to the fragmentation reaction can result in incomplete or over fragmented aRNA and cause hybridization effects. This aRNA yield can be used as a check point to ensure that all the preceding steps have been successfully completed and sufficient aRNA yield has been generated.

aRNA Quantitation and Expected Yield

- **Assessing aRNA Yield by UV Absorbance**

  The concentration of an aRNA solution can be determined by measuring its absorbance at 260 nm. We recommend using DTX 880 for convenience.

  Alternatively, the aRNA concentration can be determined by diluting an aliquot of the preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and reading the absorbance in a traditional spectrophotometer at 260 nm. Find the concentration in µg/mL using the equation shown below. (1 A_{260} = 40 µg RNA/mL)

  Conc = ((Abs)(40 µg/mL RNA)(Dilution Factor))/path length (cm)

- **Expected Yield**

  The aRNA yield will depend on the quality and amount of poly(A) RNA in the input total RNA. Since the proportion of poly(A) RNA in total RNA is affected by influences such as health of the organism and the organ from which it is isolated, aRNA yield from equal amounts of total RNA may vary considerably. Figure 4.46 shows yield and size data for aRNA produced with the kit from several different types of input RNA.

- **Concentrate the Purified aRNA (Optional)**

  If necessary, concentrate the aRNA by vacuum centrifugation. If the heater on the vacuum centrifuge has different settings, use medium or low. Check the progress of drying every 5–10 minutes, and remove the sample from the concentrator when it reaches the desired volume. Avoid drying aRNA samples to completion.

  If you are concentrating aRNA off-deck, after quantitation please follow the steps listed below to repeat quantitation and import DTX file with new OD values into the template. When complete proceed to normalization.

  A. Use a new optical plate to obtain OD values for concentrated samples.

  B. Manually pipette 95 µL of water into the well.

  C. Manually pipette 5 µL of normalized sample into the well.

  D. Mix well by pipetting up and down at least 10 times.

  E. Read with an OD Plate reader at Absorbance 260nm.

  F. Follow Step 26 - Step 27 on page 68 to import new data using the reread button.

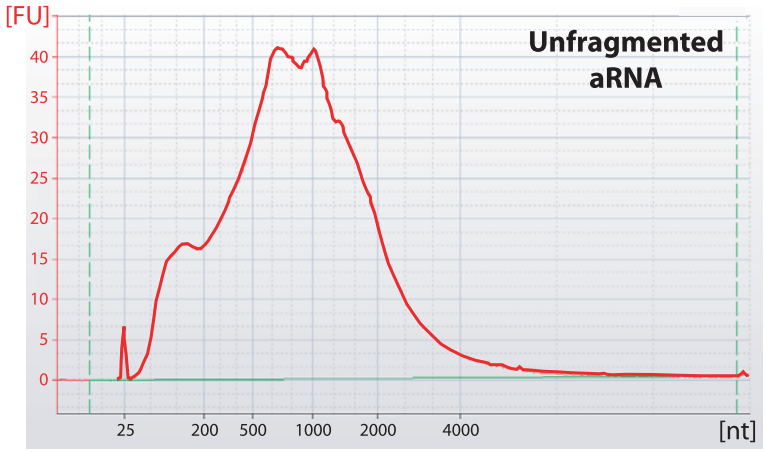
Analysis of aRNA Size

The size distribution of aRNA can be evaluated using an Agilent 2100 bioanalyzer with the Agilent RNA 6000 Nano Kit (P/N 5067-1511), or by conventional denaturing agarose gel analysis. The bioanalyzer can provide a fast and accurate size distribution profile of aRNA samples, but aRNA yield should be determined by UV absorbance or RiboGreen analysis. To analyze aRNA size using a bioanalyzer, follow the manufacturer's instructions for running the assay using purified aRNA.

Expected aRNA Size

We recommend analyzing aRNA size distribution using an Agilent bioanalyzer and RNA 6000 Nano Kit loaded with 300 ng of aRNA per well. The expected aRNA profile is a distribution of sizes from 250–5500 nt with most of the aRNA between 600–1500 nt. Average aRNA size may vary slightly depending on RNA quality and total RNA input amount.

Figure 4.46 Example Agilent Bioanalyzer Electropherogram of un-fragmented aRNA generated from 50 ng of HeLa total RNA.



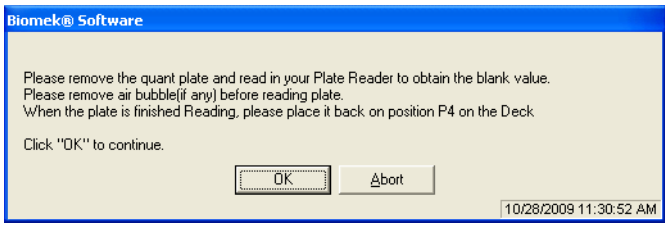
The Biomek FX^P TPE System uses spectrophotometric analysis to determine the aRNA yield. The convention that 1 absorbance unit at 260 nm equals 40 µg/mL RNA is used.

- The absorbance at 260 nm and 280 nm is checked to determine sample concentration and purity.
- The A_{260}/A_{280} ratio is maintained close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).

Continuing the Run - Quantitation

11. After the Biomek FXP TPE System prepares a blank OD plate a message appears prompting you to read a blank (Figure 4.47).

Figure 4.47



NOTE: The following method assumes the use of the recommended microplate reader as part of the Biomek FXP TPE System. If a different microplate reader is used, refer to instructions in that user guide.

A. Remove the optical plate from position P4 on the deck.

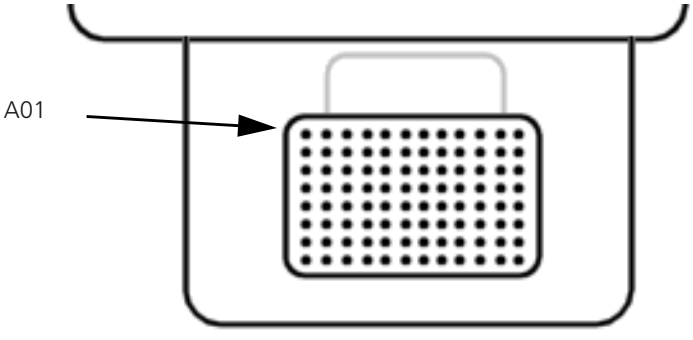
B. Turn on the DTX 880 Multimode Detector.

Figure 4.48 Beckman Coulter DTX 880 Multimode Detector

C. Open the door by pressing the ▲ button.

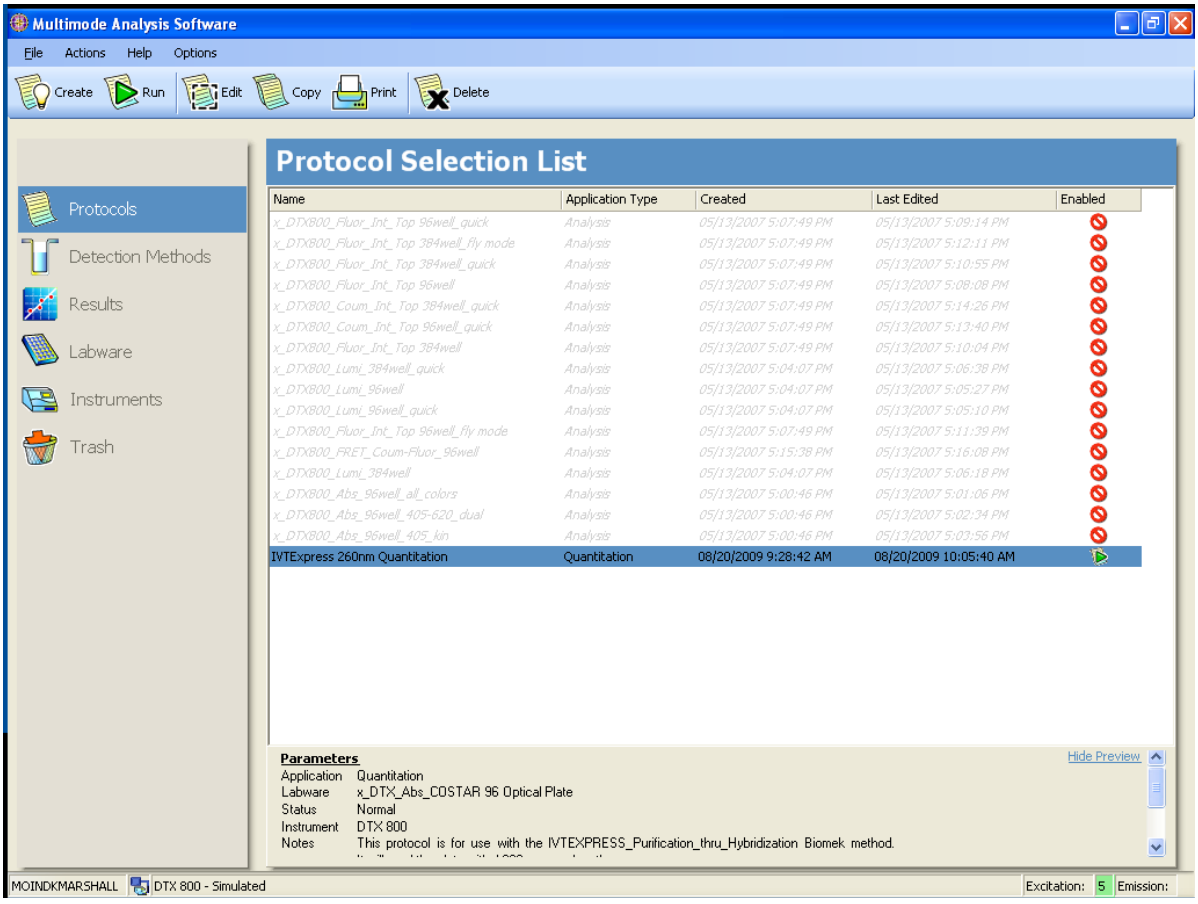
D. Place the optical plate into the DTX 880 Multimode Detector. (Refer to Figure 4.49 for correct orientation of optical plate in DTX 880.) Inspect the UV plate for bubbles. If bubbles are present, carefully remove them by centrifuging or by using a clean pipette tip.

Figure 4.49 Correct orientation of optical plate in DTX880



E. Click **Start** → **All Programs** → **Beckman Coulter** → **Multimode Analysis Software** → **Multimode Analysis Software** to open the Multimode Analysis Software (Figure 4.50).

Figure 4.50



F. From the **Protocol Selection List**, select either **IVT Express 260nm Quant S2** or **IVT Express 260nm Quant S5** to read the absorbance at 260 nm.

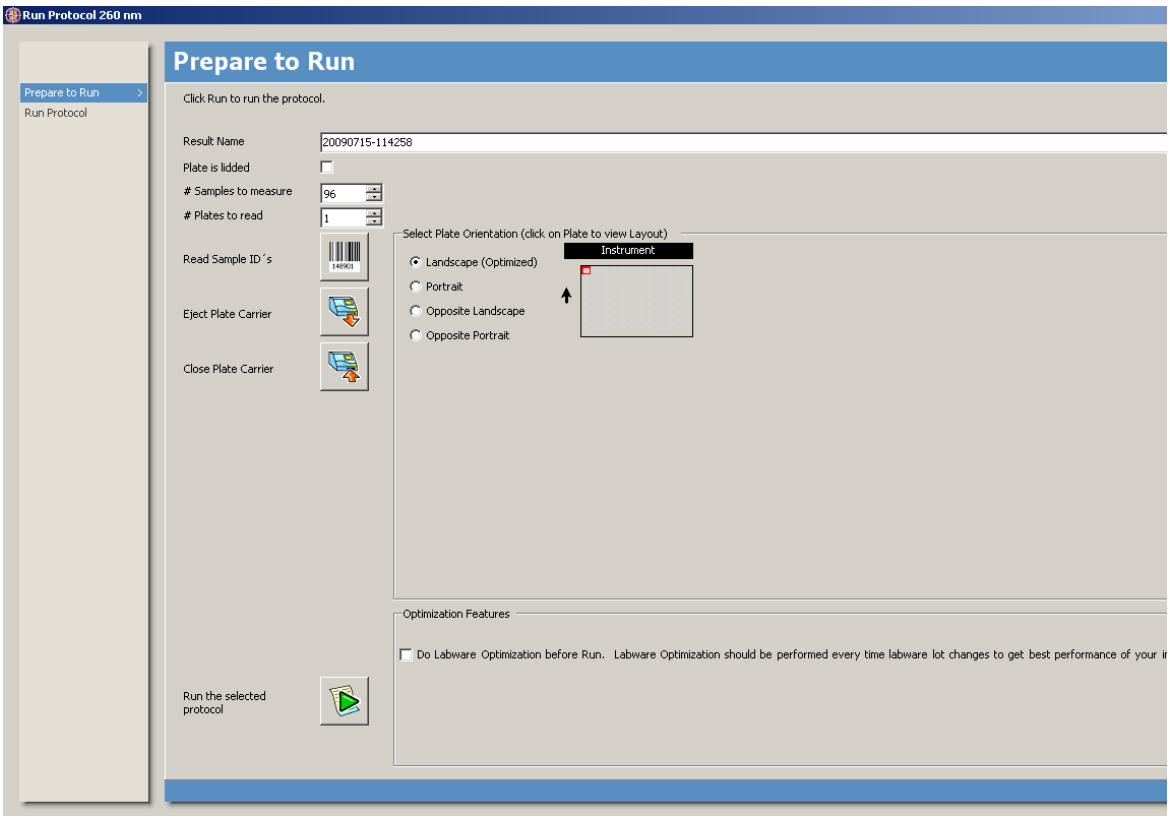
NOTE: The selection of either the S2 or S5 protocol option depends on which filter slider is installed on your microplate reader. S2 = slider 2, S5 = slider 5.

G. Close the door by pressing the ▲ button.

H. Click the **Run** icon found below the menubar

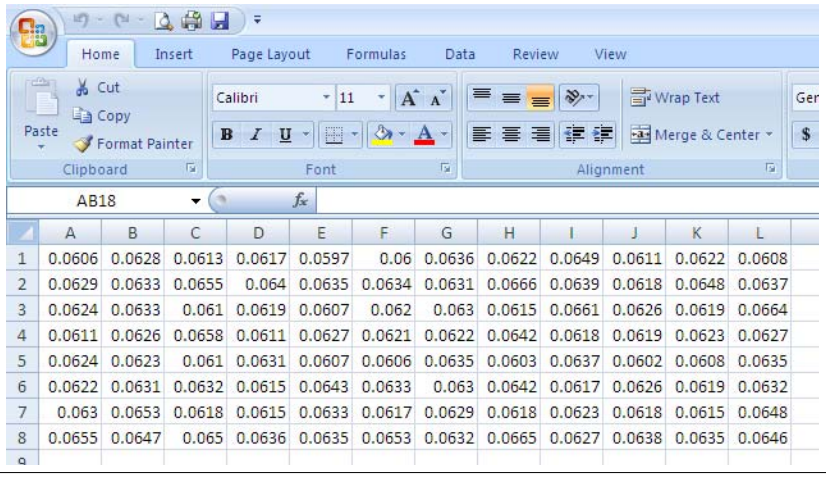
.
The Prepare to Run window appears (Figure 4.51).

Figure 4.51 Prepare to Run window



I. Click the **Run** icon found in the lower left section of the window .
The DTX 880 reads the blank and exports data to an Excel file, which opens on screen Figure 4.52.

Figure 4.52 UV Blank reading



	A	B	C	D	E	F	G	H	I	J	K	L
1	0.0606	0.0628	0.0613	0.0617	0.0597	0.06	0.0636	0.0622	0.0649	0.0611	0.0622	0.0608
2	0.0629	0.0633	0.0655	0.064	0.0635	0.0634	0.0631	0.0666	0.0639	0.0618	0.0648	0.0637
3	0.0624	0.0633	0.061	0.0619	0.0607	0.062	0.063	0.0615	0.0661	0.0626	0.0619	0.0664
4	0.0611	0.0626	0.0658	0.0611	0.0627	0.0621	0.0622	0.0642	0.0618	0.0619	0.0623	0.0627
5	0.0624	0.0623	0.061	0.0631	0.0607	0.0606	0.0635	0.0603	0.0637	0.0602	0.0608	0.0635
6	0.0622	0.0631	0.0632	0.0615	0.0643	0.0633	0.063	0.0642	0.0617	0.0626	0.0619	0.0632
7	0.063	0.0653	0.0618	0.0615	0.0633	0.0617	0.0629	0.0618	0.0623	0.0618	0.0615	0.0648
8	0.0655	0.0647	0.065	0.0636	0.0635	0.0653	0.0632	0.0665	0.0627	0.0638	0.0635	0.0646

J. Using the Excel file calculate an average reading of data:
   - For a 24-sample run use the data in the first three columns to calculate an average.
   - For a 96-sample run, average data in all columns.

K. Record the average value for future reference.

L. Close Excel. DO NOT save changes. Close Results Viewer.

NOTE: The next step requires a minimum of 20 µg of cRNA.

M. Open the door of the DTX 880 Multimode Detector by pressing the ▲ button to open. Return the plate to deck position P4 and click **OK** in Figure 4.53.

Figure 4.53

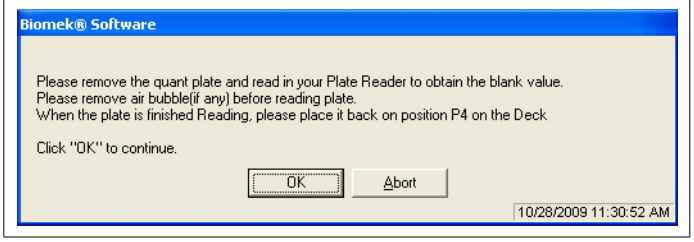


Figure 4.54 Biomek® FXP TPE System Deck / Pod Locations

12. The Biomek FXP TPE System adds the sample to the plate. Figure 4.55 appears.

Figure 4.55

13. Open the door of the DTX 880 Multimode Detector by pressing the ▲ button.

14. Take Quant plate from deck position P4 and inspect for bubbles. If bubbles are present, carefully remove them using a clean pipette tip. When ready, put the UV plate in DTX 880 Multimode Detector to read the absorbance at 260 nm.

15. Close the door of the DTX 880 Multimode Detector by pressing the ▲ button.

A. Return to the Multimode Analysis Software and click the **Run** icon found below the menubar

.

The Prepare to Run window appears.

B. Click the **Run** icon found in the lower left section of the window
.

The DTX 880 reads the plate. A Microsoft Excel file opens. This file is named the same as the Result Name, as seen in Figure 4.51.

C. In the Excel file, click the Microsoft Office Button → **Save**.

NOTE: Please Save the Excel file to a desired location for future use.

D. Close the Excel file.

E. If you selected to run a 2nd quant plate, leave the DTX turned on.

F. Close the Multimode Result Viewer by clicking the Close button in the lower right corner. Minimize the Multimode Analysis Software.

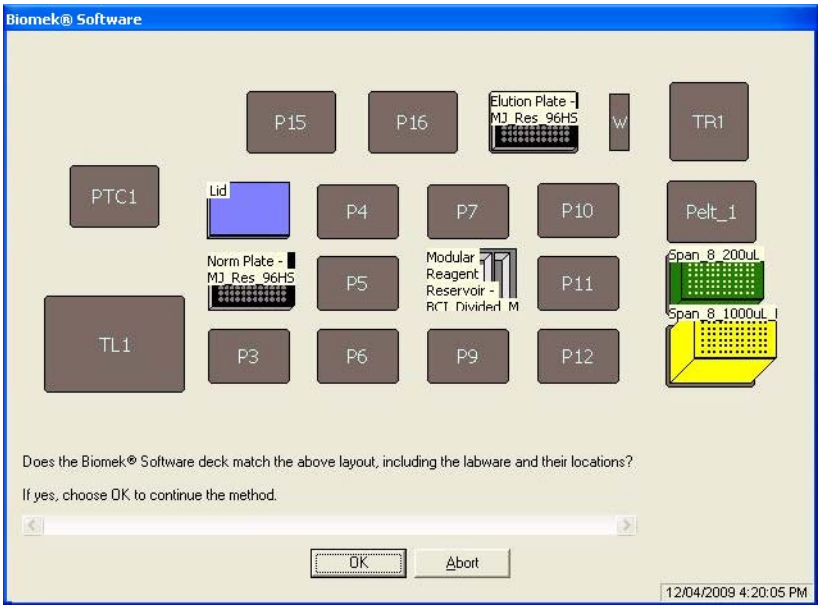
16. Open the door of the DTX 880 Multimode Detector by pressing the ▲ button and remove the Quant plate.

17. Click **OK** in Figure 4.55.

The deck setup window appears (Figure 4.56).

NOTE: Your deck layout may be different than the image shown, depending on your starting point.

Figure 4.56 Deck Setup window. 24 and 96 Sample



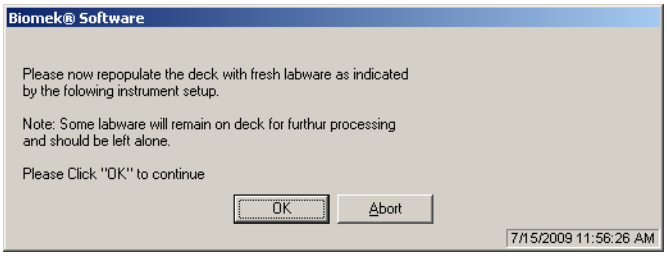
CAUTION: Do not click Abort.

Clear deck of the following labware:
This removes the used tips and clears the deck of plates that are not required or have been used.

- TL1P50 Quant Tips
- P16Sample Plate
- P5, P6, P9 P250 Tips
- P7Magnet
- P10Waste Plate
- P11Cleanup Plate

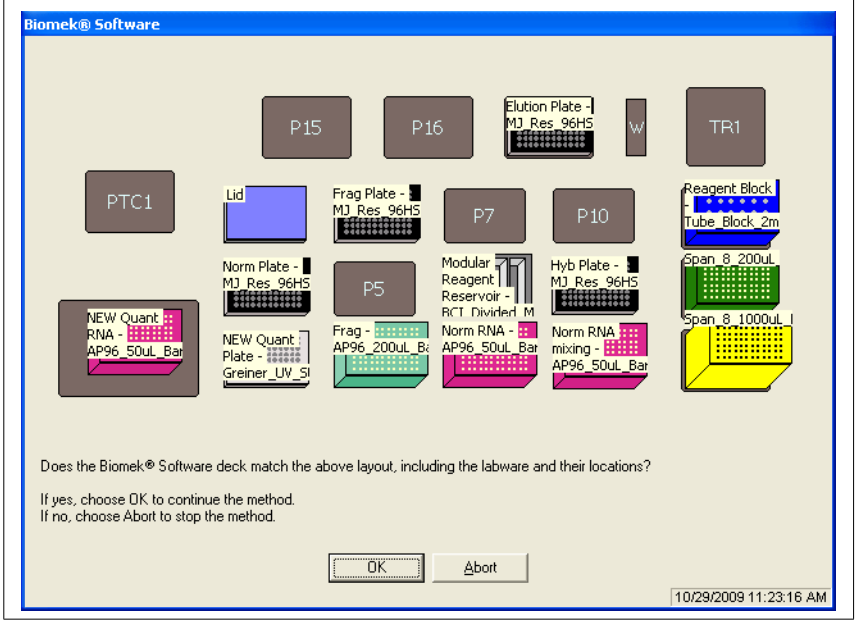
18. Click **OK**.
A message window appears (Figure 4.57).

Figure 4.57



19. Click **OK**.
The new deck layout image appears (Figure 4.57).

Figure 4.58 Deck Layout, 24 and 96 Sample



A. Set up the deck with labware according to the image onscreen (Figure 4.58).
- TL1P50 Barrier Tips
- P3Quant Plate
- P4Frag Plate
- P6200 µL Frag Tips
- P8Quarter modular reservoir (further instruction in Step B below).
- P9P50 Barrier Tips
- P11Hyb Plate
- P12P50 Barrier Tips
- Empty cold block with template

B. Setup the Quarter Modular Reservoir Holder as follows:
- In column 1: remove existing and replace with a fresh empty quarter modular reservoir.
- In column 2, remove existing and replace with a fresh quarter modular reservoir divided by width.

- Columns 3 & 4 remain the same.

C. Click **OK**.
The Purification Reagent Module Reservoir image appears (Figure 4.59 for cartridge arrays or Figure 4.60 for array plates).

Figure 4.59 Cartridge Arrays - Purification Module Reservoir 24X

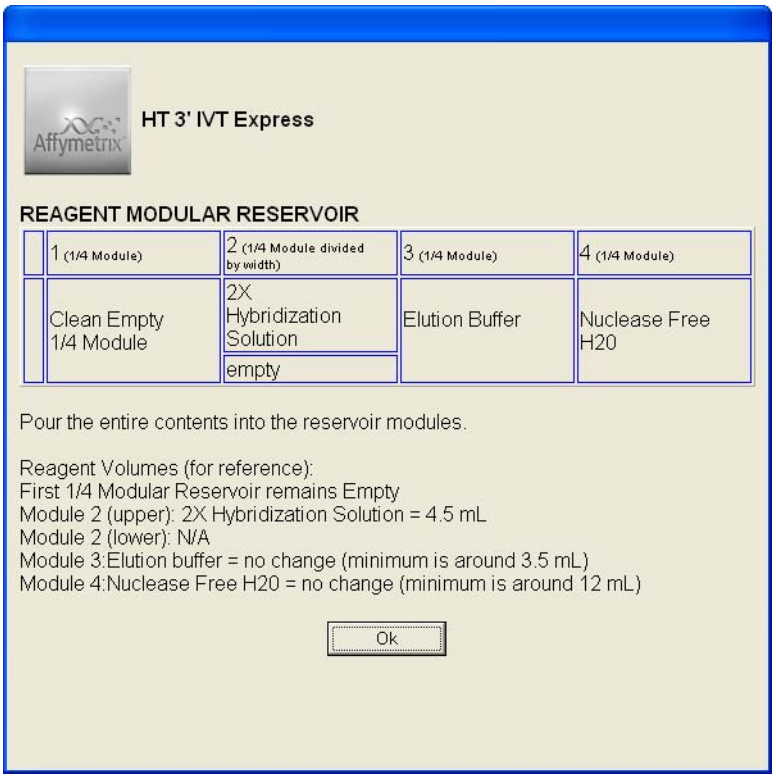
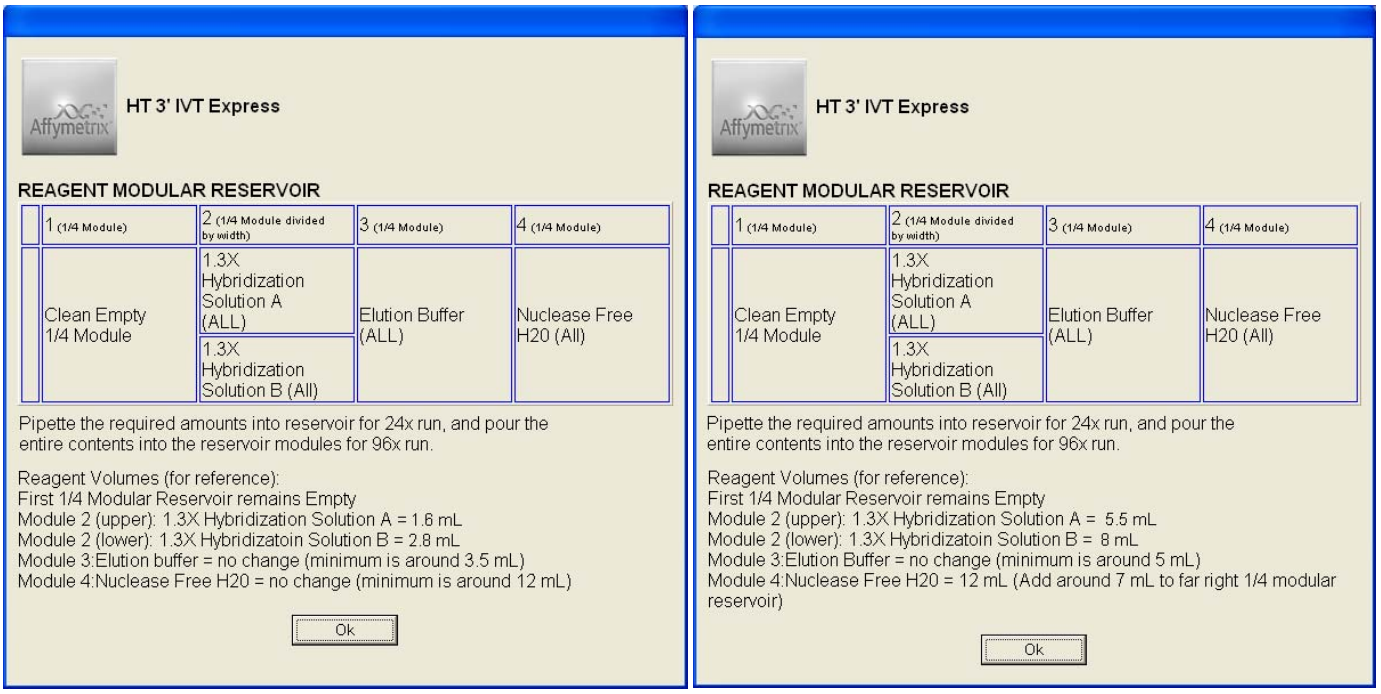


Figure 4.60 Array Plates - Purification Module Reservoir windows
Left: 24 samples, 120 (1 plate);
Right: 96 samples, 120 (1 plate)



20. Pour the reagents into the designated columns, following the image onscreen.
When done, click **OK**.
The Chilled Reagent Block window appears (Figure 4.61 for cartridge arrays and Figure 4.63 for array plates).

The Chilled Reagent Block - Cartridge Arrays

DMSO must be at room temperature.

21. Heat 20X Controls on heat block at 65°C for 5 minutes.

IMPORTANT: Mix solutions by gently flicking the reagent tube a few times. Briefly spin ALL tubes before inserting them into the reagent cold block.

(For Array Plates see *The Chilled Reagent Block - Array Plates* on page 66.)

Figure 4.61 Cartridge Arrays - Chilled Reagent Block

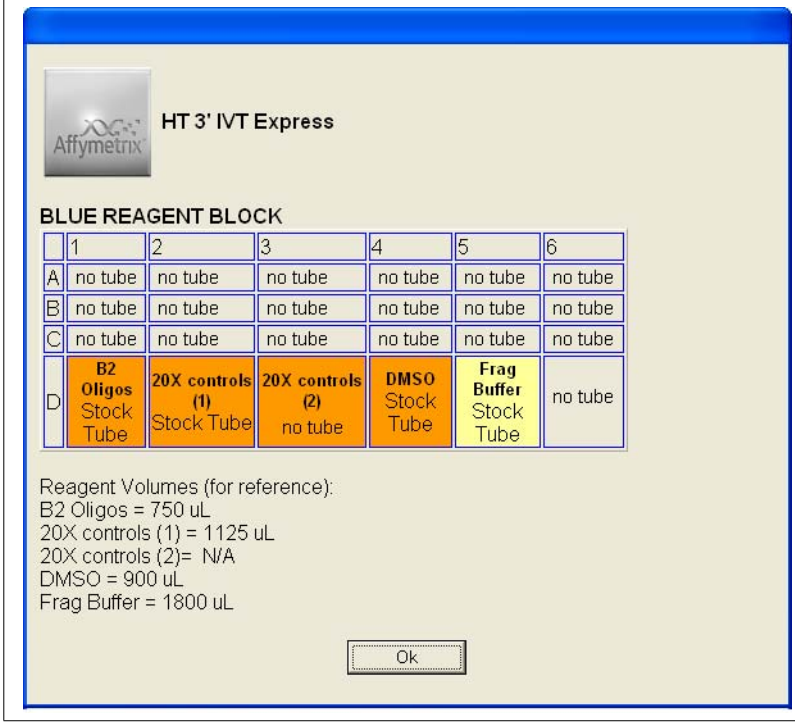
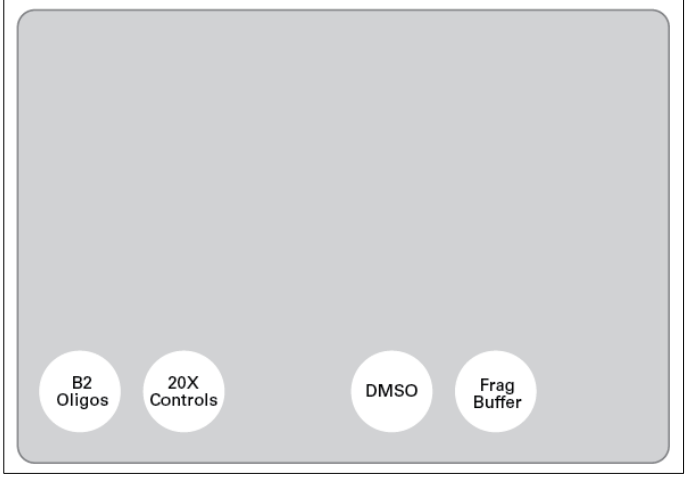


Figure 4.62 Cartridge Arrays - Cold Block Reagent Layout for 24-Sample Plate - Day 2



The Chilled Reagent Block - Array Plates

IMPORTANT: **Mix solutions by gently flicking the reagent tube a few times. Briefly spin ALL tubes before inserting them into the reagent cold block.**

(For Cartridge Arrays see *The Chilled Reagent Block - Cartridge Arrays* on page 65.)

Figure 4.63 Array Plates - Chilled Reagent Block
24 samples, 120 (1 plate); 96 samples, 120 (1 plate)

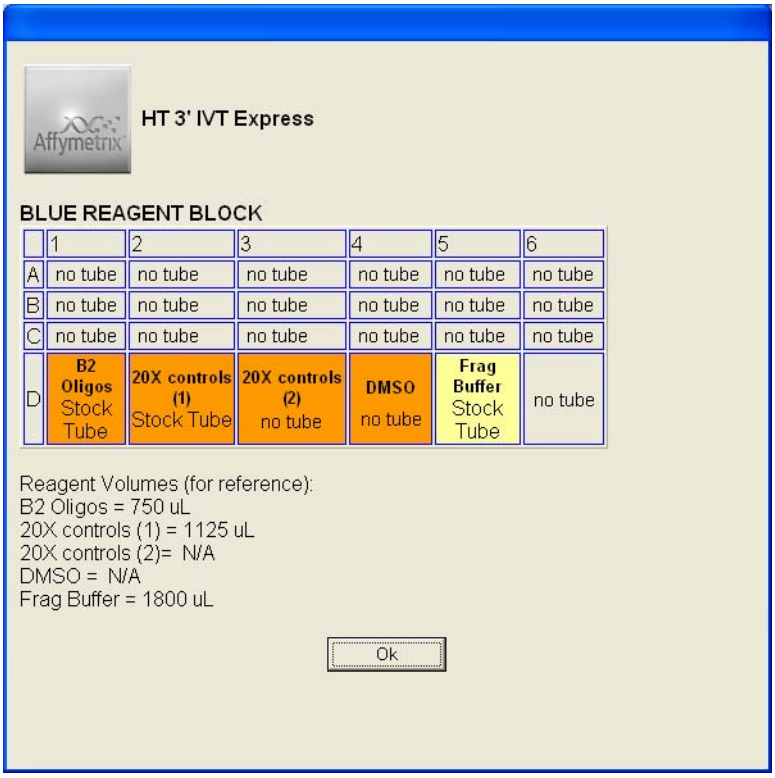
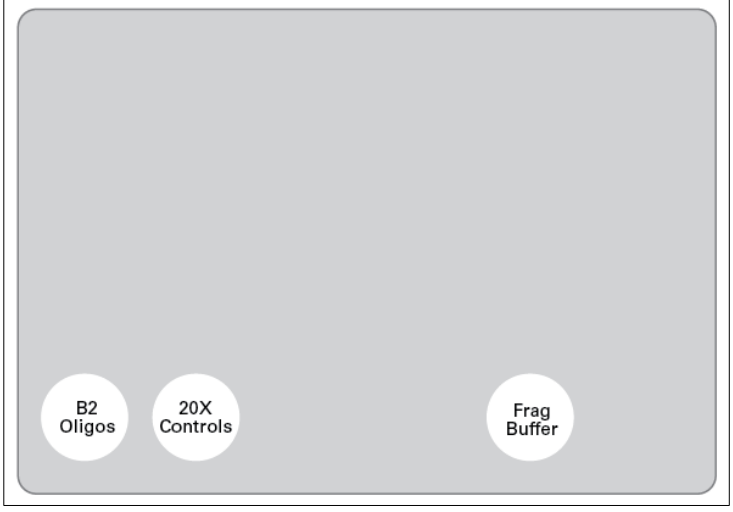


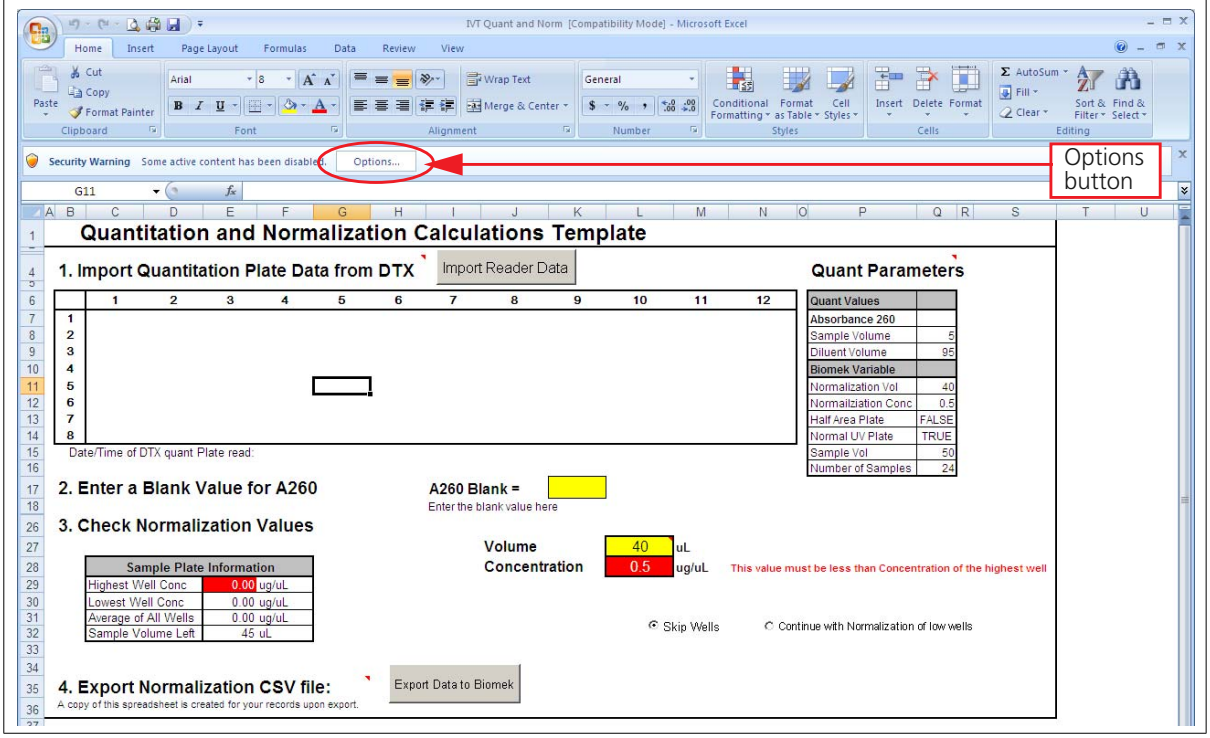
Figure 4.64 Array Plates - Cold Block Reagent Layout Day 2 for 24 samples - 1 plate and 96 samples 1 plate



22. Fill the cold reagent block as in Figure 4.62 for cartridge arrays, or Figure 4.64 for Array Plates. When finished click **OK**.
The Quantitation and Normalization Calculations template file appears, Figure 4.65.

NOTE: If you accidently exit out of the Excel spreadsheet, you can re-open it by navigating to the following location: C:\Documents and Settings\ All Users\Documents\IVT 2\ IVT Quant and Norm.xls

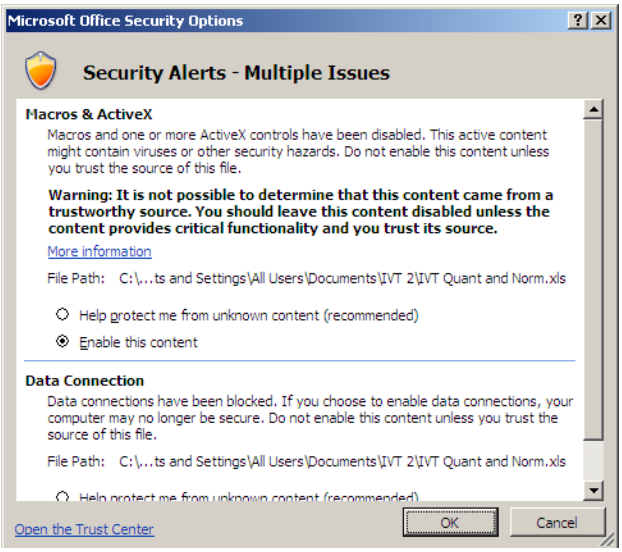
Figure 4.65 IVT Express Quantitation and Normalization Calculations template with Options button highlighted



23. Click the **Options** button (see Figure 4.65).
The Microsoft Office Security Options window appears (Figure 4.66).

NOTE: Office 2007 users will see the Microsoft security dialog box as shown in Figure 4.66, while Office 2003 users will see a security warning that prompts the user to Enable Macros. Please click the Enable Macros button.

Figure 4.66 Security Options window

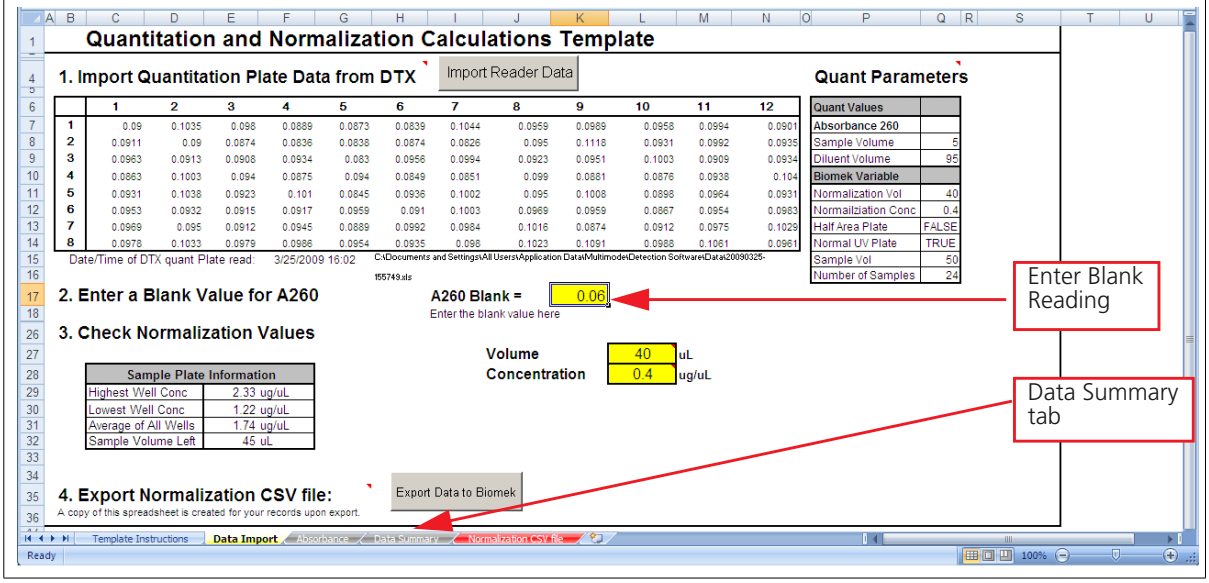


24. Click **Enable this content** in the Macros and ActiveX section of the window. Also enable data Control.
25. Click **OK**.
26. Click **Import Reader Data** in the IVT Quant and Norm template file.

> **IMPORTANT:** **Use the export function to save the file in text format. If you are using other software, export or save data as a text file and ensure that the file is reformatted to the appropriate format. If you are having trouble importing the file, open it and make sure it looks like the Quantitation Plate Template.xml data (data starts in A1). The file can be found at: C:\Documents and Settings\All Users\Documents\IVT 2**

27. Navigate to import data from DTX 880.
    If the DTX 880 Multimode Detector is connected to your workstation the data may be found at: C:\Documents and Settings\All Users\ Application Data\ Multimode\Detection Software\Data
28. Enter the average previously calculated from the blank reading in the **A260 Blank** cell at position K17, as shown in (Figure 4.67).

Figure 4.67



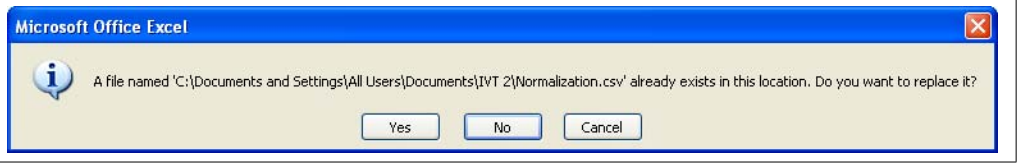
29. Click the **Data Summary** tab (Figure 4.67) to review the data for any obvious outliers. When done click the **Data Import** tab.

NOTE:

- **Samples which are outside the range of normalization (20 to 200 µg) will be carried through the remainder of the procedure but will not be properly normalized. Hybridization results from improperly normalized samples may not be valid.**
- **Low OD readings may be due to bubbles in sample wells. Check plate and inspect the UV plate for bubbles. If bubbles are present, carefully remove them using a clean pipette tip.**
- **Reread the plate for samples with unexpected low or high OD reading so that samples are properly normalized. Prior to rereading the plate please complete the following instructions:**
  - **a. Empty the well in the optical plate that has the unexpected low or high OD reading.**
  - **b. Manually pipette 95 µL of water into the well.**
  - **c. Manually pipette 5 µL of normalized sample into the well.**
  - **d. Mix well by pipetting up and down at least 10 times.**
  - **e. Read with the spectrophotomer.**
  - **f. Follow Step 26 - Step 27 on page 68 to import new data using the reread button.**

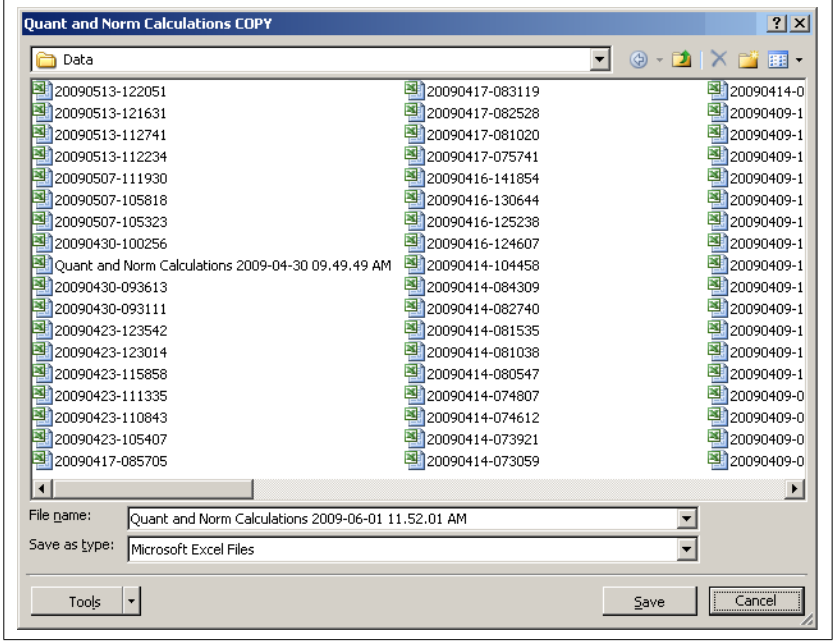
30. Click **Export Data to Biomek** button on the Data Import tab.
A Warning message appears (Figure 4.68).

Figure 4.68 Warning Message window



31. Click **Yes** in the message window.
Biomek FXP TPE System prepares the normalization plate.
Figure 4.69 appears.

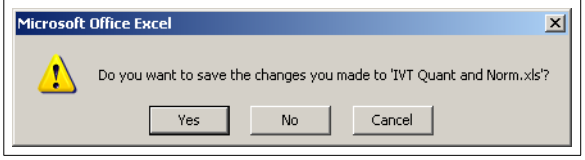
Figure 4.69



32. Click **Save** to save a copy of the data file for your use.

33. Close the Quantitation and Normalizations Template.
A warning message appears (Figure 4.70).

Figure 4.70 Warning Message



34. Click **No** in the warning message.

NOTE: You have already saved a copy as seen in figure Figure 4.69. Do not overwrite the IVT Quant and Norm.xls file.

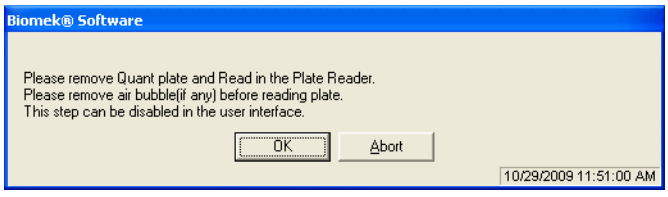
The Quantitation and Normalizations Template closes.

35. Close Excel.

Normalization

1. After the normalization plate is prepared, if you selected to run a 2nd Quant plate a user message appears (Figure 4.71).

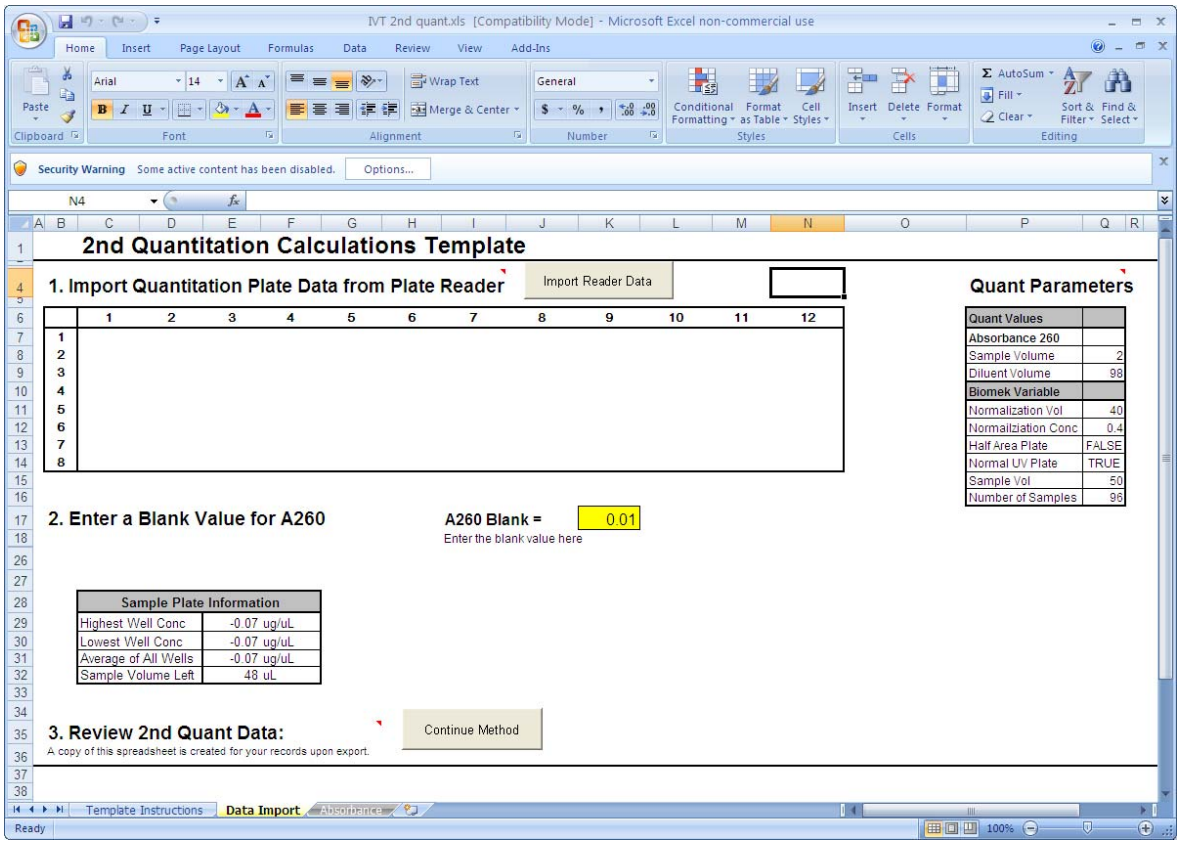
Figure 4.71



2. Remove the UV plate from position P3 and inspect for bubbles. If bubbles are present carefully remove them using a clean pipette tip.
3. Open the door of the DTX 880 Multimode Detector by pressing the ⏏ button.
4. When ready put the UV plate into the DTX 880 and read the absorbance at 260 nm
5. Close the door of the DTX 880 Multimode Detector by pressing the ⏏ button.
   A. Return to the Multimode Analysis Software and click the **Run** icon found below the menubar

   .
   The Prepare to Run window appears.
   B. Click the **Run** icon found in the lower left section of the window .
   The DTX 880 reads the plate. A Microsoft Excel file opens.
   C. In the Excel file, click the Microsoft Office Button → **Save**.
   D. Close the Excel file.
   E. Close the Multimode Result Viewer by clicking the Close button in the lower right corner. Minimize the Multimode Analysis Software.
6. Click **OK** in Figure 4.71.
   The Quantitation and Normalization Calculations Template appears (Figure 4.72).

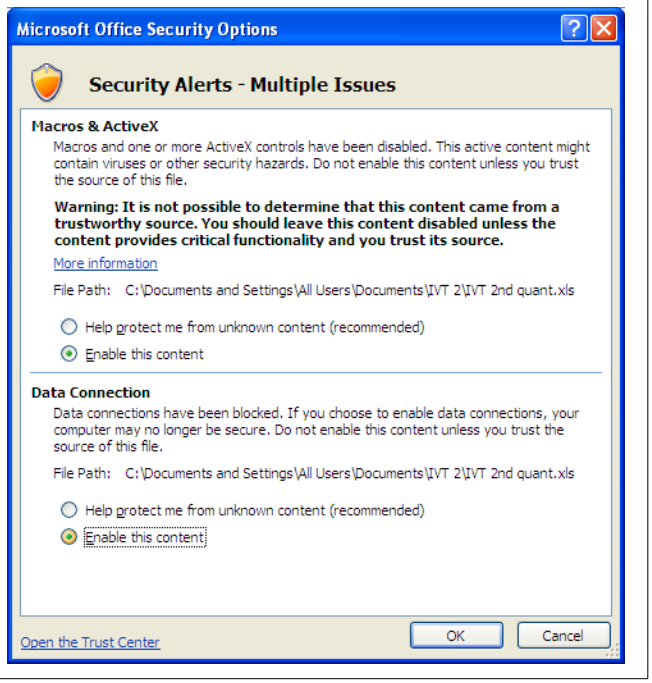
Figure 4.72



7. Click **Options**.
   The Microsoft Office Security Options window appears (Figure 4.73).

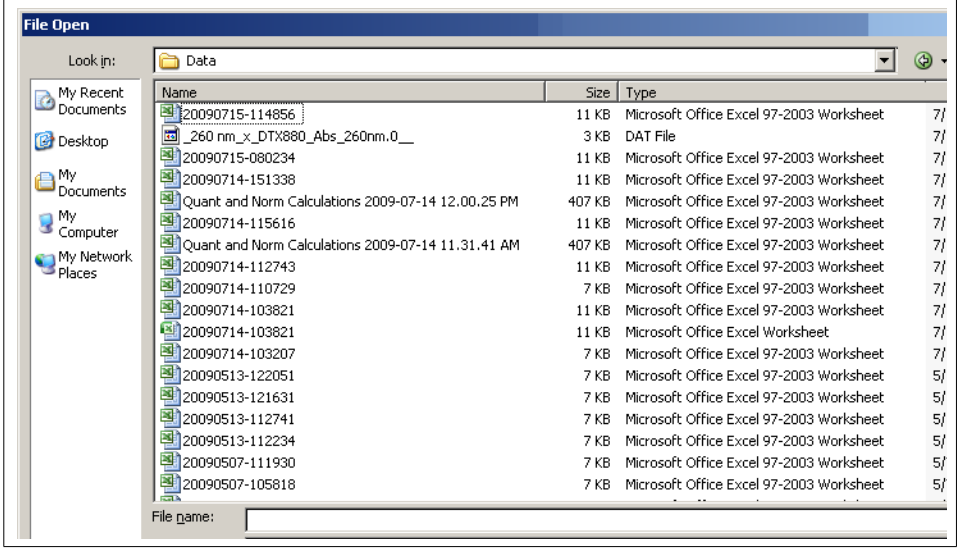
NOTE: Office 2007 users will see the Microsoft security dialog box as shown in Figure 4.73, while Office 2003 users will see a security warning that prompts the user to **Enable Macros**. Please click the **Enable Macros** button.

Figure 4.73



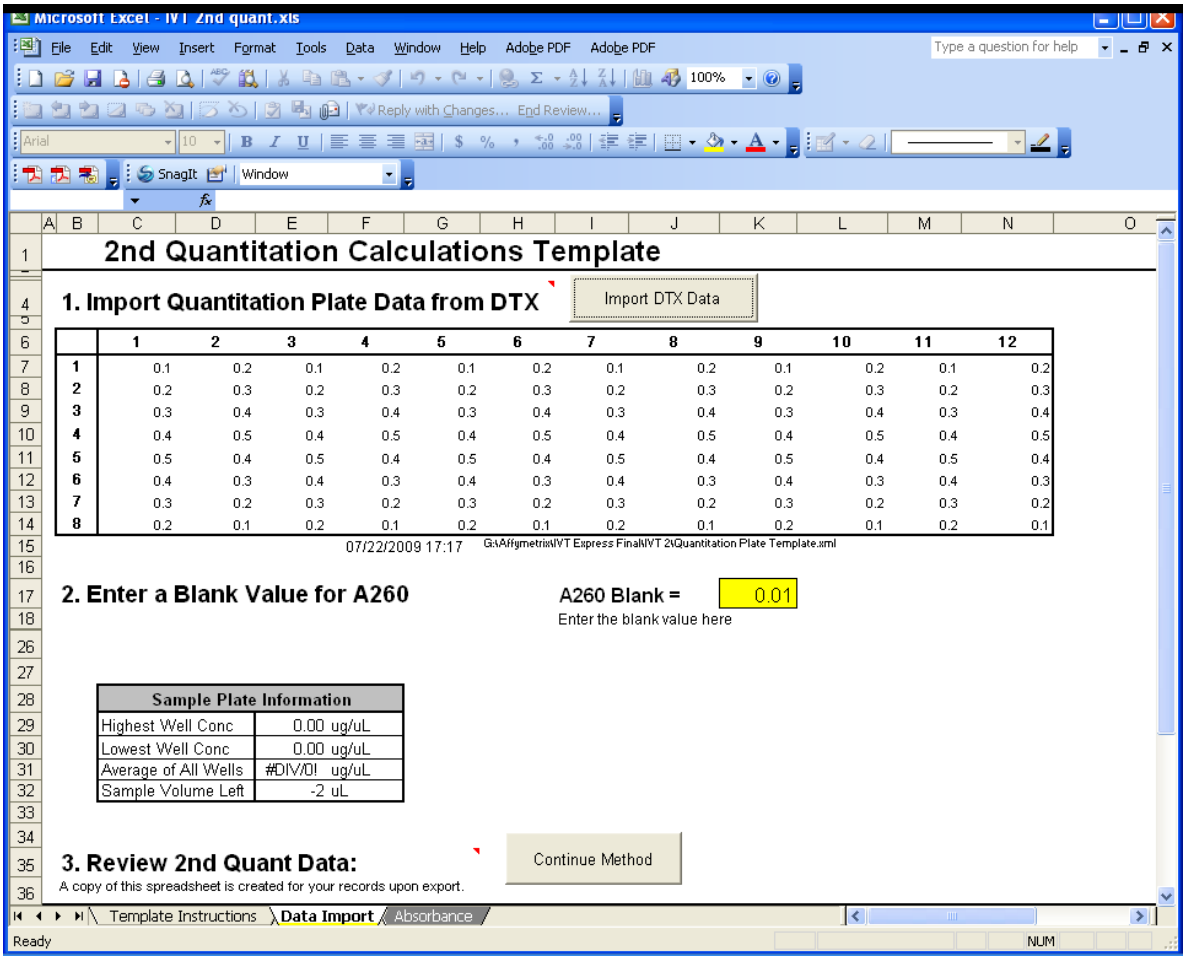
8. Click **Enable this content**, then click **OK**.
9. Click **Import Reader Data**.
10. Navigate to import data from DTX 880.
    If the DTX 880 Multimode Detector is connected to your workstation the data may be found at: C:\Documents and Settings\All Users\ Application Data\ Multimode\Detection Software\Data

Figure 4.74



11. Double-click to select your data file from the list.
    The 2nd Quantitation Calculations Template is populated with data (Figure 4.75).

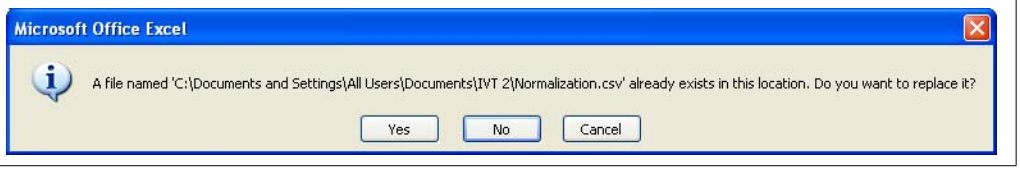
Figure 4.75



12. Enter the average previously calculated from the blank reading from the first quantitation.

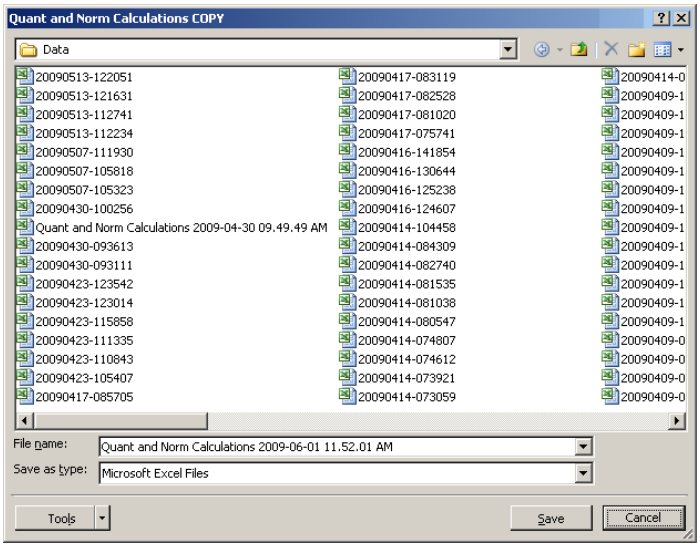
13. Click **Continue Method** button.
A Warning message appears (Figure 4.76).

Figure 4.76



14. Click **Yes** in the warning message window.
Figure 4.77 appears.

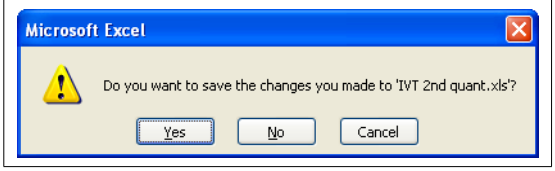
Figure 4.77



15. Click **Save** to save a copy of the data file for your use.

16. Close the 2nd Quantitation Calculations Template.
A warning message appears (Figure 4.78).

Figure 4.78 Warning Message

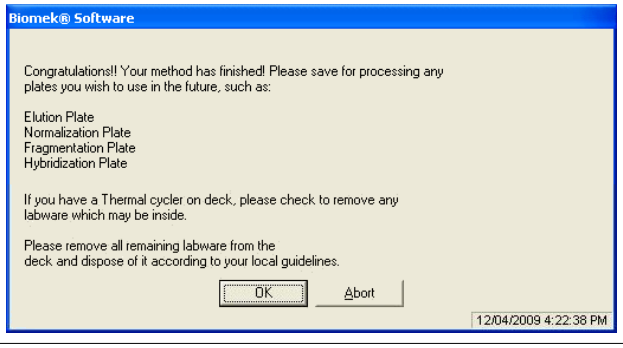


17. Click **No** in the warning message.

NOTE: You have already saved a copy as seen in figure Figure 4.77. Do not overwrite the IVT 2nd Quant.xls file.

The 2nd Quantitation Calculations Template closes.
The Biomek FXP TPE System proceeds with Fragmentation. When complete Figure 4.79 appears.

Figure 4.79



18. If using the GeneChip HT 3' IVT Express 4x24 reaction kit (P/N 901225), the 20X Controls may be used up to two times. If appropriate remove the vial of 20X control stock immediately after use from the reagent block and store at –20°C for future use.

Fragmentation and Preparation of Hybridization-Ready Sample

After the aRNA yield calculation, the Biomek FXP TPE System automatically proceeds through Fragmentation setup and makes the hybridization-ready sample. The instrument will place the plate in the on-deck thermal cycler.

Fragmentation of aRNA target before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay sensitivity. After fragmentation, the Biomek FXP TPE System will make the Hybridization cocktail mix (see Table C.3 or Table C.5) on deck. The Biomek FXP TPE System will then proceed to make the hybridization ready sample by mixing appropriate amounts of fragmented aRNA and Hybridization Cocktail Master Mix (see Table C.3 or Table C.5) in a U-bottom plate labeled ‘Hybridization Ready Sample’. After this process is completed, the hybridization-ready samples can be stored or used for hybridizing cartridges (Chapter 5) or array plates (refer to *GeneTitan® Instrument User Guide for Expression Array Plates*, P/N 702933).

If the **Setup the GeneTitan Plates** option was selected at the beginning of the run please proceed to *GeneTitan Hybridization Setup* on page 82. The result is that the hybridization-ready sample will be put in the hybridization plate, the stain trays will be prepared and the scan tray will be prepared and ready to have the lids put on and placed in the GeneTitan Instrument.

If starting a run from the hybridization step, proceed to *Starting a Run at Hybridization* on page 77.

IMPORTANT: After the Fragmentation Plate is placed into the thermal cycler, immediately remove the Elution Plate containing aRNA from the deck and seal the plate with an adhesive plate seal. Use a plate roller to secure the seal onto the plate to prevent evaporation then store the plate in a freezer at –20°C.

For short-term storage:

1. Cover the hybridization-ready sample plate with an adhesive plate sealer.
2. Store the sealed plate at –20°C.

For long-term storage:

1. Cover the hybridization-ready sample plate with an adhesive plate sealer.
2. Store the sealed plate at –80°C.

Starting a Run at Hybridization

Materials Required

Table 4.14 Materials Required for Run Starting at Hybridization

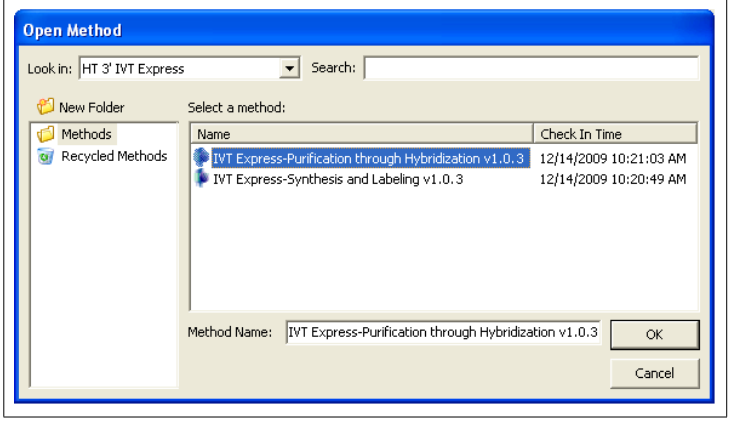
# 24X	# 96X	Description
		(Plates) GeneTitan Hybridization, Wash, and Stain Kit for 3' IVT Arrays (Affymetrix P/N 901530) Components needed: ▪ 1.3X Hybridization Solution A ▪ 1.3X Hybridization Solution B
		(Cartridges) GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix P/N 900720) Components needed: ▪ 2X hybridization Solution ▪ Nuclease-free Water ▪ DMSO
		GeneChip HT 3' IVT Express Kit Components needed: ▪ Control Oligo B2 ▪ 20X Hybridization Controls
		Nuclease-free water
		Sample plate and lid
96 tips *192 tips**	96 tips *192 tips**	P250 Barrier Tips Sterile (AP 96 Multichannel Pipettor, lt. blue), Beckman P/N 717253
24 tips	0	P250 Barrier Tips Sterile (Span 8 Pipettor, green), Beckman P/N 379503
16 tips *40 tips**	20 tips *44 tips**	P1000 LLS Barrier Tips (Span 8 Pipettor, yellow), Beckman P/N 987925
1	1	Cold Reagent Block
1	1	Cold Reagent Block template
1	1	Plate, Costar Brand Serocluster U-bottom
1	1	Quarter Reservoir, Divided by Width, Beckman P/N 372792
1 *4**	1 *4**	Quarter Reservoir, Beckman P/N 372790
1	1	Frame for Reservoirs, Beckman P/N 372795

** Number required if running GeneTitan setup.*

Procedure

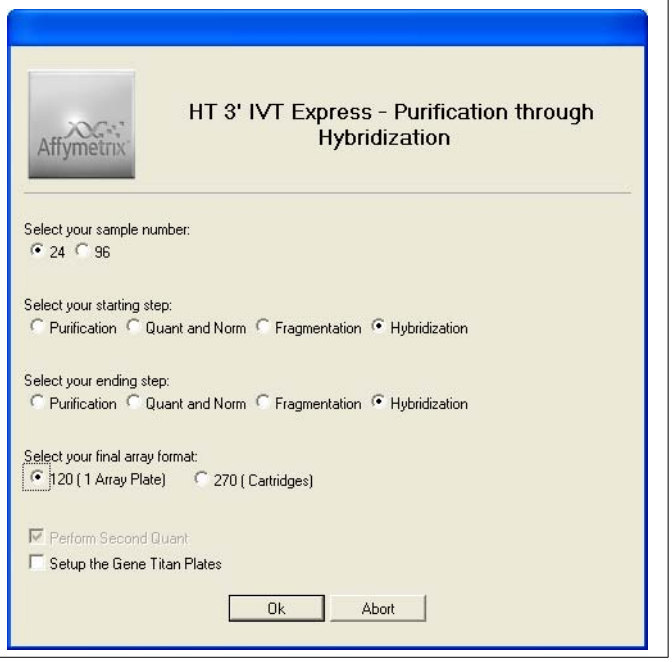
1. If necessary, click **Start** → **Programs** → **Beckman Coulter** → **Biomek Software** to launch the Biomek FXP TPE System software application.
2. Select **File** → **Open** from the menubar.
   The Open Method dialog box appears (Figure 4.41).

Figure 4.80



3. From the Open Method dialog box:
   A. Click the **Look in** drop down box and select the **HT 3’ IVT Express** project name.
   B. In the **Select a method** list box, click to select **IVT Express - Purification through Hybridization**.
   C. Click **OK**.
4. Click the Run icon.
   A summary of the run is displayed (Figure 4.81).

Figure 4.81 Run Summary



   A. **Select Sample Number** for your run: (24, or 96).

B. Select your starting step:
   - Hybridization

> **NOTE: When selecting the 24 rxn run to prepare the hybridization cocktail for the array plates, the GeneTitan Hybridization, Wash and Stain kit is sufficient for only 3x24 reactions.**

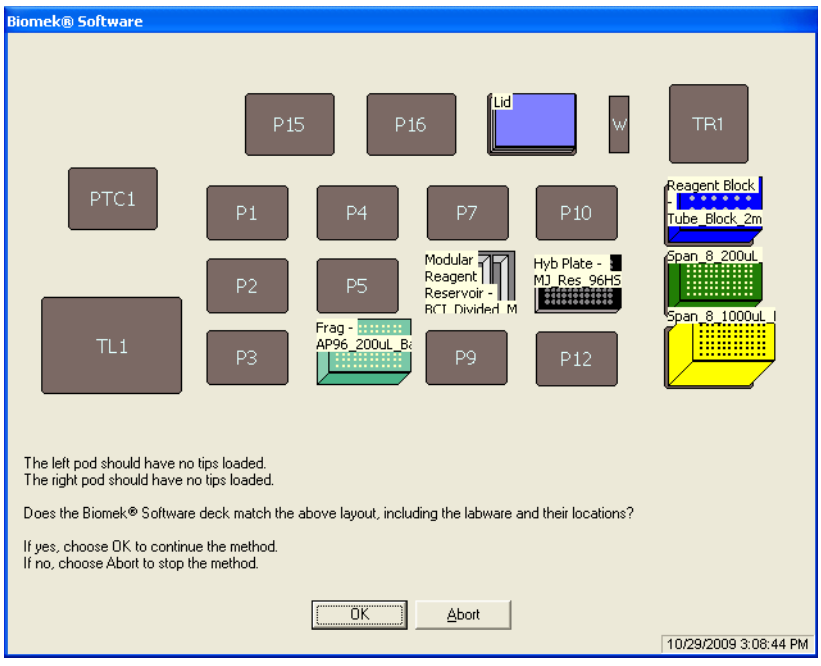
C. Select your ending step:
   - Hybridization

D. Select your final array format:
   - **120 (1 array plate)**: When preparing the hybridization cocktail for the 3' IVT PM only array plates, select the 120 (1 Array Plate) option. This option will produce 120 µL of hybridization cocktail per well.
   - **270 (cartridges)**: When preparing the hybridization cocktail for use on cartridges, select the 270 (Cartridge) option. This will create 270 µL of hybridization cocktail per well.

E. **Setup the GeneTitan Plates** - Select this option to aliquot the hybridization cocktail, stains, and array holding buffer into the hybridization tray, stain trays and scan tray. This option is only available if plates are the array format.

5. Remove sample from 4°C storage and place on the shaking Peltier.
6. When ready click **OK** in Figure 4.81 to proceed with the run.
   The Deck Setup window appears (Figure 4.82).

Figure 4.82 Deck Setup -- Cartridges



7. Setup the deck according to the deck layout onscreen. Click **OK** when you are ready to proceed.
   The Hybridization Reagent Modular Reservoir Setup window appears (Figure 4.83 cartridges, Figure 4.84 plates).

Figure 4.83 Modular Reservoir Setup - Cartridges

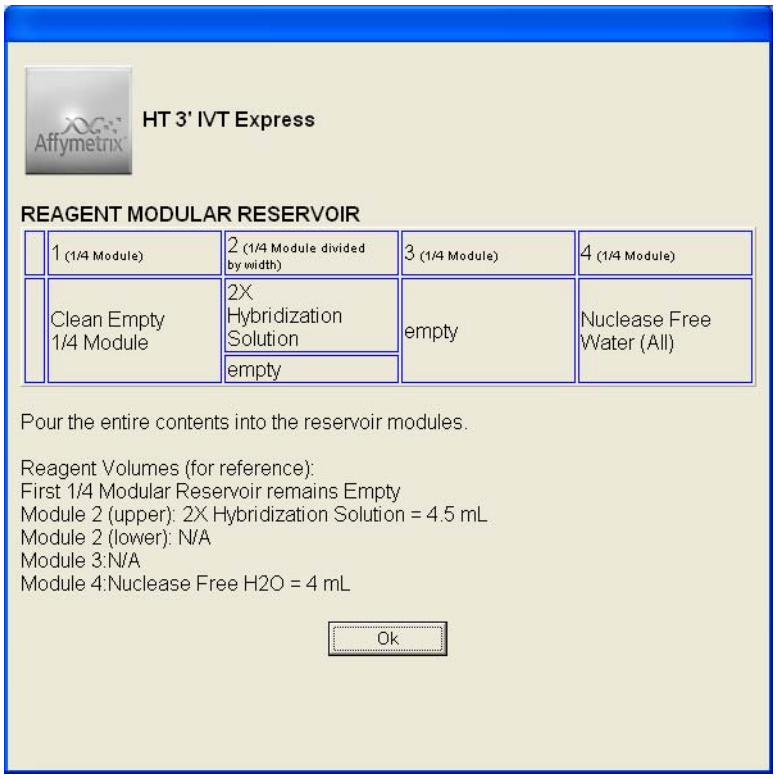
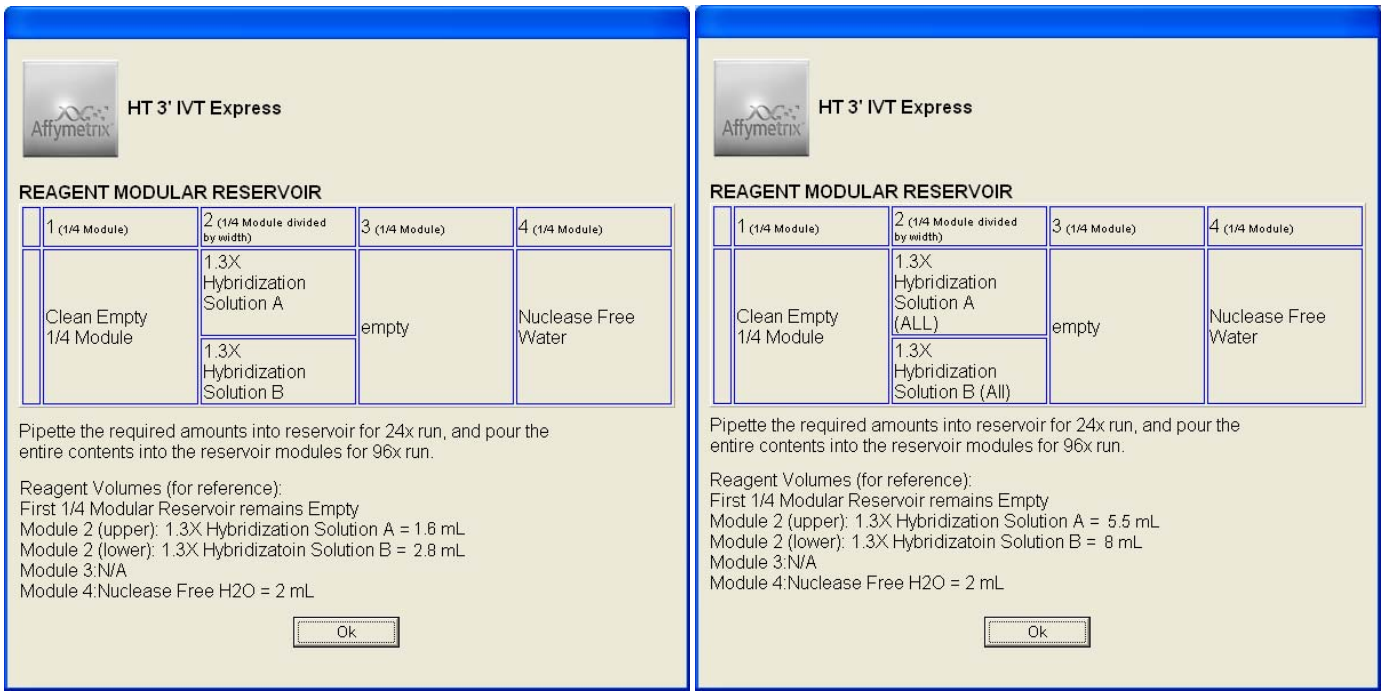


Figure 4.84 Modular Reservoir Setup: 24 Sample 1 plate (left); 96 Sample 1 plate (right)



8. Setup the Hybridization Reagent Modular Reservoir as shown in Figure 4.83 or Figure 4.84. Click **OK** when ready to proceed.

The Reagent Block Setup window appears (Figure 4.85 or Figure 4.86).

Figure 4.85 Cold Block Setup - Cartridges

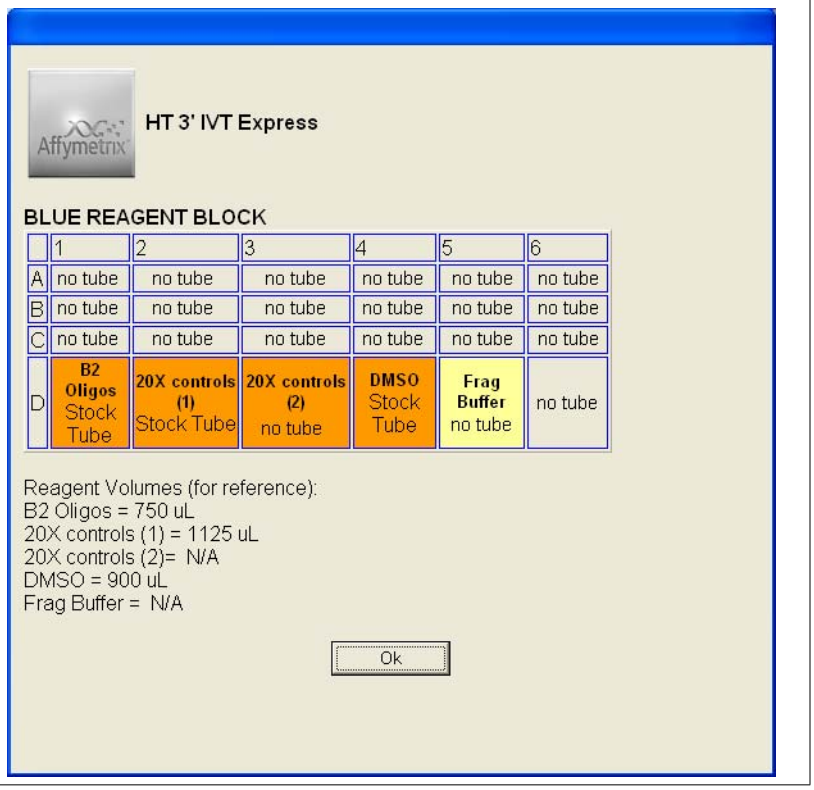
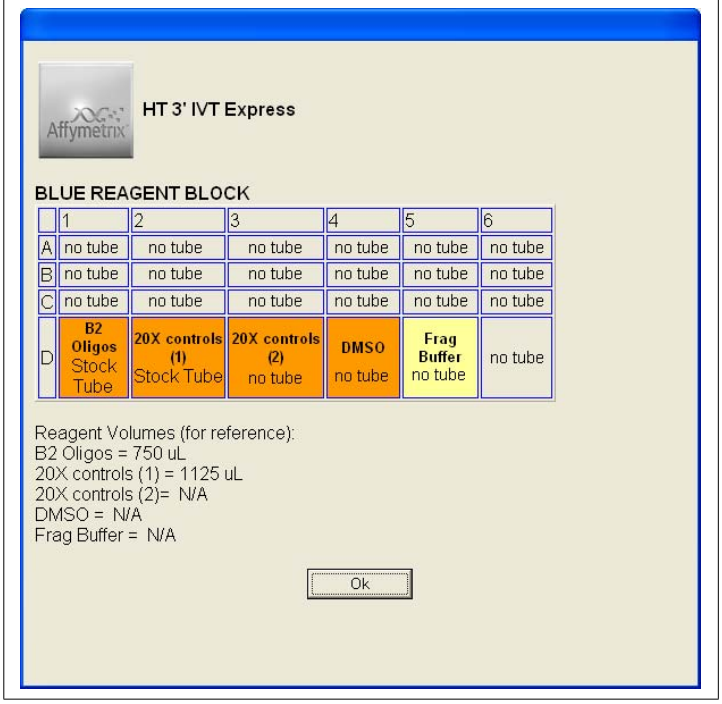


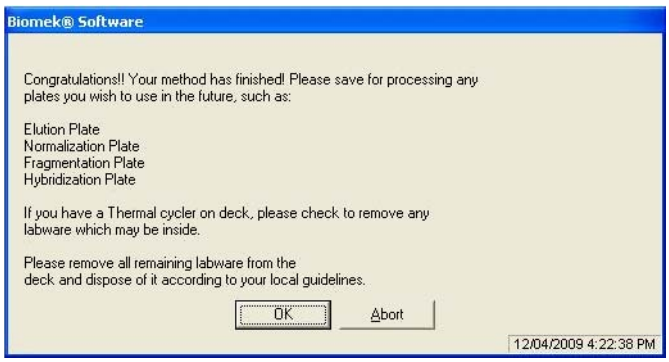
Figure 4.86 Reagent Block Setup: 24 Sample and 96 Sample 1 plate



9. Setup the Reagent Block as shown in Figure 4.85 or Figure 4.86. Click **OK** when ready to proceed.

10. The Biomek FXᴾ TPE System proceeds to make the hybridization cocktail at position 1 in the Modular Reservoir. After the hybridization cocktail has been made the Biomek FXᴾ TPE System dispenses the cocktail to the hybridization plate at deck position P11. Next the FXᴾ TPE adds the sample on the Peltier shaker to the Hybridization plate. When finished Figure 4.87 appears. If you have selected the GeneTitan Setup option, proceed to *GeneTitan Hybridization Setup* on page 82.

Figure 4.87



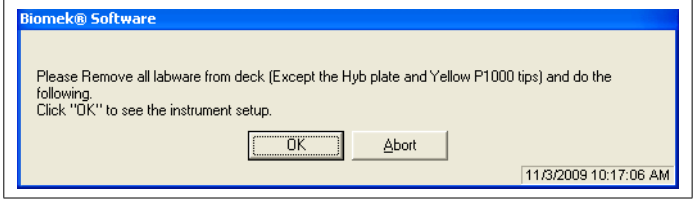
11. Click **OK** to complete method.
12. If using the GeneChip HT 3’ IVT Express 4x24 reaction kit (P/N 901225), the 20X Controls may be used up to two times. If appropriate remove the vial of 20X control stock immediately after use from the reagent block and store at –20°C for future use.

GeneTitan Hybridization Setup

The Biomek FXᴾ TPE System will proceed through Hybridization without user intervention and finishes by mixing the sample with the hybridization cocktail.

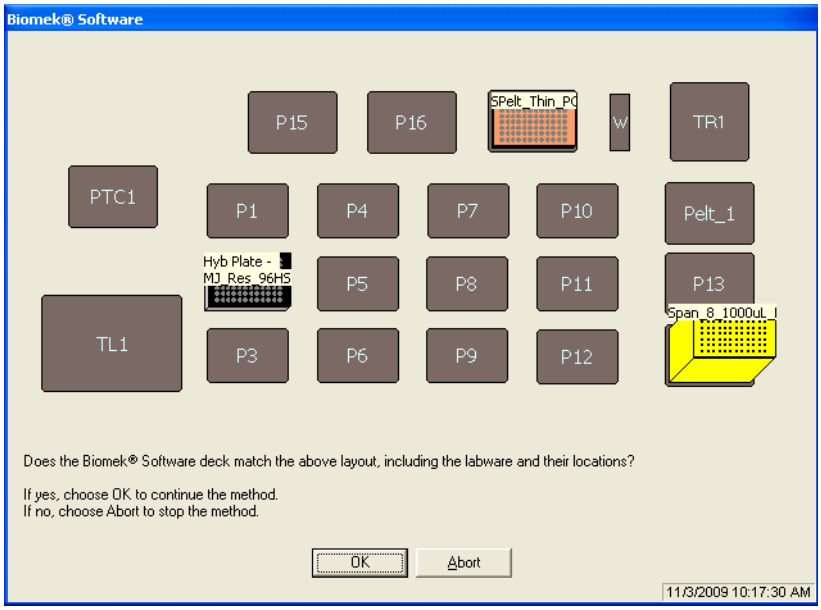
1. If you selected the option **Setup the GeneTitan Plates** at the beginning of the run, Figure 4.88 appears.

Figure 4.88



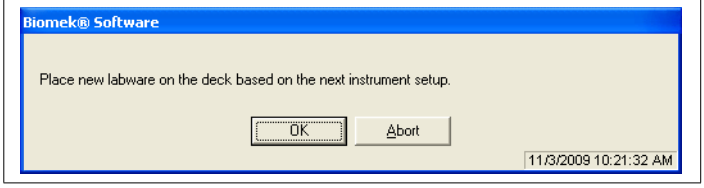
2. Click **OK**.
   Figure 4.89 appears.

Figure 4.89



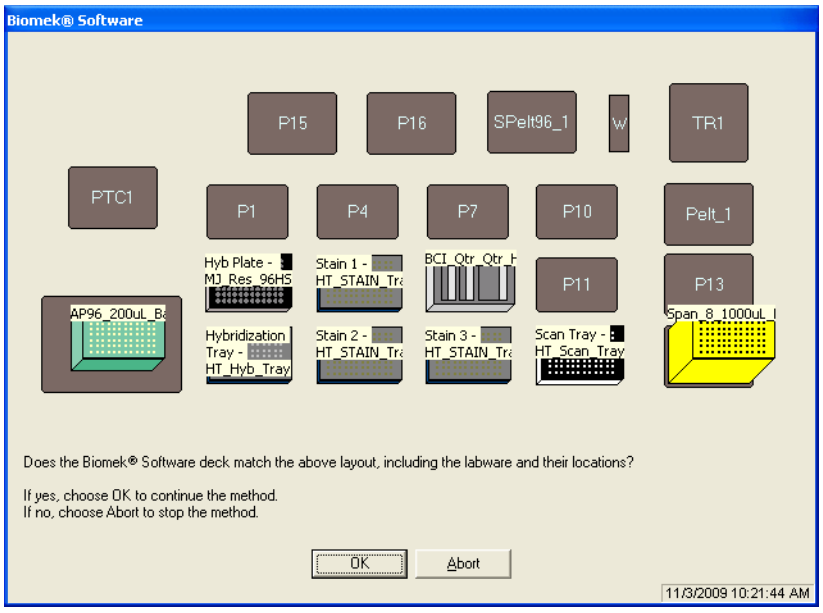
3. Remove labware from deck to match Figure 4.89. Click **OK** when ready.
   A message window appears (Figure 4.90).

Figure 4.90



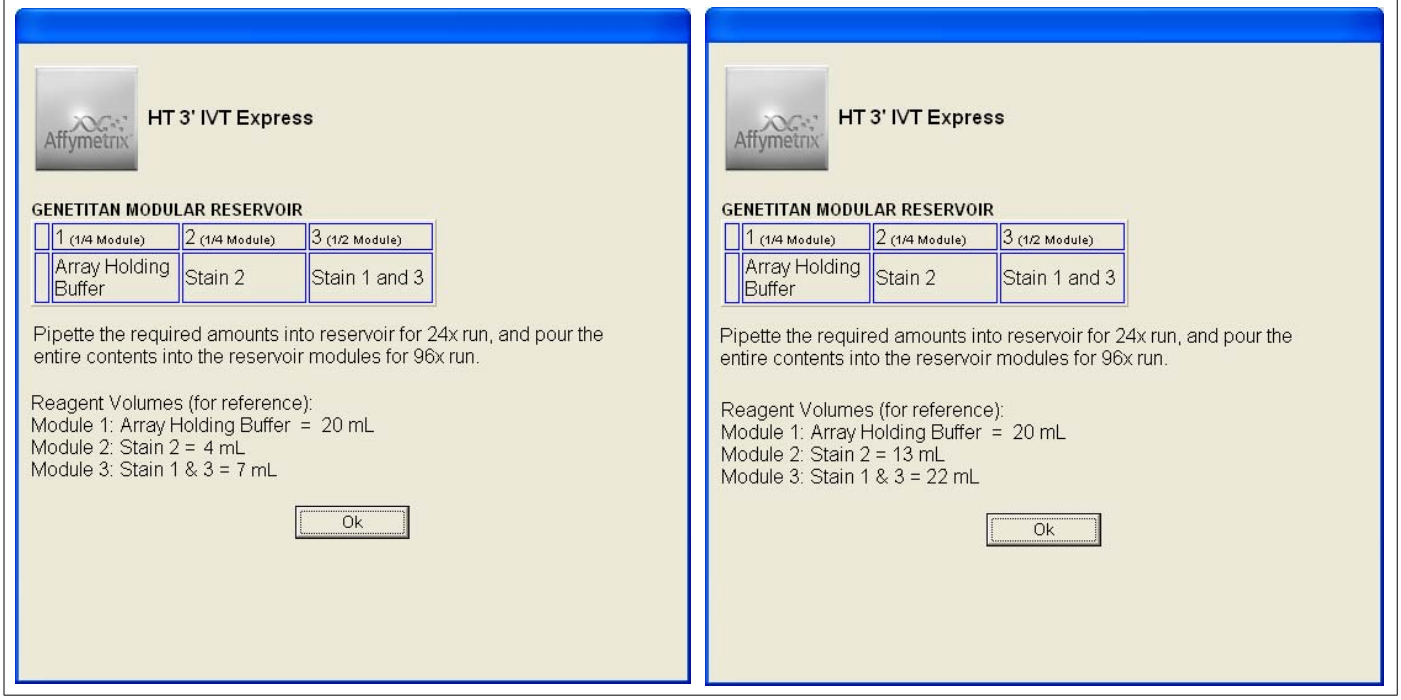
4. Click **OK**.
   Decklayout window appears, Figure 4.91 one plate.

Figure 4.91 Deck Layout -- One Plate



5. Set up the deck to match Figure 4.91 array plates. Click OK when ready. The GeneTitan Modular Reservoir window appears.

Figure 4.92 Modular Reservoir, 24 Sample 1 Plate (left) and 96 Sample 1 Plate (right)



6. Setup the Modular Reservoir Holder as follows:
   - Remove all modular reservoirs from holder.
   - In columns 1, and 2: insert a fresh quarter modular reservoir.
   - In columns 3/4: insert a fresh half modular reservoir.
   - Fill the Modular Reservoir with reagents as instructed onscreen as shown in Figure 4.92.
7. Click **OK**.

   The robot performs the following actions:
   - Denature the targets in the Bio-Rad plate
   - Aliquots Stain 1, Stain 2 and Stain 3 to the appropriate stain trays.
   - Aliquot array holding buffer to the scan tray.
   - Aliquots the hybridization cocktail from the Bio-Rad plate to the GeneTitan hybridization tray.
8. When the method finishes, please refer to the *GeneTitan® User Guide for Expression Array Plates* (P/N 902933) for for further instructions on array processing.

Hybridization Protocol for GeneChip® Cartridge Arrays

This chapter is for users of the **GeneChip® Hybridization, Wash, and Stain Kit**, (30 rxns).

NOTE: Affymetrix does not recommend the use of user-prepared reagents for the 3' IVT Express Assay. Please use the GeneChip® Hybridization, Wash and Stain Kit (P/N 900720) for all the reagents needed for the hybridization, wash and stain steps of this assay.

The GeneChip® Hybridization, Wash, and Stain Kit (P/N 900720) has been developed to complete the hybridization, wash, and stain processes for several types of GeneChip® arrays in cartridge format, specifically 3' Eukaryotic, Prokaryotic, Exon, and Tiling Arrays. For convenience, the kit contains the fewest individual components possible, minimizing the number of preparation and handling steps. Most of the reagents are ready-to-use solutions.

We recommend the use of the Hybridization, Wash, and Stain Kit when preparing GeneChip® 3' Expression Arrays using the GeneChip® Array Station or the Biomek FXᵖ TPE System for automated target preparation. This chapter provides protocol information referring to components of the Hybridization, Wash, and Stain Kit.

NOTE: The GeneChip® Hybridization, Wash, and Stain Kit contains sufficient reagents and buffers to process 30 GeneChip® 3' Expression Arrays in cartridge format when prepared for *manual* target preparation. When used for automated target preparation on the GeneChip® Array Station or the Biomek FXᵖ TPE System, the kit will process fewer GeneChip Arrays. Please refer to the following guidelines for Hybridization, Wash, and Stain Kit reagent consumption when used with the Array Station.

Table 5.1 Automated Target Preparation and Hybridization, Wash, and Stain Kit Reagent Consumption

GeneChip 3' Expression Arrays in Cartridge Format	Number of Hybridization, Wash, and Stain Kits
Up to 24	1
Up to 48	2
Up to 96	4

Target Hybridization

Target preparation on GCAS will result in a U-bottom 'Hybridization-Ready Sample' plate. Target preparation on the Biomek FXP TPE System will also result in a U-bottom 'Hybridization-Ready Sample' plate if the **Set up GeneTitan Plates** option is NOT selected at the begining of the run. Transfer the entire volume from each well into an appropriately labeled 1.5 mL microfuge tube. See the procedure below for denaturing and loading the sample into a cartridge array.

Reagents and Materials Required

- GeneChip® Hybridization, Wash, and Stain Kit: Affymetrix, P/N 900720 (30 rxns)
  From the Hybridization Module, Box 1:
  - Pre-Hybridization Mix
  - 2X Hybridization Mix
- Hybridization Oven 640: Affymetrix, P/N 800138 (110V) or 800139 (220V)
- Micropipettors, (P-2, P-20, P-200, P-1000): Rainin Pipetman (or equivalent)
- Sterile-barrier pipette tips and non-barrier pipette tips
- Heatblock

Procedure

1. Equilibrate probe array to room temperature immediately before use.

NOTE: It is important to allow the arrays to equilibrate to room temperature completely. Specifically, if the rubber septa are not equilibrated to room temperature, they may be prone to cracking, which can lead to leaks.

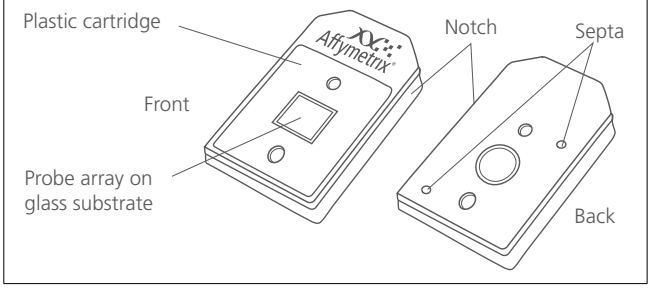
2. Heat the hybridization-ready sample (previously transferred from U-bottom plate to 1.5 mL tubes) to 99°C for 5 minutes in a heat block.
3. Meanwhile, wet the array with an appropriate volume of Pre-Hybridization Mix (see Table 5.2) by filling it through one of the septa.

Table 5.2 Probe Array Cartridge Volumes for Pre-Hybridization Mix and Hybridization Cocktail

Array	Volume
49 Format (Standard)	200 µL
64 Format	200 µL
100 Format (Midi)	130 µL
169 Format (Mini)	80 µL
400 Format (Micro)	80 µL

NOTE: Each array has two septa (see Figure 5.1 for location of the probe array septa). In order to fill the array, first vent the array chamber by inserting a clean, unused pipette tip into one of the septa; then insert the pipette tip of a micropipettor into the remaining septum to fill.

Figure 5.1 GeneChip® Probe Array



4. Incubate the probe array filled with Pre-Hybridization Mix at 45°C for 10 minutes with rotation.
5. Transfer the hybridization cocktail that has been heated at 99°C, in Step 2, to a 45°C heat block for 5 minutes.
6. Spin the hybridization cocktail at maximum speed in a microcentrifuge for 5 minutes to collect any insoluble material from the hybridization mixture.
7. Remove the array from the hybridization oven. Vent the array with a clean pipette tip and extract the Pre-Hybridization Mix from the array with a micropipettor. Refill the array with the appropriate volume of the clarified hybridization cocktail, avoiding any insoluble matter at the bottom of the tube (see Table 5.2).
8. Place probe array into the hybridization oven, set to 45°C.
9. To avoid stress to the motor, load probe arrays in a balanced configuration around the axis. Rotate at 60 rpm.
10. Hybridize for 16 hours.

    During the latter part of the 16-hour hybridization, refer to the *GeneChip Expression Wash, Stain and Scan manual for Cartridge Arrays* (P/N 702731) to prepare reagents for the washing and staining steps required immediately after completion of hybridization.

Washing, Staining and Scanning

Please refer to the *GeneChip Expression Wash, Stain and Scan User Manual for Cartridge Arrays* (P/N 702731) for the washing and staining steps required immediately after completion of hybridization.

The GeneChip Array Station

Summary of the Array Station Run

Target preparation on the Array Station takes approximately 25 hours (including incubation time) and requires two human interventions if hold options and second quantitation of normalized aRNA are not selected: initial sample preparation and aRNA quantitation. At the end of target preparation, the sample is ready to be denatured and hybridized onto cartridge arrays. Hybridization takes 16 hours. Additional time is required for scanning and is dependent on the number of samples processed and the array type. The typical workflow and chapter references are provided below.

1. Isolate high quality total RNA (refer to Chapter 2).
2. Prepare reagents required for target preparation (refer to Appendix C).
3. Perform Array Station system check (refer to Appendix D).
4. Set-up deck layout required for cDNA synthesis reaction through preparation of the hybridization-ready sample (refer to Appendix D).
5. Denature sample and hybridize overnight
   - **GeneChip Cartridge Arrays** using the GeneChip® Hybridization, Wash and Stain Kit (refer to Chapter 5).
   - **GeneChip HT Array Plates** using the GeneTitan® Hybridization, Wash and Stain Kit processed on GCAS (refer to Appendix E).
   - **GeneChip HT Array Plates** using the GeneTitan® Hybridization, Wash and Stain Kit processed on the GeneTitan Instrument (refer to *GeneTitan® Instrument User Guide for Expression Array Plates*, P/N 702933).

   NOTE: Affymetrix does not recommend the use of user-prepared reagents for the 3′ IVT Express Assay. Please use the GeneTitan® Hybridization, Wash and Stain Kit for 3′ IVT Arrays (array plates, P/N 901530) or the GeneChip® Hybridization, Wash and Stain Kit (cartridge arrays, P/N 900720) for all the reagents needed for the hybridization, wash and stain steps of this assay.
6. Wash, stain and scan cartridge arrays (refer *GeneChip® Expression Wash, Stain and Scan User Manual*, P/N 702731).
7. Scan HT Array Plates using the HT Scanner (refer *Affymetrix GeneChip® HT Scanner User's Guide*, P/N 701978).

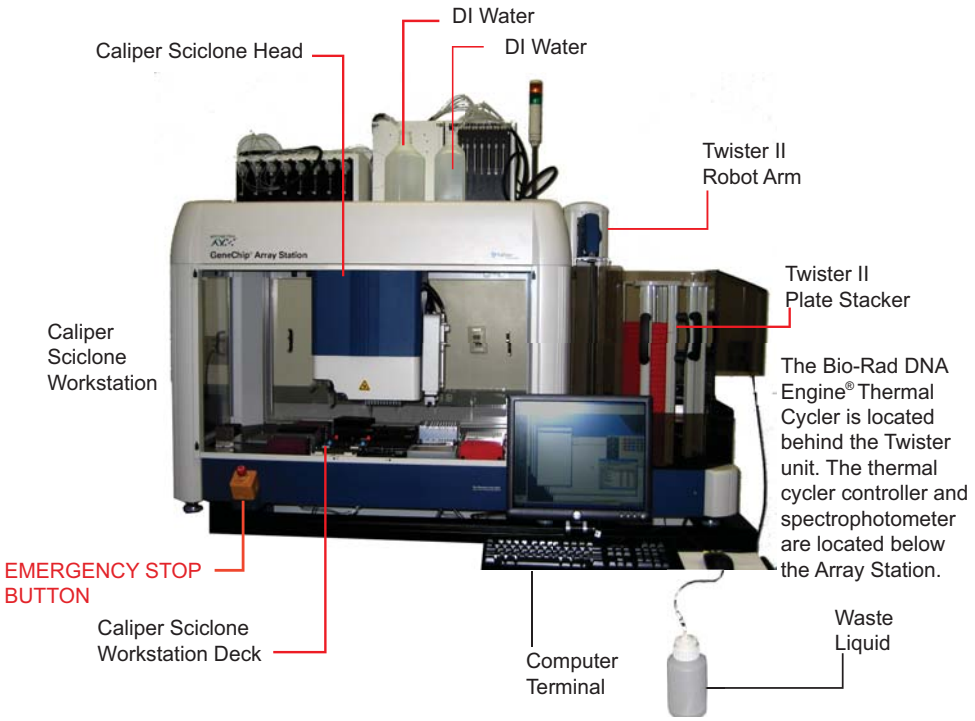
Subsystems

The array station is composed of both mechanical and software subsystems. Refer to the *GeneChip® Array Station User's Guide* (P/N 701859) for more information.

Typical Core System

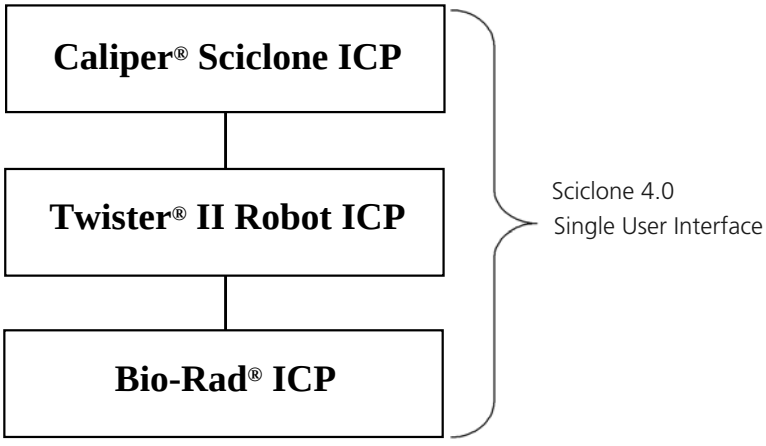
Figure A.1 illustrates the core mechanical subsystems of the GeneChip Array Station including the Caliper Sciclone workstation, Bio-Rad DNA Engine® Thermal Cycler, and Twister® II microplate handler. For detailed information on the various mechanical components of the system, please refer to the *GeneChip® Array Station User's Guide* (P/N 701859).

Figure A.1 The GeneChip® Array Station



The Sciclone 4.0 control software provides a graphical user interface (GUI) to run and track the various mechanical components that comprise the application. The Sciclone 4.0 software has integrated all instrument control programs (ICP's) into one GUI, thus enhancing software ease of use. See Figure A.2 for a schematic of the software product configuration for the Array Station.

Figure A.2 Sciclone 4.0 Software



User Documentation and Quick Reference Cards

The operation of the Array Station requires familiarity with the following user documentation.

The manuals that are relevant will depend on your system configuration. For the Array Station configuration, the following list presents the relevant guides:

1. *GeneChip® Array Station Site Preparation Guide* (P/N 702020)
2. GeneChip® Array Station User's Guide (P/N 701859)
3. *GeneChip® Array Station Deck Layout Quick Reference Card* (P/N 702013)
4. *GeneChip® HT 3' IVT Express Kit Deck Layout Quick Reference Card* (P/N 702629)
5. *GeneChip® Array Station Stain Dispensing Setup Protocol Deck Layout Quick Reference Card* (P/N 702616)
6. *GeneChip® Array Station Reagent Preparation Quick Reference Card* (P/N 702032)
7. *Affymetrix GeneChip® Operating Software User's Guide* (P/N 701439): if you are using Affymetrix GeneChip® analysis software, you should be familiar with Affymetrix' GeneChip® Operating System in order to interpret the assay results.
8. *GeneChip Expression Wash, Stain and Scan User Manual for Cartridge Arrays* P/N 702731
9. *GeneTitan Hybridization Cocktail Preparation Instructions Quick Reference Card* (P/N 08-0303)
10. *Caliper Sciclone User's Manual* (from Caliper Life Sciences).
11. *Bio-Rad MJ Thermal Cycler ICP Guide* (from Bio-Rad/MJ Research).

Regulatory Compliance

Please refer to the *GeneChip® Array Station User's Guide* (P/N 701859) for regulatory compliance and safety information for the Array Station.

RNA Preparation - GCAS

Total RNA Isolation for the GeneChip 3' IVT Express Assay

This chapter describes the general requirements for RNA isolation methods and poly-A control preparation for spiking into your RNA sample.

IMPORTANT: **The quality of the RNA is essential to the overall success of the analysis. Since the most appropriate protocol for the isolation of RNA can be source dependent, we recommend using a protocol that has been established for the tissues or cells being used. In the absence of an established protocol, using one of the commercially available kits designed for RNA isolation is suggested.**

When using a commercial kit, follow the manufacturer's instructions for RNA isolation.

Materials List

Total RNA Isolation Reagents

- TRIzol® Reagent: Invitrogen Life Technologies, P/N 15596-018, or QIAzol™ Lysis Reagent: QIAGEN, P/N 79306
- RNeasy® Mini Kit: QIAGEN, P/N 74104

Miscellaneous Reagents

- 80% ethanol (stored at –20°C)
- Pellet Paint®: Novagen, P/N 69049-3 (optional)
- Glycogen: Ambion, P/N 9510 (optional)
- 3M Sodium Acetate (NaOAc): Sigma-Aldrich, P/N S7899

Isolation of RNA from Yeast

Total RNA

Quality total RNA has been isolated successfully from yeast cells using a hot phenol protocol described by Schmitt, *et al. Nucl Acids Res* **18**:3091-3092 (1990).

Isolation of RNA from Arabidopsis

Total RNA

TRIzol® Reagent from Invitrogen Life Technologies has been used to isolate total RNA from Arabidopsis. Follow the instructions provided by the supplier and, when necessary, use the steps outlined specifically for samples with high starch and/or high lipid content. QIAzol™ Lysis Reagent from QIAGEN can also be used.

Isolation of RNA from Mammalian Cells or Tissues

Total RNA

High-quality total RNA has been successfully isolated from mammalian cells (such as cultured cells and lymphocytes) using the RNeasy® Mini Kit from QIAGEN.

If mammalian tissue is used as the source of RNA, it is recommended to isolate total RNA with a commercial reagent, such as TRIzol® or QIAzol™ reagent.

IMPORTANT: If going directly from TRIzol- or QIAzol™-isolated total RNA to cDNA synthesis, it may be beneficial to perform a second cleanup on the total RNA before starting. After the ethanol precipitation step in the Lysis Reagent extraction procedure, perform a cleanup using the QIAGEN RNeasy Mini Kit. Much better yields of labeled aRNA are obtained from the *in vitro* transcription-labeling reaction when this second cleanup is performed.

Precipitation of RNA

Total RNA

It is not necessary to precipitate total RNA following isolation or cleanup with the RNeasy Mini Kit. Adjust elution volumes from the RNeasy column to prepare for cDNA synthesis based upon expected RNA yields from your experiment. Ethanol precipitation is required following TRIzol or QIAzol reagent isolation and hot phenol extraction methods; see methods on page 96 for details.

Precipitation Procedure

1. Add 1/10 volume 3M NaOAc, pH 5.2, and 2.5 volumes ethanol.*
2. Mix and incubate at –20°C for at least 1 hour.
3. Centrifuge at ≥ 12,000 x g in a microcentrifuge for 20 minutes at 4°C.
4. Wash pellet twice with 80% ethanol.
5. Air dry pellet. Check for dryness before proceeding.
6. Resuspend pellet in DEPC-treated H_2O. The appropriate volume for resuspension depends on the expected yield and the amount of RNA required for the cDNA synthesis. Please read ahead to the cDNA synthesis protocol in order to determine the appropriate resuspension volume at this step.

*Addition of Carrier to Ethanol Precipitations

Adding carrier material has been shown to improve the RNA yield of precipitation reactions.

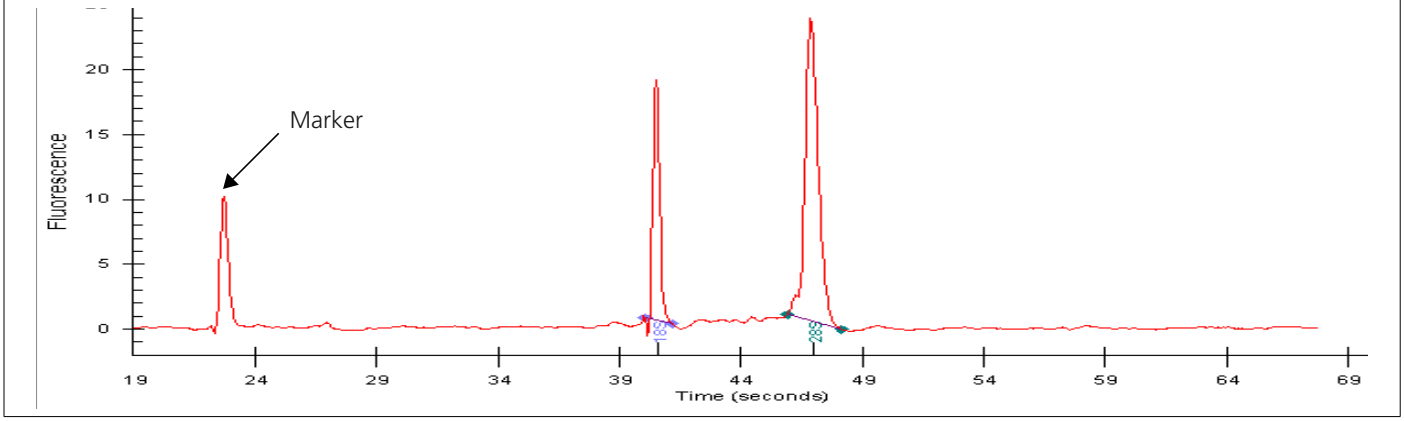
- **Pellet Paint®**
  Addition of 0.5 µL of Pellet Paint per tube to nucleic acid precipitations makes the nucleic acid pellet easier to visualize and helps reduce the chance of losing the pellet during washing steps. The pellet paint does not appear to affect the outcome of subsequent steps in this protocol; however, it can contribute to the absorbance at 260 nm when quantifying the total RNA.
- **Glycogen**
  Addition of 0.5 to 1 µL of glycogen (5 mg/mL) to nucleic acid precipitations aids in visualization of the pellet and may increase recovery. The glycogen does not appear to affect the outcome of subsequent steps in this protocol.

Quantitation of RNA

Quantify RNA yield by spectrophotometric analysis using the convention that 1 absorbance unit at 260 nm equals 40 µg/mL RNA.

- The absorbance should be checked at 260 and 280 nm for determination of sample concentration and purity.
- The A_{260}/A_{280} ratio should be close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).
- Integrity of total RNA samples can also be assessed qualitatively on an Agilent 2100 Bioanalyzer. Refer to Figure B.1 for an example of good-quality total RNA sample.

Figure B.1 Electropherogram (from the Agilent 2100 Bioanalyzer) for HeLa Total RNA. For a high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands.



Preparation of Poly-A RNA Controls

Reagents and Equipment

- GeneChip® HT 3' IVT Express Kit components: Affymetrix, P/N 901225 (4 x 24 rxn), or P/N 901253 (96 rxn)

  Components used in this step:

  - Eukaryotic Poly-A RNA Controls

Designed specifically to provide exogenous positive controls to monitor the entire eukaryotic target labeling process, a set of poly-A RNA controls is supplied in the GeneChip HT 3' IVT Express Kit.

Each eukaryotic GeneChip probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys*, *phe*, *thr*, and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are pre-mixed at staggered concentrations. The concentrated Poly-A Control Stock can be diluted with the Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final dilutions (relative to estimated copy number of total mRNA population) summarized in Table B.1.

Table B.1 Final Dilutions of Poly-A RNA Controls in Samples

Poly-A RNA Spike	Final Dilution (estimated ratio of copy number)
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:6,667

The controls are then amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples. Anticipated relative signal strength follows the order of *lys* < *phe* < *thr* < *dap*.

The **Poly-A RNA Control Stock** and **Poly-A Control Dil Buffer** are provided in the GeneChip HT IVT Express Kit to prepare the appropriate serial dilutions based on Table B.2. This is a guideline when 100, 250 or 500 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

IMPORTANT: **Use non-stick RNase-free microfuge tubes to prepare all of the dilutions (not included).**

Table B.2 Serial Dilution of Poly-A RNA Control Stock

Total RNA Input Amount	Serial Dilutions				Volume of 4th dilution to add to total RNA
	First Dilution	Second Dilution	Third Dilution	Fourth Dilution	
100 ng	1:20	1:50	1:50	1:10	2 µL
250 ng	1:20	1:50	1:50	1:4	2 µL
500 ng	1:20	1:50	1:50	1:2	2 µL

Recommendation: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.

For example, to prepare the Poly-A RNA dilutions for 1 µg of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 38 µL of Poly-A Control Dil Buffer for the First Dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the First Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the Second Dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 2 µL of the Second Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the Third Dilution (1:50).
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of this Third Dilution to 1 µg of total RNA sample directly. The final volume of the total RNA with the diluted poly-A controls should not exceed 5 µL.

NOTE: **The first dilution of the Poly-A RNA controls can be stored up to six weeks in a frost-free freezer at –20°C and freeze-thawed up to eight times.**

Preparation of Total RNA Plates for Processing on GCAS

The Target Preparation protocol starts with 5 µL of material in a Bio-Rad 96-Well Hard-Shell PCR Plate. This plate can either be prepared offline (manually) or on the GeneChip Array Station (GCAS), as described below.

User-prepared Plate Preparation

Preparing Samples with PolyA Controls

Pipet 3 µL of the total RNA sample (100 - 500 ng) and 2 µL of the appropriate PolyA spike control solution into the 96-well plate. Samples should be placed into the plate in a column-wise fashion starting from the left side of the plate. For example, if preparing 24 samples, pipet the samples into the sample wells for columns 1, 2, and 3.

Optional – Running without PolyA Controls

Affymetrix highly recommends that you utilize PolyA controls as described above. However, if you choose not to utilize these controls, the total RNA sample in the Bio-Rad 96-Well Hard-Shell PCR Plate must be adjusted to a final volume of 5 µL.

If using GCAS, once the Bio-Rad 96-Well Hard-Shell PCR Plate has been prepared with 5 µL of the material as described above, select the **Manual Sample Transfer** option when starting on the Array Station. Please refer to Appendix D for detailed information.

Automated Plate Preparation (GCAS Only)

If there is at least 20 µL of purified total RNA (0.2 to 0.4 µg/µL) in a Greiner-U-Bottom plate, the Array Station may be used to transfer 5 µL of this material to the Bio-Rad 96-Well Hard-Shell PCR Plate. Please refer to the instructions in Appendix G for an explanation of how to have the Array Station transfer 5 µL of sample to the starting plate at the beginning of a run.

Reagent Preparation for the GeneChip Array Station

This chapter presents reagent preparation instructions for GCAS users necessary before proceeding to Appendix D, *GeneChip® Array Station Setup and Target Preparation*.

IMPORTANT: **This chapter is for users of the GeneChip Array Station only. If using the Beckman Biomek® FXᴾ TPE System please proceed to Chapter 4 on page 17.**

Target Preparation Reagents

The following reagent preparation is required for both cartridge arrays and HT array plates and will be used in the procedures described in Appendix D, *GeneChip® Array Station Setup and Target Preparation* on page 107.

Reagents and Equipment Required

- GeneChip® HT 3' IVT Express Kit: Affymetrix, P/N 901225 (4 x 24 rxn) or P/N 901253 (96 rxn)
  Components used in this step:
  - aRNA Wash Solution Concentrate
  - RNA Binding Beads
  - aRNA Binding Buffer Concentrate
  - First-Strand Buffer Mix
  - Second-Strand Buffer Mix
  - IVT Labeling Buffer
- Ethanol, 100% (ACS reagent grade or equivalent): Various suppliers
- 15 mL RNAse-free bottle
- V-Tech Cold Reagent Block: Affymetrix, P/N 11-1541

Obtain First-Strand, Second-Strand and IVT Reagents

1. From the GeneChip® HT 3' IVT Express Kit, thaw the First-Strand, Second-Strand and IVT buffers.
2. Reagent block should be cooled to 4 °C.
3. The cooled reagent block is loaded with reagents in *Reagent Setup* on page 119.

Prepare aRNA Wash Solution

1. Add 6 mL 100% ethanol (ACS reagent grade or equivalent) to the bottle containing the 7.5 mL aRNA Wash Solution Concentrate.
2. Mix well and mark the label to indicate that you have added the ethanol. Refer to this solution as **aRNA Wash Solution** throughout this protocol. Store at room temperature.

Prepare aRNA Binding Mix

1. At room temperature in a nuclease-free tube, mix the aRNA Binding Beads and the aRNA Binding Buffer Concentrate in a 1:5 ratio (Table C.1).

Table C.1 aRNA Binding Mix

Component	Volume	GCAS	
		24 Rxn	96 Rxn
RNA Binding Beads*	10 µL	360 µL	1.14 mL
aRNA Binding Buffer Concentrate	50 µL	1800 µL	5.7 mL

** Mix the RNA Binding Beads by vortexing before dispensing.*

2. Refer to this solution as **aRNA Binding Mix** throughout this protocol. Store at room temperature.

Hybridization Cocktail Master Mix for Users of the GeneChip® Hybridization, Wash, and Stain Kit (Cartridge Arrays)

The following reagent preparation is required for use in the procedures described in Appendix D *GeneChip® Array Station Setup and Target Preparation*. When following the procedure described below, please select the 270 µL Hybridization Mix option during Target Preparation Protocol setup (Figure D.25).

NOTE: Affymetrix does not recommend the use of user-prepared reagents for the 3' IVT Express Assay. Please use the GeneChip® Hybridization, Wash and Stain Kit (P/N 900720) for all the reagents needed for the hybridization, wash and stain steps of this assay.

Prepare the Hybridization Cocktail Master Mix

Reagents and Materials Required

- GeneChip® Hybridization, Wash, and Stain Kit: Affymetrix, P/N 900720 (30 rxns)

  Components needed from the Hybridization Module, Box 1:
  - DMSO
  - Nuclease-free water
  - 2X Hybridization Mix
- GeneChip® HT 3' IVT Express Kit Components: Affymetrix, P/N 901225 (4 x 24 rxn), or P/N 901253 (96 rxn)

  Components used in this step:
  - Control Oligo B2 (3 nM)
  - 20X Hybridization Controls
- BD Falcon™ Test Tube, 14 mL: VWR International, P/N 60819-761
- Polypropylene Centrifuge Tubes with Caps, 50 mL: VWR International, P/N 20171-028

Preparing the Hybridization Cocktail Master Mix

NOTE: Refer to Table C.2 for the Hybridization Cocktail Master Mix composition.

1. Obtain a 14 mL BD Falcon Test Tube or a 50 mL centrifuge tube for larger volumes and label as "Hyb Mix."
2. Referring to Table C.2, combine the components as indicated in a 14 mL BD Falcon Test Tube or a 50 mL centrifuge tube, depending on the total volume.
3. Place the hybridization cocktail master mix into the appropriate columns of the 12-column reservoir (see Table C.2).
4. If necessary, remove air bubbles from the hybridization cocktail master mix by briefly pipetting the reaction mix out of the 12-column reservoir and slowly pipetting it back into to the 12-column reservoir.

Table C.2 Cartridge Hybridization Cocktail Master Mix for the 12-Column Reservoir using the GeneChip® Hybridization, Wash and Stain Kit P/N 900720

	Volume per Rxn	Adjusted Volumes (with dead volume in 12 column reservoir): 24 Rxns	96 Rxns
3 nM B2 Oligo	4.5 µL	158.0 µL	553.1 µL
20X *BioB, C, D, Cre* (controls)*	13.5 µL	474.1 µL	1,659.4 µL
2X Hybridization Mix	135.0 µL	4,741.0 µL	16,593.5 µL
DMSO (100%)	27.0 µL	948.2 µL	3,318.7 µL
Nuclease-free Water	47.8 µL	1,678.7 µL	5,875.3 µL
Total Volume	227.8 µL	8,000 µL	28,000 µL

* *Please refer to Important note below.*

IMPORTANT: It is imperative that frozen stocks of 20X GeneChip® Eukaryotic Hybridization Controls are heated to 65°C for 5 minutes to completely resuspend the aRNA before aliquoting.

Table C.3 Hybridization Cocktail Master Mix Volumes for the 12-Column Reservoir (cartridge arrays)

	Adjusted Volumes (with dead volume in 12 column reservoir): 24 Rxns	96 Rxns
Volume in Column 8*	2,600.0 µL	8,900.0 µL
Volume in Column 9*	2,600.0 µL	8,900.0 µL
Volume in Column 10*	2,600.0 µL	8,900.0 µL

* *Please refer to Table D.2 on page 122 for a complete listing of reagents and reagent volumes aliquoted into the 12 column reservoir.*

Hybridization Cocktail Master Mix for Users of the GeneTitan® HT Hybridization, Wash, and Stain Kit (Array Plates)

The following reagent preparation is required for use in the procedures described in Appendix D *GeneChip® Array Station Setup and Target Preparation*. When following the procedure described below, please select either the 120 µL (one HT Array Plate) or 240 µL (two HT Array Plates) Hybridization Mix option during Target Preparation Protocol setup (Figure D.25).

NOTE: Affymetrix does not recommend the use of user-prepared reagents for the 3' IVT Express Assay. Please use the GeneTitan Hybridization, Wash and Stain Kit for all the reagents needed for the hybridization, wash and stain steps of this assay.

Prepare the Hybridization Cocktail Master Mix

Reagents and Materials Required

- GeneTitan Hybridization, Wash, and Stain Kit for 3' IVT Arrays: Affymetrix, P/N 901530

  NOTE: The GeneTitan Hybridization, Wash, and Stain Kit was formulated with volumes for processing array plates on the GeneTitan Instrument, but contains volumes sufficient for processing array plates on GCAS.

  Components needed:
  - Nuclease-free water
  - 1.3X Hybridization Solution A
  - 1.3X Hybridization Solution B
- GeneChip® HT 3' IVT Express Kit Components: Affymetrix, P/N 901225 (4 x 24 rxn), or P/N 901253 (96 rxn)

  Components needed:
  - Control Oligo B2 (3 nM)
  - 20X Hybridization Controls

Preparing the Hybridization Cocktail Master Mix

NOTE: Refer to Table C.4 for the Hybridization Cocktail Master Mix composition.

1. Obtain a 15 mL BD Falcon Test Tube or a 50 mL centrifuge tube for larger volumes and label as "Hyb Mix."
2. Take 1.3X Hybridization Solution A and Solution B from the GeneTitan Hybridization, Wash, and Stain Kit and place on the bench. It is not necessary to warm the buffers to room temperature before making the Hybridization Master Mix.
   A. Vortex.
3. Remove Control Oligo B2 and 20X Hybridization Controls from –20°C and thaw at room temperature.
   A. Vortex and spin.
   B. Keep on ice.
4. Make a Hybridization Cocktail Master Mix according Table C.4 in a 15 mL Falcon tube.
   A. Vortex well.

Table C.4 HT Array Plate Hybridization Cocktail Master Mix for the 12-Column Reservoir using the GeneTitan Hybridization, Wash and Stain Kit P/N 901530

		1 Array/Sample		2 Arrays/Samples	
	Volume per Array (µL)	24 Array Plate	96 Array Plate	24 Array Plate	96 Array Plate
3 nM B2 Oligo	2 µL	80 µL	248 µL	154 µL	498 µL
20X *BioB, C, D, Cre* (controls)*	6 µL	240 µL	744 µL	462 µL	1,494 µL
1.3X Hybridization Solution A	32.3 µL	1,292 µL	4,005 µL	2,487 µL	8,043 µL
1.3X Hybridization Solution B	60 µL	2,400 µL	7,440 µL	4,620 µL	14,940 µL
Nuclease-free Water	0.95 µL	38 µL	118 µL	73 µL	237 µL
Total Volume	101.25 µL	4,050 µL	12,555 µL	7,796 µL	25,212 µL

** Please refer to Important note below.*

IMPORTANT: It is imperative that frozen stocks of 20X GeneChip® Eukaryotic Hybridization Controls are heated to 65°C for 5 minutes to completely resuspend the aRNA before aliquoting.

5. Place the Hybridization Cocktail Master Mix into the appropriate columns of the 12-column reservoir (see Table C.5).
6. If necessary, remove air bubbles from the Hybridization Cocktail Master Mix by briefly pipetting the reaction mix out of the 12-column reservoir and slowly pipetting it back into to the 12-column reservoir.

Table C.5 Hybridization Cocktail Master Mix Volumes for the 12-Column Reservoir

	Volume of Hybridization Master Mix (µL)			
	1 Array/Sample		2 Arrays/Samples	
	24 Array Plate	96 Array Plate	24 Array Plate	96 Array Plate
Volume in Column 8*	3,400 µL	4,000 µL	7,000 µL	8,200 µL
Volume in Column 9*	empty	4,000 µL	empty	8,200 µL
Volume in Column 10*	empty	4,000 µL	empty	8,200 µL

** Please refer to Table D.2 on page 122 for a complete listing of reagents and reagent volumes aliquoted into the 12 column reservoir.*

Thermal Cycler Programs

All HT 3' IVT Express reactions are incubated in a thermal cycler on the Array Station. Set up the thermal cycler programs for each incubation before starting the procedure. Table C.6 shows the specifications for each incubation.

Table C.6 Thermal Cycler Programs for RNA Amplification

Program (or Method)	First Incubation	Second Incubation	Third Incubation
1st-IVT2	42 °C for 2 hours	4 °C for 5 minutes	
2ND-IVT2	16 °C for 1 hours	65 °C for 10 min	4 °C for 5 minutes
40C-4HR	40 °C for 4 hours		
40C-8HR	40 °C for 8 hours		
40C-16HR	40 °C for 16 hours		
55C	55 °C for 5 minutes		
FRAG	94 °C for 35 minutes	20 °C for 5 minutes	

GeneChip® Array Station Setup and Target Preparation

This chapter describes a typical GeneChip® Array Station (GCAS) run. A schematic of the automated target preparation protocol is provided at the beginning to outline the steps in the procedure.

This chapter also describes the procedures for using the GeneChip® Array Station Software, setting up the Array Station deck, and performing quantitation and normalization of aRNA.

Reagents and Materials Required

- GeneChip® HT 3' IVT Express Kit: Affymetrix (P/N 901225, 4 x 24 rxn) (P/N 901253, 96 rxn)
- Hybridization Cocktail Master Mix (see Appendix C)
- Affymetrix® HT GCAS Accessory Kit for Target Preparation: Affymetrix, P/N 901235, 4 runs[1]
  - 96-Well Hard-Shell PCR Plate (5)
  - Costar Polystyrene U-Bottom Plates (2)
  - UV Star Plates (2)
  - High Profile 300 mL Reservoir (1)
  - Self-standing Conical Bottom Tubes, 1.5 mL (12)
  - Universal Microtiter Plate Lids (2)
  - 12 Column, Partitioned, Deep Well reservoir
- BioRad Arched Microplate Lids (3): BioRad, P/N MSL-2032
- BioRad P-pads (3): BioRad, P/N MSP-1001
- SPRIPlate 96R Magnet Plate: Agencourt, P/N 000219
- V-Tech Cold Reagent Block: Affymetrix, P/N 11-1541
- Cold Reagent Block template: Affymetrix, P/N 90-0798 (24 samples) or P/N 90-0846 (96 samples)
- Stacker tips 200 µL non-sterile: Caliper LifeSciences, P/N 78641
- Anti-Static Gun: Affymetrix, P/N 74-0014
- DNAZap: Ambion, P/N 9890
- RNaseZap: Ambion, P/N 9786
- 100% ETOH

[1] *The sub list of materials under the HT GCAS Accessory Kit indicates the numbers needed for one run and does not indicate the total number of items in the kit.*

Automated Target Preparation Schematic

Figure D.1 through Figure D.6 outlines the complete sample preparation protocol.

Figure D.1 Steps 1 through 5: First-strand cDNA Synthesis

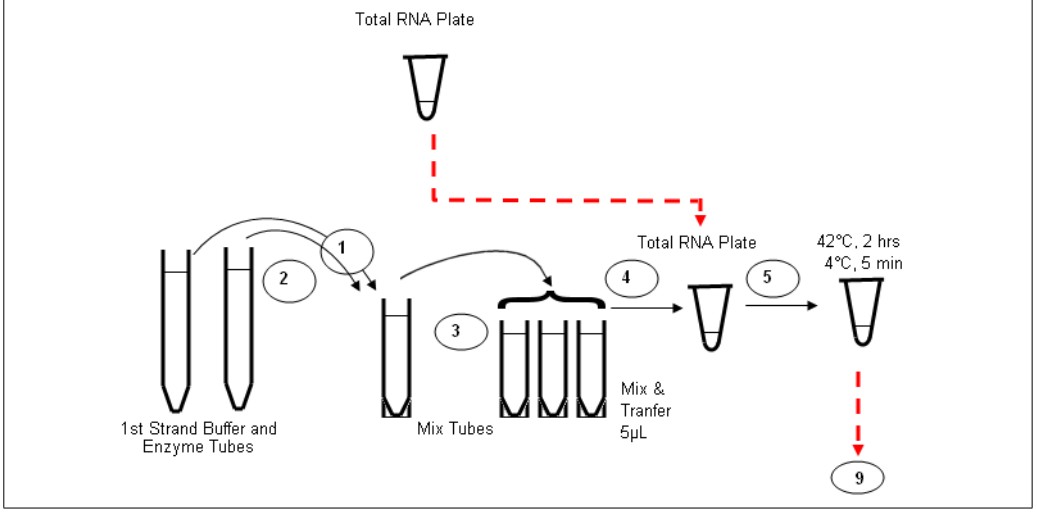


Figure D.2 Steps 6 through 11: Second Strand cDNA Synthesis

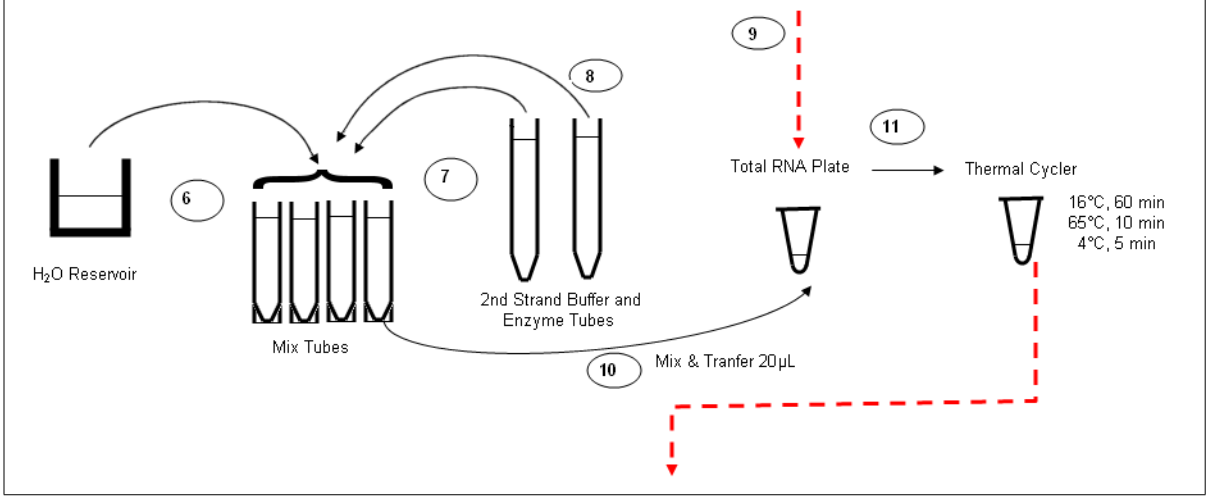


Figure D.3 Steps 12 through 17: IVT

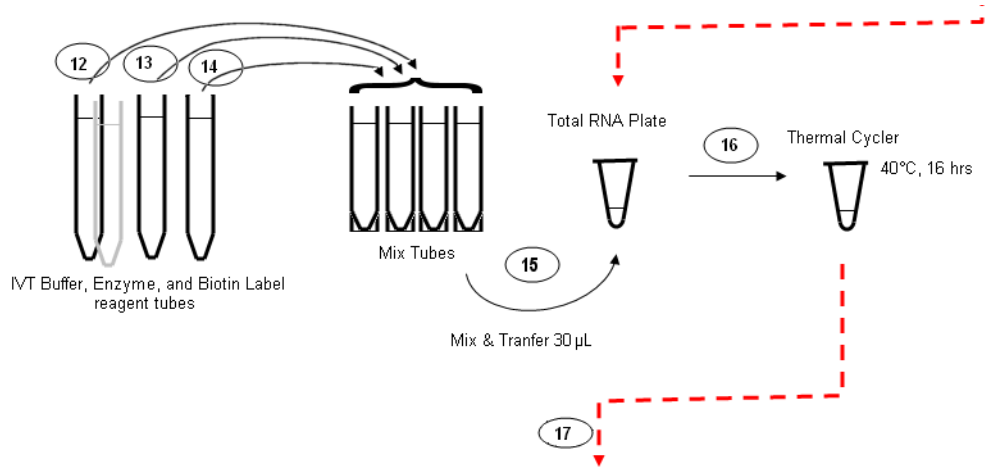


Figure D.4 Step 18 through 35: aRNA Clean-up and Elution

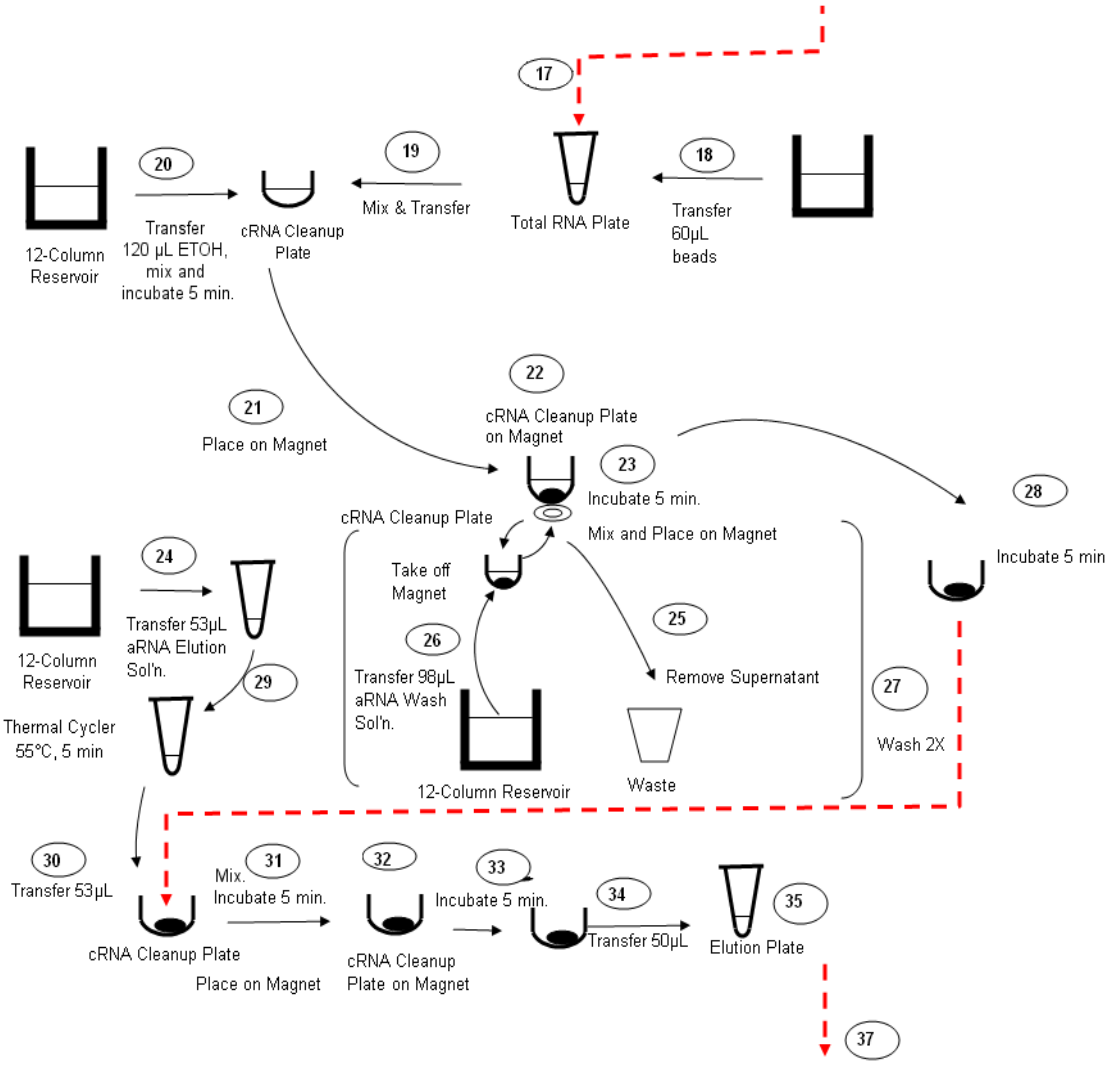


Figure D.5 Steps 36 through 40: Quantitation -- Step 41: Normalization

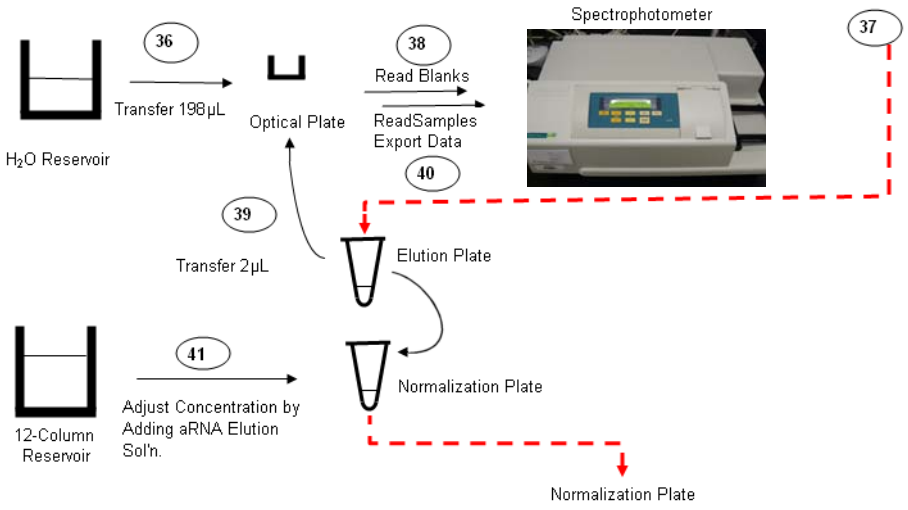
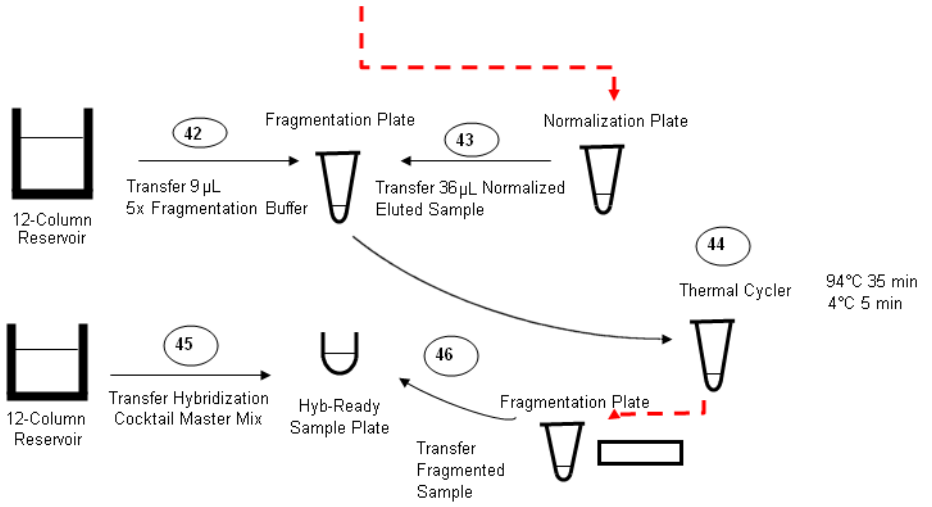


Figure D.6 Steps 42 through 44: Fragmentation -- Step 45 through 46: Hyb-Ready Sample



Check List Before a Run

Before beginning a sample preparation run, you must make the following checks of the system.

1. Ensure that the water supply connections and waste water drainage are properly installed. In addition, the bottles (illustrated in Figure D.7) must be filled with distilled or de-ionized water and the liquid waste container (illustrated in Figure D.8) must be empty.

Figure D.7 Tubing lines for water supply

Figure D.8 Liquid waste drainage

2. Ensure that all the pipette tip boxes are oriented correctly, fit snugly into the holders, and the holders fit into the Twister II Rack.

   Figure D.9 to Figure D.13 illustrate how to load the pipette tips into the Twister II Rack.

CAUTION: Tip Loading Requirement — The number of tip boxes needed for each run may vary depending on the deck layout used and the number of samples processed. Refer to Table D.1 to determine the number of tip boxes to load.

CAUTION: Clean out empty tip boxes in Twister II® Rack 2.

CAUTION: Do not remove tip boxes while run is in progress.

CAUTION: Tip boxes should not extend above the top of the Rack.

Table D.1 Pipette Tip Usage - Number of Tip Boxes Needed

	Number of Rxns	
Starting Point	**24***	**96***
First Strand	8	25
Second Strand	7	24
IVT	7	22
aRNA Clean Up	6	21
Quantitation	3	9
Fragmentation	1	3

** Numbers include Second Quantitation method except for the option of starting at Fragmentation.*

Note: All run type options ends in Hyb-Ready Samples.

3. Controlling static electrical interference: Static attraction can cause pipette tips to cling to each other. To control static attraction, use non-sterile, RNase/DNase-free tips and use a static gun to remove static discharge. See Figure D.9 and Figure D.10.
4. Another method used to control static electricity is the use of metal spacers. Spacers should be placed in between each and on top of the last RNase/DNase-free tip rack after the use of the anti-static gun. See Figure D.11.

Figure D.9 Removing static discharge from tip boxes with an anti-static gun

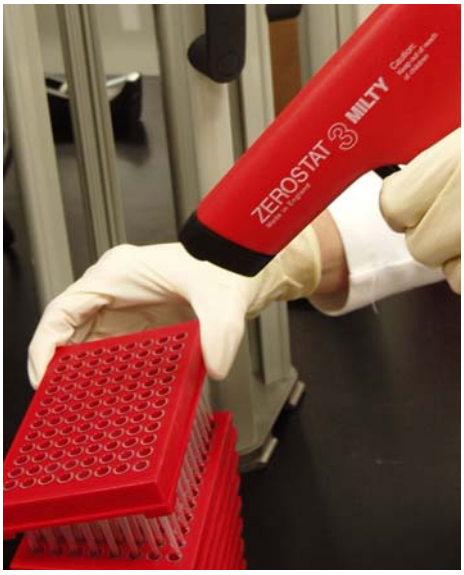


Figure D.10 Removing static discharge from tips with an anti-static gun

Figure D.11 Tips with anti-static metal spacers

Figure D.12 Loading the pipette tips into the holder and into the rack

Figure D.13 Loading the pipette boxes into the rack

Clean the Bio-Rad 96-Well Hard-Shell PCR Plate Lids

NOTE: The disposable pad under the arched lids should be cleaned before every run.

Material required

- Ambion DNAZap™
- Ambion RNaseZap® Wipes

Procedure

1. Rinse the pad with DI water.
2. Wipe the pad with RNaseZap.
3. Rinse the pad with DI water.
4. Wipe the pad with DNAZap.
5. Thoroughly rinse the pad with DI water.
6. Dry the pad with pressurized clean air or nitrogen.

NOTE: The disposable pad under the arched lids should be replaced every 15 runs. Refer to the *GeneChip® Array Station User's Guide* (P/N 701859) for further details.

Beginning a Run

This section shows you how to set up the deck and use the software to begin a sample preparation run.

Preparing the Deck

1. Set up the deck with the appropriate consumables. Refer to Figure D.14.

   NOTE: The deck layout shown in Figure D.14 is for runs starting at the beginning of the target prep process. Custom target prep runs can also be started at various other places in the protocol. Please refer to layouts in Appendix H for runs starting at other steps in the process.

2. Assemble the Peltier adaptor and prechilled cold reagent block. Ensure that the unit is securely tightened. Refer to Figure D.15 and Figure D.16.
3. Turn on the Watlow Temperature Controller to 4°C. Refer Figure D.17.

   IMPORTANT: The prechilled cold reagent block and Peltier adaptor are assembled prior to the addition of the reagent tubes. It is important that the adaptor be at room temperature when joining with the cold reagent block as it is difficult to fit the cold reagent block flush against the adaptor if the adaptor is cold. Once the chilled cold reagent block is assembled into the adapter securely, adjust the control unit to 4°C for the remainder of the run.

Figure D.14 Target Preparation Deck Layout. Refer to Table D.1 on page 112 to determine the number of tip boxes to load.

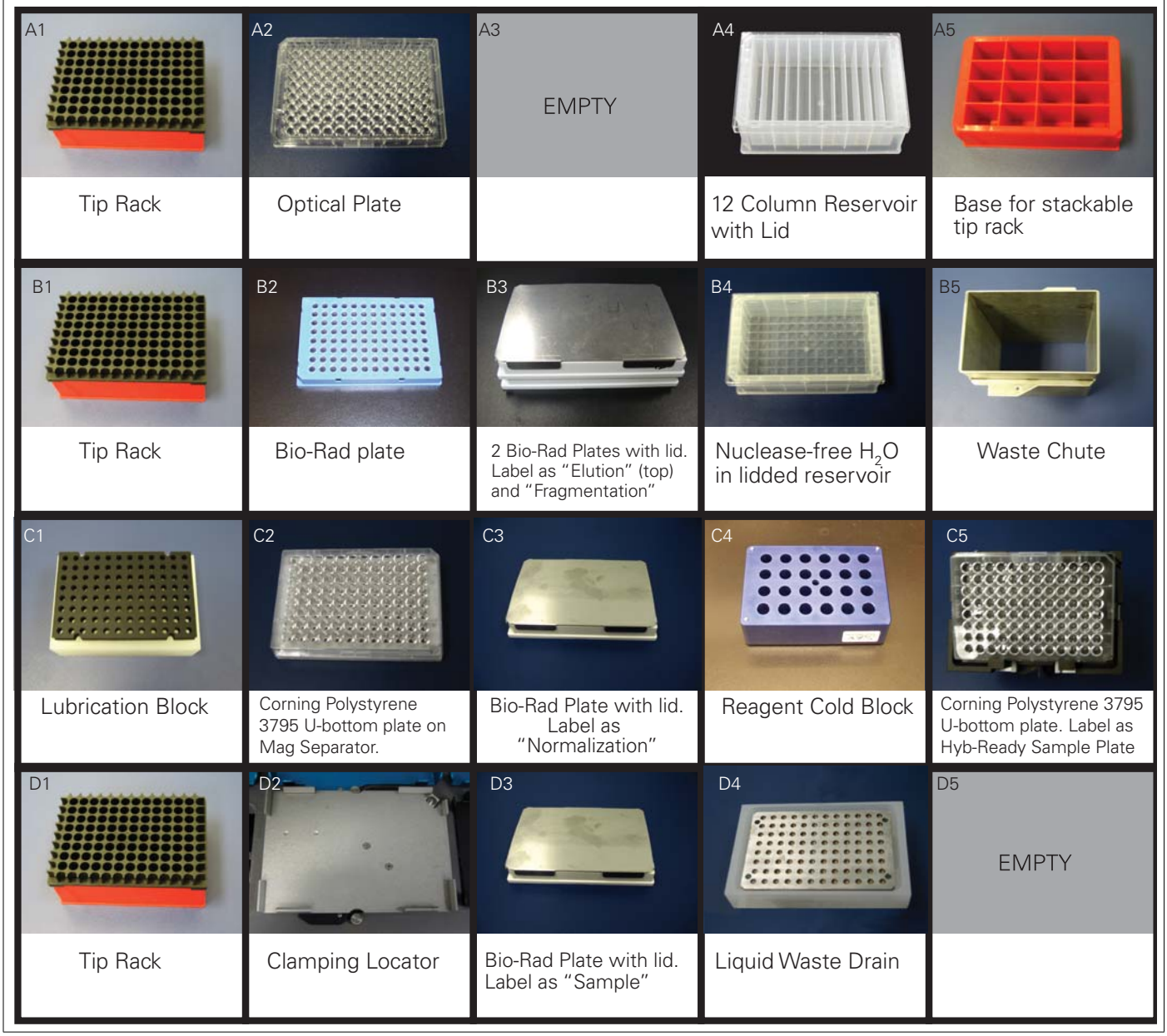


Figure D.15 Cold reagent block on the deck fixture

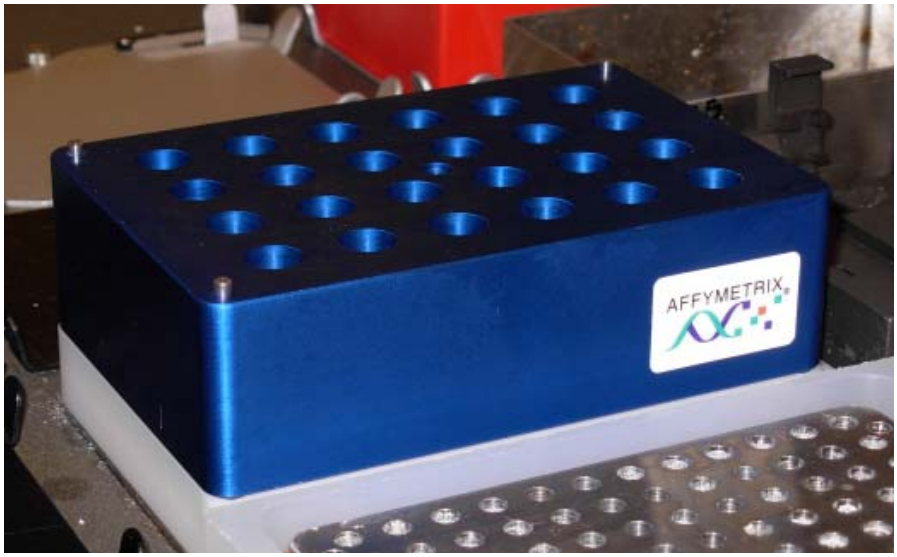


Figure D.16 Securing the cold reagent block. Do not fully tighten

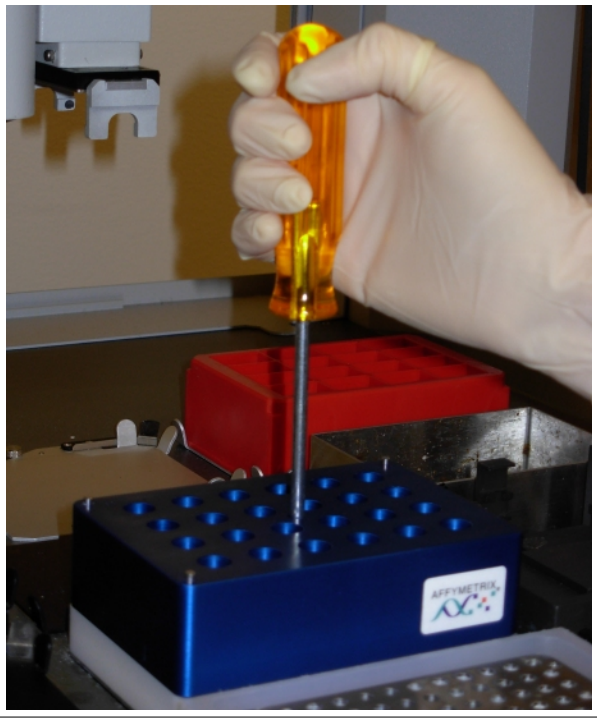


Figure D.17 Setting the Watlow Temperature Controller for the Peltier at 4°C

Reagent Setup

Reagent setup for the HT 3' IVT Express Assay involves manually loading the cold block with reagents, empty mixing tubes, and filling the 12 column reservoir. The initial reagent setup must be performed prior to starting the Target Preparation Protocol.

Cold Block

First-strand, Second-strand and IVT buffers should be thawed, as explained on page 101.

1. Place the reagent block template appropriate for your run onto the cooled (4 °C) reagent block.
2. Uncap the First-Strand, Second-Strand and IVT reagent tubes and insert them into the cooled reagent block following the color codes on the template, or as shown in Figure D.18 or Figure D.19.
3. Uncap and load the reagents and mixing tubes onto the assembled cold reagent block and adaptor following the color codes on the template, or as shown in Figure D.18 and Figure D.19.

> **!** **IMPORTANT: Before loading the tubes, please mix enzyme solutions by gently flicking the reagent tube a few times and centrifuging before inserting them into the reagent cold block.**

4. Place the reagent block on the deck in position C4.

Figure D.18 Cold Block Reagent Layout for 96-Sample Plate

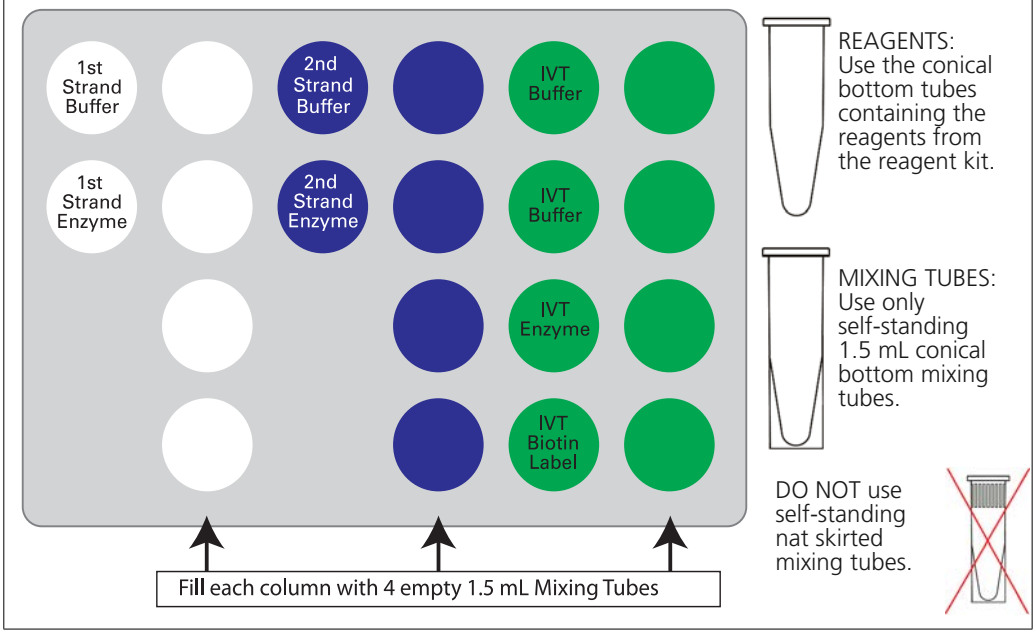


Figure D.19 Cold Block Reagent Layout for 24-Sample Plate

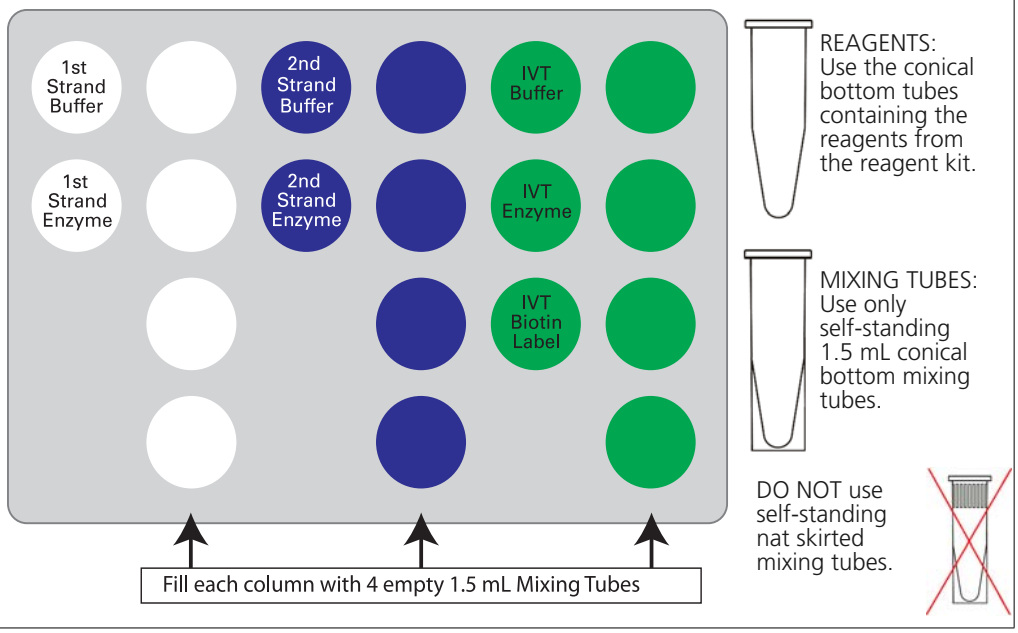
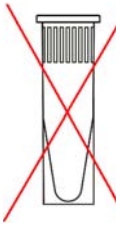


Figure D.20 Reagent Cold Block loaded for 96-sample run (top) and a 24-sample run (bottom).

REAGENTS: Use the conical bottom tubes containing the reagents from the reagent kit.

MIXING TUBES: Use only self-standing 1.5 mL conical bottom mixing tubes.

DO NOT use self-standing nat skirted mixing tubes.



! IMPORTANT: All mixing tubes used should be self-standing 1.5 mL conical bottom tubes without (textured surface). For reagents, use the conical bottom tubes (non self-standing) containing the reagents in the GeneChip® HT 3' IVT Express Kit. For the location of the reagents and mixing tubes in the cold block please refer to the cold block template or Figure D.18 / Figure D.19 on page 120.

Nuclease-free Water

1. Add >100 mL nuclease-free water to the high reservoir in deck position B4.
2. Cover with a microtiter plate lid.

12-column Reservoir

1. Fill the 12 column reservoir with reagents at room temperature as indicated in Table D.2. Please refer to *Reagent Preparation for the GeneChip Array Station* on page 101.

Table D.2 Reagents for the 12 Column Reservoir

Column	Reagent		24 Rxn	96 Rxn
1	RNA Binding Beads and aRNA Binding Buffer		2.1 mL	6.8 mL
2	100% ETOH		3 mL	7.7 mL
3	100% ETOH		3 mL	7.7 mL
4	aRNA Wash Solution*		3.1 mL	11.1 mL
5	aRNA Wash Solution*		3.1 mL	11.1 mL
6	aRNA Elution Solution		4.3 mL	15.6 mL
7	5X Array Fragmentation Buffer		1.8 mL	1.8 mL
8	Hybridization Cocktail Master Mix	cartridge arrays	2.6 mL	8.9 mL
		1 array plate	3.4 mL	2.4 mL
		2 array plates	12 mL	8.2 mL
9	Hybridization Cocktail Master Mix	cartridge arrays	2.6 mL	8.9 mL
		1 array plate	—	2.4 mL
		2 array plates	—	8.2 mL
10	Hybridization Cocktail Master Mix	cartridge arrays	2.6 mL	8.9 mL
		1 array plate	—	2.4 mL
		2 array plates	—	8.2 mL
11	Empty		—	—
12	Empty		—	—

** Prior to use, the aRNA Wash Solution Concentrate should be mixed with 100% Ethanol, as directed on label.*

2. When finished, cover reservoir with lid return to deck position A4.

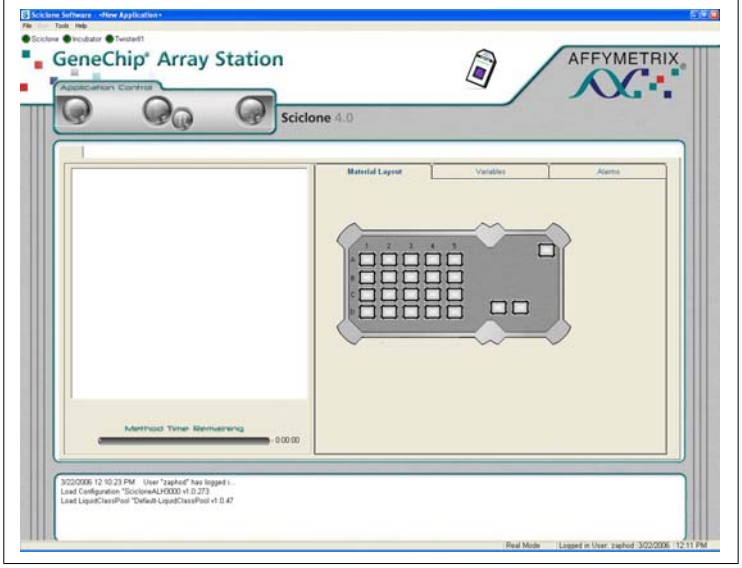
Running the Target Preparation Protocol

1. Check to confirm that the deck and racks are correctly populated with well plates, tips, etc. and that the cold reagent block is loaded with the correct reagents (Figure D.14 and Figure D.20).
2. Open the GeneChip Array Station Software by double-clicking the desktop icon, or click **Start** → **Programs** → **Caliper Life Sciences** → **Instruments** → **Sciclone** → **Sciclone Workstation Software**.
3. A Login window appears (Figure D.21). Enter your **User Name** and **Password** and click **OK**. All runs should be performed in Operator Mode. See Appendix I, *User Level Configuration* for a description of operational modes.

Figure D.21 Sciclone Workstation Software Login Window

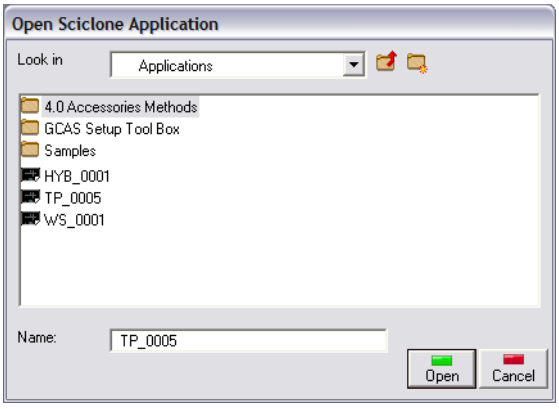
4. Once you have logged in, the main Operator window appears, as shown in Figure D.22. This window is referred to as the Runtime Window.

Figure D.22 Runtime window



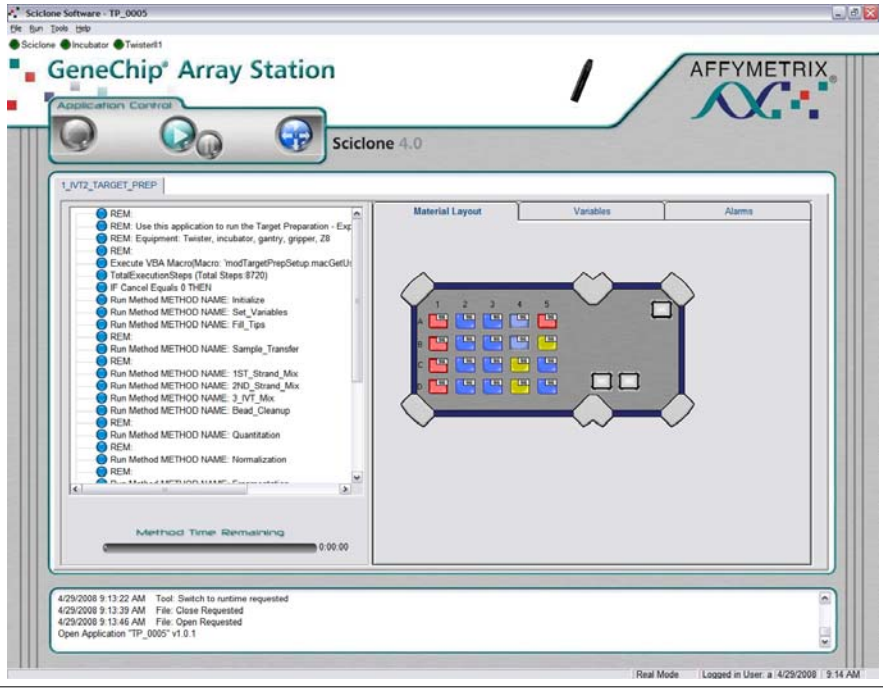
5. To load the Affymetrix Target Preparation protocol, select **File** → **Open**. The Open Sciclone Application dialog box appears (Figure D.23).

Figure D.23 Open Sciclone Application window



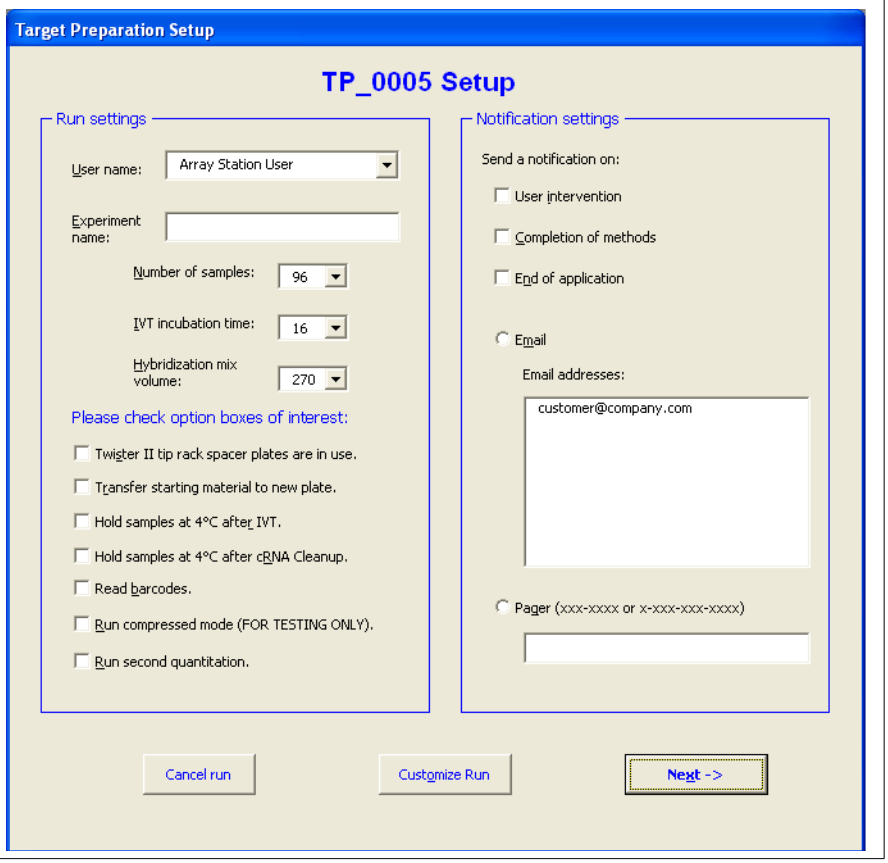
6. Select the protocol **TP_0005** and click **Open**. When the Target Preparation Protocol has completed loading, the window as shown in Figure D.24 appears.

Figure D.24 Target Preparation Protocol Loaded



7. To begin a run, click the green arrow button in the Application Control console of the Runtime window.
   The Target Preparation Setup dialog box appears (Figure D.25).

Figure D.25 The Target Preparation Setup dialog box



8. Under **Run Settings** in the Target Preparation dialog box, enter the following information:
   A. **User name**: Select the appropriate user name from the drop down menu. Please contact Affymetrix support personnel for assistance with customizing this window.
   B. **Experiment name**: Enter a name for your experiment.
   C. **Number of samples**: Select either 24 or 96. Be sure your samples are laid out in the plate in column-wise fashion starting column 1.
   D. **IVT incubation time**: Select either 4, 8 or 16 hours. Refer to Table 3.3 on page 14 for recommended incubation times based on the amount of input RNA.
   E. **Hybridization Mix Volume**: Select the appropriate option:
      - 120 µL = one HT Array Plate
      - 240 µL = two HT Array Plates
      - 270 µL = cartridge arrays
9. In the **option boxes of interest** area of the Target Preparation dialog box, select the following optional items:
   A. **Twister II tip rack spacer plates are in use**: Select this option if you are using the tip rack spacer plates to help control static. Affymetrix strongly suggests you utilize the tip rack spacer plates. A spacer plate should be placed between each rack of tips as well as on the top rack of tips before loading tips into the Twister II Rack 1.

B. **Transfer starting material to new plate** (>5 µL/well at 10-20 ng/µL) Check this box to have the Array Station transfer 5 µL of starting total RNA into the Bio-Rad 96 Well Hard Shell PCR plate. To use automated sample transfer, a minimum of 20 µL of your sample (0.2 to 0.4 µg/µL) must be in a Greiner U-bottom plate. If you select **Transfer starting material to new plate**, you will be prompted when to place and remove plates as needed. Please refer to Appendix G and Chapter 2 for more information on using the automated sample transfer option.

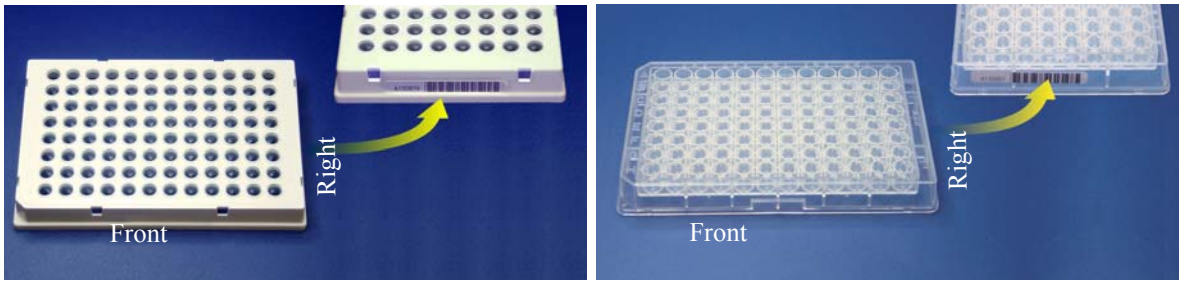
NOTE: If you select the automatic sample transfer option you must use the initial deck layout shown in **Figure G.1** of **Appendix G**. Do not use the layout shown in **Figure D.14**.

C. **Hold samples at 4 ºC after IVT**: If you select this option, the plate will be held in the thermal cycler at 4°C after the IVT step until you prompt the Array Station to resume the process. Once prompted, the process will proceed to the IVT cleanup steps.

D. **Hold samples at 4 ºC after cRNA Cleanup**: If you select this option, the plate will be held in the thermal cycler at 4°C after cRNA Cleanup Step until you prompt the Array Station to resume the process. Once prompted, the process will proceed to the Quantitation step.

E. **Read barcodes**: Check this box if you want the system to automatically read and track the barcodes of various plates used during the run. You must supply the barcodes (code 128c format) and affix them to any or all of the following plates for tracking total RNA, unfragmented aRNA, normalized aRNA, fragmented aRNA, and hyb-ready sample. Barcodes should be affixed to the right side of the plate (see Figure D.26). Barcodes recorded during the run will be reported in the Target Preparation Summary Report for your run.

Figure D.26 Barcode Positioning on Plate



F. **Run compressed method (FOR TESTING ONLY):**
Do not select this option unless you are performing a blank test run.

NOTE: Please see **Appendix H** for a description of how to use the "Customize Run" option.

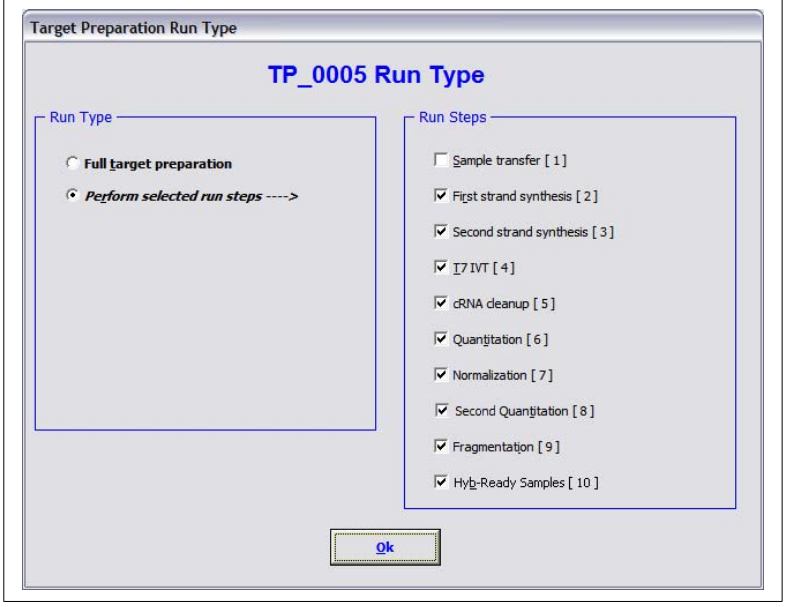
G. **Run second quantitation (OPTIONAL):**
Do not select this option unless you require the post normalization yield. Selecting this option requires one additional user intervention and placing a new OD plate on D2.

H. **Notification Settings**: Select the points at which you would like to receive notification. Notification can be received via email or pager. Ensure that the email address or pager information in the selection is correct and active. Please contact Affymetrix personnel for assistance with configuring email or pager notification

- **User intervention** —notifies you when the system requires your attention
- **Completion of methods** — notifies you when a method, or run step, is completed
- **End of application**

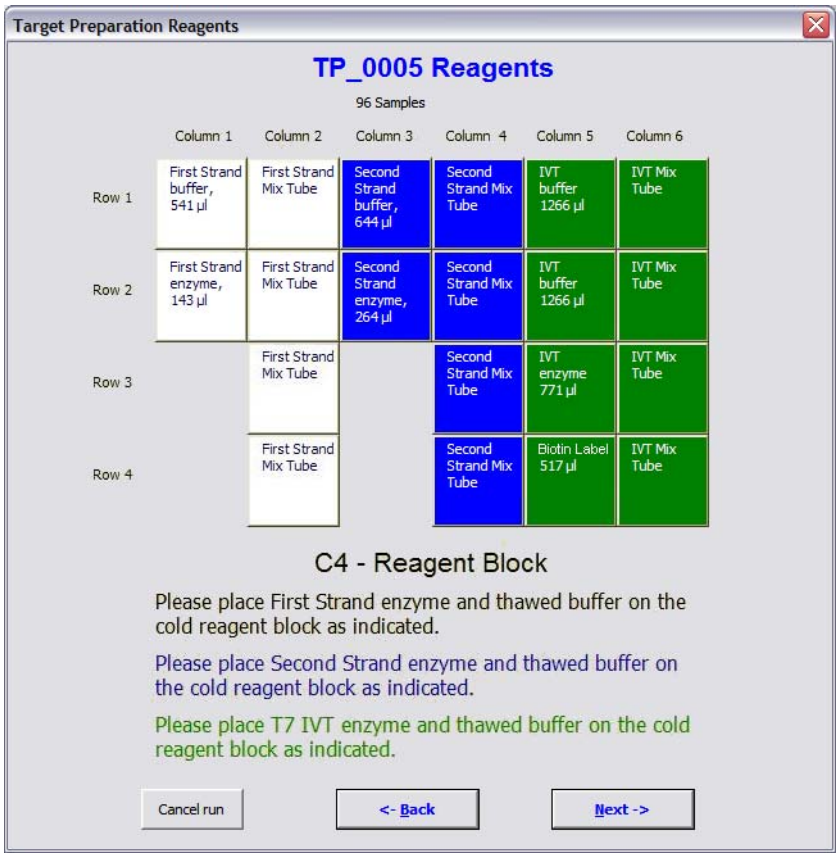
10. After you have entered the necessary information in this setup window, click one of the following options:
    - **Cancel run**: Click **Cancel run** to exit the application.
    - **Next →**: If your protocol requires all the standard methods, click **Next →** and go to Step 13 on page 127 to continue with the Target Preparation Protocol.
    - **Customize run**: If your protocol does not require all the standard methods click Customize run to select specific run steps for your protocol.
      The Target Preparation Run Type dialog box appears (Figure D.27).

Figure D.27 The Target Preparation Run Type dialog box



11. In the **Run Type** section select the **Perform selected run steps →** option
    - In the **Run Steps** section select those methods, or Run Steps, designed for your protocol.
12. Click **OK**.
    The Target Preparation Setup dialog box window returns (Figure D.25).
13. Click **Next →** .
    The Target Preparation Reagents window appears (Figure D.28).

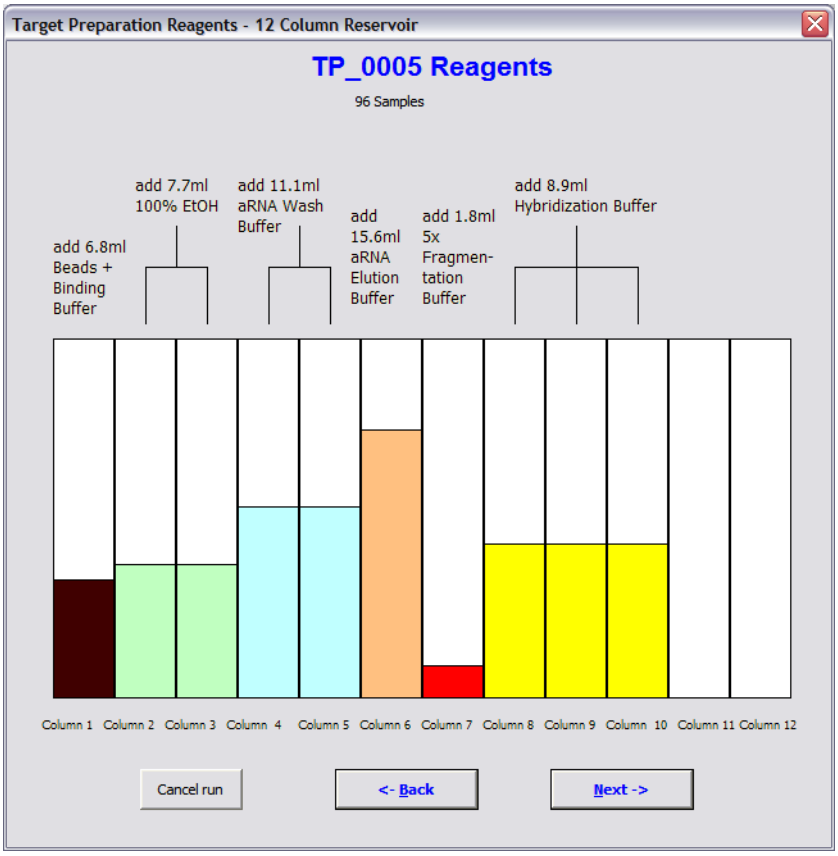
Figure D.28 Target Preparation Reagents window (96-sample)



14. The Target Preparation Reagents window graphically displays the correct setup for the Reagent Cold Block according to your selections. Verify that the reagent cold block is setup as indicated. Click **Next** → .

The Target Preparation Reagents - 12 Column Reservoir window appears (Figure D.29).

Figure D.29 Target Preparation Reagents - 12 Column Reservoir window (96-sample)



15. The Target Preparation Reagents - 12 Column Reservoir window graphically displays the correct setup for the 12 column reservoir according to your selections. Verify that reservoir is setup as indicated. Click **Next** →.

16. The deck layout window appears (Figure D.30).

Figure D.30 The Target Preparation Deck Layout window (96-sample)

NOTE: The deck layout picture in Figure D.30 is for a specific configuration including post-normalization quantitation and may not exactly reflect what is seen on your display due to the options selected in Figure D.25.

17. The TP_0005 Deck Layout window shown in Figure D.30 shows a window for the **target preparation** 96 samples. In this window the user has NOT selected the **Automatic sample transfer** option, but has selected the second quantitation option. Be sure the deck layout shown in the Target Preparation Deck Layout window matches the layout appropriate for your starting selection.
18. Ensure that you have complied with the **Notes for Starting with First Strand Synthesis**.
    - Add required reagents to 12-column reservoir in position A4 and add microtiter plate lid.
    - Add >100 mL nuclease-free water to high reservoir in position B4 and add microtiter plate lid.
    - Place reagent block in position C4 and adjust temperature controller to 4°C.
    - If running a 24-sample run: Confirm that at least 8 tip boxes are in Twister rack 1 and that none are in rack 2.
    - If running a 96-sample run: Confirm that at least 25 tip boxes are in Twister rack 1 and that none are in rack 2.
19. Click **Continue run** to proceed.

    The GCAS application starts the priming protocol, transfers tips and starts the methods:
    - *Reverse Transcription to Synthesize First-Strand cDNA*
      No user intervention required.
    - *Second-strand cDNA Synthesis*
      No user intervention required.

- *In Vitro Transcription to Synthesize Labeled aRNA*
  User intervention is required if the option "Hold samples at 4°C after IVT" is selected during Target Preparation Setup (Figure D.25).
- *aRNA Purification*
  User intervention is required if the option "Hold samples at 4°C after cRNA cleanup" is selected during Target Preparation Setup (Figure D.25).
- *aRNA Quantitation and Normalization*
  User intervention required.
- *Second Quantitation*
  User intervention required.
- *Fragmentation and Preparation of Hybridization-Ready Sample*
  No user intervention required.
- Hyb-ready Samples

Reverse Transcription to Synthesize First-Strand cDNA

The Array Station begins the First-Strand Synthesis step without user intervention. This step takes approximately 3 hours to complete and then proceeds to Second-strand cDNA Synthesis.

The composition of the First-Strand Master Mix is shown in Table D.3.

Table D.3 First-strand Master Mix/RNA Volumes

Component	Amount
First Strand Buffer Mix	4 µL
First Strand Enzyme Mix	1 µL
RNA	5 µL
Total volume	10 µL

Second-strand cDNA Synthesis

After the First-strand Synthesis, the Array Station continues to the Second-strand cDNA Synthesis step. This step takes approximately 2 hours to complete. The composition of the Second-strand Master Mix is shown in Table D.4. The Array Station proceeds to the next step without user intervention.

Table D.4 Second-strand Master Mix (for a single reaction)

Component	Amount
Nuclease-free Water	13 µL
Second Strand Buffer Mix	5 µL
Second Strand Enzyme Mix	2 µL
Total volume	20 µL

In Vitro Transcription to Synthesize Labeled aRNA

After the Second-strand Synthesis, the Array Station continues to the IVT Synthesis step. This step takes approximately 5, 9, or 17 hours to complete, depending on the IVT incubation time defined during Target Preparation Setup. The composition of the IVT Master Mix is shown in Table D.5.

The Array Station assembles the **IVT Master Mix**, mixes the component reagents, then aliquots 30 µL IVT Master Mix to 10 µL sample in Bio-Rad hardshell PCR plate.

Table D.5 IVT Master Mix (for a single reaction)

Component	Amount
IVT Biotin Label	4 µL
IVT Labeling Buffer	20 µL
IVT Enzyme Mix	6 µL
Total volume	30 µL

While the reaction plate incubates in the thermal cycler, GCAS continues mixing beads. After incubation is complete the reaction plate is held at 4°C while IVT reagent mixing commences. After incubation the reaction plate is returned to deck and placed in position D3.

NOTE: If you selected "Hold After IVT" during setup, the plate will stay in thermal cycler at 4 °C until you press OK in the dialog box.

TIP: STOPPING POINT: The aRNA can be stored overnight at –20 °C at this point, if desired.

aRNA Purification

After the IVT synthesis is complete, the aRNA is purified to remove enzymes, salts, and unincorporated nucleotides. The composition of the aRNA Binding Mix is shown in Table D.6. At the completion of this step, the Array Station proceeds to aRNA Quantitation and Normalization without user intervention if the hold option was not selected.

Table D.6 aRNA Binding Mix

Component	Amount
RNA Binding Beads*	10 µL
aRNA Binding Buffer Concentrate	50 µL

** Mix the RNA Binding Beads by vortexing before dispensing.*

NOTE: If you selected "Hold After cRNA Cleanup" during setup, the plate will stay in thermal cycler at 4 °C until you press OK in the dialog box.

aRNA Quantitation and Normalization

This section of the protocol details the user intervention steps necessary for calculating the yield of aRNA generated from the *in vitro* transcription reaction. The accurate calculation of the yield is necessary so that the correct amount of aRNA is added to the fragmentation reaction. Too much or too little aRNA added to the fragmentation reaction can result in incomplete or over fragmented aRNA and cause hybridization effects. This aRNA yield can be used as a check point to ensure that all the preceding steps have been successfully completed and sufficient aRNA yield has been generated.

aRNA Quantitation and Expected Yield

- **Assessing aRNA Yield by UV Absorbance**

  The concentration of an aRNA solution can be determined by measuring its absorbance at 260 nm. We recommend using SpectraMax Spectrophotometers for convenience.

  Alternatively, the aRNA concentration can be determined by diluting an aliquot of the preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and reading the absorbance in a traditional spectrophotometer at 260 nm. Find the concentration in µg/mL using the equation shown below. (1 A_{260} = 40 µg RNA/mL)

- **Expected Yield**

  The aRNA yield will depend on the quality and amount of poly(A) RNA in the input total RNA. Since the proportion of poly(A) RNA in total RNA is affected by influences such as health of the organism and the organ from which it is isolated, aRNA yield from equal amounts of total RNA may vary considerably. Figure D.31 shows yield and size data for aRNA produced with the kit from several different types of input RNA.

- **Concentrate the Purified aRNA (Optional)**

  If necessary, concentrate the aRNA by vacuum centrifugation. If the heater on the vacuum centrifuge has different settings, use medium or low. Check the progress of drying every 5–10 minutes, and remove the sample from the concentrator when it reaches the desired volume. Avoid drying aRNA samples to completion.

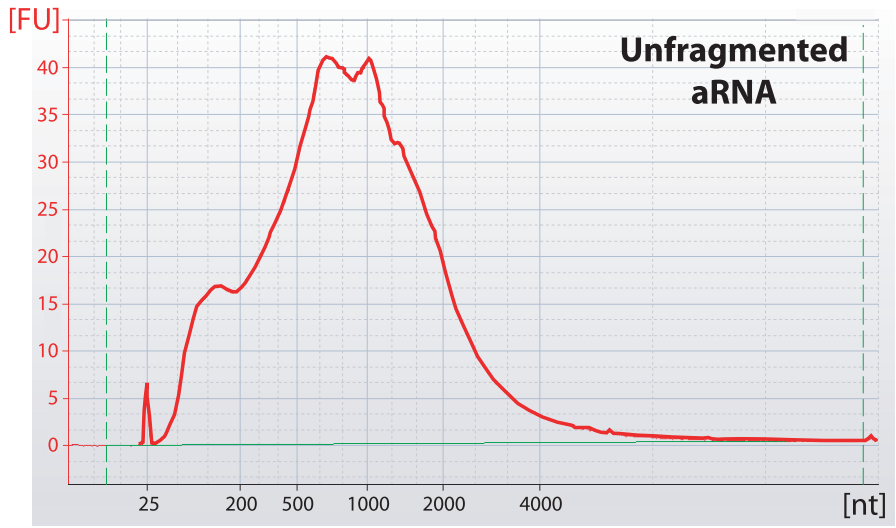
Analysis of aRNA Size

The size distribution of aRNA can be evaluated using an Agilent 2100 bioanalyzer with the Agilent RNA 6000 Nano Kit (P/N 5067-1511), or by conventional denaturing agarose gel analysis. The bioanalyzer can provide a fast and accurate size distribution profile of aRNA samples, but aRNA yield should be determined by UV absorbance or RiboGreen analysis. To analyze aRNA size using a bioanalyzer, follow the manufacturer's instructions for running the assay using purified aRNA.

Expected aRNA Size

We recommend analyzing aRNA size distribution using an Agilent bioanalyzer and RNA 6000 Nano Kit loaded with 300 ng of aRNA per well. The expected aRNA profile is a distribution of sizes from 250–5500 nt with most of the aRNA between 600–1500 nt. Average aRNA size may vary slightly depending on RNA quality and total RNA input amount.

Figure D.31 Example Agilent Bioanalyzer Electropherogram of un-fragmented aRNA generated from 50 ng of HeLa total RNA.



The GeneChip Array Station uses spectrophotometric analysis to determine the aRNA yield. The convention that 1 absorbance unit at 260 nm equals 40 µg/mL RNA is used.

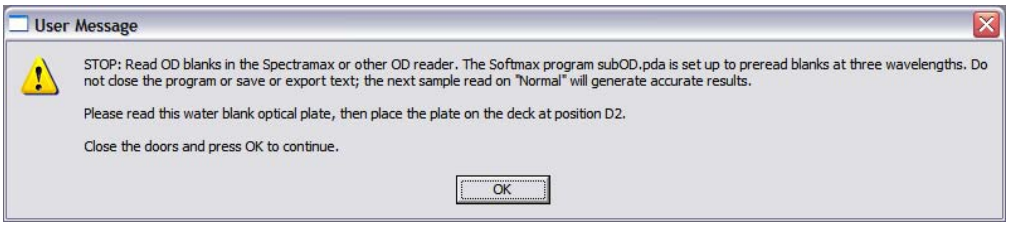
- The absorbance at 260 nm and 280 nm is checked to determine sample concentration and purity.
- The A_{260}/A_{280} ratio is maintained close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).

NOTE: **The following method assumes the use of the recommended spectrophotometer as part of the Array Station system. If a different spectrophotometer is used, refer to instructions in that user guide.**

aRNA First Quantitation and Normalization

After the Purification step the robot will remove the reaction plate from the thermal cycler and put 198 µL of water into the optical plate. A User message window appears (Figure D.32).

Figure D.32 User Message window indicating to read Blank plate.



CAUTION: **When the User Message appears, as illustrated in Figure D.32, do not click "OK" until Step 6.**

1. Turn on the spectrophotometer. Before proceeding, ensure that the LCD screen on the spectrophotometer indicates that the UV lamp has warmed up successfully.
2. Start the SoftMax® Pro software. Either click the SoftMax Pro icon on the desktop or click **Start** → **Programs** → **SoftMax Pro** → **SoftMax Pro**.

   TIP: **.Please be sure to clear any prior data that may appear on the screen. Click "Setup" → "OK". Click "Delete" in the warning message window.**

3. Open the **subOD.pda** application.
4. Remove the optical plate from deck position D2 and place the plate on the spectrophotometer tray.
5. Press the **Read** button in the SoftMax Pro software and verify that the **Pre Read** option is selected. Click **OK**.

   The spectrophotometer will read the plate as a blank.
6. Return the plate on the deck to position D2 and click **OK**.

   NOTE: **The Array Station will now add 2 µL of sample to the 198 µL of water in the optical plate and will mix the sample with pipette mixes.**

   CAUTION: **When the User Message appears, as illustrated in Figure D.33 or Figure D.34, do not click "OK" until Step 13 on page 135.**

Figure D.33 If running **First Quantitation ONLY**: User Message window indicating reading optical plate and disposing OD plate after reading.

Figure D.34 If running **Second Quantitation**: User Message window indicating reading optical plate and placing a clean optical plate.

7. Remove the optical plate and place in the spectrophotometer.
8. Press the **Read** button in the SoftMax Pro software and verify that the **Normal** option is selected. Click **OK**.

   The spectrophotometer will now read the plate.
9. When the spectrophotometer completes the optical read, export the data as "IVT2sample.txt" to the folder c:\Affymetrix\Reader Data. Save the sample data on a disc as a backup.
10. Go to c:\Affymetrix\Reader Data to check that the software has updated "IVT2sample.txt" with the correct date and time.

IMPORTANT: **Use the export function to save the file in text format. If you are using other software, export or save data as a text file and ensure that the file is reformatted to the appropriate format.**

NOTE: **If the "Run Compressed" option was selected IVT2sample.txt will not be renamed with time and date stamp.**

11. Discard optical plate.
12. Place a clean optical plate on the Array Station deck at position D2 if Second Quantitation was selected during Target Preparation Setup.
13. Click **OK** in the window shown in Figure D.33.

    The Array Station will calculate the concentrations and yields of your samples. A graphical output will be produced to show you the pre-normalization yields. An example is shown in Figure D.35.

If any wells fail to generate an amount of sample that can be correctly normalized, a red "X" will mark that well.

Figure D.35 Graphic displaying aRNA yields (in µg)

NOTE:

- **Samples which are outside the range of normalization (20 to 200 µg) will be carried through the remainder of the procedure but will not be properly normalized. Hybridization results from improperly normalized samples may not be valid.**
- **Low OD readings may be due to bubbles in sample wells. Check plate and centrifuge if bubbles are observed.**
- **Use the reread option as described in Step 14 for samples with unexpected low or high OD reading so that samples are properly normalized. Prior to proceeding to Step 14 please complete the following instructions:**

  a. Empty the well in the optical plate that has the unexpected low or high OD reading.

  b. Manually pipette 198 µL of water into the well.

  c. Manually pipette 2 µL of normalized sample into the well.

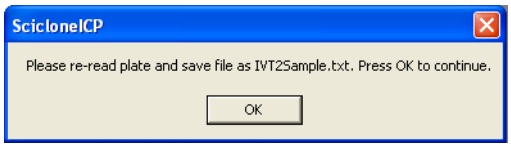
  d. Mix well by pipetting up and down at least 10 times.

  e. Read with the spectrophotomer. Export file.

  f. Follow Step 14 to import new data using the reread button.

14. Click **Reread** in the window shown in Figure D.35 if you would like to import new data into C:\Affymetrix folder so that the normalization process is corrected. When **Reread** is clicked, the following dialog will appear. Please import the new file as IVT2sample in C:\Affymetrix\Reader Data.

Figure D.36 Reread dialog box



15. Click **Resume Run** and the robot will add the correct amount of elution buffer to each well to make the dilution of aRNA ready for fragmentation. This process takes approximately 25 minutes to complete for a full 96 sample plate.

NOTE: The IVT2sample.txt file you saved for the First Quantitation above, will be renamed with the following convention:

IIVT2sample_TP_0005_cRNA_3_28_2008_5_44_59_PM.txt

Where
TP_0005 = application used
cRNA or Post-Norm (dependent on when it is used)
3_28_2008 = date stamp of when the TP_0005 *starts*
5_44_59_PM = time stamp of when the TP_0005 *starts*

These renamed files can still be found in the folder c:\Affymetrix\Reader Data after the run. In addition, all processed OD readings will be automatically saved to a Data Report at the end of the run and can be found in the folder c:\Affymetrix\reports\Data with the following naming convention: ODs_TP_0005_3_28_2008_5_44_59_PM.txt

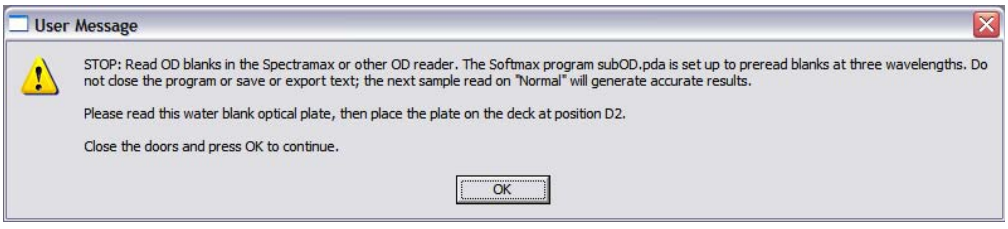
Second Quantitation

If Second Quantitation was selected during Target Preparation Setup please follow the directions below.

1. Following normalization, the Array Station will prepare a blank plate so that the normalized samples can be read to verify normalization. The blank plate will contain 198 µL of water in each well.

CAUTION: When the User Message appears, as illustrated in Figure D.37, do not click "OK" until Step 4 below.

Figure D.37 User Message window indicating to read Blank plate.



2. Remove the optical plate from deck position D2 and place the plate on the spectrophotometer tray.
3. Press the **Read** button in the SoftMax Pro software and verify that the **Pre Read** option is selected. Click **OK**.

   The spectrophotometer will read the plate as a blank.
4. Return the plate to the deck at position D2 and click **OK**.

NOTE: The Array Station will now add 2 µL of sample to the 198 µL of water in the optical plate and will mix the sample with pipette mixes.

CAUTION: When the User Message appears, as illustrated in Figure D.38, do not click "OK" until Step 8.

Figure D.38 User Message window indicating reading optical plate and placing a clean optical plate.

5. Remove the optical plate and place in the spectrophotometer.
6. Press the **Read** button in the SoftMax Pro software and verify that the **Normal** option is selected. Click **OK**.

   The spectrophotometer will now read the plate.
7. When the spectrophotometer completes the optical read, export the data as "IVT2sample.txt" to the folder c:\Affymetrix\Reader Data. Save the sample data on a disc as a backup.
8. Click **OK** in the window shown in Figure D.38.
   The array station calculates the concentrations and yields of the sample. A graphical output is produced displaying the post-normalization yields (Figure D.39).
9. Discard optical plate.

Figure D.39 Graphic displaying aRNA yields (in µg)

10. Click **Resume Run** in the Yield After Normalization window (Figure D.39).

Fragmentation and Preparation of Hybridization-Ready Sample

After the aRNA yield calculation, the Array Station continues to the fragmentation step and makes the hybridization-ready sample without user intervention. Fragmentation of aRNA target before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay sensitivity. After fragmentation, GCAS proceeds to make the hybridization ready sample by mixing appropriate amounts of fragmented aRNA and Hybridization Cocktail Master Mix (see Table C.3 or Table C.5) in a U-bottom plate labeled 'Hybridization Ready Sample'. This process takes approximately two hours for a full 96-sample plate. After this process is completed, the hybridization-ready samples can be stored or used for hybridizing cartridges (Chapter 5) or array plates (Appendix E or the *GeneTitan® Instrument User Guide for Expression Array Plates*, P/N 702933).

IMPORTANT: After the Fragmentation Plate is placed into the thermal cycler, immediately remove the Elution Plate containing aRNA from the deck and seal the plate with an adhesive plate seal. Use a plate roller to secure the seal onto the plate to prevent evaporation then store the plate in a freezer at –20°C.

For short-term storage:

1. Cover the hybridization-ready sample plate with an adhesive plate sealer.
2. Store the sealed plate at –20°C.

For long-term storage:

1. Cover the hybridization-ready sample plate with an adhesive plate sealer.
2. Store the sealed plate at –80°C.

Target Preparation Final Reports

After completion of the target preparation, three report files are saved automatically.

1. **Main Summary Report**: This report is found in the folder c:\Affymetrix\Reports\Summary. This is a user readable report that captures user information, start and stop times, set up information, lot numbers (if input), barcodes (if used), wells flagged as outside the pre-normalization range limits, and reports of all errors encountered during run. An example of a Summary Report is shown in Figure D.40.

   The summary report will be named with the following convention (example):
   SummaryReport_TP_0005_7_31_2008_6_14_52_PM.rtf
   SummaryReport: type of report
   TP_0005: type of run
   3_21_2008: date of run
   6_14_52_PM: time run started
2. **Yield Data Summary**: The Yield Data Summary is found in the folder c:\Affymetrix\Reports\Data. This report captures the A260 readings subtracted background, cRNA pre-normalization concentrations, cRNA pre-normalization yields, sample volumes for normalization, diluent volumes for normalization, and projected input amount for fragmentation. If second quantitation is completed, the report captures the normalized OD260 readings subtracted background, cRNA post-normalization concentrations, cRNA post-normalization yields, and the input amount for fragmentation.

   The naming convention for the Data Summary report is (example):
   ODs_TP_0005_7_31_2008_6_14_52_PM.txt
   ODs: indicates yield data report
   TP_0005: type of run
   3_21_2008: date run started
   6_14_52_PM: time run started

Figure D.40 Example of Final Summary Report for a Target Preparation Run

```
                Target Preparation Summary Report
                          TP_0005 IVT Express



User: Array Station User                Start Time: 4:55:44 PM
Date: Sunday, July 06, 2008             End Time: 2:11:57 PM
                                        Run Duration: 45:16:13



Tracking Identifier:
Ambion MessageAmpTM Premier Kit: 0
Number of Samples:   96
Transferred Starting Material:     YES
Tip Spacers:    NO
Hold After IVT:     NO

First Step: First Strand Synthesis [ 2 ]
Last Step:  Hybridization setup [ 10 ]
Steps Completed:


Barcodes Read:  NO

Error Report: No errors were detected.
```

GeneChip® Array Station Clean Up

After completion of the target preparation, follow the steps below to clean up the Array Station.

1. Cover the elution plate (if not already done - see page 139) and the fragmented aRNA plate with an adhesive plate sealer. Use the plate roller to securely cover the sealer on the plate to prevent evaporation.
2. Store the sealed plates at –20°C.
3. Dispose of the EtOH, water, pipette tips, and waste water appropriately.
4. Wipe up any spills that may have occurred.
5. Close the GeneChip Array Station Software.
6. Check and refill Z8 water reservoir level.

Hybridization, Wash and Stain Kit Protocol for GeneChip® HT Array Plates on GCAS

This chapter is for users of the GeneTitan® Hybridization, Wash, and Stain Kit for 3' IVT Arrays (P/N 901530) processing array plates on GCAS. For processing array plates on the GeneTitan Instrument please refer to the *GeneTitan® Instrument User Guide for Expression Array Plates*, P/N 702933.

NOTE: Affymetrix does not recommend the use of user-prepared reagents for the 3' IVT Express Assay. Please use the GeneTitan® Hybridization, Wash and Stain Kit (P/N 901530) for all the reagents needed for the hybridization, wash and stain steps of this assay.
The GeneTitan® Hybridization, Wash, and Stain Kit was formulated with volumes for processing array plates on the GeneTitan Instrument, but contains volumes sufficient for processing array plates on GCAS.

This chapter is divided into three main sections:

- *Section 1: Hybridization Setup* on page 143
- *Section 2: Hybridization* on page 151
- *Section 3: Washing and Staining* on page 152

Reagents and Materials Master List for Hybridization, Wash and Stain: Processing HT Array Plates on GCAS

The following is a comprehensive list of the reagents and materials required for this entire chapter. Each subsection in this chapter will define the specific reagents and materials used in that section.

All reagents and materials listed have been tested and evaluated by Affymetrix. Information and part numbers listed are based on U.S. catalog information. For supplier information, please refer to the Supplier Reference List in Appendix K of this manual.

IMPORTANT: Affymetrix has determined that Array Plates do not require a pre-hybridization step. Therefore, the new GeneTitan Hybridization, Wash, and Stain Kit for 3' IVT Arrays (901530) does not contain pre-hybridization mix. For those customer who still wish to include a pre-hybridization step, we recommend that you purchase the separate 1.3X Hybridization Module (901403) and dilute it to 1X for use as a pre-hybridization mix. A protocol for preparing the pre-hybridization mix is provided in Table E.1.

- GeneTitan® Hybridization, Wash, and Stain Kit for 3' IVT Arrays (P/N 901530). Kit includes:
  - Box 1 of 2:
    - 1.3X Hybridization Solution A
    - 1.3X Hybridization Solution B
    - Stain Cocktail 1 & 3
    - Stain Cocktail 2
    - Array Holding Buffer
  - Box 2 of 2:
    - Wash Buffer A
    - Wash Buffer B
- (Optional, for pre-hybridization) 1.3X Hybridization Module (P/N 901403)
- GeneChip® HT Wash Buffer A and HT Wash Buffer B (P/N 901220)[1]
- GeneChip® HT Array Holding Buffer (P/N 901218)[1]

[1] *Available for purchase separately. For users of GeneChip HT 24-array plate products, additional **HT Wash Buffers A and B** and **HT Array Holding Buffer** need to be purchased for each run of additional plates on GCAS.*

- De-ionized water
- DNAZap: Ambion, 9890
- RNaseZap: Ambion, 9786
- HT Hybridization Tray[1] (qty. 1): Affymetrix, P/N 900747 (may be ordered separately)
- HT Wash Tray[2] (qty. 5), Affymetrix P/N 900752 (may be ordered separately)
- HT Stain Tray[2] (qty. 4): Affymetrix, P/N 900745 (may be ordered separately)
- HT Scan Tray[2] (qty. 1), Affymetrix P/N 900746 (may be ordered separately)
- 96-Well Hard-Shell PCR Plate (qty. 2): Bio-Rad, P/N HSP-9601
- Omnitray (qty. 1): VWR, P/N 4660-638 - or - Greiner Round Bottom Clear Polypropylene Plate (qty. 1): E&K Scientific Products, P/N 20261 (for Hyb Setup)
- Omnitray (qty. 2): VWR International 4660-638 (for Wash & Stain)
- Round Bottom Clear Polypropylene Plate (qty. 1): E&K Scientific Products, 20261
- Quarter Reservoir Modules (qty. 2): Beckman, 372790
- Quarter Reservoir Frame (qty. 1): Beckman, 372795
- Stacker Tips 200 µL non-sterile (qty. 3): Caliper LifeSciences, P/N 78642
- Stacker Tips 200 µL non-sterile (1 box): Caliper LifeSciences, 78641
- BD Falcon™ Test Tube, 14 mL (qty. 2): VWR International 60819-761
- BD Falcon™ Test Tube, 5 mL[2] (qty. 1): VWR International 60819-728
- Polypropylene Centrifuge Tubes with Caps, Sterile 50 mL (qty. 2): VWR International, 20171-028
- RNase-Free 1.5 mL Microfuge tube (qty. 1): Ambion, 12400
- Aluminum Foil Lids: Beckman, 538619

[1] *HT Hybridization, Wash, Stain and Scan Trays are packaged with the HT Array Plates.*

[2] *Or equivalent.*

Section 1: Hybridization Setup

This section describes the GeneChip Array Station Hybridization Setup protocol for HT Array Plates using GeneChip HT Hybridization, Wash, and Stain Kit reagents.

Additionally, this section provides details on how to set up reagents on the Array Station Deck, and how to use the GeneChip Array Station Software to hybridize a HT Array Plate.

Reagents and Materials for Hybridization Setup

The following reagents and materials required in the Hybridization Setup protocol have been tested and evaluated by Affymetrix. Information and part numbers listed below are based on U.S. catalog information. For supplier information, please refer to the Supplier Reference List in Appendix K of this manual.

- Pre-hybridization Mix (Optional) Order 1.3X Hybridization Module (P/N 901403) and prepare as directed below:

Table E.1 Pre-hybridization Mix (Optional)

Reagent	Volume
HT 1.3X Hybridization Solution A	32.3 µL
HT 1.3X Hybridization Solution B	60 µL
Nuclease-free Water	27.7 µL
Total Volume (µL)	120 µL

- HT Hybridization Tray[1] (qty. 1): Affymetrix, P/N 900747 (may be ordered separately)
- HT Stain Tray[1] (qty. 1): Affymetrix, P/N 900745 (may be ordered separately)
- 96-Well Hard-Shell PCR Plate (qty. 1): Bio-Rad, P/N HSP-9601
- Omnitray (qty. 1): VWR, P/N 4660-638 - or - Greiner Round Bottom Clear Polypropylene Plate (qty. 1): E&K Scientific Products, P/N 20261
- Stacker Tips 200 µL non-sterile (qty. 3): Caliper LifeSciences, P/N 78642
- DNAZap: Ambion, 9890
- RNaseZap: Ambion, 9786
- De-ionized water

Clean the Bio-Rad Plate Lids with DNAZap™ and RNaseZap®

The disposable pad under the lids should be cleaned before the run.

1. Rinse the pad with DI Water.
2. Wipe the pad with RNaseZap.
3. Rinse the pad with DI Water.
4. Wipe the pad with DNAZap.
5. Thoroughly rinse the pad with DI Water.
6. Dry the pad with pressurized clean air or nitrogen.

[1] *Hybridization and Stain Trays are packaged with the HT Array Plates.*

Beginning a Run – Deck Layout for Hybridization Setup

This section describes how to assemble the deck and use the GeneChip Array Station Software to begin a Hybridization Setup run. The Hybridization Setup protocol will perform the following methods:

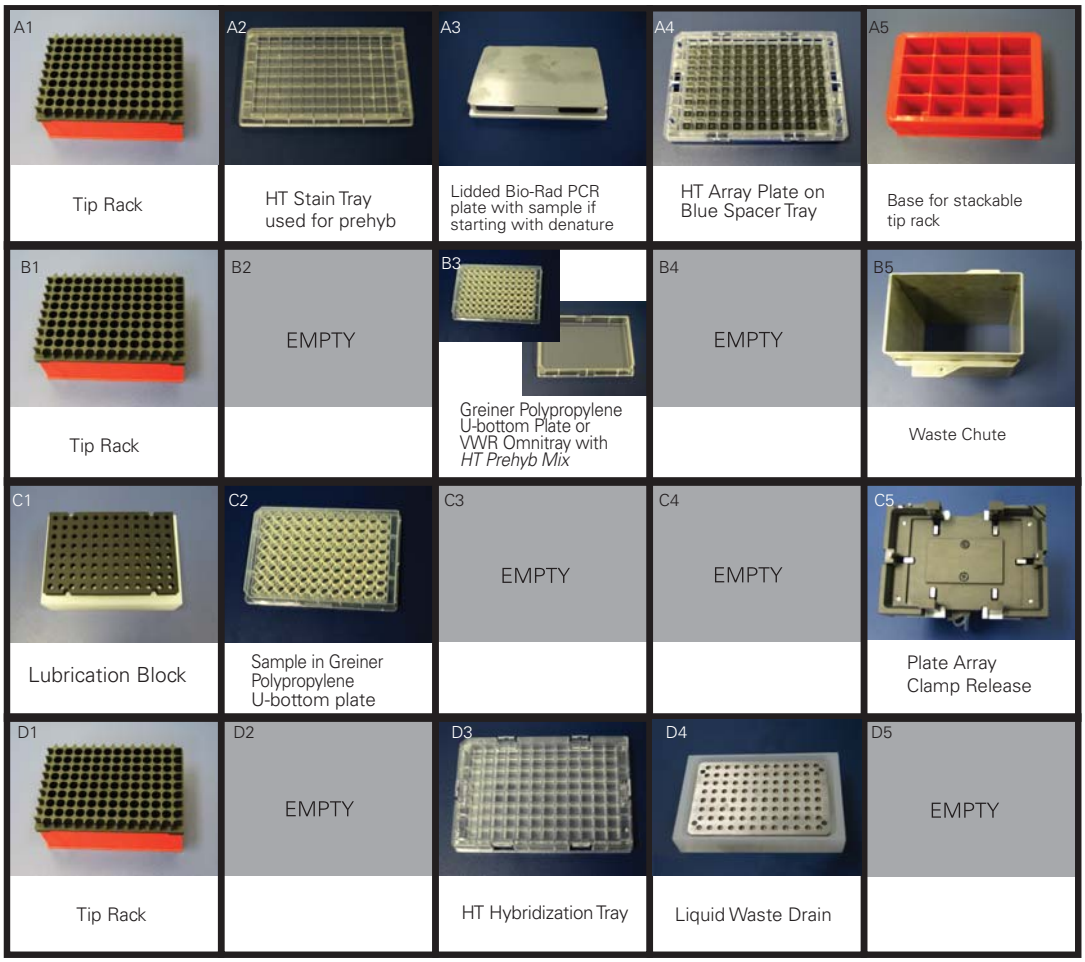
- Denature the sample for hybridization
- Assemble the HT Array Plate and the Hybridization Tray containing the hybridization-ready sample

Procedure - Assembling the Deck

1. Setup the deck with the appropriate consumables. Refer to Figure E.1.
2. Load at least four tip boxes into Rack 1.
3. Remove the cold reagent block.
4. Place the hybridization-ready samples on the deck at location C2.

NOTE: If using a HT 24-Array Plate, it is NOT necessary to re-array the samples into columns 5, 7 and 9. Samples should be in columns 1, 2, and 3 of the Greiner plate. The Array Station will transfer samples to columns 5, 7, and 9 during the protocol.

Figure E.1 The deck layout for the Hybridization Setup protocol.
Load four tip boxes into Rack 1. Empty Rack 2, leaving the tip rack base there.



NOTE: If you are using recovered samples and your samples are already in the Bio-Rad 96-Well plate, you will need to leave position C2 empty. Your samples will be in the Bio-Rad plate placed at A3.

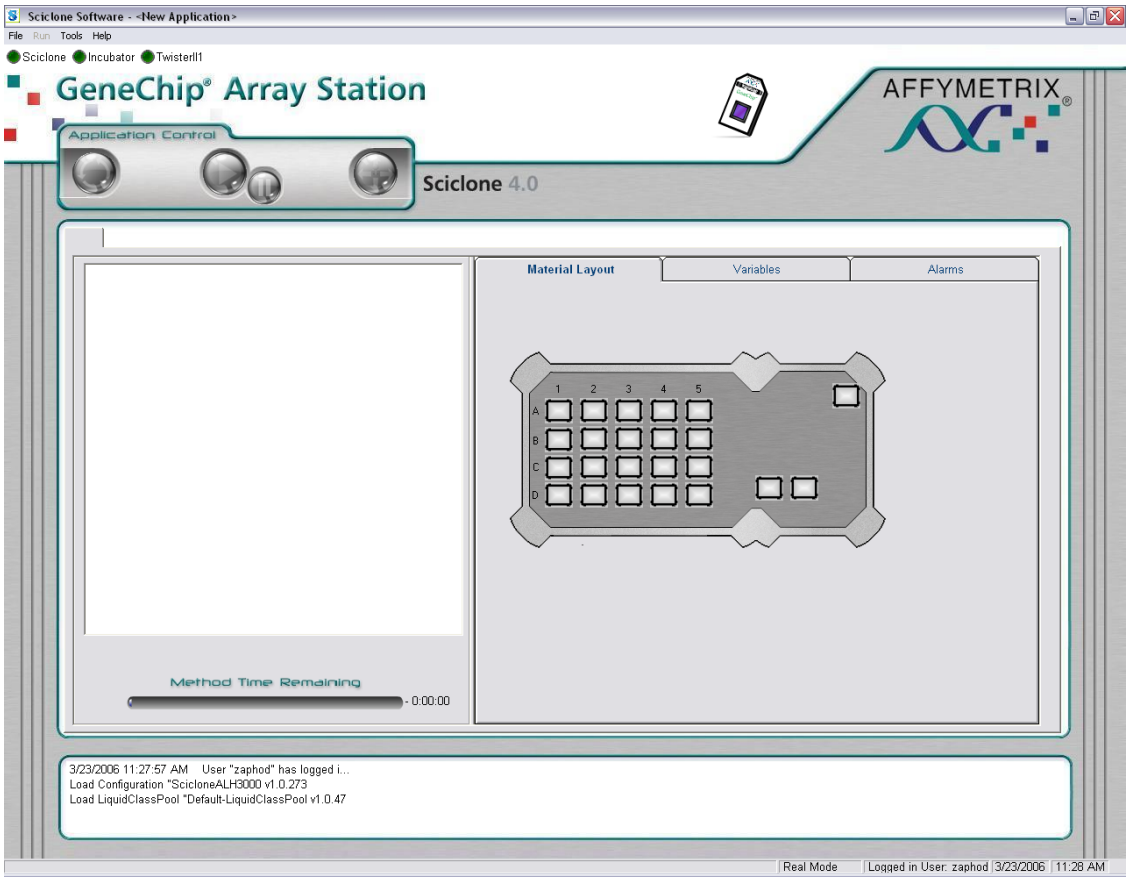
Running the Hybridization Setup Protocol

1. Check to confirm that the deck and racks are correctly populated with well plates, tips, etc.
2. Open the software by double clicking the GeneChip Array Station desktop icon, or click **Start** → **Programs** → **Caliper Life Sciences** → **Instruments** → **Sciclone** → **Sciclone Workstation Software**. The **login** window appears (Figure E.2).

Figure E.2 Sciclone Workstation Software Login window

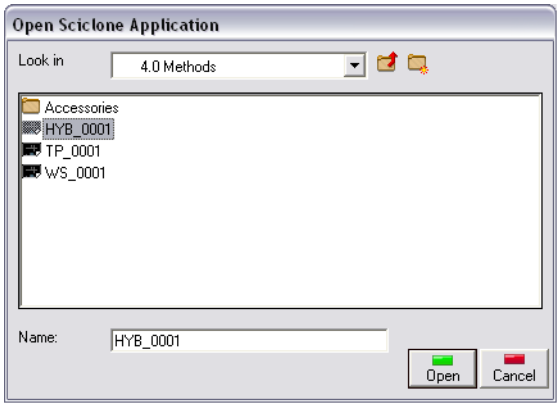
3. Enter your **User Name** and **Password** and click **OK**. The Operator Runtime interface window appears (Figure E.3).

Figure E.3 Sciclone Workstation Software Operator window



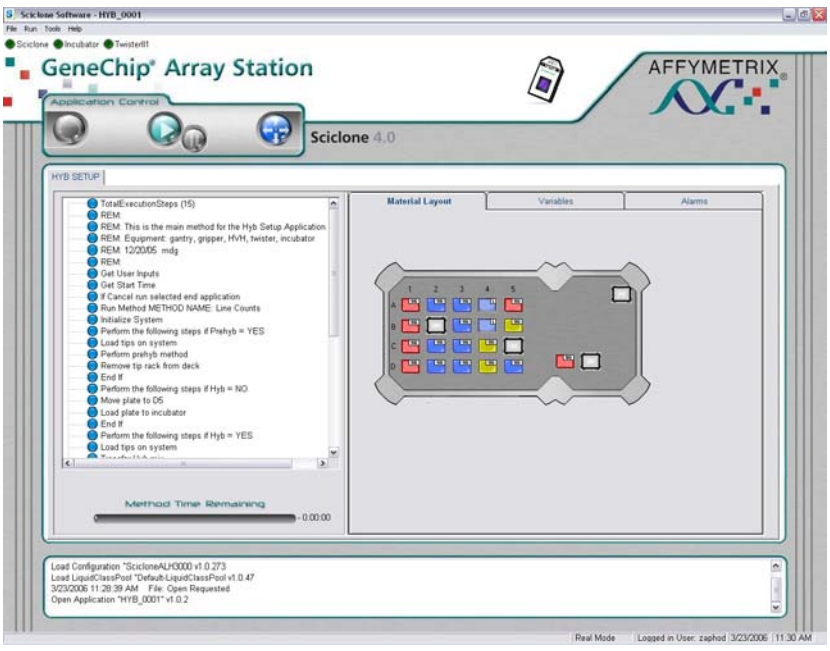
4. Select **File** → **Open** to access the load application window (Figure E.4).

Figure E.4 Open Sciclone Application window



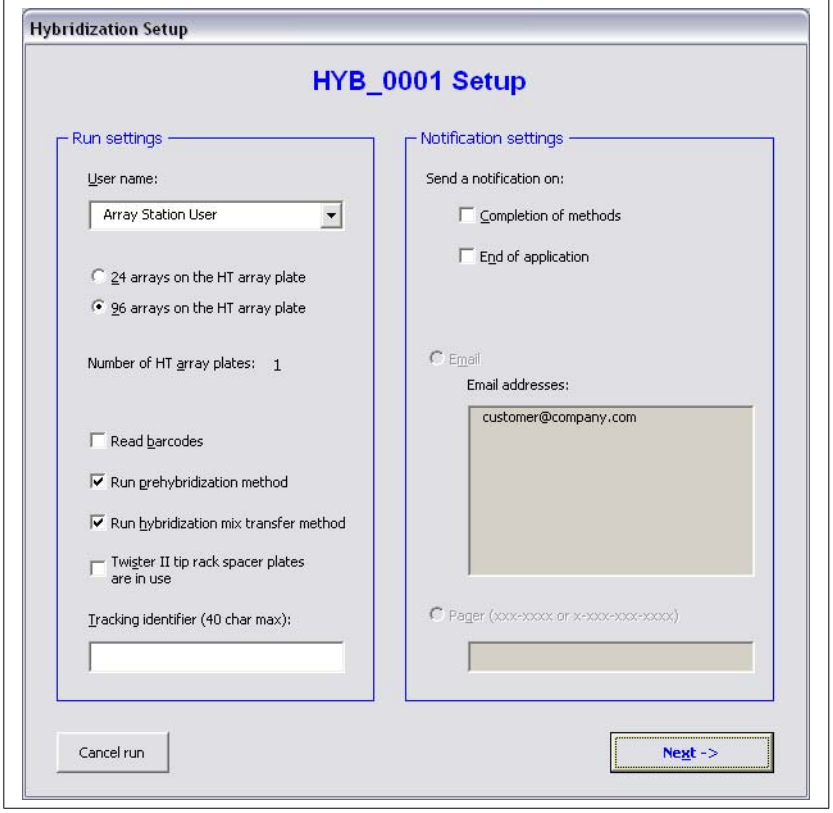
5. Select **HYB_0001** and click **Open**.
   The hybridization setup application is loaded into Sciclone Workstation Software.

Figure E.5 Hybridization Setup Protocol Loaded



6. Click the green **Start** arrow to begin the application.
   The application will prompt you to select several options through the interactive window shown in Figure E.6.

Figure E.6 Hybridization Setup window



7. Select the protocol parameters.
   - **A.** Select your **User name** from the drop-down menu.
   - **B.** Select either 24 or 96 well HT array plate. It is important that you select the proper plate type to ensure samples are transferred to the correct wells.
   - **C.** If you wish the protocol to read and store the sample plate barcode and the HT array plate barcode, check the **Read barcodes** option. Barcodes will be reported in the user summary at the end of the run.
   - **D.** Select **Run prehybridization method**.
   - **E.** Selection of the **Run hybridization mix transfer method** depends on if your samples are in the Greiner plate or are recovered samples in the Bio-Rad plate.
     1) If **Run hybridization mix transfer method** is selected, the Array Station will transfer the hyb-ready sample from a Greiner U-bottom Plate at C2 to a Bio-Rad Plate at A3. If you are running a HT 24-Array Plate, your samples should be in columns 1, 2, and 3 of the Greiner plate. The Array Station will automatically transfer these samples to columns 5,7, and 9 of the hybridization tray to match the HT 24-Array Plate.
     2) If **Run hybridization mix transfer method** is NOT selected, it is required that 100 µL of Hyb-Ready sample is placed in a Bio-Rad Plate at position A3. If you are running a HT 24-Array Plate, your samples should be in columns 1, 2, and 3 of the Bio-Rad plate. The Array Station will automatically transfer these samples to columns 5, 7, and 9 of the hybridization tray to match the HT 24-Array Plate.

NOTE: In most cases you will select and run the hybridization mix transfer method. However, if you are re-hybridizing samples recovered from a previous hybridization, your samples will already be in a Bio-Rad Plate. You should select NOT to run the hybridization mix transfer if your samples are in the Bio-Rad Plate.

F. If you are using spacer plates for tip static control (recommended), check the box **Twister tip rack spacer plates are in use**. If this box is checked, ensure you have placed spacer plates between each nested tip rack and on the top rack.

G. Enter a **Tracking identifier** (limited to 40 characters) if desired. The **Tracking identifier** will be recorded in the Hybridization Setup Summary Report at the end of the run (refer to Figure E.10 on page 150 for a description of the Summary Report).

H. Select the desired notification settings. Be sure the email address or pager information is properly configured.

8. After selecting the settings parameters, click **Next**.

You will see one of the following windows, depending on whether you have selected **Run hybridization mix transfer method**.

Figure E.7 Hybridization Deck Layout when **Run hybridization mix transfer method** is selected

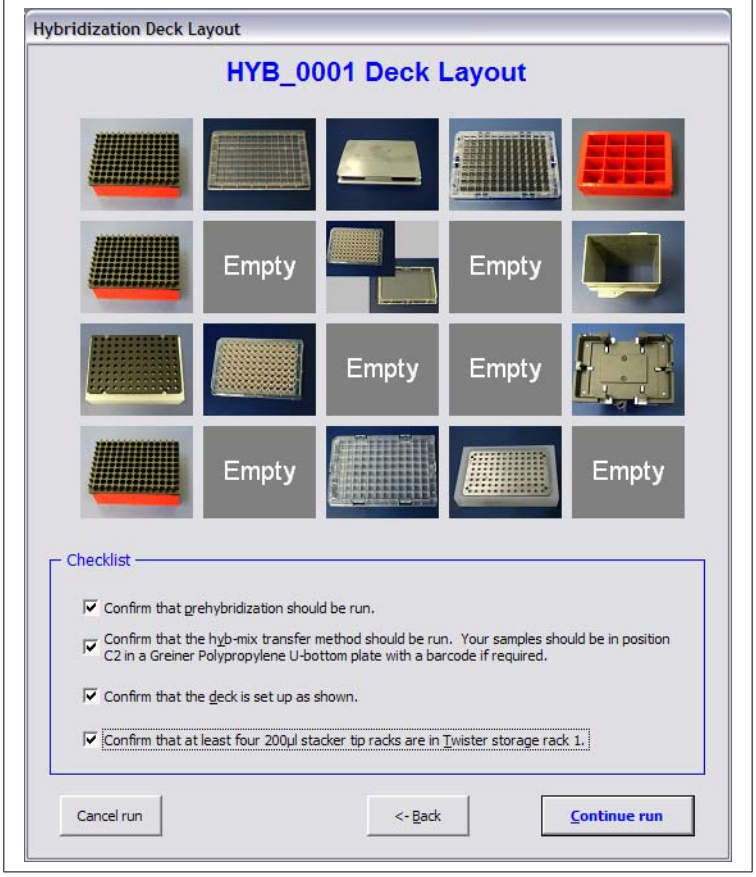
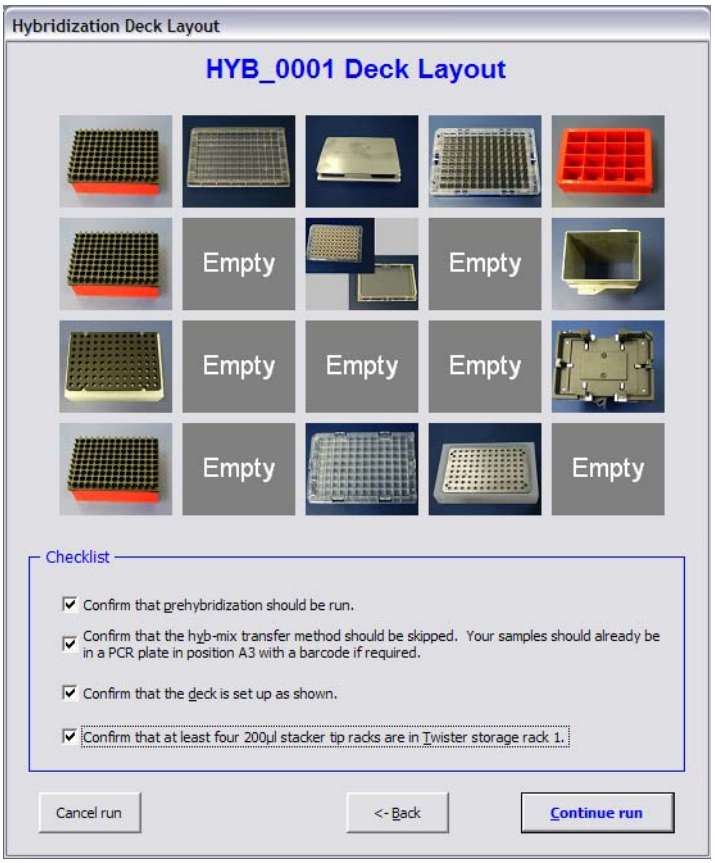
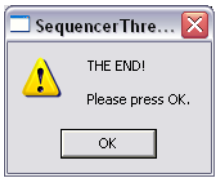


Figure E.8 Hybridization Deck Layout when **Run hybridization mix transfer method** is **NOT** selected



9. Verify that you have configured the deck properly and that you have completed and checked the items on the checklist. Click **Continue** and the Hybridization Setup protocol continues until the HT Array Plate/Hybridization Tray Sandwich is assembled.
10. At the end of the protocol, The End message appears (Figure E.9). Click **OK**.

Figure E.9 Run Complete message



You must place the HT Array Plate hybridization sandwich in an incubator equilibrated to 48°C. Please refer to page 151.

11. At the conclusion of each run, two summary reports are automatically saved by the program.

A. A user readable summary of the run is saved to the folder C:\Affymetrix\Reports\Summary. This file has the following naming convention:
SummaryReport_HYB_0001_3_23_2006_10_03_36_AM.rtf

HYB_0001 = method used
3_23_2006 = date stamp of when the HYB_0001 starts
10_03_36_AM = time stamp of when the HYB_0001 starts Figure E.10 shows an example of a Hybridization Setup Summary Report.

Figure E.10 Hybridization Setup Summary Report Example

Hybridization Setup Summary Report
HYB_0001

User: Array Station User
Date: Tuesday, April 11, 2006

Start time: 2:50:02 PM
End time: 3:12:54 PM
Run duration: 0:22:52

Tracking identifier:

Tip spacers used: NO
Number of arrays: 96

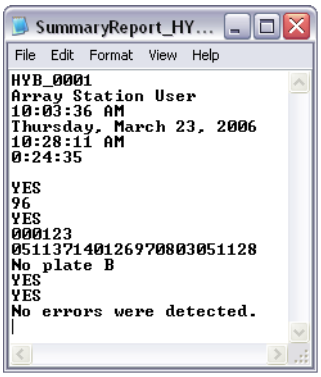
Barcode reader enabled: NO
Hyb sample barcode: 5456421231
HT array plate barcode: 654564521

Prehybridization option: YES
Hybridization mix transfer: YES

Error Report
No errors were detected.

12. A tab-delimited text report useful for transferring data to many LIMS systems is also saved. This report is saved in the folder C:\Affymetrix\Reports\Data. An example if this report is shown in Figure E.11.

Figure E.11 LIMS Summary Report Example



13. The HT Array Plate/Hybridization Tray Sandwich is ready to be placed into a 48°C incubator for 16 hours.

CAUTION: Take care in transferring the Hyb Sandwich to the hybridization oven. The sandwich should remain close to level to prevent sample contamination.

Section 2: Hybridization

This section describes how to incubate a HT Array Plate/Hybridization Tray Sandwich for hybridization.

Overnight Hybridization

1. Remove the HT Array Plate/Hybridization Tray Sandwich from the Array Station, taking care to keep the sandwich level, and place in the hybridization oven.
2. Incubate the HT Array Plate/Hybridization Tray Sandwich for 16 hours at 48°C.

IMPORTANT: **The hybridization oven should be monitored with a digital thermometer (such as VWR, P/N 23226-656) to ensure that the temperature is stabilized at 48°C.**

IMPORTANT: **The HT Array Plate/Hybridization Tray Sandwich must remain level during hybridization. A bubble level can be placed on top of the sandwich to ensure that all four corners are level.**

Section 3: Washing and Staining

Introduction

This section describes the GeneChip Array Station Wash and Stain protocol for HT Array Plates. A schematic of the automated Wash and Stain is provided to outline the steps of the procedure (Figure E.19 to Figure E.25). Also provided in this section are details on how to set up the Array Station deck and use the Sciclone 4.x software to perform the Wash and Stain protocol.

For convenience, prepackaged and ready-to-use components in the GeneChip HT Hybridization, Wash, and Stain Kit replace the previous reagent preparation protocols. HT Wash Buffer A is used for Low Stringency Wash, and HT Wash Buffer B is used for High Stringency Wash. These reagents have the same formulations as the previous low and high stringency wash buffers except for the addition of preservative. Pre-formulated HT Stain Cocktails 1 & 3 and HT Stain Cocktail 2 replace the previous stain cocktails of the same name.

The volumes contained in the HT Stain Cocktail bottles are sufficient to process 96 HT arrays and must be aliquoted appropriately when running either 24 or 96 HT array plates. HT Wash Buffer A and B are sufficient to process any single plate of either 24 or 96 HT arrays. For users processing additional 24 HT array plates, one additional HT Wash Buffer A and B (P/N 901220) and HT Array Holding Buffer (P/N 901218) must be purchased separately from Affymetrix for each additional plate to be processed.

Reagents and Materials for Wash and Stain

The following reagents and materials required in the Wash and Stain protocol have been tested and evaluated by Affymetrix. Information and part numbers listed below are based on U.S. catalog information. For supplier information, please refer to the Supplier Reference List in Appendix K of this manual.

- From the GeneChip HT Hybridization, Wash, and Stain Kit for use with 3' IVT Express on GCAS (P/N 901405):
  - HT Wash Buffer A – one 2L bottle
  - HT Wash Buffer B – one 150 mL bottle
  - HT Stain Cocktail 1 & 3 – one 22 mL bottle
  - HT Stain Cocktail 2 – one 13 mL bottle
  - HT Array Holding Buffer – one 20 mL bottle
- Quarter reservoir modules (qty. 2), Beckman Coulter P/N 372790
- Quarter reservoir frame, Beckman Coulter P/N 372795
- HT Wash Tray[1] (qty. 5), Affymetrix P/N 900752
- HT Stain Tray[1] (qty. 3), Affymetrix P/N 900745
- HT Scan Tray[1] (qty. 1), Affymetrix P/N 900746
- 96-Well Hard-Shell PCR Plate (qty 1): Bio-Rad, HSP-9601
- Omnitray (qty. 2): VWR International 4660-638
- Round Bottom Clear Polypropylene Plate (qty. 1): E&K Scientific Products, 20261
- Quarter Reservoir Modules (qty. 2): Beckman, 372790
- Quarter Reservoir Frame (qty. 1): Beckman, 372795
- BD Falcon™ Test Tube, 14 mL (qty. 2): VWR International 60819-761
- BD Falcon™ Test Tube, 5 mL[2] (qty. 1): VWR International 60819-728
- Polypropylene Centrifuge Tubes with Caps, Sterile 50 mL (qty. 2): VWR International, 20171-028
- RNase-Free 1.5 mL Microfuge tube (qty. 1): Ambion, 12400
- Stacker Tips 200 µL non-sterile (1 box): Caliper LifeSciences, 78641
- Aluminum Foil Lids: Beckman, 538619
- DNAZap: Ambion, 9890
- RNaseZap: Ambion, 9786
- De-ionized water

[1] *HT Stain, Wash, and Scan Trays are packaged with the HT Array Plates.*

[2] *Or equivalent.*

Wash and Stain Preparation

Prior to running the Wash and Stain protocol, deck preparation is required. This involves manually filling the appropriate consumable. The reagent setup must be performed prior to starting the Wash and Stain protocol.

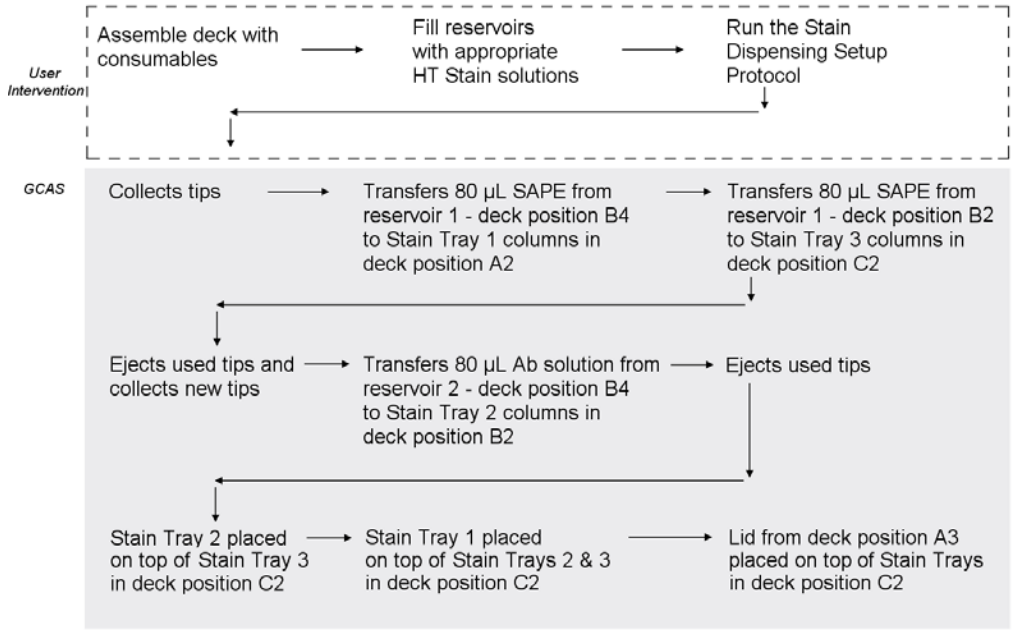
New with this version of the manual is an automated Stain Dispensing Setup Protocol. Alternatively, you may perform this function manually.

- If you plan to use the automated Stain Dispensing Setup Protocol, please go to *Stain Dispensing Setup Protocol* on page 154.
- If you plan to perform the stain dispensing manually, please go to *Manual Stain Dispensing Setup* on page 159.

Stain Dispensing Setup Protocol

This version of the manual features a new Stain Dispensing Setup Protocol provided to facilitate efficiency of time, accuracy, and ease of use. This new step is an alternative option to manual dispensing and automates the aliquoting of stain reagents prior to the Wash and Stain protocol (WS_0001).

Figure E.12 Stain Dispensing Setup Schematic

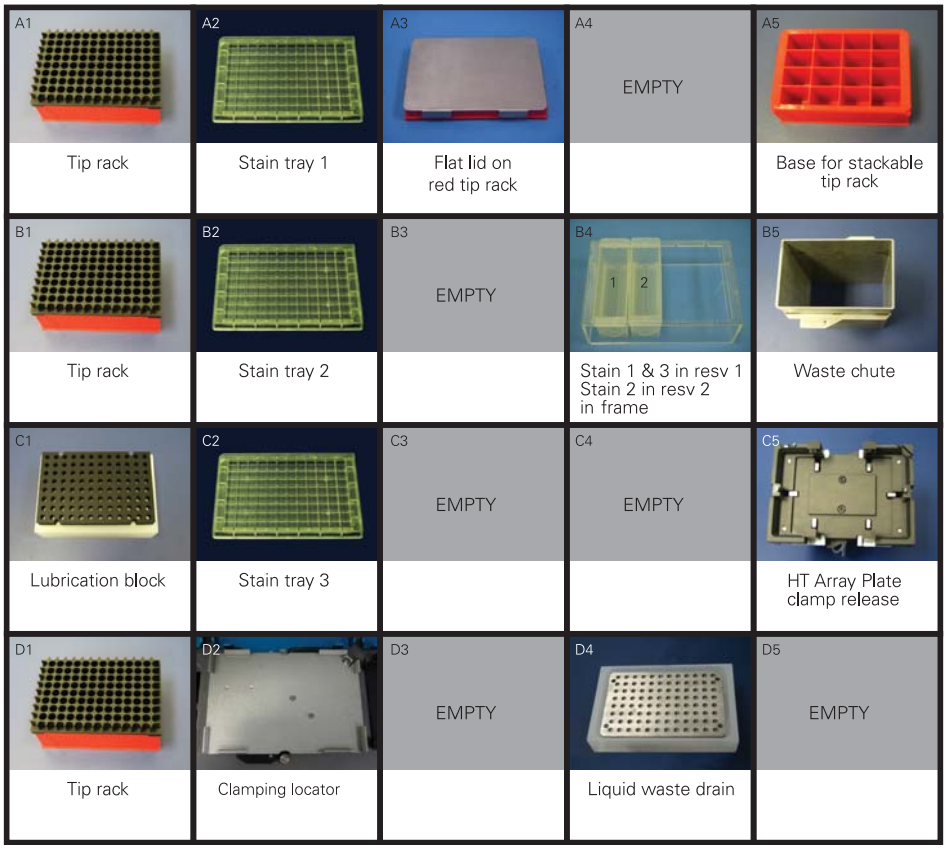


Procedure - Assembling the Deck for the Stain Dispensing Setup Protocol

1. Set up deck with appropriate consumables. Refer to Figure E.13.

NOTE: The red tip rack at position A3 is covered with the flat lid.

Figure E.13 The deck layout for Stain Dispensing Setup



2. Fill the quarter reservoirs at position B4 as follows:
   - **A.** If processing a 24-array plate, aliquot 5 mL HT Stain Cocktail 1 & 3 (SAPE) into the first quarter reagent reservoir at B4. Aliquot 3 mL for HT Stain Cocktail 2 (Ab) into the second quarter reservoir at B4.
   - **B.** If processing a 96-array plate, pour the entire contents of HT Stain Cocktail 1 & 3 bottles (SAPE) into the first quarter reagent reservoir at B4. Pour the entire contents of the HT Stain Cocktail 2 bottle (Ab) into the second quarter reservoir at B4.
3. Load at least one tip box in Rack 1.
4. Place the buffer lines in correct liquid carboys.
   - The bulk dispenser buffer line should be placed in the carboy containing HT Wash Buffer A.
   - The Z8 buffer line should be placed in DI water.

Clean the Bio-Rad Plate Lids with DNAZap™ and RNaseZap®

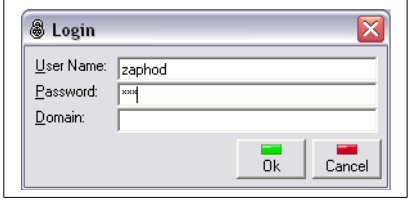
The disposable pad under the lids should be cleaned before the run.

1. Rinse the pad with DI Water.
2. Wipe the pad with RNaseZap.
3. Rinse the pad with DI Water.
4. Wipe the pad with DNAZap.
5. Thoroughly rinse the pad with DI Water.
6. Dry the pad with pressurized clean air or nitrogen.

Running the Stain Dispensing Setup Protocol

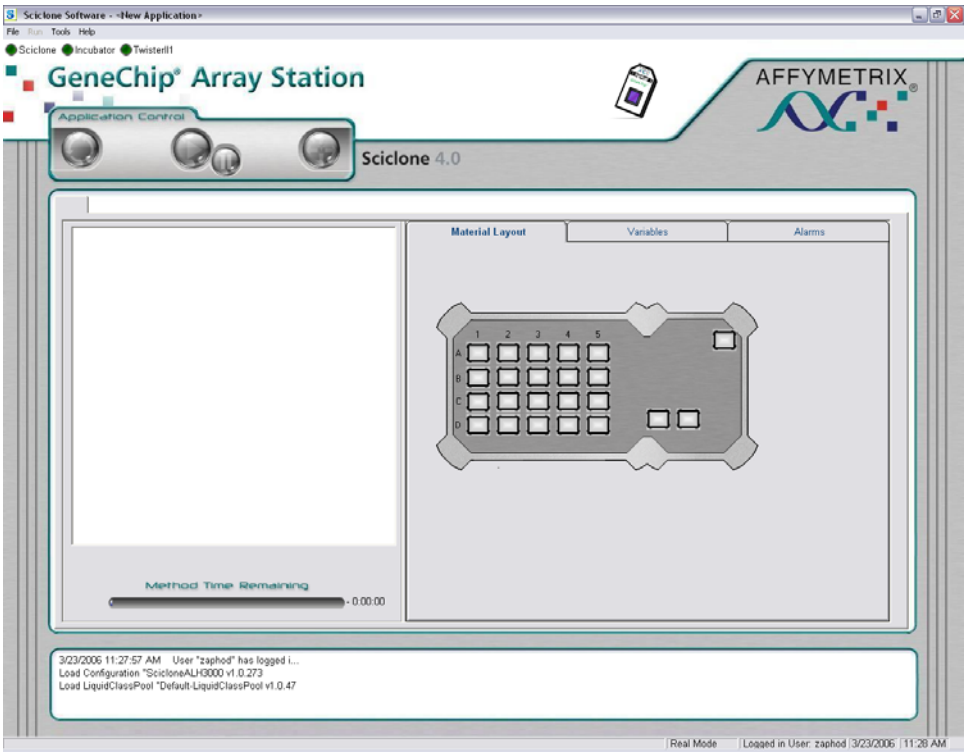
1. Check to confirm that the deck and racks are correctly populated with well plates, tips, etc.
2. Open the software by double-clicking the GeneChip Array Station desktop icon, or click **Start** → **Programs** → **Caliper Life Sciences** → **Instruments** → **Sciclone** → **Sciclone Workstation Software**. The login window appears **(**Figure E.14).

Figure E.14 Sciclone Workstation Software Login window



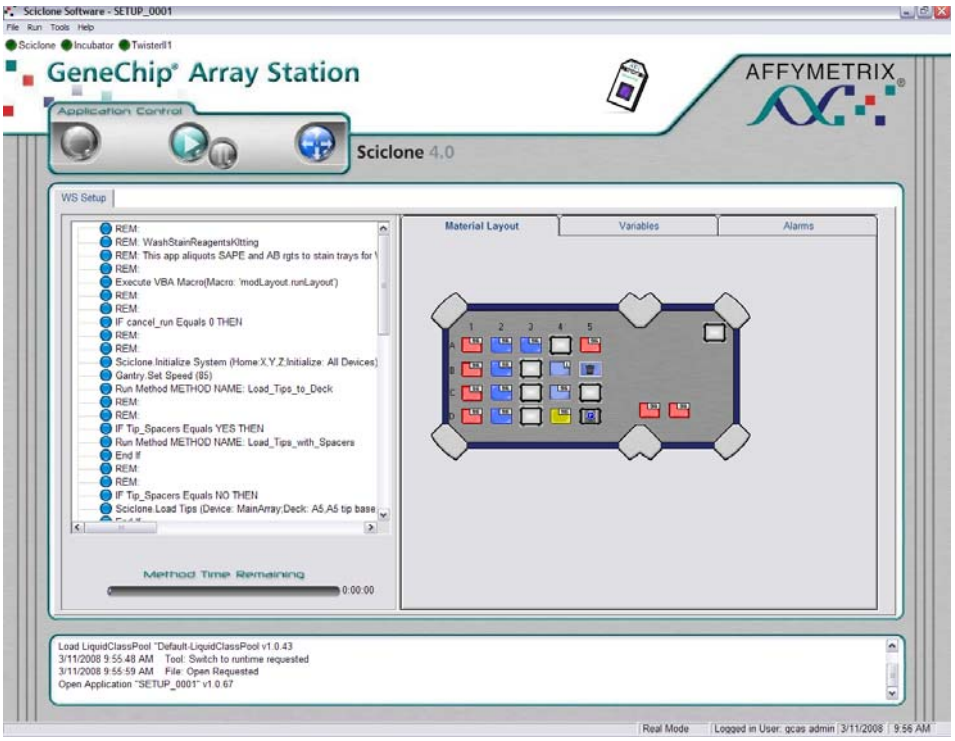
3. Enter your **User name** and **Password** and click **OK**.
   The Operator Runtime interface appears **(**Figure E.15).

Figure E.15 Sciclone Workstation Software Operator window



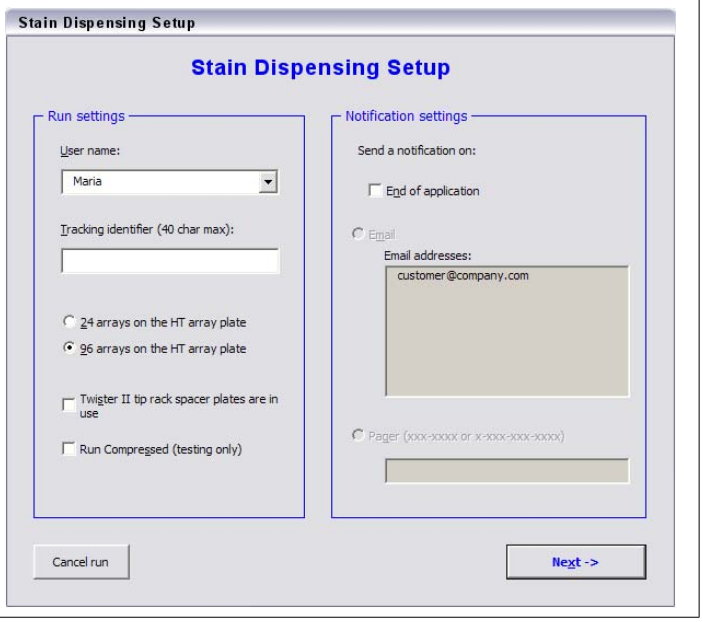
4. Select **File** → **Open** to access the load application window.
5. Select **Setup_0001** and click **Open**.
   The Stain Dispensing Setup application is loaded into Sciclone Workstation software (Figure E.16).

Figure E.16 Wash and Stain Protocol Loaded



6. Click the green **Start** arrow to begin the application.
   The Application will prompt you to select several options through the interactive window shown in Figure E.17.

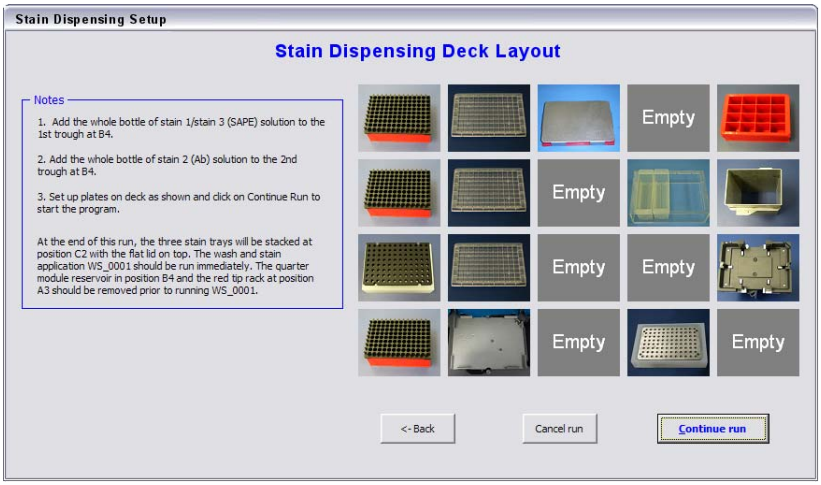
Figure E.17 Stain Dispensing Setup window



7. Select the protocol parameters: Select either a HT 24- or 96-Array Plate. It is important that you select the proper plate type to ensure samples are transferred to the correct wells.

8. Click **Next**.
   The Stain Dispensing Setup deck layout window appears (Figure E.18).

Figure E.18 Stain Dispensing Setup Deck Layout



9. Verify that you have set the deck up properly and that you have completed all items in the **Notes** section.
10. Click **Continue run** and the Array Station will perform the Stain Dispensing Setup protocol.
11. When completed, remove the red tip rack at A3 and the reagent reservoirs at B4 before continuing on to the WS_0001 application (see *Beginning a Run – Wash and Stain Deck Layout* on page 162).

Manual Stain Dispensing Setup

HT Stain Cocktail 1 & 3

1. Obtain two HT Stain trays and label one “Stain 1” and the other “Stain 3.”
2. Aliquot 80 µL of HT Stain Cocktail 1 & 3 into the appropriate wells of both labeled HT Stain Trays.

NOTE: If using a HT 24-Array Plate, the stain mixture should be aliquoted into columns 5, 7, and 9 of the HT Stain Trays.

HT Stain Cocktail 2

1. Obtain one HT Stain tray and label it “Stain 2.”
2. Aliquot 80 µL of HT Stain Cocktail 2 into the appropriate wells of the labeled HT Stain Tray.

NOTE: If using a HT 24-Array Plate, the stain mixture should be aliquoted into columns 5, 7, and 9 of the HT Stain Trays.

Clean the Bio-Rad Plate Lids with DNAZap™ and RNaseZap®

The disposable pad under the lids should be cleaned before the run.

1. Rinse the pad with DI Water.
2. Wipe the pad with RNaseZap.
3. Rinse the pad with DI Water.
4. Wipe the pad with DNAZap.
5. Thoroughly rinse the pad with DI Water.
6. Dry the pad with pressurized clean air or nitrogen.

Automated Wash and Stain Protocol

Automated Wash and Stain Schematic

Figure E.19 through Figure E.25 summarize the steps in the Wash and Stain protocol.

Figure E.19 Step 1: Fill HT Wash Trays with HT Wash Buffer A

Figure E.20 Step 2: Low Stringency Wash (LSW) using HT Wash Buffer A

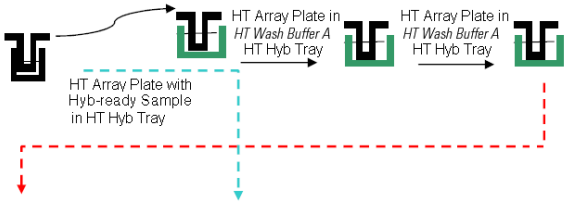


Figure E.21 Step 3: High Stringency Wash (HSW) using HT Wash Buffer B; Transfer of Hybridization Cocktail; Drain/Refill HT Wash Trays

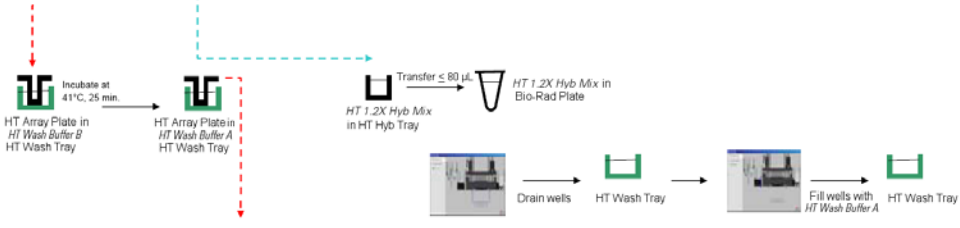


Figure E.22 Step 4: First Stain (using HT Stain Cocktail 1 & 3); Low Stringency Wash; Drain/Refill HT Wash Trays

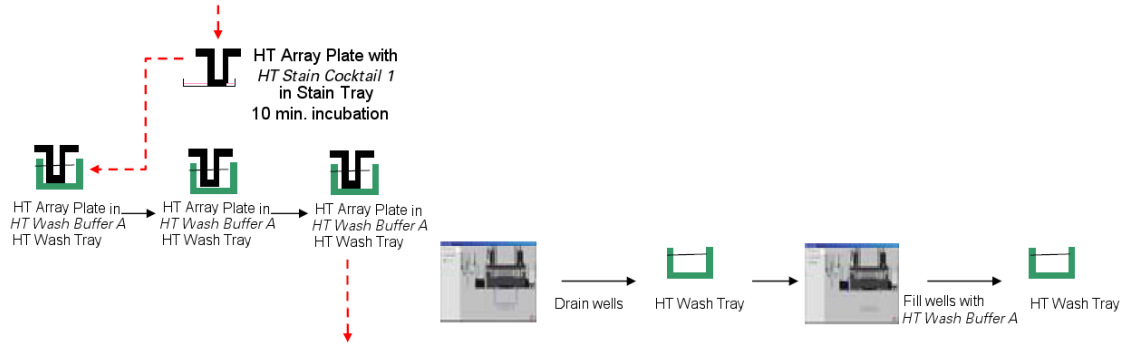


Figure E.23 Step 5: Second Stain (using HT Stain Cocktail 2); Low Stringency Wash; Drain/Refill HT Wash Tray

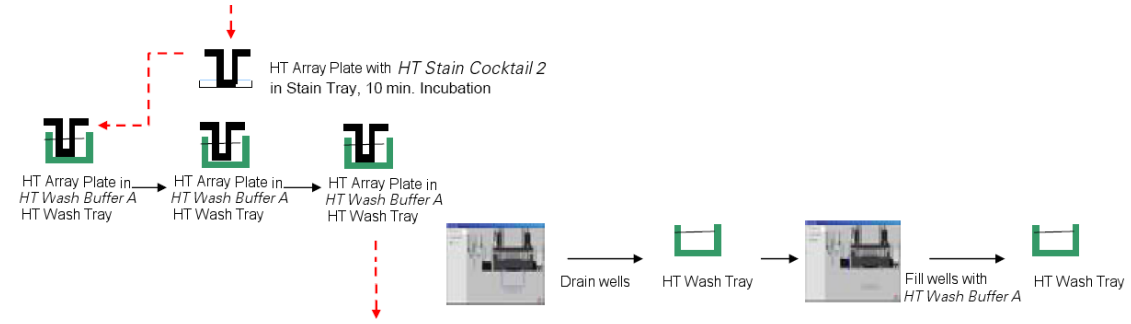


Figure E.24 Step 6: Third Stain (using HT Stain Cocktail 1 & 3); Low Stringency Wash

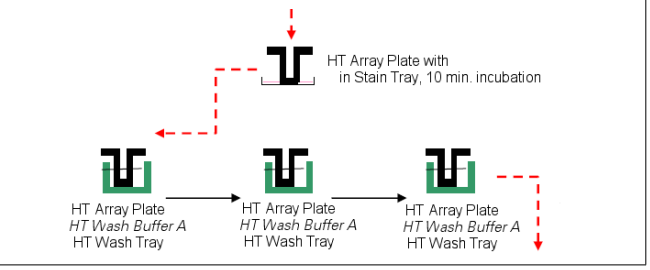
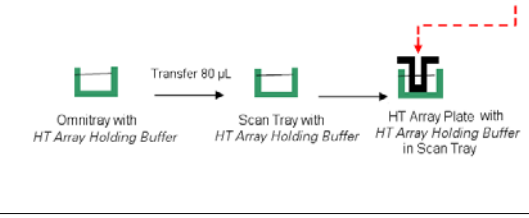


Figure E.25 Step 7: Filling HT Scan Tray with HT Array Holding Buffer and Insertion of the HT Array Plate



Beginning a Run – Wash and Stain Deck Layout

This section describes how to assemble the deck and use the Sciclone Workstation Software to begin a Wash and Stain run. The Wash and Stain protocol will perform the following methods on the GeneChip Array Station:

- Wash the hybridized HT Array Plate in HT Wash Buffer A and B
- Stain the hybridized HT Array Plate
- Assemble the hybridized HT Array Plate for scanning

Wash and Stain Deck Layout Procedure

1. Set up deck with appropriate consumables. Refer to Figure E.26.

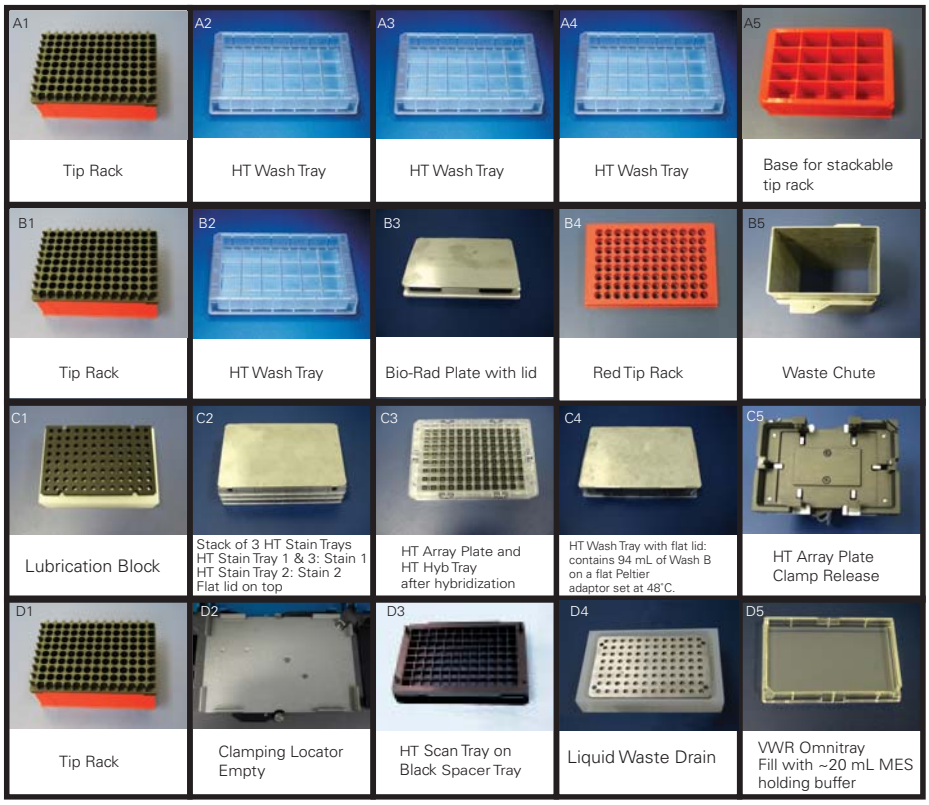
   NOTE: The HT Stain Trays containing Stain 1, Stain 2, and Stain 3 are stacked and covered with a flat lid. The three trays should be stacked in ascending order, with Stain 1 on top, and Stain 3 on bottom. Stain 1 is covered with the flat lid.

2. Load at least three tip boxes in Rack 1.
3. Assemble the Peltier adaptor and Hybridization Block Fixture. Refer to Figure E.27 and Figure E.27.
4. Set the temperature of the Watlow Temperature Controller to 48°C. Refer to Figure E.28.

   TIP: HT Wash Buffer B must be between 41°C and 42°C for the Wash and Stain protocol. During the Wash and Stain deck setup, HT Wash Buffer B is placed in a HT Wash Tray and placed on the Peltier adaptor to reach a temperature between 41°C and 42°C. The temperature adjustment between 41°C and 42°C takes a long time. To decrease this time requirement, place 100 mL of HT Wash Buffer B in a 48°C incubator the night before so it is prewarmed.

5. Place the buffer lines in correct liquid carboys.
   - The bulk dispenser buffer line should be placed in the carboy containing HT Wash Buffer A bottle.
   - The Z8 buffer line should be placed in DI water.

Figure E.26 The deck layout for the wash and stain protocol—load at least three tip racks in rack 1.



NOTE: The red tip rack used in B4 should be used only once to prevent the High Stringency Wash lid from adhering to it.

Reagent Setup for Wash and Stain Protocol

The Wash and Stain protocol requires reagent setup. This involves loading consumables on to the GCAS deck and filling the appropriate consumables with reagents. The reagent setup must be performed prior to starting the Wash and Stain protocol.

1. HT Wash Buffer A: Transfer Bulk Dispenser Buffer Line to container containing 2 Liters of HT Wash Buffer A.
2. HT Wash Buffer B: Dispense 94 mL of HT Wash Buffer B into one HT Wash Tray.
3. HT Array Holding Buffer: Pour 20 mL of HT Array Holding Buffer into a VWR Omnitray.

Figure E.27 Installing (left) and Securing (right) the hybridization block fixture

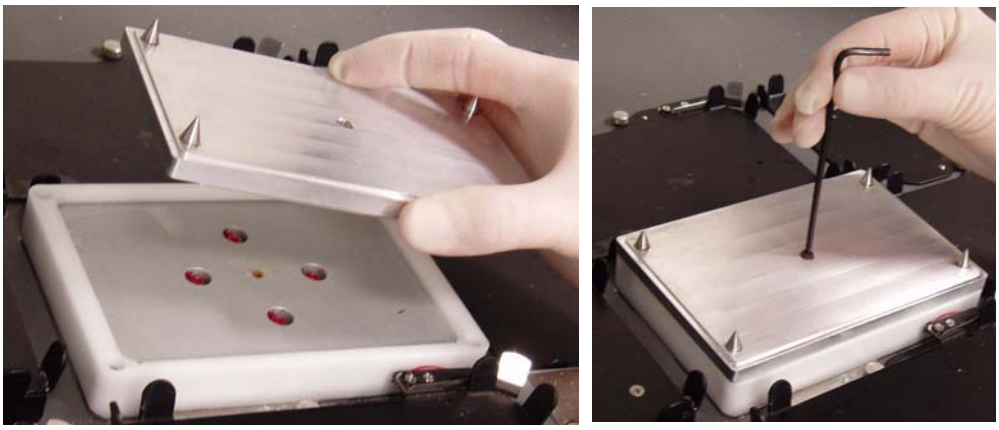


Figure E.28 Setting the 48°C temperature on the Watlow Temperature Controller

Running the Wash and Stain Protocol

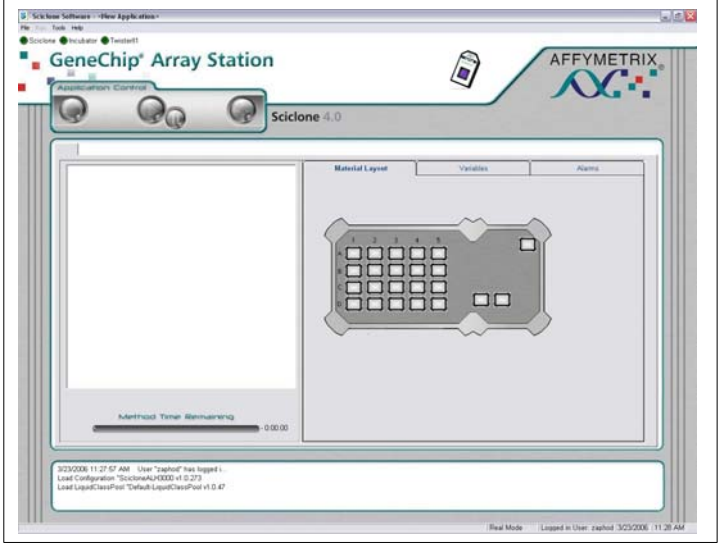
Running the Protocol

1. Check to confirm that the deck and racks are correctly populated with well plates, tips, etc.
2. Remove the HT Array Plate/Hybridization Tray Sandwich from the incubator and place on the deck at location C3.
3. Open the software by double-clicking the GeneChip Array Station desktop icon, or click **Start** → **Programs** → **Caliper Life Sciences** → **Instruments** → **Sciclone** → **Sciclone Workstation Software**. The login window appears **(**Figure E.29).

Figure E.29 Sciclone Workstation Software Login window

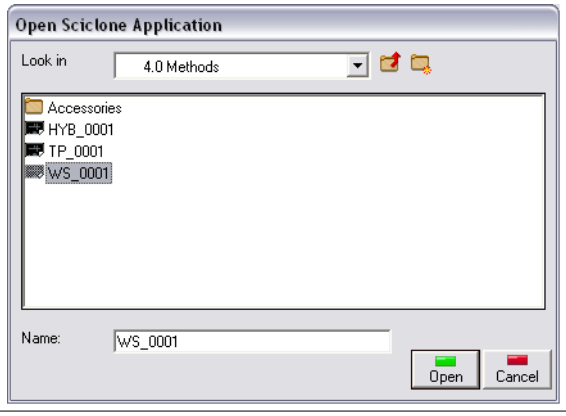
4. Enter your **User name** and **Password** and click **OK**. The Operator Runtime interface appears **(**Figure E.30).

Figure E.30 Sciclone Workstation Software Operator window



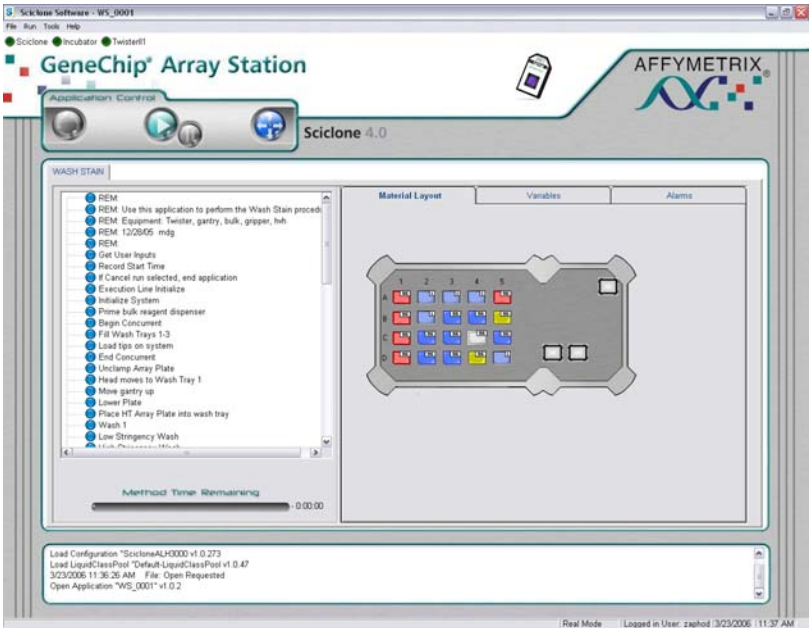
5. Select **File** → **Open** to access the load application window (Figure E.31).

Figure E.31 Open Sciclone Application window



6. Select **WS_0001** and click **Open**.
   The Affymetrix Wash Stain Protocol is loaded into Sciclone Workstation software (Figure E.32).

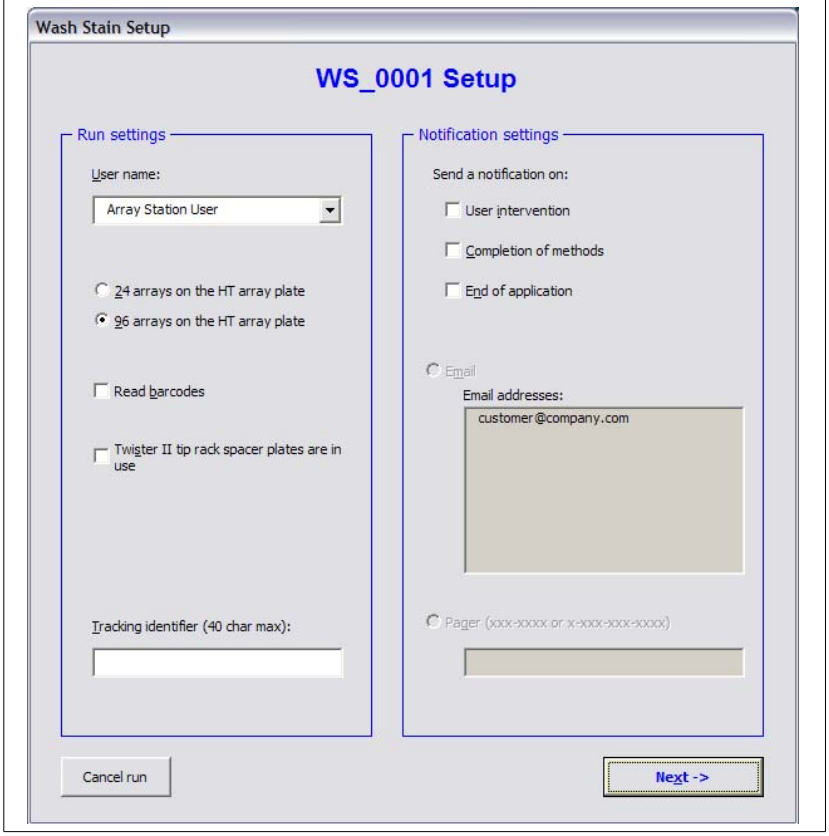
Figure E.32 Wash and Stain Protocol Loaded



7. Click the green **Start** arrow to begin the application.

8. The Application will prompt you to select several options through the interactive window shown in Figure E.33.

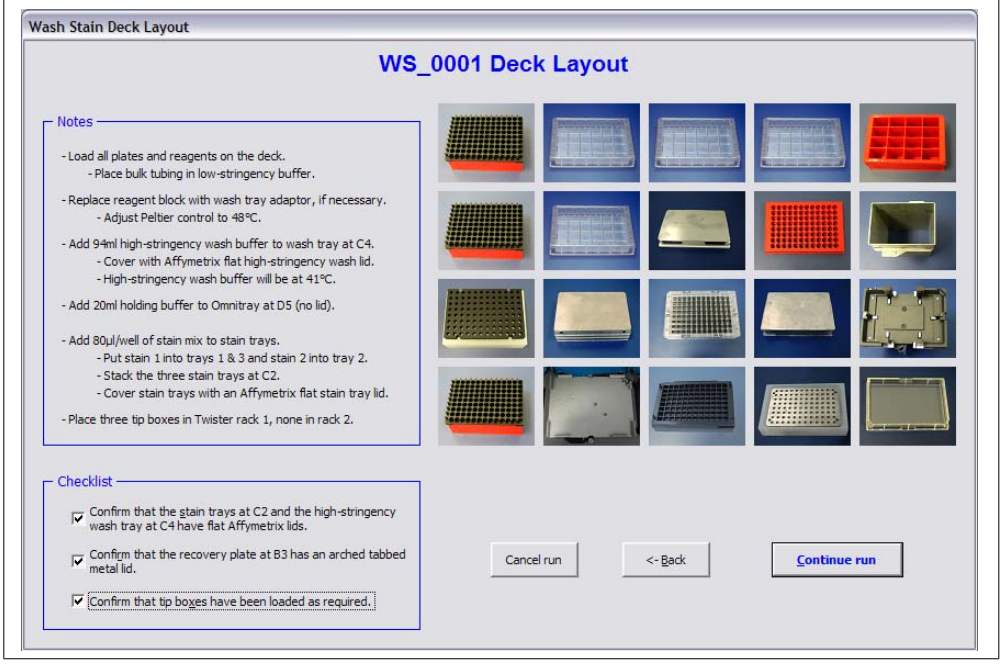
Figure E.33 Wash and Stain Setup window



9. Select the protocol parameters:
   - **A.** Select your **User name** from the drop down menu.
   - **B.** Select either a HT 24- or 96-Array Plate. It is important that you select the proper plate type to ensure samples are transferred to the correct wells.
   - **C.** If you wish the protocol to read and store the HT array plate barcode and the recovered sample plate barcode, check the **Read barcodes** option. Barcodes will be reported in the Wash/ Stain Summary Report at the end of the run.
   - **D.** If you are using spacer plates for tip static control (recommended), check the box **Twister tip rack spacer plates are in use**. If this box is checked, ensure you have placed spacer plates between each nested tip rack and on the top tip rack.
   - **E.** Enter a **Tracking identifier** (limited to 40 characters) if desired. The **Tracking identifier** will be recorded in the Summary report at the end of the run.
   - **F.** Select the desired notification settings. Be sure the email address or pager information is properly configured.

10. After selecting the settings parameters, click **Next**. The Wash Stain Deck Layout window appears (Figure E.34).

Figure E.34 Wash/Stain Deck Layout Setup window



11. Verify that you have setup the deck properly and that you have completed all items in the **Notes** section. Verify and check the boxes that you have completed all items in the **Checklist** section.

12. Click **Continue run** and the Array Station will perform the Wash/Stain protocol.

The Wash/Stain protocol continues until the HT Array Plate is stained and ready to be scanned. When the protocol has completed, the following message appears (Figure E.35).

Figure E.35 Ready to Scan window

13. Remove the stained HT Array Plate for scanning. Take care not to disturb or disrupt the array from the scan tray. Refer to the HT Scanner User's Guide (P/N 701978) for instructions on scanning the HT Array Plate.

14. Remove the sample recovery plate. Recovered hyb sample can be sealed and stored at –80°C for further use.

15. Change the Bulk Dispenser tubing back to the water bottle, Click **OK**. The Array Station will proceed to drain the wash trays and clean up the deck.

16. At the end of the protocol, The End message appears (Figure E.36). Click **OK**.

Figure E.36 Run Complete window

17. At the conclusion of each run, two summary reports are automatically saved by the program:

A. A user readable summary of the run is saved to the folder C:\Affymetrix\Reports\Summary. This file has the following naming convention:
SummaryReport_WS_0001_4_4_2006_9_56_08_AM.rtf

WS_0001 = method used
4_4_2006 = date stamp of when the HYB_0001 starts
9_56_08_AM = time stamp of when the HYB_0001 starts
Figure E.37 shows an example of a Summary Report.

Figure E.37 Wash/Stain Summary Report Example

Wash/Stain Summary Report
WS_0001

User: Array Station User
Date: Tuesday, April 04, 2006

Start time: 9:56:08 AM
End time: 11:36:37 AM
Run duration: 1:40:29

Tracking identifier:

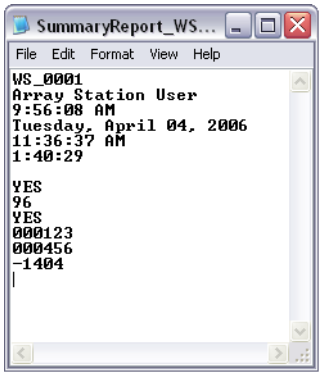
Tip spacers used: NO
Number of arrays: 96

Barcode reader enabled: NO
HT array plate barcode: Barcode not read
Recovered sample plate barcode: Barcode not read

Error Report
Error -1404: Please contact your local Affymetrix Technical Support.

B. A report useful for transferring data to many LIMS systems is also saved. This report is saved in the folder C:\Affymetrix\Reports\Data. An example if this report is shown in Figure E.38.

Figure E.38 LIMS-friendly Report Example



Troubleshooting

Positive Control Reaction

Control RNA Amplification Instructions

To verify that the process is working as expected, the HT 3' IVT Express Kit provides a control RNA sample isolated from HeLa cells. The procedure for amplifying the control RNA is as follows:

1. Dilute 2 µL of the control RNA into 18 µL of nuclease-free Water.
2. Use 1 µL of the diluted control RNA (100 ng) in a HT 3' IVT Express reaction; follow the protocol starting at *Reverse Transcription to Synthesize First-Strand cDNA* on page 131.
3. At *In Vitro Transcription to Synthesize Labeled aRNA* on page 132, use an 8 hour incubation for the IVT reaction.
4. Continue with the procedure for making biotin-modified aRNA thorough *aRNA Purification* on page 132.

Expected Results

- The positive control reaction should produce ≥ 40 µg of aRNA.
- The average size of the aRNA should be ~1000 nucleotides.

Factors that Affect Both Positive Control and Experimental Samples

If the positive control reaction yield or amplification product size does not meet expectations, consider the following possible causes and troubleshooting suggestions. These suggestions also apply to problems with amplification of experimental RNA.

Incubation Temperature(s) Were Incorrect

The incubation temperatures are critical for effective RNA amplification. Use only properly calibrated thermal cyclers for the procedure.

Condensation formed in the tube during the reaction incubation(s)

Condensation occurs when the cap of the reaction vessel is cooler (e.g., room temperature) than the bottom of the tube. As little as 1–2 µL of condensate in an IVT reaction tube throws off the concentrations of the nucleotides and magnesium, which are crucial for good yield.

If you see condensation, check to make sure that the heated lid feature of the thermal cycler is working properly.

Nuclease Contamination

Using pipettes, tubes, or equipment that are contaminated with nucleases can cleave the RNA or DNA being generated at each step in the procedure. This will reduce the size of the aRNA products and decrease aRNA yield. Both RNases and DNases can be removed from surfaces using a RNase decontamination solution such as RNaseZap®.

Troubleshooting Low Yield and Small Average aRNA Size

Consider the following troubleshooting suggestions if the positive control reaction produced the expected results, but amplification of your experimental samples results in less or smaller (average <500 nt) aRNA than expected.

Lower Than Expected Input RNA Concentration

Take another A_{260} reading of your RNA sample or, if it is available, try using 100–200 ng of RNA in the amplification procedure.

Impure RNA Samples

RNA samples with significant amounts of contaminating DNA, protein, phenol, ethanol, or salts are reverse transcribed poorly and subsequently generate less aRNA than pure RNA samples. Phenol extract and ethanol precipitate your RNA, or use a commercially available RNA cleanup kit to further purify your RNA before reverse transcription.

RNA Integrity is Compromised

RNA that is partially degraded generates cDNA that is relatively short. This will reduce the average size of the aRNA population and subsequently reduce the yield of aRNA. You can assess the integrity of an RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA (See *RNA Integrity* on page 14 for more information).

The mRNA Content of Your Total RNA Sample is Lower Than Expected

Different RNA samples contain different amounts of mRNA. In healthy cells, mRNA constitutes 1–10% of total cellular RNA (Johnson 1974, Sambrook and Russell 2001). The actual amount of mRNA depends on the cell type and the physiological state of the sample. When calculating the amount of amplification, the starting mass of mRNA in a total RNA prep should always be considered within a range of 10–30 ng per µg of total RNA (assuming good RNA quality).

Automated Sample Transfer

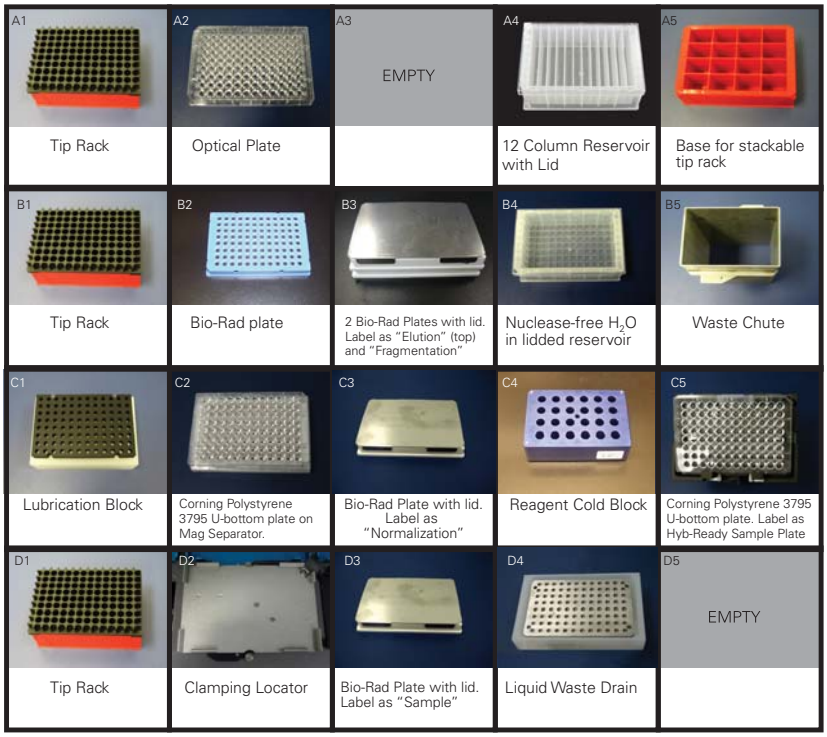
The Automated Sample Transfer function uses the Array Station to transfer 5 µL of total RNA from a Greiner U-bottom plate to a lidded Bio-Rad 96-Well Hard-Shell PCR Plate. To use this option, you must have at least 20 µL of your sample at a concentration of 0.2 to 0.4 µg/µL in a Greiner U-bottom plate. The samples should be placed in the wells in a column wise fashion in the same wells to be utilized for the Array Station Target Preparation run.

Loading Sample Plate and Initial Deck Layout for Automated Sample Transfer

Procedure

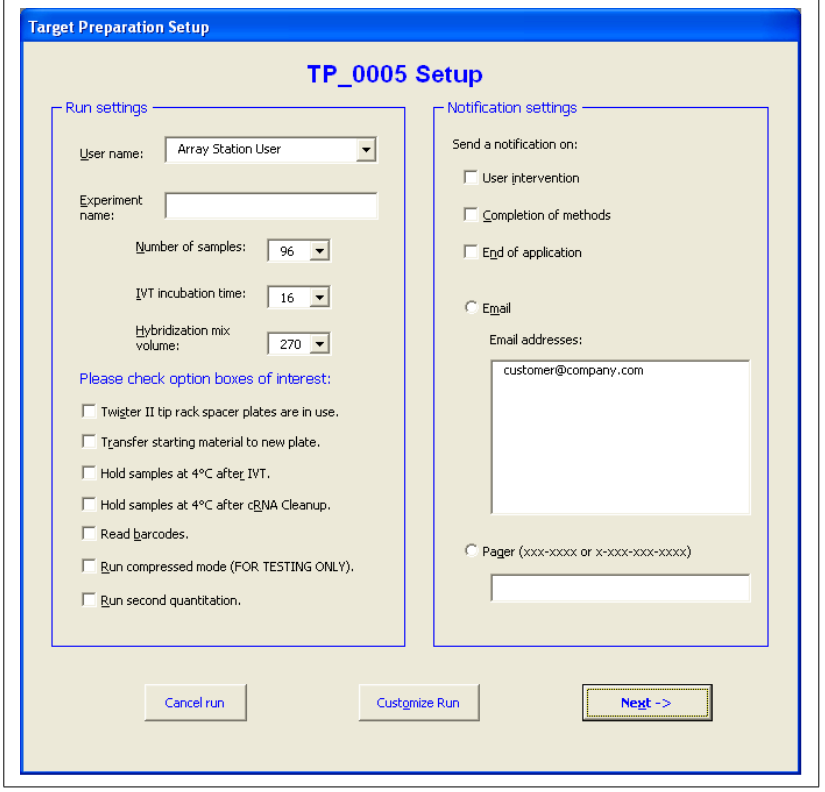
1. Set up the deck with the appropriate consumables, as illustrated in Figure G.1.

Figure G.1 Auto Starting Deck Layout



2. Start the software and load the TP_0005 application. Start the run by clicking the green arrow on the Application Control Console. The first window you see is shown in Figure G.2.

Figure G.2 Target Preparation Setup window

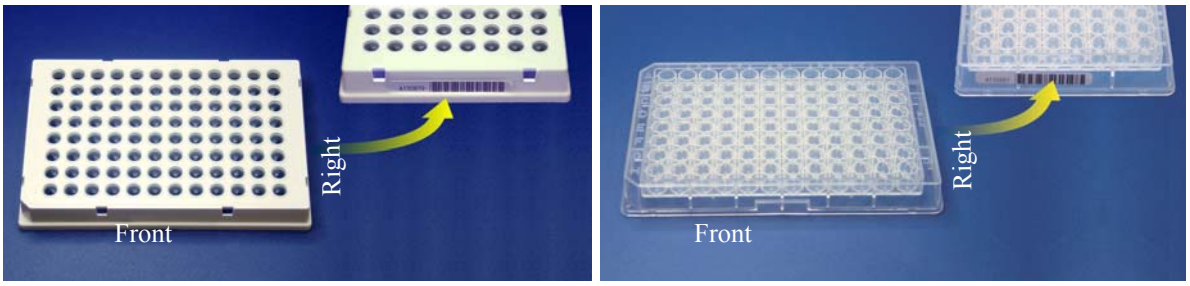


3. Under **Run Settings** in the Target Preparation dialog box, enter the following information:
   - **A. User name**: Select the appropriate user name from the drop down menu. Please contact Affymetrix support personnel for assistance with customizing this window.
   - **B. Experiment name**: Enter a name for your experiment.
   - **C. Number of samples**: Select either 24 or 96. Be sure your samples are laid out in the plate in column-wise fashion starting column 1.
   - **D. IVT incubation time**: Select either 4, 8 or 16 hours. Refer to Table 3.3 for recommended incubation times based on the amount of input RNA.
   - **E. Hybridization Mix Volume**: Select either 120, 240 or 270 µL.
     - 120 µL = one HT Array Plate
     - 240 µL = two HT Array Plates
     - 270 µL = cartridge arrays
4. In the **option boxes of interest** area of the Target Preparation dialog box, select the following optional items:
   - **A. Twister II tip rack spacer plates are in use**: Select this option if you are using the tip rack spacer plates to help control static. Affymetrix strongly suggests you utilize the tip rack spacer plates. A spacer plate should be placed between each rack of tips as well as on the top rack of tips before loading tips into the Twister II Rack 1.
   - **B. Transfer starting material to new plate** (>5 µL/well at 10-20 ng/µL). Check this box to have the Array Station transfer 5 µL of starting total RNA into the Bio-Rad 96 Well Hard Shell PCR plate. To use automated sample transfer, a minimum of 20 µL of your sample (0.2 to 0.4 µg/µL) must be in a Greiner U-bottom plate. If you select **Transfer starting material to new plate**, you will be prompted when to place and remove plates as needed. Please refer to Chapter 2 for more information on using the automated sample transfer option.

C. **Hold samples at 4 ºC after IVT**: If you select this option, the plate will be held in the incubator at 4°C after the IVT step until you prompt the Array Station to resume the process. Once prompted, the process will proceed to the IVT cleanup steps.

D. **Read barcodes**: Check this box if you want the system to automatically read and track the barcodes of various plates used during the run. You must supply the barcodes (code 128c format) and affix them to any or all of the following plates for tracking total RNA, unfragmented aRNA, normalized aRNA, fragmented aRNA, and hyb-ready sample. Barcodes should be affixed to the right side of the plate (see Figure G.3). Barcodes recorded during the run will be reported in the Target Preparation Summary Report for your run.

Figure G.3 Barcode Positioning on Plate



E. **Run compressed method (FOR TESTING ONLY):**
Do not select this option unless you are performing a blank test run.

NOTE: Please see Appendix H for a description of how to use the "Customize Run" option.

F. **Run second quantitation:**
Select this option if you would like to have a second quantitation after the samples are normalized.

G. **Notification Settings**: Select the points at which you would like to receive notification. Notification can be received via email or pager. Ensure that the email address or pager information in the selection is correct and active. Please contact Affymetrix personnel for assistance with configuring email or pager notification

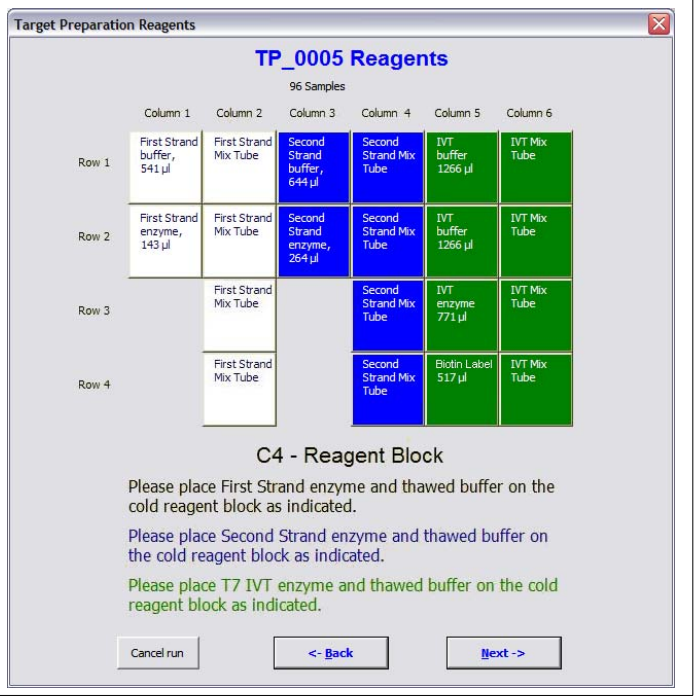
- **User intervention** —notifies you when the system requires your attention
- **Completion of methods** — notifies you when a method, or run step, is completed
- **End of application**

5. When the appropriate options are selected, click the **Next** button.

To cancel before continuing the run follow these steps:

1. **Click the "Cancel Run" button.**
2. **Two windows appear in succession.**
3. **Click "Yes" and "OK" sequentially to cancel the run.**

Figure G.4 TP_0005 Reagents window - 96 sample



6. Use the TP_0005 Reagents window to ensure all reagents are loaded. After you have verified that your reagents are loaded, click **Next**.

Figure G.5 TP_0005 Deck Layout Checklist window

To cancel before continuing the run follow these steps:

1. **Click the "Cancel Run" button.**
2. **Two windows appear in succession.**
3. **Click "Yes" and "OK" sequentially to cancel the run**

7. Ensure that you have complied with the **Notes for Starting with Sample Transfer**.
   - Add required reagents to 12-column reservoir in position A4 with microtiter plate lid.
   - Add >100 mL nuclease-free water to high reservoir in position B4 with microtiter plate lid.
   - Place reagent block in position C4 and adjust temperature controller to 4°C.
   - Sample Transfer: put at least 5 µL starting material per well in lidded Bio-Rad plate in position D2.
   - Confirm that at least 26 tip boxes are in Twister rack 1 and that none are in rack 2.
8. Click **Continue Run**.
9. After transfer is complete, Figure G.6 appears.

Figure G.6 User message to replace sample plate with lidded PCR plate.

10. Remove the Greiner plate from the D2 position and replace it with the "unfrag aRNA" plate (Bio-Rad 96-well Hard Shell PCR Plate). Close the doors to the Array Station and select **OK** to proceed.
11. Please Refer to *Running the Target Preparation Protocol* on page 123 for instructions for the remaining steps of the process.

Array Station Customized Applications and Deck Layouts

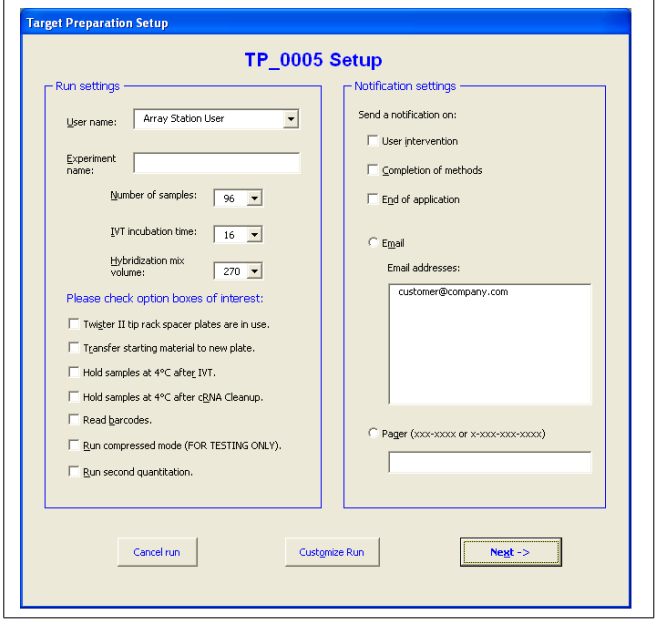
Deck layouts for the Array Station Target Preparation Options

The Array Station Automated Target Preparation Application offers options for running all or portions of the HT 3' IVT Express Assay.

You will need to configure the Deck Layout properly depending upon where in the process you elect to start. Deck layouts for all possible starting options are presented in this Appendix.

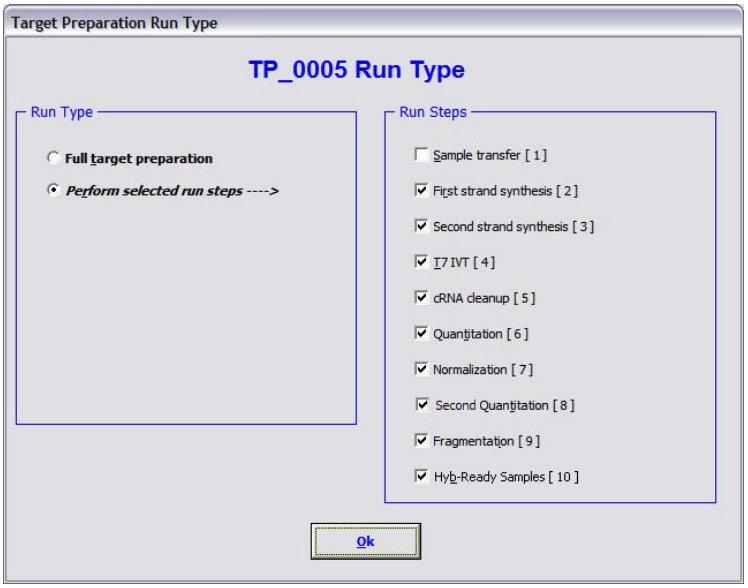
1. In order to run customized applications click the **Customize run** button as shown in Figure H.1.

Figure H.1 Target Preparation Setup window



You are prompted for information on the Run Type as shown in Figure H.2.

Figure H.2 Customized Run Setup window



2. For the **Run Type** window, you will need to select one of the following options:

   NOTE: The Sample Transfer [1] option directs the Array Station to transfer your sample from one Bio-Rad 96 Well plate to another Bio-Rad 96 Well plate. This option is available for all run types. If 5 µL of your sample is already in the Bio-Rad plate, this box should be unchecked.

   A. **Full target preparation**: When this option is selected, the protocol will run all methods associated with target preparation from Sample Transfer through Hybridization Mix preparation. Notice that when you select this option, all methods from **Sample Transfer [1]** to **Hybridization setup [10]** are selected for you in the **Run Steps**. When running the **Full target preparation** selection, you will be prompted to change the deck, read plates and transfer plate reader data to the Array Station at indicated times.

   B. **Perform selected run steps**: When this option is selected, only the methods selected under **Run Steps** will be preformed.

3. Once you have selected the **Run Type** you wish to perform, click **OK** to continue.

   The Target Preparation Setup window returns (Figure H.1).

4. Click **Next -->** in the Target Preparation Setup window to continue with your customized run.

Table H.1 lists the correct Deck Layout configurations for the typical starting selections. Figure H.3 through Figure H.5 illustrate the various deck layouts. Be sure to refer to the deck layout configuration corresponding to your starting selection for your run.

Table H.1 The Array Station Starting Deck Layouts for Major Run Types

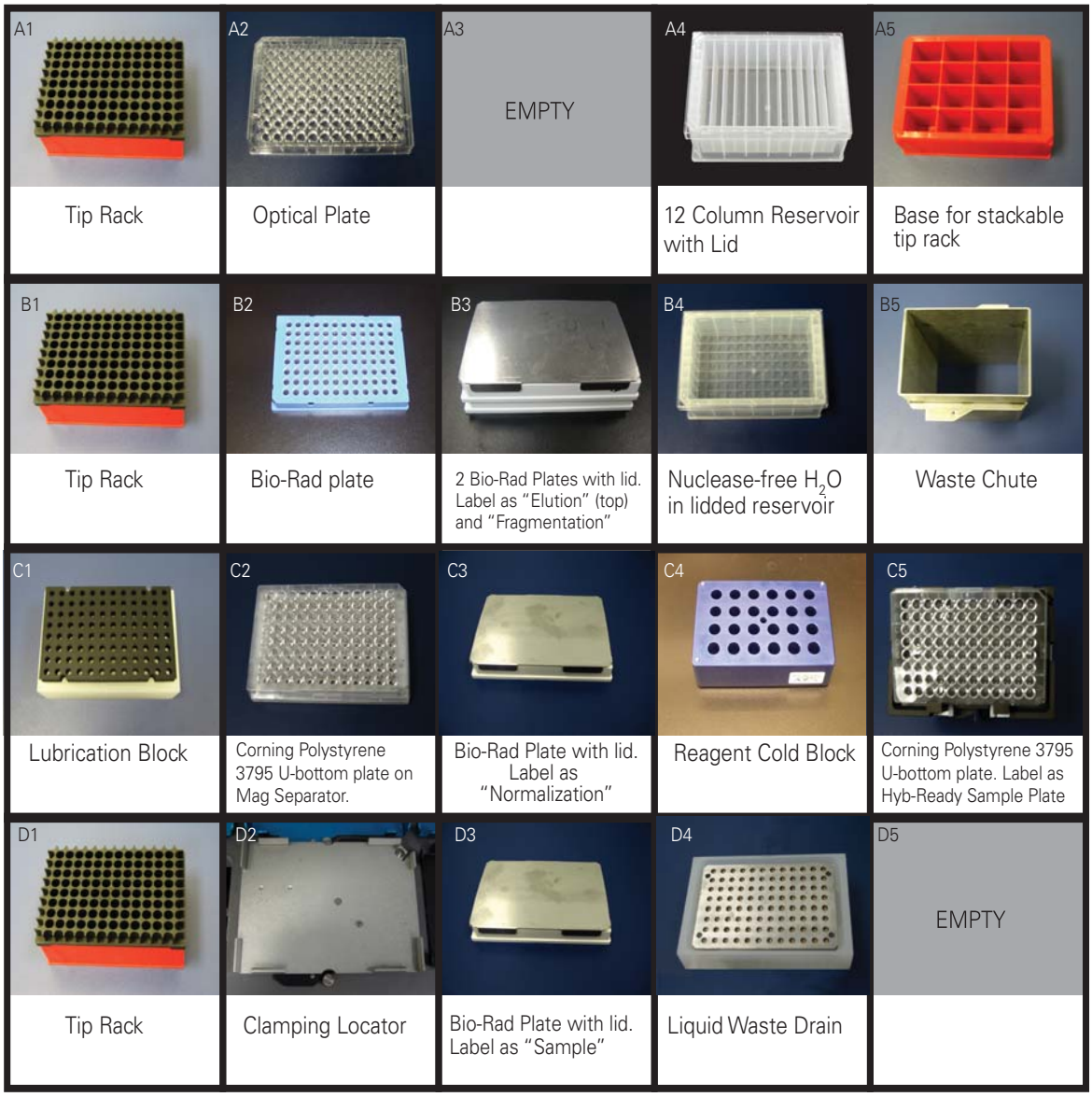
Starting Selection	Deck Layout
Full Target Prep Starting Layout – Manual Transfer	Layout A
Full Target Prep Starting Layout – Automated Transfer	Layout A
First Strand Synthesis	Layout A
Second Strand Synthesis	Layout A
IVT	Layout A
cRNA Cleanup	Layout B
Quantitation	Layout C
Fragmentation	Layout D

NOTE: Please be sure to place all plates on deck as indicated in layouts. Even if the process for which that plate is used is complete, the robot expects the plate to be in that position. Serious errors can occur if plates are missing.

Array Station Deck Layouts

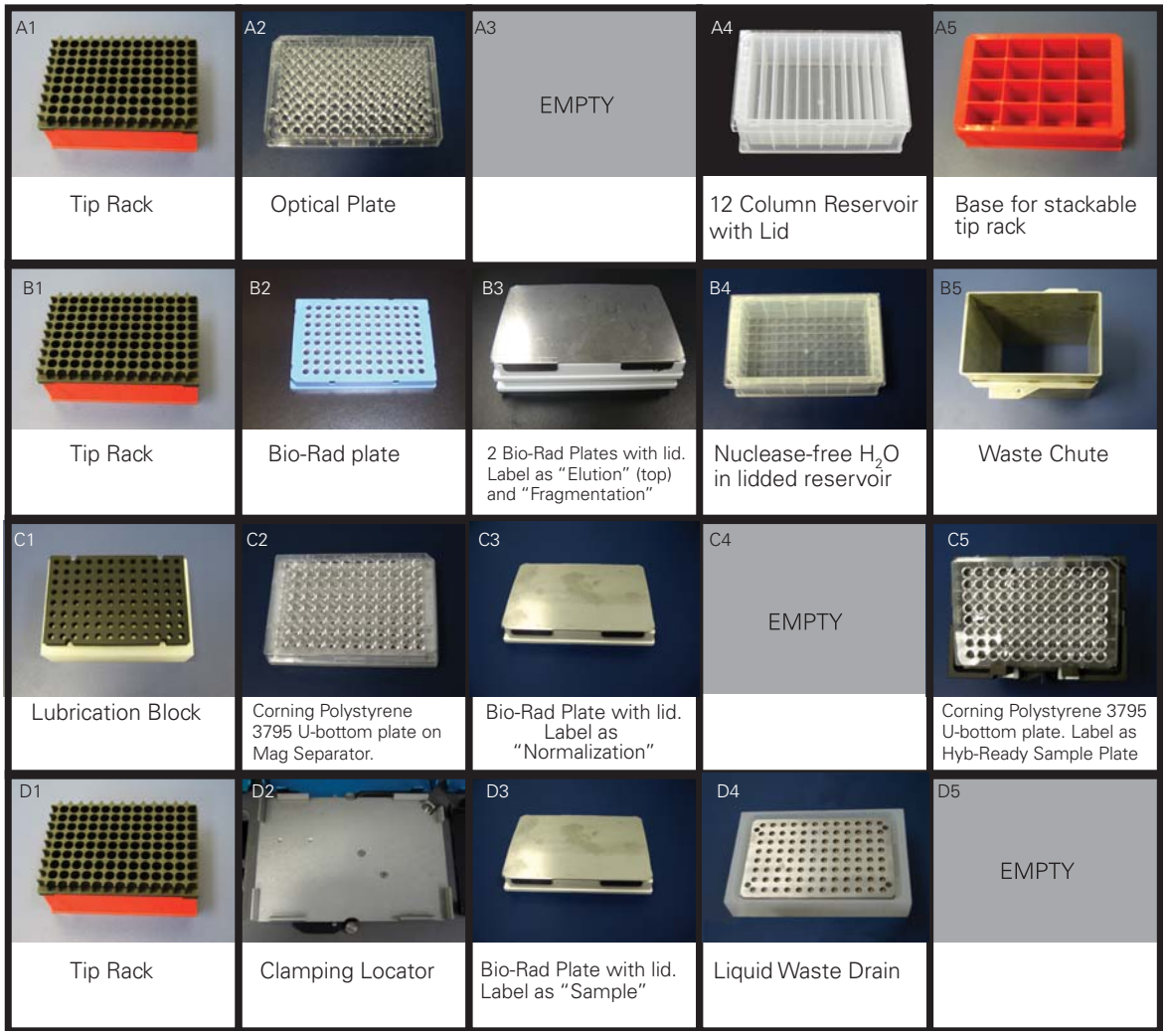
Deck Layout A

Figure H.3 Array Station Deck Layout Configuration A



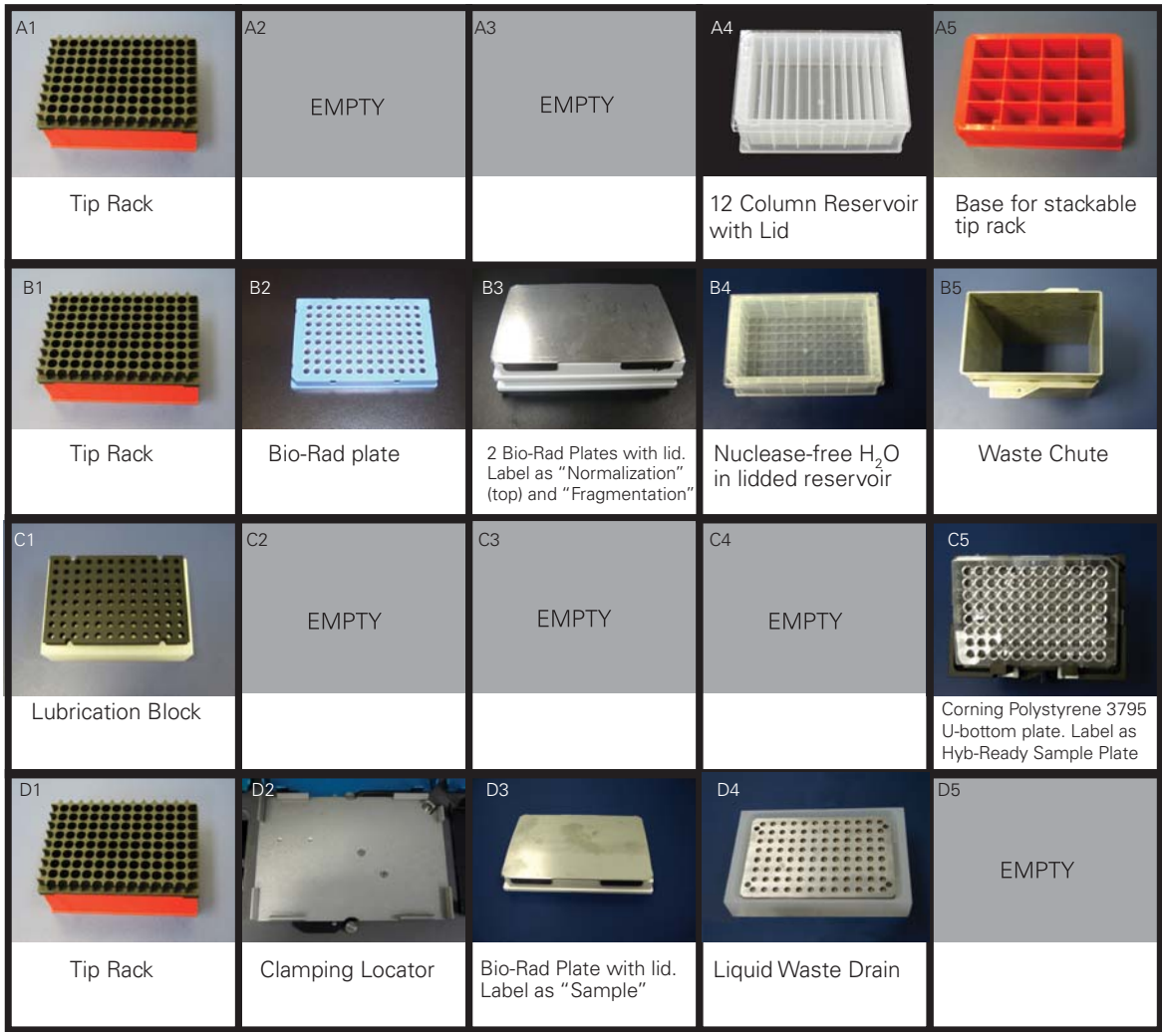
Deck Layout B

Figure H.4 Array Station Deck Layout Configuration B



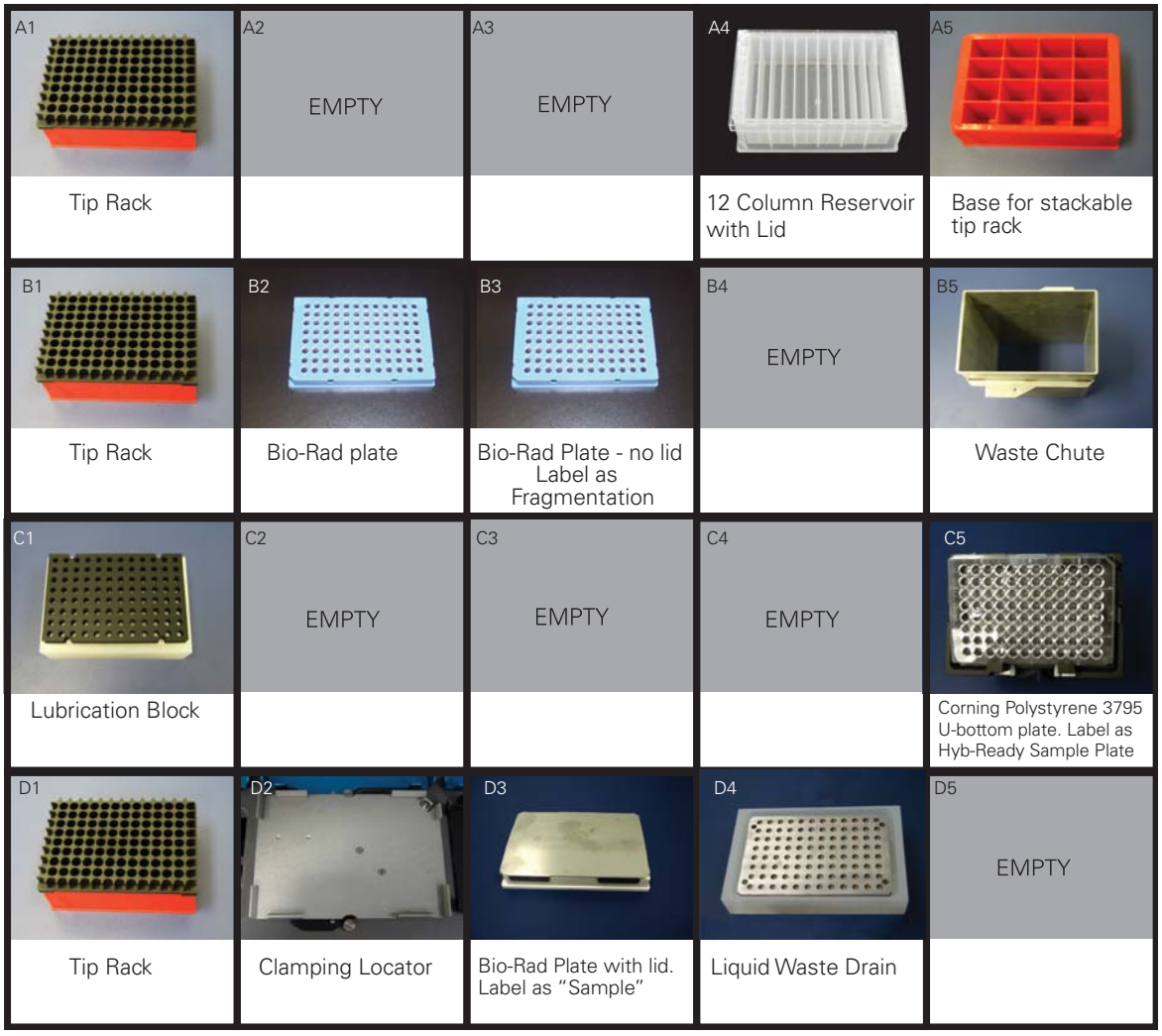
Deck Layout C

Figure H.5 Array Station Deck Layout Configuration C



Deck Layout D

Figure H.6 Array Station Deck Layout Configuration D



Sciclone Workstation Software User Information

User Level Configuration

Sciclone Workstation Software has three user groups with different permissions. These groups are Operators, Developers and Administrators.

Table I.1 Permissions for Sciclone Workstation Software

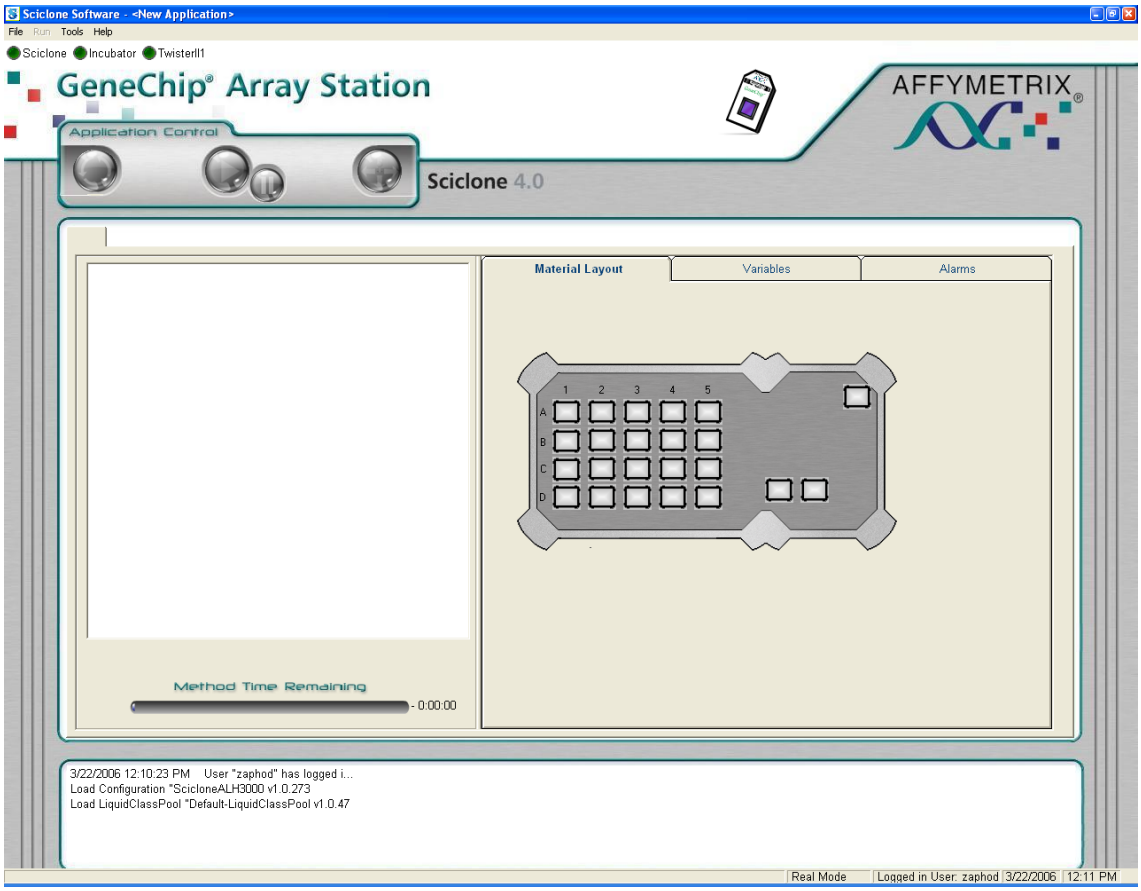
User Group	Permissions
Sciclone Operators	Access Runtime Window, Run Methods, Access Direct Control Window, Error Recovery
Sciclone Method Developers	All Operator Privileges, Access Method Editor Window, Create, Edit, Save and Delete Methods, Layouts and Liquid Classes, Import and Export Applications and Methods
Sciclone Administrators	Access all Sciclone systems

The level of access is determined at User Login. A **User Name** and **Password** is required at login (see Figure I.1). The level of access for each authorized user will be set up during installation of your Sciclone Workstation Software by Affymetrix personnel.

Figure I.1 Sciclone Workstation Software Login window

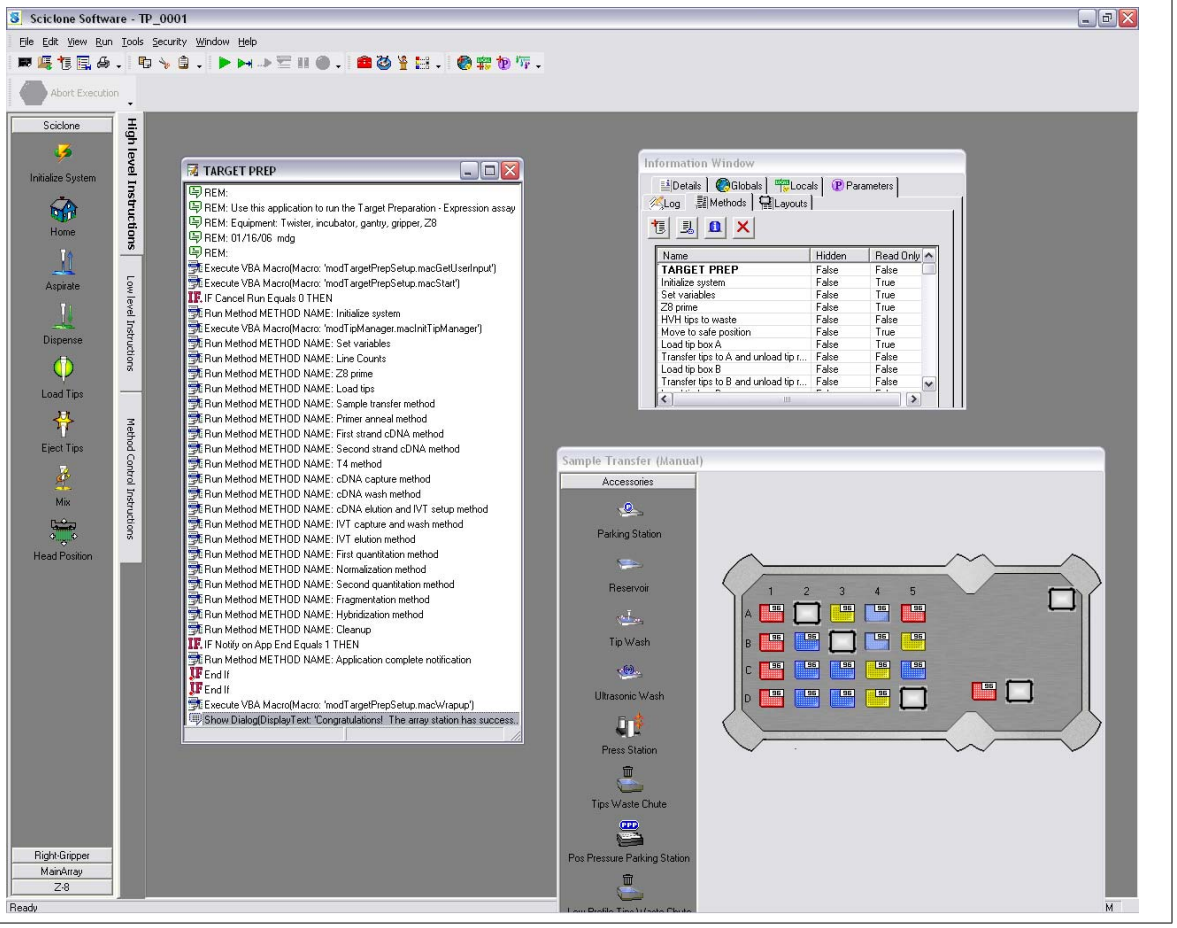
If the User logged into the system is in the Operator User Group, he will have access to the Runtime window (Figure I.2).

Figure I.2 Sciclone Workstation Software Operator window



If the User logged into the system is in the Developer or Administrator User Group, he will have access to the Method Editor window (Figure I.3).

Figure I.3 Sciclone Workstation Software Developer window



NOTE: When running the Affymetrix applications you should login as an Operator and run the applications from the Operator window.

More information on the User groups can be found in the Sciclone Users Manual.

Fluidics Station Care and Maintenance

This chapter provides instructions on caring for and maintaining the instrument, and on troubleshooting if problems arise.

Instrument Care

- Use a surge protector on the power line to the fluidics station.
- Always run a Shutdown protocol when the instrument will be off or unused overnight or longer. This will prevent salt crystals from forming within the fluidics system.
- When not using the instrument, leave the sample needles in the lowered position. Each needle should extend into an empty vial. This will protect them from accidental damage.
- Always use deionized water to prevent contamination of the lines. Change buffers with freshly prepared buffer at each system startup.
- The fluidics station should be positioned on a sturdy, level bench away from extremes in temperature and away from moving air.

IMPORTANT: **Before performing maintenance, turn off power to the station to avoid injury in case of a pump or electrical malfunction.**

Instrument Maintenance

To ensure proper functioning of the fluidics station, you should perform periodic maintenance.

Fluidics Station Bleach Protocol

Affymetrix recommends a weekly cleaning protocol for the fluidics station. This protocol uses commonly purchased sodium hypochlorite bleach.

This protocol is designed to eliminate any residual SAPE-antibody complex that may be present in the fluidics station tubing and needles. The protocol runs a bleach solution through the system followed by a rinse cycle with deionized (DI) water. This protocol takes approximately one hour and forty minutes to complete. Affymetrix recommends running this protocol weekly. You can find the current version of the protocol at:
www.affymetrix.com/support/technical/fluidics_scripts.affx.

The Bleach Cycle

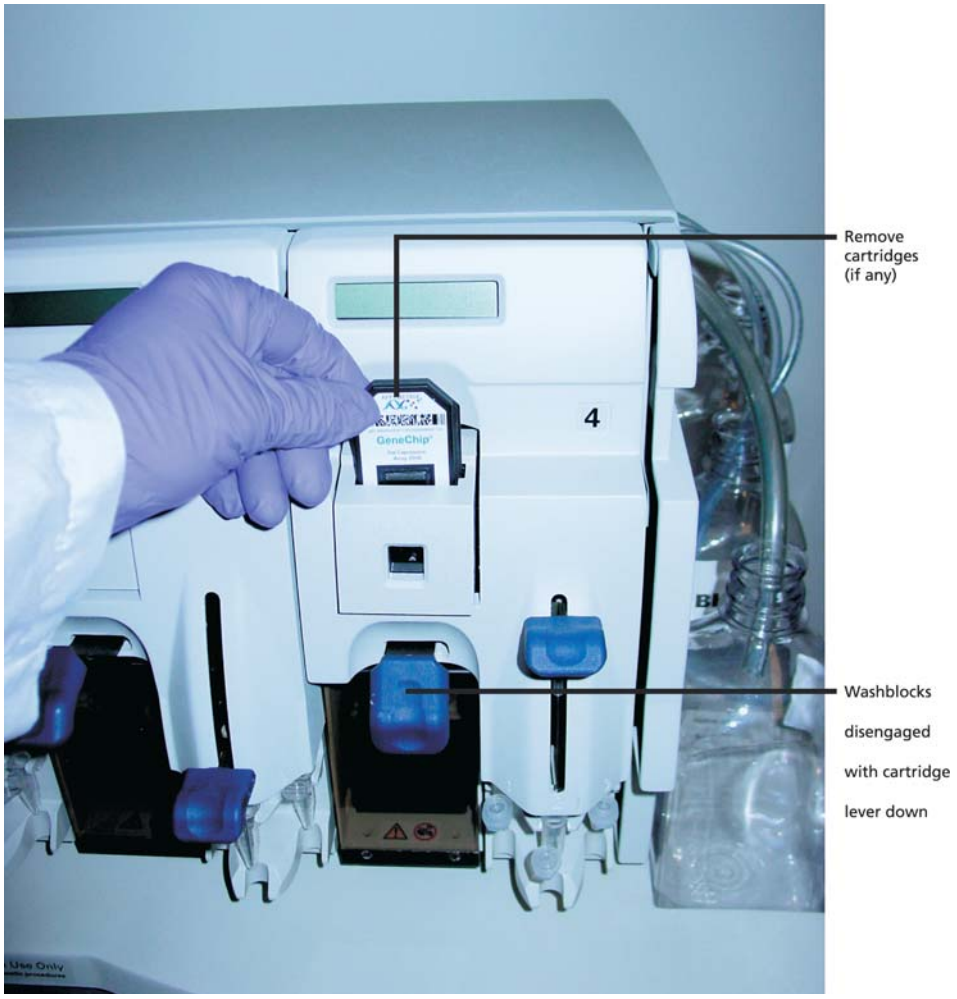
To avoid carryover, or cross contamination, from the bleach protocol, Affymetrix recommends the use of dedicated bottles for bleach and DI water. You can obtain additional bottles from Affymetrix.

Table J.1 Affymetrix Recommended Bottles

Part Number	Description
400118	Media Bottle, SQ, 500 mL
400119	Media Bottle, SQ, 1000 mL

1. Disengage the washblock for each module by pressing down on the cartridge lever. Remove any probe array cartridge (Figure J.1).

Figure J.1 Disengaged washblocks showing cartridge levers in the down position, remove any cartridges



2. Prepare 500 mL of 0.525% sodium hypochlorite solution using deionized water. For example: follow these directions to make 500 mL of bleach.

   In a 1 liter plastic or glass graduated cylinder combine 43.75 mL of commercial bleach (such as Clorox bleach, which is 6% sodium hypochlorite) with 456.25 mL of DI H_2O, mix well. Pour the solution into a 500 mL plastic bottle, and place the plastic bottle on fluidics station.

   IMPORTANT: **The shelf life of this solution is 24 hours. After this period, you must prepare a fresh solution.**

   NOTE: **Each fluidics station with four modules requires 500 mL of the 0.525% sodium hypochlorite solution.**

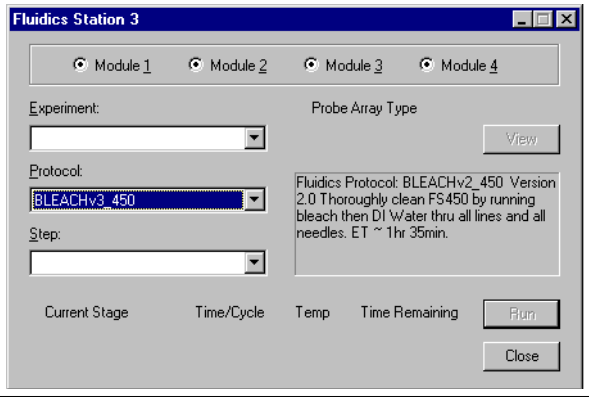
3. Place on the fluidics station an empty one liter waste bottle, a 500 mL bottle of bleach and a one liter bottle of DI water as shown in Figure J.2. Insert the waste line into the waste bottle (Figure J.2).
4. Immerse all three wash and water lines of the fluidics station into the 500 mL of bleach solution (Figure J.2). DO NOT IMMERSE THE WASTE LINE INTO THE BLEACH.

   NOTE: **The BLEACH protocol requires approximately one liter of DI water.**

Figure J.2 The bleach cycle. Immerse the tubes into the 0.525% sodium hypochlorite solution. The waste line remains in the waste bottle.

5. Open GeneChip Operating Software (GCOS).
Click **Run** → **Fluidics...** from the menu. Alternatively, click the down arrow Protocol list on the toolbar. The protocol window appears (Figure J.3).

Figure J.3 The Fluidics Station protocol window: select all modules.



6. Choose the current bleach protocol (in the current example, it is BLEACHv3_450) for each of the respective modules in the Protocol drop-down list. Select all four modules, 1 to 4, and click **Run**. The fluidics station will not start the bleach protocol until you press down on the needle lever (Figure J.4).

NOTE: Temperature will ramp up to 50°C.

7. Follow the prompts on each of the LCD. Load empty 1.5 mL vials onto each module if you have not already done so.
8. Press down on each of the needle levers to start the bleach protocol (Figure J.4).

Figure J.4 Press down on the needle levers to start the bleach protocol.

9. The fluidics station will begin the protocol and begin to empty the lines and perform the cleaning cycles using bleach solution.
10. After approximately 30 minutes, the LCD will prompt you when the bleach cycle is over and the rinse cycle about to begin.

The Rinse Cycle

Once the bleach cycle has finished, the second part of the protocol is a rinse step. This step is essential to remove all traces of bleach from the system. Failure to complete this step can result in damaged arrays.

1. Follow the prompts on the LCD for each module. Lift up on the needle levers and remove the bleach vials. Load clean, empty vials onto each module.
2. Remove the three wash and water lines from the bleach bottle and transfer them to the DI water bottle (Figure J.5). At this step, you need not be concerned regarding the bleach that remains in the lines.

Figure J.5 Immerse the three wash and water lines in the DI water bottle.

3. Press down on the needle levers to begin the rinse cycle. The fluidics station will empty the lines and rinse the needles.
4. When the rinse is completed after approximately one hour, the fluidics station will bring the temperature back to 25°C and drain the lines with air. The LCD display will read CLEANING DONE.
5. Discard the vials employed for the bleach protocol.
6. Follow these suggestions after you have completed the bleach protocol (Table J.2).

Table J.2 Quick Reference Guide to Using the FS-450

If you are:	Then do this:
Planning to use the system immediately	After running the bleach protocol, remove the DI water supply used in the rinse phase and install the appropriate reagents for use in your next staining and washing protocol (including fresh DI water). ▪ Perform a prime protocol without loading your probe arrays. **Failure to run a prime protocol will result in irreparable damage to the loaded hybridized probe arrays.**
Not planning to use the system immediately	Since the system is already well purged with water, you need not run an additional shutdown protocol. Just remove the old DI water bottle and replace it with a fresh bottle.
Not planning to use the system for an extended period of time (longer than one week)	Remove the DI water and perform a "dry" protocol shutdown. This will remove most of the water from the system and prevent unwanted microbial growth in the supply lines. Also, remove the pump tubing from the peristaltic pump rollers.

NOTE: After you have completed the bleach protocol, discard the vials.

Peristaltic Tubing Replacement

Periodically the peristaltic tubing requires replacement because of wear, contamination, or in order to avoid salt buildup. Inspect the tubing, if you see evidence of these conditions, follow the procedure outlined below.

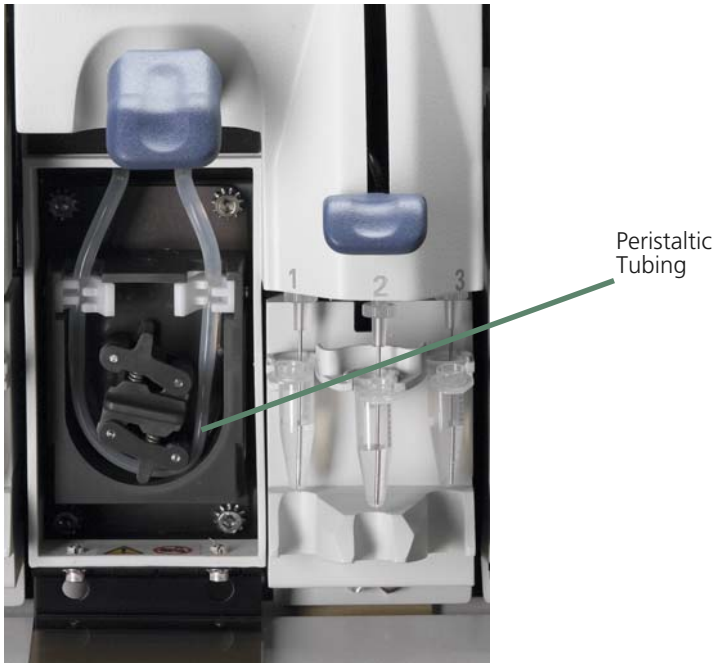
IMPORTANT: For systems in routine use, Affymetrix recommends monthly replacement of the tubing. To ensure proper performance, use only tubing available from Affymetrix. This tubing is manufactured to the required specifications to ensure proper fluid delivery and array performance. You can obtain additional tubing by ordering from Affymetrix:

Part Number	Description	Quantity
400110	Tubing, Silicone Peristaltic, 8.5	1

Wear gloves when changing tubing. Do not allow fluid from old tubing to spill onto surfaces.

1. Open the module door (Figure J.6).

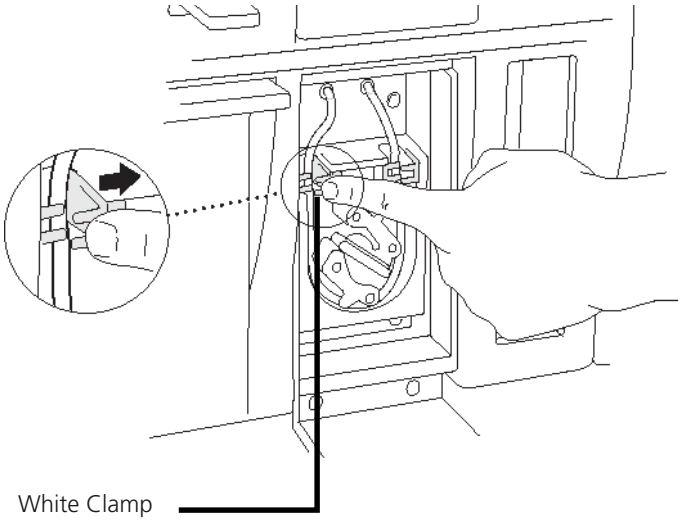
Figure J.6 Module door open showing peristaltic tubing



2. Open the white clamps to release tubing on both sides. See Figure J.7.

WARNING: Do not attempt to replace the tubing on a module where the module has been removed from the case of the fluidics station. In this case, rotating the pump may damage the motor driver circuitry.

Figure J.7 Releasing peristaltic tubing



3. Pull tubing off while gently turning the peristaltic pump head. Discard old tubing.

4. Replace tubing with new peristaltic tubing supplied with the accessory kit as described below:
   A. Attach one end of the new tubing to the fitting on the right at the top of the pump enclosure.
   B. Insert the tubing into the clamp under the fitting without stretching the portion of the tubing between the fitting and the clamp. There should be a small amount of slack in that portion of the tubing.
   C. Work the tubing into the pump head while slowly turning the pump.
   D. Insert the free end of the tubing into the other clamp, and attach it to the other fitting.
   E. Close the drop-down module door.
5. Order more replacement tubing (P/N 400110).

Troubleshooting and Assistance

If problems arise with the fluidics station, use the following tables to locate the description that matches the problem. If you cannot find a solution, call Affymetrix Technical Support for assistance.

Troubleshooting Decision Tree

The following simple flow charts (Figure J.8 and Figure J.9) show you how to begin troubleshooting the FS450/250 for a Missing Fluid Error (MFE).

Figure J.8 Troubleshooting decision tree, page 1

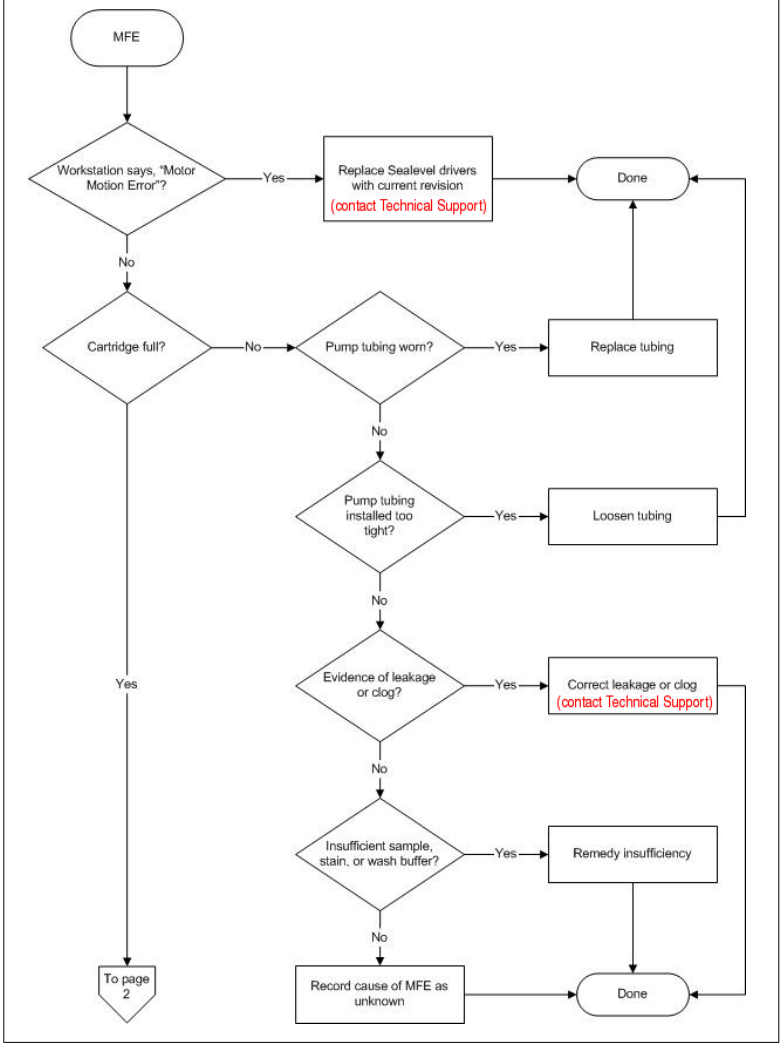
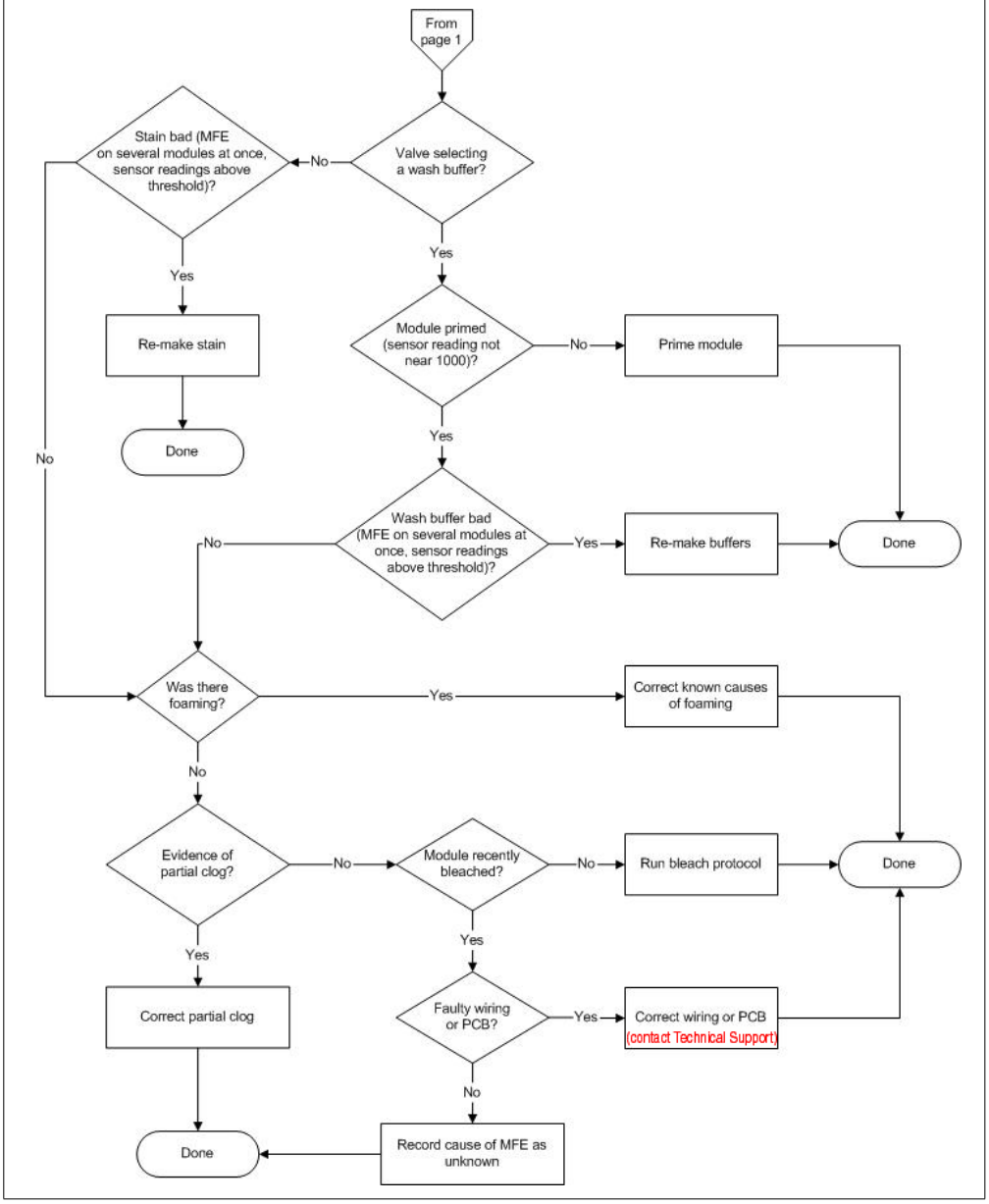


Figure J.9 Troubleshooting decision tree, page 2



Problems and Solutions

Table J.3 Common error messages, their meanings, probable causes and solutions

Error Message	Problem	Possible Cause	Solution
Missing Fluid Error	Cartridge not filling completely with sample solution or buffer during initial stages of hybridization wash or staining protocol.	Possible holes in the septa of the cartridge.	Run Recover script, and then use another cartridge.
		Sample or staining solution not in place properly.	Run Recover script. Make sure sample or stain vial is in the sample holder.
		Insufficient volume of sample or staining solution (500 µL).	Run Recover script. Add more sample solution to the sample vial.
		Blocked sampling tube or line of the fluidics station.	Run Recover script. Run the Clean or Prime script with fresh deionized (DI) water to flush out salt blockage.
		Failure of one of the fluidics sensors.	Call Affymetrix Technical Support for service.
		Pump tubing stretched too tightly around the pump.	Loosen the tubing clamps, allow tubing to relax, close the clamps.
	Cartridge not filling completely with buffer during wash script	Buffer bottle empty.	Fill buffer bottles.
		Module not primed.	Prime module.
	System detects improper conditions while filling. Note where in protocol error occurred.	▪ Missing or insufficient stain or antibody in vial? ▪ Wash empty? ▪ Air bubbles in line? ▪ Leaks?	Identify if chip is filled ▪ If important to recover fluid in chip, and then run **Recovery** script, followed by **Resume** function ▪ If not important to recover fluid in chip, run **Resume** function
	Recovered less sample than initial input during Recover script.	Loose tubing attachments inside the fluidics station.	Call Affymetrix Technical Support for service.
Fluidics Station X Does Not Respond		Power not switched on at the fluidics station.	Turn fluidics station power on, and then try to connect again.
		Incorrect fluidics station designated for communication.	Designate correct fluidics station on workstation.
		Loose cables.	Firmly connect cables to fluidics station.
Sensor Timeout	"Sensor Timeout" error message on workstation.	No user response to "Remove Vial" prompt or other prompt.	Start the selected script again.

Table J.3 (Continued)Common error messages, their meanings, probable causes and solutions

Error Message	Problem	Possible Cause	Solution
Error While Draining **Error While Filling**	Cartridge is not filling or draining properly.	Defective septa in cartridge. Insufficient sample or stain volume. Excessive bubbling in cartridge. Buffer conductivity too low. Failure of one of the fluid sensors.	Use a new cartridge. Add more sample solution to sample vial. Change the buffer: reduce detergent. Change the buffer: increase salt. Call Affymetrix Technical Support for service.
Error While Filling	System detects improper conditions while filling. Note where in protocol error occurred.	▪ Missing or insufficient stain or antibody in vial? ▪ Wash or DI water empty? ▪ Air bubbles in line? ▪ Leaks?	Identify if chip is filled: ▪ If important to recover fluid in chip, and then run **Recovery** script, followed by **Resume** function ▪ If not important to recover fluid in chip, run **Resume** function
Invalid Command	Communications error detected Note where in protocol error occurred		Identify if chip is filled. ▪ If important to recover fluid in chip, and then run **Recovery** script. ▪ Attempt to rerun script if sample loss can be tolerated. If problem persists, contact Affymetrix for service ▪ If sample loss cannot be tolerated, do not attempt to rerun script. Contact Affymetrix for service
Temperature Timeout	Temperature does not reach specified temperature.	Temperature has not reached required level in expected time if ambient temperature is within operating specifications (15 – 30°C).	Call Affymetrix Technical Support for service.
Improper Script	Script does not work.	User is attempting to run a FS400 script on FS450Dx	Download proper FS450Dx script and continue
Valve Motion Error			Run **Home** script and run desired script again If problem persists, contact Affymetrix for service.
Valve Not Homed			Run **Home** script and run desired script again If problem persists, contact Affymetrix for service.
Valve Out of Position			Run **Home** script and run desired script again If problem persists, contact Affymetrix for service.

Meaning of Error Messages

The following table lists some common error messages and their meaning (Table J.4).

Table J.4 Common Error Messages

Error Message	Meaning
"Invalid Command"	The script contains a command that can not be executed because its command code is either undefined or has a format error.
"**Improper Script"**	The first command of the script is not the required FS450 command.
"**Temperature Timeout"**	The Re-attempt command timed out before the set point temperature was reached.
"**Sensor Timeout"**	The Await Sensors command timed out before the anticipated sensor pattern was seen.
"**Valve not Homed"**	The Home command did not result in the valve reaching it HOME position.
"**Valve Motion Error"**	The Valve command did not result in the valve reaching it target valve position.
"**Valve out of Position"**	According to the outer valve encoder, the valve did not reach a valid position when it was last rotated.
"**Error while Filling"**	While filling the cartridge, the AwaitMotor command terminated because of the step count not the expected sensor pattern, and that the same error had occurred several times consecutively.
"Error while Draining"	While draining the cartridge, the AwaitMotor command terminated because of the step count not the expected sensor pattern, and that the same error had occurred several times consecutively.
"Missing Fluid Error" **Examples:** **"Stage C" "WashA"** **"Sense/Threshold" "960/890"**	"Stage C" "WashA" "Sense/Threshold" "960/890" The Pump command completed its step count before the conductivity sensor determined that the cartridge contained a solution with conductivity below the set threshold value. The Missing Fluid Error (MFE) Display not only gives a visual notification of an error condition to the operator, but gives the operator information that enables him/her to determine the cause of the error. It does this by displaying information about the sensor value and the fluid that caused the error. It shows this internal information in a continuous loop until the machine is powered down or a script is started. For example: Missing Fluid Error for 4 seconds Stage A valvePos WashA for 4 seconds Sense/Threshold 820/600 for 4 seconds

Other Problems and Solutions

Table J.5 lists other problems, causes and solutions that you may encounter.

Table J.5 Other Problems

Problem	Possible Cause	Solution
Air bubbles left in cartridge at the end of a hybridization-wash script.	Air bubble in wash line	Manually fill cartridge with Buffer A.
Buffer leaking inside the fluidics station.	Loose tubing attachments inside the fluidics station.	Call Affymetrix Technical Support for service.
	Washblock requires replacement.	Call Affymetrix Technical Support for service.
	Salt buildup in the lines of the fluidics station.	Run the Clean or Prime script with fresh DI water to flush out salt blockage.
Cartridge needles of the fluidics station not engaging with the cartridge.	Possible defective septa on the cartridge.	Use another cartridge.
	Extra flashing on the cartridge.	Use another cartridge, or call Affymetrix Technical Support for service.
	Salt buildup on the cartridge needles.	Run the Clean script with fresh DI water to flush out salt blockage. Clean cartridge needles with a wet cotton swab.
	Cartridge holder aligned and attached to the fluidics station improperly.	Call Affymetrix Technical Support for service.
	Cartridge holder not properly engaged to the fluidics station.	Place the cartridge into the cartridge holder. Push the holder door shut, and firmly lift the lever to engage the cartridge needles.
Sample needles do not properly enter vial.	Bent sample needle	Replace sample needle.
	User may be pressing the needle lever down to quickly or with too much force.	Engage sample needle lever more slowly and/or with less force.

Instrument Specifications

Fluidics Station Dimensions:

(height, depth, width)
40.2 x 41.0 x 71.1 cm or 15 13/16 x 16 1/8 x 28 inches

Product Weight:

Approximately 80 pounds or 36.3 kg

Power Input:

100 to 240 V~, 3 A
300 watts or less. Main supply voltage fluctuations not to exceed 15% of the nominal supply voltage.

Temperature:

Operating: 15° to 30°C
Storage (non-operating):-10° to 60°C

Humidity:

Operating: 10-90% RH, non-condensing
Storage (non-operating):10% to 95% RH

Other:

Pollution degree, 2

Installation category, II

Electrical Supply

The electrical supply shall meet the input specified on the instrument label. Voltage fluctuations shall not exceed 15% nominal supply voltage.

Altitude

<2000 m

Reagents, Equipment, and Supplier Contact Information - GCAS

Master List - Consumables, Reagents, and Equipment

You will need the following reagents and supplies to complete the target preparation on the GeneChip Array Station system. The reagent quantities listed are for one plate of 96 well reactions. For users of the Beckman® Biomek® FXᵖ Target Prep Instrument, please refer to the GeneChip® HT 3' IVT Expresss Method on the Beckman FXᵖ Site Preparation Guide (P/N 702895).

IMPORTANT: All labware, including pipettes, must be RNase/DNase-free.

Instruments

Table K.1 Instruments

Instruments	Supplier	P/N	Quantity / Run
GeneChip® Array Station	Affymetrix	00-0162 (110V) or 00-0235 (220v)	1
GeneChip® HT Scanner	Affymetrix	00-0172	1
Allen wrench (2.5mm)	Multiple		1
Dual Channel Thermocouple	VWR International	61220-605	1
12-multichannel 20 µL Pipetman	Rainin	L12-20	1
12-multichannel 200 µL Pipetman	Rainin	L12-200	1
8-multichannel 20 µL Pipetman	Rainin	L8-20	1
8-multichannel 200 µL Pipetman	Rainin	L8-200	1
Auto-sealing microplate lid, arched, wide tab	Bio-Rad	MSL 2032	4
V-Tech Cold Reagent Block	Affymetrix	11-1541	1
SPRIPlate 96R Magnet Plate	Agencourt	000219	1
Gripper Pads	Caliper LifeSciences	52071	8
Heatblock	Multiple		
Microseal P pads ADHESIVE	Bio-Rad	MSP-1001	1 (10 seals)
Multidispensing pipette - 1,000 µL	Rainin	E3-1000	
Multidispensing pipette - 200 µL	Rainin	E3-200	
Sealing Roller	Bio-Rad	MSR-0001	
Anti-Static Gun	Affymetrix	74-0014	
15 mL RNAse-free bottle	Various		

Consumables – Target Preparation

Table K.2 Consumables List for Target Preparation

Item	Source	P/N	Quantity / Run
Affymetrix® HT GCAS Accessory Kit for Target Preparation Containing: ▫ 96-Well Hard-Shell PCR Plate (5) ▫ Costar Polystyrene U-Bottom Plates (2) ▫ UV Star Plates (2) ▫ High Profile 300 mL Reservoir (1) ▫ Conical Bottom Tubes, 1.5 mL (12) ▫ Universal Microtiter Plate Lids (2) ▫ 12 Column, Partitioned, Deep Well reservoir	Affymetrix	901235 4 runs)	1
Arched Microplate Lids	BioRad	MSL-2032	3
BioRad P-pads	BioRad	MSP-1001	3
Cold Reagent Block template	Affymetrix	90-0798 (24 samples) or 90-0846 (96 samples)	1
Stacker tips 200 µL non-sterile	Caliper LifeSciences	78641	1 box
DNAZap™	Ambion	9890	1 (2 bottles)
RNaseZap® wipes	Ambion	9786	1 (100 wipes)

Consumables – Hybridization Setup

Table K.3 Consumables List for Hybridization Setup

Item	Source	P/N	Quantity / Run
HT Hybridization Tray*	Affymetrix	202111	1
HT Stain Tray*	Affymetrix	900745	1
96-Well Hard-Shell PCR Plate	Bio-Rad	HSP-9601	1
Omnitray or Greiner Round Bottom Clear Polypropylene Plate	VWR E&K Scientific Products	4660-638 20261	1 1
Stacker Tips 200 µL non-sterile	Caliper LifeSciences	78642	3

** Stain and Hybridization Tray are packaged with the HT Array Plates.*

Consumables – Wash and Stain

Table K.4 Consumables List for Wash and Stain of HT Array Plate

Item	Source	P/N	Quantity / Run
HT Scan Tray*	Affymetrix	900746	1
HT Stain Tray*	Affymetrix	900745	3
HT Wash Tray*	Affymetrix	900752	5
Aluminum Foil Lids	Beckman	538619	1 pack
Quarter Reservoir Modules	Beckman	372790	2
Quarter Reservoir Frame	Beckman	372795	1
BD Falcon™ Test Tube, 14 mL	VWR International	60819-761	2
BD Falcon™ Test Tube, 5 mL†	VWR International	60819-728	1
Polypropylene Centrifuge Tubes with Caps, Sterile 50 mL	VWR International	20171-028	2
RNase-Free 1.5 mL Microfuge tube†	Ambion	12400	1
Round Bottom Clear Polypropylene Plate	E&K Scientific Products	20261	1
Stacker Tips 200 µL non-sterile	Caliper LifeSciences	78641	1 Box
96-Well Hard-Shell PCR Plate	Bio-Rad	HSP-9601	1
Omnitray	VWR International	4660-638	2

** HT Stain, Wash, and Scan Trays are packaged with the HT Array Plates.*

† or equivalent

Reagents – Total RNA Isolation

Table K.5 Reagent List for Total RNA Isolation

Material	Source	P/N
TRIzol® Reagent	Invitrogen Life Technologies	15596-018
Ethanol, 80% (stored at –20°C)	Multiple	
Glycogen (optional)	Ambion	9510
Pellet Paint® (optional)	Novagen	69049-3
QIAzol™ Lysis Reagent	QIAGEN	79306
RNeasy® Mini Kit	QIAGEN	74104
Sodium Acetate (NaOAc), 3M	Sigma-Aldrich	S7899

Reagents – Target Preparation

Table K.6 Reagent List for Target Preparation

Material	Source	P/N
GeneChip® HT 3' IVT Express Kit Components needed: ▫ aRNA Binding Buffer Concentrate ▫ RNA Binding Beads ▫ aRNA Wash Solution Concentrate ▫ aRNA Elution Solution ▫ 5X Array Fragmentation Buffer ▫ First-Strand Enzyme Mix ▫ First-Strand Buffer Mix ▫ Second-Strand Enzyme Mix ▫ Second-Strand Buffer Mix ▫ IVT Enzyme Mix ▫ IVT Labeling Buffer ▫ IVT Biotin Label ▫ Control RNA (1 mg/mL HeLa total RNA) ▫ Poly-A Control Stock ▫ Poly-A Control Dilution Buffer	Affymetrix	901225 (4 x 24 rxn) 901253 (96 rxn)
Nuclease-free Water	Ambion	9932
Ethanol, 100% (ACS reagent grade or equivalent)	Various	

Reagents – Hybridization, Wash and Stain

Table K.7 Reagent List for Hybridization, Wash, and Stain

Material	Source	P/N
GeneTitan Hybridization, Wash, and Stain Kit for 3' IVT Arrays	Affymetrix	901530
GeneChip® HT 3' IVT Express Kit Components needed: ▫ Control Oligo B2 ▫ 20X Hybridization Controls	Affymetrix	901225 (4 x 24 rxn), or 901253 (96 rxn)
Bovine Serum Albumin (BSA) solution (50 mg/mL)	Invitrogen Life Technologies	15561-020
EDTA Disodium Salt, 0.5M solution (100 mL)	Sigma-Aldrich	E7889
Herring Sperm DNA	Promega Corporation	D1811
MES hydrate SigmaUltra	Sigma-Aldrich	M5287
MES Sodium Salt	Sigma-Aldrich	M5057
Nuclease-free Water, 1 L	Ambion	9932
Surfact-Amps® 20 (Tween-20), 10%	Pierce Chemical	28320
TMAC (5M)	Sigma-Aldrich	T3411
Nuclease-free Water	Ambion	9932
20X SSPE (3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA)	Cambrex	51214
5M NaCl, RNase-free, DNase-free	Ambion	9760G
Anti-Streptavidin Antibody (Goat), Biotinylated	Vector Laboratories	BA-0500
Bovine Serum Albumin (BSA) solution (50 mg/mL)	Invitrogen Life Technologies	15561-020
Distilled Water	Invitrogen Life Technologies	15230-147
Goat IgG, Reagent Grade	Sigma-Aldrich	I 5256
PBS, pH 7.2	Invitrogen Life Technologies	20012-027
R-Phycoerythrin Streptavidin	Molecular Probes	S-866
Surfact-Amps® 20 (Tween-20), 10%	Pierce Chemical	28320

Supplier Contact Information

Table K.8 Supplier Contact Information

Source	Web Site
ABGene/Marsh	www.marshbio.com
Ambion	www.ambion.com
Affymetrix	www.affymetrix.com
Agencourt	www.agencourt.com
Aldrich	www.sigmaaldrich.com
Ambion	www.ambion.com
Audioadvisor.com	www.audioadvisor.com
Beckman	www.beckman.com
Bio-Rad	www.bio-rad.com
Caliper	www.caliperls.com
Cambrex	www.cambrex.com
Cole-Parmer	www.coleparmer.com
E&K Scientific Products	www.eandkscientific.com
Fisher Scientific	www.fishersci.com
Invitrogen Life Technologies	www.invitrogen.com
Molecular Probes	www.probes.com
Novagen	www.emdbiosciences.com/html/NVG/home.html
Phenix	www.phenix1.com
Pierce Chemical	www.piercenet.com
Promega Corporation	www.promega.com
QIAGEN	www.qiagen.com
Rainin	www.rainin-global.com
Sigma-Aldrich	www.sigma-aldrich.com
USA Scientific	www.usascientific.com
Vector Laboratories	www.vectorlabs.com
VWR International	www.vwr.com

Affymetrix Contact Information

When to Contact Technical Support

Under any of the following conditions, unplug the instrument from the power source and contact Affymetrix Technical Support:

- when the power cord is damaged or frayed;
- if any liquid has penetrated the instrument;

If the instrument must be returned for repair, call Affymetrix Technical Support.

IMPORTANT: Make sure you have the model and serial number.

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Santa Clara, CA 95051
USA

E-mail: support@affymetrix.com
Tel: 1-888-362-2447 (1-888-DNA-CHIP)
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Formats of Summary Reports from the GeneChip® Array Station

Each of the applications being written for the GeneChip® Array Station produces a summary report suitable for importing into a Laboratory Information Management System (LIMS). The report will tell how the operator chose to set up the run; what the plate barcodes were (if the operator turned barcode reading on); what errors occurred during the run; and, where applicable, which wells in the plate being processed are outside an acceptance criteria. To make importation uncomplicated, the report is a text file in a standard format easily parsed by LIMS software. Because some of the items in the report are expected to contain commas, using commas as delimiters would be undesirable, so the tab-delimited text-file format will be utilized over the comma-separated-variables format. In that format the text is divided into lines, and fields within a line are separated from each other by tab characters.

File Names

The summary-report files are given names that identify the application that generates them as well as the particular run of that application being summarized. Accordingly, the file names are constructed by appending the application name to “SummaryReport_”, following that with a date-and-time stamp constructed out of the starting time of the run, and appending “.txt” as the file extension. For example, the name of a report file generated by a run of application TP_0001 on April 3, 2006, at 4:30:31pm would be “SummaryReport_TP_0001_4_3_06_4_30_31_PM.txt” on a workstation set up to use the North American date-and-time format. Note that the underscore character is used in place of other punctuation to ensure that the file name conforms to operating-system rules.

File Contents

Thus far three applications have been written, all for gene expression: Target Preparation 0001, Hybridization Setup 0001, and Wash Stain 0001. The contents of the report files from all three applications are found in the following Tables.

Table M.1 Contents of Summary Report File for Target Preparation 0001

Line Number	Contents	Possible Values or *Example*
1	Application name	TP_0001
2	Name of Array Station user	*Doe, John*
3	Run start time	*4:30:31 PM*
4	Run start date	*Monday, April 03, 2006*
5	Run end time	*8:53:41 PM*
6	Run duration	*4:23:10*
7	Tracking ID	User-entered string*
8	Tip-spacers-used flag	YES or NO
9	Number of samples	8, 16, 24, …, 96
10	In-vitro transcription time	4, 8, or 16
11	Target destination	*One HT array plate*
12	First run step	*Primer annealing [2]*†
13	Last run step	*Hybridization mix [15]*†
14	Initial sample transfer	Manual or Automatic
15	Hold-after-IVT flag	YES or NO
16	cDNA kit lot number	User-entered value‡
17	IVT kit lot number	User-entered value‡
18	Barcode-reader-enabled flag	YES or NO
19	Total RNA barcode	Value read during run**
20	Unfragmented aRNA barcode	Value read during run**
21	Normalized aRNA barcode	Value read during run**
22	Fragmented aRNA barcode	Value read during run**
23	Hybridization-ready barcode	Value read during run**
24	Limits for pre-normalization yields	*25<tab>250*
25	Wells outside pre-normalization limits	*A01<tab>C12<tab>E05*
26	Robot errors	Tab-delimited set of numbers

* *The Tracking ID is a string entered by the user when the run is being set up. It may have up to forty characters and may also be blank.*

† *The names of the run steps are followed by their step numbers enclosed in square brackets.*

‡ *Kit lot numbers will typically be in barcodes read from the kits with a hand-held barcode reader attached to the workstation.*

** *These barcodes will be read from the indicated items during the run by a barcode reader on the deck of the robot provided that the barcode-reader-enabled flag is set to YES. If the flag is set to NO or if the attempt to read the barcode failed, a phrase to that effect will replace the barcode on the corresponding line of the report.*

Table M.2 Contents of Summary Report File for Hybridization Setup 0001

Line Number	Contents	Possible Values or *Example*
1	Application name	HYB_0001
2	Name of Array Station user	*Doe, John*
3	Run start time	*4:30:31 PM*
4	Run start date	*Monday, April 03, 2006*
5	Run end time	*8:53:41 PM*
6	Run duration	*4:23:10*
7	Tracking ID	User-entered string*
8	Tip-spacers-used flag	YES or NO
9	Number of arrays	24 or 96
10	Barcode-reader-enabled flag	YES or NO
11	Hybridization sample barcode	Value read during run†
12	HT array plate A barcode	Value read during run†
13	HT array plate B barcode	No plate B‡
14	Pre-hybridization flag	YES or NO
15	Hybridization-mix-transfer flag	YES or NO
16	Robot errors	Tab-delimited set of numbers

* *The Tracking ID is a string entered by the user when the run is being set up. It may have up to forty characters and may also be blank.*

† *These barcodes will be read from the indicated items during the run by a barcode reader on the deck of the robot provided that the barcode-reader-enabled flag is set to YES. If the flag is set to NO or if the attempt to read the barcode failed, a phrase to that effect will replace the barcode on the corresponding line of the report.*

‡ *This is a place holder for a future application that will perform hybridization setup for two HT array plates in one run.*

Table M.3 Contents of Summary Report File for Wash Stain 0001

Line Number	Contents	Possible Values or *Example*
1	Application name	WS_0001
2	Name of Array Station user	*Doe, John*
3	Run start time	*4:30:31 PM*
4	Run start date	*Monday, April 03, 2006*
5	Run end time	*8:53:41 PM*
6	Run duration	*4:23:10*
7	Tracking ID	User-entered string*
8	Tip-spacers-used flag	YES or NO
9	Number of arrays	24 or 96
10	Barcode-reader-enabled flag	YES or NO
11	HT array plate barcode	Value read during run†
12	Recovered sample plate barcode	Value read during run†
13	Robot errors	Tab-delimited set of numbers

* *The Tracking ID is a string entered by the user when the run is being set up. It may have up to forty characters and may also be blank.*

† *These barcodes will be read from the indicated items during the run by a barcode reader on the deck of the robot provided that the barcode-reader-enabled flag is set to YES. If the flag is set to NO or if the attempt to read the barcode failed, a phrase to that effect will replace the barcode on the corresponding line of the report.*

File Location

By convention, the applications will write the summary files into C:\Affymetrix\Reports\Data.