Affymetrix® Gene Profiling Reagents User Guide

For In Vitro Diagnostic Use.

P/N 702749 Rev. 1

For In Vitro Diagnostic Use.

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Introduction

Affymetrix® Gene Profiling Reagents are intended for the preparation of labeled complementary RNA target from purified total RNA from fresh or frozen clinical tissue specimens for hybridization to Affymetrix GeneChip® microarrays and the measurement of fluorescence signals of labeled RNA target using the Affymetrix GeneChip® Microarray Instrumentation System.

Intended for use with separately FDA-cleared Affymetrix GeneChip microarray assays specifying the use of Affymetrix Gene Profiling Reagents.

The Affymetrix Gene Profiling Reagents use the latest technology in RNA target preparation for microarray expression analysis. Three kits provide the complete solution for total RNA and probe array processing.

- Affymetrix RNA Control Kit (P/N 901285)
- Affymetrix Transcript Synthesis and Labeling Kit (P/N 901286)
- Affymetrix Transcript Detection Kit (P/N 901299)

These kits feature:

- 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 to a single day with appropriate inputs of total RNA
- Master mixes and a simple protocol for ease of use, convenience and a high rate of success
- Magnetic-bead cRNA purification for high recovery and ease of use.

Expression Analysis Overview

The following major steps outline the steps for using the Affymetrix® Gene Profiling Reagents, also shown in Figure 1.1:

- *Step A: Target Preparation*
- *Step B: Target Hybridization*
- *Step C: Probe Array Washing and Staining*
- *Step D: Probe Array Scan*

Step A: Target Preparation

This manual describes procedures using Gene Profiling reagent kits for preparing biotinylated target from purified eukaryotic total RNA samples suitable for hybridization to GeneChip expression probe arrays.

Double-stranded cDNA is synthesized from total RNA. An *in vitro* transcription (IVT) reaction is then done to produce biotin-labeled cRNA from the cDNA. After purification the cRNA is fragmented before hybridization. Refer to Chapter 2, *Procedure 1: Setting-up the Thermal Cycler* through *Procedure 8: Preparation of the cRNA Fragmentation Reaction*.

Step B: Target Hybridization

A hybridization cocktail is prepared, including the fragmented target, and probe array controls. It is then hybridized to the probe array during a 17±1 hour incubation. Refer to *Procedure 9: Preparation of the Target Hybridization Cocktail* on page 19.

Step C: Probe Array Washing and Staining

Immediately following hybridization, the probe array undergoes an automated washing and staining protocol on the fluidics station. Specific experimental information is defined using Affymetrix® Molecular Diagnostic Software (AMDS) on a PC-compatible workstation. The probe array type, sample description, and comments are entered and saved with a unique experiment name.

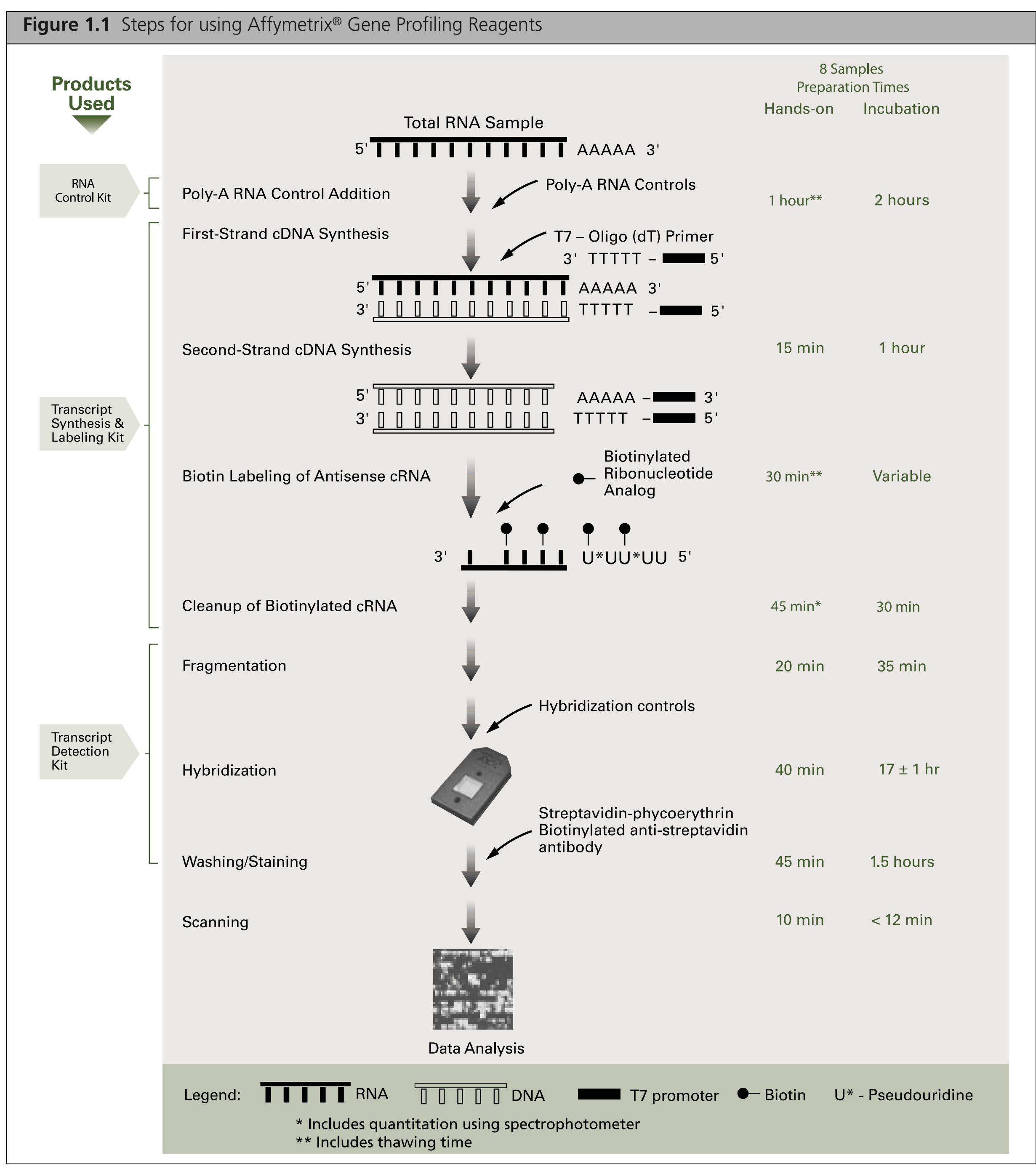
The fluidics station is then prepared for use by priming with the appropriate buffers. Refer to *Procedure 10: Probe Array Processing* on page 22. For more information on the fluidics station, refer to the *Affymetrix® Molecular Diagnostic Software User's Guide*.

Step D: Probe Array Scan

Once the probe array has been hybridized, washed, and stained, it is scanned. Each workstation running AMDS can control one scanner. The software defines the probe cells and computes an intensity for each cell. Each complete probe array image is stored in a separate data file identified by the experiment name and is saved with a data image file (.dat) extension. Refer to *Procedure 10: Probe Array Processing* on page 22.

Review the *Affymetrix® Molecular Diagnostic Software User's Guide* for safety precautions and for more information on using the scanner.

Figure 1.1 Steps for using Affymetrix® Gene Profiling Reagents



Precautions

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. Use powder-free gloves whenever possible to minimize introduction of powder particles into sample or probe array cartridges. 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.

Avoid microbial and nuclease contamination when handling the reagents.

Exercise standard precautions when obtaining, handling, and disposing of potentially carcinogenic reagents.

Dispose of unused reagents in accordance with your country, federal, state and local regulation.

Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.

Interfering Conditions

Proper storage and handling of reagents and samples is essential for robust performance.

Do not store enzymes in a frost-free freezer.

All laboratory equipment used to prepare the target during this procedure should be calibrated and carefully maintained to ensure accuracy. For example, incorrect measurement of reagents may affect the outcome of the procedure.

Do not pool reagents from different lots or from different bottles of the same kit.

Do not use a reagent kit after the expiration date.

Ensure the Relative Centrifugal Forces (RCF) of the centrifuge is 370xg during the centrifugation steps.

Dispose the used pipette tip(s) prior to proceeding to the next step to avoid contamination.

Materials Required

Reagents

Table 1.1 Reagent

Reagent	Supplier	P/N
Affymetrix® RNA Control Kit, containing: ▫ Poly-A Control ▫ Dilution Buffer	Affymetrix	901285
Affymetrix® Transcript Synthesis and Labeling Kit, containing: **Affymetrix® Transcript Synthesis and Labeling Kit A** ▫ 1st Strand Synthesis Buffer ▫ 2nd Strand Synthesis Buffer ▫ *In Vitro* Transcription Buffer ▫ Magnetic Beads ▫ Beads Wash Buffer ▫ Nuclease-free Water **Affymetrix® Transcript Synthesis and Labeling Kit B** ▫ 1st Strand Synthesis Enzyme Mix ▫ 2nd Strand Synthesis Enzyme Mix ▫ *In Vitro* Transcription Enzyme Mix ▫ RNA Label	Affymetrix	901286 901293 901298
Affymetrix® Transcript Detection Kit, containing: **Affymetrix® Transcript Detection Kit A** ▫ Pre-Hybridization Mix ▫ 2X Hybridization Mix ▫ Stain Cocktail 1 ▫ Stain Cocktail 2 ▫ DMSO ▫ Nuclease-free Water ▫ Array Holding Buffer ▫ 5X Fragmentation Buffer **Affymetrix® Transcript Detection Kit B** ▫ Wash Buffer A ▫ Wash Buffer B **Affymetrix® Transcript Detection Kit C** ▫ Oligo B2 ▫ 20X Hybridization Control	Affymetrix	901299 901307 901310 901312
Ethanol, 200 proof (to prepare the Wash solution)		

Laboratory Equipment

- Affymetrix GCS 3000Dx v.2 Instrument System with Data Transfer Server (P/N: 00-0349), or
- Affymetrix GCS 3000Dx v.2 Instrument System (P/N: 00-0334). Data Transfer Server for GCS 3000Dx v.2 available separately (P/N: 00-0345)
- Affymetrix GeneChip Hybridization Oven 645 (P/N: 00-0331)
- Thermal cycler capable of holding 96-well plates.
- LabLine Plate Shaker (P/N: 4625), or equivalent
- Heatblocks capable of holding temperatures from 45 to 99°C
- Eppendorf Centrifuge 5804 with A-2 DWP Rotor (P/N: 5804000.013) or Beckman Allegra6 centrifuge (P/N: BK366802), or equivalent
- Spectrophotometer
- Vortex Mixer
- Benchtop cooler kept in –15 to –30°C freezer
- 96-well Plate Magnetic Stand (for easy pipetting recommended magnets on the side of the wells)
- Single-channel electronic repeater pipette capable of dispensing 20 to 200 µL
- Multichannel pipette capable of dispensing 20 to 200 µL
- Single-channel pipettes capable of dispensing 0.5 to 10 µL, 2 to 20 µL, 20 to 100 µL, 100 to 1000 µL
- Brayer for sealing plates with aluminum adhesive foil

Miscellaneous Supplies

- 96-well plate compression pad
- Aluminum adhesive foil for sealing of 96-well plates
- 96-well PCR plate for incubation in thermal cycler, polypropylene
- 96-well U-bottom plate with lid for bead purification, untreated polystyrene
- Disposable two-compartment divided polystyrene reagent reservoirs, sterile, 25 mL capacity, for use in multichannel pipetting.
- Nuclease-free, non-stick 1.5 mL microcentrifuge tubes
- Nuclease-free, sterile, 1.5 mL microcentrifuge tubes
- Nuclease-free, sterile, amber 1.5 mL microcentrifuge tubes
- Polypropylene conical tubes, 15 mL
- Adhesive label dots
- Pipette tips, barrier-resistant, nuclease-free

Procedures for Using Affymetrix® Gene Profiling Reagents

Procedure 1: Setting-up the Thermal Cycler

NOTE: Turn on the thermal cycler at least 15 minutes before use. Refer to the thermal cycler user's manual for manufacturer's recommendation.

If using these procedures for the first time, it is recommended to set up and store the programs in the thermal cycler for each incubation. The specifications for each incubation are shown in Table 2.1.

Table 2.1 Thermal Cycler Programs

Step / Method	Incubation Program	Incubation Program	Hold Program	Reaction Volume
1st Strand cDNA Synthesis	42°C for 2 hours	4°C for 10 minutes	4°C hold	10 µL
2nd Strand cDNA Synthesis	16°C for 1 hour	4°C for 10 minutes	4°C hold	30 µL
IVT Reaction	40°C for 16 hours*		4°C hold	60 µL
Fragmentation	94°C for 35 minutes		4°C hold	30 µL

**Incubation time is specific for 100 ng of total RNA. The time should be adjusted based on the amount of starting total RNA used in these procedures. Refer to* *Table 2.5 on page 13*.

Refer to the thermal cycler user's manual for additional information on programming and operation.

Procedure 2: Preparation of Poly-A RNA Controls

Affymetrix® RNA Control Kit (P/N 901285) is used for this procedure.

IMPORTANT: Closely adhere to the recommendation below to obtain the desired final concentrations of the controls. Use non-stick Nuclease-free microcentrifuge tubes to prepare the dilutions.

Designed specifically to provide exogenous positive controls to monitor the entire target labeling process, a set of poly-A RNA controls is supplied in the RNA Control Kit. After the appropriate dilutions of the poly-A RNA controls, they are added to the total RNA and then amplified and labeled together. Examining the hybridization intensities of these controls on the probe array helps to monitor the labeling process.

The Poly-A Control and Dilution Buffer are provided with the kit to prepare the appropriate serial dilutions based on Table 2.2. This is a guideline when 100, 200, 500, or 1000 ng of total RNA is used as starting material. For other intermediate starting sample amounts, 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.

Instruction for Poly-A Dilution Calculation

If the starting amount of total RNA is between 100 ng and less than 250 ng, follow the preparation of the first (1:20), second (1:50) and third (1:50) serial dilutions as described in Table 2.2 and the paragraph describing the dilutions for 200 ng of total RNA, below. The formula provided is used to calculate the fourth dilution:

1: (500/starting amount of total RNA in ng)

For example, if using 125 ng the fourth dilution will be:

1: (500/125) = 1:4

If the starting amount of total RNA is between 250 ng and 1000 ng, follow the preparation of the first (1:20) and second (1:50) serial dilutions as described in Table 2.2 and the paragraph describing the dilutions for 200 ng of total RNA, below. The formula provided is used to calculate the third dilution:

1: (25000/starting amount of total RNA in ng)

For example, if using 600 ng, the third dilution will be:

1: (25000/600) = 1: 42

Table 2.2 Serial Dilutions of Poly-A Control

Starting Amount of Total RNA	Serial Dilutions				Volume to Add to the Total RNA
	First	Second	Third	Fourth	
100 ng	1:20	1:50	1:50	1:5	1 µL
200 ng	1:20	1:50	1:50	1:2.5	1 µL
500 ng	1:20	1:50	1:50		1 µL
1000 ng	1:20	1:50	1:25		1 µL

TIP: 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 controls dilutions for 200 ng of total RNA:

1. Label 4 non-stick Nuclease-free microcentrifuge tubes with1^{st}, 2nd, 3rd and 4th Dilution.
2. Thaw Dilution Buffer at room temperature. After the Dilution Buffer is thawed, pipet 38 µL of the Dilution Buffer in the 1st Dilution tube, 98 µL of the Dilution Buffer in the 2nd and 3rd Dilution tubes, and 6 µL of Dilution Buffer to the 4th Dilution tube. Return Dilution Buffer to the freezer. Keep tubes at room temperature.
3. Thaw the Poly-A Control at room temperature. After it has thawed, mix by flicking the tube and spin down to collect the liquid at the bottom of the tube. Immediately proceed to Step 4.
4. Add 2 µL of the Poly-A Control solution to the 38 µL of Dilution Buffer in the tube labeled 1st Dilution (1:20). Return the Poly-A Control to the freezer.
5. Mix thoroughly by flicking the tube and spin down to collect the liquid at the bottom of the tube.
6. Add 2 µL of the 1st Dilution to 98 µL of Dilution Buffer in the tube labeled 2nd Dilution (1:50).
7. Mix thoroughly by flicking and spin down to collect the liquid at the bottom of the tube.
8. Add 2 µL of the 2nd Dilution to 98 µL of Dilution Buffer in the tube labeled 3rd Dilution (1:50).
9. Mix thoroughly by flicking and spin down to collect the liquid at the bottom of the tube.
10. Add 4 µL of the 3rd Dilution to 6 µL of Dilution Buffer in the tube labeled 4th Dilution (1:2.5).
11. Mix thoroughly by flicking and spin down to collect the liquid at the bottom of the tube.
12. Use the 4th Dilution to prepare the solution described next in *Procedure 3: Preparation of 1st Strand cDNA Synthesis Reaction*. Discard 1st, 2nd, and 3rd Dilutions.

Procedure 3: Preparation of 1st Strand cDNA Synthesis Reaction

Affymetrix® Transcript Synthesis and Labeling Kit A (P/N 901293) and Kit B (901298) are used for this procedure.

NOTE: Turn on the thermal cycler at least 15 minutes before use. Refer to the thermal cycler user's manual for manufacturer's recommendation.

1. Prepare 1st Strand Master Mix at room temperature according to Table 2.3. Label a Nuclease-free 1.5 mL non-stick tube as *1st Strand Master Mix*.

Table 2.3 1st Strand Master Mix Preparation

Component	Working Master Mix Volumes Sufficient for 1 Reaction (V)	Working Master Mix Volumes Sufficient for 1 Reaction X 1.15 (V x 1.15)	Desired Number of Reaction(s) (R)	Total Volume Required (V x 1.15) x R
1st Strand Synthesis Buffer	4 µL	4.6 µL		
1st Strand Synthesis Enzyme Mix	1 µL	1.15 µL		
Diluted Poly-A Control (from Procedure 2)	1 µL	1.15 µL		
Total Volume	**6 µL**	**6.9 µL**		

A. Remove the 1st Strand Synthesis Buffer from the storage at 2 to 8°C. Mix the 1st Strand Synthesis Buffer by gentle vortexing, and briefly spin down to collect the contents at the bottom of the tube. Add the 1st Strand Synthesis Buffer (calculated based on Table 2.3) to the tube labeled as *1st Strand Master Mix*. Return remaining 1st Strand Synthesis Buffer to storage at 2 to 8°C.

B. Remove the 1st Strand Synthesis Enzyme Mix from the freezer using a benchtop cooler. Mix the 1st Strand Synthesis Enzyme Mix by gentle vortexing and spin down to collect the contents at the bottom of the tube. Add the 1st Strand Synthesis Enzyme Mix (calculated based on Table 2.3) to the tube labeled as *1st Strand Master Mix*. Return remaining 1st Strand Synthesis Enzyme Mix to storage in the freezer.

C. Transfer the appropriate amount of the diluted Poly-A Control from Procedure 2 to the tube labeled as *1st Strand Master Mix* (calculated based on Table 2.3). Discard the left over diluted Poly-A Control.

D. Mix the 1st Strand Master Mix well by gentle vortexing, and briefly spin down to collect the contents at the bottom of the tube.

2. Immediately transfer 6 µL of 1st Strand Master Mix (room temperature), to the bottom of the appropriate wells of the 96-well plate sitting on a plastic rack. Discard any remaining 1st Strand Master Mix.
3. Add 4 µL of the total RNA samples into the appropriate wells of the 96-well plate (total of 100 to 1000 ng per reaction; if the total RNA is contained in less than 4 µL, add Nuclease-free Water up to 4 µL). Gently mix by pipetting up and down several times.
4. Cover the plate with aluminum adhesive foil and carefully seal tops with a brayer.
5. Centrifuge at 370 x g for no more than 10 seconds at room temperature to collect the solution at the bottom of the wells.
6. Remove the plate from the centrifuge and transfer to a thermal cycler. Add the compression pad over the aluminum foil following the manufacturer's recommendation. Cover with the heated lid. Select the appropriate method for the 1st Strand cDNA Synthesis (refer to Table 2.1 on page 7). Start the method and confirm the appropriate volume of the reaction for this step: 10 µL.

7. Remove the plate from the thermal cycler after the completion of the 42°C incubation, within 10 minutes of incubation at 4°C.
8. Centrifuge at 370 x g for no more than 10 seconds at room temperature to collect the solution at the bottom of the wells. Remove plate from the centrifuge and keep at room temperature.
9. Immediately proceed to 2nd Strand cDNA synthesis as described in *Procedure 4: Preparation of 2nd Strand cDNA Synthesis Reaction* on page 12.

Procedure 4: Preparation of 2nd Strand cDNA Synthesis Reaction

Affymetrix® Transcript Synthesis and Labeling Kit A (P/N 901293) and Kit B (901298) are used for this procedure.

1. Prepare 2nd Strand Master Mix at room temperature according to Table 2.4. Label a Nuclease-free 1.5 mL non-stick tube as *2nd Strand Master Mix*.

Table 2.4 2nd Strand Master Mix Preparation

Component	Working Master Mix Volumes Sufficient for 1 Reaction (V)	Working Master Mix Volumes Sufficient for 1 Reaction X 1.10 (V x 1.10)	Desired Number of Reaction(s) (R)	Total Volume Required (V x 1.10) x R
2nd Strand Synthesis Buffer	18 µL	19.8 µL		
2nd Strand Synthesis Enzyme Mix	2 µL	2.2 µL		
Total Volume	**20 µL**	**22.0 µL**		

A. Remove the 2nd Strand Synthesis Buffer from the storage at 2 to 8°C. Mix the 2nd Strand Synthesis Buffer by gentle vortexing, and briefly spin down to collect the contents at the bottom of the tube. Add the 2nd Strand Synthesis Buffer (calculated based on Table 2.4) to the tube labeled as *2nd Strand Master Mix*. Return remaining 2nd Strand Synthesis Buffer to storage at 2 to 8°C.

B. Remove the 2nd Strand Synthesis Enzyme Mix from the freezer using a benchtop cooler. Mix the 2nd Strand Synthesis Enzyme Mix by gentle vortexing and briefly spin down to collect the contents at the bottom of the tube. Add the 2nd Strand Synthesis Enzyme Mix (calculated based on Table 2.4) to the tube labeled as *2nd Strand Master Mix*. Return remaining 2nd Strand Synthesis Enzyme Mix to the freezer.

C. Mix the 2nd Strand Master Mix well by gentle vortexing, and briefly spin down to collect the contents at the bottom of the tube. Immediately proceed to Step 2, below.

2. Carefully remove the aluminum adhesive foil from the plate sitting on a plastic rack.
3. Transfer 20 µL of 2nd Strand Master Mix into the side wall of the appropriate wells of the 96-well plate containing the 10 µL of 1st strand reaction. Discard any remaining 2nd Strand Master Mix.
4. Cover the plate with a new piece of aluminum adhesive foil and carefully seal tops with a brayer.
5. Centrifuge at 370 x g for no more than 10 seconds at room temperature to collect the solution at the bottom of the wells.
6. Remove the plate from the centrifuge and transfer to a thermal cycler.

   ! IMPORTANT: Do not cover the plate with the heated lid during the 16°C incubation, or turn the heat off (only some thermal cyclers have this capability).

   Select the appropriate method for the 2nd Strand cDNA Synthesis (refer to Table 2.1 on page 7). Start the method and confirm the appropriate volume of the reaction for this step: 30 µL.

7. Remove the plate from the thermal cycler after the completion of the 16°C incubation, within 10 minutes of incubation at 4°C.
8. Centrifuge at 370 x g for no more than 10 seconds at room temperature to collect the solution at the bottom of the wells. Remove plate from the centrifuge and keep at room temperature.
9. Immediately proceed to *Procedure 5: Preparation of the In Vitro Transcription (IVT) Reaction* on page 13.

Procedure 5: Preparation of the *In Vitro* Transcription (IVT) Reaction

Affymetrix® Transcript Synthesis and Labeling Kit A (P/N 901293) and Kit B (901298) are used for this procedure.

1. When programming the thermal cycler for the IVT reaction, determine the best time that conforms with the starting amount of total RNA. The times in Table 2.5 are for guidance only.

Table 2.5 Recommended Incubation Times

Starting Total RNA	Recommended Incubation Time
100 ng	16 hours
500 ng	4 to 8 hours
1000 ng	2 to 4 hours

2. Prepare IVT Master Mix at room temperature according to Table 2.6. Label a Nuclease-free 1.5 mL non-stick tube as *IVT Master Mix*.

Table 2.6 IVT Master Mix Preparation

	Working Master Mix Volumes Sufficient for 1 Reaction	Working Master Mix Volumes Sufficient for 1 Reaction X 1.10	Desired Number of Reaction(s)	Total Volume Required
Component	**(V)**	**(V x 1.10)**	**(R)**	**(V x 1.10) x R**
In Vitro Transcription Buffer	22 µL	24.2 µL		µL
RNA Label	2 µL	2.2 µL		µL
In Vitro Transcription Enzyme Mix	6 µL	6.6 µL		µL
Total Volume	**30 µL**	**33.0 µL**		µL

   A. Remove the RNA Label tube from the freezer and the *In Vitro* Transcription Buffer from the storage at 2 to 8°C. Keep RNA Label at room temperature to allow the solution to thaw.

   B. Mix the *In Vitro* Transcription Buffer by gentle vortexing and briefly spin down to collect the contents at the bottom of the tube. Add the *In Vitro* Transcription Buffer (calculated based on Table 2.6) to the tube labeled as *IVT Master Mix*. Return remaining *In Vitro* Transcription Buffer to storage at 2 to 8°C.

   C. After verifying that the RNA Label solution has completely thawed, mix by gentle vortexing and briefly spin down to collect the contents at the bottom of the tube. Add the RNA Label solution (calculated based on Table 2.6) to the tube labeled as *IVT Master Mix*. Return the RNA Label tube to storage in the freezer.

   D. Remove the *In Vitro* Transcription Enzyme Mix from the freezer using a benchtop cooler. Mix the *In Vitro* Transcription Enzyme Mix by gentle vortexing and briefly spin down to collect the contents at the bottom of the tube. Add the *In Vitro* Transcription Enzyme Mix (calculated based on Table 2.6) to the tube labeled as *IVT Master Mix*. Return the remaining *In Vitro* Transcription Enzyme Mix to storage in the freezer.

   E. Mix the IVT Master Mix well by gentle vortexing, and briefly spin down to collect the contents at the bottom of the tube. Immediately go to Step 3, below.

3. Carefully remove the aluminum adhesive foil from the plate sitting on a plastic rack. Keep plate at room temperature.

4. Transfer 30 µL of IVT Master Mix into the side wall of the appropriate wells of the 96-well plate containing the 30 µL of 2nd strand reaction. Discard any remaining IVT Master Mix.

5. Cover the plate with a new aluminum adhesive foil and carefully seal tops with a brayer.
6. Centrifuge at 370 x g for no more than 10 seconds at room temperature to collect the solution at the bottom of the wells.
7. Remove the plate from the centrifuge and transfer to a thermal cycler. Add the compression pad over the aluminum foil following the manufacturer's recommendation. Cover with the heated lid. Select the appropriate method for the IVT Reaction (refer to Table 2.1 on page 7 and Table 2.5 on page 13). Start the method and confirm the appropriate volume of the reaction for this step: 60 µL.
8. Remove the plate from the thermal cycler after the completion of the 40°C incubation.
9. Centrifuge at 370 x g for no more than 10 seconds at room temperature to collect the solution at the bottom of the wells. Remove plate from the centrifuge and keep at room temperature.
10. Proceed to purification of the cRNA as described in *Procedure 6: Purification of the cRNA from the In Vitro Transcription (IVT) Reaction* on page 15.

Procedure 6: Purification of the cRNA from the *In Vitro* Transcription (IVT) Reaction

Affymetrix® Transcript Synthesis and Labeling Kit A (P/N 901293) is used for this procedure.

IMPORTANT: Before starting the purification:

- **Aliquot 400 µL of Nuclease-free Water in a Nuclease-free 1.5 mL tube. This volume is sufficient to process 8 reactions.**
- **Place the Nuclease-free Water at 60°C for at least 10 minutes on a heatblock.**
- **Before the first use of the Beads Wash Buffer from the kit, add 12.6 mL of 100% ethanol to the bottle of Beads Wash Buffer and mix by inversion. Place a large check mark on the bottle, initial and date to indicate that this step has been completed.**

1. Carefully remove the aluminum adhesive foil from the plate sitting on a plastic rack.
2. Remove the bottle of Magnetic Beads from 2 to 8°C. Gently shake the bottle of Magnetic Beads to resuspend any magnetic particles that may have settled.
3. Transfer 975 µL of Magnetic Beads into the small compartment of a clean trough to process 8 reactions and return to storage at 2 to 8°C.

   This amount is sufficient to pipet 2 times FOUR 108 µL with an 8 or 12 multichannel pipette.
4. Resuspend the Magnetic Beads in the trough by pipetting the suspension up and down a few times. Draw 108 µL of Magnetic Beads with the pipette and dispense into each 60 µL IVT reaction mixture. Mix by gently pipetting up and down.
5. Using the multichannel pipette, transfer samples from the 96-well plate to an U-bottom 96-well plate. Place the plate cover over the plate.
6. Transfer the U-bottom plate to the plate shaker and shake at medium speed (setting 6 on the Labline Titer Plate Shaker) for 2 minutes at room temperature. Cover the plate with the lid before putting it on the shaker.

   IMPORTANT: The various brands and models of plate shakers do not have standardized settings. If using the plate shaker for the first time in these procedures, it is recommended to gradually increase the speed until reaching the required speed to avoid splashing solutions among wells or plates falling off the shaker. The speed should be high enough to keep the beads in solution.
7. Place the plate on the magnetic stand and allow the beads to pellet for 5 to 10 minutes until the solution is clear and the beads form a pellet against the magnet.
8. Once the mixture is transparent, using a multichannel pipette remove and discard the supernatant without disturbing the beads.
9. Remove the bottle of Beads Wash Buffer from 2 to 8°C. Ensure that ethanol has been added to the Beads Wash Buffer. Mix the Beads Wash Buffer by inversion and transfer 4.0 mL into the large compartment of a clean trough. This amount is sufficient to wash 8 samples using a multichannel pipette by pipetting out Beads Wash Buffer for 8 samples twice. Return the Beads Wash Buffer to storage at 2 to 8°C.
10. Leave the plate situated on the magnetic stand and wash each sample with Beads Wash Buffer two times as follows:

    A. From the trough, using a multichannel pipette take 200 µL of Beads Wash Buffer and add to the side wall of each sample well without disturbing the beads. Incubate for 25 to 35 seconds at room temperature.

    B. Using the multichannel pipette, aspirate off the supernatant without disturbing the beads and discard.

    C. Repeat wash steps Step A and Step B above once more. For the last wash ensure that all the supernatant is removed without disturbing the beads.

D. Allow the plate to air-dry for 5 to 7 minutes sitting on the magnetic stand. Do not cover the plate.

NOTE: Allow sufficient time for the wash solution to evaporate but do not over dry the beads.

11. Remove the plate from the magnetic stand.
12. Remove the Nuclease-free Water from the heat block at 60°C and immediately add 30 µL of Nuclease-free Water to each sample well using a repeater pipette. Add to the side wall of each well without disturbing the pellet.

! **IMPORTANT: Due to the high temperature of the Nuclease-free Water, when using the repeater pipette, allow for two extra 30 µL volumes: discard the first 30 µL before adding to the samples and do not use the last 30 µL of the elution solution.**
It is important to follow the recommendation described here to ensure that correct volumes are dispensed to each sample.

13. Cover the plate with the lid and transfer the U-bottom plate to a shaker. Shake at high speed for one minute (setting 10 on the Labline Titer Plate Shaker).
14. Check that the pellet of beads is fully dispersed. Pipette up and down to fully disrupt the pellet if needed.
15. Move the plate to the magnetic stand and allow beads to settle for 3 to 4 minutes. The solution should be clear and all the beads pelleted against the magnet.
16. Once the solution is transparent and the beads have settled, very carefully remove 30 µL of the supernatant without disturbing the pellet. Transfer the supernatant containing the eluted cRNA to a new 96-well plate or individual tubes.
17. Proceed to measure the cRNA concentration as described in *Procedure 7: Quantitation of the cRNA* on page 17 or store the cRNA sample at –80°C.

Procedure 7: Quantitation of the cRNA

Determine the concentration of the eluted cRNA by measuring its absorbance at 260 nm in a spectrophotometer.

1. If the eluted cRNA is frozen, thaw the sample. Once thawed, gently vortex the tube and briefly spin down to collect the contents at the bottom of the tube.
2. Measure the concentration of the cRNA by following the spectrophotometer manufacturer recommendation. If concentration is outside of the recommended range, perform a dilution using Nuclease-free water and measure concentration again.
3. To calculate total cRNA yield, apply the formula below:

   cRNA yield (µg)= [cRNA concentration (ng/µL)/1000] x eluate (µL) x d

   where:

   cRNA concentration is the measurement taken from the spectrophotometer in ng/µL

   eluate is the volume in µL obtained from the elution of the beads

   d is the dilution factor applied to the sample if it was needed.
4. When using total RNA as starting material, an adjusted cRNA yield must be calculated to reflect carryover of unlabeled total RNA. Using an estimate of 100% carryover, use the formula below to determine adjusted cRNA yield:

   adjusted cRNA yield = cRNA yield (µg) - (total RNAi)

   where:

   cRNA yield (µg) is the amount of cRNA calculated in Step 3.

   total RNAi is the initial amount of total RNA (µg)

   Example: Starting with 100 ng of total RNA and assuming a cRNA yield of 30 µg, therefore, adjusted cRNA yield = 30 µg cRNA - (0.1 µg total RNA) = 29.9 µg.

   Use adjusted yield in *Procedure 8: Preparation of the cRNA Fragmentation Reaction*.

NOTE: The cRNA yield will depend on the amount, sample type and the quality of the total RNA used in the reaction. It is common to observe different cRNA yields from equal amounts of total RNA from different sample types or from different preparations from the same sample type.

Procedure 8: Preparation of the cRNA Fragmentation Reaction

Nuclease-free Water and the 5X Fragmentation Buffer from the Transcript Detection Kit A (P/N 901307) are used for this procedure. If the cRNA was stored at –80°C, allow the cRNA to thaw completely at room temperature.

1. Mix the 5X Fragmentation Buffer by gentle vortexing, and briefly spin down to collect the contents at the bottom of the tube.
2. Table 2.7 shows the fragmentation reaction mix for cRNA samples at a final concentration of 0.5 µg/µL. Using the adjusted cRNA yield from Procedure 7, Step 4, calculate the volume of cRNA required to add 15 µg to the fragmentation reaction.

Table 2.7 Sample Fragmentation Reaction Preparation

Component	Amount or Volume
cRNA	15 µg
5X Fragmentation Buffer	6 µL
Nuclease-free Water (variable)	to 30 µL final volume
Total Volume	**30 µL**

3. Set up the reaction in a 0.2 mL strip tube.
4. Mix by gentle vortexing, and briefly spin down to collect the contents at the bottom of the tube.
5. Transfer the strip tubes to a thermal cycler. Select the appropriate method for the Fragmentation (refer to Table 2.1 on page 7). Cover with the heated lid. Start the method and confirm the appropriate volume of the reaction for this step: 30 µL.
6. Proceed to *Procedure 9: Preparation of the Target Hybridization Cocktail* on page 19.

Procedure 9: Preparation of the Target Hybridization Cocktail

Affymetrix® Transcript Detection Kit A (P/N 901307) and Kit C (P/N 901312) are used for this procedure.

This section describes preparation for a 49-format array.

NOTE: DMSO will solidify when stored at 4°C. Please ensure that the reagent is completely thawed prior to use.

NOTE: Set the temperature of heatblocks to 45°C, 65°C and 99°C.

1. Remove Oligo B2 and 20X Hybridization Control from the freezer and thaw at room temperature.

   ! **IMPORTANT: It is imperative that stocks of 20X Hybridization Control are heated to 65°C for 5 minutes to completely resuspend the cRNA before aliquoting.**

2. Prepare Hybridization Master Mix at room temperature for one or multiple probe arrays as outlined in Table 2.8.

Table 2.8 Hybridization Master Mix

Component	Working Master Mix Volumes Sufficient for 1 Probe Array	Working Master Mix Volumes Sufficient for 1 Probe Array X 1.10	Desired Number of Probe Arrays	Total Volume Required	Final Dilution / Concentration
	(V)	(V x 1.10)	(R)	(V x 1.10) x R	
Oligo B2 (3 nM)	4.2 µL	4.62 µL			50 pM
20X Hybridization Control (*bioB*, *bioC*, *bioD*, *cre*)	12.5 µL	13.75 µL			1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	125 µL	137.5 µL			1X
DMSO	25 µL	27.5 µL			10%
Nuclease-free Water	58.3 µL	64.13 µL			
Total Volume	**225.0 µL**	**247.5 µL**			

   A. Aliquot 225 µL of the Hybridization Master Mix into a Nuclease-free 1.5 mL tube.
   B. Add 25 µL of fragmented cRNA from Procedure 8, Step 6 to prepare the Hybridization Cocktail for one probe array. Final concentration of cRNA in the Hybridization Cocktail is 0.05 µg/µL

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

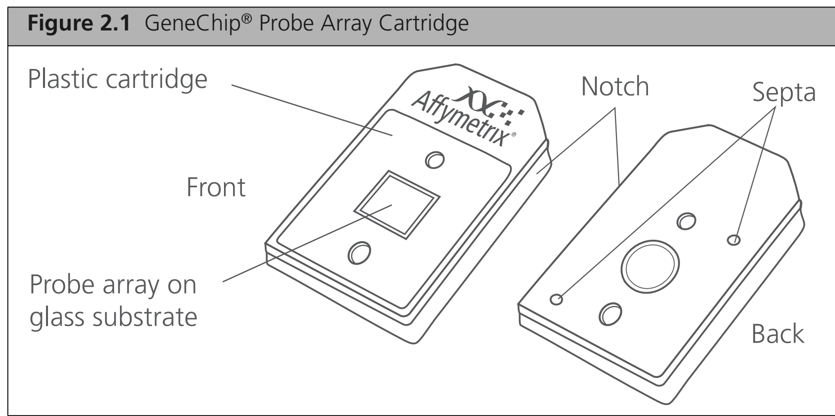
NOTE: It is important to allow the probe arrays to equilibrate to room temperature completely. Specifically, if the rubber septa are not equilibrated to room temperature, they may be prone to cracking, which can lead to leaks.

4. Heat the hybridization cocktail to 99°C for 5 minutes in a heatblock.

5. Meanwhile, wet the probe array with 200 µL of Pre-Hybridization Mix by filling it through one of the septa.

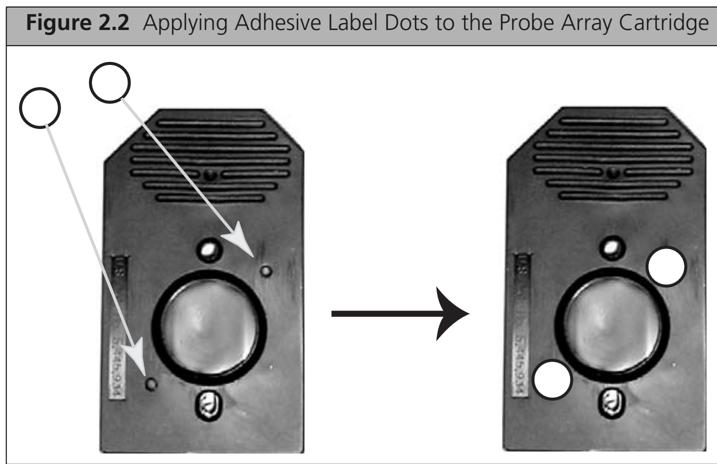
NOTE: Each probe array has two septa (see Figure 2.1 for location). In order to fill the probe array, first vent the probe array chamber by inserting a clean, unused pipette tip into one of the septa; then insert the pipette tip of a micropipettor into the remaining septum to fill.

Figure 2.1 GeneChip® Probe Array Cartridge



6. Incubate the probe array filled with Pre-Hybridization Mix at 45°C for 10 minutes at 60 rpm in the hybridization oven.
7. Transfer the hybridization cocktail that has been heated at 99°C, in Step 4 above, to a 45°C heatblock for 5 minutes.
8. Spin the hybridization cocktail at maximum speed in a microcentrifuge for 5 minutes at room temperature to collect any insoluble material from the hybridization mixture.
9. Remove the probe array from the hybridization oven. Vent the probe array with a clean pipette tip and extract the Pre-Hybridization Mix from the probe array with a micropipettor. Refill the probe array with 200 µL of the clarified hybridization cocktail from Step 8, avoiding any insoluble matter at the bottom of the tube.
10. Carefully apply one adhesive label dot to each of the two septa. Press to ensure that the label dots remain flat. If the adhesive label dots do not apply smoothly, that is, if you observe bumps, bubbles, tears, or curled edges, do not attempt to smooth out the label dot. Remove the label dot and apply a new one. See Figure 2.2.

Figure 2.2 Applying Adhesive Label Dots to the Probe Array Cartridge



11. Place probe array into the hybridization oven, set to 45°C. Rotate at 60 rpm.

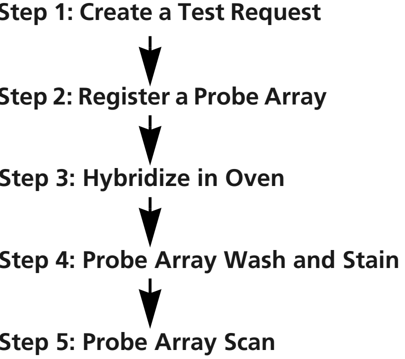
NOTE: To avoid stress to the motor, load probe arrays in a balanced configuration around the axis.

12. Hybridize for 17 ± 1 hours.
13. During the latter part of the 17-hour hybridization, proceed to *Procedure 10: Probe Array Processing* on page 22 to prepare reagents for the washing and staining steps required immediately after completion of hybridization.

Procedure 10: Probe Array Processing

Affymetrix® Transcript Detection Kit A (P/N 901307) and Kit B (901310) are used for this procedure.

The Affymetrix Molecular Diagnostics Software (AMDS) automates instrument control and provides a central infrastructure to acquire and track test requests. The following steps are required for processing a probe array.



Step 1: Creating the Test Request

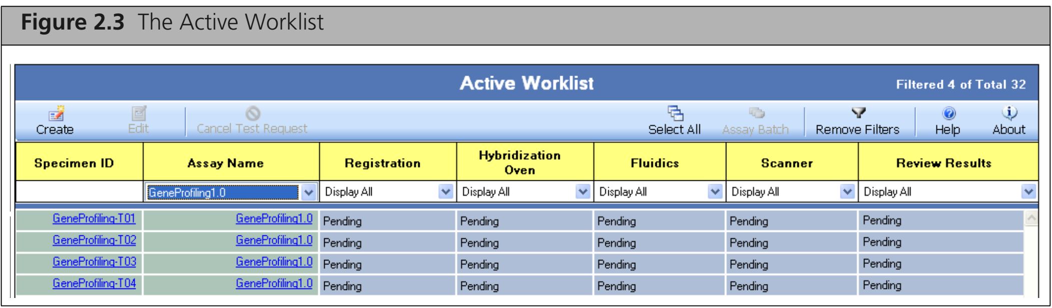
Before you can process a specimen on AMDS, you must create a Test Request (TR) in the system. You manually create the test request using the Create Test Request function within the Active Worklist in AMDS.

The test request will have a Specimen ID, which identifies the patient's specimen, and the name of the assay type that AMDS will process (Assay Name). AMDS will create a TestRequestID and manage the assay using this ID. You can view, but not edit, the TestRequestID once a probe array is associated with the Specimen ID.

1. If you are not already in the Active Worklist window, select **Active Worklist** from the left workflow panel.

   The Active Worklist panel opens (Figure 2.3).

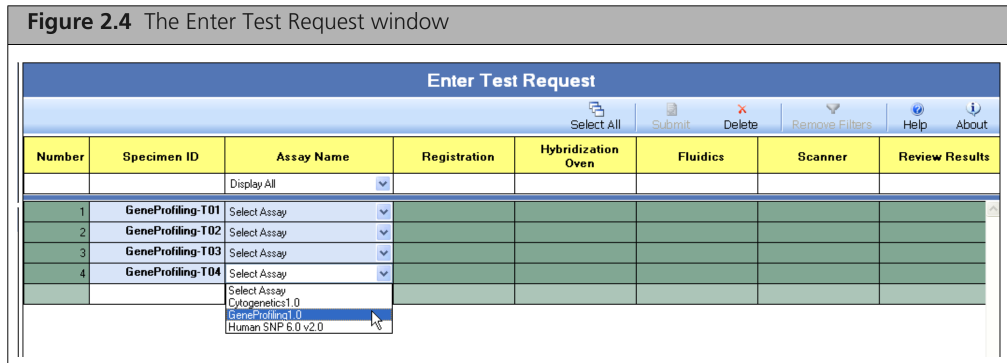
Figure 2.3 The Active Worklist



2. Click the **Create** button Create on the toolbar of the Active Worklist.

   The Enter Test Request screen appears (Figure 2.4).

Figure 2.4 The Enter Test Request window



3. In the Enter Test Request screen enter a **Specimen ID** and select **Gene Profiling Assay** from the **Assay Name** drop-down list.
4. Enter additional **Specimen ID** and **Assay Name** combinations for each probe array to be processed.
5. After all probe arrays have been entered, click the **Submit** button in the Enter Test Request screen toolbar. The Enter Test Request screen closes.

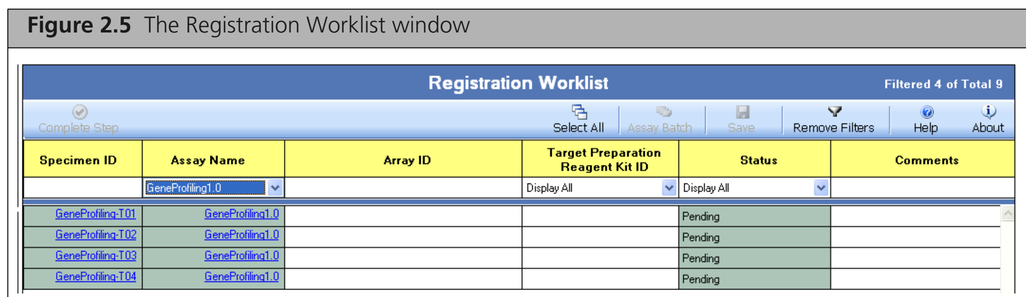
Step 2: Registering a Probe Array

Once you have created a test request, the next step in the workflow is registration. The registration step is where you associate the test request with a specific and unique probe array (Array ID). The **Array ID** identifies the unique ID of a physical array that comprises the probe array.

1. Click the **Registration** button in the left workflow panel.

   The Registration Worklist appears in the center panel (Figure 2.5).

Figure 2.5 The Registration Worklist window



2. Highlight the desired test request record (under the **Specimen ID** column) and scan the barcode on the probe array.

   The AMDS locates the proper field and adds the information.

   NOTE: To manually enter information, click in the **Array ID** field and manually enter the appropriate Array ID barcode.

3. Optional - Entering reagent lot numbers:
   Select one or more test requests and enter the same reagent lot number for all test requests by scanning the barcode on the reagent kit.

   NOTE: To manually enter information, shift+click and select all the test requests. Select one **Target Preparation Reagent Kit ID** field of a test request, enter the value manually, click **Enter** and AMDS will fill in all the selected test requests.

4. Click **Save** button in the Registration Worklist toolbar to save your registration.

5. Click the **Complete Step** button [Complete Step] in the Registration Worklist toolbar to move the test requests with complete information from the Target Registration Worklist to the next step in the workflow, Hybridization Oven step.

Step 3: Hybridizing a Probe Array

When probe array hybridization is performed in the Affymetrix Hybridization Oven 645, the AMDS manages the hybridization by using a separate workflow step with a separate worklist. The system monitors the oven temperature and duration during the hybridization process. Follow instructions in this section for hybridizing the test request.

NOTE: The procedure outlined in this section assumes that your workstation is connected to the Affymetrix Hybridization Oven 645. Refer to the *GeneChip Hybridization Oven 645 User's Guide*, P/N 08-0255, for detailed instructions.

NOTE: The Hybridization Oven 645 needs to be turned ON at least 30 minutes prior to hybridization for the oven to reach the set temperature for the procedure. If the oven is monitored via AMDS, the software will not allow the hybridization to start if the oven temperature has not reached within the specified temperature range of the procedure.

Figure 2.6 The Hybridization Worklist button

Hybridization Procedure

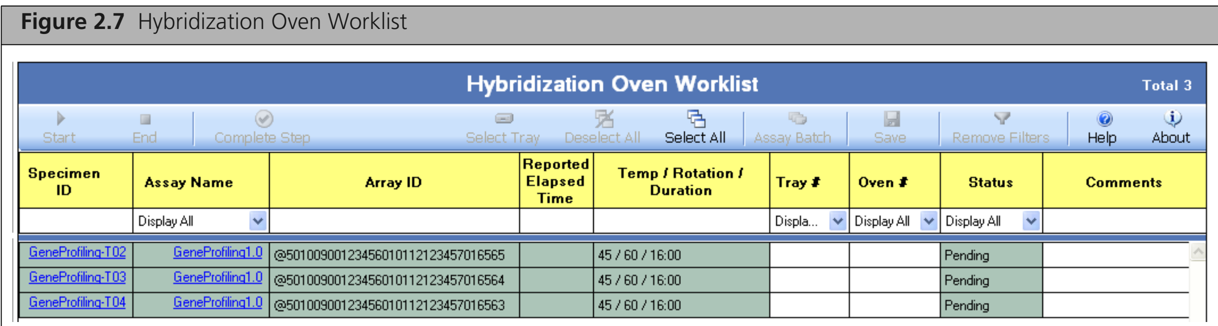
IMPORTANT: If you are using the Hybridization Oven 645, you must manually enter the oven number. Optionally, the Tray # can also be entered for tracking purposes. If you are using a hybridization oven that is not connected to the system, manually enter "E" for the external oven in the Oven # field before starting the hybridization incubation.

1. Click the **Hybridization Oven** button from the worklist (Figure 2.6).

   The Hybridization Oven Worklist appears (Figure 2.7).

   NOTE: You cannot change any parameter in the Time/Rotation/Duration field. Technicians set these when they originally designed the particular procedure.

Figure 2.7 Hybridization Oven Worklist



2. Associate test requests with a particular tray (optional).
   - A. Shft+click or Ctl+click to select several test requests.
   - B. Ctl+click in the **Tray** # field.
   - C. Enter the tray number.
   - D. Press **Enter**.

     AMDS associates all the selected test requests with the tray number.
   - E. If you want to select all the test requests that were earlier associated with a particular tray, select one test request with the newly added tray number and click the **Select Tray** button. All the test requests associated with that tray will be selected.
3. Associate test requests with a particular oven.
   - A. Shft+click or Ctl+click to select several test requests
   - B. Ctl+click in the **Oven** # field.
   - C. Enter the oven number. If you are using the Hybridization Oven 645, this number may be either 1 or 2 depending on your oven configuration.
   - D. Press **Enter**.
     AMDS associates all the selected test requests with the oven number.

     NOTE: If you are using a third party oven or an oven that is not connected to the system, enter "E" for external in the Oven # field
4. Select one or more test requests.

   All test requests that run at the same time in the same oven must have the same required temperature, rotation and duration.
5. Place the probe arrays in the tray then in the oven. Refer to your oven documentation for detailed instructions if necessary.

6. Click the **Start** button from the Hybridization Oven Worklist toolbar.

   If you are using the GeneChip® Hybridization Oven 645, AMDS will constantly display the status of the oven in the **Device Status** panel on the right side of the worklist, Figure 2.7.

7. Once the hybridization is complete, select the specimens that have completed hybridization and click the **End** button from the Hybridization Oven Worklist toolbar.

! **IMPORTANT: The color of the reported elapsed hybridization time will be yellow if the time is less than the minimum time required by the assay parameters. If the elapsed time falls within the acceptable range (determined by the assay), the color will be green. If the elapsed time is greater than that required by the assay parameters, the color will be red.**

8. Select **Complete Step** from the Hybridization Oven Worklist toolbar to advance the test request to the next step in the workflow.

9. When the system completes the hybridization, you can remove the probe array and proceed to *Step 4: Probe Array Wash and Stain*.

Step 4: Probe Array Wash and Stain

After 17± 1 hours of hybridization remove the probe array from the hybridization oven. Remove the adhesive label dots and vent the probe array by inserting a clean pipette tip into one of the septa, and extract the hybridization cocktail with a pipettor through the remaining septum. Refill the probe array completely with 250 µL of Wash Buffer A.

NOTE: If necessary, at this point the probe array can be stored at 4°C, protected from light, for up to 3 hours before proceeding with washing and staining. Equilibrate to room temperature before washing and staining.

The wash and stain procedure takes approximately 90 minutes to complete.

Preparing the Stain Reagents

Prepare the following reagents. Volumes given are sufficient for one probe array.

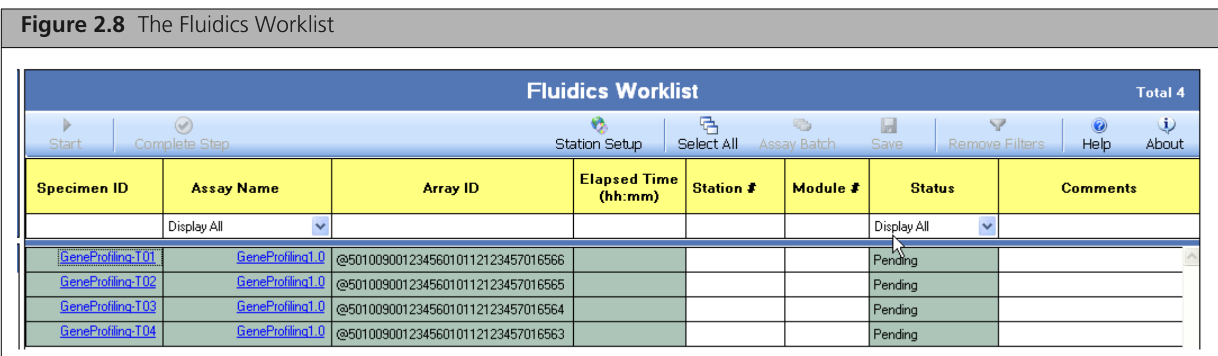
1. Remove Stain Cocktail 1, Stain Cocktail 2, and Array Holding Buffer from storage at 2° to 8°C.
2. Gently tap the bottles to mix well.
3. Aliquot the following reagents:
   - A. 600 µL of Stain Cocktail 1 into a 1.5 mL amber microcentrifuge vial.
   - B. 600 µL of Stain Cocktail 2 into a 1.5 mL (clear) microcentrifuge vial.
   - C. 800 µL of Array Holding Buffer into a 1.5 mL (clear) microcentrifuge vial.
4. Spin down all vials to remove the presence of any air bubbles.

NOTE: Stain Cocktail 1 is light-sensitive. Please be sure to use amber microcentrifuge vials when aliquoting.

Setting Up the Fluidics Station

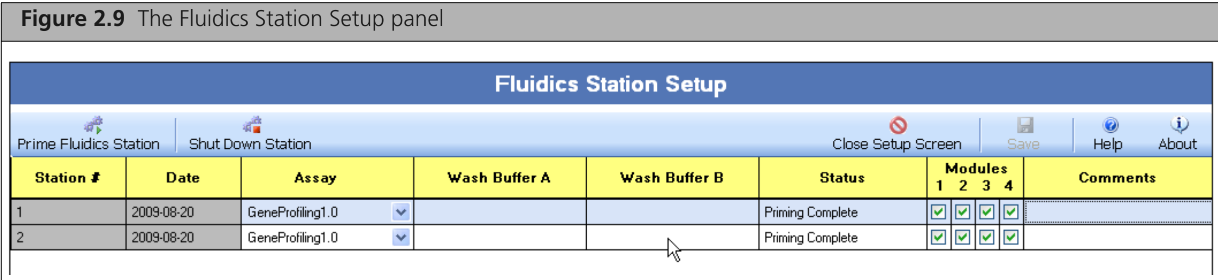
1. Click the Fluidics button in the left workflow panel.
   The Fluidics Worklist appears. (Figure 2.8).

Figure 2.8 The Fluidics Worklist



2. Click the **Station Setup** button Station Setup .
   The Fluidics Station Setup window appears (Figure 2.9).

Figure 2.9 The Fluidics Station Setup panel



3. For each Fluidics Station (**Station #**), select **Gene Profiling Assay** from the **Assay** drop-down list.
4. Add the Wash Buffer A and Wash Buffer B to the fluidics station and enter the names in the **Wash Buffer A** and **Wash Buffer B** fields.

> **NOTE: Use the module check boxes only if the fluidic station contains faulty modules.**

5. Click the **Close Setup Screen** in the Fluidics Worklist toolbar to return to the Fluidics Worklist.

Priming the Fluidics Station

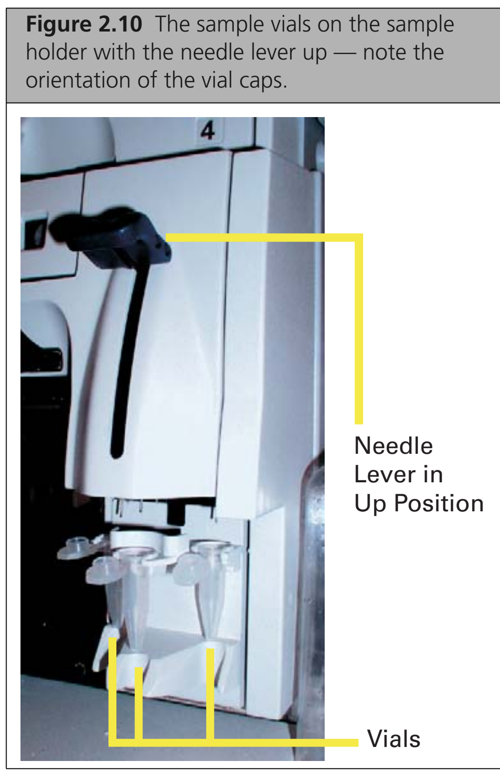
Priming fills the fluidics station lines with wash buffers designed for that assay and deionized water. You must prime the Fluidics Station FS450Dx before you can use it to run assay protocols.

You should prime the fluidics station:

- when you first start the fluidics station,
- when you change the wash solutions,
- before processing a cartridge if you have performed a shutdown on any module, and
- if the LCD window instructs you to run a prime protocol.

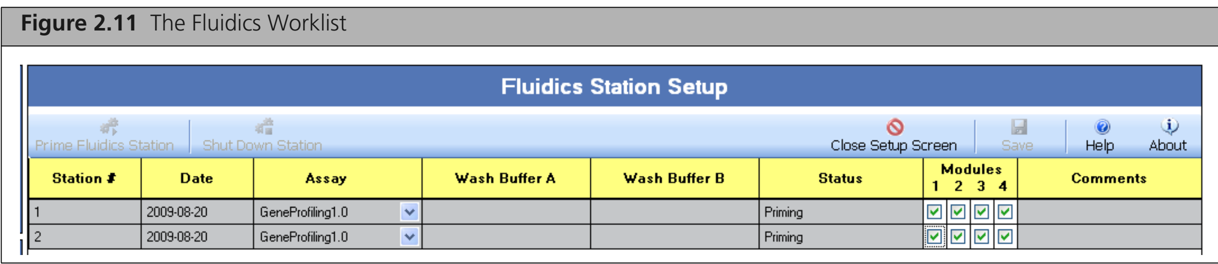
1. Check to ensure that all the wash lines are in the appropriate wash bottles. Please refer to the probe array package insert that came with the cartridge kit for the appropriate wash buffer solutions, or contact your Affymetrix technical support representative.
2. Load three empty standard 1.5 mL vials in the sample holders of each module that is to be primed (Figure 2.10).

Figure 2.10 The sample vials on the sample holder with the needle lever up — note the orientation of the vial caps.



3. In the Active Worklist (left workflow panel) click the **Fluidics** icon button.
   The Fluidics Worklist appears (Figure 2.11).

Figure 2.11 The Fluidics Worklist



Fluidics Station Setup

Prime Fluidics Station | Shut Down Station | Close Setup Screen | Save | Help | About

Station #	Date	Assay	Wash Buffer A	Wash Buffer B	Status	Modules 1 2 3 4	Comments
1	2009-08-20	GeneProfiling1.0			Priming	☑ ☑ ☑ ☑	
2	2009-08-20	GeneProfiling1.0			Priming	☑ ☑ ☑ ☑	

4. Click the **Station Setup** button.
   The Fluidics Station Setup window appears (Figure 2.12).

Figure 2.12 The Fluidics Station setup window

Fluidics Station Setup

Prime Fluidics Station | Shut Down Station | Close Setup Screen | Save | Help | About

Station #	Date	Assay	Wash Buffer A	Wash Buffer B	Status	Modules 1 2 3 4	Comments
1	2009-08-20	GeneProfiling1.0			Priming Complete	☑ ☑ ☑ ☑	
2	2009-08-20	GeneProfiling1.0			Priming Complete	☑ ☑ ☑ ☑	

5. In the Fluidics Station Setup window, select the **Station #** (top left button), the **Assay** and the **Modules** to be primed.
6. Click the **Prime Station** button.
   The **Status** field displays the priming message.

7. Follow the instructions in the LCD window ***on the fluidics station*** as the prime protocol progresses. The LCD window on the fluidics station and the fluidics station dialog box will indicate the status of the prime and when AMDS has completed the priming protocol.

After you have primed the fluidics station, you are ready to wash and stain a sample.

Using the Fluidics Station

Once you have set up and primed the fluidics station, you can now proceed to use the fluidics station in your assay.

1. Click the **Fluidics** button from the left workflow panel.

   The Fluidics Worklist appears.

2. If you are entering the information manually, select a test request record with the desired Array ID and enter the fluidics station number in the **Station #** field and module number in the **Module #** field for the test request.
3. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the down, or eject, position. When finished, verify that the cartridge lever is returned to the up, or engaged, position.
4. Remove any microcentrifuge vial remaining in the sample holder of the fluidics station module(s) being used.
5. If you are using a barcode reader, scan each probe array then immediately scan the fluidics station module that will process the probe array. The Array ID on the probe array will identify the proper test request registered to that Array ID.

   The status of the test request will change to **Ready**.

6. Select the test requests in AMDS.
7. Click the **Start** button on the toolbar of the Fluidics Worklist panel.
8. Follow the instructions on the LCD window ***on the fluidics station*** by placing the three experiment sample vials (the microcentrifuge vials) into the sample holders 1, 2, and 3 on the fluidics station.

   A. Place one vial containing 600 µL Stain Cocktail 1 in sample holder 1.

   B. Place one vial containing 600 µL Stain Cocktail 2 in sample holder 2.

   C. Place one vial containing 800 µL of Array Holding Buffer in sample holder 3.

   D. Press down on the needle lever to snap needles into position and to start the run.

   As the run begins, the Fluidics Station dialog box at the workstation terminal and the LCD window display the status of the washing and staining as the protocol progresses.

9. When the protocol is complete, the LCD window ***on the fluidics station*** displays the message **EJECT & INSPECT CARTRIDGE**.
10. Remove the probe arrays from the fluidics station modules by first pressing down the cartridge lever to the eject position.
11. Check the probe array window for large bubbles or air pockets.
    - If the probe array has no large bubbles, it is ready to scan on the GeneChip® Scanner. Pull up on the cartridge lever to engage washblock and proceed to *Step 5: Probe Array Scan* on page 30.
    - If bubbles are present, do the following:
      - Return the probe array to the probe array holder. Add 800 µL of Array Holding Buffer to vial #3 in sample holder 3. Follow instructions on the LCD window. Engage the washblock by gently pushing up on the cartridge lever to the engaged, or closed, position.
      - The fluidics station will drain the probe array and then fill it with a fresh volume of Array Holding Buffer. When it is finished, the LCD window will display **EJECT & INSPECT CARTRIDGE**. Again, remove the probe array and inspect it for bubbles. If no bubbles are present, it is ready to scan. Pull up on the lever to close the washblock and proceed to *Step 5: Probe Array Scan* on page 30.

NOTE: If the attempt to fill the probe array without bubbles is unsuccessful, the probe array should be filled manually with Array Holding Buffer using a micropipette. Excessive washing will result in a loss of signal intensity.

12. After removing a probe array from the probe array holder, the LCD window displays the message **ENGAGE WASHBLOCK**.
13. Engage the washblock by gently pulling up on the probe array lever to the up position.
14. Remove the previous vials and load three empty 1.5 mL vials.
15. The fluidics station automatically performs a Cleanout protocol. The LCD window indicates the progress of the Cleanout protocol.
16. When the fluidics station LCD window indicates **REMOVE VIALS**, the Cleanout protocol is complete.
17. Remove the sample vials from the sample holder.
18. If you do not scan the probe arrays right away, keep the probe arrays at 4°C and in the dark until ready for scanning.
19. If there are no more samples to hybridize, shut down the fluidics station following the procedure outlined in *Shutting Down the Fluidics Station* on page 34.
20. When the status indicates complete, click the **Complete Step** button to advance the test request to the next workflow step.

Step 5: Probe Array Scan

The scanner is also controlled by AMDS. The probe array is scanned after the wash protocols are complete. Make sure the laser is warmed up prior to scanning by turning it on at least 10 minutes before use. Refer to the AMDS online help and the scanner user's manual for more information on scanning.

WARNING: The scanner uses a laser and is equipped with a safety interlock system. Defeating the interlock system may result in exposure to hazardous laser light.

NOTE: You must have read and be familiar with the operation of the scanner before attempting to scan a probe array. Please refer to the *Affymetrix® Molecular Diagnostic Software User's Guide* (P/N 08-0261).

Handling the GeneChip® Probe Array

Before you scan the probe array, follow the directions in this section on handling the probe array. If necessary, clean the glass surface of the probe array with a non-abrasive towel or tissue before scanning. Do not use alcohol to clean glass.

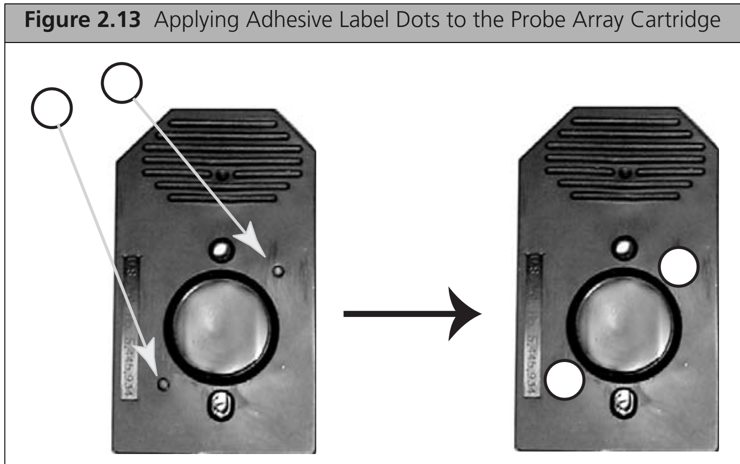
Before scanning the probe array cartridge, apply adhesive label dots to each of the two septa on the probe array cartridge to prevent the leaking of fluids from the cartridge during scanning.

IMPORTANT: Apply the dots just before scanning.

1. On the back of the probe array cartridge, clean excess fluid from around septa.

2. Carefully apply one adhesive label dot to each of the two septa. Press to ensure that the label dots remain flat. If the adhesive label dots do not apply smoothly, that is, if you observe bumps, bubbles, tears, or curled edges, do not attempt to smooth out the label dot. Remove the label dot and apply a new one. See Figure 2.13.

Figure 2.13 Applying Adhesive Label Dots to the Probe Array Cartridge

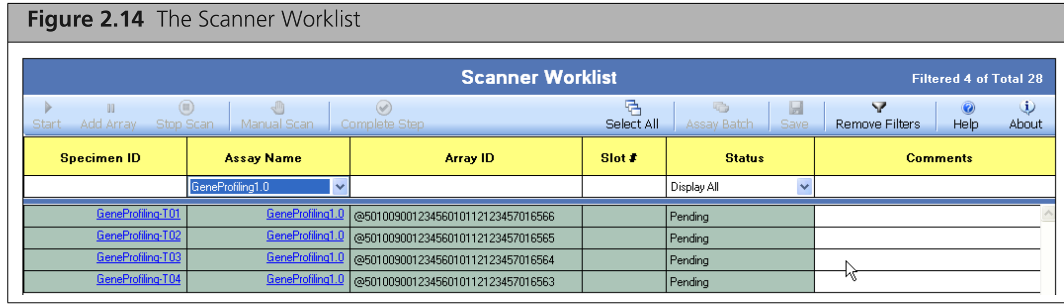


Scanning the Test Request Assay—the AutoLoaderDx

After you have hybridized, washed, and/or stained a probe array, you can now scan the probe array. AMDS automates control of the AutoLoaderDx and uses the assay information (stored in the Assay Name file) to manage the scanner settings for each type of assay. The software enables you to start a scan and collect intensity data. After the AutoLoaderDx completes the scan, AMDS creates the raw data for computing intensities.

1. Select **Scanner** from the left workflow panel.
   The Scanner Worklist in the center panel will appear (Figure 2.14).

Figure 2.14 The Scanner Worklist



2. Load the probe array into the AutoLoaderDx.
3. Click the **Start** button from the toolbar of the Scanner Worklist panel.

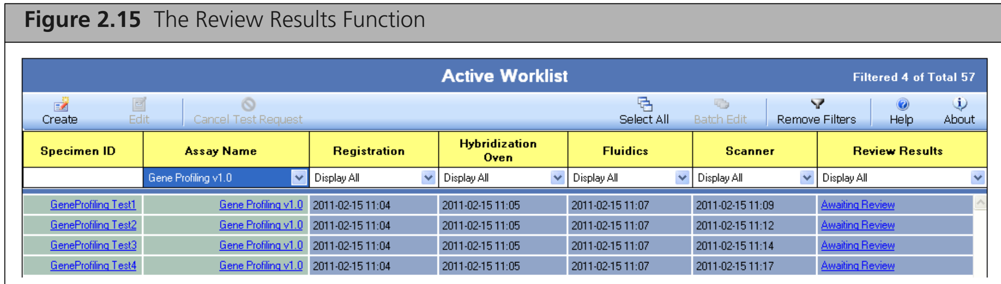
   It is not necessary to select test requests or enter any information or read any barcode. The AutoLoaderDx uses a built-in barcode reader to identify the Array ID and thus the test request that you had earlier registered with this Array ID.
4. When the scan completes, as indicated by the status for the test request showing complete, click the **Complete Step** button to advance the test request to the next step in the workflow.

NOTE: Certain assays may require additional information for each test request for that test request to run successfully. Failure to enter the additional information prior to completion of the scanning step will cause a failure while attempting to execute the analysis algorithm following the scanning step. The assay manufacturer should provide this information as part of the assay documentation. Refer to the *Affymetrix® Molecular Diagnostic Software User's Guide* (P/N 08-0261) for additional information.

Reviewing Test Results

After the scan, GRD and CEL files are generated automatically. Upon successful CEL file generation, data is transferred to the Data Transfer Server (DTS). A report is created which lists the files transferred and their destination. To view this report follow the instructions provided below.

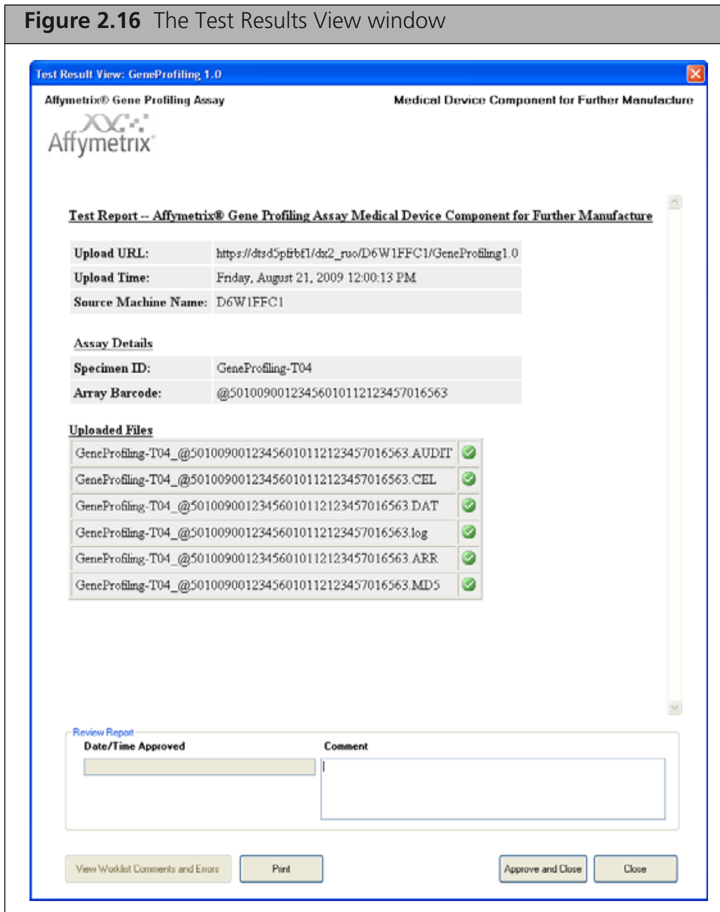
Figure 2.15 The Review Results Function



1. On the Active Worklist panel, in the **Review Results** column (Figure 2.15), select the **Awaiting Review** hyperlink for any of the completed test requests to view the Assay Report.

   The partner designed software will create a particular Results window (Figure 2.16) that is tailored for your particular assay. Each partner created software may display different Results windows.

Figure 2.16 The Test Results View window



The Test Results View window contains summary information on the assay.

In this example, the information can include but is not limited to:

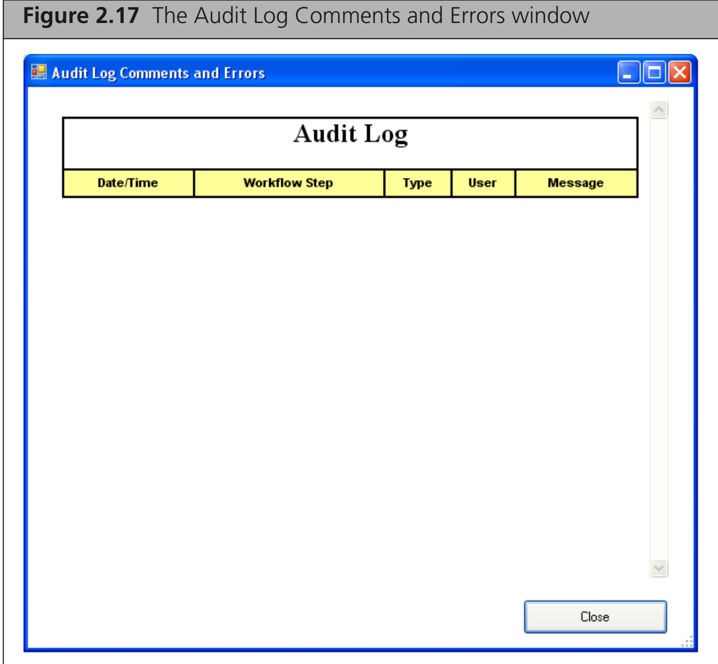
- **Upload URL**: the location in the workstation of the uploaded file
- **Upload Time**: the time and date of the upload
- **Specimen ID**

- **Patient Name**
- **Target Preparation Date**: when a technician prepared the target from the specimen
- **Test Date**: when the technician performed the AMDS assay
- **Array ID**: the ID of the specific GeneChip probe array that AMDS used to perform the assay
- **Uploaded Files**: the .cel, .dat and audit files that AMDS created from the assay

NOTE: This example is only for illustrative purposes. The actual Test Results View will be different for each partner assay. The Additional Info, the Assay Batch and the Assay Home (Assay Landing) windows may also be different. For information on the particular Test Results View for your test result, see the partner assay documentation for your assay.

2. Click the **View Worklist Comments and Errors** button in the lower left corner of the Test Results View window (Figure 2.16) to see any added information regarding the assay.
   The Comments and Errors window appears (Figure 2.17).

Figure 2.17 The Audit Log Comments and Errors window



3. Click the **Close** button.
4. In the **Comments** field of the Test Results View window, add any relevant comments regarding this assay. AMDS requires this to complete the review.
5. Click the **Print** button if you want to print the report.
6. Click **Approve**, **Reject** or **Close** (if you would like to exit without completing the review).

   AMDS moves the completed assay test request to the Non-Active Worklist and archives the assay results in a folder on the workstation.

Shutting Down the Fluidics Station

You should perform the Shutdown protocol at the end of a session. Do not keep the fluidics station ON if you will not use it again within the next 12 hours. This will reduce the risk of salt buildup in the instrument.

1. As with the prime protocol, the shutdown protocol requires three 1.5 mL (empty) vials for each module.
2. If no other hybridizations are to be performed, place wash lines into a bottle filled with deionized water.
3. From the **Fluidics** menu, select **Fluidics Station Setup**, then select the fluidics station to be shut down. Click the Shutdown Station button.
4. Follow the instructions on the fluidics station LCD screen to complete the shutdown process.
5. After the Shutdown protocol is complete, flip the ON/OFF switch on the side of the fluidics station to the **OFF** position.

IMPORTANT: To maintain the cleanliness of the fluidics station and obtain the highest quality image and data possible, a weekly bleach protocol and a monthly decontamination protocol are highly recommended.

Labeling Symbols

Table 2.9 Graphic Symbols for use in Labeling

Symbol / Label	Statement
REF	Part/Catalog Number
LOT	Lot Number
	Expiration Date YYYY–MM Kit will expire on the last day of the month.
	Temperature Limitation
Σ	Contains Sufficient for < n > Tests
Xi	Irritant
X	Hazards
	Consult Instructions for Use
	Manufacturer
IVD	*In vitro* Diagnostic Medical Device

Table 2.9 Graphic Symbols for use in Labeling

Symbol / Label	Statement
CE	European Conformity
EC REP	Authorized Representative in the European Community

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