QuickReferenceCard

affymetrix

PharmacoScan™ Assay 24-Array Format Manual Protocol Introduction and Stage 1A—Multiplex PCR and 1B—DNA Amplification

Introduction to PharmacoScan Assay 24-Array Format Manual Target Preparation

Running the PharmacoScan Assay 24-Array Format Manual Protocol requires the following sets of steps:

1. Genomic DNA Prep, described in the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286).
2. 24-Array Format Manual Target Prep, described in this QRC.
3. Array Processing, described in *GeneTitan® MC Protocol for Axiom® 2.0 Array Plate Processing QRC* (P/N 702988).

IMPORTANT: This QRC contains an abbreviated set of instructions used to perform target preparation. You must carefully read all the instructions in the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286) before performing manual target preparation.

NOTE: Array handling and processing protocols still require the use of a GeneTitan MC Instrument, as described in Chapter 5, Array Processing with the GeneTitan® Multi-Channel Instrument of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286) and the QRC (P/N 702988) described above.

Additional Notes:

- This manual assay format allows the user to run the PharmacoScan Assay for 24 Samples four (4) times using one PharmacoScan™ Reagent Kit 4x24 Reactions (P/N 902908). All samples will be processed in Columns 5, 7, and 9.
- This manual assay utilizes disposable divided reservoirs with a "trough within a trough" design which maximizes the amount of liquid accessible to pipette tips when using small amounts of reagent. During the assay, dispense reagents into either the 8-channel, 4-channel, or both sides, as instructed.
- We recommend that you prepare your genomic DNA sample plate in a clean room.
- Remove seals from plates carefully and discard used seals. Do not reuse seals.
- Use 8- or 12-channel pipettes for all sample transfers and additions of reagents and master mixes to the samples and GeneTitan trays.
- Change pipette tips after each sample transfer or addition to the samples.
- Unless otherwise specified, all reagent Modules are from the PharmacoScan Reagent Kit 4x24 Reactions (P/N 902908). Stage 1A requires the QIAGEN Multiplex PCR Plus Kit (P/N 206152) and is sufficient to process 24 samples four times.
- See Chapter 3 of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286) for a complete list of equipment and consumables required for each stage.

NOTE: PharmacoScan arrays require a total of 150 ng of gDNA. mPCR requires 50 ng and DNA Amplification requires 100 ng.

Stage 1A—Multiplex PCR

1. Preparation for Stage 1A—Multiplex PCR (mPCR)

Supplies Required

- mPCR Sample Plate (10 µL volumes of gDNA at 5 ng/µL, with Control DNAs)
- 10X Primer Mix from PharmacoScan Module A, –20°C, P/N 902896
- Reagents from QIAGEN Multiplex PCR Plus Kit, –20°C, P/N 206152

Instruments and Setup

- Approved thermal cycler
  - Must be programmed with **PharmacoScan mPCR** protocol
    - 95°C for 5 min
    - 95°C for 30 sec, 60°C for 180 sec, and 72°C for 45 sec - cycled 35 times
    - 68°C for 10 min
    - 4°C hold
  - Make sure heated lid option is used and thermal cycler is programmed to run in "9600 Mode" for ABI 9700, ABI Veriti™, ABI ProFlex™ and "Safe" mode for Eppendorf Mastercycler™ pro S.
- Plate centrifuge at room temperature

Reagent Preparation

1. Prepare reagents as shown in Table 1.1.

   NOTE: It is important that reagents are well mixed right before use.

Table 1.1 Reagent Preparation

Reagent	Quantity	Treatment
PharmacoScan 10X Primer Mix	1 tube	Thaw, vortex, pulse-spin, keep on ice
QIAGEN Multiplex PCR Master Mix, 2X	1 tube	Thaw, invert 10X to thoroughly mix, pulse-spin, keep on ice
QIAGEN Q-Solution, 5X	1 tube	Thaw, vortex, pulse-spin, keep on ice
QIAGEN RNase-free water	1 tube	Thaw, vortex, pulse-spin, keep on ice

2. Thaw mPCR Sample Plate

1. Bring mPCR Sample Plate to room temperature.
2. Vortex, pulse-spin, and place on ice or cold aluminum block.

3. Prepare mPCR Master Mix

1. Prepare mPCR Master Mix as shown in Table 1.2.
2. Add water, Q-Solution, and primers to microcentrifuge tube. Vortex and pulse spin.
3. Add QIAGEN 2X Multiplex PCR Master Mix to the tube and mix thoroughly but gently by pipetting the reaction mixture up and down five times using a P1000 pipette set to 700 µL.
4. Pulse spin and quickly proceed to next step.

Table 1.2 mPCR Master Mix

Reagent	28 reactions (>16% extra)
RNase-free Water	56 µL
Q-solution	112 µL
10X mPCR Primer Mix	112 µL
2X QIAGEN Multiplex PCR Master Mix	560 µL
TOTAL	**840 µL**

4. Add mPCR Master Mix to Samples

1. Transfer 100 µL of mPCR Master Mix to 8 wells of a strip tube.
2. Cap strip tubes and pulse spin.
3. Use multichannel pipette to transfer 30 µL of mPCR Master Mix to each sample in columns 5, 7, and 9 of mPCR Sample Plate.
4. Seal plate. Gently vortex. Pulse spin.
5. Load plate onto thermal cycler within five minutes.

5. Freeze QIAGEN Reagents

1. Return QIAGEN reagents tubes to –20°C for re-use.
2. Discard any remaining 10X Primer Mix and Control DNA reagents.

6. Run the PharmacoScan mPCR Thermal Cycler Protocol

Load the plate on the thermal cycler and run the **PharmacoScan mPCR** protocol

7. Freeze the mPCR Reaction Plate or Proceed

After the mPCR protocol finishes, you can either:

- Store the mPCR Reaction Plate at –20°C.
- Proceed to *Stage 2—Fragmentation and Precipitation* if *Stage 1B—DNA Amplification* is complete.

Stage 1B—DNA Amplification

1. Preparation for Stage 1B—DNA Amplification

Supplies Required

- Reagents from the PharmacoScan Reagent Kit 4x24 Reactions, Module 1, –20°C, P/N 902908
- Amplification Sample Plate (20 µL volumes of gDNA at 5 ng/µL)

Instrument Setup

- Pre-equilibrate the oven temperature at 37°C.
- Set the centrifuge temp at room temperature.

Reagent Preparation

1. Prepare reagents as shown in Table 1.3.

Table 1.3. Reagents Preparation

Reagent	Treatment
Axiom 2.0 Denat Soln 10X	Thaw, vortex, pulse-spin, and keep at room temperature
Axiom 2.0 Neutral Soln	Thaw (see Note below), vortex for 30 sec, and keep at room temperature
Axiom 2.0 Amp Soln	Thaw (see Note below), vortex for 30 sec, and keep at room temperature
Axiom Water	Thaw (see Note below), vortex and keep at room temperature
Axiom 2.0 Amp Enzyme	Flick tube 3X, pulse-spin, and keep in –20°C cooler until ready to use

NOTE: Allow ~1 hour for Axiom 2.0 Amp Soln to thaw on the benchtop at room temperature. If the solution is not completely thawed after 1 hour, vortex briefly and return to the benchtop to complete thawing. The bottles can also be thawed in a dish with Millipore water. The Axiom 2.0 Amp Soln and Neutral Soln must be vortexed for 30 sec to thoroughly mix before use.

2. Thaw Samples in Amplification Sample Plate:

A. Bring your gDNA samples to room temperature on the bench top.

B. Vortex, pulse-spin, and leave at room temperature.

NOTE: Carry out the master mix preparations and additions to the sample plate at room temperature.

2: Prepare Denaturation Master Mix

1. To a 1.7 mL microcentrifuge tube marked D MM, prepare the Denaturation Master Mix as shown in Table 1.4.

2. Vortex well.

Table 1.4. Denaturation Master Mix

Reagent	per Sample	Master Mix 24+
Axiom 2.0 Denat Soln 10X	2 µL	116 µL
Axiom Water	18 µL	1044 µL
Total Volume	**20 µL**	**1160 µL**

3: Add Denaturation Master Mix to Samples

1. Gently pour or pipet the Denaturation Master Mix into the 8-channel side of the reagent reservoir.

2. Add **20 µL of Denaturation Master Mix** to each sample in columns 5, 7, and 9, pipetting directly into the liquid. Do not mix by pipetting up and down.

3. Incubate the plate for **10 minutes** at room temperature. Seal, vortex, and pulse-spin in a room temperature centrifuge as soon as possible during the incubation period.

4. After incubation, immediately add the Neutralization Master Mix as described below.

4: Add Axiom 2.0 Neutral Soln to Samples

1. Gently pipet 3.64 mL of Neutral Soln into the 8-channel side of the reagent reservoir.
2. Add **130 µL of Axiom 2.0 Neutral Soln** to each sample in columns 5, 7, and 9, pipetting down the wall of the well. Do not mix by pipetting up and down.
3. Seal, vortex, and pulse-spin the Sample plate.
4. Proceed immediately to steps 5 and 6 below.

5: Prepare the Amplification Master Mix

Prepare Amplification Master Mix as shown in Table 1.5.

Table 1.5. Amplification Master Mix

Reagent	per Sample	Master Mix 24+
To a 15 mL tube marked Amp MM, add:		
Axiom 2.0 Amp Soln	225 µL	6.75 mL
Axiom 2.0 Amp Enzyme	5 µL	0.15 mL
Total Volume	**230 µL**	**6.90 mL**

Vortex the Amplification master mix well, then invert the tube 2 times, and then vortex again.

6: Add Amplification Master Mix to Samples

1. Slowly pour the Amplification Master Mix into the 8-channel side of the reagent reservoir labeled Amp MM.
2. Slowly add **230 µL Amplification Master Mix** to columns 5, 7, and 9 of the Sample plate, pipetting down the wall of the well. Do not mix by pipetting up and down.
3. Blot the top of the plate with Kimwipe, seal tightly, vortex twice, and spin the plate for one minute at 1000 rpm.
4. Place the sealed plate in an oven set at 37°C and leave undisturbed for 23 ±1 hr.
5. Gather all the reagents from Module 1 and tighten all caps. Mark reagent pouches, tubes, and bottles to track use. Store at –20°C.

7: Freeze or Proceed

After the incubation finishes, you can either:

- Proceed to *Stage 2—Fragmentation and Precipitation*.
- Store the Sample plate at –20°C.

NOTE: If freezing, do not perform the mPCR spike-in or stop amplification reaction step described in Stage 2 before you store the Sample plate at –20°C. The mPCR Spike-In and Stop Amplification Reaction step will be performed after thawing the frozen plate.

Stage 2—Fragmentation and Precipitation

Preparation for Stage 2: Fragmentation and Precipitation

Supplies Required

- Selected reagents from PharmacoScan™ Reagent Kit 4x24 Reactions (see Table 2.1):
  - Module 2-1, –20°C, P/N 901528
  - Module 2-2, 2–8°C, P/N 901529
- Isopropanol (supplied by user)

Instrument Setup

- Prepare the following instruments for this stage before you begin the assay:
  - One oven at 65°C
  - One oven at 37°C
  - One centrifuge at room temperature

NOTE: If the plate of amplifed DNA samples or mPCR Reaction Plate was frozen at the end of Stage 1, thaw the plates before beginning Stage 2. See instructions in Chapter 2 of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286) for notes on thawing and spinning down prior to changing the seal to avoid cross contamination.

TIP: Keep a balance plate ready to avoid delays during the fragmentation steps.

1: Spike mPCR Reaction into Amplification Plate

1. Vortex mPCR Reaction Plate and pulse spin.
2. Carefully transfer 10 µL of the mPCR reaction into the corresponding well of the Amplification plate. Ensure complete transfer of liquid from pipette tip.
3. Securely seal Amplification plate to minimize evaporation during next steps. Vortex, and pulse spin.
4. Immediately proceed to next step: *2: Stop DNA Amplification Reaction*.

2: Stop DNA Amplification Reaction

1. Place the Sample plate in the **65°C oven and incubate for 20 minutes**.
2. Prepare reagents at the start of the 65°C incubation of the amplification plate as shown in Table 2.1.

Table 2.1 Reagent Preparation

Reagent	Module	Treatment
Axiom 10X Frag Buffer	2-1	Thaw, vortex, and keep on ice.
Axiom Frag Enzyme	2-1	Flick tube 3X, pulse-spin, and keep in –20°C cooler until ready to use.
Precip Soln 2	2-1	Thaw, vortex, pulse-spin, and keep at room temperature.
Axiom Frag Diluent	2-2	Thaw, vortex, pulse-spin, and keep on ice.
Axiom Frag Rxn Stop	2-2	Thaw, vortex, and keep at room temperature.
Precip Soln 1	2-2	Thaw, vortex, and keep at room temperature.
Isopropanol	N/A	Keep at room temperature.

3. Transfer the Sample plate from the 65°C oven to the **37°C oven and incubate for 45 minutes**.

3: Prepare Fragmentation Master Mix

1. Start making the Fragmentation Master Mix when there is still five minutes to the finish of the 37°C incubation, using the values in Table 2.2.
   A. Add the Axiom Frag Enzyme to the Fragmentation Master Mix at the end of the 45 minute 37°C incubation.
2. Vortex twice and pour in the 8-channel side of a reagent reservoir placed at room temperature.

Table 2.2. Fragmentation Master Mix

Reagent	per Sample	Master Mix 24+
Axiom 10X Frag Buffer	45.7 µL	1.69 mL
Axiom Frag Diluent	10.3 µL	381 µL
Axiom Frag Enzyme	1.0 µL	37 µL
Total Volume	**57 µL**	**2.11 mL**

4: Add Fragmentation Master Mix to Wells

IMPORTANT: Work quickly to perform this set of steps to minimize the time that the Fragmentation plate is out of the 37°C oven.

1. Carefully remove the Sample plate from the 37°C oven and place on the bench top at room temperature.
2. Do not place the Sample plate on ice.
3. **Add 57 µL of Fragmentation Master Mix to each sample, pipetting directly into the liquid. Do not mix by pipetting up and down.**
4. Seal and vortex twice.
5. Start the timer for 30 min.
6. Quick spin the Sample plate in the room temperature plate centrifuge.
7. Quickly transfer plate to 37°C oven and incubate for 30 min.

   CAUTION: Be watchful for the end of the thirty minute incubation period. Fragmentation is an exact 30 minute incubation step. Longer or shorter incubation times may lead to poor performance.

5: Aliquot the Stop Solution to the Plate

1. A few minutes before the end of the 30 minute incubation period, carefully transfer 988 µL of Axiom Frag Rxn Stop solution in the 8-channel side of the reagent reservoir.
   - Leave the Stop solution reservoir at room temperature.
2. Remove the Sample plate from the oven and place on the bench top.
3. At the end of the 30 minute fragmentation incubation period, **add 19 µL of Stop Solution to each sample, pipetting directly into the liquid. Do not mix by pipetting up and down.**
4. Seal, vortex, and spin.
5. Keep the Sample plate at room temperature while you prepare the Precipitation Master Mix.

6: Prepare and Add Precipitation Master Mix

Carry out the following steps at room temperature.

1. Prepare Precipitation Master Mix in a 15 mL tube. Add the reagents in the order and volumes shown in Table 2.3, vortex to mix and pour into the 8-channel side of a reagent reservoir.

   Table 2.3. Precipitation Master Mix

Reagent	per Sample	Master Mix 24+
Axiom Precip Soln 1	238 µL	6.19 mL
Axiom Precip Soln 2	2 µL	52 µL
Total Volume	**240 µL**	**6.24 mL**

2. **Add 240 µL Precipitation Master Mix to each sample, pipetting down the wall of the well.** You do not need to mix up and down.
3. Seal the Sample plate, vortex, and spin.

7: Prepare and Add Isopropanol

1. Pour 20 mL isopropanol into both sides of the reservoir.
2. **Add 600 µL isopropanol to each sample** and mix well by pipetting up and down within the solution to ensure mixing. The solution should look homogeneous in the tips after pipetting 5-7 times. If not, repeat mixing a few more times until the solution looks mixed. **DO NOT vortex** the plate after isopropanol addition to avoid cross-contamination of the samples.
3. Blot the top of the plate with Kimwipe and seal tightly with a Microamp seal.
4. Carefully **transfer the Sample plate into the –20°C freezer and incubate overnight** (16-24 hours).
5. Gather all the reagents from Module 2-1 and Module 2-2 and tighten all caps. Mark reagent pouches, tubes, and bottles to track use. Store Module 2-1 at –20°C and Module 2-2 at 4°C.
6. After incubation, proceed to *Stage 3—Drying, Resuspension and QC*.

Stage 3—Centrifuge and Drying, Resuspension and Hybridization Preparation, and Sample QC

Preparation for Stage 3—Centrifuge and Drying, Resuspension and Hybridization Preparation, and Sample QC

Supplies Required

- Selected Reagents from the PharmacoScan™ Reagent Kit 4x24 Reactions (see Table 3.1):
  Module 2-1, –20°C, P/N 901528
  Module 2-2, 2–8°C, P/N 901529
- Other Reagents Required for QC steps (optional)
  - Invitrogen TrackIt™ Cyan/Orange Loading Buffer (Thermo Fisher Scientific P/N 10482-028)
  - Invitrogen TrackIt™ 25 bp DNA Ladder (Thermo Fisher Scientific P/N 10488-022)
  - Nuclease-free water, ultrapure MB Grade (USB, P/N 71786)
  - Invitrogen E-Gel® 48 4% agarose gels (Thermo Fisher Scientific P/N G8008-04)

Instrument Setup

- Prepare the following instruments for this stage:
  - Oven preheated to 37°C
  - Plate centrifuge set at 4°C
  - Jitterbug or Microplate shaker

Reagent Preparation

1. Prepare the Gel Diluent for Sample QC (1000-fold dilution of TrackIt™ Cyan/Orange Loading Buffer): Mix 49.95 mL of nuclease-free water with 50 µL of TrackIt Cyan/Orange Loading Buffer.
2. Prepare reagents as shown in Table 3.1.

Table 3.1. Reagent Preparation

Reagent	Module	Treatment
Axiom Hyb Buffer	2-1	Vortex and keep at room temperature
Axiom Hyb Soln 1	2-1	Thaw, vortex, spin, and keep at room temperature
Axiom Hyb Soln 2	2-2	Vortex, spin, and keep at room temperature
Axiom Resusp Buffer	2-2	Warm to room temperature (1 hour)

CAUTION: Some of the steps in this stage should be performed under a fume hood.

3A: Centrifuge Precipitation Plate and Dry the DNA Pellet

1. Begin thawing/warming the reagents used in this stage as shown in Table 3.1.
2. Remove the Sample plate from the –20°C freezer and **centrifuge the plate at 3200 xg at 4°C for 40 min**.
3. During centrifugation, prepare the resuspension and hybridization reagents as shown in Table 3.1.
4. Following centrifugation, empty the liquid from the Sample plate as follows:
   A. Carefully remove the seal from the Sample plate and discard the seal.
   B. **Invert the plate over a waste container and allow the liquid to drain**.
   C. While still inverted, gently press the plate on a pile of Kimwipes on a bench and **leave it for 5 min**.

   CAUTION: During this step, handle the Sample plate gently to avoid disturbing the pellets. Do not bump or bang the plate.
5. Turn the plate top side up and **place in an oven for 20 min at 37°C to dry**. If using an GeneChip® Hybridization Oven 645, turn off the rotor during the 20 min drying time.

   NOTE: If you are proceeding directly to *2: Resuspension and Hybridization Master Mix Preparation*, you can prepare the Hybridization Master Mix at this time.
6. After 20 min remove the plate from the oven, even if some droplets of liquid remain, and either:
   - Proceed directly to *3B: Resuspension and Hybridization Master Mix Preparation*. Leave the Sample plate at room temperature.
   - Tightly seal the plate and store at –20°C.

3B: Resuspension and Hybridization Preparation

NOTE:

- If a plate was stored at –20°C after drying the pellets, allow the plate to sit at room temperature for 1.5 hour before carrying out resuspension.
- Make sure the Axiom Resusp Buffer has equilibrated to room temperature before adding to dry pellets in Step 1, below.
- Carry out these steps at room temperature:

1. Pipet 1.4 mL of Axiom Resusp Buffer into the 8-channel side of a reagent reservoir. **Transfer 35 µL Axiom Resusp Buffer to each well** of the sample plate with a dry pellet. Avoid touching pellets with the pipette tips.
2. Seal the Sample plate and place the plate on one of the following shakers:
   - Thermo Scientific™ Compact Digital Microplate Shaker: at speed 900 rpm for 10 min
   - Jitterbug: at speed 7 for 10 min

   CAUTION: Perform the rest of the steps in this stage under a fume hood.
3. While the Sample plate is shaking, prepare the Hybridization Master Mix in a 15 mL tube as shown in Table 3.2. Vortex well to mix and pour contents in the 8-channel side of a reagent reservoir.

Table 3.2. Hybridization Master Mix

Reagent	per Sample	Master Mix 24+
Axiom Hyb Buffer	70.5 µL	2.26 mL
Axiom Hyb Soln 1	0.5 µL	16 µL
Axiom Hyb Soln 2	9 µL	288 µL
Total Volume	**80 µL**	**2.56 mL**

4. Inspect the Sample plate from the bottom. If the pellets are not dissolved, repeat Step 2. Pulse-spin.
5. Select a PCR plate appropriate to the type of approved thermal cycler you will use in Stage 4 and label as "Hyb Ready Plate [plate ID].".
6. Transfer the entire contents of each well in columns 5, 7, and 9 of the Resuspension Plate to the corresponding wells of the labeled Hyb Ready plate.
7. **Add 80 µL of the Hyb Master Mix to each well** in columns 5, 7, and 9 of the Hyb Ready plate.
8. Seal tightly, vortex, and pulse-spin.
9. Gather all the reagents from Module 2-1 and Module 2-2 and tighten all caps. Mark reagent pouches, tubes, and bottles to track use. Store Module 2-1 at –20°C and Module 2-2 at 4°C.

3C: Recommended: Perform Quantitation and Fragmentation Quality Control Checks

Before proceeding to *Stage 4: Denaturation and Hybridization*, we recommend that you perform quantitation and fragmentation QC checks.

To Perform the Sample QC Checks:

1. Make Dilution QC Plate:
   - **A.** Add 33 µL nuclease-free water to columns 5, 7, and 9 of a PCR plate labeled "Dil QC".
   - **B.** Transfer 3 µL of the Hyb Ready sample from each well of the Hyb Ready plate to the corresponding well of the Dil QC plate.
   - **C.** Seal, vortex, and pulse-spin.
2. Make and read OD Plate:
   - **A.** Add 90 µL nuclease-free water to columns 5, 7, and 9 of the OD Plate (96-well UV Star plate, E&K Scientific P/N 25801).
   - **B.** Transfer 10 µL of each Dilution QC Plate sample to the OD Plate and mix by pipetting up and down.
   - **C.** Read absorbance on a plate reader. See Appendix B, *Sample Quantitation after Resuspension* of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286) for more information.
3. Make and run Gel QC Samples:
   - **A.** Add 120 µL Gel Diluent (1000-fold dilution of TrackIt Cyan/Orange Loading Buffer) to columns 5, 7, and 9 of the Gel QC Plate.
   - **B.** Transfer 3 µL of each Dilution QC Plate sample to the Gel QC Plate.
   - **C.** Seal, vortex, and pulse-spin.
   - **D.** Run Gel: Consult Appendix A, *Fragmentation Quality Control Gel Protocol* of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286) for more information.

4: Freeze or Proceed to Stage 4

At this point you can:

- Proceed to *Stage 4—Denaturation and Hybridization*, or
- Store the Hyb Ready samples at –20°C.

Stage 4—Denaturation and Hybridization

Preparation for Stage 4—Denaturation and Hybridization

Supplies Required

- Reagents from from the PharmacoScan™ Reagent Kit 4x24 Reactions Kit, Module 3, Wash Buffer A (P/N 901446), Wash Buffer B (P/N 901447), Axiom Water (P/N 901578)
- PharmacoScan™ 24-array plate in a protective base (P/N 902994)
- Hyb tray from the Axiom® GeneTitan® Consumables Kit (P/N 901606)

Instruments and Setup

- GeneTitan MC Instrument
- Approved Thermal Cycler
  - Must be programmed with the **PharmacoScan Denature** protocol of 95°C for 10 min; 48°C for 3 min; 48°C for hold.
  - Use the heated lid option when setting up or running protocols.
- Hyb ready samples in plate appropriate to the thermal cycler model used
- 96-well metal chamber pre-heated in a 48°C oven

CAUTION: Some of the steps of this stage should be performed under a fume hood.

1: Prepare Hyb Ready Samples Stored at –20°C

Warm up the Hyb Ready plate at room temperature for 5 minutes.

1. Make sure the Hyb Ready plate is sealed well. If not, centrifuge the plate and change the seal.
2. Vortex the Hyb Ready plate briefly, then spin at 1000 rpm for 30 seconds.
3. Leave the Hyb Ready plate at room temperature.

2: Prepare the GeneTitan® MC Instrument and Denature Hyb Ready Sample Plate

1. **Warm up the array plate on the bench top for a minimum of 25 minutes** before setting up hybridization on the GeneTitan MC Instrument.
2. At the end of the array warm up time, open the pouch and scan the array plate barcode into the Batch Registration file.
3. Before you denature your Hyb Ready samples, ensure that the GeneTitan MC Instrument is ready for use by following the instructions given in Chapter 5, *Stage 2—Hybridization* and Appendix C, *Registering Samples in Affymetrix GeneChip® Command Console®* of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286)
   - **A.** Prepare the reagents from Module 3 by inverting the bottles 2 to 3 times to mix.
   - **B.** Upload the Batch Registration File.
   - **C.** Set up the GeneTitan MC Instrument. For more information, see:
     - *GeneTitan® MC Protocol for Axiom® 2.0 Array Plate Processing QRC* (P/N 702988).
     - Chapter 5, *Array Processing with the GeneTitan® Multi-Channel Instrument* of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286).
4. Place Hyb Ready plate in thermal cycler block, secure lid, and start the **PharmacoScan Denature** protocol.

3: Prepare Hybridization Tray and Load into the GeneTitan® MC Instrument

1. Remove the hyb tray (from Axiom GeneTitan® Consumables Kit) from packaging.
2. Label the hyb tray; please refer to Figure 4.1 and the IMPORTANT note below the figure.
3. After the **Axiom Denature** protocol has completed, remove the Hyb Ready plate from the thermal cycler and place into the preheated 96-well metal chamber.

   CAUTION: Perform the next set of steps under a fume hood.
4. **Using a pipette set at 105 µL, slowly transfer the denatured samples columns 5, 7, and 9 from the Hyb Ready plate into the respective columns (columns 5, 7, or 9) of the hyb tray**. Dispense to the first stop to avoid creating bubbles. If air bubbles are present after transferring all samples, puncture using a clean pipette tip.
5. **Load the array plate and hyb tray into GeneTitan MC Instrument**.

   The array plate is shipped with a clear top lid and a blue protective base (Figure 4.2). Before loading, the top lid must be removed.

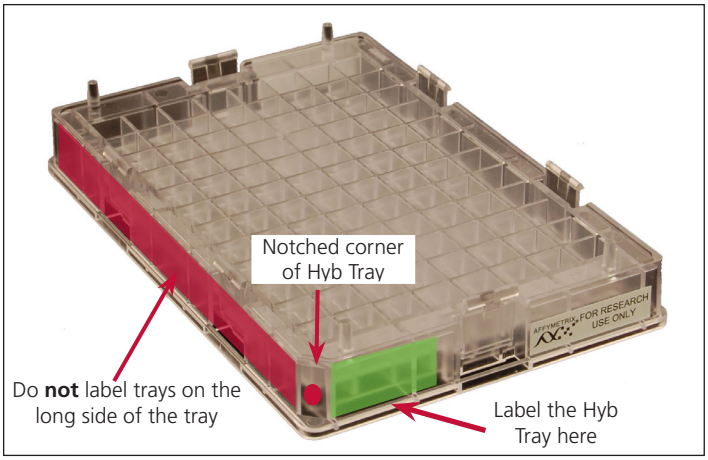
   The clear plastic lid on top of the array plate SHOULD NOT be loaded in the GeneTitan MC Instrument.

   The hyb tray should not have any bubbles and there is no need to spread the liquid around the bottom of the wells.

Hybridization continues on the GeneTitan MC Instrument for 23.5 to 24 hours before you will load the Ligation/Staining/Stabilization reagent trays into the GeneTitan MC Instrument.

You must wait until the hybridization step on the GeneTitan MC Instrument is approximately 1.5 hours from completion (22 hours after the start of hybridization) to begin Stage 5 of the Manual Target Prep.

Figure 4.1. Labeling Hyb Tray



IMPORTANT: It is critical that you write only on the proper location of the hyb tray, as shown above. Do NOT write on any other side, as this can interfere with sensors inside of the GeneTitan MC Instrument and result in experiment failure.

Figure 4.2. Array Plate as Shipped

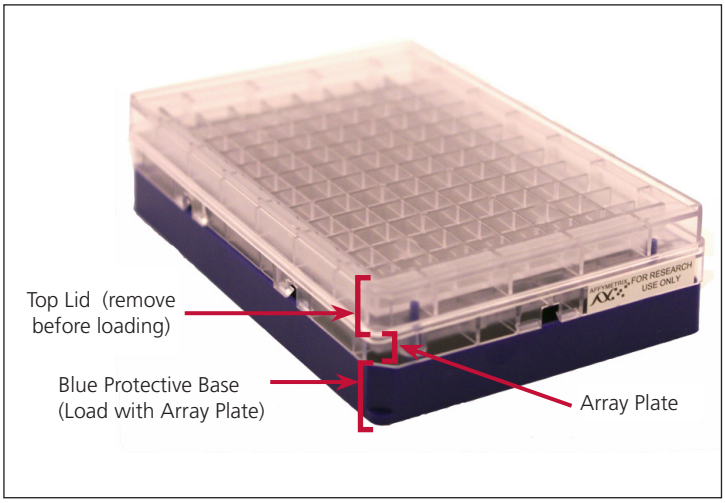
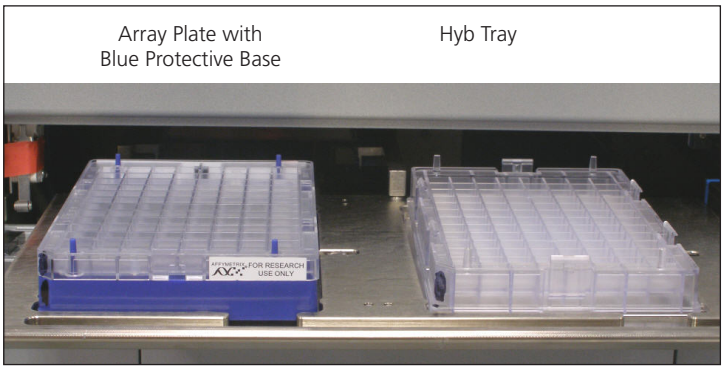


Figure 4.3. Array Plate and Hyb Tray Loaded in GeneTitan Drawer



IMPORTANT:

- The array plate must be loaded on its protective blue base, as shown above.
- After the GeneTitan MC Instrument has stacked the array plate and hyb tray, manually check the stacking by gently pressing the six latching points to confirm that the two parts are clamped properly, and check underneath the arrays to make sure there are no bubbles. If bubbles are found, attempt to remove them by gently tapping the plate on top.

Stage 5—GeneTitan® Reagent Preparation

Preparation for Stage 5—GeneTitan® Reagent Preparation

Reagents

(from PharmacoScan™ Reagent Kit 4x24 Reactions)

- Module 4-1, –20°C, P/N 901278
- Module 4-2, 2-8°C, P/N 901276
- Module 5-1, –20°C, P/N 902796
- Module 5-2, 2-8°C, P/N 902797

Instrument

- GeneTitan® MC Instrument

Consumables

- Aluminum foil (optional) to help protect reagents from light
- Items from the Axiom GeneTitan® Consumables Kit (P/N 901606):
  - Scan Tray (1)
  - Stain Tray (5)
  - Covers for Trays (6)

1: Prepare for GeneTitan Reagent Preparation

1. Prepare the reagents from Module 4 and 5 as described in the table below:

Table 5.1. Reagents from Module 4

Reagent	Temp Out of Module*	Treatment	Storage before Master Mix
Module 4-1 (P/N 901278)			
Axiom Ligate Buffer	Thaw at Room Temp	Place on bench top at room temp for 30 min. Vortex twice for 30 seconds. Examine for precipitate. If any: Warm bottle with your hands and vortex again for 30 seconds.	Place on ice
Axiom Ligate Enzyme†	Keep at –20°C until ready to use	Just before use: Flick and invert tube 2 to 3 times to mix Pulse-spin. Place in –20°C portable cooler until use.	Place in –20°C portable cooler
Axiom Ligate Soln 1	Thaw at Room Temp	Vortex and Spin	Place on Ice
Axiom Probe Mix 1	Thaw at Room Temp	Vortex and Spin	Place on Ice
Axiom Stain Buffer	Thaw at Room Temp	Vortex and Spin	Place on Ice
Axiom Stabilize Soln	Thaw at Room Temp	Vortex and Spin	Place on Ice
Module 4-2 (P/N 901276)			
Axiom Ligate Soln 2	Thaw at Room Temp (do not place on ice!)	Vortex and Spin	Store at Room Temp.
Axiom Probe Mix 2#	Place on Ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Wash A	Leave on bench	Vortex twice Place on Bench for 30 min. Look for precipitate. Vortex again if necessary.	Place on bench top at room temp
Axiom Stain 1-A#	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 1-B#	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 2-A#	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 2-B#	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stabilize Diluent	Place on ice	Vortex and pulse-spin Look for precipitate If any: Warm tube to room temperature and vortex again.	Place on ice
Axiom Water	Leave on bench	N/A	Store at Room Temp
Axiom Hold Buffer# ‡	Room Temp	Vortex	Store at Room Temp away from light

Notes:
#: These solutions are light sensitive. Do not expose tubes to direct light for a prolonged period of time.
*Temp Out of Module: the temperature the reagent is held at immediately after removal from module.
N/A: not applicable in this case
† An extra tube of Axiom Ligate Enzyme is in Module 5-1 in the event that it is needed.
‡ Axiom Hold Buffer for preparing the Scan Tray for the 2nd, 3rd, and 4th plate are provided in Module 5-2.

NOTE: The presence of some precipitate in Axiom Ligate Buffer will not adversely impact assay performance. Follow the instructions above to resuspend any precipitate before use.

NOTE: Occasionally, crystals are observed in Axiom Wash A and Axiom Stabilize Diluent upon removal from 2-8°C storage. Before using these solutions, the crystals should be dissolved by warming the solutions to room temperature and then vortexing.

2: Prepare the Stain, Ligation and Stabilization Master Mixes

Prepare Stain 1 Master Mix

1. Add reagents in the order shown in Table 5.2 in a 15 mL conical tube. This recipe will provide enough for both S1 reagent trays.
2. Gently invert the tube 10 times to mix. Place on ice and protect from direct light.

Table 5.2. Stain 1 Master Mix (for both S1 trays)

Reagent	per Array	Master Mix 24+
Axiom Wash A	201.6 µL	5.242 mL
Axiom Stain Buffer	4.2 µL	109.2 µL
Axiom Stain 1-A	2.1 µL	54.6 µL
Axiom Stain 1-B	2.1 µL	54.6 µL
Total	**210 µL (105 µL x 2)**	**5.46 mL**

Prepare Stain 2 Master Mix

1. Add reagents in the order shown in Table 5.3 in a 15 mL conical tube.
2. Gently invert the tube 10 times to mix. Place on ice and protect from direct light.

Table 5.3. Stain 2 Master Mix

Reagent	per Array	Master Mix 24+
Axiom Wash A	100.8 µL	2.62 mL
Axiom Stain Buffer	2.1 µL	54.6 µL
Axiom Stain 2-A	1.05 µL	27.3 µL
Axiom Stain 2-B	1.05 µL	27.3 µL
Total	**105 µL**	**2.73 mL**

Prepare Stabilization Master Mix

1. Add reagents in the order shown in Table 5.4 in a 15 mL conical tube.
2. Vortex the master mix at high speed for 3 sec. Place on ice.

Table 5.4. Stabilization Master Mix

Reagent	per Array	Master Mix 24+
Axiom Water	93.19 µL	2.38 mL
Axiom Stabilize Diluent	10.50 µL	268 µL
Axiom Stabilize Soln	1.31 µL	33.4 µL
Total	**105 µL**	**2.68 mL**

Prepare Ligation Master Mix

IMPORTANT: Note About the Second Tube of Axiom Ligate Enzyme: Module 5-1 contains an extra tube of Axiom Ligate Enzyme which has been provided for back-up purposes. If there is insufficient volume of the Axiom Ligate Enzyme for the preparation of the fourth (4th) 24 Sample Ligation Master Mix, discard the tube and its contents and proceed to use the second tube of Axiom Ligate Enzyme (Module 5-1). The remaining contents of the second tube of Ligate Enzyme may be discarded or stored in the freezer for future use.

The Ligation Master Mix is prepared in two stages.

Ligation Master Mix: Stage 1

1. Place the Ligation Master Mix tube on ice (15 mL conical tube).
2. Add reagents to the tube in the order shown in Table 5.5.
3. Mix well by vortexing the tube for 3 seconds. Place back on ice.

Table 5.5. Ligation Master Mix: Stage 1

Reagent	per Array	Master Mix 24+
Axiom Ligate Buffer	66.15 µL	1.75 mL
Axiom Ligate Soln 1	13.12 µL	348 µL
Axiom Ligate Soln 2	3.15 µL	83.5 µL
Sub–Total	**82.42 µL**	**2.18 mL**

Ligation Master Mix: Stage 2

1. Remove the Axiom Ligation Enzyme from the –20°C freezer and place in a cooler chilled to –20°C.
2. Add reagents in the order shown in Table 5.6.
3. Gently flick the Axiom Ligate Enzyme tube 2-3 times, then pulse-spin the tube just prior to adding the enzyme to the Master Mix.
4. Gently invert the Master Mix tube 10 times to mix (do not vortex).
5. Place on ice and protect from direct light.

Table 5.6. Ligation Master Mix: Stage 2

Reagent	per Array	Master Mix 24+
Ligation Master Mix from Stage 1	82.42 µL	2.18 mL
Axiom Probe Mix 1	10.5 µL	278 µL
Axiom Probe Mix 2	10.5 µL	278 µL
Axiom Ligate Enzyme	1.58 µL	42 µL
Total	**105 µL**	**2.78 mL**

3: Aliquot Master Mixes and Axiom Hold Buffer into Trays

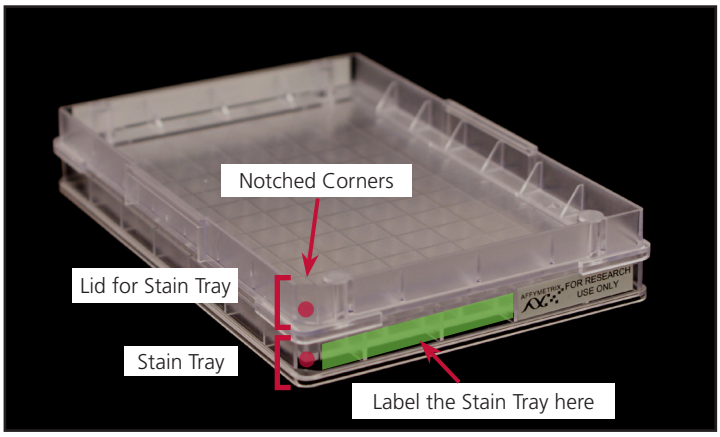
NOTE: It is not necessary to change pipette tips between additions of the same reagents to stain trays and scan trays.

Prepare Trays and Lids

1. Label two stain trays S1 (for Stain 1 Master Mix)
2. Label the remaining stain trays:
   - S2 (for Stain 2 Master Mix)
   - Stbl (for Stabilization Master Mix)
   - Lig (for Ligation Master Mix)
3. Destatic the inside of each tray and cover.

   See Appendix D, *Deionization Procedure for GeneTitan® Trays and Covers* of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286) for the recommended technique.

Figure 5.1. Stain Tray with Lid



IMPORTANT: It is critical that you write only on the proper location of the proper edge of the stain trays, as shown above. Do NOT write on any other side, as this can interfere with sensors inside of the GeneTitan MC Instrument and result in experiment failure.

Aliquoting Reagents to Stain Trays

You will need to aliquot the appropriate master mix into columns 5, 7, and 9 of the S1, S2, Stbl, and Lig trays labeled in the previous step:

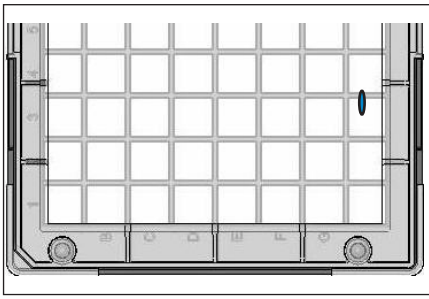
1. Pipet or pour the Master Mix into the 4- or 8-channel side of the reagent reservoir:
   - Stain 1 Master Mix: 8-channel side
   - Stain 2 Master Mix: 4-channel side
   - Stabilization Master Mix: 4-channel side
   - Ligation Master Mix: 4-channel side
2. Aliquot **105 µL per well** of the appropriate Master Mix — dispense to the first stop only to avoid creating bubbles.
3. If:
   - Bubbles are present, puncture them with a pipette tip.
   - Droplets of liquid splashed onto the well dividers, place a Kimwipe on top of the tray to blot and remove.
4. Place covers on the trays. Orient cover correctly on the tray with the notched corners together.
5. Protect the trays from light if not immediately loading onto the GeneTitan MC Instrument.

About Aliquoting Reagents to Trays

IMPORTANT: Always aliquot reagents to the bottom of the tray. Avoid touching the sides or the top of the wells with the pipette tips. Droplets close to or on the top of the well dividers may cause the lid to stick to the tray during GeneTitan processing.

When aliquoting ligation, staining, and stabilization reagents to the trays, it is not necessary to spread the reagent to each corner of the well. The reagent will spread evenly when the array plate is inserted into the reagent tray during processing with the GeneTitan MC Instrument.

Figure 5.2. Blotting Drops of Liquid on Dividers



Droplet of liquid that has splashed onto the divider of a stain tray during aliquoting.

- Ensure no droplets of liquid are on top of the wells dividers.
- Blot with a Kimwipe to remove.

Aliquoting Hold Buffer to the Scan Tray

The scan tray is shipped with two covers, a bottom protective base and a top lid (Figure 5.3).

The top cover is removed to fill the tray during the target prep process, while the scan tray is left on the protective base during this part of the process (Figure 5.4).

NOTE: Module 5-2 (P/N 902797) has 3 Axiom Hold Buffer bottles that should be used to prepare the Scan Tray for second, third, and fourth plate.

1. Pour all the contents of the Axiom Hold Buffer into both sides of the 25 mL divided reagent reservoir, placed on the bench top at room temperature.
2. Remove the scan tray from its pouch.
3. Remove the top scan tray lid, but leave the scan tray on its protective black base.
4. Aliquot **150 µL to EACH of the 96 wells** of the 96 Plate Scan Tray — dispense to the first stop and avoid touching the bottom of the tray.

   IMPORTANT: The scan tray has an open-bottom design, so it is very important that all 96 wells of the scan tray receive 150 µL of Axiom Hold Buffer.
5. If droplets of liquid splashed onto the well dividers, place a Kimwipe on top of the tray to blot and remove.
6. Cover the tray by orienting the notched corner of the lid over the notched edge of the tray, and leave on the bench top.

For more information on loading the reagent and scan trays, see:

- *GeneTitan® MC Protocol for Axiom® 2.0 Array Plate Processing QRC* (P/N 702988)
- Chapter 5, *Array Processing with the GeneTitan® Multi-Channel Instrument* of the *PharmacoScan™ Assay 24-Array Format Manual Protocol User Guide* (P/N 703286)

Figure 5.3. Scan Tray with Top Lid and Black Protective Base

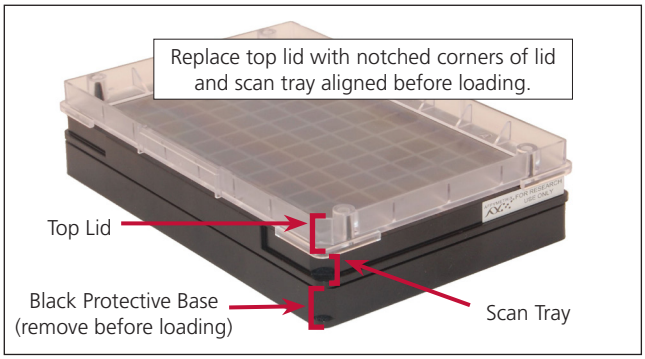
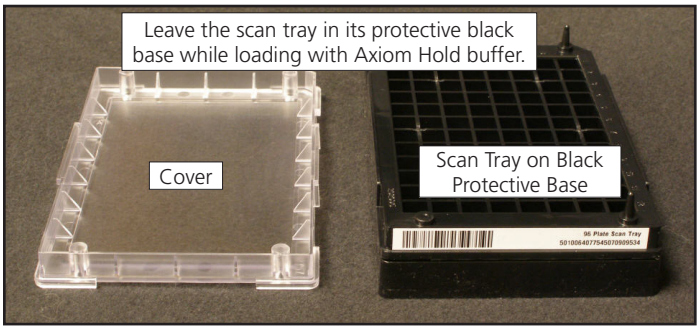


Figure 5.4. Scan Tray with Cover Removed



4. Store Remaining Reagents

Gather all the reagents from Module 4-1 and Module 4-2 and tighten all caps. Mark reagent pouches, tubes, and bottles to track use. Store Module 4-1 at –20°C and Module 4-2 at 4°C.

Affymetrix, Inc: (US) +1-888-362-2447, +1-408-731-5000 ■ (EU) +44-(0)1628-552550 ■ (JP) +81-(0)3-6430-4020 ■ (CN) +86-21-63915511
eBioscience Products: (US) +1-888-999-1371, +1-858-642-2058 ■ (EU) +43 1 796 40 40 305 ■ (JP) +81-(0)3-6430-4020
USB Products: (US) +1-800-321-9322, +1-216-765-5000 ■ (EU) +44-(0)1628-552600

www.affymetrix.com Please visit our website for international distributor contact information.

For Research Use Only. Not for use in Diagnostic Procedures.

P/N 703288 Rev. 1