PharmacoScan™ Assay 96-Array Format Manual Workflow

Pub. No. 703461 **Rev.** 2

Introduction and Stage 1A: Multiplex PCR and 1B: DNA Amplification

Introduction to PharmacoScan Assay 96-Array Format Manual Target Preparation

Running the PharmacoScan Assay 96-Array Format Manual Workflow requires the following sets of steps:

1. Genomic DNA Prep, described in the *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459).
2. 96-array format manual target reparation, described in this document.
3. Array Processing, described in *GeneTitan™ MC Protocol for Axiom™ 2.0 Array Plate Processing Quick Reference* (Pub. No. 702988).

IMPORTANT! This document contains an abbreviated set of instructions used to perform target preparation. You must carefully read all the instructions in the *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) 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 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) and the GeneTitan Quick Reference (Pub. No. 702988) described above.

New: An option for a three-hour DNA precipitation step is now available. Refer to *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) for details.

Additional notes:

- This manual assay format allows the user to run the PharmacoScan Assay for 96 Samples once using one PharmacoScan™ Reagent Kit 96 Reactions (Cat. No. 913025).
- This manual assay utilizes disposable reservoirs with a "trough within a trough" design which maximizes the amount of liquid accessible to pipette tips when using small amounts of reagent.
- 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 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 96 Reactions (Cat. No. 913025). Stage 1A requires the QIAGEN Multiplex PCR Plus Kit (Cat. No. 206152) and is sufficient to process 96 samples.
- See Chapter 3 of the *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) 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, Part No. 912896
- Reagents from QIAGEN Multiplex PCR Plus Kit, –20°C, Cat. No. 206152

Instruments and setup

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

Reagent preparation

1. Prepare reagents as shown in Table 1.

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

   Table 1 Reagent preparation

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

3. Prepare mPCR Master Mix

1. Prepare mPCR Master Mix as shown in Table 2.
2. Add water, Q-Solution, and primers to 15-mL conical tube. Vortex and pulse spin.
3. Add 2,400 µL of the QIAGEN 2X PCR Master Mix to the 15-mL tube.
   - All three QIAGEN 2X Multiplex PCR Master Mix supplied vials will be needed.
   - It is recommended to set a P-1000 single-channel pipette to 800 µL and remove this volume from the first vial.
   - Transfer this solution to the 15-mL tube. Change tips, and repeat this step for the remaining two vials.
4. Mix thoroughly, but gently, by inverting tube ten times.
5. Pulse spin and quickly proceed to next step.

   Table 2 mPCR Master Mix

Reagent	120 reactions
RNase-free Water	240 µL
Q-solution	480 µL
10X mPCR Primer Mix	480 µL
2X QIAGEN Multiplex PCR Master Mix	2,400 µL
Total	**3,600 µL**

4. Add mPCR Master Mix to samples

1. Carefully pour the prepared mPCR Master Mix into a 25-mL reservoir.
2. Use a P-200 multichannel pipette to transfer 30 µL of mPCR Master Mix to each well of the mPCR Sample Plate.
3. Seal plate. Gently vortex. Pulse spin.
4. Load plate onto thermal cycler within five minutes.

5. Discard any leftover 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 96 Reactions, Module 1, –20°C, Part No. 901711
- Amplification Sample Plate (20 µL volumes of gDNA at 5 ng/µL, with control DNAs)

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 3.

   Table 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 seconds, and keep at room temperature
Axiom 2.0 Amp Soln	Thaw (see Note below), vortex for 30 seconds, 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 ~one hour for Axiom 2.0 Amp Soln to thaw on the benchtop at room temperature. If the solution is not completely thawed after one 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 seconds 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 15 mL tube marked D MM, prepare the Denaturation Master Mix as shown in Table 4.
2. Vortex well.

   Table 4. Denaturation Master Mix

Reagent	per sample	Master mix 96+
Axiom 2.0 Denat Soln 10X	2 µL	400 µL
Axiom Water	18 µL	3.6 mL
Total volume	**20 µL**	**4.0 mL**

3: Add Denaturation Master Mix to samples

1. Gently pour or pipet the Denaturation Master Mix into the reagent reservoir.
2. Add **20 µL of Denaturation Master Mix** to each sample, pipetting directly into the liquid. Do not mix by pipetting up and down.
3. Incubate the plate for **ten 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. Pour the Neutral Soln into the reagent reservoir.
2. Add **130 µL of Axiom 2.0 Neutral Soln** to each sample, 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

1. Prepare Amplification Master Mix as shown in Table 5.

   Table 5. Amplification Master Mix

Reagent	per sample	Master mix 96+
To a 50 mL tube marked *Amp MM*, add:		
Axiom 2.0 Amp Soln	225 µL	26.0 mL
Axiom 2.0 Amp Enzyme	5 µL	578 µL
Total volume	**230 µL**	**26.58 mL**

2. Vortex the Amplification Master Mix well, then invert the tube two times, and then vortex again.

6: Add Amplification Master Mix to samples

1. Slowly pour the Amplification Master Mix into the reagent reservoir labeled Amp MM.
2. Slowly add **230 µL Amplification Master Mix** to 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 a Kimwipe laboratory tissue, seal tightly, vortex twice, and spin the plate for one minute at 1,000 rpm.
4. Place the sealed plate in an oven set at 37°C and leave undisturbed for 23 ±1 hr.

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 96 Reactions (see Table 2.1):
  - Module 2-1, –20°C, Part No. 901528
  - Module 2-2, 2–8°C, Part No. 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 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) 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 6.

   Table 6 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 7.
   - 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 reagent reservoir placed at room temperature.

Table 7. Fragmentation Master Mix

Reagent	per sample	Master mix 96+
Axiom 10X Frag Buffer	45.7 µL	6.0 mL
Axiom Frag Diluent	10.3 µL	1.35 mL
Axiom Frag Enzyme	1.0 µL	131 µL
Total volume	**57 µL**	**7.48 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 minutes.
6. Quick spin the Sample Plate in the room temperature plate centrifuge.
7. Quickly transfer plate to 37°C oven and incubate for 30 minutes.

   Caution: Be watchful for the end of the 30-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, pour the Axiom Frag Rxn Stop solution in 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 by adding 218 µL of Axiom Precipitation Solution 2 directly to the Axiom Precipitation Solution 1 bottle.
2. Vortex and pour the Precipitation Master Mix into reagent reservoir.

   Table 8. Precipitation Master Mix

Reagent	per sample	Master mix 96+
Axiom Precip Soln 1	238 µL	26 mL
Axiom Precip Soln 2	2 µL	218 µL
Total volume	**240 µL**	**26.22 mL**

3. **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.
4. Seal the Sample Plate, vortex, and spin.

7: Prepare and add isopropanol

1. Pour 65 mL of isopropanol into a 100-mL reagent 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 6-7 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 Kimwipes laboratory tissue and seal tightly with a Microamp seal.
4. Carefully **transfer the Sample Plate into the –20°C freezer and incubate overnight** (16-24 hours).
   A three-hour precipitation workflow option is also available. See the *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) for details.
5. After incubation, proceed to *Stage 3: Centrifuge and drying, resuspension and hybridization preparation, and sample QC*.

Stage 3: Centrifuge and drying, resuspension and hybridization preparation, and sample QC

Supplies required

- Selected Reagents from the PharmacoScan™ Reagent Kit 96 Reactions (see Table 9):

  Module 2-1, –20°C, Part No. 901528

  Module 2-2, 2–8°C, Part No. 901529
- Other Reagents Required for QC steps (optional)
  - Invitrogen TrackIt™ Cyan/Orange Loading Buffer (Thermo Fisher Scientific, Cat. No. 10482-028)
  - Invitrogen TrackIt™ 25 bp DNA Ladder (Thermo Fisher Scientific, Cat. No. 10488-022)
  - Nuclease-free water, ultrapure MB Grade (Thermo Fisher Scientific, Cat. No. 71786)
  - Invitrogen E-Gel® 48 4% agarose gels (Thermo Fisher Scientific, Cat. No. 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 (1,000-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 9.

Table 9. 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 (one 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 9.
2. Remove the Sample Plate from the –20°C freezer and **centrifuge the plate at 3200 x *g* at 4°C for 40 minutes**.
3. During centrifugation, prepare the resuspension and hybridization reagents as shown in Table 9.
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 clean waste container and allow the liquid to drain.
   c. While still inverted, gently press the plate on a pile of Kimwipes laboratory tissues on a bench and **leave it for five minutes**.

   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 minutes at 37°C to dry**. If using an GeneChip™ Hybridization Oven 645, turn off the rotor during the 20 minutes 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 minutes 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. Pour Axiom Resusp Buffer into 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 ten minutes
   - Jitterbug: at speed 7 for ten minutes

   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 10. Vortex well to mix and pour contents into the reagent reservoir.

   Table 10. Hybridization Master Mix

Reagent	per sample	Master mix 96+
Axiom Hyb Buffer	70.5 µL	7.8 mL
Axiom Hyb Soln 1	0.5 µL	55.6 µL
Axiom Hyb Soln 2	9 µL	1.0 mL
Total volume	**80 µL**	**8.86 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 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** of the Hyb Ready Plate.
8. Seal tightly, vortex, and pulse-spin.

3C: Perform quantitation and fragmentation quality control checks

Before proceeding to *Stage 4: Denaturation and Hybridization*, we highly 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 a PCR plate labeled "Dil QC".
   b. Transfer 3 µL of the hybridization 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 the OD Plate (96-well UV-Star® plate, E&K Scientific Cat. No. 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 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) for more information.
3. Make and run gel QC samples:
   a. Add 120 µL gel diluent (1,000-fold dilution of TrackIt™ Cyan/Orange Loading Buffer) to 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: See Appendix A, *Fragmentation Quality Control Gel Protocol* of the *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) 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 hybridization ready samples at –20°C.

Stage 4: Denaturation and hybridization

Supplies required

- Reagents from from the PharmacoScan™ Reagent Kit 96 Reactions Kit, Module 3, Wash Buffer A (Part No. 901446), Wash Buffer B (Part No. 901447), Axiom Water (Part No. 901578)
- PharmacoScan™ 96-array plate in a protective base (Cat. No. 903160)
- Hyb tray from the Axiom™ GeneTitan™ Consumables Kit (Cat. No. 901606)

Instruments and setup

- GeneTitan MC Instrument
- Approved thermal cycler
  - Must be programmed with the **PharmacoScan Denature** protocol of 95°C for ten minutes; 48°C for three minutes; 48°C for hold.
  - Use the heated lid option when setting up or running protocols.
- Hybridization 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 hybridization ready samples stored at –20°C

Warm up the Hyb Ready Plate at room temperature for five 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 1,000 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 GeneTitan Array Plate Registration file.
3. Before you denature your hybridization 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 Applied Biosystems™ GeneChip™ Command Console™* of the *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459).
   a. Prepare the reagents from Module 3 by inverting the bottles two to three times to mix.
   b. Upload the GeneTitan Array Plate Registration file.
   c. Set up the GeneTitan MC Instrument. For more information, see:
      - *GeneTitan™ MC Protocol for Axiom™ 2.0 Array Plate Processing QR* (Pub. No. 702988).
      - Chapter 5, *Array Processing with the GeneTitan™ Multi-Channel Instrument of the PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459).
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 hybridization tray (from Axiom GeneTitan™ Consumables Kit) from packaging.
2. Label the hybridization tray; please refer to Figure 1 and the IMPORTANT note below the figure.
3. After the **PharmacoScan 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 from the Hyb Ready Plate into the respective columns of the hybridization 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 hybridization tray into GeneTitan MC Instrument**.

   The array plate is shipped with a clear top cover and a blue protective base (Figure 2). Before loading, the top cover must be removed.

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

   The hybridization 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 preparation.

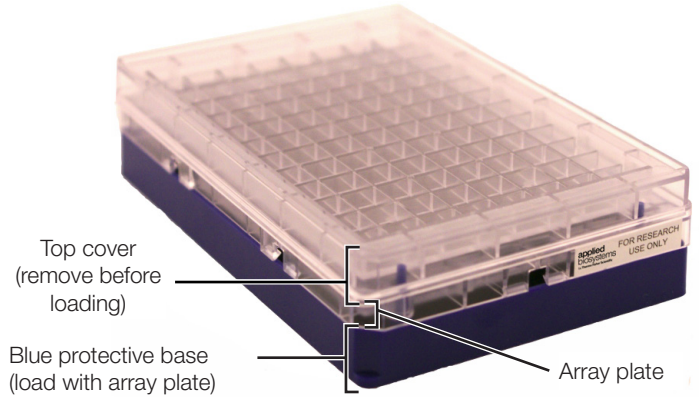


Figure 2. Array plate as shipped

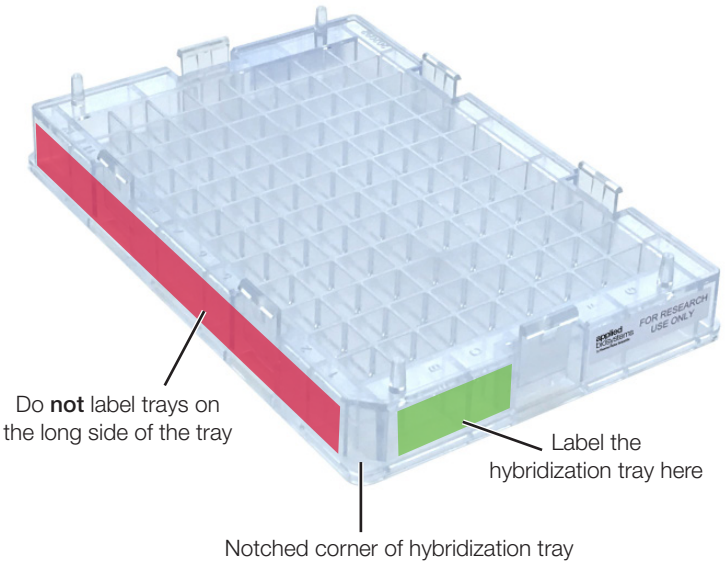


Figure 1. Labeling hybridization tray

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.

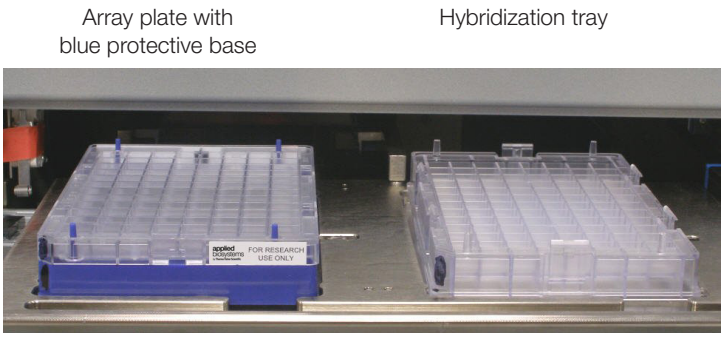


Figure 3. Array plate and hybridization tray loaded in GeneTitan drawer

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 hybridization 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 top of the plate.

Stage 5: GeneTitan™ reagent preparation

Preparation for Stage 5: GeneTitan™ reagent preparation

Reagents (from PharmacoScan™ Reagent Kit 96 Reactions)

- Module 4-1, –20°C, Part No. 901278
- Module 4-2, 2-8°C, Part No. 901276

Instrument

- GeneTitan™ MC Instrument

Consumables

- Aluminum foil (optional) to help protect reagents from light
- Items from the Axiom GeneTitan™ Consumables Kit (Cat. No. 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:

Reagent	Temp out of module[1]	Treatment	Storage
Module 4-1 (Part No. 901278)			
Axiom Ligate Buffer	Thaw at room temp	Place on bench top at room temp for 30 minutes. 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 (Part No. 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[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 minutes. Look for precipitate. Vortex again if necessary.	Place on bench top at room temp
Axiom Stain 1-A[2]	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 1-B[2]	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 2-A[2]	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 2-B[2]	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 [2]	Room temp	Vortex	Store at room temp away from light

[1] The temperature the reagent is held at immediately after removal from module.
[2] These solutions are light sensitive. Do not expose tubes to direct light for a prolonged period of time.

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 11 in a 50-mL conical tube. This recipe will provide enough for both S1 reagent trays.
2. Gently invert the tube ten times to mix. Place on ice and protect from direct light.

Table 11. Stain 1 Master Mix (for both S1 trays)

Reagent	per array	Master mix 96+
Axiom Wash A	201.6 µL	22.2 mL
Axiom Stain Buffer	4.2 µL	463 µL
Axiom Stain 1-A	2.1 µL	231 µL
Axiom Stain 1-B	2.1 µL	231 µL
Total	**210 µL (105 µL x 2)**	**23.13 mL**

Prepare Stain 2 Master Mix

1. Add reagents in the order shown in Table 12 in a 15-mL conical tube.
2. Gently invert the tube ten times to mix. Place on ice and protect from direct light.

Table 12. Stain 2 Master Mix

Reagent	per array	Master mix 96+
Axiom Wash A	100.8 µL	11.1 mL
Axiom Stain Buffer	2.1 µL	231 µL
Axiom Stain 2-A	1.05 µL	115.6 µL
Axiom Stain 2-B	1.05 µL	115.6 µL
Total	**105 µL**	**11.56 mL**

Prepare Stabilization Master Mix

1. Add reagents in the order shown in Table 12 in a 15-mL conical tube.
2. Vortex the master mix at high speed for three seconds. Place on ice.

Table 13. Stabilization Master Mix

Reagent	per array	Master mix 96+
Axiom Water	93.19 µL	10.3 mL
Axiom Stabilize Diluent	10.50 µL	1.16 mL
Axiom Stabilize Soln	1.31 µL	144.8 µL
Total	**105 µL**	**11.61 mL**

Prepare Ligation Master Mix

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 14.
3. Mix well by vortexing the tube for three seconds. Place back on ice.

Table 14. Ligation Master Mix: Stage 1

Reagent	per array	Master mix 96+
Axiom Ligate Buffer	66.15 µL	7.3 mL
Axiom Ligate Soln 1	13.12 µL	1.45 mL
Axiom Ligate Soln 2	3.15 µL	348 µL
Subtotal	**82.42 µL**	**9.10 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 15.
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 ten times to mix (do not vortex).
5. Place on ice and protect from direct light.

Table 15. Ligation Master Mix: Stage 2

Reagent	per array	Master mix 96+
Ligation Master Mix from Stage 1	82.42 µL	9.10 mL
Axiom Probe Mix 1	10.5 µL	1.16 mL
Axiom Probe Mix 2	10.5 µL	1.16 mL
Axiom Ligate Enzyme	1.58 µL	174.4 µL
Total	**105 µL**	**11.59 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 covers

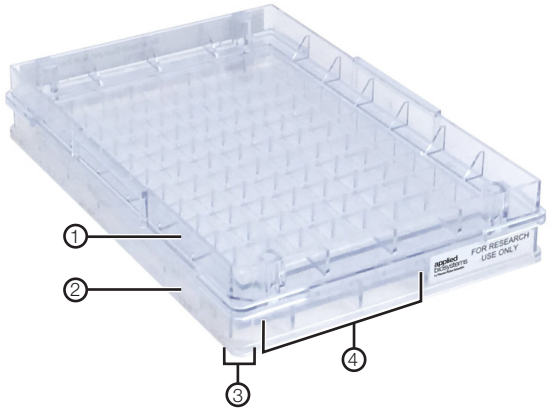
1. Label two stain trays S1 (for Stain 1 Master Mix)
2. Label the remaining stain trays:
   - S2 (for Stain 2 Master Mix)
   - Stbl (for Stabilization Master Mix)
   - Lig (for Ligation Master Mix)
3. Destatic the inside of each tray and cover.

   See Appendix D, *Deionization Procedure for GeneTitan™ Trays and Covers* of the *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459) for the recommended technique.

Aliquoting reagents to stain trays

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

1. Pour the Master Mix into the appropriately labeled 25-mL reagent reservoir:
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.



① Lid for Stain Tray
② Stain Tray
③ Notched corners aligned
④ Label stain tray in this area only

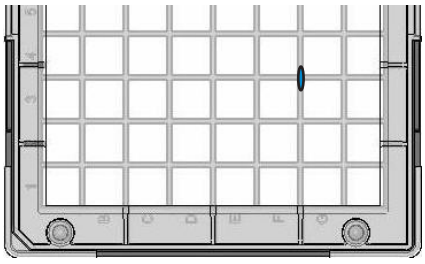
Figure 4. Stain Tray with lid

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.

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 can 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.



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 Kimwipes laboratory tissue to remove.

Figure 5. Blotting drops of liquid on dividers

Aliquoting Hold Buffer to the scan tray

The scan tray is shipped with two covers, a bottom protective base and a top lid (Figure 6).

The top cover is removed to fill the tray during the target preparation process, while the scan tray is left on the protective base during this part of the process (Figure 7).

1. Pour all the contents of the Axiom Hold Buffer into the 25-mL 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 Kimwipes laboratory tissue 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 QR* (Pub. No. 702988)
- Chapter 5, *Array Processing with the GeneTitan™ Multi-Channel Instrument* of the *PharmacoScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703459).

Replace top cover with notched corners of lid and scan tray aligned before loading.

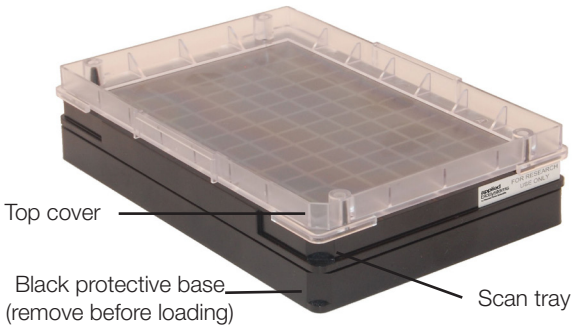


Figure 6. Scan tray with top cover and black protective base

Leave the scan tray in its protective black base while loading with Axiom Hold Buffer.

Cover

Scan tray on black protective base

Figure 7. Scan tray with cover removed

Thermo Fisher Scientific Baltics UAB | V.A. Graiciuno 8, LT-02241 | Vilnius, Lithuania — Products: PharmacoScan™ Reagent Kit

Affymetrix Pte Ltd | 7 Gul Circle #2M-01 | Keppel Logistics Building | Singapore 629563 — Products: PharmacoScan™ Array Plates

For support visit **thermofisher.com/support** or email **techsupport@lifetech.com**

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