

PharmacoScan™ Assay 24-Array Format Manual Workflow

Pub. No. 703288 Rev. 3

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 Workflow requires the following sets of steps:

1. Genomic DNA Prep, described in the *PharmacoScan™ Assay 24-Array Format Manual Workflow User Guide* (Pub. No. 703286).
2. 24-Array Format Manual Target Prep, described in this quick reference.
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 24-Array Format Manual Workflow User Guide* (Pub. No. 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 Workflow User Guide* (Pub. No. 703286) and the quick reference (Pub. No. 702988) described above.

New: An option for a three-hour DNA precipitation step is now available. See the *PharmacoScan™ Assay 24-Array Format Manual Workflow User Guide* (Pub. No. 703286) for details.

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 (Cat. No. 902908TS). All samples are 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 (Cat. No. 902908TS). Stage 1A requires the QIAGEN Multiplex PCR Plus Kit (Cat. No. 206152) and is sufficient to process 24 samples four times.
- See Chapter 3 of the *PharmacoScan™ Assay 24-Array Format Manual Workflow User Guide* (Pub. No. 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

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. 902896
- Reagents from QIAGEN Multiplex PCR Plus Kit, -20°C, Part 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
- Ensure the heated lid option is used and thermal cycler is programmed to run in “9600 Mode” for Applied Biosystems 9700, Applied Biosystems Veriti™, Applied Biosystems ProFlex™ and “Safe” mode for Eppendorf Mastercycler™ pro S.

Reagent preparation

1. Prepare reagents as shown in the following table.

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

Reagent	Quantity	Treatment
PharmacoScan 10X Primer Mix	one tube	Thaw, vortex, pulse-spin, keep on ice
QIAGEN Multiplex PCR Master Mix, 2X	one tube	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 ice or cold aluminum block.

3. Prepare mPCR Master Mix

1. Prepare mPCR Master Mix as shown in the following table.
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 μ L.
4. Pulse spin and quickly proceed to next step.

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

4. Add mPCR Master Mix to samples

1. Transfer 100 μ L of mPCR Master Mix to eight wells of a strip tube.
2. Cap strip tubes and pulse spin.
3. Use multichannel pipette to transfer 30 μ L 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

Supplies required

- Reagents from the PharmacoScan Reagent Kit 4x24 Reactions, Module 1, -20°C, Part No. 902908TS
- 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

- Prepare reagents as shown in the following table.

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.

- Thaw Samples in Amplification Sample Plate:
 - Bring your gDNA samples to room temperature on the bench top.
 - 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

- To a 1.7 mL microcentrifuge tube marked D MM, prepare the Denaturation Master Mix as shown in the following table.
- Vortex well.

Reagent	per sample	Master mix 24+
Axiom 2.0 Denat Soln 10X	2 µL	116 µL
Axiom Water	18 µL	1,044 µL
Total volume	20 µL	1,160 µL

3: Add Denaturation Master Mix to samples

- Gently pour or pipet the Denaturation Master Mix into the 8-channel side of the reagent reservoir.
- 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.
- 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.
- After incubation, immediately add the Neutralization Master Mix as described in the following section.

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 the following table.

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 a Kimwipes 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.
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 are performed after thawing the frozen plate.

Stage 2: Fragmentation and precipitation

Preparation for Stage 2

Supplies required

- Selected reagents from PharmacoScan™ Reagent Kit 4x24 Reactions (see Table 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 amplified 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 Workflow User Guide* (Pub. No. 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

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

2: Stop DNA amplification reaction

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

Table 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.

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

3: Prepare Fragmentation Master Mix

- 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.
 - Add the Axiom Frag Enzyme to the Fragmentation Master Mix at the end of the 45 minute 37°C incubation.
- Vortex twice and pour in the 8-channel side of a reagent reservoir placed at room temperature.

Table 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 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 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 the following table. Vortex to mix and pour into the 8-channel side of a reagent reservoir.

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 a 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 24-Array Format Manual Workflow User Guide* (Pub. No. 703286) for details.
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: Centrifuge and drying, resuspension & hybridization preparation, and sample QC.*

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

Supplies required

- Selected Reagents from the PharmacoScan™ Reagent Kit 4x24 Reactions (see Table 3):
 - 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

- 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.
- Prepare reagents as shown in Table 3.

Table 3. 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

- Begin thawing/warming the reagents used in this stage as shown in Table 3.
- Remove the Sample Plate from the -20°C freezer and **centrifuge the plate at 3,200 x g at 4°C for 40 minutes**.
- During centrifugation, prepare the resuspension and hybridization reagents as shown in Table 3.
- Following centrifugation, empty the liquid from the Sample Plate as follows:
 - Carefully remove the seal from the Sample Plate and discard the seal.
 - Invert the plate over a waste container and allow the liquid to drain.**
 - 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.

- 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.

- 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 hours before carrying out resuspension.
 - Ensure 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 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 the following table. Vortex well to mix and pour contents in the 8-channel side of a reagent reservoir.

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 used 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.

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 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 columns 5, 7, and 9 of 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 24-Array Format Manual Workflow User Guide* (Pub. No. 703286) 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 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 Workflow User Guide* (Pub. No. 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 hybridization ready samples at -20°C .

Stage 4: Denaturation and hybridization

Preparation for Stage 4

Supplies required

- Reagents from the PharmacoScan™ Reagent Kit 4x24 Reactions Kit, Module 3, Wash Buffer A (Part No. 901446), Wash Buffer B (Part No. 901447), Axiom Water (Part No. 901578)
- PharmacoScan™ 24-array plate in a protective base (Cat. No. 902994)
- Hybridization 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.
- 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 hybridization ready samples stored at –20°C

1. Warm up the Hyb Ready Plate at room temperature for five minutes.
2. Ensure the Hyb Ready Plate is sealed well. If not, centrifuge the plate and change the seal.
3. Vortex the Hyb Ready Plate briefly, then spin at 1,000 rpm for 30 seconds.
4. 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 GeneChip™ Command Console™* of the *PharmacoScan™ Assay 24-Array Format Manual Workflow User Guide* (Pub. No. 703286)
 - 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 24-Array Format Manual Workflow User Guide* (Pub. No. 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 hybridization tray (from Axiom GeneTitan™ Consumables Kit) from packaging.
2. Label the hybridization tray; see to Figure 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 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 loading 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.

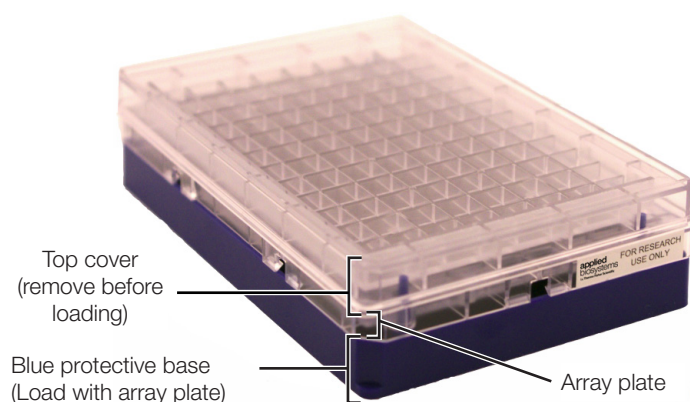


Figure 2. Array plate as shipped.

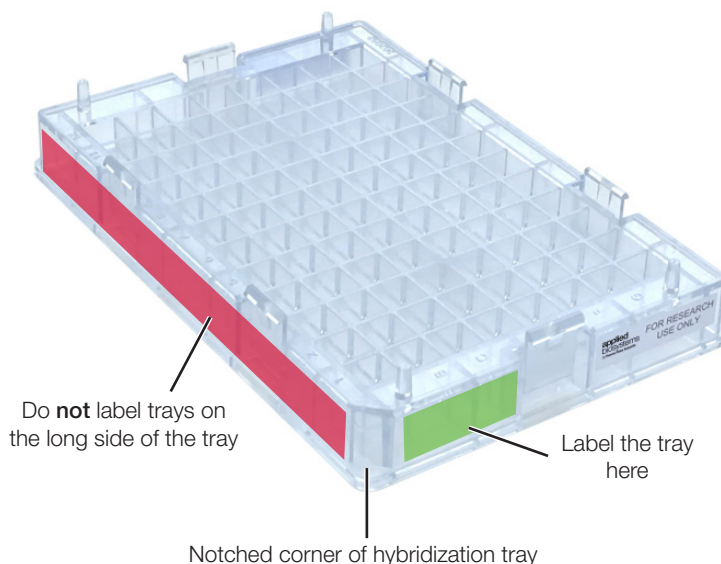


Figure 1. Correct area to label the hybridization tray.

Important! It is critical that you write only on the proper location of the hybridization 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.

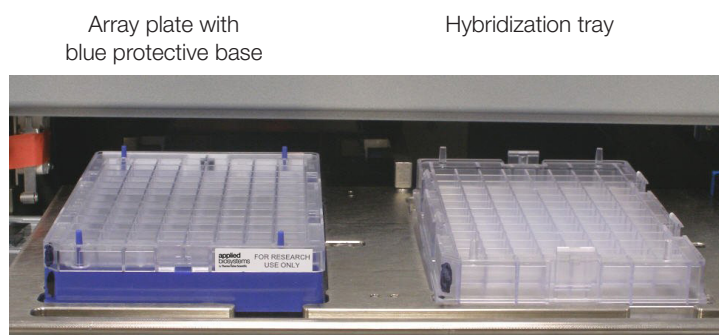


Figure 3. Array plate and hybridization tray loaded in GeneTitan drawer.

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 ensure there are no bubbles. If bubbles are found, attempt to remove them by gently tapping the plate on top.

Stage 5: GeneTitan™ reagent preparation

Reagents (from PharmacoScan™ Reagent Kit 4x24 Reactions)

- Module 4-1, –20°C, Part No. 901278
- Module 4-2, 2–8°C, Part No. 901276
- Module 5-1, –20°C, Part No. 902796
- Module 5-2, 2–8°C, Part No. 902797

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 (one)
 - Stain tray (five)
 - Covers for trays (six)

1: Prepare for GeneTitan reagent preparation

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

Reagent	Temp out of module ^[1]	Treatment	Storage
Module 4-1 (Part No. 901278)			
Axiom Ligase 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 Ligase Enzyme ^[2]	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 Ligase 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 Ligase Soln 2	Thaw at room temp. (do not place on ice!)	Vortex and spin	Store at room temp.
Axiom Probe Mix 2 ^[3]	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 ^[3]	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 1-B ^[3]	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 2-A ^[3]	Place on ice	Flick and invert tube 2 to 3 times to mix, then pulse-spin.	Place on ice
Axiom Stain 2-B ^[3]	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	(Not applicable)	Store at room temp.
Axiom Hold Buffer ^[4]	Room temp.	Vortex	Store at room temp. away from light

^[1] The temperature the reagent is held at immediately after removal from module.

^[2] An extra tube of Axiom Ligase Enzyme is in Module 5-1 in the event that it is needed.

^[3] These solutions are light sensitive. Do not expose tubes to direct light for a prolonged period of time.

^[4] Axiom Hold Buffer for preparing the scan tray for the second, third, and fourth plate are provided in Module 5-2.

Note: The presence of some precipitate in Axiom Ligase Buffer does 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 4 in a 15-mL conical tube. This recipe provides enough for both S1 reagent trays.
2. Gently invert the tube ten times to mix. Place on ice and protect from direct light.

Prepare Stain 2 Master Mix

1. Add reagents in the order shown in Table 5 in a 15-mL conical tube.
2. Gently invert the tube ten times to mix. Place on ice and protect from direct light.

Prepare Stabilization Master Mix

1. Add reagents in the order shown in Table 6 in a 15-mL conical tube.
2. Vortex the master mix at high speed for 3 seconds. Place on ice.

Prepare Ligation Master Mix

IMPORTANT! *Regarding the second tube of Axiom Ligase Enzyme:* Module 5-1 contains an extra tube of Axiom Ligase Enzyme which has been provided for back-up purposes. If there is insufficient volume of the Axiom Ligase 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 Ligase Enzyme (Module 5-1). The remaining contents of the second tube of Ligase 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 7.
1. Mix well by vortexing the tube for three seconds. Place back on ice.

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 8.
3. Gently flick the Axiom Ligase 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 4. 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

Table 5. 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

Table 6. 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

Table 7. Ligation Master Mix: Stage 1

Reagent	per array	Master mix 24+
Axiom Ligase Buffer	66.15 µL	1.75 mL
Axiom Ligase Soln 1	13.12 µL	348 µL
Axiom Ligase Soln 2	3.15 µL	83.5 µL
Subtotal	82.42 µL	2.18 mL

Table 8. 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 Ligase Enzyme	1.58 µL	42 µL
Total	105 µL	2.78 mL

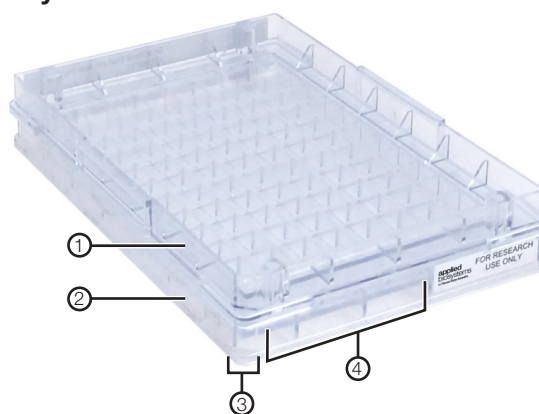
3: Aliquot Master Mixes and Axiom Hold Buffer into trays

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

Prepare trays and 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 24-Array Format Manual Workflow User Guide* (Pub. No. 703286) for the recommended technique.



- | | |
|------------------------|--------------------------------------|
| ① Cover for stain tray | ③ Notched corners aligned |
| ② Stain tray | ④ Label stain tray in this area only |

Figure 4. Stain tray with cover

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 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 Kimwipes laboratory tissue 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 cover 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 spreads evenly when the array plate is inserted into the reagent tray during processing with the GeneTitan MC Instrument.

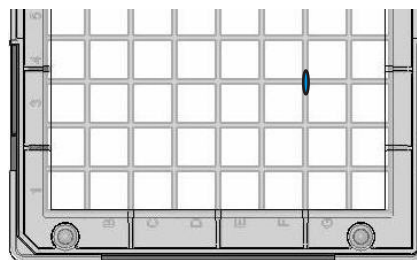


Figure 5. Blot drops of liquid on dividers.

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 Kimwipes laboratory tissue to remove.

Aliquoting Hold Buffer to the scan tray

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

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 7).

Note: Module 5-2 (Part No. 902797) has three 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 cover, 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 cover over the notched edge of the tray, and leave on the bench top.

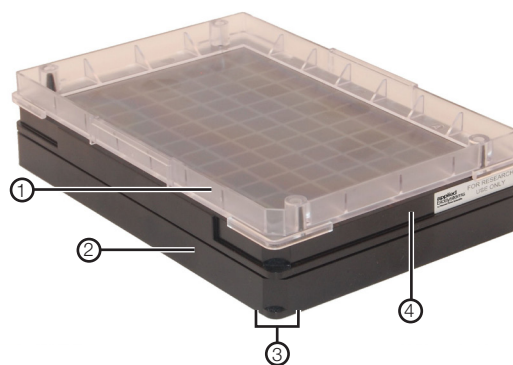
For more information on loading the reagent and scan trays, see:

- *GeneTitan™ MC Protocol for Axiom™ 2.0 Array Plate Processing Quick Reference* (Pub. No. 702988)
- Chapter 5, *Array Processing with the GeneTitan™ Multi-Channel Instrument of the PharmacoScan™ Assay 24-Array Format Manual Workflow User Guide* (Pub. No. 703286)

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.

Return top cover to the scan tray and base. Ensure that the notched corners of are aligned before loading.



- ① Cover for scan tray
- ② Black protective base (remove before loading)
- ③ Notched corners of scan tray, cover, and base aligned
- ④ Scan tray

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.



Figure 7. Scan tray with cover removed.

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Products: PharmacoScan™ Reagent Kit

Affymetrix Pte Ltd | 7 Gul Circle #2M-01 | Keppel Logistics Building | Singapore 629563

Products: PharmacoScan™ Array Plates

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