CarrierScan™ Assay 96-Array Format Manual Workflow

Pub. No. 703482 **Rev.** 3

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

Introduction to CarrierScan Assay 96-Array Format Manual Target Preparation

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

1. Genomic DNA preparation, described in the *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481).
2. 96-array format manual target preparation, described in this document.
3. Array processing, described in *GeneTitan™ MC Protocol for Axiom™ 2.0 Array Plate Processing Quick Reference* (Pub. No. MAN0017718).

IMPORTANT! This document contains an abbreviated set of instructions used to perform target preparation. You must carefully read all the instructions in the *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) 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 *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) and the GeneTitan Quick Reference (Pub. No. MAN0017718) described above.

New: An option for a 3-hour DNA precipitation step is now available. See the *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) for details.

Additional notes

- This manual workflow format allows you to run the CarrierScan Assay for 96 Samples once using 1 CarrierScan™ Reagent Kit 96 Reactions (Cat. No. 931933).
- This manual workflow 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, then 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 CarrierScan Reagent Kit 96 Reactions (Cat. No. 931933). Stage 1A requires the QIAGEN Multiplex PCR Plus Kit (Cat. No. 206152) and is sufficient to process 96 samples.
- See Chapter 3 of the *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) for a complete list of equipment and consumables required for each stage.

Note: CarrierScan arrays require a total of 150 ng of gDNA. mPCR requires 50 ng and DNA amplification requires 100 ng.

Stage 1A: Multiplex PCR

Prepare for Stage 1A

Supplies required

- mPCR Sample Plate (10 µL volumes of gDNA at 5 ng/µL, with control DNA)
- 10X Primer Mix from CarrierScan mPCR Module, –20°C, Part No. 931939
- 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 **CarrierScan mPCR** protocol
    - 95°C for 5 minutes
    - 95°C for 30 seconds, 60°C for 90 seconds, and 72°C for 45 seconds–cycled 35 times
    - 68°C for 10 minutes
    - 4°C hold
- Ensure that the heated lid option is used and the thermal cycler is programmed to run in "9600 Mode" for Applied Biosystems 9700, Veriti™, and ProFlex™ and "Safe" mode for Eppendorf® Mastercycler® pro S.

Prepare reagents

1. Prepare reagents as shown in Table 1.

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

   Table 1. Reagent handling.

Reagent	Quantity	Treatment
CarrierScan 10X Primer Mix	1 tube	Thaw, vortex, briefly centrifuge, then keep on ice.
QIAGEN Multiplex PCR Master Mix, 2X	3 tubes	Thaw, invert 10X to thoroughly mix, briefly centrifuge, then keep on ice.
QIAGEN Q-Solution, 5X	1 tube	Thaw, vortex, briefly centrifuge, then keep on ice.
QIAGEN RNase-free water	1 tube	Thaw, vortex, briefly centrifuge, then keep on ice.

Thaw mPCR Sample Plate

1. Bring mPCR Sample Plate to room temperature.
2. Vortex, briefly centrifuge, then place on cold aluminum block.

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, then briefly centrifuge.
3. Add 2,400 µL of the QIAGEN 2X PCR Master Mix to the 15-mL tube.
   - All 3 QIAGEN 2X Multiplex PCR Master Mix supplied vials are needed.
   - It is recommended to set a P-1000 single-channel pipette to 800 µL, then remove this volume from the first vial.
   - Transfer this solution to the 15-mL tube. Change tips, then repeat this step for the remaining 2 vials.
4. Mix thoroughly, but gently, by inverting tube 10 times.
5. Briefly centrifuge, then 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**

Add mPCR Master Mix to samples

1. Carefully pour the prepared mPCR Master Mix into a 25-mL reservoir.
2. Use a P200 multichannel pipette to transfer 30 µL of mPCR Master Mix to each well of the mPCR Sample Plate.
3. Seal plate. Gently vortex. Briefly centrifuge.
4. Load plate onto thermal cycler within 5 minutes.

Discard any leftover reagents

Run the CarrierScan mPCR thermal cycler protocol

Load the plate on the thermal cycler, then run the **CarrierScan mPCR** protocol.

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

Prepare for Stage 1B

Supplies required

- Reagents from the CarrierScan Reagent Kit 96 Reactions, Module 1, –20°C, Part No. 906011
- Amplification Sample Plate (20 µL volumes of gDNA at 5 ng/µL, with control DNA)

Instrument setup

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

Prepare reagents

1. Prepare reagents as shown in Table 3.

   Table 3. Reagent handling.

Reagent	Treatment
10X Denat Solution	Thaw, vortex, briefly centrifuge, then keep at room temperature.
Neutral Solution	Thaw (see Note below), vortex for 30 seconds, then keep at room temperature.
Amp Solution	Thaw (see Note below), vortex for 30 seconds, then keep at room temperature.
Water	Thaw (see Note below), vortex, then keep at room temperature.
Amp Enzyme	Flick tube 3 times, briefly centrifuge, then keep in –20°C cooler until ready to use.

   Note: Allow ~1 hour for Amp Solution to thaw on the benchtop at room temperature. If the solution is not completely thawed after 1 hour, vortex briefly, then return to the benchtop to complete thawing. The bottles can also be thawed in a dish with ultra-pure water, such as Millipore water. The Amp Solution 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 benchtop.
   b. Vortex, briefly centrifuge, then leave at room temperature.

   Note: Carry out the master mix preparations and additions to the Sample Plate at room temperature.

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+
10X Denat Solution	2 µL	400 µL
Water	18 µL	3.6 mL
Total volume	**20 µL**	**4.0 mL**

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 **10 minutes** at room temperature. Seal, vortex, then briefly centrifuge in a room temperature centrifuge as soon as possible during the incubation period.
4. After incubation, immediately add the Neutralization Master Mix as described in the following section.

Add Neutral Solution to samples

1. Pour the Neutral Soln into the reagent reservoir.
2. Add **130 µL of Neutral Solution** to each sample, pipetting down the wall of the well. Do not mix by pipetting up and down.
3. Seal, vortex, then briefly centrifuge the Sample Plate.
4. Proceed immediately to steps 5 and 6 below.

Prepare the Amplification Master Mix

1. In a 50-mL tube marked *Amp MM*, prepare Amplification Master Mix as shown in Table 5.

   Table 5. Amplification Master Mix.

Reagent	per sample	Master mix 96+
Amp Solution	225 µL	26.0 mL
Amp Enzyme	5 µL	578 µL
Total volume	**230 µL**	**26.58 mL**

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

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 laboratory tissue, seal tightly, vortex twice, then centrifuge the plate for 1 minute at 1,000 rpm.
4. Place the sealed plate in an oven set at 37°C, then leave undisturbed for 23 ±1 hour.

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

Prepare for Stage 2

Supplies required

- Selected reagents from CarrierScan™ Reagent Kit 96 Reactions:
  - HT Target Prep Module 2-1, –20°C, Part No. 906012
  - HT Target Prep Module 2-2, 2–8°C, Part No. 906013
- 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 *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) for notes on thawing and centrifuging prior to changing the seal to avoid cross-contamination.

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

Spike mPCR reaction into Amplification Plate

1. Vortex mPCR Reaction Plate, then briefly centrifuge.
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, then briefly centrifuge.
4. Immediately proceed to next step: *2: Stop DNA amplification reaction*.

Stop DNA amplification reaction

1. Place the Sample Plate in the **65°C oven, then 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. Prepare reagents.

Reagent	Module	Treatment
10X Frag Buffer	2-1	Thaw, vortex, then keep on ice.
Frag Enzyme	2-1	Flick tube 3 times, briefly centrifuge, then keep in –20°C cooler until ready to use.
Precip Soln 2	2-1	Thaw, vortex, briefly centrifuge, then keep at room temperature.
Frag Diluent	2-2	Thaw, vortex, briefly centrifuge, then keep on ice.
Frag Reaction Stop	2-2	Thaw, vortex, then keep at room temperature.
Precip Soln 1	2-2	Thaw, vortex, then 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, then incubate for 45 minutes**.

Prepare Fragmentation Master Mix

1. Start making the Fragmentation Master Mix when there is still 5 minutes to the finish of the 37°C incubation, using the values in Table 7.
   - Add the Frag Enzyme to the Fragmentation Master Mix at the end of the 45 minute 37°C incubation.
2. Vortex twice, then pour in the reagent reservoir placed at room temperature.

Table 7. Fragmentation Master Mix.

Reagent	per sample	Master mix 96+
10X Frag Buffer	45.7 µL	6.0 mL
Frag Diluent	10.3 µL	1.35 mL
Frag Enzyme	1.0 µL	131 µL
Total volume	**57 µL**	**7.48 mL**

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 then place on the benchtop 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 then vortex twice.
5. Start the timer for 30 minutes.
6. Briefly centrifuge the Sample Plate in the room temperature plate centrifuge.
7. Quickly transfer plate to 37°C oven, then 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 can lead to poor performance.

Aliquot the Stop Solution to the plate

1. A few minutes before the end of the 30-minute incubation period, pour the Frag Reaction Stop solution in the reagent reservoir.
   • Leave the Stop Solution reservoir at room temperature.
2. Remove the Sample Plate from the oven, then place on the benchtop.
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, then centrifuge.
5. Keep the Sample Plate at room temperature while you prepare the Precipitation Master Mix.

Prepare and add Precipitation Master Mix

Carry out the following steps at room temperature.

1. Prepare Precipitation Master Mix by adding 218 µL of Precip Solution 2 directly to the Precip Solution 1 bottle.
2. Vortex, then pour the Precipitation Master Mix into reagent reservoir.

   Table 8. Precipitation Master Mix.

Reagent	per sample	Master mix 96+
Precip Solution 1	238 µL	26 mL
Precip Solution 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, then centrifuge.

Prepare and add isopropanol

1. Pour 65 mL of isopropanol into a 100-mL reagent reservoir.
2. **Add 600 µL isopropanol to each sample**, then 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 laboratory tissue, then seal tightly with a Microamp seal.
4. Carefully **transfer the Sample Plate into the –20°C freezer, then incubate overnight** (16-24 hours). A 3-hour precipitation workflow option is also available. See the *CarrierScan Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) 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

Prepare for Stage 3

Supplies required

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

  HT Target Prep Module 2-1, –20°C, Part No. 906012

  HT Target Prep Module 2-2, 2–8°C, Part No. 906013
- Other Reagents Required for QC steps (optional)
  - TrackIt™ Cyan/Orange Loading Buffer (Cat. No. 10482-028)
  - TrackIt™ 25 bp DNA Ladder (Cat. No. 10488-022) or similar product.
  - Nuclease-free water, ultrapure MB grade (Cat. No. 71786)
  - E-Gel® 48 4% agarose gels (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

Prepare reagents

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 handling.

Reagent	Module	Treatment
Hyb Buffer	2-1	Vortex then keep at room temperature.
Hyb Solution 1	2-1	Thaw, vortex, centrifuge, then keep at room temperature.
Hyb Solution 2	2-2	Vortex, centrifuge, then keep at room temperature.
Resuspension Buffer	2-2	Warm to room temperature (1 hour).

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

Centrifuge Precipitation Plate, then 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, then **centrifuge the plate at 3,200 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, then discard the seal.
   b. Invert the plate over a clean waste container, then allow the liquid to drain.
   c. While still inverted, gently press the plate on a pile of laboratory tissues on a bench, then **leave it for 5 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, then **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, then either:
   - Proceed directly to *3B: Resuspension and Hybridization Master Mix preparation*. Leave the Sample Plate at room temperature.
   - Tightly seal the plate, then store at –20°C.

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 90 minutes before carrying out resuspension.
- Make sure the Resuspension Buffer has equilibrated to room temperature before adding to dry pellets in Step 1, below.
- Carry out the following steps at room temperature.

1. Pour Resuspension Buffer into a reagent reservoir. **Transfer 35 µL Resuspension Buffer to each well** of the Sample Plate with a dry pellet. Avoid touching pellets with the pipette tips.
2. Seal the Sample Plate, then place the plate on one of the following shakers:
   - Thermo Scientific™ Compact Digital Microplate Shaker: at speed 900 rpm for 10 minutes.
   - Jitterbug: at speed 7 for 10 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, then pour contents into the reagent reservoir.

   Table 10. Hybridization Master Mix.

Reagent	per sample	Master Mix 96+
Hyb Buffer	70.5 µL	7.8 mL
Hyb Solution 1	0.5 µL	55.6 µL
Hyb Solution 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. Briefly centrifuge.
5. Select a PCR plate appropriate to the type of approved thermal cycler you will use in Stage 4, then 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, then briefly centrifuge.

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.

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, then briefly centrifuge.
2. Make and read OD Plate:
   a. Add 90 µL nuclease-free water to the OD Plate (96-well UV-Star® plate, Thomas Scientific Cat. No. 25801).
   b. Transfer 10 µL of each Dilution QC Plate sample to the OD Plate, then mix by pipetting up and down.
   c. Read absorbance on a plate reader. See Appendix B, *Sample quantitation after resuspension* of the *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) for more information.
3. Make, then 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, then briefly centrifuge.
   d. Run Gel: See Appendix A, *Fragmentation quality control gel protocol* of the *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) for more information.

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

Prepare for Stage 4

Supplies required

- Reagents from from the CarrierScan™ Reagent Kit 96 Reactions Kit, Wash Buffer A (Part No. 906022), Wash Buffer B (Part No. 906023), Water (Part No. 906020)
- CarrierScan™ 1S 96-array plate in a protective base (Cat. No. 951950)
- 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 **CarrierScan Denature** protocol of 95°C for 10 minutes; 48°C for 3 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.

Prepare hybridization-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, then change the seal.
2. Vortex the Hyb-Ready Plate briefly, then centrifuge at 1,000 rpm for 30 seconds.
3. Leave the Hyb-Ready Plate at room temperature.

Prepare the GeneTitan™ MC Instrument and denature Hyb-Ready Sample Plate

1. **Warm up the array plate on the benchtop 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, then 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 *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481).
   a. Prepare the reagents from module 3 by inverting the bottles 2 to 3 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 *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481).
4. Place Hyb-Ready Plate in thermal cycler block, secure lid, then start the **CarrierScan Denature** protocol.

Prepare hybridization tray, then 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 **CarrierScan Denature** protocol has completed, remove the Hyb-Ready Plate from the thermal cycler, then 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, then hybridization tray into GeneTitan MC Instrument**.

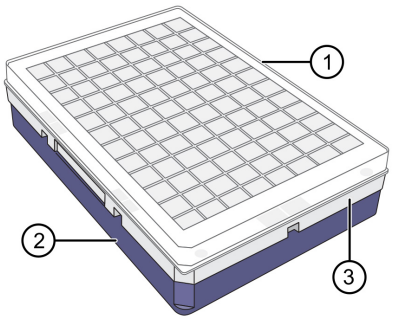
   The array plate is shipped with a clear top lid and a blue protective base (Figure 2). Before loading, the top lid must be removed.

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

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

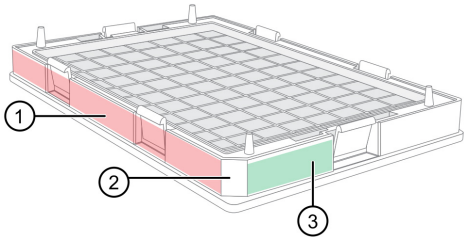
Hybridization continues on the GeneTitan MC Instrument for 23.5 to 24 hours before you load the Ligation/Staining/Stabilization reagent trays into the GeneTitan MC Instrument.

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.



1. Top lid (remove before loading)
2. Blue protective base (load with array plate)
3. Array Plate

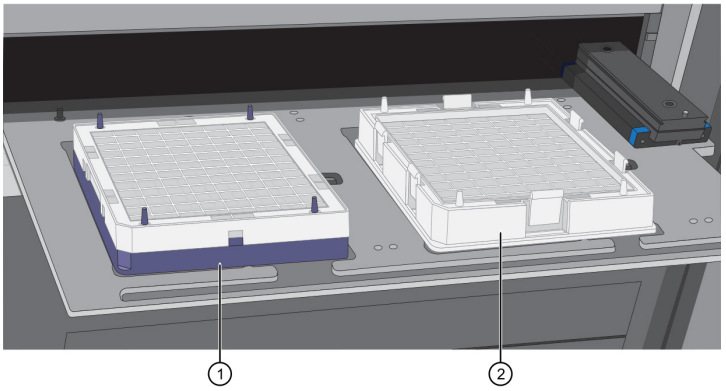
Figure 2. Array plate as shipped.



1. Do **not** label trays on the long side of the tray .
2. Notched corner of hybridization tray.
3. Label the hybridization tray here.

Figure 1. Labeling 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.



1. Array plate with blue protective base.
2. Hybridization Tray.

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 6 latching points to confirm that the 2 parts are clamped properly, then check underneath the arrays to ensure that there are no bubbles. If bubbles are found, attempt to remove them by gently tapping the plate on top.

Stage 5: GeneTitan™ Prepare reagents

Prepare for Stage 5

Reagents (from CarrierScan™ Reagent Kit 96 Reactions)

- HT Target Prep Module 3-1, –20°C, Part No. 906014
- HT Target Prep Module 3-2, 2-8°C, Part No. 906015

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)

Prepare for GeneTitan Prepare reagents

1. Prepare the reagents as described in the table below:

Reagent	Temp out of module[1]	Treatment	Storage
HT Target Prep Module 3-1 (Part No. 906014)			
Ligate Buffer	Thaw at room temperature	Place on benchtop at room temperature for 30 minutes. Vortex twice for 30 seconds. Examine for precipitate. If any, warm bottle with your hands, then vortex again for 30 seconds.	Place on ice
Ligate Enzyme[2]	Keep at –20°C until ready to use	Just before use: Flick, then invert tube 2 to 3 times to mix. Briefly centrifuge. Place in –20°C portable cooler until use.	Place in –20°C portable cooler
Ligate Solution 1	Thaw at room temperature	Vortex, then centrifuge.	Place on ice
Probe Mix 1	Thaw at room temperature	Vortex, then centrifuge.	Place on ice
Stain Buffer	Thaw at room temperature	Vortex, then centrifuge.	Place on ice
Stabilize Solution	Thaw at room temperature	Vortex, then centrifuge.	Place on ice
HT Target Prep Module 3-2 (Part No. 906015)			
Ligate Solution 2	Thaw at room temperature (do not place on ice!)	Vortex, then centrifuge.	Store at room temperature
Probe Mix 2[2]	Place on ice	Flick, then invert tube 2 to 3 times to mix, then briefly centrifuge.	Place on ice
Wash A	Leave on bench	Vortex twice. Place on Bench for 30 minutes. Look for precipitate. Vortex again if necessary.	Place on benchtop at room temperature
Stain 1-A[2]	Place on ice	Flick, then invert tube 2 to 3 times to mix, then briefly centrifuge.	Place on ice
Stain 1-B[2]	Place on ice	Flick, then invert tube 2 to 3 times to mix, then briefly centrifuge.	Place on ice
Stain 2-A[2]	Place on ice	Flick, then invert tube 2 to 3 times to mix, then briefly centrifuge.	Place on ice
Stain 2-B[2]	Place on ice	Flick, then invert tube 2 to 3 times to mix, then briefly centrifuge.	Place on ice
Stabilize Diluent	Place on ice	Vortex, then briefly centrifuge. Look for precipitate. If any: Warm tube to room temperature, then vortex again.	Place on ice
Water	Leave on bench	N/A	Store at room temperature
Hold Buffer[2]	Room temperature	Vortex.	Store at room temperature 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 Ligate Buffer will not adversely impact assay performance. Follow the instructions above to resuspend any precipitate before use.

Note: Occasionally, crystals are observed in Wash A and 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 10 times to mix. Place on ice, then protect from direct light.

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

Reagent	per array	Master mix 96+
Wash A	201.6 µL	22.2 mL
Stain Buffer	4.2 µL	463 µL
Stain 1-A	2.1 µL	231 µL
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 10 times to mix. Place on ice, then protect from direct light.

Table 12. Stain 2 Master Mix.

Reagent	per array	Master mix 96+
Wash A	100.8 µL	11.1 mL
Stain Buffer	2.1 µL	231 µL
Stain 2-A	1.05 µL	115.6 µL
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 3 seconds. Place on ice.

Table 13. Stabilization Master Mix.

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

Prepare Ligation Master Mix

The Ligation Master Mix is prepared in 2 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 3 seconds. Place back on ice.

Table 14. Ligation Master Mix: Stage 1.

Reagent	per array	Master mix 96+
Ligate Buffer	66.15 µL	7.3 mL
Ligate Solution 1	13.12 µL	1.45 mL
Ligate Solution 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, then place in a cooler chilled to –20°C.
2. Add reagents in the order shown in Table 15.
3. Gently flick the Ligate Enzyme tube 2 to 3 times, then briefly centrifuge 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, then 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
Probe Mix 1	10.5 µL	1.16 mL
Probe Mix 2	10.5 µL	1.16 mL
Ligate Enzyme	1.58 µL	174.4 µL
Total	**105 µL**	**11.59 mL**

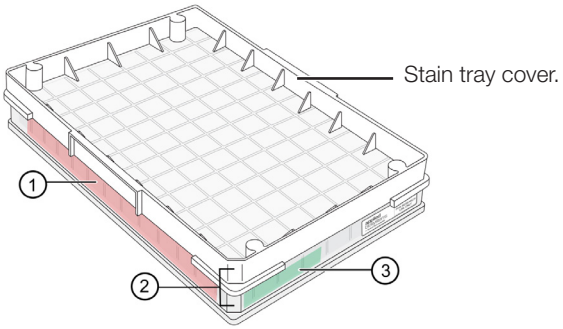
Aliquot master mixes and Hold Buffer into trays

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 2 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 *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. 703481) for the recommended technique.



① Do not label the stain tray in this area.
② Notched corners aligned
③ 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 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 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.

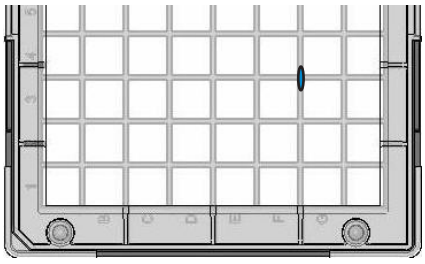
Aliquoting Hold Buffer to the scan tray

The scan tray is shipped with 2 covers, a bottom protective base and a top lid (Figure 6).

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 that no droplets of liquid are on top of the wells dividers.
- Blot with a laboratory tissue to remove.

Figure 5. Blotting drops of liquid on dividers.

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 Hold Buffer into the 25-mL reagent reservoir, placed on the benchtop 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-format 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 Hold Buffer.

5. If droplets of liquid splashed onto the well dividers, place a 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, then leave on the benchtop.

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 *CarrierScan™ Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0017718).

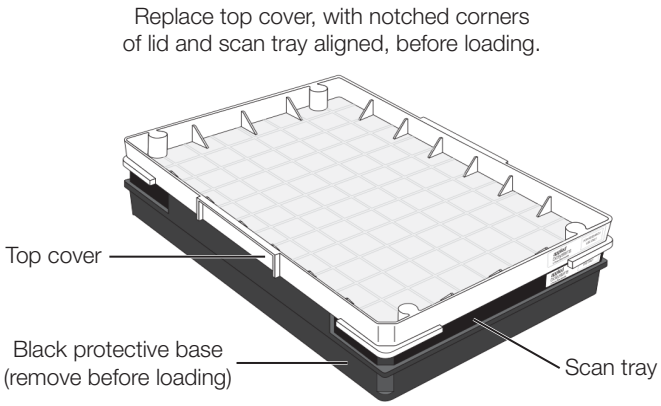


Figure 6. Scan tray with top cover and black protective base.

Figure 7. Scan tray on black base with cover removed.

For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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

Affymetrix Pte Ltd | 7 Gul Circle #2M-01 | Keppel Logistics Building | Singapore 629563 — Products: CarrierScan™ 1S Array Plates

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

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