Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol

Pub. No. 703436 **Rev.** 1

Introduction and Stage 1: DNA amplification

Introduction to manual target preparation

Running the Axiom 2.0 Assay requires the following sets of steps:

1. Genomic DNA Prep, described in the *Axiom™ 2.0 384HT gDNA Sample Prep QR*, Pub. No. 703163.
2. Target Prep of the samples, done using manual target prep, as described in this QR.
3. Array Processing, described in *GeneTitan™ MC Protocol for Axiom™ 384HT Array Plate Processing QR*, Pub. No. 703164.

Important! This QR contains an abbreviated set of instructions used to perform manual target preparation. You must carefully read all the instructions in the *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434) 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 *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434) and the QR (Pub. No. 703164) described above.

Additional notes:

- 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 Axiom 2.0 Assay Mini 96 Reagent Kit (Cat. No. 901758).
- See Chapter 3 of the *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434) for a complete list of equipment and consumables required for each stage.

1. Preparation for Stage 1: DNA amplification

Supplies required

- Reagents from Axiom 2.0 Assay Mini 96 Reagent Kit, Module 1, –20°C, Part No. 901711

Instrument setup

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

Reagent preparation

1. Prepare reagents as shown in the table below:

Reagent	Treatment
Axiom 2.0 Denat Soln 10X	Thaw, vortex, spin and keep at room temperature
Axiom 2.0 Neutral Soln	Thaw (see Note below) vortex and keep at room temperature
Axiom 2.0 Amp Soln	Thaw (see Note below) vortex 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, spin, and keep in –20°C cooler until ready to use

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

2. Thaw Samples in gDNA Plate:
   a. Bring your gDNA samples to room temperature on the bench top.
   b. Vortex, spin, and leave at room temperature.

   Note: The gDNA samples must be at a volume of 8.7 µL for each sample. Each gDNA samples must have a starting concentration of 11.5 ng/µL, 17.2 ng/µL, or 23 ng/µL, depending on the sample type and plated in an Eppendorf 96 Deep-well Plate, 2000 µL.

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

2: Prepare Denaturation Mix

1. To a 15 mL tube marked *D MM*, prepare Denaturation Master Mix as shown in the table below.

Reagent	per sample	Master Mix 96+
To the 15 mL tube marked *D MM*, add:		
Axiom Water	7.8 µL	2.2 mL
Axiom 2.0 Denat Soln 10X	0.9 µL	244 µL
Total volume	**8.7 µL**	**2.4 mL**

2. Vortex well and leave at room temperature.

3: Add Denaturation Master Mix to samples

1. Gently transfer the **Denaturation Master Mix** into the 25 mL reagent reservoir.
2. Add **8.7 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, and pulse-spin in a room temperature centrifuge
   during the incubation period.
4. After incubation, immediately add the Neutralization Master Mix as described below.

4: Add Axiom 2.0 Neutral Soln to samples

1. Gently pipet **7.5 mL of Neutral Soln** into the 25 mL reagent reservoir.
2. Add **56.6 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 on the next page.

5: Prepare the Amplification Master Mix

1. To a 15 mL tube marked Amp MM, prepare the Amplification Master Mix as shown below.
2. Vortex the Amplification Master Mix well, then invert the tube 2 times, and then vortex again.

Reagent	per sample	Master Mix 96+
To a 15 mL tube marked *Amp MM*, add:		
Axiom 2.0 Amp Soln	97.9 µL	12.0 mL
Axiom 2.0 Amp Enzyme	2.2 µL	267 µL
Total volume	**100.1 µL**	**12.3 mL**

6: Add Amplification Master Mix to samples

1. Slowly pour the Amplification Master Mix into the 25 mL reagent reservoir labeled *Amp MM*.
2. Slowly add **100.1 µL Amplification Master Mix** into each well 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 Kimwipe, seal tightly, vortex twice, and spin the plate for one minute at 1000 rpm.
4. Place the sealed plate in an oven set at 37°C and leave undisturbed for 23 ±1 hr.
5. Gather all the reagents from Module 1 and tighten all caps. Mark reagent pouches, tubes, and bottles to track use. Store at –20°C.

7: Freeze or proceed

After the incubation finishes, you can either:

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

Note: If freezing, do not perform the stop amplification reaction step before you store the Sample plate at –20°C. The Stop Amplification Reaction step is performed after thawing the frozen plate.

Stage 2: Fragmentation and precipitation

Preparation for Stage 2: Fragmentation and precipitation

Supplies required

- Selected reagents from Axiom 2.0 Assay Mini 96 Reagent Kit (see below):
  - 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:
  - One oven at 65°C
  - One oven at 37°C
  - One centrifuge at room temperature

Note: If the plate of amplified DNA samples was frozen at the end of Stage 1, thaw the plate before beginning Stage 2. See instructions in Chapter 6 of the *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434) 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: Stop 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 below.

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

3. Optional: Remove samples for quantifying amplification yield by the PicoGreen assay at a later time. See Chapter 4, *Axiom 2.0 Assay: Manual Target Preparation* for more information.
4. Transfer the Sample Plate from the 65°C oven to the **37°C oven and incubate for 45 minutes**.

2: 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 table below.

Reagent	per sample	Master Mix 96+
To the 15 mL tube marked *Frg MM*, add:		
Axiom 10X Frag Buffer	19.9 µL	3.4 mL
Axiom Frag Diluent	4.5 µL	766 µL
Axiom Frag Enzyme[1]	0.4 µL	74 µL
Total volume	**24.8 µL**	**4.2 mL**

[1] 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 a 25 mL reagent reservoir placed at room temperature.

3: Add Fragmentation Master Mix to samples

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. Do not place the Sample Plate on ice.
2. **Add 24.8 µL of Fragmentation Master Mix to each sample, pipetting directly into the liquid. Do not mix by pipetting up and down**.
3. Seal and vortex twice.
4. Start the timer for 30 min.
5. Quick spin the Sample plate in the room temperature plate centrifuge.
6. Quickly transfer plate to 37°C oven and incubate for 30 min.

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

4: Aliquot the Stop Solution to samples

1. A few minutes before the end of the 30 minute incubation period, carefully transfer **2.0 mL of Axiom Frag Rxn Stop** solution in a solution basin. Leave the *Stop* solution basin at room temperature.
2. Remove the Sample Plate from the oven and place on the benchtop.
3. At the end of the 30 minute fragmentation incubation period, **add 8.3 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.

5: Prepare and add Precipitation Master Mix

Carry out the following steps at room temperature.

1. Prepare Precipitation Master Mix in a 50 mL tube. Add the reagents in the order and volumes shown below. Vortex to mix.

Reagent	per sample	Master Mix 96+
To the 50 mL tube marked *Precip MM*, add:		
Axiom Precip Soln 1	103.5 µL	11.2 mL
Axiom Precip Soln 2	0.9 µL	94.1 µL
Isopropanol	261 µL	28.2 mL
Total volume	**365.4 µL**	**39.5 mL**

2. Pour approximately half of the Precipitation Master Mix into the reagent reservoir labeled Precip MM.

   Note: The total volume of the Precipitation Master Mix exceeds the reservoir capacity (25 mL). Pour approximately half of the Precipitation Master Mix and refill the reservoir with the rest of the Precipitation Master Mix after the first half has been exhausted.

3. **Add 365.4 µL Precipitation Master Mix to each sample**.
4. 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.
5. Blot the top of the plate with a Kimwipe and seal tightly with a Microamp seal.
6. Carefully **transfer the Sample plate into the –20°C freezer and incubate overnight** (16-24 hours).
7. 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.
8. 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

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

Supplies required

- Selected reagents from the Axiom 2.0 Assay Mini 96 Reagent Kit:
  - 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)
  - 25bp Invitrogen Ladder (Invitrogen 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 (1000-fold dilution of TrackIt™ Cyan/Orange Loading Buffer): Mix 49.95 mL of nuclease-free water with 50 µL of TrackIt Cyan/Orange Loading Buffer.
2. Prepare reagents as shown in the table below:

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

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 the table above.
2. Remove the Sample plate from the –20°C freezer and **centrifuge the plate at 3200 xg at 4°C for 40 min**.
3. During centrifugation prepare the resuspension and hybridization reagents as shown in the table above.
4. Following centrifugation, empty the liquid from the Sample plate as follows:
   a. Carefully remove the seal from the Sample plate and discard the seal.
   b. Invert the plate over a waste container and allow the liquid to drain.
   c. While still inverted, gently press the plate on a pile of Kimwipes on a bench and **leave it for 5 min**.

CAUTION! During this step, handle the sample plate gently to avoid disturbing the pellets. Do not bump or bang the plate.

5. Turn the plate top side up and **place in an oven for 20 min at 37°C to dry**. If using an GeneChip™ Hybridization Oven 645, turn off the rotor during the 20 min drying time.

   Note: If you are proceeding directly to *3B: Resuspension and Hybridization Preparation*, you can prepare the Hybridization Master Mix at this time.
6. After 20 min remove the plate from the oven , even if some droplets of liquid remain, and either:
   - Proceed directly to *3B: Resuspension and Hybridization 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 all the reagents have equilibrated to room temperature before preparing the master mix in step 1, below.
- Carry out these steps at room temperature.

CAUTION! Perform the rest of the steps in this stage under a fume hood.

1. Prepare the Hybridization Cocktail in a 15 mL tube as shown in the table. Vortex twice to mix. Transfer contents into a 25 mL reagent reservoir.
2. **Transfer 50 µL Hyb Cocktail to each well** of the Precipitation Plate. Avoid touching the pellets with the pipette tips.
3. Seal the Resuspension Plate and put on one of the following shakers:
   - Thermo Scientific™ Compact Digital Microplate Shaker: at speed 900 for 15 min
   - Jitterbug: at speed 7 for 15 min
4. Inspect the Sample Plate from the bottom. If the pellets are not dissolved, repeat Step 3. Pulse-spin.

Reagent	per sample	Master Mix 96+
to the 15 mL tube labeled *Hyb C*, add:		
Axiom Resuspension Buffer	15.2 µL	1.99 mL
Axiom Hyb Buffer	30.7 µL	4.0 mL
Axiom Hyb Soln 1	0.22 µL	28.4 µL
Axiom Hyb Soln 2	3.9 µL	511 µL
Total volume	**50 µL**	**6.5 mL**

5. Select a PCR plate appropriate to the type of approved thermal cycler you will use in Stage 4 and label as "Hyb Ready Plate [plate ID]".
6. Transfer the entire contents of each well in the Resuspension Plate to the corresponding wells of the labeled Hyb Ready Plate.
7. Seal tightly, vortex and pulse-spin.
8. 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: Sample QC (recommended): Perform quantitation and fragmentation quality control checks

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

To perform the sample QC checks:

1. Make Dilution QC Plate:
   a. Add **33 µL nuclease-free water** to each well of a PCR plate labeled *Dil QC*.
   b. Transfer **3 µL of the Hyb Ready sample** from each well of the Hyb Ready Plate to the corresponding well of the Dil QC Plate.
   c. Seal, vortex, and spin.
2. Make and read OD Sample Plate:
   a. Add **90 µL nuclease-free water** to each well of the OD Plate.
   b. Transfer **10 µL of each Dilution QC Plate** sample to the OD Plate (96-well UV Star plate, E&K Scientific Cat. No. 25801). Mix well by pipetting up and down.
   c. Read absorbance on a plate reader. See Appendix B, Sample Quantitation after Resuspension of the *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434) for more information.
3. Make and run Gel QC samples:
   a. Add **120 µL Gel Diluent** (gel loading dye diluted 1000-fold) to each well 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 *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434) for more information.

4: Freeze or proceed to Stage 4

At this point you can:

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

Stage 4: Denaturation and hybridization

Preparation for Stage 4: Denaturation and hybridization

Supplies required

- Reagents from the Axiom 2.0 Assay Mini 96 Reagent Kit, Module 3:
  - Wash Buffer A (Part No. 901446) - 2 bottles
  - Wash Buffer B (Part No. 901447) - 1 bottle
  - Axiom Water (Part No. 901578) - 1 bottle
- Axiom Mini 96 array plate in a protective base
- 384 Layout GeneTitan™ Hyb Tray (Part No. 902278) from the Axiom™ 384HT High Volume Consumables Kit (Cat. No. 902629)

Instruments and setup

- GeneTitan MC Instrument
- Approved thermal cycler
  - Must be programmed with the **Axiom 2.0 Denature** protocol of 95°C for 10 min; 48°C for 3 min; 48°C for hold.
  - Use the heated lid option when setting up or running protocols.
- Hyb ready samples in plate appropriate to the thermal cycler model used
- 96-well metal chamber pre-heated in a 48°C oven

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

1: Prepare hyb ready samples stored at –20°C

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

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

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

1. **Warm up the array plate on the bench top for a minimum of 25 minutes** before setting up hybridization on the GeneTitan MC Instrument.
2. At the end of the array warm up time, open the pouch and scan the array plate barcode into the Batch Registration file.
3. Before you denature your Hyb Ready samples, ensure that the GeneTitan MC Instrument is ready for use by following the instructions given in Chapter 5, Stage 2—Hybridization and Appendix C, Registering Samples in Applied Biosystems™ GeneChip™ Command Console™ of the *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434).
   a. Prepare the reagents from Module 3 by inverting the bottles 2 to 3 times to mix.
   b. Upload the Batch Registration file.
   c. Set up the GeneTitan MC Instrument. For more information, see:
      - *GeneTitan™ MC Protocol for Axiom™ 2.0 Array Plate Processing QR* (Pub. No. 702988).
      - Chapter 5, Array Processing with the GeneTitan™ Multi-Channel Instrument of the *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434).
4. Place Hyb Ready Plate in thermal cycler block, secure lid, and start the **Axiom 2.0 Denature** protocol.

CAUTION! Perform the sample transfer steps in this stage under a fume hood.

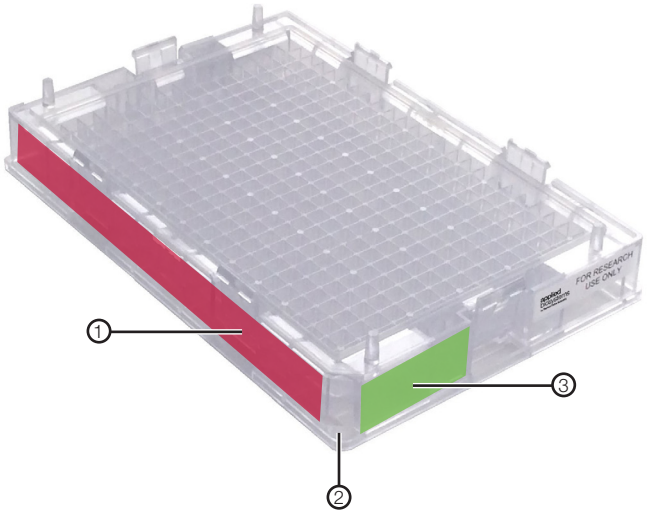
3: Prepare Hybridization Tray and load into GeneTitan™ MC Instrument

During this step, you will be switching plate formats: from 96-format (96-well Hyb Ready PCR Plate) to 384-format (384 Hyb Tray). Please refer to Chapter 4, "Stage 4: Denaturation and hybridization" of the *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434) for a detailed explanation on the location of the Quadrant 1 position on a 384-format plate.

1. Obtain one 384 layout GeneTitan™ Hyb Tray from the Axiom 384HT High Volume Consumables Kit and remove from packaging. The hybridization tray is packaged with a protective cover which should be discarded prior to use.
2. Label the hyb tray; please refer to Figure 1 and the IMPORTANT note below the figure. Remove and discard the clear hyb tray cover.
3. After the **Axiom 2.0 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 **35 µL**, slowly transfer the denatured samples from the 96-well Hyb Ready Plate into the corresponding Quadrant 1 wells of the 384 Hyb Tray as instructed and illustrated below. Dispense to the first stop to avoid creating bubbles. If air bubbles are present after transferring all samples, puncture using a clean pipette tip.



① Do not label trays on the long side of the tray
② Notched corner of Hyb Tray
③ Label the Hyb Tray here

Figure 1. Labeling Hyb Tray

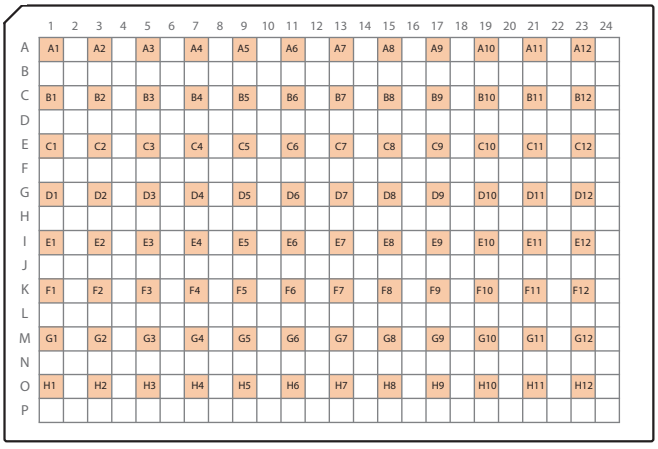
IMPORTANT! It is critical that you write only on the proper location of the hyb tray, as shown above. Do NOT write on any other side, as this may interfere with sensors inside of the GeneTitan MC Instrument and result in experiment failure.

Final layout of 384 Hyb Tray with denatured samples

- Orange wells in the figure below are Quadrant 1.
- Well designation within the highlighted wells correspond to the sample's location on the 96 format Hyb Ready Plate.

Plate format switching guidance:
Transfer denatured samples from a 96-well format PCR plate to wells in quadrant 1 of a 384-well format hyb tray.

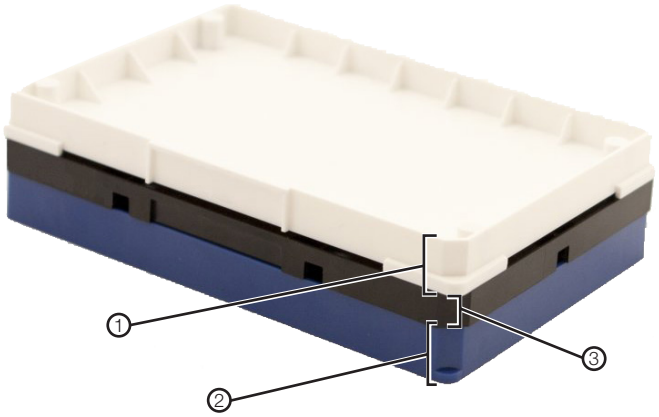
96-well format hyb ready PCR plate	384-well format hyb tray
Row A	Row A, odd wells
Row B	Row C, odd wells
Row C	Row E, odd wells
Row D	Row G, odd wells
Row E	Row I, odd wells
Row F	Row K, odd wells
Row G	Row M, odd wells
Row H	Row O, odd wells



5. Load the array plate and hyb tray into GeneTitan MC Instrument The software starts the process for placing the array plate on to the hybridization tray.
   - The array plate is shipped with a white top lid and a blue protective base (Figure 2). Before loading, the top lid must be removed.
   - The white plastic lid on top of the array plate SHOULD NOT be loaded in the GeneTitan MC Instrument.
   - The 384 Hyb Tray should not have any bubbles and there is no need to spread the liquid around the bottom of the wells.
   - The clear hyb tray cover SHOULD NOT be loaded in the GeneTitan MC Instrument.

   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.



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

Figure 2. Array plate as shipped

Array plate with blue protective base

Hyb Tray

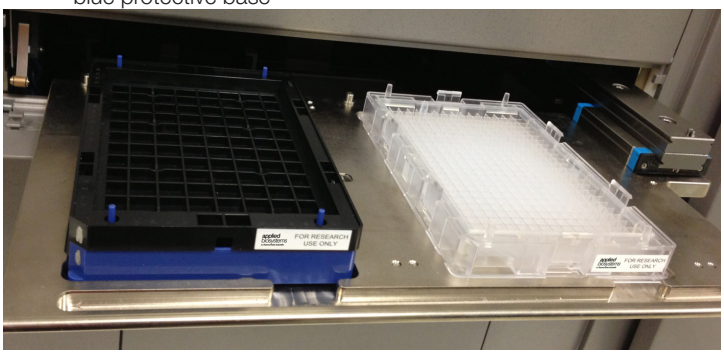


Figure 3. Array plate and Hyb Tray loaded in a GeneTitan drawer

IMPORTANT!

- The array plate must be loaded on its protective blue base, as shown above.
- After the GeneTitan MC Instrument has stacked the array plate and hyb tray, execute the array plate/hyb tray clamping and verification procedure to ensure that the two parts are properly clamped together.
- **Keeping the plate level**, inspect the bottom of the plate stack for bubbles under the arrays. If bubbles are found, attempt to remove them by gently tapping the plate. **Do NOT unclamp the plate stack**.

Stage 5: Manually preparing reagent trays for the GeneTitan™ MC Instrument

Preparation for Stage 5: Manually preparing reagent trays for the GeneTitan MC Instrument

Reagents

- Module 4-1, –20°C, Part No. 901278
- Module 4-2, 2-8°C, Part No. 901276
- Axiom Hold Buffer, 2-8°C: P/N 903012 (if running second plate)

Instrument

- GeneTitan MC Instrument

Consumables

- Aluminum foil (optional)
- Obtain the following items from the Axiom™ 384HT High Volume Consumables Kit, Cat. No. 902629:
  - 384HT GeneTitan Scan Tray and cover (1)
  - 384 Layout GeneTitan Stain Tray (2)
  - 384 Layout Axiom Stain2 Tray (1)
  - 384 Layout Axiom Ligation Tray (1)
  - 384 Layout Axiom Stab Tray (1)
  - Covers for trays (5)

1: Prepare reagents

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

Reagent	Temp out of module[1]	Treatment	Storage before Master Mix
Module 4-1 (Part No. 901278)			
Axiom Ligate Buffer	Thaw at room temp	1. Place on bench top at room temp for 30 min. 2. Examine for precipitate. 3. Vortex twice. 4. Examine for precipitate. If any, warm bottle with your hands and vortex again for thirty seconds.	Place on ice
Axiom Ligate Enzyme	Keep at –20°C until ready to use	1. Just before use, flick 2 to 3 times to mix. 2. Spin. 3. 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!)	1. Place on benchtop at room temperature for 30 to 40 min. 2. Vortex and spin	Store at room temp.
Axiom Probe Mix 2[2]	Place on ice	Flick 2 to 3 times to mix, then spin	Place on ice
Axiom Wash A	Leave on benchtop	1. Vortex twice. 2. Place on benchtop at room temperature for 30 min. 3. Look for precipitate. 4. Vortex again if necessary.	Place on benchtop at room temp
Axiom Stain 1-A[2]	Place on ice	Flick 2 to 3 times to mix, then spin	Place on ice
Axiom Stain 1-B[2]	Place on ice	Flick 2 to 3 times to mix, then spin	Place on ice
Axiom Stain 2-A[2]	Place on ice	Flick 2 to 3 times to mix, then spin	Place on ice
Axiom Stain 2-B[2]	Place on ice	Flick 2 to 3 times to mix, then spin	Place on ice
Axiom Stabilize Diluent	Place on ice	1. Vortex and spin. 2. Look for precipitate. If any, warm tube to RT and vortex again.	Place on ice
Axiom Water	Room temp	N/A	Place on benchtop 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 the table. This recipe provides enough for both S1 reagent trays.
2. Gently invert the tube 10 times to mix. Place on ice and protect from direct light.

Stain 1 Master Mix (for both S1 trays)

Reagent	per array	Master Mix 96+
To a 15 mL tube marked *S1*, add:		
Axiom Wash A	67.2 µL	7.8 mL
Axiom Stain Buffer	1.4 µL	163 µL
Axiom Stain 1-A	0.7 µL	81 µL
Axiom Stain 1-B	0.7 µL	81 µL
Total	**70 µL (35 µL x 2)**	**8.1 mL**

Prepare Stain 2 Master Mix

1. Add reagents in the order shown in the table.
2. Gently invert the tube 10 times to mix. Place on ice and protect from direct light.

Stain 2 Master Mix

Reagent	per array	Master Mix 96+
To a 15 mL tube marked *S2*, add:		
Axiom Wash A	33.6 µL	4.3 mL
Axiom Stain Buffer	0.70 µL	90 µL
Axiom Stain 2-A	0.35 µL	45 µL
Axiom Stain 2-B	0.35 µL	45 µL
Total	**35 µL**	**4.5 mL**

Prepare Stabilization Master Mix

1. Add reagents in the order shown in the table.
2. Vortex the master mix at high speed for 3 sec.
3. Place on ice.

Stabilization Master Mix

Reagent	per array	Master Mix 96+
To a 15 mL tube marked *Stbl*, add:		
Axiom Water	31.1 µL	4.0 mL
Axiom Stabilize Diluent	3.5 µL	451 µL
Axiom Stabilize Soln	0.4 µL	56 µL
Total	**35 µL**	**4.5 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.
2. Add reagents to the tube in the order shown in the table.
3. Mix well by vortexing the tube for 3 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 the table.
3. Gently flick the Axiom Ligate Enzyme tube 2-3 times, then perform a quick spin immediately prior to adding the enzyme to the Master Mix.
4. Gently invert the Master Mix tube 10 times to mix (do not vortex).
5. Place on ice and protect from direct light.

Ligation Master Mix: Stage 1

Reagent	per array	Master Mix 96+
To a 15 mL tube marked *Lig*, add:		
Axiom Ligate Buffer	22.1 µL	2.9 mL
Axiom Ligate Soln 1	4.4 µL	575 µL
Axiom Ligate Soln 2	1.1 µL	138 µL
Subtotal	**27.5 µL**	**3.6 mL**

Ligation Master Mix: Stage 2

Reagent	per array	Master Mix 96+
Ligation Master Mix from Stage 1	27.5 µL	3.6 mL
Axiom Probe Mix 1	3.5 µL	460 µL
Axiom Probe Mix 2	3.5 µL	460 µL
Axiom Ligate Enzyme	0.53 µL	69 µL
Total	**35.03 µL**	**4.6 mL**

3: Aliquot Master Mixes and Axiom Hold Buffer into trays

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

Prepare trays and lids

1. Gather the following stain trays from the Axiom 384 HT High Volume GeneTitan Consumables Kit and label as follows:

Type of tray	Part No.	Qty	Label color	Master mix/reagent	Label the tray
384 Layout GeneTitan Stain Tray	501279	1	White	Stain 1 Master Mix	*S1-1*
384 Layout GeneTitan Stain Tray	501279	1	White	Stain 1 Master Mix	*S1-2*
384 Layout Axiom Stain2 Tray	501394	1	Blue	Stain 2 Master Mix	*S2*
384 Layout Axiom Ligation Tray	501398	1	Yellow	Ligation Master Mix	*Lig*
384 Layout Axiom Stab Tray	501396	1	Green	Stabilization Master Mix	*Stbl*

2. Obtain one boxed Scan Tray (Part No. 902279) from the Axiom 384 HT High Volume GeneTitan Consumables Kit.

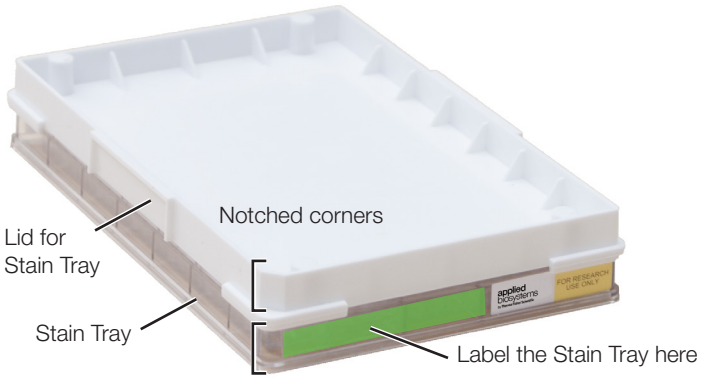


Figure 4. Stain Tray with lid

IMPORTANT! It is critical that you write only on the proper location of the proper edge of the stain trays, as shown in Figure 4. 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 quadrant 1 (Q1) of the S1, S2, Stbl, and Lig trays labeled in the previous step:

1. Pipet or pour the Master Mix into the 25 mL reagent reservoir.
2. **Aliquot 35 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. Correctly orient the cover 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 two covers, a bottom protective base and a top lid (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: The Axiom Hold Buffer pouch (Part No. 903012) holds a single Axiom Hold Buffer bottle that should be used to prepare the Scan Tray for second plate.

1. Pour all the contents of the Axiom Hold Buffer into the 25 mL divided reagent reservoir, placed on the bench top at room temperature.
2. Remove the scan tray from its pouch.
3. Remove the top scan tray lid, but leave the scan tray on its protective black base.
4. Aliquot **170 µL to EACH of the 96 wells** of the 384 HT GeneTitan Scan Tray — dispense to the first stop and avoid touching the bottom of the tray.

   WARNING! The Scan Tray requires 170 µL of Hold Buffer per well.

5. If droplets of liquid splashed onto the well dividers, place a Kimwipe on top of the tray to blot and remove.
6. Cover the tray by orienting the notched corner of the lid over the notched edge of the tray, and leave on the bench top.

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

- *GeneTitan™ MC Protocol for Axiom™ 2.0 Array Plate Processing QR* (Pub. No. 702988)
- Chapter 5, *Array Processing with the GeneTitan™ Multi-Channel Instrument* of the *Axiom™ 2.0 Assay Mini 96-Array Format Manual Protocol User Guide* (Pub. No. 703434)

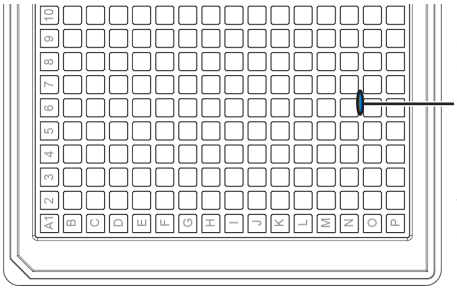
4. Store remaining reagents

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.

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.

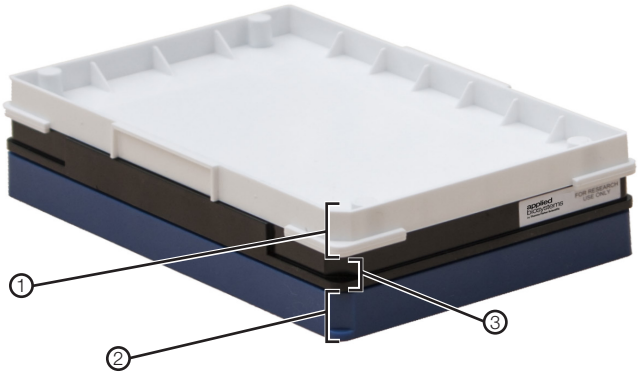
When aliquoting ligation, staining, and stabilization reagents to the trays, it is not necessary to spread the reagent to each corner of the well. The reagent will spread evenly when the array plate is inserted into the reagent tray during processing with the GeneTitan MC Instrument.



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 Kimwipe to remove.

Figure 5. Blotting drops of liquid on dividers



Replace top lid with notched corners aligned before loading.

① Top Lid
② Blue Protective Base (remove before loading)
③ Scan Tray

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

Cover

Scan tray on blue protective base

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

Figure 7. Scan tray with cover removed

The information in this guide is subject to change without notice.

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