Axiom™ 2.0 Assay 96-Array Format Manual Workflow

Catalog Numbers 901758

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Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. no. MAN0018000). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Introduction to manual target preparation

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

1. Genomic DNA preparation, described in the *Axiom™ 2.0 gDNA Sample Preparation Quick Reference* (Pub. No. MAN0017720), the *Axiom™ gDNA Sample Prep for Genome-Wide BOS 1 Array Plate Quick Reference* (Pub. No. 702975), Chapter 2 in the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000), or Chapter 2 in Section 1, in the Axiom™ Microbiome Assay Protocol in the *Axiom™ Microbiome Solution User Guide* (Pub. No. 703408).
2. Manual target preparation of the samples, described in this document.
3. Array processing, described in *GeneTitan™ MC Protocol for Axiom™ Array Plate Processing Quick Reference* (Pub. No. MAN0017718).

IMPORTANT! This quick reference contains an abbreviated set of instructions that are used to perform target preparation without the use of a liquid handling robot. Carefully read all the instructions in the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000) before performing manual target preparation.

Note: Array handling and processing protocols still require the use of a GeneTitan™ MC Instrument, as described in the appropriate user guide or quick reference.

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 Reagent Kit (Cat. No. 901758).
- See Chapter 4 of the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000) for a complete list of equipment and consumables that are required for each stage.

Stage 1: Amplify the genomic DNA

Prepare for Stage 1: Amplify the genomic DNA

Supplies required

- Reagents from the Axiom™ 2.0 Reagent Kit, Cat. No. 901758: Module 1, –20°C, Part No. 901711.

Set up the instruments

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

Prepare for DNA amplification

1. Thaw and prepare reagents from Module 1 of the Axiom™ 2.0 Reagent Kit.

Table 1 Reagent preparation.

Reagent	Treatment
Axiom™ 2.0 Denat Soln 10X	Thaw, vortex, spin and keep at room temperature
Axiom™ 2.0 Neutral Soln	Thaw, vortex, and keep at room temperature[1]
Axiom™ 2.0 Amp Soln	Thaw, vortex, and keep at room temperature[1]
Axiom™ Water	Thaw, vortex, and keep at room temperature[1]
Axiom™ 2.0 Amp Enzyme	Flick tube 3X, spin, and keep in –20°C cooler until ready to use

[1] 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 the gDNA plate:
   a. Bring your gDNA samples to room temperature on the benchtop.
   b. Centrifuge the plate, then leave at room temperature.

Note: The Sample Plate for **genotyping studies** must have 20 µL of each gDNA diluted to a concentration of 5 ng/µL or 10 ng/µL, as required according to the sample type, into an **ABgene 96 square well storage plate, 2.2 mL** or **Eppendorf™ DeepWell™ Plate 96, 2,000 µL**.

The Sample Plate for **microbiome studies** must have 20 µL of stool gDNA diluted to a concentration of 2.5 ng/µL and/or 17.5 µL cDNA must be diluted with 2.5 µL reduced EDTA TE buffer into the **Eppendorf™ DeepWell™ Plate 96, 2,000 µL**. A no template control (NTC) and Genomic DNA Standard (Ref 103) must be plated.

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

3. Label a 15-mL and 50-mL conical tube as indicated in the following table.

Label	Tube size	Temperature	Contents
D MM	15 mL	Leave tube at room temperature	Denaturation Master Mix
Amp MM	50 mL	Leave tube at room temperature	Amplification Master Mix

4. Label three 25-mL reagent reservoirs as indicated in the following table.

Label	Temperature	Contents
D MM	Leave reagent reservoir at room temperature	Denaturation Master Mix
N Soln	Leave reagent reservoir at room temperature	Neutralization Solution
Amp MM	Leave reagent reservoir at room temperature	Amplification Master Mix

Prepare Denaturation Master Mix

1. To the 15-mL centrifuge tube labeled “D MM”, use the following table to dilute the appropriate volume of Axiom™ 2.0 Denat Soln 10X using the Axiom™ Water.

Table 2 Denaturation Master Mix.

Reagent	per sample	Master mix 96+
Axiom™ Water	18 µL	3.6 mL
Axiom™ 2.0 Denat Soln 10X	2 µL	400 µL
Total volume	**20 µL**	**4 mL**

2. Vortex, centrifuge briefly, then leave at room temperature.

Add Denaturation Master Mix to samples

1. Pour the Denaturation Master Mix into the reagent reservoir marked *D MM*.
2. Using a P20 12-channel pipette, add 20 µL of Denaturation Master Mix to each sample.
   - Pipet directly into the liquid of each well. Do not mix by pipetting up and down.
   - Change tips between each addition.
   - This plate is now known as the **Denaturation Plate**.
3. Seal, then vortex the Denaturation Plate. Start the timer for a 10-minute incubation.
4. Briefly centrifuge the Denaturation Plate in a room-temperature centrifuge by bringing the centrifuge speed to 1,000 rpm (takes ~1 minute).

   The centrifuge time is included in the 10-minute incubation.
5. After incubation, immediately add the Neutralization Master Mix as described in the next step "Add Axiom™ 2.0 Neutral Soln to samples" on page 3.

Add Axiom™ 2.0 Neutral Soln to samples

1. Pour the Axiom™ 2.0 Neutral Soln into the reagent reservoir marked *N Soln*.
2. Using a P200 12-channel pipette, pipetting down the wall of each well, add **130 µL of Axiom™ 2.0 Neutral Soln** to each sample (total volume 170 µL/well).
   - Change tips between each addition.
   - The plate is now known as the **Neutralization Plate**.
3. Seal, vortex, then centrifuge the Neutralization Plate.
4. Proceed immediately to the next step "Prepare and add the Amplification Master Mix" on page 3.

Prepare and add the Amplification Master Mix

1. To a 50-mL tube marked *Amp MM*, add the reagents that are listed in the following table.

   Table 3 Amplification Master Mix.

Reagent	per sample	Master mix 96+
Axiom™ 2.0 Amp Soln	225 µL	26.0 mL
Axiom™ 2.0 Amp Enzyme	5 µL	578 µL
Total volume	**230 µL**	**26.58 mL**

2. Vortex the Amplification Master Mix well, invert the tube 2 times, then vortex again.
3. Slowly pour the Amplification Master Mix to the reagent reservoir labeled *Amp MM*.
4. Using a P1200 12-channel pipette, *slowly* add **230 µL Amplification Master Mix** to each well of the Neutralization Plate, pipetting down the wall of the well (there is now a total volume of 400 µL/well).
   - Do not mix by pipetting up and down.
   - Change tips between each addition.
   - The plate is now known as the **Amplification Plate**.
5. Seal tightly, vortex twice, then centrifuge the Amplification Plate for 1 minute at 1,000 rpm.
6. Place the sealed Amplification Plate in an oven set at **37°C** and leave **undisturbed for 23 ±1 hour**.

Freeze or proceed

After the incubation finishes, you can either:

- Proceed to “Stage 2: Fragment and precipitate the DNA” on page 4.
- Store the Amplification Plate at –20°C.

Note: If freezing, do not perform the stop amplification reaction step in Stage 2 before you store the Amplification Plate at –20°C. The stop amplification reaction step is performed after thawing the frozen plate.

Stage 2: Fragment and precipitate the DNA

Prepare for Stage 2: Fragment and precipitate the DNA

Supplies required

- Selected reagents from the Axiom™ 2.0 Reagent Kit (see Table 4):
  - Module 2-1, –20°C, Part No. 901528
  - Module 2-2, 2–8°C, Part No. 901529
- Isopropanol (supplied by user)

Set up the ovens and centrifuge

- Set 2 incubators/ovens as follows, preferably the night before:
  - One oven at 37°C
  - One oven at 65°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 starting Stage 2. See instructions in Chapter 6 of the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000) for notes on thawing and centrifuging before changing the seal to avoid cross-contamination.

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

Thaw and prepare the fragmentation reagents

Prepare reagents as listed in the following table.

Table 4 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, centrifuge, and keep in –20°C cooler until ready to use.
Axiom™ Precip Soln 2	2-1	Thaw, vortex, centrifuge, and keep at room temperature.
Axiom™ Frag Diluent	2-2	Thaw, vortex, centrifuge, and keep on ice.
Axiom™ Frag Rxn Stop	2-2	Thaw, vortex, and keep at room temperature.
Axiom™ Precip Soln 1	2-2	Thaw, vortex, and keep at room temperature.
Isopropanol	N/A	Keep at room temperature.

Label tubes and reagent reservoirs

1. Label one 15-mL conical tube *Frag MM* and place on ice. Label one 50-mL conical tube *Precip MM* and leave at room temperature.
2. Label the 4 reagent reservoirs as indicated in the following table.

Label	Size	Temperature	Contents
Frag MM	25 mL	Room temperature	Fragmentation Master Mix
Stop	25 mL	Room temperature	Frag Rxn Stop
Precip MM	25 mL	Room temperature	Precipitation Master Mix
ISO	100 mL	Room temperature	Isopropanol

Incubate samples in preheated ovens

1. Stop the DNA amplification reaction.
   a. Place the Amplification Plate in the 65°C oven.
      - If proceeding directly from the end of Stage 1, transfer the Amplification Plate from the 37°C oven to the 65°C oven. Ensure that the seal is still securely attached to the plate to minimize evaporation.
      - If working with a frozen Amplification Plate, follow the guidelines in “Thaw and prepare the fragmentation reagents” on page 4 before placing it in the 65°C oven.

   b. Incubate for 20 minutes.
   c. Prepare the fragmentation reagents as detailed in Table 4 in “Thaw and prepare the fragmentation reagents” on page 4 after starting the 65°C incubation of the Amplification Plate.
2. Prepare for fragmentation.
   a. Remove the Amplification Plate from the 65°C oven, then transfer the plate to the 37°C oven.
   b. Incubate for 45 minutes.

Prepare the Fragmentation Master Mix

1. Start making the Fragmentation Master Mix when there are 5 minutes remaining of the 37°C incubation, using the values in the following table.

Table 5 Fragmentation Master Mix.

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

   a. Add the Axiom™ Frag Enzyme to the Fragmentation Master Mix at the end of the 45-minute 37°C incubation.
2. Vortex twice, then pour into a reagent reservoir on the benchtop at room temperature.

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 Amplification Plate from the 37°C oven, then place on the benchtop at room temperature.

   Do not place the Amplification Plate on ice.

2. Use a P200 12-channel pipette to add 57 µL of Fragmentation Master Mix to each reaction.
   - Pipet directly into the liquid of each well.
   - Change tips after each addition.
   - After adding the Fragmentation Master Mix to the plate, the plate is now known as the **Fragmentation Plate**.

3. Seal, then vortex twice.

4. Start the timer for 30 minutes.

5. Briefly centrifuge the Fragmentation Plate in the room temperature plate centrifuge.

6. Quickly transfer the plate to a 37°C oven, then incubate for 30 minutes.

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

Add the Axiom™ Frag Rxn Stop solution to the Fragmentation Plate

1. A few minutes before the end of the 30-minute incubation, pour the Axiom™ Frag Rxn Stop solution into a reagent reservoir.

   Leave the reagent reservoir at room temperature.

2. Remove the Fragmentation Plate from the oven, then place on the benchtop at room temperature.

3. At the end of the 30-minute fragmentation incubation, add 19 µL of Axiom™ Frag Rxn Stop solution to each reaction.
   - Pipette directly into the liquid of each well.
   - Change tips after each addition.
   - Proceed immediately to the next step.

4. Seal, vortex, then centrifuge.

5. Keep the Fragmentation Plate at room temperature while you prepare the Precipitation Master Mix.

Prepare and add the Precipitation Master Mix

Carry out the following steps at room temperature.

1. Prepare the Precipitation Master Mix in a 50-mL tube. Add the reagents in the order and volumes listed in Table 6.

   Table 6 Precipitation Master Mix.

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

2. Vortex the Precipitation Master Mix, then pour into the reagent reservoir labeled “Precip MM”.

3. Using a P1200 12-channel pipette, add 240 µL Precipitation Master Mix to each sample. Rest each pipette tip against the wall of each well while delivering.
   - You do not need to mix up and down.
   - Change tips after each addition.
   - After adding the Precipitation Master Mix, the plate is now known as the **Precipitation Plate**.

4. Seal the Precipitation Plate, vortex, then centrifuge briefly.

Prepare and add isopropanol to Precipitation Plate

1. Remove the Precipitation Plate from the centrifuge and place on the benchtop at room temperature.

2. Pour isopropanol into the reagent reservoir labeled *ISO*.

3. Carefully remove the seal from the Precipitation Plate and discard the seal.

4. Using a P1200 12-channel pipette, 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 homogenous 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.**
   - Change the tips after each addition.

5. Blot the top of the plate with a laboratory tissue, then seal tightly with a MicroAmp™ seal.

6. Carefully transfer the Precipitation Plate into the –20°C freezer, then incubate overnight (16–24 hours).

   Note: A new option for DNA target precipitation is to incubate the plate in the –20°C freezer for 3 hours, instead of overnight. This shortened precipitation time allows you to proceed to “Stage 3: Centrifuge and dry pellets, resuspension and hybridization preparation, and sample QC” followed by “Stage 4: Denature and hybridize the Hyb-Ready Plate” on day 2 of the assay workflow.

7. After incubation, proceed to “Stage 3: Centrifuge and dry pellets, resuspension and hybridization preparation, and sample QC” on page 8.

IMPORTANT! The 3-hour DNA precipitation workflow extends the day 2 assay schedule. Approximately 9 hours are required to complete Stage 2 through Stage 4.

Stage 3: Centrifuge and dry pellets, resuspension and hybridization preparation, and sample QC

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

Supplies required

Consumables:

- 96-well PCR plate for making the Hyb-Ready Plate
  - MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates
  - Bio-Rad™ Hard-Shell™ 96-Well PCR Plate, high profile, semi skirted
  - Bio-Rad™ Hard-Shell™ 96-Well PCR Plate, low profile, full skirted
- Any 96-well PCR plate for making the following QC plates:
  - Dilution QC Plate
  - Gel QC Plate
- OD QC Plate: Greiner Bio-One™ 96-well UV-Star™ Plate

Selected reagents from the Axiom™ 2.0 Reagent Kit (see Table 7):

- Module 2-1, –20°C, Part No. 901528
- Module 2-2, 2–8°C, Part No. 901529

Other reagents required for QC steps (optional):

- Gel Diluent: 100-fold dilution of TrackIt™ Cyan/Orange Loading Buffer (Cat. No. 10482028)
- 25 bp DNA Ladder: 6-fold dilution of Applied Biosystems™25 bp DNA Ladder (Cat. No. 931343)
- UltraPure™ DNase/RNase-Free Distilled Water Cat. No 10977023
- E-Gel™ *48* Agarose Gels, 4% (Cat. No. G800804)

Set up the instruments

Prepare the following instruments for this stage:

- Oven preheated to 37°C
- Plate centrifuge set at 4°C
- Thermo Scientific™ microplate shaker or Jitterbug™ shaker

Stage 3A: Centrifuge and dry pellets and thaw reagents

1. Start thawing/warming the reagents that are used in this stage as listed in Table 7.
2. Remove the Precipitation Plate from the –20°C freezer, then centrifuge the plate at 3,200 × *g* at 4°C for 40 minutes.
3. During centrifugation, prepare the resuspension and hybridization reagents as listed in Table 7.
4. Following centrifugation, empty the liquid from the Precipitation Plate:
   a. Carefully remove the seal from the Precipitation Plate, then discard the seal.
   b. Invert the plate over a waste container, then allow the liquid to drain.
   c. While still inverted, gently press the plate on a pile of laboratory tissues on the benchtop, then leave it for 5 minutes.

   CAUTION! During this step, handle the Precipitation Plate gently to avoid disturbing the pellets. Do not bump or bang the plate.
5. Turn the plate top-side up and place in an oven for 20 minutes at 37°C to dry.
6. After 20 minutes, remove the plate from the oven, even if some droplets of liquid remain, and either:
   - Proceed directly to “Add Axiom™ Resusp Buffer to DNA pellets” on page 10. Leave the Precipitation Plate at room temp.
   - Tightly seal the plate and store at –20°C.
     - If resuspension is carried out within 4 hours, keep the plates at room temperature.
     - If resuspension is carried out after more than 4 hours, store the plates in a refrigerator (2–8°C).
   - Store the plates for resuspension on another day. Tightly seal the plate and store at –20°C.

Stage 3B: Resuspension and hybridization preparation

Prepare for resuspension and hybridization

Set the centrifuge to room temperature.

Prepare DNA pellets and warm the Axiom™ Resusp Buffer

IMPORTANT! The plate of pelleted DNA and the Axiom™ Resusp Buffer must be at room temperature before proceeding with this step.

1. Prepare the DNA pellets.
   - Frozen pellets: 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.
   - Cold pellets: If a plate was stored in a refrigerator, it is critical to equilibrate the plate to room temperature for at least 30 minutes before carrying out resuspension.
2. Warm the Axiom™ Resusp Buffer.
   - Ensure the Axiom™ Resusp Buffer has equilibrated to room temperature before adding to dry DNA pellets.
   - Carry out the resuspension steps at room temperature.

Thaw and prepare the reagents

During the centrifugation time, prepare the resuspension and hybridization reagents as shown in the following table.

Table 7 Reagent preparation.

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

CAUTION! Some steps in this stage must be performed under a fume hood.

Label tubes and reservoirs

1. Label the 15-mL tube as indicated in the following table.

Label	Tube size	Temperature	Contents
Hyb MM	15 mL	Room temperature in fume hood	Hybridization Master Mix

2. Label two 25-mL reagent reservoirs as indicated in the following table.

Label	Temperature	Contents
Resus	Room temperature	Axiom™ Resusp Buffer
Hyb MM	Room temperature in fume hood	Hybridization Master Mix

Add Axiom™ Resusp Buffer to DNA pellets

Note:

- Ensure the Axiom™ Resusp Buffer has equilibrated to room temperature before adding to dry pellets in step 2.
- Carry out these steps at room temperature.

1. Pour Axiom™ Resusp Buffer in the 25-mL reagent reservoir labeled *Resus*.
2. Using a P200 12-channel pipette, transfer **35 µL Axiom™ Resusp Buffer** to each well of the Precipitation Plate. Avoid touching the pellets with the pipette tip.
   - Change pipette tips after each addition.
   - After adding Axiom™ Resusp Buffer, the plate is known as the **Resuspension Plate**.
3. Seal the Resuspension Plate, then place on one of the following shakers:
   - Thermo Scientific™ Digital Microplate Shaker: at **speed 900 rpm for 10 minutes**.
   - Thermo Scientific™ Compact Digital Microplate Shaker: at **speed 900 rpm for 10 minutes**.
   - Jitterbug™: at **speed 7 for 10 minutes**.

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

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4. While the Resuspension Plate is shaking, prepare the Hybridization Master Mix in a 15-mL tube as described in the following table. Vortex twice to mix.

   Table 8 Hybridization Master Mix.

Reagent	per sample	Master mix 96+
Axiom™ Hyb Buffer	70.5 µL	7.8 mL
Axiom™ Hyb Soln 1	0.5 µL	55.6 µL
Axiom™ Hyb Soln 2	9 µL	1.0 mL
Total volume	**80 µL**	**8.86 mL**

5. Inspect the Resuspension Plate from the bottom. If the pellets are not dissolved, repeat step 3. Briefly centrifuge.
6. Select a PCR plate appropriate to the type of approved thermal cycler to be used in Stage 4 and label as *Hyb-Ready Plate [plate ID]*.
7. Pour the Hybridization Master Mix to the reagent reservoir labeled *Hyb MM*.
8. Using a P200 12-channel pipette, add **80 µL of the Hybridization Master Mix** to each well of the Hyb-Ready Plate.
9. Set a P200 12-channel pipette to **45 µL**. Transfer the **entire contents of each well** of the Resuspension Plate to the corresponding wells of the labeled Hyb-Ready Plate.
   - Pipet up and down to mix, then change pipette tips after each transfer.
10. Seal tightly, vortex twice, then centrifuge.
11. Do one of the following:
    - Proceed to “Stage 3C: Perform quantitation and fragmentation quality control checks (recommended)” on page 11.
    - Proceed to “Stage 4: Denature and hybridize the Hyb-Ready Plate” on page 12.
    - Store the Hyb-Ready Plate at –20°C.

Stage 3C: Perform quantitation and fragmentation quality control checks (recommended)

Prepare the reagents

Obtain the reagents for sample QC:

- Water: UltraPure™ DNase/RNase-Free Distilled Water, Cat. No. 10977023
- Gel Diluent: a 100-fold dilution of the TrackIt™ Cyan/Orange Loading Buffer, Cat. No. 10482028
- 25 bp DNA Ladder: A 6-fold dilution of the Applied Biosystems™ 25 bp DNA Ladder, Cat. No. 931343
- E-Gel™ *48* Agarose Gels, 4%, Cat. No. G800804

Label reservoirs

Label two 25-mL reagent reservoirs as indicated:

- Label 1 reservoir as *Water*.
- Label the second reservoir as *Gel Diluent*.

Table 9 Label reagent reservoirs for QC.

Label	Temperature	Contents
Water	Leave reagent reservoir at room temperature	Nuclease-free water
Gel Diluent	Leave reagent reservoir at room temperature	Gel diluent

Prepare sample QC plates

1. Make the Dilution QC Plate.
   a. Add 33 µL of nuclease-free water to each well of a 96-well PCR plate labeled *Dil QC*.
   b. Transfer 3 µL from each well of the Hyb-Ready Plate to the corresponding well of the Dilution QC Plate.
   c. Seal, vortex, then centrifuge.
2. Make, then read the OD QC Plate.
   a. Add 90 µL of nuclease-free water to each well of the OD QC Plate.
   b. Transfer 10 µL of each *Dil QC* 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 *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000) for more information.
3. Prepare, then run the Gel QC samples.
   a. Add 120 µL of Gel Diluent (100-fold dilution of TrackIt™ Cyan/Orange Loading Buffer) to each well of a 96-well PCR plate labeled *Gel QC*.
   b. Transfer 3 µL from each well of the *Dil QC* plate to the corresponding well of the *Gel QC* plate.
   c. Seal, vortex, then centrifuge.
   d. Run the gel: See Appendix A, *Fragmentation Quality Control Gel Protocol* of the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000) for more information.

Freeze or proceed

Do one of the following:

- Proceed to “Stage 4: Denature and hybridize the Hyb-Ready Plate” on page 12, or
- Store the Hyb-Ready Plate at –20°C.

Stage 4: Denature and hybridize the Hyb-Ready Plate

Supplies required

- Reagents from the Axiom™ 2.0 Reagent Kit, Cat. No. 901758
  - Axiom™ Wash Buffer A (Part No. 901446)
  - Axiom™ Wash Buffer B (Part No. 901447)
  - Axiom™ Water (Part No. 901578)
- One the following Axiom™ 96-array format plates:
  - Axiom™ human or non-human 96-array plate in a protective base
  - Axiom™ Microbiome Array Plate in a protective base
  - Axiom™ myDesign™ genotyping 96-array plate in a protective base
- 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 **Axiom 2.0 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 a plate appropriate to the thermal cycler model used
- 96-well metal chamber preheated in a 48°C oven

CAUTION! Some steps in this stage must be performed under a fume hood.

Prepare for denaturation and hybridization

1. Preheat the 96-well metal chamber in a 48°C oven.
2. Allow the array plate to equilibrate to room temperature for at least 25 minutes.
   a. Leave the array plate in the pouch at room temperature for 25 minutes before opening and loading on the GeneTitan™ MC Instrument to allow the plate to come to room temperature.
   b. At the end of the array warm up time, open the pouch and scan the array plate barcode into the GeneTitan™ Array Plate Registration file.
3. Power on the thermal cycler, then prepare for the **Axiom 2.0 Denature** program to run with the heated lid option selected.

Prepare Hyb-Ready Plate stored at –20°C

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

1. Ensure that the Hyb-Ready Plate is sealed well. If not, centrifuge the plate, then carefully 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

Before you denature the Hyb-Ready Plate:

1. Prepare the reagents from Module 3 (Axiom™ Wash Buffer A, Axiom™ Wash Buffer B, and Axiom™ Water) by inverting the bottles 2 to 3 times to mix.
2. Upload the GeneTitan Array Plate Registration file.

   See Appendix C, "Registering samples in GeneChip™ Command Console™" of the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide*.
3. Set up the GeneTitan™ MC Instrument for hybridization. For more information, see:
   - *GeneTitan™ MC Protocol for Axiom™ Array Plate Processing Quick Reference* (Pub. No. MAN0017718).
   - Chapter 5, Array Processing with theGeneTitan™ MC Instrument of the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000).

Denature the Hyb-Ready Plate

1. Open the lid of the thermal cycler, then place the sealed Hyb-Ready Plate on the thermal cycler. Check the integrity of the seal because evaporation during denaturation can negatively affect assay performance.
2. Start the **Axiom 2.0 Denature** protocol.

Prepare hybridization tray and load into GeneTitan™ MC Instrument

1. Remove the hybridization tray (from the Axiom™ GeneTitan™ Consumables Kit) from packaging.
2. Label the hybridization tray; see Figure 1 and the IMPORTANT note below the figure.

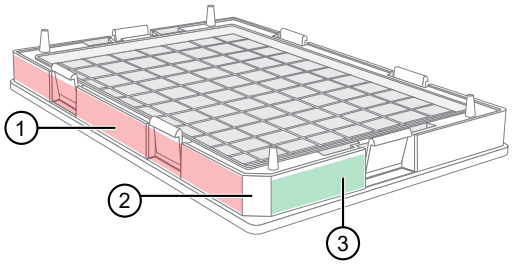

Figure 1 Label the hybridization tray.

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

IMPORTANT! It is critical that you write only on the proper location of the hybridization tray. Do *not* write on any other side, because this writing can interfere with sensors inside the GeneTitan™ MC Instrument and result in experiment failure.

3. After the **Axiom 2.0 Denature** program 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 P200 12-channel pipette, set at **105 µL**, slowly transfer the denatured samples from the Hyb-Ready Plate into the hybridization tray.
   - Dispense to the first stop to avoid creating bubbles.
   - Change pipette tips after each transfer; discard the tip even if it shows some volume remaining.
5. Puncture any air bubbles that you see using a clean pipette tip.

   Note: There is no need to spread the sample around the bottom of the hybridization tray wells. Sample distribution across the well occurs when the array plate and hybridization tray are clamped together.
6. Load the array plate and 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 cover must be removed.

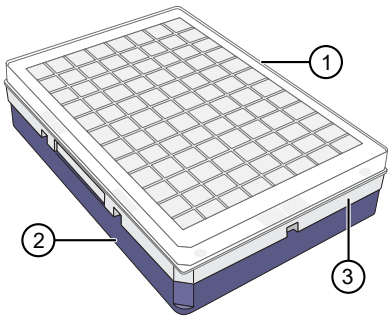

Figure 2 Array plate as shipped.

① Cover. Remove before loading.
② Blue protective base
③ Array plate

The clear plastic lid on top of the array plate *must not* be loaded in the GeneTitan™ MC Instrument.

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

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

Wait until the hybridization step on the GeneTitan™ MC Instrument is approximately 1.5 hours from completion (22 hours after the start) before starting Stage 5 of the manual target preparation.

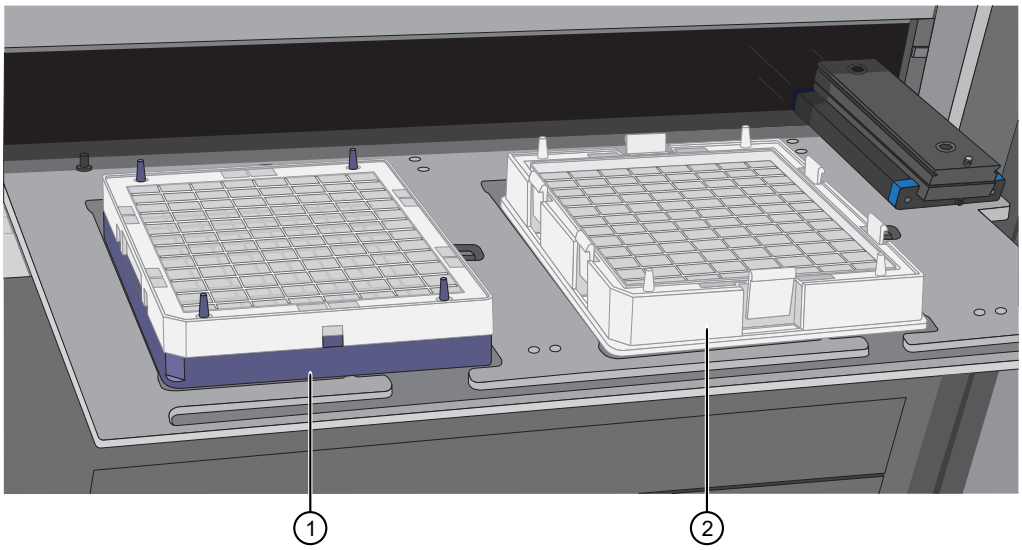

Figure 3 Array plate and hybridization tray loaded in GeneTitan™ drawer.

① Array plate on blue protective base.
② Hybridization tray

IMPORTANT!

- The array plate must be loaded on its protective blue base.
- 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. And 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: Manually prepare reagent trays for the GeneTitan™ MC Instrument

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

Equipment required

Quantity	Item
1	GeneTitan™ MC Instrument
1	GeneTitan™ ZeroStat AntiStatic Gun
1	Microcentrifuge
1	Electronic pipettor for serological pipettes
1 each	Pipettes • Single-channel P200 • Single-channel P1000 • Multichannel P200
As required	Disposable 10-mL serological pipettes
1	Vortexer
1	Portable cooler for enzyme

Consumables required

Quantity	Item
As required	Aluminum foil (optional)
• 1 • 5 • 5	Axiom™ GeneTitan™ Consumables Kit (Cat. No. 901606) • Scan Tray with top cover and protective base • Stain Tray • Covers for trays
As required	Pipette tips
5	Matrix™ Reagent Reservoir, 25 mL (Cat. No. 809311)
1	15-mL conical tube
1	50-mL conical tube
5	10-mL serological pipette

Reagents required and reagent handling

Prepare reagents according to the following table.

Module	Reagent and cap color	Thaw, then place on ice	Place on ice	Place at room temp	Reagent handling
Module 4-1 –20°C	Axiom™ Ligate Buffer	✓			Place on bench top at room temperature for 30 minutes. Vortex twice for 30 seconds. Examine for precipitate. If any, warm bottle with your hands and vortex again for 30 seconds.
	Axiom™ Ligate Enzyme	⊗ Do not thaw. Keep at –20°C until ready to use.			Immediately before use: Gently flick tube 3 times, then centrifuge briefly. Place in a –20°C portable cooler until use.
	Axiom™ Ligate Soln 1	✓			Vortex, then centrifuge briefly.
	Axiom™ Probe Mix 1	✓			Vortex, then centrifuge briefly.
	Axiom™ Stain Buffer	✓			Vortex, then centrifuge briefly.
	Axiom™ Stabilize Soln	✓			Vortex, then centrifuge briefly.
Module 4-2 2°C to 8°C	Axiom™ Ligate Soln 2			✓	Vortex, then centrifuge briefly.
	Axiom™ Probe Mix 2[1]		✓		Gently flick tube 3 times, then centrifuge briefly.
	Axiom™ Wash A			✓	Vortex well. Place on bench for 30 minutes. Look for precipitate. Vortex again if necessary.
	Axiom™ Stain 1-A[1]		✓		Gently flick tube 3 times, then centrifuge briefly.
	Axiom™ Stain 1-B[1]		✓		Gently flick tube 3 times, then centrifuge briefly.
	Axiom™ Stain 2-A[1]		✓		Gently flick tube 3 times, then centrifuge briefly.
	Axiom™ Stain 2-B[1]		✓		Gently flick tube 3 times, then centrifuge briefly.
	Axiom™ Stabilize Diluent		✓		Vortex, then centrifuge briefly. Look for precipitate. If any, warm tube to room temperature and vortex again.
	Axiom™ Water		✓		
	Axiom™ Hold Buffer[1]			✓	Vortex for 30 seconds. Place at room temperature away from light.
Estimated reagent thawing time is ~1 hour.					

[1] These solutions are light sensitive. Keep tubes out of direct light for a prolonged length 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.

Label master mix tubes and reagent reservoirs

1. Label side of each conical tube as indicated in the following table.

Tube size	Label	Contents
50 mL	*S1*	Stain 1 Master Mix
15 mL	*S2*	Stain 2 Master Mix
15 mL	*Stbl*	Stabilization Master Mix
15 mL	*Lig*	Ligation Master Mix

2. Place the 4 tubes on ice.

3. Label the five Matrix™ Reagent Reservoirs, 25-mL, as indicated in the following table.

Label	Contents
S1	Stain 1 Master Mix
S2	Stain 2 Master Mix
Stbl	Stabilization Master Mix
Lig	Ligation Master Mix
Stop	Axiom™ Hold Buffer

Prepare the stain, ligation, and stabilization master mixes

Prepare Stain 1 Master Mix

1. Add reagents in the order listed in the following table. This recipe provides enough for both S1 reagent trays.

Table 10 Stain 1 Master Mix (for both S1 trays).

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

2. Gently invert the tube 10 times to mix. Place on ice, then protect from direct light.

Prepare Stain 2 Master Mix

1. Add reagents in the order listed in the following table.

Table 11 Stain 2 Master Mix.

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

2. Gently invert the tube 10 times to mix. Place on ice and protect from direct light.

Prepare Stabilization Master Mix

1. Add reagents in the order listed in the following table.

Table 12 Stabilization Master Mix.

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

2. Vortex the master mix at high speed for 3 seconds. Place on ice.

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.
2. Add reagents to the tube in the order listed in the following table.

Table 13 Ligation Master Mix: Stage 1.

Reagent	per array	Master mix 96+
Axiom™ Ligate Buffer	66.15 µL	7.3 mL
Axiom™ Ligate Soln 1	13.12 µL	1.45 mL
Axiom™ Ligate Soln 2	3.15 µL	348 µL
Subtotal	**82.42 µL**	**9.10 mL**

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

Ligation Master Mix: Stage 2

1. Remove the Axiom™ Ligate Enzyme from the –20°C freezer and place in a cooler that is chilled to –20°C.
2. Add reagents in the order listed in the following table.

Table 14 Ligation Master Mix: Stage 2.

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

3. Gently flick the Axiom™ Ligate Enzyme tube 2–3 times, then briefly centrifuge immediately before 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.

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 *Stain1* or *S1-1* and *S1-2* (for Stain 1 Master Mix).
2. Label the remaining stain trays.
   - *Stain2* (for Stain 2 Master Mix)
   - *Stbl* (for Stabilization Master Mix)
   - *Lig* (for Ligation Master Mix)
3. Destatic the inside of each tray and cover.

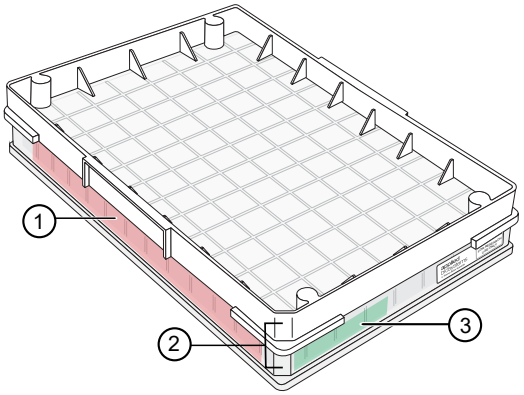

Figure 4 Stain tray with lid.

① Do not label along the long side of the tray.
② Notched corners.
③ Label the stain tray here.

IMPORTANT! It is critical that you write only on the proper location of the proper edge of the stain trays. Do *not* write on any other side, because the writing can interfere with sensors inside the GeneTitan™ MC Instrument and result in experiment failure.

See Appendix E, *Deionization procedure for GeneTitan™ trays and covers* of the *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000) for the recommended technique.

Aliquot reagents to stain trays

In this step, you aliquot the appropriate master mix into the *S1*, *S2*, *Stbl*, and *Lig* trays labeled in the previous step.

1. Aliquot 105 µL per well of the appropriate master mix; dispense to the first stop only to avoid creating bubbles.
2. Inspect the wells of the trays.
   - If bubbles are present, puncture them with a pipette tip.
   - If droplets of liquid have splashed onto the well dividers, place a laboratory tissue on top of the tray, then blot to remove.
3. Place covers on the trays. Orient cover correctly on the tray with the notched corners together.
4. Protect the trays from light if not immediately loading onto the GeneTitan™ MC Instrument.

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 reagents spread evenly when the array plate is inserted into the reagent tray during processing in the GeneTitan™ MC Instrument.

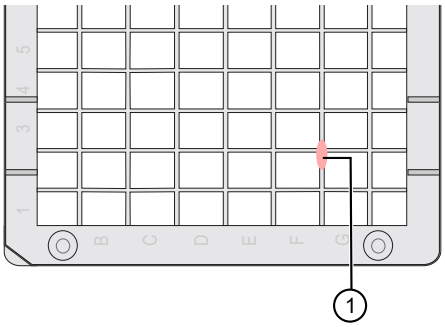

Figure 5 Check for drops of liquid on dividers.

① 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 well dividers. Blot with a laboratory tissue to remove.

Aliquoting Axiom™ Hold Buffer to the scan tray

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

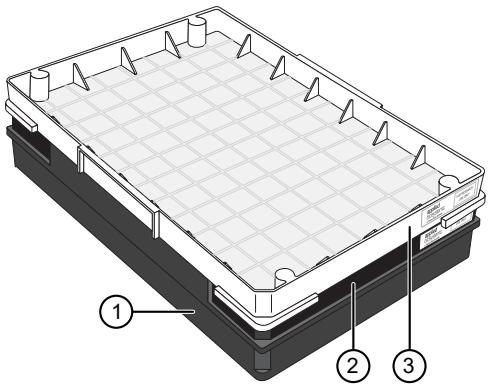

Figure 6 Scan tray with top lid and black protective base.

① Black protective base. Remove the scan tray and cover from the base before loading.

② Scan tray

③ Top cover. Replace the cover on the scan tray before loading, aligning the notched corners.

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.

1. Pour the Axiom™ Hold Buffer into a reagent reservoir, placed on the benchtop 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. Deionize the barcoded scan tray cover (Part No. 202757) that came with the scan tray.
5. Aliquot 150 µL to each well of the scan tray; dispense to the first stop and avoid touching the bottom of the tray.

   You do not need to change pipette tips between additions of the Axiom™ Hold Buffer.

   IMPORTANT! The Axiom™ Hold Buffer requires 150 µL per well.

6. If droplets of liquid splashed onto the well dividers, place a laboratory tissue on top of the tray to blot and remove.
7. Cover the tray by orienting the notched corner of the lid over the notched edge of the tray, then leave on the benchtop.
8. Continue to the array processing section for instructions on loading the reagent trays and scan tray.

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

- *GeneTitan™ MC Protocol for Axiom™ Array Plate Processing Quick Reference* (Pub. No. MAN0017718)
- *Axiom™ 2.0 Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018000)

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    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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Axiom™ 2.0 Reagent Kit

Manufacturer:
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Keppel Logistics Building |
Singapore 629563

Products:
Axiom™ Array Plates
Axiom™ myDesign™ Array Plates

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