CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow

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Note: For safety and biohazard guidelines, see the "Safety" appendix in the *CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018214). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Introduction

Running the CytoScan™ HT-CMA Assay requires the following sets of steps:

1. Genomic DNA preparation, described in the *CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018214).
2. Target preparation of the samples, performed according to the instructions in this document.
3. Array processing, described in the *GeneTitan™ MC Protocol for Axiom™ Array Plate Processing Quick Reference* (Pub. No. MAN0017718).

This document describes manual target preparation, performed using the reagents and equipment described in the *CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow Site Preparation Guide* (Pub. No. MAN0018215).

IMPORTANT! This quick reference contains an abbreviated set of instructions. Carefully read all the instructions in the *CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow User Guide* (Pub. No. MAN0018214) before running the target preparation method described here.

Note: An option for a 3-hour DNA precipitation step is available. See the user guide for details.

Target Preparation

Manual target preparation for the CytoScan™ HT-CMA Assay enables you to perform target preparation to process 96 samples at a time without the use of automation equipment. Array handling and processing procedures still require the use of a GeneTitan™ MC Instrument. See the GeneTitan™ MC Instrument array processing chapter in the *CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow User Guide*, Pub. No. MAN0018214.

IMPORTANT! Before performing manual target preparation, read the instructions in the assay preparation chapter in the user guide.

For a list of equipment and resources required for the CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow, see the *CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow Site Preparation Guide*, Pub. No. MAN0018215.

Stage 1: Amplify the genomic DNA

Input required

The gDNA Sample Plate, with 20 µL of each gDNA diluted to a concentration of 5 ng/µL in an Eppendorf™ DeepWell™ Plate 96, 2,000 µL.

Reagents required for Stage 1

Reagent and cap color	Module
From the Applied Biosystems™ HT Target Prep Reagent Kit 96F	
10X Denat Solution	HT Target Prep Module 1, –20°C (Part. No. 906011)
Neutral Solution	
Amp Solution	
Amp Enzyme	
Water	

Prepare for DNA amplification

1. Set the incubator or oven temperature to 37°C.
2. Set the centrifuge to room temperature.
3. Thaw the sample plate on the benchtop at room temperature, then vortex it, centrifuge it briefly, and leave it at room temperature.
4. Prepare reagents as shown in the following table.

Reagent and cap color	Treatment
10X Denat Solution	Thaw, vortex, and centrifuge, then keep at room temperature.
Neutral Solution	Thaw and vortex, then keep at room temperature.
Amp Solution	Thaw and vortex, then keep at room temperature.
Water	Thaw and vortex, then keep at room temperature.
Amp Enzyme[1]	Flick the tube 3 times, centrifuge it, then keep it in a –20°C cooler until ready to use. Just before using it, gently flick the tube 3 times to mix, then centrifuge it briefly.

[1] Leave at –20°C until ready to use.

Note: Allow ~1 hour for the Amp Solution to thaw on the benchtop at room temperature. If the solution is not thawed after 1 hour, vortex it briefly and return it to the benchtop to complete thawing. The reagent bottles can also be thawed in a dish with ultra-pure water such as Millipore™ water. The Amp Solution must be thoroughly mixed before use.

Prepare the Denaturation Master Mix

Carry out the following steps at room temperature.

1. To the 15-mL conical tube labeled “D MM”, add the amount of 10X Denat Solution shown in the following table, then dilute it with the amount of Water shown.

Reagent and cap color	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**

2. Vortex the tube, then leave it at room temperature.

Add Denaturation Master Mix to samples

1. Gently pipet the Denaturation Master Mix using a P1000 or pour it into the reagent reservoir labeled “D MM”.
2. 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.

   Note: This plate is now the Denaturation Plate.
3. Seal and vortex the Denaturation Plate. After vortexing, start the timer for a 10-minute incubation.
4. Centrifuge the Denaturation Plate in a room-temperature centrifuge for 1 minute at 1,000 rpm.

   Note: The centrifuge time is included in the 10-minute incubation.
5. After incubation, immediately add the Neutral Solution.

Add Neutral Solution to samples

1. Pour the Neutral Solution into a reagent reservoir labeled “N Soln”.
2. Add 130 µL of Neutral Solution to each sample. Pipet down the wall of each well. Do not mix by pipetting up and down.

   Note: This plate is now the Neutralization Plate.
3. Seal the Neutralization Plate, vortex it, then briefly centrifuge it.
4. Proceed immediately to Amplification Master Mix preparation.

Prepare the Amplification Master Mix

1. Pipet the amount of Amp Solution shown in the table into a 50-mL tube.

Reagent and cap color	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, invert the tube 2 times, then vortex it again.

Add the Amplification Master Mix to samples

1. *Slowly* pour the Amplification Master Mix into a reagent reservoir labeled “Amp MM”.
2. Slowly add 230 µL Amplification Master Mix to each well of the Neutralization Plate. Pipet down the wall of the well. Do not mix by pipetting up and down.

   Note: This plate is now the Amplification Plate.
3. Blot the top of the plate with a laboratory tissue. Seal the plate tightly, vortex it twice, then centrifuge it for 1 minute at 1,000 rpm.
4. Place the sealed Amplification Plate in an oven set at 37°C, then leave it undisturbed for 23 ±1 hours.

Freeze the plate or proceed

After the incubation finishes, do one of the following:

- 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 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

Input required

The Amplification Plate from stage 1.

Reagents required for Stage 2

Reagent and cap color	Module
From the HT Target Prep Reagent Kit 96F	
Frag Enzyme (leave at –20°C until ready to use)	HT Target Prep Module 2-1, –20°C (Part No. 906012)
10X Frag Buffer	
Precip Solution 2	
Frag Diluent	HT Target Prep Module 2-2 , 2–8°C (Part No. 906013)
Frag Reaction Stop	
Precip Solution 1	
User-supplied	
Isopropanol, 99.5%, 70 mL	—

Prepare for fragmentation and precipitation

Set oven and centrifuge temperatures

1. Set up 2 incubators or ovens. Set one oven set at 37°C. Set one oven at 65°C.
2. Set the centrifuge to room temperature.

Incubate the samples in preheated ovens

Note: If the Amplification Plate is frozen, thaw the plate before beginning Stage 2. See instructions on thawing amplified DNA samples in the *CytoScan™ HT-CMA Assay 96-Array Format Manual Workflow User Guide*, Pub. No. MAN0018214. If the Amplification Plate is not frozen, continue with the instructions in this section.

Stop the DNA amplification reaction

1. Place the Amplification Plate in the 65°C oven.
2. Incubate for 20 minutes.

Prepare for fragmentation

1. Remove the Amplification Plate from the 65°C oven, then check the seal. Press the seal, if needed.
2. Transfer the Amplification Plate to the 37°C oven.
3. Incubate for 45 minutes.

Reagent preparation

Reagent and cap color	Treatment
Frag Enzyme	Flick the tube 3X, centrifuge, and keep in –20°C cooler until ready to use.
10X Frag Buffer	Thaw, vortex, and keep on ice.
Precip Solution 2	Thaw, vortex, centrifuge, and keep at room temperature.
Frag Diluent	Thaw, vortex, centrifuge, and keep on ice.
Frag Reaction Stop	Warm to room temperature. Vortex before use.
Precip Solution 1	Warm to room temperature. Vortex before use.
Isopropanol, 99.5%, 70 mL	Keep at room temperature.

Prepare the Fragmentation Master Mix

Start making the Fragmentation Master Mix 5 minutes before completion of the 37°C incubation.

1. Add the reagents, in the order shown in the following table, to a 15-mL tube.
   a. Add the Frag Enzyme to the Fragmentation Master Mix at the end of the 45-minute 37°C incubation.

Reagent and cap color	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**

2. Vortex the Frag MM tube twice, then place it on ice.
3. Slowly pour the Fragmentation Master Mix into a reagent reservoir labeled "Frag MM". Place the reservoir 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 and place it on the benchtop at room temperature.

   Do *not* place the Amplification Plate on ice.

2. Add 57 µL of Fragmentation Master Mix to each reaction. Pipet directly into the liquid of each well. Do *not* mix by pipetting up and down.

   Note: This plate is now the Fragmentation Plate.

3. Seal the Fragmentation Plate, then vortex it twice.
4. Start the timer for 30 minutes.
5. Briefly centrifuge the Fragmentation Plate at room temperature.
6. Quickly transfer the plate to the 37°C oven, then incubate for 30 minutes.

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

7. Prepare the Frag Reaction Stop solution a few minutes before the end of the 30-minute incubation.

Add the Frag Reaction Stop solution to the Fragmentation Plate

1. A few minutes before the end of the 30-minute incubation, pour the Frag Reaction Stop solution into a reagent reservoir labeled "Stop".
2. At the end of the 30-minute fragmentation incubation, remove the Fragmentation Plate from the oven. Place it on the benchtop at room temperature.
3. End the fragmentation reaction by adding 19 µL of Frag Reaction Stop to each reaction. Pipette directly into the liquid of each well. Do not mix by pipetting up and down.
4. Seal the Fragmentation Plate, then vortex and briefly centrifuge it at 1,000 rpm.
5. Leave the Fragmentation Plate on the benchtop while you prepare the Precipitation Master Mix.

Prepare the Precipitation Master Mix

Carry out the following steps at room temperature.

1. Prepare the Precipitation Master Mix (Precip MM) by adding 218 µL of Precip Solution 2 directly to the Precip Solution 1 bottle.

Reagent and cap color	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**

2. Vortex the Precip MM bottle. Place it on the benchtop at room temperature.

Add Precipitation Master Mix to samples

Carry out the following steps at room temperature.

1. Pour the Precipitation Master Mix into a reagent reservoir labeled “Precip MM”.
2. Add 240 µL Precipitation Master Mix to each sample. Rest the pipette tip against the wall of the well while delivering. Do not mix by pipetting up and down.

   Note: This plate is now the Precipitation Plate.
3. Seal the Precipitation Plate, vortex it, then briefly centrifuge it.

Prepare and add isopropanol to the Precipitation Plate

1. Remove the Precipitation Plate from the centrifuge. Place it on the benchtop at room temperature.
2. Pour 65 mL of isopropanol into thje 100-mL reagent reservoir labeled “ISO”.
3. Add 600-µL of isopropanol to each sample, then mix well by pipetting up and down 6–7 times. Observe the solution in the tips. It should look homogeneous after pipetting 6–7 times. If not, repeat mixing a few more times until the solution looks homogeneous. Do not vortex the plate after adding the isopropanol to avoid cross-contamination of the samples.
4. Blot the top of the plate with laboratory tissue, then seal tightly with MicroAmp™ Clear Adhesive Film.

Freeze the Precipitation Plate

Designate a shelf in a –20°C freezer where the plates can be left undisturbed. In addition, the freezer must not be subjected to frequent temperature excursions.

1. Carefully transfer the Precipitation Plate into the –20°C freezer.
2. Incubate the Precipitation Plate for the desired length of time, either overnight (16–24 hours) or 3 hours.
3. After the overnight incubation finishes, proceed to “Stage 3A–3C: Centrifuge and dry, resuspension and hybridization preparation, and sample QC” on page 8.

   Note: The shortened 3-hour precipitation allows you to proceed to “Stage 3A–3C: Centrifuge and dry, resuspension and hybridization preparation, and sample QC” on page 8 followed by “Stage 4: Denaturation and hybridization” on page 12 on day 2 of the assay workflow.

Stage 3A–3C: Centrifuge and dry, resuspension and hybridization preparation, and sample QC

Input required

The Precipitation Plate from Stage 2.

Reagents required for Stages 3A, 3B, and 3C

Reagent and cap color	Module
From the HT Target Prep Reagent Kit 96F	
Hyb Buffer	HT Target Prep Module 2-1, –20°C (Part No. 906012)
Hyb Solution 1	
Resuspension Buffer	HT Target Prep Module 2-2, 2–8°C (Part No.906013)
Hyb Solution 2	
Other reagents and gel required for QC steps (optional)	
Gel Diluent, 15 mL 100-fold dilution of TrackIt™ Cyan/Orange Loading Buffer	—
E-Gel™ *48* Agarose Gels, 4%	—
25 bp DNA Ladder (Cat. No. 931343) or a similar product prepared as instructed by manufacturer.	—
Nuclease-free water, ultrapure MB Grade 15 mL (for OD QC Plate and Dilution QC Plate preparation)	—

Reagent preparation

Reagent and cap color	Preparation
Hyb Buffer	Vortex, then keep at room temperature.
Hyb Solution 1	Thaw, vortex, centrifuge, then keep at room temperature.
Hyb Solution 2	Vortex, centrifuge, then keep at room temperature.
Resuspension Buffer	Warm to room temperature (1 hour). Vortex before use.

Stage 3A: Centrifuge the Precipitation Plate and dry the DNA pellets

Centrifuge the Precipitation Plate and dry the DNA pellets

1. Preheat the oven to 37°C.
2. Transfer the Precipitation Plate from the –20°C freezer to a pre-chilled centrifuge.
3. Centrifuge the plate for 40 minutes at 4°C at 3,200 x *g*.
4. Immediately after the 40-minute centrifugation time, empty the liquid from the plate using the following steps:

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

   a. Carefully remove the seal from the Precipitation Plate. Discard the seal.
   b. Invert the plate over a clean waste container. Allow the liquid to drain. Collect the liquid, then discard it according to local, state, and federal regulations.
   c. While still inverted, gently press the plate on a pile of laboratory tissues on a bench. Allow it to drain for 5 minutes. Transfer the plate to a new pile of tissues twice during the 5-minute drain.
5. Turn the plate right side up and place it in an oven for 20 minutes at 37°C to dry.
6. Seal the plate tightly.
7. Do one of the following:
   - Proceed directly to the next stage. See “Stage 3B: Resuspend the pellets and prepare for hybridization” on page 9. Leave the sample plate at room temperature.
   - Tightly seal the Precipitation Plate, then store at –20°C.

Stage 3B: Resuspend the pellets and prepare for hybridization

Prepare for resuspension and hybridization

Set the centrifuge to room temperature.

Add the Resuspension Buffer to the DNA pellets

A plate stored at –20°C after drying the pellets must be allowed to sit at room temperature for 1.5 hours before carrying out resuspension.

Ensure that the Resuspension Buffer has equilibrated to room temperature before adding it to the dry pellets in step 2.

Carry out the following steps at room temperature.

1. Pour the Resuspension Buffer into a reagent reservoir labeled "Resus".
2. Transfer 35 µL of Resuspension Buffer to each well of the Precipitation Plate. Avoid touching the pellets with the pipette tips.

   Note: This plate is now the Resuspension Plate.
3. Seal the plate tightly.

   Note: Blue pellets should be visible at the bottom of the wells.

Prepare the Hybridization Master Mix

CAUTION! Perform the remainder of Stage 3B under a fume hood.

1. While the Resuspension Plate is shaking, prepare the Hybridization Master Mix in a 15-mL tube.
2. Add the reagents, in the order shown in the following table, to the 15-mL tube labeled “Hyb MM”.

Reagent and cap color	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	**80 µL**	**8.86 mL**

3. Vortex the tube twice to mix.

Prepare the Hyb-Ready Plate

1. Select a 96-well plate that is compatible with the thermal cycler model that is used for sample denaturation. See the user guide for compatibiity details.
2. Label the 96-well PCR plate “Hyb Ready [Plate ID]”.
3. Transfer the entire contents of each well of the Resuspension Plate to the corresponding wells of the Hyb-Ready Plate.
4. Pour the Hybridization Master Mix into a reagent reservoir labeled “Hyb MM”.
5. Add 80 µL of the Hybridization Master Mix to each well of the Hyb-Ready Plate.

   Note: Change pipette tips after each addition.
6. Seal the Hyb-Ready Plate, vortex it twice, then centrifuge it briefly.

Freeze or proceed

Do one of the following:

- Proceed to “Stage 3C: Perform quantification and fragmentation QC checks” on page 10. We strongly recommend performing the check.
- Proceed to “Stage 4: Denaturation and hybridization” on page 12.
- Store the Hyb-Ready Plate at –20°C.

Stage 3C: Perform quantification and fragmentation QC checks

Before proceeding to “Stage 4: Denaturation and hybridization” on page 12, we highly recommend that you perform quantification and fragmentation quality control checks.

Prepare the reagents

Obtain the following reagents for Sample QC.

- Nuclease-free water, 15 mL, for the water reservoir
- Gel diluent, 15 mL
- 25 bp DNA Ladder, or similar product prepared as instructed by the manufacturer.
- Two E-Gel™ *48* Agarose Gels, 4%

Perform QC checks

1. Prepare the Dilution QC Plate and OD QC Plate.
   a. Pour 15 mL of Nuclease-free Water into a reagent reservoir.

b. Add 33 µL of Nuclease-free Water to each well of the Dilution QC Plate.

c. Add 90 µL of Nuclease-free Water to each well of the OD QC Plate (96-well UV-Star™ plate).

2. Prepare the Dilution QC Plate.
   a. Transfer 3 µL of the hybridization-ready sample from each well of the Hyb-Ready Plate to the corresponding well of the Dilution QC Plate.
   b. Seal, vortex, then briefly centrifuge the plate.

3. Prepare the OD QC Plate.
   a. Transfer 10 µL of each QC Dilution sample to the corresponding wells of the OD QC Plate and mix by pipetting up and down. Change pipette tips after each transfer.

4. Prepare the Gel QC Plate.
   a. Pour 15 mL of gel diluent into a reagent reservoir.
   b. Add 120 µL of gel diluent to each well of the Gel QC Plate.
   c. Transfer 3 µL of each QC Dilution sample to the corresponding wells of the Gel QC Plate.
   d. Seal, vortex, then briefly centrifuge the plate.

5. Run the gel.
   a. Tightly seal the Gel QC Plate, vortex, and briefly centrifuge.
   b. Onto a 4% agarose e-gel load:
      - 20 µL from each well of the Gel QC Plate.
      - 25 bp DNA Ladder into the marker wells. (Follow the product instructions for dilution method.)
      - 20 µL of water to any unused wells.
   c. Run for 22 minutes.

Freeze or proceed

Do one of the following:

- Proceed to “Stage 4: Denaturation and hybridization” on page 12.
- Store the Hyb-Ready Plate at –20°C.

Stage 4: Denaturation and hybridization

Input required

The Hyb-Ready Plate from Stage 3B.

Reagents, equipment, and labware required

Quantity	Item	Instruction
Reagents from the Applied Biosystems™ HT Target Prep Reagent Kit 96F		
2 bottles/1 L	Wash Buffer A	Room temperature. Invert 2-3X for mixing before filling the GeneTitan™ bottle.
1 bottle	Wash Buffer B	Room temperature. Invert 2-3X for mixing before filling GeneTitan™ bottle.
1 bottle	Water	Room temperature.
Equipment		
1	GeneTitan™ MC Instrument	Available for hybridization.
1	Thermal cycler programmed with the CytoScan HT-CMA Denature protocol	CytoScan HT-CMA Denature protocol Use the heated lid option when setting up or running the protocol. • 95°C for 10 minutes • 48°C for 3 minutes • 48°C hold
1	96-well metal chamber warmed in a 48°C oven[1]	Keep in a 48°C oven.
Labware		
1	CytoScan™ HT-CMA Array Plate (96-array format)	Warm the array plate in the pouch at room temperature for at least 25 minutes.
1	Hybridization tray[2]	Room temperature.

[1] The metal chamber coming out the 48°C oven is warm to the touch. Gloves and mitts can be used if it feels too hot.
[2] From the Axiom™ GeneTitan™ Consumables Kit (Cat. No. 901606).

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. 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.

> **CAUTION!** Do not remove the array plate from the protective base or touch the surface of any arrays.

Prepare hybridization-ready samples stored at –20°C

1. Warm the Hyb-Ready Plate at room temperature for 5 minutes.
2. Ensure that the Hyb-Ready Plate is sealed well.
3. Vortex the Hyb-Ready Plate briefly, then centrifuge it at 1,000 rpm for 30 seconds.
4. Place the Hyb-Ready Plate at room temperature.

Prepare the GeneTitan™ MC Instrument

Before you denature the Hyb-Ready Plate, ensure that the GeneTitan™ MC Instrument is ready for use. If needed, review and follow the instructions in the user guide.

1. Prepare the reagents from the HT Target Prep Reagent Kit 96F as described in “Reagents, equipment, and labware required” on page 12.
2. Launch the GeneChip™ Command Console™ software (GCC), then select **GCC GeneTitan Control**.
3. From the Launcher window, open **GCC Portal ▸ Samples ▸ GeneTitan™ Array Plate Registration**.
4. Upload your GeneTitan™ Array Plate Registration file.

   Note: If you do not upload your GeneTitan™ Array Plate Registration file after scanning the array plate barcode, the software assigns names to your sample.
5. Select the **System Setup** tab.
6. Configure the software.
   a. For **Setup Option**, select **Hyb-Wash-Scan**.
   b. Click **Next**.
   c. Scan or manually enter the array plate barcode, then click **Next**.
   d. Select the protocol name, then click **Next**.
7. Fill the Wash A, Wash B, and Rinse bottles with Wash Buffer A, Wash Buffer B, and Water, respectively.
8. Empty the waste bottle.
9. Press the blue confirmation button on the GeneTitan™ MC Instrument to continue.
10. Open the trash bin and empty, then press the blue confirmation button to continue.
11. When the drawers open, remove consumable trays and plates, then press the blue confirmation button to continue.

    If no consumables to remove, the **Status** pane reads “Drawers are empty”.
12. When prompted by the GeneChip™ Command Console™ software, load the array plate and hybridization tray into the GeneTitan™ MC Instrument.

    See the user guide for instructions.

Denature the Hyb-Ready Plate

1. Ensure that the thermal cycler is powered on and the **CytoScan HT-CMA Denature** protocol with the heated-lid option has been selected.
2. Place the sealed Hyb-Ready Plate on the thermal cycler, close the lid, and start the **CytoScan HT-CMA Denature** protocol.

Prepare the hybridization tray and load it into the GeneTitan™ MC Instrument

CAUTION! Perform the next set of steps under a fume hood.

1. Remove the hybridization tray from the Axiom™ GeneTitan™ Consumables Kit.
2. Label the hybridization tray.

   IMPORTANT! It is critical that you write only on the proper location of the hybridization tray, on the edge in front of wells A1 and B1. Do *not* write on any other side, as the writing can interfere with sensors inside the GeneTitan™ MC Instrument and result in experiment failure.
3. After the **CytoScan HT-CMA Denature** protocol has completed, remove the Hyb-Ready Plate from the thermal cycler and place it in a 96-well metal chamber that has been warmed in an oven at 48°C.
4. Move the metal chamber containing the denatured Hyb-Ready Plate to a fume hood.

5. Using a pipette set at 105 µL, slowly transfer the denatured samples in the Hyb-Ready Plate into the corresponding wells of the hybridization tray. Dispense to the first stop to avoid creating bubbles. Change pipette tips after each transfer. Puncture any air bubbles with a clean pipette tip for each sample.

6. Load the array plate and hybridization tray into the GeneTitan™ MC Instrument.

   IMPORTANT! After the GeneTitan™ MC Instrument has stacked the array plate and hybridization tray, the instrument extends the drawer. Manually check the stacking by gently pressing the 6 latching points to ensure that the 2 parts are clamped properly, and check underneath the arrays to ensure that there are no bubbles. If bubbles are found, gently tap the plate on top to eliminate the bubbles. Do *not* tip/tilt the array plate/hybridization tray stack as you are inspecting the bottom for bubbles.

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

7. Near the end of the 23.5- to 24-hour hybridization, proceed to Stage 5. See “Stage 5: Prepare GeneTitan™ reagents and trays” on page 14.

Stage 5: Prepare GeneTitan™ reagents and trays

Reagents required and reagent handling for Stage 5

Prepare reagents according to the following table.

Module	Reagent and cap color	Thaw, then place on ice	Place on ice	Place at room temperature
HT Target Prep Module 3-1 –20°C	Ligate Buffer[1]			✓
	Ligate Enzyme[2]	⊗ Do not thaw. Keep at –20°C until ready to use.		
	Ligate Solution 1	✓		
	Probe Mix 1	✓		
	Stain Buffer	✓		
	Stabilize Solution	✓		
HT Target Prep Module 3-2 2°C to 8°C	Ligate Solution 2			✓
	Probe Mix 2[3]		✓	
	Wash A[1]			✓
	Stain 1-A[3]		✓	
	Stain 1-B[3]		✓	
	Stain 2-A[3]		✓	
	Stain 2-B[3]		✓	
	Stabilize Diluent[1]		✓	
	Water			✓
	Hold Buffer[3], [4]			✓
Estimated reagent thawing time is ~30 minutes.				

[1] Check for precipitate. If precipitate is present, repeat the vortex and centrifuge step.
[2] If needed, an extra tube of Ligate Enzyme is in HT Target Prep Module 4-1.
[3] These solutions are light sensitive. Keep tubes out of direct light for a prolonged length of time.
[4] Hold Buffer for preparing the second, third, and fourth plates is provided in HT Target Prep Module 4-2.

Guidelines for handling reagents with precipitates

When the HT Target Prep Module 3-2 is stored at 4°C, precipitation in the form of clear crystals can sometimes occur in the Wash A and Stabilize Diluent. See information in the user guide about handling reagents with precipitates for instructions to ensure that any precipitate is returned to solution before use.

Note: The presence of some precipitate does not adversely impact assay performance.

Prepare the stain, ligation, and stabilization master mixes

Prepare the Stain 1 Master Mix

1. Add reagents to a 50-mL conical tube in the order shown in the following table. This recipe provides enough for both S1 reagent trays.

Reagent and cap color	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**

2. Gently invert the tube 10 times to mix. Do not vortex.
3. Place the tube on ice and protect it from direct light.

Prepare the Stain 2 Master Mix

1. Add reagents to a 15-mL conical tube in the order shown in the following table.

Reagent and cap color	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**

2. Gently invert the tube 10 times to mix. Do not vortex.
3. Place on ice, then protect from direct light.

Prepare the Stabilization Master Mix

1. Add reagents to a 15-mL conical tube in the order shown in the following table.

Reagent and cap color	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**

2. Vortex the tube at high speed for 3 seconds. Place it on ice.

Prepare the Ligation Master Mix—part 1

The Ligation Master Mix is prepared in 2 stages.

1. Add reagents to a 15-mL conical tube in the order shown in the following table.

Reagent and cap color	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
Total	**82.42 µL**	**9.10 mL**

2. Mix well by vortexing the Lig tube for 3 seconds, then plate it on ice.

Prepare the Ligation Master Mix—part 2

The Ligation Master Mix is prepared in 2 stages.

1. Remove the Ligate Enzyme from the –20°C freezer. Place it in a cooler chilled to –20°C.
2. Add reagents to a 15-mL conical tube in the order shown in the following table.

   Note: Gently flick the Ligate Enzyme tube 2-3 times, then centrifuge it briefly immediately before adding the enzyme to the master mix.

Reagent and cap color	Per array	Master mix 96+
Ligation Master Mix from stage 1 preparation	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**

3. Gently invert the tube 10 times to mix. Do not vortex.
4. Place the tube on ice and protect it from direct light.

Aliquot master mixes and Hold Buffer into trays

Label the GeneTitan™ reagent trays

1. Label 2 stain trays “S1”.
2. Label the remaining stain trays as shown below:
   - “S2”
   - “Stbl”
   - “Lig”

Deionize the GeneTitan™ reagent trays and covers

1. Deionize the inside of each tray and cover.
2. Return the trays and covers to the bench top after deionizing.

Aliquot the Stain 1 Master Mix

1. Pour the S1 Master Mix into a reagent reservoir labeled “S1”.
2. Aliquot 105 µL per well to both trays labeled “S1”.
   - Dispense to the first stop only to avoid creating bubbles.
3. If bubbles are present, puncture them with a pipette tip.
4. If droplets of liquid have splashed onto the well dividers, place a laboratory tissue on top of the tray to blot, then remove.
5. Place covers on the S1 trays. Orient the covers correctly on the trays with the notched corners together.
6. Protect the trays from light if not immediately loading onto the GeneTitan™ MC Instrument.

Aliquot the Stain 2 Master Mix

1. Pour the S2 Master Mix into a reagent reservoir labeled “S2”.
2. Aliquot 105 µL per well to the tray labeled "S2". Dispense to the first stop only to avoid creating bubbles.
3. If bubbles are present, puncture them with a pipette tip.
4. If droplets of liquid have splashed onto the well dividers, place a laboratory tissue on top of the tray to blot, then remove.
5. Place a cover on the S2 tray. Orient the cover correctly on the tray with the notched corners together.
6. Protect the tray from light if not immediately loading onto the GeneTitan™ MC Instrument.

Aliquot the Stabilization Master Mix

1. Pour the Stabilization Master Mix into a reagent reservoir labeled “Stbl”.
2. Aliquot 105 µL per well to the tray labeled “Stbl”. Dispense to the first stop only to avoid creating bubbles.
3. If bubbles are present, puncture them with a pipette tip.
4. If droplets of liquid have splashed onto the well dividers, place a laboratory tissue on top of the tray to blot, then remove.
5. Place a cover on the tray. Orient the cover correctly on the tray with the notched corners together.

Aliquot the Ligation Master Mix

1. Pour the Ligation Master Mix into a reagent reservoir labeled “Lig”.
2. Aliquot 105 µL per well to the tray labeled “Lig”. Dispense to the first stop only to avoid creating bubbles.
3. If bubbles are present, puncture them with a pipette tip.
4. If droplets of liquid have splashed onto the well dividers, place a laboratory tissue on top of the tray to blot, then remove.
5. Place a cover on the Lig tray. Orient the cover correctly on the tray with the notched corners together.
6. Protect the tray from light if not immediately loading onto the GeneTitan™ MC Instrument.

Aliquot the Hold Buffer to the scan tray

1. Vortex the Hold Buffer, then pour it into a reagent reservoir labeled “Hold”.
2. Remove the scan tray from its pouch.
3. Remove the scan tray cover, but leave the scan tray on its protective black base.
4. Deionize the barcoded scan tray cover that came with the scan tray.
5. Aliquot 150 µL of Hold Buffer to *every* well of the 96-plate scan tray. Dispense to the first stop and avoid touching the bottom of the tray.
6. If droplets of liquid have splashed onto the well dividers, place a laboratory tissue on top of the tray to blot, then remove.
7. Cover the tray by orienting the notched corner of the scan tray cover over the notched edge of the tray with the flat side of the cover against the scan tray.

IMPORTANT! The scan tray has an open-bottom design, so it is important that all 96-wells of the scan tray receive 150 µL of Hold Buffer.

Manufacturer:
Thermo Fisher Scientific Baltics UAB |
V.A. Graiciuno 8, LT-02241 |
Vilnius, Lithuania

Products:
Applied Biosystems™ HT Target Prep Reagent Kit 96F

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

Products:
CytoScan™ HT-CMA 96-Array Plate

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

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