Oligonucleotide Array-Based CGH for Genomic DNA Analysis – Enzymatic Labeling for Blood, Cells, or Tissues

Quick Reference Guide for 16-pack microarrays

This Quick Reference Guide is an abbreviated version of publication G4132-90000, "*High-Throughput aCGH Analysis using Agilent HT Microarrays- Enzymatic Labeling of gDNA with the SureTag HT Kit Protocol*." It contains instructions for enzymatic labeling of gDNA from blood, cells, or frozen tissues and hybridization of that labeled gDNA to Agilent HT CGH and CGH+SNP microarrays in a 16-pack (i.e., 16 microarrays/slide) format.

Before You Begin

Using this Guide

This Quick Reference Guide is intended for experienced users who are already familiar with processing Agilent HT Microarrays arrayed on 16-pack slides for Comparative Genomic Hybridization (CGH). If you are a new user, refer to publication G4132-90000, *High-Throughput aCGH Analysis using Agilent HT Microarrays- Enzymatic Labeling of gDNA with the SureTag HT Kit Protocol*, which is the full-length version of this Quick Reference Guide. The full-length protocol includes additional instructions and details, as well as procedural notes, information on kit contents, required materials and equipment, and troubleshooting tips.

Safety Notes

WARNING

Wear appropriate personal protective equipment (PPE) when working in the laboratory.

For Agilent reagent safety information, consult the safety data sheets and any product hazard labels. Agilent safety data sheets are available at www.agilent.com.

Step 1. DNA Quantitation and Quality Analysis

After you have isolated genomic DNA (gDNA) from your test samples and, if applicable, your reference samples, verify the quantity and quality of the DNA samples using fluorometry, UV-VIS spectrophotometry, or agarose gel electrophoresis.

Fluorometry is the recommended approach. Use the Qubit dsDNA BR Assay Kit at room temperature (22°C to 28°C) to determine the gDNA concentration. Use 1 to 20 µL of your DNA sample for the Qubit dsDNA BR assay.

Step 2. Sample Preparation

Use either the enzymatic fragmentation method (below) or the heat fragmentation (**page 4**). If you are using CGH+SNP arrays, you must use enzymatic fragmentation.

Sample Preparation using Enzymatic Fragmentation Method

The method uses the restriction enzymes and other reagents provided in the Agilent SureTag HT or SureTag HT Complete DNA Labeling Kit to fragment DNA samples. It requires 125 ng of starting gDNA as input.

1. Pre-program the thermal cycler for the fragmentation reactions using the program below. Reaction volumes are 6.5 µL. *Start the program, then immediately pause the program as soon as the thermal cycler reaches 37°C.*
   - 37°C for 2 hours
   - 65°C for 20 minutes
   - 4°C hold
2. For each gDNA sample, use Nuclease Free Water to prepare a diluted stock at a concentration of 35.7 ng/µL.
3. Add 3.5 µL of each diluted gDNA sample to a PCR tube or to the well of a 96-well PCR plate.
4. Prepare the appropriate volume of Enzymatic Fragmentation Master Mix by combining the components in **Table 1**. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

Table 1 Enzymatic Fragmentation Master Mix

Component	Per reaction	× 32rxns (including excess)	× ___ rxns
Nuclease Free Water	1.85 µL	64.8 µL	
10× Restriction Enzyme Buffer	0.65 µL	22.8 µL	
Diluted BSA (2 µg/µL)*	0.25 µL	8.75 µL	
Alu I	0.125 µL	4.4 µL	
Rsa I	0.125 µL	4.4 µL	
Total volume	**3.0 µL**	**105.15 µL**	

* Diluted with Nuclease Free Water from stock concentration of 10 µg/µL

5. Add 3.0 µL of Enzymatic Fragmentation Master Mix to each tube or well containing a gDNA sample to make a total reaction volume of 6.5 µL. Cap or seal the tubes or plate.
6. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep the reactions on ice.
7. Place the tubes or plate in the thermal cycler. Resume the paused thermal cycler program.

8 Upon completion of the program, remove the tubes or plate, then spin briefly. Keep reactions on ice.

Optional Stopping Point Digested gDNA can be stored up to 30 days at –20°C.

9 Pre-program the thermal cycler for random primer annealing using the program below. Reaction volumes are 7.75 µL. *Start the program, then immediately pause the program as soon as the thermal cycler reaches 98°C.*

- 98°C for 3 minutes
- 4°C for 5 minutes
- 4°C hold

10 Add 1.25 µL of Random Primers to each gDNA reaction. Cap or seal the tubes or plate.

11 Mix the reactions on a vortex mixer at high speed for 10–15 seconds. Spin briefly.

12 Place the tubes or plate in the thermal cycler. Resume the paused thermal cycler program.

13 Upon completion of the program, remove the tubes or plate, then spin briefly. Keep on ice. Continue to **"Step 3. Sample Labeling"** on page 5.

Sample Preparation using Heat Fragmentation Method

CAUTION If you are using CGH+SNP arrays, do not use heat fragmentation to prepare the samples. Use the enzymatic fragmentation method on **page 3**.

The method uses the Nuclease Free Water and Random Primers included in the SureTag HT Complete Kit and SureTag HT Kit. It requires 125 ng of starting gDNA as input.

1 Pre-program the thermal cycler for random primer annealing using the program below. Reaction volumes are 7.75 µL. *Start the program, then immediately pause the program as soon as the thermal cycler reaches 98°C.*

- 98°C for 10 minutes
- 4°C for 5 minutes

2 For each gDNA sample, use Nuclease Free Water to prepare a diluted stock at a concentration of 19.2 ng/µL.

3 Add 6.5 µL of each diluted gDNA sample to a PCR tube or to the well of a 96-well PCR plate.

4 Add 1.25 µL of Random Primers to make a total reaction volume of 7.75 µL. Cap or seal the tubes or plate.

5 Mix on a vortex mixer for 5 seconds, then spin briefly.

6 Place the tubes or plate in the thermal cycler. Resume the paused thermal cycler program.

7 Upon completion of the program, remove the tubes or plate, then spin briefly. Keep on ice. Continue to **"Step 3. Sample Labeling"** on page 5.

Step 3. Sample Labeling

During sample labeling, the test sample is labeled with one dye while the reference sample is labeled with the other dye. The "polarity" of the sample labeling is a matter of experimental choice. Treat the test/reference sample pairs identically during labeling. **Minimize light exposure throughout the procedure.**

Fluorescent Labeling of gDNA

1 Pre-program the thermal cycler for the labeling reactions using the program below. Reaction volumes are 12.5 µL. *Start the program, then immediately pause the program as soon as the thermal cycler reaches 37°C.*

- 37°C for 2 hours
- 65° for 10 minutes
- 4°C hold

2 Prepare two Labeling Master Mixes (one with Cyanine 3-dUTP and one with Cyanine 5-dUTP) by mixing the components in **Table 2** in the order listed. Mix on a vortex mixer for 5 seconds, then spin briefly. Keep on ice.

Table 2 Labeling Master Mixes

Component	Per reaction	× 16 rxns (including excess)	× ___ rxns
5× Reaction Buffer	2.5 µL	42.5 µL	
10× dNTPs	1.25 µL	21.3 µL	
Cyanine 3-dUTP *or* Cyanine 5-dUTP	0.75 µL	12.8 µL	
Exo (-) Klenow	0.25 µL	4.25 µL	
Total volume	**4.75 µL**	**80.85 µL**	

3 Add 4.75 µL of appropriate Labeling Master Mix to each tube or well containing fragmented gDNA stored on ice. Final volume of each reaction is 12.5 µL. Cap or seal the tubes or plate after addition of the Labeling Master Mix.

4 Mix on a vortex mixer for 5 seconds, then spin briefly.

5 Place the tubes or plate in the thermal cycler. Resume the paused thermal cycler program.

6 Upon completion of the program, remove the tubes or plate, then spin briefly. Keep on ice and protect from light.

30 days **Optional Stopping Point** Labeled DNA can be stored up to 30 days at –20°C in the dark.

Purification of Labeled gDNA

1 Prepare enough 80% ethanol to wash all of the reactions, including sufficient overage.

The required volume of 80% ethanol is 200 µL per reaction. The 80% ethanol is used in step 9 below.

2 Prepare the SurePure Beads Master Mix by combining the components in **Table 3**.

NOTE Because the stock vials of SurePure Beads and SureTag HT Buffer include volume overage, the SureTag HT and SureTag HT Complete Kits provide sufficient quantities of these components to prepare the master mix with the excess volumes indicated in the table.

Table 3 SurePure Beads Master Mix

Component	Per reaction	× 32 rxns (including excess)	× ___ rxns
SureTag HT Buffer	30 µL	1.1 mL	
100% ethanol	120 µL	4.42 mL	
SurePure Beads	5 µL	184 µL	
Total volume	**155 µL**	**5.704 mL**	

3 Make sure that the SurePure Beads Master Mix is well mixed. Then add 155 µL of SurePure Beads Master Mix to each tube or well containing labeled gDNA.

   Final volume of each reaction is 167.5 µL.

4 Mix the reactions on a vortex mixer until the SurePure Beads are fully suspended.

5 Briefly spin the reactions in a centrifuge. Make sure that no liquid remains on the walls and lid and that the beads are still suspended.

6 Incubate the reactions at room temperature for 5 minutes to allow binding between gDNA and SurePure Beads.

7 Put the plate or tubes onto a magnetic separator device (e.g., the Permagen 96-well PCR Post Magnet Low Elution Plate, p/n LE400) and incubate at room temperature for 5 minutes to pellet the SurePure Beads.

8 With a pipettor, carefully remove and discard the supernatant in each reaction without disturbing the pellet.

9 Add 200 µL of 80% ethanol to each reaction to wash the beads. Do not remove the reactions from the magnetic separator device and do not mix. Incubate at room temperature for 1 minute.

10 With a pipettor, carefully remove and discard the supernatant in each reaction without disturbing the pellet.

11 Remove the plate or tubes from the magnetic separator device. Spin briefly.

12 Without capping or sealing the tubes or plate, incubate the reactions in an open thermal cycler set to 37°C until beads are dry (typically <5 minutes). Do not close the lid of the thermal cycler. Check the samples every minute to monitor dryness of the pellets.

13 Remove the plate or tubes from the thermal cycler as soon as the pellets are completely dry. Keep at room temperature.

14 Add 5.5 µL of 1×TE buffer (pH 8.0) to each bead pellet. Mix well by vortexing or by pipetting up and down.

NOTE If you observe a faint trail of beads on the side of the tubes or wells, use the pipettor to gently rinse the bead trail with the 1× TE buffer. This action helps to elute residual gDNA from the beads trailing along the side. Do not attempt to scrape the bead trail off the side of the tubes/wells.

15 Spin the reactions in a centrifuge for 10–15 seconds, or long enough to drive the contents off the walls and lid.

16 Incubate the reactions at room temperature for 5 minutes to elute the labeled gDNA from the SurePure Beads.

17 Put the plate or tubes onto a magnetic separator device and incubate at room temperature for 5 minutes to pellet the SurePure Beads.

   The labeled gDNA is in the supernatant.

18 With a pipettor, carefully transfer the entire volume of supernatant from each reaction to the well of a fresh 96-well plate or a fresh tube. Keep the purified labeled gDNA samples on ice.

19 Refer to **Table 4** for expected yield of labeled gDNA and specific activity after labeling and purification, when starting with high quality gDNA. The cyanine-3 and cyanine-5 yield after labeling should be nearly the same.

Table 4 **Expected Yield and Specific Activity after Labeling and Clean-up**

Measurement	Expected result
Yield	2.0 to 4.5 µg per reaction*
Specific Activity of Cyanine 3 and Cyanine 5 Labeled Sample	20–50 pmol/µg

* If the average yield is outside of this range, Agilent recommends repeating the sample fragmentation and sample labeling steps with a fresh aliquot of the DNA sample.

30 days **Optional Stopping Point** Purified, labeled DNA can be stored up to 30 days at –20°C in the dark.

Step 4. Microarray Hybridization

Preparation of labeled gDNA for hybridization

1 In a fresh PCR tube or the well of a fresh 96-well plate, combine the purified labeled gDNA from the test sample with the purified labeled gDNA from the corresponding reference sample. Use the entire volume of both samples. Keep the mixture on ice and protected from light. The total volume of the mixture is approximately 9 µL.

2 Pre-program the thermal cycler for the hybridization reactions using the program below. Reaction volumes are 24.5 µL. *Start the program, then immediately pause the program as soon as the thermal cycler reaches 98°C.*

- 98°C for 3 minutes
- 37° for 30 minutes
- 37°C hold (no longer than 1 hour)

3 Prepare the appropriate volume of Hybridization Master Mix by combining the components in **Table 5**. Mix well by pipetting up and down. Spin briefly and keep on ice.

Table 5 **Hybridization Master Mix**

Component	Volume per hybridization rxn	× 16 rxns (including excess)	× ___ rxns (µL)
Nuclease-free water	2.51 µL	50.2 µL	
Cot-1 DNA (1.0 mg/mL)	1.11 µL	22.2 µL	
40× aCGH Blocking Agent*	0.61 µL	12.2 µL	
2× HI-RPM Hybridization Buffer	12.22 µL	244.4 µL	
Total Volume	**16.45 µL**	**329.0 µL**	

* Make sure that the aCGH Blocking Agent has been reconstituted to a 40× concentration.

4 In a fresh PCR tube or the well of a fresh 96-well plate, add 16.45 µL of Hybridization Master Mix and 8.0 µL of labeled gDNA mixture. **Table 6** lists the required volumes per hybridization reaction, as well as the final total volume of each reaction and the volume that will be loaded onto the gasket slide.

Table 6 Volumes per hybridization reaction

Component	Volume per hybridization rxn
Hybridization Master Mix	16.45 µL
Labeled gDNA mixture	8.0 µL
Total Volume	**24.45 µL**
Volume to be added to gasket well	**18.0 µL**

5 Mix the hybridization reactions on a vortex mixer for 2–3 seconds or until thoroughly mixed, then briefly spin in a centrifuge. The reactions will contain bubbles but they do not interfere with hybridization.

6 Cap or seal the tubes or plate and place in the thermal cycler. Resume the paused thermal cycler program. While the thermal cycler program is running, confirm that the hybridization oven is set to 67°C. As soon as the thermal cycler program reaches the 37°C hold step, begin **"Preparation of the hybridization assembly"**, below, processing samples in batches of 16. Keep the remaining samples in the 37°C thermal cycler

Preparation of the hybridization assembly

Agilent recommends using the Agilent Hyb Station (p/n G5765A) to prepare the hybridization assembly.

For detailed instructions on proper technique, refer to the full-length protocol (publication G4132-90000).

NOTE Agilent strongly recommends using a single channel pipette (e.g., Pipetman P10 or P20) to load the samples into the gasket wells. If you have significant experience processing Agilent HT microarrays, then you may consider using a 4-channel pipette to load the gasket wells horizontally. Agilent does not recommend using an 8-channel pipette unless you are highly experienced with loading Agilent HT microarrays.

1 Remove a gasket slide from its packaging and, with tweezers, remove the protective film.

2 Insert the gasket slide into the chamber base with the barcode label resting over the base's barcode guide.

3 Slowly dispense 18.0 µL of sample mixture into each gasket well so that the liquid forms a bead in the center of the well.

   If the surface tension of the bead breaks, causing the liquid to come into contact with the gasket, spread the liquid evenly across the area of the gasket well using the procedure described in **"Sample Loading Rescue Procedure"** on page 11.

   Do not leave any of the gasket wells empty. If needed, load unused wells with 18.0 µL of 1× HI-RPM Hybridization Buffer. Make sure that the gasket wells in the four corners of the slide contain hybridization sample mixture.

4 Once all of the gasket wells have been loaded, place a microarray slide on top of the gasket slide with the numeric barcode facing up and the Agilent label facing down. Do not move the hybridization chamber once the microarray slide has been placed.

5 Place the chamber cover onto the chamber base.

6 From the rounded corner of the chamber base, slip the clamp onto the chamber base and cover until it stops in the center. Keep the chamber assembly flat on the lab bench at all times.

7 Firmly tighten the thumbscrew on the clamp.

8 Rotate the final assembled chamber in a *vertical orientation*, clockwise, 2 to 3 times. If necessary, rotate and/or tap the assembled chamber to dislodge any small or stationary bubbles.

9 Load the assembled chamber into the oven rotator rack, starting from the center of the rack (i.e., position 3 or 4). Close the oven door and set the rotator speed to 20 rpm.

10 Repeat **"Preparation of the hybridization assembly"** for the remaining microarray slides.

Hybridization

1 With all assembled chambers loaded into the rotator rack, hybridize at 67°C, with a rotator speed of 20 rpm, for 24 hours.

During hybridization, perform **"Prewarming of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2"**.

Prewarming of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2

Wash up to 5 microarray slides per group. Prewarm enough buffer for all groups (approximately 250 mL per group).

To prewarm the buffer overnight:

1 Add the required volume of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 to a new or well-cleaned storage bottle and place in an incubator or circulating water bath set to 37°C.

2 Allow the buffer to warm overnight.

To prewarm the buffer the day of the wash procedure:

1 Add the required volume of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 to a new or well-cleaned storage bottle and place in a water bath sitting on a hot plate.

2 Monitor the temperature of the water bath until the temperature is stabilized to 37°C.

3 Allow the buffer to warm for approximately 2 to 3 hours.

Step 5. Microarray Washing

Table 7 summarizes the wash procedure.

Table 7 Wash conditions

	Dish	Wash buffer	Temperature	Time
Disassembly	#1	Agilent Oligo Wash Buffer 1	Room temperature	
1st wash	#2	Agilent Oligo Wash Buffer 1	Room temperature	5 minutes, with stirring
2nd wash	#3	Agilent Oligo Wash Buffer 2	37°C	5 minutes, with stirring

Refer to the full-length protocol (publication G4132-90000) for instructions on cleaning slide-staining dishes, slide racks, and stir bars.

1 Prepare the three slide-staining dishes using the preparation instructions provided in **Table 8**.

Table 8 Preparation of slide-staining dishes for washing

Dish	Preparation
#1	1 Fill completely with Agilent Oligo Wash Buffer 1 at room temperature.
#2	1 Add a slide rack and a magnetic stir bar. 2 Fill with enough Agilent Oligo Wash Buffer 1 at room temperature to cover the slide rack. 3 Put dish on a magnetic stir plate.
#3	1 Put the prewarmed 1.5 L glass dish on a magnetic stir plate with heating element. 2 Put slide-staining dish #3 into the 1.5 L glass dish. 3 Fill the 1.5 L glass dish with pre-warmed Milli-Q ultrapure water. 4 Fill the slide-staining dish approximately three-fourths full with Agilent Oligo Wash Buffer 2 (warmed to 37°C). 5 Add a magnetic stir bar. 6 Turn on the heating element and maintain temperature of Wash Buffer 2 at 37°C. Monitor with a thermometer.

2 Remove one hybridization chamber from the oven and resume rotation of the others.

3 With the hybridization chamber assembly on a flat surface, remove the clamp assembly and the chamber cover.

4 Remove the microarray-gasket sandwich from the chamber base, keeping the microarray slide numeric barcode facing up. Without letting go of the slides, submerge the microarray-gasket sandwich into slide-staining dish #1 containing Wash Buffer 1.

5 Use forceps to pry the slides apart on the barcode end, gently twisting the forceps until the slides separate. Then, quickly transfer the microarray slide to the slide rack in slide-staining dish #2.

6 Repeat **step 2** through **step 5** for up to four additional slides in the group.

7 When all slides in the group are put into the slide rack in slide-staining dish #2, stir at 350 rpm for 5 minutes. Adjust the setting to get good but not vigorous mixing. The buffer is being well mixed when it is just forming a dimple at the surface

8 At the end of the 5-minute wash, transfer the entire slide rack to slide-staining dish #3, which contains Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37°C.

9 Activate the magnetic stirrer. Adjust the setting to get thorough mixing without disturbing the microarray slides.

10 Wash microarray slides in slide-staining dish #3 for 5 minutes.

11 Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack. Place the slide rack on a clean stack of lint-free, disposable laboratory wipes.

12 Remove the slides from the slide rack one at a time, tapping the edge of each slide on the stack of laboratory wipes to remove any remaining droplets, and then putting it in a slide holder.

13 Repeat **step 1** through **step 12** for the next group of five slides using fresh Wash Buffer 1 and fresh Wash Buffer 2 warmed to 37°C.

14 Scan slides immediately. If necessary, store slides in orange slide boxes in a N_2 purge box, in the dark.

Step 6. Microarray Scanning and Analysis

1 Put assembled slide holders into the scanner cassette of the Agilent SureScan Microarray Scanner and proceed with scanning.

2 Analyze the scanned images using the Agilent CytoGenomics software (version 5.3 or higher) or Agilent Feature Extraction software (version 12.2 or higher).

Array/Sample Tracking on Microarrays

Use the form below to make notes to track your samples.

Arrays

BARCODE	Array 1_1	Array 1_2	Array 1_3	Array 1_4	Array 1_5	Array 1_6	Array 1_7	Array 1_8
	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:
	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:	Sample:
	Array 2_1	Array 2_2	Array 2_3	Array 2_4	Array 2_5	Array 2_6	Array 2_7	Array 2_8

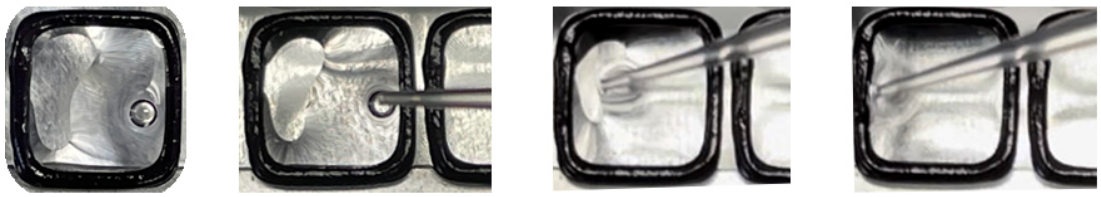
Barcode Number _____

Sample Loading Rescue Procedure

When dispensing the hybridization sample mixtures into the gasket wells of the gasket slide, the optimal outcome is for the mixture to form a bead of liquid that sits in the center of the well. The presence of a small air bubble is not a concern as long as the liquid bead is intact (i.e., the liquid is not in contact with the gasket).

If the surface tension of the liquid bead breaks, causing the sample mixture to come into contact with the edge of the gasket (as in **panel A** in the image), use the sample loading rescue procedure described below.

- First, pop any air bubbles that may be present in the sample mixture (see **panel B**). Use a clean, small-gauge pipette tip (e.g., a 10-µL tip) to gently pop the bubble. Keep the pipette tip nearly parallel with the gasket slide and gently tap the bubble with the edge of the pipette tip opening. Use a fresh pipette tip for each sample. Never attempt to pop a bubble using a wet or previously used pipette tip as capillary action will aspirate the sample mixture.
- Then, use a NEW pipette tip to spread the sample mixture evenly across the area of the gasket well (see **panel C**). Make sure that the entire surface inside the gasket is covered with sample mixture (see **panel D**).



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In This Book

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April 2025
Revision B0

G4132-90001