

Agilent GenetiSure Pre-Screen Kit for Single Cell Analysis

**Whole Genome Amplification,
Labeling, and CGH Microarray
Hybridization**

Protocol

Version B0, June 2015

**For Research Use Only. Not for use in diagnostic
procedures.**

Before you begin, view hands-on
videos of SurePrint procedures at
<http://www.agilent.com/genomics/protocolvideos>.



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In This Guide...

This guide describes the Agilent recommended operational procedures to analyze DNA copy number variations in single cells using the GenetiSure Pre-Screen Kit. This protocol is specifically developed and optimized to amplify and enzymatically label DNA from single cells and to hybridize to 8×60K and 4×180K CGH microarrays to obtain same day results.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Reference Cells Preparation

This chapter describes the method to isolate lymphocytes from female and male blood samples to be used as references in the experiment.

3 Sample Amplification

This chapter describes the method to amplify the whole genome of single cells and of the male and female reference samples prior to labeling.

4 Sample Labeling

This chapter describes the steps to differentially label the amplified DNA samples with fluorescently labeled nucleotides.

5 Microarray Processing

This chapter describes the steps to hybridize, wash, and scan the GenetiSure Pre-Screen microarrays, and to extract data using Agilent CytoGenomics version 3.0 or higher.

6 Troubleshooting

This chapter contains potential reasons for assay failure.

7 Reference

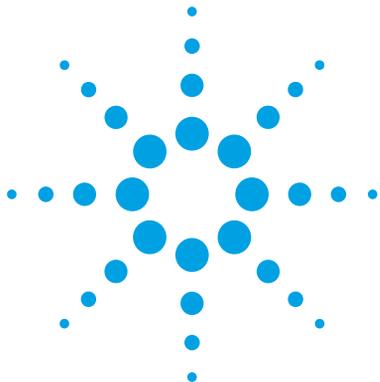
This chapter contains reference information related to the amplification, labeling, hybridization, and wash kits, and reference information on working with Agilent microarrays.

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Make sure that you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.



Procedural Notes

- Follow the procedure described in this document to amplify DNA from single cells, to increase the likelihood of a successful experiment.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettes with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing DNA or enzymes on a vortex mixer. Instead, mix the solutions and reactions by gently tapping the tube with your finger.
- Avoid repeated freeze-thaw cycles of solutions containing DNA or enzymes.
- When preparing frozen reagent stock solutions for use:
 - 1** Thaw the aliquot as quickly as possible without heating above room temperature.
 - 2** Mix briefly on a vortex mixer, and then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.
 - 3** Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

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

WARNING

- **Cyanine reagents are considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material that causes damage to the following organs: kidneys, liver, cardiovascular system, respiratory tract, skin, eye lens or cornea, stomach. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
 - **2× HI-RPM Hybridization Buffer is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Contains material that causes damage to the following organs: skin, central nervous system. May be harmful if swallowed. Avoid contact with eyes, skin and clothing.**
 - **Triton is harmful if swallowed. Risk of serious damage to eyes. Wear suitable PPE. Triton is a component of the Agilent 2× HI-RPM Hybridization Buffer.**
 - **Stabilization and Drying Solution* is considered hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). Flammable liquid and vapor. Keep away from heat, sparks and flame. Keep container closed. Use only with adequate ventilation. This solution contains material which causes damage to the following organs: kidneys, liver, cardiovascular system, upper respiratory tract, skin, central nervous system (CNS), eye, lens or cornea.**
-

1 Before You Begin

GenetiSure Pre-Screen Kit Part Numbers and Storage Conditions

GenetiSure Pre-Screen Kit Part Numbers and Storage Conditions

The Agilent part numbers, sub-kit components, and storage conditions for the GenetiSure Pre-Screen Kits are listed in [Table 1](#).

Table 1 GenetiSure Pre-Screen Kits

Kit P/N and Name	Sub-Kits	Quantity Provided	Storage Conditions
P/N G5964A GenetiSure Pre-Screen Kit, 4×180K	GenetiSure Pre-Screen Amplification and Labeling Kit*	Sufficient reagents and columns for 36 experimental samples and 12 reference samples	Store the Post Labeling Purification columns at room temperature. Store all other components at –20°C.
	GenetiSure Pre-Screen Array Kit, 4×180K	Six 1-inch × 3-inch microarray slides	Store at room temperature. After the microarray foil pouch is opened, store the slides at room temperature (in the dark) under a vacuum desiccator or N ₂ purge box. Do not store slides in open air after breaking foil.
	Hybridization Chamber Gasket Slide Kit	Six gasket slides	Store at room temperature.
P/N G5965A GenetiSure Pre-Screen Kit, 8×60K	GenetiSure Pre-Screen Amplification and Labeling Kit*	Sufficient reagents and columns for 42 experimental samples and 6 reference samples	Store the Post Labeling Purification columns at room temperature. Store all other components at –20°C.
	GenetiSure Pre-Screen Array Kit, 8×60K	Three 1-inch × 3-inch microarray slides	Store at room temperature. After the microarray foil pouch is opened, store the slides at room temperature (in the dark) under a vacuum desiccator or N ₂ purge box. Do not store slides in open air after breaking foil.
	Hybridization Chamber Gasket Slide Kit	Three gasket slides	Store at room temperature.

* The full list of components provided in the GenetiSure Pre-Screen Amplification and Labeling Kit is available in [Table 23](#) on page 60.

GenetiSure Pre-Screen Kit Part Numbers and Storage Conditions

The GenetiSure Pre-Screen Amplification and Labeling Kit and the GenetiSure Pre-Screen Array Kit can be ordered separately. Part numbers are listed in Table 2.

Table 2 GenetiSure Pre-Screen Kit, order-able part numbers for individual sub-kits

Part Number	Description
5190-7731	GenetiSure Pre-Screen Amplification and Labeling Kit
G5962A	GenetiSure Pre-Screen Array Kit, 6 slides, 4×180K
G5963A	GenetiSure Pre-Screen Array Kit, 3 slides, 8×60K
G5966A	GenetiSure Pre-Screen Array Kit, 1 slide, 8×60K
G5967A	GenetiSure Pre-Screen Array Kit, 1 slide, 4×180K
G2534-60017	Hybridization Chamber Gasket Slide Kit, 3 slides, 4-pack format
G2534-60018	Hybridization Chamber Gasket Slide Kit, 3 slides, 8-pack format

Required Reagents and Equipment

In addition to the reagents in the GenetiSure Pre-Screen Kit, the reagents and equipment listed in [Table 3](#), [Table 4](#), and [Table 5](#) are needed for the whole genome amplification, labeling, and CGH microarray hybridization protocol.

Table 3 Required reagents for reference cell preparation, amplification, and labeling with the GenetiSure Pre-Screen Kit

Description	Vendor and part number
1×TE (pH 8.0), Molecular grade	Promega p/n V6231
ISolate ready-to-use density gradient solutions, <i>or</i> ISolate concentrate, <i>or</i> Percoll, <i>or</i> Ficoll-Paque PLUS	Irvine Scientific p/n 99264 Irvine Scientific p/n 99306 Sigma-Aldrich p/n P1644-25ML GE Healthcare Life Sciences, p/n 17-1440-02
1× Phosphate Buffered Saline (pH 7.4)	Life Technologies p/n 10010-023 or equivalent
Qubit dsDNA BR Assay Kit, for use with the Qubit fluorometer, 100 assays (Optional)	Life Technologies p/n Q32850

Table 4 Required reagents for hybridization and washing

Description	Vendor and part number
Oligo aCGH/ChIP-on-chip Wash Buffer Kit <i>or</i> Oligo aCGH/ChIP-on-chip Wash Buffer 1 <i>and</i> Oligo aCGH/ChIP-on-chip Wash Buffer 2	Agilent p/n 5188-5226 Agilent p/n 5188-5221 Agilent p/n 5188-5222
Stabilization and Drying Solution*	Agilent p/n 5185-5979
Oligo aCGH/ChIP-on-chip Hybridization Kit	Agilent p/n 5188-5220 (25 slides) or p/n 5188-5380 (100 slides)
Human Cot-1 DNA	Agilent p/n 5190-3393
DNase/RNase-free distilled water	Life Technologies p/n 10977-015
Milli-Q ultrapure water	Millipore
Acetonitrile*	Sigma-Aldrich p/n 271004-1L
70% 2-Propanol (molecular biology grade)	Sigma-Aldrich p/n 563935-4L

* Optional components recommended if wash procedure B is selected.

Table 5 Required equipment

Description	Vendor and part number
Thermal cycler with heated lid	Agilent p/n G8800A or equivalent
96-well PCR plate	Agilent p/n 401334 or equivalent
Centrifuge for 96-well plates	Eppendorf p/n 5810 or equivalent
PCR plate heat sealer	Eppendorf p/n 951023078
Peel-it-lite microplate foil (removable)	Eppendorf p/n 951023205
200- μ L thin-wall tubes	Agilent p/n 410091 or equivalent
Agilent Microarray Scanner Bundle	Agilent p/n G4900DA or G2565CA, or Agilent p/n G5761AA (available in the EU, Singapore, and S. Korea)
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization oven; temperature set at 67°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Ozone-barrier slide covers (box of 20)*	Agilent p/n G2505-60550
1.5 mL RNase-free Microfuge Tube	Ambion p/n AM12400 or equivalent
Magnetic stir plate ($\times 1$ or $\times 3$) [†]	Corning p/n 6795-410 or equivalent
Magnetic stir plate with heating element	Corning p/n 6795-420 or equivalent
Microcentrifuge with rotor for 200- μ L tubes (or 500- μ L tubes if single cells were collected in these tubes) and 1.5-mL tubes	Eppendorf p/n 5430 or equivalent
Qubit Fluorometer [‡]	Life Technologies p/n Q32857
Thin wall, clear 0.5 mL PCR tubes [‡]	Life Technologies p/n Q32856 or VWR p/n 10011-830
Sterile storage bottle	Nalgene 455-1000 or equivalent
Inverted microscope, magnification 10 \times 10	Nikon TMS or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
1.5 L glass dish	Pyrex p/n 213-R or equivalent
Magnetic stir bar, 7.9 \times 38.1 mm ($\times 2$ or $\times 4$) [†]	VWR p/n 58948-150 or equivalent

1 Before You Begin

Required Hardware and Software

Table 5 Required equipment

Description	Vendor and part number
Levy Counting Chamber (Hemocytometer)	VWR p/n 15170-208
250 mL capacity slide-staining dish, with slide rack (×3 or ×5) [†]	Wheaton p/n 900200 <i>or</i> Thermo Shandon p/n 121
Circulating water baths or incubator, to warm Wash Buffer 2 and dishes for microarray wash step	
Ice bucket	
Clean forceps	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vacuum desiccator or N ₂ purge box for slide storage	
Vortex mixer	

* Optional. Recommended when processing arrays with a G2565CA scanner in environments in which ozone levels are 5 ppb or higher.

[‡] Optional.

[†] The number varies depending on if wash procedure A or B is selected.

Required Hardware and Software

The protocol requires the software needed to operate the Agilent scanner and the Agilent CytoGenomics software (version 3.0 or higher).

- Refer to the Agilent Scanner manual and Agilent CytoGenomics manuals for minimum memory requirements and other specifications for the PC used to run these software programs.
Go to <http://www.genomics.agilent.com> to download the manuals.
- You can download the design files needed for data extraction and analysis in CytoGenomics 3.0 from the Agilent SureDesign website.
Go to <http://www.agilent.com/genomics/suredesign>.

Overview of the GenetiSure Pre-Screen Workflow

The Agilent array-based Comparative Genomic Hybridization (aCGH) application uses a “two-color” process to measure DNA copy number changes (CNC).

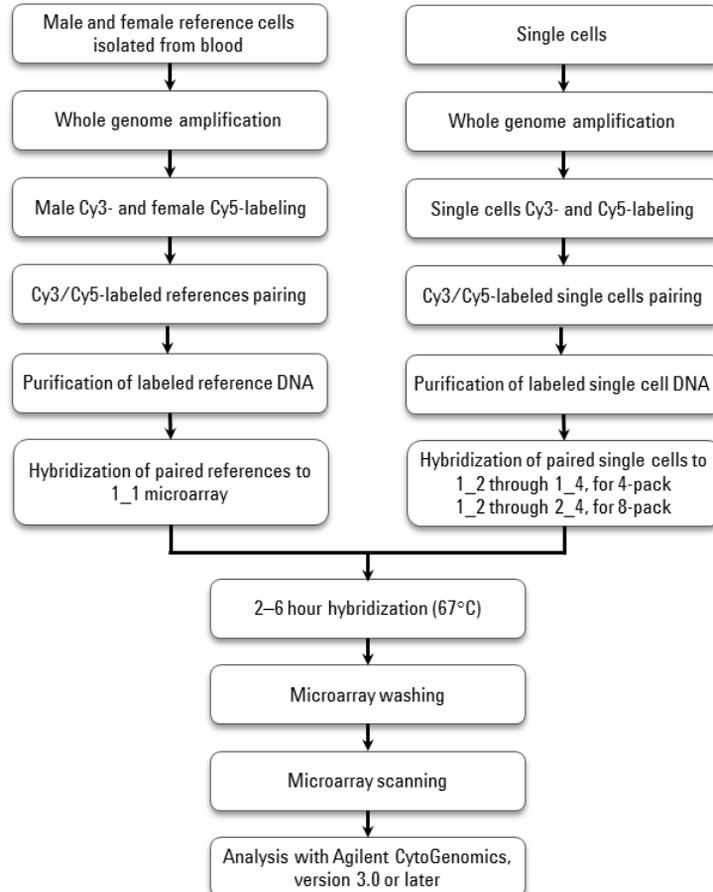
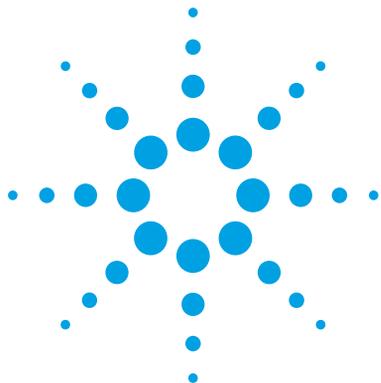


Figure 1 Overview of the GenetiSure Pre-Screen workflow for single cell analysis

1 Before You Begin
Overview of the GenetiSure Pre-Screen Workflow



2 Reference Cells Preparation

Reference Cells Preparation 18

This chapter describes the method to isolate lymphocytes from female and male blood samples that will be used as references.



Reference Cells Preparation

Use the procedure below to isolate male and female lymphocytes from blood collected in a purple top tube or from a finger stick. The lymphocyte samples will be used as references in the single cell analysis. For each microarray slide, one male and one female reference sample will be hybridized against each other on one array (Figure 1 on page 15).

NOTE

The reference cell preparation procedure uses ISolate Concentrate from Irvine Scientific, but if ISolate solution is not available, Percoll or Ficoll-Paque PLUS can be used instead. Follow the manufacturer's protocol for Percoll or Ficoll-Paque PLUS.

- 1 If using ISolate Concentrate, prepare the 90% and 50% ISolate solutions in 1.5-mL microcentrifuge tubes. (If using ISolate ready-to-use density gradient solutions, proceed to step 2. If using Percoll or Ficoll-Paque PLUS, follow the manufacturer's protocol.)
 - a Prepare a 90% Isolate solution, by diluting 900 μL of ISolate Concentrate with 100 μL of Phosphate Buffered Saline, pH 7.4 (PBS). *Do not use the PBS provided in the GenetiSure Pre-Screen Kit for this step.*
 - b Prepare a 50% Isolate solution, by diluting 500 μL of ISolate Concentrate with 500 μL of PBS. *Do not use the PBS provided in the GenetiSure Pre-Screen Kit for this step.*
 - c Let both ISolate solutions equilibrate to room temperature.
- 2 In a sterile safety cabinet, isolate the lymphocytes in a 1.5-mL centrifuge tube.
 - a Add 500 μL of lower layer solution (90% ISolate) to the 1.5 ml tube.
 - b Slowly add 500 μL of upper layer solution (50% ISolate) without disturbing the lower layer.
 - c Slowly add 100–150 μL of whole blood from a purple top blood tube or about three drops from a finger stick to the ISolate reagents without disturbing the gradient layers. See panel A in Figure 2 on page 19.
 - d Centrifuge for 20 minutes at $300 \times g$.

The sample separates into four layers. The top layer contains plasma. The second layer from the top contains lymphocytes. See panel B in Figure 2 on page 19.

- e Using a P1000 pipette, remove and discard the top plasma layer. With a fresh pipette tip, carefully transfer the lymphocyte layer, about 250 μL , to a fresh 1.5-mL tube. Avoid disturbing the layer of red blood cells.
 - f Dilute the collected lymphocytes with PBS by adding 1 volume of PBS to 1 volume of lymphocytes. *Do not use the PBS provided in the GenetiSure Pre-Screen Kit for this step.*
 - g Centrifuge for 5 minutes at $300 \times g$. Remove the supernatant and resuspend the pelleted lymphocytes in 500 μL of PBS. (Use the PBS provided in the GenetiSure Pre-Screen Kit.)
- 3 Count cells using a microscope and hemacytometer. Dilute cells to a concentration of ~ 100 cells/ μL with PBS. (Use the PBS provided in the GenetiSure Pre-Screen Kit.)
 - 4 Aliquot 10 μL of the diluted lymphocytes into fresh tubes and store at -80°C .

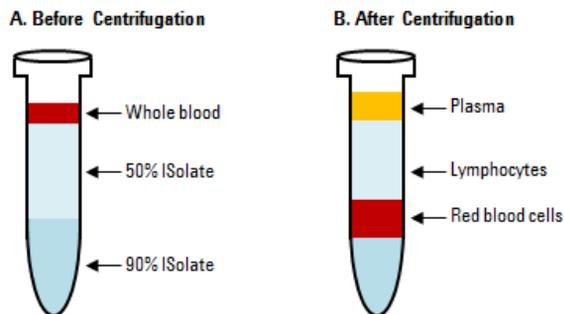
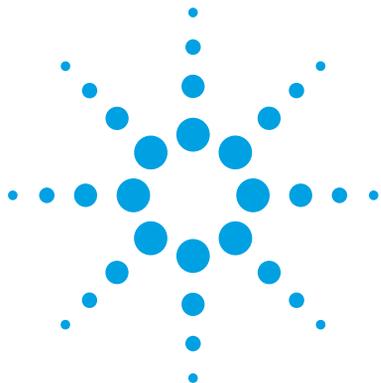


Figure 2 The layers seen in a 1.5-mL tube containing ISolate solution and blood cells before centrifugation (panel A) and after centrifugation (panel B).

2 Reference Cells Preparation

Reference Cells Preparation



3 Sample Amplification

Step 1. Whole Genome Amplification 22

Step 2. Quantitation of Amplified DNA using Qubit Fluorometer
(Optional) 25

This chapter describes the recommendations to amplify DNA from single cells and reference lymphocytes prior to labeling. For each slide, one male and one female lymphocyte sample is amplified along with the single cells. Cells are subjected to multiple displacement-based whole genome amplification to increase the amount of DNA while maintaining its genomic representation.



Step 1. Whole Genome Amplification

This section describes the Agilent recommended procedure to amplify DNA from single cells and male and female isolated lymphocyte cells.

- 1** Prepare samples of single cells.
 - If the single cell is stored in a 500 μ L-tube, use a pipette to remove the mineral oil and leave the cell in the tube.
 - If the single cell is stored in a 4-well dish or petri dish, use a denudation pipette under the microscope to transfer the cell from the dish into a 200- μ L thin-wall tube containing 3 μ L of 1 \times PBS.
- 2** Prepare reference samples of male and female lymphocytes by transferring 3 μ L of the diluted male and female lymphocytes (\sim 300 cells each) to separate 200- μ L thin-wall tubes (or 500- μ L tubes if single cells were collected in 500- μ L tubes).

NOTE

To avoid DNA contamination, do not culture the cells in serum-containing media.

- 3** Prepare a negative control sample by adding 3 μ L of 1 \times PBS to a reaction tube. (Use the PBS provided in the GenetiSure Pre-Screen Kit.)
- 4** Prepare Buffer DLB.
 - a** Add 500 μ L of nuclease-free distilled water to the provided tube of Buffer DLB to resuspend the lyophilized pellet. Mix thoroughly then spin briefly in a centrifuge.
 - b** Prepare aliquots of the reconstituted Buffer DLB. Store the aliquots at -20°C for up to 6 months. Buffer DLB is pH-labile and should not be stored longer than 6 months.

- 5 Prepare Buffer D2 (denaturation buffer).
 - a Mix the components listed in Table 6.

Table 6 Preparation of Buffer D2

Component	Volume (μL)	×9 reactions (μL) including excess*	×17 reactions (μL) (including excess)*
DTT, 1 M	0.25	2.25	4.5
Buffer DLB, reconstituted	2.75	24.75	49.5
Final volume of Buffer D2	3	27	54

*Includes a negative control reaction

- 6 Add 3 μL of Buffer D2 to each sample tube (single cell samples, male and female reference samples, and negative control) for a total volume of 6 μL. Mix carefully by flicking the tubes. *Do not pipet up and down.*

CAUTION

Do not mix samples containing single cells by pipetting up and down; cells could get stuck to the pipette tip.

- 7 Transfer the tubes to a thermal cycler with the lid temperature set to 70°C. If the lid cannot be set to 70°C, then use the lid's hot setting. Incubate the samples in the thermal cycler according to the protocol listed in Table 7. If your samples are in 500-μL tubes, and your thermal cycler does not accommodate tubes of this size, perform the 65°C incubation in a heated water bath, then transfer the tubes to ice.

Table 7 Incubation protocol to lyse the cells

Step	Temperature	Duration
Step 1	65°C	10 minutes
Step 2	4°C	hold until ready to proceed

- 8 Remove the tubes from the thermal cycler and spin briefly in a microcentrifuge.

3 Sample Amplification

Step 1. Whole Genome Amplification

- 9 Add 3 μL of Stop Solution to each sample for a total volume of 9 μL . Mix carefully by flicking the tubes. Keep the samples on ice.
- 10 Prepare the Amplification Master Mix by mixing the components in Table 8 on ice.

NOTE

Thaw the Amplification DNA Polymerase on ice. For all other components, thaw at room temperature, vortex to mix, then spin briefly in a centrifuge.

The Amplification Reaction Buffer may form a precipitate after thawing. Vortexing for 10 seconds dissolves the precipitate.

Table 8 Amplification Master Mix

Component	Volume (μL)	x9 reactions (μL) including excess*	x17 reactions (μL) including excess*
Nuclease-Free Water	4.5	42.75	81.0
Amplification Reaction Buffer	14.5	137.75	261.0
Amplification DNA Polymerase	1	9.5	18.0
Final volume of Amplification Master Mix	20	190	360

* Includes a negative control reaction

- 11 Add 20 μL of Amplification Master Mix to each 9 μL sample from the previous step to make a total volume of 29 μL .
- 12 Mix carefully by flicking the tubes then spin the tubes briefly in a centrifuge to drive the contents off the walls and lid.
- 13 Transfer the tubes to a thermal cycler with the lid temperature set to 70°C. If the lid cannot be set to 70°C, then use the lid's hot setting. Run the program in Table 9 to amplify the DNA in the samples. If your samples are in 500- μL tubes, and your thermal cycler does not accommodate tubes of this size, perform the 31°C and 65°C incubations in heated water baths, then transfer the tubes to ice.

Step 2. Quantitation of Amplified DNA using Qubit Fluorometer (Optional)

Table 9 Whole Genome Amplification

Step	Temperature	Duration
Step 1	31°C	85 minutes
Step 2	65°C	10 minutes
Step 3	4°C	hold until ready to proceed

14 Maintain the reactions at 4°C for short term storage (up to 2 days), or store at -20°C for long term storage until ready for labeling.

Step 2. Quantitation of Amplified DNA using Qubit Fluorometer (Optional)

Use Quant-iT dsDNA Broad-Range Assay Kit to measure the concentration of amplified DNA. Additionally, measuring the concentration in the negative control sample allows you to screen for contamination.

Allow the Qubit dsDNA BR Assay Kit to equilibrate to room temperature (22–28°C) before use. Temperature fluctuations can affect the accuracy of the assay.

- 1** Set up clear, thin-walled 0.5-mL PCR tubes for all samples (the two standards, the amplified DNA samples that you are processing, and the negative control).
- 2** Make a Qubit working solution. For each standard and amplified DNA sample to be quantified, mix the components in [Table 10](#).

Table 10 Qubit Working Solution

Component	Volume
Qubit dsDNA BR reagent	1 µL
Qubit dsDNA BR buffer	199 µL

3 Sample Amplification

Step 2. Quantitation of Amplified DNA using Qubit Fluorometer (Optional)

- 3 Add 190 μL of Qubit working solution to the two 0.5-mL tubes labeled for the standards.
- 4 Add 199 μL of Qubit working solution to the 0.5-mL tubes labeled for your amplified DNA samples.
- 5 Add 10 μL of Qubit dsDNA BR standard #1 to the tube labeled for standard #1, and add 10 μL of Qubit dsDNA BR standard #2 to the tube labeled for standard #2.
- 6 Add 1 μL of amplified DNA sample to the remaining 0.5-mL tubes.
- 7 Mix the contents of all tubes on a vortex mixer for 2–3 seconds, taking care not to create bubbles.
- 8 Incubate the tubes at room temperature for 2 minutes.
- 9 Calibrate the Qubit.
 - a On the home screen of the Qubit 1.0, use the up or down arrow to select dsDNA Broad Range Assay as assay type, and then press **GO**. The standard screen is automatically displayed.
 - b Select **Run new calibration**, and then press **GO**.
 - c Insert the tube with the first standard into the Qubit Fluorometer, close the lid and press **GO**. After the reading is done, remove the standard.
 - d Insert the tube with the second standard into the Qubit Fluorometer, close the lid, and press **GO**. After the reading is done remove the standard.

The calibration is complete after the second standard has been read.

- 10 Measure the concentrations of the amplified DNA samples.
 - a Insert a sample and press **GO**.
 - b When the measurement is complete (approximately 5 seconds later), make a note of the reading. The result is displayed on the screen. The number displayed is the concentration of the nucleic acid in the assay tube.
 - c Remove the sample from the instrument, insert the next sample, and press **GO**.
 - d Repeat sample readings until all samples have been read.
 - e Calculate the concentration of your original sample.

Step 2. Quantitation of Amplified DNA using Qubit Fluorometer (Optional)

The Qubit Fluorometer gives a value for the Qubit dsDNA BR assay in $\mu\text{g/mL}$. This value corresponds to the concentration after your samples were diluted into the assay tube. To calculate the concentration of your sample, use the equation below.

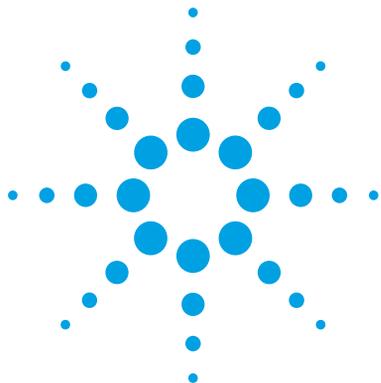
$$\text{Sample concentration} = QF \text{ value} \times 200$$

where

QF value = the value given by the Qubit Fluorometer

3 Sample Amplification

Step 2. Quantitation of Amplified DNA using Qubit Fluorometer (Optional)



4 Sample Labeling

Step 1. Fluorescent Labeling of DNA	30
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This chapter describes the steps to differentially label the amplified DNA samples with fluorescent-labeled nucleotides using reagents from the GenetiSure Pre-Screen Amplification and Labeling Kit.

The procedure uses random primers and the *exo(-)* Klenow fragment to differentially label amplified DNA samples with fluorescent-labeled nucleotides. For the GenetiSure Pre-Screen application, you first differentially label your unknown samples with cyanine-3 (Cy3) and cyanine-5 (Cy5) dyes, and then combine the samples in Cy3-Cy5 pairs. You also differentially label the reference male and female lymphocyte samples with Cy3 and Cy5 dyes and combine as a Cy3-Cy5 pair. If you have an odd number of unknown samples, or an insufficient number of unknown samples to fill all of the arrays on a 4×180K or 8×60K slide, you need to label one or more additional male lymphocyte samples to pair with the odd unknown sample or to fill the empty arrays.



Step 1. Fluorescent Labeling of DNA

NOTE

Only label the unknown samples and the reference samples. Do not label the negative control sample.

Cyanine 3-dUTP and cyanine 5-dUTP are light sensitive and are subject to degradation by multiple freeze thaw cycles. Minimize light exposure throughout the labeling procedure.

- 1 Spin the amplified DNA samples in a centrifuge for 1 minute at $5,000 \times g$ to drive the contents off the walls and lid.
- 2 Transfer 13 μL of each sample into a new 200- μL tube or into a well of a 96-well PCR plate.

NOTE

If you have an odd number of unknown samples, you need to set up an additional tube for a second male reference sample. This additional male reference sample will be used for pairing with the odd unknown sample in [step 10](#).

Similarly, if you have an insufficient number of unknown samples to fill all of the arrays on a $4 \times 180\text{K}$ or $8 \times 60\text{K}$ slide, you need to set up additional tubes for reference samples (enough to fill the empty arrays).

- 3 Add 2.5 μL of [Random Primers](#) to each reaction tube that contains 13 μL of amplified DNA sample to make a total volume of 15.5 μL . Mix well by pipetting up and down gently.
- 4 Transfer tubes to a thermal cycler with a heated lid. Program the thermal cycler according to [Table 11](#) and run the program.

Table 11 DNA denaturation using a thermal cycler

Step	Temperature	Duration
Step 1	98°C	3 minutes
Step 2	4°C	at least 3 minutes (hold at 4°C until ready to proceed)

- 5 Spin the tubes in a centrifuge for 1 minute at $5,000 \times g$ to drive the contents off the walls and lid.

- 6 Mix the components in Table 12 on ice in the order indicated to prepare one cyanine-3 (Cy3) and one cyanine-5 (Cy5) Labeling Master Mix.

Table 12 Labeling Master Mix

Component	Per reaction (μL)	× 4 rxns (μL) (including excess)	× 8 rxns (μL) (including excess)
5× Reaction Buffer	5.0	22.5	42.5
10× dNTP Mix	2.5	11.25	21.25
Cyanine 3-dUTP <i>or</i> Cyanine 5-dUTP	1.5	6.75	12.75
Exo (-) Klenow	0.5	2.25	4.25
Final volume of Labeling Master Mix	9.5	42.75	80.75

- 7 Add 9.5 μL of Labeling Master Mix to each reaction tube that contains the amplified DNA to make a total volume of 25 μL. Mix well by gently pipetting up and down.
- For the male reference sample and the female reference sample, use the Cy3 Labeling Master Mix for one of the two samples and use the Cy5 Labeling Master Mix for the other sample.
 - For the unknown samples, use the Cy3 Labeling Master Mix for half of the samples and use the Cy5 Labeling Master Mix for the other half of the samples. If you have an odd number of unknown samples, use the second male lymphocyte sample to create an even number.
- 8 Transfer sample tubes to a thermal cycler with a heated lid. Program the thermal cycler according to Table 13 and run the program.

Table 13 DNA labeling using a thermal cycler

Step	Temperature	Time
Step 1	37°C	45 minutes
Step 2	65°C	10 minutes
Step 3	4°C	hold

4 Sample Labeling

Step 2. Purification of labeled DNA

- 9 Spin the labeled DNA samples in a centrifuge for 1 minute at $5,000 \times g$ to drive the contents off the walls and lid.
- 10 Combine the differentially labeled male and female reference samples to create a Cy3-Cy5 pair with a final volume of 50 μL . Similarly, for the unknown samples, combine two differentially labeled samples to create Cy3-Cy5 pairs with a final volume of 50 μL . If you have an odd number of unknown samples, use the second male reference sample to create a Cy3-Cy5 pair.

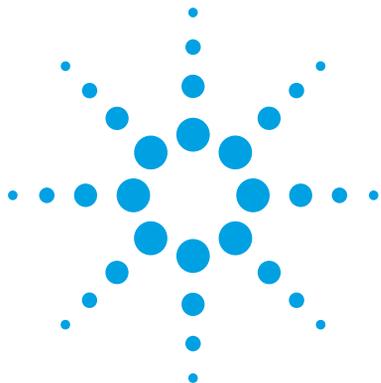
NOTE

Paired samples of labeled DNA can be stored for up to a month at -20°C in the dark.

Step 2. Purification of labeled DNA

Labeled DNA is purified using the Post Labeling Purification Columns and Collection Tubes provided with the GenetiSure Pre-Screen Amplification and Labeling Kit.

- 1 Spin the labeled DNA samples in a centrifuge for 1 minute at $5,000 \times g$ to drive the contents off the walls and lid, then transfer each 50- μL sample into a fresh 1.5-mL tube.
- 2 Add 430 μL of $1\times$ TE (pH 8.0) to each sample tube.
- 3 For each paired Cy3-Cy5 sample to be purified, label a purification column and place it into a 1.5-mL collection tube. Load each labeled sample onto the appropriate column.
- 4 Cap the columns and spin for 15 minutes at $9,000 \times g$ in a microcentrifuge at room temperature. Discard the flow-through.
- 5 Invert the columns into fresh 1.5-mL collection tubes that have been appropriately labeled. Spin for 2 minutes at $2,500 \times g$ in a microcentrifuge at room temperature to collect purified samples.
- 6 Measure the volume of each paired purified sample using a pipette. Adjust the sample volumes as needed:
 - For samples with a volume $\geq 15 \mu\text{L}$, transfer 15 μL of the sample to a fresh 200- μL reaction tube or into the well of a 96-well PCR plate.
 - For samples with a volume $< 15 \mu\text{L}$, add enough $1\times$ TE (pH 8.0) to bring the total volume to 15 μL . Then, transfer the entire 15- μL sample to a fresh 200- μL reaction tube or into the well of a 96-well PCR plate.



5 Microarray Processing

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Microarray processing consists of hybridization, washing, and scanning.



Hybridization

If you are new to microarray processing, and want to practice hybridization, prepare a 1:1 2× Hi-RPM Hybridization Buffer and water mix and use a microscope slide or used microarray slide, and a gasket slide. You can use the same slide to practice wash and placement of slide in the slide holder.

Before you begin, make sure you read and understand “Microarray Handling Tips” on page 61.

Step 1. Prepare the 10× Blocking Agent

- 1 Add 1.35 mL of DNase/RNase-free distilled water to the vial containing lyophilized 10× aCGH Blocking Agent (included in the Oligo aCGH/ChIP-on-chip Hybridization Kit, p/n 5188-5220 (25 slides) or 5188-5380 (100 slides)).
- 2 Leave at room temperature for 60 minutes and mix on a vortex mixer to reconstitute sample before use or storage.

NOTE

The 10× Blocking Agent can be prepared in advance and stored at -20°C.

Step 2. Prepare labeled DNA for hybridization

- 1 Mix the components according to the microarray format to prepare the Hybridization Master Mix.

Table 14 Hybridization Master Mix for **4-pack** microarray

Component	Volume (μL) per hybridization	$\times 4$ rxns (μL) (including excess)
1 \times TE (pH 8.0), Molecular grade	20	90
Human Cot-1 DNA (1.0 mg/mL)	10	45
10 \times aCGH Blocking Agent*	10	45
2 \times Hi-RPM Hybridization Buffer*	55	247.5
Final Volume of Hybridization Master Mix	95	427.5

* Included in the Agilent Oligo aCGH/ChIP-on-chip Hybridization Kit, p/n 5188-5220 (25 slides) or 5188-5380 (100 slides)

Table 15 Hybridization Master Mix for **8-pack** microarray

Component	Volume (μL) per hybridization	$\times 8$ rxns (μL) (including excess)
Human Cot-1 DNA (1.0 mg/mL)	5	42.5
10 \times aCGH Blocking Agent*	5	42.5
2 \times Hi-RPM Hybridization Buffer*	25	212.5
Final Volume of Hybridization Master Mix	35	297.5

* Included in the Agilent Oligo aCGH/ChIP-on-chip Hybridization Kit, p/n 5188-5220 (25 slides) or 5188-5380 (100 slides)

- 2 Add the appropriate volume of the Hybridization Master Mix to each 200- μL tube or plate well that contains 15 μL of labeled DNA to make the total volume listed in Table 16.

5 Microarray Processing

Step 2. Prepare labeled DNA for hybridization

Table 16 Volume of Hybridization Master Mix per hybridization

Microarray format	Volume of Hybridization Master Mix	Total volume
4-pack	95 μ L	110 μ L
8-pack	35 μ L	50 μ L

- 3 Mix the samples by pipetting up and down, then quickly spin in a centrifuge to drive contents to the bottom of the tubes or wells.
- 4 Transfer sample tubes or plate to a thermal cycler with a heated lid. Program the thermal cycler according to the following table and run the program:

Table 17 DNA preparation for hybridization

Step	Temperature	Time
Step 1	98°C	2 minutes exactly
Step 2	37°C	18 minutes

- 5 Remove tubes or plate from the thermal cycler. Spin 1 minute at 5,000 \times g in a centrifuge to collect the sample at the bottom of the tube.

The samples are ready to be hybridized.

CAUTION

The samples must be hybridized immediately. If not, keep the temperature of hybridization sample mixtures as close to 37°C as possible in a thermal cycler.

Step 3. Prepare the hybridization assembly

CAUTION

All arrays within a slide must be hybridized to a sample. Leaving an array empty may cause a gridding failure during feature extraction.

Refer to the Agilent Microarray Hybridization Chamber User Guide (G2534-90001) for in-depth instructions on how to load samples, assemble and disassemble chambers, as well as other helpful tips. This user guide can be downloaded from the Agilent Web site at www.agilent.com/genomics.

Before you begin, make sure you read and understand “Microarray Handling Tips” on page 61.

- 1 Load a clean gasket slide into the Agilent SureHyb chamber base with the gasket label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
- 2 Slowly dispense the appropriate volume of hybridization sample mixture onto the gasket well.
 - For 4-pack microarrays, slowly dispense 100 μL of the mixture in a “drag and dispense” manner.
 - For 8-pack microarrays, slowly dispense 47 μL of the mixture onto the center of the gasket slide so as to avoid contact between the mixture and the rubber gasket edges.

Load all gasket wells before you load the microarray slide.

CAUTION

Keep the temperature of hybridization sample mixtures as close to 37°C as possible. To do this, process them in small batches and/or put them in a thermal cycler or in an oven.

- 3 Put a microarray slide “active side” down onto the gasket slide, so the numeric barcode side is facing up and the “Agilent”-labeled barcode is facing down. Assess that the sandwich-pair is properly aligned.
- 4 Put the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
- 5 Hand-tighten the clamp firmly onto the chamber.

5 Microarray Processing

Step 4. Hybridize

- 6 Vertically rotate the assembled chamber to wet the slides and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.

Step 4. Hybridize

- 1 Load each assembled chamber into the oven rotator rack. Start from the center of the rack (position 3 or 4 when counting from the left). Set your hybridization rotator to rotate at 20 rpm.
- 2 Hybridize at 67°C for 2–6 hours.

CAUTION

If you are not loading all the available positions on the hybridization rotator rack, be sure to *balance* the loaded hybridization chambers on the rack similar to a centrifuge to prevent unnecessary strain on the oven motor.

CAUTION

You must calibrate the hybridization oven regularly for accuracy of the collected data. Refer to *Agilent G2545A Hybridization Calibration Procedure* (p/n G2545-90002) for more information.

For more information on the effects of hybridization temperature and time, as well as the rotation speed on the final microarray results, please refer to the application note titled “60-mer Oligo-Based Comparative Genomic Hybridization” (publication 5989-4848EN) from the Agilent Web site at www.agilent.com/genomics.

NOTE

The Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 that is used in the microarray wash procedure needs to be warmed overnight. While you are waiting for the microarray slides to hybridize, do the steps in “Step 1. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (overnight)” on page 40.

Microarray Wash

NOTE

The microarray wash procedure must be done in environments where ozone levels are 5 ppb or less. For Scanner C, if ozone levels are between 5 to 10 in your laboratory, use the Agilent Ozone Barrier Slide Cover. SureScan microarray scanner uses a slide holder with a built-in ozone barrier. If ozone levels exceed 10 ppb, use the Stabilization and Drying Solution together with the ozone barrier.

You can also use Carbon Loaded Non-woven Filters to remove ozone from the air. These filters can be installed in either your HVAC system, or as part of small Ozone Controlled Enclosures. These free-standing enclosures can be installed either on a lab bench or as a walk-in room within your lab. These products are available through filter suppliers listed in Agilent Technical Note 5989-0875EN.

Before you begin, determine which wash procedure to use:

Table 18 Wash procedure to follow

Ozone level in your lab	Wash Procedure	Ozone-Barrier Slide Cover
< 5 ppb	“Wash Procedure A (without Stabilization and Drying Solution)” on page 43	No
> 5 ppb < 10 ppb	“Wash Procedure A (without Stabilization and Drying Solution)” on page 43	Yes
> 10 ppb	“Wash Procedure B (with Stabilization and Drying Solution)” on page 45	Yes

Step 1. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (overnight)

The temperature of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 must be at 37°C for optimal performance.

- 1 Add the volume of buffer required to a sterile storage bottle and warm overnight in an incubator or circulating water bath set to 37°C.
- 2 Put a slide-staining dish with a lid, a 1.5-L glass dish, and one to two liters of Milli-Q ultrapure water in an incubator or water bath set at 37°C to warm overnight.

Step 2. Prepare the equipment

Always use clean equipment when doing the hybridization and wash steps.

Use only dishes that are designated and dedicated for use in GenetiSure Pre-Screen experiments.

Solvent wash

Wash staining dishes, racks and stir bars with acetonitrile or 2-Propanol to avoid wash artifacts on your slides and images.

- Use acetonitrile for equipment that was exposed to Stabilization and Drying Solution (i.e. was used in Wash Procedure B).
- Use 2-Propanol for equipment that was not exposed to Stabilization and Drying Solution (i.e. was used in Wash Procedure A).

WARNING

Conduct solvent washes in a vented fume hood.

- 1 Add the slide rack and stir bar to the slide-staining dish.
- 2 Transfer the slide-staining dish with the slide rack and stir bar to a magnetic stir plate.
- 3 Fill the slide-staining dish with 100% acetonitrile or 2-Propanol.

Step 3. Prewarm Stabilization and Drying Solution (Wash Procedure B Only)

- 4** Turn on the magnetic stir plate and adjust the speed to 350 rpm (medium speed).
- 5** Wash for 5 minutes at room temperature.
- 6** Discard the solvent as is appropriate for your site.
- 7** Repeat **step 1** through **step 6**.
- 8** Air dry all of the equipment in the vented fume hood, then proceed to “**Milli-Q ultrapure water wash**”, below.

Milli-Q ultrapure water wash

Wash all slide-staining dishes, slide racks, and stir bars thoroughly with high-quality Milli-Q ultrapure water.

- 1** Run copious amounts of Milli-Q ultrapure water through the slide-staining dishes, slide racks, and stir bars.
- 2** Empty out the water collected in the dishes.
- 3** Repeat **step 1** and **step 2** at least 5 times until all traces of contaminating material are removed.

CAUTION

Some detergents may leave fluorescent residue on the dishes. Do not use any detergent in the washing of the staining dishes, slide racks, or stir bars. If detergent is used, all traces must be removed by copiously rinsing with Milli-Q ultrapure water.

Step 3. Prewarm Stabilization and Drying Solution (Wash Procedure B Only)

The Stabilization and Drying Solution contains an ozone scavenging compound dissolved in acetonitrile. The compound in solution is present in saturating amounts and may precipitate from the solution under normal storage conditions. If the solution shows visible precipitation, warming of the solution will be necessary to redissolve the compound. Washing slides using Stabilization and Drying Solution showing visible precipitation will have profound adverse effects on microarray performance.

5 Microarray Processing

Step 3. Prewarm Stabilization and Drying Solution (Wash Procedure B Only)

WARNING

The Stabilization and Drying Solution is a flammable liquid. Warming the solution will increase the generation of ignitable vapors. Use gloves and eye/face protection in every step of the warming procedures.

WARNING

Do not use a hot plate, oven, an open flame or a microwave. Do not increase temperature rapidly. Warm and mix the material away from ignition sources.

WARNING

Failure to follow the outlined process will increase the potential for fire, explosion, and possible personal injury.

- 1 Put a clean magnetic stir bar into the Stabilization and Drying Solution bottle and recap.
- 2 Partially fill a plastic bucket with hot water at approximately 40°C to 45°C (for example from a hot water tap).
- 3 Put the Stabilization and Drying Solution bottle into the hot water in the plastic bucket.
- 4 Put the plastic bucket on a magnetic stirrer (*not a hot-plate*) and stir.
- 5 The hot water cools to room temperature. If the precipitate has not all dissolved replenish the cold water with hot water.
- 6 Repeat [step 5](#) until the solution is clear.
- 7 After the precipitate is completely dissolved, allow the solution to equilibrate to room temperature prior to use.

CAUTION

Do not filter the Stabilization and Drying Solution, or the concentration of the ozone scavenger may vary.

Step 4. Wash microarrays

Wash Procedure A (*without Stabilization and Drying Solution*)

Always use fresh Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 for each wash group (up to five slides).

Table 19 lists the wash conditions for the Wash Procedure A without Stabilization and Drying Solution.

Table 19 Wash conditions

	Dish	Wash buffer	Temperature	Time
Disassembly	#1	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	
1st wash	#2	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	5 minutes
2nd wash	#3	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2	37°C	1 minute

- 1 Completely fill slide-staining dish #1 with Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 at room temperature.
- 2 Prepare dish #2:
 - a Put a slide rack into slide-staining dish #2.
 - b Add a magnetic stir bar. Fill slide-staining dish #2 with enough Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 at room temperature to cover the slide rack.
 - c Put this dish on a magnetic stir plate.

5 Microarray Processing

Step 4. Wash microarrays

- 3 Prepare dish #3:
 - a Put the prewarmed 1.5-L glass dish on a magnetic stir plate with heating element.
 - b Put the slide-staining dish #3 into the 1.5-L glass dish.
 - c Fill the 1.5-L glass dish with pre-warmed Milli-Q ultrapure water.
 - d Fill the slide-staining dish #3 approximately three-fourths full with Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (warmed to 37°C).
 - e Add a magnetic stir bar.
 - f Turn on the heating element and maintain temperature of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37°C. Monitor with a thermometer.
- 4 Remove one hybridization chamber from the incubator and resume rotation of the others. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.
- 5 Prepare the hybridization chamber disassembly.
 - a Put the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.
 - b Slide off the clamp assembly and remove the chamber cover.
 - c With gloved fingers, remove the microarray-gasket sandwich from the chamber base by lifting one end and then grasping in the middle of the long sides. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.
 - d Without letting go of the slides, submerge the microarray-gasket sandwich into slide-staining dish #1 containing Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1.
- 6 With the sandwich completely submerged in Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1, pry the sandwich open from the barcode end only:
 - a Slip one of the blunt ends of the forceps between the slides.
 - b Gently twist the forceps to separate the slides.
 - c Let the gasket slide drop to the bottom of the staining dish.
 - d Remove the microarray slide, grasp it from the upper corners with thumb and forefinger, and quickly put into slide rack in the slide-staining dish #2 containing Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

- 7** Repeat **step 4** through **step 6** for up to four additional slides in the group. A maximum of five disassembly procedures yielding five microarray slides is advised at one time in order to facilitate uniform washing.
- 8** 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.
- 9** Wash the slides in Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2:
 - a** Transfer slide rack to slide-staining dish #3, which contains Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37°C:
 - a** Activate the magnetic stirrer.
 - b** Wash microarray slide for at least 1 minute and no more than 2 minutes. Adjust the setting to get thorough mixing without disturbing the microarray slides.
- 10** Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.
- 11** Discard used Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2.
- 12** Repeat **step 1** through **step 11** for the next group of five slides using fresh Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 warmed to 37°C.
- 13** Scan slides immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a N₂ purge box, in the dark.

Wash Procedure B (*with* Stabilization and Drying Solution)

Cyanine reagents are susceptible to degradation by ozone. Use this wash procedure if the ozone level exceeds 10 ppb in your laboratory.

Always use fresh Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 for each wash group (up to five slides).

The acetonitrile (dish #4) and Stabilization and Drying Solution (dish #5) below may be reused for washing up to 4 batches of 5 slides (total 20 slides) in one experiment. Do not pour the Stabilization and Drying Solution back in the bottle.

5 Microarray Processing

Step 4. Wash microarrays

WARNING

The Stabilization and Drying Solution must be set-up in a fume hood. Put the Wash Buffer 1 and Wash Buffer 2 set-up areas close to, or preferably in, the same fume hood. Use gloves and eye/face protection in every step of the washing procedure.

Table 20 lists the wash conditions for the Wash Procedure B with Stabilization and Drying Solution.

Table 20 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	#1	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	
1st wash	#2	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	5 minutes
2nd wash	#3	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2	37°C	1 minute
Acetonitrile wash	#4	Acetonitrile	Room temperature	10 seconds
3rd wash	#5	Stabilization and Drying Solution	Room temperature	30 seconds

- 1 In the fume hood, fill slide-staining dish #4 approximately three-fourths full with acetonitrile. Add a magnetic stir bar and put this dish on a magnetic stir plate.
- 2 In the fume hood, fill slide-staining dish #5 approximately three-fourths full with Stabilization and Drying Solution. Add a magnetic stir bar and put this dish on a magnetic stir plate.
- 3 Do step 1 through step 9 in “Wash Procedure A (without Stabilization and Drying Solution)” on page 43.
- 4 Remove the slide rack from Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 and tilt the rack slightly to minimize wash buffer carry-over. Quickly transfer the slide rack to slide-staining dish #4 containing acetonitrile, and stir at 350 rpm for 10 seconds.
- 5 Transfer slide rack to slide-staining dish #5 filled with Stabilization and Drying Solution, and stir at 350 rpm for 30 seconds.

- 6** Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.
- 7** Discard used Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2.

NOTE

The acetonitrile and the Stabilization and Drying Solution may be reused for washing of up to four batches of five slides (that is, total 20 microarray slides) in one experiment. Pour the Stabilization and Drying Solution to a different marked bottle, and protect from light with other flammables. After each use, rinse the slide rack and the slide-staining dish that were in contact with the Stabilization and Drying Solution with acetonitrile followed by a rinse in Milli-Q ultrapure water.

- 8** Repeat [step 1](#) through [step 7](#) for the next group of five slides using fresh Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 prewarmed to 37°C.
- 9** Dispose of acetonitrile and Stabilization and Drying Solution as flammable solvents.

Step 5. Put slides in a slide holder

Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in the original slide boxes in a N₂ purge box, in the dark.

For SureScan microarray scanner

- 1** Carefully place the end of the slide without the barcode label onto the slide ledge.
- 2** Gently lower the microarray slide into the slide holder. Make sure that the active microarray surface faces up, toward the slide cover.
- 3** Close the plastic slide cover, pushing on the tab end until you hear it click.

For more detailed instruction, refer to the *Agilent G4900DA SureScan Microarray Scanner System User Guide*.

5 Microarray Processing

Step 5. Put slides in a slide holder



Figure 3 Slide in slide holder for SureScan microarray scanner

For Agilent Scanner C

- In environments in which the ozone level exceeds 5 ppb, immediately put the slides with Agilent barcode facing up in a slide holder. Make sure that the slide is not caught up on any corner. Put an ozone-barrier slide cover on top of the array as shown in [Figure 4](#). Refer to the *Agilent Ozone-Barrier Slide Cover User Guide* (p/n G2505-90550), included with the slide cover, for more information.

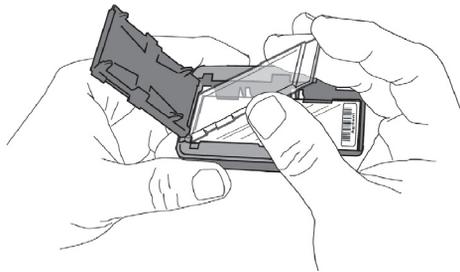


Figure 4 Inserting the ozone-barrier slide cover

- In environments in which the ozone level is below 5 ppb, put the slides with Agilent barcode facing up in a slide holder.

Microarray Scanning and Analysis

Step 1. Scan the microarray slides

An Agilent SureScan or Agilent C microarray scanner is required for the GenetiSure Pre-Screen microarrays.

Agilent SureScan Microarray Scanner

- 1 Put assembled slide holders into the scanner cassette.
- 2 Select **Protocol AgilentG3_CGH**.
- 3 Verify that the Scanner status in the main window says Scanner Ready.
- 4 Click **Start Scan**.

Agilent C Scanner Settings

- 1 Put assembled slide holders with or without the ozone-barrier slide cover into scanner carousel.
- 2 Select Start Slot *m* End Slot *n* where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.
- 3 Select **Profile AgilentG3_CGH**.
- 4 Verify scan settings. See Table 21.

Table 21 C Scanner Scan Settings

Dye channel	R+G (<i>red and green</i>)
Scan region	Agilent HD (61 x 21.6 mm)
Scan resolution	3 μm
Tiff file dynamic range	16 bit
Red PMT gain	100%
Green PMT gain	100%
XDR	<No XDR>

- 5 Check that **Output Path Browse** is set for desired location.
- 6 Verify that the Scanner status in the main window says Scanner Ready.
- 7 Click **Scan Slot m - n** on the Scan Control main window where the letter m represents the Start slot where the first slide is located and the letter n represents the End slot where the last slide is located.

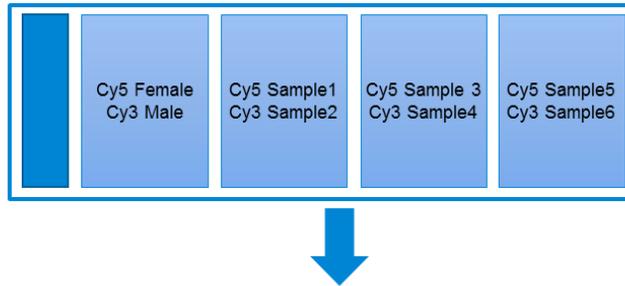
Step 2. Analyze microarray image

- After scanning is completed, load the microarray TIF images into the Agilent CytoGenomics software (version 3.0 or higher) for feature extraction and single cell analysis.

Agilent CytoGenomics software is a complete and streamlined CGH microarray data analysis solution that has Feature Extraction (FE) software built in and is able to run FE as an integral part of the analysis workflow.

Feature extraction is the process by which data is extracted from the scanned microarray image (.tif). After feature extraction of each array, Cy5- and Cy3-labeled single cell samples are compared to both female and male references that are co-hybridized to a different array on the same slide and the data are used to calculate QC metrics.

Each single cell sample will be compared to one reference with the opposite labeling (e.g. a Cy3-labeled single cell sample compared to a Cy5-labeled reference) and one reference with the same labeling (e.g. a Cy3-labeled single cell sample compared to a Cy3-labeled reference). To facilitate comparisons between single cell samples and reference samples that are labeled with the same dye, CytoGenomics transposes the signal data from Cy3-labeled single cell samples from the green channel to the red channel, while also transposing the signal data from Cy5-labeled references from the red channel to the green channel (see [Figure 5](#) for an example). The resulting log ratios are computed by Agilent CytoGenomics to identify CN changes, which are recorded in aberration reports that are saved in the Workflow Output folder in the software directory.



	Cy5 Sample1	Cy3 Sample2
Compared to Cy5 Female reference	Red channel: Cy5 Sample1	Red channel: Cy3 Sample2
	Green channel: Cy5 Female Reference (transposed from red channel)	Green channel: Cy5 Female Reference
Compared to Cy3 Male reference	Red channel: Cy5 Sample1	Red channel: Cy3 Sample2 (transposed from green channel)
	Green channel: Cy3 Male Reference	Green channel: Cy3 Male Reference

Figure 5 Example slide and resulting comparisons for two single cells samples on the slide (Sample1 and Sample2)

Microarray QC Metrics for single cell samples

These metrics are only appropriate for single cell samples analyzed with GenetiSure Pre-Screen microarrays by following the standard operational procedures provided in this user guide. The metrics can be used to assess the relative data quality from a set of single cell samples in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics, including the microarray format (4-pack or 8-pack), amplification reaction, experimental processing, scanner sensitivity, and image processing. The value guidelines presented below represent the thresholds that Agilent has observed when analyzing samples using this protocol.

To export the metrics for a single cell sample, select the sample record on the Sample Review screen, then click **QC metrics** at the bottom of the screen.

5 Microarray Processing

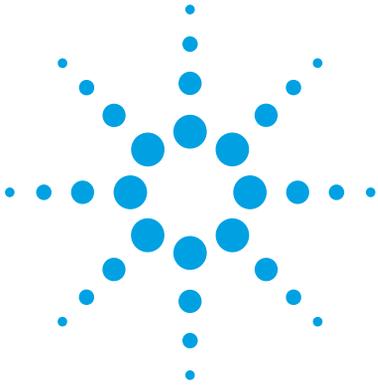
Step 2. Analyze microarray image

Table 22 QC metric thresholds for single cell samples

Metric	Excellent	Good	Evaluate
DerivativeLR_Spread	na	≤ 0.7	>0.7
gRepro	0 to 0.10	0.10 to 0.20	< 0 or >0.2
g_BGNoise	na	≤ 15	>15
g_Signal2Noise	na	≥ 10	< 10
g_SignalIntensity	na	≥ 30	< 30
rRepro	0 to 0.10	0.10 to 0.20	>0.2
r_BGNoise	na	≤ 15 or $\leq 20^*$	>15 or $>20^\dagger$
r_Signal2Noise	na	≥ 8	< 8
r_SignalIntensity	na	≥ 25	< 25

* On the Agilent SureScan microarray scanner (model G4900DA or G5761AA), the threshold is ≤ 15 . On the Agilent microarray C scanner model G2565CA, the threshold is ≤ 20 .

† On the Agilent SureScan microarray scanner (model G4900DA or G5761AA), the threshold is >15 . On the Agilent microarray C scanner model G2565CA, the threshold is >20 .



6 Troubleshooting

- If the whole genome amplification fails 54
- If the labeling efficiencies for Cy3 and Cy5 are dissimilar (i.e. the Cy3/Cy5 paired sample is not purple) 55
- If you have post-labeling signal loss 56
- If you have high BGNoise values 57
- If you have poor reproducibility 58

This chapter contains potential causes for an array failure.



If the whole genome amplification fails

If you have low post-amplification yield, as determined using the Qubit dsDNA BR kit, the whole genome amplification may have been inefficient.

- ✓ Do not mix solutions containing single cells by pipetting up and down, as this may cause the cells to adhere to the pipette tip. Instead, mix the samples by flicking the tubes.
- ✓ When preparing cells for lysis (after adding the denaturation buffer), avoid vigorous mixing, as this may shear the DNA.
- ✓ After cell lysis, proceed immediately to the next step of the protocol (the addition of the Stop Solution, followed by amplification).
- ✓ Make sure that the cell lysis and amplification incubations are performed at the correct temperatures. Use a thermal cycler with a heated lid that is set to at least 70°C.
- ✓ To avoid degradation of the DNA within the single cells, do not store the cells for extended periods of time, and make sure that the cells are always stored at the appropriate temperature.
- ✓ Do not use reconstituted Buffer DLB that has been stored at -20°C for longer than 6 months. After 6 months, prepare new aliquots of the reconstituted Buffer DLB.

If you have high concentration values for the negative control, as determined using the Qubit dsDNA BR kit, contamination was introduced during the procedure.

- ✓ The cells and amplified DNA might be compromised. Start fresh with new cell samples and reagents.

If the labeling efficiencies for Cy3 and Cy5 are dissimilar (i.e. the Cy3/Cy5 paired sample is not purple)

If the labeling efficiencies for Cy3 and Cy5 are dissimilar (i.e. the Cy3/Cy5 paired sample is not purple)

After you pair the Cy3- and Cy5-labeled samples, the paired sample should be purple in color. A paired sample that is too pink indicates inefficient Cy5 labeling. A paired sample that is too blue indicates inefficient Cy3 labeling. Inefficient labeling can result from sub-optimal whole genome amplification and labeling conditions such as too many freeze thaws of the buffers or Cyanine dUTP, enzyme degradation due to being left warm for too long, wrong temperatures or times, volume mistakes, or too much exposure of the dyes to light or air.

- ✓ Inefficient labeling can be caused by sub-optimal whole genome amplification. See the troubleshooting suggestions in “If the whole genome amplification fails” on page 54.
- ✓ Keep amplification and labeling enzymes on ice while setting up reactions, and return them to -20°C as quickly as possible. Make sure to store Cyanine dUTP at -20°C .
- ✓ Double check incubation times and temperatures (use a calibrated thermometer), and use a thermal cycler with heated lid.
- ✓ Evaporation can be a problem when you process samples at high temperatures. Make sure that sample tubes are well closed or use a plate heat sealer to avoid evaporation.
- ✓ Make sure that the pipettes are not out of calibration.
- ✓ Make sure that the reagents and master mixes are well mixed. Tap the tube with your finger or use a pipette to move the entire volume up and down. Then spin in a microcentrifuge for 5–10 seconds to drive the contents off the walls and lid. Do not mix the stock solutions and reactions that contain amplified DNA or enzymes on a vortex mixer.

If you have post-labeling signal loss

Signal loss can be due to wash or hybridization conditions that are too stringent, or degradation of the cyanine-5 signal.

Cyanine-5 signal degradation can be caused by ozone or NO_x compounds coming from pollution and/or compressors and centrifuges. Cyanine-5 signal degradation can result in less red signal around the edges of the features, a visible gradient of red intensity (especially on the left side of the slide and on slides scanned later in a batch), and poor red reproducibility of the cyanine 5-labeled single cell samples and green reproducibility of the cyanine 5-labeled reference.

- ✓ Check that the oven temperature is 67°C. If needed, recalibrate the hybridization oven. Follow the steps in *Agilent G2545A Hybridization Calibration Procedure* (p/n G2545-90002).
- ✓ Check that the temperature of Wash 2 is 37°C.
- ✓ Check that Wash 2 was not accidentally used instead of Wash 1.
- ✓ Wash and scan slides in an ozone controlled environment (<5 ppb), such as an ozone tent.
- ✓ Use small batches that can be washed and scanned in about 40 minutes to minimize exposure to air.
- ✓ For Agilent Scanner C, use the Agilent Ozone-Barrier Slide Cover (p/n G2505-60550). The SureScan scanner has built-in ozone protection.
- ✓ Use the Stabilization and Drying Solution* as described in “Wash Procedure B (with Stabilization and Drying Solution)” on page 45.

If you have high BGNoise values

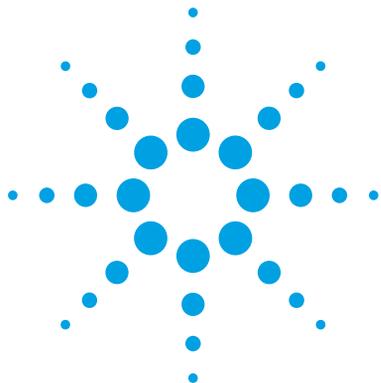
High BGNoise can cause lower signal-to-noise values (see Table 42 for thresholds) and higher DLRSD values. BGNoise is defined as the standard deviation of the signals on the negative controls. If the BGNoise is high, examine the microarray image for visible non-uniformities. High BGNoise is often introduced during hybridization steps or washes.

- ✓ Make sure that the oven is calibrated. Follow the steps in *Agilent G2545A Hybridization Calibration Procedure* (p/n G2545-90002).
Sample hybridization at incorrect temperatures affects the stringency of the hybridization.
- ✓ Make sure that wash dishes, racks and stir bars are clean. Do not use tap water or detergents to clean wash equipment. If needed, rinse wash equipment with 2-Propanol (for equipment that was not exposed to Stabilization and Drying Solution) or acetonitrile (for equipment that was exposed to Stabilization and Drying Solution) followed by rinses with MilliQ water.
- ✓ If high background is observed, perform an additional acetonitrile wash of the slides and then rescan:
 - 1 In the fume hood, fill a slide-staining dish approximately three-fourths full with acetonitrile.
 - 2 Add a magnetic stir bar and put this dish on a magnetic stir plate.
 - 3 Put the slides in a slide rack and transfer the slide rack to the slide-staining dish containing acetonitrile, and stir at 350 rpm for 1 minute.
 - 4 Slowly remove the slide rack and scan the slides immediately.

If you have poor reproducibility

Poor reproducibility (see [Table 42](#) for thresholds), defined as high CVs of signals of replicated probes may indicate that the hybridization volume was too low or that the oven stopped rotating during the hybridization. Only very high scores on this metric will affect the DLRSD.

- ✓ Take care when setting up the gasket-slide hybridization sandwich. For 4×180K arrays, dispense the hybridization sample mixture slowly in a “drag and dispense” manner to prevent spills. For 8×60K arrays, dispense the hybridization sample mixture onto the center of the gasket slide so as to avoid contact between the solution and the rubber gasket edges.
- ✓ Make sure to hand-tighten the screw of the hybridization chamber as much as possible.
- ✓ Check that the oven is rotating.



7 Reference

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This chapter contains reference information that pertains to this protocol.



Reagent Kit Components

The contents of the Agilent reagent kits used in this protocol are listed here.

Table 23 GenetiSure Pre-Screen Amplification and Labeling Kit, p/n 5190-7731

Component
Nuclease-Free Water
PBS, pH 7.5
Exo (-) Klenow
5× Reaction Buffer
Cyanine 5-dUTP
Cyanine 3-dUTP
10× dNTP Mix
Random Primers
Buffer DLB
DTT, 1 M
Stop Solution
Amplification Reaction Buffer
Amplification DNA Polymerase
Post Labeling Purification Columns
Post Labeling Purification Collection Tubes

Table 24 Oligo aCGH/ChIP-on-chip Hybridization Kit, p/n 5188-5220 (25 slides) or 5188-5380 (100 slides)

Component
2× HI-RPM Hybridization Buffer
10× aCGH Blocking Agent

Table 25 Oligo aCGH/ChIP-on-chip Wash Buffer Kit, Agilent p/n 5188-5226

Component
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2

Microarray Handling Tips

Each microarray is printed on the side of the glass slide containing the “Agilent”-labeled barcode. This side is called the “active” side. The numeric barcode is on the inactive side of the slide.

CAUTION

You must familiarize yourself with the assembly and disassembly instructions for use with the Agilent Microarray Hybridization Chamber (G2534A) and gasket slides. Practice slide kits are available.

In this “processing and hybridization” procedure, the hybridization mixture is applied directly to the gasket slide, and not to the active side of the oligo microarray. Instead, the active side of the oligo microarray is placed on top of the gasket slide to form a “sandwich slide” pair.

To avoid damaging the microarray, always handle glass slides carefully by their edges. Wear powder-free gloves. Never touch the surfaces of the slides. If you do, you may cause irreparable damage to the microarray.

Never allow the microarray surface to dry out during the hybridization process and washing steps.

Agilent Microarray Layout and Orientation

Agilent oligo microarray (1 microarray/slide format) as imaged on the Agilent microarray scanner

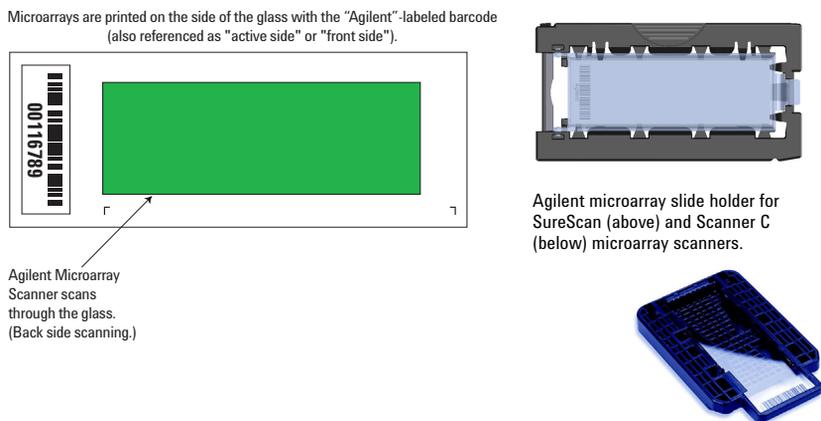


Figure 6 Agilent microarray slide and slide holder

Agilent oligo microarrays formats and the resulting "microarray design files" are based on how the Agilent microarray scanner images 1-inch \times 3-inch glass slides. Agilent designed its microarray scanner to scan through the glass slide (back side scanning). The glass slide is securely placed in an Agilent microarray slide holder with the "Agilent" labeled barcode facing the opening of the slide holder (on SureScan Microarray Scanner) or facing the inside of the slide holder (Scanner C). In this orientation, the "active side" containing the microarrays is protected from potential damage by fingerprints and other elements. Once securely placed, the numeric barcode, non-active side of the slide, is visible from the outside of the slide holder.

Figure 6 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the "microarray design files" that Agilent generates during the manufacturing process of its *in situ*-synthesized oligonucleotide microarrays.

Array/Sample tracking on microarray slides

Use the forms below to make notes to track your samples on microarray slides.

Position the gasket slide in the SureHyb chamber base with the label to the left and load the samples: top row, left to right, then lower row, left to right. The array suffix assignments from Feature Extraction will then occur in the order shown.

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
B	Sample:	Sample:	Sample:	Sample:
A				
R				
C				
O				
D				
E				

Barcode Number _____

Figure 7 4-pack microarray slides

7 Reference

Array/Sample tracking on microarray slides

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
B A R C O D E	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>
	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>	Sample: <hr/> <hr/> <hr/>

Array 2_1

Array 2_2

Array 2_3

Array 2_4

Barcode Number _____

Figure 8 8-pack microarray slide

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In This Book

This guide contains information to run the GenetiSure Pre-Screen Kit – Whole Genome Amplification, Labeling, and CGH Microarray Hybridization protocol.

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