

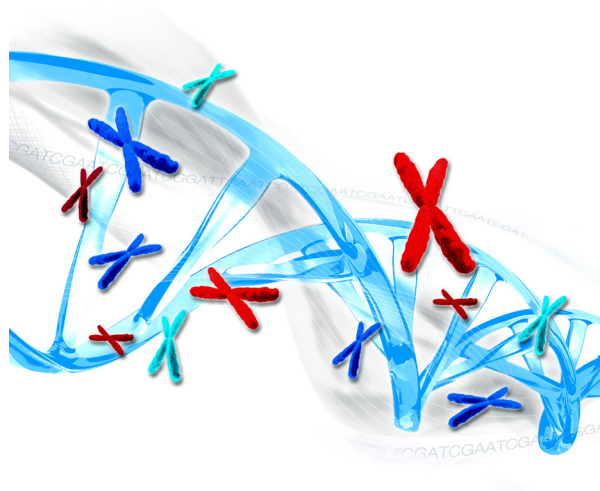
GenetiSure Pre-Screen Kit for Single Cell Analysis

Whole Genome Amplification with the REPLI-g Single Cell Kit,
Fluorescent Labeling, and CGH Microarray Hybridization

Protocol

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

Revision D0



Notices

Manual Part Number

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In This Guide...

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

1 Before You Begin

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.

2 Sample Amplification

This chapter describes the protocol to amplify DNA from single cell samples alongside samples of reference DNA (male and female). For each slide, one male and one female reference sample is amplified along with the single cells. The reference DNA samples are provided with the GenetiSure Pre-Screen Kits (P/N G9500A and G9501A) and the GenetiSure Pre-Screen Labeling Kit (P/N G9502A).

3 Sample Labeling

This chapter describes the steps to differentially label the amplified DNA samples with fluorescent-labeled nucleotides using reagents from the SureTag DNA Labeling Kit that is included in the GenetiSure Pre-Screen Kit.

4 Microarray Processing

This chapter describes the steps for microarray processing, which consists of hybridization, washing, and scanning.

5 Troubleshooting

This chapter contains potential causes for an array failure.

6 Reference

This chapter contains reference information that pertains to this protocol.

What's new in Version D0

- Correction to Human Reference DNA storage temperatures for P/N G9502A (see **Table 1** on page 10).
- Addition of Cot-1 DNA to list of sub-kits provided with P/N G9500A and G9501A (see **Table 1** on page 10).
- Updated "**Safety Notes**" on page 9.

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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

- Do not use a pipette to transfer or mix single cell samples until the whole genome amplification is complete as cells may adhere to the pipette tip.
- 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.

NOTE

If you are using this product for embryo screening please make sure you adhere to your country specific laws and regulations for human assisted reproductive technologies. Your country might have banned sex selection for non-medical purposes, as well as the commercial use of gametes, zygotes, and embryos. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Safety Notes

WARNING

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

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

Materials Provided with the GenetiSure Pre-Screen Kits

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

Table 1 Agilent GenetiSure Pre-Screen Kits

Kit P/N and Name	Sub-Kits	Quantity Provided	Storage Conditions
P/N G9500A GenetiSure Pre-Screen Kit, 8x60K, 48 Reactions	REPLI-g Single Cell Kit*, P/N 5191-4065	Sufficient reagents for whole genome amplification of 42 experimental samples and 6 reference samples	Store at -20°C
	GenetiSure Pre-Screen Microarray Kit, 8x60K, P/N G5963-60510	Three glass microarray slides each formatted with eight 60K arrays	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.
	GenetiSure Pre-Screen Purification Columns, P/N 5190-7730	25 columns and 50 collection tubes	Store at room temperature
	Human Reference DNA, Female, P/N 5190-3797	25 µg	Store at 4°C
	Human Reference DNA, Male, P/N 5190-3796	25 µg	Store at 4°C
	Human Cot-1 DNA, P/N 5190-3393	625 µL (1.0 mg/mL)	Store at -20°C
	SureTag DNA Labeling Kit, -20°C Components**, P/N 5190-3399	Sufficient reagents for 50 labeling reactions.	Store at -20°C
	Hybridization Chamber Gasket Slide Kit, P/N G2534-60018	Three gasket slides, 8 gaskets per slide	Store at room temperature

Before You Begin

Materials Provided with the GenetiSure Pre-Screen Kits

Table 1 Agilent GenetiSure Pre-Screen Kits (continued)

Kit P/N and Name	Sub-Kits	Quantity Provided	Storage Conditions
P/N G9501A GenetiSure Pre-Screen Kit, 4x180K, 48 Reactions	REPLI-g Single Cell Kit*, P/N 5191-4065	Sufficient reagents for whole genome amplification of 36 experimental samples and 12 reference samples	Store at –20°C
	GenetiSure Pre-Screen Microarray Kit, 4x180K, P/N G5962-60510	Six glass microarray slides each formatted with four 180K arrays	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.
	GenetiSure Pre-Screen Purification Columns, P/N 5190-7730	25 columns and 50 collection tubes	Store at room temperature
	Human Reference DNA, Female, P/N 5190-3797	25 µg	Store at 4°C
	Human Reference DNA, Male, P/N 5190-3796	25 µg	Store at 4°C
	Human Cot-1 DNA, P/N 5190-3393	625 µL (1.0 mg/mL)	Store at –20°C
	SureTag DNA Labeling Kit, –20°C Components**, P/N 5190-3399	Sufficient reagents for 50 labeling reactions	Store at –20°C
	Hybridization Chamber Gasket Slide Kit, P/N G2534-60018	Six gasket slides, 4 gaskets per slide	Store at room temperature
P/N G9502A GenetiSure Pre-Screen Labeling Kit, 48 Reactions	REPLI-g Single Cell Kit*, P/N 5191-4065	Sufficient reagents for whole genome amplification of 36 experimental samples and 12 reference samples	Store at –20°C
	GenetiSure Pre-Screen Purification Columns, P/N 5190-7730	Sufficient columns and tubes for 48 purification reactions	Store at room temperature
	Human Reference DNA, Female, P/N 5190-3797	25 µg	Store at 4°C
	Human Reference DNA, Male, P/N 5190-3796	25 µg	Store at 4°C
	SureTag DNA Labeling Kit, –20°C Components**, P/N 5190-3399	Sufficient reagents for 50 labeling reactions	Store at –20°C

* The full list of components provided in the REPLI-g Single Cell Kit is available in [Table 23](#) on page 62.

** The full list of components provided in the SureTag DNA Labeling Kit, –20°C Components, is available in [Table 24](#) on page 62.

Materials Required But Not Provided

Table 2, **Table 3**, and **Table 4** list the materials that are required for the whole genome amplification, labeling, and CGH microarray hybridization protocol, but are not provided in a GenetiSure Pre-Screen Kit.

Table 2 Required reagents

Description	Vendor and part number
1× TE (pH 8.0), Molecular grade	Promega p/n V6231 or equivalent
1× Phosphate Buffered Saline (pH 7.4)	Thermo Fisher Scientific p/n 10010-023 or equivalent
Qubit dsDNA BR Assay Kit, for use with the Qubit fluorometer, 100 assays (Optional. Used for quantitation of amplified DNA on Qubit Fluorometer.)	Thermo Fisher Scientific p/n Q32850
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)
DNase/RNase-free distilled water	Thermo Fisher Scientific p/n 10977-015 or equivalent
Milli-Q ultrapure water	Millipore
Acetonitrile*	Sigma-Aldrich p/n 271004-1L or equivalent
70% 2-Propanol (molecular biology grade)	Sigma-Aldrich p/n 563935-4L or equivalent

* Only needed if wash procedure B is selected.

Table 3 Required equipment

Description	Vendor and part number
Thermal cycler with heated lid	Agilent p/n G8800A or equivalent
96-well PCR plates, centrifuge for 96-well plates, and thermal plate sealer (Optional. Can substitute with 200- μ L PCR tubes.)	Plates: Agilent p/n 401334, or equivalent Centrifuge: Eppendorf p/n 5810, or equivalent Plate sealer: Agilent p/n G5402A/G, or equivalent
200- μ L PCR 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)

Before You Begin

Materials Required But Not Provided

Table 3 Required equipment (continued)

Description	Vendor and part number
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 (×1 or ×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-µL tubes and 1.5-mL tubes (and 500-µL tubes if single cells were collected in these tubes)	Eppendorf p/n 5430 or equivalent
Qubit Fluorometer (Optional. Used for quantitation of amplified DNA on Qubit Fluorometer.)	Thermo Fisher Scientific p/n Q32857
Thin wall, clear 0.5 mL PCR tubes (Optional. Used for quantitation of amplified DNA on Qubit Fluorometer.)	Thermo Fisher Scientific p/n Q32856 or VWR p/n 10011-830
Sterile storage bottle	Nalgene 455-1000 or equivalent
Inverted microscope, magnification 10 × 10, and denudation pipette (Optional. Only needed if single cell sample must be collected from a 4-well dish or petri dish.)	Microscope: 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 × 38.1 mm (×2 or ×4) [†]	VWR p/n 58948-150 or equivalent
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 or 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	

Before You Begin

Materials Required But Not Provided

Table 3 Required equipment (continued)

Description	Vendor and part number
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.

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

Table 4 Required software

Description	Vendor and part number
Agilent Microarray Scan Control program	Agilent Technologies The Agilent Microarray Scan Control program is included with the Agilent SureScan Microarray Scanner.
Agilent CytoGenomics software, version 3.0 or later	Agilent Technologies The CytoGenomics software can be downloaded free-of-charge from the Agilent website (www.agilent.com/en/download-agilent-cytogenomics-software). This website also contains a link for requesting the necessary software license.

- 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 www.agilent.com to download the manuals.
- You can download the design files needed for data extraction and analysis in CytoGenomics (version 3.0 or later) from the Agilent SureDesign website. Go to 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 copy number variants (CNVs).

GenetiSure Pre-Screen WGA workflow

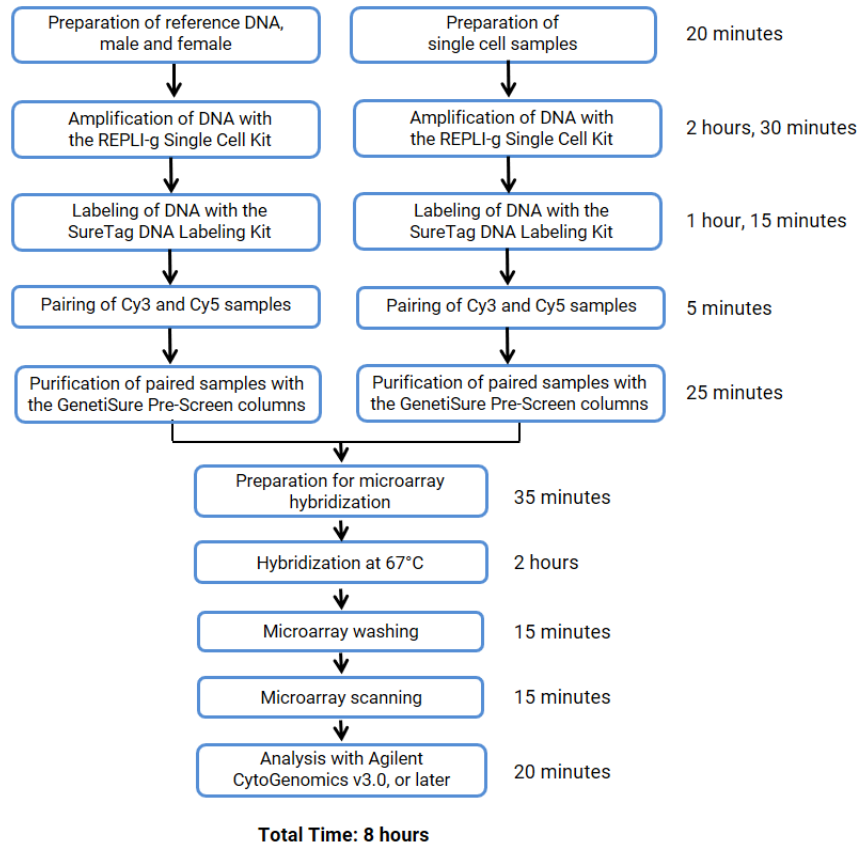


Figure 1. Overview of the GenetiSure Pre-Screen workflow for single cell analysis

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Sample Amplification

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This chapter describes the protocol to amplify DNA from single cell samples alongside samples of reference DNA (male and female). For each slide, one male and one female reference sample is amplified along with the single cells. The reference DNA samples are provided with the GenetiSure Pre-Screen Kits (P/N G9500A and G9501A) and the GenetiSure Pre-Screen Labeling Kit (P/N G9502A).

The single cell samples can be any of the following:

- Single cell collected from a day-3 human embryo biopsy
- Cells (3–10 cells total) collected from a day-5 human embryo biopsy
- Single cell sample (1–2 cells) of tissue-culture cells
- Small tissue-culture sample consisting of 5–15 cells
- Multi-cell tissue-culture sample consisting of 15–50 cells or more

Step 1. Preparation of Samples

This section describes the preparation of the male and female reference DNA samples and the single cell samples.

- 1 Prepare the reference DNA samples. Perform these steps for both the **Human Reference DNA, Female, P/N 5190-3797** and **Human Reference DNA, Male, P/N 5190-3796** that are provided in the GenetiSure Pre-Screen Kit.
 - a In a fresh 200- μ L PCR tube, combine 2 μ L of Human Reference DNA with 104.6 μ L of PBS. Mix briefly on a vortex mixer.
 - b Transfer 4 μ L of the diluted Human Reference DNA into a fresh PCR tube. Keep on ice until required.
- 2 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, petri dish, or other similar culture vessel, use a denudation pipette under the microscope to transfer the cell from the dish into a PCR tube containing 3 μ L of PBS (for a total volume of approximately 4 μ L).

Step 2. Whole Genome Amplification

This section describes the Agilent recommended procedure to amplify DNA from single cells and male and female reference DNA using the REPLI-g Single Cell Kit.

- 1 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.
- 2 Prepare Buffer D2 (denaturation buffer).
 - a Mix the components listed in **Table 5**.

Sample Amplification

Step 2. Whole Genome Amplification

Table 5 Preparation of Buffer D2

Component	Volume (μL)	×8 reactions (μL) including excess	×16 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

- 3 Add Buffer D2 to the samples (single cell samples and reference samples).
 - a Briefly spin all sample tubes in a microcentrifuge.
 - b Add 3 μL of Buffer D2 to each sample tube for a total volume of 7 μL.
 - c Mix carefully by flicking the tubes. *Do not mix on a vortex mixer or by pipetting up and down.*
 - d Briefly spin all sample tubes in a microcentrifuge then transfer to ice.
- 4 Set the thermal cycler (with heated lid) to run the program listed in **Table 6**.

Table 6 Incubation protocol to lyse the cells

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

- 5 Transfer the sample tubes to the thermal cycler, and then start the program.

NOTE

If your samples are in 500-μL tubes, and your thermal cycler does not accommodate tubes of this size, use a 65°C water bath for step 1 then transfer the samples to an ice bath.

- 6 At the end of the thermal cycler program, remove the tubes from the thermal cycler. Briefly spin them in a microcentrifuge, then transfer to ice.
- 7 Without allowing the pipette tip to contact the sample, add 3 μL of Stop Solution to each sample for a total volume of 10 μL. Mix carefully by flicking the tubes. Briefly spin them in a microcentrifuge, then transfer to ice.
- 8 Prepare the **Amplification Master Mix** by mixing the components in **Table 7** on ice. Mix the components in the order listed in the table, and briefly vortex the master mix *before* adding the Amplification DNA Polymerase. Once you add the Amplification DNA Polymerase, mix the Amplification Master Mix by flicking the tube or pipetting up and down. Then, immediately proceed to

Sample Amplification

Step 2. Whole Genome Amplification

step 9. The components are included in the REPLI-g Single Cell Kit that is included in the GenetiSure Pre-Screen Kit.

NOTE

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

The Amplification Reaction Buffer may form a precipitate after thawing. Mixing it on a vortex mixer for 10 seconds dissolves the precipitate.

Table 7 Amplification Master Mix

Component	Volume (µL) per reaction	x8 reactions (µL) including excess	x16 reactions (µL) including excess
Nuclease-Free Water	9	76.5	153
Amplification Reaction Buffer	29	246.5	493
Amplification DNA Polymerase	2	17	34
Final volume of Amplification Master Mix	40	340	680

9 Without allowing the pipette tip to contact the sample, add 40 µL of **Amplification Master Mix** to each 10 µL sample for a total volume of 50 µL per sample. Mix carefully by flicking the tubes. Briefly spin the tubes in a microcentrifuge, then transfer to ice.

10 Set the thermal cycler (with heated lid) to run the program listed in **Table 8**.

Table 8 Incubation protocol for whole genome amplification

Step	Temperature	Duration
Step 1	30°C	120 minutes
Step 2	65°C	3 minutes
Step 3	4°C	hold until ready to proceed

11 Transfer the sample tubes to the thermal cycler, and then start the program.

12 At the end of the thermal cycler program, remove the tubes from the thermal cycler. Briefly spin them in a microcentrifuge, then transfer to ice.

Step 3. Quantitation of Amplified DNA using Qubit Fluorometer

Use Quant-iT dsDNA Broad-Range Assay Kit to measure the concentration of amplified DNA.

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 and the amplified DNA samples that you are processing).
- 2 Make a Qubit working solution. For each standard and amplified DNA sample to be quantified, mix the components in **Table 9**.

Table 9 Qubit Working Solution

Component	Volume
Qubit dsDNA BR reagent	1 μ L
Qubit dsDNA BR buffer	199 μ L

- 3 Add 190 μ L of Qubit working solution to the two 0.5-mL tubes labeled for the standards.
- 4 Add 199 μ L of Qubit working solution to the 0.5-mL tubes labeled for your amplified DNA samples.
- 5 Add 10 μ L of Qubit dsDNA BR standard #1 to the tube labeled for standard #1, and add 10 μ L of Qubit dsDNA BR standard #2 to the tube labeled for standard #2.
- 6 Add 1 μ L 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, 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.

Sample Amplification

Step 3. Quantitation of Amplified DNA using Qubit Fluorometer

- 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.

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

$$QF \text{ value} = \text{the value given by the Qubit Fluorometer}$$

The expected concentration for all samples of amplified DNA is $\geq 300 \text{ ng}/\mu\text{L}$.

NOTE

If all of your samples, including the reference samples, have a low concentration (i.e., lower than $300 \text{ ng}/\mu\text{L}$) then the whole genome amplification failed and Agilent does not recommend proceeding to sample labeling. If some, but not all, of the samples have a low concentration, then Agilent recommends repeating the whole genome amplification of those samples, if possible.

Maintain the samples at 4°C for short-term storage (up to 3 days), or store at -20°C for long-term storage (up to 1 year) until ready for labeling. When ready, proceed to **Chapter 3**, "Sample Labeling."

3

Sample Labeling

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Step 2. Purification of labeled DNA 26

This chapter describes the steps to differentially label the amplified DNA samples with fluorescent-labeled nucleotides using reagents from the SureTag DNA Labeling Kit that is included in the GenetiSure Pre-Screen Kit.

The procedure uses random primers and the *exo(-)* Klenow fragment to differentially label amplified DNA samples with fluorescent-labeled nucleotides. For your single cell samples, you differentially label the amplified DNA with cyanine-3 (Cy3) and cyanine-5 (Cy5) dyes, and then combine the samples in Cy3-Cy5 pairs. You also differentially label the amplified DNA from your male and female reference samples with Cy3 and Cy5 dyes and combine as a Cy3-Cy5 pair.

CAUTION

Each array on the microarray slide needs to be hybridized with a Cy3-Cy5 pair. If you have an odd number of single cell samples, you need to label an additional reference sample with the appropriate dye in order to pair it with the odd single cell sample. If you have an insufficient number of samples to fill all of the arrays on a 4×180K or 8×60K slide, label additional reference samples with Cy3 and Cy5 in order to create enough pairs to fill the empty arrays.

Step 1. Fluorescent Labeling of DNA

NOTE

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 microcentrifuge for 1 minute at $5,000 \times g$.
- 2 Transfer 13 μL of each sample into a new PCR tube or into a well of a 96-well PCR plate.

NOTE

Each array on the microarray slide needs to be hybridized with a Cy3-Cy5 pair. If you have an odd number of single cell samples, you need to set up an additional tube for a second male reference sample and label it with the appropriate dye in order to pair it with the odd single cell sample. Pairing of Cy3- and Cy5-labeled single cells samples is described in [step 12](#).

Similarly, if you have an insufficient number of samples to fill all of the arrays on a 4x180K or 8x60K slide, set up additional tubes of references samples and label them with Cy3 and Cy5 in order to create enough Cy3-Cy5 pairs to fill the empty arrays.

- 3 Add 2.5 μL of **Random Primers** to each PCR 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. Briefly spin the tubes in a microcentrifuge, then transfer to ice.
- 4 Set the thermal cycler (with heated lid) to run the program listed in [Table 10](#).

Table 10 Incubation protocol for random primer annealing

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

- 5 Transfer the PCR tubes to the thermal cycler, and then start the program.

Sample Labeling

Step 1. Fluorescent Labeling of DNA

- 6 At the end of the thermal cycler program, remove the tubes from the thermal cycler. Briefly spin them in a microcentrifuge, then transfer to ice.
- 7 Mix the components in **Table 11** on ice in the order indicated to prepare one cyanine-3 (Cy3) and one cyanine-5 (Cy5) Labeling Master Mix. The components are included in the SureTag DNA Labeling Kit that is included in the GenetiSure Pre-Screen Kit.

Table 11 Labeling Master Mix

Component	Per reaction (μL)	× 4 reactions (μL) (including excess)	× 8 reactions (μL) (including excess)
5× gDNA Reaction Buffer	5.0	22.5	42.5
10× dNTP Mix	2.5	11.25	21.25
Cyanine 3-dUTP or 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

- 8 Add 9.5 μL of **Labeling Master Mix** to each sample tube that contains the amplified DNA to make a total volume of 25 μL. Mix well by gently pipetting up and down. Briefly spin them in a microcentrifuge, then transfer to ice.
 - 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 single cell 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 single cell samples, use the second male reference sample to create an even number.
- 9 Set the thermal cycler (with heated lid) to run the program listed in **Table 12**.

Table 12 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

- 10 Transfer the sample tubes to the thermal cycler, and then start the program.

Sample Labeling

Step 2. Purification of labeled DNA

- 11 At the end of the thermal cycler program, remove the tubes from the thermal cycler. Briefly spin them in a microcentrifuge, then transfer to ice.
- 12 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 single cell 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 single cell 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 GenetiSure Pre-Screen Purification Columns and Collection Tubes provided with the GenetiSure Pre-Screen Kit.

- 1 Spin the Cy3-Cy5 paired DNA samples in a centrifuge for 1 minute at $5,000 \times g$, then transfer each 50- μL sample into a fresh 1.5-mL tube.
- 2 Add 430 μL of molecular grade $1\times$ TE (pH 8.0) to each paired DNA sample.
- 3 For each paired DNA sample to be purified, label a purification column and place it into a 1.5-mL collection tube. Load each paired DNA sample onto the appropriate column.
- 4 Cap the columns and spin for 15 minutes at $14,000 \times g$ in a microcentrifuge at room temperature. Discard the flow-through and place the columns back in the collection tubes.
- 5 Add 15 μL of $1\times$ TE (pH 8.0) to each column.
- 6 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.
- 7 Transfer 15 μL of each sample to a fresh PCR tube or into the well of a 96-well PCR plate.

These 15 μL -samples will be used for hybridizing to the GenetiSure Pre-Screen microarrays. See **Chapter 4**, "Microarray Processing."



4

Microarray Processing

Hybridization 28

Microarray Wash 40

Microarray Scanning and Analysis 49

This chapter describes the steps for microarray processing, which 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 **“Secure Fit” Slide Box Opening Instructions** on page 64 and **“Microarray Handling Tips”** on page 66.

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

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

Table 13 Hybridization Master Mix for 4-pack microarray

Component	Volume (μL) per hybridization	× 4 reactions (μL) (including excess)
1× TE (pH 8.0), Molecular grade	20	90
Human Cot-1 DNA (1.0 mg/mL)	10	45
10× aCGH Blocking Agent*	10	45
2× 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 14 Hybridization Master Mix for 8-pack microarray

Component	Volume (μL) per hybridization	× 8 reactions (μL) (including excess)
Human Cot-1 DNA (1.0 mg/mL)	5	42.5
10× aCGH Blocking Agent*	5	42.5
2× 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)

- Add the appropriate volume of the Hybridization Master Mix to each PCR tube or plate well that contains 15 μL of paired DNA to make the total volume listed in **Table 15**.

Microarray Processing

Step 2. Prepare labeled DNA for hybridization

Table 15 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

- Mix the samples by pipetting up and down, then briefly spin in a microcentrifuge.
- Set the thermal cycler to run the program listed in **Table 16**.

Table 16 Incubation protocol for preparing DNA for hybridization

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

- Transfer the PCR tubes to the thermal cycler, and then start the program.
- At the end of the thermal cycler program, remove the tubes from the thermal cycler. Briefly spin them in a microcentrifuge and proceed to the hybridization steps.

CAUTION

The samples must be hybridized as quickly as possible. 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.

Before you begin, make sure you read and understand **“Secure Fit” Slide Box Opening Instructions** on page 64 and **“Microarray Handling Tips”** on page 66.

Remove gasket slide from its packaging

NOTE

- Do not remove gasket slide from protective sleeve until ready for use.
- Do not slice or cut open the gasket slide protective packaging.
- Handle only the edges of the gasket slide.
- Prior to use, inspect gasket slides for visible gaps or cuts through the gaskets or any debris within the hybridization areas as these are indications of instability. Do not use gasket slides that have these features.

- 1 With tweezers, carefully lift up the corner of the clear plastic covering and slowly pull back the protective film.



Figure 2. Removal of clear plastic covering

- 2 With clean, powder-free gloved fingers, remove the gasket slide from its package. Handle the slide only on its edges.

Microarray Processing

Step 3. Prepare the hybridization assembly

To avoid any potential contamination from surrounding surface materials, immediately insert the gasket slide in the chamber base using the instructions below.

Insert the gasket slide into the chamber base

- 1 Hold the gasket slide so that the barcode label is facing towards you. This side of the slide is the gasket side.

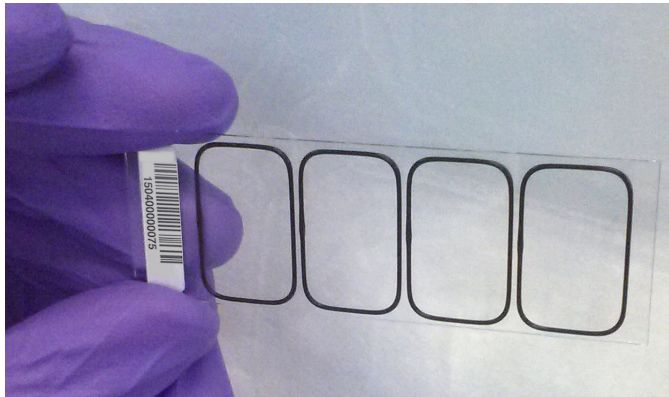


Figure 3. Gasket slide, gasket side

- 2 Locate the four chamber base guideposts and rectangular barcode guide in the chamber base.
- 3 Position the gasket slide between the 4 chamber base guide posts (see **Figure 4**) with the barcode label resting over the base's rectangular barcode guide.

Microarray Processing

Step 3. Prepare the hybridization assembly

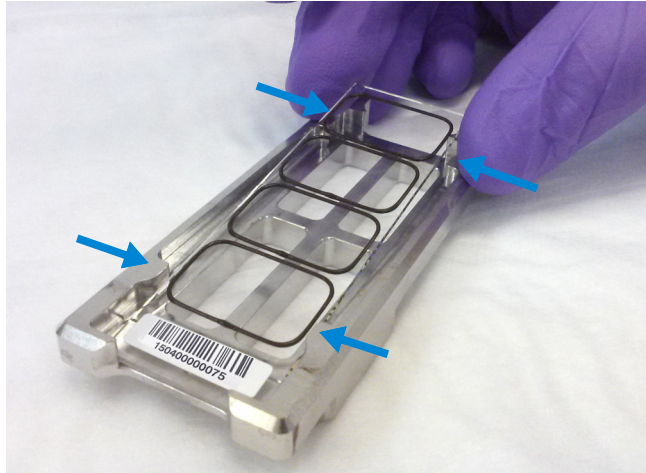


Figure 4. Chamber base, guide posts denoted with arrows

- 4 Gently place the gasket slide into the chamber base.
- 5 Make sure the gasket slide rests flush against the chamber base. Re-adjust to a flush position against the chamber base if needed.



Slide and gasket are flush

Figure 5. Correct positioning of gasket slide in chamber base

Load the sample

- 1 Slowly dispense hybridization sample mixture onto the gasket well. Load all gasket wells before you add the microarray slide.
 - For 4-pack microarrays, slowly dispense 100 μL of the mixture in a “drag and dispense” manner (described below).
 - For 8-pack microarrays, slowly dispense 50 μL of the mixture onto the center of the gasket slide so as to avoid contact between the mixture and the rubber gasket edges

The “drag and dispense” method helps to distribute the sample evenly across the surface of the well and avoids spillover of sample over the gasket edge. Start with the pipette tip near the top edge of the well. *Do not directly touch the gasket or the glass with the pipette tip.* Then, dispense the mixture while you move your pipette tip to the opposite end of the well so that the sample is distributed across the well space. Avoid creating large air bubbles as you dispense the mixture as they could lead to spillover.



This image is for demonstration purposes only. Always put the gasket slide in the chamber base before you dispense the hybridization sample mixture.

Figure 6. Drag and dispense method – Start dispensing when the pipette tip is near the top of the well. Finish dispensing when the pipette tip is near the bottom of the well.

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.

Add the microarray slide

- 1 Remove a microarray slide from the storage box between your thumb and index finger, *numeric barcode side facing up and Agilent label facing down.*
- 2 Use the four chamber base guideposts and rectangular end of the base to position the microarray slide as you lower it to within 3 mm (1/8”) above the gasket slide, making sure the microarray slide is not tilted with respect to the gasket slide. Barcode ends of both the gasket slide and the microarray slide must line up at the corners of the chamber base. Once positioned, gently rest the microarray slide on the lower gasket slide. Refer to **Figure 7** for proper technique on holding the microarray slide with both hands.

Microarray Processing

Step 3. Prepare the hybridization assembly

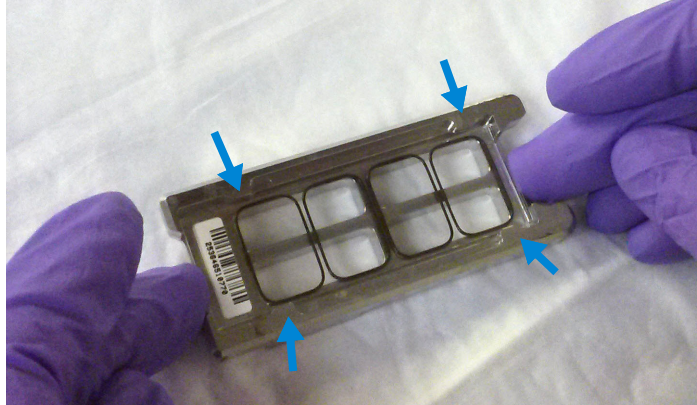


Figure 7. Chamber base with gasket and microarray slide applied, guide posts denoted with arrows

CAUTION

Do not drop the microarray slide onto the gasket slide as this increases the chances of sample mixing between gasket wells.

Once placed, do not attempt to move the chamber and sandwiched slides as this can cause leakage of the hybridization solution.

Assemble the chamber

- 1 Place the chamber cover, correct side facing up, onto the chamber base which contains the “sandwiched” slides.

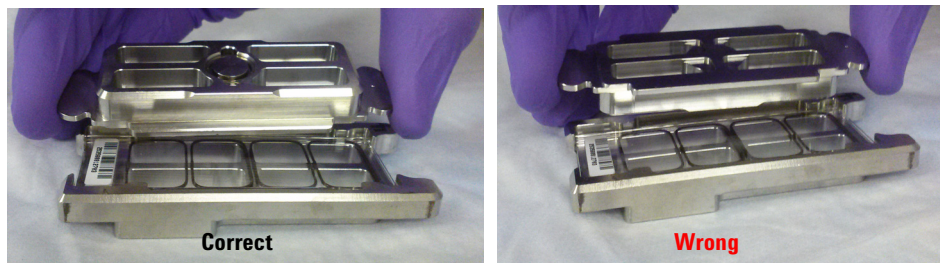


Figure 8. Chamber cover in correct (left) and incorrect (right) orientations

- 2 From the rounded corner of the chamber base, slip the clamp onto the chamber base and cover until it stops firmly in place, resting at the center of the two pieces.

Keep the chamber assembly flat on the lab bench to avoid spilling the hybridization solution.

Microarray Processing

Step 3. Prepare the hybridization assembly

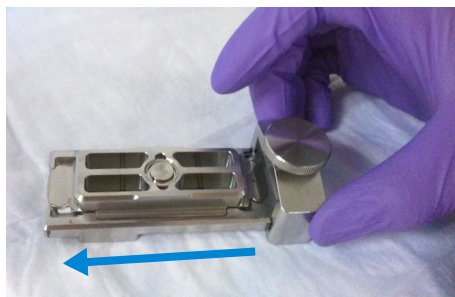


Figure 9. Slipping the clamp onto the chamber base

- 3 Firmly tighten the thumbscrew fully.
The slides will not be harmed by hand-tightening.

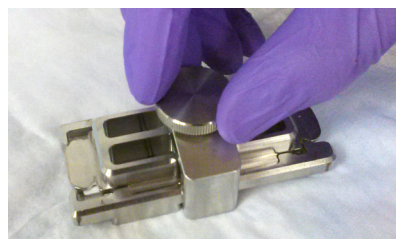


Figure 10. Tightening of the thumbscrew on the clamp

CAUTION

If you do not completely tighten the thumbscrew, hybridization solution can leak out during hybridization.

Do not use tools to tighten the thumbscrew. The use of pliers or other tools can damage the parts and will void the warranty.

- 4 Rotate the final assembled chamber in a *vertical orientation*, clockwise, 2 to 3 times to wet the gaskets (see **Figure 11**).
Rotation helps ensure that the hybridization solution will coat the entire surface of the microarray during the incubation process.

Microarray Processing

Step 3. Prepare the hybridization assembly

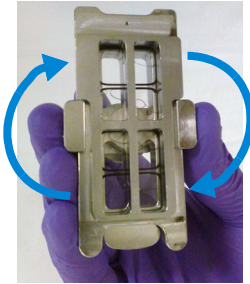


Figure 11. Rotation of the final assembled chamber

5 Inspect for good bubble formation.

- Hold the chamber vertically and inspect for stray or small bubbles that do not move as you rotate the chamber.
- Use the “large mixing bubble” to dislodge small stray or stationary bubbles.
- If the small stray or stationary bubbles persist, gently tap the assembled chamber on a firm surface. Rotate the chamber on its sides as you tap. Inspect again and repeat if needed until the small stray or stationary bubbles dissipate.

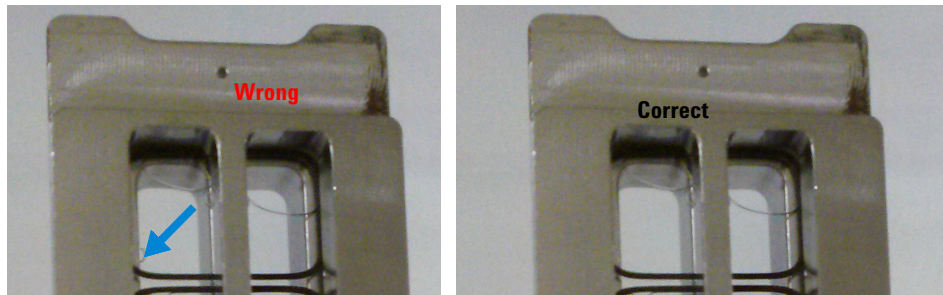


Figure 12. The slide on the left shows a stray, stationary bubble (denoted with arrow), which must be removed before hybridization. The slide on the right shows only large mixing bubbles, which move freely around the chamber when rotated. Bubbles are acceptable, as long as they move freely when you rotate the chamber.

Step 4. Hybridize

- 1 Load each assembled chamber into the oven rotator rack, starting from the center of the rack (position 3 or 4 when counting from left to right) Refer to the figure below for correct and incorrect orientations.

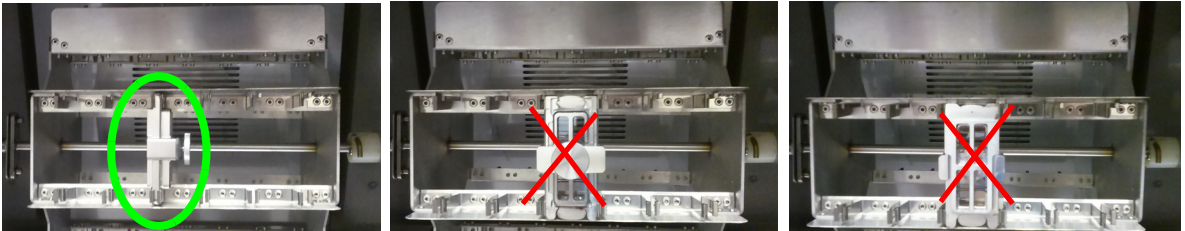


Figure 13. Assembled chambers in correct (left) and incorrect (middle and right) orientations

- 2 Hybridize at 67°C at 20 rpm for at least 2 hours, or as long as overnight.

A 2-hour hybridization is sufficient and allows for a 1-day workflow. You can also hybridize samples overnight if it better accommodates your schedule.

During hybridization, prewarm the Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 to 37°C so that it is ready for use during microarray washing. See **“Step 5. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (during hybridization)”** on page 39.

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* (publication G2545-90002) for more information.

Step 5. Prewarm Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (during hybridization)

The temperature of Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 must be at 37°C for optimal performance. Prewarm the buffer while the slides are in the hybridization oven.

- 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 during the 2–6 hour hybridization step.

After 2–6 hours of hybridization, proceed to washing the microarrays. See **“Microarray Wash”** 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 17 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. Prepare the equipment

Always use clean equipment when doing the hybridization and wash steps to avoid wash artifacts on your slides and images.

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

Acetonitrile wash (only for equipment exposed to Stabilization and Drying Solution)

For equipment that was exposed to Stabilization and Drying Solution (i.e., was used in Wash Procedure B), wash all staining dishes, racks and stir bars with acetonitrile using the procedure below. Follow up with the **“Milli-Q ultrapure water wash (all equipment)”**.

For equipment that was not exposed to Stabilization and Drying Solution (i.e., was used in Wash Procedure B), proceed directly to **“Milli-Q ultrapure water wash (all equipment)”**.

WARNING

Conduct acetonitrile 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.
- 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 Slowly remove the slide rack from the slide-staining dish.

The liquid tension created by removing the slide rack slowly helps to limit the amount of acetonitrile that adheres to the slides.

- 7 Repeat **step 1** through **step 5** with fresh acetonitrile. Discard the used acetonitrile as is appropriate for your site.
- 8 Air dry all of the equipment in the vented fume hood, then proceed to **“Milli-Q ultrapure water wash (all equipment)”**, below.

Milli-Q ultrapure water wash (all equipment)

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 2. 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.

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.

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.

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 3. 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 18 lists the wash conditions for the Wash Procedure A without Stabilization and Drying Solution.

Table 18 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.
- 3 Prepare dish #3:
 - a Put the prewarmed 1.5-L glass dish on a magnetic stir plate with heating element.

- 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 Prior to transferring the slide rack, activate the magnetic stirrer in slide-staining dish #3, which contains Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37°C. Adjust the setting to get thorough mixing.
 - b Transfer slide rack to slide-staining dish #3. If necessary, further adjust the setting on the magnetic stirrer to get thorough mixing without disturbing the microarray slides.
 - c Wash microarray slide for at least 1 minute and no more than 2 minutes.
- 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.

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 19 lists the wash conditions for the Wash Procedure B with 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
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 two batches of five slides (that is, total 10 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 4. 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*.

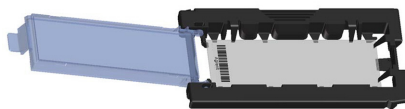


Figure 14. Slide in slide holder for SureScan microarray scanner

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 15**. Refer to the *Agilent Ozone-Barrier Slide Cover User Guide* (publication G2505-90550), included with the slide cover, for more information.

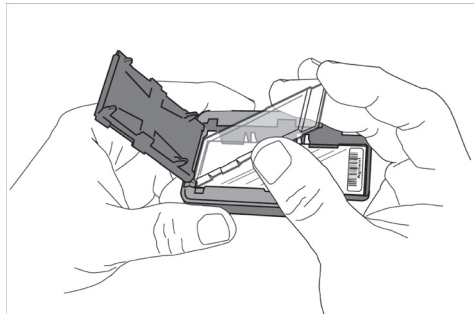


Figure 15. Inserting the ozone-barrier slide cover

- 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 is **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 20**.

Table 20 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 μ m
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 is **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.

Innopsys InnoScan 710 Microarray Scanner Settings

- 1 Load slides into the scanner with the Agilent label facing up.
- 2 In the Mapix software (version 7.4 or later), open the Scan Configuration window.
- 3 Click the Slide tab and select the appropriate format from the drop-down list (either **Oligo 8×60K slide** or **Oligo 8×60K slide**).
- 4 Click the General tab and select the **Auto** scan mode.

In this mode, the Mapix software automatically sets the laser power and PMT gain to optimize signal intensities. The scanner generates a 16-bit TIFF image with these settings.

- 5 Click the Scan icon to start scanning.

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 analysis using one of the preloaded analysis methods designed for single cell samples. These analysis methods are described in **Table 21**. Details on the analysis method parameters are accessible from the Configure Settings screen of the CytoGenomics software.

For users of the Innopsys InnoScan 710 Microarray Scanner If you are using CytoGenomics version 3.0 or 4.0, then you must first open the scanner-generated TIFF image in the Feature Extraction for CytoGenomics software module (see the *Feature Extraction for CytoGenomics User Guide* for instructions on launching the software module and opening TIFF files from within the software). As the Feature Extraction for CytoGenomics software opens the TIFF image, it converts the image to the Agilent format and saves the converted image to the original file folder (with “converted” appended to the file name). Use this converted image as the sample file in your analysis workflow in CytoGenomics 3.0 or 4.0. If you have already upgraded to CytoGenomics 5.0, then this separate conversion step for InnoScan files is not necessary.

Table 21 CytoGenomics analysis methods for single cell analysis

Analysis Method	Description/Use
Single Cell Recommended Analysis Method	Agilent's recommended analysis method for analysis of single cell samples. This analysis method is appropriate for most single cell analyses.
Single Cell Small Aberration Analysis Method	This analysis method is appropriate for analysis of single cell sample in which you want to focus on a few particular loci of interest because the Aberration Filter used in this analysis method has a less stringent threshold for aberration size than that used in the Single Cell Recommended Analysis Method. However, because of the reduced stringency, the risk for false positives is higher with this analysis method compared to the Single Cell Recommended Analysis Method.
Single Cell Long Low Aberration Analysis Method	This analysis method is appropriate for analysis of mosaic samples consisting of just a few cells. The Aberration Filter used in the analysis method is capable of finding large aberrations with a compressed \log_2 ratio.

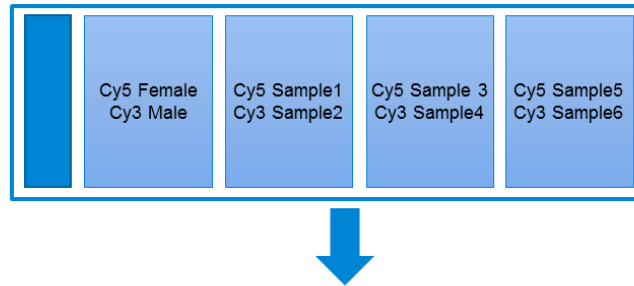
Agilent CytoGenomics software is a complete and streamlined CGH microarray data analysis solution that has Feature Extraction (FE) software built in and is able to run FE as an integral part of the analysis workflow.

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 16** 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.

Microarray Processing

Step 2. Analyze microarray image



	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 16. 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.

Table 22 QC metric thresholds for single cell samples

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

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5 Troubleshooting

- If the whole genome amplification fails 56
- If the labeling efficiencies for Cy3 and Cy5 are dissimilar 57
- If you have post-labeling signal loss 58
- If you have high BGNoise values 59
- If you have poor reproducibility 60

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 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 56.
- 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 22** on page 53 for thresholds) and higher DLRS values. 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. See **“Milli-Q ultrapure water wash (all equipment)”** on page 41.
- 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 22** on page 53 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.



6

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 REPLI-g Single Cell Kit, p/n 5191-4065

Component	Quantity
DNA Polymerase	2 × 55 µL
Reaction Buffer	3 × 700 µL
Buffer DLB (provided lyophilized)	2 tubes
Stop Solution	1.8 mL
PBS	2 × 1.5 mL
DTT, 1M	1 mL
H ₂ O	2 × 1.5 mL

Table 24 SureTag DNA Labeling Kit, -20°C Components, p/n 5190-3399

Component	Quantity
Nuclease-Free Water	1.5 mL
Exo (-) Klenow	55 µL
5× gDNA Reaction Buffer	550 µL
Cyanine 5-dUTP	78 µL
Cyanine 3-dUTP	78 µL
10× dNTP Mix	265 µL
Random Primers	265 µL
10× Restriction Enzyme Buffer*	142 µL
BSA*	15 µL
<i>A</i> <i>lu</i> I Restriction Digest Enzyme*	28 µL
<i>R</i> <i>sa</i> I Restriction Digest Enzyme*	28 µL

* This component is not used in the GenetiSure Pre-Screen Kit for Single Cell Analysis protocol.

Reference

Reagent Kit Components

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

Component	Quantity
2× HI-RPM Hybridization Buffer	p/n 5188-5220: 5 × 1.4 mL p/n 5188-5380: 25 mL
10× aCGH Blocking Agent (provided lyophilized)	p/n 5188-5220: 1 vial p/n 5188-5380: 4 vials

Table 26 Oligo aCGH/ChIP-on-chip Wash Buffer Kit, Agilent p/n 5188-5226

Component	Quantity
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	4 L per container
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2	4 L per container

"Secure Fit" Slide Box Opening Instructions

Agilent now ships all microarray slides in a newly designed "secure fit" slide box. The instructions below describe how to remove the slide box from the shipping pouch, how to open the slide box, and how to properly remove a microarray slide.

- 1 Use scissors to cut below the seal and remove box from its foil pouch.

After breaking foil on microarray pouch, store microarray slides in the slide box at room temperature (in the dark) under a vacuum desiccator or nitrogen purge box.

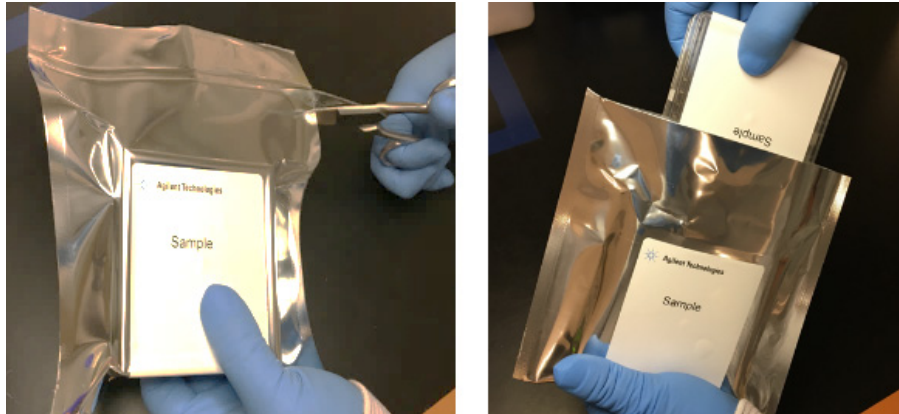


Figure 17. Opening foil pouch (left) and removing the slide box (right)

- 2 Place the slide box on a flat surface. While stabilizing the box from the top with one hand, use a sharp edge to cut the sealing tape on both sides of the box before opening.

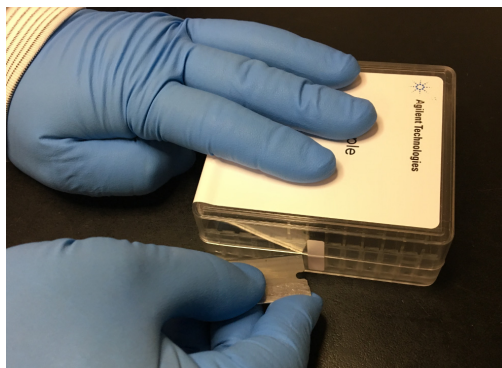


Figure 18. Cutting the sealing tape

Reference**“Secure Fit” Slide Box Opening Instructions**

- 3 With one hand, firmly hold the base of the box on the sides with the indentations (or dimples) for added grip.

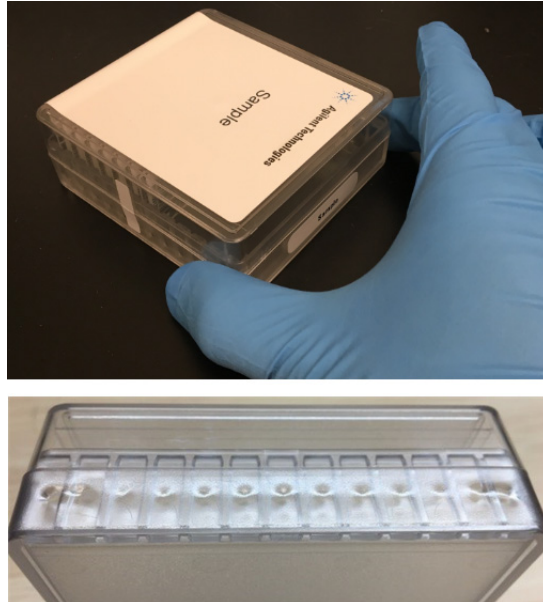


Figure 19. Gripping the base at the indentations (top) and close-up of the indentations (bottom)

- 4 Using your free hand, grasp the lid and gently lift it away from the base as if it is hinged. Set the lid aside.

Depending on your preference, you can reverse the hand positions so that the left hand holds the base while the right hand grasps the lid.

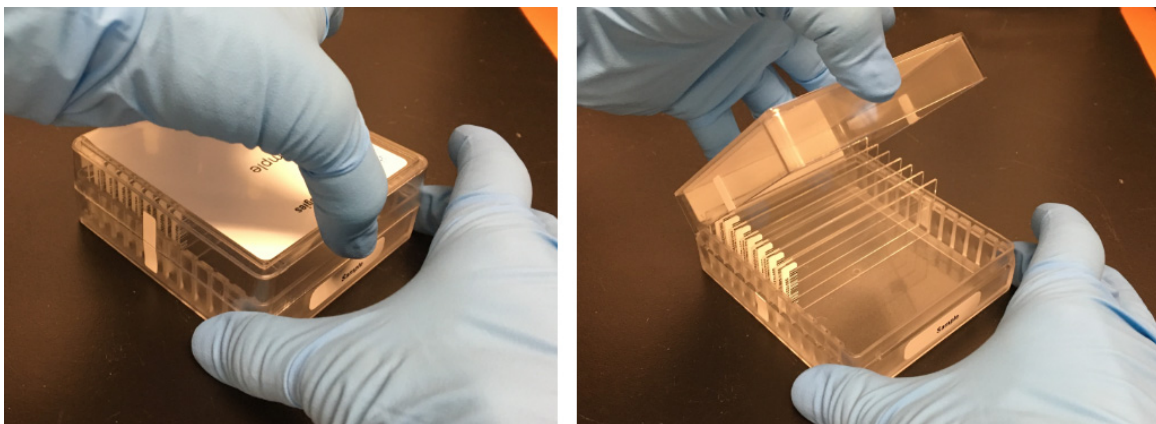


Figure 20. Grasping the lid (left) and lifting the lid from the base (right)

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.

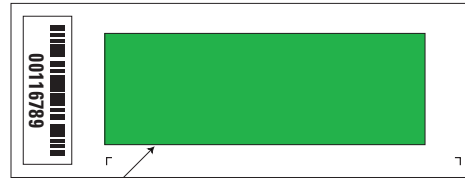
To avoid damaging the microarray, always handle glass slides carefully by their edges. Wear powder-free gloves. Never touch the surfaces of the slides. If you do, you may cause irreparable damage to the microarray.

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

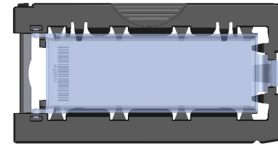
Agilent Microarray Layout and Orientation

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

Microarrays are printed on the side of the glass with the "Agilent"-labeled barcode (also referenced as "active side" or "front side").



Agilent Microarray Scanner scans through the glass. (Back side scanning.)



Agilent microarray slide holder for SureScan (above) and Scanner C (below) microarray scanners.



Figure 21. 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 x 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 21 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 A R C O D E	Sample:	Sample:	Sample:	Sample:

Barcode Number _____

Figure 22. 4-pack microarray slides

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
B A R C O D E	Sample:	Sample:	Sample:	Sample:
	Sample:	Sample:	Sample:	Sample:
	Array 2_1	Array 2_2	Array 2_3	Array 2_4

Barcode Number _____

Figure 23. 8-pack microarray slide

In This Book

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

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