EpiSwitch® Explorer Array Kit

Handbook

Array processing

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Handbook Version:	1.00

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1. Purpose

The purpose of this user guide is to describe the technology adapted by Oxford BioDynamics (OBD) to perform array analysis of EpiSwitch® markers from EpiSwitch® libraries.

This guide is adapted from the Agilent Protocol: Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Enzymatic Labeling For Blood, Cells, or Tissues (Agilent Technologies G4410-90010 Version 8.0) and describes the recommended operational procedures to interrogate EpiSwitch® markers using Agilent SurePrint microarray technology.

This guide assumes familiarity` with Agilent aCGH equipment and protocols for the handling of 1M (1-pack) SurePrint arrays. A list of Agilent user guides is included in the Reference section.

https://www.agilent.com/cs/library/usermanuals/public/GEN-MAN-G4410-90010.pdf

1.1 About EpiSwitch®

1.1.1 Chromosome Conformation Signatures

Oxford BioDynamics' EpiSwitch® technology is based on epigenetics, mechanisms that alter gene expression without altering the underlying DNA sequence and whose deregulation plays a role in the development of cancer, autoimmune, and neurologic diseases. Although DNA is often illustrated as a simple linear strand, the reality is that it is packaged into a complex three-dimensional structure that brings DNA sequences that are distant from each other in linear genomic space into close physical proximity. These long-range interactions, occurring both within and between gene loci, reflect genetic epistasis and the regulatory network imposed on the genome and as result directly modulate gene expression in the context of the established phenotype.

Collectively, functionally relevant combinations of discrete chromosomal conformations (CC) across the genome (chromatin "bar-codes") are called a chromosome conformation signature (CCS). CCSs can be monitored by means of molecular biology and therefore represent an ideal biological marker for assessing disease pathophysiology and response to therapeutic intervention.

1.1.2 EpiSwitch® Technology

EpiSwitch® proprietary industrial platform for detecting CCSs uses well-accepted principles of CC detection, proprietary molecular biology techniques and operates under ISO compliant standards. The platform generates readouts within hours and can handle diverse types of source material (peripheral blood, tissue biopsies and cellular isolates) in high throughput at high resolution and sensitivity, using a manual or robotic/automated platform. Detection is based on established molecular 3C biology techniques. In short, without disrupting the cells, the CCs are chemically stabilized, extracted as a chromatin template and the distant genomic sites found in proximity by stable juxtaposition are converted into adjacent sequence tags on an artificially generated chromatin template. Quantification of the segments can be done by comparative genomic hybridization (CGH) on an Agilent array platform, by NGS and by nested or Real-Time PCR and provides the readout of the CCS present in a sample.

1.1.3 EpiSwitch® Arrays, Performance and Stated Use

This procedure has been developed for enzymatic cyanine dye labelling of 0.5-1 µg EpiSwitch® template DNA that has been extracted from blood, tissue or cells using manual or robotic EpiSwitch® extraction protocols. This protocol can be used for single-color or dual-color array experiments.

Identical quantities of all test and reference samples should be used for labelling within a project. Each sample must first be successfully extracted using the EpiSwitch® method. Successful EpiSwitch® extraction can be confirmed using an EpiSwitch® positive control PCR assay.

2. Before you begin

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

2.1 Safety Notices

CAUTION

A CAUTION notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a CAUTION notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or

the like that, if not correctly performed or adhered to, could result in personal injury or death.

Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

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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.
- Acetonitrile is a flammable liquid and vapor. Harmful if inhaled, swallowed, or contacts skin. Target organs: liver, kidneys, cardiovascular system, and CNS.
- 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.

2.2 Procedural Notes

- Follow the EpiSwitch® extraction protocol: EXT-WI-015 EpiSwitch® library Preparation to isolate EpiSwitch® libraries prior to array processing.
- EpiSwitch® arrays are only to be used with EpiSwitch® libraries. Genomic DNA cannot be meaningfully interrogated with the EpiSwitch® platform.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- **Do not vortex** stock solutions and reactions containing **EpiSwitch® libraries or enzymes**. Instead, mix the solutions and reactions by gently tapping the tube with your finger or by pipetting up and down. **Avoid creating bubbles** wherever possible.
- Avoid repeated freeze-thaw cycles of solutions containing EpiSwitch® libraries, enzymes or Cyanine dyes.
- When preparing frozen **reagent stock solutions** for use (i.e. not enzymes or EpiSwitch® libraries):

  1. Thaw the aliquot as quickly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, and then briefly spin in a microcentrifuge (5 - 10 seconds) to collect the contents from the walls and lid.
  3. Store on ice, or in a cold block until use.

- In general, follow Biosafety Level 1 (BL1) safety rules.

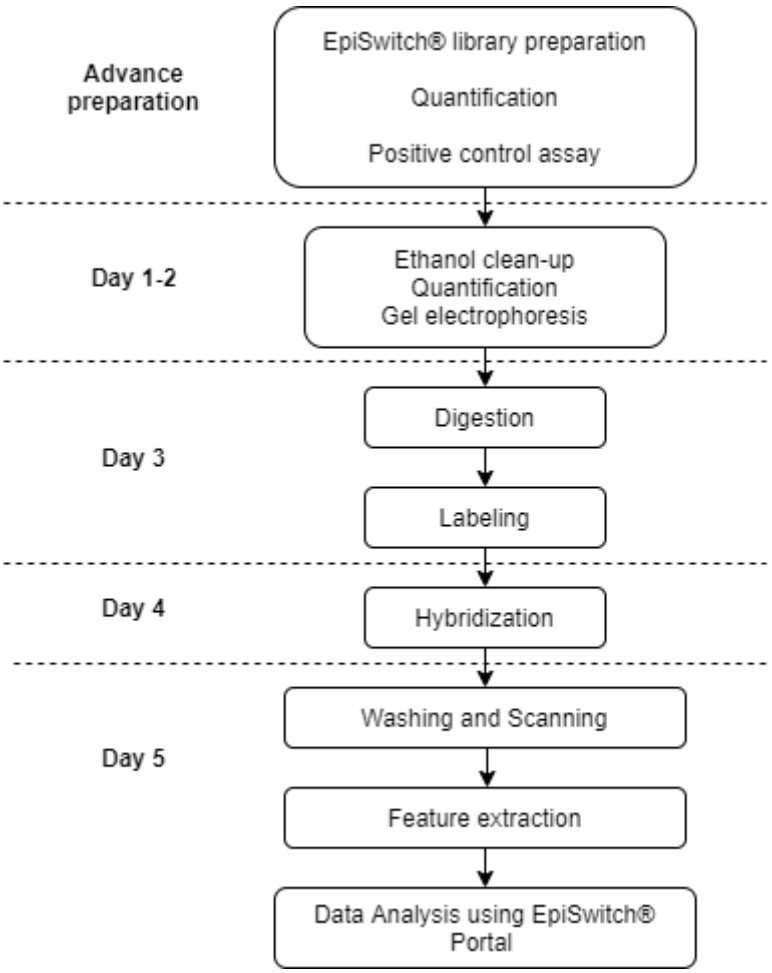
2.3 EpiSwitch® Template Requirements

EpiSwitch® library template must be extracted, quantified and PCR-validated for the presence of control chromosome conformation signatures before array processing begins. EpiSwitch® template is cleaned up and concentrated using ethanol precipitation and quantified using UV-Vis absorbance at 260 nm.

Optional: DNA may also be quantified using a fluorescence method (PicoGreen, or similar) to indicate the proportion of double stranded DNA in a sample.

It is expected that higher concentrations will be seen with UV-Vis absorbance quantitation than with fluorescence methods that measure double stranded DNA only. EpiSwitch® Explorer Array labeling reactions require 800 ng template DNA, as measured by UV-Vis absorbance. Where 800 ng is not available, labeling may proceed with a minimum of 500 ng template however it is strongly recommended that **equal** ng amounts of DNA are used for each labelling reaction **within** a project.

EpiSwitch® template quality may be assessed prior to labelling by agarose gel electrophoresis. EpiSwitch® libraries typically appear as a smear of DNA, with the bulk of the smear between 1kb-15kb on a 1% agarose gel. High molecular weight complexes may be visible as a distinct band which migrates with the 15 kb marker.



Outline of the workflow with suggested timings.

2.4 OBD EpiSwitch® Array Kit Contents

Store the microarray storage box at room temperature until ready for hybridization. After the microarray foil pouch is opened, if not used immediately, store the microarray slide(s) at room temperature (in the dark) under a vacuum desiccator or in a nitrogen gas purge box. Do not store microarray slides in open air after breaking foil.

- 1-inch × 3-inch array slide, 1 per box.
- Array Design File (.xml) for use with Feature Extraction software.
- EpiSwitch® Reagents

2.4.1 EpiSwitch® Reagents

The EpiSwitch® reagents are shipped at ambient temperature and should be transferred to the storage conditions detailed in the table below on receipt. Each EpiSwitch® Array Explorer kit is supplied with enough reagents for 50 individual reactions for ease of use. Full instructions for EpiSwitch® Library Preparation (EXT-WI-014) are available for download with this kit.

Full Name	Supplier	Part number	Molecular Weight or Concentration	Storage
EpiMix Buffer DE-A	OBD	DE-A-50	-	Ambient
EpiMix Buffer DE-B	OBD	DE-B-50	-	Ambient
10X EpiMix Buffer DE-C	OBD	DE-C-50	10x	2˚C to 8°C
EpiMix Buffer DE-D	OBD	DE-D-50	-	2˚C to 8°C
EpiMix Buffer DE-E	OBD	DE-E-50	-	2˚C to 8°C
25X EpiMix Buffer DE-F	OBD	DE-F-50	25x	Ambient
2X EpiMix Buffer DE-G	OBD	DE-G-50	2x	2˚C to 8°C

All EpiSwitch® reagents are supplied as general laboratory reagents manufactured in accordance with OBD's ISO13485:2016 certification.

WARNING

Please consult the safety data sheets and warning signs for all EpiSwitch® reagents.

2.5 Third Party Reagents

2.5.1 Sample Preparation Reagents

Description	Vendor and part number
Nuclease-free water	Thermo Fisher Scientific p/n AM9937 or similar
Ethyl alcohol, Pure, 200 proof	Sigma p/n 439836 or similar
Sodium Acetate Solution (3 M), pH 5.2	Thermo Fisher Scientific p/n R1181
UltraPure™ Glycogen	Thermo Fisher Scientific p/n 10814010
1x TE (pH 7.5) (prepared from 20x stock)	Thermo Fisher Scientific p/n T11493 or P7589 (20X TE from Quant-iT PicoGreen Kit)
Molecular biology grade water	Lonza Accugene p/n LZBE51223 or similar
1Kb plus Ladder	Thermo Fisher Scientific p/n 10787-018
Gel Loading Dye, Purple (6X)	NEB p/n B7025S
SYBR Safe	Thermo Fisher Scientific p/n S33102, or similar
Novex TBE Running buffer (5X)	Thermo Fisher Scientific p/n LC6675, or similar
UltraPure™ Agarose	Thermo Fisher Scientific p/n 16500-100, or similar
E-Gel General Purpose Agarose Gels, 1.2%	Thermo Fisher Scientific p/n G501801
SYBR Gold Nucleic Acid Gel Stain	Thermo Fisher Scientific p/n S11494
SYBR photographic filter	Thermo Fisher Scientific p/n S7569
TrackIt 1 Kb Plus DNA Ladder	Thermo Fisher Scientific p/n 10488085

2.5.2 Sample Digestion and Labeling Reagents

Description	Vendor and part number
SureTag DNA Labeling Kit*	Agilent p/n 5190-3400
1×TE (pH 8.0), Molecular grade	Promega p/n V6231

* SureTag DNA Labelling Kit contents: 10× Restriction Enzyme Buffer, BSA, Alu I, Rsa I, Purification Columns and collection tubes, Nuclease-Free Water, Exo (-) Klenow, 5× Reaction Buffer, Cyanine 5-dUTP, Cyanine 3-dUTP, 10× dNTPs, Random Primers.

2.5.3 Microarray Processing Reagents

Description	Vendor and part number
Oligo aCGH/ChIP-on-chip Hybridization Kit*	Agilent p/n 5188-5220 (25) or p/n 5188-5380 (100)
Human Cot-1 DNA (1.0 mg/mL)	Agilent p/n 5190-3393
Oligo aCGH/ChIP-on-chip Wash Buffer Kit *or* Oligo aCGH/ChIP-on-chip Wash Buffer 1 *and* 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
Acetonitrile†	Merck (Sigma-Aldrich) p/n 271004-1L

Nuclease-free water	Thermo Fisher Scientific p/n AM9937 or similar
Milli-Q ultrapure water	Merck Millipore, or similar distilled water with low TOC**

* Oligo aCGH/ChIP-on-chip Hybridization Kit* contents: 2× HI-RPM Hybridization Buffer, 10× aCGH Blocking Agent.

† Optional components recommended if wash procedure B is selected (see Agilent protocol: Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Enzymatic Labeling For Blood, Cells, or Tissues (with High Throughput Option) for wash procedure B instructions).

**TOC: Total Organic Carbon

2.6 Equipment

The list of equipment used in this protocol is given below. Alternative suitable equipment may be used.

Description	Vendor and part number
Microarray Scanner with Feature Extraction software*	Agilent p/n G4900DA
Hybridization Chamber, stainless steel	Agilent p/n G2534A
Hybridization oven: temperature set at 67°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray	Agilent p/n G2530-60029
Hybridization Chambers	Agilent p/n G2534A
SureScan slide holders	Agilent p/n G49000-60035
Thermal cycler with heated lid	Eppendorf p/n EP6311000010 or equivalent
UV-Transilluminator with SYBR photographic filter	Bio-Rad p/n 12009077 or equivalent
Gel electrophoresis cell e.g., Sub-Cell GT (optional)	Bio-Rad p/n 1704402, or similar
Magnetic stir plate (×1 or ×3) ‡‡	various
Magnetic stir plate with heating element	various
Microcentrifuge	Eppendorf 5430 or equivalent
Mini centrifuge e.g., Mini Fuge Plus	Starlab p/n N2631-0017 or equivalent
UV-VIS spectrophotometer**	Nanodrop 8000 or 2000, Tecan Infinite 200 Pro M-Nano or equivalent
1.5 L glass dish	Pyrex p/n 213-R or equivalent
Refrigerated centrifuge with fixed angle rotor	Hettich Mikro 220R or similar
Vacuum Concentrator ††	Thermo Scientific Savant SpeedVac p/n DNA 130-115 or equivalent
37^{o}C incubator (optional)	Labnet p/n I5110A, or similar
Digital balance (optional)	Sartorius PRACTUM224-1S, or similar
Magnetic stir bar, 7.9 × 38.1 mm (×2 or ×4) ‡‡	VWR p/n 58948-150 or equivalent
250 mL capacity slide-staining dish, with slide rack (×3 or ×5) ‡‡	Wheaton p/n 900200 *or* Thermo Fisher Scientific p/n 121
Circulating water baths or heat blocks. For restriction digestion and/or labeling, set to 37°C, 65°C and 98°C. For hybridization preparation, set to 37°C and 98°C. ***	Various
Ice bucket or chill blocks	Various
Clean forceps	Various
Powder-free gloves	Various
Sterile, nuclease-free barrier pipette tips	Various – to match pipette system used
Timer	Various
Vortex mixer	Various

* Other scanner systems may be used, check for compatibility.

** Either Nanodrop, Tecan Infinite M200 Pro with Nanoquant plate or equivalent may be used for UV-Absorbance based quantification and assessment of labeling efficiency.
†† Optional. Recommended for ethanol clean-up and sample concentration.

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

*** For optimal performance, OBD recommends using a thermal cycler, rather than water baths or heat blocks, for restriction digestion and, labeling steps. In the absence of a thermal cycler compatible with 520 µL reaction volumes, hybridization preparation (98°C and 37°C incubations) for 1M arrays may be best achieved using the central wells of heat blocks with pre-heated water added to the wells immediately prior to use.

2.7 Required Consumables

The disposable plasticware used in this protocol are included below. All plasticware described are RNase, DNase, DNA and pyrogen free. Alternative plasticware may be used as required.

Description	Vendor and part number
200-µL Thin-Wall Tube with flat cap	Starlab p/n L1402-4308
Hybridization gasket slides, 5-pack (20 and 100 packaging sizes are available)	Agilent p/n G2534-60003 (5), G2534-60008 (20), G2534-60005 (100)
1.5 mL LoBind Microcentrifuge Tube (sustainable at 98°C)	VWR (Eppendorf) p/n 525-0130
Thin wall, clear 0.5 mL PCR tubes**	VWR p/n 10011-830
Sterile storage bottle e.g., Nalgene 455-1000 or equivalent	Thermo Fisher Scientific p/n 10546361
50 ml Centrifuge Tube, Skirted, Loose (Sterile)	Starlab p/n E1450-0500, or similar
1.5 ml Crystal Clear Microcentrifuge Tube	Starlab p/n E1415-1510, or similar
Extended Fine Tip Mini Pastette	SLS p/n LW4231

** For large volume thermal cyclers, if available.

2.8 Thermal Cycler Incubation Programs

The following programs should be added to an Eppendorf MasterCycler X50s thermal cycler (or equivalent).

Program 1: Digestion

Parameters	
Heated Lid:	105°C
Turn off Lid at low	Yes
Ramp Rate	100%
Program: Digestion	
Step 1	37°C hold
Step 2	37°C for 2 hours
Step 3	65°C for 20 minutes
Step 4	4°C hold

Program 2: Denaturation

Parameters	
Heated Lid:	105°C
Turn off Lid at low	Yes
Ramp Rate	100%
Program: Denaturation	
Step 1	98°C hold
Step 2	98°C for 3 minutes
Step 3	4°C for 5 minutes
Step 4	4°C hold

Program 3: Labeling

Parameters	
Heated Lid:	105°C
Turn off Lid at low	Yes
Ramp Rate	100%
Program: Labeling	
Step 1	37°C hold
Step 2	37°C for 2 hours
Step 3	65°C for 10 minutes
Step 4	4°C hold

Program 4: Pre-hybridization (for thermal cyclers that can handle 520 μL reaction volumes).

Parameters	
Heated Lid:	105°C
Turn off Lid at low	Yes
Ramp Rate	100%
Program: Pre-hybridization	
Step 1	98°C hold
Step 2	98°C for 3 minutes
Step 3	37°C for 30 minutes
Step 4	37°C hold

3. Sample Preparation

This chapter describes the method to process EpiSwitch® libraries prior to digestion.

EpiSwitch® DNA extracts must be cleaned-up and concentrated prior to processing for microarray. This section describes the procedure for the clean-up and concentration of EpiSwitch® DNA by ethanol precipitation, followed by UV-absorbance-based quantification and quality control by agarose gel electrophoresis.

3.1 Ethanol clean-up and concentration

3.1.1 Advance preparation

The method requires cold 200 proof ethanol and cold 70% ethanol prepared with nuclease-free water.

1) Place an aliquot of molecular biology grade 200 proof ethanol at -20 ^{0}C (spark-free freezer).
2) Prepare 70% ethanol in a 50 mL centrifuge tube: e.g., 35 mL molecular biology grade 200 proof ethanol + 15 mL nuclease-free water. Smaller volumes may be prepared. Use within 6 months and keep air space to a minimum. Place at -20 ^{0}C (spark-free freezer).
3) Calculate the volumes of DNA, TE buffer, 3M NaAc pH 5.2 and 200% proof EtOH required to precipitate e.g., 1.6-2.0 μg (by UV-absorbance) of each EpiSwitch® library sample (see section 3.1.2).
4) Label 1.5 mL Crystal Clear microcentrifuge tubes, 1 for each sample.

3.1.2 Ethanol precipitation – Day 1

1) Add Glycogen to the base of each tube using reverse pipetting. Use 1 µL glycogen for each 50-100 µL of DNA + TE (i.e., 0.2 µg-0.4 µg UltraPure Glycogen per 1 µL of aqueous sample).
2) Add TE buffer (pH 7.5) and EpiSwitch® DNA to each tube such that TE + DNA = 100 µl.
3) Add 1/10th the aqueous volume of 3M Sodium Acetate (NaAc) pH 5.2, (i.e., 10 μL NaAc to 100 μL DNA + TE)
4) Add 2.5x the aqueous volume of cold (pre-chilled) 200 proof ethanol (i.e., 250 μL per 100 μL DNA + TE). Ensure caps are firmly closed, then invert tubes 6 times to mix. Place the tubes in a freezer (-80 ºC over-night, or at -80/-20 ºC for a few days e.g., over a weekend).

3.1.3 Ethanol precipitation – Day 2

1) Pre-chill a refrigerated centrifuge to 4 ºC. Remove the samples from the freezer and allow to thaw then place into the centrifuge immediately. Align the tube hinges to outer edge of the rotor so that you know where the pellet should form. Spin for 20 min at 18620 x g, 4 ºC.
2) Carefully remove the tubes from the centrifuge and place in a frozen cold block. Using a p1000 pipettor, remove the supernatant in a single movement – pipette slowly to avoid disturbing the pellet. NOTE: The pellet may be visible as a small and opaque white or translucent smudge near the base of the tube on the hinge side. Pellets can be difficult to see.
3) Rinse the pellet by adding 500 µL cold 70% ethanol (pre-chilled at -20ºC). Dispense the ethanol in a circular motion just inside the top of the tube so that it washes down the insides of the tube and covers the pellet. The pellet may become dislodged. Allow the tubes to stand for 5 min in the cold block.
4) Centrifuge for 20 min, at 18620 x g, 4 ºC. Align the tube hinges to outer edge of the rotor as before.
5) Carefully remove all the supernatant by pipetting without disturbing the pellet (use a p1000 set to 800-900 µL to remove all the ethanol). Keep the tubes on a frozen cold block until ready for Speedvac.
6) Dry the pellet in the Savant Speedvac DNA 130, for 10 min at 35 ºC (10 min heating time, 10 min spin time, do not preheat –leave the sample tube caps open!).
7) To resuspend the pellet, pipette a volume of nuclease-free water into the tube without touching the pellet with the pipette tip. Choose a volume between 20-40 µL, depending on the expected yield, aiming for a minimum concentration of 50 ng/µL by UV-absorbance at 260 nm. Do not mix by pipetting, briefly centrifuge to collect the nuclease-free water layer over the pellet.
8) Incubate the pellets in a 37 ºC water bath for 30 min-2 hours to resuspend the DNA. Flick the tubes with your fingers to fully resuspend the DNA and briefly centrifuge to collect the contents.
9) DNA can be kept at 4 ºC for up to 24 hours if necessary, but preferably place at -20 ºC as soon as possible.
10) Quantify the cleaned-up EpiSwitch® DNA using UV-absorbance at 260 nm. Remember to blank using nuclease-free water instead of TE. Record the 260:280 and 260:230 ratios in order to assess sample quality. Fluorescence-based (e.g., PicoGreen) quantification may also be performed, sample quantity permitting. Array processing uses the UV-absorbance at 260 nm-derived concentration for labeling calculations.

3.1.4 Agarose Gel Electrophoresis

It is recommended to assess the size profile of the EpiSwitch® library samples following ethanol clean-up to later assess sample digestion. Any 1-1.2% agarose gel electrophoresis system may be used to check 50-100 ng EpiSwitch® library using e.g., SYBR Safe or similar.

Where sample is limiting, it may be preferable to assess 20 ng EpiSwitch® library template using e.g., the Invitrogen E-Gel system with SYBR Gold Nucleic Acid Gel Stain.

4. Sample Digestion

This chapter describes how to digest EpiSwitch® libraries prior to labeling.

Restriction enzymes Alu1 and Rsa1 fragment the EpiSwitch® library DNA. The DNA size profile after digestion may be assessed using agarose gel electrophoresis. Digested libraries can be stored for up to 1 month at -20 ºC.

CAUTION

Agilent recommends using a thermal cycler for all sample incubation steps during restriction digestion, labeling, and hybridization preparation where possible. Heat blocks may increase sample-to-sample variability and water baths may fluctuate in temperature, especially at high temperatures. The 98°C incubations in particular, benefit from the use of a thermal cycler in place of a water bath or heat block.

Thermal cyclers with capacity for the 520 μL reaction volumes used with the 1M array format are not widely available. While it is recommended to use a thermal cycler where possible, modified heat block instructions are included with this protocol.

4.1 Restriction digestion with SureTag DNA labelling kit

CAUTION

EpiSwitch® Explorer arrays must only be used with EpiSwitch® libraries where successful extraction has been confirmed by positive control PCR or qPCR testing. Genomic DNA cannot be meaningfully interrogated with EpiSwitch® arrays. EpiSwitch® libraries should be purified by ethanol precipitation and resuspension in nuclease-free water prior to digestion and labelling.

If the EpiSwitch® library concentration is greater than 350 ng/μL (by UV-Vis absorbance), dilute 1:2 with nuclease-free water and requantify to make sure quantitation is accurate.

NOTE

- For dual-channel arrays, you must use equal nanogram amounts of EpiSwitch® library for both the test and reference channels.
- This direct labeling protocol requires 0.8 μg (0.5-1μg) EpiSwitch® library template per sample in a total volume of 20.2 μL.
- Agilent SureTag DNA labelling kit contents may be thawed a maximum of 3 times for optimal use. **Keep reagents in chill blocks while in use and return promptly to -20 ºC.**
- Calculate the volumes of EpiSwitch® library and nuclease-free water required for each reaction. Use equal nanograms of EpiSwitch® library in each labelling reaction within a study. The minimum recommended DNA concentration after ethanol clean-up is >=39.6 ng/μL.

4.1.1 Restriction digestion reaction set-up

1. Thaw nuclease-free water, 10x Restriction Enzyme Buffer and BSA (included in the SureTag DNA Labeling Kit). Flick the tubes briefly to mix, then briefly centrifuge to collect the contents.

2. Set up 200 µl strip tubes, 1 per reaction on ice/chill block.

3. Add SureTag nuclease-free water first, then add cleaned-up EpiSwitch® template to each tube according to the following table.

Pair ID	Tube label	EpiSwitch® Library (500-1000 ng)	DNA volume (µL)	Nuclease-free water (µL)	Total (µL)
1	Test 1	800			20.2
2	Test 2	800			20.2
3	Test 3	800			20.2
4	Test 4	800			20.2
1	Reference 1	800			20.2
2	Reference 2	800			20.2
3	Reference 3	800			20.2
4	Reference 4	800			20.2

Labelling set-up table (example)

4. On a chill block, prepare Digestion Master Mix by combining the components in the order listed in the following table. Keep enzymes at -20 ºC until ready for use, flick to mix then briefly spin and keep in a frozen chill block. Prepare 1 extra reaction per 8 samples e.g., for 8 samples, make a 9x master mix. Mix well by pipetting the whole volume up and down 12x.

Component	Per reaction (µl)	……..x reactions (µL) Master Mix
Nuclease-Free water	2.0	
10x Restriction enzyme buffer	2.6	
BSA	0.2	
Alu I	0.5	
Rsa I	0.5	
Final volume of digestion master mix	5.8	

Digestion master mix

5. Add **5.8 µl** mastermix of Digestion master mix to each 200 µl tube containing EpiSwitch® template on ice/chill block. Mix well by pipetting up and down 12x (be careful not to lose any volume). Briefly spin to collect contents.

6. Use **program 1: Digestion** on the Thermal Cycler: Start the program and hold at 37 ºC before adding the reaction tubes directly from the ice/chill block, then **Resume** to run the program.

7. **QC:** Assess DNA digestion by electrophoresis. Take 2 µL digested EpiSwitch® DNA, combine with 2µL gel loading dye purple (NEB, diluted to 2X with nuclease-free water) and run on a 1% agarose gel containing SYBRSafe (or use 1.2% E-Gels). After 30 minutes run time (130 V for a 15 cm gel), digested products should be visible as a smear mostly in the 200-1000 bp region. Keep digestion products on ice/cold block. In the event of digestion failure, do not proceed with labelling. Troubleshoot the digestion and repeat.

8. If QC is passed, then either proceed directly to sample labelling or store at -20 ºC for up to 1 month.

5. Sample Labeling

This chapter describes the steps to differentially label digested EpiSwitch® libraries with fluorescent-labeled nucleotides using random primers and the Exo (-) Klenow fragment from the SureTag Labeling Kit.

The “polarity” of the sample labeling is a matter of experimental choice. For dual-channel applications, OBD recommend that the test samples are labelled with Cy3 and reference samples with Cy5. For single channel applications the default recommendation is to use Cy3 only for all samples.

Labelled DNA may be stored at -20 ºC for up to 1 month prior to purification.

NOTE 1: Cyanine dyes are **light-sensitive** and subject to degradation by ozone and multiple freeze-thaw cycles. **Minimize exposure to light** throughout the labelling procedure.

CAUTION: All samples must be **treated identically** when they are processed, or the data quality can be adversely affected.

5.1 Denaturation

If working from frozen, first thaw the digested EpiSwitch® libraries and keep on ice/chill block.

1. Briefly centrifuge samples to collect contents in the bottom of the tubes (e.g., 1 min at 6000 x g). Keep the sample tubes cold during reaction set-up.

2. Add random primers:
   - Add **5µl Random Primers** to each reaction.

   Use a separate tip for each reaction. Mix well by gently pipetting up and down 12x. Take care to minimize sample loss during pipetting.

3. Use the program **Denaturation** on the thermal cycler: Begin the program to preheat the block and hold at 98ºC**.** Transfer the tubes directly from the ice/chill block to the pre-heated block and press **Resume** to release the hold.

NOTE: For fewer than 4 labeling reactions, it is recommended to place 2-4 0.2 mL tubes containing water or TE buffer in each of the corner positions of the thermal cycler block to distribute the lid pressure and prevent crushing of the reaction tubes.

4. Briefly spin the samples to collect contents then add labelling master mix prepared according to the table below.

5.2 Labelling Master Mix(es) preparation

- **Minimize exposure to light, avoid strong sunlight**

For dual-color: Prepare **2 master mixes,** one for Cy3 and one for Cy5, according to the following table (keep cold). Combine reagents in the order listed in the table.

For Single-color arrays: Prepare **1 Cy3 master mix** for **all** samples.

Make 1 reaction volume excess per 8 samples (e.g., 9x MM for 8 samples, 18x MM for 16 samples etc.). Ensure individual components (except Klenow) are thawed and well mixed before use. Keep Exo (-) Klenow in a frozen cold block then **gently** flick the tube to mix and briefly spin before taking enzyme. Return reagents to -20ºC promptly after use.

Component	Per reaction (µL)	Per …….X master mix (µL)
5x Reaction buffer	10.0	
10x dNTPs	5.0	
Cyanine 3-dUTP **OR** Cyanine 5-dUTP	3.0	
Exo (-) Klenow	1.0	
Final volume of Labeling master mix	**19.0**	

Labelling Master mixes

5. Briefly spin the samples to remove contents from the walls and lids.
6. Pipette master mix up and down gently to mix immediately before adding to the samples. Add **19 µL** of pre-made, ice cold, labelling master mix to each respective tube (For dual-color: Cy3 for the Test samples and Cy5 for the Reference samples). The total reaction volume should now be 48 µL. Mix well by gently pipetting up and down 12x.
7. Preheat the thermal cycler to 37 ºC by starting program: **Labeling.** Transfer samples to the pre-warmed block and press **Resume** to release the hold. Incubate for 2 hours at 37 ºC, followed by 10 min at 65 ºC to inactivate enzymes, then hold at 4 ºC. Transfer tubes to ice/cold block (protect from light). Reactions can be stored for up to 1 month at -20 ºC **protected from light.**

5.3 Column Purification of Labeled Samples

The purpose of this step is to remove excess, unincorporated cyanine dyes, dNTPs and reaction components prior to hybridization, resulting in purified, fluorescently-labeled EpiSwitch® libraries.

Labelled EpiSwitch® template is purified using the purification columns supplied with the

SureTag DNA Labelling Kit. Keep samples awaiting purification frozen at -20 until needed (if already frozen) or cold in chill blocks/on ice and protected from light if proceeding directly from the labeling step.

After purification, the yield and labelling efficiency may be assessed using UV-Vis absorbance.

Purified, labelled DNA may be stored at -20 ºC for up to 1 month prior to hybridization.

CAUTION: Minimize sample exposure to light. Avoid bright sunlight.

1. For each sample, label one 1.5 mL LoBind tube and two Agilent 2 mL collection tubes and arrange in a rack. Place a purification column into one collection tube per sample.

2. OPTIONAL: Weigh the final collection tubes and record in column B of the suggested Labelling Results table.

3. Briefly spin the Cy-labelled EpiSwitch® samples to collect contents, then transfer each to the correct pre-labelled 1.5 mL LoBind tube. **Make sure all the reaction volume is transferred.**

4. For each sample in turn, add 430 µL 1x TE (Promega, pH 8.0) to the Cy-labelled sample, mix by pipetting up and down and load onto the correct spin column.

5. Once all spin columns are loaded, make sure all caps are firmly in place and centrifuge for 10 min at 14,000 *xg,* room temperature. Use a small pastette to remove the flow-through and place the column back into the same collection tube (collect Cy-dye waste for safe disposal according to local regulations). An intense pink or blue color should be seen at the base of the column.

6. Wash the columns by adding 480 µL Promega 1x TE (pH 8.0) onto each column. Spin for 10 min at 14,000 *xg*, room temperature. Intense pink or blue color should still be visible at the base of the column.

7. Invert the column into a new (OPTIONAL pre-weighed), labelled collection tube. Centrifuge for 1 min at 1000 xg, room temperature. The recovered sample volume should be approx. 20-32 µL.

8. OPTIONAL: Weigh the collection tubes with purified sample and record the values in column A of the Labeling Results table. Calculate the recovered sample volume and record in column A-B. Use the volume to calculate the yield of labeled DNA after checking Cy dye incorporation by UV-Vis Absorbance (e.g. Nanodrop or NanoQuant).

5.4 To determine yield, and degree of labeling or specific activity

5.4.1 Nanodrop instructions

Nanodrop instructions for measuring yield and degree of labeling and/or specific activity (NanoDrop 8000 or 2000 UV-VIS Spectrophotometer).

1) From the main menu, select MicroArray Measurement, then from the Sample Type menu, select DNA-50.
2) Use 1.5 µL of 1×TE (Promega, pH 8.0) to blank the instrument.
3) Use 1.5 µL of purified labeled gDNA for quantitation. Measure the absorbance at A_{260nm} and A_{280nm} (DNA), A_{550nm} (cyanine 3), and A_{650nm} (cyanine 5).
4) Calculate the Degree of Labeling or Specific Activity of the labeled EpiSwitch® library.

5.4.2 Tecan Infinite M200 Pro

1) Follow Tecan instrument user guide to create a program to measure the absorbance at A_{260nm} and A_{280nm} (DNA), $A310_{nm}$ (background), A_{550nm} (cyanine 3), and A_{650nm} (cyanine 5).
2) Follow Tecan NanoQuant Plate user instructions to assess the samples for labeling efficiency.

5.4.3 Formulae for labelling assessment

$$\text{Degree of labelling} = \frac{340 \times \text{pmol/µL dye}}{\text{ng/µL DNA} \times 1000} \times 100\%$$

$$\text{Specific Activity} = \frac{\text{pmol/µL dye}}{\text{µg/µL DNA}}$$

$$\text{Yield (µg)} = \frac{\text{DNA concentration (ng/µL)} \times \text{Sample Volume (µL)}}{1000 \text{ ng/µg}}$$

The expected range of incorporation is between 15-25 pmol/µL for both dyes, however Cy5 usually gives lower incorporation than Cy3. The yield (total µg DNA) after labelling should be approximately equal for Cy3 and Cy5. Calculate the specific activity = pmol per µL of dye/ µg per µL DNA.

Combine results from the nanodrop or Tecan Infinite M200 Pro output with the calculated sample volumes to calculate yields and specific activity.

ID	A Tube + sample (mg)	B Tube weight (mg)	A-B Vol (µL)	Cy3 pmol/µL	Cy5 pmol/µL	Conc ng/µL	260:280	yield (µg) (conc x vol /1000)	Specific activity (pmol Cy3 or Cy5/µg)	QC pass/ fail ☑/☒
T1					-					
T2					-					
T3					-					
T4					-					
R1				-						
R2				-						
R3				-						
R4				-						

Labelling results table: adapt according to experimental design. “T” are Cy3-labelled Test samples and “R” are Cy5-labelled Reference samples. For dual-channel arrays T and R samples are paired for hybridization).

Input DNA (µg)	Yield (µg)	Specific Activity (pmol/µg)
0.2 (8-pack arrays)	3 - 6	15 - 50
0.5	8 - 13	20 – 60
1	9 - 14	20 - 60

Expected Yield and Specific Activity after labeling and column purification (Agilent data for gDNA). EpiSwitch® library template generates similar labelling results to gDNA with this protocol.

- For dual-color arrays only, combine the paired test and reference samples. The sample color should now appear violet.

- Proceed directly to hybridization or store combined labelled samples at -20 °C for up to 1 month, protected from light.

6. Array Processing

This chapter describes the steps to hybridize, wash and scan OBD EpiSwitch® Arrays.

Labelled samples are prepared for hybridization, loaded onto EpiSwitch® Explorer Arrays and incubated for 24 hours at 67 º with constant rotation.

Microarrays are washed and scanned immediately after hybridization is complete.

Agilent Feature Extraction Software is used to create text file data for analysis using the OBD EpiSwitch® Portal.

6.1 Hybridization

See Agilent protocol: Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Enzymatic Labeling For Blood, Cells, or Tissues (Agilent Technologies G4410-90010 Version 8.0), section 5 for in depth guidance on array processing. Follow instructions for 1-pack arrays.

6.1.1 Advance Preparation for Hybridization using modified heat blocks:

- Ensure the Hybridization oven is calibrated correctly according to the manufacturer's instructions (**allow 2-3 days for calibration**).
- Turn on the hybridization oven, set temperature to 67 ºC, activate and attach the data-logger to the rotor. Set rotor speed to maximum (ensure balanced or empty) and allow to equilibrate for **at least 2 hours (preferably overnight**).
- Ensure 10x Blocking Agent is reconstituted, in date and within freeze-thaw cycle limits prior to use (requires **1 hour** to reconstitute lyophilized 10x blocking agent).

- Ensure heat blocks are prepared and calibrated to 98 ºC and 37 ºC. Use the 1.5 mL tube holders with 1.5 mL LoBind tubes to ensure good conductivity. Allow **at least 1 hr** to equilibrate before use.

- Heat water in water baths to >75 ºC and 37 ºC.

6.1.2 Prepare labelled EpiSwitch® DNA for hybridization

Minimize sample exposure to light. Avoid direct sunlight.

Adjust the volume of the labeled samples (single-channel or dual-channel combined) to 158μL by the addition of 1xTE (Promega, pH 8.0) prior to setting up the hybridization cocktails.

Use the table below to calculate out how much 1x TE (Promega, pH 8.0) to add to each combined test and reference sample pair. For single channel arrays use the relevant Cy-dye column.

Hyb sample or pair ID	Cy3 vol (µL)*	Cy5 vol (dual only, µL)*	Total combined sample (µL)	Required vol (µL)	Required 1x TE pH 8 to add (µL)
1				158	
2				158	
3				158	
n				158	

Sample combination and TE volume calculation

*Sample volume after assessment of labelling efficiency.

Set up hybridization mixes in 1.5 mL LoBind microcentrifuge tubes and use 2 heat blocks calibrated to 98 ºC and 37 ºC with 1.5 mL tube adapters for incubations. **Pre-heat for at least 1 hour before use.** Add 750 µL 37ºC distilled water to the central wells of the 37ºC heat block.

Component	Volume required per sample (µL)
Human Cot-1 DNA (1mg/mL)	50
10x aCGH Blocking Agent	52
2x HI-RPM Hyb. Buffer	260
Total vol added	**362**

Hybridization components for 1M arrays

1. Add **50 µL** Human Cot-1 DNA to each tube (do not pipette up and down).
2. Add **52 µL** 10x Blocking Agent to each tube (do not pipette up and down).
3. Add **260 µL** 2x HI-RPM hybridization buffer. Avoid making bubbles and pipette slowly for greater accuracy (do not pipette up and down). **Close lids securely** and invert the tubes 4-6 times to mix. Briefly spin down to collect contents.
4. Pre-hyb set up:

   CAUTION – STEAM RISK. Use eye protection.

   Add 750 µL pre-heated distilled water (75-80 ºC) to the central tube holders of the 98ºC heat block immediately prior to adding the hybridization sample tubes. The water may begin to boil off rapidly.

   Check tube caps are securely in place then place tubes in the 98ºC “water bath” (pre-heated heat block + water), and time for precisely 3 minutes before transferring to the 37 ºC heat block water bath for 30 min. **Use metal forceps to move tubes.** Maintain the order of tube loading/transfer between the heat blocks to aid consistent timing. **Do the 98º C step in groups of max. 4 tubes at once.**

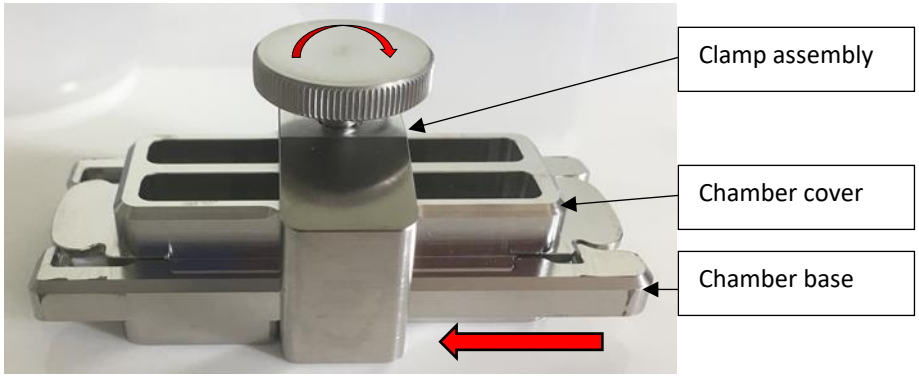
5. Prepare the Hybridization chamber assembly.

   It is recommended that a second person be present during hybridization set-up to independently verify slide numbers and record the sample loading details.

- Double check and record the barcodes of the slides and the order of the samples to be loaded.

- Prepare numbered hyb chambers, gasket slides and arrays to be ready to use with Agilent plastic tweezers and lint-free wipes pipette and tips readily to hand.

6. Once the Pre-Hyb program is complete the samples are ready to hybridize and must be loaded onto the arrays immediately. Set up one slide at a time and work in small batches (1-4), keeping samples at 37 °C on the thermal cycler or heat block until ready to load.
7. Load a clean gasket slide into the hyb chamber base with the gasket barcode label facing **up** and aligned with the rectangular section of the chamber base. Ensure the gasket slide is flush with the chamber base and not ajar.
8. Slowly dispense the hybridization sample onto the gasket slide using “drag and dispense” without touching the glass with the pipette tip. **Avoid the creation of bubbles.**
9. With clean gloved hands, carefully remove an array slide from its protective box and hold between thumb and forefinger, (or two forefingers if using both hands) with the “active side” (“Agilent” label) facing down and the numeric barcode face up. **Read the barcode aloud to the second person.** Take care not to touch the array area of the slide. Very carefully, lower the array slide (“Agilent” label face down) to within 3 mm of the loaded gasket slide using the four-chamber base guideposts and the rectangular end to ensure correct alignment. The barcode ends of both slides must line up at the rectangular end of the chamber base. Once lowered into position, gently rest the array onto the gasket slide. Do not attempt to move the slide sandwich or the chamber until the chamber cover has been secured in place otherwise there is a strong likelihood of leakage.
10. Place the hyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.



12. Hand-tighten the clamp clockwise **firmly** onto the chamber. **Only tighten, do not turn to loosen at all until hyb is complete or bubbles may be introduced and there is risk of sample leakage.**

13. Once tightened, vertically rotate the assembled chamber to wet the slide and check the mobility of the mixing bubble. There should ideally be only one large mixing bubble. Smaller bubbles are not a problem so long as they are not stationary. Tap the assembly on a hard surface if necessary, to dislodge any stationary bubbles.

Check once more that the assembly is firmly tightened, then load into the oven rotator rack before setting up the next chamber. Start from the center (position 3 or 4 when counting from the left) and balance the loading to prevent strain on the motor. Set rotation speed to maximum (20 rpm). Use an empty hyb chamber as a balance as and when required.

14. Record the hybridization start time for each chamber so that you can begin washing and scanning in the same order.

15. Hybridize at 67 ºC for 24 hours (optimization of hybridization time may be required, 24-48 hours recommended for 1M array format).

6.2 Array Washing

NOTE: Ozone

The array wash procedure must be done in environments where ozone levels are 5 ppb or less. The SureScan D array scanner uses a slide holder with a built-in ozone barrier.

For Agilent Scanner C, if ozone levels are between 5 to 10 in your laboratory, use the Agilent Ozone Barrier Slide Cover. If ozone levels in your lab exceed 10 ppb, use the Stabilization and Drying Solution together with the ozone barrier (Wash protocol B).

WARNING Wash protocol B (if used) must be performed in a ducted fume hood.

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.

- Before you begin, determine which wash procedure to use:
- Always use clean equipment when conducting the wash procedures.
- Use only dishes that are designated and dedicated for use with Agilent oligo aCGH wash buffers.

CAUTION

Do not use detergent to wash the staining dishes as some detergents may leave fluorescent residue on the dishes. If you do, you must ensure that all traces are removed by thoroughly rinsing with Milli-Q ultrapure water.

Details are given below for Agilent Wash protocol A.

For Agilent Wash protocol B (with Stabilization and Drying Solution) please see Agilent protocol: Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Enzymatic Labeling For Blood, Cells, or Tissues (Agilent Technologies G4410-90010 Version 8.0), section 5 “Array Wash”.

6.2.1 Preparation for array washing (24 hr in advance)

- Ensure glassware is clean, dry and free-from dust, ready for use.
- Pre-warm Wash buffer 2 at 37 °C overnight. Fill the buffer #2 staining dish to approx. ¾ full of Wash buffer 2. Cover with a lid and place in the 37 ºC incubator overnight. If multiple washes are needed and glassware is limited, take another 300-350 mL aliquot of wash buffer 2 in a clean bottle and warm in a water bath at 37 ºC overnight.
- Rinse the stir bars with Accugene water.

6.2.2 Set up on the day:

Before beginning washing, ensure the **scanner** is turned on. Switch the computer on first, then the scanner. **Wait** for the scanner light to switch off before activating the Agilent Microarray Scan Control software. The scanner light will turn back on when the software starts up. Check the status log at the bottom of the screen to ensure the scanner is ready for use.

- Ensure there is enough space on the hard drive for the number of scans planned. A typical, dual channel, full slide scan without compression will be in the region of 635,500 KB or 531,900 KB with loss-less compression. Single channel compressed scans are approximately 300,000 KB.
- Once array washing is completed scanning should begin **immediately**.
- Recommended scan resolution is 20-bit tif.
- Once slides are loaded into the scanner, the barcodes are read automatically, and it becomes possible to assign the scan protocol to the slide and add to the scanning queue. The SureScan D scanner has internal ozone-protection when the door is closed.

- Turn on the hotplate of the heated stirrer for wash #2 and set to 37 ºC.

Agilent wash procedure A

Step	Dish	Wash buffer	Temperature	Time
Disassembly	#1	Wash buffer 1	Room temperature	-
1st wash	#2	Wash buffer 1	Room temperature	5 min
2nd wash	#3	Wash buffer 2	37 °C	1 min (max 2 min)
Withdraw from Wash buffer 2				20-30 sec

Wash procedure A overview

Wash steps must be timed **precisely.**

Preparation:

1. Fill a glass staining dish (#1) with wash buffer 1 at room temp, ready for disassembly of the slides.
2. Prepare dish #2:
    a. Place a slide rack into dish #2 containing 1 clean blank microscope slide.
    b. Add a magnetic stir bar. Fill with enough wash buffer 1 to completely cover the slide rack and dummy slide.
    c. Place dish #2 on a magnetic stirrer (room temperature).
3. Prepare dish #3:
    a. The heated magnetic stirrer should be set to 37 °C.
    b. Keep dish #3 containing wash buffer 2 and magnetic stir bar in the 37 ºC incubator until 30 seconds before use, then bring out and place on the heated stirrer.* If processing more than 4 slides (1 wash group), ensure further 300-350 mL aliquots of wash buffer 2 in clean Nalgene bottles are prewarmed in a 37 ºC water bath.

* The temperature of the wash 2 step may be regulated using a pyrex dish waterbath set-up. See Agilent protocol: Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Enzymatic Labeling For Blood, Cells, or Tissues (Agilent Technologies G4410-90010 Version 8.0), section 5 “Array Wash” for in-depth details on this.

4. Prepare a timer set for 5 min and 1 min.

Have the correct number of slide holders ready for the slides to go into immediately after the washing procedure is finished. **Check the catches of the slide holders are in good condition and close with a “click”.** Keep slide holders clean, dry and free from dust.

Work quickly but carefully throughout the wash procedure.

Begin the washes:

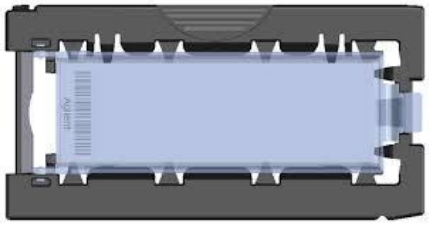
5. Remove the first hyb chamber from the oven and resume rotation of any others – add a balance chamber if necessary. Inspect the hyb chamber and record any features of note to assist with troubleshooting later e.g., presence of fixed bubbles, low volume/leakage etc.

6. Hyb chamber disassembly:
    a. Place the hyb chamber on a flat surface and loosen the thumbscrew, turning counterclockwise.
    b. Slide off the clamp assembly and remove the chamber cover.
    c. Using forceps and clean gloved fingers, remove the array-gasket sandwich from the chamber base by lifting from the barcode end and then grasping in the middle of the long sides (edges only). Keep the array slide numeric barcode facing up as you quickly transfer the sandwich to slide staining dish #1 containing aCGH/ChIP-on-Chip Wash buffer 1 (Wash Buffer 1). Keep hold of the barcode end with forceps or fingertips while the whole sandwich is submerged.

7. With the sandwich completely submerged in Wash Buffer 1, pry the sandwich open from the **barcode end only as follows:**
    a. Grip the array (top) slide with finger and thumb.
    b. Slip one of the blunt ends of the forceps between the slides.
    c. Gently twist the forceps to separate the slides.
    d. Let the gasket slide drop to the bottom of the dish while keeping hold of the array slide.
    e. Remove the microarray slide, grasp it from the upper corner at the barcode end with thumb and forefinger, and quickly put it into slide rack in the slide-staining dish #2 containing Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. Handle only the barcode portion of the array slide or its edges. **It is strongly advised to practice this maneuver beforehand.**

8. Repeat steps 5-7 for the remaining hyb chambers in turn, for up to 4 slides in any wash group. When all the slides are in the slide rack in dish #2 (Wash Buffer 1, room temp) begin stirring at 350 rpm for **5 min** (use timer). Adjust the speed to achieve good but not vigorous mixing (avoid creating bubbles!).

    When only 30 seconds of the first wash incubation remain, remove the pre-warmed staining dish #3 containing Wash Buffer 2 from the 37 ºC incubator and place on the pre-heated stir plate.
9. Wash in Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2 (Wash Buffer 2):
    a. Transfer the slide rack to staining dish #3 containing wash buffer 2 at 37 °C. Do not allow slides to dry out.
    b. Activate the stirrer and time for at least 1 minute (maximum 2 minutes). Adjust the speed to get thorough mixing without disturbing the array slides.
    c. Withdraw the slide rack **slowly** from Wash buffer 2 (take 5-10 seconds) so the slides dry evenly, minimizing droplet formation.
10. Immediately transfer the slides into the slide holders, ready for scanning.

    a. With the active surface (“Agilent” barcode) facing up, carefully place the end of the slide with the barcode label onto the slide ledge at the hinge end of the slide holder (right hand side in the image above left).
    b. Gently lower the array slide into the slide holder then use the Agilent tweezers at the very edges of the slide to push it flat down into place.
    c. Close the plastic slide cover, pushing on the tab end until you hear it **click**. Turn the slide holder through 180º so that the finger hold position is at the top and the hinge towards the left.

Scan slides immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a nitrogen purge box, in the dark until scanning can proceed.

6.3 Array scanning with SureScan D Scanner

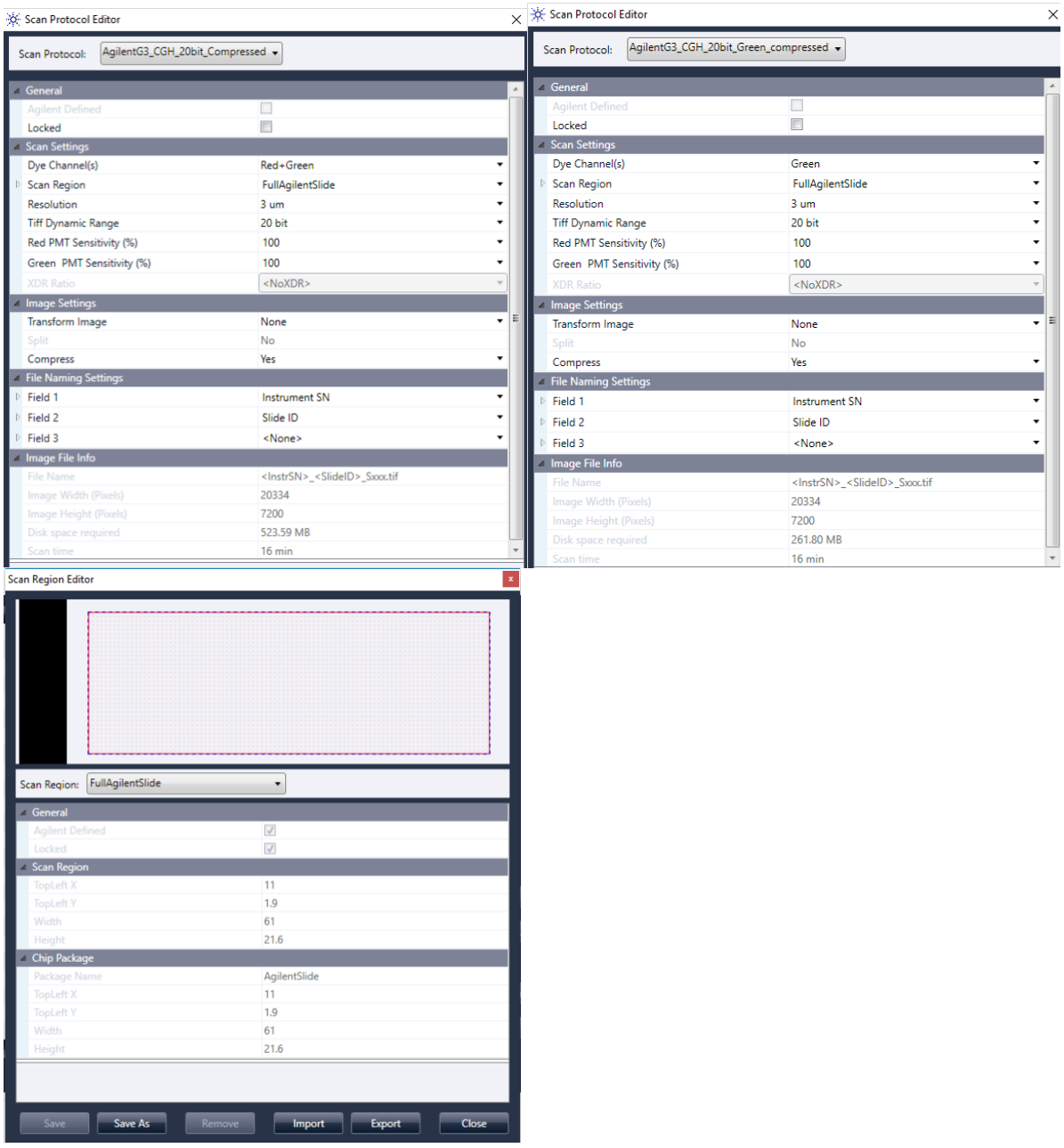
1. Use the scan control software **Open Door** button to access the scanner cassette. Place the slide holder into any open slot within the cassette with the barcodes oriented to the left and the “Agilent” label facing towards the operator. The slot number for the loaded slide blinks blue (e.g., positions 1 and 24 in the example below). Click on **Close Door**. The slides are now in an ozone-protected environment.

Note: Do not load empty slide holders (i.e., without slides) into the SureScan scanner!

Once the slide cassette door is closed the barcodes are detected in the scan slot positions (positions 1 and 24 in the example above).

2. Assign the scan protocol to be used by selecting from the drop-down list.
   Use protocol **AgilentG3_CGH_20bit_Compressed** for dual-color arrays.
   Use protocol **AgilentG3_CGH_20bit_Green_compressed** for Cy3-only labelled arrays.
   Use 20-bit scans as default.

Scan protocol details for AgilentG3_CGH_20bit_Compressed and AgilentG3_CGH_20bit_Green_compressed.



3. Check the output folder is correctly assigned to the local hard drive rather than a networked drive to minimize the risk of data interruption mid-scan.
4. Either click the button **All to Queue** to assign slides to the scanning queue or select queue positions manually using the drop-down menu under **Slide**.
5. **Start Scan** to begin scanning. Scan time is usually 15-16 minutes per slide.

6. When scanning is complete, check in the destination folder that the files are of the expected size and open the tiffs to view in Feature Extraction software. Check the Scan log at the bottom of the screen for any yellow or red warnings that may have occurred during scanning. Inspect the images to make that the full region has been scanned, that the washes look good and that there are no problems.

- If there is high background and/or the slides look smeared the slides may benefit from repeating the wash procedure. Set up fresh wash buffer 1 and 2 and go through the process again before rescanning. Repeated washing will cause a reduction in overall signal so this must be considered against any potential benefit of background/artefact reduction.

When scanning is complete, perform Feature Extraction. Add the .xml array design file (grid template) to the FE library – the array design ID is included within each array slide barcode.

To inspect an image file in Agilent FE software

1 Click on the toolbar **Open image file** button, or click **File > Open > Image**, to display the Open Dialog Box.

2 In the Open Dialog Box, double-click a .tif file from the scanner, or select a file and click **Open**.

You can also open an image file by dragging the file to the Feature Extraction desktop icon.

An image of the scanned array appears in the Workspace. Use the crop and zoom tools to inspect the image. Use the Log/Linear Color scale button to switch between views. The log scale allows low intensity portions of the array to be seen more clearly at the expense of detail at high intensities. Look for uneven washing, photo- or chemical-bleaching of the probes, good spot morphology etc.

6.4 Feature Extraction

Data are extracted from array images in **Feature Extraction (FE) projects**.

A **project** consists of one or more **extraction sets**, which in turn consist of an **array image file**, an .xml **grid file** and a **protocol**.

6.4.1 Running Feature Extraction

Add the grid template .xml array design file to the Grid template browser – the array design ID is included within each slide's individual barcode and must be checked to match before analysis.

For dual channel arrays, use the default feature extraction protocol for Agilent G3 CGH arrays: **CGH_1201_Sep17**

For single channel arrays, use a Green-only feature extraction protocol e.g., GE1_1200_Jun14.

Assign the correct design ID from the **Grid Name** panel in the protocol window.

After feature extraction is complete, the scan data text files and QC files will be ready to view in the destination folder and upload to the EpiSwitch® Portal for analysis

6.5 Washing up

Rinse slide-staining dishes, slide racks and stir bars thoroughly with high-quality Milli-Q ultrapure water before use and in between washing groups.

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 at least five times.
3) Repeat step 1 and step 2 until all traces of contaminating material are removed.

7. References

EpiSwitch® library preparation, quantification and positive control assays are detailed in the following technical protocols available for download with the EpiSwitch® Explorer array kit.

Advance Preparation Step	Protocol
Library Preparation	EXT-WI-014
Quantification and Normalisation	EXT -WI-017
Real Time PCR Detection	EXT -WI-035 – RFA control section

The following Agilent user guides provide in depth instructions for the use of aCGH Arrays, reagents, equipment, and software.

Agilent User guide name	url (as of Mar2021)
Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Enzymatic Labeling For Blood, Cells, or Tissues (with High Throughput Option)	https://www.agilent.com/cs/library/usermanuals/public/GEN-MAN-G4410-90010.pdf
Agilent G2534A Microarray Hybridization Chamber User Guide	https://www.agilent.com/cs/library/usermanuals/public/G2534-90004_HybridizationChamber_A1.pdf
Agilent G2545A Hybridization Oven	https://www.agilent.com/cs/library/usermanuals/public/G2545-90002_HybOven_Calibration_A1.pdf
Agilent SureScan microarray slide holders: Instructions for cleaning, storage, and use	https://www.agilent.com/cs/library/usermanuals/public/GEN-MAN-G5761-90004.pdf
Agilent G4900DA SureScan Microarray Scanner System Microarray Scan Control Software 9.1	https://www.agilent.com/cs/library/usermanuals/public/G4900-90000_SureScan_User.pdf
Agilent Feature Extraction 12.2	https://www.agilent.com/cs/library/usermanuals/public/G4460-90065.pdf

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