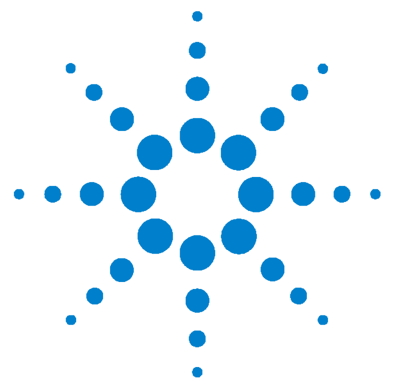

Agilent GenetiSure Dx Postnatal Assay

Part Number K1201A

For In Vitro Diagnostic Use

Rx Only

Instructions for Use

K1201-90001

Version H0, March 2026

Symbol Legend

Symbol	Description	Symbol	Description
REF	Catalog / code number	IVD	In Vitro Diagnostic Medical Device
	Temperature limitation		Manufacturer
	Consult Instructions for Use		Use-by date
Σ	Contains sufficient for <N> tests		Caution
EC REP	Authorized representative in the European Community	LOT	Batch code
	Do not reuse	%	Humidity limitation
UDI	Unique Device Identifier	CE 0123	European Conformity
	Corrosive	RCNS	Reconstitute with
CH REP	Authorized representative in Switzerland		Importer
Rx Only	Federal law restricts this device to sale by or on the order of a licensed healthcare provider		

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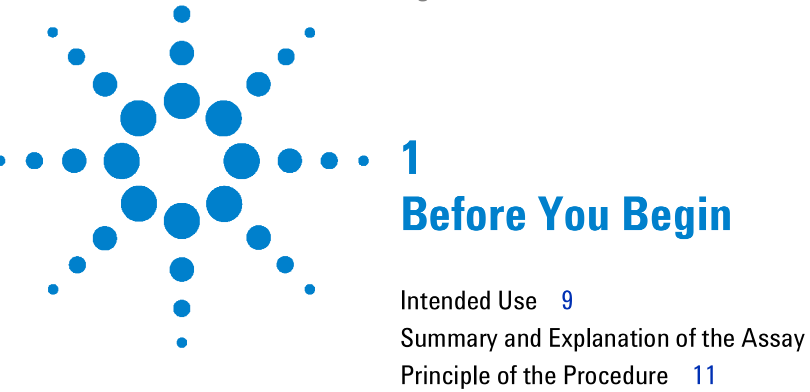

1 Before You Begin

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This chapter contains introductory information on the assay.

Carefully read all instructions prior to use.

Intended Use

For In Vitro Diagnostic Use

Intended Use

GenetiSure Dx Postnatal Assay is a qualitative assay intended for the postnatal detection of copy number variations (CNV) and copy-neutral loss of heterozygosity (cnLOH) in genomic DNA obtained from peripheral whole blood in patients referred for chromosomal testing based on clinical presentation. GenetiSure Dx Postnatal Assay is intended for the detection of CNVs and cnLOH associated with developmental delay, intellectual disability, congenital anomalies, or dysmorphic features. Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, parental evaluation, clinical genetic evaluation, and counseling, as appropriate. Interpretation of assay results is intended to be performed only by healthcare professionals, board certified in clinical cytogenetics or molecular genetics. The assay is intended to be used on the SureScan Dx Microarray Scanner System and analyzed by CytoDx Software.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing or screening, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

Summary and Explanation of the Assay

Comparative genomic hybridization (CGH) is a molecular cytogenetic method of screening cells for genomic DNA (gDNA) gains and losses at a sub-chromosomal level. Unlike traditional techniques used to detect CNVs, which rely on the examination of a single target and prior knowledge of the region under investigation, CGH produces a map of gDNA sequence copy number as a function of chromosomal location throughout the entire genome. In traditional CGH, differentially labeled test gDNA and reference

gDNA are hybridized simultaneously to chromosome spreads. The hybridization is detected with two different fluorochromes. CNVs are seen as changes in the ratio of the intensities of the two fluorochromes along the target chromosomes [Kallioniemi et al. (1992), Science, 258: 818-21]. The resolution of traditional CGH is limited to alterations of approximately 5-10 Mb.

Array CGH (aCGH) combines the principles of CGH with the use of microarrays [Schena et al. (1995), Science, 270: 467-470]. This approach overcomes the resolution limitations associated with traditional CGH. Instead of using metaphase chromosomes, glass slides arrayed with small segments of DNA are used [Lucito et al. (2003), Genome Research, 13: 2291-2305]. On an Agilent aCGH microarray, the DNA segments (known as probes) are created *in situ* directly on a glass slide. Because probes are several orders of magnitude smaller than metaphase chromosomes, the theoretical resolution of aCGH is proportionally higher than that of traditional CGH. The true level of resolution is determined by considering both probe size and the genomic distance between DNA probes.

Despite its advantages in CNV detection, aCGH alone cannot detect copy-neutral loss of heterozygosity (cnLOH). In cnLOH, a person’s genome includes a chromosome or part of a chromosome that lacks heterozygosity. For example, the person may receive two copies of a chromosome, or part of a chromosome, from one parent and no copies from the other parent due to errors in meiosis I or meiosis II. If the gDNA in a cnLOH interval is imprinted such that the genes in that region are monoallelically active (i.e., only the maternal or paternal allele of the pair is expressed), the resulting phenotype will be abnormal. Standard aCGH microarrays cannot detect cnLOH because they do not contain probes designed to detect single nucleotide polymorphisms (SNPs). To overcome this barrier, Agilent extended their aCGH microarrays to include a set of SNP probes on the same microarray (called a CGH+SNP microarray). Restriction digestion of the sample gDNA allows genotyping of SNPs located in the enzymes’ recognition sites. For each SNP probe, gDNA that has been cut at the restriction site results in a different fluorescent signal than that produced by uncut gDNA. Genotyping of SNPs allows for subsequent detection of cnLOH intervals. Thus, Agilent CGH+SNP microarrays, like those provided in the GenetiSure Dx Postnatal Assay, allow for the simultaneous, high-resolution detection of CNVs and cnLOH intervals.

Principle of the Procedure

The assay performed with the GenetiSure Dx Postnatal Assay is an *in vitro* diagnostic assay for use in a clinical laboratory. The assay is based on aCGH (molecular karyotyping).

Molecular karyotyping is a modified *in situ* hybridization technique that allows detection and mapping of gDNA sequence copy differences between two genomes in a single experiment. In molecular karyotyping analysis, two differentially labeled gDNA (patient sample and reference) are co-hybridized to complementary nucleic acid sequences synthesized *in situ* on a microarray slide.

Locations of copy number variants (CNV) in the gDNA segments of the patient sample genome are revealed by variable fluorescence intensity on the microarray.

The CGH+SNP microarray included in the GenetiSure Dx Postnatal Assay uses approximately 107,000 probes for CNV detection. Half of these probes were chosen to selectively hybridize to targeted regions designated by ISCA (the International Standards for Cytogenomic Arrays) to be of clinical interest and the other half were chosen to hybridize to sequences evenly spaced across the whole genome, commonly termed backbone probes.

The probes are distributed on the array, targeting overall 94% of the genome with at least 5 copy number probes per 400 kb, resulting in a median resolution of approximately 150 kb. Regions identified to be clinically relevant are targeted with increased probe density resulting in a median resolution of approximately 25 kb.

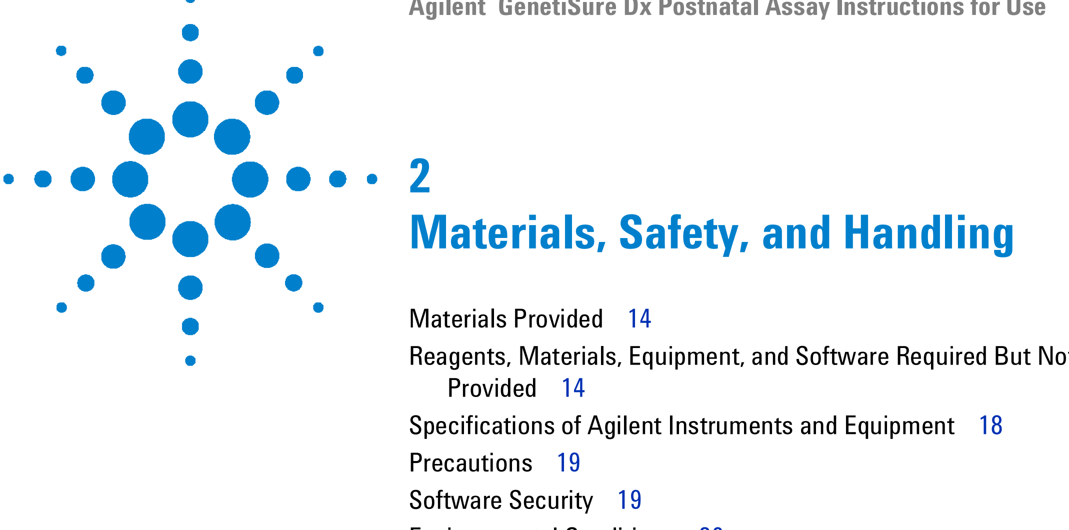
The assay compares the patient sample against a sex-matched reference sample. gDNA is extracted from the patient's whole blood and then is fluorescently labeled in parallel with the reference sample using two different fluorescent dyes. The two labeled samples are hybridized to complementary sequences (probes) that are printed on a CGH+SNP microarray.

After hybridization, the microarrays are washed and then scanned. The data from the microarray images are converted to numeric data. The relative abundance of the target sequences is computed based on the relative intensities of the fluorophores in the patient and reference samples hybridized to each of the probe sequences.

The numeric data is then processed using software specifically designed to report CNVs by chromosomal location. The reported CNVs are interpreted by a Board Certified Cytogeneticist, Molecular Geneticist, Molecular Pathologist, or similarly qualified clinician who has been trained to identify the clinically relevant CNVs, determine clinical significance, and report out these findings. cnLOH in patient samples is also reported to the clinician based upon allele data from the additional 59,647 SNP probes present on the Agilent microarray.

Regions of cnLOH are identified in the software by locating genomic regions with a statistically significant scarcity of heterozygous calls. First, the software's algorithm uses total and allele-specific copy numbers to identify each SNP site as having a homozygous or heterozygous genotype. Then, it uses a binomial probability distribution to report regions that contain an unusually high fraction of homozygous SNPs.

Software to support clinical interpretation of the results of the GenetiSure Dx Postnatal Assay diagnostic test is NOT a component of this device.



2
Materials, Safety, and Handling

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This chapter describes the reagents and other materials used in the assay and provides information for safely performing the assay.

Materials Provided

Table 1 lists the materials provided with the Agilent GenetiSure Dx Postnatal Assay. See "Product Storage" on page 22 for instructions on storing the materials.

Table 1 GenetiSure Dx Postnatal Assay (Agilent p/n K1201A)

Component	Quantity
4x180K microarray slides	6 slides; 4 assays per slide
Gasket slides	6 slides; 4 assays per slide

Reagents, Materials, Equipment, and Software Required But Not Provided

Table 2 through Table 6 list the reagents, materials, equipment, and software that are required but not provided with the Agilent GenetiSure Dx Postnatal Assay.

Table 2 Agilent kits required but not provided

Kit	Agilent p/n	Quantity	Form
Agilent GenetiSure Dx DNA Labeling Kit	K1201-64100	25 sample and 25 reference labeling reactions	Reagents and columns
Agilent GenetiSure Dx Hybridization Kit	K1201-64200	25 hybridization slides	Reagents
Agilent GenetiSure Dx Wash Buffer Set	K1201-64300	8 L Wash Buffer 1, 4 L Wash Buffer 2	Reagents
Agilent GenetiSure Dx Cot-1 Human DNA	K1201-64400	625 µL, 1 µg/µL	Reagent

Table 3 Other reagents and materials required but not provided

Reagent or Material	Form
1× TE (pH 8.0), Molecular biology grade (10 mM Tris-HCl containing 1 mM EDTA·Na_2)	Reagent
Ethanol, Molecular biology grade, 200 proof	Reagent
QIAamp DSP DNA Blood Mini kit (Qiagen p/n 61104)	Reagent
dsDNA-based fluorometric reagents	Reagent
Nuclease-free water (not DEPC-treated)	Reagent
Ultrapure water (resistivity at 25°C of 18.2 MΩ·cm)	Reagent/Materials
DNase-free tube strips, 200 µL, strips of 8, and DNase-free domed tube cap strips, strips of 8 OR DNase-free 96-well plates, 200 µL, semi-skirted OR DNase-free PCR tubes, 200 µL	Materials
Disposable plastic bottle for prewarming GenetiSure Dx Wash Buffer 2	Materials
Nuclease-free 1.5-mL microcentrifuge tubes (sustainable at 98°C)	Materials
Compression mats for thermal cycler (if needed)	Materials
Cold rack for 200-µL tubes or plates	Materials
Powder-free gloves	Materials
Sterile, nuclease-free, aerosol-barrier pipette tips	Materials

Table 4 Agilent instruments and equipment required but not provided (specifications provided in Table 7)

Instrument or Equipment	Form
SureScan Dx Microarray Scanner (Agilent p/n G5761AA)	Instrument
Hybridization Chamber Kit, Stainless	Equipment
Microarray Hybridization Oven	Equipment
Hybridization Oven Rotator Rack	Equipment
Hyb Station (Agilent p/n G5765A) - recommended but not required	Equipment
Thermal cycler	Instrument

Table 5 Other instruments and equipment required but not provided

Equipment	Form
Freezer, set to –20°C	Equipment
Refrigerator, set to 4°C	Equipment
1.5 L glass dish, Pyrex or equivalent	Equipment
250-mL capacity, glass slide-staining dish, with slide rack (×3)	Equipment
Heat blocks (optional)	Equipment
Clean forceps	Equipment
Ice bucket or cold rack	Equipment
Magnetic stir bar, 7.9 × 38.1 mm (×2)	Equipment
Magnetic stir plate	Equipment
Magnetic stir plate with heating element	Equipment
Microcentrifuge	Equipment
P10, P20, P200 and P1000 micropipettors	Equipment
Multi-channel pipettors (10 µL, 20 µL, and 200 µL) (optional)	Equipment
Programmable water bath or incubator set to 37°C	Equipment
DNase-free disposable troughs, 50 mL (optional)	Equipment
Timer	Equipment
Fluorometer	Instrument
UV-VIS spectrophotometer	Instrument
Vacuum desiccator or N_2 purge box for slide storage (see "Product Storage" on page 22)	Equipment
Vortex mixer with foam pad attachment	Equipment
Vacuum concentrator	Equipment
Mini plate spinner centrifuge	Equipment
96-well plate rack (if needed)	Equipment

Table 6 Software required but not provided

Component	Source
Agilent Microarray Scan Control program, including the GenetiSure_Dx_Postnatal scan protocol	Agilent Technologies The Agilent Microarray Scan Control program is included with the Agilent SureScan Dx Microarray Scanner. The GenetiSure_Dx_Postnatal scan protocol is available for download from the Agilent website; see “Set up the GenetiSure Dx Scan Control Protocol” on page 148.
Agilent CytoDx software	Agilent Technologies The Agilent CytoDx software can be downloaded free-of-charge from the Agilent website at: https://www.agilent.com/en/product/cgh-cgh-snp-microarray-platform/cgh-diagnostic-testing-ivd/genetisure-dx-postnatal-assay-4091940/download-cytodx-software This website also contains a link for a requesting the necessary software license.
Adobe Reader or other PDF viewing software	Adobe or other
Microsoft Excel or other spreadsheet or text editing software	Microsoft or other

Specifications of Agilent Instruments and Equipment

Table 7 lists the specifications of the Agilent instruments and equipment that are required for the assay.

Table 7 Agilent instrument and equipment specifications

Component	Specifications	
SureScan Dx Microarray Scanner (Agilent p/n G5761AA)	Dynamic Range	10^6 (with XDR scanning)
	Dynamic Autofocus	Continually adjusts scanner's focus, keeping features in focus at all times
	Compatible Dyes	Cyanine 3 and Cyanine 5, and Alexa 647, 555, and 660
	Laser Information	Green solid-state laser, 532 nm Red solid-state laser, 640 nm Power: controlled at 13mW
	Scan Window Maximum	71 mm × 21.6 mm
	PMT Adjustment	Allows adjustment of signal levels from 100% (default) to 1%
	Detection Limit	0.01 chromophores per square micron
	Pixel Placement Error	<1 pixel @ 5 micron resolution
	Uniformity	5% CV global non-uniformity; average local non-uniformity is typically 1% based on 100 micron features
Thermal Cycler; base unit and 96-well module	Block format	96 wells
	Block Temperature Range	4°C to 99°C
	Temperature Accuracy	±0.2°C (@95°C)
	Temperature Uniformity	±0.4°C (@95°C)
Hybridization Chamber Kit, Stainless	Apparatus	Stainless steel chamber top, base, clamp, thumbscrew, plastic tweezers, and Hybridization Chamber User Guide
Hybridization Oven	Rotor Motor Speed	2 to 20 RPM
	Operating Temperature Range	Ambient +5° to 70°C (+/– 0.1°C)
Hybridization Oven Rotator Rack	Capacity	Holds up to 24 hybridization chambers during hybridization oven incubation

Precautions

1. For In Vitro Diagnostic Use
2. Specimens should be handled as if infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories and in the CLSI Document M29-A. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 70% ethanol in deionized or distilled water.
3. This assay is for use only with gDNA extracted from human blood collected in the anticoagulant EDTA.
4. Wear appropriate personal protective equipment (PPE) – including disposable gloves, laboratory coat, and eye protection – when working in the laboratory or when handling specimens and reagents.
5. Do not pipette by mouth.
6. Do not eat, drink or smoke in laboratory work areas.
7. Wash hands thoroughly after handling specimens and reagents. Avoid contact of these materials with the skin, eyes, or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated.
8. If spills of these reagents occur, dilute with water before wiping dry.
9. Material Safety Data Sheets (MSDS) are available from the Agilent website at: www.agilent.com.
10. To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free, aerosol-barrier tips.

Software Security

The CytoDx software is part of the GenetiSure Dx Postnatal Assay, an in vitro diagnostic medical device.

The CytoDx software is intended for use in an environment in which system access is controlled by persons who are responsible for the content of electronic records that are on the system.

The Health Care Organization (HCO) where CytoDx is installed is responsible for maintaining system security on computer systems where the CytoDx software is installed.

The CytoDx software is a clinical laboratory application appropriate for use on computers and networks that meet all relevant security requirements for Electronic Protected Health Information (EPHI). Applicable administrative, physical, and technical safeguards must be in place prior to the installation and use of the CytoDx application.

- Computer systems must be physically and electronically protected
- HCO policies must be in place allowing only authorized users access to these systems
- An endpoint security solution must be installed on these systems that includes Virus, Spyware, Proactive, and Network threat protection
- Systems must be set to automatically secure themselves after periods of inactivity
- System and application security logs and audit trails must be monitored and evaluated against potential threats

Environmental Conditions

The Agilent GenetiSure Dx Postnatal Assay is designed for use in a clinical laboratory under controlled environmental conditions.

Operating Temperature	15° to 30°C
Operating Humidity	15% to 85% RH at 30°C

For equipment, instruments, and other materials that are required for the assay but not provided, verify that the environmental conditions are within specifications provided by the manufacturer.

Hybridized microarray slides are subject to ozone-induced dye degradation. Atmospheric ozone most significantly impacts the dyes during “Step 6. Microarray Washing” through “Step 8. Microarray Slide Scanning” in Chapter 6, “Microarray Processing Instructions”. The laboratory area used for the labeling, hybridization, washing, and scanning of the

microarray slides requires active ozone mitigation such as gas phase filtration using a carbon based media if atmospheric ozone concentration exceeds 5 ppb.

Ozone Level	Below 5 ppb

Cyanine dyes, predominantly cyanine 5 (red), and to a lesser extent, cyanine 3 (green), are subject to degradation by atmospheric ozone. As the two dyes used in the assay degrade at different rates, ozone exposure will first impact the QC metrics related to red (cyanine 5) signal intensity. These metrics include *rRepro*, *rSignalIntensity* and *rSignal2Noise*. Exposure to higher levels of ozone can also impact QC metrics related to green (cyanine 3) signal intensity, including *gRepro*, *gSignalIntensity* and *gSignal2Noise*. If microarray data were significantly impacted by ozone, then one or more of the above referenced metrics will be in the evaluate range, indicating that data may be compromised. If one or more of the QC metrics are “evaluate” and the ozone level was possibly elevated while the microarray slides were being washed and scanned, then the results are compromised and the assay should be repeated.

In addition to ozone, other oxidizing agents, such as sodium hypochlorite, can also promote dye degradation. Do not use sodium hypochlorite in the laboratory area used for the processing of the microarray slides.

Storage and Handling

Product Packaging

Upon receipt of the Agilent GenetiSure Dx Postnatal Assay, carefully inspect the product box for any visible signs of damage. After opening the product box, carefully inspect the silver pouch containing the box of microarray slides to make sure that the pouch is completely sealed.

If damage to the product box is detected, or the pouch’s vacuum seal has been compromised, contact Agilent Technical Support (see https://www.agilent.com/en/contact-us/page).

Product Storage

Store the entire Agilent GenetiSure Dx Postnatal Assay at room temperature (15°C to 30°C). After the microarray foil pouch is opened, store the microarray slides in the dark at room temperature in an environment protected from ozone and humidity (e.g., under a vacuum desiccator or N_2 purge box) for use until the expiration date. Do not store microarray slides in open air after breaking the foil.

Do not use microarrays or gasket slides past their expiration date.

Handling During Assay Execution

1. Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent bottles.
2. The use of sterile, nuclease-free pipettors and pipette tips is recommended.
3. Do not pool reagents from different lots or from different bottles of the same lot.
4. Do not use assay materials after their expiration dates.
5. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the gDNA isolation area and moving to the hybridization area.
6. Follow your institution's procedures or common practices for tracking samples throughout the assay, including procedures for tracking which sample is hybridized to each microarray.
7. Supplies and equipment for DNA isolation must be dedicated to that activity and not used for other activities or moved between areas.
8. Powder-free gloves must be worn in each area and must be changed before leaving that area.
9. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA.
10. Supplies and equipment must remain in their respective areas at all times.
11. Avoid repeated freeze-thaw cycles of solutions containing gDNA or enzymes.

12 When preparing frozen reagent stock solutions not containing gDNA or enzymes for use:

   a Thaw the aliquot as quickly as possible without heating above room temperature (15°C to 30°C).

   b Mix briefly on a vortex mixer, then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.

   c Store on ice or in a cold rack until use.

13 When preparing 96-well plates or 8x strip tubes for the thermal cycler, use domed cap strips (8x strips) to cap the strip tubes or the wells of the plate. *If you must use sealing tape, instead of cap strips, to seal plate wells, use a compression mat to avoid melting of the tape in the thermal cycler.*

14 When mixing reagents in a strip tube or 96-well plate:

   a On a vortex mixer, move the strip tubes or plate in a rocking motion, back and forth across the foam pad. Continue for at least 5 seconds.

   b Spin the plate or strip tubes in a centrifuge for 10 seconds or until all of the liquid is removed from the caps.

15 Sterile 200-µL PCR tubes can be used as an alternative to 96-well plates or 8x strip tubes in the thermal cycler.

16 All volumes stated in the instructions are intended to be used as specified within the tolerance ranges for standard micropipettors. Make sure that all pipettors are calibrated and operating within manufacturer’s specifications.

17 Never reuse a microarray slide.

Disposal

Dispose of unused reagents, waste and specimens in accordance with country, federal, state and local regulations.

Overview of the Workflow

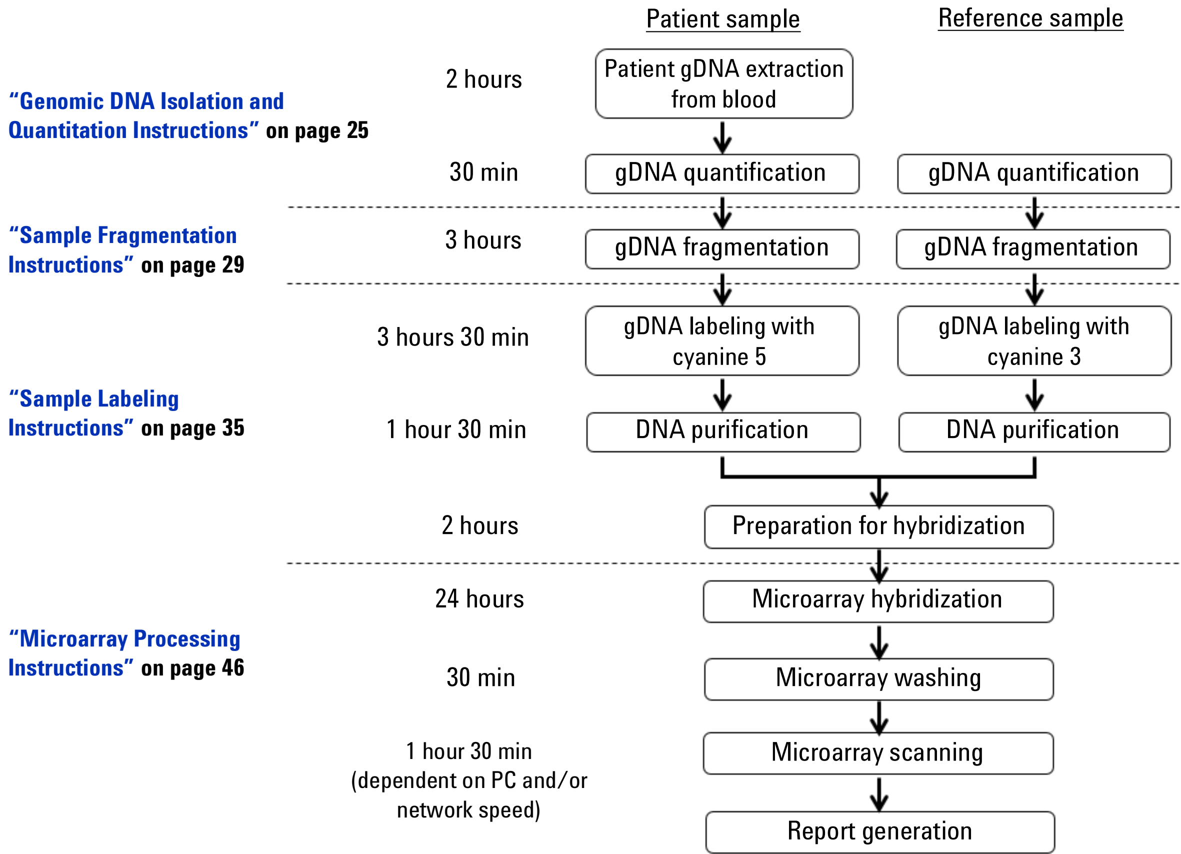
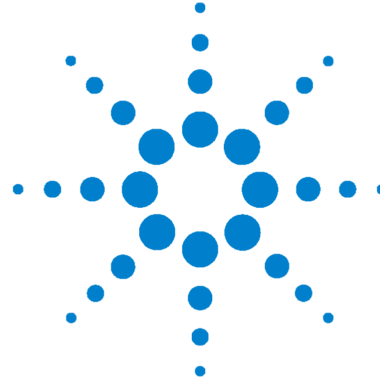


Figure 1 Agilent GenetiSure Dx Postnatal Assay workflow; time estimates are based on processing 24 patient samples



3
Genomic DNA Isolation and Quantitation Instructions

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Agilent's microarray-based GenetiSure Dx Postnatal Assay uses a "two-color" process to assess gDNA CNV and cnLOH in a patient sample relative to a sex-matched reference sample.

The gDNA used in this test is isolated from a patient's blood sample using the QIAamp DSP DNA Blood Mini kit from Qiagen (p/n 61104). The QIAamp DSP DNA Blood Mini kit requires 200 µL of blood to perform the extraction and the gDNA needs to be eluted in 100 µL.

The input amount of gDNA for the patient sample labeling reaction must be the same as for the reference sample labeling reaction. Inaccurate DNA quantitation can lead to different gDNA inputs into the patient and reference labeling reactions, which increases assay noise (as measured by the DerivativeLR_Spread QC metric).

Use only the QIAamp DSP DNA Blood Mini kit for gDNA isolation from blood. Other gDNA isolation methods are not supported.

Reagents, Materials, and Equipment List

Table 8 Required Reagents, Materials, Instruments, and Equipment for Chapter 3

Component	Form
200 µL of blood (patient sample)	—
QIAamp DSP DNA Blood Mini kit (Qiagen p/n 61104)	Reagent
Ethanol, Molecular biology grade, 200 proof	Reagent
dsDNA-based fluorometric reagents	Reagent
1× TE (pH 8.0), Molecular biology grade (10 mM Tris-HCl containing 1 mM EDTA·Na_2)	Reagent
Sterile, nuclease-free aerosol-barrier pipette tips	Materials
Nuclease-free 1.5-mL microcentrifuge tubes (sustainable at 98°C)	Materials
Personal Protective Equipment (PPE), such as lab coat, goggles, and disposable gloves	Equipment
Biosafety cabinet / fume hood	Equipment
50-mL graduated cylinder, or serological pipettes and pipette controller or bulb	Equipment
Test tube rocker	Equipment
Vortex mixer	Equipment
Heat blocks set to 56°C	Equipment
Microcentrifuge or Vacuum concentrator	Equipment
P20, P200 and P1000 micropipettors	Equipment
Timer	Equipment
Fluorometer	Instrument

Specimen Handling

Specimen Collection

The GenetiSure Dx Postnatal Assay is for use with gDNA from whole blood specimens only. Blood must be collected in tubes using EDTA as the anticoagulant.

Specimen Transport

Whole blood must be transported on wet ice in the same tube in which it was collected. Transportation of whole blood must comply with country, federal, state and local regulations for the transport of etiologic agents.

Specimen Storage

Blood specimens can be stored at 2–8°C for up to 7 days.

gDNA Extraction and Quantitation

gDNA Extraction

The gDNA must be isolated from 200 µL of the patient's whole blood using the QIAamp DSP DNA Blood Mini Kit from Qiagen (p/n 61104) following the instructions for use included with that kit. Use an elution volume of 100 µL. A total of 0.5 µg of gDNA is required for the labeling reaction. The lower limit of detection for the assay is 0.375 µg of gDNA for LOH calls and 0.250 µg of gDNA for CNV calls.

gDNA Quantitation

The concentration of gDNA must be confirmed using a double-stranded DNA-based fluorometric method. *Do not use absorbance based measurements (e.g., Nanodrop) to determine gDNA concentration.*

Inaccurate quantitation of the input gDNA can result in the purified, labeled gDNA failing the in-process QC measurements for yield or specific activity. Follow the manufacturer's protocol for the DNA-based fluorometric method and make sure that the calibration status of the fluorometer is up to date.

Prior to measuring the gDNA concentration, make sure that the gDNA is completely in solution by pipetting up and down. If the gDNA sample does not appear to be homogeneous after pipetting up and down, incubate at 37°C ±1°C for 25–30 minutes to assist with resuspension. Do not mix the gDNA sample on a vortex mixer.

The resulting gDNA sample must be at a measured concentration of 25 to 250 ng/µL to meet the sample fragmentation volume requirements. For the labeling reaction, a total of 0.5 µg of gDNA is required.

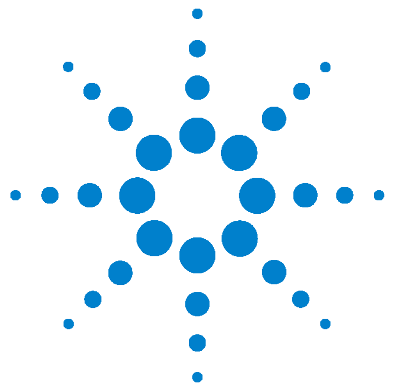
- If the gDNA sample concentration is less than 25 ng/µL, concentrate the sample by drying it with a vacuum concentrator. Then, reconstitute the gDNA in the needed volume of TE (pH 8.0) to obtain a concentration between 25 and 250 ng/µL. After reconstitution, quantitate the gDNA again to make sure the concentration is in the desired range.
- If the gDNA sample concentration is greater than 250 ng/µL, dilute the sample in TE (pH 8.0) to a concentration between 25 and 250 ng/µL. Then, quantitate the gDNA again to make sure the concentration is in the desired range of 25–250 ng/µL.

NOTE

An accurate measurement of the concentration of the Human Reference DNA is also critical. Confirm the concentration of the Human Reference DNA using a double-stranded DNA-based fluorometric method each time you perform the protocol. See “Step 1. Sample Preparation” on page 31 for instructions.

Sample Storage

Extracted gDNA may be stored at 2°C to 8°C for up to one year and at −15°C to −25°C for storage greater than one year.



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Sample Fragmentation Instructions

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Step 2. Restriction Digestion with the GenetiSure Dx DNA Labeling Kit 32

This chapter describes the fragmentation of gDNA for both the patient and the reference sample. The reference sample must be the same sex as the patient. If the sex of the patient is undetermined, use the male reference DNA.

Use ONLY the designated Agilent Reference DNA samples provided in the GenetiSure Dx DNA Labeling Kit (p/n K1201-64100).

In order to obtain high quality data, it is essential that the patient and reference samples that will be paired together on the microarray are treated identically as they are being processed.

Reagents, Materials, and Equipment List

Table 9 Required Reagents, Materials, Instruments, and Equipment for Chapter 4

Component	Form
Reagents from the GenetiSure Dx DNA Labeling Kit (Agilent p/n K1201-64100) Human Reference DNA Female Human Reference DNA Male 10X Restriction Enzyme Buffer BSA Nuclease Free Water Alu I Restriction Enzyme Rsa I Restriction Enzyme	Reagent
gDNA (0.5 µg in 20 µL of Qiagen Buffer AE or TE buffer, pH 8.0; isolated from patient sample)	—
DNase-free tube strips, 200 µL, strips of 8, and DNase-free domed tube cap strips, strips of 8 OR DNase-free 96-well plates, 200 µL, semi-skirted OR DNase-free PCR tubes, 200 µL	Materials
Nuclease-free 1.5-mL microcentrifuge tubes	Materials
Sterile, nuclease-free aerosol-barrier pipette tips	Materials
Personal Protective Equipment (PPE), such as lab coat, goggles, and disposable gloves	Equipment
Thermal cycler	Instrument
Cold rack for 200-µL tubes or plates	Equipment
96-well plate rack	Equipment
Vortex mixer with foam pad attachment	Equipment
Mini plate spinner centrifuge	Equipment
Microcentrifuge	Equipment
P10, P20, P200 and P1000 micropipettors	Equipment
Multi-channel pipettor (capable of pipetting 6µL), optional	Equipment
DNase-free disposable troughs, 50 mL, optional	Materials
Timer	Equipment
Ice bucket filled with ice, or cold rack for 1.5-mL tubes	Equipment/Materials

Step 1. Sample Preparation

NOTE

Sample pairs (consisting of one patient sample and one reference sample) must be processed in sets of four so that all four microarrays on a given microarray slide are hybridized. If you are processing fewer than four sample pairs, or the number being processed is not a multiple of four, then process one or more control pairs in order to obtain a multiple of four. A control pair consists of an Agilent Reference DNA Male sample and an Agilent Reference DNA Female sample.

Additionally, regardless of the number of sample pairs being processed, you may find it helpful to include one or more control pairs in the fragmentation and labeling steps. In the event that one of the sample pairs does not meet the necessary yield or specific activity requirements following labeling, you can continue to process the remaining sample pairs by replacing the failed sample pair with a labeled control pair.

1 Select the appropriate sex-matched reference sample based on the sex of the patient. See Table 10.

Table 10 Sex-matched reference sample

Patient sex	Reference sample
Female	Female Human Reference DNA
Male	Male Human Reference DNA

2 Make sure you have the required gDNA input amounts and volumes; see Table 11 for guidelines.

Confirm the gDNA concentration in the reference sample and in the patient sample using a double stranded DNA-based fluorometric method. See "gDNA Quantitation" on page 27 *for details.*

Table 11 Requirement of gDNA Input Amount and Volume per Microarray

gDNA type	gDNA input amount requirement (µg)	Volume of gDNA (µL)
Patient sample	0.5	20
Reference sample	0.5	20

Step 2. Restriction Digestion with the GenetiSure Dx DNA Labeling Kit

1 Thaw the 10X Restriction Enzyme Buffer and BSA (included with the GenetiSure Dx DNA Labeling Kit (p/n K1201-64100)). Briefly mix on a vortex mixer and spin in a microcentrifuge.

   Keep all reagents on ice or in a cold rack while in use and return them promptly to –25°C to –15°C.

2 For each patient sample and reference sample, add 0.5 µg of sample gDNA to a labeled nuclease-free tube (200-µL strip tube or individual 200-µL PCR tube) or to the well of a 96-well plate. Add enough nuclease-free water to bring the final volume to 20.0 µL. Put the reaction tubes or plate on ice or in a cold rack.

NOTE To ensure that each sample is tracked throughout the test, mark or label the tubes or plate with a waterproof pen.

3 Prepare the Digestion Master Mix by mixing the components in Table 12, based on the number of reactions required, in the order indicated in the table. Keep the mixture on ice.

Table 12 Preparation of Digestion Master Mix

Component	Per reaction (µL)	× 16 rxns (µL) (including excess)	× 32 rxns (µL) (including excess)	× 48 rxns (µL) (including excess)
Nuclease Free Water	2.2	37.4	74.8	110
10X Restriction Enzyme Buffer	2.6	44.2	88.4	130
BSA	0.2	3.4	6.8	10
Alu I Restriction Enzyme (10 U/µL)	0.5	8.5	17	25
Rsa I Restriction Enzyme (10 U/µL)	0.5	8.5	17	25
Final volume of Digestion Master Mix	**6.0**	**102**	**204**	**300**

4 Add 6.0 µL of Digestion Master Mix to each tube or well containing the gDNA sample to make a total volume of 26 µL.

5 Mix the samples using the appropriate procedure for the sample container.

For strip tubes and 96-well plates:

a Use domed cap strips (8x strips) to cap the strip tubes or the wells of the plate.

b On a vortex mixer, move the strip tubes or plate in a rocking motion, back and forth across the foam pad. Continue for at least 5 seconds.

c Spin the plate or strip tubes in a centrifuge for at least 10 seconds or until all of the liquid is removed from the caps. Put the samples back on ice or in a cold rack.

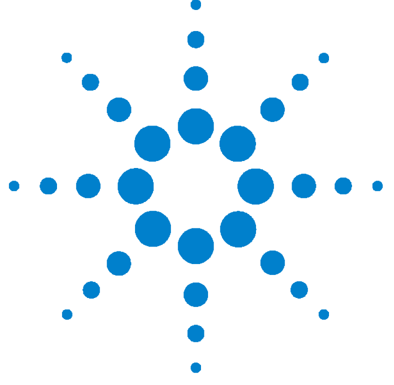
For individual 200-µL PCR tubes:

a Close the tubes, and then mix on a vortex mixer for at least 5 seconds.

b Spin the tubes in a microcentrifuge for at least 10 seconds. Put the samples back on ice or in a cold rack.

6. Set the thermal cycler to run the program below.
   - 37°C for 2 hours
   - 65°C for 20 minutes
   - 4°C hold
7. Transfer the plate or tubes into the thermal cycler, and then start the program. If prompted for the reaction volume, enter 26 µL.
8. At the end of the thermal cycler program, remove the samples from the thermal cycler and spin them in a centrifuge for at least 10 seconds to drive the contents off the walls and lid. Put samples on ice or in a cold rack.

At this point, you can store the digested gDNA for up to one month at –25°C to –15°C, or continue to "Sample Labeling Instructions" on page 35.



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Sample Labeling Instructions

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Step 2. Clean-up of Labeled gDNA 40
Step 3. Verification/Adjustment of Volumes 43
Step 4. Determination of Yield and Specific Activity 44

The GenetiSure Dx DNA Labeling Kit (p/n K1201-64100) contains sufficient two-color labeling reaction reagents, clean-up columns, and human reference DNA for the six 4x180K microarray slides included with the GenetiSure Dx Postnatal Assay.

The labeling kit uses Random Primers and the Exo (-) Klenow fragment to differentially label gDNA samples with fluorescently-labeled nucleotides. Label the patient sample with cyanine 5 and label the sex-matched reference sample with cyanine 3.

Reagents, Materials, and Equipment List

Table 13 Required Reagents, Materials, Instruments, and Equipment for Chapter 5

Component	Form
Fragmented gDNA (patient sample and reference sample)	—
Reagents and Materials from the GenetiSure Dx DNA Labeling Kit (Agilent p/n K1201-64100) Random Primers 5X gDNA Reaction Buffer 10X dNTP Mix Cyanine 3-dUTP Cyanine 5-dUTP Exo (-) Klenow Purification columns and 2-mL collection tubes	Reagent and Materials
1× TE (pH 8.0), Molecular biology grade (10 mM Tris-HCl containing 1 mM EDTA·Na_2)	Reagent
DNase-free tube strips, 200 µL, strips of 8, and DNase-free domed tube cap strips, strips of 8 OR DNase-free 96-well plates, 200 µL, semi-skirted OR DNase-free PCR tubes, 200 µL	Materials
Nuclease-free 1.5-mL microcentrifuge tubes (if performing "Step 2. Preparation of Labeled gDNA for Hybridization" on page 51 in a heat block)	Materials
Sterile, nuclease-free aerosol-barrier pipette tips	Materials
Laboratory tissue, such as KimWipes wipers	Materials
Personal Protective Equipment (PPE), such as lab coat, goggles, and disposable gloves	Equipment
Thermal Cycler	Instrument
Fluorometer	Instrument
Vacuum concentrator	Equipment
Cold rack for 200-µL tubes or plates	Equipment
96-well plate rack	Equipment

Table 13 Required Reagents, Materials, Instruments, and Equipment for Chapter 5

Component	Form
Vortex mixer with foam pad attachment	Equipment
Mini plate spinner centrifuge	Equipment
Microcentrifuge	Equipment
P10, P20, P200 and P1000 micropipettors	Equipment
Multi-channel pipettors (capable of pipetting 19 µL), optional	Equipment
DNase-Free disposable troughs, 50 mL, optional	Materials
Timer	Equipment
Ice bucket filled with ice, or cold rack	Equipment/ Materials

Step 1. Fluorescent Labeling of gDNA

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 fragmented gDNA samples in a microcentrifuge for a minimum of 10 seconds to drive the contents off the walls and lid.
2 Add Random Primers:
   - Add 5 µL of Random Primers (included in the GenetiSure Dx DNA Labeling Kit (p/n K1201-64100)) to each reaction tube or well containing a gDNA sample to make a total volume of 31 µL.
3 Mix the samples using the appropriate procedure for the sample container.

For strip tubes and 96-well plates:

- **a** Use domed cap strips (8x strips) to cap the strip tubes or the wells of the plate.
- **b** On a vortex mixer, move the strip tubes or plate in a rocking motion, back and forth across the foam pad. Continue for at least 5 seconds.
- **c** Spin the plate or strip tubes in a centrifuge for at least 10 seconds or until all of the liquid is removed from the caps. Put the samples back on ice or in a cold rack.

For individual 200-µL PCR tubes:

- **a** Close the tubes, and then mix on a vortex mixer for at least 5 seconds.
- **b** Spin the tubes in a microcentrifuge for at least 10 seconds. Put the samples back on ice or in a cold rack.

4. Set the thermal cycler to run the program below.
   - 98°C for 3 minutes
   - 4°C for 5 minutes
   - 4°C hold
5. Transfer the plate or tubes into the thermal cycler, and then start the program. If prompted for the reaction volume, enter 31 µL.
6. At the end of the thermal cycler program, remove the samples from the thermal cycler and spin them in a centrifuge for at least 10 seconds to drive the contents off the walls and lid. Put samples on ice or in a cold rack.

NOTE To avoid prolonged exposure to light, keep the tubes of Cyanine 3-dUTP and Cyanine 5-dUTP and the tubes of the Labeling Master Mixes covered with foil or a darkened lid.

7. Prepare the Labeling Master Mixes and add to samples:
   - **a** Prepare two Labeling Master Mixes by mixing the components in Table 14 in the order indicated. Use Cyanine 5-dUTP for the Labeling Master Mix for patient samples, and use Cyanine 3-dUTP for

the Labeling Master Mix for reference samples. Keep the Labeling Master Mixes on ice or in a cold rack during preparation.

b Mix the Labeling Master Mixes thoroughly on a vortex mixer, then spin them in a microcentrifuge for at least 10 seconds to drive the contents off the walls and lid.

c On ice or in a cold rack, add 19 µL of the appropriate Labeling Master Mix (cyanine 3 or cyanine 5) to each reaction tube or well containing a gDNA sample to make a total volume of 50 µL.

Table 14 Preparation of Labeling Master Mixes

Component	Per reaction (µL)	× 8 rxns (µL) (including excess)	× 16 rxns (µL) (including excess)	× 24 rxns (µL) (including excess)
5X gDNA Reaction Buffer	10.0	85	170	250
10X dNTP Mix	5.0	42.5	85	125
Cyanine 3-dUTP, *or* Cyanine 5-dUTP	3.0	25.5	51	75
Exo (-) Klenow	1.0	8.5	17	25
Final volume of Labeling Master Mix	**19.0**	**161.5**	**323**	**475**

8 Mix the samples using the appropriate procedure for the sample container.

For strip tubes and 96-well plates:

a Use domed cap strips (8x strips) to cap the strip tubes or the wells of the plate.

b On a vortex mixer, move the strip tubes or plate in a rocking motion, back and forth across the foam pad. Continue for at least 5 seconds.

c Spin the plate or strip tubes in a centrifuge for at least 10 seconds or until all of the liquid is removed from the caps. Put the samples back on ice or in a cold rack.

For individual 200-µL PCR tubes:

a. Close the tubes, and then mix on a vortex mixer for at least 5 seconds.
b. Spin the tubes in a microcentrifuge for at least 10 seconds. Put the samples back on ice or in a cold rack.

9. Set the thermal cycler to run the program below.
   - 37°C for 2 hours
   - 65°C for 10 minutes
   - 4°C hold
10. Transfer the plate or tubes into the thermal cycler, and then start the program. If prompted for the reaction volume, enter 50 µL.
11. At the end of the thermal cycler program, remove the samples from the thermal cycler and spin them in a centrifuge for at least 10 seconds to drive the contents off the walls and lid. Put the samples on ice or in a cold rack.

At this point, you can store the labeled gDNA for up to one month at –25°C to –15°C, or continue to “Step 2. Clean-up of Labeled gDNA”.

Step 2. Clean-up of Labeled gDNA

Labeled gDNA is purified using the purification columns and 2-mL collection tubes provided with the GenetiSure Dx DNA Labeling Kit (p/n K1201-64100).

NOTE Throughout this clean-up step, keep cyanine 3 and cyanine 5 labeled gDNA samples separated and protected from prolonged light exposure.

1. Spin the labeled gDNA samples in a centrifuge for at least 10 seconds to drive the contents off the walls and lid.
2. For each gDNA sample to be purified, place a reaction purification column into a 2-mL collection tube.

NOTE To ensure that each sample is tracked throughout the test, label the spin columns and collection tubes with a waterproof pen.

3 Add 330 µL of 1× TE (pH 8.0) to each spin column.

4 To each reaction tube or well, add 100 µL of 1× TE (pH 8.0). Pipette the contents up and down 5 times to mix.

5 Transfer each sample to the appropriately labeled spin column. Pipette the contents up and down 10 times to mix.

6 Close the tube lids and spin the samples for 10 minutes at approximately 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through and place the columns back in the same collection tubes.

NOTE Be sure to thoroughly discard all flow-through. If necessary, wipe the rim of the collection tubes with a clean laboratory tissue (e.g., KimWipes wipers) to remove excess liquid. Use appropriate technique to avoid contaminating the gDNA samples loaded on the columns.

7 Add 480 µL of 1× TE (pH 8.0) to each column. Spin for 10 minutes at approximately 14,000 × g in a microcentrifuge at room temperature.

8 Invert the column into a fresh, appropriately labeled 2-mL collection tube using the following procedure.

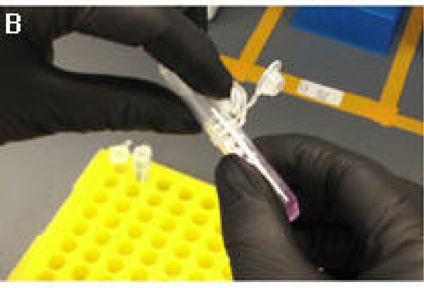
   a Keep the old collection tube upright throughout this step. With the fresh collection tube in one hand and the old collection tube in the other hand (panel A in Figure 2 on page 42), invert the fresh collection tube and use it as tweezers to grasp the edge of the column (panel B in Figure 2). Pull the column out of the old collection tube (panel C in Figure 2).

   b Maintain the grip on the column as you flip the fresh collection tube from an inverted to an upright position. Then, release the grip on the column to allow it to slide into the fresh collection tube in the inverted position (panel D in Figure 2). Discard the old collection tube containing the flow-through.

9 Spin the collection tubes containing the inverted columns for 1 minute at approximately 1,000 × g in a microcentrifuge at room temperature to collect the purified sample.

At this point, you can store the labeled gDNA samples at –25°C to –15°C overnight, or continue to “Step 3. Verification/Adjustment of Volumes” on page 43.

In the user's right hand is the old collection tube containing the column. In the user's left hand is the fresh collection tube.



The user inverts the fresh collection tube and places it over the top of the column. The column is still seated in the old collection tube and that tube remains upright.

The user squeezes the fresh collection tube, using it as tweezers to grasp the edge of the column and pull the column out of the old collection tube. The user returns the fresh collection tube to an upright position.

With the fresh collection tube in an upright position (left hand), the user releases the grip on the column, allowing it to slide into the tube. The old collection tube (right hand) containing the flow-through can now be discarded.

Figure 2 Inversion of the column into a fresh collection tube.

Step 3. Verification/Adjustment of Volumes

Make sure that the volume of each purified sample is within the desired range (18–28 µL) using one of the two approaches described below.

- Concentrate all samples using the procedure in Table 15, then proceed to "Step 4. Determination of Yield and Specific Activity" on page 44.

  OR

- Measure and record the volumes of each purified sample and then take the following actions:
  - For samples with a volume between 18 and 28 µL, proceed directly to "Step 4. Determination of Yield and Specific Activity" on page 44 without concentrating the samples.
  - For samples with a volume greater than 28 µL, concentrate the samples using the procedure in Table 15, then proceed to "Step 4. Determination of Yield and Specific Activity" on page 44.
  - For samples with a volume less than 18 µL, bring the volume up to 21 µL with 1× TE (pH 8.0), then proceed to "Step 4. Determination of Yield and Specific Activity" on page 44.

Table 15 Concentration of gDNA

1	Concentrate the gDNA sample to dryness and resuspend in 21 µL of 1× TE (pH 8.0). Do not excessively dry the gDNA because the pellets will become difficult to resuspend.
2	Put the tube or plate that contains the labeled gDNA sample on ice for at least 5 minutes, and then pipette the solution up and down 10 times to fully resuspend the pellet.

Step 4. Determination of Yield and Specific Activity

NOTE Throughout this step, keep the samples of labeled gDNA on ice and protected from light when not in use.

Use a UV-VIS spectrophotometer to measure the A260nm, A550nm (cyanine 3), A650nm (cyanine 5) and the pmol per µL of dye to determine the yield and specific activity. Use 1× TE (pH 8.0) to blank the spectrophotometer.

1. Use 1.5 µL of purified labeled gDNA for quantitation. Measure the absorbence at 260 nm (DNA), 550 nm (cyanine 3), and 650 nm (cyanine 5).
2. Calculate the Specific Activity of the labeled gDNA:

$$\text{Specific Activity*} = \frac{\text{pmol per } \mu\text{L of dye}}{\mu\text{g per } \mu\text{L genomic DNA}}$$

*pmol dye per µg gDNA

3. Record the gDNA concentration (ng/µL) for each sample. Calculate the yield as:

$$\text{Yield } (\mu\text{g}) \quad \frac{\text{DNA Concentration (ng/}\mu\text{L)} \cdot \text{Sample Volume (}\mu\text{L)}}{1000 \text{ ng/}\mu\text{g}}$$

Refer to Table 16 for expected yield of labeled gDNA and specific activity after labeling and clean-up, when starting with high quality gDNA.

Table 16 Expected Yield and Specific Activity after Labeling and Clean-up

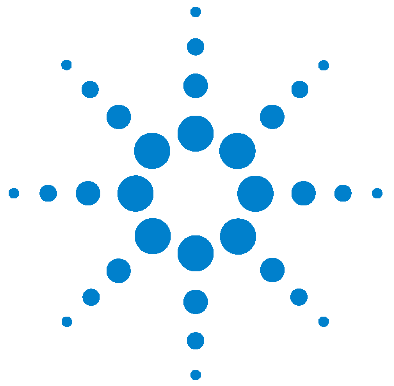
Input gDNA (µg)	Yield (µg)	Specific Activity of Cyanine 3-Labeled Sample (pmol/µg)	Specific Activity of Cyanine 5-Labeled Sample (pmol/µg)
0.5	8 to 15	20 to 50	20 to 45

After labeling, the yields of the cyanine 3-labeled sample and the cyanine 5-labeled sample should be approximately the same. If not, refer to “Troubleshooting” on page 136.

Calculations of yield and specific activity are in-process QC measurements that must meet the expected values in order to proceed to microarray hybridization. To help avoid sub-optimal hybridization, which could lead to failure of downstream actionable array QC metrics, do not proceed if the yield or specific activity of the purified, labeled gDNA is not within the expected range specified in Table 16.

All four microarrays on a given microarray slide must be hybridized with labeled gDNA that is within the expected yield and specific activity range. If a sample does not meet either the yield or specific activity range, relabel it before proceeding. Alternatively, if the other samples need to be processed urgently, replace the sample pair that contains the failed sample with a labeled control pair (if a labeled control pair is available).

At this point, you can store the labeled gDNA for up to one month at –25°C to –15°C in the dark, or continue to "Microarray Processing Instructions" on page 46. Alternatively, you can first combine the patient and reference samples (see "Step 1. Mixing of the Patient and Reference Sample" on page 50), and then store for up to one month at –25°C to –15°C in the dark.



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Microarray Processing Instructions

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Microarray processing consists of hybridization, washing, preparation for scanning, and scanning.

Reagents, Materials, and Equipment List

Table 17 Required Reagents, Materials, Instruments, and Equipment for Chapter 6

Component	Form
Labeled gDNA (patient sample and reference sample)	—
Reagents from the GenetiSure Dx Hybridization Kit (Agilent p/n K1201-64200) 10X Oligo aCGH Blocking Agent 2X CGH Hybridization Buffer	Reagent
GenetiSure Dx Cot-1 Human DNA (Agilent p/n K1201-64400)	Reagent
GenetiSure Dx Wash Buffer Set (Agilent p/n K1201-64300) Includes GenetiSure Dx Wash Buffers 1 and 2	Reagent
DNase-free tube strips, 200 µL, strips of 8, and DNase-free domed tube cap strips, strips of 8 OR DNase-free 96-well plates, 200 µL, semi-skirted OR DNase-free PCR tubes, 200 µL	Materials
Nuclease-free 1.5-mL microcentrifuge tubes	Materials
Sterile, nuclease-free aerosol-barrier pipette tips	Materials
Ultrapure water (resistivity at 25°C of 18.2 MΩ·cm), pre-warmed overnight at 37°C	Reagent/Materials
Disposable plastic bottle for prewarming GenetiSure Dx Wash Buffer 2	Materials
Black waterproof marking pen	Materials
Personal Protective Equipment (PPE), such as lab coat, goggles, and disposable gloves	Equipment
SureScan Dx Microarray Scanner* (Agilent p/n G5761AA) Includes integral 24-slide cassette, 24 slide holders, computer workstation with recovery software, power cords and network cable, Agilent Scan Control software, and Agilent Installation Qualification Tool Software	Instrument
GenetiSure_Dx_Postnatal scan protocol for the Agilent Microarray Scan Control software (see page 148 for instructions)	Software
GenetiSure Dx Postnatal Assay (Agilent p/n K1201A) Includes microarray slides and gasket slides	Equipment
Hybridization Oven**	Equipment

Table 17 Required Reagents, Materials, Instruments, and Equipment for Chapter 6

Component	Form
Hybridization Oven Rotator Rack	Equipment
Hybridization Chamber Kit, Stainless	Equipment
Vacuum desiccator or N_2 purge box for slide storage (see "Product Storage" on page 22)	Equipment
Forceps	Equipment
Thermal Cycler	Instrument
Incubator (set to 37°C)	Equipment
Programmable water bath, if using (set to 37°C)	Equipment
Heat blocks set to 37°C and 98°C	Equipment
Microcentrifuge	Equipment
P10, P20, P200 and P1000 micropipettors	Equipment
Multi-channel pipettors (capable of pipetting 71 µL), optional	Equipment
DNase-Free disposable troughs, 50 mL, optional	Materials
Timer	Equipment
Magnetic stir bar, 7.9 × 38.1 mm (×2)	Equipment
Magnetic stir plate	Equipment
Magnetic stir plate with heating element (set to 37°C)	Equipment
1.5 L glass dish	Equipment
250-mL capacity slide-staining dish, with slide rack (×3)	Equipment

* All components of the SureScan Dx Microarray Scanner need to be set up in order to perform these instructions. Refer to the *Agilent G5761A SureScan Dx Microarray Scanner System User Guide* for set up instructions.

** Agilent recommends periodic calibration of the hybridization oven following manufacturer's procedures.

10X Oligo aCGH Blocking Agent (5190-7319) WARNING. Contains: 2-Amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride. Causes skin irritation. May cause respiratory irritation. Causes serious eye irritation. Wear protective gloves. Wear eye or face protection. Avoid breathing dust. IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. Call a POISON CENTER or physician if you fell unwell. Store locked up. Dispose of contents and container in accordance with all local, regional, national and international regulations.

2X CGH Hybridization Buffer (5190-7320) DANGER. Contains: 4-Morphonlineethanesulfonic acid, monohydrate; Lithium chloride; lithium dodecyl sulphate. Causes skin irritation. Causes serious eye damage. Wear protective gloves. Wear eye or face protection. Wash hands thoroughly after handling. IF IN EYES: Rinse cautiously with water for several minutes. Immediately call a POISON CENTER or physician.

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 glass slide. See Figure 3.

The hybridization sample mixture is applied directly to the gasket slide and not to the microarray slide. Then the active side of the microarray slide is put on top of the gasket slide to form a "sandwich" made up of the gasket slide and the microarray slide.

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.

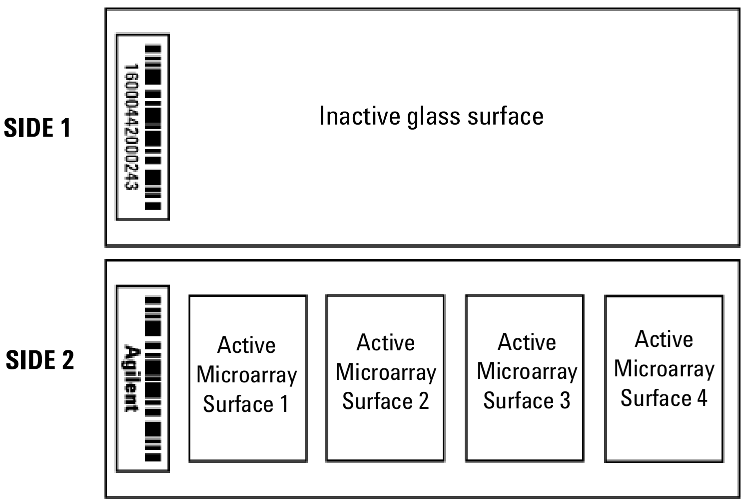


Figure 3 Slide orientation

Always hybridize a sample to all four microarrays on a given microarray slide. Failure to do so will result in a failed assay with no report generated by CytoDx.

Step 1. Mixing of the Patient and Reference Sample

1 Label the tubes in which you will be mixing the patient and reference samples so that you know which sample pair is in each tube or well.

- If you will be performing "Step 2. Preparation of Labeled gDNA for Hybridization" in a thermal cycler, use strip tubes or PCR tubes. If using strip tubes, first prepare the strip tubes for the thermal cycler incubation by cutting 8x strip tubes in half to make 4x strip tubes. Make enough 4x strip tubes for all sample pairs.
- If you will be performing "Step 2. Preparation of Labeled gDNA for Hybridization" on a heat block, use 1.5-mL microcentrifuge tubes.

2 In the appropriately labeled tube or well, combine patient and reference sample using the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample. Use the entire volume of both samples. The total volume of the mixture will either be approximately 39 µL (if the

samples were concentrated in “Step 3. Verification/Adjustment of Volumes”) or between 33 µL and 53 µL (if the samples were not concentrated in “Step 3. Verification/Adjustment of Volumes”). Keep the combined samples on ice or in a cold rack.

Step 2. Preparation of Labeled gDNA for Hybridization

1 Prepare the **10X Blocking Agent**:

   a Add 1.35 mL of nuclease-free water to the vial containing lyophilized 10X Oligo aCGH Blocking Agent, Lyophilized (included in the GenetiSure Dx Hybridization Kit (p/n K1201-64200)).

   b Leave at room temperature for at least 60 minutes and mix on a vortex mixer to reconstitute sample before use or storage. Make sure that the 10X Oligo aCGH Blocking Agent has completely gone into solution.

NOTE The 10X Oligo aCGH Blocking Agent can be prepared in advance and stored at –25°C to –15°C for up to 120 days. Do not leave it at room temperature for longer than 3 hours.

2 Confirm that you have the necessary number of chamber bases and clamps, gasket slides, and microarray slides. Confirm that the chamber bases, chamber covers, and clamps are clean and ready to use.

3 Confirm that the hybridization oven is set to 67°C.

4 If using 1.5-mL microcentrifuge tubes for the incubations, prepare two heat blocks: one at 98°C and one at 37°C.

5 Prepare the samples for hybridization:

   a Mix the components in Table 18 at room temperature to prepare the Hybridization Master Mix. Briefly and gently mix the Hybridization Master Mix on a vortex mixer and then briefly spin it in a centrifuge.

NOTE The 2X CGH Hybridization Buffer is viscous. Pipette the buffer slowly to ensure the accuracy of the volume.

Table 18 Preparation of Hybridization Master Mix

Component	Per reaction (µL)	x 8 rxns (µL) (including excess)	x 16 rxns (µL) (including excess)	x 24 rxns (µL) (including excess)
GenetiSure Dx Cot-1 Human DNA (1.0 µg/µL)	5	45	87.5	130
10X Oligo aCGH Blocking Agent (prepared in step 1 on page 51)	11	99	192.5	286
2X CGH Hybridization Buffer	55	495	962.5	1430
Final volume of Hybridization Master Mix	**71**	**639**	**1242.5**	**1846**

b Add 71 µL of the Hybridization Master Mix to each combined sample for a total volume of approximately 110 µL.

c Mix the samples by pipetting up and down, then briefly spin in a centrifuge to drive contents to the bottom of the reaction tubes.

d Incubate the samples at 95°C to 98°C for 3 minutes then at 37°C ±2°C for at least 30 minutes but not longer than 75 minutes.

- If using the thermal cycler, transfer the 4x strip tubes or PCR tubes into the thermal cycler. Set the thermal cycler to run the following program.
  - 98°C for 3 minutes
  - 37°C for 75 minutes

  If prompted for the reaction volume, enter 100 µL.
- If using a heat block, transfer the 1.5-mL microcentrifuge tubes to 95°C to 98°C for 3 minutes and then to 37°C ±2°C for at least 30 minutes but not longer than 75 minutes.

While the samples are incubating, perform “Step 3. Preparation of Chamber Assemblies” (below) within the elapse of 30 minutes.

Step 3. Preparation of Chamber Assemblies

NOTE Perform "Step 3. Preparation of Chamber Assemblies" while the samples are incubating at 37°C.

1 Check that the work area is flat, clean, and free from potential contaminants.
2 Prepare the needed quantity of labeled chamber assemblies and gasket slides to process samples. Disassemble chambers, if needed, and line up the chamber bases and chamber clamps.

Figure 4 Chamber assemblies (left) and packaged gasket slides (right)

3 Prepare pipettors and tips for sample loading.
4 To better track samples, mark your chamber bases and both sides of the clamps (see arrows in Figure 5) with a black waterproof marking pen.

   Marking the sides of the clamps will allow you to see the mark while the chamber assemblies are in the hybridization oven.

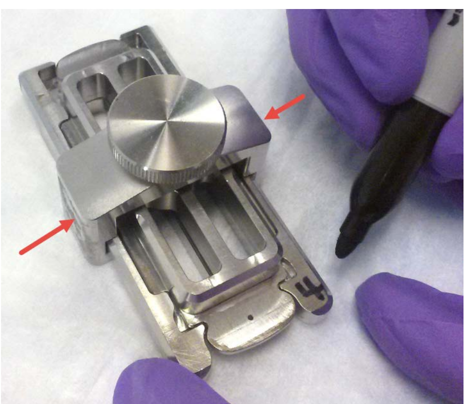


Figure 5 Labeling of chamber bases

Step 4. Microarray Hybridization

Perform "Step 4. Microarray Hybridization" for the first set of four samples to be removed from the 37°C incubation and processed. Then, repeat the step for the remaining sets of four samples. Make sure that none of samples are allowed to incubate at 37°C for longer than 75 minutes.

Agilent recommends using the Agilent Hyb Station (p/n G5765A) to prepare the hybridization assembly. Use of the Hyb Station simplifies the preparation process. For instructions, refer to the Agilent Hyb Station user guide (publication G5765-90000) and view the online video at: https://www.agilent.com/en/product/mirna-microarray-platform/mirna-microarray-supplies/microarray-hybridization-oven-228515/hyb-station-video.

Remove gasket slide from its packaging

- 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 6 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.

   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.

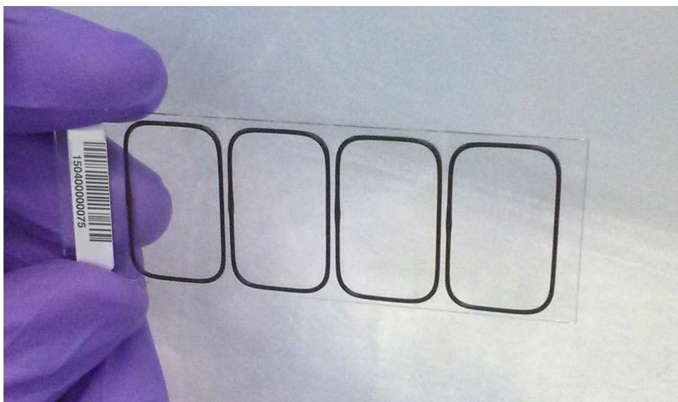


Figure 7 Gasket slide, gasket side

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 8) with the barcode label resting over the base's rectangular barcode guide.

Figure 8 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.

Figure 9 Correct positioning of gasket slide in chamber base

Remove a set of 4 samples from the thermal cycler or heat block

- After the samples have been incubating at 37°C for at least 30 minutes, remove four samples (enough for one microarray slide) from the 37°C thermal cycler or heat block.
  - If using 4x strip tubes or individual PCR tubes, open the thermal cycler (without pausing the program) and remove one 4x strip, or four PCR tubes, at a time. Promptly re-close the thermal cycler lid so that condensation does not form on the remaining strip tubes. Spin the removed tubes for at least 10 seconds in a centrifuge to collect the sample at the bottom of the tube. Proceed immediately to "Load the sample".
  - If using 1.5-mL microcentrifuge tubes, remove four samples at a time from the heat block. Spin the samples for at least 10 seconds in a microcentrifuge to collect the sample at the bottom of the tube. Proceed immediately to "Load the sample".

The samples must be kept at 37°C until they are loaded on the microarray (but not for longer than 75 minutes). Do not allow centrifuged samples to sit at room temperature for longer than 5 minutes before proceeding to the next step.

Load the sample

1 Slowly dispense 100 µL of hybridization sample mixture into a well of the gasket slide using the “drag and dispense” method (described below). Start with the well closest to the barcode.

   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 10 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.

2 With a fresh pipette tip, load 100 µL of the next hybridization sample mixture into the next gasket well using the “drag and dispense” method described in step 1. Repeat for the remaining hybridization sample mixtures. *Always load all 4 gasket wells on each microarray slide.*

NOTE To ensure accurate sample tracking, make sure to note which sample is applied to each gasket well. The sample loaded into the gasket well closest to barcode will be hybridized to microarray #1 on the microarray slide. For ease of sample tracking, Agilent recommends loading samples into the gasket wells in order, starting with microarray #1 and ending with microarray #4.

Accurate sample tracking is critical. Failure to correctly track each sample throughout each step of the assay will result in errors during data analysis.

Add the microarray slide

Never reuse a microarray slide.

1 With gloved fingers, remove a microarray slide from the slide storage box between your thumb and index finger, *numeric barcode side facing up and Agilent label facing down.*

Figure 11 Slide storage box containing microarray slides

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 12 for proper technique on holding the microarray slide with both hands.

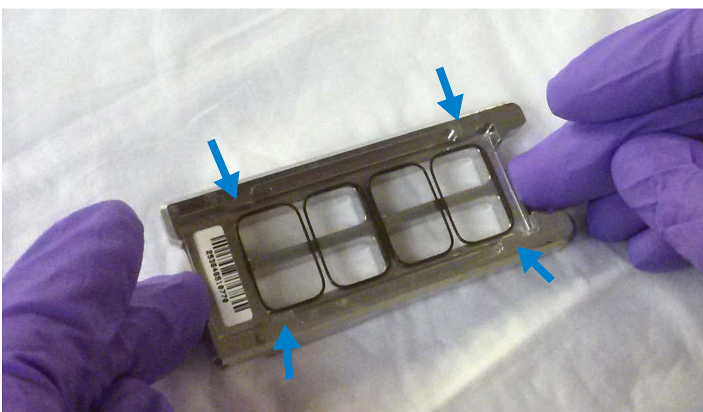


Figure 12 Chamber base with gasket and microarray slide applied, guide posts denoted with arrows

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.

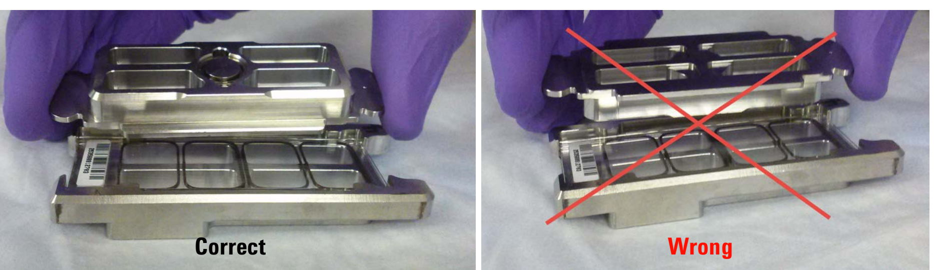


Figure 13 Chamber cover in correct (left) and incorrect (right) orientations

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.

Figure 14 Slipping the clamp onto the chamber base

3 Firmly tighten the thumbscrew fully.

The slides will not be harmed by hand-tightening.

Figure 15 Tightening of the thumbscrew on the clamp

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 16).

Rotation helps ensure that the hybridization solution will coat the entire surface of the microarray during the incubation process.

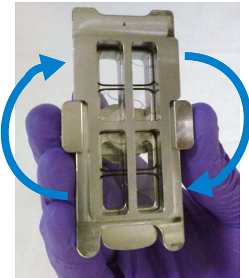
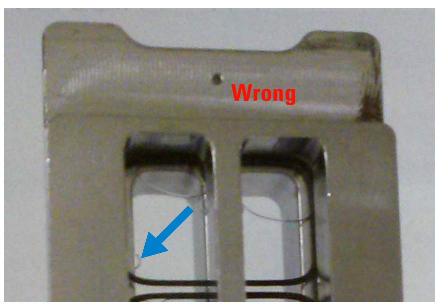


Figure 16 Rotation of the final assembled chamber

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.



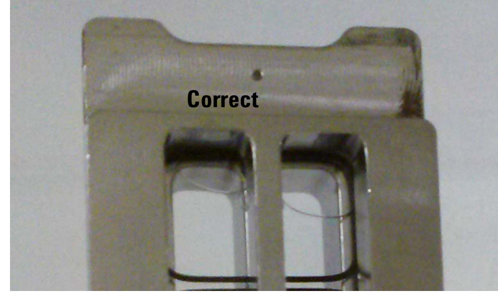


Figure 17 The slide on the left shows a stray, stationary bubble (denoted with arrow), which must be removed before hybridization. The slide on the right shows only large mixing bubbles, which move freely around the chamber when rotated. Bubbles are acceptable, as long as they move freely when you rotate the chamber.

Load assembled chamber into the hybridization oven

1 Ensure that the hybridization oven is at 67°C ±2°C.
2 Load the 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 18 Assembled chambers in correct (left) and incorrect (middle and right) orientations

3 Close the door and set the rotator speed to 20 rpm.
4 At this point, repeat “Step 4. Microarray Hybridization” on page 54 for the next set of four samples until all samples have been processed and loaded into the hybridization oven.
5 Continue to hybridize all samples at 67°C ±2°C for 24 hours (± 30 minutes).

Hybridization must occur at 67°C ± 2°C for 24 hours (± 30 minutes), or data quality will be compromised. Make sure that the oven is properly calibrated.

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.

Prewarm GenetiSure Dx Wash Buffer 2 (overnight)

The temperature of GenetiSure Dx Wash Buffer 2 must be at 37°C ±1°C for optimal wash performance.

Initiate the prewarming of the GenetiSure Dx Wash Buffer 2 on the same day that you load the assembled chambers into the hybridization oven. Prewarm enough GenetiSure Dx Wash Buffer 2 for all groups (approximately 250 mL per group). Agilent recommends washing no more than 5 microarray slides per group.

1 Add the volume of buffer required to a disposable plastic bottle and warm overnight in an incubator or water bath set to 37°C.

2 Put a slide-staining dish with a clean lid, a 1.5-L glass dish, and 1–2 L of Ultrapure water in an incubator set to 37°C to warm overnight. Then, proceed to “Step 5. Equipment Cleaning” on page 64.

Step 5. Equipment Cleaning

Always use clean equipment when conducting the wash procedures. Use only dishes that are designated and dedicated for use in GenetiSure Dx assays. Designate dishes, magnetic stir bars, and slide racks for use with either Wash Buffer 1 or Wash Buffer 2, and only use them with their designated buffer.

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 Ultrapure water.

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

1 Run copious amounts of 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.

Step 6. Microarray Washing

Prior to starting the washing procedure, turn on the SureScan Dx Microarray Scanner and the associated computer equipment and launch the Agilent Microarray Scan Control software.

Always use fresh GenetiSure Dx Wash Buffer 1 and GenetiSure Dx Wash Buffer 2 for each wash group (up to five slides per wash group).

Table 19 summarizes the wash conditions for the Wash Procedure.

Cyanine 5 has been shown to be particularly sensitive to ozone degradation. Ensure that ozone levels are no greater than 5 ppb in laboratory areas used for washing of the microarray slides.

Table 19 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	#1	GenetiSure Dx Wash Buffer 1	Room temperature	
1st wash	#2	GenetiSure Dx Wash Buffer 1	Room temperature	5 minutes
2nd wash	#3	GenetiSure Dx Wash Buffer 2	37°C ±1°C	1 minute

1. Fill slide-staining dish #1 with GenetiSure Dx Wash Buffer 1 at room temperature so that the dish is at least 3/4 full.
2. Put a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough GenetiSure Dx Wash Buffer 1 at room temperature to cover the slide rack. Put this dish on a magnetic stir plate.
3. Put the prewarmed 1.5 L glass dish on a magnetic stir plate with a heating element. Place the prewarmed slide-staining dish #3 into the 1.5 L glass dish. Fill the 1.5 L glass dish with water prewarmed to 37°C ±1°C. Fill the slide-staining dish #3 approximately 3/4 full with GenetiSure Dx Wash Buffer 2 (warmed to 37°C ±1°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of GenetiSure Dx Wash Buffer 2 at 37°C ±1°C; monitor the temperature using a thermometer.

The hybridization chambers are hot. Use caution when removing the chambers from the hybridization oven and during chamber disassembly.

4 Remove one hybridization chamber from the hybridization oven and resume rotation of the others. Make sure that the hybridization oven is still at 67°C ±2°C.

5 Examine the hybridization chamber that you removed. Make sure that any bubbles formed during hybridization are rotating freely. Also make sure that the volume of the hybridization solution does not appear to have decreased.

NOTE

If you do see any stationary bubbles or any decrease in the volume of the hybridization solution, make note of those particular samples. The QC Report generated by the Agilent CytoDx software includes metrics designed to measure failures due to these issues. See "Possible hybridization solution volume loss or leakage during hybridization due to improper hybridization assembly" on page 145 in Chapter 11, "Troubleshooting".

6 Remove the clamp from the hybridization chamber assembly.

a Put the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.

Figure 19 Loosening of the thumbscrew

b Slide off the clamp and remove the chamber cover.

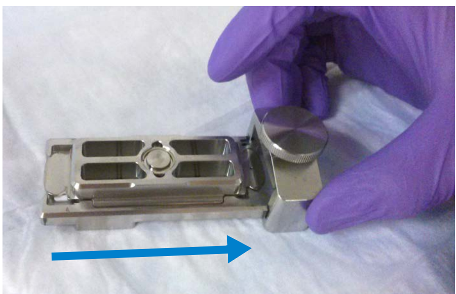


Figure 20 Removal of the clamp

7 Remove the microarray-gasket sandwich from the chamber base by lifting one end and then grasping in the middle of the short sides (see Figure 21). Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1 containing GenetiSure Dx Wash Buffer 1. Submerge the microarray-gasket sandwich into slide-staining dish #1 without letting go of the slides.

Figure 21 Removal of the microarray-gasket sandwich from the chamber base

8 With the sandwich completely submerged in GenetiSure Dx Wash Buffer 1, pry the sandwich open from the barcode end only. Do this by slipping one of the blunt ends of the forceps between the slides and then gently twist the forceps to separate the slides. Let the gasket slide drop to the bottom of the staining dish (see Figure 22). Remove the microarray slide, grasp it from the upper corners with thumb and forefinger, and quickly put into the slide rack in the slide-staining dish #2 containing GenetiSure Dx Wash Buffer 1 at room temperature.

Minimize the exposure of the slide to air. Touch only the barcode portion of the microarray slide or its edges.

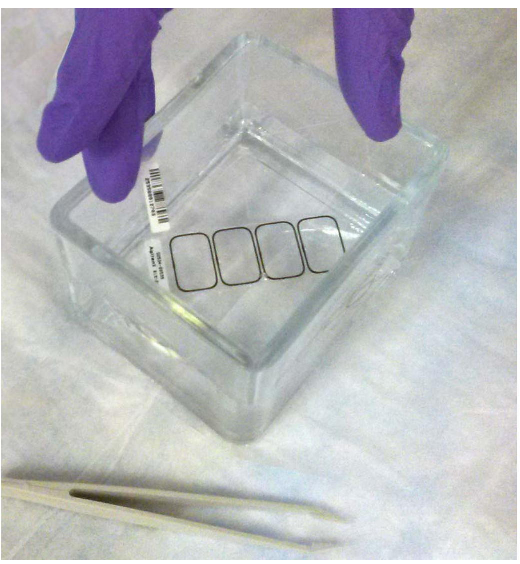


Figure 22 Removal of the microarray slide from staining dish #1

9. Repeat step 4 through step 8 for up to four additional slides in the group. To facilitate uniform washing, wash no more than five microarray slides per group.
10. When all slides in the group are put into the slide rack in slide-staining dish #2, stir at 350 rpm for 5 minutes (tolerance ± 1 minute). Adjust the setting to get thorough mixing without disturbing the microarray slides. During the 5-minute interval, prepare slide-staining dish #3 by activating the magnetic stirrer.
11. Quickly transfer slide rack to slide-staining dish #3 containing GenetiSure Dx Wash Buffer 2 at 37°C (tolerance ± 1°C). Wash the microarray slides for at least 1 minute and not exceeding 2 minutes. Adjust the setting to get thorough mixing without disturbing the microarray slides.

12 Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5–10 seconds to remove the slide rack.

13 Proceed immediately to "Step 7. Microarray Slide Preparation for Scanning" on page 69 for each wash group before repeating step 1 through step 12 for the next group. For each group, use fresh GenetiSure Dx Wash Buffer 1 and fresh GenetiSure Dx Wash Buffer 2 prewarmed to 37°C ±1°C, and rinse the dishes with Ultrapure water before using again.

Step 7. Microarray Slide Preparation for Scanning

Fingerprints cause errors in the fluorescence detection. For accurate readings, touch only the edges of the slide and always use gloves when handling slides.

Do not write on the slides with markers or place any labels on the slide other than the appropriate barcode.

The slide is inserted into a slide holder before loading it into the scanner.

1 Before you insert the slide into a slide holder, inspect the slide holder for any dust or fingerprints. If found, remove the dust or fingerprints with compressed air or a soft, dust-free cloth. If the slide holder is scratched, worn, or damaged, has a lid that does not close tightly, or has a hinge that does not move freely, discard the slide holder and select a different one.

2 Place the slide holder on a flat surface, with the clear cover facing up, and the tab on the right. This helps to ensure that you have the slide aligned properly when you insert it into the slide holder.

3 Gently push in and pull up on the tabbed end of the clear plastic cover to open the slide holder.

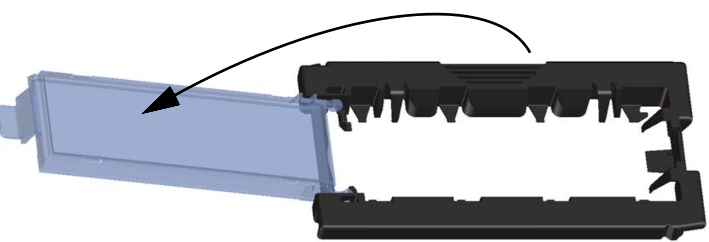


Figure 23 Opening the slide holder

4 Insert the slide into the holder.

a Hold the slide at the barcode end and position the slide over the open slide holder. Make sure that the active microarray surface faces up with the barcode on the left, as shown in Figure 24. Review Figure 3 on page 50 for a description of the active side.

b Carefully place the end of the slide without the barcode label onto the slide ledge. See Figure 24.

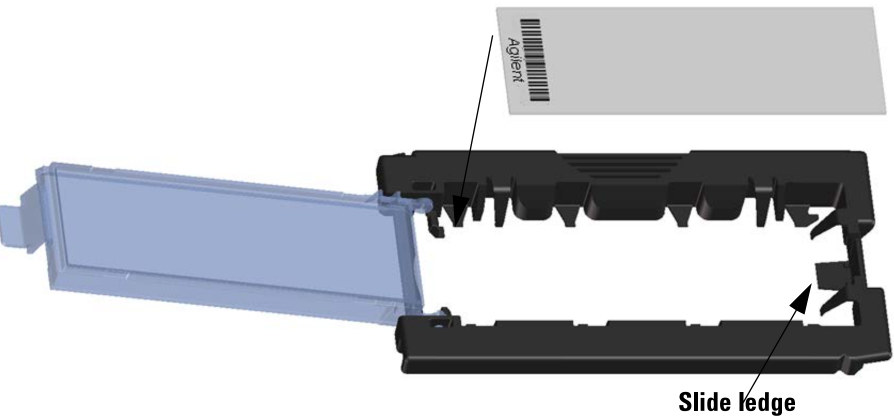


Figure 24 Inserting slide into the slide holder

c Gently lower the slide into the slide holder. See Figure 25.

d Close the plastic slide cover, pushing on the tab end until you hear it “click.” This moves the slide into position in the holder.

An improperly inserted slide can damage the SureScan Dx scanner.

e Gently push in and pull up on the tabbed end of the clear plastic cover to open it again and verify that the slide is correctly positioned.

Once inserted, the slide lies flat and matches up with the alignment points on the slide holder.

f Close the plastic slide cover, gently pushing on the tab end until you hear it “click”. See Figure 26. Make sure that the slide holder is completely closed.

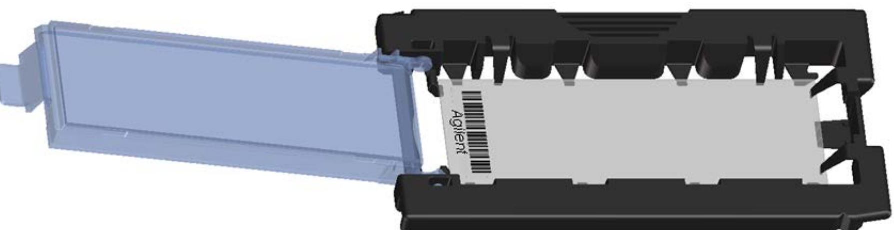


Figure 25 Slide inserted in slide holder – cover open

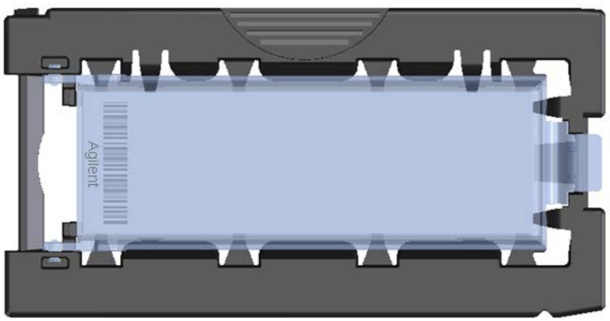


Figure 26 Slide inserted in slide holder – cover closed

For instructions on removing the slides, see “Remove the slides” on page 77.

Step 8. Microarray Slide Scanning

Cyanine 5 has been shown to be particularly sensitive to ozone degradation. Ensure that ozone levels are no greater than 5 ppb in laboratory areas used for scanning of the microarray slides.

Load the slide holders into the cassette

Once the slides are properly inserted in the slide holders, you can load the slide holders into the cassette of the SureScan Dx Microarray Scanner. The cassette and slide holders are designed to ensure that the slide holders are inserted correctly.

NOTE Do not load slide holders that do not contain slides into the SureScan Dx scanner.

1 Turn on the SureScan Dx scanner and the computer workstation that is connected to the scanner. Then, launch the Scan Control program.

   Refer to the *Agilent G5761A SureScan Dx Microarray Scanner System Instructions for Use* for instructions on how to perform this step.

2 In the Scan Control program window, click **Open Door** to open the scanner door.

The correct way to open the scanner door is using the Open Door button in the Scan Control program. Do not attempt to open the door manually.

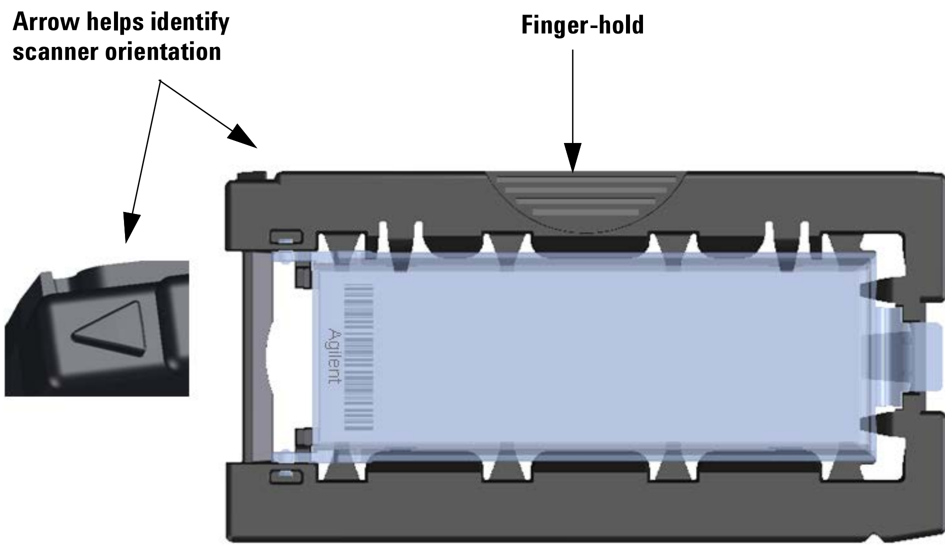


Figure 27 Slide holder helps you to insert slides correctly

3 Pick up the slide holder using the finger hold. The arrow on top of the slide holder points to the left when you pick up the slide holder correctly. See Figure 27.

4 Slowly and carefully insert the slide holder into any open slot of the cassette. See Figure 28. The slot numbers are clearly labeled on the slide cassette. Do not force or drop the slide holder into the cassette; it inserts easily if properly aligned with the finger hold on top and the arrow facing to the left.

NOTE The SureScan Dx scanner scans slides in the order set in the scan queue. The scanner skips over any empty slots.

Figure 28 Inserting slide holder into cassette

5 Make sure that the slide holder is seated in the bottom of the cassette slot.

   The slot number for the loaded slide blinks blue.

6 Repeat step 3 through step 5 until all slide holders are loaded in the cassette.

The slide numbers next to the cassette and in the slot table of the Scan Control program window change color to indicate the state of the slot.

Improper placement of the slide holder in the cassette can result in severe damage to the SureScan Dx scanner.

7 In the Scan Control program, click **Close Door**. The following events happen:

   - The scanner door closes.
   - The scanner reads the barcode for each slide.
   - The barcode is displayed under **Slide ID** in the Scan Control software slot table.
   - The default output folder is applied. (Once the slides are added to the scan queue, the output folder cannot be changed unless you remove the slides from the queue, change the output folder location, and then add the slides back to the queue prior to scanning.)

- The Scan Control protocol **GenetiSure_Dx_Postnatal** is assigned in the slot table.
- The *State* slot changes to "Ready for queue."

NOTE

If the scanner fails to read the barcode, remove the slide holder and gently reposition it in the scanner, ensuring that the barcode is not obstructed. If you see condensation on the barcode, wait for the condensation to clear before repositioning the slide in the scanner.

If the barcode still cannot be read by the scanner, manually enter the barcode for that slide. Refer to the SureScan Dx User Guide for instructions.

8 Confirm that the *Scan Protocol* slot is set to the **GenetiSure_Dx_Postnatal** protocol for all slides. Ensure that the scanning parameters for this protocol (which are displayed on the right side of the screen) match those shown in Table 20.

Table 20 Parameters for the GenetiSure_Dx_Postnatal Scan Control protocol

Parameter	Value
General	
Agilent Defined	Yes
Locked	Yes
Scan Settings	
Dye Channel(s)	Red+Green
Scan Region	FullAgilentSlide
Resolution	3 µm
Tiff Dynamic Range	16 bit
Red PMT Sensitivity (%)	100
Green PMT Sensitivity (%)	100
XDR Ratio	<NoXDR>
Image Settings	
Transform Image	None
Split	No

Table 20 Parameters for the GenetiSure_Dx_Postnatal Scan Control protocol

Parameter	Value
Compress	No
File Naming Settings	
Field 1	Instrument SN
Field 2	Slide ID
Field 3	<None>
Image File Info	
File Name	<InstrSN>_<SlideID>_Sxxx.tif
Image Width (Pixels)	20334
Image Height (Pixels)	7200
Disk space required	558.49 MB
Scan time	16 min

Add slides to the scan queue

NOTE Once you add a slide to the scan queue, you cannot change its scan settings. To change the scan settings, remove the slide from the queue.

To add a slide to the scan queue, its State must be "Ready for queue."

1 Add slides to the queue in the Scan Control software.
   - In the Scan Control main window, click **All to Queue** to add all slides in the slot table with a State of "Ready for queue" to the scan queue. In the confirmation dialog box that opens, click **Yes** to add the slides to the queue.

   OR
   - In the Scan Control slot table, click the **State** cell for the first slide to scan and click **Add to Queue**.
2 For each additional slide you want to scan:
   - Click the **State** cell and select **Add to queue first** to add the slide to the top of the scan queue.

OR

- Click the **State** cell and select **Add to queue last** to add the slide to the bottom of the scan queue.

As each slide is added to the queue, its **State** indicates that it is in the queue and the order in which the slide is scanned. (In queue 1, In queue 2, for example.) The status indicator light changes to solid blue.

Do not click **All to Queue** at this step as it will cause all slides to be scanned, including those that have already been scanned.

Scan your slides

NOTE A *scan* refers to creating an image of the probes on a single slide.

1 If necessary, in the Scan Control main window, click **Close Door**.

   Wait until the door closes and the **Start Scan** button is enabled.

2 In the Scan Control main window, click **Start Scan** to begin scanning the slides that were added to the queue. The scanner scans the slides in their order in the scan queue.

   During a scan, you see the following:

   - The slot status indicator light for the current slide blinks green during the scan process, and the scan progress (for example, Scanning 50%) is displayed in the slot State.
   - The remaining scan time and required disk space are displayed at the bottom of the Scan Control main window.
   - Events during the scan are logged in the Scan Log and Status Log.

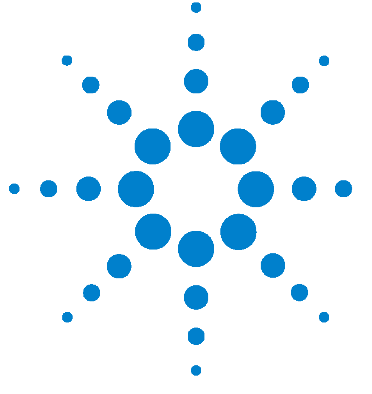
Remove the slides

When the scan is complete and the Open Door button is enabled, you can unload the slide holders from the cassette and then remove the slides from the slide holders.

1 In the Scan Control main window, click **Open Door** to open the scanner door.

   If the Open Door button is not available, you cannot open the door. Check to make sure that scanning process is finished.

2 Remove the slide holders from the cassette.
3 Remove the slides from the slide holders.
   a Hold the slide holder on the sides with the Agilent logo facing up.
   b Gently push in and pull up on the tabbed end of the clear plastic cover to open it.
   c Push up on the barcode end of the slide from underneath the slide holder to avoid fingerprints on the sample area.
   d Grasp the slide from the sides and remove from the slide holder.
4 In case a rescan is needed, store the slides in a vacuum desiccator or N_2 purge box protected from light.
5 At the completion of the scanning, the microarray TIF images are ready to load into the Agilent CytoDx Software for feature extraction, CNV and cnLOH identification, and reporting. Proceed to Chapter 7, “Microarray Image Analysis Instructions”.



7. Microarray Image Analysis Instructions

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Step 8. Signing Off of Sample Results and Generation of Final Report 100

The microarray TIF images generated by the scanner are analyzed by the Agilent CytoDx Software to perform feature extraction, CNV and cnLOH identification, and reporting.

NOTE

If the assay failed any of the nine actionable QC metrics, the software will generate a report for review of the QC metrics but "sign-off" will not be allowed for the report. See Table 22 on page 89 for a list of the actionable QC metrics.

CytoDx users with the role of Technician, Cytogeneticist, or Lab Director can run standard analysis workflows. Typically, the Technician runs the analysis and the Cytogeneticist or Lab Director reviews and interprets the results. The Lab Director is ultimately responsible for signing off on sample results.

The check boxes displayed at the start of each step (as shown below) indicate which user roles are permitted to perform that step. Roles with a green check mark are the ones with the necessary privileges.

☑ Technicians ☑ Cytogeneticists ☑ Lab Directors ☑ System Admins

Reagents, Materials, and Equipment List

Table 21 Required Reagents, Materials, and Equipment for Chapter 7

Component	Source	Form
TIFF images of microarray slides	SureScan Dx Microarray Scanner	Image File
Agilent CytoDx software	Agilent Technologies	Software
Adobe Reader or other PDF viewing software	Adobe or other	Software
Microsoft Excel or other spreadsheet or text editing software	Microsoft or other	Software

NOTE

Before continuing, make sure the CytoDx software is installed and set up. Refer to the *Agilent CytoDx Software Installation and Set Up Guide* for instructions on installing and setting up the CytoDx software.

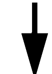
The instructions in this chapter apply to CytoDx version 2.0. If you are working in a version other than 2.0, some of the steps for performing microarray image analysis may differ from the steps shown here. Consult the help system within your CytoDx application.

Overview of the image analysis process

The process of analyzing microarray images in order to identify aberrations in a patient gDNA sample is summarized in Figure 29. In a typical laboratory, a Technician is responsible for the early steps in the process, specifically, setting up and submitting the analysis workflow job. The Cytogeneticist then reviews the results and classifies the aberrations. The results must be signed off by a Lab Director, which results in generation of the final Cyto report. The Cyto report can then be incorporated into the clinical report that is provided to the ordering physician.

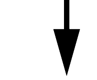
CytoDx Technician

- Sets up and submits the analysis workflow to analyze the tiff images from the scanner



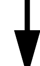
CytoDx Cytogeneticist

- Reviews the aberration results and QC metrics in Triage View
- Edits aberration calls as needed
- Classifies aberrations, making use of external databases as appropriate



CytoDx Lab Director

- Reviews the aberration results and classifications made by the Cytogeneticist in Triage View
- Modifies input from the Cytogeneticist as needed
- Signs off on the results and generates final Cyto report



Ordering physician

- Reviews the clinical report which includes the Cyto report

Figure 29 The image analysis process organized by user role

Step 1. Preparation of the Sample Attribute File

☐ Technicians ☐ Cytogeneticists ☑ Lab Directors ☐ System Admins

About sample attribute files

A sample attribute file (SAF) is a text file, typically prepared in Microsoft Excel, that defines the attributes for a list of samples. The file must follow these guidelines:

- The first line of the file contains the names of the attributes that are being defined in the file (e.g., Array ID, Global Display Name, etc.). The attributes are separated by tabs. You can create a template of the SAF that has the first line of the file completed. See "Export a SAF template" on page 83.
- The rest of the lines of the file contain the values of each attribute, one line per microarray. The values must be in the same order as the attributes in the first line of the file.
- The file must contain the following five attributes:

  Array ID: The Array ID is the barcode number of the microarray slide followed by the number 1 and the number of the microarray on the 4x slide. An example of an Array ID would be 254457710741_1_4, where 254457710741 is the barcode number and 4 is the number of the microarray.

  Global Display Name: The Global Display Name can be identical to the Array ID, or it can be a custom name, such as a sample or patient identifier. If you use a custom name, make sure it is unique to that microarray. This Global Display Name is displayed throughout CytoDx for the microarray (e.g., in reports) and cannot be repeated for another microarray, even if that microarray was hybridized to the same sample.

  Green Sample: This attribute designates the genotype reference file to be used in the analysis. If you used the female reference DNA, type **Agilent Female Reference**. If you used the male reference DNA, type **Agilent Male Reference**.

  Red Sample: This attribute is the identifier for the patient sample.

  Polarity: For the Polarity, enter **1**. A polarity of 1 confirms to the software that the Green Sample is the reference sample.
- If you want the Cyto report to include ISCN nomenclature for the sex chromosomes in addition to the autosomes, then the SAF must also include the Gender attribute.

An example of a sample attribute file created in Microsoft Excel is shown in Figure 30.

	A	B	C	D	E	F
1	Array ID	Global Display Name	Green Sample	Red Sample	Gender	Polarity
2	254457710738_1_1	254457710738_1_1	Agilent Male Reference	Sample 1	Male	1
3	254457710738_1_2	254457710738_1_2	Agilent Female Reference	Sample 2	Female	1
4	254457710738_1_3	254457710738_1_3	Agilent Female Reference	Sample 3	Female	1
5	254457710738_1_4	254457710738_1_4	Agilent Male Reference	Sample 4	Male	1
6	254457710739_1_1	254457710739_1_1	Agilent Male Reference	Sample 5	Male	1
7	254457710739_1_2	254457710739_1_2	Agilent Female Reference	Sample 6	Female	1
8	254457710739_1_3	254457710739_1_3	Agilent Male Reference	Sample 7	Male	1
9	254457710739_1_4	254457710739_1_4	Agilent Female Reference	Sample 8	Female	1
10	254457710740_1_1	254457710740_1_1	Agilent Male Reference	Sample 9	Male	1
11	254457710740_1_2	254457710740_1_2	Agilent Female Reference	Sample 10	Female	1
12	254457710740_1_3	254457710740_1_3	Agilent Female Reference	Sample 11	Female	1
13	254457710740_1_4	254457710740_1_4	Agilent Female Reference	Sample 12	Female	1
14	254457710741_1_1	254457710741_1_1	Agilent Male Reference	Sample 13	Male	1
15	254457710741_1_2	254457710741_1_2	Agilent Male Reference	Sample 14	Male	1
16	254457710741_1_3	254457710741_1_3	Agilent Female Reference	Sample 15	Female	1
17	254457710741_1_4	254457710741_1_4	Agilent Male Reference	Sample 16	Male	1
18	254457710821_1_1	254457710821_1_1	Agilent Male Reference	Sample 17	Male	1
19	254457710821_1_2	254457710821_1_2	Agilent Male Reference	Sample 18	Male	1
20	254457710821_1_3	254457710821_1_3	Agilent Male Reference	Sample 19	Male	1
21	254457710821_1_4	254457710821_1_4	Agilent Male Reference	Sample 20	Male	1

Figure 30 Example of a sample attribute file

Export a SAF template

NOTE

With the release of CytoDx 2.0, the process for SAF template creation was simplified to eliminate the need for mapping the columns of the SAF to individual sample attributes in the software. If you are working in a version of CytoDx prior to version 2.0, this mapping step is still required prior to exporting a SAF template. See the topic “Map columns of the sample attribute files” in your CytoDx help system for instructions.

Before creating the SAF, export a template of the SAF. The instructions below describe how to export the default SAF template. Only CytoDx users with the role of Lab Director can export a SAF template.

1 At the top of the CytoDx program window, click **Configure Settings**.

2 In the command navigator on the left side of the Configure Settings screen, click **Sample Attributes**.

3 Near the top of the screen, click the Sample Attribute Mapping tab.

The table lists the available SAF templates. CytoDx comes preloaded with a default SAF template that includes the five required sample attributes. For instructions on creating a custom SAF template, consult the CytoDx help system.

4 In the row for the desired SAF template, click **Export SAF**.

   A dialog box opens.

5 In the dialog box, browse to the folder where you want to save the template. In the file name field, type a name for the template. Templates are saved as tab-delimited text files (*.*txt*). Click **Save**.

   A message box opens notifying you that the template was successfully exported.

Prepare the sample attribute file

1 Open the SAF template in Microsoft Excel or another text editor program.

   The first row or first line of text contains the names of the sample attributes, separated by tabs.

2 In the next row or line of text, type the attributes for the first Array ID that you want to analyze.

3 Repeat step 2 for all Array IDs that you want to analyze.

4 Save the file as a tab-delimited text file.

Entering incorrect sample information in the sample attribute file will result in errors in the results. Make sure that all sample information is associated with the correct Array ID.

Step 2. Setup and Submission of the Analysis Workflow

☑ Technicians ☑ Cytogeneticists ☑ Lab Directors ☐ System Admins

NOTE

The instructions provided here describe how to manually set up and run an analysis workflow. The CytoDx software is also capable of Auto-Processing microarray tiff image files that are saved to a designated folder. See the CytoDx help system for instructions on how to use the CytoDx Auto-Processing feature.

Once you have created the SAF, you can set up the workflow in CytoDx using the tools on the Analysis Workflow screen. CytoDx users with the role of Technician, Cytogeneticist, or Lab Director can run analysis workflows.

1 At the top of the CytoDx program window, click **Analysis Workflow**.

   The Analysis Workflow screen opens to the Import Samples step.

2 Click **Select Samples**.

   The Open dialog box opens.

3 Browse to the microarray image file (*.*tif*). Select the file and click **Open**.

   The Image Information dialog box opens.

4 Click **Add Images**.

   The dialog box closes and the program adds the samples to the table on the Analysis Workflow screen. The program assigns a global display name to each sample, which by default is the barcode number.

5 (Optional) To view the tiff image for a sample, click on the row for that sample in the table and then click **View Image**.

   The Agilent Feature Extraction for CytoDx software module opens, displaying the tiff image of the slide. See the CytoDx help system for information on the tools available in this software module. When you are finishing viewing the image, close the Feature Extraction for CytoDx software module to return to setting up the workflow in the CytoDx program.

6 Click **Next** to go to the Describe Samples step.

7 Click **Import Sample Attribute File**.

   The Import Sample Attribute Files dialog box opens.

8 Select the sample attribute file and click **Open**.

A message box opens informing you of the number of rows in the sample attribute file that were successfully imported and the number of rows that were ignored. CytoDx ignores any rows for samples that you did not import.

9 Click **OK** to close the message box.

The program populates the table on the Describe Samples screen with the values from the sample attribute file.

10 Click **Next** to go to the Run Analysis step.

11 In the Job Name field, type a name for the analysis workflow job, or use the default job name.

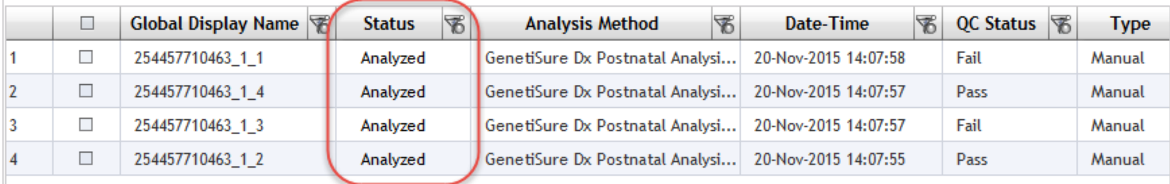
12 (Optional) In the Job Description field, type a description of the analysis workflow job that will help you identify it later.

13 At the bottom of screen, click **Run Analysis**.

A message box opens notifying you that the workflows are in the queue.

14 Click **OK** to close the message box.

The program directs you to the Sample Review screen. The samples for the workflow job are listed in the table and their status is displayed in the Status column. When a workflow job is in-progress, its status is *Running*. Once the job is complete, the status changes to *Analyzed*, as shown in Figure 31.



	☐	Global Display Name	Status	Analysis Method	Date-Time	QC Status	Type
1	☐	254457710463_1_1	Analyzed	GenetiSure Dx Postnatal Analysi...	20-Nov-2015 14:07:58	Fail	Manual
2	☐	254457710463_1_4	Analyzed	GenetiSure Dx Postnatal Analysi...	20-Nov-2015 14:07:57	Pass	Manual
3	☐	254457710463_1_3	Analyzed	GenetiSure Dx Postnatal Analysi...	20-Nov-2015 14:07:57	Fail	Manual
4	☐	254457710463_1_2	Analyzed	GenetiSure Dx Postnatal Analysi...	20-Nov-2015 14:07:55	Pass	Manual

Figure 31 Sample Review Screen – Status column circled

NOTE

If the Status column changes to *Fail* instead of *Analyzed*, then the sample could not be processed. As soon as you see that a sample has failed, rescan the slide in a different slide holder as quickly as possible. The failure could be due to the slide not being straight within the slide holder and a rescan of the slide in the proper position may yield usable results.

15 Make note of any samples in the Sample Review table that have *Fail* in the QC Status column (see Figure 32). This indicates that the sample

did not pass all nine of the critical QC metrics. The Lab Director cannot sign off on samples that did not pass the critical QC metrics.

	☐	Global Display Name	Status	Analysis Method	Date-Time	QC Status	Type
1	☐	254457710463_1_1	Analyzed	GenetiSure Dx Postnatal Analysi...	20-Nov-2015 14:07:58	Fail	Manual
2	☐	254457710463_1_4	Analyzed	GenetiSure Dx Postnatal Analysi...	20-Nov-2015 14:07:57	Pass	Manual
3	☐	254457710463_1_3	Analyzed	GenetiSure Dx Postnatal Analysi...	20-Nov-2015 14:07:57	Fail	Manual
4	☐	254457710463_1_2	Analyzed	GenetiSure Dx Postnatal Analysi...	20-Nov-2015 14:07:55	Pass	Manual

Figure 32 Sample Review Screen – QC Status column circled

Step 3. Accessing of the Analysis Results in Triage View

Once the workflow job is complete, the results of the analysis are ready to view in the Triage View window. CytoDx users with the role of Technician, Cytogeneticist, or Lab Director can open sample results in Triage View, but only Cytogeneticists and Lab Directors can check out sample results.

Open sample results in Triage View

1. On the Sample Review screen, locate the sample of interest in the table and mark the check box in that row.

   You can mark multiple samples and open their results in the same Triage View session.

2. At the bottom of the screen, click **Triage View**.

   The Triage View window opens displaying the sample results from the selected workflow(s).

OR

1. On the Sample Review screen, locate the sample of interest in the table and double-click directly on that row.
2. In the message box that opens, click **Yes** to confirm that you want to open Triage View.

Check out/check in sample results in Triage View (if needed)

If you want to make any edits and notes to the sample results in Triage View, you need to check out the results. Checking out the sample results is only necessary if you want to make edits; it is not required to view the results. Samples that are checked out to one CytoDx user cannot be edited by another CytoDx user.

1 Click **Change Status > Check Out**.

   The Authentication dialog box opens.

2 Enter your user name and password and click **Ok**.

   The sample is now checked out to you. You can review the results, edit the aberration calls, and classify the aberrations as necessary.

3 To check in sample results, click **Change Status > Check In**. Once checked in, you can close Triage View and the program will save the changes you made to the sample results. You will need to check out the results again in order to make additional changes.

Step 4. Review of the QC Metrics

☐ Technicians	☑ Cytogeneticists	☑ Lab Directors	☐ System Admins

CytoDx measures several QC metrics to help assess the quality of the data. Nine of the QC metrics are classified as actionable. Actionable QC metrics are those that are the most critical indicators of assay performance. If any of these nine actionable QC metrics do not pass, then CytoDx sets the status of that metric to *Fail*, and the sample results for that microarray cannot be signed off. The actionable QC metrics are listed in Table 22.

Non-actionable QC metrics are classified as informational. For informational QC metrics, if the metric does not pass, then CytoDx sets to the status to *Evaluate*. A metric in the *Evaluate* category does not disallow sign-off of the sample results. However, if you find multiple informational QC metrics are rated as *Evaluate* in a single sample, examine the data quality before proceeding. The informational QC metrics are listed in Table 23.

1 At the top of the Triage View, click **QC Metrics > QC Metrics**.

   The CGH & SNP QC Metrics dialog box opens with the values of the actionable metrics displayed on the left and the values of the informational metrics displayed on the right. Color coding in the table indicates the rating (Excellent, Good, or Fail/Evaluate) for each metric.

2 Review the metrics. Make note of any metrics in the Fail or Evaluate category.

3 (Optional) Generate the QC Metric report.

   a At the top of the Triage View, click **Reports > QC Metrics**. Microsoft Excel launches and opens the QC report.

   b Save the QC report to the desired location. Make note of this location so you can later send the report to Agilent, if required.

Table 22 Actionable QC metrics with values for *Excellent*, *Good*, and *Fail*

Metric Name	Excellent	Good	Fail
IsGoodGrid	=1	NA	<1
AnyColorPrcntFeatNonUnifOL	<1	1 to 5	>5
DerivativeLR_Spread	<0.20	0.20 to 0.30	>0.30
g_Signal2Noise	>100	25 to 100	<25
r_Signal2Noise	>100	30 to 100	<30
LogRatioImbalance	–0.16 to 0.16	(–0.26 to –0.16) or (0.16 to 0.26)	<–0.26 or >0.26
Reference Correct	≥0.8	NA	<0.8
SNP Call Rate	≥0.6	NA	<0.6
Call Ambiguity	<0.07	NA	≥0.07

Table 23 Informational QC metrics with values for *Excellent*, *Good*, and *Evaluate*

Metric Name	Excellent	Good	Evaluate
gRepro	0 to 0.05	0.05 to 0.20	<0 or >0.20
g_BGNoise	<5	5 to 20	>20
g_SignalIntensity	>150	50 to 150	<50

Table 23 Informational QC metrics with values for *Excellent*, *Good*, and *Evaluate*

Metric Name	Excellent	Good	Evaluate
rRepro	0 to 0.05	0.05 to 0.20	<0 or >0.20
r_BGNoise	<5	5 to 20	>20
r_SignalIntensity	>150	50 to 150	<50
RestrictionControl		0.80 to 1	<0.80 or >1
Heterozygosity	NA	0.15 to 0.35	<0.15 or >0.35
Goodness of Fit (Diploid Region)	No ranges established; always rated as *Good*		
Separability	NA	>0.8 and <1.0	≤0.8 or ≥1.0
Clonal Fraction	No ranges established; always rated as *Good*		
cnLOH Fraction	No ranges established; always rated as *Good*		

Step 5. Review of the Aberrations

☐ Technicians ☑ Cytogeneticists ☑ Lab Directors ☐ System Admins

This step covers the tasks involved in viewing the CNVs and cnLOH intervals identified in a sample. It also describes how to suppress, edit, and add aberration calls. Although the tasks in this step are not required in order to sign off on the sample results, they may help provide context for assessment of the aberrations.

Aberration calls must always be reviewed carefully. Unexpected results, such as a much larger than expected number of aberrations as compared to other patient samples, could indicate an assay failure that was not identified by the CytoDx QC metrics or filters. In such instances, re-run the assay to confirm the results.

NOTE The laboratory reporting out the assay results is responsible for including in its final report any information related to report updates (i.e., aberrations that were suppressed, edited, or added by a reviewer).

View results in the Triage View panels

NOTE

If you are working in a version of CytoDx prior to version 2.0, the Triage View panels in your software may differ slightly from the example figures shown in this section. Additionally, some functionalities (e.g., viewing multiple samples in a single Triage View session) are not available in versions prior to 2.0

Triage View displays the aberrations identified in one or more samples. Data are displayed in the Genomic Viewer, which consists of 5 distinct panels: Genome View, Chromosome View, Gene View, Tracks View, and the Tab View, which includes the Interval Table. Review the panels to see the gains, losses, and cnLOH intervals.

When multiple samples are open in Triage View, data in the Genome View, Chromosome View, and Gene View can be displayed as stacked or overlaid. In stacked view, the data from each open sample are plotted separately and displayed in side-by-side panes. In overlaid view, the data from all samples are plotted together in a single pane. Some of the Tab View tables (CGH Probes and SNP Probes) can also toggle between stacked and overlaid. When in overlaid view, the table data are specific only to the selected sample tab. In stacked view, data from multiple samples are displayed side-by-side in separate columns.

Genome View The Genome View panel shows images of each chromosome. The selected chromosome is highlighted in blue, and the cursor appears as a blue line across the chromosome. The locations of LOH intervals are denoted as aqua-shaded regions. The top left corner of the view displays the number of gains, losses, and LOH intervals (LOH) detected in the sample. If multiple samples are open in Triage View and the data are displayed as overlaid, then the numbers reflect the totals across all open samples.

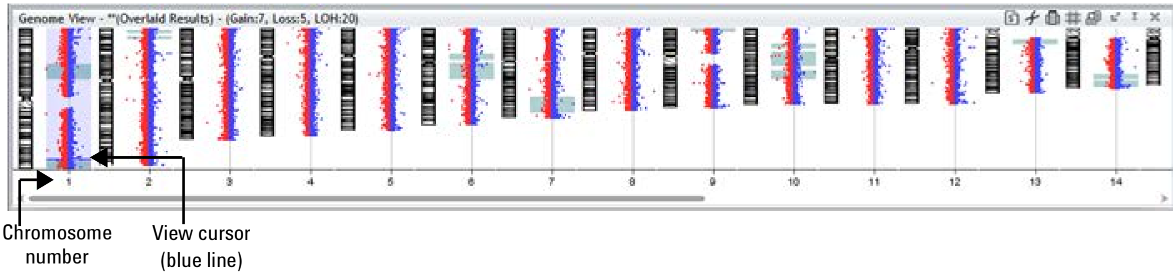


Figure 33 Example of the Genome View panel in Triage View, chromosome 1 selected

Chromosome View The Chromosome View panel shows a more detailed diagram of the chromosome selected in Genome View. In the default view, the cytobands appear to the left of the plot area. The view cursor appears as a solid blue line across the plot area. The selected region of the chromosome appears as a dotted blue box centered on the view cursor.

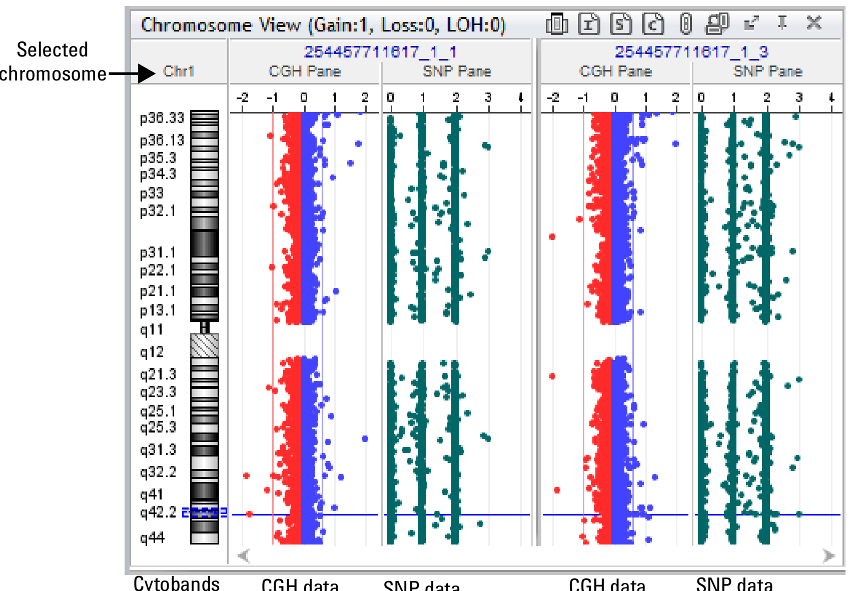


Figure 34 Example of the Chromosome View panel in Triage View

Gene View The Gene View panel shows a more detailed view of the chromosomal region selected in Chromosome View. The CGH log ratio data appear as a scatter plot. Within the scatter plot, the regions occupied by genes appear as small boxes (gene locations are determined by the Genes track that comes preloaded with the CytoDx software). The location of the cursor matches the location of the cursors in other views. The name of the chromosome, the coordinates, and size of the displayed chromosomal region appear at the top of the view.

To zoom in on a region in the Gene View, click and drag the cursor on the desired region or click the Zoom In icon located in the Triage View toolbar. To zoom out, click the Zoom Out icon.

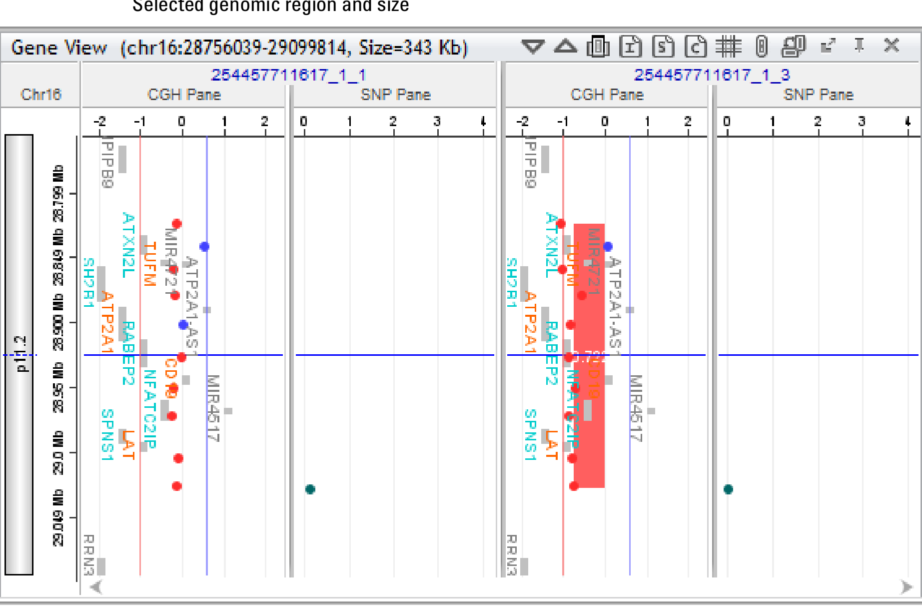


Figure 35 Example of the Gene View panel in Triage View

Tracks View The Tracks View displays intervals of chosen tracks within the selected genomic region, or provides a graphic representation of the interval density across that region. The Tracks View includes all tracks that are currently marked for display. Depending on the data orientation, the tracks are either stacked vertically with the track names in the left margin, or they are stacked horizontally with the track names at the top (as shown in Figure 36).

To select which tracks are marked for display, click the icon in the toolbar to view a menu of available tracks.

NOTE

The tracks listed in the menu are those that have already been imported into the software. CytoDx users with the role of Lab Director can import tracks from the Configure Settings screen. For instructions, see the CytoDx help system or Agilent publication K1201-90000, CytoDx Installation and Set Up Guide.

Users are responsible for ensuring that tracks are updated on a regular basis per laboratory procedures.

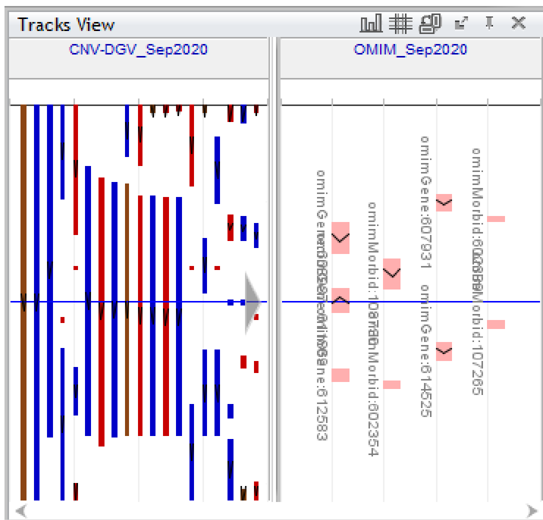


Figure 36 Example of the Tracks View panel in Triage View

Interval Table The Interval Table, which is one of the tables available for display in the Tab View of the Genomic Viewer, lists all the aberrations that the program identified in the sample. The table lists the start and stop positions of the aberrations, the size of the aberrations, the number of probes in the aberration intervals, and the names of any genes that overlap the aberrations. The table also includes a Classification column that notes any classifications that you or another CytoDx user assigned to the aberrations.

Interval Table

Chromosome	Start	Stop	Cytoband	Gene Name	Size(kb)	Type	#Probes	Mean Log ...	State	Suppr...	Classification	Count	Action
chr13	19,463,637	60,245,473	q11 - q21.2	TUBA3C,TPTE2,MPHOSPH8,P	40,781.837	Gain	1,543	0.567	Algorithm ...	☐	[+]	Click on h...	Edit Notes
chr14	19,562,141	20,421,677	q11.2	POTEG,LOC101929572,POTEH	859.537	Loss	14	-0.452	Algorithm ...	☐	[+]	Click on h...	Edit Notes
chr22	22,701,977	23,228,483	q11.22	PRAME,GGTLC2,MIR650,ZNF2	526.507	Loss	19	-0.418	Algorithm ...	☐	[+]	Click on h...	Edit Notes

Figure 37 Example of the Interval Table in Triage View

View gene annotations in external databases (optional)

The links in the Gene Name column of the Interval Table provide access to gene annotation information in online databases such as OMIM and Ensembl.

NOTE Agilent has not validated the information provided in any of the online databases. Validation of that information must be performed by the user.

1 In the Tab View, click the tab for the Interval Table (if not already selected).

2 In the Interval Table, locate the row for the aberration of interest. Right-click within that row. In the menu, go to **View in External Databases** and select one of the databases. If you select UCSC, DGV, or Ensembl as the database, then select the **By Gene Names** option.

   The View Gene dialog box or OMIM dialog box (if you selected OMIM) opens.

3 Next to the gene of interest, click **Search** to open the selected database and locate information for the gene. (For OMIM, double-click the OMIM Id.)

   The selected database opens in your internet browser and displays the information for the selected gene.

View genomic regions in an external genome browser (optional)

Links accessible through the Gene View provide access to online genome browsers.

1. Set the Gene View to the genomic region of interest.

   See the CytoDx help system for detailed instructions on how to set the Gene View to display a desired genomic region.

2. Right-click within the Gene View. In the menu, select **UCSC**, **DGV**, or **Ensembl**, then click **By Genomic Locations**.

   The View Coordinates in UCSC/DGV/Ensemble Browser dialog box opens.

3. Complete the dialog box with the track parameters and click **OK**.

   The default internet browser application opens to the appropriate browser website, with the selected region displayed.

Suppress aberrations (optional)

In some cases, you may want to suppress an aberration call in the sample. For example, you may wish to suppress LOH intervals that are not copy neutral, e.g., LOH intervals within large deletions.

1. In the Tab View, click the tab for the Interval Table (if not already selected).

2. In the Interval Table, mark the check box in the Suppress column for each call that you want to suppress.

   The program suppresses the aberration. Once the CytoDx Lab Director signs off on the sample results, CytoDx removes the suppressed calls from the Interval Table.

Edit aberrations (optional)

You can edit the start and stop positions of an aberration call.

1. In the Tab View, click the tab for the Interval Table (if not already selected).

2. In the Interval Table, locate the CNV or LOH call that you want to edit. In the row for that call, click **Edit**.

   The Edit Aberration dialog box opens.

3. Use the fields in the dialog box to edit the start and stop positions. Click **Apply** to apply your changes and close the dialog box.

Add aberrations (optional)

You can add an aberration call to a specified genomic interval.

1 At the top of the Triage View, click **Change Call > Add Call**.

   The Add Aberration/SNP Interval call dialog box opens.

2 In the Call drop-down list, select the type of aberration to be added (Gain, Loss, or LOH).

3 In the Chromosome drop-down list, select the chromosome number where the aberration is located.

4 In the Start and Stop fields, type the nucleotide positions of the start and stop locations of the aberration.

5 (Optional) If the new aberration is a gain or loss, type values for the mean log ratio and p-value into the Mean and Pvalue fields. If the new aberration is an LOH interval, type the LOH score into the LOH Score field.

6 Click **Find Probes**.

   The table at the bottom of the dialog box lists the probes that fall within the specified aberration interval (highlighted in green) and the five most adjacent upstream and downstream probes (highlighted in gray).

7 Click **Add**.

   The dialog box closes and the new aberration is added to the sample. In the Interval Table, the aberration is listed with a state of *Added*.

Step 6. Classification of Aberrations

☐ Technicians ☑ Cytogeneticists ☑ Lab Directors ☐ System Admins

Classifications are user-defined annotations (up to 70 characters) used to classify the CNVs and LOH intervals identified in a sample.

NOTE

The Lab Director must first create the classifications in CytoDx in accordance with existing laboratory practices. For instructions on creating classifications, see the CytoDx help system or the *CytoDx Installation and Set Up Guide*. Commonly used classifications are listed in Table 24.

Table 24 Common classifications

Classification
Pathogenic
Likely pathogenic
VOUS (variant of unknown significance)
Likely benign
Benign

Classifying aberrations (e.g., as Pathogenic, Likely pathogenic, VOUS, Likely benign, or Benign) requires clinical interpretation of the aberration results. Clinical interpretation is performed by a Board Certified Cytogeneticist, Molecular Geneticist, Molecular Pathologist, or similarly qualified clinician who has been trained to identify the clinically relevant CNVs and cnLOH intervals, determine clinical significance, and report out these findings.

1 If you have not yet checked out the sample results, do that now. See "Check out/check in sample results in Triage View (if needed)" on page 88 for instructions.

2 (Optional) Set the Tracks View to display the aberration track (*Aberration_Track_hg19*) which contains all the aberrations that are present in the previously signed-off samples in the database.

Displaying the aberration track alongside sample results can be helpful when deciding how to classify aberrations. You can easily determine if an aberration was present in any of the previously analyzed samples and, if so, see how it was classified. To view the classification assigned to an aberration track interval, hover the cursor over the interval to view the tooltip. Note that you may need to customize the tooltips so that they include classification information. Lab Directors can customize tooltips from the **Configure Settings > Tracks** screen.

3 In the Tab View, click the tab for the Interval Table (if not already selected).

4 In the row for the aberration of interest, expand the drop-down list in the Classification column.

5 In the menu that opens, mark the classifications that you want to apply to the aberration.

6 Click **OK**.

The menu closes and the classifications appears in the Classification column for the aberration. CytoDx saves your changes when you check in or sign off on the sample results.

Step 7. Generation of the Pre-final Report (optional)

☐ Technicians ☑ Cytogeneticists ☑ Lab Directors ☐ System Admins

The program generates a final report when the Lab Director signs off on the sample results, but you can view a pre-final copy of the report before the results are signed off. A banner at the bottom of the pre-final report PDF states that the document is an intermediate report.

1 At the top of the Triage View, click **Reports > Cyto Report**.

The Intermediate Cyto Report dialog box opens.

2 Select the template for the Cyto report and click **Proceed**.

The program begins generating the report, and a progress bar opens on the screen. When the report is finished, the PDF opens. If you want to save a copy, do so before closing the PDF.

Step 8. Signing Off of Sample Results and Generation of Final Report

☐ Technicians ☐ Cytogeneticists ☑ Lab Directors ☐ System Admins

Only Lab Directors can perform this task. Once sample results are signed off, no further changes can be made by any CytoDx user.

1 Before signing off, review the sample attributes to ensure that the attributes were correctly assigned. Incorrect sample information can lead to incorrect results.

   a At the top of the Triage View, click **Sample Info > Attributes**.

      The Attributes dialog box opens.

   b Verify that the values for the sample attributes are correct.

      If the Array ID, Green Sample, Red Sample, or Polarity attribute is incorrectly assigned, do not sign off on the sample. Delete the sample record and repeat the analysis workflow using the correct sample information.

   c Click **Close** to close the dialog box.

2 At the top of the Triage View, click **Change Status > Sign Off**.

   The Authentication dialog box opens.

3 Enter your user name and password and click **Ok**.

   The Confirm dialog box opens.

4 Click **OK** to confirm that you want to sign off and finalize the results.

   The Generate Sign Off Reports dialog box opens.

5 In the drop-down list under **Cyto Report**, select the template to be used for the report.

NOTE

The templates in the drop-down list include the default template as well as any custom templates that have already been created in the software.

CytoDx users with the role of Lab Director can create custom templates from the Configure Settings screen or by clicking **Create New Template** from within the Generate Sign Off Reports dialog box. For instructions, see the CytoDx help system.

6 Under **Other reports that will be generated**, mark the additional reports that you want to generate.

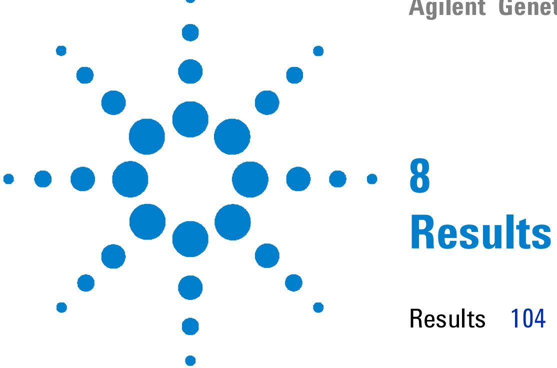
NOTE Some of the options for these additional reports are configurable. CytoDx users with the role of Lab Director can configure these options from the Configure Settings screen. For instructions, see the CytoDx help system.

7 Click **Proceed** to begin report generation.

CytoDx generates the reports and saves the files to the output folder. The Cyto report is generated in PDF and XML formats. When the program finishes the reports, a dialog box opens asking if you want to open the folder containing the reports. Click **Yes** to open the folder, or click **No** to close the dialog box and return to the Triage View without opening the folder. The reports can also be accessed from the Sample Review screen of the software.

The final Cyto report generated during sign-off contains the final results of the assay. It lists the gains, losses, and cnLOH intervals in the sample and the classification assigned to each aberration, as well as other information specified in the report template.

Follow your institutional procedures to ensure security of the report.



8
Results

Results 104

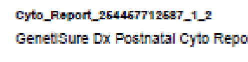
This chapter describes the principles for interpretation of the assay results.

Results

In an analytical accuracy study with 600 samples, a total of 2479 aberrations were used for evaluation of aberration calling accuracy. The average confirmation rates for the GenetiSure Dx Postnatal Assay were calculated to be 93.5% for larger CNVs (>20 probes per aberration), 92.5% for smaller CNVs (5–20 probes), and 90.1% for cnLOH intervals. See "Accuracy" on page 121 of Chapter 10 for details about the calculations for aberration calling accuracy and the aberrations included in the evaluation.

The aberrations identified in a patient sample by the CytoDx algorithms can be viewed from the Triage View screen of the CytoDx software. The Interval Table near the bottom of this screen lists the aberrations in table format, with each aberration listed as an individual row in the table. Aberrations with a state of *Algorithm Generated* were identified by the CytoDx aberration detection algorithm, while aberrations with a state of *Added* were manually added by a CytoDx user. Aberrations with a state of *Edited* were either identified by the CytoDx algorithm or added by a CytoDx user, but the start and/or stop positions of the aberration were manually changed by a user. Aberrations that have been suppressed by a CytoDx user are still listed in the Interval Table, but the check box in the Suppress column is marked. In the final Cyto Report, which is generated when the Lab Director signs off on the sample results, the aberrations that are listed in the Amp/Del Intervals Table and the LOH Intervals Table reflect the aberrations listed in the Triage View Interval Table at the time of sign-off (see Figure 38 on page 105). Note that suppressed aberrations are not listed in the Cyto Report.

Clinical interpretation of the aberration results takes place during classification of the aberrations (e.g., as Pathogenic, Likely pathogenic, VOUS, Likely benign, or Benign) in the CytoDx software. Clinical interpretation is performed by a Board Certified Cytogeneticist, Molecular Geneticist, Molecular Pathologist, or similarly qualified clinician who has been trained to identify the clinically relevant CNVs and cnLOH intervals, determine clinical significance, and report out these findings. To select the appropriate classification, the clinician can rely on prior knowledge, comparisons to tracks or to other samples, and references to gene ontology database that describe the functions and disease associations for genes impacted by the aberrations. The classification assignments appear in the Cyto Report in the Classifications section (see Figure 38 on page 105).



Cyto_Report_254457712587_1_2
GenetiSure Dx Postnatal Cyto Report

Sample Information

Array ID	:	254457712587_1_2
Global Display Name	:	254457712587_1_2
Green Sample	:	Agilent Male Reference (Track : Agilent Male CNV Reference, GenotypeRef : AGILENT MALE)
Red Sample	:	NA13475
SignedOff by	:	AGILENT\dhuffman

Classifications

Gain/Loss Interval Classifications

Interval	Classification
chr15:102161480-102252128	Benign
chr7:72841632-74133332	Pathogenic
chr14:20203610-20421677	VOUS

LOH Interval Classifications

No classification available.

Gain/Loss Intervals Table

Chr	Start-Stop(bp)	Size(kb)	Cytoband	#Probes	Gain/Loss	Annotations
chr7	72841632-74133332	1,291.701	q11.23	84	-0.794880	FZD9, BAZ1B, BCL7B, TBL2, MLXIPL, VPS37D, DNAJC30, BUD23, STX1A, MIR4284...
chr14	20203610-20421677	218.068	q11.2	8	-0.543895	OR4Q3, OR4M1, OR4N2, OR4K3, OR4K2, OR4K5, OR4K1
chr15	102161480-102252128	90.649	q26.3	21	0.541340	TM2D3, TARSL2

Gain=Gain
Loss=Loss
Total Gain/Loss Intervals: 3

LOH Intervals Table

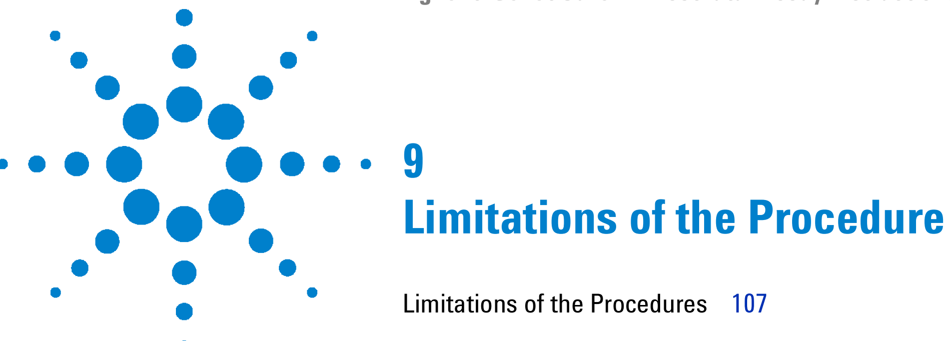
No data available.

Total LOH Intervals: 0

For In Vitro Diagnostic Use

Page 1 Analysis Method : GenetiSure Dx Postnatal Analysis Method_v1 14-Aug-2017 11:18:13

Figure 38 Page 1 of a sample Cyto Report



9
Limitations of the Procedure

Limitations of the Procedures 107

This chapter describes the limitations of the procedure.

Limitations of the Procedures

1. The CytoDx software does not perform aberration detection in the pseudoautosomal regions (PAR) of the Y chromosome. As the PAR sequences are the same on the X and Y chromosomes, in male samples, CytoDx cannot distinguish between a PAR aberration on the X chromosome and a PAR aberration on the Y chromosome. Consequently, any aberrations identified in a PAR are assigned to the X chromosome. For male patient samples (or any patient sample paired with a male reference sample), probes in Y chromosome PAR regions are included in the Triage View display of the CytoDx software for visualization purposes only. In patient samples that contain an abnormality of the Y chromosome, the abnormalities in the PAR1 and PAR2 regions at the ends of the short and long arms of the Y chromosome are only displayed on the X chromosome. For female patient samples (or any patient sample paired with a female reference sample), probes on the Y chromosome are included in the Triage View display of the CytoDx software for visualization purposes, but the samples are not analyzed for Y chromosome aberrations.
2. The assay should be performed in a licensed clinical laboratory.
3. The assay is for use with gDNA from whole blood specimens only. Blood must be collected in tubes using EDTA as the anticoagulent. It has not been validated for any other specimen type.
4. The assay was validated for use with 500ng of gDNA. Less than 375 ng gDNA may not work.
5. Do not quantify the DNA using absorbance.
6. Mosaicism CN less than 50% may not be reliably detected.
7. The GenetiSure Dx Postnatal Assay cannot identify balanced chromosomal rearrangements, such as translocations or inversions.
8. The smallest regions that the software will report are 20 kb and 5 probes for gains, and 10 kb and 5 probes for losses. At this size range, reproducibility for copy number gains between 20–50 kb is 82%, and reproducibility for copy number losses between 10–50 kb is approximately 76% (refer to the analytical performance in Table 25 on page 111). Performance of the assay has not been assessed for CNVs with size and marker number below these settings for reporting.
9. The smallest regions the software will report for cnLOH are 5 Mb and 100 SNP probes.

10 Links to external databases have not been evaluated or curated by Agilent.

11 GenetiSure Dx Postnatal Assay is limited to personnel trained in this assay.

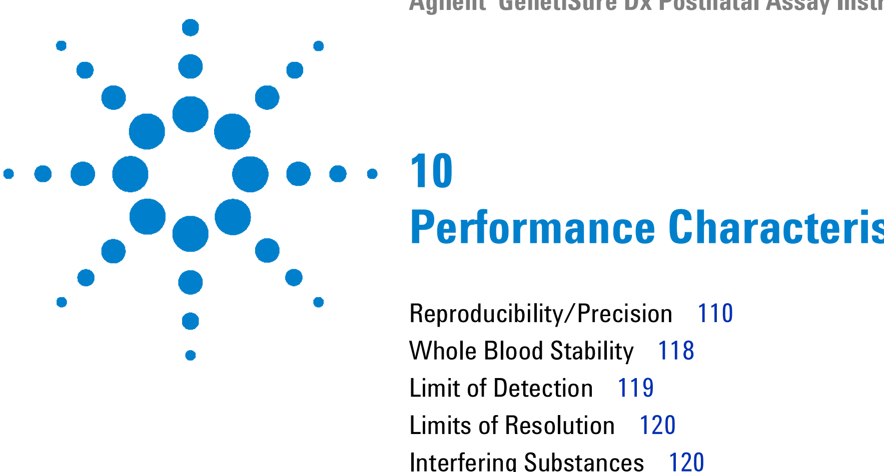
12 This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing or screening, population screening, or for the detection of, or screening for, acquired or somatic aberrations.

13 Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, parental evaluation, clinical genetic evaluation, and counseling, as appropriate. Confirmation of microarray results using qPCR, FISH, or another approach is recommended.

14 CNVs that are present in regions with no or low probe coverage may not be detected, including regions in which probes were masked during the CytoDx analysis. The CytoDx software masks probes in regions in which the reference sample contains a CNV and in some regions of hypervariability. The preloaded tracks *Agilent Female CNV Reference* and *Agilent Male CNV Reference* contain the masked intervals for the female and male reference samples respectively. You can view these tracks from the **Configure Settings > Tracks** screen in the CytoDx software.

15 The GenetiSure Dx Postnatal Assay is for use on the SureScan Dx Microarray Scanner.

16 The GenetiSure Dx Postnatal Assay was designed and validated to categorize CNVs as “Gains” or “Losses,” not to report quantitative values for copy number. The CytoDx software may report two or more embedded or adjacent CNVs as a single aberration.



10
Performance Characteristics

Reproducibility/Precision 110
Whole Blood Stability 118
Limit of Detection 119
Limits of Resolution 120
Interfering Substances 120
Cross Contamination 121
Accuracy 121
Clinical Validity 129

This chapter describes the performance characteristics of the assay.

Reproducibility/Precision

Three reproducibility/precision studies were conducted. The first was a multi-site reproducibility study, and the second and third were two single-site precision studies that assessed assay variabilities across multiple reagent lots, scanners, and repeated DNA extractions.

Reproducibility

The aim of the reproducibility study was to demonstrate that GenetiSure Dx Postnatal Assay achieves acceptable, reproducible results when performed at multiple laboratory sites by multiple operators over multiple days. Replicates of forty-eight (48) test samples containing a wide range of chromosomal aberrations (gains, losses, and cnLOH intervals) were processed by two separate operators, at each of three individual clinical laboratories, in three (3) one-week intervals for a total of 864 data points.

Individual aberrations called within each processed test sample were compared to their respective replicates (18 replicates for each aberration, operator by site by week) by pairwise replicate analysis (PRA), requiring at least 50% overlap of chromosomal coordinates for confirmation. Positive agreement was assessed separately for small CNVs (5–20 probes contained with the aberration), larger CNVs (>20 probes), or cnLOH regions. The results demonstrate that the pre-defined acceptance criteria were met for each category with a pairwise replicate agreement of 80.22%, 95.83%, and 89.08%, respectively. Using a more stringent 80% overlap criteria for pairwise replicate agreement, acceptance criteria were also met.

Data were further refined by size, probe number, aberration type, and study variable (e.g., operator, site, test sample). The results demonstrate that the assay is reproducible and suitable for implementation in a clinical laboratory environment.

Table 25 Reproducibility of Aberrations Categorized by Size (in kb) and Type Based on Call Rate, Pairwise Agreement between Replicates and Positive Percent Agreement (PPA) for Two Criteria (50% and 80% Overlap) in All Regions in the Site-to-Site Study

Aberration Type	Aberration Range (kb)	# Aberrations	Call Rate (%)	Pairwise Replicate Agreement (%)		PPA (%)	
				Overlap			
				50%	80%	50%	80%
Gain	10–50	5	51.2	82.5	82.5	82.9	82.9
	50–100	3	68.7	96.3	96.3	97.3	97.3
	100–200	13	50.5	79.9	79.8	80.1	80.0
	200–500	26	82.7	86.3	84.6	91.5	89.4
	500–1000	9	79.7	80.2	78.9	87.6	86.0
	1000–2000	7	72.1	90.7	82.8	92.4	81.6
	2000–5000	11	65.1	75.9	75.9	79.7	79.7
	5000+	13	93.2	98.4	98.4	99.1	99.1
	Total	87	73.8	85.7	84.4	89.9	88.2
Loss	10–50	14	51.6	76.8	76.1	77.6	76.2
	50–100	2	100.0	100.0	89.5	100.0	89.5
	100–200	23	81.4	82.3	78.1	88.1	82.9
	200–500	31	82.6	81.8	75.5	86.3	78.7
	500–1000	55	72.8	81.2	76.2	85.2	78.5
	1000–2000	30	83.3	86.4	85.9	91.5	91.0
	2000–5000	18	88.9	87.4	85.1	89.9	87.3
	5000+	20	100.0	100.0	100.0	100.0	100.0
	Total	193	80.1	84.6	81.3	89.0	84.8
All CNVs (Gain & Loss)	Total	280	78.1	85.0	82.3	89.3	85.8
cnLOH	5000–10,000	21	50.6	77.1	76.8	77.4	76.8
	10,000–20,000	11	91.5	99.0	96.4	99.4	96.7
	20,000+	13	100.0	100.0	98.4	100.0	98.4
	Total	45	74.9	89.1	87.9	92.7	91.1

Table 26 Reproducibility of Aberrations Categorized by Probe Number and Type Based on Call Rate, Pairwise Agreement between Replicates and Positive Percent Agreement (PPA) for Two Criteria (50% and 80% Overlap) in All Regions in the Site-to-Site Study

Aberration Type	Aberration Range (# probes)	# Aberrations	Call Rate (%)	Pairwise Replicate Agreement (%)		PPA (%)	
				Overlap			
				50%	80%	50%	80%
Gain	5–7	11	38.0	76.5	76.5	69.0	69.0
	7–10	15	54.1	70.6	69.4	72.8	70.6
	10–15	23	87.9	89.4	88.3	94.0	92.7
	15–20	11	66.5	82.4	79.9	86.8	83.1
	20–30	9	89.6	97.5	97.5	97.9	97.9
	30–100	3	70.3	93.0	93.0	95.0	95.0
	100–500	3	72.3	90.2	90.2	93.2	93.2
	500+	12	100.0	100.0	100.0	100.0	100.0
	Total	87	73.8	86.1	85.3	90.5	89.4
Loss	5–7	36	61.1	76.6	75.6	80.9	79.1
	7–10	39	65.5	77.6	75.4	82.8	79.6
	10–15	42	81.9	85.5	81.0	90.5	85.0
	15–20	18	96.9	95.9	94.0	97.6	95.7
	20–30	16	87.5	89.1	85.7	91.6	87.9
	30–100	10	92.2	93.2	92.9	96.3	96.0
	100–500	17	100.0	100.0	100.0	100.0	100.0
	500+	15	100.0	100.0	100.0	100.0	100.0
	Total	193	80.1	86.3	84.2	91.1	88.5
All CNVs (Gain & Loss)	Total	280	78.1	86.2	84.6	90.9	88.8
cnLOH	100–200	25	54.8	80.3	80.3	82.0	82.0
	200–500	13	100.0	100.0	97.7	100.0	97.7
	500+	7	100.0	100.0	100.0	100.0	100.0
	Total	45	74.9	89.1	88.4	92.7	91.8

For endpoint analysis, only those CNVs detected were assessed for endpoint agreement (i.e., 'no calls' in replicates could not be included). The CNVs had to have the same copy number state (gain/loss) in order to be included in the endpoint agreement calculation. Endpoint agreement is assessed by median % absolute endpoint deviation, standard deviation of left endpoint, and standard deviation of right endpoint. The results are shown in Table 27 and Table 28.

For copy number aberrations (combined gains and losses), the Median % Absolute Endpoint Deviation was 3% (mean) when analyzed by both probe number and size (in kb). For cnLOH calls, the Median % Absolute Endpoint Deviation was 1% (mean) for both analyses. In addition, the average % overlap for all pairwise confirmed aberrations was 82.1% (by probe number) and 80.8% (by size) for CNVs, and 84.7% (by probe number) and 84.5% (by size) for cnLOH calls.

Table 27 Reproducibility of Aberration Breakpoints by Probe Number

Type	Aberration Range (# probes)	N	% CV Aberration Length	Average % Overlap	Median % Absolute Endpoint	SD Left Endpoint (# Probes)	SD Right Endpoint (# Probes)
			Mean (Min, Median, Max)		Mean (Min, Median, Max)		
Gain	5–7	11	3.0 (0.0, 0.0, 11.7)	51.9	0.01 (0.00, 0.00, 0.13)	0.1 (0.0, 0.0, 0.6)	0.1 (0.0, 0.0, 0.5)
	7–10	15	9.0 (0.0, 6.5, 35.4)	55.1	0.05 (0.00, 0.00, 0.31)	0.5 (0.0, 0.4, 2.0)	0.5 (0.0, 0.0, 2.9)
	10–15	23	5.1 (0.0, 4.5, 19.0)	86.6	0.01 (0.00, 0.00, 0.08)	0.4 (0.0, 0.2, 2.7)	0.2 (0.0, 0.0, 1.5)
	15–20	11	5.0 (0.0, 3.0, 17.0)	74.8	0.02 (0.00, 0.00, 0.11)	0.8 (0.0, 0.4, 2.8)	0.1 (0.0, 0.0, 0.5)
	20–30	9	3.0 (0.0, 1.5, 19.5)	96.5	0.01 (0.00, 0.00, 0.05)	0.8 (0.0, 0.0, 5.1)	0.4 (0.0, 0.3, 1.7)
	30–100	3	0.9 (0.0, 1.3, 1.4)	90.2	0.00 (0.00, 0.00, 0.01)	0.1 (0.0, 0.0, 0.3)	0.3 (0.0, 0.5, 0.5)
	100–500	3	0.1 (0.0, 0.2, 0.2)	87.3	0.00 (0.00, 0.00, 0.00)	0.2 (0.0, 0.0, 0.5)	0.0 (0.0, 0.0, 0.0)
	500+	12	0.1 (0.0, 0.1, 0.3)	99.9	0.00 (0.00, 0.00, 0.00)	1.0 (0.0, 0.5, 3.6)	0.8 (0.0, 0.2, 3.2)
	Total	87	4.3 (0.0, 2.4, 35.4)	81.4	0.02 (0.00, 0.00, 0.31)	0.5 (0.0, 0.3, 5.1)	0.3 (0.0, 0.0, 3.2)

Table 27 Reproducibility of Aberration Breakpoints by Probe Number (continued)

Type	Aberration Range (# probes)	N	% CV Aberration Length	Average % Overlap	Median % Absolute Endpoint	SD Left Endpoint (# Probes)	SD Right Endpoint (# Probes)
			Mean (Min, Median, Max)		Mean (Min, Median, Max)		
Loss	5–7	36	6.9 (0.0, 7.4, 21.1)	65.2	0.03 (0.00, 0.00, 0.20)	0.1 (0.0, 0.0, 1.1)	0.4 (0.0, 0.3, 1.5)
	7–10	39	6.6 (0.0, 4.5, 28.6)	68.2	0.02 (0.00, 0.00, 0.22)	0.1 (0.0, 0.0, 0.9)	0.5 (0.0, 0.3, 2.2)
	10–15	42	9.0 (0.0, 3.6, 66.1)	81.0	0.05 (0.00, 0.00, 0.60)	0.8 (0.0, 0.0, 6.8)	0.5 (0.0, 0.3, 3.0)
	15–20	18	7.4 (0.0, 1.7, 53.6)	93.2	0.10 (0.00, 0.00, 1.42)	1.2 (0.0, 0.1, 9.4)	0.7 (0.0, 0.2, 9.8)
	20–30	16	8.5 (0.0, 0.4, 47.3)	87.7	0.01 (0.00, 0.00, 0.18)	1.4 (0.0, 0.1, 6.9)	0.7 (0.0, 0.0, 5.1)
	30–100	10	3.9 (0.0, 0.3, 24.0)	92.1	0.01 (0.00, 0.00, 0.04)	0.1 (0.0, 0.0, 1.0)	1.2 (0.0, 0.0, 8.9)
	100–500	17	0.3 (0.0, 0.1, 1.3)	99.9	0.00 (0.00, 0.00, 0.00)	0.3 (0.0, 0.0, 2.6)	0.2 (0.0, 0.2, 0.4)
	500+	15	0.1 (0.0, 0.1, 0.2)	100.0	0.00 (0.00, 0.00, 0.00)	0.3 (0.0, 0.0, 3.7)	0.3 (0.0, 0.0, 1.1)
	Total	193	6.2 (0.0, 1.3, 66.1)	82.4	0.03 (0.00, 0.00, 1.42)	0.5 (0.0, 0.0, 9.4)	0.5 (0.0, 0.0, 9.8)
All CNVs (Gain & Loss)	Total	280	5.6 (0.0, 1.9, 66.1)	82.1	0.03 (0.00, 0.00, 1.42)	0.5 (0.0, 0.0, 9.4)	0.4 (0.0, 0.0, 9.8)
cnLOH	100–200	25	4.8 (0.0, 4.8, 13.4)	67.8	0.01 (0.00, 0.00, 0.11)	4.7 (0.0, 0.3, 25.6)	3.5 (0.0, 0.5, 17.2)
	200–500	13	7.0 (4.1, 6.1, 12.5)	98.2	0.00 (0.00, 0.00, 0.02)	9.7 (0.0, 5.7, 32.3)	8.0 (0.0, 2.9, 28.4)
	500+	7	6.0 (4.1, 5.4, 9.6)	98.6	0.01 (0.00, 0.00, 0.04)	32.2 (1.1, 36.4, 74.8)	9.3 (0.0, 2.6, 42.9)
	Total	45	5.6 (0.0, 5.4, 13.4)	84.7	0.01 (0.00, 0.00, 0.11)	10.4 (0.0, 3.0, 74.8)	5.7 (0.0, 0.9, 42.9)

Table 28 Reproducibility of Aberration Breakpoints by Size (kb)

Type	Aberration Range (kb)	N	% CV Aberration Length	Average % Overlap	Median % Absolute Endpoint Deviation	SD Left Endpoint (kb)	SD Right Endpoint (kb)
			Mean (Min, Median, Max)		Mean (Min, Median, Max)		
Gain	10–50	5	5.0 (0.0, 0.7, 14.7)	69.3	0.00 (0.00, 0.00, 0.02)	0.8(0.0, 0.2, 3.5)	0.5 (0.0, 0.0, 2.5)
	50–100	3	0.0 (0.0, 0.0, 0.0)	94.7	0.00 (0.00, 0.00, 0.00)	0.0 (0.0, 0.0, 0.0)	0.0 (0.0, 0.0, 0.0)
	100–200	13	2.2 (0.0, 0.0, 7.5)	65.5	0.00 (0.00, 0.00, 0.01)	2.1 (0.0, 0.0, 10.6)	1.7 (0.0, 0.0, 11.0)
	200–500	26	10.1 (0.0, 4.8, 62.9)	82.4	0.06 (0.00, 0.00, 1.04)	21.7 (0.0, 7.2, 248.9)	16.4 (0.0, 3.4, 186.3)
	500–1000	9	8.5 (0.0, 3.4, 27.8)	76.6	0.01 (0.00, 0.00, 0.05)	15.8 (0.0, 0.0, 72.9)	38.2 (0.0, 0.0, 195.6)
	1000–2000	7	8.9 (0.0, 0.0, 27.5)	83.9	0.00 (0.00, 0.00, 0.03)	81.0 (0.0, 0.0, 359.0)	36.4 (0.0, 0.0, 210.8)
	2000–5000	11	7.3 (0.0, 1.5, 58.6)	68.2	0.01 (0.00, 0.00, 0.07)	169.0 (0.0, 28.3, 1437.3)	8.1 (0.0, 0.0, 54.3)
	5000+	13	0.3 (0.0, 0.1, 1.2)	98.2	0.00 (0.00, 0.00, 0.01)	33.0 (0.0, 24.2, 147.2)	49.8 (0.0, 0.3, 471.7)
	Total	87	6.2 (0.0, 1.5, 62.9)	80.8	0.02 (0.00, 0.00, 1.04)	41.3 (0.0, 2.5, 1437.3)	20.5 (0.0, 0.0, 471.7)

Table 28 Reproducibility of Aberration Breakpoints by Size (kb) (continued)

Type	Aberration Range (kb)	N	% CV Aberration Length	Average % Overlap	Median % Absolute Endpoint Deviation	SD Left Endpoint (kb)	SD Right Endpoint (kb)
			Mean (Min, Median, Max)		Mean (Min, Median, Max)		
Loss	10–50	14	3.3 (0.0, 0.0, 29.6)	62.6	0.00 (0.00, 0.00, 0.00)	0.3 (0.0, 0.0, 1.4)	0.7 (0.0, 0.0, 8.2)
	50–100	2	11.4 (0.0, 11.4, 22.9)	97.0	0.00 (0.00, 0.00, 0.00)	6.9 (0.0, 6.9, 13.9)	0.0 (0.0, 0.0, 0.0)
	100–200	23	13.7 (0.0,0.0, 139.6)	78.8	0.07 (0.00, 0.00, 0.82)	11.8 (0.0, 0.0, 220.5)	10.3 (0.0, 0.0, 87.5)
	200–500	31	19.2 (0.0, 3.3, 141.8)	78.4	0.03 (0.00, 0.00, 0.35)	18.2 (0.0, 0.0, 391.8)	40.3 (0.0, 0.0, 186.3)
	500–1000	55	13.2 (0.0, 6.5, 53.4)	74.0	0.01 (0.00, 0.00, 0.14)	41.5 (0.0, 0.0, 448.0)	57.2 (0.0, 6.8, 271.0)
	1000–2000	30	4.4 (0.0, 1.2, 27.2)	83.6	0.02 (0.00, 0.00, 0.16)	16.2 (0.0, 0.0, 124.1)	60.2 (0.0, 13.5, 446.5)
	2000–5000	18	11.7 (0.0, 1.7, 69.2)	86.1	0.11 (0.00, 0.00, 1.42)	251.5 (0.0, 6.2, 1715.0)	276.3 (0.0, 25.6, 1892.8)
	5000+	20	0.0 (0.0, 0.0, 0.2)	100.0	0.00 (0.00, 0.00, 0.00)	6.6 (0.0, 0.0, 114.7)	2.7 (0.0, 0.0, 39.4)
	Total	193	10.6 (0.0, 1.3, 141.8)	80.9	0.03 (0.00, 0.00, 1.42)	42.9 (0.0, 0.0, 1715.0)	59.5 (0.0, 0.0, 1892.8)
All CNVs (Gain & Loss)	Total	280	9.3 (0.0, 1.4, 141.8)	80.8	0.03 (0.00, 0.00, 1.42)	42.4 (0.0, 0.0, 1715.0)	47.4 (0.0, 0.0, 1892.8)
cnLOH	5000–10000	21	5.2 (0.0, 4.5, 22.2)	61.3	0.02 (0.00, 0.00, 0.24)	160.7 (0.0, 0.3, 25.6)	3.5 (0.0, 0.5, 17.2)
	10000–20000	11	5.6 (0.0, 6.2, 10.3)	96.3	0.00 (0.00, 0.00, 0.00)	431.8 (0.0, 346.5, 961.0)	8.0 (0.0, 2.9, 28.4)
	20000+	13	3.3 (0.3, 3.3, 10.9)	98.5	0.01 (0.00, 0.00, 0.04)	1323.1 (0.0, 810.2, 6338.1)	9.3 (0.0, 2.6, 42.9)
	Total	45	4.7 (0.0, 3.4, 22.2)	84.5	0.01 (0.00, 0.00, 0.24)	562.8 (0.0, 263.6, 6338.1)	336.0 (0.0, 67.8, 1947.4)

Between-Lot Reagent and Scanner Precision

The aim of these precision studies was to demonstrate that GenetiSure Dx Postnatal Assay achieves acceptable, precise results when performed using multiple reagent manufacturing lots and when analyzed on multiple scanner instruments. Forty-eight (48) test samples containing a range of chromosomal aberrations (gains, losses, and cnLOH) were processed by multiple operators, using combinations of three (3) reagent lots and three (3) scanner instruments across three (3) processing weeks at a single site for a total of 432 data points.

Individual aberrations called within each processed test sample were compared to their respective replicates (9 replicates for each aberration, representing 3x3 reagent-lot/scanner combinations) by pairwise replicate analysis (PRA), requiring at least 50% overlap of chromosomal coordinates for confirmation. Agreement was assessed separately for small CNVs (5-20 probes contained with the aberration), larger CNVs (>20 probes), or cnLOH regions. The results demonstrate that the pre-defined acceptance criteria were met for each category with a pairwise replicate agreement of 83.33%, 98.39%, and 80.80%, respectively. Results were similar when using a more stringent 80% overlap criteria for pairwise replicate agreement. In addition, no substantial differences were observed when the pairwise replicate agreement was assessed separately for inter-lot vs. intra-lot replicate pairs, or for inter-scanner vs. intra-scanner replicate pairs.

Data were further refined by size, probe number, aberration type, and study variable (e.g., reagent lot, scanner, processing week). The results demonstrate that the assay produces consistent aberration calls across multiple reagent lots and scanners, and is suitable for implementation in a clinical laboratory environment.

Precision – DNA Extraction

The GenetiSure Dx Postnatal Assay is for use with gDNA from whole blood specimens only. The gDNA is extracted from the patient's whole blood using the QIAamp DSP DNA Blood Mini Kit from Qiagen (p/n 61104) following the Instructions For Use (IFU) included with the Qiagen kit.

The DNA extraction precision study was performed to assess the aberration calling concordance of the assay across repeated DNA extractions from the same blood sample performed by different operators in multiple runs, to determine repeatability and precision.

A panel of twenty-four (24) samples was tested by each operator. The gDNA from the samples was extracted using the same lot of the Qiagen kit, at one site, in duplicate, by 3 operators, on 3 separate days, for a total of 432 extractions (3 operators × 3 days × 2 duplicates × 24 samples).

Primary analysis was performed using pairwise comparison of aberration results on each of the 18 replicates (3 operators × 3 days × 2 duplicates) for each sample. An aberration was considered confirmed if at least 50% of the region of aberration overlapped between the replicates being compared.

The results of the 50% overlap analysis demonstrated that results obtained from multiple extractions of the same sample were highly concordant, regardless of operator and day upon which the samples were extracted. The individual Pairwise Replicate Agreement % values stratified by week or operator were similar to each other and to the overall averages shown (82.17% for CNVs with 5–20 probes, 98.47% for CNVs with >20 probes, and 81.15% for cnLOH), which further supports that similar assay performance can be expected from different extractions, personnel, day, and sample.

Whole Blood Stability

The GenetiSure Dx Postnatal Assay is for use with gDNA extracted from whole blood specimens only. Blood is collected in tubes containing EDTA as the anticoagulant.

Whole blood specimens from the collection site may be stored prior to processing in batch, or may need to be transferred to a remote laboratory for processing.

To determine the stability of whole blood specimens prior to gDNA isolation, 24 whole blood specimens, 12 male and 12 female, were obtained from a blood bank and gDNA was isolated from the specimens at 1, 3, 7, and 10 days after initial collection.

A list of aberrations for each sample extracted on Days 3, 7 and 10 were reported and compared with the 'Day 1' list for the same sample. An aberration from the 'Day 1' sample was considered confirmed in the stored samples if the test result identifies a region of aberration that overlaps the 'Day 1' region by at least 50%.

For small CNVs (those with 5–20 probes), the confirmation rate was >90% for all tested storage times. For larger CNVs (those with >20 probes), the confirmation rate was >98% for all tested storage times. When a more stringent confirmation criteria of 80% overlap was applied, similar results were obtained (>90% for small CNVs and >97% for large CNVs).

The results of both the 50% and 80% overlap analysis methods demonstrated that whole blood specimens may be stored for up to 10 days at 2–8ºC prior to gDNA isolation. Samples stored for this period of time and processed with the GenetiSure Dx Postnatal Assay produced acceptable results.

Limit of Detection

To determine the analytical sensitivity, or the Limit of Detection (LOD) of the GenetiSure Dx Postnatal Assay, a study was conducted to evaluate the minimum and maximum amounts of DNA acceptable as the assay input to detect CNVs and cnLOH intervals accurately. Twenty-four (24) gDNA samples with known chromosomal aberrations were obtained from Coriell Institute for Medical Research (Coriell). These DNA samples were tested in the assay using two (2) lots of reagents across a range of varied DNA input levels from 0.125 µg (125 ng) to 1 µg (1000 ng), with 0.5 µg (500 ng) as the recommended input quantity (standard).

The study assessed the impact of various DNA input on aberration calling and determined the upper and lower limits of detection (ULOD and LLOD) of the assay by comparing the percentage of aberrations confirmed at each non-standard DNA input level against pre-defined acceptance criteria. Data from this study support the use of 500 ng as the recommended input amount. The study data, and supplemental data generated under similar study conditions, demonstrate that performance does not decline down to 375 ng. The data support a conservative LLOD at 375 ng and a common ULOD at 1000 ng for both CNVs and cnLOH intervals. For CNVs only, the LLOD could be further reduced to 250 ng.

The assay performs robustly at the recommended input amount of 500 ng and is stable at considerably lower and higher amounts.

Limits of Resolution

The limits of resolution (i.e., minimum aberration size detectable) for the GenetiSure Dx Postnatal Assay are set by the thresholds used in the CytoDx analysis method. These thresholds vary by aberration type.

Gains must be at least 20 kb and contain at least 5 CGH probes. Losses must be at least 10 kb and contain at least 5 CGH probes. LOH intervals must be at least 5 Mb and contain at least 100 SNP probes.

For mosaic samples, the sensitivity of CNV detection is reduced, especially for CNVs containing ≤ 100 probes. However, CNVs containing ≥ 100 probes can be reliably detected in mosaic samples in which the aberration is present in ≥50% of the sample cells.

Interfering Substances

The GenetiSure Dx Postnatal Assay uses gDNA isolated from patient whole blood. Specimens may be obtained from patients with endogenous conditions resulting in hemolysis, bilirubinemia, or lipemia.

To determine the effects of these conditions on the results of the test, the study evaluated the impact of hemoglobin, conjugated bilirubin, unconjugated bilirubin and triglycerides (triolein) spiked into whole blood prior to gDNA isolation. The level of each interferent in the spiked whole blood samples is listed in Table 29.

Table 29 Final levels of interferents in the whole blood samples

Interferent	Final level
Hemoglobin	~2× the level of the non-adulterated condition
Conjugated bilirubin	20 mg/dL
Unconjugated bilirubin	20 mg/dL
Triglycerides (triolein)	>3000 mg/dL

Blood drawn from twelve (12) phenotypically normal males and twelve (12) phenotypically normal females was used in the testing.

The list of aberrations for each sample containing a given interferent was reported and compared with the 'non-adulterated control' list for the same sample. For small CNVs (those with 5–20 probes), the confirmation rate was >90% for all tested interferents. For larger CNVs (those with >20 probes), the confirmation rate was >98% for all tested interferents.

The results demonstrated that the test results are not altered by the presence of excessive hemoglobin, triglycerides (triolein), or bilirubin (conjugated or unconjugated) in the patient whole blood specimen.

Cross Contamination

The GenetiSure Dx Postnatal Assay consists of a glass slide composed of four (4) independent microarrays that are sealed by a gasket slide during sample hybridization. Cross-contamination can arise during sample processing, especially during hybridization set up, when samples are loaded onto each of the 4 adjacent arrays or during the overnight incubation due to gasket leakage. The presence of contamination could result in corrupt and inaccurate patient data.

This study was designed to determine if cross contamination occurs during the routine assay workflow and, if so, what the impact on data would be. For this study, two (2) male and two (2) female Coriell DNA samples, each with distinctive sets of known chromosomal aberrations, were tested across multiple microarray slides under conditions that would either allow or prevent detection of cross contamination between the adjacent arrays on the slides.

No suspected cross contamination was detected. This supports the appropriateness of the sample handling workflow, the accuracy of the data collected, and the integrity of the gasket slide materials for use with the assay.

Accuracy

Accuracy of the GenetiSure Dx Postnatal Assay results was assessed by comparing the CNVs identified by GenetiSure Dx Postnatal Assay to the results obtained using comparator microarray methods. A total of 556 out

of 626 samples were eligible for testing. The sample panel consisted of 451 aberrant genomic DNA (gDNA) samples derived from established commercial cell lines, 76 archived clinical gDNA samples isolated from whole blood specimens of anonymized patients, 5 globally recognized syndrome reference panel gDNA samples, and 24 fresh blood-derived gDNA samples extracted from whole blood of phenotypically normal subjects. The samples were selected to maximize the variation across the genome with consideration for gain and loss segments of various sizes/number of probes, chromosomal representation, CNV regions in genic and non-genic regions, and in telomeric and centromeric regions. A total of 2187 CNV regions and 292 cnLOH regions covered 91% of the genome. These aberrations were more prevalent in non-telomeric/non-centromeric regions than in telomeric/centromeric (1337 regions vs 1130 regions). A total of 23% (508 out of 2187 regions) of the CNVs had high (>45%) GC content.

Due to the diversified sample panel composition and lack of an applicable universal comparator, independent (non-Agilent) commercially available microarray based assays analytically validated for copy number detection were employed to assist accuracy assessment of CNV aberrations and resolve discrepancies. The samples were tested through the GenetiSure Dx Postnatal Assay using standard procedures in a designated Agilent laboratory.

A target Agilent aberration was deemed "confirmed" if a minimum percent overlap was found with comparator aberration call(s) of the same type (gain, loss, or cnLOH). For the data presented below, a 50% overlap was required, with the Agilent aberrations being compared independently to the other platforms. All eligible Agilent aberrations were assessed, one at a time (Figure 39).

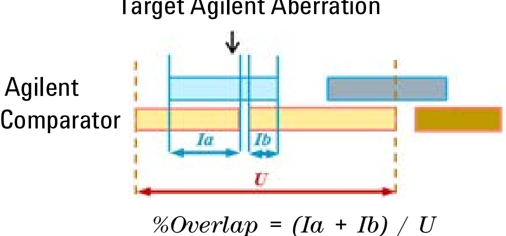


Figure 39 Method for comparison of Agilent detected aberrations with those of comparator platform(s)

If an Agilent CNV aberration could not be “confirmed” by the microarray-based comparator, another analytically validated method (qPCR) was employed to adjudicate the results. To avoid bias in the assessment, an additional 5% randomly selected “confirmed” CNV aberrations (separately selected for CNVs >20 probes and CNVs with 5-20 probes) were also included in this discrepancy resolution testing. Other CNVs directly subject to a third method confirmation included CNVs near the limit of resolution selected from normal whole blood samples.

The results are summarized and stratified by copy number state, size or probe number, and genomic region. The results shown in Table 30 and Table 31 are presented as stratified by probe number, either including indeterminate CNVs as unconfirmed (scheme a) or excluding indeterminate CNVs (scheme b). In Table 32 and Table 33, the results are presented using the same schemes for treatment of indeterminate CNVs, with the results stratified by length in kb.

Table 30 GenetiSure Dx Postnatal Assay Accuracy for all aberration regions Stratified by Gain/Loss and CNV size (# of probes) when compared to the comparator method – Scheme a: Including Indeterminate CNVs as “Not Confirmed”

Type	Aberration Range (# of probes)	Sample Size (N)*	# Confirmed	Confirmation Rate (95% CI)	FPR (95% CI)
Gain	5–7	48	38	79.2% (65.7%, 88.3%)	20.8% (11.7%, 34.3%)
	7–10	101	85	84.2% (75.8%, 90.0%)	15.8% (10.0%, 24.2%)
	10–15	197	152	77.2% (70.8%, 82.5%)	22.8% (17.5%, 29.2%)
	15–20	101	83	82.2% (73.6%, 88.4%)	17.8% (11.6%, 26.4%)
	20–50	148	104	70.3% (65.2%, 77.0%)	29.7% (23.0%, 37.5%)
	50–500	82	64	78.0% (67.9%, 85.6%)	22.0% (14.4%, 32.1%)
	500+	169	166	98.2% (94.9%, 99.4%)	1.8% (0.6%, 5.1%)
	Total	846	692	81.8% (79.1%, 84.3%)	18.2% (15.7%, 20.9%)

Table 30 GenetiSure Dx Postnatal Assay Accuracy for all aberration regions Stratified by Gain/Loss and CNV size (# of probes) when compared to the comparator method – Scheme a: Including Indeterminate CNVs as "Not Confirmed" (continued)

Type	Aberration Range (# of probes)	Sample Size (N)*	# Confirmed	Confirmation Rate (95% CI)	FPR (95% CI)
Loss	5–7	216	196	90.7% (86.1%, 93.9%)	9.3% (6.1%, 13.9%)
	7–10	202	165	81.7% (75.8%, 86.4%)	18.3% (13.6%, 24.2%)
	10–15	257	216	84.0% (79.1%, 88.0%)	16.0% (12.0%, 20.9%)
	15–20	125	90	72.0% (63.6%, 79.1%)	28.0% (20.9%, 36.4%)
	20–50	130	95	73.1% (64.9%, 80.0%)	26.9% (20.0%, 35.1%)
	50–500	225	217	96.4% (93.1%, 98.2%)	3.6% (1.8%, 6.9%)
	500+	186	180	96.8% (93.1%, 98.5%)	3.2% (1.5%, 6.9%)
	Total	1341	1159	86.4% (84.5%, 88.2%)	13.6% (11.8%, 15.5%)
All CNVs	Total	2187	1851	84.6% (83.1%, 86.1%)	15.4% (13.9%, 16.9%)
cnLOH	100–200	132	94	71.2% (63.0%, 78.2%)	28.8% (21.8%, 37.0%)
	200–500	102	96	94.1% (87.8%, 97.3%)	5.9% (2.7%, 12.2%)
	500+	58	58	100.0% (93.8%, 100.0%)	0.0% (0.0%, 6.2%)
	Total	292	248	84.9% (80.4%, 88.6%)	15.1% (11.4%, 19.6%)

* The number of aberrations analyzed in each range bin, including indeterminate CNVs as "not confirmed"

Table 31 GenetiSure Dx Postnatal Assay Accuracy for all aberration regions Stratified by Gain/Loss and CNV size (# of probes) when compared to the comparator method – Scheme b: Excluding Indeterminate CNVs

Type	Aberration Range (# of probes)	Sample Size (N)*	# Confirmed	Confirmation Rate (95% CI)	FPR (95% CI)
Gain	5–7	43	38	88.4% (75.5%, 94.9%)	11.6% (5.1%, 24.5%)
	7–10	91	85	93.4% (86.4%, 96.9%)	6.6% (3.1%, 13.6%)
	10–15	175	152	86.9% (81.1%, 91.1%)	13.1% (8.9%, 18.9%)
	15–20	91	83	91.2% (83.6%, 95.5%)	8.8% (4.5, 16.4%)
	20–50	124	104	83.9% (76.4%, 89.3%)	16.1% (10.7%, 23.6%)
	50–500	72	64	88.9% (79.6%, 94.3%)	11.1% (5.7%, 20.4%)
	500+	169	166	98.2% (94.9%, 99.4%)	1.8% (0.6%, 5.1%)
	Total	765	692	90.5% (88.2%, 92.3%)	9.5% (7.7%, 11.8%)
Loss	5–7	197	196	99.5% (97.2%, 99.9%)	0.5% (0.1%, 2.8%)
	7–10	183	165	90.2% (85.0%, 93.7%)	9.8% (6.3%, 15.0%)
	10–15	231	216	93.5% (89.6%, 96.0%)	6.5% (4.0%, 10.4%)
	15–20	102	90	88.2% (80.6%, 93.1%)	11.8% (6.9%, 19.4%)
	20–50	114	95	83.3% (75.4%, 89.1%)	16.7% (10.9%, 24.6%)
	50–500	222	217	97.7% (94.8%, 99.0%)	2.3% (1.0%, 5.2%)
	500+	184	180	97.8% (94.5%, 99.2%)	2.2% (0.8, 5.5%)
	Total	1233	1159	94.0% (92.5%, 95.2%)	6.0% (4.8%, 7.5%)
All CNVs	Total	1998	1851	92.6% (91.4%, 93.7%)	7.4% (6.3%, 8.6%)
cnLOH	100–200	132	94	71.2% (63.0%, 78.2%)	28.8% (21.8%, 37.0%)
	200–500	99	96	97.0% (91.5%, 99.0%)	3.0% (1.0%, 8.5%)
	500+	58	58	100.0% (93.8%, 100.0%)	0.0% (0.0%, 6.2%)
	Total	289	248	85.8% (81.3%, 89.4%)	14.2% (10.6%, 18.7%)

* The number of aberrations analyzed in each range bin, excluding indeterminate CNVs

Table 32 GenetiSure Dx Postnatal Assay Accuracy for all aberration regions Stratified by Gain/Loss and CNV size (kb) when compared to the comparator method – Scheme a: Including Indeterminate CNVs as "Not Confirmed"

Type	Aberration Range (kb)	Sample Size (N)*	# Confirmed	Confirmation Rate (95% CI)	FPR (95% CI)
Gain	20–100	69	37	53.6% (42.0%, 64.9%)	46.4% (35.1%, 58.0%)
	100–200	94	75	79.8% (70.6%, 86.7%)	20.2% (13.3%, 29.4%)
	200–300	136	120	88.2% (81.7%, 92.6%)	11.8% (7.4%, 18.3%)
	300–500	123	92	74.8% (66.5%, 81.6%)	25.2% (18.4%, 33.5%)
	500–1000	90	70	77.8% (68.2%, 85.1%)	22.2% (14.9%, 31.8%)
	1000–10,000	168	136	81.0% (74.3%, 86.2%)	19.0% (13.8%, 25.7%)
	10,000+	166	162	97.6% (94.0%, 99.1%)	2.4% (0.9%, 6.0%)
	Total	846	692	81.8% (79.1%, 84.3%)	18.2% (15.7%, 20.9%)
Loss	10–100	88	59	67.0% (56.7%, 76.0%)	33.0% (24.0%, 43.3%)
	100–200	207	180	87.0% (81.7%, 90.9%)	13.0% (9.1%, 18.3%)
	200–300	129	114	88.4% (81.7%, 92.8%)	11.6% (7.2%, 18.3%)
	300–500	116	103	88.8% (81.8%, 93.3%)	11.2% (6.7%, 18.2%)
	500–1000	209	164	78.5% (72.4%, 83.5%)	21.5% (16.5%, 27.6%)
	1000–10,000	398	351	88.2% (84.6%, 91.0%)	11.8% (9.0%, 15.4%)
	10,000+	194	188	96.9% (93.4%, 98.6%)	3.1% (1.4%, 6.6%)
	Total	1341	1159	86.4% (84.5%, 88.2%)	13.6% (11.8%, 15.5%)
All CNVs	Total	2187	1851	84.6% (83.1%, 86.1%)	15.4% (13.9%, 16.9%)
cnLOH	5000–10,000	93	61	65.6% (55.5%, 74.5%)	34.4% (25.5%, 44.5%)
	10,000–20,000	94	84	89.4% (81.5%, 94.1%)	10.6% (5.9%, 18.5%)
	20,000+	105	103	98.1% (93.3%, 99.5%)	1.9% (0.5%, 6.7%)
	Total	292	248	84.9% (80.4%, 88.6%)	15.1% (11.4%, 19.6%)

* The number of aberrations analyzed in each range bin, including indeterminate CNVs as "not confirmed"

Table 33 GenetiSure Dx Postnatal Assay Accuracy for all aberration regions Stratified by Gain/Loss and CNV size (kb) when compared to the comparator method – Scheme b: Excluding Indeterminate CNVs

Type	Aberration Range (kb)	Sample Size (N)*	# Confirmed	Confirmation Rate (95% CI)	FPR (95% CI)
Gain	20–100	53	37	69.8% (56.5%, 80.5%)	30.2% (19.5%, 43.5%)
	100–200	87	75	86.2% (77.4%, 91.9%)	13.8% (8.1%, 22.6%)
	200–300	123	120	97.6% (93.1%, 99.2%)	2.4% (0.8%, 6.9%)
	300–500	107	92	86.0% (78.2%, 91.3%)	14.0% (8.7%, 21.8%)
	500–1000	77	70	90.9% (82.4%, 95.5%)	9.1% (4.5%, 17.6%)
	1000–10,000	153	136	88.9% (82.9%, 92.9%)	11.1% (7.1%, 17.1%)
	10,000+	165	162	98.2% (94.8%, 99.4%)	1.8% (0.6%, 5.2%)
	Total	765	692	90.5% (88.2%, 92.3%)	9.5% (7.7%, 11.8%)
Loss	10–100	76	59	77.6% (67.1%, 85.5%)	22.4% (14.5%, 32.9%)
	100–200	187	180	96.3% (92.5%, 98.2%)	3.7% (1.8%, 7.5%)
	200–300	115	114	99.1% (95.2%, 99.8%)	0.9% (0.2%, 4.8%)
	300–500	105	103	98.1% (93.3%, 99.5%)	1.9% (0.5%, 6.7%)
	500–1000	179	164	91.6% (86.6%, 94.9%)	8.4% (5.1%, 13.4%)
	1000–10,000	379	351	92.6% (89.5%, 94.8%)	7.4% (5.2%, 10.5%)
	10,000+	192	188	97.9% (94.8%, 99.2%)	2.1% (0.8%, 5.2%)
	Total	1233	1159	94.0% (92.5%, 95.2%)	6.0% (4.8%, 7.5%)
All CNVs	Total	1998	1851	92.6% (91.4%, 93.7%)	7.4% (6.3%, 8.6%)
cnLOH	5000–10,000	93	61	65.6% (55.5%, 74.5%)	34.4% (25.5%, 44.5%)
	10,000–20,000	92	84	91.3% (83.8%, 95.5%)	8.7% (4.5%, 16.2%)
	20,000+	104	103	99.0% (94.8%, 99.8%)	1.0% (0.2%, 5.2%)
	Total	289	248	85.8% (81.3%, 89.4%)	14.2% (10.6%, 18.7%)

* The number of aberrations analyzed in each range bin, excluding indeterminate CNVs

Analysis was also conducted using alternative methods of comparing the aberrations to the comparator calls. Results using those methods were similar to those presented (data not shown).

When calculated using one of the alternative methods, which included a predefined 65% minimum overlap criterion and a composite of aberrations from two platforms as a comparator, and a definitive scheme where by “indeterminate” calls were excluded, the aberration confirmation rates (% agreement with comparator) were 93.5% for larger CNVs (>20 probes), 92.5% for smaller CNVs (5–20 probes,) and 90.1% for all cnLOH aberrations.

The endpoint agreements were also analyzed. Agreements for one comparator are shown in Table 34. Analysis by other methods were found to have similar results (data not shown).

Table 34 Endpoint Agreement for comparator platform 1; Binning by Number of Probes; Start/Stop Breakpoints Combined; Endpoint Agreement Criteria: ≤2 probes for CNVs (Gain/Loss), ≤50 probes for cnLOH

Type	Aberration Range (# of probes)	Breakpoints, N	Breakpoint Agreement, N	Breakpoint Agreement, % (95% CI)
Gain	5–7	48	47	97.9% (89.1%, 99.6%)
	7–10	126	116	92.1% (86.0%, 95.6%)
	10–15	258	223	86.4% (81.7%, 90.1%)
	15–20	110	88	80.0% (71.6%, 86.4%)
	20–50	128	115	89.8% (83.4%, 94.0%)
	50–500	124	104	83.9% (76.4%, 89.3%)
	500+	296	258	87.2% (82.9%, 90.5%)
	Total	1090	951	87.2% (85.1%, 89.1%)

Table 34 Endpoint Agreement for comparator platform 1; Binning by Number of Probes; Start/Stop Breakpoints Combined; Endpoint Agreement Criteria: ≤2 probes for CNVs (Gain/Loss), ≤50 probes for cnLOH (continued)

Type	Aberration Range (# of probes)	Breakpoints, N	Breakpoint Agreement, N	Breakpoint Agreement, % (95% CI)
Loss	5–7	236	234	99.2% (97.0%, 99.8%)
	7–10	124	117	94.4% (88.8%, 97.2%)
	10–15	116	89	76.7% (68.3%, 83.5%)
	15–20	84	69	82.1% (72.6%, 88.9%)
	20–50	138	110	79.7% (72.2%, 85.6%)
	50–500	420	382	91.0% (87.8%, 93.3%)
	500+	350	283	80.9% (76.4%, 84.6%)
	Total	1468	1284	87.5% (85.7%, 89.1%)
All CNVs	Total	2558	2235	87.4% (86.0%, 88.6%)
cnLOH	100–200	188	176	93.6% (89.2%, 96.3%)
	200–500	194	176	90.7% (85.8%, 94.1%)
	500+	116	104	89.7% (82.8%, 94.0%)
	Total	498	456	91.6% (88.8%, 93.7%)

Clinical Validity

A retrospective clinical study was performed to characterize the clinical performance characteristics of GenetiSure Dx Postnatal Assay for the purpose of reporting the pathogenic detection rate (potential diagnostic yield) of the assay. A total of 800 gDNA samples from patients suspected of having pathogenic aberrations (SPA samples) were collected from three regionally distinct clinical institutions that offer postnatal array testing for the detection of chromosomal abnormalities. One hundred (100) samples from phenotypically normal individuals were also processed using the GenetiSure Dx Postnatal Assay and were used to assess the aberrations that might be expected to be found in a normal (non-patient) population. The aberrations detected in each sample, for all nine hundred (900)

samples, were interpreted by one of four cytogeneticists as Benign, Likely Benign, Variant Of Unknown Significance (VOUS), Likely Pathogenic, or Pathogenic.

The interpretations of the calls were in agreement if the cytogeneticists at both the GenetiSure Dx Postnatal Assay processing site and at the collection site determined an aberration to be of the same pathogenicity.

The test results, per sample, were compared to historical array data from the respective collection site, which were generated using the methods established at each laboratory. All reported Pathogenic and Likely Pathogenic copy number variants (CNVs), gains and losses, were subject to confirmation by alternative methods. Confirmation methods used at the collection sites were selected by the sites based on the assay availability as well as the nature of the aberration being confirmed. The methods used included one or more of the following:

a. G-banded karyotyping

b. Fluorescence in situ hybridization (FISH)

c. Multiplex Ligation-dependent Probe Amplification (MLPA)

d. Quantitative Polymerase Chain Reaction (qPCR)

e. Non-Agilent Comparative Genomic Hybridization oligonucleotide microarrays (molecular karyotyping)

The diagnostic yield based on the samples evaluated with the GenetiSure Dx Postnatal Assay, when considering only copy number aberrations, was 15%. This increased to 20% when cnLOH aberrations were also considered.

Table 35 Diagnostic Yield by Collection Site (95% CI)

Collection Site	Number of Samples	Collection Site: Number of Pathogenic Calls	Collection Site: Diagnostic Yield	GenetiSure Dx Postnatal Assay: Number of Pathogenic Calls	GenetiSure Dx Postnatal Assay: Diagnostic Yield
Copy Number Aberrations Only					
Site 1	257	29	11% (8.0%, 15.7%)	39	15% (11.3%, 20.1%)
Site 2	313	35	11% (8.2%, 15.2%)	33	11% (7.6%, 14.4%)
Site 3	230	48	21% (16.1%, 26.6%)	45	20% (15.0%, 25.2%)

Table 35 Diagnostic Yield by Collection Site (95% CI) (continued)

Collection Site	Number of Samples	Collection Site: Number of Pathogenic Calls	Collection Site: Diagnostic Yield	GenetiSure Dx Postnatal Assay: Number of Pathogenic Calls	GenetiSure Dx Postnatal Assay: Diagnostic Yield
Total	800	112	14% (11.8%, 16.6%)	117	15% (12.3%, 17.2%)
All Aberrations (Copy Number and cnLOH)					
Site 1	257	29	11% (8.0%, 15.7%)	48	19% (14.4%, 23.9%)
Site 2	313	39	12% (9.2%, 16.6%)	60	19% (15.2%, 23.9%)
Site 3	230	48	21% (16.1%, 26.6%)	51	22% (17.3%, 28.0%)
Total	800	116	15% (12.2%, 17.1%)	159	20% (17.3%, 22.8%)

Results of the PPA and NPA analysis are presented considering only copy number aberrations (Table 36) or considering both copy number and cnLOH aberrations (Table 37). For the copy number aberration only analysis, samples with pathogenic cnLOH aberrations were considered as non-pathogenic, unless they also included a pathogenic copy number aberration.

Table 36 Comparison of Sample Classification between the Collection Site and the GenetiSure Dx Postnatal Assay, Considering only Copy Number Aberrations

GenetiSure Dx Postnatal Assay Interpretation		Collection Site Aberration Interpretation					Total
		Pathogenic Interpretation		Non-Pathogenic Interpretation			
		Pathogenic	Likely Pathogenic	VOUS	Likely Benign[1]	Normal[2]	
Pathogenic Interpretation	Pathogenic	56	14	9	0	3	82
	Likely Pathogenic	12	4	11	0	8	35
Non-Pathogenic Interpretation	VOUS	5	8	35	0	32	80
	Normal[2]	6	7	80	1	509	603
Total		79	33	135	1	552	800

Table 36 Comparison of Sample Classification between the Collection Site and the GenetiSure Dx Postnatal Assay, Considering only Copy Number Aberrations (continued)

GenetiSure Dx Postnatal Assay Interpretation	Collection Site Aberration Interpretation					Total
	Pathogenic Interpretation		Non-Pathogenic Interpretation			
	Pathogenic	Likely Pathogenic	VOUS	Likely Benign[1]	Normal[2]	
PPA[3]	86/112 = 76.8% (95% CI = 68.2% – 83.6%)					
NPA[4]	657/688 = 95.5% (95% CI = 93.7% – 96.8%)					

[1]One Site 2 sample was presented with the interpretation on Likely Benign.

[2]Samples from the GenetiSure Dx Postnatal Assay or Site 1 with either only Benign or Likely Benign aberrations, or samples without aberrations, are classified as "Normal". Site 3 and Site 2 provided sample classifications of "Normal".

[3]Positive Percent Agreement (PPA): Percent [(Agilent GenetiSure Dx Postnatal Assay = Pathogenic & Collection site classification = Pathogenic)/(Collection site classification = Pathogenic)]

[4]Negative Percent Agreement (NPA): Percent [(Agilent GenetiSure Dx Postnatal Assay = Non-pathogenic & Collection site classification = Non-Pathogenic)/(Collection site classification = Non-Pathogenic)]

When considering only copy number aberrations in the sample classification, PPA was 76.8% and NPA was 95.5%. In total, 26 samples which were determined to have Pathogenic or Likely Pathogenic copy number aberrations by the collection sites were reported as non-pathogenic by the GenetiSure Dx Postnatal Assay. Most of these aberrations were either detected by GenetiSure Dx Postnatal Assay, but interpreted differently by the cytogeneticist, or below the detection limit of the Assay.

Table 37 Comparison of Sample Classification between the Collection Site and the GenetiSure Dx Postnatal Assay, Considering Copy Number and cnLOH Aberrations

GenetiSure Dx Postnatal Assay Interpretation		Collection Site Aberration Interpretation					Total
		Pathogenic Interpretation		Non-Pathogenic Interpretation			
		Pathogenic	Likely Pathogenic	VOUS	Likely Benign[1]	Normal[2]	
Pathogenic Interpretation	Pathogenic	56	14	9	0	3	82
	Likely Pathogenic	14	5	23	0	35	77
Non-Pathogenic Interpretation	VOUS	5	10	59	0	46	120
	Normal[2]	7	5	74	1	434	521

Table 37 Comparison of Sample Classification between the Collection Site and the GenetiSure Dx Postnatal Assay, Considering Copy Number and cnLOH Aberrations (continued)

GenetiSure Dx Postnatal Assay Interpretation	Collection Site Aberration Interpretation					Total
	Pathogenic Interpretation		Non-Pathogenic Interpretation			
	Pathogenic	Likely Pathogenic	VOUS	Likely Benign[1]	Normal[2]	
Total	82	34	165	1	518	800
PPA[3]	89/116 = 76.7% (95% CI = 68.3% – 83.5%)					
NPA[4]	614/684 = 89.8% (95% CI = 87.3% – 91.8%)					

[1]One Site 2 sample was presented with the interpretation on Likely Benign.

[2]Samples from the GenetiSure Dx Postnatal Assay or Site 1 with either only Benign or Likely Benign aberrations, or samples without aberrations, are classified as "Normal". Site 3 and Site 2 provided sample classifications of "Normal".

[3]Positive Percent Agreement (PPA): Percent [(Agilent GenetiSure Dx Postnatal Assay = Pathogenic & Collection site classification = Pathogenic)/(Collection site classification = Pathogenic)]

[4]Negative Percent Agreement (NPA): Percent [(Agilent GenetiSure Dx Postnatal Assay = Non-pathogenic & Collection site classification = Non-Pathogenic)/(Collection site classification = Non-Pathogenic)]

A list of observed syndromes was compiled based on the pathogenic and likely pathogenic calls detected from the 800 SPA samples (Table 38). In total, 36 distinct syndromes were identified, which encompassed 73 cases from the clinical study sample set.

Table 38 List of Syndromes in the Clinical Study Sample Set

Syndrome Association	Number of Cases
10q26 Deletion Syndrome	1
13q Deletion Syndrome	1
15q11.2 Deletion Syndrome	5
15q13.3 Microdeletion Syndrome	1
15q25 Deletion Syndrome	1
16p11.2 Microdeletion	1
16p11.2 Microduplication	2
16p12.1 Deletion Syndrome	1
16p13.11 Microdeletion	2

Table 38 List of Syndromes in the Clinical Study Sample Set (continued)

Syndrome Association	Number of Cases
16p13.11 Microduplication neurocognitive disorder susceptibility locus	3
1q21.1 Deletion Syndrome	2
1q21.1 Duplication Syndrome	2
22q11.2 Duplication Syndrome	3
2q37 Deletion Syndrome	4
3q29 Deletion Syndrome	1
7q11.23 Duplication Syndrome	1
8p23.1 Microdeletion/CDH syndrome	1
8p23.1 Microduplication	1
Angelman/Prader-Willi Syndrome	6
Charcot-Marie-Tooth Neuropathy, Type 1a	1
DiGeorge Syndrome	7
Distal 22q11.2 Deletion Syndrome	1
Down Syndrome/Trisomy 21	6
Ichthyosis, X-Linked/ STS Deficiency	1
Isodicentric Chromosome 15 Syndrome	1
Jacobsen/ 11q Deletion	1
Klinefelter Syndrome	4
Mental Retardation-Hypotonic Facies Syndrome, X-Linked/ Smith-Fineman-Myers	1
Neuropathy, Hereditary, With Liability to Pressure Palsies; HNPP	1
Sotos Syndrome-1/ 5q35 Deletion Syndrome	1
Triple X Syndrome	2
Trisomy 9 mosaicism	1

Table 38 List of Syndromes in the Clinical Study Sample Set (continued)

Syndrome Association	Number of Cases
Turner Syndrome	1
Williams-Beuren Syndrome	2
Xq26.3 Duplication Syndrome	1
Other	2
Total	**73**

Expected values/Reference range:

The prevalence of CNVs in patient populations depends on risk factors such as age, gender, presence of symptoms, and family history. A blinded study was conducted to assess the potential impact of the GenetiSure Dx Assay CNV results on interpretation using 100 phenotypically normal individual samples in the clinical specimen evaluation described above. The results showed that 8 samples with aberrations detected by the GenetiSure Dx Postnatal Assay were interpreted as Likely Pathogenic or Pathogenic by the cytogeneticist. Of the 8 samples with Pathogenic or Likely Pathogenic aberrations reported, 2 of those were cnLOH aberrations reported as Likely Pathogenic. These samples were not confirmed by an independent method. The other six (6) samples contained copy number changes, of which 5 were confirmed by qPCR. One (1) of the copy number changes, a 100 kb gain on Chromosome 22, was not confirmed by qPCR.

11 Troubleshooting

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This chapter contains potential reasons for an assay failure.

Samples are not within the Specific Activity or Yield range

If, at the end of the gDNA labeling step, your samples do not meet the Specific Activity and/or Yield ranges due to factors other than poor sample quality, it could be for one of the reasons below. Troubleshooting for poor sample quality is included under "DerivativeLR_Spread metric failed" on page 140.

If you have low specific activity not due to poor sample quality

Low specific activity can result from sub-optimal labeling conditions such as using Cyanine 3-dUTP or Cyanine 5-dUTP that has undergone more than eight freeze-thaw cycles, using enzymes that have degraded due to being left warm for too long, performing a step at the wrong temperature or duration, incorrect volume, or allowing the reactions to be exposed to excessive light or air.

Store cyanine dUTP at –25°C to –15°C and limit the number of freeze/thaw cycles to eight or less. Keep enzymes on ice while in use and return to –25°C to –15°C as quickly as possible.

Double check incubation times and temperatures (use a calibrated thermometer).

Make sure that the pipettors are not out of calibration.

Do not thaw gDNA or reagents at temperatures above 30°C.

Make sure that the gDNA, reagents, and master mixes are well mixed. Quickly mix the samples on a vortex mixer, tap the tube with your finger, or use a pipettor to move the entire volume up and down. Then spin in a microcentrifuge for 5 to 10 seconds to drive the contents off the walls and lid.

If you have low yield not due to poor sample quality

Possible sample loss during clean-up after labeling.

See "Step 2. Clean-up of Labeled gDNA" on page 40 to remove unreacted dye.

If you have high specific activity

Inaccurate quantitation of the input gDNA can contribute to high specific activity.

Follow the manufacturer's protocol for the DNA-based fluorometric method and make sure that the calibration status of the fluorometer is up to date. The following best practices can help ensure the accuracy of the measurements.

- After the gDNA samples have been diluted or concentrated to achieve an expected concentration in the desired range, make sure that the samples are well mixed. Then, allow the samples to incubate at 4°C for approximately 1 hour, or for the duration optimized by your laboratory for your fluorometer.
- After mixing the gDNA samples with the required reagent(s) for the fluorometric assay, promptly measure the fluorescence on the fluorometer.

CytoDx workflow failed

If the Agilent CytoDx software shows a status of *Failed* for the analysis workflow job, then the entire job failed and results cannot be accessed.

If the tiff image that was analyzed in the workflow was imported from a network drive, then a sporadic network connection may have caused the failure. Delete the failed workflow, then save the image to a local drive and reprocess.

Check if the slide was scanned with the incorrect Scan Protocol. The correct Scan Protocol is **GenetiSure_Dx_Postnatal**. If another protocol was used, quickly rescan the slide using **GenetiSure_Dx_Postnatal.**

The failure could be due to the slide not being straight within the slide holder. This slant of the slide relative to the slide holder can sometimes occur with older slide holders that do not properly secure the slide. The result is a skewed image of the array that CytoDx cannot process. Use the instructions below to view the image in the Feature Extraction software module by initiating a new workflow for the image file.

a Make note of the location of the microarray image file (**.tif*) that was used in the failed analysis workflow. If the workflow was run using Auto-Processing, then the file is in the Tiff Image Archive Folder (as specified on the **Configure Settings > Auto-Processing Settings** screen of the CytoDx software). If the workflow was run manually, then the file is still in its original location (unless it has been manually moved).

b At the top of the CytoDx program window, click **Analysis Workflow**.

The Analysis Workflow screen opens to the Import Samples step.

c Click **Select Samples**.

The Open dialog box opens.

d Browse to the microarray image file. Select the file and click **Open**.

The Image Information dialog box opens.

e Click **Add Images**.

The dialog box closes and the program adds the samples to the table on the Analysis Workflow screen.

f In the table, click on the row for the failed sample, then click **View Image**.

The Agilent Feature Extraction for CytoDx software module opens, displaying the tiff image of the slide. See the CytoDx help system for further information on the tools available in this software module.

g Review the image of the slide. **If the slide appears to be rotated or incomplete, rescan the slide in a different slide holder as soon as possible. Discard the slide holder that was used in the original scan.**

h Close the Feature Extraction for CytoDx software module and delete the samples from the Analysis Workflow screen in CytoDx. (Do not continue to set up or submit a workflow for the samples.)

Slide holder stuck in the scanner

Occasionally, when removing a slide holder from the microarray scanner, the slide holder may not easily eject.

Restart the scanner then try again to remove the slide holder. Do not use that particular slide holder again. Additional slide holders can be purchased from Agilent (part number G4900-60035).

DerivativeLR_Spread metric failed

The Agilent CytoDx Software will generate an intermediate report if the QC metric thresholds were not met. However, sign-off of the document will not be permissible in the software if any of the nine critical QC metrics are called as failed.

If your DerivativeLR_Spread is in the “fail” range, it could be due to the gDNA quality and/or concentration. The sections below describe indicators and ways of determining the possible reason(s) for the high DerivativeLR_Spread.

Low OD260/230 or OD260/280 value

Sample quality can be evaluated using a spectrophotometer. A low OD260/230 value can indicate contaminants, such as residual salt or organic solvents (which would inhibit enzyme). A low OD260/280 value indicates residual protein. Either condition can result in low specific activity (pmol dye/µg gDNA). See “Step 4. Determination of Yield and Specific Activity” on page 44.

Repurify the gDNA using the QIAamp DSP DNA Blood Mini Kit following the instructions for use included with that kit.

Make sure to calibrate the spectrophotometer with the appropriate buffer.

Poor sample quality due to degradation

Degraded gDNA results in biased labeling.

Analyze gDNA on a 1 to 1.5% agarose gel to determine if it is degraded. Intact gDNA appears as compact, high-molecular weight bands with no lower molecular weight smears.

Estimated concentration of gDNA is too high or too low

The input amount of gDNA for the experimental labeling reaction must be the same as for the reference sample labeling reaction. Precipitated gDNA or gDNA that is at a very high concentration cannot be quantitated accurately, resulting in an inaccurate estimated concentration.

Make sure that the gDNA is completely in solution by pipetting up and down. If needed, incubate at 37°C ±1°C for 25–30 minutes. If the gDNA concentration is >250 ng/µL, dilute 1:2 in TE (pH 8.0) and re-quantitate to make sure quantitation is accurate.

Different DNA isolation methods can create different quantitation artifacts. Verify that the patient gDNA samples were isolated using the QIAamp DSP DNA Blood Mini kit.

Repurify the gDNA using the QIAamp DSP DNA Blood Mini Kit following the instructions for use included with that kit.

Make sure that the reagents used for quantitation are fresh and that the fluorometer is appropriately calibrated. If necessary, requantitate with fresh reagents.

Make sure that the reference DNA and patient DNA are quantitated at the same time, using the same fluorometer and reagents.

g_Signal2Noise and/or r_Signal2Noise metric failed

If your g_Signal2Noise metric or r_Signal2Noise metric is too low and is called as “fail”, then either the background noise is too high or the signal is too weak. Weak signal could be the result of ozone-induced dye degradation, insufficient sample labeling, leakage of the hybridization solution during hybridization of the microarray slide, or other factors.

High background noise

See the suggestions in “g_BGNoise and/or r_BGNoise metric is “Evaluate”” on page 143 for tips on reducing background due to insufficient washing of the glassware.

During microarray washing, make sure that the temperature of GenetiSure Dx Wash Buffer 2 is 37°C ±1°C.

Possible insufficient labeling

See “Step 4. Determination of Yield and Specific Activity” on page 44 for instructions on measuring the specific activity of the labeled gDNA samples. If the specific activity is not in the range indicated in Table 16, review the troubleshooting recommendations in “Samples are not within the Specific Activity or Yield range” on page 137.

Possible ozone degradation

See “Environmental Conditions” on page 20 for maximum ozone levels during assay processing.

When repeating the assay, make sure that ozone levels are no greater than 5 ppb in laboratory areas used for the labeling and hybridization of the microarray slides.

Possible hybridization solution volume loss or leakage during hybridization due to improper hybridization assembly

See “Step 4. Microarray Hybridization” on page 54 for hybridization assembly steps. If needed, Agilent recommends practicing the hybridization assembly steps with 1× CGH Hybridization Buffer.

g_BGNoise and/or r_BGNoise metric is “Evaluate”

If your g_BGNoise metric or r_BGNoise metric is in the “Evaluate” range, it could be due to insufficient washing of the glassware.

Run copious amounts of Ultrapure water through the slide-staining dishes, slide racks, and stir bars. Empty out the water collected in the dishes at least ten times.

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 Ultrapure water.

Use only dishes that are designated and dedicated for use in GenetiSure Dx assays. Designate dishes, magnetic stir bars, and slide racks for use with either Wash Buffer 1 or Wash Buffer 2, and only use the dishes with their designated buffer.

Reference Correct metric failed

If the signals measured from the reference channel do not match the expected patterns based on the reference genotype, the Reference Correct metric will fail.

Check if the reference sample was incorrectly identified in the sample attribute file. Refer to “Step 1. Preparation of the Sample Attribute File” on page 81 for information on sample attribute files.

A misidentified reference sample could indicate an error in sample loading or sample tracking.

SNP Call Rate and/or Call Ambiguity metric failed

The SNP Call Rate metric will fail if it is too low, and the Call Ambiguity metric will fail if it is too high. In either case, the SNP calling algorithm was not able to call enough of the SNPs with high enough confidence.

The quality of the SNP data is highly dependent on the quality of the CGH data. Check the DerivativeLR_Spread, g_Signal2Noise, and r_Signal2Noise metrics. If any of these metrics is close to failing, the SNP data quality can be impacted. See the troubleshooting sections for those particular metrics for ways to improve their performance.

SNP data quality is also impacted by the quality of the restriction digest. Check the Restriction Control metric. If the value for this metric is in the evaluate range, the restriction digest might not have been adequate for the samples or reference. Make sure that gDNA is properly in solution prior to the restriction digest, and that the reaction is properly mixed. Make sure that the restriction enzymes are stored properly and are kept on ice while setting up the restriction digest reactions.

Separability metric is “Evaluate”

The Separability metric can be affected by elevated values for the DerivativeLR_Spread metric, even when the DerivativeLR_Spread metric is in the “Good” range.

Follow the recommendations outlined in “DerivativeLR_Spread metric failed” on page 140.

Alternatively, a Separability metric in the “Evaluate” range could indicate a decrease in the hybridization stringency, especially if the DerivativeLR_Spread metric is in the “Excellent” range.

Make sure that the hybridization oven is properly calibrated and that hybridization is allowed to proceed for the full 24 hrs.

Heterozygosity metric is “Evaluate”

The Heterozygosity metric is rated as “Evaluate” if the SNP heterozygosity of the sample is measured as being significantly higher or lower than that expected for typical samples.

If any of the other SNP metrics failed or was rated as “Evaluate”, follow the troubleshooting recommendations provided for that metric.

If all other SNP metrics passed, carefully review your SNP data. If the Heterozygosity metric is below the expected range, the low level of SNP heterozygosity could be explained by the characteristics of the sample (e.g., if the sample has a high level of identity by descent). Conversely, in samples in which the Heterozygosity metric is above the expected range, the cause is frequently a failure in the SNP analysis. In such cases, interpret the SNP data with caution.

Other non-critical QC metric is “Evaluate”

The Agilent CytoDx Software will generate a report if the QC metric thresholds were not met, however “Sign-off” of the document will not be permissible in the software.

If any of the non-critical QC metrics is in the “evaluate” range, it could be for the reasons below. See Table 23 on page 89 for a list of the non-critical QC metrics.

Possible hybridization solution volume loss or leakage during hybridization due to improper hybridization assembly

See “Step 4. Microarray Hybridization” on page 54 for hybridization assembly steps. If needed, Agilent recommends practicing the hybridization assembly steps with 1× CGH Hybridization Buffer.

Possible presence of stationary bubbles in the assembled hybridization chamber that were not resolved prior to hybridization

See “Step 4. Microarray Hybridization” on page 54 for hybridization assembly steps. Make sure to rotate the assembled hybridization chamber to check for bubble motion prior to loading the assembled chamber into the hybridization oven.

Possible hybridization temperature variation

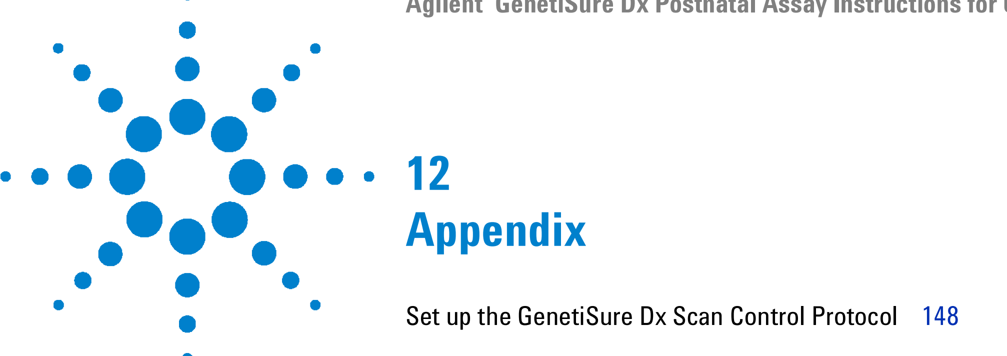
See “Load assembled chamber into the hybridization oven” on page 63 for hybridization oven temperature. Make sure the hybridization oven is calibrated.

Possible wash temperature variation

See “Step 5. Equipment Cleaning” on page 64 and “Step 6. Microarray Washing” on page 65 and follow instructions for maintenance of dishes and wash buffers, especially Wash Buffer 2.

Possible ozone degradation

See “Environmental Conditions” on page 20 for maximum ozone levels during assay processing.



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Appendix

Set up the GenetiSure Dx Scan Control Protocol 148

This chapter contains set up instructions for the GenetiSure Dx Postnatal Scan Protocol, which is used by the Agilent SureScan Dx Microarray Scanner to scan the GenetiSure Dx Postnatal microarrays. The scan protocol must be loaded into the Scan Control software prior to scanning.

Set up the GenetiSure Dx Scan Control Protocol

The Scan Control software that operates the Agilent SureScan Dx Microarray Scanner comes preloaded with multiple scan protocols. However, when scanning microarray slides from the GenetiSure Dx Postnatal Assay, you must use the GenetiSure_Dx_Postnatal scan protocol, which is not preloaded in the software. To obtain this scan protocol, download it from the SureScan download page on the Agilent website and then import it into the Scan Control software using the instructions provided in this guide.

These instructions describe how to download the scan protocol that is to be used for scanning microarrays from the Agilent GenetiSure Dx Postnatal Assay and how to import that scan protocol into the Scan Control software (version 9.1) that operates your SureScan Dx Microarray Scanner (model number G5761A).

Step 1. Download the GenetiSure Dx Postnatal protocol file

If the scanner-connected computer has an internet connection, you can perform this set of steps on that computer. In such cases, you can bypass the steps in "Step 2. Transfer the protocol file to the scanner-connected computer".

1 On an internet-connected computer, go to the Agilent SureScan download page on the Agilent website at https://www.agilent.com/en/download-surescan-scan-control-software.

2 Click the link for downloading the GenetiSure_Dx_Postnatal scan protocol.

3 Save the file to a selected location.

Step 2. Transfer the protocol file to the scanner-connected computer

This set of steps is necessary if the protocol file was not downloaded directly to the computer that is connected to the scanner.

1 Save the scan protocol file to a USB drive or other portable media that is compatible with the scanner-connected computer.

2 Insert the USB drive into the scanner-connected computer. Without opening the file, copy and paste it onto the hard drive of the scanner-connected computer.

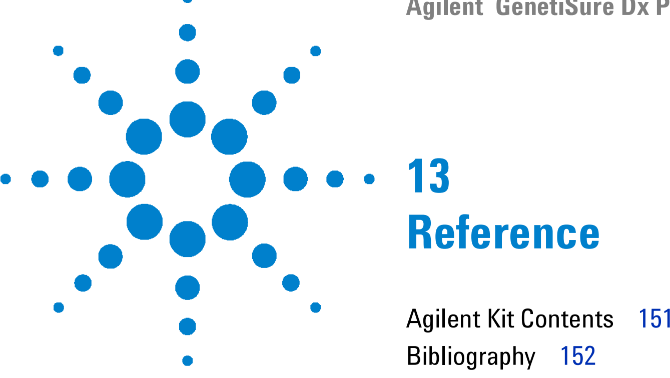
Step 3. Import the protocol into the Scan Control program

1. Launch Scan Control software 9.1 on the scanner-connected computer.
2. Click **Tools > Scan Protocol Editor**.

   The Scan Protocol Editor dialog box opens.
3. Click **Import**.

   The Open dialog box opens.
4. Browse to the location where you saved the GenetiSure_Dx_Postnatal scan protocols file.
5. Select the scan protocols file, and click **Open**.

   The scan protocol in the file is imported.



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Reference

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This chapter contains information on the reagents provided in the GenetiSure Dx DNA Labeling Kit, the GenetiSure Dx Hybridization Kit, the GenetiSure Dx Wash Buffer Set, and the GenetiSure Dx Cot-1 Human DNA. It also contains a bibliography.

Agilent Kit Contents

Table 39 GenetiSure Dx DNA Labeling Kit (p/n K1201-64100)
Sufficient reagents for 25 sample and 25 reference labeling reactions

Component	Quantity
Human Reference DNA Female	1 tube of 125 µL; 0.2 µg/µL
Human Reference DNA Male	1 tube of 125 µL; 0.2 µg/µL
10X Restriction Enzyme Buffer	1 tube of 142 µL
BSA	1 tube of 15 µL
Alu I Restriction Enzyme	1 tube of 265 µL; 10 U/µL
Rsa I Restriction Enzyme	1 tube of 265 µL; 10 U/µL
Nuclease Free Water	1 tube of 1500 µL
Exo (-) Klenow	1 tube of 55 µL
5X gDNA Reaction Buffer	1 tube of 550 µL
Cyanine 5-dUTP	1 tube of 78 µL
Cyanine 3-dUTP	1 tube of 78 µL
10X dNTP Mix	1 tube of 265 µL
Random Primers	1 tube of 265 µL
Purification columns and 2-mL collection tubes	1 bag of 50 columns

Table 40 GenetiSure Dx Hybridization Kit (p/n K1201-64200)
Sufficient reagents for hybridization of up to 25 slides

Component	Quantity
2X CGH Hybridization Buffer	5 tubes of 1400 µL each
10X Oligo aCGH Blocking Agent, Lyophilized	1 tube of lyophilized reagent

Table 41 GenetiSure Dx Wash Buffer Set (p/n K1201-64300)

Component	Quantity
GenetiSure Dx Wash Buffer 1	2 boxes of 4 L
GenetiSure Dx Wash Buffer 2	1 box of 4 L

Table 42 GenetiSure Dx Cot-1 Human DNA (p/n K1201-64400)

Component	Quantity
GenetiSure Dx Cot-1 Human DNA	1 tube of 625 µL; 1 µg/µL

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3. Hutton M. Kearney, PhD, Erik C. Thorland, PhD, Kerry K. Brown, PhD, Fabiola Quintero-Rivera, MD, and Sarah T. South, PhD, A Working Group of the American College of Medical Genetics (ACMG) Laboratory Quality Assurance Committee, "American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants," *Genetics in Medicine* 2011;13(7):680-5.

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Revision Log

Revision	Change
Revision H0	Added new limitation of the procedure

Manufacturer

Agilent Technologies, Inc.
5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA
www.agilent.com

Authorized Representative for the European Union

Agilent Technologies Denmark ApS
Produktionsvej 42
2600 Glostrup, Denmark

Authorized Representative for Switzerland

Agilent Technologies (Schweiz) AG,
Lautengartenstrasse 6
4052 Basel, Switzerland

European Union and Switzerland Importer

Agilent Technologies Deutschland GmbH
Hewlett-Packard-Str. 8
76337 Waldbronn, Germany

Agilent Technologies (Schweiz) AG,
Lautengartenstrasse 6
4052 Basel, Switzerland

Agilent Worldwide Technical Support

Visit www.agilent.com/en/contact-us/page to find country-specific phone numbers, or send an email to one of the email addresses below.

United States: clinical.support@agilent.com

Europe: genomics_dx_tech_europe@agilent.com

Japan: email_japan@agilent.com

Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the country in which the user and/or the patient is established.

A Summary of Safety and Performance (SSP) is located on the Agilent product website at https://www.agilent.com/en/product/cgh-cgh-snp-microarray-platform/cgh-diagnostic-testing-ivd/genetisure-dx-postnatal-assay-4091940, after launch of the European Database on Medical Devices (Eudamed), where it is linked to the Basic UDI-DI (570057R0403P3011C040000N8).

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