Agilent SureSelect DNA Capture Array

Protocol

Version 1.0, July 2009

SureSelect platform manufactured with Agilent SurePrint Technology

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Agilent Technologies

Notices

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CAUTION

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In this Guide...

Agilent SureSelect DNA Capture Array System provides an alternate approach to Agilent's DNA Target Enrichment System. Like the SureSelect Target Enrichment System, the DNA Capture Array is capable of isolating user-defined genomic regions from complex genomes, lowering sequencing costs by focusing only on regions of interest. This array-based target enrichment method is also ideal for researchers who need to test a few samples with various DNA microarray capture designs.

If you have comments about this protocol, send an e-mail to feedback_genomics@agilent.com.

1 Before You Begin

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

2 DNA Sample Preparation

This chapter contains instructions for DNA library production specific to the Illumina single-read sequencing platform.

3 Microarray Processing

This chapter describes the steps to hybridize and wash the DNA Capture microarray. It also describes how to elute, lyophilize and check the quality of your sample.

Content

1 Before You Begin

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

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing gDNA on a vortex mixer. Instead, gently tap the tube with your finger to mix the sample.
- Avoid repeated freeze-thaw cycles of stock and diluted gDNA solutions.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

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

Microarray

- The G4458A Agilent SureSelect DNA Capture Arrays can be custom designed and ordered in the SureSelect DNA Capture application space of eArray, a free, Web-based software tool. Go to https://earray.chem.agilent.com/earray/ for more information or to get started.
- The SureSelect DNA Capture Array includes 244,000 probes on a 1" by 3" glass slide. There is no control grid on this microarray.
- Store microarrays at room temperature.

Required Reagents

Table 1 Required Reagents

Description	Company and part no.
Agilent Oligo aCGH Hybridization Kit	Agilent p/n 5188-5220 (25) or p/n 5188-5380 (100)
Agilent Oligo aCGH Wash Buffer 1 and 2 set *or* Agilent Oligo aCGH Wash Buffer 1 (4 L) Agilent Oligo aCGH Wash Buffer 2 (4 L)	Agilent p/n 5188-5226 Agilent p/n 5188-5221 Agilent p/n 5188-5222
Agilent DNA 1000 Kit	Agilent p/n 5067-1504
Nuclease-free Water (not DEPC-treated)	Ambion, Cat # AM9930 or equivalent
Phusion Master Mix with HF Buffer	Finnzymes Cat #F-531
Illumina Genomic DNA Sample Prep Kit Oligo Only Kit	Illumina, Cat # FC-102-1001 Illumina, Cat # FC-102-1003
Illumina Genomic DNA Sample Prep Oligo Only Kit	Illumina, Cat # FC-102-1003
Human Cot-1 DNA *or* Mouse Cot-1 DNA *or* Rat Hybloc	Invitrogen p/n 15279-011 *or* Kreatech p/n EA-020 Invitrogen p/n 18440-016 Applied Genetics p/n RHB
Trackit Cyan/Orange Loading Dye (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose)	Invitrogen p/n 10482-028
Trackit 50 BP DNA ladder	Invitrogen p/n 10488-043
NuSeive GTG Agarose	Lonza p/n 50080
MinElute PCR Purification Kit	Qiagen, Cat # 28004
QIAquick PCR Purification Kit	Qiagen, Cat # 28104
QIAquick or MinElute Gel Extraction Kit	Qiagen p/n 28704 or Qiagen p/n 28604
Ethidium Bromide	Sigma Cat # 46067
50x TAE buffer (molecular biology grade)	
Distilled water	
100% Ethanol, molecular biology grade	

Genomic DNA Oligonucleotide Sequences

Table 2 PCR Primers – 50 µM each

PCR Primer	Sequence
PCR primer 1.1	5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'
PCR primer 2.1	5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT 3'

Table 3 Blocking Oligonucleotide (BO) Mix – 200 µM each

PCR Primer	Sequence
BO 1 (PCR primer 1.1)	5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'
BO 2 (PCR primer 2.1)	5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT 3'
BO 3 (rev. comp. 1.1)	5' AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT 3'
BO 4 (rev. comp. 2.1)	5' AGATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG 3'

Required Equipment

Table 4 Required Equipment

Description	Company and part no.
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization Chamber gasket slides, 5-pack (alternative packaging sizes are available) for 1x microarrays	Agilent p/n G2534-60003
Hybridization oven; temperature set at 65°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Agilent 2100 Bioanalyzer	Agilent p/n G2938C
UV-Transilluminator with SYBR photographic filter	Alpha Innotech p/n Alphalmager 2000 or equivalent
Nuclease-free 1.5 mL microfuge tubes (sustainable at 95°C)	Ambion p/n AM12400 or equivalent
Dark Reader transilluminator	Clare Chemical Research, Inc. p/n DR45M
Magnetic stir bar (×2)	Corning p/n 401435 or equivalent
Magnetic stir plate	Corning p/n 6795-410 or equivalent
Magnetic stir plate with heating element	Corning p/n 6795-420 or equivalent
Covaris microTUBE with AFA fiber and snap cap	Covaris p/n520045
Microcentrifuge	Eppendorf p/n 5417C or equivalent
Nuclease-free 0.2 mL PCR tubes, thin-walled	Eppendorf p/n 951010006 or equivalent
Vortex mixer	Fisher-Scientific Cat # 02215365
UV-VIS spectrophotometer	NanoDrop p/n ND-1000 or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
1.5 L glass dish	Pyrex p/n 213-R or equivalent

Table 4 Required Equipment (continued)

Description (continued)	Company and part no.
Series 700 Microarray Oven	SciGene p/n 1070-00-01 (115 V) SciGene p/n 1070-00-02 (230 V)
250 mL capacity slide-staining dish, with slide rack (×3)	Wheaton p/n 900200 or equivalent
Circulating water baths or heat blocks set to 30°C, 37°C, 65°C, and 95°C	
Ice bucket	
Clean forceps	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Disposable scalpels or razor blades	
Electrophoresis unit	
Gel trays and tank	
Electrophoresis power supply	
Water bath	
1-mL 30G syringes	

Table 5 Recommended Equipment

Description	Company and part no.
Sample Preparation System	Covaris S-series Single Tube Sample Preparation System, Model S2 *or* Diagenode Bioruptor UCD-200
Real Time PCR Machine, Plates and Accessories	Bio-Rad Dyad Disciple Dual-Bay Thermal Cycler Chassis #PTC-0221G
Tetrad Thermocycler	MJ Research PTC-225 Tetrad PCR
Vacuum Concentrator	Savant SpeedVac SVC100
UV-VIS Spectrophotometer	Thermo Fisher Scientific NanoDrop 1000

2
DNA Sample Preparation

This chapter contains instructions for prepped library production specific to the Illumina single-read sequencing platform. It is intended for use with the Illumina prep kit (p/n FC-102-1001).

Refer to the Illumina protocol *Preparing Samples for Sequencing Genomic DNA* (p/n 11251892 Rev. A) for more information.

NOTE Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0. If the DNA is not of high enough quality, you need to purify the DNA further. Check the size of genomic DNA both before and after sonication.

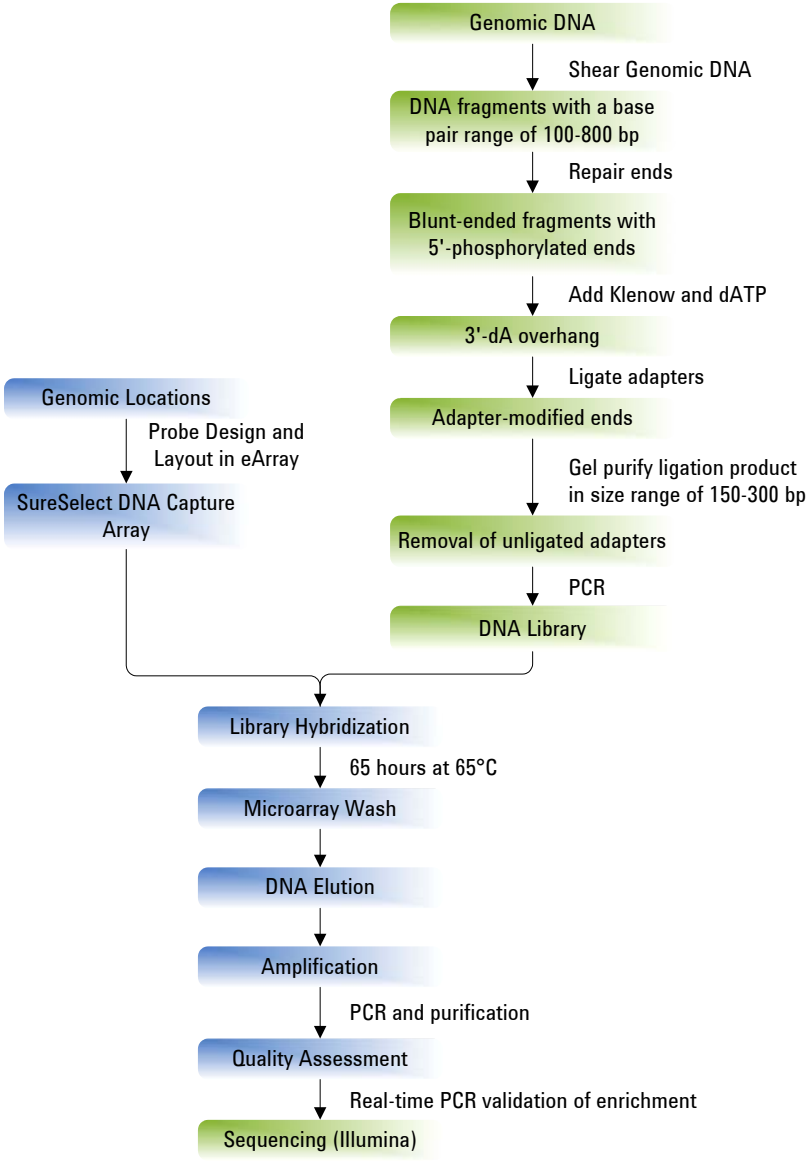

Figure 1 Overall sequencing sample preparation workflow.

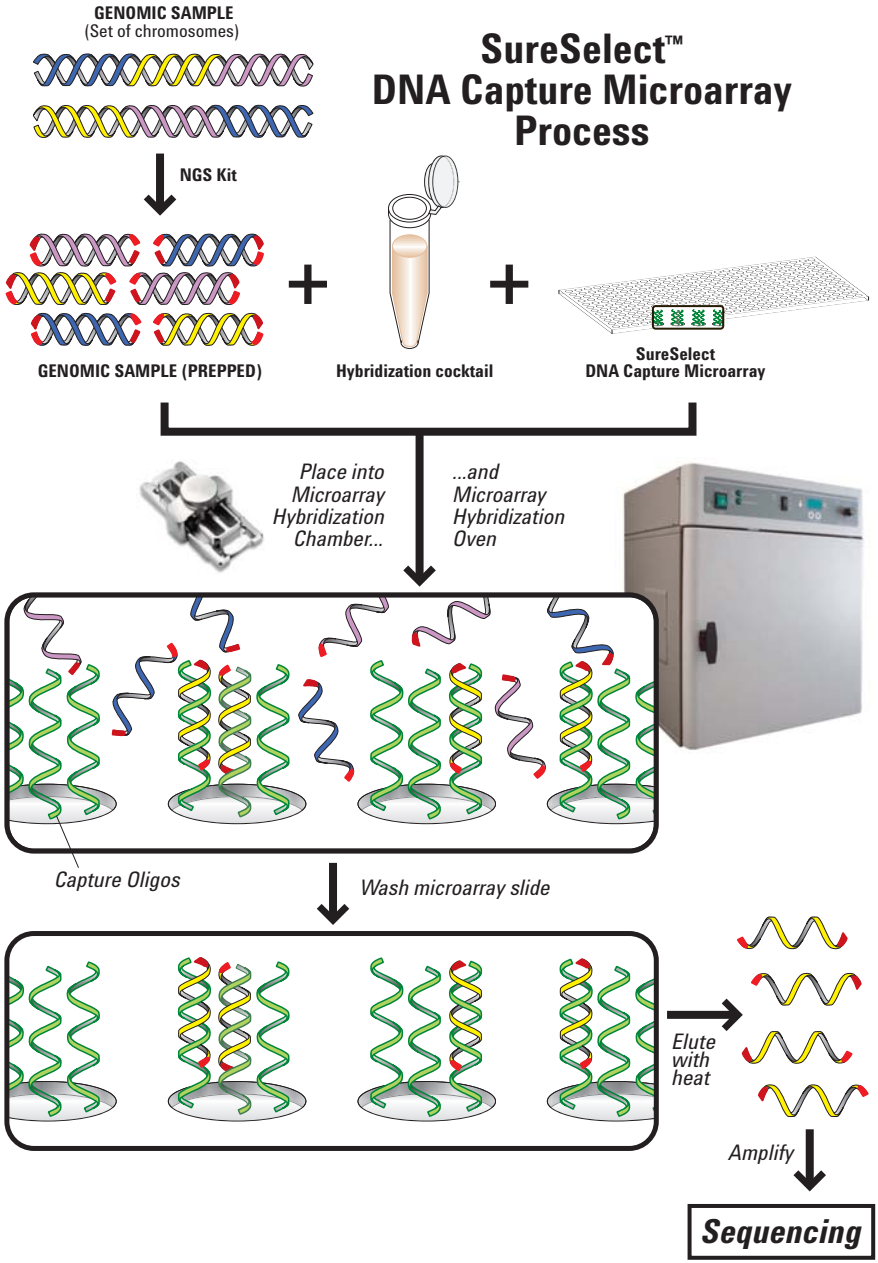

Figure 2 SureSelect Target Enrichment System Capture Process

Table 6 DNA Sample Preparation

Step	Time Required
Step 1. Shear DNA	1.5 hours
Step 2. Purify the sample with the QIAquick PCR Purification Kit	15 minutes
Step 3. Assess quality with Agilent 2100 Bioanalyzer	1 hour
Step 4. Repair the fragmented DNA to generate blunt ends	30 minutes
Step 5. Purify the repaired DNA with the QIAquick PCR Purification Kit	15 minutes
Step 6. Add 3' A overhang to the end of blunt ended DNA fragments	30 minutes
Step 7. Purify the sample with a Qiagen MinElute PCR Purification Column	15 minutes
Step 8. Ligate the adapter	30 minutes
Step 9. Purify the sample with the QIAquick PCR Purification Kit	15 minutes
Step 10. Select DNA size using a gel	1 hour
Step 11. Purify the gel	30 minutes
Step 12. Amplify DNA library	1 hour
Step 13. Purify the sample	15 minutes
Step 14. Check gDNA library quantity and quality	30 minutes
Step 15. Assess size, quality with Agilent 2100 Bioanalyzer	30 minutes

Table 7 Microarray Processing

Step 1. Preparation of Labeled Genomic DNA for Hybridization	1 hour
Step 2. Microarray Hybridization	65 hours
Step 3. Wash Preparation	overnight
Step 4. Microarray Washing	30 minutes
Step 5. Elution	30 minutes
Step 6. Lyophilization	2 to 4 hours
Step 7. PCR Amplification	1 hour
Step 8. Real-Time PCR	3.5 hours

Step 1. Shear DNA

1 Adjust the genomic DNA concentration to 2 to 5 µg in 100 µL with TE.

2 Load the Covaris microTUBE into the Covaris S-Series sample preparation system. Refer to the Covaris instrument user guide to set up the instrument.

3 Shear with the settings in Table 8. The target peak for base pair size is 200.

Stopping Point If you do not continue to the next step, store the sheared DNA overnight at -20°C.

Table 8 Covaris shear settings

Setting	Value
Duty Cycle	10%
Intensity	10
Cycles per Burst	500
Time	60 seconds
Set Mode	Frequency sweeping
Temperature	4°C

NOTE

You can use the Diagenode Bioruptor UCD-200 instead of the Covaris sample preparation system.

You can use a nebulizer, but significant DNA losses occur when a nebulizer is used.

To check DNA shear distribution, you can also use a 2% Agarose gel /w 50-bp ladder instead of Bioanalyzer.

Step 2. Purify the sample with the QIAquick PCR Purification Kit

1. Add 500 µL of PB to the sample and mix well by pipetting.
2. Place a QIAquick spin column in a 2 mL collection tube.
3. Transfer the 600 µL sample to the QIAquick column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
4. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
5. Place the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
6. Transfer the QIAquick column to a new 1.5 mL collection tube to elute the cleaned sample. Add 32 µL of buffer EB (10 mM Tris·Cl, pH 8.5) directly onto the QIAquick filter membrane. Wait 60 seconds, then centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
7. Collect the eluate.
8. If the DNA that you collect is between 1.5 to 2 µg, continue at "Step 3. Assess quality with Agilent 2100 Bioanalyzer".

This purification step can result in a loss of DNA. You may need to quantify with a spectrophotometer to check that DNA is not lost.

Step 3. Assess quality with Agilent 2100 Bioanalyzer

Use a Bioanalyzer DNA 1000 chip and reagent kit.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 expert software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Check that:
   - The size range of the DNA is between 100 to 800 bp.
   - The OD is greater than 1.8
   - The concentration of DNA is between 1 to 2 µg.

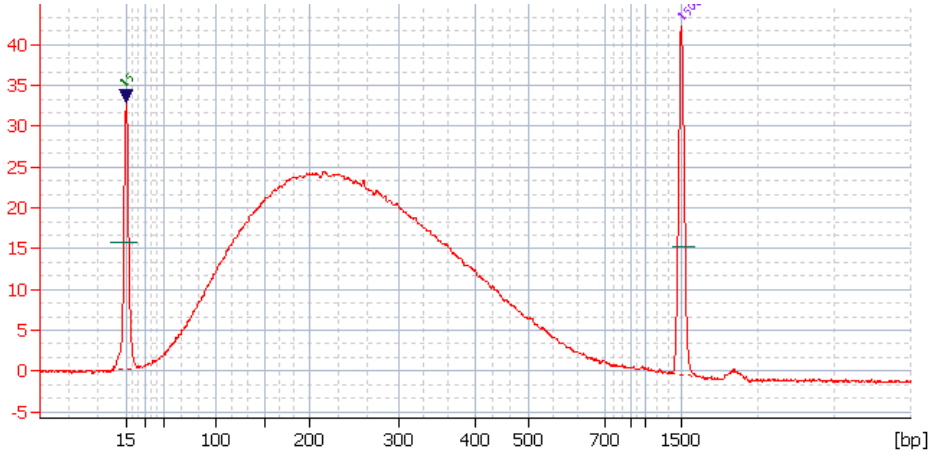

Figure 3 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows a distribution with a peak size of 200 nucleotides.

Step 4. Repair the fragmented DNA to generate blunt ends

1 In PCR tubes or strip tubes, prepare the reaction mix in Table 9 for each prepped library, on ice. Mix well by gently pipetting up and down.

NOTE If a precipitate forms in the T4 DNA ligase buffer with 10mM ATP, heat at 37°C for 5 minutes, and mix on a vortex mixer.

Table 9 End Repair

Reagent	Volume for 1 Library
DNA sample	29 µL*
Nuclease-free water	46 µL
T4 DNA ligase buffer with 10mM ATP	10 µL
dNTP mix	4 µL
T4 DNA polymerase	5 µL
Klenow enzyme	1 µL
T4 PNK	5 µL
Total Volume	100 µL

* This is the amount of DNA sample needed after bioanalyzer and spectrophotometer readings. The amount of DNA needed before the readings is at least 1 µg.

2 Incubate in a thermal cycler for 30 minutes at 20°C.

If you use a heated lid, make sure that the lid temperature does not exceed 50°C.

Step 5. Purify the repaired DNA with the QIAquick PCR Purification Kit

1. Add 500 µL of PB to the sample and mix well by pipetting.
2. Place a QIAquick spin column in a 2 mL collection tube.
3. Transfer the 600 µL sample to the QIAquick column Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
4. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
5. Place the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
6. Transfer the QIAquick column to a new 1.5 mL collection tube to elute the cleaned sample.
7. Add 32 µL of buffer EB directly onto the QIAquick filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
8. Collect the eluate.

Stopping Point If you do not continue to the next step, store the eluate overnight at 4°C or longer at -20°C.

Step 6. Add 3' A overhang to the end of blunt ended DNA fragments

1 In PCR tubes or strip tubes, prepare the reaction mix in Table 10 for each prepped library, on ice. Mix well by gently pipetting up and down.

Table 10 Adding "A" Bases

Reagent	Volume for 1 Library
DNA sample	32 µL
Klenow buffer	5 µL
dATP	10 µL
Klenow exo (3′ to 5′ exo minus)	3 µL
Total Volume	**50 µL**

2 Incubate in a thermal cycler for 30 minutes at 37°C.

Step 7. Purify the sample with a Qiagen MinElute PCR Purification Column

1. Allow the MinElute columns (stored at 4°C) to come to room temperature.
2. Add 500 µL of PB to the sample and mix well by pipetting.
3. Place a MinElute spin column in a 2 mL collection tube.
4. Transfer the 300 µL sample to the MinElute column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
5. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
6. Place the MinElute column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
7. Transfer the MinElute column to a new 1.5 mL collection tube to elute the cleaned sample. Add 10 µL buffer EB directly onto the MinElute filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
8. Collect the eluate.

Stopping Point If you do not continue to the next step, store the eluate overnight at 4°C or longer at -20°C.

Step 8. Ligate the adapter

This step uses a 10:1 molar ratio of adapter to genomic DNA insert, based on a starting quantity of 5 µg of DNA before fragmentation.

If you used 1 to 2.5 µg of input DNA, then adjust the adapter oligo mix to 5 µL of adapter. For 2.5 to 5 µg, use 10 µL adapter oligo mix.

1 Prepare the reaction mix in Table 11 on ice. Mix well by gently pipetting up and down.

Table 11 Ligation master mix

Reagent	Volume for 1 Library
DNA sample	10 µL
Nuclease-free water	5 µL
DNA ligase buffer	25 µL
Genomic Adapter oligo mix	5 µL
DNA ligase	5 µL
Total Volume	**50 µL**

2 Incubate for 15 minutes at room temperature.

Step 9. Purify the sample with the QIAquick PCR Purification Kit

This step is optional. Do this step to reduce the volume and to eliminate unligated adapters.

1. Add 500 µL of PB to the sample and mix well by pipetting.
2. Place a QIAquick spin column in a 2 mL collection tube.
3. Transfer the 300 µL sample to the QIAquick column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
4. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
5. Place the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
6. Transfer the QIAquick column to a new 1.5 mL collection tube to elute the cleaned sample. Add 30 µL of buffer EB directly onto the QIAquick filter membrane. Wait 60 seconds, then centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
7. Collect the eluate.

Step 10. Select DNA size using a gel

WARNING **Prolonged exposure to UV light can damage your DNA.**

Ethidium Bromide is carcinogenic. Handle with care.

Excise as narrow a band as possible from the gel during gel purification.

CAUTION Do not purify multiple samples on a single gel. Cross-contamination between libraries can occur.

1. Prepare a 0.4 to 0.5 µg/mL Ethidium Bromide gel with distilled water and TAE. Use TAE with a final concentration of 1X. Use a large well comb that holds 70 µL volume to prevent overloading.
2. Add 3 µL of loading buffer to 8 µL of the low molecular weight DNA ladder.
3. Add 10 µL loading buffer to 30 µL of the DNA from the purified ligation reaction, or adjust as appropriate for 50 µL reaction.
4. Load all of the ladder solution to one lane of the gel.
5. Load the entire sample in another lane of the gel, leaving at least a gap of one empty lane between ladder and sample. Electrophoresis parameters may need to be optimized.
6. Run the gel at 100V for 30 to 60 minutes.
7. View the gel on a Dark Reader transilluminator, which is a safer alternative to a UV transilluminator.
8. Use a clean scalpel or razor blade to excise the region of gel that contains DNA fragments in the 150 to 300 bp range.

   Keep the slice weight under 400 mg to avoid the need to use two purification columns.

Step 11. Purify the gel

Use a Qiagen Gel Extraction Kit (Qiagen, p/n 28704) to purify the DNA from the agarose slices.

1. Weigh the gel slice. If the gel slice exceeds 400 mg, use 2 Qiagen QIAquick spin columns for purification.
2. Add 6 volumes of Buffer QG to 1 volume of gel (100 mg = 100 µL).

   You may need to do this in a 15 mL conical tube or in two tubes.
3. Incubate at 50°C for 10 minutes (or until the gel slice has completely dissolved). To help dissolve gel, mix the tube in a vortex mixer every 2 to 3 minutes during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose), or adjust pH per manufacturer's instruction.
5. Add 1 gel volume of isopropanol to the sample and mix.

   For example, if the agarose gel slice is 100 mg, add 100 µL isopropanol.

   Do not spin the sample in a centrifuge at this time.
6. Place a QIAquick spin column in a provided 2 mL collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and spin in a centrifuge for 1 minute at 17,900 x g (13,000 rpm).

   The maximum volume of the column reservoir is 800 µL. For sample volumes of more than 800 µL, simply load and spin again.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. To wash, add 750 µL of Buffer PE to QIAquick column and spin in a centrifuge for 2 minutes at 17,900 x g (13,000 rpm).
10. Discard the flow-through and spin the QIAquick column in a centrifuge for an additional 1 minute at 17,900 x g (13,000 rpm).

    Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
11. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.

12 To elute DNA, add 30 µL of Buffer EB to the center of the QIAquick membrane. Wait 60 seconds, then spin the column in a centrifuge for 1 minute.

If 2 Qiagen MinElute columns were used for a single sample, elute each one in 15 uL of EB, for a total of 30 uL per DNA sample.

Step 12. Amplify DNA library

This step uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends, and to amplify the amount of DNA in the library. The PCR is done with two primers that anneal to the ends of the adapters. Eighteen cycles of PCR are used.

You will do 12 parallel reactions for each sample to amplify. Each PCR yields about 2 to 3 µg DNA, which creates approximately 25 µg of DNA per sample.

1 Prepare the PCR reaction mix in Table 12, on ice. Mix well by gently pipetting up and down.

Table 12 PCR Components

Reagent	Volume for 1 Library
DNA	1 µL
Nuclease-free Water	22 µL
PCR primer 1.1	1 µL
PCR primer 2.1	1 µL
DNA Polymerase (from Illumina Kit or Finnzymes Phusion HF Master Mix 2X)	25 µL
Total Volume	**50 µL**

2 Amplify using the following PCR program:

Table 13 PCR protocol

Step	Temperature	Time
Step 1	98°C	30 seconds
Step 2	98°C	10 seconds
Step 3	65°C	30 seconds
Step 4	72°C	30 seconds
Step 5		Repeat Step 2 through Step 4 for a total of 17 times.
Step 6	72°C	5 minutes
Step 7	4°C	Hold

Step 13. Purify the sample

1. Combine 3 PCR reactions so that the 12 parallel reactions result into 4 reaction pools.
2. Add 500 µL of PB to each of the four reaction pools and mix well by pipetting.
3. Place a QIAquick spin column in a 2 mL collection tub.
4. Transfer the 300 µL pool to the QIAquick column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
5. Add 750 µL of buffer PE to the column. Spin the sample in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm). Discard the flow-through.
6. Place the QIAquick column back in the 2 mL collection tube and spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
7. Transfer the QIAquick column to a new 1.5 mL collection tube to elute the cleaned sample. Add 30 µL of buffer EB directly onto the QIAquick filter membrane. Wait 60 seconds, then spin in a centrifuge for 60 seconds at 17,900 x g (13,000 rpm).
8. Collect the eluate, which can be stored at 4°C.

Step 14. Check gDNA library quantity and quality

Use the NanoDrop ND-1000 UV-VIS Spectrophotometer (or equivalent) to assess gDNA library concentration and purity.

1. Select **Nucleic Acid Measurement**, then select **Sample Type** to be **DNA-50**.
2. Use 1.5 µL of EB buffer to blank the instrument.
3. Use 1.5 µL of each gDNA sample to measure DNA concentration. Record the gDNA concentration (ng/µL) for each sample. Calculate the yield (µg) by multiplying DNA concentration (ng/µL) by the sample volume and dividing by 1000.
4. Record the **A260/A280** and **A260/A230** ratios. High-quality gDNA samples should have an **A260/A280** ratio of 1.8 to 2.0, indicating the absence of contaminating proteins, and an **A260/A230** ratio of >2.0, indicating the absence of other organic compounds such as guanidinium isothiocyanate, alcohol and phenol as well as cellular contaminants such as carbohydrates.

NOTE A minimum of 10 µg of library is required for hybridization. For optimal results, use 20 µg gDNA library.

Step 15. Assess size, quality with Agilent 2100 Bioanalyzer

Use a Bioanalyzer DNA 1000 chip and reagent kit to assess the quality and size distribution of the PCR products.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 expert software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results.

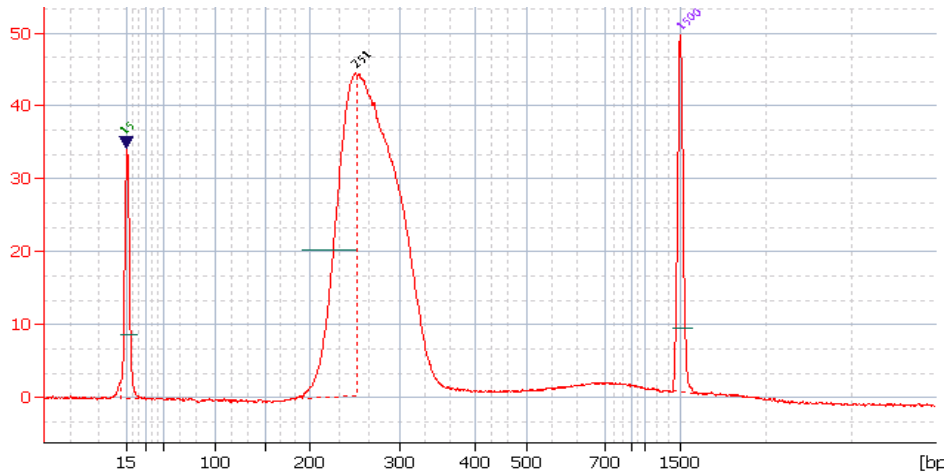

Figure 4 Analysis of amplified prepped library DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows single peak in the size range of 200 to 300 nucleotides.

3 Microarray Processing

This chapter describes the steps to hybridize and wash the DNA Capture microarray. It also describes how to elute, lyophilize and check the quality of your sample.

Step 1. Preparation of Labeled Genomic DNA for Hybridization

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

   a Add 1,350 µL of nuclease-free water to the vial containing lyophilized 10X Blocking Agent (supplied with Agilent Oligo aCGH Hybridization Kit).

   b Leave at room temperature for 60 minutes and mix on a vortex mixer to reconstitute sample before use or storage.

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

2 Prepare the samples for hybridization:

   a Equilibrate water baths or heat blocks to 95°C and 37°C or use a PCR machine.

   b Mix the components listed in Table 14 to prepare the Hybridization Master Mix.

Table 14 Preparation of Hybridization Sample Mixture

Component	Volume (µL) per hybridization
Sample DNA (10 to 20 µg in nuclease-free water)	138
Blocking oligo 1 (200 µM) Forward Primer	5
Blocking oligo 2 (200 µM) Reverse Primer	5
Blocking oligo 3 (200 µM) reverse complement of Forward Primer	5
Blocking oligo 4 (200 µM) reverse complement of Reverse Primer	5
Cot-1 DNA (1.0 mg/mL)*	50
Agilent 10X Blocking Agent†	52
Agilent 2X Hi-RPM Hybridization Buffer†	260
Final Volume of Hybridization Master Mix	520

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

c Mix the sample by pipetting up and down, then quickly spin in a centrifuge to drive contents to the bottom of the reaction tube.

d Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes, then immediately transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 30 minutes.

or

Transfer sample tubes to a PCR machine. Program the machine according to the following table and run the program:

Table 15

Step	Temperature	Time
Step I	95 °C	3 minutes
Step II	37 °C	30 minutes

e Remove sample tubes from the water bath, heat block or PCR machine. Spin 1 minute at 17,800 × g in a centrifuge to collect the sample at the bottom of the tube.

Step 2. Microarray Hybridization

NOTE

Familiarize yourself with the assembly and disassembly instructions for use with the Agilent microarray hybridization chamber and gasket slides. Please refer to the Agilent Microarray Hybridization Chamber User Guide (G2534-90001) for in-depth instructions on how to load samples, assemble and disassemble chambers, as well as other helpful tips. This user guide can be downloaded from the Agilent Web site at www.agilent.com/chem/dnamanuals-protocols.

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.

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 placed on top of the gasket slide to form a "sandwich slide pair".

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

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

Hybridization Assembly

1. Load a clean gasket slide into the Agilent SureHyb chamber base with the gasket label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
2. Slowly dispense 490 µL of hybridization sample mixture onto the gasket well in a "drag and dispense" manner.
3. Place a microarray slide "active side" down onto the gasket slide, so the numeric barcode side is facing up and the "Agilent"-labeled barcode is facing down. Check that the sandwich-pair is properly aligned.
4. Hand-tighten the clamp onto the chamber.

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

6 Place assembled slide chamber in the rotator rack in a hybridization oven set to 65°C. Set your hybridization rotator to rotate at 20 rpm.

7 Hybridize at 65°C for 65 hours.

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

Step 3. Wash Preparation

Do this step the day before you wash the microarray slides.

Cleaning with Milli-Q Water Wash

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

a Run copious amounts of Milli-Q water through the slide-staining dishes, slide racks and stir bars.

b Empty out the water collected in the dishes at least five times.

c Repeat step a and step b until all traces of contaminating material are removed.

Prewarming Oligo aCGH Wash Buffer 2 (Overnight)

The temperature of Oligo aCGH Wash Buffer 2 must be at 37°C for optimal performance.

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

2 Put a slide-staining dish into a 1.5 L glass dish three-fourths filled with water and warm to 37°C by storing overnight in an incubator set to 37°C.

Step 4. Microarray Washing

Always use fresh Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2 for each wash group (up to five slides).

Table 16 lists the wash conditions for the DNA Capture Array.

Table 16 Wash conditions

	Dish	Wash buffer	Temperature	Time
Disassembly	#1	Oligo aCGH Wash Buffer 1	Room temperature	
1st wash	#2	Oligo aCGH Wash Buffer 1	Room temperature	10 minutes
2nd wash	#3	Oligo aCGH Wash Buffer 2	37°C	5 minute

1. Completely fill slide-staining dish #1 with Oligo aCGH Wash Buffer 1 at room temperature.
2. Place a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH Wash Buffer 1 at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.
3. Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Oligo aCGH Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH Wash Buffer 2 at 37°C; monitor using a thermometer.
4. Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.
5. Prepare the hybridization chamber disassembly.
   a. Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter-clockwise.
   b. Slide off the clamp assembly and remove the chamber cover.
   c. With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the

microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

d Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Oligo aCGH Wash Buffer 1.

6 With the sandwich completely submerged in Oligo aCGH 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 turn the forceps upwards or downwards to separate the slides. Let the gasket slide drop to the bottom of the staining dish. Remove the microarray slide and place into slide rack in the slide-staining dish #2 containing Oligo aCGH Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

7 Repeat step 4 through step 6 for up to four additional slides in the group. A maximum of five disassembly procedures yielding five microarray slides is advised at one time in order to facilitate uniform washing.

8 When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 10 minutes. Adjust the setting to get good but not vigorous mixing.

9 Set oven temperature to 95°C, or check that the Scigene oven is at 95°C.

10 Transfer slide rack to slide-staining dish #3 containing Oligo aCGH Wash Buffer 2 at 37°C, and stir using setting 4 for 5 minutes.

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

12 Discard used Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2.

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

Step 5. Elution

1. Dry the slides in a centrifuge at 600 rpm for 30 seconds.
2. Load a clean gasket slide into the Agilent SureHyb chamber base with the gasket label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
3. Slowly dispense 490 µL of nuclease-free water onto the gasket well in a "drag and dispense" manner.
4. Place a microarray slide "active side" down onto the gasket slide, so the numeric barcode side is facing up and the "Agilent"-labeled barcode is facing down. Check that the sandwich-pair is properly aligned.
5. Hand-tighten the clamp onto the chamber.
6. Vertically rotate the assembled chamber to wet the slides and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.
7. Place the hybridization chambers that contain the slide-gasket sandwiches in a 95°C Scigene 700 series oven for 10 minutes.

CAUTION The hybridization chambers will be hot. Use care when you handle the hybridization chambers.

8. Quickly and carefully remove the hybridization chambers from the oven.
9. For each chamber, grasp the slide sandwich and chamber base with a paper towel to avoid the heat, then loosen the chamber screw a quarter turn.
10. Tilt the chamber so that the narrow end (no label) points upward.
11. Insert a 1-mL 30G syringe into the narrow end of the slide sandwich, through the rubber ring between the gasket and the slide.
12. Remove as much of the eluate as possible with the syringe.

Step 6. Lyophilization

1 Use a vacuum concentrator such as a Savant Speed Vac to lyophilize the eluted DNA down to 50 µL. Set the vacuum concentrator on high for approximately 2.5 to 4 hours.

2 For each sample, set up five independent PCR reactions according to Table 17.

Table 17 Preparation of PCR Sample Mixture

Component	Volume (µL) per hybridization
Phusion HF Master Mix	25
Forward Primer (50 µM)	1
Reverse Primer (50 µM)	1
Eluted Template	5
Nuclease-free water	18
Final Volume	50

3 Transfer sample tubes to a PCR machine. Program the machine in Table 18 and run the program:

Table 18 PCR Program

Step	Temperature	Time
Step 1	98°C	30 seconds
Step 2	98°C	10 seconds
Step 3	65°C	30 seconds
Step 4	72°C	30 seconds
Step 5		Repeat Step 2 through Step 4 17 times.
Step 6	72°C	5 minutes
Step 7	4°C	Hold

Step 7. PCR Amplification

1. Remove sample tubes from the water bath, heat block or PCR machine.
2. Spin 1 minute at 17,800 × g in a centrifuge to collect the sample at the bottom of the tube.
3. Purify the sample. See "Step 13. Purify the sample" on page 34.
4. Quantify the DNA. See "Step 14. Check gDNA library quantity and quality" on page 35.
5. Dilute the sample to a working concentration of 10 nM.

Step 8. Real-Time PCR

Do this step to check whether target regions are getting enriched.

1 Check that the primers are around 20 bases long with a T_m of 58 to 60°C.

Refer to the SYBR Green PCR Master Mix (Applied Biosystems) product insert for more information and guidelines.

2 For each target region and a non-target region, set up 3 PCR reactions for the pre-hybridization template and 3 PCR reactions for the post-hybridization (amplified eluate) template. See Table 19.

Make sure both sets of DNA are at 20 ng/µL.

Table 19 Preparation of qPCR Sample Mixture

Component	Volume (µL) per hybridization
SYBR Green PCR Master Mix (Applied Biosystems)	10
Nuclease free water	8
For primer (10 µM)	0.5
Rev primer (10 µM)	0.5
Template (20 ng/µL)	1
Total Reaction Volume	**20**

3 Transfer sample tubes to a PCR machine. Program the machine in Table 20 and run the program:

Table 20 PCR Program

Step	Temperature	Time
Step 1	98°C	10 seconds
Step 2	98°C	15 seconds
Step 3	60°C	1 minute
Step 4		Repeat Step 2 and Step 3 for a total of 40 cycles.
Step 5	4°C	Hold

4 Evaluate the deltaC_T between the pre-hybridization and post-hybridization DNA to determine the level of enrichment.

For example, if you use GAPDH as the non-targeted region and "Gene X" as the targeted region (a region printed on the array), then use primers directed to those regions to compare the pre-hybridized and post-hybridized DNA populations and determine whether hybridization-based enrichment succeeded for Gene X.

If Gene X is enriched after hybridization-based capture, the number of cycles needed (the cycle threshold, or C_t) after enrichment for the non-targeted GAPDH region to reach the required threshold will be greater than that of the targeted Gene X region.

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In This Book

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