applied biosystems

CytoScan™ Optima Assay Manual Workflow

User Guide

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Chapter 1 Safety

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Introduction

CytoScan™ Optima assay has been optimized for the detection of chromosomal abnormalities associated with miscarriages (products of conception, [POC]) and pre-natal samples. The assay is designed to be used with genomic DNA of prenatal and perinatal origin such as Amniotic Fluid, Chorionic Villi, POC as well as peripheral blood, in conjunction with CytoScan Optima array, GeneChip™ System 3000 and Chromosome Analysis Suite (ChAS). CytoScan Optima array consists of features covering control, copy number (CN) and single-nucleotide polymorphism (SNP) probes. There are a total of 18,018 CN markers and 148,450 SNP markers uniformly spaced over the genome with enhanced interrogation of 396 regions of prenatal interest. Cumulatively, through the collection of SNPs and non-polymorphic probes the application provides the ability to support detection of CNVs, enable the elucidation of allelic imbalance (i.e., the pattern of inheritance deviates from expected Mendelian inheritance patterns), identify copy number neutral abnormalities such as absence (AOH) or loss of heterozygosity (LOH) and characterize unbalanced translocation events in the samples of interest.

The product is for research use only. Not for use in diagnostic procedures.

Assay Warnings and Precautions

Follow universal precautions for laboratory and assay procedures, and waste disposal. Follow federal, state, local, and within-country regulations.

Before you begin, go to Appendix A, *Workflow and Practices* for specific setup instructions, and equipment and technique illustrations and Appendix B, *Thermal Cycler Programs* for specific Thermal Cycler Program set ups.

For additional specific warnings, precautions, and procedures, see:

- *GeneChip™ Command Console™ 3.2 User Guid*e (Pub. No. 702569)
- *GeneChip™ Fluidics Station 450 User Guide for AGCC* (Pub. No. 08-0295)
- *GeneChip™ Hybridization Oven 645 User Guide* (Pub. No. 08-0255)

See our web site for all user guides (*CytoScan™ Optima Assay User Guide*, *Chromosome Analysis Suite (ChAS) User Guide*, *GeneChip™ Command Console™ User Guide*, *GeneChip™ Fluidics Station 450 User Guide*, *GeneChip™ Hybridization Oven 645 User Guide*), and training materials (containing Training kit and positive control DNA), SDS for all hazards in CytoScan™ Optima assay reagents.

Precautions

The physical and toxicological properties of the products in this kit(s) have not been thoroughly investigated. Follow prudent laboratory practices and use general laboratory safety equipment (eye protection, labcoat, and lab gloves) and good personal hygiene when working with these or any laboratory reagents. Refer to the Safety Data Sheet for more information.

Proper laboratory practice is necessary as previously amplified PCR product is the most likely potential source of contamination. We strongly recommend two separate work areas be used to minimize the risk of cross contamination during the assay procedure. It is essential to adhere to workflow recommendations. PCR reactions should be set up in the Pre-PCR Area only. Personnel should not re-enter the Pre-PCR Clean Area once exposed to PCR products without first showering and changing into clean clothes.

Carefully reading and following the protocol as written is essential. The CytoScan™ Optima assay has been verified using the reagents and suppliers listed. Substitution of reagents and not following detailed procedures are not recommended as your results could be suboptimal.

Pre-/Post-PCR

- Follow standard procedures and single-direction workflow for the Pre-PCR laboratory area.
- Use dedicated equipment for each area (e.g., thermal cyclers, microfuges, pipettes and tips, ice buckets, etc.)
- Place all reagents and master stocks in use area. Do not move equipment between Pre-and Post-PCR Rooms, e.g., ice buckets, pipettes, etc.
- Use separate copy of assay procedure in Pre-and Post-PCR areas.
- Follow procedures for re-entry of Pre-PCR Clean Room from post-PCR.
- If pre-PCR work is done in a laminar flow hood or PCR cabinet, then additionally ensure:
  - laminar flow hood is always on
  - UV lamp is on when not in use

Do Not

- Use kit after its expiration date.
- Use reagents after more than four freeze-thaw cycles.
- Use any water other than Nuclease-free Water supplied with the CytoScan™ Optima Kit.
- Reuse a plate seal.

Do

- Use 250 ng of double-stranded genomic DNA (gDNA) that is not degraded (size >10 kb by gel analysis), not contaminated, and free of PCR inhibitors; verify concentration using quantitation method specific to dsDNA. The purity ratio (A_{260}/A_{280} ratio) of input DNA must be between 1.7–2.1.
- Follow procedures for gowning, etc.
- Use nuclease-free pipette tips with aerosol barriers for all pipetting steps.
- Use extraction methods that yield DNA compatible with DNA quality as specified above.
- Chill essential equipment such as cooling blocks and reagent coolers before use.
- Keep all tubes, master mixes, and working solutions in chilled cooling blocks on ice.
- Keep enzymes at –25 to –15°C until needed, then immediately place in reagent cooler chilled to –15 to –25°C. Do not store enzymes at –80°C.
- Where indicated, keep reagents chilled at 2 to 8°C and place on ice during use. After thawing, immediately place on ice and use within 1.5 hours.
- Maintain sample consistency; ensure all transitions to incubation temperatures are rapid and well-controlled. Enzyme activity is a function of temperature.

- Enzyme handling:
  - Maintain tube in –15 to –25°C cooler
  - Quick spin and vortex 1 to 3 seconds
  - Place back in cooler for use
- Because Fragmentation Reagent activity can decline over time after dilution on ice, add it to the samples as quickly as possible.
- Maintain 15 to 30°C (RT) throughout the procedure.
- Always use freshly prepared master mixes.
- Use only Nuclease-free Water supplied with the kit.
- Follow instructions for sealing, vortexing, and centrifuging. Ensure plates are tightly sealed to prevent sample loss and cross-well contamination. Always use a new seal.
- Pipet accurately using calibrated pipettors.
- Use equipment calibrated according to manufacturer instructions
- Use only specified assay stopping points.
- Check that your spectrophotometer or Nanodrop is accurately calibrated, and be sure the OD measurement is within the linear range of the instrument as per the manufacturer's recommendations.
- Hybridization oven temperature is critical to the performance of the assay. Use the GeneChip™ Hybridization Oven 645 only. Hybridization ovens should be serviced at least once a year to ensure that they are operating within specification.

Controls

- Use of a pre-qualified sample [e.g., Ref 103 (P/N 900421)] as a positive control is highly recommended. These controls are effective troubleshooting tools that will help you confirm the successful completion of each stage of the assay.
- Use a negative control. Use a blank well with Low EDTA TE Buffer (P/N 902424). The absence of bands on your PCR gel for the negative control confirms no previously amplified PCR product has contaminated your samples.
- Oligonucleotide controls are included in the reagent kit. These controls are added to the target samples prior to hybridization and act to confirm successful hybridization, washing, staining, and scanning of the array.

GeneChip™System 3000

- *GeneChip™ Fluidics Station 450 User Guide for AGCC* (Pub. No. 08-0295)
- *GeneChip™ Hybridization Oven 645 User Guide* (Pub. No. 08-0255)

Safety Symbols and Information

Refer to the SDS for each component provided at

www.ThermoFisher.com/support/technical/safetydatasheetsmain.affx. Wear protective clothing to include gloves and eye protection.

Table 1.1 Safety Contents and Warnings/Precautions for Reagents

Reagent	Contains	Warnings/Precautions
Hyb Buffer Part 1 	Tetramethylammonium chloride	DANGER. Toxic if swallowed and in contact with skin; causes skin irritation, serious eye irritation; may cause respiratory irritation. Avoid breathing and avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.
TdT Buffer 	Cacodylic acid	DANGER. Harmful if swallowed, may cause cancer. Toxic if swallowed and in contact with skin; causes skin irritation, serious eye irritation; may cause respiratory irritation. Avoid breathing and avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.
BSA 	Glycerol	WARNING. Causes minor skin irritation and eye irritation. Avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.
DNA Ligase 	Glycerol	WARNING. Causes minor skin irritation and eye irritation. Avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.
Fragmentation Buffer 	Tris [Tris(hydroxmethyl) aminomethane]	WARNING. Causes minor skin irritation and eye irritation. Avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.
Fragmentation Reagent 	Glycerol	WARNING. Causes minor skin irritation and eye irritation. Avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.
Nsp I 	Glycerol	WARNING. Causes minor skin irritation and eye irritation. Avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.
CytoScan DNA Polymerase 	Glycerol	WARNING. Causes minor skin irritation and eye irritation. Avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.
TdT 	Glycerol	WARNING. Causes minor skin irritation and eye irritation. Avoid contact with skin or eyes; if contact occurs wash immediately. If skin irritation or rash occurs, seek medical advice.

These materials contain ≤0.1% sodium azide: Array Holding Buffer, Stain Buffer 1, Stain Buffer 2, and Purification Beads. Exercise caution in disposal: buildup of sodium azide in lead and copper plumbing can form highly explosive metal azides.

Chapter 2 CytoScan™ Optima Assay Procedure

Before You Begin

Read all warnings and precautions and refer to Table C.2 for module and component part number and labeling.

Table 2.1 Temperature Range Definitions

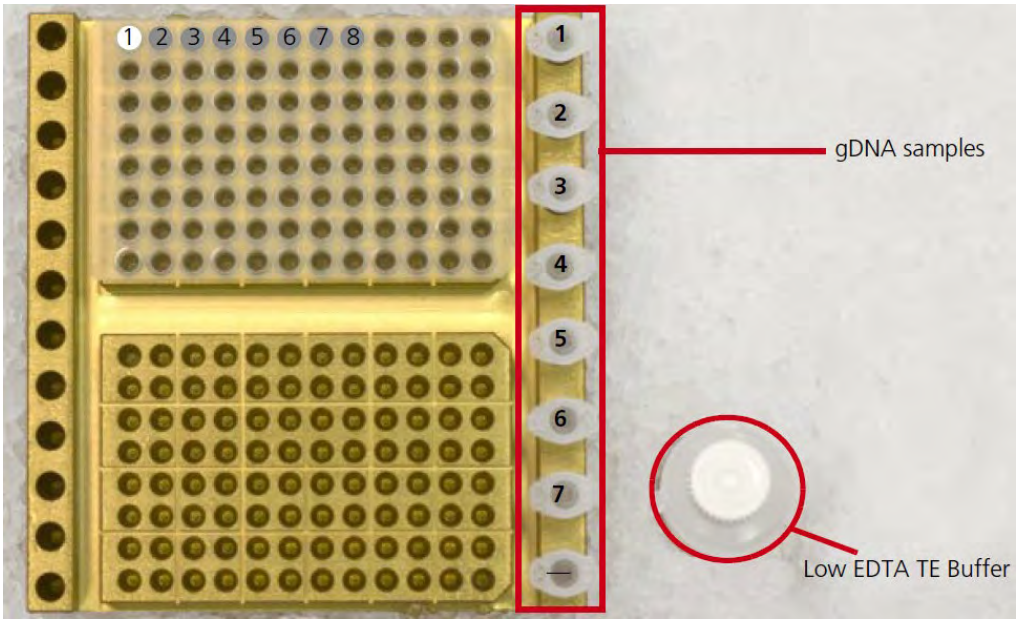
Temperature	Range
Freeze	–15 to –25°C
Ice	2 to 8°C
RT (Room Temperature)	15 to 30°C

Section 1. Prepare gDNA Plate

Dilute Stock gDNA to Working Concentration

1. Place a 96-well plate in the upper half of the cooling block on ice (Figure 2.1).

Figure 2.1 96-well Plate in Cooling Block, on Ice



2. Place the gDNA at 15 to 30°C (RT) until thawed (≤30 minutes); then place in the cooling block on ice.

IMPORTANT: Use within 1 hour.

3. Vortex the gDNA samples for 3 seconds.
4. Centrifuge 650 x *g* for 1 minute; then place in the cooling block.
5. Dilute each sample to 50 ng/µL with Low EDTA TE in separate wells of the 96-well plate or in 1.5 mL eppendorf tubes.

NOTE: Use double-strand-specific quantitation method to determine sample concentration.

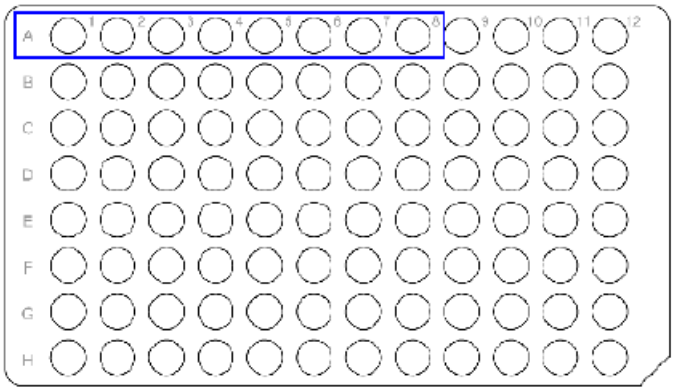
6. Tightly seal the plate.
7. Centrifuge 650 x *g* for 1 minute.
8. Place on cooling block.

Aliquot Prepared gDNA into Assay Plate

Set up Work Area

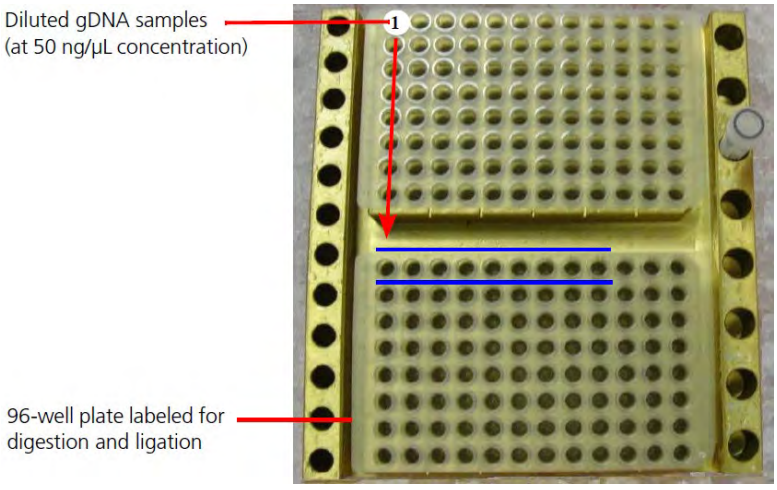
1. Mark 96-well plate (Table 2.1, Figure 2.2).

Figure 2.2 Plate Marking



2. Place the plate on the lower half of the cooling block.
3. Vortex gDNA 3 seconds; centrifuge.
4. Transfer **5 µL** of first sample from gDNA stock plate to well A1 of DIG-LIG plate; transfer all samples in same way (Figure 2.3).
5. For the controls, aliquot 5 µL of:
   a. Positive Control gDNA to well A7.
   b. Low EDTA TE buffer [negative control] to well A8.

Figure 2.3 Transfer Samples from gDNA Plate to DIG-LIG Plate



6. Tightly seal DIG-LIG plate with new seal; centrifuge 650 x *g* for 1 minute.

Section 2. Restriction Enzyme Digestion

Prepare Reagents, Equipment, and Consumables

Turn on the thermal cycler to preheat lid. Leave block at RT.

Set up Work Area

1. Place the cooling block and water on ice.

NOTE: For more than 8 samples, place 8-12 tube strip on upper half of cooling block.

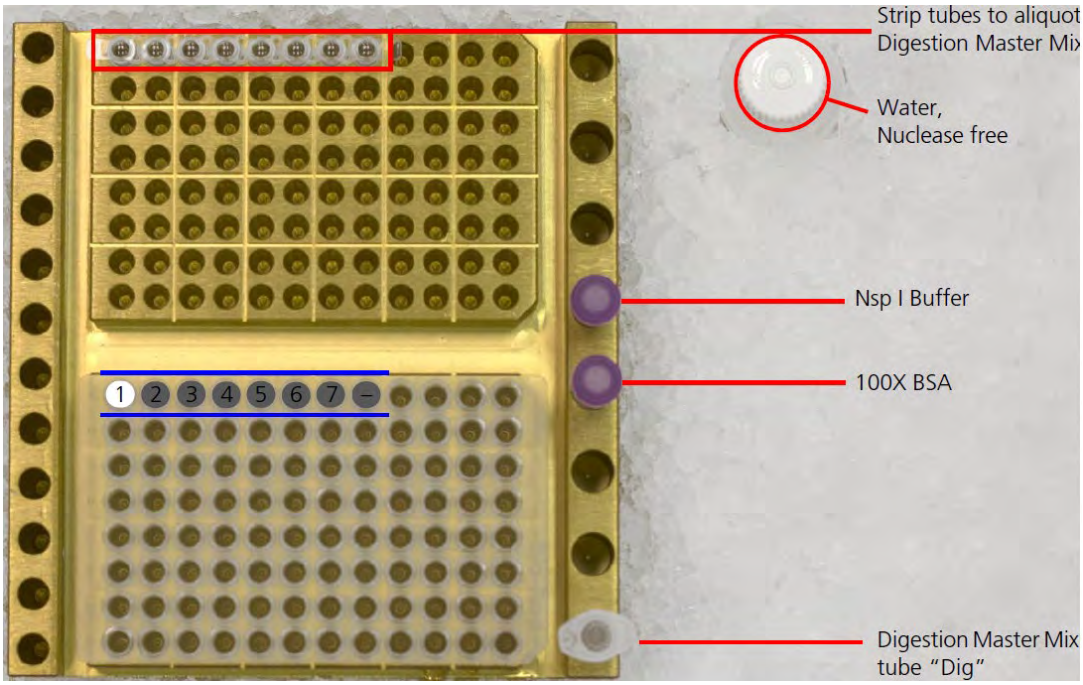
2. Label a 1.5 mL tube "Dig" and place it in the cooling block.
3. Cut adhesive film into strips wide enough to seal 8 or 12 strip tubes.

Prepare Reagents

1. Thaw the following reagents at RT (≤30 minutes), immediately place on ice:
   - Nsp I Buffer
   - 100X BSA

IMPORTANT: Set up reaction within 1 hour.

Figure 2.4 Digestion setup (Nsp I enzyme not pictured, still at –15 to –25°C)



2. Prepare Nsp I Buffer and 100X BSA:
   a. Vortex 3 times for 1 second each.
   b. Quick spin 3 seconds.
   c. Place in cooling block on ice.
3. Place Nuclease-free Water on ice.

Prepare Digestion Master Mix

Prepare and store all reagents, tubes, and cooling block on ice. Dispense within 10 minutes.

1. To the 1.5 mL Eppendorf tube ("Dig") add per Table 2.2:
   - Water, Nuclease free, chilled (2 to 8°C)
   - Nsp I Buffer
   - 100X BSA

Table 2.2 Digestion Master Mix(≥ 8 samples, 20% overage)

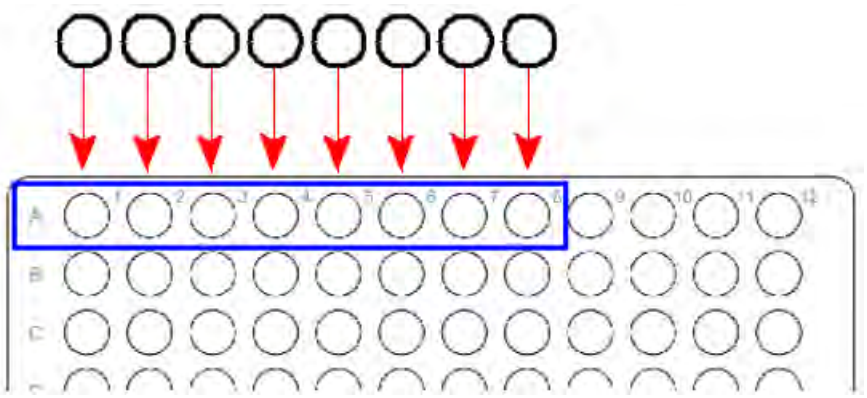
Reagent	1 Sample	8 Samples	16 Samples 24	Samples
Water, Nuclease free	11.55 µL	110.9 µL	221.8 µL	332.6 µL
Nsp I Buffer	2.00 µL	19.2 µL	38.4 µL	57.6 µL
100X BSA	0.20 µL	1.9 µL	3.8 µL	5.8 µL
Nsp I	1.00 µL	9.6 µL	19.2 µL	28.8 µL
Total	**14.75 µL**	**141.6 µL**	**283.2 µL**	**424.8 µL**

2. Vortex 3 times for 1 second each.
3. Quick spin 3 seconds.
4. Place Digestion Master Mix in chilled cooling block (2 to 8°C).
5. Remove Nsp I enzyme from the freezer and immediately place in a chilled cooler (–15 to –25°C).
6. Mix Nsp I.
   a. Quick spin 1 second.
   b. Vortex 1 second.
   c. Quick spin 3 seconds.
7. Return to –15 to –25°C cooler while preparing Digestion Master Mix.
8. Immediately add Nsp I to Digestion Master Mix.
9. Return enzyme to –15 to –25°C cooler.
10. Vortex Digestion Master Mix 3 times for 1 seconds each.
11. Quick spin 3 seconds.
12. Place in chilled cooling block (2 to 8°C).

Add Digestion Master Mix to Samples

1. Divide the Digestion Master Mix equally into 8 or 12 strip tubes on cooling block on ice.
2. Seal strip tube with adhesive film strip (or strip caps).
3. Centrifuge 650 x *g* for 1 minute and place on cooling block on ice.
4. Remove and discard seal.
5. Unseal the plate and discard seal.
6. Use multichannel P20 pipette and add **14.75 µL** of the Digestion Master Mix to each sample in row A (Figure 2.5).

Figure 2.5 Addition of Digestion Master Mix to Samples



7. Seal the plate tightly with a new seal.

Load Samples on Thermal Cycler

1. Vortex the plate 1 second all corners and center.
2. Centrifuge 650 x *g* for 1 minute.
3. Load the plate on thermal cycler, ensure thermal cycler lid is preheated, and run **CytoScan Optima Digest** program.

Table 2.3 CytoScan Optima Digest

Temperature	Time
37°C	2 hours
65°C	20 minutes
4°C	5 minutes
4°C	∞

4. Discard the remaining Digestion Master Mix and return reagents to the freezer. When done, leave the plate in thermal cycler. Process within 2.5 hours.
5. Ensure the plate is sealed tightly and centrifuge 650 x *g* for 1 minute.
6. Place the plate in cooling block on ice and immediately go to *Section 3. Ligation.*

Section 3. Ligation

Turn on the thermal cycler to preheat lid. Leave block at RT.

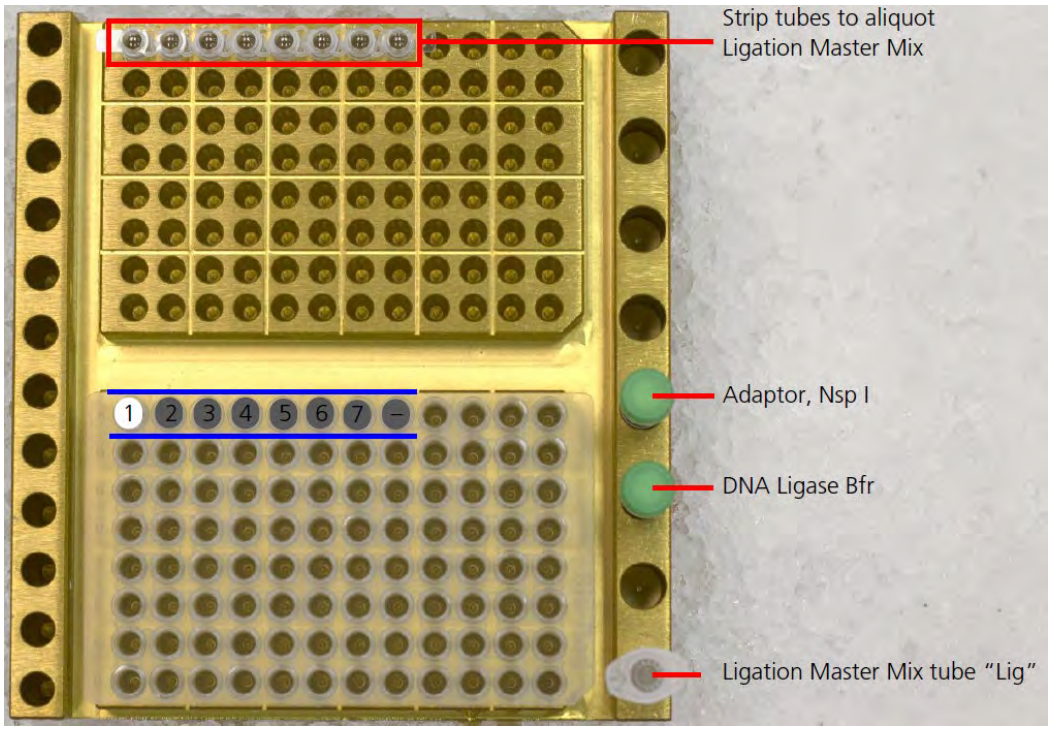
Set up Work Area

1. Place cooling block on ice.
2. Label a 1.5 mL tube "Lig" and place it in cooling block.
3. Place 8-tube strip on upper half of cooling block.
4. Cut adhesive film into strips wide enough to seal 8 or 12 strip tubes.

   Thaw Reagents and Digested Samples
5. Allow following to thaw at RT (≤30 minutes)
6. Immediately place on ice and use within **1 hour.**

- Adaptor, Nsp I
- DNA Ligase Bfr

Figure 2.6 Ligation setup (DNA Ligase not pictured, still at –15 to –25°C)



Prepare Digested Samples and Reagents

1. Prepare samples:
   a. Centrifuge 650 x g for 1 minute.
   b. Place in lower half of cooling block on ice.
2. Prepare reagents:
   a. Vortex DNA Ligase Bfr and Adaptor, Nsp I 3 times for 1 second each.

NOTE: If precipitate is present in DNA Ligase Bfr, vortex until precipitate is dissolved.

   b. Quick spin 3 seconds.
   c. Place in chilled cooling block (2 to 8°C).

Prepare Ligation Master Mix

Prepare and store all reagents, tubes, and cooling block on ice. Dispense within 10 minutes.

1. Label a 1.5 mL tube "Lig" and add per Table 2.4:
   - DNA Ligase Buffer
   - Adaptor, Nsp I

Table 2.4 Ligation Master Mix (≥8 samples, 25% overage)

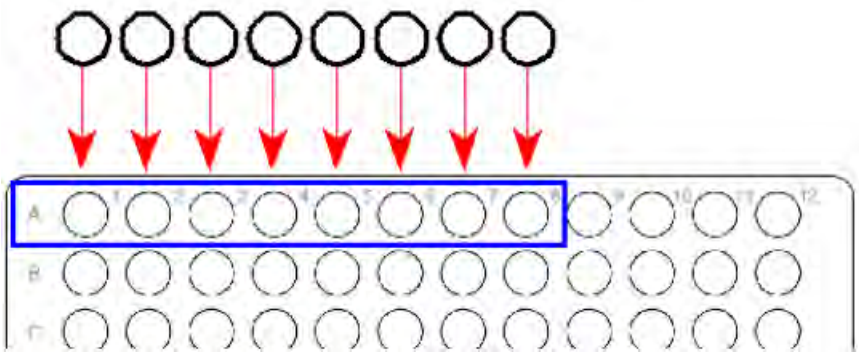
Reagent	1 Sample	8 Samples	16 Samples	24 Samples
DNA Ligase Bfr	2.50 µL	25.0 µL	50.0 µL	75.0 µL
Adaptor, Nsp I	0.75 µL	7.5 µL	15.0 µL	22.5 µL
DNA Ligase	2.00 µL	20.0 µL	40.0 µL	60.0 µL
Total	**5.25 µL**	**52.5 µL**	**105.0 µL**	**157.5 µL**

2. Vortex 3 times for 1 second each.
3. Quick spin 3 seconds.
4. Remove the DNA Ligase from freezer and immediately place in chilled cooler (–15 to –25°C).
5. Mix DNA Ligase.
   a. Quick spin 1 second.
   b. Vortex 1 second.
   c. Quick spin 3 seconds.
6. Place in –15 to –25°C cooler.
7. Immediately add the DNA Ligase to the Ligation Master Mix; place back in –15 to –25°C cooler.
8. Vortex the Ligation Master Mix 3 times for 1 second each.
9. Quick spin 3 seconds.
10. Place the Ligation Master Mix in cooling block on ice.
11. Immediately go to next step.

Add Ligation Master Mix

1. Divide the Ligation Master Mix equally into 8 or 12 strip tubes on ice.
2. Seal strip tube with adhesive film strip (or strip caps) and quick spin.
3. Place back in cooling block on ice.
4. Remove and discard the strip tube seal.
5. Unseal the digested sample plate and discard seal.
6. Use a multi-channel P20 pipette to add **5.25 µL** of the Ligation Master Mix to each digested sample (Figure 2.7).

Figure 2.7 Addition Ligation Master Mix



Load Samples on Thermal Cycler

Ensure thermal cycler lid is preheated.

1. Seal the plate tightly with new seal.
2. Vortex for 1 second in all corners and center.
3. Centrifuge 650 x *g* for 1 minute.
4. Load the plate on the thermal cycler and run **CytoScan Optima Ligate** program (Table 2.5).

Table 2.5 CytoScan Optima Ligate Thermal Cycler Program

Temperature	Time
16°C	3 hours
70°C	20 minutes
4°C	5 minutes
4°C	∞

5. Discard the remaining Ligation Master Mix and return reagents to the freezer.
6. When program finishes, leave the plate in thermal cycler.

NOTE: You can hold plate in thermal cycler at 4°C for up to 16-20 hours (overnight).

7. After removing from thermal cycler, ensure the plate is sealed tightly.
8. Centrifuge 650 x *g* for 1 minute.
9. Store at –15 to –25°C.

TIP: Optional stopping point. The plate may be frozen at –15 to –25°C for up to 1 week.

Section 4. PCR

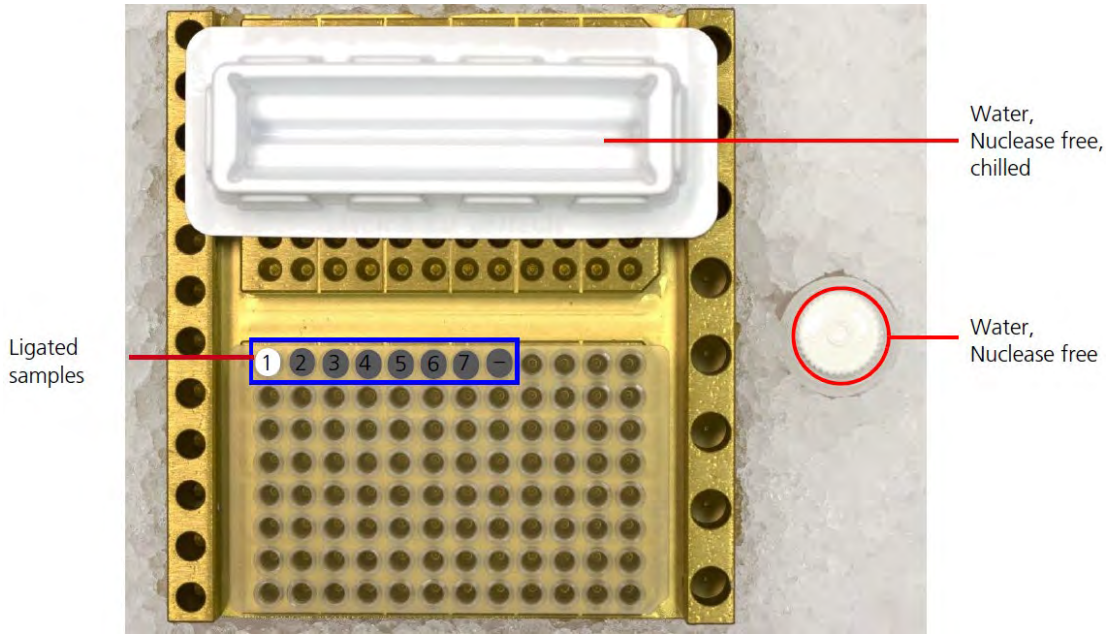
Turn on the thermal cycler in the Post-PCR Room to preheat lid.

Dilute Ligated Samples

Twenty minutes before use, place Nuclease-free Water on ice.

1. Place cooling block on ice.
2. Place reagent reservoir on upper half of cooling block on ice (Figure 2.8).

Figure 2.8 Reagent Reservoir on Upper Half of Cooling Block on Ice



3. Pour the chilled Nuclease-free Water into reagent reservoir.
4. Place the plate in lower half of the cooling block on ice.

NOTE: If samples are frozen, thaw at RT (≤ 30 minutes). Centrifuge 650 x *g* for 1 minute. Immediately place plate on the lower half of the cooling block. Process within 1 hour.

5. Unseal the ligated sample plate and discard seal.
6. Use a P200 pipette to add **75 µL** of Nuclease-free Water to each reaction.

Table 2.6

Sample	Volume/Sample
Ligated DNA	25 µL
Water, Nuclease free (chilled)	75 µL
Total	**100 µL**

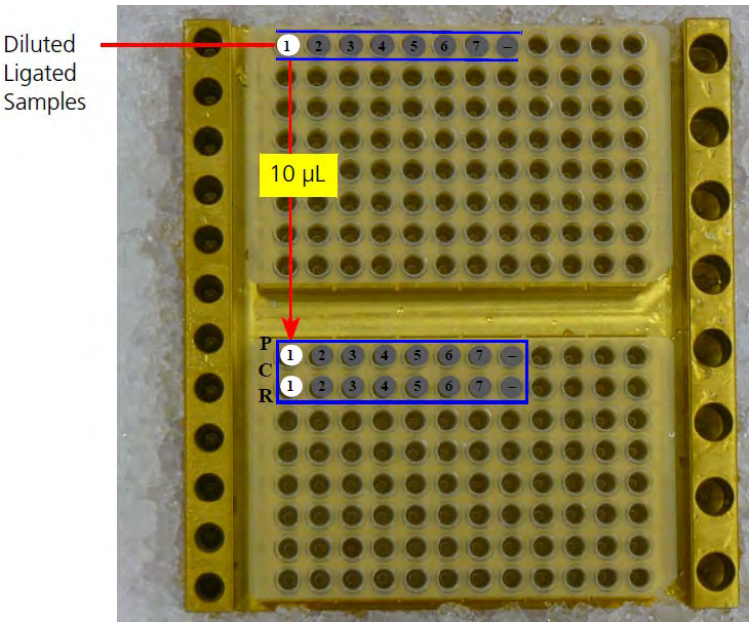
7. Tightly seal plate with new seal.
8. Vortex 1 second in all corners and center.
9. Centrifuge 650 x *g* for 1 minute.
10. Go to *Run PCR*.

Run PCR

Transfer to PCR Plate

1. Place cooling block on ice.
2. Keep the diluted ligated sample plate on the upper half of the cooling block.
3. Label a new plate "PCR" and the place in the lower half of the cooling block.
4. Unseal the ligated and diluted sample plate and discard seal.
5. Use a multi-channel P20 pipette to transfer **10 µL** of each sample to the corresponding well of the PCR plate (transfer 2 times). Store leftover samples at –15 to –25°C.

Figure 2.9



6. Seal the plate tightly with a new seal and centrifuge 650 x *g* for 1 minute.

Thaw Reagents and Samples

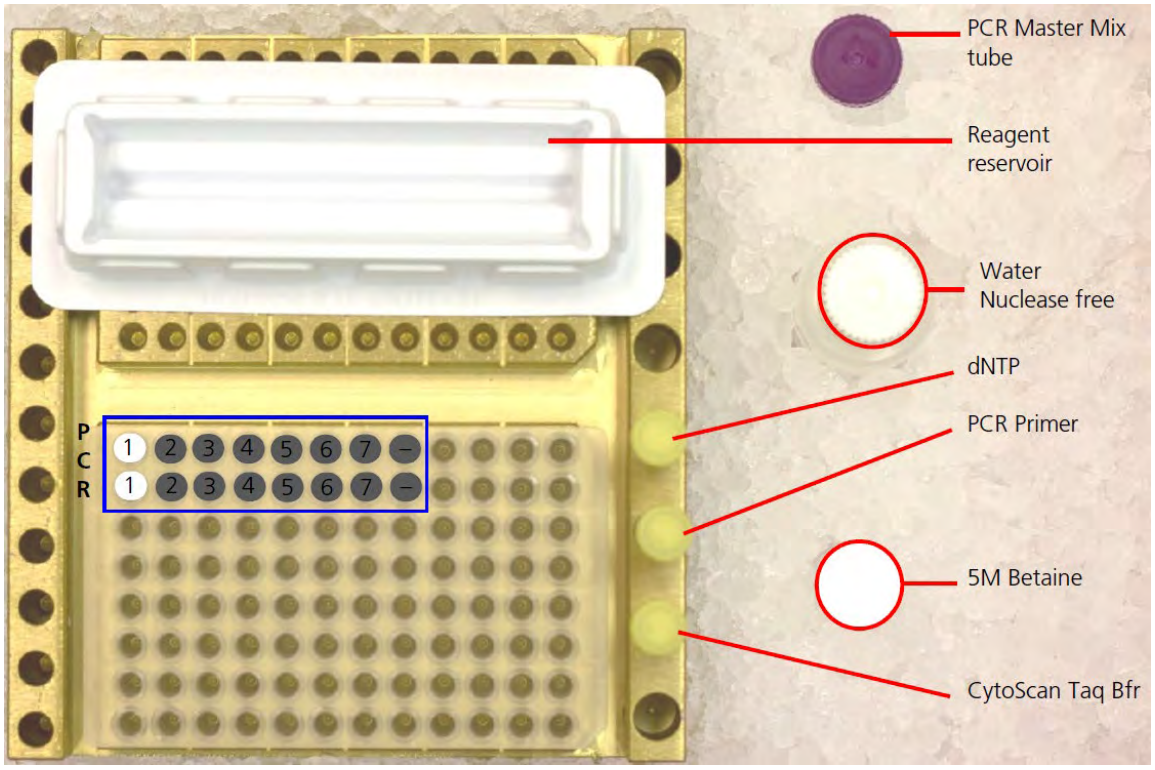
Thaw at RT (≤30 minutes); immediately place on ice and use within 1 hour:

- CytoScan Taq Buffer
- dNTP
- PCR Primer

Prepare Reagents and Ligated Samples

1. Label 15 mL centrifuge tube "PCR".
2. Place on ice (Figure 2.10):
   - Water, Nuclease free (chilled on ice for 10 minutes)
   - 5M Betaine (If precipitate is present, vortex until precipitate is dissolved)
   - Reagent reservoir on upper half of cooling block on ice

Figure 2.10



3. Immediately place in the lower half of the cooling block on ice.
4. Prepare reagents (not enzyme):
   a. Vortex 3 times for 1 second each.
   b. Quick spin 3 seconds.
   c. Place in chilled cooling block (2 to 8°C).

Prepare PCR Master Mix

Prepare and store all reagents, tubes, and cooling block on ice. Dispense within 20 minutes.

1. Keep 15 mL centrifuge tube on ice, add reagents in order shown in Table 2.7.

IMPORTANT: Leave the CytoScan Taq DNA Polymerase at –20°C until ready to use.

Table 2.7 PCR Master Mix (≥8 samples, 20% overage)

Reagent	1 Reaction	8 Sample	16 Samples	24 Samples
Water, Nuclease free (chilled)	50.3 µL	965.8 µL	1931.5 µL	2897.3 µL
CytoScan Taq Bfr	10.0 µL	192.0 µL	384.0 µL	576.0 µL
5M Betaine	20.0 µL	384.0 µL	768.0 µL	1152.0 µL
dNTP	3.5 µL	67.2 µL	134.4 µL	201.6 µL
PCR Primer	4.2 µL	80.6 µL	161.3 µL	241.9 µL
CytoScan Taq DNA Polymerase	2.0 µL	38.4 µL	76.8 µL	115.2 µL
Total	**90 µL**	**1728 µL**	**3456 µL**	**5184 µL**

2. Vortex 3 seconds.
3. Take the CytoScan Taq DNA Polymerase from the freezer and immediately place in a chilled cooler (–15 to –25°C).
4. Mix CytoScan Taq DNA Polymerase.
   a. Quick spin 1 second.
   b. Vortex 1 second.
   c. Quick spin 3 seconds
5. Immediately add to the PCR Master Mix, then return to –15 to –25°C cooler.
6. Vortex the PCR Master Mix 3 times for 1 second each.
7. Pour the PCR Master Mix into the reagent reservoir. Keep cooling block on ice.

Add PCR Master Mix to Samples

1. Unseal the PCR sample plate and discard seal.
2. Use a multi-channel P200 pipette to add **90 µL** of the PCR Master Mix to each sample on the PCR plate.
   - Avoid contamination: Change pipette tips after each transfer.
   - 8 samples: Tilt reagent reservoir so each pipette tip picks up 90 µL.

Table 2.8

Sample	Volume/Sample
Ligated and diluted DNA	10 µL
PCR Master Mix	90 µL
Total	**100 µL**

3. Tightly seal the plate with a new seal.
4. Vortex for 1 second in all corners and center.
5. Repeat vortex 1 time.
6. Centrifuge at 650 x *g* for 1 minute.
7. Immediately load the plate on the thermal cycler.

Load Plate and Run PCR Program

Ensure thermal cycler lid is preheated. Ensure block is RT (15 to 30°C).

1. Move the plate on ice to Post-PCR Room.
2. Load the plate on the thermal cycler.
3. Run the **CytoScan Optima PCR** program.

NOTE: The plate maybe held 16-20 hours (overnight) in the thermal cycler at 4°C.

Table 2.9 CytoScan Optima PCR Thermal Cycler Program

Temperature	Time	Cycles
95°C	1 minute	1X
95°C 60°C 68°C	30 seconds 45 seconds 60 seconds	30X
68°C	7 minutes	1X
4°C	5 minutes	
4°C	Hold (can be held overnight)	
Volume: 100 µL Specify *Maximum* Mode		

4. When done, leave the plate in the thermal cycler. If proceeding to purification, the plate must be processed within 2.5 hours.
5. After removing from thermal cycler, keep the plate on a 96-well plate rack.
6. Ensure that the plate is tightly sealed.
7. Centrifuge 650 x *g* for 1 minute.

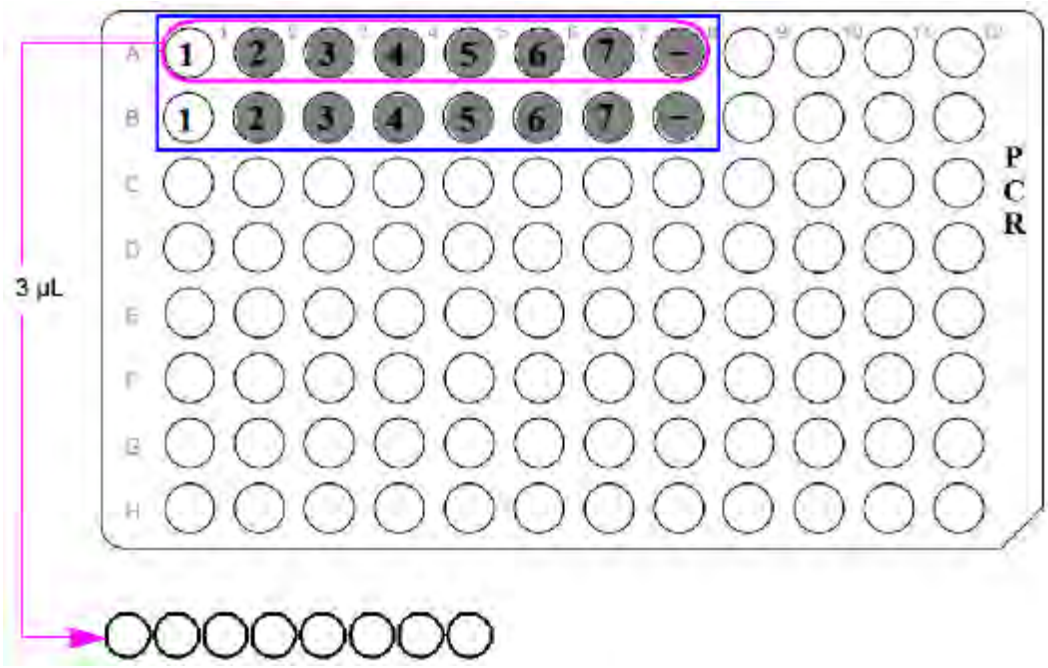
TIP: OPTIONAL STOPPING POINTS: If not proceeding immediately to the next step, the plate maybe stored at –20°C for up to 1 week.

Check PCR Reaction

1. Unseal the PCR plate and discard seal.
2. Transfer **3 µL** of the PCR product from each well of row A to corresponding wells of the strip tube (Figure 2.11) for in-process QC.

IMPORTANT: For >12 samples, transfer 3 µL PCR product from first row for every 2 rows of same samples.

Figure 2.11 Transfer of PCR products to gel plate



3. Seal the PCR plate tightly with a new seal.
4. Seal the gel strip tubes tightly with an adhesive film strip or strip tube caps.
5. Vortex gel strip tubes at maximum setting for 1–3 seconds.
6. Centrifuge in microfuge.
7. Prepare the QC samples by adding 17 µL of diluted loading buffer (see *Section 11. In-Process Quality Control*) and run samples on 2% agarose gel to resolve bands.
8. Inspect gel and follow *Section 11. In-Process Quality Control*. The majority of PCR target distribution is 150 to 2200 bp (note higher intensities at 300 to 2200 bp).

Section 5. PCR Product Purification

Prepare Purification Wash Buffer

1. Add **45 mL** absolute ethanol to the Purification Wash Buffer bottle.
2. Cap the bottle tightly and mix by inverting unopened Purification Wash Buffer bottle 10 times.
3. Write the date of the ethanol addition on the bottle.

Prepare PCR Products

Pool PCR Products

NOTE:

- Requires transfer of assay intermediate from plate to independent tubes.
- Perform these steps at Room Temperature.

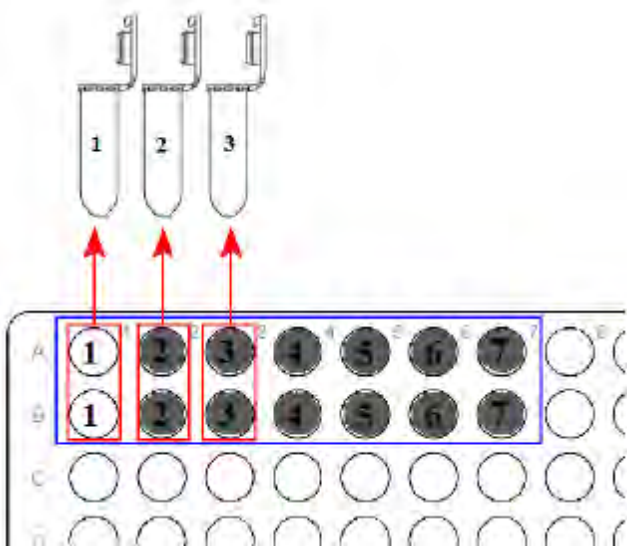
1. Mark each 2.0 mL tube with the sample number using a permanent marker.
2. Ensure that the plate is tightly sealed.
3. Centrifuge 650 x *g* for 1 minute.

4. Remove and discard plate seal.
5. Use a P200 pipette to transfer all 2 well aliquots of each sample to marked 2.0 mL tube (Figure 2.12).

NOTE: If using a multi-channel P200 pipette, organize tips to ensure multi-channel pipetting into wells of plate and tubes to match well/tube and pipette.

IMPORTANT: To avoid cross-contamination, use new pipette for each transfer and take care with pipette tips when pooling samples for purification.

Figure 2.12 Pool PCR products



6. Ensure total volume in each well is transferred and pooled.

Volume in each PCR well	= 100 µL
Total volume in 2 PCR wells	= 200 µL
Total Volume in Each 2.0 mL Tube	**= 200 µL/tube –3 µL aliquoted for PCR gel**

Purify PCR Products

Change tips between pipetting steps.

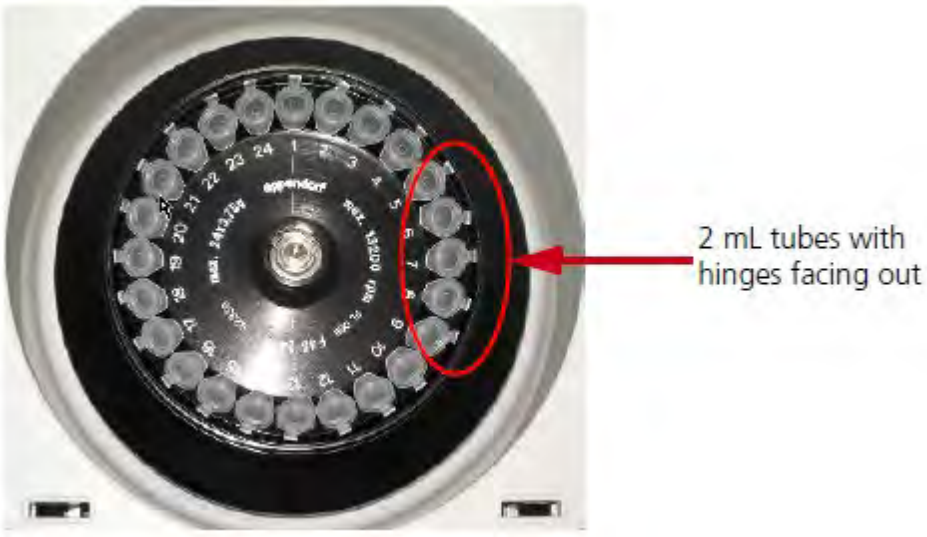
1. Thoroughly mix the Purification Beads stock by inverting bottle 10 times. Examine the bottom of the bottle to ensure that the solution appears homogeneous.
2. Slowly open tube caps to prevent spill.
3. Add Purification Beads to each pooled sample:
   - **8 Samples:** Use a single-channel P1000 pipette to add **360 µL** Purification Beads per sample directly from bottle.
   - **16 and 24 Samples:** Use a multi-channel P1000 pipette to add **360 µL** Purification Beads per sample.
     - 16 samples: Add **8 mL** Purification Beads to reagent reservoir.

- 24 samples: Add **12.5 mL** Purification Beads to reagent reservoir.

- Add **360 µL** Purification Beads to each pooled sample, 3 to 4 samples at a time. Organize tips to enable multi-channel pipetting into tubes to match tube and pipette.

4. Securely cap each tube and invert 10 times.
5. Incubate at RT for 10 minutes.
6. Load tubes onto centrifuge with cap hinge facing out and centrifuge 16,000 x *g* for 3 minutes.

Figure 2.13 2 mL Tubes Loaded into Centrifuge



7. Place the tube on magnetic stand so cap hinge is over magnet. Ensure pellet is completely pulled towards magnet.
8. Use a P1000 pipette to remove supernatant without disturbing bead pellet. Discard supernatant.

- **16 and 24 Samples:** Use a multi-channel P1000 pipette. Remove supernatant from 3 to 4 samples at a time.

Add Purification Wash Buffer

1. Use a P1000 pipette to add **800 µL** Purification Wash Buffer to each tube.

- **16 and 24 Samples:** Pour Purification Wash Buffer into reservoir. With multi-channel pipette add **800 µL** Purification Wash Buffer to each tube, 3 to 4 samples at a time.

2. Cap the tubes and load into foam tube adapter. Fully insert the tubes into foam to secure. Space sample tubes evenly. Use balance if necessary.
3. Vortex 2 minutes.

NOTE: Bead pellet may not be completely resuspended; this is acceptable.

4. Centrifuge tubes, cap hinges facing out, 16,000 x *g* for 3 minutes.
5. Place the tubes on magnetic stand and visually verify that beads are completely pulled towards magnet with no beads present in Purification Wash Buffer supernatant at bottom of tubes.
6. Visually verify that bead pellet is adjacent to magnet when removing supernatant.
   a. Use P1000 pipette to remove supernatant without disturbing bead pellet.
   b. Discard supernatant.

- **16 and 24 Samples:** Use multi-channel pipette to remove supernatant from 3 to 4 samples at a time.

7. Centrifuge tubes, cap hinges facing out, 16,000 x *g* for 30 seconds; place tubes back on magnetic stand so cap hinge is over magnet. Ensure pellet is completely pulled towards magnet.
8. Use a P20 pipette to remove remaining drops of Purification Wash Buffer from each tube bottom, 1 sample at a time. Do not disturb or break off any bead pellet.
9. Take tubes **OFF** magnetic stand and allow remaining Purification Wash Buffer to evaporate by leaving tubes uncapped at RT for 7 minutes.

Add Elution Buffer

1. Use a P100 pipette to add **27 µL** Elution Buffer to each tube directly onto beads.
   - **16 and 24 Samples:** Use a multi-channel P200 pipette. Add to 3 to 4 samples at a time.
2. Cap tubes and load into foam tube adapter. Balance tubes.
3. Vortex 10 minutes to resuspend beads.
4. Examine each tube to be sure beads are resuspended in homogeneous slurry. (If beads are not fully resuspended, flick tube to dislodge pellet, vortex 2 minutes at maximum speed. Re-examine tubes and dislodge and vortex pellet until beads are resuspended.)
5. With cap hinges facing out, centrifuge tubes 16,000 x *g* for 3 minutes.
6. Place the tubes on magnetic stand for 10 minutes so beads are pulled to side of tube.

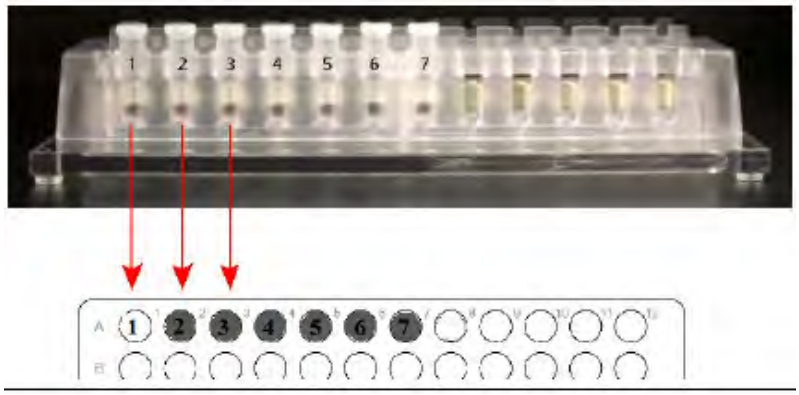
NOTE: Visually verify that bead pellet is completely pulled towards magnet with no beads present in Elution Buffer at bottom of tubes.

7. Transfer **25 µL** of eluted sample to correct well on new 96-well plate (Figure 2.14).

IMPORTANT: Ensure correct volume is pipetted as fragmentation step is sensitive to input mass.

NOTE: Eluate may appear brownish. Sometimes a brown residue is seen at pipette tip, but usually remains behind on tip when sample is pipetted out.

Figure 2.14 Purified Sample Transfer to Fresh Plate



8. Tightly seal plate, vortex 1 second in all corners and center.
9. Centrifuge 650 x *g* for 1 minute.
10. Ensure plate is tightly sealed.
11. Proceed to *Section 6. Quantitation*.

Section 6. Quantitation

This assay was optimized using a UV spectrophotometer for quantitation. OD measurement accuracy is critical. Be sure OD measurement is within instrument linear range.

Prepare Reagents, Equipment, and Consumables

Turn spectrophotometer on at least 10 minutes before use.

Place on bench top:

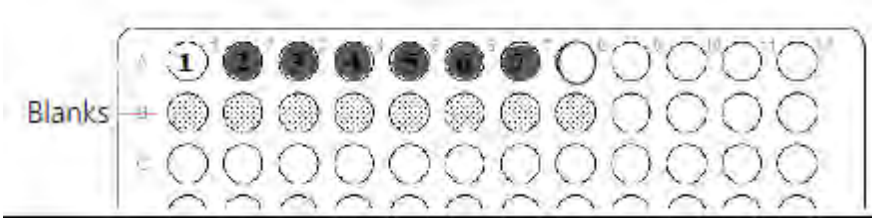
- Water, Nuclease free
- UV plate or 96-well plate
- **Optional:** conical tube or reagent reservoir

Microplate Spectrophotometer

Prepare Diluted Aliquots of Purified Samples

1. Use a multi-channel P200 pipette to add **198 µL** Nuclease-free Water to sample wells of the UV plate.
2. Make blank: Add **200 µL** Nuclease-free Water to each well of empty row (Figure 2.15).

Figure 2.15 Row for blanks



3. Use a multi-channel P20 pipette to:
   a. Transfer **2 µL** of each purified sample to the corresponding well of the UV plate.
   b. Pipet up and down 2 times so all sample is dispensed (100-fold dilution).
4. Use new seal, tightly seal plate with purified samples, and use lint-free lab wipe on adapter surface.
5. Vortex, then centrifuge 650 x *g* for 1 minute. Proceed immediately to quantitation or store at –15°C to –25°C.

IMPORTANT: If stored at –15 to –25°C the plate must be removed within 20 hours.

Quantitate Diluted Purified PCR Product

NOTE: One absorbance unit at 260 nm = 50 µg/mL (equivalent to 0.05 µg/µL) for double-stranded PCR products (for path length = 1 cm).

1. Measure OD of each sample at 260, 280, and 320 nm. Use OD_{280} and OD_{320} as controls.
2. Determine OD_{260} measurement for water blank and calculate average.
3. Determine concentration of each PCR product:
   a. Calculate one OD260 reading for every sample: OD260 = (sample OD260) – (average water blank OD260)
   b. Calculate undiluted concentration for each sample in µg/µL: OD_{260} x 0.05 µg/µL x 100
4. Determine acceptable DNA yield.

NOTE: For each sample the DNA yield must be ≥ 2.5 µg/µL. Average DNA yield for 8 or more samples must be ≥3.0 µg/µL. Do not further process any samples that do not meet this specification.

These OD ranges are based on use of conventional UV spectrophotometer plate readers and assume path length = 1 cm.

- OD_{260}/OD_{280} ratio = 1.7 to 2.1.
- OD_{320} measurement very close to zero (≤0.1).

5. Immediately go to *Section 7. Fragmentation* or store plate at –15 to –25°C for 16-20 hours.

TIP: OPTIONAL STOPPING POINT: The plate maybe stored at –15 to –25°C for up to 1 week.

Section 7. Fragmentation

Perform all additions, dilutions, and mixing on ice. Ensure all reagents reach equilibrium before use.

Precautions

- Enzyme is temperature sensitive:
  - Handle tube by cap or base *only*. Do not touch tube sides.
  - Keep at –15 to –25°C except when aspirating enzyme volume.
  - Centrifuge to ensure contents of tube are uniform.
  - Perform all steps rapidly and without interruption.
- Add enzyme to Fragmentation Master Mix last.
- Enzyme is **viscous:**
  - Pipet slowly.
  - Avoid excess solution outside pipette tip.

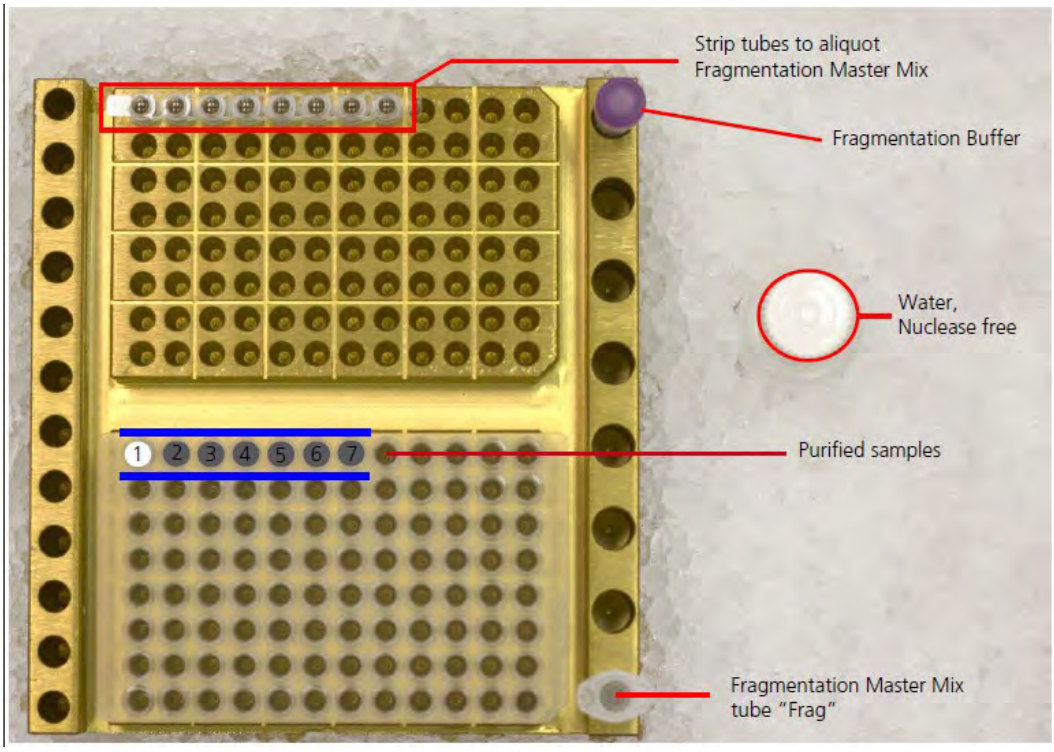
Preparation

Turn on thermal cycler to preheat lid. Leave block at RT.

Set up Work Area

1. Set the plate centrifuge to 2 to 8°C at least 15 to 20 minutes before fragmentation step and close lid.
2. Place cooling block and Nuclease-free Water on ice.

Figure 2.16 Fragmentation Setup (Fragmentation Reagent not pictured, still at –15 to –25°C)



3. Place 8-tube strip in chilled cooling block (2 to 8°C) at least 10 minutes before use.
4. Label a 1.5 mL Eppendorf tube "FRAG" and keep chilled in cooling block.
5. Cut adhesive film into strips wide enough to seal 8 or 12 strip tubes.

Thaw and Prepare Reagents

1. Thaw the plate of purified, quantitated samples at RT (≤30 minutes).
2. Ensure a tight seal and centrifuge plate 650 x *g* for 1 minute.
3. Place the plate on the lower half of cooling block on ice for at least 10 minutes. Process within 1 hour.
4. Thaw Fragmentation Buffer at RT (≤30 minutes); place on cooling block on ice.

IMPORTANT: Use within 1 hour.

5. Prepare Fragmentation Buffer:
   a. Vortex 3 times for 1 second each.
   b. Quick spin 3 seconds.
   c. Place in chilled cooling block (2 to 8°C).

Prepare Fragmentation Master Mix

Prepare and store all reagents, tubes, and cooling block on ice. Dispense within 10 minutes.Always prepare Fragmentation Master Mix according to Table 2.10 even when processing less than 24 samples.

1. Add Nuclease-free Water and Fragmentation Buffer per Table 2.10.
   a. Vortex tube 3 times for 1 second.
   b. Quick spin 3 seconds.

Table 2.10 Fragmentation Master Mix

Reagent	Volume
Water, Nuclease free	215 µL
Fragmentation Buffer	275 µL
Fragmentation Reagent	10.0 µL
Total	**500 µL**

2. Remove the Fragmentation Reagent from the freezer and immediately place in cooler chilled to –15 to –25°C.
   a. Immediately quick spin 3 seconds to bring down reagent from top of tube.
   b. Vortex 1 second.
   c. Quick spin 3 seconds.
   d. Immediately place in –15 to –25°C cooler.
3. Add Fragmentation Reagent per Table 2.10.
4. Immediately place back in –15 to –25°C cooler.
5. Vortex the Fragmentation Master Mix 3 times for 1 seconds each.
6. Quick spin 3 seconds and immediately place in cooling block.

Fragmentation

Add Fragmentation Master Mix to Samples

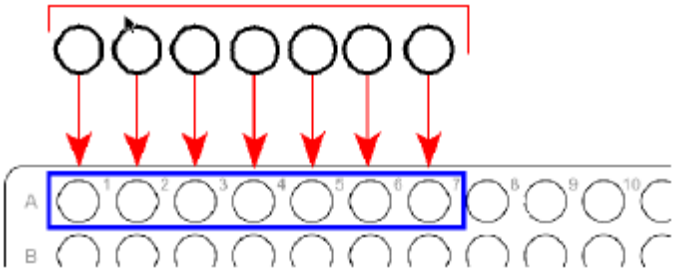
1. Quickly add **40 µL** of the Fragmentation Master Mix to each well of strip tubes placed in cooling block on ice.
2. Seal the strip tubes with adhesive film strip or strip caps.
3. Centrifuge the strip tubes and place back in cooling block on ice. Remove and discard seal.
4. Remove and discard the plate seal.
5. Use a multi-channel P20 pipette to transfer 5 µL Fragmentation Master Mix to each sample (Figure 2.17). Do not pipet up and down. Do not introduce air bubbles at bottom of tubes.

TIP: It is recommended to use a multi-channel pipette to dispense the Fragmentation Master Mix for any number of samples since Fragmentation is a time and temperature sensitive step.

Table 2.11

Sample	Volume/Sample
Purified PCR product	23 µL
Fragmentation Master Mix	5 µL
Total	**28 µL**

Figure 2.17 Addition of Fragmentation Master Mix



6. Seal the plate tightly with new seal.
7. Vortex 1 second in all corners and center.
8. Bring the sample plate to the centrifuge on cooling block in ice box (2 to 8°C).
9. Centrifuge the plate in pre-chilled centrifuge 650 x *g* for 1 minute. Quickly remove the plate from centrifuge and place in cooling block in ice box.
10. Carry the sample plate on the cooling block and immediately load the Fragmentation plate on thermal cycler with preheated lid.
11. Run the **CytoScan Optima Fragment** program.

Table 2.12 CytoScan Optima Fragment Thermal cycler Program

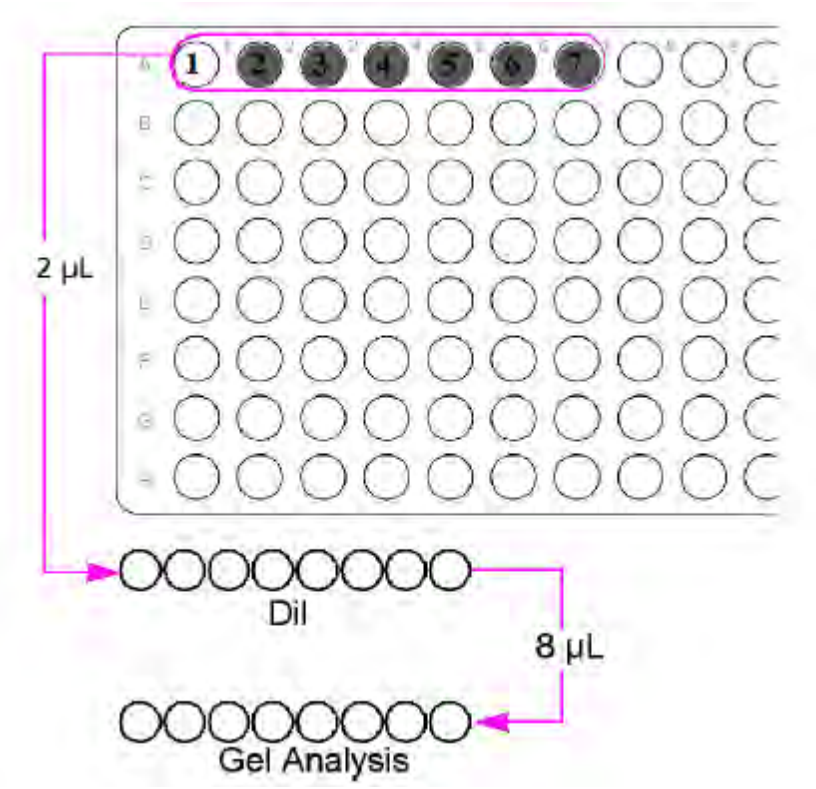
Temperature	Time
37°C	35 minutes
95°C	15 minutes
4°C	5 minutes
4°C	∞

12. When done, leave the plate in the thermal cycler. Process within 2.5 hours.
13. After removing from thermal cycler, ensure that the plate is sealed tightly and centrifuge 650 x *g* for 1 minute and transfer to chilled cooling block on ice.
14. Remove and discard any remaining Fragmentation Master Mix. Never re-use Fragmentation Master Mix.

Check Fragmentation Reaction

1. Ensure that the plate is tightly sealed; centrifuge 650 x *g* for 1 minute. Place on the lower half of the cooling block on ice.
2. Label an 8-tube strip tube "Dil".
3. Add **14 µL** Nuclease-free Water to each well of strip tube.
4. Unseal the fragmented DNA plate and discard seal.
5. Add **2 µL** of fragmented samples from plate to corresponding wells of strip tube "Dil" (Figure 2.18).

Figure 2.18 Transfer 2 µL Fragmented Product to Strip Tube



6. Seal fragmented DNA plate with new seal and keep on lower half of cooling block on ice.
7. Label a fresh 8-or 12-well strip tube "Gel Analysis". Add 12 µL of diluted loading buffer to each well.
   a. Add 8 µL diluted fragmented product from Step 6 to 12 µL diluted loading buffer in Step 8.
   b. Pulse vortex.
   c. Centrifuge 650 x *g* for 1 minute.
8. Run samples on 4% agarose gel to resolve bands.
9. Inspect gel and follow *Section 11. In-Process Quality Control*. The majority of fragment size distribution must be **25 to 125 bp.**
10. Proceed immediately to *Section 8. Labeling*.

TIP: Optional Stopping point: If not proceeding to the next step the fragmented DNA plate maybe held at –15 to –20°C for up to 60 hours.

Section 8. Labeling

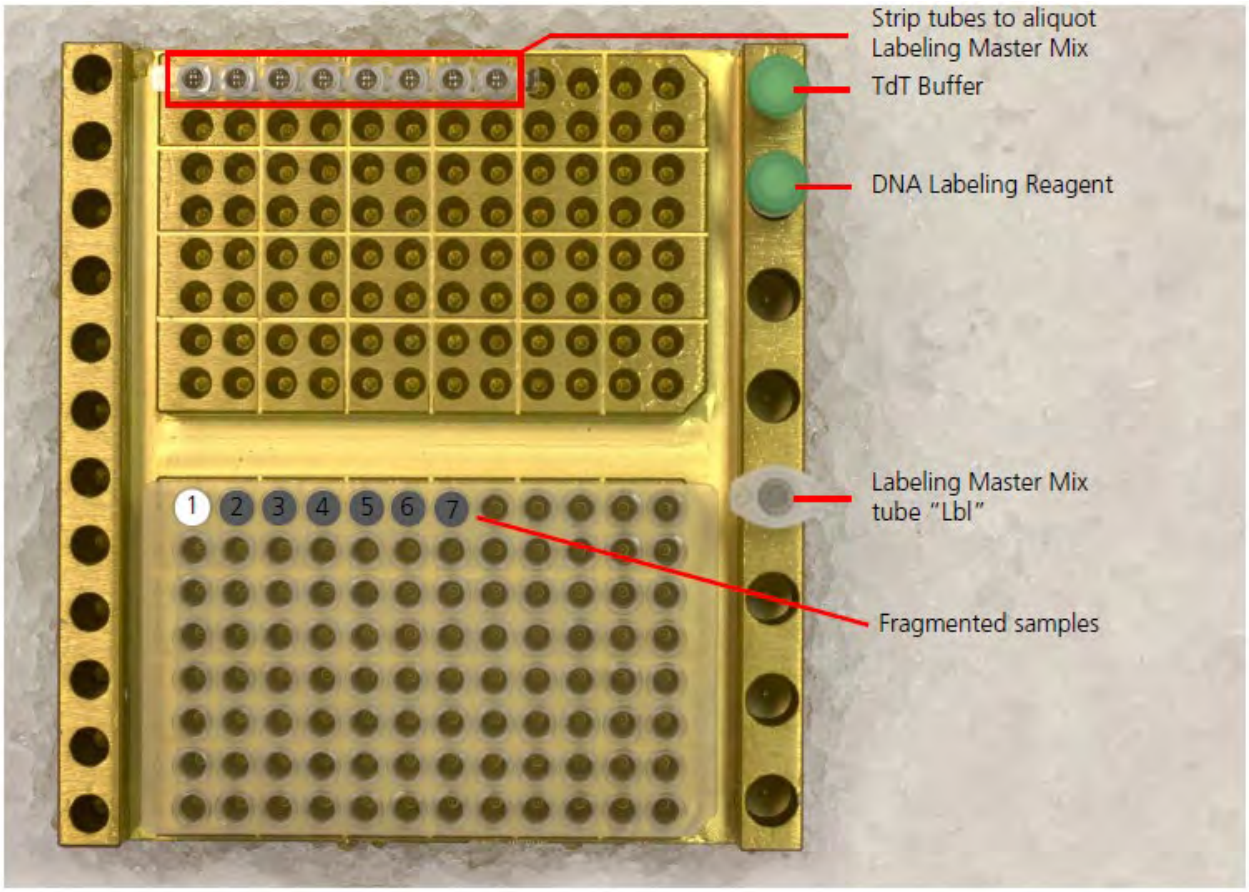
Preparation

Turn on the thermal cycler to preheat lid. Leave block at RT.

Set up Work Area

1. Place the cooling block on ice.
2. Place a 8-tube strip in upper half of cooling block on ice.
3. Label a 1.5 mL microfuge tube "LBL" and place in cooling block.

Figure 2.19 Labeling Setup (TdT Enzyme at –15 to –20°C and hence not shown)



4. Cut adhesive film into strips wide enough to seal 8 or 12 strip tubes.

Thaw and Prepare Reagents

1. Thaw at RT (≤30 minutes), then immediately place on ice and use within 1 hour:
   - TdT Buffer
   - DNA Labeling Reagent
2. Prepare TdT Buffer and DNA Labeling Reagent:
   a. Vortex both 3 times for 1 second each.
   b. Quick spin 3 seconds; place in cooling block.

Prepare Labeling Master Mix

Prepare and store all reagents, tubes, and cooling block on ice. Dispense within 10 minutes.

1. Add to the 1.5 mL tube on ice per Table 2.13:
   - TdT Buffer
   - DNA Labeling Reagent

Table 2.13 Labeling Master Mix (≥8 samples, 20% overage)

Reagent	1 Sample	8 Samples	16 Samples	24 Samples
TdT Buffer	7 µL	67.2 µL	134.4 µL	201.6 µL
DNA Labeling Reagent	1 µL	9.6 µL	19.2 µL	28.8 µL
TdT Enzyme	1.8 µL	16.8 µL	33.6 µL	50.4 µL
Total	**9.8 µL**	**93.6 µL**	**187.2 µL**	**280.8 µL**

2. Vortex 3 times for 1 second each.
3. Quick spin 3 seconds.
4. Remove the TdT Enzyme from freezer and immediately place it in a cooler pre-chilled to –15 to –25°C.
5. Mix TdT Enzyme.
   a. Quick spin 1 second.
   b. Vortex 1 second.
   c. Quick spin 3 seconds, place in cooling block.
6. Immediately place in –15 to –25°C cooler.
7. Add the TdT Enzyme to the Labeling Master Mix. Place in –15 to –25°C cooler.
8. Vortex Labeling Master Mix 3 times for 1 second each.
9. Quick spin 3 seconds.

Labeling

Add Labeling Master Mix

Keep samples in cooling block and all tubes on ice when making additions.

1. Add Labeling Master Mix to pre-chilled strip tubes on cooling block on ice.
2. Seal strip with adhesive film strip or strip caps and quick spin.
3. Place back in cooling block, remove and discard seal.
4. Remove and discard the plate seal.
5. Use a P20 multi-channel pipette to add **9.8 µL** Labeling Master Mix to each sample.

Table 2.14

Sample	Volume/Sample
Fragmented DNA (less 2.0 µL for gel analysis)	26 µL
Labeling Master Mix	9.8 µL
Total	**38.5 µL**

6. Use a new seal and tightly seal the plate.
7. Vortex 1 second each in all corners and center.
8. Centrifuge 650 x *g* for 1 minute.
9. Place the labeling plate in thermal cycler and run **CytoScan Optima Label** program.

Table 2.15 CytoScan Optima Label Thermal Cycler Program

Temperature	Time
37°C	4 hr
95°C	15 minutes
4°C	5 minutes
4°C	∞

10. When program finishes, leave the plate in the thermal cycler or transfer to a chilled cooling block on ice.
11. Ensure that the plate is sealed tightly and centrifuge 650 x *g* for 1 minute.

NOTE: If not proceeding to the next step, the plate maybe held at –15 to –20°C for up to 1 week.

Section 9. Target Hybridization via AGCC

Important Information About This Stage

To help ensure the best results, carefully read the information below before you begin this stage of the protocol. Since this user guide is intended as an assay protocol manual, there is no specific section on all of the various features and workflows available in the Command Console software. If you would like to learn more about Command Console, please refer to the *GeneChip™ Command Console™ 3.2 User Guide* (Pub. No. 702569).

Prepare the Equipment

Turn On the Thermal Cycler

Power on the thermal cycler to preheat the lid. **Leave the block at room temperature.**

Preheat the Hybridization Oven 645

NOTE: Confirm that the Hybridization Oven 645 is calibrated. The hybridization oven should be serviced at least once per year to ensure operation within specification.

To preheat the hybridization ovens:

1. Turn on the oven at least 1 hour before hybridization with the temperature set to 50°C.
2. Set the rpm to 60.
3. Turn the rotation on and allow to preheat for 1 hr before loading arrays.

Prepare the Arrays and Create a Batch Registration File

NOTE: Remove array from storage 1 hour before hybridization to equilibrate to RT. Do not remove arrays from pouch. During this time you may scan the barcode which will be used in batch registration.

To prepare the arrays:

1. Unwrap the arrays and place on the bench top, septa side up.
2. Mark the front and back of each array with a designation that will identify which sample is loaded onto each array (Figure 2.21).

Create a Batch Registration File

To register a new sample using AGCC:

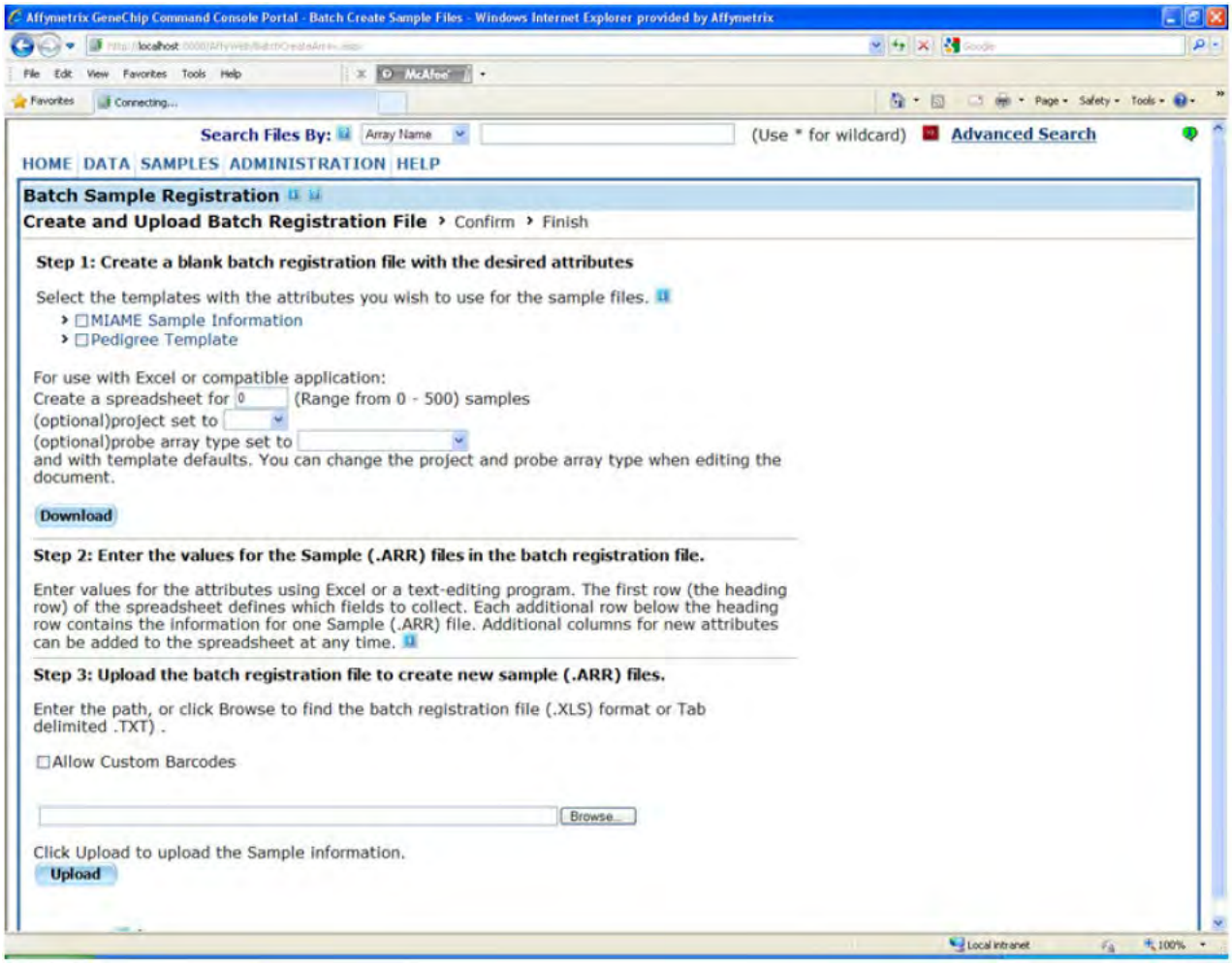
1. From the Command Console, launch the AGCC Portal.

> **IMPORTANT:** Confirm that you are running AGCC v.3.2.2 or higher. If not, please update your version of AGCC to v.3.2.2 or latest available.

2. Under the Samples tab, select Batch Registration.

   The Batch Registration window opens (Figure 2.20).

Figure 2.20 Batch Registration Window



3. Within Step 1:
   a. Enter the number of samples for which a spreadsheet needs to be created under Create a Spreadsheet for.
   b. Select Default from the Project Set to drop-down list.
   c. Select CytoScanOptima_Array from the Probe Array type set to drop-down list:
   d. Click Download.

      An Excel spreadsheet opens.

4. Within Step 2:
   a. Name the experiment file using the following convention: "SampleName_PlateCoordinate_ExperimentDescriptionString_ArrayType_OperatorInitials_yyyymmdd".
   b. The sample file name and the 'Array name' would be identical.
   c. Scan the corresponding barcodes for each Sample name.
   d. Save the Excel file in "Excel 97-2003 workbook" format.
5. Within Step 3:
   a. Browse to the location of the Batch registration file that was saved.
   b. Upload the Batch registration file by clicking the tab to create new sample (.ARR) files. A new window opens.
6. Click **Save** to save the new sample files.

Prepare the Arrays

1. Place the arrays on a clean bench top area designated for hybridization.
2. Insert a 200 µL pipette tip into the upper right septum of each array.
3. Paste two 1/2" Tough-Spots on the top edge of the array for later use (Figure 2.21).

IMPORTANT: To ensure that the data collected during scanning is associated with the correct sample, mark each array in a meaningful way. It is critical that you know which sample is loaded onto each array.

Figure 2.21 Arrays Prepared for Sample Loading

Prepare the Reagents and Consumables

Set Up the Work Area

To set up the work area:

1. Place a double cooling block on ice (Figure 2.22).
2. Place a reagent reservoir on the upper half of the cooling block on ice.
3. Label the 15 mL centrifuge tube as Hyb Master Mix, and place on the ice.

Prepare the Samples

1. If the labeled samples from the previous stage were frozen, allow them to thaw on the bench top to room temperature and spin down at 2000 rpm for 1 minute.
2. Immediately place the plate in the lower half of the cooling block on ice.

Thaw and Prepare the Reagents

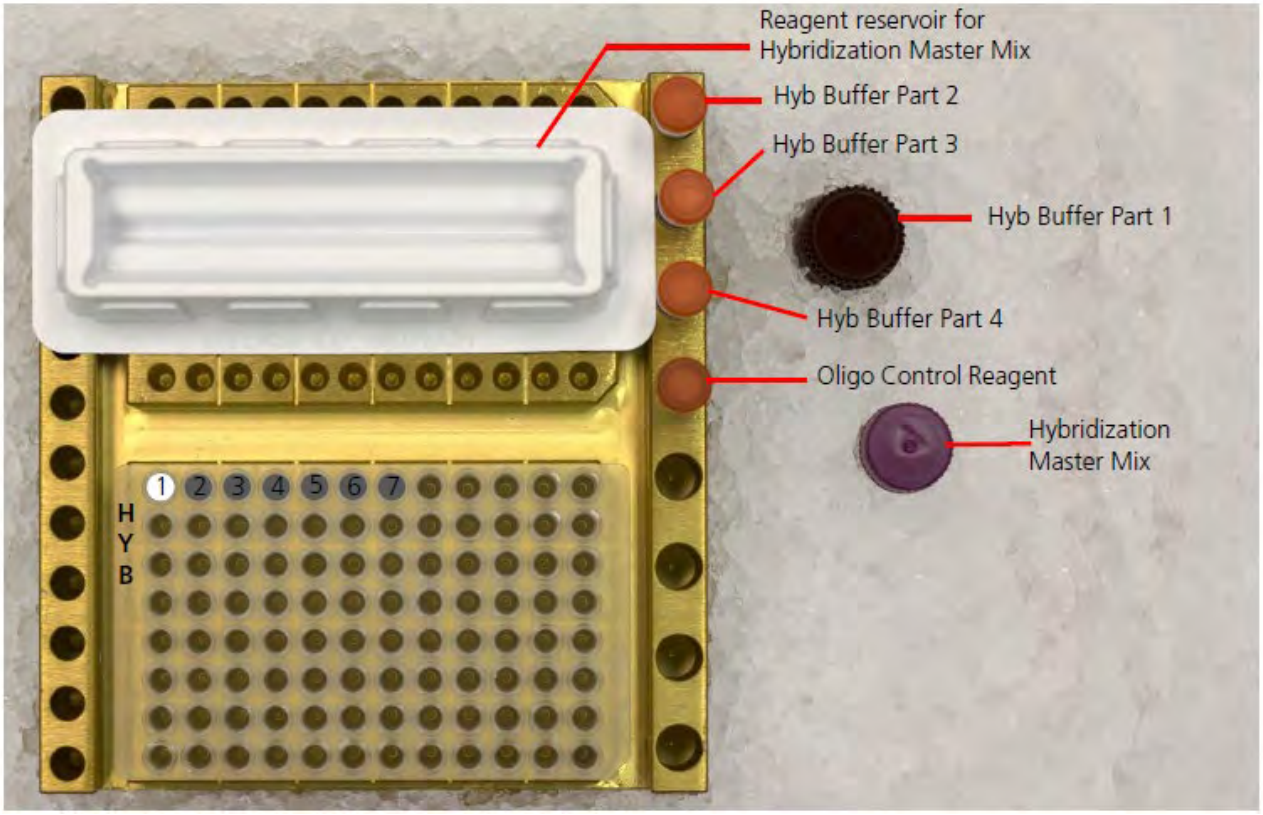
Thaw the following reagents at room temperature. Immediately place on cooling block on ice when thawed.

- Hyb Buffer Part 1
- Hyb Buffer Part 2
- Hyb Buffer Part 3
- Hyb Buffer Part 4
- Oligo Control Reagent

Prepare the reagents as follows:

1. Vortex each reagent at high speed 3 times, 1 second each time.
2. Pulse spin for 3 seconds, then place in the cooling block as shown in Figure 2.22.
3. Quick spin 3 seconds, then place in cooling block.

Figure 2.22 Hybridization Setup



Prepare the Hybridization Master Mix

1. To the 15 mL Hyb Master Mix centrifuge tube on ice, add the appropriate volume of each reagent in the order shown in Table 2.16.

IMPORTANT: Some of the Hyb Buffer components are viscous; carefully pipet and dispense when preparing the master mix.

2. Mix well by vortexing the master mix at high speed 3 times, 3 seconds each time (until the mixture is homogeneous).

Table 2.16 Hybridization Master Mix (≥8 Samples, 20% overage)

Reagent	1 Sample	8 Samples	16 Samples	24 Samples
Hyb Buffer Part 1	82.5 µL	792 µL	1584 µL	2376.0 µL
Hyb Buffer Part 2	7.5 µL	72 µL	144.0 µL	216.0 µL
Hyb Buffer Part 3	3.5 µL	33.6 µL	67.2 µL	100.8 µL
Hyb Buffer Part 4	0.5 µL	4.8 µL	9.6 µL	14.4 µL
Oligo Control Reagent	1.0 µL	9.6 µL	19.2 µL	28.8 µL
Total	**95 µL**	**912 µL**	**1824 µL**	**2736 µL**

IMPORTANT: Make sure the Hybridization Master Mix is adequately vortexed.

Add Hybridization Master Mix and Denature

NOTE: When working with more than 8 samples, we strongly recommend transferring the master mix to a reservoir and dispensing the master mix from the reservoir into the samples using a multi-channel pipette.

To add Hybridization Master Mix and denature the samples:

1. Remove and discard the plate seal.
2. Pour the Hybridization Master Mix into a reagent reservoir placed on the upper half of the cooling block on ice. Use a multi-channel pipette to add 95 µL of Hybridization mix to the samples.

IMPORTANT: The Hybridization Master Mix is viscous; pipette carefully when dispensing to samples.

3. Tightly seal the plate with a new seal and carefully check to confirm that the plate is well sealed.

IMPORTANT: Ensure that the plate is vortexed to mix sample and hybridization buffer well.

4. Vortex the plate at high speed for 1 second each in all corners and in the center. REPEAT vortexing to ensure that the plate is well mixed, then spin down at 650 x *g* for 1 minute.

TIP: Optional stopping point. Seal the plate tightly and store with samples in Hybridization Master Mix at –15 to –25°C for up to 1 week. When ready to use the frozen plate, follow the instruction in the note below.

NOTE: When ready to use a previously frozen plate:

1. **Thaw at RT.**
2. **Ensure plate is sealed tightly.**
3. **Centrifuge 650 x *g* for 1 minute.**
4. **Remove and replace seal with new seal.**
5. **Seal tightly.**
6. **Vortex to ensure complete mixing.**
7. **Centrifuge 650x *g* for 1 minute.**
8. **Place plate on cooling block on ice.**

5. Place the plate onto the pre-heated thermal cycler and run the CytoScan Optima Hyb program.

Table 2.17 CytoScan Optima Hyb Thermal Cycler Program

Temperature	Time
95°C	10 minutes
49°C	3 minutes
49°C	∞

Load the Samples onto Arrays

To load the samples onto arrays:

1. When the thermal cycler reaches 49°C, leave the samples at 49°C for at least three minutes, and then open the lid.

IMPORTANT: Load only 4 to 6 arrays at a time. Remove the seal from the hybridization plate for only 4 to 6 samples at a time.

2. If you are hybridizing more than eight samples, cut and remove the seal from 4 to 6 samples at a time only.

   Leave the remaining wells covered. Keeping these wells covered helps prevent cross-contamination and evaporation.

Figure 2.23 Loading Samples onto Arrays

IMPORTANT: The hybridization mix is very viscous. Pipet slowly to ensure that all of the volume is loaded into the chip.

3. Using a P200 pipette, remove 100 µL of the first sample and immediately inject it into an array.
4. Cover the septa on the array with the 1/2" Tough-Spots that were previously placed on the top edge of the array (Figure 2.23).

   Press firmly to ensure a tight seal to prevent evaporation and leakage.
5. When 4 to 6 arrays are loaded and the septa are covered:
   a. Load the arrays into an oven tray evenly spaced.
   b. Immediately place the tray into the hybridization oven.

      Do not allow loaded arrays to sit at room temperature for more than approximately 1 minute. Ensure that the oven is balanced as the trays are loaded, and ensure that the trays are rotating at 60 rpm at all times.
6. Repeat this process until all samples are loaded onto arrays and are placed in the hybridization oven.

   All samples should be loaded within 30 minutes.
7. Allow the arrays to rotate at 50°C, 60 rpm for 16 to 18 hours.

IMPORTANT: Allow the arrays to rotate in the hybridization oven for 16 to 18 hrs at 50°C and 60 rpm. This temperature is optimized for this product, and should be stringently followed.

Section 10. Washing, Staining, and Scanning Arrays

Prime the Fluidics Station

Priming ensures the lines of the fluidics station are filled with the appropriate buffers and the fluidics station is ready to run fluidics station protocols.

Priming should be done:

- When the fluidics station is first started
- When wash solutions are changed
- Before washing, if a shutdown has been performed
- If the LCD window instructs the user to prime

The Fluidics Station 450 is used to wash and stain the arrays; it is operated using AGCC software.

To prime the Fluidics Station:

1. Turn on the Fluidics Station.
2. Prime the Fluidics Station.
   - From Command Console application, start the 'Launcher'.
   - From the Launcher, open 'AGCC Fluidics Control' application.
   - From the AGCC Fluidics Control panel, select 'PRIME_450' script for the specific fluidics stations and the modules.
   - Intake buffer reservoir A: use Wash A.
   - Intake buffer reservoir B: use Wash B.
3. To initiate the fluidics script, click the "Run" icon for each module or click the "Run All" icon, for all the selected stations and modules.

Washing and Staining Arrays

1. Briefly vortex the stain bottles before aliquoting the reagents.
2. Aliquot the following reagents into 1.5 mL microfuge tubes for each array:
   a. Aliquot 500 µL Stain Buffer 1 into 1.5 mL microfuge tubes (use amber color tubes as Stain Buffer 1 is light sensitive).
   b. Aliquot 500 µL Stain Buffer 2 into 1.5 mL microfuge tubes (clear/natural tubes).
   c. Aliquot 800 µL Array Holding Buffer into 1.5 mL microfuge tubes (blue tubes).
3. Select **CytoScan_Optima_Array_450** from the AGCC Fluidics Control Panel.
4. Start the protocol and follow the instructions in the LCD on the Fluidics Station.

   If you are unfamiliar with inserting and removing arrays from the fluidics station modules, refer to the appropriate Fluidics Station User Guide or Quick Reference Card (P/N 08-0093 for the Fluidics Station 450).

   Eject the wash block to avoid sensor time out.
5. Remove any previously loaded empty vials.
6. When prompted to "Load vials 1-2-3":
   a. Place one vial containing 500 µL Stain Buffer 1 in position 1.
   b. Place one vial containing 500 µL Stain Buffer 2 in position 2.
   c. Place one vial containing 800 µL Array Holding Buffer in position 3.
7. After 16 to 18 hrs of hybridization, remove no more than 6 arrays at a time from the oven. Remove the Tough-Spots from the arrays.

IMPORTANT: Once the arrays are removed from the hybridization oven, quickly load them onto the Fluidics Station. Delays during this step will impact data quality.

8. Immediately insert the arrays into the designated modules of the fluidics station while the cartridge lever is in the Down or Eject position and engage the wash block.
9. Press down on the needle lever to snap needles into position and to start the run.

   The fluidics protocol begins. The Fluidics Station dialog box at the workstation terminal and the

   LCD window display the status of the washing and staining steps.
10. When the wash and stain procedure is completed, remove the arrays from the fluidics station by first pressing down the cartridge lever to the Eject position.
11. Check the array window for bubbles or air pockets. If air bubbles are present, return the array to the fluidics station. Follow the instructions on the LCD panel of the fluidics station. Pull the lever up and load to remove bubbles.
12. If air bubbles are still present after repeating the above process a few times, use the manual process.
    a. Insert a 200 µL pipette tip into the upper right septum of the array.
    b. Using a pipette, remove half of the solution.
    c. Manually fill the array with Array Holding Buffer.
13. If the array has no bubble, it is ready for scanning. Proceed to *Scanning Arrays*.

    If the arrays cannot be scanned promptly, store them at 4°C in the dark until ready for scanning. Scan must be performed within 24 hr.

14. Pull up on the cartridge lever to engage wash block. Remove the microcentrifuge vials containing stain and replace with three empty vials as prompted.
15. When washing and staining are complete, shut down the fluidics station following the procedure.

Scanning Arrays

The GeneChip Scanner 3000 7G is controlled by AGCC software.

Prepare the Scanner

Turn on the scanner at least 10 minutes before use.

WARNING: The scanner uses a laser and is equipped with a safety interlock system. Defeating the interlock system may result in exposure to hazardous laser light.

Read and be familiar with the operation of the scanner before attempting to scan an array. Refer to the *GeneChip™ Scanner 3000 Quick Reference Card* (P/N 08-0075).

Prepare Arrays for Scanning

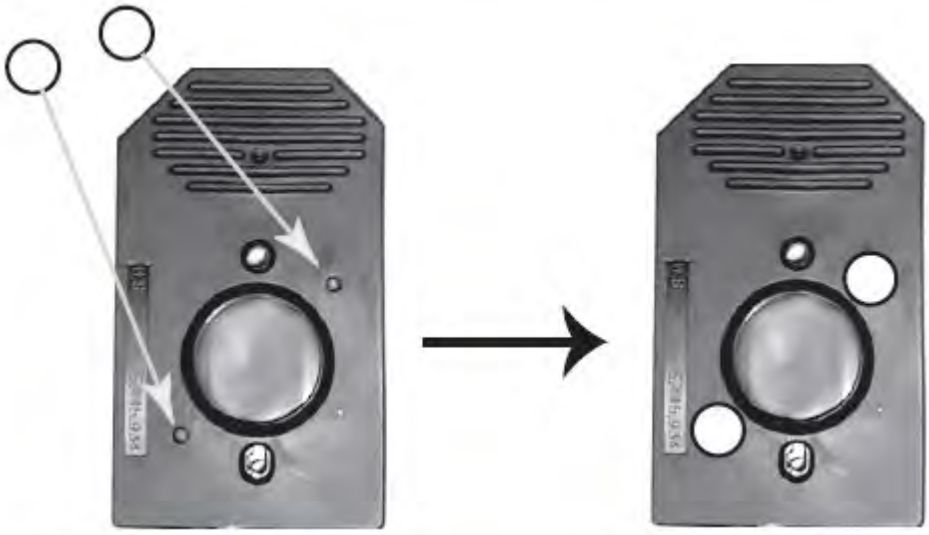
To prepare arrays for scanning:

1. If the arrays were stored at 4°C, allow them to warm to room temperature before scanning.
2. If necessary, clean the glass surface of the array with a non-abrasive towel or tissue before scanning.

   Do not use alcohol to clean the glass surface.
3. On the back of the array cartridge, clean excess fluid from around the septa.
4. Carefully cover both septa with Tough-Spots (Figure 2.24).

   Press to ensure the spots remain flat. If the spots do not apply smoothly (e.g., if you see bumps, bubbles, tears or curled edges) do not attempt to smooth out the spot. Remove the spot and apply a new spot.

Figure 2.24 Applying Tough-Spots™ to Arrays



Scanning the Array

NOTE: Customers using the Autoloader should refer to the Autoloader User Guide.

To scan arrays:

1. Open the 'AGCC Scan Control' application from the 'Launcher'.
2. Load the arrays onto the Autoloader of the scanner.
3. Once all the arrays are loaded, click the **Start** icon to initiate the scan.
4. Select the check box **arrays in carousel positions 1-4 at room temperature**. If the arrays are not at room temperature, do not select this option. The scanner will wait 10 minutes before scanning begins to allow the arrays to reach room temperature.

   Only one scan per array is required. Pixel resolution and wavelength are preset and cannot be changed.

WARNING: The door is locked while the instrument is scanning. Do not attempt to open the door manually.

Adding Arrays During an Autoloader Run

To add arrays while an AutoLoader run is in progress:

1. Click the **Add Chips** icon  .

   The GeneChip Scanner message appears.

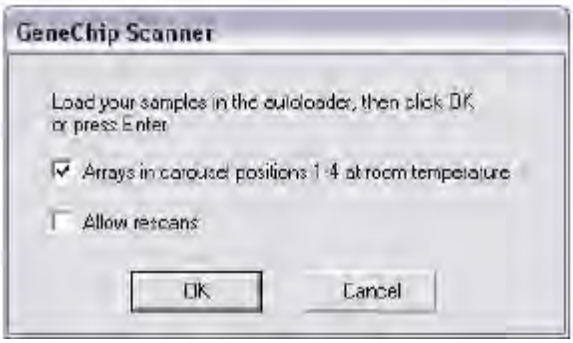
Figure 2.25 GeneChip Scanner Message

2. Click Add after Scan.

IMPORTANT: Do not use the Add Now feature. Use only the Add after Scan feature when working with CytoScan™ Arrays.

3. When the status on the scanner reads **Autoloader Door Unlocked**, open the scanner and add the arrays.
4. Close the scanner.
5. When the following message is displayed, click **OK**.

Figure 2.26 GeneChip Scanner Message



6. After you click **OK**, click the **Resume** icon.
7. If any arrays in the carousel are to be rescanned, select the check box **Allow rescans**.

Shutting Down the Fluidics Station

To shut down the Fluidics Station:

1. Gently lift up the cartridge lever to engage (close) the washblock.

   After removing an array from the holder, the LCD window displays the message **ENGAGE WASHBLOCK**. The instrument automatically performs a Cleanout procedure. The LCD window indicates the progress of this procedure.
2. When **REMOVE VIALS** is displayed in the LCD, remove the vials.
3. The **REMOVE VIALS** message indicates the cleanout procedure is complete.
4. If no other processing is to be performed, place the wash lines into a bottle filled with deionized water.
5. Using AGCC, choose the **Shutdown_450** protocol for all modules.
6. Run the protocol for all modules.

   The Shutdown protocol is critical to instrument reliability. Refer to the instrument User Guide for more information.
7. When the protocol is complete, turn the instrument off.
8. Empty the waste bottle.

IMPORTANT: To maintain the cleanliness of the fluidics station and obtain the highest quality image and data possible, a weekly bleach protocol is highly recommended. Follow the procedure outlined in Appendix E, *Fluidics Station Care and Maintenance* for weekly maintenance of the fluidics stations.

Section 11. In-Process Quality Control

IMPORTANT: The "In-Process Quality Control" is a critical in-process step and must include PCR and fragmentation gels, and purified PCR DNA yield.

Use strip tubes or 96-well plates.

PCR Product

1. Prepare the loading buffer dilution.
2. From the first row, add:

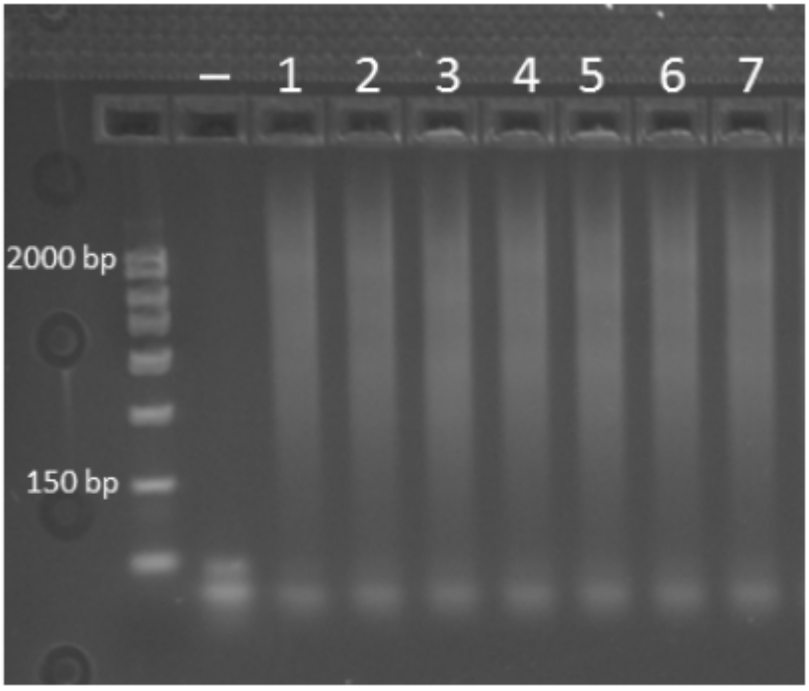
a. **3 µL** PCR product

   to

b. **17 µL** diluted loading buffer (total = 20 µL)

3. Vortex.
4. Centrifuge 650 x *g* for 1 minute.
5. Load onto gel.
6. Prepare the PCR marker dilutions.
7. Follow manufacturer's instructions to run gel that meets requirements (Figure 2.27 and Figure 2.28).

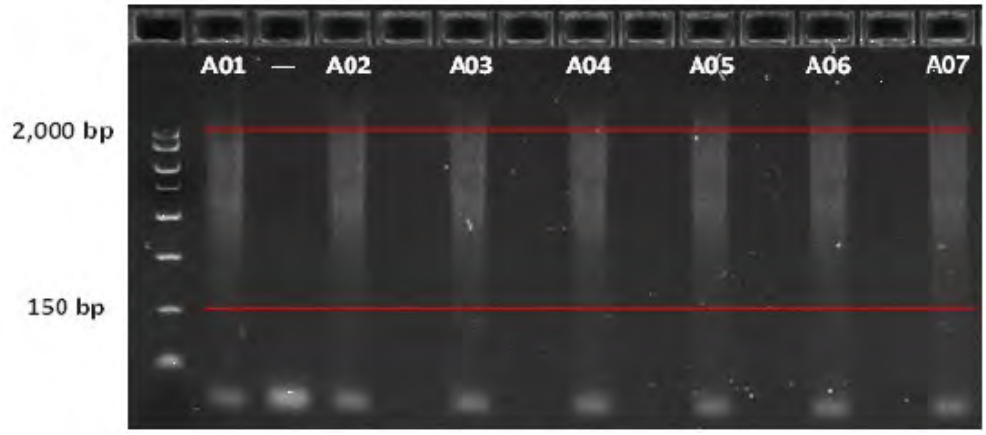
Figure 2.27 Example PCR gel image on 2% gel with negative control.



Requirements – PCR Product Gel Quality Control Interpretation

- Target distribution of smear covers **150 to 2,200 bp**
- Higher intensities at **300 to 2,200 bp**

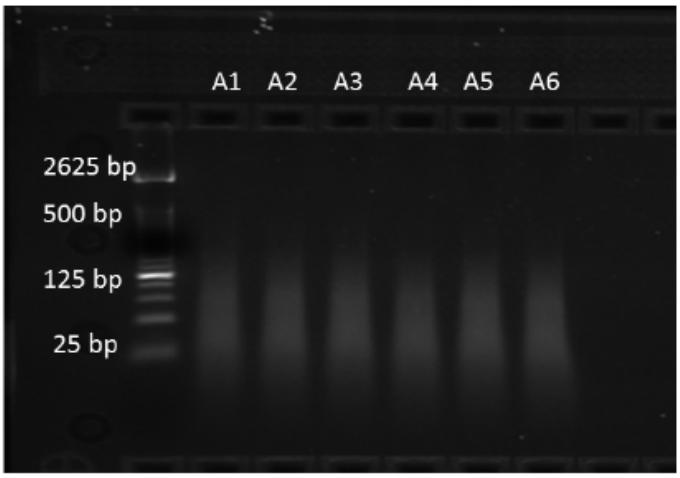
Figure 2.28 PCR size range with positive and negative control.



Fragmented Product

1. Prepare the loading buffer dilution.
2. Add:
   a. **2 µL** fragmented product
      to
   b. **14 µL** water (1:8 dilution)
3. Add:
   a. **8 µL** diluted fragmented product to
   b. **12 µL** diluted loading buffer
4. Pulse vortex.
5. Centrifuge 650 x *g* for 1 minute.
6. Load onto gel.
7. Prepare DNA marker dilutions.
8. Follow manufacturer's instructions and run on 4% agarose gel to meet requirements (Figure 2.29 and Figure 2.30).

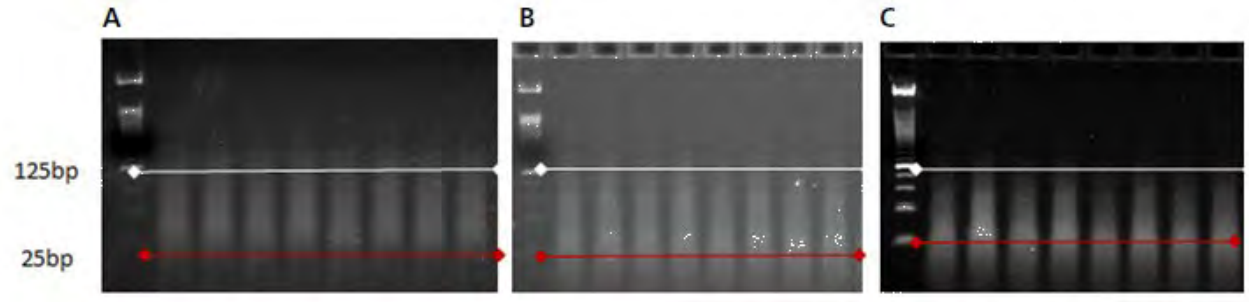
Figure 2.29 Example Fragmentation Gel Image on 4% Gel



Requirements for Fragmentation Product Gel Quality Control Interpretation

- Target distribution of smear covers **25 to 125 bp**
- High intensity at **25 to 125 bp**

Figure 2.30 Examples of Good Fragmentation Gels: Passing Performance Besides Variability in Smear Patterns



The three gels in Figure 2.31 have the majority of high intensity bands above 125 bp (left); at 125 bp (middle); or below 25 bp (right).

Figure 2.31 Examples of Failing Fragmentation Gels

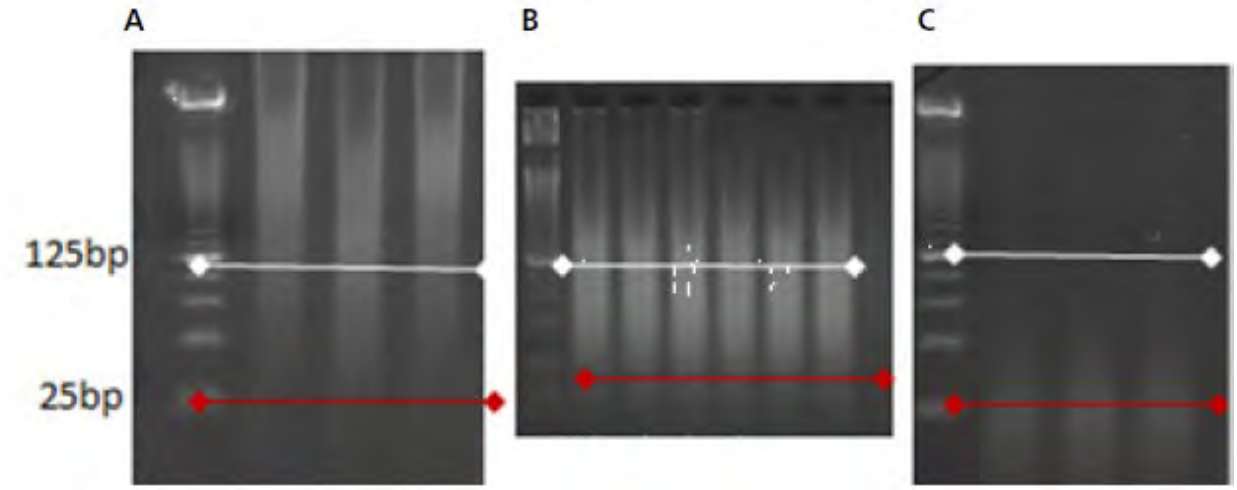


Table 2.18

If majority of target distribution is...	Then sample is...
At 125 bp or extends to 2652 bp	Under fragmented (Figure 2.31, A and B)
Below 25 bp	Over fragmented (Figure 2.31, C)

Section 12. Array Quality Control Threshold

After processing the arrays on the Chromosome Analysis Suite, the following thresholds are applicable:

- MAPD ≤0.29
- SNPQC ≥8.5

For samples not passing these QC threshold, please refer to the troubleshooting section in this user guide (Appendix D). Other metrics can be used as guidance for sample performance. For more information on how these or other metrics are calculated please refer to the *Chromosome Analysis Suite (ChAS) User Guide* (Pub. No. 702943).

Appendix A Workflow and Practices

Workflow

The 3.5-day workflow for one operator and 8 to 24 samples is given in Figure A.1. The 2.5-day workflow for one operator and 8 to 24 samples is given in Figure A.2.

Figure A.1 3.5-day Workflow

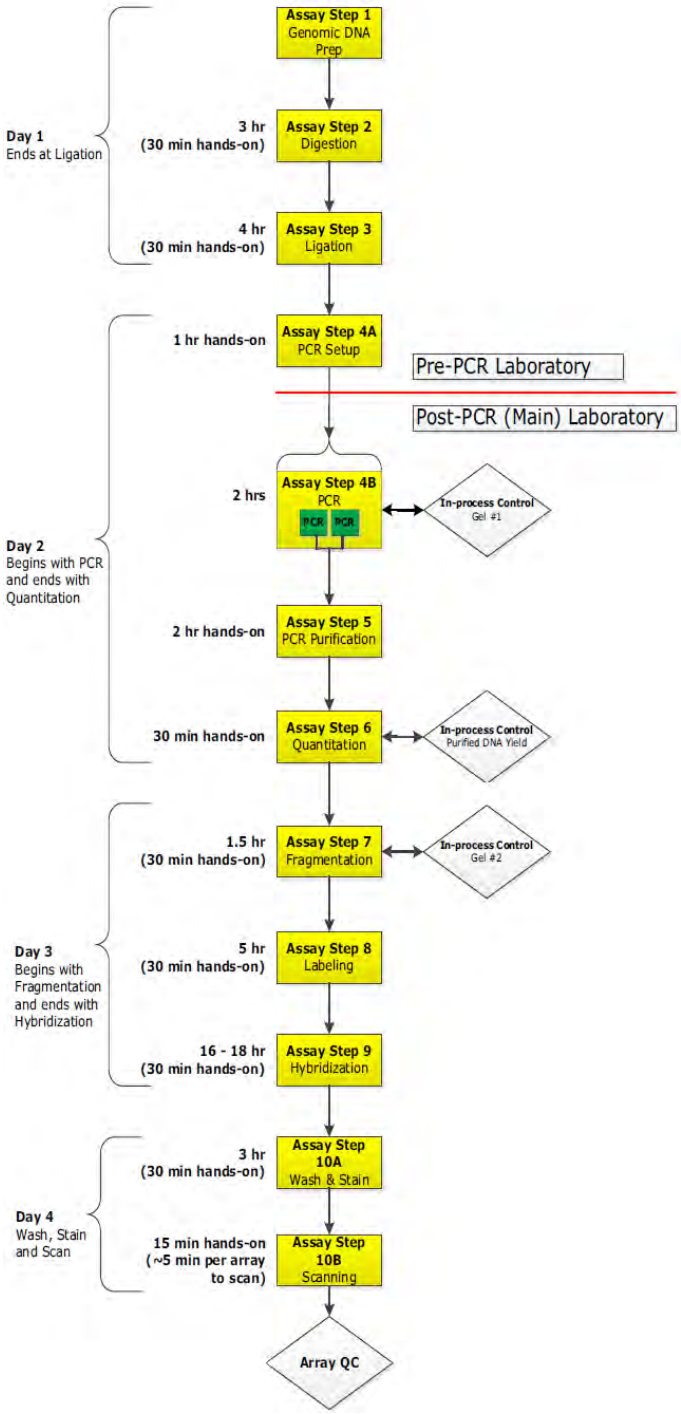
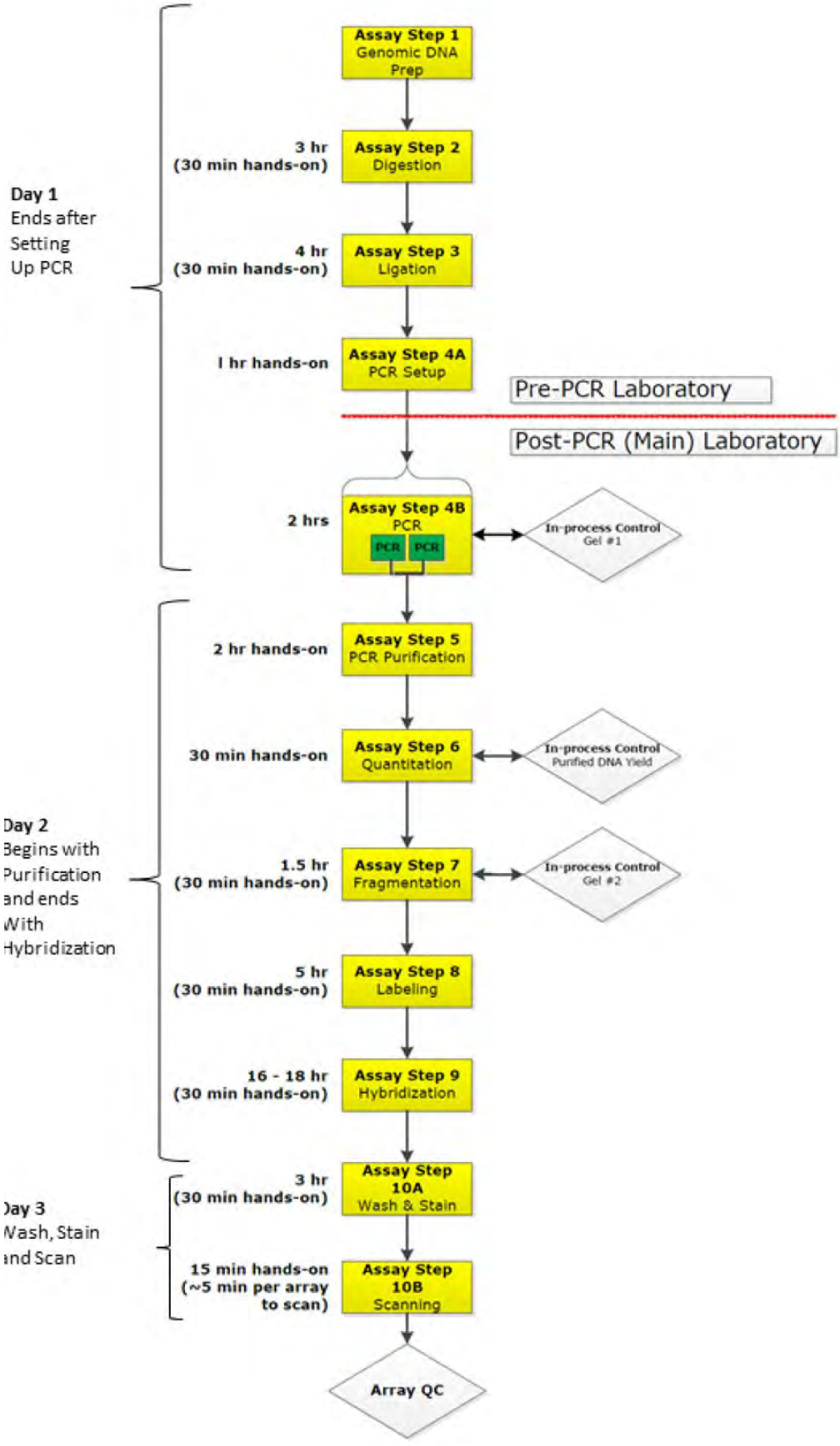


Figure A.2 2.5-day Workflow



Specific Laboratory Practices

Seals

Avoid cross-contamination.

Plate

- Seal plate with adhesive film applicator for each well and plate edge surface.
- Ensure tight seals all over the plate and well edges
- Use PCR-certified clear adhesive films for 96-well plates
  - Use only once and never reuse

NOTE: The seal on the plate may become loose due to the high temperature in the thermal cycler.

- When removing plate from freezer:
  1. Thaw plate at RT
  2. Ensure tight seal
  3. Centrifuge 650 x *g* for 1 minute
  4. Place plate on pre-chilled cooling block (2 to 8°C)

Strip Tubes

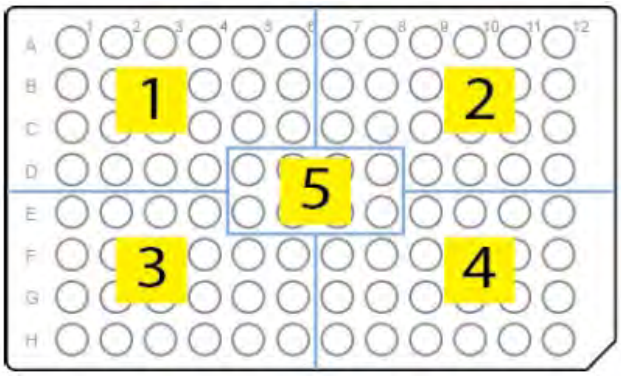
- Cut adhesive film into strips wide enough to seal 8 or 12 strip tubes
- For master mix, use adhesive film strips or strip caps to seal before centrifuging

Vortex

Always vortex on maximum setting:

- **Enzyme:** Vortex 1 to 3 seconds once
- **Reagents:** Vortex 3 times for 1 to 3 seconds each
- **Plates:** Vortex for 1 to 3 seconds in all corners and in center (Figure A.3), repeat once for high volume reagents: PCR (100 µL), hybridization-ready samples (131 µL)
- **Master Mix Tubes:** Vortex 3 times before and after adding enzyme, 1 to 3 seconds each
- During purification, use vortex with foam tube adapter for 1.5 mL microfuge tubes on maximum vortex setting

Figure A.3 Plate vortexing



Centrifuge

Always centrifuge at maximum setting unless otherwise noted.

- Enzymes: for 1 second
- Reagents: for 1 to 3 seconds
- Plates: 650g for 1 minute
- Master Mix tubes and strips: for 1 to 3 seconds.
- QC strips: for 1 to 3 seconds.

PCR Practices

Samples

IMPORTANT: Dilute ligated DNA with chilled Nuclease-free Water before PCR.

- PCR volume = 100 µL
- Keep PCR plates at 2 to 8°C (ice or chilled cooling block) before purification
- Avoid cross-contamination

PCR Product Purification

PCR Pooling

- Avoid cross-contamination
- Process tubes in same order as samples being pooled
- Change pipette tips after each sample transfer
- Thoroughly mix purification bead solution by shaking and inverting bottle several times so mixture appears homogeneous. **Do not** vortex bead bottle.

Pellet Washing

- Use molecular biology grade ethanol to prepare Purification Wash Buffer
- Allow supernatant to clarify before removal
- Replace vortex foam tube adapters at signs of wear
- Always use:
  - Fresh tips
  - Multi-channel pipette to remove the initial Purification Wash Buffer
  - Single-channel pipette to remove:
    - Residual purification buffer
    - Supernatant
- Remove stand from magnetic rack when drying pellets. Dry for exactly 7 minutes; do not under-or over-dry.

Elution

- Ensure pellets are completely suspended in elution buffer before centrifuging for 3 minutes.
- Final elution step. If beads do not form tight pellet within 10 minutes, verify rack is placed correctly on magnetic stand and extend time until eluant is clear and all beads are pulled towards magnet.

Appendix B Thermal Cycler Programs

Before you begin processing samples, enter and save these programs into the appropriate thermal cycler(s).

Pre-and Post-PCR Programs

Table B.1 Pre-PCR Clean Room

Number of Thermal Cyclers Required	Program Name
1	CytoScan Optima Digest
	CytoScan Optima Ligate

Table B.2 Post-PCR Room

Number of Thermal Cyclers	Required Program Name
1	CytoScan Optima PCR
	CytoScan Optima Fragment
	CytoScan Optima Label
	CytoScan Optima Hyb

Thermal Cycler Programs

CytoScan Optima Digest

Table B.3 CytoScan Optima Digest Thermal Cycler Program

Temperature	Time
37°C	2 hours
65°C	20 minutes
4°C	5 minutes
4°C	∞

CytoScan Optima Ligate

Table B.4 CytoScan Optima Ligate Thermal Cycler Program

Temperature	Time
16°C	3 hours
70°C	20 minutes
4°C	5 minutes
4°C	∞

CytoScan Optima PCR

You must use thermal cyclers with silver or gold-plated silver blocks. Do not use thermal cyclers with aluminum blocks.

- Ramp speed: Max
- Volume: 100 µL

Table B.5 CytoScan Optima PCR Thermal Cycler Program

Temperature	Time	Cycles
95°C	1 minute	1X
95°C 60°C 68°C	30 seconds 45 seconds 60 seconds	} 30X
68°C	7 minutes	1X
4°C	5 minutes	1X
4°C	∞	

CytoScan Optima Fragment

Table B.6 CytoScan Optima Fragment Thermal Cycler Program

Temperature	Time
37°C	35 minutes
95°C	15 minutes
4°C	5 minutes
4°C	∞

CytoScan Optima Label

Table B.7 CytoScan Optima Label Thermal Cycler Program

Temperature	Time
37°C	4 hr
95°C	15 minutes
4°C	5 minutes
4°C	∞

CytoScan Optima Hyb

Table B.8 CytoScan Optima Hyb Thermal Cycler Program

Temperature	Time
95°C	10 minutes
49°C	3 minutes
49°C	∞

Appendix C Required Equipment, Consumables, and Reagents

Table C.1 Equipment and Software, Required

Item	Part Number
Equipment	
GeneChip™ Fluidics Station 450	00-0079
Tubing, Silicone peristaltic for GeneChip™ Fluidics Station 450	400110
GeneChip™ Hybridization Oven 645	00-0331
GeneChip™ Scanner 30007G	00-0213
Software	
GeneChip Command Console Software	Version 3.2.2 or higher
Chromosome Analysis Suite (ChAS 2.1 or higher)	901394

Table C.2 CytoScan™ Optima Assay Reagents, Required

Module P/N	Module	Item/Reagent	Qty	Part Number
902527	CytoScan™ Optima – Module 1: Pre-Lab Restriction, Ligation, and Amplification	• Nsp I	• 1	• 902421
		• Nsp I Buffer	• 1	• 902422
		• 100X BSA	• 1	• 902423
		• DNA Ligase	• 1	• 902379
		• DNA Ligase Bfr	• 1	• 902378
		• CytoScan Taq Buffer	• 1	• 902582
		• CytoScan Taq DNA Polymerase	• 1	• 902581
		• dNTP	• 1	• 902401
		• 5M Betaine	• 1	• 902402
		• Adaptor, Nsp I	• 1	• 902426
		• PCR Primer	• 1	• 902427
902528	CytoScan™ Optima – Module 2: Pre-Lab TE Buffer and Water	• Low EDTA TE Buffer	• 1	• 902424
		• Water, Nuclease free	• 1	• 902425
902529	CytoScan™ Optima – Module 3: Main Lab Fragmentation, Labeling, and Hybridization	• Fragmentation Reagent	• 1	• 902428
		• Fragmentation Buffer	• 1	• 902301
		• TdT Enzyme	• 1	• 902429
		• TdT Buffer	• 1	• 902430
		• DNA Labeling Reagent	• 1	• 902431
		• Oligo Control Reagent	• 1	• 902432

Module P/N	Module	Item/Reagent	Qty	Part Number
		• Hyb Buffer Part 1	• 1	• 902433
		• Hyb Buffer Part 2	• 1	• 902434
		• Hyb Buffer Part 3	• 1	• 902435
		• Hyb Buffer Part 4	• 1	• 902436
Module P/N	**Module**	**Item/Reagent**	**Qty**	**Part Number**
902530	CytoScan™ Optima – Module 4: Main Lab Stain, Holding Buffer, Beads, and Water	• Stain Buffer 1	• 1	• 902440
		• Stain Buffer 2	• 1	• 902441
		• Array Holding Buffer	• 1	• 902442
		• Purification Beads	• 1	• 902443
		• Water, Nuclease free	• 1	• 902425
902531	CytoScan™ Optima – Module 5: Main Lab Elution Buffer, and Purification Wash Buffer	• Elution Buffer	• 1	• 902444
		• Purification Wash Buffer	• 1	• 902437
		• Wash A	• 4	• 902438
		• Wash B	• 4	• 902439
Arrays Required				
902507	CytoScan Optima Array Kit, 6 pack			

From Other Suppliers

Reagents Required but not provided.

Table C.3 Reagents from Other Suppliers, Required

Item
Bleach, 6.15% sodium hypochlorite
PCR gel DNA ladder: 50 to 2000 bp
Fragmentation gel DNA ladder: 25 to 2600 bp
Ethanol, absolute
Gel-loading buffer

Pre-PCR Clean Room – Equipment Required but Not Provided

Table C.4 Pre-PCR Clean Room Equipment, Required

Item
If assay is to be performed in one room: • Laminar Flow Cabinet, 6 foot or • PCR Cabinet
Benchtop Cooler, with the capacity to hold 8 to 32 tubes (1.5 mL) and ability to maintain temperature below –15°C for 2 hours.
Centrifuge, plate, multipurpose, 330 µL capacity
Cooling chamber, double-block, with the capacity to hold 96 well plates with a maximum volume capacity of 330 µL
Freezer, –15 to –25°C; deep freeze; manual defrost; 17 cu ft
Microfuge (for tubes and strip tubes)
Pipettors: • 12-channel, 2 to 20 µL • 12-channel, 20 to 200 µL • single-channel, 100 to 1000 µL • single-channel, 2 to 20 µL • single-channel, 20 to 200 µL
Rectangular Ice Tray Large, 9L (16 x 13 in, 41 x 33cm)
Storage Racks, Tube, 96-well
Thermal cycler: capable of holding 200 µL volume and 96-well plate; heat block capable of holding temperature of 4 to 99.9°C; temperature accuracy of ±0.25°C (at 35 to 99.9°C); heating and cooling block ramp rate of ≤4°C per second.
Vortexer, 60 Hz, 75 W, 600 to 3200 RPM

Post-PCR Room – Equipment Required but Not Provided

Table C.5 Post-PCR Room Equipment Required

Item
Adhesive film applicator (hard plastic)
Anti-vibration pad, used with vortexer to prevent movement during operation
Cooler, benchtop, with capacity to hold 8 to 32 tubes (1.5 mL) and maintain temperature below –15°C for 2 hours
Cooling chamber, double-block, with capacity to hold 96-well plates with a maximum volume capacity of 330 µL
Electrophoresis supplies
Freezer, –15 to –25°C; deep freeze; manual defrost; 17 cu ft
Gel imager
Magnetic rack with magnet on the side and capable of holding 8 to 12 tubes of 1.5 to 2 mL capacity
Microcentrifuge, non-refrigerated with capacity to hold 24 tubes and maximum rotation speed of 16,200 x g
Microfuge (for tubes and strip tubes)
Microtube foam insert
Pipettors: • 12-channel, 100 to 1200 µL • 12-channel, 2 to 20 µL • 12-channel, 20 to 200 µL • single-channel, 100 to 1000 µL • single-channel, 2 to 20 µL • single-channel, 20 to 200 µL
Plate centrifuge, refrigerated multipurpose, plate carriers for 4 x 96-well assay plates
Platform Head, 6-inch, for microtube foam insert
Rectangular ice tray, large, 9L (16 x 13in; 41 x 33cm)
Refrigerator, 2 to 8°C, 6 cu ft
Spectrophotometer, UV/VIS, single or multichannel
Storage racks, tube, 96-well with lid, 1.5 mL/2 mL tubes
Thermal cycler: capable of holding 200 µL volume and 96-well plate; heat block capable of holding temperature of 4 to 99.9°C; temperature accuracy of ±0.25°C (at 35 to 99.9°C); heating and cooling block ramp rate of ≤4°C per second; thermal uniformity of ±0.5°C.
Vortexer, 60 Hz, 75 W, 600-3200 RPM

Consumables Required but Not Provided

Table C.6 Consumables from other Suppliers, Required

Item
Adhesive films, clear, PCR-certified, 96-well plates
Adhesive label dot, 1/2-inch and 3/8-inch
Agarose gel, 2%
Agarose gel, 4%
Microcentrifuge tubes, nuclease-free, sterile, 1.5 mL polypropylene
Microcentrifuge tubes, nuclease-free, sterile, 2.0 mL polypropylene
Microcentrifuge tubes, nuclease-free, sterile, non-stick, 1.5 mL amber polypropylene
Microcentrifuge tubes, nuclease-free, sterile, non-stick, 1.5 mL blue polypropylene
Microcentrifuge tubes, nuclease-free, sterile, non-stick, 1.5 mL natural polypropylene
Microcentrifuge tubes, nuclease-free, sterile, non-stick, 50 mL polypropylene microcentrifuge tubes
Pipette tips with aerosol barriers, 20 µL, 200 µL, and 1000 µL
Plate, OD for UV, 96-well (required only if using microplate spectrophotometer)
Plates, unskirted PCR with a maximum volume of 330 µL
Reagent reservoir, 25 mL
Tube strips, nuclease-free, sterile, 8-well, 0.2 mL polypropylene

Symbols

Table C.7

Symbol / Label	Statement / Meaning
	Part/Catalog Number
	Lot Number
	Expiration Date YYYY-MM-DD
	Temperature Limitation

Appendix D Troubleshooting the CytoScan Optima Assay

Table D.1

PCR Gel QC Step	Likely Cause	Solution
Faint or no PCR product visible on gel. Both samples and positive control affected.	Failed restriction digest or adapter ligation.	• Repeat the assay from the beginning with Genomic Control DNA after reviewing best practices, ensuring that all equipment is correctly calibrated, and reagents are handled and stored properly. • If available, include ligated material from a previous successful experiment as a positive control for the PCR step. If it fails again, repeat with fresh reagents.
		Ensure that the ligation buffer is thoroughly resuspended before use.
		Ensure that the reaction plates are sealed tightly in all steps.
	Non-optimal PCR conditions.	Use only calibrated thermal cyclers. Double-check PCR programs to ensure that they have been entered correctly.
		Check the PCR reagents. Use only the recommended reagents.
		Verify pipette calibration and function. Repeat PCR from the remaining digestion / ligation material if available, otherwise restart from the beginning.
		Take care with preparation of master mixes. Ensure accurate pipetting and thorough mixing.
		• Use the recommended 96-well PCR plates and plate seals. • Ensure that the plates are sealed tightly in all steps.
	Ligation reaction not diluted or diluted ligation reaction not mixed properly prior to PCR.	Be sure to correctly dilute the ligation reaction with the water provided in the kit and mix properly before proceeding with PCR.

PCR Gel QC Step	Likely Cause	Solution
Faint or no PCR product visible on the gel. Samples are affected but positive control is OK.	Insufficient or degraded genomic DNA.	• Starting amount of 250 ng genomic DNA should be used. • Confirm the concentration using a calibrated spectrophotometer. • Confirm that the genomic DNA sample meets the quality and integrity guidelines. See page 8.
	Sample DNA contains enzymatic or chemical inhibitors. Nsp I can be inhibited by high concentrations of salts.	Ensure that genomic DNA is extracted using procedures that are compatible with DNA quality defined in Chapter 1. See Chapter 4.

Table D.1

PCR Gel QC Step	Likely Cause	Solution
Wrong size distribution of PCR product.	Mispipetting of PCR primer volume in the master mix. Mispipetting of Taq polymerase in the master mix.	• Verify pipette calibration and function. • Repeat PCR from the remaining digestion/ligation material if available; otherwise restart from the beginning.
PCR product evident in the negative control.	Reagents or equipment contaminated with ligated product or amplified product.	• Always use filter tips. • Clean the pre-PCR lab area and equipment thoroughly using 10% bleach. • Decontaminate the pipettes following manufacturer's recommendation. • Retrain personnel on pre-lab best practices. • Repeat the assay using fresh reagents and sample.

Table D.2

Purification Yield QC Step	Likely Cause	Solution
Low eluate volume (<25 µL)	Insufficient volume due to pipetting error or pipet out of calibration.	Check pipette calibration. Make sure 27 µL of elution buffer is added to the beads for elution and the tubes are centrifuged before placing on the magnet.
	Over drying of beads.	Follow the recommended time for drying.

Purification Yield QC Step	Likely Cause	Solution
Low yields (the average purification yield of 7 or more samples is <3.0 µg/µL or individual yield is <2.5 µg/µL)	Loss of sample prior to purification.	If the yield is not adequate, repeat the assay.
	Possible problems with input genomic DNA.	• Use collection and purification procedures that meet DNA quality specifications as described in Chapter 1 to avoid carryover of inhibitors such as heme, EDTA, etc. • Starting amount of 250 ng genomic DNA should be used. • Confirm the concentration using a calibrated spectrophotometer. • Confirm that the genomic DNA sample meets the quality and integrity guidelines provided in Chapter 4.
	Purification Wash Buffer was prepared incorrectly.	Verify that the correct volume of absolute ethanol was added to the Purification Wash Buffer before use.
	Inadequate mixing of Purification Beads and PCR reactions during binding.	Take care to completely mix the PCR reactions and the Purification Beads during sample binding.
	Inadequate bead washing prior to elution.	Repeat purification with attention towards complete removal of the binding eluate before the bead wash.
	Excess Elution Buffer added to beads.	Verify pipette calibration and function.
	Incorrect buffer was used for elution.	Verify that the Elution Buffer was used during the elution step and not the Purification Wash Buffer.
	Purification Beads were over dried.	Do not dry Purification Beads longer than the recommended time.
	The eluted DNA plate was inadequately vortexed before taking an aliquot for an OD reading.	Eluted DNA can be heterogeneous. Repeat the dilution followed by an OD reading, making sure to vortex the eluted DNA and the OD plate thoroughly at each step.

Purification Yield QC Step	Likely Cause	Solution
PCR reaction volume was inaccurate.		Repeat the assay and confirm that the PCR reaction is set up correctly.
High yields (>4.5 µg/µL)	Too little Elution Buffer added to the Purification Beads.	Verify pipette calibration and function. Make sure 27 µL of Elution Buffer is added to the Purification Beads for elution.
	Eluted DNA plate inadequately vortexed before OD reading is taken.	Eluted DNA can be heterogeneous. Repeat the dilution followed by OD reading, being sure to vortex the eluted DNA and the OD plate thoroughly at each step.
	Instruments or pipettes may be out of calibration or incorrectly set.	Verify instrument and pipette calibration and settings during operation.
	Yield calculation formula within the software template may be incorrect.	Verify the formula used to calculate the yields from a given O.D.

Table D.3

Fragmentation QC Step (Gel)	Likely Cause	Solution
Over fragmentation: Majority of fragmented sample appears <50 bp on a 4% agarose gel.	Excess Fragmentation Reagent was added during preparation of the Fragmentation Master Mix.	• Carefully observe the pipette tip and the shaft during pipetting of the fragmentation reagent. Touch the tip to the inside of the vial to help remove any droplets of enzyme clinging to the exterior of the tip. • Make sure pipettes are calibrated.
	Purified samples or assembled reactions were allowed to warm to room temperature during reaction assembly or prior to incubation.	Ensure that the plate centrifuge is completely chilled to 4°C before spinning the assembled fragmentation plate.
		Keep the master mix, samples, and reaction components on ice or in a cooling block at all times during master mix assembly and dispensing of the master mix to the samples.
		Check that the fragmentation reaction temperature and incubation time are correctly programmed on the thermal cycler and that the fragmentation mix is made correctly as per the guidelines.

Fragmentation QC Step (Gel)	Likely Cause	Solution
		Ensure that the master mix tube and strip tubes are pre-chilled before reaction setup.
Under fragmentation: PCR product is still visible in 150-2200 bp size region on a 4% agarose gel.	Improper storage or handling of the Fragmentation Reagent.	• The Fragmentation Reagent should be stored at –15°C to –25°C at all times. • Handle the Fragmentation Reagent as minimally as possible, holding the vial at the cap rather than the center. Return the Fragmentation Reagent to the cooler as soon as the reagent has been dispensed. We recommend storing the Fragmentation Reagent at –15°C to –25°C inside a cooler to preserve its activity. • Do not over vortex the Fragmentation Reagent.
	• Insufficient Fragmentation Reagent or Fragmentation Buffer was added during assembly of the Fragmentation Master Mix. • Improper mixing of the Fragmentation Master Mix.	Verify pipette calibration and function. Take care when preparing the master mix to ensure accurate pipetting and thorough mixing.
	The Fragmentation Master Mix was not made fresh or was allowed to warm to room temperature before use.	• Keep the Fragmentation Master Mix on ice at all times to preserve activity. • Work quickly during reaction assembly. • Do not save or reuse a previously assembled Fragmentation Master Mix.
	Samples were frozen during fragmentation reaction assembly or centrifugation.	• Make sure that cold blocks are not chilled to –15°C to –25°C as sample freezing can occur. • Before centrifugation, ensure that the interior of the chilled plate centrifuge is not lower than 2-8°C.

Fragmentation QC Step (Gel)	Likely Cause	Solution
	Thermal cycler was not programmed correctly or is out of calibration.	• Confirm that the fragmentation reaction temperature and incubation time are correctly programmed on the thermal cycler. • Confirm that the fragmentation mix is made correctly as per the guidelines. • Verify that the thermal cycler is within calibration.

Table D.4

.CEL File Generation	Likely Cause	Solution
.CEL file is not generated	Signal from the corner checkerboards is absent.	Verify that the Oligo Control Reagent was added to the Hybridization Master Mix during assembly. The Oligo Control Reagent must be present during hybridization to ensure proper grid alignment.
	Signal from corner checkerboards is dim.	• Verify that the correct amount of the Oligo Control Reagent was added to the Hybridization Master Mix during assembly. • Ensure that GeneChip™ Hybridization Oven 645 is calibrated and set to the correct temperature. • Ensure that Hybridization Master Mix was correctly assembled and added at the correct volume to the fragmented samples. • Confirm that Stain Buffer 1 and Stain Buffer 2 are placed in the correct order on the fluidics station. Stain Buffer 1 is light sensitive. Be sure to store Stain Buffer 1 in the dark when not in use. • Use only the recommended staining reagents.
	Bright hybridization artifact(s) obscure gridding oligo locations on the array.	Try to manually align the grid. See the *GeneChip™ Command Console™ User Guide* (Pub. No. 702569) for instructions. If manual grid alignment fails to produce a .CEL file, repeat the experiment.

Table D.5

Data QC Failures	Likely Cause	Solution

Low or failing SNPQC	• Maternal cell contamination (MCC), triploidy or whole genome mosaic samples.
	• Cross-contamination between samples within a plate.
	• Contaminated reagents, equipment, or input DNA.
		• Analyze the allelic difference track on ChAS, if it shows altered patterns compatible with MCC, triploidy or whole genome mosaic, do not discard and continue as usual.
		• Repeat assay using a control sample of known integrity.
		• Review and follow best practices.
		  • Ensure a tight plate seal at every step.
		  • Use fresh filter tips at each pipetting step.
		  • Use caution when pooling PCR product.
		• If the problem persists use fresh reagents and fresh input DNA.
		• Decontaminate the pre-PCR room and equipment if necessary.

		Process only 4 to 6 arrays at a time. When processing arrays for washing it is important to work quickly as delays in this step will impact data quality.
		Perform all steps after removal of arrays from the oven to the time the washing begins with minimal delays.
	Over or under fragmentation of the PCR product	See above.
	Hybridization oven out of calibration or oven model is not compatible with this assay.	• Ensure that only the GeneChip™ Hybridization Oven 645 is used for this assay. • Have the oven serviced.

Table D.5

Data QC Failures	Likely Cause	Solution
Elevated or failing MAPD	Assay drift due to variation in assay execution.	• Recalibrate pipettes to ensure accurate delivery of reagent volumes. • Consider operator retraining or review by an Field Applications Scientist if the problem persists. • Review Best practices under Chapter 1 and Appendix A.
	Over fragmentation	See above.
	Degraded starting material.	• Perform a QC gel of input DNAs to assess samples for degradation. • Ensure that the DNA samples are of high quality (for example, run in a 1 to 2% agarose gel and compare to a Genomic DNA Control).
	Reference is inappropriate for the sample.	Use only the recommended sample types.
High MAPD with low SNPQC	Error during washing the array.	• Ensure that the Wash A and B lines of the Fluidics Station are placed in the correct wash buffers during priming and array washing. • Ensure that the Fluidics Stations are maintained according to the guidelines in the Fluidics Station User's Guide.

Appendix E Fluidics Station Care and Maintenance

General Fluidics Station Care

1. Use a surge protector on the power line to the fluidics station.
2. Always run a Shutdown protocol when the instrument will be off or unused overnight or longer. This will prevent salt crystals from forming within the fluidics system.
3. To ensure proper functioning of the instrument, perform periodic maintenance.
4. When not using the instrument, leave the sample needles in the lowered position. Each needle should extend into an empty vial. This will protect them from accidental damage.
5. Always use deionized water to prevent contamination of the lines. Change buffers with freshly prepared buffer at each system startup.
6. The fluidics station should be positioned on a sturdy, level bench away from extremes in temperature and away from moving air.

WARNING: Before performing any maintenance, turn off power to the fluidics station to avoid injury in case of a pump or electrical malfunction.

Fluidics Station Bleach Protocol

We recommend a weekly cleaning protocol for the fluidics station. This protocol uses commonly purchased sodium hypochlorite bleach.

This protocol is designed to eliminate any residual SAPE-antibody complex that may be present in the fluidics station tubing and needles. The protocol runs a bleach solution through the system followed by a rinse cycle with deionized (DI) water. This protocol takes approximately one hour and forty minutes to complete. We recommend running this protocol weekly, regardless of the frequency of use. The current version of the protocol can be found at: **thermofisher.com**.

The Bleach Cycle

To avoid carryover, or cross contamination, from the bleach protocol, we recommend the use of dedicated bottles for bleach and DI water. Additional bottles can be obtained from Thermo Fisher.

Table E.1 Recommended Bottles

Part Number	Description
400118	Media Bottle, SQ, 500 mL
400119	Media Bottle, SQ, 1000 mL

1. Disengage the washblock for each module by pressing down on the cartridge lever. Remove any probe array cartridge (Figure E.1).
2. Prepare 500 mL of 0.525% sodium hypochlorite solution using deionized water.

   You can follow these directions to make 500 mL of bleach:

   In a 1 liter plastic or glass graduated cylinder, combine 43.75 mL of commercial bleach (such as

   Clorox™ bleach, which is 6.15% sodium hypochlorite) with 456.25 mL of DI H_2O, mix well. Pour the solution into a 500 mL plastic bottle, and place the plastic bottle on fluidics station.

IMPORTANT:

- **Prepare beach solution fresh at the time of use.**
- **Each fluidics station with 4 modules requires 500 mL of 0.525% sodium hypochlorite solution.**

Figure E.1 Disengaged Washblocks Showing Cartridge Levers in the Down Position. Remove any cartridges.

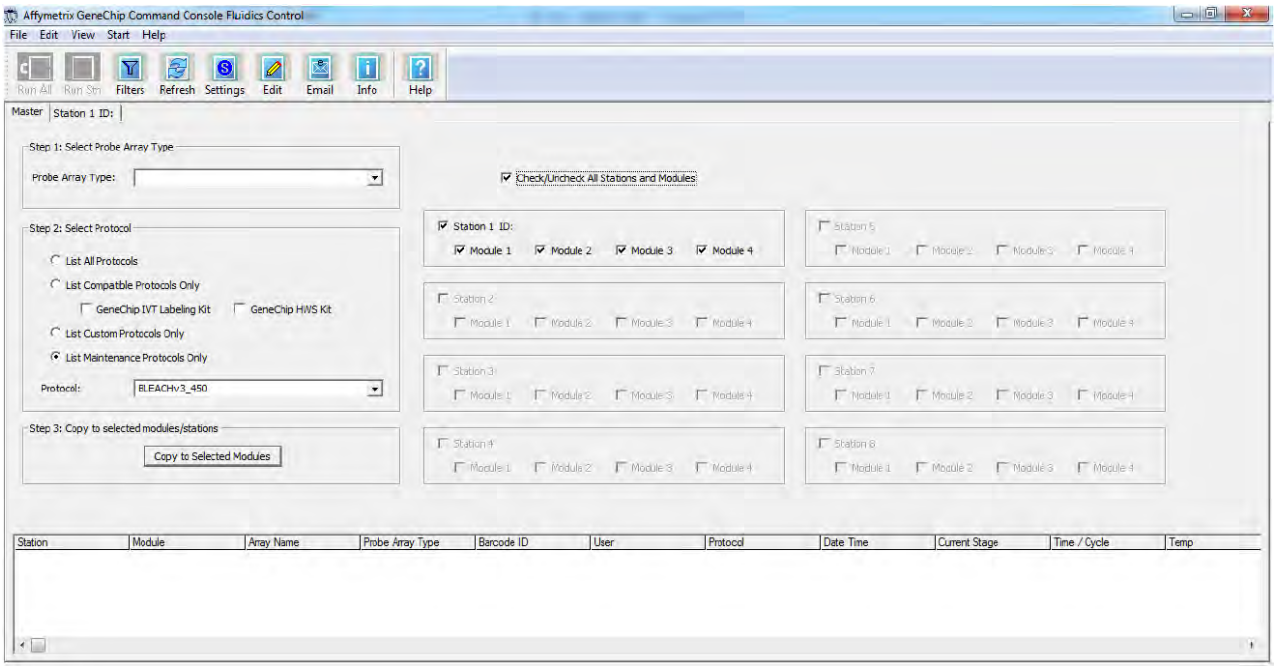
3. As shown in Figure E.2:
   a. Place on the fluidics station an empty one liter waste bottle, a 500 mL bottle of bleach and a one liter bottle of DI water.
   b. The Bleach protocol requires approximately one liter of DI water.
   c. Insert the waste line into the waste bottle.
   d. Immerse all three wash and water lines into the bleach solution.

IMPORTANT: Do NOT immerse the waste line into the bleach.

4. Open the instrument control software (AGCC).
5. Choose the current bleach protocol for each module.

Figure E.2 Bleach Cycle

Figure E.3 Fluidics Station Protocol Window: Select All Modules



6. In AGCC, run the protocol for all modules.

NOTE: The fluidics station will not start until the needle lever is pressed down (Figure E.4). The temperature will ramp up to 50°C.

7. Follow the prompts on each LCD. Load empty 1.5 mL vials onto each module if not already done so.
8. Press down on each of the needle levers to start the bleach protocol (Figure E.4).

Figure E.4 Press Down on the Needle Levers to Start the Bleach Protocol

9. The fluidics station will begin the protocol, emptying the lines and performing the cleaning cycles using bleach solution.
10. After approximately 30 minutes, the LCD will prompt you when the bleach cycle is over and the rinse cycle is about to begin.

The Rinse Cycle

Once the bleach cycle has finished, the second part of the protocol is a rinse step. This step is essential to remove all traces of bleach from the system. Failure to complete this step can result in damaged arrays.

1. Follow the prompts on the LCD for each module. Lift up on the needle levers and remove the bleach vials. Load clean, empty vials onto each module.
2. Remove the three wash and water lines from the bleach bottle and transfer them to the DI water bottle (Figure E.5).

   At this step, there is no need to be concerned about the bleach remaining in the lines.

Figure E.5 Immerse the Three Wash and Water Lines in the DI Water Bottle

3. Press down on the needle levers to begin the rinse cycle.
4. The fluidics station will empty the lines and rinse the needles.
5. When the rinse is completed after approximately one hour, the fluidics station will bring the temperature back to 25°C and drain the lines with air.
6. The LCD display will read CLEANING DONE.
7. Discard the vials used for the bleach protocol.
8. After completing the bleach protocol, follow the suggestions for storage of the Fluidics Station 450 in Table E.2.

Table E.2 Storage Suggestions for the Fluidics Station 450

If:	Then do this:
Planning to use the system immediately	After running the bleach protocol, remove the DI water supply used in the rinse phase and install the appropriate reagents for use in the next staining and washing protocol (including fresh DI water). • Perform a prime protocol without loading your probe arrays. **Failure to run a prime protocol will result in irreparable damage to the loaded hybridized probe arrays.**
Not planning to use the system immediately	Since the system is already well purged with water, there is no need to run an additional shutdown protocol. Remove the old DI water bottle and replace it with a fresh bottle.
Not planning to use the system for an extended period of time (longer than one week)	Remove the DI water and perform a "dry" protocol shutdown. This will remove most of the water from the system and prevent unwanted microbial growth in the supply lines. Also, remove the pump tubing from the peristaltic pump rollers.

Documentation and support

Obtaining support

Technical support

For the latest services and support information for all locations, visit **www.thermofisher.com**.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**thermofisher.com/support)**
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **thermofisher.com/support**.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.