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UserGuide

OncoScan® CNV FFPE Assay Kit

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P/N 703302 Rev. 1

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Contents

Chapter 1

Introduction

Topics in this chapter include:

About the OncoScan® CNV FFPE Assay Kit

Obtaining genome wide copy number and loss of heterozygosity (LOH) profiles from solid tumor samples is a significant challenge due to the difficulty of working with limited amounts of highly modified and degraded DNA derived from heterogeneous FFPE samples.

The list of clinically relevant and actionable copy number aberrations is growing and along with this, a recognition of the importance of genome-wide copy number and LOH profiles for solid tumor sample analysis. Genome wide copy number can also be used to detect sub clones and clonal evolution. The number and complexity of copy number aberrations has also been shown to be an indicator of patient prognosis.

Traditional FFPE sample analysis techniques such as fluorescent *in situ* hybridization (FISH) are limited to locus specific, low-resolution copy number information. Next generation sequencing approaches require target preparative methods that bias copy number determination and deep coverage to provide accurate copy number information from heterogeneous FFPE samples and may not be a practical option for most researchers.

The OncoScan® CNV Assay utilizes the Molecular Inversion Probe (MIP) assay technology which was originally developed for SNP genotyping, but has subsequently been used for identifying other types of genetic variation including focal insertions and deletions, larger copy number alterations and loss of heterozygosity (LOH).

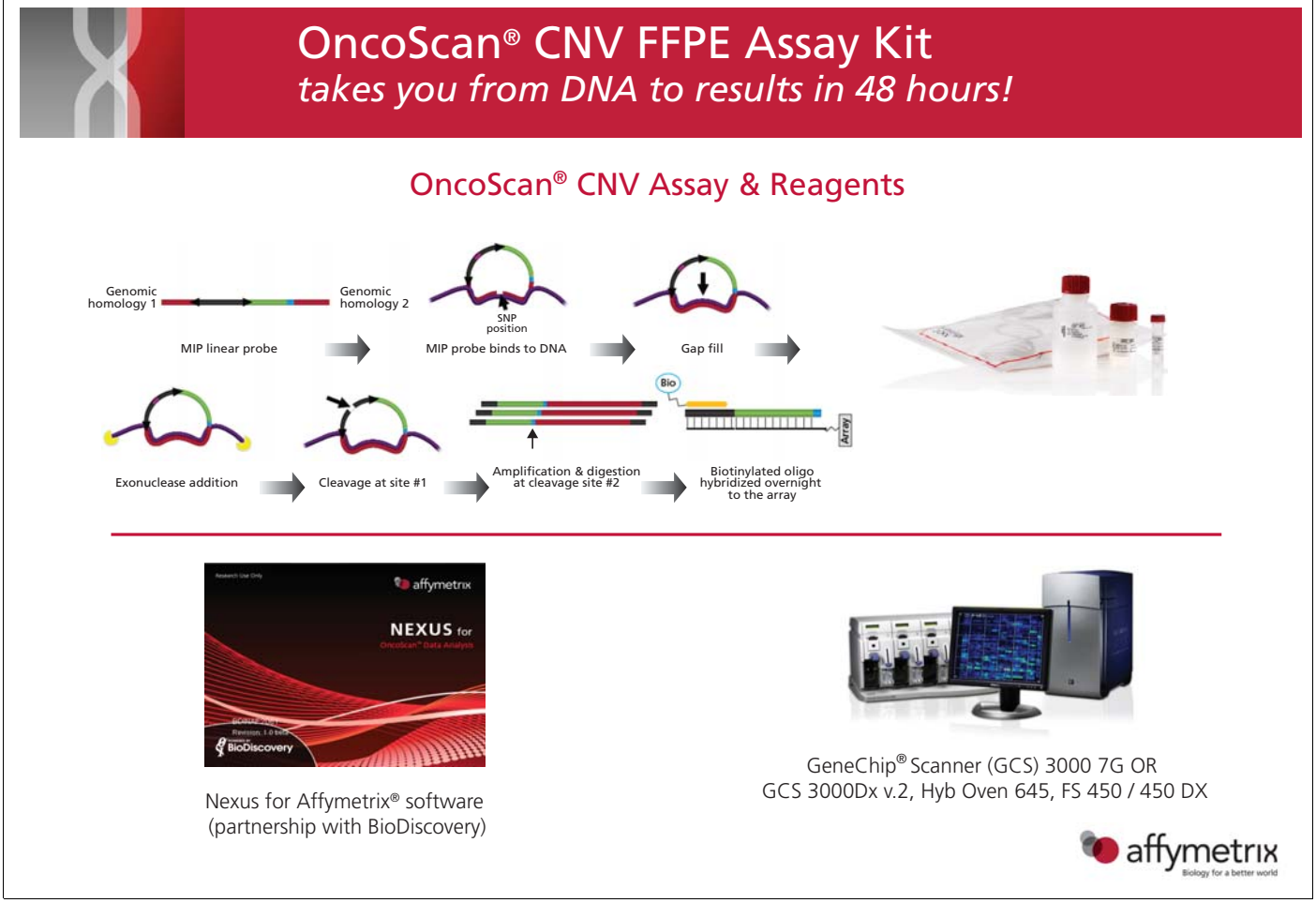
This assay has been shown over time to perform well with highly degraded DNA, such as that derived from FFPE- preserved tumor samples of various ages and with <100 ng DNA of starting material, thus - making the assay a natural choice in cancer clinical research.

The OncoScan® CNV FFPE Assay Kit is a complete and robust solution for degraded FFPE sample analysis for solid tumor tissues. The OncoScan CNV Assay can be run on existing Affymetrix' instruments, and offers genome wide copy number coverage with high resolution on cancer genes, within a 48 hour turnaround time period from DNA to results.

The optimized solution consists of:

- OncoScan® CNV reagents and CNV arrays
- OncoScan® Console, Nexus for Affymetrix® software, and Chromosome Analysis Suite (ChAS)
- GeneChip® Scanner 3000 7G or GeneChip® Scanner 3000 Dx2 instrumentation

Figure 1.1 OncoScan® CNV FFPE Solution



About this User Guide

This document is a guide for technical personnel conducting the Affymetrix® OncoScan® CNV Assay experiments in the laboratory. It contains:

- Best practices recommended by Affymetrix
- Laboratory setup
- FFPE sample preparation and quantification protocols recommended by Affymetrix
- Equipment and consumables required for each step
- Step-by-step protocols for the assay
- Protocols for washing, staining, and scanning arrays
- Fluidics Station care and maintenance
- Guidelines for processing 7, 9, 13 and 25 sample formats

Chapter 2

Laboratory Setup and Recommendations

This chapter provides an overview of the recommended laboratory setup to be used when performing the Affymetrix® OncoScan® CNV FFPE Kit Protocol.

Laboratory Setup — Two Separate Rooms

Laboratory

The use of two separate rooms greatly reduces the risk of sample contamination due to previously-amplified PCR products. These rooms are referred to as the:

- *Pre-PCR Lab*
- *Post-PCR Lab*

For more information on laboratory requirements and the equipment required to perform this protocol, refer to the *OncoScan® CNV FFPE Assay Kit Site Preparation Guide*, P/N 703346.

The high-level steps performed in each room are presented in Table 2.1.

Table 2.1 Assay Workflow When Two Separate Rooms are Used

Room	Template (Genomic DNA)	PCR Product
Pre-PCR Clean Lab Assay steps: ▫ Genomic DNA preparation and Picogreen quantitation of the genomic DNA and dilution ▫ Anneal ▫ First Stage PCR		
Post-PCR Lab Assay steps: ▫ Second Stage PCR ▫ HaeIII digest ▫ GeneChip Array hybridization ▫ Array washing and staining ▫ Array scanning		

Pre-PCR Lab

The Pre-PCR Lab should be a low copy DNA template lab, and should be free of PCR product (amplicons). Hence, no amplified product may be taken into the Pre-PCR Lab. Do not open the seal of the First stage PCR plate in the Pre-PCR Lab.

NOTE: The Post-PCR Lab has airborne contamination with the amplified MIP-annealed template. It is strongly recommended not to enter the Post-PCR Lab while doing the pre-PCR steps of the assay. (After entering the Post-Amp Lab, do not re-enter the Pre-Amp Lab without first showering and changing into freshly laundered clothes).

Contamination Prevention

1. All of the reagents and master stocks required for the steps performed in the Pre-PCR Lab should be stored in this lab under the appropriate conditions.
2. All of the equipment required for the steps performed in this lab should be dedicated. Do not move any equipment including ice buckets and pipettes between the Pre- and Post-PCR Labs.
3. Always wear a fresh gown, booties, hair net, and gloves to prevent PCR carryover, and to minimize the risk of trace levels of contaminants being brought into the lab.
4. Proper gowning procedure must be followed in the Pre-PCR Lab by using the steps given below:
   - Using the tacky mats to remove any dust and particulates from the bottom of shoes.
   - Putting on disposable booties.
   - Putting on bouffant head cover (cap).
   - Putting on the right size disposable laboratory gloves.
   - Putting on disposable sterile lab coats.
   - Wiping down the work bench area with 70% Ethanol before and after use.
   - Frequently changing the gloves throughout the assay as needed.

Activities in the Pre-PCR Lab

1. Preparation of non-amplified genomic DNA
   A. Extraction and Purification of genomic DNA
   B. Picogreen® quantitation of the genomic DNA

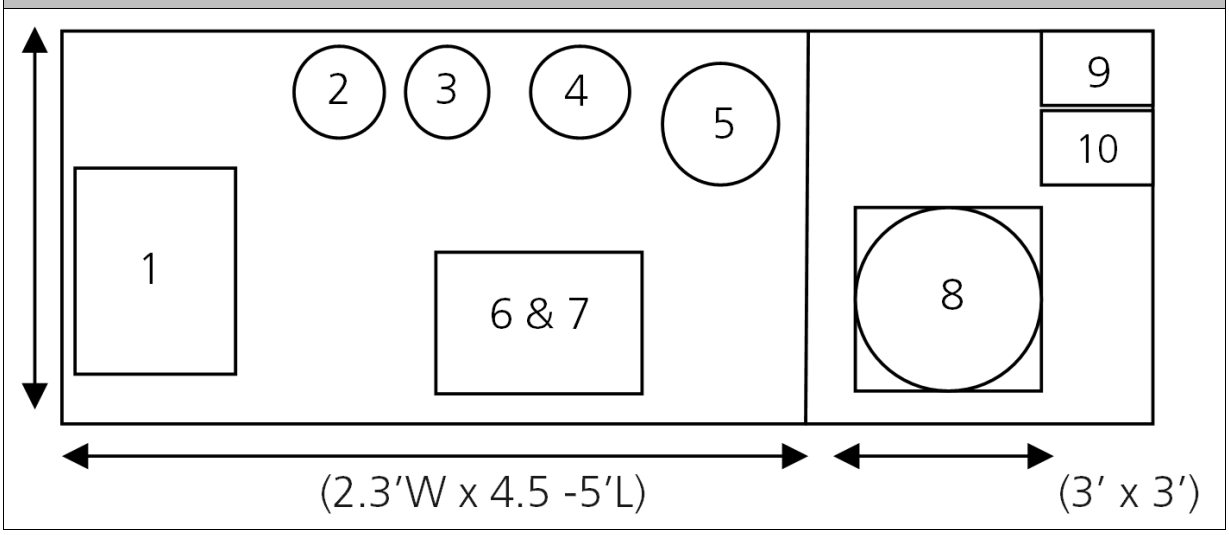
   NOTE: If the Qubit® or Fluoroskan instrument is located in the Post-PCR Lab, transfer only the volume needed for Picogreen quantitation and store the rest of the DNA in Pre-PCR Lab.

   C. Dilution or concentration of the genomic DNA as needed
2. Anneal reaction
3. Gap-Fill reaction
4. Exonuclease digestion reaction
5. Cleavage reaction
6. First Stage PCR reaction

Equipment Required in Pre-PCR Lab

The major pieces of equipment required for this lab are given below. Refer to Figure 2.1, Figure 2.2 and Figure 2.3.

Figure 2.1 Pre-PCR Lab Bench Equipment Requirements



Pre-PCR Lab Equipment

1. Approved thermal cyclers, see Table 4.2 or Appendix C for validated thermal cyclers
2. Microfuge for 1.5 mL Eppendorf vials
3. Microfuge for strip tubes
4. Vortexer
5. Single/Multi- Channel Pipettes on stand [L-20, L-200, L-1000, L12-20, and L12-200]
6. Ice bucket
7. Cold blocks for 96-well PCR plates - 4 each
8. Plate centrifuge - refrigerated or non-refrigerated
9. Freezer, –20˚C
10. Refrigerator, 2-8˚C

Figure 2.2 Pre-PCR Lab Bench Equipment - Recommended Setup

Figure 2.3 Pre-PCR Lab - Plate Centrifuge

Post-PCR Lab

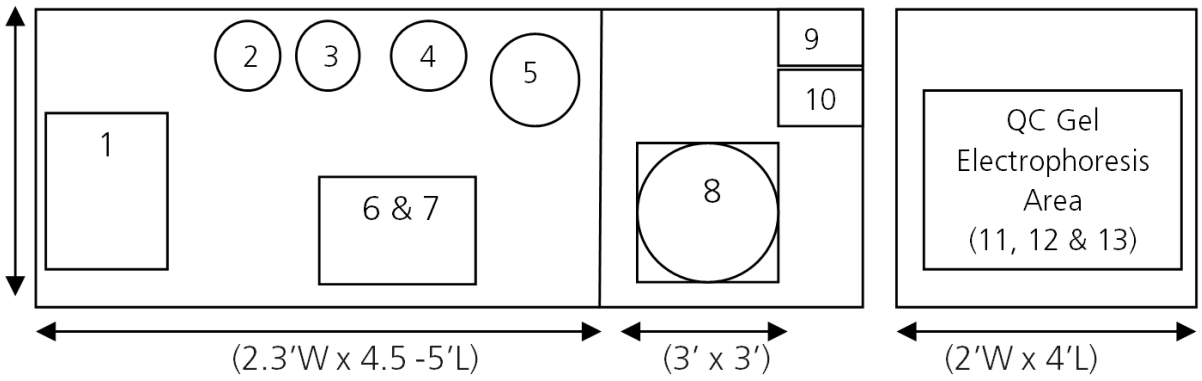
Activities in the Post-PCR Lab

1. Second Stage PCR amplification
2. QC gel of First stage PCR by agarose gel electrophoresis
3. HaeIII digest of Second Stage PCR
4. QC gel of HaeIII digest by agarose gel electrophoresis
5. Preparation of hybridization target
6. Sample labeling and hybridization onto GeneChip® Arrays
7. Washing and staining of arrays
8. Scanning of arrays

Equipment Required in Post-PCR Lab

The major pieces of equipment required for this lab are given below. The bench top equipment requirements are same as for the Pre-PCR Lab, however additional equipment is needed for processing QC gels. Refer to Figure 2.4.

Figure 2.4 Post-PCR Lab Bench Equipment Requirements



Post-PCR Lab Equipment

1. Approved thermal cyclers, see Table 4.2 or Appendix C for validated thermal cyclers
2. Microfuge for 1.5 mL Eppendorf vials
3. Microfuge for strip tubes
4. Vortexer
5. Single/Multi- Channel Pipettes on stand [L-20, L-200, L-1000, L12-20 (or L24-20), and L12-200 (or L24-200)
6. Ice bucket
7. Cold blocks for 96-well PCR plates - 3 each
8. Plate centrifuge - refrigerated or non-refrigerated
9. Freezer, –20°C
10. Refrigerator, 2-8°C
11. Electrophoresis gel box
12. Electrophoresis power supply
13. Gel Imager
14. GeneChip® Hybridization Oven 645
15. Fluidics Station 450 or Fluidics Station 450Dx connected to an Affymetrix® GeneChip® workstation
16. GeneChip® Scanner 3000 7G or GeneChip® Scanner 3000 Dx2 connected to an Affymetrix® GeneChip® workstation

Safety Precautions

The Affymetrix® OncoScan® CNV FFPE Assay Kit as well as the Affymetrix® OncoScan® CNV Arrays are for research use only. All DNA and other potentially infectious materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Some components required for this assay may pose significant health risks. Follow prudent laboratory practices when handling and disposing of carcinogens and toxins.

Refer to the manufacturer's Safety Data Sheet for additional information.

Chapter 3

OncoScan® CNV Assay Overview

This chapter provides an overview of the Affymetrix® OncoScan® CNV Assay, including information about assay configuration and workflows.

Assay and Reagent Configuration

Reagents Required

For a complete list of reagents, instruments, software and consumables required, please refer to Appendix C on page 111.

OncoScan® CNV Reagent Kit — P/N 902692

The OncoScan® CNV FFPE Assay Kit is designed to process 24 samples plus a negative control. (Note: the negative control is evaluated in the gel QC steps, but is not hybridized to arrays). The OncoScan CNV FFPE Assay Kit may also be used to process samples in multiple runs as given in Table 3.1 and Table 3.2, below.

Table 3.1

Number of Runs per Assay Kit	Number of Test Samples per Run	Positive Control Sample per Run	Negative Control per Run	Total Number of Samples Processed per Run	Total Number of Samples Hybridized per Run (excludes negative control)	Number of Arrays Required per run	Total number of Arrays Needed for All Runs
1	23	1	1	25	24	48	48
2	11	1	1	13	12	24	48
3	7	1	1	9	8	16	48
4	5	1	1	7	6	12	48

Table 3.2

	7 Reactions per Run	9 Reactions per Run	13 Reactions per Run	25 Reactions per Run
Number of Runs per Assay Kit	4	3	2	1
Total Number of Reagent Reactions per Assay Kit	28	27	26	25
Number of Samples (includes 1 positive control per run)	6	8	12	24
Negative Control per Run	1	1	1	1

OncoScan CNV Reagent Kit Components

Table 3.3 OncoScan® CNV Reagent Kit and Components

Module	Storage	Part Number*
OncoScan® CNV Buffer C	–20°C	902687
▪ Buffer C		▪ 902696
OncoScan® CNV Copy Number Probe Mix 1.0 & Controls	–20°C	902688
▪ Positive Control (12 ng/µL) ▪ Negative Control ▪ Copy Number Probe Mix 1.0 ▪ Buffer A		▪ 902249 ▪ 902250 ▪ 902248 ▪ 902246
OncoScan® CNV Gap Fill and 1st Stage PCR	–20°C	902689
▪ Buffer A ▪ Gap Fill Enzyme Mix ▪ SAP, Recombinant (1 U/µL) ▪ dNTP Mix (A/T) ▪ dNTP Mix (G/C) ▪ Nuclease-free Water ▪ Exo Mix ▪ Cleavage Buffer ▪ Cleavage Enzyme (2 U/µL) ▪ PCR Mix ▪ Taq Polymerase (5 U/µL)		▪ 902246 ▪ 902252 ▪ 902251 ▪ 902254 ▪ 902255 ▪ 902253 ▪ 902256 ▪ 902257 ▪ 902258 ▪ 902259 ▪ 902260
OncoScan® CNV 2nd Stage PCR and Post PCR Processing	–20°C	902690
▪ PCR Mix ▪ Taq Polymerase (5 U/µL) ▪ Buffer B ▪ HaeIII Enzyme (10 U/µL) ▪ Exo I Enzyme (20 U/µL) ▪ Nuclease Free Water ▪ Hybridization Mix		▪ 902259 ▪ 902260 ▪ 902261 ▪ 902262 ▪ 902263 ▪ 902253 ▪ 902264
OncoScan® CNV Stain Reagents	2-8°C	902691
▪ Stain 1 ▪ Stain 2 ▪ Array Holding Buffer		▪ 902265 ▪ 902266 ▪ 901733
Individual Bottles	Room Temp	
▪ Wash A ▪ Wash B		▪ 901680 ▪ 901681

* *Part Numbers are for identification purposes only. Individual kit components cannot be ordered separately.*

OncoScan® CNV Assay Workflow

This section provides an overview of the 48 hour workflow spanning 3 days, segregated by Pre-PCR and Post-PCR Lab space.

- Day-1_ PM (Pre-PCR Lab): The DNA sample plate is prepared with the normalized DNA at 12 ng/µL. The DNA samples are then incubated overnight to anneal the MIP probe. (2-3 hours hands-on time depending on the number of samples, anneal overnight for 16-18 hours)
- Day-2_ AM (Pre-PCR Lab): The annealed DNA samples are processed through the Pre-PCR assay steps; (Total time: ~2.5 to 3 hours)
  - Addition of Gap-fill master mix to the overnight annealed DNA
  - Divide the total volume of each reaction into two wells on different rows each containing equal volume (10 µL), referred to as AT and GC channels (Channel Split)
  - Gap-Fill the annealed probe with specific dNTP mixes
  - Exonuclease reaction to remove the unligated (not gap-filled, linear) probes
  - Cleavage enzyme reaction to linearize the gap-filled circular MIP Probes
  - 1st PCR amplification of the gap-filled, linearized MIP probe
- Day-2_ PM (Post-PCR Lab): The amplified MIP products from the 1st PCR reaction are taken through the Post-PCR assay steps that end in Array hybridization. (Total time: ~3.5 to 4.5 hours, depending on the number of samples and overnight Array hybridization for 16-18 hours)
  - Second PCR amplification to enrich the MIP product
  - HaeIII digestion of the second PCR product
  - QC Gels are run to determine the size distribution of the 1st PCR reaction, and the HaeIII digested products
  - Preparation of hybridization target with the digested HaeIII product
  - Denature target for hybridization
  - Hybridization of target onto the OncoScan® Array
- Day-3_ AM (Post-PCR Lab): (1.5 hours to wash and 15 minutes scanning per sample (2 arrays per sample))
  - Array Wash and Stain in GeneChip Fluidics Station
  - Array Scan in GeneChip Scanner
- Day-3_ PM (Post-PCR Lab): The CEL files are available for data analysis

Figure 3.1 Summary of **OncoScan®** CNV Assay Workflow

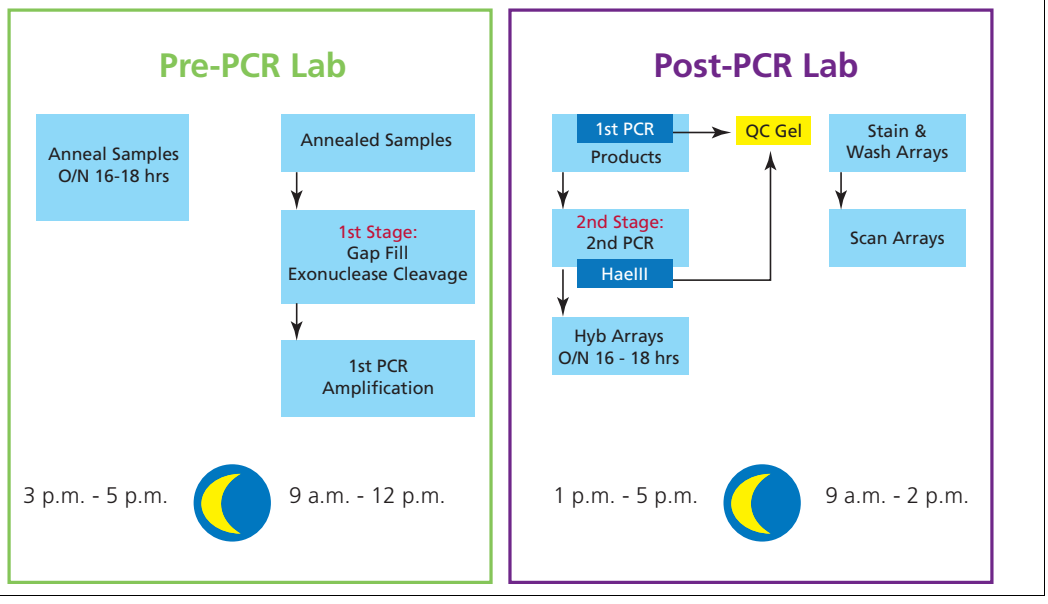
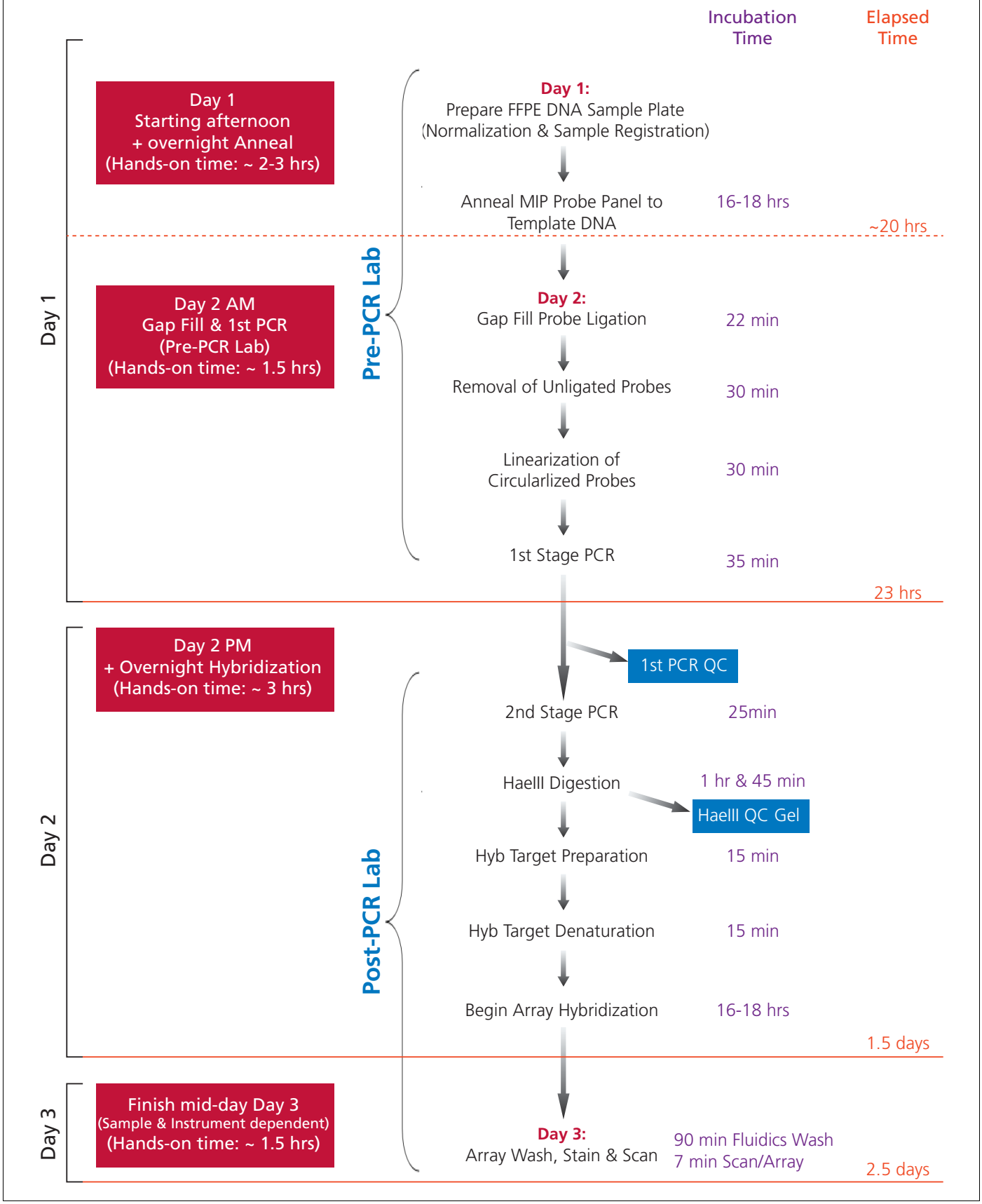


Figure 3.2 Detailed **OncoScan®** CNV Assay Workflow



Chapter 4

Best Practices for Running the OncoScan® CNV FFPE Assay Kit Protocol

This section provides tips for ensuring successful performance of the protocol. Topics in this section include:

Laboratory Workflow

1. The first half of the assay will take place in a Pre-PCR Lab (Anneal through 1st PCR) and the second half will take place in the Post-PCR Lab (2nd PCR through Array Scanning).
2. Maintain a single direction workflow. It is acceptable to move from the Pre-PCR Lab to the Post-PCR Lab, but moving from the Post-PCR Lab to the Pre-PCR Lab should be avoided.
3. Never bring amplified products into the Pre-PCR Lab. Never open the seal of the 1st PCR plate in the Pre-PCR Lab. Do not spin down the 1st PCR plate in the Pre-PCR Lab.
4. Keep dedicated equipment in each room or area used for this protocol. To avoid contamination, do not move equipment between the Pre-PCR and the Post-PCR Labs.

Preparing the Work Area for Each Stage

Many of the stages in the OncoScan® CNV FFPE Assay Kit Protocol must be performed rapidly and on ice to carefully control enzyme activity and temperature transitions. Therefore, we recommend that you set up all of the equipment, consumables and reagents (except for the enzymes) prior to beginning each stage.

Other Important Guidelines

1. During the Pre-PCR portion of the assay the thermal cycler program will be paused and resumed multiple times. It is recommended to set an additional timer for notification so that the thermal cycler can be paused before the program moves onto the next step.
2. It is mandatory to use compression pads on the thermal cyclers (both Veriti and ABI 9700 models) throughout the assay in both labs.

Preparation & Quantification of DNA Samples

Sample preparation and quantification method play a critical role in the success of the OncoScan® CNV Assay. Refer to the following sections that describe the protocols recommended by Affymetrix:

- Chapter 6, *OncoScan® CNV FFPE Assay Kit Protocol* on page 30
- Appendix E, *FFPE DNA Extraction Protocol for OncoScan® CNV Assay* on page 122
- Appendix F, *PicoGreen® dsDNA Quantification Protocol for OncoScan® CNV Samples* on page 127
- Appendix G, *Qubit® dsDNA Quantification Protocol for OncoScan® CNV Samples* on page 130

Handling the Normalized DNA Sample Plate (Reaction-ready DNA Sample Plate (6.6 µL/well at 12 ng/µL))

If a reaction-ready DNA sample plate was previously prepared with a volume of 6.6 µL/well at 12 ng/µL prior to running the assay, follow the instructions below to thaw the plate.

- Thaw the reaction-ready DNA sample plate (6.6 µL/well at 12 ng/µL) at room temperature for 10 - 15 minutes.
- Ensure the plate seal is tight and spin the plate down at 2400 rpm for 30 seconds.
- Vortex the plate at max speed for 4 seconds and spin down at 2400 rpm for 30 seconds.
- Keep it on a cold block until ready to be used in the Anneal reaction.

Controls

Using positive and negative controls is strongly recommended to assess the performance of each run. We recommend assaying the OncoScan® Positive Control supplied in the OncoScan® CNV FFPE Assay Kit as a positive control throughout the entire assay, starting from Anneal stage to Array Hybridization stage. We recommend processing the OncoScan® Negative Control supplied in the OncoScan® CNV FFPE Assay Kit as a negative control for the assay through the HaeIII gel QC stage only.

Reagent Handling and Storage

Proper storage and handling of reagents is essential for robust performance. Follow these guidelines to ensure best results:

- Use reagents that are not provided in this kit from the recommended vendors only.
- Store all reagents at the recommended temperatures and conditions. Do not use reagents that have been improperly stored. Storage methods can profoundly impact activity.
- Upon receipt of the reagent kit, store the Affymetrix® Nuclease-free water (P/N 902253) at 4°C for your convenience.
- Do not use expired reagents or reagents that have undergone more than the recommended number of freeze-thaw cycles.
- Seal all vials and bottle caps well after use to prevent evaporation.
- Do not store enzymes in a frost-free freezer.

! IMPORTANT: Always use the 24 reaction OncoScan® CNV Reagent Kit for this protocol. You can freeze/thaw the reagents in the 24 reaction kit up to 4 times.

- Store the reagents used for Anneal, Gap-fill and 1st PCR only in the Pre-PCR Lab.

When Using Reagents at the Lab Bench

- Properly chill essential equipment such as cooling blocks and reagent coolers before use.
- Unless otherwise indicated, keep all reagents (except for enzymes) on ice, or in a cooling block that has been chilled to 4°C and placed on ice during use.
- Ensure that enzymes are kept at –20°C until needed. When removed from the freezer, immediately place in a bench top reagent cooler that has been chilled to –20°C.
- Keep all tubes, master mixes and working solutions on ice or in chilled cooling blocks on ice.
- Since enzyme activity is a function of temperature, ensure that all temperature transitions to incubation temperatures are rapid and/or well controlled to help maintain consistency across samples.

Master Mix Preparation

Carefully follow each master mix recipe. Use pipettes that have been calibrated as per the manufacturer's specifications. Use only the Affymetrix Nuclease-free Water that is supplied with the kit. Do not use any other water. If the master mix runs out during the aliquoting process on any of these procedures, it suggests that a volume error has been made or the pipettes are not accurate. In this situation we strongly recommend repeating the master mix preparation.

Chilling the Assay Plate Before Reagent Addition

The reagents are ALWAYS added to chilled PCR plates (placed on the cold block) throughout the assay. Whenever a chilling step is called for, chill the plate on the cold block for 1 minute and then spin down at 2400 rpm for 30 seconds before adding the reagents.

Equipment and Calibration

Keep dedicated equipment for this protocol in each of the lab areas, including pipettes, ice buckets, coolers, etc. It is critical to use equipment that conforms to the guidelines and specifications detailed in this manual. To avoid contamination, do not move equipment back and forth from the Post-PCR Lab to the Pre-PCR Lab. Lab instrumentation plays an important role in the successful execution of this assay. To help maintain consistency across samples and operators, all equipment must be well maintained and routinely calibrated per manufacturer recommendations, including:

- All thermal cyclers
- GeneChip® Hybridization Oven 645
- GeneChip® Fluidics Station 450 or 450Dx
- GeneChip® Scanner 3000 7G or 3000Dx2
- Fluorescence Plate Reader or Qubit
- All single and multi-channel pipettes

Pipettes and Pipetting Recommendations

The types of pipettes specified for use throughout this protocol are:

- Single channel, manual
- 12-channel, manual or electronic
- Optional: 24-channel, manual or electronic

General Pipetting Recommendations

Since the OncoScan® CNV Assay protocol involves a series of ordered stages, the output of one stage directly impacts the performance of the subsequent stage. Many of the reagents in the OncoScan® CNV FFPE Assay Kit are in very viscous solutions. For best results:

- Always use pipettes that have been calibrated as per the manufacturer's specifications.
- It is essential that operators be proficient with the use of single and multi-channel pipettes.
- Always use filter tips for pipetting. This is essential to reduce sample contamination.
- Pipet slowly to allow enough time for the correct volume of solution to enter the pipette tip.
- Avoid excess solution on the outside of pipette tips.
- To ensure full volume transfer, check pipette tips after each pickup and dispense.
- To avoid the formation of air bubbles, dispense liquids at the bottom of each well.
- Always use the type and volume of pipette specified in the protocol.

To familiarize with the use of multi-channel pipettes, we strongly recommend practicing several times before processing actual samples. Water can be used to get a feel for aspirating and dispensing solutions to multiple wells simultaneously. Take special care to observe complete evacuation of liquid from all pipette tips when using a multichannel pipette.

Electronic Pipetting Recommendations

Follow the instructions provided with the pipettes for the dispense/mix program that:

- Allows reagents to be aspirated and dispensed at a set volume.
- Mixes automatically upon dispensing, wherein the mix volume can be different from the dispense volume.

Two options are available for tracking the number of mixes when using Rainin EDP3-Plus electronic pipettes: the counter option, or the beep option (pipette beeps after each mix). We recommend using the beep option, since the counter does not start at zero with each use. Instead, it counts pipette operations sequentially. Refer to the instructions provided with your pipettes for more information.

Seal, Vortex, and Spin

Unless otherwise noted, follow the instructions below when the protocol instructs you to seal, vortex and spin.

Handling the Plate Seal

- To minimize sample cross contamination and to ensure tight seals, use each seal only once. NEVER REUSE A SEAL. Discard used seals immediately to avoid contaminating equipment or working surfaces with DNA.
- The seal may become loose due to high temperature in the thermal cycler. Always ensure tight sealing before vortexing a plate.
- Whenever a plate is taken out of the thermal cycler, before continuing on to the next step, ensure that the seal is tight, spin the plate in the centrifuge, then remove the seal and discard.
- Whenever a plate is taken out of the freezer, first thaw the plate, ensure that the seal is tight, centrifuge, and only then remove the plate seal.
- When reaction setup is completed, always use a new seal to seal the plate.
- When applying the seal to a plate, press the seal tightly onto the plate using an adhesive film applicator. Using a plastic lid or a plastic tube rack is a potential source of contamination. Make sure that the seal is tight around all plate/well edges.

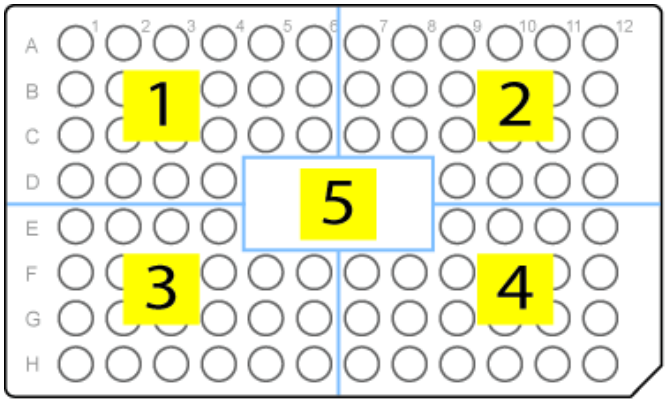
Sealing Strip Tubes

Cut adhesive seal into strips wide enough to seal 8 or 12 strip tubes. Alternatively, strip caps can also be used for sealing. Seal the strip tubes containing master mix with the adhesive strips or strip caps before spinning in the bench top quick spin microfuge.

Vortex

- **Master Mix tubes**: Vortex the master mix at high speed 3 times, 1 second each time.
- **Vortex reagents**: Vortex all reagents except the enzymes at high speed 3 times, 1 second each time. Ensure there is no precipitate. If a precipitate is observed, repeat vortexing.
- **Vortex enzyme**: Quick vortex once, 1 second.
- **Vortex plates**: High speed for 1 second each in all corners and in the center (Figure 4.1).

Figure 4.1 Vortex Plates at the Corners and Center



NOTE: We recommend using MicroAmp® Clear Adhesive Films to seal your plates.

IMPORTANT: Always ensure that your plates are tightly sealed. A tight seal will prevent sample loss and cross-well contamination, particularly when plates are being vortexed. NEVER REUSE A SEAL. ALWAYS USE A NEW SEAL.

Spin

When instructed to spin down plates or reagent vials, follow these guidelines unless otherwise instructed.

- **Plates**:
  - Spin at room temperature or at 4°C in a refrigerated centrifuge if available.
  - Start the centrifuge, allow it to reach 2400 rpm and spin at that speed for 1 minute.
- **Reagent Vials**: 3 seconds using bench top mini-centrifuge.
- **Enzyme Vials**: 3 seconds using bench top mini-centrifuge.

Guidelines for All Stages of the Assay

- Pre-chill the thawed reagents on ice.
- Pre-chill empty tubes for master mix on ice and empty strip tubes in a cold block on ice.
- Leave the enzymes at –20°C until ready to use and add the enzyme as the last reagent at any step.
- All reagent additions in all steps must be performed on ice.
- Always carry the sample plate to the centrifuge or the thermal cycler on the cooling block on ice.
- Always prepare the reagents ahead for the next step. Add the reagents for the next step immediately after chilling and spinning down the plate. Vortex, Spin down and proceed to the next step with 10 minutes of removing from the thermal cycler until the end of HaeIII step.
- Do not let the plate sit on the cold block for more than 10 minutes at any Pre-PCR assay steps including the Cleavage Reaction step.
- Do not let the plate sit on the thermal cycler for more than 30 minutes at the PCR and HaeIII steps.

Stopping Points on the Assay

- First PCR plate can be frozen if the assay can not be continued.
- HaeIII plate can be frozen at the end of HaeIII reaction, if the assay can not be continued. (DO NOT ADD hybridization cocktail to the HaeIII plate.)

Running Gels

- 3% Pre-cast Agarose 96-well format gels: Run gels at 150V for 15 minutes in the appropriate electrophoresis chamber.

Hybridization

- Load only 6 to 8 arrays at a time. Remove the seal from the hybridization plate for only 6-8 samples at a time.
- Preheat the hybridization oven to 49°C at least one hour prior to use.

Washing Arrays

It is important to work quickly when processing arrays for washing. Delays during this step will impact data quality. To optimize this step, we suggest the following:

- 30 minutes before hybridization is complete, prime the fluidics stations with the correct wash buffers.
- Start the Fluidics Protocol and follow the directions on the LCD panel of the fluidics station.
- Load Stain 1, Stain 2, and the Array Holding buffer in their respective positions on the fluidics station. Eject the wash block to avoid sensor time out.
- Process only 6-8 arrays at a time.
- Minimize delays when performing all steps after the arrays are removed from the oven, up to the time when washing begins.

Thermal Cyclers, 96-Well Plate, and Adhesive Seals

To run the OncoScan CNV FFPE Assay Kit Protocol at a throughput of 48 assays/day, you will need 2 thermal cyclers: 1 in the Pre-PCR Lab; 1 in the Post-PCR Lab.

The OncoScan CNV FFPE Assay Kit Protocol has been optimized using the following thermal cyclers, 96-well plate, and adhesive films.

! IMPORTANT: Use only the 96-well plate and adhesive seals listed in Table 4.1, and only the thermal cyclers listed in Table 4.2. Using other plates and seals that are incompatible with these thermal cyclers can result in loss of sample or poor results.

Please refer to Appendix B for critical information about programing and operating Veriti Thermal Cyclers.

Table 4.1 96-Well Plate and Adhesive Seals

Item	Vendor	Part Number
96-well Half-skirted PCR Plate	E&K Scientific	289196
MicroAmp® Clear Adhesive Film	Applied Biosystems	4306311

Table 4.2 Thermal Cyclers

Laboratory	Thermal Cyclers Validated for Use
Pre-PCR Clean Area Use one of these units.	Applied Biosystems Units: ▪ GeneAmp® PCR System 9700 (silver block or gold-plated silver block), P/N 4314878 ▪ Veriti® 96-Well Thermal Cycler, 0.2 mL, P/N 4375786
Post-PCR Area Use one of these units	Applied Biosystems Units: ▪ GeneAmp® PCR System 9700 (silver block or gold-plated silver block), P/N 4314878 ▪ Veriti® 96-Well Thermal Cycler, 0.2 mL, P/N 4375786

Program Your Thermal Cyclers

Use only calibrated thermal cyclers. We recommend that thermal cyclers be serviced at least once per year to ensure that they are operating within the manufacturer's specifications. The thermal cycler programs listed below are used in this protocol. Enter and save the following programs prior to processing samples on the appropriate thermal cycler in the Pre-PCR Lab and Post-PCR Lab.

Thermal cycler program details are listed in Appendix B, *Thermal Cycler Programs* on page 101. For Veriti Thermal Cyclers, please pay attention to the information in the section, *Important Information on Using the Veriti® Thermal Cycler* on page 102 regarding programming and operating the Veriti Thermal Cycler BEFORE creating any programs.

Pre-PCR Lab Thermal Cycler Programs

Set up the thermal cyclers in the Pre-PCR Lab to run the following programs:

- OncoScan Anneal
- OncoScan Gap Fill
- OncoScan 1st PCR

Post-PCR Lab Thermal Cycler Programs

Set up the thermal cyclers in the Post-PCR Lab to run the following programs:

- OncoScan 2nd PCR
- OncoScan HaeIII
- OncoScan Hybridization

Hybridization Oven

Confirm that the GeneChip® Hybridization Oven 645 is calibrated before starting the hybridization step. Accurate hybridization temperature is critical for this assay. We recommend servicing hybridization ovens at least once per year to ensure that they are operating within the manufacturer's specifications.

Quality Control Gel Recommendations

We recommend running two quality control gels during the execution of this assay protocol. Knowing in advance that a sample is unlikely to provide data of acceptable quality through in-process QC failures will save arrays. The purpose of each gel is described below.

- Gel 1, 1st Stage PCR: Run to identify any samples that did not amplify. For samples in which successful amplification has occurred, one single band at approximately120 base pairs should be seen. No distinct band at approximately 120 base pairs should be visible in the Negative Control and in samples in which amplification has not occurred. A smear or another unexpected band indicates the PCR did not work. These samples should not be processed for Array Hybridization.
- Gel 2, HaeIII Digest: Run after HaeIII digestion at 37°C to confirm acceptable gel banding pattern. The gel indicates both successful amplification during the second stage PCR reaction and HaeIII digestion. The predominant pattern should be two bands at approximately 40 and 70 bp.

NOTE: If the QC gel was run at the end of the HaeIII digest thermal cycler program (after the deactivation of the enzyme at 95°C), a smear, around 50-100 bp, will be observed instead of the two bands (40 bp and 70 bp). Hence, it is strongly recommended to run the HaeIII digest gel at the end of 37°C.

Safety Information

WARNING: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

CAUTION: All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as lab coat, safety glasses and gloves. Care should be taken to avoid contact with skin and eyes. In case of contact with skin or eyes, wash immediately with water. See SDS (Safety Data Sheet) for specific advice.

Chapter 5

FFPE DNA General Requirements for OncoScan® CNV Assay

The general requirements for the FFPE DNA extraction, purification and quantification methods are described in this chapter. The success of this assay requires accurate quantification of the FFPE DNA by PicoGreen® quantification method. Hence, Affymetrix strongly recommends using the tested protocols provided below. Affymetrix also recommends using the OncoScan® Positive Control supplied in the OncoScan® CNV Reagent Kit (Module P/N: 902688, Reagent P/N: 902249) as a positive control carried through the entire assay from Anneal up to hybridization on the arrays.

FFPE DNA Extraction and Purification for OncoScan® CNV Assay

IMPORTANT: Affymetrix strongly recommends using the QIAamp® DNA FFPE Tissue Kit protocol for purifying DNA from FFPE Blocks that will be used in the OncoScan® CNV Assay. For improved DNA yields, we also recommend a modification to the QIAamp DNA FFPE Tissue Kit protocol. The modified procedure adds a heating step at 98°C for 15 minutes to improve the tissue digestion process to release DNA from tissue sections.

The protocol found in Appendix E, *FFPE DNA Extraction Protocol for OncoScan® CNV Assay* is **required and critical** to generate good quality samples for the OncoScan® CNV Assay. DNA must be eluted using the elution reagent provided in the recommended kit (ATE buffer). DNA MUST NOT BE eluted in Water.

Other optional tested elution reagents:

- 1x TE Buffer with low EDTA at pH 8.0 (with 10 mM Tris at pH 8.0 and 0.1 mM EDTA)

Please refer to the QIAamp DNA FFPE Tissue Kit protocol for more information on the QIAGEN web page given below.

http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/Genomic-DNA/QIAamp-DNA-FFPE-Tissue-Kit#resources

NOTE: Affymetrix highly discourages the use of any sample preparation that is not silica based in OncoScan® CNV FFPE Assay.

dsDNA Quantification Protocol for OncoScan® CNV FFPE Samples

IMPORTANT: It is mandatory to determine the sample concentration using a dsDNA specific quantification method. We strongly recommend using either Quanit-iT™ PicoGreen Assay or Qubit dsDNA Quantification, both by Life Technologies, as both of these methods have been validated for use in the OncoScan® CNV FFPE Assay. Sample concentration determined by UV absorbance or NanoDrop must not be used at all in this assay.

Perform either of the two Affymetrix recommended and tested dsDNA quantitation protocols to measure the concentration of the eluted FFPE DNA. These protocols are provided in:

- Appendix F, *PicoGreen® dsDNA Quantification Protocol for OncoScan® CNV Samples* on page 127
- Appendix G, *Qubit® dsDNA Quantification Protocol for OncoScan® CNV Samples* on page 130,

NOTE: There are other dsDNA quantitation kits available that may deliver results different than the above mentioned kits. Hence, Affymetrix strongly recommends not using any kit other than the ones recommended above.

Sample Normalization (dilution to a working concentration of 12 ng/µL)

All genomic DNA samples should be normalized to a single concentration of 12 ng/µL using low EDTA 1X TE buffer (10mM Tris, 0.1mM EDTA, pH8.0). The positive control DNA that is included in the OncoScan® CNV FFPE Assay Kit is already normalized to a working concentration of ~12 ng/µL.

Preparing a Plate of Normalized FFPE Genomic DNA

A normalization plate is generated so that all DNA samples used in the assay are at the required volume (6.6 µL) and concentration (12 ng/µL) from the Stock DNA sample plate or Stock DNA vials.

Handling the Stock (original concentration) DNA Sample Plate for Normalization:

The steps provided below describe how to process a stock (original) DNA plate for sample normalization if the samples were already distributed in a "stock DNA sample plate". This is to ensure a complete resuspension of the DNA samples.

1. Thaw the stock DNA sample plate at room temperature for 15 minutes.
2. Ensure the plate seal is tight and spin the plate down at 2400 rpm for 30 seconds.
3. Vortex the plate at max speed for 4 seconds and spin down at 2400 rpm for 30 seconds.
4. Repeat Step 2 and Step 3 above.
5. Keep it on the cold block until ready to be used for preparing the normalized DNA plate.
6. When preparing the dilution or normalization plate, add the volume of reduced EDTA TE buffer (10mM Tris, pH8.0, 0.1mM EDTA, pH8.0) first before adding the volume of the stock DNA.
7. After addition of both reduced EDTA TE buffer and DNA, tightly seal and vortex the plate at max speed for 4 seconds. Spin the plate down at 2400 rpm for 30 seconds.
8. Keep the plate on the cold block until ready to be used in the Anneal reaction.

Handling the Stock (original concentration) DNA Sample Vials for Normalization:

The steps provided below describe how to process the stock DNA vials for sample normalization if the samples were stored in vials at –20°C. This is to ensure a complete resuspension of the DNA samples.

1. Thaw the DNA vials at room temperature for 15 minutes.
2. Vortex the vials at max speed for 4 seconds and spin down briefly.
3. Leave the vials at room temperature for 5 minutes and then repeat Step 2, above, again.
4. Keep the vials on ice until ready to be used for sample normalization.
5. When preparing the dilution or normalization plate, add the volume of low EDTA TE buffer (10mM Tris, pH8.0, 0.1mM EDTA, pH8.0) first before adding the volume of the stock DNA.
6. After addition of both reduced EDTA TE buffer and DNA, tightly seal and vortex the normalized sample plate (or vials) at max speed for 4 seconds.
7. Spin down the plate briefly and keep on ice until ready to be used in the Anneal reaction.

Chapter 6

OncoScan® CNV FFPE Assay Kit Protocol

The OncoScan® CNV FFPE Assay Kit Protocol is presented in stages. The stages are:

- *Stage 1 — Anneal*
- *Stage 2 — Gap Fill Through 1st PCR*
- *Stage 3 — First QC Gel and 2nd PCR (Post-PCR Lab)*
- *Stage 4 — HaeIII Digest and Second QC Gel*
- *Stage 5 — Hybridization*
- Chapter 7, *Washing, Staining and Scanning Arrays*

Preparing a Plate of Genomic DNA and a Batch Registration File

Genomic DNA Preparation

To perform the OncoScan® CNV FFPE Assay Kit Protocol, you will need to prepare a plate of genomic DNA (gDNA). The protocol is written for 24 samples (23 samples and 1 positive control) plus 1 negative control.

As Part of gDNA Plate Preparation:

1. Determine sample concentrations (we recommend using the *Quant-iT™ PicoGreen® dsDNA Assay Kit* from Life Technologies). Refer to *dsDNA Quantification Protocol for OncoScan® CNV FFPE Samples* on page 28.
2. Normalize all gDNA samples to a single concentration of 12 ng/µL using 1X TE (pH 8.0) with Reduced EDTA (0.1 mM EDTA). Refer to *Sample Normalization (dilution to a working concentration of 12 ng/µL)* on page 29.

   The OncoScan Positive Control included in the OncoScan® CNV Reagent Kit has already been normalized to a working concentration based on the concentration determined by PicoGreen quantification.

NOTE: Positive Control DNA may be retired from time to time as dictated by availability. New Positive Control DNA will be introduced if this occurs.

IMPORTANT: We strongly recommend you determine your sample concentration using the Quant-IT PicoGreen Assay by Life Technologies. Sample concentration determined by UV absorbance should not be used at all in this assay.

Batch Registration File Preparation

Sample information can be recorded in a Batch Registration File and uploaded into Affymetrix® GeneChip® Command Console® (AGCC). It is recommended to enter the sample information into a batch registration file when preparing the genomic DNA plate. During array preparation, array barcodes must be added to this file prior to uploading the information into AGCC.

Instructions on completing the sample information spreadsheet are listed in Appendix A, *Registering Samples in Affymetrix® GeneChip® Command Console®* on page 93.

NOTE: Make sure to select the OncoScan CNV array type to avoid issues with analysis.

Stage 1 — Anneal

About this Stage

This stage begins in the Pre-PCR Lab. During this stage, genomic DNA samples and controls, and reagents (Anneal Master Mix) are combined in an *Anneal Plate*.

The Anneal Plate is then placed on a thermal cycler and the program, ***OncoScan Anneal***, is run. Because the samples must be left to anneal for 16 to 18 hr, this stage is typically performed at the end of the day, and the program is allowed to run overnight.

Location and Duration

- Pre-PCR Lab
- Hands-on time: approximately 45 min
- Thermal cycler time: 16 to 18 hr

Equipment and Materials Required

The following equipment and materials are required to perform this stage.

In the Pre-PCR Lab

Table 6.1 Equipment and Materials Required in Pre-PCR Lab for *Stage 1 — Anneal*

Quantity	Item
2	Cold block, 96-well, chilled in 4°C refrigerator
1	Centrifuge, plate
1	Eppendorf Nuclease-free tubes, 1.5 mL
As required	Strip tubes, strip caps
1	Ice container, rectangular, filled with ice
1	Marking pen, extra fine point, permanent
As required	MicroAmp Clear Adhesive Films
1	Microcentrifuge for vials
1	Microfuge for strip tubes
1	Half-skirt 96-well plate
1 each	Pipette: ▪ Single-channel P20 ▪ Single-channel P200 ▪ Single-channel P1000 ▪ 12-channel P200 ▪ 12- or 24-channel P20
As required	Pipette tips for the pipettes listed above
1	Thermal Cycler, 96-well GeneAmp PCR System 9700 (gold or silver block) or Veriti Thermal Cycler
1	Compression Pad for Thermal Cycler
1	Vortexer
5-23	Customer dependent DNA

OncoScan® CNV Reagent Components Required

Table 6.2 OncoScan® CNV Reagent Components Required

OncoScan® CNV Reagents from Module OncoScan® CNV Buffer C
Buffer C

Table 6.3 OncoScan® CNV Reagent Components Required

OncoScan® CNV Reagents from Module OncoScan® CNV Copy Number Probe Mix 1.0 & Controls
Copy Number Probe Mix 1.0
Positive Control
Negative Control
Buffer A

Thaw Reagents and Prepare for Anneal Program

Location: Pre-PCR Lab

1. Ensure the thermal cycler is setup to run the program at the appropriate ramp speed (max for the 9700 and 9700-Max-Mode setting for the Veriti Thermal Cycler. Refer to *Ramp Speed Settings for Veriti® Thermal Cycler* on page 102.
2. Ensure all 3 programs (***OncoScan Anneal, OncoScan Gapfill, OncoScan 1stPCR***) have been created in the thermal cycler.

   NOTE: Refer to Appendix B, *Setting the Ramp Speed and Volume for Each Program* for detailed instructions. For Veriti Thermal Cyclers, refer to the information on converting a method to create a method for the Veriti Thermal Cycler with ramp rates that simulate those on the 9700 Thermal Cycler at Max mode.

3. If the DNA sample plate has been prepared as an Anneal-ready plate (containing 6.6 µL of DNA per well at 12 ng/µL), do not transfer the samples to a new Anneal plate which could result in sample loss. Label the same sample plate as "Anneal" and mark the sample wells (Row A, or Rows A & E) as specified in the *OncoScan® CNV FFPE Assay Kit Quick Reference Card*, (7 Sample QRC, P/N 703303; 9 Sample QRC, P/N 703304; 13 Sample QRC, P/N 703305; 25 Sample QRC, P/N 703306).

4. The Master Mix preparation tables throughout the assay contain the information on the appropriate volume of the reagent for processing 7, 9, 13 or 25 reactions in columns 4, 5, 6, and 7 respectively. Hence,
   - For processing 7 reactions (5 gDNA samples plus 1 positive control and 1 negative control), use information from column-4 in the reagent master mix tables at each step of the assay. This column will provide the reagent volume needed for processing 7 anneal reactions, 14 Post-GapFill reactions after channel-split and hybridizing 12 arrays.
   - For processing 9 reactions (7 gDNA samples plus 1 positive control and 1 negative control), use information from column-5 in the reagent master mix tables at each step of the assay. This column will provide the reagent volume needed for processing 9 anneal reactions, 18 Post-GapFill reactions after channel-split and hybridizing 16 arrays.
   - For processing 13 reactions (11 gDNA samples plus 1 positive control and 1 negative control), use information from column-6 in the reagent master mix tables at each step of the assay. This column will provide the reagent volume needed for processing 13 anneal reactions, 26 Post-GapFill reactions after channel-split and hybridizing 24 arrays.
   - For processing 25 reactions (23 gDNA samples plus 1 positive control and 1 negative control), use information from column-7 in the reagent master mix tables at each step of the assay. This column will provide the reagent volume needed for processing 25 anneal reactions, 50 Post-GapFill reactions after channel-split and hybridizing 48 arrays.

Table 6.4

Number of DNA Samples Processed	Number of Tubes per Tube Strip Needed to Aliquot Master Mix	Master Mix Column (from the table) to be Used
7 Samples	7 Tube Strip	Column 4
9 Samples	9 Tube Strip	Column 5
13 Samples	12 Tube Strip	Column 6
25 Samples	12 Tube Strip	Column 7

Prepare for Anneal Program

1. Turn on a thermal cycler and allow the lid to heat up.
2. Setup an ice pan and place two cold blocks on ice.
3. Label a 96-well PCR plate *ANN* that will be used for Anneal. Place it on a cold block and keep it covered.

Thawing the DNA Sample Plate (Normalized at 12 ng/µL)

1. Remove the DNA sample plate (prepared in section *Genomic DNA Preparation* on page 30 and has been normalized to 12 ng/µL) from –20°C and thaw at room temperature for 15-30 minutes. Ensure the seal is tight.
2. Once the DNA has been completely thawed, make sure the seal is tight and then vortex the plate at max speed for 5 seconds and spin the sample plate and place it on the cold block.
3. Aliquot 6.6 µL of the DNA to the "ANNEAL" plate in the corresponding wells of the Anneal Plate as entered in the Batch Registration File.
4. Mark the reaction wells with a permanent marker for easier addition of reagents. Keep the plate covered with a plate sealing film on a cold block until ready to use.

To thaw the reagents:

1. Remove the following reagents from the OncoScan® CNV Reagent Kit.
   - Buffer A
   - Copy Number Probe Mix 1.0
   - Buffer C
2. Thaw them at room temperature for 10-15 minutes.
3. After the vials have been completely thawed, vortex them at max speed for 3 seconds, spin down briefly and place them on ice.

Prepare the Anneal Master Mix

Location: Pre-PCR Lab

To Prepare the Anneal Master Mix:

1. Label a 1.5 mL Eppendorf tube with *Ann*.
2. Place a tube strip as required by the number of samples in a cold block and the *Ann* Eppendorf tube on ice until ready to use.
3. To the tube labeled *Ann*, add the reagents listed in Table 6.5 in the order shown.
4. Vortex between additions. Vortex entire Anneal Master Mix for 3 sec at max speed and spin down briefly.
5. Using the column appropriate to the number of reactions, aliquot the specific volumes of Anneal Master Mix from Table 6.5 to a corresponding tube strip.
6. Keep the aliquoted strip tube covered on a cold block until ready to use.

Table 6.5 Anneal Master Mix

Number of Runs per Assay Kit			**4**	**3**	**2**	**1**
Number of Samples (includes 1 positive control per run)			6	8	12	24
Negative Control per Run			1	1	1	1
Reagent	**P/N**	**1 Reaction**	**7 Reactions (~60% overage)**	**9 Reaction (~60% overage)s**	**13 Reaction (~60% overage)**	**25 Reaction (~60% overage)**
Buffer A	902246	1.53 µL	17.1 µL	22.0 µL	31.8 µL	61.2 µL
Copy Number Probe Mix 1.0	902248	1.37 µL	15.3 µL	19.7 µL	28.5 µL	54.8 µL
Buffer C	902696	0.5 µL	5.6 µL	7.2 µL	10.4 µL	20.0 µL
Total Volume		**3.40 µL**	**38.1 µL**	**49.0 µL**	**70.7 µL**	**136.0 µL**
Volume per Tube for Tube Strip			5.0 µL 7-Tube Strip	5.0 µL 9-Tube Strip	5.0 µL 12-Tube Strip	10 µL 12-Tube Strip

Addition of Anneal Master Mix to Anneal Plate

Location: Pre-PCR Lab

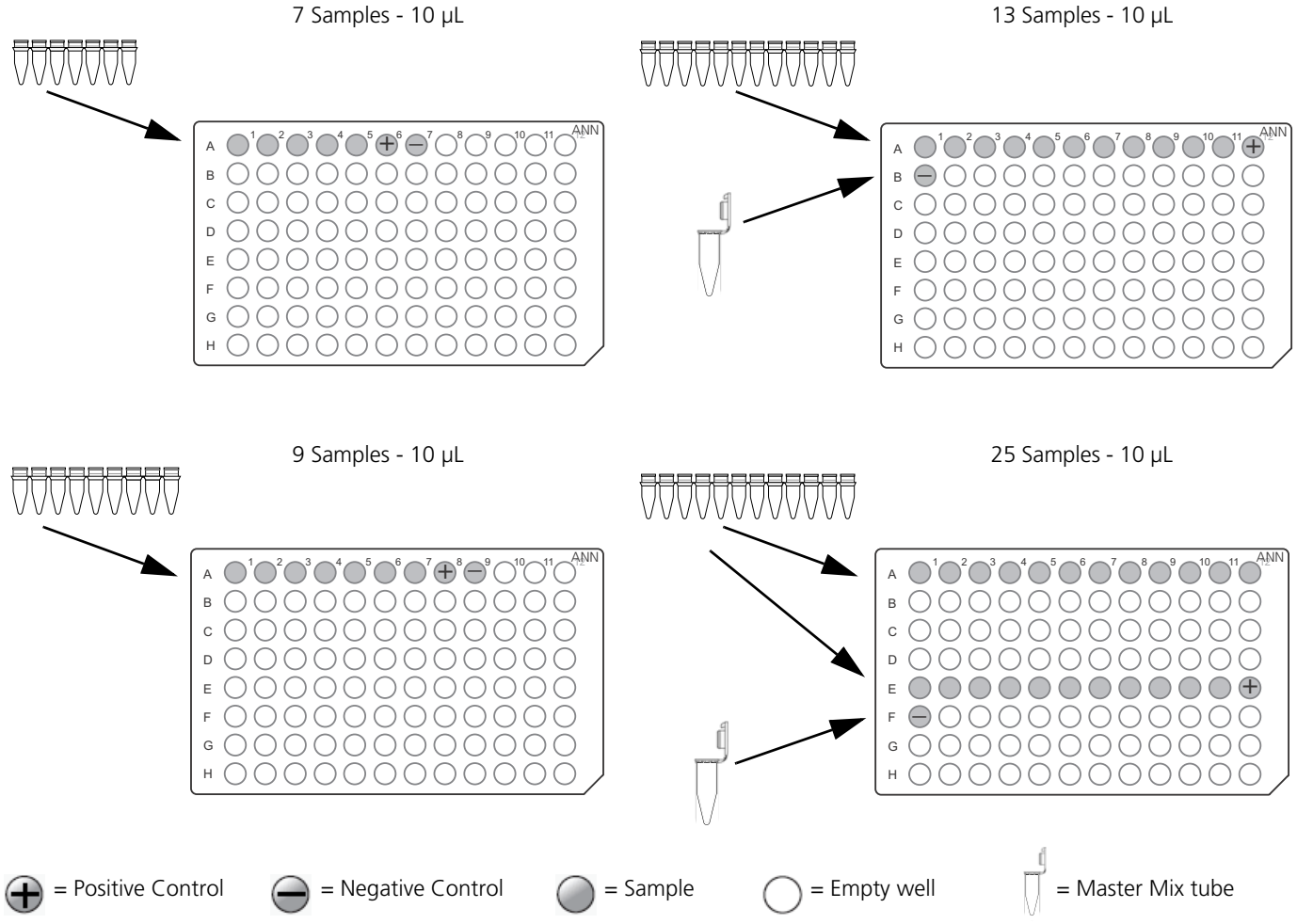
To Transfer Anneal Master Mix to Samples in Anneal Plate:

1. Using a 12-channel P20 pipette, transfer 3.4 µL of Anneal Master Mix from the strip tube to specific rows depending on the sample size being processed on the Anneal Plate. Pipet up and down 3 times to rinse tips (Figure 6.1).

NOTE: For the 13 or 25 Sample Reaction, add 3.4 µL of Anneal Master Mix from the Master Mix tube to the Negative Control sample (Figure 6.1).

NOTE: Now the current volume in the Anneal Plate is 10 µL.

Figure 6.1 Adding Anneal Master Mix to Anneal Plate



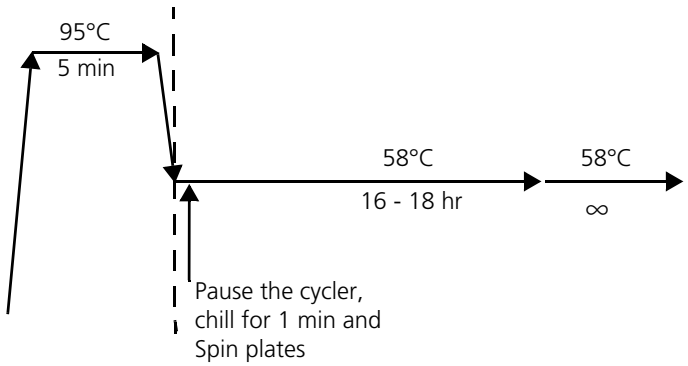
2. Tightly seal the Anneal Plate with a clear adhesive film. Vortex the Anneal Plate at max speed for 5 seconds using the 5-point plate vortexing method. Spin down the plate at 2400 rpm for 60 seconds.
3. Place the plate on the thermal cycler and start the ***OncoScan Anneal*** program.
4. After 6 minutes into the run, pause the program at 58°C (1 min into 58°C). Remove the ANNEAL plate from the thermal cycler and place on a chilled cold block on ice for 1 minute. Ensure the seal is tight.

5. Spin down the plate at 2400 rpm for 30 seconds.
6. Place the ANNEAL plate back on the thermal cycler and press **Start** one more time to *Resume*.

NOTE: It is good practice to double check that the thermal cycler program did resume. Check all programs after pressing Resume to make sure the program is indeed running.

7. Incubate the samples for 16 to 18 hours. Do not incubate samples for more than 18 hours.

Figure 6.2 OncoScan Anneal Thermal Cycler Program



IMPORTANT: Throughout the protocol, when carrying any 96 well plate to and from the centrifuge, carry the plate in the chilled cold block to keep the reactions as cold as possible.

Stage 2 — Gap Fill Through 1st PCR

About this Stage

During this stage, Gap Fill Mix is added to each reaction. Then the samples are transferred from the Anneal Plate to 1st PCR Plate.

The 1st PCR Plate is then placed on a thermal cycler and the program ***OncoScan Gap Fill*** is started. During the first 82 min of this program, five additional reagents are added to the Assay Plate, one reagent at a time. Prior to each addition, the plate is removed from the thermal cycler and cooled on the cold block for 1 min and spun down at 2400 rpm for 30 sec.

The OncoScan® CNV Reagents that the operator is adding during thermal cycling are found in the module **Gap Fill and 1st stage PCR**:

1. Gap Fill Mix
2. dNTP Mix
3. Exo Mix
4. Cleavage Mix
5. PCR Mix

Location and Duration

- Pre-PCR Lab
- Hands-on time: 1 hour 15 min
- Thermal cycler time: 1 hr 20 min

Input Required from Previous Stage

Table 6.6

Item
Anneal Plate

Equipment and Materials Required

Table 6.7 Equipment and Materials Required for *Stage 2 — Gap Fill Through 1st PCR*

Quantity	Item
4	Cold block, chilled in 4°C refrigerator
1	Anneal Plate from previous stage
1	Centrifuge, plate
1	Ice container, rectangular, filled with ice
As required	MicroAmp Clear Adhesive Films
As required	Microcentrifuge for tubes and strips

Table 6.7 Equipment and Materials Required for *Stage 2 — Gap Fill Through 1st PCR* (Continued)

Quantity	Item
1 each	Pipettes: ▪ Single-channel P20 ▪ Single-channel P200 ▪ Single-channel P1000 ▪ 12-channel P200 ▪ 12- or 24-channel P20
As required	Filtered Pipette tips fitted for the above pipettes
1	Half-skirt PCR Plate
1	Bench top Cooler
1	Thermal Cyclers, 96-well GeneAmp PCR System 9700 (gold or silver block), Veriti Thermal Cycler
As required	Tubes, Eppendorf 2.0 mL, Nuclease-free
As required	Tubes, Eppendorf 1.5 mL, Nuclease-free
4	Tube strips with caps, PCR 12-well
1	Blue Permanent Marker, Extra fine tip
1	Red Permanent Marker, Extra fine tip
1	Timer

OncoScan® CNV Reagent Components Required

Table 6.8 OncoScan® CNV Reagent Components Required

OncoScan® CNV Reagents from Module OncoScan® CNV Gap Fill and 1st Stage PCR
Nuclease-free Water
Buffer A
Gap Fill Enzyme Mix
SAP, Recombinant
dNTP Mix (AT), dNTP Mix (GC)
Exo Mix
Cleavage Buffer
Cleavage Enzyme
PCR Mix
Taq Polymerase

Thaw OncoScan® CNV Reagents

IMPORTANT: Leave the Exo Mix, Cleavage Enzyme, Gap Fill, SAP and the Taq Polymerase at –20°C until ready to use.

To Thaw the Reagents:

1. Place the Buffer A, dNTP AT Mix and dNTP GC Mix, Cleavage Buffer, water and PCR Mix on the bench top at room temperature for 10-15 minutes to thaw.
2. Vortex all of the reagents at max speed for 3-6 seconds, spin down and keep on ice until ready to use.

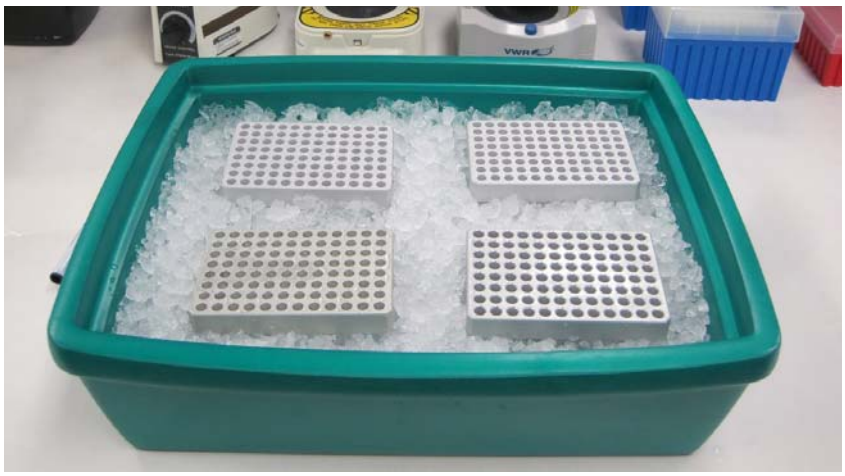
NOTE: It is highly recommended to prepare the AT Mix and GC Mix separately and using a new pair of gloves for each mix. It is also useful to label all wells, tubes and strips for dNTP AT mix in a different colored lab marker relative to the dNTP GC mix. This will help in easy identification of the different dNTP mixes and avoid a mix-up.

Prepare the AT Mix and GC Mix

To Prepare the AT Mix and GC Mix

Place 4 chilled cold blocks in a rectangular ice bucket filled to the rim with ice.

Figure 6.3 Cold Blocks in Ice Bucket



NOTE: Avoid any type of contamination between the AT and GC mixes during preparation. Mark the tubes and strips as specified below and keep them in separate cold blocks.

1. Label two 1.5 mL Eppendorf tubes for each dNTP master mix as follows: label one tube AT with a blue colored marker and the other tube GC with a red colored marker. Place these tubes on ice until ready to use.
2. Label two sets of strip tubes as follows: label one set of strip tubes AT in blue colored marker and the other set of strip tubes GC in red colored marker. Place both sets of labeled strip tubes in a cold block on ice until ready to use.
3. To the tube labeled AT, use the appropriate column in Table 6.9 to add the reagents in the order shown. Rinse the pipette tips three times.
4. Vortex the entire AT Master Mix tube for 3 sec at maximum speed. Spin down briefly in microfuge.
5. Using the column appropriate to the number of reactions from Table 6.9, aliquot the specific volumes of the AT Mix from Table 6.9 to the strip tubes labeled AT. Cover the strip tube with caps or a strip from the MicroAmp adhesive film. Spin down the strip tubes if needed in case of bubbles.

6. Keep the strip tube on chilled cold blocks until ready to use.

NOTE: In Table 6.9 and Table 6.10, you will notice the master mix volumes are the same for the 7, 9 and 13 sample workflows. Irrespective of the number of samples processed, the A/T and G/C master mixes are always prepared for 24 samples, due to the small volume of the reagents used in master mix preparation. Discard any leftover master mixes and always prepare them fresh on the day of Gap Fill.

Table 6.9 A/T Mix

Reagent	P/N	1 Reaction	7 Reactions (~20% overage)	9 Reactions (~20% overage)	13 Reactions (~20% overage)	25 Reactions (~20% overage)
Water	902253	3.93 µL	113 µL	113 µL	113 µL	118 µL
dNTPs (A/T)	902254	0.07 µL	2.1 µL	2.1 µL	2.1 µL	2.2 µL
Total		**4.0 µL**	**115 µL**	**115 µL**	**115 µL**	**120 µL**
Volume per Tube for Tube Strip			9.6 µL 7-Tube Strip	9.6 µL 9-Tube Strip	9.0 µL 12-Tube Strip	9.6 µL 12-Tube Strip

NOTE: CHANGE GLOVES. Use fresh gloves while preparing the G/C mix to avoid contamination.

7. To the tube labeled GC, using the column appropriate to the number of reactions add the reagents listed in Table 6.10 in the order shown. Rinse the pipette tips three times.
8. Vortex the entire GC Master Mix tube for 3 sec each at maximum speed. Spin down briefly at 2400 rpm.
9. Using the column appropriate to the number of reactions from Table 6.10, aliquot the specific volumes of the GC Mix from Table 6.10 to the strip tube labeled GC. Cover the strip tube with caps or strips of plate seal. Spin down the strip tube if needed in case of bubbles.
10. Keep the strip tube on chilled cold blocks until ready to use.

Table 6.10 G/C Mix

Reagent	P/N	1 Reaction	7 Reactions (~20% overage)	9 Reactions (~20% overage)	13 Reactions (~20% overage)	25 Reactions (~20% overage)
Water	902253	3.93 µL	113 µL	113 µL	113 µL	118 µL
dNTPs (G/C)	902255	0.07 µL	2.1 µL	2.1 µL	2.1 µL	2.2 µL
Total		**4.0 µL**	**115 µL**	**115 µL**	**115 µL**	**120 µL**
Volume per Tube for Tube Strip			9.6 µL 7-Tube Strip	9.6 µL 9-Tube Strip	9.0 µL 12-Tube Strip	9.6 µL 12-Tube Strip

Prepare Gap Fill Mix

To Prepare the Gap Fill Mix:

NOTE: CHANGE GLOVES. Use fresh gloves to prepare the Gap-Fill Master Mix.

1. Label a 1.5 mL Eppendorf tube and one set of strip tubes with the letter *G*.
2. Place the stripe tubes in a chilled cold block and the labeled 1.5 mL Eppendorf tube on ice until ready to use.

3. To the tube labeled *G*, using the column appropriate to the number of reactions add the reagents in Table 6.11 in the order shown. Pipet up and down 3 times to rinse tips.

NOTE: Gap Fill Mix contain 50% glycerol, so pipet slowly.

4. Vortex between additions. Vortex the entire Gap Fill Mix for 3 sec at maximum speed. Spin down briefly in microfuge.
5. Using the column appropriate to the number of reactions, aliquot the specific volumes of the Gap Fill Master Mix from Table 6.11 to a 12-tube strip. Cover the strip tube with cap. Spin down the strip tube and place on a chilled cold block until ready to use.

NOTE: In case user has a 8-strip tube microfuge, user can cut 12-tube strip accordingly to fit 8-tube microfuge.

Table 6.11 Gap Fill Master Mix

Reagent	P/N	1 Reaction	7 Reactions (~20% overage)	9 Reactions (~20% overage)	13 Reactions (~20% overage)	25 Reactions (~20% overage)
Water	902253	10.58 µL	89 µL	114 µL	165 µL	318 µL
Buffer A	902246	1.18 µL	9.9 µL	12.7 µL	18.3 µL	35.3 µL
SAP, Recombinant (1U/µL)	902251	0.84 µL	7.1 µL	9.1 µL	13.1 µL	25.2 µL
Gap Fill Enzyme Mix	902252	1.40 µL	11.8 µL	15.1 µL	21.8 µL	42.0 µL
Total		**14.0 µL**	**117.6 µL**	**151.2 µL**	**218.4 µL**	**420 µL**
Volume per Tube for Tube Strip			16.0 µL 7-Tube Strip	16.0 µL 9-Tube Strip	16.5 µL 12-Tube Strip	33 µL 12-Tube Strip

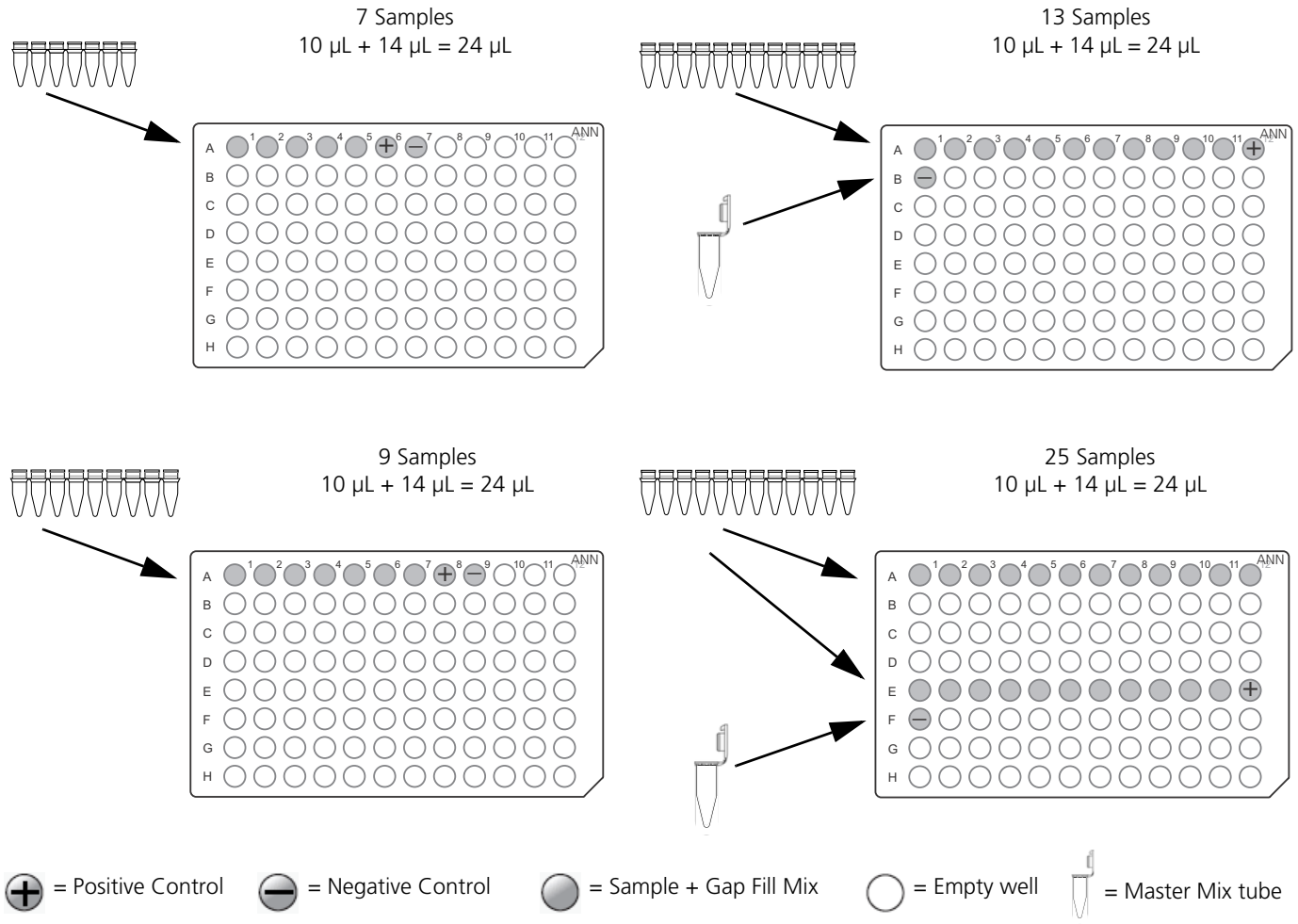
Add Gap Fill Mix

CAUTION: Make sure the plate is in the correct orientation (well A1 at the top left position) before pipetting the reagents from the strip tube to the plate.

To Add the Gap Fill Mix:

1. Remove plate ANNEAL from the thermal cycler, place in a cold block on ice for 1 min; as soon as you put the plate on ice, stop the *OncoScan Anneal* program and start the *OncoScan Gap Fill* program on the thermal cycler and wait for the thermal cycler to reach 58˚C, then pause the program.
2. After the 1 min incubation on ice, spin the *ANNEAL* Plate at 2400 rpm for 60 sec and return it to the cold block.
3. Slowly and carefully remove the adhesive seal in order to avoid any contamination through sample splashing.
4. Using a 12-channel P20 pipette, add 14.0 µL Gap Fill Mix to each reaction in the appropriate rows, as shown in Figure 6.4 below. Pipet up and down 3 times to rinse tips.
   - For the 13 or 25 Sample Reaction, add 14 µL of Gap-Fill Master Mix from the G Master Mix Tube to the Negative Control sample.
5. Tightly seal the plate, vortex at high speed for 5 sec, then spin down the Anneal Plate at 2400 rpm for 60 sec. Keep the Anneal Plate on the cold block. The current volume in the Anneal Plate is now 24.0 µL.

Figure 6.4 Adding Gap Fill Mix to ANNEAL PLATE



Channel Split

CAUTION: When transferring sample volumes from one 96-well plate to another 96-well plate, make sure BOTH plates are at the correct orientation (well A1 at top left) before pipetting.

1. Label a fresh 96-well half-skirt PCR plate *1st PCR*, clearly marking the alternating AT and GC rows with blue and red colored markers respectively for each row of sample used. **(Label rows A & E as "AT" with a Blue marker and Rows C & G as "GC" with a Red marker (see Figure 6.6). Mark the reaction wells in the same colors).**
   - The samples from the A row on the ANNEAL Plate will be split into the A and C rows on the *1st PCR Plate*.
   - The samples from the E row on the ANNEAL Plate will be split into rows E and G on the *1st PCR Plate*.

   NOTE: For samples less than or equal to 12 on the anneal plate, it will be split into two rows on the Assay Plate for AT and GC in row A & C respectively, as shown in Figure 6.6.
2. Using a 12-channel P20 pipette, transfer 10.0 µL each reaction from the ANNEAL Plate to the *1st PCR* Plate, as shown in Figure 6.5 and Figure 6.6.
   - For the 13 Sample Reaction, add 10 µL of the Negative Control sample into well B01 & 10 µL into well D01.
   - For the 25 Sample Reaction, add 10 µL of the Negative Control sample into well F01 & 10 µL into well H01.
3. Tightly seal the *1st PCR* Plate; then spin down at 2400 rpm for 60 sec.
4. Load the plate on the thermal cycler and resume the program to run (Figure 6.7 on page 45).

Figure 6.5

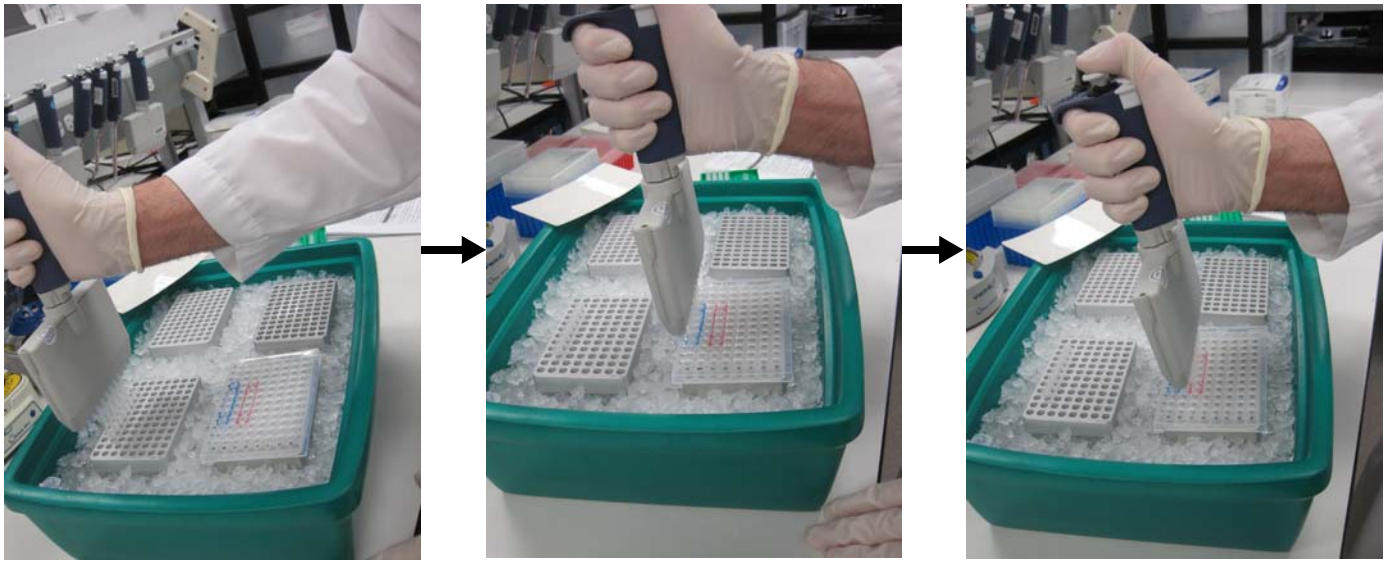


Figure 6.6 Preparing the Gap Fill - 1st PCR Plate

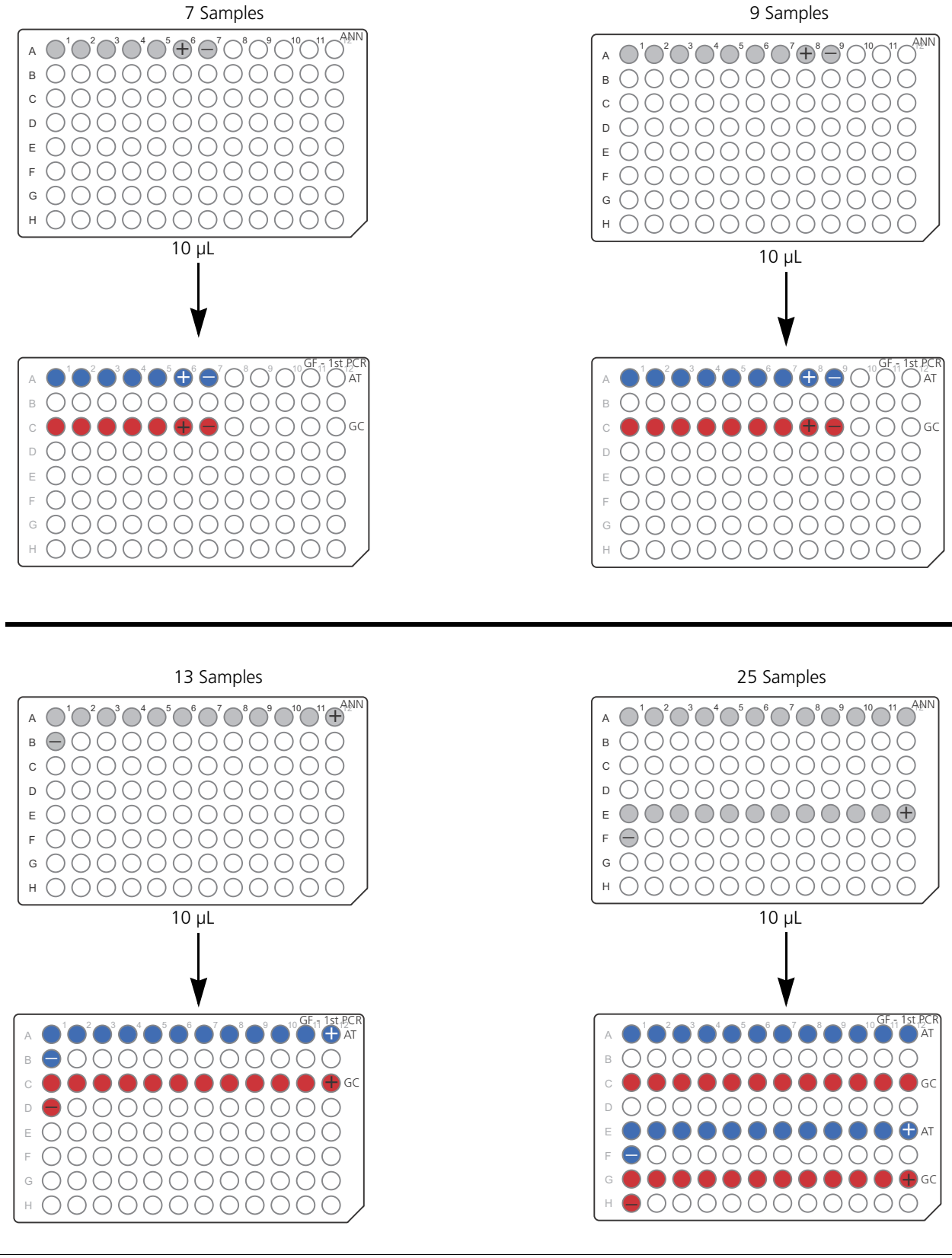


Figure 6.7 OncoScan Gap Fill Thermal Cycler Program (for GeneAmp® PCR System 9700)

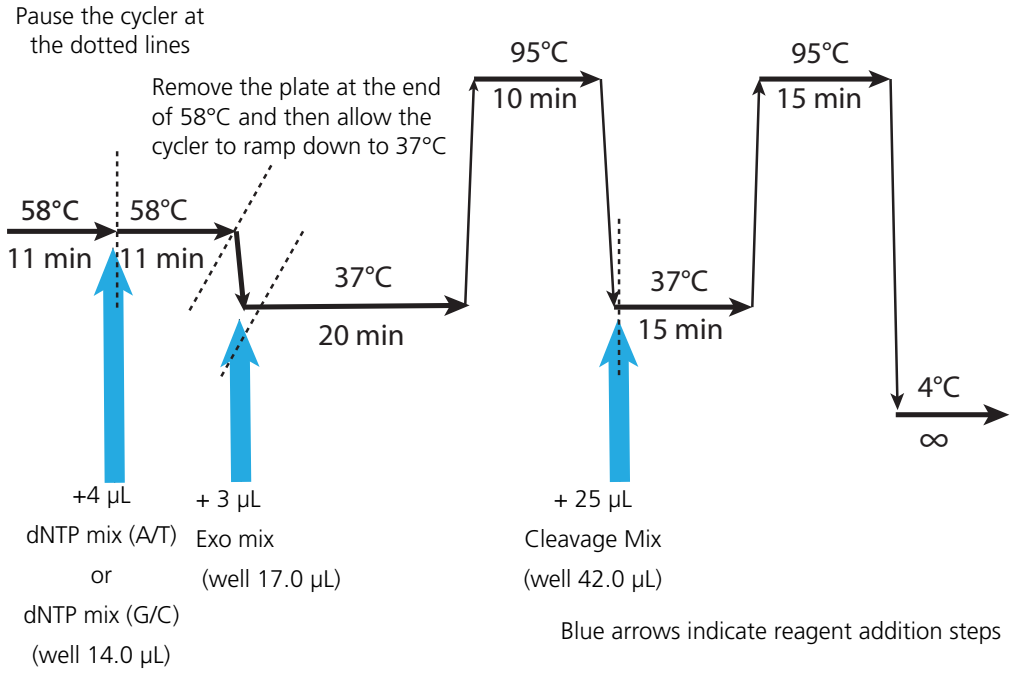
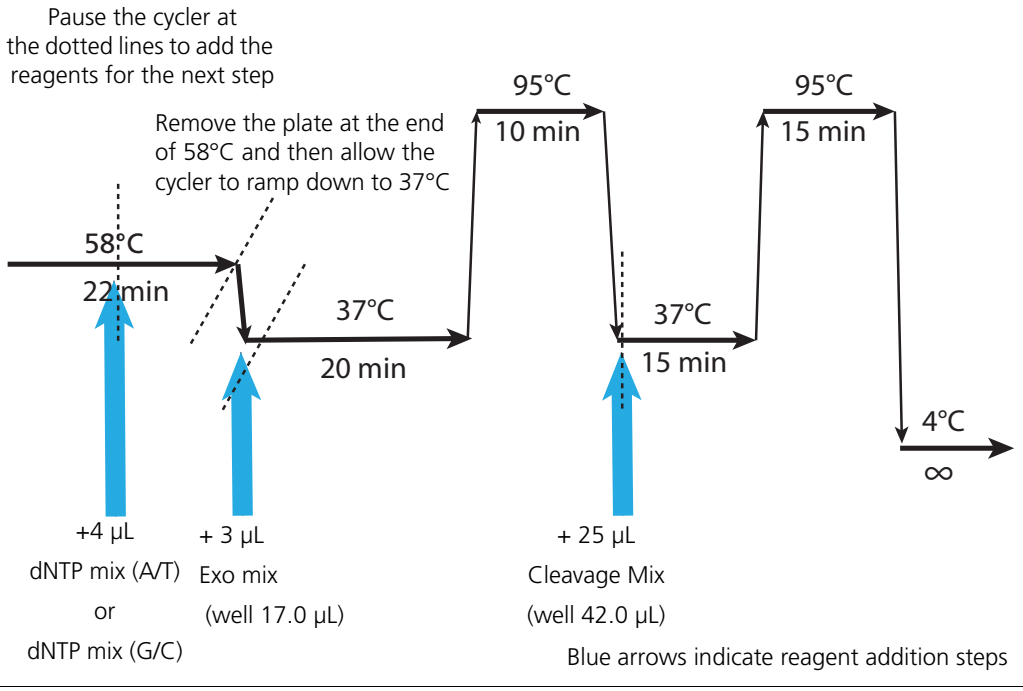


Figure 6.8 OncoScan Gap Fill Thermal Cycler Program (for **Veriti Thermal Cycler**)



NOTE: For the Veriti Thermal Cycler, when programming the "OncoScan Gap Fill" program, combine the first two 58°C incubations for 11 min each into one stage at 58°C for 22 min (see Table B.2 on page 105 and Figure B.5 on page 106). This change is recommended because it has been noticed that the touch screen does not consistently respond to the "PAUSE RUN" function when paused between the two stages of 58°C. (The touch screen may not pause at the end of the 58°C at 11 min and will move on to the next 58°C stage). This will ruin the Gap Fill reaction, leading to assay failure. Using a laboratory timer is critical during the first two 58°C incubations. Make sure the program pauses when the "PAUSE RUN" is touched.

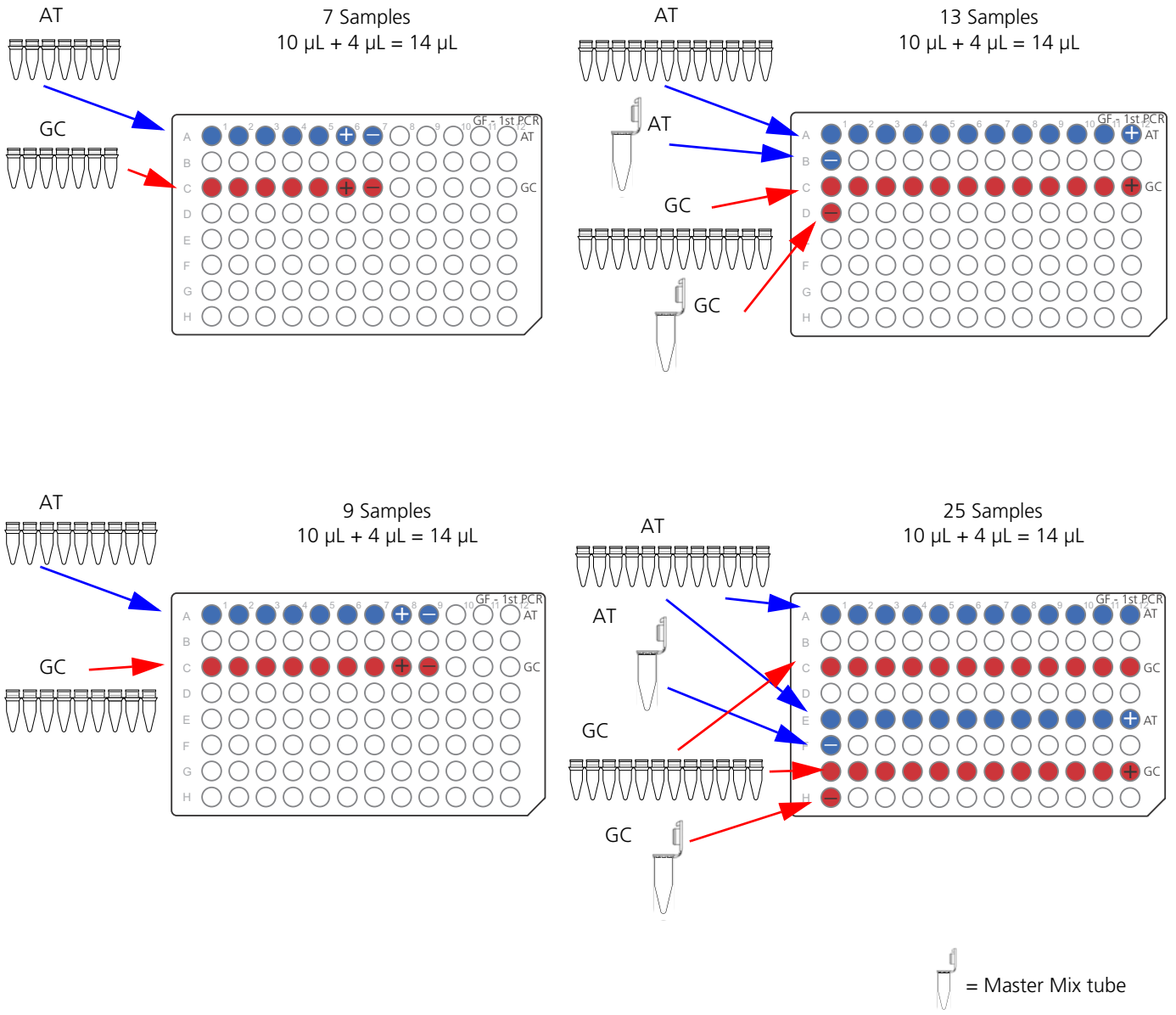
NOTE: It is strongly recommended to use a timer during this stage of the OncoScan® CNV FFPE Assay Protocol so as not to miss pausing the thermal cycler or important reagent additions.

Add the dNTP AT Mix and dNTP GC Mix

To Add the AT Mix and GC Mix to the Assay Plate:

1. After 11 min at 58°C, press *Pause* on the thermal cycler and remove the *1st PCR* Plate.
2. Place the plate in a cold block on ice for 1 min.
3. Spin down at 2400 rpm for 30 sec. Remove the *1st PCR* Plate from the centrifuge and return the plate to the cold block on ice.
4. Ensure the plate is at the correct orientation (Well A1 at top left). Carefully remove the adhesive film in a slow motion to avoid any contamination between samples in wells.
5. Using a 12-channel P20 pipette, add 4 µL of AT Mix to:
   - row A on the *1st PCR* Plate for 7, 9 and 13 reactions. Pipet up and down 3 times to rinse tips (Figure 6.9).
     - For the 13 Sample Reaction, add 4 µL of AT Mix from the Master Mix tube to the well B01 on the *1st PCR* Plate (Figure 6.9).
   - rows A and E on the *1st PCR* Plate for 25 reactions. Pipet up and down 3 times to rinse tips (Figure 6.9).
     - For the 25th sample reaction, add 4 µL of the AT Mix from the Master Mix tube to the well F01 on the *1st PCR Plate*.
6. Using a 12-channel P20 pipette, add 4 µL of GC Mix to:
   - row C on the *1st PCR* Plate for 7, 9 and 13 reactions. Pipet up and down 3 times to rinse tips (Figure 6.9).
     - For the 13 Sample Reaction, add 4 µL of GC Mix from the Master Mix tube to the well D01 on the *1st PCR* Plate (Figure 6.9).
   - rows C and G on the *1st PCR* Plate for 25 reactions. Pipet up and down 3 times to rinse tips.
     - For the 25 Sample Reaction, add 4 µL of GC Mix from the Master Mix tube to the well H01 on the *1st PCR* Plate (Figure 6.9).

Figure 6.9 Adding the dNTP AT Mix and dNTP GC Mix



NOTE: The current volume in the 1st PCR Plate for each sample in the wells is 14 µL.

7. Tightly seal the plate, vortex at high speed for 5 sec; then spin down at 2400 rpm for 60 sec.
8. Place the *1st PCR* Plate back on the thermal cycler and press *Resume*.
9. Set a timer for 10 min to be back in the lab to pause the thermal cycler **at 58**°C immediately after the 11 min incubation.

NOTE: During the 58°C incubation for 11 min, prepare the Exo mix.

NOTE: It is CRITICAL to remove the assay plate at 58°C, BEFORE the cycler ramps down to 37°C to avoid possible non-specific Gapfill.

Prepare and Add the Exo Mix

NOTE: Starting from this step onward until the end of the assay, the number of reactions will double due to AT & GC Channel split. Hence all of the master mixes, from the addition of Exo Mix and beyond, will be prepared to account for twice the number of reactions. The reactions must be carried out in the same plate layout until the end of the assay.

- **7 anneal reactions (6 samples including a positive control + 1 Negative control) will be processed as 14 reactions.**
- **9 anneal reactions (8 samples including a positive control + 1 Negative control) will be processed as 18 reactions.**
- **13 anneal reactions (12 samples including a positive control + 1 Negative control) will be processed as 26 reactions.**
- **25 anneal reactions (24 samples including a positive control + 1 Negative control) will be processed as 50 reactions.**

To Prepare and Add the Exo Mix:

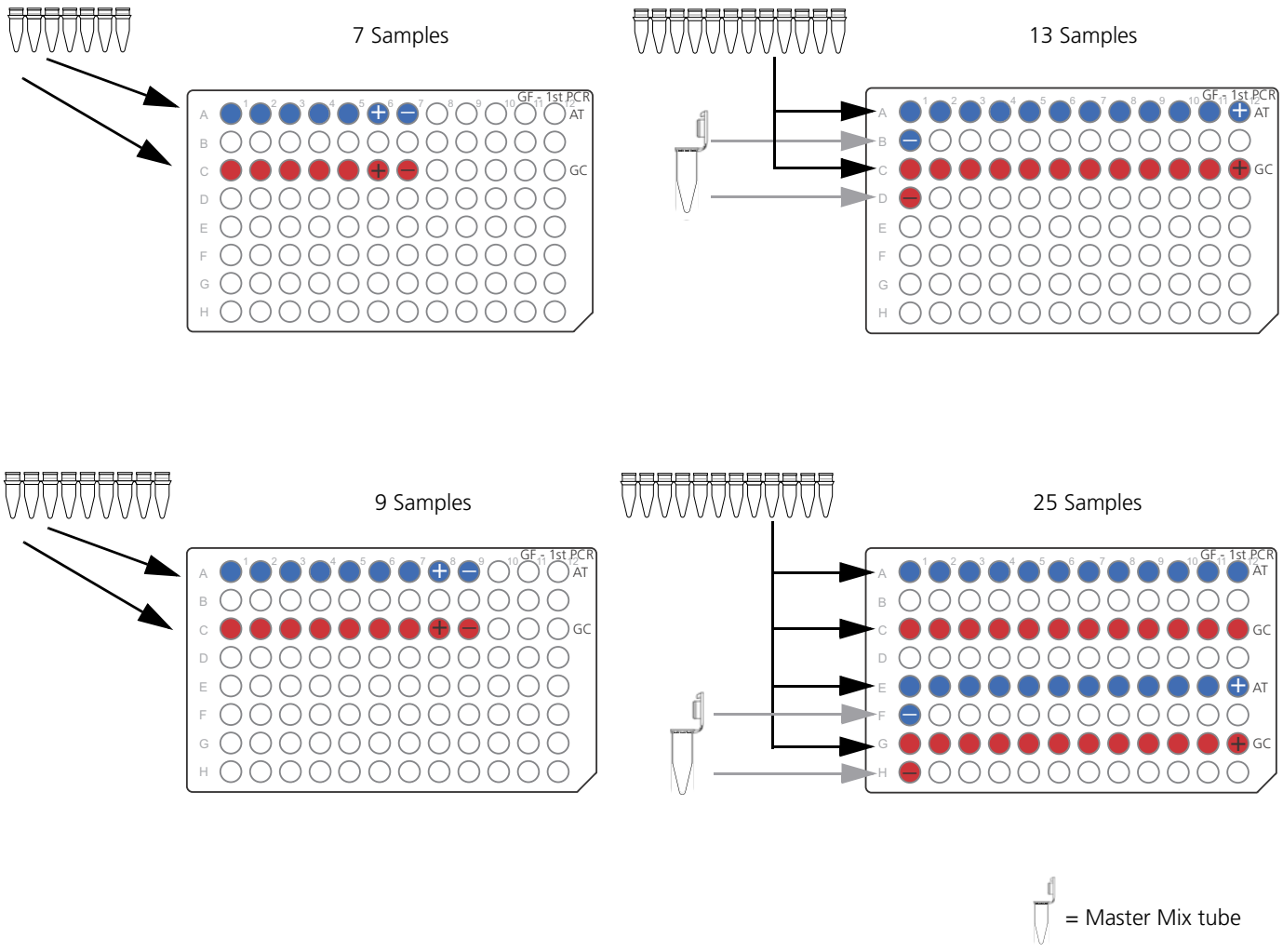
1. Use a bench top cooler to carry the Exo Mix from the –20°C freezer. Vortex for 1 second and spin down. Place back in cooler.
2. Label one set of strip tubes with the Letter *E*.
3. Aliquot the appropriate volume of the Exo mix directly into each strip tube.
   - For 7, 9 or 13 sample reaction, aliquot 7.5 µL of the Exo mix directly into the appropriate number of wells in the strip tube.
   - For 25 sample reaction, aliquot 16.0 µL of the Exo mix directly into each well of the 12-strip tube.
4. Cap the strip tubes and spin down to remove any bubbles.
5. Place the strip tubes in a cold block on ice.
6. To add the Exo Mix to the Assay Plate:
   A. When the thermal cycler temperature reaches the end of the 58°C incubation, press *Pause* and remove the *1st PCR* Plate.

      NOTE: Remove the assay plate at 58°C, BEFORE the cycler ramps down to 37°C to avoid possible non-specific Gapfill.

   B. Place the plate in a cold block on ice for 1 min.
   C. Resume the program without a plate in the thermal cycler and allow it to ramp down to start of the 37°C incubation. Pause thermal cycler at 37°C.
   D. After 1 min on ice, spin down at 2400 rpm for 30 sec. Remove the plate from the centrifuge and place the *1st PCR* Plate back on the cold block on ice.
   E. Ensure the plate is at the correct orientation (Well A1 at top left). Carefully remove the adhesive film.
   F. Using a 12-channel P20 pipette, add 3 µL Exo Mix to each reaction. Pipet up and down 3 times to rinse tips (Figure 6.10).
      - For the 13 or 25 Sample Reaction, add 3.0 µL of Exo Mix reagent to each of the Negative Control wells (Figure 6.10).

      NOTE: The current volume in the Assay Plate is 17 µL per well.

Figure 6.10 Adding Exo Mix to Plate



7. Tightly seal the plate, vortex at high speed for 5 sec; then spin down at 2400 rpm for 60 sec.
8. Place the *1st PCR* Plate back on the thermal cycler and press *Resume*.

Prepare and Add the Cleavage Master Mix

To Prepare and Add the Cleavage Master Mix:

1. Label a set of strip tubes with *CM* and place in a cold block until ready to use. Label a 1.5 mL Eppendorf tube as *CM* and place on ice until ready to use.
2. During the last 5 min at 95°C on the thermal cycler, prepare the Cleavage Master Mix as follows:
3. Use a bench top cooler to carry the Cleavage Enzyme from the –20°C freezer.
   A. Vortex (at max speed for 3 seconds) and spin down the Cleavage Buffer that has been sitting on ice. Return to ice.
   B. Vortex the Cleavage Enzyme for 1 sec. Spin down the Cleavage Enzyme and keep on ice.
   C. Add the Cleavage Buffer and Cleavage Enzyme needed for the appropriate number of samples according to the Table 6.12 to the 1.5 mL (or 2.0 mL) Eppendorf tube (*CM*).

D. Pipet up and down 3 times to rinse tips. Vortex the *CM* tube for 3 seconds (3 times - 1 second at a time) at maximum speed and spin down. Keep on ice.

E. Using the column appropriate to the number of reactions, aliquot the specific volumes of the Cleavage Master Mix from Table 6.12 to the strip tube labeled *CM*. Cover the strip tube with cap. Spin down the strip tube and place on a chilled cold block until ready to use.

NOTE: The master mix tables from Table 6.12 onward give the volumes needed for the number of reactions as opposed to the number of samples given in previous tables. Since there are now 2 reactions for every 1 sample.

Table 6.12 Cleavage Master Mix

Number of Runs per Assay Kit			**4**	**3**	**2**	**1**
Number of Samples (includes 1 positive control per run)			6	8	12	24
Negative Control per Run			1	1	1	1
AT & GC Channels			x 2 Channels	x 2 Channels	x 2 Channels	x 2 Channels
Reagent	**P/N**	**1 Reaction**	**14 Reactions (~25% overage)**	**18 Reactions (~25% overage)**	**26 Reactions (~25% overage)**	**50 Reactions (~25% overage)**
Cleavage Buffer	902257	25.0 µL	438 µL	563 µL	813 µL	1563 µL
Cleavage Enzyme	902258	0.2 µL	3.5 µL	4.5 µL	6.5 µL	12.5 µL
Total		**25.2 µL**	**441.0 µL**	**567.0 µL**	**819.0 µL**	**1575.0 µL**
Volume per Tube for Tube Strip			60.0 µL 7-Tube Strip	60.0 µL 9-Tube Strip	60.0 µL 12-Tube Strip	120.0 µL 12-Tube Strip

4. To add the Cleavage Master Mix to the Assay Plate:

A. When the thermal cycler temperature reaches the start of the 37°C step, press *Pause* and remove the *1st PCR* Plate.

B. Place the *1st PCR* Plate in a cold block on ice for 1 min.

C. Spin down at 2400 rpm for 30 sec. Remove the Assay Plate from the centrifuge and place it on the cold block. Carefully remove the adhesive seal from the plate.

D. Using a 12-channel P200 pipette, add 25 µL Cleavage Master Mix to each reaction. Pipet up and down 3 times to rinse tips (Figure 6.11).

- For the 13 or 25 Sample Reaction, add 25 µL of Cleavage Mix from the Master Mix tube to each of the Negative Control wells (Figure 6.11).

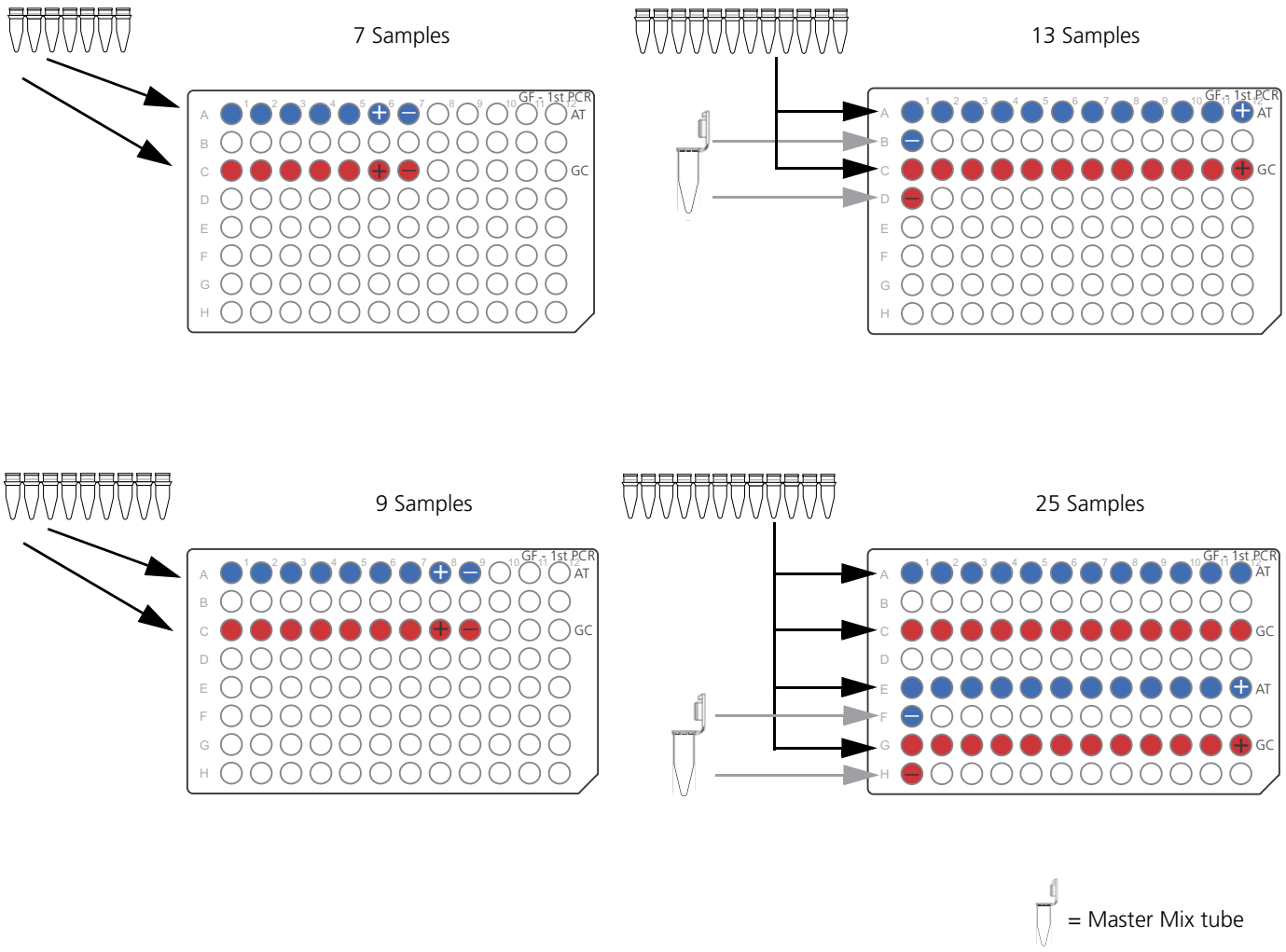
NOTE: The current volume in the Assay Plate is 42.0 µL.

5. Tightly seal the plate, vortex at high speed for 5 sec; then spin down at 2400 rpm for 60 sec.

6. Place the *1st PCR* plate back on the thermal cycler and press *Resume*.

7. Set a timer for 25 min to leave 5 min to make the master mix for the next *PCR* step.

Figure 6.11 Adding Cleavage Master Mix to Plate



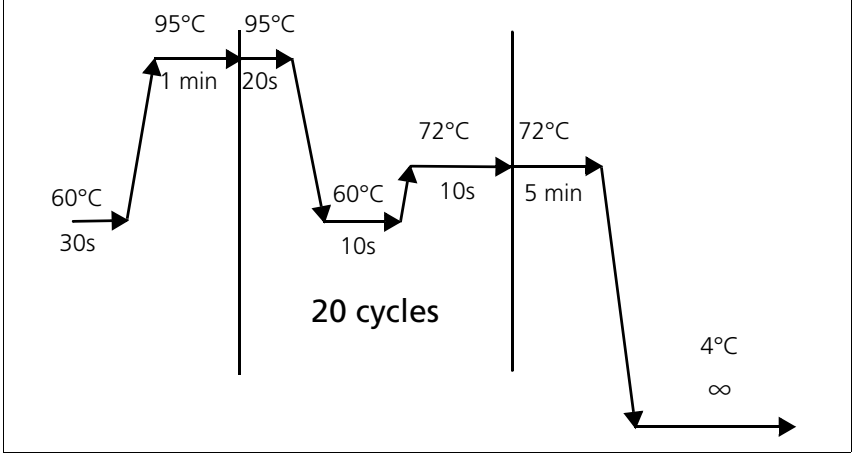
Prepare and Add the PCR Mix

1. Label one set of strip tubes *PCR* and place in a cold block until ready to use. Label a 1.5 mL Eppendorf tube (or 2.0 mL Eppendorf tube for 50 reactions) as *PCR* and place on ice until ready to use.
2. During the last 5 min at 95°C on the thermal cycler, prepare the PCR Mix as follows:
   - **A.** Using a bench top cooler, carry the Taq Polymerase from the –20°C freezer.
   - **B.** Vortex and spin down the PCR Mix that has been sitting on ice.
     Vortex the Taq Polymerase for 1 sec and spin down. Return to ice.
   - **C.** Add the PCR Mix and Taq Enzyme needed for the appropriate number of samples according to the Table 6.13 to the 1.5 mL (or 2.0 mL) Eppendorf tube labeled as *PCR*. Pipet up and down 3 times to rinse tips. Vortex the PCR Master Mix tube for 3 seconds (3 times - 1 second at a time) at maximum speed and spin down. Keep on ice.
   - **D.** Using the column appropriate to the number of reactions, aliquot the specific volumes of the 1st PCR Master Mix from Table 6.13 to the strip tube labeled *PCR*. Cover the strip tube with caps. Spin down the strip tube and place on a chilled cold block until ready to use.

Table 6.13 1st PCR Master Mix

Number of Runs per Assay Kit			**4**	**3**	**2**	**1**
Number of Samples (includes 1 positive control per run)			6	8	12	24
Negative Control per Run			1	1	1	1
AT & GC Channels			x 2 Channels	x 2 Channels	x 2 Channels	x 2 Channels
Reagent	**P/N**	**1 Reaction**	**14 Reactions (~25% overage)**	**18 Reactions (~25% overage)**	**26 Reactions (~25% overage)**	**50 Reactions (~25% overage)**
PCR Mix	902259	24.4 µL	428 µL	550 µL	794 µL	1528 µL
Taq Polymerase	902260	0.56 µL	9.8 µL	12.6 µL	18.1 µL	34.9 µL
Total		**25.0 µL**	**437 µL**	**562 µL**	**812 µL**	**1562 µL**
Volume per Tube for Tube Strip			60.0 µL 7-Tube Strip	60.0 µL 9-Tube Strip	60.0 µL 12-Tube Strip	120.0 µL 12-Tube Strip

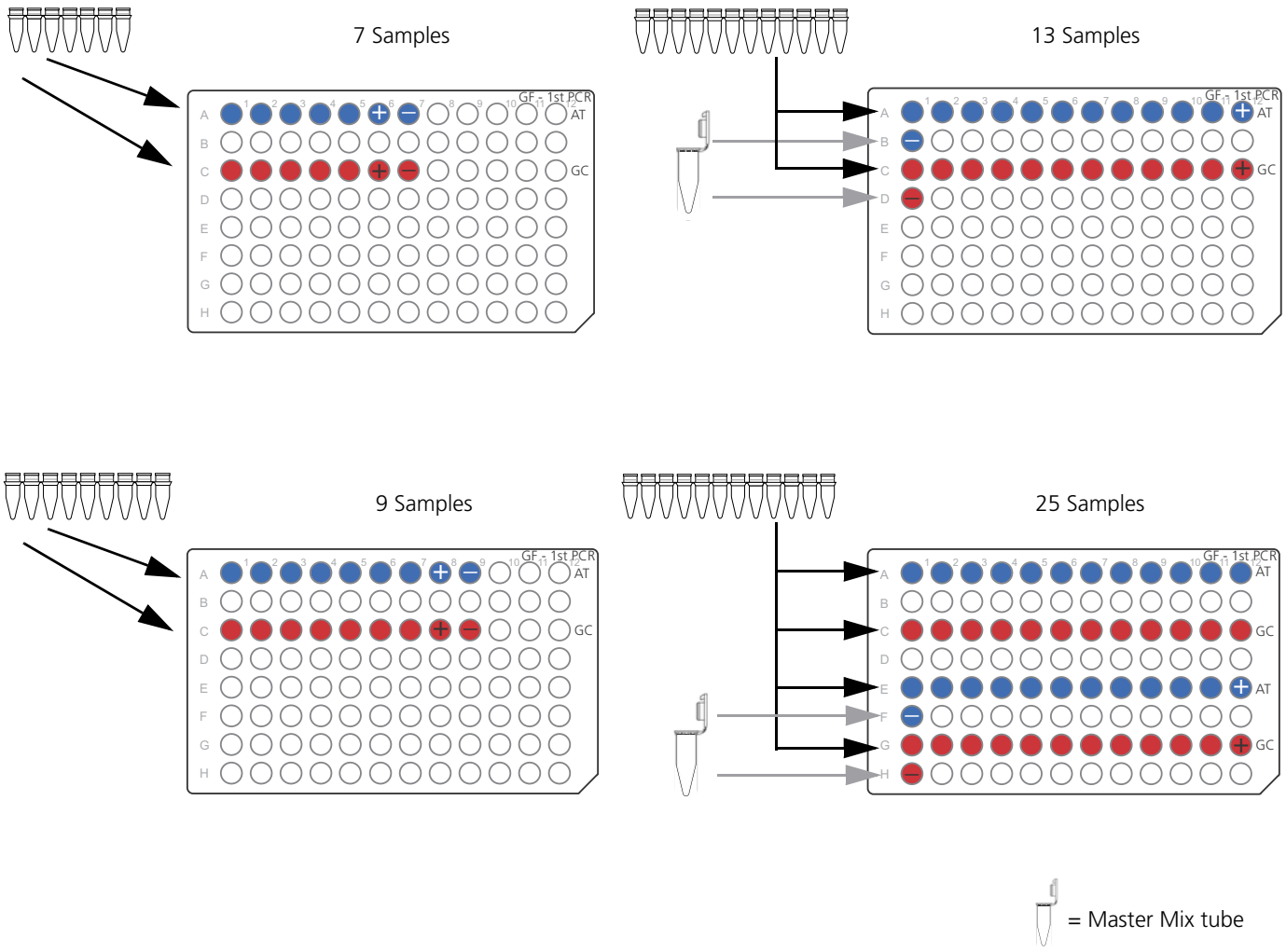
Figure 6.12 OncoScan 1st PCR Thermal Cycler Program



3. Once the ***OncoScan GAP Fill*** program completes, remove the *1st PCR* plate and place on a chilled cold block for 1 min. After the 1 min incubation, make sure the seal is tight on the plate, then vortex and spin down the *1st PCR* plate for 30 sec.
4. Stop the ***OncoScan GAP Fill*** program and start the ***OncoScan 1st PCR*** program, pause program when thermal cycler reaches 60°C.
5. Add the PCR mix to the Assay plate and remember to carefully remove the adhesive. Using a 12-channel P200 pipette, add 25 µL PCR Master Mix to each reaction. Pipet up and down 3 times to rinse tip (Figure 6.13).
   - For the 13 or 25 Sample Reaction, add 25 µL of the PCR master Mix reagent from the Master Mix tube to each of the Negative Control wells (Figure 6.13).

NOTE: The currently volume in the Assay Plate is 67.0 µL.

Figure 6.13 Adding 1st PCR Master Mix to Plate



6. Tightly seal the plate, vortex at high speed for 5 sec; then spin down at 2400 rpm for 60 sec.
7. Place the plate on the thermal cycler and press **Resume**.
8. When the program has ended, transfer the sealed *1st PCR* Plate to the Post-PCR Lab and place on ice.

IMPORTANT: To prevent contamination from PCR products, the Assay Plate must remain tightly sealed until it has been transferred to the Post-PCR Lab. DO NOT open the seal of the Assay Plate after PCR or spin down in the Pre-PCR Lab.

Stage 3 — First QC Gel and 2nd PCR (Post-PCR Lab)

About this Stage

This stage begins in the Post-PCR Lab. During this stage, the sealed *1st PCR* Plate will be transferred to the Post-PCR Lab. A 2nd PCR amplification will be set up and run in the Post-PCR Lab. An aliquot from each sample of the *1st PCR* Plate will be taken and a QC gel will be performed to check the amplified products.

NOTE: Make sure all three required thermal cycler programs (OncoScan 2nd PCR, OncoScan HaeIII, OncoScan Hybridization) are set up on the thermal cycler BEFORE starting this stage. Refer to Appendix B for programs and detailed instructions for programming the Veriti Thermal Cyclers.

Location and Duration

- Post-PCR Lab
- Hands-on time: 20 min
- Thermal cycler time: 25 min

Input Required from Previous Stage

Table 6.14

Item
1st PCR Plate with amplified DNA from the Pre-PCR Lab

Equipment and Materials Required

Table 6.15 Equipment and Materials Required for *Stage 3 — First QC Gel and 2nd PCR (Post-PCR Lab)*

Quantity	Item
3	Cold block, chilled in 4°C refrigerator
1	Centrifuge, plate
1	Ice container, rectangular, filled with ice
As required	MicroAmp Clear Adhesive Films
As required	Eppendorf tube, 2.0 mL, Nuclease-free
As required	Eppendorf tube, 1.5 mL, Nuclease-free
1	Microcentrifuge for tubes
1	Microcentrifuge for strip tubes
1 each	Pipettes: ▪ Single-channel P200 ▪ Single-channel P1000 ▪ 12-channel P20
As required	Pipette tips
1	Thermal Cycler, 96-well GeneAmp PCR System 9700 (gold or silver block), Veriti Thermal Cycler

Table 6.15 Equipment and Materials Required for *Stage 3 — First QC Gel and 2nd PCR (Post-PCR Lab)* (Continued)

Quantity	Item
1	Tube strips with caps, PCR 12-well
2	Plate, 96-well half-skirt PCR
1	Gel Box (Wide-Mini sub cell GT basic system)
1	Power Supply for Gel Electrophoresis
1	Alphalmager HP System for gel imaging (with software)
1	Bench top cooler
1	Blue Permanent Marker, Extra fine tip
1	Red Permanent Marker, Extra fine tip
1	Compression Pad
1	Timer

OncoScan® CNV Reagent Components Required

Table 6.16 OncoScan® CNV Reagent Components Required

OncoScan® CNV Reagents from Module OncoScan® CNV 2nd Stage PCR and Post PCR Processing
PCR Mix
Taq Polymerase

QC Gel Materials Required

Table 6.17 QC Gel Materials Required (Not included in the OncoScan® CNV FFPE Assay Kit)

Quantity	Item	Vendor and Part Number
1	NEB Low Molecular Weight Ladder	New England Biolabs, P/N N3236S
1	Gel loading Dye, Blue (6X)	New England Biolabs, P/N B7021S
1	100% Glycerol	Affymetrix, P/N 16374
1	EDTA, 0.5 M solution	Affymetrix, P/N 15694
1	3% Agarose gel	Bio-Rad Precast ReadyAgarose™ Wide-Mini Gel, P/N 161-3040

Transfer Assay Plate to Post-PCR Lab

If you have not already done so, transfer *1st PCR* Plate to the Post-Amp Lab and place on ice.

IMPORTANT: To prevent contamination from PCR products, the *1st PCR* Plate must remain tightly sealed until it has been transferred to the Post-Amp Lab.

Preparing the 1st PCR Plate for the 2nd PCR Stage

1. Ensure the seal is tight. Vortex the *1st PCR* Plate at max speed for 5 sec. Spin down the plate at 2400 rpm for 60 sec. Keep on cold block until ready to use.

Prepare the 2nd PCR Mix and Setup 2nd PCR

CAUTION: When transferring sample volumes from one 96-well plate to another 96-well plate, make sure BOTH plates are at the correct orientation (well A1 at top left) before pipetting.

1. Turn on thermal cycler to heat lid.
2. Remove the PCR mix from the –20˚C freezer and thaw at room temperature for 10-15 minutes.
3. Fill a rectangular ice bucket with ice and place 3 chilled cold blocks on the ice.
4. Label a new 96-well half-skirt PCR plate as *2nd stage PCR* and keep in a cold block on ice. **Following the same layout as the *1st PCR* Plate, label row A (or A & E for 25 reactions) as "AT" with a Blue marker and Row C (or Rows C & G for 25 reactions) as "GC" with a Red marker. Mark the reaction wells in the same colors.** Cover the plate with a plate seal until ready for use.
5. Label a strip tube and a 1.5 mL Eppendorf tube as *PCR 2*. Place the labeled strip tube in a chilled cold block and the 1.5 mL tube on ice until ready to use.
6. Start the ***OncoScan 2nd PCR*** program and pause when the thermal cycler reaches 60˚C.
7. Prepare the PCR Mix as follows:
   - **A.** Use a bench top cooler to carry the Taq Polymerase from the –20˚C freezer.
   - **B.** Once thawed, vortex and spin down the PCR Mix. Keep on ice.
   - **C.** Vortex the Taq Polymerase for 1 sec. Spin down the Taq Polymerase. Keep on ice.
   - **D.** Add the PCR Mix and Taq Polymerase as needed for the appropriate number of samples according to the Table 6.18 to the 1.5 mL Eppendorf tube (*PCR 2*). Pipet up and down 3 times to rinse tips. Vortex the PCR Master Mix tube for 3 seconds (3 times - 1 second at a time) at maximum speed and spin down. Keep on ice.
   - **E.** Using the column appropriate to the number of reactions, aliquot the specific volumes of the 2nd PCR Master Mix from Table 6.18 to a 12-tube strip. Cover the strip tube with cap. Spin down the strip tube and place on a chilled cold block until ready to use.
   - **F.** Using a 12-channel P200 pipette, add 25 µL 2nd PCR Master Mix from the strip tube to the appropriate wells in the new 2nd PCR plate (Figure 6.14).
     - For the 13 or 25 Sample Reaction, add 25 µL of the 2nd PCR Master Mix from the Master Mix tube to each of the Negative Control wells (Figure 6.14).

Figure 6.14 2nd Stage PCR

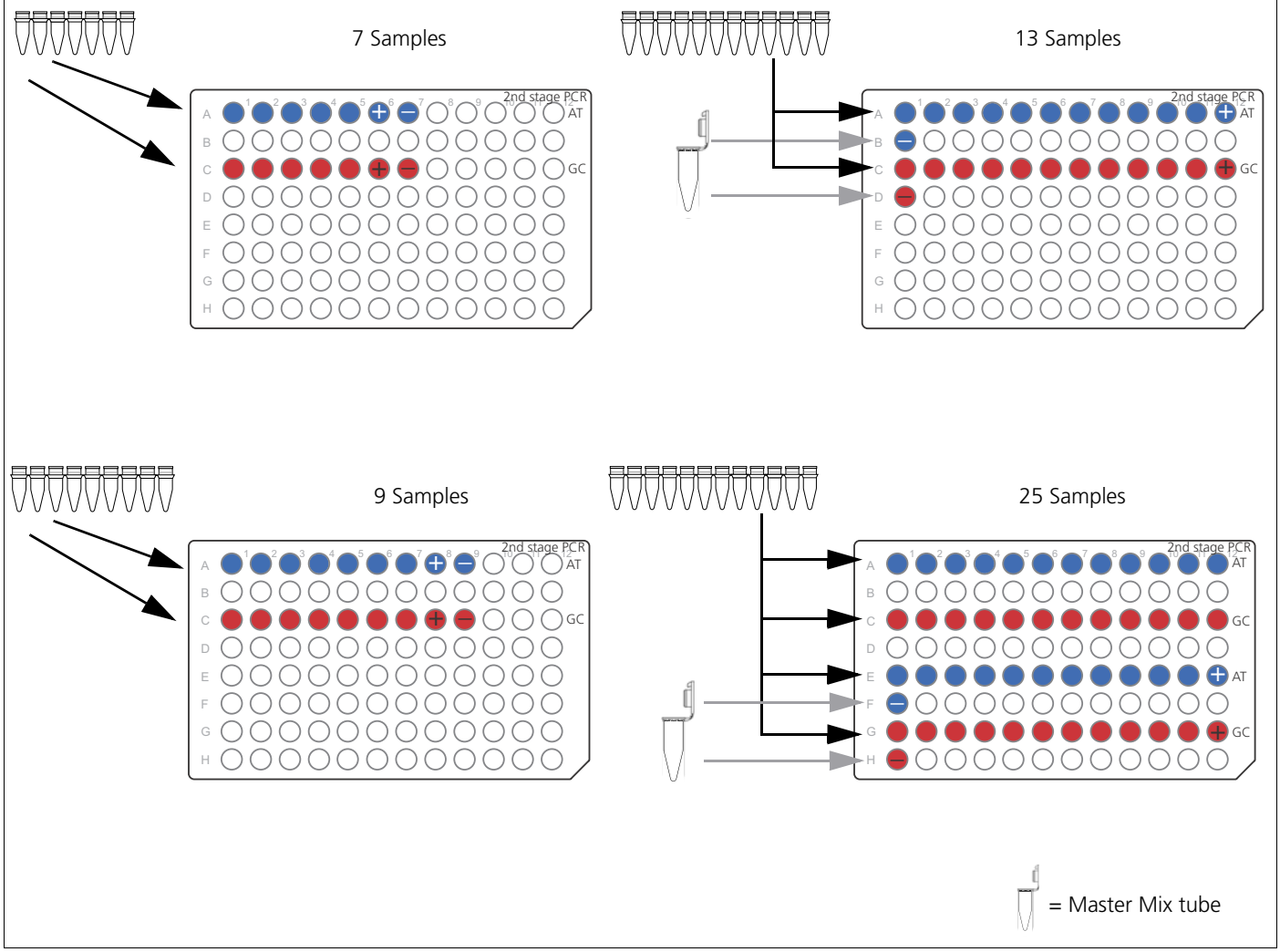
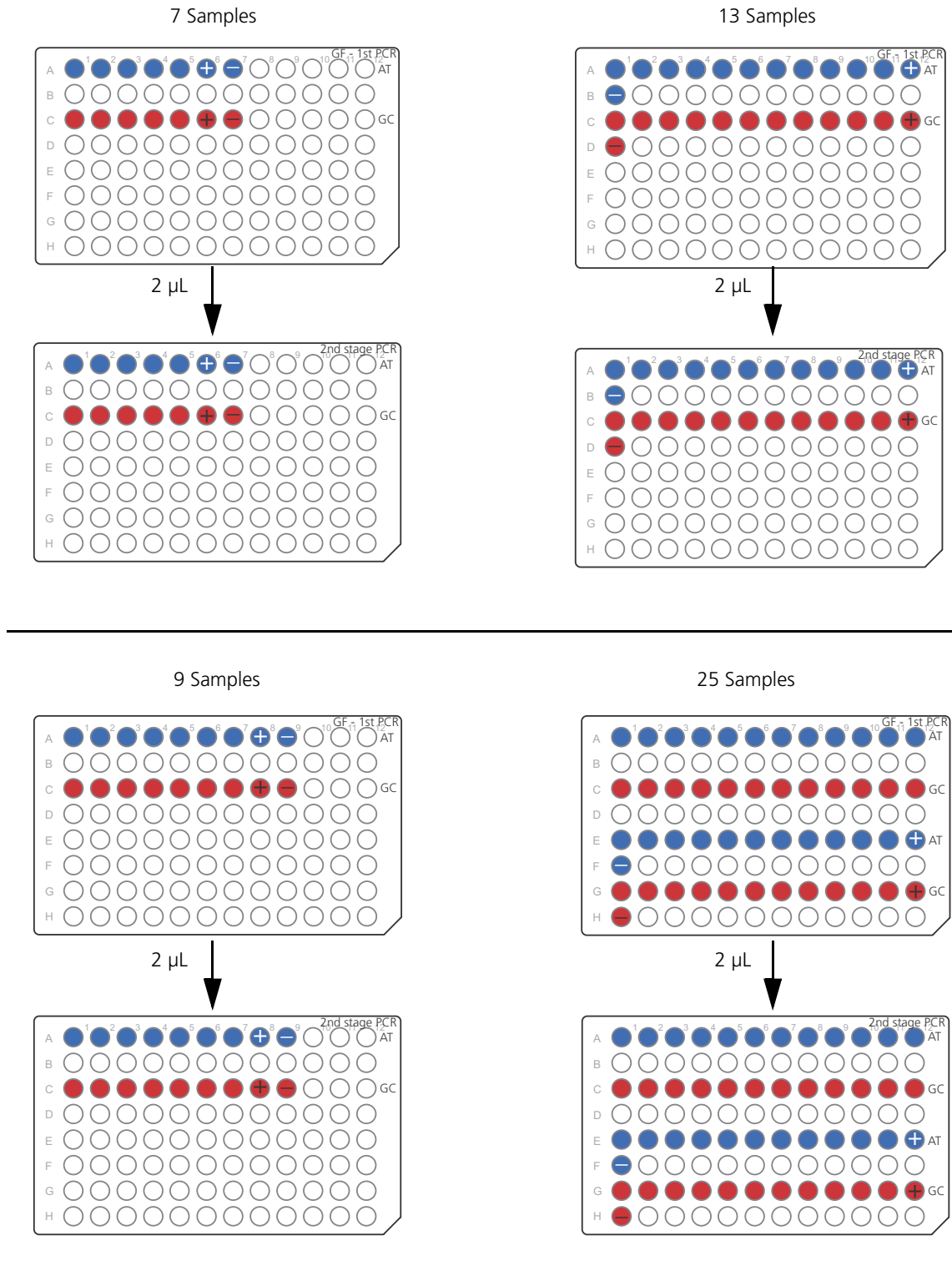


Table 6.18 2nd PCR Master Mix

Number of Runs per Assay Kit			**4**	**3**	**2**	**1**
Number of Samples (includes 1 positive control per run)			6	8	12	24
Negative Control per Run			1	1	1	1
AT & GC Channels			x 2 Channels	x 2 Channels	x 2 Channels	x 2 Channels
Reagent	**P/N**	**1 Reaction**	**14 Reactions (~25% overage)**	**18 Reactions (~25% overage)**	**26 Reactions (~25% overage)**	**50 Reactions (~25% overage)**
PCR Mix	902259	24.4 µL	428 µL	550 µL	794 µL	1528 µL
Taq Polymerase	902260	0.56 µL	9.8 µL	12.6 µL	18.1 µL	34.9 µL
Total		**25.0 µL**	**437 µL**	**562 µL**	**812 µL**	**1562 µL**
Volume per Tube for Tube Strip			60.0 µL 7-Tube Strip	60.0 µL 9-Tube Strip	60.0 µL 12-Tube Strip	120.0 µL 12-Tube Strip

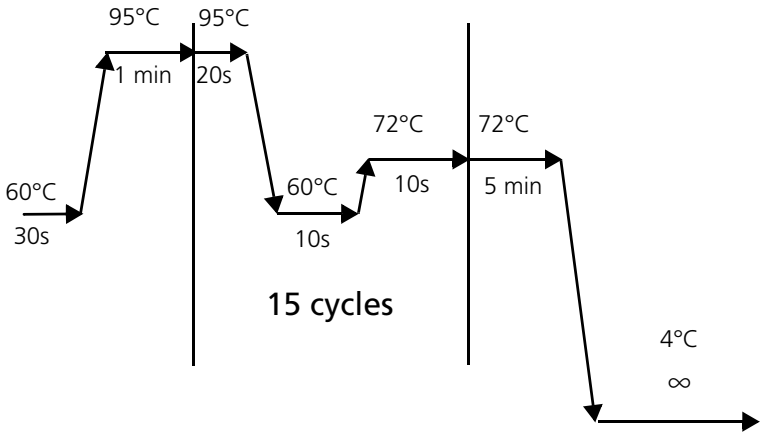
8. Remove the seal from the *1st PCR* Plate carefully and add 2 µL from each well to the corresponding wells in the *2nd PCR* Plate. Pipet mix 3 times up and down to rinse pipette tips. Cover the *1st PCR* Plate loosely with a seal until ready to use for QC gel procedure. Keep *1st PCR* Plate on a chilled cold block (Figure 6.15).
   - For the 13 or 25 Sample Reaction, add 2 µL of the Negative Control 1st PCR product to the appropriate negative control wells (Figure 6.15).

Figure 6.15



9. Seal the *2nd PCR* Plate. Vortex for 5 sec at max speed and spin down at 2400 rpm for 60 seconds.
10. Place the *2nd PCR* Plate on the thermal cycler and resume the ***OncoScan 2nd PCR*** program (Figure 6.16)

Figure 6.16 OncoScan 2nd PCR Thermal Cycler Program



Prepare and Run the First QC Gel

The first quality control gel is used to check for first PCR product.

NOTE: The instructions below are specific for running the First PCR QC gel on 3% Agarose gel and electrophoresis chamber mentioned in Table 6.25. Adjust the gel running conditions based on the Gel chamber used to run the gels. Run gels at 5V/cm (5 volts × Distance in cm between electrodes). For example, run a 33 cm electrophoresis box at 165V; run a 16 cm electrophoresis box at 80V. Please refer to Appendix D for instructions on running on 4% E-Gels.

Prepare the Gel Materials

While the ***OncoScan 2nd PCR*** program is running, prepare the materials required for the first QC gel.

Reagents for Gel Loading

1. Glycerol-EDTA buffer (50% Glycerol + 50 mM EDTA) (Table 6.19):

Table 6.19 Glycerol-EDTA Buffer (50% Glycerol + 50 mM EDTA)

Reagent	Initial Concentration	Final Concentration	Volume
100% Glycerol	100%	50%	1000.0 µL
0.5 M EDTA	500 mM	50 mM	200.0 µL
Nuclease-free Water	N/A	N/A	800.0 µL
Total Volume			**2000.0 µL**

2. 1:10 Dilution of 6X Gel Loading Dye (Table 6.20): (Store at 4°C for long-term storage)

Table 6.20 1:10 Dilution of 6X Gel Loading Dye

Reagent	Volume
Glycerol-EDTA buffer (50% Glycerol + 50 mM EDTA)	900.0 µL
NEB 6X Loading Dye	100.0 µL
Total Volume	**1000.0 µL**

3. Dilution of the 50 bp ladder (Table 6.21):
(1 mL of the diluted ladder can be used in ~200 lanes, Store at –20°C for long-term storage)

Table 6.21 Dilution of the 50 bp Ladder

Reagent	Volume
Glycerol-EDTA buffer (50% Glycerol + 50 mM EDTA)	830.0 µL
NEB 6X Loading Dye	100.0 µL
NEB 50 bp Ladder	70.0 µL
Total Volume	**1000.0 µL**

Prepare the QC Gel 1 Plate

CAUTION: When transferring sample volumes from one 96-well plate to another 96-well plate, make sure BOTH plates are in the correct orientation (well A1 at top left) before pipetting.

To Prepare the Gel Plate:

1. Label one fresh PCR plate *Gel1* (the gel plate) and load it as follows:
   A. Aliquot 8 µL of *1st PCR* Plate product to the appropriate wells.
   B. Add 2 µL of 1/10th diluted gel loading dye. Pipet up and down three times to rinse the tips.
2. Seal *1st PCR* Plate tightly and keep in a cold block on ice.
   Total volume left in each well is approximately 56.0 µL
3. Tightly seal the gel QC plate, vortex and spin down.
4. Load 10 µL of each reaction onto a 3% agarose gel.
5. Load 5.0 µL of diluted 50 bp ladder on both sides of the gel marked as “M”.
6. Run the gel at 150V for 15 min.
7. Examine the gel in a gel imager to ensure PCR products are approximately 120 bp.
   Figure 6.17 illustrates good results for each sample.

Figure 6.17 For samples in which successful amplification has occurred, one single band at approximately120 base pairs should be seen. No distinct band at approximately 120 base pairs should be visible in the Negative Control and in samples in which amplification has not occurred.

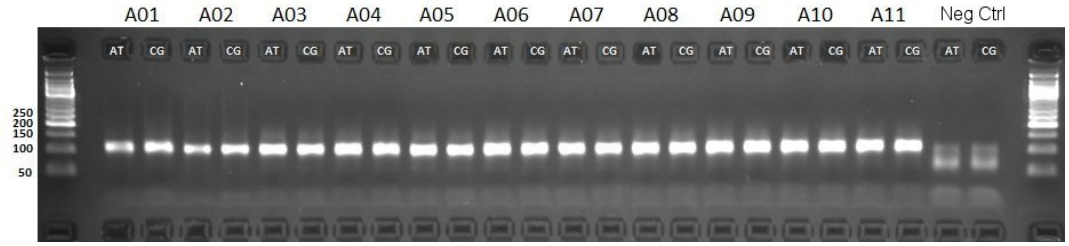
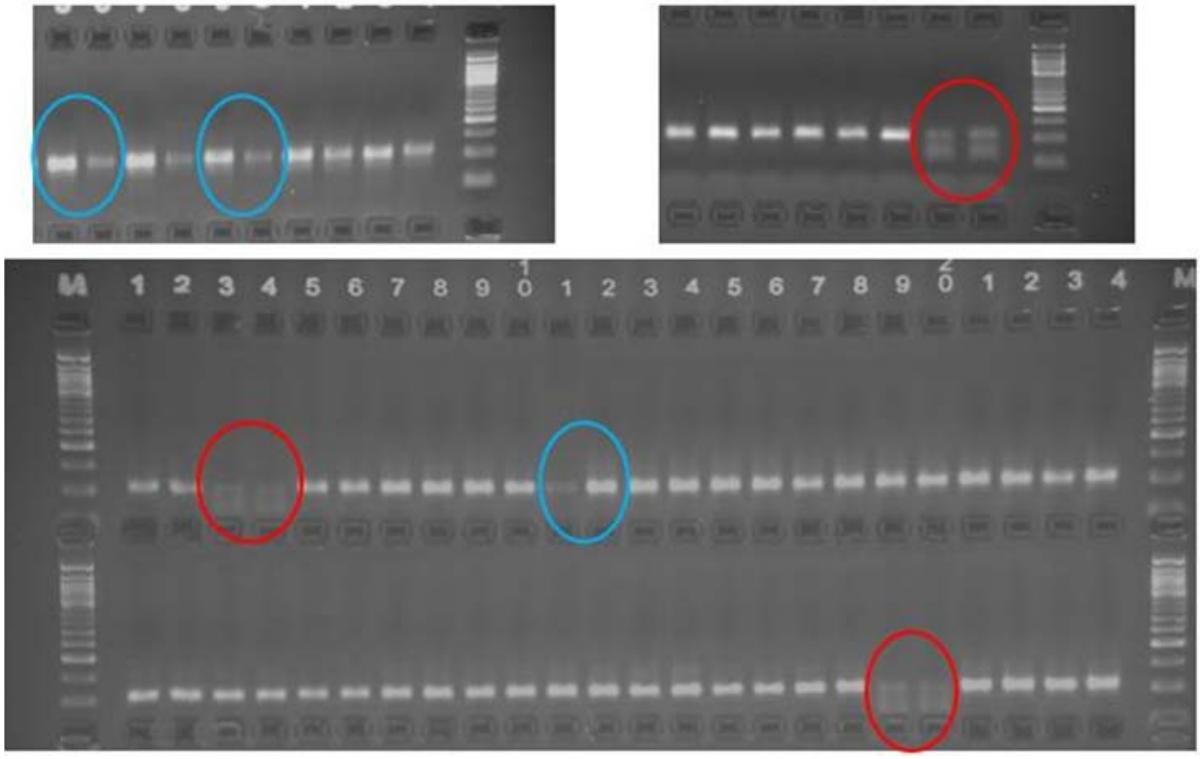


Figure 6.18 Poor QC Gel for First QC Gel (1st PCR)



Blue Circles:

QC gel pattern: Faint or no PCR product visible on the 1st PCR QC gel image in AT or GC channel for a given sample.

Possible Causes: Gel loading error or mis-pipetting.

Solution:

- Eliminate the gel loading error by running the QC gel again.
- If the repeat gel shows the same faint/no PCR band in a given channel, there was likely a mis-pipetting step after the Channel Split in the Pre-PCR stage. Repeat this sample from the beginning of the assay.

DO NOT hybridize these samples onto the Array.

Red Circles:

QC gel pattern: Faint or no PCR product visible on the 1st PCR QC gel image in BOTH AT and GC channel for a given sample.

Possible Causes: Poor DNA quality, inaccurate DNA quantitation or mis-pipetting the DNA or MIP probe.

Solution:

- Recheck the quantification for the sample by using the recommended PicoGreen protocol to make sure you have 80 ng of starting material.
- Mis-pipetting in the Anneal step by not adding the probe mix or the input DNA can result in no PCR bands. Repeat Assay from the beginning.

DO NOT hybridize these samples onto the Array. (Refer to the Troubleshooting section for details.)

QC Check Point 1

If any of the samples have the similar pattern shown in the "poor QC" results (Figure 6.18), run the gel again to make sure there was no gel loading error.

If the repeat gel shows the same pattern, do not proceed with the assay for the problematic samples. Refer to the troubleshooting guidelines and repeat the assay again. **DO NOT hybridize these samples onto the arrays.**

Stage 4 — HaeIII Digest and Second QC Gel

About this Stage

During this stage, smaller DNA fragments are generated to improve sample hybridization onto the OncoScan® CNV Arrays. The DNA fragment size is then checked on the second QC gel.

Location and Duration

- Post-PCR Lab
- Hands-on time: 30 min
- Thermal cycler time: 1 hr 45 min

Input Required from Previous Stage

Table 6.22

Item
2nd PCR Plate with Amplified DNA

Equipment and Materials Required

Table 6.23 Equipment and Materials Required for *Stage 4 — HaeIII Digest and Second QC Gel*

Quantity	Item
2	Cold blocks, chilled in 4°C refrigerator
1	Centrifuge, plate
1	Ice container, rectangular, filled with ice
As required	MicroAmp Clear Adhesive Films
1	Microcentrifuge for tubes
1	Microcentrifuge for strips
1 each	Pipettes: ▪ Single-channel P200 ▪ Single-channel P1000 ▪ 12-channel P20 ▪ 12-channel P200 ▪ Optional: 24-channel; P20
As required	Pipette tips
2	Plate, 96-well PCR
1	Thermal Cycler, 96-well GeneAmp PCR System 9700 (gold or silver block), Veriti Thermal Cycler
1	Tube, Eppendorf 1.5 mL
2	Tube strips with caps, PCR 12-well
1	Bench Top Cooler
1	Blue Permanent Marker, Extra fine tip

Table 6.23 Equipment and Materials Required for *Stage 4 — HaeIII Digest and Second QC Gel* (Continued)

Quantity	Item
1	Red Permanent Marker, Extra fine tip
1	Compression Pad
1	Timer

OncoScan® CNV Reagent Components Required

Table 6.24 OncoScan® CNV Reagent Components Required

OncoScan® CNV Reagents from Module OncoScan® CNV 2nd Stage PCR and Post PCR Processing
Buffer B
HaeIII Enzyme
Exonuclease I

Gel Materials Required

Table 6.25 Gel Materials Required

Quantity	Item	Supplier
1	1X TE Buffer	Affymetrix P/N 75792
1	NEB Low Molecular Weight Ladder, diluted	Diluted 50 bp ladder, prepared as instructed on page 60.
2 µL/rxn	1:10 Loading buffer	1:10 diluted Gel Loading Dye prepared as instructed on page 60.
1	3% Agarose gel	Bio-Rad Precast Ready Agarose Wide-Mini Gel, P/N 161-3040

Thaw the Reagents

To Thaw the Reagents:

1. Place the Buffer B on the bench top for 10-15 minutes to thaw at room temperature.
2. Once thawed, vortex Buffer B at max speed for 3-5 seconds, spin down briefly and place on ice.

Prepare and Run the HaeIII Digest

To Prepare and Run the HaeIII Digest:

1. Label one fresh 96-well half-skirt PCR Plate *HaeIII*. **Following the same layout as the *1st PCR* Plate, label row A (or A & E if running 25 reactions) as "AT" with a Blue marker and Row C (or C & G if running 25 reactions) as "GC" with a Red Marker. Mark the reaction wells in the same colors**. Keep it loosely covered with seal on chilled cold block.
2. Stop the PCR program after the completion of the 2nd PCR and remove the *2nd PCR* Plate from the thermal cycler. Place the *2nd PCR* Plate on chilled cold block for 1 min. After 1 min, ensure the seal is tight and Vortex for 5 seconds at max speed. Spin down at 2400 rpm for 60 sec. Return the plate to the cold block until ready to use for the HaeIII digest.
3. Label a strip tube and a 1.5 mL Eppendorf tube with *HaeIII*.
4. Place the labeled strip in a chilled cold block and the labeled 1.5 mL Eppendorf tube on ice.
5. Using a bench top cooler, carry the *Hae III* and Exo enzymes from the –20°C. Vortex briefly and spin down. Place on ice.
6. Add the Hae III Mix and Exo Enzyme needed for the appropriate number of samples according to the Table 6.26 to the 1.5 mL Eppendorf tube (*Hae III*). Pipet up and down 3 times to rinse tips. Vortex the HaeIII Master Mix tube briefly at maximum speed and spin down. Keep on ice.
7. Using the column appropriate to the number of reactions, aliquot the specific volumes of the HaeIII Master Mix from Table 6.26 to a 12-tube strip. Cover the strip tube with cap. Spin down the strip tube and place on a chilled cold block until ready to use.

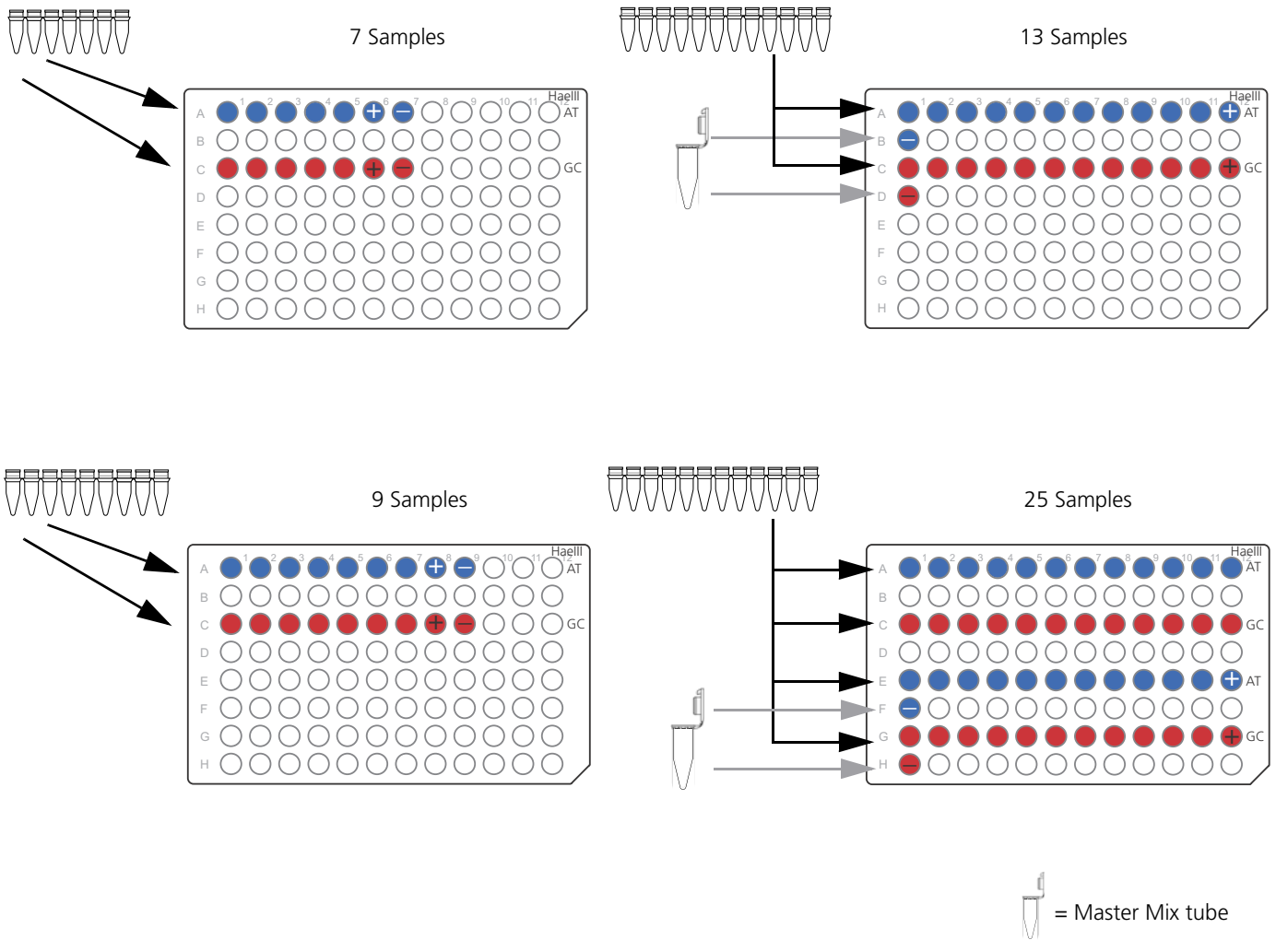
Table 6.26 HaeIII Master Mix

Reagent	P/N	1 Reaction	14 Reactions (~20% overage)	18 Reactions (~20% overage)	26 Reactions (~20% overage)	50 Reactions (~20% overage)
Buffer B	902261	19.10 µL	321 µL	413 µL	596 µL	1146 µL
HaeIII Enzyme	902262	0.40 µL	6.7 µL	8.6 µL	12.5 µL	24.0 µL
Exo Enzyme	902263	0.50 µL	8.4 µL	10.8 µL	15.6 µL	30.0 µL
Total		**20.0 µL**	**336 µL**	**432 µL**	**624 µL**	**1200 µL**
Volume per Tube for Tube Strip			45.0 µL 7-Tube Strip	45.0 µL 9-Tube Strip	48.0 µL 12-Tube Strip	96.0 µL 12-Tube Strip

CAUTION: When transferring sample volumes from one 96-well plate to another 96-well plate, make sure BOTH plates are at the correct orientation (well A1 at top left) before pipetting.

8. Using a 12-channel P20 pipette, add 20 µL of HaeIII Master Mix to corresponding well of the *HaeIII* Plate (Figure 6.19).
   - For the 13 or 25 Sample Reaction, add 20 µL of the HaeIII Master Mix from the Master Mix tube to each of the Negative Control wells (Figure 6.19).

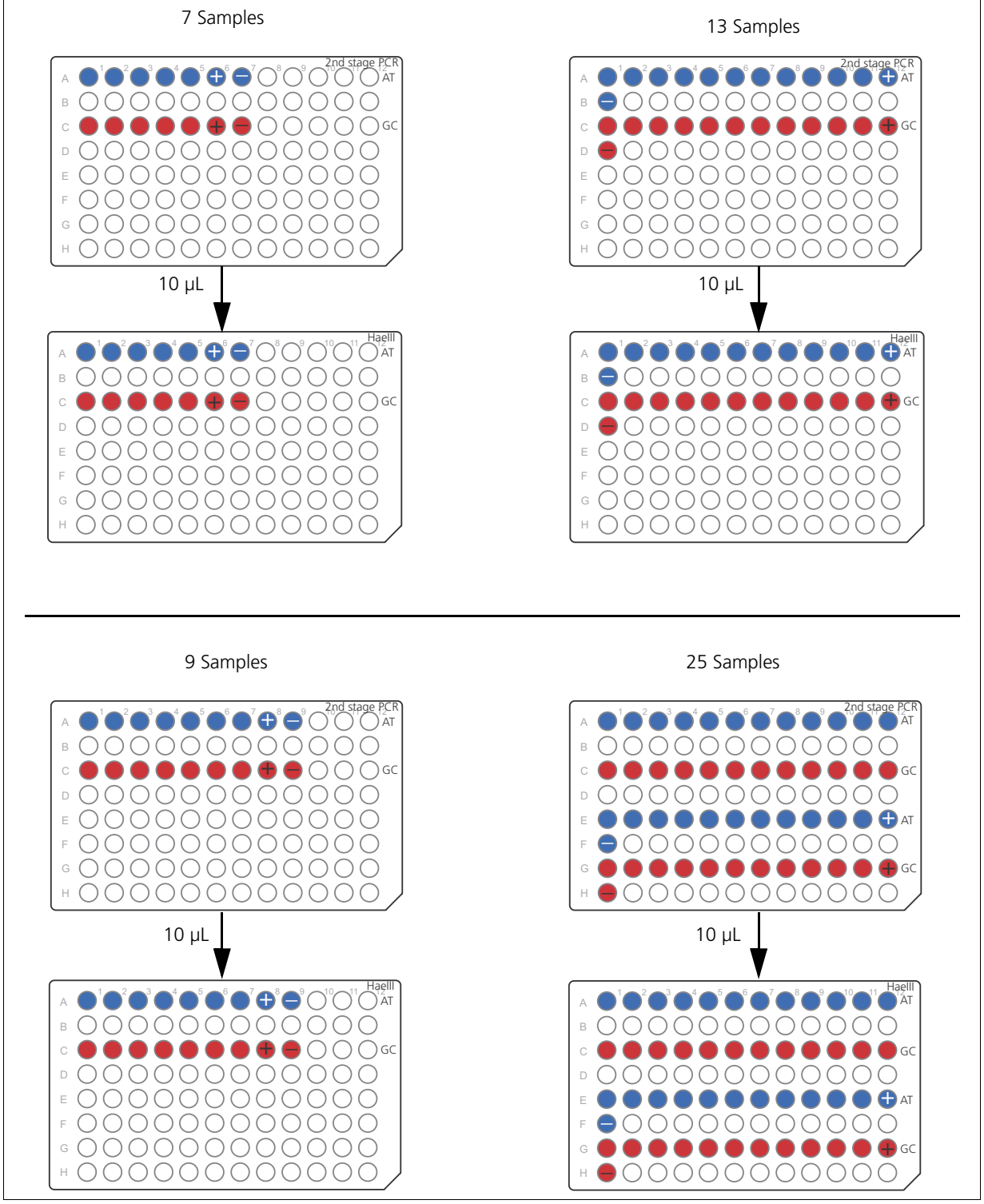
Figure 6.19 Adding HaeIII Master Mix to Plate



9. Using a 12-channel P20 pipette, add 10 µL of 2nd PCR amplified DNA from 96 well plate labeled 2nd stage PCR to the corresponding wells of the *HaeIII* Plate. Pipet up and down 3 times to rinse the tips (Figure 6.20).
   - For the 13 or 25 Sample Reaction, add 10 µL of the 2nd PCR from the Negative control to each of the appropriate Negative Control wells (Figure 6.20).

NOTE: Total volume each well: 30 µL.

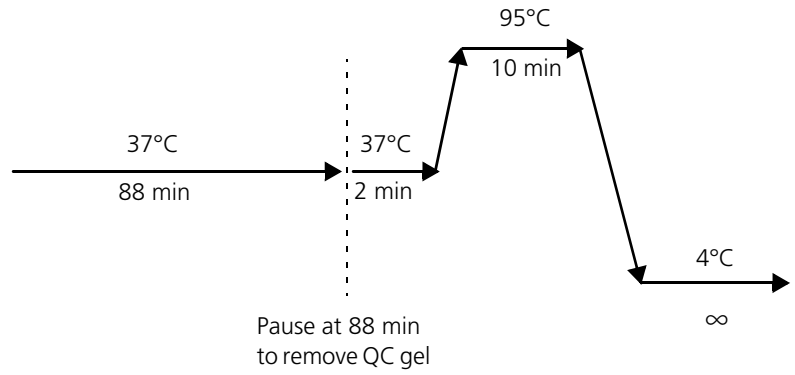
Figure 6.20



10. Seal the *HaeIII* Plate tightly while the plate is in a chilled cold block.
11. Vortex the plate at high speed for 5 sec, and spin down at 2400 rpm for 60 sec.
12. Place the plate on a thermal cycler and run the ***OncoScan HaeIII*** program (Figure 6.21).

13. Set a timer for 85 min to pause the thermal cycler at 37˚C to remove the aliquot for QC gel. The *HaeIII* Plate needs to be paused **before** the 95˚C at 10 min in order to observe the size of the DNA after HaeIII digest. Setting the timer for 85 min will allow enough time to return to the thermal cycler and pause the program at 37˚C at 88 minutes (2 minutes before the program reaches 95˚C).

Figure 6.21 HaeIII Thermal Cycler Program



Prepare and Run the HaeIII Gel (Second QC Gel)

Run the second quality control gel to check the HaeIII Digestion reaction.

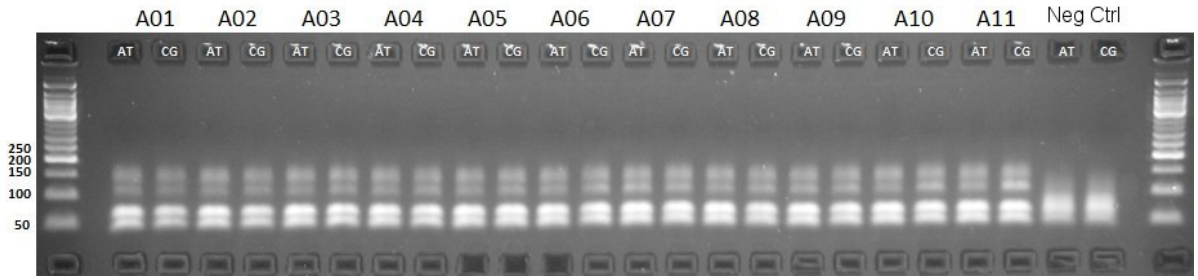
NOTE: The instructions below are specific for running the HaeIII QC gel on 3% Agarose gel and electrophoresis chamber mentioned in Table 6.25. Adjust the gel running conditions based on the Gel chamber used to run the gels. Run gels at 5V/cm (5 volts × Distance in cm between electrodes). For example, run a 33 cm electrophoresis box at 165V; run a 16 cm electrophoresis box at 80V. Please refer to Appendix D for instructions on running the HaeIII gel QC.

To Prepare and Run the Second Quality Control Gel:

1. Label a 96-well PCR plate *Gel2* (the gel plate). Ensure the plate is at the correct orientation (well A1 at top left).
2. Add 4 µL of 1X TE to the gel plate.
3. Add 2 µL of 1:10 Gel Loading Dye to each reaction, pipetting up and down 3 times to rinse the tips.
4. When the cycler reaches 88 min at 37˚C, pause the cycler.
5. Remove the *HaeIII* Plate and place it on a cold block for **1 minute**.
6. Ensure the *HaeIII* Plate is tightly sealed and vortex at **max speed for 5 seconds**. Spin down the plate at **2400 rpm for 60 seconds**.
7. Ensure the plate is at the correct orientation (well A1 at top left). Remove the plate seal slowly and carefully.
8. Remove **4 µL of HaeIII digest sample** and add it to the *Gel 2* Plate.
9. Seal the *HaeIII* plate and place it back on the thermal cycler. Resume the ***OncoScan HaeIII*** program.
10. Seal the QC Gel 2 sample plate, Vortex and Spin down briefly.
11. Load 10 µL of each reaction and the ladder onto a 3% agarose gel.
12. Load 3.5 µL of diluted 50 bp ladder on both sides of the gel marked as “M”.
13. Run the gel at 150V for 15 min.
14. Examine the gel in a gel imager to ensure that a doublet bands around 40-70 bp are observed.

Figure 6.22 illustrates a good HaeIII digest gel results.

Figure 6.22 The gel indicates both successful amplification during the second stage PCR reaction and HaeIII digestion. The predominant pattern should be two bands at approximately 40 and 70 bp.



QC Check Point 2

If any of the samples DO NOT have the similar gel banding pattern shown in the "Good QC" results (Figure 6.22) or have a gel banding pattern similar to the negative control, run the gel again to make sure there was no gel loading error.

If the repeat gel of a given sample shows a smear similar to that of the negative control, do not proceed with the assay. **DO NOT hybridize these samples onto the arrays.**

Refer to the troubleshooting guidelines and repeat the assay again, as mentioned below.

1. Prepare and set up a new 2nd PCR.
2. Prepare and set up a new HaeIII using the newly setup PCR.
3. Run the HaeIII gel QC again

Stage 5 — Hybridization

About this Stage

During this stage, each reaction is denatured then loaded onto an OncoScan® CNV Array – one well (either AT or GC) per array. The arrays are then placed into a hybridization oven that has been preheated to 49°C. Samples are left to hybridize for 16 to 18 hours.

To help ensure the best results, carefully read the information below before you begin this stage.

IMPORTANT:

- **Make sure the QC check point criteria are met before processing this stage. For a sample to be hybridized onto the array, BOTH 1st PCR QC gel and HaeIII QC gel must meet the QC criteria.**
- **Be sure to equilibrate the arrays to room temperature; otherwise, the rubber septa may crack and the array may leak.**
- **An accurate hybridization temperature is critical for this assay. Therefore, we recommend that your hybridization oven(s) be serviced at least once per year to ensure that they are operating within specifications.**
- **If you will be running more than 2 batches through a given Fluidics Stations, we recommend that you stagger the hybridization of the arrays by 2 hours so that the arrays in the third or fourth batch will not be in the hyb oven for more than 18 hours.**

Location and Duration

- Post-PCR Lab
- Hands-on time: approximately 1 hr
- Thermal cycler time: 15 min
- Hybridization time: 16 to 18 hours

Input Required from Previous Stage

Table 6.27

Item
HaeIII Digest Plate containing HaeIII Digested DNA

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 6.28 Equipment and Consumables Required for *Stage 5 — Hybridization*

Quantity	Item
1	Cold block, chilled to 4°C (do not freeze)
2 per sample	OncoScan® CNV Array
1	GeneChip® Hybridization Oven 645
1	Ice bucket, filled with ice
1	Razor blade
As required	MicroAmp Clear Adhesive Film

Table 6.28 Equipment and Consumables Required for *Stage 5 — Hybridization* (Continued)

Quantity	Item
1 each	Pipettes: ▫ Single channel P200 ▫ Single-channel P1000 ▫ 12-channel P200
As required	Pipette tips for pipettes listed above; full racks
1	Plate, 96-well PCR
1	Plate centrifuge
1	Plate holder
1	Reagent Reservoir, 25 mL
1	Thermal Cycler, 96-well GeneAmp PCR System 9700 (gold block), Veriti Thermal Cycler
1	Compression Pad for Thermal Cycler
2 per array	Tough-Spots®, 1/2 in. diameter

OncoScan® CNV Reagent Components Required

Table 6.29 OncoScan® CNV Reagent Components Required

OncoScan® CNV Reagents from Module OncoScan® CNV 2nd Stage PCR and Post PCR Processing
Hybridization Mix
Water

Preheat the Hybridization Ovens

To Preheat the Hybridization Ovens:

1. Turn each Hybridization Oven 645 on and press the “Pause” button to start.
2. Set the temperature to 49°C.
3. Set the rpm to 60.
4. Press the run button once to allow hybridization oven to start. Allow to preheat.
5. Allow approximately 30 minutes to 1 hour for the oven to preheat.

Prepare the Arrays

To Prepare the Arrays:

1. Allow the OncoScan CNV arrays to equilibrate to room temperature (10 to 15 min).
2. Unwrap the arrays and place on the bench top.
3. For each array:
   A. Based on the sample size, mark the front side of the array 1A to 24A (for AT channel) and 1C to 24C (for GC channel) only if all 24 samples are being hybridized. Each sample uses two arrays so take care in labeling the array clearly to ensure that you know which sample is loaded onto each array.

B. Open the Batch Registration Excel File and scan the Array barcodes onto the "Barcode" column of the Batch Registration File. Save the Batch Registration File and close it.

NOTE: Double-check that the array type is assigned as OncoScan CNV to avoid analysis issues.

C. Flip the arrays face down and label the array in the same manner as described in Step 3A above on the back side of the array as well.

D. Insert a 200 µL pipette tip (unfiltered tips are preferred) into the upper right septum.

IMPORTANT: To ensure that the data collected during scanning is associated with the correct sample, mark the pairs of arrays by sample number (1A through 24A or 1C through 24C).

Figure 6.23 Preparing the Arrays for Hybridization

Prepare the Hybridization Master Mix

To Prepare the Hybridization Master Mix:

NOTE: Prepare only enough hybridization cocktail for the arrays that are being hybridized. Hyb plate must be prepared fresh right before hybridization. If the samples are not going to be hybridized on the arrays, store them as HaeIII digest at –20°C.

The number of arrays to be hybridized will depend on the availability of the GeneChip® Hybridization Oven 645, the number of Fluidics Stations (FS450) for the Wash/Stain procedure and the Scanner. Please plan ahead for array hybridizations.

- **The Negative Control sample that was processed until the HaeIII digest will not be hybridized on the arrays. Hence the total number of arrays hybridized per run will be two less than the respective Post-GapFill reactions.**

Table 6.30 Hybridization Master Mix

Number of Runs per Assay Kit			**4**	**3**	**2**	**1**
Number of Samples (includes 1 positive control per run)			6	8	12	24
Negative Control per Run			1	1	1	1
AT & GC Channels			x 2 Channels	x 2 Channels	x 2 Channels	x 2 Channels
Reagent	**P/N**	**For 1 Array**	**12 Arrays**	**16 Arrays**	**24 Arrays**	**48 Arrays**
Water	902253	30.0 µL	0.504 mL	0.648 mL	0.936 mL	1.80 mL
Hybridization Mix	902264	118.0 µL	1.9824 mL	2.5488 mL	3.6816 mL	7.08 mL
Total		148.0 µL	2.4864 mL	3.1968 mL	4.6176 mL	8.88 mL

1. Label a 15 mL conical tube as *Hyb* and place on ice until ready to use.
2. Using the appropriate column in Table 6.30, make the hybridization cocktail by adding volume of water and hybridization mix to the *Hyb* tube.
3. Vortex at maximum speed for 3 seconds (1-2 seconds at a time), spin down briefly, and place it on ice until ready to be used.

Prepare the Hyb Plate and Denature the Samples

To Prepare the Hyb Plate and Denature the Samples:

1. Remove the *HaeIII* Plate from the thermal cycler after the program is complete. Ensure the seal is secure. Vortex at maximum speed for 5 sec. Spin down the plate at 2400 rpm for 60 sec, and place in a cold block on ice.
2. Label a fresh 96-well plate with *Hyb* and **following the same layout as the *1st PCR* Plate, label row A (or A & E if running 24 samples) as "AT" with a Blue marker and Row C (or C & G if running 24 samples) as "GC" with a Red marker. Mark the reaction wells in the same colors**. Place on ice covered loosely with a seal.

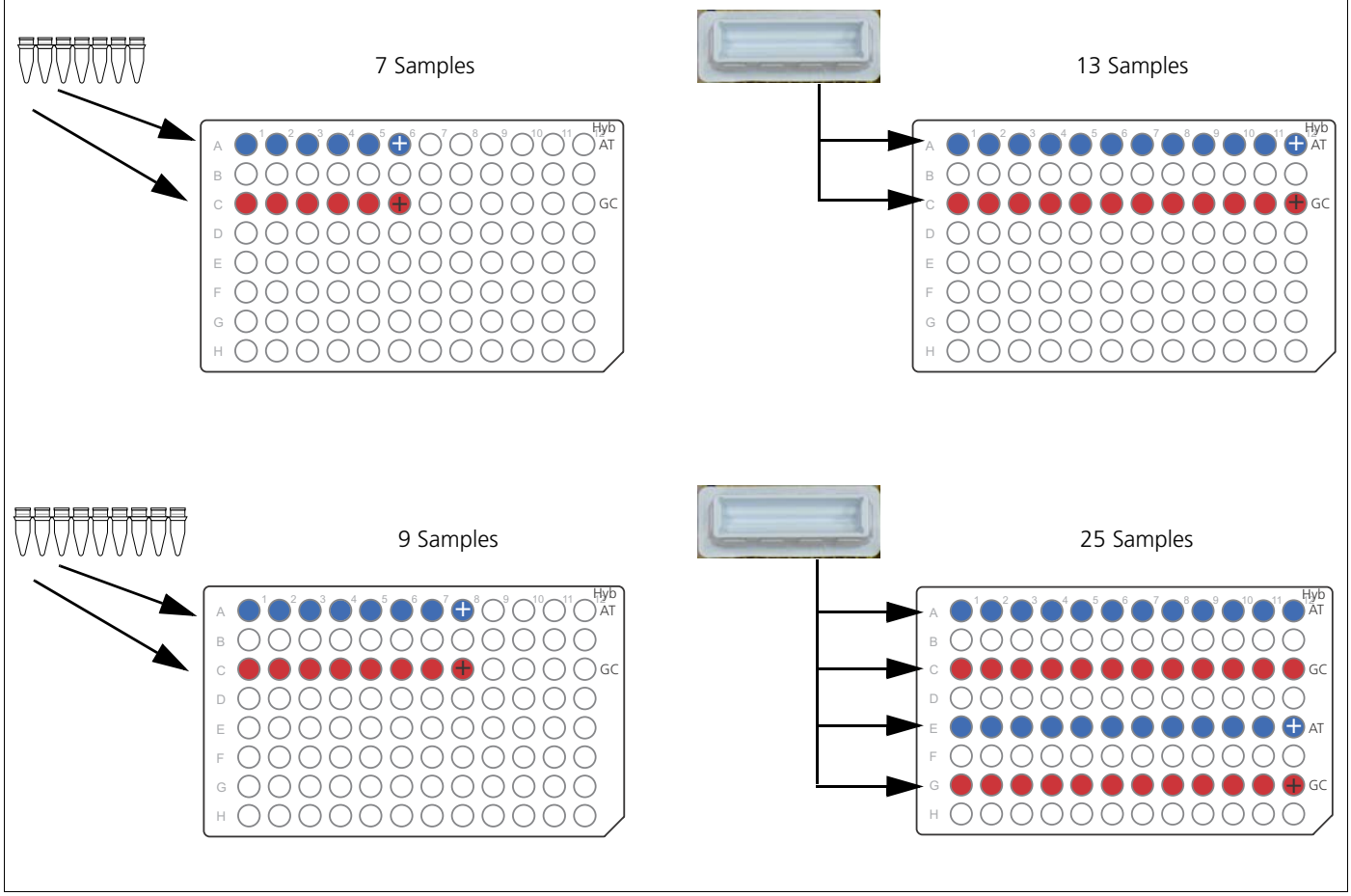
NOTE: Depending on the volume, if necessary use a reagent reservoir to aliquot the hybridization cocktail. For smaller volumes, aliquot the hybridization cocktail directly into the hybridization plate.

NOTE: The negative control is assessed only through the HaeIII gel and will not be carried through to the Hyb Plate.

3. If using a reservoir, place a reagent reservoir on ice, and pour the Hybridization Master Mix slowly and carefully into the reservoir.
4. Using a 12-channel P200 pipette:
   A. Aliquot 148 µL of Hybridization Master Mix to the appropriate wells of the Hyb Plate (Figure 6.24).

IMPORTANT: Any time fresh pipette tips are used, aspirate and dispense three times to wet the tips prior to aliquoting Hybridization Master Mix.

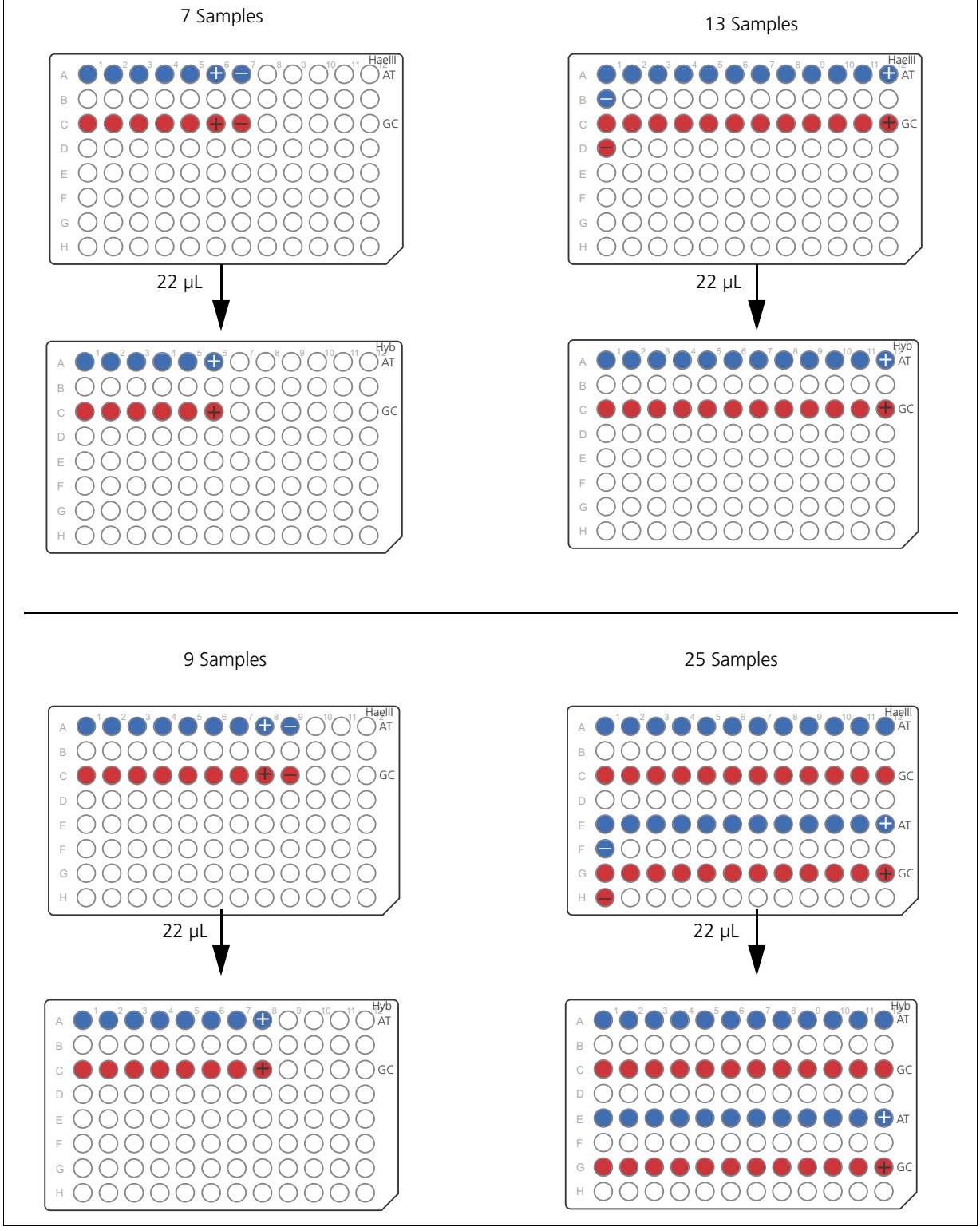
Figure 6.24 Adding Hybridization Master Mix to Plate



5. Ensure the *HaeIII* Plate and the *Hyb* Plate are at the correct orientation (Well A1 at top left). Remove the seal from the *HaeIII* Plate carefully.
6. Using a 12-channel P200 pipette, transfer 22 µL of each reaction from the *HaeIII* Plate to the *Hyb* Plate. Pipet up and down 3 times to rinse tips (Figure 6.25).

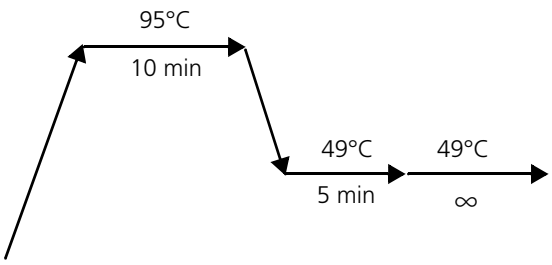
NOTE: Total volume in each well: 170 µL.

Figure 6.25 Transfer from HaeIII Plate to Hyb Plate



7. Tightly seal the *Hyb* Plate, vortex at maximum speed for 5 sec, then spin down at 2400 rpm for 60 sec.
8. Place the *Hyb* Plate on the thermal cycler and make sure the plate is at the correct orientation (well A1 at top left).
9. Start the ***OncoScan Hybridization*** program and allow the program to run.

Figure 6.26 OncoScan Hybridization Thermal Cycler Program



Generate and Upload a Sample Batch Registration File (if it was done earlier)

To Generate and Upload a Sample Batch Registration File:

1. If using Affymetrix® GeneChip® Command Console® (AGCC) software generate and enter information into a sample batch registration file.

   NOTE: Sample nomenclature is extremely important when using the "auto-generate results file name" function in OncoScan® Console. Please refer to the *OncoScan® Console User Guide* (P/N 703195) for recommended sample nomenclature.

   A. Scan the array barcodes. Verify that the correct array type was assigned as OncoScan CNV.
   B. Upload the sample and array information to AGCC. For more information, see *Generating a Sample Batch Registration File* on page 93.
2. If using a Dx2 system, enter each individual sample as described in the AMDS software manual on page 33.

Load the Samples onto OncoScan® CNV Arrays

To Load the Samples Onto Arrays:

1. At the end of the 5 min incubation at 49°C while leaving the plate on the thermal cycler at 49°C, use a razor blade to carefully cut the adhesive seal to expose 8 wells at a time (1A through 4A and 1C through 4C).
2. Working with 8 arrays at a time:
   - **A.** Aspirate 160 µL from one well and slowly inject it into the lower left septum of the correspondingly marked array.
   - **B.** Remove the pipette tip from the upper right septum of each array.
   - **C.** Repeat Step A and Step B until 8 arrays are loaded.
   - **D.** Cover both septa with large Tough-Spots as shown in Figure 6.27.
     Using larger Tough-Spots that overlap the window makes them easier to remove later on.

Figure 6.27 Covering the septa with Tough-Spots® that overlap the window

   - **E.** Place the arrays into hybridization oven trays.
   - **F.** Load the trays into the hybridization oven.
   - **G.** Repeat these steps until all of the samples are loaded onto arrays and placed in the oven.
3. Allow the arrays to incubate at 49°C and 60 rpm for 16 to 18 hours.
4. Discard the HaeIII Plate ONLY if all of the samples were used for hybridization. If there are samples remaining on the plate, seal the plate well and store it at –20°C.
5. Discard the Hyb Plate in a hazardous waste bin.

IMPORTANT: Arrays must rotate in the oven for 16 to 18 hours at 49°C and 60 rpm (18 hr maximum before you begin Chapter 7, *Washing, Staining and Scanning Arrays*).

Chapter 7

Washing, Staining and Scanning Arrays

This chapter describes how to wash, stain and scan the Affymetrix® OncoScan® CNV Arrays. The instruments that you will use include the:

- GeneChip® Fluidics Station 450 to wash and stain the arrays
- GeneChip® Scanner 3000 7G or GeneChip® Scanner 3000 Dx2 to scan the arrays

Once the arrays are scanned, the array image (.dat file) is ready for analysis.

NOTE: Affymetrix recommends a weekly cleaning protocol (Bleach_450) for the fluidics station using sodium hypochlorite bleach. Failing to run the Bleach protocol may result in reduced signal on the OncoScan® CNV Arrays. Refer to Chapter 8, *Fluidics Station Care and Maintenance* on page 87 for instructions on maintaining the Fluidics Stations.

Equipment and Consumables Required

The following equipment and consumables are required for washing, staining and scanning Affymetrix® OncoScan® CNV Arrays.

Table 7.1 Equipment and Consumables Required for Washing, Staining and Scanning Arrays

Item	Vendor	Part Number
GeneChip® Scanner 3000 7G or 3000 Dx2	Affymetrix	—
GeneChip® Fluidics Station 450 or 450Dx	Affymetrix	—
The instrument control application: Affymetrix GeneChip® Command Console v.3.2.2 or higher or AMDS v.1.3 and higher	Affymetrix	—
Tube, Safe-Lock Tube 1.5 m, Amber	Eppendorf	022363221
Tube, Safe-Lock Tube 1.5 mL, Blue	Eppendorf	022363247
Tube, Safe-Lock Tube 1.5 mL, Natural	Eppendorf	022363352
Pipets, (P-2, P-20, P-200, P-1000)	Rainin Pipetman® (or equivalent)	—
Sterile-barrier pipette tips and non-barrier pipette tips	—	—
Tygon® Tubing, 0.04" inner diameter	Cole-Parmer	H-06418-04
Tough-Spots®, Label Dots (3/8")	USA Scientific	9185-0000

Reagents Required

The following reagents are required for washing and staining arrays. These reagents have been tested and evaluated by Affymetrix scientists.

Table 7.2 Reagents Required for Washing and Staining Arrays

Reagent
Stain Buffer 1
Stain Buffer 2
Affymetrix® GeneChip® Array Holding Buffer
Affymetrix® GeneChip® Wash A
Affymetrix® GeneChip® Wash B

Fluidics Station and Scanner Control Software

If you are using the Affymetrix GeneChip Command Console software (version 3.2.2 or higher) to operate the fluidics and scanner, please refer to the *Affymetrix® GeneChip® Command Console® User Guide* (P/N 702569).

If you are using the Affymetrix Molecular Diagnostic Software (version 1.3 or higher) for washing, staining and scanning the OncoScan arrays, please refer to the AMDS User Guide.

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 protocols.

Priming should be done:

- When the fluidics station is turned on to wash the arrays
- 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 or 450 Dx is used to wash and stain the OncoScan® CNV Arrays.

To Prime the Fluidics Station:

1. Turn on the Fluidics Station.
2. Put the wash buffer lines in the appropriate wash buffers (Wash-A line in Wash Buffer-A and Wash-B line in Wash Buffer-B.
3. Ensure there is a minimum of 400 mL of Wash-A and 300 mL of Wash-B in the bottles before starting the “Prime” protocol.
4. Ensure the water line is in the Deionized Water bottle and there is at least 300 mL of water in the bottle and that the waste container has been emptied.
5. Prime the Fluidics Station.
   - From Affymetrix Command Console application, start the ‘Affymetrix Launcher’ on the desktop (Figure 7.1).
   - From the Affymetrix Launcher, open ‘AGCC Fluidics Control’ application (Figure 7.2).

- From the AGCC Fluidics Control panel, select **PRIME_450** script for the specific fluidics stations and the modules.

IMPORTANT: Use the Affymetrix® GeneChip® Wash A and Wash B buffers that are designated for the OncoScan® Assay only. These wash buffers differ from the GeneChip® Expression buffers.

Figure 7.1 Launching the AGCC Fluidics Station Control Software

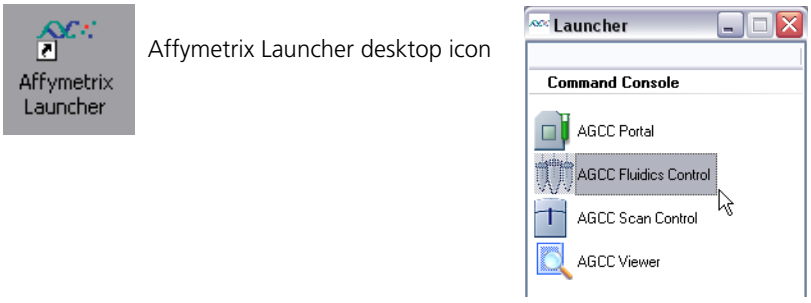
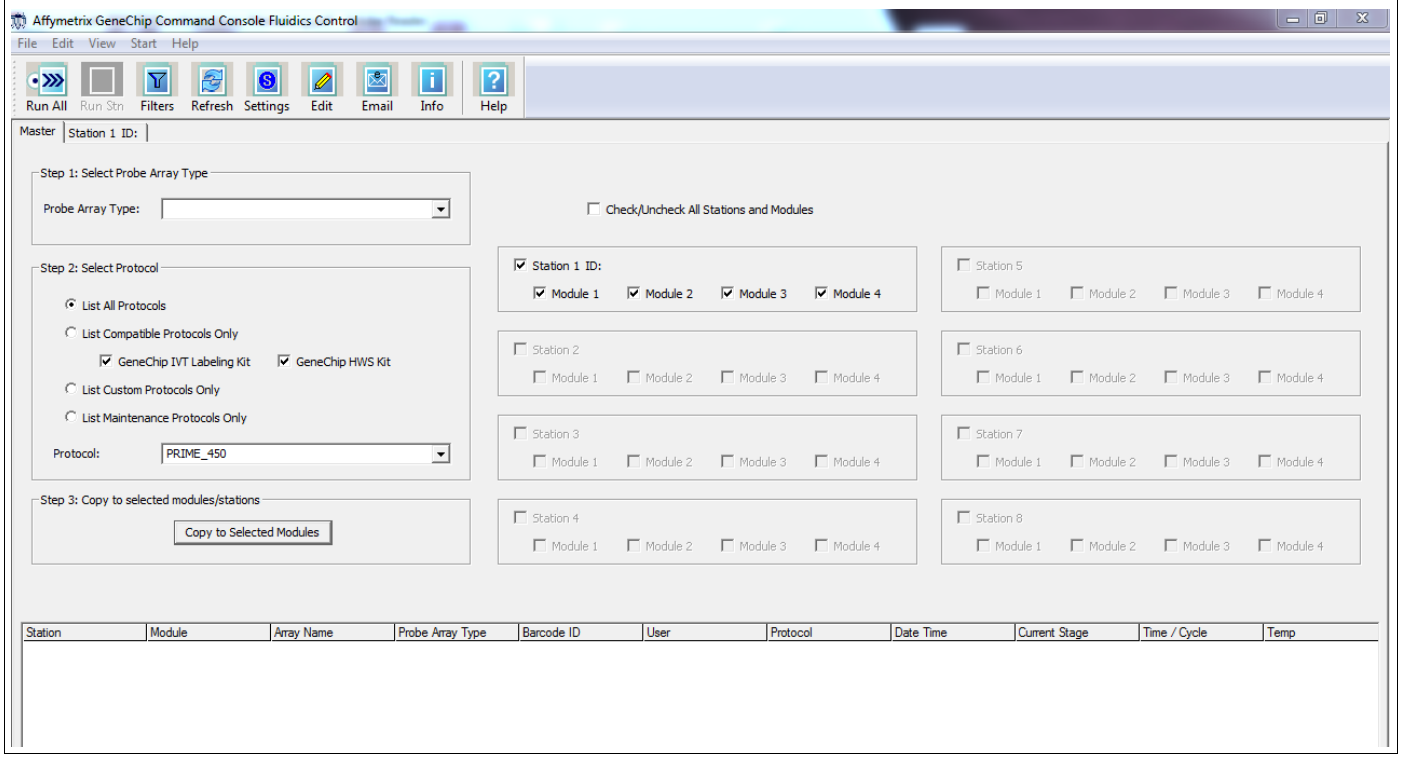


Figure 7.2 Affymetrix GeneChip® Command Console® Fluidics Control window

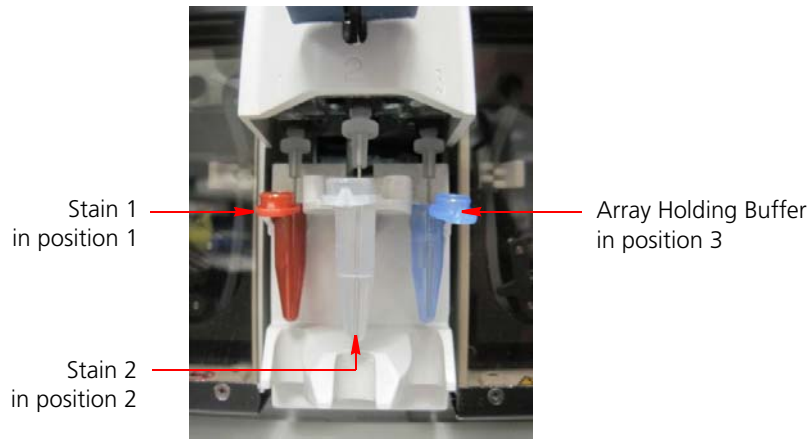


6. To initiate the fluidics script, click the “Run” icon for each module or click the “Run All” icon, for all the selected stations and modules. (“Run All” can be performed only after selecting the stations and modules, pressing the “Copy to Selected Modules” button to copy the protocol to all of the modules.)
7. Once the “prime” protocol is done, lift the needle lever and remove the vials used for priming.

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 1000 µL Array Holding Buffer into 1.5 mL microfuge tubes (blue tubes).
3. Select the **OncoScan_450** wash protocol from the AGCC Fluidics Control Panel.
4. Start the protocol and follow the instructions displayed on the LCD panel on the Fluidics Station module.
   If you are unfamiliar with inserting and removing arrays from the fluidics station modules, refer to the appropriate Fluidics Station User's Guide or Quick Reference Card (P/N 08-0093 for the Fluidics Station 450).
5. Eject the wash block to avoid sensor time out.
6. Remove any previously loaded empty vials.
7. 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 1000 µL Array Holding Buffer in position 3 (Figure 7.3).

Figure 7.3 Reagent positions on the Fluidics Station



8. After 16 to 18 hrs of hybridization, remove only the number of arrays that will be washed at this time. Leave the remaining arrays in the hyb oven at 49°C until the Fluidics Station(s) is ready for another run. Remove the Tough-Spots from the arrays that are ready to be washed and stained.

IMPORTANT: Tough-Spots must be removed from arrays prior to washing and staining. Failure to do so can cause inadequate seating of the array in the washblock and failure of the washing and staining process.

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.

9. Immediately insert the arrays into the designated modules of the fluidics station while the cartridge lever is in the Down or Eject position. Pull the handle to engage the array in the washblock, as directed on the LCD panel.

10. When the LCD panel instructs, 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.

    NOTE: Watch the fluidics for 2 minutes to make sure the protocol begins. If the user engages the washblock or presses the needles down before instructed on the LCD panel, the protocol may not begin. The user should watch for each module to start the process before walking away from the instrument.

    It is strongly recommended to check on the Fluidics Stations every 30 minutes to make sure there are no errors while washing or staining.

11. When the wash and stain procedure is completed, remove the arrays from the fluidics station by pressing down the cartridge lever to the Eject position.
12. 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.
13. If air bubbles are still present then repeat the above process or use the manual process.
    A. Insert a 200 µL pipette tip into the upper right septum of the array.
    B. Using a P-200 pipette, remove the entire volume of the holding buffer from the array.
    C. Manually fill the array with 170 µL of Array Holding Buffer.
14. If the array has no bubble, it is ready for scanning. Proceed to *Scanning Arrays* on page 82.

    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 hours.
15. Pull up on the cartridge lever to engage wash block. Remove the microcentrifuge vials containing stain and replace with three empty vials as prompted.
16. If there are more arrays to wash and stain, repeat Step 3 through Step 15 above.
17. When all array washing and staining is complete, shut down the fluidics station following the procedure on page 85.

Scanning Arrays

The GeneChip Scanner 3000 7G is controlled by AGCC software. The GeneChip Scanner 3000 Dx2 is controlled by AMDS.

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).

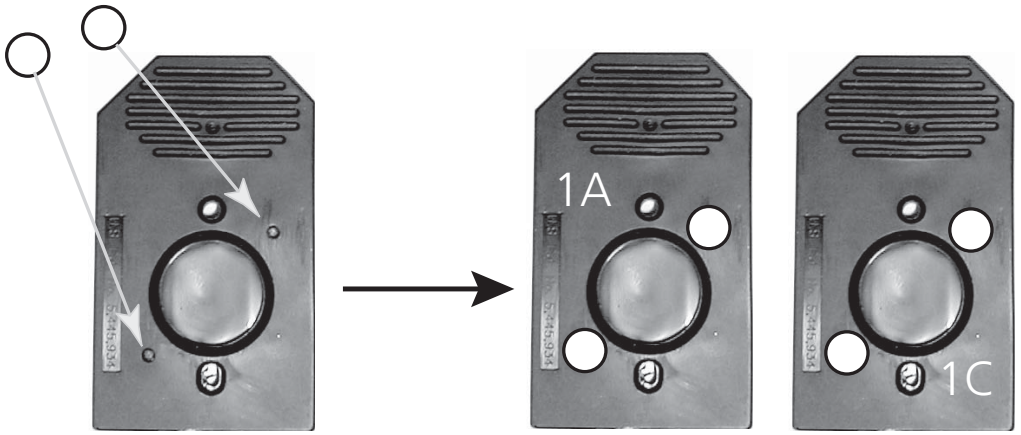
Prepare Arrays for Scanning

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, if present.
4. Carefully cover both septa with Tough-Spots (Figure 7.4).
   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.

NOTE: We recommend using the smaller 3/8" Laser Tough-Spots for this step. When applying the Tough-Spots do NOT overlap the center area as the Tough-Spots should be flat against the array to avoid being pulled off inside the scanner.

Figure 7.4 Applying Tough-Spots® to Arrays



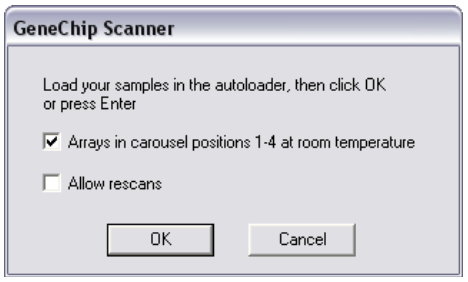
Scanning the Array

To Scan Arrays:

Ensure the completed OncoScan Batch Registration (Excel file with array barcodes) has been uploaded to the scanner computer.

1. Open the 'AGCC Scan Control' application from the 'Affymetrix Launcher'.
2. Load the arrays into 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" (Figure 7.5). 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.

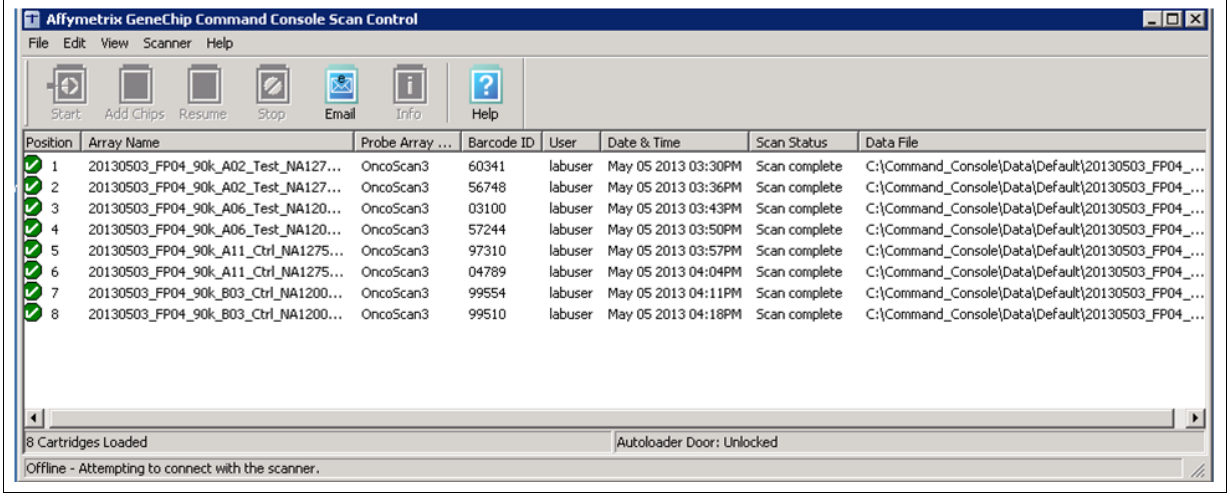
Figure 7.5 GeneChip Scanner Message



Only one scan per array is required. Pixel resolution and wavelength are preset and cannot be changed. Once the scan starts, the scanning status is shown in the Scan Status column (Figure 7.6). Each array takes approximately 7 minutes to scan.

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

Figure 7.6 Scan Status



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.

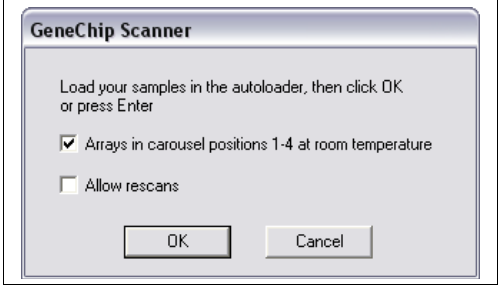
Figure 7.7 GeneChip Scanner Message

2. Click **Add after Scan**.

> **IMPORTANT: Do not use the Add Now feature. Use only the Add after Scan feature when working with OncoScan® CNV 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 7.8 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**.

> **NOTE: If you select Allow rescans, all arrays in the carousel will be scanned. Remove arrays that have already been scanned and do not need to be rescanned. If you do not select Allow rescans and you leave already scanned arrays in the scanner, you will see an error message in the scanner status stating the array has already been scanned and has been skipped. This is normal.**

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 on the LCD display window, remove the vials.
   The **REMOVE VIALS** message indicates the cleanout procedure is complete.
3. If no other processing is to be performed, place the wash lines into a bottle filled with deionized water.
4. Using AGCC, choose the **Shutdown_450** protocol for all modules.
5. Run the protocol for all modules.
   The Shutdown protocol is critical to instrument reliability. Refer to the instrument User's Guide for more information.
6. When the protocol is complete, turn the instrument off. Close the lid on the Wash Buffer bottles and save them at room temperature for later use.
7. 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 (see Chapter 8, *Fluidics Station Care and Maintenance* on page 87.**

Chapter 8

Fluidics Station Care and Maintenance

General Fluidics Station Care

- Use a surge protector on the power line to the fluidics station.
- Always run a Shutdown protocol when the instrument is unused overnight or longer. This will prevent salt crystals from forming within the fluidics system.
- To ensure proper functioning of the instrument, perform periodic maintenance.
- 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.
- Always use deionized water to prevent contamination of the lines. Change buffers with freshly prepared buffer at each system startup.
- 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

Affymetrix recommends 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. Affymetrix recommends running this protocol weekly, regardless of the frequency of use. The current version of the protocol can be found at:

www.affymetrix.com/support/technical/fluidics_scripts.affx

The Bleach Cycle

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

Table 8.1 Affymetrix 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 8.1 on page 88.
2. Prepare 500 mL of 0.525% sodium hypochlorite solution using deionized water.

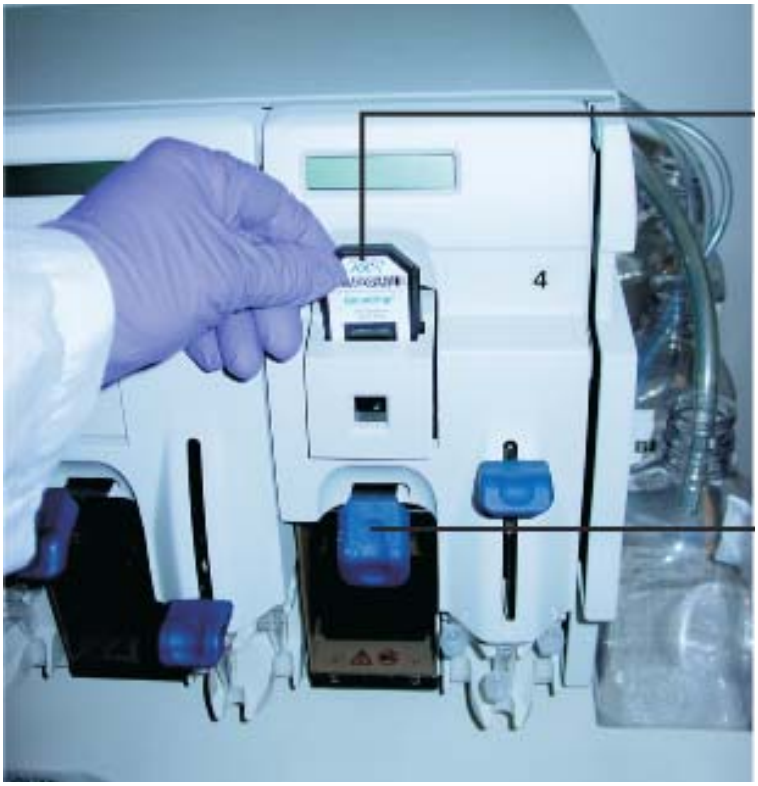
   You can follow these directions to make 500 mL of bleach:

   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:

- **The shelf life of this solution is 24 hr. After this period, you must prepare fresh solution.**
- **Each fluidics station with 4 modules requires 500 mL of 0.525% sodium hypochlorite solution.**

Figure 8.1 Disengaged Washblocks Showing Cartridge Levers in the Down Position. Remove any cartridges.



3. As shown in Figure 8.2 on page 89:
   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.
      The Bleach protocol requires approximately one liter of DI water.
   B. Insert the waste line into the waste bottle.
   C. Immerse all three wash and water lines into the bleach solution.

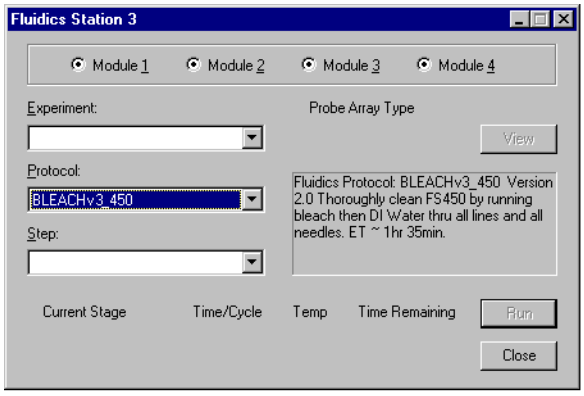
   IMPORTANT: Do NOT immerse the waste line into the bleach.
4. Open the instrument control software (AGCC or AMDS).
5. Choose the current bleach protocol for each module.

Figure 8.2 Bleach Cycle

Immerse the tubes into the 0.525% sodium hypochlorite solution.

The waste line remains in the waste bottle.

Figure 8.3 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 8.4 on page 90). The temperature will ramp up to 50°C.

7. Follow the prompts on each LCD display window. 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 8.4).

Figure 8.4 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 display window 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 display window 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 8.5).

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

Figure 8.5 Immerse the Three Wash and Water Lines in the DI Water Bottle

3. Press down on the needle levers to begin the rinse cycle.
   The fluidics station will empty the lines and rinse the needles.
4. 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.
   The LCD display will read CLEANING DONE.
5. Discard the vials used for the bleach protocol.
6. After completing the bleach protocol, follow the suggestions for storage of the Fluidics Station 450 in Table 8.2.

Table 8.2 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.

Appendix A

Registering Samples in Affymetrix® GeneChip® Command Console®

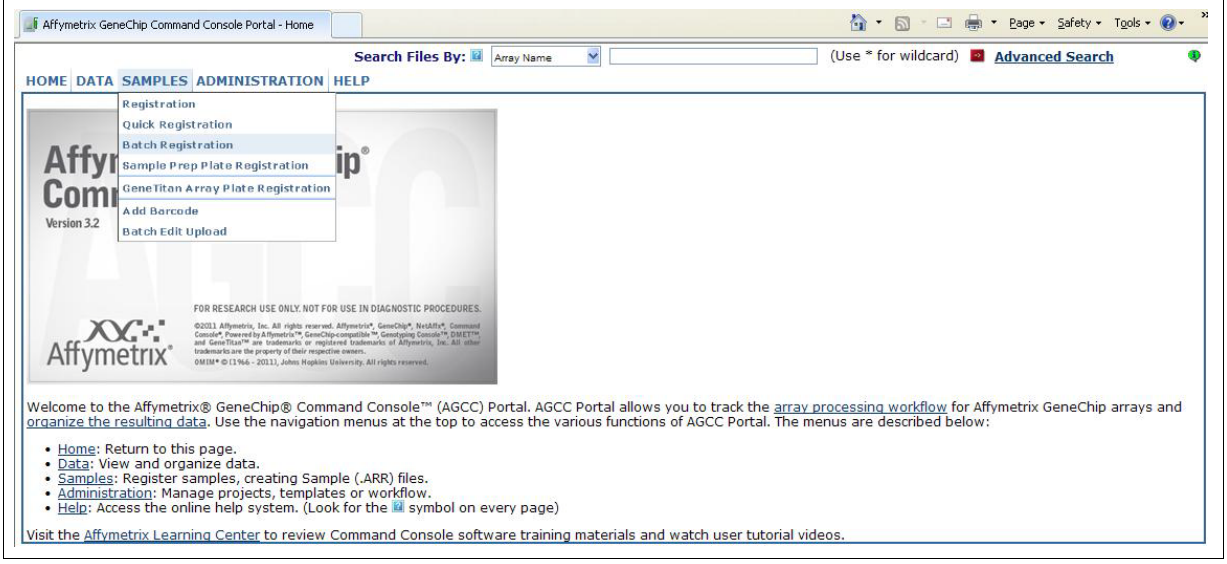
IMPORTANT: We strongly recommend that you *batch register* your sample and array information *prior to washing and scanning*. If you accidentally wash and scan your arrays without first registering them, the intensity filename (.CEL files) will not include channel-specific information needed to properly pair the intensity files in OncoScan Console. Before you can genotype the intensity files, you will have to manually edit these files to include the required information.

Generating a Sample Batch Registration File

To Generate a Sample Batch Registration File:

1. From the Launcher, open **AGCC Portal**.
2. Hold the cursor over **Samples** tab and select **Batch Register** from the drop-down menu (Figure A.1).

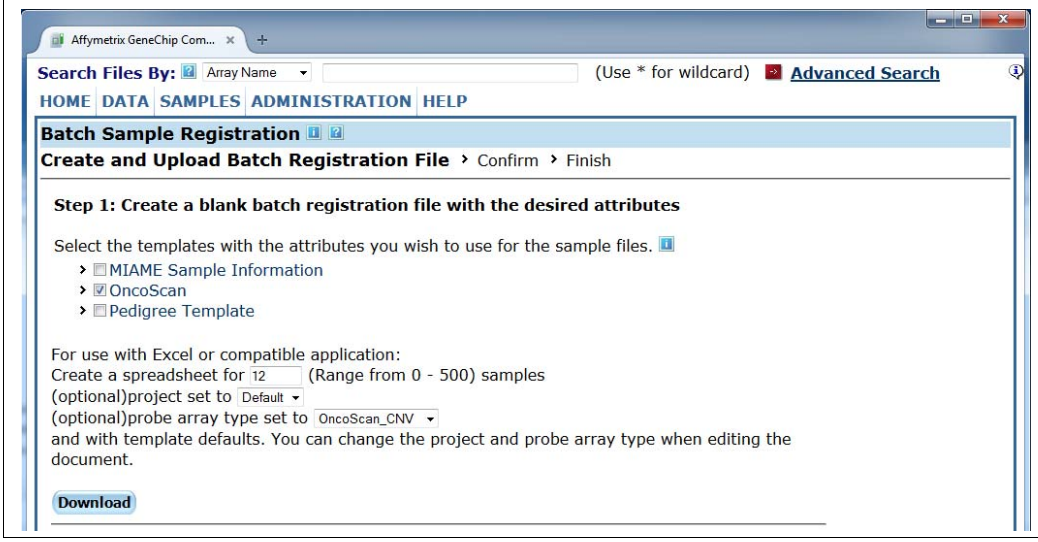
Figure A.1 Selecting Batch Register to open a spreadsheet template.



3. Create a blank Batch Registration File by selecting (Figure A.2 on page 94):
   - **A.** A template (this example uses the OncoScan.TEMPLATE included with the .ZIP file that also includes the OncoScan_CNV AGCC library file installer).
   - **B.** The number of samples to be recorded on the spreadsheet (one per chip).
   - **C.** Optional: A project name from the drop-down menu.
   - **D.** Optional: The array type from the drop-down menu (select OncoScan_CNV).

IMPORTANT: Make sure to select the correct OncoScan CNV array type to avoid issues with analysis.

Figure A.2 Creating a blank batch sample registration file.



4. Click **Download**.
   A blank registration file is displayed.

IMPORTANT: Sample nomenclature is very important for downstream use of Auto-Generate OSCHP file names in OncoScan Console. Common root names should be consistent all the way up to the last character of the CEL file name. Affymetrix recommends using an "A" or "C" as the last character to designate the channel in the CEL file naming convention. Example: "SampleName_05A.CEL" is an AT Channel file, while "SampleName_05C.CEL" is a GC Channel file.

5. Enter the sample information (Figure A.3 on page 94).
   Required fields:
   - Sample File Path
   - Project
   - Sample File Name (name that AGCC will assign to the .ARR file)
   - Array Name
   - Probe Array Type (Click in an empty cell to open the drop-down menu. Select OncoScan CNV.)
   - Barcode
   - (Optional) any sample attributes (like Channel) supplied by the selected template
6. Open **File → Save As** and:
   A. Select the location where the file will be stored.
   B. Enter a name for the file.

Figure A.3 Enter information into the batch registration file.

	A	B	C	D	E	F	G
1	Sample File Path	Project	Sample File Name	Array Name	Probe Array Type	Barcode	Channel:OncoScan:SingleSelect:Required
2		Default	20150407_prjct011_P01_A01_A	20150407_prjct011_P01_A01_A	OncoScan_CNV	@5211910095733812311642502844513 4	AT
3		Default	20150407_prjct011_P01_A02_A	20150407_prjct011_P01_A02_A	OncoScan_CNV	@5211910095733412311642502844419 0	AT
4		Default	20150407_prjct011_P01_A03_A	20150407_prjct011_P01_A03_A	OncoScan_CNV	@5211910095733512311642502844432 1	AT
5		Default	20150407_prjct011_P01_A04_A	20150407_prjct011_P01_A04_A	OncoScan_CNV	@5211910095733112311642502848638 4	AT
6		Default	20150407_prjct011_P01_A05_A	20150407_prjct011_P01_A05_A	OncoScan_CNV	@5211910095733712311642502844495 8	AT
7		Default	20150407_prjct011_P01_A06_A	20150407_prjct011_P01_A06_A	OncoScan_CNV	@5211910095733712311642502844497 6	AT
8		Default	20150407_prjct011_P01_A01_C	20150407_prjct011_P01_A01_C	OncoScan_CNV	@5211910095733812311642502844511 4	GC
9		Default	20150407_prjct011_P01_A02_C	20150407_prjct011_P01_A02_C	OncoScan_CNV	@5211910095733212311642502848663 8	GC
10		Default	20150407_prjct011_P01_A03_C	20150407_prjct011_P01_A03_C	OncoScan_CNV	@5211910095805801311742528517 5508	GC
11		Default	20150407_prjct011_P01_A04_C	20150407_prjct011_P01_A04_C	OncoScan_CNV	@5211910095807101311742528517 0434	GC
12		Default	20150407_prjct011_P01_A05_C	20150407_prjct011_P01_A05_C	OncoScan_CNV	@5211910095807101311742528517 0356	GC
13		Default	20150407_prjct011_P01_A06_C	20150407_prjct011_P01_A06_C	OncoScan_CNV	@5211910095805901311742528517 5615	GC

Upload the Batch Registration File to AGCC

To Upload the Batch Registration File to AGCC:

1. Open the batch registration file created for this set of samples.
2. Scan the array barcodes into this file.
3. Ensure selected array type is OncoScan CNV.
4. In the Batch Sample Registration window, Step 3 (Figure A.4 on page 95):
   A. If custom barcodes have been affixed to the arrays, select the check box **Allow Custom Barcodes**.
   B. Click **Browse**; then navigate to and open the batch registration file.
   C. Click **Upload**.
   D. When you see the message "Do you want to save samples?" click **Save** (Figure A.5 on page 96). Figure A.6 on page 96 appears.

> **IMPORTANT: You must click Save once you have uploaded the batch registration file. If you do not click Save, the information is not uploaded.**

Figure A.4 Uploading a sample batch registration file.

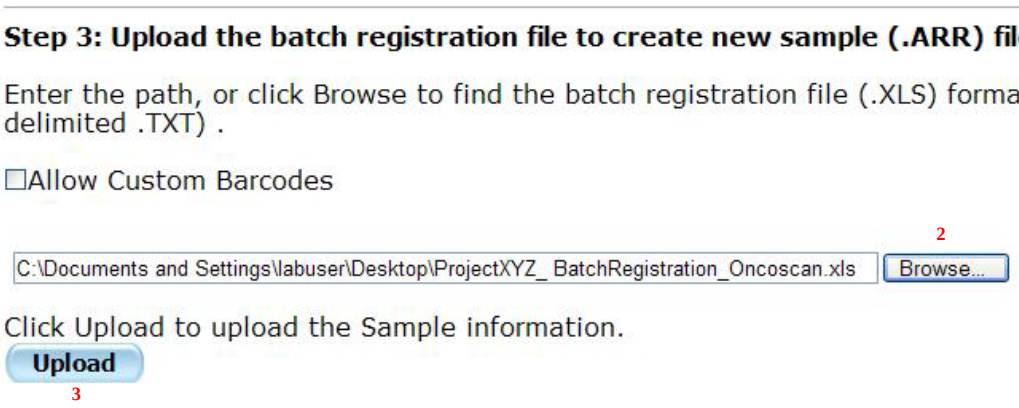
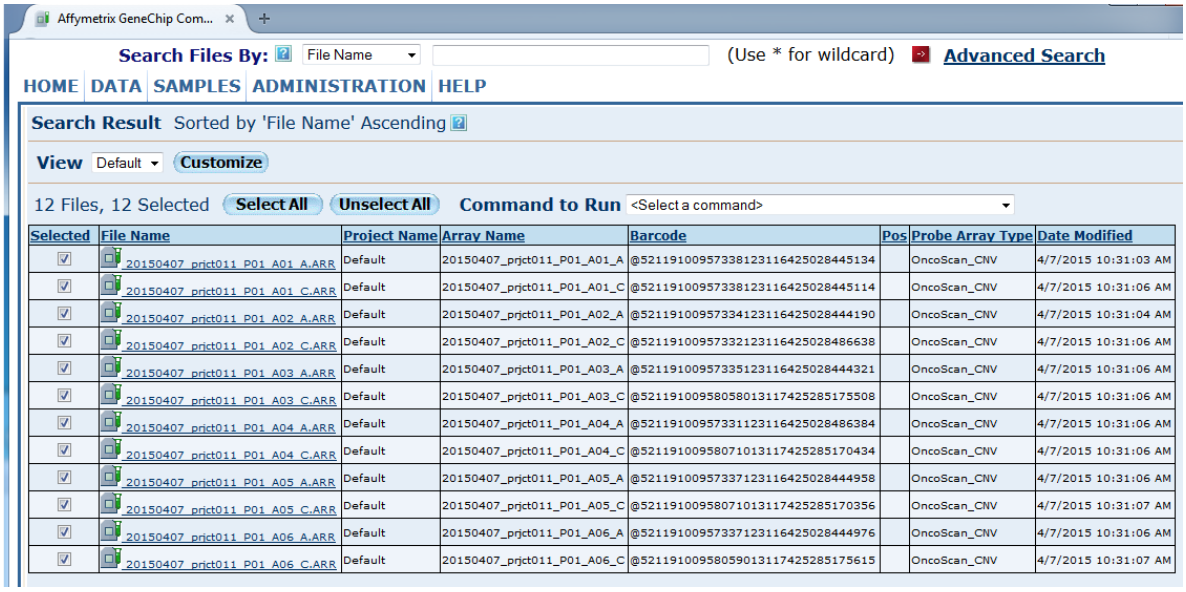


Figure A.5 Batch registration.

Figure A.6 Batch registration



Selected	File Name	Project Name	Array Name	Barcode	Pos	Probe Array Type	Date Modified
☑	20150407_prjct011_P01_A01_A.ARR	Default	20150407_prjct011_P01_A01_A	@52119100957338123116425028445134		OncoScan_CNV	4/7/2015 10:31:03 AM
☑	20150407_prjct011_P01_A01_C.ARR	Default	20150407_prjct011_P01_A01_C	@52119100957338123116425028445114		OncoScan_CNV	4/7/2015 10:31:06 AM
☑	20150407_prjct011_P01_A02_A.ARR	Default	20150407_prjct011_P01_A02_A	@52119100957334123116425028444190		OncoScan_CNV	4/7/2015 10:31:04 AM
☑	20150407_prjct011_P01_A02_C.ARR	Default	20150407_prjct011_P01_A02_C	@52119100957332123116425028486638		OncoScan_CNV	4/7/2015 10:31:06 AM
☑	20150407_prjct011_P01_A03_A.ARR	Default	20150407_prjct011_P01_A03_A	@52119100957335123116425028444321		OncoScan_CNV	4/7/2015 10:31:06 AM
☑	20150407_prjct011_P01_A03_C.ARR	Default	20150407_prjct011_P01_A03_C	@52119100958058013117425285175508		OncoScan_CNV	4/7/2015 10:31:06 AM
☑	20150407_prjct011_P01_A04_A.ARR	Default	20150407_prjct011_P01_A04_A	@52119100957331123116425028486384		OncoScan_CNV	4/7/2015 10:31:06 AM
☑	20150407_prjct011_P01_A04_C.ARR	Default	20150407_prjct011_P01_A04_C	@52119100958071013117425285170434		OncoScan_CNV	4/7/2015 10:31:06 AM
☑	20150407_prjct011_P01_A05_A.ARR	Default	20150407_prjct011_P01_A05_A	@52119100957337123116425028444958		OncoScan_CNV	4/7/2015 10:31:06 AM
☑	20150407_prjct011_P01_A05_C.ARR	Default	20150407_prjct011_P01_A05_C	@52119100958071013117425285170356		OncoScan_CNV	4/7/2015 10:31:07 AM
☑	20150407_prjct011_P01_A06_A.ARR	Default	20150407_prjct011_P01_A06_A	@52119100957337123116425028444976		OncoScan_CNV	4/7/2015 10:31:06 AM
☑	20150407_prjct011_P01_A06_C.ARR	Default	20150407_prjct011_P01_A06_C	@52119100958059013117425285175615		OncoScan_CNV	4/7/2015 10:31:07 AM

About Batch Registration Files

A batch registration file is a file that contains sample attributes, such as the sample name, source plate, source well and sample type. The options for creating this file include:

- Using a pre-defined template.
- Editing a template.
- Creating your own template.

Using the Predefined Template — OncoScan.TEMPLATE

If the predefined template is not already installed on the computer with AGCC, you must first copy it to this computer. Obtain the OncoScan_CNV AGCC library installer .ZIP file from Affymetrix. The .ZIP file containing the installer also includes the file OncoScan.TEMPLATE. Copy this file to the Templates folder used by AGCC (usually located at C:\Command_Console\Templates).

Creating or Editing a Template

Editing Templates

To Edit a Template:

1. From the AGCC Portal, open **Administration → Templates → Edit**.
2. Select the template you would like to edit (for example, you can edit OncoScan.TEMPLATE to suit your project or study requirements).
3. Delete, edit, or add attributes.
4. Click **Save**.

Creating Templates

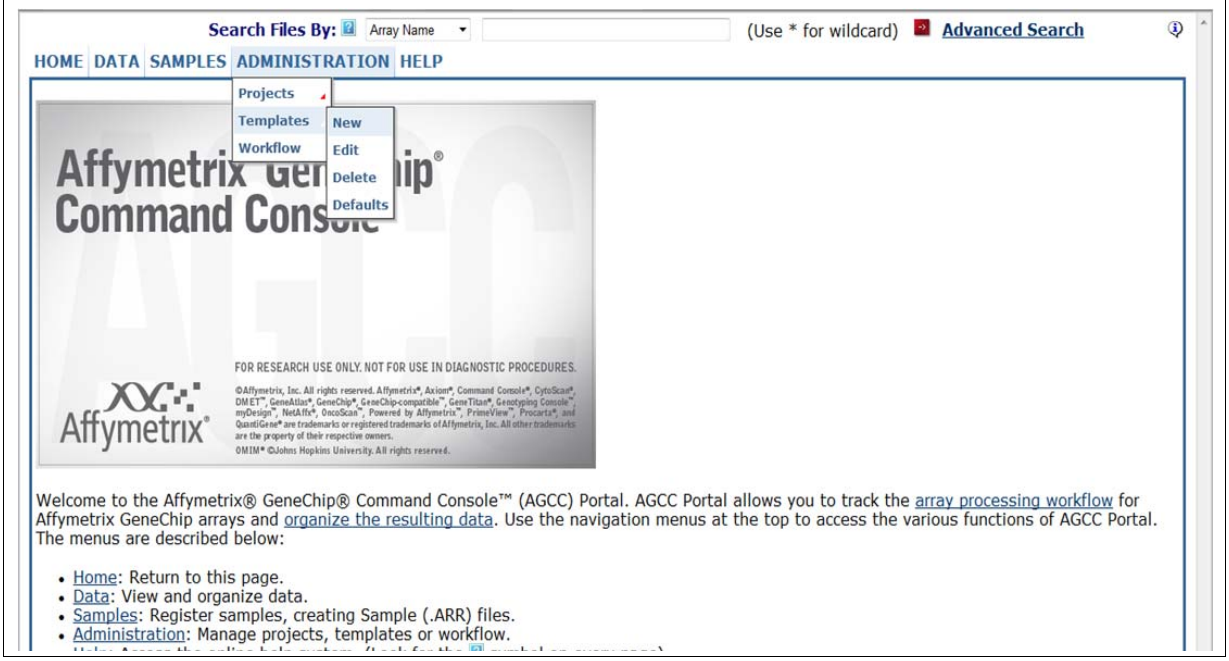
You can create and edit templates in AGCC Portal. To help with template creation, the following fields are automatically included when creating a template:

Path	Probe Array Type	Barcode
Project	**Sample File Name**	**Array Name**

To Create Your Own Template:

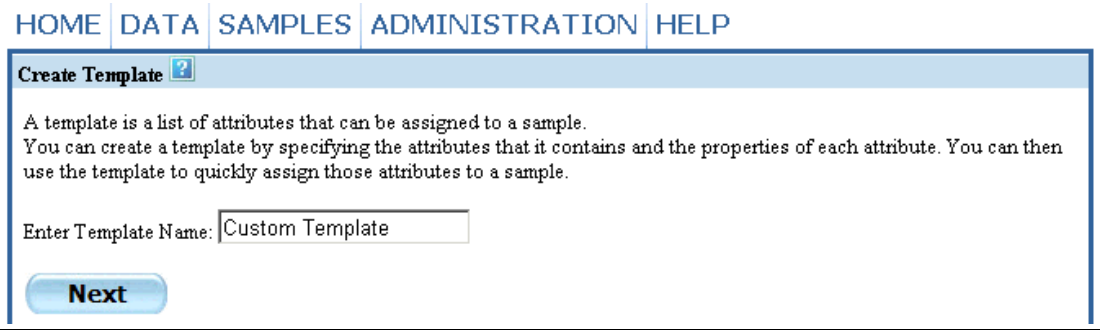
1. From the AGCC Portal, open **Administration → Templates → New**.

Figure A.7 Creating a new template for sample batch registration



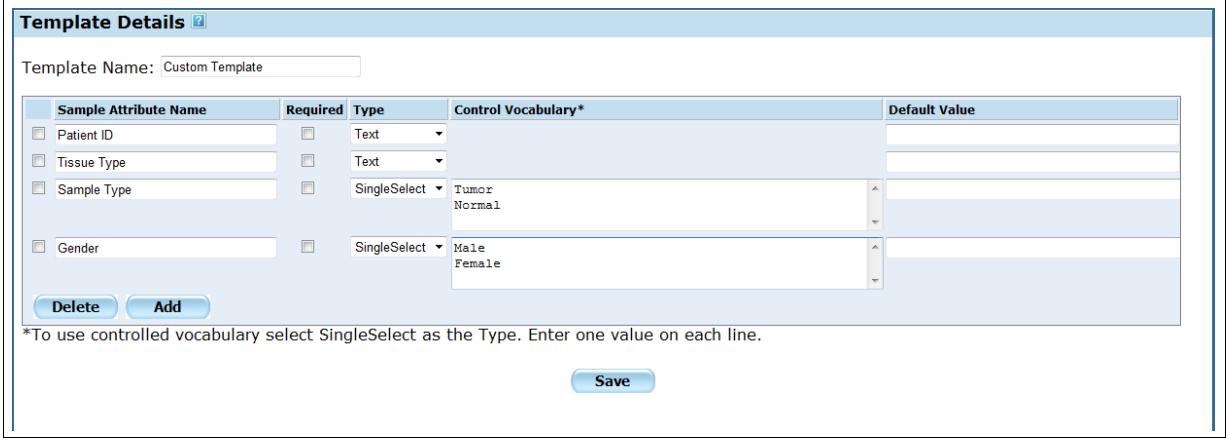
2. Enter a name for the template; then click **Next**.
   The template name is appended to each attribute that you add; therefore, you may want to keep the name as short as possible (see Figure A.11 on page 100).

Figure A.8 Entering a name for the new template



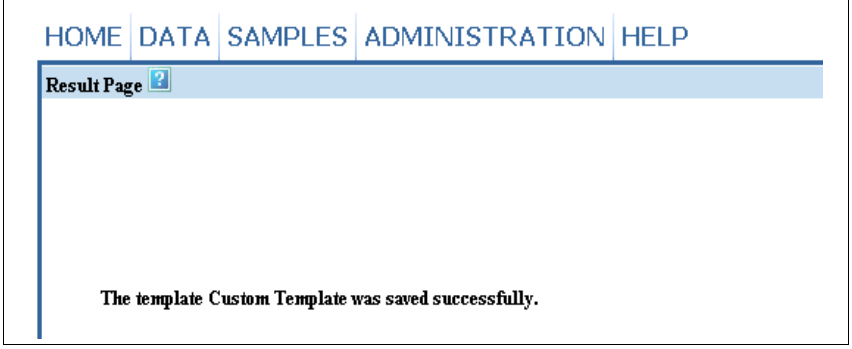
3. Add attributes to the template by clicking **Add** and defining the attribute.

Figure A.9 Adding attributes to a new template



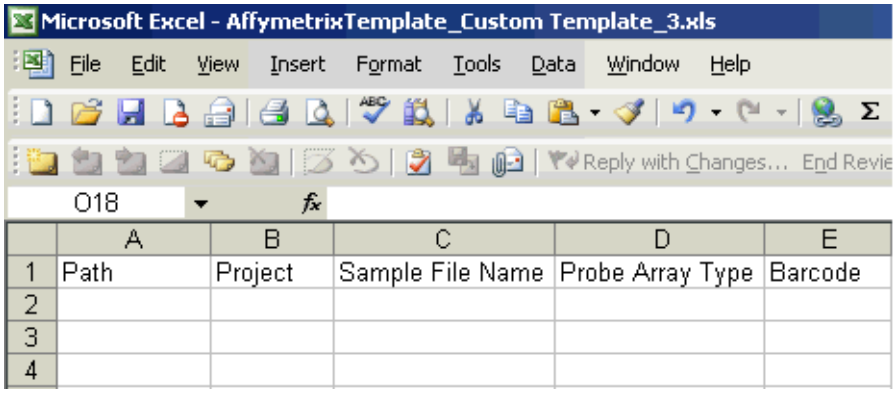
4. When finished adding attributes, click **Save**.

Figure A.10 Adding attributes to a new template



The template as described in the instructions above would look like Figure A.11 when selected for use as an Excel spreadsheet.

Figure A.11 Example of a custom template



	A	B	C	D	E
1	Path	Project	Sample File Name	Probe Array Type	Barcode
2					
3					
4					

F	G
Operator:Custom Template	Sample Type:Custom Template

Appendix B

Thermal Cycler Programs

Seven thermal cycling programs are used throughout the OncoScan® CNV FFPE Assay Kit Protocol. This appendix describes each of the programs required for the protocol.

Thermal Cyclers

To run the OncoScan® CNV FFPE Assay Kit Protocol at a throughput of 24 assays/day, you will need at least 2 thermal cyclers: 1 in the Pre-Amp Lab, and 1 in the Post-Amp Lab.

Pre-PCR Lab Thermal Cycler Programs

Set up the thermal cyclers in the Pre-PCR Lab to run the following programs:

- OncoScan Anneal
- OncoScan Gap Fill (This program is set up differently in the Veriti Thermal Cycler)
- OncoScan 1st PCR

Post-PCR Lab Thermal Cycler Programs

Set up the thermal cyclers in the Post-PCR Lab to run the following programs:

- OncoScan 2nd PCR
- OncoScan HaeIII
- OncoScan Hybridization

Setting the Ramp Speed and Volume for Each Program

IMPORTANT: Set the correct ramp speed and volume for each thermal cycler program.

Ramp Speed

Max = Ramp speed for GeneAmp® PCR System 9700 Thermal Cycler (gold or silver block). Use the instructions below for setting up the "9700-Max-Mode" ramp speed on the Veriti Thermal Cycler.

Ramp Speed Settings for Veriti® Thermal Cycler

Use the "**Convert Method**" feature of the Veriti Thermal Cycler to create a method for the Veriti Thermal Cycler with ramp rates that simulate those on the 9700 thermal cycler at Max mode. Please follow instructions on the *Veriti® Thermal Cycler User Guide*, Chapter 5 (pages 5-2 to 5-4).

To Use a Run Method From a GeneAmp® PCR System 9700 Thermal Cycler

1. Obtain a copy of the original method you want to convert, either by printing it from the instrument or copying it manually to a piece of paper.
2. In the **Main Menu** screen, touch **Tools Menu**.
3. In the **Tools Menu** screen, touch **Convert a Method** to open the Convert Method wizard.
4. Touch the check box next to the run method's original format "9700 Max Mode" then touch **Next**. *Veriti® Thermal Cycler User Guide* (page 5-3).
5. In the second page, enter the original run method. Touch **Next** when you are done.
6. In the **Save Run Method** page, enter a name and select a folder for the run method. Names are limited to 16 characters. By default, the name begins with "9700-Max-Mode-".
7. Also enter the reaction volume, the temperature for the heated cover as 105.0°C, and any notes for this method.
8. When you finish, touch **Save & Exit**. The **Tools Menu** is displayed.

Important Information on Using the Veriti® Thermal Cycler

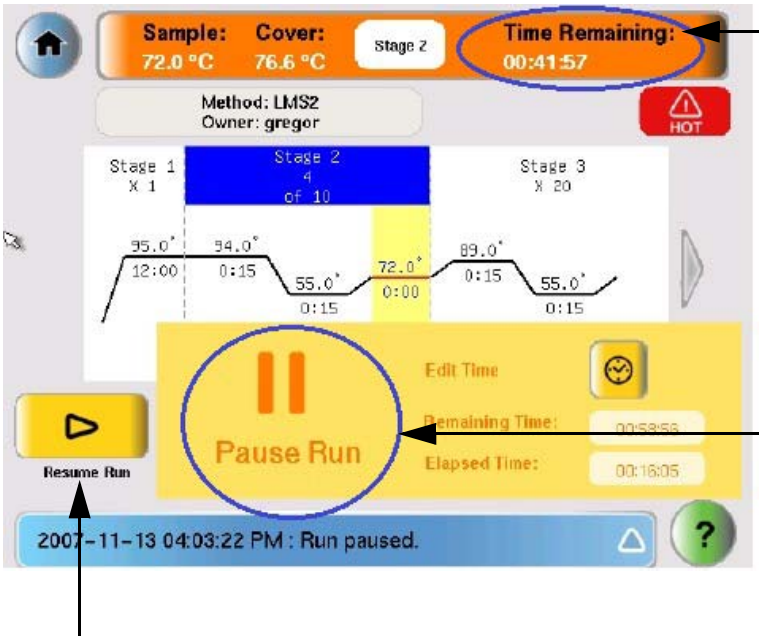
The touch screen sometimes may not respond when "**Pause Run**" or "**Resume Run**" functions are performed. Make sure the screen is only touched ONCE, firmly, and NOT TAPPED TWICE.

When the touch screen is touched with a double tap to perform "**Pause Run**", the program will momentarily pause and then immediately resume. This can create assay interruptions and possibly lead to assay failures. Ensure the touch screen is only touched ONCE, firmly.

Pay attention to the screen to make sure:

- the program pauses when "**Pause Run**" is performed. (Figure B.1)
- the program resumes when "**Resume Run**" is touched. (Figure B.1)
- When the program is Paused, the clock STOPS on "**Time Remaining**", displayed in the upper right corner of the touch screen. (Figure B.1)

Figure B.1 Veriti® Thermal Cycler Touch Screen



When the program is Paused, the clock STOPS on "**Time Remaining**" display.

The program pauses when "**Pause Run**" is performed.

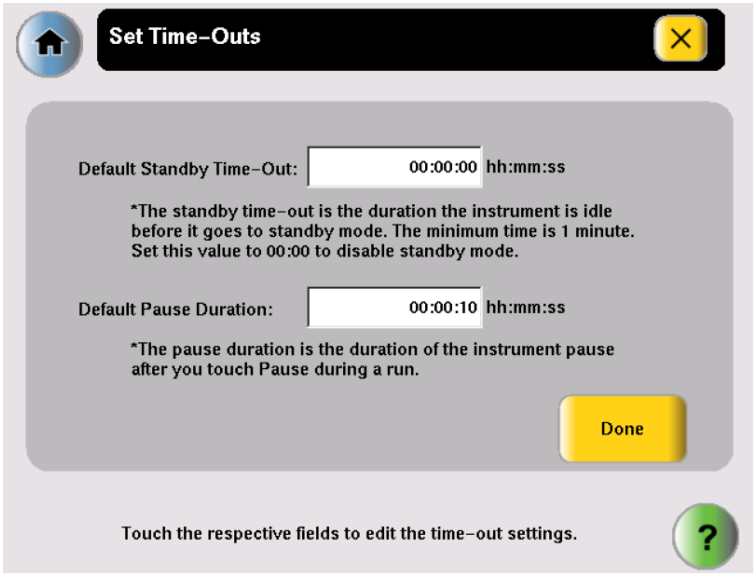
The program resumes when "**Resume Run**" is touched.

Make sure to:

- Set the **Default Standby Time-Out** to 00.00 (screen does not get turned off)
- Set the **Default Pause Duration** to 24:00:00 (pause time: 24 hours)

Refer to the Figure B.2, below, for detailed instructions on setting time-outs.

Figure B.2 Setting Time-Outs for the Veriti® Thermal Cycler



Setting Time-Outs

1. In the Main Menu screen, touch **Settings Menu**.
2. In the Settings Menu screen, touch **Set Time-Outs**.
3. In the Set Time-Outs screen, touch **Time-out**, then enter the duration.
   - **Default Standby Time-Out** sets the duration that the instrument is idle before the touchscreen is turned off. Enter a duration of **00:00** if you do not want the instrument to turn off the touchscreen.
   - **Default Pause Duration** sets the duration the instrument pauses, after you touch Pause during a run. Set to **24:00:00** (pause time: 24 hours).
4. Touch **Done**, then **OK** to save your changes and return to the Settings Menu screen.

OncoScan® Anneal Thermal Cycler Program

About the OncoScan® Anneal Program

The *OncoScan Anneal* program consists of three holds and no cycles.

Ramp speed and volume:

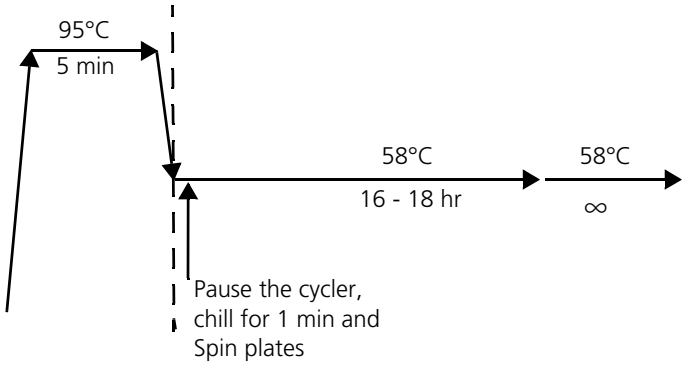
- **Ramp speed**
  - GeneAmp PCR System 9700 (gold or silver block) = Max
- **Volume**: 10 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 102.

Table B.1 Stages of the OncoScan Anneal Thermal Cycler Program

Stage	Temperature	Time
Denature	95°C	5 min
Anneal	58°C	16 - 18 hrs
Anneal	58°C	Infinity

Figure B.3 OncoScan Anneal Thermal Cycler Program



OncoScan® Gap Fill Thermal Cycler Program

About the OncoScan® Gap Fill Program

The OncoScan Gap Fill thermal cycler program consists of 10 holds and 1 cycle.

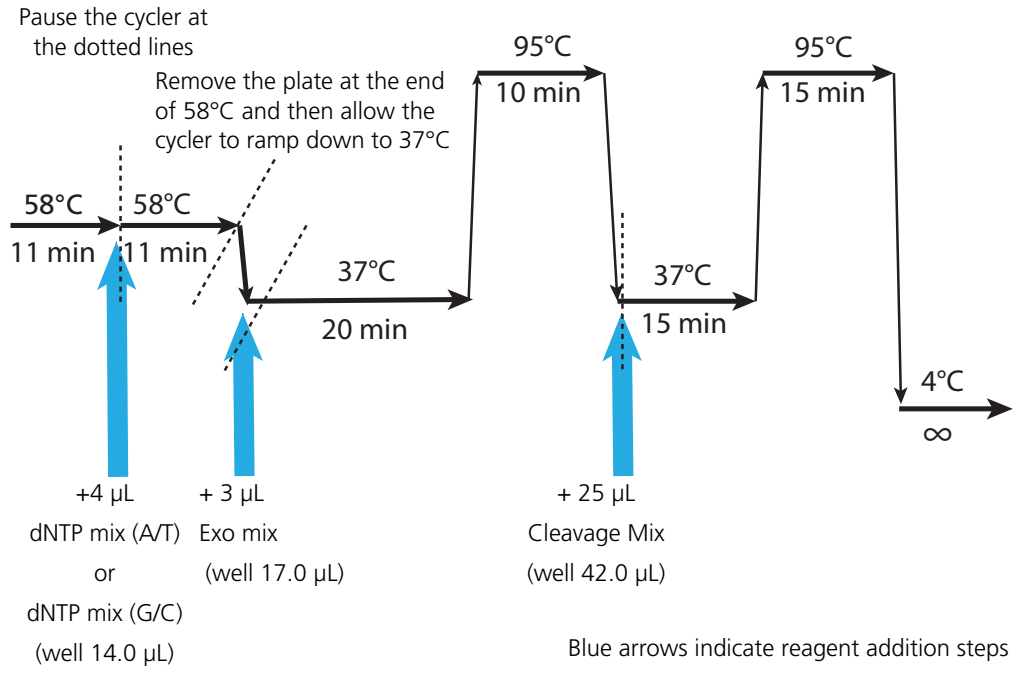
- **Ramp speed**
  - GeneAmp PCR System 9700 (gold or silver block) = Max
- **Volume**: 42 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 102.

Table B.2 Stages of the OncoScan Gap Fill Thermal Cycler Program

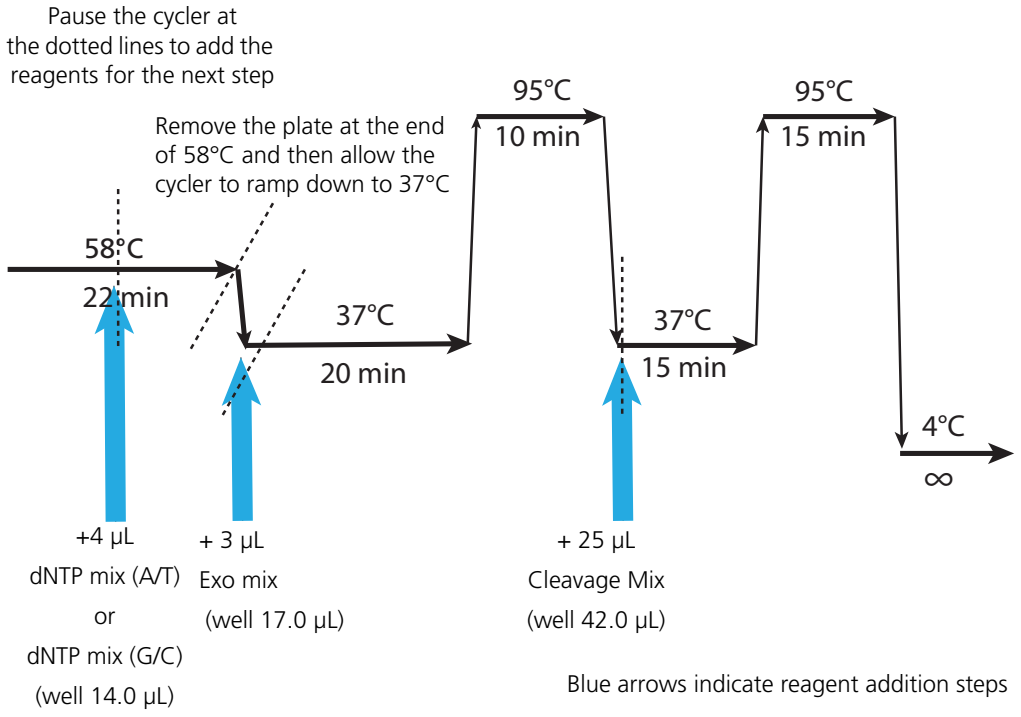
Stage	Temperature	Time	Cycles
Gap Fill	58°C	11 min	—
AT/GC Mix Addition	58°C	11 min	—
Exo Mix Addition	37°C	20 min	
Denature	95°C	10 min	
Cleavage Mix Addition	37°C	15 min	
Denature	95°C	15 min	
Hold	4°C	Infinity	

Figure B.4 OncoScan Gap Fill Thermal Cycler Program (for GeneAmp® PCR System 9700)



NOTE: For the Veriti Thermal Cycler, when programming the "OncoScan Gap Fill" program, combine the first two 58°C incubations for 11 min each into one stage at 58°C for 22 min (see Table B.2 and Figure B.5). This change is recommended because it has been noticed that the touch screen does not consistently respond to the "PAUSE RUN" function when paused between the two stages of 58°C. (The touch screen may not pause at the end of the 58°C at 11 min and will move on to the next 58°C stage). This will ruin the Gap Fill reaction, leading to assay failure. Using a laboratory timer is critical during the first two 58°C incubations. Make sure the program pauses when the "PAUSE RUN" is touched.

Figure B.5 OncoScan Gap Fill Thermal Cycler Program (for **Veriti Thermal Cycler**)



OncoScan® 1st PCR Thermal Cycler Program

About the OncoScan® 1st PCR Program

The OncoScan 1st PCR program consist of 2 holds and 1 cycle.

Ramp speeds:

- GeneAmp PCR System 9700 (gold or silver block) = Max

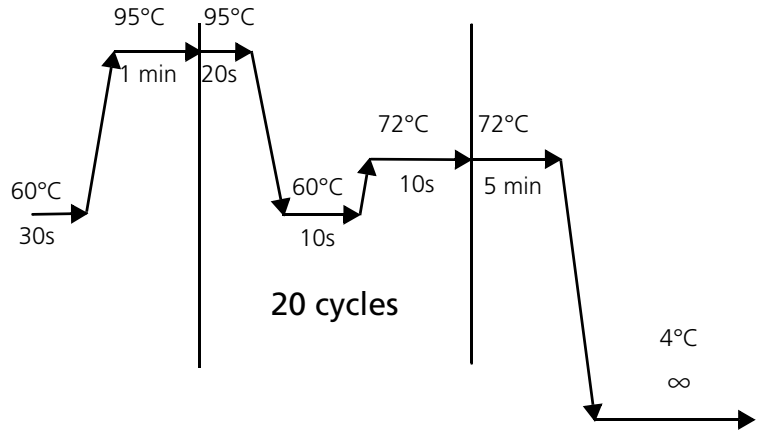
Volume: 67 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 102.

Table B.3 Stages of the OncoScan 1st PCR Thermal Cycler Program

Stage	Temperature	Time	Cycles
PCR Reaction Start	60°C	30 sec	
Template Denaturation	95°C	1 min	
Denaturation	95°C	20 sec	20 cycles
Anneal	60°C	10 sec	
Extension	72°C	10 sec	
Extension	72°C	5 min	
Hold	4°C	Infinity	

Figure B.6 OncoScan 1st PCR Thermal Cycler Program



OncoScan® 2nd PCR Thermal Cycler Program

About the OncoScan® 2nd PCR Program

The OncoScan 2nd PCR program consist of 2 holds and 1 cycle.

Ramp speeds:

- GeneAmp PCR System 9700 (gold or silver block) = Max

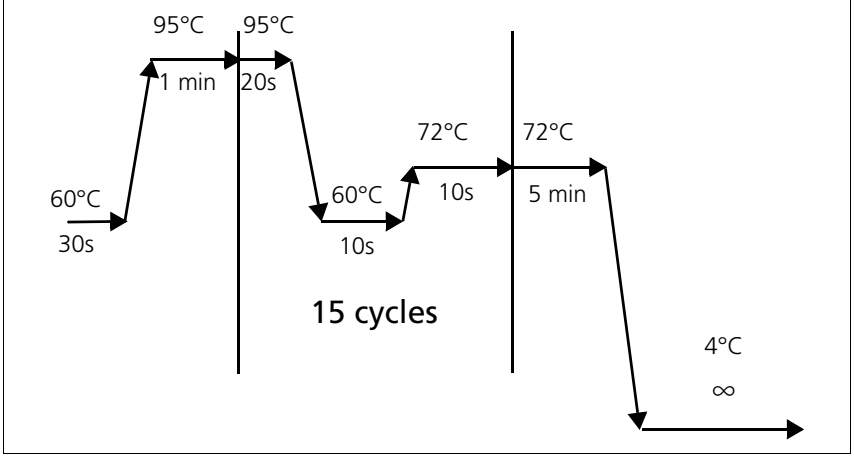
Volume: 27 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 102.

Table B.4 Stages of the OncoScan 2nd PCR Thermal Cycler Program

Stage	Temperature	Time	Cycles
PCR Reaction Start	60°C	30 sec	
Template Denaturation	95°C	1 min	
Denaturation	95°C	20 sec	15 cycles
Anneal	60°C	10 sec	
Extension	72°C	10 sec	
Extension	72°C	5 min	
Hold	4°C	Infinity	

Figure B.7 OncoScan 2nd PCR Thermal Cycler Program



OncoScan® HaeIII Digest Thermal Cycler Program

About the OncoScan® HaeIII Digest Program

The *OncoScan HaeIII Digest* program consists of 2 holds and no cycles.

Ramp speeds:

- GeneAmp PCR System 9700 (gold or silver block) = Max

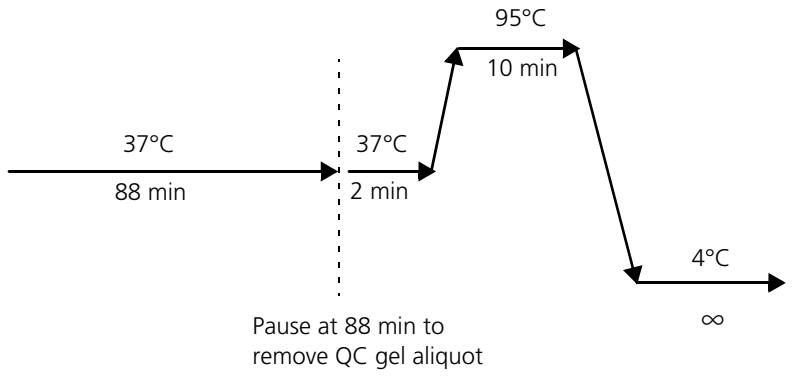
Volume: 30 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 102.

Table B.5 Stages of the HaeIII Digest Thermal Cycler Program

Stage	Temperature	Time
HaeIII Digestion	37°C	88 min
Continuation of HaeIII Digestion after pausing to get the QC gel aliquot	37°C	2 min
Deactivation of the Enzyme	95°C	10 min
Hold	4°C	Infinity

Figure B.8 HaeIII Digest Thermal Cycler Program



OncoScan® Hybridization Thermal Cycler Program

About the OncoScan® Hybridization Program

The *OncoScan Hybridization* program consists of 2 holds and no cycles.

Ramp speeds:

- GeneAmp PCR System 9700 (gold or silver block) = Max

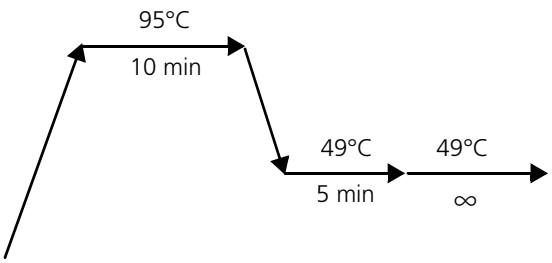
Volume: 170 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 102.

Table B.6 Stages of the OncoScan Hybridization Thermal Cycler Program

Stage	Temperature	Time
Denature	95°C	10 min
	49°C	5 min
Standby	49°C	Infinity

Figure B.9 OncoScan Hybridization Thermal Cycler Program



Appendix C

Equipment, Software, Consumables and Reagents List

This chapter lists the equipment, software, consumables and reagents required to perform the OncoScan® CNV FFPE Assay Protocol.

Required from Affymetrix®

Affymetrix Equipment and Software Required

Table C.1 Affymetrix Equipment and Software Required

✓	Item	Part Number
❑	GeneChip® Fluidics Station 450 — two or more units required	00-0079
❑	Tubing, Silicone peristaltic for GeneChip® Fluidics Station 450	Contact Affymetrix
❑	GeneChip® Hybridization Oven 645	00-0331
❑	GeneChip® 3000 Scanner 7G System with Workstation and Autoloader	00-0215
❑	GeneChip® Command Console® Software (AGCC)	Version 3.2 or higher
❑	OncoScan® Console (OC) Software analysis (AGCC)	Latest version
❑	Chromosome Analysis Suite Software (ChAS)	Latest version
❑	Nexus for Affymetrix Software	Latest version

OncoScan® CNV Reagents and OncoScan® CNV Arrays Required

Table C.2 OncoScan® CNV Reagent Kit and OncoScan® CNV Arrays

✓	Item	Part Number
❑	OncoScan® CNV FFPE Assay Kit Array and Reagent Kit Bundle, Sufficient for 24 samples and includes 48 OncoScan CNV Arrays — Components in this kit are listed below.	902695
	OncoScan® CNV Buffer C ▪ Buffer C	
	OncoScan® CNV Copy Number Probe Mix 1.0 & Controls ▪ Positive Control (12ng/µL) ▪ Negative Control ▪ Copy Number Probe Mix 1.0 ▪ Buffer A	
	OncoScan® CNV Gap Fill and 1st Stage PCR ▪ Buffer A ▪ Gap Fill Enzyme Mix ▪ SAP, Recombinant (1 U/µL) ▪ dNTP Mix (A/T) ▪ dNTP Mix (G/C) ▪ Nuclease-free Water ▪ Exo Mix ▪ Cleavage Buffer ▪ Cleavage Enzyme (2 U/µL) ▪ PCR Mix ▪ Taq Polymerase (5 U/µL)	
	OncoScan® CNV 2nd Stage PCR and Post PCR Processing ▪ PCR Mix ▪ Taq Polymerase (5 U/µL) ▪ Buffer B ▪ HaeIII Enzyme (10 U/µL) ▪ Exo I Enzyme (20 U/µL) ▪ Nuclease-free Water ▪ Hybridization Mix	
	OncoScan® CNV Stain Reagents ▪ Stain 1 ▪ Stain 2 ▪ Array Holding Buffer	
	Individual Bottles ▪ Wash A ▪ Wash B	
❑	**Arrays** ▪ OncoScan® CNV Array, 4 x 12 pack	

From Other Suppliers — Reagents, Equipment and Consumables Required

Reagents Required for Gel QC

(Not included in the OncoScan® CNV FFPE Assay Kit)

Table C.3 Reagents Required for Gel QC (Not included in the OncoScan CNV FFPE Assay Kit)

Item	Supplier	Part Number
3% 96-well pre-cast agarose gel (as needed, based on the number of samples)	Bio-Rad	161-3062
50 bp DNA ladder	New England Biolabs	N3236S
Gel loading Dye, Blue (6X)	New England Biolabs	B7021S
100% Glycerol	Affymetrix	16374
EDTA, 0.5 M Solution	Affymetrix	15694
1X TE buffer Low EDTA, pH 8.0	Affymetrix	75793

Table C.4 Reagents and Equipment Required for Gel QC using E-Gels (Not included in the OncoScan CNV FFPE Assay Kit)

Item	Supplier	Part Number
Mother E-Base™ Device	Life Technologies	EB-M03
Daughter E-Base™ Device (optional for running multiple gels simultaneously)	Life Technologies	EB-D03
E-Gel® 48 4% Agarose Gels	Life Technologies	G8008-04
TrackIt™ Cyan/Orange Loading Buffer	Life Technologies	10482-028
50 bp DNA ladder	New England Biolabs	N3236S
Nuclease-free Water	Affymetrix	71786

Miscellaneous Consumables Required

Table C.5 Miscellaneous Consumables Required

Item	Qty Needed per 24 Samples	Part Number	Supplier
0.2 mL PCR 12-tube strip with 12-cap strips, natural	10	1402-2400	USA Scientific
1/2" Laser Tough-Spots	as needed	RNBW-1100	Diversified Biotech
3/8" Laser Tough-Spots	as needed	RNBW-1000	Diversified Biotech
Micro-Amp Clear Adhesive Film	as needed	4306311	Applied Biosystems
Optical 96 Well Half-skirt plate	5	289196	E&K Scientific
MicroAmp Optical 96 well reaction plate (Optional for UK; compatible with ABI thermal cyclers)	5	N8010560	Invitrogen
VWR PCR Plate, 96-Well, Flat Plate	Optional (for gel QC)	82006-636	VWR
1.5 mL Safe-Lock Tubes, Natural	as needed	022363204	Eppendorf
1.5 mL Safe-Lock Tubes, Blue	as needed	022363247	Eppendorf
1.5 mL Safe-Lock Tubes, Amber	as needed	022363221	Eppendorf
Non-Stick RNase-free Microfuge Tubes (1.5 mL)	as needed	AM12450	Life Technologies
Non-Stick RNase-free Microfuge Tubes (2.0 mL)	as needed	AM12475	Life Technologies
25 mL Reagent Reservoir	as needed	89094-662	VWR
Pipette Tip Refills, 20 µL	as needed	GP-L10F	Rainin
Pipette Tip Refills, 200 µL	as needed	GP-L200F	Rainin
Pipette Tip Refills, 1000 µL	as needed	GP-L1000F	Rainin
15 mL Centrifuge tubes		21008-103	VWR

Equipment Required

When performing the pre-PCR stages of the OncoScan CNV FFPE Assay Kit Protocol, great care should be taken to avoid sample contamination with PCR products.

Table C.6 Equipment Required

Item	Part Number	Supplier	Optional Part Number/ Manufacturer	Needed In	Needed In
Vortexer Maxi Mix II	M37615	Thermo Scientific	Vortex Genie G560 /VWR	Pre-PCR Lab	Post-PCR Lab
Picofuge Galaxy Mini	C1213	VWR	—	Pre-PCR Lab	Post-PCR Lab
Striptube Centrifuge Galaxy Mini	C1213	VWR	—	Pre-PCR Lab	Post-PCR Lab
Ice Pan, Maxi	89231-998	VWR	—	Pre-PCR Lab	Post-PCR Lab
96-well Cold Blocks	81001		—	Pre-PCR Lab	Post-PCR Lab
GeneAmp PCR system 9700 Thermal Cycler (with Gold block)	N050200	Life Technologies	4375786* (Veriti 96-well) / Life Technologies	Pre-PCR Lab	Post-PCR Lab
Single Channel Pipettes (entire LTS set)	L-2, L-20, L-200, L-1000	Rainin	—	Pre-PCR Lab	Post-PCR Lab
12-Channel LTS Pipette (20 µL and 200 µL)	L12-20, L12-200	Rainin	—	Pre-PCR Lab	Post-PCR Lab
24-Channel LTS Pipette (20 µL and 200 µL)	L24-20, L24-200	Rainin	—	Pre-PCR Lab	Post-PCR Lab
MicroAmp Clear Adhesive Film	4306311	Life Technologies	—	Pre-PCR Lab	Post-PCR Lab
Plate Centrifuge 5810 or 5804	5810 / 5804	Eppendorf	—	Pre-PCR Lab	Post-PCR Lab
Benchtop Cooler	89004-558	VWR	—	Pre-PCR Lab	Post-PCR Lab
Gel Box (Wide-Mini sub cell GT basic system)	170-4489	Bio-Rad	—	—	Post-PCR Lab
Power Supply for Gel Electrophoresis	164-5070	Bio-Rad	—	—	Post-PCR Lab
AlphaImager HP System for gel imaging (with software)	Varies (Inquire)	Protein Simple	—	—	Post-PCR Lab
Hybridization Oven 645	00-0331	Affymetrix	—	—	Post-PCR Lab
Probe Array Cartridge Carriers	90-0356 to 90-0359	Affymetrix	—	—	Post-PCR Lab
GeneChip® Fluidics Station 450	Varies depending on location	Affymetrix	—	—	Post-PCR Lab
GeneChip® Scanner 3000 7G System with Workstation and AutoLoader	Varies depending on location	Affymetrix	—	—	Post-PCR Lab
GeneChip® Command Console® Software (AGCC)	Inquire	Affymetrix	—	—	Post-PCR Lab

* The Veriti 96-well Fast Thermal Cycler, P/N 4375305 is not compatible with this assay protocol. Use only one of the two recommended thermal cyclers listed above.

Table C.6 Equipment Required (Continued)

Item	Part Number	Supplier	Optional Part Number/ Manufacturer	Needed In	Needed In
If preparing samples using QIAGEN FFPE Kit					
Fume Hood			—	Sample Preparation Lab (Clean or Pre-PCR)	
Thermomixer® R with 1.5 mL adapter	5355-31595	Eppendorf	—		
Centrifuge 5415D	5425 50003	Eppendorf	5415C		
Vortexer Maxi Mix II	M37615	Thermo Scientific	Vortex Genie G560 /VWR		
Picofuge Galaxy Mini	C1213	VWR	—		
DNA120 Speedvac	DNA120-115	Thermo Scientific	DNA110		
Fluoroskan Ascent Microplate Fluorometer	5210470	Thermo Scientific	—		
Single Channel Pipettes (entire LTS set)	L-2, L-20, L-200, L-1000	Rainin	—		

Supplier Contact List

Table C.7 Supplier Contact List

Supplier	Web Site Address (www not required for some addresses)
Affymetrix	www.affymetrix.com
Applied Biosystems	www.appliedbiosystems.com
Bio-Rad	www.bio-rad.com
Bio-Smith	www.biosmith.com
C.B.S. Scientific	www.cbsscientific.com
Clontech	www.clontech.com
Diversified Biotech	divbio.com
E&K Scientific	www.eandkscientific.com
Eppendorf	www.eppendorf.com
ESCO	www.escoglobal.com
Fisher Scientific	www.fishersci.com
Invitrogen	www.lifetechnologies.com
Lonza	www.lonza.com
Life Technologies	www.lifetechnologies.com
New England Biolabs	www.neb.com
QIAGEN	www.qiagen.com
Rainin	www.rainin.com
TekNova	www.teknova.com
VWR	www.vwr.com

Appendix D

Running QC Gels on E-Gel

Procedure for Running First (1st PCR) and Second (HaeIII) QC Gel on E-Gel

The instructions given below are guidelines to run E-gels to check the quality of the PCR and HaeIII digest products that are generated in the OncoScan® CNV FFPE Assay. For additional information, please refer to the E-Gel® Pre-Cast Agarose Electrophoresis System.
http://tools.lifetechnologies.com/content/sfs/productnotes/F_071215_E-Gel-RD-MKT-TL-HL.pdf

Equipment, E-Gels, and Reagents Required

Table D.1 Equipment, E-Gels, and Reagents Required

Item	Supplier	Part Number
Mother E-Base™ Device	Life Technologies	EB-M03
Daughter E-Base™ Device (optional for running multiple gels simultaneously)	Life Technologies	EB-D03
E-Gel® 48 4% Agarose Gels	Life Technologies	G8008-04
TrackIt™ Cyan/Orange Loading Buffer	Life Technologies	10482-028
50 bp DNA ladder	New England Biolabs	N3236S
Nuclease-free Water	Affymetrix	71786

CAUTION: The E-Gel® contains Ethidium Bromide. Always wear gloves when handling E-Gels and dispose of the gel and gloves in a hazardous waste container.

Prepare and Run the First QC Gel (1st PCR)

CAUTION: When transferring sample volumes from one 96-well plate to another 96-well plate, make sure BOTH plates are in the correct orientation (well A1 at top left) before pipetting.

Prepare the Gel QC Reagents

1. Prepare 1:1000 dilution of TrackIt™ Cyan/Orange Loading Buffer by adding 50 µL of TrackIt Cyan/Orange Loading Buffer to 49.5 mL of Nuclease-free water.
2. Prepare a working stock of 1:10 dilution of NEB 50 bp DNA ladder as given below.

Table D.2 Preparation of 1:10 Dilution of NEB 50 bp DNA Ladder (Working Stock)

Reagent	Volume
Water	85.0 µL
TrackIt Cyan/Orange Loading Buffer (Undiluted)	5.0 µL
NEB 50 bp DNA ladder (1000 ng/ µL)	10.0 µL
Total Volume	**100.0 µL**

The final concentration of the ladder is 100 ng/µL. The volume prepared above (100 µL) is adequate for loading ~30 lanes. Store at 2-8°C and use it within one month.

3. Prepare the diluted 50 bp DNA ladder enough to load 20 µL per lane.
   - Volume per lane: Add 3 µL of diluted ladder prepared above (Step 2) to17 µL of water.
   - If less than 25 samples were run on the assay, prepare 80 µL of the diluted ladder. (The ladder will be loaded on both sides of a 48-well gel in the Marker lane.)
   - If 25 samples were run on the assay, prepare 40 µL of the diluted ladder. (It is recommended to load the diluted ladder in the Marker lane on left side of a 48-well gel only, so the Marker lane on the right side of the gel can be used to run negative control.)

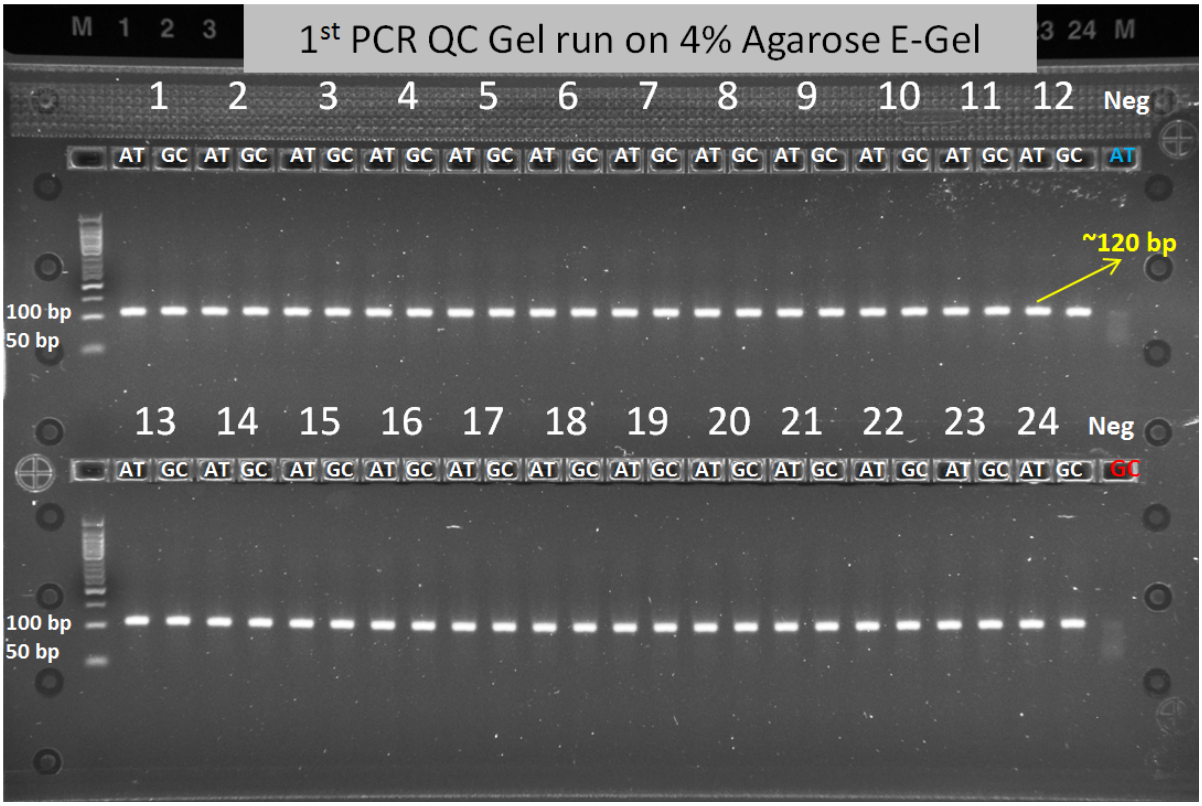
Prepare the Gel Plate

1. Label a new PCR plate *Gel1* (the 1st QC Gel Plate) and load it as follows:
   A. Add 16 µL of 1:1000 diluted TrackIt Cyan/Orange Loading Buffer in the appropriate wells
   B. Add 4 µL of 1st PCR product in the wells of the *Gel1* Plate that contains loading buffer. Pipet up and down three times to rinse the tips.
2. Seal 1st PCR Plate tightly and keep in a cold block on ice.
3. Tightly seal the first gel QC plate, vortex and spin down.

Run the E-Gel

1. Turn on the power for the E-Base (red light).
2. Push the **Power/Prg** button to make sure the program is set to EG mode (not EP).
3. Remove the comb(s) from a 48-well 4% Agarose E-Gel and wipe away any buffer that comes out of the gel or is on the surface.
4. Insert the E-Gel into the slot.
5. Load 18 µL of the 1st PCR sample onto the 48-well 4% agarose E-Gel.
6. Load 18 µL of the diluted marker prepared above (Refer to Step 3 in *Prepare the Gel QC Reagents* section) into each of the marker wells on either both sides or one side of the gel as needed.
7. Fill all empty wells with 20 µL of water.
8. Ensure program is set to EG mode and Set run time to 20 min.
9. Push the **Power/Prg** button again to start (light will change from red to green).
10. When the run time is reached, the system will automatically shut off (the dye should be near the end of the lane). The gel is now ready for imaging.
11. Examine the 1st PCR QC gel in a gel imager to ensure one single band of the PCR product at approximately 120 bp is observed.
    Figure D.1 illustrates good QC gel results.

Figure D.1 For samples in which successful amplification has occurred, one single band at approximately120 base pairs should be seen. No distinct band at approximately 120 base pairs should be visible in the Negative Control and in samples in which amplification has not occurred. The image below was taken with a 1.0 second exposure setting.



Prepare and Run the Second QC Gel (HaeIII Digest)

CAUTION: When transferring sample volumes from one 96-well plate to another 96-well plate, make sure BOTH plates are in the correct orientation (well A1 at top left) before pipetting.

Prepare the Gel QC Reagents

1. Use the 1:1000 dilution of TrackIt Cyan/Orange Loading Buffer prepared in Step 1 of *Prepare the Gel QC Reagents* on page 117.
2. Use the 1:10 dilution of NEB 50 bp DNA ladder prepared in Step 2 of *Prepare the Gel QC Reagents* on page 117.
3. Prepare the diluted 50 bp DNA ladder enough to load 20 µL per lane.
   - Volume per lane: Add 3 µL of diluted ladder prepared above (Step 2) to17 µL of water.
   - If less than 25 samples were run on the assay, prepare 80 µL of the diluted ladder. (The ladder will be loaded on both sides of a 48-well gel in the Marker lane.)
   - If 25 samples were run on the assay, prepare 40 µL of the diluted ladder. (It is recommended to load the diluted ladder in the Marker lane on left side of a 48-well gel only, so the Marker lane on the right side of the gel can be used to run negative control.)

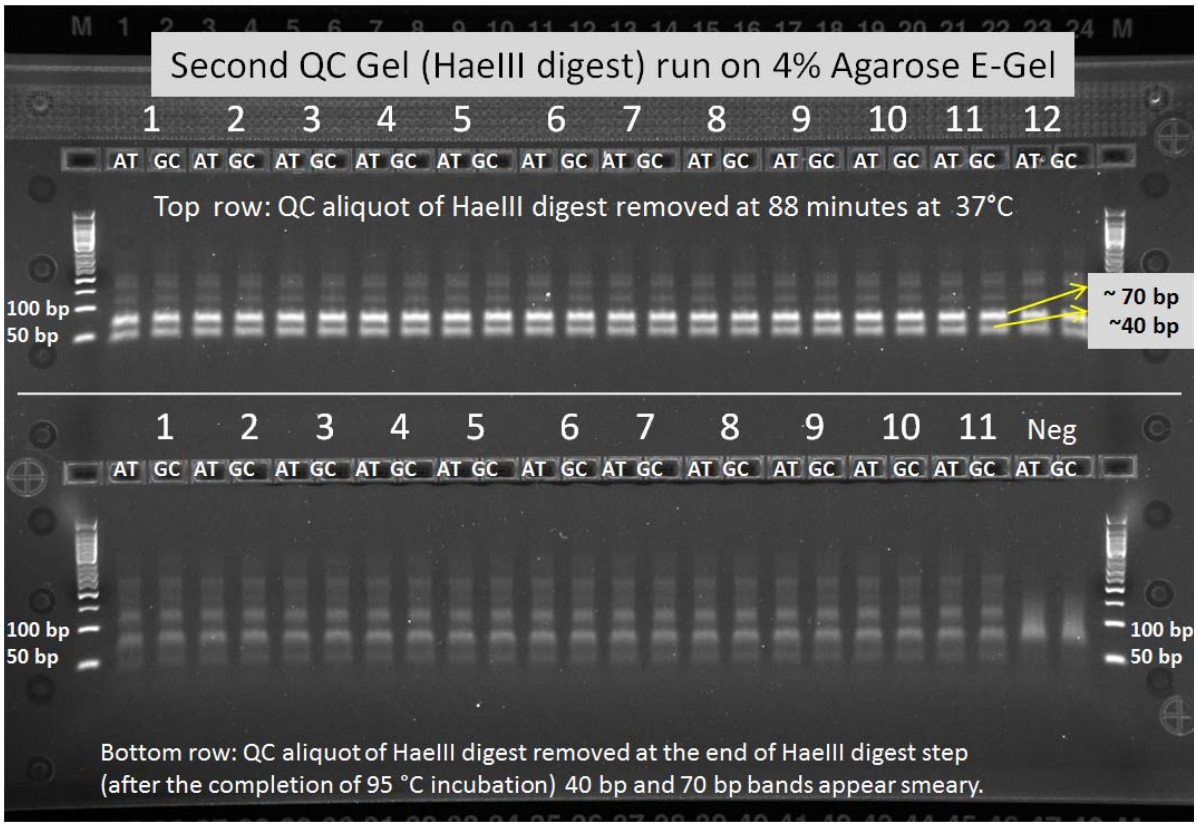
Prepare the Second QC Gel (HaeIII Digest) Plate

1. Label a new PCR plate *Gel2* (the HaeIII QC Gel Plate) and load it as follows:
2. Add 16 µL of 1:1000 diluted TrackIt Cyan/Orange Loading Buffer to the appropriate wells.
3. When the cycler reaches 88 min at 37°C, pause the cycler.
4. Remove the *HaeIII* Plate and place it on a cold block for 1 minute.
5. Ensure the *HaeIII* Plate is tightly sealed and vortex at max speed for 5 seconds. Spin down the plate at 2400 rpm for 60 seconds.
6. Ensure the plate is at the correct orientation (well A1 at top left). Remove the plate seal slowly and carefully.
7. Remove 4 µL of HaeIII digest sample and add it to the *Gel2* Plate into the appropriate wells of the *Gel1* Plate containing the loading buffer. Pipet up and down three times to rinse the tips.
8. Seal the *HaeIII* Plate and place it back on the thermal cycler. Resume the **OncoScan HaeIII** program.
9. Tightly seal the first gel QC plate, vortex and spin down.

Run the E-Gel

1. Turn on the power for the E-Base (red light).
2. Push the **Power/Prg** button to make sure the program is set to EG mode (not EP).
3. Remove the comb(s) from a 48-well 4% Agarose E-Gel and wipe away any buffer that comes out of the gel or is on the surface.
4. Insert the E-Gel into the slot.
5. Load 18 µL of the HaeIII digest sample onto the 48-well 4% Agarose E-Gel.
6. Load 18 µL of the diluted marker prepared above (Refer to Step 3 in *Prepare the Gel QC Reagents* section) into each of the marker wells on either both sides or one side of the gel as needed.
7. Fill all empty wells with 20 µL of water.
8. Ensure program is set to EG mode and Set run time to 20 min.
9. Push the **Power/Prg** button again to start (light will change from red to green).
10. When the run time is reached, the system will automatically shut off (the dye should be near the end of the lane). The gel is now ready for imaging.
11. Examine the HaeIII QC gel in a gel imager to ensure that a predominant pattern of doublet bands around **40 bp and 70 bp** are observed.
    Figure D.2 illustrates good QC gel results.

Figure D.2 The gel indicates both successful amplification during the second stage PCR reaction and HaeIII digestion. The predominant pattern should be two bands at approximately 40 and 70 bp. The image below was taken with a 1.0 second exposure setting.



Appendix E

FFPE DNA Extraction Protocol for OncoScan® CNV Assay

NOTE: Affymetrix strongly recommends using the QIAamp DNA FFPE Tissue Kit protocol for purifying DNA from FFPE Blocks that will be used in OncoScan® Assay. For improved DNA yields, we also recommend a modification to the QIAamp DNA FFPE Tissue Kit protocol. The modified procedure adds a heating step at 98°C for 15 minutes to improve the tissue digestion process to release DNA from tissue sections

Please refer to the QIAamp DNA FFPE Tissue Kit protocol for more information on the QIAGEN web page given below.

http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/Genomic-DNA/QIAamp-DNA-FFPE-Tissue-Kit#resources

Equipment and Reagents Required, but not Provided with OncoScan® CNV FFPE Assay Kit

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Equipment Required

Table E.1 Equipment Required

Equipment	Vendor	P/N
Microtome (HM340E Electronic Rotary Microtome Package, with Universal Cassette Clamp and Disposable Blade Carrier E (ThermoFisher Cat#: 905190A))	VWR International	89219-568
Vortex Genie 2 (G560)	VWR International	58815-234
Microcentrifuge, 5424 (EPPE022620401)	VWR International	80094-126
Thermomixer R (22670107) (Qty Required: 2)	VWR International	21516-166
Block for 24 x 1.5 mL Tubes for Thermomixer R (22670522) (Qty Required: 2)	VWR International	21516-176
Pipet-Lite LTS Pipette, Single Channel, 0.5-10 µL	Rainin	L-10
Pipet-Lite LTS Pipette, Single Channel, 20-200 µL	Rainin	L-200
Pipet-Lite LTS Pipette, Single Channel, 100-1000 µL	Rainin	L-1000
Pipet-Lite LTS Pipette, 12-Channel, 2-20 µL	Rainin	L12-20

Consumables Required

Table E.2 Consumables Required

Equipment	Vendor	P/N
HP-35 High-Profile blades with PTFE coating	Fischer Scientific	3153735
Eppendorf tubes, 1.5 mL microcentrifuge tubes	VWR International	022363204
Non-Stick RNase-free Microfuge Tubes (1.5 mL)	Life Technologies	AM12450
Greenpak LTS 20 µL Filter Tip, 960 Tips	Rainin	GP-L10F
Greenpak LTS 200 µL Filter Tip, 960 Tips	Rainin	GP-L200F
Greenpak LTS 1000 µL Filter Tip, 768 Tips	Rainin	GP-L200F
VWR PCR Plate, 96-Well, Flat Plate	VWR	82006-636

Reagents Required

Table E.3 Reagents Required

Equipment	Vendor	P/N
QIAamp DNA FFPE Tissue Kit ▪ QIAamp MinElute Columns ▪ Collection Tubes - 2 mL ▪ Buffer ATL ▪ Proteinase K ▪ Buffer AL ▪ Buffer AW1 ▪ Buffer AW2 ▪ Buffer ATE	QIAGEN	56404 ▪ 1020901 ▪ 1016810 ▪ 1014758 ▪ 19133 ▪ 1014604 ▪ 1014790 ▪ 1014592 ▪ 1049476
Xylene	Sigma Aldrich	534056-500ML
Ethanol (96-100%)	Sigma Aldrich	459836
RNase A	QIAGEN	1007885

Optional Prerequisite

The yield of tissue from FFPE is highly dependent on the type of tissue and method used for the initial fixation. For optimal DNA yield, samples submitted for extraction should have a tissue size of 400-700 square mm.

Preparation of Buffers

Preparing Buffer ATL

- Check whether precipitate has formed in Buffer ATL. If necessary, dissolve by heating to 70˚C with gentle agitation.

Preparing Buffer AL

- Check whether precipitate has formed in Buffer AL. If necessary, dissolve by heating to 70˚C with gentle agitation.

Preparing Buffer AW1

- Add 25 mL ethanol (96-100%) to the bottle containing 19 mL Buffer AW1 concentrate. Can be stored in room temperature up to 1 year.

Preparing Buffer AW2

- Add 30 mL ethanol (96-100%) to the bottle containing 13 mL Buffer AW2 concentrate. Can be stored in room temperature up to 1 year.

Deparaffinization

1. From an FFPE block, prepare 10 micron slices. Place 5 slices in a 1.5 mL Eppendorf Safe-Lock Tube.

   NOTE: The Eppendorf Tubes must have the centrifugation stability of up to 30,000 x g to prevent tube breakage. Please refer to the consumables list.

2. Turn on two thermal mixers. Set one to 56˚C and the other to 98˚C.
3. Add 1 mL of Xylene to the tube.
4. Vortex the tube at max speed for 10 seconds.
5. Spin down the tube at full speed (~14000 rpm) for 5 minutes.
6. Without disturbing the pellet remove the Xylene.

   NOTE: Be sure to place waste Xylene in the appropriate container.

7. Add 1 mL of Ethanol to the tube.
8. Vortex the tube at max speed for 10 seconds.
9. Spin down the tube at full speed (~14000 rpm) for 5 minutes.
10. Without disturbing the pellet remove the Ethanol by using a P1000 pipette.

    NOTE: Be sure to place waste Ethanol in the appropriate container.

11. Repeat Step 7 through Step 10 once more.
12. Spin down the tube at full speed (~14000 rpm) for 3 minutes.
13. Use a p20 or P200 pipette to completely remove any residual Ethanol without disturbing the pellet.
14. Allow any remaining Ethanol to evaporate by letting the tube air dry for 10 minutes at room temperature.

Tissue Lysis

1. Add 180 µL of ATL Buffer to the tube after ensuring the residual ethanol has completely evaporated.
2. Vortex at full speed for 10 seconds.
3. Spin down the tube briefly and then place it onto the thermal mixer that is set at 98˚C.
4. Incubate the tube for 15 minutes with a 15 second mix at 1400 rpm every 1 minute.

   NOTE: Thermomixer settings: Ensure the thermomixer is programmed to shake the samples for 15 sec after each minute at 1400 RPM.

5. After 15 minutes, stop the thermomixer program and turn off the thermomixer. Let the tube cool down for 5 minutes in the thermomixer before removing them.
   This is to ensure the vials do not pop open due to high heat and pressure.
6. Remove the tube carefully and slowly (as they may still pop open) from the thermal mixer and allow it cool at room temperature for 10 minutes.
7. Spin down the tube to remove any solution from the top of the tube.

8. Add 20 µL of Proteinase K to the tube.
9. Vortex the tube at max speed for 10 seconds, then spin down briefly.
10. Place tube on the thermal mixer that is set at 56˚C.
11. Incubate the tube for at least 3.5 hours with a 15 second mix at 1400 rpm every 1 minute.
12. After 3.5 hours, verify that all tissue has lysed.
    A. If tissue remains, incubate the samples overnight. If tissue still remains, add an additional 20 µL of Proteinase K and continue incubation for a minimum of 1 hour.
13. Spin down the tube and place them onto the thermal mixer that is set at 90˚C.
14. Incubate the tube for 1 hour with a 15 second mix at 1400 rpm every 1 minute.

NOTE: Thermomixer settings: Ensure the thermomixer is programmed to shake the samples for 15 sec after each minute at 1400 RPM.

15. After 1 hour, remove the tube from the thermal mixer and allow to cool at room temperature for 10 minutes.
16. Spin down the tube to remove any solution from the top of the tube.
17. Add 2 µL of RNase A to each tube.
18. Vortex the tube at max speed for 10 seconds, then spin down briefly. Allow to incubate for 2 minutes.

DNA Purification

1. Remove a QIAamp MinElute column from the refrigerator and allow to warm to room temperature for 15 minutes.
2. Remove the ATL tube and equilibrate at room temperature.
3. Add 200 µL of Buffer AL to the sample tube, vortex at max speed for 10 seconds, then spin down briefly.
    A. If processing multiple samples, ensure that after adding Buffer AL, Ethanol is added as quickly as possible.
    B. Precipitate may form at this step, which does not affect the DNA yield.
4. Add 200 µL of Ethanol to each tube, vortex at max speed for 10 seconds, then spin down briefly.
5. Label the QIAamp MinElute column (in a 2 mL collection tube) appropriately.
6. Carefully transfer the entire lysate to the QIAamp MinElute column (in a 2 mL collection tube) without wetting the rim, close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min.
    A. Check to see that all the lysate has moved through the column. If lysate is still in the column centrifuge again at a higher speed for 1 minute.
7. Place the column into new collection tube and discard eluate.
8. Open the column and add 500 µL of Buffer AW1.
9. Load the column onto the centrifuge and spin at 8000 rpm (6000g) for 1 minute.
    A. Check to see that the entire Buffer AW1 has moved through the column. If Buffer AW1 is still in the column centrifuge again at a higher speed for 1 minute.
10. Place the column into new collection tube and discard eluate.
11. Open the column, and add 500 µL of Buffer AW2.
12. Load the column onto the centrifuge and spin at 8000 rpm (6000g) for 1 minute.
    A. Check to see that the entire Buffer AW2 has moved through the column. If Buffer AW2 is still in the column centrifuge again at a higher speed for 1 minute.
13. Place the column into new collection tube and discard eluate.
14. Load the column onto the centrifuge and spin at 14000 rpm (20000g) for 3 minutes to dry the membrane completely.

DNA Elution

1. Label a clean Nuclease-free 1.5 mL tube for DNA elution.
2. Place the column into the labeled 1.5 mL tube prepared for elution.
3. Add 50 µL of Buffer ATE to the center of the column membrane.
4. Close the lid and incubate the column at room temperature for 5 minutes.
5. Load columns onto the centrifuge and spin at 14000 rpm (20000g) for 1 minute to elute the DNA.
   A. Note: Caps for 1.5 mL tube will not be able to close at this step due to the columns. When loading onto the centrifuge rotate caps to the right to keep them from breaking during the spin.
   B. When eluting multiple samples, alternate positions in centrifuge to make room for caps.

Quantitation of Eluted FFPE DNA

1. Perform either of the two Affymetrix recommended and tested dsDNA quantitation protocols that are included in the user guide, to measure the concentration of the eluted FFPE DNA. Affymetrix strongly recommends using these protocols that have been tested at Affymetrix using the following kits for DNA quantitation.
   - Quant-iT™ PicoGreen® dsDNA Assay Kit (Catalog Number: P7589, LifeTechnologies ™)
   - Qubit® dsDNA HS Assay Kit (Catalog Number: Q32851, LifeTechnologies™)
2. Record the results and the volume information in a spreadsheet.

Appendix F

PicoGreen® dsDNA Quantification Protocol for OncoScan® CNV Samples

Materials Required but not Provided with OncoScan® CNV FFPE Assay Kit

Quant-iT™ PicoGreen® dsDNA Assay Kit: Invitrogen (Life Technologies) P/N P7589

- Equivalent Catalog Number: P/N P11496 (dye product size: 10 x 100 µL)
- Quant-iT™ PicoGreen dsDNA Reagent (200× concentrate, frozen at +4°C in dark): In order to avoid repeated freeze thawing of the reagent, divide the 1 mL stock solution into 50~100 µL aliquots and use within one year, or until expiration date. Avoid more than 3 freeze-thaw cycles on any one aliquot vial.
- Lambda DNA Standard Stock (100 ng/µL aqueous at +4°C in screw-cap tube)

Other User Supplied Reagent

- 1X TE pH 7.5~8.0 (for blank samples and as diluent for DNA samples): Affymetrix P/N 75893 or equivalent

Consumables

- P-20 & P-200 pipette tips
- 96-well microtiter plates
- 384-well dark plate for fluorescence reading: E&K Scientific, P/N EK-30076
- Screw-cap Eppendorf tubes
- 8 or 12-length strip tubes
- 15 mL conical tubes
- Plate centrifuge

Equipment

- P-20 & P-200 single and multiple-channel pipettes
- Vortexer
- Bench-top mini-centrifuge
- Fluorescence Plate Reader

Genomic DNA Samples

Purified gDNA Samples should be kept refrigerated for no more than 2 weeks, or frozen at –20°C or lower for long-term storage. If frozen, thaw samples at room temperature for 20-30 minutes; spin down condensate; vortex 3 seconds; re-spin; vortex an additional 3 seconds; re-spin (it is essential that the DNA samples are fully resuspended and homogenous. Ensure any DNA that might have dried on the side of the wells have been collected and completely resuspended.)

Procedure

NOTE: Follow manufacturer's instructions on how to perform the Quant-iT™ PicoGreen® dsDNA Assay and how to operate the Fluorescence Plate Reader with appropriate software for data collection and analysis. It is recommended to follow the specific instructions provided below for accurate and reliable quantification of FFPE-derived DNA samples.

Prepare Lambda DNA Standards

Use the table below to make stock solutions of the lambda DNA standards in screw-cap tubes. Store in no more than 200 µL aliquots at +4°C as working stock for use within a week, or at –20°C for longer-term. Use 1X TE as diluent. The optimal range of standards may need to be modified for different fluorescence reading systems.

Table F.1 Picogreen Standards: from Lambda DNA (100 ng/µL in kit)

Standard	Final Conc.	µL Diluent	DNA Used	µL DNA
A	2.0 ng/µL	980	Lambda	20
B	1.5 ng/µL	50	Std A	150
C	1.0 ng/µL	100	Std A	100
D	800 pg/µL	120	Std A	80
E	600 pg/µL	140	Std A	60
F	400 pg/µL	160	Std A	40
G	200 pg/µL	180	Std A	20
H	100 pg/µL	190	Std A	10

Prepare Sample Dilutions

Dilute high concentration DNA samples with 1x TE such that the final concentration is within the dynamic range of the PicoGreen dsDNA Assay. It is usually acceptable to have diluted DNA concentrations in the range of 0.3 ~2.0 ng/µL. However, the actual dynamic range can vary significantly depending on the fluorescence reading system used. It is recommended for each customer to carefully characterize the appropriate limits of PicoGreen quantification for their specific system.

NOTE: For FFPE-derived DNA samples, it is usually sufficient to start with a 1:50 dilution, or try multiple dilution levels for each sample. Prepare TWO independent replicate dilution series of each sample. If a sample is tested at multiple dilution levels, only measured concentrations within the dynamic range determined above for your instrument will be accepted.

Mix sample dilutions thoroughly by vortexing for 5 seconds followed by spinning down briefly in a centrifuge.

Prepare 1:200 Dilution of PicoGreen® Stock in 1X TE

Thaw PicoGreen 200× stock in the dark at room temperature, vortex & spin down.

Determine the total amount of diluted PicoGreen reagent needed.

Mix diluted PicoGreen working solution in Eppendorf or 15 mL conical tube by vortexing. Store the diluted PicoGreen working solution in dark at room temperature until needed.

Perform the PicoGreen® dsDNA Quantification Assay

Mix each diluted DNA sample with PicoGreen working solution in a 384-well dark plate suitable for fluorescence reading. Vortex the sealed plate at max speed (2400-3000 rpm) for 5-8 seconds, mixing for 1 second at each corner and then the center. and spin at 2000 rpm for 30 seconds. Incubate the plate in dark for 5 minutes before reading fluorescence. Be sure to include standards and blanks on the same plate. Verify the excitation and emission filters of correct wavelengths are selected.

Use appropriate software associated with the fluorescence plate reader for data collection and analysis.

! **IMPORTANT: When generating the standard curve, it is recommended to use direct linear fit without logarithmic transformation. If an option is available in the software to specify the intercept of the linear fit, it is usually beneficial to force the intercept to be zero. Enforce the quality of the fitted curve by only accepting standard curves with R^2 >0.98.**

Appendix G

Qubit® dsDNA Quantification Protocol for OncoScan® CNV Samples

Materials Required but not Provided with OncoScan® CNV FFPE Assay Kit

Qubit® dsDNA HS Reagent Kit: Invitrogen (Life Technologies) P/N Q32851 or P/N Q32854

- Qubit® dsDNA HS Reagent (Component A): 250 µL or 1.25 mL 200x concentrate in DMSO (stored at room temperature in dark)
- Qubit® dsDNA HS Buffer (Component B): 50 mL or 200 mL (stored at room temperature)
- Qubit® dsDNA HS Standard #1 (Component C): 1 mL or 5 mL, 0 ng/µL in TE (stored at +4˚C)
- Qubit® dsDNA HS Standard #2 (Component D): 1 mL or 5 mL, 10 ng/µL in TE (stored at +4˚C)

Other User-Supplied Reagent

- 1X TE pH 7.5~8.0 (as diluent for DNA samples): Affymetrix P/N 75893 or equivalent

Consumables

- P-20 & P-200 pipette tips
- 15 mL or 50 mL falcon tube (disposable) for mixing the Qubit® working solution
- 0.5 mL Qubit® assay tubes: Invitrogen P/N Q32856 (500 tubes) or Axygen PCR-05-C tubes (VWR, P/N 10011-830)

Equipment

- Vortexer
- Bench-top mini-centrifuge
- P-20 & P-200 pipettes
- Qubit® 2.0 Fluorometer: Invitrogen (Life Technologies) P/N Q32866

Genomic DNA Samples

Purified gDNA Samples should be kept refrigerated for no more than 2 weeks, or frozen at –20˚C for long-term storage. If frozen, thaw samples at room temperature for 20-30 minutes; spin down condensate; vortex 3 seconds; re-spin; vortex an additional 3 seconds; re-spin (It is essential that the DNA samples are fully resuspended and homogeneous. Ensure any DNA that might have dried on the side of the wells have been collected and completely resuspended.).

Procedure

NOTE: Follow manufacturer's instructions on how to use the Qubit® dsDNA HS Assay Kit and the Qubit® 2.0 Fluorometer. It is recommended to follow the specific instructions provided below for accurate and reliable quantification of FFPE-derived DNA samples.

Sample Pre-dilution

Dilute high concentration DNA samples with 1x TE such that the final concentration is estimated to be within the range of 0.3 ng/µL ~2.0 ng/µL (equivalent to Qubit measured concentration, or QF value, between 15 ng/mL and 100 ng/mL).

NOTE: For FFPE-derived DNA samples, it is usually sufficient to start with a 1:50 dilution, or try multiple dilution levels for each sample to insure that the DNA being measured is within the 0.3 ng/µL ~2.0 ng/µL range.

Mix sample dilutions thoroughly by vortexing for at least 3 seconds followed by spinning down in a microcentrifuge.

Use pre-diluted DNA in the Qubit dsDNA HS Assay within one hour at room temperature.

Qubit® dsDNA HS Assay

Follow manufacturer's instructions on how to perform the Qubit® dsDNA HS Assay Kit, with following notes:

- Although the kit allows a variable sample input volume from 2 to 20 µL, it is recommended to always use a constant sample volume of 10 µL.
- After DNA has been incubated with the Qubit® working solution for a minimum of 2 minutes, read fluorescence signal in the Qubit® 2.0 Fluorometer within 30 minutes, or store the assay tube protected from light for up to 2 hours at room temperature before reading. Do not refrigerate.
- Always generate a new calibration curve using standards processed in parallel with test samples and with fresh prepared Qubit working solution.
- For accurate quantification, the Qubit measured concentration (QF value) should be between 15 ng/mL and 100 ng/mL (equivalent to sample pre-dilution to 0.3 ng/µL ~2.0 ng/µL, when 10 µL is used in the assay). If the Qubit measured concentration falls out of the range, adjust the dilution factor and measure again.

Normalization of Qubit®-determined FFPE DNA Concentration (Optional)

This additional step is applicable to FFPE-derived DNA samples when it is desired to convert the Qubit-determined dsDNA concentration to the equivalent concentration determined by the PicoGreen® dsDNA quantification assay (Invitrogen P/N P11496). Although the two DNA quantification platforms work by the same mechanism, minor discrepancies may exist in the reported concentration for FFPE-derived DNA samples. The PicoGreen® dsDNA quantification assay has been used primarily to determine the amount of assay input DNA during the development of OncoScan product. Therefore for best consistency, a normalization factor is recommended to convert the Qubit-determined DNA concentration to PicoGreen equivalent value.

For Qubit measurements within the recommended dynamic range (QF value between 15 ng/mL and 100 ng/mL; see Sample Pre-dilution above), the following equation can be used:

PicoGreen Equivalent Concentration = Qubit-determined Concentration * 0.8

Example

An FFPE DNA sample is first diluted 50 times with 1X TE. 10 µL of the diluted sample is tested in the Qubit dsDNA HS assay with 190 µL Qubit working solution (total assay volume 200 µL). The QF value is 32 ng/mL, *without using the "Dilution Calculator" feature of the instrument*.

Then -

(Qubit) Concentration of the diluted sample = 32 * (200/10) = 640 ng/mL

NOTE: This value can also be obtained directly using the "Dilution Calculator" feature.

To convert this value into PicoGreen Equivalent, a normalization factor of **0.8** if applied:

Corresponding **PicoGreen Equivalent Concentration** = 640 * 0.8 = 512 ng/mL (0.512 ng/µL)

For testing in the OncoScan Assay, the original sample concentration will be stated as:

0.512 * 50 = **25.6 ng/µL**

Appendix H

Troubleshooting

General Assay Performance Recommendations

As with any assay requiring PCR, the OncoScan® CNV FFPE Assay Kit protocol has an inherent risk of contamination from PCR product from the previous reactions. In Chapter 2, *Laboratory Setup and Recommendations* (page 10), two separate work areas are strongly recommended to minimize the risk of cross contamination during the assay procedure. 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 OncoScan® CNV FFPE Assay Kit protocol has been validated using the reagents and suppliers listed. Substitution of reagents and/or taking protocol shortcuts could result in sub-optimal results.

Success of the OncoScan CNV assay depends on the following critical steps recommended in the user guide:

- Accurate sample quantitation by PicoGreen® method
- Proper storage and use of the reagents
- Proper use of the workflow
- Proper use and maintenance of the equipment including
  - Calibrated thermal cyclers
  - Calibrated Pipettes
  - Using filter pipette tips and recommended plate seals
  - Maintenance of Fluidics stations – Bleach protocol performed every week and tubing changed every 5-6 weeks

Additional recommendations are as follows:

- Plan ahead to ensure that the reagents and equipment you require, including pipettes, are in the correct work area. Ensuring the proper equipment is available in the proper laboratory areas will make the workflow easier, and will help reduce the risk of sample contamination.
- If Veriti Thermal Cyclers are used to run the OncoScan CNV FFPE Assay, pay particular attention to the details in Appendix B such as converting the programs to 9700-max mode, caution on the touch screen use while pausing and resuming the program, setting the default Pause time, etc...
- Pay particular attention to the storage and handling of reagents. Proper storage and handling is particularly important for enzymes such as Gap-fill, Exo Mix and the Taq Polymerase enzyme.

  To prevent loss of enzyme activity:
  - Store the enzymes in a cooler placed in a –20˚C freezer to preserve activity. When taking out enzymes for reaction setup, always use a cooler chilled to –20˚C.
  - Take care when pipetting enzymes stored in glycerol, which is viscous. Do not store enzymes at –80˚C.
- Preparing the Master Mixes with 20% to 25% overages as provided in this user guide and the Quick Reference Card ensures consistency in reagent preparation by minimizing pipetting errors and reducing handling time of temperature sensitive reagents. The success of this assay depends on the accurate pipetting and subsequent thorough mixing of small volumes of reagents.
- The PCR reaction for this assay has been validated using the specified thermal cyclers. We highly recommend that your PCR thermal cyclers be calibrated regularly. Take care when programming your thermal cycler and ensure all programs in this assay runs at “Max mode” (9700) setting. Use the recommended 96-well plate and the plate seal. Substitution of thermal cyclers, plates or plate seals are not recommended as the results could be suboptimal.

- It is essential to run gels to monitor both the First PCR and HaeIII reactions.

  For the first PCR reaction, individual PCR products from both AT and GC channels are run on a gel in adjacent wells. A single band should be visible around 120 bp. See *Prepare and Run the First QC Gel* on page 59 for more information.

  Following HaeIII digestion, remove the volume required for the gel from each well BEFORE the cycler reaches 95°C and run the samples on a gel. Successful digestion is confirmed by the presence of a doublet band at 40 bp and 70 bp. See *Prepare and Run the HaeIII Gel (Second QC Gel)* on page 68 for more information.
- Always run positive and negative controls in parallel with each group of samples.

  The absence of a 120 bp band on the First PCR gel for the negative control confirms no previously amplified PCR product has contaminated the samples or reagents. Use Genomic DNA from the OncoScan® CNV Reagent Kit as a positive control in the assay. These controls are effective troubleshooting tools that will help you confirm the successful completion of each stage of the assay.
- Regularly calibrate all single channel and multi-channel pipettes and always use filter pipette tips.
- Hybridization oven temperature and the RPM 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.

OncoScan® CNV FFPE Assay Kit Protocol Troubleshooting

Table H.1 OncoScan® CNV FFPE Assay Kit Protocol Troubleshooting

Problem	Potential Cause	Solution
PCR Gel QC Step		
Faint or no PCR product visible on the 1st PCR QC gel image in both AT and GC channel for a given sample.	Low input starting material.	Recheck the quantification for the sample by using the recommended PicoGreen protocol to make sure you have 80 ng of starting material.
	Failed anneal reaction.	Mis-pipetting in the Anneal step by not adding the probe mix or the input DNA can result in no PCR bands. Repeat Assay from the beginning.
	Sample type.	Check to see if this sample might contain chemical or enzymatic inhibitors. If so, try cleaning sample over a column and starting assay from the beginning.
Faint or no PCR product visible on the 1st PCR QC gel image in AT or GC channel for a given sample.	Pipetting error during Gel QC.	Run the first PCR from both channels again to ensure there was not a pipetting error in loading the gel.
	Pipetting error in Pre-PCR step.	If the repeat gel shows the same faint/no PCR band in a given channel, there was likely a mis-pipetting step after the Channel Split in the Pre-PCR stage. Repeat this sample from the beginning of the assay.
Smeared or multiple bands in PCR Gel	Low input starting material.	Recheck the quantification for the sample by using the recommended PicoGreen protocol to make sure you have 80 ng of starting material.
PCR product in the negative control	Reagents or equipment contaminated with amplified product.	Always use filter pipette tips. Clean the Pre-PCR Lab 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. DO NOT OPEN the seal of the amplified 1st PCR Plate in the Pre-PCR Lab. Do not store the 1st PCR Plate in the Pre-PCR Lab.
Hae III gel smeared, no distinct double bands	Forgot to pause the thermal cycler at 88 min and pull an aliquot for Gel QC.	Check your gel against the example gel in which the aliquot was taken after the 95°C incubation.

Table H.1 OncoScan® CNV FFPE Assay Kit Protocol Troubleshooting (Continued)

Problem	Potential Cause	Solution
Dim array	Fluidics Stations needs bleach protocol run.	Dim arrays (low signal on the array) might indicate that the fluidics stations need the bleach maintenance performed. We recommend bleaching the fluidics station once a week. The peristaltic tubing needs to be changed every 5-6 weeks.
The .cel file is not generated	Signal from corner checkerboards is dim.	Ensure that GeneChip® Hybridization Oven 645 is calibrated and set to the correct temperature. Confirm that Stain 1 and Stain 2 are placed in the correct order on the fluidics station. Ensure that Stain Buffer 1 is stored in the dark when not in use. Use only those reagents provided by Affymetrix.
	One or more of the sub-grids in the .dat file image were not gridded by the software.	Follow the AGCC manual griding procedure to fix the grids and re-generate the CEL file.