

Copy Number Analysis Using Biomark HD with the 96.96 IFC and TaqMan CNV Assays

Introduction

Copy number variations (CNVs) are inherited or acquired (*de novo*) structural changes in the natural complement of DNA segments within the genome, resulting in a loss (deletion) or gain (duplication) of nucleotides. CNVs can vary in size from a few bases to a complete gene or chromosome, and they represent an important genetic variation component that can have an advantageous, deleterious, or neutral phenotypic outcome. Research applications that analyze CNV biomarkers include disease association studies (for example autoimmunity), pharmacogenomics, and cancer research. Additionally, CNV analysis is routinely employed within the crop and animal sciences to guide the selection of traits that affect nutritional quality or yield, promote disease resistance, and enable adaptation to environmental conditions.

In this technical note, we describe an analysis method that may be used in a copy number workflow. This method includes the 96.96 Dynamic Array™ IFC for Gene Expression and Applied Biosystems® TaqMan® assays on Biomark™ HD. CopyCount-CNV™ software may be used for subsequent analysis of the data generated, as shown through the study conducted to demonstrate proof of principle. Although the 96.96 Dynamic Array integrated fluidic circuit (IFC) enables analysis of up to 96 individual research samples, it is advisable to run samples in replicate when performing CNV analysis to achieve the best results. The total number of samples analyzed with the 96.96 Dynamic Array IFC depends on the number of replicates. For example, 24 samples can be analyzed when running replicates of 4, and 32 samples can be analyzed with replicates of 3.

The workflow for CNV analysis on Biomark HD described here is a guideline. Optimization in your laboratory is recommended.

Study Design

To assess workflow and performance of the protocol, 48 samples that include one duplicate sample and one no template control (NTC) were run with two TaqMan assays and 8 replicates per assay. Samples were sourced from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research (see [Table 1](#) for repository ID numbers) and an external laboratory, which extracted and processed the samples in accordance with the protocol described in this technical note. The assays were sourced from Thermo Fisher Scientific (see [Table 2](#)).

Table 1. Study samples obtained from Coriell Institute

Repository ID	
NA17104	NA17128
NA17107	NA17194
NA17109	NA17227
NA17117	

Table 2. Assays used to conduct the copy number analysis study

Assay ID	Gene Target	Assay Type
Hs00010001_cn	CYP2D6, exon 9	TaqMan Copy Number
Hs03297761_s1	RPPH1	TaqMan Gene Expression

Raw fluorescence data collected from Biomark HD was processed by Fluidigm with an alternative commercially available software package to establish a reference copy number.

The same data was subsequently blinded, parsed into separate files (.csv) by assay, and provided to DNA Software along with the following information to facilitate configuration of the CopyCount-CNV

software: PCR volume, amplicon length, primer and probe concentrations, dye type, and chemistry format.

Analysis was conducted by DNA Software using CopyCount-CNV in uncalibrated mode. The software generated a copy number result for each sample-assay combination, as well as metrics for the CV of ratio, Z-score, and confidence value. Results were subsequently unblinded by Fluidigm to compare the observed results with the predicted copy number.

Results and Discussion

CopyCount-CNV software generated copy number results that were 100% concordant with the results of the alternate software product for this dataset of 47 sample-assay pairs (see Figure 1). The one sample represented twice in the sample set produced the same copy number result using either software package. While the results concordance was high, the CopyCount-CNV data had a smaller standard deviation and greater separation between bins used to define copy number (see Figure 2). This indicates results generated with CopyCount-CNV have greater precision.

Conclusions

Both software packages generated the same copy number call for 100% of the samples as part of a blind test. As a result of the tighter standard deviation, CopyCount-CNV had more separation between bins than the alternate software package used for comparison. For high-throughput applications, it is expected that CopyCount-CNV would make fewer false copy number determinations relative to the comparative software used to conduct this study.

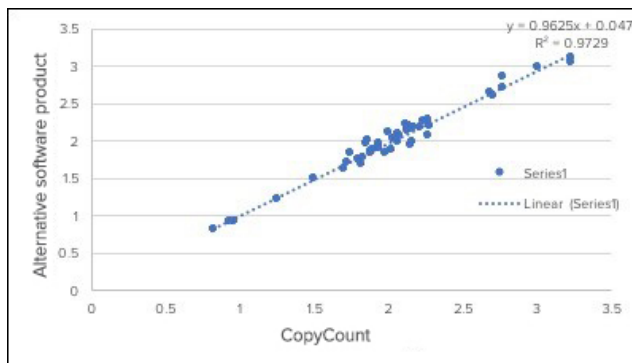
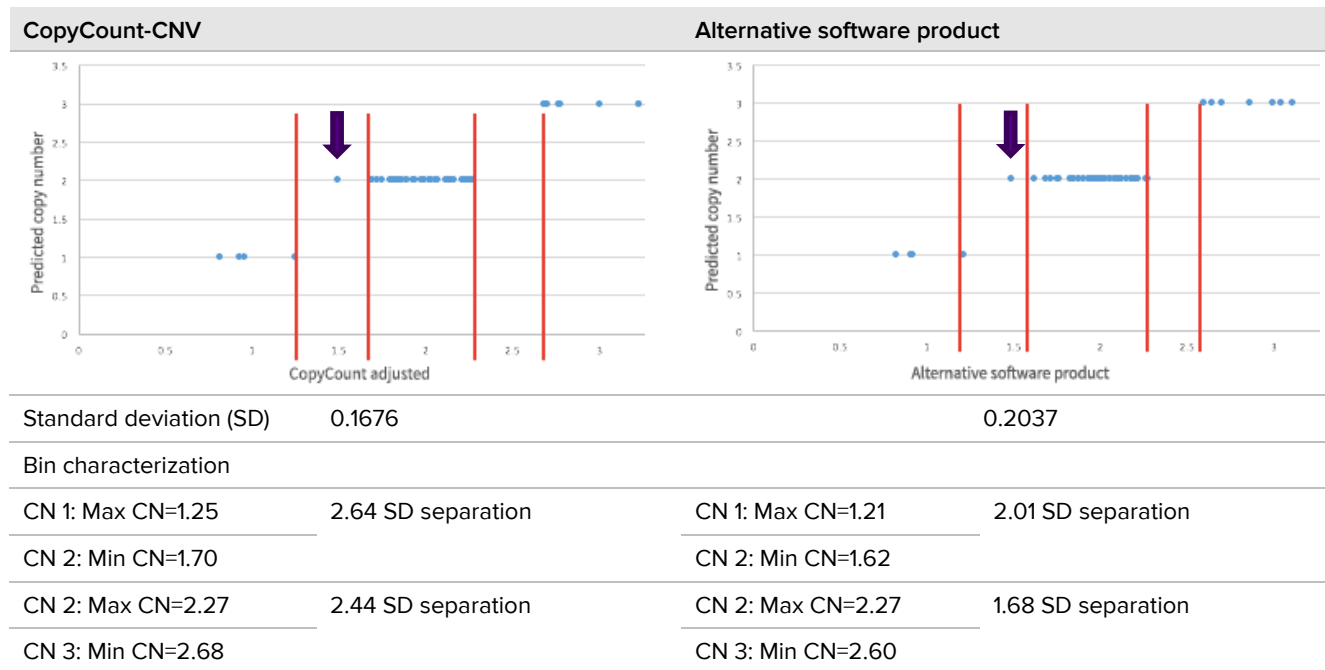


Figure 1. Copy number results for CYP2D6 for the 47 study samples, as determined by CopyCount-CNV and a commercially available alternative software package



Arrows indicate the same sample generated an ambiguous result for both software packages.

Figure 2. Comparison of the standard deviation and bin characterization parameters for the two software packages

Materials

Required Reagents

- 2X Assay Loading Reagent (Fluidigm PN 85000736, part of kit PN 100-7611)
- 20X Fast GT Sample Loading Reagent (Fluidigm 100-3065, part of kit PN 100-7606)
- TaqMan GTXpress™ Master Mix (2X) (Thermo Fisher Scientific PN 4403311)
- TaqMan PreAmp Master Mix (2X) (Thermo Fisher Scientific PN 4391128)
- TaqMan CNV Target Assays with FAM™ MGB probe (Thermo Fisher Scientific)
- TaqMan CNV Reference Assay with FAM MGB probe (Thermo Fisher Scientific)

Do not use a reference assay labeled with VIC® dye.

- DNA Suspension Buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) (Teknova PN T0221, 1 mL)
- PCR-certified water
- Human gDNA samples

Required Consumables

- 96.96 Dynamic Array IFC for Gene Expression (Fluidigm PN BMK-M-96.96)
- Control Line Fluid for 96.96 IFCs (Fluidigm PN 89000021)
- 1.5 mL Eppendorf® microtubes
- 8-well PCR tube strips
- 96-well PCR plates and sealing films

Required Equipment

- Biomark HD system
NOTE The IFC run requires a customized thermal protocol. See [Appendix A](#) for custom protocol parameters.
- Juno™ system or IFC Controller HX
- HX Interface Plate (Fluidigm PN 101-6116), if using Juno
- Pipettes (single- and multichannel) and appropriate low-retention filter tips
- Vortexer

- Two centrifuges: one for microtubes, one for 96-well PCR plates
- Standard thermal cycler [if using specific target amplification (STA)]

Best Practices

For the overall success of the protocol, we recommend the following best practices.

- Avoid creating bubbles while vortexing reagents and when transferring reagents to the IFC.
 - Vortex gently (low speed) but thoroughly (at least 5 sec) to ensure all reagents and reagent mixes are homogeneous.
 - After vortexing, centrifuge to collect all mixes at the bottoms of the wells before pipetting into the IFC inlets. Failure to do so may result in a decrease in data quality.
 - Check the source plate or tube for bubbles before pipetting.
 - Check pipette tips for air gaps while pipetting.
 - Pipet reagents slowly and carefully to transfer entire volumes and to minimize bubbles.
 - To avoid creating bubbles in the IFC inlets, pipet into the inlets at an angle and do not go past the first stop on the pipette. If a bubble is introduced, ensure that it floats to the top of the inlet.
 - If necessary, remove bubbles from an IFC inlet by removing the contents of the inlet by pipette and then carefully re-pipetting the contents into the inlet.
- Unless otherwise specified, thaw reagents at room temperature (15–30 °C), and then use them at room temperature.
- Mix and centrifuge reagents as directed.
- For better traceability, assemble DNA samples in a 96-well PCR plate and record in a sample map.
- Place the sample mixes on ice when not in use.
- To reduce the number of pipetting steps, we recommend first aliquoting reagents into an 8-well PCR tube strip to enable transfer into a 96-well plate using an 8-channel pipette.

- Inspect the IFC for any signs of visible damage before use. Ensure that the barcode label is intact and the IFC surfaces are clear of particulates.
 - During use, take care to avoid the introduction of particulates, reagents, and fluids to the surface of the IFC.
 - Use clear tape to remove any particulates from the IFC surface.

Protocol

For detailed instructions on instrument and software operation, see the appropriate instrument user guide.

IMPORTANT

- Before using reagents, read and understand the detailed instructions and safety guidelines in this document. For complete safety information, see [Appendix C](#).
- Ensure that all equipment is turned on and operational at least 30 min before use.
- Generate sample mix and assay mix plate maps in advance to facilitate reagent deliveries and maintain traceability throughout the protocol.

Preamplify the Samples Using STA

Specific target amplification (STA) is recommended to ensure abundance of each CNV target or reference prior to distribution of sample within the IFC.

IMPORTANT Use only Applied Biosystems TaqMan Copy Number assays at 20X concentration.

- Vortex and centrifuge all reagents.
- Denature all genomic DNA samples at 98 °C for 5 min followed by snap cooling on ice for 2 min.
- Label a 1.5 mL microcentrifuge tube 0.2X Pooled Assays and combine the 20X TaqMan assays and DNA Suspension Buffer to prepare a primer pool mix, where each primer is at a final concentration of 0.2X. See the example in [Table 3](#).
- Add DNA Suspension Buffer to bring final volume to 400 µL. This will be enough for STA of at least 192 samples.

Table 3. Example primer pool mix. This example shows the volumes needed for four assays.

Component	Vol. per Assay (µL)*	Vol. in 0.2X Primer Pool (µL)*
Four 20X TaqMan assays	4	16
DNA Suspension Buffer (Teknova PN T0221)	—	384
Total	—	400

* Includes overage

- Prepare the preamplification mix in a 1.5 mL microcentrifuge tube by combining the components in [Table 4](#).

Table 4. Preamplification mix

Component	Vol. per Reaction (µL)*	Vol. for 96 Samples (µL)* n=120 reactions
TaqMan PreAmp Master Mix (2X)	2.5	300
Pooled assay mix (0.2X)	1.25	150
Total	3.75	450

* Includes overage

- Transfer 54 µL of the preamplification mix into each well of the strip.
- Using an 8-channel pipette, transfer 3.75 µL of preamplification pre-mix to each well of a 96-well plate. For 48 samples, fill only 48 wells. For 96 samples, fill the entire plate.
- Using an 8-channel pipette, transfer 1.25 µL of genomic DNA to each well, to bring the total volume to 5 µL.
- Seal the plate, vortex briefly, and then centrifuge at 2,000 x g for 1 min.

- 10 Place the plate in a standard thermal cycler and cycle using the following thermal protocol:

Temperature	Time	Cycle	
95 °C	10 min	Hold	
95 °C	15 sec	Denaturation	15 cycles*
60 °C	4 min	Annealing/ extension	
4 °C	∞	Hold	

* Recommended starting point (within a range of 10–20 cycles). The appropriate number of cycles may need to be determined empirically because sample sets may vary with respect to sample amount and quality.

- 11 After cycling, dilute the reaction 1:5 by adding 20 µL of DNA Suspension Buffer to the preamplification product.

Prime the IFC

For detailed instructions about injecting Control Line Fluid, see the Control Line Fluid Loading Procedure (PN 68000132).

IMPORTANT

- Use the GE 96.96 IFC within 24 hr of opening the package.
- Use only 96.96 syringes with 150 µL of Control Line Fluid.
- Do not evacuate air from syringes prior to injecting Control Line Fluid.
- Avoid bending the syringe tip. Be careful when removing the syringe cap to prevent drips.
- Avoid getting Control Line Fluid on the exterior of the IFC or in the inlets because this makes the IFC unusable. If this occurs, use a new IFC.

- 1 Turn on the Juno instrument or IFC Controller HX, if necessary. Initialization takes approximately 5 min.
- 2 If using Juno, ensure that the HX Interface Plate is installed in the instrument.

- 3 Inject Control Line Fluid into each accumulator on the IFC (see Figure 3). Use the entire contents of one syringe in each accumulator.

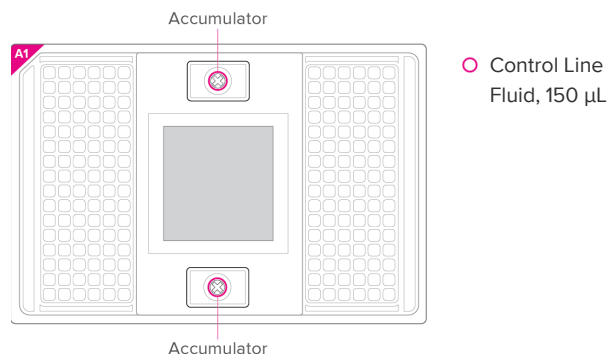


Figure 3. Accumulators on the GE 96.96 IFC

- 4 Place the IFC into the controller:
 - Juno: Tap **OPEN** to open the instrument tray and align the notched corner of the IFC to the white notch on the tray. Tap **LOAD**.
 - IFC Controller HX: Press **EJECT** to open the instrument tray and align the notched corner of the IFC to the A1 mark. Press **Load Chip**.
- 5 Run the prime script:
 - Juno: Tap **Prime 96.96 GE** and tap **Run**.
 - IFC Controller HX: Select **Prime (136x)** and press **Run Script**.

Priming the IFC takes approximately 20 min. During that time, prepare the assays and samples.


IMPORTANT Load the IFC within 60 min of completing the Prime script.

Prepare 10X TaqMan Assays

IMPORTANT Use only Applied Biosystems TaqMan Copy Number assays at 20X concentration.

- 1 Allow the 2X Assay Loading Reagent to thaw at room temperature before use.
- 2 Briefly vortex and centrifuge reagents before use.
- 3 Label a new 96 well plate 10X Assays.
- 4 In each well, prepare the assay mixes using volumes shown in Table 5 (sufficient volume to include four replicates per assay). Volumes can be scaled to include more replicates as necessary.

Table 5. Assay mix for each 20X CNV assay. Each 20X CNV assay will be replicated four times in the IFC.

Component	Vol. per Inlet (μL)*	Vol. for 4 Replicates (μL) n=5 for coverage
20X TaqMan CNV assay	3	15
2X Assay Loading Reagent 	3	15
Total	6	30


* Includes coverage

- Seal the 10X Assay plate well using an adhesive film, and then vortex to mix and centrifuge.

Prepare Sample Mixes

- Thaw all reagents to room temperature. Ensure that the 20X GT Fast Sample Loading Reagent is at room temperature before use.
- Briefly vortex and centrifuge reagents before use.
- In a DNA-free hood, prepare the sample pre-mix in a 1.5 mL sterile tube using volumes shown in Table 6 (sufficient volume to process 96 individual samples).

Table 6. Sample pre-mix

Component	Vol. per Inlet (μL)*	Sample Pre-Mix for One 96.96 IFC (μL)*
PCR-certified water	0.27	32.4
TaqMan GTXpress Master Mix	4	480
20X GE Sample Loading Reagent 	0.4	48
Total	4.67	560.4

* Includes coverage

- Transfer 68 μL of sample pre-mix into each well of 8-well PCR tube strip.
- Dispense the sample pre-mix and add the DNA sample as shown in Table 7. Use an 8-channel pipette to transfer sample pre-mix from the tube strip into each well of a 96-well PCR plate.

Avoid creating bubbles. Remove the sample pre-mix plate from the DNA-free hood, and then add diluted, preamplified genomic DNA to each aliquot of sample pre-mix.

Assign at least 1 well as NTC (no template control). Do not add genomic DNA to this well. Instead, add 3.33 μL of PCR-certified water.

Table 7. Sample mix

Component	Vol. per Well (μL)*
Sample pre-mix (see Table 6)	4.67
Diluted, preamplified genomic DNA	3.33
Total	8.0

* Includes coverage

- Seal the plate well using an adhesive film.
- Mix the reactions by briefly vortexing, and then centrifuge.

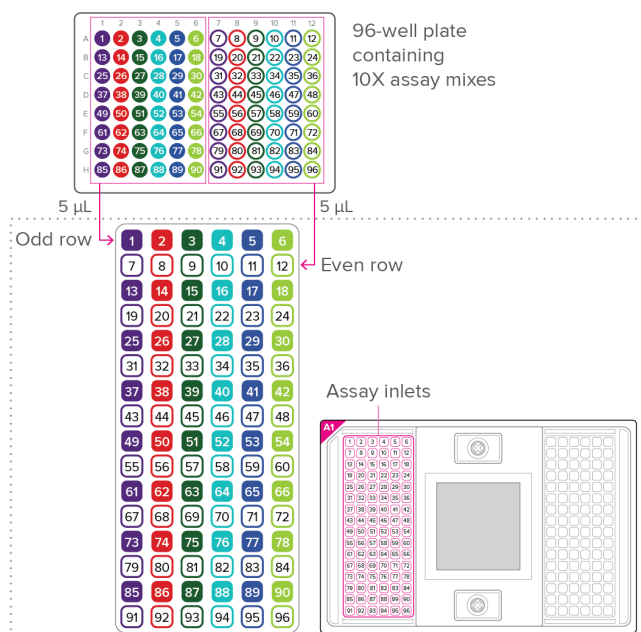
Load the IFC

IMPORTANT

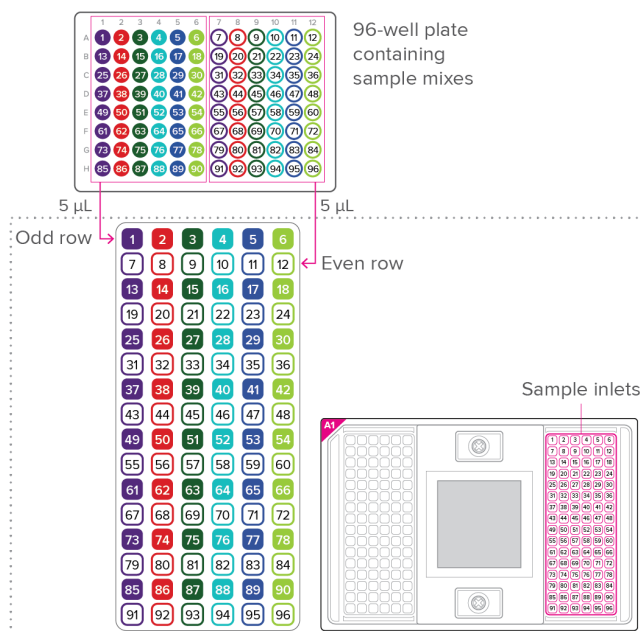
- Avoid creating bubbles (see Best Practices on page 3).
- Note the IFC orientation based on the A1 corner before you start pipetting.
- Each assay will be replicated four times, so be sure to dispense each CNV 10X assay mix into four separate inlets.

- Turn on the Biomark HD system (computer and instrument), if necessary, and launch the Data Collection software to allow the instrument to start up and the camera to cool to the appropriate temperature. Temperature stabilization takes up to 20 min.
- Ensure that the 10X assay and sample mix plates have been vortexed and centrifuged within 30 min of use. Repeat the vortex and centrifuge process if the plates are unused for >30 min.
- When the prime script is complete, remove the primed IFC from the controller.

- Pipet 5 μ L of each 10X assay mix into the assay inlets of the IFC.



- Pipet 5 μ L of each sample mix into the sample inlets of the IFC.



- Place the IFC into the controller and run the load mix script:

- Juno: Tap **Load Mix 96.96 GE**, and then tap **Run**.
- IFC Controller HX: Select **Load Mix (136x)** and press **Run Script**.

Loading the IFC takes approximately 90 min.

- After loading is complete, eject the IFC from the controller and collect real-time PCR data on Biomark HD.

IMPORTANT Start the IFC run on Biomark HD immediately after completing the Load Mix script.

Collect Real-Time PCR Data on Biomark HD

Transfer the loaded IFC from the controller to the Biomark HD and collect real-time PCR data.

IMPORTANT The IFC run requires a customized thermal protocol. See [Appendix A](#) for instructions.

- Remove and discard the protective film from the bottom of the IFC.
- Use clear tape to remove any dust particles or debris from the IFC surface.
- If necessary, double-click the **Data Collection** icon on the desktop of the Biomark HD system computer to launch the software.
- Click **Start a New Run**.
- Confirm that the camera status indicator at the bottom of the window is green.
- Place the IFC on the instrument tray, aligning the notched A1 corner on the IFC with the A1 on the tray, and click **Load**.
- Complete the Chip Barcode and Type section:
 - Verify IFC barcode and IFC type.
 - Choose project settings (if applicable) and click **Next**.
- Complete the Chip Run section by selecting either a new or a pre-defined run.
- Complete the Chip Run Name and Location section:
 - Enter a run name or select the checkbox to use the IFC barcode as the run name.
 - Select a file storage location for a new IFC run or browse to select a pre-defined run file and click **Next**.

- Complete the Application, Reference and Probes section and then click **Next**.

For...	Select...
Application	Gene Expression
Passive reference	ROX
Assay	Single Probe
Probes	FAM-MGB

- Complete the Thermal Protocol section:
 - Browse to and select the thermal protocol: **GE 96x96 Standard GTXpress v1.pcl**
 - Confirm that **Auto Exposure** is selected and click **Next**.
- Verify the IFC run information and click **Start Run**.
The IFC run takes approximately 2 hr.

Analyze the Data

After the run is complete, process your data using the Real-Time PCR Analysis software, followed by analysis using software that specializes in copy number variant determination, such as CopyCount-CNV software.

Appendix A: Customize the Thermal Protocol

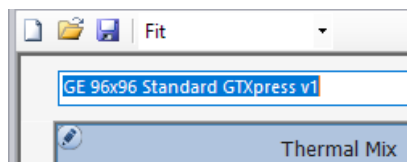
For detailed instructions for using the Protocol Editor, see the Biomark HD Data Collection User Guide (PN 100-2451).

- Start the Biomark HD Data Collection software.
- Select **Tools > Protocol Editor**.
- In the Protocol Editor, select **File > Open** to browse to and open the protocol **GE 96x96 Standard v2.pcl**.
- Make the following changes:
 - Remove the UNG slice.
 - Change the Duration for the Hot Start slice to **20 sec**.

Table 8. Custom protocol parameters

Temp.	Time	Cycles	Description	
50 °C	2 min	1	Thermal mix	
70 °C	30 min			
25 °C	10 min			
96.5 °C	20 sec	1	Hot start	
96 °C	15 sec	40	PCR	Denaturation
60 °C	60 sec			Annealing

- Change the name in the title bar to: **GE 96x96 Standard GTXpress v1**



- Select **File > Save As** and save the protocol with the name: **GE 96x96 Standard GTXpress v1.pcl**

Appendix B: Related Documents

Go to fluidigm.com to download these related documents.

Title	Part Number
Control Line Fluid Loading Procedure	68000132
IFC Controller MX and IFC Controller HX User Guide	68000112
Juno System User Guide	100-7070
Biomark HD Data Collection User Guide	100-2451
Real-Time PCR Analysis User Guide	68000088
CopyCount-CNV user guide (for software v2.1.9.2 or later)	—

NOTE This document is available for download from fluidigm.com on the product page for CopyCount-CNV (Fluidigm PN 101-7966).

Appendix C: Safety

General Safety

In addition to your site-specific safety requirements, Fluidigm recommends the following general safety guidelines in all laboratory and manufacturing areas:

- Use the appropriate personal protective equipment (PPE): safety glasses, fully enclosed shoes, lab coats, and gloves, according to your laboratory safety practices.
- Know the locations of all safety equipment (fire extinguishers, spill kits, eyewashes/showers, first-aid kits, safety data sheets, etc.), emergency exit locations, and emergency/injury reporting procedures.
- Do not eat, drink, or smoke in lab areas.
- Maintain clean work areas.
- Wash hands before leaving the lab.

Instrument Safety

For complete instrument safety information, including a full list of the symbols on the instrument, refer to the appropriate instrument user guide (see [Appendix B](#)).



WARNING BIOHAZARD. If you are putting biohazardous material on the instrument, use appropriate personal protective equipment and adhere to Biosafety in Microbiological and Biomedical Laboratories (BMBL), a publication from the Centers for Disease Control and Prevention, and to your lab's safety protocol to limit biohazard risks. If biohazardous materials are used, properly label the equipment as a biohazard. For more information, see the BMBL guidelines online at cdc.gov/biosafety/publications/index.htm.

Chemical Safety

The responsible individuals must take the necessary precautions to ensure that the surrounding workplace is safe and that instrument operators are not exposed to hazardous levels of toxic substances. When working with any chemicals, refer to the applicable safety data sheets (SDSs) provided by the manufacturer or supplier.

Disposal of Products

Used IFCs should be handled and disposed of in accordance with federal, state, regional, and local laws for hazardous waste management and disposal.

Appendix D: Instrument Software Requirements and Ordering Information

Fluidigm Instrument and Software Requirements

Operation	Instruments	Software Version
IFC loading and PCR	Juno	v3.11.1 or later
	IFC Controller HX	v2.8 or later
Imaging	Biomark HD	v4.2.2 or later with customized thermal protocol
Data analysis	—	Real-Time PCR Analysis v4.5.1 or later

Recommended Third-Party Software

Operation	Software Version
Copy number analysis	CopyCount-CNV 2.1.9.2 or later NOTE CopyCount-CNV was developed by DNA Software, and is available for purchase through Fluidigm for use with Biomark HD.

Ordering Information

Vendor	Name	Part Number
IFC and Control Line Fluid		
Fluidigm	96.96 Dynamic Array IFC for Gene Expression	BMK-M-96.96
Fluidigm	Control Line Fluid Kit—96.96	89000021
Fluidigm reagents		
Fluidigm	2X Assay Loading Reagent—5 Tubes	100-7611
Fluidigm	20X Fast GT Sample Loading Reagent—5 Tubes	100-7606
Third-party reagents		
Thermo Fisher Scientific	TaqMan GTXpress Master Mix (2X)	4403311
Thermo Fisher Scientific	TaqMan PreAmp Master Mix (2X)	4391128
Thermo Fisher Scientific	TaqMan CNV Target Assays with FAM-MGB probe	—
Thermo Fisher Scientific	TaqMan CNV Reference Assay with FAM-MGB probe	—
Teknova	DNA Suspension Buffer	T0221
Major laboratory supplier	PCR-certified water	—

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