Real-Time PCR Analysis
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About this User Guide

How to Use This Guide

The following chapters provide information about the analysis software and protocols for Real-Time PCR on the BioMark™ or BioMark™ HD Systems.

Safety Alert Conventions

Fluidigm documentation uses specific conventions for presenting information that may require your attention. Refer to the following safety alert conventions.

Safety Alerts for Chemicals

For hazards associated with chemicals, this document follows the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and uses indicators that include a pictogram and a signal word that indicates the severity level:

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="example.png" alt="Pictogram" /> DANGER</td>
<td>Pictogram (see example) consisting of a symbol on a white background within a red diamond-shaped frame. Refer to the individual safety data sheet (SDS) for the applicable pictograms and hazards pertaining to the chemicals being used.</td>
</tr>
<tr>
<td>WARNING</td>
<td>Signal word that indicates less severe hazards.</td>
</tr>
</tbody>
</table>
Safety Alerts for Instruments

For hazards associated with instruments, this document uses indicators that include a pictogram and signal words that indicate the severity level:

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="example" alt="Pictogram" /></td>
<td>Pictogram (see example) consisting of a symbol on a white background within a black triangle-shaped frame. Refer to the instrument user guide for the applicable pictograms and hazards pertaining to instrument usage.</td>
</tr>
<tr>
<td><strong>DANGER</strong></td>
<td>Signal word that indicates an imminent hazard that will result in severe injury or death if not avoided.</td>
</tr>
<tr>
<td><strong>WARNING</strong></td>
<td>Signal word that indicates a potentially hazardous situation that could result in serious injury or death if not avoided.</td>
</tr>
<tr>
<td><strong>CAUTION</strong></td>
<td>Signal word that indicates a potentially hazardous situation that could result in minor or moderate personal injury if not avoided.</td>
</tr>
<tr>
<td><strong>IMPORTANT</strong></td>
<td>Signal word that indicates information necessary for proper use of products or successful outcome of experiments.</td>
</tr>
</tbody>
</table>

Safety Data Sheets

Read and understand the SDSs before handling chemicals. To obtain SDSs for chemicals ordered from Fluidigm, either alone or as part of this system, go to fluidigm.com/sds and search for the SDS using either the product name or the part number.

Some chemicals referred to in this user guide may not have been provided with your system. Obtain the SDSs for chemicals provided by other manufacturers from those manufacturers.
Related Documents

This document is intended to be used in conjunction with these related documents:

- Fluidigm BioMark HD Data Collection Software User Guide (PN 100-2451)
- Biomark HD System Site Requirements Guide (PN 100-2595)
- Fluidigm IFC Controller Setup Quick Reference (PN 68000117)
- Fluidigm IFC Controller Usage Quick Reference (PN 68000126)
- Fluidigm Control Line Fluid Loading Procedure Quick Reference (PN 68000132)
- Gene Expression Analysis with the GE 24.192 IFC Using TaqMan Assays Protocol (PN 101-7571)
- Gene Expression Analysis with the GE 24.192 IFC Using TaqMan Assays Quick Reference (PN 101-7916)
- Fluidigm Flex Six™ IFC TaqMan® Fast/Standard Gene Expression Workflow (PN 100-7251)
- Fluidigm 48.48 Fast Real-Time PCR Workflow Quick Reference (PN 100-2637)
- Fluidigm 48.48 Real-Time PCR Workflow Quick Reference (PN 68000089)
- Fluidigm 96.96 Fast Real-Time PCR Workflow Quick Reference (PN 100-2638)
- Fluidigm 96.96 Real-Time PCR Workflow Quick Reference (PN 68000130)
- Fluidigm 192.24 Real-Time PCR Workflow Quick Reference (PN 100-6170)
- Fluidigm 192.24 Fast Real-Time PCR Workflow (BioMark™ HD only) Quick Reference (PN 100-6174)
- Fluidigm Gene Expression Specific Target Amplification Quick Reference (PN 68000133)
- Fluidigm 192.24 Delta Gene™ Fast/Standard Gene Expression Workflow (PN 100-7222)
- cDNA Preparation with Fluidigm® Reverse Transcription Master Mix Quick Reference (PN 100-6472)
- Fluidigm Gene Expression PreAmp with Fluidigm 5x PreAmp Master Mix and Delta Gene Assays Quick Reference (PN 100-5875)
- Fluidigm Gene Expression PreAmp with Fluidigm 5x PreAmp Master Mix and TaqMan Assays Quick Reference (PN 100-5876)
Real-Time qPCR

Real-time quantitative PCR (qPCR) is a powerful technique for quantifying changes in gene expression by producing millions of copies of specific, targeted regions of complementary DNA (cDNA) that has been reverse transcribed from messenger RNA (mRNA).

Advantages of Real-Time qPCR

Historically, qPCR has been a time-consuming process because of the time required to get gel-based end-point-measured (plateau phase) results. These results tended to be less accurate, and did not have as wide a dynamic range as real-time PCR. With the advent of quantitative data collection during the exponential phase of PCR, real-time quantification is a reality.

PCR Fundamentals

To appreciate the advantages of real-time PCR, a short review of PCR fundamentals is in order. At the start of a PCR reaction, reagents are in excess, both template and product are at low enough concentrations that product renaturation does not compete with primer binding, and amplification proceeds at a constant, exponential rate. The point at which the reaction rate ceases to be exponential and enters a linear phase of amplification is variable, and, at the plateau phase, the amplification rate drops to near zero.
The Exponential Phase

To ensure accuracy and precision, quantitative data is best when collected at a point in which every reaction is in the exponential phase of amplification—this being the only phase in which amplification is easily reproducible.

Advantages of Real-Time qPCR TaqMan® Chemistry

The BioMark™ Systems use dual-labeled probes, such as TaqMan® probes, for real-time qPCR amplification.

Dual-labeled probes are oligonucleotides that contain a fluorescent reporter dye on the 5’ base, and a quencher located on the 3’ base. When irradiated, the excited fluorescent reporter dye transfers energy to the nearby quencher molecule rather than fluorescing, resulting in a non-fluorescent substrate. Dual-labeled probes are designed to hybridize to a complementary region of the cDNA. The probe is flanked by an upstream and downstream primer pair that generates a PCR product. During PCR, when the polymerase extends the PCR product from the upstream primer, the 5’ exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent quencher and reporter dyes and Fluorescence Resonance Energy Transfer (FRET) no longer occurs. The increase in fluorescence intensity is proportional to the number of probe molecules that are cleaved.
Advantages of Delta Gene™ Chemistry

Delta Gene™ Assays have sensitivity and linearity similar to probe-based assays. They enable users to take full advantage of the BioMark HD System with minimal experimental setup time using validated protocols. With Delta Gene™ assays, flexible groups of biologically-related genes are available for specific requirements, eliminating the need to use fixed content. Amplicons are designed to cross an intron whenever possible to avoid genomic DNA amplification.
BioMark™ Systems for Genetic Analysis

The BioMark™ Systems include the optical, thermal cycling, and software components necessary to perform real-time quantitative PCR (qPCR) analysis on Dynamic Array™ IFCs.

The BioMark™ Systems provide orders of magnitude higher throughput for real-time qPCR compared to conventional platforms due to its Dynamic Array™ IFCs — nanofluidic chips that contain fluidic networks that automatically combine sets of samples with sets of assays. This innovative solution for real-time qPCR provides reaction densities far beyond what is possible with microtiter plates and significantly reduces the number of liquid-handling steps and the volume per reaction.

High-End Detection Optics

The BioMark™ Systems includes a high-resolution CCD camera that covers 30mm by 30mm, an area sufficiently large to simultaneously image all reactions in Dynamic Array™ IFCs. The BioMark™ System optics and analysis software is available for different applications, which are compatible with a variety of Fluidigm chip families for TaqMan® chemistry. The system’s computer-controlled chip tray automatically loads the chip into the instrument for ease of use. A barcode reader tracks experiments, reducing the chance of errors.
BioMark™ System Components

BioMark™ Systems include an internal thermal cycler, flat panel monitor, keyboard, and mouse. (The BioMark™ HD System is pictured.)

Controllers for IFCs

Juno

Juno™ is a universal controller that primes, loads, and thermal cycles assay-sample mixes.
IFC Controllers

The controllers were designed specifically to work with specific chips:

- **IFC Controller HX**—for priming and loading the Flex Six™ Gene Expression IFC and the 96.96 Dynamic Array™ IFC.
- **IFC Controller MX** (pictured)—for priming and loading the 48.48 Dynamic Array™ IFC.
- **IFC Controller RX**—for loading the 192.24 Gene Expression IFC.

Integrated Fluidic Circuit (IFC) Components

Although chip architect varies, the essential components, such as sample and assay wells and accumulators are common to all.

Flex Six™ Gene Expression IFC

The Fluidigm Flex Six™ Gene Expression IFC addresses the requirement for substantial variation in sample and assay numbers during target selection while allowing complete use of the IFC. It utilizes a completely new architect which incorporates six 12 X 12 partitions that can be organized in any configuration, in up to six separate experimental runs. This new IFC adjusts to customers’ experimental needs during target selection and largely eliminates the need for microplate-based experiments.
48.48 Dynamic Array™ IFC

The Fluidigm 48.48 Dynamic Array™ IFC is an efficient solution for large-scale, real-time qPCR. The key to this efficiency is the matrix of channels, chambers, and integrated valves finely patterned into layers of silicone. This material is gas permeable, allowing the blindfill of fluids into valve-delimited chambers. The valves partition samples and reagents and allow them to be systematically combined into 2,304 assays.

The significance of this approach to operational efficiency is immense. Managing a gene expression study involving 2,000 samples against a set of 48 genes would require 1,000 96-well plates as compared to 42 Dynamic Array™ IFCs. Managing the same study would require 192,000 steps on microplates but only 4,032 liquid-transfer steps on the chips. Comparative time required to complete such a study would typically involve 100 days on plates but just 4 1/2 days on chips. In addition, the running cost is reduced by half or more.

96.96 Dynamic Array™ IFC

The Fluidigm 96.96 Dynamic Array™ IFC is similar to the 48.48 Dynamic Array™ IFC but with high throughput. On one side of the frame are 96 wells to accept the samples and, on the other, 96 wells to accept the probe and primer pairs. Once in the wells, the components are pressurized into the chip using an IFC controller. The components are then systematically combined into 9,216 parallel reactions.
24.192 Dynamic Array™ IFC for Gene Expression

The Fluidigm 24.192 Dynamic Array IFC for gene expression supports the analysis of up to 192 markers per sample, the largest panel size of any Fluidigm gene expression IFC. The GE 24.192 IFC is well suited for customers who want to maximize data collection from rare samples. It is also a valuable tool in custom panel development, where it can be used to validate performance of potential gene expression assays prior to final panel selection and testing on a larger number of samples (for example, by using the 96.96 Dynamic Array IFC).

192.24 Gene Expression IFC

The Fluidigm 192.24 Gene Expression IFC meets the needs of production users who have narrowed their gene panel and require high sample throughput. Together with the BioMark™ Systems, this IFC enables maximum throughput with minimal hands-on time. The 192.24 Gene Expression IFC for can be particularly useful in clinical research and production environments, where users will benefit from the low-cost per sample, high reliability, and simple workflow of the IFC.
BioMark™ System Process Overview

The simplicity of running experiments on either BioMark™ System is illustrated in the process below.
1. Prime the chip.
2. Add the samples and assays to the chip.
3. Load and mix samples and assays.
4. Run your real-time experiment on the BioMark™ System.

Before You Begin

To ensure good experimental results, follow the guidelines listed below.

Organizing Your Work

- Label all reagent and reaction tubes.
- Maintain a separate DNA-free laminar flow hood—do not use nucleic acid samples in this hood.
- Use dedicated pipettes, tubes, and gloves for all manipulations that do not involve nucleic acid samples, which never leave the DNA-free (“Sample”) laminar flow hood.

Preventing Contamination

- Manipulate DNA samples under a dedicated laminar flow hood (for example, name it “Sample”).
- Use separate dedicated pipettes, tubes, and gloves for all manipulations involving nucleic acid samples, which never leave the DNA-dedicated laminar flow hood.
- Change gloves frequently.
- Use aerosol-resistant disposable pipette tips. Discard tips after each use.
- Use disposable, UV-irradiated plastic ware.
- Ensure that all equipment, including paper, pens, and lab coats are dedicated for use only in a particular laboratory. For example, dedicated laboratory coats for each of the PCR rooms.
- Do not bring contaminated workbooks into clean PCR areas.
- Aliquot PCR reagents.
- Wipe PCR hoods daily with DNAZap™ (Ambion) or a similar DNA decontaminate.
- Use ultra-violet radiation to complete decontamination.
- Ensure that only authorized users work in PCR areas and handle PCR equipment.
• Prepare reagents in a dedicated DNA-free laminar flow hood. DNA-free areas prohibit any biological material, including DNA or RNA extracts, and PCR products. Also, in the DNA-free area, prepare and aliquot reagent stocks and reaction mixes.

Handling Nucleic Acid, PCR Mixes, and PCR Reactions

• Prevent carry-over of amplified DNA sequences by setting up PCR reactions in a dedicated laminar flow hood, while keeping post-PCR manipulations separate.
• Add extracted DNA to the PCR reaction mixes in the DNA-dedicated (“Sample”) laminar flow hood. Be sure to prepare the PCR reaction mixes in the DNA-free laminar flow hood.
• Keep the amplification room—where PCR machines are housed—separate from the room in which PCR reactions are assembled (DNA free laminar flow hood).

Using Controls

• Include—whenever possible—a positive control that amplifies weakly but consistently. Using a strongly positive control sample may result in excess amplified product which may serve as a source of contamination.
• Use well-characterized negative samples such as lambda DNA.
• Include reagent controls containing all the necessary reagent components but excluding test DNA.
• Use decontaminating enzymes such as uracil N-glycosylase (UNG) or Uracil-DNA Glycosylase (UDG) to further minimize the likelihood of contamination.

What You Need for Experiments

This section describes the materials that you need to perform your experiments including reagents we support and sample requirements. In addition, you need the following:
• BioMark™ System or BioMark™ HD System
• IFC Controller
• Flex Six IFC, 48.48 Dynamic Array™ IFC, 96.96 Dynamic Array™ IFC, or 192.24 Dynamic Array™ IFC
• 20X GE Sample Loading Reagent (Fluidigm PN 85000735, 85000746)—store at -20 ºC.
• 20X DNA Binding Dye Sample Loading Reagent (for EvaGreen protocols) (Fluidigm, PN 100-3738)—store at -20ºC
• 2X Assay Loading Reagent (Fluidigm PN 85000736)—store at -20 ºC.
• Deionized DNA-free, DNase-free, RNase-free water—store at room temperature.
• DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) (TEKnova, PN T0221)—store at room temperature.
• Sample Mix
• Prime/probes sets
• Samples of interest

Supported Detection Reagents

We support the following detection reagents with the BioMark™ Systems.

Probe Types

• FAM-MGB
• VIC-MGB
• FAM-TAMRA
• FAM-non fluorescent quencher

Additional Probe Types

Fluidigm does not support other probe types at this time, however, additional probe types may be run with the BioMark™ Systems using the following guidelines:

<table>
<thead>
<tr>
<th>Fluorophores With...</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Excitation Wavelengths</strong></td>
<td><strong>Emission Wavelengths</strong></td>
</tr>
<tr>
<td>between 465 and 505 nm</td>
<td>And</td>
</tr>
<tr>
<td>between 510 and 550 nm</td>
<td>And</td>
</tr>
</tbody>
</table>

PCR Master Mixes

If you choose to use master mixes other than those specified in this guide, you may have to alter the protocol described in this guide. Contact Fluidigm Technical Support for additional information.

**IMPORTANT:** You must use a passive reference.
Sample Requirements

RNA Quality

Your RNA should have an 260:280 Ratio between 1.5 and 1.8. Prior to use on a chip, monitor the integrity of your RNA on a system such as the Agilent® 2100 bioanalyzer.

cDNA Preparation

Synthesize cDNA as described in Appendix A, cDNA Preparation with Fluidigm® Reverse Transcription Master Mix.

cDNA Input

The exact amount of cDNA to be used for each experiment depends on the relative abundance of the target gene. Unless you have concentrations in excess of 1,000 copies of your target template per µL of sample, increase the your target concentration by using preamplification as described in Fluidigm Gene Expression PreAmp with Fluidigm 5x PreAmp Master Mix and Delta Gene Assays Quick Reference (PN 100-5875) and the Fluidigm Gene Expression PreAmp with Fluidigm 5x PreAmp Master Mix and TaqMan Assays Quick Reference (PN 100-5876).

cDNA Storage

Avoid multiple freeze-thaw cycles by storing cDNA at 4°C. For longer storage, aliquots may be stored at -20°C.

Reagent Storage

Consult manufacturers’ product inserts for storing specific reagents.
Using Real-Time PCR Analysis Software

Launching the Software

1. Double-click the Real-Time PCR Analysis Software icon on your desktop.

   The Start screen opens.
Menus and Icons

Top Menu Bar

Secondary Menu Bar

File

The File menu has the following options:

<table>
<thead>
<tr>
<th>Option</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>New</td>
<td>Open the Chip Run Setup Wizard</td>
</tr>
<tr>
<td>New From Current Chip Run...</td>
<td>Open a new version of the chip run you are currently analyzing</td>
</tr>
<tr>
<td>Open</td>
<td>Open location of .bml chip run data files</td>
</tr>
<tr>
<td>Open Multiple Chip Runs...</td>
<td>Open two or more chip runs and combine them in one view</td>
</tr>
<tr>
<td>Save</td>
<td>Save your current run data file with any changes</td>
</tr>
<tr>
<td>Convert to More Samples Chip Run...</td>
<td>Convert your chip run to a more samples chip run</td>
</tr>
</tbody>
</table>
### Edit

The Edit menu options depend on the active window.

<table>
<thead>
<tr>
<th>If the Active Window Is...</th>
<th>Your Options Are...</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample Setup</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Detector Setup</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Details Views</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Results Table</strong></td>
<td></td>
</tr>
</tbody>
</table>

#### Email Chip Run Information...

This function collects chip run information (.bml file, first and last cycle images) and attaches to an email that can be sent to Fluidigm Technical Support or other party to share information conveniently.

#### Close

Close your current run data file.

#### Export

Export **Results** table data or **Heat Map** data as .csv text file.

#### C:\...

Open the location of recently viewed/used .bml files.

#### Exit

Close the application.
View

Select Chip Explorer and/or Task to display these panes in your window.

Report

Two reports are available:

- Chip Preparation Report
  This report records the loading pattern for a chip run. After creating a new chip run file, use the Chip Preparation Report to record the data for hand-pipetting.
• Install Test Report
This report is only available after a chip run (.bml) file has been opened and analyzed in the software.
Install Test Report


   A warning about properly setting up the chip appears. Click OK if the setup is correct. The report is generated.

2. Go to File > Export Document.... to select a file format. PDF is the default.
3. Select a folder location to save the file.
4. Change the Name and/or file type, if needed.
5. Click Save.

Tools

The Tools menu has the following options:

- Dispense Map Editor…
- Options…

See “Using the Dispense Map Editor” on page 46.
Options

You can modify your report options and customize your analysis parameters (see “Customizing the Analysis Parameters” on page 32) by selecting Tools > Options.

You can change the following report export options:

- **Default folder.** In the Default Folder drop-down menu, you can select Chip Run Folder or User Specified Folder. If you select User Specified Folder, you can browse to the folder where you want to save the results or report.

- **Export comments.** When this option is enabled, the report includes the user-defined comments in the exported data.

- **Substitute “Flag” with “Fail.”** When this option is enabled, the Flag label for Ct calls is substituted with Fail.

- **Mark User Calls in Table Results.** When this option is enabled, manually changed calls are shown in the report as “mPass” or “mFail.”

- **Export Reagent Reference Count.** When this option is enabled, the report includes the Reagent Reference Count columns.

- **Export Sample and Reagent Plate Information.** When this option is enabled, the report includes the sample and reagent plate names and barcodes.

- **Use Post Export Command.** This option saves the results in a custom format. Contact Technical Support for assistance.
Customizing the Analysis Parameters

You can customize the Ct and MCA Analysis Parameters before you open a chip run. This change will apply only to chip runs that have not been previously analyzed.

IMPORTANT: If you customize the Analysis Parameters on a shared workstation, this will cause default setting to be changed for all users. Please use caution.

After launching the Real-Time PCR Analysis software:
1. Select Tools > Options.
2. In the Options dialog box, click Analysis Parameters.
3. Check the checkboxes for the parameters you want to customize, then select or enter the desired settings. All unchecked boxes will use the original default settings.
Creating a New Chip Run

1. Click creating a new chip run under Chip Run Summary or click Create a New Chip Run under Task.

   The Chip Run Setup Wizard opens.

2. Follow the steps to complete the setup.

3. Complete the wizard and go to:
   - “Setting Up a Sample Plate” on page 38 to set up a sample plate.
   - “Setting up a Detector (Assay) Plate” on page 49 to set up an assay plate.

Opening an Existing Chip Run

1. To analyze a previous chip run, click opening an existing chip run in the Welcome screen (Chip Run Summary) or click Open a Chip Run under Task or click File > Open.
2 Double-click the chip run file (.bml extension).

The chip run file opens.

NOTE: You cannot open a chip run if another use is analyzing it.

3 Click Details Views.
4 Click Analyze.

**IMPORTANT:** You must click Analyze each time you change parameters. A reminder dialog appears if you do not click Analyze after each change.

The first time a chip is analyzed, the chamber-finding algorithm locates the chamber boundaries of each captured image. This may take some time.

Continue to:

- “Setting Up a Sample Plate” on page 38 to set up a sample plate.
  OR
- “Setting up a Detector (Assay) Plate” on page 49 to set up an assay plate.
Finding Corners Manually (if required)

NOTE: If the chamber finding algorithm cannot locate the four corner cells of the chip during the first analysis, the following error message will appear.

If this occurs, you can manually set the corners and then analyze the chip.

1  Click OK.
   The Set Corners of the Chambers Area dialog box appears.
2  Zoom in to see the corner cells.

NOTE: If you cannot see the four corner cells, adjust the Contrast slider. (If an insufficient amount of ROX dye was used in setting up the IFC corners, it will be difficult to see the corner cells.

3  Position the corners of the red box at the perimeter of the chip image.
4 Make sure each corner is placed on the outer edges of each corner cell (see below).

5 Click Done.

**NOTE:** If no ROX is present, the corner cells are very dark. You may have to count the number of rows and columns (48 down, 48 across for the 48.48 IFC for example) to make sure you are placing cross hairs correctly.

If the algorithm cannot detect ROX in the cells, it displays the chambers as blue dots.
1. You can drag the red box corners to the corner chamber locations to match the cells to chambers.

Forced Manual Corner Find

If the automated manual corner find results are not satisfactory, you can perform a forced manual corner find by pressing the CTRL key and simultaneously clicking on the Analyze button.

Setting Up a Sample Plate

Use this table as a guide when annotating your samples:

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>An unused position. Nothing in the chamber.</td>
</tr>
<tr>
<td>NAC</td>
<td>No Amplification Control: usually the Taq polymerase is left out of the reaction; this is a negative control confirming that positives cannot occur without the PCR working.</td>
</tr>
</tbody>
</table>
1 In the Chip Explorer window, select Sample Setup.

The Sample Plate Setup Wizard opens.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTC</td>
<td>No Template Control (negative control): everything included except the sample; to show that a positive result cannot be obtained when the sample is left out.</td>
</tr>
<tr>
<td>Unknown</td>
<td>An experimental sample.</td>
</tr>
<tr>
<td>Reference</td>
<td>A sample against which the unknown samples are compared or normalized.</td>
</tr>
<tr>
<td>Standard</td>
<td>A sample against which unknown samples are compared in a standard curve analysis.</td>
</tr>
</tbody>
</table>
1 Choose the appropriate **Container type** and **Container format**.

<table>
<thead>
<tr>
<th>Container type</th>
<th>Container format</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SBS Plate</strong>: the plates where samples and detectors are stored before being pipetted into chip.</td>
<td><strong>SBS96</strong>: a 96-well plate. For 192.24 IFCs, two source plates are provided for annotating samples.</td>
</tr>
<tr>
<td><strong>Sample Inlets</strong>: location where samples enter the chip.</td>
<td><strong>SBS384</strong>: a 384-well plate.</td>
</tr>
</tbody>
</table>

2 Click **OK**.

3 Select the cells to use as a reference.
   - Click the upper left corner to select all the cells.
   - Click and hold while dragging your cursor through cells.
   - Click individual cells while pressing the **CTRL** key.
4 OPTIONAL: Click the Map icon.

The map shows selected cell(s) relative to the entire sample plate.
NOTE: If you selected SBS Plate as your container type, clicking the Map icon opens a sample plate map. If you selected Sample Inlets, clicking the Map icon opens a sample inlet map.

5 Click Editor. The Sample Editor opens.
6 Select the appropriate type.

NOTE: To identify a reference, see “Calculating Delta Ct Sample Values” on page 114.

7 Enter the sample name.
8 Enter the relative concentration.
9 Click Update. The Sample Plate Setup reflects the updates.
10 Close the Sample Editor.

11 Click the Open Mapping File icon.
Double-click either left or right sample mapping file to determine dispense location.

**NOTE:** If you are analyzing a 192.24 Gene Expression IFC, you have several options: 192-Sample-SBS384 (left or right).dsp, 192-Sample-SBS96 (left or right).dsp, or 192-Sample-SBS96 (Even or Odd).dsp

**NOTE:** For Flex Six chip runs, you must manually annotate according to inlets.

Your selection is displayed in light blue (left or right).

Your sample plate setup is complete. Next, go to “Using the Sample Mapping Viewer,”

**Using the Sample Mapping Viewer**

Use the Sample Mapping Viewer to view or record the loading pattern after setting up the sample plate.

1. Click Sample Mapping View. The dispensing map opens.
2. Click a cell in the Source Plate to see where it loads on the Target Plate.
NOTE: If you click an unused cell, the “Well not used” warning appears.

Using the Replay Control

Use the Replay Control to show where and in what sequence the Target Plate receives the samples from the Source Plate.

Plays the sequence from start to finish, one row at a time. Click it once to pause and then click again to continue.

Advances the loading one row at a time with each click toward the end

Moves the loading to the end position

Moves the loading back one row at a time with each click toward the start

Clears the map.

Returns the loading to the start position

Start position

End position
Using the Dispense Map Editor

Use the Dispense Map Editor to record custom load maps for future use. After recording your loading sequence, you can save it and play it back anytime.

1. Click Tools > Dispense Map Editor.
2. Click New. The New Dispense Map window opens.
3. Complete the New Dispense Map using the following as a guide.

4. Click OK to open the new dispense map in the Dispense Map Editor.

**Source Plate.** Graphical representation of the plate from which the samples and/or detectors are pipetted.

**Dispense Map.** This table shows you where the samples and detectors are on the chip.

**Target Plate.** This is a graphical representation of the plate into which the samples and/or detectors are pipetted.
5 Click **Begin Editing** in the recording control pane.

![Recording Control](image)

a Click the first cell from the **Source Plate**. Then, click the location in the **Target Plate**.

b Continue clicking appropriate cells (from the **Source Plate** to the **Target Plate**) until your custom loading map has been recorded.

**NOTE:** When you click **Begin Editing**, the dispensing pane becomes inactive.

6 Click **Stop Editing**.
These graphics show custom loading and how it looks as you proceed.

7 Review the loading pattern you have recorded by clicking the green arrow button in the playback control pane.
Setting up a Detector (Assay) Plate

Use this table as a guide when annotating detectors (assays):

<table>
<thead>
<tr>
<th></th>
<th>Experiment reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>A control or reference gene</td>
</tr>
<tr>
<td>NRC</td>
<td>No Reagent Control: negative control using only buffer, no primers/probes (detectors)</td>
</tr>
</tbody>
</table>

To Set Up the Detector:

1. Click Detector Setup.
2. Click New.
3. Choose the appropriate Container type and Container format. For 24.192 IFCs, two source plates are provided for annotating detectors.
4. Click OK to open the Detector Plate screen. OPTIONAL: Double-click between columns to expand.
5 Select cells by performing one of the following:
- Click and hold while dragging your cursor through cells.
- Click the upper left corner to select all the cells.
- Click individual cells while pressing the CTRL key.
6 OPTIONAL: Click the Map icon 🗺. The map opens and shows selected cell(s) relative to the entire detector plate.
NOTE: If you selected SBS Plate as your container type, clicking the Map icon opens a detector plate map. If you selected assay detector Inlets, clicking the Map icon opens an assay detector inlet map.

1 Click Editor.

The Detector Editor opens.

2 Complete the Detector Editor:
   a Select the appropriate type.

   NOTE: If you want to identify a reference before moving on, see “Calculating Delta Ct Detector Values” on page 118.

   b Enter the Detector Name.
3 Click **Update**.
The Detector Plate Setup reflects the updates.

4 Close the Detector Editor.

5 Click the **Open Mapping File** icon.

6 Double-click either left or right sample mapping file.
Your selection is displayed in light blue (left or right) in the **Mapping Viewer**.

Your detector plate setup is complete.

**NOTE:** You can also copy and paste sample/assay names directly from Microsoft® Excel® spreadsheets.

## Advanced User Options

### Converting a Chip Run to a More Samples Run

A More Samples run requires its own sample and assay setup, and Fluidigm provides the necessary Microsoft Excel setup templates for you.

Follow these steps to convert a chip run to a More Samples run:

- **Step 1:** Set up your samples
- **Step 2:** Set up your assays
- **Step 3:** Import the samples and assays to the Analysis software

**Step 1: Set Up Samples**

1. Go to C:\Program Files\Fluidigm\BioMarkDataAnalysis\ApplicationData\FileFormats.
2. Open SamplePlateDefinitionForMoreS.
3. Click Options to enable Active X (if prompted).

4. If prompted, select Enable this content in the Microsoft® Office Security Options dialog.
5. Click OK.
6. Edit the Microsoft Excel template to match your experiment.
7. Click Create Plate CSV File.
8 Open the new CSV file tab and double-check your annotations.

9 Click **Save to a CSV file** to save the file and select a location to save it.

**Step 2: Set Up Assays**

1 Open **AssayPlateDefinitionForMoreS**.
2 Edit the Microsoft Excel file to match your experiment.
3 Click **Create Plate CSV File**. A second CSV file tab is added to the file.
4 Open the new CSV file tab and double-check your annotations.
5 Click **Save to a CSV file** to save the file and select a convenient location for future retrieval.

**Step 3: Import the Sample and Assay Templates**

**To Import the Sample template files:**

1 From the Data Analysis software, open a chip run you want to annotate.
2 Select **Sample Setup**.
3 Click **Import** under **Task**.
4 Browse to the location where you saved your sample template.
5 Click **Open**.
6 Go to **File > Convert to More Samples Chip Run**.

**To Import the Assay template files:**

1 From the Data Analysis software, open a chip run you want to annotate.
2 Select **Assay Setup**.
3 Click **Import** under **Task**.
4 Browse to the location where you saved your assay template.
5 Click **Open**.
6 Go to **File > Convert to More Samples Chip Run**.
Importing Multiple Chip Runs

You can import multiple chip runs to increase data points. This can be done to combine the individual arrays of Flex Six IFCs, or to combine multiple 48.48, 96.96 or 192.24 Dynamic Array™ IFCs.

This feat requires some pre-planning on your part, however. The function enables combining multiple chip runs in a two dimensional layout, meaning you can add chip runs down the sample axis and/or the assay axis.

**NOTE:** There should be uniformity across the chip runs. Samples and Assays of the chip runs must be set up the same, annotated the same, run on the same machine and in a similar time frame.

**NOTE:** To ensure consistent annotation, you can annotate the samples/assays of the first chip, then export the .plt file. Then, import the same .plt file to annotate the other chip runs.

1. Click File > Open Multiple Chip Runs.

2. Click the folder containing the multiple chip runs.
3 Click the arrow to move all the chip runs in the folder to the other pane. Alternately, expand the multiple chip run folder and choose specific .bml run files, clicking the arrow for each file that you want to add to the other pane. Alternatively, you can select files from various folders.

4 Type a name.

5 Click **Browse**.

6 Navigate to a location to store the data.

7 Click **OK**.
Viewing Chip Run Data in the Data Analysis Software

Working with Analysis Settings

You can customize these analysis settings:

- Quality Threshold
- Baseline Correction
- C<sub>t</sub> Threshold Method

To change the settings:
1. Launch the Real-Time PCR Analysis software.
2. Click Details Views. Analysis settings are located under Task.

Changing the Quality Threshold

The Quality Threshold in the BioMark™ Analysis software is a qualitative tool designed to measure the “quality” of each amplification curve. Basically, each amplification curve is compared to an ideal exponential curve and as the quality score approaches 1 the closer it is to ideal. The further the curve is from ideal, its quality score approaches 0.
The default cutoff of 0.65 is an arbitrary value set by Fluidigm. Any curve above 0.65 passes. Any curve below, fails. This does not mean the curve or Ct value is invalid because the quality threshold is merely a tool that flags curves that may be problematic or suboptimal so you can assess if they are true growth curves or artifacts. Once reviewed, you can change the quality value to Pass.

To change the quality threshold:
1. Click Details View.
2. Under Analysis Settings, enter a different value in the Quality Threshold field.

**Changing the Baseline Correction**

To change the baseline correction:
1. Click Details View.
2. Under Analysis Settings, select a different option in the Baseline Correction field.

<table>
<thead>
<tr>
<th>Baseline Correction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>Produces higher Ct values when the amplification is low. Linear baseline correction eliminates baseline “drift” by flattening the baseline.</td>
</tr>
<tr>
<td>Linear (Derivative) [default]</td>
<td>An additional method of baseline correction with a more robust handling of chamber-to-chamber baseline variation.</td>
</tr>
</tbody>
</table>

The difference between Constant and Linear baseline corrections is shown here:
Changing the Ct Threshold Method

To change the quality threshold:

1. Click Details View.
2. Under Analysis Settings, select a different option in the Ct Threshold Method field.

Baselines are rising in the Constant correction (shown as black fill).

Baselines are flattened in the Linear correction.
### Auto (Global)
Automatically calculates a threshold that is applied to the entire chip.

### Auto (Detectors)
Independently calculates a threshold for each detector on a chip.
You must enter a unique detector name in the Detector Editor during detector setup.

### User (Global)
Allows you to manually adjust the threshold when searching for the $C_T$ rise in slope. The value is applied to all the detectors.

### User (Detectors)
Allows for tighter control when searching for the $C_T$ curve’s rise in the slope.
You can individually set the threshold for each detector on the Ct Thresholds tab.

Select the Initialize with Auto checkbox to reset the threshold values using the Auto Detector method. Click Analyze to update the results.
Saving or Loading Threshold Settings

To save your detector threshold settings for use with another chip run, right-click anywhere in the threshold table and select **Save Table**. Name the file and choose the location where you want to save the file.

To load your saved detector threshold settings, select **Load Table**. Browse to the location of the settings file you want.

Working with Details Views

There are three different views:

- **Results Table** -- view results in one table
- **Image View** -- view images from individual cycles
- **Heat Map View** -- view color-coded $C_T$ values
- **Dual Views** -- view two primary views (Results and Heat Map, for example) side by side

Click the **Expand/Collapse** button to display any view at full size or as split screen.

Using the Results Table

To access the Results Table:

1. Click **Details Views**.
2. Select **Results Table**, if it is on a different view.
Viewing Chip Run Data in the Data Analysis Software

In the **Results Table** view, right-click a column header to:

- Adjust columns, see page 64
- Group columns, see page 65
- Sort columns, see page 71
- Column Chooser, see page 72
- Customize search filters, see page 74
- Add user defined comments, see page 75

**Resizing Columns**

**Using the Cursor**

- Position the cursor on a column edge. When the cursor changes to a double arrow, hold and drag the column bigger or smaller.

  Or

- Double-click the column edge to adjust the column to precisely fit the contents.

**Using “Best Fit”**

- One column: right-click a column header and select Best Fit. The column automatically adjusts to precisely fit the contents.
• All columns: right-click a column header and select **Best Fit (all columns)** to adjust all columns to precisely fit the contents.

**Grouping Two or More Columns**

1. Right-click on any column header.
2. Click **Group by Box**.

The grouping bar appears.
3 Click the column header that you want to group and, while holding down the mouse button, drag it to the bar as shown below.

   a. Click and hold mouse button on header

   b. Drag to any place on the bar

   c. Release mouse button and header snaps to position at the left

The data are now grouped by name in the Results Table.
4 Group as many elements as you like by dragging and dropping, as in the example below.

Click + (plus) or – (minus) to expand/collapse the windows.
5 Drag and drop one header element over another as shown below to change places (hierarchy). The hierarchy dictates how the data displays as you expand windows.

In this example, the **ID** header is dragged over the **Type** header and then dropped. They exchange places as a result.
Ungrouping One Header

1. Right-click a header within a group.
2. Click Ungroup to remove the header from the group.

Ungrouping All Headers

1. Right-click anywhere on the grouping bar.
2. Click Clear Grouping.
Expanding and Collapsing All

1. Right-click anywhere on the grouping bar.
2. Click Full Expand. The grouped windows expand as shown below.

3. Collapse all by right-clicking anywhere on the grouping bar.
4 Select Full Collapse.

Before full collapse

After full collapse

### Sorting Columns

1. Right-click a column header.
2. Choose either Ascending or Descending to sort that column accordingly.
Unsorting Columns

1. Right-click a sorted column header.
2. Click Clear Sorting.

Column Chooser

Depending on how you set up your sample plate and detector plate, you can have 20+ columns in the Results Table, all of which are not viewable at once. To temporarily remove columns not of immediate interest, follow the procedure below.

1. Right-click a header.
2. Click Column Chooser. The Customization dialog opens.

3. Drag and drop unwanted column headers onto the Customization dialog.
4. Replace the column headers by dragging them from the Customization dialog to their original position.

Dropdown Menus on the Column Headers

Each column header has a dropdown menu. Place your cursor over a header to reveal the symbol.

1. Click the dropdown menu symbol to display the menu.
2. Click a location to go to that location.
3 Click and drag the menu to size it.

![Custom Filters](image)

Use filters to narrow your search for a particular parameter. In the following example, we isolate C_t quality values below 0.8.

1 Click the **Quality** header menu.
2 Click **Custom**.

The Custom AutoFilter dialog box opens.

3 Delimit your search:
   a Select a filter.
   b Enter the target value (0.8 in this example).
   Or
   c Click the **Field** checkbox to activate the menu and select a filter.
4 Optional: Continue delimiting your search by clicking And/Or and then selecting filters from the menus.

5 Click OK.

Adding User Defined Comments

A user can add comments to each chamber in the Results table. In addition, macros can be set up to apply common comments to multiple chambers.

1 Click on the Results Table menu.
2 Click on the Comments button.

The Add Comments and Accelerator Key Definitions dialog box appears.

3 Enter a comment, such as “C_t”.
4 Click OK to apply to first chamber.
5 Or, click the Show Accelerator Keys button.
6 Enter a comment that will be used frequently, “Ct”, for example, in the Enter Comments box.

7 Select the preferred Accelerator Key and repeat the comment. F2 was used in this example.

8 Click the Save As Default button.
   Now you can select chambers you wish to add this comment to and simply press the F2 key.

Using the Image View

View images from individual cycles in this window.

1 Click the Results Table menu.

2 Click Image View.

The default Image View opens.
3 Select a dye.

4 Select a cycle number from the Cycle Selection menu.

IMPORTANT: An image displays only after you have selected a dye and a cycle number. A representation of the chip displays in the Image View.
5 Optional: Click the double arrow to expand the image.

Image View Tool Bar

Elements of the Image View tool bar are shown below.

- Expand Image button
- Click to enlarge to 100%
- Displays local reference map
- Auto contrast adjustment
- Manual adjust contrast slider
- Zoom
- Dye selector
- Toggles overlay On and Off
- Cycle image drop-down menu
- Fit image by auto, width, or height

Zoom

You can increase or decrease the image view size in several ways:
- Multi-clicking the magnifying glass buttons (+ and -).
- Click the 100% button.
- Clicking the Fit button to fit image to width.
- Clicking inside Image View and then rolling the mouse scroll wheel (up/backward = larger, down/forward = smaller).
Location Reference Map

Use the location map to reference your cell of interest within the entire framework of the chip.

1. Click the Location Reference map icon to open the map.
2. Click and drag the blue rectangle to a location of interest. In the example below, the blue rectangle within the Location Reference map is dragged to the green cells which enlarges the green cells in the Image Viewer.

Drag blue rectangle to area of interest

Dragging the blue rectangle to an area of interest enlarges that area in the Image Viewer as shown here.
Adjusting the Size of the Location Reference Map

The size of the image in the Image Viewer determines the size of the blue rectangle in the Location Reference Map.

In the example below left, the image is has not been zoomed so the blue rectangle on the map is large. In the example below right, the image has been enlarged (by clicking in the Image View and then rolling the scroll wheel on the mouse).

![Image View](image1.png)

Here, the blue rectangle is large and cannot be dragged with much accuracy.

![Image View](image2.png)

Here, the image has been enlarged so that the blue rectangle is smaller and easily dragged to an area of interest.

**NOTE:** Selected cells in the Image View are also displayed in the Graph View as shown below.
Overlay

1. Click the Overlay icon to activate the red-square grid.
2. Click the Overlay icon again to inactivate the red-square grid.
Contrast (Auto or Manual)

You can apply contrast adjustments to all the dyes at once or to each dye individually by selecting All or Individual from the Contrast drop-down menu.

To adjust image contrast:
- Click the Auto-Contrast icon.
- Or,
- Move the contrast sliders by placing your cursor over a slider, then click and drag.

Dyes

Change the dyes.

View Image in Each Cycle

Use the menu to select an image to view. Select number 7 in the menu, for example, and the image taken at cycle 7 displays in the Image Viewer.

Fused Image View

If the chip run you are analyzing has more than one color, the Fuse option will be available in the dye selection drop-down menu. This allows you to see the difference in intensity across multiple dyes in a single image.

To utilize the fuse image view:
1. Click the Results Table menu.
2. Click Image View.
3 Select **Fuse** from the dye drop-down menu.

The Assign Color Values dialog box appears.

4 Select a color for each dye.

5 Select which dye you wish to overlay on top of the other.

6 Click **OK**.

7 You can click on Fuse again to change any parameter.

---

**Using the Heat Map**

The heat map color codes $C_t$ values for easy reference.

To access the heat map view:

1. Click **Details Views** in the Real-Time PCR Analysis software.
2. Go to **Heat Map View**.

The default heat map opens.
**NOTE:** A black square indicates no C_t value or a value outside of the spectrum range, as shown in the example below. Also, negative controls that do not show amplification appear as black squares.

**NOTE:** An X signifies an amplification curve marked as Flag.

3  *Optional:* Click the double arrow 🔄 to expand the image.
4 **Optional:** Hold your cursor over a cell of interest and an information dialog box opens; click the cell and the information appears on the task bar.

Hold the cursor over a cell to open the information dialog box...

...click the cell to display the information on the bar also.

5 **Optional:** select a row or a column by clicking an inlet or using the right-click menu as shown below.

Click to select a column

Click to select a row

Hover cursor over cell and right-click to open options menu.
6 Optional: Click the upper left corner in the heat map to select all cells.

Heat Map View Tool Bar

Color Lookup Editor

You can define a range of valid Ct or Tm values using the Color Lookup Editor.

1 Click the Color Lookup Editor button �┃.
2 Choose Ct-YellowToBlue or Tm-YellowToBlue.
3 Click Edit.

The Spectrum Editor opens.

4 Choose RGB (red, green, blue) or HSL (hue, saturation, lightness).

5 Optional: Change the percentage increments between colors by changing the number.
   a Click Edit.
   b Change the value (from 1 to 20).
   c Click OK.
d  Click OK.

The change is reflected in the heat map and in the legend.

6  Optional: Click Invalid Color Data to change the color of failed cells.
   a  Click a color square.
   Or
   b  Click Define Custom Color to pick a color other than a basic color.
c Click OK.

Color Range Pane in the Color Lookup Editor

You can change the following parameters:
- Number of color segments
- Minimum value
- Maximum value
- Auto Range

Changing the Number of Color Segments

Change the segments shown in the heat map.
1 Type a value (2 minimum).
2 Click OK to reflect changes in the legend.

The examples below illustrate that the greater the number of color segments, the finer distinction between legend values.

1 Color Segment
Changing Minimum Values

Change the minimum value when you want to exclude a segment from the lower range. For example, changing the value from 1 to 10, excludes any $C_t$ value from 1 to 10 as the example below illustrates.

1. Enter a value.
2. Click OK.

Segments with values from 1 to 10 have been eliminated as the minimum acceptable value is now greater than 10. As a result, the lowest value on the legend is now over 10.

Changing Maximum Values

Change the maximum value when you want to exclude a segment from the higher range (from 1 to 39). For example, changing the maximum value from 35 (default value) to 10, any $C_t$ or $T_m$ values above 10 are excluded (gray area), as the example below illustrates.

1. Enter a value.
2. Click OK.
Using the Heat Map

When you click OK, the heat map and the legend reflect the change also.

Using Auto Range

Auto range allows you to eliminate a percentage of the upper and lower ranges of all valid Ct or Tm values.

1. Click Auto Range.

2. Change the values.
   In the example below, the minimum and maximum values have been changed to 3.0. Therefore, after discarding the lower and upper 3% of valid values, you are left with a range of 11.86 to 28.18.

This range is represented in the Color Lookup Editor illustrated below. Note the eliminated values (from 1 to 11.86, and 28.18 to 35) are now gray areas.
3 Click OK to see the changes in the heat map and the heat map legend.

**Saving Changes**

To save custom parameters that you have set:

1. Click Save.
2. Enter a name for your custom parameters.
3. Click Save.

The Color Lookup Editor opens.

4. Click the Color Scheme menu to see the saved parameters.

**Location Reference Map**

Use the location map to reference your cell of interest within the entire framework of the chip.

- Click the Location Reference map icon to open the map.
Legend

The legend is a color representation of the $C_t$ or $T_m$ values displayed on the heat map.

- Click the Legend icon.

Using the Heat Map
Preferences

Click the Preferences button.

- Show Grid
- Show Cell Text
- Preferences
- Show Column Headers (192.24 IFCs only)
- Show Panel Titles (192.24 IFCs only)

Changing Grid and Selected Cell Color Preferences

To change heat map grid lines:
1. Click Preferences.
2. Click the Grid line color rectangle.
   The color palette opens.
3 Click on a color.
4 Click OK.
5 Click the **Show Grid** box.
6 Click OK. The new color grid lines display in the heat map.

7 *Optional*: Click **Show Grid** again to toggle the grid on and off.

**To change the color of the borders of selected cells:**
1 Click the **Preferences** button.
2 Click the **Selection frame color** rectangle.
   The color palette opens.
3 Click a color.
4 Click OK. Frames of selected cells now show the new color in the heat map.
**Viewing Chip Run Data in the Data Analysis Software**

**Show Cell Text**

To show the details of a cell in text:

1. Click **Preferences**.
2. Click **Show Cell Text**. Heat map cells are enlarged and text is now visible.

3. **Optional**: Click **Show Cell Text** again to toggle the text view on and off.

**Zoom**

Increase or decrease the image view size by multi-clicking the magnifying glass icons (+ and -).
Layout View

Toggle between inlet-based, chip-based, and custom views in the heat map.

Inlet-Based View

The inlet-based view shows the cell in the same numbered sequence as the inlets on the chip, as shown below.

Chip-Based View

The chip-based view shows a sequence of numbers assigned to chambers on a chip counting from top-left corner to the right and then, top to bottom.
Custom View

The Custom View selection adds two buttons to the tool bar, one to modify the column layout and one to modify the row format. Both function in the same manner (see detailed description below). Any modifications are rendered immediately in the heat map. The changes will be saved with the chip run.

From within the B dialog, customize the heat map layout by changing the way rows and columns are displayed. Use the graphic below as a guide.
To change row order:
1. Click the row order button. The Row Order [Sample] dialog opens. Group, reorder, and hide rows and columns as needed.

- Sort names in ascending order
- Sort names in descending order
- Sort indices in descending order
- Sort numbers in ascending order

When enabled, will hide or show all rows and columns that share the same name as the selection.

Click and drag the cursor to select a group
Using the Graph View

The graph view displays the curve data and information about the current selection of chambers. The secondary tool bar allows you to change the display of the data.

Graph Viewer Tool Bar

Expand/collapse pane

Change threshold by clicking and dragging threshold line

Clear or Clear All manual changes

Full Range, Auto Range or Manual Range
Toggling the Threshold

Click the **Threshold** button to apply a $C_t$ threshold line to the amplification graph.

Using the Graph Edit Button

**IMPORTANT:** The **Edit** button is enabled only when the **CT Threshold Method** is either **Auto (Detectors)** or **User (Detectors)**.
Toggle Edit

Click **Toggle Edit** in conjunction with **Toggle Threshold** enable moving the threshold bar to a new position by clicking and dragging it in the lower graph. This can only be done in **User Data Global** or **User Data Detector** threshold analysis methods.

Threshold number changes as you re-position the threshold line.

C\textsubscript{t} Threshold Method can be only **User Data (Global)** or **User (Data Detectors)** for **Toggle Edit** to be enabled.

Toggle Log Graph

The log graph shows more detail of the same view of the amplification curve. Note the finer scale on the log graph y axis below left.
Changing Pass/Flag

If the heat map reveals a problematic experiment, you can manually change the call to exclude the experiment. Change cells to pass or flag as appropriate. After reviewing the data, you can manually change the call to Pass or Flag. In the example below, the passing cell is manually set to Flag.

1. Click a cell to activate it.
2. Click the Pass or the Flag icon. Or, click Edit > Pass or Edit > Flag.

NOTE: You can substitute the label Fail for Flag. See “Tools” on page 30.
If you used two probes, make sure the appropriate graph tab is active. In the example below, FAM-MGB and VIC-MGB probes each have a tab on the graph view. Click the appropriate graph tab before changing the call.

NOTE: You can use the Call Redo or Undo buttons to revert back to the original call state.

**Using the AnimateFeat**

In the Graph Views, watch an animation of each cell on the entire chip in sequence. Use this feat while in the Results Table, Image View, and/or Heat Map.

1. Click a cell or row.
2. Click Play.
   
   Watch the Normalized Intensity and Amplification graphs as each cell is displayed in sequence.
3 **Optional:** Adjust the animation speed.
   a. Click ![3fps](image).
   b. Choose a viewing speed.

4 Click **Stop** to stop the animation.
5 Click **Play** to continue the animation.
Selecting a Single Cell

In the Results Table—click a cell to activate its data in the graphs and on the Information bar.

In the Image View—when the cursor becomes crosshairs, click the cell to activate the data in the graphs and on the Information bar.

In the Heat Map—click a cell to activate its data in the graphs and on the Information bar.

Selecting More Than One Cell

Isolate data for a single cell or for multiple cells in any details view (Results Table, Image View, or Heat Map) using the following methods.
## Using the Graph View

<table>
<thead>
<tr>
<th>In the Details Views Window</th>
<th>Procedure</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Results Table</td>
<td>Press and hold the keyboard <strong>SHIFT</strong> key and click the 2 outer cells for a continuous range of cells. The data for the range of cells display in the Graph Views. Or, Press and hold the keyboard <strong>CTRL</strong> key while clicking individual cells.</td>
<td><img src="image1.png" alt="Image 1" /></td>
</tr>
<tr>
<td>Image View</td>
<td>Press and hold the keyboard <strong>CTRL</strong> key while clicking individual cells of interest. You cannot select a contiguous range in this view.</td>
<td><img src="image2.png" alt="Image 2" /></td>
</tr>
<tr>
<td>Heat Map</td>
<td>Click a cell and then hold and drag to highlight a range of cells. Or, Press and hold the keyboard <strong>CTRL</strong> key while clicking individual cells. Or, Click on the Column or Row heading and select all chambers in the column or row.</td>
<td><img src="image3.png" alt="Image 3" /></td>
</tr>
</tbody>
</table>
Cross Highlighting and Selecting

When you select multiple chambers, they are displayed as a line in the lower graphs.

To Cross Highlight: hover the mouse over a graph line and its chamber will be highlighted in the other graphs.
Double-clicking on the graph line selects it in the primary view and the graph view.

Using Show Dual Views

The Show Dual Views button allows you to view two primary views side-by-side, such as a Results Table View and a Heat Map View.

1. From the Details Views, click the Show Dual Views button.
2 Select which type of primary view you’d like the software to display.

3 Click Hide Dual Views to go back to only one primary view, if desired.

Export IFC Run Data

You can export IFC run data from the Heat Map and the Results Table views in a comma-separated value format (.csv) that can be viewed in programs such as Microsoft Excel.

NOTE: The raw data output is used by advanced users to develop alternative analysis methods. This export option will report the fluorescence intensity values for each reaction chamber for each dye and for each PCR cycle (for real-time protocols). It includes Amplification and MCA curve data (if the IFC run contains a melt curve protocol).

NOTE: The comments, user calls, and Reagent Reference Count columns are not exported by default. Go to Tools > Options to enable exporting these columns.

1 Click File > Export.

2 Enter a name for the file.
3. Select the type of view. You can export data from:
   - Table results
   - Heat map view
   - Table results, with raw data

4. Navigate to the desired folder.
   **NOTE:** By default, the file is saved in the same folder as the IFC run (.bml) file.

5. Click Save.

6. To open the saved data, navigate to the .csv file.

7. Double-click the .csv file to open it in a program such as Microsoft Excel.

Below is an example of exported data (Table Results) in a .csv file, opened in an Microsoft Excel spreadsheet.

Double-click between columns to expand them.
Opening Exported Data (.csv files)

NOTE: The data looks different in the .csv file depending on the view from which you exported.

1. Double-click the saved .csv file of interest.

Name the file.csv

The exported .csv file below was saved from the Results Table view.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample Name</td>
<td>Name</td>
<td>Type</td>
<td>Value</td>
<td>Quality</td>
<td>Call</td>
<td>Thres.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>123</td>
<td>chip001</td>
<td>Test</td>
<td>0.5</td>
<td>Pass</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>chip002</td>
<td>Test</td>
<td>0.00001</td>
<td>Fail</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>
The exported .csv file below was saved from the **Heat Map** view.
Calculating Delta $C_t$ Sample Values

To calculate the delta ($\Delta$) $C_t$ samples:

1. Click Sample Setup.

2. Select 1 to 3 cells where the reference sample was added.

3. Click Editor.

4. Click Reference.

5. Enter a sample name.

6. Click Update.

The Sample Setup reflects the change.
Calculating Delta Ct Sample Values

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7 Select all cells that you want to reference. Typically, you select all the cells (except for the three reference cells) as in the example below.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Type Name:</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
</tr>
<tr>
<td>B</td>
<td>Type Name:</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
</tr>
<tr>
<td>C</td>
<td>Type Name:</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
</tr>
<tr>
<td>D</td>
<td>Type Name:</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
</tr>
<tr>
<td>E</td>
<td>Type Name:</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
</tr>
<tr>
<td>F</td>
<td>Type Name:</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
</tr>
<tr>
<td>G</td>
<td>Type Name:</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
</tr>
<tr>
<td>H</td>
<td>Type Name:</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
<td>Reference: 00001</td>
</tr>
</tbody>
</table>

8 Click Editor.
9 Enter a sample name.
10 Select Unknown.
11 Select the reference you created.
12 Click Update.
The changes are recorded as shown in the example below.

13 Click the mapping icon.

The Open Sample Mapping File dialog opens.

14 Double-click left or right mapping.

15 Click Details Views.
16 Click Analyze.

\( \Delta C_t \) sample values are now available in the Results Table view.

### Calculating Delta \( C_t \) Detector Values

To calculate \( \Delta C_t \) detector values, follow the procedure described in the previous section, “Calculating Delta Ct Sample Values” on page 114.

Steps that are specific to the procedure for calculating \( \Delta C_t \) detector values are described below.

- Click Detector Setup.

- Select 1 or more cells to use as a reference and select Reference in the Editor.
• Select the cells you want to reference and select Test in the Editor. You can annotate the selected cell with one reference or all of the references (if there are more than one reference detector).

The Reagent Reference Count group in the Results Table shows the number of reference detectors used to calculate the $\Delta C_t$ values.

The $\Delta C_t$ values are calculated using the average of each reference detector used for a chamber. If there are replicates for each reference detector, the delta $C_t$ is calculated as the average of the averages of each reference detector. For each reference detector selected for a test detector, the Details Views Results Table shows the following information:

<table>
<thead>
<tr>
<th>Results Table Column</th>
<th>Shows for each reference detector…</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>The total number of chambers (replicates) that correspond to each reference detector annotated in the detector setup plate.</td>
</tr>
<tr>
<td>Passed</td>
<td>Out of the “Total” number of chambers, how many had a $C_t$ value that passed the quality threshold (default setting is 0.65) or were manually passed.</td>
</tr>
<tr>
<td>Used</td>
<td>How many valid values were used for calculating $\Delta C_t$. Note: If at least one replicate passes the quality threshold, the number of “Used” chambers equals the number of “Pass” chambers. Otherwise, the “Used” count equals the number of chambers containing a valid $C_t$ value (less than 999.0).</td>
</tr>
</tbody>
</table>

If you are using multiple reference detectors for a single chamber, the number of replicates for each detector are shown in the Total, Passed, and Used columns separated by commas.

The Delta $C_t$ quality score of a chamber is calculated as the minimum of the $C_t$ quality of the chamber and the quality score of the $C_t$ of the reference detectors, which is the average of the average of the “Used”.
For a chamber that is manually called, the Ct values used for calculating the quality score are 1 for Pass and 0 for Flag.

Delta-Delta C_t Values

The ΔΔC_t values are available to you after the sample and the detector ΔC_t values are calculated (see “Calculating Delta Ct Detector Values” on page 118 and, “Calculating Delta Ct Sample Values” on page 114). When sample and detector ΔC_t values have been calculated, click Details Views to see ΔΔC_t data:

Viewing Delta C_t Data in the Heat Map

In addition to viewing ΔC_t and ΔΔC_t data in the Results Table, view ΔC_t data in the Heat Map.

1. Click Details Views.
2. Click Analyze, if necessary.
3. Click Heat Map View from the Results Table menu.
4 Select FAM-MGB Delta C_t Sample from the menu.

Reference cells:

FAM-MGB Delta-Delta C_t Heat Map Data with Inlet-Based View

Reference cells:

FAM-MGB Delta-Delta C_t Heat Map Data with a Chip-Based View

Reference cells:

Congratulations, you have successfully viewed your analyzed chip run data.
Introduction

The Calibration Curve View Module (CCVM) (also known as “standard curve”) is a view that allows the user to create calibration curves based on the $C_t$ and known concentration differences of samples on the chip. After calibration curves are created, they are used to determine the approximate concentration of unknown samples on the chip. The approximate values are displayed in a table format.

For the CCVM to appear:

1. Open an unanalyzed chip run. See “Opening an Existing Chip Run” on page 33 for more information.
2. Select Details Views.
3. Click the Analyze button.
4. Select Sample Setup.
5. Click New to set up a new sample plate. Choose SBS plate or Sample Inlet for your container type. (For more detail, see “Setting Up a Sample Plate” on page 38.)
6. Select a Mapping option (left- or right-side maps).

7. Use the Editor to annotate your sample cells. Make sure at least two wells are Standard type (essential for CCVM), are named, and have concentration values.
8. Set up Detector plate. For more detailed information see “Setting up a Detector (Assay) Plate” on page 49.
9. Select Details Views.
10 Click the **Analyze** button.
   The Details Views item now has a plus sign in front of it.

11 Expand the plus sign (+) next to **Details Views** and the **Calibration View** option appears on the tree.

12 Select **Calibration View** to launch the CCVM page.

**CCVM Page Example**

Below is the CCVM page, which consists of five individual panes and two tool bars.
Using CCVM to Determine Concentration Levels of Unknown Samples

The objective of using CCVM, is to set up a chip run with at least two standard type wells (where the concentration of DNA is known) and to predict the concentration of the unknown type samples. Four to six standard type samples are recommended. The calls of experiments that include standard samples are then plotted on the graph pane.

CCVM also allows you to modify calls associated with calibrators. This action is the same as modifying calls in the other views, but also has effect of adding or removing datapoints from the regression line (calibration curve) calculation.

When first launched, CCVM displays the contents and calibration curves of the data. These curves are created with a default fitting method (weighted linear).

1 In the Primary View Tool Bar, select a probe type, such as FAM MGB.

2 On the CCVM page, click on a detector in the Detector Table pane.
   The Detector Table displays the attributes of a detector in three columns: Name of detector, Style of fitting method and Count of Calibrators for this row.
   If detectors are named the same name, they are listed on one row in the table and the total of all calibrators are listed in the third column.

3 Adjacent to the Detector Table is a list of the calibrators applied to that detector. In the graph area below, valid detectors are plotted.
   The Calibrator Table displays six attributes of a calibrator: Name, Chamber ID, C_t value, Concentration, Error, and Call.
Select a detector and its corresponding calibrators are listed on the right pane. The selected experiments are plotted on the graph area below.

4 Select a row in the Calibrator Table and the corresponding data point in the calibration curve becomes larger. Conversely, you can lasso or click on a data point in the chart and the corresponding row in the Calibration Table is highlighted.

NOTE: You can lasso a point by pressing the left mouse button and dragging the mouse around the data point(s) to create a circle.

Only calibrators with valid $C_t$ values are plotted in the calibration curve. Invalid $C_t$ values are listed as 999. Calibrators that are auto or manual passed are plotted as blue dots. They are considered valid calibrators. Calibrators that are manually flagged or passed are plotted as red dots and are considered invalid calibrators. CCVM only uses blue data points to create calibration curves. If there are no valid calibrators, no calibration curve is drawn.
5 You can modify the calls by manually changing the calibrators’ calls to Pass or Flag via the secondary view tool bar.

- Select a calibrator you wish to change (you can select the row in the Calibrator Table or lasso a data point on the chart).
b Click either Pass or Flag on the secondary tool bar. Flag turns the points red. Pass turns the points blue. The corresponding calibrator in the Calibration Table changes its call accordingly and the Call column is updated.

**Manual Flag selected**

**Corresponding plotted points changes to red**

**NOTE:** You can use the Call Redo or Undo buttons to revert back to the original call state.

c Go to the Details Views page.

d Click the Analyze button to re-analyze the chip with these new parameters.
The resulting calibration curve is slightly modified.

The analysis software can now use the calibration curve data to predict the approximate concentration of unknown sample types.

6 Go to the Details Views page.
7 Select the Results Table.
8 The approximate values are listed in the “Calibrated rConc” (Calibrated relative concentration) column.
Viewing Multiple Calibration Curves

You can also view multiple calibration curves at once on the CCVM page. To select multiple rows of assay in the Detector Table, Ctrl + left mouse click the rows of interest. Individual curves are rendered in the Graph Area.

Note, however, that the Calibrator Table behaves differently when multiple rows are selected in the Detector Table. The Calibrator Table is masked gray and the user cannot perform any actions on it. This is because the Calibrator Table was designed to only show calibrators for one detector at a time.
Introduction to qPCR + MCA Chip Runs

When a DNA binding dye is used for detecting PCR products, the products of the reaction can be analyzed by following the run with Melt Curve Analysis (MCA). This chapter provides a brief overview for when you analyze a chip run that has been run with a qPCR+MCA protocol.

Running a Chip with a qPCR + MCA Protocol

When you run a chip with a qPCR and MCA protocol, the Tm peak detected inside the Tm detection range validates the amplification curve of the PCR cycle. If no Tm peak is detected, then any amplification that may exist is not considered valid and the quality of the Ct is set to zero (0), making the chamber Fail.

You analyze data by clicking the Analyze button.
The T_m Ranges

The T_m peak detection range is set for each detector. By default, each detector range is the temperature range of the protocol.

To identify the temperature range in which you expect to see a T_m peak, select a region of the temperature range. A T_m peak in range validates the C_t curve and a T_m peak outside of range invalidates it, failing the chamber.

The MCA tab allows you to change these parameters:

- **Peak Sensitivity** - adjust how sensitive the algorithm is for detecting a peak, with 1 being the least sensitive and 10 being the most sensitive.

- **Peak Ratio Threshold** - determine if a peak outside of range should cause the chamber to fail when multiple peaks are detected (one in range and one out of range).

- **T_m Ranges** - adjust the detection range for each detector.

### Viewing the T_m Ranges

Toggle the Threshold button to display the T_m detection range (light blue).

### Editing the T_m Ranges

Toggle the Threshold / Edit button to display the MCA tab and directly change the T_m ranges for each detector.
You can also click and drag the T_m ranges.

### Working with Views

There are three views:
- Results Table
- Heat Map
- Graph

### Results Table View

A T_m section appears for each probe type. The first column is the value for the T_m peak detected in range. The second column is the value for the out of range T_m peak. A value of 999 means no peak was detected.

<table>
<thead>
<tr>
<th>T_m</th>
<th>In Range</th>
<th>Out Range</th>
<th>Peak Ratio</th>
<th>Peak Call</th>
</tr>
</thead>
<tbody>
<tr>
<td>71.39</td>
<td>999.00</td>
<td>1.00</td>
<td></td>
<td>✔️</td>
</tr>
<tr>
<td>74.10</td>
<td>999.00</td>
<td>1.00</td>
<td></td>
<td>✔️</td>
</tr>
<tr>
<td>74.73</td>
<td>999.00*</td>
<td>1.00</td>
<td></td>
<td>✔️</td>
</tr>
<tr>
<td>82.00</td>
<td>999.00</td>
<td>1.00</td>
<td></td>
<td>✔️</td>
</tr>
<tr>
<td>82.20</td>
<td>999.00</td>
<td>1.00</td>
<td></td>
<td>✔️</td>
</tr>
</tbody>
</table>

### Heat Map View

For MCA runs, there are two additional data views: Inside T_m and Outside T_m. Also, the spectrum is adjusted for T_m values.

### Graph View

In this view, there are two layouts: Combined and Tabbed.
the Combined view shows the visible Amplification ($C_t$) and Melting ($T_m$) graphs.

The Tabbed view has two tabs of graphs for each probe type defined in the chip run: qPCR and MCA.

The qPCR tab shows Normalized Intensity (PCR) and Amplification.

The MCA tab shows graphs for Normalized Intensity (MCA) and Melting.

The Melting Graph

The Melting graph displays the $T_m$ curve for the selected chambers. A green vertical line represents a $T_m$ peak inside the $T_m$ detection range.
A black vertical line represents a $T_m$ peak detected outside the $T_m$ detection range.

Exporting Data

CSV Table data -- includes the $T_m$ columns from the Table View.
CSV Heat Map data -- includes visible data in the heat map ($C_t$, Inside $T_m$, Outside $T_m$.)
cDNA Preparation with Fluidigm® Reverse Transcription Master Mix

Overview

The Fluidigm Reverse Transcription Master Mix is a 5x master mix containing all components required for cDNA synthesis, including buffer, dNTPs, primers, a ribonuclease inhibitor and an engineered RNaseH+ MMLV reverse transcriptase. A mixture of random primers and oligo dT is used for priming. cDNA prepared using the Reverse Transcription Master Mix is suitable for preamplification using the Fluidigm PreAmp Master Mix (100-5580, 100-5581).

The range of total RNA that can be used in a 5-µL reverse transcription reaction is 2.5 pg to 250 ng. However, the success with a given sample in qPCR will depend on the level of gene expression for the genes of interest, the percentage of mRNA in the total RNA, and the number of cycles of preamplification performed prior to qPCR. In general, for total RNA input in the range of 2 ng to 250 ng, 10-14 cycles of preamplification should be sufficient. For a total RNA input less than 2 ng, increasing the number of preamplification cycles to 18-20 may improve performance.

Preparation of RT Reactions

1. Thaw all reagents on ice. Briefly vortex and centrifuge the reagents before using.

2. On ice, prepare a Pre-Mix of the Reverse Transcription Master Mix and water as indicated in the table below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume / Reaction (µL)</th>
<th>Volume for 48 Reactions + 10% Overage (µL)</th>
<th>Volume for 96 Reactions + 10% Overage (µL)</th>
<th>Volume for 192 Reactions + 10% Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>1.0</td>
<td>52.8</td>
<td>105.6</td>
<td>211.2</td>
</tr>
<tr>
<td>Master Mix</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase-free Water</td>
<td>3.0</td>
<td>158.4</td>
<td>316.8</td>
<td>633.6</td>
</tr>
<tr>
<td>RNA (2.5 pg/µL - 250 ng/µL)</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>5.0</td>
<td>211.2</td>
<td>422.4</td>
<td>844.8</td>
</tr>
</tbody>
</table>

3. In a PCR plate (on ice) aliquot 4 µL of Pre-Mix for each sample.

4. Add 1 µL of RNA to each well containing Pre-Mix, making a total volume of 5 µL.

5. Properly seal and gently vortex to mix the reverse transcription reactions.

6. Centrifuge the reactions and place in a standard thermal cycler.
Thermal Cycling

1. In a thermal cycler, incubate using the following protocol:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reverse Transcription</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>25 ºC</td>
<td>85 ºC</td>
</tr>
<tr>
<td>Time</td>
<td>5 min</td>
<td>30 min</td>
</tr>
</tbody>
</table>

**NOTE:** After the reverse transcription reaction is complete, the reactions can either be stored at -20 ºC or used immediately for preamplification reactions with the Fluidigm PreAmp MasterMix. cDNA prepared with the Fluidigm Reverse Transcription Master Mix can be used in preamplification with either TaqMan Gene Expression Assays or Delta Gene Assays. Up to 1.25 µL of the reaction can be used in a 5-µL Preamplification reaction.
Introduction

The use of DNA binding dyes for gene expression analysis is a lower cost alternative to the use of labeled probes. The method is sensitive and when coupled with melt curve analysis the specificity of the primers can be confirmed. For this protocol we are recommending the use of EvaGreen® dye, which has several advantages over SYBR® Green I (1, 2). This document provides a fast cycling protocol that can be used on either the BioMark™ HD with fast ramp rates (5.5°C/s) or the BioMark with the normal ramp rate (2°C/s). This protocol can be used with the 48.48 Dynamic Array™ integrated fluidic circuit (IFC), 96.96 Dynamic Array™ IFC, and the 192.24 Gene Expression IFC.

NOTE: This appendix only specifies reagent prep for the 48.48 Dynamic Array™ IFC and the 96.96 Dynamic Array™ IFC. When using a 192.24 Gene Expression IFC, refer to the Fluidigm 192.24 Delta Gene™ Fast/Standard Gene Expression Workflow (PN 100-7222).

The use of the fast ramp rate on the BioMark HD System requires the use of a PCR master mix that has been optimized for fast cycling. The fast master mix recommended for use in this protocol includes both EvaGreen® and ROX in the master mix, which makes it convenient to use. This master mix also works well on the BioMark System with the normal ramp of 2°C/s. The total cycling time on the BioMark System will be longer than the cycling time on the BioMark HD System, but still faster than standard protocols.

Primers need to be designed to reduce the potential for primer dimer formation and to be highly specific for the target of interest. For the development of this protocol, we used Delta Gene™ Assays, a set of assays designed by the Assay Design Group at Fluidigm which avoid SNPs and are highly specific for the gene of interest.

We recommend preamplification to increase the number of copies of target DNA. Prior to qPCR reactions the preamplification reaction is treated with Exonuclease I to eliminate the carryover of unincorporated primers.

References

1 SsoFast™ EvaGreen® Supermix With Low ROX product literature (http://www.bio-rad.com)
Required Reagents

- Exonuclease I (New England BioLabs, PN M0293L)
- SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, PN 172-5211)
- PreAmp Master Mix (Fluidigm, PN 100-5580, 100-5581)
- 20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738)
- 2X Assay Loading Reagent (Fluidigm, PN 85000736)
- 100 µM each Forward and Reverse Primer Stock Mixture for each assay of interest
- TE Buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA) (TEKnova, PN T0221)
- DNA Suspension Buffer (TEKnova, PN T0021)
- PCR Certified Water (TEKnova, PN W3330)

Required Equipment

- BioMark or BioMark HD System
- IFC Controller MX (for the 48.48 Dynamic Array IFC) or HX (for the 96.96 Dynamic Array IFC)
- Standard 96-well Thermal Cycler

Software Requirements

Fluidigm® Real-Time PCR Analysis Software v.3.0.2 or higher and BioMark HD Data Collection Software v.3.1.2 or higher is required for this protocol.

Gene Expression PreAmp with Fluidigm® PreAmp Master Mix and Delta Gene™ Assays

In the BioMark HD System, samples are loaded into individual inlets and then distributed across multiple reaction chambers in nanoliter volume aliquots. With these small volumes, detecting the specific targets requires a minimum of 800 copies/µL in the final sample mix. For genes with lower expression levels, there are too few copies to detect adequately in cDNA samples. Preamplification is used to increase the number of copies to a detectable level for a greater number of genes.

Preamplification allows for multiplex amplification of up to 96 targets. A pool of primers is prepared from the same gene expression assays to be used for qPCR. By using the real-time qPCR assays in the preamplification reaction, only the targets of interest are amplified. A limited number of cycles is used, generally 10-14. Under these conditions of low primer concentration and a limited number of cycles, the cDNA is amplified without significant bias for the majority of genes.
Pooling the Delta Gene Assays

1. In a microcentrifuge tube, combine 1 µL of each 100 µM stock Delta Gene Assay, up to a total of 96 assays.

2. Add DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA; TEKnova, PN T0221) to make the final volume 200 µL. The concentration of each assay will be 500 nM.

**NOTE:** Volume can be adjusted proportionally based on the number of samples to be amplified.

Preparing Sample Pre-Mix and Samples

1. In a DNA-free hood, prepare a Pre-Mix for the reactions as indicated in the table below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume / Reaction (µL)</th>
<th>Volume for 48 Reactions + 10% Overage (µL)</th>
<th>Volume for 96 Reactions + 10% Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PreAmp Master Mix</td>
<td>1.00</td>
<td>52.8</td>
<td>105.6</td>
</tr>
<tr>
<td>Pooled Delta Gene Assay Mix (500 nM)</td>
<td>0.50</td>
<td>26.4</td>
<td>52.8</td>
</tr>
<tr>
<td>Water</td>
<td>2.25</td>
<td>118.8</td>
<td>237.6</td>
</tr>
<tr>
<td>cDNA</td>
<td>1.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>5.00</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. In a PCR plate, aliquot 3.75 µL of Pre-Mix for each sample.

3. Remove the plate from the DNA-free hood and add 1.25 µL of cDNA to each well containing Pre-Mix, making a total volume of 5 µL.

4. Mix the reactions by briefly vortexing, then centrifuge.

Thermal Cycling

1. Place the plate in the thermal cycler and cycle using the following table as a guide:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hold</th>
<th>Cycle (10 cycles)</th>
<th>Annealing/Extension</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong></td>
<td></td>
<td><strong>Denaturation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>95 ºC</td>
<td>2 min</td>
<td>15 s</td>
<td>4 min</td>
<td>4 ºC</td>
</tr>
</tbody>
</table>

**NOTE:** Ten (10) cycles are recommended as a starting point, but this number can be increased up to 20 cycles, if necessary. The appropriate number of cycles should be determined empirically.
Exonuclease I Treatment to Remove Unincorporated Primers

1. Dilute the Exonuclease I to 4U/µL as shown below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 5 µL Sample (µL)</th>
<th>48 Samples with Overage (µL)</th>
<th>96 Samples with Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.4</td>
<td>84.0</td>
<td>168.0</td>
</tr>
<tr>
<td>Exonuclease I Reaction Buffer*</td>
<td>0.2</td>
<td>12.0</td>
<td>24.0</td>
</tr>
<tr>
<td>Exonuclease I at 20 Units/µL</td>
<td>0.4</td>
<td>24.0</td>
<td>48.0</td>
</tr>
<tr>
<td>Total Volume</td>
<td>2.0</td>
<td>120.0</td>
<td>240.0</td>
</tr>
</tbody>
</table>

*Exonuclease I and buffer from New England BioLabs, PN M0293S or PN M0293L

2. Add 2 µL of diluted Exonuclease I at 4 U/µL to each preamplification reaction, vortex, centrifuge, place in a thermal cycler, and incubate with the following program:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Digest</th>
<th>Inactivate</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37 ºC</td>
<td>80 ºC</td>
<td>4 ºC</td>
</tr>
<tr>
<td>Time</td>
<td>30 min</td>
<td>15 min</td>
<td>∞</td>
</tr>
</tbody>
</table>

3. Dilute the final products in TE Buffer (10 mM Tris-Cl, 1.0 mM EDTA, TEKnova, PN T0221) as shown in the table below:

<table>
<thead>
<tr>
<th>Volume of Preamplification Reaction + Exonuclease I dilution</th>
<th>5-fold dilution</th>
<th>10-fold dilution</th>
<th>20-fold dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 µL</td>
<td>18 µL</td>
<td>43 µL</td>
<td>93 µL</td>
</tr>
</tbody>
</table>

**NOTE:** Diluted reaction products can either be assayed immediately or stored at -20ºC for later use. Diluted reaction products should be stable for at least one week.
Preparing Sample Pre-Mix and Samples

The following protocol only specifies reagent prep for the 48.48 Dynamic Array™ IFC and the 96.96 Dynamic Array™ IFC. When using a 192.24 Gene Expression IFC, refer to the Fluidigm 192.24 Delta Gene™ Fast/Standard Gene Expression Workflow (PN 100-7222).

1 Prepare the Sample Pre-Mix as shown below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Volume for 48.48 Dynamic Array IFC (µL)</th>
<th>Volume for 96.96 Dynamic Array IFC (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X SsoFast EvaGreen Supermix with Low ROX (Bio-Rad, PN 172-5211)</td>
<td>2.5</td>
<td>3.0</td>
<td>180.0</td>
<td>360.0</td>
</tr>
<tr>
<td>20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738)</td>
<td>0.25</td>
<td>0.3</td>
<td>18.0</td>
<td>36.0</td>
</tr>
<tr>
<td>PreAmp and Exo I-treated sample</td>
<td>2.25</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.0</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Sample Pre-Mix solution

2 Aliquot 3.3 µL of Pre-Mix for each sample and add 2.7 µL of PreAmp and Exo I-treated sample.

3 Vortex the Sample Mix solution for a minimum of 20 seconds, and centrifuge for at least 30 seconds. Prepared reactions can be stored for short times at 4°C until the samples are ready to be loaded into the chip.

IMPORTANT: Use caution when pipetting the 20X DNA Binding Dye Sample Loading Reagent as bubbles can be introduced.
Preparation of the Assay Mix

The following protocol only specifies reagent prep for the 48.48 Dynamic Array™ IFC and the 96.96 Dynamic Array™ IFC. When using a 192.24 Gene Expression IFC, refer to the Fluidigm 192.24 Delta Gene™ Fast/Standard Gene Expression Workflow (PN 100-7222).

1. Dilute the 100 µM stocks of combined Forward and Reverse Primers for each assay to a final concentration of 5 µM as shown:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Volume for 50 µL Stock (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Assay Loading Reagent</td>
<td>2.5</td>
<td>3.0</td>
<td>25</td>
</tr>
<tr>
<td>1X DNA Suspension Buffer</td>
<td>2.25</td>
<td>2.7</td>
<td>22.5</td>
</tr>
<tr>
<td>100 µM each mixed Forward and Reverse Primers</td>
<td>0.25</td>
<td>0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Total Volume</td>
<td>5</td>
<td>6</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2 Assay Mix solution

2. Vortex the Assay Mix for a minimum of 20 seconds and centrifuge for at least 30 seconds to spin down all components.

IMPORTANT: Vortex thoroughly and centrifuge all sample and assay solutions before pipetting into the chip inlets. Failure to do so may result in a decrease in data quality.

NOTE: The final concentration of each primer is 5 µM in the inlet and 500 nM in the final reaction.

Priming and Loading the Dynamic Array IFC

The following protocol only specifies priming and loading for the 48.48 Dynamic Array™ IFC and the 96.96 Dynamic Array™ IFC. When using a 192.24 Gene Expression IFC, refer to the Fluidigm 192.24 Delta Gene™ Fast/Standard Gene Expression Workflow (PN 100-7222).

IMPORTANT: Due to different accumulator volumes, use the appropriate control syringe for your chip type: 300 µL for the 48.48 Dynamic Array IFC or 150 µL for the 96.96 Dynamic Array IFC.

1. Inject control line fluid into each accumulator on the chip.

2. Remove and discard the blue protective film from the bottom of the chip.

3. Place the chip into the IFC controller MX for the 48.48 Dynamic Array IFC or the IFC Controller HX for the 96.96 Dynamic Array IFC, then run the Prime (113x) script for the 48.48 Dynamic Array IFC or the Prime (136x) script for the 96.96 Dynamic Array IFC.
4 When the script has finished, press **Eject** to remove the primed chip from the IFC Controller.

**IMPORTANT:** While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into the inlets.

5 Pipette 5 µL of each assay and 5 µL of each sample into their respective inlets on the chip.

6 Return the chip to the IFC Controller.

7 Using the IFC controller software, run the **Load Mix (113x)** script for the 48.48 Dynamic Array IFC or **Load Mix (136x)** script for the 96.96 Dynamic Array IFC to load the samples and assays into the chip.

8 When the Load Mix script has finished, remove the loaded chip from the IFC Controller.

You are now ready for your chip run.

---

**Figure 3** 48.48 Dynamic Array IFC sample and assay inlets
Figure 4 96.96 Dynamic Array IFC sample and assay inlets

Using the Data Collection Software

1. Double-click the Data Collection Software icon on the desktop to launch the software.
2. Click Start a New Run.
3. Check the status bar to verify that the camera has a green light to indicate that it is ready.
4. Remove the blue tape from the back of the chip if this was not done previously. Place the chip into the reader.
5. Click Load.
6. Verify chip barcode and chip type.
7. Choose project settings (if applicable).
8. Click Next.
9. Chip Run file:
   a. Select New.
   b. Browse to a file location for data storage.
   c. Click Next.
10. Application, Reference, Probes:
    a. Select Application Type—Gene Expression.
    b. Select Passive Reference—ROX.
    c. Select Probe—Single probe.
    d. Select Probe type—EvaGreen.
Click **Next**.

11 Click **Browse** to find the thermal cycling protocol files:

For BioMark HD:
- GE Fast 48x48 PCR+Melt v2.pcl
- GE Fast 96x96 PCR+Melt v2.pcl

For BioMark:
- GE 48x48 PCR+Melt v2.pcl
- GE 96x96 PCR+Melt v2.pcl

12 Confirm **Auto Exposure** is selected.

13 Click **Next**.

14 Verify the chip run information.

15 Click **Start Run**.

The cycling parameters are given for the two different chip types:

<table>
<thead>
<tr>
<th>Segment</th>
<th>Type</th>
<th>Temperature (°C)</th>
<th>Duration (seconds)</th>
<th>BioMark HD Ramp Rate (°C/s)</th>
<th>BioMark Ramp Rate (°C/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48x48 chip</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1</strong></td>
<td>Hot Start</td>
<td>95</td>
<td>60</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>PCR (30 Cycles)</strong></td>
<td>96</td>
<td>5</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>Melting Curve</strong></td>
<td>60</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60-95</td>
<td></td>
<td>1°C/3 s</td>
<td>1°C/3 s</td>
</tr>
<tr>
<td>96x96 chip</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1</strong></td>
<td>Thermal Mix</td>
<td>70</td>
<td>2400</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>Hot Start</strong></td>
<td>95</td>
<td>60</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td><strong>2</strong></td>
<td>PCR (30 Cycles)</td>
<td>96</td>
<td>5</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>Melting Curve</strong></td>
<td>60</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60-95</td>
<td></td>
<td>1°C/3 s</td>
<td>1°C/3 s</td>
</tr>
</tbody>
</table>

Table 5 Thermal cycle parameters

### Using the Real-Time PCR Analysis Parameters

1 Double-click the Real-Time PCR Analysis software icon on the desktop to launch the software.
2 Click **Open Chip Run**.
3 Double-click a ChipRun.bml file to open it in the software.
4 Enter detector and sample information.
5 Select **Analysis Views**. We recommend using the **AutoGlobal** method to set the threshold.
6 We recommend using **Linear Derivative** as the baseline correction method. For more information about baseline correction methods, contact Fluidigm Technical Support.
7 Always compare the T<sub>m</sub> of the intended products to a positive control sample.
8 Click **Analyze**.

**NOTE:**

**NOTE:** For more information about melting curve analysis, see the Fluidigm Real-Time PCR Analysis Software v 3.0 User Guide (PN 68000088).
Two-Step Single-Cell Gene Expression Using EvaGreen® Supermix on the BioMark™ and BioMark™ HD Systems

Introduction

This protocol includes a separate reverse transcription step and a specific target amplification (STA) step, hence its “two-step” title. The following protocol enables the use of a DNA binding dye for quantitative PCR gene expression. DNA binding dyes offer flexibility at a very low upfront cost relative to probe-based assays, and can be used for a variety of applications. This specific protocol has been tested for gene expression targeting 1 and 10 cells on the BioMark™ and BioMark™ HD System and should serve only as a guideline for any customers interested in qPCR dye experiments. We recommend examining melting curve (T_m) and C_q for all assays alongside a positive control sample.

The protocol involves performing Specific Target Amplification (STA), which enriches samples for loci of interest. STA retains relative abundance between loci and permits quantitative C_q information to be derived. See Devonshire et al. BMC Genomics 2011, 12:118 for more information on STA (preamplification). Quantitative PCR is then performed in the presence of a DNA binding dye, known as EvaGreen® dye. Quantitative PCR thermal cycling protocols are immediately followed by acquisition of a melting curve (T_m) to allow assessment of reaction quality. See Mao et al., BMC Biotechnology 2007, 7:76, for further information on the physicochemical properties of EvaGreen dye.

This two-step protocol is optimized for both the BioMark™ and BioMark™ HD systems. It includes a fast master mix and a relatively short qPCR protocol. We have validated a supermix that has EvaGreen® and ROX already incorporated. The protocol also uses a 2-step VILO™ cDNA synthesis kit.

Required Reagents

- SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, PN 11754-250)
- SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, PN 172-5211)
- SUPERase-In™ RNase Inhibitor (Ambion, PN AM2696)
- 2X Assay Loading Reagent (Fluidigm, PN 85000736)
- 20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738)
- DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) (TEKnova, PN T0221)
- Exonuclease I (New England BioLabs, PN M0293S or M0293L)
- 0.5M EDTA, pH 8.0 (Invitrogen, PN Am9260G)
- T4 Gene 32 Protein (New England BioLabs, PN M0300S or M0300L)
- TaqMan® PreAmp Master Mix, (Applied Biosystems, PN 4391128)
Two-Step Single-Cell Gene Expression Using EvaGreen® Supermix on the BioMark™ and BioMark™ HD Systems

- NP-40 Detergent Surfact-Amps Solution (Fisher Scientific, PN PI-28324, or Thermo Scientific, PN 28324)
- Nuclease-free Water (Teknova, PN W3330)

**Required Equipment**

- BioMark™ or BioMark™ HD System
- IFC Controller MX (for the 48.48 Dynamic Array IFC), IFC Controller HX (for the 96.96 Dynamic Array IFC), IFC Controller RX and Juno
- Standard 96-well thermal cycler
- 96-well plates that are compatible with the FACS instrument (if FACS sorting) and thermal cycler
- Adhesive plate seals (Applied Biosystems, PN 4311971)

**Software Requirements**

Fluidigm Real-Time PCR Analysis Software v.3.1.3 or higher and Fluidigm Data Collection software v.3.1.2 or higher is recommended for this protocol. For earlier versions, contact Technical Support. Call 1-866-358-4354 (within U.S.) or 1-650-266-6100 (outside U.S.), or email techsupport@fluidigm.com.

**Preparing the Reverse Transcription (RT) Reaction Assembly**

1. For each well of a 96-well plate that will be used for sorting, prepare RT mix solution.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
<th>48 Samples with Overage (µL)</th>
<th>96 Samples with Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X VILO™ Reaction Mix</td>
<td>1.2</td>
<td>72.0</td>
<td>144.0</td>
</tr>
<tr>
<td>20U/µL SUPERase-In™</td>
<td>0.3</td>
<td>18.0</td>
<td>36.0</td>
</tr>
<tr>
<td>10% NP40</td>
<td>0.25</td>
<td>15.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>3.25</td>
<td>195.0</td>
<td>390.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5.0</strong></td>
<td><strong>300.0</strong></td>
<td><strong>600.0</strong></td>
</tr>
</tbody>
</table>

**Table 1 RT Mix Solution 1**

2. Pipette 5 µL of RT Mix Solution 1 into each well to be used of the 96-well PCR plate.

3. Sort individual cells or sort up to 10 cells directly into the same plate containing RT Mix Solution 1.

**NOTE:** Sort cells into the same 96-well plate that will be used for thermal cycling.
4 Seal the plate and vortex thoroughly for 15 seconds.
5 Pre-chill centrifuge to 4°C.
6 Centrifuge plate briefly at 4°C.
7 Immediately freeze the plate on dry ice.
8 Store plate at -80°C or thaw plate to use immediately.

Denaturation of RNA

1 When you are ready to perform RT cycling:
   a Thaw samples on ice.
   b Use a pre-chilled centrifuge maintained at 4°C to spin the plate briefly.
   c Preheat thermal cycler to 65°C.
   d Transfer the samples to the thermal cycler.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Denature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>65 °C</td>
</tr>
<tr>
<td>Time</td>
<td>90 seconds</td>
</tr>
</tbody>
</table>

2 Snap chill the plate on ice immediately for 5 minutes and centrifuge briefly at 4 °C.
3 Prepare enough RT Mix Solution 2 for all sorted wells, according to the table below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
<th>48 Samples with Overage (µL)</th>
<th>96 Samples with Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X SuperScript® Enzyme Mix</td>
<td>0.15</td>
<td>9.0</td>
<td>18.0</td>
</tr>
<tr>
<td>T4 Gene 32 Protein</td>
<td>0.12</td>
<td>7.2</td>
<td>14.4</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>0.73</td>
<td>43.8</td>
<td>87.6</td>
</tr>
<tr>
<td>Total</td>
<td>1.00</td>
<td>60.0</td>
<td>120.0</td>
</tr>
</tbody>
</table>

Table 2  RT Mix Solution 2

4 Aliquot 1 µL into each of the wells and centrifuge briefly at 4 °C.
RT Cycling

Follow the thermal cycling conditions below on a standard thermal cycler:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reverse Transcription</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>25 ºC</td>
<td>50 ºC</td>
</tr>
<tr>
<td>Time</td>
<td>5 minutes</td>
<td>30 minutes</td>
</tr>
</tbody>
</table>

1. Centrifuge and store the first strand cDNA samples at -20ºC or proceed directly to PCR.

Preparing 10X STA Primer Mix

1. We recommend using Delta Gene™ Assays from Fluidigm. These assays come as a Forward and Reverse primer mix with each primer at a concentration of 100 µM.

   **NOTE:** If you obtain primers from another source, combine the Forward primer and Reverse primer for each assay so that the concentration of each primer is 100 µM. Proceed to step 2.

2. In a DNA-free hood, combine equal volumes of each 100 µM primer pair.

3. Dilute using 1X DNA Suspension Buffer so that each primer is at a final concentration of 500 nM. This mix represents a 10X concentration of pooled STA Primer Mix.

4. Vortex for 20 seconds and centrifuge for 30 seconds to spin down all components.

5. Store 10X STA Primer Mix at 4ºC for repeated usage up to six months or store at -20ºC for long-term storage.

<table>
<thead>
<tr>
<th>48 Primer Pairs (EXAMPLE)</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µL each primer pair (100 µM each)</td>
<td>1 µL (x 48 = 48 µL)</td>
</tr>
<tr>
<td>1X DNA Suspension Buffer</td>
<td>152 µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>200</strong></td>
</tr>
</tbody>
</table>

Table 3 Preparation of 500 nM (10X) pooled STA Primer Mix
Preparing STA Reaction Mix

1. For each well of a 96-well PCR plate that was used for sorting, prepare the following mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 9 µL Sample (µL)</th>
<th>48 Samples with Overage (µL)</th>
<th>96 Samples with Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® PreAmp Master Mix (Invitrogen, PN 4391128)</td>
<td>7.5</td>
<td>390.0</td>
<td>780.0</td>
</tr>
<tr>
<td>10X STA Primer Mix (500 nM)</td>
<td>1.5</td>
<td>78.0</td>
<td>156.0</td>
</tr>
<tr>
<td>0.5M EDTA, pH 8.0 (Invitrogen, PN AM9260G)</td>
<td>0.075</td>
<td>3.90</td>
<td>7.80</td>
</tr>
<tr>
<td>Total Volume</td>
<td>9.0</td>
<td>471.9</td>
<td>943.8</td>
</tr>
</tbody>
</table>

Table 4  STA Reaction Mix

2. Aliquot 9 µL of the STA reaction mix to each of the first strand cDNA samples.

STA Cycling

1. Follow the thermal cycling conditions below on a standard thermal cycler.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Enzyme Activation</th>
<th>20 Cycles (1-10 cells)</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
<td>Annealing/Extension</td>
</tr>
<tr>
<td>Temperature</td>
<td>95 ºC</td>
<td>96 ºC</td>
<td>60 ºC</td>
</tr>
<tr>
<td>Time</td>
<td>10 minutes</td>
<td>5 seconds</td>
<td>4 minutes</td>
</tr>
</tbody>
</table>

Exonuclease I (Exo I) Treatment Method

For best results, we recommend using a cleanup step to remove unincorporated primers. This can be done with Exonuclease I (E.coli).

1. Just before use, dilute the Exonuclease I to 4U/µL as shown:
Two-Step Single-Cell Gene Expression Using EvaGreen® Supermix on the BioMark™ and BioMark™ HD Systems

Table 5  Exo I Reaction Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 15 µL Sample (µL)</th>
<th>48 Samples with Overage (µL)</th>
<th>96 Samples with Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.2</td>
<td>252.0</td>
<td>504.0</td>
</tr>
<tr>
<td>Exonuclease I Reaction Buffer (10X)</td>
<td>0.6</td>
<td>36.0</td>
<td>72.0</td>
</tr>
<tr>
<td>Exonuclease I at 20 units/µL</td>
<td>1.2</td>
<td>72.0</td>
<td>144.0</td>
</tr>
<tr>
<td>Total Volume</td>
<td>6.0</td>
<td>360.0</td>
<td>720.0</td>
</tr>
</tbody>
</table>

2 Add 6 µL of diluted Exo I at 4 U/µL to each 15 µL STA reaction, vortex, centrifuge and place in a thermal cycler.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Digest</th>
<th>Inactivate</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37°C</td>
<td>80°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Time</td>
<td>30 minutes</td>
<td>15 minutes</td>
<td>infinity</td>
</tr>
</tbody>
</table>

3 Dilute the final products to an appropriate concentration for testing. The minimum amount of dilution that should be used is 5-fold but if the Ct (also known as Cq) values are consistently below 6 for some of the assays this may need to be increased to 10-fold or 20-fold. Use low EDTA TE or DNA Suspension Buffer (TEKnova, PN T0221) to dilute the products as shown below:

Table 1: Dilution Table

<table>
<thead>
<tr>
<th>Volume of STA Reaction + Exonuclease I</th>
<th>5-fold dilution</th>
<th>10-fold dilution</th>
<th>20-fold dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.0 µL</td>
<td>54.0 µL</td>
<td>129.0 µL</td>
<td>279.0 µL</td>
</tr>
</tbody>
</table>

Table 6  Dilution Table

4 Store diluted STA products at -20 ºC or use immediately for on-chip PCR.

NOTE: For larger volume STA reactions adjust the amounts of materials proportionally.
Preparing the Sample Pre-Mix and Samples

The following protocol only specifies reagent prep for the 48.48 Dynamic Array™ IFC and the 96.96 Dynamic Array™ IFC. When using a 192.24 Gene Expression IFC, refer to the Fluidigm 192.24 Fast/Standard Gene Expression Workflow Quick Reference (PN 100-7222).

We recommend calculating overages when preparing the Sample Pre-Mix solution. The volumes in the table below apply to a Fluidigm 48.48 Dynamic Array IFC and a Fluidigm 96.96 Dynamic Array IFC.

1 Combine the following to make the Sample Pre-Mix solution:

<table>
<thead>
<tr>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Sso Fast EvaGreen Supermix With Low ROX (Bio-Rad Laboratories, PN 172-5211)</td>
</tr>
<tr>
<td>20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738) green cap</td>
</tr>
<tr>
<td>STA and Exo I-treated sample</td>
</tr>
</tbody>
</table>

2 In a 96-well plate, combine 3.3 µL of Sample Pre-Mix with 2.7 µL of STA and Exo I-treated sample to make a final volume of 6 µL Sample Mix solution.

3 Vortex the Sample Mix solution for a minimum of 20 seconds, and centrifuge for at least 30 seconds.

Prepared reactions can be stored at 4 °C overnight.

Table 2: Sample Pre-Mix solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Volume for 48.48 Dynamic Array IFC (µL) (60 samples)</th>
<th>Volume for 96.96 Dynamic Array IFC (µL) (120 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Sso Fast EvaGreen Supermix With Low ROX (Bio-Rad Laboratories, PN 172-5211)</td>
<td>2.5</td>
<td>3.0</td>
<td>180.0</td>
<td>360.0</td>
</tr>
<tr>
<td>20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738) green cap</td>
<td>0.25</td>
<td>0.3</td>
<td>18.0</td>
<td>36.0</td>
</tr>
<tr>
<td>STA and Exo I-treated sample</td>
<td>2.25</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**IMPORTANT:** Use caution when pipetting the Fluidigm 20X DNA Binding Dye Sample Loading Reagent as bubbles can be introduced.
Preparing the 5 µM (10X) Assay Mix

The following protocol only specifies reagent prep for the 48.48 Dynamic Array™ IFC and the 96.96 Dynamic Array™ IFC. When using a 192.24 Gene Expression IFC, refer to the Fluidigm 192.24 Fast/Standard Gene Expression Workflow Quick Reference (PN 100-7222).

The same preparation of primers can be used for the Fluidigm 48.48 Dynamic Array IFC and the Fluidigm 96.96 Dynamic Array IFC. Prepare primers as shown below.

1. Combine the following:

   Table 3: Assay Mix solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for per Inlet (µL)</th>
<th>Volume for per Inlet with Overage (µL)</th>
<th>Volume for 50 µL Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Assay Loading Reagent (Fluidigm, PN 85000736)</td>
<td>2.5</td>
<td>3.0</td>
<td>25.0</td>
</tr>
<tr>
<td>1X DNA Suspension Buffer (Teknova, PN T0221)</td>
<td>2.25</td>
<td>2.7</td>
<td>22.5</td>
</tr>
<tr>
<td>100 µM each of Forward and Reverse Primer Mix</td>
<td>0.25</td>
<td>0.3</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5.0</strong></td>
<td><strong>6.0</strong></td>
<td><strong>50.0</strong></td>
</tr>
</tbody>
</table>

2. Vortex the Assay Mix for a minimum of 20 seconds, and centrifuge for at least 30 seconds to spin down all components.

**IMPORTANT:** Vortex thoroughly and centrifuge all sample and assay solutions before pipetting into the chip inlets. Fail to do so may result in a decrease in data quality.

**NOTE:** The final concentration of each primer is 5 µM in the inlet and 500 nM in the final reaction.
Priming the Chip and Loading Assay and Samples

For instructions on priming and loading the 192.24 Gene Expression IFC, refer to the Fluidigm 192.24 Fast/Standard Gene Expression Workflow Quick Reference (PN 100-7222).

IMPORTANT: Due to different accumulator volumes, use the appropriate control syringe for your chip type: 300 µL (for the 48.48 Dynamic Array IFC) or 150 µL (for the 96.96 Dynamic Array IFC).

1. Inject control line fluid into each accumulator on the chip (see Fig 1 for the 48.48 Dynamic Array IFC or Fig 2 for the 96.96 Dynamic Array IFC).
2. Remove and discard the blue protective film from the bottom of the chip.
3. Place the chip into the IFC Controller MX (for the 48.48 Dynamic Array IFC) or the IFC Controller HX (for the 96.96 Dynamic Array IFC), then run the Prime script (for the 48.48 Dynamic Array IFC) or the Prime script (for the 96.96 Dynamic Array IFC).
4. When the Prime script has finished, press Eject to remove the primed chip from the IFC Controller.

IMPORTANT: While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into the inlets.

5. Pipette 5 µL of each assay and 5 µL of each sample into their respective inlets on the chip.
6. Return the chip to the IFC Controller.
7. Using the IFC Controller software, run the Load Mix script (for the 48.48 Dynamic Array IFC) or Load Mix script (for the 96.96 Dynamic Array IFC) to load the samples and assays into the chip.
8. When the Load Mix script has finished, remove the loaded chip from the IFC Controller.
9. Remove any dust particles or debris from the chip surface using scotch tape. You are now ready for your chip run.
Figure 1  48.48 Dynamic Array IFC sample and assay inlets

Figure 2  Fig 2. 96.96 Dynamic Array IFC sample and assay inlets
Using the Data Collection Software

1. Double-click the Data Collection Software icon on the desktop to launch the software.
2. Click **Start a New Run**.
3. Check the status bar to verify that the camera has a green light to indicate that it is ready.
4. Remove the blue tape from the back of the chip if this was not done previously. Place the chip into the reader.
5. Click **Load**.
6. Verify chip barcode and chip type.
7. Choose project settings (if applicable).
8. Click **Next**.
9. Chip Run file:
   a. Select **New**.
   b. Browse to a file location for data storage.
   c. Click **Next**.
10. Application, Reference, Probes:
    a. Select Application Type—**Gene Expression**.
    b. Select Passive Reference—**ROX**.
    c. Select Probe—**Single probe**.
    d. Select Probe type—**EvaGreen**.
    e. Click **Next**.
11 Click **Browse** to find the thermal cycling protocol files:

For BioMark™ HD:
- GE Fast 48x48 PCR+Melt v2.pcl
- GE Fast 96x96 PCR+Melt v2.pcl

For BioMark™:
- GE 48x48 PCR+Melt v2.pcl
- GE 96x96 PCR+Melt v2.pcl

<table>
<thead>
<tr>
<th>Segment</th>
<th>Type</th>
<th>Temperature (°C)</th>
<th>Duration (seconds)</th>
<th>BioMark™ HD Ramp Rate (°C/s)</th>
<th>BioMark™ Ramp Rate (°C/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thermal Mix</td>
<td>70</td>
<td>2400</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>30</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Hot Start</td>
<td>95</td>
<td>60</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>PCR (30 Cycles)</td>
<td>96</td>
<td>5</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>20</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Melting Curve</td>
<td>60</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60-95</td>
<td></td>
<td>1°C/3 s</td>
<td>1°C/3 s</td>
</tr>
</tbody>
</table>

96x96 chip

<table>
<thead>
<tr>
<th>Segment</th>
<th>Type</th>
<th>Temperature (°C)</th>
<th>Duration (seconds)</th>
<th>BioMark™ HD Ramp Rate (°C/s)</th>
<th>BioMark™ Ramp Rate (°C/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hot Start</td>
<td>95</td>
<td>60</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>PCR (30 Cycles)</td>
<td>96</td>
<td>5</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>20</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Melting Curve</td>
<td>60</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60-95</td>
<td></td>
<td>1°C/3 s</td>
<td>1°C/3 s</td>
</tr>
</tbody>
</table>

48x48 chip

12 Confirm **Auto Expos** is selected.

13 Click **Next**.

14 Verify the chip run information.
15 Click Start Run.

The cycling parameters are given for the two different chip types:

<table>
<thead>
<tr>
<th></th>
<th>Type</th>
<th>Temperature (°C)</th>
<th>Duration (seconds)</th>
<th>BioMark™ HD Ramp Rate (°C/s)</th>
<th>BioMark™ Ramp Rate (°C/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thermal Mix</td>
<td>70</td>
<td>2400</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>30</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Hot Start</td>
<td>95</td>
<td>60</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>PCR (30 Cycles)</td>
<td>96</td>
<td>5</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>20</td>
<td>5.5</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Melting Curve</td>
<td>60</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60-95</td>
<td></td>
<td>1 °C/3 s</td>
<td>1 °C/3 s</td>
</tr>
</tbody>
</table>

48x48 chip

|       | Hot Start                     | 95               | 60                | 5.5                          | 2                        |
| 2     | PCR (30 Cycles)               | 96               | 5                 | 5.5                          | 2                        |
|       |                               | 60               | 20                | 5.5                          | 2                        |
| 3     | Melting Curve                 | 60               | 3                 | 1                            | 1                        |
|       |                               | 60-95            |                   | 1 °C/3 s                     | 1 °C/3 s                 |

Using the Real-Time PCR Analysis Parameters

1 Double-click the Real-Time PCR Analysis software icon on the desktop to launch the software.
2 Click Open Chip Run.
3 Double-click a ChipRun.bml file to open it in the software.
4 Enter detector and sample information.
5 Select Details Views. We recommend using the AutoGlobal method to set the threshold.
6 We recommend using Linear Derivative as the baseline correction method. For more information about baseline correction methods, contact Fluidigm Technical Support.
7 Always compare the Tm of the intended products to a positive control sample.
8 Click Analyze.

NOTE: For more information about melting curve analysis, see the Fluidigm Real-Time PCR Melting Curve Analysis User Guide (PN 68000118).
Two-Step Single-Cell Gene Expression Using EvaGreen® Supermix on the BioMark™ and BioMark™ HD Systems
Fast Gene Expression Analysis Using TaqMan Gene Expression Assays on the BioMark™ HD System

Introduction

This protocol is intended to be used for fast gene expression analysis on the BioMark™ HD System using TaqMan® Gene Expression Assays. The protocol is suitable for use with either 48.48 or 96.96 Dynamic Array™ IFCs and appropriate cycling protocols are provided for each chip type. This protocol requires a BioMark™ HD System, which includes a thermal cycler with fast cycling capabilities. The protocol also requires the use of a master mix that does not require a long hot start and that works well with the shortened cycling times. Three master mixes that we have found to work well are Quanta PerfeCTa™ qPCR Fast Mix from Quanta Biosciences (also available from VWR), TaqMan Fast Universal Master Mix from Applied Biosystems, and TaqMan GTXpress Master Mix from Applied Biosystems. In preliminary testing, the new TaqMan Fast Advanced Master Mix from Applied Biosystems also appears to be suitable for use with this protocol. Although the four master mixes above are recommended because they work well for fast gene expression analysis, they also can be used with standard cycling conditions. This protocol was thoroughly tested on a wide variety of assays and good results can be expected from the majority of assays. For especially difficult assays, the cycling conditions can be modified.

Required Reagents

- PreAmp Master Mix (Fluidigm, PN 100-5580, 100-5581)
- 20X TaqMan Gene Expression Assays (Applied Biosystems)
- 2X Assay Loading Reagent (Fluidigm, PN 85000736)
- 2X Master Mix for Fast Cycling:
  - Quanta PerfeCTa® qPCR Fast Mix®, low ROX™ (Quanta Biosciences, PN 95078-012 or VWR, PN 101419-220) or
  - TaqMan Fast Universal PCR Master Mix (Applied Biosystems, PN 4352042) or
  - TaqMan GTXpress Master Mix (Applied Biosystems, PN 4401892) or
  - TaqMan Fast Advanced Master Mix (Applied Biosystems, PN 4444557)
- 20X GE Sample Loading Reagent (Fluidigm, PN 85000735, 85000746)

Required Equipment

- Standard 96-well Thermal Cycler
• IFC Controller MX (for the 48.48 Dynamic Array IFC) or HX (for the 96.96 Dynamic Array IFC) or RX (for the 192.24 Gene Expression IFC)
• BioMark™ HD System

Required Software

Fluidigm® Real-Time PCR Analysis Software v.3.0.2 or higher and BioMark™ HD Data Collection Software v.3.0.2 or higher is required for this advanced development protocol.

Gene Expression PreAmp with Fluidigm® PreAmp Master Mix and TaqMan® Assays

Overview

In the BioMark HD System, samples are loaded into individual inlets and then distributed across multiple reaction chambers in nanoliter volume aliquots. With these small volumes, detecting the specific targets requires a minimum of 800 copies/µL in the final sample mix. For genes with lower expression levels, there are too few copies to detect adequately in cDNA samples. Preamplification is used to increase the number of copies to a detectable level for a greater number of genes.

Preamplification allows for multiplex amplification of up to 96 targets. A pool of primers is prepared from the same gene expression assays to be used for qPCR. By using the real-time qPCR assays in the preamplification reaction, only the targets of interest are amplified. A limited number of cycles is used, generally 10-14. Under these conditions of low primer concentration and a limited number of cycles, the cDNA is amplified without significant bias for the majority of genes.

Pooling the TaqMan Gene Expression Assays

1. In a microcentrifuge tube, combine equal volumes of each 20x TaqMan gene expression assay, up to a total of 96 assays.

2. Dilute the pooled assays using DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA; TEKnova, PN T0221) so that each assay is at a final concentration of 0.2x (180 nM).

3. The chart below provides an example using 96 assays:

<table>
<thead>
<tr>
<th>96 Assays</th>
<th>DNA Suspension Buffer</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µL each assay (20x)</td>
<td>8 µL</td>
<td>200 µL</td>
</tr>
</tbody>
</table>

NOTE: Volume can be adjusted proportionally based on the number of samples to be amplified.
Preparing Sample Pre-Mix and Samples

1. In a DNA-free hood, prepare a Pre-Mix for the reactions as indicated in the table below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume / Reaction (µL)</th>
<th>Volume for 48 Reactions + 10% Overage (µL)</th>
<th>Volume for 96 Reactions + 10% Overage (µL)</th>
<th>Volume for 192 Reactions + 10% Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PreAmp Master Mix</td>
<td>1.00</td>
<td>52.8</td>
<td>105.6</td>
<td>211.2</td>
</tr>
<tr>
<td>Pooled TaqMan assay mix (0.2x)</td>
<td>1.25</td>
<td>66.0</td>
<td>132.0</td>
<td>264.0</td>
</tr>
<tr>
<td>Water</td>
<td>1.50</td>
<td>79.2</td>
<td>158.4</td>
<td>316.8</td>
</tr>
<tr>
<td>cDNA</td>
<td>1.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>5.00</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. In a PCR plate, aliquot 3.75 µL of Pre-Mix for each sample.
3. Remove the plate from the DNA-free hood and add 1.25 µL of cDNA to each well containing Pre-Mix, making a total volume of 5 µL.
4. Mix the reactions by briefly vortexing, then centrifuge.

Thermal Cycling

1. Place the plate in the thermal cycler and cycle using the following table as a guide:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hold</th>
<th>Cycle (14 cycles)</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperat</td>
<td>95 °C</td>
<td>95 °C 60 °C 4 °C</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>2 min</td>
<td>15 s 4 min ∞</td>
<td></td>
</tr>
</tbody>
</table>

2. After cycling, dilute the reaction 1:5 by adding 20 µL TE Buffer (10 mM Tris-HCl, 1.0 mM EDTA, TEKnova, PN T0221) to the final 5 µL reaction volume for a total volume of 25 µL.

**NOTE:** Fourteen (14) cycles are recommended as a starting point, but this can be decreased down to 10 cycles or increased up to 20 cycles, if necessary. The appropriate number of cycles should be determined empirically.

**NOTE:** Diluted reaction products can either be assayed immediately or stored at -20 °C for later use. Diluted reaction products should be stable for at least one week.
Preparing 10X Assays

The following protocol only specifies reagent prep for the 48.48 Dynamic Array™ IFC and the 96.96 Dynamic Array™ IFC. When using a Flex Six™ Gene Expression IFC, refer to the Fluidigm Flex Six™ IFC TaqMan® Fast/Standard Gene Expression Workflow (PN 100-7251). When using a 192.24 Gene Expression IFC, refer to the Fluidigm 192.24 Real-Time PCR Workflow Quick Reference (PN 100-6170) or the Fluidigm 192.24 Fast Real-Time PCR Workflow (BioMark™ HD only) Quick Reference (PN 100-6174).

1. In a DNA-free hood, prepare aliquots of 10X assays using volumes in the table below (scale up appropriately for multiple runs).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Volume per 50 µL Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X TaqMan Gene Expression Assay (Applied BioSystems)</td>
<td>2.5</td>
<td>3.0</td>
<td>25</td>
</tr>
<tr>
<td>2X Assay Loading Reagent (Fluidigm, PN 85000736)</td>
<td>2.5</td>
<td>3.0</td>
<td>25</td>
</tr>
<tr>
<td>Total Volume</td>
<td>5.0</td>
<td>6.0</td>
<td>50</td>
</tr>
</tbody>
</table>

Final Concentration at 10X: Primers: 9 µM, Probe: 2.5 µM

Preparing Sample Pre-Mix and Samples

1. Prepare a Sample Pre-Mix solution containing the Master Mix and 20X GE Sample Loading Reagent sufficient for the number and type of chips to be run.

The following table provides the component amounts for one 48.48 or one 96.96 chip.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Sample Pre-Mix for 48.48 (µL)</th>
<th>Sample Pre-Mix for 96.96 (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Master Mix*</td>
<td>2.5</td>
<td>3.0</td>
<td>180.0</td>
<td>360.0</td>
</tr>
<tr>
<td>20X GE Sample Loading Reagent (Fluidigm, PN 85000735, 85000746)</td>
<td>0.25</td>
<td>0.3</td>
<td>18.0</td>
<td>36.0</td>
</tr>
<tr>
<td>cDNA</td>
<td>2.25</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.0</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Quanta PerfeCTa® qPCR Fast Mix®, low ROX™ (Quanta BioSciences, PN 95078-012 or VWR, PN 101419-220) or TaqMan Fast Universal PCR Master Mix (Applied Biosystems, PN 4352042) or TaqMan GTXpress Master Mix (Applied Biosystems, PN 4401892) or TaqMan Fast Advanced Master Mix (Applied Biosystems, PN 4444557)
These volumes include some overage to account for pipetting error.

2 In a DNA-free hood, combine the two Sample Pre-Mix components in a 1.5 mL sterile tube—enough volume to fill an entire chip. Aliquot 3.3 µL of the Sample Pre-Mix for each sample.

3 Remove the aliquots from the DNA-free hood and add 2.7 µL of cDNA to each, to make a total volume of 6 µL in each aliquot.

**Priming and Loading the Dynamic Array IFC**

- For instructions on priming and loading the 48.48 Dynamic Array IFC, see *Fluidigm 48.48 Fast Real-Time PCR Workflow Quick Reference* (PN 100-2637).
- For instructions on priming and loading the 96.96 Dynamic Array IFC, see the *Fluidigm 96.96 Real-Time PCR Workflow Quick Reference* (PN 68000130).
- For instructions on priming and loading the Flex Six™ Gene Expression IFC, see the *Fluidigm Flex Six™ IFC TaqMan® Fast/Standard Gene Expression Workflow* (PN 100-7251).
- For instructions on priming and loading the 192.24 Gene Expression IFC, see the *Fluidigm 192.24 Real-Time PCR Workflow Quick Reference* (PN 100-6170) or the *Fluidigm 192.24 Fast Real-Time PCR Workflow (BioMark™ HD only) Quick Reference* (PN 100-6174).
Using the Data Collection Software

The protocols used for data collection are fast protocols.

- **48.48**: Select GE 48X48 Fast v1.pcl in the GE folder. This cycling protocol is 95°C for 1 minute, followed by 35 cycles of 96°C for 5 seconds and 60°C for 20 seconds. This protocol takes approximately 26 minutes.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hot Start (Taq Activation)</th>
<th>Amplification - 35 Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>95°C</td>
<td>96°C, 60°C</td>
</tr>
<tr>
<td>Time</td>
<td>1 min</td>
<td>5 sec, 20 sec</td>
</tr>
</tbody>
</table>

- **96.96**: Select GE 96x96 Fast v2.pcl in the GE folder. The cycling protocol portion includes the Thermal Mix protocol for the 96.96 Dynamic Array IFC. The total program runs approximately 66 minutes. The complete program is as follows: 70°C for 40 minutes, 60°C for 30 seconds, 98°C for 1 minute, followed by 35 cycles of 97°C for 5 seconds and 60°C for 20 seconds.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ramp Rate: Fast 5.5°C/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>1 1 1 35</td>
</tr>
<tr>
<td>Temperature</td>
<td>70°C 60°C 98°C 97°C 60°C</td>
</tr>
<tr>
<td>Time</td>
<td>40 min 30 s 1 min 5 s 20 s</td>
</tr>
</tbody>
</table>

Using UNG for Preventing Carryover Contamination

The Quanta PerfeCTa qPCR Fast Mix and the TaqMan Fast Advanced Master Mix both contain UNG. If using these master mixes, the cycling program can be modified to include a UNG step to protect against carryover contamination. Note the following:

- For the Quanta PerfeCTa qPCR Fast Mix, a 2-minute incubation at 45°C is recommended by the manufacturer. For the 48.48 Dynamic Array IFC, this should be added at the beginning of the program. For the 96.96 Dynamic Array IFC, this should be added before the Hot Mix step.

- For the TaqMan Fast Advanced Master Mix, a 2-minute incubation at 50°C is recommended. This should be added at the beginning of the program for the 48.48 Dynamic Array IFC and before the Hot Mix step for the 96.96 Dynamic Array IFC.
The TaqMan Fast Universal PCR Master Mix contains dUTP and can be modified by the addition of UNG. The TaqMan GTXpress Master Mix was designed for genotyping applications and does not contain any dUTP.
Introduction

This protocol is intended to be used for fast gene expression analysis of single cells using TaqMan™ Gene Expression Assays on the BioMark™ HD System. The protocol includes three sections: 1) single cell sorting; 2) reverse transcription and specific target amplification (RT-STA); and 3) real-time PCR on either 48.48 or 96.96 Dynamic Array™ integrated fluidic circuits (IFCs). Individual cells are sorted by Fluorescence Activated Cell Sorting (FACS) into a 96-well PCR plate. RT-STA is carried out on a 96-well thermal cycler using the CellsDirect™ One-Step qRT-PCR kit and gene-specific primers included in the TaqMan assays. This reaction generates sufficient template cDNA for TaqMan real-time analysis on Dynamic Array IFCs of hundreds of genes from hundreds of single cells in parallel. Fast real-time PCR for gene expression analysis requires a BioMark™ HD System, which includes a thermal cycler with fast cycling capabilities. Quanta PerfeCTa™ qPCR Fast Mix from Quanta Biosciences (also available from VWR) is used in combination with TaqMan Gene Expression Assays from Life Technologies Corporation for real-time PCR analysis.

This protocol has been verified on both 48.48 and 96.96 Dynamic Array IFCs.

Required Reagents

- CellsDirect One-Step qRT-PCR Kit (Invitrogen, catalog numbers 11753-100 and 11753-500)
- SUPERase-In (Ambion, PN AM2694)
- DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) (TEKnova, PN T0221)
- 20X TaqMan Gene Expression Assays (Applied Biosystems)
- 2X Assay Loading Reagent (Fluidigm, PN 85000736)
- Quanta PerfeCTa qPCR Fast Mix, low ROX (Quanta Biosciences, PN 95078-012 or VWR, PN 1014190-220)
- 20X GE Sample Loading Reagent (Fluidigm, PN 85000735, 85000746)
- PCR certified water (TEKnova, PN W3330)

Required Equipment

- FACS instrument
- Standard 96-well Thermal Cycler
- 96-well PCR plates that are compatible with the FACS instrument and thermal cycler
- Adhesive plate seals (ABI, PN 4311971)
- IFC Controller MX (for the 48.48 Dynamic Array IFC) or HX (for the 96.96 Dynamic Array IFC)
- BioMark™ HD System

Software Requirements

Fluidigm® Real-Time PCR Analysis Software v.3.0.2 or higher and BioMark™ HD Data Collection Software v.3.0.2 or higher is required for this protocol.

Cell Sorting Procedure

1. For each 96-well PCR plate, prepare 2X reaction reagent by mixing 580 µL of the CellsDirect 2X Reaction Mix with 11.6 µL of Ambion SUPERase-In.
2. Pipette 5.1 µL of the 2X reaction reagent into each well of the PCR plate and seal the plate with adhesive film. Store the plate on ice if cells are sorted within one day. Otherwise, store at -20°C.
3. Using a FACS instrument, sort cells of interest directly into the plate containing the 2X reaction reagent.
4. Seal plates with adhesive film, vortex for 10 seconds, and centrifuge at 1500 RPM for 1 minute.
5. Use immediately or store at -80°C.

NOTE: 1) The FACS instrument needs to be carefully calibrated to deposit single cells in the center of each well of the PCR plate. 2) Sort in batch mode using a FACS machine. 3) Sort cells into the same plate that will be used for thermal cycling.

Reverse Transcription-Specific Target Amplification (RT-STA)

This reverse transcription preamplification procedure works for both standard and fast TaqMan gene expression applications.

1. In a DNA-free hood, pool all TaqMan Gene Expression Assays and dilute with DNA Suspension Buffer so that each assay is at a final concentration of 0.2X. For example, pipette 4 µL of each of the 96 TaqMan Gene Expression Assays (384 µL total) into a 1.5 ml sterile tube and add 16 µL of DNA Suspension Buffer, resulting in 400 uL of 0.2X primer/probe mix.
For each 96-well PCR plate containing sorted cells, prepare the reaction mix by combining 300 µL of the 0.2X Primer/Probe mix, 24 µL of SuperScript III RT/Platinum® Taq Mix and 144 µL of PCR certified water into a 1.5 ml sterile tube. Vortex for 10 seconds.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Well (µL)</th>
<th>Volume per 96-well Plate with Overage (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2X Primer/Probe Mix</td>
<td>2.5</td>
<td>300.0</td>
</tr>
<tr>
<td>SuperScript™ III RT/Platinum® Taq Mix</td>
<td>0.2</td>
<td>24.0</td>
</tr>
<tr>
<td>PCR certified water</td>
<td>1.2</td>
<td>144.0</td>
</tr>
<tr>
<td>Total</td>
<td>3.9</td>
<td>468.0</td>
</tr>
</tbody>
</table>

Table 1 Reaction Mix

To each tube of an 8-well PCR strip, add 50 µL of the reaction mix. Transfer 3.9 µL of the reaction mix to each well of the PCR plate containing cells from the strip using an 8-channel pipette, seal, vortex for 10 seconds and centrifuge at 1500 RPM for 1 minute. Place the plate onto a 96-well thermal cycler and proceed to RT-STA using the thermal cycling protocol below:

<table>
<thead>
<tr>
<th>Condition</th>
<th>RT</th>
<th>Taq Activation</th>
<th>STA - 18 Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Denaturation</td>
</tr>
<tr>
<td>Temperat</td>
<td>50°C</td>
<td>95°C</td>
<td>95°C</td>
</tr>
<tr>
<td>Time</td>
<td>15 min</td>
<td>2 min</td>
<td>15 sec</td>
</tr>
</tbody>
</table>

Table 2 Thermal cycle conditions

Dilute the resulting cDNA product 1:5 with DNA Suspension Buffer.
Preparing 10X Assays

1 In a DNA-free hood, prepare aliquots of 10X assays using volumes in the table below (scale up appropriately for multiple runs).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Volume per 50 µL Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X TaqMan Gene Expression Assay</td>
<td>2.5</td>
<td>3.0</td>
<td>25.0</td>
</tr>
<tr>
<td>2X Assay Loading Reagent</td>
<td>2.5</td>
<td>3.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Total Volume</td>
<td>5.0</td>
<td>6.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Table 3 Assay preparation

Preparing Sample Pre-Mix and Samples

1 Prepare a Sample Pre-Mix solution containing the Master Mix and 20X GE Sample Loading Reagent sufficient for the number and type of chips to be run.

The following table provides the component amounts for one (1) 48.48 or one (1) 96.96 chip.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Sample Pre-Mix for 48.48 (µL)</th>
<th>Sample Pre-Mix for 96.96 (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Master Mix*</td>
<td>2.5</td>
<td>3.0</td>
<td>180</td>
<td>360</td>
</tr>
<tr>
<td>20X GE Sample Loading Reagent</td>
<td>0.25</td>
<td>0.3</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>Diluted RT-STA Sample</td>
<td>2.25</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Volume</td>
<td>5.0</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4 Sample Pre-Mix and Samples

*Quanta PerfeCTa™ qPCR Fast Mix, low ROX (Quanta BioSciences, PN 95078-012 or VWR, PN 1014190-220)

These volumes include some overage to account for pipetting error.

1 In a DNA-free hood, combine the two Sample Pre-Mix components (A) and (B) from the table above in a 1.5 mL sterile tube--enough volume to fill an entire chip. Aliquot 3.3 µL of the Sample Pre-Mix for each sample to be analyzed in an empty 96-well PCR plate.
2 Remove the Sample Pre-Mix aliquots from the DNA-free hood and add 2.7 µL of the diluted RT-STA sample to each, to make a total volume of 6 µL, then seal, vortex for 10 seconds and centrifuge at 1500 RPM for 1 minute.

**Priming and Loading the Dynamic Array IFC**

For instructions on loading the 48.48 Dynamic Array IFC, see *Fluidigm 48.48 Real-Time PCR Workflow Quick Reference* (PN 68000089). For instructions on loading the 96.96 Dynamic Array IFC, see *Fluidigm 96.96 Real-Time PCR Workflow Quick Reference* (PN 68000130).

**Using the Data Collection Software**

The protocols used for data collection are fast protocols.

- **48.48**: Select GE 48X48 Fast v1.pcl in the GE folder. This protocol takes approximately 26 minutes. This cycling protocol is described below:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hot Start (Taq Activation)</th>
<th>Amplification - 35 Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
</tr>
<tr>
<td>Temperature</td>
<td>95°C</td>
<td>96°C</td>
</tr>
<tr>
<td>Time</td>
<td>1 min</td>
<td>5 sec</td>
</tr>
</tbody>
</table>

  **Table 5 48.48 protocol**

- **96.96**: Select GE 96x96 Fast v2.pcl in the GE folder.
  - The cycling protocol includes the Thermal Mix protocol for the 96.96 Dynamic Array IFC. The total program runs approximately 66 minutes. The complete program is described below:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ramp Rate: Fast 5.5°C/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thermal Mix</td>
</tr>
<tr>
<td>Cycles</td>
<td>1</td>
</tr>
<tr>
<td>Temperature</td>
<td>70°C</td>
</tr>
<tr>
<td>Time</td>
<td>40 min</td>
</tr>
</tbody>
</table>

  **Table 6 96.96 protocol**
Overview of the Flex Six IFC

The Fluidigm® Flex Six™ Gene Expression IFC provides substantial flexibility in sample and assay numbers during target selection and genotyping while allowing complete use of the IFC. The six 12 X 12 partitions can be organized in any configuration in up to six separate experimental runs.

Note: The chemistry (for example, EvaGreen or TaqMan) for an experimental run can differ from run to run. However, all partitions in use for a single experimental run must use the same chemistry.

- **Sequential Runs.** You can run any combination of experimental partitions sequentially. As a simple example, you can run a single partition, store the IFC, and then run additional partitions after storing the IFC again until all six partitions are used or until the IFC passes its 90-day expiration date:

- **Parallel Runs.** You can run experimental partitions in parallel. Each partition can contain an independent experiment, can be grouped together to form tiled experiments, or can be a mixture of both.
Components of the Flex Six IFC

Partitions and Inlets

- There are a total of six independent partitions in each Flex Six IFC (partitions 1-6).
- Each partition has a 12 X 12 format (12 assay and 12 sample inlets).
- Each partition can be run independently as a separate experimental run (at different times or on different days) or simultaneously (up to six partitions per run).

**IMPORTANT:** At minimum, all 12 assay inlets and all 12 sample inlets for an active partition must be filled.

The recommended volume of fluid in each inlet for the Flex Six Gene Expression IFC is 3 µL for assays and 3 µL for samples.
Barcodes

Each Flex Six IFC has a unique barcode. The first three digits of the barcode determine which applications are listed in the Applications menu in the software:

- 153x - Flex Six Gene Expression (GE) IFC
- 154x - Flex Six Genotyping (GT) IFC

**IMPORTANT:** Do not combine GE and GT runs in the same IFC. Each IFC type is for a specific purpose.

Barrier Plugs

Barrier plugs allow tracking of which partitions are used and prevent pipetting into the wrong wells. Do not discard the barrier plugs.

- Each plug is labeled according to its location (A for assay or S for sample, plus a number between 1 and 6).
- You can write on the plugs to denote which partitions have been used.
- Do not interchange the locations of the barrier plugs.
- During an experimental run, leave barrier plugs on the unused inlets of the IFC.
- When an IFC is being stored, ensure that there are barrier plugs on all unused inlets to eliminate dust or other contaminants in the inlets and to serve as visual aids for tracking partitions that can be used at a later date.
Configuring an Experiment

You can configure experiments in several ways.

**Note:** The chemistry (for example, EvaGreen or TaqMan) for an experimental run can differ from run to run. However, all partitions in use for a single experimental run must use the same chemistry.

12 Assays X 12 Samples
(One partition)

12 Assays X 24 Samples
(Two partitions)

Note: Scale up the amount of assays and samples appropriately.
Configuring an Experiment

24 Assays X 12 Samples (Two partitions)
Note: Scale up the amount of assays and samples appropriately.

24 Assays X 36 Samples (Six partitions)
Note: Scale up the amount of assays and samples appropriately.
## Flex Six IFC Workflows

### Required Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Part number</th>
<th>Store at ...</th>
</tr>
</thead>
<tbody>
<tr>
<td>PreAmp Master Mix</td>
<td>Fluidigm, PN 100-5580, 100-5581</td>
<td>-20 °C</td>
</tr>
<tr>
<td>2X Assay Loading Reagent</td>
<td>Fluidigm, PN 100-5359</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PCR Certified Water</td>
<td>TEKnova, PN W3330</td>
<td>Room temperature</td>
</tr>
<tr>
<td>2 Control Line Fluid Syringes (150 µL each)</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

#### For Delta Gene® Assays

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Part number</th>
<th>Store at ...</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta Gene™ Primers (Forward and Reverse combined; 100 µM each)</td>
<td>ASY-GE, ASY-GE WET</td>
<td>-20 °C</td>
</tr>
<tr>
<td>PreAmp and Exo I-treated sample:</td>
<td>Fluidigm, PN 100-5580, 100-5581</td>
<td>-20 °C</td>
</tr>
<tr>
<td>PreAmp Master Mix</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Delta Gene Assay Mix</td>
<td>New England BioLabs, PN M0293L</td>
<td></td>
</tr>
<tr>
<td>Exonuclease I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SsoFast™ EvaGreen® Supermix with Low ROX (2X)</td>
<td>Bio-Rad Laboratories, PN 172-5211</td>
<td>-20 °C (frozen) 4 °C (thawed)</td>
</tr>
<tr>
<td>DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA)</td>
<td>TEKnova, PN T0221</td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

#### For TaqMan® Assays

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Part number</th>
<th>Store at ...</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Gene Expression Assays (20X)</td>
<td>Life Technologies</td>
<td>-20 °C</td>
</tr>
<tr>
<td>20X GE Sample Loading Reagent</td>
<td>Fluidigm, PN 100-6311</td>
<td></td>
</tr>
<tr>
<td>Fast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TaqMan Fast Advanced Master Mix (2X)</td>
<td>Life Technologies, PN 4444557</td>
<td>4 °C</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TaqMan Gene Expression PCR Master Mix (2X)</td>
<td>Life Technologies, PN 4369016</td>
<td></td>
</tr>
</tbody>
</table>
Required Equipment

- IFC Controller HX
- BioMark™ System, or BioMark™ HD System
- Microcentrifuge
- Vortex mixer
- Plate centrifuge
- Single-channel P2-P1000 pipettes (Rainin recommended)

Required Firmware and Software

- Fluidigm Data Collection Software v4 or later
- Fluidigm Real-Time PCR Analysis Software v4 or later
- IFC Controller HX
  - Firmware 52 or later
  - Software 2.5 or later
**Flex Six IFC EvaGreen Fast/Standard Gene Expression Workflow**

**Prime the Flex Six IFC**

**IMPORTANT:** Use the Flex Six integrated fluidic circuit (IFC) within three months of opening the package. Load the IFC within 60 minutes of priming on first use.

**IMPORTANT:** Dripping control line fluid on the chip or in the inlets makes the IFC unusable.

**Note:** It is not necessary to evacuate air from the syringe prior to injection of control line fluid.

**Note:** You only need to prime the IFC on the first run. On subsequent use, skip this step.

For information on how to inject control line fluid, see the Fluidigm® Control Line Fluid Loading Procedure Quick Reference (PN 68000132).

For information on using the IFC Controller HX, see the IFC Controller MX-HX User Guide (PN 68000112).

During the first usage of each Flex Six IFC, prime the new Flex Six IFC:

1. Using the included syringes, inject 150 µL of control line fluid into each accumulator on the IFC. **Do not** remove the barrier plugs until you load the IFC.
2. Remove and discard the blue protective film from the bottom of the IFC.
3. Place the IFC into the IFC Controller HX, then run the Prime (153x) script. This should take about 15 minutes.

**Prepare 10X Assays**

**IMPORTANT:** Due to the small pipetting volumes necessary for preparing a single assay mix, preparing a 10X assay stock is recommended. Unused 10X Assays can be stored at -20°C for up to three weeks.

**Note:** When preparing for an experiment that uses more than one partition, scale up the amount of assays appropriately.

In a DNA-free hood, prepare aliquots of 10X assays using volumes in Table 1.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Volume for 40 µL Stock (µL) (10 replicates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µM each Delta Gene Primers (Forward and Reverse combined; 100 µM each), non wet-lab tested (ASY-GE) or wet-lab tested (ASY-GE WET)</td>
<td>0.15</td>
<td>0.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Prepare Sample Pre-Mix and Samples

**IMPORTANT:** Pipet with care! The Delta Gene Sample Reagent is extremely viscous. **DO NOT VORTEX the Delta Gene Sample Reagent by itself at its stock concentration.**

**IMPORTANT:** Vortex thoroughly and centrifuge all assay and sample solutions **EXCEPT the Flex Six Delta Gene Sample Reagent.** You can thaw the Flex Six Delta Gene Sample Reagent a maximum of SIX times only. Fail to follow the above caution statements may result in a decrease in data quality.

**Note:** When preparing for an experiment that uses more than one partition, scale up the amount of samples appropriately.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Volume for 40 µL Stock (µL) (10 replicates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Suspension Buffer</td>
<td>1.35</td>
<td>1.8</td>
<td>18.0</td>
</tr>
<tr>
<td>2X Assay Loading Reagent (Fluidigm, PN 100-5359)</td>
<td>1.5</td>
<td>2.0</td>
<td>20.0</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>3.0</td>
<td>4.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>
Combine components in Table 2 to make the Sample Pre-Mix and final Sample Mixture in a 96-well plate, tubes, or tube strips.

1. In a DNA-free hood, combine the Sample Pre-Mix components to make enough for your experiment (33 µL/partition). Aliquot 2.2 µL of the pre-mix for each sample.

2. Remove the aliquots from the DNA-free hood and add 1.8 µL of PreAmp and Exo I-treated sample to each, making a total volume of 4 µL in each aliquot. Vortex and spin down the final Sample Mixture.

Table 2  Sample Pre-Mix and Sample Mixture for EvaGreen Fast/Standard Gene Expression

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Sample Pre-Mix for 1 Partition (µL) (15 reactions for ease of pipetting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SsoFast EvaGreen Supermix with Low ROX (2X) (Bio-Rad)</td>
<td>1.50</td>
<td>2.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Flex Six™ Delta Gene™ Sample Reagent (Fluidigm, PN 100-7673)</td>
<td>0.15</td>
<td>0.2</td>
<td>3.0</td>
</tr>
<tr>
<td>PreAmp and Exo I-treated sample* (Added individually to Sample Pre-Mix)</td>
<td>1.35</td>
<td>1.8</td>
<td>−</td>
</tr>
<tr>
<td>Total Volume</td>
<td>3.00</td>
<td>4.0</td>
<td>−</td>
</tr>
</tbody>
</table>

*. For more information about PreAmp and Exonuclease I treatment, refer to the Gene Expression PreAmp with Fluidigm® PreAmp Master Mix and Delta Gene™ Assays Quick Reference (PN 100-5875).

**Load the IFC**

**IMPORTANT:** Vortex thoroughly and centrifuge all assay and sample solutions before pipetting into IFC inlets. Fail to do so may result in a decrease in data quality.

**IMPORTANT:** While pipetting, do not go past the first stop on the pipet. Doing so may introduce bubbles into inlets, which can cause load fails.

**IMPORTANT!** At minimum, all 12 assay inlets and all 12 sample inlets for a partition must be filled.

- For unused assay inlets in active partitions, prepare 2.0 µL Assay Loading Reagent and 2.0 µL water per inlet.
- For unused sample inlets in active partitions, prepare 2.2 µL Sample Pre-Mix and 1.8 µL water per inlet.

After assay reagents and samples are pipetted into the inlets, they are loaded into their respective partitions in the thermo-conductive frame during the Load Script step. The reactions occur in the partitions. All usage of partitions and inlets is tracked by the Data Collection software for use with all IFCs.
To load the Flex Six IFC:
1 Make sure barrier plugs are placed on unused inlets to mitigate pipetting into the wrong wells and to track used/unused partitions.

2 Pipet one partition at a time by removing the barrier plugs for that particular partition.
3 Pipet 3 µL of each assay and each sample into their respective inlets. Do not replace the barrier plugs after pipetting.
4 Place the IFC in the IFC Controller HX.
5 Using the IFC Controller HX software, run the Load Mix (153x) script to load the samples and assays into the IFC. This should take 50 minutes.
6 When the Load Mix (153x) script has finished, remove the loaded IFC from the IFC Controller HX. Do not replace barrier plugs after loading.

You are now ready for data collection on the BioMark or BioMark HD System.

Data Collection Using the BioMark or BioMark HD System

Setting Up a Tracking File

If this is the first time you are running a Flex Six IFC after installing the Data Collection software or after restarting the computer, you will need to set up a tracking file. After the tracking file is set up initially, the software creates a directory and stores all Flex Six data in this location.

NOTE: If the directory containing tracking files changes, the Data Collection software will no longer be able to track IFC usage.

NOTE: If you have multiple BioMark Systems, you should create the tracking file in a location that can be accessed by all the BioMark Systems. Only usage tracking files should be stored this way; we do not recommend storing chip run data on a network.

1 On the Home page, select Tools > Flex Six Usage Tracking.
2 Click **New** to create a new tracking file, or you can select an existing tracking file by using the **Browse** button.

3 If you are creating a new tracking file, enter a name for the file and navigate to the desired storage location.

4 Click **Done**.

**Run the Flex Six IFC**

1 Double-click the Data Collection Software icon on the desktop.
2 Click **Start a New Run**.
3 Remove debris from the top of the IFC with clear tape.
4 Place the loaded IFC into the BioMark or BioMark HD System.
   a Choose project settings (if applicable).
   b Click **Next**.
5 Click **Load**.
6 Select the partitions you wish to run.
7 Select the Application, Reference, Probes:
   a Select the Application Type: **Gene Expression**.
   b Select the Passive Reference: **ROX**.
   c Select **single probe assay** and the probe type **EvaGreen**.
   d Click **Next**.
8 Select the thermal cycling protocol:
   - BioMark (Standard): **GE Flex six Melter v1**

<table>
<thead>
<tr>
<th>Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal Mix</td>
<td>25 °C</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>70 °C</td>
<td>60 min</td>
</tr>
<tr>
<td>Hot Start</td>
<td>95 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>PCR Cycle</td>
<td>Denaturation</td>
<td>96 °C</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>60 °C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ramp Rate:</th>
<th>Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
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<tr>
<td>Normal 2 °C/s</td>
<td>Thermal Mix</td>
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<td>30 min</td>
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<tr>
<td></td>
<td>70 °C</td>
<td>60 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hot Start</td>
<td>95 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>30 Cycles</td>
<td>Denaturation</td>
<td>96 °C</td>
<td>5 s</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>60 °C</td>
<td>20 s</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ramp Rate:</th>
<th>Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow 1 °C/s</td>
<td>Melting</td>
<td>60 °C—95 °C</td>
<td>3 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 °C/3 s</td>
</tr>
</tbody>
</table>

- BioMark (Fast): **GE Flex six Fast Melter v1**

<table>
<thead>
<tr>
<th>Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal Mix</td>
<td>25 °C</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>70 °C</td>
<td>60 min</td>
</tr>
<tr>
<td>Hot Start</td>
<td>95 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>PCR Cycle</td>
<td>Denaturation</td>
<td>96 °C</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>60 °C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ramp Rate:</th>
<th>Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Thermal Mix</td>
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<td>30 min</td>
</tr>
<tr>
<td></td>
<td>70 °C</td>
<td>60 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hot Start</td>
<td>95 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>30 Cycles</td>
<td>Denaturation</td>
<td>96 °C</td>
<td>5 s</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>60 °C</td>
<td>20 s</td>
</tr>
</tbody>
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<tr>
<th>Ramp Rate:</th>
<th>Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
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<tbody>
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<td>Slow 1 °C/s</td>
<td>Melting</td>
<td>60 °C—95 °C</td>
<td>3 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 °C/3 s</td>
</tr>
</tbody>
</table>

9 Confirm that Auto Expos is selected.
10 Click Start Run.

**Post Chip Run**

The Flex Six IFC requires a Post Run Process to ensure the 90-day life time. Run the five-minute Post Run script immediately after the BioMark or BioMark HD System run, prior to any storage of the IFC.

1 Immediately after the chip run, take out the IFC, load it into the IFC Controller HX, then run the Post Run (153x) script to relax the valves. This takes approximately five minutes.

2 You can now put the barrier plugs back into the used inlets. Remember to label the used barrier plugs so that you have a record of which partitions/inlets have been used.
Store a Used Flex Six IFC

**IMPORTANT:** Use the entire IFC within 90 days of first use.

**IMPORTANT:** After storage, you can load any unused partitions without the need to re-prime the used IFC.

Store the IFC at room temperature and protect it from dust until the next use.
- The IFC can be stored at room temperature on the bench top or in a drawer. It is not necessary to store the IFC in the dark, inside the silver wrapper, or inside the box.
- Lay the IFC flat with inlets facing up when storing. Do not store the IFC on its side or upside-down.
- After a run, put the barrier plugs back into the used inlets. Remember to label used barrier plugs so that you have a record of which partitions/inlets have been used.
- Between runs, be certain that the barrier plugs are in their proper positions for all unused partitions and that the IFC remains free of dust.
Flex Six IFC TaqMan Fast/Standard Gene Expression Workflow

Prime the Flex Six IFC

**IMPORTANT:** Use the Flex Six integrated fluidic circuit (IFC) within three months of opening the package. Load the IFC within 60 minutes of priming on first use.

**IMPORTANT:** Dripping control line fluid on the chip or in the inlets makes the IFC unusable.

**Note:** It is not necessary to evacuate air from the syringe prior to injection of control line fluid.

**Note:** You only need to prime the IFC on the first run. On subsequent use, skip this step.

For information on how to inject control line fluid, see the Fluidigm® Control Line Fluid Loading Procedure Quick Reference (PN 68000132).

For information on using the IFC Controller HX, see the IFC Controller MX-HX User Guide (PN 68000112).

During the first usage of each Flex Six IFC, prime the new Flex Six IFC:

1. Using the included syringes, inject 150 µL of control line fluid into each accumulator on the IFC. **Do not** remove the barrier plugs until you load the IFC.
2. Remove and discard the blue protective film from the bottom of the IFC.
3. Place the IFC into the IFC Controller HX, then run the Prime (153x) script. This should take about 15 minutes.

Preparing 10X Assays

**Note:** When preparing for an experiment that uses more than one partition, scale up the amount of assays appropriately.

**Note:** If you prefer to prepare a 10X assay stock, unused 10X assays can be stored at -20°C for up to three weeks.

In a DNA-free hood, prepare aliquots of 10X assays using volumes in Table 3 (scale up appropriately for multiple runs).
Prepare Sample Pre-Mix and Samples

Note: When preparing for an experiment that uses more than one partition, scale up the amount of samples appropriately.

Combine components in Table 4 to make the Sample Pre-Mix and final Sample Mixture in a 96-well plate, tubes, or tube strips.

1 In a DNA-free hood, combine the Sample Pre-Mix components to make enough for your experiment (33 µL/partition). Aliquot 2.2 µL of the pre-mix for each sample.

2 Remove the aliquots from the DNA-free hood and add 1.8 µL of preamplified cDNA to each, making a total volume of 4 µL in each aliquot.

Table 4 Sample Pre-Mix and Sample Mixture for TaqMan Fast/Standard Gene Expression

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Inlet (µL)</th>
<th>Volume per Inlet with Overage (µL)</th>
<th>Sample Pre-Mix for 1 Partition (µL) (15 reactions for ease of pipetting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STANDARD: TaqMan Gene Expression PCR Master Mix (2X) (Life Technologies, PN 4369016)</td>
<td>1.50</td>
<td>2.0</td>
<td>30.0</td>
</tr>
<tr>
<td>OR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAST: TaqMan Fast Advanced Master Mix (2X) (Life Technologies, PN 4444557)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20X GE Sample Reagent (Fluidigm, PN 100-6311)</td>
<td>0.15</td>
<td>0.2</td>
<td>3.0</td>
</tr>
<tr>
<td>Preamplified cDNA * (Added individually to Sample Pre-Mix)</td>
<td>1.35</td>
<td>1.8</td>
<td>–</td>
</tr>
<tr>
<td>Total Volume</td>
<td>3.00</td>
<td>4.0</td>
<td>–</td>
</tr>
</tbody>
</table>

* For more information about PreAmp and Exonuclease I treatment, refer to Gene Expression PreAmp with Fluidigm® PreAmp Master Mix and TaqMan® Assays (PN 100-5876)
Load the IFC

- There are a total of six independent partitions in each Flex Six™ IFC (partitions 1-6).
- Each partition has a 12 X 12 format (12 assay inlets and 12 sample inlets).
- Each partition can be run independently as a separate experimental run (at different times or on different days) or simultaneously (up to six partitions per run).

IMPORTANT: Vortex thoroughly and centrifuge all assay and sample solutions before pipetting into IFC inlets. Fail to do so may result in a decrease in data quality.

IMPORTANT: While pipetting, do not go past the first stop on the pipette. Doing so may introduce bubbles into inlets, which can cause load fails.

IMPORTANT: At minimum, all 12 assay inlets and all 12 sample inlets for a partition must be filled.

- For unused assay inlets in active partitions, prepare 2.0 µL Assay Loading Reagent and 2.0 µL water per inlet.
- For unused sample inlets in active partitions, prepare 2.2 µL Sample Pre-Mix and 1.8 µL water per inlet.

After assay reagents and samples are pipetted into the inlets, they are loaded into their respective partitions in the thermo-conductive frame during the Load Script step. The reactions occur in the partitions. All usage of partitions and inlets is tracked by the Data Collection software for use with all IFCs.

To load the Flex Six IFC:

1. Make sure barrier plugs are placed on unused inlets to mitigate pipetting into the wrong wells and to track used/unused partitions.
2. Pipet one partition at a time by removing the barrier plugs for that particular partition.
3. Pipet 3 µL of each assay and each sample into their respective inlets.
4. Do not replace the barrier plugs after pipetting.
5. Place the IFC in the IFC Controller HX.
6. Using the IFC Controller HX software, run the Load Mix (153x) script to load the samples and assays into the IFC. This should take 50 minutes.
7. When the Load Mix (153x) script has finished, remove the loaded IFC from the IFC Controller HX.

Do not replace barrier plugs after loading.

You are now ready for data collection on the BioMark or BioMark HD System.
Data Collection Using the BioMark or BioMark HD System

Setting Up a Tracking File

If this is the first time you are running a Flex Six IFC after installing the Data Collection software or after restarting the computer, you will need to set up a tracking file. After the tracking file is set up initially, the software creates a directory and stores all Flex Six data in this location.

**NOTE:** If the directory containing tracking files changes, the Data Collection software will no longer be able to track IFC usage.

**NOTE:** If you have multiple BioMark Systems, you should create the tracking file in a location that can be accessed by all the BioMark Systems. Only usage tracking files should be stored this way; we do not recommend storing chip run data on a network.

1. On the Home page, select **Tools > FLEXsix Usage Tracking**.

2. Click **New** to create a new tracking file, or you can select an existing tracking file by using the **Browse** button.

3. If you are creating a new tracking file, enter a name for the file and navigate to the desired storage location.

4. Click **Done**.

Run the Flex Six IFC

1. Double-click the Data Collection Software icon on the desktop.
2 Click Start a New Run.
3 Remove debris from the top of the IFC with clear tape.
4 Place the loaded IFC into the BioMark or BioMark HD System.
   a Choose project settings (if applicable).
   b Click Next.
5 Click Load.
6 Select the partitions you wish to run.
7 Select the Application, Reference, Probes:
   a Select Application Type: Gene Expression.
   b Select Passive Reference: ROX.
   c Select probe types.
   d Click Next.
8 Select a thermal cycling protocol:
   - BioMark™ or BioMark™ HD (Standard): GE FLEXsix Standard v1
     | Type               | Temperature | Time  |
     |-------------------|-------------|-------|
     | Thermal Mix       | 25 °C       | 30 min|
     |                   | 70 °C       | 60 min|
     | UNG and Hot Start | UNG         | 50 °C | 2 min |
     |                   | Hot Start    | 95 °C | 10 min|
     | PCR Cycle 40 Cycles | Denaturation | 95 °C | 15 s  |
     |                   | Annealing   | 60 °C | 1 min |
   - BioMark™ HD ONLY (Fast): GE FLEXsix Fast v2
     | Type               | Temperature | Time  |
     |-------------------|-------------|-------|
     | Thermal Mix       | 25 °C       | 30 min|
     |                   | 70 °C       | 60 min|
     |                   | 50 °C       | 2 min |
     | Hot Start         | 95 °C       | 1 min |
     | PCR Cycle 35 Cycles | Denaturation | 96 °C | 5 s   |
     |                   | Annealing   | 60 °C | 20 s  |
9 Confirm Auto Expos is selected.
10 Click Start Run.

Post Chip Run

The Flex Six IFC requires a Post Run Process to ensure the 90-day life time. Run the five-minute Post Run script immediately after the BioMark or BioMark HD System run, prior to any storage of the IFC.
1 Immediately after the chip run, take out the IFC, load it into the IFC Controller HX, then run the Post Run (153x) script to relax the valves. This takes approximately five minutes.

2 You can now put the barrier plugs back into the used inlets. Remember to label the used barrier plugs so that you have a record of which partitions/inlets have been used.

Store a Used Flex Six IFC

**IMPORTANT:** Use the entire IFC within 90 days of first use.

**IMPORTANT:** After storage, you can load any unused partitions without the need to re-prime the used IFC.

Store the IFC at room temperature and protect it from dust until the next use.

- The IFC can be stored at room temperature on the bench top or in a drawer. It is not necessary to store the IFC in the dark, inside the silver wrapper, or inside the box.
- Lay the IFC flat with inlets facing up when storing. Do not store the IFC on its side or upside-down.
- After a run, put the barrier plugs back into the used inlets. Remember to label used barrier plugs so that you have a record of which partitions/inlets have been used.
- Between runs, be certain that the barrier plugs are in their proper positions for all unused partitions and that the IFC remains free of dust.
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

WARNING! Do not modify the instruments. Unauthorized modifications may create a safety hazard.

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.

CAUTION! The FC1™ Cycler chuck gets hot and can burn your skin. Please use caution when working near the chuck.

For a full list of the symbols on the instrument, refer to the BioMark™ HD Data Collection Software User Guide (PN 100-2451) or the BioMark™/EP1™ Data Collection Software User Guide (PN 68000127).
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 and reagents should be handled and disposed of in accordance with federal, state, regional, and local laws for hazardous waste management and disposal.

Do not dispose of this product in unsorted municipal waste. This equipment may contain hazardous substances that could affect health and the environment. Use appropriate take-back systems when disposing of materials and equipment.

Learn more at fluidigm.com/compliance
For technical support visit fluidigm.com/support.