

QuickGuide: RealFast™ Genotyping on Bio-Rad CFX96

Setup for Genotyping Assays:

- Open the Bio-Rad CFX Maestro software (QuickGuide is based on version 2.2).
- In the Startup Wizard select instrument **CFX96** and run type **User-defined**.
- In the Run Setup select **Create New** within the **Protocol** tab. The **Protocol Editor** opens.
 - Select a sample volume of **20 µl** and setup the PCR program: **3 min at 95°C** followed by **40 cycles of 15 sec at 95°C** and **1 min at 60°C**. Press **OK** and save the protocol file. Press **Next**.
- In the tab called **Plate** select **Create New**. The **Plate Editor** opens.
 - Select **Settings > Plate Type** and choose the correct type of plate.
 - Select Scan Mode **All Channels**.
 - Click **Select Fluorophores** and select
 - **FAM** and **HEX** in case you use **singleplex** RealFast™ Assays, or
 - **FAM, HEX, ROX** and **Cy5** in case you use **multiplex** RealFast™ Assays.
 - Select wells by clicking in the well selector and choose the sample type (**NTC** or **Unknown**); a minimal setup should contain 2 replicates of a No Template Control (NTC), a control homozygous for allele 1 (control 1), a control homozygous for allele 2 (control 2) and an unknown sample.
 - Click **Load** check boxes to load fluorophores
 - **FAM** and **HEX** for **singleplex**, or
 - **FAM, HEX, ROX** and **Cy5** for **multiplex** RealFast™ Assays.
 - Type target names and sample name and press **Enter**.
 - Click check box to load **Replicate** number.
 - Define **Well Groups** in case you are running several assays at the same time.
 - Press **OK** and save the plate file.
- Press **Next** and load your PCR tubes; start the run.

| | | |
|--------------|--------|----------------|
| 1 | 95.0 | C for 3:00 |
| 2 | 95.0 | C for 0:15 |
| 3 | 60.0 | C for 1:00 |
| + Plate Read | | |
| 4 | GOTO 2 | .39 more times |
| END | | |

Analysis of Genotyping Assays: Single Threshold Method (recommended)

- Open the data file: **File > Open > Data File**.
- Select the **Allelic Discrimination** tab.
 - Select the well group (top right) in case you were running several assays at the same time.
 - Select **Fluorophores**: choose either
 - **FAM** and **HEX** (for one marker) or
 - **ROX** and **Cy5** (for the other marker).
 - In **Settings** choose **Cq Determination Mode > Single Threshold**.
 - The software automatically analyses the raw data and generates an Allelic Discrimination chart and genotyping results are displayed in the results table.



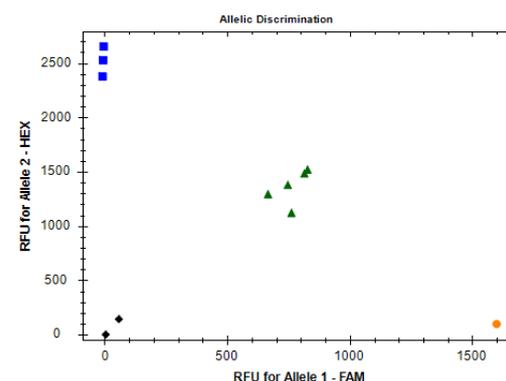
Selected Fluorophores

X: FAM Y: HEX

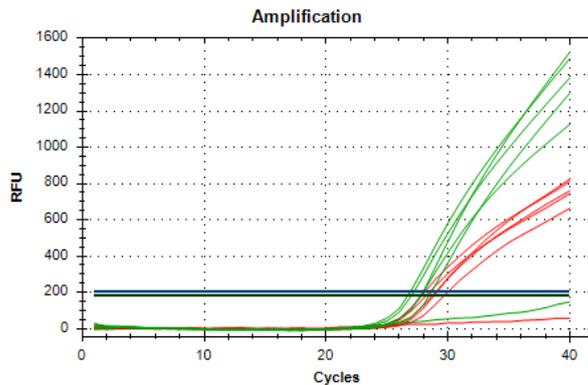
Select cycle: 40

Positives for allele 1 or allele 2 group along the horizontal and vertical axis, while heterozygotes are displayed in the middle of the Allelic Discrimination chart.

- The correct assignment for each well should be verified in the **Quantification** tab. Each control displays amplification in the respective channel only. In most assays **WT-Controls** are **HEX** or **Cy5** labeled, whereas **MUT-Controls** are **FAM** or **ROX** labeled.



The WT-Control shows an amplification in the HEX or Cy5 channel, no amplification in the FAM or ROX channel should be visible. If the threshold line is below the background signal of FAM or ROX, then manually move the threshold line above the background. Do the same for MUT-Control and verify that there is an amplification in the FAM or ROX channel but no signal in the HEX or Cy5 channel.

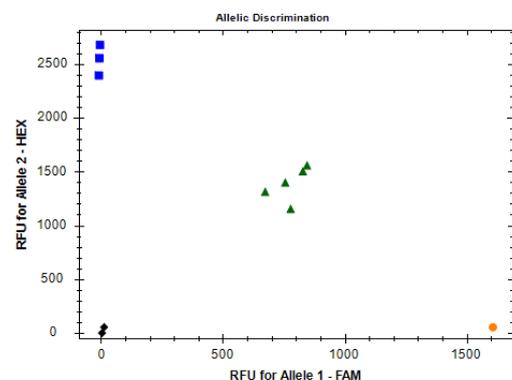


» **Note:** For assignment of fluorophores to hydrolysis probes see *Instructions for Use of the respective RealFast™ Assay*. «

- To generate a report, select **Tools > Reports**. In the pop-up window **Report** select **Allelic Discrimination** and adjust the report according to your needs.

Analysis of Genotyping Assays: Regression (optional)

- Open the data file: **File > Open > Data File**
- Select the **Allelic Discrimination** tab.
 - Select the well group (top right) in case you were running several assays at the same time. In **Settings** choose **Cq Determination Mode > Regression**
 - The software automatically analyses the raw data and generates Allelic Discrimination chart and genotyping results are displayed in the results table.
- The correct assignment for each well should be verified in the **Quantification** tab.
- To generate a report, select **Tools > Reports**. In the pop-up window **Report** select **Allelic Discrimination** and adjust the report according to your needs.



Optional - Fluorescence Drift Correction

In the rare event that clusters were not correctly assigned to the corresponding genotype in the allelic discrimination tab, a Fluorescence Drift Correction may be beneficial:

- In **Settings** choose **Baseline Setting > Apply Fluorescence Drift Correction**.

