QuickGuide: RealFast™ Genotyping on AB QuantStudio 5

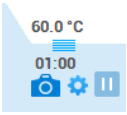
Setup for Genotyping Assays:

- Open the QuantStudio Design & Analysis Software and click **Create New Experiment**.

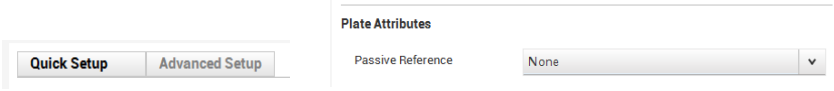
- Define **Experiment Properties**
  Name: Name of experiment
  Instrument type: **QuantStudio 5 System**
  Block type: **96-Well 0,2 ml Block**
  Experiment type: **Genotyping**
  Chemistry: **TaqMan® Reagents**
  Run mode: **Standard**

- In **Method** select a sample volume of **20 µl** and setup the PCR program:
  *optional but recommended: include Pre-PCR Read **30 sec** at **60°C.***

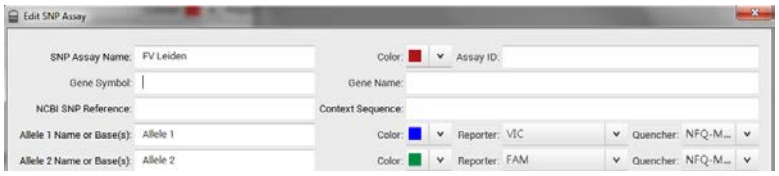
  - Holding Stage: **3 min** at **95°C**
  - Cycling Stage: **40 cycles** of **15 sec** at **95°C** and **1 min** at **60°C**.
    Make sure **Data Collection On** is enabled.

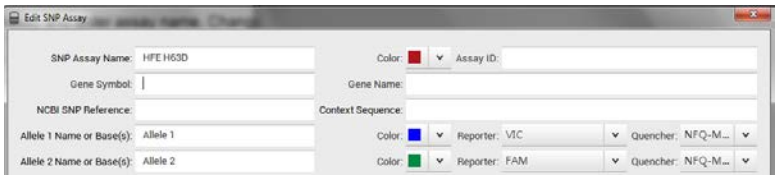


- In **Plate Quick Setup** define **None** as **Passive Reference** dye.



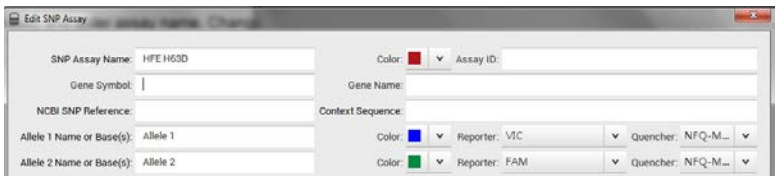
- In **Plate Advanced Setup** assign **SNP Assay(s)** and **Samples** to selected wells.
  - Select **SNP Assay 1** and click **Action** and **Edit** and enter assay name. Keep default settings for reporter dyes:
    Allele 1 = VIC (corresponds to HEX-labeled probe)
    Allele 2 = FAM (corresponds to FAM-labeled probe)
    Quencher = NFQ-MGB



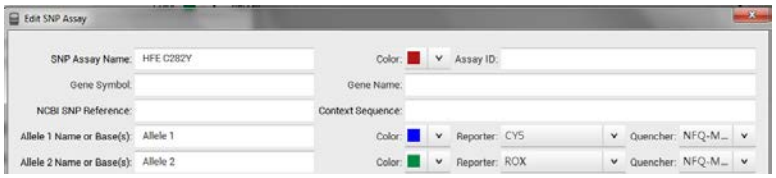


*» **Note**: In case you want to run several **different singleplex** RealFast™ Assays in parallel, create New SNP Assay for each of the markers to be analyzed. «*

*» **Note**: For **multiplex** RealFast™ Assays **two SNP Assays have to be created** – one for each marker. «*



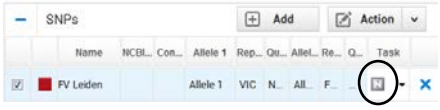
  - Click **Add** to open a **New SNP Assay** and enter assay name. Change reporter dyes:
    Allele 1 = CY5 (corresponds to CY5-labeled probe)
    Allele 2 = ROX (corresponds to ROX-labeled probe)
    Quencher = NFQ-MGB



- Click **Add** repeatedly to enter all your samples and controls.

- Select the total number of wells per assay (2 Negative Controls + Positive Controls + number of samples) by click+drag in the grid in the Plate Layout.
- Assign SNP assay to selected wells by ticking the box next to the **SNP Assay(s)**.

- Define your **Negative Control:**
  - Select a replicate (2 wells) in the plate layout by click+drag.
  - Assign Negative Control to corresponding well by ticking the box next to the sample name.
  - Select “N” in the pull-down menu **Task**.



- Define your **Positive Controls:**
  - In the plate layout select a well for each **Positive Control** by mouse click.
  - Assign Positive Control to corresponding well by ticking the box next to the sample name.
  - In the pull-down menu **Task** select
    - **“1/1”** for **HEX-** or **Cy5-positive Control** (in most cases WT-Control),
    - **“2/2“** for **FAM-** or **ROX-positive Control** (in most cases MUT-Control),
    - **“1/2”** for **HEX-/FAM-** or **CY5-/ROX-positive Control** (mix WT- and MUT-Control 1:1)

  Alternatively, you can define your Positive Controls as “U” like your samples (see below).

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

- Define your **Samples:**
  - In the plate layout select a well for each sample by mouse click.
  - Assign sample to corresponding well by ticking the box next to the sample name.
  - Select “U” in the **Task** pull-down menu.

- Load your reaction plate into the QuantStudio 5 instrument. Go to **Run** and press **START RUN**, select the serial number of the machine to start the run.

Analysis of Genotyping Assays:

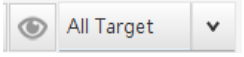
After completing a run or after opening an **Existing Experiment** the software displays the **Results**:

- The **Amplification Plot** appears automatically**.**

- If you have **different** RealFast™ Assays in one run, select appropriate samples by clicking **Action** and selecting **Select SNP Assay** in the dropdown menu.

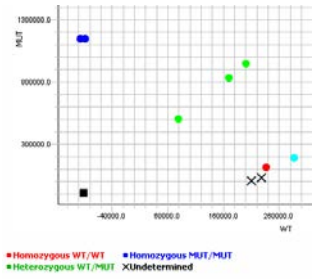
*» **Note**: With **multiplex** RealFast™ Assays all 4 fluorophores can be depicted in one **Amplification Plot**!*

- To change the threshold according to the IFU click **All Targets,** select a target and move the threshold with the curser, then click **Analyze**.

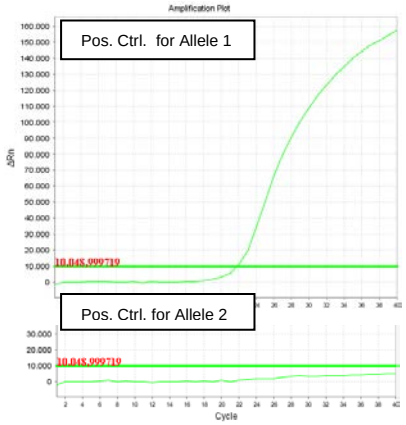


- **Allelic Discrimination Plot**: Cartesian Plot type

  - Always verify correct assignment of samples in the **Allelic Discrimination Plot**: e.g. select WT-Control in the **Plate Advanced Setup** > corresponding point on the Allele 1 axis turns from red to turquoise.
  - In case a sample appears as undetermined = **x**, verify correct amplification in the **Amplification Plot** and manually assign genotype with **Apply Call** in dropdown menu at the eye icon.



- Click **Amplification Plot** to control correct amplification of <u>all controls</u>:
  - Select the following plot settings at the eye icon:
    > **Plot Type**: ΔRn vs Cycle
    > **Graph Type**: Linear
    > **Plot Color**: Target.
  - In the dropdown menu **All Target** choose **Allele 1.** In **View Plate Layout** select your **Positive Control** for **Allele 1** (mostly WT-Control) - an amplification curve should be visible in the Amplification Plot. Select your **Positive Control** for **Allele 2** (mostly MUT-Control) - NO amplification curve should be visible.
  - The threshold for **Allele 1** should be above the background signal of the **Positive Control** for **Allele 2**. If not, click on the eye icon and disable tickbox for **Auto** Threshold. Set the threshold manually by clicking on the threshold line in the plot and moving it above the background signal.
  - In the dropdown menu **All Target** choose **Allele 2.** Select your **positive Control** for **Allele 2** (mostly MUT- Control) - an amplification curve should be visible in the Amplification Plot. Select your **Positive Control** for **Allele 1** (mostly WT-Control) - NO amplification curve should be visible.



  - The threshold for **Allele 2** should be above the background signal of the **Positive Control** for **Allele 1**. If not proceed as described above for threshold setting.
  - Verify absence of any contamination in the **Negative Control**. No amplification should be visible, neither for Allele 1 nor for Allele 2.
  - Choose **All Target.** Select your samples one by one and verify positive amplification.

- To show results as table: click 
  - Adjust the table according to your needs by selecting/deselecting the listed features in **View**.

- To print a report select **File > Print Report** in the upper menu bar:
  - Select data for the report according to your needs.

  - If you wish to export your data go to the **Export** tab and choose the file type and content