QuickGuide: RealFast™ Genotyping on AB 7500 Fast

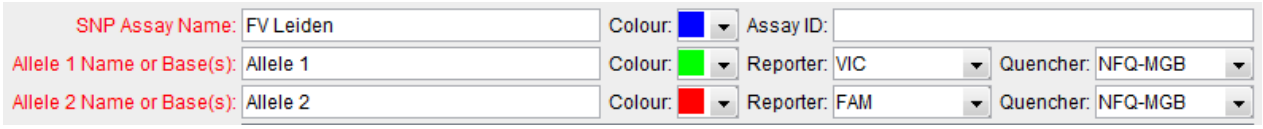
Setup for Genotyping Assays:

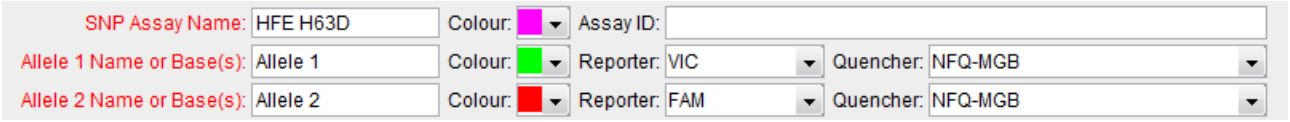
- Open the **AB 7500 software** (QuickGuide is based on version 2.0.6) and click **Advanced Setup.** In the Experiment Menu go to **Setup.**

- In **Experiment Properties** define Experiment: **Name**
  Instrument: **7500 Fast**
  Type of experiment: **Genotyping**
  Reagents: **TaqMan® Reagents**
  Ramp speed: **Standard**

- In **Plate Setup** assign **SNP Assay(s)** and **Samples** to selected wells.
  - Select **SNP Assay 1** and click **Edit > Edit SNP Assay** to 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

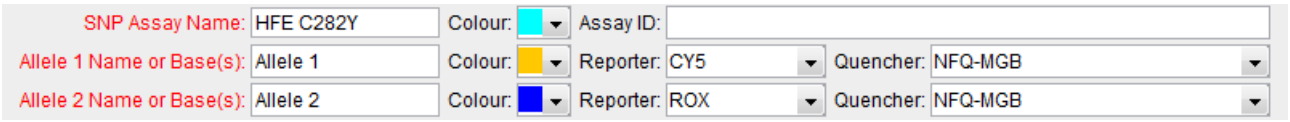
*» **Note**: In case you want to run several **different singleplex** RealFast™ Assays in parallel, create New SNP Assay for each of the markers to be analyzed. «*



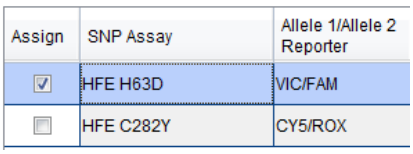


*» **Note**: For **multiplex** RealFast™ Assays **two SNP Assays have to be created** – one for each marker. «*

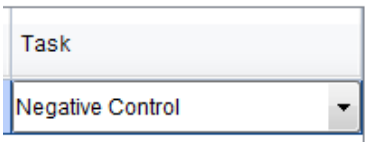
- Click **Create New SNP** Assay and enter assay name. Change reporter dyes:
  Allele 1 = CY5 (corresponds to CY5-labeled probe)
  Allele 2 = ROX (corresponds to ROX-labeled probe)
  Quencher = NFQ-MGB



- Click **Add New Sample** repeatedly to enter all your samples and controls.
- Select **None** as passive reference dye.
- In the **View Plate Layout** select the total number of wells per assay (2 Negative Controls + Positive Controls + number of samples) by click+drag in the grid.
- Assign SNP assay to selected wells by ticking the **Assign** box in the field **Assign SNP Assay(s) to ….**



- Define your **Negative Control:**
  - Select a replicate (2 wells) in the plate layout by click+drag.
  - Select **Negative Control** in the pull-down menu **Task**.



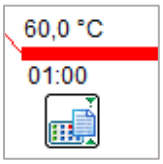
- Define your **Positive Controls:**
  - In the plate layout select a well for each **Positive Control** by mouse click.
  - Assign Positive Control to corresponding well by ticking the **Assign** box in the field **Assign Sample to ….**
    In the pull-down menu **Task** select
    Positive Control Allele 1/Allele1 for **HEX-** or **Cy5-positive Control** (in most cases WT-Control),
    Positive Control Allele 2/Allele2 for **FAM-** or **ROX-positive Control** (in most cases MUT-Control),
    Positive Control Allele 1/Allele2 for **HEX-/FAM-** or **CY5-/ROX-positive Control** (mix WT- and MUT-Control 1:1)
    Alternatively, you can define your Positive Controls as "Unknown" 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 **Assign** box in the field **Assign Sample to ….**

- In **Run Method** select a sample volume of **20 µl** and setup the PCR program:
  *optional but recommended: include Pre-PCR Read **1 min** 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.
  - Post-PCR Read: **1 min** at **60°C.** Make sure **Data Collection On** is enabled.

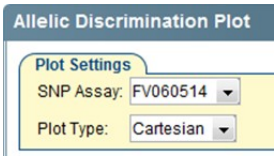


- **Save** the experiment.

- Load your PCR tubes/plate and press **START RUN** (green button) to start the run.

Analysis of Genotyping Assays:

After completing a run or after opening a genotyping data file the software displays the Experiment Menu **Analysis:**

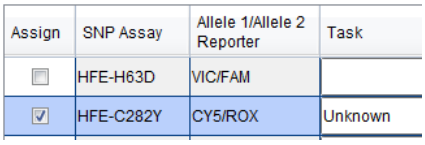


- Results automatically appear in the **Allelic Discrimination Plot.**

- If you have **different** RealFast™ Assays in one run, switch between assays in the dropdown menu **SNP Assay**.

*» **Note**: With **multiplex** RealFast™ Assays the two markers (corresponding to HEX/FAM and Cy5/ROX, respectively) have to be analyzed **one after the other** ! «*

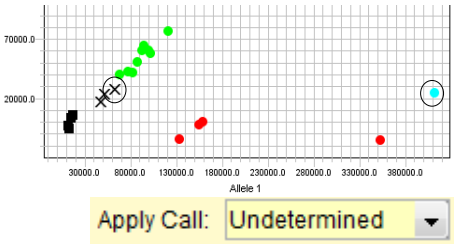
  - *Switch between the markers by going back to the Experiment Menu **Setup**.*
  - *Select all samples and deselect the SNP assay for the marker chosen at the beginning, then tick the box of the SNP assay for the other marker.*
  - *The information about the Task will be lost, therefore define the Negative Controls and the Positive Controls (optional) again by selecting from the dropdown menu.*
  - *Go back to **Analysis** and press **Reanalyse** (green buttom top right).*



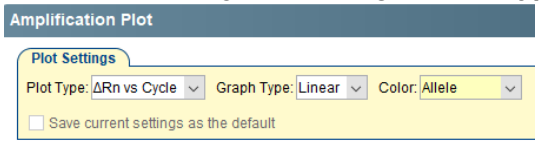
Assign	SNP Assay	Allele 1/Allele 2 Reporter	Task
☐	HFE-H63D	VIC/FAM	
☑	HFE-C282Y	CY5/ROX	Unknown

- Use **Plot Type**: Cartesian

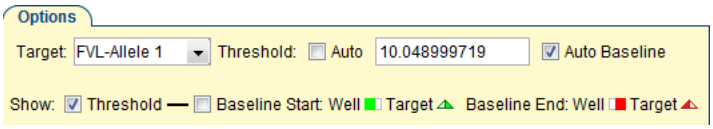
  - Always verify correct assignment of samples in the **Allelic Discrimination Plot**: e.g. select WT-Control in the **View Plate Layout** > corresponding point on the Allele 1 axis turns from red to turquoise.
  - In case a sample appears as undetermined = **x**, verify correct amplification in the **Amplification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

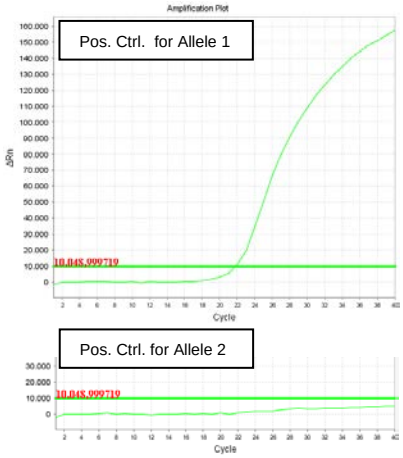


- Click **Amplification Plot** to control correct amplification of <u>all controls</u>:
  - Select the following **Plot Settings:** > **Plot Type**: ΔRn vs Cycle > **Graph Type**: Linear > **Color**: Allele.



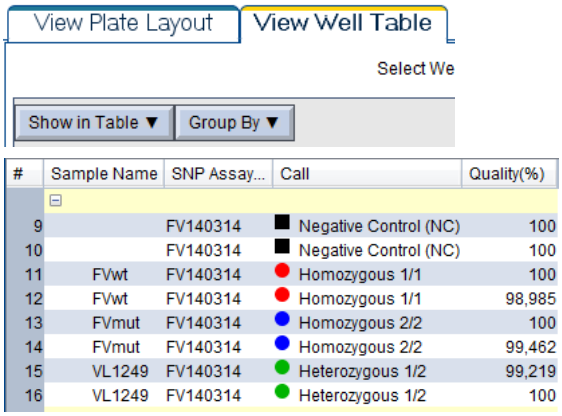
  - In the dropdown menu **Target** choose **Allele 1.** In **View Plate Layout** select your **Positive Control** for **Allele 1** (mostly WT-Control) - an amplification curve should be visible in the Amplification Plot.
    Select your **Positive Control** for **Allele 2** (mostly MUT-Control) - NO amplification curve should be visible.
  - The threshold for **Allele 1** should be above the background signal of the **Positive Control** for **Allele 2**. If not, disable tickbox for **Auto** Threshold and set threshold manually by clicking on the threshold line in the plot and moving it above the background signal.





  - In the dropdown menu **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.
  - In the dropdown menu **Target** choose **All.** Select your samples one by one and verify positive amplification.

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



#	Sample Name	SNP Assay...	Call	Quality(%)
9		FV140314	Negative Control (NC)	100
10		FV140314	Negative Control (NC)	100
11	FVwt	FV140314	Homozygous 1/1	100
12	FVwt	FV140314	Homozygous 1/1	98,985
13	FVmut	FV140314	Homozygous 2/2	100
14	FVmut	FV140314	Homozygous 2/2	99,462
15	VL1249	FV140314	Heterozygous 1/2	99,219
16	VL1249	FV140314	Heterozygous 1/2	100

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

- To export results in an Excel or Text file click **Export ….** in the upper menu bar:
  - Define **Export Properties** and **Customise Export**.