

QuickGuide: RealFast™ Genotyping on AB 7500 Fast

Setup for Genotyping Assays:

- Open the **AB 7500 software** 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. «

SNP Assay Name:	FV Leiden	Colour:	█	Assay ID:	
Allele 1 Name or Base(s):	Allele 1	Colour:	█	Reporter:	VIC
Allele 2 Name or Base(s):	Allele 2	Colour:	█	Reporter:	FAM
				Quencher:	NFQ-MGB

SNP Assay Name:	HFE H63D	Colour:	█	Assay ID:	
Allele 1 Name or Base(s):	Allele 1	Colour:	█	Reporter:	VIC
Allele 2 Name or Base(s):	Allele 2	Colour:	█	Reporter:	FAM
				Quencher:	NFQ-MGB

- » **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

SNP Assay Name:	HFE C282Y	Colour:	█	Assay ID:	
Allele 1 Name or Base(s):	Allele 1	Colour:	█	Reporter:	CY5
Allele 2 Name or Base(s):	Allele 2	Colour:	█	Reporter:	ROX
				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**

Assign	SNP Assay	Allele 1/Allele 2 Reporter
<input checked="" type="checkbox"/>	HFE H63D	VIC/FAM
<input type="checkbox"/>	HFE C282Y	CY5/ROX

- 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**.

Task
Negative Control

- 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)

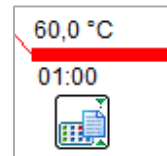
Alternatively, you can define your Positive Controls as "Unknown" like your samples (see below).

» **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.

Include: Pre-PCR Read Amplification Post-PCR Read

- 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.

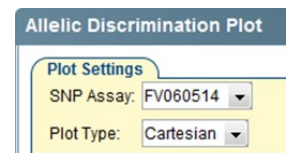


- **Save** the experiment.
- Load your PCR tubes/plate and press **START RUN** (green button) to start the run.

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**.



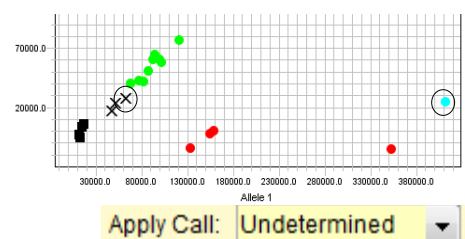
» **Note:** With **multiplex** RealFast™ Assays the two markers (corresponding to HEX/FAM and Cy5/ROX, respectively) have to be analyzed **one after the other** ! «

- 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 button top right).

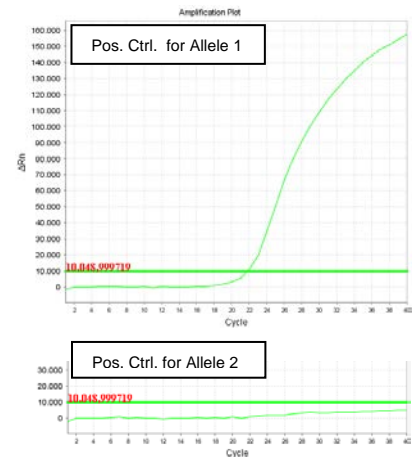
Assign	SNP Assay	Allele 1/Allele 2 Reporter	Task
<input type="checkbox"/>	HFE-H63D	VIC/FAM	
<input checked="" type="checkbox"/>	HFE-C282Y	CY5/ROX	Unknown

- 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 all controls:
 - 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.



Options

Target: FVL-Allele 1 Threshold: Auto 10.048999719 Auto Baseline

Show: Threshold Baseline Start: Well Target Baseline End: Well Target

- 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**.
- 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**.

View Plate Layout View Well Table

Select We

Show in Table ▼ Group By ▼

#	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