

QuickGuide: RealFast™ Genotyping on StepOne™

Setup for Genotyping Assays:

- Open the StepOne™ Software and click **Advanced Setup**. In the Experiment Menu go to **Setup**.
- In **Experiment Properties** define Experiment: **Name**
Instrument: **StepOne™** (48 Wells) or **StepOnePlus™** (96 Wells)
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

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

- Click **Create New SNP Assay** in case you wish to analyze several SNP assays in parallel.
- Click **Add New Sample** repeatedly to enter all your samples and controls.
- Select **ROX** 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 assay to selected wells by ticking the **Assign** box in the field **Assign SNP Assay(s) to....**

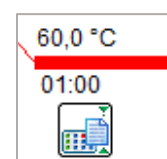
Assign	SNP Assay
<input checked="" type="checkbox"/>	FV Leiden

- 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-positive Control** (in most cases WT-Control),
Positive Control Allele 2/Allele2 for **FAM-positive Control** (in most cases MUT-Control),
Positive Control Allele 1/Allele2 for **HEX-/FAM-positive Control** (mix WT- and MUT-Control 1:1)
Alternatively, you can define your Positive Controls as „Unknown“ like your samples (see below).
- 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 30 sec at 60 °C.

Task
Negative Control

- 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.

Include:	<input checked="" type="checkbox"/> Pre-PCR Read	<input checked="" type="checkbox"/> Amplification	<input checked="" type="checkbox"/> Post-PCR Read
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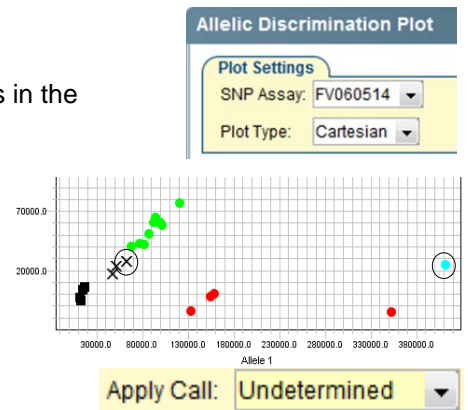


- **Save** the experiment.
- Load your PCR tubes/plate and press **START RUN** (green button) to start the run.

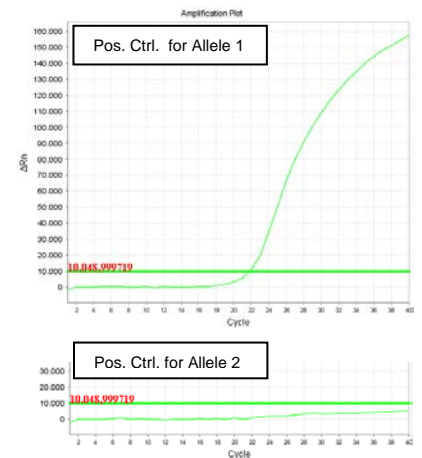
Analysis of Genotyping Assays:

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 more than one SNP assay per run, switch between assays in the dropdown menu **SNP Assay**.
 - 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**: ΔR_n 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