QuickGuide: RealFast™ Genotyping on Rotor-Gene® 6000

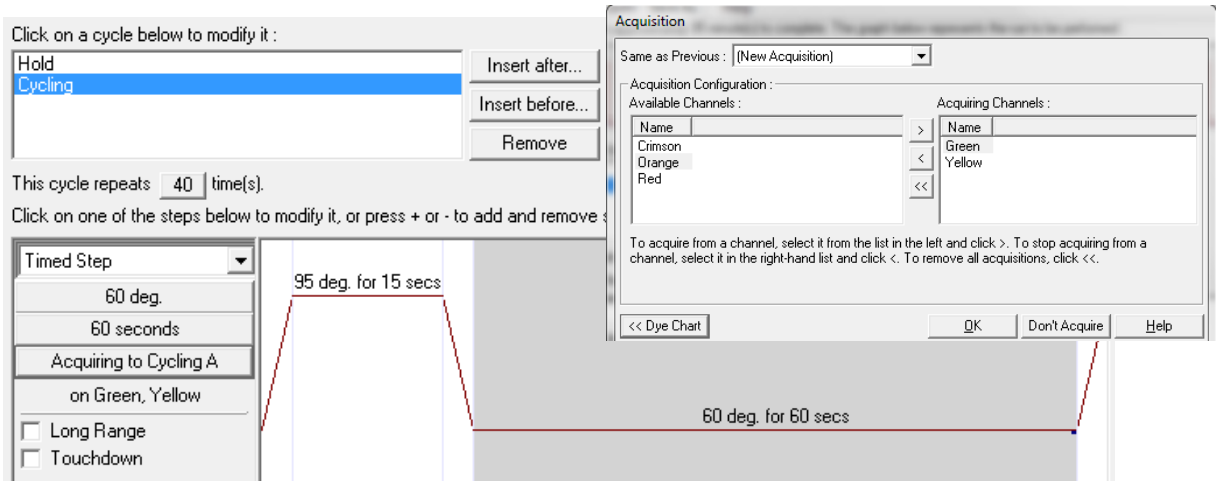
Setup for Genotyping Assays:

- Launch the **Rotor-Gene® 6000 software**.
- From the upper menu press **New.**
  - Within the New Run window select the **Advanced** tab.
  - Choose **Two Step** and press **New**.

- The **New Run Wizard** window will appear.
  - Select the **Rotor Type** and tick the box **Locking Ring Attached**. Press **Next.**
  - Define a Reaction Volume of **20 µl.** *Optional: enter an operator name and run specific notes.* Press **Next.**
  - Click **Edit Profile ….**, enter the following PCR program and confirm with **OK**:

For **singleplex** RealFast™ Assays use:

	Cycles	Temperature [C°]	Duration [mm:ss]	Acquiring
Hold	-	95	03:00	-
Cycling	40	95	00:15	-
		36-well rotor: **56** 72-well rotor: **60**	01:00	Acquiring to Cycling A on **Green** and **Yellow**



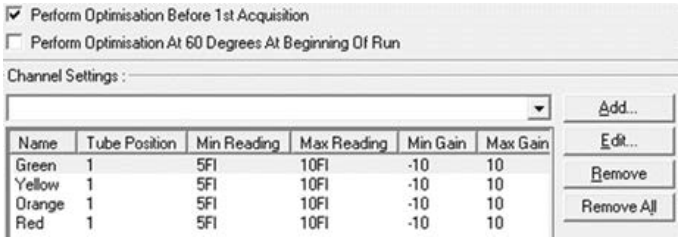
For **multiplex** RealFast™ Assays use:

	Cycles	Temperature [C°]	Duration [mm:ss]	Acquiring
Hold	-	95	03:00	-
Cycling	40	95	00:15	-
		36- well & 72-well rotor: **60**	01:00	Acquiring to Cycling A on **Green, Yellow, Red, Orange**

- Click **Gain Optimisation ....**

- Select **Green** from the drop down menu **Channel Settings.** Press **Add ....**
  Keep the default values in **Auto-Gain Optimisation Channel Settings** and confirm.

  Select **Yellow** from the drop down menu **Channel Settings.** Press **Add ....**
  Keep the default values in **Auto-Gain Optimisation Channel Settings** and confirm.



Repeat this procedure with **Orange** and **Red** for **multiplex** RealFast™ Assays.
Tick the box **Perform Optimisation Before 1st Acquisition.** Close window.

Setting	Value
FAM1 Gain	5
Green Gain	5
HEX1 Gain	5
Yellow Gain	5
Auto-Gain Optimisation	Before First Acquisition
Rotor	72-Well Rotor
Sample Layout	1, 2, 3, ...
Reaction Volume (in microliters)	20

*» **Note**: In case you carry out different RealFast™ Assays within one run, it is advisable to do gain optimisation individually for each of the assays. Create new channels, e.g. FAM1, HEX1,……for data acquisition. «*

- Proceed with **Next.**

- Press **Start Run**.
- Define a file name and press **Save**.
- The **New Run Wizard** window will pop up, where the sample **Names** and **Types** (NTC, Unknown and Positive Controls) can be defined.
- Press **Finish** and the **Run Progress** window will appear.

Analysis of Genotyping Assays using Allelic Discrimination:

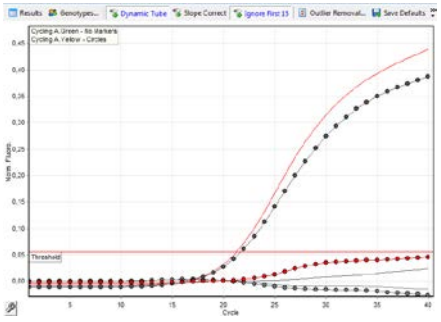
- Launch the **Rotor-Gene® 6000 software**.
- In the upper menu press **Open** and load your genotyping data file.
- The software displays the **Raw Channel (Cycling A. Green)** window.

- Press the **Analysis** button from the upper menu and choose **Allelic Discrimination**.
  - Select **Cycling A. Green** and **Cycling A. Yellow** and press **Show**.

- In the **Allelic Discrimination Analysis** window press **Genotypes.**
  - According to your assay, select the appropriate channels for **wild type (WT)** (mostly **Cycling A. Yellow**), **mutant (MUT)** (mostly **Cycling A. Green**) and **Heterozygous** (**Cycling A. Green** and **Cycling A. Yellow**). Confirm with **OK.**

Genotype	Reacting Channels	
Wild Type		Cycling A.Yellow
Heterozygous	Cycling A.Green	Cycling A.Yellow
Mutant	Cycling A.Green	

  - Press **Dynamic Tube.**
    *Optional: If necessary, use either **Slope Correct** or **Ignor First** for optimization of amplification curves, whatever fits best.*



- Set the **Discrimination Threshold** as following:
  - Click on the threshold button in the lower right panel. In the analysis graph click and drag the red threshold line above the background signals of the positive controls.

- Results are shown in the **Allelic Discrimination Results** window.

- To generate a report press **Reports** form the upper menu and select the relevant report in the **Report Browser** to be shown and printed.

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

- Repeat the analysis for the other marker by pressing **Analysis → Allelic Discrimination**. Then select **Cycling A. Orange** and **Cycling A. Red** and press **Show**.

Genotype	Reacting Channels	
Wild Type		Cycling A.Red
Heterozygous	Cycling A.Orange	Cycling A.Red
Mutant	Cycling A.Orange	

- Proceed as described above. For most assays **wild type (WT)** corresponds to Cycling A. Red and **mutant (MUT)** to Cycling A. Orange.

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

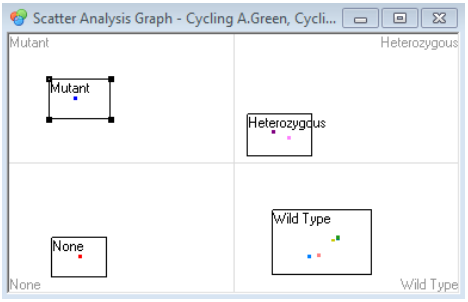
Analysis of Genotyping Assays using Scatter Plot:

- Launch the **Rotor-Gene® 6000 software**.
- In the upper menu press **Open** and load your genotyping data file.
- The software displays the **Raw Channel (Cycling A. Green)** window.

- Press the **Analysis** button from the upper menu and choose **Scatter Graph Analysis**.
  - Select **Cycling A. Green** and **Cycling A. Yellow** and press **Show**.

- In the **Scatter Analysis** window press **Genotypes.**
  - According to your assay, select the appropriate channels for **wild type (WT)** (mostly **Cycling A. Yellow**), **mutant (MUT)** (mostly **Cycling A. Green**) and **heterozygous** (**Cycling A. Green** and **Cycling A. Yellow**). Confirm with **OK.**
  - Press **Dynamic Tube.**
    *Optional: If necessary, use either **Slope Correct** or **Ignor First** for optimization of amplification curves, whatever fits best.*

Genotype	Reacting Channels	
Wild Type		Cycling A.Yellow
Heterozygous	Cycling A.Green	Cycling A.Yellow
Mutant	Cycling A.Green	



- Data points are shown in the **Scatter Analysis Graph** window.
  - Draw a rectangle around the data points in each quarter > define the corresponding genotypes as **Wild Type**, **Mutant** and **Heterozygous**, respectively, and the No-Template Control as **None**.

- Samples and their corresponding genotypes are shown in the **Scatter Analysis Result** window.

- To generate a report press **Reports** form the upper menu and select the relevant report in the **Report Browser** to be shown and printed.

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

- Repeat the analysis for the other marker by pressing **Analysis → Allelic Discrimination**. Then select **Cycling A. Orange** and **Cycling A. Red** and press **Show**.

- Proceed as described above. For most assays Wild Type corresponds to Cycling A. Red and Mutant to Cycling A. Orange.

Genotype	Reacting Channels	
Wild Type		Cycling A.Red
Heterozygous	Cycling A.Orange	Cycling A.Red
Mutant	Cycling A.Orange	

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