


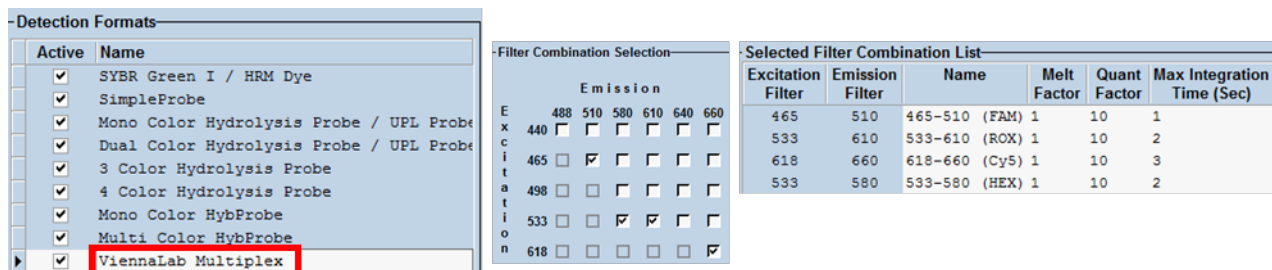
# QuickGuide: RealFast™ Genotyping on LightCycler® 480II

## Setup for Genotyping Assays:

- Open the **LightCycler® 480 software** and login with your username and password.

### » Note for using multiplex RealFast™ Assays:

- For correct analysis of multiplex RealFast™ Assays, it is inevitable to create a **Color Compensation (CC) File** in order to avoid crosstalk between detection channels.  
We recommend to use LightMix® Universal Color Compensation Hexaplex (TIB MOLBIO) for performing a CC experiment. Follow the manufacturer's instructions. Note that CC Files are unique for the instrument they were created on and cannot be used for other instruments.
- Open the **LightCycler® 480 software** and login with your username and password.
- Open **Tools** by clicking on the Tool-Button  then select: **Detection Formats**.
  - Define a new **Detection Format** with following filter combinations and change Quant Factor and Max Integration Time according to the table below:



Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
465	510	465-510 (FAM)	1	10	1
533	610	533-610 (ROX)	1	10	2
618	660	618-660 (Cy5)	1	10	3
533	580	533-580 (HEX)	1	10	2

- Name your Detection Format, e.g. ViennaLab Multiplex.
- Close the Tool menu. «

- Choose **New Experiment**.
- Define your PCR program in the **Run Protocol** tab.
  - For **singleplex** RealFast™ Assays select **Dual Color Hydrolysis Probe / UPL Probe** as Detection Format.
  - For **multiplex** RealFast™ Assays select correct Detection Format, i.e. **ViennaLab Multiplex**.
  - Select a reaction volume of **20 µl** and setup the **Program**:

Program Name	Cycles	Analysis Mode	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)
pre-incubation	1	None	95°C	none	00:03:00	4.4
amplification	40	Quantification	95°C	none	00:00:15	4.4
			60°C	single	00:01:00	2.2
cooling	1	None	40°C	none	00:00:30	2.2

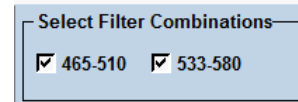
- Add program steps in the **Programs** window with "+" and edit **Cycles** and **Analysis Mode**. To edit **Target (°C)**, **Acquisition Mode** and **Hold**, click on the corresponding step in the **Program Name** window and change parameters or add steps ("+" in the **Temperature Targets** window.

- Click on **Subset Editor** button on the left side of the window.
  - Press "+" to create a new subset and rename your subset.
  - Select wells in the grid and press **Apply**.

- Click on **Sample Editor** button on the left side of the window.

- **Select Workflow > Endpt Geno.**

- For **singleplex** RealFast™ Assays **select Filter Combinations > 456-510 nm (FAM) and 533-580 nm (HEX).**



- For **multiplex** RealFast™ Assays **select Filter Combinations > 456-510 nm (FAM), 533-580 nm (HEX), 618-660 nm (Cy5) and 533-580 nm (ROX).**



- Choose your **Subset** of Samples.

- Define your **No Template Control (NTC)**:

- **Select Samples** field: select well by mouse-click or two wells by ctrl+mouse click.

- **Edit Endpt Geno Properties** field: Type **NTC** in the **Sample Name** field and press **Enter**. Choose **Negative Control** as **Sample Type**.

Pos	Color	Repl Of	Sample Name	EndPt Sample Type	EndPt Genotype
A1	<span style="color: blue;">■</span>		NTC	Negative Con	
A2	<span style="color: red;">■</span>		NTC	Negative C ▾	

- Define your **Positive Controls** as Standard (*alternatively: Define your Positive Controls as Unknown*):

- **Select Samples** field: select a well by mouse click.

- **Edit Endpt Geno Properties** field: Type the name of your **Positive Control** for **HEX/Cy5** in the **Sample Name** field. Choose **Standard**.

- In the chart (EndPt Genotype) type in the genotype corresponding to your

Pos	Color	Repl Of	Sample Name	EndPt Sample Type	EndPt Genotype
B3	<span style="color: green;">■</span>		Pos Ctrl HEX	Standard	WT
B4	<span style="color: magenta;">■</span>		Pos Ctrl FAM	Standard ▾	MUT

**Positive Control HEX** or **Cy5**. The genotype will be in most cases **wild type (WT)** for the fluorophore HEX or Cy5.

- Repeat the steps above with your **Positive Control FAM** or **ROX**. The genotype will be in most cases **mutant (MUT)** for the fluorophore FAM or ROX.

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

- Define your **Samples**:

- **Select Samples** field: select a well by mouse click.

- **Edit Endpt Geno Properties** field: Type the name of your first sample in the corresponding field and press **Enter**. Check **Unknown**.

- Define the rest of your samples.

- Save** or **export** your experiment by pressing the corresponding button:



- Load your samples and start the experiment.



### **Analysis of Genotyping Assays:**

- Open the **LightCycler® 480 software** and login with your username and password. The **Overview** window appears.

- Click on  or choose **Navigator** in the flip-window on the top left.

- Choose an experiment from the data bank, or

- import an experiment located outside the data bank by pressing **Import**.

- After the file is loaded the **Summary** window of your experiment is displayed.

- Press the **Analysis** button to reach the analysis window.

- Within the **Create New Analysis** field choose **Endpoint Genotyping**.



- A pop-up window will be launched. If applicable select a **Subset** of samples or analyze **All Samples** in case your plate contains only one type of assay. Give a **Name** to your analysis. Press the **OK** button.

#### For singleplex RealFast™ Assays:

- Assign **Allele X** to **FAM** (465-510) and **Allele Y** to **HEX** (533-580) and press **OK**.
- A Scatter Plot with the signals for HEX (y-axis) and FAM (x-axis) is displayed.

*Optional: Press **Color Comp** and choose **In Use** or – if available – **In Database** for color compensation of FAM (510) and VIC (580). Press the **OK** button.*

- Press the **Calculate** button and review your results in the chart bottom-left. The column **Results > Call** displays the genotype of your samples.  
In the **Scatter Plot** points for homozygous samples for the allele Y (HEX) group along the vertical axis, homozygous samples for allele X (FAM) along the horizontal axis. Heterozygotes will generate a cluster in the middle.

- **Save** or **Export** your data by pressing the corresponding button.  or 
- After saving your data you can customize and generate a report via the **Report** button.



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

#### For multiplex RealFast™ Assays:

- Assign **Allele X** to **FAM** (465-510) and **Allele Y** to **HEX** (533-580) and press **OK**.
- A Scatter Plot with the signals for HEX (Y-axis) and FAM (x-axis) is displayed.
- Press **Color Comp** and choose **In Database**. Select the Color Compensation file created with the LightMix® Universal Color Compensation Hexaplex (TIB MOLBIO) Kit and select all channels. Press the **OK** button.

- Press the **Calculate** button and review your results in the chart bottom-left. The column **Results > Call** displays the genotype of your samples.  
In the **Scatter Plot** points for homozygous samples for the allele Y (HEX) group along the vertical axis, homozygous samples for allele X (FAM) along the horizontal axis. Heterozygotes will generate a cluster in the middle.

- For analysis of the other marker press the **Filter Comb** Button on the bottom of the page.
  - Assign **Allele X** to **ROX** (533-610) and **Allele Y** to **Cy5** (618-660) and press **OK**.
  - A Scatter Plot with the signals for Cy5 (Y-axis) and ROX (x-axis) is displayed.
  - Press **Color Comp** and choose **In Database**. Select the same Color Compensation File as before.
  - Press the **Calculate** button and review your results in the chart bottom-left. The column **Results > Call** displays the genotype of your samples.  
In the **Scatter Plot** points for homozygous samples for the allele Y (Cy5) group along the vertical axis, homozygous samples for allele X (ROX) along the horizontal axis. Heterozygotes will generate a cluster in the middle.

- **Save** or **export** your data by pressing the corresponding button.  or 
- After saving your data you can customize and generate a report via the **Report** button.