QuickGuide: RealFast™ Genotyping on LightCycler® 480II

Setup for Genotyping Assays:

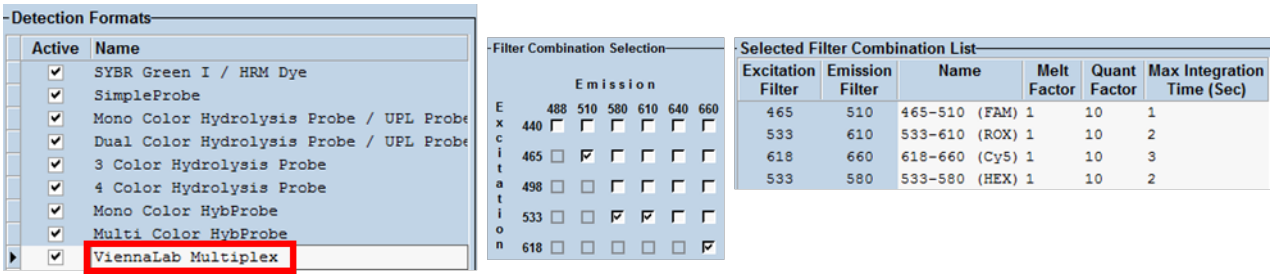
- Open the **LightCycler® 480 software** (QuickGuide is based on version 1.5.1) and login with your username and password.

  » ***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:*



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

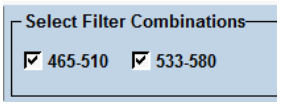
- Choose **New Experiment**.
- Define your PCR program in the **Run Protocol** tab.
  - For **singleplex** RealFast™ Assays select **Dual Color Hydrolysis Probe / UPL Probe** as Detection Format.
  - For **multiplex** RealFast™ Assays select correct Detection Format, i.e. **ViennaLab Multiplex**.
  - 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

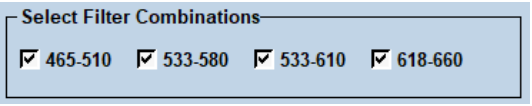
  - Add program steps in the **Programs** window with "**+**" and edit **Cycles** and **Analysis Mode**. To edit **Target (°C)**, **Acquisition Mode** and **Hold**, click on the corresponding step in the **Program Name** window and change parameters or add steps ("**+**") in the **Temperature Targets** 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 > 465-510** nm (FAM) and **533-580** nm (HEX).



  - For **multiplex** RealFast™ Assays **select Filter Combinations > 465-510** nm (FAM), **533-580** nm (HEX), **533-610** nm (ROX) and **618-660** nm (Cy5).



  - 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			NTC	Negative Con	
A2			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 **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.

Pos	Color	Repl Of	Sample Name	EndPt Sample Type	EndPt Genotype
B3			Pos Ctrl HEX	Standard	WT
B4			Pos Ctrl FAM	Standard	MUT

*» **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.

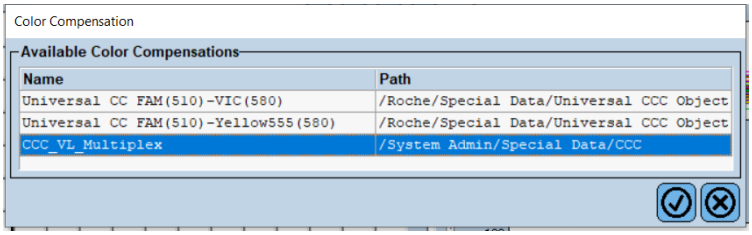
- **Save** or **export** your experiment by pressing the corresponding button:
- Load your samples and start the experiment.

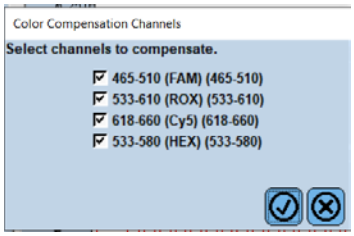
<u>Analysis of Genotyping Assays:</u>

- 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.**

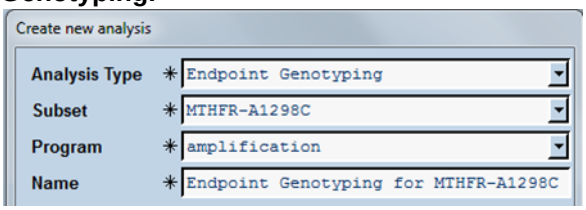
- After the file is loaded the **Summary** window of your experiment is displayed.
- For **singleplex RealFast™ Assays** proceed with “press the **Analysis** button” as explained below.
- For multiplex RealFastTM Assays press the Experiment button first, then proceed to the Data tab:
  - Select **Color Compensation “in Database”** (below the graphic “Temperature History”)
  - Select color compensation file suitable for ViennaLab multiplex RealFast™ Assays

➢ Select channels to compensate





- 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.

For **multiplex** RealFast™ Assays:

*» **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 !** «*

- ➢ 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.