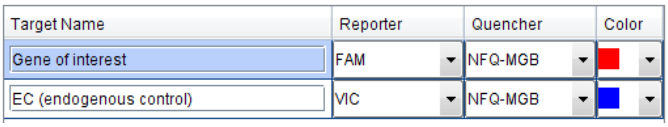
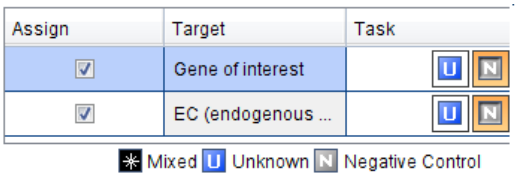
QuickGuide: RealFast™ CNV on AB 7500 Fast

Setup of Relative Quantitation Assays:

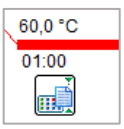
- Open the ABI 7500 Software (QuickGuide is based on version 2.0.6) and click **Advanced Setup.**
- In **Setup > Experiment Properties** select:
  - Instrument: **7500 Fast** (96 Wells)
  - Type of experiment: **Quantitation – Comparative C_T ($\Delta\Delta C_T$)**
  - Reagents: **TaqMan® Reagents**
  - Ramp speed: **Standard**
- In **Setup > Plate Setup** go to **Define Targets and Samples**:
  - **Define Targets** in the corresponding field**:**
    - Provide a name for your gene of interest and choose **FAM** as **Reporter** and **NFQ-MGB** as **Quencher**.
    - Add the second target by pushing the **Add New Target** button.
    - Type **EC** (endogenous control) as **Target Name** and choose **VIC** as **Reporter** and **NFQ-MGB** as **Quencher**.



  - **Define Samples** in the corresponding field:
    - Type **Calibrator** in the field for the **Sample Name**. This represents the positive control which is included in the assay kit.
    - **Add New Sample**(s) by pushing the corresponding button and rename the field(s) according to the sample(s) you want to analyze.
- In **Setup > Plate Setup** go to **Assign Targets and Samples**
  - Define the **Negative Control Template**:
    - Select a replicate of three wells by ctrl-click.
    - Within the field **Assign Target(s) to the selected wells** check boxes for the gene of interest (e.g. CYP21A2) and **EC**.
      Click on the button **N** (Negative Control) in **Task**.



  - Define your **Calibrator**:
    - Select a replicate of three wells by ctrl-click.
    - Within the field **Assign Target(s) to the selected wells** check boxes for the gene of interest (e.g. CYP21A2) and **EC**. Click on the button **U** (Unknown) in **Task**.
    - Check the box for the **Calibrator** within the field **Assign Sample(s) to the selected wells**.
  - Within the field **Select relative quantitation settings** choose **Calibrator** as your **Reference Sample** and **EC** as **Endogenous Control**.
  - Within the field called **Select the dye to use as the passive reference** select **ROX**.
  - Define your **Samples**:
    - Select a replicate of three wells by ctrl-click.
    - Within the field **Assign Target(s) to the selected wells** check boxes for the gene of interest (e.g. CYP21A2) and **EC**. Click on the button **U** (Unknown) in **Task**.
    - Check the box for the **Sample** you wish to assign within the field called **Assign Sample(s) to the selected wells**.
- In **Setup > Run Method** go to **Graphical View**
  - Select a reaction volume of **20 µl**
  - Define your PCR program:
    - Holding Stage: **10 min at 95°C**
    - Cycling Stage: **40 cycles 15 sec** at **95°C** and **1 min** at **60°C**. Make sure **Data Collection On** is enabled
    - Delete first Holding Stage at 50°C, if present.

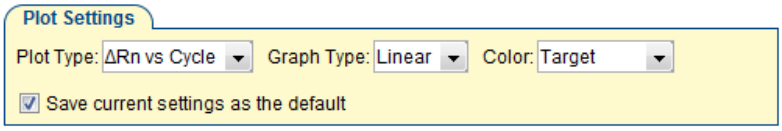


- Load your reaction plate into the AB 7500 Fast instrument and press **START RUN** (green button).

Analysis of Relative Quantitation Assays:

After completing a run or after opening a genotyping data file the software displays the Experiment Menu **Analysis:**

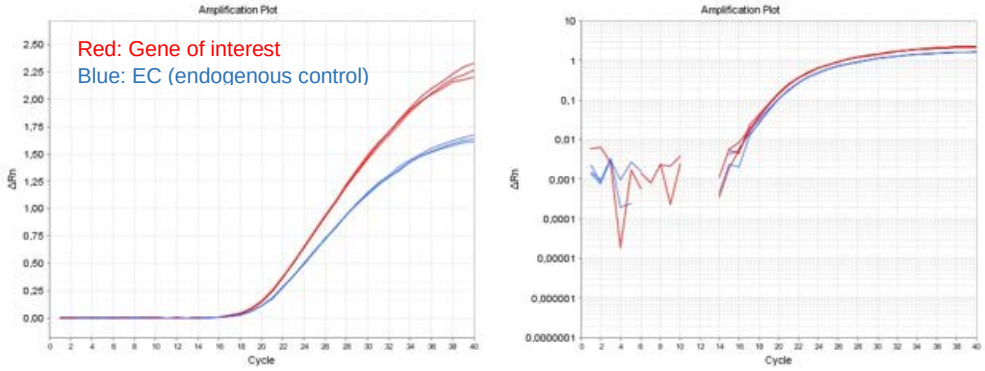
- Results automatically appear in the **Amplification Plot.**
  - Adjust the **Plot Settings** to **ΔRn vs Cycle** (Plot Type), **Linear** or **Log** (Graph Type), **Target** (Color)



  - Tick the box for **Show Threshold** in the **Options** field.

- Press the **Analysis Settings** Button and go to **C_T Settings**.
  - Adjust the **Threshold** for both targets according to the settings in the **Assay Description** and press the button Apply Analysis Settings.

- Select individual replicates in the **View Plate Layout** field and review your samples.
  - The interval between the curve for the gene of interest and for the **Endogenous Control** (**EC**) is related to the copy number variation.



Example: Amplification Plot of the Calibrator sample. Linear (left) and log (right) graph type.

- Go to **View Well Table**.
  - Press the **Show in Table** button and customize the table.
  - Review the **Relative Quantities (RQ)** and define the CNV status of your samples according to the Assay Description.
  - Go to **Gene Expression** (left) and select **RQ vs Sample** in the **Plot settings**. The relative quantities of each sample are displayed as bar chart.

- To print a report click **Print Report** in the upper menu bar:
  - Select data for the report according to your needs.