Instruction for Use

Color Compensation Kit Multiplex 1

Color Compensation for the LightCycler® 480 II Instruments.

G070MP1-CC

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1 Intended Use

Color Compensation Kit Multiplex 1 is intended for generating a Color Compensation File for multiplex real-time PCR experiments on the LightCycler® 480 II instruments using Hydrolysis Probes.

2 Principle of the Kit

Due to the emission spectra overlap of Cyan500 (Blue), FAM (Green), HEX (Yellow), ROX (Orange) and Cy5 (Red) the Roche LightCycler® 480 II can pick up signals from dyes measured by another channel. This so called 'crosstalk' can lead to incorrect results. To correct the crosstalk impact, Roche recommends to create a Color Compensation File. This File permits the LightCycler® 480 software to subtract fluorescence crosstalk from a reporter dye into inappropriate channels.

3 Package Contents

The reagents supplied are sufficient for 3 reactions (one run).

Table 1: Components of the Color Compensation Kit Multiplex 1.

Label	Lid Color	Content
Blank Mix	clear	64 µl
Blue Mix	blue	64 µl
Green Mix	green	64 µl
Orange Mix	orange	64 µl
Red Mix	red	64 µl
Yellow Mix	yellow	64 µl
Blank Control	clear	16 µl
Positive Control	violett	80 µl

4 Equipment and Reagents to be Supplied by User

- Sterile microtubes
- Disposable powder-free gloves
- Pipets (adjustable volume)
- Sterile pipet tips with filters
- Plate centrifuge
- Vortexer
- LightCycler® 480 II
- LightCycler® 480 II 96 well plate (white) and plate sealer (optical foil)

5 Transport, Storage and Stability

Color Compensation Kit Multiplex 1 is shipped on dry ice or cool packs. All components must be stored at ≤-18°C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package.
For convenience, opened reagents can be stored at +2-8°C for up to 6 months.
Protect kit components from direct sunlight during the complete test run.

6 Important Notes

- Color Compensation Kit Multiplex 1 must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Stick to the protocol described in the Instruction for Use.
- Regularly decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine Color Compensation Kit Multiplex 1 components of different lot numbers.

7 Creating a Color Compensation File for the LightCycler® 480 II

7.1 Important Points Before Starting

- Please pay attention to the chapter 6 ‚Important Notes'.
- Before setting up the real time PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR setup.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed and centrifuged briefly.

7.2 Preparation of the Color Compensation Reaction Mixes

Before starting the Color Compensation experiments it is important to prepare the Reaction Mixes. These Mixes must be combined in the corresponding tubes according to Table 2.

Table 2: Prepare mixes of Color Compensation Kit Multiplex 1.

Blank	n=1	n=3 (3+1)
Blank Mix	16 µl	64 µl
Blank Control	4 µl	16 µl
Total	20 µl	80 µl

Blue	n=1	n=3 (3+1)
Blue Mix	16 µl	64 µl
Positive Control	4 µl	16 µl
Total	20 µl	80 µl

Green	n=1	n=3 (3+1)
Green Mix	16 µl	64 µl
Positive Control	4 µl	16 µl
Total	20 µl	80 µl

Orange	n=1	n=3 (3+1)
Orange Mix	16 µl	64 µl
Positive Control	4 µl	16 µl
Total	20 µl	80 µl

Red	n=1	n=3 (3+1)
Red Mix	16 µl	64 µl
Positive Control	4 µl	16 µl
Total	20 µl	80 µl

Yellow	n=1	n=3 (3+1)
Yellow Mix	16 µl	64 µl
Positive Control	4 µl	16 µl
Total	20 µl	80 µl

7.3 Preparation of the Color Compensation Plate

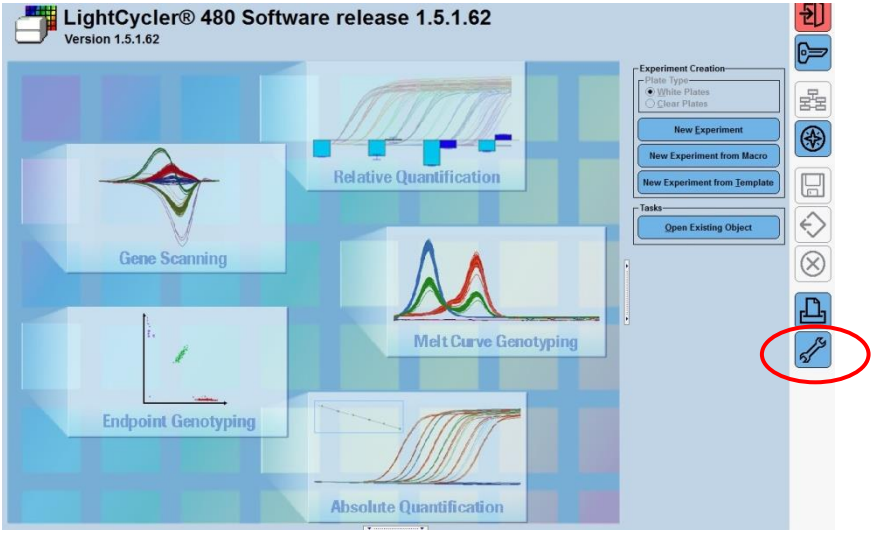
For the Color Compensation experiment pipette 3 reactions with 20 µl of each dye into the microwell plate as shown in Table 3. Seal the plate with optical foil, centrifuge the plate for 1 min at 1500 x g.

Table 3: Pipetting scheme for Color Compensation experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank		Blue		Green		Orange		Red		Yellow	
B												
C												
D												
E												
F												
G												
H												

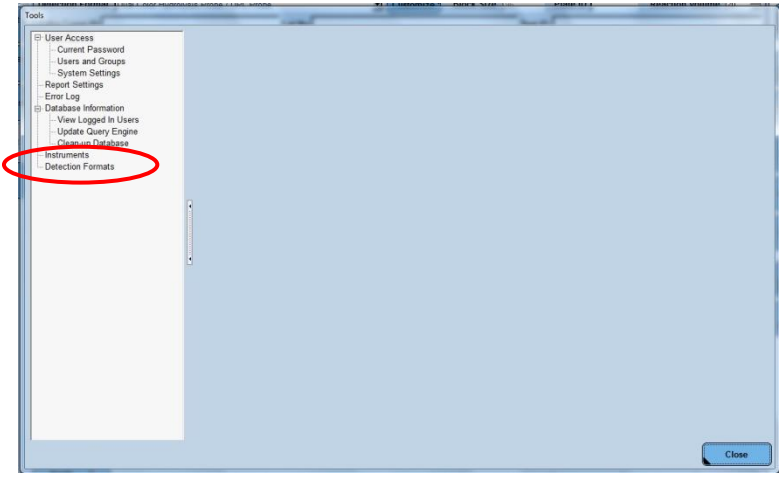
7.4 Run a Color Compensation experiment

Open the LightCycler® 480 Software and click on ‘open Tools’.



Detection Format

Select ‘Detection Formats’ in the new window.



Click ‘New’ in the folder ‘Detection Formats’ and choose a new name, e.g. G070MP1-CC for your new detection format. Use the checkboxes in ‘Filter Combination Selection’ to mark the required wavelengths. Set ‘Melt Factor’ to 1 and ‘Quant Factor’ to 5 for 440-488 nm, and 10 for the other channels. ‘Max integration Time’ is set on 1 for 440–488 nm and 465-510 nm, on 2 for 533–580 nm and 533–610 nm and on 3 for 618–660 nm. Close the window.

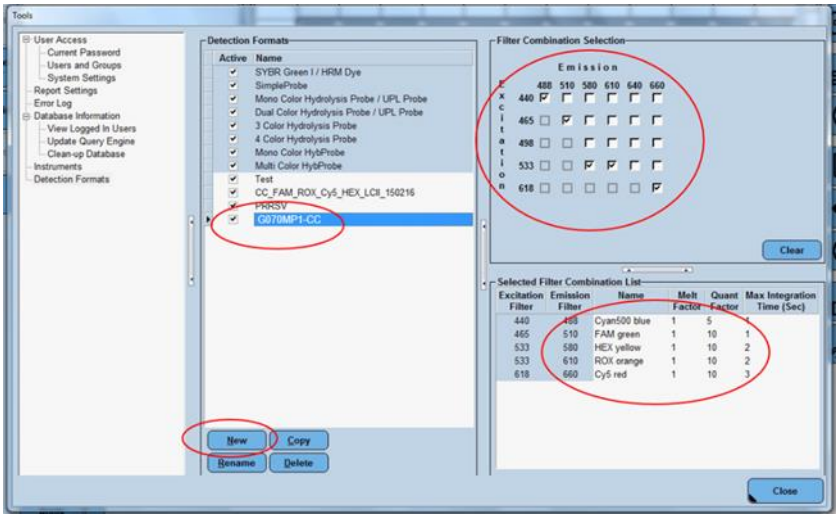
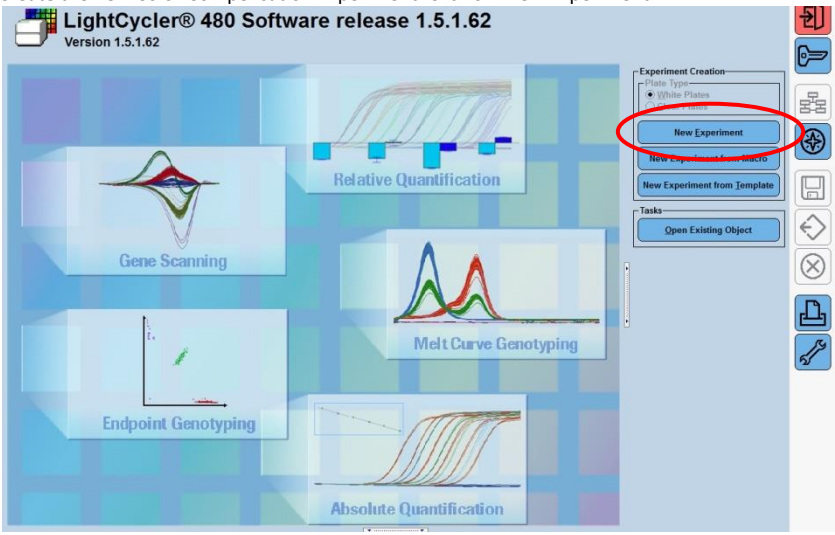


Table 4: Filter Setting and Filter Combination List

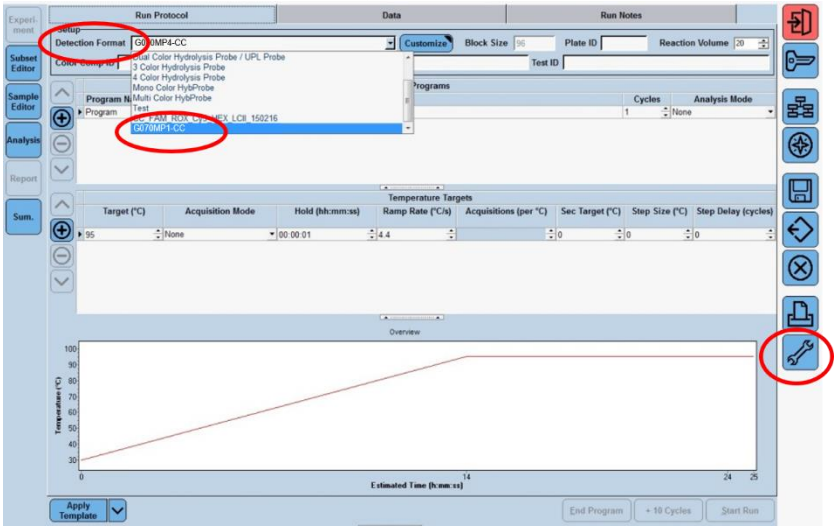
		Emission Filters					
		488	510	580	610	640	660
Excitation Filters	440	Blue					
	465		Green				
	498						
	533			Yellow	Orange		
	618						Red

	Name	Melt Factor	Quant Factor	Max integration time
440 – 488 nm	Blue	1	5	1
465 – 510 nm	Green	1	10	1
533 – 580 nm	Yellow	1	10	2
533 – 610 nm	Orange	1	10	2
618 – 660 nm	Red	1	10	3

Create the new Color Compensation Experiment. Click on ‘New Experiment’.

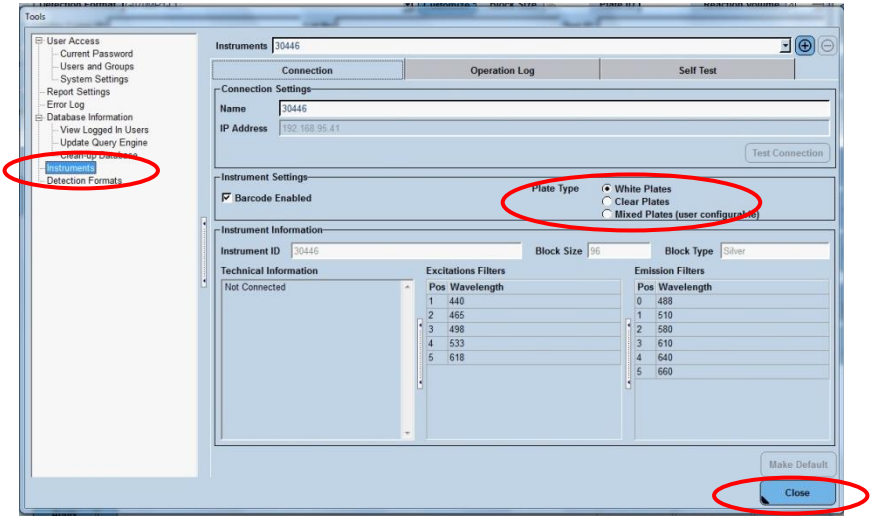


Choose the new detection format in ‘Setup’. Click ‘Open Tools’.



Instrument Settings

Select ‘Instruments’. Make sure to enter the correct plate type (white or clear). The Color Compensation is developed for white plates. The use of transparent plates or stripes is not recommended. The Color Compensation is bound to this kind of plate, if the plate type is changed, an additional Color Compensation experiment will be needed. Close the window.



Program Settings

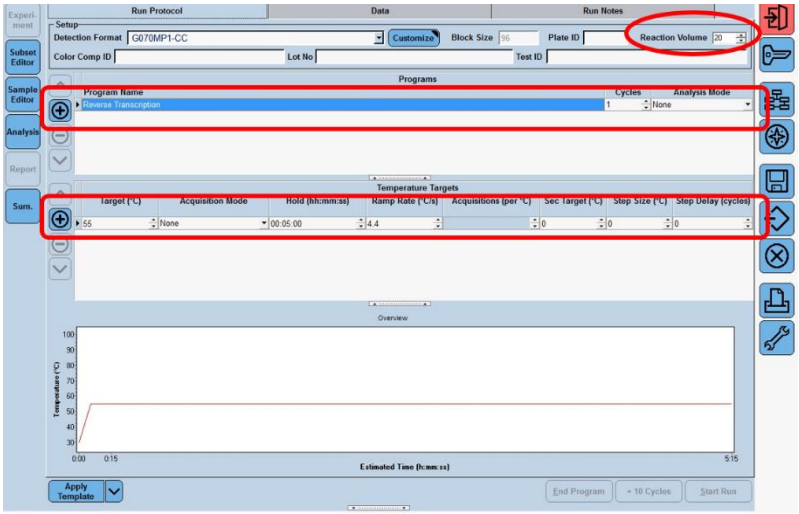
Please refer to screenshots starting from page 12. Press ‘+’ to add further steps to the PCR protocol. Change the ‘Program Name’, ‘Cycles’, ‘Analyses Mode’, ‘Target’, ‘Acquisition Mode’, ‘Hold’, ‘Ramp Rate’ as shown in Table 6.

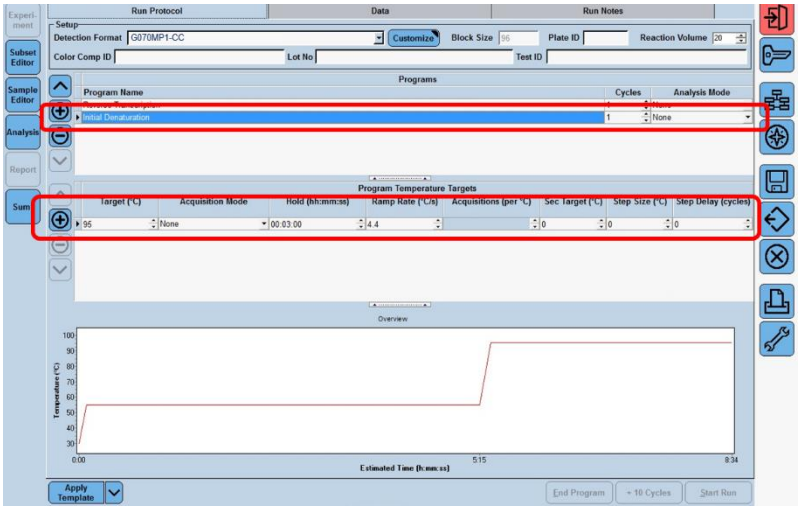
Table 5: Cycler steps for Color Compensation

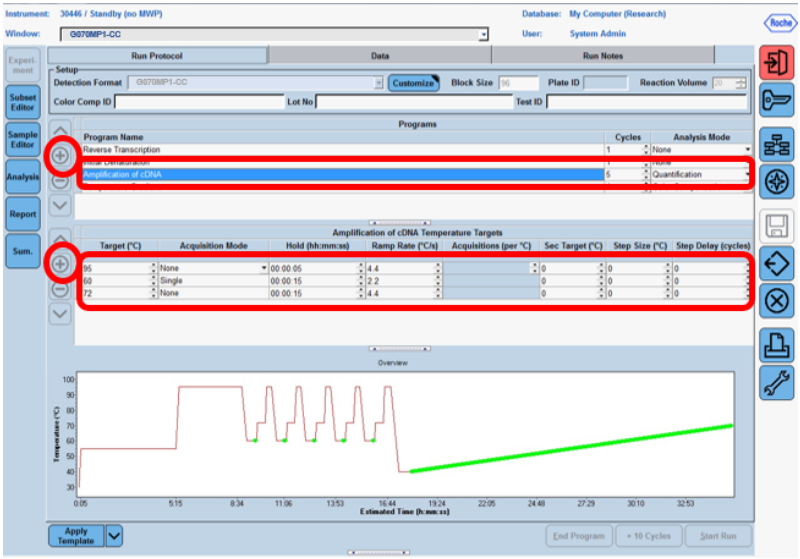
Program Name	Cycles	Analysis Mode
Reverse Transcription	1	None
Initial Denaturation	1	None
Amplification	5	Quantification
Temperature Gradient	1	Color Compensation

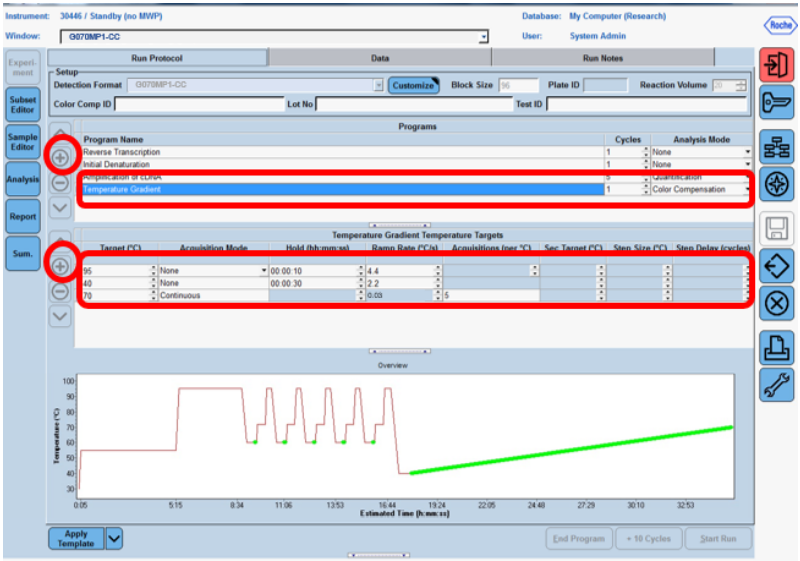
Table 6: Program Temperature Targets

Description	Temp.	Acquisition Mode	Time	Ramp Rate (°C / s)	Acquisition (per °C)
Reverse Transcription	55°C	none	5 min	4.4	
Initial Denaturation	95°C	none	3 min	4.4	
Amplification	95°C	none	5 s	4.4	
	60°C	single	15 s	2.2	
	72°C	none	15 s	4.4	
Temperature Gradient	95°C	none	10 s	4.4	
	40°C	none	30 s	2.2	
	70°C	continuous		0.03	5


Run Protocol
Data
Run Notes
Setup
Detection Format
G070MP1-CC
Customize
Block Size
Plate ID
Reaction Volume
Color Comp ID
Lot No
Test ID
Programs
Program Name
Cycles
Analysis Mode
Reverse Transcription
None
Temperature Targets
Target (°C)
Acquisition Mode
Hold (hh:mm:ss)
Ramp Rate (°C/s)
Acquisitions (per °C)
Sec Target (°C)
Step Size (°C)
Step Delay (cycles)
55
None
00:05:00
4.4
Overview
Temperature (°C)
Estimated Time (h:mm:ss)
0:00
0:15
5:15
Apply Template
End Program
+ 10 Cycles
Start Run


Run Protocol
Data
Run Notes
Setup
Detection Format
G070MP1-CC
Customize
Block Size
Plate ID
Reaction Volume
Color Comp ID
Lot No
Test ID
Programs
Program Name
Cycles
Analysis Mode
Initial Denaturation
None
Program Temperature Targets
Target (°C)
Acquisition Mode
Hold (hh:mm:ss)
Ramp Rate (°C/s)
Acquisitions (per °C)
Sec Target (°C)
Step Size (°C)
Step Delay (cycles)
95
None
00:03:00
4.4
Overview
Temperature (°C)
Estimated Time (h:mm:ss)
0:00
5:15
8:34
Apply Template
End Program
+ 10 Cycles
Start Run

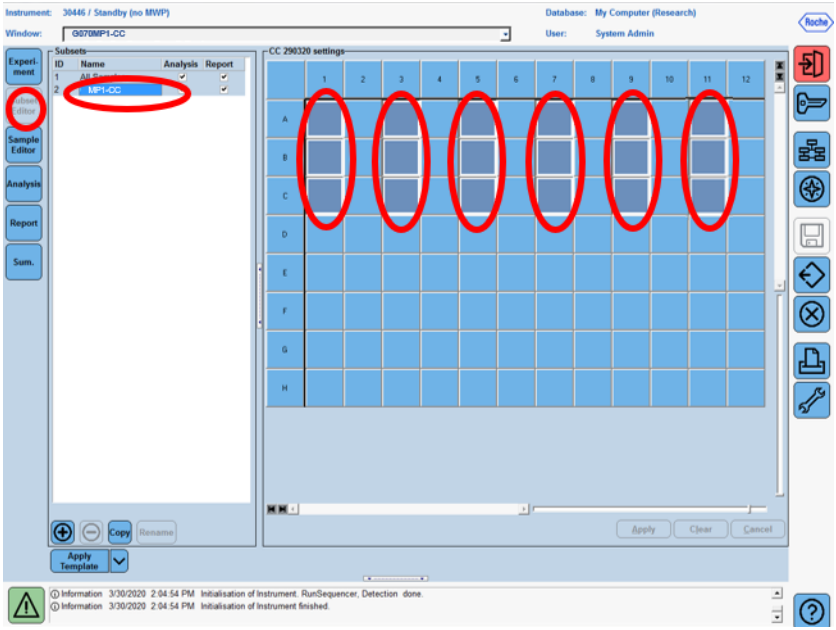

Instrument: 30446 / Standby (no MWP)
Database: My Computer (Research)
Window: G070MP1-CC
User: System Admin
Run Protocol
Data
Run Notes
Setup
Detection Format
Customize
Block Size
Plate ID
Reaction Volume
Color Comp ID
Lot No
Test ID
Programs
Program Name
Cycles
Analysis Mode
Reverse Transcription
Amplification of cDNA
Quantification
Amplification of cDNA Temperature Targets
Target (°C)
Acquisition Mode
Hold (hh:mm:ss)
Ramp Rate (°C/s)
Acquisitions (per °C)
Sec Target (°C)
Step Size (°C)
Step Delay (cycles)
Overview
Temperature (°C)
Estimated Time (h:mm:ss)
Apply Template
End Program
+ 10 Cycles
Start Run


Instrument: 30446 / Standby (no MWP)
Database: My Computer (Research)
Window: G070MP1-CC
User: System Admin
Run Protocol
Data
Run Notes
Setup
Detection Format
Customize
Block Size
Plate ID
Reaction Volume
Color Comp ID
Lot No
Test ID
Programs
Program Name
Cycles
Analysis Mode
Reverse Transcription
Initial Denaturation
Temperature Gradient
Color Compensation
Temperature Gradient Temperature Targets
Overview
Temperature (°C)
Estimated Time (h:mm:ss)
Apply Template
End Program
+ 10 Cycles
Start Run

Sample setting

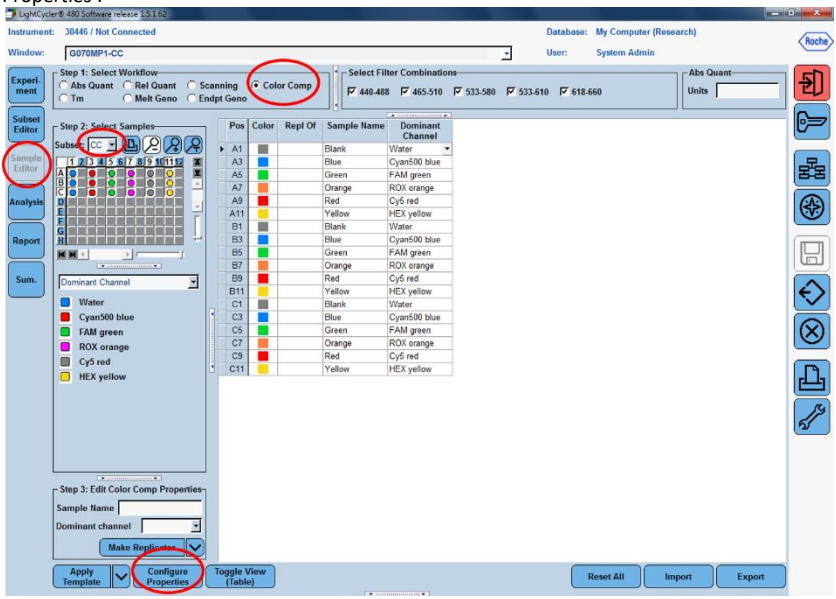
Subset Editor:

For optimal measurement and calibration of the LightCycler® 480 II it is necessary to create a subset for each well which is included in the Color Compensation experiment. Choose 'Subset Editor', click on '+' and add the name of the new subset (e.g. MP1-CC). Press 'Strg' (Ctrl) and select the wells included in the experiment and click 'Apply'.

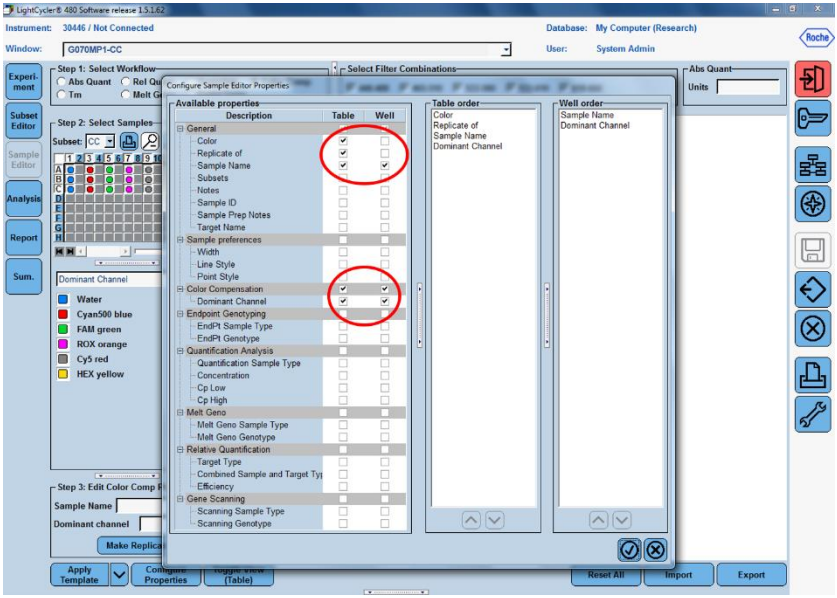


Sample Editor:

Choose the ‘Sample Editor’, tick ‘Color Comp’ and choose the created subset. Select ‘Configure Properties’.



Click ‘Color’, ‘Replicate of’, ‘Sample Name’, ‘Color Compensation’ and ‘Dominant Channel’. Click ‘ok’.



Insert ‘Sample Name’ and ‘Dominant Channel’ into ‘Step 3: Edit Color Comp Properties’ according to Table 7. Coloring of the respective rows can be edited in ‘Color’.

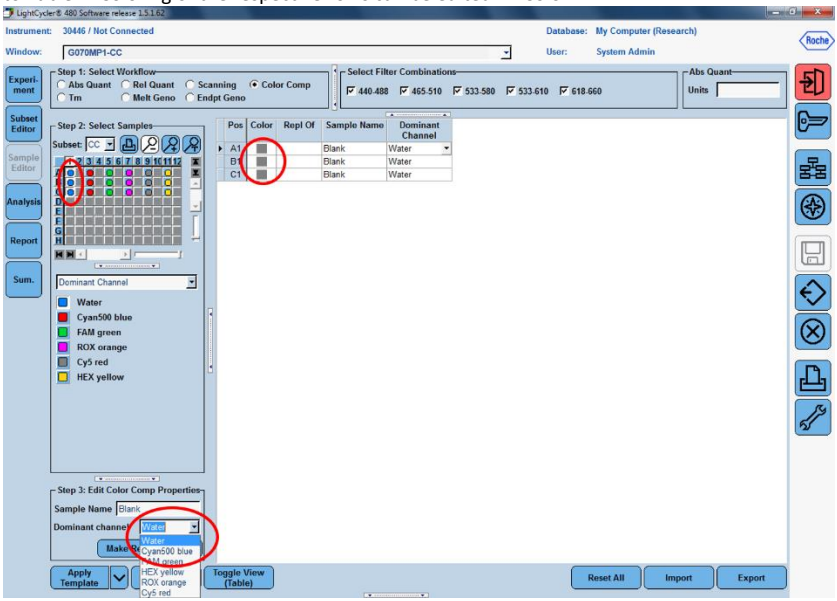
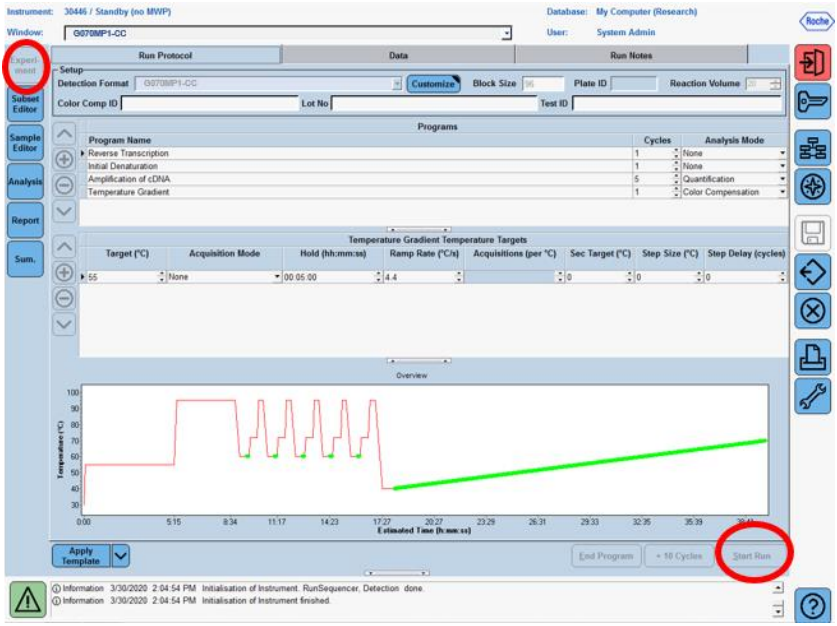


Table 7: Dominant channels for Color Compensation

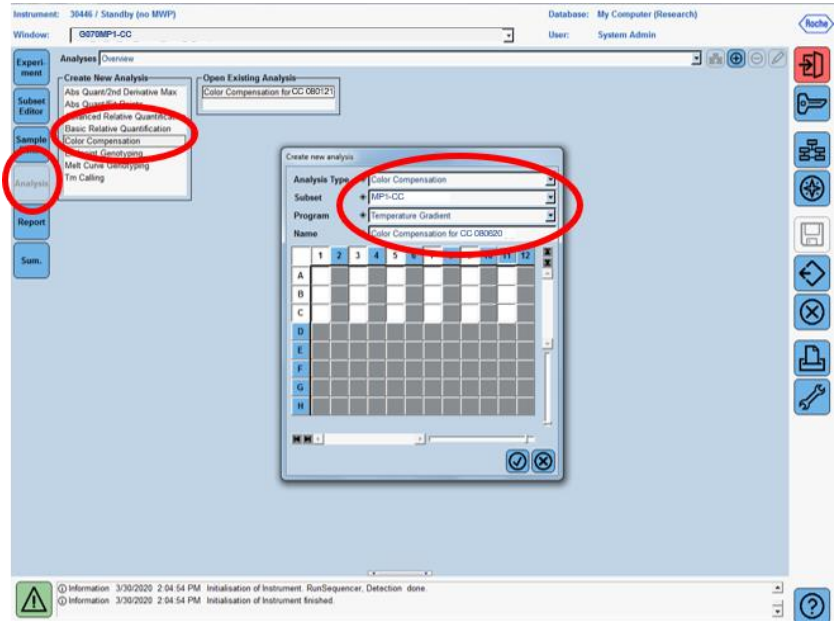
Row	Reagent	LightCycler® 480 II
1	Blank	Water
3	Blue	440 / 488
5	Green	465 / 510
7	Orange	533 / 610
9	Red	618 / 660
11	Yellow	533 / 580

Select ‘Experiment’. Open tray of the LightCycler® 480 II instrument. Insert centrifuged plate and close tray. Start the run on the ‘start run’ button and save the experiment.

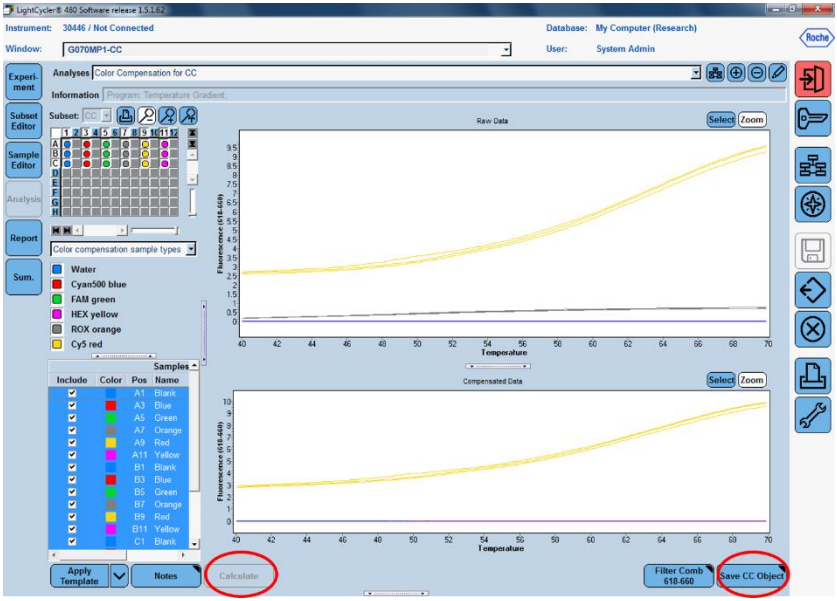


7.5 Creation of the Color Compensation File

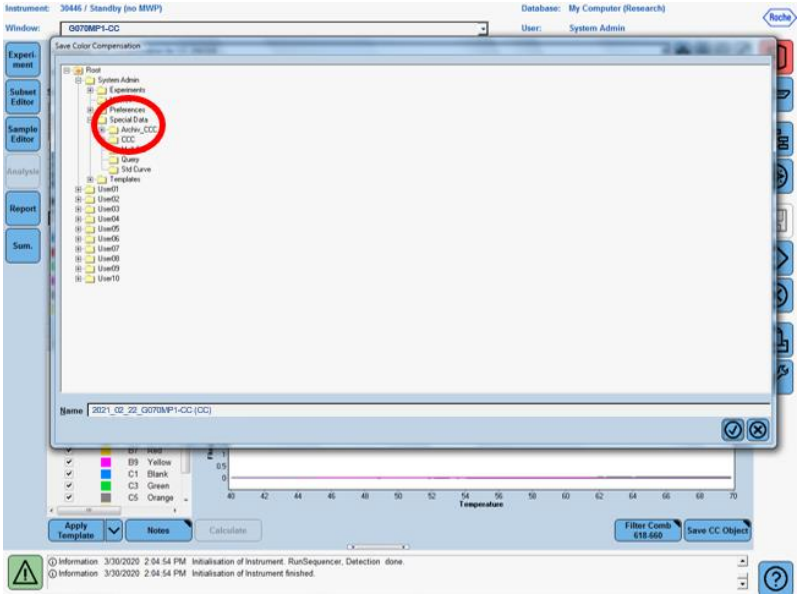
After the run has finished click on ‘Analysis’ on the module bar and choose the digital box ‘Create New Analysis’ the field ‘Color Compensation’. Choose and confirm your subset (e.g. MP1-CC) in the new window. Click ‘ok’.



Click on ‘Calculate’ to perform the Color Compensation analysis. Confirm the analysis with ‘Save CC Object’ in the folder ‘Special Data’ - ‘CCC’. The Color Compensation file can now be used in other LightCycler® 480 II experiments, the creation of the file is finished.



Check all detection channels, by clicking on ‘Filter Comb’. The ‘Raw Data’ shows a baseline (blank), a channel specific curve and in the ROX-channel and the Cy5 channel a crosstalk curve. The ‘Compensated Data’ (lower picture) must show only the channel specific curve.



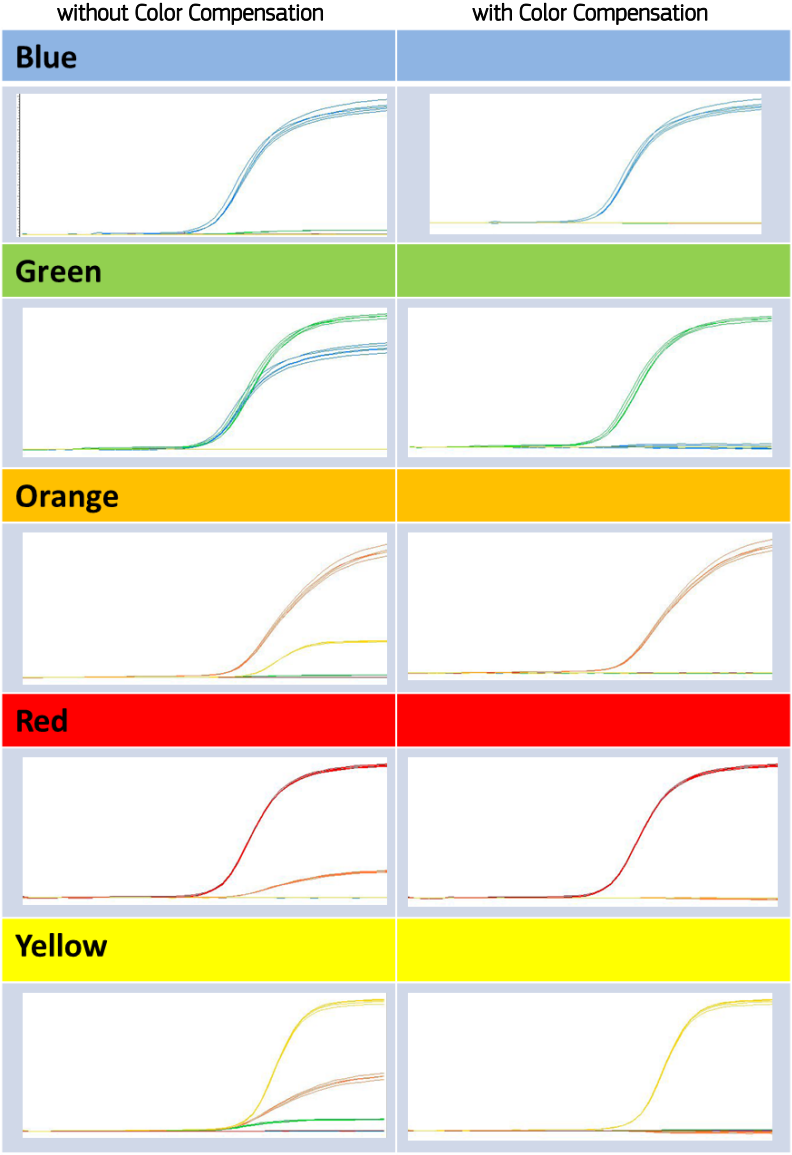
7.6 Verification of the received Color Compensation File

Open the runfile of the respective real time (RT-) multiplex PCR and take a look at the Positive Control. After addition of the Color Compensation, the crosstalk between the channels should be minimized and a difference as shown in Figure 1 should be visible.

8 Installation on multiple LightCycler® 480 II instruments

The Color Compensation Kit Multiplex 1 can be used on multiple LightCycler® 480 II instruments. Therfore use the same plate, that was already used for a Color Compensation Experiment and place it in the next LightCycler® 480 II. Then perform the program again as shown in Table 6 and follow the manual step by step.

Figure 1: Overview of verification of Color Compensation.



8.1 The Use of the Color Compensation File

To apply the Color Compensation open a multiplex PCR assay and click the 'Analysis' button to select the appropriate filter combination. Click the Color Compensation dropdown menu and select 'in Database'. Choose the stored Color Compensation File (G070MP1-CC) you want to apply to the assay. The 'Color Compensation (off)' button switches to 'Color Compensation (on)'. This confirms that the Color Compensation is active and the multiplex assay can now be analyzed.

9 Abbreviations and Symbols

BLANK MIX	Blank Mix	REF	Catalog number
BLUE MIX	Blue Mix	Σ	Contains sufficient for <n> test
GREEN MIX	Green Mix	- 18°C	Upper limit of temperature
ORANGE MIX	Orange Mix		Manufacturer
RED MIX	Red Mix		Use by YYYY-MM-DD
YELLOW MIX	Yellow Mix	LOT	Batch code
BLANK CONTROL	Blank Control	CONT	Content
CONTROL +	Positive Control		Consult instructions for use
		PCR	Polymerase Chain Reaction