

Instruction for Use

Color Compensation Kit SARS-CoV-2 seqc

Color Compensation for the use of specific PCR kits (e.g. virellaSARS-CoV-2 seqc real time RT-PCR) on LightCycler[®] 480 II Instruments.

REF

G070MP2-CC



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1 Intended Use

Color Compensation Kit SARS-CoV-2 seqc is intended for generating a Color Compensation File for the multiplex real-time RT-PCRs virellaSARS-CoV-2 seqc and other specific PCR kits on the LightCycler® 480 II instruments.

2 Principle of the Kit

Due to the emission spectra overlap of 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 SARS-CoV-2 seqc.

Label	Lid Color	Content 3 Reactions
Blank Mix	clear	55,2 µl
Green Mix	green	55,2 µl
Orange Mix	orange	55,2 µl
Red Mix	red	55,2 µl
Yellow Mix	yellow	55,2 µl
Blank Control	clear	24 µl
Positive Control	violet	96 µl

4 Equipment and Reagents to be Supplied by User

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

5 Transport, Storage and Stability

Color Compensation Kit SARS-CoV-2 seqc is shipped on dry ice or cool packs. All components must be stored at $\leq -18^{\circ}\text{C}$ 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 $+2-8^{\circ}\text{C}$ for up to 6 months.

Protect kit components from direct sunlight during the complete test run.

6 Important Notes

- Color Compensation Kit SARS-CoV-2 seqc 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 SARS-CoV-2 seqc 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 – except the enzyme - 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 SARS-CoV-2 seqc.

Blank	n=1	n=3 (3+1)
Blank Mix	13,8 µl	55,2 µl
Blank Control	6 µl	24 µl
Total	19,8 µl	79,2 µl

Green	n=1	n=3 (3+1)
Green Mix	13,8 µl	55,2 µl
Positive Control	6 µl	24 µl
Total	19,8 µl	79,2 µl

Orange	n=1	n=3 (3+1)
Orange Mix	13,8 µl	55,2 µl
Positive Control	6 µl	24 µl
Total	19,8 µl	79,2 µl

Red	n=1	n=3 (3+1)
Red Mix	13,8 µl	55,2 µl
Positive Control	6 µl	24 µl
Total	19,8 µl	79,2 µl

Yellow	n=1	n=3 (3+1)
Yellow Mix	13,8 µl	55,2 µl
Positive Control	6 µl	24 µl
Total	19,8 µl	79,2 µl

Seal the plate with optical foil, centrifuge the plate for 1 min at 1500 x g.

7.3 Preparation of the Color Compensation Plate

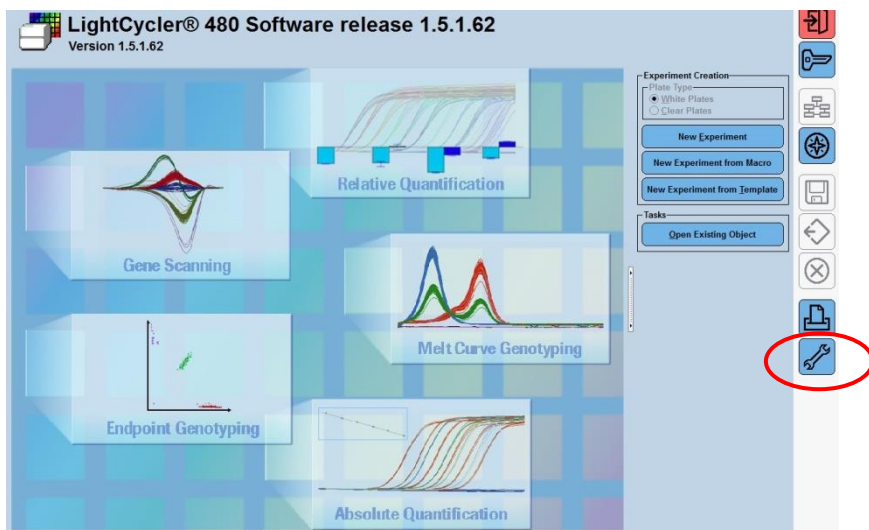
For the Color Compensation experiment pipette 3 reactions with 20 μ l of each dye into the microwell plate as shown in Table 3. Seal the plate with the optical foil and centrifuge briefly.

Table 3: Pipetting scheme for Color Compensation experiment.

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

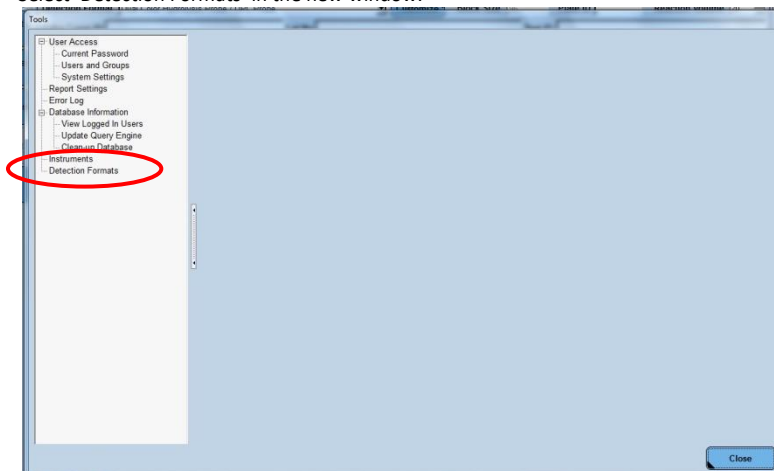
7.4 Run a Color Compensation experiment

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. G070MP2-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 10. 'Max integration Time' is set on 1 for 465-510 nm, on 2 for 533–580 nm and 533–610 nm and on 3 for 618–660 nm. Close the window.

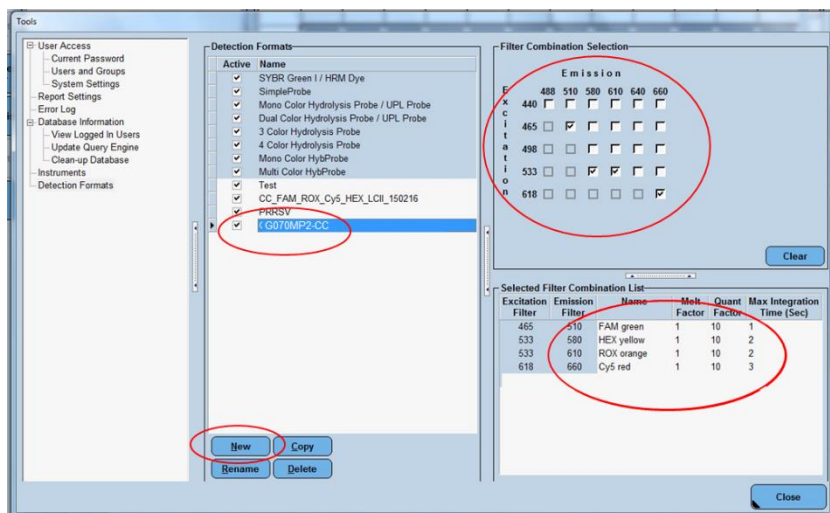
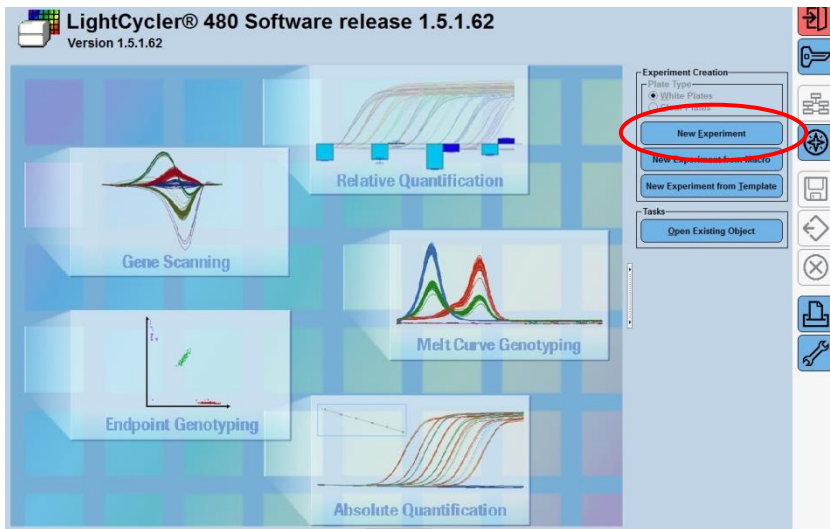


Table 4: Filter Setting and Filter Combination List

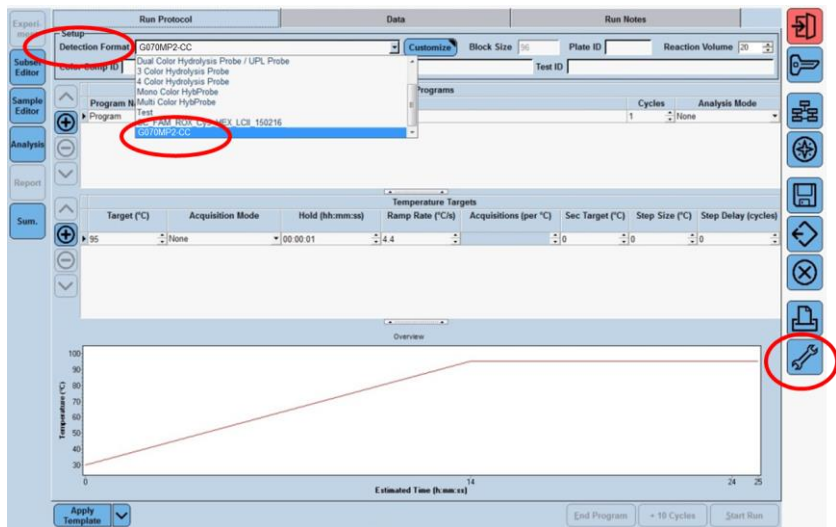
		Emission Filters					
		488	510	580	610	640	660
Excitation Filters	440						
	465		Green				
	498						
	533			Yellow	Orange		
	618						Red

	Name	Melt Factor	Quant Factor	Max integration time
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'.

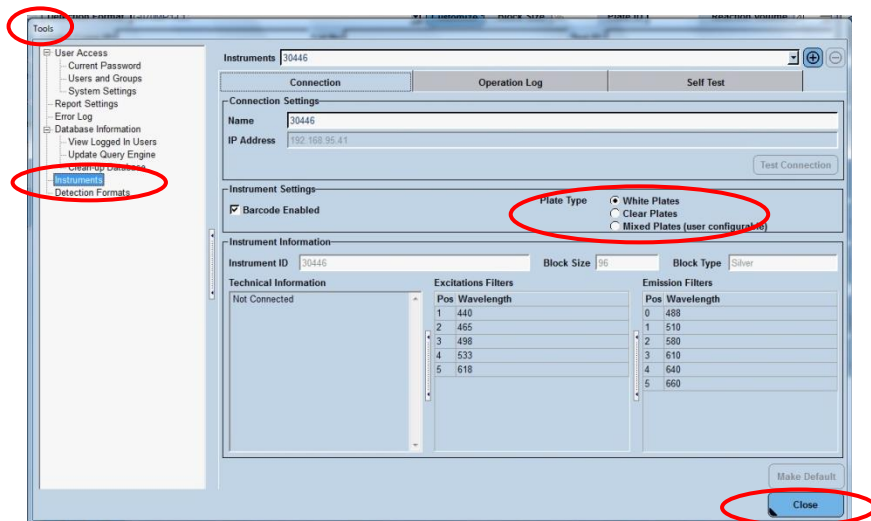


Choose the new detection format in 'Setup'. Click 'Open Tools'.



Instrument Settings

Select 'Instruments'. Make sure to enter the correct plate type (white or clear). The Color Compensation is 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	
	95°C	none	5 s	4.4	
Amplification	60°C	single	15 s	2.2	
	72°C	none	15 s	4.4	
	95°C	none	10 s	4.4	
Temperature Gradient	40°C	none	30 s	2.2	
	70°C	continuous		0.02	5

Run Protocol Setup: Detection Format: G070MP2-CC, Block Size: 96, Plate ID: [blank], Reaction Volume: 20

Programs:

Program Name	Cycles	Analysis Mode
Reverse Transcription	1	None

Temperature Targets:

Target (°C)	Acquisition Mode	Hold (h: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	0	0	0	0

Overview Graph: Temperature (°C) vs. Estimated Time (h:mm:ss). The graph shows a ramp from 30°C to 55°C at 4.4°C/s, reaching 55°C at 0:05:00.

Run Protocol Setup: Detection Format: G070MP2-CC, Block Size: 96, Plate ID: [blank], Reaction Volume: 20

Programs:

Program Name	Cycles	Analysis Mode
Initial Denaturation	1	None

Program Temperature Targets:

Target (°C)	Acquisition Mode	Hold (h: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	0	0	0	0

Overview Graph: Temperature (°C) vs. Estimated Time (h:mm:ss). The graph shows a ramp from 30°C to 95°C at 4.4°C/s, reaching 95°C at 0:03:00.

Expert
Setup

Run Protocol Data Run Notes

Setup
Detection Format: 0070MP2-CC Customize Block Size: 96 Plate ID: Reaction Volume: 20

Subset Editor
Color Comp ID: Lot No: Test ID: [Key Icon]

Sample Editor
Programs

Program Name	Cycles	Analysis Mode
Reverse Transcription	1	None
Initial Denaturation	1	None
Amplification of cDNA	5	Quantification

Analysis

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)
95	None	00:00:05	4.4	0	0	0	0
60	Single	00:00:15	2.2	0	0	0	0
72	None	00:00:15	4.4	0	0	0	0

Report

Overview

Apply Template End Program + 10 Cycles Start Run

Expert
Setup

Run Protocol Data Run Notes

Setup
Detection Format: 0070MP2-CC Customize Block Size: 96 Plate ID: Reaction Volume: 20

Subset Editor
Color Comp ID: Lot No: Test ID: [Key Icon]

Sample Editor
Programs

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

Analysis

Temperature Gradient 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	00:00:10	4.4	0	0	0	0
40	None	00:00:30	2.2	0	0	0	0
70	Continuous		0.03	5	0	0	0

Report

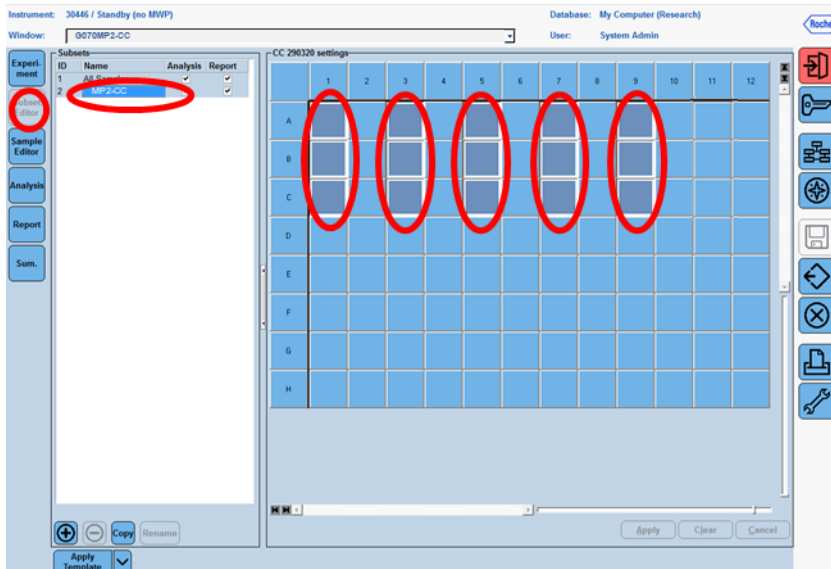
Overview

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. MP2-CC. Press 'Strg' (Ctrl) and select the wells included in the experiment and click 'Apply'.



Sample Editor:

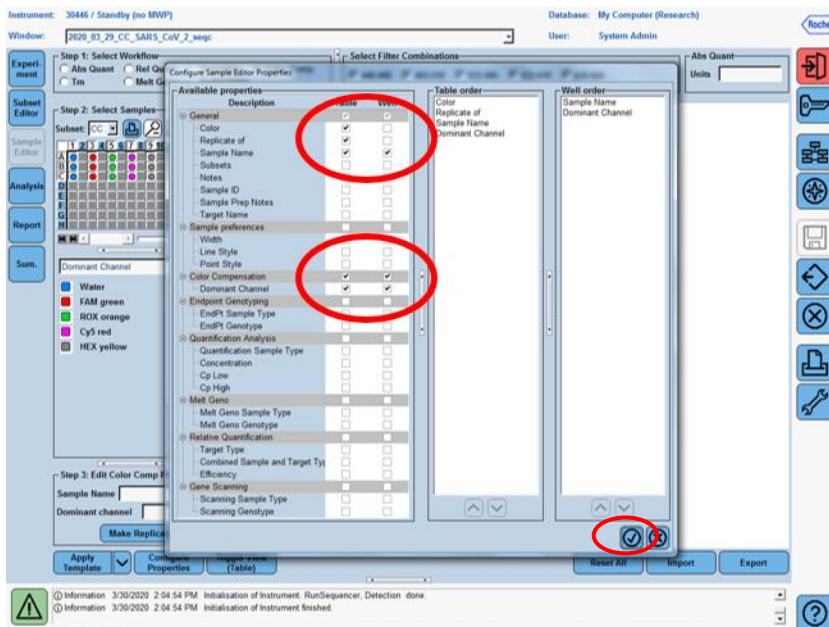
Choose the 'Sample Editor', tick 'Color Comp' and choose the created subset. Select 'Configure Properties'.

The screenshot shows the 'Sample Editor' software interface. The top bar displays 'Instrument: 30446 / Standby (no MWP)', 'Database: My Computer (Research)', and 'User: System Admin'. The main window is titled 'Step 1: Select Workflow' and shows a workflow step 'Color Comp' selected. Below this, a table lists sample positions (A1-A9, B1-B9, C1-C9) with their corresponding colors and sample names. The 'Configure Properties' button is highlighted with a red circle.

Pos	Color	Repl Of	Sample Name	Dominant Channel
A1			Blank	Water
A3	Green		Green	FAM green
A5	Orange		Orange	ROX orange
A7	Red		Red	Cy5 red
A9	Yellow		Yellow	HEX yellow
B1			Blank	Water
B3	Green		Green	FAM green
B5	Orange		Orange	ROX orange
B7	Red		Red	Cy5 red
B9	Yellow		Yellow	HEX yellow
C1			Blank	Water
C3	Green		Green	FAM green
C5	Orange		Orange	ROX orange
C7	Red		Red	Cy5 red
C9	Yellow		Yellow	HEX yellow

The interface also includes a 'Sample Editor' sidebar on the left, a 'Dominant Channel' dropdown menu, and a 'Step 3: Edit Color Comp Properties' section at the bottom. The 'Configure Properties' button is highlighted with a red circle.

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'.

The screenshot shows the software interface for a LightCycler 480 II. The 'Step 3: Edit Color Comp Properties' section is active, showing a table for 'Sample Name' and 'Dominant Channel'. The 'Color' selection table is also visible, with rows corresponding to the dominant channels. The 'Apply Template' button is highlighted with a red circle.

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

Table 7: Dominant channels for Color Compensation

Row	Reagent	LightCycler® 480 II
1	Blank	Water
3	Green	465 / 510
5	Orange	533 / 610
7	Red	618 / 660
9	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.

The screenshot displays the software interface for the LightCycler 480 II instrument. The window title is '2020_03_29_CC_SARS_CoV_2_seq'. The instrument is identified as '30446 / Standby (no MWP)'. The user is 'System Admin' and the database is 'My Computer (Research)'. The interface is divided into several sections:

- Setup:** Includes 'Detection Format' (FAST/SMART-CC), 'Block Size' (96), 'Plate ID', and 'Reaction Volume'.
- Programs:** A table listing the run steps:

Program Name	Cycles	Analysis Mode
Reverse Transcription	1	None
Initial Denaturation	1	None
Amplification of cDNA	5	Quantification
Temperature Gradient	1	Color Compensation
- Temperature Gradient Temperature Targets:** A table with columns: Target (°C), Acquisition Mode, Hold (h:mm:ss), Ramp Rate (°C/s), Acquisitions (per °C), Sec Target (°C), Step Size (°C), and Step Delay (cycles). The first row shows: 55, None, 00:05:00, 4.4, 0, 0, 0, 0.
- Overview:** A graph showing 'Temperature (°C)' on the y-axis (30 to 100) and 'Estimated Time (h:mm:ss)' on the x-axis (0:00 to 20:00). The graph shows a red line representing the temperature profile, which includes a series of steps between 55°C and 95°C, followed by a green line showing a linear ramp from approximately 40°C to 60°C.
- Buttons:** 'Apply Template', 'End Program', '+ 10 Cycles', and 'Start Run' (circled in red).
- Log:** Information messages at the bottom: '3/30/2020 2:04:54 PM Initialisation of Instrument. Run/Sequencer, Detection done' and '3/30/2020 2:04:54 PM Initialisation of Instrument finished'.

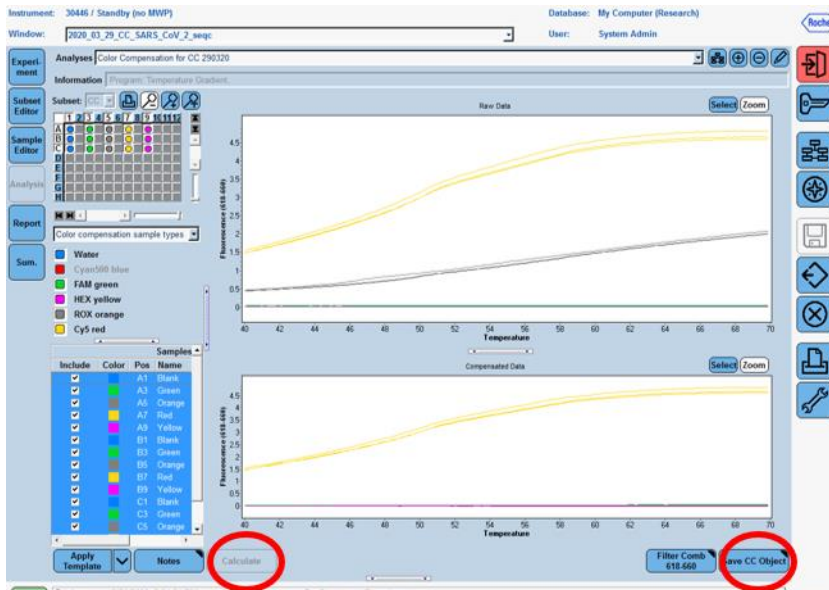
7.5 Creation of the Color Compensation File

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. MP2-CC) in the new window. Click 'ok'.

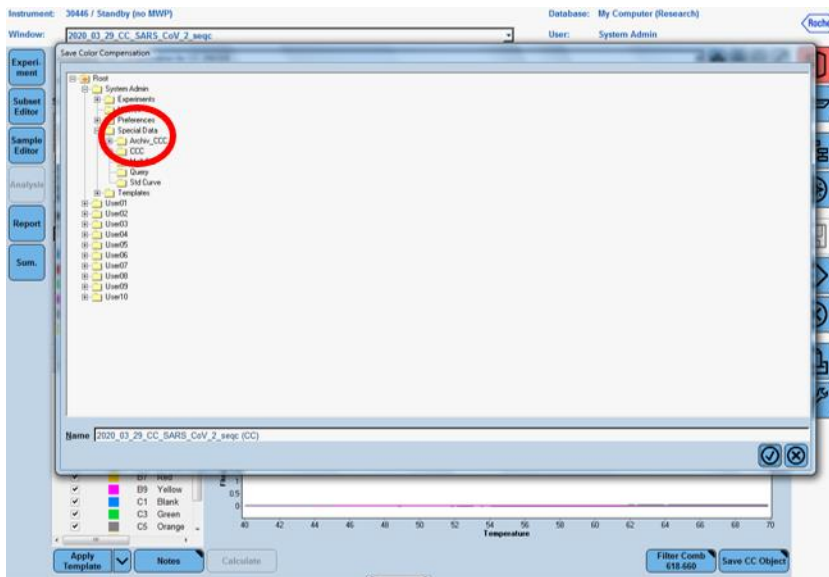
The screenshot shows the software interface with the following elements:

- Top Bar:** Instrument: 30446 / Standby (no MWP), Database: My Computer (Research), User: System Admin.
- Window:** 0070MP2-CC
- Left Panel:** Experiment, Subset Editor, Sample, Analysis, Report, Sum.
- Main Area:**
 - Analyses Overview:**
 - Create New Analysis:** Abs Quant/2nd Derivative Max, Abs Quant/Bi-Exponential, Basic Relative Quantification, **Color Compensation**, Report Geotyping, Melt Curve Overlaying, Tm Calling.
 - Open Existing Analysis:** Color Compensation for CC 080123
 - Create new analysis dialog:**
 - Analysis Type:** Color Compensation
 - Subset:** MP2-CC
 - Program:** Temperature Gradient
 - Name:** Color Compensation for CC 080620
 - Grid:** A 12x8 grid with columns 1-12 and rows A-H.
- Bottom Panel:** Information 3/30/2020 2:04:54 PM Initialisation of Instrument, RunSequencer, Detection done. Information 3/30/2020 2:04:54 PM Initialisation of Instrument finished.

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

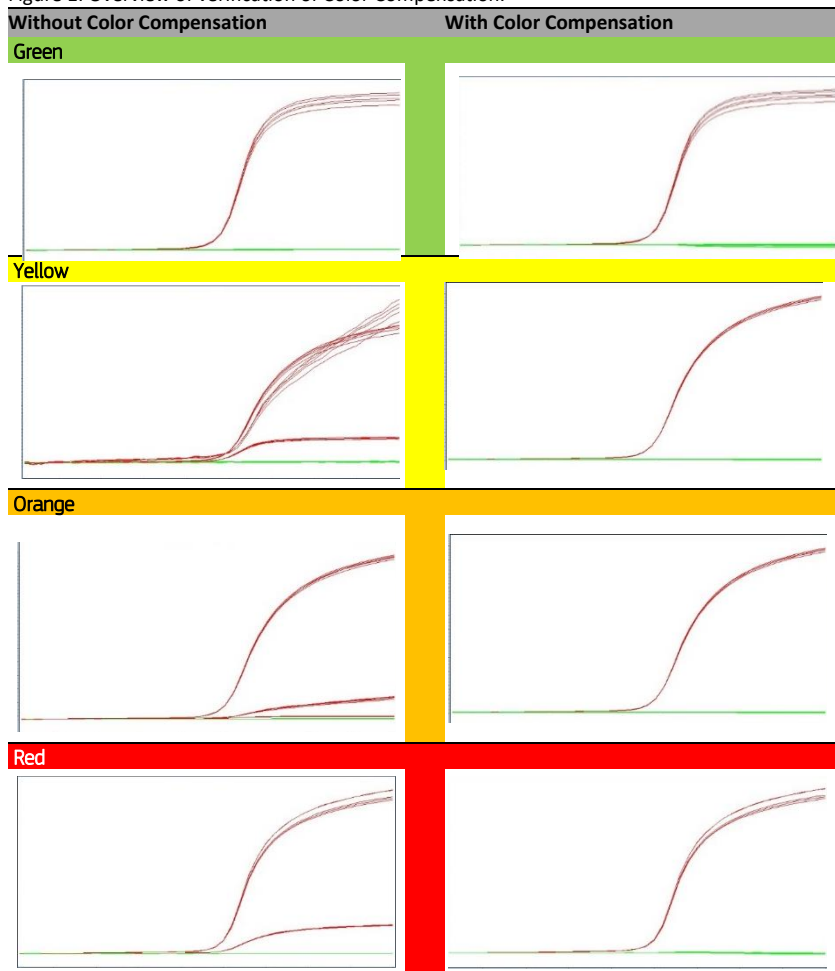
Open a runfile of the respective real time (RT-) multiplex PCR and take a look on the Positive Control. After addition of the Color Compensation, the crosstalk between the channels should be minimized and a difference as shown in Figure 1 should be visible.

8 Installation on multiple LightCycler® 480 II instruments

The Color Compensation Kit SARS-CoV-2 seqc can be used on multiple LightCycler® 480 II instruments.

Therefore 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.

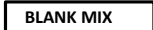







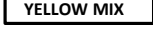



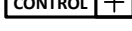


Figure 1: Overview of verification of Color Compensation.



8.1 The Use of the Color Compensation File

To apply the Color Compensation open virellaSARS-CoV-2 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 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		Catalog number
	Green Mix		Contains sufficient for <n> test
	Orange Mix		Upper limit of temperature
	Red Mix		Manufacturer
	Yellow Mix		Use by YYYY-MM-DD
	Blank Control		Batch code
	Positive Control		Content
			Consult instructions for use
		DNA	Deoxyribonucleid Acid
		PCR	Polymerase Chain Reaction
		RNA	Ribonucleic Acid