User manual

4-Color Compensation Set
for Check-Direct CPE

Version 1.2
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REF 18-0070 Σ 1 IFU 070-05

For use on the Roche Light Cycler®480 system I&II

CE IVD

Intended use

The 4-Color Compensation Set is used to create an application-specific color compensation object (or file) on the Light Cycler®480 system I&II. The 4-Color Compensation Set is to be used in combination with the Check-Direct CPE real-time PCR diagnostic kit (Ref 18-0080). The Check-Direct CPE assay requires a color compensation run once a year after the calibration of the optical parts of the LC®480 system I&II. Once the application-specific color compensation object has been performed and the data file created, it is used to analyze all the data generated with the Check-Direct CPE real-time PCR diagnostic test.

Product purpose and function

The Check-Direct CPE real-time PCR diagnostic kit (Ref 18-0080) simultaneously detects four different colors on the LC®480 System I&II. Due to the overlap of the emission spectra of organic dyes, crosstalk emission between detector channels can occur. This phenomenon is described as the overspill of one dye into the next detector channel which may result in the misinterpretation of the data. To correct for cross-talk emission between detector channels, color compensation can be applied when analyzing the data.

The dye calibrators used in the color compensation set are identical to the dyes used in the Check-Direct CPE diagnostic kit. During a color compensation run, the LC®480 instrument measures the fluorescence of each dye calibrator in all the channels and generates an instrument-specific color compensation file or object. When analyzing Check-Direct CPE experimental data, the software uses this color compensation file/object data to reassign the fluorescence in each detector channel to the appropriate dye. As a result, only one dye signal is detected in each channel.

Kit contents for color compensation run

Components (Mat. No.)	Description	Storage conditions
FAM calibrator (9-0092)	1 brown tube (green cap 🟢) 50 µl	- 20°C, **store in the dark**
VIC calibrator (9-0094)	1 brown tube (yellow cap 🟡) 50 µl	
Red calibrator (9-0093)	1 brown tube (red cap 🔴) 50 µl	
Cy5 calibrator (9-0091)	1 brown tube (blue cap 🔵) 50 µl	
FAM control (9-0096)	1 tube (green inlay 🟢) 100 µl	
VIC control (9-0098)	1 tube (yellow inlay 🟡) 100 µl	
Red control (9-0097)	1 tube (red inlay 🔴) 100 µl	
Cy5 control (9-0095)	1 tube (blue inlay 🔵) 100µl	
CPE PCR Mastermix (9-0081)	1 transparent tube and cap (450 µl)	+ 4 °C
User manual (9-0099)	Leaflet – download from website	Not critical

Storage, handling and stability

The product is shipped cooled. The CPE PCR Mastermix should be stored at +4°C upon receipt. All other reagents should be stored at -20°C upon receipt. Please visually inspect the box upon initial opening to ensure that its contents are intact. Please contact the Check-Points office at *support@check-points.com* if you have any further questions. Store kit reagents at indicated temperature until expiration date.

Materials required but not supplied with the kit

Supplies	Equipment
• PCR-grade water (e.g. Milli-Q) • Disposable laboratory (powder-free) gloves • Lab coat • Pipettes & disposable sterile filter- tips for volumes of 1 to 1000 µl • 1.5 ml tubes (“Eppendorf tubes”) • LightCycler® 480 multiwell plate 96 (Product no. 04729692001) • PCR plate seal	• LightCycler®480 Real-time PCR system I/II (Roche, CH) • Vortex mixer • Plate centrifuge • Mini-centrifuge

Good laboratory practices

The quality of the results depends on strict compliance with the following good laboratory practices, especially:

- The test must be performed by adequately trained personnel.
- Do not use reagents after their expiration date.
- Before use, thaw frozen reagents completely at room temperature and vortex briefly to obtain a homogeneous solution. After vortexing briefly, spin down the solution to avoid contamination when opening the lid.
- Follow recommendations for storage, handling and freeze-thaw cycles to preserve the quality of the kit’s reagents.
- Protect reagents from light to avoid photo-bleaching of the dyes.
- Periodically, verify the accuracy and precision of pipettes, as well as correct functioning of the instruments.

Prevention of contaminations

Use separate rooms: a pre-PCR room and a post-PCR room.

- The preparation of the amplification reactions are carried out in the pre-PCR room.
- Incubation in the real-time PCR thermocycler is carried out in the post-PCR room.
- Never transfer items from the post-PCR room to the pre-PCR room.

To keep laboratory free of PCR product contamination:

- Use pipettes with hydrophobic filter tips.
- Make sure to always use a new pipette tip when adding solutions, test samples, and controls to wells of a 96-well plate.
- Follow proper pipette-dispensing techniques to prevent aerosols.
- Wear clean disposable gloves and clean lab coats for the different steps of the test.
- Change gloves whenever you suspect that they are contaminated.
- Keep the tubes of all kit components and samples closed as much as possible.
- Clean the lab benches and all equipment regularly with a 0.5% sodium hypochlorite solution.

Users are strongly advised to read the full protocol before starting the test

Performing the color compensation

1. Important points before starting

- Follow the manual instructions to create the color compensation object. For further information, refer to the LC®480 Instruments Operator's Manual, Software version 1.5, section Advanced Software Functionalities, Color Compensation Analysis.
- On the LC®480 System I&II, the color compensation reactions can be run in parallel to experimental samples, *e.g*. Check-Direct CPE real-time PCR assay.
- Color compensation (CC) is instrument-specific, thus it is necessary to generate a CC object for every LC®480 instrument. A new object has to be created after the optical system has been checked.

2. Reaction setup

Prepare five reaction mixes: one for each calibrator dye and one for the blank. The reaction mixes are prepared in order to have enough volume to load 5 replicates of 25 µL for each reaction mixes on the 96-well plate (see Table 1 and Figure 1).

Prepare the following five reaction mixes according to Table 1:

1) **Mix Blank**: add the CPE PCR Mastermix and adjust final volume with MilliQ water.
2) **Mix FAM**: prepare the reaction mix with the FAM Calibrator dye solution (green cap ●), the FAM Positive Control (green inlay ●), and the CPE PCR Mastermix.
3) **Mix VIC**: prepare the reaction mix with the VIC Calibrator dye solution (yellow cap ●), the VIC Positive Control (yellow inlay ●), and the CPE PCR Mastermix.
4) **Mix Red**: prepare the reaction mix with the Red Calibrator dye solution (red cap ●), the Red Positive Control (red inlay ●), and the CPE PCR Mastermix.
5) **Mix Cy5**: prepare the reaction mix with the Cy5 Calibrator dye solution (blue cap ●), the Cy5 Positive Control (blue inlay ●), and the CPE PCR Mastermix.

3. Detection Format

A new Detection Format needs to be generated when using the 4-Color Compensation Set for the first time. If the format has already been generated, continue to step 4.

- Go to "open tools" in the LC®480 Software 
- Select "Detection Formats" and click on "New"
- Re-name the new format to *Check-Points 4-Color Set*
- Check the boxes of the Excitation/Emission for the corresponding Instrument as outlined in Figure 2.

Table 1: *The five reaction mixes setup*

25µl/reaction	Blank (µl)	FAM (µl)	VIC (µl)	Red610 (µl)	Cy5 (µl)
Calibrator dye solution	0	15	15	15	15
Control	0	60	60	60	60
CPE PCR Mastermix	75	75	75	75	75
Sterile MilliQ water	75	0	0	0	0
Total Reaction Mix	*150*	*150*	*150*	*150*	*150*

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank		FAM		VIC		Red		Cy5			
B												
C												
D												
E												
F												
G												
H												

Figure 1: *Example of 96-well plate setup for LC®480*

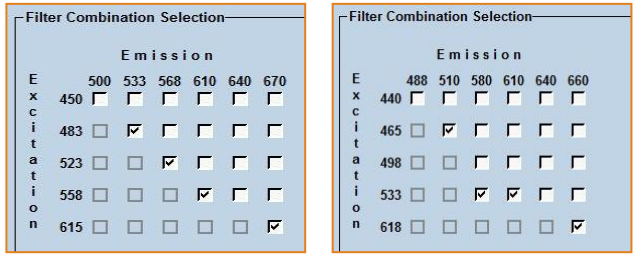

Figure 2: *Filter Combination Selection for LC®480 I & II*

- Change the “Quant Factor” for all the filter combinations to 10 and the “Max Integration Time (Sec)” to 2 as outlined in Figure 3. As a reference also change the name of the filtercombination (e.g. FAM, VIC, RED, CY5)

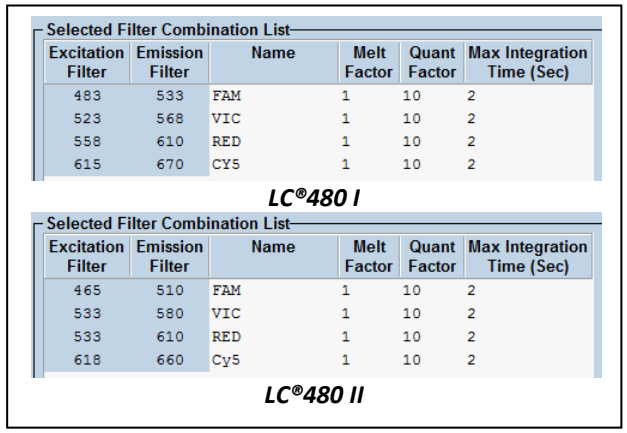

Figure 3: *Filter Combination List for LC®480 I & II*

- Close the Tools window

4. Real-time PCR instrument settings

- In the LC®480 software version 1.5, select **New Experiment**.
- In “Experiment”/“Setup”, select **Detection Format: Check-Points 4-Color Set.**
- In “Experiment”/“Setup”, select **Customize** and make sure all 4 filter combinations are active and the ”Integration Time Mode” is set to “Dynamic”.
- In “Experiment”/“Setup”, enter “Reaction Volume”: “25”.
- In “Experiment”/“Programs”, create the color compensation PCR program as presented in Table 2.

Table 2: *Color compensation program setup*

Program Name: Hotstart Step							
Cycles : 1				Analysis Mode: none			
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (°C)
50	None	00:02:00	4.40		0	0	0
95	None	00:10:00	4.40		0	0	0
Program Name: Amplification Step							
Cycles : 45				Analysis Mode: quantification			
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (°C)
95	None	00:00:15	4.40		0	0	0
60	Single	00:01:00	2.20		0	0	0
Program Name: Color Compensation Step							
Cycles : 1				Analysis Mode: color compensation			
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (°C)
95	None	00:00:10	4.40				
40	None	00:00:30	2.20				
80	Continuous		0.03	5			
Program Name: Cooling Step							
Cycles : 1				Analysis Mode: none			
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (°C)
40	None	00:00:30	2.20		0	0	0

Data analysis

Important points before starting: For a detailed description of how to operate your real-time PCR instrument and how to analyze data, please refer to the real-time PCR instrument's instruction manual.

1. In **Sample Editor**, select work-flow: **Color Comp**.
2. Define the properties of the samples. Enter the "Dominant Channel" corresponding to the sample calibrator loaded on the plate for the color compensation. As described in Figure 4, for samples in position A1 to E1 select Dominant Channel **Water**; for samples in position A3 to E3 select Dominant Channel **FAM**; for samples in position A5 to E5 select Dominant Channel **VIC**; for samples in position A7 to E7 select Dominant Channel **Red** for samples in position A9 to E9 select Dominant Channel **Cy5**
3. Select **Analysis**
4. Select **Color Compensation** from the Analysis menu
5. Select the subset of samples corresponding to the color compensation experiment.
6. Select **Calculate**
7. Select **Save CC Object**. The stored *Color Compensation Object* should be used for the analysis of runs conducted with the following product: Check-Direct CPE Real-time PCR kit (Ref 18-0080).

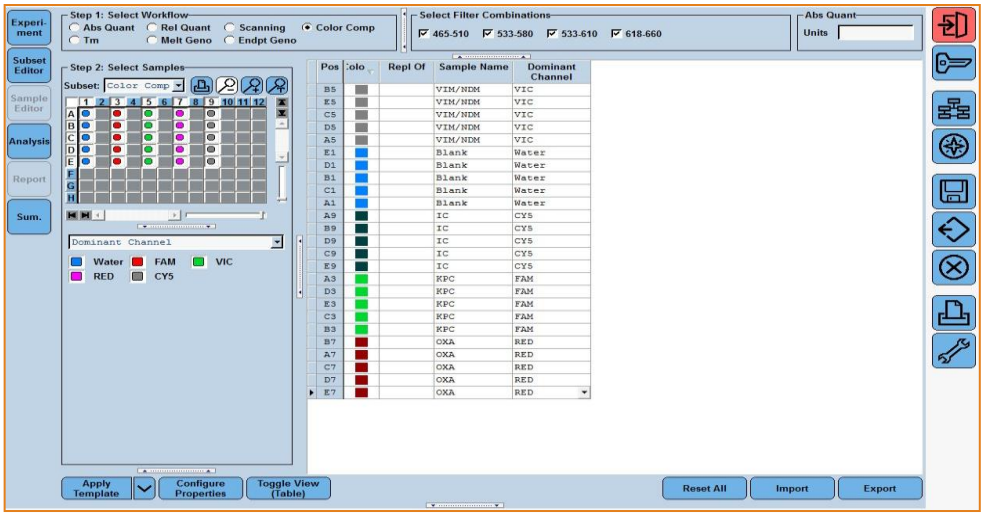

Figure 4: *Screen shot of LC®480 Software version 1.5. Sample editor selection for the 4-color compensation sample set with LC®480.*

Frequently asked questions (FAQ) & Troubleshooting

1. **The real-time PCR gives an error message.**
   Refer to the real-time PCR instrument user manual or contact the local technical support of the real-time PCR instrument company.

2. **I left Solutions out of the -20°C (-4˚F) storage.**
   These reagents must be stored at -20°C (-4°F) for proper performance of the test. The performance of the product cannot be fully guaranteed if these solutions were left out of -20°C (-4˚F) for more than 24 hours.

3. **Real-time results show no detection or very low fluorescent signals detection for all calibrators and in all detector channels.**
   Possible causes and troubleshooting:
   - The Calibrator dye solutions containing the fluorescent probes and primers are degraded. Please check expiration date, the number of thaw/freezing cycles that CPE solution tube have undergone, and if the kit was stored correctly.
   - The real-time PCR system may be responsible for these results. Please refer to instrument User's manual or contact your real-time PCR instrument local representative.

4. **Check-Direct CPE results show cross-talk emission signals in one or more detector channels.**
   - The Calibrator dye solutions containing the fluorescent probes and primers is degraded. Please check expiration date, the number of thaw/freezing cycles that CPE solution tube have undergone, and if the kit was stored correctly.
   - The color compensation run was not performed well, repeat the assay.
   - The CPE PCR Mastermix did not perform well. Check expiration date and if the solution was stored correctly.

5. **Data Analysis and Interpretation.**
   If you encounter difficulties with the data analysis and interpretation please refer to LC®480 Instruments Operator's Manual– Software version 1.5, section Advanced Software Functionalities (Chapter 7) Color Compensation Analysis (pp.248-256). Alternatively contact Check-Points Technical Support at **support@check-points.com**

Key to symbols used

IVD	For *In Vitro* Diagnostic Use	CAL RED	Red calibrator
REF	Catalog number	CAL VIC	VIC calibrator
LOT	Batch code	CONTROL CY5	Cy5 control
IFU	IFU number	CONTROL FAM	FAM control
CAL CY5	Cy5 calibrator	CONTROL RED	Red control
CAL FAM	FAM calibrator	CONTROL VIC	VIC control
PCR Mastermix CPE	CPE PCR Mastermix		
	Temperature limitation		Use before YYYY-MM
	Contains sufficient for < n > tests		Manufacturer

Technical assistance

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