# User manual

## 4-Color Compensation Set
## for Check-Direct CPE

**Version 1.0**
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REF 18-0070 Σ 2 IFU 070-03

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

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INTENDED USE ..... 2
PRODUCT PURPOSE AND FUNCTION ..... 2
KIT CONTENTS FOR 2 COLOR COMPENSATION RUNS ..... 2
STORAGE, HANDLING AND STABILITY ..... 2
MATERIALS REQUIRED BUT NOT SUPPLIED WITH THE KIT ..... 3
GOOD LABORATORY PRACTICES ..... 3
PERFORMING THE COLOR COMPENSATION ..... 4
1. IMPORTANT POINTS BEFORE STARTING ..... 4
2. REACTION SETUP ..... 4
3. REAL-TIME PCR INSTRUMENT SETTINGS ..... 4
DATA ANALYSIS ..... 5
FREQUENTLY ASKED QUESTIONS (FAQ) & TROUBLESHOOTING ..... 6
KEY TO SYMBOLS USED ..... 6
TECHNICAL ASSISTANCE ..... 6

## 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) detects simultaneously 4 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 2 color compensation runs

| 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 | |
| Red610 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 | |
| Red610 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 (550 µl) | + 4 °C |
| Manual (9-0099) | Leaflet – download from website | Not critical |

## Storage, handling and stability

Check-Direct CPE reagents are 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. Check-Direct CPE solution should not be exposed to more than 12 freeze-thaw cycles. 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 |
|---|---|
| • Sterile MilliQ water<br>• Disposable laboratory (powder-free) gloves<br>• Lab coat<br>• Pipettes & disposable sterile filter- tips for volumes of 1 to 1000 µl<br>• 1.5 ml tubes (“Eppendorf tubes”)<br>• 96-well PCR white plate<br>• PCR plate seal | • LightCycler®480 Real-time PCR system I&II (Roche, CH)<br>• Vortex mixer<br>• Plate centrifuge<br>• 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.
- The 4-Color Compensation Set contains sufficient reagents to run 2 color compensation run.
- 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 3 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 Red610**: prepare the reaction mix with the Red610 Calibrator dye solution (red cap ●), the Red610 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.

***Table 1:*** *The five reaction mixes setup*

| 25µl/reaction | Blank (µl) | FAM (µl) | VIC (µl) | Red610 (µl) | Cy5 (µl) |
|---|---|---|---|---|---|
| Calibrator dye solution | 0 | 10 | 10 | 10 | 10 |
| Control | 0 | 40 | 40 | 40 | 40 |
| CPE PCR Mastermix | 50 | 50 | 50 | 50 | 50 |
| Sterile MilliQ water | 50 | 0 | 0 | 0 | 0 |
| *Total Reaction Mix* | *100* | *100* | *100* | *100* | *100* |

## 3. Real-time PCR instrument settings

- In the LC®480 software version 1.5, select **New Experiment**.
- In "Experiment"/"Setup", select **Detection Format**
  - LC®480 I: **Multi Color Hydrolysis Probe**
  - LC®480 II: **4 color FAM VIC/HEX RED610 CY5**.

- In "Experiment"/"Setup", select **Customize** and then select the four detectors specific to the instrument in use indicated in Table 2.
- In "Experiment"/"Setup", enter "Reaction Volume": "25".
- In "Experiment"/"Programs", create the color compensation PCR program as presented in Table 3.

***Table 2:*** *Color compensation dyes filter combination to select*

| LC®480 I Detector | LC®480 II Detector |
|---|---|
| FAM (483-533) | FAM (465-510) |
| VIC/HEX/Yellow555 (523-568) | VIC/HEX (533-580) |
| Red610 (558-610) | Red610 (533-610) |
| Cy5 (615-670) | Cy5 (618-660) |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blank | | FAM | | VIC | | Red 610 | | Cy5 | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

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

***Table 3:*** *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 2, for samples in position A1 to C1 select Dominant Channel **Water**; for samples in position A3 to C3 select Dominant Channel **FAM**; for samples in position A5 to C5 select Dominant Channel **VIC/HEX**; for samples in position A7 to C7 select Dominant Channel **Red610** for samples in position A9 to C9 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).

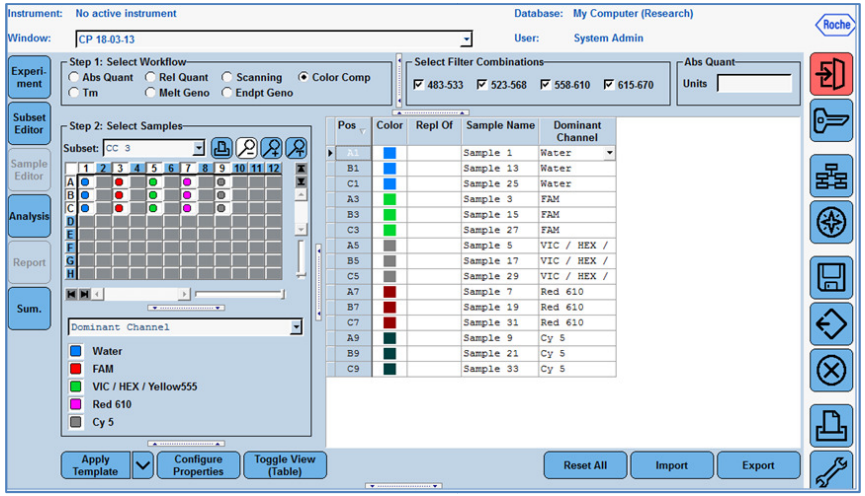


***Figure 2:*** *Screen shot of LC®480 Software version 1.5. Sample editor selection for the 4-color compensation sample set with LC®480*

## 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 RED610 | Red610 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 RED610 | Red610 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 |
| | Consult instructions for use | | |

## Technical assistance

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