QIAGEN Supplementary Protocol

Generating and applying color compensation files for the LightCycler® 480 system

This supplementary protocol is intended for users of the LightCycler 480 system who are performing multiplex, real-time PCR using QuantiFast® Multiplex Kits, QuantiTect® Multiplex Kits, or QuantiTect Virus Kits. The protocol describes how to generate and apply a color compensation file, which is required for accurate analysis of multiplex, real-time PCR data for most combinations of reporter dyes.

IMPORTANT: Please read the handbook supplied with the QuantiFast Multiplex Kit, QuantiTect Multiplex Kit, or QuantiTect Virus Kit, paying careful attention to the "Safety Information" and "Important Notes" sections, before beginning this procedure.

Introduction

The LightCycler 480 system has detection channels that allow detection of multiple reporter dyes in the same reaction. However, even when reporter dyes with well separated emission spectra are used, each reporter dye will be detected by more than one detection channel. Therefore, multiplex, real-time PCR results will be inaccurate for most combinations of reporter dyes unless a correction is made. This is achieved by using a color compensation file, which contains information that corrects the crosstalk between the detection channels:

- Color compensation files can be generated before or after carrying out multiplex, real-time PCR and can be stored for later use.
- Each color compensation file is specific for a specific combination of reporter dyes. It is necessary to generate a new color compensation file if a new combination of reporter dyes is used.
- A color compensation file is specific for the instrument it was created on. Therefore, if you want to repeat a multiplex assay on another LightCycler 480 system, you will also need to generate a new color compensation file on the same instrument.

If you are carrying out duplex PCR assays, certain combinations of reporter dyes are spectrally well separated and may not require a color compensation file. For details, see Table 4 (page 7).

The procedure below describes how to generate and apply color compensation files for duplex, triplex, or 4plex PCR assays using TaqMan® probes or other dual-labeled probes. The following steps are required:

- Preparing a source of reporter dyes for the color compensation experiment. This is achieved by running replicate singleplex reactions for each of your reporter dyes until they reach the plateau phase. In addition, replicate control reactions containing master mix only are also prepared.
- Performing a color compensation experiment. Fluorescence data are collected and used to generate a color compensation file containing information for correcting crosstalk between detection channels.
- Applying the color compensation file after carrying out a multiplex, real-time PCR experiment when performing data analysis.

  Note: The color compensation file is applied to all samples in the experiment. If the experiment contains more than one combination of reporter dyes, several color compensation files (one for each combination of dyes) will be required.

Procedure

Creating samples for a color compensation experiment

1. **For each reporter dye, set up at least 5 replicate singleplex reactions containing 1x master mix, template, and primer–probe set. In addition, set up at least 5 replicate control reactions containing 1x master mix only.**

   Use the same master mix as the one that will be used in your multiplex assay. For reaction setup, refer to the handbook supplied with the QIAGEN multiplex PCR kit you are using, and follow the duplex protocol for the LightCycler 480. Be sure to use the specified reaction volumes for a 96- or 384-well plate.

2. **Run the singleplex reactions until they reach the plateau phase. Check whether PCR amplification was successful before using the completed reactions as a source of reporter dyes in the next part of this procedure.**

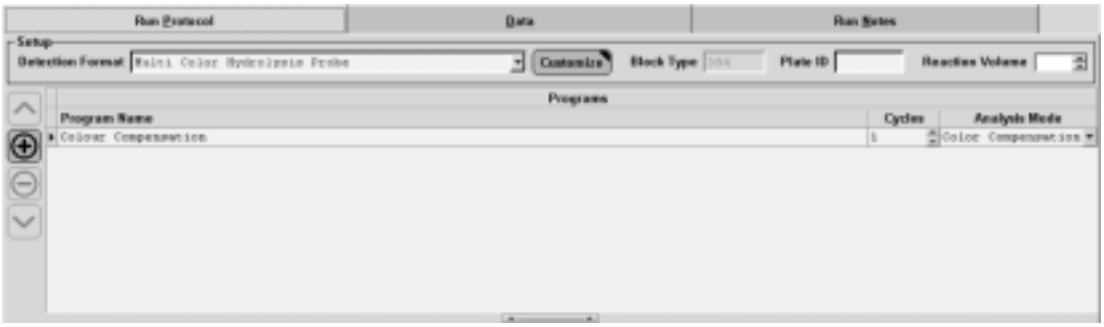
Performing a color compensation experiment

3. **Start the LightCycler 480 Software (version 1.2).**

   Note: To be able to generate a color compensation file, you first need to log on to the software using a user account that includes access rights for *Administrator* or *Expert User*.

4. **In the drop-down menu at the top of the window, select *New Experiment*. Then click the "Experiment" button on the left-hand side of the window.**

5. **In the "Programs" panel, enter in the "Program Name" dialog field a name for the temperature protocol. Be sure that the "Cycles" dialog field contains a value of 1. In the "Analysis Mode" dialog field, select *Color Compensation*.**



6. **In the "Temperature Targets" panel, enter the temperature protocol according to the parameters in Table 1.**

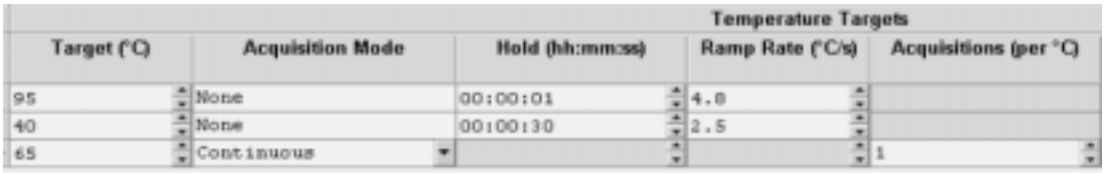
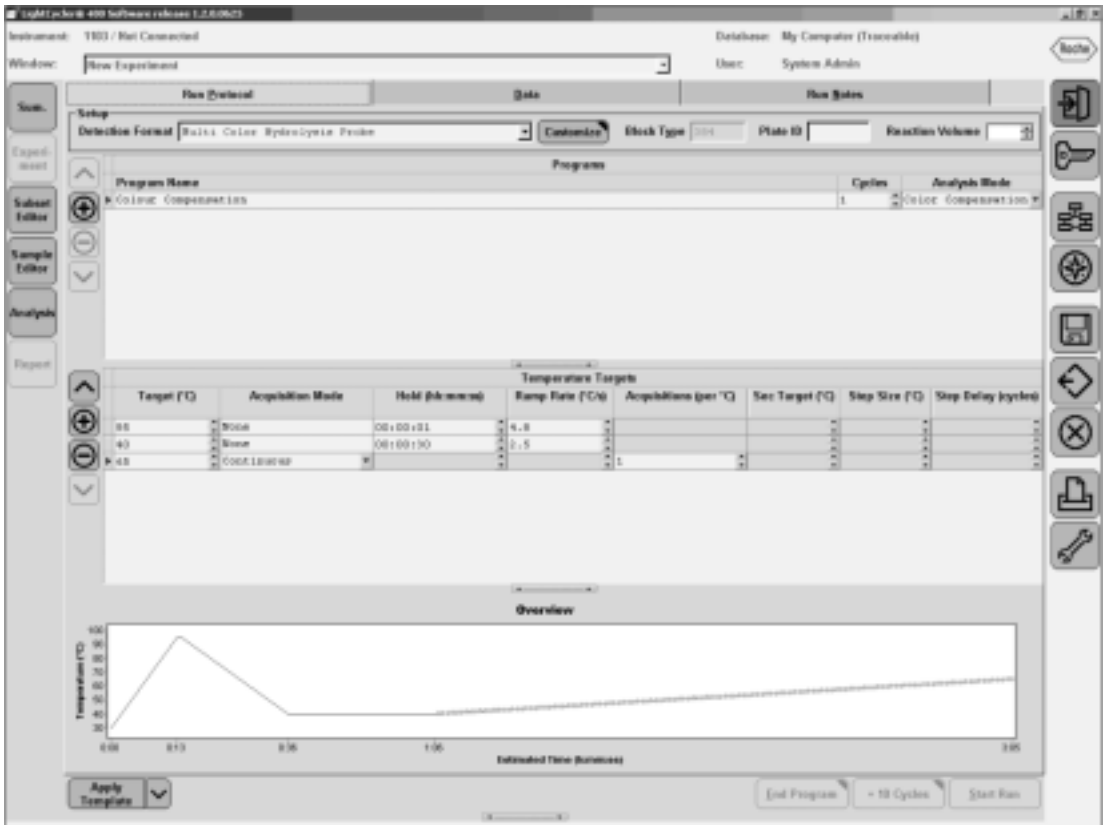


Table 1. Parameters for "Temperature Targets" panel

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:00	4.8	
40	None	00:00:30	2.5	
x*	Continuous	00:00:00		1

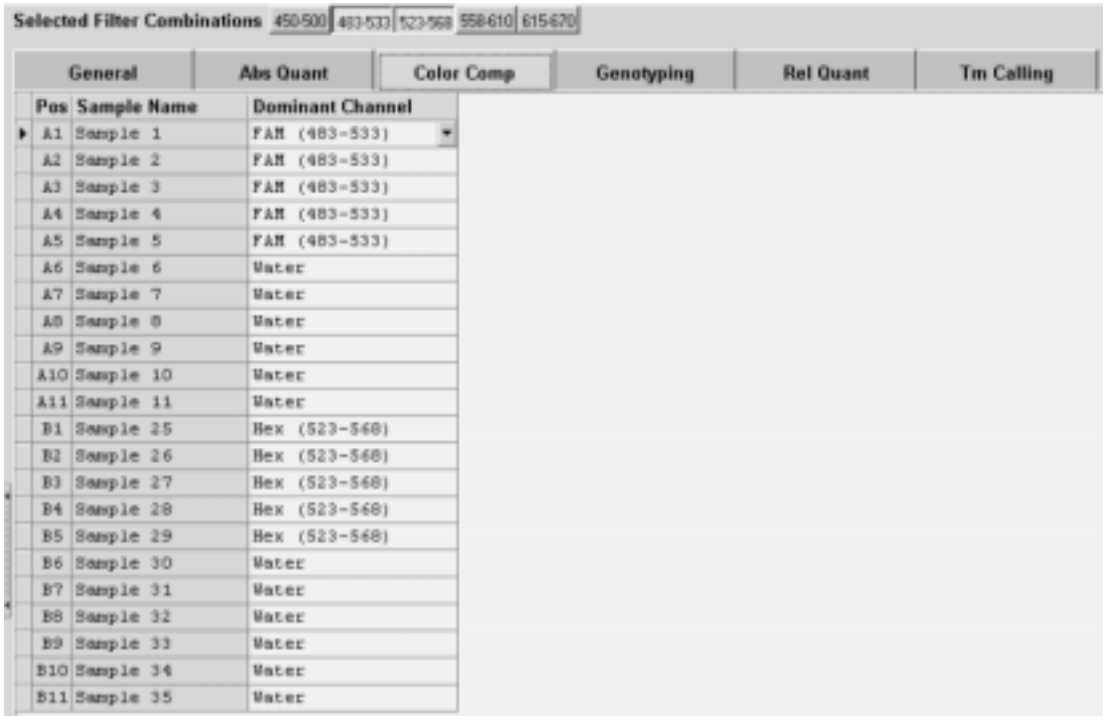
* x = Temperature of annealing/extension step in multiplex assay (°C) + 5°C

The final screen should look like the screenshot below.



7. Click the "Sample Editor" button on the left-hand side of the window. Then select the "Color Comp" tab.

8. **In the "Dominant Channel" dialog fields, select the dyes contained in the different samples. See Table 2 for reporter dyes and the corresponding dominant channels. For the replicate samples containing no dye, select *Water*.**



Pos	Sample Name	Dominant Channel
A1	Sample 1	FAM (483-533)
A2	Sample 2	FAM (483-533)
A3	Sample 3	FAM (483-533)
A4	Sample 4	FAM (483-533)
A5	Sample 5	FAM (483-533)
A6	Sample 6	Water
A7	Sample 7	Water
A8	Sample 8	Water
A9	Sample 9	Water
A10	Sample 10	Water
A11	Sample 11	Water
B1	Sample 25	Hex (523-568)
B2	Sample 26	Hex (523-568)
B3	Sample 27	Hex (523-568)
B4	Sample 28	Hex (523-568)
B5	Sample 29	Hex (523-568)
B6	Sample 30	Water
B7	Sample 31	Water
B8	Sample 32	Water
B9	Sample 33	Water
B10	Sample 34	Water
B11	Sample 35	Water

Table 2. Suitable combinations of reporter dyes for the LightCycler 480 system

Type of assay	Filter (Channel 1) Cyan 500 (450–500)*	Filter (Channel 2) FAM (483–533)*	Filter (Channel 3) HEX (523–568)*	Filter (Channel 4) Red 610 (558–610)*	Filter (Channel 5) Cy5 (615–670)*
Duplex		**6-FAM**	**HEX** JOE VIC®		
Duplex		**6-FAM**		**Texas Red®** **ROX**	
Duplex		**6-FAM**			**Cy®5**
Triplex		**6-FAM**	**HEX** JOE VIC	**Texas Red** **ROX**	
Triplex		**6-FAM**	**HEX** JOE VIC		**Cy5**
Triplex		**6-FAM**		**Texas Red** **ROX**	**Cy5**
4plex		**6-FAM**	**HEX** JOE VIC	**Texas Red** **ROX**	**Cy5**
5plex	**Cyan 500**	**6-FAM**	**HEX**	**Texas Red**	**Cy5**

* The numbers in parentheses indicate the band path wavelengths of the detection filters. Reporter dye combinations marked in bold have been successfully tested by QIAGEN.

Table 3. Suitable combinations of reporter dyes for the LightCycler 480 system which require no color compensation file

Type of assay	Filter (Channel 1) Cyan 500 (450–500)*	Filter (Channel 2) FAM (483–533)*	Filter (Channel 3) HEX (523–568)*	Filter (Channel 4) Red 610 (558–610)*	Filter (Channel 5) Cy5 (615–670)*
Duplex	Cyan 500			Texas Red ROX	
Duplex	Cyan 500				Cy5
Duplex		6-FAM		Texas Red ROX	
Duplex		6-FAM			Cy5
Duplex			HEX JOE VIC		Cy5

9. **Save your changes by clicking the**  **icon.**
10. **Place the PCR plate (which contains the samples prepared in step 2) in the LightCycler 480.**
11. **Click the "Start Run" button at the bottom of the window to start the experiment.**
12. **After the experiment ends, click the "Analysis" button in the left-hand side of the window to open the "Create New Analysis" dialog box. Select "Color Compensation". Then click "OK".**
13. **Click the "Calculate" button to perform color compensation analysis.**
14. **Click the "Save CC Object" button to save the color compensation file.**

    The color compensation file is now ready to use.

Applying a color compensation file for data analysis

15. **Open the assay, and click the "Filter Combination" button to select the filter combination you want to display.**

16. **In the following drop-down menu, select *In Database*, and choose the stored color compensation file you want to apply to the assay.**

The "Color Comp" button switches to "Color Comp (On)" to confirm that color compensation is applied.

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