QIAGEN Supplementary Protocol

Generating and applying color compensation files for the LightCycler® 2.0 system

This supplementary protocol is intended for users of the LightCycler 2.0 system who are performing multiplex, real-time PCR using QuantiFast® Multiplex Kits or QuantiTect® Multiplex 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.

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

Introduction

The LightCycler 2.0 system has detection channels that allow detection of multiple reporter dyes in the same capillary. 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 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 2.0 system, you will also need to generate a new color compensation file on the same instrument.

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

- Preparing samples, each containing 1x master mix and one of the reporter dyes to be used in a multiplex, real-time PCR experiment (use the master mix supplied with the QuantiFast or QuantiTect Multiplex Kit). In addition, a sample containing 1x master mix only (the background sample) is needed.

The samples containing reporter dyes should exhibit significant fluorescence. Possible sources of such dyes include single-dye real-time PCR samples that have reached the PCR plateau phase or oligonucleotides labeled with reporter dye.

The most convenient source of reporter dyes are the probes to be used in your multiplex assay. Although TaqMan probes are also labeled with a quencher, the fluorescence from the reporter dye is not absolutely quenched. When the concentrations of probes described in the procedure below are used, the residual fluorescence from the reporter dyes is sufficient for generating a color compensation file. Although we recommend using probes labeled with a nonfluorescent quencher, the procedure below is also compatible with probes labeled with TAMRA dye as quencher.

- 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 before carrying out a multiplex, real-time PCR experiment or afterwards 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. **Set up 2–4 samples, each containing a single reporter dye in 1x master mix, according to Table 1 (use the master mix supplied with the QuantiFast or QuantiTect Multiplex Kit). Each sample corresponds to one of the reporter dyes used in your multiplex PCR assay. Also prepare a sample containing 1x master mix only.**

   Therefore, you need to prepare 3 samples for a duplex assay, 4 samples for a triplex assay, or 5 samples for a 4plex assay.

Table 1. Preparing samples containing TaqMan probes for a color compensation experiment*†

Component	Volume	Final concentration
2x master mix (from QuantiFast or QuantiTect Multiplex Kit)	10 µl	1x
TaqMan probe labeled with:		
6-FAM, HEX, JOE, or VIC®, or	Variable	0.5 µM
Texas Red® or ROX, or	Variable	2 µM
Alexa Fluor® 660 or Pulsar® 650	Variable	2 µM
RNase-free water	Variable	–
Total volume	**20 µl**	**–**

* Each sample contains one TaqMan probe only. In addition, a control sample containing 1x master mix only must be prepared.

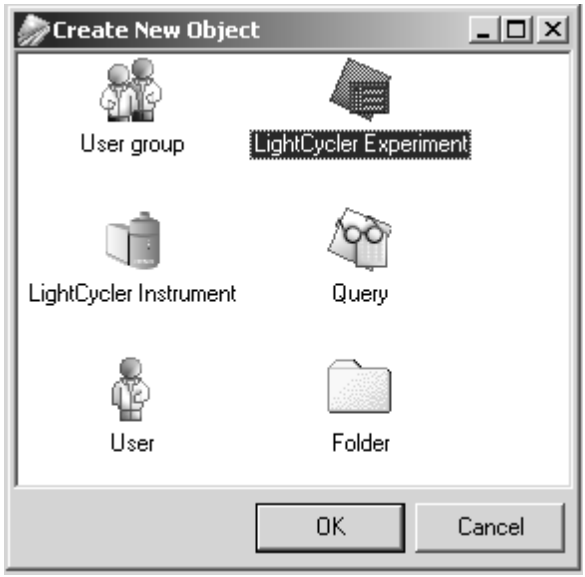
† If using 100 µl capillaries, increase the amounts of all components by 5-fold.

Performing a color compensation experiment

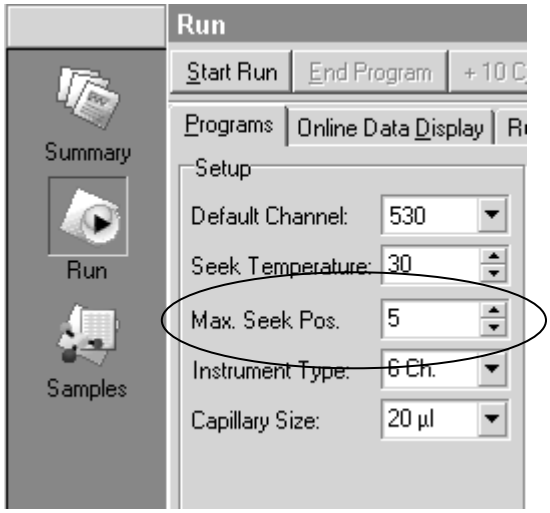
2. **Start the LightCycler Software 4.**

   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*.

3. **Click the "File" menu, and select "New" to open the "Create New Object" dialog box. Double-click the "LightCycler Experiment" icon to open a new LightCycler experiment file.**



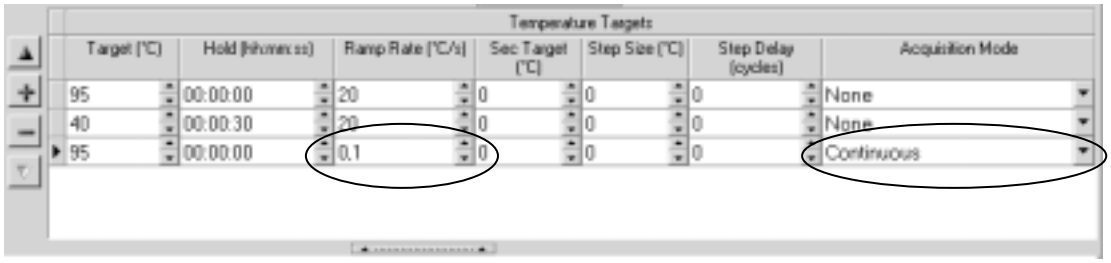
4. **Select the "Programs" tab, and enter in the "Max. Seek Pos." dialog field the number of samples that will be analyzed in the experiment.**



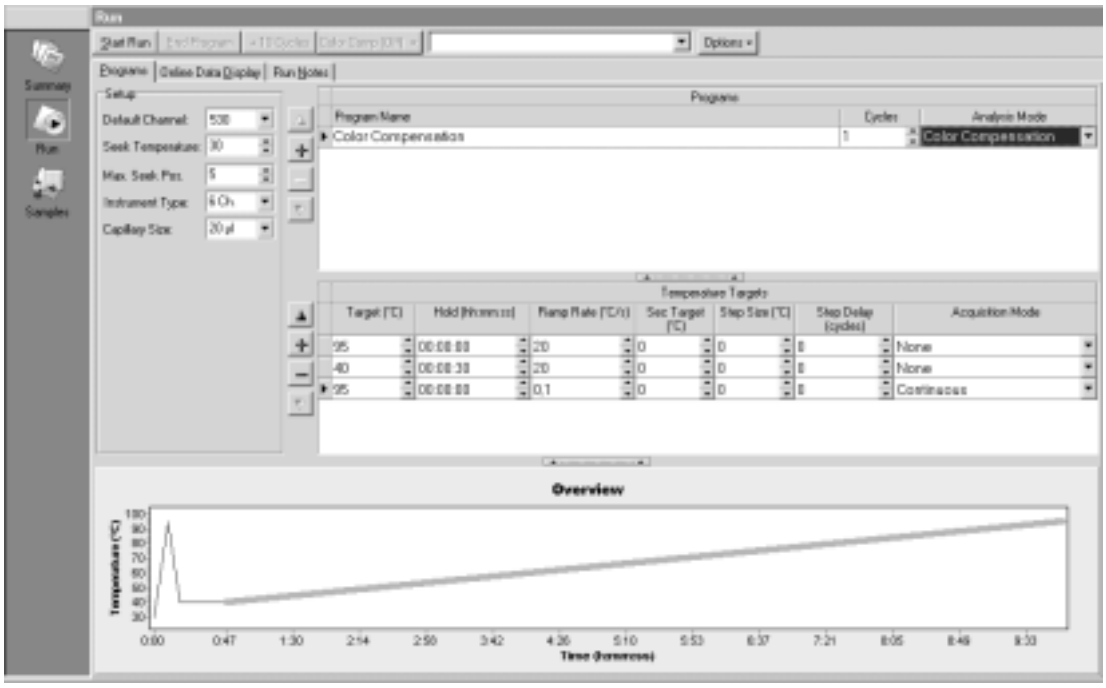
Enter a value of 3 for duplex assays, a value of 4 for triplex assays, or a value of 5 for 4plex assays.

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*.**

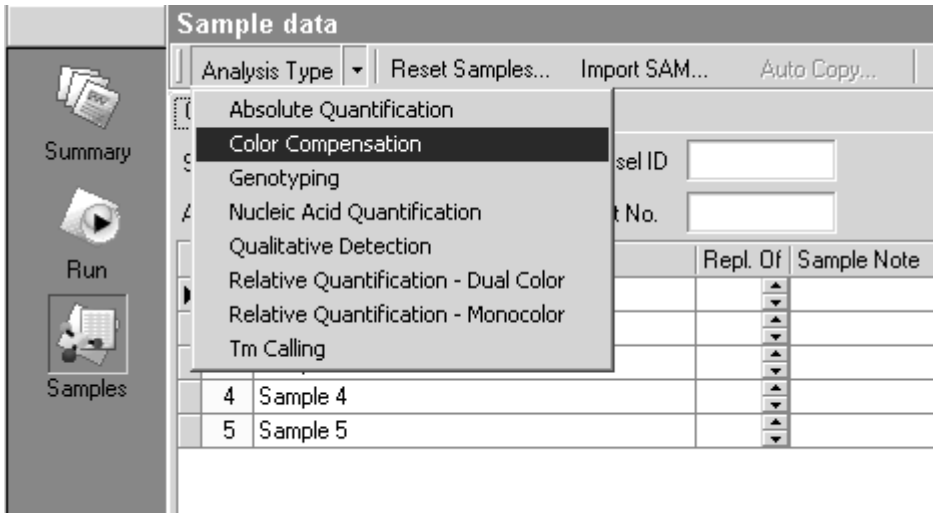
6. **In the "Temperature Targets" panel, enter the temperature protocol as shown below. In the last step, make sure that "Ramp Rate" is set to a value of 0.1°C/s and "Acquisition Mode" is set to *Continuous*.**



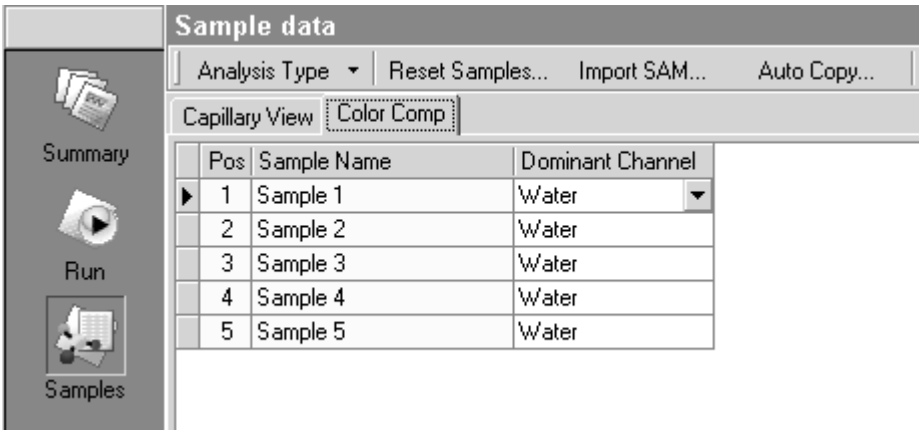
The final screen should look like the screenshot below.



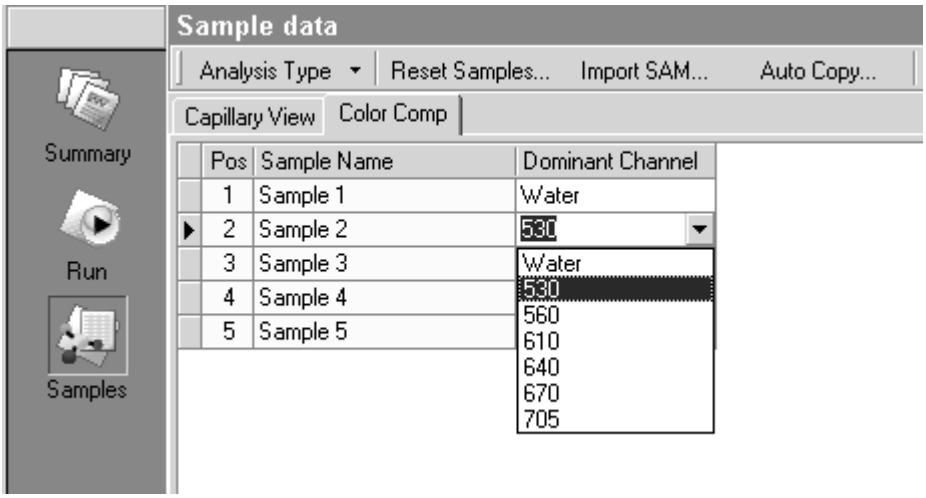
7. Click the "Samples" icon on the left-hand side of the window. Then click the "Analysis Type" menu, and select "Color Compensation".



The "Color Comp" tab appears.



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.**



Note: *Sample 1* must be 2x master mix diluted with water to a 1x concentration (use the master mix supplied with the QuantiFast or QuantiTect Multiplex Kit). For *Sample 1*, the "Dominant Channel" must be set to *Water*. Be sure to set a different "Dominant Channel" for each sample.

Table 2. Reporter dyes tested for use on the LightCycler 2.0 system, and corresponding dominant channels

Dye	Excitation maximum (nm)	Emission maximum (nm)*	Dominant channel
FAM	494	518	530 nm
JOE	520	548	560 nm
VIC	538	552	560 nm
Yakima Yellow®	526	552	560 nm
HEX	535	553	560 nm
ROX	587	607	610 nm
Texas Red	596	615	610 nm
Alexa Fluor 660	660	690	705 nm
Pulsar 650†	460	650	705 nm

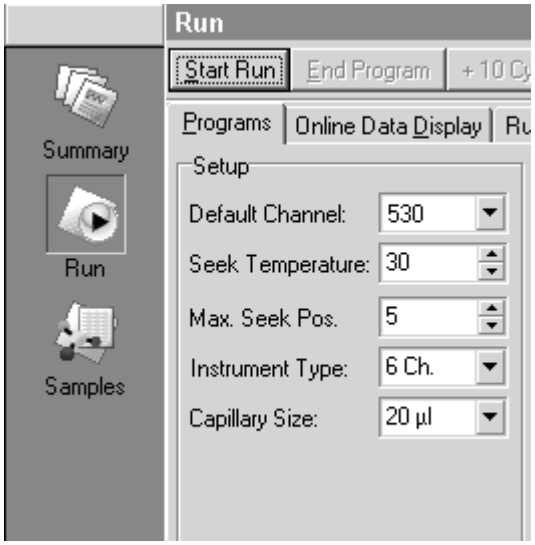
* Emission spectra may vary depending on the buffer conditions.

† This dye exhibits a broad emission peak. The greatest signal can be detected using the 705 nm channel.

9. **Place the capillaries (which contain the samples prepared in step 1) in the LightCycler 2.0. Sample 1 (i.e., master mix diluted with water) must be placed in position 1 of the carousel. The other samples must be placed in order of increasing emission wavelength. For example, if creating a color compensation file for a 4plex PCR assay, place sample 2 (containing the dye with the lowest emission wavelength) in position 2, and place sample 5 (containing the dye with the highest emission wavelength) in position 5.**

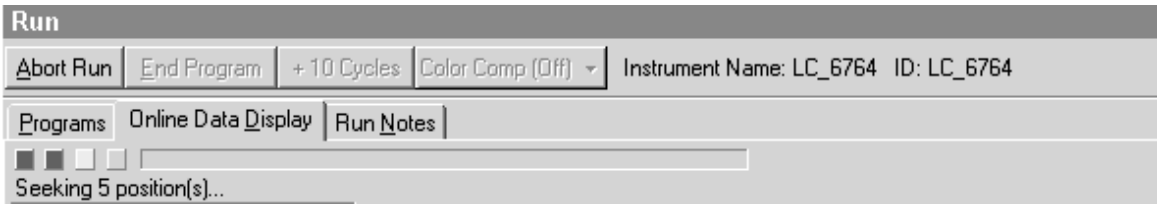
   Ensure that at least one button in the "Selected Channels" panel is selected.

10. Click the "Run" icon. Then click the "Start Run" button to start the experiment.

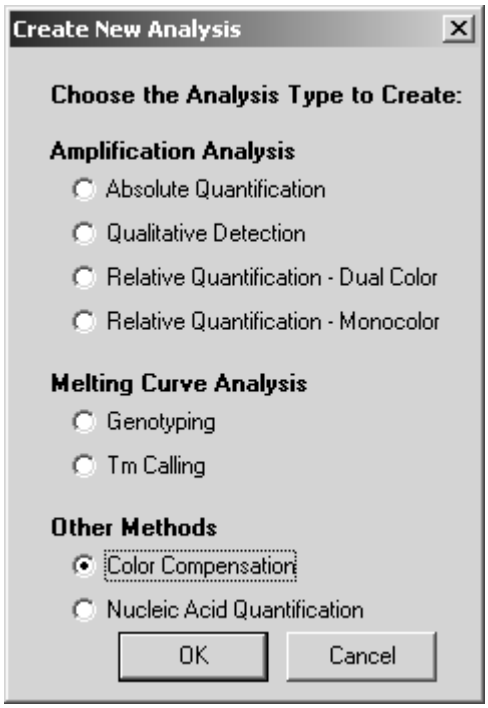


11. Click the "Save" button" to save the experiment. In the dialog box which appears, enter a name for the file, and specify the location in which to save the file.

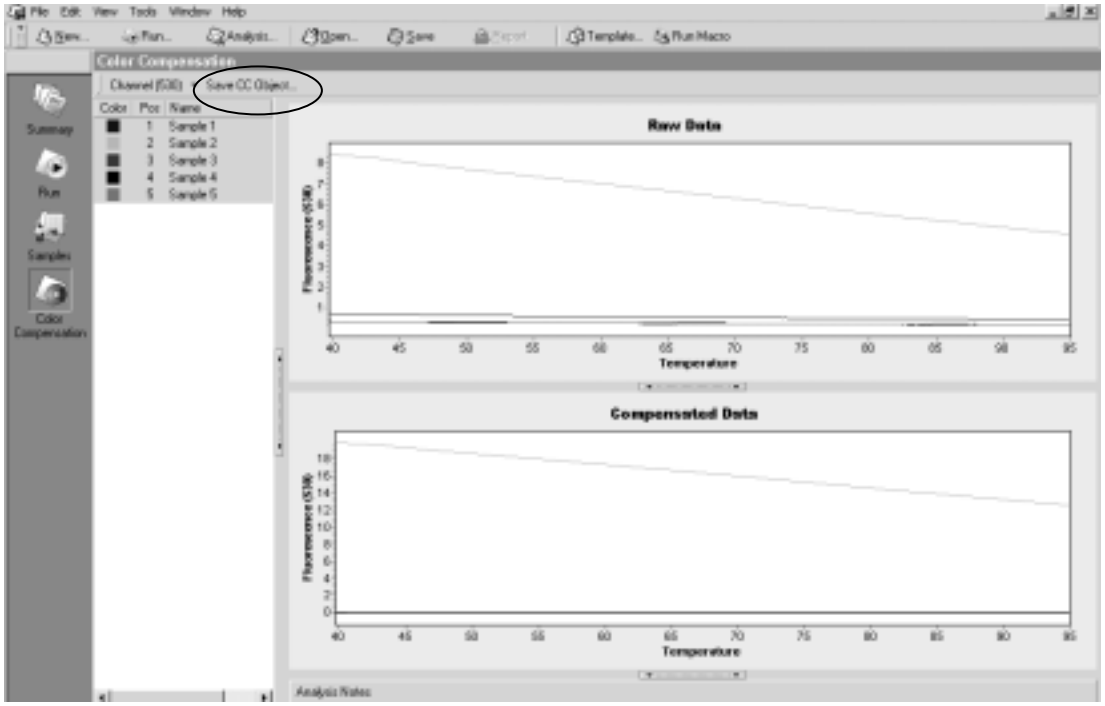
12. During the experiment, a progress bar appears.



13. After the experiment ends, click the "Analysis" button to open the "Create New Analysis" dialog box. Under "Other Methods", select "Color Compensation". Then click "OK".



14. In the "Color Compensation" window which appears, click the "Save CC Object" button to save the color compensation file.

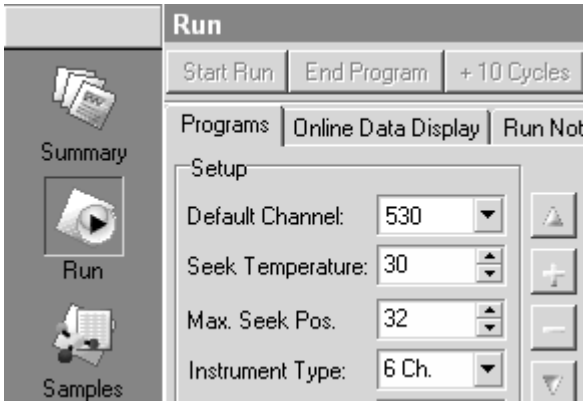


Applying a color compensation file for data analysis

15. Follow steps 16–20 if you want to apply a color compensation file <u>before</u> carrying out a multiplex, real-time PCR experiment. Alternatively, follow steps 21–26 if you want to apply a color compensation file <u>after</u> a multiplex, real-time PCR experiment.

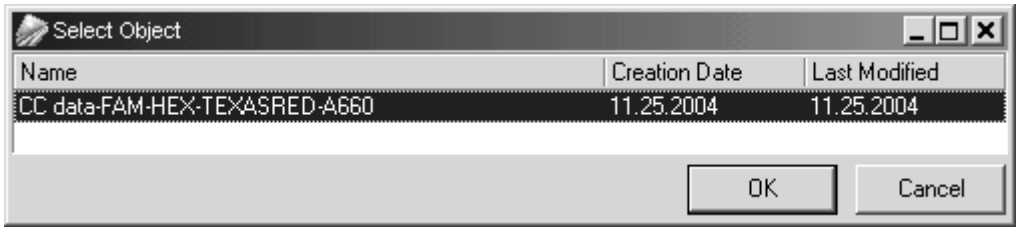
Applying a color compensation file before a multiplex, real-time PCR experiment

16. **Click the "Run" icon on the left-hand side of the window.**

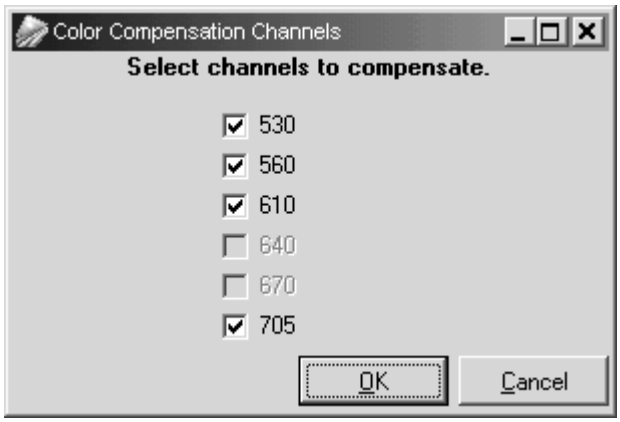


17. **Click the "Color Comp" button, and select "Select Color Compensation…" to open the "Select Object" dialog box.**

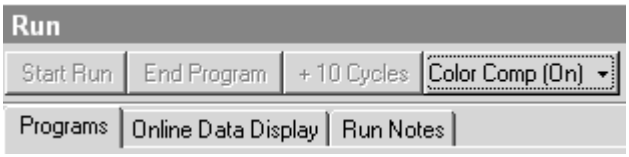
18. **Select a color compensation object from the list displayed in the "Select Object" dialog box. Then click "OK".**



19. **The "Color Compensation Channels" dialog box opens. The channels entered for the generation of the color compensation file are selected by default. Click "OK" to confirm the selection.**

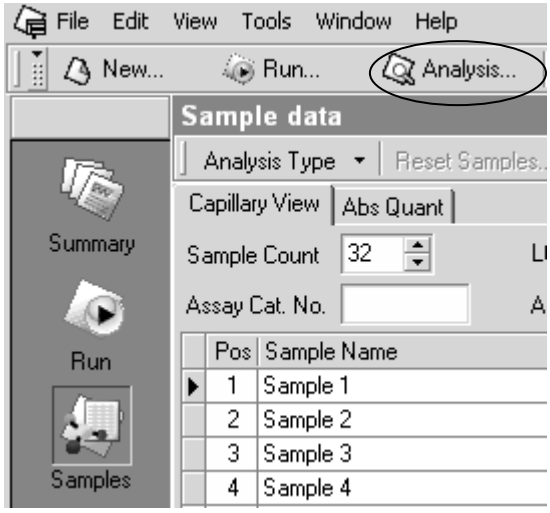


20. The "Color Comp" button switches to "Color Comp (On)" to confirm that color compensation is active.

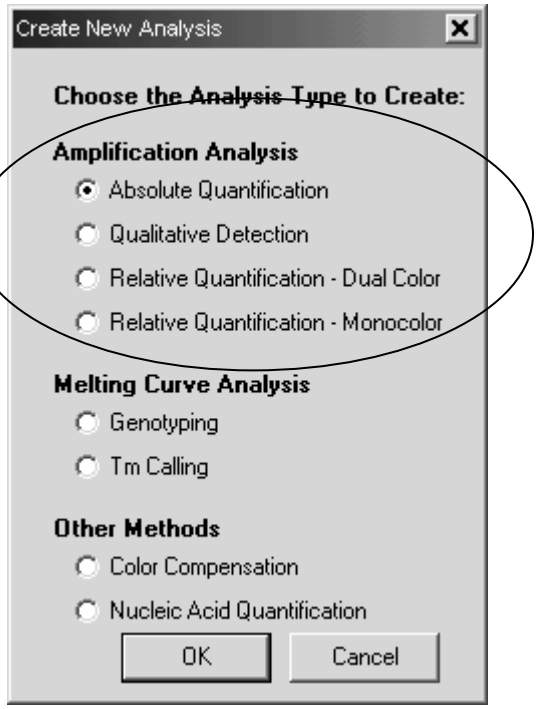


Applying a color compensation file <u>after</u> a multiplex, real-time PCR experiment

21. After the experiment has ended, click the "Analysis" button to open the "Create New Analysis" dialog box.



22. Select the type of analysis required, and click "OK".

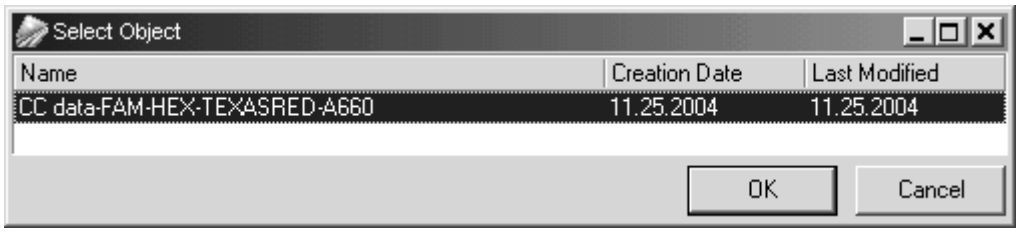


For details on the different analysis modes, refer to the *LightCycler 2.0 Instrument Operator's Manual*.

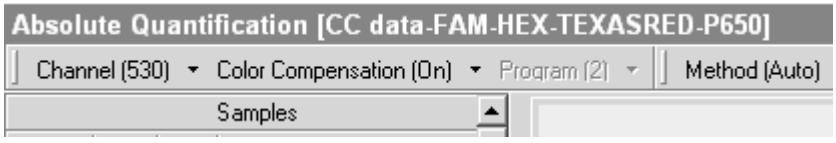
23. Click the appropriate "Quantification" button on the left-hand side of the window.

24. Click the "Color Comp" button, and select "Select Color Compensation..." to open the "Select Object" dialog box.

25. Select a color compensation object from the list displayed in the "Select Object" dialog box. Then click "OK".



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



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