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Color Compensation on the Roche LightCycler 2.0 Instrument using LightCycler 4.0 Software

How do I use this guide?

This guide is formatted in a FAQ style; however, simply following the individual questions from beginning to end will provide the step-by-step instructions to perform a Color Compensation on the LightCycler 2.0 instrument.

Why should I color compensate my data?

The basic premise of color compensation for any instrument, be it real-time PCR or otherwise, is that samples which contain more than one fluorescent signal must be segregated into the contributing signals. This is necessary due to the fact that fluorescent dyes do not simply emit fluorescence at a single wavelength; rather, their fluorescence is emitted over a broad range of wavelengths. The vast majority of fluorescent dyes have emission spectra ranging in the $100\text{-}200^{+}$ nm range. Thus each emission will be producing at least some signal in each of the higher wavelength channels.

So the color compensation file makes it possible for the LightCycler 2.0 instrument to exclusively 'see', in any one channel, the fluorescent signal from the dye designated for that channel.

Do I need to color compensate my data?

Unless you are running reactions which contain more than one signal producing dye in a single capillary you will **NOT** need to perform color compensation.

I have a two color fluorescent system, how do I set up the color compensation reactions?

Two colors; three colors...six colors the basic protocol is the same. You will want to have a reaction capillary dedicated to each dye, and one for a blank (no dye). In the case of a two color system you will need three reactions. Use dye concentrations similar to those used in the actual PCR. Set up the reactions as follow:

Reaction Blank Capillary	Dye 1 Capillary	Dye 2 Capillary
2µL Master Mix	2µL 10x Master Mix	2µL 10x Master Mix
	2µL Probe or Free Dye*	2µL Probe or Free Dye*
16µL Water	14µL Water	14µL Water

*Choose the free dye if you are using a self-quenched probe (i.e. TaqMan or Molecular Beacon)

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Do I actually need to run a PCR to make a color compensation file?

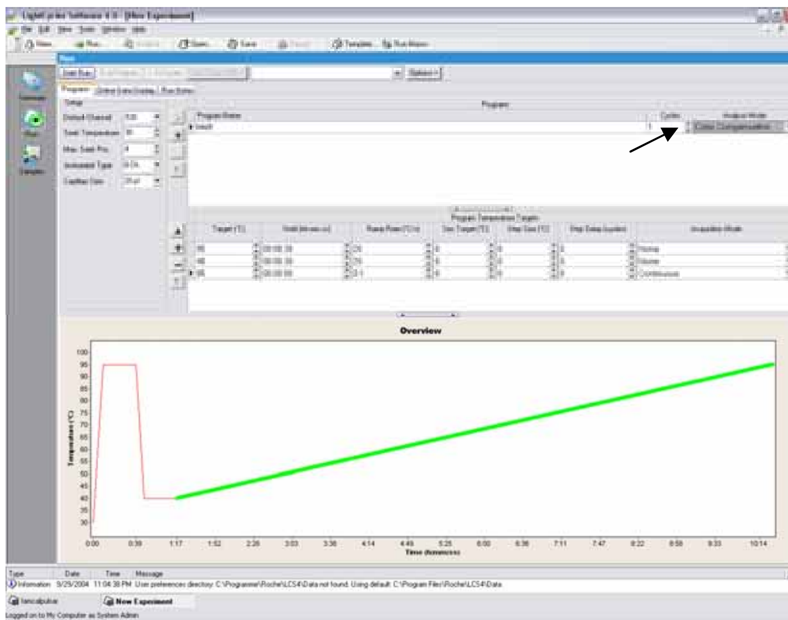
For most users it will simply be necessary to run a melt (temperature ramp with acquisition) on the reactions that you have set up and the color compensation data from this will be sufficient.

There are some reactions which can be challenging to produce good reaction sensitivities. In such cases it can be beneficial to actually use the specific probes in a true PCR with template, primers, and one probe per reaction and then perform the melt at the end of the PCR.

How do I generate Color Compensation Data?

Simply, run a melt program on the reactions that have been set up for your dyes. The melt program that can be run as a stand-alone or tacked onto any existing PCR profile should be:

Hold Temp (°C)	Hold Time (sec)	Ramp Rate (°C/sec)	Acquisition
95	30	20	
40	30	20	
95	0	0.1	Continuous



The run screen should appear similar to the figure (right).

***Note**: The Analysis Mode for this Program is "Color Compensation" (Arrow). Be sure that is option is selected so the instrument will be able to read all of the capillaries during the initial seek procedure.

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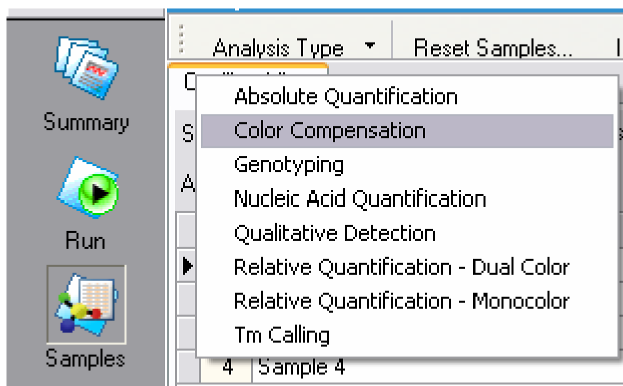
How do I assign the appropriate dyes to channels?

In the Run Module be sure to assign the appropriate number of samples to be detected. Load the capillaries as follows: Capillary 1= Water Blank (no dye), Capillary 2= lowest wavelength emission, Capillary 3=next highest wavelength emission, etc.

Then simply select the Sample module and under the Analysis Type menu will be Color Compensation (highlighted below). Select this option.

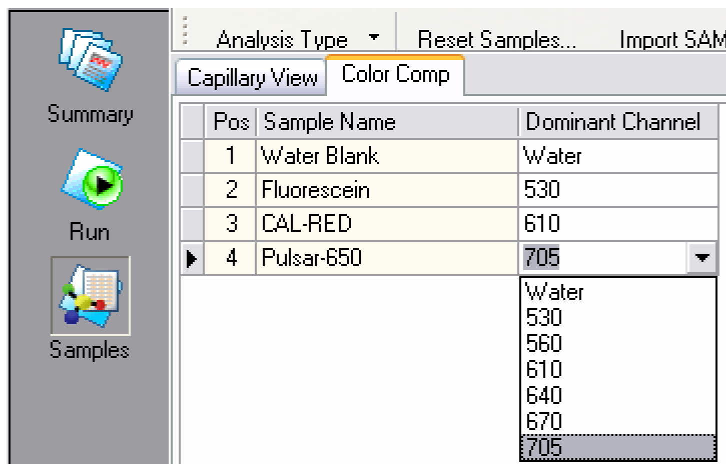


A new window will open with the samples listed as 1-X (whatever number of samples have been assigned in the Run Module)

For each Sample it will be necessary to assign the corresponding dye or emission maximum for that dye.

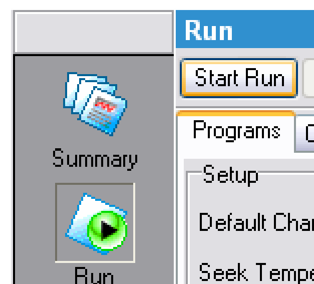
The selected channels can be changed during the run, if you are unsure of the 'best' channel to use for any particular dye.

In the Figure below a three dye system is being color compensated.



Select any channels that you think are appropriate so long as the dyes are loaded in order of increasing emission maximum, then you will have no trouble adjusting the specific "Dominant Channel" during the run.

Now you are ready to select the Run Module and begin the color compensation run by selecting the "Start Run" button. The run program will begin automatically and at this point just wait until it is completed.



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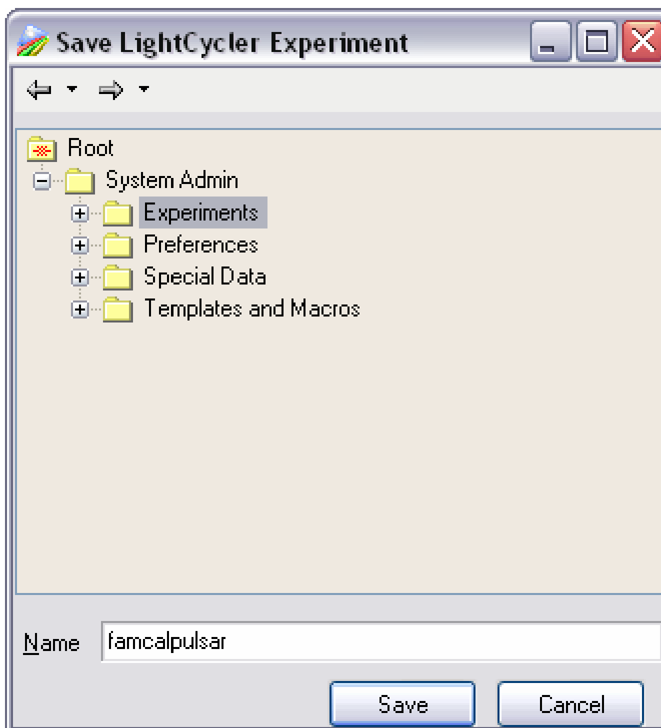
What do I do after the run has completed?

Be sure to save the run data. This is done in the same way that any other run is done.

Select the Save title bar button. The "Save LightCycler Experiment" dialog box should appear.

Select the Experiments folder, expand it if necessary and assign the data to an appropriate folder.

Name the file and select the Save button in the dialog box.

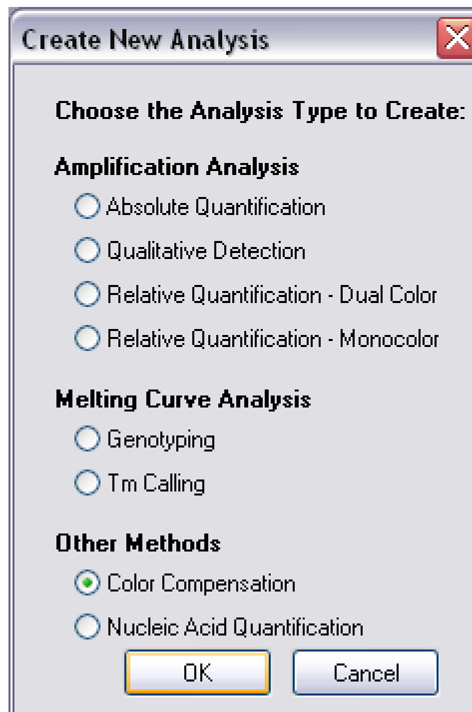


How do I convert the run data into a color compensation file?

With the run data file open, select from the title bar menu options "Analysis". The following window will open:

Select "Color Compensation" and then select OK. A new module button will appear on the left of the database screen showing that the color compensation has been completed.

A this point the file is ready to use to color compensate any data that has the same dyes as were used to generate this color compensation file.



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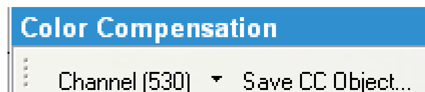
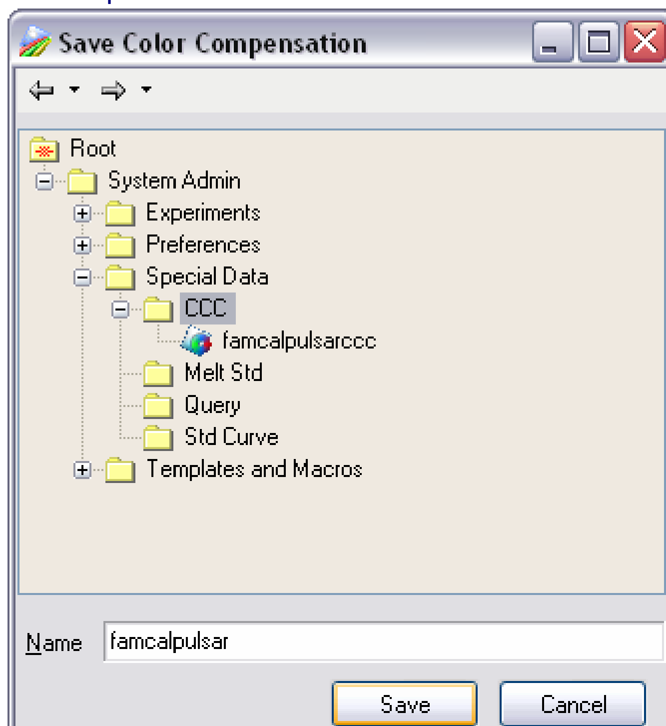
How can I use this color compensation later?

Saving your color compensation file for later use is only slightly different than saving any other file.

Select the "Color Compensation" module button. Then select the "Save CC Object" button just above the sample names.



This will open the Save Color Compensation Dialog Box.



Expand the Special Data folder and then select the CCC folder. In the Name input box give the file a name and then select the Save button.

How can I tell how well the color compensation worked?

The easiest way is to check the actual data for the dyes in each of the channels. Simply select the "Color Compensation" module button on the left of the database screen. This will bring up the Color Compensation window, with the detector channel set to the default or to whichever channel was selected on the Run screen for this program.

In this example the default channel is 530nm, or Channel 1 for Fluorescein. Select a single sample from the sample list.

The data screen should have two large windows, the top window will contain the "Raw Data" for the selected channel, and bottom window will show the "Compensated Data" and the effect of performing color compensation on the selected channel.

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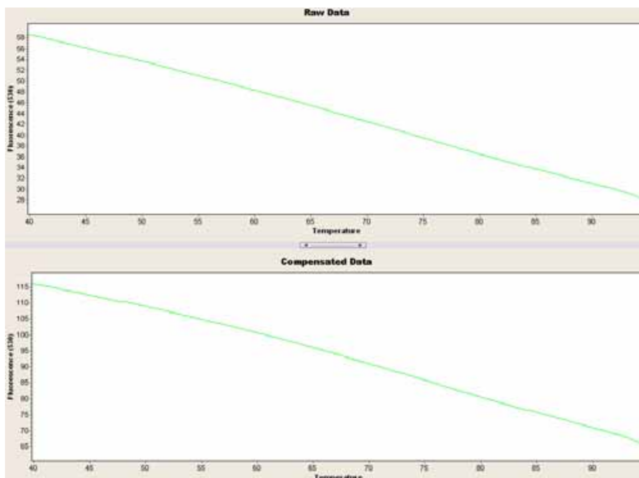
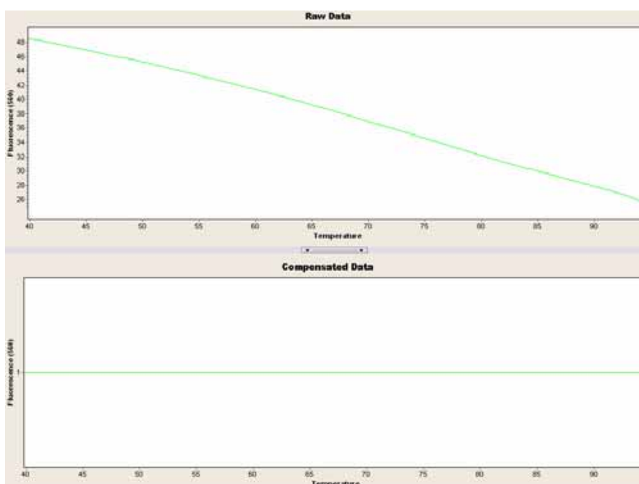
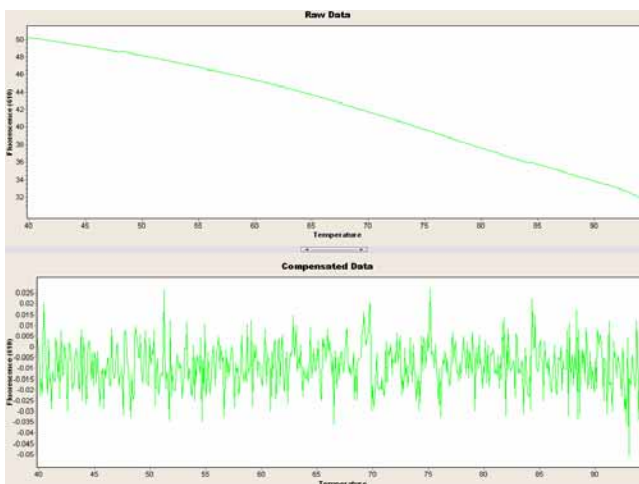


In this example (right) the signal shows little change other than in magnitude. This is expected for this channel with the dye used here. The signal here is from fluorescein dye and it is being detected in channel 1 (530), as it was assigned before the run.



Looking into a different channel (right) where no other dyes had been assigned, in this case (560nm), the Compensated Data graph indicates that no signal is being contributed by the fluorescein dye into this channel, straight, zero slope line.



Looking into a channel (right) where another dye has been assigned, in this case (610nm), the Compensated Data graph typically will show a jagged line. This indicates that some signal compensation between the assigned dye for this channel and the selected sample dye is occurring. To evaluate how well the compensation worked simply see the Y-axis and note the magnitude of the signal changes. Typically, the Y-axis values are very low, and this in turn makes for jagged lines in the output graph.

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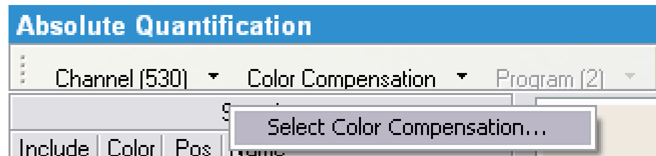
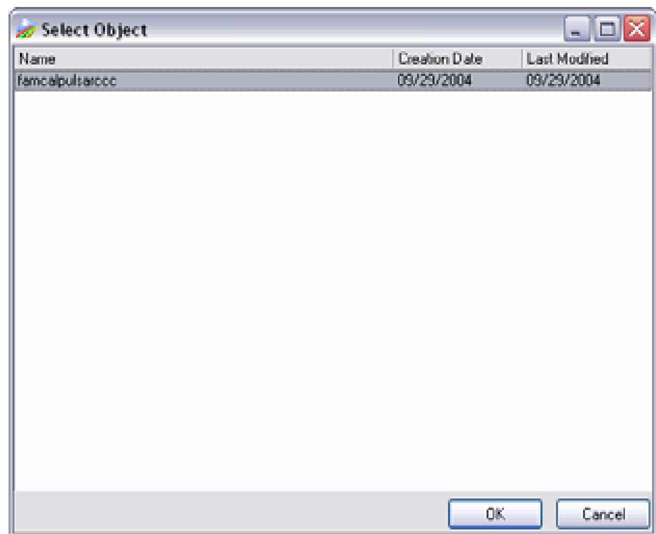
I've got a color compensation file that looks good, how do I use it to compensate an actual data file?

Once you have the color compensation file made, using it is rather simple.

Simply, perform the dual color, or more PCR experiment. After the run has completed, select the Analysis module as usual for the experiment. Then on the title menu bar select the "Color Compensation" Button and then choose the "Select Color Compensation" button.





A Select Object dialog window will open. If the color compensation file has been saved to the Special Data, CCC folder the file that will be used to perform the color compensation should be an option.

Select the file to use and then select OK. This will return you to the data analysis window. An hourglass cursor will indicate that the data is being recalculated.

When the standard mouse cursor returns the data will have been recalculated and color compensated, allowing the analysis of each sample for each dye included I the sample.

At this point the color compensation of the data is completed, and any analyses that need to be performed can be done so with little concern for the impact of signal from one dye bleeding into the signal from another dye.

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