

2. After the reactions have been assembled in capillaries, seal capillaries and centrifuge briefly following the instrumentation instructions to collect contents at the bottom of each capillary.

Note: Keep the reactions on ice during reaction setup until loading the LightCycler® carousel.

III. Generating the Color Compensation Object

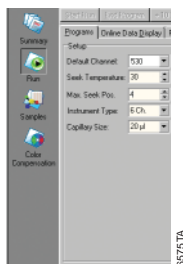
For multiplex assays, a color compensation object (cc object) must be created and applied to multicolor data to allow proper interpretation. To generate the cc object, labeled Plexor™ primers are used as dye calibrators in an initial color compensation cycling experiment. Any subsequent runs performed with the same dyes and similar cycling conditions can be analyzed using this color compensation object.

Notes:

1. The LightCycler® instrument should be programmed before preparing the Plexor™ primers that will be used as the dye calibrators (Section III.D).
2. The color compensation object can be generated after the experimental run and applied to the multiplex experiment before final analysis.
3. A list of LightCycler®-compatible dyes is available at:
www.promega.com/plexorresources/

III.A. Color Compensation Thermal Cycling Program

1. Open LightCycler® software (version 4.0).
2. Select the “Run” module from the global toolbar.
3. Select the “Programs” tab.
4. Under “Setup,”
 - a. Set “Default Channel” to “530.”
 - b. Set the “Seek Temperature” to “30.”
 - c. Set “Max. Seek Pos.” equal to the number of capillaries to be used. This will be equal to the number of dyes in the calibration plus one for the calibrator blank.
 - d. Set “Instrument Type” to “6 Ch.” (switch to “3 Ch.” for LightCycler® 1.5 using software version 4.0).
 - e. Set the “Capillary Size” to “20µl”. Adjust the volume accordingly if other capillary sizes are used.



III.A. Color Compensation Thermal Cycling Program (continued)

5. Thermal cycling will consist of four programs: heating, cycling, temperature gradient and a final cooling step. In the “Programs” section, name the first program of this experiment “Heating” or a similar title.
6. Change “Cycles”, “Analysis Mode” and “Temperature Targets” for the program to the values indicated in Figure 1. New steps are added by selecting the lower “+” button.

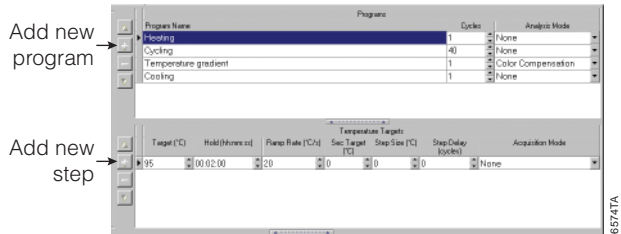


Figure 1. Heating program.

7. Select the “+” button next to the program names.
8. In the dialog box that appears, name the second program “Cycling” or a similar title.
9. Change “Cycles,” “Analysis Mode” and “Temperature Targets” for the program to the values indicated in Figure 2. New steps are added by selecting the lower “+” button.

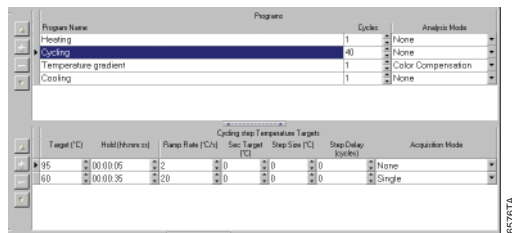
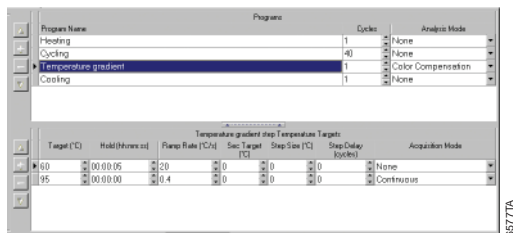


Figure 2. Cycling program.

10. Select the “+” button next to the program names.
11. In the dialog box that appears, name the third program “Temperature Gradient” or a similar title.

- Change “Cycles,” “Analysis Mode” and “Temperature Targets” for the program to the values indicated in Figure 3. New steps are added by selecting the lower “+” button.



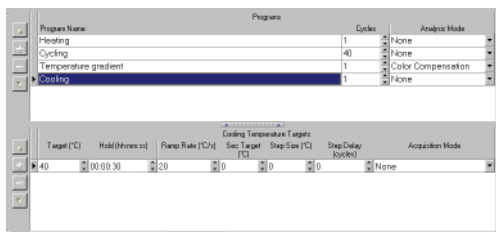
Program									
Program Name	Cycles	Analysis Mode							
Heating	1	None							
Cycling	40	None							
Temperature gradient	1	Color Compensation							
Cooling	1	None							

Temperature gradient step Temperature Targets									
Target (°C)	Hold (Hours:00)	Ramp Rate (°C/h)	Sec. Target (°C)	Step Size (°C)	Step Delay (cycles)	Acquisition Mode			
60	00:00:05	20	0	0	0	None			
95	00:00:00	0.4	0	0	0	Continuous			

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Figure 3. Temperature gradient program.

- Select the “+” button next to the program names.
- In the dialog box that appears, name the fourth program “Cooling” or a similar title.
- Change “Cycles,” “Analysis Mode” and “Temperature Targets” for the program to the values indicated in Figure 4. New steps are added by selecting the lower “+” button.



Program									
Program Name	Cycles	Analysis Mode							
Heating	1	None							
Cycling	40	None							
Temperature gradient	1	Color Compensation							
Cooling	1	None							

Cooling Temperature Targets						
Target (°C)	Hold (Hours:00)	Ramp Rate (°C/h)	Sec. Target (°C)	Step Size (°C)	Step Delay (cycles)	Acquisition Mode
40	00:00:30	20	0	0	0	None

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Figure 4. Cooling program.

III.B. Color Compensation Sample Editing

1. Open the “Samples” module from the left task bar.
2. Select “Color Compensation” as the “Analysis Type.”
3. Indicate the most suitable dye channels for detection from the “Selected Channels”.
4. Select the “Capillary View” tab.
5. Enter the “Sample Name” of the dye (or blank) in each position.

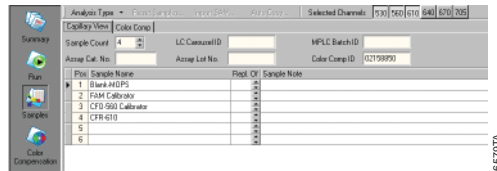


Figure 5. Capillary view tab.

6. Select the “Color Comp” tab.
7. Choose the appropriate “Dominant Channel” for each dye based on the optimal emission wavelength of each dye. Choose “water” for the blank.

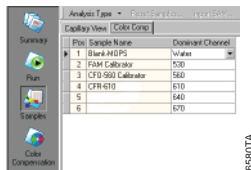


Figure 6. Color compensation tab.

III.C. Dye Calibrator Setup

You will need a separate dye calibrator for each dye in your multiplex, as well as a blank calibrator containing no dye. The same labeled Plexor™ primers that will be used in your experimental amplifications should be used as the dye calibrators. When using Plexor™ primers as dye calibrators, it is not necessary to add template to the color compensation reactions.

Set up calibration samples on ice.

1. For each dye label being used in the multiplex, dilute the labeled Plexor™ primer in 1X Plexor™ Master Mix as follows (20µl needed per dye calibrator if 20µl capillaries are used to prepare the dye calibrators):

Table 1. Calibrator Setup.

	Dye Calibrator	Blank Calibrator (no dye)
2X Plexor™ Master Mix	12.5µl	12.5µl
25X labeled Plexor™ Primer*	1µl	—
MOPS/EDTA buffer	11.5µl	12.5µl
Total volume	25µl	25µl

* FAM-labeled primers should be run at 0.3µM final concentration. All other dye-labeled primers should be run at 1µM final concentration

2. Mix each dye calibrator well.
3. For each dye being calibrated, set aside one 20µl capillary and one additional capillary for the dye calibrator blank.
4. Dispense 20µl of the calibrator blank (1X Plexor Master Mix) to the first capillary. The blank must be run in the first position of the instrument.
5. Dispense 20µl of each dye calibrator to the subsequent capillaries. Make note of the capillary numbers for each sample (i.e., capillary 2: FAM; capillary 3: CAL Fluor® Orange 560; capillary 4: CAL Fluor® Red 610).
6. Seal capillaries with the plastic stoppers.
7. Briefly centrifuge the capillaries using the LightCycler® Carousel centrifuge. Alternatively, use a standard benchtop centrifuge at $700 \times g$ using the LightCycler® centrifuge adapters, and place capillaries in LightCycler® Carousel after centrifugation.
8. Close lid after loading carousel.
9. Select the green “Run” button.
10. When run is complete, select the “Save CC Object” button.
11. Save the object with a descriptive name (date and dyes calibrated) to the “Special Data\CCC” folder.