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Methods of Color Compensation on the LightCycler Instruments FAM/Pulsar 650 Duplexed Assays

Color compensation is used to subtract fluorescent bleed-through from a reporter into inappropriate channels outside of its dominant channel. While unnecessary for singleplex amplifications, color compensation is critical for multiplexed assays to resolve overlapping signals.

LightCycler™ 1.2 Color Compensation using the provided CC File

Our partner, Fluorescentric (www.fluorescentric.com) has kindly provided a universal CC File that can be imported into your LightCycler 1.2 software to ensure that the FAM and Pulsar 650 signals are resolved from one another. This file is available for download at no charge from the Biosearch website at <http://www.biosearchtech.com/download/calibration.asp>

1. Save the CC file (cccFAMPULSAR-042004.ccc) to the hard drive of your LightCycler computer within the user/calibration folder
2. When you are ready to analyze your FAM/P-650 multiplexing data, click on the **Select CC Data** button and choose this file.

In addition to using this provided file, color compensation can also be performed using Taqman probes directly or using T10 calibration dyes (inquire about availability). The LightCycler 2.0 instructions for each of these methods of calibration (listed below) can be adapted to the LightCycler 1.2. For more information on calibrating the LightCycler 1.2, please refer to the LightCycler Operator's Manual (version 3.5) on pages 115-119, and the product insert for the LightCycler Color Compensation Set.

LightCycler™ 2.0 Color Compensation using the provided .ixo File

This experimental file can be used to generate a color compensation object (CC) to apply to FAM/P-650 duplexed assays on the LightCycler 2.0. Because it was created using T10 calibration dyes, it is considered appropriate for most duplex assays. This file is available for download at no charge from the Biosearch website at <http://www.biosearchtech.com/download/calibration.asp>

1. Download and save "cccFAMPULSAR-102804.ix0" to the computer operating your LightCycler 2.0
2. Open this experimental file by importing into the LightCycler software (version 4.0)
3. Color Compensation will be displayed as a chosen analysis. At the top of this color compensation module, select **Save CC Object** and you will be prompted to name the object. An appropriate name might be "FAM/P-650 Duplex (T10)"
4. This CC object can now be applied to FAM/P-650 duplexed assays to subtract out any crosstalk. Within the **Analysis** module of your duplexed experiment, select the **Color Compensation** drop-down menu from the title bar. Choose **Select Color Compensation** and finally choose the CC object you have just saved.

Notice: Both the LightCycler 1.2 and 2.0 files can only be applied to FAM/P-650 duplexed assays. They cannot be applied to multiplexed assays that use different or additional reporters. For these assays, a separate CC object will need to be generated using either the TaqMan probes themselves or T10 calibration dyes.

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LightCycler™ 2.0 Color Compensation using TaqMan Probes

Taqman™ probes can be used for calibration because fluorescence is permanently released during the amplification through a cleavage mechanism, after which the color compensation data is extracted. Other probe designs such as Molecular Beacons™ do not release their fluorescence through this cleavage mechanism and so cannot be used directly for calibration

1. TaqMan™ reactions must be separately amplified for FAM and for Pulsar 650. Prepare these two reactions in individual capillaries exactly as you will prepare them in your subsequent experimental assays. Include a sufficient quantity of template so that the amplifications are expected to plateau. You do not need any replicates. Finally, prepare a capillary to represent the blank, containing only PCR buffer.
2. Cap and Centrifuge each of these capillaries and then place them into the sample carousel. Place the buffer capillary into position 1, the FAM capillary into position 2, and the Pulsar 650 capillary into position 3. Placing them in this order is a requirement of the LightCycler's color compensation procedure.
3. Close the instrument and start the LightCycler software application. Prepare a **New Experiment**, and within the **Run Module** enter the thermal cycling parameters according to the details of your assay. Set the seek temp to 45°C. Set the **Max. Seek Pos.** to "3" indicating the number of capillaries you have loaded into the instrument. Do not add a melt curve or choose **color compensation** from the **Analysis Mode** menu as this will be programmed in a subsequent run. Start the first amplification run.
4. During this thermal cycle program your targets will be amplified and the TaqMan probes cleaved, releasing the fluorescence necessary for color compensation. It is not critical that the LightCycler be able to "seek" the capillaries during this process. In fact, since the instrument seeks FAM fluorescence as part of its optics optimization, it will likely be unable to recognize the buffer blank capillary or the Pulsar 650 reaction. Proceed with the run despite any warning prompts, and follow the course of the amplification by monitoring the FAM capillary. After the amplification has completed you can collect the data necessary for color compensation.
5. Select **New Experiment** and you will be presented with a default **Run Module**. Create a single program (1 cycle) with the following segments:

Segment 1:	95°C for 0 seconds at 20°C/second	Acquisition Mode: None
Segment 2:	40°C for 30 seconds at 20°C/second	Acquisition Mode: None
Segment 3:	95°C for 0 seconds at 0.1°C/second	Acquisition Mode: Continuous

Choose **Color Compensation** from the **Analysis Mode** drop-down menu.
6. Set the **seek temp** to 45°C. Set the **Max. Seek Pos.** to "3" indicating the number of capillaries you have loaded into the instrument, and then switch to the **Sample Module**.
7. Give the samples in each position appropriate names, eg. Position 1 = Buffer, Position 2 = FAM, Position 3 = Pulsar 650. This naming convention should be used in your future experiments.
8. After naming the samples, select the **Analysis Type** drop-down menu and choose **Color Compensation**.
9. You will now need to select the dominant channel (filter) that should be used to measure the fluorescent reporter for each reaction. For your capillary containing buffer in sample position 1, the dominant channel should be **Water**. For the FAM amplification, the dominant channel should be **530**. For Pulsar 650, the dominant channel should be **705**.

Important: this decision will determine the channels used to detect this combination of fluorescent reporters in your future experiments.
10. After choosing the dominant channels for each of your samples, switch back to the **Run Module** and click **Start Run**. You will be prompted to save the experimental file with an appropriate name and the run will begin.
11. Before beginning the thermal cycle, the instrument will "seek" the position of the capillaries as a part of optimization of the optics. If the instrument fails to locate one or more of the samples, it may be necessary to abort the run so that it may be reprogrammed, this time with a higher **seek temperature**.
12. Once the run is completed, click on the **Analysis** pane and select **Color Compensation** beneath the **Other Methods** category. You will be presented with a color compensation analysis module where you can view the fluorescence of each reporter with respect to temperature.

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13. At the top of this **Color Compensation Module**, select **Save CC Object**. You will be prompted to name the color compensation file, and the location where it will be saved (usually the CCC directory).
14. This color compensation is now available to apply to your future FAM/P-650 duplexed assay. While this CC Object is most accurate when applied to the same amplifications used to generate it, it might also be applicable to other FAM/P-650 duplexed assays.
15. To apply this CC Object to your results, Select **Color Compensation** from the **Color Compensation** drop-down menu of any analysis module. Cross-talk of fluorescent reporters will be subtracted out of their non-dominant channels and allow the results to be precisely quantitated. However, if crosstalk is not completely remedied when applying this file to different FAM/P-650 duplexed assays, a separate assay-specific CC object will need to be generated for these in the same manner as above.

LightCycler™ 2.0 Color Compensation using T10 Calibration Dyes

1. T10 oligonucleotides, singly labeled with either FAM or Pulsar 650, can also be used to calibrate your LightCycler. Please inquire about the availability of these calibration dyes.
2. Prepare 3 capillaries: one containing only PCR buffer to represent a blank, one containing a 300 nM solution of FAM T10 diluted in PCR buffer, and the final one containing a 1 µM solution of Pulsar 650 diluted in PCR buffer.
3. Cap and Centrifuge each of these capillaries and then place them into the sample carousel. Place the buffer capillary into position 1, the FAM capillary into position 2, and the Pulsar 650 capillary into position 3. Placing them in this order is a requirement of the LightCycler's color compensation procedure. Close the instrument and start the LightCycler software application
4. Continue from Step 5 of the TaqMan procedure listed above.

For additional information on calibrating the LightCycler 2.0, please consult the **LightCycler 2.0 Instrument Operator's Manual (Version 4.0)**, explaining the procedure on pages 149-151, and the product insert for the LightCycler Multicolor Demo set. Also, a FAQ clearly explaining calibration of the LightCycler has been kindly provided by our partner, Fluorescentric. This FAQ is available for download at <http://www.biosearchtech.com/download/calibration.asp>