

Manual

Thermal cycler spectral calibration instructions

CAL FLUOR and Quasar dyes

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Thermal cycler spectral calibration instructions

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1. Why is spectral calibration necessary?

Most real-time qPCR instruments require dye calibration to distinguish the fluorescent signals from one another. Although unnecessary for simple singleplex amplifications, spectral calibration is critical for multiplexed assays (whether real-time qPCR or SNP genotyping) so that overlapping fluorescent signals can be resolved from one another. On certain instruments, all dyes that will be multiplexed together must be calibrated together. In addition, in order to successfully use dual-labeled probes containing [CAL Fluor™](#) and/or [Quasar™ dyes](#) in multiplexed assays, certain real-time PCR instruments must first be calibrated to recognise the pure dye spectra. This document provides detailed, step-by-step instructions for calibrating some of these instruments. You can refer to our [Multiplexing recommendations table](#) to see if your instrument needs dye calibration, or consult the instrument instruction manual.

2. Instrument-specific calibration instructions offered in this guide

Currently supported:

- Thermo Fisher Scientific (Applied Biosystems) ABI **Prism® 7700**
- Thermo Fisher Scientific (Applied Biosystems) ABI **Prism 7500**
- Bio-Rad Laboratories **iCycler® iQ**
- Bio-Rad Laboratories **iQ™ 5**
- Bio-Rad Laboratories **Opticon® 2**
- Danaher Corporation (Cepheid) **SmartCycler® II**
- Eppendorf **Mastercycler® ep Realplex**
- Roche **LightCycler® 480**

If your Applied Biosystems instrument is not listed above (e.g. **Viiia™ 7** or **QuantStudio™**), please refer to [these instructions](#) from ThermoFisherScientific that detail how to calibrate their instruments for custom dyes.

The Bio-Rad **CFX** instrument does not require custom dye calibration for CAL Fluor and Quasar dyes as it is factory-calibrated for these.

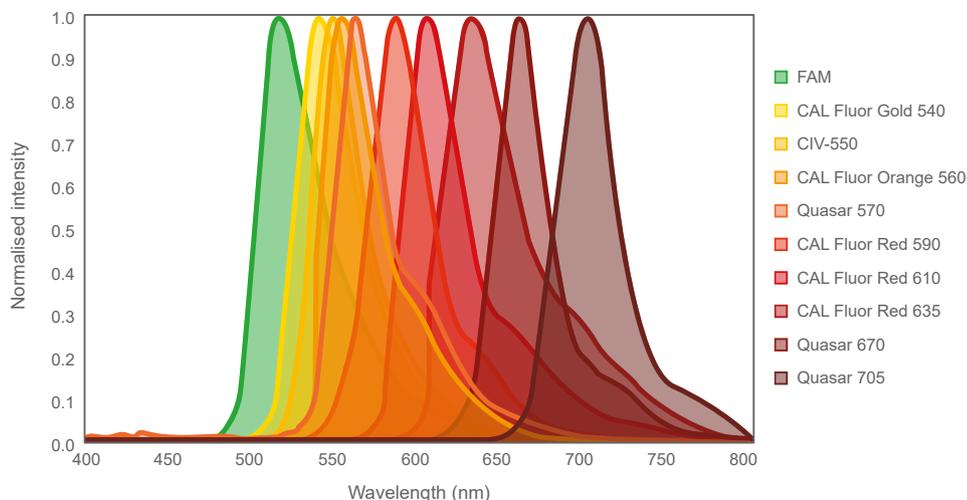
The [IntelliQube™](#) from LGC Biosearch Technologies™ will be calibrated for CAL Fluor and Quasar dyes by our service team during installation. Calibration is not required for the Biosearch Technologies [Nexar™](#) (**Araya™**) instrument. The gains settings can be adjusted by our service team to optimise the instrument for the chemistry used.

NOTE: If your model is not included in this document, please consult the instruction manual that came with your instrument for guidelines on custom dye calibration.

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3. Normalised emission spectra of calibration dyes



4. Calibration standards - tube contents

Each calibration standard is supplied in an individual tube containing 5 nmol of lyophilised calibration dye covalently linked to a 10 base oligonucleotide comprised of poly T residues (T10).

Biosearch Technologies provides calibration standards in the specifications shown below:

Calibration standard	Catalogue number	Amount provided
FAM	RD-5025-5	5 nmol
CAL Fluor Gold 540	RD-5080-5	5 nmol
CIV-550	RD-5030-5	5 nmol
CAL Fluor Orange 560	RD-5081-5	5 nmol
CAL Fluor Red 590	RD-5083-5	5 nmol
CAL Fluor Red 610	RD-5082-5	5 nmol
CAL Fluor Red 635	RD-5084-5	5 nmol
Quasar 570	RD-5063-5	5 nmol
Quasar 670	RD-5065-5	5 nmol
Quasar 705	RD-5067-5	5 nmol

If the desired calibration dye is not listed above, simply order a T10 oligo labeled with the dye of your choice at the 5' end, and select RP-HPLC purification as a [Custom oligo](#), to mimic the calibration dyes offered.

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5. Calibration concentrations

Commercially available real-time thermal cycler models differ substantially in their optics. Furthermore, several instruments of the same model can also differ in fluorescence detection sensitivity. Calibration concentrations required for each of the CAL Fluor and Quasar dyes will therefore vary according to instrument. Recommended concentrations (determined empirically at Biosearch Technologies and with selected scientific collaborators working in the field) are provided within the instrument-specific instructions included below. We suggest that you “fine-tune” these values using the procedure specified by your instrument manufacturer.

If your model is not included in this document, please consult the instruction manual that came with your instrument for guidelines on custom dye calibration. A dilution series can establish an appropriate concentration, which also may be different for each dye included in the calibration. We recommend a dilution series of 25 nM, 50 nM, 100 nM, 200 nM and 300 nM, if this information is not provided in the instruction manual. Based on the fluorescent intensities that result, select a concentration that is within the instrument's linear range of detection and does not saturate the optics. That concentration should be appropriate for calibration by dispensing into either a subset of wells or the whole reaction plate, depending upon instrument requirements.

6. Preparation of calibration standards for use

After determining the final working concentration within your instrument-specific instructions, create a stock solution at a higher concentration by resuspending this calibration dye in a 1X dilution buffer containing: 10 mM Tris-HCl pH 8.0, 50 mM NaCl, and 5 mM MgCl₂. This dilution buffer should not contain an internal reference dye (without ROX). Store at -20 °C until you are ready to dilute to the working concentration and perform spectral calibration.

7. Storage and handling of calibration standards

Calibration standards should be subjected to a minimum number of freeze-thaw cycles. Therefore, we recommend that you aliquot your calibration standard into individual microvials, each having sufficient material for one dye calibration, and store them frozen at -20 °C or -80 °C. Calibration standards can be stored frozen in solution for over one year.

8. Protection from photobleaching

To ensure optimum activity and to safeguard maximum performance lifetime, calibration standards should always be protected from light to avoid photobleaching.

9. Optional reaction plate centrifugation

When dispensing calibration standards into a reaction plate, care should be taken to ensure that bubbles are not introduced and that solution has not collected on the side of any wells. A brief centrifugation of the plate (<1500 x g for 5 seconds) may correct these problems, but is otherwise not required.

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10. Storing and saving calibration plates for future use

After successfully calibrating your real-time PCR instrument, the reaction plate containing your calibration standard can be frozen at -20 °C and re-used in the future for subsequent calibrations. Most instruments recommend periodic recalibration. Consult your instrument's manual for the recommended frequency of recalibration.

11. Customer service and technical support

If you would like to purchase any calibration standards, please contact our customer service team:

Region	Customer service email
North America, Latin America	Genomics.americas@lgcgroup.com
Europe, Middle East and Africa	Genomics.emea@lgcgroup.com
Asia Pacific, excluding China	Genomics.apac@lgcgroup.com
China	Genomics.china@lgcgroup.com

If you require technical assistance, please do not hesitate to contact our technical support team at: techsupport@lgcgroup.com

12. Instrument-specific calibration instructions

12.1. Thermo Fisher Scientific ABI Prism 7700 sequence detection system

Following is a general procedure for calibrating compatible dyes on the ABI 7700 and a table listing the recommended dye concentrations. For further details, please refer to the **ABI User Bulletin #4**: Part Number: 904989, as well as **ABI Prism 7700 User's Manual**, Part Number: 903564.

Calibration standard	Calibration concentration on the Prism 7700	Number of calibrations possible (approximate)
FAM	200 nM	125
CAL Fluor Gold 540	200 nM	125
CAL Fluor Orange 560	250 nM	100
CAL Fluor Red 590	Incompatible	Incompatible
CAL Fluor Red 610	Incompatible	Incompatible
CAL Fluor Red 635	Incompatible	Incompatible
Quasar 570	Incompatible	Incompatible
Quasar 670	Incompatible	Incompatible

- 1) Prior to running spectral calibration with a calibration standard you will need to generate a background component file. This procedure allows the instrument to measure the background signal of each well and to compensate for any residual fluorescence contaminating the heating block;

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- 2) Dispense 50 μL of deionised water into each well of an optical PCR reaction plate. Seal the plate with an optical adhesive cover and place a compression pad on top of the plate. Position the plate into the instrument and tighten the lid;
- 3) Start the SDS software application and close the plate layout that opens automatically. Select **New Plate** from the **File** menu, designate the plate type as **Background**, and click **OK**. Click **Show Analysis** and the wells depicted in the plate layout will turn black. Click **Run**;
- 4) After the run has completed, select **Calibrate** from the **Instrument** menu. Within the **Calibrate** submenu, select **Extract Background Component**. You will then be prompted to quit the application. The background data has been stored regardless of whether you choose to save or discard the file containing the plate setup. You are now ready to prepare your calibration plate containing the calibration standard;
- 5) Dilute the calibration standard in 1X dilution buffer (without ROX) to the necessary working concentration (see table above) and a volume of 250 μL . Mix well;
- 6) For each calibration standard, dispense 50 μL of this dye solution into each of four wells in an optical PCR reaction plate. This will leave 50 μL excess which can be discarded. Seal the plate with an optical adhesive cover and place a compression pad on top of the plate. Position the plate into the instrument and tighten the lid;
- 7) Open the SDS software application, close the existing plate layout and select **New Plate** from the **File** menu. Designate plate type as **Pure Spectra** and click **OK**;
- 8) Within the **Sample Type** field, select **Sample Type Setup**. You will need to create a new entry for each dye by clicking **Add**. A blank entry will be added to the bottom of the list. Within the **Name** field for this entry, type **Pure Dye** exactly as spelled;
- 9) Within the **Acronym** field, type a short name for the new dye name using the following conventions: the letter “Z” followed by the acronym that you wish to use. For example, **ZORG** could be used to describe CAL Fluor Orange 560;
NOTE: This naming convention is important for the following reasons: the ABI 7700 allows you to calibrate for an unlimited number of custom dyes, but a software bug requires users to maintain FAM, TAMRA, and ROX within the first seven entries alphabetically. This list of calibrated dyes can be viewed by selecting **Edit Pure Dye** within the **Instrument**→**Calibrate** menus. Adding a Z in front of your dye acronym ensures that this custom entry will be appended to the end of the list and not prevent the software from recognising the previously mentioned dyes;
- 10) Make sure that the **Quencher** box is unchecked. Create entries for any additional calibration standards you will calibrate on the same reaction plate, and then exit **Sample Type Setup** by clicking **OK**;
- 11) In the plate layout, select the wells that correspond to the wells in your reaction plate containing your dye calibration solution. Once these wells are selected, label them as the appropriate pure calibration standard that is now an entry in the **Sample Type** pop-up menu. Repeat for any other calibration standards included on the plate. Click the **Show Analysis** button and your plate layout will turn entirely black. Click **Run** and the instrument will begin collecting the calibration data;
- 12) When the instrument has completed the data collection, select the four wells in the plate layout that correspond to a calibration standard. Select **Calibrate** from the **Instrument** menu, and then select **Extract Pure Dye**. Click **OK** on the following confirmation screen;

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- 13) You will now be presented with a screen showing the emission spectra of each of your four replicate wells, as well as an emission spectrum representing the average of these replicates. If any of the spectra deviate from the rest of the group, remove these from the average and then click **OK**. Repeat for the other dyes you are calibrating simultaneously. Quit the application;
- 14) Calibration has been completed and a file titled **Pure Dye** has now been updated to include the new dye(s) in addition to the previous standard dyes. This file is stored in the following directory: **System Folder/Preferences/SDS/Spectra Components**. Importantly, backup this **Pure Dye** file with an appropriate name such as **Master Pure Dye**;
- 15) The **Pure Dye** file will serve as your 'dye palette' when running future experiments and can be viewed by selecting **Edit Pure Dye** within the **Instrument** → **Calibrate** menus. Just prior to performing a real-time PCR experiment with these newly calibrated dyes, you will need to delete dye entries that will remain unused during the experiment, using the **Edit Pure Dye** window;
IMPORTANT NOTE: filtering out unused dyes places the required dye within the first seven entries, enabling the SDS software to recognise it. This will permanently modify the **Pure Dye** file, which is why the backup described in step 16 is essential. **IF THE PURE DYE FILE HAS NOT BEEN BACKED UP, DELETED ENTRIES CAN ONLY BE REGAINED THROUGH RECALIBRATION!** Due to the software limitation described in step 9 above, the entries for FAM, TAMRA, and ROX should **NEVER** be removed or the application will not open properly;
- 16) Once you have completed real-time PCR with your required custom dye(s), you must restore the dye entries that were removed. Quit the SDS application and replace the current **Pure Dye** file with the **Master Pure Dye** backup file, making the appropriate name changes. Always keep a copy of the **Master Pure Dye** file elsewhere on the computer for future use.

12.2. Thermo Fisher Scientific ABI 7500 sequence detection system

Following is a general procedure for calibrating compatible dyes on the ABI 7500 and a table listing the recommended dye concentrations. For further details, please refer to the **ABI 7300/7500/7500 Fast Real Time PCR System Installation and Maintenance Guide, Part number: 4347828 Rev. E**.

Calibration standard	Calibration concentration on the 7500	Number of calibrations possible (approximate)
FAM	Pre-calibrated	Pre-calibrated
CAL Fluor Gold 540	100 nM	25
CAL Fluor Orange 560	75 nM	33
CAL Fluor Red 590	100 nM	25
CAL Fluor Red 610	60 nM	41
CAL Fluor Red 635	40 nM	62
Quasar 570	Unknown	Unknown
Quasar 670	75 nM	33

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- 1) Before calibrating this instrument for your selected dyes, a background and optical calibration must be performed. Please refer to the **ABI 7300/7500/7500 Fast Real Time PCR System Installation and Maintenance Guide** for further instructions. The background calibration allows the software to measure the fluorescence intrinsic to the 7500 instrument and subtract this out from the raw spectral data of future runs. The optical calibration is unique to the ABI 7500 and is required because of the optical effects of the additional Cy 5 filter. Once these calibrations have been completed, begin preparing the dye calibration plate(s), as described below;
- 2) For each of the dyes that you wish to calibrate, dilute an aliquot of the stock dye(s) to the recommended working concentration (see table above) using 1X dilution buffer (without ROX) - The total volume of this dilution should be 2.0 mL;
- 3) Starting with one dye, dispense 20 μ L from this dilution into all wells of an optical PCR reaction plate. Seal the plate with an optical adhesive seal. Prepare additional plates in the same manner for each additional dye that you wish to calibrate;
- 4) Open the 7500 SDS software application. In the **Quick Startup** dialog box, click **Create New Document**. Under the **Assay** drop-down menu, select **Pure Spectra**, choose **96-well clear** as the container and designate **Blank Document** as the template;
- 5) Once the **Pure Spectra Calibration Manager** opens, click **Add Dye** to create a new entry for this custom dye. Enter the name of the appropriate dye and click **OK**. Repeat this procedure to add a new entry for each dye that will be calibrated;
- 6) Next, select one of these new entries in the **Dye List** field and click **Calibrate**. When prompted to disconnect the plate document, click **Yes**. Load the reaction plate containing the pure calibration dye into the instrument when directed by the software. Then click **Yes**. The software will automatically begin the calibration run;
- 7) Upon completion of the calibration run, remove the reaction plate from the instrument. Repeat step 6 for all remaining dyes;
- 8) After completing all calibrations, it is important to examine the calibration data in order to screen for inconsistencies in dye spectra. Select the **Results** tab and then the **Spectra** sub-tab. Highlight all of the wells by clicking the upper-left corner box of the plate map. Under the **Analysis** menu, choose **Extract Pure Spectra** and click **OK**. The software will now present a plot of the emission spectra for each of the 96 replicate wells. Verify that the peak of the spectra occurs in the correct filter.
- 9) The calibration is complete and the data is automatically saved as an SDS document titled **Pure Dye** in the following directory: **Applied Biosystems/SDS Documents**;
- 10) Repeat steps 8-9 to analyse the calibration data for the remaining dyes, which are presented in plate documents beneath the active one, and then quit the application;
- 11) Before the newly calibrated dyes can be added to a plate document, a detector must be created for each dye. Within the 7500 SDS Software, select **Detector Manager** under the **Tools** menu. Under the **File** menu, choose **New**. Enter a name and select a detector colour. Make sure to select **None** within the **Quencher Dye** field. After a detector has been created for each dye, click **Done**. You are now ready to run these dyes on the 7500 instrument.

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12.3. Bio-Rad Laboratories iCycler IQ

Following is a general procedure for calibrating compatible dyes on the Bio-Rad iCycler. For further details, please refer to the **iCycler IQ Real-Time Detection System Instruction Manual**, Part number: 170-8740, and the following table for recommended concentrations.

Calibration standard	Calibration concentration on the iCycler	Number of calibrations possible (approximate)
FAM	300 nM	33
CAL Fluor Gold 540	300 nM	33
CAL Fluor Orange 560	300 nM	33
CAL Fluor Red 590	300 nM	33
CAL Fluor Red 610	300 nM	33
CAL Fluor Red 635	300 nM	33
Quasar 570	300 nM	33
Quasar 670	300 nM	33

- 1) Calibration data for the iCycler is saved to the file **RME.ini** located in the directory **C:\Program Files\Bio-Rad\iCycler\ini**. By opening this file in a word processor you can view the spectral profile for each of the dyes that are currently calibrated. More information on this file is available in **Appendix I** of the **Resource Guide**. Before performing calibration of any additional dyes, it is important to make a backup copy of this file with an appropriate name such as **Original RME.ini**;
- 2) To ensure that the iCycler collects spectral data on all relevant channels, it is necessary to anticipate which fluorescent dyes you might use together on a reaction plate and calibrate for all of those simultaneously in one run. For example, if you multiplex a FAM, CAL Fluor Orange 560, and CAL Fluor Red 610 assay, but had calibrated for each of these dyes on separate days, an error message might result stating, "Response Matrix element not found in RME database..." Recalibration is then necessary;
- 3) Dilute each calibration standard in 1X dilution buffer (without ROX) to the working concentration of 300 nM and a volume of 550 μ L. Mix well;
- 4) For each dye, dispense 50 μ L of the prepared calibration solution into each of ten wells in an optical PCR reaction plate. This will leave you with a 50 μ L excess for each calibration solution, which you can discard. Seal the plate with an optical adhesive cover and then save it until you are prompted to place it into the iCycler;
- 5) Start the iCycler software application. In the **Library** mode, check to make sure that you are in the **View Protocol** pane and select the **PureDyeCalibration.tmo** thermal cycling protocol;
- 6) Switch to the **View Plate Setup** pane and select the **Pure Dye.pts** plate setup. Click the button **Edit this Plate Setup** and you will be transferred to the **Workshop** mode of the application;
- 7) Clear the existing well labels by selecting the **Erase** function and clicking the top left corner of the plate layout;

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- 8) Next, select the **Pure Dye** button and label the wells on the plate layout that correspond to the wells in the reaction plate containing the calibration dye(s);
- 9) Switch to the **Select and load fluorophores** pane and deselect any of the fluorophore entries that are currently checked;
- 10) The fluorophore entries are labeled with standard dye names followed by the excitation filter through which they are detected. Make a note of those standard dye entries that are coupled with excitation filters appropriate for the dye(s) you will be calibrating. Ideally, your choice should be an entry that is infrequently used and whose calibration information can be overwritten to accommodate the alternative dye. CAL Fluor Gold 540, CAL Fluor Orange 560, and Quasar 570 are each optimally excited using the 530/30X filter. CAL Fluor Red 590 is optimally excited using the 545/30X filter. CAL Fluor Red 610 is optimally excited using the 575/30X filter. CAL Fluor Red 635 and Quasar 670 are each optimally excited using the 635/30X filter.

IMPORTANT NOTE: the iCycler does not permit you to create new dye entries beyond their list of standard dyes. Therefore, to use a custom dye on this instrument requires calibrating a standard entry using the custom dye calibration solution. It will be a good idea to leave a note near the instrument outlining the dyes that have been calibrated for the various entries. This calibration will overwrite the previous settings for that entry, which is why the backup described in step 1 is essential! If the need ever arises to use the original dye that was replaced by the alternative dye, the backup **RME.ini** file can be substituted for the modified one.

- 11) Check the box of the standard dye entry for which you will calibrate the new dye. You will be immediately prompted to select a colour representing this dye. On the plate layout, select the wells that you designated as **Pure Dye** and they will be filled in with the colour of this entry. Repeat for any additional dyes that you will be calibrating simultaneously;
- 12) After you have designated the location of the calibration dye on your plate layout, click **Save this plate setup** and finally, **Run with selected protocol**;
- 13) The screen will switch to the **Run Prep** pane, and at this point you should place an **external well factor plate** into the instrument. Preparation of an external well factor plate is described on page 54 of the **Resource Guide**. Select **Begin Run** and the instrument will prompt you to enter a name for the optical data file that will be generated. The iCycler will first collect optical data for the well factors. When the iCycler pauses, open the instrument, remove the well factor plate, and replace it with the reaction plate containing your calibration dyes. Click **Continue Running Protocol**;
- 14) When calibration has completed, you need to make a backup of the now-modified **RME.ini** file with an appropriate name such as **Current RME.ini**. You are now prepared to run real-time PCR using your custom dye(s) on the iCycler. In setting up the plate layouts for these future reactions, remember to use the standard dye entries upon which your alternative dyes are calibrated.

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12.4. Bio-Rad Laboratories iQ5

Following is a general procedure for calibrating compatible dyes on the Bio-Rad iQ5. For additional information, refer to the **Instruction Manual, iQ5 Optical System Software, Rev. C**; Bulletin # 10005604.

Calibration standard	Calibration concentration on the iCycler	Number of calibrations possible (approximate)
FAM	300 nM	41
CAL Fluor Gold 540	300 nM	41
CAL Fluor Orange 560	300 nM	41
CAL Fluor Red 590	300 nM	41
CAL Fluor Red 610	300 nM	41
CAL Fluor Red 635	Incompatible	Incompatible
Quasar 570	Incompatible	Incompatible
Quasar 670	300 nM	41

NOTE: these recommended dye concentrations and predicted numbers of calibrations assume 50 μ L reaction volumes. On the iQ5 it is necessary to perform dye calibration using the same volume as in your future PCR reactions, and so some adjustment to the following procedure may be necessary. Re-calibration is necessary each time the reaction volume is changed.

- 1) Calibration data files for the iQ5 instrument are stored in the directory **C:\Program Files\Bio-Rad\iQ5\RMEData**. These XML-formatted files are best viewed within the **iQ5 Optical System Software** application by selecting **Calibration Data** under the **View** menu.
- 2) The iQ5 can calibrate for up to five different dyes simultaneously assuming that they are each detected on different channels/filter sets. Dilute each calibration standard using 1X dilution buffer (without ROX) to a concentration of 300 nM and a total volume of 440 μ L;
- 3) For each calibration dye, aliquot 50 μ L of the diluted solution into eight wells of an optical PCR reaction plate. An excess 40 μ L of each solution will remain, which can then be discarded. Seal the reaction plate with an optical adhesive cover and store the plate until the instrument is ready to measure its fluorescence profile;
- 4) Start the **iQ5 Optical System Software** and click the Calibration button in the dialog box on the left. Then choose the **Pure Dye** button toward the top of the screen. If the instrument has not yet had its masks aligned, the background fluorescence measured, or the well factors collected, please refer to the calibration module tab in the **iQ5 Multicolor Real Time PCR Detection System Online Manual** before proceeding;
- 5) In the **Selected Plate Setup** field, select **Create New**;
- 6) Edit the **Sample Volume**, **Seal Type** and **Vessel Type** fields to correspond with the features of your reaction plate;

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- 7) Click the **Select/Add Fluorophores** button. Choose a name for the fluorophore up to eight characters long, and carefully choose the filter-set through which it should be detected. CAL Fluor Gold 540 and CAL Fluor Orange 560 are optimally detected using filter position 3. CAL Fluor Red 590 is optimally detected using filter position 4, while CAL Fluor Red 610 is optimally detected using filter position 5. Quasar 670 is optimally excited using filter position 6. Finally, click **Add New Fluor** to make this dye a permanent entry. Repeat this process for the remaining custom dyes you wish to calibrate;
- 8) Before leaving the **Fluor Selector** screen, check the box corresponding to each fluorophore that will be calibrated during this run. Select a colour to identify each fluorophore on the plate map. Label the wells on the plate layout that correspond to the reaction plate wells that contain the calibration dyes;
- 9) Select **Save and Exit Plate Editing**. The window will revert back to the **Pure Dye Plate Setup** screen;
- 10) Load the reaction plate containing the calibration standards into the iQ5 instrument console. Click the **Run Pure Dye Calibration** button. The programme will prompt you to name the calibration data file that will be produced;
- 11) When the pure dye run has finished, a dialog box will appear stating **Pure Dye Calibration Run Complete**. Click **OK**. You are now able to run real-time PCR using your newly calibrated dyes on the iQ5.

12.5. Bio-Rad Laboratories Opticon 2

Following is a general procedure for calibrating compatible custom dyes on the Bio-Rad Opticon 2. For further details of the calibration procedure, please refer to the **Instruction Manual, DNA Engine Opticon 2 System, Rev. F**, Part number: 07218. The table below details the recommended concentrations for each calibration standard.

Calibration standard	Calibration concentration on the iCycler	Number of calibrations possible (approximate)
FAM	300 nM	3
CAL Fluor Gold 540	500 nM	2
CAL Fluor Orange 560	1000 nM	1
CAL Fluor Red 590	Incompatible	Incompatible
CAL Fluor Red 610	Incompatible	Incompatible
CAL Fluor Red 635	Incompatible	Incompatible
Quasar 570	Incompatible	Incompatible
Quasar 670	Incompatible	Incompatible

- 1) The Opticon 2 first measures an empty reaction plate to subtract blank values from the subsequent calibration measurements. Obtain an empty plate of the same type you will use for future real-time PCR reactions and seal it using an optical adhesive cover. Place a compression pad on top of the plate and place it into the instrument;

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- 2) Start the **Opticon Monitor 2** application and select **Dye Calibration** from the **Tools** drop-down menu. You will be presented with a **Dye Calibration Wizard**. In the **Plate type** field, choose the appropriate reaction plate (white or clear). Within the **Dye name** field, type a name for the dye that you wish to calibrate, eg. "CALGold." Click **Next**;
- 3) Click **Go** and the instrument will begin acquiring the data. Shortly, the instrument will pause and prompt you to rotate the plate 180° (angular degrees). After doing so, click **Go**;
- 4) Once the measurements have finished, inspect the results. A problem with the plate will be indicated by anomalous values for one or more wells. If this is observed, discard the plate and start over. Otherwise, click the **Next** button. Note the general signal intensity of the blank plate in each of the detection channels;
- 5) Remove the empty plate from the instrument and discard the optical cover or caps. Dilute the calibration standard in 1X dilution buffer (without ROX) to the necessary working concentration – (see table above) and a volume of 5 mL. Mix well;
- 6) Dispense 50 µL of this calibration solution into all 96 wells of the same reaction plate that was just removed from the instrument. Seal the plate with a new optical adhesive cover, position a compression pad on top, and place it back into the instrument. Click **Go**. Shortly, the instrument will pause and prompt you to rotate the plate 180° (angular degrees). After doing so, click **Go**;
- 7) Once the measurements have finished, again inspect for anomalous results. The signal detected by the dye in at least one of the channels should be over 10 times the signal produced by the blank plate in that channel. Importantly, the ratio of intensities of channel 1 to channel 2 should be consistent across the plate. After inspection, click **Next**;
Click **Finish**. The calibration procedure is now complete. To repeat the procedure for any additional custom dyes that you wish to calibrate on this instrument, click **Yes** at the final prompt.

12.6. Danaher Corporation (Cepheid) SmartCycler II

Following is a general procedure for calibrating compatible dyes on the SmartCycler II and a table listing the recommended dye concentrations. For further details about the calibration procedure, please refer to the **Operator's Manual SC DX 3.0**, Part number: 950-0150.

Calibration standard	Calibration concentration on the iCycler	Number of calibrations possible (approximate)
FAM	200 nM	60
CAL Fluor Gold 540	150 nM	80
CAL Fluor Orange 560	100 nM	125
CAL Fluor Red 590	1000 nM	12
CAL Fluor Red 610	200 nM	60
CAL Fluor Red 635	1200 nM	10
Quasar 570	600 nM	20
Quasar 670	300 nM	40

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NOTE: there is considerable variation in the sensitivity of fluorescence detection from one I-CORE® reaction site to the next. While we have strived to determine a universal calibration concentration for each dye, it is possible that these recommendations might produce too low a fluorescent signal to successfully calibrate one or more sites on your instrument. Alternatively, the concentration might be too high resulting in a low gain setting and future amplifications that are poorly detected. We recommend fine-tuning these suggested concentrations based on the characteristics of your specific instrument, aiming for a consensus concentration that achieves a gain setting of 2 across most reaction sites.

- 1) These calibration instructions are specific to 25 µL reaction volumes. If you use different reaction sizes, the recommended concentrations listed in the above table may no longer be appropriate for your application and the instructions will need to be adjusted accordingly;
- 2) The **SmartCycler Operator Manual** recommends preparing sufficient tubes to simultaneously calibrate all reaction sites on a processing block - 16 tubes for each custom dye and an additional 16 tubes containing only buffer (the instruction to prepare 16 tubes that contain a mixture of all dyes is specific to the SmartCycler but unnecessary for the SmartCycler II). With the possibility that our recommended concentrations are unsuitable for your specific processing block, we recommend only preparing enough tubes to calibrate four reaction sites at a time, using these same tubes to sequentially calibrate the remaining sites. Using this strategy to calibrate for a quadruplexed assay would only require 16 tubes in total, and if one dye proves to be inappropriate only four tubes would need to be prepared at a new concentration;
- 3) All dyes that you plan on using simultaneously as reporters in a multiplexed assay will need to be calibrated in the same run to produce a single “dye set.” If you then change or eliminate one of these reporters in your assay it is strongly recommended that you recalibrate your instrument for this new arrangement;
- 4) For each custom dye to be calibrated, prepare solutions by diluting into 1X dilution buffer (without ROX) to the appropriate concentration (see above table) and a final volume of 125 µL. This should leave you with 25 µL excess. Also prepare 125 µL of dilution buffer alone. Ensure that each of these solutions is uniformly mixed;
- 5) Dispense 25 µL of each dye solution into each of four reaction tubes. Do the same for the dilution buffer. Label the tubes accordingly and spin them down in the minifuge so that the solution collects into the diamond reservoir. If any of these tubes have bubbles remaining in the reservoir, spin them down again. Set these tubes aside for future use;
- 6) Turn on the Smart Cycler II instrument and open the Smart Cycler application. From the **Tools** drop-down menu, select **Optical Calibration**;
- 7) From the **Dye Set Location** drop-down menu, select one of the two user-accessible entries to calibrate, either **Dye Set-5** or **Dye Set-6**. Type a name for this dye set to remind you which dyes were calibrated and the volumes involved. For example, a 25 µL volume calibration for the dyes FAM, CAL Fluor Orange 560, CAL Fluor Red 610, and Quasar 670 could be titled FORQ25;
- 8) Select a **Hold Temp** and a **Read Temp** that is identical to the temperature at which you will be acquiring fluorescence data during your actual real-time PCR amplifications. For a typical 2-step reaction, this is 60 °C. Select the 25 µL tube size. The **Hold Time** and the **Read Time** can be left at 10 seconds each;

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- 9) In the **Dyes** pane, type acronyms for the names of the dyes you will be calibrating across the four channels, and the concentration associated with each. FAM should be calibrated on channel 1. CAL Fluor Gold 540, CAL Fluor Orange 560, CAL Fluor Red 590 and Quasar 570 should all be calibrated on channel 2. CAL Fluor Red 610 should be calibrated on channel 3. CAL Fluor Red 635 and Quasar 670 should be calibrated on channel 4. If any channels will be left blank (such as for a duplex or triplex assay) type “unused” or “empty” into the **Name** field of the appropriate channel;
- 10) In the **Excitation/Emission Mapping** pane, make sure that the wavelength range associated with each channel is correctly positioned for the associated dye. However, if any channel will be left un-calibrated, the excitation and emission mapping for that channel should be left at its default values. Otherwise, channel 1 should have an excitation of 450-495 nm and an emission of 510-527 nm, channel 2 should have an excitation of 500-550 nm and an emission of 565-590 nm, channel 3 should have an excitation of 565-590 nm and an emission of 606-650 nm, and channel 4 should have an excitation of 630-650 nm and an emission of 670-750 nm;
- 11) Click the **Select Sites** button at the bottom of the window and select the first four reaction sites that you will be calibrating, typically A1-A4. Click **OK**;
- 12) Click the **Start Calibration** button at the bottom of the window and click **Proceed** at the prompt that follows;
- 13) A new popup window will appear, prompting you to place the four buffer-only tubes into the appropriate reaction sites. After doing so, click **OK**. Four LED's associated with the reaction sites you are calibrating should light up;
- 14) After fluorescence readings of the buffer tubes have been obtained, the instrument will prompt you to replace these tubes with those containing your first calibration dye. After doing so, click **OK**. Follow the instrument prompts for the remaining dyes you will be calibrating. Each time you place and remove tubes from the instrument, use care in handling so that the sides of the tubes are not scratched and bubbles are not introduced into the diamond reservoir. These same tubes will be required later in the procedure;
- 15) If instrument calibration was successful you will be prompted with the choice to either begin or skip the verification process. This process confirms that the signal intensity of each fluor is as expected, and that bleed-through into adjacent channels can be successfully subtracted out. It is recommended that you conduct verification after calibration. Calibration can fail if either the Gain Normalised Signal (GNS) or the Signal-to-Background ratio (S/B) is too low. Both of these errors indicate that calibration needs to be repeated with the concentration of one of the dyes boosted to a higher value;
- 16) The verification procedure will require the same set of tubes to be placed into the instrument again, but it is highly recommended that you shuffle their placement so that the same exact tube will not be placed back into the same exact reaction site. Before beginning verification, we recommend taking this moment to spin them down in the minifuge so that any bubbles introduced during the previous steps can be removed;
- 17) Follow the prompts during the verification procedure. Upon completion you will be prompted to restart the SmartCycler II application;

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- 18) After the application has finished starting up, we recommend returning to the **Optical Calibration** window and clicking the **Report** button to view the results of the last calibration procedure. Make a note of the **Gains** associated with each channel to understand the intensity of your calibration dyes within the instrument's range of detection. The gains run from 0 → 3. If you ever witness amplifications that abruptly plateau in your future PCR reactions, this is a sign that the optics have been saturated and that the gain setting was too high for your amplification on that channel. If endpoint fluorescence is important for your data analysis then it will be necessary to recalibrate that channel using a lower concentration that elicits a reduced gain setting;
- 19) Repeat this calibration procedure (step 6 → step 17) for the remaining 12 reactions sites on the instrument. Once again, use care to not scratch the tubes or introduce bubbles. It is also important to keep all dye names and settings the same as with your previous calibration;
- 20) Once these are complete, you are ready to run your multiplexed assay on the calibrated reaction sites.

12.7. Eppendorf Mastercycler ep realplex

Following is a general procedure for calibrating compatible dyes on the Eppendorf Mastercycler ep realplex and a table listing the recommended dye concentrations. For further details, please refer to the **Mastercycler ep realplex manual, EDP No: 6302 900.378-07/0608**, which describes dye administration and custom calibration procedures.

Calibration standard	Calibration concentration on the iCycler	Number of calibrations possible (approximate)
FAM	250 nM	10
CAL Fluor Gold 540	350 nM	7
CAL Fluor Orange 560	Incompatible	Incompatible
CAL Fluor Red 590	Incompatible	Incompatible
CAL Fluor Red 610	Incompatible	Incompatible
CAL Fluor Red 635	Incompatible	Incompatible
Quasar 570	Incompatible	Incompatible
Quasar 670	Incompatible	Incompatible

NOTE: these recommended dye concentrations and predicted number of calibrations assume 20 μ L reaction volumes. We recommend performing dye calibration using the same volume as in your future PCR reactions, and so adjustments to these concentrations and the following procedure may be necessary.

- 1) New dye entries need to be added into the Realplex system software for each custom dye that you will be calibrating. This is done using the dye administration window by clicking the **Dyes** button within the toolbar;

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- 2) Click the button **New Dye** to create an entry for the first dye to calibrate. Type in a name for the dye and select the principle filter for its detection. FAM has an emission maximum at 521 nm and is best detected using filter 1 (520 nm); CAL Fluor Gold 540 has an emission maximum at 544 nm and is best detected using filter 2 (550 nm). Confirm this new dye entry by clicking **OK**;
- 3) Note that the sensitivity code for a newly created dye is currently blank, and will not be determined until calibration is complete. Once determined, each of the four digits in this sensitivity code reflect the gain setting applied on each of the four channels;
- 4) Prior to calibrating your custom dye, it will be necessary to perform background calibration. Prepare a background calibration plate by dispensing 1 x dilution buffer (without ROX) into each well of a 96-well reaction plate. We recommend using the same reaction volume as will be used for dye calibration and your subsequent PCR reactions. Before proceeding to the next steps, follow the background calibration procedure starting on page 43 of the Realplex manual;
- 5) If any wells fail the background calibration, prepare a new reaction plate and repeat the procedure until all wells register as "OK." Once calibration is successfully completed, click the **Default** button and then click **Close**. You are now ready to perform dye calibration;
- 6) Dilute the calibration standard in 1X dilution buffer (without ROX) to the necessary working concentration (see table above) and a final volume of 3 mL. Mix well;
- 7) Dispense 20 μ L of this calibration solution into each of the 96 wells of a PCR reaction plate. Seal the plate with an optical adhesive cover and place it into the heating block;
- 8) Select **Color Calibration** from within the **Setup** menu. A colour calibration window will be presented in which you select the dye to be calibrated and also the appropriate background calibration (such as the one performed immediately previous). If the dye entry has not already been created for this dye, you will first need to do so by clicking the **Dyes** button in the toolbar.
- 9) The **Block Temp** and **Lid Temp** can also be adjusted at this point. We recommend leaving the lid temperature at its default value, but adjusting the block temperature to the same temperature that will be used to detect fluorescence in your future PCR reactions. For example, in most real-time reactions it is customary to measure fluorescence at the annealing/extension temperature of 60 °C, and so the block temperature should be set as such;
- 10) After selecting the appropriate dye and adjusting the block temperature, click the **Calibrate** button. Confirm this calibration run by clicking **OK**. After the instrument heats and measures the fluorescence in every well, the software will prompt you to rotate the plate 180°(angular degrees). After doing so, click **OK**;
- 11) A successful calibration is indicated by all 96 wells in the plate layout turning green. If the procedure fails for any reason, we recommend preparing a new plate and repeating the procedure. If the procedure fails again, this might be an indication that the dye was too dilute and calibration should be repeated at a higher concentration;
- 12) Click **Close** and the calibration procedure is complete. Even if the procedure was successful, it is now important to inspect the sensitivity code assigned to the dye just calibrated. If any of these digits is a "1," the calibration procedure will need to be repeated, this time using half the dye concentration as previous. Also, inspect the digit corresponding to the optimal channel for dye detection. Ideally, this digit should be between 2 and 7, or the calibration procedure will need to be repeated using a higher or lower dye concentration;

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- 14) Repeat this procedure for each additional dye that you wish to calibrate;
- 15) Once calibration of all dyes is complete, the effectiveness of dye calibration can be empirically tested by running each of your multiplexed assays individually as singleplex reactions. Importantly, instruct the software to expect all signals within all wells as though they were multiplexed. If any amplification is recorded on a channel that does not contain that fluorophore, this represents crosstalk, which might interfere with subsequent quantitation, but it might not be significant enough to impact results. Compare the intensity of the false amplification to that of the correct amplification. If the false amplification rises to a final fluorescence (ΔR_n) less than 3% the correct amplification, it should not present problems to your future PCR reactions;
- 16) You are now all set to run your multiplexed assay on the Eppendorf Mastercycler ep *realplex*.

12.8. Roche LightCycler 480

Following is a general procedure for calibrating compatible dyes on the Roche LightCycler 480 and a table listing the recommended dye concentrations. For further details, please refer to the **LightCycler 480 Instrument Operator's Manual**, which describes colour compensation analysis under the section "Advanced Software Functionalities."

Calibration standard	Calibration concentration on the LightCycler 480	Number of calibrations possible (approximate)
FAM	100 nM	500
CAL Fluor Gold 540	Not recommended	Not recommended
CAL Fluor Orange 560	100 nM	500
CAL Fluor Red 590	Not recommended	Not recommended
CAL Fluor Red 610	100 nM	500
CAL Fluor Red 635	Not recommended	Not recommended
Quasar 570	Not recommended	Not recommended
Quasar 670	100 nM	500

NOTE: these recommended dye concentrations and predicted numbers of calibrations assume 20 μ L reaction volumes. We recommend performing dye calibration using the same volume as in your future PCR reactions, and so adjustments to these concentrations and the following procedure may be necessary.

IMPORTANT NOTE: It is required that the detector format used to create the Colour Compensation file is identical to the arrangement you intend to use for all of your subsequent experiments, and for which colour compensation (CC) will be necessary. The settings presented below are recommendations only. Colour compensation assays may either be run in parallel to an experiment or else on a separate plate. If running an experiment in parallel to a colour compensation run, each set of wells must be designated as a subset. Only the CC subset should be saved as a Colour Compensation Object (CCO). Please see below.

- 1) Create a stock solution of the calibration standard by adding 50 μ L of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8), to create a 100 μ M stock solution;

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- 2) For each calibration dye, create a working stock at 1 μM concentration by diluting 1 in 100 with TE buffer;
- 3) The CC experiment must contain:
 - a. Five replicates of a water blank, containing 10 μL nuclease-free water and 10 μL of your 2X master mix of choice
 - b. Five replicates for each calibration standard, containing 2 μL of your working dye stock (1 μM), 10 μL of 2X master mix, and 8 μL of nuclease free water, to a total volume of 20 μL .

IMPORTANT NOTE: We recommend use of the same qPCR master mix that you intend to use in your subsequent assays. However, this master mix must not contain a ROX passive reference dye.

- 4) Turn on the LC480 and allow it to warm up for 10 minutes;
- 5) In the software window, select the **Tools Icon** (wrench);
 - a. Under **Detection Format**, choose **New**;
 - b. Select appropriate filter combinations for each of the calibration dyes you wish to use. They are listed by absorption/emission pairs as shown in the table below:

Calibration dye	Detector format	Melt factor	Quant factor	Max integration time (sec)
FAM	465-510	1	1	2
CAL Fluor Orange 560	533-580	1	1	2
CAL Fluor Red 610; LC Red	533-610	1	1	2
Quasar 670; Cy5	618-660	1	1	2

NOTE: the 3 colour hydrolysis probe detection format is pre-set for those identified in grey

- c. Type in a name that describes your unique calibration series;
 - d. Click **Close**.
- 6) From the software homepage; select **New Experiment**;
- 7) The software will open the **Experiment Editor tab** for you. Under **Detection Format** choose the name of the calibration series you just created in step 6, or else a pre-set series if appropriate;
- 8) Click the **Customize tab** and ensure that all desired filters are selected; you do not need to use all of them;
- 9) To run the Colour Compensation assay alone, set up the Melt Curve programme as below.
To run a Colour Compensation assay in parallel to an experimental assay, set the following four programmes to run in series:

Note: You will need to add three programmes, and to do so press the (+) symbol for each.

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Action (add programme)	Programme name	Cycles	Analysis
	Initial Denaturation	1	None
+	qPCR	40	Quantification
+	Melt Curve	1	Color Compensation
+	Cool Down	1	None

Target (°C)	Acq. mode	Hold	Ramp rate (°C/sec)	Acq./°C
95	None	00:10:00	None	
95	None	00:00:15 (or appropriate)	4.8	
60	Single	00:01:00 (or appropriate)	2.5	
95	None	00:00:30	4.8	
40	None	00:00:30	2.5	
95	Continuous			1
40	None	00:00:30	2.5	

- 10) Once you have finished setting the thermal cycling conditions, pull down the menu arrow next to Apply Template and click **Save As Template**. The software will direct you to a pre-labeled folder called **Templates**. You may use this template as the thermal cycling settings for future experiments where the same detection format is appropriate;
- 11) Click on the **Subset Editor** tab in the left-hand column, below the Experiment Editor tab. Here is where you select only the wells you will use for colour compensation. Hold down the control button to select several wells or rows. Add a subset by clicking (+) in the subset name window. Type in an appropriate name for the calibration set. Do the same for your experimental wells if running an experiment in parallel. Give this a separate subset name. Click **Apply**;
- 12) Click on the **Sample Editor** tab located below the Subset Editor tab;
- 13) In the Sample Editor window, find the **Select Workflow** window and click either:
 - Colour Compensation** – if the assay is for colour compensation purposes only; or
 - Absolute Quantitation** – if the assay includes a qPCR run;
 - a. In the **Select Filter Combinations**, ensure all desired channels are checked;
 - b. In the **Select Samples** window, select the subset you are using.
- 14) The table to the right allows you to assign sample names and to designate wells as replicates if you desire;
- 15) While all the channels listed will be collecting data, it is recommended that you select the dominant channel for each calibration dye using the pull down menu next to each sample name. The water blanks for the colour calibration set must use 'Water' as the dominant channel;
- 16) To start, the run a plate must be inserted into the machine. Well A1 must be located in the top left-hand corner;

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- 17) In the right-hand column, click on the Save icon (**floppy disk**) to save the experiment;
 - a. The software will direct you to a pre-labeled folder called **Experiments**;
 - b. Save the experiment with a name representative of the calibration set and/or experimental assay;
 - c. Click **Start Run**.
- 18) Once the run is completed, open the **Analysis** tab located below the Sample Editor tab;
 - a. In the Select Analysis window, select **Colour Compensation**;
 - b. If necessary, in the **Subset Editor** select only the calibration series of wells;
 - c. Click **Calculate**.
- 19) Press the **Save As CCO** (Colour Compensation Object). This will save the colour compensation run in a pre-labeled folder named **Special Data** and a subfolder named **CCC**. Once saved, you may use the CCO to colour compensate for an experimental assay with the same detector format, as described below;

12.8.1. To create a new experiment for which colour compensation is available

- 1) From the software main window select **New Experiment From Template**;
 - a. Select the template you created for subsequent experimental assays;
 - b. Click **OK**.
- 2) In the **Experiment Editor**;
 - a. Make no changes to the Detection Format. This must remain unchanged;
 - b. Add or modify the programmes suitable to your experimental design.
- 3) In the **Subset Editor**, create one large subset which includes those wells to be used in the experiment;
 - a. Click **(+)** to add a new subset if desired;
 - b. Click **Apply** to save it to the experiment.
- 4) In the **Sample Editor**, select **Absolute Quantitation** in the **Select Workflow** window;
 - a. Select the subset you just created which should represent the wells containing sample;
 - b. Assign sample names, or designate as replicates, for each well.
- 5) Click on the **Experiment Editor** tab;
 - a. Insert the sealed qPCR plate, (plate should be centrifuged to remove bubbles);
 - b. Save the file by clicking on the **Floppy disk** icon;
 - c. Press **Start Run**.

12.8.2. To analyse a run using a CCO file

- 1) Open the **Analysis** tab after the run is complete;
- 2) Choose **Absolute Quantitation** in the **Select Workflow** window;
- 3) Press **Calculate**;
- 4) Press the **Filter Combination** tab. Select the filters you wish to compensate (must select at least two);

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- 5) Press the **Colour Comp** tab and select **In Database** to call up the CCO files you have saved earlier;
- 6) Choose the CCO file which contains the dye compensation information appropriate for the current experiment;
- 7) Click **OK**;

Cross-talk of fluorescent reporters will be subtracted out of their non-dominant channels and allow the results to be quantified more precisely.

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