

Primerdesign™ Ltd

LightCycler® 480 II
SARS-CoV-2 Winterplex
Colour Calibration
Instructions For Use

Generating and applying colour compensation files for using the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay with the LightCycler® 480 II System

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G E N E S I G

Kits by Primerdesign



This supplementary protocol is intended for users of the LightCycler® 480 II Instrument System who are performing Real-time PCR using the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay. The protocol describes how to generate and apply a colour compensation file, which is required for accurate analysis of the data produced by the reporter dye combination used in the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay.

Important: Please read the handbook supplied with the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay, paying careful attention to the “Warnings and Precautions” section, before beginning this procedure.

1. Introduction

The Lightcycler® 480 II System has detection channels that allow detection of multiple reporter dyes in the same reaction. However, even when reporter dyes with well separated emission spectra are used, dyes can still be detected by multiple detection channels. Therefore, a correction must be made to ensure Real-Time PCR results are accurate. This is achieved by using a colour compensation file, which contains information that corrects the bleed through between the detection channels.

- Colour compensation files can be generated before or after carrying out a run with the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay and can be stored for later use.
- A colour compensation file is specific for the instrument it was created on. Therefore, if you want to use the genesig® SARS-CoV-2 Winterplex assay on a different LightCycler 480 II System, you will also need to generate a new colour compensation file on the second instrument.

To generate and apply colour compensation files for the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay the following steps are required:

- Prepare a source of reporter dyes for the colour compensation experiment. This is achieved by running 5 replicate reactions for each of the reporter dyes used in the assay until they reach the plateau phase.
- Perform a colour compensation experiment. Fluorescence data is collected and used to generate a colour compensation file containing information for correcting bleed through between detection channels.
- Apply the colour compensation file after performing a run with the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay when performing data analysis.

2. Materials

Most of the necessary reagents are present in the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay and can be used in this supplementary protocol. However, additional reagents supplied with the calibration kit are listed below:

- 1 x Flu A Positive control template (FAM)
- 1 x Flu B Positive control template (Cy5)
- 1 x RSV Positive control template (ROX)
- 1 x genesig® Winterplex Internal extraction control (VIC/HEX)
- 3 x 1.5 mL Template preparation buffer

3. Procedure

3.1 Creating samples for the colour compensation experiment

3.1.1. Reaction mix set up

For the FluA/FluB/RSV primer & probe mix included in the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay, set up at least 5 replicate reactions for every target present and 5 NTC reactions

Prepare a reaction mix according to the directions found in the IFU for the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay, enough for 25 wells:

Reaction mix Component	1 x volume required (µl) *
Onestep Lyophilised Master Mix	10*
Primer & Probe mix (Flu A/FluB/RSV)	2*

Add 12 µl of the FluA/FluB/RSV reaction mix into x25 wells in an appropriate well plate.

Using aseptic technique, resuspend each tube of dried template (Flu A (FAM), Flu B (Cy5), RSV (ROX), genesig® Winterplex Internal Extraction Control (VIC/HEX)) in 800 µl of Template preparation buffer and vortex thoroughly. Resuspended PCT is stable for one month when stored at -20°C. Freeze/ thaw cycles should be minimized and not exceed 5 freeze/thaws. The reagent once resuspended can be aliquoted into smaller volumes if required and stored at -20°C.

Caution: This reagent contains a high copy number of positive control material and should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination of other kit reagents and clinical specimens.

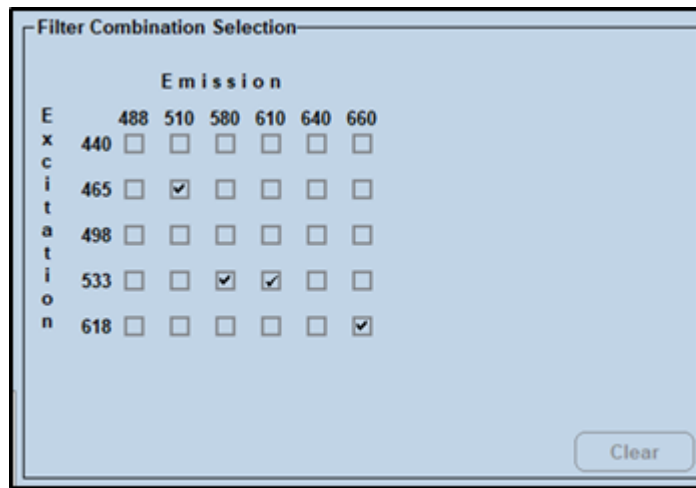
Each PCT contains synthetic DNA representing the genomic region of interest of the assay targets: Flu A, Flu B, RSV, and Internal Extraction Control.

Add 8 µl of each PCT to 5 wells to produce 5 replicate reactions for each reporter dye.
Add 8 µl of water RNase/DNase water to 5 wells to produce 5 NTC replicates.

Seal the plate and place in the LightCycler® 480 II System.

3.1.2. qPCR machine set up and amplification program

Run the genesig® SAR-CoV-2 Winterplex Flu/RSV primer & probe plate following the amplification program and custom filter combination outlined in the IFU for the genesig® Real-Time PCR SARS-CoV-2 Winterplex (CE IVD) assay.



Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
465	510	FAM	1	10	2
533	580	VIC / HEX / Y1	10	10	2
618	660	Cy 5 / Cy 5.5 1	10	10	2
533	610	Red 610	1	10	2

Steps	Time	Temperature	Cycles
Reverse Transcription	10 min	55° C	1
Initial Denaturation (Taq Activation)	2 min	95° C	1
Denaturation	10 sec	95° C	45
Annealing and Extension	60 sec	60° C*	

*Acquisition must be performed at the end of this stage

At the end of the run check whether PCR amplification was successful before using the completed reactions as a source of reporter dyes for generating the colour compensation file.

3.2 Performing a colour compensation experiment

- Open the LightCycler® 480 II System software. To generate a colour compensation file, the software must be opened by a user account that includes access rights for *Administrator* or *Expert User*.
- In the drop-down menu at the top of the window, select *New Experiment*. Then click the “Experiment” button on the left-hand side of the window.
- In the “Programs” panel, enter in the “Program Name” dialog field a name for the temperature protocol. The “Cycles” dialog field must contain a value of 1. In the “Analysis Mode” dialog field, select “Color Compensation”.

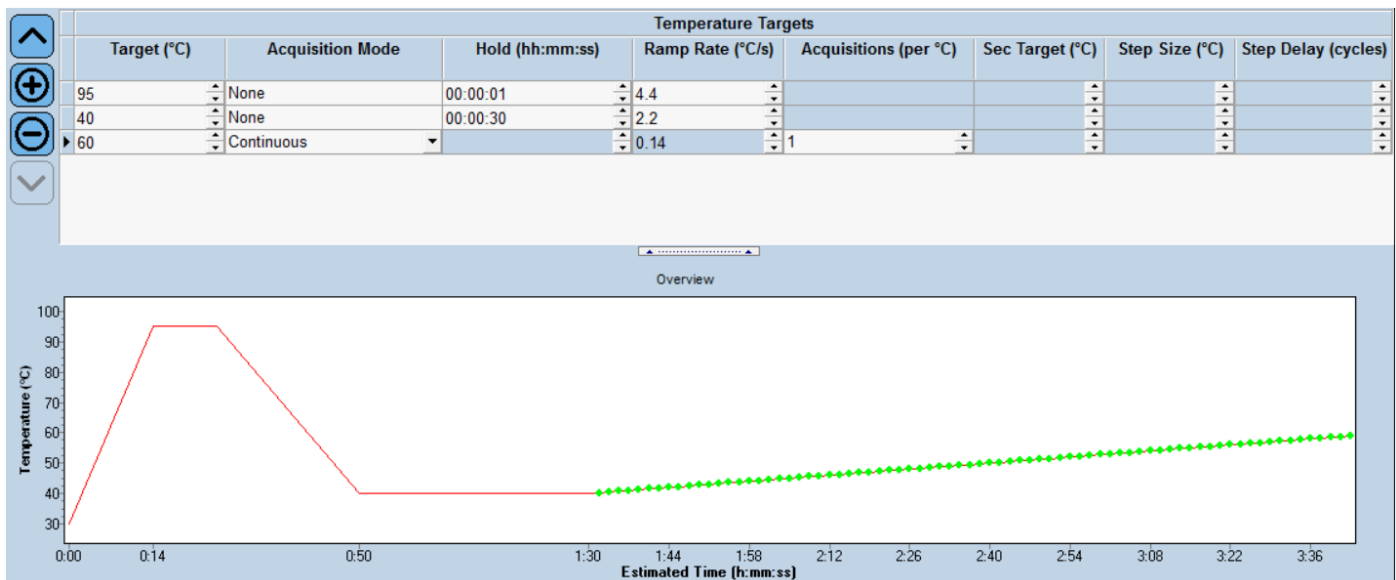
Setup
 Detection Format Block Size Plate ID Reaction Volume
 Color Comp ID Lot No Test ID

Programs		Cycles	Analysis Mode
Program Name	Name for Temperature Protocol	1	Color Compensation

d) In the “Temperature Targets” panel, enter the temperature protocol according to the table below

Temperature (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:01	4.4	N/A
40	None	00:00:30	2.2	N/A
60	Continuous		0.14	1

The final screen should look like the screenshot below:




e) Click the “Sample Editor” button on the left-hand side of the window. Then select the ‘Color Comp’ tab.

f) In the “Dominant Channel” select dyes for each set of replicates.

For the **FluA/FluB/RSV** primer & probe mix calibration, Flu A PCT replicates should be set to FAM, Flu B PCT replicates to Cy5, RSV PCT replicates to ROX and genesig® Winterplex Internal extraction control PCT replicates to HEX/VIC. For the NTC replicates select Water.



g) Save your changes by clicking the  icon.

h) Click the “Start Run” button at the bottom of the window to start the experiment.

i) After the experiment ends, click the “Analysis” button on the left-hand side of the window to open the “Create New Analysis” dialog box. Select “Color Compensation” then click “OK”.

j) Click the “Calculate” button to perform colour compensation analysis.

k) Click the “Save CC Object” button to save the colour compensation file. The colour compensation file is now ready to use.

3.3 Applying a colour compensation file for data analysis

- a) Open the completed assay and click the “Filter Combination” button to select the filter combination you want to display.
- b) In the following drop-down menu, select *In Database*, and choose the stored colour compensation file you want to apply to the assay.



The “Color Comp” button switches to “Color Comp (On)” to confirm, that colour compensation is applied.

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