Supplementary Protocol March 2022

Using the QIAGEN® QIAquant® 96/384 with the Multitarget Detection Assay for Respiratory Viruses: SARS-CoV-2, Flu A, Flu B, RSV A/B

The multitarget assay is optimized to work with QIAprep&amp™ Viral RNA UM Kit (**www.qiagen.com/qiaprepamp-viral-rna-um-kit**) on human samples. To ensure optimal performance of the multitarget detection assay for respiratory viruses on your cycler of choice, we recommend to check the settings of your instrument in use and adjust them if needed.

Important note: This supplementary protocol is a general guideline. There might be fluctuations between single instruments. The process requires validation under specific laboratory settings.

Further information

- *QIAprep&amp Viral RNA UM Kit Handbook*: **www.qiagen.com/HB-2830**
- *Multitarget Detection Assay for Respiratory Viruses: SARS-CoV-2, Flu A, Flu B, RSV A/B – Quick-Start Protocol*: **www.qiagen.com/HB-3005**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Instrument settings – before running the experiment

1. Thermal Cycler: Adjust the PCR protocol according the quick-start protocol and check the "Scan" box next to the 58° anneal/extension step.
2. Scan: Select the channels that are going to be used (FAM, JOE, ROX, Cy5, and Cy5.5). Furthermore, color compensation can be applied/removed via dropdown menu (please refer to Section 3 of "after the run" for installation).
3. Optional: Samples -> Define your Samples (can be done after the run)
4. Run the experiment or save as template for quick access to above options.

Instrument settings for results interpretation – after the run

1. For easy CT-Analysis: Monitoring -> Calculate CT
2. Set threshold:

   Automatic threshold setting is recommended for results analysis. In case there are samples in the same plate with very strong and very weak fluorescence, the automatic threshold can be

calculated based on the samples with weak fluorescence for more accurate results. For this, select samples with weak fluorescence in the plate view on the left side, and then, go to the "gear symbol" (display options). Select "Ok-Auto Thr" to calculate an automatic threshold considering just the selected samples.

If it is desired to have a fixed threshold value, this should be experimentally determined. A serial dilution of the target of interest is recommended (Figure 1) and negative controls (NTC). Viral positive material for the serial dilution can be obtained from several providers (e.g., Zeptometrix, ATCC), as well as in vitro transcribed RNA. The threshold should be set in the exponential phase of the PCR and above the background noise of the NTC. Be aware that a signal with a very early CT but extremely low fluorescence might be an artefact due to incorrect baseline setting and belongs to the background noise.

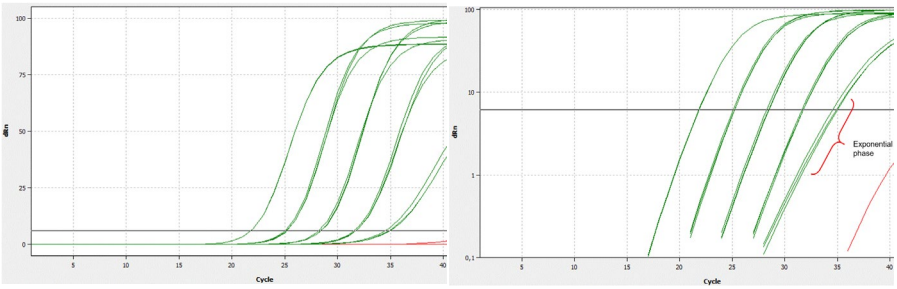

Figure 1. Amplification plot of a serial dilution obtained with the Flu A assay for threshold (black line) setting. In red=NTC. Left: linear scale, right: log scale

3. Dealing with crosstalk

When a very strong fluorescent signal (early CT) is detected in a specific channel, there is a chance that this signal is detected (bleeds) in some proportion in the adjacent channel while performing multiplexing. In this case, we talk about crosstalk, and it is recognized by unusual early CTs with very low fluorescence in the raw data from the affected channel. This is unavoidable due to an overlap in the detection range specified for each channel. The probes used in our assay are **double-quenched,** which assures a reduced background fluorescence, helping to minimize crosstalk.

Using the **QIAquant 96/384**, crosstalk has been detected affecting adjacent channels: from FAM into JOE, from JOE into ROX, from ROX into JOE and into Cy5, and from Cy5.5 into Cy5. Figure 2 shows an example of how crosstalk can be detected in an adjacent channel (JOE), when there is a strong signal detected in FAM.

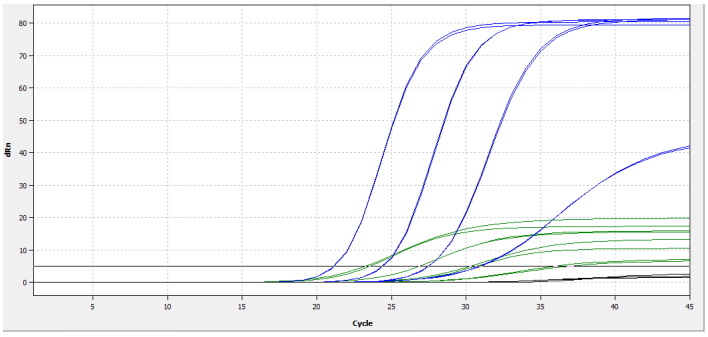

Figure 2. Amplification plots showing strong signals in the FAM channel (blue curves) that bleed into the adjacent channel (JOE-green curves). Color compensation off.

To make sure that the signals detected in a specific channel are not coming from an adjacent channel, a **color compensation file** (in the following text shortened: "CC") must be manually installed. This CC file has been specially designed for this assay and can be applied via the dropdown menu in the "Scan" setting before or after the run. To obtain this file, please contact our technical support (it will be necessary to provide the serial number of your instrument).

Installing color compensation file

1. Before starting (disclaimer): if a CC file is already in use for other purposes, it is necessary to perform a backup of this file (specified later on). Otherwise, other CC files will be deleted in the process.
2. Software version 1.0.3 or above is necessary and can be downloaded from our website. Previous software versions do not support this function.
3. Navigate to: "C:\ProgramData\QIAGEN\QIAquant 96 Software\1.0.3\Dev3" (The 384-Cycler software uses a similar folder: "QIAquant 384 Software").
4. Program Data is a hidden folder (follow 1 below if not done already). Drive C: will be used as default. If changed during installation, "ProgramData" must be searched in set location. (Optional) In Windows File Explorer: "View" Tab -> check "Hidden Items" (Figure 3).

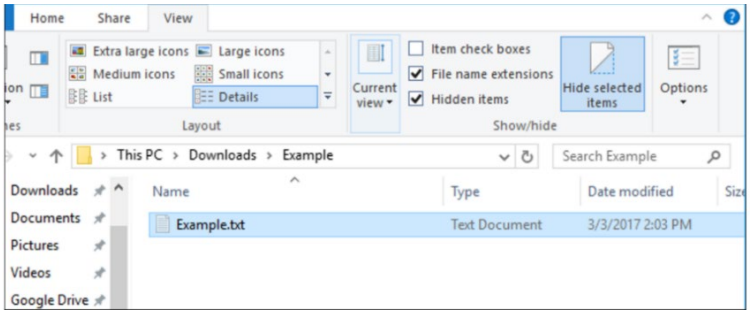

Figure 3. Location of the color compensation file.

5. Replace (in case there is already one) “compensation.cmp” with new file sent by our technical support. Be aware, that the file that you received should be renamed as “compensation.cmp” when saved in the folder. This file contains previously done color compensations. If those are needed for future use, it is recommended to back up or rename the old file (e.g., “compensation_old.cmp” and save it apart for quick exchange. Merging two compensation.cmp files into one is currently not supported.
6. Apply CC in the software under “Scan” -> Dropdown Menu “Select” -> Popup window “multitarget assay for respiratory viruses” -> “OK”.

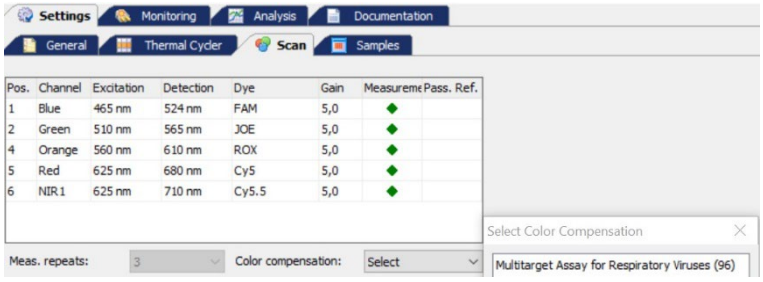

Figure 4. Selection of CC-file.

The results should be analyzed after applying the color compensation to make sure that the effect of the crosstalk has been removed.

Important note: To identify potential crosstalk in your instruments and under your specific settings, it is strongly recommended to perform a dilution series of a positive material for each channel individually. Special attention should be given to the adjacent channels to be able to discard any crosstalk or to take measures to deal with it (threshold setting).

Document Revision History

Date	Changes
03/2022	Initial release

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.