This document outlines several clarifications and helpful tips identified during the implementation of the CDC Flu SC2 assay. All laboratories should refer to the [instructions for use](#) (IFU) for detailed information, but this document offers some clarifications and tips to complement the IFU.

Thermocyler Settings and Calibration

Q1: Texas Red vs Texas Red-X calibration

It is important to recognize that the Texas Red-X dye calibrator provided by IDT DNA Inc. is **not the same** as the Texas Red dye calibration provided in the 7500 Real Time PCR Systems Spectra Calibration Kit II used for the ABI7500 Fast Dx biannual calibration service. You should perform the Texas Red-X dye calibration using the calibration protocol provided by the CDC.

Q2: I performed the standard calibration but still have background noise. How can I further cleanup background noise?

The calibration probe kit from IDT currently contains four calibration probes (FAM, Yakima Yellow, Texas Red X and CY5). ABI7500 Fast Dx instruments are typically already calibrated for FAM and CY5, and we recommend that you **do not** recalibrate your instruments to the FAM and CY5 probes contained within the calibration probe kit, as it is not necessary and could impact multiplex test performance. When performing the multiplex assay, use the FAM and CY5 detectors that are calibrated by ThermoFisher during the ABI7500 Fast Dx biannual calibration service. We **do** recommend that you calibrate to the Yakima Yellow and Texas Red X dyes for optimal performance of the multiplex.

If you performed the standard calibration to Yakima Yellow and Texas Red X and you detect background noise/signal during your Flu SC2 multiplex testing, please review your dye calibration results to ensure that the calibration was successful. A successful dye calibration will demonstrate consistent signals across the wells, with a spectra peak that occurs at the same wavelength and does not diverge significantly. On an ABI 7500, the Yakima Yellow spectra peak occurs in filter 2/filter B, and the Texas Red X spectra peak occurs in filter 4/filter D. If you observe irregular peaks within other wavelengths during the dye calibration, please perform a background calibration to ensure that there is no contaminating signal in the instrument and repeat the dye calibration.

If continued background noise is experienced, consider updating the detection filter for the influenza B probe from VIC to JOE. This adjustment moves the instrument data collection for the second filter away from the FAM channel (filter 1) and can help reduce background noise/signal. You can retroactively apply this filter update to any SDS files created on ABI 7500 instruments that were calibrated to JOE when the SDS file was generated.

To apply an updated filter retroactively to an SDS file, please perform the following steps:

1. Open your SDS file and click on the **Setup** tab and the **Plate** tab. This will show you your plate layout.
2. Select all wells containing samples/controls within the plate (e.g., if you have master mix in all 96 wells, then you would select all wells of the plate; do **not** select empty wells).
3. With the relevant wells selected, right-click on the plate layout and select **Well Inspector**. A new, smaller window will open that lists the detectors/reporters currently assigned to the wells in your plate.
4. Click the **Add Detector** button at the bottom left corner of the Well Inspector window. This will open up the Detector Manager window.
5. Click the **File** drop-down menu button in the bottom left corner of the Detector Manager window and select **New.**
6. A **New Detector** window will open. Use this window to define your new detector. For example, to make a InfB_JOE detector, type "**InfB_JOE**" into the **Name** box, select **JOE** from the **Reporter Dye** drop down menu, keep **Quencher Dye** set to **None**, and select the color you would like the amplification curves for this detector to be displayed in (e.g. green for InfB_JOE). Once you have defined these parameters, click **OK** at the bottom of the New Detector screen.

7. Closing the New Detector window will take you back to the Detector Manager window. Find your new detector that you just created within the list (e.g., InfB_JOE) and click the **Add To Plate Document** button at the bottom of the Detector Manager window.
8. A small SDS Software box will pop-up that states that your selected detector is Added to the Document. Click **OK**.
9. To close the Detector Manager window, click the **Done** button in the bottom right corner of the Detector Manager window.
10. At this point, you should now see your plate in the background with the relevant wells selected, and the **Well Inspector** window should still be open.
11. In the Well Inspector window, deselect the detector that you want to swap out by clicking on the check-mark box (e.g., InfB_VIC or InfB_m). This will remove the check mark next to that detector and remove that detector from the selected wells.
12. Once the old detector is removed, select the updated detector (e.g., InfB_JOE) within the Well Inspector window by clicking on the check mark box to add a check mark next to the new detector. This will add in the new detector/reporter to the highlighted wells.
13. To close the Well Inspector window, click the **Close** button at the bottom of the Well Inspector window.
14. To reanalyze the data with this updated detector, go to the **Analysis** menu at the very top of the SDS file and click **Analyze**. Note that once you have clicked Analyze, you will need to set the threshold for the new detector data in the same manner that you set the threshold for your previous analysis.

CDC Flu SC2 Assay Verification

Q3: **While verifying the performance of the Flu SC2 assay, I observed a drop out or negative result for the Influenza A or influenza B targets in the low SIPC samples during verification. Why did this occur?**

Do not use diluted SIPC (1:10 SIPC) as your starting material for verification. The SIPC provided with your Flu SC2 Multiplex kit has an extra 0.2ml for use in verification testing. Use this undiluted (neat) SIPC as your starting material for verification. Diluted SIPC (1:10 SIPC) is for use as the diagnostic testing positive control. If your laboratory already diluted SIPC for diagnostic testing (1:10 SIPC), this diluted material cannot be used for verification.

Q4: **Why are there two components to the Flu SC2 Multiplex positive control and why can you not combine them prior to extraction?**

There are two positive control components in the Flu SC2 multiplex: 1) seasonal influenza positive control (SIPC) and 2) SARS-CoV2 Positive Control (SC2PC). The combined FluSC2PC control serves as both an extraction and a PCR control. SIPC serves as the extraction control, and is a combination of inactivated influenza A and influenza B seasonal viruses and A549 cells. The SC2PC contains a synthetic RNA of the SARS-CoV-2 genome. When you combine SIPC and SC2PC without the presence of an RNA lysis buffer, RNAses from the A549 cells can chew up the SARS-CoV-2 RNA. Therefore, you should never combine these two controls before extraction or without the presence of lysis buffer.

Watch CDC Flu SC2 Multiplex Assay Training Videos
CDC published brief videos (<15 min) to demonstrate how the test should be set up and how the results of testing should be analyzed on the ABI 7500 Fast Dx. All laboratories performing the Flu SC2 Multiplex should encourage testing staff to watch these videos, preferably prior to implementing testing with the Flu SC2 Multiplex.

Diagnostic Testing

Q5: **The sensitivity of the Flu SC2 Multiplex assay seems low or variable.**

Make sure you **analyze and set the threshold for each primer/probe set individually.** Please carefully follow the instructions for analyzing test results presented on pages 21-24 of the [Instructions for Use](#). CDC has also produced a [brief video](#) (<15 min) demonstrating results analysis for this assay. If sensitivity of your Flu SC2 Multiplex is still an issue, please contact CDC by emailing CDCSARS2FluAB@cdc.gov and include "Flu SC2 Multiplex" in the subject line.

Q6: **How should we prepare SIPC aliquots? Can you clarify the final SIPC aliquot volume?**

You should make 20 aliquots of diluted SIPC (1:10) by adding 50µl of stock SIPC into 450µl of nuclease-free water for a total of 500µl in each of the 20 aliquot tubes. The 500µl aliquots allow for sufficient volume and flexibility across different extraction platforms given different starting volume requirements.

Informatics Considerations

Q7: **Our lab uses the Lab Web Portal for COVID-19 test ordering and resulting. Will the Flu SC2 Multiplex be available in Lab Web Portal?**

APHL is rolling out support to add influenza and SARS-CoV-2 multiplex assay ordering capabilities to Lab Web Portal instances. APHL will contact laboratories in December to begin implementation.

Q8: **Where can I find more information on LOINC, SNOMED and allowable code combinations?**

Please refer to the [SARS-CoV2 Encoding Guidelines and CDC Flu SC2 Multiplex Assay Code tables and guidance](#).

Q9: **Are we required to report the influenza A or influenza B subtyping results within the same HL7 message as the initial influenza A, influenza B and SARS-CoV2 Multiplex Screen?**

CDC will accept a separate HL7 message containing the influenza A or influenza B subtyping/lineage results if the Patient ID and Specimen ID remain the same between both HL7 messages.

Q10: **Are we required to report the RP result in our HL7 message?**

If your PHL chooses to report the multiplex targets, then you must report the human RP as well. The RP is important for determining the result interpretation.

Q11: **Can we map and report Flu and SARS-CoV-2 pathogens in the same OBX message segment?**

If your PHL chooses to report the multiplex conclusions and more than one organism is detected, you will need to send each organism as a separate OBX segment. The LOINC code 95423-0 can be populated in the OBX-3 segment and OBX-4 can be used to differentiate the pathogens. Refer to the [assay code guidance](#) for more information.

General Reminders & Considerations

Q12. **What kit and enzyme should I use with the Flu SC2 Multiplex?**

Only EUA-labeled primers and probes distributed by the [International Reagent Resource](#) may be used for viral testing with the Flu SC2 Multiplex Assay. The IRR catalog number for the kit is RR-1 Influenza SARS-CoV-2 Multiplex Assay (EUA). There are two enzyme master mixes available for use with the Flu SC2 Multiplex:

rRT-PCR Enzyme Master Mix Options		
Reagent	**Quantity**	**Catalog No.**
Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX)	1 X 0.5 mL/80 reactions A28521	1 X 0.5 mL/80 reactions A28521
	5 X 1 mL/ 800 reactions A28522	5 X 1 mL/ 800 reactions A28522
	1 X 10 mL/1600 reactions A28523	1 X 10 mL/1600 reactions A28523
Quantabio UltraPlex™ 1-Step ToughMix® (4X)	1 x 0.5 mL/80 reactions	95166-100 (VWR 10804-944)
	5 x 0.5 mL/400 reactions	95166-500 (VWR 10804-946)

Q13: Can I use the same enzyme for both the Flu SC2 Multiplex and the original CDC 2019-nCoV assay?

The TaqPath enzyme authorized for use with the Flu SC2 Multiplex is **not the same TaqPath enzyme** authorized for use with the original CDC 2019-nCoV assay. Laboratories should carefully review the name and catalog numbers on the enzymes before use.

Q14: What is the supply outlook for the CDC Flu SC2 kits?

CDC is not able to manufacture the Flu SC2 multiplex assay at a level to support all PHL testing; unfortunately, the demand currently outstrips production capabilities. CDC encourages laboratories to continue to utilize a variety of platforms to support their overall state testing plans.