

CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay

For Emergency Use Only

Instructions for Use

Catalog # Flu SC2-EUA

500 reactions

For *In Vitro* Diagnostic (IVD) Use

Rx Only

Centers for Disease Control and Prevention
Influenza Division
1600 Clifton Rd NE
Atlanta GA 30329-4027



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Intended Use

The Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay is a real-time RT-PCR multiplexed test intended for the simultaneous qualitative detection and differentiation of SARS-CoV-2, influenza A virus, and/or influenza B virus nucleic acid in upper or lower respiratory specimens (such as nasopharyngeal, oropharyngeal and nasal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals suspected of respiratory viral infection consistent with COVID-19 by a healthcare provider¹. Symptoms of respiratory viral infection due to SARS-CoV-2 and influenza can be similar.

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, that meet requirements to perform high complexity tests.

The Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay is intended for use in the detection and differentiation of SARS-CoV-2, influenza A, and/or influenza B viral RNA in patient specimens, and is not intended to detect influenza C. RNA from influenza A, influenza B, and/or SARS-CoV-2 viruses is generally detectable in upper and/or lower respiratory specimens during infection. Positive results are indicative of active infection but do not rule out bacterial infection or co-infection with other viruses; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all SARS-CoV-2 positive results to the appropriate public health authorities.

Negative Flu SC2 Multiplex Assay results do not preclude SARS-CoV-2, influenza A, and/or influenza B infection and should not be used as the sole basis for diagnosis, treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and/or epidemiological information.

Negative results obtained from individuals who are not exhibiting clinical signs and symptoms associated with respiratory viral infection at the time of specimen collections should be interpreted with particular caution. Negative results in asymptomatic individuals cannot be used as definitive evidence that an individual has not been exposed to SARS-CoV-2 or influenza viruses and has not been infected with any of these viruses.

Testing with the Flu SC2 Multiplex Assay is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The Flu SC2 Multiplex Assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

¹For this EUA, a healthcare provider includes, but is not limited to, physicians, nurses, pharmacists, technologists, laboratory directors, epidemiologists, or any other practitioners or allied health professionals.

Summary and Explanation

An outbreak of pneumonia of unknown etiology in Wuhan City, Hubei Province, China was initially reported to WHO on December 31, 2019. Chinese authorities identified a novel coronavirus (i.e., SARS-CoV-2), which has now resulted in millions of confirmed human infections globally. Cases of asymptomatic infection, mild illness, severe illness, and deaths have been reported.

The Flu SC2 Multiplex Assay is a molecular *in vitro* diagnostic test that aids in the detection and differentiation of RNA from SARS-CoV-2, influenza A virus, and/or influenza B virus and is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers and dual-labeled hydrolysis probes (TaqMan[®]), and control material used in rRT-PCR for the *in vitro* qualitative detection and differentiation of SARS-CoV-2 virus RNA, influenza A virus RNA and/or influenza B virus RNA in upper and lower respiratory specimens.

The term “qualified laboratories” refers to laboratories certified under CLIA, meeting the CLIA requirements to

perform high complexity testing, and in which all users, analysts, and any person reporting results from use of this device are trained to perform and interpret the results from this procedure prior to use.

Principles of the Procedure

The Flu SC2 Multiplex Assay is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test. The Flu SC2 Multiplex Assay contains three primer/probe sets (InfA, InfB, and SC2) that target the RNA of influenza A virus, influenza B virus, and SARS-CoV-2 virus, respectively. The assay also contains primers and a probe to detect the human RNase P gene (RP) in clinical specimens or control samples. The oligonucleotide primers and probe for detection of SARS-CoV-2 were selected from an evolutionarily conserved region of the 3' terminus of SARS-CoV-2 genome and include part of the carboxy-terminal portion of the nucleocapsid (N) gene. Primers and probes for the detection of influenza A viruses were selected from an evolutionarily well conserved region of the matrix (M1) gene. The primers and probe selected for detection of influenza B viruses were selected from a conserved region of the nonstructural 2 gene (NS2). The assay is a multiplex assay, run in a single well/vessel, designed for detection and differentiation of RNA from SARS-CoV-2 virus, influenza A viruses, and/or influenza B viruses.

Regions of the nucleic acids extracted from upper and lower respiratory specimens that are complementary to the oligonucleotide primers are reverse transcribed into cDNA and amplified by polymerase chain reaction using either the Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX), or Quantabio UltraPlex™ 1-Step ToughMix® (4X), using an Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with SDS version 1.4.1 software. If the target nucleic acids are present, they are amplified, and the probe(s) anneal to specific complementary sequences located between the corresponding forward and reverse primers during the PCR process. During the extension phase of the PCR, the 5' nuclease activity of DNA polymerase degrades the probe bound to the specific target, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. Probes specific to each virus generate a fluorescent signal at different wavelengths, enabling the instrument to differentiate between the signals. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by Applied Biosystems 7500 Fast Dx Real-Time PCR System with SDS version 1.4.1 software.

Detection of viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.

Materials Required (Provided)

Table 1. CDC Catalog # Flu SC2-EUA – Influenza SARS-CoV-2 Multiplex Assay Kit

Component	Part Number	Vials Per Kit	Quantity Per Vial	State	Application
FluSC2 Combined Primer Mix	MR-665	1	80 nmol	1.5 mL aqueous	Includes targets for InfA, InfB, SC2, and RP
FluSC2 Combined Probe Mix	MR-666	1	10 nmol	1.5 mL aqueous	Includes targets for InfA, InfB, SC2, and RP
SIPC Seasonal Influenza Positive Control (non-infectious)	MR-541	1	1.2mL	aqueous	Yields a positive result for InfA, InfB, and RP
SC2PC Positive Control (non-infectious)	MR-669	1	1 x 10 ⁴ copies/μL	dried	Yields a positive result for SC2

Materials Required (But Not Provided)

Table 2. CDC Catalog # KT0189 – Human Specimen Control (HSC)

Description	Quantity	CDC Catalog No.
The HSC consists of noninfectious (beta-propiolactone treated) cultured human cell material supplied as a liquid suspended in 0.01 M PBS at pH 7.2-7.4.	10 vials x 500 µL	KT0189

Acceptable alternatives to HSC:

- Negative human specimen material: Laboratories may prepare a volume of human specimen material (e.g., human sera or pooled leftover negative respiratory specimens) to extract and run alongside clinical samples as an extraction control. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these Instructions for Use.
- Contrived human specimen material: Laboratories may prepare contrived human specimen materials by suspending any human cell line (e.g., A549, Hela, or 293) in PBS. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these Instructions for Use.

rRT-PCR Enzyme Master Mix Options

Table 3. 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
	5 X 1 mL/ 800 reactions	A28522
	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)
	1 x 5.0 mL/800 reactions	95166-01K (VWR 76121-382)

RNA Extraction Options

Table 4. RNA Extraction Options

Instrument/Manufacturer	Extraction Kit	Catalog No.
QIAGEN	¹ QIAamp DSP Viral RNA Mini Kit	50 extractions (61904)
	¹ QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
QIAGEN EZ1 Advanced XL	¹ EZ1 DSP Virus Kit	48 extractions (62724) Buffer AVL (19073) EZ1 Advanced XL DSP Virus Card (9018703)
	¹ EZ1 Virus Mini Kit v2.0	48 extractions (955134) Buffer AVL (19073) EZ1 Advanced XL Virus Card v2.0 (9018708)
Roche MagNA Pure 96	¹ DNA and Viral NA Small Volume Kit	576 extractions (06 543 588 001) External Lysis Buffer (06 374 913 001, 12 239 469 103, 03 246 779 001 or 03 246 752 001)
QIAGEN QIAcube	¹ QIAamp DSP Viral RNA Mini Kit	50 extractions (61904)
	¹ QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)

¹CDC has confirmed that the external lysis buffer used with this extraction method is effective for inactivation of SARS-CoV-2, Influenza A viruses, and Influenza B viruses.

CAUTION: Lysis buffers used with these extraction methods contain guanidinium thiocyanate or guanidine-containing materials. Highly reactive and/or toxic compounds may form if combined with sodium hypochlorite (bleach).

Qualifying Alternative Components

If a laboratory modifies this test by using unauthorized, alternative components (e.g., extraction methods or PCR instruments), the modified test is not authorized under this EUA. FDA's Policy for Diagnostic Tests for Coronavirus Disease-2019 during the Public Health Emergency, updated May 11, 2020, does not change this.

Equipment and Consumables Required (But Not Provided)

- 1.5 mL polypropylene microcentrifuge tubes (DNase/RNase free) and/or 5 mL polypropylene microcentrifuge tubes (DNase/RNase free)
- Racks for 1.5 mL/5 mL microcentrifuge tubes
- 70% ethanol (EtOH)
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- DNAZap™ (ThermoFisher Scientific, catalog #AM9890), or equivalent
- RNase AWAY™ (Fisher Scientific, catalog #21-236-21), or equivalent
- Disposable powder-free gloves and surgical gowns
- Molecular-grade nuclease-free water (RNase/DNase Free) or 10mM Tris pH 7.4-8.2
- -70°C and -20°C freezer(s)
- 4°C refrigerator
- 2 x 96-well cold blocks (-20°C)
- Micropipettors (range between 1-10 µL, 10-200 µL and 100-1000 µL)
- Multichannel Micropipettors (1-10 µL, 5-50 µL)
- Aerosol barrier pipette tips
- MicroAmp™ Fast 8-tube strip 0.1 mL (ThermoFisher Scientific, catalog #4358293) or MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (alternate to 8-tube strips) (ThermoFisher Scientific, catalog #4346906, 4346907, or 4366932)
- MicroAmp™ Optical 8-cap strip (required, do not use film) (ThermoFisher Scientific, catalog #4323032)
- Strip Tubes and Caps 0.1 mL (4 tubes and caps) (QIAGEN, catalog #981103 or 981106)
- Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with SDS v1.4.1 Software (ThermoFisher Scientific, catalog #4406985 or #4406984)
- Extraction systems (instruments): QIAGEN EZ1 Advanced XL, QIAGEN QIAcube, Roche MagNA Pure 96
- Benchtop microcentrifuge
- Vortex mixer

Warnings and Precautions

- For *In Vitro* Diagnostic Use (IVD).
 - This test has not been FDA cleared or approved; this test has been authorized by FDA under an EUA for use by laboratories certified under CLIA, 42 U.S.C. § 263a, that meet requirements to perform high complexity tests.
 - This test has been authorized only for the simultaneous qualitative detection and differentiation of nucleic acid from SARS-CoV-2, influenza A virus and influenza B virus and not for any other viruses or pathogens.
 - This test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostic tests for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.
- Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.
- Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.
- Handle all specimens as if infectious using safe laboratory procedures. Refer to Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition BMBL (<http://www.cdc.gov/biosafety/publications/bmbl5/index.htm>) for standard biological safety guidelines for all procedures.

- Specimen processing should be performed in accordance with national biological safety regulations.
- If infection with SARS-CoV-2 is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected using appropriate infection control precautions.
- If infection with a novel influenza A virus is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected using appropriate infection control precautions for novel virulent influenza viruses and sent to state health departments for testing **IMMEDIATELY**. Virus culture should not be attempted in these cases unless a BSL-3E facility is available to receive and culture specimens.

Note: Novel influenza A viruses are new or re-emergent human strains of influenza A virus that cause cases or clusters of human disease, as opposed to those strains commonly circulating in humans that cause seasonal epidemics. Human populations have residual or limited immunity (either by vaccination or previous infection) to novel influenza A viruses.

- Performance characteristics have been determined with human upper respiratory specimens and lower respiratory tract specimens from human patients with signs and symptoms of respiratory infection.
- Perform all manipulations of potentially infectious virus specimens within a Class II (or higher) biological safety cabinet (BSC).
- Use personal protective equipment such as (but not limited to) gloves and lab coats when handling kit reagents while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.
- Amplification technologies such as PCR are sensitive to accidental introduction of product from previous amplification reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon). Workflow in the laboratory should proceed in a unidirectional manner.
 - Maintain separate areas for assay setup and handling of nucleic acids.
 - Always check the expiration date of reagents prior to use. Do not use expired reagent(s). Do not substitute or mix reagents from different kit lots or from other manufacturers.
 - Change aerosol barrier pipette tips between all manual liquid transfers.
 - During preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with nucleic acids.
 - Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
 - Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
 - Change gloves between samples and whenever contamination is suspected.
 - Keep reagent and reaction tubes capped or covered as much as possible.
 - Primers, probes (including aliquots), and enzyme master mix must be thawed and maintained on cold block at all times during preparation and use.
 - Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, “DNAzap™” or “RNase AWAY™” to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
- Reagents, master mix, and RNA should be maintained on a cold block or on ice during preparation and use to ensure stability.
- Dispose of unused kit reagents and human specimens according to local, state, and federal regulations.
- External Lysis Buffers may contain guanidinium thiocyanate or guanidine-containing materials which can create highly reactive and/or toxic compounds if combined with sodium hypochlorite (bleach).

Reagent Storage, Handling, and Stability

- Store all primers and probes, HSC, and SIPC positive control at $\leq -20^{\circ}\text{C}$; Store the SC2PC control material at $2-8^{\circ}\text{C}$ until rehydrated for use.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on a cold block at all times during preparation and use.
- Do not refreeze primers and probes.
- Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use.

Specimen Collection, Handling, and Storage

Inadequate or inappropriate specimen collection, storage, and transport are likely to yield false negative test results. Training in specimen collection is highly recommended due to the importance of specimen quality. CLSI MM13-A may be referenced as an appropriate resource.

- Collecting the Specimen
 - Refer to *Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons for Coronavirus Disease 2019 (COVID-19)* <https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html>
 - Follow specimen collection device manufacturer instructions for proper collection methods.
 - Swab specimens should be collected using only swabs with a synthetic tip, such as nylon or Dacron®, and an aluminum or plastic shaft. Calcium alginate swabs are unacceptable and cotton swabs with wooden shafts are not recommended.
 - Respiratory specimens should be collected and placed into appropriate transport media, such as viral transport media (VTM), as described by CDC and WHO guidelines. See <https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html>
- Transporting Specimens
 - Specimens must be packaged, shipped, and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential SARS-CoV-2 or influenza virus specimens. Store specimens at $2-8^{\circ}\text{C}$ and ship overnight on ice pack. If a specimen is frozen at $\leq -70^{\circ}\text{C}$, ship overnight on dry ice.
- Storing Specimens
 - Specimens received cold should be stored refrigerated ($2-8^{\circ}\text{C}$) for up to 72 hours before processing.
 - If a delay in specimen extraction is expected, store specimens at $\leq -70^{\circ}\text{C}$.
 - Specimens received frozen should be stored at $\leq -70^{\circ}\text{C}$ until processing.
 - Store any residual specimens at $\leq -70^{\circ}\text{C}$.
- Storing Purified Nucleic Acid
 - Store purified nucleic acids at $\leq -70^{\circ}\text{C}$.

Specimen Referral to CDC

Referring a specimen to the CDC

- Ship all specimens overnight to CDC.
- Ship frozen specimens on dry ice and non-frozen specimens on cold packs. Ship extracted RNA on dry ice.
- Refer to the International Air Transport Association (IATA - www.iata.org) for requirements for shipment of human or potentially infectious biological specimens. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential SARS-CoV-2 or influenza specimens.
- Questions on submissions should be directed to CDCSARS2FluAB@cdc.gov.
- Prior to shipping specimens to CDC, notify CDCSARS2FluAB@cdc.gov.
- Fill out the CDC Specimen Submission Form 50.34. Include specimen type and Ct values detected by your laboratory, if available.
- Send all samples to the following recipient:

Attention: Triage and Reporting Laboratory (Unit 66)
c/o STAT Lab
Centers for Disease Control and Prevention
1600 Clifton Rd., Atlanta, GA 30329-4027
Phone: (404) 639-4710

The emergency contact number for CDC Emergency Operations Center (EOC) is

770-488-7100

Reagent and Controls Preparation

Primer and Probe Preparation

The Flu SC2 Combined Primer Mix (Part# MR-665) and the Flu SC2 Combined Probe Mix (Part# MR-666) should be aliquoted and frozen upon receipt. Do not refreeze primers and probes.

1. If frozen, thaw completely.
2. Completely mix and centrifuge primer and probe tubes for 15 seconds at 2000 X g.
3. Label 10 new, nuclease-free, sterile tubes for the Flu SC2 Combined Primer Mix (Part# MR-665) with the following information:
 - Flu SC2 Multiplex Primers
 - Kit lot #
 - Expiration date
4. Label 10 new, nuclease-free, sterile tubes for the Flu SC2 Combined Probe Mix (Part# MR-666) with the following information:
 - Flu SC2 Multiplex Probes
 - Kit lot #
 - Expiration date
5. Aliquot 150 µL of primer mix into each of the 10 tubes labeled Flu SC2 Multiplex Primers and freeze at $\leq -20^{\circ}\text{C}$.
6. Aliquot 150 µL of probe mix into each of the 10 tubes labeled Flu SC2 Multiplex Probes and freeze at $\leq -20^{\circ}\text{C}$.

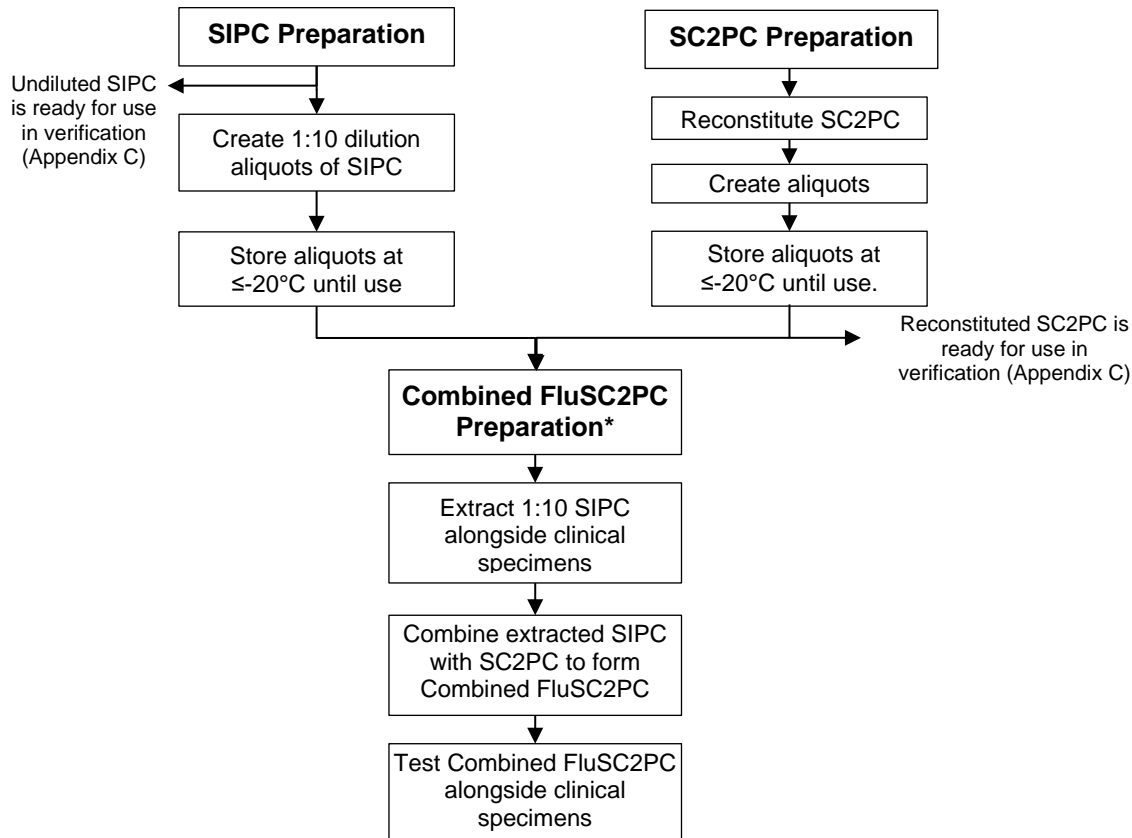
Note: Aliquots of primers and probes are stored at $\leq -20^{\circ}\text{C}$ until expiration date as long as QC requirements are met.

7. Aliquots of primers and probes that have been thawed for use may be stored at $2-8^{\circ}\text{C}$ for up to 3 weeks. Aliquots of primers and probes should be stored in the dark. Do not refreeze.

Positive Control Preparation

The Combined FluSC2PC serves as both an extraction and a PCR control. One component of the Combined FluSC2PC, Seasonal Influenza Positive Control (SIPC) is extracted with clinical specimens to ensure successful RNA extraction. SARS-CoV2 Positive Control (SC2PC) is then added to the extracted SIPC material to create the Combined FluSC2PC and serve as a positive PCR control for all targets in the Flu SC2 Multiplex Assay.

Figure 1: Overview of Combined FluSC2PC Preparation



*Combined FluSC2PC is to be prepared alongside each batch of clinical specimens.

Seasonal Influenza Positive Control (SIPC) Preparation

1. Label 20 new nuclease-free, sterile, tubes for each single-use aliquot with the following information:
 - 1:10 SIPC
 - Kit lot #
 - Expiration Date
2. Create 20 single use 1:10 dilutions of SIPC by adding 50 µl SIPC to 450 µl molecular-grade nuclease-free water in the labeled nuclease-free, sterile, tubes.
3. Mix by pipetting up and down 3X
4. Store at -20°C or below.

SARS-CoV-2 Positive Control (SC2PC) Preparation

1. Remove SC2PC from 2-8°C.
2. Allow to sit at ambient temperature for 15 minutes.
3. Briefly centrifuge dried control for 30 seconds at 2000 x g to collect pellet in the bottom of the tube.

4. Add 0.2 mL (200 µL) of 10 mM Tris, pH 7.4-8.2, or molecular-grade nuclease-free water.
5. Allow to fully rehydrate for at least 15 minutes at room temperature.
6. After the control is fully rehydrated, pulse vortex to ensure a homogenous solution.
7. Label 10 new nuclease-free, sterile 0.5 mL tubes with the following information:
 - SC2PC
 - Kit lot#
 - Expiration Date
8. Aliquot reconstituted SC2PC by pipetting 20 µL SC2PC into each of the 10 labeled tubes.
9. Store SC2PC aliquots at -70°C or below.

Combined FluSC2PC Preparation

(prepared alongside each batch of clinical specimens to be extracted and tested)

1. Thaw one tube of the 1:10 SIPC.
2. Mix the appropriate ratio of 1:10 SIPC to external lysis buffer for the approved RNA extraction method being used.
3. Extract SIPC with a qualified RNA extraction method per these instructions for use alongside clinical specimens.
4. In a new nuclease-free tube, combine 10 µL of the SC2PC with 90 µL of the extracted SIPC to create the Combined FluSC2PC.
5. Pulse vortex to ensure a homogenous solution and briefly spin for 15 seconds at 2000 X g.
6. 5 µL of Combined FluSC2PC is used per reaction.
7. Combined FluSC2PC must be created and tested alongside each batch of clinical specimens.

Human Specimen Control (HSC) Preparation (not provided)

1. Human Specimen Control (HSC) or one of the listed acceptable alternative extraction controls must be extracted and processed with each specimen extraction run.
2. Extract HSC with an RNA extraction method per these Instructions for Use alongside each batch of clinical specimens.

No Template Control (NTC) (not provided)

1. Sterile, molecular-grade nuclease-free water.
2. Aliquot in small volumes.
3. Used to check for contamination during specimen extraction and/or plate setup.

General Preparation

Equipment Preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use. Decontamination agents should be used including 10% bleach, 70% ethanol, and **DNAzap™** or **RNase AWAY®** to minimize the risk of nucleic acid contamination.

Reagent Preparation

NOTE: All reagents should be kept on ice or cold block during assay preparation.

Primers and Probes

1. Thaw frozen aliquots of each primer/probe mix. Thawed aliquots may be stored at 2-8°C in the dark for up to 3 weeks. **Do not re-freeze.**
2. Gently mix each primer/probe aliquot by inversion 10 times.
3. Briefly centrifuge each primer/probe mix aliquot for 15 seconds at 2000 X g.
4. Place each primer/probe mix aliquot in cold rack during master mix preparation.

Real-time RT-PCR Reagents

TaqPath™ 1-Step Multiplex Master Mix (No ROX) or Quantabio UltraPlex™ 1-Step ToughMix® (4X)

1. Place TaqPath™ 1-Step Multiplex Master Mix (No ROX) OR Quantabio UltraPlex™ 1-Step ToughMix® (4X) in a cold rack at 2-8°C.
2. Completely thaw the Master Mix vial.
3. Mix the Master Mix by inversion 10 times.
4. Briefly centrifuge Master Mix for 15 seconds at 2000 X g and place in cold rack.

Nucleic Acid Extraction

Performance of the Influenza SARS-CoV-2 Multiplex Assay is dependent upon the amount and quality of template RNA purified from human specimens. The following commercially available RNA extraction kits and procedures have been qualified and validated for recovery and purity of RNA for use with the assay.

- **Manufacturer's recommended procedures (except as noted below) are to be used for sample extraction.**
- **HSC must be extracted and processed alongside patient specimens in each run.**
- **Combined FluSC2PC must be prepared alongside patient specimens in each run.**

QIAGEN QIAamp® DSP Viral RNA Mini Kit or QIAamp® Viral RNA Mini Kit

Options and recommendation(s):

- 100 µL input and elution volume
 - Utilize 100 µL of sample with 560 µL of Buffer AVL (with carrier RNA)
 - Elute with 100 µL of elution buffer
- 140 µL input and elution volume
 - Utilize 140 µL of sample with 560 µL of Buffer AVL (with carrier RNA)
 - Elute with 140 µL of elution buffer

QIAGEN EZ1 Advanced XL

Options and recommendation(s):

- Option 1
 - Kit: QIAGEN EZ1 DSP Virus Kit and Buffer AVL with carrier RNA (supplied separately) for offboard lysis
 - Card: EZ1 Advanced XL DSP Virus Card
 - Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL with carrier RNA (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 120 µL.
- Option 2
 - Kit: QIAGEN EZ1 Virus Mini Kit v2.0 and Buffer AVL with carrier RNA (supplied separately) for offboard lysis
 - Card: EZ1 Advanced XL Virus Card v2.0
 - Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL with carrier RNA (total input volume is 400 µL). Proceed with extraction on the EZ1 Advanced XL. Elution volume is 120 µL.

NOTE: In both options, carrier RNA is to be added to Buffer AVL for offboard lysis following manufacturer's instructions.

Roche MagNA Pure 96

- Kit: Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit
- Protocol: Viral NA Plasma Ext Lys SV 4.0 Protocol or Viral NA Plasma Ext Lys SV Protocol
- Recommendation(s): Add 100 µL of sample to 350 µL of pre-aliquoted External Lysis Buffer (supplied separately) (total input sample volume is 450 µL). Proceed with the extraction on the MagNA Pure 96. (**Internal Control = None**). Elution volume is 100 µL.

QIAGEN QIAcube

- Kit: QIAGEN QIAamp® DSP Viral RNA Mini Kit or QIAamp® Viral RNA Mini Kit
- Recommendations: Utilize 140 µL of sample and 560 µL of Buffer AVL with carrier RNA and elute with 100 µL of elution buffer.

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources. Inclusion does not imply endorsement by the Centers for Disease Control and Prevention.

Assay Setup for Applied Biosystems™ 7500 Fast Dx

Master Mix Preparation / Plate Setup

1. In the assay preparation area, label a sterile, nuclease-free, 1.5 mL tube or sterile, nuclease-free 5 mL tube for the Flu SC2 Multiplex Assay reaction master mix.
2. Determine the number of reactions (N) to be prepared per assay.
3. Calculate the amount of each reagent to be added to the tube for each master mix by multiplying the number of reactions (samples plus controls) per marker by the volume of reagent indicated in **Table 5**.

NOTE: It is necessary to make excess reaction master mix to allow for pipetting error.

Example: If number of samples (n) including controls ≤ 48, then $N = n + 2$

If number of samples (n) including controls > 48, then $N = n + 4$

Table 5. Steps and Calculations for Master Mix Preparation Using TaqPath™ 1-Step Multiplex Master Mix (No ROX) or Quantabio UltraPlex™ 1-Step ToughMix®

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Molecular-Grade Nuclease-free Water	$N \times 7.75 \mu\text{L}$
2	FluSC2 Combined Primer Mix	$N \times 3 \mu\text{L}$
3	FluSC2 Combined Probe Mix	$N \times 3 \mu\text{L}$
4	TaqPath™ 1-Step Multiplex Master Mix (No ROX) OR Quantabio UltraPlex™ 1-Step ToughMix® (4X)	$N \times 6.25 \mu\text{L}$
	Total Volume	$N \times 20.0 \mu\text{L}$

4. Dispense reagents into labeled 1.5 mL or 5 mL tube. After addition of the reagents, mix reaction mixtures by pipetting repeatedly 5 times. **Do not vortex.**
5. Briefly centrifuge for 15 seconds at 2000 X g to collect contents at the bottom of the tube, and then place the tube in a cold rack.
6. Set up reaction strip tubes or plates in a 96-well cooler rack.
7. Dispense 20 μL of prepared master mix into all sample and control wells.
8. Prior to moving to the nucleic acid handling area, prepare the NTC reactions for well A12 in the assay preparation area.
9. Pipette 5 μL of molecular-grade nuclease-free water into the NTC sample well A12. Securely cap NTC well A12 but leave the rest of the wells of column 12 uncapped before proceeding.
10. Cover the entire reaction plate and move the reaction plate to the specimen nucleic acid handling area.

Sample Addition

1. Gently mix nucleic acid sample tube(s)/wells and centrifuge to collect contents.
2. After centrifugation, place extracted nucleic acid samples in the cold rack.
3. Carefully pipette 5.0 μL of sample into its designated well (i.e. Sample "S1" into well A1). Samples should be added consecutively down each column, as illustrated in **Figure 2**. Keep other sample wells covered

during additions. Change tips after each addition. Samples should not be pipetted into well A12 (NTC control), B12 (HSC control) or H12 (Combined FluSC2PC well).

4. Securely cap each column once all 8 samples have been added to prevent cross contamination and to ensure sample tracking.
5. Change gloves often and when necessary to avoid contamination.
6. Repeat steps #3 and #4 for the remaining samples.
7. Add 5 μ L of Human Specimen Control (HSC) extracted sample to the HSC well B12 (**Figure 2**, column 12), If more HSC wells are needed due to multiple extractions batches add HSC to C12-D12 as needed. Securely cap well B12, and any well containing sample for wells C12 – G12 after addition before proceeding. Leave well H12 uncapped.
8. Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

Assay Positive Control Addition

1. Pipette 5 μ L of Combined FluSC2PC to the positive template control sample well H12 (**Figure 2**). If more FluSC2PC wells are needed due to multiple extractions batches add additional FluSC2PC to F12-G12 as needed. Securely cap wells after addition of the Combined FluSC2PC.

NOTE: *If using 8-tube strips, add 8 samples, cap, and label the TAB of each strip to indicate sample position.*

DO NOT LABEL THE TOPS OF THE REACTION TUBES!

2. Briefly centrifuge reaction tube strips for 10-15 seconds. After centrifugation return to cold rack.

NOTE: *If using 96-well plates, centrifuge plates for 30 seconds at 500 x g, 4°C.*

Figure 2 - Flu SC2 Multiplex Assay: Example of Sample and Control Set-up

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	NTC
B	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	HSC
C	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	S89 or HSC
D	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	S90 or HSC
E	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85	S91
F	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86	S92 or PC
G	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87	S93 or PC
H	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88	PC

Running a Test on the Applied Biosystems™ 7500 Fast Dx

If the most current Flu SC2 Multiplex Assay template does not already exist on your instrument, please proceed to Appendix A: Instrument Setup for Applied Biosystems™ 7500 Fast Dx Using Optimal Filter Calibrations or Appendix B: Instrument Setup for Applied Biosystems™ 7500 Fast Dx If Optimal Filter Calibrations Are Not Available for instructions to create a template, then return to this section.


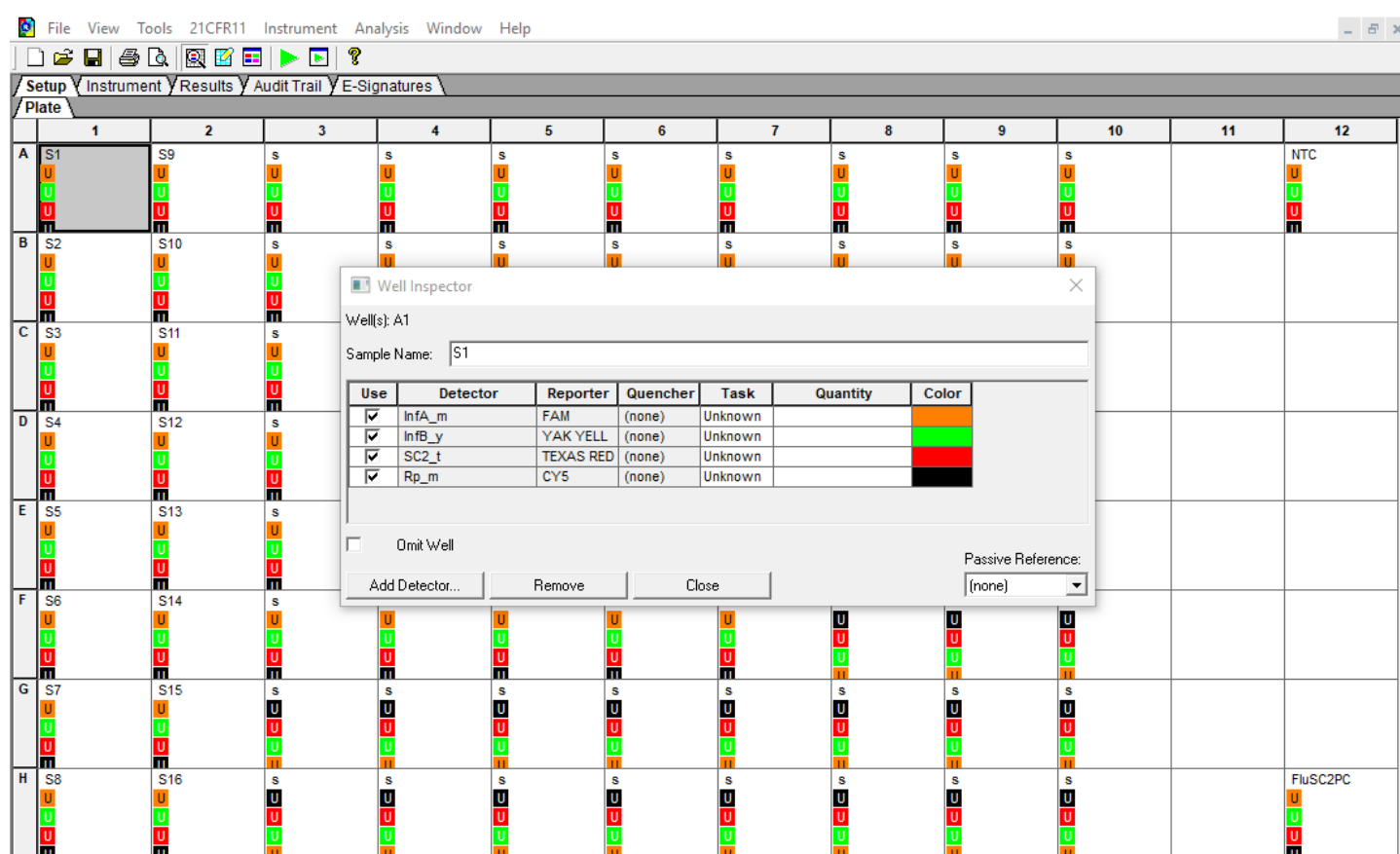
1. Turn on the 7500 Fast Dx Real-Time PCR Instrument and computer.
2. Launch the 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
3. A new window should appear, select **Open Existing Document** from the menu.
4. Navigate to select the ABI Run Template folder from the desktop.
5. Double click on the appropriate template file (**Flu SC2 Multiplex Assay TaqPath™** or **Flu SC2 Multiplex Assay UltraPlex™**).
6. There will be a brief pause allowing the 7500 Fast Dx Real-Time PCR Instrument to initialize. This initialization is followed by a clicking noise. **Note: The machine must be turned on for initialization.**
7. After the instrument initializes, a plate map will appear (**Figure 3**). The detectors and controls should already be labeled as they were assigned in the original template.
8. Click the **Well Inspector** icon  from the top menu.
9. Highlight specimen wells of interest on the plate map.
10. Type sample identifiers to **Sample Name** box in the **Well Inspector** window (**Figure 3**).
11. Select all four detectors (Targets) as shown in Figure 3.

Figure 3 - Selecting Targets



12. Repeat steps 9-11 until all sample identifiers are added to the plate setup.
13. Once all specimen and control identifiers are added, click the Close button on the Well Inspector window to return to the Plate set up tab.
14. Click the Instrument tab at the upper left corner.
15. The reaction conditions, volumes, and type of 7500 reaction should already be loaded (Figure 4a – Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX) and 4b - Quantabio UltraPlex™ 1-Step ToughMix® (4X)).

Figure 4a - Instrument Settings for use with Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX)

The screenshot displays the 'Instrument' tab of the Applied Biosystems 7500 software. The interface is divided into several sections:

- Instrument Control:** Contains buttons for 'Start', 'Stop', 'Disconnect', and 'Extend...'. It also shows 'Estimated Time Remaining (hh:mm):' and 'Status:' fields.
- Temperature:** Fields for 'Sample:', 'Cover:', 'Heat Sink:', and 'Block:'.
- Cycle:** Fields for 'Stage:', 'Rep:', 'Time (mm:ss):', and 'State:'.
- Thermal Cycler Protocol:** This section is active and shows a thermal profile graph. It includes tabs for 'Thermal Profile', 'Auto Increment', and 'Ramp Rate'. The graph shows three stages:
 - Stage 1:** 1 rep, 25.0°C, 2:00.
 - Stage 2:** 1 rep, 50.0°C, 15:00.
 - Stage 3:** 45 reps, 95.0°C, 2:00; 95.0°C, 0:15; 55.0°C, 0:30.
- Settings:** Includes 'Sample Volume (µL):' set to 25, 'Run Mode' set to 'Standard 7500', and 'Data Collection' set to 'Stage 3, Step 2 (55.0 @ 0:30)'.

Figure 4b - Instrument Settings for use with Quantabio UltraPlex™ 1-Step ToughMix® (4X)

Setup Instrument Results Audit Trail E-Signatures

Instrument Control

Start Stop Disconnect Extend...

Estimated Time Remaining (hh:mm):

Status:

Temperature

Sample: Heat Sink:
Cover: Block:

Cycle

Stage: Rep:
Time (mm:ss): Step:
State:

Thermal Cycler Protocol

Thermal Profile Auto Increment Ramp Rate

Stage 1 Stage 2 Stage 3

Reps: 1 Reps: 1 Reps: 45

95.0 95.0
3:00 0:15
50.0 55.0
15:00 0:30
25.0
2:00

Add Cycle Add Hold Add Step Add Dissociation Stage Delete Help

Settings

Sample Volume (µL): 25

Run Mode: Standard 7500

Data Collection: Stage 3, Step 2 (55.0 @ 0:30)

16. Ensure settings are correct (refer to Appendix A: Instrument Setup for Applied Biosystems™ 7500 Fast Dx Using Optimal Filter Calibrations or Appendix B: Instrument Setup for Applied Biosystems™ 7500 Fast Dx If Optimal Filter Calibrations Are Not Available).
17. Before proceeding, the run file must be saved; from the main menu, select **File**, then **Save As**. Save in appropriate run folder designation.
18. Load the plate into the plate holder in the instrument. Ensure that the plate is properly aligned in the holder.
19. Once the run file is saved, click the **Start** button. Note: The run should take approximately 1hr and 30 minutes to complete

Results Analysis

1. After the run has completed, select the **Results** tab at the upper left corner of the software.
2. Select the **Amplification Plot** tab to view the raw data (**Figure 6**). If the data is in linear view, please update to log view by right clicking within the Amplification plot. This will bring up a Graph Settings control box (**Figure 5**). For Y-axis, if linear is currently selected, to switch to log view, select log and then select Apply.

Figure 5 - Graph Settings Control Box

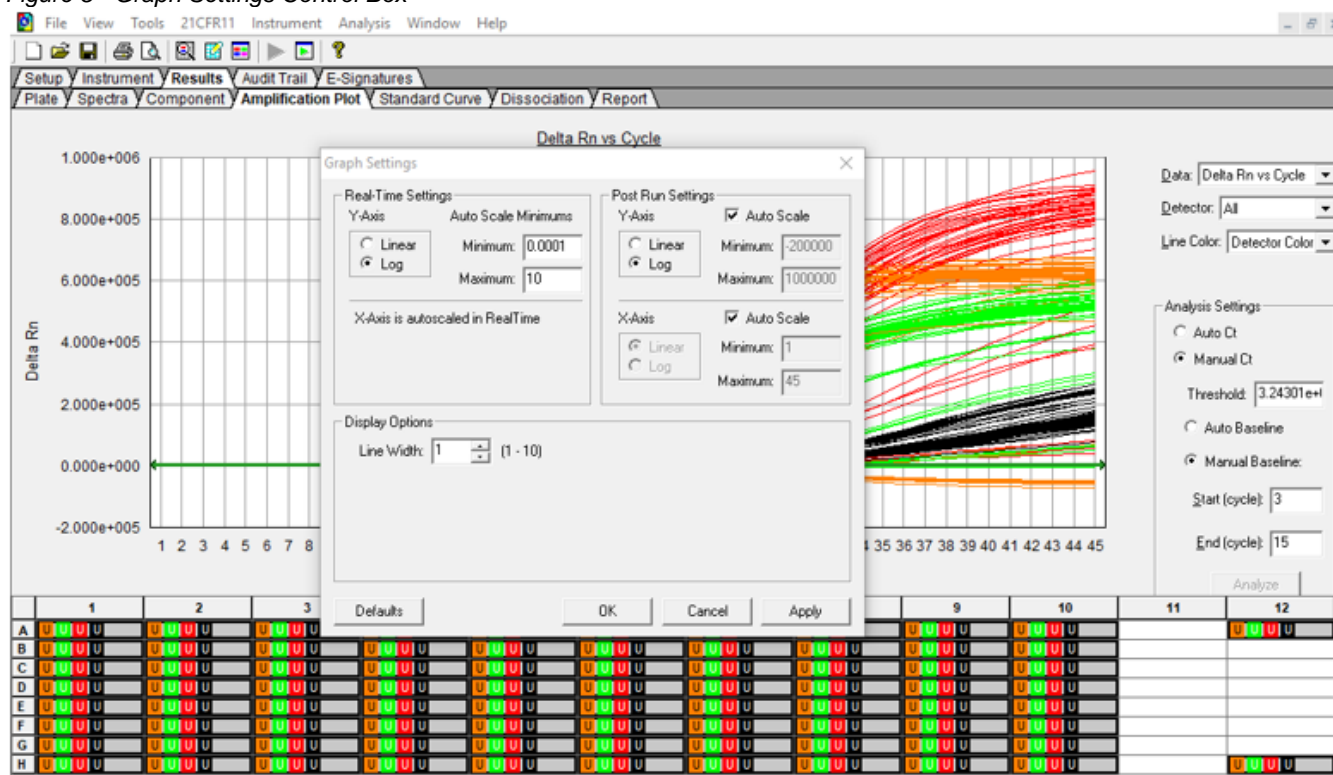
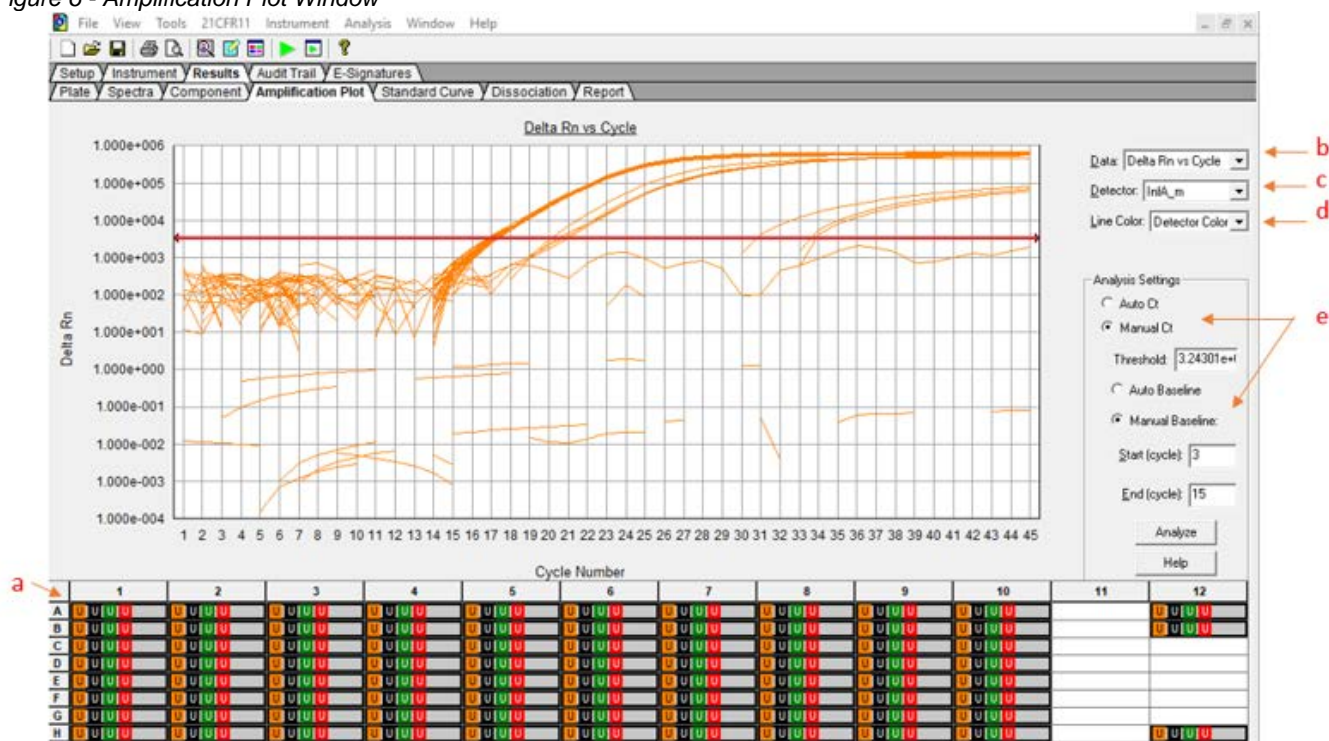
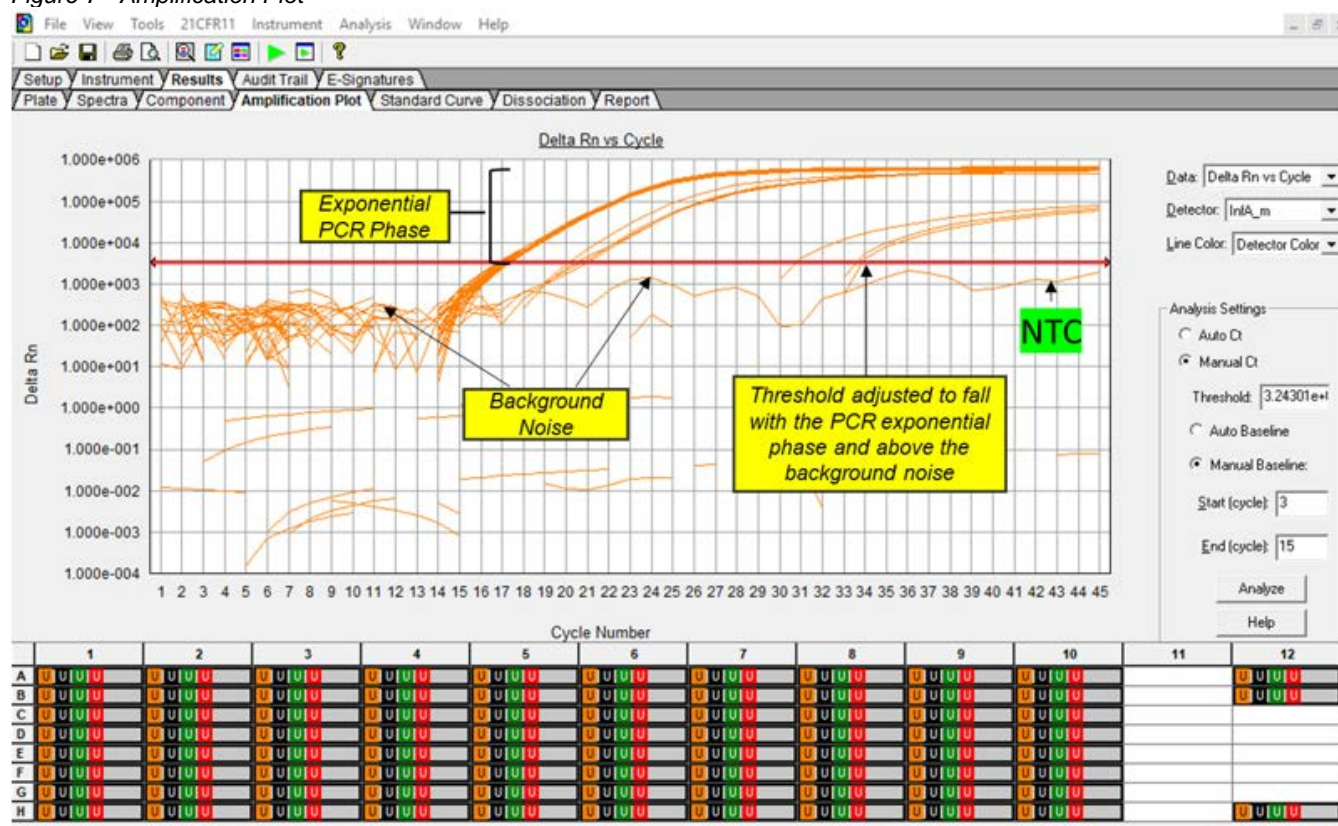


Figure 6 - Amplification Plot Window



- Start by highlighting all the samples from the run; to do this, click on the upper left box (a) of the sample wells (Figure 6). All of the amplification curves will appear on the graph.
- On the right side of the window (b), the **Data** drop down selection should be set to **Delta Rn vs. Cycle**.
- Select detector (e.g., **InfA_m**) from (c), the **Detector** drop down menu, using the downward arrow.
 - Please note that each multiplex target (detector) should be analyzed individually to reflect different performance profiles of each primer and probe set.
- In the **Line Color** drop down (d), the **Detector Color** should be selected.
- Under **Analysis Settings** select **Manual Ct** (e).
 - Do not change the **Manual Baseline** default numbers.
- Click and drag the red threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal (Figure 7).

Figure 7 - Amplification Plot



- Click the **Analyze** button in the lower right corner of the window. The red threshold line will turn to green, indicating the data has been analyzed.
- Repeat steps 5-9 to analyze results generated for each set target of FluSC2 multiplex markers (e.g., SC2_t, InfB_y, Rp_m).
- Save analysis file by selecting **File** then **Save As** from the main menu.
- After completing analysis for each of the markers, select the **Report** tab above the graph to display the Ct values (**Figure 8**). To filter report by sample name in ascending or descending order, simply click on **Sample Name** in the table.

Figure 8 – Report

File View Tools 21CFR11 Instrument Analysis Window Help

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Interpretation of Results and Reporting

Extraction and Positive Control Results and Interpretation

No Template Control (NTC)

The NTC consists of using molecular-grade nuclease-free water in the rRT-PCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence growth curves that cross the threshold line. If any of the NTC reactions exhibit a growth curve that crosses the cycle threshold, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

Combined FluSC2PC

The Combined FluSC2PC consists of two components, SIPC (Part # MR-541) and SC2PC (Part # MR-667), that need to be combined as directed in the Reagent and Controls Preparation section to make the final control sample for the reaction. Combined FluSC2PC consists of RNA from inactivated influenza A virus, influenza B virus, a synthetic SARS-CoV-2 RNA, and nucleic acid extracted from A549 human lung epithelial cells. The Combined FluSC2PC is prepared alongside each batch of clinical specimens and should be positive for all targets in the Flu SC2 Multiplex Assay InfA, InfB, SC2, and RP.

- If the Combined FluSC2PC is positive for SC2, but generates negative results for InfA, InfB and/or RP, this indicates a possible problem during specimen extraction or a problem with the RT-PCR reaction(s) for InfA, InfB and/or RP. Repeat extraction and testing of any clinical specimens that had been extracted alongside the failed Combined SC2PC.
- If the Combined FluSC2PC is positive for RP, but negative for InfA, InfB and SC2, this could indicate a possible problem with the RT-PCR reaction, or the control may have been contaminated with RNase. Repeat the rRT-PCR run with fresh enzyme. If the Combined FluSC2PC is still only positive for RP, repeat extraction and testing of any clinical specimens that had been extracted alongside the failed Combined SC2PC.

Human Specimen Control (HSC) (Extraction Control)

HSC is used as an RNA extraction procedural control to demonstrate successful recovery of nucleic acid, extraction reagent integrity and as a control for cross-contamination during the extraction process. The HSC control consists of non-infectious cultured human cell (A549) material. Purified nucleic acid from the HSC should yield a positive result with the RP primer and probe set and negative results for influenza A, influenza B, and SARS-CoV-2 agent-specific markers. If the HSC generates a negative result for RP, this indicates a potential problem with the extraction process. If HSC generates positive results for any of the viral markers (InfA, InfB or SC2), this may be an indication of possible cross-contamination during extraction or RT-PCR reaction set-up. If the NTC is also positive it strongly suggests the cross-contamination occurred in the reaction setup, and if NTC is negative then cross-contamination likely occurred during extraction. If the HSC fails to give the expected results, invalidate the run, repeat extraction and RT-PCR testing for all specimens that had been extracted alongside the failed HSC.

Table 6. Extraction and Positive Control Results and Interpretation

Control Type	Control name	Used to Evaluate	InfA	InfB	SC2	RP	Expected Ct
Positive	Combined FluSC2PC	Extraction and RT-PCR reagent integrity including primers and probes for all targets	+	+	+	+	< 40.00 Ct for InfA, InfB, Sc2 <35.00 Ct for RP
Extraction	HSC	Extraction reagent integrity, contamination	-	-	-	+	< 35.00 Ct
Negative	NTC	Contamination of reagents or environmental contamination	-	-	-	-	None detected (≥40.00 Ct for InfA, InfB, SC2 and ≥35.00 Ct for RP)

Controls should be evaluated before any patient data is reported or recorded. If the controls do not react as described above, the run should be considered invalid and a retest is needed

Flu SC2 Multiplex Assay Results Interpretation Guide

If a laboratory obtains unexpected results for assay controls or if inconclusive or invalid results are obtained and cannot be resolved through the recommended retesting, please contact CDC Technical Support for consultation and possible specimen referral. See pages 10 and 42 for referral and contact information.

- Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the patient specimen results cannot be interpreted.
- The Flu SC2 Multiplex Assay should be evaluated as 4 separate targets (an example of potential results is shown in Table 7). Thus, the cycle threshold line should be set for each target in the multiplex (InfA, InfB, SC2, and RP) above the background signal and within the exponential phase of the fluorescence curves, as described in the Instructions for Use.

RNase P (Internal Specimen Control)

The RNase P (RP) in the Flu SC2 Multiplex Assay serves as an internal control for the assay that is used in conjunction with the data from other targets for interpretation of an individual specimen.

- RP should be positive (<35.00 Ct) for all clinical specimens in the absence of a signal for one of the viral targets. If RP is negative in the presence of a positive result for one of the viral targets, the viral target result should be considered valid. However, if all viral targets generate negative results and RP is also negative, the test is considered invalid. Failure to detect RP in clinical specimens could indicate:
 - Insufficient nucleic acid extraction from clinical samples
 - Poor specimen quality or loss of specimen integrity
 - Improper assay execution
 - Reagent or equipment malfunction
- If all viral targets are negative (≥40.00 Ct) and RP generates a Ct ≥35.00 for a specimen in the Flu SC2 Multiplex Assay, the result should be considered invalid for the specimen. Repeat testing of specimen nucleic acid and/or re-extract and repeat RT-PCR. If all targets are negative after retest, report the specimen as invalid. Collection of a new specimen and subsequent testing should be considered.

Positive result

When all controls exhibit the expected results and one or more of the viral targets (InfA, InfB, and/or SC2) DOES cross the threshold line BEFORE 40.00 Ct then the specimen is considered positive for those virus(es). Multiple viruses may be detected in a single specimen.

Negative result

When all controls exhibit the expected results, a specimen is considered negative if all viral targets' cycle threshold curves (InfA, InfB and SC2) DO NOT cross the threshold line BEFORE 40.00 Ct (< 40.00 cycles) AND the RP internal control DOES cross the threshold line at ≤ 35.00 Ct.

Invalid result

When all controls exhibit the expected results, and no curves cross the threshold line BEFORE 40.00 Ct (< 40.00 cycles) for InfA, InfB, SC2, and BEFORE 35.00 Ct (<35.00 cycles) for RP, then the result is invalid. Repeat testing of specimen nucleic acid and/or re-extract and repeat RT-PCR. If the specimen remains invalid upon retest, collection of a new specimen and subsequent testing should be considered.

Table 7. Flu SC2 Multiplex Assay Interpretation

InfA Result	InfB Result	SC2 Result	RP Result	Interpretation	Report	Actions
+	-	-	+ or -	influenza A RNA detected	Positive for influenza A	Report results to sender
-	+	-	+ or -	influenza B RNA detected	Positive for influenza B	Report results to sender
-	-	+	+ or -	SC2 RNA detected	Positive for COVID-19	Report results to sender
-	+	+	+ or -	influenza B and SC2 RNA detected	Positive for influenza B and COVID-19	Report results to sender
+	+	-	+ or -	influenza A and influenza B RNA detected	Positive for influenza A and Influenza B	Report results to sender
+	-	+	+ or -	influenza A and SC2 RNA detected	Positive for Influenza A and COVID-19	Report results to sender
+	+	+	+ or -	influenza A, influenza B and SC2 RNA detected	Positive for influenza A, influenza B and COVID-19	Report results to sender
-	-	-	+ (<35 Ct)	Not Detected	Negative	Report results to sender. Consider testing for other respiratory viruses
-	-	-	- (≥ 35 Ct)	Invalid result	Invalid	Consider repeat of extraction and/or rRT-PCR or collecting a new specimen

^aLaboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

^bOptimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest that 2019-nCoV infection is possible, and diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If 2019-nCoV infection is still suspected, re-testing should be considered in consultation with public health authorities.

Standards-Based Electronic Laboratory Reporting for Influenza

Background

This section contains the recommendations for uniform coding and vocabulary for the CDC Flu SC2 Multiplex Assay.

The following information is provided to assist the performing laboratory in complying with new federal guidelines for the meaningful use of electronic health information systems. The implementation of adopted standards should be harmonized across all performing laboratories to ensure semantic interoperability to better support electronic data exchange.

The CDC, developer of this assay through collaboration, has established Standard English terminology for the test name and test results with the testing community and expert knowledge of the processes involved. It is recognized that this terminology will differ in countries outside the United States. However, through the use of national and international agreements, it is possible to establish a universal set of codes and terms to accurately characterize laboratory observations. Recommendations in this package insert apply to the reporting of results of this assay only within the United States.

Process for achieving uniformity in laboratory test results

The laboratory performing the assay may utilize a Laboratory Information Management Systems (LIMS) with connections to a hospital or medical system Electronic Health Record (EHR). The coding systems include LOINC - Logical Observation Identifiers Names and Codes (LOINC® - <http://www.loinc.org>) and SNOMED CT – Systematic Nomenclature of Medicine--Clinical Terms (<http://www.ihtsdo.org/>). These coding systems have specific capabilities that are essential for achieving uniformity. The test request and results are to be incorporated into a standard Health Level 7 (HL7) electronic format for laboratory test messaging. More information about HL7 can be found at <http://www.hl7.org>.

LOINC provides for a common understanding of the medical procedure or process related to the specific assay, in this case, the process of detecting the presence of SARS-CoV-2 and/or influenza viruses and the potential sub-typing of the detected influenza virus. The LOINC codes specified here describe the important information about the methodology employed by the assay including recovery and amplification of one or more RNA targets. Multiple LOINC codes are utilized to convey that the assay is composed of multiple components, i.e. it is a panel or a battery of subtests. In the case of the CDC Flu SC2 Multiplex Assay, the LOINC also provides a means for conveying that an interpretive test summary is appropriate.

SNOMED CT codes provide for unambiguous representation of the test results and allow the application of specific concepts such as “detected” or “positive” or the identification of detected organism names. Though not further defined in this document, SNOMED CT can also be used to provide a description of the type and source/ location of the specimen being tested, or for conveying information about failures of the test procedure or the lack of adequate specimen.

Specific Recommendations for Standards-Based Electronic Data Exchange for Influenza

Laboratories can find more information regarding implementation of HL7 messaging for CDC Flu rRT-PCR Dx Panel, including applicable LOINC test codes and SNOMED result codes at <http://www.cdc.gov/flu/professionals/diagnosis/rtpcr-test-kits.htm>

Quality Control

- Quality control requirements must be performed in conformance with local, state, and federal regulations or accreditation requirements and the user's laboratory standard quality control procedures. For further guidance on appropriate quality control practices, refer to 42 CFR 493.1200.
- Quality control procedures are intended to monitor reagent and assay performance.
- Test all positive controls prior to running diagnostic samples and with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice (cGLP) recommends including a positive extraction control in each nucleic acid isolation batch.
- HSC extraction control must proceed through nucleic acid isolation with each batch of specimens to be tested.
- Prepared SIPC must proceed through nucleic acid isolation with each batch of clinical specimens. The resulting nucleic acid must be used to create the Combined FluSC2PC to be run alongside the specimens in the RT-PCR.
- Always include a negative control (NTC), and the appropriate positive control (i.e., Combined FluSC2 PC) in each amplification and detection run.

Limitations

- This assay is for *in vitro* diagnostic use under FDA Emergency Use Authorization only.
- All users, analysts, and any person reporting diagnostic results should be trained to perform this procedure by a competent instructor. They should demonstrate their ability to perform the test and interpret the results prior to performing the assay independently.
- Performance of the CDC Flu SC2 Multiplex Assay has only been established in upper and lower respiratory specimens.
- Negative results do not preclude influenza or SARS-CoV-2 virus infection and should not be used as the sole basis for treatment or other patient management decisions.
- A false negative result may occur if a specimen is improperly collected, transported, or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- Performance evaluations have shown decreased sensitivity of the influenza A target when a high titer of SARS-CoV2 or influenza B is also present in the sample. If a negative influenza A result is obtained with this assay and co-infection is suspected, additional testing of the sample with a cleared influenza A diagnostic is recommended.
- In the case of influenza A and B viruses, children tend to shed virus more abundantly and for longer periods of time than adults. Therefore, testing specimens from adults for the presence of RNA from influenza viruses will have lower sensitivity than testing specimens from children.
- Positive and negative predictive values are highly dependent on prevalence. False negative test results are more likely during peak activity when prevalence of disease is high. False positive test results are more likely during periods of low influenza or SARS-CoV-2 activity when prevalence is moderate to low.
- The performance of the assay has not been established in individuals who received nasally administered influenza vaccine. Individuals who received nasally administered influenza A vaccine may have positive influenza A test results for up to three days after vaccination.
<http://www.cdc.gov/mmwr/preview/mmwrhtml/rr57e717a1.htm>
- Do not use any reagent past the expiration date, as this may affect performance of the assay.
- Optimum specimen types and timing for peak viral levels during infections caused by a novel influenza A or SARS-CoV-2 virus have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the viruses.

- If SARS-CoV-2 or an influenza A or B virus mutates in the rRT-PCR target region, the specific novel virus may not be detected or may be detected less predictably. Inhibitors or other types of interference may produce a false negative result. An interference study evaluating the effect of common cold medications was not performed.
- The potential for the epidemiology and pathology of disease caused by a specific novel influenza A virus or SARS-CoV-2 virus to affect test performance is not fully known. For example, clinicians and laboratories may not know the optimum types of specimens to collect, and when during the course of infection these specimens are most likely to contain levels of virus that can be readily detected.
- Detection of viral RNA may not indicate the presence of infectious virus or that influenza or SARS-CoV-2 viruses are the causative agent for clinical symptoms.
- The performance of this test has not been established for monitoring treatment of influenza A, influenza B, or SARS-CoV-2 infection.
- The performance of this assay has not been established for screening of blood or blood products for the presence of influenza A, influenza B or SARS-CoV-2.
- This assay cannot rule out diseases caused by other bacterial or viral pathogens.

Conditions of Authorization for the Laboratory

The CDC Flu SC2 Multiplex Assay Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and other authorized labeling are available on the FDA website: <https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas>.

However, to assist clinical laboratories using the CDC Flu SC2 Multiplex Assay (“your product” in the conditions below), the relevant Conditions of Authorization are listed below:

- A. Authorized laboratories¹ using your product will include with test result reports, all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- B. Authorized laboratories using your product will use your product as outlined in the authorized labeling. Deviations from the authorized procedures, including the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to use your product are not authorized under this EUA.
- C. Authorized laboratories that receive your product will notify the relevant public health authorities of their intent to run your product prior to initiating testing.
- D. Authorized laboratories using your product will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- E. Authorized laboratories will collect information on the performance of your product and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and CDC (via email: CDCSARS2FluAB@cdc.gov) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of your product of which they become aware.
- F. Authorized laboratories will report adverse events, including problems with test performance or results, to MedWatch by submitting the online FDA Form 3500 (<https://www.accessdata.fda.gov/scripts/medwatch/index.cfm?action=reporting.home>) or by calling 1-800-FDA-1088.
- G. All laboratory personnel using your product must be appropriately trained in PCR techniques and use

appropriate laboratory and personal protective equipment when handling this kit and use your product in accordance with the authorized labeling.

- H. CDC, authorized distributors, and authorized laboratories using your product will ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to FDA for inspection upon request.

¹ The letter of authorization refers to, “Laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests” as “authorized laboratories.”

Performance Characteristics

Analytical Sensitivity:

Limit of Detection (LoD):

Analytical sensitivity of the Flu SC2 Multiplex Assay was determined in limit of detection studies using live viral stocks of an influenza A virus, an influenza B virus and a SARS-COV-2 virus (Table 8) to determine the lowest concentration of each virus at which 95% of all replicates are positive. Serial, 5-fold dilutions of each virus were prepared with a diluent consisting of a suspension of human A549 cells and viral transport medium (VTM). Specific titers for each dilution of these viruses in 50% infectious doses per ml are listed in Tables 9-11. Range-finding was done to determine an estimated LoD. Triplicate samples of each serial dilution were tested using the Flu SC2 Multiplex Assay by extracting 120 µL of each virus dilution, eluting the resulting nucleic acid in 120 µL elution buffer, and 5 µL was used as input for the reactions. The estimated LoD was defined as the lowest concentration at which each target (InfA, InfB, and SC2) demonstrated 100% positivity (3 out of 3 replicates). Extraction was performed using the QIAGEN EZ1 DSP Virus Kit on the QIAGEN EZ1 Advanced XL. Subsequent testing was done using both the TaqPath™ Multiplex Master Mix (No ROX) and the Quantabio UltraPlex™ 1-Step ToughMix® (4X). Testing was conducted side by side with either the FDA-authorized CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel using the ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix or the FDA-cleared CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza A/B Typing Kit using the Invitrogen™ SuperScript™ III Platinum™ One-Step qRT-PCR Kit to demonstrate the relative detection of the Flu SC2 Multiplex Assay compared to these assays. All assay controls performed as expected, and results of range-finding across all three targets are presented in Tables 9-11 below.

Table 8. Virus Selection for LoD Studies

Virus Lineage	Virus	Titer of Stock (TCID₅₀/mL)	Virus Species
A(H1N1)pdm09	A/Illinois/20/2018	10 ^{7.8}	Influenza A
B-Victoria	B/Colorado/06/2017	10 ^{8.3}	Influenza B
SARS-CoV-2	2019-nCoV/USA-WA1/2020	10 ^{6.5}	SARS-COV-2

Table 9. Range Finding LoD (InfA) – Flu SC2 Multiplex vs Flu IVD

ID ₅₀ Titer/mL	ID ₅₀ Titer/rxn	Flu SC2 Multiplex (Ct)						Flu IVD (Ct)		
		InfA (TaqPath)			InfA (Ultrplex)			InfA_V2		
10 ^{4.8}	3.15E+02	21.05	20.66	20.89	21.45	21.55	21.55	22.18	22.20	22.11
2 X 10 ^{3.8}	6.31E+01	23.50	23.59	23.17	24.25	24.26	24.31	24.97	24.97	24.66
4 X 10 ^{2.8}	1.26E+01	26.57	26.5	26.81	27.41	27.60	27.72	27.71	27.49	27.50
8 X 10 ^{1.8}	2.52E+00	29.90	30.20	30.15	30.45	30.57	30.82	30.50	29.97	29.74
1.6 X 10 ^{1.8}	5.05E-01	35.58	35.24	36.17	37.28	36.32	33.76	32.32	32.43	33.63
3.2X10 ^{0.8}	1.01E-01	42.23	37.28	0.00	0.00	0.00	0.00	36.01	34.61	34.96
6.4X10 ^{-0.2}	2.02E-02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	36.24	36.34
1.28X10 ^{-0.2}	4.04E-03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 10. Range Finding LoD (InfB) – Flu SC2 Multiplex vs. Flu IVD

ID ₅₀ Titer/mL	ID ₅₀ Titer/rxn	Flu SC2 Multiplex (Ct)						Flu IVD (Ct)		
		InfB (TaqPath)			InfB (Ultrplex)			InfB		
10 ^{4.3}	9.98E+01	24.47	24.44	24.31	24.03	23.69	24.12	25.8	25.68	25.93
2 X 10 ^{3.3}	2.00E+01	27.57	27.45	27.71	27.82	27.16	27.35	28.84	28.98	29.30
4 X 10 ^{2.3}	3.99E+00	31.09	30.17	30.47	30.89	30.08	29.31	32.10	32.10	32.38
8 X 10 ^{1.3}	7.98E-01	34.38	33.49	34.43	34.09	33.61	32.98	35.19	35.49	35.99
1.6 X 10 ^{1.3}	1.60E-01	39.75	0.00	0.00	0.00	41.32	42.59	0.00	0.00	0.00
3.2X10 ^{0.3}	3.19E-02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6.4X10 ^{-0.7}	6.38E-03	0.00	0.00	0.00	38.16	0.00	0.00	0.00	0.00	0.00
1.28X10 ^{-0.7}	1.28E-03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 11. Range Finding LoD (SC2) – Flu SC2 Multiplex vs. 2019-nCoV EUA

ID ₅₀ Titer/mL	ID ₅₀ Titer/rxn	Flu SC2 Multiplex (Ct)						2019-nCoV Real-Time RT PCR(Ct)					
		SC2 (TaqPath)			SC2 (Ultrplex)			2019-nCoV N1			2019-nCoV N2		
10 ^{4.5}	1.58E+02	18.34	18.55	18.41	19.17	19.27	19.14	19.33	19.46	19.46	20.11	20.15	20.18
2 X 10 ^{3.5}	3.16E+01	20.72	20.81	20.21	21.27	21.38	21.28	21.33	21.43	21.32	22.29	22.11	22.27
4 X 10 ^{2.5}	6.32E+00	22.98	23.03	22.96	23.77	23.85	23.60	24.03	24.01	23.85	24.83	24.67	25.00
8 X 10 ^{1.5}	1.26E-01	25.41	25.80	25.42	26.18	26.41	26.14	26.48	26.57	26.46	27.56	27.45	27.77
1.6 X 10 ^{1.5}	2.53E-01	28.69	28.87	28.50	29.19	29.28	28.59	30.26	29.77	29.51	31.35	31.17	31.31
3.2X10 ^{0.5}	5.06E-02	31.31	31.42	31.32	32.14	32.64	32.42	32.74	33.17	32.27	34.27	33.96	33.83
6.4X10 ^{-0.5}	1.01E-02	35.14	36.36	34.58	37.86	0.00	0.00	36.10	35.34	35.81	37.56	39.27	37.26
1.28X10 ^{-0.5}	2.02E-03	0.00	0.00	0.00	0.00	0.00	0.00	37.16	0.00	0.00	0.00	0.00	0.00

Limit of Detection Confirmation:

A confirmation of the LoD for the Flu SC2 Multiplex Assay was demonstrated using live viral stocks of an influenza A virus, an influenza B virus and a SARS-CoV-2 virus (Table 8) to determine the lowest concentration of each virus at which 95% of all replicates test positive. Twenty (20) individually extracted samples at and above the estimated LoD were prepared with a diluent consisting of a suspension of human A549 cells and viral transport medium (VTM) and extracted using the QIAGEN EZ1 Advanced XL with the QIAGEN EZ1 DSP Virus Kit. Extracted samples were then tested using the Flu SC2 Multiplex Assay using both the TaqPath™ Multiplex Master Mix (No ROX) and the Quantabio UltraPlex™ 1-Step ToughMix® (4X). Acceptance criteria for LoD were defined as the lowest concentration demonstrating at least 95% positivity (19 out of 20 replicates). LoD results are summarized in Table 12.

Table 12. Flu SC2 Multiplex Assay Limit of Detection Summary

Viral Target	LoD (ID ₅₀ / reaction)					
	TaqPath 1-step Multiplex			Ultraplex 1-step ToughMix		
	InfA	InfB	SC2	InfA	InfB	SC2
Influenza A (InfA)	5.05E-01	---	---	5.05E-01	---	---
Influenza B (InfB)	---	7.98E-01	---	---	7.98E-01	---
SARS-CoV-2 (SC2)	---	---	1.01E-02	---	---	5.06E-02

Inclusivity (analytical sensitivity):***Inclusivity – SARS-CoV-2 In Silico Analysis:***

The inclusivity/exclusivity of each primer and probe oligonucleotide sequence for the SC2 target of the Flu SC2 Multiplex Assay was tested via NCBI BLAST+ against the nr/nt database. The database search parameters were as follows: 1) The nucleotide collection consists of GenBank+EMBL+DDBJ+PDB+RefSeq sequences, but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences and sequences longer than 100Mb; 2) The database is non-redundant. Identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry; 3) Database was updated on 04/25/2020 (N=57791697 sequences analyzed); 4) The search parameters automatically adjust for short input sequences and the expect threshold is 1000; 5) The match and mismatch scores are 1 and -3, respectively; 6) The penalty to create and extend a gap in an alignment is 5 and 2 respectively. In addition, each primer sequence was also tested against the NCBI β Coronaviridae nucleotide database to test for non-specific hits within a targeted search space. The database search parameters were the same as described above with the following differences: 1) The nucleotide collection consists of only Betacoronavirus nucleotide sequence data; 2) The database is non-redundant. Identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry; 3) Database was updated on 04/26/2020 (N= 9186 sequences analyzed NCBI). Hit results were analyzed and assessed for potential non-SARS-CoV2 target matches. Results were categorized as perfect matches to the primer/probe sequences, close matches (within 2 nucleotide edit distance), and distant matches (> 2 nucleotide edit distance). Results confirmed only perfect matches to SARS-CoV-2 and close matches to SARS-CoV-2 ancestors (i.e., no genomes identified with more than 2 nt mismatches).

Inclusivity – Influenza and SARS-CoV-2 Viruses:

The inclusivity of the Flu SC2 Multiplex Assay was evaluated using 13 influenza A and 2 influenza B viruses representing temporal, geographic, and genetic diversity within the subtype and lineage, and one SARS-CoV-2 virus. These were prepared at two concentrations, to test the specificity of the primer and probe sets against the three viral targets in the Flu SC2 Multiplex Assay. Samples were tested in triplicate using the Qiagen EZ1 DSP virus kit on the QIAGEN EZ1 Advanced XL extraction platform and tested using the Applied Biosystems TaqPath™ Multiplex Master Mix (No ROX). The influenza A target (InfA) generated positive results in both concentrations of the influenza A viruses tested. The influenza B target (InfB) generated positive results in both concentrations of the influenza B viruses tested. The SC2 target generated positive results with the SARS-CoV-2 strain at both concentrations tested. All controls performed as expected. Results are presented below in Table 13.

Table 13. Inclusivity and Cross-Reactivity

No.	Lineage	Strain designation	Conc ID ₅₀ /mL	FluSC2 Multiplex Assay (Ct)								
				InfA			InfB			SC2		
1	A(H1N1) pdm09	A/Florida/81/2018	10 ^{2.1}	36.82	0.00	35.22	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{3.1}	27.48	28.16	27.74	0.00	0.00	0.00	0.00	0.00	0.00
2	A(H3N2)	A/Kansas/14/2017	10 ^{2.9}	31.06	30.65	29.88	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{3.9}	26.53	26.90	26.38	0.00	0.00	0.00	0.00	0.00	0.00
3	A(H1N2)v	A/Ohio/35/2017	10 ^{1.9}	30.91	31.25	30.99	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{2.9}	27.03	26.98	26.58	0.00	0.00	0.00	0.00	0.00	0.00
4	A(H2N2)	A/chicken/Pennsylvania/ 298101-4/2004	10 ^{3.5}	33.40	33.20	34.71	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{4.5}	28.93	29.26	29.29	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{5.5}	25.07	25.00	25.01	0.00	0.00	0.00	0.00	0.00	0.00
5	A(H3N2)v	A/Ohio/13/2017	10 ^{1.6}	35.48	35.49	33.98	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{2.6}	28.48	28.45	28.51	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{3.6}	24.59	24.47	24.60	0.00	0.00	0.00	0.00	0.00	0.00
6	A(H3N8)	A/equine/Ohio/01/2003	10 ^{3.4}	31.01	31.55	31.07	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{4.4}	27.07	27.28	27.09	0.00	0.00	0.00	0.00	0.00	0.00
7	A(H3N2)	A/canine/Florida/43/2004	10 ^{3.1}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{4.1}	34.10	35.45	36.87	0.00	0.00	0.00	0.00	0.00	0.00
8	A(H5N2)	A/Northern pintail/ Washington/40964/2014	10 ^{3.4}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{4.4}	36.89	36.45	34.43	0.00	0.00	0.00	0.00	0.00	0.00
9	A(H5N8)	A/gyrfalcon/Washington/ 41088-6/2014	10 ^{3.75}	38.48	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{4.75}	29.60	29.38	29.90	0.00	0.00	0.00	0.00	0.00	0.00
10	A(H6N2)	A/chicken/California/32213- 1/2000	10 ^{3.2}	34.19	32.52	32.49	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{4.2}	28.22	27.96	28.23	0.00	0.00	0.00	0.00	0.00	0.00
11	A(H7N2)	A/feline/New York/ 16-040082-1/2016	10 ^{5.2}	28.16	28.30	28.46	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{6.2}	24.61	24.86	24.80	0.00	0.00	0.00	0.00	0.00	0.00
12	A(H7N9)	A/Taiwan/1/2017	10 ^{3.5}	32.20	33.11	32.53	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{4.5}	28.18	28.04	28.05	0.00	0.00	0.00	0.00	0.00	0.00
13	A(H9N2)	A/Bangladesh/0994/2011	10 ^{4.5}	34.25	35.32	36.90	0.00	0.00	0.00	0.00	0.00	0.00
			10 ^{5.5}	29.25	29.37	29.19	0.00	0.00	0.00	0.00	0.00	0.00
14	B-VIC	B/Maryland/15/2016	10 ^{2.5}	0.00	0.00	0.00	30.82	31.07	31.20	0.00	0.00	0.00
			10 ^{3.5}	0.00	0.00	0.00	27.03	27.25	27.18	0.00	0.00	0.00
15	B-YAM	B/Phuket/3073/2013	10 ^{2.9}	0.00	0.00	0.00	31.87	31.77	32.18	0.00	0.00	0.00
			10 ^{3.9}	0.00	0.00	0.00	28.05	28.25	28.45	0.00	0.00	0.00
16	SCoV2	2019-nCoV/USA- WA1/2020	10 ^{0.5}	0.00	0.00	0.00	0.00	0.00	0.00	33.11	34.15	34.88
			10 ^{1.5}	0.00	0.00	0.00	0.00	0.00	0.00	29.25	29.30	29.44

Cross-reactivity (non-targeted influenza types and coronavirus types):

Cross-reactivity of each primer probe set to viruses targeted by another component of the Flu SC2 Multiplex Assay was also evaluated as part of the inclusivity study (above) and during testing of the panel of viruses and viral transcript listed in Table 14 (below). The SC2 primer and probe set generated negative results for all influenza A and B viruses tested. InfA generated negative results with all influenza B viruses and SARS-CoV-2. InfB generated negative results for all influenza A and SARS-CoV-2. All assay controls performed as expected, and results are shown in Table 13 (above), Table 14 (below).

Table 14. Cross-Reactivity Evaluation – (non-targeted influenza A/B)

No.	Origin	Subtype	Strains Designation	Conc ID ₅₀ / mL	Flu SC2 Multiplex Assay (Ct)								
					InfA			InfB			SC2		
1	Human	A(H1N1) pdm09	A/Florida/81/2018	10 ^{8.1}	13.97	14.00	14.02	0.00	0.00	0.00	0.00	0.00	0.00
2	Human	A(H3N2)	A/Kansas/14/2017	10 ^{8.9}	13.62	13.66	13.68	0.00	0.00	0.00	0.00	0.00	0.00
3	Swine	A(H1N2) v	A/Ohio/35/2017	10 ^{6.9}	14.71	14.90	14.84	0.00	0.00	0.00	0.00	0.00	0.00
4	Avian	A(H2N2)	A/chicken/Pennsylvania/ 298101-4/2004	10 ^{9.5}	15.60	15.66	15.74	0.00	0.00	0.00	0.00	0.00	0.00
5	Swine	A(H3N2) v	A/Ohio/13/2017	10 ^{6.6}	20.85	20.96	20.86	0.00	0.00	0.00	0.00	0.00	0.00
6	Equine	A(H3N8)	A/equine/Ohio/01/2003	10 ^{8.4}	16.50	16.70	16.68	0.00	0.00	0.00	0.00	0.00	0.00
7	Canine	A(H3N2)	A/canine/Florida/43/2004	10 ^{8.1}	19.61	19.69	19.44	0.00	0.00	0.00	0.00	0.00	0.00
8	Avian	A(H5N2)	A/Northern pintail/ Washington/40964 /2014	10 ^{9.4}	16.39	16.43	16.49	0.00	0.00	0.00	0.00	0.00	0.00
9	Avian	A(H5N8)	A/gyrfalcon/Washington/ 41088 -6/2014	10 ^{9.75}	14.12	14.10	14.13	0.00	0.00	0.00	0.00	0.00	0.00
10	Avian	A(H6N2)	A/chicken/California/ 32213-1/2000	10 ^{9.2}	14.97	15.05	15.08	0.00	0.00	0.00	0.00	0.00	0.00
11	Avian	A(H7N2)	A/feline/New York/16- 040082-1/2016	10 ^{10.2}	15.76	15.94	16.00	0.00	0.00	0.00	0.00	0.00	0.00
12	Avian	A(H7N9)	A/Taiwan/1/2017	10 ^{9.5}	16.69	16.90	16.99	0.00	0.00	0.00	0.00	0.00	0.00
13	Avian	A(H9N2)	A/Bangladesh/0994/2011	10 ^{10.5}	18.03	18.14	18.21	0.00	0.00	0.00	0.00	0.00	0.00
14	Human	B-VIC	B/Maryland/15/2016	10 ^{8.5}	0.00	0.00	0.00	13.46	13.49	13.47	0.00	0.00	0.00
15	Human	B-YAM	B/Phuket/3073/2013	10 ^{8.9}	0.00	0.00	0.00	13.66	13.67	13.68	0.00	0.00	0.00

Cross-reactivity (assay exclusivity):

The Flu SC2 Multiplex Assay was evaluated for cross-reactivity with influenza C viruses, non-targeted coronaviruses, and other human respiratory viruses, bacteria, and yeast (Table 15-Table 17). Nucleic acids from high titer preparations (typically $\geq 10^6$ TCID₅₀/mL or EID₅₀/mL, $\geq 10^6$ CFU/mL) of 35 organisms (16 viruses, 18 bacteria, and 1 yeast) representing respiratory pathogens or flora commonly present in human respiratory specimens and genetic near neighbors of viruses targeted by the assay were extracted with QIAGEN EZ1 Advanced XL extraction method using the QIAGEN EZ1 DSP Virus Kit. An RNA transcript which included an entire SARS-CoV (2003 era) Nucleocapsid (N) gene region through the 3' terminus was also tested to demonstrate specificity of the assay. Additionally, five (5) No template controls (NTC) and five (5) HSC controls were performed in triplicate to illustrate there is no reactivity of the Flu SC2 Multiplex Assay in the NTC's or mock Human specimen controls (Table 18). Testing was performed using the Applied Biosystems TaqPath™ Multiplex Master Mix (No ROX). All controls performed as expected, and results are shown below in Table 15-Table 18.

Table 15. Exclusivity Evaluation – Influenza C and Coronaviridae

No.	Strain Designation	Type/Subtype	ng/μL	FluSC2 Multiplex Assay (Ct)								
				InfA			InfB			SC2		
1	C/Minnesota/1/2016	Influenza C	nd ¹	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	C/Minnesota/4/2015	Influenza C	nd ¹	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	C/Minnesota/29/2015	Influenza C	nd ¹	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	MERS-CoV	Zoonotic beta coronavirus	5.4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	HCoV OC43	Endemic beta coronavirus	1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	HCoV 229E	Endemic alpha coronavirus	6.2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	HCoV NL63	Endemic alpha coronavirus	2.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	HCoV HKU1	HKU1	3.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9	NA	RNA transcript of the 3' Terminus (~2KB) of SARS-CoV/Urbani	NA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

¹Infectious dose titer not determined for influenza C

Table 16. Exclusivity Evaluation – Bacteria and Yeast

No.	Aliquoted	Strain	CFU/mL or ng/uL	FluSC2 Multiplex Assay (Ct)		
				InfA	InfB	SC2
1	<i>Bordetella pertussis</i>	Tohama I	10 ¹⁰	0.00	0.00	0.00
2	<i>Candida albicans</i> (yeast)	3147	10 ^{8.5}	0.00	0.00	0.00
3	<i>Chlamydia pneumoniae</i> ¹	CM-1	40 IFU/mL	0.00	0.00	0.00
4	<i>Corynebacterium diphtheriae</i> ²	NCTC 13129	57.4 ng/uL	0.00	0.00	0.00
5	<i>Escherichia coli</i>	K12	10 ^{9.6}	0.00	0.00	0.00
6	<i>Streptococcus pyogenes</i>	7790-06	10 ^{7.5}	0.00	0.00	0.00
7	<i>Haemophilus influenzae</i>	M15709	10 ^{6.4}	0.00	0.00	0.00
8	<i>Lactobacillus plantarum</i>	N/A ³	10 ^{8.8}	0.00	0.00	0.00
9	<i>Legionella pneumophila</i>	Philadelphia-1	10 ^{8.4}	0.00	0.00	0.00
10	<i>Moraxella catarrhalis</i>	M15757	10 ^{9.5}	0.00	0.00	0.00
11	<i>Mycobacterium tuberculosis</i>	H37Ra	10 ⁵	0.00	0.00	0.00
12	<i>Mycoplasma pneumoniae</i>	PI 1428	10 ⁹	0.00	0.00	0.00
13	<i>Neisseria elongata</i>	N/A ³	10 ⁵	0.00	0.00	0.00
14	<i>Neisseria meningitidis</i>	M2578	10 ^{7.9}	0.00	0.00	0.00
15	<i>Pseudomonas aeruginosa</i>	N/A ³	10 ^{10.5}	0.00	0.00	0.00
16	<i>Staphylococcus aureus</i>	N/A ³	10 ^{10.7}	0.00	0.00	0.00
17	<i>Staphylococcus epidermidis</i>	N/A ³	10 ^{10.5}	0.00	0.00	0.00
18	<i>Streptococcus pneumoniae</i>	249-06	10 ^{6.6}	0.00	0.00	0.00
19	<i>Streptococcus salivarius</i> ²	DSM 13084	109 ng/uL	0.00	0.00	0.00

¹Organism quantified by Infectious Forming Units (IFU)

²Organism quantified by spectrophotometry (ng/μL)

³NA = not applicable

Table 17. Exclusivity Evaluation – Non-flu Respiratory Viruses

No.	Strain	Non-Influenza Respiratory Viruses	Concentration (TCID ₅₀ /ml)	FluSC2 Multiplex Assay (Ct)		
				InfA	InfB	SC2
1	Ad.71	Human Adenovirus, type 1	10 ^{9.2}	0.00	0.00	0.00
2	S-1058	Human Adenovirus, type 7a	10 ^{7.1}	0.00	0.00	0.00
3	NA	Human parainfluenza 1 ¹	3.0 ng/μL	0.00	0.00	0.00
4	Greer	Human parainfluenza 2	10 ^{3.1}	0.00	0.00	0.00
5	C-243	Human parainfluenza 3	10 ^{7.9}	0.00	0.00	0.00
6	CH93-18b	Respiratory syncytial virus	10 ^{6.8}	0.00	0.00	0.00
7	1A	Human Rhinovirus A	10 ^{5.8}	0.00	0.00	0.00
8	Echo 6	Enterovirus	10 ^{6.9}	0.00	0.00	0.00
9	KOS	Herpes Simplex virus	5 X 10 ^{7.75}	0.00	0.00	0.00
10	AV92-3:H	Varicella-zoster virus	5 X 10 ^{3.75}	40.45	0.00	0.00
11	B95-8	Epstein Barr virus ¹	1.7 ng/μL	0.00	0.00	0.00
12	Edmonston	Measles	5 X 10 ^{4.5}	0.00	0.00	0.00
13	Enders	Mumps	5 X 10 ^{6.5}	0.00	0.00	0.00
14	AD-169	Cytomegalovirus	5 X 10 ^{6.25}	0.00	0.00	0.00

¹Organism quantified by spectrophotometry (ng/μL)

Table 18. Exclusivity Evaluation – NTC and HSC

No.	Sample	FluSC2 Multiplex Assay (Ct)											
		InfA			InfB			SC2			RP		
1	No Template Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	No Template Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	No Template Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	No Template Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	No Template Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	Human Specimen Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	29.98	29.71	30.03
2	Human Specimen Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	28.36	28.28	28.89
3	Human Specimen Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	29.47	29.76	29.88
4	Human Specimen Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	29.35	29.46	29.57
5	Human Specimen Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	29.82	29.87	29.73

Co-Infection Sensitivity:

Analytical sensitivity of the Flu SC2 Multiplex Assay in the context of a co-infection scenario was evaluated by performing a series of serial dilution experiments using mock co-infection specimens for each of the viral targets in the assay. To create the mock co-infection specimens, RNA was extracted from three high-titer virus isolates using the Qiagen EZ1 DSP Virus Kit on the QIAGEN EZ1 Advanced XL platform. Concentrations for these isolates were selected to represent a consistent Ct that ranged between 21-23 (Table 20), and A549 RNA was added to simulate human respiratory specimens. The extracted RNA corresponding to each viral target in the Flu SC2 Multiplex Assay was serially diluted in the presence of high-titer RNA background concentrations of RNA corresponding to the other targets in the assay; configurations tested are shown in Table 21. Triplicate samples of each serial dilution were tested with the Flu SC2 Multiplex Assay using both the TaqPath™ Multiplex Master Mix (No ROX) and the Quantabio UltraPlex™ 1-Step ToughMix® (4X) to determine the sensitivity of the assay when multiple viral targets are present in a sample. All controls performed as expected, and summary data are presented in Table 22 below.

Table 19. Virus selected for Co-infection study and Background Titer

Virus	Type	Background titer ID₅₀/mL
2019-nCoV/USA-WA1/2020	SARS-CoV-2	10 ^{3.5}
A/Illinois/20/2018	Influenza A	10 ^{4.8}
B/Colorado/06/2017	Influenza B	10 ^{4.3}

Table 20. Combinations tested for Co-infection study

Strains	Titer		
InfA	high	high	serial dilution
InfB	high	serial dilution	high
SC2	serial dilution	high	high

Table 21. Observed LoD – Simulated Co-infection Study

Virus type	Strains	LoD (ID₅₀/reaction)					
		TaqPath 1-step Multiplex			Ultrplex 1-step ToughMix		
		InfA	InfB	SC2	InfA	InfB	SC2
A(H1N1)pdm09	A/Illinois/20/2018	1.26E+01	---	---	1.26E+01	---	---
B-Victoria	B/Colorado/06/2017	---	7.92E-01	---	---	7.92E-01	---
SARS-CoV-2	2019-nCoV/USA-WA1/2020	---	---	5.00E-02	---	---	5.00E-02

Clinical Evaluation:

Clinical performance of the SARS-CoV-2 Influenza Multiplex Assay was evaluated using a panel of upper and lower respiratory, residual human clinical specimens. A total of 104 positive specimens (33 SARS-CoV-2 positive, 30 influenza A positive, 30 influenza B positive) and 11 negative specimens were extracted using the QIAGEN EZ1 Advanced XL with the QIAGEN EZ1 DSP Virus kit. Extracted samples were tested using the Flu SC2 Multiplex Assay with both the Applied Biosystems TaqPath™ Multiplex Master Mix (No ROX) and the Quantabio UltraPlex™ 1-Step ToughMix® (4X). SARS-CoV-2 positive specimens were confirmed using the FDA-authorized CDC 2019-nCoV-Real-Time RT-PCR Diagnostic Panel, and Influenza A and B virus positive specimens (NP/OP) were confirmed using the FDA-cleared CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza A/B Typing Kit. Specimens negative for SARS-CoV-2, influenza A virus and influenza B virus were confirmed by negative result(s) on both the CDC 2019-nCoV-Real-Time RT-PCR Diagnostic Panel and the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza A/B Typing Kit. All controls performed as expected and a summary of the results is presented in Table 23.

Table 22. Clinical performance: Specimen type and results (SARS-CoV-2)

Specimen type	Virus type	Number	Positive	% Agreement
NP	SCoV2	18	18/18	100
OP	SCoV2	1	1/1	100
Oral	SCoV2	7	7/7	100
Sputum	SCoV2	3	3/3	100
NP/ Oral	SCoV2	1	1/1	100
Th/ NP	SCoV2	3	3/3	100

Results of testing with the Flu SC2 Multiplex Assay were compared to the expected result for each specimen to determine percent agreement and a summary of results is shown in Table 24.

Table 23. Clinical performance: Summary of Results

Flu SC2 Target	Clinical Specimen Panel			
	Influenza A Positive	Influenza B Positive	SARS-CoV-2 Positive	Negative
InfA Positive	30/30	0/30	0/33	71/71
InfB Positive	0/30	30/30	0/33	71/71
SC2 Positive	0/30	0/30	33/33	74/74
Negative	0/71	0/71	0/74	11/11
Total:	30	30	33	227

¹ Negative results are represented by analyte in each clinical specimen tested. Eleven samples negative for all three viruses were included.

Overall Percent Agreement:100%

Extraction Method Bridging:

Analytical performance comparison

An extraction bridging study was conducted to evaluate the performance of the QIAGEN EZ1 Advanced XL with EZ1 DSP Virus Kit extraction method used in Flu SC2 Multiplex Assay performance characterization studies with the following additional extraction methods: QIAGEN QIAamp Viral RNA Mini Kit (manual) and the Roche MagNA Pure 96 with Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit.

Contrived specimens were prepared using quantified SARS-CoV-2 virus (2019-nCoV/USA-WA1/2020) prepared in sputum. Three-fold serial dilutions were created and subsequently extracted five times using the QIAGEN EZ1 Advanced XL with QIAGEN EZ1 DSP Virus Kit, the Roche MagNA Pure 96 with the Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit, and with the QIAGEN QIAamp DSP Viral RNA Mini Kit. Resulting nucleic acid samples were tested with the Flu SC2 Multiplex using the Applied Biosystems TaqPath™ Multiplex Master Mix (No ROX). All candidate methods performed comparably, and controls performed as expected. Summary data are presented in Table 25.

Table 24. RNA Extraction Bridging: Analytical sensitivity summary

Extraction Platform	LoD (ID ₅₀ /mL)	
	LoD Confirmed	Sputum
Qiagen- EZ1 DSP Virus kit	6.4X10 ^{-0.5}	3.2X10 ^{0.5}
Qiagen QIAamp Viral RNA Mini Kit	Not tested	6.4X10 ^{-0.5}
Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit	Not tested	3.2X10 ^{0.5}

Contact Information, Ordering, and Product Support

For ordering contact the International Reagent Resource:

Website: InternationalReagentResource.org

Email: contact@internationalreagentresource.org

For technical support contact CDC:

Email: CDCSARS2FluAB@cdc.gov and include “Flu SC2 Multiplex” in the subject line

Appendix A: Instrument Setup for Applied Biosystems™ 7500 Fast Dx Real-time PCR Instrument Using Optimal Filter Calibrations

Creating a Flu SC2 Multiplex Assay template for the 7500 Fast Dx with optimal filter calibrations

The recommended, optimal calibration settings for running the Flu SC2 Multiplex Assay are FAM, Yakima Yellow, Texas Red X, and CY5. This section outlines how to set up a Flu SC2 Multiplex Assay template with these recommended filter settings that can be used for all Flu SC2 Multiplex Assay testing on this instrument.

1. Launch the 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
2. A new window should appear, select **Create New Document** from the menu.

Figure 1. New Document Wizard Window

New Document Wizard

Define Document
Select the assay, container, and template for the document, and enter the operator name and comments.

Assay: Standard Curve (Absolute Quantitation)

Container: 96-Well Clear

Template: Blank Document

Run Mode: Standard 7500

Operator: Training

Comments: SDS v1.4.1

Plate Name: Plate1

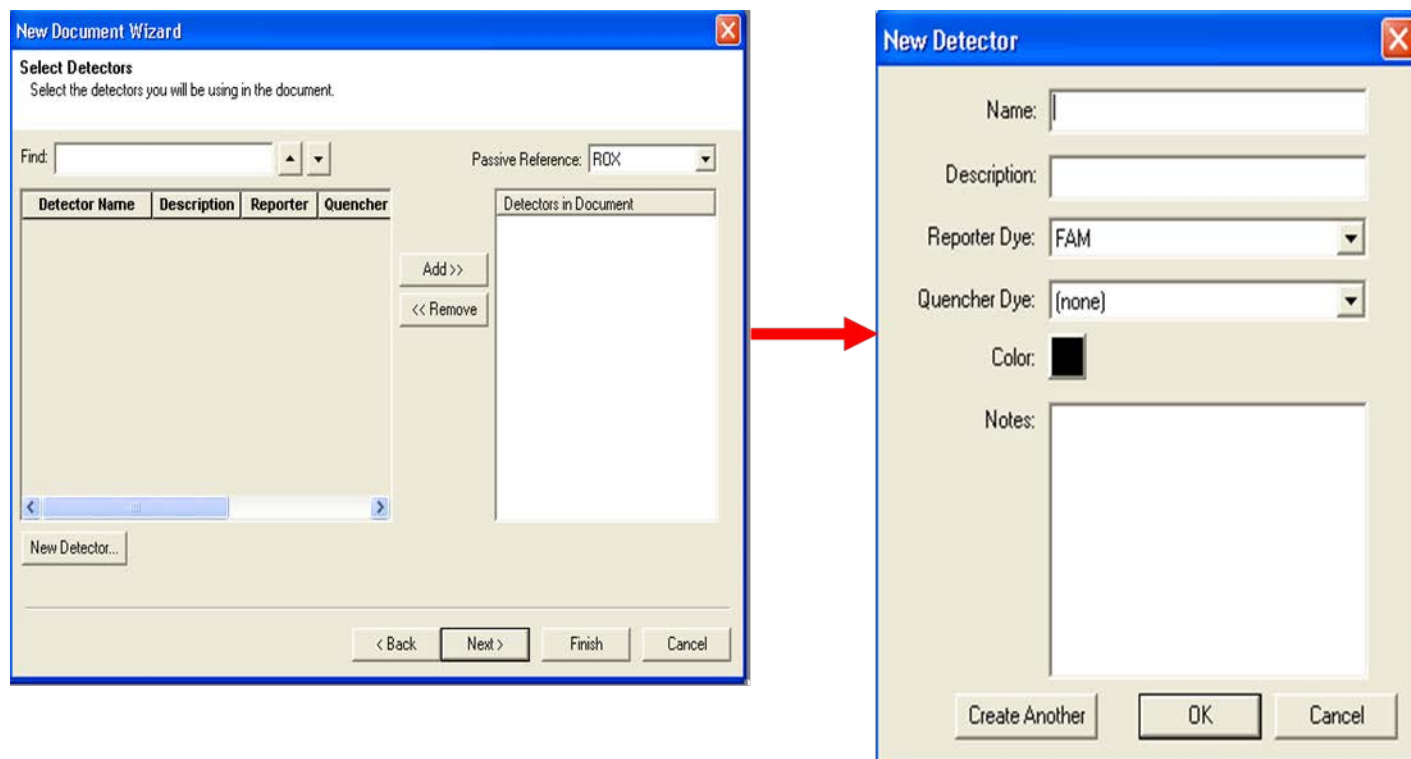
< Back Next > Finish Cancel

Make sure to change Run Mode to: Standard 7500

3. The **New Document Wizard** screen in **Figure 1** will appear. Select:
 - a. Assay: **Standard Curve (Absolute Quantitation)**
 - b. Container: **96-Well Clear**
 - c. Template: **Blank Document**
 - d. Run Mode: **Standard 7500**
 - e. Operator: **Your Name**
 - f. Comments: **SDS v1.4.1**
 - g. Plate Name: **Your Choice**
4. After making selections click **Next** at the bottom of the window.
5. After selecting next, the **Select Detectors** screen (**Figure 2**, left image) will appear.

6. Click the **New Detector** button (**Figure 2**).
7. The **New Detector** window will appear (**Figure 2**, right image). A new detector will need to be defined for each target within the Flu SC2 Multiplex Assay. Creating these detectors will enable the analysis of each primer and probe set individually at the end of the reaction.

Figure 2. Creating New Detectors

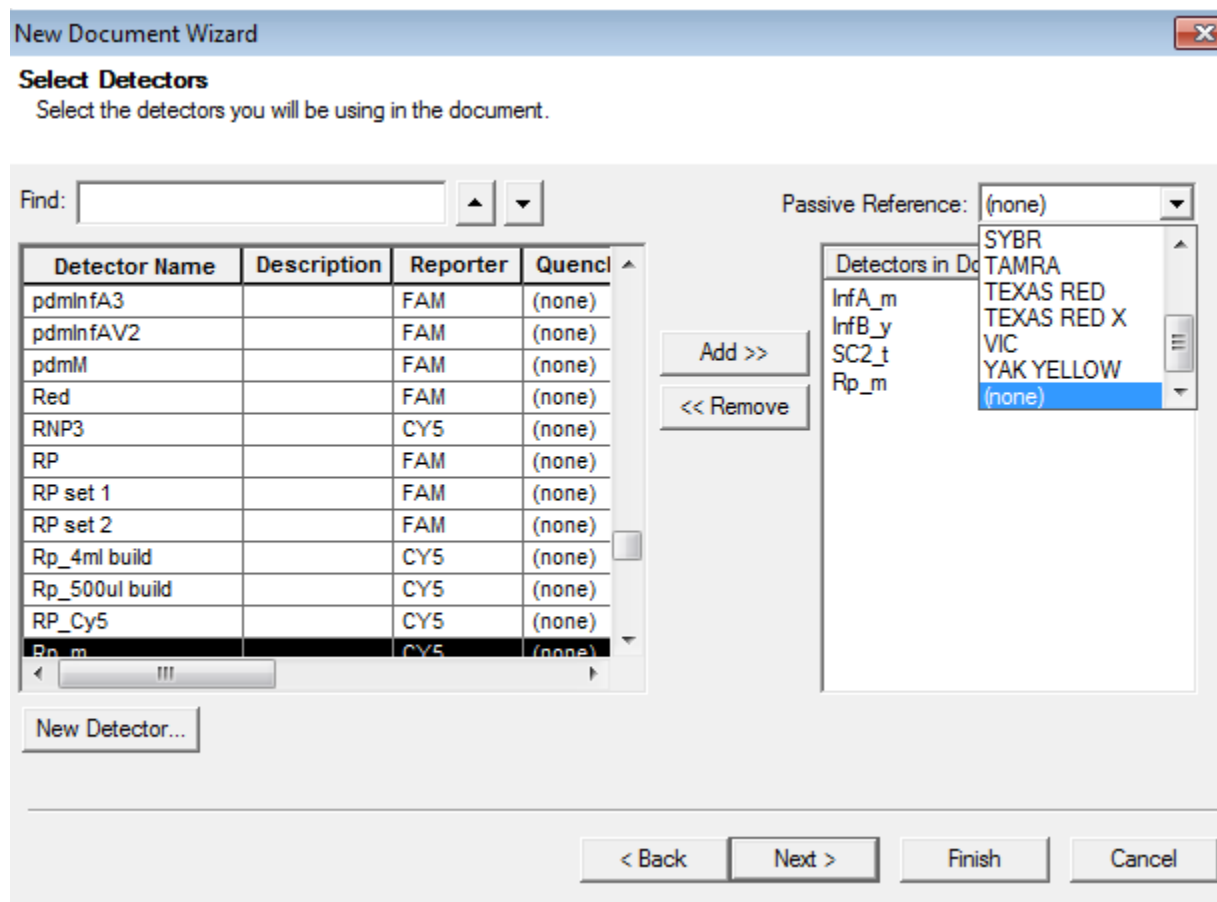


8. Start by creating the InfA Detector (**Figure 2**, left image). Include the following:
 - a. Name: **InfA_m**
 - b. Description: *leave blank*
 - c. Reporter Dye: **FAM**
 - d. Quencher Dye: **(none)**
 - e. Color: *to change the color of the detector indicator do the following:*
 - ⇒ Click on the color square to reveal the color chart
 - ⇒ Select a color by clicking on one of the squares
 - ⇒ After selecting a color click **OK** to return to the New Detector screen
 - f. Click the **OK** button of the **New Detector** screen to return to the screen shown in the left image in **Figure 2**.
9. Repeat step 6-8 for each target in the Flu SC2 Multiplex Assay.

Name	Reporter Dye	Quencher Dye
InfA_m	FAM	(none)
InfB_y	Yakima Yellow	(none)
SC2_t	Texas Red X	(none)
RP_m	CY5	(none)

- After each Detector is added, the **Detector Name**, **Description**, **Reporter**, and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 3**).
- Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (**Figure 3**). Detector names will appear on the right side of the **Select Detectors** window (**Figure 3**).
- Once all detectors have been added, select **(none)** for **Passive Reference** at the top right drop down menu (**Figure 3**).

Figure 3. Adding New Detectors to Document and Selecting Passive Reference



Passive reference should be set to “(none)” as described above.

13. Click **Next** at the bottom of the **Select Detectors** window (**Figure 3**) to proceed to the **Set Up Sample Plate** window (**Figure 4**).
14. In the **Set Up Sample Plate** window (**Figure 4**), select all the wells in the plate.
15. In the top portion of the window, select **the four detectors in the Flu SC2 Multiplex Assay (InfA_m, InfB_y, SC2_t, and RP_m)**. A check will appear next to the detector that was selected (**Figure 4**). The selected wells will be populated with four colored “U” icons to indicate that the detectors have been selected.
16. Select **Finish** after detectors have been assigned to each well (**Figure 4**).

Figure 4. Sample Plate Set-up

New Document Wizard

Set Up Sample Plate
Setup the sample plate with tasks, quantities and detectors.

Use	Detector	Reporter	Quencher	Task
<input checked="" type="checkbox"/>	InfA_m	FAM	(none)	Unknown
<input checked="" type="checkbox"/>	InfB_y	YAK YELLOW	(none)	Unknown
<input checked="" type="checkbox"/>	SC2_t	TEXAS RED X	(none)	Unknown
<input checked="" type="checkbox"/>	Rp_m	CY5	(none)	Unknown

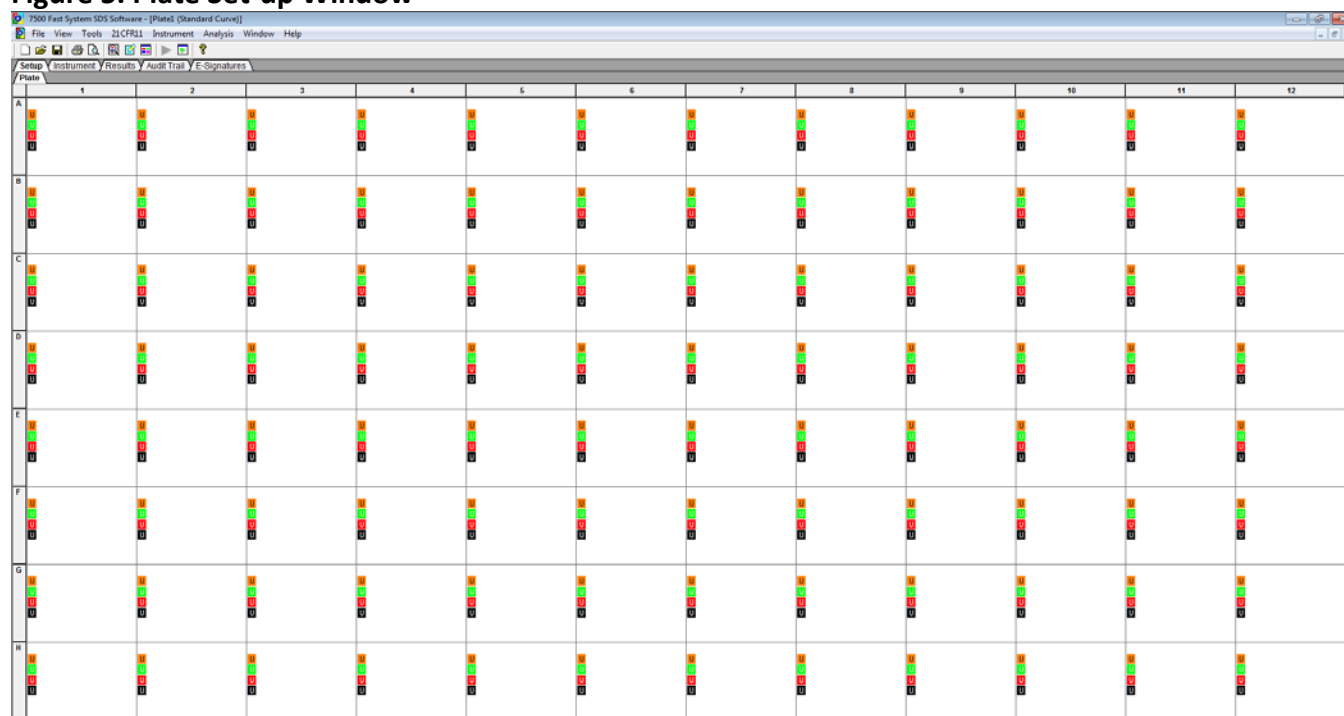
111

	1	2	3	4	5	6	7	8	9	10	11	12
A	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
B	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
C	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
D	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
E	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
F	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
G	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
H	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U

< Back Next > Finish Cancel

17. After clicking **Finish**, there will be a brief pause allowing the 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. **Note: The machine must be turned on for initialization.**
18. After initialization, the **Plate** tab of the **Setup (Figure 5)** will appear.
19. Each well of the plate should contain colored U icons that correspond with the detector labels that were previously chosen. To confirm detector assignments, select **Tools** from the file menu, then select **Detector Manager**.

Figure 5. Plate Set-up Window



20. The **Detector Manager** window will appear (**Figure 6**).

Figure 6. Detector Manager Window

The screenshot shows the 'Well Inspector' window. At the top, there is a 'Well(s):' label and a 'Sample Name:' text box. Below this is a table with the following columns: Use, Detector, Reporter, Quencher, Task, Quantity, and Color. The table contains four rows of data, all with the 'Use' checkbox checked. The 'Color' column shows orange, green, red, and black for the four rows respectively. Below the table is a large empty area with a scrollbar. At the bottom, there is an 'Omit Well' checkbox, three buttons ('Add Detector...', 'Remove', 'Close'), and a 'Passive Reference:' dropdown menu set to '(none)'.

Use	Detector	Reporter	Quencher	Task	Quantity	Color
<input checked="" type="checkbox"/>	InfA_m	FAM	(none)	Unknown		Orange
<input checked="" type="checkbox"/>	InfB_y	YAK YELL	(none)	Unknown		Green
<input checked="" type="checkbox"/>	SC2_t	TEXAS RED	(none)	Unknown		Red
<input checked="" type="checkbox"/>	Rp_m	CY5	(none)	Unknown		Black

Well(s):
Sample Name:

☐ Omit Well

Add Detector... Remove Close

Passive Reference: (none) ▼

21. Confirm all Flu SC2 Multiplex Assay detectors are included and that each target has a **Reporter** set **appropriately as per instructed in step 9** and the **Quencher** is set to **(none)**.
22. If all detectors are present, select **Done**. The detector information has been created and assigned to wells on the plate.

Defining the Instrument Settings:

(Important: Use Appropriate Settings for Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX) or Quantabio UltraPlex™ 1-Step ToughMix® (4X))

1. After detectors have been created and assigned, proceed to instrument set up.
2. Select the **Instrument** tab to define thermal cycling conditions.
3. Modify the thermal cycling conditions as follows (**Figure 7** and **Figure 8**):

Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX) (Figure 7)

- a. In Stage 1, Step 1 set to **2 min** at **25°C**; **1 Rep.**
- b. In Stage 1, Step 2 set to 15 min at 50°C
- c. In Stage 2, Set to **2.0 min** at **95°C**; **1 Rep.**
- d. In Stage 3, Step 1 set to **15 sec** at **95°C**.
- e. In Stage 3, Step 2 set to **30 sec** at **55.0°C**.
- f. In Stage 3, Reps should be set to **45**.
- g. Under **Settings (Figure 7)**, bottom left box, change volume to 25 µL.
- h. Under **Settings, Run Mode** selection should be **Standard 7500**.
- i. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 7**).

Quantabio UltraPlex™ 1-Step ToughMix® (4X) (Figure 8)

- a. In Stage 1, Step 1 set to 2 min at 25°C; 1 Rep.
- b. In Stage 1, Step 2 set to 15 min at 50°C
- c. **In Stage 2, Set to 3.0 min at 95°C; 1 Rep.**
- d. In Stage 3, Step 1 set to **15 sec** at **95°C**.
- e. In Stage 3, Step 2 set to **30 sec** at **55.0°C**.
- f. In Stage 3, Reps should be set to **45**.
- g. Under **Settings (Figure 8)**, bottom left box, change volume to 25 µL.
- h. Under **Settings, Run Mode** selection should be **Standard 7500**.
- i. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 8**).

Figure 7. Instrument Window For Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX)

Setup

Instrument

Results

Audit Trail

E-Signatures

Instrument Control

Start

Stop

Disconnect

Extend...

Estimated Time Remaining (hh:mm):

Status:

Temperature

Sample:

Cover:

Heat Sink:

Block:

Cycle

Stage:

Time (mm:ss):

Rep:

Step:

State:

Thermal Cycler Protocol

Thermal Profile

Auto Increment

Ramp Rate

Stage 1

Reps: 1

25.0

2:00

50.0

15:00

Stage 2

Reps: 1

95.0

2:00

Stage 3

Reps: 45

95.0

0:15

55.0

0:30

Add Cycle

Add Hold

Add Step

Add Dissociation Stage

Delete

Help

Settings

Sample Volume (µL):

25

Run Mode

Standard 7500

Data Collection :

Stage 3, Step 2 (55.0 @ 0:30)

Figure 8. Instrument Window For Quantabio UltraPlex™ 1-Step ToughMix® (4X)

Setup **Instrument** **Results** **Audit Trail** **E-Signatures**

Instrument Control

Start Estimated Time Remaining (hh:mm):

Stop

Disconnect Status:

Extend...

Temperature

Sample: Heat Sink:

Cover: Block:

Cycle

Stage: Rep:

Time (mm:ss): Step:

State:

Thermal Cycler Protocol

Thermal Profile Auto Increment Ramp Rate

Stage 1 Repts: 1

25.0 2:00

50.0 15:00

Stage 2 Repts: 1

95.0 3:00

Stage 3 Repts: 45

95.0 0:15

55.0 0:30

Add Cycle Add Hold Add Step Add Dissociation Stage Delete Help

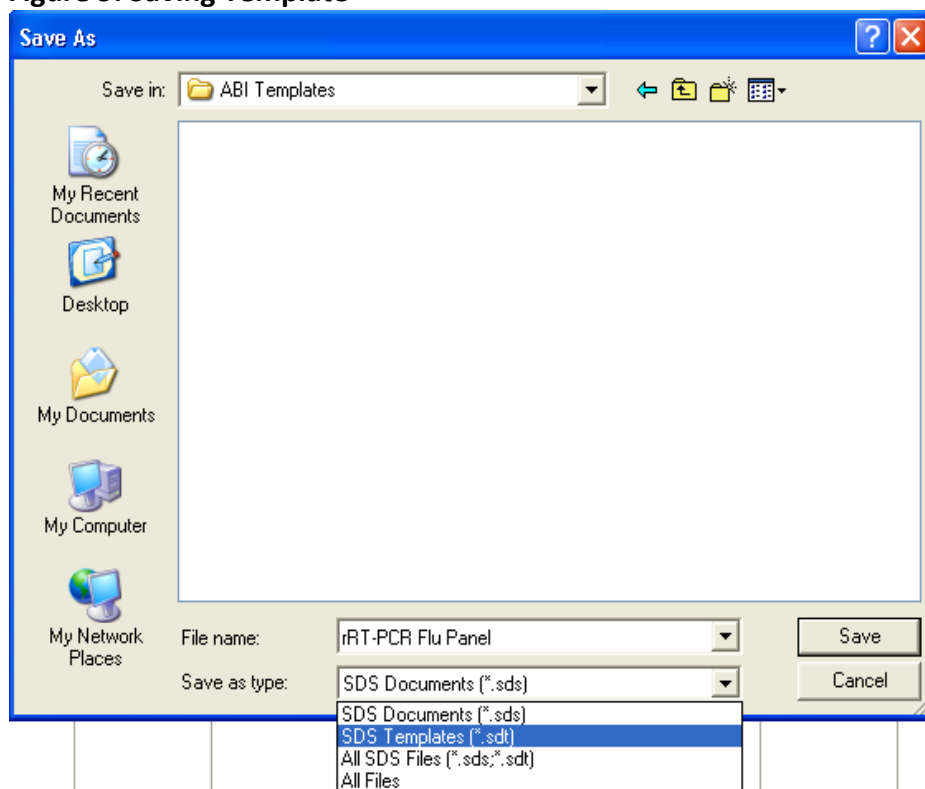
Settings

Sample Volume (µL): 25

Run Mode: Standard 7500

Data Collection: Stage 3, Step 2 (55.0 @ 0:30)

4. After making changes to the Instrument tab, the template file is ready to be saved. To save the template, select File from the top menu, then select Save As. Since the two enzyme options have different instrument settings it is recommended that the template be saved with a name indicating the enzyme option.
5. Save the template as Flu SC2 Multiplex TaqPath or Flu SC2 Multiplex Ultrplex as appropriate in the desktop folder labeled “ABI Run Templates” (user must create this folder). Save as type should be SDS Templates (*.sdt) (Figure 9).

Figure 9. Saving Template

Appendix B: Instrument Setup for Applied Biosystems™ 7500 Fast Dx Real-time PCR Instrument If Optimal Filter Calibrations Are Not Available

Creating a Flu SC2 Multiplex Assay template for the 7500 Fast Dx if optimal filter calibrations are not available

The recommended, optimal calibration settings for running the Flu SC2 Multiplex Assay are FAM, Yakima Yellow, Texas Red X, and CY5. However, if these optimal calibration settings are not available, the Flu SC2 Multiplex Assay can be performed using the alternative filter calibrations FAM, VIC, ROX, and CY5. This section outlines how to set up a Flu SC2 Multiplex Assay template using these alternative filter calibrations.

1. Launch the 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
2. A new window should appear, select **Create New Document** from the menu.

Figure 1. New Document Wizard Window

New Document Wizard

Define Document
Select the assay, container, and template for the document, and enter the operator name and comments.

Assay: Standard Curve (Absolute Quantitation)

Container: 96-Well Clear

Template: Blank Document Browse...

Run Mode: Standard 7500

Operator: Training

Comments: SDS v1.4.1

Plate Name: Plate1

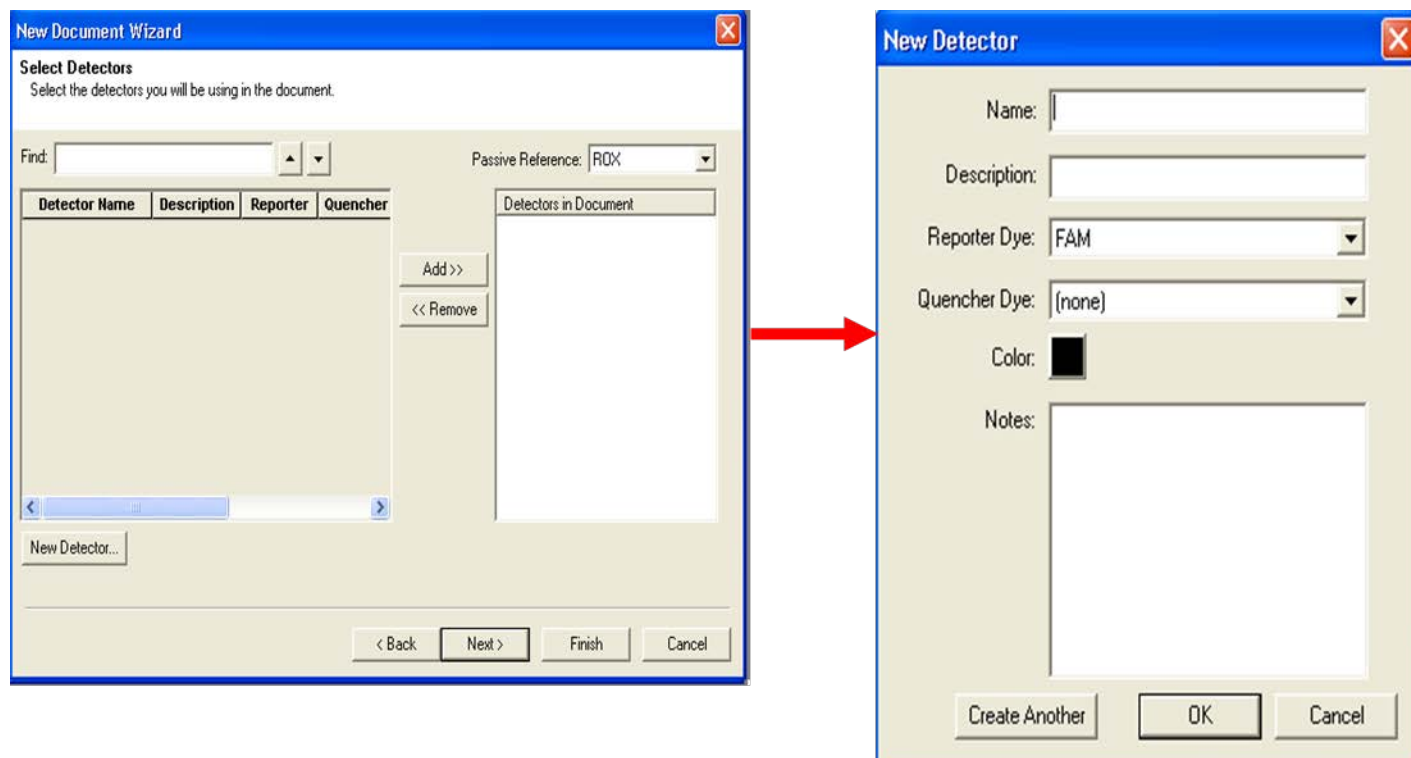
< Back Next > Finish Cancel

Make sure to change Run Mode to: Standard 7500

3. The **New Document Wizard** screen in **Figure 1** will appear. Select:
 - a. Assay: **Standard Curve (Absolute Quantitation)**
 - b. Container: **96-Well Clear**
 - c. Template: **Blank Document**
 - d. Run Mode: **Standard 7500**
 - e. Operator: **Your Name**
 - f. Comments: **SDS v1.4.1**
 - g. Plate Name: **Your Choice**

4. After making selections click **Next** at the bottom of the window.
5. After selecting next, the **Select Detectors** screen (**Figure 2**, left image) will appear.
6. Click the **New Detector** button (**Figure 2**).
7. The **New Detector** window will appear (**Figure 2**, right image). A new detector will need to be defined for each target within the Flu SC2 Multiplex Assay. Creating these detectors will enable the analysis of each primer and probe set individually at the end of the reaction.

Figure 2. Creating New Detectors

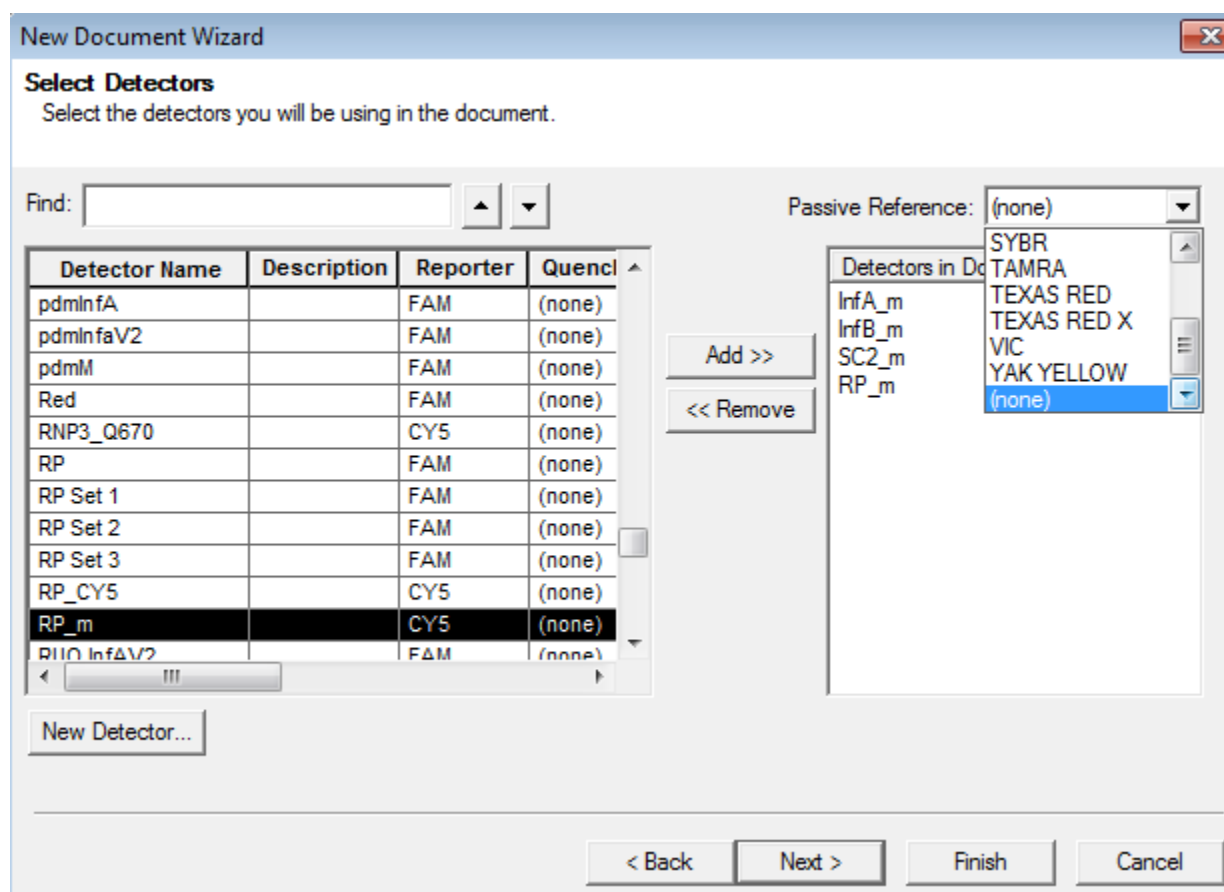


8. Start by creating the InfA Detector (**Figure 2**, left image). Include the following:
 - a. Name: **InfA_m**
 - b. Description: *leave blank*
 - c. Reporter Dye: **FAM**
 - d. Quencher Dye: **(none)**
 - e. Color: *to change the color of the detector indicator do the following:*
 - ⇒ Click on the color square to reveal the color chart
 - ⇒ Select a color by clicking on one of the squares
 - ⇒ After selecting a color click **OK** to return to the New Detector screen
 - f. Click the **OK** button of the **New Detector** screen to return to the screen shown in the left image in **Figure 2**.
9. Repeat step 6-8 for each target in the Flu SC2 Multiplex Assay.

Name	Reporter Dye	Quencher Dye
InfA_m	FAM	(none)
InfB_m	VIC	(none)
SC2_m	ROX	(none)
RP_m	CY5	(none)

- After each Detector is added, the **Detector Name**, **Description**, **Reporter**, and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 3**).
- Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (**Figure 3**). Detector names will appear on the right side of the **Select Detectors** window (**Figure 3**).
- Once all detectors have been added, select **(none)** for **Passive Reference** at the top right drop down menu (**Figure 3**).

Figure 3. Adding New Detectors to Document and Selecting Passive Reference



Passive reference should be set to “(none)” as described above.

- Click **Next** at the bottom of the **Select Detectors** window (**Figure 3**) to proceed to the **Set Up Sample Plate** window (**Figure 4**).

14. In the **Set Up Sample Plate** window (**Figure 4**), select all the wells in the plate.
15. In the top portion of the window, select **the four detectors in the Flu SC2 Multiplex Assay (InfA_m, InfB_m, SC2_m, and RP_m)**. A check will appear next to the detector that was selected (**Figure 4**). The selected wells will be populated with four colored “U” icons to indicate that the detectors have been selected.
16. Select **Finish** after detectors have been assigned to each well (**Figure 4**).

Figure 4. Sample Plate Set-up

New Document Wizard X

Set Up Sample Plate
Setup the sample plate with tasks, quantities and detectors.

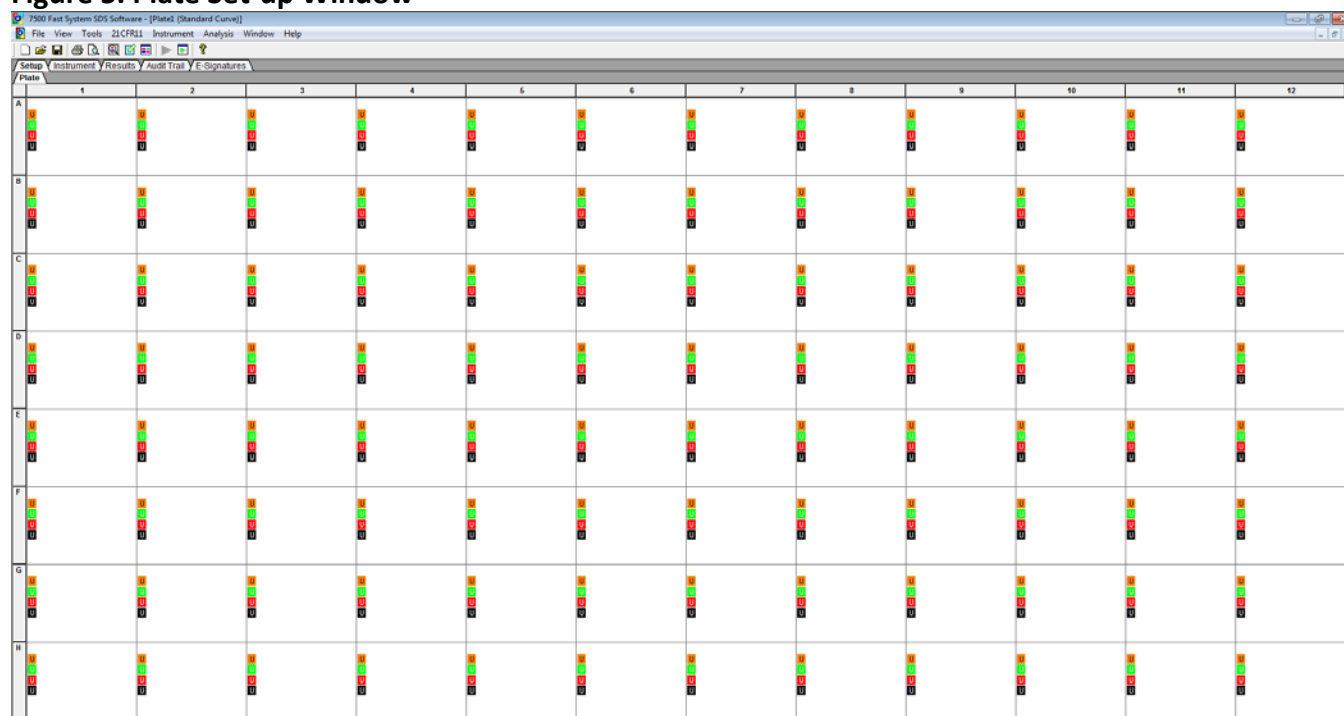
Use	Detector	Reporter	Quencher	Task	Qua
<input checked="" type="checkbox"/>	InfA_m	FAM	(none)	Unknown	
<input checked="" type="checkbox"/>	InfB_m	VIC	(none)	Unknown	
<input checked="" type="checkbox"/>	SC2_m	ROX	(none)	Unknown	
<input checked="" type="checkbox"/>	RP_m	CY5	(none)	Unknown	

III

	1	2	3	4	5	6	7	8	9	10	11	12
A	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
B	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
C	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
D	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
E	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
F	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
G	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U
H	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U	U U U

17. After clicking Finish, there will be a brief pause allowing the 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. Note: The machine must be turned on for initialization.
18. After initialization, the Plate tab of the Setup (**Figure 5**) will appear.
19. Each well of the plate should contain colored U icons that correspond with the detector labels that were previously chosen. To confirm detector assignments, select Tools from the file menu, then select Detector Manager.

Figure 5. Plate Set-up Window



20. The **Detector Manager** window will appear (**Figure 6**).

Figure 6. Detector Manager Window

The screenshot shows the 'Well Inspector' window. At the top, there is a 'Well(s):' label and a 'Sample Name:' text box. Below this is a table with the following data:

Use	Detector	Reporter	Quencher	Task	Quantity	Color
<input checked="" type="checkbox"/>	InfA_m	FAM	(none)	Unknown		Orange
<input checked="" type="checkbox"/>	InfB_m	VIC	(none)	Unknown		Green
<input checked="" type="checkbox"/>	SC2_m	ROX	(none)	Unknown		Red
<input checked="" type="checkbox"/>	RP_m	CYS	(none)	Unknown		Black

Below the table is a large empty area. At the bottom left, there is a checkbox labeled 'Omit Well'. At the bottom right, there is a 'Passive Reference:' label and a dropdown menu showing '(none)'. At the bottom center, there are three buttons: 'Add Detector...', 'Remove', and 'Close'.

21. Confirm all Flu SC2 Multiplex Assay detectors are included and that each target has a **Reporter** set **appropriately as per instructed in step 9** and the **Quencher** is set to **(none)**.
22. If all detectors are present, select **Done**. The detector information has been created and assigned to wells on the plate.

Defining the Instrument Settings:

(Important: Use Appropriate Settings for Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX) or Quantabio UltraPlex™ 1-Step ToughMix® (4X))

1. After detectors have been created and assigned, proceed to instrument set up.
2. Select the **Instrument** tab to define thermal cycling conditions.
3. Modify the thermal cycling conditions as follows (**Figure 7** and **Figure 8**):

Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX) (Figure 7)

- a. In Stage 1, Step 1 set to **2 min** at **25°C**; **1 Rep.**
- b. In Stage 1, Step 2 set to 15 min at 50°C
- c. In Stage 2, Set to **2.0 min** at **95°C**; **1 Rep.**
- d. In Stage 3, Step 1 set to **15 sec** at **95°C**.
- e. In Stage 3, Step 2 set to **30 sec** at **55.0°C**.
- f. In Stage 3, Reps should be set to **45**.
- g. Under **Settings (Figure 7)**, bottom left box, change volume to 25 µL.
- h. Under **Settings, Run Mode** selection should be **Standard 7500**.
- i. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 7**).

Quantabio UltraPlex™ 1-Step ToughMix® (4X) (Figure 8)

- a. In Stage 1, Step 1 set to 2 min at 25°C; 1 Rep.
- b. In Stage 1, Step 2 set to 15 min at 50°C
- c. **In Stage 2, Set to 3.0 min at 95°C; 1 Rep.**
- d. In Stage 3, Step 1 set to **15 sec** at **95°C**.
- e. In Stage 3, Step 2 set to **30 sec** at **55.0°C**.
- f. In Stage 3, Reps should be set to **45**.
- g. Under **Settings (Figure 8)**, bottom left box, change volume to 25 µL.
- h. Under **Settings, Run Mode** selection should be **Standard 7500**.
- i. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 8**).

Figure 7. Instrument Window For Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX)

Setup Instrument Results Audit Trail E-Signatures

Instrument Control

Start Estimated Time Remaining (hh:mm):

Stop

Disconnect Status:

Extend...

Temperature

Sample: Heat Sink:

Cover: Block:

Cycle

Stage: Rep:

Time (mm:ss): Step:

State:

Thermal Cycler Protocol

Thermal Profile Auto Increment Ramp Rate

Stage 1 Stage 2 Stage 3

Reps: 1 Reps: 1 Reps: 45

95.0 95.0

2:00 0:15

50.0 55.0

15:00 0:30

25.0

2:00

Add Cycle Add Hold Add Step Add Dissociation Stage Delete Help

Settings

Sample Volume (µL): 25

Run Mode Standard 7500

Data Collection: Stage 3, Step 2 (55.0 @ 0:30)

Figure 8. Instrument Window For Quantabio UltraPlex™ 1-Step ToughMix® (4X)

Instrument Control

Start Stop Disconnect Extend...

Estimated Time Remaining (hh:mm):

Status:

Temperature

Sample: Heat Sink:
Cover: Block:

Cycle

Stage: Rep:
Time (mm:ss): Step:
State:

Thermal Cycler Protocol

Thermal Profile Auto Increment Ramp Rate

Stage 1	Stage 2	Stage 3
Reps: 1	Reps: 1	Reps: 45
25.0	95.0	95.0
2:00	3:00	0:15
50.0		55.0
15:00		0:30

Add Cycle Add Hold Add Step Add Dissociation Stage Delete Help

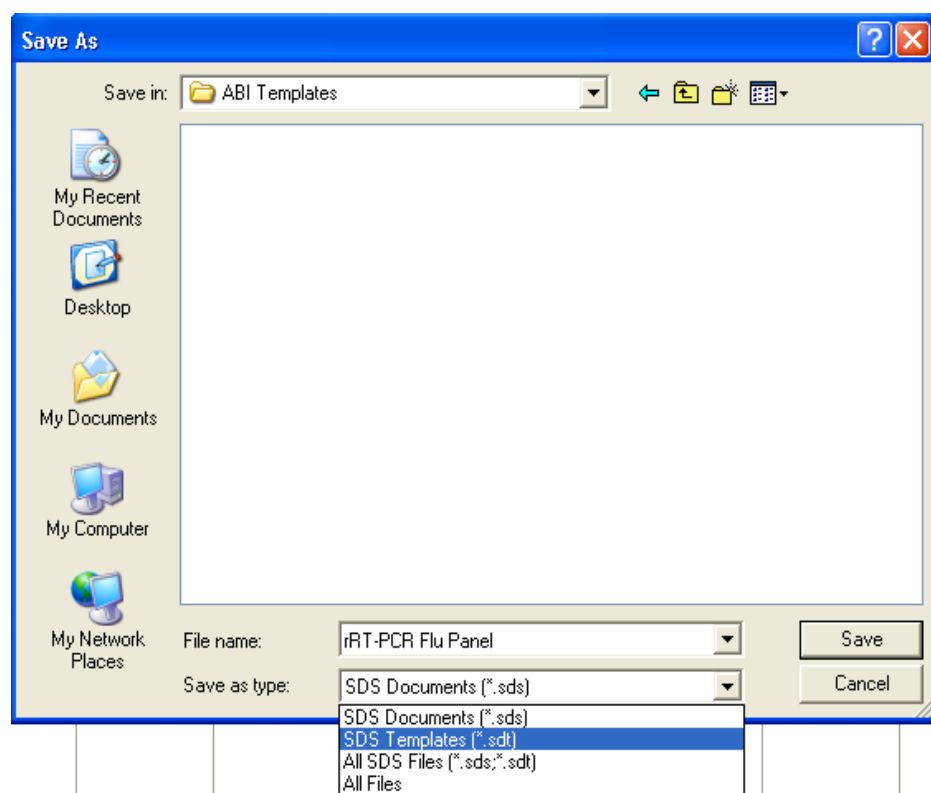
Settings

Sample Volume (µL): 25

Run Mode: Standard 7500

Data Collection: Stage 3, Step 2 (55.0 @ 0:30)

- After making changes to the **Instrument** tab, the template file is ready to be saved. To save the template, select **File** from the top menu, then select **Save As**. Since the two enzyme options have different instrument settings it is recommended that the template be saved with a name indicating the enzyme option.
- Save the template as Flu SC2 Multiplex TaqPath or Flu SC2 Multiplex Ultrplex as appropriate in the desktop folder labeled **"ABI Run Templates"** (user must create this folder). Save as type should be SDS Templates (*.sdt) (**Figure 9**).

Figure 9. Saving Template

Appendix C: Verification Recommendations

Purpose

This appendix is intended to provide CDC recommendations for the preparation of a panel of mock specimens for use in verifying performance of the CDC Influenza SARS-CoV-2 Multiplex Assay by end user laboratories. However, it is up to each facility's CLIA Director to determine the appropriate CLIA verification approach for the laboratory.

Please consult the following guidance from CMS regarding diagnostic tests authorized by FDA for emergency use: <https://www.cms.gov/Medicare/Provider-Enrollment-and-Certification/SurveyCertificationGenInfo/Policy-and-Memos-to-States-and-Regions-Items/QSO18-19-CLIA>.

Required Materials

The Seasonal Influenza Positive Control (SIPC) is provided with the Flu SC2 Multiplex Assay Kit. The SIPC consists of noninfectious influenza positive control materials as well as cells from the A549 human epithelial cell line. SIPC is extracted before use and ensures RNA is correctly released in the extraction process. SIPC will yield a positive result with the following primer and probe sets: InfA, InfB, and RP.

The SARS-CoV-2 positive control (SC2PC) provided with the Flu SC2 Multiplex Assay should be prepared according to the Instructions for Use. The SC2PC consists of 5 kilobase fragments of synthetic RNA representing the SARS-CoV-2 virus genome. SC2PC will yield a positive result with the SC2 primer and probe sets in the Flu SC2 Multiplex Assay. The SC2PC is added to the extracted SIPC to make the final positive control (FluSC2PC) for the Flu SC2 Multiplex Assay.

Approximately 2 mL of a respiratory specimen (upper or lower), that is negative for influenza A, B and SARS CoV-2, will be needed for testing. Specimens meeting this criterion may be pooled if less than 2 mL of one specimen is available. If it is necessary to use pooled specimens, it is advisable to use specimens in similar transport media.

Refer to the Flu SC2 Multiplex Assay Instructions for Use for additional reagents, materials, and instructions.

Precautions

Positive controls should be handled in an approved BSL-2 handling area to avoid contamination of laboratory equipment and reagents that could cause false positive results. The positive controls provided with the Flu SC2 Multiplex Assay are made from inactivated influenza virus, human cells and SARS-CoV-2 RNA transcript and are non-infectious. However, the Flu SC2 Multiplex Assay should be handled in accordance with Good Laboratory Practices.

Store reagent at appropriate temperatures (as described in the Product Information Sheet and Instructions for Use) and hold on ice once thawed.

Please use standard precautions when handling respiratory specimens.

Instructions for Preparing Mock Specimens Before Extraction with the QIAGEN EZ1 Advanced XL

- Refer to the Flu SC2 Multiplex Assay Instructions for Use for reconstitution and preparation of the materials for use. RNA should be kept cold during preparation and use.
Note: For verification, use undiluted SIPC provided with the Flu SC2 Multiplex Assay.
- Aliquot 280 µL of Buffer AVL (with carrier RNA) into each of nine tubes labeled 1-9.
NOTE: Carrier RNA should be added to Buffer AVL following the manufacturer's instructions.
- Make 1:100 dilution of SIPC by adding 10 µL of SIPC into 990 µL molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2.
- Make a 1:10 dilution of SC2PC (rehydrated according to specifications in the Flu SC2 Multiplex Instructions for Use) by adding 5 µL of SC2PC into 45 µL of molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-

8.2.

- Add 98 µL of respiratory specimen into each of the nine labeled tubes with 280 µL of Buffer AVL previously added.
- To prepare samples with SIPC at a moderate concentration, add 12 µL of undiluted SIPC into each tube labeled 1-3, with Buffer AVL and respiratory specimen previously added.
- To prepare samples with SIPC at a low concentration, add 12 µL of 1:100 dilution of SIPC into each tube labeled 4-6, with Buffer AVL and respiratory specimen previously added.
- To prepare negative control samples, add 12 µL of molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2 into each tube labeled 7-9, with Buffer AVL and respiratory specimen previously added.
- To finish preparation of the moderate concentration samples (tubes 1-3), add 10 µL undiluted SC2PC to each tube.
- To finish preparation of the low concentration samples (tubes 4-6), add 10 µL of the 1:10 SC2PC to each tube.
- To finish preparation of the negative samples (tubes 7-9), add 10 µL of the molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2 to each tube.
- Perform extractions of all nine samples according to the Flu SC2 Multiplex Assay Instructions for Use.

Instructions for Preparing Mock Specimens Before Extraction with the QIAamp DSP Viral RNA Mini Kit or the QIAamp Viral RNA Mini Kit

- Refer to the Flu SC2 Multiplex Assay Instructions for Use for reconstitution and preparation of the materials for use. RNA should be kept cold during preparation and use.
Note: For verification, use undiluted SIPC provided with the Flu SC2 Multiplex Assay.
- Aliquot 560 µL of Buffer AVL (with carrier RNA) into each of nine tubes labeled 1-9.
NOTE: Carrier RNA should be added to Buffer AVL following manufacturer's instructions.
- Make 1:100 dilution of SIPC by adding 10 µL of SIPC into 990 µL molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2.
- Make a 1:10 dilution of SC2PC (rehydrated according to specifications in the Flu SC2 Multiplex Assay Instructions for use) by adding 5 µL of SC2PC into 45 µL of molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2.
- Add 116 µL of respiratory specimen into each of the nine labeled tubes containing the 560 µL Buffer AVL added earlier.
- To prepare samples with SIPC at a moderate concentration, add 14 µL of undiluted SIPC into each tube labeled 1-3, with Buffer AVL and respiratory specimen previously added.
- To prepare samples with SIPC at a low concentration, add 14 µL of 1:100 dilution of SIPC into each tube labeled 4-6, with Buffer AVL and respiratory specimen previously added.
- To prepare negative control samples (tubes 7-9), add 14 µL of molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2 into each tube labeled 7-9, with Buffer AVL and respiratory specimen previously added.
- To finish preparation of the moderate concentration samples (tubes 1-3), add 10 µL undiluted, rehydrated SC2PC to each tube.
- To finish preparation of the low concentration samples (tubes 4-6), add 10 µL of the 1:10 SC2PC to each tube.
- To finish preparation of the negative samples (tubes 7-9), add 10 µL of the molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2 to each tube.
- Perform extractions of all nine samples according to the Flu SC2 Multiplex Instructions for Use.

Instructions for Preparing Mock Specimens Before Extraction with the Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit

- Refer to the Flu SC2 Multiplex Assay Instructions for Use for reconstitution and preparation of the materials for use. RNA should be kept cold during preparation and use.

Note: For verification, use undiluted SIPC provided with the Flu SC2 Multiplex Assay.

- Aliquot 350 µL of external lysis buffer into each of nine tubes labeled 1-9.
- Make 1:100 dilution of SIPC by adding 10 µL of SIPC into 990 µL molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2.
- Make a 1:10 dilution of SC2PC (rehydrated according to specifications in the Flu SC2 Multiplex Assay Instructions for use) by adding 5 µL of SC2PC into 45 µL molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2.
- Add 80 µL of respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with external lysis buffer previously added.
- To prepare samples with SIPC at a moderate concentration, add 10 µL of undiluted SIPC into each tube labeled 1-3, with external lysis buffer and respiratory specimen previously added.
- To prepare samples with SIPC at a low concentration, add 10 µL of 1:100 dilution of SIPC into each tube labeled 4-6, with external lysis buffer and respiratory specimen previously added.
- To prepare negative control samples, add 10 µL of molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2 into each tube labeled 7-9, with external lysis buffer and respiratory specimen previously added.
- To finish preparation of the moderate concentration samples (tubes 1-3), add 10 µL undiluted, rehydrated SC2PC to each tube.
- To finish preparation of the low concentration samples (tubes 4-6), add 10 µL of the 1:10 SC2PC to each tube.
- To finish preparation of the negative samples (tubes 7-9), add 10 µL of the molecular-grade nuclease-free water or 10 mM Tris, pH 7.4-8.2 to each tube.
- Perform extractions of all nine samples according to the Flu SC2 Multiplex Instructions for Use.

Procedure

Follow the Flu SC2 Multiplex Assay Instructions for Use for testing each of the moderate concentration, low concentration, and negative samples at least once, but consult state and local jurisdiction requirements to ensure compliance with CLIA regulations.

Expected Results

Moderate concentration samples (tubes 1-3) should be positive for SARS-CoV-2 (SC2), influenza A virus (InfA) and influenza B virus (InfB), and RnaseP (RP).

Low concentration samples (tubes 4-6) should be positive for SARS-CoV-2 (SC2), influenza A virus (InfA) and influenza B virus (InfB), and RnaseP (RP).

Negative samples (tubes 7-9) should be negative for SARS-CoV-2 (SC2), influenza A virus (InfA), and influenza B virus (InfB), but positive for RNase P (RP).

≥ 89% of test results should be in agreement with the expected results. If percent agreement is less than 89%, contact CDC at CDCSARS2FluAB@cdc.gov. Successful verification is required prior to use of the Flu SC2 Multiplex Assay for diagnostic testing.

Questions

Please send questions or comments about these instructions by email to CDCSARS2FluAB@cdc.gov. Please use subject line: Flu SC2 Multiplex Verification



FluSC2-EUA

**Influenza SARS-CoV-2 Multiplex Assay
Product Information Sheet**

DO NOT DISCARD: Important product-specific information

Influenza SARS-CoV-2 Multiplex Assay
For use under EMERGENCY USE AUTHORIZATION (EUA) only
Rx only

CATALOG: FluSC2-EUA

KIT LOT:

EXPIRATION DATE: YYYY-MM-DD (2 Years from DOM)

INTENDED USE

The Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay is a real-time RT-PCR multiplexed test intended for the simultaneous qualitative detection and differentiation of SARS-CoV-2, influenza A virus, and/or influenza B virus nucleic acids in upper or lower respiratory specimens (such as nasopharyngeal, oropharyngeal and nasal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals suspected of respiratory viral infection consistent with COVID-19 by a healthcare provider¹. Symptoms of respiratory viral infection due to SARS-CoV-2 and influenza can be similar.

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, that meet requirements to perform high complexity tests.

The Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay is intended for use in the detection and differentiation of SARS-CoV-2, influenza A, and/or influenza B viral RNA in patient specimens, and is not intended to detect influenza C. RNA from influenza A, influenza B, and/or SARS-CoV-2 viruses is generally detectable in upper and/or lower respiratory specimens during infection. Positive results are indicative of active infection but do not rule out bacterial infection or co-infection with other viruses; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all SARS-CoV-2 positive results to the appropriate public health authorities.

Negative Flu SC2 Multiplex Assay results do not preclude SARS-CoV-2, influenza A, and/or influenza B infection and should not be used as the sole basis for diagnosis, treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and/or epidemiological information.

Negative results obtained from individuals who are not exhibiting clinical signs and symptoms associated with respiratory viral infection at the time of specimen collections should be interpreted with particular caution. Negative results in asymptomatic individuals cannot be used as definitive evidence that an individual has not been exposed to SARS-CoV-2 or influenza viruses and has not been infected with any of these viruses.

Testing with the Flu SC2 Multiplex Assay is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The Flu SC2 Multiplex Assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

¹For this EUA, a healthcare provider includes, but is not limited to, physicians, nurses, pharmacists, technologists, laboratory directors, epidemiologists, or any other practitioners or allied health professionals.



FluSC2-EUA

**Influenza SARS-CoV-2 Multiplex Assay
Product Information Sheet****PACKAGE CONTENTS**

PACKAGING	COMPONENT	PART NUMBER	COMPONENT LOT NUMBER	VIALS PER KIT	QUANTITY /VIAL	STATE
Box	Flu SC2 Combined Primer Mix	MR-665		1	80 nmol	1.5 mL aqueous
	Flu SC2 Combined Probe Mix	MR-666		1	10 nmol	1.5 mL aqueous
	SIPC Seasonal Influenza Positive Control (non-infectious)	MR-541		1	1.2mL	aqueous
	SC2PC Positive Control (non-infectious)	MR-669		1	1 x 10 ⁴ copies/μL	Dried

STORAGE INSTRUCTIONS

Upon receipt, store all primers, probes, and SIPC positive control at ≤-20°C. Store the SC2PC control material at 2-8°C until rehydrated for use. Refer to the CDC Influenza SARS-CoV-2 Multiplex Assay Instructions for Use before opening and preparing reagents for use.

PROCEDURE/INTERPRETATION/LIMITATIONS

Users should refer to the **CDC Influenza SARS-CoV-2 Multiplex Assay Instructions for Use** posted on the FDA website for all IVD products used under Emergency Use Authorization, <http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm>.

IVD**PRECAUTIONS**

This reagent should be handled in an approved BSL-2 handling area to avoid contamination of laboratory equipment and reagents that could cause false positive results. This product is non-infectious. However, this product should be handled in accordance with Good Laboratory Practices.

This test has not been FDA cleared or approved; this test has been authorized by FDA under an EUA for use by laboratories certified under CLIA, that meet requirements to perform high complexity tests.

This test has been authorized only for the simultaneous qualitative detection and differentiation of nucleic acids from SARS-CoV-2, influenza A virus and influenza B virus, and not for any other viruses or pathogens.

This test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostic tests for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.

REAGENT COMPLAINTS/QUESTIONS

If you have a question/comment about this product, please contact the CDC by email at CDCSARS2FluAB@cdc.gov. Please include "Flu SC2 Multiplex" in the subject line.

DISTRIBUTED BY

Manufactured by the Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, Georgia, 30329, USA