CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (CDC Flu rRT-PCR Dx Panel)

Influenza A Subtyping Kit (VER 4)

Instructions for Use
Package Insert

Catalog # FluIVD03-12
1000 reactions

For *In-vitro* Diagnostic (IVD) Use

Rx Only

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

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Intended Use

The Influenza A Subtyping Kit contains reagents and controls of the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel and is intended for use in real-time RT-PCR (rRT-PCR) assays on an *in vitro* diagnostic real-time PCR instrument that has been FDA-cleared for use with this kit in conjunction with clinical and epidemiological information:

- For determination of the subtype of seasonal human influenza A viruses as seasonal A(H3) and/or A(H1)pdm09 from viral RNA in upper respiratory tract clinical specimens (including nasopharyngeal swabs [NPS], nasal swabs [NS], throat swabs [TS], nasal aspirates [NA], nasal washes [NW] and dual nasopharyngeal/throat swabs [NPS/TS]) and lower respiratory tract specimens (including bronchoalveolar lavage [BAL], bronchial wash [BW], tracheal aspirate [TA], sputum, and lung tissue) from human patients with signs and symptoms of respiratory infection and/or from viral culture;

- To provide epidemiologic information for surveillance of circulating influenza viruses.

Performance characteristics for influenza were established during a season when seasonal influenza viruses A(H1N1) and A(H3N2) were the predominant influenza A viruses in circulation and during a season when the A(H1N1)pdm09 influenza virus was the predominant influenza A virus in circulation. Performance characteristics may vary with other emerging influenza A viruses.

Negative results do not preclude influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions. Conversely, positive results do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease.

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 with appropriate infection control precautions for novel virulent influenza viruses and sent to state or local health department for testing. Viral culture should not be attempted unless a BSL 3E facility is available to receive and culture specimens.

All users, analysts, and any person reporting results from use of this device should be trained to perform and interpret the results from this procedure by a competent instructor prior to use. CDC Influenza Division will limit the distribution of this device to only those users who have successfully completed a training course provided by CDC instructors or designees.

Summary and Explanation

Influenza viruses are the causative agents of influenza infection, an acute respiratory illness that is highly contagious and results in significant morbidity each year with flu-related deaths ranging from a low of about 3,000 to a high of about 49,000 people (https://www.cdc.gov/flu/about/disease/index.htm). Influenza virus types A and B primarily infect the nasopharyngeal and oropharyngeal cavities. Influenza A viruses are further categorized into subtypes based on two major surface proteins, hemagglutinin (HA) and neuraminidase (NA). Similarly, Influenza B viruses can also be separated into two major lineages. The infective potential of influenza is frequently underestimated and can result in high morbidity and mortality rates, especially in elderly persons and in high risk patients, such as the very young and immuno-compromised. Typical influenza viral infections in humans have a relatively short incubation period of 1 to 2 days, with symptoms that last about a week including an abrupt onset of fever, sore throat, cough, headache, myalgia, and malaise. When a subject is infected with a highly virulent strain of influenza, these symptoms can progress rapidly to pneumonia and in

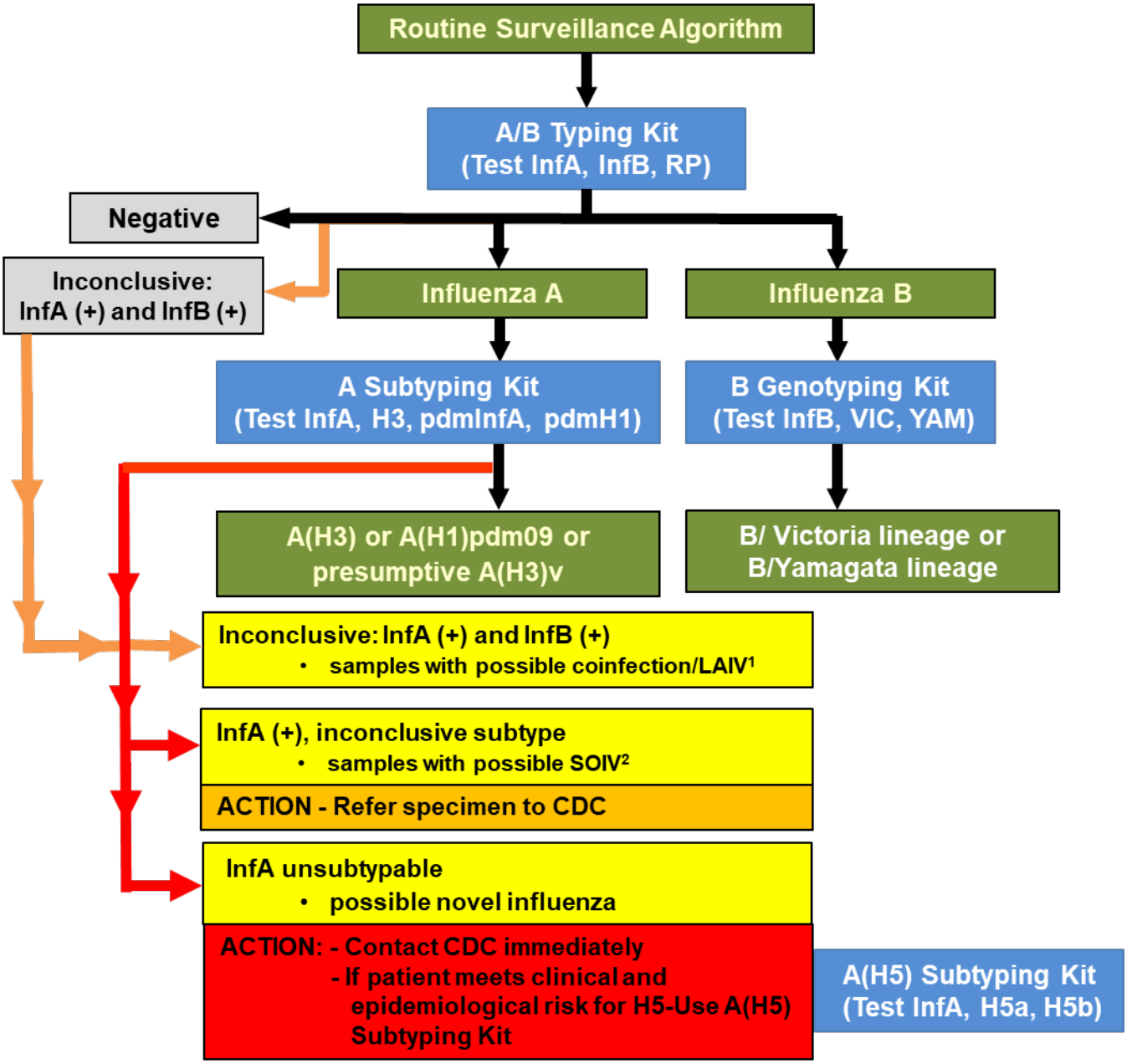
some circumstances death. Pandemic outbreaks of highly virulent influenza present a serious risk to human and animal health worldwide.

Influenza viruses may be detected and characterized from clinical specimens by several methods that vary in sensitivity, speed, and capability with regard to distinguishing influenza types and subtypes. The CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel is a nucleic acid amplification assay that detects influenza A and B viruses and further characterizes influenza A subtypes A(H1)pdm09, A(H3), and A(H5) (Asian lineage) and influenza B lineages B/Victoria and B/Yamagata. Components of the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel can be obtained in several kit configurations.

CDC Human Influenza Real-Time RT-PCR Diagnostic Panel		
Kit	**Contents**	**Application**
A/B Typing Kit	**Primer and Probe Sets:** InfA, InfB, RP **Controls:** Seasonal Influenza Positive Control (SIPC), Human Specimen Control (HSC)	• Detects all influenza A and B viruses
A Subtyping Kit	**Primer and Probe Sets:** InfA, H3, pdmInfA, pdmH1, RP **Controls:** SIPC	• Specifically detects and differentiates seasonal influenza subtypes H3 and H1pdm09 • Detects classical swine influenza viruses and swine triple reassortant viruses[1]
A/H5 Subtyping Kit	**Primer and Probe Sets:** InfA, H5a, H5b, RP **Controls:** H5VC, HSC	• Specifically detects influenza A(H5) Asian lineage viruses
B Lineage Genotyping Kit	**Primer and Probe Sets:** InfB, VIC, YAM, RP **Controls:** Influenza B Positive Control (IBPC)	• Specifically detects and differentiates influenza B lineage genotypes; B/Victoria, B/Yamagata

[1]Classical and triple reassortant swine viruses are not seasonal viruses and must be referred to CDC for further confirmation.

Laboratories may use the influenza A, B, A(H1)pdm09, and A(H3) and A(H5) (Asian Lineage) primer and probe sets simultaneously to type and/or subtype suspected influenza positive clinical specimens. Additionally, influenza B viruses may be further characterized as B/Victoria or B/Yamagata lineage using the VIC and YAM primer and probe sets. The CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel assists in routine surveillance of seasonal influenza following an algorithm as follows:



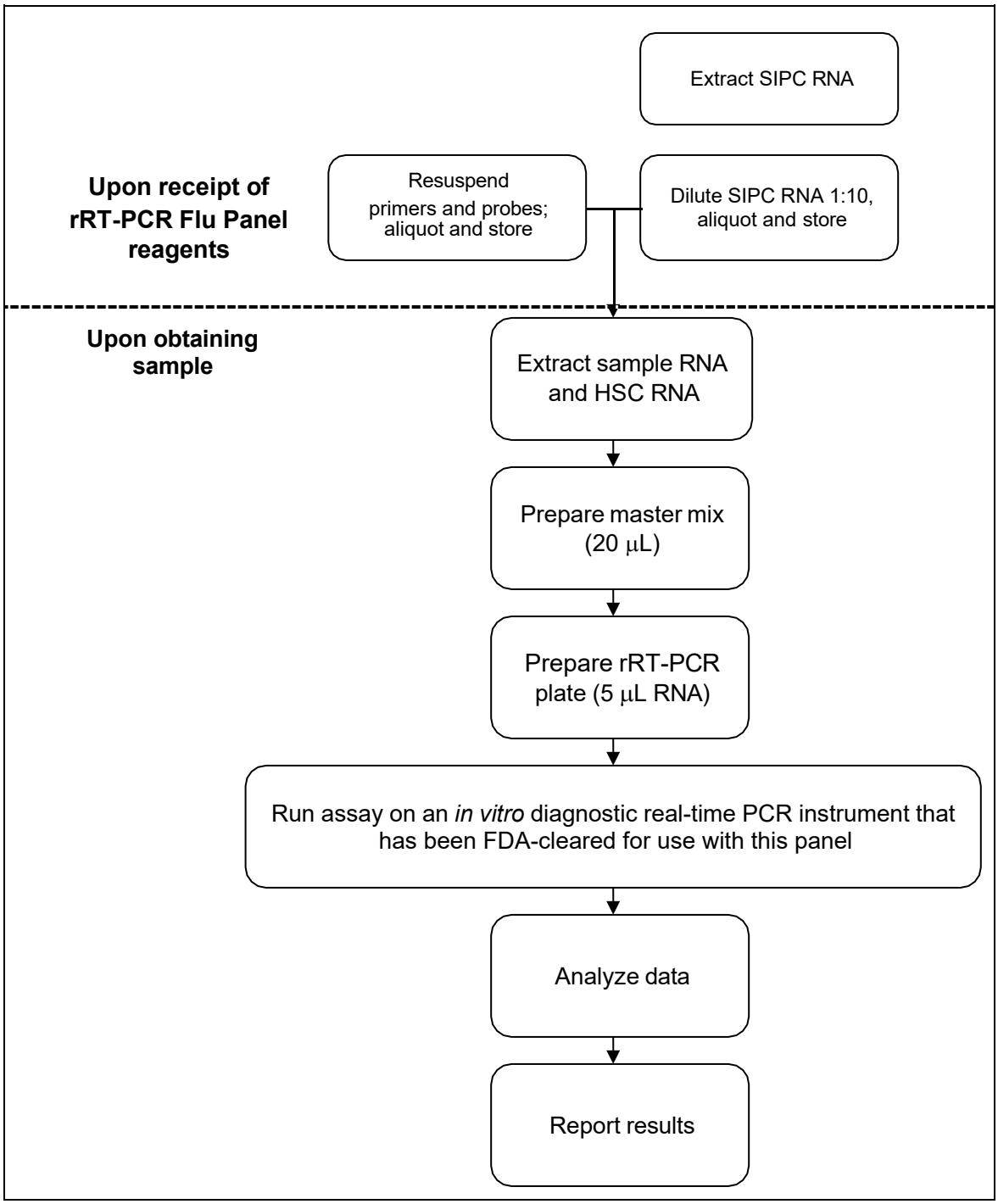
[1]LAIV=Live Attenuated Influenza Virus
[2]SOIV=Swine Origin Influenza Virus

Principles of the Procedure

The Influenza A Subtyping Kit contains components of the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel that is used in rRT-PCR assays on an FDA-cleared *in vitro* diagnostic real-time PCR instrument. The primer and probe sets contained in the Influenza A Subtyping Kit are designed for the detection and characterization of influenza type A viruses that infect humans.

The Influenza A Subtyping Kit consists of oligonucleotide primers and dual-labeled hydrolysis (TaqMan®) probes and controls, which may be used in rRT-PCR assays for the in vitro qualitative detection and characterization of the human influenza virus RNA in respiratory specimens from patients presenting with influenza-like illness (ILI). The oligonucleotide primers and probes for detection of Influenza A and 2009 Influenza A (swine origin) were selected from highly conserved regions of the matrix (M), and the nucleoprotein (NP), respectively. Oligonucleotide primers and probes for characterization and differentiation of seasonal influenza A(H3) and A(H1)pdm09 viruses were selected from highly conserved regions of their respective HA genes. Detection of viral RNA not only aids in the diagnosis of illness caused by seasonal, newly emerging, and novel influenza viruses in patients with ILI, but also provides epidemiological and surveillance information on influenza and aids in the presumptive laboratory identification of specific novel influenza A viruses.

Summary of Influenza Testing Process



Materials Required (Provided)

Influenza A Subtyping Kit (VER 4) Contents: Catalog # FluIVD03-12

Box#1: Primers and Probes

Reagent Label	*Part #*	*Description*	*Quantity / Tube*	*Reactions / Tube*
InfA-F	MR-637	Influenza A Forward Primer	20 nmol	1000
InfA-R	MR-638	Influenza A Reverse Primer	20 nmol	1000
InfA-P	SO3285 or MR-431	Influenza A Probe (FAM)	5 nmol	1000
H3-F	MR-719	Influenza A(H3) Forward Primer	20 nmol	1000
H3-R	MR-720	Influenza A(H3) Reverse Primer	20 nmol	1000
H3-P	MR-721 or MR-722	Influenza A(H3) Probe (FAM)	5 nmol	1000
pdmInfA-F	MR-640	Influenza A pdm09 Forward Primer	20 nmol	1000
pdmInfA-R	MR-641	Influenza A pdm09 Reverse Primer	20 nmol	1000
pdmInfA-P	MR-038 or MR-529	Influenza A pdm09 Probe (FAM)	5 nmol	1000
pdmH1-F	MR-644	Influenza A(H1)pdm09 Forward Primer	20 nmol	1000
pdmH1-R	MR-518	Influenza A(H1)pdm09 Reverse Primer	20 nmol	1000
pdmH1-P	MR-639	Influenza A(H1)pdm09 Probe (FAM)	5 nmol	1000
RP-F	SO3313	Human RNase P Forward Primer	20 nmol	1000
RP-R	SO3314	Human RNase P Reverse Primer	20 nmol	1000
RP-P	SO3315 or MR-432	Human RNase P Probe (FAM)	5 nmol	1000

Box #2: Seasonal Influenza Positive Control

Reagent Label	*Part #*	*Description*	*Quantity / Tube*	*Notes*
SIPC	MR-541	Seasonal Influenza Positive Control (SIPC): For use as a positive control with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel procedure to ensure the detection of seasonal influenza virus A(H3), A(H1)pdm09, and influenza type B. The SIPC contains noninfectious positive control materials supplied as a liquid, 500 µL per vial, suspended in 0.01 M phosphate buffer saline (PBS) at pH 7.2-7.4. SIPC consists of three (3) different beta-propiolactone treated influenza viruses (influenza A(H3), A(H1)pdm09, and influenza B) and cultured human cells (A549). SIPC will yield a positive result with the following primer and probe sets: InfA, InfB, H3, pdmInfA, pdmH1, and RP.	1 tube x 900 µL / tubes	One thousand eight hundred, 5 µL reactions per tube

Critical Equipment and Materials Required (But Not Provided)

Real-Time PCR Instruments and Software

Manufacturer	Instrument	Software
ThermoFisher Scientific	Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument	SDS Software version 1.4
ThermoFisher Scientific	Applied Biosystems™ QuantStudio™ Dx (QSDx) Real-Time PCR Instrument	QuantStudio™ Dx software version 1.0.3
QIAGEN	Rotor-Gene Q MDx (QMDx)	Rotor-Gene AssayManager version 1.0.4.1 Epsilon (US) Plug-In version 1.0.1

rRT-PCR Enzyme Master Mix Options

Reagent	Quantity	Catalog No.
Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (without Rox)	100 reactions	11732-020
	500 reactions	11732-088
Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (with Rox)	100 reactions	11745-100
	500 reactions	11745-500
Quanta BioSciences qScript™ One-Step qRT-PCR Kit, Low ROX	50 reactions	95059-050
Quanta BioSciences qScript™ One-Step qRT-PCR Kit, Low ROX	200 reactions	95059-200

RNA Extraction Options

Instrument/Manufacturer	Extraction Kit	Catalog No.
Roche MagNA Pure LC	Total Nucleic Acid Kit	192 extractions: 03 038 505 001
Roche MagNA Pure 96	DNA and Viral NA Small Volume Kit	576 extractions 06 543 588 001 External Lysis Buffer 06 374 913 001
QIAGEN	QIAmp DSP Viral RNA Mini Kit	50 extractions: 61904
QIAGEN QIAcube	QIAmp DSP Viral RNA Mini Kit	50 extractions: 61904
QIAGEN EZ1 Advanced XL	EZ1 DSP Virus Kit	48 extractions (62724) Buffer AVL (19073) EZ1 Advanced XL DSP Virus Card (9018703)

Instrument/Manufacturer	Extraction Kit	Catalog No.
	EZ1 RNA Tissue Mini Kit	48 extractions: 959034 EZ1 Advanced XL RNA Card: 9018705
bioMérieux NucliSENS® easyMAG®and bioMérieux EMAG® (Automated magnetic extraction reagents sold separately. Both instruments use the same reagents and disposables, with the exception of tips.)		EasyMAG® Magnetic Silica (280133) EasyMAG® Disposables (280135) EasyMAG® Lysis Buffer (280134) EasyMAG® Wash Buffers 1,2, and 3 (280130, 280131, 280132) EMAG® 1000µL Tips (418922)

CDC Approved Ancillary Reagents with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel

Specific lots for the ancillary reagents that are not manufactured under Quality System Regulations (Invitrogen SuperScript™ and Quanta BioSciences qScript™) will be qualified for use with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel by the CDC Influenza Division. Any lots not specifically qualified by the CDC Influenza Division for use with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel are not valid for use with this device, and may affect device performance.

A supplemental cumulative list of the qualified ancillary reagents lots for use with the Flu rRT-PCR Dx Flu Panel is available and can be requested by sending an email to FluSupport@cdc.gov or downloaded directly from the FluSupport SharePoint website at https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.

Any issues related to assay performance or test failure that are suspected to involve ancillary reagents should be reported to the CDC Influenza Division by emailing FluSupport@cdc.gov.

General Equipment and Consumables Required (But Not Provided)

Manufacturer	Consumables	Catalog No.
ThermoFisher Scientific	MicroAmp™ Fast 8-tube strip 0.1 mL	#4358293
	MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (alternate to 8-strip tubes)	#4346906, #4346907, or #4366932
	MicroAmp™ Optical 8-cap strip (required, do not use film)	#4323032
Applied Biosystems™ (ThermoFisher Scientific)	96-well Fast Plate	4481190
	96-well Fast Plate with Barcode (alternative)	4481194
	Optical Adhesive Covers	4481193
QIAGEN	4-Strip Tubes and Caps 0.1 mL (4 tubes and caps)	#981103 or #981106

- Plasticware and consumables
- RNase/DNase-free 1.5 mL polypropylene microcentrifuge tubes
- 100% ethanol (EtOH)
- Disposable gloves
- Molecular grade water (RNase/DNase Free)
- -70°C and -20°C freezer(s)
- 4°C refrigerator
- 96-well cold block
- Micropipettors (range between 1-10 µL, 10-200 µL and 100-1000 µL)
- Benchtop microcentrifuge

Warnings and Precautions

- For *in vitro* diagnostic use (IVD).
- 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) current edition for standard biological safety guidelines for all procedures (Search: CDC BMBL).
- Specimen processing should be performed in accordance with national biological safety regulations.
- 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 with 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 that cause cases or clusters of human disease, as opposed to those strains commonly circulating in humans that cause seasonal epidemics and to which**

human populations have residual or limited immunity (either by vaccination or previous infection).

- Performance characteristics have been determined with human upper respiratory specimens (including NPS, NS, TS, NA, NW, NPS/TS) and lower respiratory tract specimens (including BAL, BW, TA, sputum and lung tissue) from human patients with signs and symptoms of respiratory infection and/or from viral culture.
- 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 amplifications 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 prior to use. Do not use expired reagent. Do not substitute or mix reagent 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 5% 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 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.

Reagent Storage, Handling, and Stability

- Store all primers and probes at 2-8°C until re-hydrated for use; Store all control materials (SIPC) at ≤ -20°C.
- 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 cold block at all times during preparation and use.
- Do not refreeze 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
  - Follow specimen collection devices 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.
- Transporting Specimens
  - Ensure that, when transporting human respiratory specimens, all applicable regulations for the transport of etiologic agents are met. Human respiratory specimens to be tested within 72 hours post-collection should be transported refrigerated at 2–8°C. Alternatively, specimens may be frozen and transported for testing.
  - Respiratory specimens should be collected and placed into viral transport media (VTM)) or universal transport media (UTM) as described by CDC and WHO guidelines. Lavage specimens in phosphate buffered saline (PBS) are accepted.. See the FluSupport SharePoint site for more information: FluSupport SharePoint site at **https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx** .
- Storing Specimens
  - Specimens received cold should be stored refrigerated (2–8°C) for up to 72 hours before processing. Store any residual specimens at ≤ -70°C.
  - Although optimal performance is met when testing fresh specimens within 72 hours of collection, performance has been demonstrated with frozen specimens. If testing of a fresh specimen is not possible within 72 hours storage at 2–8°C, the specimen may be frozen at ≤ -70°C and tested at a later time.
  - Specimens received frozen should be stored at ≤ -70°C until processing. Store any residual specimens at ≤ -70°C.
- Storing Purified Nucleic Acid
  - Store purified nucleic acids at ≤ -70°C.

Specimen Referral to CDC

- Referring a specimen to the CDC
  - Ship all specimens overnight to CDC.
  - Ship specimens 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.
  - Prior to shipping, notify CDC Influenza Division that you are sending specimens by sending an email to FluSupport@cdc.gov.
  - Fill out the Influenza Specimen Submission Form completely – include specimen type and Ct values detected by your laboratory. Specimen Submission Form can be found on the FluSupport SharePoint site at https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.
  - Send all samples to the following recipient:

Attention: Genomics and Diagnostics Team (Unit 200)
c/o STAT Lab
Virology, Surveillance and Diagnosis Branch, Influenza Division
Centers for Disease Control and Prevention
1600 Clifton Rd., Atlanta, GA 30329-4027
Phone: (404) 639-2434

The emergency contact number for CDC Emergency Operations Center (EOC) is 770-488-7100

Reagent and Controls Preparation

Primer and Probe Preparation:

1. Upon receipt, store lyophilized primers and probes at 2-8°C.
2. Rehydration
   a. Remove primers and probes from 2-8°C.
   b. Allow primers and probes to sit at ambient temperature for 15 minutes.
   c. Quickly centrifuge dried primers and probes to collected pellet in the bottom of the tube.
   d. Pipette 0.5 ml (500 µL) of 10 mM Tris, pH 7.4-8.2 or PCR-grade water into each dried PCR primer or probe.
   e. Allow primers and probes to fully rehydrate for at least 15 minutes at room temperature.
   f. After primers and probes are fully rehydrated, pulse vortex to ensure a homogenous solution.
3. Prepare a Combined Oligonucleotide Mix for Each Marker Set
   a. For each marker set (e.g. InfA), pipette the entire volume (0.5 mL) of the reconstituted forward and reverse primers and probe into a single new, nuclease-free, sterile tube (henceforth, the primer/probe mix).
   b. Pulse vortex the combined primer/probe mix to ensure a homogenous solution.
4. Aliquot
   a. Label five new, nuclease-free, sterile tubes for each combined primer/probe mix with the following information:
      - Marker set name
      - Kit lot #
      - Expiration date
   b. Aliquot 300 µL of each combined primer/probe mix into respective labeled tubes and store at -20°C.
5. Storage
   a. After rehydration
      i. Aliquots of primers and probes are stored at -20°C or below until expiration date as long as QC requirements are met.
      ii. Thawed aliquots of primers and probes may be stored at 2-8°C for up to 3 months. Combined primers/probe mix aliquots should be stored in the dark.

Seasonal Influenza Positive Control (SIPC) Preparation:

1. Reagent
   a. Inactivated, noninfectious influenza virus preparation supplied as a liquid suspension in 0.01 M PBS.
   b. Control contains three different influenza viruses representing influenza A(H3), A(H1)pdm09, and influenza B viruses in cultured human cells.
   c. Volume: 0.9 mL yields approximately 9 mL of positive control RNA.
2. Storage
   a. Store at -20°C or below upon receipt. Do not dilute.
3. Procedure
   a. SIPC must be extracted prior to use. The final volume of eluted RNA should equal the volume of extracted control material. For example, 100 µL of control material should result in 100 µL of RNA extract (1:1).
   b. Dilute the RNA 1:10 with nuclease free water.
   c. Label one new nuclease-free, sterile, tube for each single-use aliquot with the following information:
      - Control RNA name and 1:10 dilution
      - Kit lot #
      - Expiration date
   d. Dispense the 1:10 diluted RNA into single-use aliquots and store at -20°C or below for up to 6 months. Recommended aliquot volumes (but not required):
      - 40 µL aliquots for use with the Influenza A Subtyping Kit assays.

e. Use one aliquot per run. Discard after use. Do not use residual RNA.

General Preparation

Equipment Preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use. Decontamination agents should be used including 5% 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 rack during assay preparation.

Primers and Probes Reagents

- Thaw frozen aliquots of combined primer/probe mix. *Thawed aliquots may be stored at 2-8°C in the dark for up to 3 months.* ***Do not re-freeze****.*
- Vortex each combined primer/probe mix for 15 seconds.
- Briefly centrifuge each primer/probe mix.
- Place combined primer/probe mix in cold rack during master mix preparation.

Real-time RT-PCR Reagents (Important: Select Appropriate Enzyme System)

Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System

- Place Invitrogen 2X PCR Master Mix and Superscript III RT/Platinum Taq enzyme mix in a cold rack at 2-8°C.
- Completely thaw the 2X PCR Master Mix vial.
- Mix the 2X PCR Master Mix by inversion 10 times.
- Briefly centrifuge 2X PCR Master Mix and Superscript III RT/Platinum Taq enzyme mix then place in cold rack.

OR

Quanta BioSciences qScript™ One-Step qRT-PCR Kit, Low ROX

- Place Quanta qScript™ One-Step Master Mix (2X) and qScript™ One-Step Reverse Transcriptase in a cold rack at 2-8°C.
- Completely thaw the One-Step Master Mix (2X) vial.
- Mix the One-Step Master Mix (2X) by inversion 10 times.
- Briefly centrifuge One-Step Master Mix (2X) and qScript™ One-Step Reverse Transcriptase then place in cold rack.

Nucleic Acid Extraction

Performance of the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel 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 panel:

QIAGEN QIAamp® DSP Viral RNA Mini Kit

Recommendation(s): Utilize 100 µL of sample and elute with 100 µL of buffer, or utilize 140 µL of sample and elute with 140 µL of buffer.

QIAGEN QIAcube with the QIAamp DSP Viral RNA Mini Kit
Kit: QIAGEN QIAamp® DSP Viral RNA Mini Kit
Recommendations: Utilize 140 µL of sample and elute with 100 µL of buffer.

QIAGEN EZ1 Advanced XL
Kit: QIAGEN EZ1 DSP Virus Kit and Buffer AVL (supplied separately) for off-board lysis
Card: EZ1 Advanced XL DSP Virus Card
Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 120 µL.

Roche MagNA Pure LC
Kit: Roche MagNA Pure Total Nucleic Acid Kit
Protocol: Total NA External_lysis
Recommendation(s): Add 100 µL of sample to 300 µL of pre-aliquoted TNA isolation kit lysis buffer (total input sample volume is 400 µL). Elution volume is 100 µL.

Roche MagNA Pure 96
Kit: Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit
Protocol: 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 (**Note: Internal Control = None)**. Elution volume is 100 µL.

bioMérieux NucliSENS® easyMAG® Instrument
Protocol: General protocol (not for blood) using "Off-board Lysis" reagent settings
Recommendation(s): Add 100 µL of sample to 1000 µL of pre-aliquoted easyMAG lysis buffer (total input sample volume is 1100 µL). Incubate for 10 minutes at room temperature. Elution volume is 100 µL.

bioMérieux EMAG® Instrument
Protocol: Custom protocol: CDC Flu V1 using "Off-board Lysis" reagent settings.
Recommendation(s): Add 100 µL of samples to 2000 µL of pre-aliquoted easyMAG lysis buffer (total input sample volume is 2100 µL). Incubate for 10 minutes at room temperature. Elution volume is 100 µL. The custom protocol, CDC Flu V1, is programmed on the bioMérieux EMAG® instrument with the assistance of a bioMérieux service representative. Installation verification is documented at the time of installation. Laboratories are recommended to retain a record of the step-by-step verification of the bioMérieux custom protocol installation procedure.

Manufacturer's recommended procedures (except as noted in recommendations above) are to be used for sample extraction.

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 BiosystemsTM 7500 Fast Dx or Applied BiosystemsTM QuantStudioTM Dx

IMPORTANT INFORMATION!

The CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel consists of individual assays that can be utilized to meet laboratory testing algorithms, for example to perform high throughput screening using the Influenza A/B Typing Assay. However, CDC strongly recommends that laboratories perform subtype characterization of all the influenza A positives with the Influenza A Subtyping Kit. Current recommendations can be found on the FluSupport SharePoint site at https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.

Master Mix Preparation / Plate Setup

If extracted specimen RNA has been previously tested with the Influenza A/B Typing Kit and generated valid results for the RP marker set and HSC extraction control, the RP marker and HSC control need not be repeated when using the Influenza A Subtyping Kit. However, in the event that specimens need to be re-extracted or if the Influenza A Subtyping Kit is used directly, then RP and the HSC control should be included in the run. **Additional plate setups that include HSC are available on the FluSupport SharePoint site (**https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx).

1. In the assay preparation area, label a sterile, nuclease-free, 1.5 mL tube for each specific master mix / marker to be prepared (i.e. InfA, pdmH1, H3, etc.).
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 **Figure 1**.

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

Example: If number of samples (n) including controls = 1 to 14, then N = n + 1
If number of samples (n) including controls > 15, then N = n + 2

Figure 1. Steps and Calculations for Master Mix Preparation (Important: Select Appropriate Enzyme System)

Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 5.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	SuperScript™ III RT/Platinum® *Taq* Mix	N x 0.5 µL
4	2X PCR Master Mix	N x 12.5 µL
	Total Volume	**N x 20.0 µL**

OR

Quanta BioSciences qScript™ One-Step qRT-PCR Kit, Low ROX

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 5.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	Quanta qScript™ One-Step Reverse Transcriptase	N x 0.5 µL
4	One-Step Master Mix (2X)	N x 12.5 µL
	Total Volume	**N x 20.0 µL**

4. Dispense reagents into each respective labeled 1.5 mL microcentrifuge tube. After addition of the reagents, mix reaction mixtures by pipetting up and down. ***Do not vortex***.
5. Centrifuge for 5 seconds 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 each master mix into the appropriate wells going across the row as shown below (**Figure 2**).

Figure 2. Influenza A Subtyping Kit: Example of Reaction Master Mix Plate Set-up

	1	2	3	4	5	6	7	8	9	10	11	12
A	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA
B	H3	H3	H3	H3	H3	H3	H3	H3	H3	H3	H3	H3
C	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA
D	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1
E	Empty	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	Empty
F	Empty	H3	H3	H3	H3	H3	H3	H3	H3	H3	H3	Empty
G	Empty	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	pdm InfA	Empty
H	Empty	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	pdm H1	Empty

NOTE: If specimens were not already tested with the Influenza A/B Typing Kit and produced valid RP results, the specimen will need to be tested with RP. Additional plate setup options and assay profiles that include RP are available on the FluSupport SharePoint site (https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.)

8. Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reactions for column #1 in the assay preparation area.
9. Pipette 5 µL of nuclease-free water into the NTC sample wells. Securely cap NTC wells before proceeding.
10. Cover the entire reaction plate and move the reaction plate to the specimen nucleic acid handling area.

Sample Addition

1. Gently vortex nucleic acid sample tubes for approximately 5 seconds.
2. After centrifugation, place extracted nucleic acid sample tubes in the cold rack.
3. Samples should be added to column 2-11 (column 1 and 12 are for controls) to the specific assay that is being tested as illustrated in **Figure 3**. Carefully pipette 5.0 µL of the first sample into all the wells labeled for that sample (i.e. Sample "S1" down column #2). *Keep other sample wells covered during addition. Change tips after each addition.*
4. Securely cap the column to which the sample has 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. If necessary, add 5 µL of Human Specimen Control (HSC) extracted sample to the HSC wells (**Figure 3**, column 11). Securely cap wells after addition. **NOTE: HSC is not included with the Influenza A Subtyping Kit (FluIVD03-6). HSC must be obtained from another kit in the CDC Human Influenza Real-Time RT-PCR Diagnostic Panel. Please use the Package Insert for the Influenza A/B Typing Kit for instructions on the preparation and addition of HSC. Additional plate setups with the addition of HSC are available on the FluSupport SharePoint site (https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx).**
8. Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

Assay Control Addition

1. Pipette 5 µL of SIPC RNA to the sample wells of column 12 (**Figure 3**)**.** Securely cap wells after addition of the control RNA.
   NOTE: *If using 8-tube strips, 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 3. Influenza A Subtyping Kit: Example of Sample and Control Set-up

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	SIPC
B	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	SIPC
C	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	SIPC
D	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	SIPC
E	empty	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	empty
F	empty	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	empty
G	empty	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	empty
H	empty	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	empty

NOTE: HSC needs to be tested with each nucleic acid extraction run. If samples are from an extraction run not tested with HSC, testing of HSC is necessary. Additional plate setup options and assay profiles that include HSC are available on the FluSupport SharePoint site (https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.)

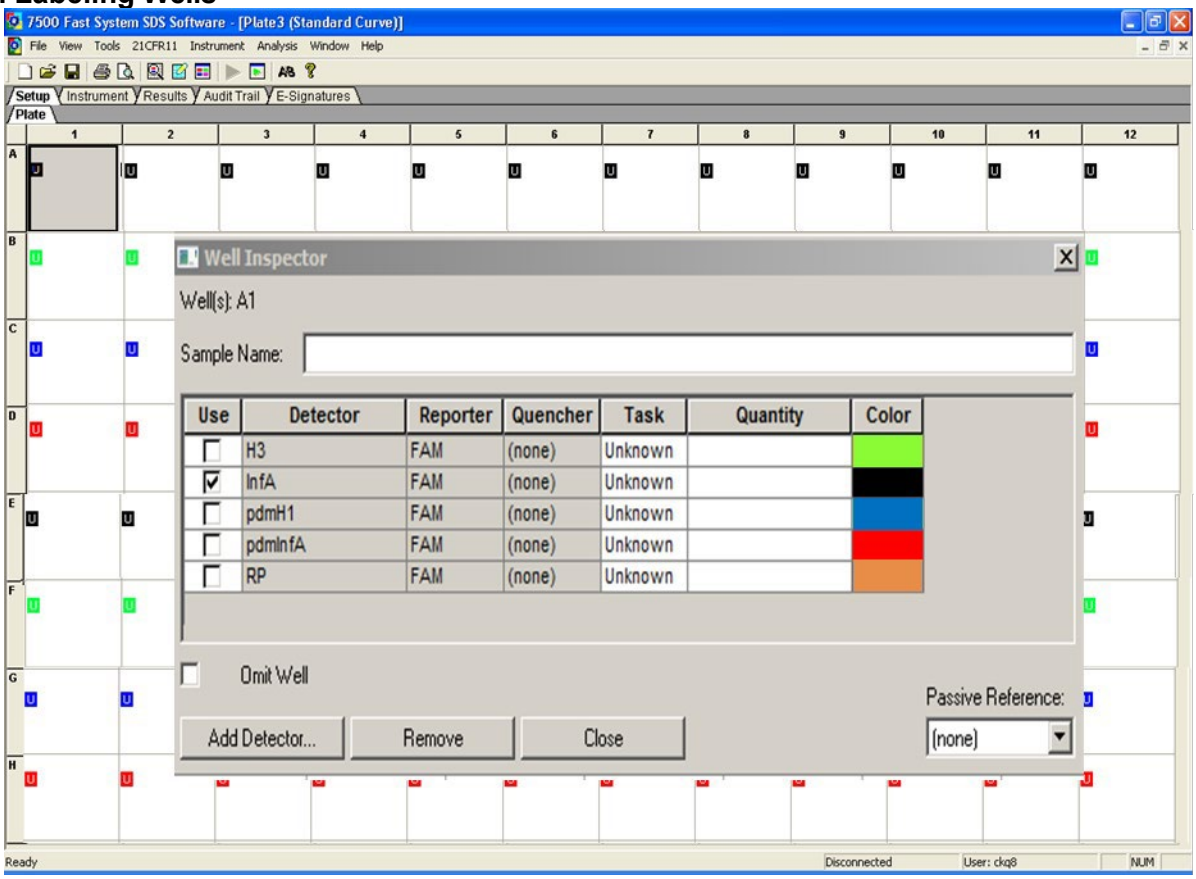
Running a Test on the Applied BiosystemsTM 7500 Fast Dx

If the most current Influenza A Subtyping Kit template does not already exist on this instrument, please proceed to *Appendix A: Instrument Setup for Applied Biosystems™ 7500 Fast Dx.*

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 (**Influenza A Subtyping Kit SuperScript** or **Influenza A Subtyping Kit qScript**).
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 4)**. The detectors and controls should already be labeled as they were assigned in the original template.

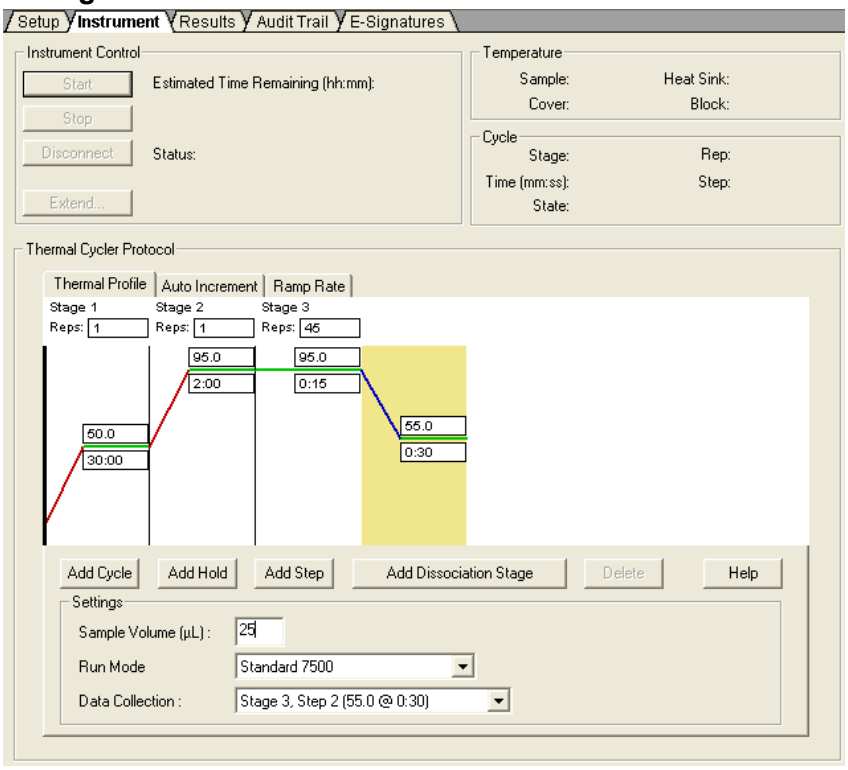
8. Click the **Well Inspector** icon  from the top menu.
9. Highlight specimen wells of interest on the plate map.
10. Type sample identifiers to **Sample Name** box in the **Well Inspector** window (**Figure 4**).

Figure 4. Labeling Wells



11. Repeat steps 9-10 until all sample identifiers are added to the plate setup.
12. 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.
13. Click the **Instrument** tab at the upper left corner.
14. The reaction conditions, volumes, and type of 7500 reaction should already be loaded. (**Figure 5**).

Figure 5. Instrument Settings

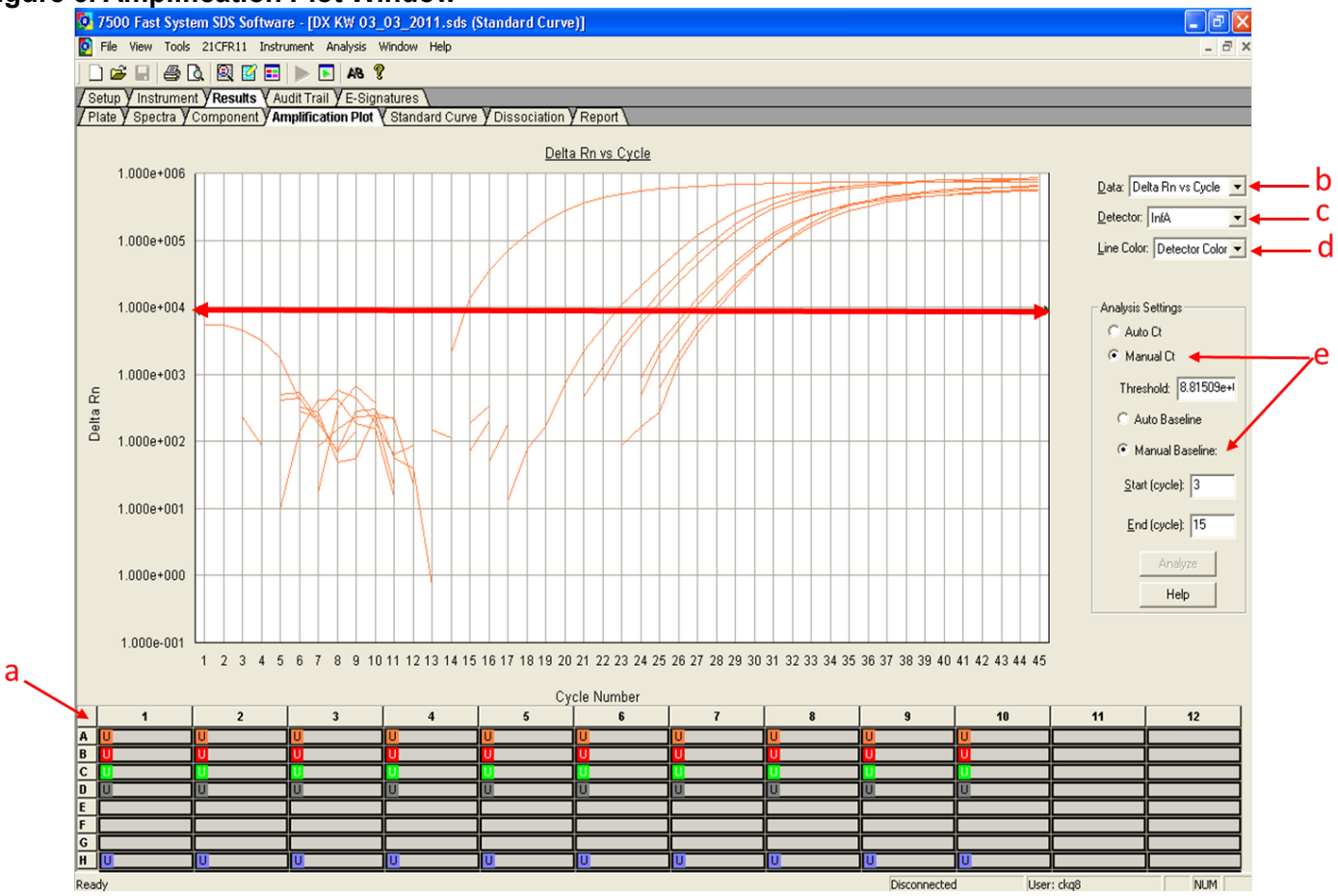


15. Ensure settings are correct (refer to *Appendix A: Instrument Setup for Applied Biosystems™ 7500 Fast Dx*).
16. Before proceeding, the run file must be saved; from the main menu, select **File,** then **Save As**. Save in appropriate run folder designation.
17. Load the plate into the plate holder in the instrument. Ensure that the plate is properly aligned in the holder.
18. Once the run file is saved, click the **Start** button. *Note: The run should take approximately 1hr and 45 minutes to complete.*

Results Analysis

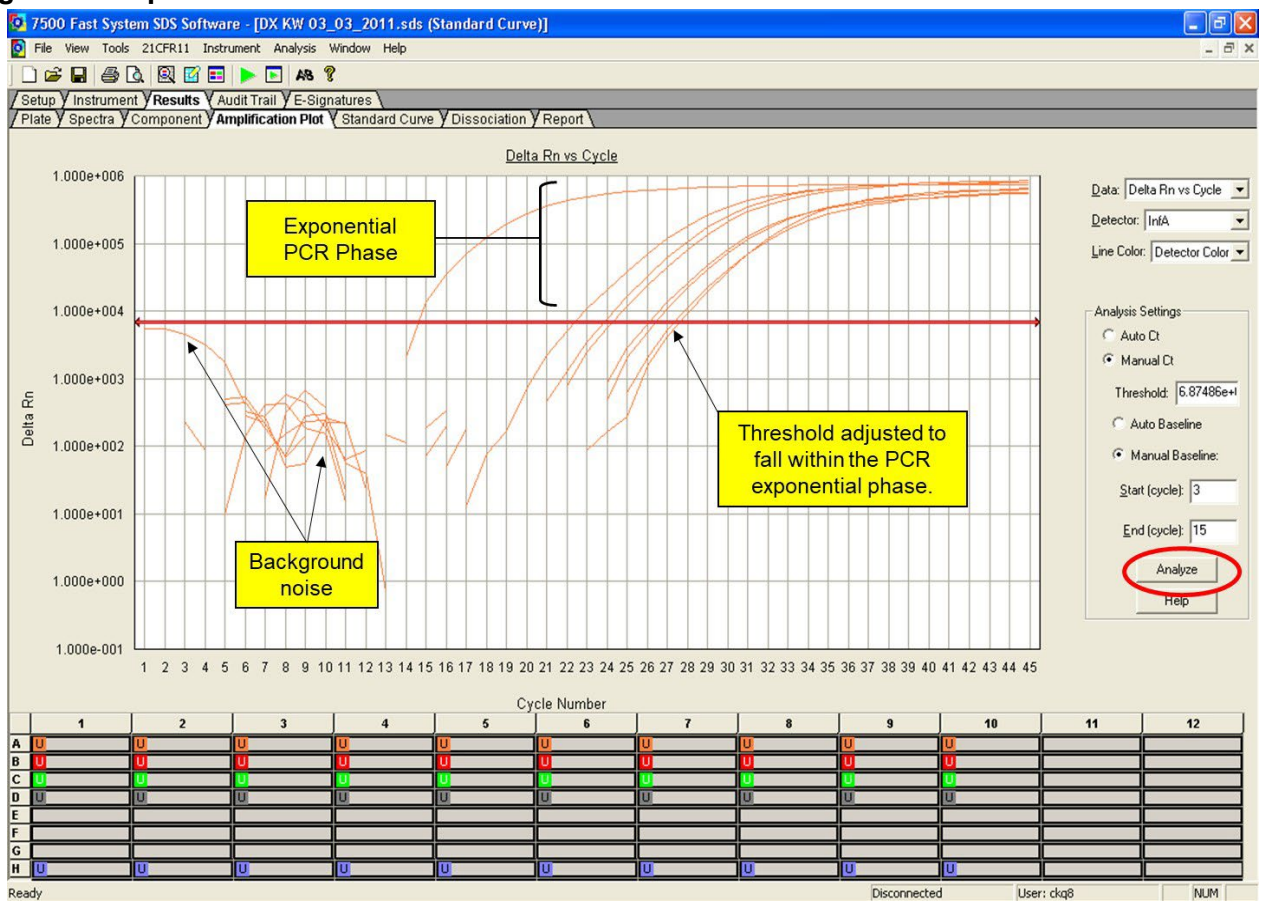
1. After the run has completed, select the **Results** tab at the upper left corner of the software.
2. Select the **Amplification Plot** tab to view the raw data (**Figure 6**).

Figure 6. Amplification Plot Window



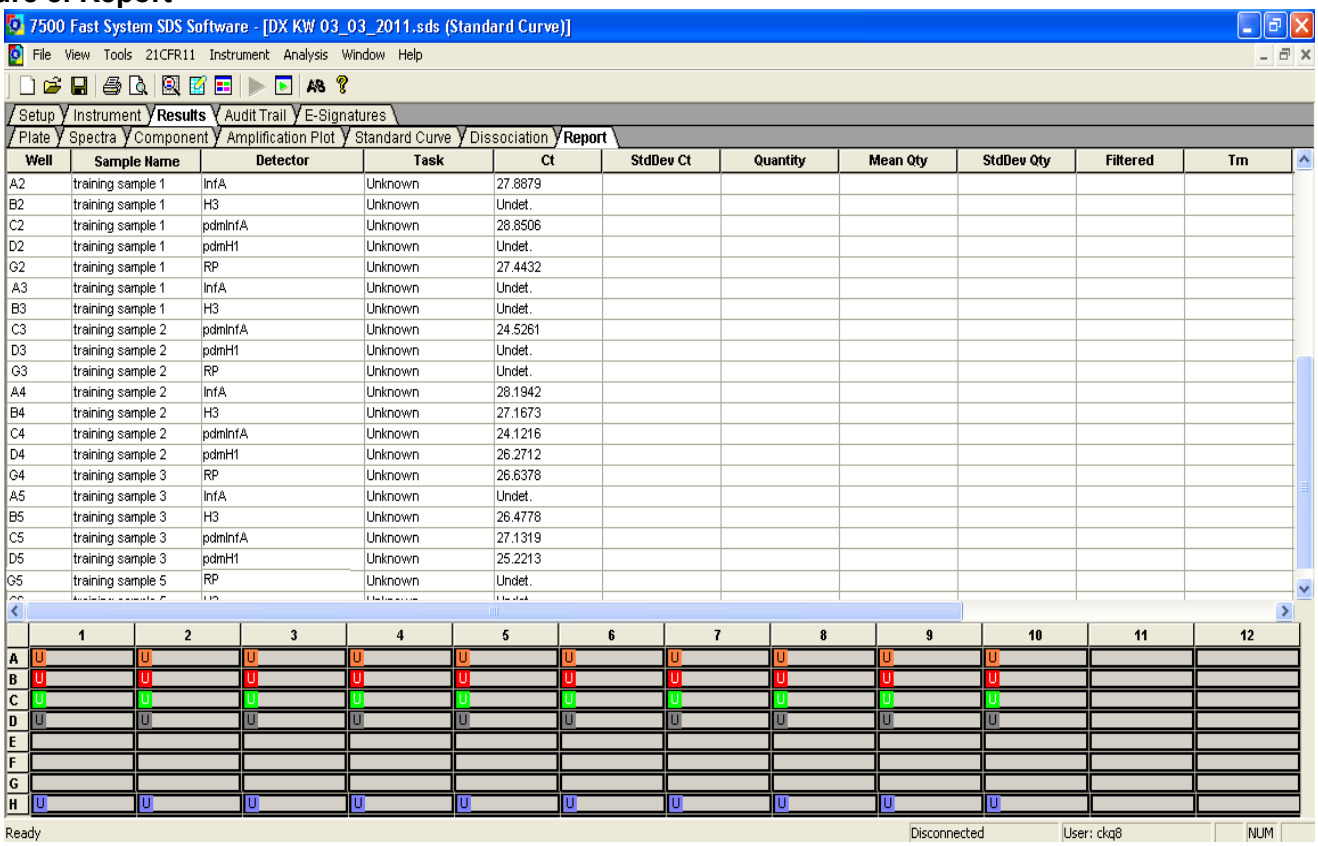
3. Start by highlighting all the samples from the run; to do this, click on the upper left box (**a**) of the sample wells (**Figure 6**). All the growth curves should appear on the graph.
4. On the right side of the window (**b**), the **Data** drop down selection should be set to **Delta Rn vs. Cycle**.
5. Select **InfA** from (**c**), the **Detector** drop down menu, using the downward arrow.
    a. Please note that each detector is analyzed individually to reflect different performance profiles of each primer and probe set.
6. In the **Line Color** drop down (**d**), **Detector Color** should be selected.
7. Under **Analysis Settings** select **Manual Ct** (**e**).
    a. Do not change the **Manual Baseline**.
8. Using the mouse, 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



9. 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.
10. Repeat steps 5-9 to analyze results generated for each set of markers (i.e. InfA, pdmH1, H3, etc.).
11. Save analysis file by selecting **File** then **Save As** from the main menu.
12. 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



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
A2	training sample 1	InfA	Unknown	27.8879						
B2	training sample 1	H3	Unknown	Undet.						
C2	training sample 1	pdmInfA	Unknown	28.8506						
D2	training sample 1	pdmH1	Unknown	Undet.						
G2	training sample 1	RP	Unknown	27.4432						
A3	training sample 1	InfA	Unknown	Undet.						
B3	training sample 1	H3	Unknown	Undet.						
C3	training sample 2	pdmInfA	Unknown	24.5261						
D3	training sample 2	pdmH1	Unknown	Undet.						
G3	training sample 2	RP	Unknown	Undet.						
A4	training sample 2	InfA	Unknown	28.1942						
B4	training sample 2	H3	Unknown	27.1673						
C4	training sample 2	pdmInfA	Unknown	24.1216						
D4	training sample 2	pdmH1	Unknown	26.2712						
G4	training sample 3	RP	Unknown	26.6378						
A5	training sample 3	InfA	Unknown	Undet.						
B5	training sample 3	H3	Unknown	26.4778						
C5	training sample 3	pdmInfA	Unknown	27.1319						
D5	training sample 3	pdmH1	Unknown	25.2213						
G5	training sample 5	RP	Unknown	Undet.						

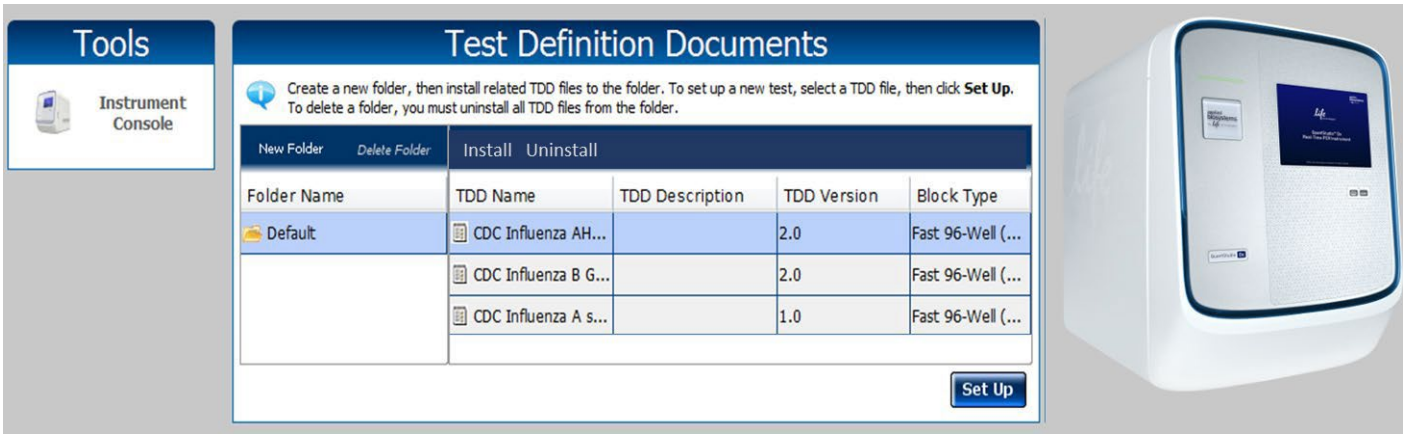
	1	2	3	4	5	6	7	8	9	10	11	12
A	U	U	U	U	U	U	U	U	U	U		
B	U	U	U	U	U	U	U	U	U	U		
C	U	U	U	U	U	U	U	U	U	U		
D	U	U	U	U	U	U	U	U	U	U		
E												
F												
G												
H	U	U	U	U	U	U	U	U	U	U		

Running a Test on the Applied BiosystemsTM QuantStudioTM Dx

If the test definition document file (.tdd) for the Influenza A Subtyping Kit does not already exist on this instrument, please proceed to *Appendix B: Instrument Setup for Applied Biosystems™ QuantStudio™ Dx*.

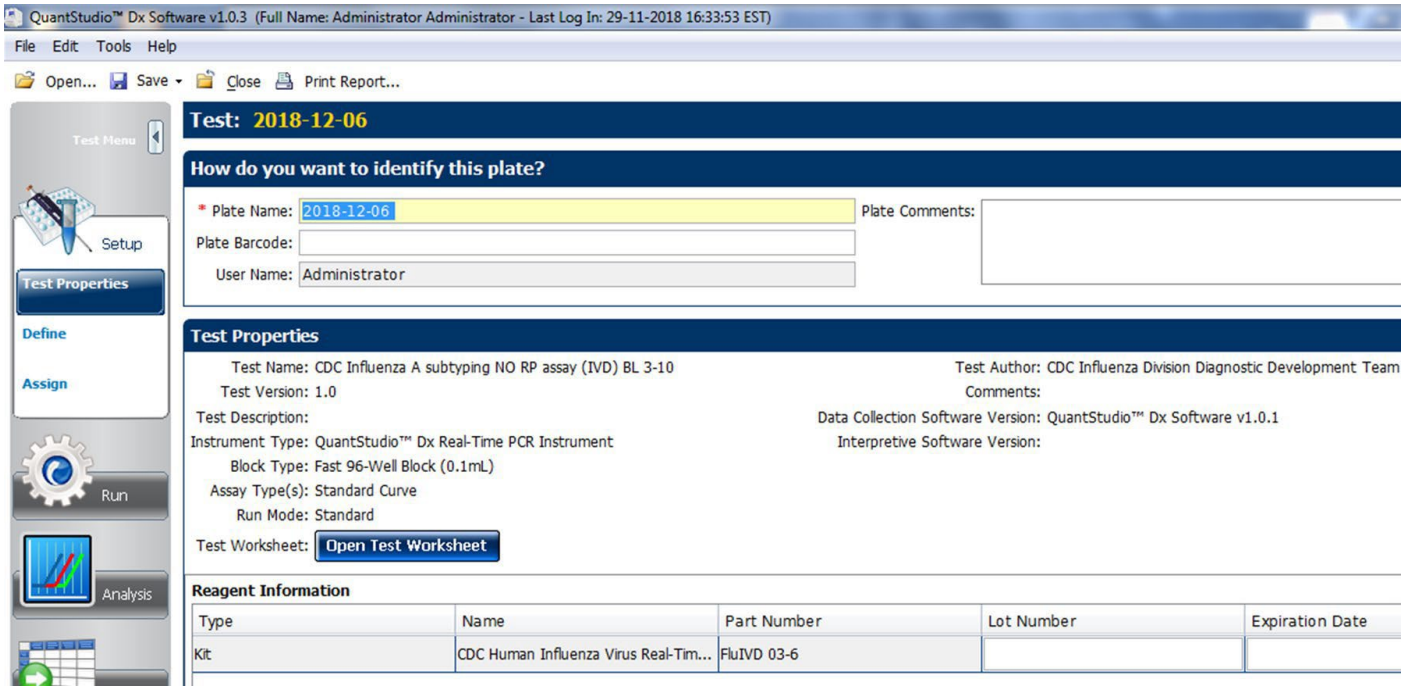
1. Turn on the QuantStudio™ Dx Real-Time PCR Instrument and computer.
2. On the instrument touch screen select **IVD Mode**, then select **Set Instrument Mode**.
3. Launch the QuantStudio™ Dx Real-time PCR System by double clicking on the QuantStudio™ Dx software icon on the computer desktop.
4. Enter username and password into the **Log In** window and select **Log In**.
5. The **Show Security Events** window will appear. Select **Close.**
6. Navigate to select the appropriate CDC Influenza A Subtyping Kit .tdd file, then select **Set Up** (**Figure 9**).
7. There will be a brief pause allowing the QuantStudio™ Dx Real-Time PCR Instrument to initialize. ***Note: The machine must be turned on for initialization.***

Figure 9. Test Definition Document



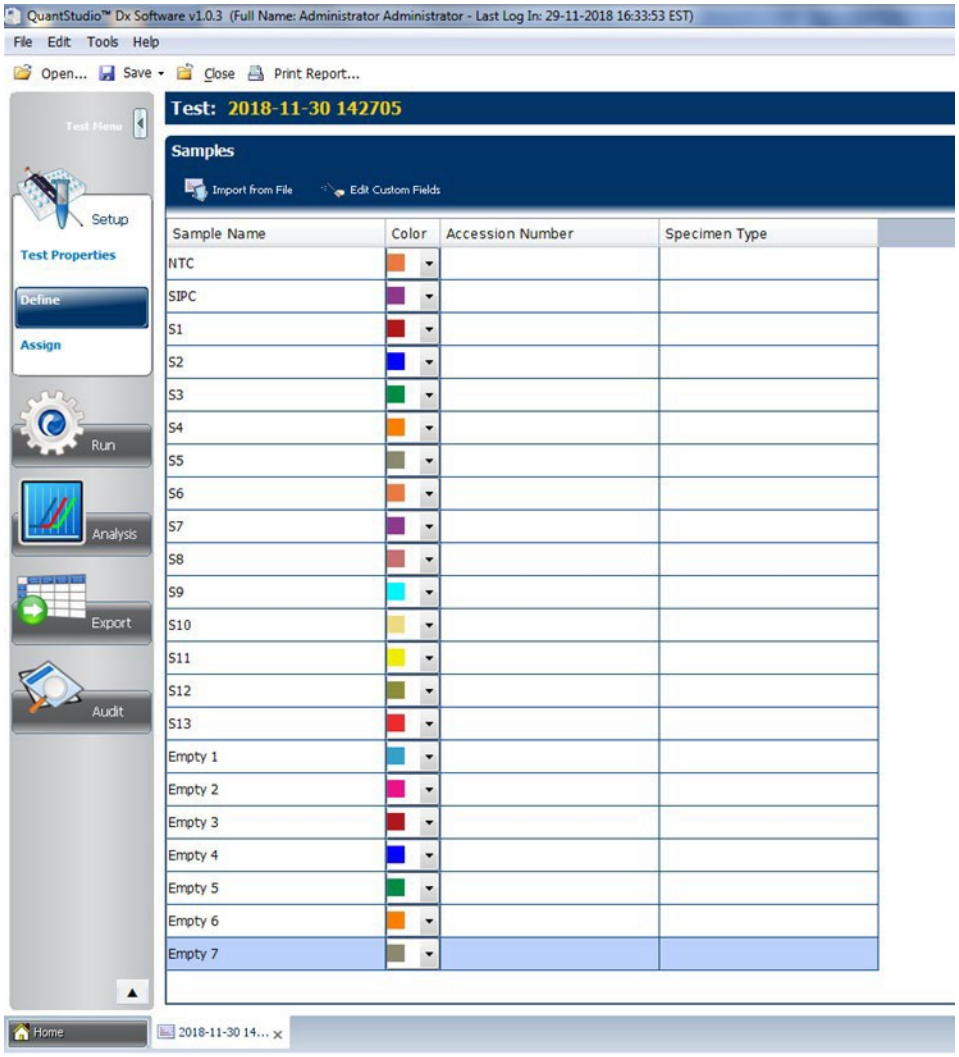
8. After the instrument initializes, the **Test Properties** window will appear (**Figure 10**).
9. Enter the reagent lot number and reagent expiration date.

Figure 10. Test Properties Set-up Window



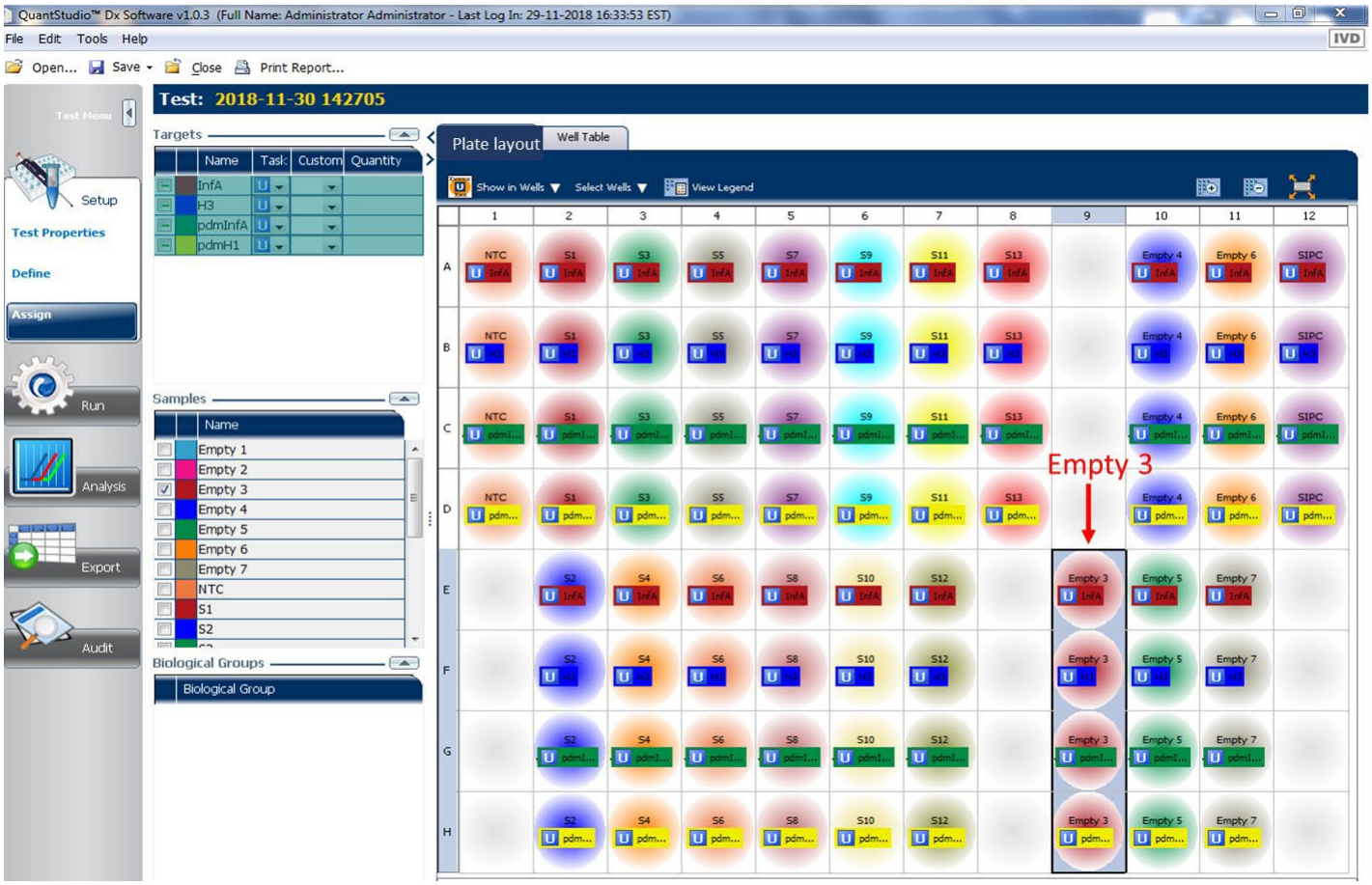
10. Select **Define** (**Figure 10)** in the left margin to assign sample identifiers in the **Samples** window (**Figure 11**).
11. Enter a test sample identifier into each **Sample Name** field to replace the default sample identifiers “Sample 1”, “Sample 2”, etc.
12. Replace “No Template Control” with “NTC” and replace “Positive Control” with “SIPC”.
13. Enter “empty1”, “empty2”, etc. into each corresponding **Sample Name** field for any empty wells.

Figure 11. Defining Sample Wells



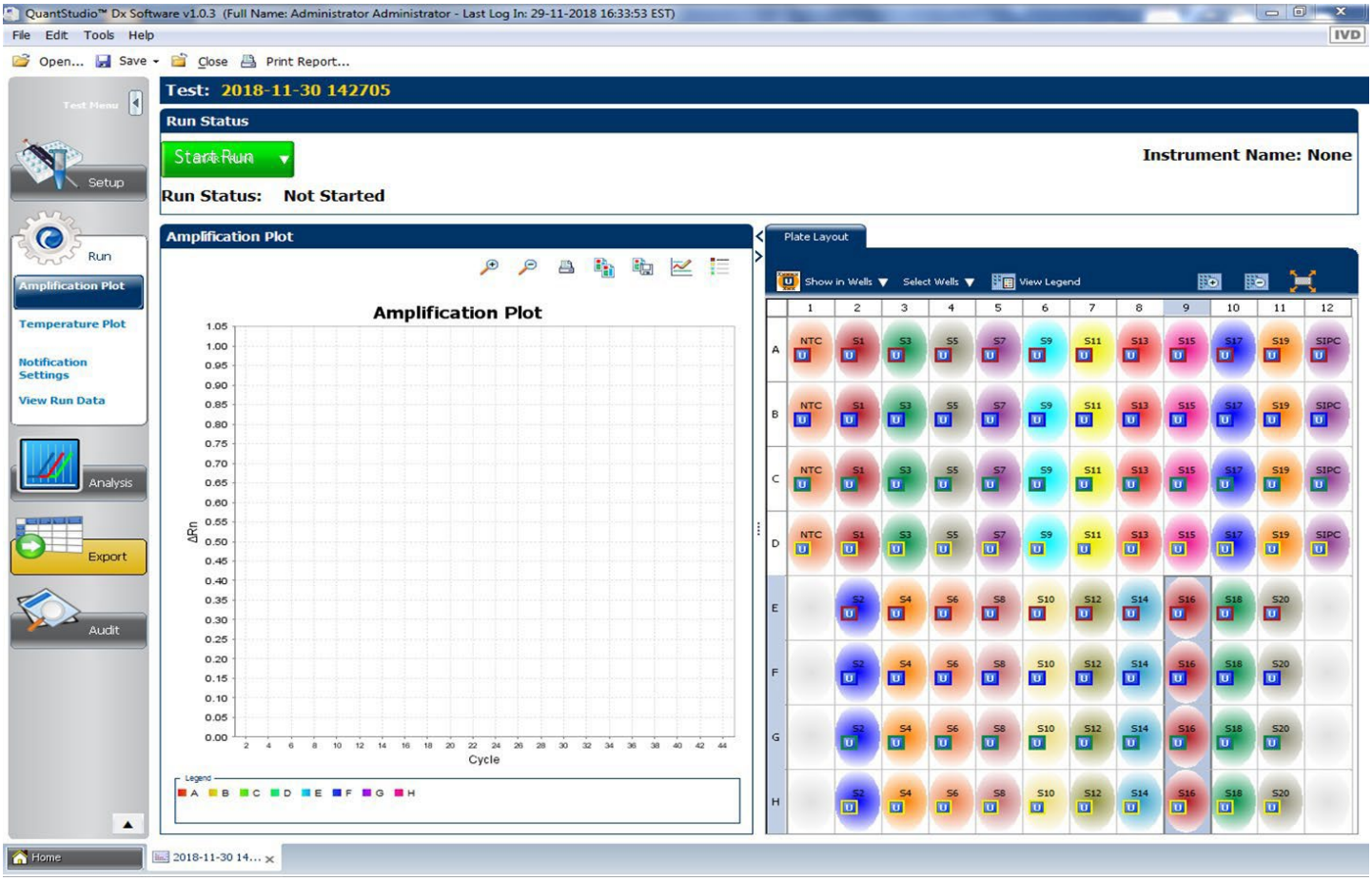
14. Once all control and sample identifiers have been added, select **Assign** (**Figure 12**) in the left margin to review the plate assignment.
15. If desired, the user may remove the empty wells from the plate layout by selecting the column corresponding to the empty sample position on the plate layout and deselecting the wells labeled “empty” on the **Samples** list to the left of the plate layout.

Figure 12. Finalizing Plate Layout



16. Select the **Run** tab from the left margin.
17. The amplification plot and plate layout will load automatically (**Figure 13**).
18. Load the plate into the plate holder in the instrument. Ensure that the plate is properly aligned in the holder.
19. Ensure the setup is correct, then click the **Start Run** green tab in the upper left corner. The serial number of the connected QuantStudio™ Dx Real-Time PCR Instrument will appear. Select the appropriate serial number.
20. In the window that appears select any reason from **Reason** drop down menu, then select **OK.**
21. Save the run file to the designated folder. Once the run file is saved, the run will start. *Note: The run should take approximately 1hr and 45 minutes to complete.*

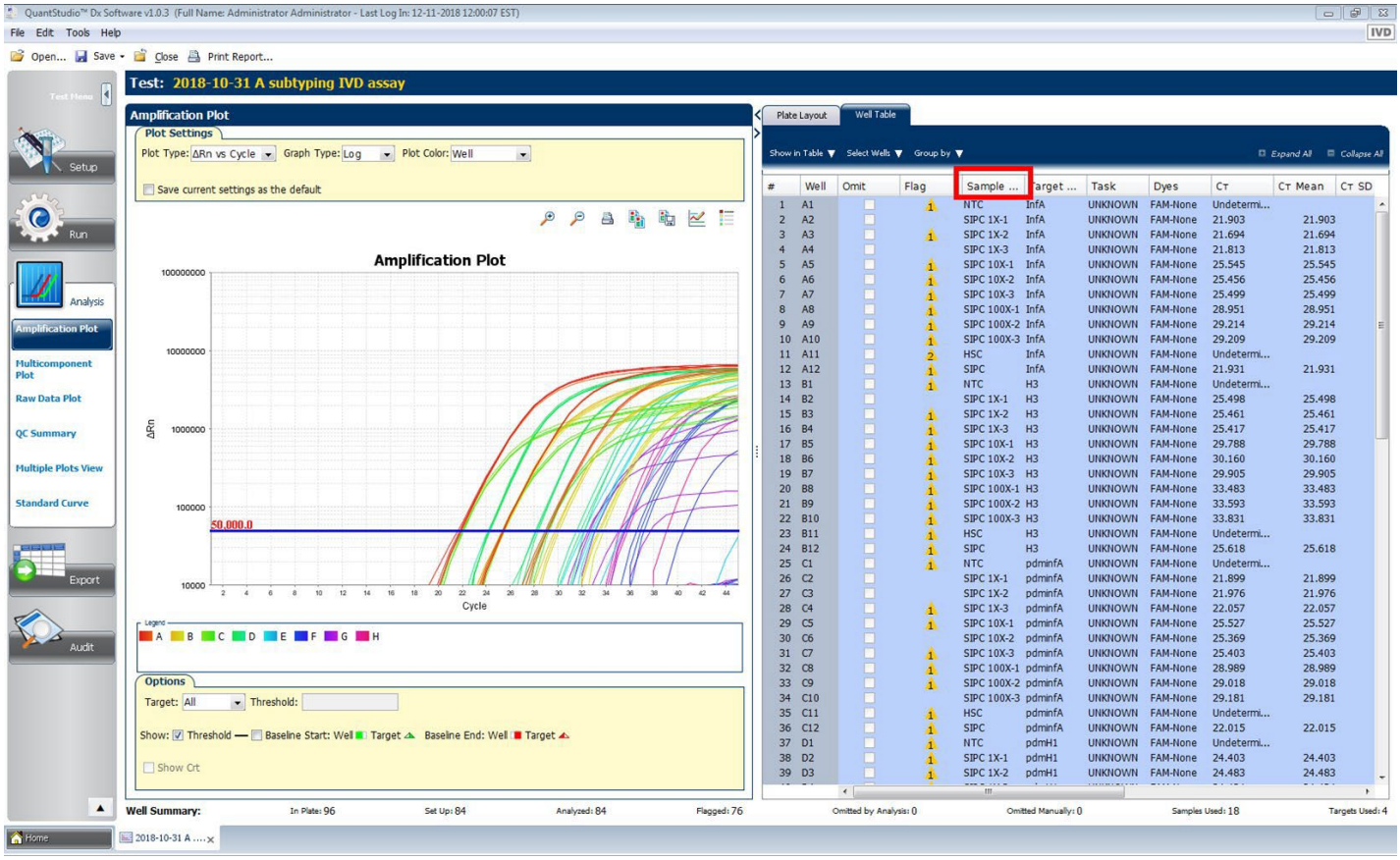
Figure 13. Starting the Run



Results Analysis

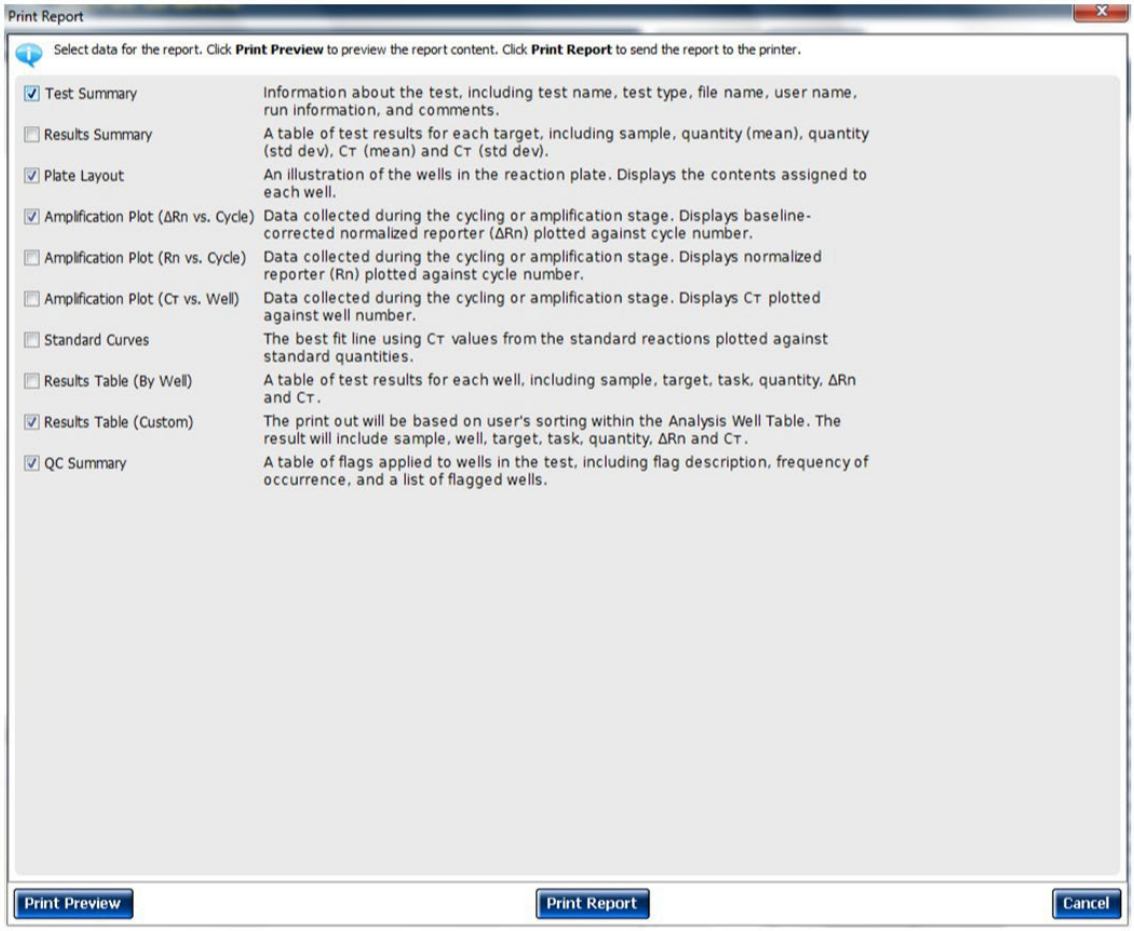
1. After the run has completed, the **Amplification Plot** tab in the **Analysis** window will display (**Figure 14**).
2. In the **Analysis** view, select the **Well Table** tab at the upper left side of the plate layout and review the analyzed data.
3. Sort the wells by sample clicking the **Sample** column (**Figure 14**).

Figure 14. Amplification Plot



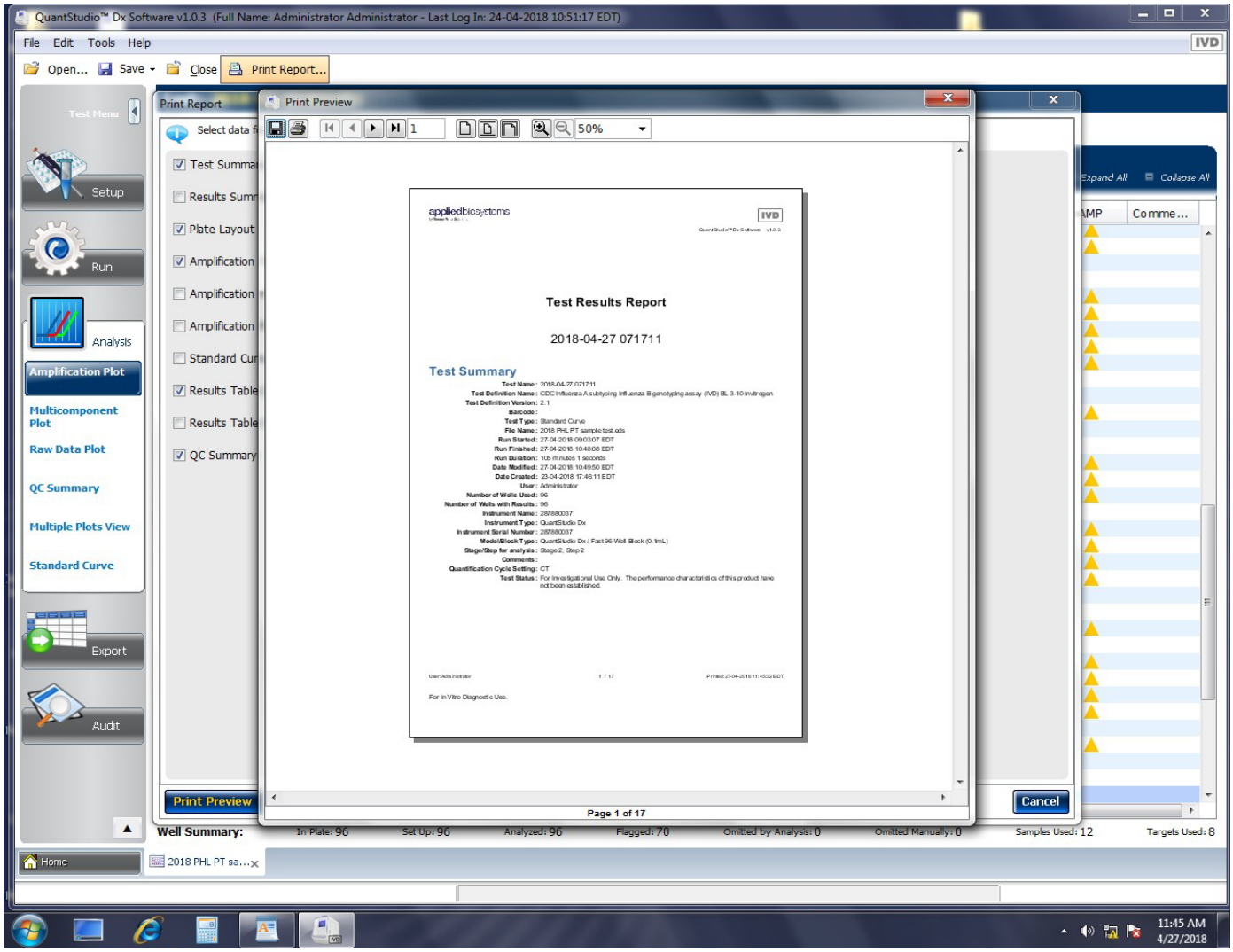
4. Select the **Print Report** icon in the tool bar at the top of the screen (**Figure 14**) to open the **Print Report** window (**Figure 15**).
5. Select **Test Summary, Plate Layout, Amplification Plot (ΔRn vs. Cycle), Results Table (Custom), and QC Summary**.

Figure 15. Report Settings



6. Select **Print Preview** at the bottom left of the window to preview the report (**Figure 16).**
7. Select **Print** icon at the top of the print preview window. The report can be printed to a printer or pdf.

Figure 1. Print Report



Assay Setup on the QIAGEN Rotor-Gene Q MDx

IMPORTANT INFORMATION!

The CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel consists of individual assays that can be utilized to meet laboratory testing algorithms, for example to perform high throughput screening using the Influenza A/B Typing Assay. However, CDC strongly recommends that laboratories perform subtype characterization of all the influenza A positives with the Influenza A Subtyping Kit. Current recommendations can be found on the FluSupport SharePoint site at: https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.

Master Mix Preparation / Plate Setup

If extracted specimen RNA has been previously tested with the Influenza A/B Typing Kit and generated valid results for the RP marker set and HSC extraction control, the RP marker and HSC control need not be repeated when using the Influenza A Subtyping Kit. However, in the event that specimens need to be re-extracted or if the Influenza A Subtyping Kit is used directly, then RP and the HSC control should be included in the run. **Additional plate setups that include HSC are available on the FluSupport SharePoint site (**https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.)

1. In the assay preparation area, label a sterile, nuclease-free, 1.5 mL tube for each marker specific master mix to be prepared (i.e. InfA, pdmH1, H3, etc.).
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 **Figure 17.**

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

Example: If number of samples (n) including controls = 1 to 14, then N = n + 1
If number of samples (n) including controls > 15, then N = n + 2

Figure 17. Steps and Calculations for Master Mix Preparation (Important: Select Appropriate Enzyme System)

Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 5.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	SuperScript™ III RT/Platinum® *Taq* Mix	N x 0.5 µL
4	2X PCR Master Mix	N x 12.5 µL
	Total Volume	**N x 20.0 µL**

OR

Quanta BioSciences qScript™ One-Step qRT-PCR Kit, Low ROX

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 5.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	Quanta qScript™ One-Step Reverse Transcriptase	N x 0.5 µL
4	One-Step Master Mix (2X)	N x 12.5 µL

Step #	Reagent	Vol. of Reagent Added per Reaction
	Total Volume	**N x 20.0 µL**

1. Dispense reagents into the appropriate 1.5 mL labeled microcentrifuge tube. After addition of the reagents, mix reaction mixtures by pipetting up and down. ***Do not vortex***.
2. Centrifuge for 5 seconds to collect contents at the bottom of the tube and place the tube in a cold block.
3. Set up reactions in 4-tube strip tubes on a 72-well cold block.
4. Dispense 20 µL of each master mix into the appropriate wells as shown below (**Figure 18).**

Figure 18. Influenza A Subtyping Kit: Example of Reaction Master Mix 72-Well Strip Tube Set-up

1		2		3		4		5		6		7		8		9	
1	InfA	**9**	InfA	**17**	InfA	**25**	InfA	**33**	InfA	**41**	InfA	**49**	InfA	**57**	InfA	**65**	InfA
2	H3	**10**	H3	**18**	H3	**26**	H3	**34**	H3	**42**	H3	**50**	H3	**58**	H3	**66**	H3
3	pdm InfA	**11**	pdm InfA	**19**	pdm InfA	**27**	pdm InfA	**35**	pdm InfA	**43**	pdm InfA	**51**	pdm InfA	**59**	pdm InfA	**67**	pdm InfA
4	pdm H1	**12**	pdm H1	**20**	pdm H1	**28**	pdm H1	**36**	pdm H1	**44**	pdm H1	**52**	pdm H1	**60**	pdm H1	**68**	pdm H1
5	InfA	**13**	InfA	**21**	InfA	**29**	InfA	**37**	InfA	**45**	InfA	**53**	InfA	**61**	InfA	**69**	InfA
6	H3	**14**	H3	**22**	H3	**30**	H3	**38**	H3	**46**	H3	**54**	H3	**62**	H3	**70**	H3
7	pdm InfA	**15**	pdm InfA	**23**	pdm InfA	**31**	pdm InfA	**39**	pdm InfA	**47**	pdm InfA	**55**	pdm InfA	**63**	pdm InfA	**71**	pdm InfA
8	pdm H1	**16**	pdm H1	**24**	pdm H1	**32**	pdm H1	**40**	pdm H1	**48**	pdm H1	**56**	pdm H1	**64**	pdm H1	**72**	pdm H1

NOTE: If specimens were not already tested with the Influenza A/B Typing Kit and produced valid RP results, the specimen will need to be tested with RP. Additional plate setup options and assay profiles that include RP are available on the FluSupport SharePoint site. (https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.)

1. Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reaction for tubes 1-4 in the assay preparation area.
2. Pipette 5 µL of nuclease-free water into the NTC sample wells. Securely cap and label the first strip cap tab for the position to which the NTC has been added.

   NOTE*: *DO NOT LABEL THE SIDES OF THE REACTION TUBES!
3. Cover the entire cold block and move to the specimen nucleic acid handling area.

Sample Addition

1. Gently vortex nucleic acid sample tubes for approximately 5 seconds.
2. After centrifugation, place extracted nucleic acid sample tubes in the cold rack.

3. Carefully pipette 5 µL of the appropriate sample to the wells labeled for that sample (**Figure 18**). *Keep other sample wells covered during addition. Change tips after each addition.*
4. Securely cap and label the first strip cap tab for the position to which the sample has been added to prevent cross contamination.
   NOTE*: *DO NOT LABEL THE SIDES OF THE REACTION TUBES!
5. Change gloves often when necessary to avoid contamination.
6. Repeat steps 3 and 4 for the remaining samples.
7. Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

Assay Control Addition

1. Pipette 5 µL of SIPC RNA to the appropriate sample wells (**Figure 19**). Securely cap and label the first strip cap tab for the position to which the SIPC has been added. ***NOTE*: *DO NOT LABEL THE SIDES OF THE REACTION TUBES!***
2. Centrifugation is NOT needed for QMDx tubes.
3. Keep reaction tubes on the cold block until the instrument is ready to receive the tubes. Ensure tubes are properly labeled.

Figure 19. Influenza A Subtyping Kit: Sample and Control Set-up

1		2		3		4		5		6		7		8		9	
1	NTC	**9**	S2	**17**	S4	**25**	S6	**33**	S8	**41**	S10	**49**	S12	**57**	S14	**65**	S16
2	NTC	**10**	S2	**18**	S4	**26**	S6	**34**	S8	**42**	S10	**50**	S12	**58**	S14	**66**	S16
3	NTC	**11**	S2	**19**	S4	**27**	S6	**35**	S8	**43**	S10	**51**	S12	**59**	S14	**67**	S16
4	NTC	**12**	S2	**20**	S4	**28**	S6	**36**	S8	**44**	S10	**52**	S12	**60**	S14	**68**	S16
5	S1	**13**	S3	**21**	S5	**29**	S7	**37**	S9	**45**	S11	**53**	S13	**61**	S15	**69**	SIPC
6	S1	**14**	S3	**22**	S5	**30**	S7	**38**	S9	**46**	S11	**54**	S13	**62**	S15	**70**	SIPC
7	S1	**15**	S3	**23**	S5	**31**	S7	**39**	S9	**47**	S11	**55**	S13	**63**	S15	**71**	SIPC
8	S1	**16**	S3	**24**	S5	**32**	S7	**40**	S9	**48**	S11	**56**	S13	**64**	S15	**72**	SIPC

NOTE: HSC needs to be tested with each nucleic acid extraction run. If samples are from an extraction run not tested with HSC, testing of HSC is necessary. Additional plate setup options and assay profiles that include HSC are available on the FluSupport SharePoint site (https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.)

Running a Test on the QIAGEN Rotor-Gene Q MDx

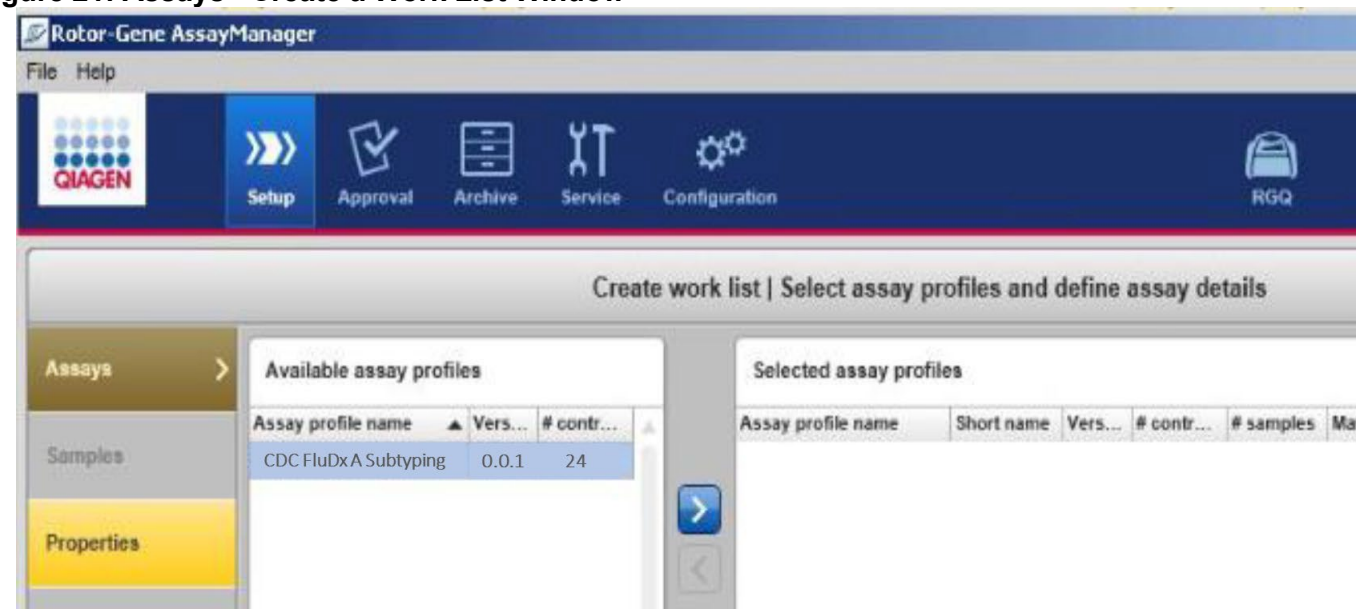
If the most current assay profile for the Influenza A Subtyping Kit does not already exist on this instrument, please proceed to *Appendix C: Instrument Setup for QIAGEN Rotor-Gene Q MDx.*

1. Turn on the QIAGEN Rotor-Gene Q MDx instrument and computer.
2. Launch the QIAGEN Rotor-Gene AssayManager by double clicking on the Assay Manager icon on the desktop.
3. Enter User ID and Password into the Rotor-Gene Assay Manager login window (Figure 20), then select Closed from the Mode drop down menu and click on the OK button.

Figure 20: Rotor-Gene Assay Manager Login Window

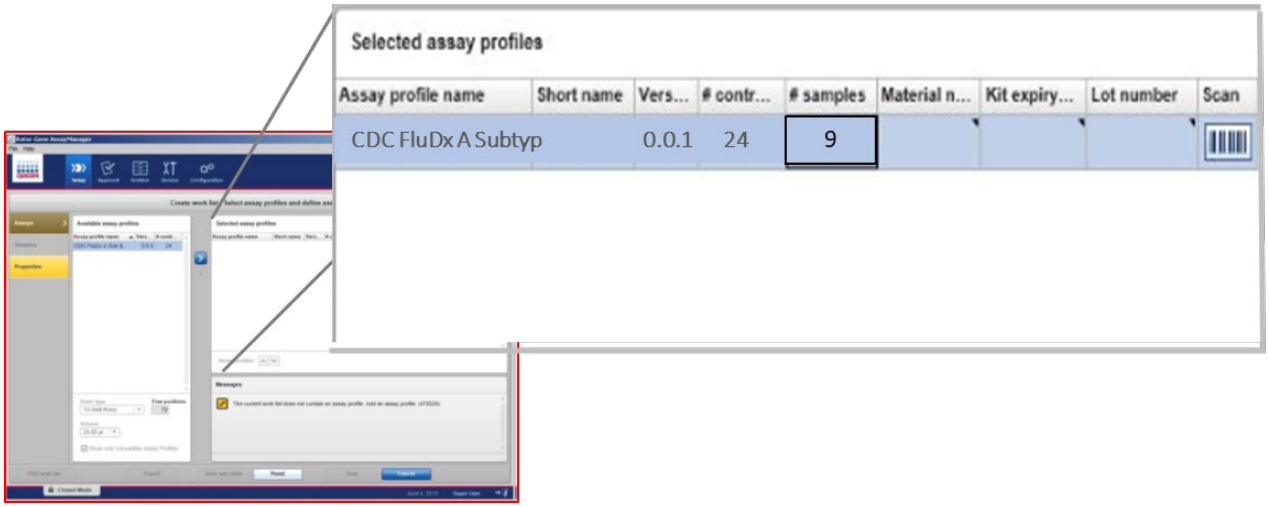
4. The **Available Work List** window will appear under the **Setup** tab. Select **New Work List** button at the bottom right corner of the screen.
5. Select the appropriate assay profile in the **Create a work list** window under the **Setup** tab (**Figure 21**):
    a. Select the **Assays** tab from the left margin.
    b. Select the appropriate assay profile from the **Available assay profiles** window on the left.
    c. Use the blue **arrow** button to move the selected assay profile to the **Selected assay profiles** window to the right.

Figure 21: Assays - Create a Work List Window



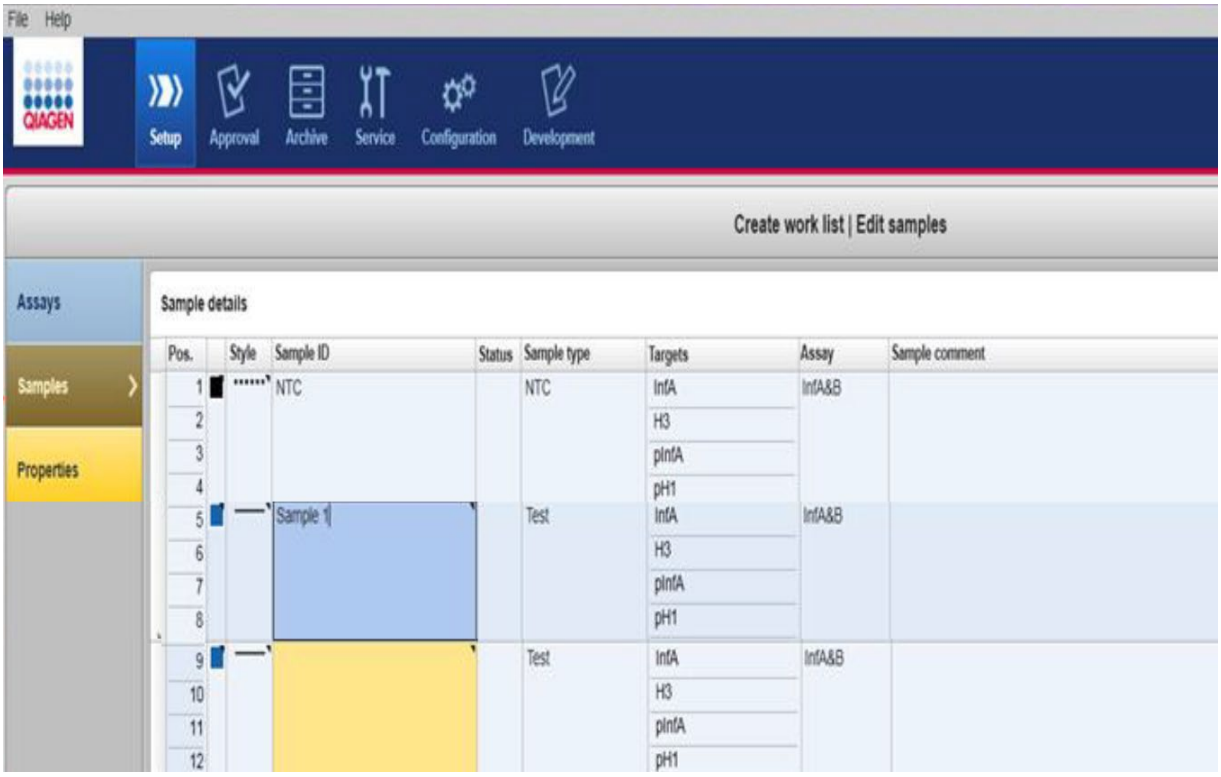
6. In the **Selected assay profiles** window enter the number of samples to be tested (*do not include controls: NTC and Positive Controls).* Rows will change from yellow to blue after the number is entered (**Figure 22**).

Figure 22: Selected Assay Profiles Window



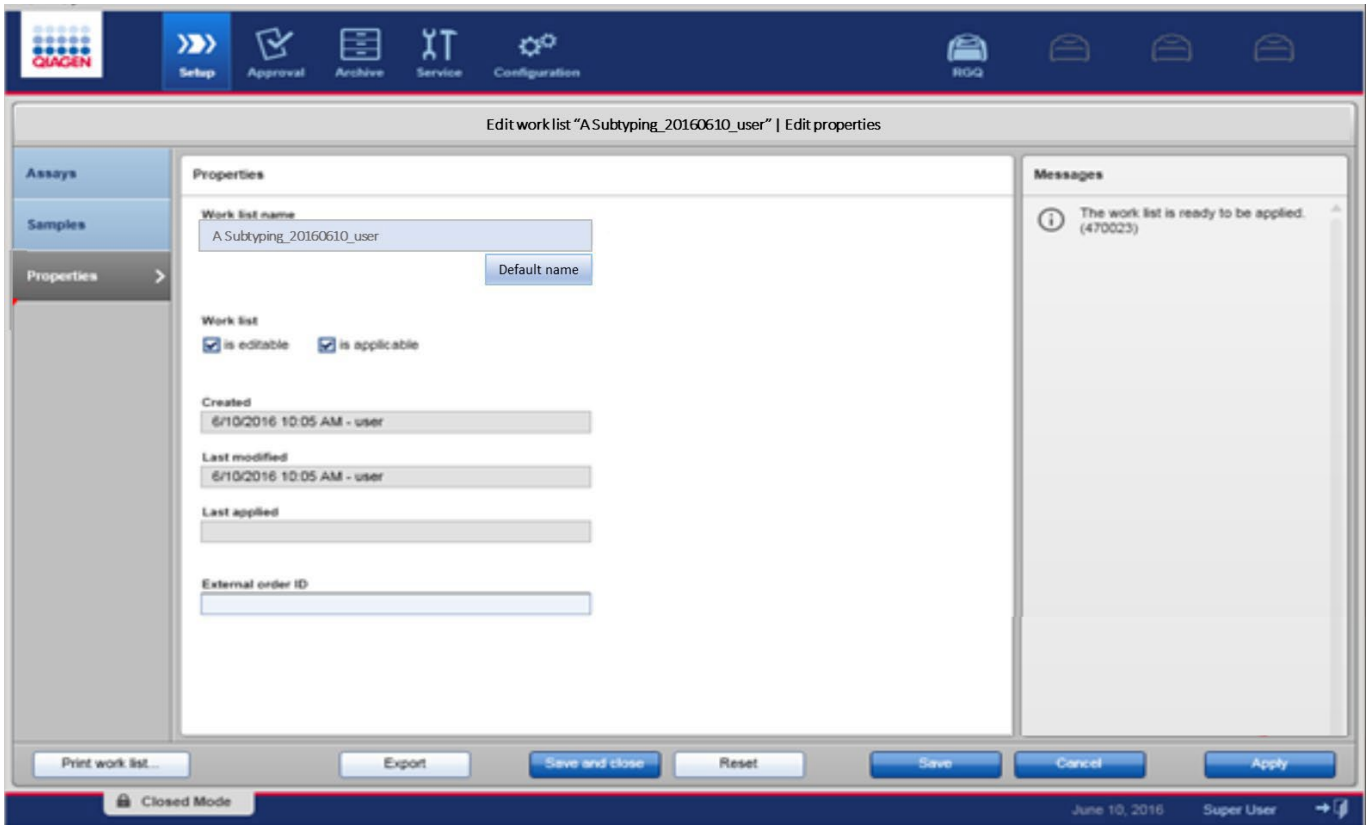
7. Select the **Samples** tab in the left margin. Enter samples names into the **Sample ID** column; controls are automatically added. *On the sample details table, the column heading "Pos." corresponds to the position on the 72-Well Rotor* (**Figure 23**).

Figure 23. Samples - Create a Work List Window | Edit Samples



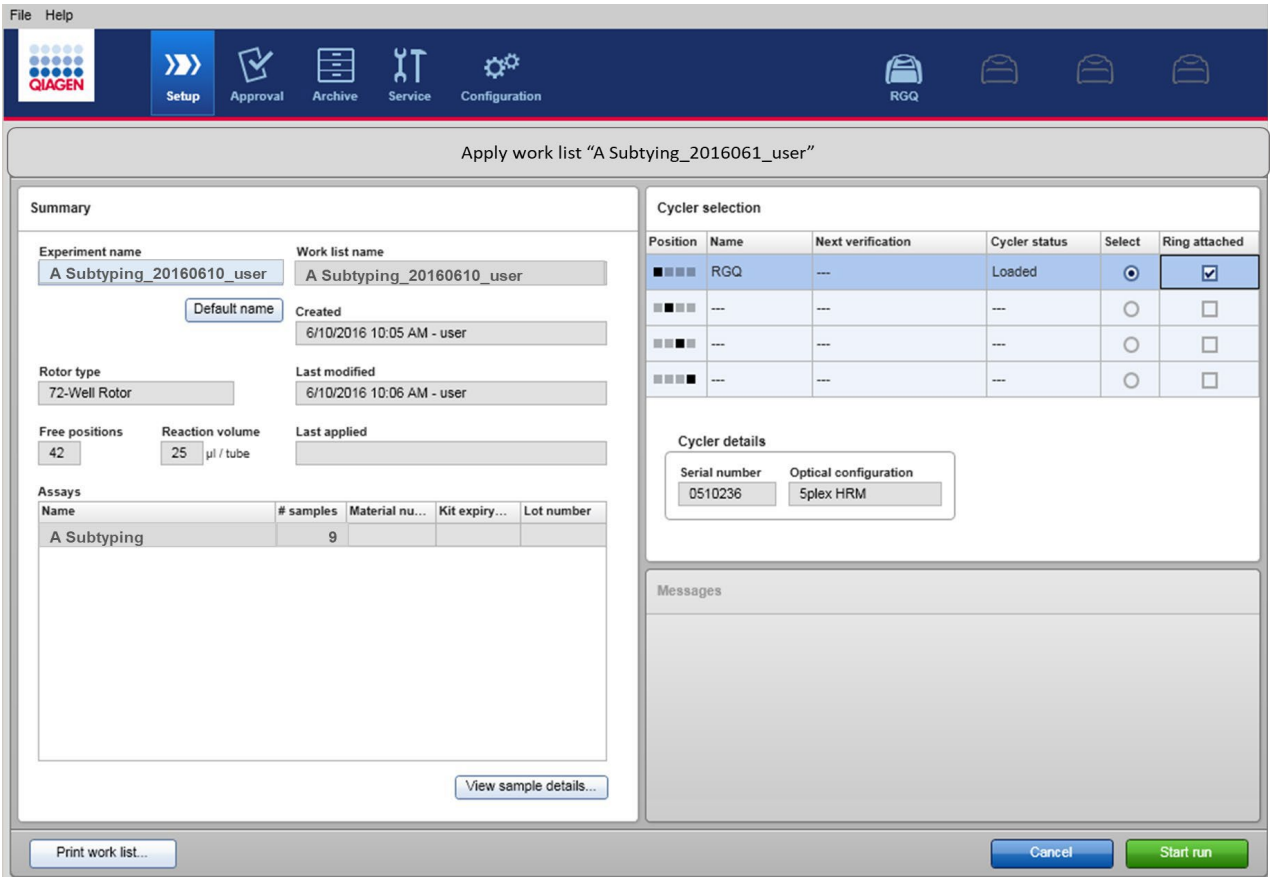
8. Select the **Properties** tab in the left margin (**Figure 24**)**:**
    a. Enter a name in the **Work list name** field or use the **Default name** button.
    b. Click the box next to "**is applicable**" under **Work list**.
    c. Click **Apply** in the bottom right corner.

Figure 24. Properties – Edit Work List Window



9. The **Apply work list** window will appear (**Figure 25**).
    a. Enter the experiment name into the **Experiment name** box or use the **Default name** button.
    b. Prior to adding reactions to the 72-Well rotor, confirm that the first tab of each set of four reaction tubes is labeled with the appropriate position number.
    c. Add samples and control tubes to the positions on the rotor that correspond to their position on the aluminum block. Fill the empty positions with tubes containing an equal volume of water to balance the rotor.
    d. Attach the locking ring to the 72-Well rotor then insert the rotor into the Rotor-Gene Q MDx instrument.
    e. Ensure the correct instrument is selected in the **Name** column of the **Cycler selection** table.
    f. In the **Cycler selection** table, check the box under **Ring attached**.
    g. Before proceeding ensure all samples are loaded into the instrument and the locking ring is securely attached. Securely close the instrument top.
10. Select the **Start Run** button in the bottom right corner of the screen. *Note: The run should take approximately 1hr and 45 minutes to complete.*

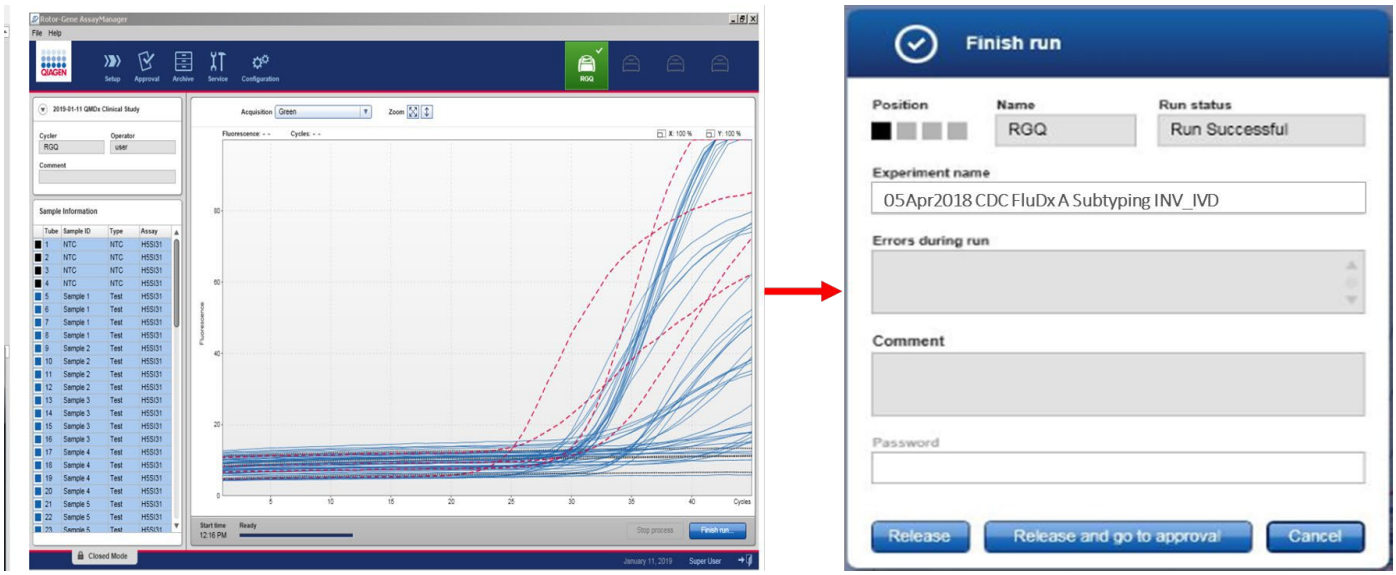
Figure 25. Apply Work List Window



Results Analysis

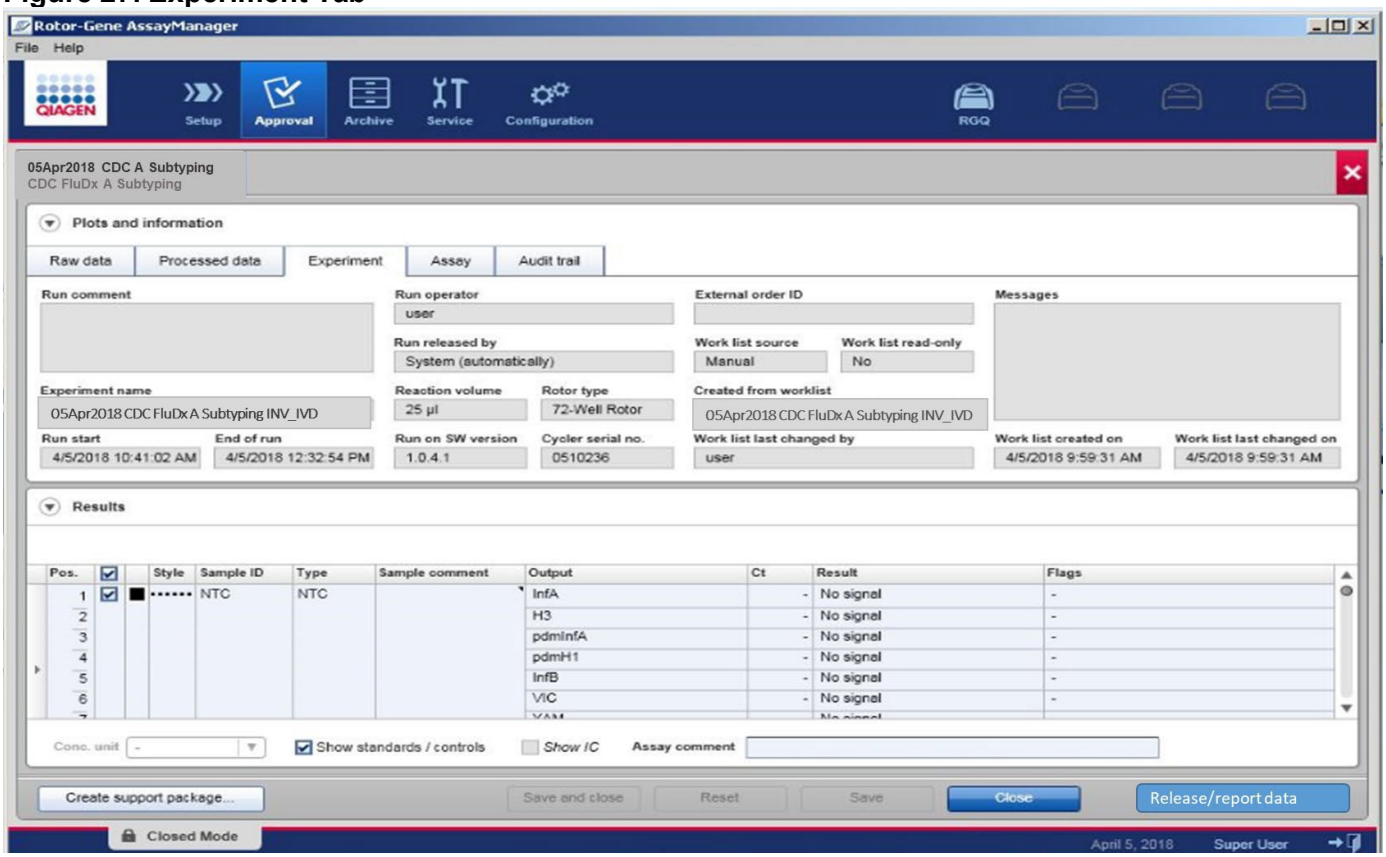
1. Once the run is complete, click the **Finish run** button at the bottom right of the screen.
2. The **Finish run** window will appear (**Figure 26**).

Figure 26. Finish Run Window



3. Click **Release and go to approval** at the bottom of the **Finish run** window.
4. The data analysis window will appear automatically on the **Experiment** tab in the **Approval** menu (**Figure 27**).
5. Review all results and look for any flags.
    a. Result report of “Invalid” and a Flag report of “Multiple Threshold crossings” will invalidate the specimen or control result for that specific influenza target.
    b. Amplification curves can be viewed for each target under the Processed Data Tab.

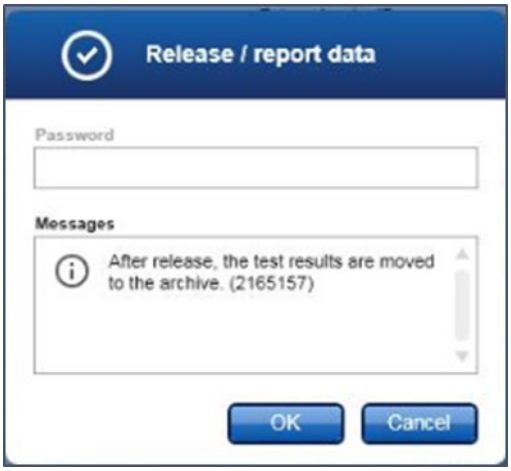
Figure 27. Experiment Tab



6. After reviewing the data, click the **Release/report data** button at the bottom right of the window.

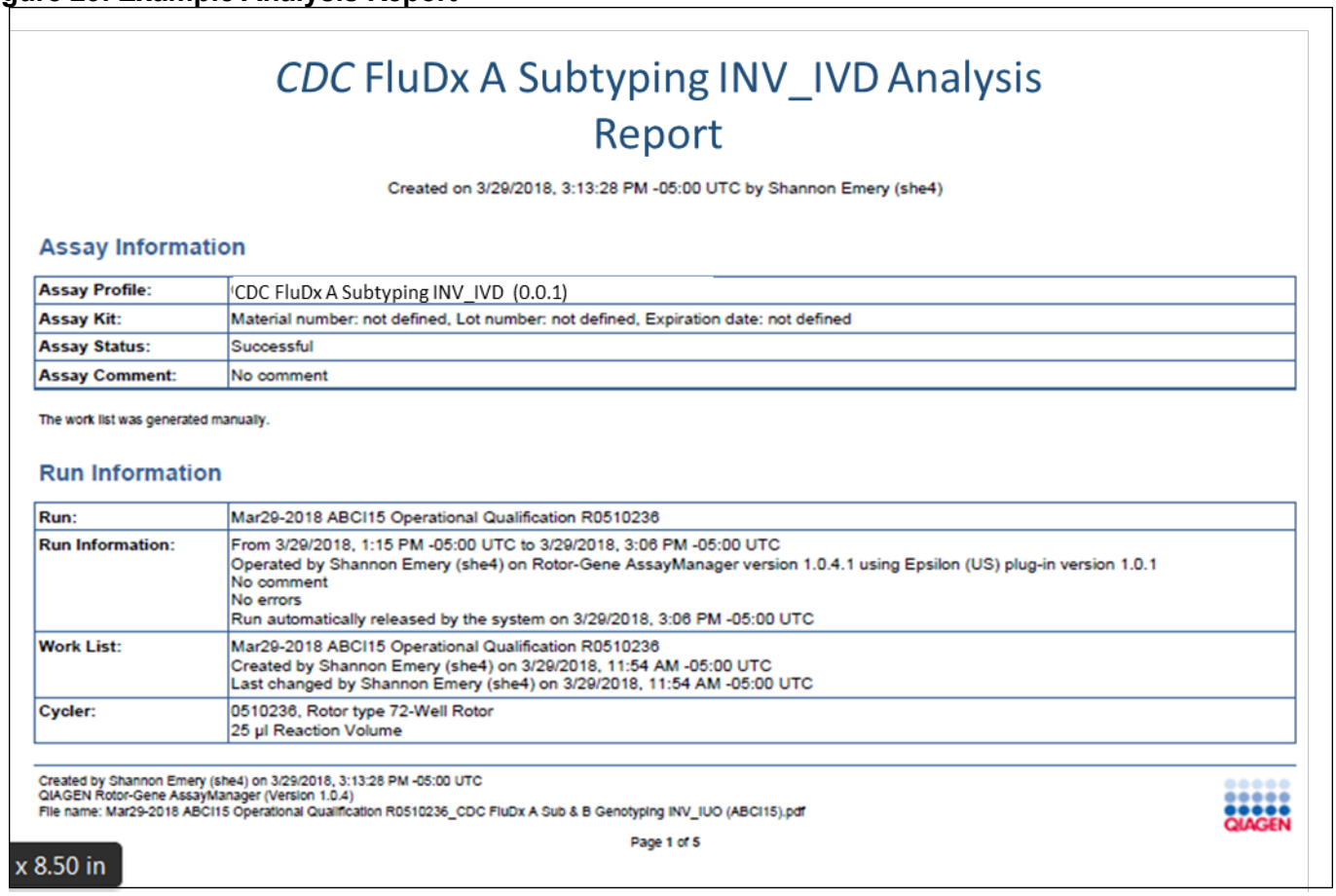
The Release/report data window will appear (Figure 28).

Figure 28. Release/Report Data Window



7. Click **OK**
8. The software will generate a pdf report of the results (**Figure 29**).

Figure 29. Example Analysis Report



CDC FluDx A Subtyping INV_IVD Analysis Report

Created on 3/29/2018, 3:13:28 PM -05:00 UTC by Shannon Emery (she4)

Assay Information

Assay Profile:	CDC FluDx A Subtyping INV_IVD (0.0.1)
Assay Kit:	Material number: not defined, Lot number: not defined, Expiration date: not defined
Assay Status:	Successful
Assay Comment:	No comment

The work list was generated manually.

Run Information

Run:	Mar29-2018 ABCI15 Operational Qualification R0510236
Run Information:	From 3/29/2018, 1:15 PM -05:00 UTC to 3/29/2018, 3:06 PM -05:00 UTC Operated by Shannon Emery (she4) on Rotor-Gene AssayManager version 1.0.4.1 using Epsilon (US) plug-in version 1.0.1 No comment No errors Run automatically released by the system on 3/29/2018, 3:06 PM -05:00 UTC
Work List:	Mar29-2018 ABCI15 Operational Qualification R0510236 Created by Shannon Emery (she4) on 3/29/2018, 11:54 AM -05:00 UTC Last changed by Shannon Emery (she4) on 3/29/2018, 11:54 AM -05:00 UTC
Cycler:	0510236, Rotor type 72-Well Rotor 25 µl Reaction Volume

Created by Shannon Emery (she4) on 3/29/2018, 3:13:28 PM -05:00 UTC
QIAGEN Rotor-Gene AssayManager (Version 1.0.4)
File name: Mar29-2018 ABCI15 Operational Qualification R0510236_CDC FluDx A Sub & B Genotyping INV_IUO (ABCI15).pdf

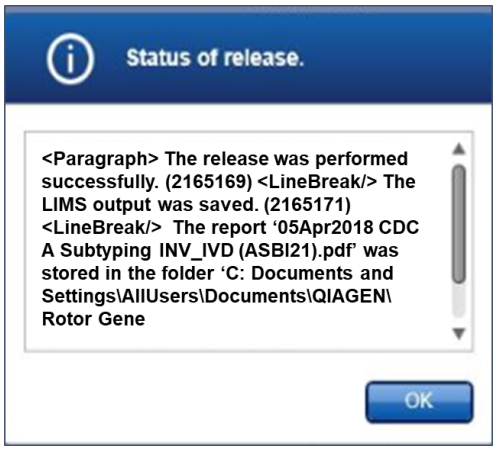
Page 1 of 5

QIAGEN

x 8.50 in

9. Print the pdf report.
10. Close the **Status of release** window (**Figure 30**) to archive the data by clicking **OK**.

Figure 30. Status of Release Window



Interpretation of Results and Reporting

Extraction and Positive Control Results and Interpretation

No Template Control (NTC)

- The NTC consists of using 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.

Seasonal Influenza Positive Control (SIPC)

- The SIPC consists of three different influenza viruses representing influenza A(H3), A(H1)pdm09, and influenza B viruses suspended in cultured human cells (A549). Purified RNA from the SIPC will yield a positive result with the following primer and probe sets: InfA, InfB, H3, pdmInfA, pdmH1, and RP.

Human Specimen Control (HSC) (Extraction Control)

- When HSC is run with the Influenza A Subtyping Kit (see previous section on Assay Set Up), the HSC is used as an RNA extraction procedural control to demonstrate successful recovery of RNA as well as extraction reagent integrity. The HSC control consists of noninfectious cultured human cell (A549) material. Purified RNA from the HSC should yield a positive result with the RP primer and probe set and negative results with all influenza specific markers.

Expected Performance of Controls Included in the Influenza A Subtyping Kit

Control Type	Internal Control Name	Used to Monitor	InfA	H3	pdm InfA	pdm H1	RP	Expected Ct Values
Positive	SIPC	Substantial reagent failure including primer and probe integrity	+	+	+	+	+	< 38.00 Ct
Negative	NTC	Reagent and/or environmental contamination	-	-	-	-	-	None detected
Extraction	HSC	Failure in lysis and extraction procedure	-	-	-	-	+	< 38.00 Ct

If the controls do not exhibit the expected performance as described, the assay may have been set up and/or executed improperly, or reagent or equipment malfunction could have occurred. Invalidate the run and re-test.

RNase P (Extraction Control)

- All clinical samples should exhibit fluorescence growth curves in the RNase P reaction that cross the threshold line within 38.00 cycles (< 38.00 Ct), thus indicating the presence of the human RNase P gene. Failure to detect RNase P in any clinical specimens may indicate:
  - Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
  - Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
  - Improper assay set up and execution.
  - Reagent or equipment malfunction.
- If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:
  - If the InfA along with H3, pdmInfA, pdmH1, are positive even in the absence of a positive RP, the influenza result should be considered valid. It is possible, that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RP signal does not

preclude the presence of influenza virus RNA in a clinical specimen.

- If all influenza markers AND RNase P are negative for the specimen, the result should be considered inconclusive for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-test, report the results as inconclusive and a new specimen should be collected if possible.

➢ The RP assay may be negative when testing virus culture samples.

Influenza Markers (InfA, H3, pdmInfA, pdmH1)

➢ When all controls exhibit the expected performance, a specimen is considered negative if all influenza markers (InfA, H3, pdmInfA, pdmH1) cycle threshold growth curves **DO NOT** cross the threshold line within 38.00 cycles (< 38.00 Ct) **AND** the RNase P growth curve **DOES** cross the threshold line within 38.00 cycles (< 38.00 Ct).

➢ When all controls exhibit the expected performance, a specimen is considered positive for influenza if the influenza marker (InfA, H3, pdmInfA, pdmH1) cycle threshold growth curve crosses the threshold line within 38.00 cycles (< 38.00 Ct). The RNase P may or may not be positive as described above, but the influenza result is still valid. When testing tissue culture derived samples, the RNase P result is likely to yield negative / not detected result due to the absence of the human RNase P target.

➢ When all controls exhibit the expected performance and the growth curves for the influenza markers (InfA, H3, pdmInfA, pdmH1) **AND** the RNase P marker **DO NOT** cross the cycle threshold growth curve within 38.00 cycles (< 38.00 Ct), the result is inconclusive. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the re-tested sample is negative for all markers and all controls exhibit the expected performance, the result is "Inconclusive."

➢ When all controls exhibit the expected performance and the cycle threshold growth curve for influenza A (InfA) marker **ONLY** crosses the threshold line within 38.00 cycles (< 38.00 Ct) without any subtype markers indicating detection (InfA positive without H3, pdmInfA, pdmH1 subtypes detected), the sample has the potential for containing a novel and/or newly emerging influenza A virus. The extracted RNA from the specimen should be re-tested **IMMEDIATELY**. If the fresh unfrozen residual RNA is not available, re-extract RNA from the residual specimen and re-test. If the re-tested sample is again positive for InfA only and all controls exhibit the expected performance, the state public health laboratory director, or designee, should contact the CDC Influenza Division **IMMEDIATELY** at flusupport@cdc.gov to coordinate the transfer of the specimen to CDC as quickly as possible for confirmatory testing. NOTE: Do not test this sample using the Influenza A/H5 Subtyping Kit unless the patient meets the most current U.S. Department of Health and Human Services (DHHS) clinical and epidemiologic criteria for testing suspect A/H5 specimens.

➢ When all controls exhibit the expected performance and growth curves for InfA, pdmInfA, and H3 cross the threshold line within 38.00 cycles (< 38.00 Ct) and the growth curve for pdmH1 **DOES NOT** cross the cycle threshold growth curve within 38.00 cycles (< 38.00 Ct), extracted RNA should be re-tested immediately. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the original result is confirmed:

- Report the specimen to be "Presumptive Positive for Influenza A(H3N2) Variant Virus" and contact the CDC Influenza Division **IMMEDIATELY** at flusupport@cdc.gov to coordinate the transfer of the specimen to CDC as quickly as possible for confirmation.

Influenza A Subtyping Kit Results Interpretation Guide

The table below lists the expected results for the Influenza A Subtyping Kit. If results are obtained that do not follow these guidelines, re-extract and re-test the sample. If repeat testing yields similar results, contact CDC Technical Support for consultation and possible specimen referral as some results may indicate novel or emerging influenza viruses. See pages 12 and 67 for referral and contact information.

Influenza A Subtyping Kit

InfA	H3	pdm InfA	pdm H1	RP	Result Interpretation[a]	Report for CDC Surveillance	Notes and Special Guidance
+	+	-	-	±	Influenza A detected Subtype: H3 detected	Influenza A(H3)	
+	-	+	+	±	Influenza A detected Subtype: H1pdm09 detected	Influenza A(H1)pdm09	If one, but not both, of the pdm markers is positive, re-extract and test. If same result is obtained, report as Inconclusive and refer to the CDC guidance on referral specimens.
-	-	-	-	+	Influenza A not detected	Not Detected	
-	-	-	-	-	Inconclusive Result	Inconclusive	Re-extract specimen and test. If results are similar, report inconclusive.
-	If any of these markers are positive and InfA is negative			±	Inconclusive Result	Inconclusive	Re-extract specimen and test. If results are similar, report inconclusive.

[a]Laboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

The CDC strongly recommends that laboratories subtype all the influenza A positives with the Influenza A Subtyping Kit. The CDC recommends maintaining the enhanced surveillance efforts by state and local health departments, hospitals, and clinicians to identify patients that may be transmitting a possible novel or newly emerging influenza virus.

CDC Guidance for Public Health Surveillance with Influenza Subtyping

For the most current guidelines for surveillance reporting, please contact CDC Influenza Division – Epidemiology and Prevention Branch – Surveillance, Outbreak and Response Team at 404-639-3747.

Follow-Up Actions Regarding Non-Standard Results

If a laboratory encounters subtyping results that do not coincide with the typical expected results of the Influenza A Subtyping Kit listed in the table above, the following guidance is provided to assist in determining the appropriate course of action. Please refer to the most current guidance from CDC on the criteria for referring specimens and samples to CDC for laboratory confirmation found on the FluSupport Sharepoint site at https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx.

If the result of the Influenza A Subtyping Kit indicates an influenza A unsubtypable **AND** the patient does not meet the most current U.S. DHHS clinical and epidemiologic criteria for testing suspect A/H5 specimens for influenza A/H5, the Laboratory Director should contact CDC (flusupport@cdc.gov) or the CDC Influenza Epidemiology Division **IMMEDIATELY** after generating a repeated result. This result may be indicative of a

novel or newly emerging influenza virus and any delay in contacting CDC may result in a critical loss of response time.

Non-Standard Results with the Influenza A Subtyping Kit (After Re-Testing)

InfA	H3	pdm InfA	pdm H1	RP	Result Interpretation	Report for CDC Surveillance	Notes and Special Guidance
+	+	+	-	±	Influenza A detected; Presumptive positive for Influenza A(H3N2) variant virus	Presumptive positive for Influenza A(H3N2)v	Refer specimen to CDC immediately for confirmation.
+	+	+	+	±	Influenza A detected Subtype: H3 detected; H1pdm09 detected	Inconclusive	Refer specimen to CDC immediately for further characterization.
+	-	-	-	±	Influenza A detected Subtype undetectable	Inconclusive	Possible novel or newly emerging influenza. State Lab Director or designee should contact CDC (flusupport@cdc.gov) immediately for instructions for transferring specimen to CDC for further testing and guidance.

Standards-Based Electronic Laboratory Reporting for Influenza

Background

This section contains the recommendations for uniform coding and vocabulary for CDC Human Influenza Virus Real-time RT-PCR Diagnostic Panel.

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 influenza 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 influenza virus 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; 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 Human Influenza Virus Real-Time RT-PCR Diagnostic Panel, the LOINC also provides 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 for 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’s 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 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.
- Although HSC is not included with the Influenza A Subtyping Kit, the HSC extraction control must proceed through nucleic acid isolation per batch of specimens to be tested.
- Always include a negative control (NTC), and the appropriate positive control (SIPC) in each amplification and detection run.

Limitations

- 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. CDC Influenza Division will limit the distribution of this device to only those users who have successfully completed training provided by CDC instructors or designees. This device is subject to a special control requiring that distribution be limited to laboratories with (i) experienced personnel who has training in standardized molecular testing procedures and expertise in viral diagnosis, and (ii) appropriate biosafety equipment and containment (21 CFR866.3332(b)(2)).
- Negative results do not preclude influenza 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. Children tend to shed virus more abundantly and for longer periods of time than adults. Therefore, testing specimens from adults 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 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 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.
- Optimum specimen types and timing for peak viral levels during infections caused by a novel influenza A virus have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus.
- If the virus mutates in the rRT-PCR target region, a specific novel influenza A 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.
- Test performance can be affected because the epidemiology and pathology of disease caused by a specific novel influenza A virus 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 is the causative agent for clinical symptoms.
- The performance of this test has not been established for monitoring treatment of influenza A infection.
- The performance of this test has not been established for screening of blood or blood product for the presence of influenza A.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

Expected values

During February 25, 2012, to May 19, 2012, World Health Organization and National Respiratory and Enteric Virus Surveillance System (NREVSS) collaborating laboratories in the United States tested 47,281 respiratory specimens for influenza viruses. Of these, 9,415 (19.9%) were positive: 85% of the positive specimens were positive for influenza A viruses and 15% were positive for influenza B viruses. Among the 5,071 influenza A viruses for which subtyping was performed, 3,680 (72.6%) were influenza A(H3) viruses and 1,391 (27.4%) were A(H1N1)pdm09 influenza viruses. From August 30, 2009, through March 27, 2010, World Health

Organization (WHO) and National Respiratory and Enteric Virus Surveillance System (NREVSS) collaborating laboratories in the United States tested 422,648 specimens. Of these, 89,585 (21.1%) were positive: 89,298 (99.7%) were positive for influenza A and 287 (0.3%) were positive for influenza B. Among 66,978 influenza A viruses for which subtyping was performed, almost all (66,589 [99.4%]) were A(H1N1)pdm09 viruses.

Of the 37,260 specimens reported during February 14 - March 27, 2010, a total of 2,020 (5.4%) tested positive for influenza, of which 1,999 (98.9%) were positive for influenza A and 21 (1.0%) were positive for influenza B. Of the 1,510 influenza A viruses reported since mid-February for which subtyping was performed, almost all (1,506 [99.7%]) were A(H1N1)pdm09 viruses. No seasonal influenza A(H1N1) viruses and only three influenza A (H3N2) viruses were reported. During February 14 - March 27, states in the Southeast (Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee) accounted for approximately 55% of the influenza positives reported but only 20% of the specimens tested.

During October 1, 2006--May 19, 2007, World Health Organization and National Respiratory and Enteric Virus Surveillance System (NREVSS) collaborating laboratories in the United States tested 179,268 respiratory specimens for influenza viruses; 23,753 (13.2%) were positive. Of these, 18,817 (79.2%) were influenza A viruses and 4,936 (20.8%) were influenza B viruses. Among the influenza A viruses, 6,280 (33.4%) were subtyped; 3,912 (62.3%) were influenza A(H1N1) viruses and 2,368 (37.7%) were influenza A(H3N2) viruses (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5631a2.htm). In the rRT-PCR Flu Panel multi-center prospective clinical study during the 2006-2007 influenza season, the prevalence as determined by virus culture was as follows: influenza A(H1) (7.0%), influenza A(H3) (23.6%), and influenza B (9.9%).

Performance Characteristics

Clinical Performance

The performance characteristics of oligonucleotide primer and probe sets of the Influenza A Subtyping Kit were evaluated in multiple clinical investigations and influenza seasons. The combined summary clinical data are presented as well as an additional clinical evaluation to demonstrate the performance of an alternate enzyme chemistry.

Prospective clinical evaluations were performed at 4-8 U.S. public health laboratories during the 2006-2007 and 2007-2008 influenza seasons when A(H1N1) and A(H3N2) viruses were predominant, and during the 2009-2010 and 2011-2012 influenza seasons when A(H1N1)pdm09 and A(H3N2) viruses were predominant. In each study, samples were obtained from specimens collected for routine influenza testing at each site from individuals who were symptomatic for influenza-like illness (ILI) and included both upper respiratory and lower respiratory specimen types. Low prevalence subtypes during a particular season were also supplemented with retrospective samples from previous seasons. Reference methods for detection and identification of influenza A and influenza A(H1) and A(H3) subtypes during the 2006-2007, 2007-2008, and 2009-2010 seasons were rapid culture (shell vial) followed by direct fluorescent antibody screening and identification. The comparator method for confirmation of the influenza A(H1N1)pdm09 subtype was genetic sequence analysis of the HA gene. A prospective clinical evaluation was also performed during the 2011-2012 influenza season at 6 U.S. public health laboratories to evaluate the performance of an alternate enzyme system. In this study the comparator method was the CDC device with the original cleared enzyme system. The table below provides a summary of each year's study population by age group and by specimen type.

	Clinical Study/Season			
Patient Age	2006-2007	2007-2008	2009-2010	2011-2012
0-16	68	148	685	434
17-54	221	65	779	328
≥ 55	79	26	398	215
Not Reported	49	84	39	25
Specimen Type				
NPS	415		1021	798
NPS/TS		38	99	37
NA		14	57	13
NW			462	24
TS		84	50	8
NS			170	113
Isolates	24	187		
Lower Resp			42	5

Prospective Study Results

Influenza A Comparison

Clinical Study	Specimen Type	# of Positives[1]	% Sensitivity (95% CI)	# of Negatives[1]	% Specificity (95% CI)
2006-2007	NPS, NS	142/143	99.3 (96.1 - 99.9)	251/272	92.3 (88.5 - 94.9)
2007-2008	TS	17/17	100.0 (81.6 - 100.0)	59/60	98.3 (91.1 - 99.7)
	NA	4/4	100.0 (51.0 - 100.0)	10/10	100.0 (72.2 - 100.0)
	NPS/TS	15/15	100.0 (56.6 - 100.0)	17/20	100.0 (88.6 - 100.0)
	Isolates	10/10	100.0 (72.2 - 100.0)	108/174	62.1 (54.7 - 68.9)
2009-2010	NA, NW	19/19	100.0 (83.2 - 100.0)	281/283	99.3 (97.5 - 99.8)
	NPS, NS	61/63	96.8 (89.1 - 99.1)	783/814	96.2 (94.6 - 97.3)
	NPS/TS	6/6	100.0 (61.0 - 100.0)	59/59	100.0 (93.9 - 100.0)
	BAL, BW, TA	5/6	83.3 (43.6 - 97.0)	20/24	83.3 (64.1 - 93.3)

[1]Proportion of actual positives or actual negatives correctly identified versus the comparator (virus culture)

Influenza A(H3) Comparison

Clinical Study	Specimen Type	# of Positives[1]	% Sensitivity (95% CI)	# of Negatives[1]	% Specificity (95% CI)
2006-2007	NPS, NS	98/98	100.0 (96.2 – 100.0)	287/317	90.5 (86.8 - 93.3)
2007-2008	TS	15/16	93.8 (71.7 – 98.9)	56/61	91.8 (82.2 – 96.4)
	NA	1/1	100.0 (20.7 - 100.0)	12/13	92.3 (66.7 – 98.6)
	NPS/TS	6/7	85.7 (48.7 – 97.4)	25/28	92.9 (77.4 – 98.0)
	Isolates	1/1	100.0 (20.7 - 100.0)	154/183	84.2 (78.2 – 88.7)

[1]Proportion of actual positives or actual negatives correctly identified versus the comparator (virus culture)

Influenza A(H1)pdm09 Influenza Comparison

Clinical Study	Specimen Type	# of Positives[1]	% Sensitivity (95% CI)	# of Negatives[1]	% Specificity (95% CI)
2009-2010	NA, NW	16/16	100.0 (80.6 – 100.0)	282/286	98.6 (96.5 – 99.5)
	NPS, NS	57/57	100.0 (93.7 - 100.0)	793/820	96.7 (95.3 – 97.7)
	NPS/TS	5/5	100.0 (56.6 – 100.0)	59/60	98.3 (91.1 – 99.7)
	BAL, BW, TA	5/5	100.0 (56.6 - 100.0)	21/25	84.0 (65.3 – 93.6)

[1]Proportion of actual positives or actual negatives correctly identified versus the comparator (genetic sequence analysis)

Retrospective Study Results

Retrospective evaluations were performed with clinical specimens collected previously in the 2009-2010, 2011-2012, 2013-2014, and 2015-2016 influenza seasons. The percent positive agreement and percent negative agreement of individual assays was determined with the CDC Human Influenza rRT-PCR Diagnostic Panel and a comparator.

Influenza A Comparison

Clinical Study	Specimen Type	# of Positives[1]	% Positive Agreement (95% CI)
2009-2010	NA, NW	209/214	97.7 (94.6 – 99.0)
	NPS, NS	310/312	99.4 (97.7 – 99.8)
	NPS/TS	31/31	100.0 (89.0 – 100.0)
	BAL, BW, TA	10/10	100.0 (72.3 - 100.0)

[1]Proportion of actual positives correctly identified versus the comparator (virus culture)

Influenza A Comparison with Ver2 InfA Assay

		Invitrogen SuperScript™		Quanta qScript™	
Clinical Study	Specimen Type	# of Positives[1]	% Positive Agreement (95% CI)	# of Positives[1]	% Positive Agreement (95% CI)
2011-2012, 2013-2014	NPS, NS	51/51	100.0 (93.0-100.0)	51/51	100.0 (93.0-100.0)
	NPS/TS	2/2	100.0 (34.2-100.0)	2/2	100.0 (34.2-100.0)
	TS	4/4	100.0 (51.0-100.0)	4/4	100.0 (51.0-100.0)
	NW	3/3	100.0 (43.9-100.0)	3/3	100.0 (43.9-100.0)
	Sputum	1/1	100.0 (20.7-100.0)	1/1	100.0 (20.7-100.0)
	BW	1/1	100.0 (20.7-100.0)	1/1	100.0 (20.7-100.0)

[1]Proportion of positive samples correctly identified versus the predicate device.

		Invitrogen SuperScript™		Quanta qScript™	
Clinical Study	Specimen Type	# of Negatives[1]	% Negative Agreement (95% CI)	# of Negatives[1]	% Negative Agreement (95% CI)
2011-2012, 2013-2014	NPS	54/54	100.0 (93.4-100.0)	54/54	100.0 (93.4-100.0)

[1]Proportion of negative samples correctly identified versus the predicate device.

Influenza A(H1)pdm09 Comparison

Clinical Study	Specimen Type	# of Positives[1]	% Positive Agreement (95% CI)
2009-2010	NA, NW	203/205	99.0 (96.5 – 99.7)
	NPS, NS	301/302	99.7 (98.1 – 99.9)
	NPS/TS	29/29	100.0 (88.3 – 100.0)
	BAL, BW, TA	10/10	100.0 (72.3 - 100.0)

[1]Proportion of actual positives correctly identified versus the comparator (genetic sequence analysis)

Influenza A(H1)pdm09 Comparison with Ver2 pdmH1 Assay

Clinical Study	Specimen Type	Invitrogen SuperScript™		Quanta qScript™	
		# of Positives[1]	% Positive Agreement (95% CI)	# of Positives[1]	% Positive Agreement (95% CI)
2011-2012, 2013-2014, and 2015-2016	BW	1/1	100.0 (20.7-100.0)	1/1	100.0 (20.7-100.0)
	NPS, NS	34/35	97.1 (85.5-99.5)	33/33	100.0 (89.6-100.0)
	NW	4/4	100.0 (51.0-100.0)	4/4	100.0 (51.0-100.0)
	TS	2/2	100.0 (34.2-100.0)	2/2	100.0 (34.2-100.0)

[1]Proportion of actual positives correctly identified versus the predicate device and genetic sequence analysis.

Clinical Study	Specimen Type	Invitrogen SuperScript™		Quanta qScript™	
		# of Negatives[1]	% Negative Agreement (95% CI)	# of Negatives[1]	% Negative Agreement (95% CI)
2015-2016	NPS	53/53	100.0 (93.2-100.0)	52/52	100.0 (93.1-100.0)

[1]Proportion of negative samples correctly identified versus the predicate device.

Influenza A(H1)pdm09 Comparison with Ver3 pdmH1 Assay

Clinical Study	Specimen Type	Invitrogen SuperScript™		Quanta qScript™	
		# of Positives[1]	% Positive Agreement (95% CI)	# of Positives[1]	% Positive Agreement (95% CI)
2011-2012, 2013-2014	BW	1/1	100.0 (20.7-100.0)	1/1	100.0 (20.7-100.0)
	NPS, NS	28/28	100.0 (87.9-100.0)	28/28	100.0 (87.9-100.0)
	NW	3/3	100.0 (43.9 100.0)	3/3	100.0 (43.9-100.0)
	TS	3/3	100.0 (43.9-100.0)	3/3	100.0 (43.9-100.0)

[1]Proportion of actual positives correctly identified versus the predicate device.

Clinical Study	Specimen Type	Invitrogen SuperScript™		Quanta qScript™	
		# of Negatives[1]	% Negative Agreement (95% CI)	# of Negatives[1]	% Negative Agreement (95% CI)
2011-2012, 2013-2014	NPS	54/54	100.0 (93.4-100.0)	54/54	100.0 (93.4-100.0)

[1]Proportion of negative samples correctly identified versus the predicate device.

Analytical Performance

Analytical Sensitivity – Limit of Detection (LOD)

Analytical sensitivity was demonstrated for the Influenza A Subtyping Kit by determining the LOD of each

primer and probe set using Quanta qScript™ and Invitrogen SuperScript™ enzyme kits. The LOD for each primer and probe set was calculated to indicate the lowest detectable concentration of influenza virus measured by 50% infectious dose in eggs (EID_{50}/mL) or tissue culture ($TCID_{50}$/mL) at which ≥ 95% of all replicates tested positive. The lowest concentration of influenza virus detected was determined to be the end-point concentration where the type and subtype primer and probe sets had uniform detection. If the two end-points differed in concentration, the higher (or limiting) point was used.

Influenza Virus Tested	Influenza Strain Designation	LOD (ID_{50}/mL)	
		Invitrogen SuperScript™	Quanta qScript™
A(H1N1)pdm09	A/Michigan/45/2015[a]	$10^{2.9}$	$10^{2.9}$
	A/Illinois/20/2018[b]	$10^{2.0}$	$10^{2.0}$
A(H3N2)	A/Perth/16/2009 [a]	$10^{2.8}$	$10^{2.8}$
	A/Victoria/361/2011 [a]	$10^{2.8}$	$10^{2.8}$

[a]EID_{50}/L
[b]$TCID_{50}$/mL

Analytical Reactivity (Inclusivity)

The analytical inclusivity of the InfA, H3, pdmInfA, and pdmH1 primer and probe sets was demonstrated by testing 10 influenza virus strains of seasonal A(H3N2), and A(H1N1)pdm09 at low virus concentrations at or near the LOD. The Influenza A Subtyping Kit analytical inclusivity indicated 100% concordance with expected results for all primer and probe sets included in the device.

Analytical Specificity - Exclusivity

Non-Influenza Human Respiratory Pathogens

Analytical specificity (cross-reactivity) was evaluated by testing InfA, H3, pdmInfA, and pdmH1 primer and probe sets within the device with nucleic acids extracted from 36 organisms (16 viruses, 19 bacteria, and 1 yeast) representing common respiratory pathogens or flora commonly present in specimens collected from the nasopharynx region. Bacteria and yeast were tested at concentrations greater than or equal to10^6 cfu/ml with the exception of *Chlamydia pneumoniae* which was quantified by determining inclusion forming units and *Mycobacterium tuberculosis* where DNA was extracted from pure culture and quantified by spectrophotometry. Non-influenza respiratory viruses were tested at concentrations greater than 10^6 $TCID_{50}$/mL with the exception of human parainfluenza type 2 ($10^{3.1}$ $TCID_{50}$/ml due to difficulty generating a high titer virus stock in culture) and human parainfluenza type 1 where RNA was extracted from viral culture and quantified by spectrophotometry. Similarly, RNA was extracted from viral cultures and concentrations were determined by spectrophotometry for Coronaviruses CoV 229E and CoV OC43, and Epstein-Barr virus.

Organism Tested			Reactivity			
Bacteria and Yeast	Strain	cfu / mL	InfA	H3	pdmInfA	pdmH1
Bordetella pertussis	A639	$10^{8.3}$	-	-	-	-
	Tahoma I	$10^{10.0}$	nd[1]	nd	-	nd
Candida albicans	2001-21-196	$10^{8.8}$	-	-	-	-
	3147	$10^{8.5}$	nd	nd	-	nd
Chlamydia pneumoniae[2]	TW183	40 IFU/mL	-	nd	-	-
	CM-1	40 IFU/mL	nd	nd	-	nd
Corynebacterium diphtheriae[4]	NA[3]	10^{10}	-	-	-	-
	NCTC 13129	57.4 ng/ µL	nd	nd	-	nd
Escherichia coli	K12	$10^{9.6}$	-	-	-	-
Haemophilus influenzae	M15709	$10^{6.4}$	-	-	-	-
Lactobacillus plantarum	NA[3]	$10^{8.8}$	-	-	-	-
Legionella pneumophila	NA[3]	$10^{10.3}$	-	-	-	-
	Philadelphia-1	$10^{8.4}$	nd	nd	-	nd
Moraxella catarrhalis	M15757	$10^{9.5}$	-	-	-	-
Mycobacterium tuberculosis[4]	BCG	0.25 ng /µL	-	-	nd	nd
	H37Rv	95 ng/ µL	-	nd	-	-
Mycobacterium tuberculosis	H37Ra	$10^{5.0}$	nd	nd	-	nd
Mycoplasma pneumoniae	MI-29	$10^{7.7}$	-	-	-	-
	PI 1428	$10^{9.0}$	**nd**	**nd**	-	**nd**
Neisseria elongata	NA[3]	$10^{8.6}$	-	-	-	-
	NA[3]	$10^{5.0}$	**nd**	**nd**	-	**nd**
Neisseria meningitidis	M2578	$10^{7.9}$	-	-	-	-
Pseudomonas aeruginosa	NA[3]	$10^{10.5}$	-	-	-	-
Staphylococcus epidermidis	NA[3]	$10^{10.5}$	-	-	-	-
Staphylococcus aureus	NA[3]	$10^{10.7}$	-	-	-	-
Streptococcus pneumoniae	249-06 (Thailand)	$10^{6.6}$	-	-	-	-
Streptococcus pyogenes	7790-06	$10^{7.5}$	-	-	-	-
Streptococcus salivarius	SS1672	$10^{8.4}$	-	-	-	-
	DSM 13084	109 ng/ µL	**nd**	**nd**	-	**nd**
Viruses	Strain	$TCID_{50}$/mL	InfA	H3	pdmInfA	pdmH1
Enterovirus	Echo 6	$10^{6.9}$	-	-	-	-
Human Adenovirus, type 1	Ad.71	$10^{9.2}$	-	-	-	-
Human Adenovirus, type 7a	S-1058	$10^{7.1}$	-	-	-	-
Human Coronavirus virus[4]	OC43	50.4 ng /µL	-	-	-	-
Human Coronavirus virus[4]	299E	31.6 ng /µL	-	-	-	-
Human Rhinovirus A	1A	$10^{5.8}$	-	-	-	-
Human Parainfluenza 1 virus[4]	NA[3]	3.0 ng/ µL	-	-	-	-
Human Parainfluenza 2 virus	Greer	$10^{3.1}$	-	-	-	-
Human Parainfluenza 3 virus	C-243	$10^{7.9}$	-	-	-	-
Respiratory Syncytial virus	CH93-18b	$10^{6.8}$	-	-	-	-
Herpes Simplex Virus	KOS	$10^{8.4}$	-	nd	-	-
Varicella-zoster Virus	AV92-3	$10^{4.4}$	-	nd	-	-
Epstein Barr Virus[4]	B95-8	1.7 ng/µL	-	nd	-	-
Measles Virus	Edmonston	$10^{5.2}$	-	nd	-	-
Mumps Virus	Enders	$10^{7.2}$	-	nd	-	-
Cytomegalovirus	AD-169	$10^{6.9}$	-	nd	-	-

[1] nd = not determined; [2] Organism quantified by Infectious Forming Units (IFU); [3]NA = not applicable;
[4] Organism quantified by spectrophotometry (ng/µL)

The study demonstrated that the primer and probe sets contained within the Influenza A Subtyping Kit did not cross-react with any of the non-influenza respiratory pathogens or commensal organisms and demonstrated 100% concordance with the expected results.

Exclusivity with Influenza Viruses with Pandemic Potential

To demonstrate the ability of the InfA primer and probe set to detect influenza A(H5) viruses with pandemic potential and exclusivity of the pdm primer and probe sets, 10 avian A(H5) influenza viruses that have been shown to infect humans were tested. The avian A(H5) influenza viruses were grown and harvested from allantoic/amniotic fluid from infected embryonated chicken eggs (ECE) and titered. Each virus was extracted using the Roche MagNA Pure Compact RNA Isolation kit, and the purified RNA was tested in triplicate.

Avian A(H5) Influenza Viruses Tested at High Virus Titer

Type	Virus	Clade	Titer (log EID_{50}/mL)	InfA	pdm InfA	pdm H1
H5N1	A/Japanese white eye/Hong Kong/1038/2006	2.3.4	9.2	+ (3/3)	-	-
	A/Duck/Hunan/795/2002	2.1	9.9	+ (3/3)	-	-
	A/Chicken/Yunnan/1251/2003	1	9.3	+ (3/3)	-	-
	A/Common magpie/Hong Kong/645/2006	2.3.2	9.2	+ (3/3)	-	-
	A/Vietnam/1194/2004	1	9.3	+ (3/3)	-	-
	A/Egypt/321/2007	2.2	9.2	+ (3/3)	-	-
	A/Anhui/1/2005	2.3.4	9.3	+ (3/3)	-	-
	A/Chicken/India/NIV3487/2006	2.2	9.3	+ (3/3)	-	-
	A/Chicken/Vietnam/NCVD-016/2008	7	9.1	+ (3/3)	-	-
	A/Cambodia/R040505/2007	1	8.5	+ (3/3)	-	-

To demonstrate the ability of the InfA primer and probe set to detect potential pandemic influenza A viruses and exclusivity of the other primer and probe sets in the Influenza A Subtyping Kit, 15 non-human influenza viruses that have been shown to infect humans were tested. The non-human influenza viruses were propagated in ECE, grown to high titer, and harvested as described previously. Each virus was extracted using Roche MagNA Pure Compact RNA kit. Cross-reactivity was observed with the pdmInfA assay with A/gyrfalcnon/Washington/41088-6/2014.

Animal Influenza Viruses Tested at High Virus Titer

Species	Virus	Subtype	Titer (log EID_{50}/mL)	InfA	H3	pdmInfA	pdmH1
EQUINE	A/equine/Ohio/01/2003	EQ-H3N8	7.5	+ (3/3)	-	-	-
SWINE	A/Iowa/1/2006	H1N1v	8.2	+ (3/3)	nd[1]	+ (3/3)	+ (3/3)

Species	Virus	Subtype	Titer (log EID_{50}/mL)	InfA	H3	pdmInfA	pdmH1
SWINE	A/Texas/14/2008	H1N1v	8.3	+ (3/3)	nd	+ (3/3)	+ (3/3)
SWINE	A/Ohio/09/2015	H1N1v	7.7	+ (3/3)	nd	+ (3/3)	+ (3/3)
SWINE	A/Ohio/35/2017	H1N2v	6.9	+ (3/3)	nd	+ (3/3)	-
SWINE	A/Minnesota/19/2011	H1N2v	7.1[2]	+ (3/3)	nd	+ (3/3)	-
AVIAN	A/chicken/Pennsylvania/298101-4/2004	H2N2	9.5	+ (3/3)	-	-	-
AVIAN	A/fowl/New Jersey/38092/2014	H2N2	9.2	+ (3/3)	nd	-	-
SWINE	A/Ohio/13/2017	H3N2v	6.6	+ (3/3)	nd	+ (3/3)	-
CANINE	A/canine/Florida/43/2004	H3N8	7.2	+ (3/3)	-	-	-
AVIAN	A/chicken/Alabama/1975	H4N8	10.0	+ (3/3)	-	-	-
AVIAN	A/chucker/Minnesota/14591-7/1998	H5N2	7.2	+ (3/3)	-	-	-
AVIAN	A/Northern pintail/Washington/40964/2014	H5N2	9.4	+ (3/3)	nd	-	-
AVIAN	A/duck/Singapore-Q/F119-3/1997	H5N3	8.5	+ (3/3)	-	-	-
AVIAN	A/gyrfalcon/Washington/41088-6/2014	H5N8	9.8	+ (3/3)	nd	+ (3/3)	-
AVIAN	A/duck/Pennsylvania/1969	H6N1	9.2	+ (3/3)	-	-	-
AVIAN	A/chicken/California/32213-1/2000	H6N2	9.1	+ (3/3)	-	-	-
AVIAN	A/chicken/New York/13237-6/1998	H6N8	10.0	+ (3/3)	-	-	-
AVIAN	A/mallard/NL/12/2000 IB-CDC-1	H7N7	9.5	+ (3/3)	-	-	-
AVIAN	A/Anhui/01/2013	H7N9	10.9	+ (3/3)	nd	-	-
AVIAN	A/turkey/Wisconsin/66	H9N2	9.7	+ (3/3)	nd	-	-
AVIAN	A/Bangladesh/0994/2011	H9N2	10.5	+ (3/3)	nd	-	-
AVIAN	A/chicken/New Jersey/15906-9/1996	H11N1	6.5	+ (3/3)	nd	-	-

[1] nd = not determined
[2]Titer of A/Minnesota/19/2011 is $TCID_{50}$/mL units

Influenza A Subtyping Kit Reproducibility/Precision

The reproducibility and precision of the primer and probe sets used in the Influenza A Subtyping Kit were evaluated in two studies prior to the merger of the CDC Human Influenza Virus Real-Time RT-PCR Detection and Characterization Panel (K080570) and the CDC rRT-PCR 2009 A(H1N1)pdm Flu Panel (K101564).

The first study utilized three separate laboratory sites in a collaborative bridging study with Applied Biosystems™

using the 7500 Fast Dx Real-time PCR instruments and SDS software version 1.4. Instrument functionality was validated at each site by Applied Biosystems™ prior to the reproducibility assessment. The panel of 9 simulated samples that were used included influenza A(H1N1), A(H3N2), A(H5N1) (reassortant), and Influenza B at two viral RNA concentrations each (a low viral RNA concentration near the assay lower LOD range and a 1:10 dilution of the same sample). The low viral RNA concentration generally was one log above the assay cutoff for all analytes, whereas the 1:10 dilution of the same sample approximated a sample at the cutoff or a "high negative" for the different analytes. Four RNA purification methods were used to evaluate reproducibility of the CDC rRT-PCR Flu Panel on the validated 7500 Fast Dx Real-time PCR instruments: the automated MagNA Pure LC RNA Isolation Kit III (Roche Applied Science), the manual QIAGEN RNeasy® RNA extraction (QIAGEN Ltd.), the Roche MagNA Pure LC Automated Isolation System with Total RNA isolation method, and the QIAGEN QIAamp® Viral RNA manual extraction method. The manufacturer's instructions for use provided in the package insert were followed.

The panels and assay controls were tested at each site by 2 operators on 5 different days within a 10-day period. Each site tested one of the four extraction methods associated with this device. Simulated samples in the panel used in the reproducibility evaluation of the Influenza A Subtyping Kit were:

- **Sample #1** Influenza A(H1N1) at low viral RNA titer range
- **Sample #2** Influenza A(H1N1) at a 1:10 dilution of sample 1
- **Sample #3** Influenza A(H3N2) at low viral RNA titer range
- **Sample #4** Influenza A(H3N2) at a 1:10 dilution of sample 3
- **Sample #9** Influenza Negative (Uninfected A549 cells)

Bridging Study Statistical Analysis of the CDC Human Influenza Virus Real-Time RT-PCR Detection and Characterization Panel (K080570) on the 7500 Dx Fast Real-time PCR Instrument

Sample and Analyte Tested	Roche MagNA Pure TNA			QIAGEN QIAamp® RNA			QIAGEN RNeasy®			Roche MagNA Pure RNA			Total Agreement w/ Expected Results	95 % CICI
	Agreement w/ expected Result	Avg. Ct	% CV	Agreement w/ expected Result	Avg. Ct	% CV	Agreement w/ expected Result	Avg. Ct	% CV	Agreement w/ expected Result	Avg. Ct	% CV		
Sample 1 (low) - **InfA**	10/10	33.73	3.12	10/10	32.24	5.65	10/10	34.34	1.94	10/10	31.55	3.69	40/40	91.2–100.0
Sample 1 (low) - **RNaseP**	10/10	28.62	4.00	10/10	30.08	5.63	10/10	27.67	3.28	10/10	28.22	3.13	40/40	91.2–100.0
Sample 2 (1:10 of Sample 1) - **InfA**	9/10	36.59	3.22	10/10	35.30	5.93	10/10	37.03	4.07	10/10	33.97	3.32	39/40	86.8 - 99.9
Sample 2 (1:10 of Sample 1) - **RNaseP**	10/10	29.51	3.80	10/10	31.13	9.58	10/10	28.46	2.47	10/10	28.65	2.12	40/40	91.2–100.0
Sample 3 (low) - **InfA**	10/10	35.39	3.74	10/10	33.32	7.09	10/10	34.01	7.16	10/10	32.29	1.82	40/40	91.2–100.0
Sample 3 (low) - **H3**	9/10	36.25	2.52	10/10	34.98	7.60	10/10	34.07	2.86	10/10	33.28	1.71	39/40	86.8 - 99.9
Sample 3 (low) - **RNaseP**	10/10	29.17	5.32	10/10	30.02	5.33	10/10	28.30	3.05	10/10	28.50	3.34	40/40	91.2–100.0
Sample 4 (1:10 of Sample 3) - **InfA**	7/10	38.44	4.05	10/10	37.03	5.08	5/10	39.05	3.46	10/10	36.18	2.41	32/40	64.4 - 91.0
Sample 4 (1:10 of Sample 3) - **H3**	6/10	38.82	3.34	8/10	39.90	6.14	6/10	38.01	4.81	9/10	36.82	3.49	29/40	56.1 – 85.4
Sample 4 (1:10 of Sample 3) - **RNaseP**	10/10	29.75	4.32	10/10	31.11	7.89	10/10	28.56	4.60	10/10	28.68	3.35	40/40	91.2–100.0
Sample 9 Influenza (-) **RNaseP**	10/10	29.79	3.17	10/10	30.08	5.91	10/10	28.27	4.83	10/10	28.86	3.68	40/40	91.2–100.0

A second reproducibility and precision study was conducted with the CDC rRT-PCR 2009 A(H1N1)pdm Flu Panel (K101564) at six separate laboratory sites. Each testing site assessed a panel of four simulated samples

at relative moderate, low (near the assay lower LOD range), and “high negative” viral RNA concentration, and a negative sample. The panels and assay controls were tested at each site by two operators on five different days within a 10-day period. Each site tested one of the six extractions methods associated with this device. Simulated samples in the qualification panel used in the reproducibility evaluation of the Influenza A Subtyping Kit were:

- **Sample #1** Influenza A(H1N1)pdm09 moderate viral RNA titer range
- **Sample #2** Influenza A(H1N1)pdm09 low viral RNA titer range
- **Sample #3** Influenza A(H1N1)pdm09 “high negative” RNA titer range
- **Sample #4** Influenza Negative (uninfected A549 cells)

Summary of Reproducibility Study for the CDC rRT-PCR A(H1N1)pdm09 Flu Panel (K101564)

N=10 tests		bioMerieux NucliSENS easyMAG			Roche MagNA Pure LC TNA			Roche MagNA Pure Compact NA			QIAGEN QIAcube Viral RNA			QIAGEN Manual Viral RNA			Roche MagNA Pure Compact RNA				
		Agreement with expected result	Avg. Ct	% CV	Agreement with expected result	Avg. Ct	% CV	Agreement with expected result	Avg. Ct	% CV	Agreement with expected result	Avg. Ct	% CV	Agreement with expected result	Avg. Ct	% CV	Agreement with expected result	Avg. Ct	% CV	**Total Agreement**	**95% CI**
No Template Control		10/10	NA	NA	10/10	NA	NA	10/10	NA	NA	10/10	NA	NA	10/10	NA	NA	10/10	NA	NA	**60/60**	**94.0-100**
Sample 1 moderate	**InfA**	10/10	28.52	2.59	10/10	30.53	2.55	10/10	31.99	4.23	10/10	27.92	2.06	10/10	28.85	2.00	10/10	30.54	2.12	**60/60**	**94.0-100**
	pdm InfA	10/10	27.49	2.10	10/10	29.98	2.69	10/10	30.63	3.10	10/10	27.68	2.25	10/10	28.72	1.59	10/10	29.81	2.57	**60/60**	**94.0-100**
	pdm H1	10/10	30.21	2.12	10/10	32.23	3.17	10/10	32.98	2.31	10/10	30.63	1.48	10/10	31.09	1.69	10/10	32.38	2.57	**60/60**	**94.0-100**
	RP	10/10	28.78	1.33	10/10	30.79	2.65	10/10	32.27	2.51	10/10	27.55	2.43	10/10	27.82	2.45	10/10	29.43	1.77	**60/60**	**94.0-100**
Sample 2 low	**InfA**	10/10	33.10	3.26	8/10	35.28	5.54	8/10	36.12	4.11	10/10	32.95	1.04	10/10	33.49	3.14	10/10	34.48	2.88	**56/60**	**88.5-99.6**
	pdm InfA	10/10	32.50	2.48	9/10	33.80	2.97	9/10	35.00	2.88	10/10	32.91	3.32	10/10	33.89	3.27	10/10	34.48	2.92	**58/60**	**88.5-99.6**
	pdm H1	10/10	35.19	3.10	7/10	36.06	4.49	7/10	36.68	2.43	10/10	34.66	1.27	10/10	36.12	2.18	10/10	36.57	3.70	**54/60**	**79.5-96.2**
	RP	10/10	28.44	1.34	10/10	30.33	2.78	10/10	31.44	3.27	10/10	27.41	1.95	10/10	28.34	1.55	10/10	28.57	5.11	**60/60**	**94.0-100**
Sample 3 "high negative"	**InfA**	8/10	NA	NA	10/10	NA	NA	9/10	NA	NA	10/10	NA	NA	7/10	NA	NA	10/10	NA	NA	**54/60**	**79.5-96.2**
	pdm InfA	10/10	NA	NA	10/10	NA	NA	10/10	NA	NA	9/10	NA	NA	8/10	NA	NA	10/10	NA	NA	**57/60**	**86.1-99.0**
	pdm H1	10/10	NA	NA	9/10	NA	NA	10/10	NA	NA	9/10	NA	NA	10/10	NA	NA	10/10	NA	NA	**58/60**	**88.5-99.6**
	RP	10/10	28.04	2.13	10/10	30.08	4.92	10/10	31.12	2.60	10/10	26.89	2.09	10/10	28.33	1.41	10/10	28.45	3.31	**60/60**	**94.0-100**
Sample 4 A549 cells	**RP**	10/10	28.68	2.75	10/10	30.20	5.68	10/10	32.10	1.74	10/10	27.06	2.65	10/10	28.60	2.29	10/10	29.33	4.00	**60/60**	**94.0-100**
HSC	**RP**	10/10	28.51	2.30	10/10	29.80	4.48	10/10	29.55	3.11	10/10	25.89	2.04	10/10	27.30	1.61	10/10	27.35	4.98	**60/60**	**94.0-100**
Influenza 2009 A(H1N1) pdm Positive Control	**InfA**	10/10	23.71	2.10	10/10	21.95	2.97	10/10	23.54	4.10	10/10	21.62	1.39	10/10	23.66	2.63	10/10	24.95	2.21	**60/60**	**94.0-100**
	pdm InfA	10/10	22.81	2.68	10/10	22.27	1.91	10/10	23.79	2.78	10/10	21.82	2.62	10/10	23.63	1.57	10/10	24.93	2.03	**60/60**	**94.0-100**
	pdm H1	10/10	25.76	1.55	10/10	24.35	4.78	10/10	26.36	2.42	10/10	25.31	1.19	10/10	25.89	2.13	10/10	27.52	2.53	**60/60**	**94.0-100**
	RP	10/10	28.84	1.66	10/10	27.44	3.23	10/10	29.03	4.03	10/10	27.35	1.84	10/10	28.59	2.35	10/10	28.82	3.26	**60/60**	**94.0-100**

Additional Nucleic Acid Extraction Platforms: Reproducibility/Precision

The bioMérieux EasyMAG and EMAG systems which use the same chemistry and reagents were compared and determined to perform equivalently. The reproducibility and precision of additional nucleic acid extraction instruments and chemistries were evaluated with the CDC Human Influenza Real-Time RT-PCR Diagnostic Panel using three testing sites for each instrument. Each site assessed a panel of three simulated samples consisting of a moderate positive, low positive (near the assay LOD range), and negative sample. The panel was tested at each site by two different analysts over the course of 5 different days. Samples extracted by the platform or chemistry under evaluation were tested using the InfA, H3, and RP assays.

Reproducibility Summary - QIAGEN EZ1 Advanced XL, EZ1 DSP Virus Kit

Panel Sample	Primer/ Probe Set	Site 1			Site 2			Site 3			Agreement Total	95%CI
		Agreement	Ave. Ct	%CV	Agreement	Ave. Ct	%CV	Agreement	Ave. Ct	%CV		
A(H3) Moderate	InfA	10/10	25.46	2.36	10/10	26.45	2.80	10/10	28.46	4.12	30/30	100.0 (88.7-100.0)
	H3	10/10	27.15	2.92	10/10	28.22	3.25	10/10	28.61	4.40	30/30	100.0 (88.7-100.0)
	RP	10/10	22.45	1.44	10/10	23.47	2.42	10/10	24.50	5.68	30/30	100.0 (88.7-100.0)
A(H3) Low	InfA	10/10	28.96	3.41	10/10	30.35	1.87	10/10	32.21	2.55	30/30	100.0 (88.7-100.0)
	H3	10/10	30.56	2.60	10/10	31.70	1.96	10/10	32.63	3.18	30/30	100.0 (88.7-100.0)
	RP	10/10	22.26	1.08	10/10	23.53	1.12	10/10	24.58	4.63	30/30	100.0 (88.7-100.0)
Negative	InfA	10/10	0.00	n/a	10/10	0.00	n/a	10/10	0.00	n/a	30/30	100.0 (88.7-100.0)
	H3	10/10	0.00	n/a	10/10	0.00	n/a	10/10	0.00	n/a	30/30	100.0 (88.7-100.0)
	RP	10/10	24.80	1.49	10/10	25.35	3.91	10/10	26.93	4.83	30/30	100.0 (88.7-100.0)

n/a = not applicable

Reproducibility Summary - QIAGEN EZ1 Advanced XL, EZ1 RNA Tissue Mini Kit

Panel Sample	Primer/ Probe Set	Site 1			Site 2			Site 3			Agreement Total	95%CI
		Agreement	Ave. Ct	%CV	Agreement	Ave. Ct	%CV	Agreement	Ave. Ct	%CV		
A(H3) Moderate	InfA	10/10	25.97	3.48	10/10	26.40	4.06	10/10	28.59	2.01	30/30	100.0 (88.7-100.0)
	H3	10/10	27.40	4.13	10/10	27.56	2.35	10/10	28.66	0.79	30/30	100.0 (88.7-100.0)
	RP	10/10	22.08	3.31	10/10	23.52	1.56	10/10	24.77	2.14	30/30	100.0 (88.7-100.0)
A(H3) Low	InfA	10/10	30.07	2.04	10/10	30.30	3.26	10/10	32.37	2.44	30/30	100.0 (88.7-100.0)
	H3	10/10	31.02	3.14	10/10	30.94	2.06	10/10	32.59	1.15	30/30	100.0 (88.7-100.0)
	RP	10/10	21.88	2.37	10/10	23.29	2.32	10/10	24.30	2.93	30/30	100.0 (88.7-100.0)
Negative	InfA	10/10	0.00	n/a	10/10	0.00	n/a	10/10	0.00	n/a	30/30	100.0 (88.7-100.0)
	H3	10/10	0.00	n/a	10/10	0.00	n/a	10/10	0.00	n/a	30/30	100.0 (88.7-100.0)
	RP	10/10	24.36	2.57	10/10	25.32	1.55	10/10	27.38	1.85	30/30	100.0 (88.7-100.0)

Reproducibility Summary – Roche MagNA Pure 96, DNA and Viral NA Small Volume Kit

Panel Sample	Primer/ Probe Set	Site 1			Site 2			Site 3			Agreement Total	95%CI
		Agreement	Ave. Ct	%CV	Agreement	Ave. Ct	%CV	Agreement	Ave. Ct	%CV		
A(H3) Moderate	InfA	10/10	31.29	3.97	10/10	30.05	3.37	10/10	27.61	3.93	30/30	100.0 (88.7-100.0)
	H3	10/10	32.00	3.36	10/10	31.92	3.32	10/10	28.44	4.05	30/30	100.0 (88.7-100.0)
	RP	10/10	27.16	4.07	10/10	24.25	1.66	10/10	23.35	3.48	30/30	100.0 (88.7-100.0)
A(H3) Low	InfA	10/10	34.91	2.97	10/10	33.49	2.86	10/10	31.40	3.07	30/30	100.0 (88.7-100.0)
	H3	10/10	35.45	2.54	10/10	35.35	2.75	10/10	32.37	3.30	30/30	100.0 (88.7-100.0)
	RP	10/10	26.97	3.85	10/10	24.22	0.98	10/10	23.11	2.70	30/30	100.0 (88.7-100.0)
Negative	InfA	10/10	0.00	n/a	10/10	0.00	n/a	10/10	0.00	n/a	30/30	100.0 (88.7-100.0)
	H3	10/10	0.00	n/a	10/10	0.00	n/a	10/10	0.00	n/a	30/30	100.0 (88.7-100.0)
	RP	10/10	29.40	3.66	10/10	26.64	1.36	10/10	25.58	2.91	30/30	100.0 (88.7-100.0)

Enzyme Equivalency

Clinical Comparison

A prospective clinical study was conducted during the 2011-2012 influenza season to compare the performance of the CDC Human Influenza rRT-PCR Diagnostic Panel using Quanta BioSciences qScript™ One-Step qRT-PCR Kit, Low ROX (Quanta qScript™) and Invitrogen Superscript™ III Platinum® One-Step Quantitative RT-PCR Kit (Invitrogen Superscript™). Residual material from a total of 1,002 respiratory specimens from patients who were symptomatic for influenza-like illness (ILI) was collected and tested at 6 clinical sites. Nine hundred thirty-one specimens were included in the data analysis after exclusion of samples with inconclusive results (42), technician or instrument error (25), or unspecified specimen type (4). The results from the prospective study are summarized in the table below showing the percent sensitivity or specificity with the two-sided 95% confidence interval.

Prospective Study Comparison

Assay Result	# of Positives[1]	% Positive Agreement (95% CI)	# of Negatives[1]	% Negative Agreement (95% CI)
A(H3)	331/335	98.8 (97.0 – 99.5)	595/596	99.8 (99.1 – 100.0)
A(H1)pdm09	43/43	100.0 (91.8 – 100.0)	888/888	100.0 (99.6 – 100.0)

[1]Proportion of actual positives or actual negatives correctly identified versus the comparator

The analytical and clinical performance of the CDC Human Influenza rRT-PCR Diagnostic Panel using Quanta qScript™ is substantially equivalent when compared with the Invitrogen Superscript™. Both enzyme kits are cleared for use with the CDC Human Influenza rRT-PCR Diagnostic Panel.

Real-Time PCR Instrument Equivalency

Clinical Comparison

A retrospective clinical study was conducted to assess the equivalency between the FDA-cleared Applied Biosystems™ 7500 FastDx and either the Applied Biosystems™ QuantStudio™ Dx or the QIAGEN Rotor-Gene Q MDx instruments using specimens collected during the 2011-2012 and 2013-2014 influenza seasons. Specimens were tested using the CDC Human Influenza rRT-PCR Diagnostic Panel and Invitrogen Superscript™ III Platinum® One-Step Quantitative RT-PCR Kit (Invitrogen Superscript™). Due to the lack of available clinical specimens containing influenza A(H5) viruses, the clinical study specimens were supplemented with contrived samples of stock virus added in different concentrations to an A549 cell suspension in virus transport medium (VTM). A total of 50 positive natural clinical specimens and contrived samples and 50 negative natural clinical specimens were used. Both the Applied Biosystems™ QuantStudio™ Dx and the QIAGEN Rotor-Gene Q MDx instruments performed equivalently to the Applied Biosystems™ 7500 FastDx with 100% positive and 100% negative agreement and a 95% confidence interval of 92.9-100.0.

Carryover and Cross-contamination

The potential for carryover and cross-contamination when testing samples of high viral RNA concentration using either the Applied Biosystems™ QuantStudio™ Dx or the QIAGEN Rotor-Gene Q MDx instruments was examined. An alternating pattern of high positive and negative contrived samples was analyzed using assays from the CDC Human Influenza Real-Time RT-PCR Diagnostic Panel. A total of five individual runs were performed on each instrument. The percent agreement with the expected result was 100% for all samples. No carryover or cross –contamination effect was seen with either instrument.

Analytical sensitivity

The limit of detection equivalence between the FDA-cleared Applied Biosystems™ 7500 FastDx and either the Applied Biosystems™ QuantStudio™ Dx or the QIAGEN Rotor-Gene Q MDx instrument was examined. Each primer and probe set of the CDC Human Influenza Virus Real-Ttime RT-PCR Diagnostic Panel was used to test for influenza virus RNA extracted from serial dilutions of characterized reference stocks of influenza A(H3N2), A(H1N1)pdm09, A(H5N8), B/Victoria lineage, and B/Yamagata lineage viruses. The lowest detectable concentration of each virus at which all replicates tested positive using the Applied Biosystems™ QuantStudio™ Dx or the QIAGEN Rotor-Gene Q MDx was the same or within one 5-fold dilution when compared to the FDA-cleared Applied Biosystems™ 7500 FastDx.

Reproducibility/Precision

Studies were performed to assess the reproducibility of the Applied Biosystems™ QuantStudio™ Dx and the QIAGEN Rotor-Gene Q MDx instruments with the CDC Human Influenza Real-Time RT-PCR Diagnostic Panel. A blinded panel of contrived influenza A and influenza B samples containing a background of betapropiolactone (BPL) -treated A549 cells in VTM was assembled by adding a BPL-treated influenza A(H3N2) virus, A/Hong Kong/4801/2014, or an influenza B/Victoria virus, B/Nevada/03/2011, respectively. The samples included a moderate positive sample and a low positive sample near the established assay LOD as well as a negative sample consisting of background A549 cells and VTM. Testing of each instrument was performed over five different days at three sites by two different analysts at each site. Analysts extracted nucleic acid from the contrived samples and performed rRT-PCR with the InfA, H3, pdmInfA, pdmH1, InfB, YAM, VIC and RP assays from the CDC Human Influenza Real-Time RT-PCR Diagnostic Panel using Invitrogen SuperScript™ enzyme. The results for the reproducibility study are summarized in tables below. Both instruments demonstrated high reproducibility with ≥ 93.3% agreement across different sites, analysts, and days.

Reproducibility Summary – Applied Biosystems™ QSDx instrument

Panel Sample	Primer/ Probe Set	Site 1			Site 2			Site 3			Agreement Total	95% CI
		Agreement	AVG Ct	%CV	Agreement	AVG Ct	%CV	Agreement	AVG Ct	%CV		
sample 1 Moderate A/H3N2	InfA	10/10	27.70	2.46	10/10	29.08	2.53	10/10	29.80	1.25	30/30	100.0 (88.7 - 100.0)
	H3	10/10	28.53	2.96	10/10	29.45	2.98	10/10	30.93	1.27	30/30	100.0 (88.7 - 100.0)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	9/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	29/30	96.7(83.3 - 99.4)
	VIC	9/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	29/30	96.7(83.3 - 99.4)
	YAM	9/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	29/30	96.7(83.3 - 99.4)
	RP	10/10	23.41	6.51	10/10	24.52	3.01	10/10	25.50	1.63	30/30	100.0 (88.7 - 100.0)
sample 2 Moderate B/Victoria	InfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	H3	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	24.70	3.99	10/10	25.54	1.39	10/10	27.17	1.20	30/30	100.0 (88.7 - 100.0)
	VIC	10/10	27.31	8.60	10/10	25.30	3.20	10/10	28.19	2.28	30/30	100.0 (88.7 - 100.0)
	YAM	9/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	29/30	96.7(83.3 - 99.4)
	RP	10/10	23.62	8.17	10/10	24.46	3.46	10/10	25.41	1.57	30/30	100.0 (88.7 - 100.0)
sample 3 Negative	InfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	H3	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	VIC	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	YAM	9/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	29/30	96.7(83.3 - 99.4)
	RP	10/10	25.50	3.50	10/10	26.55	3.91	10/10	28.10	1.11	30/30	100.0 (88.7 - 100.0)
sample 4 Low A/H3N2	InfA	8/10	31.78	1.84	10/10	33.10	2.27	10/10	33.96	1.70	28/30	93.3(78.7 - 98.2)
	H3	10/10	33.18	6.32	10/10	33.75	2.66	10/10	35.01	1.97	30/30	100.0 (88.7 - 100.0)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	VIC	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	YAM	9/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	29/30	96.7(83.3 - 99.4)
	RP	10/10	23.05	10.76	10/10	24.76	1.90	10/10	25.75	1.53	30/30	100.0 (88.7 - 100.0)
sample 5 Low B/Victoria	InfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	H3	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	29.41	0.92	10/10	30.26	1.99	10/10	31.73	1.24	30/30	100.0 (88.7 - 100.0)
	VIC	8/10	31.37	6.26	10/10	30.00	1.91	10/10	32.89	2.80	28/30	93.3(78.7 - 98.2)
	YAM	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	RP	10/10	21.98	10.97	10/10	24.46	2.53	10/10	25.75	0.95	30/30	100.0 (88.7 - 100.0)

n/a = not applicable

Reproducibility Summary –QIAGEN Rotor-Gene Q MDx instrument

Panel Sample	Primer/ Probe Set	Site 1			Site 2			Site 3			Agreement Total	95% CI
		Agreement	AVG Ct	%CV	Agreement	AVG Ct	%CV	Agreement	AVG Ct	%CV		
sample 1 Moderate A/H3N2	InfA	10/10	27.59	1.73	10/10	25.69	1.02	10/10	25.95	0.95	30/30	100.0 (88.7 - 100.0)
	H3	10/10	28.86	2.03	10/10	26.54	1.28	10/10	26.80	0.88	30/30	100.0 (88.7 - 100.0)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	VIC	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	YAM	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	RP	10/10	23.74	1.71	10/10	21.88	0.50	10/10	22.21	1.30	30/30	100.0 (88.7 - 100.0)
sample 2 Moderate B/Victoria	InfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	H3	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	26.02	1.46	10/10	24.06	0.69	10/10	24.26	0.85	30/30	100.0 (88.7 - 100.0)
	VIC	10/10	27.06	2.21	10/10	25.17	2.54	10/10	25.36	2.71	30/30	100.0 (88.7 - 100.0)
	YAM	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	RP	10/10	23.73	0.85	10/10	21.92	0.30	10/10	22.14	1.41	30/30	100.0 (88.7 - 100.0)
sample 3 Negative	InfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	H3	9/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	29/30	96.7 (83.3-99.4)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	VIC	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	YAM	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	RP	10/10	26.17	2.96	10/10	23.87	0.98	10/10	24.52	1.21	30/30	100.0 (88.7 - 100.0)
sample 4 Low A/H3N2	InfA	10/10	31.61	1.69	10/10	29.75	1.16	10/10	29.93	1.97	30/30	100.0 (88.7 - 100.0)
	H3	10/10	32.75	2.64	10/10	30.61	2.05	10/10	30.81	0.93	30/30	100.0 (88.7 - 100.0)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	VIC	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	YAM	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	RP	10/10	23.90	1.65	10/10	21.90	0.54	10/10	22.32	1.83	30/30	100.0 (88.7 - 100.0)
sample 5 Low B/Victoria	InfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	H3	10/10	n/a	n/a	10/10	n/a	n/a	9/10	n/a	n/a	29/30	96.7 (83.3-99.4)
	pdmInfA	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	pdmH1	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	InfB	10/10	30.71	4.70	10/10	28.25	1.04	10/10	28.53	1.08	30/30	100.0 (88.7 - 100.0)
	VIC	9/10	32.85	3.41	10/10	29.94	2.08	10/10	30.58	3.52	29/30	96.7 (83.3-99.4)
	YAM	10/10	n/a	n/a	10/10	n/a	n/a	10/10	n/a	n/a	30/30	100.0 (88.7 - 100.0)
	RP	10/10	23.70	0.92	10/10	21.88	0.31	10/10	22.18	1.18	30/30	100.0 (88.7 - 100.0)

n/a = not applicable

Amendment to H3 Primers and Probe Set

Limit of Detection (LoD):

The influenza A(H3) primers and probe set was modified to ensure comprehensive detection of recently circulating seasonal influenza A(H3) viruses, which contain a new mutation near the 3' end of the target gene sequence. LoD was reverified using two influenza A(H3) strains: one legacy strain and one representative strain of the newly circulating virus. Viruses used are listed below.

Viruses Used in the H3 Amendment LoD Reverification Studies

Virus	Type	3' Mutation
A/Darwin/09/2021	Influenza A/H3	Yes
A/HongKong/4801/2014	Influenza A/H3	No

The LoD for the modified influenza A(H3) primer/probe set was found to be equivalent.

Inclusivity, Cross-Reactivity, and Exclusivity:

Cross-reactivity, and exclusivity studies were performed to reverify performance and were found to show equivalent performance with the modified influenza A(H3) primer/probe set. Inclusivity studies were also performed to reverify performance and were found to show comparable performance.

Clinical Evaluation

A retrospective clinical study was performed by testing 60 banked positive influenza specimens that were confirmed to either contain the 3' mutation or not by next generation sequencing (NGS), and 60 banked negative influenza specimens. Specimens were tested using both the modified primer/probe set (H3_v2) and the current primer/probe set (H3_IVD). Both positive percent agreement (PPA) and negative percent agreement were found to be equivalent with the modified primer/probe set.

Agreements with Positive Retrospective Clinical Specimens

Primer/Probe Set	Invitrogen Superscript™ III		Quanta qScript™	
	# of Positives[1]	% Positive Agreement (95% CI)	# of Positives[1]	% Positive Agreement (95% CI)
H3_v2 (ZEN)	60/60	100 (93.4-100)	58/60	96.67 (89-99)
H3_v2 (BHQ)	60/60	100 (93.4-100)	58/60	96.67 (89-99)
H3_IVD	60/60	100 (93.4-100)	57/60	95 (86-98)

[1]Proportion of positive samples correctly identified versus the comparator

Agreements with Negative Retrospective Clinical Specimens

Primer/Probe Set	Invitrogen Superscript™ III		Quanta qScript™	
	# of Negatives[1]	% Negative Agreement (95% CI)	# of Negatives[1]	% Negative Agreement (95% CI)
H3_v2 (ZEN)	60/60	100 (93.4-100)	60/60	100 (93.4-100)
H3_v2 (BHQ)	60/60	100 (93.4-100)	60/60	100 (93.4-100)
H3_IVD	60/60	100 (93.4-100)	60/60	100 (93.4-100)

[1]Proportion of negative samples correctly identified versus the comparator

Disposal

Dispose of hazardous or biologically contaminated materials according to the practices of your institution.

References

1. Ballew, H. C., *et al*. "Basic Laboratory Methods in Virology," DHHS, Public Health Service 1975 (Revised 1981), Centers for Disease Control and Prevention, Atlanta, Georgia 30333.

2. Clinical Laboratory Standards Institute (CLSI), "Collection, Transport, Preparation and Storage of Specimens for Molecular Methods: Approved Guideline," MM13-A, Vol. 25, No. 31.

3. Clinical Laboratory Standards Institute (CLSI), "User Protocol for Evaluation of Qualitative Test Performance," Guideline EP-12A.

4. Clinical Laboratory Standards Institute (CLSI), "Genotyping for Infectious Disease: Identification and Characterization; Approved Guideline,' MM10-A, Vol. 26, No. 9.

5. Lieber, M., *et al*. "A Continuous Tumor Cell Line from a Human Lung Carcinoma with Properties of Type II Alveolar Epithelial Cells." *International Journal of Cancer* 1976, 17(1), 62-70.

6. Szretter, K. J., *et al*. " Influenza: Propagation, Quantification, and Storage" in Current Protocols in Microbiology 2006 15G.1.1-15G.1.22; Editors: R. Coica, T. Kowalik, J. M. Quarles, B. Stevenson, R. K. Taylor, A. E. Simon and T. Downey; John Wiley & Sons, Inc. Hoboken, NJ.

7. White L. A. "Standard Operating Procedure BPP/041 (Innocuity Testing of Viral Reagents)," DHHS, Public Health Service 1982, Centers for Disease Control and Prevention, Atlanta, Georgia 30333.

Contact Information, Ordering, and Product Support

For technical and product support, contact the CDC Influenza Division Support team directly.

For Ordering contact the International Reagent Resource:

Website: InternationalReagentResource.org
Email: contact@internationalreagentresource.org

For technical support contact CDC:

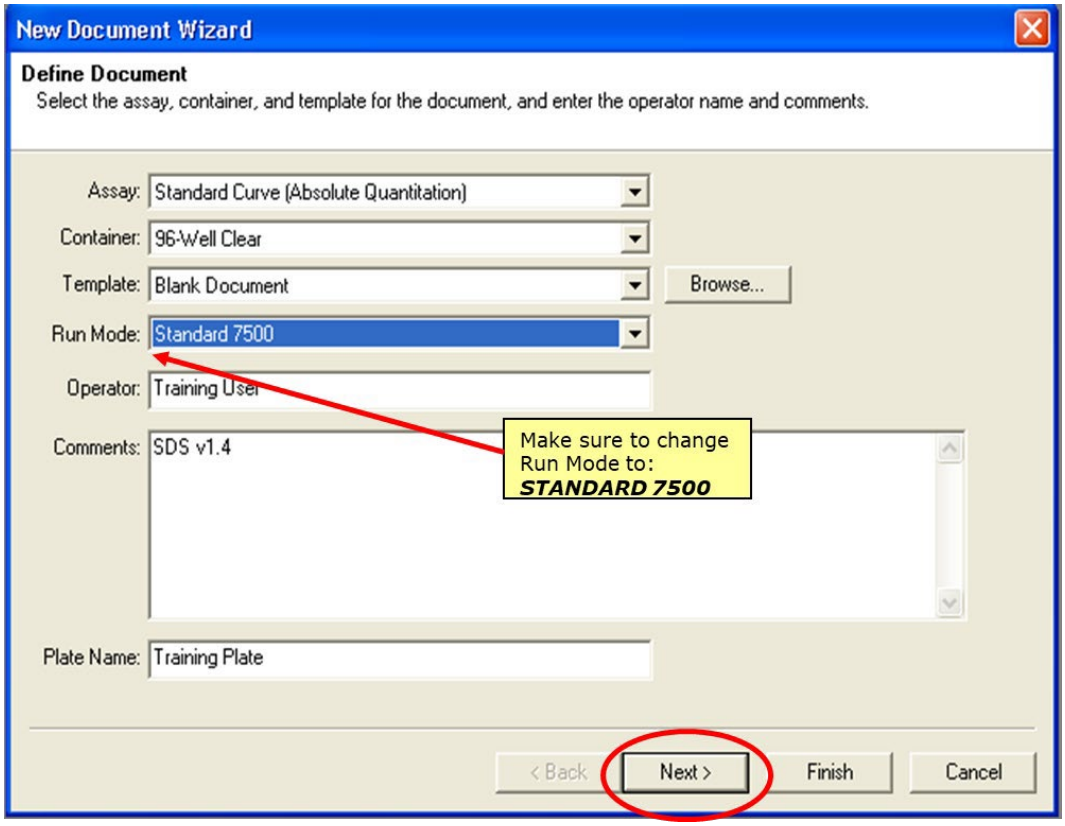
Send email to: FluSupport@cdc.gov

Appendix A: Instrument Setup for Applied BiosystemsTM 7500 Fast Dx Real-time PCR Instrument

This section describes how to create an Influenza A Subtyping Kit template for the 7500 Fast Dx. This template will be saved and used for all Influenza A Subtyping Kit testing on this instrument.

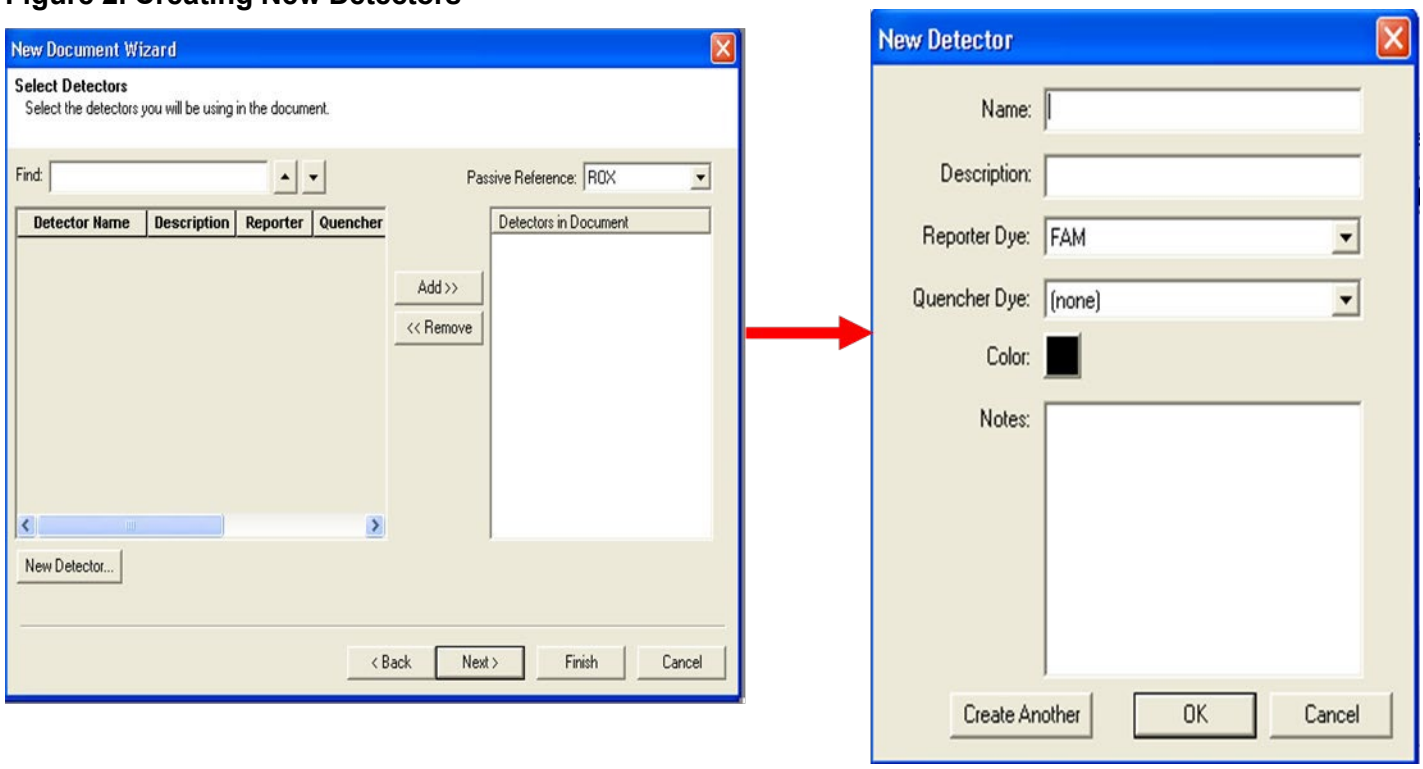
1. Launch the 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
2. A new window should appear, select **Create New Document** from the menu.

Figure 1. New Document Wizard Window



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**
   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 influenza primer and probe set. 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



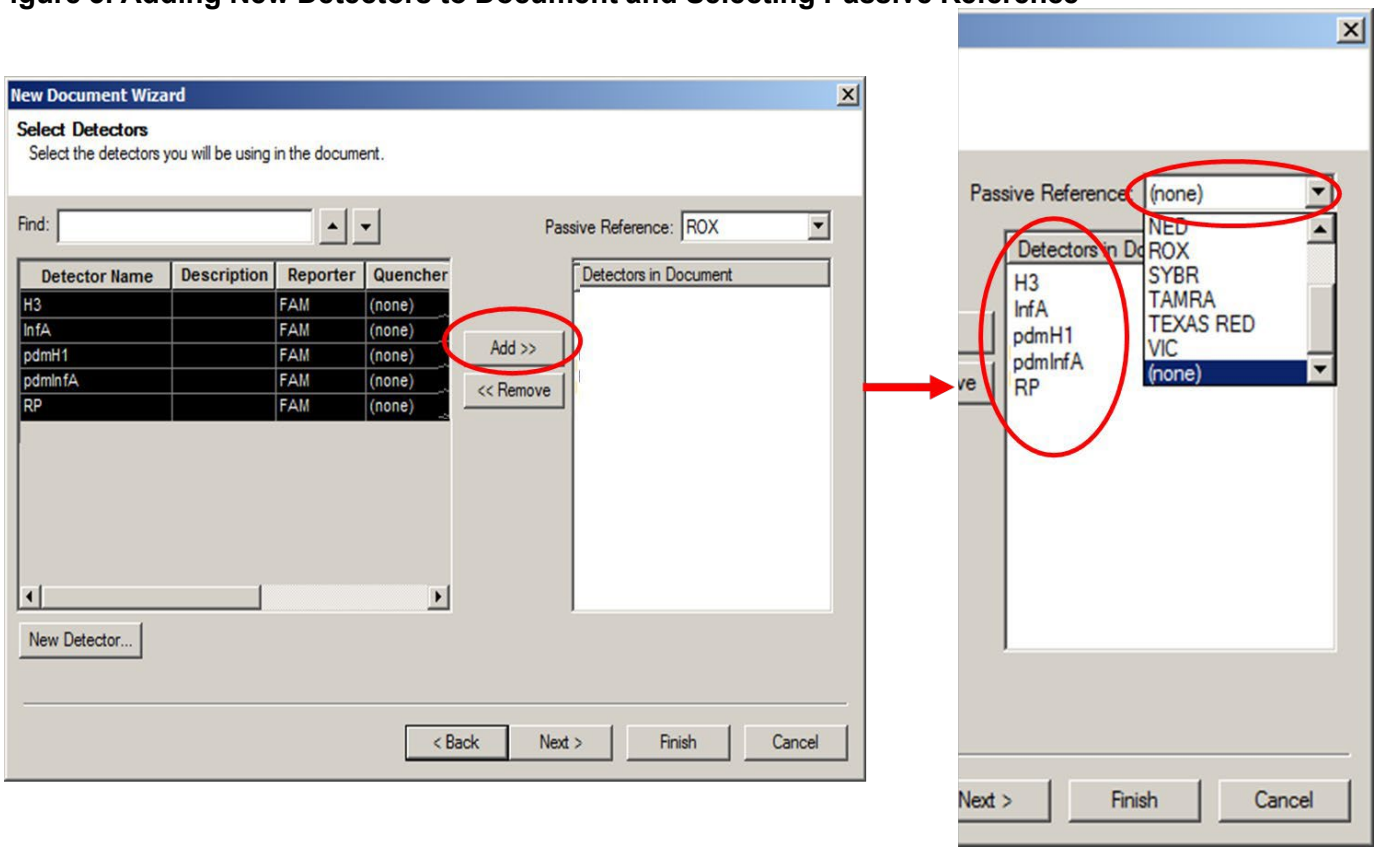
8. Start by creating the InfA Detector (**Figure 2**, right image). Include the following:
   a. Name: **InfA**
   b. Description: *leave blank*
   c. Reporter Dye: **FAM**
   d. Quencher Dye: **(none)**
   e. Color: *to change the color of the detector indicator do the following:*
      ⇒ Click on the 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 influenza target in the panel.

Name	Reporter Dye	Quencher Dye
InfA	FAM	(none)
H3	FAM	(none)
RP	FAM	(none)
pdmInfA	FAM	(none)
pdmH1	FAM	(none)

10. After each Detector is added, the **Detector Name**, **Description**, **Reporter** and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 3**).

11. Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (**Figure 3**, left image). Detector names will appear on the right side of the **Select Detectors** window (**Figure 3**, right image).
12. Once all detectors have been added, select **(none)** for **Passive Reference** at the top right drop down menu (**Figure 3**, right image).

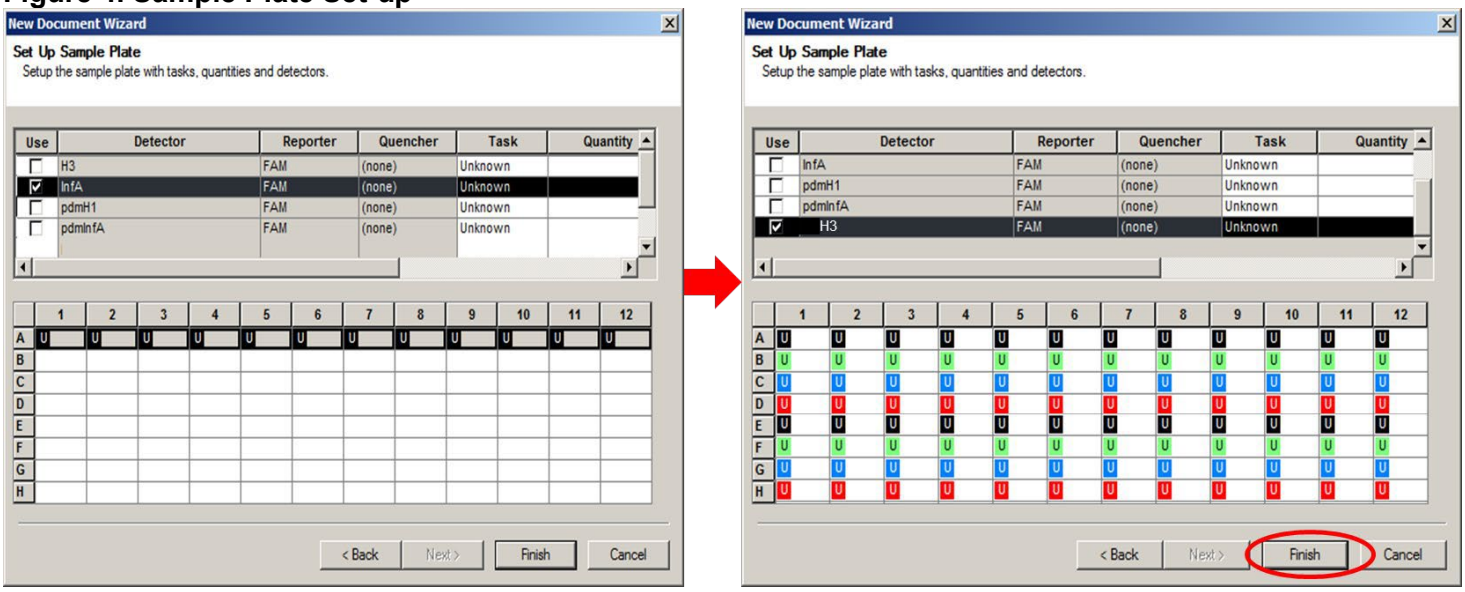
Figure 3. Adding New Detectors to Document and Selecting Passive Reference



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

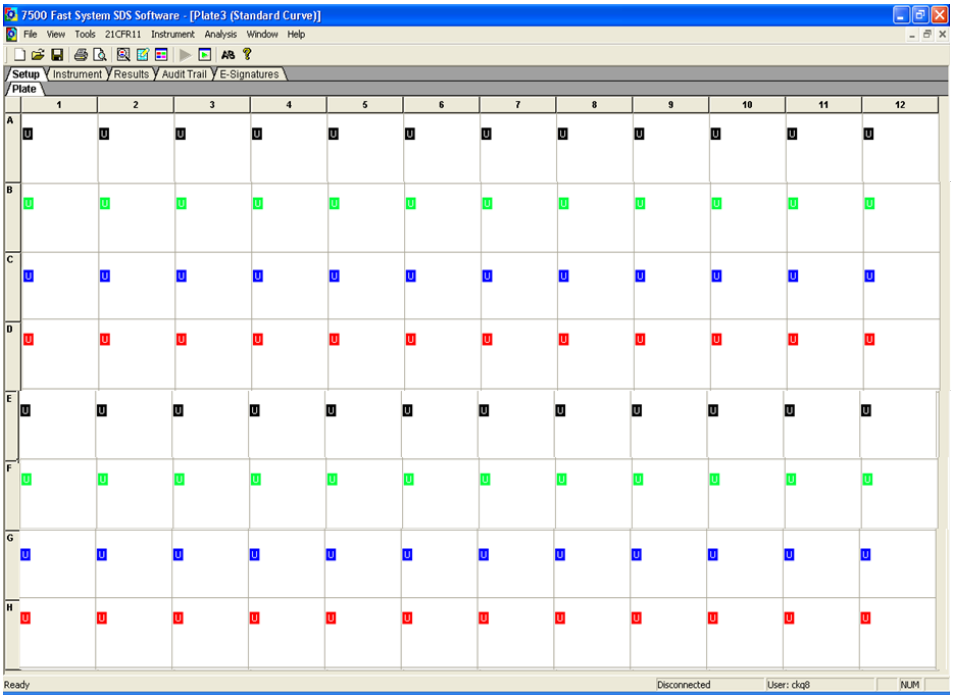
13. Click **Next** at the bottom of the **Select Detectors** window (**Figure 3,** right image) to proceed to the **Set Up Sample Plate** window (**Figure 4**).
14. In the **Set Up Sample Plate** window (**Figure 4**), select row **A** from the in the spreadsheet in the lower portion of the window.
15. In the top portion of the window, select detector **InfA**. A check will appear next to the detector that was selected (**Figure 4**). The row in the spreadsheet that corresponds with **InfA** will be populated with a colored "U" icon to indicate which detector has been selected.
16. Repeat step 14-15 for each detector that will be used in the assay.
17. Select **Finish** after detectors have been assigned to their respective rows (**Figure 4**, right image).

Figure 4. Sample Plate Set-up



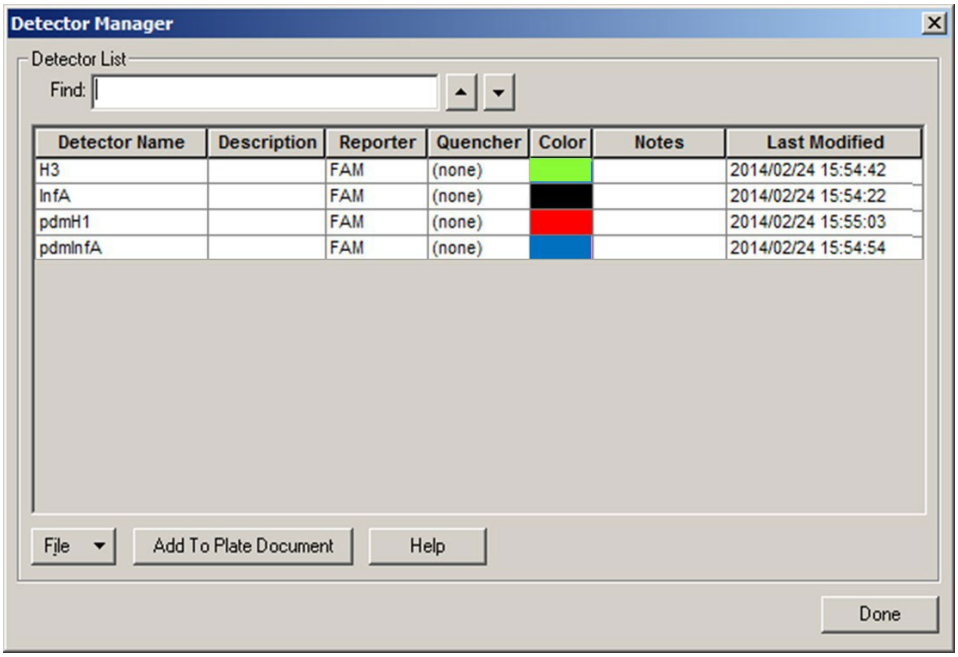
18. After clicking **Finish**, there will be a brief pause allowing the 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***
19. After initialization, the **Plate** tab of the **Setup** (**Figure 5)** will appear.
20. 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



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

Figure 6. Detector Manager Window



22. Confirm all influenza detectors are included and that each influenza target has a **Reporter** set to **FAM** and the **Quencher** is set to **(none)**.
23. 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 Invitrogen SuperScript™ III or Quanta qScript™ Enzyme)

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**):

Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System

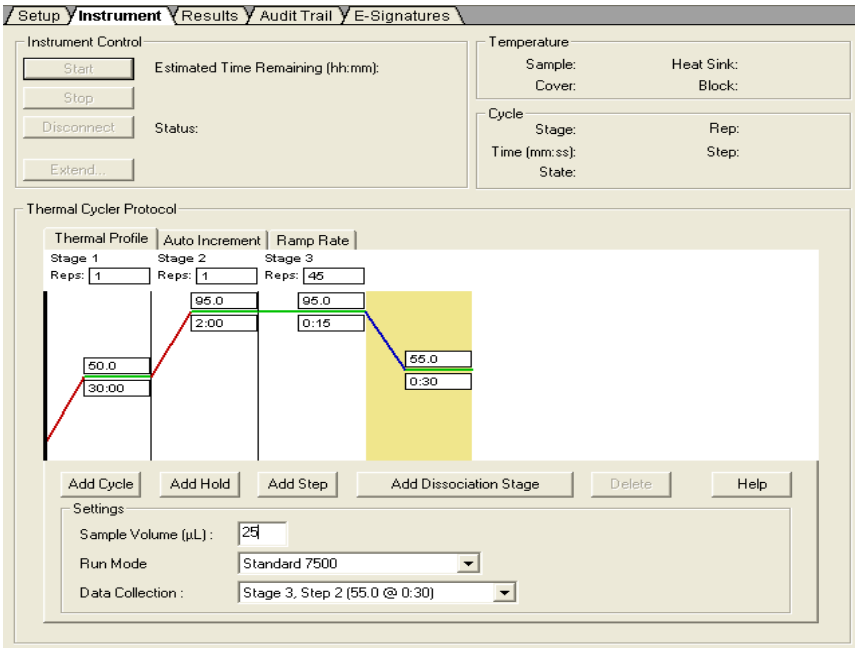
a. In Stage 1, Set to **30 min** at **50°C**; **1 Rep**.
b. In Stage 2, Set to **2 min** at **95°C**; **1 Rep**.
c. In Stage 3, Step 1 set to **15 sec** at **95°C**.
d. In Stage 3, Step 2 set to **30 sec** at **55.0°C.**
e. In Stage 3, Reps should be set to **45.**
f. Under **Settings** (**Figure 7**),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

OR

Quanta BioSciences qScript™ One-Step qRT-PCR Kit, Low ROX

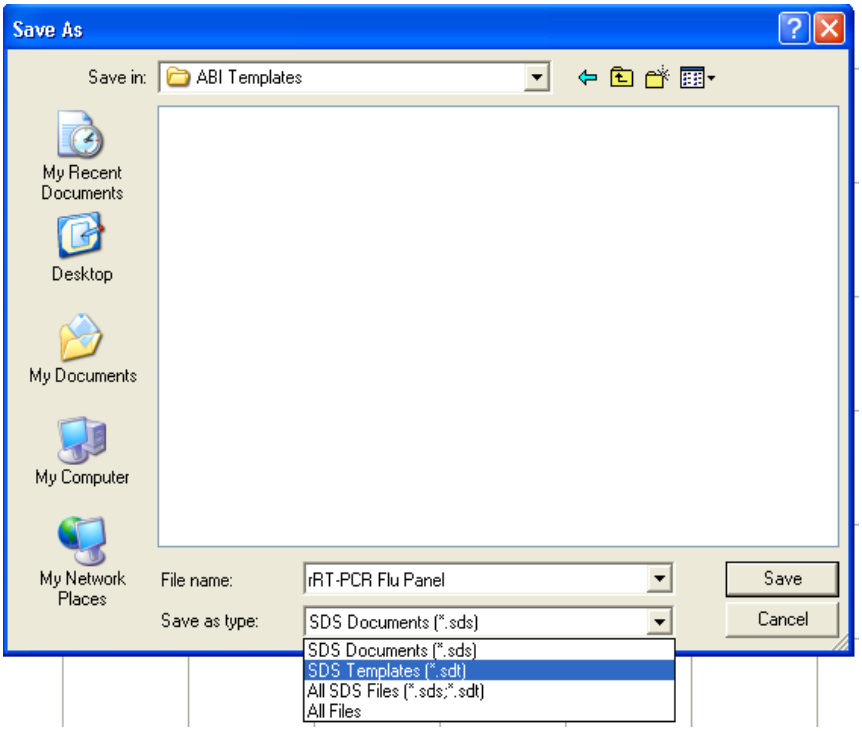
a. In Stage 1, Set to **30 min** at **50°C**; **1 Rep**.
b. In Stage 2, Set to **5 min** at **95°C**; **1 Rep**.
c. In Stage 3, Step 1 set to **15 sec** at **95°C**.
d. In Stage 3, Step 2 set to **30 sec** at **55.0°C**.
e. In Stage 3, Reps should be set to **45**.
f. Under **Settings (Figure 7)**, bottom left box, change volume to 25 µL.
g. Under **Settings**, **Run Mode** selection should be **Standard 7500**.
h. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 7**).

Figure 7. Instrument Window



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 **Influenza A Subtyping Kit SuperScript** or **Influenza A Subtyping Kit qScript** as appropriate in the desktop folder labeled "***ABI Run Templates***" (*user must create this folder*). Save as type should be SDS Templates (*.sdt) (**Figure 8**).

Figure 8. Saving Template

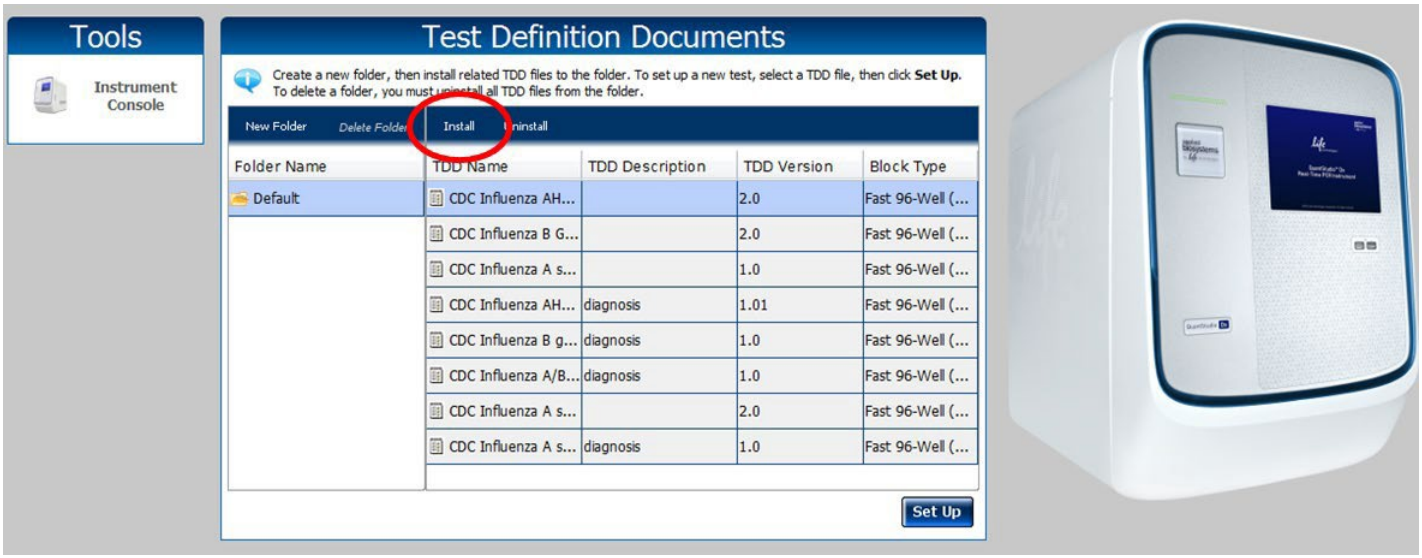


Appendix B: Instrument Setup for Applied Biosystems™ QuantStudio™ Dx (QSDx)

This section describes how to install a test definition document (.tdd) for the Influenza A Subtyping Kit onto the QuantStudio™ Dx. This .tdd file will be saved and used for all Influenza A Subtyping Kit testing on this instrument.

1. Go to the FluSupport Sharepoint site at https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx and download the appropriate test definition document file (*.tdd) for the Influenza A Subtyping Kit for use with the QuantStudio™ Dx Real-time PCR system and save to a designated folder.
2. Launch the QuantStudio™ Dx Real-time PCR System by double clicking on the QuantStudio™ Dx software icon on the desktop.
3. Enter the default username and password into the **Log In** pop-up window and select **Log In**.
4. The **Show Security Events** window will open. Select **Close.** The **Test Definition Document** window (**Figure 1)** will automatically open.

Figure 1. Installing New Tests



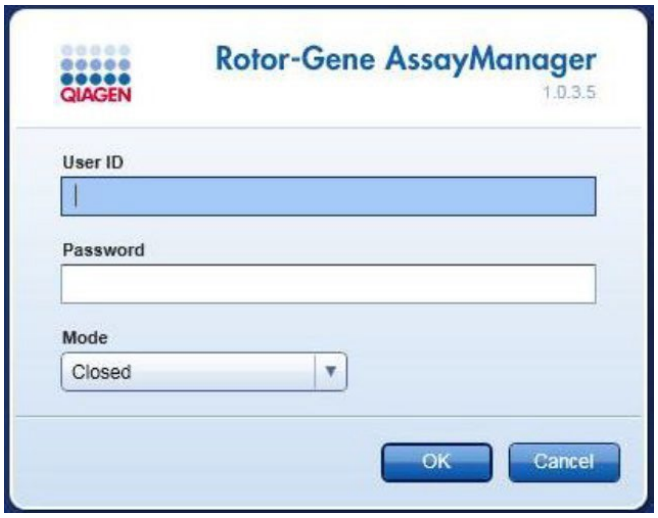
5. In the **Test Definition Document** window select **Install** to display the **Open** window.
6. Navigate to the folder containing the test definition document files (.tdd).
7. Select the appropriate Influenza A Subtyping Kit file (.tdd) and click **Open**. The file name will now be displayed in the **Test Definition Document** table.
8. If necessary, install another test definition document by clicking **Install** and repeat steps 6-7.
9. Once a test definition document is installed it will persist until deleted. This .tdd file should be used for all Influenza A Subtyping Kit testing.

Appendix C: Instrument Setup for QIAGEN Rotor-Gene Q MDx (QMDx)

This section describes how to import an assay profile for the Influenza A Subtyping Kit to the Rotor-Gene Q MDx. This assay profile will be saved and used for all Influenza A Subtyping Kit testing on this instrument.

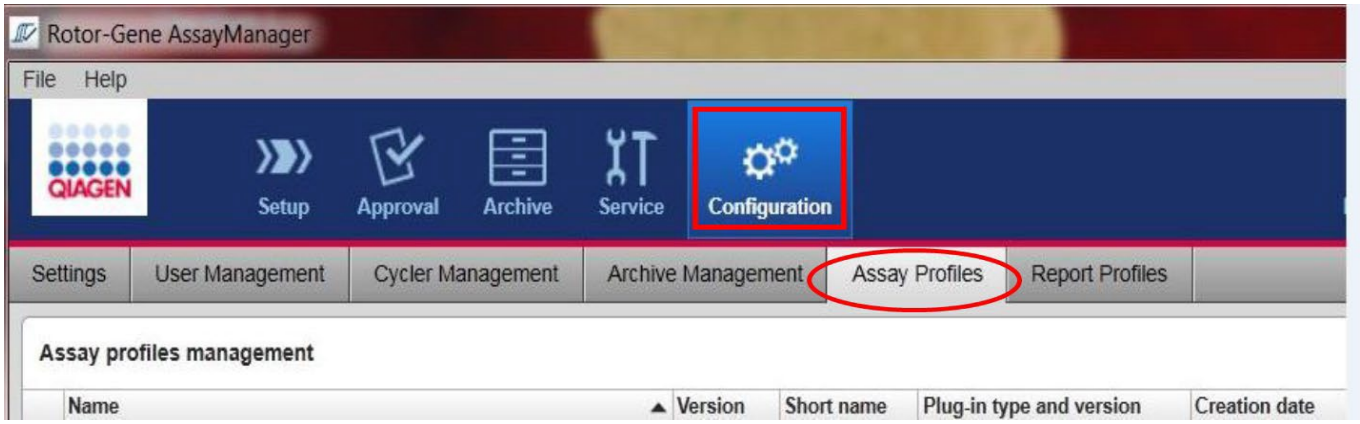
1. Go to the FluSupport Sharepoint site at https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx and download the appropriate assay profile for the Influenza A Subtyping Kit for use with the QIAGEN Rotor-Gene Q MDx instrument and save to a designated folder.
2. Launch the QIAGEN Rotor-Gene Assay Manager software by double clicking on the icon on the desktop.
3. Enter **User ID** and **Password** into the **Rotor-Gene Assay Manager** login window (**Figure 1**).
4. Select **Closed** from the **Mode** drop down menu.

Figure 1. Rotor-Gene AssayManager Login Window



5. Select **OK** and the Rotor-Gene AssayManager main navigation window (**Figure 2**) will open.
6. Select the **Configuration** tab from the top menu, then select the **Assay Profiles** tab (**Figure 2**).

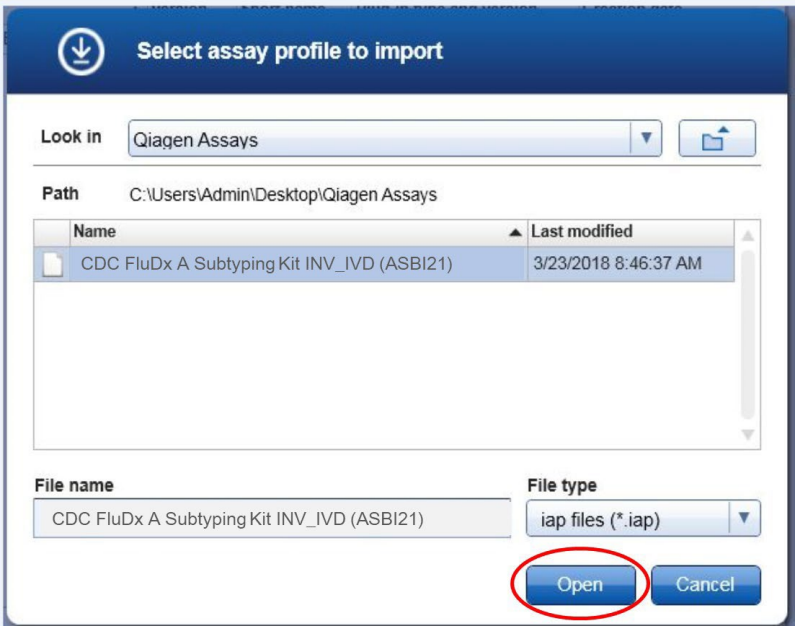
Figure 2. Rotor-Gene Assay Manager – Main Navigation Window



7. Click the **Import** button at the bottom right of the **Assay Profiles** tab to display the **Select assay profile to import** window (**Figure 3**).
8. Navigate to the folder that contains the assay profiles using the **Look in** drop down menu.

9. Select the appropriate Influenza A Subtyping Kit assay profile (.iap) file and click **Open.**

Figure 3. Select Assay Profile to Import Window



10. The **Import Successful** window (**Figure 4**) will appear. Click **OK** to return to the **Assay Profiles** tab. The new assay will be listed under **Assay profiles management (Figure 4).**
11. Once a test definition document is imported, it will persist in the **Assay Profiles** tab. This assay profile should be used for all Influenza A Subtyping Kit testing.

Figure 4. Import Successful Window and Assay Manager Configuration Screen