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

Influenza A/H5 Subtyping Kit (VER 4)

Instructions for Use
Package Insert

Catalog # FluIVD03-11
1000 reactions

For *In-vitro* Diagnostic (IVD) Use

Rx Only

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

CENTERS FOR DISEASE CONTROL AND PREVENTION

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Intended Use

The Influenza A/H5 Subtyping Kit contains reagents and controls of the Centers for Disease Control and Prevention (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 the presumptive identification of virus in patients who may be infected with influenza A subtype A(H5) (Asian lineage) from viral RNA in human respiratory specimens, conjunctival swabs and viral culture in conjunction with clinical and epidemiological risk factors;

- 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.

Testing with the influenza H5a and H5b primer and probe sets should not be performed 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. The definitive identification of influenza A(H5) (Asian lineage) either directly from patient specimens or from virus cultures requires additional laboratory testing, along with clinical and epidemiological assessment in consultation with national influenza surveillance experts.

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/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

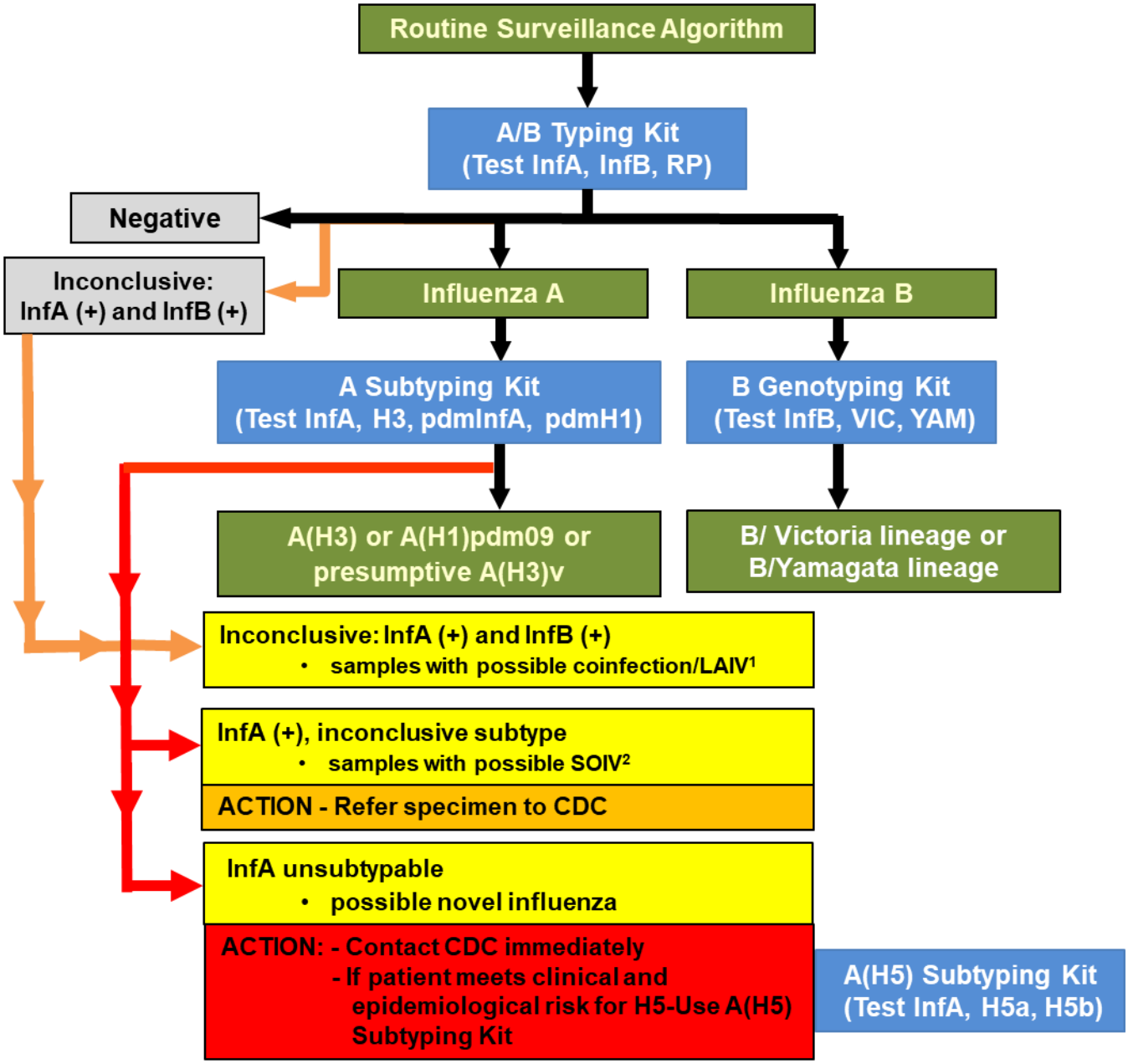
including an abrupt onset of fever, sore throat, cough, headache, myalgia, and malaise. When a subject is infected with a highly virulent strain of influenza, these symptoms can progress rapidly to pneumonia and in some circumstances death. Pandemic outbreaks of highly virulent influenza present a serious risk to human and animal health worldwide. Symptoms of conjunctivitis have also been associated with avian influenza infection.

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 A(H3) and A(H1)pdm09 • Detects classical swine influenza viruses and swine triple reassortant viruses[1]
A/H5 Subtyping Kit	**Primer and Probe Sets:** InfA, H5a, H5b, RP **Controls:** Influenza A/H5N1 Positive Control with Human Cell Material (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:



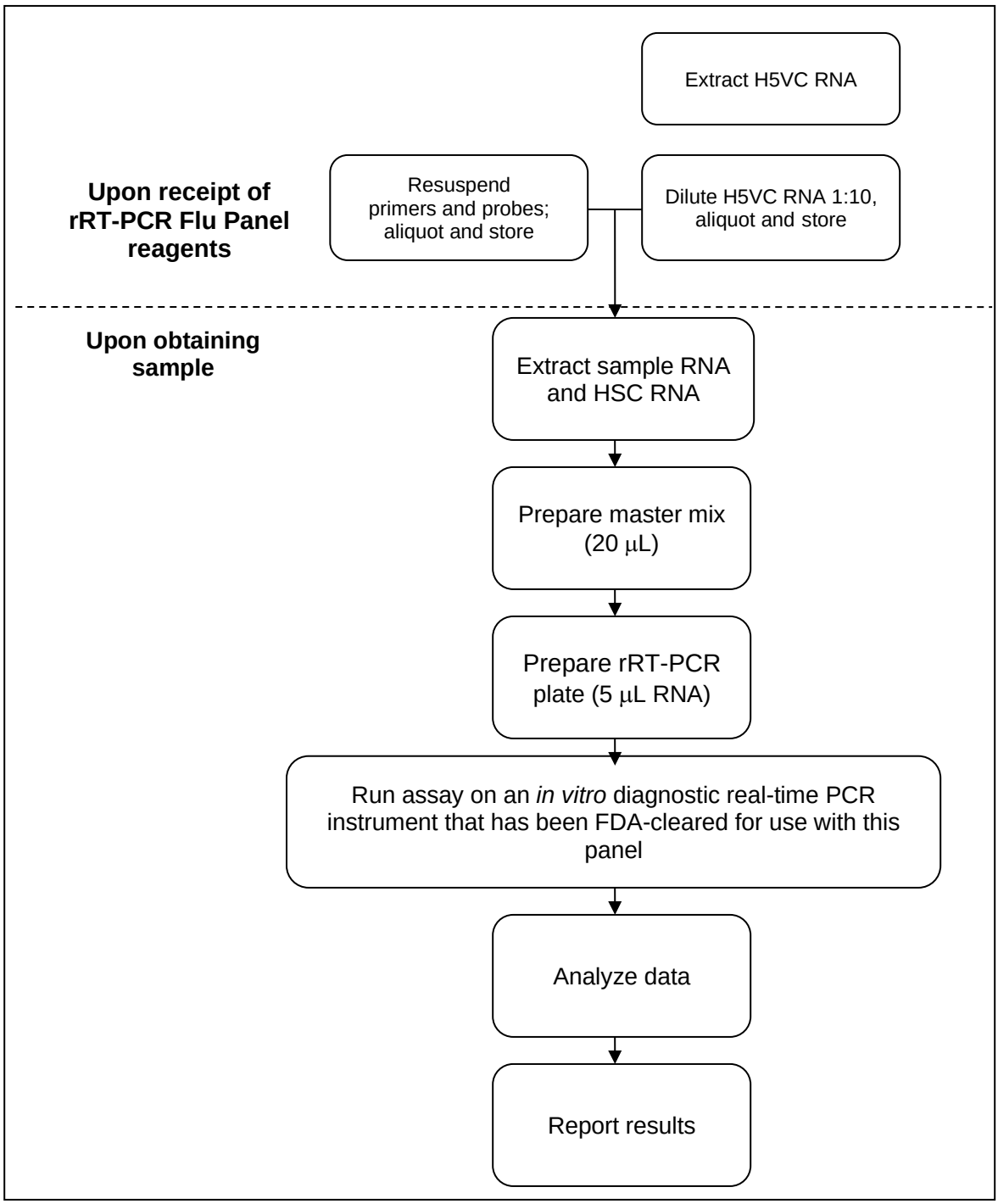
[1]LAIV=Live Attenuated Influenza Virus
[2]SOIV=Swine Origin Influenza Virus

Principles of the Procedure

The Influenza A/H5 Subtyping Kit contains components of the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel that are 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/H5 Subtyping Kit are designed for the detection and characterization of influenza type A(H5) (Asian lineage) viruses.

The Influenza A/H5 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 influenza virus RNA in respiratory specimens and conjunctival swabs from patients presenting with influenza-like illness (ILI) and/or symptoms associated with influenza A(H5) infection. The oligonucleotide primers and probes for detection of influenza A viruses were selected from highly conserved regions of the matrix (M) protein. Oligonucleotide primers and probes for characterization and differentiation of avian influenza A(H5) (Asian lineage) viruses were selected from highly conserved regions of the 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/H5 Subtyping Kit (VER 4) Contents: Catalog # FluIVD03-11

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
H5a-F	MR-409	Influenza H5a Forward Primer	30 nmol	1000
H5a-R	MR-433	Influenza H5a Reverse Primer	20 nmol	1000
H5a-P	MR-410	Influenza H5a Probe (FAM)	7.5 nmol	1000
H5b-F	MR-411	Influenza H5b Forward Primer	20 nmol	1000
H5b-R	MR-443	Influenza H5b Reverse Primer	20 nmol	1000
H5b-P	MR-434	Influenza H5b 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: Human Specimen Extraction Controls and Influenza A/H5N1 Positive Control

Reagent Label	Part #	Description	Quantity / Tube	Notes
Human Specimen Control (HSC)	HS0096	Human Specimen Control (HSC): For use as a RNA extraction procedural control to demonstrate successful recovery of RNA as well as extraction reagent integrity. Purified RNA from the HSC material should yield a positive result with the RP primer and probe set and negative results with all influenza specific markers. The HSC consists of noninfectious (beta propiolactone treated) cultured human cell material supplied as a liquid suspended in 0.01 M PBS at pH 7.2-7.4.	17 tubes x 500 µL / tubes	Five 100 µL extractions per tube

Reagent Label	Part #	Description	Quantity / Tube	Notes
H5VC	VA2850	Influenza A/H5N1 Positive Control with Human Cell Material: For use as a positive control to ensure proper detection of Influenza A(H5) virus (Asian lineage). The contents are noninfectious (beta-propiolactone treated) positive control material suspended in 0.01 M phosphate buffer saline (PBS) at pH 7.2-7.4. The H5VC control consists of a reassortant human vaccine candidate virus (A/Vietnam/1203/04 x PR/8/34) that was generated by reverse genetics and cultured human cell material. The H5VC will yield a positive result with the following primer and probe sets: InfA, H5a, H5b, and RP.	1 tubes 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 Compact	Nucleic Acid Isolation Kit I	32 extractions: 03 730 964 001
	RNA Isolation Kit	32 extractions: 04 802 993 001
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	QIAamp DSP Viral RNA Mini Kit	50 extractions: 61904
QIAGEN QIAcube	QIAamp DSP Viral RNA Mini Kit	50 extractions: 61904

Instrument/Manufacturer	Extraction Kit	Catalog No.
QIAGEN EZ1 Advanced XL	EZ1 DSP Virus Kit	48 extractions (62724) Buffer AVL (19073) EZ1 Advanced XL DSP Virus Card (9018703)
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.)	**N/A**	EasyMAG® Magnetic Silica (280133) EasyMAG® Lysis Buffer (280134) EasyMAG® Lysis Buffer, 2 mL (200292) EasyMAG® Wash Buffers 1,2, and 3 (280130, 280131, 280132) EasyMAG® Disposables (280135) Biohit Pipette Tips (*easyMAG® only*) (280146) 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	ABI MicroAmp™ Fast 8-tube strip 0.1 mL	#4358293
	ABI MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (alternate to 8-strip tubes)	#4346906, #4346907, or #4366932
	ABI MicroAmp™ Optical 8-cap strip (required, do not use film)	#4323032
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.
- Identification of patients for testing of conjunctival swab specimens, and collection procedures for this type of specimen, should follow up-to-date CDC guidance. Conjunctival swab collection has the potential to cause ocular injury and/or introduce bacterial infection and should be performed by appropriately trained staff using the indicated sterile swabs only.
- 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 nasopharyngeal swabs [NPS], nasal swabs [NS], throat swabs [TS], nasal aspirates [NA], nasal washes [NW] and dual nasopharyngeal/throat swabs [NPS/TS]),lower respiratory tract specimens (including

bronchoalveolar lavage [BAL], bronchial wash [BW], tracheal aspirate [TA], sputum and lung tissue) and conjunctival swabs from human patients with signs and symptoms associated with influenza A/H5 infection and/or from viral culture. Human respiratory specimens, conjunctival swabs and/or virus culture samples are acceptable specimens for influenza A(H5) testing. Due to the rarity of these specimen types, performance was determined in accordance with recommendations for A(H5) testing procedures.

- Influenza A/H5 is a relatively novel and evolving agent of infection in humans, the full clinical spectrum and viral dynamics of which are still being investigated and may change over time. Test performance may vary depending on specimen type and may not fully correlate with symptoms. Conjunctival swab specimen testing may be sufficient to identify influenza A/H5 viruses in patients with conjunctivitis and a strong epidemiological reason to suspect this infection. For patients with respiratory or non-specific symptoms (e.g., fever, headache), paired testing of multiple respiratory tract specimen types following CDC recommendations should be attempted whenever feasible.
- Perform all manipulations of live virus samples within a Class II (or higher) biological safety cabinet (BSC).
- Use personal protective equipment such as (but not limited to) gloves and lab coats when handling kit reagents while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.
- Amplification technologies such as PCR are sensitive to accidental introduction of product from previous 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.

Important Public Health and Surveillance Information Regarding Testing with the Influenza A/H5 Subtyping Kit

CDC recommends maintaining the enhanced surveillance efforts by state and local health departments, hospitals, and clinicians to identify patients at increased risk for infection with avian influenza A (A(H5)). Guidelines for enhanced surveillance and testing are as follows:

- Testing for avian influenza A(H5) is indicated for patients with medically-attended influenza-like-illness and/or symptoms associated with influenza A/H5 infection who meet any of the following exposure criteria:

1. Patients who have had recent contact (within <10 days of illness onset) with potentially infected birds (i.e., sick or dead birds, or flocks where highly pathogenic avian influenza A(H5) virus infection has been confirmed) in any of the following categories:
    - Domestic poultry (e.g., chickens, ducks, geese)
    - Wild aquatic birds (e.g., ducks, geese, swans)
    - Other bird species that have been confirmed to be infected with highly pathogenic avian influenza A(H5) viruses (Refer to current CDC guidance below)
2. Patients who have had recent close contact (within <10 days of illness onset) with confirmed or suspected cases of human infection with avian influenza. Close contact may be regarded as coming within approximately 6 feet of a confirmed or suspected case while the case was ill (beginning 1 day prior to illness onset until resolution of illness).
3. Recent travel (within <10 days of illness onset) to areas outside the United States where known human cases of highly pathogenic avian influenza infection have become infected or to areas where highly pathogenic avian influenza viruses are known to be circulating in animals (for a regularly updated listing of A(H5)-affected countries, see the OIE website).
4. Unprotected exposure to infectious highly pathogenic avian influenza in a laboratory.

- Testing for avian influenza A(H5) should be considered on a case-by-case basis in consultation with state and local health departments.

CDC Reference: Interim Guidance on Testing, Specimen Collection, and Processing for Patients with Suspected Infection with Novel Influenza A Viruses with the Potential to Cause Severe Disease in Humans: Interim Guidance on Specimen Collection and Testing for Patients with Suspected Infection with Novel Influenza A Viruses Associated with Severe Disease or with the Potential to Cause Severe Disease in Humans | Bird Flu | CDC

These recommendations are subject to change; please refer to current recommendations posted on the CDC website: http://www.cdc.gov/flu/avianflu/

Reagent Storage, Handling, and Stability

- Store all primers and probes at 2-8°C until re-hydrated for use; Store all control materials (HSC, H5VC) 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 and/or conjunctival specimens, all applicable regulations for the transport of etiologic agents are met. 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 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 CDC 50.34 Specimen Submission Form completely or use CSTOR.
  - Send all samples to the following recipient:

Attention: Genomics and Diagnostics Team
c/o STATT Unit 198
Virology, Surveillance and Diagnosis Branch, Influenza Division
Centers for Disease Control and Prevention
1600 Clifton Rd., Atlanta, GA 30329-4027
Phone: +1 (470) 604-5078

The emergency contact number for CDC Emergency Operations Center (EOC) is +1 (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.

Human Specimen Control (HSC) Preparation:

1. Reagent
    a. Noninfectious cultured human cell material supplied as a liquid suspension in 0.01 M PBS.
    b. Volume: 0.5 mL per vial.
2. Storage
    a. Store at -20°C or below upon receipt. Do not dilute.
3. Procedure
    a. Human Specimen Control must be extracted and processed with each batch of samples to be tested. 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.
    b. Do not dilute extracted RNA prior to testing.

Influenza A/H5N1 (Asian Lineage) Positive Control (H5VC) Preparation:

1. Reagent
    a. Inactivated, noninfectious positive control material is supplied as a liquid, 900 µL per vial, suspended in 0.01 M PBS.
    b. Control contains a reassortant human vaccine candidate virus (A/Vietnam/1203/04 x PR/8/34) that was generated by reverse genetics in cultured human cell material.
2. Storage
    a. Store at -20°C or below upon receipt. Do not dilute.

3. Procedure
    a. H5VC 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 extracted RNA into aliquots and store at -20°C or below for up to 6 months. Recommended aliquot volumes (but not required):
        - 25 µL aliquots for use with the Influenza A/H5 Assay

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 combined 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 Compact
Kit: Roche MagNA Pure Nucleic Acid Isolation Kit I
Protocol: Total_NA_Plasma100_400
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 Compact
Kit: Roche MagNA Pure RNA Isolation Kit
Protocol: RNA_Tissue Protocol
Recommendation(s): Add 100 µL of sample to 250 µL of pre-aliquoted RNA isolation kit lysis buffer (total input sample volume is 350 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 Biosystems™ 7500 Fast Dx or Applied Biosystems™ QuantStudio™ 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 or Influenza A subtyping 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/H5 Subtyping Kit. However, in the event that specimens need to be re-extracted or if the Influenza A/H5 Subtyping Kit is used directly, then RP and the HSC control should be included in the run. The Plate Set-Ups described below include RP and HSC for demonstration purposes.

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, H5a, H5b, 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	Volume 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	Volume 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/H5 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	H5a	H5a	H5a	H5a	H5a	H5a	H5a	H5a	H5a	H5a	H5a	H5a
C	H5b	H5b	H5b	H5b	H5b	H5b	H5b	H5b	H5b	H5b	H5b	H5b
D	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
E	empty	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	InfA	empty
F	empty	H5a	H5a	H5a	H5a	H5a	H5a	H5a	H5a	H5a	H5a	empty
G	empty	H5b	H5b	H5b	H5b	H5b	H5b	H5b	H5b	H5b	H5b	empty
H	empty	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	empty

NOTE: If specimens were already tested with the A/B Typing Kit or A Subtyping Kit and produced valid RP results,then the specimen does not need to be repeat tested with RP.

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 2-10 (columns 1, 11, 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.
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 H5VC 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/H5 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	H5VC
B	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	H5VC
C	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	H5VC
D	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	H5VC
E	empty	S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	empty
F	empty	S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	empty
G	empty	S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	empty
H	empty	S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	empty

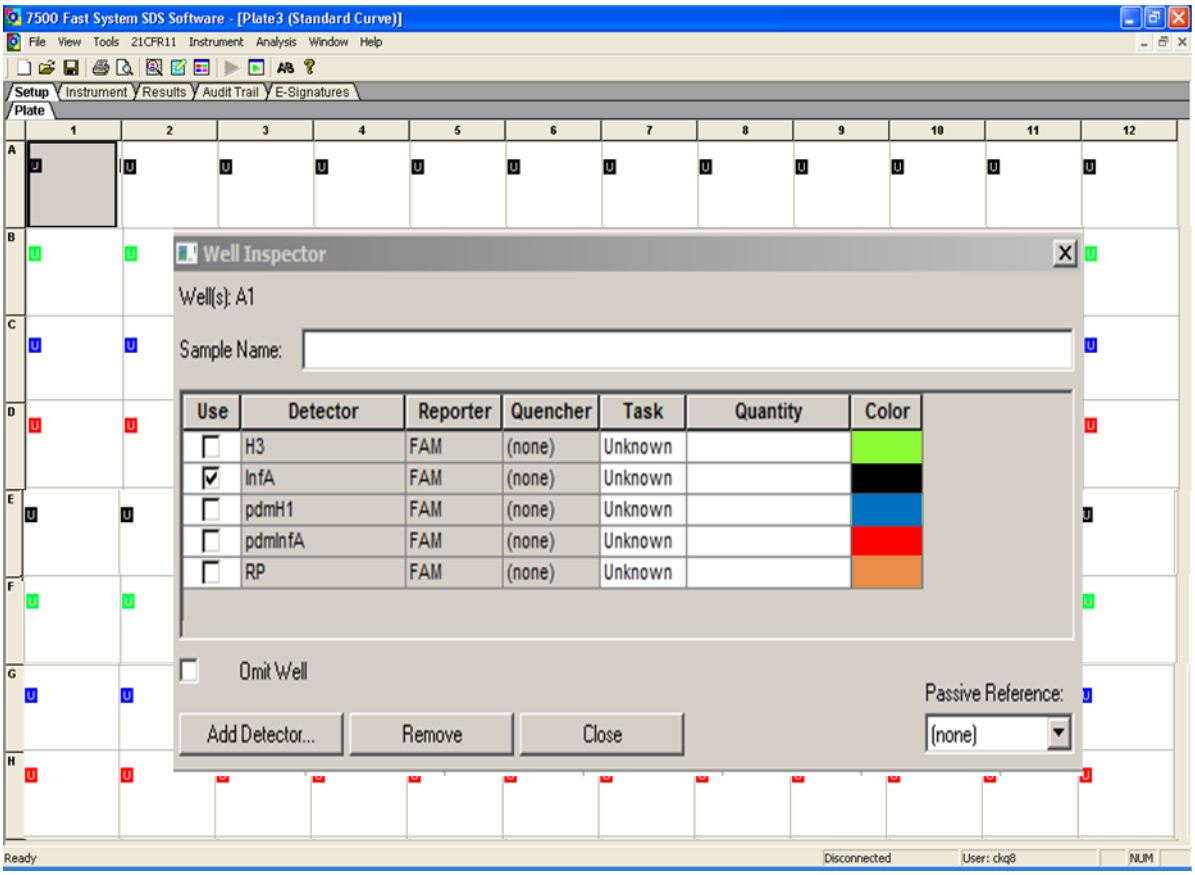
NOTE: HSC needs to be tested with each nucleic acid extraction run. If samples are from an extraction run already tested with HSC, repeat testing of HSC is not necessary.

Running a Test on the Applied Biosystems™ 7500 Fast Dx

If the most current Influenza A/H5 Subtyping Kit template does not already exist on this instrument, please proceed to *Appendix A: Instrument Setup for Applied Biosystems™ 7500 Fast Dx.*

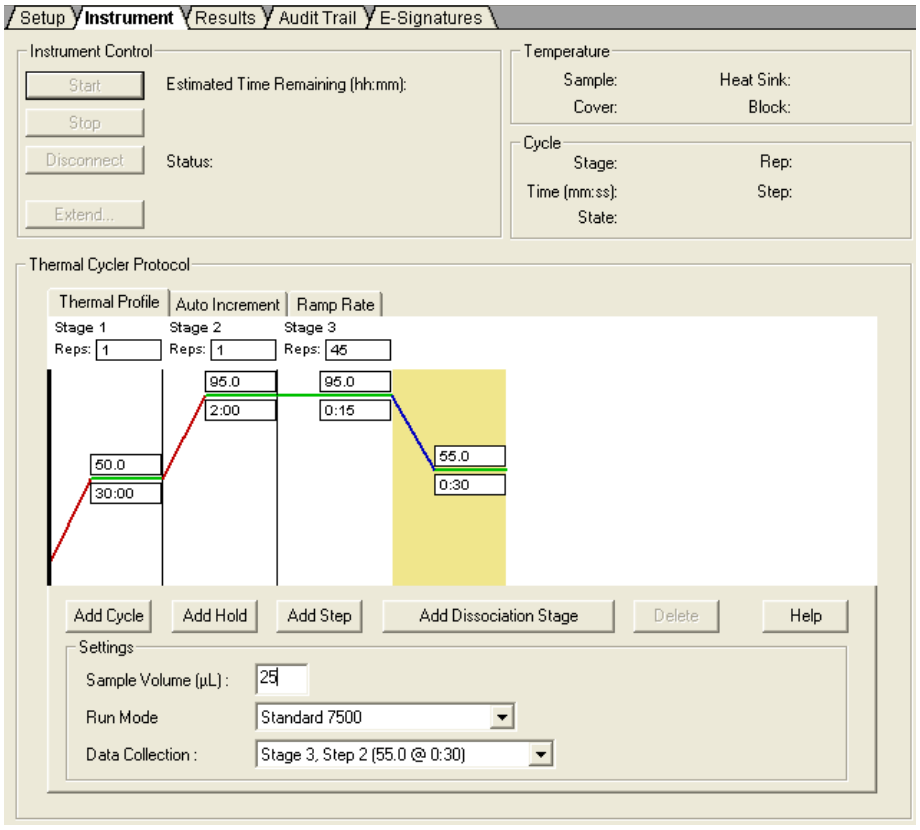
1. Turn on the 7500 Fast Dx Real-Time PCR Instrument and computer.
2. Launch the 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
3. A new window should appear, select **Open Existing Document** from the menu.
4. Navigate to select the ABI Run Template folder from the desktop.
5. Double click on the appropriate template file (**Influenza AH5 Kit SuperScript** or **Influenza AH5 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

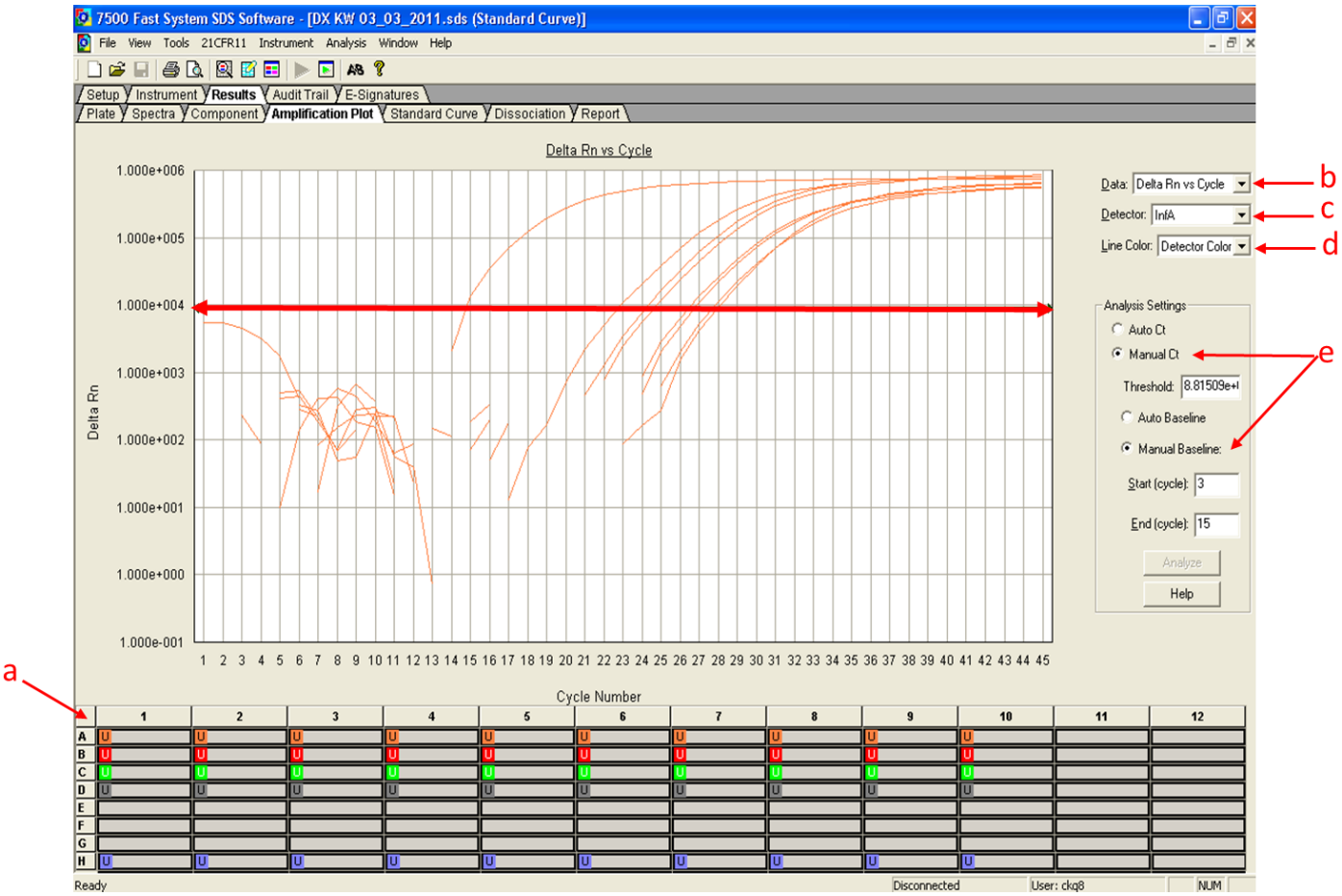


15. Ensure settings are correct (refer to *Appendix A: Instrument Setup for Applied Biosystems™ 7500 Fast Dx*).
16. Before proceeding, the run file must be saved; from the main menu, select **File,** then **Save As**. Save in appropriate run folder designation.
17. Load the plate into the plate holder 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 1hour 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.

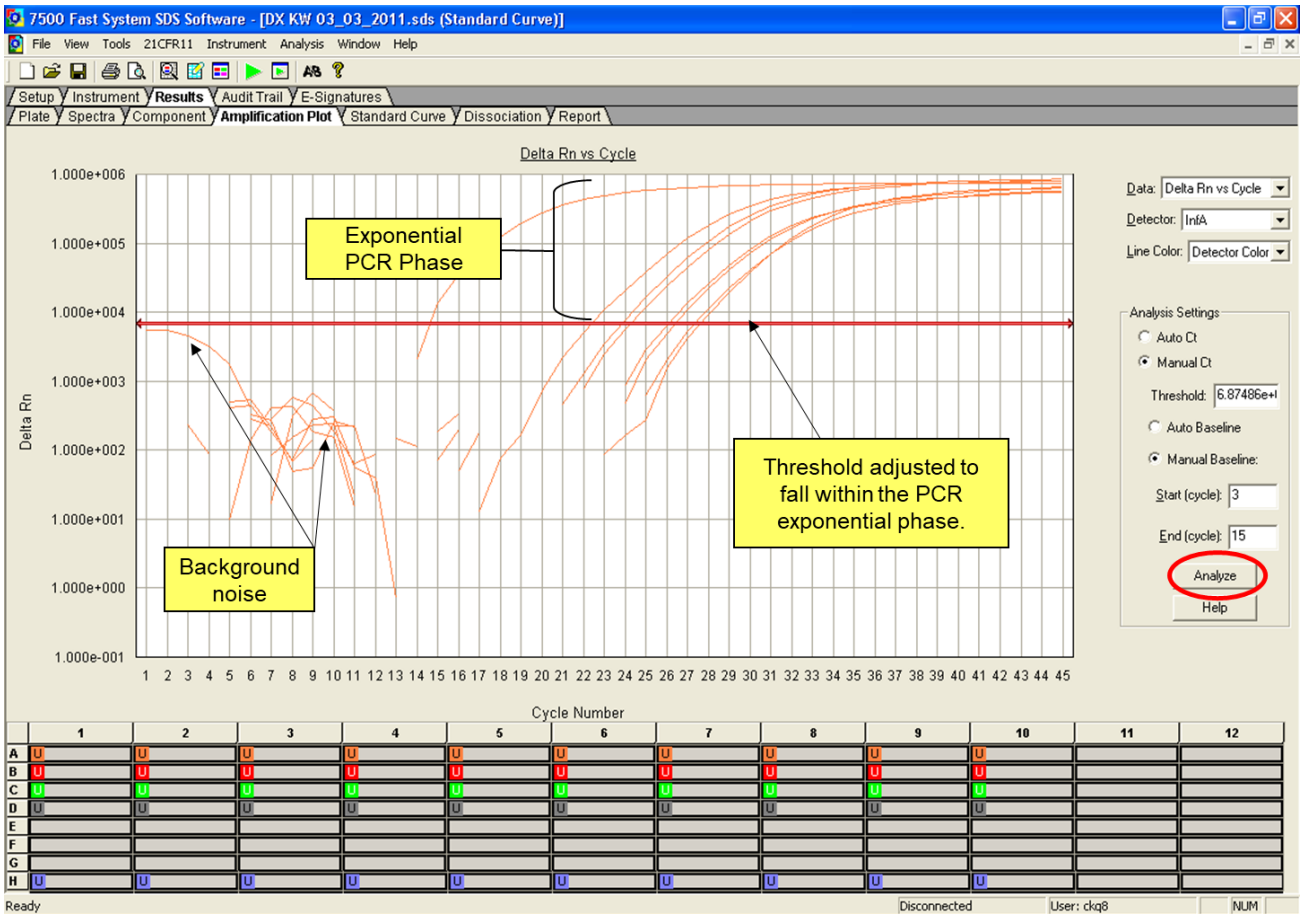
a. Please note that each detector is analyzed individually to reflect different performance profiles of each primer and probe set.

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** default numbers.

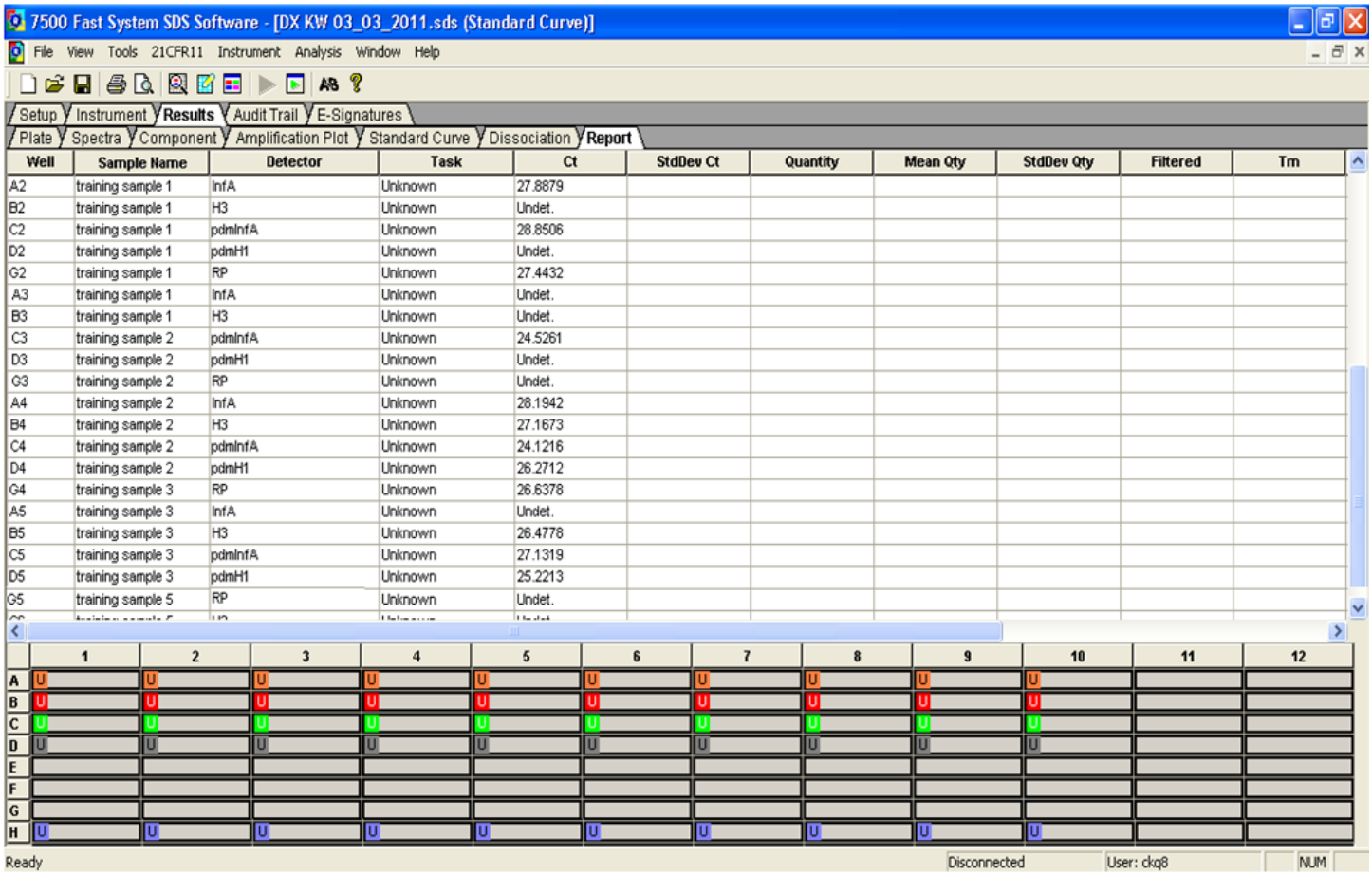
8. Click and drag the red threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal (**Figure 7)**.

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, H5a, H5b, 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



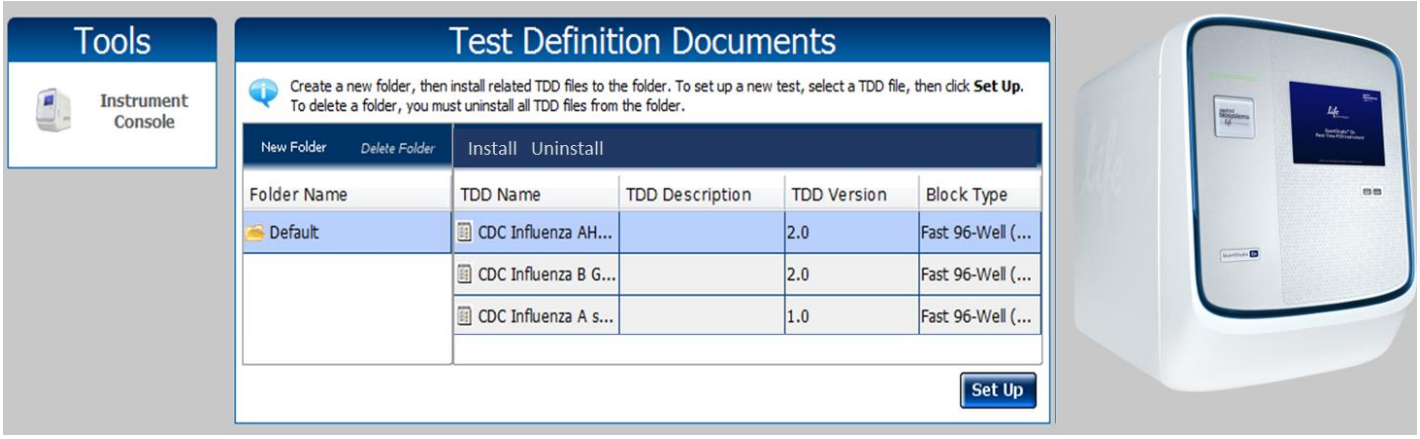
Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
A2	training sample 1	InfA	Unknown	27.8879						
B2	training sample 1	H3	Unknown	Undet.						
C2	training sample 1	pdmInfA	Unknown	28.8506						
D2	training sample 1	pdmH1	Unknown	Undet.						
G2	training sample 1	RP	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.						

Running a Test on the Applied BiosystemsTM QuantStudioTM Dx

If the test definition document file (.tdd) for the Influenza A/H5 Subtyping Kit does not already exist on this instrument, please proceed to *Appendix B: Instrument Setup for Applied Biosystems™ QuantStudio™ Dx*.

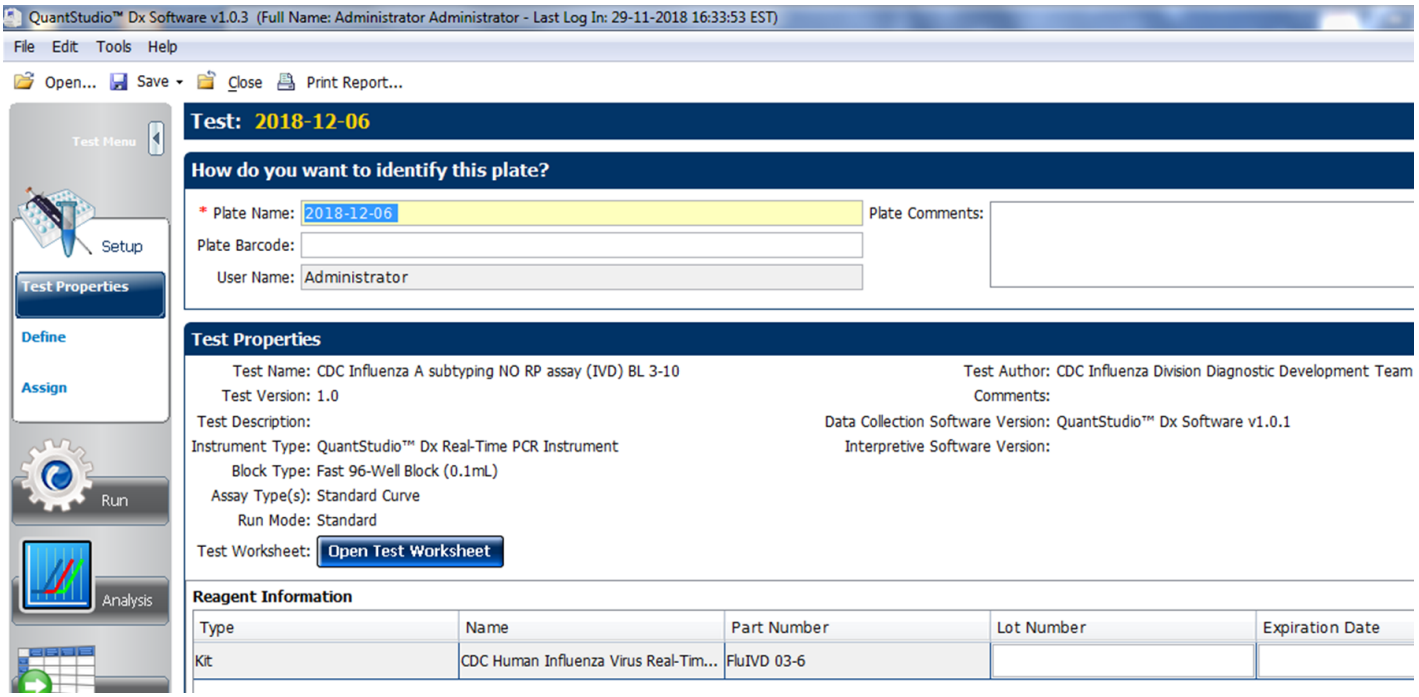
1. Turn on the QuantStudio™ Dx Real-Time PCR Instrument and computer.
2. On the instrument touch screen select **IVD Mode**, then select **Set Instrument Mode**.
3. Launch the QuantStudio™ Dx Real-time PCR System by double clicking on the QuantStudio™ Dx software icon on the computer desktop.
4. Enter 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/H5 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



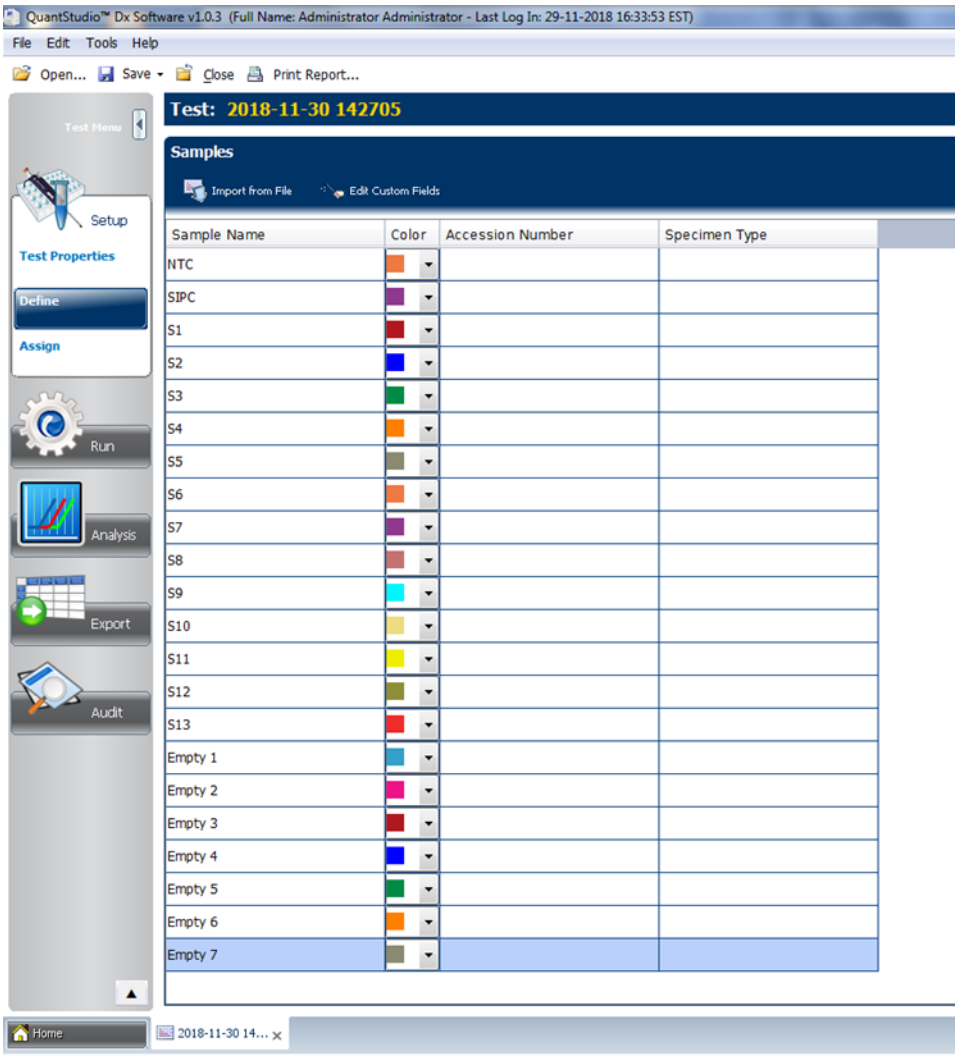
8. After the instrument initializes, the **Test Properties** window will appear (**Figure 10**).
9. Enter the reagent lot number and reagent expiration date.

Figure 10. Test Properties Set-up Window



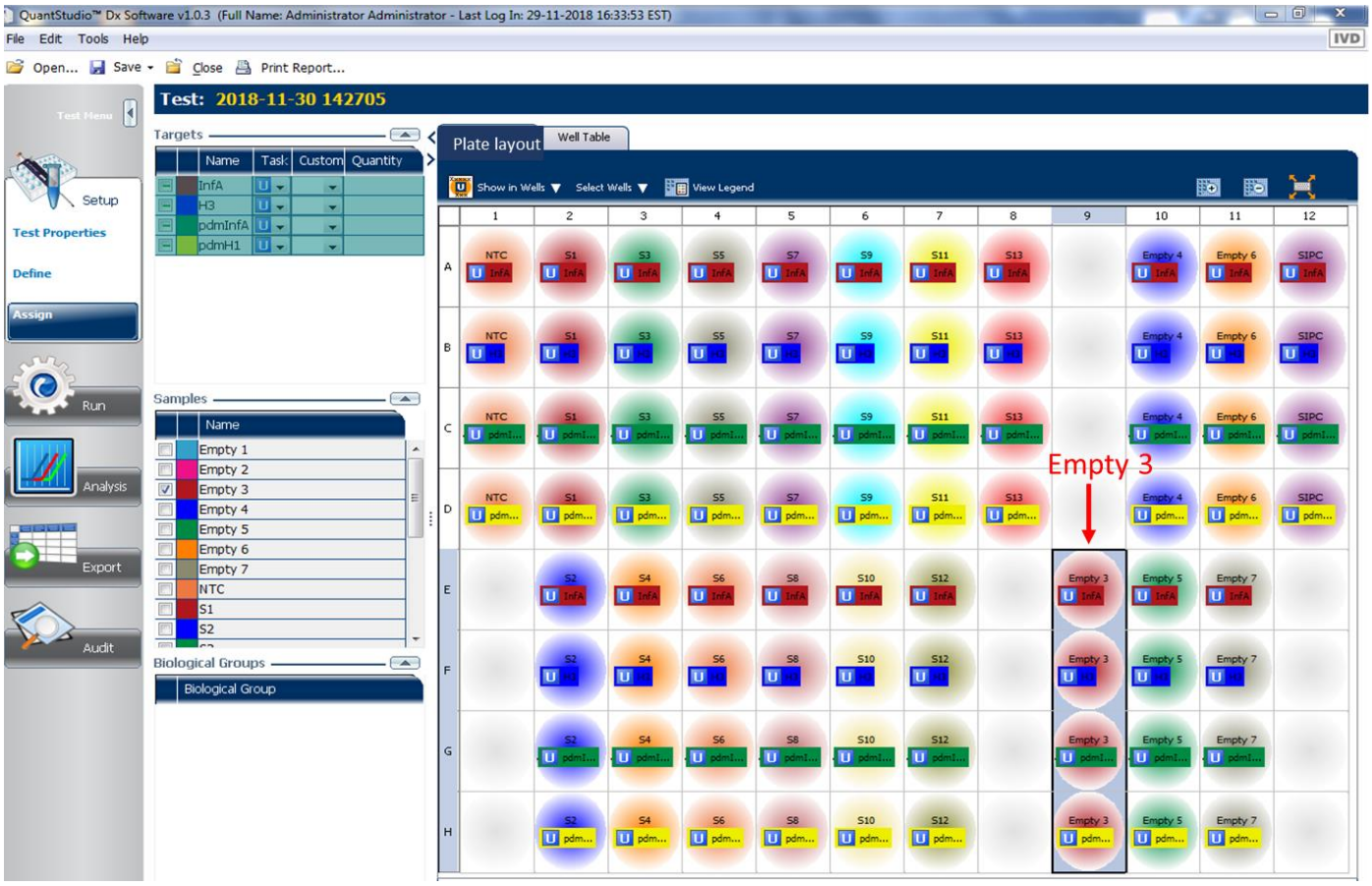
10. Select **Define** (**Figure 10)** in the left margin to assign sample identifiers in the **Samples** window (**Figure 11**).
11. Enter a test sample identifier into each **Sample Name** field to replace the default sample identifiers “Sample 1”, “Sample 2”, etc.
12. Replace “No Template Control” 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



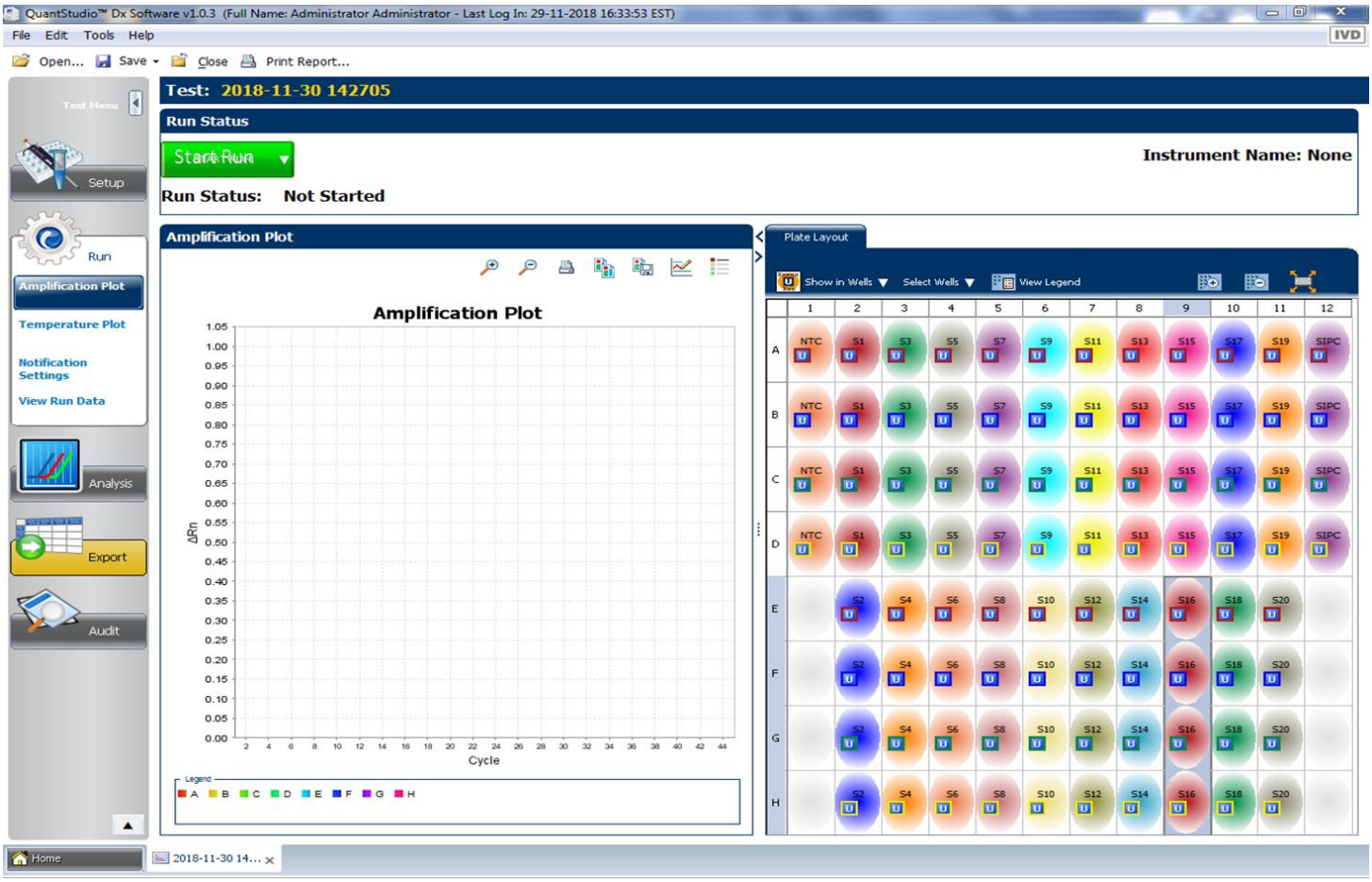
14. Once all control and sample identifiers have been added, select **Assign** (**Figure 12**) in the left margin to review the plate assignment.
15. If desired, the user may remove the empty wells from the plate layout by selecting the column corresponding to the empty sample position on the plate layout 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 1hour and 45 minutes to complete.*

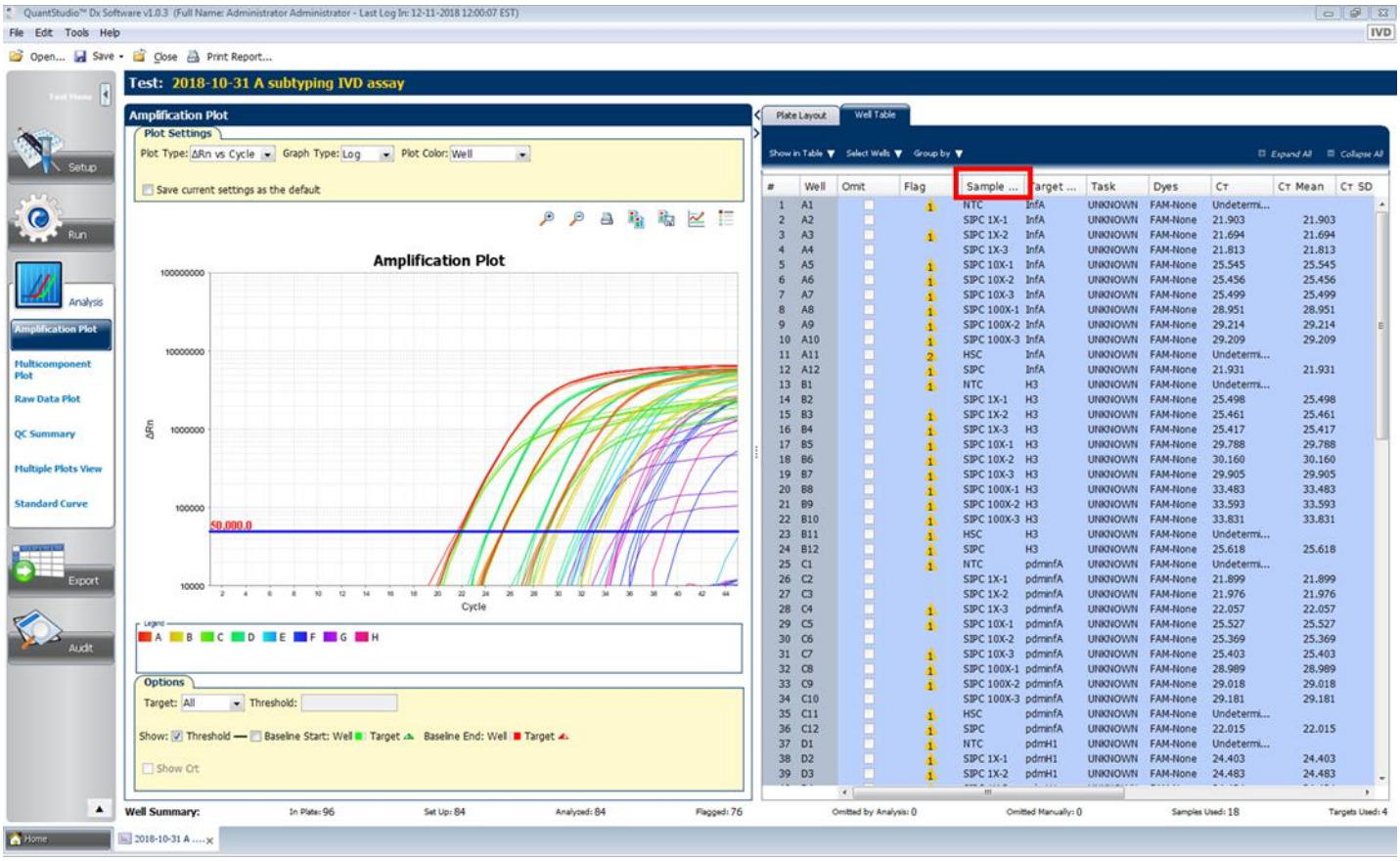
Figure 13. Starting the Run



Results Analysis

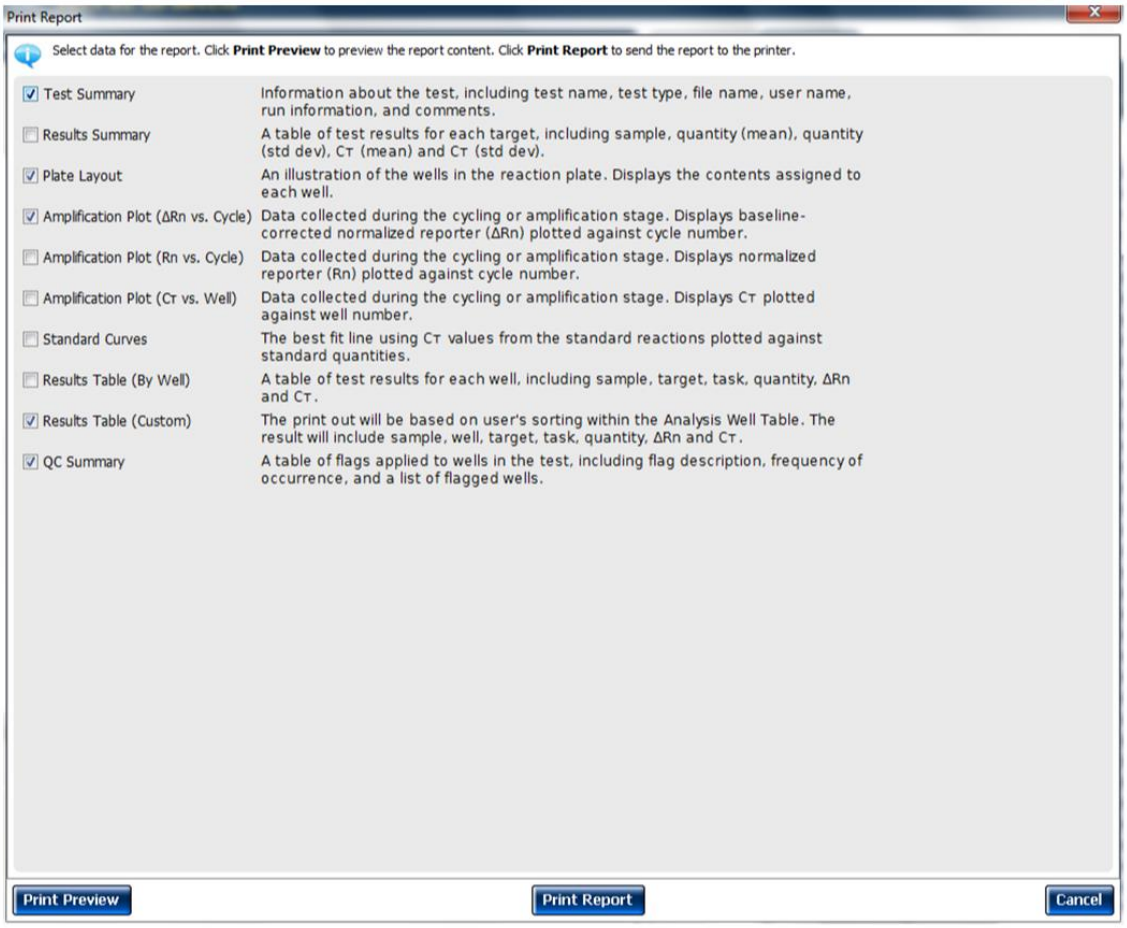
1. After the run has completed, the **Amplification Plot** tab in the **Analysis** window will display (**Figure 14**).
2. In the **Analysis** view, select the **Well Table** tab at the upper left side of the plate layout review the analyzed data.
3. Sort the wells by sample, clicking the **Sample**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

Figure 14. Amplification Plot



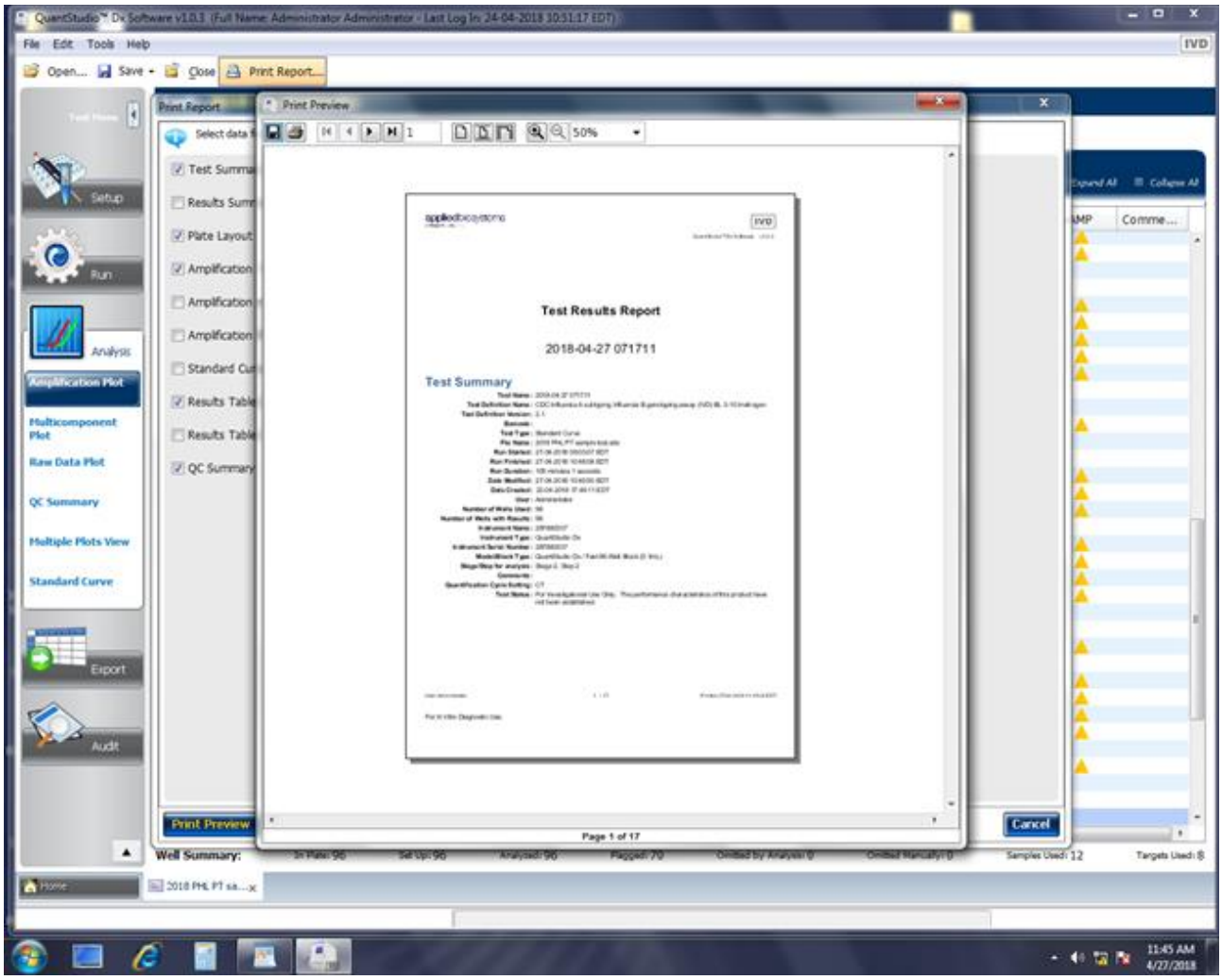
4. Select the **Print Report** icon in the tool bar at the top of the screen (**Figure 14**) to open the **Print Report** window (**Figure 15**).
5. Select **Test Summary, Plate Layout, Amplification Plot (ΔRn vs. Cycle), Results Table (Custom), and QC Summary**.

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 16. 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/H5 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/H5 Subtyping Kit. However, in the event that specimens need to be re-extracted or if the Influenza A/H5 Subtyping Kit is used directly, then RP and the HSC control should be included in the run. The Plate Set-Ups described below include RP and HSC for demonstration purposes.

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, H5a, H5b, 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	Volume 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	Volume 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**

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/H5 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	H5a	**10**	H5a	**18**	H5a	**26**	H5a	**34**	H5a	**42**	H5a	**50**	H5a	**58**	H5a	**66**	H5a
3	H5b	**11**	H5b	**19**	H5b	**27**	H5b	**35**	H5b	**43**	H5b	**51**	H5b	**59**	H5b	**67**	H5b
4	RP	**12**	RP	**20**	RP	**28**	RP	**36**	RP	**44**	RP	**52**	RP	**60**	RP	**68**	RP
5	InfA	**13**	InfA	**21**	InfA	**29**	InfA	**37**	InfA	**45**	InfA	**53**	InfA	**61**	InfA	**69**	InfA
6	H5a	**14**	H5a	**22**	H5a	**30**	H5a	**38**	H5a	**46**	H5a	**54**	H5a	**62**	H5a	**70**	H5a
7	H5b	**15**	H5b	**23**	H5b	**31**	H5b	**39**	H5b	**47**	H5b	**55**	H5b	**63**	H5b	**71**	H5b
8	RP	**16**	RP	**24**	RP	**32**	RP	**40**	RP	**48**	RP	**56**	RP	**64**	RP	**72**	RP

NOTE: If specimens were not already tested with the A/B Typing Kit or A Subtyping Kit and produced valid RP results, the specimen will need to be repeat tested with RP.

5. 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.
6. 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!
7. 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/H5 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**	HSC
2	NTC	**10**	S2	**18**	S4	**26**	S6	**34**	S8	**42**	S10	**50**	S12	**58**	S14	**66**	HSC
3	NTC	**11**	S2	**19**	S4	**27**	S6	**35**	S8	**43**	S10	**51**	S12	**59**	S14	**67**	HSC
4	NTC	**12**	S2	**20**	S4	**28**	S6	**36**	S8	**44**	S10	**52**	S12	**60**	S14	**68**	HSC
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, then testing of HSC is necessary.

Running a Test on the QIAGEN Rotor-Gene Q MDx

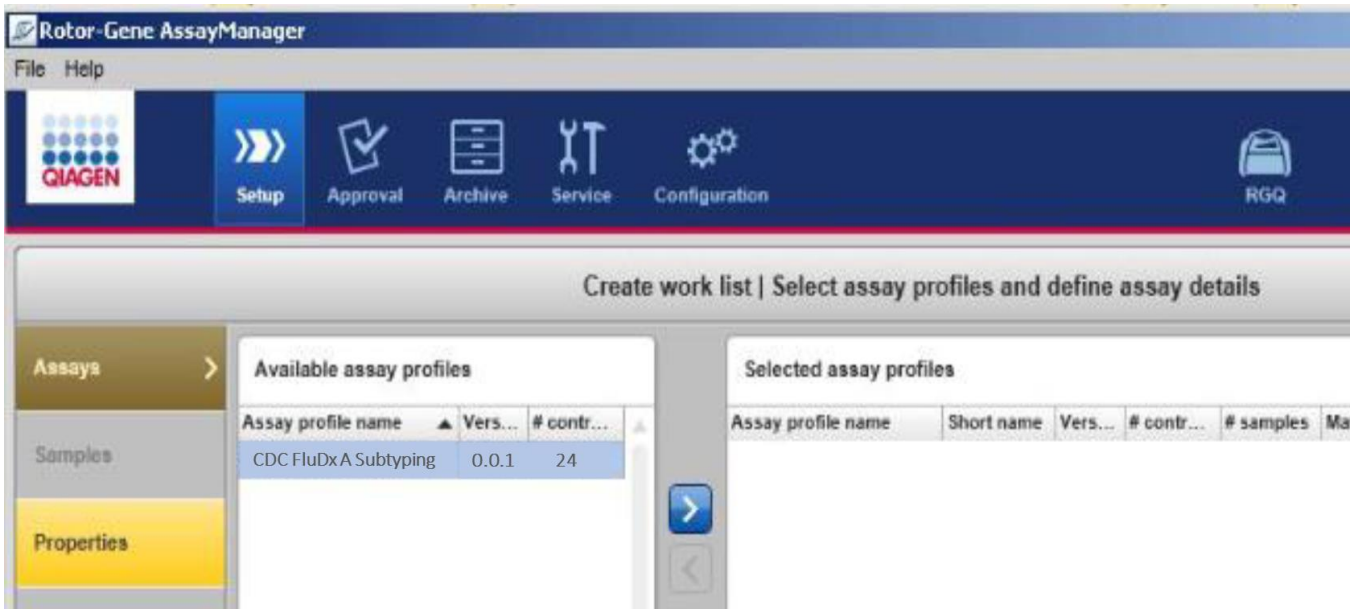
If the most current assay profile for the Influenza A/H5 Subtyping Kit does not already exist on this instrument, please proceed to *Appendix C: Instrument Setup for QIAGEN Rotor-Gene Q MDx.*

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

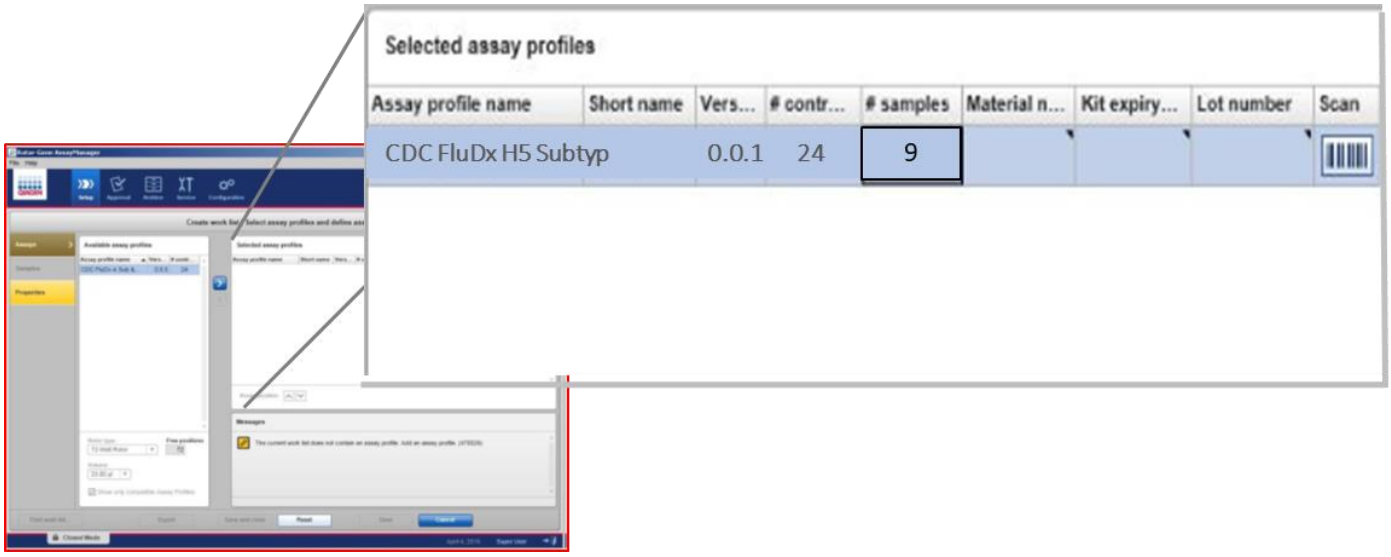
4. The **Available Work List** window will appear under the **Setup** tab. Select **New Work List** button at the bottom right corner of the screen.
5. Select the appropriate assay profile in the **Create a work list** window under the **Setup** tab (**Figure 21**):
    a. Select the **Assays** tab from 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. Create a Work List Window



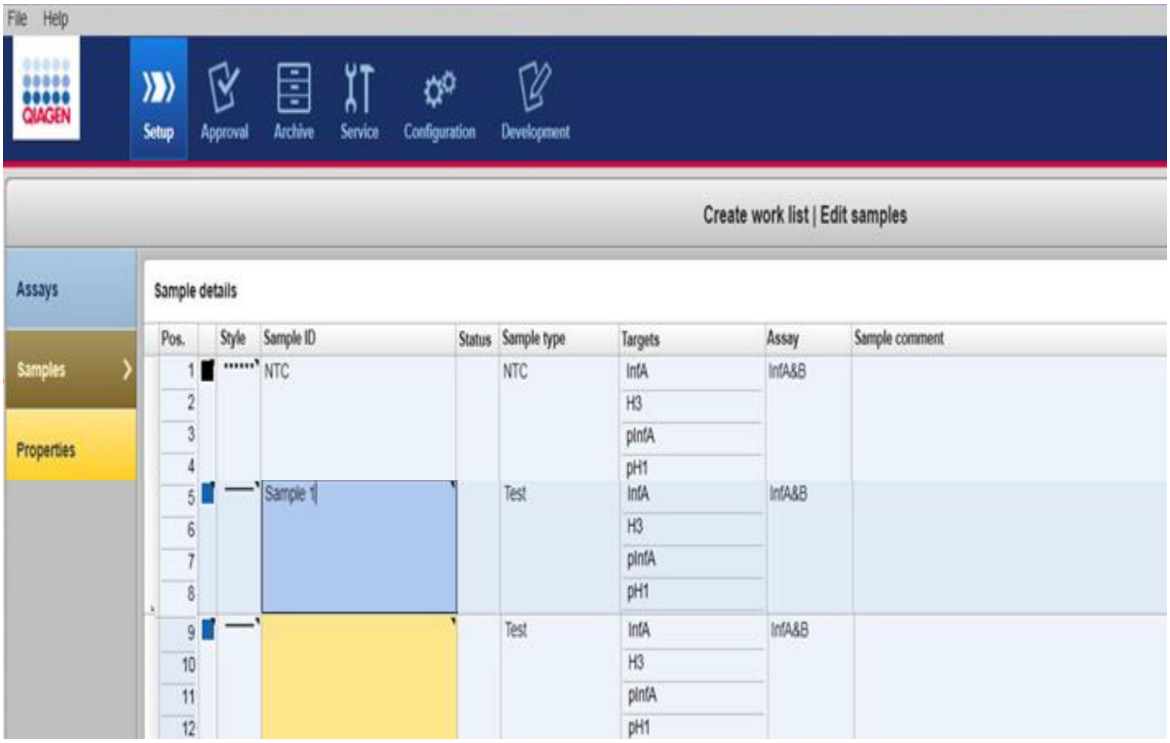
6. In the **Selected assay profiles** window enter the number of samples to be tested (*do not include controls: NTC and Positive Controls*). Rows will change from yellow to blue after the number is entered (**Figure 22**).

Figure 22. Selected Assay Profiles Window



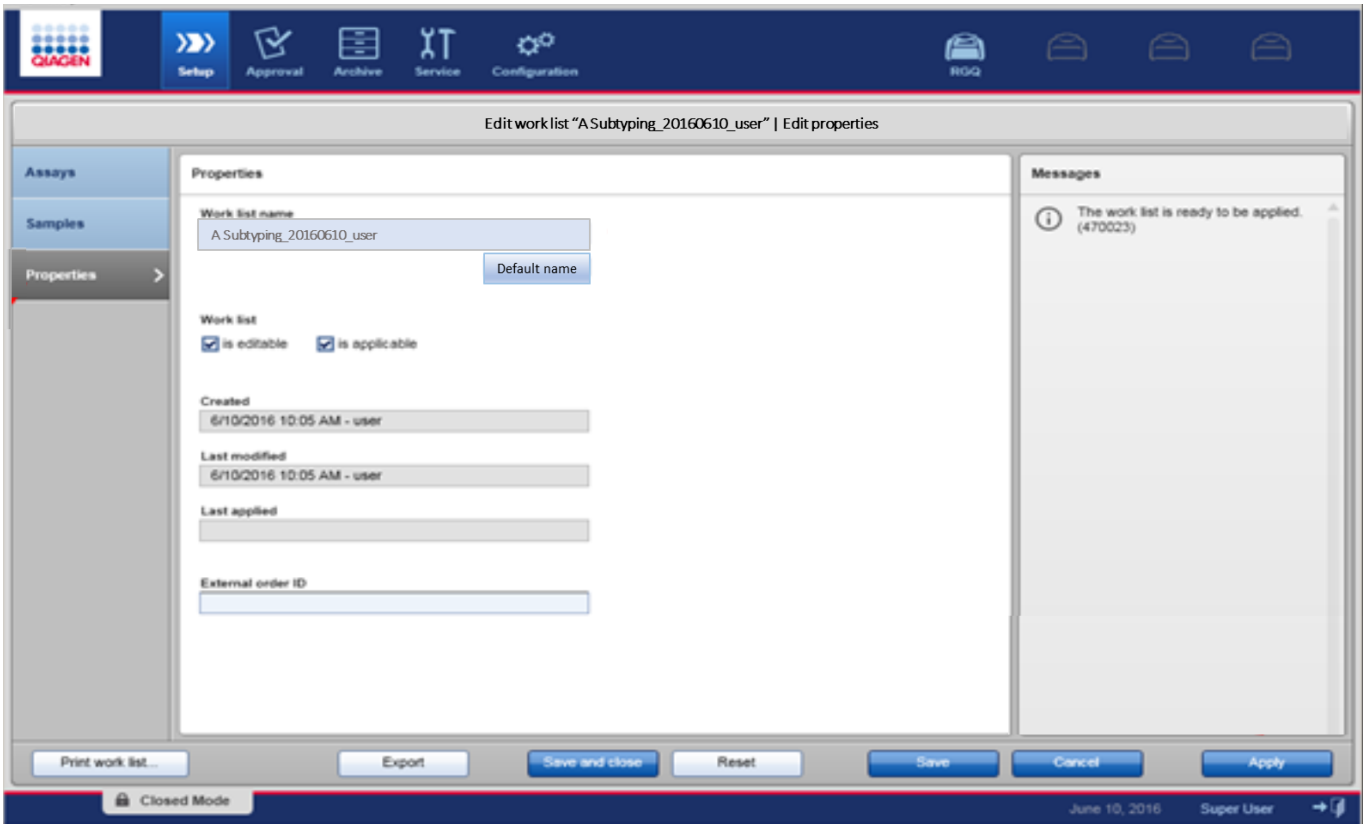
7. Select the **Samples** tab in the left margin. Enter samples names into the **Sample ID** column; controls are automatically added. *On the sample details table, the column heading "Pos." corresponds to the position on the 72-Well Rotor* (**Figure 23**).

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



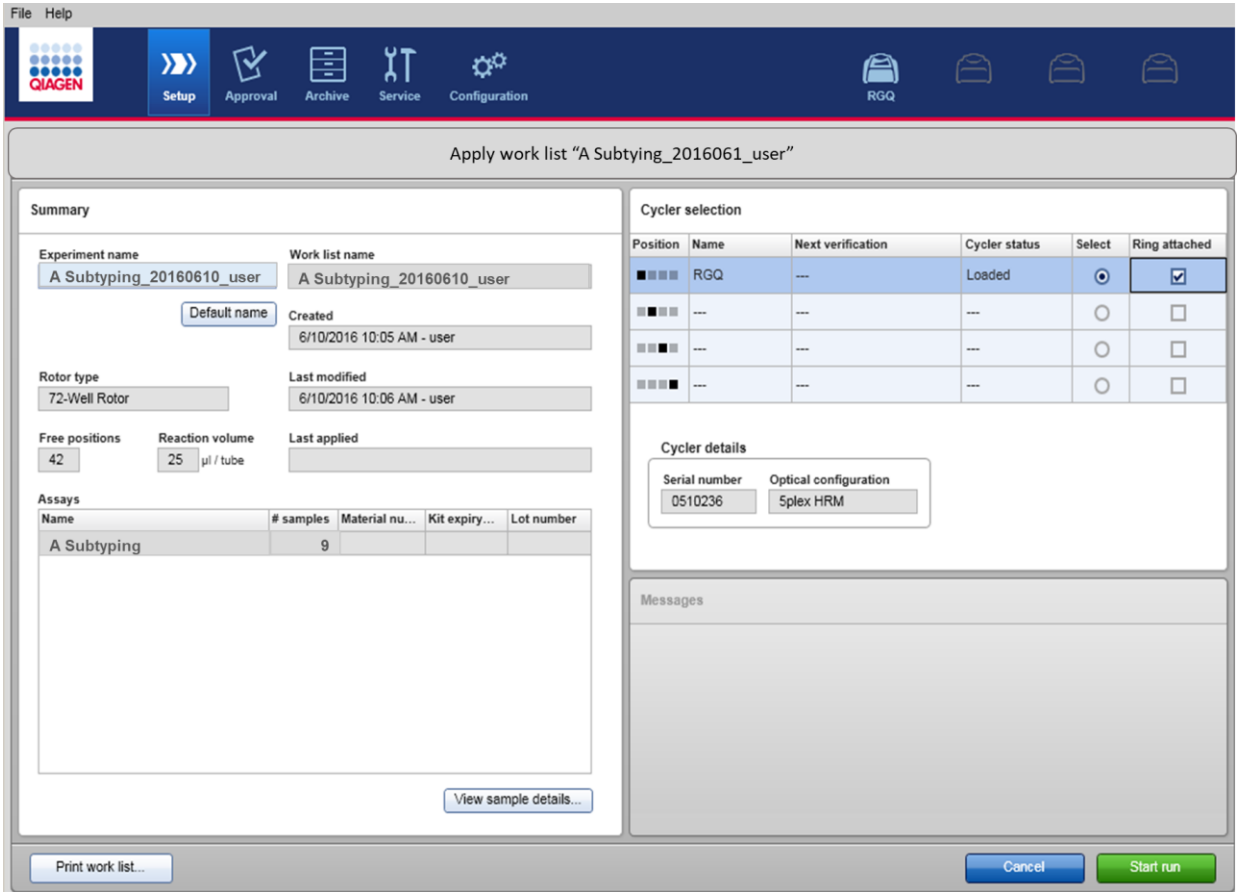
8. Select the **Properties** tab in the left margin (**Figure 24**)**:**
    a. Enter a name in the **Work list name** field or use the **Default name** button.
    b. Click the box next to "**is applicable**" under **Work list**.
    c. Click **Apply** in the bottom right corner.

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 1hour and 45 minutes to complete.*

Figure 25. Apply Work List Window



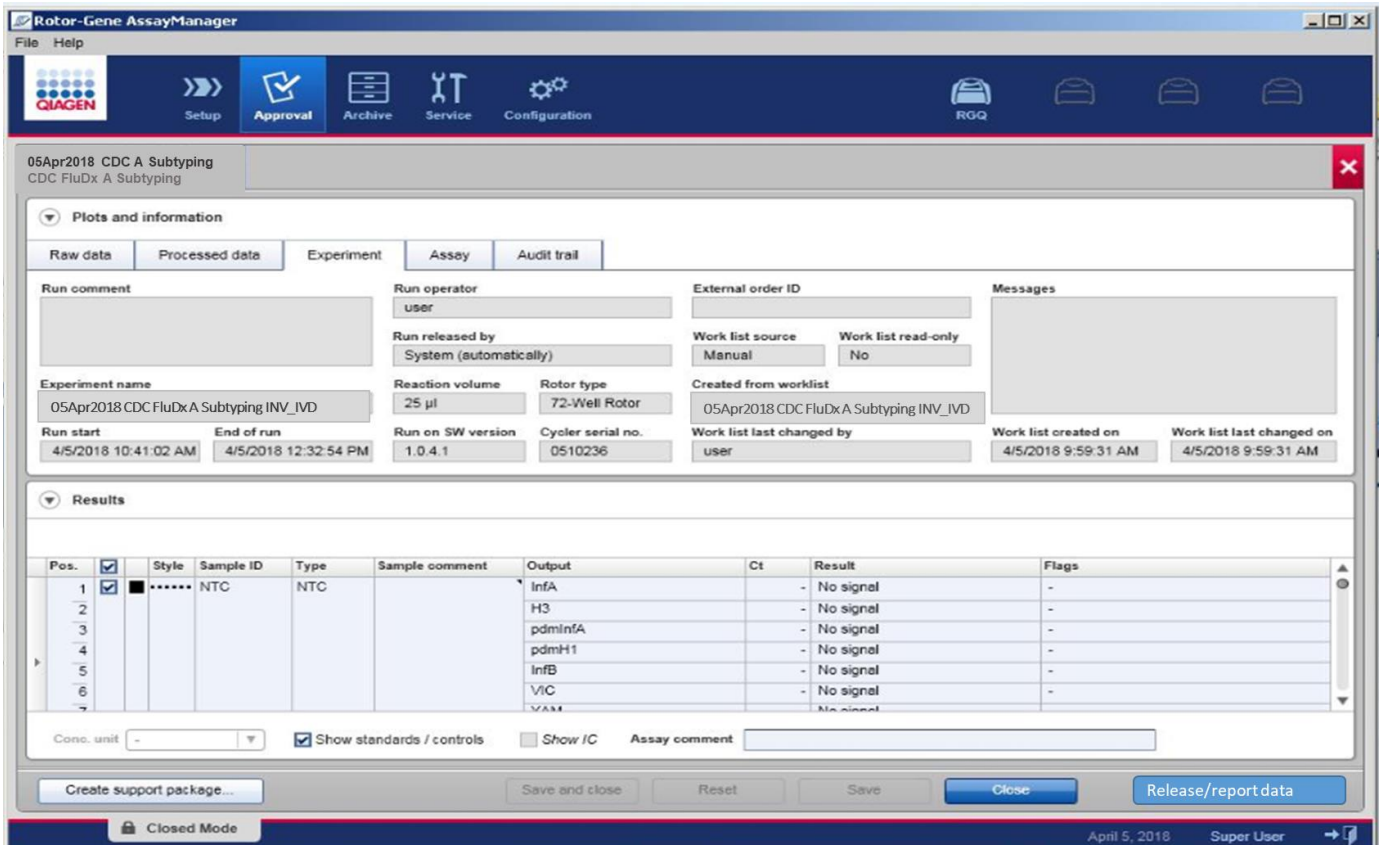
Results Analysis

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

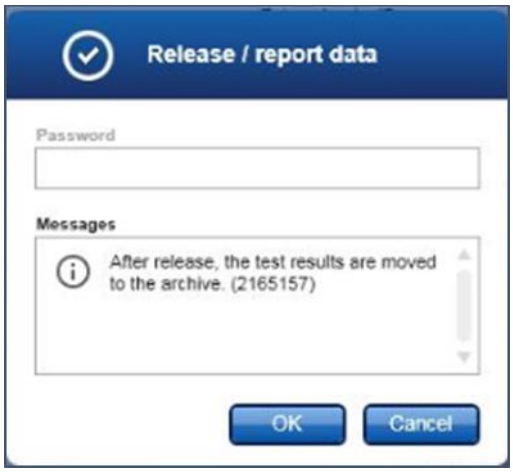
3. Click **Release and go to approval** at the bottom of the **Finish run** window.
4. The data analysis window will appear automatically on the **Experiment** tab in the **Approval** menu (**Figure 27**).
5. Review all results and look for any flags.
    a. Result report of “Invalid” and a Flag report 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



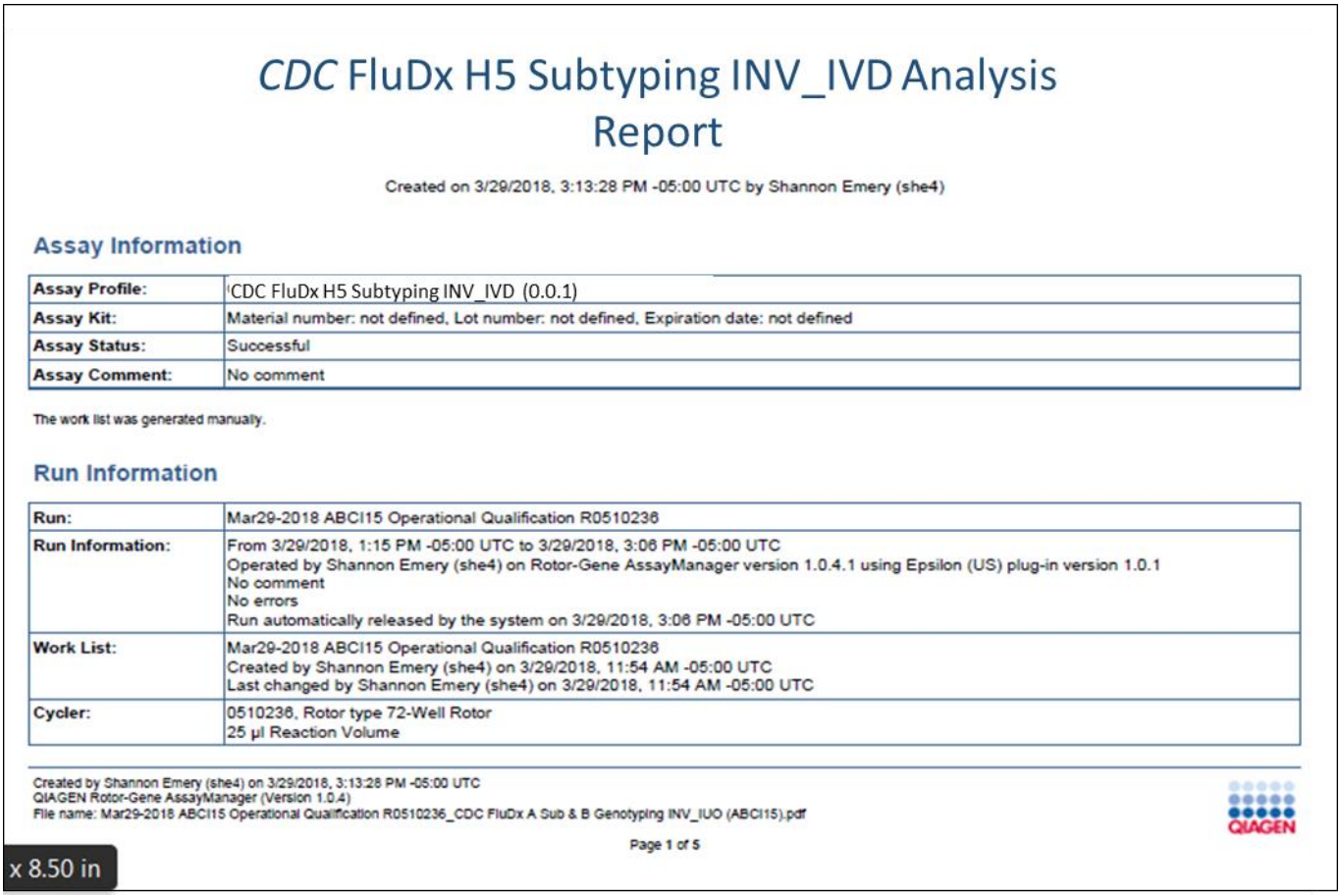
6. After reviewing the data, click the **Release/report data** button at the bottom right of the window.
7. The **Release/report data** window will appear (**Figure 28**).

Figure 28. Release/Report Data Window



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

Figure 29. Example Analysis Report



CDC FluDx H5 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 H5 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

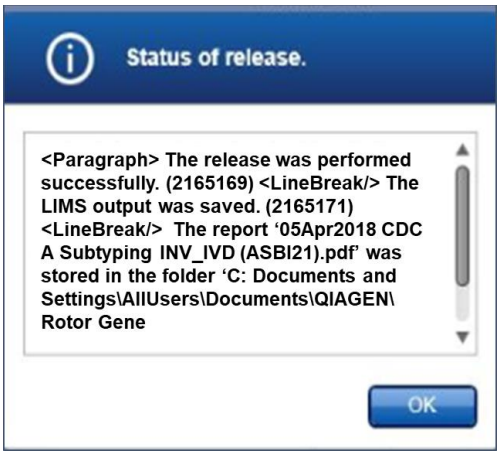
Created by Shannon Emery (she4) on 3/29/2018, 3:13:28 PM -05:00 UTC
QIAGEN Rotor-Gene AssayManager (Version 1.0.4)
File name: Mar29-2018 ABCI15 Operational Qualification R0510236_CDC FluDx A Sub & B Genotyping INV_IUO (ABCI15).pdf

Page 1 of 5

x 8.50 in

10. Print the pdf report.
11. 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.
-

H5 Virus Control (H5VC)

- The H5VC control consists of a reassortant human vaccine candidate virus (A/Vietnam/1203/04 x PR/8/34) that was generated by reverse genetics and cultured human cell (A549) material. Purified RNA from the H5VC will yield a positive result with the following primer and probe sets: InfA, H5a, H5b, and RP.
-

Human Specimen Control (HSC) (Extraction Control)

- When HSC is run with the Influenza A/H5 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/H5 Subtyping Kit

Control Type	Internal Control Name	Used to Monitor	InfA	RP	H5a	H5b	Expected Ct Values
Positive	H5VC	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, then 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 H5a and H5b 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, H5a, H5b)

- When all controls exhibit the expected performance, a specimen is considered negative if all influenza marker (InfA, H5a, H5b) 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 expected performance and growth curves for InfA, H5a **AND** H5b markers cross the threshold line within 38.00 cycles (< 38.00 Ct):
  - Report the specimen to be "Presumptive Positive for Influenza A/H5 Virus" and contact the CDC Influenza Division **IMMEDIATELY** to coordinate transfer of the specimen to CDC for additional testing. Note: the RNase P marker result may or may not be positive as described above, but the influenza result is still valid.
- When all controls exhibit the expected performance and growth curves for InfA and **EITHER** H5a **OR** H5b markers cross the threshold line within 38.00 cycles (< 38.00 Ct), extracted RNA from the specimen should be re-tested immediately. If residual RNA is not available, re-extract RNA from residual specimen and re-

test. If all controls exhibit the expected performance on the repeated test and either H5a **OR** H5b reactions cross the threshold line within 38.00 cycles (< 38.00 Ct):

- Report the specimen to be "Inconclusive for Influenza A/H5 virus" and contact the CDC Influenza Division **IMMEDIATELY** to coordinate transfer of the specimen to CDC for additional testing.

- When all controls exhibit the expected performance and the growth curves for the influenza markers (InfA, H5a, H5b) **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 the H5 markers indicating detection (InfA positive without H5a and H5b), the sample should be tested for other influenza subtypes using the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel. If no subtype is detected with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel, 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.

Influenza A/H5 Subtyping Results Interpretation Guide

The table below lists the expected results for the Influenza A/H5 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. 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.

Influenza A/H5 Subtyping Kit

InfA	H5a	H5b	RP	Result Interpretation[a]	Report for CDC Surveillance	Notes and Special Guidance
+	+	+	±	Influenza A Detected Presumptive positive for Influenza A(H5) virus	Presumptive Positive A(H5)	Contact CDC immediately for instructions for coordination of transferring the specimen to CDC for additional testing and further guidance.
+	+	-	±	Influenza A Detected Inconclusive for Influenza A(H5) virus	Inconclusive A(H5)	Re-Test; Contact CDC immediately for instructions for coordination of transferring the specimen to CDC for additional testing and further guidance.
+	-	+	±	Influenza A Detected Inconclusive for Influenza A(H5) virus	Inconclusive A(H5)	
-	If either marker is positive while Inf A is Negative		±	Inconclusive Result	Inconclusive	Re-test; Notify CDC

InfA	H5a	H5b	RP	Result Interpretation[a]	Report for CDC Surveillance	Notes and Special Guidance
+	-	-	±	Influenza A Detected; Influenza A(H5) virus not detected	Influenza A	If the specimen has not been tested for A(H3) and A(H1)pdm09 subtypes, proceed with this testing by using the FDA cleared CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel-A/Subtyping kit (Cat# FluIVD03-12). If no subtype is detected, the result may indicate a 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.
-	-	-	±	Influenza A NOT Detected	Influenza A Not Detected	Not Applicable
-	-	-	-	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.

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 +1 (404) 639-3747.

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 42CFR 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.
- 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 (H5VC) 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 CFR 866.3332(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.
- 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.
- If a patient specimen tests negative and there is strong epidemiological or clinical indication of influenza A/H5 infection, collection and testing of additional specimens may be appropriate.
- Performance of this test with conjunctival swabs was evaluated in specimens collected from persons with known influenza A/H5 exposure exhibiting flu-like and/or conjunctival symptoms.
- 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 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/H1 viruses and 2,368 (37.7%) were influenza A/H3 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 clinical performance of oligonucleotide primer and probe sets of the Influenza A/H5 Subtyping Kit were evaluated using contrived samples of grown virus added to an A549 cell suspension to simulate positive clinical

samples. A total of fifty positive contrived samples in high, moderate, and low concentrations were evaluated. In addition, sixty-five specimens that tested negative for influenza A with the CDC Human Influenza rRT-PCR Diagnostic Panel that were obtained from a clinical study conducted during the 2011-2012 influenza season were evaluated. Testing was performed with both enzymes approved for use with the kit. The results are summarized below.

Enzyme Utilized	# of Positives[1]	% Positive Agreement (95% CI)	# of Negatives[2]	% Negative Agreement (95% CI)
Quanta BioSciences qScript™	50/50	100 (92.9 – 100.00)	65/65	100 (94.4 – 100.00)
Invitrogen SuperScript™	44/50	88.0 (76.2 - 94.4)	65/65	100 (94.4 – 100.00)

[1]Proportion of contrived samples correctly identified as positive by both influenza A H5a and H5b primer and probe sets
[2]Proportion of negative samples correctly identified versus the comparator

Analytical Performance

Analytical Sensitivity – Limit of Detection (LOD)

Analytical sensitivity of the Influenza A/H5 Subtyping Kit was demonstrated by determining the LOD of each primer and probe set. The LOD for each primer and probe set was calculated to indicate the lowest detectable concentration of influenza virus (EID_{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 (EID_{50}/ML)	
		Invitrogen SuperScript™	Quanta qScript™
A/H5N1	A/Vietnam/1203/2004×A/Puerto Rico/8/34 reassortant (A/Vietnam/1203/2004 PR8- VNH5N1- PR8/CDC-RG)	$10^{3.8}$	$10^{2.4}$
	A/duck/Vietnam/NCVD-1544/2012	$10^{3.1}$	$10^{3.1}$
A/H5N8	A/gyrfalcon/Washington/41088-6/2014	$10^{3.35}$	$10^{3.35}$

Analytical Reactivity (Inclusivity)

The analytical inclusivity of the InfA, H5a and H5b primer and probe sets was demonstrated by testing 16 Influenza A(H5) viruses representative of different geographic locations and phylogenetic clades at concentrations at or near the established LOD. The Influenza A/H5 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, H5a, and H5b primer and probe sets with nucleic acids extracted from 35 organisms (16 viruses, 18 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 of Coronaviruses CoV 299E and CoV OC43, and Epstein-Barr virus and concentrations were determined by spectrophotometry.

Organism Tested			Reactivity	
Bacteria and Yeast	**Strain**	**cfu / mL**	**Inf A**	**A/H5[1]**
Bordetella pertussis	A639	$10^{8.3}$	-	-
Candida albicans	2001-21-196	$10^{8.8}$	-	-
Chlamydia pneumoniae[2]	TW183	40 IFU/mL	-	-
Corynebacterium diphtheriae	NA[3]	10^{10}	-	-
Escherichia coli	K12	$10^{9.6}$	-	-
Haemophilus influenzae	M15709	$10^{6.4}$	-	-
Lactobacillus plantarum	NA	$10^{8.8}$	-	-
Legionella pneumophila	NA	$10^{7.1}$	-	-
Moraxella catarrhalis	M15757	$10^{9.5}$	-	-
Mycobacterium tuberculosis[4]	H37Rv	100 ng/ µL	-	-
Mycoplasma pneumoniae	M129	$10^{7.7}$	-	-
Neisseria elongata	NA	$10^{8.6}$	-	-
Neisseria meningitidis	M2578	$10^{7.9}$	-	-
Pseudomonas aeruginosa	NA	$10^{10.5}$	-	-
Staphylococcus epidermidis	NA	$10^{10.5}$	-	-
Staphylococcus aureus	NA	$10^{10.7}$	-	-
Streptococcus pneumoniae	249-06 (Thailand)	$10^{6.6}$	-	-
Streptococcus pyogenes	7790-06	$10^{7.5}$	-	-
Streptococcus salivarius	SS1672	$10^{8.4}$	-	-
Viruses	**Strain**	**$TCID_{50}$/mL**	**Inf A**	**A/H5[1]**
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	3.0 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	$5 \times 10^{7.75}$	-	-
Varicella-zoster Virus	AV92-3:H	$5 \times 10^{3.75}$	-	-
Epstein Barr Virus[4]	B95-8	1.7 ng/µL	-	-
Measles Virus	Edmonston	$5 \times 10^{4.5}$	-	-
Mumps Virus	Enders	$5 \times 10^{6.5}$	-	-
Cytomegalovirus	AD-169	$5 \times 10^{6.25}$	-	-

[1] The result of both A/H5a and A/H5b primer probe sets were concordant for all organisms tested
[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/H5 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

To demonstrate the ability of the influenza A primer and probe set to detect influenza A viruses and exclusivity of the H5a and H5b primer and probe sets in the Influenza A/H5 Subtyping Kit, 20 non-human and 10 human influenza viruses were tested. The 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.

Human Influenza Viruses Tested at High Virus Titer

Virus	Subtype	Titer (log EID_{50}/mL)	InfA	A/H5[1]
A/Brisbane/59/07	A(H1N1)	8.4	+ (3/3)	-
A/Fujian Gulou/1896/2009	A(H1N1)	9.1	+ (3/3)	-
A/Perth/16/2009	A(H3N2)	8.9	+ (3/3)	-
A/Texas/50/2012	A(H3N2)	9.2	+ (3/3)	-
A/California/07/09	A(H1N1)pdm09	8.4	+ (3/3)	-
A/Washington/24/2012	A(H1N1)pdm09	8.5	+ (3/3)	-
B/Brisbane/60/2008	B(Victoria lineage)	9.3	+ (3/3)	-
B/Montana/5/2012	B(Victoria lineage)	8.4	+ (3/3)	-
B/Wisconsin/01/2010	B(Yamagata lineage)	9.2	+ (3/3)	-
B/Massachusetts/02/2012	B(Yamagata lineage)	9.2	+ (3/3)	-

[1] The result of both A/H5a and A/H5b primer probe sets were concordant for all organisms tested

Animal Influenza Viruses Tested at High Virus Titer

Species	Virus	Type	Titer (log EID_{50}/mL)	InfA	A/H5[1]
SWINE	A/swine/Wisconsin/125/1997	SW-H1N1	7.9	+ (3/3)	-
SWINE	A/Maryland/12/1991	SW-H1N1	9.0	+ (3/3)	-
SWINE	A/Iowa/1/2006	SW-H1N1	9.0	+ (3/3)	-
AVIAN	A/chicken/Pennsylvania/298101-4/2004	H2N2	9.5	+ (3/3)	-
SWINE	A/Indiana/21/2012	H3N2v	9.4	+ (3/3)	-
SWINE	A/Minnesota/11/2010	H3N2v	9.2	+ (3/3)	-
CANINE	A/canine/Florida/43/2004	H3N8	7.2	+ (3/3)	-
EQUINE	A/equine/Ohio/01/2003	EQ-H3N8	7.5	+ (3/3)	-
AVIAN	A/chicken/Alabama/1975	H4N8	10.0	+ (3/3)	-
AVIAN	A/duck/Singapore-Q/F119-3/1997	H5N3	8.5	+ (3/3)	+ (3/3)
AVIAN	A/duck/Pennsylvania/1969	H6N1	9.2	+ (3/3)	-
AVIAN	A/Taiwan/2/2013	H6N1	10.2	+ (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/Netherlands/12/2000	H7N3	9.5	+ (3/3)	-
AVIAN	A/chicken/Arkansas/10/2008	H7N3	9.9	+ (3/3)	-
AVIAN	A/Anhui/1/2013	H7N9	10.9	+ (3/3)	-
AVIAN	A/Bangladesh/0994/2011	H9N2	10.5	+ (3/3)	-
AVIAN	A/chicken/New Jersey/15906-9/1996	H11N1	6.5	+ (3/3)	-
AVIAN	A/duck/Memphis/546/1974	H11N9	9.8	+ (3/3)	-

[1] The result of both A/H5a and A/H5b primer probe sets were concordant for all organisms tested

Influenza A/H5 Subtyping Kit Reproducibility/Precision

The reproducibility and precision of the oligonucleotide primer and probe sets of the Influenza A/H5 Subtyping Kit were evaluated in a collaborative bridging study with Applied Biosystems™ using the 7500 Fast 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 simulated samples that were used included influenza A(H5N1) (reassortant) 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 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 to evaluate the reproducibility of the Influenza A/H5 Subtyping Kit were:

- **Sample #5** Influenza A(H5N1) WT at low viral RNA titer range
- **Sample #6** Influenza A(H5N1) WT at a 1:10 dilution of sample 5
- **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				
	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	Total Agreement w/ Expected Results	95 % CI
Sample 5 (low) - **InfA**	**10/10**	**33.68**	**2.70**	**10/10**	**31.90**	**6.66**	**10/10**	**33.07**	**3.14**	**10/10**	**30.80**	**1.74**	**40/40**	**91.2–100.0**
Sample 5 (low) - **H5a**	**10/10**	**35.69**	**3.76**	**10/10**	**33.65**	**5.52**	**10/10**	**34.58**	**4.94**	**10/10**	**33.03**	**4.55**	**40/40**	**91.2–100.0**
Sample 5 (low) - **H5b**	**9/10**	**37.91**	**3.58**	**10/10**	**36.27**	**9.56**	**10/10**	**36.09**	**4.49**	**10/10**	**34.54**	**3.11**	**39/40**	**86.8 - 99.9**
Sample 5 (low) - **RNaseP**	**10/10**	**29.44**	**4.05**	**10/10**	**30.16**	**7.18**	**10/10**	**27.97**	**4.84**	**10/10**	**28.46**	**2.78**	**40/40**	**91.2–100.0**
Sample 6 (1:10 of Sample 5) - **InfA**	**8/10**	**36.90**	**6.60**	**10/10**	**34.84**	**4.89**	**8/10**	**37.10**	**5.15**	**10/10**	**33.54**	**2.61**	**36/40**	**76.3 – 97.2**
Sample 6 (1:10 of Sample 5) - **H5a**	**5/10**	**37.90**	**3.24**	**9/10**	**36.5**	**4.91**	**7/10**	**37.77**	**7.35**	**10/10**	**35.71**	**2.51**	**31/40**	**61.6 – 89.2**
Sample 6 (1:10 of Sample 5) - **H5b**	**4/10**	**39.29**	**ND***	**5/10**	**39.43**	**3.74**	**7/10**	**38.74**	**3.35**	**10/10**	**35.71**	**5.30**	**27/40**	**50.9 – 81.4**
Sample 6 (1:10 of Sample 5) - **RNaseP**	**10/10**	**30.05**	**3.58**	**10/10**	**30.12**	**3.39**	**10/10**	**28.59**	**4.37**	**10/10**	**28.66**	**3.45**	**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**

Notes: * The CV% is not determined because more than 5 of the samples were negative and the analyte was not detected

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)

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 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-time 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

Conjunctival Swab Studies

Conjunctival Swab Clinical Evaluation

Human upper respiratory tract (URT) swab specimens, including nasopharyngeal swab (NPS) specimens, combined nasal swab (NS) and oropharyngeal swab (OS) specimens, NS and/or OS specimens, as well as human conjunctival swab (CS) specimens were collected by health care professionals (HCPs) as part of public health investigations into outbreaks of influenza A/H5 in dairy cows and poultry in the US from March to November 2024. All available URT specimens and CS specimens from individuals with either a presumptive influenza A/H5 positive laboratory result (in any specimen) at the state public health laboratories, or a strong epidemiological link and clinical suspicion that indicate suspected influenza A/H5 infection, were sent to the CDC laboratory in Atlanta for confirmatory testing and evaluation.

URT specimens and CS specimens collected from a total of 44 confirmed and 2 probable human cases of A/H5N1 were received and tested using the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza A/H5 Subtyping Kit (VER 4) (i.e., the CDC A/H5 assay). The influenza A/H5N1 confirmed or probable case status for each case was adjudicated based on the criteria described in the current Council of State and Territory Epidemiologists (CSTE) document 24-ID-09.

Of the 44 confirmed human cases and 2 probable cases, 42 (95.5%) and 2 (100%), respectively, reported conjunctivitis symptoms. While 28 confirmed cases and 1 probable case reported both respiratory and/or systemic symptoms and conjunctivitis symptoms, 16 confirmed cases and 1 probable case reported conjunctivitis symptoms only. The mean age of the 46 confirmed and probable cases was 36.5 years (range: 18-57 years); 87.0% were male and 13.0% were female.

Due to a field correction regarding the H5b component assay reagents required for testing and result interpretation using the CDC A/H5 assay, clinical specimens from 9 of the 44 confirmed cases were tested without the H5b component assay reagents. As a result, clinical specimens from these 9 confirmed cases were excluded from the performance assessment (i.e., 35 confirmed cases and 2 probable cases were assessed).

Of the 35 confirmed cases assessed, 33/35 (94.3%) reported conjunctivitis symptoms. Of those 33 cases, 26/33 (78.8%) tested positive and 3/33 (9.1%) tested inconclusive for A/H5 on CS specimens using the CDC A/H5 assay. Four (4) of the 33 confirmed cases with conjunctivitis symptoms (15.2%) tested positive for A/H5 on URT specimens only using the CDC A/H5 assay.

Of the 2 confirmed cases without conjunctivitis symptoms reported, 1/2 (50%) tested positive for A/H5 on a CS specimen, and 1/2 (50%) tested positive for A/H5 on a URT specimen only using the CDC A/H5 assay.

Of the 2 probable cases with conjunctivitis symptoms reported, 2/2 (100%) tested negative on CS specimens using the CDC A/H5 assay.

Subject matched paired URT swab specimens and CS specimens were available for testing in 32 out of 35 confirmed cases and 1 of the 2 probable cases. For 3 of the 35 confirmed cases and 1 of the 2 probable cases, only CS specimens were available for testing.

Of the subject matched paired URT and CS specimens from 32 confirmed human cases, 10/32 (31.3%) were positive on at least one URT and the CS specimens;15/32 (46.9%) were positive in CS specimens only and 4/32 (12.5%) were positive in at least one URT specimen only. For 3/32 (9.4%) confirmed human cases the CS specimens tested inconclusive and URT specimens tested negative.

Of the subject matched paired URT and CS specimens from 1 probable human case, all URT and the CS specimen tested negative.

For 3 of the 35 confirmed cases where only CS specimens were available for testing, the CS specimens tested positive in 2 cases, and inconclusive in 1 case.

For 1 of the 2 probable cases where only a CS specimen was available for testing, the result was negative.

Comparisons of subject-matched paired conjunctival swab (CS) and upper respiratory tract (URT) swab specimens testing results using the CDC A/H5 assay in confirmed and probable A/H5N1 cases (n=33) across all symptoms are presented in the two tables below:

Patients with Subject Matched Paired URT Specimens and CS Specimens (n=33)				
	Patient Symptoms			
	Conjunctivitis Only	Conjunctivitis + Flu-like Symptoms	Flu-like Symptoms Only	All Symptoms
A/H5 Positive CS Only	8	6	1	15
A/H5 Positive URT Swab Only	1	2	1	4
Positive in Both CS and URT Swab	2	8	0	10
Positive in Neither CS or URT Swab	1	3	0	4
Total Patients	12	19	2	33

Patients with Subject Matched Paired URT and CS Specimens (n=33)				
	Patient Symptoms			
	Conjunctivitis Only	Conjunctivitis + Flu-like Symptoms	Flu-like Symptoms Only	All Symptoms
A/H5 Positive CS	10 (out of 12)	14 (out of 19)	1 (out of 2)	25 (out of 33)
A/H5 Positive URT Swab	3 (out of 12)	10 (out of 19)	1 (out of 2)	14 (out of 33)

Conjunctival Swab Analytical Evaluation

Confirmation of Limit of Detection

To assess the Limit of Detection (LoD) of the Influenza A/H5 Subtyping Kit testing conjunctival swab collected in VTM/UTM specimens, an initial range-finding experiment using BPL inactivated A/American Wigeon/South Carolina/22-000345-001/2021 H5N1 was performed in a background of VTM + A549 human lung epithelial cells (a simulated respiratory VTM matrix). A 5-fold dilution series of the virus was prepared using this simulated matrix as the diluent. Each dilution step was tested in triplicate using the CDC Flu rRT-PCR Dx Panel Influenza A/H5 Subtyping assay. The range finding limit of detection was defined as the viral titer of the last dilution step demonstrating 100% positive results (3/3) in this experiment. Results demonstrated a preliminary LoD with an approximate titer of $10^{5.5}$ EID_{50}/ml.

Due to limited availability of negative conjunctival swab matrix material, the estimated LoD was further evaluated by testing replicates at concentrations of 2x and 5x preliminary LoD using contrived specimens in either influenza A/H5 negative conjunctival swab matrix or simulated respiratory VTM matrix. Contrived specimens were spiked with BPL inactivated A/American Wigeon/South Carolina/22-000345-001/2021 H5N1 virus. Five replicates of each contrived specimen type at either 2x LoD or 5x preliminary LoD were tested along with 10 negatives for each matrix. Each specimen was extracted on the Qiagen EZ1 Advanced XL with the EZ1 DSP Virus kit, amplified by the Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (without Rox) and run on the Applied Biosystems 7500 Fast Dx PCR platform. Detection levels and Ct values of the conjunctival swab matrix were similar to that observed in the simulated respiratory matrix in VTM at both the 2x and 5x preliminary LoD concentrations. Based on this data, the LoD for the conjunctival swab specimens was determined be $10^{5.5}$ EID_{50}/ml.

Limit of Detection for Conjunctival Swab in VTM/UTM Specimens

Concentration	Matrix Sample	Assay Target								Replicates Tested
		InfA		H5a		H5b		RP		
		Avg Ct	St Dev	Avg Ct	St Dev	Avg Ct	St Dev	Avg Ct	St Dev	
2x $10^{5.5}$ EID_{50}/ml	Conjunctival Swab Matrix	29.49	0.40	33.81	0.31	33.22	0.63	26.72	0.23	5
	Respiratory Matrix in VTM	29.35	0.79	33.47	0.89	33.38	0.40	27.72	1.17	5
5x $10^{5.5}$ EID_{50}/ml	Conjunctival Swab Matrix	26.77	0.19	31.06	0.25	30.91	0.37	26.54	0.28	5
	Respiratory Matrix in VTM	26.26	0.26	30.37	0.20	30.76	0.20	26.77	0.23	5
Negative	Conjunctival Swab Matrix	0.00	0.00	0.00	0.00	0.00	0.00	25.76	0.24	5
	Respiratory Matrix in VTM	0.00	0.00	0.00	0.00	0.00	0.00	27.86	0.43	5

Interfering Substances

Two studies were performed to evaluate the potential impact of exogenous interfering substances in conjunctival swab specimen matrix. Studies were conducted using two common over the counter eye drops containing olopatadine or naphazoline hydrocholoride.

For the first study, to evaluate the effect of eyedrops on the assay, a set of contrived specimens (N=5) was constructed with a measured volume of each eyedrop plus A/American Wigeon/South Carolina/22-000345-001/2021 H5N1 at 2x LoD in conjunctival swab matrix. An additional set of contrived specimens (N=5) was constructed with a measured volume of each eyedrop plus A/American Wigeon/South Carolina/22-000345-001/2021 H5N1 at 2x LoD in VTM + A549 cells (a simulated respiratory matrix). Each contrived specimen contained 2.31% ClearEye eyedrop (naphazoline hydrochloride as the main ingredient) and 2.08% Pataday eyedrop (olopatadine hydrochloride as the main ingredient), and was extracted on the Qiagen EZ1 Advanced XL with the EZ1 DSP Virus kit, amplified by the Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (without Rox) and run on the Applied Biosystems 7500 Fast Dx PCR platform. Due to a lack of sufficient volume of natural conjunctival swabs, the study was conducted by mixing both the olopatadine and naphazoline hydrochloride into a single reaction. Cycle threshold (Ct) results were compared to contrived specimens tested at 2x LoD without the addition of eyedrops as the control condition. No interference was seen with the addition of olopatadine and naphazoline hydrochloride in either the contrived specimens in conjunctival matrix or the contrived specimens in VTM + A549 cells matrix in this study.

Interfering Substance Evaluation of Eyedrops (Olopatadine and Naphazoline hydrochloride) Added to Contrived Conjunctival Matrix Specimens and Contrived Specimens in VTM + A549 Matrix

Matrix at 2x LoD	Interfering Substance	Assay Target								Replicates Tested
		InfA		H5a		H5b		RP		
		Avg Ct	St Dev	Avg Ct	St Dev	Avg Ct	St Dev	Avg Ct	St Dev	
Conjunctival Swab Matrix	Without Eyedrops	29.49	0.40	33.81	0.31	33.22	0.63	26.72	0.23	5
	With Eyedrops	29.35	0.37	33.50	0.61	33.12	0.31	26.36	0.21	5
Respiratory Matrix in VTM	Without Eyedrops	29.35	0.79	33.47	0.89	33.38	0.40	27.72	1.17	5
	With Eyedrops	28.26	0.33	32.24	0.31	32.73	0.60	27.16	0.17	5

The second study was performed by adding 5 µl of Pataday (olopatadine hydrochloride as the main ingredient) or Clear Eyes (naphazoline hydrochloride as the main ingredient) eyedrop dilutions in molecular-grade-water (2.22% Pataday and 2.46% Clear Eyes, respectively) directly into the RT-PCR enzyme master mix and tested in 20 replicates to evaluate the effect of the eyedrop chemistry on the A/H5 assay and determine if autofluorescence is produced due to the eyedrop additions that could generate a false positive A/H5 assay result. No false positive A/H5 assay result was seen when the eyedrops were added directly to the RT-PCR reaction in this study.

Interfering Substance Evaluation of Olopatadine and Naphazoline hydrochloride Added Directly to the RT-PCR Reaction

Replicate	Olopatadine Hydrochloride Added Directly to the RT-PCR Reactions				Naphazoline Hydrochloride Added Directly to the RT-PCR Reactions			
	Ct Values				Ct Values			
	InfA	H5a	H5b	RP	InfA	H5a	H5b	RP
REP- 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
REP- 20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

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 30329.

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. Ng *et al*. "Influenza A H5N1 Detection,” *Emerging Infectious Diseases*, August 2005, 11(8), 1303-1305.

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8. 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 30329.

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:

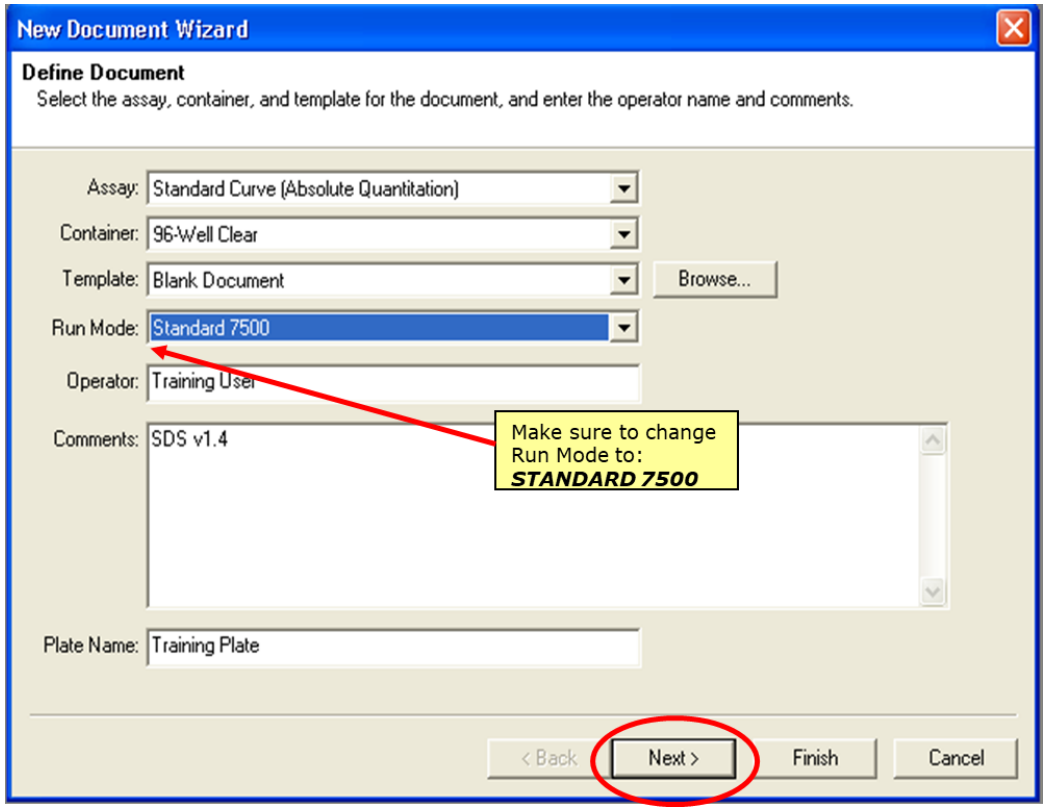
Send email to: FluSupport@cdc.gov

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

This section describes how to create an Influenza A/H5 Subtyping Kit template for the 7500 Fast Dx. This template will be saved and used for all Influenza A/H5 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 A1. New Document Wizard Window

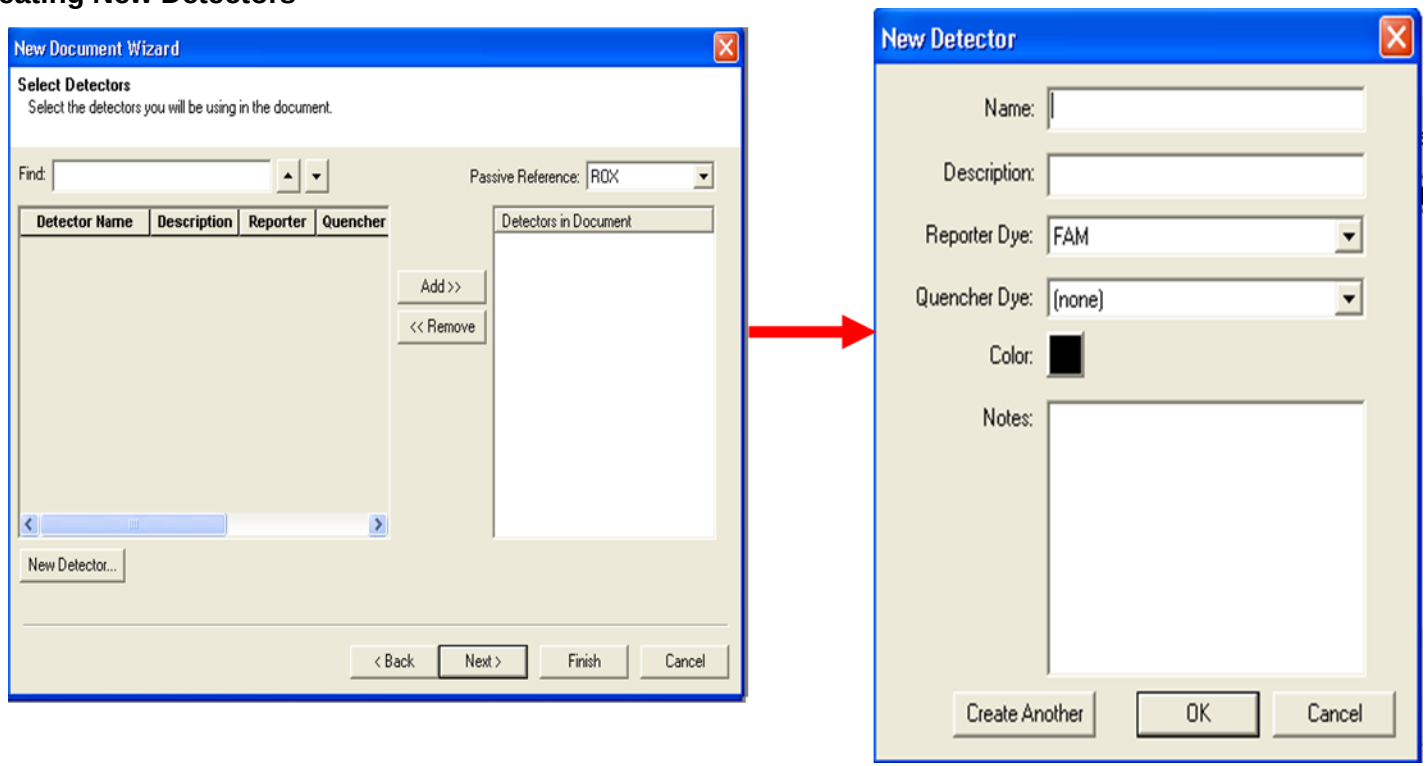


3. The **New Document Wizard** screen in **Figure A1** will appear. Select:
   a. Assay: **Standard Curve (Absolute Quantitation)**
   b. Container: **96-Well Clear**

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 A2**).
7. The **New Detector** window will appear (**Figure A2**, 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 A2. Creating New Detectors



8. Start by creating the InfA Detector (**Figure A2**, left image). Include the following:
a. Name: **InfA**
b. Description: *leave blank*

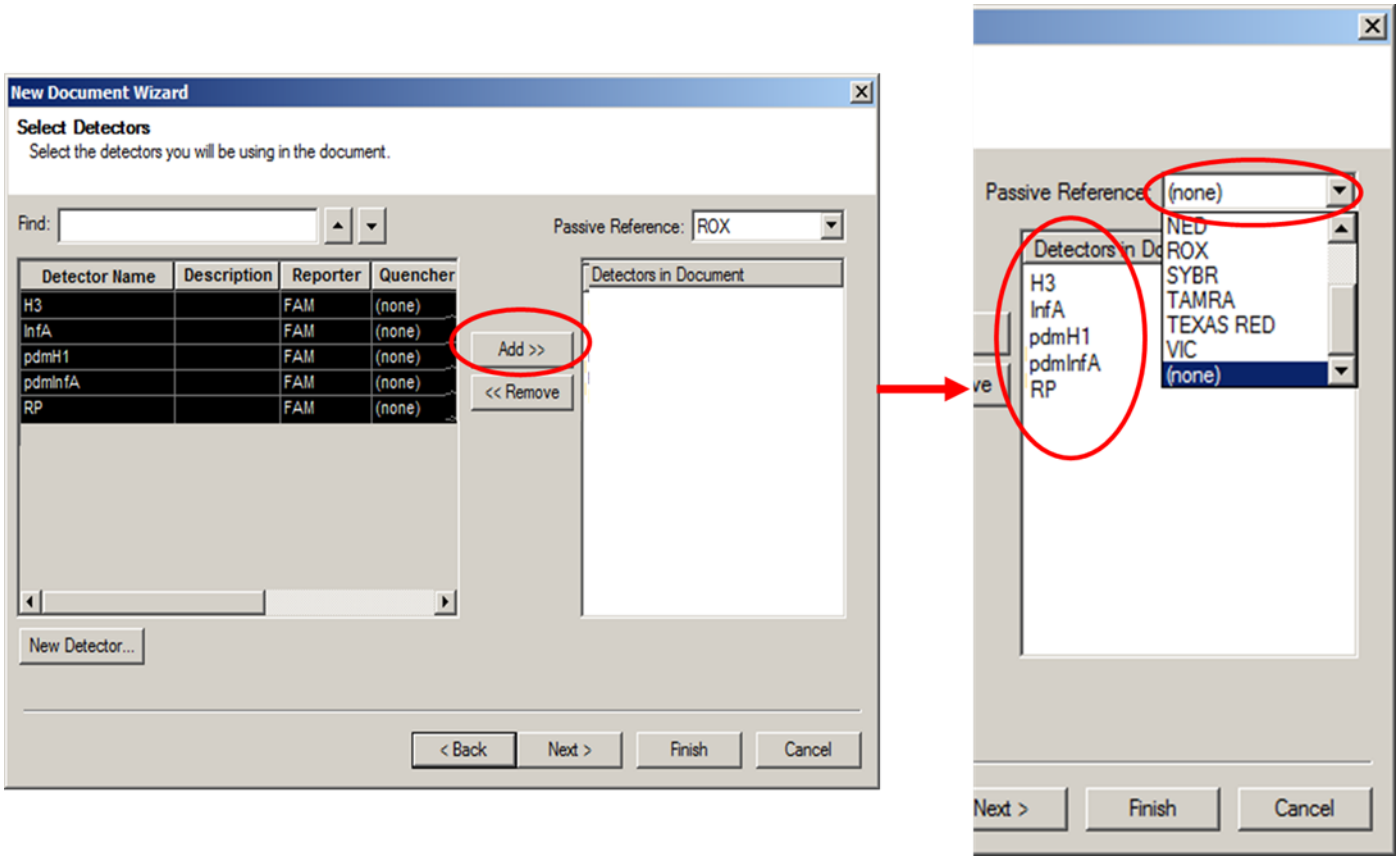
c. Reporter Dye: **FAM**
d. Quencher Dye: **(none)**
e. Color: *to change the color of the detector indicator do the following:*
    - ⇒ Click on the color square to reveal the color chart
    - ⇒ Select a color by clicking on one of the squares
    - ⇒ After selecting a color click **OK** to return 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 A2**.

9. Repeat step 6-8 for each influenza target in the panel.

Name	Reporter Dye	Quencher Dye
InfA	FAM	(none)
H5a	FAM	(none)
H5b	FAM	(none)
RP	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 A3**).
11. Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (**Figure A3**, 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 A3**, right image).

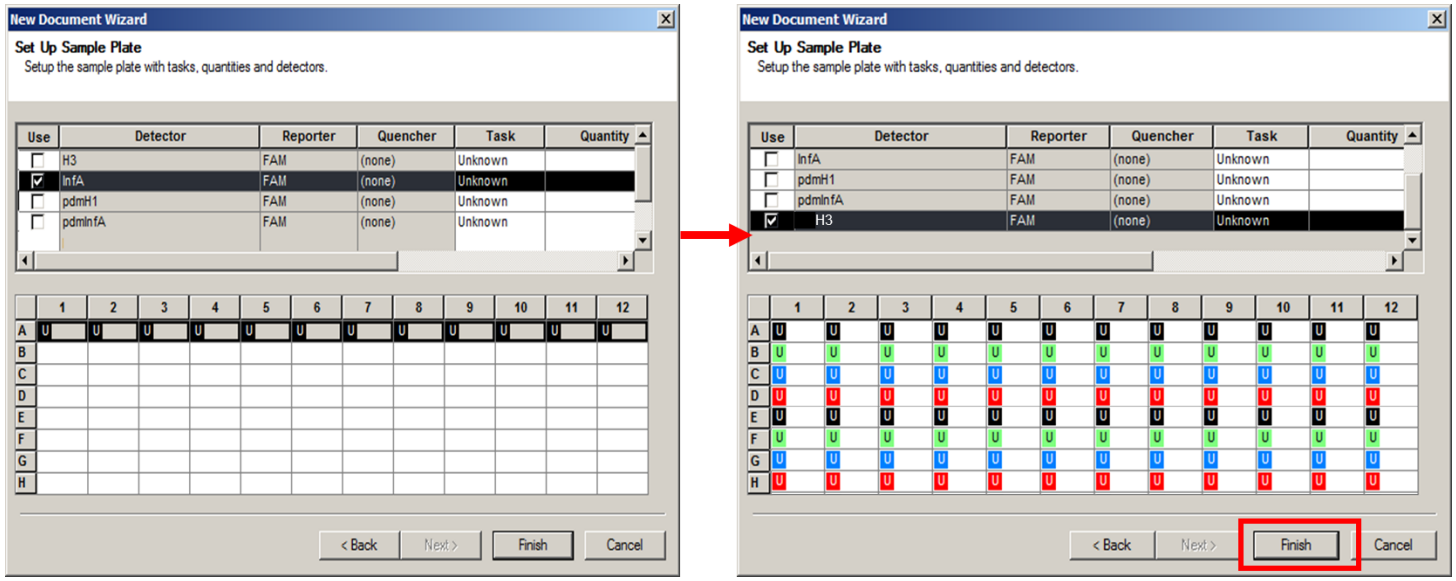
Figure A3. Adding New Detectors to Document and Selecting Passive Reference



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

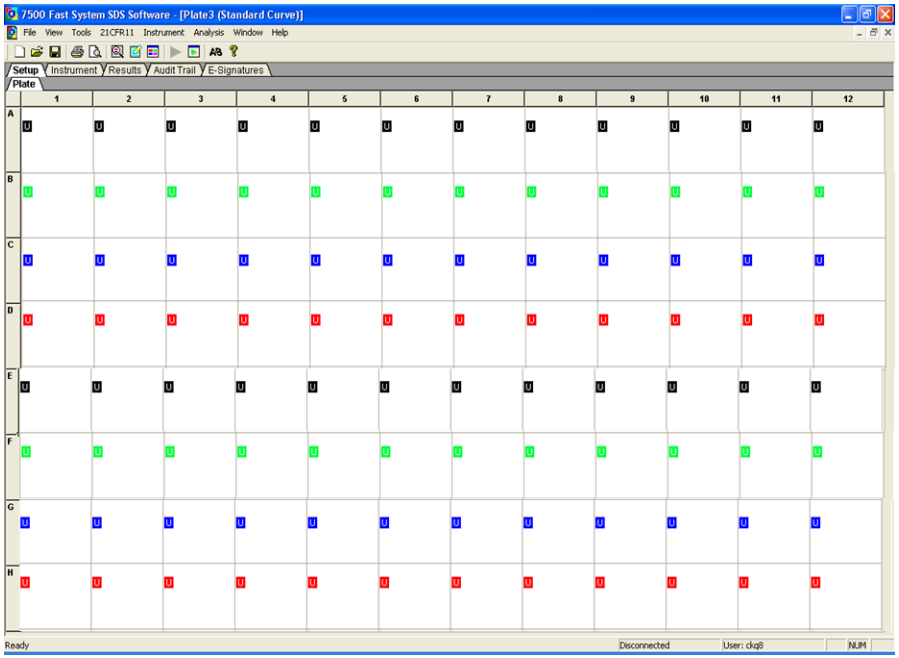
13. Click **Next** at the bottom of the **Select Detectors** window (**Figure 3**) to proceed to the **Set Up Sample Plate** window (**Figure A4**).
14. In the **Set Up Sample Plate** window (**Figure A4**), select row **A** from the in the spreadsheet in the lower portion of the window.
15. In the top portion of the window, select detector **InfA**. A check will appear next to the detector that was selected (**Figure A4**). 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 A4**, right image).

Figure A4. Sample Plate Set-up



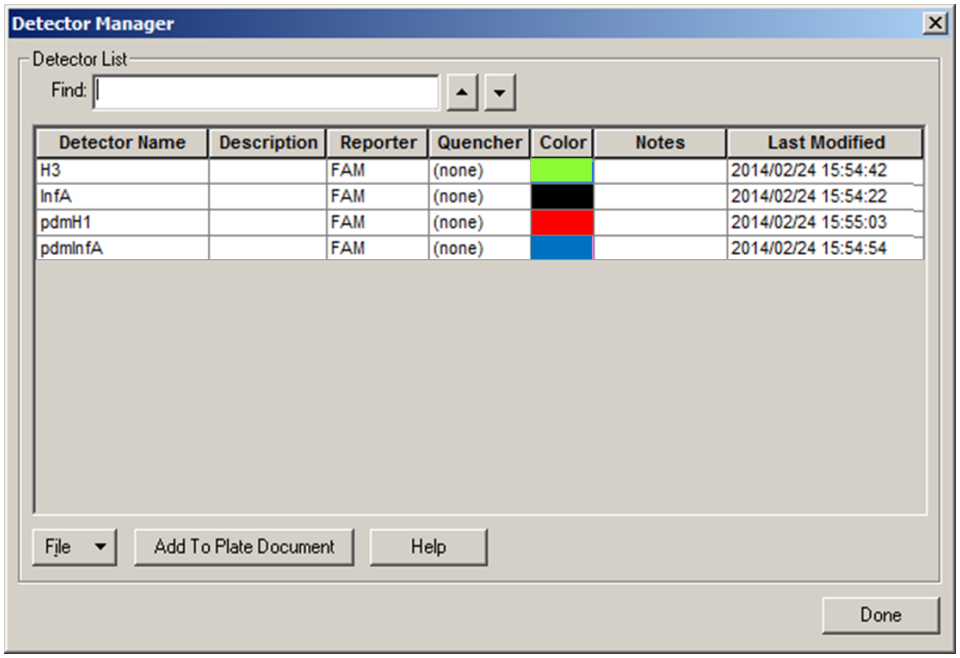
18. After clicking **Finish**, there will be a brief pause allowing the 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***
19. After initialization, the **Plate** tab of the **Setup** (**Figure A5)** will appear.
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 A5. Plate Set-up Window



21. The **Detector Manager** window will appear (**Figure A6**).

Figure A6. 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 A7**):

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 **2.0 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.**

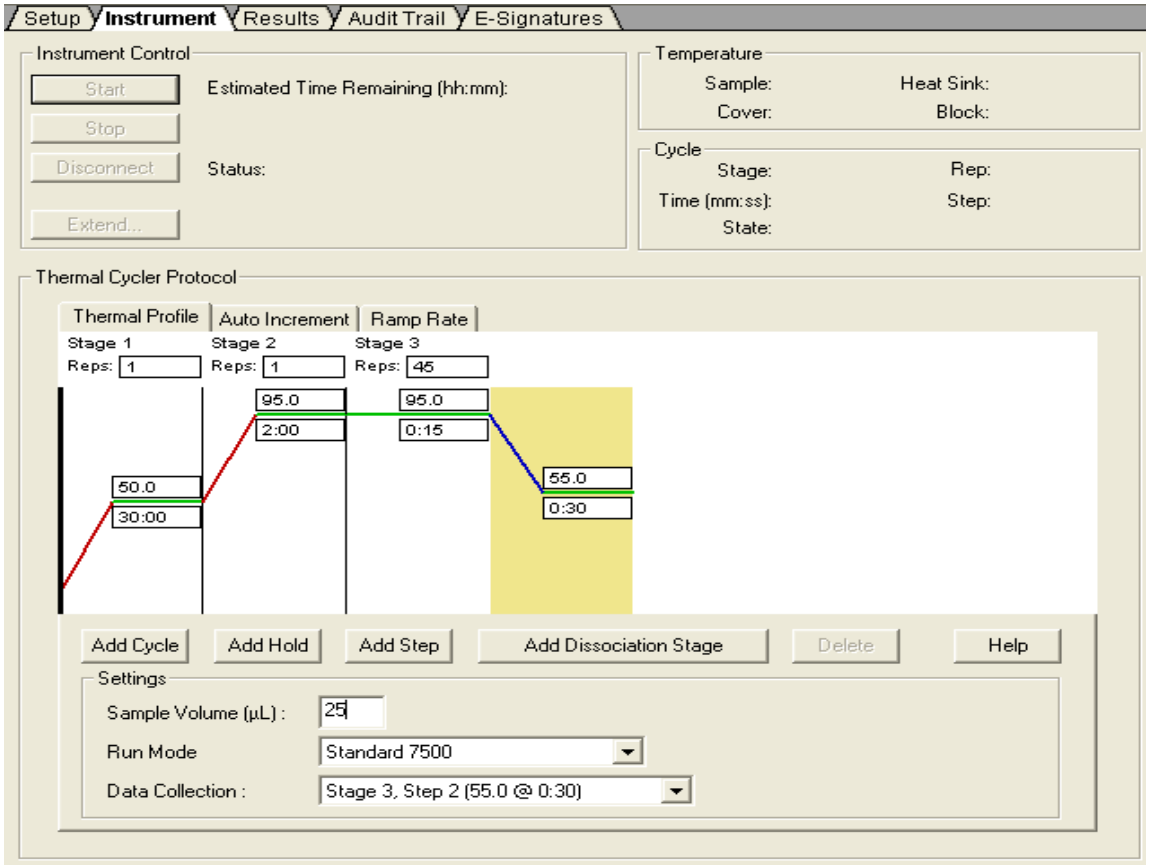
e. In Stage 3, Reps should be set to **45.**
f. Under **Settings** (**Figure 7**), bottom left box, change volume to 25 µL.
g. Under **Settings**, **Run Mode** selection should be **Standard 7500**.
h. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure A7**).

OR

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

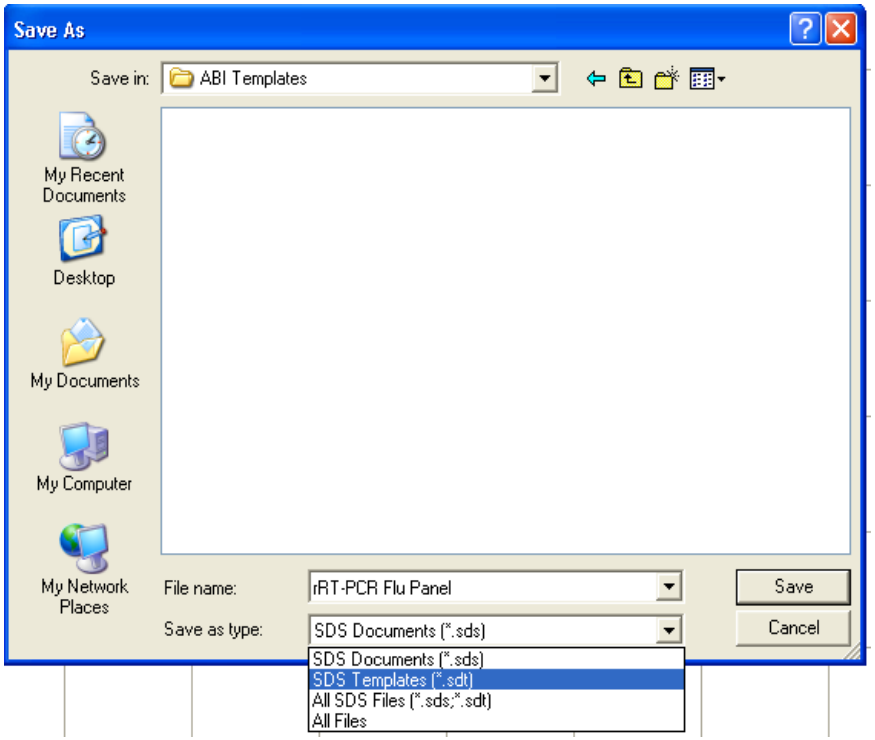
a. In Stage 1, Set to **30 min** at **50°C**; **1 Rep**.
b. In Stage 2, Set to **5.0 min** at **95°C**; **1 Rep**.
c. In Stage 3, Step 1 set to **15 sec** at **95°C**.
d. In Stage 3, Step 2 set to **30 sec** at **55.0°C**.
e. In Stage 3, Reps should be set to **45**.
f. Under **Settings (Figure A7)**, 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 A7**).

Figure A7. 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/H5 Subtyping Kit SuperScript** or **Influenza A/H5 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 A8**).

Figure A8. Saving Template

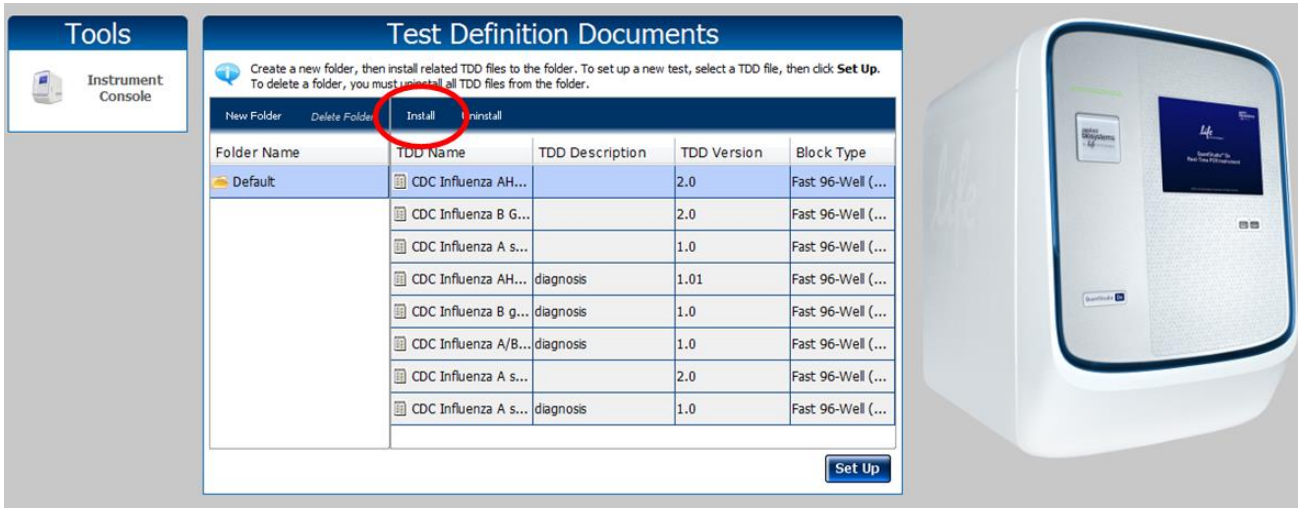


Appendix B: Instrument Setup for Applied Biosystems™ QuantStudio™ Dx (QSDx)

This section describes how to install a test definition document (.tdd) for the Influenza A/H5 Subtyping Kit onto the QuantStudio™ Dx. This .tdd file will be saved and used for all Influenza A/H5 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/H5 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 B1)** will automatically open.

Figure B1. Installing New Tests



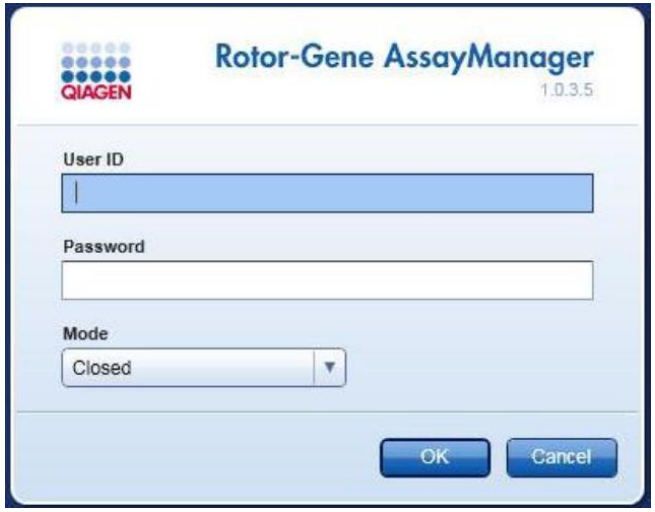
5. In the **Test Definition Document** window select **Install** to display the **Open** window.
6. Navigate to the folder containing the test definition document files (.tdd).
7. Select the appropriate Influenza A/H5 Subtyping Kit file (.tdd) and click **Open**. The file name will now be displayed in the **Test Definition Document** table.
8. If necessary, install another test definition document by clicking **Install** and repeat steps 8-9.
9. Once a test definition document is installed it will persist until deleted. This .tdd file should be used for all Influenza A/H5 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/H5 Subtyping Kit to the Rotor-Gene Q MDx. This assay profile will be saved and used for all Influenza A/H5 Subtyping Kit testing on this instrument.

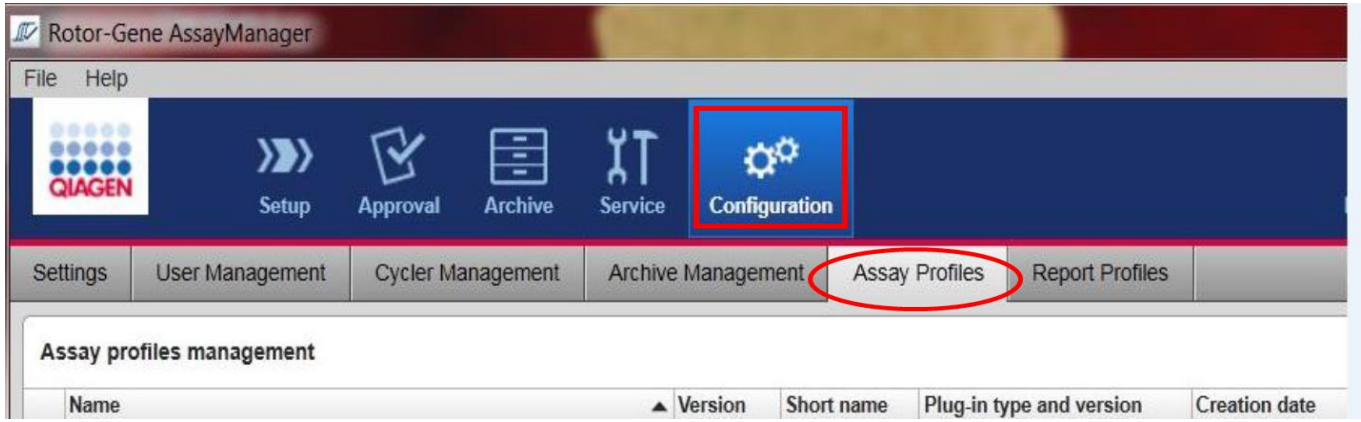
1. Go to the FluSupport Sharepoint site at https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx and download the appropriate assay profile for the Influenza A/H5 Subtyping Kit for use with the QIAGEN Rotor-Gene Q MDx instrument and save to a designated folder.
2. Launch the QIAGEN Rotor-Gene 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 C1**).
4. Select **Closed** from the **Mode** drop down menu.

Figure C1. Rotor-Gene AssayManager Login Window



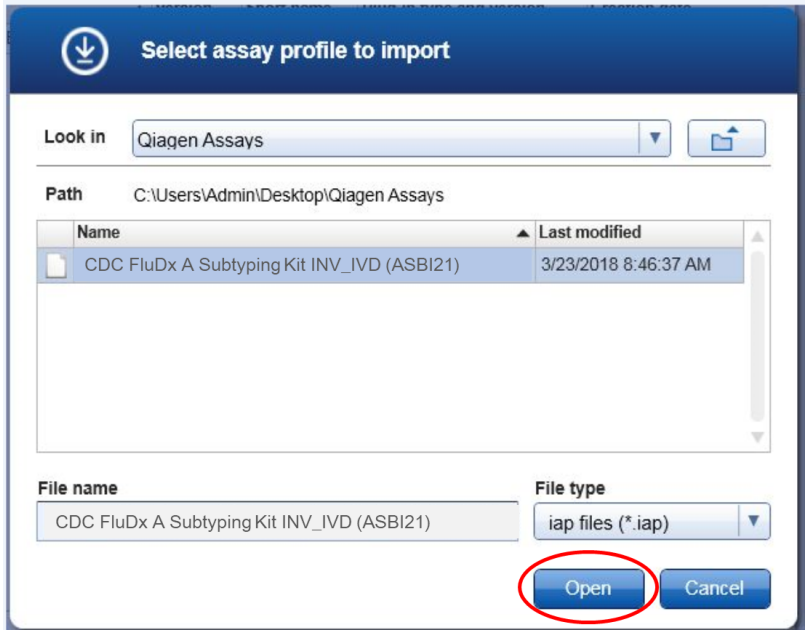
5. Select **OK** and the Rotor-Gene AssayManager main navigation window (**Figure C2**) will open.
6. Select the **Configuration** tab from the top menu, then select the **Assay Profiles** tab (**Figure C2**).

Figure C2. 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 C3**).
8. Navigate to the folder that contains the assay profiles using the **Look in** drop down menu.
9. Select the appropriate Influenza A/H5 Subtyping Kit assay profile (.iap) file and click **Open.**

Figure C3. Select Assay Profile to Import Window



10. The **Import Successful** window (**Figure C4**) will appear. Click **OK** to return to the **Assay Profiles** tab. The new assay will be listed under **Assay profiles management (Figure C4).**
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/H5 Subtyping Kit testing.

Figure C4. Import Successful Window and Assay Manager Configuration Screen