

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

Influenza B Lineage Genotyping Kit (VER 1.1)

**Instructions for Use
Package Insert**

**Catalog # FluIVD03-7
1000 reactions**

For *In-vitro* Diagnostic (IVD) Use

Rx Only

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



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Intended Use

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

- For the determination of the genetic lineage of human influenza B viruses as B/Victoria or B/Yamagata lineage from viral RNA in upper respiratory tract clinical specimens (including nasopharyngeal swabs [NPS], nasal swabs [NS], throat swabs [TS], nasal aspirates [NA], nasal washes [NW] and dual nasopharyngeal/throat swabs [NPS/TS]) from human patients with signs and symptoms of respiratory infection and/or from viral culture;
- To provide epidemiologic information for surveillance of circulating influenza viruses.

Performance characteristics for influenza B lineage genotyping were established during a season when influenza B/Victoria and B/Yamagata lineages were in circulation.

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.

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

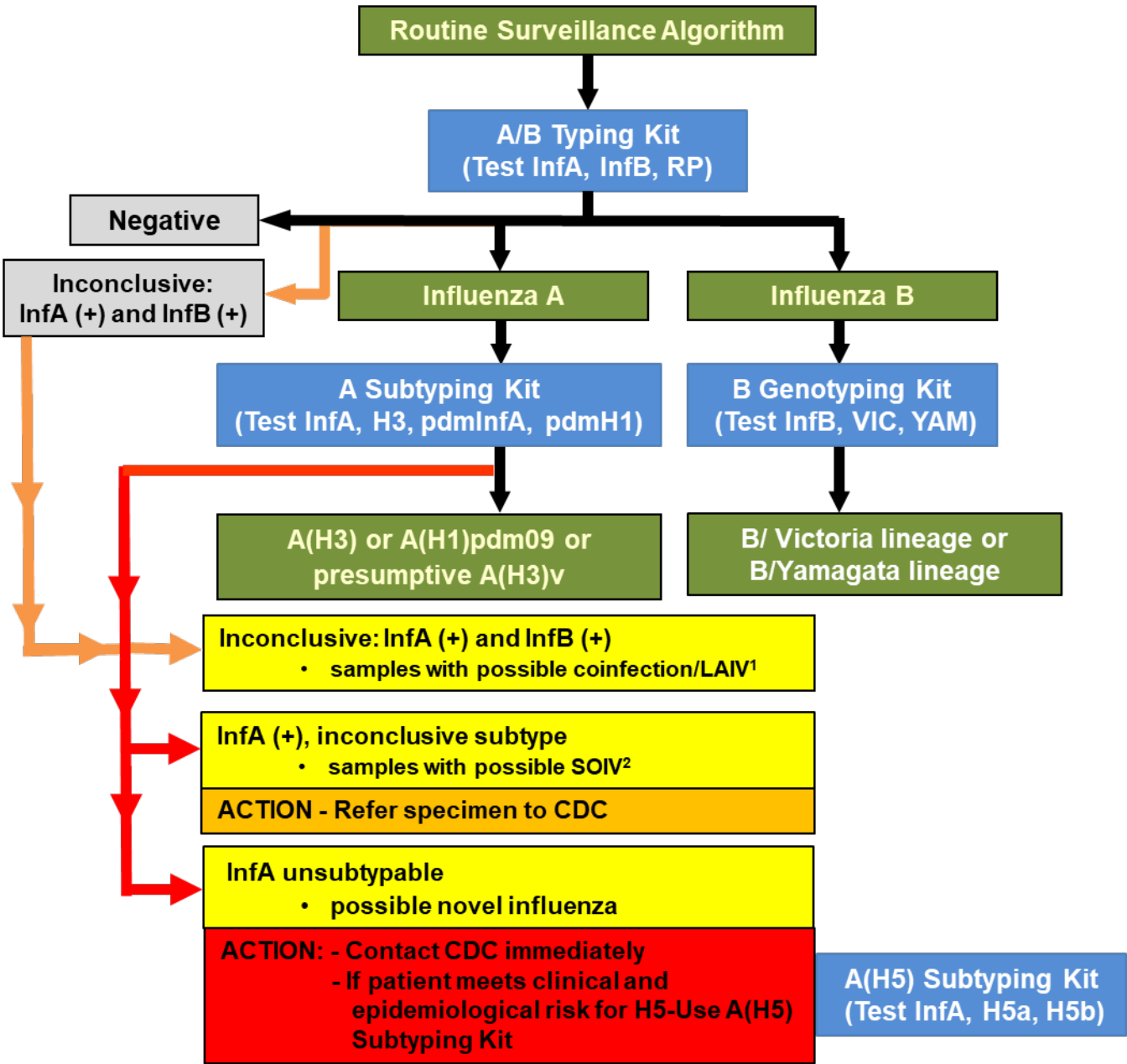
Influenza viruses are the causative agents of influenza infection, an acute respiratory illness that is highly contagious and results in significant morbidity each year with flu-related deaths ranging from a low of about 3,000 to a high of about 49,000 people (<https://www.cdc.gov/flu/index.htm>). Influenza 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.

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 viruses¹
A/H5 Subtyping Kit	Primer and Probe Sets: InfA, H5a, H5b, RP Controls: H5VC, HSC	• Specifically detects influenza A(H5) Asian lineage viruses
B Lineage Genotyping Kit	Primer and Probe Sets: InfB, VIC, YAM, RP Controls: Influenza B Positive Control (IBPC)	• Specifically detects and differentiates influenza B lineage genotypes; B/Victoria, B/Yamagata

¹Classical 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:



¹LAIV=Live Attenuated Influenza Virus

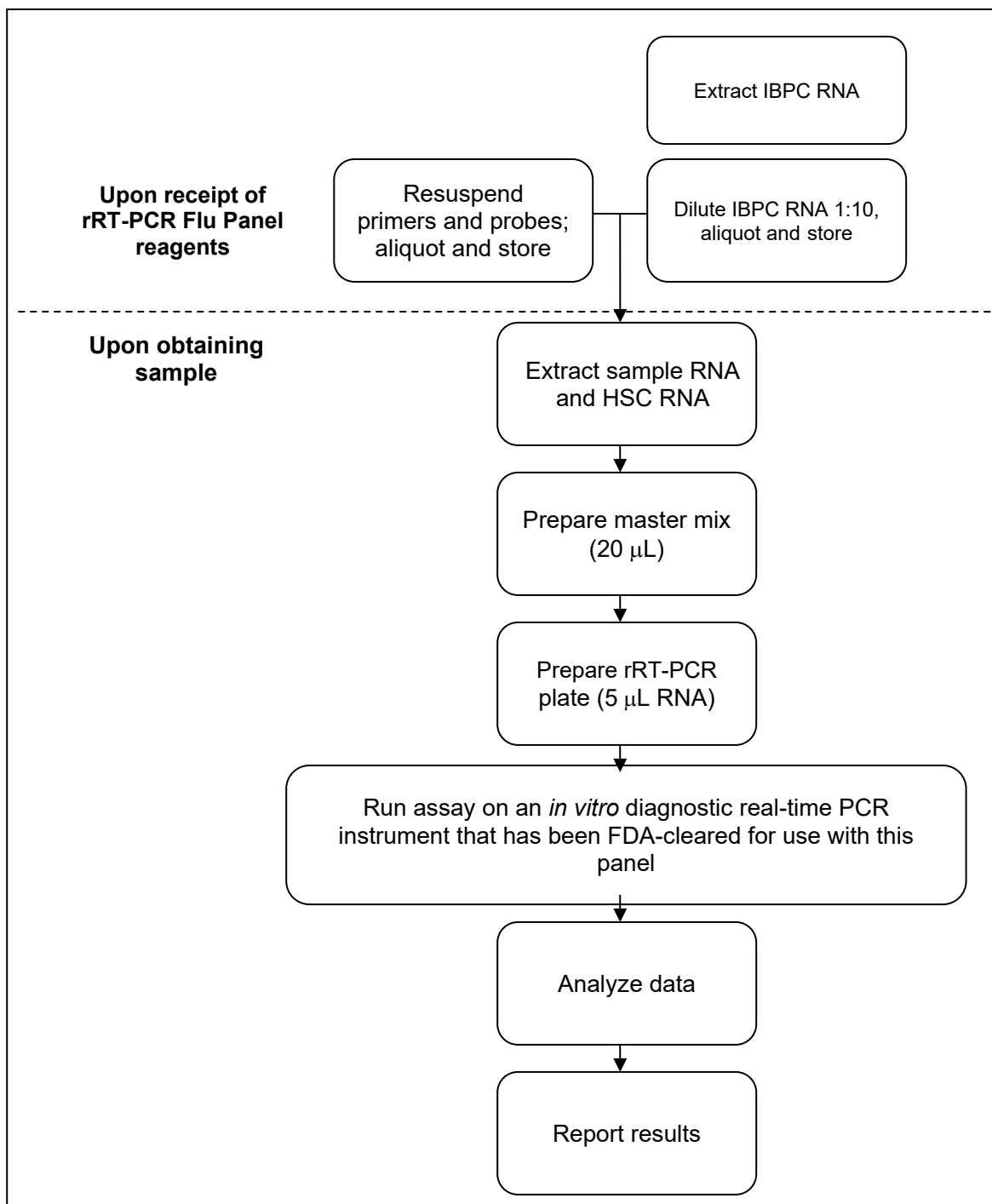
²SOIV=Swine Origin Influenza Virus

Principles of the Procedure

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

The Influenza B Lineage Genotyping 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 from patients presenting with influenza-like illness (ILI). The oligonucleotide primers and probes for detection of influenza B viruses were selected from highly conserved regions of the non-structural (NS) protein gene segment. Oligonucleotide primers and probes for characterization and differentiation of influenza B/Victoria and B/Yamagata viruses were selected from highly conserved regions of their 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 viruses.

Summary of Influenza Testing Process



Materials Required (Provided)

Influenza B Lineage Genotyping Kit (VER 1.1) Contents: Catalog # FluIVD03-7

Box #1: Primers and Probes

Reagent Label	Part #	Description	Quantity / Tube	Reactions / Tube
InfB-F	SO3311	Influenza B Forward Primer	20 nmol	1000
InfB-R	SO3299	Influenza B Reverse Primer	20 nmol	1000
InfB-P	SO3300 or MR-619	Influenza B Probe (FAM)	5 nmol	1000
VIC-F	MR-610	Influenza B/Victoria Lineage Forward Primer	20 nmol	1000
VIC-R	MR-261	Influenza B/Victoria Lineage Reverse Primer	20 nmol	1000
VIC-P	MR-611	Influenza B/Victoria Lineage Probe (FAM)	5 nmol	1000
YAM-F	MR-262	Influenza B/Yamagata Lineage Forward Primer	20 nmol	1000
YAM-R	MR-612	Influenza B/Yamagata Lineage Reverse Primer	20 nmol	1000
YAM-P	MR-258	Influenza B/Yamagata Lineage 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: Influenza B Positive Control

Reagent Label	Part #	Description	Quantity / Tube	Notes
IBPC	MR-257	Influenza B Positive Control (IBPC): For use as a positive control with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel procedure to ensure the detection of seasonal influenza B virus of both the B/Yamagata and B/Victoria lineages. The IBPC contains noninfectious positive control materials supplied as a liquid, 900 µl per vial, suspended in 0.01 M phosphate buffer saline (PBS) at pH 7.2–7.4. IBPC consists of two different beta-propiolactone treated influenza viruses, representing influenza B/Yamagata and B/Victoria lineages, suspended in cultured human cells (A549). IBPC will yield a positive result with the following primer and probe sets: InfB, VIC, YAM, and RP	1 tube x 900 µL / tubes	One thousand eight hundred 5 µL reactions per tube

Critical Equipment and Materials Required (But Not Provided)

Real-Time PCR Instruments and Software

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

rRT-PCR Enzyme Master Mix Options

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

RNA Extraction Options

Instrument/Manufacturer	Extraction Kit	Catalog No.
Roche MagNA Pure LC	Total Nucleic Acid Kit	192 extractions: 03 038 505 001
Roche MagNA Pure 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	DSP Viral RNA Mini Kit	50 extractions: 61904
QIAGEN QIAcube	DSP Viral RNA Mini Kit	50 extractions: 61904
QIAGEN EZ1 Advanced XL	EZ1 DSP Virus Kit	48 extractions (62724) Buffer AVL (19073) EZ1 Advanced XL DSP Virus Card (9018703)

Instrument/Manufacturer	Extraction Kit	Catalog No.
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 (<i>easyMAG[®] only</i>) (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.
- 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.
- Performance characteristics of the Influenza B Lineage Genotyping Kit have been determined with human upper respiratory specimens (including NPS, NS, TS, NA, NW, NPS/TS) from human patients with signs and symptoms of respiratory infection and/or from viral culture.
- 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.

Reagent Storage, Handling, and Stability

- Store all primers and probes at 2–8°C until re-hydrated for use; Store all control materials (IBPC) at ≤ -20°C.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on cold block at all times during preparation and use.
- Do not refreeze probes.
- Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use.

Specimen Collection, Handling, and Storage

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

➤ Collecting the Specimen

- Follow specimen collection devices manufacturer instructions for proper collection methods.
- Swab specimens should be collected using only swabs with a synthetic tip, such as nylon or Dacron®, and an aluminum or plastic shaft. Calcium alginate swabs are unacceptable and cotton swabs with wooden shafts are not recommended.

➤ Transporting Specimens

- Ensure that when transporting human respiratory specimens, all applicable regulations for the transport of etiologic agents are met. Human respiratory specimens, to be tested within 72 hours post-collection, should be transported refrigerated at 2–8°C. Alternatively, specimens may be frozen and transported for testing.
- Respiratory specimens should be collected and placed into viral transport media (VTM) or universal transport media (UTM) as described by CDC and WHO guidelines. Lavage specimens in phosphate buffered saline (PBS) are accepted. See the FluSupport SharePoint site for more information: <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 RDSB/STATT Unit 198
Virology, Surveillance and Diagnosis Branch, Influenza Division
Centers for Disease Control and Prevention
1600 Clifton Rd., Atlanta, GA 30329-4027
Phone: (404) 639-2434

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

Reagent and Controls Preparation

Primer and Probe Preparation:

1. Upon receipt, store lyophilized primers and probes at 2-8°C.
2. Rehydration
 - a. Remove primers and probes from 2-8°C.
 - b. Allow primers and probes to sit at ambient temperature for 15 minutes.
 - c. Quickly centrifuge dried primers and probes to collected pellet in the bottom of the tube.
 - d. Pipette 0.5 ml (500 µL) of 10 mM Tris, pH 7.4-8.2 or PCR-grade water into each dried PCR primer or probe.
 - e. Allow primers and probes to fully rehydrate for at least 15 minutes at room temperature.
 - f. After primers and probes are fully rehydrated, pulse vortex to ensure a homogenous solution.
3. Prepare a Combined Oligonucleotide Mix for Each Marker Set
 - a. For each marker set (e.g. InfB), 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 primer/ probe mix aliquots should be stored in the dark.

Influenza B Positive Control (IBPC) Preparation:

1. Reagent
 - a. Inactivated, noninfectious positive control material is supplied as a liquid suspension in 0.01 M PBS.
 - b. Control contains an influenza B virus of each major lineage (B/Victoria and B/Yamagata)
 - c. Volume: 0.9 mL yields approximately 9 mL of positive control RNA.
2. Storage
 - a. Store at -20°C or below upon receipt. Do not dilute.
3. Procedure
 - a. IBPC must be extracted prior to use. The final volume of eluted RNA should equal the volume of extracted control material. For example, 100 µL of control material should result in 100 µL of RNA extract (1:1).
 - b. Dilute the RNA 1:10 with nuclease free water.
 - c. Label one new nuclease-free, sterile, tube for each single-use aliquot with the following information:
 - Control RNA name and 1:10 dilution
 - Kit lot #
 - Expiration date
 - d. Dispense the 1:10 diluted RNA into 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 B Lineage Genotyping Kit
 - e. Use one aliquot per run. Discard after use. Do not use residual RNA.

General Preparation

Equipment Preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use. Decontamination agents should be used including 5% bleach, 70% ethanol, and **DNAzap™** or **RNase AWAY®** to minimize the risk of nucleic acid contamination.

Reagent Preparation

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

Primers and Probes Reagents

- Thaw frozen aliquots of combined primer/probe mix. *Thawed aliquots may be stored at 2-8°C in the dark for up to 3 months. **Do not re-freeze.***
- Vortex each combined primer/probe mix for 15 seconds.
- Briefly centrifuge each combined primer/probe mix.
- Place combined primer/probe mixes 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 recommends that laboratories perform genotyping characterization of all the influenza B positives with the Influenza B Lineage Genotyping Kit. Current testing 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 B Lineage Genotyping Kit. However, in the event that specimens need to be re-extracted or if the Influenza B Lineage Genotyping 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. InfB, VIC, YAM, etc.).
2. Determine the number of reactions (N) to be prepared per assay.
3. Calculate the amount of each reagent to be added to the tube for each master mix by multiplying the number of reactions (samples plus controls) per marker by the volume of reagent indicated in **Figure 1**.

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

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

If number of samples (n) including controls > 15, then $N = n + 2$

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

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

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

OR

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

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

4. Dispense reagents into each respective labeled 1.5 mL microcentrifuge tube. After addition of the reagents, mix reaction mixtures by pipetting up and down. Do not vortex.
5. Centrifuge for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
6. Set up reaction strip tubes or plates in a 96-well cooler rack.
7. Dispense 20 µL of each master mix into the appropriate wells going across the row as shown below (Figure 2).

Figure 2. Influenza B Lineage Genotyping Kit: Example of Reaction Master Mix Plate Set-up

	1	2	3	4	5	6	7	8	9	10	11	12
A	InfB	InfB	InfB	InfB	InfB	InfB	InfB	InfB	InfB	InfB	InfB	InfB
B	VIC	VIC	VIC	VIC	VIC	VIC	VIC	VIC	VIC	VIC	VIC	VIC
C	YAM	YAM	YAM	YAM	YAM	YAM	YAM	YAM	YAM	YAM	YAM	YAM
D	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
E	empty	InfB	InfB	InfB	InfB	InfB	InfB	InfB	InfB	InfB	InfB	empty
F	empty	VIC	VIC	VIC	VIC	VIC	VIC	VIC	VIC	VIC	VIC	empty
G	empty	YAM	YAM	YAM	YAM	YAM	YAM	YAM	YAM	YAM	YAM	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, 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 2-11 (column 1, 11 (bottom half), and 12 are for controls) to the specific assay that is being tested as illustrated in **Figure 3**. Carefully pipette 5.0 µL of the first sample into all the wells labeled for that sample (i.e. Sample “S1” down column #2). *Keep other sample wells covered during addition. Change tips after each addition.*
4. Securely cap the column to which the sample has been added to prevent cross contamination and to ensure sample tracking.
5. Change gloves often and when necessary to avoid contamination.
6. Repeat steps #3 and #4 for the remaining samples.
7. If necessary, add 5 µL of Human Specimen Control (HSC) extracted sample to the HSC wells (**Figure 3**, column 11). Securely cap wells after addition. **NOTE: HSC is not included with the Influenza B Lineage Genotyping Kit (FluIVD03-7). HSC must be obtained from another kit in the CDC Human Influenza Real-Time RT-PCR Diagnostic Panel. Please use the Package Insert for the Influenza A/B Typing Kit for instructions on the preparation and addition of HSC.**
8. Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

Assay Control Addition

1. Pipette 5 µL of IBPC 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!

- 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 B Lineage Genotyping 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	IBPC
B	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	IBPC
C	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	IBPC
D	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	IBPC
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

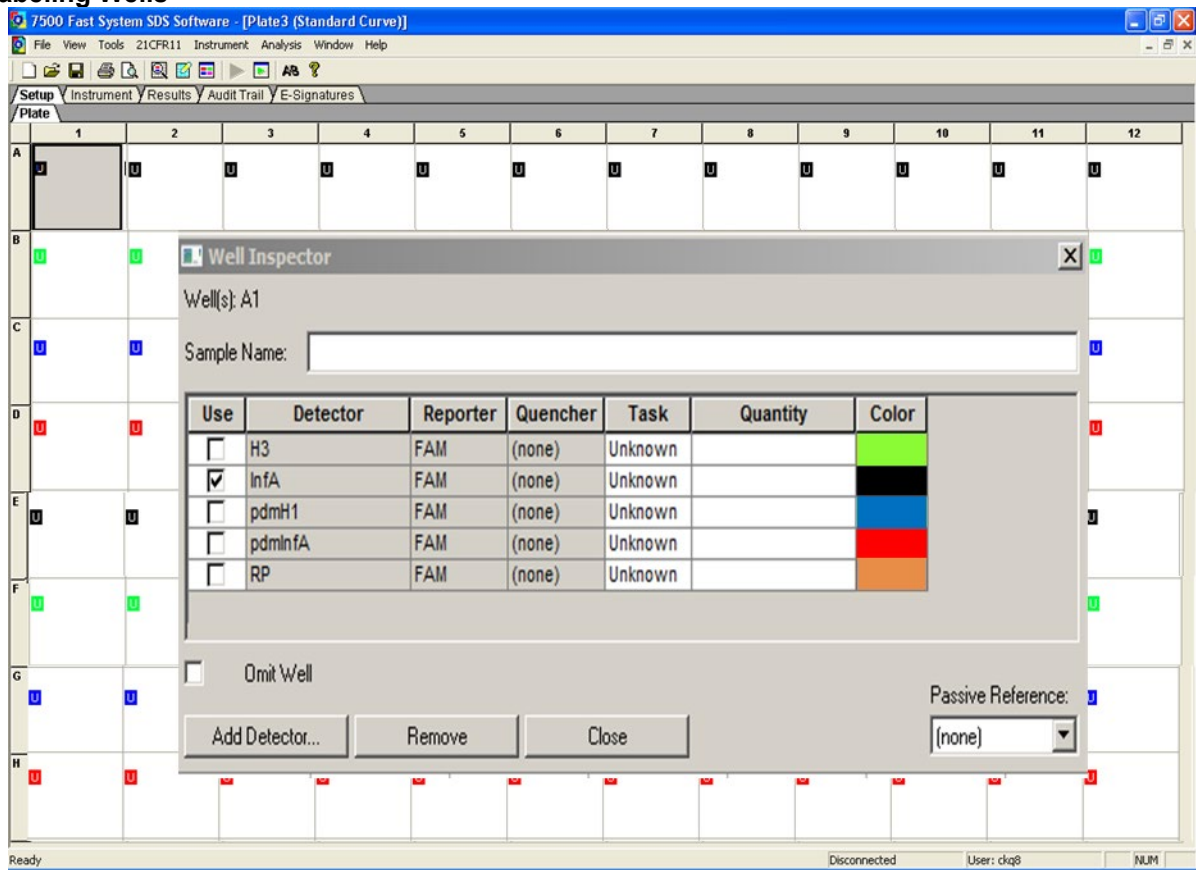
NOTE: HSC needs to be tested with each nucleic acid extraction run. If samples are from an extraction run already tested with HSC, repeat testing of HSC is not necessary.

Running a Test on the Applied Biosystems™ 7500 Fast Dx

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

- Turn on the 7500 Fast Dx Real-Time PCR Instrument and computer.
- Launch the 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
- A new window should appear, select **Open Existing Document** from the menu.
- Navigate to select the ABI Run Template folder from the desktop.
- Double click on the appropriate template file (**Influenza B Lineage SuperScript** or **Influenza B Lineage qScript**).
- 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.**
- 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.
- Click the **Well Inspector** icon  from the top menu.
- Highlight specimen wells of interest on the plate map.
- 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

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

Instrument Control

Start Stop Disconnect Extend...

Estimated Time Remaining (hh:mm):

Status:

Temperature

Sample: Cover: Heat Sink: Block:

Cycle

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

Thermal Cycler Protocol

Thermal Profile	Auto Increment	Ramp Rate
Stage 1	Stage 2	Stage 3
Reps: 1	Reps: 1	Reps: 45
50.0	95.0	95.0
30:00	2:00	0:15
		55.0
		0:30

Add Cycle Add Hold Add Step Add Dissociation Stage Delete Help

Settings

Sample Volume (µL): 25

Run Mode: Standard 7500

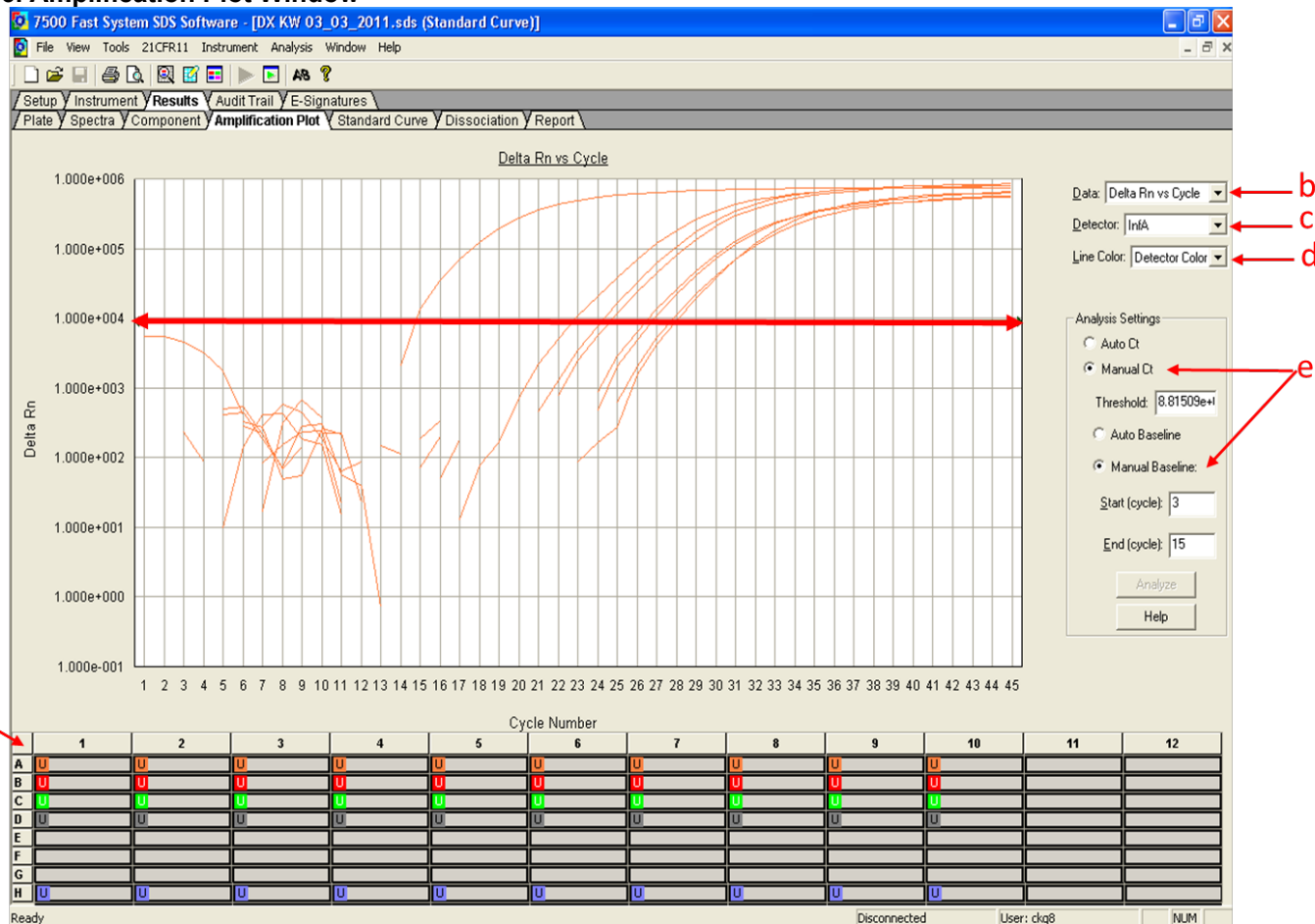
Data Collection: Stage 3, Step 2 (55.0 @ 0:30)

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

Results Analysis

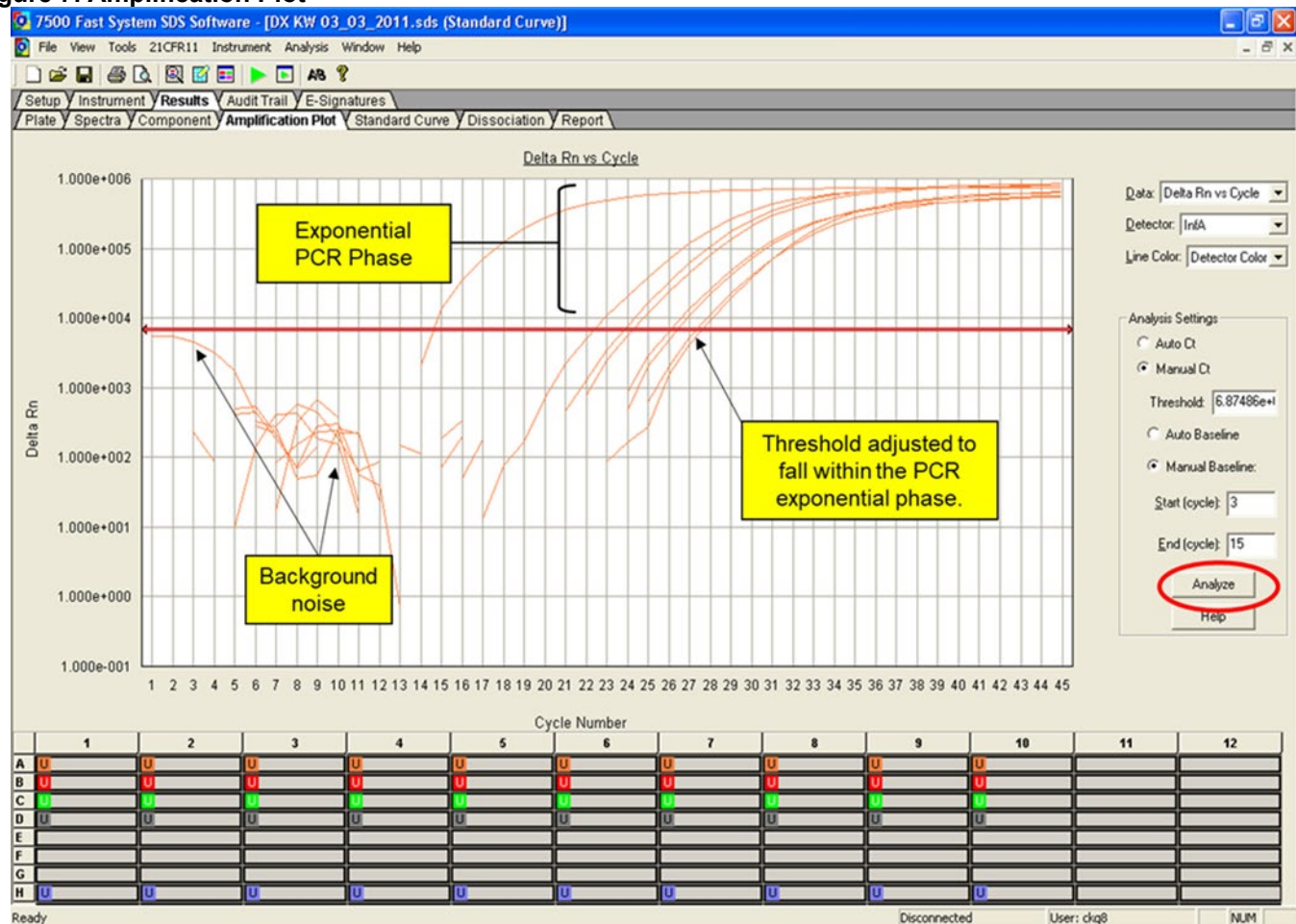
1. After the run has completed, select the **Results** tab at the upper left corner of the software.
2. Select the **Amplification Plot** tab to view the raw data (**Figure 6**).

Figure 6. Amplification Plot Window



- 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.
- On the right side of the window (b), the **Data** drop down selection should be set to **Delta Rn vs. Cycle**.
- Select **InfB** from (c), the **Detector** drop down menu, using the downward arrow.
 - Please note that each detector is analyzed individually to reflect different performance profiles of each primer and probe set.
- In the **Line Color** drop down (d), **Detector Color** should be selected.
- Under **Analysis Settings** select **Manual Ct** (e).
 - Do not change the **Manual Baseline** default numbers.
- Click and drag the red threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal (Figure 7).

Figure 7. Amplification Plot



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

Figure 8. Report

7500 Fast System SDS Software - [DX KW 03_03_2011.sds (Standard Curve)]

File View Tools 21CFR11 Instrument Analysis Window Help

Setup Instrument Results Audit Trail E-Signatures

Plate Spectra Component Amplification Plot Standard Curve Dissociation 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	pdnInfA	Unknown	28.8506						
D2	training sample 1	pdnH1	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	pdnInfA	Unknown	24.5261						
D3	training sample 2	pdnH1	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	pdnInfA	Unknown	24.1216						
D4	training sample 2	pdnH1	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	pdnInfA	Unknown	27.1319						
D5	training sample 3	pdnH1	Unknown	25.2213						
G5	training sample 5	RP	Unknown	Undet.						

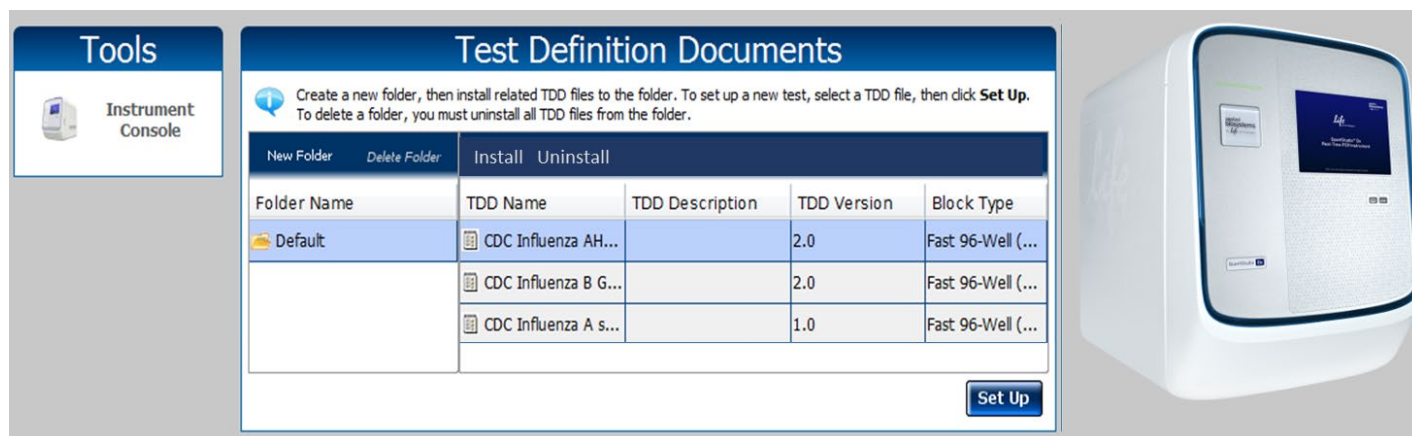
Ready Disconnected User: dlo8 NUM

Running a Test on the Applied Biosystems™ QuantStudio™ Dx

If the test definition document file (.tdd) for the Influenza B Lineage Genotyping 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 B Lineage Genotyping 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

QuantStudio™ Dx Software v1.0.3 (Full Name: Administrator Administrator - Last Log In: 29-11-2018 16:33:53 EST)

File Edit Tools Help

Open... Save Close Print Report...

Test Menu

Setup

Test Properties

Define

Assign

Run

Analysis

Test: 2018-12-06

How do you want to identify this plate?

* Plate Name: 2018-12-06 Plate Comments:

Plate Barcode:

User Name: Administrator

Test Properties

Test Name: CDC Influenza A subtyping NO RP assay (IVD) BL 3-10 Test Author: CDC Influenza Division Diagnostic Development Team

Test Version: 1.0 Comments:

Test Description: Data Collection Software Version: QuantStudio™ Dx Software v1.0.1

Instrument Type: QuantStudio™ Dx Real-Time PCR Instrument Interpretive Software Version:

Block Type: Fast 96-Well Block (0.1mL)

Assay Type(s): Standard Curve

Run Mode: Standard

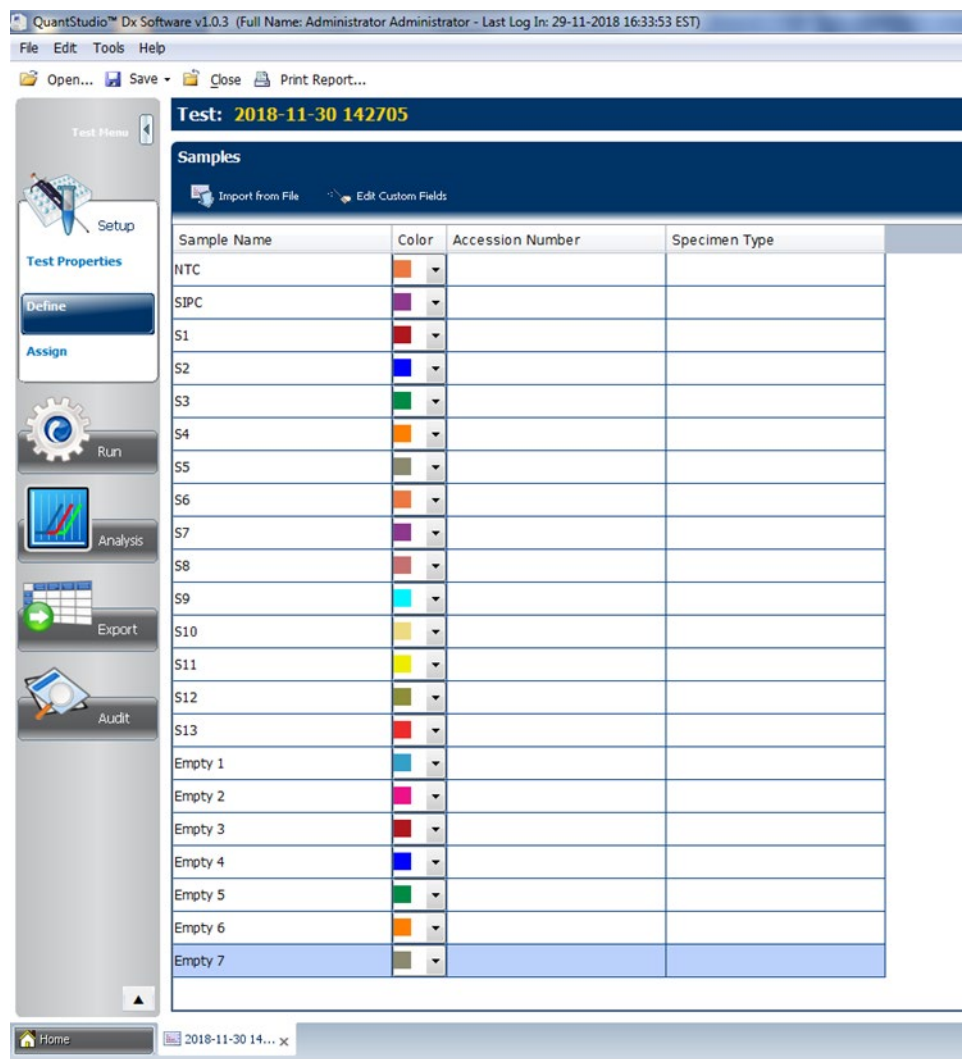
Test Worksheet: [Open Test Worksheet](#)

Reagent Information

Type	Name	Part Number	Lot Number	Expiration Date
Kit	CDC Human Influenza Virus Real-Tim...	FluIVD 03-6		

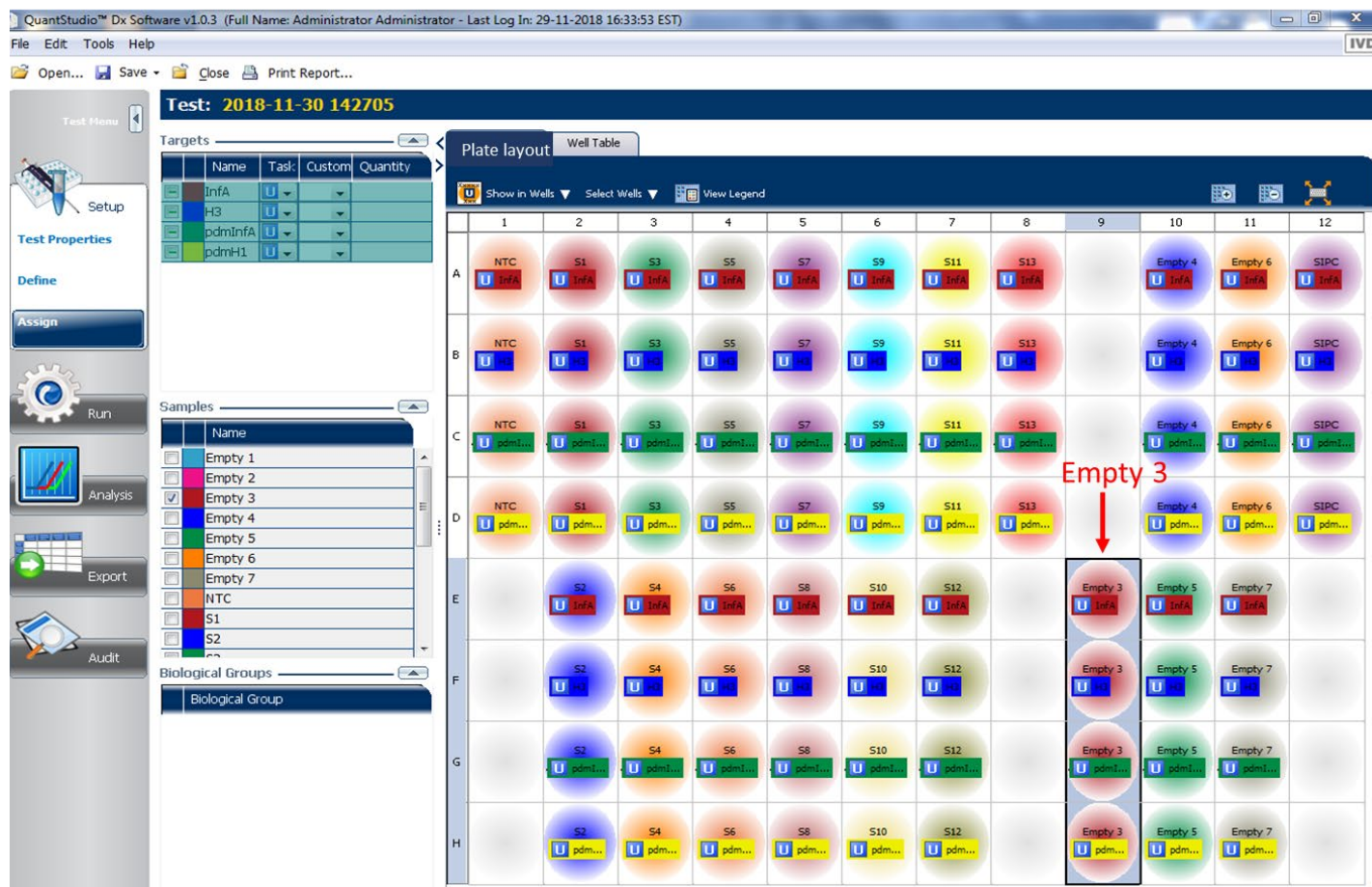
10. Select **Define (Figure 10)** in the left margin to assign sample identifiers in the **Samples** window (**Figure 11**).
11. Enter a test sample identifier into each **Sample Name** field to replace the default sample identifiers "Sample 1", "Sample 2", etc.
12. Replace "No Template Control" with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

Figure 11. Defining Sample Wells



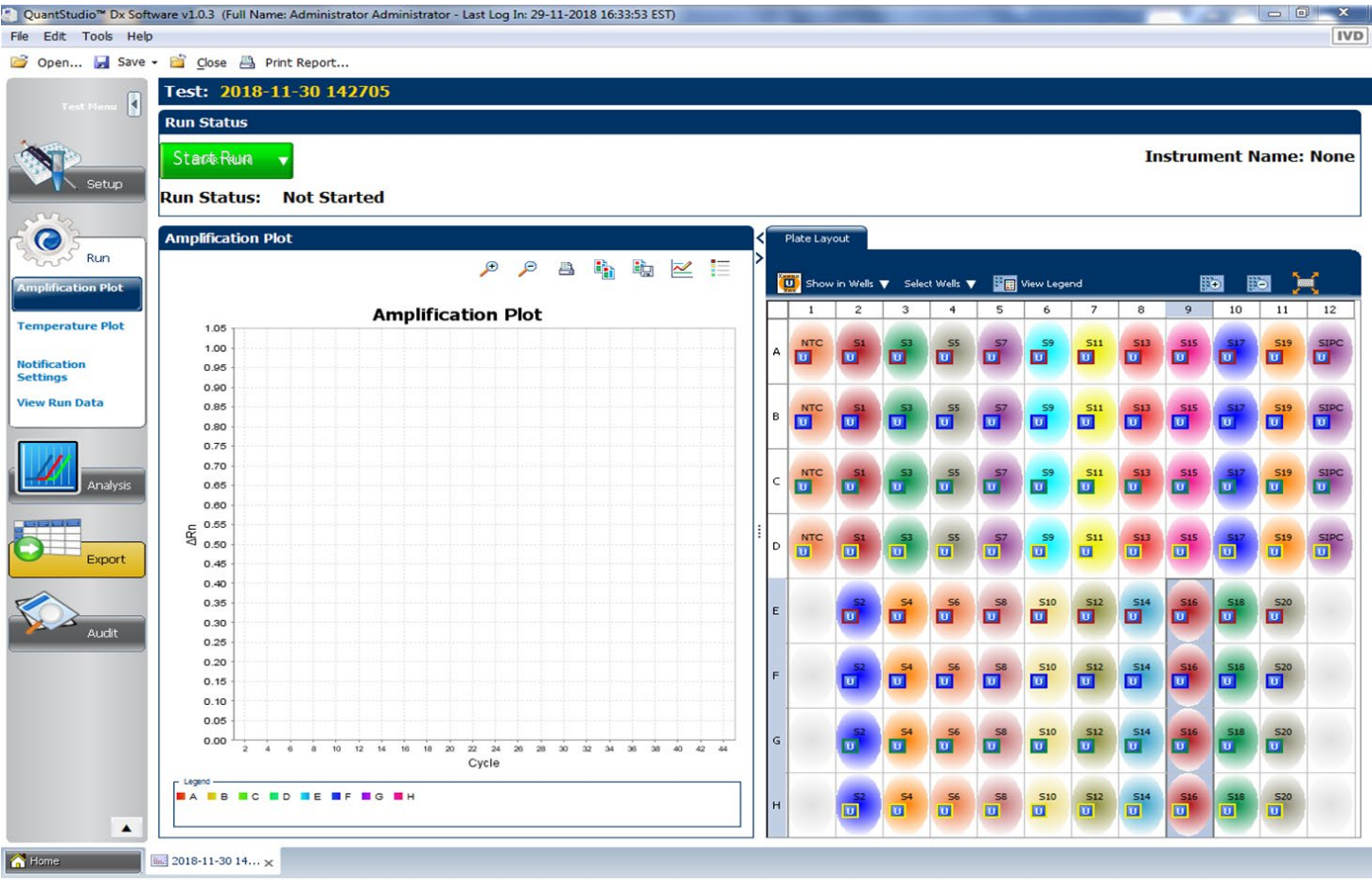
- 14. Once all control and sample identifiers have been added, select **Assign (Figure 12)** in the left margin to review the plate assignment.
- 15. If desired, the user may remove the empty wells from the plate layout by selecting the column corresponding to the empty sample position on the plate layout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

Figure 12. Finalizing Plate Layout



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

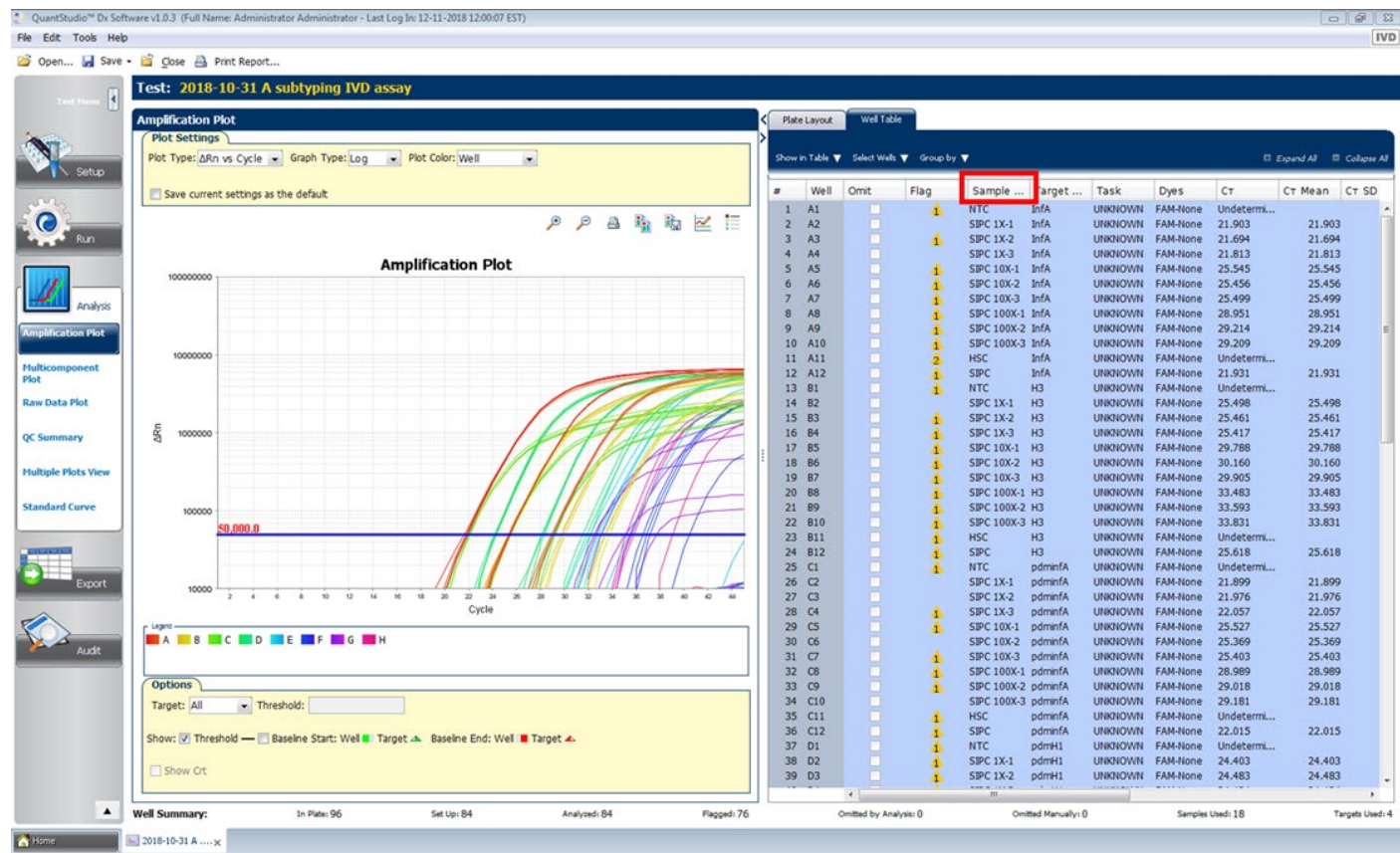
Figure 13. Starting the Run



Results Analysis

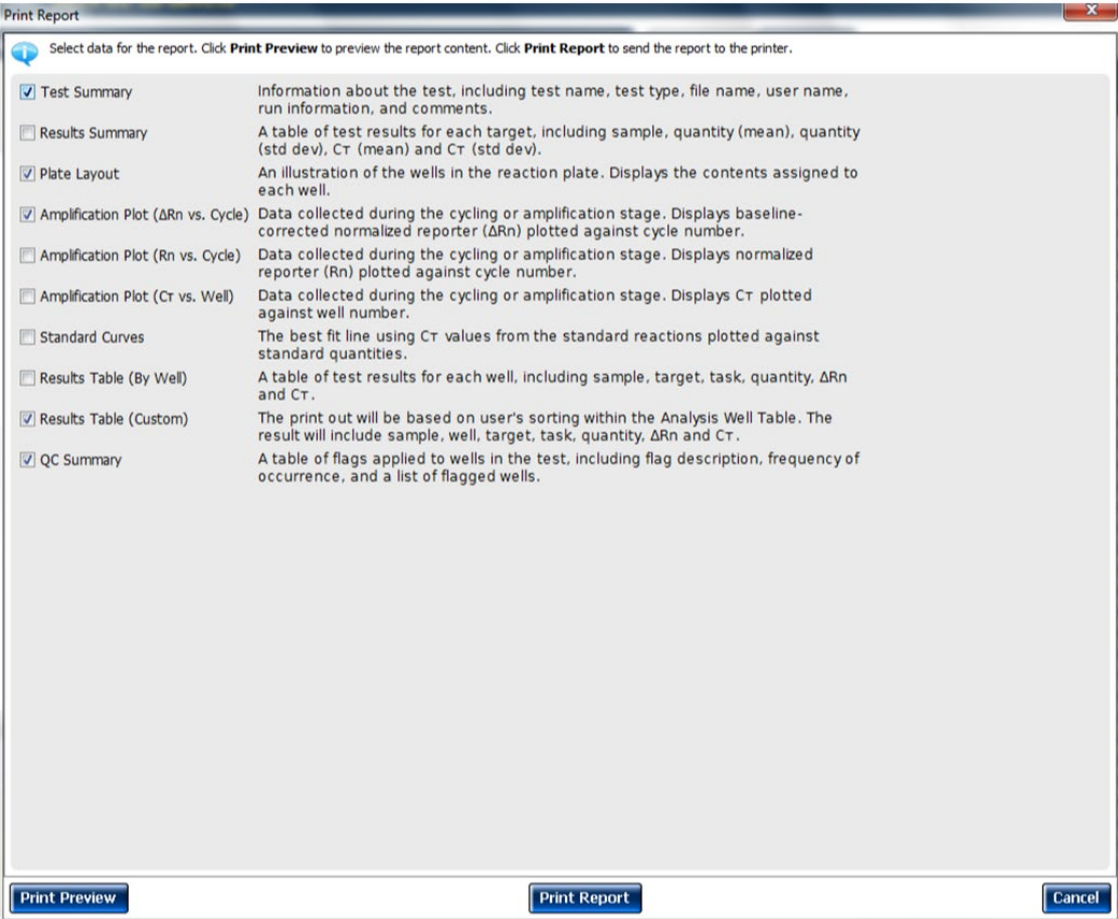
1. After the run has completed, the **Amplification Plot** tab in the **Analysis** window will display (**Figure 14**).
2. In the **Analysis** view, select the **Well Table** tab at the upper left side of the plate layout review the analyzed data.
3. Sort the wells by sample clicking the **Sample** column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

Figure 14. Amplification Plot



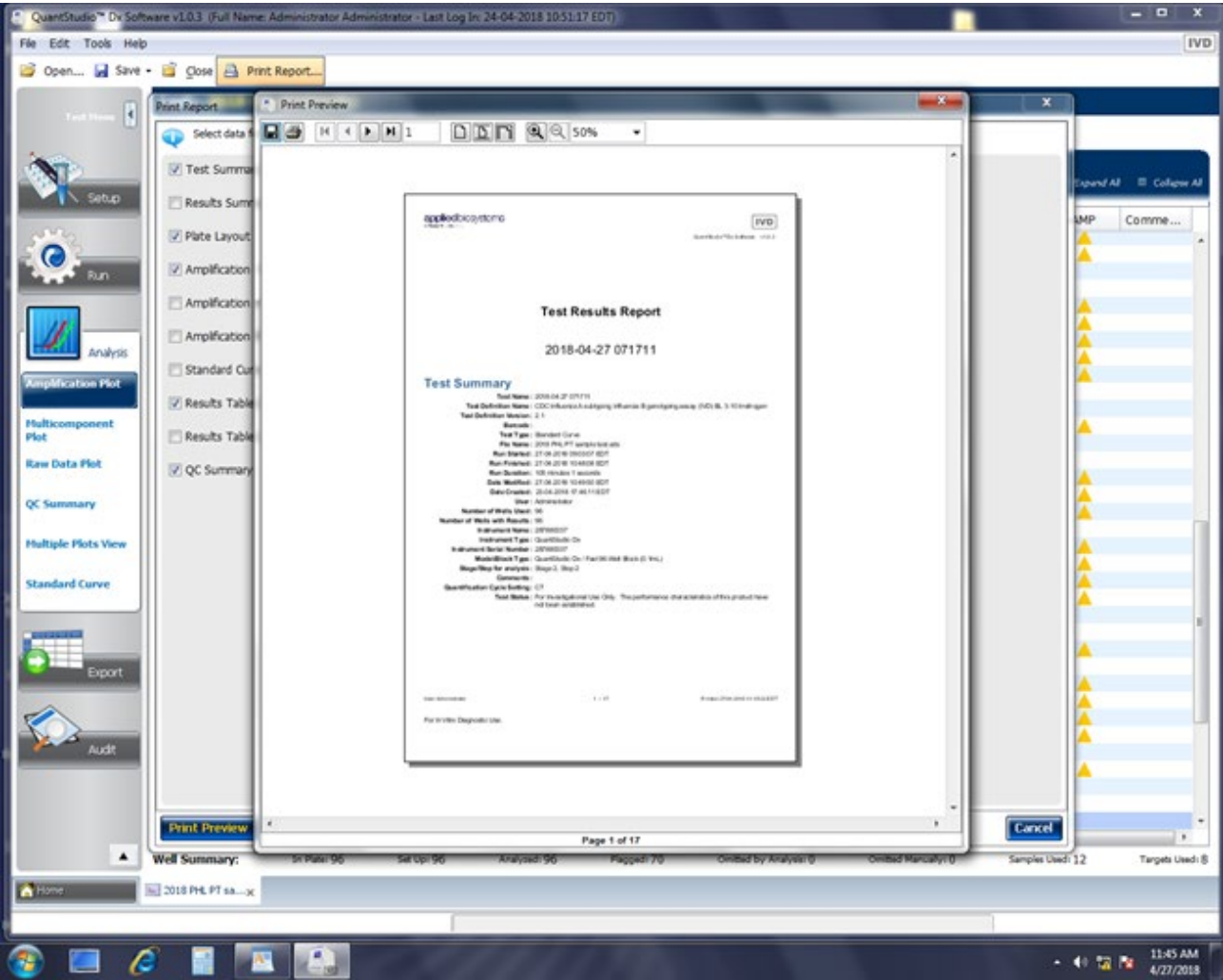
4. Select the **Print Report** icon in the tool bar at the top of the screen (**Figure 14**) to open the **Print Report** window (**Figure 15**).
5. Select **Test Summary**, **Plate Layout**, **Amplification Plot (ΔRn vs. Cycle)**, **Results Table (Custom)**, and **QC Summary**.

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

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 B Lineage Genotyping Kit. However, in the event that specimens need to be re-extracted or if the Influenza B Lineage Genotyping 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. InfB, VIC, YAM, etc.).
2. Determine the number of reactions (N) to be prepared per assay.
3. Calculate the amount of each reagent to be added to the tube for each master mix by multiplying the number of reactions (samples plus controls) per marker by the volume of reagent indicated in **Figure 17**.

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

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

If number of samples (n) including controls > 15, then $N = n + 2$

Figure 17. Steps and Calculations for Master Mix Preparation (Important: Select Appropriate Enzyme System)
Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System

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

OR

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

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 5.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	Quanta qScript™ One-Step Reverse Transcriptase	N x 0.5 µL
4	One-Step Master Mix (2X)	N x 12.5 µL
	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 B Lineage Genotyping Kit: Example of Reaction Master Mix 72-Well Strip Tube Set-up

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

NOTE: If specimens were already tested with the A/B Typing Kit or A Subtyping Kit and produced valid RP results, the specimen will not 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 to 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 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 B Lineage Genotyping 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 already tested with HSC, repeat testing of HSC is not necessary.

Running a Test on the QIAGEN Rotor-Gene Q MDx

If the most current assay profile for the Influenza B Lineage Genotyping 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

QIAGEN Rotor-Gene AssayManager 1.0.3.5

User ID
[text box]

Password
[text box]

Mode
Closed [dropdown arrow]

OK Cancel

- 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

Rotor-Gene AssayManager

File Help

QIAGEN Setup Approval Archive Service Configuration RGQ

Create work list | Select assay profiles and define assay details

Assays Samples Properties

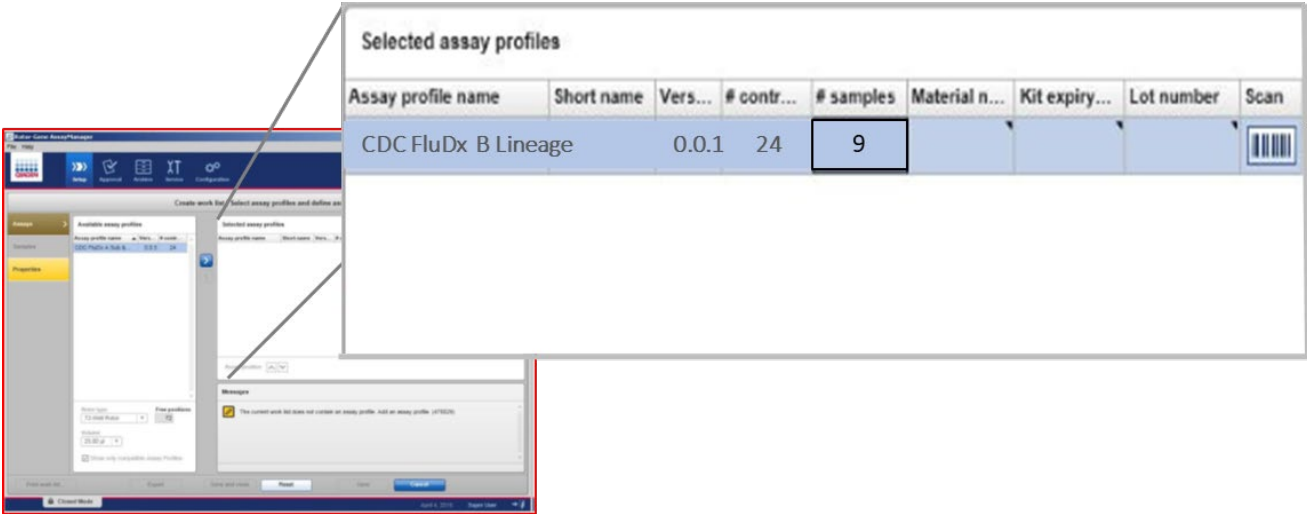
Available assay profiles		
Assay profile name	Vers...	# contr...
CDC FluDxA Subtyping	0.0.1	24

[Blue Arrow Button]

Selected assay profiles				
Assay profile name	Short name	Vers...	# contr...	# samples

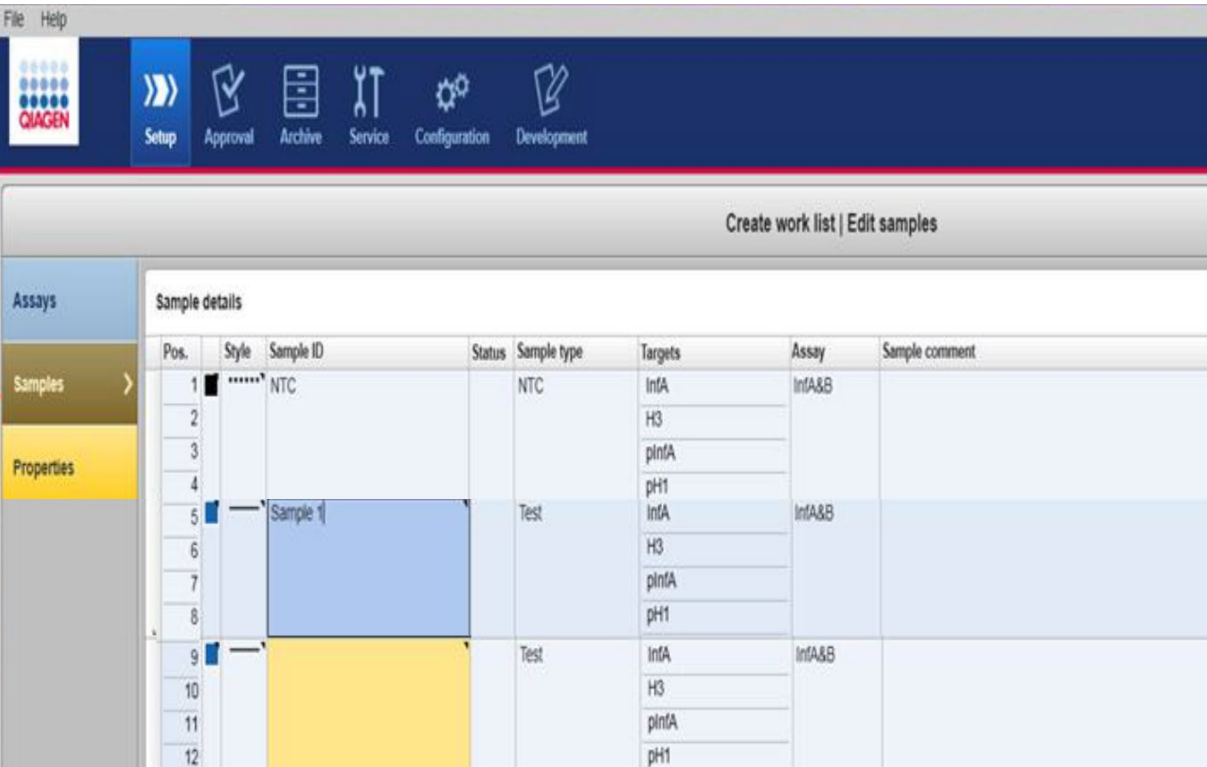
- 7. In the **Selected assay profiles** window enter the number of samples to be tested (do not include controls: NTC and Positive Controls). Rows will change from yellow to blue after the number is entered (Figure 22).

Figure 22: Selected Assay Profiles Window



8. 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



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

Figure 24. Properties – Edit Work List Window

10. 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.
11. Select the **Start Run** button in the bottom right corner of the screen. *Note: The run should take approximately 1hr and 45 minutes to complete.*

Figure 25. Apply Work List Window

File Help

QIAGEN Setup Approval Archive Service Configuration RGQ

Apply work list "A Subtyping_2016061_user"

Summary

Experiment name: A Subtyping_20160610_user
Work list name: A Subtyping_20160610_user
Default name
Created: 6/10/2016 10:05 AM - user
Rotor type: 72-Well Rotor
Last modified: 6/10/2016 10:06 AM - user
Free positions: 42
Reaction volume: 25 µl / tube
Last applied:
Assays:
Name: A Subtyping # samples: 9 Material nu... Kit expiry... Lot number
View sample details...

Cycler selection

Position	Name	Next verification	Cycler status	Select	Ring attached
■ ■ ■ ■	RGQ	---	Loaded	☒	☒
■ ■ ■ ■	---	---	---	☐	☐
■ ■ ■ ■	---	---	---	☐	☐
■ ■ ■ ■	---	---	---	☐	☐

Cycler details

Serial number: 0510236 Optical configuration: Splex HRM

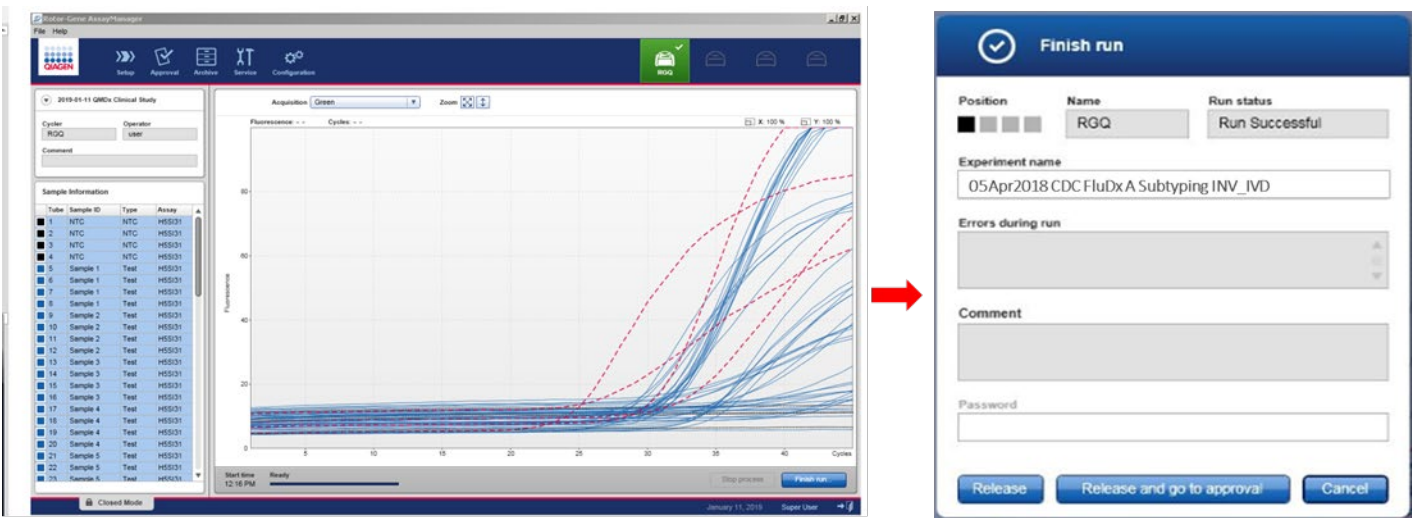
Messages

Print work list... Cancel Start run

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

The screenshot shows the Rotor-Gene AssayManager software interface. The top menu bar includes File, Help, and a toolbar with icons for Setup, Approval, Archive, Service, Configuration, and RGQ. The main window title is "05Apr2018 CDC A Subtyping CDC FluDx A Subtyping". Below the title bar, there are tabs for Plots and information, Raw data, Processed data, Experiment, Assay, and Audit trail. The Experiment tab is selected, showing a form with fields for Run comment, Run operator, Run released by, Experiment name, Run start, End of run, Reaction volume, Rotor type, Run on SW version, Cyclor serial no., External order ID, Work list source, Work list read-only, Created from worklist, Work list last changed by, Work list created on, and Work list last changed on. Below the form is a Results section with a table of results. The table has columns for Pos., Style, Sample ID, Type, Sample comment, Output, Ct, Result, and Flags. The table contains 7 rows of data, all showing "No signal" results. At the bottom of the window, there are buttons for "Create support package...", "Save and close", "Reset", "Save", "Close", and "Release/report data". The status bar at the bottom shows "Closed Mode", "April 5, 2018", and "Super User".

Pos.	Style	Sample ID	Type	Sample comment	Output	Ct	Result	Flags
1	✓	NTC	NTC		InfA	-	No signal	-
2					H3	-	No signal	-
3					pdmInfA	-	No signal	-
4					pdmH1	-	No signal	-
5					InfB	-	No signal	-
6					ViC	-	No signal	-
7					VAM	-	No signal	-

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

Release / report data

Password

Messages

After release, the test results are moved to the archive. (2165157)

OK

Cancel

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

Figure 29. Example Analysis Report

CDC FluDx B Lineage 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 B Lineage 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, 3:08 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, 3:08 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

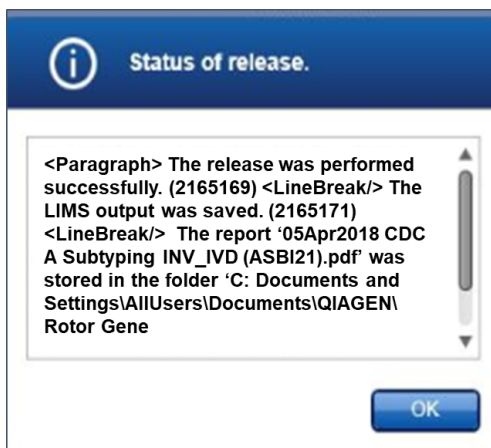
Created by Shannon Emery (she4) on 3/29/2018, 3:13:28 PM -05:00 UTC
QIAGEN Rotor-Gene AssayManager (Version 1.0.4)
File name: Mar29-2018 ABCI15 Operational Qualification R0510236_CDC FluDx A Sub & B Genotyping INV_IUO (ABCI15).pdf

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.

Influenza B Positive Control (IBPC)

- The IBPC consists of two different influenza viruses representing influenza B/Victoria and B/Yamagata lineage suspended in cultured human cells (A549). Purified RNA from the IBPC will yield a positive result with the following primer and probe sets: InfB, VIC, YAM, and RP.

Human Specimen Control (HSC) (Extraction Control)

- When HSC is run with the Influenza B Lineage Genotyping 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 B Lineage Genotyping Kit

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

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

RNase P (Extraction Control)

- All clinical samples should exhibit fluorescence growth curves in the RNase P reaction that cross the threshold line within 38.00 cycles (< 38.00 Ct), thus indicating the presence of the human RNase P gene. Failure to detect RNase P in any clinical specimens may indicate:
 - Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
 - Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
 - Improper assay set up and execution.
 - Reagent or equipment malfunction.
- If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:
 - If the InfB or InfB along with VIC, or YAM 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 (InfB, VIC, and YAM)

- When all controls exhibit the expected performance, a specimen is considered negative if all influenza marker (InfB, VIC, and YAM) cycle threshold growth curves **DO NOT** cross the threshold line within 38.00 cycles (< 38.00 Ct) **AND** the RNase P growth curve **DOES** cross the threshold line within 38.00 cycles (< 38.00 Ct).
- When all controls exhibit the expected performance, a specimen is considered positive for influenza if the influenza marker (InfB, VIC, and YAM) cycle threshold growth curve crosses the threshold line within 38.00 cycles (< 38.00 Ct). The RNase P may or may not be positive as described above, but the influenza result is still valid. When testing tissue culture derived samples, the RNase P result is likely to yield negative / not detected result due to the absence of the human RNase P target.
- When all controls exhibit the expected performance and the growth curves for the influenza markers (InfB, VIC, and YAM) **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 B crosses the threshold line within 38.00 cycles (< 38.00 Ct) without any genotype markers indicating detection (InfB positive without VIC or YAM detected), the extracted RNA should be retested. If the same result is obtained, the laboratory should coordinate transfer of the specimen to CDC for further analysis.

Influenza B Lineage Genotyping Kit Results Interpretation Guide

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

Influenza B Lineage Genotyping Kit

InfB	VIC	YAM	RP	Result Interpretation ^a	Report for CDC Surveillance	Notes and Special Guidance
+	+	-	±	Influenza B Detected; B/Victoria lineage detected	Influenza B Victoria	
+	-	+	±	Influenza B Detected; B/Yamagata lineage detected	Influenza B Yamagata	
-	-	-	+	Influenza B NOT Detected	Not Detected	
-	-	-	-	Inconclusive Result	Inconclusive	Re-extract specimen and test. If results are similar, report inconclusive
+	+	+	±	Influenza B Detected; B/Victoria lineage detected; B/Yamagata lineage detected.	Influenza B Inconclusive lineage	If original result, re-extract and re-test. If result is repeated, specimen should be referred to CDC for further characterization; possible co-infection or LAIV detection
+	-	-	±	Influenza B Detected; Lineage undetectable	Influenza B Inconclusive lineage	If original result, re-extract and/or re-test. If result is repeated, specimen should be referred to CDC for further characterization
-	If either marker is positive while InfB is negative		±	Inconclusive Result	Inconclusive	If original result, re-extract and re-test. If result is repeated, report as 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 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.
- Although HSC is not included with the Influenza B Lineage Genotyping Kit, the HSC extraction control must proceed through nucleic acid isolation per batch of specimens to be tested.
- Always include a negative control (NTC), and the appropriate positive control (IBPC) in each amplification and detection run.

Limitations

- All users, analysts, and any person reporting diagnostic results should be trained to perform this procedure by a competent instructor. They should demonstrate their ability to perform the test and interpret the results prior to performing the assay independently. CDC Influenza Division will limit the distribution of this device to only those users who have successfully completed training provided by CDC instructors or designees. This device is subject to a special control requiring that distribution be limited to laboratories with (i) experienced personnel who has training in standardized molecular testing procedures and expertise in viral diagnosis, and (ii) appropriate biosafety equipment and containment (21 CFR866.3332(b)(2)).
- Negative results do not preclude influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen. Children tend to shed virus more abundantly and for longer periods of time than adults. Therefore, testing specimens from adults will have lower sensitivity than testing specimens from children.
- Positive and negative predictive values are highly dependent on prevalence. False negative test results are more likely during peak activity when prevalence of disease is high. False positive test results are more likely during periods of low influenza activity when prevalence is moderate to low.
- The performance of the assay has not been established in individuals who received nasally administered influenza vaccine. Individuals who received nasally administered influenza vaccine may have positive test results for up to three days after vaccination. <http://www.cdc.gov/mmwr/preview/mmwrhtml/rr57e717a1.htm>
- Do not use any reagent past the expiration date.
- 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 or B infection.
- The performance of this test has not been established for screening of blood or blood product for the presence of influenza A or B.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

Expected values

During January 2017 to April 2017 the World Health Organization and National Respiratory and Enteric Virus Surveillance System (NREVSS) collaborating laboratories in the United States tested 59,279 respiratory specimens for influenza viruses. Of these, 33,743 (57.0%) were positive: 78% of the positive specimens were positive for influenza A viruses and 22% were positive for influenza B viruses. Among the 6,527 influenza B viruses for which the lineage was determined, 1,716 (26.3%) were influenza B/Victoria lineage and 4,811 (73.7%) were B/Yamagata lineage.

Performance Characteristics

Clinical Performance

The performance characteristics of the previously FDA-cleared version of the Influenza B Lineage Genotyping Kit (K132508) were evaluated using upper respiratory tract specimens from a prospective clinical investigation performed at multiple U.S. public health laboratories during the 2011-2012 influenza season. Samples were obtained from 997 upper

respiratory tract specimens collected for routine influenza testing at 6 study sites from individuals who were symptomatic for influenza-like illness (ILI). Specimens were excluded for unspecified type, inconclusive testing result, and technician error. The study population and specimen types are summarized in the table below.

Patient Age	Number of Specimens
0-16	431
17-54	326
≥ 55	215
Not Reported	25
Specimen Type	
NPS	798
NPS/TS	37
NA	13
NW	24
TS	8
NS	113
Not reported	4

Specimens were tested with the InfB and RP primer and probe set from the FDA-cleared CDC Human Influenza Real-Time RT-PCR Diagnostic Panel to establish the presence of influenza B viral RNA and confirm adequate specimen collection. Performance of Influenza B Lineage Genotyping Kit in the 2011-2012 study was compared to bi-directional nucleic acid sequence analysis of the influenza B hemagglutinin gene and the previously FDA-cleared version of the kit.

Prospective Study Results

Influenza B/Victoria Lineage Comparison

Clinical Study	Specimen Type	# of Positives ¹	% Sensitivity (95% CI)	# of Negatives ¹	% Specificity (95% CI)
2011-2012	NA, NW	2/2	100.0 (34.2 – 100.0)	37/37	100.0 (90.6 – 100.0)
	NPS, NS	45/45	100.0 (92.1 – 100.0)	862/863	99.9 (99.3 – 100.0)
	NPS/TS	12/12	100.0 (75.8 – 100.0)	25/25	100.0 (86.7 – 100.0)
	TS	0	NA ²	8/8	100.0 (67.6 – 100.0)

¹Proportion of samples correctly identified versus the comparator.

²NA = not applicable

Influenza B/Yamagata Lineage Comparison

Clinical Study	Specimen Type	# of Positives ¹	% Sensitivity (95% CI)	# of Negatives ¹	% Specificity (95% CI)
2011-2012	NA, NW	0	NA ²	39/39	100.0 (91.0 – 100.0)
	NPS, NS	19/19	100.0 (83.1 – 100.0)	889/889	100.0 (99.6 – 100.0)
	NPS/TS	2/2	100.0 (34.2 – 100.0)	35/35	100.0 (90.1 – 100.0)
	TS	0	NA ²	8/8	100.0 (67.6 – 100.0)

¹Proportion of samples correctly identified versus the comparator.

²NA = not applicable

Retrospective Study Results

The performance characteristics of the genotyping oligonucleotide primer and probe sets of the Influenza B Lineage Genotyping Kit (VER 1.1) were evaluated in a retrospective study using upper respiratory tract specimens collected from previous seasons for routine influenza testing at each site from individuals who were symptomatic for influenza-like illness (ILI). Performance was compared to the previously FDA-cleared version of the Influenza B Lineage Genotyping Kit.

Influenza B Lineage Genotyping Kit (VER 1.1): Influenza B/Victoria Lineage Comparison

Specimen Type	# of Positives ¹	% Positive Agreement (95% CI)	# of Negatives ¹	% Negative Agreement (95% CI)
NPS, NS	29/29	100.0 (88.3 – 100.0)	156/156	100.0 (97.6 – 100.0)
TS	1/1	100.0 (20.7 - 100.0)	1/1	100.0 (20.7 - 100.0)
Total	30/30	100.0 (88.7 – 100.0)	157/157	100.0 (97.6 – 100.0)

¹Proportion of samples correctly identified versus the comparator.

Influenza B Lineage Genotyping Kit (VER 1.1): Influenza B/Yamagata Lineage Comparison

Specimen Type	# of Positives ¹	% Positive Agreement (95% CI)	# of Negatives ¹	% Negative Agreement (95% CI)
NPS, NS	95/95	100.0 (96.1 – 100.0)	90/90	100.0 (95.9 – 100.0)
TS	1/1	100.0 (20.7 - 100.0)	1/1	100.0 (20.7 - 100.0)
Total	96/96	100.0 (96.2 - 100.0)	91/91	100.0 (96.0 – 100.0)

¹Proportion of samples correctly identified versus the comparator.

Analytical Performance

Analytical Sensitivity – Limit of Detection (LOD)

Analytical sensitivity of the Influenza B Lineage Genotyping Kit (VER 1.1) was demonstrated by determining the LOD using Quanta qScript™ and Invitrogen SuperScript™ enzyme kits. Characterized virus of known 50% infectious dose titer (EID₅₀/mL) was extracted and the RNA serially diluted and tested (n= 3 to 5 replicates) in order to determine an apparent endpoint range. The LOD for each primer and probe set was confirmed by testing extraction replicates (n=20) of the highest virus dilution where ≥ 95% of all replicates tested positive. Virus dilutions were prepared in virus transport medium containing human A549 cells to emulate clinical specimen matrix. The lowest concentration where the InfB and VIC or InfB and YAM primer and probe sets had uniform detection was reported as the LOD.

Influenza B Virus Lineage	Virus Designation	LOD (EID ₅₀ /mL)	
		Invitrogen SuperScript™	Quanta qScript™
B/ Victoria	B/Maryland/15/2016	10 ^{1.7}	10 ^{1.7}
B/Yamagata	B/ Texas/81/2016	10 ^{2.2}	10 ^{1.5}

Analytical Reactivity (Inclusivity)

The analytical inclusivity of the Influenza B Lineage Genotyping Kit (VER 1.1) was demonstrated by testing ten influenza B/Victoria lineage and ten influenza B/Yamagata lineage viruses from diverse geographic locations at low virus concentrations at or near the LOD. The primer and probe sets demonstrated 100% concordance with the expected results with all viruses tested.

Analytical Specificity - Exclusivity

Non-Influenza Human Respiratory Pathogens

Analytical specificity (cross-reactivity) was evaluated by testing InfB, VIC and YAM primer/probe sets with nucleic acids extracted from 36 organisms (16 viruses, 19 bacteria, and 1 yeast) representing common respiratory pathogens or flora commonly present in specimens collected from the nasopharynx region using the original FDA-cleared version of the kit (K132508). Bacteria and yeast were tested at concentrations greater than or equal to 10^6 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 TCID₅₀/mL with the exception of human parainfluenza type 2 ($10^{3.1}$ TCID₅₀/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 229E and CoV OC43 and Epstein-Barr virus and concentrations were determined by spectrophotometry. Each test was performed using Invitrogen SuperScript™ and Quanta qScript™ enzyme kits.

Organism Tested			Invitrogen SuperScript™			Quanta qScript™		
Bacteria and Yeast	Strain	cfu / mL	Inf B	VIC	YAM	InfB	VIC	YAM
<i>Bordetella pertussis</i>	A639	10 ^{8.3}	-	-	-	-	-	-
<i>Candida albicans</i>	2001-21-196	10 ^{8.8}	-	-	-	-	-	-
<i>Chlamydia pneumoniae</i> ¹	TW183	40 IFU/mL	-	-	-	-	-	-
<i>Corynebacterium diphtheriae</i>	NA	10 ¹⁰	-	-	-	-	-	-
<i>Escherichia coli</i>	K12	10 ^{9.6}	-	-	-	-	-	-
<i>Haemophilus influenzae</i>	M15709	10 ^{6.4}	-	-	-	-	-	-
<i>Lactobacillus plantarum</i>	NA	10 ^{8.8}	-	-	-	-	-	-
<i>Legionella pneumophila</i>	NA	10 ^{7.1}	-	-	-	-	-	-
<i>Moraxella catarrhalis</i>	M15757	10 ^{9.5}	-	-	-	-	-	-
<i>Mycobacterium tuberculosis</i> ²	H37Rv	95 ng/ µL	-	-	-	-	-	-
<i>Mycoplasma pneumoniae</i>	MI-29	10 ^{7.7}	-	-	-	-	-	-
<i>Neisseria elongata</i>	NA	10 ^{8.6}	-	-	-	-	-	-
<i>Neisseria meningitidis</i>	M2578	10 ^{7.9}	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	NA	10 ^{10.5}	-	-	-	-	-	-
<i>Staphylococcus epidermidis</i>	NA	10 ^{10.5}	-	-	-	-	-	-
<i>Staphylococcus aureus</i>	NA	10 ^{10.7}	-	-	-	-	-	-
<i>Streptococcus pneumoniae</i>	249-06 (Thailand)	10 ^{6.6}	-	-	-	-	-	-
<i>Streptococcus pyogenes</i>	7790-06	10 ^{7.5}	-	-	-	-	-	-
<i>Streptococcus salivarius</i>	SS1672	10 ^{8.4}	-	-	-	-	-	-
Viruses	Strain	EID ₅₀ /mL or TCID ₅₀ /mL	Inf B	VIC	YAM	InfB	VIC	YAM
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 ²	OC43	50.4 ng /µL	-	-	-	-	-	-
Human Coronavirus virus ²	299E	31.6 ng /µL	-	-	-	-	-	-
Human Rhinovirus A	1A	10 ^{5.8}	-	-	-	-	-	-
Human Parainfluenza 1 virus ²	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	10 ^{8.4}	-	-	-	-	-	-
Varicella-zoster Virus	AV92-3	10 ^{4.4}	-	-	-	-	-	-
Epstein Barr Virus ²	B95-8	1.7 ng/µL	-	-	-	-	-	-
Measles Virus	Edmonston	10 ^{5.2}	-	-	-	-	-	-
Mumps Virus	Enders	10 ^{7.2}	-	-	-	-	-	-
Cytomegalovirus	AD-169	10 ^{6.9}	-	-	-	-	-	-

¹ Organism quantified by Infectious Forming Units (IFU)

² Organism quantified by spectrophotometry (ng/µL)

The study demonstrated that the primer and probe sets contained within the Influenza B Lineage Genotyping Kit did not cross-react with any of the non-influenza respiratory pathogens or commensal organisms and demonstrated 100% concordance with the expected results. The Influenza B Lineage Genotyping Kit (VER 1.1) is expected to maintain the same analytical specificity with regard to non-influenza respiratory pathogens or commensal organisms as the original

FDA-cleared version of the kit, since the primer/probe designs of the original version and VER 1.1 of the kit are essentially identical.

Exclusivity with Influenza Viruses with Pandemic Potential

The Influenza B Lineage Genotyping Kit (VER 1.1) was evaluated for cross-reactivity with various influenza A viruses of subtypes that circulate in humans or from animal origin that infect humans. Each test was performed using Invitrogen SuperScript™ and Quanta qScript™ enzyme kits.

Origin Species	Virus Designation	Subtype	EID ₅₀ /mL or TCID ₅₀ /mL	Invitrogen SuperScript™		Quanta qScript™	
				VIC	YAM	VIC	YAM
Human	A/Michigan/45/2015	A(H1N1)pdm09	10 ^{8.3}	-	-	-	-
Human	A/Hong Kong/4801/2014	A(H3N2)	10 ^{7.9}	-	-	-	-
Swine	A/Ohio/35/2017	A(H1N2)v	10 ^{6.9}	-	-	-	-
Swine	A/Ohio/13/2017	A(H3N2)v	10 ^{6.9}	-	-	-	-
Avian	A/gyrfalcon/Washington/41088-6/2014	A(H5N8)	10 ^{9.75}	-	-	-	-
Avian	A/Northern pintail/Washington/40964/2014	A(H5N2)	10 ^{9.4}	-	-	-	-
Avian	A/Bangladesh/0994/2011	A(H9N2)	10 ^{10.5}	-	-	-	-
Avian	A/Anhui/1/2013	A(H7N9)	10 ^{10.9}	-	-	-	-

To demonstrate exclusivity of the Influenza B Lineage Genotyping Kit (VER 1.1) to detect only influenza B viruses of the corresponding lineage, 10 influenza B/Victoria and 10 B/Yamagata lineage viruses were tested at high titer with the opposite primer and probe set. Each test was performed using Invitrogen SuperScript™ and Quanta qScript™ enzyme kits.

Virus Designation	Lineage	EID ₅₀ /mL or TCID ₅₀ /mL	Invitrogen SuperScript™		Quanta qScript™	
			VIC	YAM	VIC	YAM
B/Hong Kong/259/2010	B/Vic	10 ^{8.2}	3/3*	0/3	3/3	0/3
B/Bolivia/1526/2010		10 ^{8.4}	3/3	0/3	3/3	0/3
B/Laos/89/2011		10 ^{6.2}	3/3	0/3	3/3	0/3
B/Michigan/09/2011		10 ^{8.5}	3/3	0/3	3/3	0/3
B/New Jersey/01/2012		10 ^{8.9}	3/3	0/3	3/3	0/3
B/Montana/05/2012		10 ^{8.4}	3/3	0/3	3/3	0/3
B/Texas/02/2013		10 ^{9.1}	3/3	0/3	3/3	0/3
B/Florida/103/2016		10 ^{6.3}	3/3	0/3	3/3	0/3
B/Florida/76/2016		10 ^{6.5}	3/3	0/3	3/3	0/3
B/Hong Kong/269/2017		10 ^{4.2}	3/3	0/3	3/3	0/3

*Indicates the number of positive replicates out of three

Virus Designation	Lineage	EID ₅₀ /mL or TCID ₅₀ /mL	Invitrogen SuperScript™		Quanta qScript™	
			VIC	YAM	VIC	YAM
B/Brisbane/03/2007	B/Yam	10 ^{8.4}	0/3*	3/3	0/3	3/3
B/Pennsylvania/07/2007		10 ^{8.2}	0/3	3/3	0/3	3/3
B/Hubei-Wujiagang/158/2009		10 ^{8.2}	0/3	3/3	0/3	3/3
B/Wisconsin/01/2010		10 ^{8.9}	0/3	3/3	0/3	3/3
B/Finland/39/2010		10 ^{8.1}	0/3	3/3	0/3	3/3
B/Estonia/55669/2011		10 ^{7.8}	0/3	3/3	0/3	3/3
B/Taiwan/1242/2011		10 ^{9.9}	0/3	3/3	0/3	3/3
B/Massachusetts/02/2012		10 ^{9.2}	0/3	3/3	0/3	3/3
B/Phuket/3073/2013		10 ^{9.9}	0/3	3/3	0/3	3/3
B/Guangdong-Liwan/1133/2014		10 ^{9.9}	0/3	3/3	0/3	3/3

*Indicates the number of positive replicates out of three

Influenza B Lineage Genotyping Kit Reproducibility/Precision

An internal precision study was performed with the Influenza B Lineage Genotyping Kit primer and probe sets using the original FDA-cleared version of the kit (K132508). Three samples of each influenza B lineage were tested by two operators who individually performed two separate runs per testing day over 12 testing days. This design generated 96 results per panel member. The samples used in this study were:

- **Sample #1** Influenza B/Victoria, moderate viral RNA titer range
- **Sample #2** Influenza B/Victoria, low viral RNA titer range
- **Sample #3** Influenza B/Victoria, “high negative” RNA titer range
- **Sample #4** Influenza B/Yamagata, moderate viral RNA titer range
- **Sample #5** Influenza B/Yamagata, low viral RNA titer range
- **Sample #6** Influenza B/Yamagata, “high negative” RNA titer range

Panel Sample	Primer/ Probe Set	Analyst 1			Analyst 2			Agreement Total	95%CI
		Agreement	Ave. Ct	%CV	Agreement	Ave. Ct	%CV		
B/Victoria Moderate	InfB	48/48	23.86	4.3	48/48	23.96	5.0	96/96	100.0 (96.2 – 100.0)
	VIC	48/48	26.36	3.7	48/48	25.98	5.9	96/96	100.0 (96.2 – 100.0)
B/Victoria Low*	InfB	46/48	33.28	4.7	45/48	33.68	6.0	91/96	94.8 (88.4 – 97.8)
	VIC	31/48	37.32	4.5	38/48	36.60	5.0	69/96	71.9 (62.2 – 79.9)
B/Victoria High Negative	InfB	47/48	N/A	N/A	44/48	N/A	N/A	91/96	94.8 (88.4 – 97.8)
	VIC	48/48	N/A	N/A	48/48	N/A	N/A	96/96	100.0 (96.2 – 100.0)
B/Yamagata Moderate	InfB	48/48	26.01	4.0	48/48	25.70	4.4	96/96	100.0 (96.2 – 100.0)
	YAM	48/48	26.70	4.2	48/48	26.14	4.5	96/96	100.0 (96.2 – 100.0)
B/Yamagata Low	InfB	46/48	32.14	3.6	48/48	32.46	3.2	94/96	97.9 (92.7 – 99.4)
	YAM	46/48	32.72	4.0	48/48	32.21	3.9	94/96	97.9 (92.7 – 99.4)
B/Yamagata High Negative	InfB	48/48	N/A	N/A	48/48	N/A	N/A	96/96	100.0 (96.2 – 100.0)
	YAM	47/48	N/A	N/A	48/48	N/A	N/A	95/96	99.0 (94.3 – 99.8)

N/A=not applicable

* Using information from the LoD range finding study, this low positive precision study sample was prepared using a concentration lower than the reported LoD in order to target a concentration as close to the C₉₅ as possible. However, since the assay is very close

to its analytical endpoint at this concentration, several replicates between both operators produced no measureable fluorescence above background or measurements above the cutoff.

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 the QIAGEN Rotor-Gene Q MDx instruments performed equivalently to the Applied Biosystems™ 7500 FastDx with 100% positive and 100% negative agreement and a 95% confidence interval of 92.9-100.0.

Carryover and Cross-contamination

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

Analytical sensitivity

The limit of detection equivalence between the FDA-cleared Applied Biosystems™ 7500 FastDx and either the Applied Biosystems™ QuantStudio™ Dx or the QIAGEN Rotor-Gene Q MDx instrument was examined. Each primer and probe set of the CDC Human Influenza Virus Real-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)
sample 2 Moderate B/Victoria	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)
	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)
sample 3 Negative	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)
	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)
sample 4 Low A/H3N2	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)
	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)
sample 5 Low B/Victoria	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)
	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)
sample 5 Low B/Victoria	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

Disposal

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

References

1. Ballew, H. C., *et al.* "Basic Laboratory Methods in Virology," DHHS, Public Health Service 1975 (Revised 1981), Centers for Disease Control and Prevention, Atlanta, Georgia 30333.
2. Clinical Laboratory Standards Institute (CLSI), "Collection, Transport, Preparation and Storage of Specimens for Molecular Methods: Approved Guideline," MM13-A, Vol. 25, No. 31.
3. Clinical Laboratory Standards Institute (CLSI), "User Protocol for Evaluation of Qualitative Test Performance," Guideline EP-12A.
4. Clinical Laboratory Standards Institute (CLSI), "Genotyping for Infectious Disease: Identification and Characterization; Approved Guideline," MM10-A, Vol. 26, No. 9.
5. Lieber, M., *et al.* "A Continuous Tumor Cell Line from a Human Lung Carcinoma with Properties of Type II Alveolar Epithelial Cells." *International Journal of Cancer* 1976, 17(1), 62-70.
6. Szretter, K. J., *et al.* "Influenza: Propagation, Quantification, and Storage" in *Current Protocols in Microbiology* 2006 15G.1.1-15G.1.22; Editors: R. Coica, T. Kowalik, J. M. Quarles, B. Stevenson, R. K. Taylor, A. E. Simon and T. Downey; John Wiley & Sons, Inc. Hoboken, NJ.
7. White L. A. "Standard Operating Procedure BPP/041 (Innocuity Testing of Viral Reagents)," DHHS, Public Health Service 1982, Centers for Disease Control and Prevention, Atlanta, Georgia 30333.

Contact Information, Ordering, and Product Support

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

For Ordering contact the International Reagent Resource:

Website: InternationalReagentResource.org

Email: contact@internationalreagentresource.org

For technical support contact CDC:

Send email to: FluSupport@cdc.gov

[Appendix A: Instrument Setup for Applied Biosystems™ 7500 Fast Dx Real-time PCR Instrument](#)

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

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

Figure 1. New Document Wizard Window

New Document Wizard

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

Assay: Standard Curve (Absolute Quantitation)

Container: 96-Well Clear

Template: Blank Document

Run Mode: Standard 7500

Operator: Training User

Comments: SDS v1.4

Plate Name: Training Plate

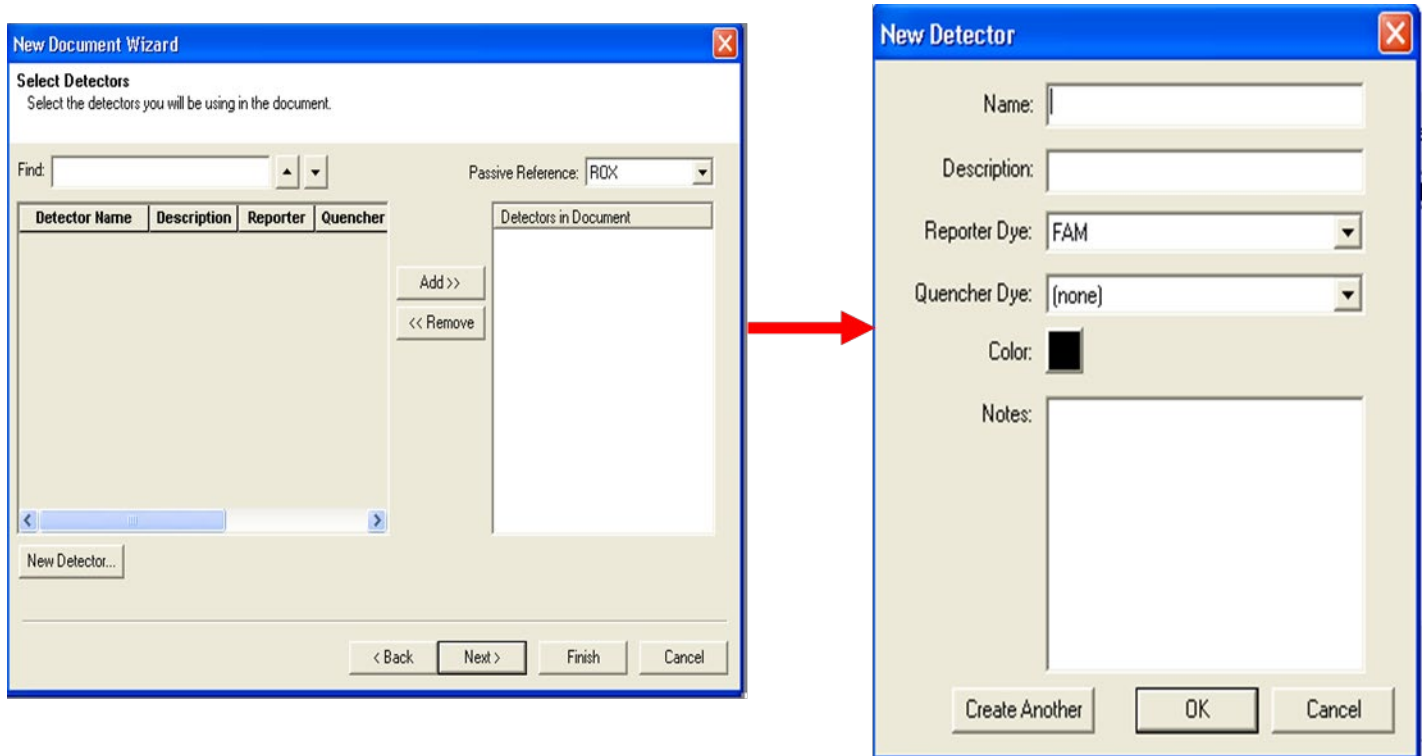
< Back **Next >** Finish Cancel

Make sure to change Run Mode to: **STANDARD 7500**

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

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

Figure 2. Creating New Detectors



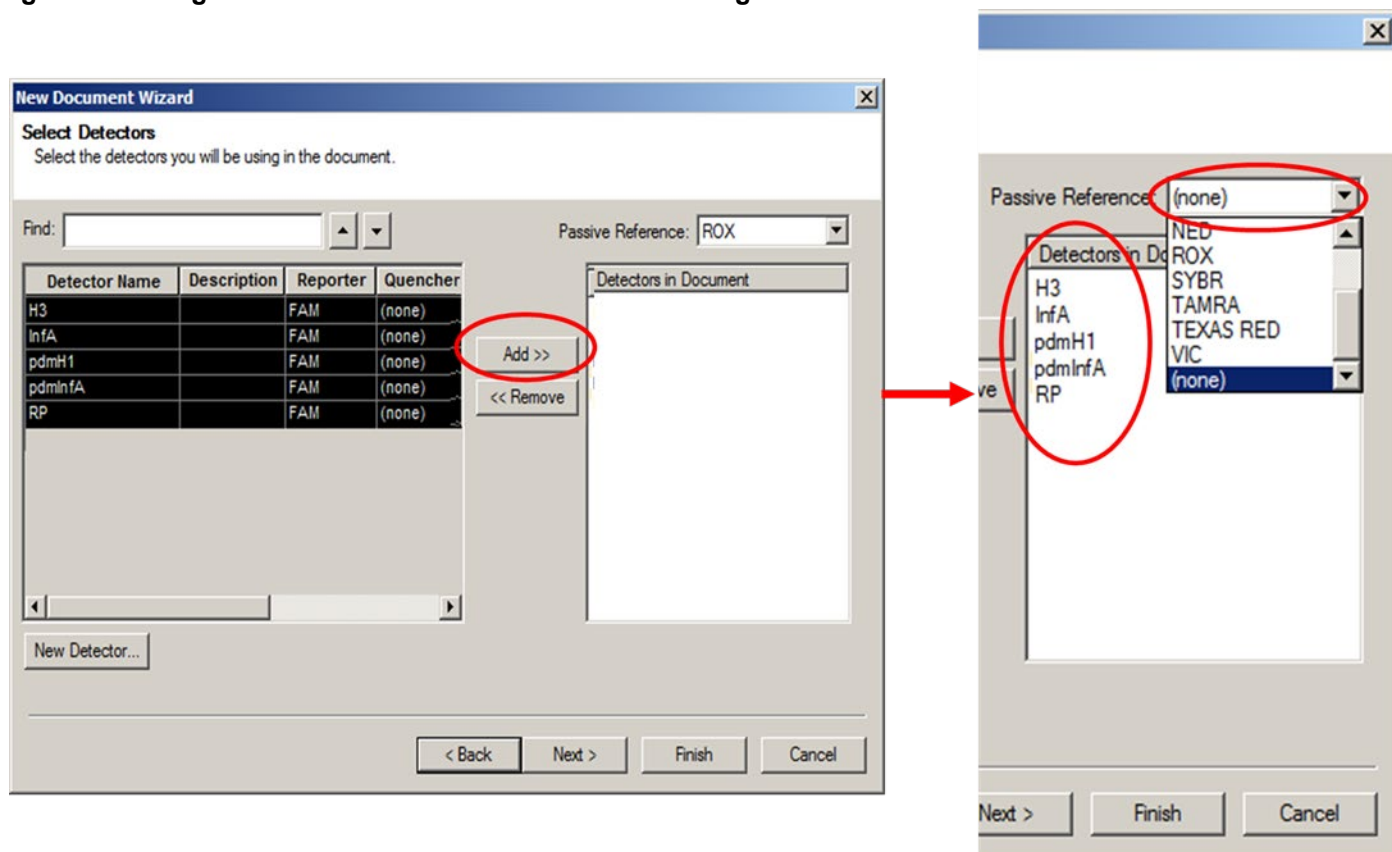
8. Start by creating the InfB Detector (**Figure 2**, left image). Include the following:
- Name: **InfB**
 - Description: *leave blank*
 - Reporter Dye: **FAM**
 - Quencher Dye: **(none)**
 - Color: *to change the color of the detector indicator do the following:*
 - ⇒ Click on the color square to reveal 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
 - Click the **OK** button of the **New Detector** screen to return to the screen shown in the left image in **Figure 2**.
9. Repeat step 6-8 for each influenza target in the panel.

Name	Reporter Dye	Quencher Dye
InfB	FAM	(none)
VIC	FAM	(none)
YAM	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 3**).

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

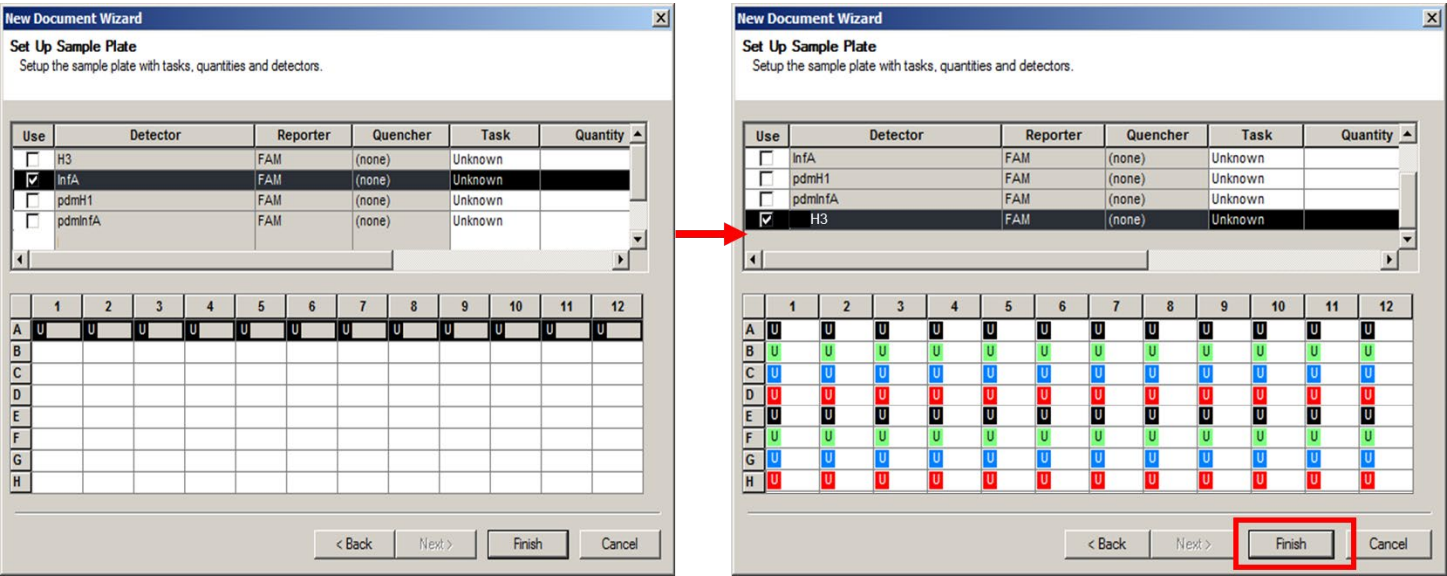
Figure 3. Adding New Detectors to Document and Selecting Passive Reference



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

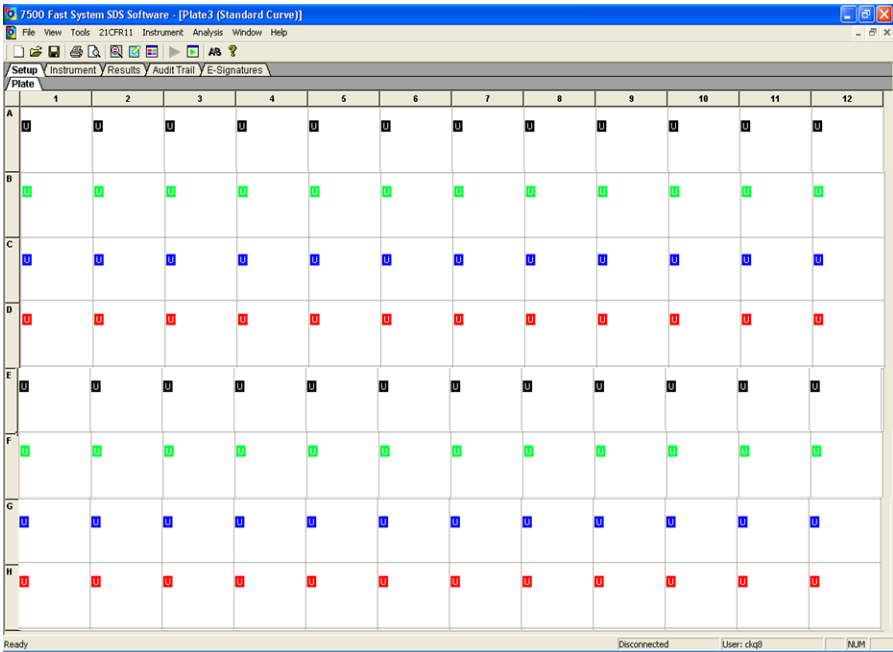
13. Click **Next** at the bottom of the **Select Detectors** window (Figure 3) to proceed to the **Set Up Sample Plate** window (Figure 4).
14. In the **Set Up Sample Plate** window (Figure 4), select row **A** from the in the spreadsheet in the lower portion of the window.
15. In the top portion of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

Figure 4. Sample Plate Set-up



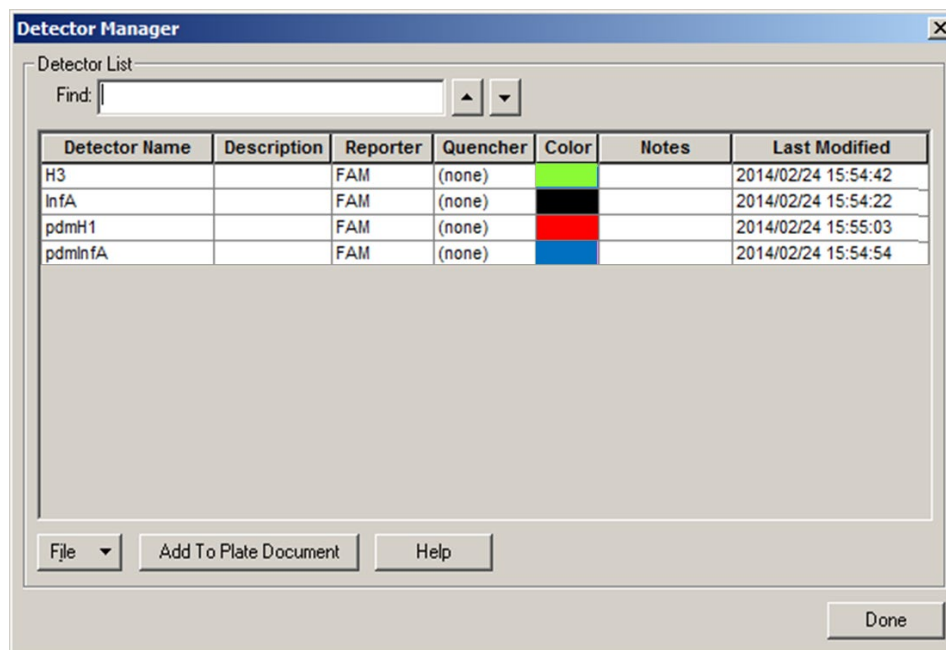
18. After clicking **Finish**, there will be a brief pause allowing the 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. **Note: The machine must be turned on for initialization.**
19. After initialization, the **Plate** tab of the **Setup** (Figure 5) will appear.
20.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

Figure 5. Plate Set-up Window



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

Figure 6. Detector Manager Window



22. Confirm all influenza detectors are included and that each influenza target has a **Reporter** set to **FAM** and the **Quencher** is set to **(none)**.
23. If all detectors are present, select **Done**. The detector information has been created and assigned to wells on the plate.

Defining the Instrument Settings

(Important: Use Appropriate Settings for Invitrogen SuperScript™ III or Quanta qScript™ Enzyme)

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

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

- a. In Stage 1, Set to **30 min at 50°C; 1 Rep.**
- b. In Stage 2, Set to **2.0 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 7**).

OR

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

- a. In Stage 1, Set to **30 min at 50°C; 1 Rep.**
- b. In Stage 2, Set to **5.0 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 7**).

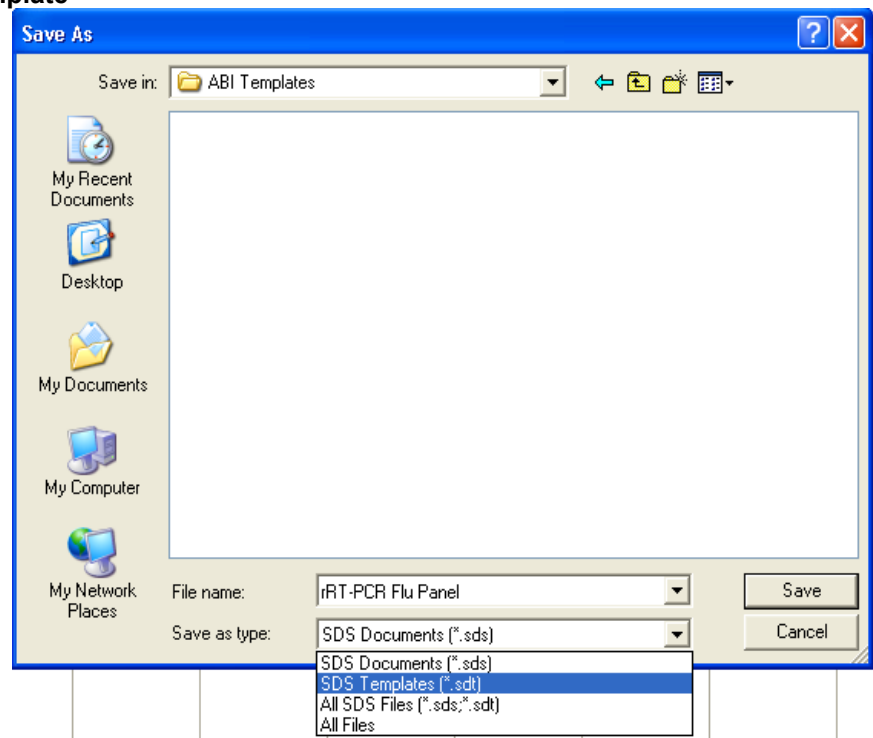
Figure 7. Instrument Window

The screenshot displays the 'Instrument' tab of a software interface. At the top, there are tabs for 'Setup', 'Instrument', 'Results', 'Audit Trail', and 'E-Signatures'. The 'Instrument' tab is active, showing 'Instrument Control' buttons (Start, Stop, Disconnect, Extend...) and 'Estimated Time Remaining (hh:mm):'. To the right, there are fields for 'Temperature' (Sample, Cover, Heat Sink, Block) and 'Cycle' (Stage, Rep, Time (mm:ss), State). Below this is the 'Thermal Cycler Protocol' section, which includes a 'Thermal Profile' graph and a table of stages. The graph shows a temperature profile starting at 50.0°C, ramping to 95.0°C, holding for 2:00, ramping to 95.0°C, holding for 0:15, ramping to 55.0°C, and holding for 0:30. The 'Stage 3' section is highlighted in yellow. Below the graph are buttons for 'Add Cycle', 'Add Hold', 'Add Step', 'Add Dissociation Stage', 'Delete', and 'Help'. At the bottom, there is a 'Settings' section with fields for 'Sample Volume (µL):' (25), 'Run Mode' (Standard 7500), and 'Data Collection:' (Stage 3, Step 2 (55.0 @ 0:30)).

Thermal Profile	Auto Increment	Ramp Rate
Stage 1	Stage 2	Stage 3
Reps: 1	Reps: 1	Reps: 45
50.0	95.0	95.0
30:00	2:00	0:15
		55.0
		0:30

4. After making changes to the **Instrument** tab, the template file is ready to be saved. To save the template, select **File** from the top menu, then select **Save As**. Since the two enzyme options have different instrument settings it is recommended that the template be saved with a name indicating the enzyme option.
5. Save the template as **Influenza B Lineage Genotyping Kit SuperScript** or **Influenza B Lineage Genotyping Kit qScript** as appropriate in the desktop folder labeled "**ABI Run Templates**" (*user must create this folder*). Save as type should be SDS Templates (*.sdt) (**Figure 8**).

Figure 8. Saving Template

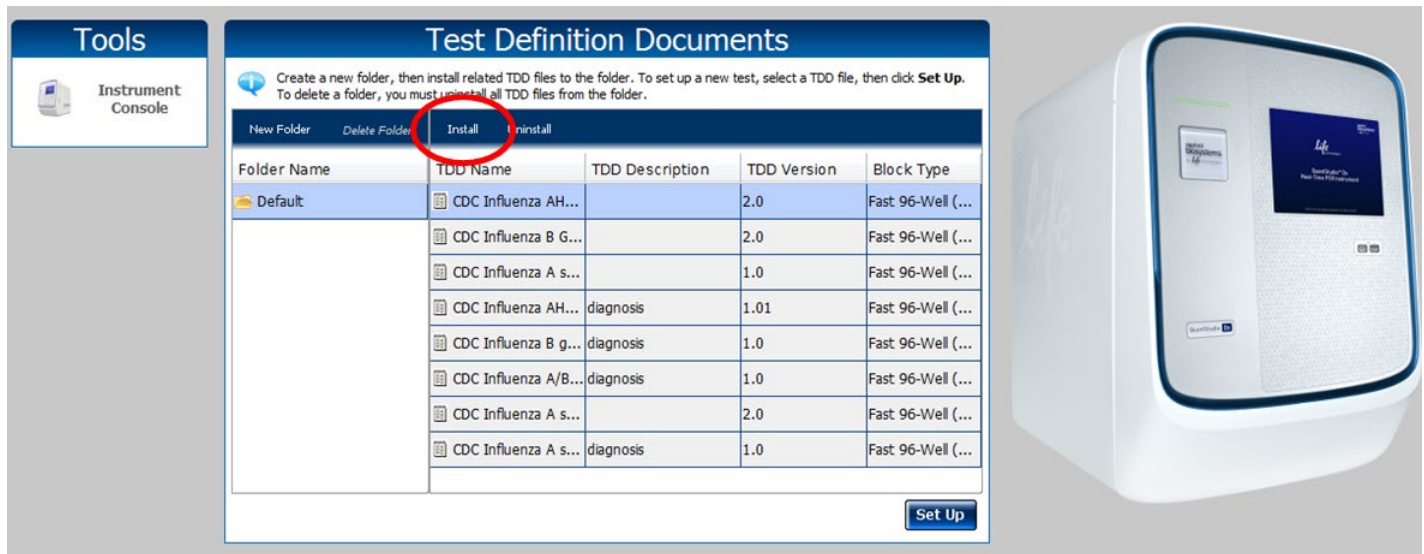


[Appendix B: Instrument Setup for Applied Biosystems™ QuantStudio™ Dx \(QSDx\)](#)

This section describes how to install a test definition document (.tdd) for the Influenza B Lineage Genotyping Kit onto the QuantStudio™ Dx. This .tdd file will be saved and used for all Influenza B Lineage Genotyping 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 B Lineage Genotyping Kit for use with the QuantStudio™ Dx Real-time PCR system and save to a designated folder.
2. Launch the QuantStudio™ Dx Real-time PCR System by double clicking on the QuantStudio™ Dx software icon on the desktop.
3. Enter the default username and password into the **Log In** pop-up window and select **Log In**.
4. The **Show Security Events** window will open. Select **Close**. The **Test Definition Document** window (**Figure 1**) will automatically open.

Figure 1. Installing New Tests



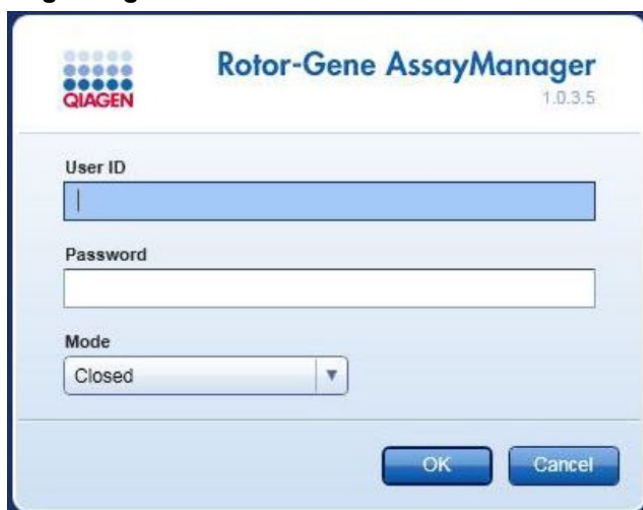
5. In the **Test Definition Document** window select **Install** to display the **Open** window.
6. Navigate to the folder containing the test definition document files (.tdd).
7. Select the appropriate Influenza B Lineage Genotyping Kit file (.tdd) and click **Open**. The file name will now be displayed 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 B Lineage Genotyping 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 B Lineage Genotyping Kit to the Rotor-Gene Q MDx. This assay profile will be saved and used for all Influenza B Lineage Genotyping Kit testing on this instrument.

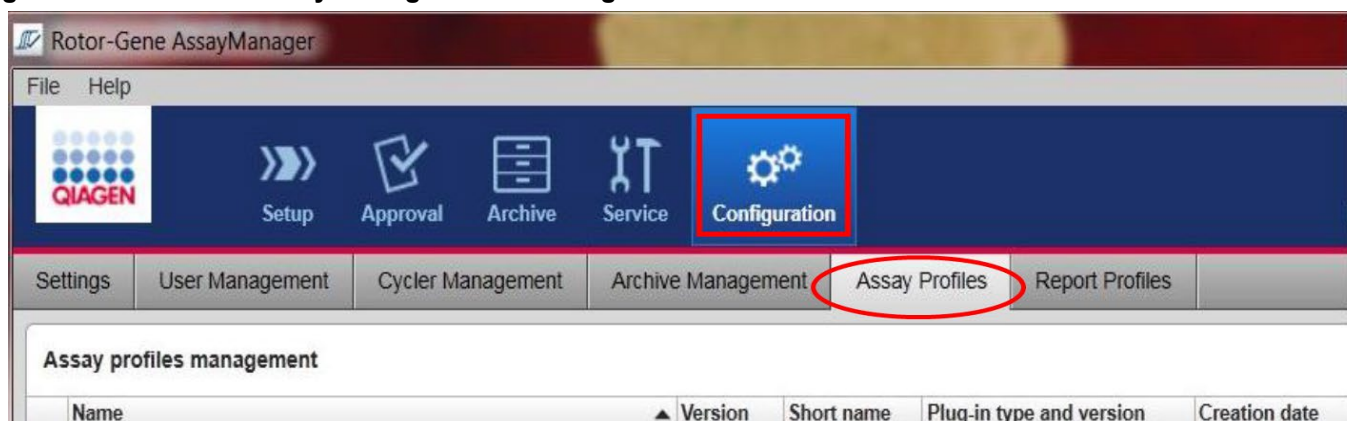
1. Go to the FluSupport Sharepoint site at <https://cdcpartners.sharepoint.com/sites/NCIRD/FS/default.aspx> and download the appropriate assay profile for the Influenza B Lineage Genotyping Kit for use with the QIAGEN Rotor-Gene Q MDx instrument and save to a designated folder.
2. Launch the QIAGEN Rotor-Gene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

Figure 1. Rotor-Gene AssayManager Login Window

The image shows the login window for Rotor-Gene AssayManager. It has a light blue background with the QIAGEN logo in the top left and the text "Rotor-Gene AssayManager 1.0.3.5" in the top right. There are three input fields: "User ID" (a blue bar), "Password" (a white bar), and "Mode" (a dropdown menu currently showing "Closed"). At the bottom right are "OK" and "Cancel" buttons.

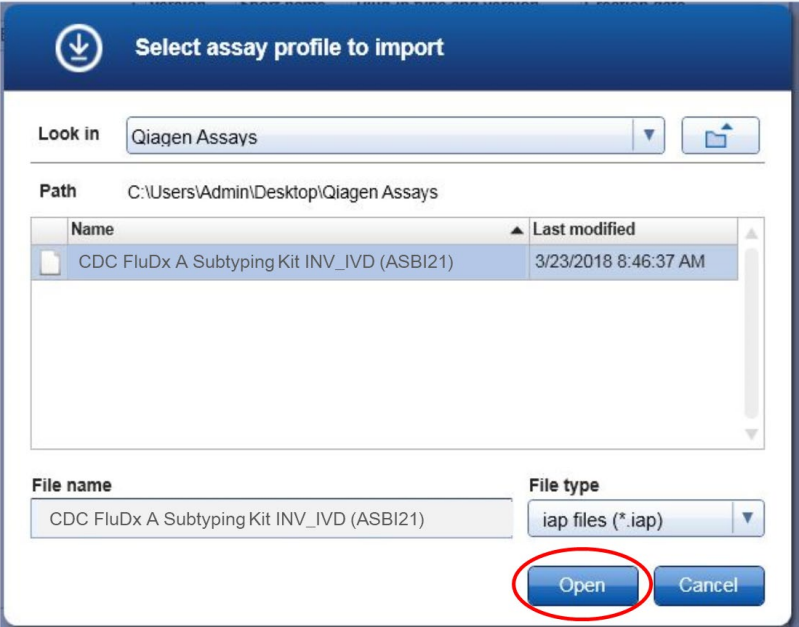
5. Select **OK** and the Rotor-Gene AssayManager main navigation window (**Figure 2**) will open.
6. Select the **Configuration** tab from the top menu, then select the **Assay Profiles** tab (**Figure 2**).

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



7. Click the **Import** button at the bottom right of the **Assay Profiles** tab to display the **Select assay profile to import** window (**Figure 3**).
8. Navigate to the folder that contains the assay profiles using the **Look in** drop down menu.
9. Select the appropriate Influenza B Lineage Genotyping Kit assay profile (.iap) file and click **Open**.

Figure 3. Select Assay Profile to Import Window



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

Figure 4. Import Successful Window and Assay Manager Configuration Screen

