TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0

Protocol

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For The TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0 (PN 4384261, 4384262, 4384263, 4384264)

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TRADEMARKS:

Part Number 4383777 Rev. B
06/2010

TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0

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This product is designed to test only for the presence of the influenza virus type and subtype(s) named on the product label. It has been verified by third party research institutions to detect a nucleic acid sequence that has been identified as present in the nucleic acid of the designated type and subtype(s) as listed in the nucleic acid sequence database maintained by the National Center for Biotechnology Information (NCBI) of the United States National Institutes of Health. This product has also been tested against certain other respiratory pathogens for selectivity. However, it has not been tested against all influenza types or subtype(s), viruses or other organisms. In addition to inherent limitations on nucleic acid sequence selection, validation, verification and testing, many other factors can affect detection efficacy and test results, including sample preparation, laboratory practices, equipment use and maintenance, reagent storage or loading, and operating and environmental conditions. Applied Biosystems cannot and does not represent, warrant or otherwise guarantee (i) that this product will always detect the presence of the designated influenza virus type and subtype(s) or the nucleic acid sequence it is designed to detect, even if the sample tested contains such type, subtype(s) or sequence, (ii) that the nucleic acid sequence that this product is designed to detect is not present in other influenza virus type or subtype(s) or organisms, or (iii) that it will not indicate a positive finding when tested against other type or subtype(s) of the influenza virus or other organisms. This product should not be used as the sole basis for determining the presence or absence of the designated influenza virus type or subtype(s). The user should always confirm and validate the presence or absence of the designated type

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Contents

Preface

This preface contains:

Safety

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word–**IMPORTANT, CAUTION, WARNING, DANGER**–implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD**. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" below.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **www.appliedbiosystems.com**, click the link for **Support**, then click the link for **MSDS Search**.
2. In the Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Chemical Waste Hazard

CHEMICAL WASTE HAZARD. Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety

WARNING **BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities utilizing the appropriate safety equipment (for example, physical containment devices). Individuals should be trained in accordance with applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and adhere to the following guidelines and/or regulatory requirements as applicable:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company/institution's Biosafety Program protocols for working/handling potentially infectious materials.

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

How to Obtain More Information

Related Documentation

See the following related documents for more information on the topics in this guide:

- User's Guide for your Applied Biosystems Sequence Detection System (SDS) or Real-Time PCR System

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documentation. You can e-mail your comments to:

techpubs@appliedbiosystems.com

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Obtain worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

Product Overview

The four TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0 are each comprised of a Real-Time PCR assay that detects one of the following: Influenza A, the H5 subtype of Influenza A, the H7 subtype of Influenza A, or the N1 subtype of Influenza A. Each kit is sufficient for 96 reactions.

The TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0 reactions use:

- Reverse transcription to convert viral RNA to cDNA.
- Polymerase chain reaction (PCR) to amplify the viral target.
- TaqMan® probes to detect the presence of a specific influenza strain.
- An Internal Positive Control (IPC) to check for the presence of PCR-inhibitors.
- One of the following Applied Biosystems instruments to perform the PCR and detect the probe cleavage:
  - ABI PRISM® 7000 Sequence Detection System
  - Applied Biosystems 7500 Fast Real-Time PCR System
  - Applied Biosystems 7900HT Fast Real-Time PCR System (using a standard block)
  - Applied Biosystems 7300 or 7500 Real-Time PCR System

Sequence submissions to NCBI beginning in 2001 were used as the basis of the design for the TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0. Bioinformatics analysis predicted that the kits will detect most Influenza A and Influenza A subtype H5, H7, and N1 isolates for which sequence data exists. See "Appendix B: Specificity and Limit of Detection" on page 33 for further discussion of predicted and demonstrated specificity.

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Chemistry Overview

Reaction Components

Reaction components of each TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0 are:

- Assay Beads (Influenza A, Influenza A subtype H5, Influenza A subtype H7, or Influenza A subtype N1) containing dNTPs, DNA polymerase, primers, TaqMan® probes, and other components for reverse transcription and PCR.
- 10× Reconstitution Buffer containing components for reverse transcription and PCR.
- Sample:
  - RNA, which you supply, isolated from environmental or epidemiological samples
  - RNA positive control
  - Negative control

Each kit provides reagents for 96 reactions.

About Negative and Positive Controls

It is good practice to include PCR-specific and process-specific controls in your experiment. A negative PCR control (PN 4340451), in the form of a nuclease-free water is provided in the kit. A negative process control is obtained by running a reaction without the addition of any sample at any step in the process. Positive PCR controls (PN 4383940), for Influenza A and the H5 subtype of Influenza A in one tube and the H7 and N1 subtypes of Influenza A in a second tube, are provided in the kit. A positive process control is obtained by running sample material similar to the real samples to be assayed, for example, blood, other fluids, or cultures.

- Negative Controls:
  - Run at least two PCR controls to confirm that kit reagents do not contain any components that amplify.
  - Run a process control to confirm that your process does not contain any components that amplify.
- Positive Controls:
  - Run a PCR control to confirm that the kit reagents amplify the expected target.

– Run a process control to test the entire process from sample preparation to detection to ensure that your process can generate a positive result.

IMPORTANT! When you run positive controls, cover sample and negative control wells before pipetting positive controls to avoid cross-contamination of samples and negative controls.

Internal Positive Control (IPC)

Applied Biosystems includes an IPC in the assay beads. A positive IPC signal:

- Demonstrates that PCR reagents amplify as expected.
- Allows accurate interpretation of negative sample results.

UNG Contamination Control System (Optional)

AmpErase® UNG is not included in the assay beads. AmpErase UNG treatment can be added in this protocol because dUTP is substituted for dTTP in the assay beads. AmpErase UNG minimizes or eliminates the reamplification of carryover PCR products by:

- Substituting dUTP for dTTP in the assay beads.
- Treating the mix with the enzyme uracil N-glycosylase (UNG, EC 3.2.2) prior to amplification (Longo et al., 1990).

The substitution of dTTP with dUTP as a dNTP substrate in PCR and the inclusion of UNG can result in the removal of up to 200,000 copies of a previously amplified product per 30 µL reaction.

For information on integrating optional AmpErase UNG treatment with the kit, refer to "Appendix C: Preparing RT-PCR with UNG" on page 37.

RT-PCR

The TaqMan® Influenza A and Influenza A subtype H5, H7, and N1 reactions use a one-step reverse transcription-polymerase chain reaction (RT-PCR) format in a single-tube, single-enzyme system. The DNA polymerase included in the assay beads functions as both a thermoreactive reverse transcriptase and a thermostable DNA polymerase and provides the 5' to 3' nuclease activity necessary for the cleavage of the fluorogenic probe.

During RT-PCR (Figure 1 on page 5):

1. RNA is reverse-transcribed to cDNA.
2. PCR cycles are performed:
   a. Primers anneal to the cDNA target sequence.

b. The DNA polymerase creates a new cDNA strand by extending the primers with nucleotides.

c. If the cDNA target of interest is present in the amplification product, the TaqMan probe hybridizes to the sequence.

d. The 5′ to 3′ nucleolytic activity of the DNA polymerase cleaves the hybridized probe between the reporter dye and the quencher dye (see "TaqMan® Probes" below for more information). The reporter dye fragments are displaced from the target, resulting in an increase in fluorescence.

Note: This step, which occurs in every cycle, does not interfere with the exponential accumulation of product.

e. The polymerization of the strand continues. The 3′ end of the probe is blocked to prevent extension of the probe during PCR.

f. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

Note: The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Nonspecific amplification is not detected in the absence of a probe-binding site.

TaqMan® Probes

The TaqMan® probe contains a fluorescent reporter dye at the 5′ end of the probe and a quencher dye at the 3′end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye suppresses the reporter fluorescence. Probe cleavage during the PCR reaction spatially separates the reporter dye from the quencher moiety and allows detection of the reporter dye fluorescence.

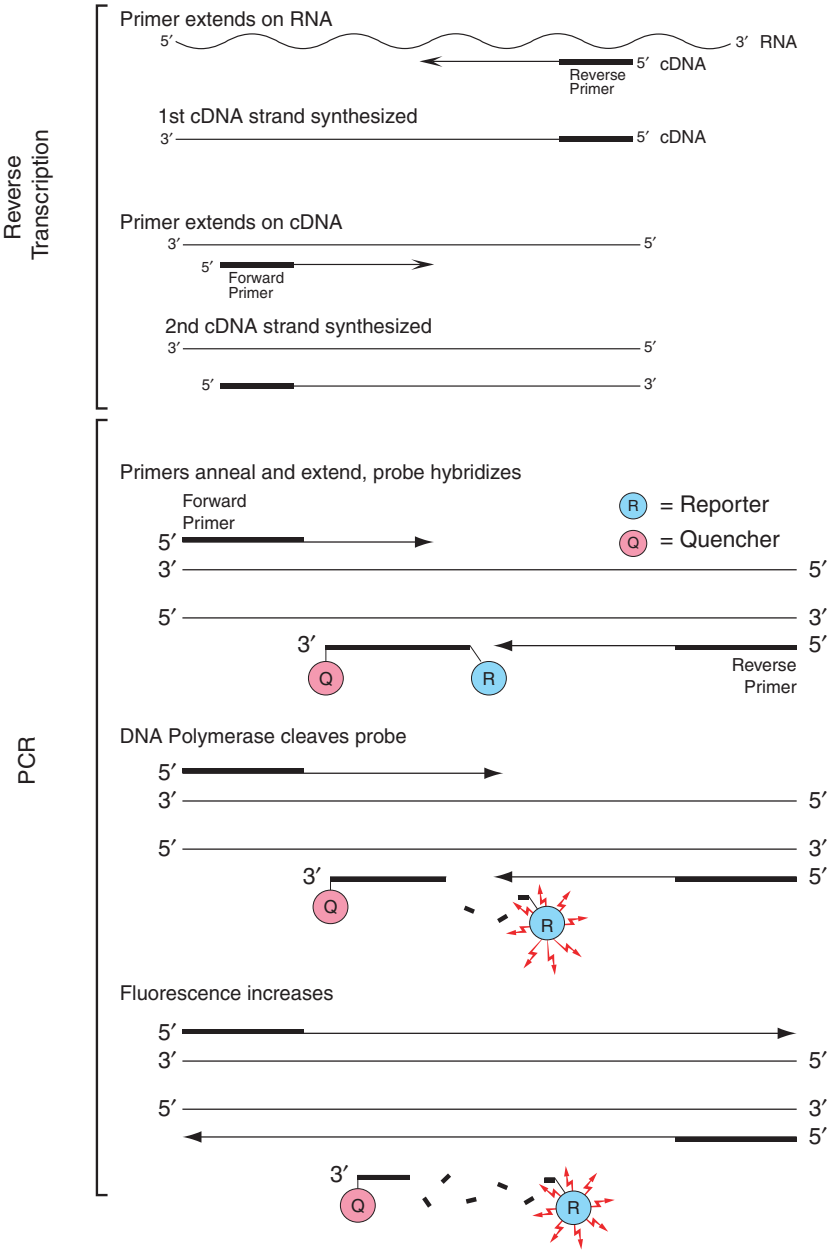


Figure 1 Reverse Transcription — Polymerase Chain Reaction (RT-PCR)

Materials and Equipment

Kit Contents

The TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0 contain reagents for 96 Influenza A or Influenza A subtype H5, H7, or N1 RT-PCR reactions. Each kit contains:

- This protocol
- One pouch containing:
  - Assay beads (in tubes) for Influenza A or Influenza A subtype H5, H7, or N1
  - One bag of 12 MicroAmp™ Optical 8-Cap Strips
- One box containing:
  - 10× Reconstitution Buffer
  - Negative control
- One box containing:
  - RNA Positive Control, Influenza A and Influenza A subtype H5
  - RNA Positive Control, Influenza A subtypes H7 and N1

Table 1 TaqMan® Influenza Detection Kits 2.0

Kit (Part Number)	Tube Cap Color	Reagent
TaqMan Influenza A Detection Kit 2.0 (PN 4384261)	Red	Influenza A Assay Beads, 96 tubes in 8-tube strips, (96 reactions/kit)
TaqMan® Influenza A subtype H5 Detection Kit 2.0 (PN 4384262)	Dark Green	Influenza A subtype H5 Assay Beads, 96 tubes in 8-tube strips, (96 reactions/kit)
TaqMan® Influenza A subtype H7 Detection Kit 2.0 (PN 4384263)	Orange	Influenza A subtype H7 Assay Beads, 96 tubes in 8-tube strips, (96 reactions/kit)
TaqMan® Influenza A subtype N1 Detection Kit 2.0 (PN 4384264)	Light Blue	Influenza A subtype N1 Assay Beads, 96 tubes in 8-tube strips, (96 reactions/kit)

Table 1 TaqMan® Influenza Detection Kits 2.0

Kit (Part Number)	Tube Cap Color	Reagent
Included in all TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0	Blue 	10× Reconstitution Buffer, 1 tube, 500 µL
	Grey 	Negative Control, nuclease-free water, 1 tube, 2.0 mL
	Purple 	RNA Positive Control, Influenza A and Influenza A subtype H5, 1 tube, 1.5 mL
	Gold	RNA Positive Control, Influenza A subtypes H7 and N1, 1 tube, 1.5 mL
	N/A	MicroAmp™ Optical 8-Cap Strips, 96 caps in strips of 8

Storage

- Upon receipt, store Assay Beads at 2 °C to 8 °C.
- Upon receipt, store all other components at -15 °C to -25 °C.
- Minimize freeze-thaw cycles.
- Protect components from light. Excessive exposure to light may affect the fluorescent probes.

Equipment and Materials Not Included in the Kit

The following tables include equipment and materials for using the TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0. Unless otherwise noted, many of the items listed are available from major laboratory suppliers (MLS).

Table 2 Reagents available from Applied Biosystems

Cap color	Reagent	Source
White 	AmpErase® UNG, 1 U/µL‡	Applied Biosystems (PN N8080096)§

‡ Upon receipt, store component at –15 °C to –25 °C. Minimize freeze-thaw cycles.

§ **UNG treatment is optional.** See "Appendix C: Preparing RT-PCR with UNG" on page 37 for the protocol.

Table 3 Instruments from Applied Biosystems

Instruments	Source
ABI PRISM® 7000 Sequence Detection System	Contact your local Applied Biosystems sales office.
Applied Biosystems 7300 Real-Time PCR System	
Applied Biosystems 7500 Real-Time PCR System	
Applied Biosystems 7500 Fast Real-Time PCR System	
Applied Biosystems 7900HT Fast Real-Time PCR System (using a standard block)	

Table 4 User-supplied materials

Materials	Source
Plates, tubes, caps and covers, as needed:	
• MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 20 plates, 0.2 mL	Applied Biosystems (PN 4306737) Not recommended for use with 7500 Fast system. For 7500 Fast system reactions, use PN 4346906.
• MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 20 plates, 0.1 mL	Applied Biosystems (PN 4346906)
• MicroAmp™ Optical 96-Well Reaction Plate with Barcode and Optical Adhesive Films, 100 plates and 100 covers	Applied Biosystems (PN 4314320)
• MicroAmp™ Optical 8-Tube Strip, 1000 tubes in strips of 8, 0.2 mL	Applied Biosystems (PN 4316567) Not recommended for use with 7500 Fast system. For 7500 Fast system reactions, use PN 4358293.
• MicroAmp™ Optical 8-Cap Strip, 300 strips	Applied Biosystems (PN 4323032)
• MicroAmp™ Optical Adhesive Film Kit	Applied Biosystems (PN 4313663)
• MicroAmp™ Optical Adhesive Film, 25 covers	Applied Biosystems (PN 4360954)
• MicroAmp™ Splash Free 96-Well Base	Applied Biosystems (PN 4312063)
Benchtop microcentrifuge	Major laboratory supplier (MLS)
Disposable gloves	MLS
Pipette tips, aerosol resistant	MLS

Table 4 User-supplied materials *(continued)*

Materials	Source
Pipettors: Positive-displacement Air-displacement Multichannel	MLS
RNase-free microfuge tubes, 1.5 ml	Applied Biosystems (PN AM12400)
RNase-free tips, 200 µL	Applied Biosystems (PN AM12650)
RNase-free tips, 1000 µL	Applied Biosystems (PN AM12660)
Ambion RT-PCR Grade Water	Applied Biosystems (PN AM9935)
Centrifuge with adapter for 96-well plate	MLS
Vortexer	MLS

Preventing Contamination

PCR Good Laboratory Practices

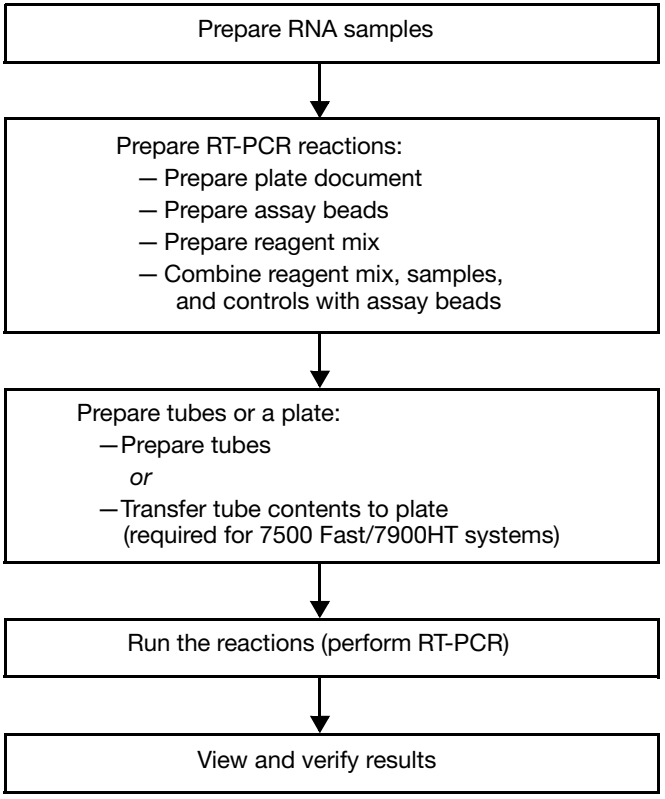
PCR assays require special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The high throughput and repetition of these assays can lead to amplification of a single DNA molecule (Saiki *et al.,* 1985; Mullis and Faloona, 1987).

Preventing Contamination

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas, dedicated equipment, and supplies for:
  - Sample preparation and PCR setup
  - PCR amplification and post-PCR analysis
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes and reaction plates carefully. Do not splash or spray PCR samples.
- Keep reactions and components sealed as much as possible.
- Use positive-displacement pipettes or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with freshly diluted 10% chlorine bleach solution.

IMPORTANT! To avoid false positives due to amplified material in your work area, do not open tubes after amplification.

Detection Procedure Overview



Preparing RNA Samples

Validating Your Own RNA Sample Preparation Procedure

It is important to use high purity viral RNA that is free of materials that can inhibit amplification in the performance of these assays. It is anticipated that most commercially available viral RNA isolation kits should satisfy the requirements of the TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0. The Ambion MagMAX™ AI/ND Viral RNA Isolation Kit was used successfully during development of these assays.

However, Applied Biosystems has not validated an RNA isolation procedure for use with this protocol. You must validate your own procedure.

Sample Handling Precautions

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities utilizing the appropriate safety equipment (for example, physical containment devices). Individuals should be trained in accordance with applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and adhere to the following guidelines and/or regulatory requirements as applicable:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR 1910.1030; **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company/institution's Biosafety Program protocols for working/handling potentially infections materials.

Additional information about biohazard guidelines is available at: **www.cdc.gov**

Preparing RT-PCR Reactions

Overview

Preparing RT-PCR reactions consists of:

- Preparing the plate document
- Preparing assay beads
- Preparing the reagent mix
- Pipetting reagent mix, samples, and controls into tubes containing assay beads

Preparing the Plate Document

For information on creating a plate document, refer to the documentation provided with your instrument.

For information on creating a plate document based on UNG treatment, refer to "Appendix C: Preparing RT-PCR with UNG" on page 37.

To prepare the plate document:

1.	Create a plate document (select **Absolute Quantification (Standard Curve)** from Assay drop-down list). Refer to the appropriate instrument user guide for details.
2.	Create or select **FAM™** dye and **VIC®** dye detectors with the Quencher Dye set to **(none)** or **Non Fluorescent**.
3.	Associate both FAM and VIC dye detectors with each reaction. **Note:** The FAM dye is used for detection of the target (for example, Influenza A subtype H7) and the VIC dye is used for detection of the internal positive control (IPC).

To prepare the plate document: ***(continued)***

4. Set thermal cycling conditions according to the following table:

Times and Temperatures				
RT Step			**PCR Step**	
			Melt	Anneal/ Extend
HOLD	HOLD	HOLD	CYCLE (each of 40 cycles)	
1 min 95 °C	15 min 60 °C	5 min 95 °C	20 sec 95 °C	1 min 60 °C

5. Set Sample Volume to **30** µL.

6. Select the appropriate Run Mode for your system according to the following table:

System	Run Mode
7500 and 7500 Fast	Standard 7500
7000	9600 Emulation
7300	Standard 7300
7900HT	Standard

Preparing the Assay Beads

To prepare the assay beads:

1.	Open the storage pouch containing the assay beads by cutting at the notch located in the upper corner of the storage pouch above the zip lock strip. **IMPORTANT!** Do not remove the desiccant from the storage pouch.
2.	Remove the appropriate number of individual tubes or 8-tube strips (one tube for each reaction you plan to run). **Note:** Applied Biosystems recommends performing three replicates for each sample.
3.	Place tubes on ice or cold block.
4.	Seal the storage pouch using the zip lock strip and store the pouch at 2 °C to 8 °C.

Preparing Reagent Mix

For information on performing reactions using the UNG treatment, refer to "Appendix C: Preparing RT-PCR with UNG" on page 37.

All reactions use the same reagent mix. Later, you combine the reagent mix with the assay beads and one of the following: unknown RNA sample, positive control, or negative control.

CHEMICAL HAZARD, 10× Reconstitution Buffer may cause eye, skin and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the reagent mix:

1.	Thaw all reagents completely. **IMPORTANT!** Keep all tubes on ice.
2.	Mix 10× Reconstitution Buffer by vortexing. Briefly spin down tube contents using a microcentrifuge. **IMPORTANT!** Keep the tube on ice.
3.	For each assay you are performing, label a sterile microcentrifuge tube with the assay name.

To prepare the reagent mix: ***(continued)***

4. Calculate the volume of components needed for the number of sample replicates and controls for one assay. The table below lists volumes for one reaction and ten reactions (three replicates each of one sample, the negative control, and the RNA positive control, plus one extra reaction volume to compensate for pipetting error).

   Note: Applied Biosystems recommends performing three replicates for each sample.

Component	Volume (µL)		Final Concentration
	One 30-µL Reaction	Ten 30-µL Reactions	
10× Reconstitution Buffer	3.0	30.0	1×
RT-PCR Grade Water	17.0	170.0	–
Total Mix	20.0	200.0	–

5. Pipette the appropriate volumes of components into the reagent mix tube, then mix by vortexing. Briefly spin down tube contents using a microcentrifuge.

   IMPORTANT! Keep all tubes on ice.

6. Repeat steps 4 and 5 to prepare a reagent mix for each remaining assay.

Pipetting Reagent Mix, Samples, and Controls to Assay Bead Tubes

To pipette:

1.	Before removing the caps from the assay bead tubes, gently tap the tubes as needed to move all of the assay beads to the bottom of all tubes.
2.	Carefully remove the caps from the assay bead tubes to avoid disturbing the beads from the bottom of the tubes, then discard the colored caps. **IMPORTANT!** Do not use colored caps or tubes for kit reactions. Colored caps or tubes are not compatible with real-time PCR.
3.	For each sample or control you plan to run, transfer 20 µL of the assay-specific reagent mix into a tube containing the appropriate influenza assay beads. **IMPORTANT!** Use a new pipette tip for each assay-specific reagent mix. **IMPORTANT!** Keep all tubes on ice.
4.	Using a new pipette tip for each assay bead tube, transfer 10 µL of RNA sample, RNA positive control, or negative control into the appropriate tubes. **IMPORTANT!** Use a new pipette tip for each assay bead tube, even when pipetting the same sample or control. **Note:** Beads dissolve in 1 to 5 seconds.

Preparing Tubes or a Plate

Perform the next step appropriate for your system. For a:

- 7000/7300/7500 system, proceed to:
  - "Preparing Tubes" on page 20 to run the reactions in the assay bead tubes.

    or
  - "Transferring Tube Contents to a Plate" on page 22 to transfer the reactions to a plate appropriate for your system.
- 7500 Fast system, proceed to "Transferring Tube Contents to a Plate" on page 22 to transfer reactions to 96-well Fast plates appropriate for the 7500 Fast system.
- 7900HT system, go to "Transferring Tube Contents to a Plate" on page 22 to transfer reactions to the 96-well standard plates appropriate for the 7900HT system.

Preparing Tubes

If using a 7500 Fast or 7900HT system, go to "Transferring Tube Contents to a Plate" on page 22.

To prepare tubes for a 7000/7300/7500 system:

1.	Place transparent optical strip caps (PN 4316567) on the tubes. **IMPORTANT!** Do not use colored caps or tubes for kit reactions. Colored caps or tubes may affect dye-signal readings during RT-PCR.

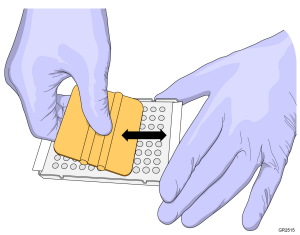
To prepare tubes for a 7000/7300/7500 system: ***(continued)***

2.	Using a sealing tool, affix caps to the tubes. **IMPORTANT!** Apply significant downward pressure on the sealing tool in all steps to form a complete seal on top of the tubes. If you are using the rolling capping tool: a. Roll the capping tool across all strips of caps on the short edge, then the long edge of the tray. b. Roll the capping tool around all outer rows of strips of caps.  If you are using the rocking capping tool: a. Slip your fingers through the handle with the holes in the tool facing down. b. Place the holes in the tool over the first eight caps in a row. c. Rock the tool back and forth a few times to seal the caps. d. Repeat for remaining caps in the row, then for all remaining rows. 
3.	Vortex the tubes on the high setting for 5 seconds, then spin the contents down at 2000 g for 20 seconds using a centrifuge (with a plate adaptor if necessary).
4.	Proceed to "Running the Reactions (Performing RT-PCR)" on page 24.

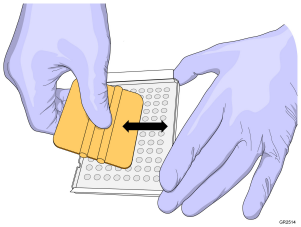
Transferring Tube Contents to a Plate

For 7500 Fast and 7900HT systems, transfer reaction contents from the assay-bead tubes to 96-well Fast or standard plates, respectively, appropriate to system.

To transfer tube contents:

1.	Examine the tubes to ensure that the entire reaction volume is available for transfer: • If all tube contents are not at the bottom of a tube, cap the tube, then tap or spin down the tube as needed. • If air bubbles are present, either: – Cap the tube, then spin down the tube contents *or* – Set the pipette volume in the 43 to 45 µL range and aspirate the entire reaction volume, including the air bubble **IMPORTANT!** Keep all tubes on ice.
2.	Use a pipette to transfer the entire reaction volume to the appropriate plate. If you use a: • 7500 Fast system, dispense the reaction mix into a Fast optical 96-well plate (PN 4346906). • 7900HT system, dispense the reaction mix into a standard optical 96-well plate (PN 4306737). **IMPORTANT!** Use a new pipette tip for each reaction, even when pipetting the same sample or control. **IMPORTANT!** Keep all tubes on ice.
3.	Place an optical adhesive cover on the plate, then rub the flat edge of the applicator back and forth along the long edge of the plate.  **IMPORTANT!** Apply significant downward pressure on the applicator in all steps to form a complete seal on top of the wells. Pressure is required to activate the adhesive on the optical cover.

To transfer tube contents: ***(continued)***

4.	Rub the flat edge of the applicator back and forth along the short edge (width) of the plate. 
5.	Rub the end of the applicator horizontally and vertically between all wells.
6.	Rub the end of the applicator around all outside edges of the plate using small back and forth motions to form a complete seal around the outside wells. 
7.	Vortex the plate on the high setting for 5 seconds, then spin the contents down at 2000 g for 20 seconds using a centrifuge (with a plate adaptor if necessary).
8.	Proceed to "Running the Reactions (Performing RT-PCR)" on page 24.

Running the Reactions (Performing RT-PCR)

Overview

Running tubes or a plate consists of using an Applied Biosystems Sequence Detection System (SDS) or Real-Time PCR System to analyze your sample.

Before You Begin

Ensure that your instrument is properly installed and calibrated. For calibration information, see the documentation provided with your instrument.

Running the Reactions

To run the reactions:

1.	Place the appropriate 8-tube strip or 96-well plate adaptor in the SDS or Real-Time PCR System.
2.	Open the plate document that corresponds to the reaction plate.
3.	Load the reaction tubes or plate into the SDS or Real-Time PCR System. If loading 8-tube strips, place strips in a vertical position in the center of the block. If columns 1 and 12 are not used, fill them with empty strips.
4.	Start the run.

IMPORTANT! To avoid false positives due to amplified material in your work area, do not open tubes after amplification.

Viewing and Verifying Results

Overview

The steps you perform to view results depend on the instrument you use. Refer to the appropriate instrument user guide for instructions on how to analyze data and view your results.

Viewing Results

To view results:

1. View the amplification plots for all reactions.
2. For all reactions, under Analysis Settings, select **Manual Baseline**. Set Start (cycle) to **6** and set End (cycle) to **15**.
3. For all reactions, under Analysis Settings, select **Manual Ct**, then enter the appropriate Manual Ct Threshold value for your assay/system combination according to the following table:

System	Influenza Kit			
	A	H5	H7	N1
7000	0.30	0.30	0.50	0.20
7300	0.20	0.40	0.40	0.20
7500/ 7500 Fast/7900HT	0.20	0.20	0.25	0.10

4. Analyze the data.
5. Verify the runs and the analysis settings according to the instructions in "Verifying Results" on page 26.

To view results: ***(continued)***

6. Check each sample for FAM™ dye signal (target-specific signal) and VIC® dye signal (IPC), then interpret results using the following table:

FAM signal (target)	VIC signal (IPC)	Result
Present	Present	Presumptive Positive. See "Verifying Results" on page 26.
Present	Absent	Invalid – repeat assay. See "Appendix A: Troubleshooting" on page 30.
Absent	Present	Presumptive Negative.
Absent	Absent	See "Appendix A: Troubleshooting" on page 30.

Verifying Results

To verify results:

1. Examine the IPC signal in all wells.

   Signal for the internal positive control (VIC® dye detector) in all wells should yield Cts in the range of 31 to 36 at the manual Ct threshold specified in step 3 on page 25 (see the example in Figure 2 below).

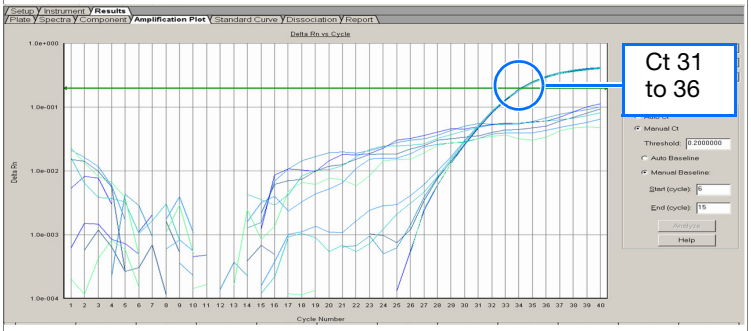
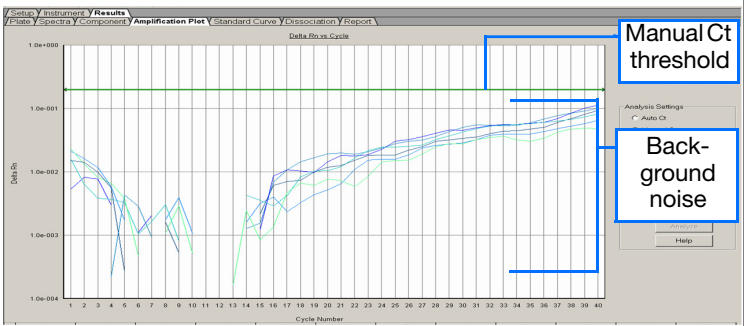
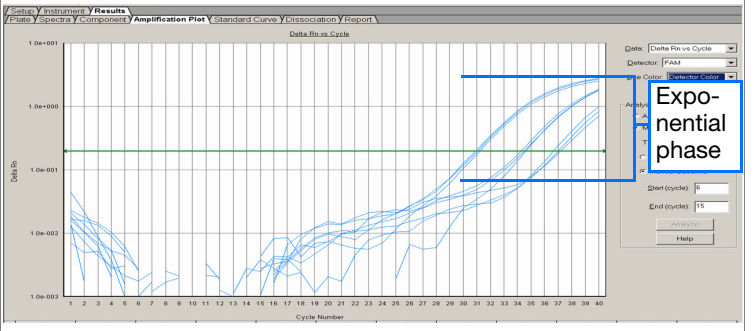


Figure 2 Influenza A subtype H5 assay IPC in negative control amplification plot (7500 SDS System)

To verify results: ***(continued)***

2.	If you observe either of the following, see "Appendix A: Troubleshooting" on page 30: • IPC signal is not present in negative control, unknown, or positive control wells • IPC signal Ct is greater than 36 in unknown or positive controls wells • IPC signal Ct is inconsistent between unknown wells
3.	Examine all negative control wells. Figure 3 below shows a typical amplification plot for the negative control for the Influenza A subtype H5 assay. **Note:** You may see a low signal in the negative control wells (NTC – No Template Control; FAM™ dye detector) even in the absence of contamination. This signal represents background noise, and it typically remains below the manual Ct threshold.  **Figure 3 Influenza A subtype H5 assay negative control amplification plot (7500 SDS System)**
4.	Verify that no negative control samples show target-specific amplification (see Figure 4 on page 28 for an example of target-specific amplification). If you observe target-specific signal in negative control wells, see "Appendix A: Troubleshooting" on page 30.

To verify results: ***(continued)***

5.	Verify that negative control well signals do not cross the manual Ct threshold specified in step 3 on page 25. **Note:** Crossing the threshold can be due to high background noise and can result in signals that do not show target-specific amplification. Take the following steps if the wells do not show target-specific amplification: a. Manually set the threshold slightly above any negative control signal. **IMPORTANT!** Do not set the threshold too high above noise. Doing so decreases the detection sensitivity of positive samples. Decrease the threshold, if the threshold does not cross the exponential phase in positive wells (see steps 6 and 7). b. Analyze again. **Note:** Setting the threshold above negative control signal changes the Cts of the negative controls to "Undetermined."
6.	Examine amplification plots for all positive wells. True positive samples yield signal (FAM™ dye detector) with amplification plots that cross the threshold within the exponential phase of the plot. Figure 4 shows an amplification plot for the RNA positive control for Influenza A subtype H5 assay detected at 1000, 100, and 10 copies per reaction.  **Figure 4 Influenza A subtype H5 assay RNA positive control amplification plot — 1000, 100, and 10 copies (7500 SDS System)**

To verify results: ***(continued)***

7.	If you adjusted the manual Ct threshold in step 5, verify that the exponential phase of the amplification plot crosses the newly adjusted threshold. If it does not: a. Decrease the manual Ct threshold so that: – In negative control wells, the threshold is above noise – In positive wells, the threshold crosses the exponential phase b. Analyze again.

Appendix A: Troubleshooting

Observation	Possible Cause	Action
No IPC or target-specific signal detected in unknown wells	RT or PCR inhibited	Repeat sample preparation, then repeat the assay reactions. If RT or PCR is still inhibited, dilute the sample (for example, 1:5 or 1:10) to dilute inhibitors, or use alternate RNA purification procedure.
	TaqMan® Influenza Assay Beads and reagents not stored properly	Repeat the assay reactions using properly stored reagents. Protect the assay beads from light.
	10× Reconstitution Buffer not stored properly	
	10× Reconstitution Buffer not included in reagent mix	Repeat the assay reactions using properly prepared reagent mix.
	Pipetting error (no reagent mix added)	Repeat the assay reactions. Make sure to pipette reagent mix into all wells.
IPC signal Ct is greater than 36 in unknown or positive controls wells	RT or PCR inhibited	Repeat sample preparation, then repeat the assay reactions. If RT or PCR is still inhibited, dilute the sample (for example, 1:5 or 1:10) to dilute inhibitors, or use alternate RNA purification procedure.
IPC signal Ct is inconsistent between unknown wells		
No IPC detected, but target-specific signal detected	High copy number of target RNA resulting in preferential amplification of the target-specific RNA	Dilute the sample (for example, 1:5 or 1:10), then repeat assay reactions.

Observation	Possible Cause	Action
Target-specific signal detected in negative control wells	Carryover contamination	Repeat the assay reactions using fresh aliquots of all reagents and clean pipetting equipment. If the negative control still shows contamination, repeat assay reactions using a new kit. If the negative control still shows contamination, contact Applied Biosystems Technical Support.
Target-specific signal and no IPC signal detected in negative control wells	Carryover contamination and one of the following: • High copy number of target RNA resulting in preferential amplification of the target-specific RNA • Problem with IPC amplification	Examine unknowns to determine if IPC signal is present. If IPC signal is present in unknown wells, you can rule out a problem with IPC amplification. Repeat the assay reactions using fresh aliquots of all reagents and clean pipetting equipment.
No IPC or target-specific signal in positive control wells	Assay beads not stored properly	Repeat the assay reactions using properly stored reagents. Protect the assay beads from light.
	10× Reconstitution Buffer not stored properly	
	10× Reconstitution Buffer not included in reagent mix	Repeat the assay reactions using properly prepared reagent mix.
No target-specific signal detected in positive control wells	Pipetting error (no positive control added)	Repeat the assay reactions. Make sure to pipette positive control into all positive control wells.

Observation	Possible Cause	Action
Replicate results for this sample are inconsistent	All replicate wells for a sample do not have the same result.	If more than two replicates yield the same result (for example, you ran three replicates and two replicates are negative, but one replicate is positive), the result associated with the larger number of replicates is probably accurate. However, your laboratory protocol may dictate that you repeat the assay using fresh samples and reagents. If you ran only two replicates and results are not consistent, repeat the assay using fresh samples and reagents.

Appendix B: Specificity and Limit of Detection

Inclusion List

Assay designs were evaluated against influenza strains representing each of the possible assay results:

- **Negative/Negative** – Non-Influenza A/Non-Influenza H5, H7, or N1 subtypes
- **Positive/Negative** – Influenza A/Non-Influenza H5, H7, or N1 subtypes
- **Positive/Positive** – Influenza A/Influenza H5, H7, or N1 subtypes
- Samples from both avian and human sources that represent both geographical and temporal diversity were evaluated. Non-influenza respiratory pathogens were also tested for cross-reactivity.

Strains that have not been submitted to NCBI databases and are not listed in this appendix may also yield positive results. The isolates listed in Tables 5 through 8 were successfully detected in independent external testing:

Table 5 Influenza A (non-H5 subtype) isolates

A/Germany/1791/05 (H3N2)
A/Germany/1558/05 (H3N2)
A/Germany/1527/05 (H1N1)
A/Germany /1456/05 (H1N1)
A/Germany/1329/05 (H1N1)
A/Coal Tit/Yokohama-aq/22/2003 (H3N8)
A/finch/Yokohama-aq/39/2003 (H3N8)
A/chicken/Yokohama-aq/301/2002 (H9N2)
A/chicken/Yokohama-aq/144/2001 (H9N2)
A/Hong Kong/1174/99 (H3N2)
A/Hong Kong/54/98 (H1N1)
A/Teal/Hong Kong/W312/97 (H6N1)
A/Quail/Hong Kong/G1/97 (H9N2)
A/Duck/Hong Kong/Y280/97 (H9N2)
A/emu/NSW/1997 (H7N4)

Table 5 Influenza A (non-H5 subtype) isolates *(continued)*

A/finch/Yokohama-aq/2/1997 (H4N6)
A/grey teal/WA/1840/1979 (H4N4)
A/shelduck/WA/1762/1979 (H15N9)
A/duck/Victoria/1976 (H7N7)

Table 6 Influenza A subtype H5 isolates

A/goose/Hungary/3413/2007 (H5N1)
A/goshawk/Germany/R60/06 (H5N1)
A/whooper swan /Germany/R65/06 (H5N1)
A/x/Romania/2910/06 (H5N1)
A/x/Romania/2912/06 (H5N1)
A/x/Romania/3076/06 (H5N1)
A/x/Romania/3077/06 (H5N1)
A/x/Romania/3258/06 (H5N1)
A/x/Romania/3282/06 (H5N1)
A/x/Romania/3293/06 (H5N1)
A/turkey/Turkey/R11/06 (H5N1)
A/x/Turkey/R12/06
A/chicken/Vietnam/P41/05 (H5N1)
A/chicken/Vietnam/P78/05 (H5N1)
A/chicken/Vietnam/P22/05 (H5N1)
A/duck/Vietnam/TG24-01/05 (H5N1)
A/duck/Vietnam/TG36-H2-01/05 (H5N1)
A/duck/Vietnam/AG40-O2/05 (H5N1)
A/chicken/GXLA/1204/05 (H5N1)
A/chicken/Indonesia/R60/05 (H5N1)
A/falco cherrug/Saudi Arabia/R324/05 (H5N1)
A/Thailand/AIV-1/2004
A/chicken/Cambodia/1A/2004 (H5N1)
A/chicken/GxU7/1204/2004
A/Ck/Indonesia/4/2004 (H5N1)

Table 6 Influenza A subtype H5 isolates *(continued)*

A/chicken/Indonesia/R132/1/04 (H5N1)
A/chicken/Indonesia/R132/2/04 (H5N1)
A/chicken/Indonesia/R132/3/04 (H5N1)
A/Thailand/MK2/2004 (H5N1)
A/Ck/Vietnam/33/04 (H5N1)
A/chicken/Vietnam/8/2004 (H5N1)
A/Vietnam/1203/2004 (H5N1)
A/duck/Yokohama-aq/10/2003 (H5N1)
A/HK/212/03 (H5N1)
A/HK/213/03 (H5N1)
A/chicken/Indonesia/R132-134/03 (H5N1)
A/mallard/Germany/Wv1310-13K/03 (H5N2)
A/teal/Germany/Wv1310-13K/03 (H5N2)
A/Gs/HK/739.2/02 (H5N1)
A/Gs/HK/4376/99 (H5N1)
A/HK/483/97 (H5N1)
A/HK/486/97 (H5N1)

Table 7 Influenza A subtype H7 isolates

A/NL/219/2003
A/CK/NL/2003

Table 8 Influenza A subtype N1 isolates

A/Vietnam/1203/2004 (H5N1)
A/HK/212/2003 (H5N1)
A/HK/213/2003 (H5N1)
A/Thailand/MK2/2004 (H5N1)
A/Thailand/AIV-1/2004
Ck/Vietnam/33/2004 (H5N1)
V05525/2416

Exclusion List

The following non-influenza respiratory pathogens and potential anti-targets have been tested and do not yield positive results:

- Adenovirus
- Influenza B
- Parainfluenza (groups 1, 2, 3, and 4)
- RSV
- SARS-CoV
- Human DNA
- Human RNA
- Chicken DNA
- Duck DNA
- Influenza A-non-H5 (for H5 assays)

Limit of Detection

The TaqMan® Influenza A/H5/H7/N1 Detection Kits 2.0 assays have been shown to reliably detect 100 copies of positive control RNA present in a 30 µL reaction.

Predicted Inclusion

Based on bioinformatics analysis, the assays included in this kit perfectly match and are predicted to detect the following strains (sequence submissions beginning with NCBI 2001):

- **Influenza A assay**: at least 1600 avian and human strains
- **Influenza A subtype H5 assay**: at least 350 avian and human strains
- **Influenza A subtype H7 assay:** at least 100 avian strains
- **Influenza A subtype N1 assay:** at least 600 avian and human strains

Note: It is anticipated that many Influenza A and Influenza A subtype H5, H7, and N1 isolates beyond those specifically predicted will yield positive results.

Appendix C: Preparing RT-PCR with UNG

Overview

Preparing RT-PCR consists of:

- Preparing the plate document, see below
- Preparing assay beads, refer to page 16
- Preparing the reagent mix, refer to page 39
- Pipetting reagent mix, samples, and controls into tubes containing assay beads, refer to page 19

Preparing the Plate Document with UNG

For information on creating a plate document, refer to the documentation provided with your instrument.

To prepare the plate document:

1. Create a plate document (select **Absolute Quantification (Standard Curve)** from Assay drop-down list). Refer to the appropriate instrument user guide for details.
2. Create or select **FAM™** dye and **VIC®** dye detectors with the Quencher Dye set to **(none)** or **Non Fluorescent**.
3. Associate both FAM and VIC dye detectors with each reaction.

   Note: The FAM dye is used for detection of the target (for example, Influenza A subtype H7) and the VIC dye is used for detection of the internal positive control (IPC).
4. Set thermal cycling conditions according to the following table:

Times and Temperatures					
Initial Step UNG Treatment		RT Step	UNG Deactivation	PCR Step	
				Melt	Anneal/ Extend
HOLD	HOLD	HOLD	HOLD	CYCLE (each of 40 cycles)	
1 min 72 °C	2 min 50 °C	15 min 60 °C	5 min 95 °C	20 sec 95 °C	1 min 60 °C

To prepare the plate document: ***(continued)***

5.	Set Sample Volume to **30** µL.
6.	Select the appropriate Run Mode for your system according to the following table:

System	Run Mode
7500 and 7500 Fast	Standard 7500
7000	9600 Emulation
7300	Standard 7300
7900HT	Standard

Preparing the Assay Beads

Proceed to "Preparing the Assay Beads" on page 16.

Preparing Reagent Mix with UNG

All reactions use the same reagent mix. The reagent mix will be combined with the assay beads and one of the following: unknown RNA sample, positive control, or negative control.

CAUTION CHEMICAL HAZARD, 10× Reconstitution Buffer may cause eye, skin and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the reagent mix:

1.	Thaw all reagents completely. **IMPORTANT!** Keep all tubes on ice.
2.	Mix 10× Reconstitution Buffer by vortexing. Using a microcentrifuge, briefly spin down the tube contents. **IMPORTANT!** Keep the tube on ice.
3.	For each assay you are performing, label a sterile microcentrifuge tube with the assay name.

To prepare the reagent mix: *(continued)*

4. Calculate the volume of components needed for the number of sample replicates and controls for one assay. The table below lists volumes for one reaction and ten reactions (three replicates each of one sample, the negative control, and the RNA positive control, plus one extra reaction volume to compensate for pipetting error).

   Note: Applied Biosystems recommends performing three replicates for each sample.

Component	Volume (µL)		Final Concentration
	One 30-µL Reaction	Ten 30-µL Reactions	
10× Reconstitution Buffer	3.0	30.0	1×
RT-PCR Grade Water	16.5	165.0	–
AmpErase® UNG (1 U/µL)	0.5	5.0	0.017 U/µL
Total Mix	20.0	200.0	–

5. Pipette the appropriate volumes of components into the reagent mix tube, then mix by vortexing. Spin down contents using a microcentrifuge.

   IMPORTANT! Keep all tubes on ice.

6. Repeat steps 4 and 5 to prepare a reagent mix for each remaining assay.

Pipetting Reagent Mix, Samples, and Controls to Assay Bead Tubes

Proceed to "Pipetting Reagent Mix, Samples, and Controls to Assay Bead Tubes" on page 19.

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