TaqMan® Influenza A/H5 Detection Kit Version 1.0

Protocol

This product is for research use only.
Not for use in diagnostic procedures.

Applied Biosystems

This product is for research use only. Not for use in diagnostic procedures.

For The TaqMan® Influenza A/H5 Detection Kit Version 1.0 (PN 4370330)

NOTICE TO PURCHASER: LIMITED LICENSE

Use of the TaqMan® Influenza A/H5 Detection Kit Version 1.0 (PN 4370330) is covered by US patent claims and corresponding patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product solely in environmental testing applications, including epidemiological studies, including reporting results of purchaser's activities for a fee or other commercial consideration, and also for the purchaser's own internal research. No right under any other patent claim (such as apparatus or system claims for real-time PCR) is conveyed expressly, by implication, or by estoppel.

Further information on purchasing licenses may be obtained from the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

For The TaqMan® EZ RT-PCR Core Reagents (PN N8080236)

NOTICE TO PURCHASER: LIMITED LICENSE

Use of this product is covered by US patent claims and corresponding patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as the patented 5' Nuclease Process claims) and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

The use of uracil-N-glycosylase for carryover prevention is licensed by Invitrogen Corporation under U.S. patent claims and foreign equivalents for research purposes only. No right for use in other applications, including the diagnosis of disease in humans, animals, or plants under any patents owned by Invitrogen Corporation is conveyed by the purchase of this product.

TRADEMARKS:

Part Number 4370566 Rev. C
06/2010

TaqMan® Influenza A/H5 Detection Kit

IMPORTANT NOTICE
PLEASE READ BEFORE USING THIS PRODUCT

PLEASE READ THE IMPORTANT INFORMATION BELOW, INCLUDING THE LIMITATION OF WARRANTY AND LIMITATION OF LIABILITY TERMS, BEFORE USING THIS PRODUCT. If the terms and conditions. limitations and statements are not acceptable, notify Applied Biosystems immediately, but in any event no later than 5 days from delivery of this product to you, and arrangements will be made for return of this product and for refund of the purchase price, less shipping costs. You must notify Applied Biosystems within 5 days of delivery in order to obtain a refund. USE OF THIS PRODUCT CONSTITUTES ACKNOWLEDGMENT THAT YOU HAVE READ AND AGREED TO, AND ACCEPT, THE INFORMATION AND TERMS BELOW, INCLUDING WITHOUT LIMITATION THE LIMITATIONS OF WARRANTY, LIABILITY AND REMEDIES. Any additional or different terms in Buyer's purchase form(s) or other documents are material alterations and are hereby rejected.

Field of Use, Limitations. This product and data obtained from use of this product should not be used for human diagnostic, human treatment or other clinical purposes, or for personal, family or household purposes. This product has not been cleared or otherwise approved by the United States Food and Drug Administration or by any other regulatory body in any country, or under the European IVD Directive, for diagnostic, treatment or any other clinical purpose. This product is for Research Use Only. Not for use in diagnostic procedures. This product should not be used as the sole basis for assessing the presence or absence of any particular organism or virus type or subtype and should be used only by or under supervision of technically qualified persons. Read the Limitation of Warranty, Liability and Remedies statements below before using this product. See additional important information below.

This product should be used only by technically qualified persons who have their own independent skill and expertise in connection with the selection and use of products such as this product, and in strict compliance with good and safe laboratory practices and all applicable instructions, warnings and other information in user manuals and other product documentation. This product should be used only with appropriate nucleic acid sequence detection equipment that has been properly maintained and calibrated by technically qualified persons.

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

or subtype(s) by other means. Applied Biosystems will not be responsible or liable for failures to detect the designated influenza virus type or subtype(s) or for false positive indications.

LIMITED WARRANTY AND LIMITATION OF LIABILITY AND REMEDIES. Applied Biosystems warrants that this product will be free of defects in materials and workmanship for a period of 90 days from the date delivered by Applied Biosystems to the buyer or its agent (“Warranty Period”), subject to the conditions set forth below. Applied Biosystems will replace, free of charge, any defective product, or refund the purchase price of the defective product, in Applied Biosystems' discretion, if a warranty claim is made within the Warranty Period or within 30 days after the end of the Warranty Period. Any warranty claim must be received by Applied Biosystems during the Warranty Period or within 30 days after the end of the Warranty Period. This product may not be returned without a return authorization number from Applied Biosystems, and then only in the manner prescribed by Applied Biosystems. Return authorization numbers may be obtained from Applied Biosystems by calling or e-mailing your Applied Biosystems salesperson or representative. Expiration dates or shelf life periods with respect to this product are included for informational purposes only and shall not be deemed a period of warranty.

Applied Biosystems' warranty does not cover, and Applied Biosystems will not replace or refund the purchase price of, any defective product if a defect is caused by failure to use the product in accordance with Applied Biosystems' instructions and good laboratory practices by technically qualified persons or by other misuse or neglect, including but not limited to improper maintenance, shipping or handling, inappropriate storage or maintenance, or storage or maintenance not in accordance with Applied Biosystems' instructions, improper or abnormal use, adulteration, degradation, any unauthorized change or modification to the product, or any defect caused by accident.

THE FOREGOING WARRANTY IS APPLIED BIOSYSTEMS' SOLE AND EXCLUSIVE WARRANTY WITH RESPECT TO THIS PRODUCT AND IS IN LIEU OF ALL OTHER WARRANTIES, EXPRESSED OR IMPLIED, ALL OF WHICH OTHER WARRANTIES ARE EXPRESSLY DISCLAIMED, INCLUDING WITHOUT LIMITATION THOSE OF MERCHANTABILITY, FITNESS OR SUITABLITY FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT, WHETHER ARISING FROM A STATUTE OR OTHERWISE IN LAW OR FROM A COURSE OF DEALING OR USAGE OF TRADE. THE REPLACEMENT OF DEFECTIVE PRODUCT OR REFUND OF THE PURCHASE PRICE AS SET FORTH ABOVE IS BUYER'S SOLE AND EXCLUSIVE REMEDY WITH RESPECT TO APPLIED BIOSYSTEMS' BREACH OF THE FOREGOING WARRANTY OR ANY CLAIMS OF ANY KIND OR NATURE WHATSOEVER RELATING TO DEFECTIVE PRODUCT.

IN ADDITION TO THE FOREGOING, THE SOLE AND EXCLUSIVE REMEDY OF BUYER AND ANY USER OF THIS PRODUCT, AND THE SOLE AND EXCLUSIVE LIABILITY OF APPLIED BIOSYSTEMS ITS AFFILIATES, DISTRIBUTORS, SUPPLIERS, LICENSORS OR REPRESENTATIVES FOR ANY AND ALL CLAIMS IN RESPECT THEREOF (OTHER THAN WARRANTY CLAIMS, WHICH SHALL BE GOVERNED BY THE PRECEDING PARAGRAPH), INCLUDING CLAIMS IN TORT, CONTRACT, STRICT LIABILITY, NEGLIGENCE OR OTHERWISE, SHALL BE LIMITED TO THE GREATER OF (i) REFUND OF THE PURCHASE PRICE OR REPLACEMENT OF THE PRODUCT OR (ii) US $2,000.00. BUYER OR ANY SUCH USER OF THIS PRODUCT SHALL NOTIFY APPLIED BIOSYSTEMS OF ANY CLAIM WITHIN 30 DAYS THEREOF AND SHALL COMMENCE ANY ACTION AGAINST APPLIED BIOSYSTEMS WITHIN ONE YEAR OF THE EVENT OR OMISSION GIVING RISE TO THE CAUSE OF ACTION OR OTHERWISE SHALL BE BARRED FROM ANY REMEDY. APPLIED BIOSYSTEMS SHALL NOT BE RESPONSIBLE FOR ANY COST, LOSS, LIABILITY OR DAMAGE (INCLUDING WITHOUT LIMITATION LOSS OF REVENUE OR PROFIT, LOST OR DAMAGED DATA OR OTHER COMMERCIAL OR ECONOMIC LOSS, OR ANY OTHER DIRECT OR ANY INDIRECT, INCIDENTAL, SPECIAL, OR CONSEQUENTIAL DAMAGES WHATSOEVER) THAT ARISES FROM BUYER'S OR ANY SUCH USER'S OPERATION OF ITS BUSINESS OR FROM THE USE OF THIS PRODUCT, WHETHER SINGLY OR IN COMBINATION WITH OTHER PRODUCTS.

EXCEPT FOR THE LIMITED REMEDIES SET FORTH ABOVE, BUYER AND ANY USER OF THIS PRODUCT ASSUME ALL RISK AND LIABILITY RESULTING FROM USE OF THIS PRODUCT, WHETHER USED SINGLY OR IN COMBINATION WITH OTHER PRODUCTS.

Some countries or jurisdictions limit the scope of or preclude limitations or exclusion of warranties or of remedies or damages. In such countries and jurisdictions, the limitation or exclusion of warranties, or remedies or damages set forth above shall apply to the fullest extent permitted by law, and shall not apply only to the extent prohibited by law.

The terms and conditions set forth above are supplemental to Applied Biosystems General Terms and Conditions of Sale or other agreement between Applied Biosystems and buyer. In case of a conflict, the terms set forth above shall take precedence and prevail.

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" on page x.)
- 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 **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, 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
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

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

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 TaqMan® Influenza A/H5 Detection Kit Version 1.0 provides a rapid procedure for detecting the presence of viral targets. The kit includes reagents sufficient to perform 100 Influenza A tests and 100 Influenza H5 tests.

The TaqMan® Influenza A/H5 Detection Kit Version 1.0 assays 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 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 Detection Kit Version 1.0. Bioinformatics analysis predicted that the Influenza kit will detect most type A and subtype H5 isolates for which sequence data exists. See "Appendix B: Specificity and Limit of Detection" on page 28 for further discussion of predicted and demonstrated specificity.

This product is for research use only. Not for use in diagnostic procedures.

Chemistry Overview

Reaction Components

Reaction components of the TaqMan® Influenza A/H5 Detection Kit Version 1.0 are:

- Reagent Mix (assembled from the TaqMan® EZ RT-PCR Core Reagents (PN N8080236)) containing dNTPs, DNA polymerase, and other components for reverse transcription and PCR.
- Target Assay Mixes (Influenza A and Influenza H5) containing primers and TaqMan probes for PCR.
- Sample:
  - RNA isolated from environmental or epidemiological samples, which you supply
  - RNA positive control provided in the kit
  - Negative control provided in the kit

The kit provides 100 Influenza A assays and 100 Influenza H5 tests.

About Negative and Positive Controls

It is good practice to include PCR-specific and process-specific controls in your experiment.

- Negative Controls:
  - Run at least two "PCR controls" (the negative control provided in the kit) to confirm that kit reagents do not contain any components that amplify.
  - Run a "process control" (from sample preparation to detection without any sample added) to confirm that your process does not contain any components that amplify.
- Positive Controls:
  - Run a "PCR control" (the positive control provided in the kit or DNA supplied by the user) to confirm that the kit reagents amplify the expected target.
  - Run a "process control" (use positive sample material similar to the real samples you will assay, for example, blood, body fluids, cultures) to test the entire process from sample preparation to detection to ensure that your process can generate the 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 Target Assay Mix. A positive IPC signal demonstrates that:

- PCR reagents amplify as expected.
- Allow accurate interpretation of negative sample results.

UNG Contamination Control System

The AmpErase® UNG treatment in this protocol minimizes or eliminates the reamplification of carryover PCR products by:

- Substituting dUTP for dTTP in the PCR Reagent Mix.
- 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 can result in the removal of up to 200,000 copies of a previously amplified product per 50 µL reaction.

r*Tth* DNA Polymerase

The r*Tth* DNA Polymerase functions as both a thermoreactive reverse transcriptase and a thermostable DNA polymerase. The r*Tth* DNA Polymerase reverse transcribes RNA efficiently in the presence of Mn^{2+} and at elevated temperatures (≥60 °C). It also provides the 5' to 3' nuclease activity necessary for the cleavage of the fluorogenic probe.

RT-PCR

The TaqMan Influenza A/H5 assay uses a one-step reverse transcription-polymerase chain reaction (RT-PCR) format. This assay combines r*Tth* DNA polymerase with the fluorogenic 5' nuclease assay in a single-tube, single-enzyme system.

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

1. RNA is reverse-transcribed to cDNA.
2. PCR cycles are performed:
   a. Primers anneal, or bind, 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 r*Tth* DNA Polymerase cleaves the hybridized probe between the reporter dye and the quencher dye (see below for more information on TaqMan probes). The reporter dye fragments are displaced from the target, resulting in an increase in fluorescence.

   This process occurs in every cycle and 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.

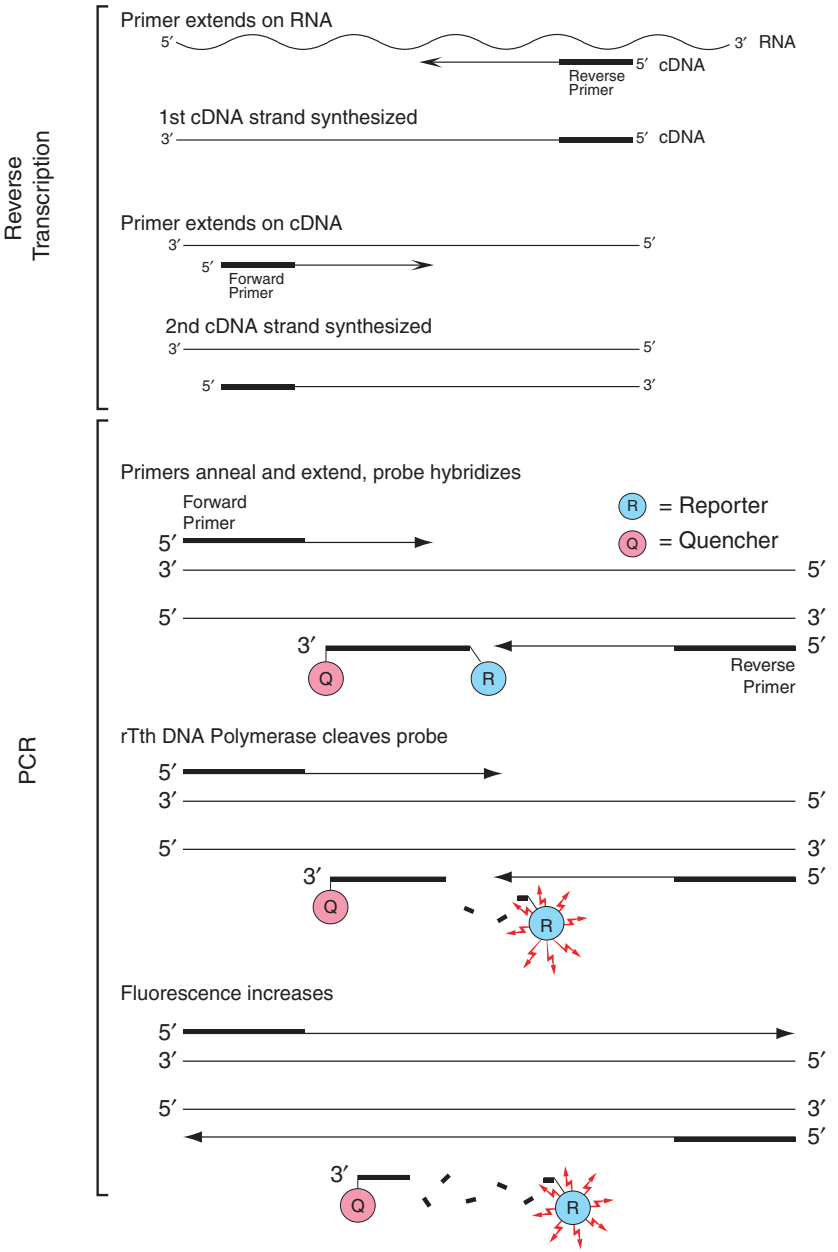


Figure 1 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Materials and Equipment

Kit Contents

The TaqMan® Influenza A/H5 Detection Kit (PN 4370676) contains reagents for 100 Influenza A RT-PCR tests and 100 Influenza H5 RT-PCR tests. The kit contains two boxes plus this protocol:

- TaqMan® EZ RT-PCR Core Reagents (PN N8080236)
- TaqMan® Influenza A/H5 Detection Kit Version 1.0 (PN 4370330)

Table 1 TaqMan® EZ RT-PCR Core Reagents (PN N8080236)

Cap Color	Reagent	Volume
Purple 	r*Tth* DNA Polymerase, 2 tubes, 1,000 units	400 µL
White 	AmpErase® UNG, 1 tube, 100 units	100 µL
Blue 	deoxy ATP, 1 tube	320 µL
White 	deoxy CTP, 1 tube	320 µL
Orange 	deoxy GTP, 1 tube	320 µL
Purple 	deoxy UTP, 1 tube	320 µL
Purple 	5X TaqMan® EZ Buffer, 2 tubes	2 mL
White 	Manganese Acetate solution, 2 tubes	2 mL

Table 2 TaqMan® Influenza A/H5 Detection Kit Version 1.0 (PN 4370330)

Cap Color	Reagent	Volume
Red 	10X Influenza A Target Assay Mix, 1 tube	500 µL
Dark Green 	10X Influenza H5 Target Assay Mix, 1 tube	500 µL
White 	Negative Control, 1 tube	1000 µL
Clear	RNA Positive Control, 1 tube	1.5 mL

Storage

- Upon receipt, store all components of all boxes at –20 °C.
- Protect components from light. Excessive exposure to light may affect the florescent probes.
- Minimize freeze-thaw cycles.

Equipment and Materials Not Included in the Kit

The following tables include equipment and materials for using the TaqMan® Influenza A/H5 Detection Kit Version 1.0. Unless otherwise noted, many of the items listed are available from major laboratory suppliers (MLS).

Table 3 Instruments from Applied Biosystems

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

Table 4 User-supplied materials

Materials	Source
Plates, tubes, caps and covers, as needed:	
• MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 20 plates	Applied Biosystems (PN 4306737)
• 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	Applied Biosystems (PN 4316567)
• MicroAmp™ Optical 8-Cap Strip, (8 caps/strip), 300 strips	Applied Biosystems (PN 4323032)

Table 4 User-supplied materials *(continued)*

Materials	Source
Plates, tubes, caps and covers, as needed *(continued)*:	
• 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
Pipet tips, aerosol resistant	MLS
Pipettors: Positive-displacement Air-displacement Multichannel	MLS
Sterile microcentrifuge tubes with attached screw-cap lid	MLS
RNase-free, sterile-filtered water	MLS
Centrifuge with adapter for 96-well plate	MLS
Vortexer	MLS

Preventing Contamination

Overview

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

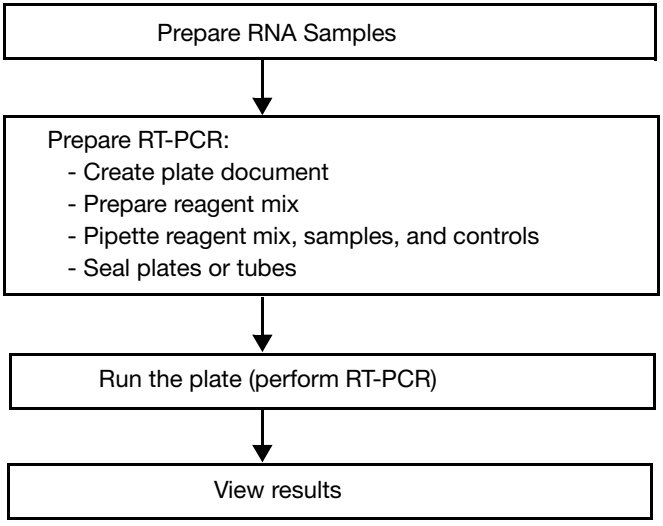
Preventing Contamination

- If possible, maintain separate work areas, dedicated equipment, and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products

  Note: Work areas can be simulated using a clean bench or PCR bench available from major laboratory suppliers.
- Do not bring amplified PCR products into the PCR setup area.
- 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 and before leaving the work area.
- Before pipetting positive controls, close all unknown sample and negative control tubes.
- Do not open sample tubes in clean room areas.
- Keep reactions and components capped as much as possible.
- Use positive-displacement pipets or aerosol-resistant pipet tips.
- Clean lab benches and equipment after use with freshly diluted 10% 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 Detection Kit Version 1.0, and the Qiagen QIAamp® Viral RNA Mini 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; **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 infections materials.

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

Preparing RT-PCR

Overview

Preparing RT-PCR consists of:

- Preparing the plate document
- Preparing the reagent mix
- Preparing the RNA positive control
- Pipetting reagent mix, samples, and controls
- Sealing plates or tubes

Preparing the Plate Document

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 (Assay type = Absolute Quantification without standard curve).
2. Create or select **FAM** dye and **VIC** dye detectors for each reaction. Leave the Quencher Dye set to (none) or Non Fluorescent.
3. Set thermal cycling conditions:

Times and Temperatures					
Initial Step UNG Treatment		RT	UNG Deactivation	Each of 40 Cycles	
				Melt	Anneal/ Extend
HOLD	HOLD	HOLD	HOLD	CYCLE	
1min 72 °C	2 min 50 °C	30 min 60 °C	5 min 95 °C	20 sec 94 °C	1 min 58 °C

Preparing Reagent Mix

Prepare separate reagent mixes for the Influenza A assay and the Influenza H5 assay. Each reagent mix contains all assay components except the target RNA, positive control, or negative control.

To prepare the reagent mix:

1.	Thaw all reagents completely. **IMPORTANT!** Keep all tubes on ice.
2.	When the reagents are thawed, mix each tube component by vortexing.
3.	Using a microcentrifuge, briefly spin down the tube contents. **IMPORTANT!** Protect the Target Assay Mix from excessive exposure to light. When finished with the kit, return it to the –20 °C freezer.
4.	Label a sterile microcentrifuge tube with the first target name.
5.	Calculate the volume of components needed for the number of samples, replicates, and controls for your run (see Table 5 on page 15). Applied Biosystems recommends performing three replicates for each sample. Table 5 on page 15 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).
6.	Pipette the appropriate volumes of components into the reagent mix tube, then mix gently by inversion. **IMPORTANT!** Keep all tubes on ice.
7.	Repeat steps 4 through 6 for the second target assay reagent mix.

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

⚠ CAUTION CHEMICAL HAZARD. AmpErase® uracil N-glycosylase may cause eye and skin irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Table 5 Reagent Mix Components and Volumes

Component	Volume for One 50-µL Reaction (µL)	Final Conc	Volume for Ten 50-µL Reactions (µL)
RNase-free water	10.5	–	105.0
5X TaqMan EZ Buffer	10	1X	100.0
Manganese acetate (25 mM)	6	3 mM	60.0
dATP (10 mM)	1.5	300 µM	15.0
dCTP (10 mM)	1.5	300 µM	15.0
dGTP (10 mM)	1.5	300 µM	15.0
dUTP (20 mM)	1.5	600 µM	15.0
r*Tth* DNA Polymerase (2.5 U/µL)	2.0	0.1 U/µL	20.0
AmpErase UNG (1 U/µL)	0.5	0.01 U/µL	5.0
10× Assay Mix	5.0	450 nM primers 200 nM probes	50.0
Total Mix	**40**	–	**400**

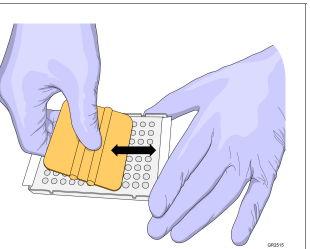
Pipetting Reagent Mix, Samples, and Controls

To pipette:

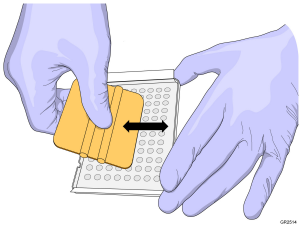
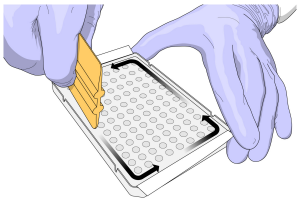
1.	For each sample or control you are assaying: a. Transfer 40 µL of the Influenza A reagent mix into the appropriate wells. b. Transfer 40 µL of the Influenza H5 reagent mix into the appropriate wells. **Note:** Use a new tip for each reagent mix. Keep plates and tubes on ice.
2.	Transfer 10 µL of RNA sample, RNA positive control, or negative control into the appropriate wells. Gently pipette up and down to mix the solution. **IMPORTANT!** Mix very gently with the pipette tip at the bottom of the tube to minimize aerosol formation and cross-contamination. **Note:** Use a new tip for each well, even when pipetting the same sample or control.
3.	Make sure reagents are in the bottom of the wells. If available, use a centrifuge with a plate adapter to briefly centrifuge the plate.

Sealing Plates or Tubes

To seal plates:

1.	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 seal plates: ***(continued)***

2.	Rub the flat edge of the applicator back and forth along the short edge (width) of the plate. 
3.	Rub the end of the applicator horizontally and vertically between all wells.
4.	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. 

To seal tubes:

1.	Place strip caps on the tubes.
2.	**IMPORTANT!** Apply significant downward pressure on the sealing tool in all steps to form a complete seal on top of the tubes. Using a sealing tool, affix caps to 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.

Running a Plate (Performing RT-PCR)

Overview

Running a plate consists of using an Applied Biosystems Sequence Detection System 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 a Plate

To run the plate:

1.	Open the plate document that corresponds to the reaction plate.
2.	Load the reaction plate into the SDS or Real-Time PCR System.
3.	Start the run.

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

Viewing 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 the entire plate.
2.	If you are using a 7300/7500 system, analyze the data using the Manual baseline option (not Automatic), and retain the default Ct baseline values (cycle 3 to 15). If you are using a 7000 or 7900HT Fast system, skip to step 3. These systems do not have an Automatic baseline option.
3.	Check each sample for FAM™ dye signal (target specific signal) and VIC® dye signal (IPC), then interpret results:

FAM signal (target)	VIC signal (IPC)	Result
Present	Present	Positive. See "Interpreting Results" on page 21.
Present	Absent	Invalid – repeat assay. See "Appendix A: Troubleshooting" on page 26.
Absent	Present	Negative
Absent	Absent	See "Appendix A: Troubleshooting" on page 26.

Interpreting Results

To interpret 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 34 at the default threshold of 0.2.

   Figures 2 and 3 show amplification plots for the IPC and negative control for Influenza A and H5 assays.

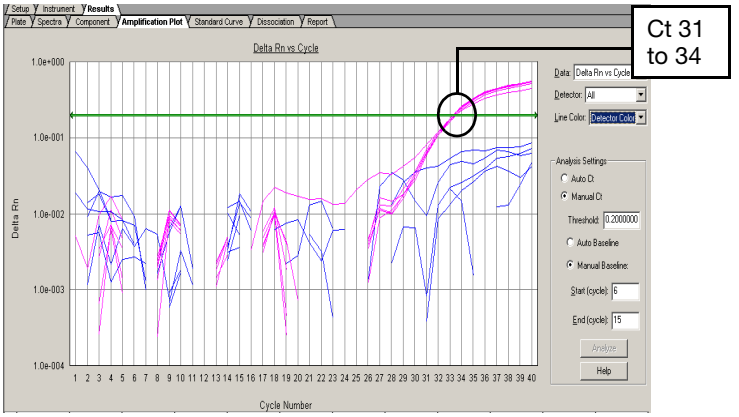


Figure 2 Influenza A Assay IPC in Negative Control Amplification Plot (7000 SDS System, Threshold 0.2)

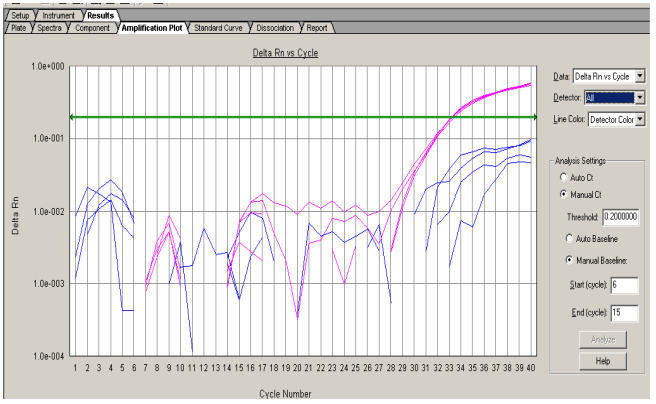
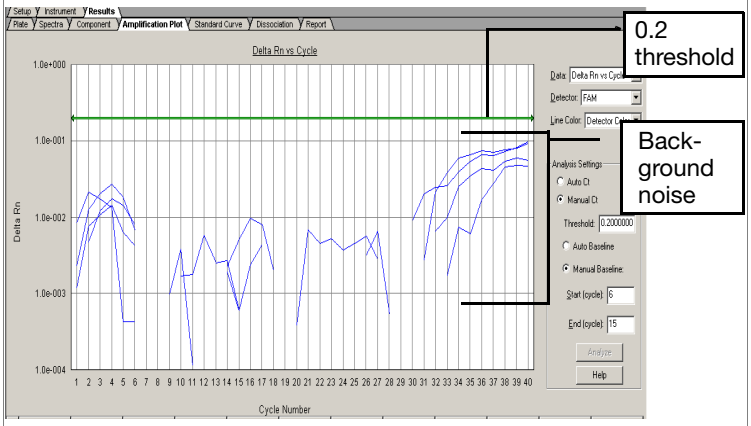


Figure 3 Influenza H5 Assay IPC in Negative Control Amplification Plot (7000 SDS System, Threshold 0.2)

To interpret results: ***(continued)***

2.	If you observe either of the following, see "Appendix A: Troubleshooting" on page 26: • IPC signal is not present in negative control, unknown, or positive control wells • IPC signal Ct is greater than 34 in unknown or positive controls wells • IPC signal Ct is inconsistent between unknown wells
3.	Examine all negative control wells. Figure 4 below and Figure 5 on page 23 show typical amplification plots for the negative control for Influenza A and Influenza H5 assays. Note that 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 typically remains below the 0.2 default threshold.  **Figure 4 Influenza A Assay Negative Control Amplification Plot (7000 SDS System, Threshold 0.2)**

To interpret results: *(continued)*

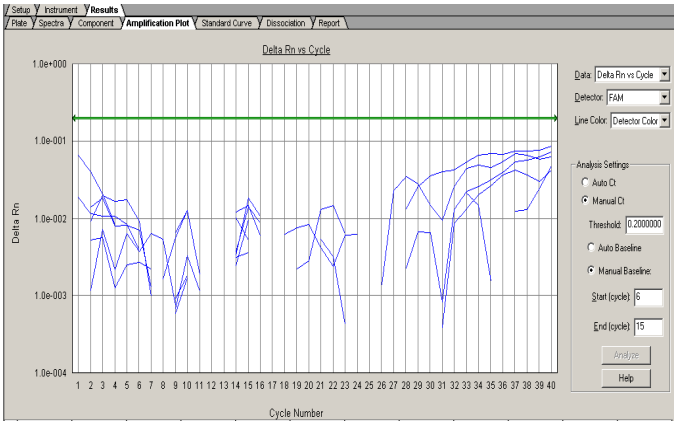


Figure 5 Influenza H5 Assay Negative Control Amplification Plot (7000 SDS System, Threshold 0.2)

4. Verify that no negative control samples show target-specific amplification (see Figure 6 on page 24 for an example of target-specific amplification).

   If you observe target-specific signal in negative control wells, see "Appendix A: Troubleshooting" on page 26.

5. If any negative control wells signals cross the default 0.2 threshold (which should occur infrequently) and 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. You will need to decrease if the threshold does not cross the exponential phase in positive wells (see steps 6 and steps 7).

   b. Analyze again.

      Note: Setting the threshold above negative control signal changes the Cts of the negative controls to "Undetermined."

To interpret results: ***(continued)***

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.

   Figures 6 and 7 show amplification plots for the RNA positive control for Influenza A and H5 assays detected at 1000, 100 and 10 copies per reaction.

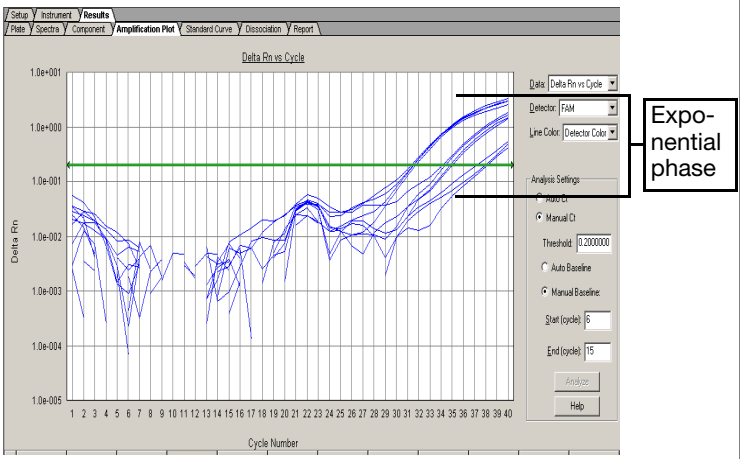


Figure 6 Influenza A Assay RNA Positive Control Amplification Plot – 1000, 100, and 10 Copies

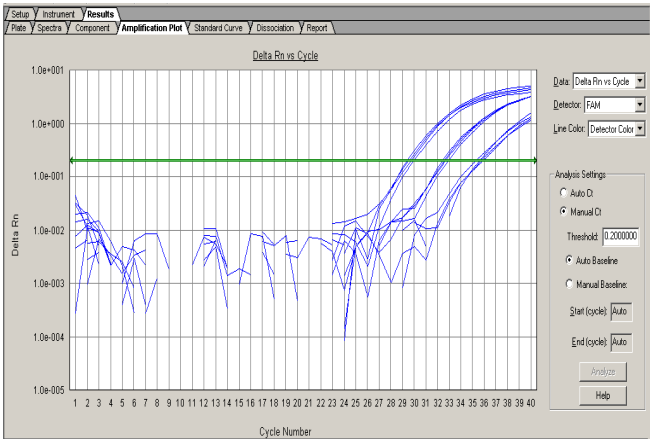


Figure 7 Influenza H5 Assay RNA Positive Control Amplification Plot – 1000, 100, and 10 Copies

To interpret results: ***(continued)***

7.	If you adjusted the 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 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 assay. 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® EZ RT-PCR reagents not stored properly	Repeat the assay using properly stored reagents. Avoid freezing and thawing assay components. Protect the Assay Mix from light.
	Target specific 10× Assay Mix not stored properly	
	Pipetting Error (no reagent mix added)	Repeat the assay. Make sure to pipette reagent solution into all wells.
IPC signal Ct is greater than 34 in unknown or positive controls wells	RT or PCR inhibited	Repeat sample preparation, then repeat assay. 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 the assay.
Target-specific signal detected in negative control wells	Carryover contamination	Repeat the assay using fresh aliquots of all reagents and clean pipetting equipment. If the negative control still shows contamination, repeat the assay using a new kit. If the negative control still shows contamination, contact Applied Biosystems Technical Support.

Observation	Possible Cause	Action
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 using fresh aliquots of all reagents and clean pipetting equipment.
No IPC or target-specific signal in positive control wells	TaqMan® EZ RT-PCR reagents not stored properly	Repeat the assay using properly stored reagents. Avoid freezing and thawing assay components. Protect the Assay Mix from light.
	Target specific 10× Assay Mix not stored properly	
No target-specific signal detected in positive control wells	Pipetting error (no positive control added)	Repeat the assay. Make sure to pipette positive control into all positive control wells.
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 duplex assay results:

- **Negative/Negative** – Non-Influenza A/Non-Influenza H5 subtype
- **Positive/Negative** – Influenza A/Non-Influenza H5 subtype
- **Positive/Positive** – Influenza A/Influenza H5 subtype

H5N1 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.

Table 6 Non-H5 Influenza A Isolates

Avian Isolates	Human Non-H5 Isolates
A/Quail/Hong Kong/G1/97 (H9N2)	A/Hong Kong/54/98 (H1N1)
A/Duck/Hong Kong/Y280/97 (H9N2)	A/Hong Kong/1174/99 (H3N2)
A/Teal/Hong Kong/W312/97 (H6N1)	———

Table 7 H5 Influenza Isolates

Non Human H5 Isolates	Human H5 Isolates
A/Gs/HK/437.6/99	A/HK/483/97
A/Gs/HK/739.2/02	A/HK/486/97
A/Dk/HK/821/02	A/HK/212/03
A/Ck/HK/31.4/02	A/HK/213/03
A/Ck/HK/96.1/02	A/Viet Nam/1203/2004
Ck/Vietnam/33/04	A/Thailand/MK2/2004

Table 7 **H5 Influenza Isolates *(continued)***

Non Human H5 Isolates	Human H5 Isolates
A/Thailand/AIV-1/04	———
CK/Indonesia/4/2004	———

Exclusion List

Non-influenza respiratory pathogens and other 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

Both the Influenza A and H5 assays included in the TaqMan® Influenza A/H5 Detection Kit Version 1.0 have been shown to detect as few as 100 copies of RNA present in a 50 µL reaction of the positive control.

Predicted Inclusion

Based on bioinformatics analysis, the assays included in this kit perfectly match and are predicted to detect the following strains (sequence submissions to NCBI 2001 through 2005):

- **Influenza A assay**: 355 human strains and 326 avian strains
- **Influenza H5 assay**:16 human strains and 273 avian strains

Note: It is anticipated that many A and A/H5 isolates beyond those specifically predicted will yield positive results.

References

Kwok, S. and Higuchi, R. 1989. Avoiding false positives with PCR. *Nature* 339:237–238.

Longo, M.C., Berninger, M.S., and Hartley, J.L. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93:125–128.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol*. 155:335–350.

Saiki, R.K., Scharf, S., Faloona, F., *et al*. 1985. Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354.

Worldwide Sales and Support

Applied Biosystems vast distribution and service network, composed of highly trained support and applications personnel, reaches 150 countries on six continents. For sales office locations and technical support, please call our local office or refer to our Web site at **www.appliedbiosystems.com**.

Applied Biosystems is committed to providing the world's leading technology and information for life scientists.

Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: +1 650.638.5800
Toll Free (In North America): +1 800.345.5224
Fax: +1 650.638.5884

06/2010

Applied Biosystems

Part Number 4370566 Rev. C