Avian Influenza Virus RNA Test Kit

VetMAX™-Gold AIV Detection Kit

Catalog Number 4485261

Pub. No. 4486415 Rev. B

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

WARNING! The 2X Multiplex RT-PCR Buffer may cause eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Wear appropriate protective eyewear, clothing, and gloves. First Aid: If inhaled, remove to fresh air. In case of contact, flush thoroughly with water. If symptoms develop, get medical attention.

WARNING! The Multiplex RT-PCR Enzyme Mix contains ethylene dioxide, which is known to the State of California to cause cancer.

Product information

Name, intended use, and principle of the procedure

The Applied Biosystems™ Avian Influenza Virus RNA Test Kit is a highly sensitive, qualitative, one-step, real-time reverse transcription PCR (real-time RT-PCR) assay to detect Avian Influenza Virus (AIV) RNA isolated from individual poultry (chicken/turkey) oropharyngeal/tracheal swab samples.

AIV is an enveloped, negative-sense RNA virus of the genus *Influenzavirus A* and family Orthomyxoviridae. AIV subtypes are defined by the surface glycoproteins hemagglutinin and neuraminidase. Low pathogenic avian influenza (LPAI) strains exist in avian reservoir hosts and can be transmitted to poultry. H5 and H7 subtypes of LPAI strains are unique in their capability to undergo host adaptation and to evolve into highly pathogenic avian influenza (HPAI). HPAI viral infections arise *de novo* in poultry infected with LPAI H5 and H7 subtypes and become rapidly fatal due to an overwhelming systemic collapse. The VetMAX™-Gold AIV Detection Kit enables diagnosis of AIV in poultry populations.

The assay consists of a single-well/tube, real-time RT-PCR assay in which RNA is reverse-transcribed into cDNA; two viral matrix targets and one nucleoprotein target are amplified and detected in real time using fluorescent TaqMan™ probes (hydrolysis probe chemistry). The assay detects sequences that are common to all AIV subtypes. The kit includes:

- Influenza Virus-Xeno™ RNA Control Mix, to serve as a positive control for the real-time RT-PCR components and it is also used to set the cycle threshold (C_t) for evaluating test results.
- Xeno™ RNA Control, to serve as an internal positive control for the RNA purification process and monitor for the presence of PCR inhibitors.
- Influenza Virus Primer Probe Mix, optimized for multiplex real-time RT-PCR amplification of Xeno™ RNA Control and AIV RNA targets.

Limitations

- The kit is not intended for differentiating AIV subtypes.
- Handle samples as recommended in Table 2 to prevent degradation of any AIV RNA that is present.
- RNA extraction methods should yield RNA free of RT-PCR inhibitors, which can prevent amplification of target RNA.
- Follow “Good laboratory practices for PCR and RT-PCR” on page 5 to prevent false positive amplifications due to contamination of test samples with PCR products.

Contents and storage

Reagents for 100 25-µL real-time RT-PCR tests are supplied.

Table 1 VetMAX™-Gold AIV Detection Kit

Component	Amount	Storage
2X Multiplex RT-PCR Buffer	1.375 mL	–30°C to –10°C
Multiplex RT-PCR Enzyme Mix	280 µL	
Influenza Virus Primer Probe Mix	110 µL	
Xeno™ RNA Control (10,000 copies/µL)	250 µL	
Influenza Virus-Xeno™ RNA Control Mix (1,000 copies/µL)	30 µL	
Nuclease-free Water	1.175 mL	–30°C to 25°C

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Required materials not supplied

Item	Source[1]
Real-time PCR instrument and accessories, one of the following:	
7500 Fast Real-Time PCR System (96-well), running SDS Software v1.4 7500 Fast Precision Plate Holder, for 0.1 mL tube strips (Cat. No. A29252), or equivalent	Contact your local sales office.
QuantStudio™ 5 Real-Time PCR System, 96-well, 0.1-mL	Contact your local sales office.
Equipment	
Microcentrifuge	MLS
Laboratory mixer (vortex or equivalent)	MLS
Nuclease-free pipettors	MLS
2 ice buckets: • One for the PCR setup area where the master mix is prepared • One for the area where RNA may be present	MLS
Plates or tubes and caps	
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	4366932 (200 plates), 4346906 (20 plates), or equivalent
MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL	4346907
MicroAmp™ Optical Adhesive Film	4311971 (100 covers), 4360954 (25 covers), or equivalent
MicroAmp™ Fast 8-Tube Strip, 0.1 mL	4358293, or equivalent
MicroAmp™ Optical 8-Cap Strips	4323032, or equivalent
Additional consumables and reagents	
Filtered pipette tips	thermofisher.com/pipettetips
Nuclease-free reagent tubes for preparing master mixes	MLS
1X Phosphate Buffered Saline (PBS), pH 7.4	MLS
Viral Transport Media	MLS

[1] Unless otherwise indicated, all materials are available through thermofisher.com. "MLS" indicates that the material is available from fisherscientific.com or another major laboratory supplier.

Isolate RNA from samples

Table 2 Sample handling recommendations

Step or process	Recommendation
Transport/storage of samples	Transport oropharyngeal/tracheal swab samples between 4°C and 25°C or in accordance with manufacturer's instructions.
Preparation of swab samples	1. Place one oropharyngeal/tracheal swab sample into a 1.5-mL tube or deep-well 96-well plate, then add 0.75 mL of Viral Transport Media. 2. Vortex vigorously for 3 minutes, then pulse-spin to remove debris from the tube cap. 3. Remove 50 µL of supernatant for RNA isolation.
Preparation of mock-purified samples (for use in extraction control PCRs)	Prepare duplicate mock-purified samples, using 1X PBS as the starting material. Process with the same RNA isolation method that is used for test samples.
Proposed RNA isolation method	MagMAX™-96 Viral RNA Isolation Kit (Cat. No. AM1836, AMB1836-5) or an equivalent RNA isolation method.
Required modifications to the RNA isolation method	• Add 2 µL of undiluted Xeno™ RNA Control per isolation to the lysis solution used for RNA isolation. • Add carrier RNA to the lysis solution according to the manufacturer recommendations. Carrier RNA is provided in the MagMAX™-96 Viral RNA Isolation Kit (Cat. No. AM1836, AMB1836-5).

Perform real-time RT-PCR

1 Determine the quantity of reactions and thaw the reagents

a. On each plate, include the following control reactions (see "Set up and run the real-time PCR instrument" on page 3):
   - Positive control (prepare duplicate reactions); use 8 µL of the Influenza Virus-Xeno™ RNA Control Mix (1,000 copies/µL).
   - No-template control (NTC) (prepare duplicate reactions); use nuclease-free water in place of sample RNA.

b. Plan the plate layout so that the wells containing NTCs are located as far as possible from positive controls and test samples to prevent accidental cross-contamination.

c. Thaw RT-PCR master mix reagents in one ice bucket and controls and samples in a separate ice bucket. Gently vortex each tube to mix the contents thoroughly, then briefly centrifuge to collect the solution at the bottom of the tube. Keep the reagents on ice.

2 Prepare the RT-PCR master mix on ice

Combine the following components for the number of reactions required plus 10% overage.

Component	Volume per 25 µL reaction
2X Multiplex RT-PCR Buffer	12.5 µL
Multiplex RT-PCR Enzyme Mix	2.5 µL
Influenza Virus Primer Probe Mix	1.0 µL
Nuclease-free water	1.0 µL
Total volume of RT-PCR master mix	**17.0 µL**

3 Set up the RT-PCR reactions

a. Dispense 17 µL of RT-PCR master mix to the appropriate wells of a PCR plate or PCR tubes on ice.

b. Add the appropriate component for the reaction type, according to the following table:

Reaction type	Component	Volume per reaction
Test sample	Sample RNA	8.0 µL
NTC	Nuclease-free water	8.0 µL
Positive control	Influenza Virus-Xeno™ RNA Control Mix (1,000 copies/µL)	8.0 µL
Extraction control	Mock-purified PBS sample	8.0 µL

c. Seal each reaction vessel, mix, then centrifuge briefly to bring the contents to the bottom.

4 Set up and run the real-time PCR instrument

For detailed information to set up and run the instrument, see the appropriate documentation for your instrument.

a. Following the manufacturer's instructions, set up the run using the following parameters:

- Experiment type: Standard curve
- Run mode: Standard
- Reaction volume: 25 µL
- ROX™ passive reference dye: Included in the RT-PCR Buffer
- TaqMan™ probe reporter dyes and quenchers:

Target	Reporter	Quencher
AIV	FAM™ dye[1]	Eclipse™ Q
Xeno™ RNA Control	VIC™ dye[2]	Eclipse™ Q

[1] Absorbance maximum of 495 nm; emission maximum of 520 nm.

[2] Absorbance maximum of 540 nm; emission maximum of 552 nm.

b. Run the thermal cycler program and collect real-time amplification data during stage 3. Use the following thermal cycler settings:

Stage		Reps.	Temp.	Time
Reverse transcription	1	1	48°C	10 minutes
RT inactivation/initial denaturation	2	1	95°C	10 minutes
Amplification	3	40	95°C 60°C	15 seconds 45 seconds

Data analysis

See your real-time PCR instrument user guide for instructions on how to analyze your data, using the following method.

Table 3 Data analysis

Method	Details
Use the **Control-Based Threshold** setting for data analysis.	1. Select **Manual CT**. 2. Export ΔR_n values for the positive control samples (Influenza Virus-Xeno™ RNA Control, 1,000 copies/µL). 3. Average the FAM™ and VIC™ dye values (separately) for the ΔR_n at cycle 40 for all replicates of the positive control reaction. 4. Set the threshold for the AIV RNA reactions at 5% of the average maximum fluorescence value of the AIV RNA amplification signal in the positive control reactions. Example: If the average maximum fluorescence value for the AIV RNA target in the positive control reactions is 3.0, set the AIV RNA threshold at 0.15. 5. Repeat step 4 for the Xeno™ RNA Control target using a 5% threshold. Example: If the average maximum fluorescence value for the Xeno™ RNA target in the positive control reactions is 2.0, set the Xeno™ RNA threshold at 0.1.
Check the raw fluorescence data.	Verify that increased fluorescence seen in the normalized data is also evident without mathematical data processing.

Interpretation of test results

Verify that your real-time RT-PCR run is valid before analyzing test sample results.

Table 4 Criteria for a valid real-time RT-PCR run

Reaction type	C_t value for AIV RNA	C_t value for Xeno™ RNA Control
Positive control	25–29	25–29
NTC	Not detected[1]	Not detected[1]
Extraction control	Not detected[1]	27.5–34

[1] If the C_t value is <40, see "Troubleshooting" on page 5.

Table 5 Interpretation of sample test results

C_t value for AIV RNA	C_t value for Xeno™ RNA	Interpretation
<38	27.5–34[1]	AIV-positive sample
Not detected	27.5–34	AIV-negative sample
38–40	27.5–34[2]	Suspect result[2]

[1] See "Troubleshooting" on page 5.
[2] See Table 6.

Table 6 Assessment of suspect results

Result	Action	
Suspect result: The sample AIV C_t value is 38–40.	Analyze suspect RNA samples for the presence/absence of RT-PCR inhibitors by calculating the Xeno™ RNA C_t shift: **Xeno™ RNA C_t Shift = SS – XEC**, where: **SS =** C_t of Xeno™ RNA in the suspect sample **XEC =** Average C_t of Xeno™ RNA in the extraction controls	
	Workflow A **Xeno™ RNA C_t shift ≥1.5**	**Workflow B** **Xeno™ RNA C_t shift <1.5**
	1. Repeat the real-time RT-PCR with 2 µL of the suspect RNA sample. (RT-PCR inhibitors may be present in the RNA.) If the AIV C_t value is: • <38—The sample is AIV positive. No further testing is required. • ≥38—Continue with steps 2 through 5 of this procedure. 2. Dilute the original diagnostic sample 1:4. 3. Repeat the RNA purification on triplicate aliquots of the diluted sample. 4. Repeat the real-time RT-PCR with 8 µL of purified RNA from step 3. 5. Determine the number of samples with a AIV C_t value <40: • 0 of 3: AIV negative • 1 of 3: Presumptive positive; confirm with secondary test method • ≥2 of 3: AIV positive	1. Repeat the RNA purification on triplicate aliquots of the original diagnostic sample. 2. Repeat the real-time RT-PCR with 8 µL of purified RNA from step 1. 3. Determine the number of samples with a AIV C_t value <40: • 0 of 3: AIV negative • 1 of 3: Presumptive positive; confirm with secondary test method • ≥2 of 3: AIV positive

Confirmatory testing

All samples generating a positive or presumptive positive test result with the VetMAX™-Gold AIV Detection Kit should be submitted to a national laboratory (or equivalent authorized laboratory) for confirmatory testing.

Troubleshooting

Observation	Possible cause	Recommended action
Positive control reaction: Influenza Virus-Xeno™ RNA Control—no signal Xeno™ RNA Control—no signal	The Influenza Virus-Xeno™ RNA Control Mix was improperly handled, resulting in RNA degradation.	Use appropriate precautions against RNase contamination when handling the control RNAs. For example, wear clean gloves and use nuclease-free barrier pipette tips.
	The Multiplex RT-PCR Enzyme Mix was stored or handled improperly, and it lost activity.	Repeat the RT-PCR with fresh reagents.
	The thermal cycler was not properly set up.	Check the thermal cycler settings. See "Set up and run the real-time PCR instrument" on page 3.
	The RT-PCR master mix was prepared incorrectly.	Repeat the test with correctly prepared RT-PCR master mix.
NTC or extraction control reaction: C_t value is <40	There was contamination during the RNA extraction or PCR.	• Repeat the RNA isolation or real-time RT-PCR with fresh reagents and freshly decontaminated pipettes. • Set up the real-time RT-PCR in an area separate from areas used for RNA isolation and PCR product analysis.
Test samples: Xeno™ RNA Control—no or low signal AIV RNA—high signal	The Xeno™ RNA Control primers and probe are at limiting concentrations in the RT-PCR. High levels of AIV RNA in a sample can reduce amplification of Xeno™ RNA Control.	No or low signal from Xeno™ RNA Control is expected in a reaction that has a strong signal for AIV RNA.
Test samples: Xeno™ RNA Control—no signal AIV RNA—no signal or suspect-range signal	Poor RNA recovery.	• Check the C_t values of Xeno™ RNA Control in the mock-purified samples. • C_t ≥38: indicates that Xeno™ RNA Control was omitted or that RNA recovery was poor. • Repeat the RNA purification of the original diagnostic sample.
	The RNA samples contain inhibitors of RT-PCR.	See Table 6.

Good laboratory practices for PCR and RT-PCR

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution.

Documentation and support

Customer and technical support

In the United States, call 1-800-955-6288.

Visit **thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support
- Order and web support
- Product documentation

Limited product warranty

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Revision	Date	Description
B	27 February 2020	• Updated the list of compatible real-time PCR systems. • Updated to the current document template, with associated updates to the warranty, trademarks, and logos. • Added new warning statements regarding the 2X Multiplex RT-PCR Buffer and Multiplex RT-PCR Enzyme Mix.
A	25 March 2014	New document.

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