1.0 DESCRIPTION

1.1 H5N1 Bird Flu Assay v1.0

1.1.1 The H5N1 Bird Flu Assay v1.0 is a single plex, RT-qPCR test that has been formulated to detect the H5 (hemagglutinin (HA) gene) in the H5N1 strain of influenza. This has been coupled with the robust Influenza A Pan Flu assay. This combination will detect all strains of Influenza but will also identify specifically the H5 (hemagglutinin (HA) gene) on those samples from positive subjects with the H5 strain of influenza. The H5N1 assay has been designed to be specific and detect multiple clades of H5N1, including clade 2.3.4.4b of highly pathogenic avian influenza viruses currently circulating in 2024. The H5N1 Bird Flu Assay v1.0 detects the following targets in Table 1.

Table 1

Targets
H5 (hemagglutinin (HA) gene Influenza H5N1)
Influenza A (pan flu)
SPRY1 Internal control

1.2 The H5N1 Bird Flu Assay v1.0 may not detect emerging novel mutated strains.

1.3 The H5N1 Bird Flu Assay v1.0 is for Research Use Only. Not intended for in-vitro diagnostics. This product has not been approved by the FDA.

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2.0 TEST PRINCIPLE

2.1 The H5N1 Bird Flu Assay v1.0 provides probes and primers required to perform a single plex RT-qPCR detection of specific targets. The primer and probe sets are designed to detect nucleic acid from targets listed in Table 1. The SPRY1 gene is used as an internal sample integrity and extraction control. This novel multi-species control will be positive for a variety of human and veterinary applications. The SPRY1 gene assay will detect DNA from most mammalian and avian species in addition to human DNA. Specific positivity for target species to be determined by end user.

2.2 Amplification master mix is not included and must be purchased separately. This product has been specifically formulated to perform optimally with the following amplification kit. The use of alternate master mixes is not supported by Bandit Biosystems.

- 2.2.1 SeqOnce OneStep RT-qPCR Multiplex Amplification Kit 1800 - Part Number: 500030

2.3 A suitable extraction method is needed for isolation of nucleic acid from the specific sample types being processed. The extracted sample is then added to a master mix containing Taq Polymerase, nuclease free water, RT mix, and H5N1 Bird Flu Assay v1.0 Primer/Probe Mix. The Reverse Transcriptase in the RT mix will transcribe the RNA into cDNA. The DNA polymerase will amplify the cDNA fragments that share homology with the primer/probe sets. During the amplification phase of the PCR cycle, the probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are separated from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by the real-time PCR instrument. The workflow consists of four steps:

- 2.3.1 Sample extraction
- 2.3.2 RT-qPCR Plate setup
  - 2.3.2.1 Master Mix setup
  - 2.3.2.2 Add extracted sample to qPCR reactions
- 2.3.3 RT-qPCR
- 2.3.4 Analysis

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3.0 REAGENTS/MEDIA

3.1 H5N1 Bird Flu Assay v1.0 Primer/Probes + Controls Kit

3.1.1 Materials Provided (See Table 2 and Table 3)

3.2 Concentration and Storage

3.2.1 15X Primer/Probe Mix, 10,000 copy/µL control

3.2.2 Store at -20°C upon arrival

Table 2

Kit Part Number	Kit Description	Component Part Number
500029	H5N1 Bird Flu Assay v1.0, 200 Reactions	400089 450038

Table 3

Component Part Number	Description	Volume	Storage
400089	15X H5N1 Bird FluPrimer Probe Mix (1) Multiplex	(2) x 110 µL	-20°C
450038	H5N1 Bird Flu Positive Control (1) Control Tube	(1) x 100 µL	-20°C

3.3 Recommended But Not Included (see Table 4 below, only one required)

Table 4

Kit Part Number	Kit Description	Volume	Storage
500030	SeqOnce OneStep RT-qPCR Multiplex Amplification Kit 1800	(3) x 0.6 mL Master Mix (1) x 1 mL Water (1) x 0.110 mL RT Mix	-20°C

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4.0 SUPPLIES/MATERIALS SUGGESTED BUT NOT PROVIED

Table 5

Item	Source
Optical 96-Well or 384-well PCR Plates	Instrument specific
Optical Adhesive Film	Instrument specific
Nucleic acid extraction method suitable to extract RNA and DNA from desired sample type	Sample type specific

5.0 GENERAL LABORATORY EQUIPMENT AND REAGENTS SUGGESTED BUT NOT PROVIDED

Table 6

Equipment	
Item	**Source**
qPCR instrument	Any capable of detection in FAM, HEX and ROX fluorescence channels or similar wavelengths to these.
Vortex Mixer	Any
Microcentrifuge	Any
Microwell plate centrifuge, with a rotor that accommodates standard microplates	Any
Laboratory mixer, vortex, or equivalent	Any
Laboratory Freezers -30°C to -10°C	Any
Micropipettes (2 or 10 µL, 200 µL and 1000 µL)	Any
Multichannel micropipettes (2-20 uL)	Any
2 x 96-well -20°C cold blocks	Any
Optional Tubes, Plates, and Other Consumables	
Racks for 1.5-mL microcentrifuge tubes	Any
Molecular grade water, nuclease-free	Any
10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)	Any
DNAZap or equivalent	Ambion AM9890

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RNAse Away or equivalent	Fisher Scientific 21-236-21
Disposable powder-free gloves and approved personal protective equipment	Any
Aerosol barrier pipette tips	Any
1.5-mL microcentrifuge tubes (DNase/RNase free)	Any
Tris-EDTA (TE) pH 8.0- molecular grade DNAse RNAse free	Any

6.0 SAFETY PRECAUTIONS AND GOOD LABORATORY PRACTICES

6.1 Wear disposable gloves, laboratory coats and protective eyewear when handling reagents and samples. Handle all samples as potentially infectious and follow all safety guidelines in accordance with Good Laboratory Practices.
6.2 Only use DNase and RNase free reagents and consumables.
6.3 Do not use reagents beyond their expiration.
6.4 Before using, inspect each tube for signs of leakage. Do not use if there is any sign of leakage.

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7.0 QUALITY CONTROL

7.1 A Negative and Positive Control must be included in each assay run to detect potential failure in specimen processing, amplification, or detection steps.

7.2 SPRY1 extraction control is included in the Primer/Probe Mix. This is an internal DNA control extracted together with the sample nucleic acid. It is amplified and detected in the same way as target nucleic acid. This demonstrates a control of the extraction procedure and the detection of potential PCR inhibition.

7.3 Controls are provided at a concentration of approximately 10,000 copies / µL. Dilutions may be made in Tris-EDTA (TE) pH 8.0 or provided dilution buffer.

7.3.1 Controls Provided (See Table 7)

Table 7

Control	
Part Number 450038	*H5N1 Bird Flu Positive Control*

8.0 RECOMMENDED PROTOCOL

8.1 Provided Primer/Probe Mix

Table 8

Primer Group/Tube	Targets	Dye
1	*H5 (hemagglutinin (HA) gene Influenza H5N1)*	FAM
	Influenza A (pan flu)	HEX
	SPRY1 internal control	ROX

8.1.1 HEX can be replaced by VIC or JOE if not present on the program.

8.1.2 ROX can be replaced by TEXAS RED if not present on the program.

8.1.3 Select all three dyes for each labeled well.

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8.2 Set-up Instructions

8.2.1 Reaction volumes have been optimized at 15 µL and may be run at this volume in either 96 or 384 well instruments.

8.2.2 Extract DNA and RNA from sample per separate protocol as determined by individual user.

8.2.3 The sample is now ready to be added to the RT-qPCR reaction (see following steps).

8.2.3.1 RT-qPCR Reagent Setup

8.2.3.1.1 Completely thaw the Master Mix (or equivalent), Nuclease free water, RT Mix, and primer/probe mix by setting on ice for ~30 minutes or thawing at room temperature for ~5 minutes.

8.2.3.1.2 Once thawed, briefly vortex the tubes at max speed to mix, then spin down briefly to collect at bottom of tube. Check that there is no precipitate at the bottom of the Master Mix tube. If any is present, vortex tube until it dissolves and then spin down.

8.2.3.1.3 In a clean room or designated setup area, combine Master Mix in a 1.5ml or larger microcentrifuge tube in the order shown below (See Table 9 below) for a 15µL reaction volume.

Table 9

Component	Volume Reaction	Final Concentration
2X Exmoor Master Mix (PN: 400084)*	7.5 µL	1X
RT Mix (PN:400090)	0.5 µL	1X
Nuclease Free Water	2.0 µL	N/A
15X Primer/Probe Mix *	1 µL	1X

*vortex to mix prior to use

8.2.3.1.4 Mix the master mix made from step above by vortexing briefly.

8.2.3.1.5 Distribute **11 µL** of the master mix using an appropriate pipette to all wells of a plate that will be used.

8.2.3.1.6 Add **4 µL** of extracted sample DNA, positive control, and NTC to appropriate wells.

8.2.3.1.7 Seal the plate and spin down in a centrifuge for 30 seconds at 3000 RPM to collect the mixed samples.

8.2.3.1.7.1 If the centrifuge does not go to speeds of 3000 RPM, then use the highest speed possible for the instrument.

8.2.3.1.8 Place the plate into the designated qPCR instrument and run the following conditions:

8.2.3.2 Thermal Cycling Conditions

8.2.3.2.1 Recommend Cycling Conditions (See Table 10 below)

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Table 10

Cycling Step	Stage	No. of Cycles	Temperature	Holding Time
Reverse Transcription	1	1	50°C	15 minutes
RT inactivation, Taq polymerase Enzyme Activation	1	1	95°C	2 minutes
Amplification*	2	40	95°C	3 seconds
			58°C	30 seconds

*Collect fluorescence during annealing/extension phase (58°C) step on FAM, HEX, and ROX channels (or equivalent channels).

9.0 RECOMMENDED INTERPRETATION OF RESULTS

9.1 Appropriate cut off for positivity and analysis must be independently established. The guidelines below are for general use only and are not to be used as the sole method of interpretation (See Table 11 and Table 12 below).

9.2 The H5 hemagglutinin (HA) gene from Influenza H5N1 is not specific to the N1 strain. Other Influenza viruses exhibiting the hemagglutinin (HA) gene such as H5N2 or other rare strains could also be detected.

9.3 The pan flu A assay is specific to a highly conserved region in the matrix protein 1 (M1) gene. This assay will be positive for all H*N* strains of influenza, including H5N1.

Table 11

Sample Interpretation		
Ct<40	+	Nucleic Acid Detected
Ct>40	-	Nucleic Acid Not Detected

Table 12

Pathogen Pan Flu (HEX)	Pathogen H5 (FAM)	SPRY1 (ROX)	Result Interpretation
-	-	-	Invalid
-	-	+	Pathogen Nucleic Acid not detected
-	+	+/-	Inconclusive
+	+	+/-	Influenza H5 specific detected
+	-	+/-	Influenza A – Not H5 detected

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10.0 LIMITATIONS

10.1 False-negative results may arise from:
- 10.1.1 Improper sample collection
- 10.1.2 Degradation of genetic material during shipping/storage
- 10.1.3 Specimen collection after pathogen genetic material can no longer be found in the specimen matrix
- 10.1.4 Unvalidated media or assay reagents
- 10.1.5 The presence of qPCR inhibitors
- 10.1.6 Mutation in the pathogen genomes
- 10.1.7 Improper vortexing and centrifuging when preparing RT-qPCR reactions
- 10.1.8 Expired reagents

10.2 False-positive results may arise from:
- 10.2.1 Cross contamination during specimen handling or preparation
- 10.2.2 Cross contamination between samples
- 10.2.3 Specimen mix-up
- 10.2.4 DNA contamination during product handling
- 10.2.5 Improper sealing of the PCR plate

11.0 SUPPORT

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