HPAI H5 Subtyping in Milk and Milk Products Using RT-qPCR

EXPIRATION 12/2025

The recent emergence of the Highly Pathogenic Avian Influenza (HPAI) in cattle in the U.S has elevated concerns over whether the virus can result in contamination of the milk supply for humans and animals and has spurred the need for effective and efficient testing of milk for HPAI. Currently, molecular diagnostic tools are being used to screen milk for potential HPAI contamination, but confirmation must be accomplished through traditional propagation methods to determine if any viable virus is present in the milk. To support response for this event, FDA will screen milk and milk products for Influenza A virus (IAV) followed by H5 subtyping and submit positive samples to a diagnostic laboratory for confirmation testing.

The method described here was developed for emergency use for HPAI H5 subtyping which includes an internal amplification control and uses the Qiagen One Step RT-qPCR kit for detection. Analysis should be completed using RNA extracted from screened milk and milk products where the IAV M gene was detected (*Standard Operating Procedure for Extraction and Detection of Influenza A Virus in Milk and Milk Products*).

Safety Considerations:

- **Prior to performing this HPAI analysis, laboratories should conduct facility and matrix specific risk assessments to determine adequate controls to implement for minimizing risks associated with analysis. FDA's ORA Safety Office has conducted general risk assessments for screening of raw and pasteurized milk product(s) for HPAI based on ORS lab information. The *HPAI Risk Assessment Guidance* has been posted for ORS laboratory reference. ORS local laboratory BSL2+ leads and management should access this site regularly for updates to assist in performing site-specific risk assessments and identify any additional risks that may be present and need to be accounted for with alternate local procedures when testing these matrices.**

  Refer to BAM Chapter 26 and Appendices for guidance on typical logarithmic curves, false positive curves and additional data analysis guidance.

Table of Contents

1. Materials and Reagents

a. DNase/RNase-free water (Life Technologies AM9937 or equivalent)
b. Deionized/Distilled water/Milli-Q water
c. DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5 ml (Life Technologies AM12350 or equivalent)
d. DNase/RNase-free microcentrifuge tubes 1.5 ml, non-stick, low retention, siliconized (Life Technologies AM12450 or equivalent)
e. DNase/RNase-free microcentrifuge tubes 2.0 ml, non-stick, low retention, siliconized (Life Technologies AM12475 or equivalent)
f. Filter barrier aerosol resistant micropipettor tips DNase/RNase-free (0.2 – 1000 µl)
g. Qiagen collection tubes (Qiagen 19201)
h. 2.0 mL microcentrifuge tubes DNase/RNase-free (USA Scientific 1620-2799 or equivalent)
i. OneStep RT-PCR Kit (Qiagen 210210 or 210212)
j. Ambion Superase·In RNase Inhibitor (20 units/µl); Life Technologies AM2694 (2,500 U) or AM2696 (10,000 U)
k. 50 mM $MgCl_2$ (BioRad 1708872 or equivalent) or 25 mM $MgCl_2$ (ThermoFisher Scientific AB0359, or equivalent)
l. Internal Control RNA (BioGX Cat No. 750-0001—contact company)
m. Standard desalted primers and HPLC probes for all RT-qPCR assays (Integrated DNA Technologies or equivalent)
n. RT-qPCR positive controls (Hem5 and Hem5 no.2 gblocks, sequences available in document, Integrated DNA Technologies or equivalent)
o. RT-qPCR no template control (Nuclease free-water Applied Biosystems AM9937)
p. ROX Reference dye (Invitrogen cat#12223-012)

2. Equipment and Supplies

a. Ultra-low freezer (-70˚C or lower)
b. Latex or nitrile gloves (powder-free)
c. Vortex mixer (Labsource S16-109 or equivalent)
d. DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5 ml (Life Technologies AM12350 or equivalent)
e. Hype-Wipe disinfecting towelettes (Fisher Scientific 14-412-56 or equivalent)
f. Adjustable micropipettors (0.2 - 1000 µl), dedicated for RNA work only
g. Filter barrier aerosol resistant micropipettor tips DNase/RNase-free (0.2 – 1000 µl)
h. AB 7500 FAST (Applied BioSystems 4351106)
i. ABI 96 well plates (Life Technologies 4346906)
j. ABI plates cover (Life Technologies 4311971)
k. Mini plate spinner (Fisher Scientific 14-100-143 or equivalent)
l. Mini centrifuge (Labsource C90-044 or equivalent)
m. 96 well cool rack (Sigma Aldrich #Z606634-1EA or equivalent)

Outlined H5 RT-qPCR Protocol ABI 7500 (refer to Table 1 for primers and probes and Tables 2-7 for master mix components. Refer to manufacturer's instructions for preparation of gblock positive controls).

Cycling Conditions

Reverse transcription: 50°C for 3000 sec Activation: 95°C for 900 sec

Cycling: 45 cycles of 95°C for 15 sec, 55°C for 20 sec, then 64°C for 70 sec with optics on

Reaction Set-Up

Note: Always use aerosol resistant pipette tips for PCR.

1. Thaw primer solutions, probe solutions, and buffer mix and place them in 4°C bench top cool block or on ice in master mix set up hood.

2. Vortex reagents for 2-3 sec at setting 7-10, and then briefly centrifuge for 3-5 sec in a mini centrifuge to settle the liquid to the bottom of the tube. Place in ice or bench top cooler. Keep Enzyme Mix in cooling block or on ice at all times, these enzymes should not be defrosted.

3. Prepare Master Mix for all sample and control reactions as listed in table B2.2 (additional master mix preparations can be found in Appendix F. Keep all thawed components, reagents, controls and master mixes in cooling block.

   Note: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared. Negative and positive controls should be added to each reaction set-up.

4. Proceed to hood/area or room where the template is added and thaw IAC RNA and sample RNA in the designated hood where the template is added. Briefly centrifuge the tubes 3-5 sec in micro-centrifuge to settle the liquid at the bottom of the tube. Add appropriate volume of IAC, (0.2 µl/rxn) to Master Mix (keep cold); Vortex briefly & Pulse spin.

5. Add 22 µl Master Mix to each designated reaction tube or sample wells.

6. Add 3 µl of sample template to three designated reaction tubes or sample wells.

7. Close reaction tubes or seal sample plate once sample and appropriate controls have been added, briefly spin to mix bring down reagents.

Instrument Set-Up

1. Open AB Software (version 2.0-2.3).

2. Fill out the “Experiment Name” field.

3. Select 7500 (FAST).

4. Select “Quantitation Standard Curve.”

5. Select “TaqMan Reagents.”

6. Select “Standard (~2.5 hours to complete run).”

7. Click “Plate Set Up.”

8. Under the “Define Targets” area click on the “Add New Target” until you have 3 targets.

9. Fill Target 1 with H5 NAEA MOD, Target 2 with H5 MX, and Target 3 with IAC.

10. Change the reporter dye to FAM for H5 NAEA MOD, Cy5 for H5 MX and JOE for IAC (do not change quenchers from the default NFQ- MGB).

11. Under Sample section click “Add Samples” until you have the requisite number of samples.

12. Fill in your sample name in each of the areas provided.

13. Click “Assign Targets & Samples” tab and name samples and assign samples to the appropriate well(s).

14. Select ROX as the passive reference dye.

15. Select “Run Method.”

16. The first holding stage is 3000 sec (50 min) at 50°C.

17. The second holding stage is 900 sec (15 min) at 95°C.
18. Cycling stage repeats 45 cycles.
19. First stage 15 sec at 95°C.
20. Second stage set at 20 sec at 55°C.
21. Third stage set at 70 sec (1 min 10 sec) at 64°C (click the icon for collecting data at "collect data on hold").
22. Save the run method – Save as H5 Multiplex.
23. Click the "Analysis" tab on the right.
24. In the upper right corner click on "Analysis Settings."
25. For "Data Analysis change settings for FAM (H5 NAEA MOD), Cy5 (H5 MX), and JOE (Internal Control): Threshold set to 0.01 for JOE (IAC) and Cy5 (H5 MX) and 0.1 for FAM (H5 NAEA MOD) and "Baseline Start Cycle" to 3 and "End Cycle" to 10.
26. Click "Apply Analysis Settings."
27. Click "Run" tab on the right.
28. Click green "Start Run" box.

Data Interpretation – H5 Multiplex Assay

For this H5 multiplex assay, FAM is the H5 NAEA MOD probe fluorescent label, Cy5 is the H5 MX probe fluorescent label, and JOE is the internal amplification control (IAC) probe fluorescent label.

1. Sample is "negative" if:
    a. RT-qPCR negative control is negative for H5 NAEA MOD and H5 MX,
    b. RT-qPCR positive control is positive for H5 NAEA MOD and H5 MX,
    c. Matrix control sample (if included) is negative for H5 NAEA MOD and H5 MX IAV,
    d. Unknown is negative for H5 NAEA MOD and H5 MX,

e. Internal amplification control (IAC) is positive. No further analysis is needed.

2. Sample is “positive” if:

   a. RT-qPCR negative control is negative for H5 NAEA MOD and H5 MX,

   b. RT-qPCR positive control is positive for H5 NAEA MOD and H5 MX,

   c. Unknown sample is positive for the detection of H5 NAEA MOD and/or H5 MX.

3. Samples are invalid if:

   a. If the negative RT-qPCR control sample demonstrates positive results crossing the FAM Cy5 threshold or if the IAC is negative, repeat the RT-qPCR assay,

   b. The average of the IAC C_t values for the sample replicates are more than 4.0 C_t’s greater than the Negative Control IAC C_t value, repeat the RT-qPCR assay using remaining RNA or RNA from a newly extracted tube with a 1 µl RT-qPCR reaction in triplicate. If the repeat of the newly extracted sample yields average IAC C_t values 4.0 C_t‘s higher than the Negative Control IAC C_t value, repeat the sample analysis from the beginning using additional food/sample. With the new repeat, the concentrate/eluate will be a 1:5 dilution and complete RT-qPCR with 1 µl reactions in triplicate.

Note: A positive sample is a result that demonstrates log amplification. Log amplification can be viewed as a graph on the ABI 7500 Fast platform. If the sample does not exhibit log amplification and crosses the threshold, the RT-qPCR reaction should be repeated.

3. Controls and Reagent Recipes

a. Working Concentration ROX Dye

ROX dye (Invitrogen)	-20°C	50 µl
Primer TE		450 µl

Add mixture to 1.5 ml DNase/RNase-free dark tube, mix well and make 50-100 µl aliquots in DNase/RNase dark tubes. **Store at 4°C** for 3 months.

b. Primer TE (10mM Tris, 0.1mM EDTA, pH 8.0)

1M Tris pH 8.0	100 µl
0.05M EDTA	20 µl
PCR-grade water (DNase/RNase-free)	9.88 ml

Prepare in sterile 50 ml conical tube. Store at room temperature.

c. gblock Positive Control

Hem5

TCTCCATGATTCAAATGTCAAGAACTTATACGATAAGGTCCGACTCCAGC
TGAGAGACAATGCAAAAGAATTAGGCAACGGGTGCTTTGAATTCTACCA
CAAGTGTGACGATGAATGCATGGAAAGTGTGAGAAATGGAACGTATGAC
TATCCACAATATCAGAAGAATCAAGACTGAACAGGGAGGAAATAGACGG
AGTCAAATTAGAATCAATGGGGACTTATCAGATACTTTCAATCTATTCAAC
AGTAGCGAGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCTTTTT
GGATGTGCTCCAATGGATCATTGCAGTGCAGAATTTGCATCTAAAATTGT
GAGTTCAGATTGTAATTAAAAACACCCTTGTTTCTACT

Hem no.2

AGACTTCCATGACTCAAATGTCAAGAACCTTTATGATAAGGTCCGACTAC
AGCTTAAGGATAATGCAAAAGAACTGGGGAATGGTTGTTTCGAGTTCTAT
CACAAATGTGATAATGAATGTATGGAAAATGTAAGAAACGGGACGTATGA
CTACCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGAGAGGAAATAAGT
GGAGTAAAATTGGAATCAATAGGAATCTACCAAATACTGTCAATTTATTCA
ACAGTGGCGAGTTCCCTAGTGCTGGCAATCATGATGGCTGGTCTATCTT
TATGGATGTGTTCCAACGGGTCGTTACAGTGCAGAATTTGCATTTAGGTT
TGTGAATTCAGATTGTAGTTAAAAACACC

Hydrate each according to manufacturer's instructions using primer TE. Dilute both controls with DNase/RNase free water to -6 for working stocks. Combine each diluted control working stocks. Store at ≥-20°C until use.

Table 1. Primer and Probe Sequences for IAV H5 and Internal Amplification Control

Identification	Primers	Location[#]
H5 NAEA MOD[†]	5’ CGT ATG ACT AYC CRC ART AYT CA 3’	1492-1515
H5F MX[†]	5’ TCA ACA GGG ACT TAT CAG ATA C 3’	1590-1613
H5R NA[@]	5’ AGA CCA GCT ACC ATG ATT GC 3’	1643-1624
IC 46F[a]	5’ GAC ATC GAT ATG GGT GCC G 3’	N/A
IC 194R[a]	5’ AAT ATT CGC GAG ACG ATG CAG 3’	N/A
H5 NAEA 1637 Pro1*[@]	FAM/ZEN - 5’ TC AAC AGT GG CGA GTT CCC TAG CA 3’ - IB-FQ	1628-1649
H5 MX 1680 Pro2[¥@]	Cy5/TAO - 5’ TC AAT CTA TT CTA CAG TAG TGA GTT CCC TAG CAC TG 3’ - IB RQ	1619-1649
IAC Probe	JOE/ZEN - 5’ TCT CAT GCG TCT CCC TGG TGA ATG TG 3’ - IB FQ	N/A

[@]Primer and probe sequences from USDA NVSL Protocol
[†]Modified sequence from USDA NVSL Protocol
[a] Depaola, Jones, Woods et. al. 2010 Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232.
[¥]IB RQ- Iowa Black RQ
*IB FQ- Iowa Black FQ
[#]based on accession no. MT325967, KJ729343, MT325967, OQ933425, MT547695

Table 2. ABI 7500 Amplification Reaction Components for IAV H5

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase-Free water		6.8 µl	
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$~	50 mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
H5 NAEA MOD	10 µM	1 µl	0.4 µM
H5F MX 1592	10 µM	1 µl	0.4 µM
H5R NA 1685	10 µM	1 µl	0.4 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
H5 NAEA Probe	10 µM	0.625 µl	0.250 µM
H5 MX Probe	10 µM	0.625 µl	0.250 µM
IAC Probe	10 µM	0.375 µl	0.15 µM
OneStep RT-PCR Enzyme Mix		1.00 µl	
Superase·in	20 Units/µl	0.25 µl	5 Units
ROX ref dye^		2 µl	
Internal Amplification Control RNA		*0.2 µl	
RNA		3 µl	

*Amount varies with concentration of IAC RNA. The amount of IAC template needs to be adjusted based on the prepared stock concentration to report Cycle threshold (C_t) of 20-25 PCR cycles when no inhibition is present in the reaction. The required concentration was provided to each laboratory participating in the validation study.
˜With the addition of 1.5 mM MgCl, the final concentration per reaction is 4.0 mM MgCl
^Concentration range 23-25 µM cat no. 12-223-012 (Invitrogen/Life Technologies) Fisher Scientific

Alternatively, primer, probe, buffer, and enzyme mixes can be prepared ahead of time and frozen as separate components for more high throughput sample analysis. See below for recipes for these mixes and master mix component tables for the use of these mixes.

Master mix components preparation for IAV H5 RT-qPCR assay on AB7500

Table 3. IAV H5 Primer Mix*

Primers/H_2O	**Volume**
H5 NAEA MOD	50.0 µl
H5F MX	50.0 µl
H5R NA	50.0 µl
IC46	9.375 µl
IC194	9.375 µl
DNase/RNase-free water	831.25 µl

*Made with 100 µM primers stocks and PCR grade water

Table 4. IAV H5 Probe Mix*

Probe/H_2O	**Volume**
H5 NAEA	31.25
H5 MX	31.25
ICP	18.75
DNase/RNase-free water	418.75

*Made with 100 µM probe stocks and PCR grade water

Table 5. Enzyme Mix*

Component	**Volume**
One-Step Qiagen enzyme	~200 µL
Superase-IN (10000U)	50 µL

Table 6. Buffer Mix*

Reagents	Volume (50 mM MgCl)	Volume (25 mM MgCl)
DNase/RNase-free water	1760 µl	1610 µl
5X Buffer	1000 µl	1000 µl
MgCl	150 µl	300 µl
dNTPs	200 µl	200 µl

*Made with components from Qiagen One-Step RTqPCR kit and PCR grade water

Table 7. Master Mix Composition using Buffer, Primer, Probe, and Enzyme Mixes

Reagents	Volume µl/rxn
Buffer mix	15.55 µl
Primer mix	2 µl
Probe mix	1 µl
Enzyme mix	1.25 µl
ROX reference dye	2 µl
IAC	0.2 µl
Total	22 µl

4. References

1. USDA National Veterinary Services Laboratory. NVSL-SOP-0068 Real-time RT-PCR Detection of Influenza A and Avian Paramyxovirus Type-1, revisions 5, May 18, 2023.

2. US Food and Drug Administration Bacteriological Analytical Manual Chapter 26 and Appendices: Concentration, Extraction and Detection of Enteric Viruses from Food, July 2022.

3. US Food and Drug Administration Bacteriological Analytical Manual. Additional Chemistry and Microbiology Resources Used by the Foods Program. SOP for Extraction and Detection of Influenza A virus in Milk and Milk Products https://www.fda.gov/media/179502/download?attachment

4. DePaola A, Jones JL, Woods J, Burkhardt W 3rd, Calci KR, Krantz JA, Bowers JC, Kasturi K, Byars RH, Jacobs E, Williams-Hill D, Nabe K. Bacterial and viral pathogens in live oysters: 2007 United States market survey. Appl Environ Microbiol. 2010 May;76(9):2754-68.

Revision History

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