Extraction and Detection of Influenza A virus in Milk and Milk Products

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) and submit positive samples to a diagnostic laboratory for confirmation testing.

The methods described here were developed for emergency use to screen milk and milk products for the IAV M gene using the Qiagen's® QIAamp Viral RNA Mini Kit and Qiagen One Step RT-qPCR kit for extraction and detection, respectively. Murine Hepatitis Virus (MHV) is used as the process/extraction control. IAV valid sample results are contingent upon the successful detection of the MHV extraction control from the sample being tested.

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

Materials and Reagents

a. 95% Ethanol (Sigma E7023 or equivalent)
b. DNase/RNase free water (Life Technologies AM9937 or equivalent)
c. Deionized/Distilled water/Milli-Q water
d. NaCl (Sigma S3014 or equivalent)
e. NaOH (Sigma S5881 or equivalent)
f. HCl (Sigma H1758 or equivalent)
g. Glycine (Sigma G7126 or equivalent)
h. 10X PBS tissue culture (t.c.) grade (Sigma P5493) to be diluted to 1X
i. KCl (Sigma P9541 or equivalent)
j. Potassium Dihydrogenphosphate (Sigma P9791 or equivalent)
k. Disodium Hydrogen Phosphate (Sigma S5011 or equivalent)
l. Glycerol (Sigma G5516 or equivalent)
m. DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5 ml (Life Technologies AM12350 or equivalent)
n. DNase/RNase-free microcentrifuge tubes 1.5 ml, non-stick, low retention, siliconized (Life Technologies AM12450 or equivalent)
o. DNase/RNase-free microcentrifuge tubes 2.0 ml, non-stick, low retention, siliconized (Life Technologies AM12475 or equivalent)
p. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)
q. Qiagen QIAamp Viral RNA Mini Kit (Qiagen 52904)
r. Qiagen collection tubes (Qiagen 19201)
s. OneStep™ PCR Inhibitor Removal Kit (Zymo Research D6030)
t. 2.0 mL microcentrifuge tubes DNase/RNase free (USA Scientific 1620-2799 or equivalent)
u. OneStep RT-PCR Kit (Qiagen 210210 or 210212)
v. Ambion Superase·In RNase Inhibitor (20 units/µl); Life Technologies AM2694 (2,500 U) or AM2696 (10,000 U)
w. Eppendorf DNA LoBind Tubes 5.0 mL Fisher Scientific 0030108310 or equivalent
x. 15 ml polypropylene conical tubes (Fisher Scientific 14-959-70C, or equivalent)
y. 50 mM $MgCl_2$ (BioRad 1708872 or equivalent) or 25 mM $MgCl_2$ (ThermoFisher Scientific AB0359, or equivalent)
z. Internal Control RNA (BioGX Cat No. 750-0001—contact company)
aa. Standard desalted primers and HPLC probes for all RTqPCR assays (Integrated DNA Technologies or equivalent)
bb. RT-qPCR positive controls (Quantitative Genomic RNA from Influenza A virus

H1N1 strain A/California/07/2009 (H1N1) pdm09 MHVATCC VR-189DQ)

cc. RT-qPCR no template control (Nuclease free-water Applied Biosystems AM9937)

dd. ROX Reference dye (Invitrogen cat#12223-012)

ee. Extraction control murine hepatitis virus MHV-A59 (ATCC VR-764)

Equipment and Supplies

a. Biological Safety Cabinet (BSC- 2 Type A2 or higher air exchange rate)

b. Ultra-low freezer (-70 ˚C or lower)

c. Latex or nitrile gloves (powder-free)

d. Vortex mixer (Labsource S16-109 or equivalent)

e. Disposable scalpels (Fisher Scientific 12-460-456) or plastic spatula (Thomas Scientific 1140H82) or equivalent

f. 150 mm x 15 mm Petri Dishes (Fisher 08-757-148 or equivalent)

g. DNase/RNase-free microcentrifuge tubes, non-stick, low retention, siliconized 0.5 ml (Life Technologies AM12350 or equivalent)

h. Nasco Whirl PakTM bags;12.5cm” 9.5cm” (Fisher Scientific 01-812-5M or equivalent)

i. Hype-Wipe Disinfecting Towelettes (Fisher Scientific 14-412-56 or equivalent)

j. Adjustable Micropipettors (0.2 – 1000 µl), dedicated for RNA work only

k. Filter barrier aerosol resistant micropipettor tips DNase/RNase free (0.2 – 1000 µl)

l. AB 7500 FAST (Applied BioSystems 4351106)

m. ABI 96 well plates (Life Technologies 4346906)

n. ABI plates cover (Life Technologies 4311971)

o. Mini Plate Spinner (Fisher Scientific 14-100-143 or equivalent)

p. Mini Centrifuge (Labsource C90-044 or equivalent)

q. 96 well cool rack (Sigma Aldrich #Z606634-1EA or equivalent)

r. Disposable scalpels (Fisher Scientific 12-460-456 or equivalent)

s. Mettler Toledo™ NewClassic ME Precision Balance, 2200 g (Fisher Scientific 01-912-408 or equivalent)

t. Centrifuge capable of speeds of up to 3,000 x g, and with rotors capable of holding 5 ml or 15 ml conical tubes (Fisher Scientific 0540061 or equivalent

u. Drummond pipette aid (Fisher Scientific or equivalent)

v. Centrifuge capable of speeds of 21,000 x g, and with rotors capable of holding 1.5 ml and 2.0 ml microcentrifuge tubes (Eppendorf 2231000655 or equivalent)

w. Disposable transfer pipettes (Fisher 13-711-22 or equivalent)

x. Orbital shaker (Fisher Scientific 11-676-231 or equivalent)

Sample Prep Procedure for Fluid Milk and Sour Cream

For dehydrated milk products, hydrate according to manufacturer's instructions and proceed with protocol as indicated below

1. Remove 1.0 g (ml) sample and add 10 µl murine hepatitis virus (MHV) extraction control to each 1.0 g (ml) sample (prepare MHV extraction control as described in BAM Chapter 26 for the murine norovirus extraction control except dilution will be 1:1000).

2. Dilute by adding 2.0 ml of t.c. PBS to the 1.0 ml sample containing the extraction control from step 1 and briefly vortex to mix.

3. ***Remove 140µl for RNA extraction. Store remainder of sample at -70° C.***

Sample Prep Procedure for Cheeses and Butter

1. Tare and weigh 1.0 ± 0.5 g sample cut from the center part of the cheese using sterile equipment to a Whirl Pak sample bag and add 10 µl murine hepatitis virus (MHV) extraction control to each 1.0 g sample (prepare MHV extraction control as described in BAM Chapter 26 for the murine norovirus extraction control except dilution will be 1:1000).

2. Add 3.0 ml of t.c. PBS to the sample and close bag tightly. Squeeze the sample by hand for 90 sec or until the sample has gone into solution.

3. Transfer sample to a 5.0ml or 15 ml conical tube. Add an additional 1.0 ml of t.c. PBS to rinse Whirl Pak bag.

4. Transfer rinse and any large particulates to conical tube in step 3 (Can use a spatula for large particulates).

5. Centrifuge at 3,000 x g for 10 min at 21 ± 2 ºC.

6. ***Remove 140µl of supernatant for RNA extraction. Store remainder of sample supernatant at -70° C.***

RNA Extraction

Note: Before starting extraction, prepare AVL with carrier RNA and place and aliquot of Buffer AVE in 70 ºC heating block.

1. Add 560 µl prepared Buffer AVL with carrier RNA (Section A6 BAM Chapter 26) to sample.
2. Vortex for 20 sec and incubate at room temperature (15-25 °C) for 10 min.
3. Add 700 µl of 95-100% ethanol to the flow through and mix immediately by inverting. Do not centrifuge. Continue without delay to next step.
4. Apply 630 µl of the solution to a QIAamp mini column.
5. Centrifuge 6000 x *g* for 1 min. Place the QIAamp spin column in a new collection tube. Discard flow through and collection tube.
6. Repeat steps 4 and 5 until the entire sample has been passed through the column, discarding the collection tube each time.
7. Transfer the QIAamp mini column into a new 2 ml collection tube. Add 500 µl Buffer AW1. Incubate at room temperature for 15 min. Centrifuge 1 min at 6000 x *g*. Discard flow through and collection tube.
8. Transfer the QIAamp mini column into a new 2 ml collection tube.
9. Add 500 µl Buffer AW2 onto the QIAamp mini column. Centrifuge at maximum speed (≥ 16,000 x *g*) for 3 min. Discard flow through and collection tube.
10. Transfer the QIAamp mini column into a new 2 ml collection tube. Centrifuge at maximum speed (≥ 16,000 x *g*) for 1 min to dry column.
11. To elute RNA, transfer the QIAamp mini column into a new 1.5 ml low retention/siliconized DNase/RNase free centrifuge tube.
12. Add 50 µl of pre-heated (70 ºC) Buffer AVE directly onto the QIAamp silica-gel membrane. Close the tube gently, and centrifuge for 1 min at 8000 x g.
13. Pipette the eluted 50 µl back to the top of the column. Add an additional 50 µl of pre-heated Buffer AVE to column. Close the tube gently, and centrifuge for 1 min at 8,000 x *g*.

14. Discard column and place tube with RNA (step 13) on ice to prepare Zymo columns.

15. Prepare one Zymo column per manufacturer's instructions.

16. Transfer Zymo column into a clean 1.5 or 2.0 ml low-retention, siliconized RNase/DNase free microcentrifuge tube.

17. Transfer RNA from step 14 to prepared Zymo One Step RT-PCR inhibitor remover column.

18. Spin at 16,000 x *g* for 3 min.

19. Proceed with RT-qPCR or freeze at -70 °C for storage.

Buffer and Reagent Recipes

a. t.c. PBS

10X PBS (Sigma P5493)	100 ml
Sterile deionized water	900 ml

Adjust pH to 7.5, sterilize at 121 °C for 15 min and store at 4 ± 2 °C.

****Note: Can be used where protocols indicate t.c. PBS or formula below can be used****

b. t.c. PBS

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.12 g
Na_2HPO_4	0.91 g

QS to make 1 L with deionized water. Adjust pH to 7.5, sterilize at 121 °C for 15 min and store at 4 ± 2 °C.

c. Working Concentration ROX Dye

ROX dye (Invitrogen)	-20ºC	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.

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

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

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

e. Carrier RNA

Buffer AVE	310 µl
310 µg carrier RNA	310 µg

Add 310 µl of buffer AVE to carrier RNA, store in 60 µl aliquots **at -20°C**. If carrier RNA is different concentration, the ratio is 1:1 buffer AVE and carrier RNA.

f. AVL Buffer and AVE/Carrier RNA mix

Hydrate carrier RNA with buffer AVE (provided in QIAamp Viral RNA kit). Volumes of Buffer AVL and carrier RNA–Buffer AVE mix required for the number of extractions to be performed (refer to table below).

Store remaining carrier RNA in 30 µl aliquots at -20 °C.

No. samples	Vol. Buffer AVL (ml)	Vol. carrier RNA–AVE (µl)	No. samples	Vol. Buffer AVL (ml)	Vol. carrier RNA–AVE (µl)
1	0.56	5.6	13	7.28	72.8
2	1.12	11.2	14	7.84	78.4
3	1.68	16.8	15	8.40	84.0
4	2.24	22.4	16	8.96	89.6
5	2.80	28.0	17	9.52	95.2
6	3.36	33.6	18	10.08	100.8
7	3.92	39.2	19	10.64	106.4
8	4.48	44.8	20	11.20	112.0
9	5.04	50.4	21	11.76	117.6
10	5.60	56.0	22	12.32	123.2
11	6.16	61.6	23	12.88	128.8
12	6.72	67.2	24	13.44	134.4

Detection of IAV and MHV via RT-qPCR using the ABI 7500 FAST

Outlined MHV RT-qPCR Assay for ABI 7500 (refer to Table 1 for primers and probes and Table 2 for master mix components. Refer to BAM Chapter 26 for preparation of positive controls)

Cycling Conditions

Reverse transcription: 50°C for 3000 sec Activation: 95°C for 900sec Cycling: 45 cycles of 95°C for 10 sec, then 64°C for 60 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 crushed 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 B2.1 (additional master mix preparations can be found in Appendix E). Keep all thawed components, reagents, controls, and master mixes in cooling block or on crushed ice.

Note: Viral RNA templates should be added to reaction tubes in a designated area separate from location where master mixes are prepared. A negative and positive control should be added to each run 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 (pulse spin) 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.

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

    b. 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 2 targets.
9. Fill Target 1 with MHV, Target 2 with IAC.
10. Change the reporter dye to FAM for MHV 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 10 sec at 95 °C.
20. Second stage set at 60 sec (1 min) at 64 °C (click the icon for collecting data at “collect data on hold”).
21. Save the run method – Save as MHV Multiplex.
22. Click the “Analysis” tab on the right.
23. In the upper right corner click on “Analysis Settings.”
24. For “Data Analysis change settings for FAM (MHV) and JOE (Internal Control): Threshold set to 0.1 for FAM and 0.01 for JOE. Set “Baseline Start Cycle” to 3 and “End Cycle” to 15.
25. Click “Apply Analysis Settings.”
26. Click “Run” tab on the right.
27. Click green “Start Run” box.

Data Interpretation – Murine Hepatitis Virus Multiplex Assay

1. Repeat any invalid sample. Sample is “invalid” if:
    a. The RT-qPCR negative control demonstrates positive C_t results for MHV in FAM or if the IAC is negative (no C_t from JOE),
    b. The RT-qPCR positive control is negative (no C_t from FAM) for MHV,
    c. The MHV RT-qPCR is negative (no C_t from FAM) for any sample,
    d. 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 template in the

RT-qPCR reaction in triplicate. If the 1 µl template reactions yields an average IAC C_t value greater than 4.0 C_t higher than the Negative Control IAC C_t value, repeat the sample analysis from the beginning using additional food sample. With the new sample, dilute 1:5 and complete RT-qPCR with 1 µl reactions in triplicate.

2. Sample is "valid" and can be reported if:

   a. RT-qPCR negative control is negative for MHV,

   b. RT-qPCR positive control is positive for MHV,

   c. RT-qPCR is positive for MHV in all spiked matrices,

   d. Internal amplification control (IAC) is positive in all reactions and average of the IAC C_t values for sample is within 4.0 C_t's of the negative control IAC C_t value.

Note: For MHV, if 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 AND the corresponding sample is positive for influenza A M gene, the MHV RTqPCR does not have to be repeated. If influenza A M gene is detected in a sample that has inhibition present in the RTqPCR reaction and has log amplification, this sample does not need to be repeated for influenza A M gene RT-qPCR and would be considered positive. Repeating RT-qPCR reactions due to inhibition is to ensure that you do not have false negatives. Labs should perform dilutions as necessary on the stock concentration of MHV to achieve an optimal working stock that generates Ct values between 30-37 from spiked food in the absence of inhibition.

Table 1. Primer and Probe Sequences for MHV and Internal Amplification Control

Identification	Primers	Location[#]
MHVF[@]	5' GGA ACT TCT CGT TGG GCA TTA TAC T 3'	101-108
MHVR	5' ACC ACA AGA TTA TCA TTT TCA CAA CAT A 3'	157-184
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
MHV Probe	FAM/ZEN - 5' CA TGC TAC GG CTC GTG TAA CCG AAC TGT 3' - IB-RQ*	123-151
IAC Probe	JOE - 5' TCT CAT GCG TCT CCC TGG TGA ATG TG -IB FQ 3'*	N/A

[@]Besselsen,et. al., 2002

[a] Depaola, Jones, Woods et. al. 2010 Internal Amplification Control (IAC) primers and probes are covered by U.S. Patent Application 0060166232.

*IB FQ- Iowa Black FQ

*IB RQ- Iowa Black RQ

[#]based on accession no. X00509

Table 2. ABI 7500 Amplification Reaction Components for MHV

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase Free H_20		9.3 µl	-
5X OneStep RT-PCR Buffer	5X	5.0 µl	1X
$MgCl_2$~	50mM	0.75 µl	1.5 mM
dNTP Mix	10 mM	1 µl	0.4 mM
MHVF	10 µM	0.5 µl	0.2 µM
MHVR	10 µM	0.5 µl	0.2 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
MHV Probe	10 µM	0.75 µl	0.300 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 4.0mM MgCl
^Concentration range 23-25 µM cat no. 12-223-012 (Invitrogen/Life Technologies) Fisher Scientific

Outlined IAV RT-qPCR Protocol ABI 7500 (refer to Table 3 for primers and probes and Table 4 for master mix components. Refer to BAM Chapter 26 for preparation of positive controls).

Cycling Conditions

Reverse transcription: 50°C for 3000 sec Activation: 95°C for 900sec
Cycling: 45 cycles of 95°C for 10sec then 64°C for 60sec 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 2 targets.
9. Fill Target 1 with IAV, Target 2 with IAC.
10. Change the reporter dye to FAM for IAV 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 10 sec at 95 °C.
20. Second stage set at 60 sec (1 min) at 64 °C (click the icon for collecting data at “collect data on hold”).
21. Save the run method – Save as IAV Multiplex.
22. Click the “Analysis” tab on the right.
23. In the upper right corner click on “Analysis Settings”
24. For “Data Analysis change settings for FAM (IAV) and JOE (Internal Control): Threshold set to 0.01 for JOE (IAC) and 0.1 for FAM (IAV) and “Baseline Start Cycle” to 3 and “End Cycle” to 15.
25. Click “Apply Analysis Settings”
26. Click “Run” tab on the right.
27. Click green “Start Run” box.

Data Interpretation – IAV Multiplex Assay

For this IAV multiplex assay, FAM is the IAV 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 IAV,
    b. RT-qPCR positive control is positive for IAV,
    c. Matrix control sample (if included) is negative for IAV,
    d. Unknown is negative for IAV,
    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 IAV,
    b. RT-qPCR positive control is positive for IAV,

c. Unknown sample is positive for the detection IAV.

3. Samples are invalid if:

   a. If the negative RT-qPCR control sample demonstrates positive results crossing the FAM or if the IAC is negative, repeat the RTqPCR 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. With the new repeat, the concentrates will be a 1:5 dilution and complete RT-qPCR with 1µl 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 RTqPCR reaction should be repeated.

Table 3. Primer and Probe Sequences for IAV M and Internal Amplification Control

Identification	Primers	Location[#]
IAVMF 25[@]	5' AGA TGA GTC TTC TAA CCG AGG TC 3'	24-47
IAVMR1 124	5' TGC AAA AAC ATC TTC AAG TCT CTG 3'	124-101
IAVMR2 124[∞]	5' TGC AAA GAC ACT TTC CAG TCT CTG 3'	124-101
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
IAV Probe	FAM/ZEN - 5' TC AGG CCC CC TCA AAG CCG A 3' - IB-RQ*	74-93
IAC Probe	JOE - 5' TCT CAT GCG TCT CCC TGG TGA ATG TG - IB FQ 3'*	N/A

[@]USDA 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. PP732870.1, PP664089, PP731283.1

Table 4. ABI 7500 Amplification Reaction Components for IAV M1

Reagent	Initial Concentration	Volume per 25 µl reaction	Final Concentration
RNase Free H_20		8.8 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
IAVMF 25	10 µM	0.5 µl	0.2 µM
IAVMR1 124	10 µM	0.5 µl	0.2 µM
IAVMR2 124	10 µM	0.5 µl	0.2 µM
IC 46F	10 µM	0.1875 µl	0.075 µM
IC 194R	10 µM	0.1875 µl	0.075 µM
IAV Probe	10 µM	0.75 µl	0.300 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 4.0mM 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 mastermix component tables for the use of these mixes.

Master mix components preparation for RT-qPCR assays on AB7500

Table 5. MHV Primer Mix*

Primers/H_2O	**Volume**
MHVF	25.0 µl
MHVR	25.0 µl
IC46	9.375 µl
IC194	9.375 µl
DNase/RNase water	931.25 µl

*Made with 100µM primers stocks and PCR grade water

Table 6. MHV Probe Mix

Probe/H_2O	**Volume**
MNVP	37.5
ICP	18.75
DNase/RNase water	443.75

*Made with 100µM probe stocks and PCR grade water

Table 7. IAV Primer Mix*

Primers/H_2O	**Volume**
IAVM1 25 F	25.0 µl
IAVMR1 124	25.0 µl
IAVMR2 124	25.0 µl
IC46	9.375 µl
IC194	9.375 µl
DNase/RNase water	906.25 µl

*Made with 100µM primers stocks and PCR grade water

Table 8. IAVM Probe Mix

Probe/H_2O	**Volume**
IAVP	37.5
ICP	18.75
DNase/RNase water	443.75

*Made with 100µM probe stocks and PCR grade water

Table 9. Enzyme Mix*

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

Table 10. Buffer Mix*

Reagents	**Volume (50mM MgCl)**	**Volume (25mM MgCl)**
DNase/RNase freeH2O	1760 µl	1910µl
5X Buffer	1000 µl	1000µl
MgCl	150 µl	300µl
dNTPs	200 µl	200µl

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

Table 11. Master Mix Composition using Buffer, Primer, Probe, and Enzyme Mixes

Reagents	**Volume** µl/rxn	[redacted]
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	
	22 µl	Total

References

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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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