



CDC protocol of realtime RTPCR for influenza A(H1N1)

28 April 2009

revision 1 (30 April 2009)

revision 2 (6 October 2009)

The WHO Collaborating Centre for influenza at CDC Atlanta, United States of America, has made available the protocol, attached, of realtime RTPCR for influenza A(H1N1).

CDC Realtime RTPCR (rRTPCR) Protocol for Detection and Characterization of Swine Influenza (version 2009)

General Comments

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources. Inclusion does not imply endorsement by the Centers for Disease Control and Prevention.

Assumptions: This procedure assumes a basic familiarity with rRT-PCR assays.

Assay principle: The *CDC Realtime RTPCR (rRTPCR) Protocol for Detection and Characterization of Swine Influenza* includes a panel of oligonucleotide primers and dual-labeled hydrolysis (Taqman[®]) probes to be used in real-time RT-PCR assays for the *in vitro* qualitative detection and characterization of swine influenza viruses in respiratory specimens and viral cultures. The InfA primer and probe set is designed for universal detection of type A influenza viruses. The swInfA primer and probe set is designed to specifically detect all swine influenza A viruses. The swH1 primer and probe set is designed to specifically detect swine H1 influenza. This assay is utilized for testing influenza A positive respiratory specimens (unsubtypable) taken from suspect swine influenza A infected patients.

Protocol Use Limitations: These protocols were optimized using quantitative one-step probe RT-PCR (Invitrogen SuperScript[™]III Platinum[®] One-Step Quantitative Kit) that have been shown to produce comparable results on 96-well format thermocycler systems such as Applied Biosystems[™] real-time PCR systems (7000, 7300, 7500, etc.), BioRad real-time PCR detection systems (iQ[™] or iQ5[™]) or Stratagene QPCR instruments (MX4000[®], MX3000[®] or MX3005[®]).

Safety Information: Specimen processing should be performed in accordance with pertaining national biological safety regulations.

Acceptable specimens: Respiratory specimens including: bronchoalveolar lavage, tracheal aspirates, sputum, nasopharyngeal or oropharyngeal aspirates or washes, and nasopharyngeal or oropharyngeal swabs. Swab specimens should be collected only on swabs with a synthetic tip (such as polyester or Dacron[®]) and an aluminum or plastic shaft. Swabs with cotton tips and wooden shafts are not recommended. Specimens collected with swabs made of calcium alginate are not acceptable.

Rejection criteria:

- Specimens not kept at 2-4°C (≤4 days) or frozen at -70°C or below.
- Inappropriate specimens not listed above.

Nucleic acid extraction: Performance of RT-PCR amplification based assays depends on the amount and quality of sample template RNA. RNA extraction procedures should be qualified and validated for recovery and purity before testing specimens. Commercially available

extraction procedures including QIAamp® Viral RNA Mini Kit, or RNeasy® Mini Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, MagNA Pure LC RNA Isolation Kit II, and Roche MagNA Pure Total Nucleic Acid Kit have been shown to generate highly purified RNA when following manufacturer's recommended procedures for sample extraction.

Materials

Reagents:

1. One-step quantitative RT-PCR probe hydrolysis (e.g., Taqman®) kit
 - Invitrogen SuperScript™III Platinum® One-Step Quantitative Kit (cat# 11732-020 or 11745-100).
2. Molecular grade sterile distilled water (RNase and DNase free)
3. Forward and reverse primers (40µM)
4. Dual-labeled probes (10µM)
5. Positive control materials

Supplies:

1. Laboratory marking pen
2. Cooler racks for 1.5 microcentrifuge tubes and 96-well 0.2ml PCR reaction tubes
3. 20µl and 200µl adjustable pipettes and aerosol barrier tips
4. 0.2ml PCR reaction tube strips or plates
5. Optical strip caps
6. Sterile, nuclease free 1.5 ml microcentrifuge tubes
7. Disposable powder-free gloves

Equipment:

1. Microcentrifuge
2. Vortex
3. Real-time PCR detection system with a 96-well format thermocycler reaction block.

Procedure

Preparation:

1. Avoiding sample contamination

Because of the sensitivity of fluorogenic 5' nuclease assays, special precautions must be taken to avoid false positive amplifications. The following precautionary steps are recommended:

- (a) Maintain separate areas for assay setup and handling of nucleic acids.
- (b) Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
- (c) Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
- (d) Change gloves between samples and whenever you suspect they may be contaminated.
- (e) Keep reagent and reaction tubes capped or covered as much as possible.

2. Equipment preparation

Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 5% bleach, “DNAzap™” or “RNase AWAY®” to minimize risk of nucleic acid contamination.

3. Reagent preparation

NOTE: Keep all reagents on cold rack during assay set up.

(a) Primers and probes

- Thaw frozen aliquots of primer and probes (Thawed aliquots of probes may be stored in the dark up to 3 months at 2-8°C. Do not re-freeze probes).
- Vortex all primers and probes.
- Briefly centrifuge all primers and probes and then place in cold rack.

(b) Realtime RTPCR reagents

- Place Master Mix and enzyme in cold rack
 - Thaw the 2X Reaction Mix vial.
 - Mix the 2X Reaction Mix by inversion.
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- Briefly centrifuge 2x Reaction Mix and enzyme then place in cold rack

Tests for each RT-PCR run

1. Each sample RNA extract is tested by separate primer/probe sets: InfA, Universal swine (swFluA), Swine H1 (swH1) and RNaseP (RP). The RNaseP primer and probe set targets the human RNase P gene and thus serves as an internal positive control for human nucleic acid.
2. No template controls (NTC) and positive template controls (PTC) for all primer/probe sets should be included in each run.
3. Human Specimen Control (HSC) provides a secondary negative control that validates the nucleic extraction procedure and reagent integrity.

Reaction setup

Reaction assay mixtures are made as a cocktail and dispensed into the 96-well reaction plate. Water and extracted nucleic acid or positive template controls are then added to the appropriate test reactions and controls.

1. Label one 1.5 ml microcentrifuge tube for each primer/probe set.
2. Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction cocktail to allow for the NTC, PTC, HSC reactions and pipetting error. See below:
 - If number of samples (n) including controls = 1 to 14, then $N = n + 1$
 - If number of samples (n) including controls > 15, then $N = n + 2$
3. Master Mix: calculate the amount of each reagent to be added for each primer/probe set reaction master mix. The calculations are as follows:

Reagent	Volume of Reagent Added per Reaction
Nuclease-free Water	N x 5.5 µl
Forward Primer	N x 0.5 µl
Reverse Primer	N x 0.5 µl
Probe	N x 0.5 µl
SuperScript™ III RT/Platinum® Taq Mix	N x 0.5 µl
2X PCR Master Mix	N x 12.5 µl
Total Volume	N x 20.0 µl

After addition of the water, mix reaction mixtures by pipetting up and down. Do not vortex.

5. Centrifuge for 5 sec to collect contents at bottom of the tube, and then place the tube in cold rack.

6. Set up reaction strip tubes or plates in 96-well cooler rack.

7. Dispense 20µl of each master mix into each well going across the row as shown below:

Example Test Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	InfA											
B	sw InfA											
C	swH1											
D	RP											
E	InfA											
F	sw InfA											
G	swH1											
H	RP											

Example Sample Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	PTC
B	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	PTC
C	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	PTC
D	NTC	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	PTC
E		S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	
F		S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	
G		S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	
H		S2	S4	S6	S8	S10	S12	S14	S16	S18	HSC	

Note: Negative template controls (NTC) should be added first (column 1) before any of the samples are added to check for contamination in the master mix. HSC should be added after the samples have been added (column 11) to check for cross-contamination during sample preparation or addition. Positive template controls (PTC) should be added last after all samples and NTCs are sealed.

8. Before moving the plate to the nucleic acid handling area, set up the NTC reactions for column 1 in the assay set-up area. As shown above, samples can be added by column.

9. Pipette 5 µl of nuclease free water into the NTC wells. Cap NTC wells.
10. Cover the reaction plate and move the reaction plate to the nucleic acid handling area.
11. Vortex the tubes containing the samples for 5 sec. Centrifuge tubes for 5 sec.
12. Set up the extracted nucleic acid samples in the cold rack.
13. As shown above, samples can be added by column. Pipette 5 µl of the first sample into all the wells labeled for that sample (for example, Sample “S1” as shown above). Change tips after each addition
14. Cap the column to which the sample has been added. This will help to prevent sample cross contamination and enable you to keep track of where you are on the plate.
15. Change gloves when necessary to avoid contamination.
16. Repeat steps 13. through 15. for the remaining samples.
17. Add 5 µl of HSC extracted sample to the HSC wells (column 11). Cap HSC wells.
18. Finally, pipette 5 µl of positive template control RNA into all PTC wells. Cap PTC wells.
19. If using 8-tube strips, label the TAB of each strip to indicate sample position (DO NOT LABEL THE TOPS OF THE REACTION TUBES!). Briefly centrifuge tube strips for 10-15 seconds. Return strip tubes to cold rack.
If using plates, centrifuge at 500 x g for 30 seconds at 4°C. Return to cold rack.

RT-PCR amplification conditions

The reaction volume is 25µl. Program the thermocycler as follows:

Reverse Transcription	50°C for 30 min
Taq inhibitor activation	95°C for 2 min
PCR amplification (45 cycles)	95°C for 15 sec 55°C for 30 sec*

* Fluorescence data (FAM) should be collected during the 55°C incubation step.

Interpretation/examination:

1. The NTC reactions for probe/primer sets should not exhibit fluorescence growth curves that cross the threshold line. If a false positive occurs with one or more of the primer and probe NTC reactions, sample contamination may have occurred. Invalidate the run and repeat the assay with stricter adherence to the procedure guidelines.
2. All clinical samples should exhibit RP reaction curves that cross the threshold line at or before 37 cycles, thus indicating the presence of sufficient RNA from human RNase P gene indicating the specimen is of acceptable quality. However, it is possible that some samples may fail to give positive reactions due to low cell numbers in the original clinical sample. Also, samples taken from animal/avian species or cell culture typically exhibit either no RP reaction, or a weak RP reaction. Failure to detect RNase P in any of the clinical samples may indicate:
 - (a) Improper extraction of nucleic acid from clinical materials resulting in loss of RNA or carry-over of RT-PCR inhibitors from clinical specimens
 - (b) Absence of sufficient human cellular material in sample to enable detection
 - (c) Improper assay set up and execution
 - (d) Reagent or equipment malfunction
3. The HSC should NOT exhibit fluorescence growth curves for primer/probe sets InfA, swFluA, or swH1 that cross the threshold line within 40 cycles. If any influenza specific primer/probes exhibit a growth curve that crosses the threshold line, interpret as follows:

- (a) Contamination of RNA extraction reagents may have occurred. Invalidate the run and confirm reagent integrity of RNA extraction reagents prior to further testing.
 - (b) Cross contamination of samples occurred during RNA extraction procedures or assay setup. Invalidate the run and repeat the assay with stricter adherence to procedure guidelines.
- 4.** PTC reactions should produce a positive result with the InfA, swInfA, swH1, and RP reactions before 40 cycles. If expected positive reactivity is not achieved, invalidate the run and repeat the assay with stricter adherence to procedure guidelines. Determine the cause of failed PTC reactivity, implement corrective actions, and document results of the investigation and corrective actions. Do not use PTC reagents that do not generate expected result.
- 5.** When all controls meet stated requirements, a specimen is considered presumptive positive for influenza A virus if the InfA reaction growth curves cross the threshold line within 40 cycles. If the reaction for influenza A is positive, it may also be positive for Univ SW and/or SW H1. A specimen is considered presumptive positive for swine influenza A/H1 if BOTH the InfA and the respective subtype (swInfA or swH1) reaction growth curves cross the threshold line within 40 cycles. If a specimen is positive for InfA and only one of the subtype reactions or positive for InfA only, contact CDC for guidance.
- 6.** When all controls meet the stated requirements, a specimen is considered negative for influenza virus if growth curves for neither InfA cross the threshold within 40 cycles.

Limitations:

1. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. A false negative result may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
3. A false negative result may occur if an excess of DNA/RNA template is present in the in the reaction. If inhibition of the RP control reaction is noted for a particular sample, extracted RNA can be tested at 2 or more dilutions (e.g., 1:10 and 1:100) to verify the result.

PRIMER AND PROBE SETS

Note: primer/probe sets may undergo periodic modification as information about current circulating viruses is known.

Primers and Probes	Sequence (5'>3')	Working Concentration
InfA Forward	GAC CRA TCC TGT CAC CTC TGA C	40 μM
InfA Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	40 μM
InfA Probe ¹	TGC AGT CCT CGC TCA CTG GGC ACG	10 μM
SW InfA Forward	GCA CGG TCA GCA CTT ATY CTR AG	40 μM
SW InfA Reverse	GTG RGC TGG GTT TTC ATT TGG TC	40 μM
SW InfA Probe ²	CYA CTG CAA GCC CA" T" ACA CAC AAG CAG GCA	10 μM
SW H1 Forward	GTG CTA TAA ACA CCA GCC TYC CA	40 μM
SW H1 Reverse	CGG GAT ATT CCT TAA TCC TGT RGC	40 μM
SW H1 Probe ²	CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A	10 μM
RnaseP Forward	AGA TTT GGA CCT GCG AGC G	40 μM
RnaseP Reverse	GAG CGG CTG TCT CCA CAA GT	40 μM
RnaseP Probe ¹	TTC TGA CCT GAA GGC TCT GCG CG	10 μM

¹ TaqMan® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) (Biosearch Technologies, Inc., Novato, CA) at the 3'-end.

² Taqman® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified "T" residue with BHQ1, with a modified 3'-end to prevent probe extension by Taq polymerase.