Detection of avian influenza A viruses of subtype N1 by real time RT-PCR

This protocol is a copy of the standard operating procedure used by the OIE/FAO International Reference laboratory for AI at the Animal and Plant Health Agency. If you have any technical queries please contact AIWRL@apha.gov.uk

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Contents

1. INTRODUCTION

1.1 Purpose/Scope of this Protocol

1.1.1 This protocol provides the details required to detect viruses of N1 avian influenza virus (AIV) by reverse transcriptase RealTime (RRT)-PCR in swabs and tissues.

1.2 Background information

1.2.1 The N1 RRT-PCR described by Payungporn *et al* (J Virol Methods (2006) **131**;143-147) has been adapted to work in RRT-PCR chemistry (Slomka *et al,* Avian Pathology (2012); 41:177-193) . It is a rapid, sensitive and specific method for the detection of N1 AIV viruses.

1.2.2 In this protocol there is considerable commonality with other AIV RRT-PCRs in use (section 3), Procedure / Method (section 4) and Results (section 5) as regards (i) Chemicals and Reagents (3.2) for primers and probes, and (ii) the results interpretation guide for the two methods, *i.e.* sub-section 5.2.

1.2.3 Note the N1 RRT-PCR thermocycling conditions (section 4.6.2) which are the same as for the H5 and H7 RRT PCRs which have been validated at APHA. Primer and probe sequences are clearly unique (section 3.2.1) where attention is drawn to the use of an MGB-probe labelled with the VIC fluorophore, *ie* the fluorophore is **not** the commonly used FAM dye.

2. SAFETY

2.1 It is your laboratory's responsibility to ensure all work described in this protocol is conducted to a high safety standard.

3. MATERIALS

3.1 Documentation and software

3.1.1 Procedures:

Procedure for isolation and detection of viruses from avian species using embryonated eggs.

RNA extraction from biological samples

Extraction of nucleic acids from swabs using the QIAGEN BioRobot Universal

Influenza-Detection of influenza A matrix gene by real time Taqman® RT-PCR

Eurasian H5 avian influenza RealTime PCR

3.2 Chemicals and reagents

3.2.1 N1 RRT- PCR primers and probe (Payungporn *et al*, 2006)

N1F2 (forward primer):	5'-GTT TGA GTC TGT TGC TTG GTC-3'
N1R1: (reverse primer):	5'-TGA TAG TGT CTG TTA TTA TGC C-3'
N1 probe:	5' VIC-TTG TAT TTC AAT ACA GCC AC-MGB 3'

3.2.2 N1 RRT-PCR master mix

This utilises the Qiagen OneStep RT-PCR kit (Cat No. 1044287). Reagents not contained in the kit can be obtained from other suppliers. Volumes indicated below will be sufficient for 10 x 25μl reactions, *ie* divide the Master Mix into 10 x 23μl volumes & add 2μl extracted RNA to each.

DEPC treated water (ie RNAse-free)	Ambion or similar	139μl
(x5) Qiagen 1 step RT PCR buffer		50μl
Rox ref dye (pre-diluted 1:500 from Stratagene stock in DEPC water)	Stratagene or similar	3.75μl
Qiagen dNTP mix		10μl
N1F2 (forward primer): (50μM)	Sigma or similar	2μl
N1R1: (reverse primer): (50μM)	Sigma or similar	2μl
N1 probe: (30μM)	Applied Biosystems (MGB probe)	1.25μl
25mM magnesium chloride	Promega or similar	12.5μl
RNAsin (40U/μl)	Promega or similar	1μl

Qiagen 1 step RT PCR enzyme mix	10μl

3.2.3 N1 controls

1. Negatives:

Include at least one “no template controls” (NTC) where 2μl RNase free water are added.

2. N1 extraction control (positive):

An Eurasian N1 specimen, for example, an aliquot of inactivated A/chicken/Scotland/59 H5N1 should serve as an RNA extraction control (manual or robotic). The Ct value of this extracted RNA has been predetermined earlier: This should be within the Ct range 28-32. Higher titre (*ie* lower Ct) controls may risk contaminating neighbouring reaction wells. **NB** No positive control is required for manual extractions from egg amplified material where positivity has been demonstrated by haemagglutination as these act as positive controls in themselves. A positive control must be included for all clinical specimens where haemagglutination activity is unknown. See Appendix 1 for preparation of new batches. Enter as “HEX positive” during experimental set-up using the software on the RealTime instrument. See Appendix 1 for preparation of new batches.

3. N1 RNA dilution series (“standards”):

Use any Eurasian N1 RNA preparation from extracted egg fluid as x10 fold dilution series (at least 4 dilutions). This series can be used to determine the N1 RRT-PCR efficiency. See Appendix 1 for preparation of new batches.

AIV RNA is prepared in batches with a designated arbitrary value of 10^7 and stored at -70°C. Take out an aliquot and thaw. Carefully prepare ten-fold dilutions of RNA in DEPC water as follows:-

Ten-fold dilution from 10^7	Designated value (label tubes with these values)
10^{-1}	10^6
10^{-2}	10^5
10^{-3}	10^4
10^{-4}	10^3
10^{-5}	10^2
10^{-6}	10^1

Mix each dilution by agitation / flicking and briefly centrifuge. Make sufficient of each dilution so that enough is made to use for all the N1 RRT-PCR runs for that day. Store the aliquots on ice. Discard at the end of the working day. Each standard to be run is indicated on the worksheet using the designated value.

If conducting semi-quantitative N1 RRT-PCR, it is important to include low positive N1 controls which correspond to 10 EID_{50}/ml and 100 EID_{50}/ml (i.e. approx. Ct 36) of H7 AIV in the sample prior to RNA extraction – this will relate to defining the N1 RRT-PCR positive / negative cut-off (section 5.2., below).

4. Quantification of N1 positive controls:

Depending on the purpose of the experiment, the N1 positive control material may have been quantified by using classical virus titre determination in embryonated chickens' eggs or by using M-gene RealTime PCR in quantitative mode with a series of standards which had themselves been obtained from AIV of known titre.

3.3 Equipment

3.3.1

Microcentrifuge tubes (1.5ml)	Biomek2000 robot or similar
-70°C or lower freezer	20µl robot barrier tips
Pipettes	-18°C or lower freezer
Sterile, Rnase-free pipette tips with aerosol barrier	Vortex mixer
Mx3000/3005 real time RT-PCR plates/strips	Microcentrifuge (with rotor for 2ml tubes)
Mx3000/3005 real time RT-PCR plate caps	Mx3000/3005 real time RT-PCR quantitative machine

4. PROCEDURE/METHOD

4.1 Test Reliability

4.1.1 Process of assuring ongoing test reliability:

Method & frequency: Internal QA (IQA) is achieved by blind testing in a Ring Trial. This consists of testing a "Mini Panel" of anonymous AIV samples, where IQA results demonstrate ongoing test reliability. In addition, an annual EU AI PCR Proficiency Panel is prepared & tested as part of an external QA (EQA) scheme, where testing of this Panel can also provide evidence of ongoing test reliability.

This and other AI RRT-PCR protocols note the importance of gauging RRT-PCR performance by monitoring the (i) Ct value observed for the positive extraction control and the (ii) Ct values and standard curve obtained from the 10-fold dilution series (RNA standards) to determine the efficiency of the RRT-PCR. This is crucial for the routine use of the AI RRT-PCRs for routine use in Reference / Diagnostic Laboratory work.

4.2 Preparation of PCR master mix and loading

4.2.1 Preparation of PCR master mix and loading of RealTime plate / strips(s) to be carried out in the PCR clean room.

4.2.2 Make up master mix sufficient for the number of samples to be tested.

4.2.3 Thoroughly mix the master mix and centrifuge for 30 secs to remove bubbles.

4.2.4 Aliquot 23µl of master mix per well of the RealTime plate / strip(s).

NB: Plastic ware for the Mx3000/3005 series are specific for that model of Stratagene machine, and are not interchangeable. Make sure the plasticware is appropriate for the chosen RealTime PCR platform.

4.2.5 Loosely place the plate caps on the portion of the plate being used. All wells will need to be covered even though only a portion of the plate may be used.

4.2.6 Bring the plate / strip(s) out of the PCR clean room and place on ice before addition of sample RNA and controls.

4.3 Preparation of N1 RNA controls

4.3.1 N1 positive and negative controls are as described above in 3.2.3.

4.3.2 Extract N1 RNA manually or robotically.

4.3.3 Include N1 RNA positive controls “standards” as a dilution series in each N1 RRT-PCR experiment (section 3.2.3, paragraph 3).

4.4 Manual addition of samples and standards

4.4.1 **NB** Take care to avoid cross contamination of samples at this stage.

Change gloves frequently and do not hold tips containing RNA above the incorrect wells. It is advised to cover strips loosely which have not yet been filled with caps (strip covers) to further minimise the risk of cross contamination.

Referring to plate layout, add **2**µl of the negative extraction control, then add the sample RNA to the master mix. Replace caps on tubes.

Where possible, use a dedicated multi-channel pipette to load multiple samples *e.g.* eight samples per strip.

4.4.2 Add **2**µl DEPC water as a no template control (NTC), replace cap on this well and then add the real time PCR RNA standards and positive RNA extraction control to appropriate wells based on your worksheet layout

4.4.3 Once RNA is added, fit caps (strip covers) to all wells.

4.4.4 It is important that the caps are fitted firmly and correctly onto the wells before insertion into the RealTime instrument.

4.4.5 If the RealTime plate/ strip(s) are not to be loaded into the instrument immediately, keep the plate / strip(s) on ice until ready to test.

4.5 Reverse transcription (RT) and PCR

4.5.1 Place the RealTime plate / strip(s) in the appropriate RealTime instrument.

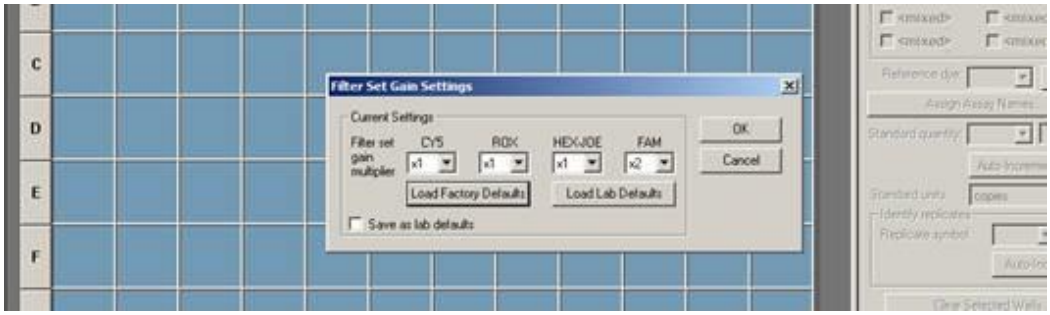
4.5.2 The thermocycling profile is:

RT step:	50C for 30 mins
	95C for 15 mins
PCR step (x40 cycles)	95C for 10 secs
	54C for 30 secs
	72C for 10 secs

These cycling conditions are identical to those employed in validated H5 and H7 RRT PCRs (see 1.2.3 above), so enabling more than one target to be addressed in a single RRT PCR experiment.

NB: Collect fluorescence data at end of 55C step using the HEX and ROX filters. The HEX channel is chosen to detect VIC fluorescence. Use endpoint read option with one read.

N.B: 'Filter Gain Setting' - After servicing or repair this setting is often changed back to the factory default of FAM x8. On return the filters for use Cy5 ROX and HEX should all be set to x1, Fam should be set to x2. Please see example below. If prompted or you wish to check the filter settings, it is possible in the MXPro software to change the 'Filter Set Gain Settings' – click Instrument, then filter Set Gain Settings.



4.5.3 Open a new file on the instrument using the MxPro software and select the RealTime quantitative PCR (multiple standards) option for the experiment. The plate setup can be re-entered for each experiment or imported from previous experiments if desired. Thermal profiles for import note that the lamp on both RealTime machines requires 20 minutes to warm up, and this can occur during the RT step (4.5.2 above).

4.5.4 When the plate is setup and thermoprofile windows have been entered, select "run" to start thermocycling. A storage window will automatically open.

4.5.5 The complete run takes approximately 2.0 hours. If the machine is not booked for immediate use by another colleague upon completion of the run, select the option to turn off the lamp at the end of the run. This is because the lamps have a limited lifespan.

5. RESULTS

5.1 Analysis and display of results by using MxPro software

5.1.1 The fluorescence data can be viewed during and / or after the PCR using the raw data plots tab in the "Run" section by using the MxPro software.

5.1.2 When the run is complete, the MxPro file should be saved.

5.1.3 To analyse the data select the Analysis section button and select the wells to be examined in the Analysis / Setup window.

5.1.4 To view the results click on the Results tab in the Analysis section and view the amplification plots. There are four options for analysing the fluorescence:

R (Multicomponent view) displays the **raw fluorescence** in arbitrary units.

dR displays the **baseline-corrected fluorescence**. As all reactions and wells will start with slightly different fluorescence readings, this option sets a baseline value of 0 to all plots. This correction is determined by the fluorescence values obtained during the initial rounds of the PCR. The adaptive baseline algorithm calculates the best baseline for each plot individually.

Rn displays the **fluorescence normalised** to the passive reference dye (ROX). This allows for fluctuations in fluorescence, which are not due to cleavage of the TaqMan probe.

dRn displays the **baseline-corrected normalised fluorescence**.

The dRn option with the ROX channel switched on is the most appropriate option for analysis of the data.

5.2 Interpretation of N1 RRT-PCR results

5.2.1 Analyse the data by comparing the results obtained for the negative (NTC) and N1 positive controls.

5.2.2 Negative controls (NTCs):

All NTCs should give "No Ct" as their final result. High Cts in all NTC wells with a linear character (*eg* >37) & giving a very low level final

fluorescence (*ie* little greater than the initial "flare" fluorescence values at early cycles) suggest that probe degradation may have occurred, *eg* the probe has been excessively frozen and thawed. Although such observations may not invalidate the experiment, it is advised to discard the aliquot & thaw-out a fresh aliquot of the relevant N1 probe for subsequent experiments & note result.

If the late Ct value (>37) has a logarithmic / sigmoidal character where clear final fluorescence values are observed, then contamination of the NTC wells with N1RNA may be considered. It is also possible that such very late Ct signals may occasionally occur spuriously. Repeat the N1 RRT-PCR experiment.

5.2.3 N1 positive controls

1. N1 extraction control (3.2.3, paragraph 2):

This should reproducibly amplify with a Ct value +/- 2 Cts either side of their predetermined Ct value. Greater increases above the predetermined Ct value suggest that:

- RNA extraction from the specimens has been suboptimal. Repeat.
- It is also possible that the N1 positive control may have degraded if stored incorrectly *eg* 4C, excessive freeze-thawing etc….
- Deviation (+/- 2 Cts) may also occur where software settings on the instrument are inaccurate / inappropriate and the fluorescence cut-off level excessively high / low & is affecting the Ct value of the predetermined N1 positive control. It may be possible to reset the software after the experiment to restore the expected Ct value provided the other controls are in order.

2. N1 RNA dilution series "standards" (3.2.5, paragraph 3)

Ten-fold dilution series should yield a straight line with R^2 value of >0.975 (ideally >0.985), slope in the range of -3.1 to –3.9 which should correspond to N1 RRT-PCR efficiency in the range 80 – 115%. Deviation from these conditions may be due to (i) inaccurate assembly of the master mix / inaccurate pipetting, (ii) inaccurate RNA addition (iii) degradation of the RNA standards by inappropriate storage or (iv) inefficient thermocycling / fluorescence reading due to an instrument problem. This indicates a need to address these matters for subsequent N1 RRT-PCR experiments.

5.2.4 If all controls are within acceptable limits, analyse the data for the test samples.

5.2.5 Criteria for assessing results on test samples are as follows in the N1 RRT-PCR:

1. Examine the N1 RNA dilution series: Note the Ct value of the control RNA extracted from a Eurasian N1 sample at a titre of 10 EID_{50}/ml (this Ct being an average of duplicate testing) – refer to section 3.2.5 paragraph 3 (above). This should yield a Ct value in the range 34 – 37 by N1 RRT-PCR. In this example, where Ct 36 is obtained from the 10 EID_{50}/ml RNA control, a Ct < 36 in the N1 RRT-PCR is considered clearly N1 positive, while a specimen recording "No Ct" value is clearly negative.

2. Samples with Ct >36 in the N1 RRT-PCR, but with a logarithmic / sigmoidal character giving clear final fluorescence greater than that observed during early cycles: These are generally considered as "inconclusive", however the following paragraph presents a possible strategy for confirmation as either "N1 [very low] positive" or "N1 negative":

3. Check the Ct value when the same RNA extract was tested by the M gene RealTime PCR: A Ct value of 34-37 by M gene RealTime PCR suggests that this may be a very weak positive N1 RRT-PCR result. A reproducible result obtained after re-extraction of RNA from the same clinical material by N1 / M gene RealTime PCRs would tend to confirm this. However, it is unlikely that such "late Ct risers" (particularly those from field submissions) would yield positive results by other tests *eg* virus isolation or conventional AIV PCRs due to their lower sensitivity.

6. REFERENCES

6.1 **Payungporn, S., Chutinimitkul, S., Chaisingh, A., Damrongwantanapokin, S., Buranathai, C., Amonsin, A., Theamboonlers, A., Poovorawan, Y.,** 2006. Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J. Virol. Methods **131**, 143-147.

Marek J Slomka, Thanh L To, Hien H Tong, Vivien J Coward, Amanda Hanna, Wendy Shell, Theo Pavlidis, Anstice L E Densham, Georgios Kargiolakis, Mark E Arnold, Jill Banks, Ian H Brown Challenges for accurate and prompt molecular diagnosis of clades of highly pathogenic avian influenza H5N1 viruses emerging in Vietnam. Avian Pathology (2012); 41:177-193.

APPENDIX 1

Preparation of TaqMan®real-time RT- PCR positive extraction controls and RNA standards

These can be prepared from either live or inactivated egg-grown antigen and can be supplied either as an aliquot of freeze-dried material or an aliquot from a wet 'antigen stock.

NB - For live antigen follow safety procedures in place for the virus type being produced. Work at the appropriate containment level for the virus being used – refer to the virus categorisation risk document

To prepare positive extraction controls

1. If the antigen is supplied freeze-dried, reconstitute the vial with 1ml molecular grade water. Ensure the solution is mixed thoroughly by gentle vortexing.
2. Prepare a 10-fold dilution series of the antigen in PBS e.g. from 10^{-1} - 10^{-6} dilutions inclusive.
3. Freeze 200 µl aliquots of each dilution at -70 ºC.
4. Thaw-out an aliquot of each dilution and extract RNA from each aliquot either manually or on the Bio-robot, RNA extraction from biological samples or protocol, Extraction of nucleic acids from swabs using the Qiagen BioRobot Universal.
5. Test the RNA from each member of the dilution series by the appropriate real-time PCR assay. Choose the optimal 10-fold dilution from the resulting Ct values.
6. Prepare the chosen dilution of the antigen in bulk using PBS, assign a batch number, dispense into 200µl aliquots, label and freeze at -70 ºC.
7. Remove an aliquot from the freezer, extract the RNA and test by the appropriate real-time RT-PCR to confirm the Ct values from the dilution fall within the required range.

To prepare RNA PCR standards

1. If the antigen is supplied freeze–dried, reconstitute the vial with 1ml molecular grade water. Ensure the solution is mixed thoroughly by gentle vortexing.
2. Manually extract the RNA from the whole reconstituted antigen from 1ml of wet stock according to the protocol, RNA extraction from biological samples.
3. Pool together the RNA extractions.
4. Freeze a 5µl aliquot at-70 ºC
5. Thaw this aliquot and prepare a 10-fold dilution series in molecular grade water from as described above from 10^{-1} - 10^{-7}
6. Test the dilution series by the appropriate real-time RT-PCR. Test the current 'in-use' RNA standard batch in parallel on the same PCR test run.
7. Directly compare the results of the two batches. The Ct values and test efficiencies should be similar i.e. Ct values +/- 2.0 per corresponding dilution. If there is an

apparent significant difference, adjust the dilution of the new batch of RNA (eg. make a 1/10 dilution) and repeat stages 3-7 above.

8. Once the required dilution is achieved, prepare 5µl aliquots, assign a batch number, label the tubes and store at -70 ºC.