

Eurasian H5 avian influenza RealTime PCR

This protocol is a copy of the standard operating procedure used by the avian influenza CRL at the Veterinary Laboratories Agency. If you have any technical queries please contact aiwrl@vla.defra.gsi.gov.uk

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1. INTRODUCTION

1.1 Purpose/Scope of this Protocol

1.1.1 This protocol provides the details required to detect Eurasian isolates of H5 avian influenza (AI) by RealTime PCR.

1.2 Background information

1.2.1 This is a rapid, sensitive and specific method for the detection of H5 AI. Validation at VLA has revealed it to be effective in detecting H5 AI directly from clinical specimens, *i.e.* there is no need to first isolate the suspect H5 isolate in embryonated eggs by growth to a high titre (biological amplification). H5 RealTime PCR has been described previously (Spackman *et al*, 2002), but validation was restricted to a largely N American collection of H5 AI isolates. For the method described in this protocol, haemagglutinin gene sequences from Eurasian H5 isolates (mainly 1996-2004) were aligned. Here the H5 primer and hydrolysis probe sequences of Spackman *et al* (2002) were modified to ensure effective detection of recent Eurasian H5 isolates.

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. This includes an awareness of risks relating to *eg* dangerous or toxic chemicals, potentially hazardous procedures etc.... Here local safety rules in your laboratory should be understood by all relevant members of staff.

3. MATERIALS

3.1 Documentation and software

3.1.1 Worksheet for RealTime plate layout.

Instruction manual for RealTime PCR platform and software in use in your laboratory.

3.2 Chemicals and reagents

3.2.1 Primers & probe (note degenerate nucleotides shown in bold):

H5LH1 ACA TAT GAC TAC CCA CAR TAT TCA G

H5RH1 AGA CCA GCT AYC ATG ATT GC

H5PRO FAM-TCW ACA GTG GCG AGT TCC CTA GCA-TAMRA

3.2.2 H5 RealTime PCR master mix

This utilises the Qiagen OneStep RT-PCR kit (Cat No. 210212). Reagents not contained in the kit can be obtained from other suppliers. Volumes indicated below will be sufficient for 10 x 25µl reactions, *i.e.* divide the Master Mix into 10 x 23µl volumes & add 2µl extracted RNA to each.

DEPC treated water (<i>ie</i> RNase-free)	Ambion or similar	137.5µl
(x5) Qiagen 1 step RT PCR buffer		50µl
Rox ref dye (pre-diluted 1:500 from Stratagene stock in DEPC water)	Stratagene similar	or 3.75µl
Qiagen dNTP mix		10µl
H5LH1 (50µM)	Sigma or similar	2µl
H5RH1 (50µM)	Sigma or similar	2µl
H5PRO (30µM)	Sigma or similar	2.5µl
25mM magnesium chloride	Promega or similar	12.5µl
RNAsin (40U/µl)	Promega or similar	1µl
Qiagen 1 stepRT PCR enzyme mix		10µl

3.2.3 H5 controls:

Negatives

Include at least two “no template controls” (NTCs) where 2ul RNase free water is added to each well.

Positives

Quantified H5 specimen *eg* aliquot of inactivated freeze-dried H5N1 A/chicken/Scotland/59 should serve as an extraction control (manual or robotic). The Ct value of this extracted RNA has been predetermined earlier, and ideally use in duplicate for each H5 RealTime experiment. Enter as “unknown” (or equivalent) during experimental set-up using the software on the RealTime instrument.

Use any other H5 RNA preparation from extracted egg fluid which has been diluted into a x10 fold dilution series (at least 4 dilutions). Ideally this should be a duplicate dilution series. This series (entered appropriately as “standards” with relative quantities indicated by using the

software while setting up the RealTime experiment) can be used to determine the H5 RealTime PCR efficiency.

4. PROCEDURE/METHOD

4.1 Preparation of PCR master mix and loading

4.1.1 Preparation of PCR master mix and loading of real time plate/strip(s) to be carried out in the PCR clean room.

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

4.1.3 Thoroughly mix the master mix and centrifuge for 30 seconds to remove bubbles.

4.1.4 Aliquot 23 μ l of master mix per reaction in the RealTime plate/strip(s).

NB: Ensure the plasticware (*ie* plates / strips and covers / caps) is appropriate for the chosen RealTime PCR platform.

4.1.5 Loosely place the plate / strip caps on the plate / strip(s). All wells will need to be covered even though only a portion of the plate may be used.

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

4.2 Preparation of H5 RNA controls

4.2.1 H5 positive controls are as described above in 3.2.3.

4.2.2 Extract RNA as per the manufacturer's protocol

4.2.3 Include H5 positive controls (ideally in duplicate) in each H5 RealTime experiment.

4.3 Manual addition of samples and standards

4.3.1 Regardless of whether RNA extraction has been conducted manually or robotically (VI 537), at VLA we manually add 2 μ l of the sample RNA to the 23 μ l of master mix. A dedicated multichannel pipette is convenient for adding robotically extracted RNA.

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.

4.3.2 Add 2 μ l of RealTime PCR controls to appropriate wells based on your worksheet layout. Re-apply caps to the rest of the plate/strip(s).

4.3.3 Once RNA is added, fit caps to all wells.

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

4.3.4 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.

4.4 Reverse transcription (RT) and PCR

4.4.1 Place the real time plate/strip(s) in the appropriate real time machine.

4.4.2 Incubate the reactions with the following thermocycling profile:

RT step: 50°C for 30 mins

95°C for 15 mins

PCR step (x40 cycles) : 95°C for 10 secs

54°C for 30 secs

72°C for 10 secs

NB: Collect fluorescence data at end of 54°C step using the ROX and FAM filters.

4.4.3 Open a new file using the instrument software to define all the necessary RealTime PCR experimental parameters. This is conducted according to the manufacturer's software instructions. Provide the file an appropriate H5 RealTime PCR name.

4.4.5 The complete run takes approximately 2 hours, but this may vary according to the make of instrument. If the machine is not booked for immediate use by another colleague upon completion of the run, select the option (if available) to turn off the lamp at the end of the run. This is because certain makes of lamp have a limited lifespan.

5. RESULTS

5.1 Analysis and display of results

5.1.1 Conduct this after familiarising yourself with your RealTime PCR software and instructions.

5.2 Interpretation of results

5.2.1 Analyse the data by comparing the results obtained for the negative (NTCs) & H5 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 (e.g. >38) & giving a very low level final fluorescence (*i.e.* little greater than the initial “flare” fluorescence values at early cycles) suggest that probe degradation may have occurred, e.g. 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 H5 probe for subsequent experiments & note result.

If the late Ct value has a logarithmic / sigmoidal character where clear final fluorescence values are observed, then contamination of the NTC wells with H5 RNA must be considered. Repeat the experiment.

5.2.3 H5 positive controls:

Quantified specimens which serve as an extraction control (e.g. A/chicken/Scotland/59 (H5N1), section 3.2.3) should reproducibly amplify with a Ct value +/- 2 Cts either side of their predetermined Ct value of e.g. 30. Greater deviations from the predetermined Ct value suggest that:

- The H5 positive control may have degraded (if Ct greater than predetermined value).
- 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 H5 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.
- Deviation (+/- 2Cts) 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 H7 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.

Ten-fold dilution series of RNA should yield a straight line with R² value of 0.975 (ideally >0.985), slope in the range of -3.0 to -3.9 which should correspond to H5 RealTime PCR efficiency in the range 80 - 110%.

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:

1. Samples with Ct <33:
H5 positive.
2. Samples with Ct >33 but with a clear logarithmic/sigmoidal character

giving clear final fluorescence greater than that observed during early cycles:

At the time of writing these are considered inconclusive by H5 RealTime PCR. However, it is important to consider the results of other tests e.g. virus isolation in embryonated eggs (followed by typing with reference antisera) and/or H5 conventional PCRs & sequencing which may reveal a H5 positive result for the specimen.

3. Samples with Ct >33 but with a linear character giving final fluorescence which is approximately the same as that observed during early cycles:
 - a. As noted above in section 5.2.2, such a result may be due to H5 probe degradation. Resort to using fresh aliquot of probe.
 - b. Alternatively, other possible explanations include inefficient hybridisation at late cycles to non-specifically amplified nucleic acid of host origin in the RNA sample.

Such results are most likely to be H5 negative, but consider the results of other tests e.g. virus isolation in embryonated eggs (followed by typing) and / or H5 conventional PCRs to exclude a H5 positive result for the specimen.

4. No Ct value:

H5 RealTime PCR negative.

5.2.6 All H5 positive results from a potential new cases of AI are repeated and for an index case should be confirmed by use of virus isolation in accord with current OIE/EU guidelines. For the latter conventional H5 PCR and sequencing before reporting is crucial in order to confirm the H5 subtype of detected AI.

5.2.7 Other considerations for the Eurasian H5 RealTime PCR:

This H5 RealTime PCR was designed & validated to detect recent Eurasian H5 isolates. However, there is some evidence that certain American lineage H5s *may* be detected by this procedure. However, it must be stressed that detection of American H5 isolates by this Eurasian H5 RealTime PCR has not been validated.

Primer & probe design for Eurasian H5 isolates:

As these viruses continue to evolve, it is possible that new isolates will emerge which will be less efficiently detected by the above method, or even not detected at all. Revision of primer / probe sequences will be necessary and this will be reflected in any future updated versions of this protocol.

6. REFERENCES

- 6.1.1 **Slomka, M. J., Pavlidis, T., Banks, J., Shell, W., McNally, A., Essen, S., Brown, I. H., 2007.** Validated H5 Eurasian real-time reverse transcriptase–polymerase chain reaction and its application in H5N1 outbreaks in 2005–2006. *Avian Dis.* 51, 373-377.