Protocol shared by Animal and Plant Health Agency (APHA), Weybridge, UK
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RealTime (RRT-) PCR testing for Eurasian H5 avian influenza viruses (AIVs) following outbreaks of a new H5N8 highly-pathogenic avian influenza virus (HPAIV) in Europe

1. Testing by the Eurasian H5 RRT-PCR protocol

Validation of the Eurasian H5 RRT-PCR (Slomka et al 2007) led to its adoption as a recommended method for H5 avian influenza virus (AIV) detection by AI National Reference Laboratories (NRLs) in the Member States (MSs) of the European Union (EU) and elsewhere. In subsequent years this test has been successful in diagnosing H5 AIV poultry outbreaks and for investigations of AIV infection in wild birds, including those caused by "Asian-lineage" H5N1 highly pathogenic (HP)AI viruses (e.g. Gaidet *et al* (2008), Baumer *et al* (2010), Slomka *et al* (2010), Śmietanka & Minta (2014), Tønnessen *et al* (2013)).

Fitness-for-purpose testing of this Eurasian H5 RRT-PCR has been reinforced through its use by EU AI NRLs and other laboratories during the annual EU AI PCR Proficiency exercise, undertaken since 2006. Participating laboratories have demonstrated their ability to successfully use this test in the identification of a variety of different H5 AIVs (both low pathogenicity (LP)AI and HPAI) of contemporary origin which are included as blind specimens in this annual EU-organised Proficiency Panel.

Continuing evolution of the Asian lineage H5N1 HPAI viruses has resulted in mismatches in the primer and probe binding sequences for the Eurasian H5 RRT-PCR, as observed for clades 2.3.2 and 2.3.4. However, it was shown that the Eurasian H5 RRT-PCR remained sensitive for the detection of these viruses in clinical specimens, demonstrating a clear degree of tolerance for mismatches in the primer / probe binding sequences (Slomka *et al* 2012).

The current need to detect newly-emerged H5N8 HPAI viruses, following their recent incursions into the EU (November 2014), has shown that there are three and two mismatches in the forward and reverse primers respectively for the Eurasian H5 RRT-PCR. Despite these mismatches, the Eurasian H5 RRT-PCR remains sufficiently sensitive to have successfully diagnosed the European H5N8 HPAI outbreaks and wild bird incidents to date. Therefore the Eurasian H5 RRT-PCR is considered a sufficiently sensitive test for the detection of the new H5N8 HPAI viruses, and no immediate modification is urgently required for the protocol presented in this document. Nevertheless, the global situation concerning new incursions and potential inter-continental spread of H5N8 HPAI viruses is being monitored, and any need to modify the Eurasian H5 RRT-PCR protocol for specific detection of this particular H5N8 HPAI virus will be communicated as and when the need may arise. It must be emphasised, however, that any H5N8-specific RRT-PCR would be designed for optimised detection of this particular H5 AI virus, and should **not** be considered as a direct replacement for the Eurasian H5 RRT-PCR outlined in this protocol which remains sensitive for the detection of a broad range of Eurasian H5 AIVs.

2. Eurasian H5 RRT-PCR protocol

Primer and probe design: This test has been included as a recommended Eurasian H5 RRT-PCR protocol in the EU Diagnostic Manual for AI (EU 2006). The nucleotide sequences of the primers and hydrolysis probe were designed at APHA by Slomka *et al* 2007:

H5LH1: 5’ ACA TAT GAC TAC CCA CA**R** TAT TCA G 3’

H5RH1: 5’ AGA CCA GCT A**Y**C ATG ATT GC 3’

H5PRO: FAM- 5’ TC**W** ACA GTG GCG AGT TCC CTA GCA 3’ - BHQ1

Note degenerate nucleotides indicated in bold. As an alternative to Black Hole Quencher-1 (BHQ-1), TAMRA may be used as the quencher at the 3’ end of the probe.

Eurasian H5 RRT- PCR Master Mix: This is based on the Methods section of Spackman *et al* (2002), which utilises the OneStep RT-PCR Kit (Cat # 210212, Qiagen). Reagents not provided in the kit can be obtained from other suppliers as indicated in below table which describes a Eurasian H5 RRT-PCR Master Mix for 10 x 25µl reactions:

Ingredients - all except primers & probe are from Qiagen OneStep RT-PCR Kit (cat # 210212) & other stated suppliers:	Volumes (µl) for a 10 reaction recipe:
DEPC-H2O (Ambion / Life Technologies or similar):	137.5
(x5) Qiagen 1-step RT PCR buffer:	50
ROX ref dye (pre-dilution of 1:500 neat stock from Stratagene / Agilent in DEPC-H2O):	3.75
Qiagen dNTP mix:	10
H5LH1 (50µM):	2
H5RH1 (50µM):	2
H5PRO (30µM): (Remember to reconstitute a freeze-dried probe in (x1) TE buffer*)	2.5
25mM MgCl2 (Promega):	12.5
RNAsin (40U/µl, Promega):	1
Qiagen 1-step RT PCR enzyme mix:	10
Total	231.25

Mix the Master Mix tube thoroughly by inversion & flicking, spin approx 30'' to remove bubbles.
Then aliquot 23µl of the Master Mix per reaction well, then add 2µ extracted RNA / appropriate controls as target to each well.

*TE buffer (x1): 10mM Tris-HCl (pH7.5) 1mM EDTA

Eurasian H5 RRT- PCR cycling conditions: These are currently in use on Stratagene (Agilent) Mx3000 & Mx3005 instruments at APHA. Other laboratories using different instrumentation platforms should first critically and carefully examine these cycling conditions as they may *not* perform optimally on non-Stratagene RealTime instruments. Hence cycling temperatures, times and ramp speeds may all need to be modified by a non-Stratagene user:
30 mins at 50C, 15 mins at 95C;
Then x40 cycles of:
10 secs at 95C
30 secs at 54C
10 secs at 72C
NB: Fluorescence data is gathered at the end of each 54C annealing step.

3. References

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