

**H7 Eurasian RealTime PCRs for the detection  
and pathotyping of Eurasian H7 avian influenza  
isolates**

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](mailto: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 (i) detect Eurasian isolates of H7 avian influenza virus (AIV) by RealTime PCR and also to (ii) pathotype any H7 positive specimen by amplicon sequencing.

### 1.2 Background information

1.2.1 Two H7 RealTime PCRs are presented in this document. Both are rapid, sensitive and specific method for the detection of Eurasian H7 AIV isolates. In brief, the two H7 RealTime PCR methods differ as follows:

1.2.2 "**HA2**" H7 RealTime PCR: Validation studies at VLA have shown this to be the more sensitive of the two H7 detection methods, amplifying a product within the HA2 region of the H7 gene. Detection utilises a H7-specific hydrolysis ("TaqMan") probe with fluorescence emission in RealTime.

1.2.3 Cleavage Site ("**CS**") H7 RealTime PCR: While validation studies have shown this to be the less-sensitive of the two H7 RealTime PCRs, this method amplifies a product which spans the H7 gene cleavage site (CS) region. Sequencing of this H7 CS amplicon allows for successful pathotyping of the H7 AIV as either low pathogenic (LP) or highly pathogenic avian influenza (HPAI).

1.2.4 Validation at VLA has revealed both H7 RealTime PCR approaches to be effective in detecting H7 AI directly from clinical specimens, *ie* there is no need to first isolate the suspect H7 isolate in embryonated eggs by growth to a high titre. H7 RealTime PCR has been described previously (Spackman *et al*, 2002), but here primer and probe design was tailored for the detection of N American H7 AI isolates.

1.2.5 For the methods described in this protocol, haemagglutinin (HA) gene sequences from Eurasian H7 isolates (mainly 1995-2005) were aligned. This served to optimise primer and probe design for the detection of Eurasian H7 AIV isolates. The **HA2** H7 RealTime PCR was designed at VLA, while the **CS** H7 RealTime PCR was designed by Mr John Voermans and Dr Guus Koch at CIDC-Lelystad in the Netherlands.

1.2.6 In this protocol there is considerable commonality for both the HA2 and CS H7 RealTime PCRs as regards Materials (section 3), Procedure / Method (section 4) and Results (section 5). However, it is important to note the differences between these two H7 RealTime PCRs as regards (i) Chemicals and Reagents (3.2) for primers and probes, and (ii) the results interpretation guide for the two methods, *ie* sub-sections 5.2.5 and 5.2.6.

1.2.7 In addition, the CS H7 RealTime PCR provides the additional opportunity to pathotype by amplicon sequencing as stated in Procedure / Method (section 4.6). Examination of this H7 CS amplicon sequence will determine whether the H7 specimen is low pathogenic (LP) or highly pathogenic avian influenza (HPAI).

## 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

3.1.4 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 "HA2" H7 RealTime PCR primers and probe

LH6H7 5'-GGC CAG TAT TAG AAA CAA CAC CTA TGA-3'

RH4H7 5'-GCC CCG AAG CTA AAC CAA AGT AT-3'

H7pro11 5'-FAM-CCG CTG CTT AGT TTG ACT GGG TCA ATC T-BHQ-3'

3.2.2 "CS" H7 RealTime PCR primers and probe

H7F 5'-CGT GCA AGT TTT CTG AGA GG-3'

H7R 5'-GAC CTT CCC ATC CAT TTT CA-3'

H7-TM1 5'-FAM-AAC CCG CTA TGG CAC CAA ATA GGC CTC-BHQ-3'

3.2.3 HA2 H7 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, *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
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(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
LH6H7 (50µM)	Sigma or similar	2µl
RH4H7 (50µM)	Sigma or similar	2µl
H7pro11 (30µM)	Sigma or similar	1.25µl
25mM magnesium chloride	Promega or similar	12.5µl
RNAsin (40U/µl)	Promega or similar	1µl
Qiagen 1 stepRT PCR enzyme mix		10µl

#### 3.2.4 **CS** H7 RealTime PCR master mix

This utilises the Qiagen OneStep RT-PCR kit as described above (3.2.3) for the HA2 H7 RealTime PCR. The only differences are the choice of primers and probe. The 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
H7F (50µM)	Sigma or similar	2µl
H7R (50µM)	Sigma or similar	2µl
H7-TM1 (30µM)	Sigma or similar	1.25µl
25mM magnesium chloride	Promega or similar	12.5µl
RNAsin (40U/µl)	Promega or similar	1µl
Qiagen 1 stepRT PCR enzyme mix		10µl

#### 3.2.5 H7 controls:

##### 1. Negatives:

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

##### 2. H7 extraction control (positive):

*Eg* an aliquot of inactivated H7 virus from your repository. This should serve as an RNA extraction control (manual or robotic). The Ct value of this extracted RNA should be predetermined earlier according to

your decision, but a “low positive” titre is advisable eg a Ct value of approximately 30. This is because a low titre extraction control is much more likely to indicate problems with RNA extraction, whereas a high titre (*ie* lower Ct) extraction may not indicate this. Higher titre extraction controls may also risk contaminating neighbouring reaction wells. Ideally use in duplicate for each H7 RealTime experiment. Enter as “unknown” (or equivalent) during experimental set-up using the software on the RealTime instrument.

3. H7 RNA dilution series (“standards”):

Use any other Eurasian H7 RNA preparation from extracted egg fluid as 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 H7 RealTime PCR efficiency. It is important to include low positive H7 controls which correspond to approximately 10 EID<sub>50</sub>/ml and 100 EID<sub>50</sub>/ml of H7 AIV in the sample prior to RNA extraction – this will relate to defining the H7 RealTime PCR positive / negative cutoff, where the corresponding Ct value will differ in the HA2 and CS versions (sections 5.2.5 and 5.2.6, below).

4. Quantification of H7 positive controls:

The H7 positive control material may have been quantified by using classical virus titre determination in embryonating chickens’ eggs or by using M-gene RealTime 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)	Correctly functioning RNA extraction robot
	-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
	Appropriate plasticware for the RealTime PCR platform of your choice.	Microcentrifuge (with rotor for 2ml tubes)
	RealTime PCR platform of your choice, where you are satisfied that both the instrument are in correct working order.	

## 4. PROCEDURE/METHOD

### 4.1 Preparation of PCR master mix and loading

4.1.1 Preparation of PCR master mix and loading of RealTime plasticware for reactions 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 secs to remove bubbles.

4.1.4 Aliquot 23µl of master mix per well of 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 Use plasticware to loosely cover the plate / strips accordingly

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

### 4.2 Preparation of H7 RNA controls

4.2.1 H7 positive and negative controls are as described above in 3.2.5.

4.2.2 Extract H7 RNA manually or robotically according to the manufacturer's instructions.

4.2.3 Include H7 RNA positive controls "standards" (ideally in duplicate) as a dilution series in each H7 RealTime PCR experiment (section 3.2.5, paragraph 3).

### 4.3 Manual addition of samples and standards

4.3.1 Referring to plate layout, add 2µl of the sample RNA to the 23µl of master mix. Where possible, use a dedicated multi-channel pipette to load multiple samples *eg* eight samples per strip.

**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.

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

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

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

4.3.5 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.

#### 4.4 Robotic addition of samples

4.4.1 Gloves to be worn at all times.

4.4.2 Work according to the manufacturer's instructions in order to extract RNA from specimens.

4.4.3 At VLA we prefer to add extracted RNA to the RealTime plate manually by using a multichannel pipette. Once addition of RNA is complete, reseal the plate containing the eluted RNA and store at -70C.

4.4.4 Manually add 2µl of RealTime PCR controls (positive and negative) to appropriate wells as described above in 4.3.2.

4.4.5 Ensure that the caps / strip covers are completely flat across the whole plate.

4.4.6 The plate is now ready to be run on a RealTime PCR instrument.

4.4.7 If the plate is not to be loaded into the RealTime instrument immediately, keep plate on ice until required.

#### 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 identical for **both** the **HA2** and **CS** H7 RealTime PCRs. These are also identical to that used for the H5 RealTime PCR which enables more than one of these assays to be ran simultaneously in one plate:

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

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

- 4.5.3 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.
- 4.5.4 Ensure that the software files are distinguished *ie* **HA2** or **CS H7** RealTime PCR. If running both H7 RealTime PCRs and / or the H5 RealTime PCR (4.5.2, above) in the same experiment, then name the file appropriately.
- 4.5.5 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.

#### **4.6 Molecular pathotyping by sequencing the CS H7 RealTime PCR amplicon**

- 4.6.1 Here the amplicon generated by the **CS H7** RealTime PCR can be directly purified from the reaction mix after completion of this RealTime PCR. There is **no** need to electrophorese this H7 amplicon through an agarose gel prior to purification. Amplicon purification and sequencing is as described by protocol "OneStep RT PCR for detection of H5 & H7 avian influenza and cleavage site sequencing", with the difference that primers H7F and H7R (3.2.2, above) are used to prime the BigDye sequencing reactions. The assembled sequence from the **CS H7** RealTime amplicon is then used to determine the H7 sample as being either low pathogenic (LP) or highly pathogenic avian influenza (HPAI).

### **5. RESULTS**

#### **5.1 Analysis and display of results by using the RealTime instrument's software**

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

#### **5.2 Interpretation of results in both H7 RealTime PCRs**

- 5.2.1 Analyse the data by comparing the results obtained for the negative (NTC) and H7 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* >38) & 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 H7 probe for

subsequent experiments & note result.

If the late Ct value (>38) has a logarithmic / sigmoidal character where clear final fluorescence values are observed, then contamination of the NTC wells with H7 RNA may be considered. It is also possible that such very late Ct signals may occasionally occur spuriously. Repeat the H7 RealTime PCR experiment.

### 5.2.3 H7 positive controls

#### 1. H7 extraction control (3.2.5, paragraph 2):

Quantified specimens 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 H7 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 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.

#### 2. H7 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 -3.0 to -3.9 which should correspond to H5 RealTime PCR efficiency in the range 80 – 110%. Deviation from these conditions may not on its own invalidate the test, provided the low positive standards have given Ct's within 2 Cts of the expected value (see sections 5.2.5 and 5.2.6 below). However, the results cannot then be interpreted quantitatively. This deviation may reflect *eg* (i) inaccurate RNA addition (ii) degradation of the RNA standards by inappropriate storage or (iii) inefficient thermocycling / fluorescence reading due to an instrument problem. This indicates a need to address these matters for subsequent H7 RealTime 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 **HA2** H7 Realtime PCR when used on Stratagene Mx3000 series RealTime instruments. The below example Ct values may **not** apply to other makes of RealTime PCR platform:

1. Examine the H7 RNA dilution series: Note the Ct value of the control RNA extracted from a Eurasian H7 sample at a titre of 10 EID<sub>50</sub>/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 both H7 RealTime PCRs. In this example, where Ct 36 is obtained from the 10 EID<sub>50</sub>/ml RNA control, a Ct < 36 in the **HA2** H7 RealTime PCR is considered clearly H7 positive, while a specimen recording “No Ct” value is clearly negative.
2. Samples with Ct >36 in the **HA2** H7 RealTime 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 two paragraphs present a possible strategy for confirmation as either “H7 [very low] positive” or “H7 negative”:
3. Check the Ct value when the same RNA extract was tested by the M gene RealTime PCR protocol: A Ct value of 34-37 by M gene RealTime PCR suggests that this may be a very weak positive **HA2** H7 RealTime result. A reproducible result obtained after re-extraction of RNA from the same clinical material by **HA2** H7 / 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 H7 PCRs due to their lower sensitivity.
4. However, if **HA2** H7 / M gene RealTime PCR retesting of re-extracted RNA yields a “No Ct” result then such specimens are considered negative.

5.2.6 Criteria for assessing results on test samples are as follows in the **CS** H7 RealTime PCR when used on Stratagene Mx3000 series RealTime instruments. The below example Ct values may *not* apply to other makes of RealTime PCR platform:

1. These criteria are similar in principle to those listed in the above four paragraphs of section 5.2.5. However, validation with clinical specimens has shown the **CS** H7 RealTime PCR may be less sensitive (up to x10 fold) in comparison to the **HA2** RealTime PCR, and this discrepancy may be even greater for Ct values of >30. Examine the H7 RNA dilution series: The 100 EID<sub>50</sub>/ml extracted RNA standard typically gives a Ct value of 32 (+/- 2) in the **CS** H7 RealTime. In this example, a Ct <32 in the **CS** H7 RealTime PCR is considered clearly H7 positive, while a specimen recording “No Ct” value is clearly negative.
2. It is possible that **CS** amplification plots (*ie* with a sigmoidal / logarithmic character) may be observed in the Ct range 32-38. Samples which give such inconclusive results should be re-tested and / or reconsidered with the results of another RealTime PCR (eg M

gene and /or HA2 H7) as outlined for the HA2 version, section 5.2.5, paragraphs 2 - 4 (above).

5.2.7 All H7 positive results from a potential new case 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 H7 PCR and sequencing before reporting is crucial in order to confirm the H7 subtype and pathotype.

5.2.8 Other considerations for the two H7 RealTime PCRs:

Both H7 RealTime PCRs described in this protocol were designed, optimised and validated to detect recent Eurasian H7 isolates. Because of clear sequence differences between American and Eurasian H7 AIVs, it was not considered likely the former H7s would be detected reliably by the two H7 RealTime PCRs described in this protocol. Results from the validation work affirmed this supposition.

Primer and probe considerations for Eurasian H7 isolates:

As Eurasian H7 viruses continue to evolve, it is possible that new isolates will emerge which will be less efficiently detected by the two described H7 RealTime PCR methods, or possibly not detected at all. Revision of H7 primer / probe sequences may be necessary and this will be reflected in any future updated versions of this protocol.

## **6. REFERENCES**

6.1.1 **Spackman, E., Senne, D.A, Myers, T.J, Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T., Suarez, D.L,** 2002. Development of a real time reverse transcriptase PCR for type A influenza virus and the avian H5 and H7 haemagglutination subtypes. *J. Clin. Microbiol.* **40**: 3256-3260.