

**One Step RT PCR for detection of H5 & H7
avian influenza & cleavage site sequencing**

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

Contents

1. INTRODUCTION	3
1.1 PURPOSE/SCOPE OF THIS PROTOCOL	3
1.2 BACKGROUND INFORMATION	3
2. SAFETY	3
2.1 LOCAL SAFETY PROCEDURES	3
3. PROCEDURE/METHOD	3
3.1 RNA EXTRACTION	3
3.2 ONESTEP RT PCR (QIAGEN KIT).....	3
3.3 H5 PCR PRIMERS	4
3.4 H7 PCR PRIMERS	5
3.5 CYCLING CONDITIONS & AMPLICON DETECTION	6
3.6 NUCLEOTIDE SEQUENCING	6
4. RESULTS	6
5. REFERENCES	7
APPENDIX 1	9
INTERPRETATION OF H5 CLEAVAGE SITE RESULTS.....	9

1. INTRODUCTION

1.1 Purpose/Scope of this protocol

1.1.1 This protocol provides details required to determine and analyse the nucleotide sequence of samples of H5 & H7 avian influenza (AI) submitted for statutory sequencing. PCR conditions outlined below include those which have been successful in generating amplicons from RNA directly extracted from clinical specimens.

1.2 Background information

1.2.1 As described in the OIE diagnostic manual, determination of the nucleotide sequence (and the deduced amino acid sequence) of specific regions of the genomes of these viruses allows an estimation of their pathogenicity to be made.

2. SAFETY

2.1 Local safety procedures

2.1.1 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. PROCEDURE/METHOD

3.1 RNA extraction

3.1.1 This should be carried out (i) manually or (ii) robotically.

3.2 OneStep RT PCR (Qiagen kit)

3.2.1 The below protocol is designed round the OneStep RT-PCR kit (Qiagen, cat # 210212):

Prepare the master mix for 50ul reaction volumes where (i) cDNA is synthesised followed by (ii) PCR in the same tube:

REAGENT	FINAL CONCENTRATION	VOL (µl) REQUIRED FOR ONE REACTION (50µl)	µl TOTAL
RNase-free water	-	28.8 µl	
PCR Buffer 5X from Qiagen OneStep RT-PCR kit	1X	10 µl	
dNTPs Mix 10mM each (from Qiagen kit)	0.4mM each	2 µl	
Forward primer: 50 pmol/µl (50µM)	1µM	1 µl	
Reverse primer: 50 pmol/µl (50µM)	1µM	1 µl	
RNase Inhibitor 40U/µl (Promega)	8U	0.2 µl	
One Step RT-PCR Enzyme Mix (Qiagen kit)		2 µl	
VOLUME MINUS TARGET		45 µl	
VOLUME EXTRACTED RNA		5µl for clinical specimens, but 2.5ul RNA plus 2.5ul RNase-free water should suffice for egg-grown AI	
FINAL REACTION VOLUME		50 µl	

3.2.2 Optional variation:

Addition of “Q-Solution” has been suggested by the manufacturer for the above kit protocol (Qiagen) to assist in the amplification of difficult templates/specimens. Here 10ul (x5) Q-Solution can be included into each 50ul reaction volume. The manufacturer’s protocol shows examples where inclusion of Q-Solution can sometimes help overcome problems in generating H5 AI amplicons, but recommends that reactions both with and without Q-Solution should be run in parallel.

3.3 H5 PCR primers

3.3.1 It is important to note the following when working with these two conventional H5 PCR approaches. Results from VLA and from collaborating EU laboratories in the AVIFLU project have revealed the following:

- H5KHA PCR:
 Produces approx 300bp amplicon. This appears to be highly sensitive and successfully amplifies H5 RNA directly extracted from clinical specimens. However, some non-H5 clinical samples can produce a band(s) of similar mobility, where amplicon sequencing is the only means of establishing whether these are derived from H5 or other microbes.
- J3 / B2a PCR:
 Produces approx 300bp amplicon. This is less sensitive than the KHAH5 PCR, but can produce H5 amplicons from higher-titre clinical specimens. H5 specificity and general robustness appears to be

better than for the KHAH5 PCR conditions. Preferable to use this PCR rather than H5 KHA (above) when amplifying RNA extracted from H5 AI which has been harvested from egg fluid.

- S1 / B2a PCR:
Produces approx 1200bp amplicon which spans the HA1 region including the cleavage site sequence. Normally used to amplify H5 RNA extracted from egg-grown H5 AI, but may also amplify from very high titre clinical specimens.

3.3.2 H5 primer sequences:

H5-kha-1: CCT CCA GAR TAT GCM TAY AAA ATT GTC

H5-kha-3: TAC CAA CCG TCT ACC ATK CCY TG

Note: the inclusion of degenerate nucleotides indicated above in bold.

J3: GAT AAA TTC TAG CAT GCC ATT CC

B2a: TTT TGT CAA TGA TTG AGT TGA CCT TAT TGG

S1: AGC AGG GGT ATA ATC TCT CA

B2a: As above

3.4 H7 PCR primers

3.4.1

- GK7.3/GK7.4:
Produces an approx 200-230 bp amplicon which spans the cleavage site. These are the most sensitive primer pair which can amplify from H7 clinical specimens.
- 397/391 and H7.5/391:
These two primer pairs produce an approx 600 and 1100bp amplicons respectively. These span the HA1 region including the cleavage site sequence. Normally used to amplify H7 RNA extracted from egg-grown H7 AI, but may also amplify from very high titre clinical specimens.

3.4.2 H7 primer sequences:

397: ACATACAGTGGGATAAGAACC

391: TCTCCTTGTGCATTTTGTATGCC

GK7.3: ATGTCCGAGATATGTTAAGCA

GK7.4: TTTGTAATCTGCAGCAGTTC

3.5 Cycling conditions & amplicon detection

3.5.1 This should be carried out as follows for the two primer pairs.

H5KHA cycling conditions:

30 min at 50°C, 94°C for 15 min; 40 cycles of 94°C for 30 sec, 58°C for 1 min and 68°C for 2min and a final extension at 68°C for 7 min.

NB: Note the above caveat (2.3.1) concerning possible non-H5 amplicons with this H5KHA PCR.

J3/B2a cycling conditions:

30 min at 50°C, 95°C for 15 min; 35 cycles of: 94°C for 45 sec, 50°C for 45 sec and 72°C for 2min, and a final extension at 72°C for 10 min.

S1/B2a; 397/391; GK7.3/GK7.4 & H7.5/391 cycling conditions:

Precisely as for J3/B2a PCR above.

3.5.2 Electrophorese in 2-2.5% agarose and detect by ethidium bromide staining.

3.6 Nucleotide sequencing

3.6.1 This should be carried out by a method of choice in your laboratory, eg ABI BigDyes, Beckman etc.:

- Respective primers used for H5 / H7 PCR as above, and / or:
- In the case of the larger amplicons (*ie* S1/B2a and H7.5/391), use additional primers within the amplicon which can provide sequence data for both the cleavage site and the HA1 region of the respective H5/H7 genes:

For H5: S1/B2a amplicon would require J3 and B2a for sequencing

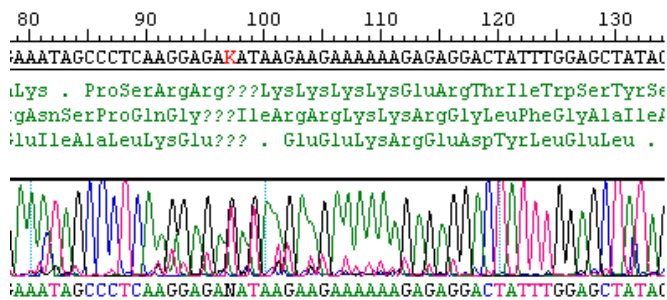
For H7: H7.5/391 amplicon would require 397 and 391, and /or GK7.3 and GK7.4 for sequencing.

4. RESULTS

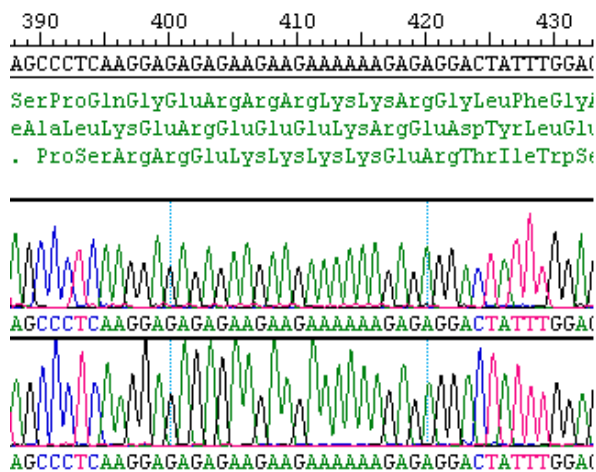
4.1.1 The nucleotide sequence data should be analysed using appropriate sequence analysis software of your choice, eg Lasergene (DNASTar). The common cleavage site nucleotide sequences (and amino acid motifs), and points for their interpretation are listed in Appendix 1.

4.1.2 Sequencing results: Fit for purpose

Where fluorescent dye sequencing chemistry is employed, the sequencing chromatograms which span the cleavage site region should be assessed critically. Two examples are shown below. The top chromatogram represents a single sequencing run (*ie* in one orientation) where in places the quality of the data is poor (*ie* superimposed peaks of more than one signal, a general background pattern of red (*ie* T) signals), as seen from the character of the plot. This results in clearly one ambiguous nucleotide call at the cleavage site, and likely errors among neighbouring nucleotides which upon translation results in poor aminoacid sequence data at the cleavage site. Such a result would be considered **“unfit for purpose”** unless there was a complementary sequencing run available (*ie* in the opposite orientation) of clearly superior quality.



The next example (below) consists of two complementary sequencing runs in opposite orientations. Compare the quality of the two below chromatograms with the above, *ie* clear and discrete peaks which agree in both orientations. There are no superimposed peaks nor is there any underlying background, and translation into aminoacid sequence is perfectly reliable in giving a polybasic highly pathogenic AI cleavage site in this example. Clearly the below data is **“fit for purpose”**.



5. REFERENCES

- 5.1.1 **Slomka, M.J, Coward, V.J., Banks, J., Löndt, B.Z., Brown, I.H, Voermans, J., Koch, G., Handberg, K.J., Jørgensen, P.H., Cherbonnel-Pansart, M., Jestin, V., Cattoli, G., Capua, I., Ejdersund, A., Thorén, P., Czifra, G., 2007a.** Identification of sensitive and specific avian influenza PCR methods through blind ring trials in the European Union. *Avian Dis.* 51, 227-234.

Appendix 1

Interpretation of H5 cleavage site results

An avian influenza virus is considered HPAI if there are multiple basic (arginine - R or lysine- K) amino acids at the HA cleavage site. Most HPAI virus HAs have the sequence motif R-X-R/K-R-G (X can be any amino acid) although there are exceptions. In practice any motif with more than two basic amino acids should be considered HPAI or to be at risk of becoming HPAI. The cleavage site always begins with a proline - P and ends with RGLF.

Table 3: Examples of H5 AI amino acid sequences across the cleavage site (not inclusive)

H5 cleavage site motifs			
Virus	Subtype	Motif	Pathogenicity ¹
Numerous LPAI Eurasian viruses	H5N*	PQRET----RGLF	-
A/turkey/Kfar Vitkin/71	H5N2	PQREA----RGLF	-
A/turkey/Ramon/73	H5N2	PQREA----RGLF	-
A/laughing gull/AK/296/75	H5N3	PSIGE----RGLF	-
A/arctic tern/AK/300/75	H5N3	PSIGE----RGLF	-
A/mallard/Alberta/271/88	H5N3	PEKQT----RGLF	-
A/chicken/Italy/RA9097/98	H5N9	PQKET----RGLF	-
A/chicken/Scotland/59	H5N1	PQRKK----RGLF	+
A/tern/South Africa/61	H5N1	PQRETRRQKRGLF	+
A/turkey/Ontario/7732/66	H5N9	PQRRKK---RGLF	+
A/chicken/PA/1370/83	H5N2	PQKKK----RGLF	+
A/duck/Ireland/113/83	H5N8	PQRKRKK--RGLF	+
A/turkey/Ireland/1378/83	H5N8	PQRKRKK--RGLF	+
A/turkey/England/91	H5N1	PQRKRKT--RGLF	+
A/chicken/Pueblo/94	H5N2	PQRKRKT--RGLF	+
A/chicken/Queretaro/20/95	H5N2	PQRKRKRKTRGLF	+
A/chicken/Hong Kong/990/97	H5N1	PQERRRRKKRGLF	+
A/Hong Kong/156/97	H5N1	PQRETRRKKRGLF	+
A/Hong Kong/486/97	H5N1	PQRRRRKK-RGLF	+
A/poultry/Italy/97	H5N2	PQRRRKK-RGLF	+
A/chicken/Italy/1487/97	H5N2	PQRRKKR-RGLF	+
A/chicken/Thailand/04 (1, 2, 3)	H5N1	PQERRRRKKRGLF	+
A/avian/Texas/04	H5N2	PQRKKRGLF	+
A/ostrich/ZA/N227/04	H5N2	PQREKRRKKRGLF	+

¹= Pathogenicity of viruses as determined by IVPI or equivalent tests. + = HPAI, - = LPAI

Table 4: Examples of H7 AI amino acid sequences across the cleavage site (not inclusive)

Virus	Subtype	Motif	Pathogenicity¹
Numerous American viruses	H7N*	PENPK----TRGLF	-
Numerous American and Eurasian	H7N*	PEIPK----GRGLF	-
A/ruddy turnstone/NJ/65/85	H7N3	PEKPK----TRGLF	-
A/duck/OH/10/88	H7N8	PESPK----TRGLF	-
A/finch/CA/28710-8/93	H7N8	PEIPK----ERGLF	-
A/pekin robin/CA/30412-5/94	H7N1	PEIPK---RRRGLF	-
A/FPV/Brescia/02	H7N1	PSKKR---KKRGLF	+
A/FPV/Dobson/27	H7N7	PELPK-KRRKRGLF	+
A/FPV/Dutch/27	H7N7	PPKKR---RKRGLF	+
A/FPV/Rostock/34	H7N1	PEPSK-KREKRGLF	+
A/FPV/Weybridge	H7N7	PELPK-KRRKRGLF	+
A/FPV/Egypt/45	H7N7	FSKKR---RKRGLF	+
A/turkey/England/63	H7N3	PETPK--RRRRGLF	+
A/chicken/Victoria/76	H7N7	PEIPK-KKEKRGLF	+
A/duck/Victoria/76	H7N7	PEIPK----KRGLF	-
A/turkey/England/199/79	H7N7	PEIPK-KREKRGLF	+
A/chicken/Leipzig/79	H7N7	PEIPK--KKGRGLF	+
A/goose/Leipzig/192/79	H7N7	PEIPKKKKKGRGLF	+
A/chicken/Victoria/1/85	H7N7	PEIPK-KREKRGLF	+
A/chicken/Pakistan/447/94	H7N3	PETPK-RKRKRGLF	+
A/chicken/Pakistan/547-99/95	H7N3	PETPK--RRNRGLF	+
A/chicken/Pakistan/1369/95	H7N3	PETPK--RRKRGLF	+
A/Teal/Taiwan/19. 2-37-2/98	H7N1	PEIPK-GRGLF	
A/poultry/Italy/1999	H7N1	PEIPKGSRVRRGLF	+
Italy/7159-V02/02	H7N3	PEIPKGRGLF	-
Pakistan/various/01	H7N3	PETPK--RRKRGLF	+
Pakistan/27/01	H7N3	PEIPKGRGLF	-
Pakistan/33/ 01	H7N3	PETPKRRKRGLF	+
A/poultry/Chile/2002	H7N3	PEKPKTCSPLSRC RETRGLF	+
A/poultry/Chile/2002	H7N3	PEKPKTRGLF	-
A/chicken/NL/2003	H7N7	PEIPKRRRRGLF	+
A/turkey/NL/2003	H7N3	PEIPKGRGLF PEIPKGSRVRRGLF	- +
A/ck/Pakistan/03	H7N3	PETPK-RRKRGLF	+