

**Detection of influenza A matrix gene by real
time Taqman® RT-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 To rapidly detect influenza RNA extracted from clinical and chick embryo amplified samples and provide an early warning for the presence of influenza virus in a sample.

1.2 Background information

1.2.1 Real time PCR is a method that has been introduced relatively recently. The technology combines DNA amplification with detection of the products in a single tube. In the case of influenza A viruses an extra reverse transcriptase step is required to convert the RNA into cDNA. This format is highly beneficial as it removes the significant contamination risk caused by opening tubes for post-PCR manipulation. It is also less time consuming than gel based analysis and can supply a quantitative result.

1.2.2 Current detection methods are based on changes in fluorescence proportional to the increase in product. Fluorescence is monitored during each PCR cycle to provide an amplification plot, allowing the user to follow the reaction.

1.2.3 The PCR is based on detection of a conserved region of the influenza A virus matrix gene using fluorogenic hydrolysis type probes and will detect all influenza A subtypes.

2. MATERIALS

2.1 Chemicals and reagents

2.1.1 Primers and probe

Sep 1 AGA TGA GTC TTC TAA CCG AGG TCG

Sep2 TGC AAA AAC ATC TTC AAG TCT CTG

SePRO FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA

2.1.2 Real time 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.

DEPC treated water	Ambion or similar	137.5µl
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(x5) Qiagen 1 step RT PCR buffer		50µl
Rox ref dye(pre diluted 1:500 in DEPC water	Stratagene or similar	3.75µl
Qiagen dNTP mix		10µl
Sep1(50µM)	Sigma or similar	2µl
Sep2(50µM)	Sigma or similar	2µl
SePRO(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

2.1.3 Real time PCR M gene standards:

These are quantified RNA standards extracted from infected allantoic fluid of known titre.

2.2 Equipment

2.2.1	Microcentrifuge tubes (1.5ml)	-18°C or lower freezer
	-70°C or lower freezer	Vortex mixer
	Pipettes tubes)	Microcentrifuge (with rotor for 2ml
	Sterile, Rnase-free pipette tips with aerosol barrier	Mx3000/4000 real time RT-PCR quantitative machine
	Mx3000/4000 real time RT-PCR plates/strips	
	Mx3000/4000 real time RT-PCR plate caps	

3. PROCEDURE/METHOD

3.1 Preparation of PCR master mix and loading

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

- 3.1.2 Make up master mix sufficient for the number of samples to be tested.
- 3.1.3 Thoroughly mix the master mix and centrifuge for 30 seconds to remove bubbles.
- 3.1.4 Aliquot 23µl of master mix per well of the real time plate/strip(s).
- 3.1.5 Loosely place the plate caps on the plate. All wells will need to be covered even though only a portion of the plate may be used.
- 3.1.6 Bring the plate/strip(s) out of the clean room and place on ice before addition of sample RNA and RNA standards.

3.2 Preparation of RNA standards

- 3.2.1 Extract RNA using appropriate method
- 3.2.2 Carefully prepare dilutions of RNA in water in the range 10^0 , 10^1 , 10^2 , 10^3 , 10^4 . Mix each dilution by vortexing and briefly centrifuge. Make sufficient of each dilution so that a number of aliquots of each dilution will be made. Aliquot in volumes that the RNA does not have more than 3 freeze/thaws. Store aliquots at -70°C .
- 3.2.3 Each standard to be run as indicated on the worksheet

3.2 Manual addition of samples and standards

- 3.3.1 Referring to plate layout, add 2µl of the sample RNA to the 23µl of master mix.

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

- 3.3.2 Add 2µl of real time PCR standards to appropriate wells based on your worksheet layout. Also include a no template control (NTC) well as a negative control, using 2µl water as your template. Re-apply caps to the rest of the plate/strip(s).

- 3.3.3 Once RNA is added, fit caps to all wells.

It is important that the caps are fitted firmly and correctly onto the wells before being used on the real time machine.

- 3.3.4 If the real time plate/strip(s) are not to be loaded onto the real time machine straight away, keep the plate/strip(s) on ice until ready to test.

3.3 Reverse transcriptase and PCR

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

dR displays the **baseline-corrected fluorescence**. As all reactions and wells will start with a slightly different fluorescence reading 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.

4.2 Interpretation of results

4.2.1 Analyse the data by comparing the results obtained for the positive standards and NTC.

4.2.2 Construct a standard curve with the positive standards. As a guideline the curve should have the following criteria:

Efficiency: >80%

Slope between -3.1 and -3.8

R2 value: >0.980

4.2.3 Check the Ct values for any robot extraction controls. These should reproducibly amplify with a Ct value +/- 2 Ct's either side of their predetermined Ct value of 30. Greater deviations from the predetermined Ct value suggest that:

- The M gene 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 M gene 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 (+/- 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 M gene 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.

- 4.2.4 If all controls are within acceptable limits analyse the data for the test samples.
- 4.2.5 An increase in fluorescence will be observed at an early stage for positive samples. The NTC and negative samples should not result in an increase in fluorescence above the baseline.
- 4.2.6 Criteria for assessing whether a sample is positive or negative are as follows:
- Ct value <30 - samples are M gene positive
- Ct value 30-35 with sigmoidal/logarithmic appearance – samples are weak positive.
- Ct value >35 samples are negative if the amplification plot has a linear shape. These plots may represent spurious probe degradation or non-specific fluorescence.
- 4.2.7 All positive results are repeated and confirmed by use of virus isolation or conventional gel based PCR and sequencing before reporting in order to establish the subtype of influenza A virus detected.

5. REFERENCES

- 5.1.1 **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 assay for type A influenza virus and the avian H5 and H7 haemagglutination subtypes. *J. Clin. Microbiol.* 40, 3256-3260.
- 5.1.2 **Chang-Won Lee, Suarez, D.L., 2004.** Application of real time RT-PCR for the quantification and competitive replication study of H5 and H7 subtype avian influenza virus. *J. Viro. Methods.* 119, 151-158.

Appendix 1

Testing new batches of reagents

New batches of probe and primers should be tested when they are first re-suspended. Probe and primers should be aliquoted once re-suspended to avoid repeated freeze thawing.

Test new reagents in the same real time PCR as the current reagents using the positive standards and NTC according to this protocol. If the new reagents are fit for purpose both sets of reagents should give equivalent results.