

**Swine Influenza - Detection of influenza A
matrix gene by "perfect match" real time
Taqman® RT-PCR**

This protocol is a copy of the standard operating procedure used by the mammalian influenza team at the CRL, Veterinary Laboratories Agency, Weybridge. 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 swine clinical and chick embryo amplified samples, and provide an early warning for the presence of influenza A virus in a sample. Clinical samples may consist of swabs or tissues.

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 transcription 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 rev-mod primer:

TGC AAA **GAC ACT** TTC **CAG** TCT CTG

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

(Black Hole Quencher 1 (BHQ1) may be used instead of TAMRA in the probe)

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 |
| (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 rev-mod(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 a European swine influenza virus (SIV) allantoic fluid. The SIV preparation had been titrated in eggs to provide its known titre in terms of 50% egg infectious doses (EID50) per ml. An example of an appropriate European SIV isolate is A/swine/England/742104/95 (H3N2).

2.2 Equipment

| | | |
|-------|--|--|
| 2.2.1 | Microcentrifuge tubes (1.5ml) | -18°C or lower freezer |
| | -70°C or lower freezer | Vortex mixer |
| | Pipettes | Microcentrifuge (with rotor for 2ml tubes) |
| | 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.
- 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 RNA is prepared in batches at a concentration of 10^5 and stored at -70°C . Take out an aliquot and thaw. 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 enough is made to use for all of the M gene RRT-PCR runs for that day. Store the aliquots on ice. Discard at the end of the working day.
- 3.2.3 Each standard to be run as indicated on the worksheet

3.3 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.

3.4 Reverse transcription and PCR

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

- 3.4.2 Incubate the reactions with the following thermocycling profile:

RT step: 50°C for 30 mins,

95°C for 15 mins

PCR step (x 40 cycles): 95°C for 10 secs

60°C for 20 secs

Collect fluorescence data during the 60°C annealing step using the ROX and FAM filters and the endpoint read option with 2 reads if using the Mx4000. The Mx3000 will only allow 1 reading per 20 seconds.

- 3.4.3 Note that the lamp on both types of real time machines requires 20 minutes to warm up. Open a new file on the machine and use the real time quantitative PCR(multiple standards) option for the experiment type. The thermocycle profile and plate setup can be re-entered for each experiment or imported from previous experiments if desired.

- 3.4.4 When the plate setup and thermoprofile windows have been entered, select run to start thermocycling. A storage window will automatically open. Save the file in an appropriate folder on the hard drive of the computer.

- 3.4.5 The complete run takes approximately 1 hour and 50 minutes. If the machine is not being used by anyone else after you, select the option to turn the lamp off at the end of the run as the lamps have a limited lifespan.

4. RESULTS

4.1 Analysis and display of results

- 4.1.1 The fluorescence data can be viewed during, and after, the PCR reaction using the raw data plots tab in the run section.

- 4.1.2 When the run is complete make a record of the file name and its storage

location and save a backup of the file in a separate location.

4.1.3 To analyse the data select the Analysis section button and select the wells to be examined in the Analysis/Setup window.

4.1.4 To view the results click on the Results tab in the Analysis section and view the amplification plots. There are four options for analysing the fluorescence:

R (Multicomponent view) displays the **raw fluorescence** in arbitrary units.

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 - 120%

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 +/- 3 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 and 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 (+/- 3 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. Record the standard efficiency and positive control values in the relevant log book.

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.

Samples registering “No Ct” are clearly negative.

Ct value >35 samples are likely negative if the amplification plot has a linear shape. These plots may represent spurious probe degradation.

When testing a herd of pigs or any other single epidemiological unit containing swine, any number of specimens giving Ct values <35 indicate influenza A infection.

4.2.7 In the case of herd testing where there are no samples yielding Ct<35, observation of any samples yielding a Ct >35 necessitates re-extraction from the original sample and re-testing on the M gene RRT-PCR together with the original RNA sample. Any subsequent positives may be further tested by other approaches and by virus isolation.

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 **Lee, C.-W., 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.