

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

This protocol is a copy of the standard operating procedure used by the OIE/FAO international reference laboratory for ND at the Animal and Plant Health Agency. If you have any technical queries please contact aiwrl@apha.gov.uk

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

Contents

1. INTRODUCTION	3
1.1 PURPOSE/SCOPE OF THIS PROTOCOL	3
1.2 BACKGROUND INFORMATION	3
2. SAFETY	3
3. MATERIALS	4
3.1 DOCUMENTATION AND SOFTWARE	4
3.2 CHEMICALS AND REAGENTS	4
3.3 EQUIPMENT	6
4. PROCEDURE/METHOD	6
5. RESULTS	10
6. REFERENCES.....	11
7. APPENDIX.....	12

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

1. INTRODUCTION

1.1 Purpose/Scope of this Protocol

1.1.1 To rapidly detect Newcastle disease virus (NDV) RNA extracted from chick embryo amplified samples and clinical material including avian tissues, swabs and fluids.

1.2 Background information

1.2.1 Real-time PCR is a technology that combines DNA amplification with the fluorescent detection of the products in a single tube. In the case of RNA viruses an extra reverse transcriptase step is required to convert the RNA into cDNA. This test combines the reverse transcription, PCR and detection in a single tube. 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 The current detection method is based upon fluorescent probe technology in which fluorescence increases proportional to an increase in product. Fluorescence is monitored during each PCR cycle to provide an amplification plot that can be analysed to confirm the presence or absence of NDV RNA. Real-time PCR machines analyse raw fluorescence data and produce a cycle threshold (Ct) value for each sample. The cycle at which the fluorescence increases above the critical threshold is reported as the Ct value. The critical threshold is an arbitrary fluorescence value that can be manipulated by the operator but must be set in the exponential phase of the reaction. The accuracy of the Ct is dependent upon the fluorescent reading of the raw data and the fluorescent value for the critical threshold.



1.2.3 AAvV-1 can be avirulent or virulent based upon the nucleotide sequence across the cleavage site of the fusion gene. The virulent forms of AAvV-1 are notifiable NDV. The PCR is based upon a 186bp region spanning the cleavage site of the NDV fusion gene, in which two probes are included to discriminate between virulent and avirulent AAvV-1. One probe detects all virulent cleavage sites while the other probe detects avirulent cleavage site sequences. Optionally an additional third probe can be added when the sample potentially contains a lineage 6 (Class I) virus.

2. SAFETY

2.1 It is the responsibility of the individual laboratory to ensure all work described in this protocol is conducted to a high safety standard. This includes an awareness of risks relating to e.g. dangerous or toxic

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

chemicals, potentially hazardous procedures etc. Here local safety rules in your laboratory should be understood by all relevant members of staff.

2.2  Areas within this procedure which refer to Safety Critical activities are denoted in the paragraph number column with the  sign to highlight these areas to users.

3. MATERIALS

3.1 Documentation and software

3.1.1 All details of rRT-PCR's carried out should be recorded.

3.1.2 For each NDV rRT-PCR test conducted all relevant log books and tracking paperwork must be filled in by the operator.

3.1.3 All scientists who are trained to perform this NDV rRT PCR on a real-time PCR platform must be sufficiently familiar with the accompanying relevant software in order to run an experiment.

3.1.4 Procedures Document References

- Virus isolation – Procedure for the extraction of samples from avian carcasses for virus isolation
- Procedure for isolation and detection of viruses from avian species using embryonated eggs.
- RNA extraction from biological samples.
- Extraction of nucleic acids from swabs using the Qiagen BioRobot Universal

3.1.5 Worksheet for real-time plate layout (See appendix 1).

3.2 Chemicals and reagents

3.2.1 Primers and probes – Sigma or similar

F4804	GCATACAACAGAACAYTGAC
RT7	GGAGGATGTTGGCAGC
VRP1[FAM]	CCTATAAA <u>A</u> ICGIT <u>T</u> CTGICTCCTTCC[BHQ1]

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

ARP3[HEX] CCTATAA**G**ICGIC**C**CTGTITCCC[BHQ1]

Optional Lin 6 primer

F4804 Lin6 GCATACAATAGGACBCTTACAAC

Optional Lin 6 probe

ARP6 [HEX] CCTACCAAACGCTCCTGCCGTTTC[BHQ1]

NOTE: I = inosine

Bold and underlined base = Locked nucleic acid (LNA)

3.2.2 Reagents –

- Molecular grade water – any supplier
- Qiagen 1 step RT-PCR kit – or comparable
- Rox reference dye (diluted to 1 in 1500) - Stratagene or similar
- 25mM magnesium chloride – Promega or similar
- RNAsin (40U/μl) (pre dilute 1 in 10) – Promega or similar

3.2.3 NDV RRT-PCR Controls

1. No template control

Include at least one no template control where only mastermix is added. Label this as “NTC” in the instrument software. This is to control for contamination of mastermix.

2. Negative extraction control

Include at least one negative extraction control. This should be labelled as “NAC” (nonamplification control) in the instrument software. This controls for cross contamination during handling of the samples.

3. Two positive extraction controls

Include two positive extraction controls from Ulster (avirulent) and AG68 (virulent) AAvV-1 pre-diluted to a determined Ct value of between 28-32. These should be labelled as “Fam positive control” in the instrument software. This controls for the efficiency of the RNA extraction.

4. Two positive RNA controls

Include previously extracted positive RNA samples of Ulster and AG68 pre-diluted and aliquoted to give a Ct of between 28-32. These

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

should be labelled as “Standards” in the instrument software. This controls the performance of the mastermix.

The positive control is prepared and crossover testing of batches is carried out to ensure fit for purpose continuity of the assay from batch to batch.

3.3 Equipment

3.3.1	Microcentrifuge tubes (1.5ml and 0.5ml)	Realtime PCR instrument (eg. MX3000P)
	-70°C or lower freezer	-18°C or lower freezer
	Sterile Rnase free tips with aerosol barrier	Microcentrifuge
	Pipettes	Vortex mixer
	Polystyrene bijou or Universal	Ultracentrifuge
	Realtime instrument plasticware	

4. PROCEDURE/METHOD

4.1 Test Reliability

4.1.1 Process of assuring ongoing test reliability:

Test reliability is assessed each time the test is run through the analysis of 6 controls. For a test to be valid the NTC and the negative extraction control must not produce a Ct value of <37. The two positive extraction controls and the two RNA controls must be within two Ct of the predetermined Ct value. All samples that have a test failure by these conditions will be repeat tested.

Real-time PCR machines are serviced annually. This service includes a temperature profile to ensure that the machine is performing at the temperatures specified.

All operators must complete training to perform the test overseen by a competent individual.

On-going competence is addressed by biennial participation by all competent staff in testing a blind ring trial, with annual assessment carried out through completion of successful tests.

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

The performance of the assay is also assessed through participation in EQA ring trials. These can be from various sources but as a minimum will be provided annually by the EU community reference laboratory for ND.

4.1.2 Frequency

- Controls are included with every test.
- All operators are required to successfully complete a test annually and a blind ring trial biennially.
- Primers, probes and controls undergo batch-to-batch fit for purpose testing.
- The real-time instruments are serviced annually.
- External ring trial annually.

4.2 Preparation of controls – The controls are identical to SOP VI.570

- 4.2.1 Stocks of Ulster and AG68 are grown according to protocol entitled “Procedure for the Isolation and Detection of Viruses from Avian Species using Embryonated Eggs”
- 4.2.2 RNA batches of Ulster and AG68 are extracted using protocol entitled “RNA extraction from biological samples”
- 4.2.3 Prepare a 10-fold dilution series of both viruses RNA and test using the rRT-PCR.
- 4.2.4 Dilute the stock of virus to the dilution that produced a Ct of between 28-32 using elution buffer AE (Qiagen RNA elution buffer) containing 5µl/ml of RNA.
- 4.2.5 Prepare 200µl aliquots of Ulster and AG68, label as U or AG. Number sequentially and store at –70°C. These are the avirulent and virulent extraction controls.
- 4.2.6 Prepare 10µl aliquots of Ulster and AG68, label as U or AG. Number sequentially and store at –70°C. These are the avirulent and virulent positive RNA controls.
- 4.2.7 Test one aliquot of each of the controls to ensure it is fit for purpose and record the batch number and Ct value obtained.
- 4.2.8 **This Ct value is the expected result that will be used to determine if all subsequent tests are valid.**

4.3 Preparation of PCR mastermix

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

4.3.2

Reagent	Volume
Molecular grade water (i.e. RNase-free)	9µl

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

(x5) Qiagen 1 step RT PCR buffer	5µl
Rox ref dye (pre-diluted 1in 1500 from Stratagene stock in molecular grade water)	1µl
dNTP mix	1µl
RT6 (10µM in molecular grade water)	1µl
RT7 (10µM in molecular grade water)	1µl
VRP1 (5µM in TE buffer)	1µl
ARP3 (5µM in TE buffer)	1µl
25mM magnesium chloride (MgCl ₂)	1µl
RNAasin (40U/µl) (pre-diluted 1in 10 from Promega stock or similar)	1µl
Qiagen 1 step RT PCR enzyme mix	1µl

Note: If a lineage 6 (Class I) AAvV-1 is suspected an additional probe (ARP6) should be added to enable detection of this divergent lineage. ARP6 is included at the same titre as ARP3 and the volume of water adjusted appropriately.

- 4.3.3 Add reagents as above. Make up master mix sufficient for the number of samples to be tested. Smaller volumes can be made in a 1.5ml eppendorf, for larger volumes use either a bijou or a universal.
- 4.3.4 Thoroughly mix the master mix. For eppendorfs pulse spin the tube to remove any bubbles. For bijous/universals no spin is needed as they are nonstick.
- 4.3.5 Aliquot 23µl of master mix per well of the real-time plate/strip(s). Be sure to use an appropriate plasticware for the real-time machine you will be using.
- 4.3.6 Loosely place the plate caps on the plate or cover with plate sealer.
- 4.3.7 Bring the plate/strip(s) out of the clean room for the addition of sample RNA and controls.

4.4 Addition of samples and controls

- 4.4.1 Referring to plate layout (see Appendix 1), add 2µl of the sample RNA to the 23µl of mastermix.
- 4.4.2 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.
- 4.4.3 Once RNA is added, fit caps to all wells except for the controls.
It is important that the caps are fitted firmly and correctly onto the wells before being used on the real-time machine.
- 4.4.4 Referring to plate layout, add 2µl of the controls (negative extraction control first) to the 23µl of mastermix.

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

- 4.4.5 Optional: If air bubbles are lodged into the tubes, pulse spin at max speed to dislodge.
- 4.4.6 Real-time plate/strip(s) can be kept at room temperature until ready to test. If they are to be stored for an extended before testing they should be kept on ice or at +4°C.

4.5 Running the reactions

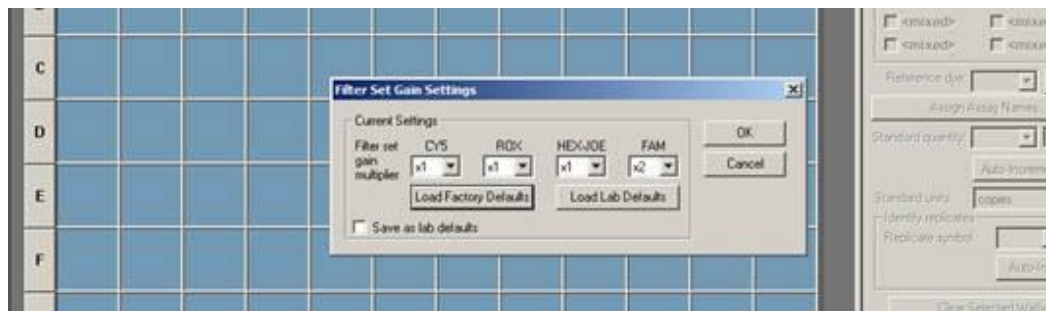
- 4.5.1 Open the real-time PCR machine and load the plate/strips according to the instrument specifications.
- 4.5.2 Access the real-time run software and label the wells according to the plate plan.
- 4.5.3 Select FAM and HEX as reporter dyes with ROX as the reference dye in all wells.
- 4.5.4 Label the controls as follows; Mastermix only=NTC, negative extraction=NAC, positive extraction controls=FAM positive control, positive RNA control=Standard
- 4.5.5 The cycling conditions for this test are
- 50°C for 30 minutes
 - 95°C for 15min
 - followed by 40 cycles of
 - 95°C for 10 seconds
 - 50°C for 30 seconds
 - 72°C for 30 seconds

Fluorescence is read at the end of the 50°C for 30 seconds annealing step.

- 4.5.6 In the MXpro software it is possible to change the 'Filter Set Gain Settings' – click Instrument, then filter Set Gain Settings.

For our use Cy5 Rox and Hex should all be set to x1, Fam should be set to x2.

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR



After servicing or repair this setting is often changed back to the factory default of Fam x8 and should be corrected.

4.5.7 Save the file in an appropriate folder on the hard drive of the computer.

5. RESULTS

5.1 For each reporter dye in every well the raw fluorescence data will be plotted by the software. The raw data will be automatically analysed by the accompanying relevant software to create a critical threshold and use this to report any Ct values. Users must be aware of how to access Ct results from appropriate real-time PCR software. If the raw fluorescence is above 35000 the accuracy of the readings is reduced. In this case check the filter gain settings to ensure they are set to the correct values as defined above.

The steps to interpreting results on MXPro software are:

- Examine the critical threshold using either dR (baseline-corrected raw fluorescence) when no background reference dye (eg. ROX) has been used and dRn (baseline-corrected normalized fluorescence) when a background reference dye has been used. The critical threshold will be set automatically by the software but you must check that the software algorithm has not set the bar too low or too high. The critical threshold must be set in the exponential phase of the reaction above the background fluorescence at a point where the amplification of the controls is vertical on a logarithmic scale (i.e. the threshold must be set roughly halfway between the chaotic signals at the bottom and where the plot starts to curve).

Finally check the reported Ct's for the controls as below.

5.2 For a valid test the six NDV RRT-PCR controls must meet the following

Molecular pathotyping of Avian Avulavirus type 1 by Real Time PCR

conditions:

1. No template control – “No Ct” or ct must be ≥ 37 .
2. Negative extraction control – “No Ct” or Ct must be ≥ 37 .
3. Two positive extraction controls - Ct value of the extraction control must be within two Ct of the predetermined batch ct. For example if the predetermined Ct value of a batch of Ulster was 30 and the predetermined Ct of the AG68 batch was 29 then on a valid test the Ulster Ct would be between 28-32 and the AG68 would be between 27-31.
4. Two positive RNA controls - Ct value of the RNA control must be within two Ct of the predetermined batch Ct in the same manner as the extraction control.

5.3 A positive sample produces a Ct value of < 37 . Negative results produce “No Ct”. A Ct value of ≥ 37 is an inconclusive result and requires repeat testing for confirmation. A Ct value of ≥ 37 on repeat testing is reported as negative. Virulent samples produce a positive Ct in the FAM channel whereas avirulent samples produce a positive Ct in the HEX channel. The results should be in agreement with those obtained with protocol entitled “AAvV-1 screening real time PCR”.

5.4 OPTIONAL ADDITIONAL WORK: Positive samples can be sequenced for confirmation of the cleavage site. To complete the sequencing purify the PCR product, sequence and identify the cleavage site according to protocol entitled “”, using F4804 and RT7 as the sequencing primers (at 1 μ m concentration). This will yield a product from which the cleavage site motif may be deduced.

6. REFERENCES

- 6.1 Fuller CM, Collins MS, Alexander DJ. 2009. Development of a real-time reverse-transcription PCR for the detection and simultaneous pathotyping of Newcastle disease virus isolates using a novel probe. Arch Virol. 2009; 154(6):929-37.

7. Appendix

Plate reference:

Batch no.
Reagent Register:
Avirulent extraction:
Avirulent RNA standard:
Virulent extraction:
Virulent RNA standard:

Samples processed:
clinical material
Date:

AVL plate:
Date:

Robotic extraction:
Date:
Manual extraction:
Date:

PCR:
Test:
Date:

	1	2	3	4	5	6	7	8	9	10	11	12	Exp Ct	Res Ct
A												Negative extraction control	No Ct	
B												No template control	No Ct	
C														
D												Avirulent extraction control		
E												Avirulent RNA Standard		
F														
G												Virulent extraction control		
H												Virulent RNA Standard		

Valid Test

NO YES