

ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE	VALIDATION DOSSIER REAL TIME RT-PCR (rRT- PCR) DETECTION OF AVIAN INFLUENZA SUBTYPE H5, H7, H9	Research & Development Department PAG. 1 DI 31
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Prepared by	.		NOTES	DATE	N. REV.
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VALIDATION DOSSIER

REAL TIME RT-PCR (rRT- PCR)
DETECTION OF AVIAN INFLUENZA
SUBTYPE
H5, H7, H9

Part I: Description of the validation process

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SUMMARY

Among the different haemagglutinin (HA) subtypes of avian influenza viruses, H5, H7 and H9 are of major interest because of the serious consequences for the poultry industry and the increasing frequency of direct transmission of these viruses to humans. The availability of new tools to detect rapidly and subtype influenza viruses can enable the immediate application of measures to prevent widespread transmission of the infection. In this document, the development and validation of a novel one step real time PCR (RRT-PCR) to detect simultaneously H5, H7 and H9 subtypes of AI from clinical samples of avian origin is presented. The sensitivity of the RRT-PCR assay was determined using *in vitro* transcribed RNA and ten-fold serial dilutions of titrated avian influenza viruses. High sensitivity levels were obtained, with limits of detection ranging from 10^1 to 10^3 RNA copies and from $10^{2.74}$ EID₅₀/100µl to 10^1 EID₅₀/100µl with titrated viruses. Excellent results were achieved in the intra- and inter-assay variability tests. The comparison of results obtained from the analysis of 725 avian samples compared to the reference method (virus isolation) showed a high level of agreement.

To date, this is the first real time PCR protocol available for the simultaneous detection of AI viruses belonging to H5, H7 and H9 subtypes reported. The results obtained indicate that this method is suitable as a routine laboratory test for rapid detection and differentiation of the three most important avian influenza virus subtypes in samples of avian origin. Based on the results of the comparative analysis performed in this study and the EU proficiency test, the present protocol provides similar sensitivity and specificity to the protocols developed at the Community Reference Laboratory. Thus, the rapidity and practicability of this novel assay do not impact negatively the quality of its performance.

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Scientific reference: the development and validation study has been published in *Journal of Clinical Microbiology*. Monne I, Ormelli S, Salviato A, De Battisti C, Bettini F, Salomoni A, Drago A, Zecchin B, Capua I, Cattoli G. 2008. Development and validation of a one step real time per assay for the simultaneous detection of H5, H7 and H9 subtype avian influenza viruses. *J Clin Microbiol.* 2008 May;46(5):1769-73.

Reference documents for PCR test validation

The validation procedure applied for this test has been developed following the guidelines provided by the following documents:

OIE, 2008 OIE Quality standard and guidelines for veterinary laboratories: Infectious diseases. 2nd edition. OIE Paris, France <http://www.oie.int>

EPA, United States Environmental Protection Agency. 2004. Quality assurance/quality control Guidance for laboratories performing PCR analyses on environmental samples.

Crowther J. R., Unger H. and Viljoen G. J. 2006. Aspects of kit validation for tests used for the diagnosis and surveillance of livestock diseases: producer and end-user responsibilities. *Rev. sci. tech. Off. Int. Epiz.* **25** (3), 913-35.

IZSve Quality Assurance. Procedura gestionale 13/9. “Validazione delle procedure di prova in PCR qualitativa”. Vers. 00 October 30th, 2006.

Laboratory performing the validation and contact persons

The whole validation procedure was carried out at the Istituto Zooprofilattico Sperimentale delle Venezie – Research & Development Department (SC-6), Laboratory of Molecular Biology. Viale dell’Università 10 – 35020 Legnaro (PD) Italy.

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Precautions

Special precautions are applied within the PCR laboratory in order to avoid false negative and false positive results. The validation procedure was carried out following the guidelines and rules provided by internal Quality Assurance documentation on Good Laboratory Practices (Istruzione Operativa 27 “Istruzioni per l’esecuzione di un test diagnostico mediante PCR – Buone pratiche di laboratorio” – vers. 00, 25th October 2006) and according to the QSOP 38 “Good laboratory practice when performing molecular amplification assays”, available at www.evaluations-standards.org.uk

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MATERIALS AND METHODS

Viruses and bacterial strains. Selected avian viruses and bacteria were used to test specificity and sensitivity of the RRT-PCR assay (table 1).

To produce viral working stocks for the standardization of the assay, all avian viruses were propagated in the allantoic cavities of 9- to 11-day-old embryonated fowls eggs whereas Avian Pneumovirus Type A and B isolates were grown in and harvested from tissue cultures. Bacterial strains were cultured and propagated using standard methods (24).

The Median Egg Infectious Dose (EID₅₀) of each of the avian influenza viruses used in the sensitivity tests was calculated according to the Reed and Muench formula (18).

RNA extraction. Viral RNA was extracted from clinical samples, supernatant of cell culture and allantoic fluid using the Qiagen Rneasy ® Mini Kit according to manufacturer's directions (Qiagen, Hilden, Germany). Two hundred µl of allantoic fluid or of PBS suspension of cloacal, tracheal swabs, faeces and organs were used in the extraction. RNA was eluted in a final volume of 50 µl and stored at -80 °C.

Primer and probe sets design. H5, H7 and H9 specific primer and probe sets for conserved regions in the HA2 subunit of the H5, H7 and H9 HA gene sequences were designed (table 2). Because of the significant sequence variability of the H5, H7 and H9 genes belonging to viruses isolated in different parts of the world, Eurasian and African H5, H7 and H9 influenza viruses were chosen as the main targets for primer and probe design. Multiple alignments of historical and recent H5, H7 and H9 subtypes were performed to minimize primer and probe mismatches. The alignment was performed using respectively 166, 81 and 131 HA nucleotide sequences for H5, H7 and H9 subtypes. Primers and probes were designed and optimised to have compatible melting temperature (T_m) enabling them to be

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used with identical thermal profile. The hydrolysis probes for H9 and H5 genes contained FAM as fluorescent reporter dye at the 5' end and TAMRA as quencher dye at the 3' end. The H7 hydrolysis probe was labelled with VIC at the 5' end and the 3' end label was TAMRA.

RRT-PCR. The reagents contained in QuantiTect Multiplex[®] RT-PCR kit (Qiagen, Hilden, Germany) were used for RRT-PCR reactions. All but one of the primers targeting the HA gene were applied to PCR at the optimized concentration of 300 nM each. The exception was the H7 specific reverse primer which was used at a concentration of 900 nM. Specific fluorescent labelled probes were used at a final concentration of 150 nM. The RRT-PCR reaction took place in a final volume of 25 µl using the RotorGene 6000 (Corbett, Australia) apparatus. Each PCR tube contained a single primer/probe set (i.e. H5 or H7 or H9). The identical thermal profile was adopted in order to detect the distinct subtypes simultaneously and within the same run. The following protocol was used for all primer/probe sets: 20 minutes at 50 °C and 15 minutes at 95 °C followed by 40 cycles at 94 °C for 45 sec and 54 °C for 45 sec.

Analytical specificity and sensitivity. The specificity of the primer/probe sets was tested on nucleic acids extracted from a diverse array of microorganisms that may be naturally present in samples of avian origin (table 1). Each strain used was tested in triplicate.

In the present study, the term “Sensitivity of the method” reflects the efficacy of the entire method applied to recover the target organism in the field specimens, including the RNA extraction procedure and the RRT-PCR protocol (10). For this reason, allantoic fluid containing ten-fold serial dilutions of titrated avian influenza viruses belonging to the H5, H7 and H9 subtypes were prepared, the RNA was extracted and then used for the sensitivity test (table 3). To establish whether the different types of sample matrixes could influence the analytical sensitivity, lungs obtained from SPF chickens were

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weighed (0.1gr) and homogenised with sterile quartz sand in 1 ml (1:10 w/v) of phosphate buffered saline (PBS, pH 7.4). Lung homogenates were then blended with ten-fold dilutions of titrated H5, H7 and H9 subtype viruses and processed for RNA extraction. Similarly, faeces obtained from SPF chickens were used for sensitivity tests. One gram samples of faeces were weighed and homogenised with sterile quartz sand to obtain a 1:5 w/v suspension in PBS. Blending and dilution were performed as described for lung samples.

Evaluation of the analytical sensitivity of the method was done by testing each dilution in five replicates. The sensitivity of the method was determined as the last dilution at which at least 4/5 replicates of each dilution was positive.

Limit of detection. In the present study, the PCR detection limit reflects the sensitivity of the RRT-PCR procedure, which includes the sensitivity of the primers and probes as well as the preparation of the master mix and the optimisation of thermocycling conditions (10). To determine the limit of detection (LoD) of the assay in terms of RNA copy numbers, in vitro transcribed RNA of the H5, H7 and H9 genes, were analysed. Briefly, the HA genes of A/chicken/Yamaguchi/7/04 (H5N1), A/turkey/Italy/4580/99 (H7N1) and A/turkey/Wisconsin/66 (H9N2) strains were amplified by RT-PCR and the amplification products were cloned into the PCR-II vector using the dual promoter TOPO TA cloning® kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids with the HA insert were isolated from positive E. coli colonies using the GenElute™ Plasmid Miniprep kit (Sigma-Aldrich, St. Luis, USA). The H5, H7 and H9 insert control plasmids were linearised using the restriction enzyme Hind III (MBI Fermentas, Lithuania) for H9 and H7 genes and Not I (New England Biolabs, MA, USA) for the H5 gene. The in vitro-transcribed RNA was generated from the T7 promoter using the RiboMax kit (Promega, Madison, WI) according to the manufacturer's recommendation and then quantified by UV BioPhotometer (Eppendorf, Hamburg, Germany).

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The number of the RNA copies was calculated following the formula reported in a previous study (12). Ten-fold dilutions of the RNA transcripts, ranging from 1 to 10^{10} copies/ μ l, were prepared. The LoD of the assay were determined by 3 independent replicates.

Intra- and inter- assay variability. The repeatability of the H5, H7 and H9 RRT-PCR assay was determined using three different concentrations (high, intermediate and low) of each viral subtype tested. Selected concentration were: $10^{5.83}$, $10^{4.83}$ and $10^{3.83}$ (H5); $10^{6.37}$, $10^{5.37}$ and $10^{3.37}$ (H7); 10^6 , 10^4 and 10^2 (H9) EID₅₀ /100 μ l. For intra-assay variability, each dilution was analysed in triplicate. For inter-assay variability, each dilution was analysed in six different runs performed by two distinct operators on different days. The coefficient of variation (CV) was determined in accordance with previously published guidelines (10).

Detection of virus RNA in samples collected from birds exposed in the field and infected experimentally. To evaluate whether or not the RRT-PCR assay could be used as a diagnostic tool in surveillance programmes for avian influenza, we analysed retrospectively by VI and RRT-PCR 725 samples collected during field and laboratory investigations from different avian species of poultry (n=234) and wild birds (n=491) in Eurasia and Africa in 2006-2007. Samples consisted of tracheal swabs (n=114), cloacal swabs (n=504) and organs (n= 107; consisting of trachea, lungs, intestines and brain) collected during necropsies. The number of samples analysed for clinical validation is in accordance with the guidelines proposed in a previous study (5).

Participation in the EU proficiency test. The capability of the assay to correctly identify AI viruses was also tested through the participation in two international EU proficiency tests organized by the Community Reference Laboratory (CRL) for AI (VLA, Weybridge, UK) in 2007 and 2008. In these proficiency tests, a panel of 10 blinded samples were submitted to the laboratory and tested with the assay evaluated in the present document.

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Comparison of the protocols: IZSVe H5 specific real time vs VLA H5 specific real time protocol.

A comparative analytical sensitivity test was performed by testing serial dilutions of *in vitro* transcribed RNA of the H5 gene by the application of the newly developed protocol and the protocol developed at the CRL for AI (20). The test was conducted using triplicates for each dilution, in the same day and by the same operator.

RESULTS

Specificity, analytical sensitivity and intra/inter- assay variability. The H5, H7 and H9 primer and probe sets were able to detect RNA of virus strains of their respective subtype only (table 1). The sensitivity of the RRT-PCR assay was determined using *in vitro* transcribed RNA and titrated reference viruses.

In terms of HA gene copy number, the LoD for the H5, H7 and H9 were 10^3 , 10^1 and 10^3 gene copies/ μ l of *in vitro*-transcribed RNA respectively. To determine linearity of the reaction and PCR efficiency, the Ct values of individual dilutions were plotted against the initial gene copy number leading to typical standard curves. Figure 1 shows quantification data and standard curve for H7 subtype as an example. The linear range of RRT-PCR assays span within 10^{10} and 10^2 copies/ μ l for H7 gene and within 10^{10} and 10^4 copies/ μ l for H5 and H9 gene. The reaction efficiencies for H5, H7 and H9 genes were 0.97, 0.98, 0.97 respectively. The correlation coefficient (R^2) was higher than 0.99 in all measurements.

The sensitivity of the method relative to infectious virus titre detectable ranged between $10^{2.74}$ EID₅₀/100 μ l and 10^1 EID₅₀/100 μ l. The results obtained for the sensitivity of the method in different samples are summarized in Table 3.

To assess the intra- and inter-assay reproducibility, three different concentrations (high, medium, and low) of each reference virus were tested in triplicate in six different runs performed by two distinct

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operators on different days. The coefficient of variation within runs (intra-assay variability) ranged from 0.12% to 2.64%. The inter- assay variation ranged from 2.26%- 4.11% (table 4).

Detection of viral RNA in samples collected from field exposed and experimentally infected birds.

A total of 725 samples was analysed by VI and RRT-PCR for H5, H7 and H9 subtypes (table 5). Of these, 141 samples tested positive for H5 by mean of RRT-PCR (4/114 tracheal swabs; 54/504 cloacal swabs; 83/107 organs). For H7, the RRT-PCR assay identified 58 positive samples (44/114 tracheal swabs; 14/504 cloacal swabs) and 30 specimens resulted positive to H9 subtype (21/114 tracheal swabs; 9/504 cloacal swabs). The comparison of the results of the two methods, summarized in table 5, showed an agreement of 94.06%, 99.17%, 98.89% for H5, H7 and H9 subtypes respectively. Cohen's kappa coefficient was 0.80, 0.94 and 0.85, respectively. The difference between the two methods was not statistically significant ($p < 0,01$). The percentage of agreement between RRT-PCR and VI was influenced by a higher number of samples tested positive by RRT-PCR but negative by VI. However, 43/55 RRT-PCR+/VI – samples were sequenced and their identity confirmed (data not shown). Only two of 26 samples that were positive by VI for H9 subtype, tested negative in the molecular assay.

Participation in the EU proficiency test. Results related to the 2007 and to the 2008 proficiency tests indicated that all the samples were correctly identified both in 2007 and in 2008.

Comparison of the protocols: IZSve H5 specific real time vs VLA H5 specific real time protocol.

In terms of HA gene copy number, the LoD for the H5 were 10^3 gene copies/ μ l of *in vitro*-transcribed RNA for both the tested protocols. For each dilution, the average Ct was similar with less than 1 Ct difference generally observed among the 2 protocols.

Applicability of the protocol to alternative real time PCR platforms (data not shown). The newly developed protocol has been applied on Applied Biosystems 7300 platform with similar results. No further modification were necessary. Preliminary tests were conducted on the Roche Light Cycler 2.0.

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In this case the results suggested the suitability of this platform for the application of the present protocol, but significant modification of the reagents were necessary. In particular, the use of reagents manufactured by Roche and optimized for this platform are in this case recommended.

DISCUSSION

Recently, molecular diagnostic tests have proven themselves to be invaluable as a first step in the identification and control of disease outbreaks. Conventional RT-PCR and Real time RT-PCR have been applied successfully to the diagnosis of AI (22, 7, 14, 15, 20, 25, 19, 8, 13). In this document we present data on the development and validation of a Real Time hydrolysis probe-based RT-PCR assay for the simultaneous detection of avian influenza viruses belonging to H5, H7 and H9 subtypes. Our results prove that the assay is highly specific and sensitive. In a previous study (22), a RRT-PCR assay was developed on the sequences of North American H5 and H7 AI subtypes. In the latter, the sensitivity data obtained were comparable to the results described here. However, the protocol described previously was a one step RT-PCR with different thermal profiles for H5 and H7 detection. In addition to its sensitivity and specificity, the method described in the present document offers several advantages over conventional diagnostic methods including rapidity, flexibility and ease of use. This assay makes the results available for the three major AI viruses currently prevalent in poultry in large areas of the World in approximately 3 hours and the use of a single step RRT-PCR procedure provides some protection against contamination events. Based on its technical characteristics, this assay could be used for large scale screening and subtyping of viral RNA during influenza A virus outbreaks and for surveillance programmes.

This RRT-PCR assay was developed and validated using the same annealing temperature in order to identify the H5, H7 and H9 subtypes in a single analytical session. In the literature, the use of

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multiplexed PCR reactions has been reported as resulting in a decreased sensitivity of the method (21, 26) and the optimisation of the concentration of the multiplex PCR components to achieve optimal amplification can pose several difficulties (9). For this reason, the development of a multiplex assay was not attempted as it was so important to identify the three subtypes and maximize the assay's performance. The application of this RRT-PCR format was also due to the necessity of having a cost-effective and flexible diagnostic tool that could be easily switched to a single subtype identification method that would be applicable during investigation of outbreaks caused by one only of these subtypes. It should also be considered that not all of the existing real time PCR platforms are capable of detecting more than two fluorophores simultaneously, making a triplex PCR protocol inapplicable.

The suitability of the RRT-PCR test described in the present study as a diagnostic tool to recognize the three most important HA subtypes of AI rapidly, is confirmed by the results obtained using samples from birds infected naturally. These clinical samples were obtained during field and laboratory investigations from a wide range of avian species and geographical areas. The assay has been used for monitoring AI in poultry and wild birds and it has proved capable of identifying the presence of several distinct genetic lineages of H5, H7 and H9 viruses, including the H5N1 sublineages circulating in Eurasia and Africa (data not shown). The comparison of the results obtained applying the conventional diagnostic method (VI) and RRT PCR assay to these clinical samples showed good agreement. The lowest level of agreement was observed in the RRT-PCR/VI results for H5 subtype. Negative results by VI for H5 could be explained considering the condition of the clinical samples at the time of the arrival. Many of the samples, that proved positive for H5 subtype by RRT-PCR, but negative by VI, were submitted to the OIE/FAO Reference Laboratory from Africa and the Middle East and in some cases the cold chain was not maintained during the shipping, compromising the viability of the viruses. Based upon the results obtained in the present study, the RRT-PCR assay for simultaneous detection of

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H5, H7 and H9 could be a useful instrument for rapid screening and surveillance in wild and domestic birds. Although the method can not replace the standard virus isolation technique, this RRT-PCR assay offers several advantages over standard methods and could be use as a reliable tool for the rapid detection of the three AI viruses, including identification of co-circulating strains. Routine application in critical environments such as live bird markets, or on samples obtained from wild birds in their breeding or resting sites could give an indication of the degree of co-infection with these subtypes providing insight on the complex eco-epidemiology of avian influenza infections in such birds.

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Table 1: Viral and bacterial strains used in the validation process study and obtained results

Microrganisms	Strain	H5	H7	H9
AIV from Europe				
AIV	A/mallard/Italy/05 H5N1	+	-	-
AIV	A/swan/Italy/06 H5N1	+	-	-
AIV	A/chicken/Poland/07 H5N1	+	-	-
AIV	A/swan/Poland/06 H5N1	+	-	-
AIV	A/chicken/Italy/98 H5N2	+	-	-
AIV	A/duck/Italy/04 H5N3	+	-	-
AIV	A/turkey/Italy/80 H5N2	+	-	-
AIV	A/chicken/Italy/99 H7N1	-	+	-
AIV	A/turkey/Italy/99 H7N1	-	+	-
AIV	A/chicken/Netherlands/ H7N7	-	+	-
AIV	A/turkey/Italy/02 H7N3	-	+	-
AIV	A/turkey/Italy/07 H7N3	-	+	-
AIV	A/mallard/Italy/94 H7N4	-	+	-
AIV	A/turkey/Scotland/70 H9N7	-	-	+
AIV	A/Guinea fowl/Italy/07 H9N2	-	-	+
AIV	A/duck/Italy/08 H9N2	-	-	+
AIV	A/cockatoo/England/72 H4N8	-	-	-

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AIV	A/gull/Denmark/02 H16N3	-	-	-
AIV from Asia and Russia				
AIV	A/chicken/Vietnam/05 H5N1	+	-	-
AIV	A/chicken/Afghanistan/07 H5N1	+	-	-
AIV	A/swan/Iran/06 H5N1	+	-	-
AIV	A/chicken/Iran/08 H5N1	+	-	-
AIV	A/falcon/Kuwait/07 H5N1	+	-	-
AIV	A/chicken/Saudi Ar/07 H5N1	+	-	-
AIV	A/chicken/Saudi Arabia/05 H9N2	-	-	+
AIV	A/chicken/Saudi Arabia/06 H9N2	-	-	+
AIV	A/chicken/Saudi Arabia/07 H9N2	-	-	+
AIV	A/chicken/Saudi Arabia/08 H9N2	-	-	+
AIV	A/turkey/Turkey/05 H5N1	+	-	-
AIV	A/avian/Macaw/80 H7N7	-	+	-
AIV	A/avian/Pakistan/95 H7N3	-	+	-
AIV	A/chicken/Jordan/04 H9N2	-	-	+
AIV	A/wild bird/Iran/07 H9N2	-	-	+
AIV	A/mallard/Gurjev/82 H14N5	-	-	-
AIV	A/avian/Turkey/2008 H5N1	+	-	-
AIV	A/avian/Afghanistan/2008 H9N2	-	-	+
AIV	A/avian/Qatar/2008 H9N2	-	-	+
AIV from Africa				

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AIV	A/chicken/Egypt/06 H5N1	+	-	-
AIV	A/duck/Nigeria/07 H5N1	+	-	-
AIV	A/chicken/Ivory Coast/06 H5N1	+	-	-
AIV	A/chicken/Benin/07 H5N1	+	-	-
AIV	A/chicken/Benin/08 H5N1	+	-	-
AIV	A/chicken/Togo/07 H5N1	+	-	-
AIV	A/chicken/Egypt/08 H5N1	+	-	-
AIV	A/duck/Nigeria/08 H5N1	+	-	-
AIV	A/chicken/Togo/08 H5N1	+	-	-
AIV	A/wild bird/Mali/06 H5N3	+	-	-
AIV	A/wild bird/Egypt/07 H7N3	-	+	-
AIV	A/wild bird/Egypt/07 H9N2	-	-	+
AIV	A/ostrich/SA/01 H10N1	-	-	-
AIV	A/wild bird/Mali/06 H11N9	-	-	-
AIV from America and Australia				
AIV	A/turkey/Canada/65 H6N2	-	-	-
AIV	A/turkey/Ontario/6118/68 H8N4	-	-	-
AIV	A/turkey/Wisconsin/66 H9N2	-	-	+
AIV	A/duck/Memphis/ H11N9	-	-	-
AIV	A/duck/Alberta/60/76 H12N5	-	-	-
AIV	A/gull/Maryland/704/77 H13N6	-	-	-
AIV	A/shearwater/Aut/79 H15N9	-	-	-

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Microorganisms AI-unrelated					
APMV-1	Ulster 2C	-	-	-	
PPMV-1	Pigeon/2875/00	-	-	-	
APMV-2	Chicken/Yucaipa/56	-	-	-	
APMV-3	Turkey/1087/82	-	-	-	
APMV-4	Duck/Hong Kong D3/75	-	-	-	
APMV-6	Duck/Hong Kong/199/77	-	-	-	
APMV-7	Dove/United Kingdom/4/75	-	-	-	
APMV-8	Goose/1053/76	-	-	-	
APMV-9	Pintail/Italy/493/04	-	-	-	
IBV	793B serotype	-	-	-	
IBV	QX-like serotype	-	-	-	
APV	Type A	-	-	-	
APV	Type B	-	-	-	
Bacteria					
<i>Salmonella spp</i>		-	-	-	
<i>Campylobacter</i>		-	-	-	
<i>spp</i>		-	-	-	
<i>Escherichia coli</i>		-	-	-	
<i>spp</i>		-	-	-	

^aAIV Avian Influenza Virus;

^bAPMV Avian Paramyxovirus;

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^cPPMV Pigeon Paramyxovirus;

^dIBV Infectious Bronchitis Virus;

^eAPV Avian Pneumovirus.

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Table 2: Real Time RT-PCR primer and probe sequences.

Target	Primer/probe	Sequence (5' - 3')
Avian H5	H5-For	TTATTCAACAGTGGCGAG
	H5NE-Rev	CCAG(T)AAAGATAGACCAGC
	H5 probe	CCCTAGCACTGGCAATCATG
Avian H7	H7-For	TTTGGTTTAGCTTCGGG
	H7- deg Rev	GAAGAA(C)AAGGCC(T)CATTG
	H7 probe	CATCATGTTTCATACTTCTGGCCAT
Avian H9	H9-For	ATGGGGTTTGCTGCC
	H9-Rev	TTATATACAAATGTTGCAC(T)CTG
	H9 probe	TTCTGGGCCATGTCCAATGG

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Table 3: Sensitivity of the method.

Strain	Titre of virus stock (EID₅₀ /100µl)	Biological Matrix	Sensitivity (EID₅₀ /100µl)
A/mallard/Italy/3401/05 H5N1 LPAI	10 ^{5.83}	allantoic fluid	10^{1.83}
		lung	10^{1.83}
		faeces	10^{1.83}
A/chicken/Yamaguchi/7/04 H5N1 HPAI	10 ^{6.74}	allantoic fluid	10^{1.74}
		lung	10^{2.74}
		faeces	10^{2.74}
A/turkey/Italy/4580/99 H7N1 HPAI	10 ^{7.37}	allantoic fluid	10^{1.37}
		lung	10^{1.37}
		faeces	10^{1.37}
A/turkey/Wisconsin/66 H9N2 LPAI	10 ⁷	allantoic fluid	10¹
		lung	10¹
		faeces	10²

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Table 4: Intra and inter-assay coefficients of variation (values in percentage).

	H5		H7		H9	
Dilution	Intra assay variability	Inter assay variability	Intra assay variability	Inter assay variability	Intra assay variability	Inter assay variability
high	<1.68	4.06	<1.66	4.02	<1.48	4.11
medium	<1.58	3.81	<1.35	3.02	<1.74	3.19
low	<1.16	2.76	<2.64	2.26	<4.11	3.92

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Table 5: Results of real Time RT-PCR and virus isolation from clinical samples.

Results by:		Number of samples		
RRT- PCR	VI/HI	H5	H7	H9
+	+	98	52	24
+	-	43	6	6
-	-	584	667	693
-	+	0	0	2

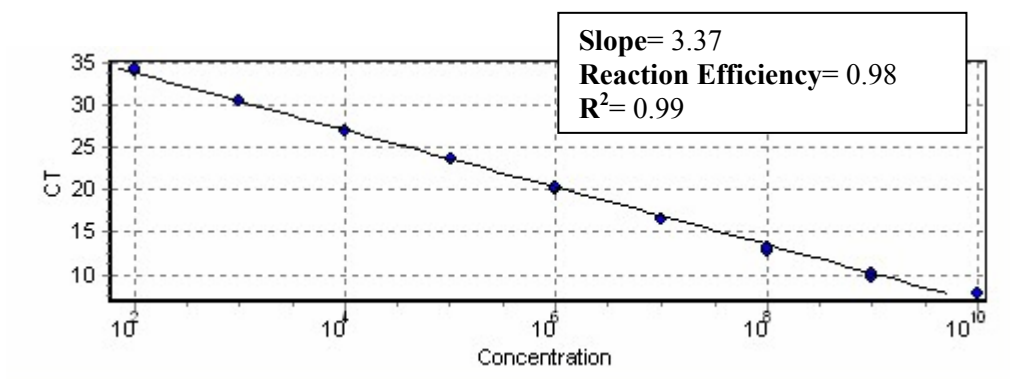
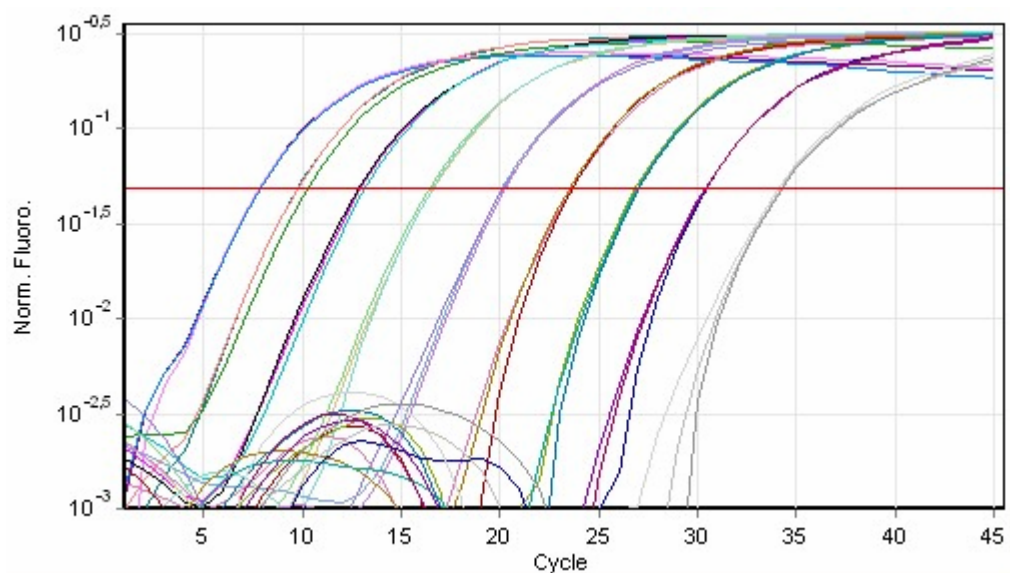


Figure 1. Construction of the standard curve of the H7 gene real time assay. Serial ten fold dilutions of in vitro transcribed RNA (from 10^2 to 10^{10} gene copies/ μ l) were plotted against threshold cycle.

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Prepared by			NOTES	DATE	N. REV.
Dr. G. Cattoli Dr. I. Monne				28/04/2008	00

VALIDATION DOSSIER

**REAL TIME RT-PCR (rRT- PCR)
DETECTION OF AVIAN INFLUENZA
SUBTYPE
H5, H7, H9**

Part II: Description of the testing protocol

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**TITLE: REAL TIME RT-PCR (rRT- PCR) DETECTION OF AVIAN INFLUENZA
SUBTYPE H5, H7, H9**

1. INTRODUCTION

The protocol describes the reagents and the procedure necessary to reveal the presence of avian influenza viruses RNA belonging to the H5, H7 and H9 subtypes in samples of avian origin by real time PCR amplification. According to users' preferences and priorities, the detection of the three subtypes can be performed simultaneously, within the same real time PCR run, in separate PCR tubes or wells. The assay is a one step RT-PCR protocol, meaning that the reverse transcription of the target RNA and the subsequent PCR amplification occur within the same reaction mix in the same tube. This will significantly reduce the processing time and the risks of sample contamination. Appendix A includes the description of the validation process performed according to the guidelines indicated in the references (5, 6, 7)

2. INTENDED USE

Protocol and reagents for the specific and simultaneous detection of avian influenza A viruses belonging to the H5, H7 and H9 subtypes. To be used in a nucleic acid amplification test, namely real time PCR, to directly detect specific virus RNA in specimens of avian origin and viral cultures.

Target: primers and probes are directed to a conserved region in the subunit HA2 of the haemagglutinin genomic segment (segment 4).

Population to be tested: avian species.

Specimen type: allantoic fluid of embryonated eggs (viral culture); cell supernatants (viral cultures); tracheal or oro-pharyngeal swab; cloacal swab; internal organs (e.g. trachea, lungs, brain); faecal material.

Important note: the primers and probes were designed on multiple alignments of sequences available in public genetic databases. Due to sequence variability among HA sequences within the same subtypes, primers and probes for H5 and H7 were optimized for the detection of H5 and H7 viruses belonging to the Eurasian/African genetic lineages.

3. References

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7. Crowther J. R., Unger H. and Viljoen G. J. 2006. Aspects of kit validation for tests used for the diagnosis and surveillance of livestock diseases: producer and end-user responsibilities. Rev. sci. tech. Off. Int. Epiz. **25** (3), 913-35.

4. REAGENTS/SOLUTIONS

Product name	notes
Sterile RNase free water	
Sterile Phosphate Buffered Solution (PBS, pH 7.4)	
Nucleospin® RNA II (Macherey Nagel) or	RNA extraction (Manual)
RNeasy® Mini Kit (QIAGEN) or	RNA extraction (Manual)
MagMAX™-96 AI/ND Viral RNA Isolation Kit (Ambion)	RNA extraction (Automatic)
Ethanol 70%	
Isopropanol 100%, Ethanol 100%	
QantiTect® Multiplex RT-PCR (Qiagen)	one-step RT-PCR
Probe FAM H5 (Applied Biosystems/Operon)	FAM-5'-CCC TAG CAC TGG CAA TCA TG-3'-TAMRA
Primer H5 F (Operon)	5'-TTA TTC AAC AGT GGC GAG-3'
Primer H5NE R (Operon)	5'-CCA KAA AGA TAG ACC AGC -3'
Probe VIC(HEX) H7 (Applied Biosystems/Operon)	VIC (HEX)5'-CAT CAT GTT TCA TAC TTC TGG CCA T-3'TAMRA
Primer H7 F (Operon)	5'-TTT GGT TTA GCT TCG GG-3'
Primer H7 R deg (Operon)	5'-GAA GAM AAG GCY CAT TG-3'
Probe FAM H9 (Applied Biosystems/Operon)	FAM-5'-TTC TGG GCC ATG TCC AAT GG-3'TAMRA
Primer H9F (Operon)	5'-ATG GGG TTT GCT GCC-3'
Primer H9R (Operon)	5'- TTA TAT ACA AAT GTT GCA YCT G-3'

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7. STANDARD OPERATING PROCEDURES

7.1 RNA EXTRACTION

This document describes the procedure and the reagents for the real time PCR assay only. It does not include reagents for the extraction of the RNA. However, the protocol was evaluated through the application of manual silica column-based RNA extraction technology (Nucleospin® RNA II; Macherey Nagel; RNeasy® Mini Kit; QIAGEN). For robotic RNA extractions on swabs and faecal suspensions, similar results were obtained through the application of MagMAX™-96 AI/ND Viral RNA Isolation Kit (Ambion).

RNA is a fragile molecule, that can be easily deteriorated in the samples improperly collected, handled and stored. Be sure to take all the precautions in order to avoid RNA degradation and to assure a proper RNA extraction procedure. Sample collection, handling, storage and RNA extraction laboratory protocols may significantly influence the performances of the real time PCR tests.

7.2 SAMPLE PREPARATION

Depending on the type of specimen to be processed, some preliminary procedures are necessary before submitting the sample to the RNA extraction protocol.

Allantoic fluids (i.e. virus isolates from SPF embryonated chicken egg):

- Transfer the sample volume requested by the RNA extraction kit of allantoic fluid in a sterile tube containing the exact amount of lysis buffer, following the manufacturer's instructions.

Organs/tissues:

- Disgregate and homogenize a small specimen (e.g. 5 mm³) of tissue such as trachea, lungs or brain in 500 µl of PBS. Transfer the suspension in microcentrifuge tube.
- Centrifuge the suspension at maximum speed (8,000 g) for 30 sec.
- Transfer the sample volume requested by the RNA extraction kit in a sterile tube containing the exact amount of lysis buffer, following the manufacturer's instructions.

Swabs (cloacal/trachea/oro-pharyngeal):

- In a sterile tube (15 ml) add 1 ml of sterile PBS;
- Submerge and accurately roll the swab into the PBS (1 to 5 cloacal swabs/ml or 1 to 5 tracheal swabs/ml);
- Vortex the suspension;
- Transfer the sample volume requested by the RNA extraction kit in a sterile tube containing the exact amount of lysis buffer, following the manufacturer's instructions.

Faeces

- In a 15 ml sterile tube, add 1ml of PBS to 1gr of faeces;
- vortex the suspension and centrifuge at 8,000g for 30 sec;
- Transfer the sample volume requested by the RNA extraction kit in a sterile tube containing the exact amount of lysis buffer, following the manufacturer's instructions.

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7.4 rRT-PCR MASTER MIX PREPARATION

7.4.1 rRT-PCR MASTER MIX PREPARATION FOR H5

REAGENTS (stock conc.)	FINAL CONCENTRATION	µl x 1 REACTION
Sterile RNase free water	/	0,55
Primer H5F (5µM)	300 nM	1,5
Primer H5NE R (5µM)	300 nM	1,5
2x RT-PCR master mix	1X	12,5
Probe FAM H5 (1µM)	150 nM	3,75
Enzyme mix		0,2
REAGENTS VOLUME		20µl
RNA		5µl
FINAL VOLUME		25µl

7.4.2 rRT-PCR MASTER MIX PREPARATION FOR H7

REAGENTS (stock conc.)	FINAL CONCENTRATION	µl x 1 REACTION
Sterile RNase free water	/	0,55
Primer H7F (10µM)	300 nM	0,75
Primer H7 Rdeg (10µM)	900 nM	2,25
2x RT-PCR master mix	1X	12,5
Probe VIC (HEX)H7 (1µM)	150 nM	3,75
Enzyme mix		0,2
REAGENTS VOLUME		20µl
RNA		5µl
FINAL VOLUME		25µl

7.4.3 rRT-PCR MASTER MIX PREPARATION FOR H9

REAGENTS (stock conc.)	FINAL CONCENTRATION	µl x 1 REACTION
Sterile RNase free water	/	0,55
Primer H9 F (5µM)	300 nM	1,5
Primer H9 R (5µM)	300 nM	1,5
2x RT-PCR master mix	1X	12,5
Probe FAM H9 (1µM)	150 nM	3,75
Enzyme mix		0,2
REAGENTS VOLUME		20µl
RNA		5µl
FINAL VOLUME		25µl

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7.5. rRT-PCR amplification Thermal Profile (AB7300; Rotor Gene 6000 Corbett)

<i>rRT-PCR H5,H7,H9</i> Thermal profile	
RT REACTION	50°C / 20 min
INITIAL DENATURATION STEP	95°C / 15 min
DENATURATION	94° C/45 sec
ANNEALING	54°C/45 sec
CYCLES N.	40

7.6 DETECTION OF THE AMPLIFIED PRODUCT

At the end of the PCR amplification reaction, data are collected and managed through the 7300AB system software (AB 7300) e Rotor Gene 6000 series software (Rotor Gene 6000 Corbett).

8. Result Interpretation§

- **Step 1. Real time PCR run validation.**
 - Check the amplification plot of the positive and negative controls. The positive control should have a clear increase of the fluorescence signal ranging between 9 and 20 Cycle threshold (Ct). The negative control should not have any increase of fluorescence above the threshold.
 - In case the controls do not provide the expected results, the run should be considered invalid and the tests repeated.

- **Step 2. Interpretation of sample results.**
 - Samples should be considered as POSITIVE when a clear and regular increasing of fluorescence is associated with a Ct ranging from 9 to 35 (for Operon's oligos, Ct 37).
 - Samples should be considered as NEGATIVE when there is no detection of increasing fluorescence associated with the specific fluorophore (FAM or HEX). Sample revealing a weak increase of fluorescence with irregular amplification plot and Ct > 35 (37) and < 40 should be considered as non-specific and the sample should be defined as NEGATIVE.
 - Samples revealing regular but weak increasing of fluorescence with a Ct > 35 (37) and < 40 should be considered as DUBIOUS/BORDERLINE and the test should be repeated or confirmed by another assay.

§ Derived from validation tests on Corbett 6000 and evaluation on Applied Biosystems 7300 real time PCR platforms carried out at the Istituto Zooprofilattico Sperimentale delle Venezie, Italian National, FAO and OIE reference laboratory for avian influenza and Newcastle disease. Other laboratories using different instrumentation platforms should first critically and carefully examine both the cycling conditions and the result interpretations as they may not perform optimally on other instruments.

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10. ANNEXES

Validation Dossier part I: description of the validation procedure.

11. RELATED DOCUMENTS (NOT INCLUDED)

Nucleospin® RNA II (Macherey Nagel) Instruction Manual vers. October 2007/ rev 08.

RNeasy® Mini Kit (QIAGEN) Instruction Manual vers. April 2006.

MagMAX™-96 AI/ND Viral RNA Isolation Kit (Ambion) Instruction Manual ver. 0608.

QuantiTect Multiplex RT-PCR kit (QIAGEN) Instruction Manual vers. October 2005.