Institut Pasteur de Lille	PROCEDURE	N°	P001RIVERS
	RT-PCR Detection of avian *Influenza* A viruses H5N1	Version	1
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This paper describes procedures for the detection of M and H5 genes by real-time RT-PCR that can be used in case of suspicion of H5N1 contamination. The following testing strategy is suggested:

- RNA extraction
- Amplification of M and H5 genes in parallel.

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1. AIM

This paper describes procedures for the detection of M and H5 genes by real-time RT-PCR that can be used in case of suspicion of H5N1 contamination.
Quantitative detection methods by RT-PCR have been optimised to detect H5N1. Detection techniques may provide sufficient sensitivity, not only for direct analysis of heavily contaminated samples (such as duck ponds) and of solid matrices (sediments, biota, food), but also for analysis of lakes and especially for drinking water after a concentration step. The methods have been validated on concentrated water samples (described in Procedure P002RIVERS) and on extracted/eluted viruses from muds (described in Procedure P003RIVERS).

2. MATERIAL

2.1. COMMERCIAL KITS AND REAGENTS

Kits or Reagents	Supplier	Reference
QIAamp Viral RNA Minikit	Qiagen	52 904
SuperScript™ III Platinum® One-Step Quantitative RT-PCR System	Invitrogen	11732-020
Non acetyled BSA 10%	Invitrogen	P2046

2.2. PRIMERS AND PROBES

The primers and probes proposed in this protocol (in Table 1), were designed to detect clades 1, 2-1, 2-2 and 2-3.

The primers and probes used target the hemagglutinin (H5) gene which has experienced a high rate of evolutionary changes. The primers and probe of the matrix (M) gene were selected from the conserved region of all subtypes of *Influenza* A viruses. Spackman *et al.* (2002) have described in detail real time reverse-transcriptase (RRT) PCR for avian influenza detection; they developed a generic M gene RRT-PCR that amplifies within this highly conserved gene and a H5 type specific detection which amplifies within the hemagglutinin HA2 region (Spackman et al., 2002). However, this method was validated on North American lineage Avian Influenza Viruses (AIV) only: molecular phylogeny studies have revealed distinct American and Eurasian lineages for H5. That's why Slomka *et al.* (2007) have adapted primers and probe sequences for the successful detection of current Highly Pathogenic (HP) H5N1 from Asian lineage and other Eurasian H5 AIVs isolated in recent years. Validation of the H5 RRT-PCR was conducted with 51 specimens of avian influenza viruses H5 (34 strains of HP viruses H5N1 isolated between 2004 and 2006 and 12 non-H5N1 viruses since 1996, such as H5N2, H5N3, H5N7, H5N8, and H5N9); 52 other clinical specimen of non-H5 AIVs were used in order to demonstrate the specificity of the detection (Slomka et al., 2007).

Table 1 – Influenza A virus H5N1 RT-PCR primers and TaqMan® TAMRA probes

Specificity	Primers/probe	Sequence (5'-3')	Reference
Influenza A virus Matrix (M) gene	Flu/A/M/25/+ Flu/A/M/124/- Flu/A/M/64/+prob	AGA TGA GTC TTC TAA CCG AGG TCG TGC AAA AAC ATC TTC AAG TCT CTG Fam-TCA GGC CCC CTC AAA GCC GA-Tamra	(Spackman et al., 2002)
Avian H5 gene	Flu/A/H5/1456/+ Flu/A/H5/1685/- Flu/A/H5/1637/+prob	ACA TAT GAC TAC CCA CAR TAT TCA G AGA CCA GCT AYC ATG ATT GC Fam-TCW ACA GTG GCG AGT TCC CTA GCA-Tamra	(Slomka et al., 2007)

2.3. POSITIVE CONTROL FOR H5 AND M REAL-TIME RT-PCR

Positive control for H5 real-time RT-PCR is an in vitro transcribed RNA derived from **A/Chicken/Cambodia/2003** (**à confirmer par CIBU)** strain (Emerging Infectious Diseases. Vol 11, No 10:1515-21). The transcript contains the Open Reading Frame of H5 gene (from ATG to base # 1,703) as negative strand. Each microtube contains about 10^{11} copies of target sequences per µL.

Positive control for M real-time RT-PCR is an in vitro transcribed RNA derived from **A/…** (**à confirmer par CIBU)** strain. The transcript contains the Open Reading Frame of M gene (from ATG to base # 982) as negative strand. Each microtube contains about 10^{11} copies of target sequences per µL.

Preparation of transcribed RNA (M and H5)
Dilute 2 µL of each transcript in 188 µL of distilled water to obtain the concentration of 10^9 copies/µL. Use this dilution to prepare working banks of reagent in order to avoid freezing/thawing cycles. Store dilutions at -80°C.

3. EXTRACTION

RNAs are purified from liquid samples according to the manufacturer's instructions (protocol in annexe). Qiamp Viral RNA Minikit™® is used for purification of viral RNA from 140 µL of sample. RNAs bound to the membrane are eluted in 60 µL of water.

4. REACTIVE MIX PREPARATION

Primers and probes were validated in the conditions described below.

The RT-PCR Mix was realized with the following kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system
- These Mixes were used on the real-time PCR instrument: LightCycler 2.0 (Roche)

It is possible to use these primers and probes with other kits, and to perform assays on other real-time PCR instruments.

RNA extract (5µL) was analyzed by RT-PCR.
Reaction mixture conditions are reported in Table 2.

Table 2 – Reaction mixture conditions

Superscript III Platinium One-Step qRT-PCR system - Invitrogen	**Final concentration**	**Volume (µL)**
H_2O	/	0
2X Reaction mix containing dNTP and 6 mM MgSO4	1X 3 mM	10
MgSO4 (50 mM)	3 mM	1.2
Forward Primer (10 µM)	0.5 µM	1
Reverse Primer (10 µM)	0.5 µM	1
Taqman Probe (25 µM)	0.2 µM	0.16
Non-acetylated BSA –INVITROGEN P2046 (10 mg/mL)	0.5 mg/mL	1
Superscript III RT/Platinium Taq mix	/	0,8
Template	/	**5**

5. CONTROLS

The transcribed RNAs are then serially diluted 10-fold, ranging from 10^6 to 10 copies / 5 µL (corresponding to the tested volume by RT-PCR) to assess the efficiency of the method. The threshold cycle -Ct or CP - is the cycle at which a significant increase in fluorescence occurs -i.e. when fluorescence becomes distinguishable from background-. A graph of Ct vs. log10 copy number of the sample from the dilution series is produced and used as a calibration curve. The slope of this graph is used to determine efficiency.

Each real-time RT-PCR assay includes in addition of unknown samples:

- One negative sample extracted during RNA extraction (negative extraction control),
- One negative amplification control (reactive mix control),
- Three quantification positive controls, at least, including 10^6, 10^4 and 10^2 copies of *in vitro* synthesized RNA transcripts of H5 or M gene.

Presence or absence of inhibitors in extracted RNAs samples are checked by spiking experiments.
1 µL of 10^6 copies of H5 or M RNA transcripts are diluted in 5 µL of analysed RNA samples before RNA amplification. Ct of spiked samples must be equal (± no more than 3 Ct) to the Ct obtained for 1 µL of 10^6 copies of H5 or M RNA transcripts tested without RNA samples, in the same operating conditions.

6. AMPLIFICATION CYCLES

Retained real-time RT-PCR conditions are reported in Table 3.

Table 3 – Cycling conditions

STEPS		Temperature	Time	Acquisition mode	Number of cycles	Analysis mode
Reverse transcription		50°C	00:15:00	None	1	None
Denaturation	Initial PCR Activation	95°C	00:02:00	None	1	None
Amplification	Denaturation	95°C	00:00:15	None	50	Quantification
	Annealing / Extension	60°C	00:00:30	Single		
Cooling		40°C	00:00:30		1	None

7. QUANTIFICATION THRESHOLDS AND SENSITIVITY

The quantification threshold corresponds to the limit log-number of copies under which crossing point values were no longer proportional to logarithmic values of copies concentration. The real-time RT-PCR specific of M and H5 genes can quantify down to 5.3 10^2 and 2.4 10^2 copies / 5 µL, respectively.
Sensitivity is about 10 copies of RNA transcript per reaction (this amount of target sequences is always detected); the fluorescent signal can be detected at RNA standard dilution of M and H5 as low as 10 copies / 5 µL in a single real-time RT-PCR assay.

8. RESULTS

RT-PCR efficiency is calculated using the Roche instrumentation software; the efficiency value has to be included in range 1.8 to 2.0, indicating an efficient rate of replication of RNA.

When RT-PCR inhibitors have been put in evidence in a sample, a second RT-PCR shall be performed using 2- or 10-fold diluted RNA extracts.

9. ANNEX

Protocol: Purification of Viral RNA (Spin Protocol)

Spin Protocol

This protocol is for purification of viral RNA from 140 µl plasma, serum, urine, cell-culture media, or cell-free body fluids using a microcentrifuge. For automated purification of viral RNA using the QIAamp Viral RNA Mini Kit on the QIAcube, refer to the *QIAcube User Manual* and the relevant protocol sheet.

Larger starting volumes, up to 560 µl (in multiples of 140 µl), can be processed by increasing the initial volumes proportionally and loading the QIAamp Mini column multiple times, as described below in the protocol. Some samples with very low viral titers should be concentrated before the purification procedure; see "Protocol: Sample Concentration" (page 30).

Alternatively, larger sample volumes can be processed using one of the following kits, which provide simultaneous purification of viral DNA and RNA.

- QIAamp MinElute® Spin Kit* 200 µl
- QIAamp MinElute Vacuum Kit 500 µl
- QIAamp UltraSens® Virus Kit 1000 µl

Important points before starting

- Read "Important Notes" (pages 15–22) before starting the protocol.
- All centrifugation steps are carried out at room temperature (15–25°C).

Things to do before starting

- Equilibrate samples to room temperature (15–25°C).
- Equilibrate Buffer AVE to room temperature for elution in step 11.
- Check that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on page 17.
- Add carrier RNA reconstituted in Buffer AVE to Buffer AVL according to instructions on page 15.

Procedure

1. **Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.**

   If the sample volume is larger than 140 µl, increase the amount of Buffer AVL–carrier RNA proportionally (e.g., a 280 µl sample will require 1120 µl Buffer AVL–carrier RNA) and use a larger tube.

* Fully automatable on the QIAcube. See www.qiagen.com/MyQIAcube for protocols.

Spin Protocol

2. **Add 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.**

   To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

3. **Incubate at room temperature (15–25°C) for 10 min.**

   Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA. Potentially infectious agents and RNases are inactivated in Buffer AVL.

4. **Briefly centrifuge the tube to remove drops from the inside of the lid.**

5. **Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.**

   Only ethanol should be used since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl of ethanol). In order to ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6. **Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.**

   Close each spin column in order to avoid cross-contamination during centrifugation.

   Centrifugation is performed at 6000 x *g* (8000 rpm) in order to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7. **Carefully open the QIAamp Mini column, and repeat step 6.**

   If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

8. **Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.**

   It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.

9. **Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 11, or to eliminate any chance of possible Buffer AW2 carryover, perform step 10, and then continue with step 11.**

   Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow-through, containing Buffer AW2, contacting the QIAamp Mini column. Removing the QIAamp Mini column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Mini column. In these cases, the optional step 10 should be performed.

10. **Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

11. **Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x *g* (8000 rpm) for 1 min.**

    A single elution with 60 µl of Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column. Performing a double elution using 2 x 40 µl of Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate.

    Viral RNA is stable for up to one year when stored at –20°C or –70°C.