



INSTITUT PASTEUR

---

Centre National de Référence  
Du Virus Influenzae  
Région Nord

Sylvie van der Werf  
Dominique Rousset  
Vincent Enouf

**Real-time PCR of Type A  
A(H1N1)2009 viruses**  
Ref: SOP/FluAswl/031209

## INTRODUCTION

---

This paper describes procedures for the detection of influenza A viruses (M gene) the novel A(H1N1)2009 virus (H1 2009 and N1 2009 gene) by real-time RT-PCR that can be used in case of suspicion of human infection by the novel A(H1N1)2009 virus. We suggest the following testing strategy:

- RNA extraction
- Amplification in parallel of M, H1 2009, N1 2009 and GAPDH (to assess quality of the specimen and extraction procedure) genes

## MATERIAL

---

### Kits

QIAamp Viral RNA (QIAGEN mini Kit 50)

Ref: QIAGEN 52 904

SuperScript™ III Platinum® One-Step Quantitative RT-PCR System  
Non acetyled BSA 10%

Ref: Invitrogen 11732-020  
Ref: Invitrogen P2046

### Primers and probes

If the sample is positive for M and negative for H1 2009/N1 2009, we suggest to use the H1N1h and H3N2h sets of primers previously sent as SOP/FluAswl/140509.

Name	Sequences	Mers	PCR Pdt	Ref
GAPDH-6Fw	GAAGGTGAAGGTCGGAGT	18		3
GAPDH-231Rv	GAAGATGGTGATGGGATTTC	20	226 bp	3
GAPDH-202Probe(-)	CAAGCTTCCCGTTCTCAGCC [5']Fam [3']BHQ-1	20		3
GRAM/7Fw	CTTCTAACCGAGGTCGAAACGTA	23		2
GRAM/161Rv	GGTGACAGGATTGGTCTTGCTTTA	25	202 bp	2
GRAM probe/52/+	TCAGGCCCCCTCAAAGCCGAG [5']Fam [3']BHQ-1	21		2
GRswH1-349Fw	GAGCTAAGAGAGCAATTGA	19		1
GRswH1-601Rv	GTAGATGGATGGTGAATG	18	253bp	1
GRswH1-538Probe(-)	TTGCTGAGCTTTGGGTATGA [5']Fam [3']BHQ-1	20		1
GRswN1-975Fw	TCCACGCCCTAATGATAA	18		1
GRswN1-1084Rv	TTCTCCCTATCCAAACAC	18	110bp	1
GRswN1-1045bProbe(-)	ATCCTTTTACTCCATTTGCTCC [5']Fam [3']Tamra	22		1

1/ National Influenza Center (Northern-France), Institut Pasteur, Paris.

3/ National Influenza Center (Southern-France), CHU, Lyon.

2/ Wong et al., 2005, J. Clin. Pathol. 58:276-280

## **NUCLEIC ACID EXTRACTION**

---

RNA is extracted from specimens using the QIAamp Viral RNA kit (QIAGEN Mini Kit 50 ref 52904). RNA extracted from 200  $\mu$ l of original sample, is eluted in 60  $\mu$ l of elution buffer.

## **MIX PREPARATION FOR ALL SEPARATE PRIMER/PROBE COMBINATIONS**

---

All primers and probes described below were validated under the following conditions.

RT-PCR Mix kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)

Real-time PCR equipments:

- LightCycler 1.5 or 2.0 (Capillaries)
- LightCycler 480

Adjustments may be required for the use of other kits or other real-time PCR instruments.

Primers and probes for the detection of influenza A viruses (M gene), GAPDH and the novel A(H1N1)v virus (H1 2009 and N1 2009 gene) were also validated under the following conditions.

RT-PCR Mix kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)

Real-time PCR equipments:

- 7500 Applied® Biosystems
- SmartCycler® Cepheid

LightCycler 1.5 or 2.0 (Capillaries)

<u>Mix :</u>	Vol ( $\mu$ l)	[final]
H <sub>2</sub> O PPI :	1.06	
Reaction mix 2X :	10	3 mM Mg
MgSO <sub>4</sub> (50mM) :	0.24	0.6 mM Mg
Forward Primer (10 $\mu$ M):	1	0.5 $\mu$ M
Reverse Primer (10 $\mu$ M):	1	0.5 $\mu$ M
Probe (10 $\mu$ M):	0.4	0.2 $\mu$ M
<b>BSA non acetylated (10mg/ml)</b>	<b>0.5</b>	<b>0.25 mg/ml</b>
SuperscriptIII RT/Platinum Taq Mix :	0.8	
<b>Final Volume:</b>	<b>15 <math>\mu</math>l</b>	

LightCycler 480 (96)

<u>Mix :</u>	Vol ( $\mu$ l)	[final]
H <sub>2</sub> O PPI :	1.56	
Reaction mix 2X :	10	3 mM Mg
MgSO <sub>4</sub> (50mM) :	0.24	0.6 mM Mg
Forward Primer (10 $\mu$ M):	1	0.5 $\mu$ M
Reverse Primer (10 $\mu$ M):	1	0.5 $\mu$ M
Probe (10 $\mu$ M):	0.4	0.2 $\mu$ M
SuperscriptIII RT/Platinum Taq Mix :	0.8	
<b>Final Volume:</b>	<b>15 <math>\mu</math>l</b>	

15  $\mu$ l of reaction mix + 5  $\mu$ l of RNA samples

7500 Applied® Biosystems or SmartCycler

<u>Mix :</u>	Vol ( $\mu$ l)	[final]
H <sub>2</sub> O PPI :	1.5	
Reaction mix 2X :	12.5	3 mM Mg
Forward Primer (10 $\mu$ M):	2	0.8 $\mu$ M
Reverse Primer (10 $\mu$ M):	2	0.8 $\mu$ M
Probe (5 $\mu$ M):	1	0.2 $\mu$ M
ROX reference dye (diluted 1/10)	0.5	
SuperscriptIII RT/Platinum Taq Mix :	0.5	
<b>Final Volume:</b>	<b>20 <math>\mu</math>l</b>	

20  $\mu$ l of reaction mix + 5  $\mu$ l of RNA samples

## CONTROLS

---

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

- Two negative samples bracketing unknown samples during RNA extraction (negative extraction controls)
- Positive controls (in duplicate); when using *in vitro* synthesized transcripts as controls include five quantification positive controls (in duplicate) including  $10^4$ ,  $10^3$  and  $10^2$  copies of *in vitro* synthesized RNA transcripts.
- For the N1 2009 RT-PCR, positive-patient cDNA or RNA can be used as a positive control.
- One negative amplification control.

## AMPLIFICATION CYCLES (LIGHTCYCLER SYSTEM)

Reverse transcription	45°C	15 min	x1
Denaturation	95°C	3 min	x1
Amplification	95°C	10 sec	
	55°C	10 sec	
	72°C	20 sec	x50
Cooling	40°C	30 sec	x1

## AMPLIFICATION CYCLES (7500 APPLIED OR SMARTCYCLER SYSTEM)

Reverse transcription	50°C	15 min	x1
Denaturation	95°C	2 min	x1
Amplification	95°C	15 sec	
	60°C	40 sec	x50

## SENSITIVITY

---

### For the M real-time RT-PCR

Sensitivity, in terms of 95% hit rate is about 100 copies of RNA genome equivalent per reaction (this amount of target sequences is always detected), the probability to detect lower amounts of virus decreases, but samples containing 10 copies could be detected.

### For the H1 2009 real-time RT-PCR

Sensitivity is comparable to that of the M real-time RT-PCR and comparable to the sensitivity of the CDC kit ( $C_p < 36$  for all positive specimens tested so far).

### For the N1 2009 real-time RT-PCR

Sensitivity is comparable to that of the M real-time RT-PCR and better than the H1 2009 real-time RT-PCR.

## SPECIFICITY

---

### For the H1 2009 and N1 2009 real-time RT-PCR

Limited testing so far showed no detection for seasonal influenza viruses (influenza A(H1N1), A(H3N2), B) nor for specimens known to be positive for other respiratory viruses (influenza C, RSV A, B, hBoV, hPIV1,3, hMPV, HRV, enterovirus, adenovirus, CMV, HSV, VZV).

For swine influenza viruses, detection was positive for A/sw/England/117316/86 (classical swine lineage) and negative for A/sw/England/502321/94 (H3N2).

For A(H1N1)v viruses, detection was positive for A/California/4/2009 as well as for more than 10 specimens positive for the novel A(H1N1)v virus

**NOTE: the H1 2009 real-time RT-PCR does not detect the positive control from the CDC kit**

### POSITIVE CONTROL FOR M AND GAPDH REAL-TIME RT-PCR

---

Positive control for M real-time RT-PCR is an *in vitro* transcribed RNA derived from strain A/Paris 650/06(H1N1). The transcript contains the Open Reading Frame of the M gene (from the ATG to nt 982) as negative strand. Each microtube contains  $10^{11}$  copies of target sequences diluted in yeast tRNA, and lyophilised.

Positive control for GAPDH real-time RT-PCR is an *in vitro* transcribed RNA. The transcript contains the Open Reading Frame of the M gene (from nt 6 (ATG = 1) to nt 231) as negative strand. Each microtube contains  $10^{11}$  copies of target sequences diluted in yeast tRNA, and lyophilised.

#### Reconstitution of transcribed RNA

Add 100  $\mu$ l of distilled water to obtain a solution at a concentration of  $10^9$  copies/ $\mu$ l. Store at  $-80^{\circ}\text{C}$ . Dilute in  $\text{H}_2\text{O}$  to prepare a master bank at  $2 \times 10^6$  copies/ $\mu$ l. Store at  $-80^{\circ}\text{C}$ . From this prepare a working bank of reagent at  $2 \times 10^4$  copies/ $\mu$ l in order to avoid freeze/thaw cycles. Working tubes may be stored at  $-20^{\circ}\text{C}$  for less than one week.

Positive controls are available upon request (grippe@pasteur.fr)

## INTERPRETATION OF RESULTS

---

GAPDH reactions should give a  $C_p < 35$ ; if higher and otherwise negative results are obtained this may result from:

- poor quality of the specimen with insufficient number of cells ; obtain a new specimen for the same patient
- presence of inhibitors; repeat the procedure with dilutions of the extracted RNA (e.g. 1:10, 1:100) and/or repeat RNA extraction.

Positive reactions for M and H1 2009/N1 2009 and negative reactions for H1h, N1h, H3h, N2h : confirmed case for A(H1N1)2009 virus

Positive reaction for M and negative for H1 2009/N1 2009 and for H1h, N1h, H3h, N2h (usually seen for low virus load in specimen); repeat reactions and/or repeat RNA extraction

Positive reaction for M and negative for H1 2009/N1 2009 but positive for either N1h, H3h and negative for H1h and N2h (usually seen for low virus load in specimen); infection with seasonal virus; repeat reactions and/or repeat RNA extraction to determine sub-types

Positive reaction for M and for H1 2009/N1 2009 and positive reaction for either N1h, H3h may reflect a cross-contamination or a possible co-infection with both the novel A(H1N1)v virus and a seasonal virus; repeat RNA extraction and repeat reactions with all necessary precautions to avoid cross-contamination.