RIVM Laboratory Protocol Library

Influenzavirus A and B PCR
LightCycler – probe test
A-Matrix-B-NS-H1pdm09-H3-H5-N1pdm09-N1avian-N2

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This protocol has been made available by:

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1. Reagents

1.1. RNA isolation

Automated MagNApure LC Total Nucleic Acid Kit (Roche cat no. 03 038 505 01)
or,
Manual High Pure RNA isolation Kit (Roche cat no. 11 828 665 001)

One-step protocol for: A-Matrix, B-NS, H3, H1pdm09 H5, N1pdm09, N1avian and N2

1.2. Lightcycler PCR

PCR reactions are validated on the LightCycler 480 with:
- one-step: TaqMan® Fast Virus 1-Step Master Mix (LifeTechnologies cat no. 4444436)

1.3. Primersets **

Matrix Protein gene (specific for all influenzavirus A subtypes, H1-H16).
INFAM Sense1   AAG ACC AAT **CC**T GTC ACC TCT GA*
INFAM Sense2   AAG ACC AAT **CT**T GTC ACC TCT GA*
INFAM Sense4   AAG ACC AAT **TC**T GTC ACC TCT GA*
INFAM a-Sense   **C**AA AGC GTC TAC GCT GCA GTC C
INFAM a-Sense2   **T**AA AGC GTC TAC GCT GCA GTC C

INFAM probe3   *5'6 Fam* TTT GT**K** TTC ACG CTC ACC GTG CC *3'EDQ1*

* INFAM sense primer is a mixture of equal amounts of the three primer variants to a final
concentration of 30 pmol/ul. The reason is the following distribution of ambiguities at positions
10 and 11 of the primer site in the matrix genome segment:
* INFAM a-sense primer is a mixture of equal amounts of the two primer variants to a final
concentration of 30 pmol/ul. The reason is the following distribution of ambiguities at position 1
in the matrix genome segment:

** Adapted primers are summarised in the appendix below.

A(H1N1)pdm09   TCTT
A(H3N2) 2004-2016   TTCT
A(H1N1) seasonal   TCCT
A(H5N1) human viruses TCCT

NS-INF B
INFB-NS779F   GTC TTA ATG AAG GAC ATT CAA AGC C
INFB-NS886R   TAA AGT TCT TCC GTG ACC AGT CTA
INFB-848P   *5'Yak Yellow* GTC AAG AGC ACC GAT TAT CAC CAG AAG AG *3'BHQ1*

HA seasonal H3 subtype
H3-1541F2   **CR**AT GT**R** TAC AG**G** GAT GAA GC**W**TTA AAC A
H3-1600R   TAG GAT CCA ATC TTT GTA CCC TGA CTT
H3-1571P1   *5'Yak Yellow* AGC TCA ACT CCC TTG ATC TGG AA**Y** CGG *3'BHQ1*

HA human H1pdm09 (H1v) subtype
H1v-1306F   TGG ACT TAC AAT GCC GAA CT
H1v-1423R   CAG CCG TTT CCA ATT TCC TT
H1v-1357P2   *5'Texas Red* **G**GAC TA**T** CAC GAT TCA AAT GTG AAG AAC T *3'BHQ2*

HA avian H5 subtype
H5-HA-960F   ATA TgT gAA ATC AAA CAR ATT AgT CC
H5-HA-1100R   gTR gAT TCT TTg TCT gCA gCg TA
H5-HA-1050P   *5'6Fam* TAC CCA TAC CAA CCA TCT ACC ATT CCC T*3'BBQ1*

NA seasonal N2 subtype (specific for human NA)
N2-278F2   GTG **G**CA TTA CAG GAT TTG CAC
N2-376R   **Y**TG TCC AAG GGC AAA TTG ATA AC
N2-327P   *5'Yak-Yellow* GAC ATC TGG GTG ACA AGA GAA CCT TAT-3'*BHQ1*

NA human N1pdm09 (N1v) subtype
N1v-1142F1   **GA**C TGG GAC **T**GA CAA TAA **A**TT CTC AA
N1v-1244R1   **C**AG CCC TGT TAG TTC TGG ATG.C
N1v-1193P1   *5'6 Fam* AGT GGT CAG G**G**T ATA GCG GGA GT *3'BHQ1*

NA avian N1 subtype (specific for H5 HPAI NA)
N1-1069F1*   CCA AAT GGG TGG ACT GRA A
N1-1069F2*   CCA AAT GGG TGG gCT GgA a
N1-1079F*   GGA CTG RAA CGG ACA GTA
N1-1207R   AGA ARC AAG GTC TTA TGC ART CTA
N1-1150P1**   *5'Yak Yellow* GGG AGT TTT GTY CAG CAT CCA GAA *3'BHQ1*
N1-1150P2**   *5'Yak Yellow* GGG AGT TTT GTC CAG CAT CCR GAA *3'BHQ1*
* mix the N1 forward (F) primers in equimolar amounts
** mix the N1 probes (P) in equimolar amounts

2. Procedure

1.   Prepare appropriate dilutions of positive controls
2.   Isolate RNA using the High pure RNA isolation Kit or MagNApure

Mixes

Influenza A Matrix /B NS	[end-conc.]	µl
PCR grade water		
Taqman FV MM (4x)		5
INFAM Sense mix 1-3	0.75 µM	
INFAM a-Sense	0.75 µM	
INFAM probe 2 (Fam)	0.25 µM	
INFB-NS779F	0.75 µM	
INFB-NS886R	0.75 µM	
INFB-848P	0.25 µM	
Total volume		15

H1pdm09-H3 Human	[end-conc.]	µl
PCR grade water		
Taqman FV MM (4x)		5
H1-sw-1306F1	0.75 µM	
H1-sw-1423R	0.75 µM	
H1 sw-1357p2	0.25 µM	
H3-1541F1	1.5 µM	
H3-1600R	0.75 µM	
H3-1571P1	0.75 µM	
Total volume		15

H5	[end-conc]	µl
PCR grade water		
Taqman FV MM (4x)		5
H5-HA-960F	0.75 µM	
H5-HA-1100R	0.75 µM	
H5-HA-1050P	0.3 µM	
Total volume		15

N1pdm09-N2	[end-conc]	µl
PCR grade water		
Taqman FV MM (4x))		5
N1pdm09-1142F	0.75 µM	
N1pdm09-1244R	0.75 µM	
N1pdm09-1193P	0.25 µM	
N2-278F	1 µM	
N2-376R	0.63 µM	
N2-327P	0.25 µM	
Total volume		20

NA-Avian H5-HPI	[end-conc]	µl
PCR grade water		
Taqman FV MM (4x))		5
H5-NA-1069F1	0.25 µM	
H5-NA-1069F2	0.25 µM	
H5-NA-1079F	0.25 µM	
H5-NA-1207R	0.75 µM	
H5-Na-1150P1	0.15 µM	
H5-NA-1150P2	0.15 µM	
Total volume		20

Mix per reaction with RNA

PCR grade water 7 µl
Taqman FV MM (4x) 5µl
Primer/probe mix 3µl
_____+
15 µl mix volume

RNA 5 µl
_____+
20 µl final volume

LC 480 One-step PCR temperature protocol:

PCR Program	Segment number	Temerature target (°C)	Hold Time	Ramp rate (°C/sec.)	Acquisition mode
Reverse transcription	1	50	15 min.	4.4	None
Reverse transcription	2	95	2 min.	4.4	None
Amplification (cycles:50)	1	94	10 sec.	4.4	None
	2	60	30sec.	2.2	Single
Cooling	1	40	30	4.4	None

Exotic avian Influenza's H7N9 en H9

HA1 avian H7 subtype*
AI-H7N9-F1   cgt gca agC ttCctg aga gg
AI-H7N9-R2   gGc ctt ccc atc cat ttt ca
AI-H7N9-TM1   *5'6 Fam*'aac ccg cta tAg cac caa ata ggc ctc *3'BHQ1*

> *Adapted from Slomka et al. Influenza Other Respir Viruses 2009 Jul.3(4) 151-64 Nucleotides in Capital adapted*

HA2 avian H7 subtype*

H7-LH6FGGC CAG TAT TAG AAA TAA CAC CTA TGA
H7-RH4   GCC CCG AAG CTA AAC CAA AGT AT
H7-pro11   *5'6 Fam* CCG CTG CTT AGT TTG ACT GGG TCA ATC T   *3'BHQ1*

NA avian (H7) N9 subtype
N9-1210F   TCT TTC ATG GAC TAT TGG GC
N9-1334R   GAA CAC ATC GAT ACT AKA CTA TTG C
N9-1271P   *5'6 Fam*'TAC GTG GAA GAC CCA AGG ARG ATA AAG *3'BHQ1*

HA avian H9 subtype
H9-FOR   ATG GGG TTT GCT GCC
H9-REV   TTA TAT ACA RAT GTT GCA YCT G
H9-PROBE   *5'6 Fam*'TTC TGG GCC ATG TCC AAT GG *3'BHQ1*

H7-HA1	[end-conc]	µl
PCR grade water		
Taqman FV MM (4x)		5
AI-H7N9-F1	0.25 µM	
AI-H7N9-R2	0.25 µM	
AI-H7N9-TM1	0.125 µM	
Total volume		15

H7-HA2	[end-conc]	µl
PCR grade water		
Taqman FV MM (4x)		5
H7-LH6F	0.25 µM	
H7-RH4	0.25 µM	
H7-pro11	0.125 µM	
Total volume		15

PCR Program **H9**	Segment number	Temerature target (°C)	Hold Time	Ramp rate (°C/sec.)	Acquisition mode
Reverse transcription	1	50	15 min.	4.4	None
Reverse transcription	2	95	2 min.	4.4	None
Amplification (cycles:50)	1	94	10 sec.	4.4	None
	2	60	30sec.	2.2	Single
Cooling	1	40	30	4.4	None

N9	[end-conc]	µl
PCR grade water		
Taqman FV MM (4x)		5
N9-1210F	0.25 µM	
N9-1334R	0.25 µM	
N9-1271	0.125 µM	
Total volume		15

H9	[end-conc]	µl
PCR grade water		
Taqman FV MM (4x)		5
H9-FOR	0.5 µM	
H9-REV	0.5 µM	
H9 probe	0.25 µM	
Total volume		15

Appendix November 2016.
Based on *in silico analysis* of Influenza sequences from the 2015-2016 season, the following primers and probes are adapted to emerged mutations.
All other primers and probes are not changed

INFAM-sense4	AAG ACC AAT **T**CT GTC ACC T**Y**T GA
INFAM A-sense2	**T**AA AGC GTC TAC GCT GCA GTC C
INFAM-probe3	TTT GT**K** TTC ACG CTC ACC GTGCC
H3-1541F2	**C**R**AT GTR TAC AG**G** GAT GAA GC**W** TTA AAC A
N2-278F2	GTG**G**CA TTA CAG GAT TTG CAC
H1-sw-1357P2	**G**GAC TA**T** CAC GAT TCA AAT GTG AAG AAC T
N1v-1142F1	GACTG GGA C**T**G ACA ATA A**A**T TCT CAA
N1v-1244R1	**C**AGC CCT GTT AGT TCT GGA TG**C**
N1v-1193P1	AGT GGT CAG G**G**T ATA GCG GGA GT