Influenza A(H1N1) M2 detailed pyrosequencing protocol for antiviral susceptibility testing

13 May 2009

The WHO Collaborating Centre for influenza at CDC Atlanta, United States of America, has made available the M2 detailed pyrosequencing protocol, attached, for antiviral susceptibility testing of influenza A(H1N1).

This document updates and replaces the Protocol for antiviral susceptibility testing by pyrosequencing, published on 29 April 2009.

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SOP Title: Swine-H1N1 M2 detailed pyrosequencing protocol
Effective Date: May 11, 2009

Pyrosequencing of M2 gene to detect resistance to adamantanes (amantadine and rimantadine)

RT-PCR primers (20 uM):
sw-M2-F670, AGC TCC AGT GCT GGT CTG AAA G
sw-M2-R900-biot, GAC TCA GGC ACT CCT TCC GTA GAA

Sequencing primer (100 uM):
sw-M2-F747-seq, GCG ATT CAA GTG ATC C

RT-PCR protocol: using Invitrogen Superscript III One step RT-PCR with HiFi Taq system.

	Volume (ul)
2X Reaction Mix	**25**
Superscript III HiFi Taq	**1**
Forward primer(20uM)	**1**
Reverse primer(20uM)	**1**
Rnase Inhibitor	**0.5**
Nuclease free water	**16.5**
Total/sampels	**45**
Template	**5**
Final volume	**50**

PCR Cycling Parameters:

Temp	Length	
50	**30min**	
94	**2min**	
94	**15sec**	
55	**30sec**	**45 cycles**
68	**60 sec**	
68	**5min**	
4	**hold**	

Pyrosequencing protocol: using PyroGold Reagents (for 96 samples)

1- Binding Reaction

1- Binding Buffer: 4 ml
2- Dist H2O: 2 ml
3- Sepharose beads (vortex well): 300 ul
4- Mix well by vortexing
5- Dispense 60 ul of binding buffer to each well of a clean 96-RT-PCR plate (Use 12 channel)
6- Transfer 20 ul of RT-PCR product to appropriate wells containing binding buffer.
7- Cover with film tape
8- Shake plate for at least 10 min

2- Annealing Reaction:

1- Annealing buffer: 4.4 ml
2- Sequencing primer: 20 ul (sw-M2-F747-seq) at 100 uM
3- Vortex and add 40 ul annealing reaction to each well of a 96-well soft pyrosequencing plate.

3- *Dispensation order: cy*clic 60 (CATG/GT)

4- Module used: SQA (sequence analysis)

5- Sequence to be analyzed:

M2 pyrosequencing target regions of seasonal H1N1 and H3N2 compared to that of the swine origin 2009 H1N1 viruses: (nucleotide and amino acid differences compared to seasonal H1N1 viruses are shown in bold font and underlined).

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          25  26  27  28  29  30  31  32  33  34  35  36  37  38
               P   L   V   V   A   A   N   I   I   G   I   L   H   L
H3:    AC CCG CTT GTT GTT GCC GCG AAT ATC ATT GGG ATC TTG CAC TTG AT
               P   L   V   V   A   A   S   I   I   G   I   V   H   L
H1:    AT CCT CTT GTT GTT GCC GCA AGT ATA ATT GGG ATT GTG CAC CTG AT
               P   L   V   I   A   A   N   I   I   G   I   L   H   L
swH1:  AT CCT CTC GTC ATT GCA GCA AAT ATC ATT GGG ATC TTG CAC CTG AT
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Established markers of resistance to M2 blockers (amantadine and rimantadine):
L26F (CTT/C to TTT)
V27A (GTT to GCT)
A30V/T (GCG to GTG), or GCN to GTN, GCG to ACG
S31N (AGT/C to AAT/C)
G34E (GGG to GAG)
The 2009 swine-origin H1N1 viruses are adamantane resistant and carry the S31N change in M2 protein (AGT----AAT).