



## **Influenza A(H1N1) NA-H274 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 NA-H274 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 NA-H274 detailed pyrosequencing protocol  
 Effective Date: May 12, 2009

**Pyrosequencing of NA gene to detect oseltamivir resistance-conferring mutation  
 H274Y (H275Y in N1 numbering)**

*RT-PCR primers (20 uM):*

Uni-sw-N1-B-F780, GGG GAA GAT TGT YAA ATC AGT YGA

Uni-sw-N1-B-R1273-biot, CWA CCCA GAA RCA AGG YCT TAT G

*Sequencing primer (100 uM):*

Uni-sw-N1-B-F804seq, GYT GAA TGC MCC TAA TT

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

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

**PCR Cycling Parameters:**

Temp	Length	
<b>50</b>	<b>30min</b>	
<b>94</b>	<b>2min</b>	
<b>94</b>	<b>15sec</b>	<b>45 cycles</b>
<b>55</b>	<b>30sec</b>	
<b>68</b>	<b>60 sec</b>	
<b>68</b>	<b>5min</b>	
<b>4</b>	<b>hold</b>	

**Pyrosequencing protocol:** using PyroGold Reagents (for 96 samples)

*1- Binding Reaction*

- 1- Binding Buffer: 4 ml
- 2- Dist H<sub>2</sub>O: 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 (Uni-sw-N1-B-F804seq) at 100 uM
- 3- Vortex and add 40 ul annealing reaction to each well of a 96-well soft pyrosequencing plate.

*3- Dispensation order:* cyclic 20 (CATG/GT)

*4- Module used:* SQA (sequence analysis)

*5- Sequence to be analyzed:*

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ATC**AC**TATGAGGAATGCTCCTGTTATCCTGATTCTAG