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.
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.

<table>
<thead>
<tr>
<th>Volume (ul)</th>
<th>PCR Cycling Parameters:</th>
</tr>
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<tbody>
<tr>
<td>2X Reaction Mix</td>
<td>25</td>
</tr>
<tr>
<td>Superscript III HiFi Taq</td>
<td>1</td>
</tr>
<tr>
<td>Forward primer(20uM)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer(20uM)</td>
<td>1</td>
</tr>
<tr>
<td>Rnase Inhibitor</td>
<td>0.5</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>16.5</td>
</tr>
<tr>
<td>Total/sampels</td>
<td>45</td>
</tr>
<tr>
<td>Template</td>
<td>5</td>
</tr>
<tr>
<td>Final volume</td>
<td>50</td>
</tr>
</tbody>
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<tbody>
<tr>
<td></td>
<td></td>
<td>45 cycles</td>
</tr>
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</table>

Temp | Length |
---|-------|
50 | 30min |
94 | 2min |
94 | 15sec |
55 | 30sec |
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 (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:**

   274
   ATCAGTATAGGAGCTCCTGTATCTTAG