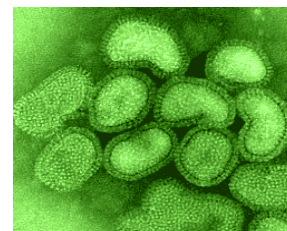


## NISN

### Panel of Influenza A Viruses for Assessment of Resistance to Neuraminidase Inhibitors



#### Panel Description:

This panel of influenza A viruses is for the evaluation of resistance to neuraminidase (NA) inhibitors and for the standardization of  $IC_{50}$  values. The package includes four human influenza A viruses isolated, plaque purified and cultured in Madin Darby canine kidney (MDCK) cells. There are two vials of each virus (each vial containing 250  $\mu$ l of virus-containing phenol red-free culture medium).

1. **A/MISSISSIPPI/3/01 (H1N1) wild-type virus (A/New Caledonia/20/99-like)** - carrying histidine at position 274 (274H) of neuraminidase glycoprotein (zanamivir and oseltamivir carboxylate susceptible).
2. **A/MISSISSIPPI/3/01 (H1N1) resistant virus (A/New Caledonia/20/99-like)** – carrying tyrosine at position 274 (274Y) of neuraminidase glycoprotein (reduced susceptibility to oseltamivir carboxylate).
3. **A/FUKUI/20/04 (H3N2) wild-type virus (A/Fujian/411/2002-like)** - carrying glutamic acid at position 119 (119E) of neuraminidase glycoprotein (zanamivir and oseltamivir carboxylate susceptible).
4. **A/FUKUI/45/04 (H3N2) resistant virus (A/Fujian/411/2002-like)** - carrying valine at position 119 (119V) of neuraminidase glycoprotein (reduced susceptibility to oseltamivir carboxylate).

#### Storage Conditions:

The influenza A viruses included in the panel were dispensed aseptically. Viruses are provided on dry ice and should be stored at  $-70^{\circ}\text{C}$  immediately upon arrival.

#### Growing the Viruses:

**Important note before proceeding:** It is strongly recommended to limit the number of passages of the influenza viruses included in the panel because some NA inhibitor-resistant viruses can be unstable following cell culture passage, resulting in a mixture of drug-resistant and drug-sensitive variants. Existence of a mixture of variants may have a direct affect on the drug susceptibility of the reference virus ( $IC_{50}$  values). Therefore, before use in a NA inhibition assay the viruses included in the panel should be cultured

**only once** in an influenza virus permissive cell line such as MDCK, aliquoted and stored at  $-70^{\circ}\text{C}$  until used.

All of the following procedures should be conducted under normal aseptic cell culture conditions:

**Note: Infection of MDCK cells with each of the four influenza A viruses must be performed separately and all appropriate safety procedures and virological requirements should be followed.**

A. For each virus, prepare two  $75\text{cm}^2$  cell culture flasks of MDCK cells to near 100% confluence.

B. Prepare 1:100 dilution of virus in sterile phosphate-buffered saline (PBS), pH 7.2-7.4. Label sterile tubes with appropriate dilutions, and add 1800  $\mu\text{l}$  PBS to the first tube (1:10 dilution) and 9000  $\mu\text{l}$  PBS to the second tube (1:100 dilution). Take 200  $\mu\text{l}$  of the virus and add it to the first tube to give 1:10 dilution, mix thoroughly. Take 1000  $\mu\text{l}$  of the 1:10 dilution and add it to the second tube to give 1:100 dilution, mix thoroughly. The remaining virus stock should be kept and stored at  $-70^{\circ}\text{C}$ .

C. Wash the MDCK cell monolayer twice with sterile PBS, and then inoculate the 5 ml of diluted virus (1:100 dilution) onto each of the two  $75\text{cm}^2$  cell culture flasks of MDCK cells.

D. Incubate the  $75\text{cm}^2$  flasks for 30 minutes at  $35^{\circ}\text{C}$  for virus adsorption. Remove the virus inoculum and add 20 ml per flask of an appropriate maintenance medium with TPCK-trypsin and then incubate at  $35^{\circ}\text{C}$  for 3 days (up to 5 days of incubation is possible). Observe the cell monolayer daily for cytopathic effect. Virus growth should be confirmed by haemagglutination assay or another assay.

## **Storage Conditions of Influenza A Viruses for Use in NA Inhibition Assays:**

To avoid repeated freeze-thaw steps it is recommended that influenza viruses for use in NA inhibition assays should be stored in cryogenic tubes at  $-70^{\circ}\text{C}$  in the following way:

From the 40 ml of cell culture supernatant harvested, the recommended volumes for the storage of each virus is:

- 20 x 100ul (100ul of virus should be sufficient for a single assay)
- 8 x 1000ul (For long term storage)
- 15 x 2000ul (For long term storage)

As needed, one of the medium sized tubes (1000ul or 2000ul) can be thawed and refrozen into volumes enough for use in only a single assay (for example 100  $\mu\text{l}$ ).

## Determination of IC<sub>50</sub> Values:

The Panel of Influenza A Viruses for Assessment of Resistance to Neuraminidase Inhibitors have been grown and tested by four different laboratories. The protocols for NA enzyme inhibition assays can be provided from NISN upon request. The drug concentration that inhibited 50% of the NA enzymatic activity (IC<sub>50</sub>) was determined from the dose-response curve. IC<sub>50</sub> values can be determined using various software programs such as GraphPad Prism® 4, GraphPad, San Diego, CA. IC<sub>50</sub> values determined by the four laboratories using both a chemiluminescence- and a fluorescence-based NA enzyme inhibition assays are listed in Table 1.

**Table 1. Evaluation of the panel of influenza A viruses in NA enzyme inhibition assays.**

Influenza A virus	Subtype	NA mutation	NA enzyme inhibition assay			
			Fluorescence-based assay <sup>a</sup> (IC <sub>50</sub> , nM)		Chemiluminescence-based assay <sup>b</sup> (IC <sub>50</sub> , nM)	
			Oseltamivir carboxylate	Zanamivir	Oseltamivir carboxylate	Zanamivir
A/Mississippi/3/01	H1N1	--	0.6-3.0	0.9-1.4	0.7 ± 0.2	0.4 ± 0.03
A/Mississippi/3/01	H1N1	274Y	325-413	0.7-1.8	148.9 ± 47.0	1.7 ± 0.2
A/Fukui/20/04	H3N2	--	0.2-0.8	1.1-2.8	0.4 ± 0.1	2.5 ± 0.5
A/Fukui/45/04	H3N2	119V	48-180	1.8-3.8	10.3 ± 1.2	1.9 ± 0.2

<sup>a</sup> IC<sub>50</sub> values presented as the range of values determined by three different laboratories.

<sup>b</sup> IC<sub>50</sub> values presented as the mean ± 1 standard deviation determined by multiple assays from one laboratory

### Use Restrictions:

The Panel of Influenza A Viruses for Assessment of Resistance to Neuraminidase Inhibitors are being provided by the Neuraminidase Inhibitor Susceptibility Network (NISN) and are distributed for internal research, non-commercial purposes only. The viruses are provided without material transfer agreement, however, NISN require that the viruses, their products or its derivatives may not be distributed to third parties. All requests for these viruses must be addressed directly to NISN. NISN should be acknowledged as the source of these viruses

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