

# Panel of Influenza A and B viruses for Assessment of Neuraminidase Inhibitor Susceptibility

## Panel Description:

This panel of influenza A and B viruses is for the evaluation of susceptibility to neuraminidase (NA) inhibitors and for the standardization of IC<sub>50</sub> values. The package includes eight human influenza A and B viruses isolated, plaque purified and cultured in Madin Darby canine kidney (MDCK) cells. There are two vials of each virus (each vial containing 250 µl of virus).

1. A/MISSISSIPPI/3/2001 wild-type virus (former seasonal H1N1; A/New Caledonia/20/99-like) - carrying histidine at position 275 (275H) of the neuraminidase glycoprotein.
2. A/MISSISSIPPI/3/2001 variant virus (former seasonal H1N1; A/New Caledonia/20/99-like) – carrying tyrosine at position 275 (275Y) of the neuraminidase glycoprotein – i.e. a H275Y substitution.
3. A/FUKUI/20/2004 wild-type virus (H3N2; A/Fujian/411/2002-like) - carrying glutamic acid at position 119 (119E) of the neuraminidase glycoprotein.
4. A/FUKUI/45/2004 variant virus (H3N2; A/Fujian/411/2002-like) - carrying valine at position 119 (119V) of the neuraminidase glycoprotein – i.e. an E119V substitution.
5. B/PERTH/211/2001 wild-type virus (B/Sichuan/379/99-like) – carrying aspartic acid at position 197 (197D) of the neuraminidase glycoprotein.
6. B/PERTH/211/2001 variant virus (B/Sichuan/379/99-like) – carrying glutamic acid at position 197 (197E) of the neuraminidase glycoprotein – i.e. a D197E substitution.
7. A/PERTH/265/2009 wild-type virus (H1N1pdm09; A/California/7/2009-like) - carrying histidine at position 275 (275H) of the neuraminidase glycoprotein.
8. A/PERTH/261/2009 variant virus (H1N1pdm09; A/California/7/2009-like) - carrying tyrosine at position 275 (275Y) of the neuraminidase glycoprotein – i.e. a H275Y substitution.

The susceptibility of these viruses to oseltamivir carboxylate, zanamivir, peramivir and laninamivir is detailed in Table 1.

# Storage Conditions:

The influenza A and B 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: Before use in a NA inhibition assay the viruses included in the panel need to be cultured in an influenza virus permissive cell line such as MDCK, aliquoted and stored at  $-70^{\circ}\text{C}$  until used. It is strongly recommended to limit the number of passages of the influenza viruses included in the panel to only two passages in MDCK cells because some NA inhibitor-resistant viruses can be unstable during cell culture passage, resulting in a mixture of drug-resistant and drug-susceptible variants. Existence of a mixture of variants may have a direct effect on the apparent drug susceptibility of the reference virus (decreased  $\text{IC}_{50}$  values, possibly associated with a decreased slope in the dose-response curve generated from the assay results). Ideally, after every passage the NA genes of viruses should be sequenced to assess maintenance of the decreased susceptibility/resistance markers in a homogeneous state and check for any additional mutations that might encode amino acid substitutions elsewhere in the NA.

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

Note: Infection of MDCK cells with each of the eight influenza A and B 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 (for 1:10 dilution) and  $9000\ \mu\text{l}$  PBS to the second tube (for 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  $-70^{\circ}\text{C}$  for any future culture that is necessary.
- C. Wash the MDCK cell monolayers twice with sterile PBS, and then inoculate each of two  $75\text{cm}^2$  cell culture flasks with 5 ml of diluted virus (1:100 dilution).
- D. Incubate the  $75\text{cm}^2$  flasks for 30 minutes at  $35^{\circ}\text{C}$  to allow virus adsorption. Remove the virus inoculum and add 20 ml per flask of an appropriate maintenance medium with TPCK-trypsin ( $1\ \mu\text{g}/\text{ml}$  final concentration) 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 and B 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°C in the following way:

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

20 x 100 µl (100 µl of virus should be sufficient for a single assay)

8 x 1000 µl (For long term storage)

15 x 2000 µl (For long term storage)

As needed, one of the medium sized tubes (1000 µl or 2000 µl) can be thawed and refrozen into volumes sufficient for use in only a single assay (for example 100 µl).

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

The Panel of Influenza A and B Viruses for Assessment of Neuraminidase Inhibitor Susceptibility have been grown and tested by seven different laboratories. The protocols for various NA enzyme inhibition assays are available from the isirv Antiviral Group (AVG) website ([www.isirv.org/avg](http://www.isirv.org/avg)). 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, or GraphPad, San Diego, CA. IC<sub>50</sub> values determined by the seven laboratories using a range of different NA enzyme inhibition assays are listed in Table 1. Four NA inhibitors zanamivir, oseltamivir carboxylate, peramivir and laninamivir were tested.

Note: Considerable variation in IC<sub>50</sub> values generated for individual viruses can occur between laboratories largely due to the use of different NA inhibition assay protocols.

## Use Restrictions:

The Panel of Influenza A and B Viruses for Assessment of Resistance to Neuraminidase Inhibitors is being provided by the isirv-AVG using viruses provided to the WHO Collaborating Centres for Reference and Research on Influenza as part of the WHO Global Influenza Surveillance and Response System.

The Panel is distributed for laboratory research, non-commercial purposes only. isirv-AVG cannot guarantee the suitability of the Panel for any other purpose and takes no responsibility for results obtained through use other than that described in the enclosed instructions. isirv-AVG also cannot guarantee against the loss of activity whilst in transit or subsequent storage.

The viruses are provided free of charge and without a material transfer agreement. However, isirv-AVG stipulates that the viruses, their products or any derivatives may not be distributed to third parties. All requests for these viruses must be addressed directly to isirv- AVG. ([avg@isirv.org](mailto:avg@isirv.org)) isirv-AVG should be acknowledged as the source of these viruses.

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

**Fluorescence-based assays<sup>a</sup>**

Strain designation	(Sub)type	Genotype <sup>c</sup>	Oseltamivir carboxylate IC <sub>50</sub>		Zanamivir IC <sub>50</sub>		Peramivir IC <sub>50</sub>		Laninamivir IC <sub>50</sub>	
			Range	Median	Range	Median	Range	Median	Range	Median
B/Perth/211/2001	B	WT	8 - 128	13.1	0.5 – 12	1.3	0.3 – 0.8	0.3	1 – 2	NA
B/Perth/211/2001	B	D197E	79 - 966	182.6	3 – 290	7.1	6 – 58	29.0	4 – 6	NA
A/Fukui/20/2004	A(H3N2)	WT	0.1 – 5	0.2	0.5 - 3	1.1	0.2 – 0.3	0.2	0.6 – 1	NA
A/Fukui/45/2004	A(H3N2)	E119V	23 – 378	42.3	0.5 - 3	1.0	0.2 – 0.3	0.3	0.7 – 1	NA
A/Mississippi/03/2001	A(H1N1)	WT	0.4 – 10	0.5	0.3 - 1	0.4	0.1	0.1	0.1 – 0.2	NA
A/Mississippi/03/2001	A(H1N1)	H275Y	257 – 3455	458.2	0.3 - 2	0.4	40-47	45.8	0.3 – 0.3	NA
A/Perth/265/2009	A(H1N1pdm09)	WT	0.2 – 10	0.6	0.2 - 1	0.2	0.1	0.1	0.1 - 0.2	NA
A/Perth/265/2009	A(H1N1pdm09)	H275Y	132 - 2179	191.3	0.2 - 2	0.4	23 - 32	26.1	0.2 – 0.3	NA

**Chemiluminescence-based assays<sup>b</sup>**

Strain designation	(Sub)type	Genotype <sup>c</sup>	Oseltamivir carboxylate IC <sub>50</sub>		Zanamivir IC <sub>50</sub>		Peramivir IC <sub>50</sub>		Laninamivir IC <sub>50</sub>	
			Range	Median	Range	Median	Range	Median	Range	Median
B/Perth/211/2001	B	WT	2 - 4	2.1	1.0 – 1.3	1.2	0.3 – 0.4	0.4	2 – 4	2.3
B/Perth/211/2001	B	D197E	10 - 30	22.4	2 – 11	4.2	0.9 – 2.8	2.2	2.3 – 7.9	4.1
A/Fukui/20/2004	A(H3N2)	WT	0.1 – 0.2	0.2	0.9 – 1.3	1.1	0.2 – 0.3	0.3	1 – 2	1.6
A/Fukui/45/2004	A(H3N2)	E119V	3 – 5	4.3	0.9 – 1.1	1.1	0.2 – 0.3	0.3	1 – 2	1.3
A/Mississippi/03/2001	A(H1N1)	WT	0.2 – 0.4	0.2	0.2 – 0.3	0.3	0.2 – 0.3	0.3	0.2 – 0.3	0.2
A/Mississippi/03/2001	A(H1N1)	H275Y	104 – 127	120.6	0.4 – 0.4	0.4	12-15	14.1	0.5 – 0.7	0.6
A/Perth/265/2009	A(H1N1pdm09)	WT	0.2 – 0.3	0.3	0.3 – 0.3	0.3	0.1 – 0.1	0.1	0.2 - 0.4	0.3
A/Perth/265/2009	A(H1N1pdm09)	H275Y	71 - 107	101.7	0.3 – 0.4	0.3	9 - 13	10.7	0.4 – 0.8	0.5

<sup>a</sup> Range and median IC<sub>50</sub> values based on results determined by seven different laboratories, except for peramivir data which is based on data from three laboratories and the laninamivir data based on two laboratories. Laboratories used a range of different fluorescence-based protocols.

<sup>b</sup> Range and median IC<sub>50</sub> values based on results determined by two different laboratories, using NA-Star or NA XTD chemiluminescence-based assays supplied by Life Technologies.

<sup>c</sup> Amino acid substitution position in the NA: D197E corresponds to D198E, and H275Y corresponds to H274Y, in N2 NA numbering. WT: wild-type.