WHO COLLABORATING CENTRE FOR REFERENCE & RESEARCH ON INFLUENZA AUSTRALIA	
Standard Operating Procedure **WHO – 025**	Page 1 of 10
Fluorometric Neuraminidase Inhibition Assay	
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A	Original	17/8/2003	Aeron Hurt
B	Minor procedural changes	13/3/2007	Aeron Hurt

1. INTRODUCTION

1.1 Purpose

To determine the sensitivity of influenza virus isolates to neuraminidase inhibitor drugs.

1.2 Principles of the Procedure

Replication of influenza viruses relies on initial attachment to cell receptors via the viral haemagglutinin (HA) and subsequent escape of newly synthesised viruses from the infected cell which is mediated by the enzymic action of the viral neuraminidase (NA). Neuraminidase inhibitor (NI) drugs, such as Zanamivir (Relenza) and Oseltamivir (Tamiflu), act by binding to the highly conserved active enzymatic site of the NA and inhibiting this key function thereby preventing release and spread of the virus. By measuring the inhibitory effect on neuraminidase enzyme activity it is possible to determine how susceptible or resistant a virus is to particular NI drugs. A fluorescence based assay which measures the fluorogenic end product 4-methylumbelliferone released from the substrate 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) by the enzymatic activity of influenza virus neuraminidase is a simple and reliable method for monitoring inhibitory effects of NI drugs. By assaying the uninhibited NA activity of a virus in comparison to the enzymatic activity following incubation with a range of NI drug concentrations, it is possible to determine the drug concentration required to reduce NA activity by 50% as the IC_{50} value. Clinically sensitive strains have been found to have an IC_{50} range of 0.001 – 15nM for zanamivir and 0.001 – 25nM for oseltamivir carboxylate. Clinically resistant strains have been reported for both inhibitors in the IC_{50} range from 43nM to as high as 8020nM.

For convenience each virus to be tested is diluted 1/10 prior to performing the inhibition assay. However some viruses possessing a high NA activity may show an exaggerated IC_{50} value and will appear on initial analysis as being resistant. To further investigate any viruses that appear resistant, serial dilutions should be made to determine the dilution level at which there is a linear relationship between NA activity and MUNANA concentration. An inhibition assay should then be repeated at this pre-determined virus concentration. This pre-dilution step is only required on viruses that sit outside of the normal IC_{50} range.

2. TRAINING

Training in the use of pipettes and preparing dilutions is required for this assay. Additionally, basic training in the use of Microsoft Excel software is also required for the analysis of data.

3. MATERIALS

3.1 Hardware

Nunc 96 well Black FluoroNunc plates, polystyrene, untreated (Cat No. 237105)
Sarstedt 96 well 'U'-bottom microtest plates, polystyrene (Cat No. 82 9923 154)
Corning Tube filter, pore size 0.22μm, cellulose acetate (Cat No. 430320)
Single and Multi-channel Pipettes ranging from 1μL to 1000μL
Greiner microrack 1.2mL tubes (Cat No. 102201)
Incubator (37°C)
Labsystems Fluoroskan II fluorometer

3.2 Reagents

2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) (Sigma Cat No. M8639)
2-[N-morpholino]ethanesulphonic acid (MES) (free acid) (Sigma Cat No. M8250)
1M Calcium Chloride ($Ca\ Cl_2$) (APS AJAX Fineham Cat No. 127-500G)
Absolute Ethanol (APS AJAX Fineham Cat No. 214-2.5L GL)
Surfactant-Amps-NP-40 (10% solution) (Pierce Cat No. 28324)
Bovine Serum Albumin (Fraction V, IgG free, low endotoxin) (Sigma Cat No. A2058)
0.824M Sodium Hydroxide (NaOH(aq)) (APS AJAX Fineham Cat No. 482-2.5KG)
Neuraminidase Inhibitors:

- Zanamivir (Relenza), purchased through pharmacies.
- Oseltamivir carboxylate (GS 4071), the active form of the ethyl ester prodrug oseltamivir phosphate, supplied by Hoffmann-La Roche

4. SAFETY

NA inhibition assays may be carried out on the bench only after treatment of the samples to inactivate viruses (see 5.1.5). If this is not done then all procedures should be carried out in a biological safety cabinet (Class II).

5. GENERAL PROCEDURE

5.1 Preparation of Reagents

5.1.1 Preparation of 1X and 2X strength Assay Buffer

2X Assay Buffer (2XAB)

13g MES
8mL 1M $Ca\ Cl_2$
992mL Distilled Water

Mix components to homogeneity then add 10M sodium hydroxide to adjust to a pH of 6.5. Then filter the buffer using a sterile cellulose acetate filter of pore size 0.2μm.

1X Assay Buffer (1XAB)

Prepare by making a 1:1 v/v dilution of 2XAB in distilled water.

1X and 2X AB can be stored at room temperature for a period of two years.

5.1.2 Preparation of Inhibitors

Prepare NI drugs at concentrations of 0.03, 0.3, 3, 30, 300, 3000 and 30000nM in 2XAB. These concentrations correspond to 0.01, 0.1, 1, 10, 100, 1000 and 10000nM in the final assay volume. Solutions should be clearly labelled indicating the type of inhibitor, inhibitor concentration, dilutent batch and the date prepared.

Zanamivir
Each blister of Relenza contains 5mg of powdered Zanamivir (MW=332.32) plus 20 mg of lactose excipient. Add the contents of one blister to 50.13ml of 2XAB to give a 300μM solution. From this stock solution make serial 1:10 dilutions to achieve the concentrations stated above.

Oseltamivir (GS 4071)
Make 300μM solution of compound GS 4071 (MW=284.36) by adding 4.266mg to 50ml of 2XAB. From this stock solution make serial 1:10 dilutions to achieve the concentrations stated above.

Store all inhibitor dilutions at 2-8°C.

Stock solutions must be replaced after 12 months and dilutions prepared from the stock after 6 months.

5.1.3 Preparation of MUNANA substrate stock and working solution

Prepare 2.5mM MUNANA stock by addition of 20mL distilled water to 25mg MUNANA and store in 800μL aliquots at -20°C. Working strength substrate (0.3mM) is prepared by addition of 720μL 2.5mM MUNANA to 5.28mL 1XAB which is sufficient volume for one plate. Keep working strength MUNANA on ice unless used immediately, any left over material should be discarded.

5.1.4 Preparation of Stop Solution

Prepare by adding 2.225mL 0.824M NaOH to 11.0mL absolute ethanol (1 plate equivalent).

Stop solution must be prepared fresh for each experiment due to precipitation on storage.

5.1.5 Preparation of virus samples

Virus dilutions are most conveniently made in Greiner microrack 1.2mL tubes, which allows the viruses to be arranged in a 96 well plate format and therefore accessible by a 12-tip multi-channel pipette.

Standard 1/10 dilutions for assay are prepared in 1xAB containing NP-40 (0.1% v/v) in a final volume of 500µL.

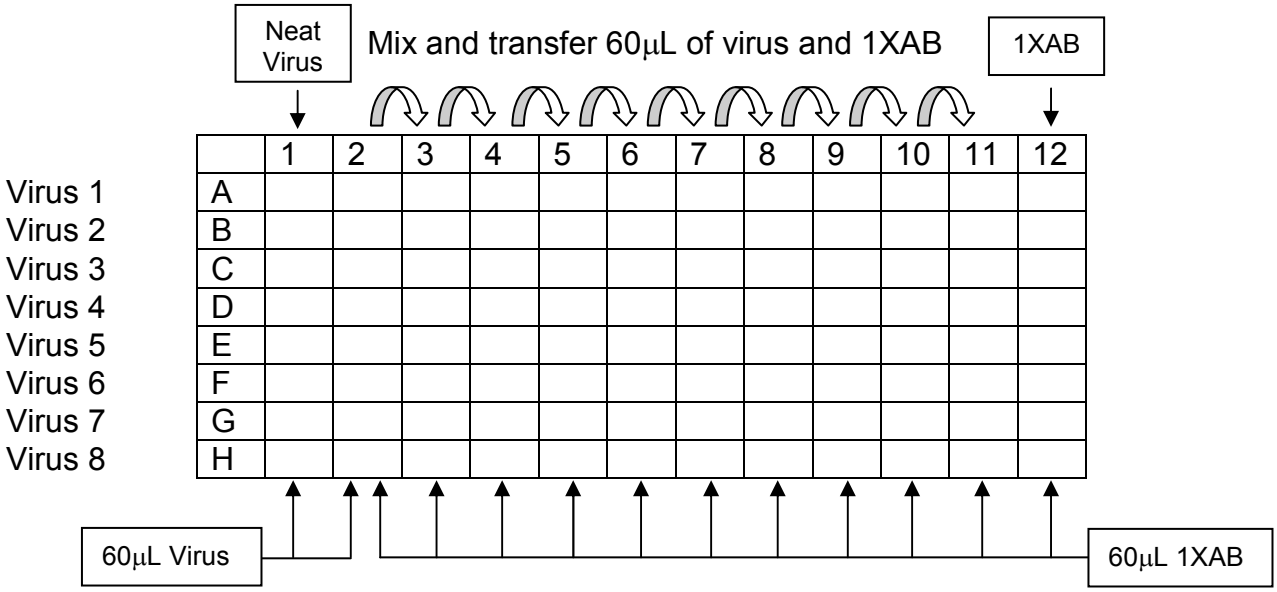
To each tube add, in order:
50µL of virus (cell culture supernatant or clinical sample)
5µL 10% NP-40
445µL 1xAB

When dealing with a large number of viruses it is more convenient to prepare a diluent master mix containing 1XAB and NP-40 (0.1% v/v) and then to add 450µL of diluent master mix to 50µL of each virus. Store virus preparations at 4°C unless used immediately.

5.2 Neuraminidase Activity Assay Procedure

Note: This procedure is used to determine the appropriate virus dilution for use in the NA inhibition assay, it is only necessary if a virus has previously been found to have an IC_{50} value outside of the normal range (mean ± standard deviation) using the standard 1/10 dilution. The form 'NA Inhibition assays' on the WHO database automatically calculates the mean and standard deviation of IC_{50} values. Otherwise proceed to section 5.3 using the standard virus dilution.

Add 60µL of neat virus to rows A-H in columns 1-2 of a U-bottom 96 well plate. Add 60µL of 1XAB (containing NP-40 as prepared above) to rows A-H columns 2-12. Using an electronic multi-tip pipette mix the viruses and 1XAB in column 2 and transfer 60µL to column 3, mix and transfer 60µL to column 4. Continue making 1 in 2 dilutions of each virus until column 11 (ensuring 60µL is discarded from this column), and then leave column 12 containing 1XAB only. The following figure illustrates the plate set up for this procedure.



After preparing the virus dilutions in a U-bottom plate transfer 50µL to the corresponding wells of a 96 well black FluoroNunc plate and then add 50µL of working strength MUNANA substrate to each well using a multichannel pipette. Tap the plate by hand to mix and then incubate for 1 hour at 37°C.

Following the incubation period, the reaction is stopped by the addition of 100µL per well of stop solution.

The plate can then be read on the fluorometer using the following parameters.

	Wavelength	**Slit Width**
Excitation	360 nm	2.5 nm
Emission	448 nm	20 nm

Microsoft Excel should be used for the processing of results.

Adjust the fluorescence readings of the plate by averaging the 1XAB values and subtracting from each virus fluorescence value. Then draw a graph plotting virus dilution against fluorescence units. From the sigmoidal curve produced for each virus it is possible to select the dilution that corresponds to the mid-point of the linear section of the curve. At this virus dilution a linear relationship between NA activity and MUNANA substrate exists, and this is the appropriate dilution for use in the following NA inhibition assay.

5.3 Neuraminidase Inhibition Assay Procedure

Add 50μL of NI drug at dilutions ranging from 0 (1XAB only) to 30,000nM to rows A to H respectively in a 96 well black FluoroNunc plate. Following the addition of the inhibitor dilutions, add 50μL per well of control and test viruses (diluted and NP-40 treated) to columns 1-11. Add 50μL of 1XAB per well to column 12 for blank readings in the absence of virus. See plate plan below. Tap the plate by hand to mix. Cover the plate and incubate at room temperature for 45 minutes.

The following figure illustrates the plate set up for this procedure.

		1	2	3	4	5	6	7	8	9	10	11	12
0 (1XAB)	A												
0.03 nM	B												
0.3 nM	C												
3 nM	D												
30 nM	E												
300 nM	F												
3,000 nM	G												
30,000nM	H												
		Control virus 1	Control virus 2	Control virus 3	Test Virus 1	Test Virus 2	Test Virus 3	Test Virus 4	Test Virus 5	Test Virus 6	Test Virus 7	Test Virus 8	No Virus 1XAB

Control Virus 1 = wba (a wildtype strain with a 'normal' IC_{50} value)
Control Virus 2 = xw (a lab derived resistant strain with a E119G mutation and a 'high' zanamivir IC_{50} value)
Control Virus 3 = yn (a lab derived resistant strain with a R292K mutation and a 'high' oseltamivir carboxylate IC_{50} value)

Note: If testing a large number of viruses it is not necessary to include control viruses on every plate, however a 'no virus' (1XAB) negative control must be included on each plate.

Following incubation add 50μL of working strength MUNANA substrate to each well using a multichannel pipette. Tap the plate by hand to mix and then incubate for 1 hour at 37°C.

Following this incubation period, the reaction is stopped by the addition of 100μL per well of stop solution.

The plate should then be read on the fluorometer using the following parameters. The reaction is stable for storage overnight at 2-8°C if nessecary.

	Wavelength	Slit Width
Excitation	360 nm	2.5 nm
Emission	448 nm	20 nm

Data is saved as a .xls file and then can be used for further analysis.

5.4 Analysis of results

Microsoft Excel is used for processing the results.

Open the 'NA Inhibition assay' Excel template located in the templates folder of all serology laboratory PC's by selecting <File>, <New>, <NA Inhibition assay>. Next open the .xls file from the floppy disc and copy and paste the data into the 'NA Inhibition assay' template. Save the template now using the file name 'NA inhibition' followed by the date of the assay (eg. NA inhibition 31.6.01) in the folder M:\WHOFLU\Serology\NA Inhibition Assays. Once the raw fluorometric data is pasted into the template, background fluorescence is subtracted automatically from the counts of each well and % uninhibited activity is determined automatically by calculating results from inhibited wells as a percentage of the uninhibited fluorescence.

Results are then automatically plotted as % uninhibited activity versus inhibitor concentration (nM) on a semi-log plot. Inhibitor concentration is expressed as 0 to 10,000nM (rather than 0 to 30,000nM) as this is the inhibitor concentration in the final assay volume.

A logistic curve fit program written by Dr. T. Rae (Supplied Hoffmann-La Roche) has been loaded into the 'Add-Ins' section of Microsoft Excel on all serology laboratory PC's and is used to produce a curve of best fit for each sample and calculate an IC_{50} value for each curve. Appendix 1 describes how to use the logistic curve fit program.

6. DOCUMENTATION

Ensure the Excel template containing the data, % uninhibited activity, and IC_{50} values for each virus is saved. IC_{50} values for each virus against each inhibitor should be recorded within the WHO database in the form 'NA Inhibition assays'. The form 'NA Inhibition assays' also automatically calculates the maximum, minimum, mean and standard deviation of IC_{50} values (based on all the data) for each subtype against each of the two NA inhibitors Zanamivir and GS 4071.

IC_{50} values that fall outside the normal distribution (Mean $\pm$ Standard Deviation) as calculated in the form 'NA Inhibition assays' should be noted and re-assayed after titrating the NA activity (see section 5.2).

7. APPENDICES

APPENDIX 1

USE OF THE LOGISTIC CURVE FIT PROGRAM IN MICROSOFT EXCEL

The following are instructions to determine 'curves of best fit' and IC_{50} values of viruses from an excel chart using a logistic curve fit program written by Dr. T. Rae (IS, Roche).

1) Right hand click mouse on grey area of graph (active graph area)

2) Select 'Curve Fit'.

3) Choose Logit (Dose Response). (Default = spline). Click OK.

4) In the text box, click on the curve to be fitted - this will highlight the curve name.

5) Click 'Guess' then 'Fit'.

6) Click OK to 'solution found'. The Lgt curve fit should now be drawn on the graph and the Lgt graph will appear in the text box.

7) Highlight the Lgt curve in the text box.

8) Place a tick in 'paste at', click in the box below 'paste at' then click on the cell in the spreadsheet where the IC50 data is to be pasted eg. cell B39.

9) Click 'Solve', then OK for solution found. The IC50 will be automatically pasted into the spreadsheet.

10) Repeat procedure for other curves.

APPENDIX 2

EXAMPLE OF NEURAMINIDASE INHIBITION DATA SET USING EXCEL TEMPLATE

Raw Data

[Zanamivir] nM	wba	xw 2/3	yn1	Virus 1	Virus 2	Virus 3	Virus 4	Virus 5	Virus 6	Virus 7	Virus 8	No Virus
0	4063	1460	900.7	4415	5019	5351	4623	4729	2502	2431	4238	108.1
0.1	3258	1490	926.5	3778	4447	4444	4106	4193	2055	1897	3617	120.9
1	3102	1501	923.2	3690	4304	4301	3400	3965	1955	1766	3569	113.2
10	2579	1494	947.8	2954	3849	3786	2803	3335	1623	1418	3023	113.6
100	902.3	1293	698.1	686.4	802	786	692	694.6	721	355.4	731.5	108.2
1000	228.1	1019	330.8	256	276.8	296.3	299.3	257.3	239.5	157.1	229.3	115.3
10000	127.4	379.4	145.4	152.9	145.5	143.2	136.2	148.9	128.1	116.6	129.9	116.3
100000	119.7	151.8	118.6	132.5	125.3	122.8	123.5	128.8	113.2	111.5	113.1	111.6

Average Background	113.4

Manipulated Data - Background Subtracted

[Zanamivir] nM	wba	xw 2/3	yn1	Virus 1	Virus 2	Virus 3	Virus 4	Virus 5	Virus 6	Virus 7	Virus 8
0	3949.6	1346.6	787.3	4301.6	4905.6	5237.6	4509.6	4615.6	2388.6	2317.6	4124.6
0.01	3144.6	1376.6	813.1	3664.6	4333.6	4330.6	3992.6	4079.6	1941.6	1783.6	3503.6
0.1	2988.6	1387.6	809.8	3576.6	4190.6	4187.6	3286.6	3851.6	1841.6	1652.6	3455.6
1	2465.6	1380.6	834.4	2840.6	3735.6	3672.6	2689.6	3221.6	1509.6	1304.6	2909.6
10	788.9	1179.6	584.7	573	688.6	672.6	578.6	581.2	607.6	242	618.1
100	114.7	905.6	217.4	142.6	163.4	182.9	185.9	143.9	126.1	43.7	115.9
1000	14	266	32	39.5	32.1	29.8	22.8	35.5	14.7	3.2	16.5
10000	6.3	38.4	5.2	19.1	11.9	9.4	10.1	15.4	-0.2	-1.9	-0.3

% Uninhibited Activity

[Zanamivir] nM	wba	xw 2/3	yn1	Virus 1	Virus 2	Virus 3	Virus 4	Virus 5	Virus 6	Virus 7	Virus 8
0.01	80	100	100	85	88	83	89	88	81	77	85
0.1	76	100	100	83	85	80	73	83	77	71	84
1	62	100	100	66	76	70	60	70	63	56	71
10	20	88	74	13	14	13	13	13	25	10	15
100	3	67	28	3	3	3	4	3	5	2	3
1000	0	20	4	1	1	1	1	1	1	0	0
10000	0	3	1	0	0	0	0	0	0	0	0

IC 50	1.572	175.684	24.995	1.818	2.704	15.124	14.108	2.084	1.942	0.944	2.221