Protocol for fluorescence-based neuraminidase assay and neuraminidase inhibition assay
(St. Jude Children's Research Hospital)

Buffers and solutions:

0.1 M MES (pH=6.5)
2.1 g MES *(Sigma, M5287-250G)*
100 ml H2O
Check pH (adjust with conc. NaOH)
MES hydrate > 99.5% titration, $C_6H_{13}NO_4SxH_2O$, FW 195.2 (Anh)

325 mM MES (pH=6.5)
6.9 g MES
100ml H2O
Check pH (adjust with conc. NaOH)

0.1 M CaCl2
1.47 g $CaCl_2x2H_20$
100 ml H20

10 mM CaCl2
0.15g $CaCl_2x2H_20$
100 ml H2O

Enzyme buffer
32.5 ml 0.1 M MES (pH=6.5)
4.0 ml 0.1 M $CaCl_2$
63.5 ml H_20
Store at +4ºC

Standard
4 mM Stock standard solution (stable for ~ 1 month at 4ºC, protect from light)
7 mg standard (4-Methyl-Umbelliferone) (*Sigma; Cat= M-1381*)
5 ml absolute ethanol
5 ml 0.9 % NaCl (wt/v)
40 µM Working standard solution (prepare right before experiments)
10 µl stock standard solution
990 µl 0.9 % NaCl (wt/v)

Stop solution (pH=10.7)
100 ml ethanol
250 ml H_2O
Mixed water and ethanol first and then add Glycin
3.004 g Glycin (*Fisher; G46-500*)
Check pH (adjust with conc. NaOH)
Add water to 400 mls

Preparation of substrate stock (5 mM solution):

Substrate: 2’-(4-Methylumbelliferyi)-α-D-N-acetylneuraminic acid, sodium salt
Sigma-Aldrich M-8639, MW 489.4

1. Prepare water-saturated methyl-tert butyl ether (*Methyl-tert butyl ether, HPLC grade; e.g., Fisher E127*) by shaking 20 ml ether with 10 ml distilled H_2O in glass-stoppered glass Erlenmeyer (KILMAX #22, 125 ml volume, Fisher Scientific). Remove (lower) water phase after separation of water and ether layers and shake again with another 10 ml dH_2O. Remove this water as well. All procedures must be done with glass pipets or pateur glass pipets.

2. Add 2.25 ml distilled H_2O to a 5 mg vial of substrate [2’-(4-Methylumbelliferyi)-α-D-N-acetylneuraminic acid, sodium salt]. It is better to order the substrate that is aluquited by 5 mg per vial/bottle; in this case it is possible to add distilled H_2O directly to the vial/bottle. The powder dissolves quickly. Transfer solution to a 15 ml polypropylene snap-cap tube or a glass tube.

3. Add 5 ml water-saturated methyl-tert butyl ether to substrate solution. Shake vigorously. Remove (upper) ether phase by paster pipet. Repeat this twice. In total, three extractions.

4. Put substrate solution on ice. To remove the remaining ether, nitrogen gas is bubbled through the substrate solution for ±2 hours, until the solution has lost almost all ether smell.

5. Aliquot substrate into 100 or 50 µl per tube and store at -20°C.

Substrate mixture for NA assay

Prepare the mixture just before the experiment and leave on ice
Concentration of substrate mixture – 167 µM, after adding to the plate – the final concentration of substrate will be 100 µM.

Buffers/Substrate	½ plate	1 plate	2 plate	3 plate
1 Volume 325 mM MES	250 µl	500 µl	1000 µl	1500 µl
3 volume 10 mM $CaCl_2$	750 µl	1500 µl	3000 µl	4500 µl
Substrate	50 µl of 5 mM MUN (from -20C) 450 µl H2O	100 µl of 5 mM MUN (from -20C) 900 µl H20	200 µl of 5 mM MUN (from -20C) 1800 µl H20	300 µl of 5 mM MUN (from -20C) 2700 µl H20

Preparation of the stocks of NA inhibitors
(Compounds to be used in NI assays)

Molecular weight:

Oseltamivir carboxylate, tartrate salt:	386.4 g/mol
Zanamivir monohydrate:	350.3 g/mol

Note: It is important to request the compounds from an appropriate source and ask about Product information. The composition and purity varies.

Steps:

1. In our experiments we are using the stock of NA inhibitor at concentration **2mM.**

   For oseltamivir carboxylate (active ingredient of oseltamivir):
   - Weight 0.01544 gram oseltamivir carboxylate and dissolve in 20 mL of sterile ddH_2O.
   - Explanations:

     1M (1000mM) – 386 gram – 1L (1000 mL)
     2 mM - X gram – 1L (1000 mL)
     X= 0.772 gram per 1L (1000 mL) or 0.015444 gram per 20 mL

   For zanamivir:
   - Weight 0.014 gram zanamivir and dissolve in 20 mL of sterile ddH_2O.
   - Explanations:

     1M (1000mM) – 350 gram – 1L (1000 mL)
     2 mM - X gram – 1L (1000 mL)
     X= 0.70 gram per 1L (1000 mL) or 0.014 gram per 20 mL

2. Vortex the mixture and the compound can be easily dissolved. The optional procedure is to incubate the compound in water bath at 37C for 15-20 minutes.

3. Filter the solution through a sterile Acrodisc Syringe Filter (0.2 µm HT Tuffryn Membrane Low Protein Binding). This procedure is performed in a hood with a syringe and allows elimination of bacteria and fungus.

4. Aliquot the NA inhibitor solution (at a concentration 2 mM) into 200µL per tube in sterile 0.5 mL tubes. This procedure is performed in a hood.

5. Store at -20ºC freezer and avoid freeze-thawing.

NA assay

1. Prepare serial 2-fold dilutions of the assayed virus with the enzyme buffer. It is more appropriate to do it on ice and in the 96-well plates used for HA assay.

   1:2 dilution → 1:4 dilution
   50 µl virus + 50 µl enzyme buffer → 50 µl virus + 50 µl enzyme buffer → etc.

2. Transfer 10 µl of each virus dilution (do it in duplicate) to the assay plate (flat bottom) starting with the most diluted column. Add 10 µl of enzyme buffer to each well of the plate. The reaction is initiated by the addition of 30 µl per well of the substrate mix (final concentration of the substrate 100 µM)

1st row	2nd row	3rd row	→ 12 row
Blank (20 µl)	Standard	10 µl virus sample	10 µl virus sample (1:1024)
Enzyme buffer		10 µl enzyme buffer	10 µl enzyme buffer
30 µl substrate mix		30 µl substrate mix	30 µl substrate mix

Standard (correlation between volume and activity)
5 µl = 200 pmol
10 µl = 400 pmol
20 µl = 800 pmol

3. Incubate the plate at 37º C for 30 minutes (with shaking sometimes).

4. The reaction is terminated by the addition of 150 µl per well of stop solution (0.014M NaOH in 83% ethanol).

5. The fluorescence of the released 4-methylumbelliferone was measured in a Fluoroskan II (Labsystems, Helsinki, Finland) spectrophotometer using excitation and emission wavelengths of 355 and 460 nm, respectively.

Plates that are recommended to use for NA and NI assays:

White/polystyrene: Costar, Cat. No. 3917
Black/polystyrene: Fisher, Cat. No. 14-245-197A

NA inhibition assay

1. Mix 10 µl virus in enzyme buffer (1500 to 2000 relative fluorescent units, as determined in NA assay) with 10 µl compound in enzyme buffer. Different concentrations of the compound must be used. Incubate the plate at 37º C for 30 minutes (with shaking sometimes).

Design of the plate

Blank	Standard 5µl = 200pmol	0.5µM + Virus 1	0.1 µM	0.05 µM	0.01 µM	0.005 µM	0.001 µM	0.0005 µM	0.0001 µM	0.00005 µM	virus + enzyme buffer
Blank	Standard 5µl = 200pmol	Virus 1	→	→	→	→	→	→	→	→	→
Blank	Standard 10µl = 400pmol	Virus 2									
Blank	Standard 5µl = 400pmol	Virus 2									
Blank	Standard 20µl = 800pmol	Virus 3									
Blank	Standard 20µl = 800pmol	Virus 3									
Blank	Empty	Virus 4									
Blank	Empty	Virus 4									

2. Add 30 µl per well of the substrate mix (final concentration of the substrate 100 µM) and further incubate 1 hour at 37º C.

3. The reaction is terminated by the addition of 150 µl per well of stop solution (Glycin in ethanol, pH=10.7).

4. The fluorescence of the released 4-methylumbelliferone was measured in a Fluoroskan II, type 371 (Labsystems, Helsinki, Finland) spectrophotometer using excitation and emission wavelengths of 355 and 460 nm, respectively.

REFERENCE:

Potier M, Mameli L, Belislem M, Dallaire L, Melanxon SB (1979) Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-α-D-N-acetylneuraminate) substrate. Anal Biochem 94: 287-296.