

NA enzyme inhibition assay for sensitivity to NA inhibitors Fluorescence Based Assay (MUNANA):

Measures the 4-methylumbelliferone (MU) released from the fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetyneuraminic acid (MUNANA) by the enzymatic activity of influenza neuraminidase (NA). NA activity assays involved serial two-fold dilutions of virus in water in white microtitre plates (Optiplates, Packard). An equal volume (50 μ L) of MUNANA substrate (0.2mM MUNANA, 0.1M NaOAc [pH 5.5], 10mM CaCl₂ in H₂O) is added. After 60 minutes at 37°C, 200 μ L of stop solution (0.2M Na₂CO₃ in H₂O) is added per well. Fluorescence is read using a Perkin Elmer LS50B luminescence spectrophotometer with an excitation and emission wavelengths of 365nm and 450nm respectively.

To determine IC₅₀ values, 25 μ L of [2x] the appropriate virus dilution and 25 μ L of [2x] inhibitor concentrations are mixed and incubated at room temperature for 30 minutes. Inhibitors used at final concentrations of 10 μ M to 0.01nM (log or half-log dilutions in H₂O). MUNANA substrate is added (50 μ L of 0.2mM) and incubated at 37°C for 60 minutes before adding 200 μ L of stop mix.

Reagents/Equipment (Fluorescence):

Substrate: 2'-(4-methylumbelliferyl)- α -D-N-acetyneuraminic acid (MUNANA)

Reaction mix: 0.2mM MUNANA + 100mM NaOAc buffer (pH 5.5) + 10mM CaCl₂ (in H₂O).

For 50mL of reaction mix add 1mL 10mM MUNANA + 25mL 0.2M NaOAc buffer (pH 5.5) + 5mL 100mM CaCl₂ + 19mL H₂O.

Note: 0.2M NaOAc buffer (pH 5.5) = 0.2M sodium acetate (in H₂O), pH adjusted with 50% acetic acid.

Stop solution: 0.2M Na₂CO₃ (in H₂O).

Microtitre Plates: Optiplate™ –96 (white, polystyrene, 96-well)

Part number: 6005290

Perkin Elmer

or black plates for Fluoroskan #6005270

Fluorimeter: Perkin-Elmer LS50B (luminescence spectrophotometer, attenuated)

Wavelength - excitation: 365 / emission: 450 nm or suitable alternative instrumentation).

Inhibitors:

Zanamivir (GG-167/Zanamivir/Relenza™)

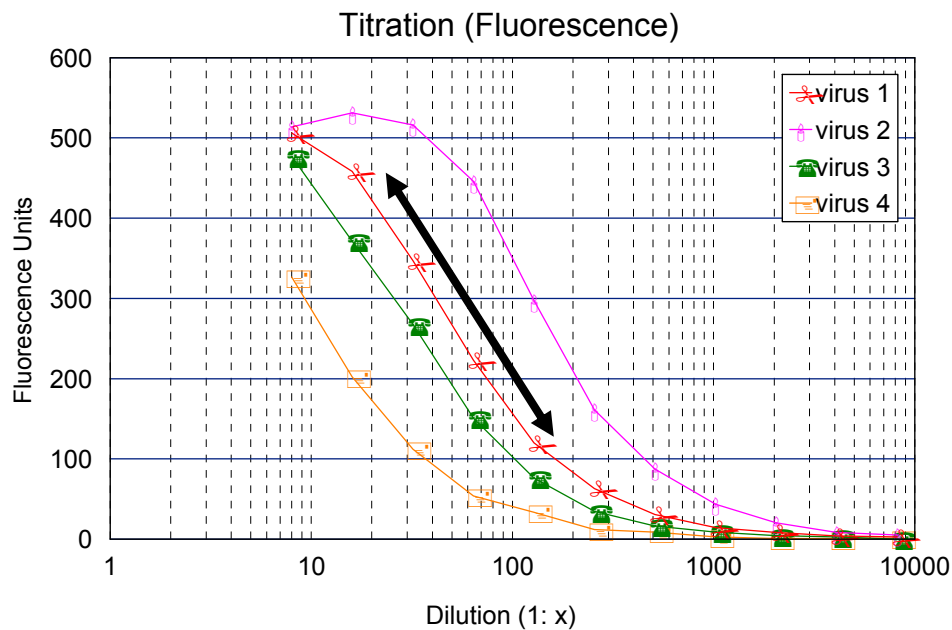
Oseltamivir carboxylate (Active ingredient of Tamiflu™, by hydrolysis)

Prepare serial dilutions of the inhibitors such that the final concentrations in the assay are 10 μ M to 0.01 nM (either log₁₀ or half-log dilutions in water). Inhibitors diluted to [2x] required concentrations (20 μ M to 0.02 nM) in water, as diluted 1:2 in assay (25 μ L [2x] inhibitor + 25 μ L [2x] virus). Make up large volume, aliquot and freeze dilutions.

Titration Procedure (Fluorescence):

- Rapidly thaw virus samples in water bath set at 37°C and vortex to mix thoroughly.
- Dilute virus 1:10 in 1st well of each row of Optiplate (11 μ L virus + 100 μ L water [Figure 1])

FIGURE 2. Fluorescence Titration (Graph).



Inhibition Procedure (Fluorescence):

1. Add 25 μL of [2x] inhibitor concentration to appropriate wells, starting with most concentrated at the start of each row. (ie start with 20 μM , 2 μM , 0.2 μM etc. for final 10 μM , 1 μM , etc.)
2. If you are doing half log dilutions do across 10 wells and then leave 2 control virus wells. This allows 8 samples per 96-well tray – in rows A-H.
3. If you do log10 dilutions then you can do them down the plate, which allows 12 samples per tray from row 1-12.
4. Add 25 μL of diluted virus to wells. Include ‘virus only’ wells (no inhibitor) for each virus (25 μL water + 25 μL of diluted virus) plus one blank well, eg H12
5. Cover or seal (adhesive film) plate and pre- incubate at room temperature for 30 minutes.
6. Add 50 μL of reaction mix to each well.
7. Cover or seal (adhesive film) plate and incubate at 37°C for 60 minutes.
8. Add 200 μL of STOP solution per well.
9. Read fluorescence using luminescence spectrophotometer.
10. The fluorescence reading is very stable and can be read when convenient.

FIGURE 3. Fluorescence - Inhibition Assay Schematic.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Con											
B	0.01 nM											
C	0.1 nM											
D	1 nM											
E	10 nM											
F	100 nM											
G	1000 nM											
H	10,000 nM											Blank

Calculate the percent inhibition by subtracting the average of the water blanks from all values, using the 'virus only' (zero inhibitor) values as the control. Note that the lowest drug concentrations should be comparable to the zero drug controls, except for resistant mutants, so can also be used as a check for the control value. Plot graph as inhibitor concentration nM vs. percent inhibition for each replicate. Determine the IC_{50} (that is, the concentration of inhibitor that inhibits 50% virus activity, Fig 4). Be aware of curves which do not reach maximum and minimum values, as curve fitting programs do not cope well with these. *Also mixed populations may not reach 100% or 0%, and curves are often flatter.* A shift of the inhibition curve to the right indicates a higher IC_{50} for that virus, ie the NA appears to be resistant. Include a known **sensitive** strain of the **same serotype**, in each assay.

Figure 4

