



**DETERMINATION OF INFLUENZA VIRUS SUSCEPTIBILITY TO
NEURAMINIDASE INHIBITORS USING A FLUORESCENT SUBSTRATE
(St. Jude Children's Research Hospital)**

OBJECTIVE AND SUMMARY:

This procedure describes the method used to determine influenza virus neuraminidase (NA) activity and sensitivity to NA inhibitors using an NA enzyme inhibition assay with a fluorescent substrate. The NA enzyme cleaves sialic acid from adjacent sugar residues. NA inhibitors block this activity by competitively binding the enzyme active site. NA activity and sensitivity to inhibitors can be determined using the fluorogenic substrate, MUNANA [2'-(4-Methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate]. This substrate is cleaved by NA to yield free 4-Methylumbelliferone which fluoresces at high pH. The increase in fluorescence is quantified to give a measure of NA activity. Performing this assay in the presence of NA inhibitors allows determine the concentration of drug required for inhibition of enzyme activity by 50% (IC₅₀).

REAGENTS:

1. Influenza virus, tissue culture- or egg-grown with an HA titer of ≥ 20 units.
2. NA subtype matched reference viruses (if available)
3. 2-Morpholinoethanesulfonic acid (MES) (Sigma – M5287)
4. Calcium Chloride (Fisher – M1612)
5. NA inhibitor - Oseltamivir Carboxylate (Roche GS4071 or Ro64-0802)
6. NA inhibitor - Zanamivir (Glaxo-Smithkline GR121167X or GG167)
7. Substrate - 2'-(4-Methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate (MUNANA) (Sigma – M8639)
8. Standard - 4-Methylumbelliferone Sodium Salt (4-MUSS) (Sigma – M1508)
9. Glycine (Sigma- G7126)
10. Absolute Ethanol
11. Sodium Hydroxide (Fisher – S318)
12. Distilled Water
13. Black Polystyrene 96-well plates (Costar-3916) recommended.
14. Synergy-2 Multi-mode microplate reader (BioTek, BioTek Instruments, Inc. Winooski, Vermont 05404-0998, USA)

PREPARATION OF BUFFERS AND SOLUTIONS:

All solutions can be stored at room temperature if desired to be used everyday, if not store at 4°C and warm to room temperature before using. MUNANA working stock should be made fresh before every assay. The buffers can be used no more than 4-5 weeks due change in pH in the buffers.

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MASTER STOCK SOLUTIONS AND BUFFERS:

325 mM MES: 6.9 g MES in 100 ml dd-Water (pH – 6.5 with Conc. NaOH)

0.1 M MES: 2.1 g MES in 100 ml dd-Water (pH – 6.5 with Conc. NaOH)

10 mM CaCl₂: 0.15 g CaCl₂ in 100 ml dd-Water

0.1 M CaCl₂: 1.47 g CaCl₂ in 100 ml dd-Water

Stop Solution: Mix 100 ml Absolute Ethanol + 250 ml of dd-Water and add 3.04 g Glycine, dissolve Glycine and adjust the pH – 10.7 with concentrated NaOH. Make up to 400 mL with sterile distilled water.

4 mM Standard Stock: 4-Methylumbelliferone sodium salt:

7.9 mg in 10 ml dd-Water (395 mg in 500 ml dd-Water for accuracy).

5 mM Oseltamivir Carboxylate:

tartrate salt (M.W. - 386.4): 38.64 mg in 20 ml of dd-Water.

5 mM Zanamivir Monohydrate:

(M.W. - 350.3): 35.03 mg in 20 ml of dd-Water.

Vortex can dissolve the compound easily, or incubate the compound at 37°C water bath for 15-20 minutes. Sterile filter the solution in the Biosafety cabinet using 0.2 µm syringe filter. Aliquot 50 µl of each NA inhibitor compounds in 0.2 or 0.5 ml sterile tubes. Store at -20°C. Avoid freeze thawing.

5 mM MUNANA Stock: 2'-(4-Methylumbelliferyi)-α-D-N-acetylneuraminic acid, sodium salt (Sigma M-8639, M.W. - 489.4).

Add 2240 µl of sterile dd-Water to 5 mg vial of MUNANA, mix well, aliquot and store at -20°C to make 5 mM Stock.

WORKING SOLUTIONS AND BUFFERS:

Enzyme Buffer: 32.5 ml of 0.1 M MES (pH-6.5)
4 ml of 0.1 M CaCl₂
63.5 ml of dd-Water (Store Solution at +4°C)

40 µM 4-Methylumbelliferone Sodium Salt Working Stock:

10 µl of 4 mM stock of 4-methylumbelliferone sodium salt is added to 990 µl of enzyme buffer.

Oseltamivir Carboxylate and Zanamivir (Concentrations 5 μ M – 0.0005 μ M):

Dilute 5 mM drug stock 1000 times with enzyme buffer to get 5 μ M compound. Dilute 5 μ M stock 5 times to get 1 μ M. Dilute these two working stocks by 10-fold serial dilutions to have the remaining drug dilutions as desired for the NI assay.

Label	A	B	C	D	E	F	G	H	I	J
Desired Conc (μM)	5	1	0.5	0.1	0.05	0.01	0.005	0.001	0.0005	0.0001
Oseltamivir (5 mM)	1:1000 of Stock	1:5 of A	1:10 of A	1:10 of B	1:10 of C	1:10 of D	1:10 of E	1:10 of F	1:10 of G	1:10 of H
Zanamivir (5 mM)	1:1000 of Stock	1:5 of A	1:10 of A	1:10 of B	1:10 of C	1:10 of D	1:10 of E	1:10 of F	1:10 of G	1:10 of H

Substrate Working Stock: Prepare the mixture just before the experiment and leave on ice. Concentration of substrate mixture – 167 μ M, after adding to the plate – the final concentration of substrate will be 100 μ M.

Buffers/Substrate	$\frac{1}{2}$ plate	1 plate	2 plates	3 plates
Enzyme Buffer	1450 μ l	2900 μ l	6800 μ l	8700 μ l
Substrate	50 μ l of 5 mM MUNANA (from -20C)	100 μ l of 5 mM MUNANA (from -20C)	200 μ l of 5 mM MUNANA (from -20C)	300 μ l of 5 mM MUNANA (from -20C)

NA ACTIVITY DETERMINATION (MUNANA ASSAY):

This assay describes how to measure the NA activity of influenza virus. The optimal virus sample dilution to standardize virus dose when measuring virus IC₅₀ to NA inhibitor can be determined using this method. **Each assay should include subtype matched validated reference viruses**, examples reference viruses and validation criteria for the assay is given in useful notes.

- i. Add 20 μ l of enzyme buffer to each well of black 96-well plate, column A1-H1 (blank).
- ii. Add 20 μ l of enzyme buffer to each well of black 96-well plate, columns A3-A12 through H3-H12.
- iii. Make duplicate two fold dilutions of virus material, with a starting dilution of $\frac{1}{2}$, by adding 20 μ l of first virus to wells A3 and B3, 20 μ l of second virus to wells C3 and D3 and so on until row 3 is filled. Mix virus and buffer by pipetting up and down several times.
- iv. Serial dilute the virus down the plate by carrying over 20 μ l from row 3 to row 4 and so on till row 12. Discard 20 μ l from row 12.
- v. Add 50 μ l of 4-Methylumbelliferone sodium salt standard in different concentrations to wells A2-F2. This serves a positive control. Follow the table below:

NA ASSAY PLATE LAYOUT:

Column	Blank	4-MUSS (μM)	Virus Dilutions									
			1:2 Dil	1:4 Dil	1:8 Dil	1:16 Dil	1:32 Dil	1:64 Dil	1:128 Dil	1:256 Dil	1:512 Dil	1:1024 Dil
	1	2	3	4	5	6	7	8	9	10	11	12
A	Enz Buff	4	Vir-1									
B	Enz Buff	4	Vir-1									
C	Enz Buff	8	Vir-2									
D	Enz Buff	8	Vir-2									
E	Enz Buff	16	Vir-3									
F	Enz Buff	16	Vir-3									
G	Enz Buff		Vir-4									
H	Enz Buff		Vir-4									

4-Methylumbelliferone Standard Concentrations:

Wells to Add	Total Volume (μl)	Stock Concentration (μM)	Volume of Stock (μl)	Volume of Enzyme Buffer (μl)	Final Concentration (μM)
A2, B2	50	40	5	45	4
C2, D2	50	40	10	40	8
E2, F2	50	40	20	30	16

- vi. To perform assay in 96-wells plate, prepare 3 mls of MUNANA substrate working stock per plate as mentioned in the preparation of working solutions and buffers section. Add 30 μl of substrate to each well including the blank column excluding the column 2, which already has 50 μl of 4-methylumbelliferone standard.
- vii. Cover the plate with plate lid; mix the contents by rocking on a shaker for a minute. Incubate at 37°C for 30 minutes in dark.
- viii. Terminate the reaction by adding 150 μl of stop solution to all the 96 wells. Read the plate within 20 minutes of adding the stop solution detecting fluorescence using wavelength of 360 nm and an excitation wavelength of 460 nm. (Use Synergy-2 multimode plate reader in Room # 16409).
- ix. The blanked data is exported from the plate reader and standard virus dose calculation has to be carried out independently and the mean of this virus dose is considered for NI assay.
- x. **Standard Virus dose calculation for NI Assay:** Define the virus dilution in which enzyme activity yields the equivalent level of fluorescence in 30 minutes as 10 μM of 4-methylumbelliferone (standard curve with 4-Methylumbelliferone). This dilution is then used in the NA inhibition assay in order to ensure equivalent activities for each virus are compared. This cut off should be within the linear range of NA enzyme activity curve.

NEURAMINIDASE INHIBITION ASSAY:

This section describes how to determine the IC₅₀ of a virus to a NAI. The NA activity of each virus should be measured (NA assay) before performing the NI assay. For best results both assays should be performed on the same day. Each assay should include NA subtype matched validated reference viruses. (Store viruses and the 96-well plate on ice while performing assay and adding all reagents).

1. Add 20 µl of enzyme buffer to each well of black 96-well plate, column A1-H1 (Blank). Add 10 µl of enzyme buffer to column A12-H12 (Virus control).
2. Add 10 µl different concentrations of NA inhibitor to each column of the plate, starting with **5 µM** in column A3-H3, **1 µM** in column A4-H4, **0.5 µM** in column A5-H5, **0.1 µM** in column A6-H6, **0.05 µM** in column A7-H7, **0.01 µM** in column A8-H8, and **0.005 µM** in column A9-H9. (Refer to NA inhibitors table below).
3. Dilute each virus in enzyme buffer according to the factor determined in the NA assay.
4. Add 10 µl of first virus to wells A3-A12 and B3-B12, 10 µl of second virus to wells C3-C12 and D3-D12 and so on until all the columns are filled with viruses.
5. Add 50 µl of 4-Methylumbelliferone sodium salt standard in to wells A2-F2. This serves a positive control. (Exactly as in NA assay above).
6. Cover the plate with plate lid; mix the contents by rocking on a shaker for a minute. Incubate at 37°C for 30 minutes in dark.
7. Prepare 3 mls of MUNANA substrate working stock per plate as mentioned in the preparation of working solutions and buffers section. Add 30 µl of MUNANA substrate to each well including the blank column A1-H1 excluding the column A2-H12, which already has 50 µl of 4-methylumbelliferone standard.
8. Cover the plate with plate lid; mix the contents by rocking on a shaker for a minute. Incubate at 37°C for 30 minutes in dark.
9. Terminate the reaction by adding 150 µl of stop solution to all the 96 wells. Read the plate within 20 minutes of adding the stop solution detecting fluorescence using wavelength of 360 nm and an excitation wavelength of 460 nm. (Use Synergy-2 multimode plate reader in Room # I6409).
10. Export the normalized data (subtracted from blank) from the plate reader and determine the IC₅₀ values for the tested viruses using the **Graph-pad Prism** program. IC₅₀ values are calculated for the replicates independently, and the mean IC₅₀ is taken as the final value.

Neuraminidase Inhibitors for NI Assay per plate: 5 mM NA inhibitors stocks (Oseltamivir carboxylate and Zanamivir) are diluted 1:1000 times in enzyme buffer, which is 5 µM and this is diluted 1:5 times to yield 1 µM. Add these two drug dilutions to the A3 and B3 wells (Oseltamivir carboxylate), and to the A4 and B4 wells (Zanamivir) in a 96-well plate. Dilute the drugs ten-fold from these concentrations to be used in the NI assay. Follow the table below:

Column	Blank	Drug Concentration (μM)										
		5	1	0.5	0.1	0.05	0.01	0.005	0.001	0.0005		
	1	2	3	4	5	6	7	8	9	10	11	12
A			150 μl	150 μl	135 μl	135 μl	135 μl	135 μl	135 μl	135 μl	135 μl	
B			150 μl	150 μl	135 μl	135 μl	135 μl	135 μl	135 μl	135 μl	135 μl	
					Add 15ul from row 3	Add 15ul from row 4	Add 15ul from row 5	Add 15ul from row 6	Add 15ul from row 7	Add 15ul from row 8	Add 15ul from row 9	

NI ASSAY PLATE LAYOUT:

Column	Blank	4-MUSS (μM)	Drug Concentration (μM)									No Drug
			5	1	0.5	0.1	0.05	0.01	0.005	0.001	0.0005	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Enz Buff	4	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir Con
B	Enz Buff	4	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir-1	Vir Con
C	Enz Buff	8	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir Con
D	Enz Buff	8	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir-2	Vir Con
E	Enz Buff	16	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir Con
F	Enz Buff	16	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir-3	Vir Con
G	Enz Buff		Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir Con
H	Enz Buff		Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir-4	Vir Con

NEURAMINIDASE INHIBITION ASSAY VALIDATION AND REFERENCE CRITERIA:

- ◆ Reference viruses are included in all NA activity and NA inhibition assays and should be NA subtype matched to the sample undergoing testing. If the sample subtype is unknown, reference strains of all subtypes should be used.
- ◆ IC_{50} calculations are carried out on replicates independently and the final IC_{50} is a mean of the two values.
- ◆ Any sample for which replicate titrations show greater than 20% difference in value should be repeated.
- ◆ Validation limits for each reference virus should be determined. Limits are defined as three standard deviations above or below the median IC_{50} . The median is calculated from the minimum of ten independent assays of the reference viruses. If any sample with valid IC_{50} value is greater than 1.65 SD above the median for the subtype and season are retested twice and if the mean value is still 1.65 SD above median the sample is classified as minor outlier. Any sample with a mean IC_{50} value greater than 3 SD above the median for the season and subtype is classified as a major outlier. Both minor and major outliers are subjected to further characterization and genotyping.
- ◆ If a reference virus IC_{50} value fails to meet validation criteria for a given drug, the test using that drug is invalidated and all samples are repeated. Trends in

reference IC₅₀ performance should be monitored. A batch that fails to meet validation criteria in three consecutive assays should be discarded.

USEFUL NOTES:

- ◆ Fluorescence is measured in Relative Fluorescence Units (RFU). Because of this different fluorescence based plate readers have different range of values and different dynamic range. Raw data measured in RFU cannot be compared from one machine to another, and can only be compared from one assay to the next if the settings are not changed.
- ◆ Virus isolates that have been stored frozen can be analyzed for IC₅₀. After thawing, virus should be stored at +4°C until the IC₅₀ determination is complete.
- ◆ Pre-warm the MUNANA substrate at 37°C for 15 minutes before adding to assay plate.
- ◆ Samples with low titers may not have sufficient NA activity for inhibition testing and may give inaccurate IC₅₀ values. Use samples with HA titers of 20 and or if the peak NA activity is equivalent to 10 µM of 4-Methylumbelliferone sodium salt can be reliably tested.
- ◆ Between performing the NA activity assay and NI assay, viruses should be stored at +4°C. No more than 24 hours should elapse between the two tests; otherwise virus dilution for the NI assay should be determined by NA assay again. This is advised because the NA activity in viruses with particular mutations in NA gene causing resistance can be unstable.

SUPPLEMENTAL INFORMATION:

Determination of standard viral dose for NA inhibition assay using Synergy-2 plate reader

4-METHYLUMBELLIFERONE SODIUM SALT SODIUM SALT STANDARD CURVE

1. Dilute 4-Methylumbelliferone sodium salt (4-MUSS) stock in stop solution to 100 µM concentration.
2. Serial dilute 4-MUSS in 2-fold dilutions in stop solution.
3. Pipette 200 µl of each dilution into same plates used for NA and NI assay (black, flat bottom 96-well plates). 200 µl is used for measurements, as this is equal to 200 µl of final volume in NA and NI assay.
4. Measure the fluorescence activity of 4-MUSS titration series in Synergy-2 multi mode plate reader.
5. Plot 4-MUSS standard curve and consider the RFU corresponding to 4-MUSS in linear range.
6. The 4-MUSS in linear range can be applied to curves generated by viral titrations to determine standard dose for IC₅₀.
7. Based on the curve below we can choose any where in between 3 µM - 12 µM of 4-MUSS using Synergy-2 multi-mode microplate reader.
8. To avoid discrepancy in using different virus doses for IC₅₀, we can choose 10 µM of 4-MUSS that corresponds to ~4000 RFU in the curve below that is well in linear range and use this to choose the standard virus dose for all NA Inhibition assays.

