Gentaur Europe BVBA Voortstraat 49, 1910 Kampenhout BELGIUM
Tel 0032 16 58 90 45 info@gentaur.com

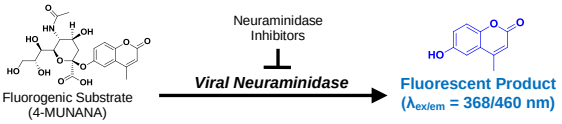
Influenza Neuraminidase Inhibitor Susceptibility Assay Kit

11/17

(Catalog # K524-1000; 1000 Reactions; Store at -20°C)

I. Introduction:

Neuraminidase (EC 3.2.1.18) is a tetrameric Ca^{2+}-dependent sialidase enzyme that cleaves α-glycosidic linkages of terminal sialic acid residues from the oligosaccharide chains of glycoproteins. Neuraminidase-family enzymes are present in most lifeforms, from bacteria to humans, and are also encoded by several viral genomes, including influenza viruses. Influenza neuraminidase plays a critical role in replication and transmission of the virus by preventing self-aggregation of replicating progeny viral particles and enabling release of active influenza virions from infected host cells. Neuraminidase inhibitors, which competitively block the enzyme-mediated release of newly synthesized viral particles, are widely used as anti-influenza drugs. Rapid mutation of viral neuraminidase can yield new influenza strains that are more pathogenic and also engender resistance to existing neuraminidase inhibitors. Hence, monitoring of neuraminidase activity and inhibitor susceptibility in influenza field isolates collected worldwide is integral to identifying new pandemic influenza strains and guiding public health strategies to control seasonal influenza epidemics. BioVision's Influenza Neuraminidase Activity/Inhibitor Susceptibility Assay Kit enables rapid measurement of viral neuraminidase activity, utilizing a fluorogenic substrate that is converted into a stable, highly fluorescent product (Ex/Em = 368/460 nm). The assay allows for high-throughput functional screening of baseline neuraminidase activity and inhibitor susceptibility patterns in influenza virus isolates in order to evaluate the possible emergence of new inhibitor-resistant strains. A straightforward, standardized protocol consistent with the latest CDC and WHO research guidelines ensures reproducible neuraminidase inhibitor IC_{50} data for influenza antiviral susceptibility surveillance. The assay may also be used for screening of novel neuraminidase inhibitors against known drug-resistant influenza strains. The kit includes sufficient reagents to perform at least 1000 reactions (ten assay plates) in a 96-well plate format.



II. Applications:

- Rapid assessment of neuraminidase activity in influenza virus isolates
- Characterization of neuraminidase inhibitor susceptibility of known or newly emergent influenza strains
- High-throughput screening of novel neuraminidase inhibitor antiviral drugs

III. Sample Type:

- Influenza virus (type A or B, from any host species) isolates (propagated from embryonated chicken eggs or cultured MDCK cells)
- Purified or recombinant neuraminidase enzyme preparations

IV. Kit Contents:

Components	K524-1000	Cap Code	Part Number
Neuraminidase Assay Buffer	225 ml	NM	K524-1000-1
Neuraminidase Stop Solution	110 ml	NM	K524-1000-2
Neuraminidase Substrate (100X)	1 vial	Red	K524-1000-3
Viral Neuraminidase Positive Control	1 vial	Violet	K524-1000-4
4-MU Standard (5 mM)	100 µl	Yellow	K524-1000-5

V. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate reader (excitation range = 355-375 nm; emission range = 450-470 nm)
- Precision multi-channel pipette and reagent reservoir
- Reference neuraminidase inhibitors (such as oseltamivir carboxylate (Cat. #1886) and zanamivir (Cat. #B1900))
- Black 96-well plates with flat, opaque bottom

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

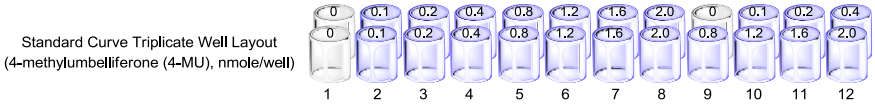
- **Neuraminidase Assay Buffer:** Warm solution to room temperature prior to use. Store solution at 4°C when not in use.
- **Neuraminidase Stop Solution:** Warm solution to room temperature prior to use. Store solution at 4°C when not in use.
- **Neuraminidase Substrate (100X):** Reconstitute with 1250 µl of ddH_2O and vortex to yield a 10 mM stock solution (100X). Divide into aliquots (100 µl is required per 96-well assay plate) and store at -20°C, protected from light. Avoid repeated freeze/thaw cycles.
- **Viral Neuraminidase Positive Control:** Reconstitute with 110 µl of Neuraminidase Assay Buffer. Aliquot if desired and store at -20°C, protected from light. Avoid repeated freeze/thaw cycles and keep aliquots on ice while in use.
- **4-MU Standard (5 mM):** Provided as a stock solution in DMSO. Store at -20°C, protected from light. Stable for 5 freeze/thaw cycles.

VII. Influenza Neuraminidase Activity/Inhibitor Susceptibility Assay Protocol:

1. Standard Curve Preparation:

a. Dilute the 4-MU Standard by adding 10 µl of the 5 mM stock to 990 µl of Neuraminidase Assay Buffer to obtain a 50 µM working solution. Add 0, 2, 4, 8, 16, 24, 32 and 40 of the 50 µM solution into a series of wells and adjust the volume of each well to 100 µl with Neuraminidase Assay Buffer, yielding 0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 nmole/well 4-MU Standard. Add 100 µl of the Neuraminidase Stop Solution to each standard curve well (bringing the final volume to 200 µl/well) and mix thoroughly.

Note: A 4-MU Standard Curve should be generated before performing the neuraminidase activity viral titration for the first time (or if any changes to the fluorescence microplate reader are made). If running multiple 96-well assay plates at the same time, a single standard curve may be used for all of the plates. A suggested template for creation of a triplicate-well 4-MU Standard Curve using two rows of a single assay plate is given below.



b. Measure the fluorescence of standard curve wells at Ex/Em = 368/460 nm. Subtract the 0 nmole/well RFU reading from all of the standard readings, plot the background-subtracted values and calculate the slope of the 4-MU Standard curve.

c. Based upon the standard curve, calculate the relative fluorescence signal (RFU value) generated by 1 nmole of 4-MU Standard. This RFU value will be used as a benchmark for normalization of neuraminidase activity of different viral samples, in order to determine the appropriate dilution factor for each viral sample to use in the inhibitor susceptibility portion of the assay.

2. Virus Titer Normalization By Neuraminidase Activity:

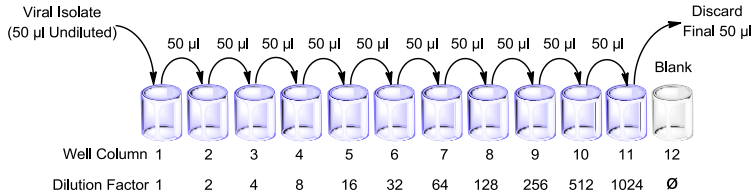
a. Sample Preparation:

For influenza strains propagated using cultured mammalian cells as a host: harvest culture supernatant once inoculated cells exhibit characteristic cytopathic effects (CPEs; for example, significant rounding and detachment of cells from the monolayer). Centrifuge supernatant at 5000 x *g* at 4°C for 15 min to pellet cellular debris and transfer the clarified supernatant to a new prechilled tube.

For strains propagated using embryonated chicken eggs: collect allantoic fluid from inoculated eggs and clarify fluid by centrifugation at 2000 x *g* at 4°C for 15 min to pellet any red blood cells or debris. Transfer clarified allantoic fluid to a new prechilled tube.

Note: Keep influenza viral isolates on ice (or at 4°C) while in use. For long-term storage, aliquot and store viral isolates at -80°C (influenza virions are unstable at -20°C and will rapidly lose infectivity) and avoid repeated freeze/thaw cycles.

b. Serial Dilution Setup: For each viral strain to be tested, prepare a series of 12 reaction wells (one row of a 96-well plate) by adding 50 µl of Neuraminidase Assay Buffer to each well in columns 1-12. Add 50 µl of undiluted viral isolate to column 1 and mix well contents thoroughly by pipetting up and down. Perform a series of 2-fold serial dilutions across the row of wells according to the diagram below. Begin by transferring 50 µl from column 1 to column 2, mix well contents by pipetting, and continue to transfer 50 µl to each successive well until reaching column 11. Discard 50 µl from column 11, leaving column 12 to serve as a background control well (no virus) to correct for any non-enzymatic substrate hydrolysis. *At this point, the volume of each well should be 50 µl.*



Note: If desired, you may prepare a positive control well in lieu of one or more of the background control wells. For each positive control well, add 10 µl of the reconstituted Viral Neuraminidase Positive Control solution and adjust the well volume to 50 µl with Neuraminidase Assay Buffer. *The Viral Neuraminidase Positive Control is intended for validation of substrate metabolism only; do not dilute serially or use as a sample for neuraminidase inhibitor susceptibility tests.*

c. Reaction Preparation: Preincubate the plate at 37°C for 10 min to equilibrate the well contents to the reaction temperature. During the preincubation, prepare a 2X concentrated Neuraminidase Substrate working solution by diluting the reconstituted Neuraminidase Substrate 100X stock with Neuraminidase Assay Buffer at a 1:50 ratio. Prepare a sufficient amount of 2X Neuraminidase Substrate working solution to add 50 µl to each reaction well, including background control and positive control wells. *Note: for one 96-well assay plate, combine 100 µl of 100X Neuraminidase Substrate stock with 4900 µl Neuraminidase Assay Buffer.*

d. Neuraminidase Reaction: Start the reaction by adding 50 µl of the 2X Neuraminidase Substrate solution to each reaction well using a multichannel pipette, bringing the volume to 100 µl/well. Incubate the plate at 37°C for 60 min, protected from light.

e. Measurement: Terminate the reaction by adding 100 µl of Neuraminidase Stop Solution to each well. Mix well contents thoroughly and measure the fluorescence of all wells at Ex/Em= 368/460 nm in endpoint mode. *The fluorescence signal generated following the addition of Neuraminidase Stop Solution is stable for at least 4 hours at room temperature (or overnight at 4°C) if the plate is protected from light and evaporation. Addition of Stop Solution will inactivate influenza virions, rendering them non-infective.*

f. Calculation: For all viral dilution sample wells, quantify the specific fluorescence (*F*) by subtracting the mean fluorescence intensity of the no virus background control wells (RFU_{blank}) from the fluorescence intensity of each sample well (RFU_{sample}): $F = RFU_{sample} - RFU_{blank}$. For each viral strain, plot the background-subtracted *F* values against the sample dilution factor and determine which dilution factor yields a specific fluorescence that most closely corresponds to the RFU value generated by 1 nmole of 4-MU Standard (as determined from the 4-MU Standard curve). *This will be the viral dilution factor employed in the inhibitor susceptibility assay*.

3. Viral Neuraminidase Inhibitor Susceptibility Assay:

a. Neuraminidase Inhibitor Reagent Preparation: For each test neuraminidase inhibitor (NI), dissolve the compound in the proper solvent to produce a 10 mM master stock solution (see note regarding NI solubility). Prepare a 1 mM working solution by diluting the master stock in Neuraminidase Assay Buffer at a 1:10 ratio. To determine IC_{50} values for NIs, 4X working solutions should be prepared in a range of concentrations by diluting the 1 mM working solution in Neuraminidase Assay Buffer (in order to generate a multi-point dose-response curve). For each NI, we recommend preparing a series of ten 4X working solutions in Neuraminidase

Assay Buffer (0.04, 0.2, 0.4, 2, 4, 20, 40, 400, 4000 and 40000 nM, corresponding to a final concentration range of 0.01 nM to 10 µM). For each viral strain to be tested for NI susceptibility, prepare a series of reaction wells containing 25 µl of each 4X NI test concentration working solution as well as corresponding no inhibitor control (containing 25 µl Neuraminidase Assay Buffer) and background control (containing 50 µl Neuraminidase Assay Buffer) wells:

Well Column	1	2	3	4	5	6	7	8	9	10	11	12
4X NI Working Solution (nM)	N/A	0.04	0.2	0.4	2	4	20	40	400	4000	40000	N/A
Final NI Concentration (nM)	0	0.01	0.05	0.1	0.5	1	5	10	100	1000	10000	Ø

Note: The NIs oseltamivir carboxylate (Cat. #B1886), zanamivir (Cat. #B1900) and peramivir are water soluble. For each of these NIs, master stock solutions (10 mM) may be prepared in ddH_2O, aliquoted and stored at -20°C. If an organic solvent is used to prepare a NI master stock solution, the final amount of solvent should be the same for all NI test concentrations and the no inhibitor (vehicle) control wells. *In our experience, DMSO does not affect neuraminidase activity at final concentrations less than 2% (v/v).*

b. Virus Sample Preparation: For each viral strain to be tested for NI susceptibility, dilute the viral isolate stock with Neuraminidase Assay Buffer according to the optimal dilution factor determined in the neuraminidase activity viral titration assay (see Section 2). Add 25 µl of diluted virus solution to the no inhibitor (vehicle) control well and to each NI test concentration well. *Do not add diluted virus to the background control wells.*

c. Reaction Preparation: Preincubate the plate at 37°C for 30 min to allow the inhibitors to interact with the viral enzymes. During the preincubation, prepare a 2X concentrated Neuraminidase Substrate working solution by diluting the reconstituted Neuraminidase Substrate 100X stock with Neuraminidase Assay Buffer at a 1:50 ratio. Prepare 50 µl of 2X Neuraminidase Substrate solution for each well (for one assay plate, combine 100 µl of 100X Neuraminidase Substrate stock with 4900 µl Neuraminidase Assay Buffer).

d. Neuraminidase Reaction: Start the reaction by adding 50 µl of the 2X Neuraminidase Substrate working solution to each reaction well using a multichannel pipette, bringing the volume to 100 µl/well. Incubate the plate at 37°C for 60 min, protected from light.

e. Measurement: Terminate the reaction by adding 100 µl of Neuraminidase Stop Solution to each well. Mix well contents thoroughly and measure the fluorescence of all wells at Ex/Em= 368/460 nm in endpoint mode. *The fluorescence signal generated following the addition of Neuraminidase Stop Solution is stable for at least 4 hours at room temperature (or overnight at 4°C) if the plate is protected from light and evaporation.*

f. Calculation: For each reaction well, including no inhibitor/vehicle controls, subtract the fluorescence intensity of the background control well (RFU_{blank}) to determine background-corrected fluorescence (denoted by F). For each NI test concentration (F_{NI}) in the dose-response curve, calculate percent inhibition (or remaining activity) relative to vehicle control (F_{VC}) using the following equation:

$$\textbf{\% Inhibition} = \frac{F_{VC}-F_{NI}}{F_{VC}} \times 100\% \quad \textbf{or} \quad \textbf{\% Relative Activity} = \frac{F_{NI}}{F_{VC}} \times 100\%$$

Plot the relative activity (or percent inhibition) at each NI concentration and calculate the IC_{50} value by non-linear logistic curve fitting.

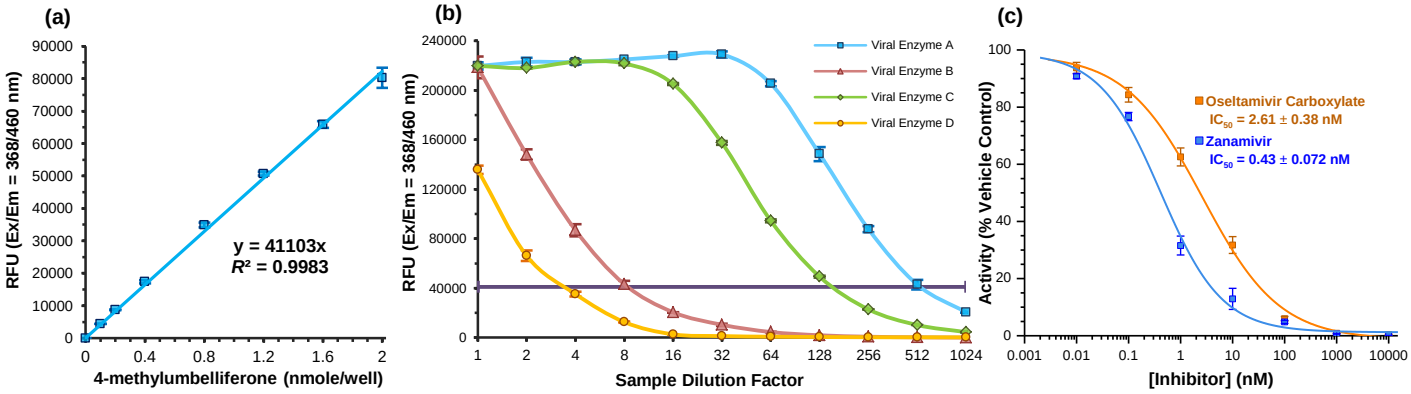


Figure: (a) Standard curve of 4-methylumbelliferone (4-MU) fluorescence. One mole of 4-MU Standard corresponds to the metabolism of one mole of Neuraminidase Substrate. **(b)** Influenza virus sample titration based upon functional assay of neuraminidase enzyme activity. The appropriate viral dilution factor for inhibitor susceptibility screening corresponds to the neuraminidase activity yielding 1000 pmole of the fluorescent metabolite 4-MU over the time course of the assay (indicated by the bold purple line). **(c)** Dose-response curve for inhibition of influenza neuraminidase (from Influenza A/Anhui/2005 (H5N1)) by the antiviral drugs oseltamivir carboxylate (the active metabolite of oseltamivir, Cat. #B1886) and zanamivir (Cat. #B1900). Percent activity at each dose was calculated by comparison to activity in the presence of vehicle (Neuraminidase Assay Buffer) only. IC_{50} values were derived by 4-parameter logistic curve fitting with each point representing the mean ± SEM of at least 4 replicates. Assays were performed according to the kit protocol.

VIII. RELATED PRODUCTS:

Neuraminidase Activity Fluorometric Assay Kit (K732)
Oseltamivir Acid (B1886)
Human CellExp™ Influenza A Virus Neuraminidase (7508)
Zanamivir (B1900)

FOR RESEARCH USE ONLY! Not to be used on humans.