ab283398 – Influenza Neuraminidase Inhibitor Susceptibility Assay Kit

For the screening of potential Influenza Neuraminidase inhibitors.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit:
http://www.abcam.com/ab283398

Storage and Stability

On receipt entire assay kit should be stored at -20°C, protected from light. Upon opening, use kit within 6 months.

Materials Supplied

Item	Quantity	Storage Condition
Neuraminidase Assay Buffer	225 mL	-20°C
Neuraminidase Stop Solution	110 mL	-20°C
Neuraminidase Substrate (100X)	1 vial	-20°C
Viral Neuraminidase Positive Control	1 vial	-20°C
4-MU Standard (5 mM)	100 µl	-20°C

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Multi-well spectrophotometer
- Black 96-well white plate with flat opaque bottom
- Precision multi-channel pipette and reagent reservoir
- Reference neuraminidase inhibitors

Reagent Preparation

- Before using the kit, spin the tubes prior to opening.

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.

Assay Protocol

Standard Curve Preparation:

1. 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.
2. 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.
3. Add 100 µl of the Neuraminidase Stop Solution to each standard curve well (bringing the final volume to 200 µl/well) and mix thoroughly.

Δ 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 the plates.*

4. 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.
5. 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, to determine the appropriate dilution factor for each viral sample to use in the inhibitor susceptibility portion of the assay.

Virus Titer Normalization by Neuraminidase Activity:

a. Sample Preparation:

1. 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 minutes to pellet cellular debris and transfer the clarified supernatant to a new prechilled tube.
2. 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 minutes to pellet any red blood cells or debris. Transfer clarified allantoic fluid to a new prechilled tube.

Δ 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:

1. 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.
2. Add 50 µl of undiluted viral isolate to column 1 and mix well. Perform a series of 2-fold serial dilutions across the row of wells, by transferring 50 µl from column 1 to column 2, mix contents by pipetting, and continue to transfer 50 µl to each successive well until reaching column 11.
3. 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.

Δ Note: *If desired, 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:

1. Preincubate the plate at 37°C for 10 minutes to equilibrate the well contents to the reaction temperature.
2. 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.
3. Prepare enough 2X Neuraminidase Substrate working solution to add 50 µl to each reaction well, including background control and positive control wells.

Δ Note: *For one 96-well assay plate, combine 100 µl of 100X Neuraminidase Substrate stock with 4900 µl Neuraminidase Assay Buffer.*

d. Neuraminidase Reaction:

1. 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.
2. Incubate the plate at 37°C for 60 minutes, protected from light.

e. Measurement:

1. Terminate the reaction by adding 100 µl of Neuraminidase Stop Solution to each well.
2. Mix contents thoroughly and measure the fluorescence of all wells at Ex/Em= 368/460 nm in endpoint mode.

Δ Note: *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:

1. 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}):
   $F = RFU_{sample} - RFU_{blank}$.
2. 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.

Viral Neuraminidase Inhibitor Susceptibility Assay:

a. Neuraminidase Inhibitor Reagent Preparation:

1. For each test neuraminidase inhibitor (NI), dissolve the compound in the proper solvent to produce a 10 mM master stock solution (see Note below).
2. Prepare a 1 mM working solution by diluting the master stock in Neuraminidase Assay Buffer at a 1:10 ratio.
3. 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 (to generate a multi-point dose-response curve).
4. For each NI, prepare 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).
5. 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 wells (containing 50 µl Neuraminidase Assay Buffer)

Δ Note: *The NIs oseltamivir carboxylate, zanamivir and peramivir are water soluble. For each of these NIs, master stock solutions (10 mM) may be prepared in ddH2O, 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. DMSO does not affect neuraminidase activity at final concentrations less than 2% (v/v).*

b. Virus Sample Preparation:

1. 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.
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:

1. Preincubate the plate at 37°C for 30 minutes to allow the inhibitors to interact with the viral enzymes.
2. 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.
3. 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:

1. 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.
2. Incubate the plate at 37°C for 60 minutes, protected from light.

e. Measurement:

3. Terminate the reaction by adding 100 µl of Neuraminidase Stop Solution to each well.
4. Mix contents thoroughly and measure the fluorescence of all wells at Ex/Em= 368/460 nm in endpoint mode.

f. Calculation:

1. 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).
2. 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:

$$\% \text{ Relative Inhibition} = \frac{F_{VC} - F_{NI}}{F_{VC}} \times 100$$

$$\text{Relative Activity (\%)} = \frac{F_{NI}}{F_{VC}} \times 100$$

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

Technical Support

For all technical or commercial enquiries please go to:

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