



Molecular Epidemiology Team
Virus Surveillance and Diagnosis Branch

*Centers for Disease Control and Prevention, NCIRD/CCID
Influenza Division*

SOP Title: Chemiluminescent Neuraminidase Activity Inhibition Assay (NAStar)
Effective Date: July 18, 2007

**Chemiluminescent (NA-Star) Neuraminidase Activity Inhibition Assay
(CDC Procedure)**

Materials Required:

- **NA-Star Kit (Reagents and Plates) – Applied Biosystems, Part No. 4374422**
- **If individually purchased:**
Assay Buffer: NA-Star Buffer (Applied Biosystems, Part No. 4374345)
Substrate Buffer: NA-Star substrate (Applied Biosystems Part No. 4374347)
Accelerator: NA-Star Accelerator (Applied Biosystems Part No. 4374346)
Plates: Opaque 96 well white plates (Applied Biosystems Part No. 4374349)
Reagent: NA-Star Reagent (Applied Biosystems Part No. 4374348)
- **Plate reader for luminescence**
- **Software for IC50 calculations**

Prior to starting the Assay:

- **Thaw virus isolates to be assayed**
- **Prepare drug dilutions**
- **Prepare a fresh working stock of NA-Star substrate reagent (from the NA-Star kit)**
Dilute Na-Star substrate 1:1000 in NA-Star Buffer
(Example: 20µl NA-Star substrate in 20 ml NA-Star Buffer)
- **Bring all reagents to room temperature**
- **Turn on plate reader and prime injector to be used with NA-Star accelerator reagent**

Part I. NA Activity Assay to determine the working dilution of virus:**16 viruses can be tested on one 96 well plate.****Inclusion of a pair of drug-sensitive and drug-resistant viruses for each NA subtype to be tested is recommended.**

- Prepare serial 2 fold dilutions of virus starting with 1:5 (in NA-Star buffer)
- Add 80µl NA-Star buffer to column 1 and 7
- Add 50µl NA-Star buffer to columns 2-5 and 8-11.
- Add 50µl NA-Star buffer to columns 6 and 12 as a blank control.
- Add 20µl of the virus to be tested to the well in column 1 of each row, one row per virus (serial dilute the virus in columns 2-5 [Pipet up and down (3x) and remove 50µl add to next well, etc. Discard last 50µl]).
- Repeat as above with the next batch of viruses to be tested in columns 7-11.
- Add 10µl freshly prepared NA-Star substrate to each well starting from the blank control (column 6 towards column 1).
- Repeat for columns 12 to 7, making sure to change tips.
- Tap plate gently on each side to mix virus and NA-Star substrate.
- Incubate plates at room temperature for 30 minutes.
- Place plate in the luminescence plate reader and start the relevant program to be used on your particular piece of equipment. The program will need to add 60µl of NA-Star Accelerator to each well of the plate and will need to measure the readout of the well after a 1 second delay.

The virus dilution that gives <40 and >10 signal to noise ratio is considered the optimal virus dilution.

NOTE: Virus must be diluted by at least 1:5 with NA-Star buffer to reduce the quenching effect seen from phenol red present in tissue culture media.

Part II. Neuraminidase Activity Inhibition Assay (to determine IC₅₀ value):

- Prepare working virus dilution (as determined in Part I) in NA-Star buffer in 1.7ml eppendorf tube (enough to test the virus against two drugs).
- Prepare drug dilutions according to the chart below in NA-Star buffer:

Inhibitor Dilutions:

Dilution #	Use	Required conc (nM)	FINAL conc (nM)*
1	30ul of 50uM stock of inhibitor + 720ul NA-Star Buffer	2000	1000
2	250ul dilution 1 + 540ul NA-Star Buffer	633	316
3	250ul dilution 2 + 540ul NA	200	100
4	250ul dilution 3 + 540ul NA	63.4	31.7
5	250ul dilution 4 + 540ul NA	20	10.0
6	250ul dilution 5 + 540ul NA	6.3	3.18
7	250ul dilution 6 + 540ul NA	2	1.01
8	250ul dilution 7 + 540ul NA	0.64	0.32
9	250ul dilution 8 + 540ul NA	0.20	0.10
10	250ul dilution 9 + 540ul NA	0.06	0.032

*The final concentration does not account for 10µl of NA-Star substrate; half-log₁₀ dilutions.

- Add 25µl of each drug dilution to each well, with the lowest drug concentration in column 10.
- Add 50µl NA-Star buffer to column 12 and 25µl NA-Star buffer to column 11.
- Add 25µl of virus dilution to each well in columns 1-11. (Start at column 11 and add towards column 1.)
- Tap plates gently on each side to mix virus and drug dilutions.
- Incubate the plates at 37°C for 30 minutes.
- Add 10µl of NA-Star substrate to each well. Start from column 12 back to column 1.
- Tap plates gently on each side to mix NA-Star substrate with virus and drug dilutions.
- Incubate the plates at room temperature for 30 minutes.
- Stagger the addition of NA-Star substrate if doing multiple plates to account for the time it takes to read each plate (~7 minutes).
- Place plate in the luminescence plate reader and start the relevant program to be used on your particular piece of equipment. The program will need to add 60µl of NA-Star Accelerator to each well of the plate and will need to measure the readout of the well after a 1 second delay.

Data Processing:

- After reading the plate export the data as an excel file and use the required validated software to calculate the IC50 values.