USER GUIDE

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NA-Fluor™ Influenza Neuraminidase Assay Kit

For use with:

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Revision B

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About this guide

IMPORTANT! Before using this product, read and understand the information the "Safety" appendix in this document.

Revision history

Revision	Date	Description
A	August 2010	
B	November 2013	Removed note that states enzyme incubation can be shortened or lengthened. Removed note that states the assay can be performed in real-time without the addition of NA-Fluor™ Stop Solution. Updated Safety appendix and Documentation and support.

NA-Fluor™ Influenza Neuraminidase Assay Kit

Product information

Purpose of the product

The NA-Fluor™ Influenza Neuraminidase Assay Kit (Cat. no. 4457091) provides the NA-Fluor™ fluorescent substrate, assay reagents, and protocols to measure neuraminidase (NA) activity from influenza virus. The assay is a direct, functional enzyme assay that detects both known and new mutations that affect neuraminidase inhibitor (NI) drug sensitivity. It uses the MUNANA substrate and generates data that can be compared with data generated using standard Neuraminidase Inhibitor Susceptibility Network (NISN) (**www.nisn.org**) IC_{50} determination protocols.

The assay kit has been optimized for monitoring the effect of NIs on neuraminidase enzyme activity, a method widely used by research or public health laboratories for influenza virus NI susceptibility screening. However, the NA-Fluor™ Influenza Neuraminidase Assay Kit can be used for any application that has been demonstrated using traditional MUNANA assays. These applications include high-throughput screening to identify novel NIs during lead discovery, monitoring NA activity from non-viral sources, or monitoring NI sensitivity in cell-based virus growth or inhibition assays. For more information, see "MUNANA-based neuraminidase assays" on page 25.

Note: Life Technologies also offers a chemiluminescence-based neuraminidase assay kit: NA-XTD™ Influenza Neuraminidase Assay Kit (Cat. no. 4457535).

About this protocol

This protocol provides procedures for:

- Viral titering based on NA activity
- Neuraminidase enzyme inhibition assays

The procedures in this protocol have been developed and optimized based upon various Neuraminidase Inhibitor Susceptibility Network (NISN) published IC_{50} protocols.

Kit contents and storage

Reagents for ten 96-well microplates are supplied.

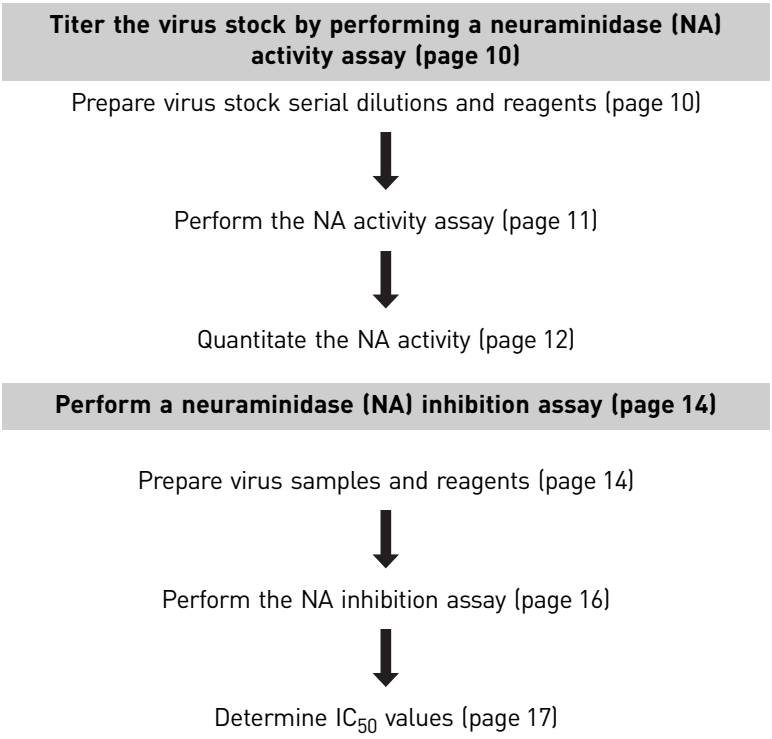
Item	Volume or Quantity	Storage Conditions
NA-Fluor™ Substrate MUNANA (4-(methylumbelliferyl)-N-acetylneuraminic acid)	6.1 mg (5 mL)	–20 °C, protect from light
NA-Fluor™ 2X Assay Buffer 66.6 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, 8 mM $CaCl_2$, pH 6.5	230 mL	2 to 8 °C
NA-Fluor™ Stop Solution, 0.2 M Na_2CO_3	110 mL	2 to 8 °C

Materials and equipment required

Item	Source
Tissue culture supplies and host cell lines for virus culture preparation	Multiple laboratory suppliers (MLS†)
Reference influenza virus strains (NI-resistant and NI-sensitive sub-type matched)	ATCC, NISN, or similar influenza reference laboratory NISN provides information for obtaining virus strain sets at **http://nisn.org/v_virus_reference_panel.html**
Neuraminidase inhibitors (NIs) • Oseltamivir carboxylate • Zanamivir • Other NIs	See **http://nisn.org/v_neuraminidase_inhibitor_drugs.html** for contact information on obtaining NIs. • Roche (Oseltamivir carboxylate) • GlaxoSmithKline (Zanamivir)
Distilled water	Life Technologies Cat. no. 4387937 or Cat. no. 10977015 at **www.invitrogen.com** or similar MLS
4-Methylumbelliferone sodium salt	Sigma-Aldrich M1508 or similar
Black, 96-well, flat-bottom plates with plastic lids	ThermoFisher Scientific NUNC (Cat. No. 237105) or similar MLS
Fluorescence plate reader (excitation range 350 to 365 nm and emission range 440 to 460 nm) (slit widths vary between instruments)	MLS†
Lab equipment • Incubator or plate warmer: 37 °C • Freezer: –20 and –80 °C • Pipettors and tips, single-channel • Pipettors and tips, multi-channel • Microcentrifuge tubes • Reagent reservoirs, single- and multi-well (optional)	MLS†
Data analysis software – Dose-response analysis software, such as GraphPad Prism	GraphPad Software or similar supplier

† For the SDS of any chemical not distributed by Life Technologies, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Workflow


Titer the virus stock by performing a neuraminidase (NA) activity assay (page 10)
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Titer the virus stock by performing a neuraminidase (NA) activity assay

Determine the NA activity of each new virus strain to determine the optimum virus dilution for the neuraminidase inhibition assay.

NA activity, which is the production of product (4-MU) over time (60 min at 37 °C for the standard assay), is determined by comparing relative fluorescence units (RFU) obtained from an NA activity assay to a standard curve of 4-MU concentrations plotted against RFU values (see "Generate a 4-MU(SS) Standard Curve" on page 23).

Before performing an NA activity assay on an instrument for the first time, generate a 4-MU(SS) standard curve to:

- Determine the linear range of fluorescence versus concentration on your instrument.
- Select an RFU range for normalizing viral strains according to NA activity.

Prepare virus stock serial dilutions and reagents

Prepare virus stock

Prepare virus culture supernatants or egg allantoic fluid from single-passage flu virus strains using established culture methods. A comprehensive protocol for influenza virus culture and preparation of viral stocks is available at **http://www.nisn.org/documents/NISN_reference_panel_leaflet.pdf** or **http://www.atcc.org**.

Prepare reagents

1. Reconstitute NA-Fluor™ Substrate to make a 2.5 mM stock solution:
   a. Reconstitute the NA-Fluor™ Substrate powder by injecting 5 mL of distilled water into the substrate vial (yields 2.5 mM stock solution).
   b. Aliquot the 2.5 mM stock solution in 500 µL volumes and store at –20 °C in a non-defrosting freezer protected from light.

      Use stock aliquots upon thaw and discard unused portions.

      Note: A volume of 480 µL of 2.5 mM stock solution is sufficient for one 96-well assay plate.

2. Prepare 20 mL of 1X assay buffer by adding 50:50 (v/v) 2X NA-Fluor™ Assay Buffer and distilled water (volume needed to prepare substrate working solution and making virus stock serial dilutions).

3. Prepare NA-Fluor™ Substrate 200 µM working solution. For one 96-well assay plate, combine 480 µL of 2.5 mM NA-Fluor™ Substrate stock solution and 5.52 mL of 1X assay buffer.

   Note: Protect NA-Fluor™ Substrate working solution from light.

Compatibility with viral inactivation methods

The NA-Fluor™ Assay is compatible with the following viral inactivation methods:

- 0.1% NP-40 added to the virus sample or viral dilution buffer
- 0.2-1% Triton X-100 added to the virus sample (Jonges et al., 2010) or viral dilution buffer
- 40% ethanol in Stop Solution added during the neuraminidase inhibition assay. For volume sufficient for one 96-well assay microtiter plate, combine 4.8 mL 100% ethanol and 7.2 mL NA-Fluor™ Stop Solution.

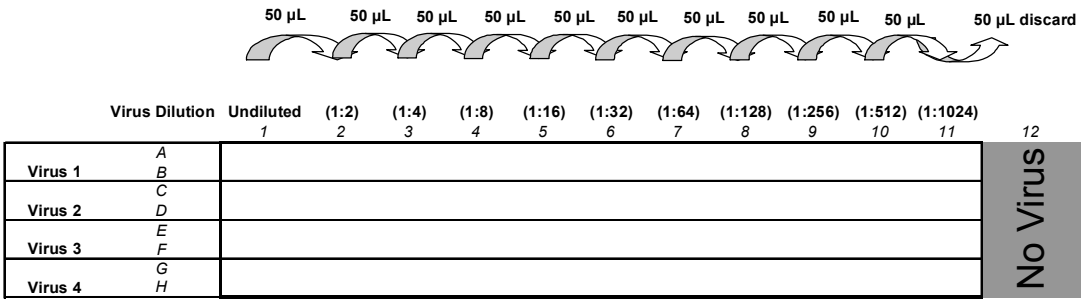
Prepare serial dilutions

Prepare virus stock serial dilutions in a black, 96-well, flat bottom plate. Mix and change tips between dilutions.

1. Add 50 µL of 1X assay buffer to columns 1 to 12 (see Figure 1).
2. Add 50 µL of undiluted virus stock to column 1.
3. Make 2-fold dilutions by transferring 50 µL of undiluted virus stock from column 1 to column 2 and so on, stopping at column 11.
4. Discard 50 µL of volume from column 11.

   Do not add virus to wells in column 12. No Virus Control wells are required on every plate.

Figure 1 Virus stock serial dilutions



Perform the NA activity assay

1. Add 50 µL of 200 µM NA-Fluor™ Substrate working solution to all wells of the plate.
2. Place the lid on the plate, then incubate at 37 °C for 60 minutes, protected from light. Shaking the plate during incubation recommended.
3. Terminate the reaction by adding 100 µL of NA-Fluor™ Stop Solution to each well.

4. Read the plate using an excitation wavelength range of 350 nm to 365 nm and an emission wavelength range of 440 nm to 460 nm.

   Note: The plate can be read immediately, up to 4 hours when stored at room temperature, or up to 3 days when stored at 4 °C and protected from light.

   Note: The NA-Fluor™ assay has been shown to have no phenol red interference.

Quantitate the NA activity

1. Average the relative fluorescence unit (RFU) values of the No Virus Control wells (column 12 in Figure 1 on page 11).

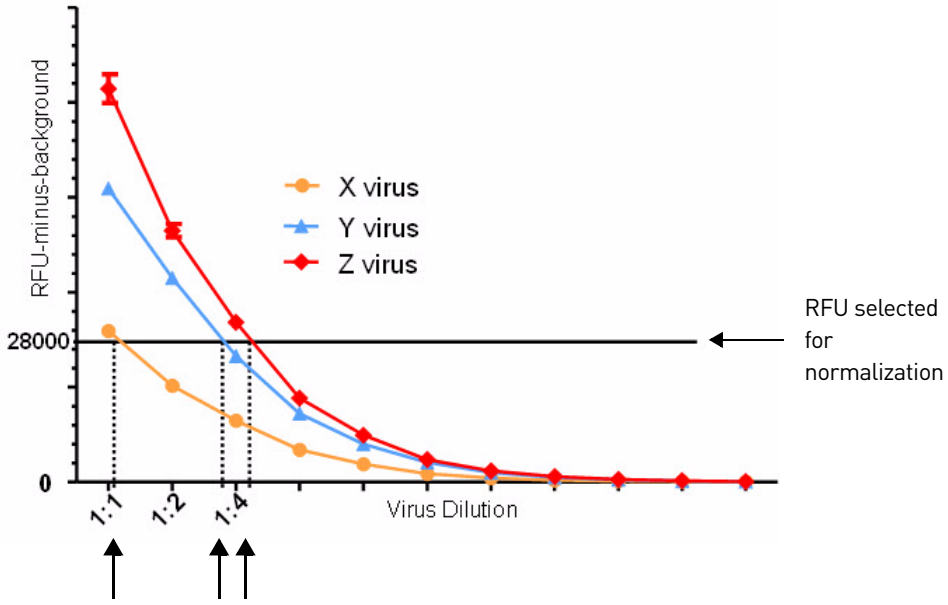
2. Subtract the averaged RFU value from the RFU value for each virus dilution to obtain RFU-minus-background values for wells in column 1 to 11.

3. Plot the virus dilutions versus RFU-minus-background values.

4. For each virus strain, select the dilution factor that yields the RFU value you have identified in your 4-MU(SS) standard curve to use for NA activity normalization.

   Figure 2 shows an example plot in which the NA activity for three viruses is plotted against the fluorescence signal. For this example, a fluorescence signal of 28000 RFU corresponding to 10 µM of 4-MU(SS) from a standard curve has been chosen to normalize NA activity. Virus dilutions of ~1:1 for X virus, <1:4 for Y virus, and >1:4 for Z virus correspond to the 28000 RFU range.

Figure 2 Example NA activity plot



Note the following:

- Samples with signal below the linear range of the 4-MU(SS) standard curve may not have sufficient NA activity for inhibition testing and may give inaccurate IC_{50} values. For best results, use samples with hemagglutinin (HA) titers ≥20 and/or NA activity within the linear range of the 4-MU(SS) standard curve.
- If it is not possible to obtain samples with ideal viral titer or NA activity, you can use low-titer viral samples for IC_{50} determination. To do so, use a viral dilution of the NI-sensitive reference virus or other reference virus strains in which the NA activity is similar to the activity of your low-titer viral sample so that the IC_{50} values can be compared under limited NA activity conditions.
- If you are not able to generate a 4-MU(SS) standard curve, use an RFU value that falls within the linear range of your virus dilution versus RFU-background value curve. Use a viral dilution of each viral strain that corresponds to this RFU value for an NA inhibition assay for best comparison between viral strains within the assay.

Perform a neuraminidase (NA) inhibition assay

Before performing the NA inhibition assay on a new virus strain, titer the virus sample by performing an NA activity assay to determine the optimum dilution of the virus strain (see page 10).

Prepare virus samples and reagents

Reference viruses

The following subtype-matched reference viruses are recommended in each assay run (use reference strains appropriate for virus isolates and NIs you are screening):

- An NI-sensitive strain
- Corresponding strains resistant to zanamivir, oseltamivir carboxylate, or other NI you are using.

Note: If you run multiple plates, reference viruses are not required on all plates.

Prepare samples and reagents

1. Prepare 20 mL of 1X assay buffer by adding 50:50 (v/v) 2X NA-Fluor™ Assay Buffer and distilled water (volume needed to prepare substrate working solution, prepare the optimum dilution of the virus strain determined on page 10, and make NI serial dilutions).
2. Dilute each virus in 1X assay buffer according to the dilution factor determined in the NA activity assay on page 10.
3. Prepare NA-Fluor™ Substrate 200 µM working solution. For one 96-well assay plate, combine 480 µL of 2.5 mM NA-Fluor™ Substrate stock solution and 5.52 mL of 1X assay buffer.

   Note: Protect NA-Fluor™ Substrate working solution from light.

Prepare neuraminidase inhibitors (NIs)

Bring NIs to ambient temperature before preparing.

Prepare master stock

Prepare a 25 mM master stock of each neuraminidase inhibitor (NI):

- Oseltamivir carboxylate (D-tartrate salt, MW = 386.4) is soluble in water. Dissolve 19.3 mg in 2 mL distilled water.
- Zanamivir (MW = 332.3) is soluble in water up to 18 mg/mL. Dissolve 17.5 mg in 2 mL distilled water.
- Other NIs: Prepare and dissolve according to manufacturer recommendations.

Aliquot and store according to manufacturer recommendations. Avoid freezing-thawing.

Prepare working stock

1. Dilute the 25 mM master stock 1:250 (40 µL 25 mM master stock + 10 mL distilled water) to make a 100 µM working stock. Aliquot in 500 µL volumes and store the working stock according to manufacturer recommendations; use working stock aliquots upon thaw and discard unused portions.

2. Prepare NI serial dilutions at 4X final assay concentration in 1X assay buffer.

Dilution	Combine	NI concentration before assay (4X)	NI concentration in final assay
1 (1:2.5)	400 µL WS† NI + 600 µL AB‡	40,000 nM	10,000 nM
2 (1:10)	100 µL Dil 1 + 900 µL AB	4000 nM	1000 nM
3 (1:10)	100 µL Dil 2 + 900 µL AB	400 nM	100 nM
4 (1:10)	100 µL Dil 3 + 900 µL AB	40 nM	10 nM
5 (1:10)	100 µL Dil 4 + 900 µL AB	4 nM	1 nM
6 (1:10)	100 µL Dil 5 + 900 µL AB	0.4 nM	0.1 nM
7 (1:10)	100 µL Dil 6 + 900 µL AB	0.04 nM	0.01 nM
8 (1:10)	900 µL AB	0 nM	0 nM

† Working Stock = 100 µM NI
‡ 1X Assay Buffer

Note: For compound screening for neuraminidase inhibition, prepare compounds at 4X the desired concentration in 1X assay buffer. A final concentration of 5% DMSO can be tolerated in the assay, although all wells should contain the same concentration of DMSO, including No Virus Control wells.

Plate layout

Table 1 Plate layout

Final NI conc (nM)		1	2	3	4	5	6	7	8	9	10	11	12
No NI control	A	Virus 1†		Virus 2†		Virus 3†		Virus 4		Virus 5		No Virus Control‡	
0.01	B												
0.1	C												
1	D												
10	E												
100	F												
1000	G												
10000	H												

† Include reference viruses as appropriate. See "Reference viruses" on page 14 for recommendations.
‡ No Virus Control wells are required on every plate.

Perform the NA inhibition assay

1. Refer to the plate layout in Table 1 on page 15 to prepare the assay plate:

   Note: This procedure adds NI dilutions to the No Virus Control wells to allow you to determine the effect of NI concentration on assay background RFUs. However, neither oseltamivir nor zanamivir at these concentrations affect NA-Fluor™ assay background. If you choose not to add the NI dilutions to the control, add 25 µl of 1X assay buffer to columns 11 and 12 in step a instead of 25 µl of the NI dilution series.

   a. Add 25 µL of the 4X NI dilution series to all rows of a 96-well plate: row A (No NI control) through row H (40000 nM NI).

   b. Add 25 µL of 1X assay buffer to No Virus Control wells in columns 11 and 12.

   c. Add 25 µL of diluted virus sample to columns 1 to 10 (add virus 1 to column 1 and 2, add virus 2 to column 3 and 4, and so on). Ensure that the virus and NI dilutions are mixed.

2. Place the lid on the plate, then incubate for 20 to 30 minutes at 37 °C. Shaking the plate during incubation is recommended.

3. Add 50 µL of diluted 200 µM NA-Fluor™ Substrate working solution to each well.

4. Place the lid on the plate, then incubate for 60 minutes at 37 °C protected from light. Shaking the plate during incubation is recommended.

5. Terminate the reaction by adding 100 µL of NA-Fluor™ Stop Solution to all wells. Stop Solution also enhances signal and stabilizes the fluorescent reaction product.

6. Read the plate using an excitation wavelength range of 350 nm to 365 nm and an emission wavelength range of 440 nm to 460 nm.

   Note: The plate can be read immediately, up to 4 hours when stored at room temperature, or up to 3 days when stored at 4 °C and protected from light.

Determine IC_{50} values

Determine IC_{50} values from dose-response data using sigmoidal curve-fitting or point-to-point plotting. You can use any software that can perform sigmoidal curve-fitting or point-to-point plotting.

See the NISN web site (**http://www.nisn.org**) for detailed information on determining, interpreting, and troubleshooting IC_{50} results, and for criteria for defining NI-resistant virus strains.

1. Average the RFU (relative fluorescence unit) values of the No Virus Control wells.
2. Subtract the averaged RFU value from the RFU value of all wells to obtain RFU-minus-background values.
3. Plot NI concentration (in nM or M) versus RFU-minus-background values using non-linear curve fitting in GraphPad Prism software or other software.

   This plot should yield a sigmoid dose-response curve.

Guidelines for GraphPad Prism Software

This section provides some guidelines for analyzing data if you use GraphPad Prism Software for sigmoidal curve-fitting of dose response data (for more information, see the *GraphPad Prism Step-by-Step Examples Guide* or contact the manufacturer).

1. Select the graph type (typically XY, points only graph with Y error bars) and number of replicates.
2. Enter data:
   a. X Values: enter NI concentrations in molar concentration in exponential notation. Enter a concentration that is 100 to 1000-fold lower than lowest NI dilution to use as the "0" NI value.
   b. Y Values: enter response data in appropriate sets of Y columns. Raw signal intensity (RFU-minus-background dose response) data is exported into software.
3. Data manipulation/analyze:
   a. Log Transform X Values: in Analyze:Transforms dialog box, select X=Log(X)
   b. Normalize Y Values: in the Analyze:Normalize dialog box, define 0% as the smallest values and 100% as the highest values (default), present results as Percentages and Create a new graph.
4. Curve-fitting:
   a. With the graph generated above displayed, click Analyze:Nonlinear regression, select Sigmoidal dose-response (variable slope), (same as "four-parameter logistic" in other graphing programs).
   b. (Optional) Constrain curve-fit parameters by clicking the Constraints tab and set BOTTOM parameter to 0.0 and TOP parameter to 100.0.

      Note: Constraining the parameters may alter the determined IC_{50} value.

5. Graph adjustments:
    a. Values/data from the Results page can be copied/pasted onto the Graph page to display curve-fit parameters on graph.
    b. X-axis and Y-axis adjustments can be made to alter range, to segment the X-axis, change concentration display, etc.

Troubleshoot

Table 2 Neuraminidase inhibition assay troubleshooting

Observation	Possible cause	Recommended action
Higher than expected background fluorescence signal in No Virus Control wells	NA-Fluor™ Substrate not stored at –20 °C	Use properly stored NA-Fluor™ Substrate.
	Reagent contamination with a neuraminidase source (viral, bacterial, fungal, or other source)	Use fresh reagents.
Low signal	Instrument incorrectly set up	Set for an excitation wavelength range of 350 nm to 365 nm and an emission wavelength range of 440 nm to 460 nm.
	Reagent contamination with a neuraminidase inhibitor (NI).	Use fresh reagents.
	Low virus concentration and/or low neuraminidase (NA) activity	Use a higher virus titer or a higher dilution of the same titer. **Note:** If it is not possible to obtain samples with ideal viral titer or NA activity, you can use low-titer viral samples for IC_{50} determination. To do so, use a viral dilution of the NI-sensitive reference virus or other reference virus strains in which the NA activity is similar to the activity of your low-titer viral sample so that the IC_{50} values can be compared under limited NA activity conditions.
		Extend the 60-minute incubation step up to 2 hours.
		Use the NA-XTD™ Influenza Neuraminidase Assay Kit (Cat. no. 4457535) which provides higher detection sensitivity.
The RFU values for the highest NI concentration are not comparable to the RFU values for the No Virus Control (which does not contain virus), and will yield inaccurate IC_{50} values	NI dilution series not optimized	Optimize the NI dilution series using a higher starting concentration of NI. **Note:** Resistant strains may require higher NI concentrations to maximize inhibition.
The RFU values for the lowest NI concentration are not comparable to the RFU values for the No NI Control, and will yield inaccurate IC_{50} values	NI dilution series not optimized	Optimize the NI dilution series so that the lowest NI concentration does not yield inhibition.

Ordering and Support Information

How to order

To place an order online, go to **www.lifetechnologies.com**.

To place an order from the U.S. or Canada, dial 1-800-345-5224, then follow the voice instructions.

To place an order outside the U.S., go to **www.lifetechnologies.com** to locate your nearest Life Technologies office.

Description	Quantity	Cat. no.
NA-Fluor™ Influenza Neuraminidase Assay Kit	Reagents sufficient for 960 assays (100 µL) or ten 96-well assay microplates	4457091

Obtain SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtain support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited product warranty

Life Technologies and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

Generate a 4-MU(SS) Standard Curve

4-Methylumbelliferone (4-MU), the end product of MUNANA cleavage by neuraminidase, can be used to determine the linear range of substrate turnover detection on your specific instrument.

Generate a 4-Methylumbelliferone sodium salt (4-MU(SS)) standard curve to identify an RFU value within the linear range of fluorescence detection on your instrument. For each viral strain, use the dilution factor that best corresponds to the chosen RFU value from the 4-MU(SS) standard curve. This method can be applied for each viral strain to normalize the NA activities of each virus strain for use in the NI assay for best intra-assay comparison of data.

IMPORTANT! Fluorescence units are relative (the total number of RFUs is different on different instruments).

1. Prepare 100 mM stock 4-MU(SS) (Sigma M1508) by adding 198.1 mg to 10 mL of distilled water. To make working solution of 100 µM, make a 1:1000 dilution in distilled water.

   Note: Note: Protect 4-MU(SS) solution from light.

2. Serially dilute 4-methylumbelliferone sodium salt (4-MU(SS)) master stock solution (100 mM, Sigma-Aldrich M1508 or similar) in 2-fold dilutions, in NA-Fluor™ Stop Solution.

Dilution	Combine	Concentration
1 (1:1000)	1 µL WS† + 1000 µL SS‡	100 µM
2 (1:2)	500 µL Dil 1 + 500 µL SS	50 µM
3 (1:2)	500 µL Dil 2 + 500 µL SS	25 µM
4 (1:2)	500 µL Dil 3 + 500 µL SS	12.50 µM
5 (1:2)	500 µL Dil 4 + 500 µL SS	6.25 µM
6 (1:2)	500 µL Dil 5 + 500 µL SS	3.12 µM
7 (1:2)	500 µL Dil 6 + 500 µL SS	1.56 µM
8 (1:2)	500 µL Dil 7 + 500 µL SS	0.78 µM
9 (1:2)	500 µL Dil 8 + 500 µL SS	0.39 µM
10 (1:2)	500 µL Dil 9 + 500 µL SS	0.20 µM
11 (1:2)	500 µL Dil 10+ 500 µL SS	0.10 µM
12 (1:2)	500 µL SS	N/A

† Working Stock (WS) = 100 mM in 4-MU(SS) in distilled water

‡ NA-Fluor™ Stop Solution (SS) is used to enhance fluorescence and provides the same conditions as the NA activity assay.

3. Pipette 200 µL of each serial dilution of 4-MU(SS) in duplicate into a black, 96-well, flat bottom plate. (The standard curve 200 µl volume is equal to the 200 µL of the NA activity assay volume.)

4. Read the plate using an excitation wavelength range of 350 nm to 365 nm and an emission wavelength range of 440 nm to 460 nm.

5. Plot RFU versus 4-MU(SS) concentrations. Figure 3 illustrates a 4-MU(SS) standard curve.

6. Determine the NA activity/RFU range you will use for the NA inhibition assay.

   Example (Figure 4): You decide to perform all NI assays using an NA activity of 10 µM 4-MU/60 min at 37 °C. On the fluorescence reader used to generate the standard curve in this example, 10 µM 4-MU corresponds to ~28,000 RFU. You would then use viral dilutions corresponding to ~28,000 RFU for each viral sample surveyed in an NI assay.

   Note: The choice of 10 µM 4-MU is arbitrary within the linear range. Lower NA-activity equivalents such as 2 µM 4-MU also fall within the linear range of detection in this example. Do not select a concentration that is outside the linear range (for example, 20 µM in the curve below), where instrument signal may be saturated and small changes in fluorescence are not detectable.

Figure 3 4-MU(SS) standard curve, linear range shown in box

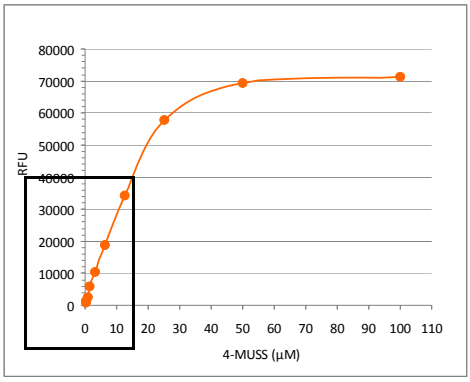
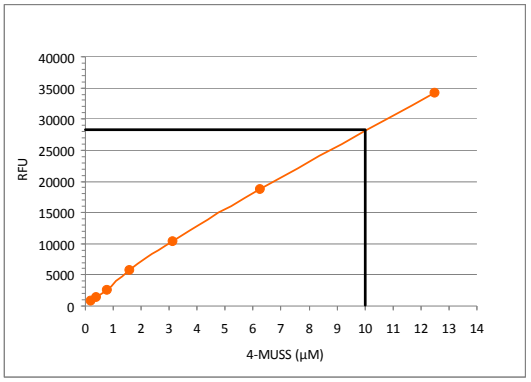


Figure 4 Example: Determine the NA activity/RFU range for the NA inhibition assay using the linear range of 4 MU(SS) standard curve shown in box in Figure 3



Example:
To normalize at 10 µM 4-MU NA activity, use an RFU range ~28,000

Background information

MUNANA-based neuraminidase assays

The NA-Fluor™ Influenza Neuraminidase Assay Kit provides a standardized fluorescence-based assay, which quantitates the fluorogenic end product 4-methylumbelliferone released from the non-fluorescent substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) by the enzymatic activity of neuraminidase. The amount of fluorescence directly relates to the amount of enzyme activity.

MUNANA-based neuraminidase (NA) assays have been used for NA activity quantitation in detection and monitoring of influenza virus growth or growth inhibition in cell-based assays, as well as NA activity from other viruses and organisms, including bacteria, mammals, parasites. MUNANA-based assays have been widely used in high throughput screening for new neuraminidase inhibitors (NIs) and influenza virus surveillance around the globe. Influenza replication has been measured with MUNANA substrate using AVINA assay (**A**ccelerated **V**iral **I**nhibition with **NA** as read-out assay) for drug toxicity evaluation (Eichelberger et al., 2008). AVINA assays developed using MUNANA substrate measures viral growth inhibition in the presence of inhibitory antibodies or compounds (Hassantoufighi, et al., (2010).

Neuraminidase inhibitor (NI) resistance monitoring

Viral neuraminidase is the target of key antiviral drugs effective in treating the influenza strains that are currently considered world-health threats. Influenza virus neuraminidase functions by allowing release of newly formed viral particles from a host cell by cleaving terminal N-acetyl neuraminic acid (sialic acid) which serves as the attachment site between the viral hemagglutinin (HA) protein and glycoconjugates on the host cell surface. Neuraminidase inhibitor (NI) drugs, including oseltamivir (Tamiflu®) and zanamivir (Relenza®), as well as drugs awaiting approval, act by binding competitively to the active site of the neuraminidase, thus inhibiting release and spread of the virus. Mutations in viral neuraminidase have been identified which render the virus resistant to NIs, resulting in the need to survey influenza-strain susceptibility to this class of antiviral drugs. Viral susceptibility or resistance can be monitored by measuring the drug inhibitory effect on NA enzyme activity.

NI resistance monitoring is critical to track treatment efficacy and global spread of resistant viral strains for seasonal, avian, animal and pandemic strains. Increasingly, since the introduction of neuraminidase inhibitor therapeutics in 1999, mutations conferring resistance to neuraminidase inhibitors are arising and spreading, with seasonal A/H1N1 isolates predominantly resistant to oseltamivir globally. Functional neuraminidase inhibition assays enable detection of any resistance mutation, and are therefore extremely important in conjunction with sequence-based screening assays for global monitoring of virus isolates for NI resistance mutations, including known and new mutations.

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at: **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**
- World Health Organization, Laboratory Biosafety Manual, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at: **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**

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