NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit

Protocol

Applied Biosystems

For Research Use Only. Not for use in diagnostic procedures.

Information in this document is subject to change without notice. Applied Biosystems assumes no responsibility for any errors that may appear in this document.

APPLIED BIOSYSTEMS DISCLAIMS ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL APPLIED BIOSYSTEMS BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

Use of this product is covered by US patent 6,555,698 and corresponding patent claims outside the US.

TRADEMARKS:

AB (Design), Applied Biosystems, and NA-*Star* are registered trademarks, and Applera and Celera Genomics are trademarks of Applera Corporation or its subsidiaries in the U.S. and/or certain other countries.

All other trademarks are the sole property of their respective owners.

Part Number 4375714 Rev. B
08/2006

Contents

Preface

This preface covers:

Safety

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below.

Definitions

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD.** Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page vi.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field of the MSDS Search page:
   a. Type in the chemical name, part number, or other information that you expect to appear in the MSDS of interest.
   b. Select the language of your choice.
   c. Click **Search**.

3. To view, download, or print the document of interest:
    a. Right-click the document title.
    b. Select:
        - **Open** – To view the document
        - **Save Target As** – To download a PDF version of the document to a destination that you choose
        - **Print Target** – To print the document
4. To have a copy of an MSDS sent by fax or e-mail, in the Search Results page:
    a. Select **Fax** or **Email** below the document title.
    b. Click **RETRIEVE DOCUMENTS** at the end of the document list.
    c. Enter the required information.
    d. Click **View/Deliver Selected Documents Now**.

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical Waste Hazards

CAUTION **HAZARDOUS WASTE.** Refer to Material Safety Data Sheets and local regulations for handling and disposal.

WARNING **CHEMICAL WASTE HAZARD.** Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

WARNING **CHEMICAL STORAGE HAZARD.** Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide 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.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument 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 **BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is only for submitting comments and suggestions relating to documentation. To order documents, download PDF files, or for help with a technical question, go to **http://www.appliedbiosystems.com**, then click the link for **Support**. (See "How to Obtain Support" below).

How to Obtain Support

Applied Biosystems is committed to meeting the needs of your research through enabling technologies such as the Tropix 1,2-dioxetane chemiluminescence technology. Our support staff is available to answer questions about using this product to its fullest potential.

Contacting Technical Support:

- Phone: 1-800-542-2369, or 1-781-271-0045, then press 2.
- Fax: 1-508-383-7855
- **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

Product Overview

Product Description

Neuraminidase Inhibitor Resistance Assays

Purpose

The purpose of this protocol is to provide instructions to health or research laboratories for using the Applied Biosystems NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit. The protocol describes the required reagents and samples, performing the assay, and how to analyze your results.

About this Protocol

The NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit provides the NA-*Star* chemiluminescent neuraminidase substrate, together with all necessary assay reagents and microplates, to measure the resistance level of influenza virus isolates to neuraminidase inhibitor antiviral therapeutics.

The NA-*Star* chemiluminescent substrate provides highly sensitive detection of neuraminidase enzyme activity from influenza virus types A and B, including human, avian, porcine, and equine viruses (1-6). NA-*Star* substrate is primarily used in screening assays to monitor strain resistance of global influenza isolates to anti-viral agents targeting flu neuraminidase.

Neuraminidase inhibitor resistance monitoring is critical to track treatment efficacy and global spread of resistant viral strains (7, 8). Influenza is a critical global health problem. Highly sensitive, rapid, facile detection assays for viral detection and monitoring, vaccine research and development, and further antiviral therapeutic development are critical for management of this global health threat.

Chemiluminescent Assay Technology

Neuraminidase assays performed with the NA-*Star* 1,2-dioxetane chemiluminescent substrate (sodium (2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5-chloro)tricyclo[3.3.1.1^{3,7}] decan}-4-yl-phenyl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranoside)onate) have been shown to provide 60-fold higher sensitivity than neuraminidase assays with traditional fluorescent substrates, such as MUNANA. Results show good correlation with fluorescent assay results (1, 2). In addition, the chemiluminescent assay provides linear results over three to four orders of magnitude compared to one to two orders of magnitude achieved with the fluorescent assay (2).

The NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit combines highly sensitive and rapid quantitation of neuraminidase activity from influenza virus isolates with a simple assay protocol to provide a convenient method for public health and research laboratories to monitor the resistance levels of influenza virus isolates to neuraminidase inhibitors.

Protocol Overview

Assay

To perform the assay, virus dilutions (from cell culture supernatant) are briefly incubated with neuraminidase inhibitor, and are then incubated for 10-30 min with NA-*Star* substrate. Assay plates are placed in a luminometer (ideally equipped with an on-board injector), and an accelerator solution is injected, immediately triggering light emission from the cleaved NA-*Star* substrate. Light signal intensity is measured within seconds of accelerator injection, using a 0.5 - 1 sec/well read. The entire assay is completed in under 1½ hours.

Data Analysis

To determine the IC50 value of the neuraminidase inhibitor with each viral isolate, use nonlinear curve-fitting, dose-response analysis software (user-supplied).

Additional Applications

The NA-*Star* Influenza Neuraminidase Inhibitor Resistance Detection Kit can also be used for:

- Neuraminidase activity assays, including screening assays for the identification and development of new neuraminidase inhibitors.
- Virus or virus-like particle neuraminidase activity assays used in virus and vaccine research and development.
- Quantitation of neuraminidase activity from other organisms, including bacteria, mammals, other viruses and parasites, in which the neuraminidase activity may play a role in pathogenesis.

Materials and Equipment

Kit Materials

Item	Description	Volume or Quantity
NA-*Star*® Substrate	10 mM substrate concentrate	100 µL (Dilute 1:1000 with NA-*Star* Assay Buffer prior to use)
NA-*Star*® Assay Buffer	26 mM MES (2-[N-Morpholino]ethanesulfonic acid) (pH 6.0), 4 mM $CaCl_2$	480 mL
NA-*Star*® Accelerator	Proprietary solution containing luminescence enhancer	2 × 70 mL
NA-*Star*™ Detection Microplates	Opaque, solid-white 96-well microplates	10 plates
NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit Protocol	–	1

Storage Conditions

Item	Storage conditions
NA-*Star*® Substrate	4 °C
NA-*Star*® Assay Buffer	
NA-*Star*® Accelerator	
NA-*Star*™ Detection Microplates	Room temperature
NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit Protocol	–

User-Supplied Materials and Equipment

Virus Culture (Sample Prep)

- Tissue culture supplies (plates, media) for influenza viral culture
- Host cell lines for influenza virus culture
- Reference influenza strains (NI-resistant and NI-sensitive strains)

Neuraminidase (purified) – Optional

Purified neuraminidase can be used to prepare dilutions for use as a positive control, or to determine dynamic detection range instrument capability.

Bovine serum albumin (BSA) should be added to NA-*Star* Assay Buffer when diluting purified neuraminidases for assays.

Suggested neuraminidase suppliers:

- New England Biolabs [#P0720L (*C. perfringens*) or #P0728S (*S. typhimurium*)]
- Calbiochem [#480717 (*V. cholerae*)]

Suggested BSA supplier: Sigma (#A4503 Fraction V) or similar

Antiviral Agents

Desired neuraminidase inhibitors

Assay Set-up

- Micropipettors (single, multi-channel)
- Pipets
- Tubes for preparing dilutions
- Reagent reservoirs

Equipment

- 37 ° C incubator
- Microplate luminometer (or multimode instrument with top-reading luminescence capability) ideally equipped with on-board injectors (1 or 2 injectors). If necessary, a luminometer without injectors can be used, as long as a 12-channel micropipettor is available (the assay signal intensity and signal/noise will be lower if instrument injector is not used).

Data Analysis

Dose response (nonlinear curve fit) analysis software

Ordering Information

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., please go to **www.appliedbiosystems.com** to locate your nearest Applied Biosystems office.

Description	Quantity	Part Number
NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit	10-plate pack of 96-well microplates + reagents sufficient for 960 assay wells	4374422
NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Reagent Set	Reagents sufficient for 960 assay wells	4374348
NA-*Star*™ Detection Microplates	10-plate pack of 96-well microplates	4374349

Preparing Reagents and Samples

Overview to Preparing Virus Samples

Viral Culture

Prepare cell culture supernatants or egg allantoic fluid from single-passage influenza virus strains using established culture methods. Ideally, the hemagglutination (HA) titer of the virus harvest should be greater than or equal to 16, to provide the most accurate results. Samples with very low virus concentration can result in false positives or unreliable readings.

Note: It is not necessary to determine HA titer if the values are anticipated to be appropriate based on prior experience.

Virus Sample Dilution

Dilute the virus sample in NA-*Star* Assay Buffer as required. Your dilution depends on the level of neuraminidase activity present in a particular virus strain, and on virus production level in the culture. Use a virus sample dilution of 1:5 (for example, 200 µL culture supernatant + 800 µL NA-*Star* Assay Buffer). This overall dilution provides sufficient dilution of phenol red-containing media such that signal interference is minimal (see Important! below). This dilution typically provides a sufficient concentration of virus for the assay. Virus samples can be diluted further if virus cultures contain high levels of virus. Accurate IC50 values have been obtained over a wide range of virus dilutions, so testing of multiple dilutions is not necessary. Total diluted virus sample volume should be 25 µL/well in wells that contain neuraminidase inhibitor. For duplicate rows, a minimum of 550 µL of diluted virus sample to fill wells 1-11 in example layout Figure 1 on page 10, is required, with additional volume needed for a pipetting reservoir.

IMPORTANT! Phenol red from the tissue culture media causes interference with the light signal emission, depending on the concentration of phenol red. Therefore, do not directly compare signal intensities from wells containing different amounts of phenol red. This is not a consideration if the virus sample dilution is identical in all wells. If you are using the detection reagents to perform a virus titration to determine an optimal dilution for IC50 determination, then grow virus cultures in phenol red-free medium. The concentration of phenol red varies within the dilution series, causing different levels of signal interference.

Virus Titration (Neuraminidase Activity) with Assay Protocol (Optional)

The optimal virus sample dilution can be determined by performing the assay protocol, using serial 1:2 dilutions of virus culture supernatant with NA-*Star* Assay Buffer, and omitting addition of neuraminidase inhibitor (use 50 µl/well sample volume). Determine signal:noise by calculating the ratio of the signal for each dilution to the assay noise (culture media from uninfected cells or reagent background). A signal:noise ratio of 40:1 has been found to be optimal for subsequent IC50 determinations (1, 3). For virus titration assays, it is necessary to use phenol red-free media (see Important! above). However, while some users prefer to first determine an optimal virus dilution, the 1:5 dilution suggested above has been found to provide highly consistent results, thus eliminating the need for phenol red-free culture media.

Purified Neuraminidase Sample Prep (Optional)

Dilute purified neuraminidase in NA-*Star* Assay Buffer containing 100 µg/mL BSA (prepare using 10 mg/mL BSA stock [100X]). Neuraminidase quantitation in the range of sub-pM to nM is typically achieved, depending on the specific activity of the enzyme preparation.

Neuraminidase Inhibitor Dilution

Prepare serial dilutions (10 half-log dilutions, or as desired) of neuraminidase inhibitors in NA-*Star* Assay Buffer, such that dilutions are 2× the final desired concentration, and total volume prepared is sufficient for the number of plates being run with excess for pipetting. Total volume of NI dilution should be 25 µL/well. Place neuraminidase inhibitor dilutions (or NA-*Star* Assay Buffer control) into wells of a source plate aligned for pipetting into the assay plate. Dilution 1 should be placed in wells in column 1 of the assay plate, dilution 2 should be placed in column 2, and so forth.

For compound screening, prepare compounds at 2× the desired concentration in a total volume of 25 µL of NA-*Star* 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 control wells.

Example of a Half-log Neuraminidase Inhibitor Dilution Series

Stock = 50 µM neuraminidase inhibitor

Dilution #	Dilution	Use	nM
1	1:25	30 µL 50 µM stock NI + 720 µL NA-*Star* Assay Buffer	2000
2	1:3.16	250 µL Dilution 1 + 540 µL NA-*Star* Assay Buffer	633
3		250 µL Dilution 2 + 540 µL NA-*Star* Assay Buffer	200
4		250 µL Dilution 3 + 540 µL NA-*Star* Assay Buffer	63.4
5		250 µL Dilution 4 + 540 µL NA-*Star* Assay Buffer	20
6		250 µL Dilution 5 + 540 µL NA-*Star* Assay Buffer	6.3
7		250 µL Dilution 6 + 540 µL NA-*Star* Assay Buffer	2
8		250 µL Dilution 7 + 540 µL NA-*Star* Assay Buffer	0.64
9		250 µL Dilution 8 + 540 µL NA-*Star* Assay Buffer	0.20
10		250µL Dilution 9 + 540 µL NA-*Star* Assay Buffer	0.06

NA-*Star* Substrate Dilution

Note: Prior to each opening of the NA-*Star* Substrate concentrate, place the tube in the microcentrifuge and spin briefly to ensure that all volume is at the bottom of the tube.

Dilute NA-*Star* Substrate 1:1000 in NA-*Star* Assay Buffer immediately before use. Prepare only enough volume for immediate use (10 µL per well plus additional volume as needed for injector priming or reagent reservoir). Do not save any unused diluted NA-*Star* substrate or return any reagent to the stock tube.

Example of Diluting NA-*Star* Substrate

For one 96-well plate, prepare a total of 4 mL of diluted NA-*Star* substrate (4 µL NA-*Star* Substrate + 4 mL NA-*Star* Assay Buffer). This volume provides 960 µL total volume required for the assay wells with ~3 mL excess volume for the reagent pipetting reservoir or instrument injector priming (excess volume required should be determined based on particular instrument priming consumption).

Assay Protocol

Plate Layout

Below is an example of a plate layout. Use column(s):

- 1-10 to determine the IC50.
- 11 to provide a readout of viral neuraminidase activity in the absence of neuraminidase inhibitor (untreated).
- 12 to provide a background or noise readout (signal resulting from combination of reagent background and presence of any non-viral neuraminidase activity).

Column contents:

- 1 through 11 contain virus dilutions
- 12 contains the identical dilution of cell culture media from uninfected cells (or NA-*Star* Assay Buffer control).

In the example shown below, the virus isolate is aliquotted in duplicate rows.

		+ vir											- vir
virus		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	**A**	1000	317	100	32	10	3.2	1	0.32	0.1	0.03	0	0
1	**B**	1000	317	100	32	10	3.2	1	0.32	0.1	0.03	0	0
2	**C**	1000	317	100	32	10	3.2	1	0.32	0.1	0.03	0	0
2	**D**	1000	317	100	32	10	3.2	1	0.32	0.1	0.03	0	0
3	**E**	1000	317	100	32	10	3.2	1	0.32	0.1	0.03	0	0
3	**F**	1000	317	100	32	10	3.2	1	0.32	0.1	0.03	0	0
4	**G**	1000	317	100	32	10	3.2	1	0.32	0.1	0.03	0	0
4	**H**	1000	317	100	32	10	3.2	1	0.32	0.1	0.03	0	0

Figure 1 Example of a plate layout showing the final concentration of neuraminidase inhibitor (nM) in each well

Adding Neuraminidase Inhibitor and Virus Sample

To add neuraminidase inhibitor and virus sample to designated wells:

1.	Add: • 25 µL of diluted inhibitor in NA-*Star* Assay Buffer to specified wells (columns 1 through 10) and • 25 µL of NA-*Star* Assay Buffer to control wells without neuraminidase inhibitor (columns 11 and 12 in Figure 1).
2.	Add: • 25 µL of diluted virus sample per well in duplicate in columns 1 through 11 (see Figure 1). and • 25 µL of uninfected culture supernatant diluted in NA-*Star* Assay Buffer to some wells (column 12 in Figure 1) as a negative control for assay background (noise). **Note:** If assay is being used for virus titration only (no inhibitor), then use 50 µL/well of virus sample.
3.	Place the lid on the plate, shake the plate for a few seconds on a plate shaker to ensure that well contents are mixed, and incubate for 10-20 min at 37 °C.

Adding NA-*Star* Substrate

Add diluted NA-*Star* Substrate using either the instrument injector or multichannel pipettor. For optimal accuracy and minimal signal variability across the plate, add NA-*Star* Substrate to each well in the same order and in a similar time frame as you will subsequently inject NA-*Star* Accelerator (such that the substrate incubation time is identical for all wells). To add diluted NA-*Star* Substrate, use a:

- Multi-channel micropipettor, timing each row or column addition to correspond to the order and time required for subsequent injection and read.

  or

- Instrument injector, timing each row or column as for micropipettor above. It is not necessary to measure light emission immediately following substrate injection (there is very minimal light emission until addition of accelerator solution to assay), but some instruments may require measurement coupled with injection (in this case, use the shortest read time possible, and discard the data).

WARNING **CHEMICAL HAZARD**. **NA-*Star* Accelerator** causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To add substrate:

1.	Add 10 µL of diluted NA-*Star* Substrate to each well.
2.	Incubate: a. Place the lid on the plate. b. Shake the plate for a few seconds on a plate shaker to ensure that the contents are mixed. c. Incubate for 10-30 min at room temperature.

Measuring the Chemiluminescent Signal

To measure the chemiluminescent signal:

1.	Prime the luminometer injector with the NA-*Star* Accelerator (required priming volume depends on your particular instrument). **Note:** Do NOT place injector tubing directly into the stock bottle of NA-*Star* Accelerator or return unused NA-*Star* Accelerator to the stock bottle. **Note:** For optimum assay signal intensity and sensitivity, use a luminometer with an on-board injector to add NA-*Star* Accelerator. NA-*Star* Accelerator is injected into a single well and light signal is measured within seconds, prior to injection/read in the next well.

To measure the chemiluminescent signal:

2.	Set up your instrument control software parameters: a. Set the injector to dispense 60 µL. b. Add a delay of 2 sec. c. Set the read time to 0.5 - 1 sec/well. **Note:** Read temperature is at ambient. Have the instrument temperature control turned off. **Note:** After the NA-*Star* Accelerator addition, the resulting light signal peaks within 10-12 seconds at ambient temperature (~25 °C) and then decays with a half-life of ~5 min. For most accurate results, read each well immediately (2 sec) after injection. **Note:** For manual addition of NA-*Star* Accelerator: manually add NA-*Star* Accelerator with a multi-channel pipettor if a luminometer with automatic injector is not available. Add NA-*Star* Accelerator quickly to the plate, adding row-wise or column-wise depending on the order that wells are read. Then, place the plate immediately into the luminometer and begin chemiluminescent signal measurement. Set read time for 1.0 sec/well. The resulting signal is being measured as it decays, so the signal intensity and signal/noise will not be as high as with the use of the instrument injector.
3.	Place the assay plate into the luminometer.
4.	Inject 60 µL of NA-*Star* Accelerator into each well and measure the chemiluminescent signal as set up in step 2.
5.	Save your data file for importing into your data analysis tool.

Analyzing Data

Quantitation of Neuraminidase Activity

To determine optimal virus titration (dilution) using the neuraminidase activity:

1.	To analyze the data, divide the signal (value for each virus dilution) by noise (value from non-virus-containing control well).
2.	Plot Signal/Noise versus enzyme amount (or virus dilution) on a log-log graph.

Calculating IC50 of Neuraminidase Inhibitor

To calculate the IC50 for neuraminidase inhibitor for a specific viral strain:

1.	Prepare averaged signal intensity data, if multiple replicates of each drug dilution have been assayed.
2.	Export averaged signal intensity (dose response) data to analysis software that enables nonlinear curve fitting and IC50 calculation (for example, GraphPad Prism).

References

1. Wetherall, NT, T Trivedi, J Zeller, C Hodges-Savola, JL McKimm-Breschkin, M Zambon and FG Hayden (2003). Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: Report of the Neuraminidase Inhibitor Susceptibility Network. *J Clin Microbiol* 41(2):742-750.

2. Mungall, BA, X Xu and A Klimov (2003). Assaying susceptibility of avian and other influenza A viruses to Zanamivir: Comparisons of fluorescent and chemiluminescent neuraminidase assays. *Avian Diseases* 47:1141-1144.

3. Buxton, RC, B Edwards, RR Juo, JC Voyta, M Tisdale and RC Bethell (2000). Development of a sensitive chemiluminescent neuraminidase assay for the determination of influenza virus susceptibility to Zanamivir. *Anal Biochem* 280:291-300.

4. McKimm-Breschkin, J, T Trivedi, A Hampson, A Hay, A Klimov, M Tashiro, F Hayden and M Zambon (2003). Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to Zanamivir and Oseltamivir. *Antimicrobial Agents and Chemotherapy* 47(7):2264-2272.

5. Boivin, G and N Goyette (2002). Susceptibility of recent Canadian influenza A and B virus isolates to different neuraminidase inhibitors. *Antiviral Research* 54:143-147.

6. Jackson, D, W Barclay and T Zurcher (2005). Characterization of recombinant influenza B viruses with key neuraminidase inhibitor resistance mutations. *J Antimicrobial Chemotherapy* 55(2):162-169.

7. Mungall BA, X Xu and A Klimov (2004). Surveillance of influenza isolates for susceptibility to neuraminidase inhibitors during the 2000-2002 influenza seasons. *Virus Research* 103:195-197.

8. The World Health Organization Global Influenza Program Surveillance Network (2005). Evolution of H5N1 avian influenza viruses in Asia. *Emerging Infectious Diseases* 11(10):1515-1521.

9. Aymard, M, O Ferraris, L Gerentes, J Jolly and N Kessler (2003). Neuraminidase assays. *Developmental Biology* 115:75-83.

10. Hurt, AC, JL McKimm-Breschkin, M McDonald, IG Barr, N Komadina and AW Hampson (2004). Identification of a human influenza type B strain with reduced sensitivity to neuraminidase inhibitor drugs. *Virus Research* 103:205-211.

Worldwide Sales and Support

Applied Biosystems vast distribution and service network, composed of highly trained support and applications personnel, reaches 150 countries on six continents. For sales office locations and technical support, please call our local office or refer to our Web site at **www.appliedbiosystems.com**.

Applera is committed to providing the world's leading technology and information for life scientists. Applera Corporation consists of the Applied Biosystems and Celera Genomics businesses.

Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: +1 650.638.5800
Toll Free (In North America): +1 800.345.5224
Fax: +1 650.638.5884

08/2006

Applied Biosystems

Part Number 4375714 Rev. B