NA-Fluor™ and NA-XTD™ Influenza Neuraminidase Assays for Neuraminidase Quantitation and Inhibition Assays

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OVERVIEW

Applied Biosystems® NA-Fluor™ and NA-XTD™ Influenza Neuraminidase Assays:

- ✶ Applications:
  - √ Measure virus sensitivity to neuraminidase inhibitors (NIs)
  - √ Identify new candidate NIs or anti-viral compounds
- ✶ Results:
  - √ High sensitivity and wide dynamic range
  - √ Neuraminidase inhibitor IC_{50} values in benchmarking to traditional assay protocols
  - √ Signal kinetics and stability provides assay format flexibility and ease-of-use
  - √ Excellent Z' values for high throughput screening assay capability
  - √ Cell-based viral growth inhibition assay demonstrated

INTRODUCTION

The Applied Biosystems® NA-Fluor™ and NA-XTD™ Influenza Neuraminidase Assay Kits are optimized for direct quantitation of neuraminidase inhibitor (NI) resistance by monitoring neuraminidase (NA) activity. These assays can also be used in the identification or characterization of novel NIs or other antiviral compounds during lead discovery. Global monitoring of influenza strains for NI-resistance is essential for understanding NI efficacy for seasonal, pandemic or avian influenzas, and studying the epidemiology of resistance mutations. The rapid spread of seasonal H1N1 virus oseltamivir-resistance to 98.5% in the US (1) highlights the importance of resistance monitoring and the need for continued development of new influenza anti-virals.

The fluorescent NA-Fluor™ assay uses the traditional 2'-[4-methylumbelliferyl]-α-D-N-acetylneuraminic acid (MUNANA) substrate. Assay reagents and protocol were optimized based on Neuraminidase Inhibitor Susceptibility Network (NISN) protocols to provide an economical, standardized and easy-to-use assay kit that generates IC_{50} data comparable to historical MUNANA assay data. The NA-Fluor™ assay signal is stable up to several days after assay termination, enabling read-time flexibility and provides a typical Z' value of 0.8, for excellent high throughput screening assay capability. In addition, the NA-Fluor™ assay can be conducted in a real-time format for enzyme kinetic studies.

The chemiluminescent NA-XTD™ assay provides the next-generation NA-XTD™ 1,2-dioxetane neuraminidase substrate. Like the first-generation chemiluminescent NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit, the NA-XTD™ assay provides highly sensitive neuraminidase quantitation with a wide assay dynamic range. The NA-XTD™ assay provides extended-glow light emission. IC50 values determined either immediately or several hours after assay completion are identical, and Z' values of 0.5 – 0.86 are obtained, providing excellent high throughput screening assay capability. The NA-XTD™ assay is also used to quantitate influenza NA activity directly in cell-based virus cultures to monitor viral growth or inhibition.

In addition to NI resistance quantitation (1-7), additional applications include identification of new NI compounds (8), NI characterization (9), studies of virus transmission (10), neuraminidase-based quantitation of virus-like particles (11), and cell-based virus quantitation (12).

MATERIALS AND METHODS

The NA-Fluor™, NA-XTD™ and NA-*Star*® assay kits are supplied by Life Technologies. The NA-Fluor™ assay (P/N 4457091) includes 1) NA-Fluor™ 2X Assay Buffer, 2) NA-Fluor™ Substrate (solid powder) and 3) NA-Fluor™ Stop Solution. The NA-XTD™ assay (P/N 4457535) includes 1) NA Sample Prep Buffer (contains Triton X-100), 2) NA-XTD™ Assay Buffer, 3) NA-XTD™ Substrate concentrate, 4) NA-XTD™ Accelerator and 5) NA-*Star*™ Detection Microplates (solid-white 96-well microplates). Influenza strains include type A/H1N1 (VR-1469™ (human), VR-1520™ (human), VR-1682™ (swine)) and B/Lee/40 (VR-1535) from ATCC (Manassas, VA) and A/H1N1/Texas/36/91, wild-type sensitive and H275Y mutant oseltamivir-resistant strains, (kindly provided by the CDC, Atlanta GA). Assays were performed according to their respective protocols.

NA-Fluor™ Assay: 25 µL diluted virus (in 1X Assay Buffer)
25 µL NI dilution (in 1X Assay Buffer), pre-incubate x 30 min at 37°C
50 µL NA-Fluor™ substrate, incubate x 60 min at 37°C
100 µL Stop Solution, read with Ex 355 nm/Em 460 nm

NA-XTD™ Assay: 25 µL diluted virus (in Assay Buffer)
25 µL NI dilution (in Assay Buffer), pre-incubate x 30 min at 37°C
25 µL 1:1000 NA-XTD™ substrate (in Assay Buffer), incubate x 30 min at r.t.
60 µL Accelerator, read 1 sec/well

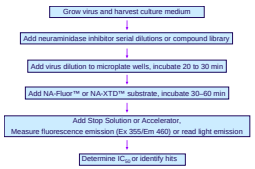
Fluorescent assays and chemiluminescent were both performed with the SpectraMax M5 multimode microplate reader (Molecular Devices, Sunnyvale, CA). IC_{50} analysis was performed using GraphPad Prism® dose-response curve-fitting.

ACKNOWLEDGEMENTS

Influenza A/Texas/36/91 (H1N1) wild-type and H275Y strains were kindly provided by Dr. Larisa Gubareva (Influenza Branch, CDC, USA).
Oseltamivir carboxylate was kindly provided by F. Hoffmann-La Roche Ltd (Basel Switzerland), and zanamivir was kindly provided by GlaxoSmithKline (Research Triangle Park, NC).

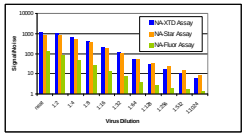
RESULTS

Figure 1. Neuraminidase Inhibitor IC_{50} Determination Assay Workflow



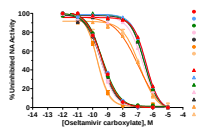
Assay workflow for use of either the NA-Fluor™ or NA-XTD™ assay for NI IC_{50} determination or lead identification with viral isolates.

Figure 2. Sensitivity Comparison of NA-Fluor™, NA-XTD™ and NA-*Star*® Assays



Serial dilutions of influenza A/WS/33 (H1N1) were measured with each assay. The NA-XTD™ and NA-*Star*® assays provide similar Signal/Noise and low-end detection sensitivity, with better sensitivity and a wider assay dynamic range than the NA-Fluor™ MUNANA-based assay. Higher enzyme detection sensitivity enables use of lower virus concentrations for IC_{50} or HTS assays.

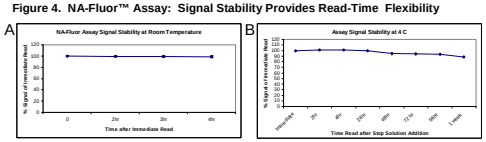
Figure 3. Comparison of NA-Fluor™ Assay to Other Assays for IC_{50} Determination



Protocols	Wild Type (WT) IC50 (nM)	Mutant (H275Y) IC50 (nM)	Fold Resistance to Oseltamivir
NA-Fluor	0.42	165	389
NISN 1	0.47	327	696
NISN 2	0.41	271	663
NISN 3	0.55	217	639
NISN 4	0.36	279	775
NA-Star	0.2	115	575
NA-XTD	0.19	101	711

Protocols were run in parallel to determine IC_{50} values of influenza A/Texas/36/91 (H1N1) oseltamivir-sensitive and resistant H275Y strains for oseltamivir carboxylate. The NA-Fluor™ assay provides IC_{50} values and sensitivity similar to NISN-published, MUNANA-based protocols (12) and can be compared with historical data sets based on these protocols. IC_{50} values obtained with the NA-XTD™ and NA-*Star*® assays are similar, but can be slightly lower than values obtained with MUNANA-based assays.

Figure 4. NA-Fluor™ Assay: Signal Stability Provides Read-Time Flexibility



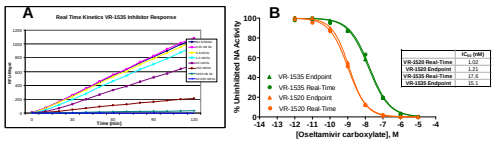
NA-Fluor™ signal read-out remains stable for hours and up to days providing flexibility and batch-mode capability for high throughput applications. A) Signal stability at room temperature (25° C) following addition of NA-Fluor™ Stop Solution. B) Signal stability with plate storage at 4° C following stop solution addition. Fluorescent signal shown as % of the immediate assay read-out. IC_{50} values determined using data collected up to 3 days after assay completion (plates stored at 4°C) are identical (data not shown).

Table 1. NA-Fluor™ Assay: Suitability for High Throughput Compound Screening

Virus Strain/Dilution	Read Time	Signal/Noise (- NI RFU/+NI RFU)	%CV (+NI)	%CV (-NI)	Z'	HTS Suitability
VR-1520/1:10	immediate	8.5	4.3	5.4	0.78	Excellent
	2 hr	8.4	3.7	5.1	0.8	Excellent
	3 hr	8.4	3.6	4.9	0.8	Excellent
	4 hr	8.4	3.6	4.9	0.8	Excellent

The NA-Fluor™ assay demonstrates low intra-assay signal variability and provides a Z' value of 0.78 or greater, consistent across multiple read times, indicating that it has excellent quality as a high throughput screening assay for identification of candidate neuraminidase inhibitor compounds.

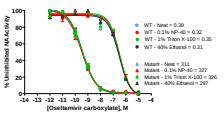
Figure 5. NA-Fluor™ Assay: Real-Time vs. End-Point Assay Format



	IC_{50} (nM)
VR-1520 Real-Time	1.02
VR-1520 Endpoint	1.21
VR-1535 Real-Time	17.6
VR-1535 Endpoint	15.1

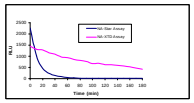
The NA-Fluor™ assay can be run in either endpoint or real-time kinetic mode. A) Substrate turn-over rate is linear for >2 hours enabling flexibility in assay format. Real-time acquired RFU values are typically 5-6 fold lower than RFUs acquired after addition of stop solution at the same time point. B) Oseltamivir IC_{50} values determined with either real-time data by slope analysis (no stop solution addition) or a 60 minute end-point read-out (with stop solution) read-time for influenza A/WS/33 (H1N1) or influenza B/Lee/40 are nearly identical.

Figure 6. NA-Fluor™ Assay: Compatibility with Viral Inactivation Methods



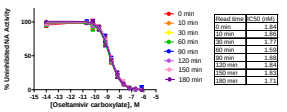
The NA-Fluor™ Assay is compatible with standard methods used to inactivate virus (13). Oseltamivir IC_{50} values with influenza A/Texas/36/91 (H1N1) sensitive and oseltamivir-resistant mutant (H275Y) strains were determined in the presence of 0.1% NP-40 or 1% Triton X-100 (in assay), and 40% ethanol (in stop solution).

Figure 7. NA-XTD™ Assay: Extended-glow Light Emission Kinetics



The half-life of light emission following addition of NA-XTD™ Accelerator (T=0) with the NA-XTD™ assay is ~2 hours, compared to ~10 minutes with the NA-*Star*® assay. The longer-lived light emission kinetics eliminates the need for accelerator injection and luminometer instrumentation equipped with on-board injectors.

Figure 8. NA-XTD™ Assay: Read-Time Flexibility



Read time	IC_{50} (nM)
0 min	1.84
10 min	1.65
30 min	1.77
60 min	1.59
90 min	1.88
120 min	1.84
150 min	1.83
180 min	1.71

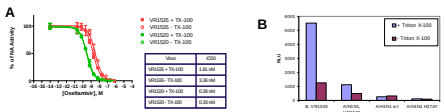
IC_{50} values were determined using data collected up to 3 hours after addition of NA-XTD™ Accelerator. The IC_{50} curves and values are nearly identical at each time point, using influenza B/Lee/40.

Table 2. NA-XTD™ Assay: Suitability for High Throughput Compound Screening

Virus Strain/Dilution	Read Time (min)	Signal/Noise (- NI RLU/+NI RLU)	%CV (+NI)	%CV (-NI)	Z'	HTS Suitability
VR-1520/1:10	10	36	13.9	4.6	0.84	Excellent
	20	36	15	3.9	0.86	
	100	38	19.6	4.8	0.83	
VR-1520/1:100	10	39	25.7	7.3	0.7	Excellent
	20	28	23.6	10	0.62	
	100	63	35.1	10.6	0.57	
VR-1682/1:50	10	27	22.2	7.1	0.67	Excellent
	20	20	32.6	7.8	0.61	
	100	14	24.2	10.6	0.5	

The NA-XTD™ assay provides Z' values of 0.5-0.86, determined with two different virus strains at multiple virus concentrations, and similar across multiple read times, indicating excellent quality as a high throughput screening assay for identification of candidate neuraminidase inhibitor compounds.

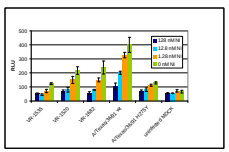
Figure 9. NA-XTD™ Assay: Compatibility with Triton® X-100 Addition to Virus Sample



Virus	IC50
VR1535 + TX-100	1.81 nM
VR1535 - TX-100	2.30 nM
VR1520 + TX-100	0.30 nM
VR1520 - TX-100	0.30 nM

Triton X-100 detergent at 1% has been shown to inactivate flu virus while increasing neuraminidase activity (13). A) NA Sample Prep Buffer (containing 10% Triton X-100) was added to virus stocks (to 1% final), virus was diluted, and IC_{50} values determined with oseltamivir carboxylate. IC_{50} values are very similar following addition of Triton X-100. B) NA activity of virus dilutions +/- Triton X-100. Typically, a 2-4-fold increase in NA activity is obtained with Triton X-100 addition to virus stock.

Figure 10. NA-XTD™ Assay: Cell-Based Virus Growth Inhibition Assay



The NA-XTD™ Assay was used to monitor cell-based virus growth inhibition similar to the described AVINA (**A**ccelerated **V**iral **I**nhibition with **NA** as read-out) assay (12). MDCK 96-well microplate cell cultures were infected with indicated virus strains, followed by incubation in the presence of varying concentrations of oseltamivir carboxylate. Samples of culture media were assayed 24 hours later. Quantitation of NA activity with the NA-XTD™ assay demonstrates inhibition of viral growth by oseltamivir in cell culture. This cell-based assay capability can be used for identification of compounds inhibiting virus growth/replication or quantitation of antibody-based viral growth inhibition.

CONCLUSIONS

The Applied Biosystems® NA-Fluor™ Influenza Neuraminidase Assay Kit, based on the widely-used fluorescent MUNANA substrate, provides a standardized, easy-to-use, cost-effective assay system ideal for high throughput viral or compound screening. The NA-XTD™ Influenza Neuraminidase Assay Kit is a next-generation chemiluminescent neuraminidase assay providing high assay sensitivity and "glow" light emission kinetics for improved ease-of-use and high throughput capability.

Together these assays offer:

- ✶ Standardized reagents and protocols for:
  - Neuraminidase inhibitor (NI) resistance screening
  - High throughput identification of new NIs
- ✶ Choice of fluorescent or chemiluminescent detection technology
- ✶ Simple instrumentation requirements
- ✶ High sensitivity for use with low virus concentrations
- ✶ Compatibility with batch-mode processing and large-scale assay throughput required for HTS
- ✶ Excellent assay quality (Z') for high-throughput screening new NI lead identification
- ✶ Compatibility with virus inactivation reagents
- ✶ Broad specificity of influenza detection
- ✶ Flexibility in assay format and read-time
- ✶ Additional NA assay applications
  - Cell-based viral growth and inhibition assays
  - Detection of NA from other organisms (bacteria, viruses)
  - Quantitation of mammalian sialidase

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TRADEMARKS/LICENSING