NA-Fluor™ Influenza Neuraminidase Assay Kit: Standardization of the classic MUNANA-based neuraminidase activity assay for monitoring anti-viral resistance in influenza

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ABSTRACT

Here, we describe a MUNANA-based assay kit, the NA-Fluor™ Influenza Neuraminidase Assay Kit, which provides standardization and reproducibility of IC_{50} methodology. The assay utilizes MUNANA substrate concentration and other conditions comparable to protocols that have been used historically to generate influenza antiviral resistance data. We have benchmarked this assay against the four different MUNANA-based protocols provided on the Neuraminidase Inhibitor Susceptibility Network (NISN) website, http://www.nisn.org/au_about_us.html. The NA-Fluor™ assay gives similar IC_{50} values for oseltamivir carboxylate and zanamivir when compared to NISN protocols for several tested oseltamivir sensitive and resistant viral strains and can be used to detect mutant virus in mixed viral populations. The assay can be performed in either the traditional end-point format or using a real-time, kinetic read and is compatible with typical methods to reduce viral contamination such as the addition of detergents or ethanol. The assay is easy to use, reproducible with a Z' of 0.8, and the fluorescence signal is stable for hours making this assay compatible with processing many different viral isolates in a surveillance screen or for high throughput screening for drug lead identification.

INTRODUCTION

Although neuraminidase inhibitors (NIs) remain an effective treatment for influenza virus strains currently considered world health threats, such as Influenza virus A which includes pandemic H1N1 and Influenza virus B, viral resistance to these drugs threatens their usefulness for future pandemics. Key antivirals oseltamivir carboxylate (Tamiflu®) and zanamivir (Relenza®) target the viral enzyme neuraminidase which is responsible for release of newly generated viral particles from an infected cell's surface, a process necessary for transmission of the virus to other cells within the organism and for transmission between organisms. Oseltamivir resistance in seasonal H1N1 increased from 12.3% during the 2007-2008 flu season to 98.5% in 2008-2009 season (1), heightening concern that drug resistance will likewise become prominent in pandemic viral strains and highlighting the need for antiviral drug resistance surveillance. The H275Y mutation in H1N1 neuraminidase is the most common mutation conferring resistance, however, due to the high mutation rates of viruses, new mutations can be expected that will also render viral neuraminidase less sensitive to antiviral drugs. PCR methods can be used to detect previously identified mutations, however, functional neuraminidase enzyme activity inhibition testing is necessary for detecting drug resistance that results from novel mutations. The two neuraminidase enzyme inhibition assays using either the fluorescent MUNANA or chemiluminescent NA-Star® substrate are robust tools for NI susceptibility testing. The MUNANA-based assay is broadly used by many groups including several world health organizations for NI susceptibility testing, yet no standardized protocol or dedicated kit is in place for this assay, making comparison of data generated between different laboratories difficult.

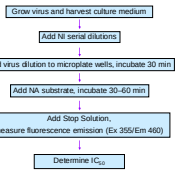
MATERIALS AND METHODS

All assays, unless otherwise noted, were conducted using the standard NA-Fluor™ Influenza Neuraminidase Assay Kit protocol. Basically, NIs were prepared in 10-fold serial dilutions at 4x final concentration in assay buffer (16.65 mM MES, 2mM $CaCl_2$, pH 6.5) and 25 µl were added to wells of a black flat-bottom 96-well microplate. Viral samples were diluted (1:3 to 1:100 depending on virus strain and titer) in assay buffer and 25 µl were added to the NI serial dilutions for preincubation (30 min at 37 C). NA-Fluor™ Substrate was added at 50 µl for a final assay concentration of 100 µM MUNANA and incubated (37 C for 60 min). No-virus controls were included on each assay plate. Assay was terminated by addition of 100 µl of NA-Fluor™ Stop Solution and plates were read on a SpectraMax® M5 or M2 (Molecular Devices) plate reader using Ex 355nm/Em 460nm settings. For data analysis, no-virus control assay well RFUs were subtracted from viral data RFUs and data was processed using either Microsoft Excel or Graphpad® Prism software. For real-time detection, the assay was performed as above but without the stop solution addition and assay reads were taken at 5 min intervals.

Virus samples were titered according to the NA-Fluor™ Assay protocol. Influenza-A/WS/33 (H1N1) (VR-1520™) and influenza B/Lee/40 (VR-1535™) were purchased from ATCC. Other viral strains were gifts (See Acknowledgements.)

RESULTS

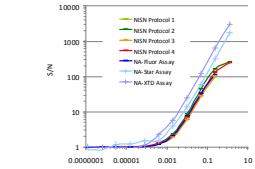
Figure 1. Neuraminidase Inhibition Assay Workflow



Standard workflow for use of the NA Fluor Assay for screening viral isolates for sensitivity to neuraminidase inhibitors.

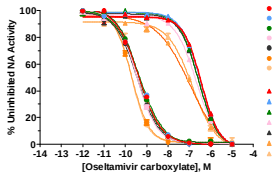
Comparison to Traditional MUNANA Assays

Figure 2. Comparison of NA-Fluor Assay Sensitivity



The NA-Fluor™ Assay provides low-end sensitivity (by signal to noise ratio) and dynamic range similar to NISN-published, MUNANA-based protocols. The assay shows ~5 fold less low-end sensitivity than our chemiluminescent assays. All protocols were run simultaneously side by side using serial dilutions of bacterial neuraminidase (C. perf.).

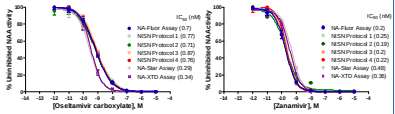
Figure 3. Comparison of NA-Fluor™ Assay to Other Assays for IC_{50} Determination



Protocols	Wild Type (WT) IC50 (nM)	Mutant (H275Y) IC50 (nM)	Fold Resistance to Oseltamivir
NA-Fluor	0.42	365	869
NISN 1	0.47	327	696
NISN 2	0.41	271	661
NISN 3	0.35	217	620
NISN 4	0.16	279	775
NA-Star	0.2	115	575
NA-XTD	0.19	135	711

The NA-Fluor™ Assay provides IC_{50} values and sensitivity for detecting sensitive virus similar to NISN-published, MUNANA-based protocols. All protocols were run in parallel to determine the IC_{50} values of influenza-A/Texas/36/91 (H1N1) sensitive and oseltamivir-resistant mutant (H275Y) strains for oseltamivir carboxylate.

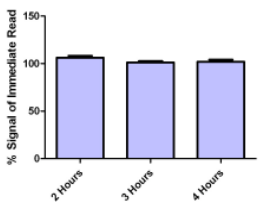
Figure 4. Additional Protocol Comparison



The NA-Fluor™ Assay provides IC_{50} values similar to NISN-published, MUNANA-based protocols. The seven protocols were run in parallel to determine the IC_{50} values of influenza-A/WS/33 (H1N1) for oseltamivir carboxylate (A) or zanamivir (B).

Compatibility for High Throughput Processing

Figure 5. NA-Fluor™ Assay Signal Stability with Time



The NA-Fluor™ Assay signal remains stable over time providing ease of use for high throughput virus processing. An oseltamivir carboxyate IC_{50} determination assay was performed with influenza B/Lee/40 (VR-1535™). Plates were read immediately after addition of NA-Fluor™ Stop Solution and again at the indicated times after storage at room temperature. IC_{50} values did not change over time with storage (data not shown).

Table 1. Reproducibility of NA-Fluor™ Assay

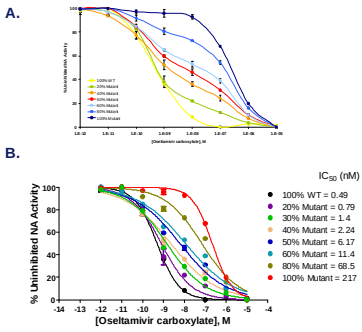
Time (hours)	0	2	3	4
NA-Fluor Ave **% Read-out** (96 reps)	100	106.3	101.2	102.0
St. Dev. (16 no virus reps, 40 no drug reps, 40 10nM drug reps)	0	6.0	1.4	2.2
$Z' = 1 - \frac{3\sigma_b + 3\sigma_s}{\lvert\mu_b - \mu_s\rvert}$	0.78	0.80	0.80	0.80

σ = St. Dev
µ = mean
S = No inhibitor
b = 100% inhibitor

The NA-Fluor™ assay is highly reproducible giving a Z' of 0.78 or above indicating that the assay can be used confidently to identify neuraminidase inhibitors in high throughput screening mode.

Detection of Mutants in Mixed Viral Populations

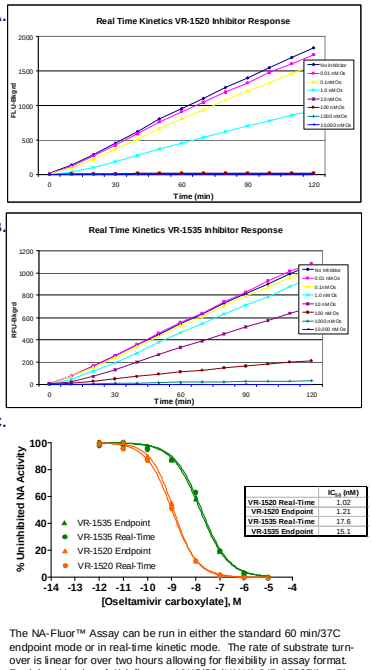
Figure 6. Detection of NI-Resistant H1N1 Virus in Mixed Populations



The NA-Fluor assay provides a large shift in IC_{50} values between sensitive and oseltamivir-resistant virus that allows detection of mutant virus in mixed viral populations. Sensitive and oseltamivir-resistant (H275Y) influenza A/Texas/36/91 (H1N1) strain dilutions were normalized by NA activity. A) Data plotted using point-by-point graphing in Microsoft Exel. B) IC_{50} determination by curve fitting performed using GraphPad® Prism software. Subpopulations displaying drug-resistant mutations can be detected in mixtures of 50:50 sensitive and resistant virus by IC_{50} values alone.

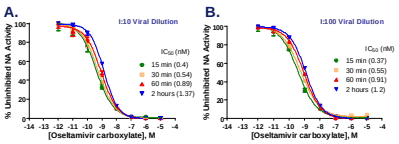
Assay Flexibility

Figure 7. Assay Time Flexibility



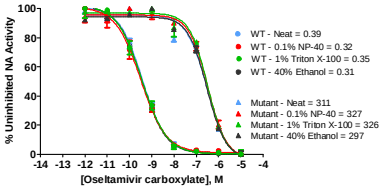
The NA-Fluor™ Assay can be run in either the standard 60 min/37C endpoint mode or in real-time kinetic mode. The rate of substrate turn-over is linear for over two hours allowing for flexibility in assay format. Real-time kinetics of A) influenza-A/WS/33 (H1N1) (VR-1520™) or B) influenza B/Lee/40 (VR-1535™) neuraminidase activity in the presence of varying concentrations of oseltamivir carboxylate. Real-time acquired RFUs are typically 5-6 fold lower than RFUs acquired after addition of stop solution at the same time point. C) Comparison of IC_{50} values for the above strains using real-time data by slope analysis (no stop solution) or a 60 minute end-point read-out (with stop solution).

Figure 8 NA-Fluor™ Assay Flexibility in Time and Virus Dilution



The NA-Fluor™ Assay can be performed over a range of assay times or viral dilutions. Influenza-A/WS/33 (H1N1) (VR-1520™) was assayed using either a 1:10 (A) or a 1:100 (B) for oseltamivir carboxylate sensitivity. Assays were terminated with stop solution at the indicated times. IC_{50} values vary slightly with assay time, likely due to inhibitor off-rates. Similar end-point data can be extracted from real-time assay data (not shown). For this virus strain, IC_{50} values are similar for the 1:10 versus 1:100 viral dilution for various time points.

Figure 9. Compatibility with Viral Inactivation Methods.



The NA-Fluor™ Assay is compatible with standard methods used to inactivate virus. Above, oseltamivir sensitivity of influenza-A/Texas/36/91 (H1N1) sensitive and oseltamivir-resistant mutant (H275Y) strains was determined in the presence of 0.1% NP-40 or 1% Triton X-100 during the assay and in the presence of 40% ethanol in stop solution added to terminate the reaction.

CONCLUSIONS

We have developed a standardized MUNANA-based neuraminidase assay kit, the NA-Fluor™ Influenza Neuraminidase Assay Kit, that has been optimized for NI susceptibility screening. The assay is:

- A standardized MUNANA-based fluorescent assay.
- Economically priced
- Provides data that can be compared to data generated using traditional MUNANA-based protocols
- Has stable signal read-out allowing for high throughput processing
- Easy to use
- Highly reproducible
- Provides flexibility in assay format
- Compatible with viral inactivation methods
- Environmentally friendly

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