# The NA-XTD™ Influenza Neuraminidase Assay Kit: Extended-glow chemiluminescence assay system for highly sensitive influenza neuraminidase assays

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## ABSTRACT

The new Applied Biosystems® NA-XTD™ Influenza Neuraminidase Assay Kit provides the next-generation NA-XTD™ 1,2-dioxetane chemiluminescent neuraminidase (NA) substrate, together with all necessary assay reagents and microplates, to quantitate sensitivity of influenza virus isolates to neuraminidase inhibitors. Like the NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit, the NA-XTD™ Influenza Neuraminidase Assay provides highly sensitive detection of influenza neuraminidase activity. In addition, the NA-XTD™ assay provides an extended-glow chemiluminescent light signal that eliminates the need for reagent injection and enables signal measurement either immediately or up to several hours after assay completion. The NA-XTD™ assay is also used to quantitate influenza NA activity directly in cell-based virus cultures to monitor viral growth or inhibition.

## INTRODUCTION

Global monitoring of influenza strains for resistance to neuraminidase inhibitors (NIs) is essential for understanding their efficacy for seasonal, pandemic or avian influenza, and studying the epidemiology of viral strains and resistance mutations. Functional neuraminidase inhibition assays enable detection of any resistance mutation, making them extremely important for global monitoring of virus sensitivity to NIs. The first-generation chemiluminescent NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit has been widely used for virus NI sensitivity assays (1-8), including identification of A/H1N1 pandemic virus resistant to oseltamivir (9,10). In addition, this assay has been used for identification of new NI compounds (11), NI characterization (12), clinical and animal studies of virus transmission (13), drug delivery (14), NA quantitation of virus-like particles (15) and cell-based virus quantitation (16).

Neuraminidase assays performed with chemiluminescent 1,2-dioxetane substrates, including NA-*Star*® and NA-XTD™ substrates, typically provide 5-to-50-fold higher sensitivity by signal-to-noise ratio than assays performed with the fluorescent MUNANA substrate. In addition, chemiluminescent assays provide linear results over 3-4 order of magnitude of neuraminidase concentration compared to 2-3 orders of magnitude with the fluorescent assay (2). The high assay sensitivity achieved with chemiluminescent assays enables use of lower concentrations (and amounts) of viral stocks, and the wide assay range minimizes the need to pre-titer virus stocks to determine optimal working dilutions for $IC_{50}$ determination.

The NA-XTD™ substrate has a single structural difference from the NA-*Star*® substrate that provides a much longer-lasting chemiluminescent signal, with a signal half-life of approximately 2 hours, eliminating the need for luminometer instruments equipped with reagent injectors and enabling more convenient batch-mode processing of assay plates. The NA-XTD™ Assay Kit also provides a new accelerator solution, containing a next-generation polymer enhancer, and a Triton® X-100-containing sample prep buffer providing enhanced NA activity.

## MATERIALS AND METHODS

The NA-XTD™, NA-*Star*® and NA-Fluor assay kits are supplied by Life Technologies. The NA-XTD™ assay kit includes 1) NA Sample Prep Buffer (Triton X-100-containing), 2) NA-XTD Assay Buffer for dilution of virus, neuraminidase inhibitors (NIs) and substrate, 3) NA-XTD Substrate, 4) NA-XTD Accelerator to trigger light emission, and 5) NA-Star Detection Microplates (solid-white 96-well microplates, optional). Influenza strains used include type A/H1N1 (VR-1469™ (human), VR-1520™ (human), VR-1682™ (swine) from ATCC (Manassas, VA) and A/H1N1/Texas/36/91, wild-type and H275Y oseltamivir-resistant mutant strains, (kindly provided by the CDC, Atlanta GA), and B/Lee/40 (VR-1535™, ATCC) viruses. NIs used include oseltamivir carboxylate (F. Hoffmann-La Roche Ltd, Basel, Switzerland) and zanamivir (GlaxoSmithKline, Research Triangle Park, NC). All assays were performed according to the respective assay protocol.

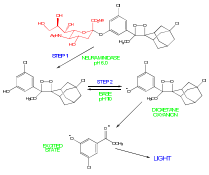
Sample prep:
1. Add 10% volume of NA Sample Prep Buffer to virus stock
2. Dilute virus as appropriate

NA-XTD Assay:
- 25 µL diluted virus
- 25 µL NI dilution, pre-incubate x 30 min at 37°C
- 25 µL 1:1000 NA-XTD, incubate x 30 min
- 60 µL Accelerator, read 1 sec/well

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

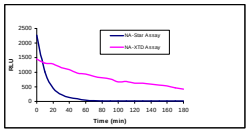
## RESULTS

**Figure 1. Light Emission Mechanism of NA-XTD™ Substrate**



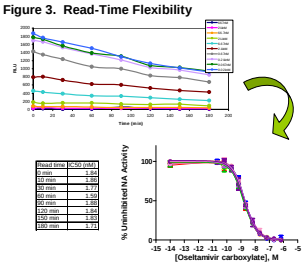

Enzymatic cleavage of the sialic acid group from the NA-XTD™ 1,2-dioxetane substrate generates a metastable reaction intermediate. Upon shifting the reaction pH with the NA-XTD™ Accelerator solution, protonation of the intermediate generates an unstable dioxetane oxyanion, which breaks down to form an excited state product that decays with energy released as light emission. The NA-XTD™ Accelerator includes a next-generation polymer enhancer that provides a large increase in the quantum yield of light emission.

**Figure 2. 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, using influenza A/WS/33 (H1N1) (strain VR-1520™). The longer-lived light emission kinetics eliminates the need for accelerator injection and luminometer instrumentation equipped with on-board injectors.

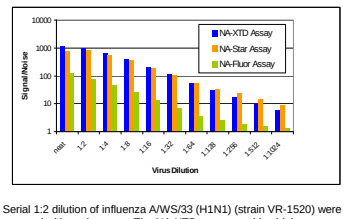
**Figure 3. Read-Time Flexibility**




| Read time | IC50 (nM) |
|---|---|
| 0 min | 1.84 |
| 10 min | 1.86 |
| 30 min | 1.77 |
| 60 min | 1.59 |
| 90 min | 1.88 |
| 120 min | 1.84 |
| 150 min | 1.83 |
| 180 min | 1.71 |

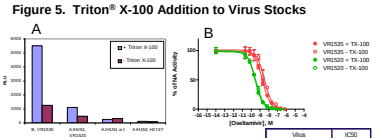
$IC_{50}$ values were determined using data collected up to 3 hours after addition of NA-XTD™ Accelerator. Although signal intensity slowly decreases over time, the $IC_{50}$ curves and values are identical at each time point, using influenza B/Lee/40 (strain VR-1535™).

**Figure 4. Sensitivity Comparison of NA-XTD™, NA-*Star*® and NA-Fluor™ Assays**




Serial 1:2 dilution of influenza A/WS/33 (H1N1) (strain VR-1520) were assayed with each assay. The NA-XTD assay provides higher Signal/Noise, higher detection sensitivity (better low-end detection), and a wider assay dynamic range than fluorescent assays with the MUNANA substrate. The higher sensitivity enables use of lower amounts of virus stock for $IC_{50}$ assays.

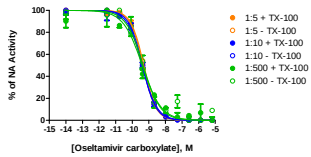
**Figure 5. Triton® X-100 Addition to Virus Stocks**




| Virus | IC50 |
|---|---|
| VR1535 + TX-100 | 1.81 nM |
| VR1535 - TX-100 | 3.36 nM |
| VR1520 + TX-100 | 0.26 nM |
| VR1520 - TX-100 | 0.33 nM |

Triton X-100 detergent at 1% has been shown to inactivate flu virus while increasing neuraminidase activity (17). A) NA Sample Prep Buffer (containing 10% Triton X-100) was added to virus stocks (at 1/10 volume, achieving a final concentration of 1%), and NA activity of virus stock dilutions was assayed. Triton X-100 may increase NA activity up to 4-fold, but is not consistently observed, and seems to be most effective with more concentrated virus stocks. B) $IC_{50}$ values are unaffected by the addition of Triton X-100.

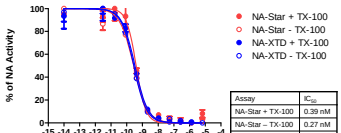
**Figure 6. $IC_{50}$ Determination at Different Virus Dilutions**




| Virus Dilution | - TX-100 | + TX-100 |
|---|---|---|
| 1:5 | 0.50 nM | 0.45 nM |
| 1:10 | 0.39 nM | 0.41 nM |
| 1:500 | 0.45 nM | 0.40 nM |

Oseltamivir carboxylate $IC_{50}$ determination assay was performed with a range of virus dilutions (influenza A/WS/33 (H1N1), strain VR-1520™). $IC_{50}$ values are nearly identical over a 100-fold difference in virus concentration.

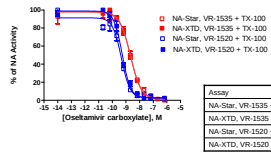
**Figure 7. Zanamivir $IC_{50}$ Determination: Comparison of NA-XTD™ and NA-*Star*® Assays (+/- Triton® X-100)**




| Assay | $IC_{50}$ |
|---|---|
| NA-Star + TX-100 | 0.39 nM |
| NA-Star – TX-100 | 0.27 nM |
| NA-XTD + TX-100 | 0.33 nM |
| NA-XTD – TX-100 | 0.28 nM |

Zanamivir $IC_{50}$ determination was performed with influenza A/Texas/36/91 (H1N1) using both assays. $IC_{50}$ values and curves are nearly identical between the NA-XTD and NA-*Star* assays. In addition, $IC_{50}$ values are unaffected by the addition of Triton X-100 to the virus stock prior to virus dilution.

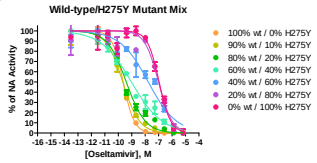
**Figure 8. Oseltamivir $IC_{50}$ Determination: Comparison of NA-XTD™ and NA-Star® Assays**




| Assay | $IC_{50}$ |
|---|---|
| NA-Star, VR-1535 + TX-100 | 2.13 nM |
| NA-XTD, VR-1535 + TX-100 | 2.13 nM |
| NA-Star, VR-1520 + TX-100 | 0.46 nM |
| NA-XTD, VR-1520 + TX-100 | 0.54 nM |

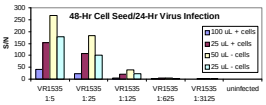
Oseltamivir $IC_{50}$ determination was performed with influenza A/WS/33 (H1N1) (strain VR-1520) and B/Lee/40 (strain VR-1535) using both assays, following addition of NA Sample Prep Buffer to virus stocks. $IC_{50}$ values and curves are nearly identical between the NA-XTD and NA-*Star* assays.

**Figure 9. Detection of NI-Resistant Virus in a Mixed Population**



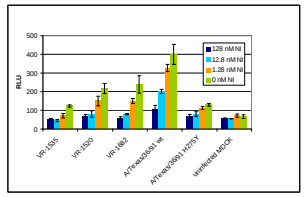

Oseltamivir-sensitive and resistant viruses (A/Texas/36/91 (H1N1) wild-type (wt) and H275Y (mutant)) were mixed in different ratios, following normalization of NA activity, and oseltamivir $IC_{50}$ values were determined. Mixtures of sensitive and resistant viruses demonstrate intermediate $IC_{50}$ values and curves with slope different from standard sigmoidal dose-response (+/-1). At 10% mutant virus, $IC_{50}$ value is similar, but slope is noticeably different from 100% sensitive virus (wt).

**Figure 10. Assay Protocol for Viral NA Quantitation in 96-Well Cell Culture**




MDCK cell were seeded in 96-well culture plate and cultured until cells were ~80% confluent (48 hours). Cells were infected with virus dilutions (B/Lee/40 (strain VR-1535™)) and incubated for 24 hours. Different volumes of culture media were assayed with the NA-XTD assay, either in the culture plate or in a separate assay plate. Performing the assay using the entire well contents (100 µL) reduces assay sensitivity due to the high concentration of phenol red. Assaying a smaller volume of culture medium (either in culture plate or a separate assay plate) provides higher sensitivity, and enables temporal monitoring or use of remaining culture medium for other assays. Viral NA quantitation provides a convenient read-out to measure viral growth or inhibition, including inhibition in the presence of inhibitory compounds (Figure 11) or antibodies, described as AVINA (Accelerated Viral Inhibition with NA as read-out assay (16).

**Figure 11. Cell-based Virus Growth Inhibition Assay**




MDCK cell cultures in a 96-well microplate were infected with different virus strains (B/Lee/40 (VR-1535™), A/WS/33 (H1N1) (VR-1520™), A/Swine/1976/31 (H1N1) (VR-1682™), A/Texas/36/91 (H1N1) wild-type and H275Y mutant), 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.

## CONCLUSIONS

The Applied Biosystems® 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. The Applied Biosystems® NA-Fluor™ Influenza Neuraminidase Assay Kit, based on the fluorescent MUNANA substrate, has also been developed to complement the NA-XTD™ and NA-*Star*® chemiluminescence assays, for users lacking luminometer instrumentation or choosing to use fluorescence assay detection. Together these kits offer:

- Standardized reagents and protocols
- Choice of detection technology
- Simple instrumentation requirements
- High sensitivity for use with low virus concentrations
- Compatibility with batch-mode processing and large-scale assay throughput
- Broad specificity of influenza detection
- Flexibility in assay format
- Additional NA assay applications – cell-based viral assays, screening for new NIs, detection of NA from other organisms

Functional neuraminidase inhibition assays enable detection of any resistance mutation so are extremely important in conjunction with sequence-based screening assays for global monitoring of virus isolates for NI resistance mutations, including known and new mutations. Together, these assays provide highly sensitive, convenient and versatile assay systems with standardized assay reagents and simple assay protocols for influenza researchers.

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## TRADEMARKS/LICENSING