THE NA-*STAR*® INFLUENZA NEURAMINIDASE INHIBITOR RESISTANCE DETECTION KIT: CHEMILUMINESCENCE ASSAY FOR DETECTION AND QUANTIFICATION OF INFLUENZA NEURAMINIDASE ACTIVITY

C.E. Miller[1], K. Skaare[1], A. Mihali, A.C. Chiulli[1], A. Lackenby[2], J. Baldevarona[2] and M. Zambon[2]
[1]Applied Biosystems, 35 Wiggins Avenue, Bedford, MA 01730 USA
[2]Health Protection Agency, Colindale, London, UK

Introduction

The NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit provides the NA-*Star*® 1,2-dioxetane 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. NA-*Star* substrate provides highly sensitive detection of neuraminidase enzyme activity from influenza virus types A and B, including human, avian, porcine and equine viruses.

Neuraminidase assays performed with the NA-*Star* 1,2-dioxetane chemiluminescent substrate provide approximately 50-fold higher sensitivity than the MUNANA fluorescence substrate. The chemiluminescent NA-*Star* assay provides linear results over 3-4 orders of magnitude of neuraminidase concentration compared to 1-2 orders of magnitude achieved with the fluorescent assay, providing a greater assay dynamic range. Virus dilutions are briefly incubated with neuraminidase inhibitor, and then the two-reagent detection assay is performed. The entire assay is completed in approximately one hour. Data analysis, using non-linear curve fitting dose response analysis software (not provided), is performed to determine the IC_{50} value of the neuraminidase inhibitor with each viral isolate. The NA-*Star* chemiluminescence assay has been compared to MUNANA fluorescence assays performed with isolates of the major influenza types, H1N1, H3N2 and influenza B strains, including NI-sensitive and resistant strains.

The NA-*Star* Influenza Neuraminidase Inhibitor Resistance Detection Kit combines highly sensitive and rapid chemiluminescent quantitation of neuraminidase activity from flu virus isolates using supplied reagents and a simple assay protocol to provide a convenient method for use in research laboratories to monitor the resistance levels of both human and animal influenza virus isolates to neuraminidase inhibitors.

Materials and Methods

Influenza strains: Influenza Type A (VR-1469, H1N1, strain A/PR/8/34), and Influenza Type B (VR-1535, strain B/Lee/40) are from ATCC and were grown on MDCK cells. Additional strains are from the strain repository at the UK Health Protection Agency Oseltamivir carboxylate was obtained from F. Hofmann-La Roche (Basel, Switzerland), and Zanamivir was from GlaxoSmithKline (UK). MUNANA was obtained from Fluka. NA-*Star* assays were performed with the NA-*Star* Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems, P/N 4374422) according to the supplied kit protocol. Briefly, virus dilution is incubated with NI dilution for 15 min, then diluted NA-*Star* substrate is added and incubated for 30 min. NA-*Star* Accelerator is injected

and light emission read immediately. MUNANA assays were performed according to standard published protocols.
Data analysis (IC50 determinations) was performed using non-linear curve fitting (GraphPad Prism 4.0) or Excel analysis using custom fitting algorithms.

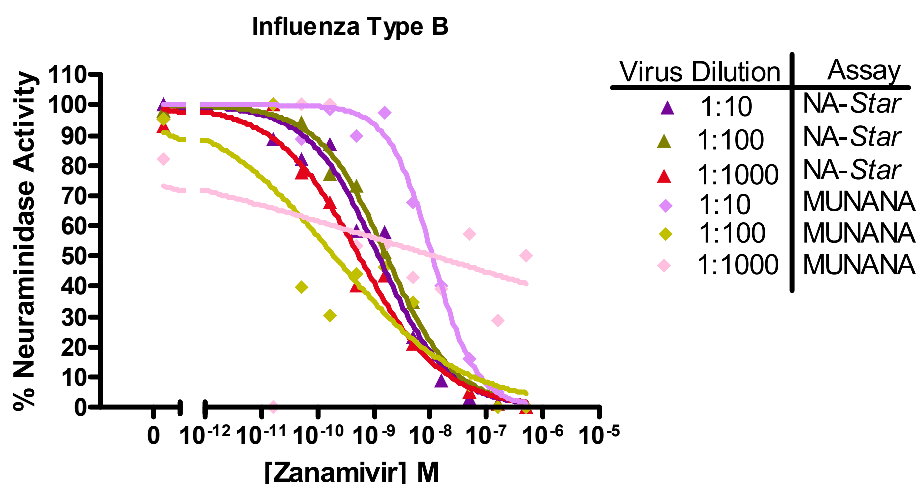
Results

Chemiluminescent reactions result in conversion of chemical energy to light energy, as light emission. The NA-*Star* substrate is a 1,2-dioxetane structure bearing a sialic acid cleavable group. To perform the NA-*Star* assay, virus dilutions (from cell culture supernatant) are first incubated in the presence of neuraminidase inhibitor. Then NA-*Star* substrate is added and incubated, under conditions optimal for neuraminidase activity, for 30 min for substrate cleavage to proceed. Finally, light emission is triggered immediately upon addition of an Accelerator (ideally performed with an on-board reagent injector), which provides required pH shift and a proprietary polymeric enhancer required for efficient light emission. Chemiluminescent assays are performed in solid white microplates, and light emission is measured in a luminometer. The NA-*Star* Influenza Neuraminidase Inhibitor Resistance Detection Kit provides the NA-*Star* Substrate, NA-*Star* Assay Buffer (used for dilution of neuraminidase inhibitors, virus samples and NA-Star Substrate), NA-*Star* Accelerator, detection microplates and a user protocol.

The NA-*Star* assay was initially compared to the MUNANA assay by quantitating neuraminidase activity in serial dilutions of both purified bacterial neuraminidases and virus culture supernatants. The chemiluminescent NA-*Star* assay provides provides linear results over 3-4 orders of magnitude of neuraminidase concentration compared to 1-2 orders of magnitude achieved with the fluorescent assay, providing a greater assay dynamic range. In addition, the sensitivity of the NA-*Star* assay is 15-50-fold higher than the MUNANA assay, enabling detection of lower concentrations of virus, and providing much higher signal-to-noise ratios (data not shown). So, with the NA-*Star* assay, virus culture supernatants can be used at higher dilutions for performing neuraminidase inhibitor quantitation assays, thus requiring much less of the virus culture supernatant.

IC_{50} values for zanamivir were determined with both the NA-*Star* and the MUNANA assay over a range of virus culture supernatant dilutions (Figure 1). The NA-*Star* assay provides a much higher signal/noise than the MUNANA assay at the same dilution. For the 1:10 virus dilution, the signal:noise value (virus dilution without drug:mock-infected media control) with NA-*Star* assay is 650:1, while that with the MUNANA assay is 5:1 (not shown). The IC_{50} variability is considerably higher for the MUNANA assay at different virus concentrations, and at least for the type B virus above, only the highest concentration provides a good curve fit. With the NA-*Star* assay, accurate quantitation of IC_{50}s over a wider range of virus concentration minimizes the need for a virus pre-titration step prior to IC_{50} quantitation assay.

Figure 1. IC50 Determination: NA-*Star* Assay vs. MUNANA Assay at Multiple Virus Concentrations



Several neuraminidase inhibitor-resistant mutant flu strains (and the corresponding wild-type sensitive strains) were assayed with both the NA-*Star* and MUNANA assays (Table 1). There is generally good correlation between the IC_{50} values determined with the NA-*Star* and MUNANA assays for the NI-sensitive strains. The IC_{50} values determined with the NA-*Star* assay tend to be slightly lower than values determined with MUNANA assay. For a few of the NI-resistant strains (H3N2 119V with oseltamivir; flu B 152K with zanamivir), the NA-*Star* IC_{50} values are significantly lower than those obtained with MUNANA, and ratio of neuraminidase activity between mutant and wild-type is not as great.

Table 1. NI-Resistant Viruses: NA-*Star* Assay vs. MUNANA Assay

		Oseltamivir		Zanamivir	
Virus Subtype	Reference Strain	NA Star	Munana	NA Star	Munana
H1N1	274H WT	0.16	0.63	0.13	0.28
	274Y Mutant	86.55	349.60	0.22	0.46
	Ratio Mt/Wt	**534.55**	**558.82**	**1.69**	**1.64**
H3N2	119E WT	0.34	0.73	0.47	1.45
	119V Mutant	2.47	38.27	0.31	2.10
	Ratio Mt/Wt	**7.30**	**52.53**	**0.66**	**1.45**
	292R WT	0.28	0.37	0.60	1.00
	292K Mutant	1776.70	1560.32	4.86	12.00
	Ratio Mt/Wt	**6352.63**	**4240.00**	**8.10**	**12.00**
Flu B	152R WT	0.24	1.91	1.70	1.70
	152K Mutant	>3000	1560.32	13.00	115.00
	Ratio Mt/Wt	**#VALUE!**	**815.38**	**7.65**	**67.65**

Summary

The NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit provides higher sensitivity detection, wider assay dynamic range, improved ease-of-use compared to fluorescent MUNANA assays. The complete reagent set supports “standardized” reaction conditions, enabling more accurate comparison of experimental results. Influenza NA assays using NA-*Star* substrate have been compared to MUNANA with a wide range of flu strains in public health labs globally, including the US, Canada, Australia, Europe, Japan and the UK (1-12). NA-*Star* chemiluminescent substrate provides highly sensitive detection of neuraminidase activity from many flu viruses, including human types A and B, avian, equine and porcine viruses. This broad assay capability makes it an important new tool for researching the global spread of influenza drug resistance, as well as for additional applications:

- Identification and development of new NIs
- NA detection in other pathogens
- Influenza virus quantitation/neuraminidase standardization (13)
- Quantitation of viral growth in culture for study of cellular infectivity/virus replication

The discrepancy in IC_{50} values obtained with the NA-*Star* and MUNANA assays has not been attributed to virus concentration or substrate concentration, but possibly could reflect differences in substrate cleavage efficiency by certain neuraminidases or assay protocol differences, and remains under investigation.

Global monitoring of influenza strains for resistance to anti-viral inhibitors is essential for studying epidemiology of viral strains and mutations and for reliably understanding the efficacy of neuraminidase inhibitor therapeutics both in the event of a significant influenza outbreak and for seasonal influenza. The NA-*Star* kit provides highly sensitive detection with ease-of-use, standardized reagents and versatile assay capability. Ongoing substrate and assay development will provide improvements in light emission kinetics and new applications.

References

1. Buxton, RC, B Edwards, RR Juo, JC Voyta, M Tisdale and RC Bethell. 2000. Anal. Biochem. 280:291-300.
2. Boivin G and N Goyette. 2002. Antiviral Research 54:143-147.
3. Aymard, M, O Ferraris, L Gerentes, J Jolly and N Kessler. 2003. Developmental Biology 115:75-83.
4. McKimm-Breschkin, J, T Trivedi, A Hampson, A Hay, A Klimov, M Tashiro, F Hayden and M Zambon. 2003. Antimicrobial Agents and Chemotherapy 47(7):2264-2272.
5. Mungall, BA, X Xu and A Klimov. 2003. Avian Diseases 47:1141-1144.
6. Wetherall, NT, T Trivedi, J Zeller, C Hodges-Savola, JL McKimm-Breschkin, M Zambon and FG Hayden. 2003. J. Clin. Microbiol. 41(2):742-750.
7. Mungall BA, X Xu and A Klimov. 2004. Virus Research 103:195-197.
8. Hurt, AC, JL McKimm-Breschkin, M McDonald, IG Barr, N Komadina and AW Hampson. 2004. Virus Research 103:205-211.
9. Jackson, D, W Barclay and T Zurcher. 2005. J. Antimicrobial Chemotherapy 55(2):162-169.

10. The World Health Organization Global Influenza Program Surveillance Network. 2005. Emerging Infectious Diseases 11(10):1515-1521.
11. Monto, AS, JL McKimm-Breschkin, C Macken, AW Hampson, A Hay, A Klimov, M Tashiro, RG Webster, M Aymard, FG Hayden and M Zambon. 2006. Antimicrobial Agents and Chemotherapy 50(7):2395-2402.
12. Zurcher, T, PJ Yates, J Daly, A Sahasrabudhe, M Walters, L Dash, M Tisdale and JL McKimm-Breschkin. 2006. J Antimicrobial Chemotherapy 58:723-732.
13. Bright, RA, DM Carter, S Daniluk, FR Toapanta, A Ahmad, V Gavrilov, M Massare, P Pushko, N Mytle, T Rowe, G Smith and TM Ross. 2007. Vaccine 25:3871-3878.

Acknowledgements

Dr. Rick Bright (Novavax, Inc., Malvern, PA); Angela Perrin (F. Hoffman-La Roche Ltd., Basel, Switzerland); Dr. Margaret Tisdale, Carol Jenkins (GlaxoSmithKline, NC, USA and UK); Dr. Larisa Gubareva, Dr. Varough Deyde (Influenza Branch, Centers for Disease Control).

Applera, Applied Biosystems, AB (Design) and NA-Star are registered trademarks of Applera Corporation or its subsidiaries in the US and/or certain other countries.
NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit: Covered under U.S. Patent No. 6,555,698.
For Research Use Only. Not for use in diagnostic procedures.