



QFlu™ NI Assay

For Detection of Resistance to Influenza Neuraminidase Inhibitors

Cellex, Inc.

I. INTENDED USE

The QFlu™ NI Assay is a rapid chemiluminescent assay for the direct and quantitative detection of influenza A and B viral neuraminidase enzyme activity and resistance of antiviral drugs targeting this enzyme.

QFlu™ NI Assay is intended for research use only.

II. SUMMARY

Influenza illness is classically characterized by sudden onset of fever, chills, headache, myalgias, and a non-productive cough. Epidemics of influenza typically occur during winter months with an estimated 114,000 hospitalizations and about 36,000 deaths per year in the U.S. Influenza viruses can also cause pandemics, during which the numbers of illnesses and deaths from influenza-related complications can increase dramatically.

Management of influenza includes use of antiviral therapy. Currently available antivirals target one of the two influenza viral components, the M2 ion channel or neuraminidase (for reviews, see ref 1-3). Influenza viral strains resistant to the M2 channel inhibitors rimantadine and amantadine are prevalent during interpandemic influenza. More strikingly, some H5N1 isolates are already resistant to one or both M2 channel inhibitors, suggesting that these inhibitors might not be effective antivirals during a pandemic (1-3). Thus, the neuraminidase inhibitors oseltamivir and zanamivir are the mainstay drugs for therapeutic intervention in cases of influenza virus infections. Since zanamivir is administered through inhalation, a cumbersome procedure, oseltamivir is the favored choice for epidemic influenza treatment or stockpiling in preparation of a pandemic influenza.

Based on widespread resistance to the M2 channel blockers, it is not unreasonable to speculate that widespread use of neuraminidase inhibitors may eventually lead to emergence of their resistance in seasonal or potential pandemic influenza virus. A number of neuraminidase inhibitor resistant mutants have been identified during preclinical or clinical studies. *In vitro* selection did lead to the isolation of NA gene mutations E119V, R292K, H274Y, and R152K, which confer resistance to oseltamivir (4-9). In a three-year surveillance (1999-2002), eight virus variants with a > 10-fold decrease in susceptibility to oseltamivir were isolated (10). These findings paint a worrisome picture for the use of influenza neuraminidase inhibitors.

Indeed, widespread of seasonal influenza virus H1N1 resistant to oseltamivir did appear during the 2008/2009 flu season in U.S., which heightens the need to more closely monitor flu antiviral drug resistance issue (11-14).

Still, recent findings of high concentrations of oseltamivir carboxylate in sewage water (15) unveiled another avenue – the avian population – from which oseltamivir resistant influenza virus strains may emerge because wild ducks live in environmental water systems and are the natural hosts of influenza virus. Although it may be a coincidence, it was reported that a resistant H5N1 strain carrying the H274Y mutation caused viremia in two patients who subsequently died from avian influenza (16).

Patients who are suspected of having influenza may benefit from treatment with an antiviral agent especially if given within the first 48

hours of onset of illness. Thus, prompt diagnosis of influenza viral infections may aid physicians in undertaking appropriate preventative and therapeutic intervention.

Influenza types A and B virus possess surface glycoproteins with neuraminidase activity, which hydrolyzes substrates that contain alpha-ketosidically linked N-acetylneuraminic acid (Neu5Ac). The QFlu™ NI Assay uses a substrate that enables the production of light signal in the presence of influenza viral neuraminidase. Thus, the QFlu™ NI Assay detects viral enzyme activity rather than the viral antigens.

The emitted light signal in the QFlu™ assay is directly proportional to influenza viral neuraminidase activity, which in turn is proportional to virus concentration in the sample. This test can detect both influenza virus type A and B, but does not differentiate between these two influenza virus types, nor does the QFlu™ NI Assay detect influenza type C, which does not possess neuraminidase. However, Type C influenza is not considered a significant clinical pathogen.

Since influenza viral neuraminidase is the target of oseltamivir carboxylate and Zanamivir and a number of flu antiviral candidates, the assay can be used to detect influenza virus that is resistant to these drugs or drug candidates or used to screening drug candidates targeting the neuraminidase.

The QFlu™ NI Assay is simple and rapid, and requires no prior titration of the sample. When used as a neuraminidase inhibition assay, the assay involves 1) mixing the master mix with the sample (40:5 ratio), 2) dispensing 45 µL of the resulting mix into multiple wells (normally 8-10) containing various concentrations of inhibitor to a final concentration of 0 to 1000 nM, 3) incubating at room temperature for 30 minutes, and 4) measuring the light signal using a luminometer that accommodates microwell plates. The entire process takes less than 10 min manual time.

III. REAGENTS AND SUPPLIES

Part Number: K22
Lyophilized master mix; each vial is sufficient for two microwell plates.

Storage conditions
-20°C or below.

Supplies not provided:

- Luminometer that accommodates microwell plates
- External positive and negative controls
- Microwell plates suitable for chemiluminescence assays

IV. APPROPRIATE SAMPLES

Sample types:

- Cultured influenza virus
- Other sample types as determined by the user

Sample Storage Media: The following media and solutions have been tested and found to be compatible with the QFlu™ NI Assay:

Earle's Minimal Essential Medium (EMEM)
EMEM with 0.5% BSA
Hanks Basal Salt Solution
Phosphate Buffered Saline (PBS)
PBS with 0.5% BSA
HEPES Buffer, pH 7.5 (up to 100 mM)
Imidazole, pH 7.0 (up to 100 mM)
Tris Buffer (<100 mM, pH 7.0-7.8)

Incompatible Solvents and Solutions: The following solvent and solutions were tested and found to be incompatible with the QFlu™ NI Assay:

DMSO in concentrations greater than 5%.

V. INHIBITION ASSAY PROTOCOL

The following protocol is designed to estimate the IC₅₀ value for the virus in a sample. Other user-specific protocol should be developed by the user accordingly. For example, drug candidate screening may use different protocol.

Step 1 – Prepare the neuraminidase inhibitors at various concentrations. The followings are examples: 0 (buffer), 5, 25, 50, 100, 250, 500, 750, 1000, 5000 and 10,000 nM. Dispense 5 µL each of the inhibitor to a microwell.

Step 2 – Add 5 µL of sample to each well

Step 3 – Dissolve the lyophilized master mix in 4 or 8 mL of deionized water for 1 plate or 2 plate kits, respectively. Place the dissolved reagent on ice if not used immediately.

Step 4 – Dispense 40 µL of the detection mix from Step 3 to the microwells. Incubate at room temperature for 30 minutes. The relatively long incubation duration is based on the observation that influenza neuraminidase inhibitor binding is a slow reaction (ref 17), but it can be optimized by the user. In our hands, short incubation time (e.g., 5-10 min) results IC₅₀ values that are similar to those with longer incubation time.

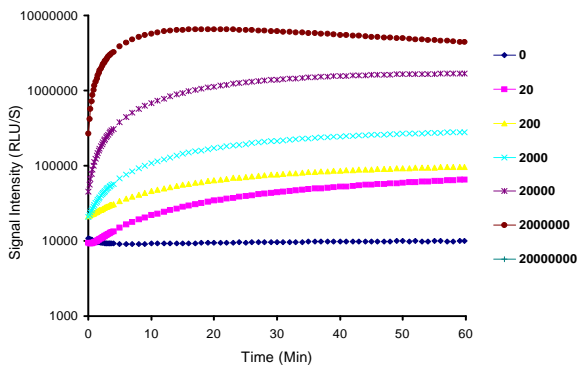
Step 5 – Place the microwell plate in the luminometer and measure the light signal.

A. REACTION KINETICS

The reactions were initiated by adding influenza virus to the detection mix as described in the Inhibition Assay Protocol except that there is no inhibitor in the reactions; instead, increasing amounts of virus were added to the reactions. The signal over a period of 60 minutes was recorded using a Bethold Sirius luminometer. The light signal was measured once every 10 seconds. The numbers in the column in Fig. 1 are the input influenza virus amounts in TCID₅₀ units.

In the presence of influenza virus, the signal increased rapidly in the first 10 minutes, after which the signal reached a plateau.

Fig 1 | Neuraminidase Reaction Kinetics



B. PRECISION

A precision study was performed to assess run-to-run and day-to-day variability of the assay. The light signal was measured at 15 minutes after addition of a sample to the detection mix using the CLX 2000 luminometer. The study was performed over a three day period (not consecutive days), during which duplicate samples for each concentration were tested each day. The raw data are presented in Table 1.

Table 1: The Raw Data (RLU) from the Precision Study

TCID ₅₀ Units	Day 1		Day 2		Day 3	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
0	3830	3899	3592	3846	2542	2761
100	5343	5436	5130	4913	5153	4700
1,000	14244	14948	12859	13137	13739	13404
10,000	94454	101067	100594	109886	89069	91993
100,000	874529	85420	819245	855301	880671	935644

The mean, standard deviation (SD) and coefficient of variation (CV) of the signal (RLU) were calculated and are presented in Table 2. Except for the background counts, the test results for all the samples containing influenza virus were quite reproducible with CV less than 8% (Table 2).

Table 2: Statistical Profile of the Data from the Precision Study

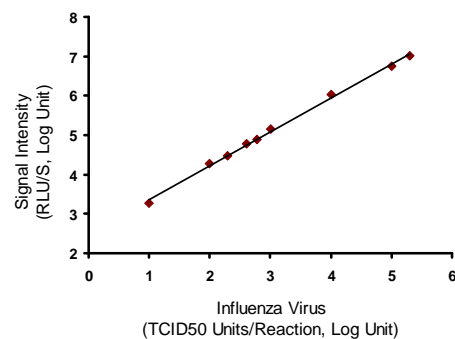
TCID ₅₀ Units	Mean	Standard Deviation	CV (%)
0	3411	602	17.65
100	5112	272	5.32
1,000	13722	769	5.61
10,000	97844	7559	7.72
100,000	869933	38691	4.45

C. LINEARITY

A linearity study was performed to assess the linearity and linear range of the assay. Sample containing 0, 10, 100, 200, 400, 600, 1000, 10,000, 100,000, or 200,000 TCID₅₀ units of cell culture-adapted influenza virus, strain A/WS/33, was mixed with the detection mix and incubated at room temperature for 15 minutes, followed by measurement of the emitted light signal using a Bethold Sirius luminometer.

The net RLU from each sample was plotted against the input TCID₅₀ units of the virus. As shown in Fig 2, the assay was linear throughout the entire tested spectrum (10 to 200,000 TCID₅₀ units) with a correlation coefficient (R²) of 0.997 (95% confidence interval: 0.986 – 1.0).

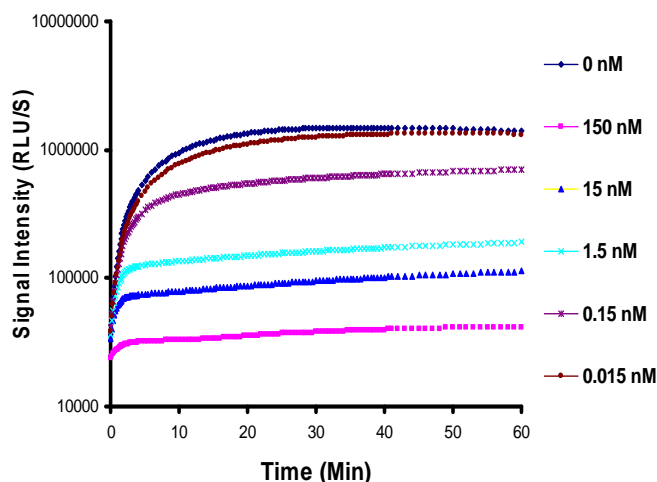
Fig 2 | Relationship between Signal Output and Virus Input



D. NEURAMINIDASE INHIBITION KINETICS

The inhibition assay was carried out by following the inhibition assay protocol using oseltamivir carboxylate as the inhibitor, which was used in final concentrations shown in Fig 3. Cultured influenza virus (A/WS/33) was used as the sample. The reactions were immediately placed in a luminometer for signal measurement. The light signal was collected over a period of 60 minutes.

Fig 3 | Oseltamivir Carboxylate Inhibition Kinetics



E. ASSAY SPECIFICITY AND CROSS-REACTIVITY

BACTERIA AND FUNGI

The following bacterial species were tested at a concentration of 2×10^8 colony forming units (CFU)/mL and found not to have cross reactivity in negative samples, or inhibition in positive samples:

Acinetobacter calcoaceticus anitratus, *Enterobacter cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Yersinia pseudotuberculosis*, and *Candida albicans*.

VI. WARNING AND PRECAUTIONS

1. Currently For Research Use Only.
2. Freezing of neuraminidase samples tend to reduce neuraminidase activity.

VII. LIMITATIONS

1. This version of QFlu™ NI Assay is not specific for influenza neuraminidase. A version specific for influenza neuraminidase will be available soon.

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MORE INFORMATION

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