Journal of Visualized Experiments

Fluorescence-based neuraminidase inhibition assay to assess the susceptibility of influenza viruses to the neuraminidase inhibitor class of antivirals

--Manuscript Draft--

Manuscript Number:	JoVE55570R2
Full Title:	Fluorescence-based neuraminidase inhibition assay to assess the susceptibility of influenza viruses to the neuraminidase inhibitor class of antivirals
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Fluorescence, neuraminidase, resistance, influenza, MUNANA, antiviral, inhibitor, phenotypic assay, oseltamivir, zanamivir
Manuscript Classifications:	3.2: Virus Diseases; 3.8: Respiratory Tract Diseases; 4.26: Pharmaceutical Preparations; 5.1: Diagnosis
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Abstract:	The neuraminidase (NA) inhibitors are the only class of antivirals approved for the treatment and prophylaxis of influenza that are effective against currently circulating strains. In addition to their use in treating seasonal influenza, the NA inhibitors have been stockpiled by a number of countries for use in the event of a pandemic. It is therefore important to monitor the susceptibility of circulating influenza viruses to this class of antivirals. There are different types of assays that can be used to assess the susceptibility of influenza viruses to the NA inhibitors, but the enzyme inhibition assays using either a fluorescent substrate or a chemiluminescent substrate are the most widely used and recommended. This protocol describes the use of a fluorescence-based assay to assess influenza virus susceptibility to NA inhibitors. The assay is based on the NA enzyme cleaving the substrate 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) to release the fluorescent product 4-methylumbelliferone (4-MU). Therefore the inhibitory effect of a NA inhibitor on the influenza virus NA is determined based on the concentration of the NA inhibitor that is required to reduce 50% of the NA activity, given as IC50 value.
Author Comments:	
Additional Information:	
Question	**Response**
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6 October 2016

Teena Metha PhD
Science Editor
Journal of Visualized Experiments (JOVE)

Dear Dr. Teena,

Please find enclosed our article “Fluorescence-based neuraminidase inhibition assay to assess the susceptibility of influenza viruses to the neuraminidase inhibitor class of antivirals” which we submit to be considered for publication in JOVE.

The manuscript describes the use of an in-house fluorescence-based neuraminidase inhibition assay to assess the susceptibility among circulating influenza A and B viruses to NA inhibitors. The assay relies on the use of a fluorescent substrate to measure the inhibitory effect of the NA inhibitors on the influenza neuraminidase enzyme.

Due to the simplicity and robustness of the assay, we believe that the digital format of this article will be a useful tool for researchers who wish to adopt the assay to assess the antiviral susceptibility of their strains of influenza viruses.

Kind regards

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TITLE:
Fluorescence-based neuraminidase inhibition assay to assess the susceptibility of influenza viruses to the neuraminidase inhibitor class of antivirals

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KEYWORDS:
Fluorescence, neuraminidase, resistance, influenza, MUNANA, antiviral, inhibitor, phenotypic assay, oseltamivir, zanamivir

SHORT ABSTRACT:
We describe the use of a phenotypic fluorescence-based neuraminidase inhibition assay to assess the susceptibility of influenza A and B viruses to the neuraminidase inhibitor class of antivirals.

LONG ABSTRACT:
The neuraminidase (NA) inhibitors are the only class of antivirals approved for the treatment and prophylaxis of influenza that are effective against currently circulating strains. In addition to their use in treating seasonal influenza, the NA inhibitors have been stockpiled by a number of countries for use in the event of a pandemic. It is therefore important to monitor the susceptibility of circulating influenza viruses to this class of antivirals. There are different types of assays that can be used to assess the susceptibility of influenza viruses to the NA inhibitors, but the enzyme inhibition assays using either a fluorescent substrate or a chemiluminescent substrate are the most widely used and recommended. This protocol describes the use of a fluorescence-based assay to assess influenza virus susceptibility to NA inhibitors. The assay is based on the NA enzyme cleaving the 2'-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) substrate to release the fluorescent product 4-methylumbelliferone (4-MU). Therefore, the inhibitory effect of an NA inhibitor on the influenza virus NA is determined based on the concentration of the NA inhibitor that is required to reduce 50% of the NA activity, given as an IC_{50} value.

INTRODUCTION:

Haemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins of influenza A and B viruses. HA binds to the sialic acid-galactose of cell surface glycoproteins or glycolipids, while the NA releases virus by cleaving the sialic acid from the galactose on the cell surface[1]. The NA inhibitors are a class of influenza antivirals that were rationally designed to bind tightly to the NA enzymatic active site, thereby preventing the release and spread of virus progeny. Oseltamivir and zanamivir are two NA inhibitors that have been approved in many countries worldwide for the treatment and prophylaxis of influenza. In recent years, two additional NA inhibitors, peramivir and laninamivir, have been approved for use in a limited number of countries. Screening of influenza viruses for susceptibility to NA inhibitors and the identification of mutations that confer resistance are important in determining and monitoring the effectiveness of this class of antivirals.

In the last 16 years, the fluorescence-based NA inhibition assay has been performed routinely at the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne (Melbourne WHOCCRRI) to monitor the changing trend of antiviral susceptibility among circulating influenza viruses. Annually, more than 2,000 influenza viruses are tested for antiviral susceptibility. In most influenza seasons, >98% of the viruses are susceptible to all four NA inhibitors[2-4], although during the 2007-2008 northern hemisphere influenza season, there was a surge in the number of former seasonal A(H1N1) viruses that had reduced susceptibility to oseltamivir[5]. This group of viruses, which contained the NA amino acid substitution H275Y, spread to the rest of the world by the end of 2008, making oseltamivir inappropriate globally for the treatment of this virus. The vast majority of currently circulating influenza B, influenza A(H3N2), and influenza A(H1N1)pdm09 strains are susceptible to oseltamivir, although community clusters of A(H1N1)pdm09 variants containing the NA amino acid substitution H275Y that confers reduced oseltamivir and peramivir susceptibility, have been reported in various parts of the world[6,7].

Because of the need for a sufficiently high virus titer, clinical specimens (including animal nasal washes) must be passaged in either cell culture or embryonated chicken eggs prior to antiviral susceptibility testing. The NA inhibition assay described in this article can be divided into three sections:

Determining the linear range for the fluorescent product 4-methylumbelliferone (4-MU) on a particular fluorometer.

Due to the inherent differences between fluorometers, the linear range for the fluorescent end-product, 4-MU, and the relative fluorescence unit (RFU), need to be established. Once the linear range for 4-MU is established, the optimal target signal is selected, to which the concentration of the influenza viruses is adjusted in the NA activity assay. Once completed for a particular fluorometer, this should not need to be repeated.

Determining the NA activity of the viruses.

The NA activity assay is a simple assay that involves the addition of the 2'-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) substrate to serially diluted

viruses. The amount of fluorescent end-product 4-MU generated from the cleavage of MUNANA by the NA is measured using a fluorometer. The appropriate virus dilution to use in the NA inhibition assay is selected by plotting fluorescence units against virus dilution. From the sigmoidal curve produced, the mid-point of the linear section should correspond to the 4-MU linear range of the fluorometer determined in section 1 and will inform the appropriate concentration of viruses to be used in section 3.

Assessing virus susceptibility to NA inhibitors using the NA inhibition assay.
To assess the susceptibility of viruses to a particular NA inhibitor, viruses at the dilution determined in section 2 are incubated with a range of NA inhibitor concentrations. Following a subsequent incubation with MUNANA, the 4-MU generated by uninhibited viruses is measured in RFU by the fluorometer. The inhibitory effect of the NA inhibitor on the NA enzyme activity of a virus is calculated according to the NA inhibitor concentration required to reduce 50% of the NA activity, given as an IC_{50} value.

PROTOCOL:

1) Determining the linear range of fluorescent product 4-MU on a fluorometer

1.1) Prepare 10 mL of 6.4 mM 4-MU stock solution by dissolving 11.3 mg of 4-MU in 5 mL of absolute ethanol. Add 5 mL of 0.9% NaCl (w/v) to the stock solution to make 10 mL. Prepare a 640 µM working solution by diluting 1 mL of 6.4 mM 4-MU in 9 mL of 1x assay buffer (33.3 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 4 mM $CaCl_2$, pH 6.5).

Note: Prepare the 2x assay buffer by adding 13 g of MES and 8 mL of 1 M $CaCl_2$ to 992 mL of distilled water. Adjust the pH to 6.5 using 10 M of NaOH. Filter the buffer using a sterile cellulose acetate filter of pore size 0.2 µm. Caution! Sodium hydroxide is caustic and may cause chemical burns to the skin and eyes. Ensure that full personal protective equipment is worn.

1.2) Prepare 5 mL of a range of 4-MU concentrations (*i.e.,* 2.5 µM, 5 µM, 10 µM, 20 µM, 40 µM, 80 µM, 160 µM, and 320 µM) through a two-fold serial dilution of 640 µM 4-MU using 1x assay buffer.

1.3) Dispense 50 µL of each serial dilution of 4-MU (two wells per dilution) into a clear, 96-well flat-bottom plate and 50 µL of 1x assay buffer into the remaining wells (which serve as blanks to measure background signals).

1.4) Prepare a 2.5 mM MUNANA stock solution by reconstituting 2.5 mg of MUNANA in 20 mL of distilled water. Mix 0.72 mL of 2.5 mM MUNANA with 5.28 mL of 1x assay buffer to obtain a 300 µM MUNANA working solution (sufficient volume for one plate). Cover the tube containing the 300 µM MUNANA working solution with aluminum foil and keep it on ice unless used immediately. Discard any leftover materials.

Note: The 2.5 mM MUNANA stock solution can be stored at -20 °C for 1 month and must be

used within one freeze/thaw cycle. The 4-MU and MUNANA solutions are light sensitive and must be protected from prolonged light exposure.

1.5) Add 50 µL of 300 µM MUNANA to each well, gently tap to mix, and incubate at 37 °C for 30 min. Cover the plate with a plate sealer to prevent evaporation.

Note: This step is to account for the background fluorescence in the NA inhibition assay.

1.6) Prepare the stop solution by mixing 11 mL of absolute ethanol with 2.225 mL of 0.824 M NaOH (sufficient volume for one plate).

1.7) Add 100 µL of stop solution to each well to stop the reaction and gently tap to mix.

1.8) Read the plate using a fluorometer with an excitation wavelength setting of 355 nm and an emission wavelength setting of 460 nm, as per the manufacturer's instructions.

1.9) Calculate the average background signal using fluorescence signals from all the wells containing no 4-MU. Subtract this average background signal from each of the wells containing 4-MU and calculate the average signals (RFU) for each 4-MU concentration. Plot a standard curve of RFU against the 4-MU concentration (µM), as shown in Figure 1a; a close-up linear section of the curve is shown in Figure 1b.

1.10) Visualize the plot of RFU against 4-MU concentration (µM) to determine the linear range and the optimal target signal; the linear range is where the fluorescence signal increases proportionately to the increasing concentrations of the 4-MU on the plot, while the optimal target signal is an arbitrary 4-MU concentration within the linear range.

Note: The linear range and the optimal target signal are fluorometer-specific. For example, the fluorometer at the Melbourne WHOCCRRI has a linear range of 2.5-40 µM 4-MU and an optimal target signal of ~30 µM 4-MU, which corresponds to ~1,500 RFU.

2) Determining the NA activity of the viruses

Note: Influenza viruses are cultured to sufficient titers in Madin-Darby Canine Kidney (MDCK) cells or embryonated chicken eggs[8].

2.1) Dispense 120 µL per well of undiluted cultured influenza viruses into column 1 and 60 µL of 1x assay buffer containing 0.1% NP-40 into the remaining 11 columns of a 96-well, U-bottom plate.

2.2) Serially perform two-fold dilution of the viruses across the plate (*i.e.,* transfer 60 µL from column 1 to column 2 and so forth, up to column 11) using a multichannel pipette, leaving column 12 as a blank containing only 1x assay buffer.

2.3) Transfer 50 µL from each of the wells (diluted viruses and blanks) into a clear, 96-well, flat-bottom plate.

Note: It is not necessary to change pipette tips if materials are transferred from column 12 through to column 1.

2.4) Add 50 µL of 300 µM MUNANA (prepared as per step 1.4) per well and gently tap the plate to mix. Incubate the plate at 37 °C for 1 h. Cover the plate with a plate sealer to prevent evaporation.

2.5) Add 100 µL of stop solution (prepared as per step 1.6) per well to terminate the reaction and gently tap the plate to mix.

2.6) Read the plate using a fluorometer.

2.6.1) Use an excitation wavelength setting of 355 nm and an emission wavelength setting of 460 nm.

2.7) Determine the average background signal based on the fluorescence readings in column 12 and subtract the average background signal from each well. Plot a graph of RFU against virus dilutions.

Note: The background values for 100 µM MUNANA at the WHOCCRRI Melbourne are typically between 50 and 120 RFU, but these will differ depending on the fluorometer being used.

2.8) View the plot of RFU against virus dilutions to determine the mid-point of the linear section of the curve for each virus (Figure 2). Use the optimal target signal (determined in step 1) as the reference point.

Note: This should correspond with the 4-MU linear range of the fluorometer determined in section 1 and will provide the appropriate concentration of viruses to be used in section 3.

3) Assessing virus susceptibility to NA inhibitors using the NA inhibition assay

3.1) Prepare master stocks of NA inhibitors at concentrations of 300 µM.

3.1.1) Prepare 300 µM zanamivir (molecular weight, MW = 332.32 g/mol) by dissolving 5.0 mg of zanamivir in 50 mL of 2x assay buffer (66.6 mM MES and 8 mM $CaCl_2$, pH 6.5).

3.1.2) Prepare 300 µM oseltamivir carboxylate (D-tartrate; MW = 386.44 g/mol) by dissolving 5.8 mg in 50 mL of 2x assay buffer.

3.1.3) Prepare 300 µM peramivir trihydrate (MW = 382.45 g/mol) by dissolving 5.7 mg in 50 mL of 2x assay buffer.

3.1.4) Prepare 300 µM laninamivir (MW = 346.34 g/mol) by dissolving 5.2 mg in 50 mL of 2x assay buffer.

Note: The NA inhibitor master stocks can be stored at -20 °C for 12 months. Check the MW of the NA inhibitors to ensure the correct weights and volumes are used in reconstitution. The oseltamivir carboxylate is the active compound of the prodrug oseltamivir phosphate. Therefore, only the oseltamivir carboxylate should be used in the NA inhibition assay.

3.2) From the master stocks, prepare working stocks of ten-fold serial dilutions of the NA inhibitors in 50-mL centrifuge tubes at concentrations of 0.03 nM, 0.3 nM, 3 nM, 30 nM, 300 nM, 3,000 nM, and 30,000 nM in 2x assay buffer (66.6 mM MES and 8 mM $CaCl_2$, pH 6.5); this is for use across multiple assays.

Note: The final concentrations of NA inhibitors in the reaction volume (50 µL of virus dilution + 50 µL of NA inhibitor + 50 µL of 300 µM MUNANA) are 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, 1,000 nM, and 10,000 nM, respectively. The final concentration does not include the 100 µL of stop solution. Store all NA inhibitors dilutions at 2-8 °C. The expiry date is the same as that of the master stocks.

3.3) Prepare the virus dilutions in 1x assay buffer containing 0.1% NP-40 surfactant in a 96-deep-well block. Use virus dilutions based on the NA activity assay results derived from section 2. Use a total volume of 2 mL per virus to test four NA inhibitors.

Note: Prepare two wells (1 mL per well) per virus in a 96-deep-well block. Examples of virus dilutions for VIRUS 1, 2, and 3 are shown in Table 1.

3.4) Dispense the required volume of NA inhibitors (prepared as per step 3.2) into an 8-deep-well reservoir. From there, dispense 50 µL of NA inhibitors at dilutions ranging from 0 nM (2x assay buffer only) to 30,000 nM in rows A to H in a clear, 96-well, flat-bottom plate.

Note: The plate layout is illustrated in Figure 3.

3.5) Add 50 µL of diluted test viruses per well to columns 1-11 and 50 µL per well of 1x assay buffer only to column 12. Gently tap the plate to mix and incubate at room temperature for 45 min. Cover the plate with a plate sealer to prevent evaporation.

3.6) Add 50 µL of 300 µM MUNANA (prepared as per step 1.4) per well and gently tap the plate to mix. Incubate the plate at 37 °C for 1 h. Cover the plate with a plate sealer to prevent evaporation.

3.7) Add 100 µL of stop solution (prepared as per step 1.6) to each well and gently tap the plate to mix.

3.8) Read the plate using a fluorometer.

3.8.1) Use an excitation wavelength of 355 nm and an emission wavelength of 460 nm, as described previously.

4. Calculation of IC_{50} values

Note: The JASPR v1.2 is curve-fitting software that enables the calculation of IC_{50} values. The software was developed by the Influenza Division at the CDC, Atlanta, USA. The software utilizes the equation: $V=V_{max}$ x(1-([*I*]/(K_i +[*I*]))), where V_{max} is the maximum rate of metabolism, [*I*] is the inhibitor concentration, *V* is the response being inhibited, and K_i is the IC_{50} for the inhibition curve.

4.1) Copy and paste the raw data outputted by the fluorometer into a spreadsheet in a 12-column plate format (96-well), starting with cell A1.

Note: Raw data from each subsequent plate must be staggered by one empty row. If each virus has been tested against four NA inhibitors, paste the raw data in a set of four (with an empty row between each plate).

4.2) Open the fitting software and click on the "Experiment" tab. Choose "Alternative 2-drug Fluoro 11 Samples."

4.3) Click on the "Options" tab and tick "Generate Graphs."

4.4) Click on the "Options" again and click on the "New Key" tab. Save the "inhibition_key" .csv file in the same folder as the raw data spreadsheet file.

4.5) In the inhibition_key file, list all sample names below the ID.

4.6) If four NA inhibitors were tested, insert spaces for two additional rows below Oseltamivir and type in "Peramivir" and "Laninamivir." Save the changes made to the inhibition_key file.

4.7) Return to the "jaspr v 1.2-Inhibition Curve fitting" window and select the raw data file as the "Experiment File" and inhibition_key as the "Key File." Run the analysis. Save the results in .csv and .pdf formats.

Note: The software will automatically plot an inhibition curve (RFU against NA inhibitor concentrations), as shown in Figure 4. The program also calculates IC_{50} values for each virus against an individual NA inhibitor (Figure 4). Additionally, JASPR presents the IC_{50} values and the signal-to-background (S/B) ratio in spreadsheet format. The background values can differ from lab to lab depending on the fluorometer used. At the Melbourne WHOCCRRI, the background values for 100 µM MUNANA range from 50 to 120 RFU. For a reliable IC_{50} value, an S/B ratio of ≥10 is preferred, although a ratio of less than 10 is still acceptable, particularly for mutant

viruses that have very low NA activity.

4.8) Inspect the IC_{50} values and the curve shapes generated by the software. All the data points should fall on or close to the curve; if they do not, repeat the NA inhibition assay.

Note: For viruses that show unusually high IC_{50} values, the assay should be repeated to confirm the result.

REPRESENTATIVE RESULTS:

Using standardized reporting guidelines from the WHO Working Group on Surveillance of Influenza Antiviral Susceptibility[9], the susceptibility of influenza viruses to the NA inhibitors are reported using the terms normal inhibition (NI), reduced inhibition (RI), and highly reduced inhibition (HRI). NI viruses are those with IC_{50} values less than 10-fold compared to the reference median IC_{50} for influenza A viruses (or less than 5-fold for influenza B viruses). RI viruses are those with IC_{50} values between 10- and 100-fold above the reference median IC_{50} for influenza A viruses (or 5- and 50-fold for influenza B). HRI viruses are those with IC_{50} values of 100-fold above the reference median IC_{50} for influenza A viruses (or above 50-fold for influenza B viruses); see Table 2.

The reference median IC_{50} values for A(H1N1)pdm09, A(H3N2), and B Yamagata/B Victoria viruses are calculated and updated annually at the Melbourne WHOCCRRI to reflect minor changes in the IC_{50} values of circulating influenza strains to the NA inhibitors (Table 3). The median IC_{50} values in influenza A(H1N1)pdm09 viruses are almost the same across the four NA inhibitors, but the median zanamivir and laninamivir IC_{50} values for A(H3N2) viruses are 2- to 4-fold higher compared to oseltamivir and peramivir IC_{50} values (Table 3). The median oseltamivir IC_{50} value for influenza B viruses is generally 5- to 10-fold higher than the zanamivir, peramivir, and laninamivir IC_{50} values (Table 3).

The NA inhibition assay is a phenotypic assay that does not provide information on the genetic changes associated with RI or HRI. Therefore, it is important that genetic analysis is performed following the identification of viruses with RI or HRI. At the Melbourne WHOCCRRI, the NA gene of variants is analyzed using Sanger sequencing and pyro-sequencing. A representative list of amino acid substitutions that can be found in the NA gene of viruses with RI and HRI variants is presented in Table 4. A more extensive list of amino acid substitutions that can alter NAI susceptibility is also available on the WHO website [10].

Figure 1 RFU against 4-MU concentration. a) Standard curve of RFU against 4-MU concentration (µM). The dotted box shows the linear range of 4-MU for the fluorometer. The fluorescence signals above the linear range may be saturated, and therefore, any small changes in fluorescence may not be detected by the fluorometer. b) Close-up linear section of the standard curve of Figure 1a for the identification of the "optimal target signal." The fluorometer at the Melbourne WHOCCRRI has a linear range of 2.5-40 µM 4-MU and an optimal target signal of ~30 µM 4-MU, which corresponds to ~1,500 RFU.

Figure 2: Example of the NA activity curves of influenza viruses. The average background value of 50.61 RFU has been subtracted from every dilution point on the NA activity curves. The arrows indicate the appropriate virus dilution to use in the NA inhibition assay for each virus. For easier preparation of virus dilutions, one may choose to perform a 1/100 dilution for VIRUS 3 instead of a 1/96 dilution.

Figure 3: Plate layout for the setup of the NA inhibition assay. Each plate includes the last column, which acts as a negative control that contains no virus but only 1x assay buffer (AB), NA inhibitor, MUNANA, and stop solution. Note: JASPR uses readings from column 12 of each plate to determine the average blank signal used in the calculation of the IC_{50} values.

Figure 4: Example of an inhibition curve and IC_{50} value of an A(H1N1)pdm09 virus, A/Perth/82/2015. The JASPR software presents the inhibition curve as fluorescence (RFU) against the increasing concentration (nM) of NA inhibitor, with every point fit within the curve. Based on the inhibition curve, the IC_{50} value is determined as the concentration of NA inhibitor to reduce 50% of the virus NA activity.

Table 1: Preparation of virus dilutions for VIRUS 1, 2, and 3 in the NA inhibition assay.

Table 2: The WHO Antiviral Working Group recommended guidelines for the classification of influenza virus susceptibility to NA inhibitors.

Table 3: Median IC_{50} and IC_{50} range of normal inhibition (NI) viruses from 2015 derived in the WHO CCRRI, Melbourne.

Table 4: Representative list of amino acid substitutions linked to reduced inhibition (RI) or highly reduced inhibition (HRI) to NA inhibitors.

Table 5: Troubleshooting for potential problems in the NA inhibition assay.

DISCUSSION:

The global monitoring of influenza virus susceptibility to NA inhibitors is currently being conducted by a number of laboratories using either fluorescent or chemiluminescent NA inhibition assays[11,12]. The fluorescent assay is more commonly used than the chemiluminescent assay. Although both assays are robust and reproducible, the IC_{50} values obtained from the fluorescence-based assay are often higher than the chemiluminescence-based assay, making a direct comparison of the data from the two assays difficult[13]. Even with the use of the same protocol, data generated from one laboratory may vary from another. Because of these variations between laboratories, the WHO Working Group on Surveillance of Influenza Antiviral Susceptibility produced a guideline to assist in inter-laboratory comparisons. Rather than comparing the absolute IC_{50} values, this guideline uses a comparison based on the IC_{50} fold difference to the median IC_{50} of the NI influenza viruses tested in each particular laboratory. The ability to compare data from the five collaborating centers has resulted in the annual publication of global influenza antiviral susceptibility data[2-4]. The availability of the large

amount of influenza susceptibility data in the public domain allows researchers to compare IC_{50} data from those studies with that generated in their own laboratories.

Other NA inhibition assays that adopt a similar concept are also commercially available. These commercial kits that contain ready-to-use reagents (NA inhibitors not included) are equally reproducible. However, the in-house NA inhibition assay is substantially cheaper than the commercial kits, because the majority of the reagents can be made in-house in larger quantities and the MUNANA substrate, which previously made up the major cost of the assay, can now be purchased from various sources at competitive prices. The cost of testing one influenza isolate per drug is approximately $1 (USD). At the Melbourne WHOCCRRI, improvements have been made to the in-house NA inhibition assay after the incorporation of a robotic platform for the liquid handling components of the assay. Apart from the manual preparation of virus dilutions, the majority of the procedures are performed using the liquid-handling robot. Not only does this minimize manual handling, but it also increases the numbers of assays that can be run in a day.

Although the NA inhibition assay is highly robust, there are a number of critical steps that need to be completed with additional care. First, any irregularity in the NA inhibitor concentrations can shift the inhibition curves and the IC_{50} values; therefore, careful attention should be paid when preparing the NA inhibitor concentrations. Second, accurate pipetting and precise incubation periods are crucial to maintaining consistent results across assays; this can be achieved by using calibrated pipettes and timers. The inclusion of control viruses in every assay also enables the monitoring of assay performance from assay to assay and over long periods of time. Third, because the NA enzyme activity of seasonal influenza viruses is optimal at pH 6.5, the correct pH of the assay buffer is important. Some reports have found that the use of lower pH conditions may improves the identification of influenza variants, such as the A(H7N9) variant containing the R292K mutation[14,15]. However, the modification to the pH of the assay buffer will shift the IC_{50} values, and this may complicate the comparison of data within laboratories and between laboratories. Other modifications and troubleshooting that can be performed are listed in Table 5.

The NA inhibitors are the only class of approved antivirals that are currently effective against circulating influenza viruses. Until other antiviral classes become available for clinical use, the antiviral susceptibility surveillance of circulating influenza viruses will be focused on NA inhibitors alone. Because of the simplicity and reproducibility of results, the use of the NA inhibition assay to assess influenza virus susceptibility to NA inhibitors will continue.

ACKNOWLEDGMENTS:
The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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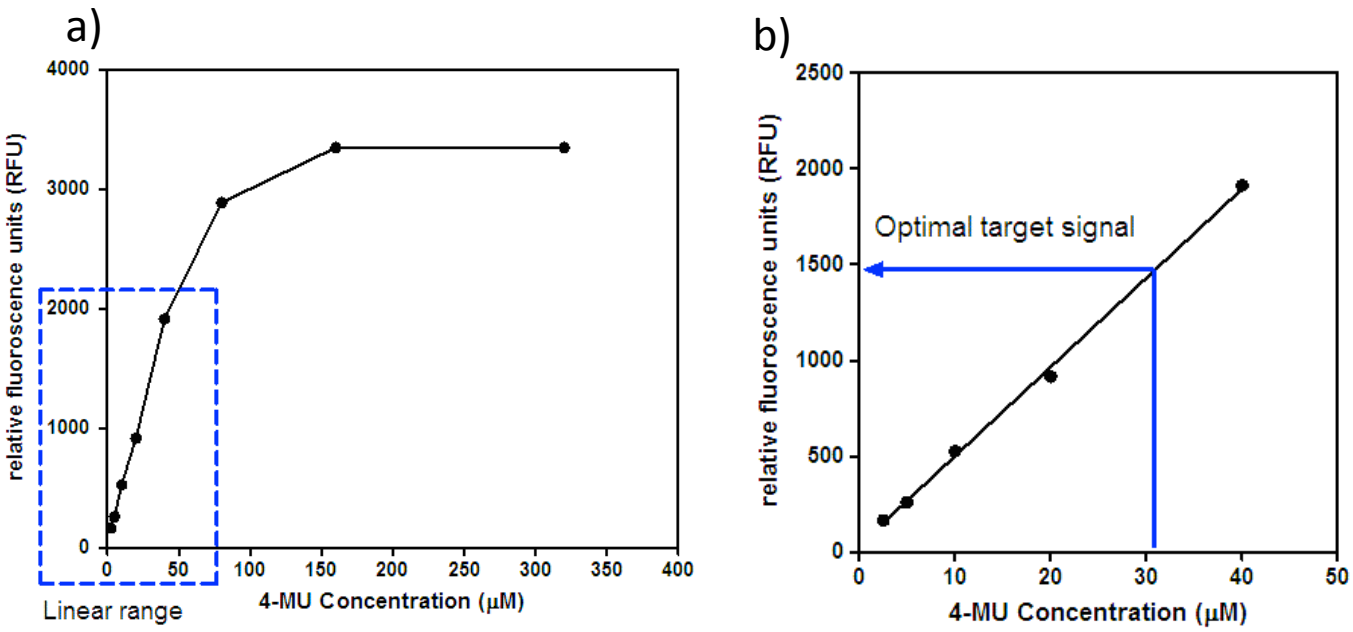

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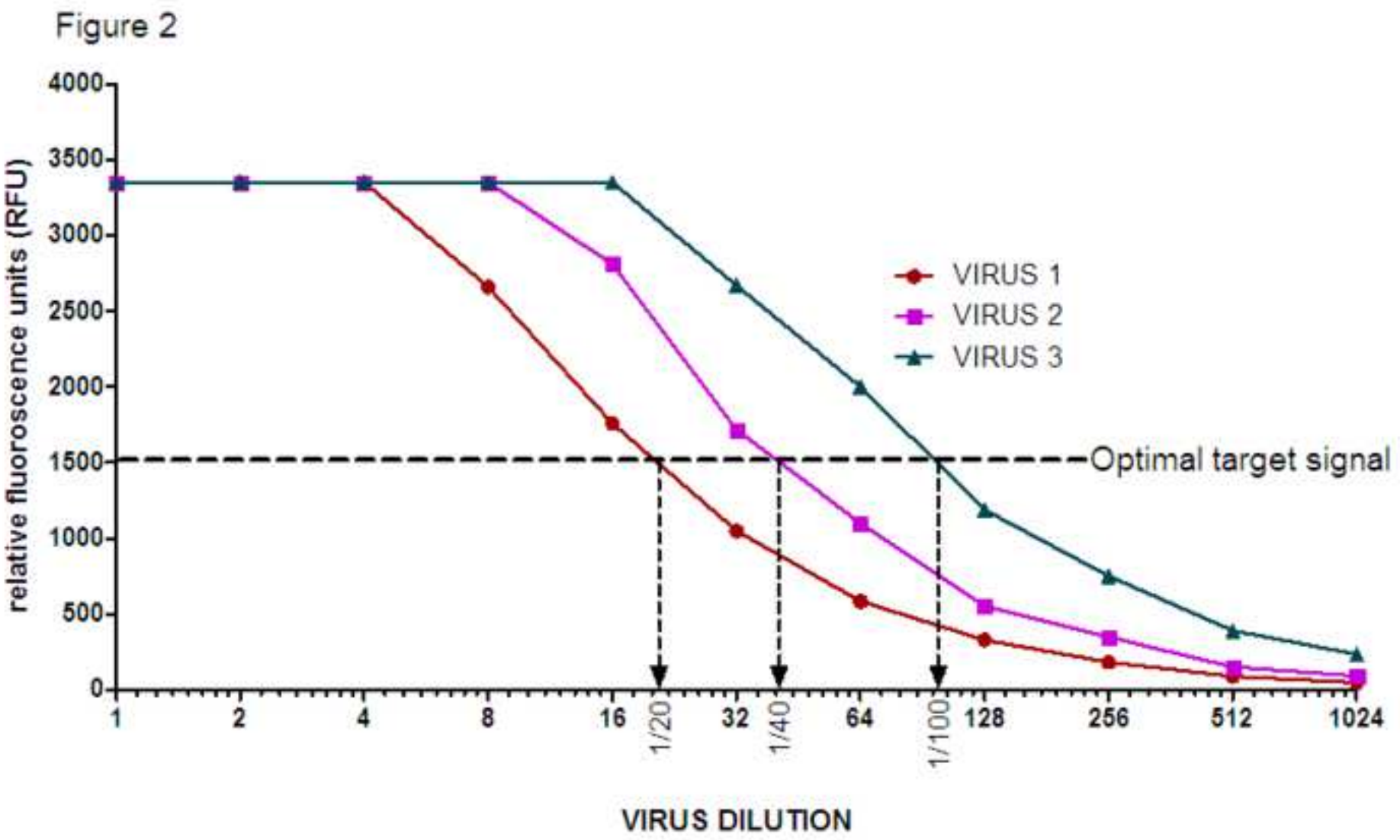

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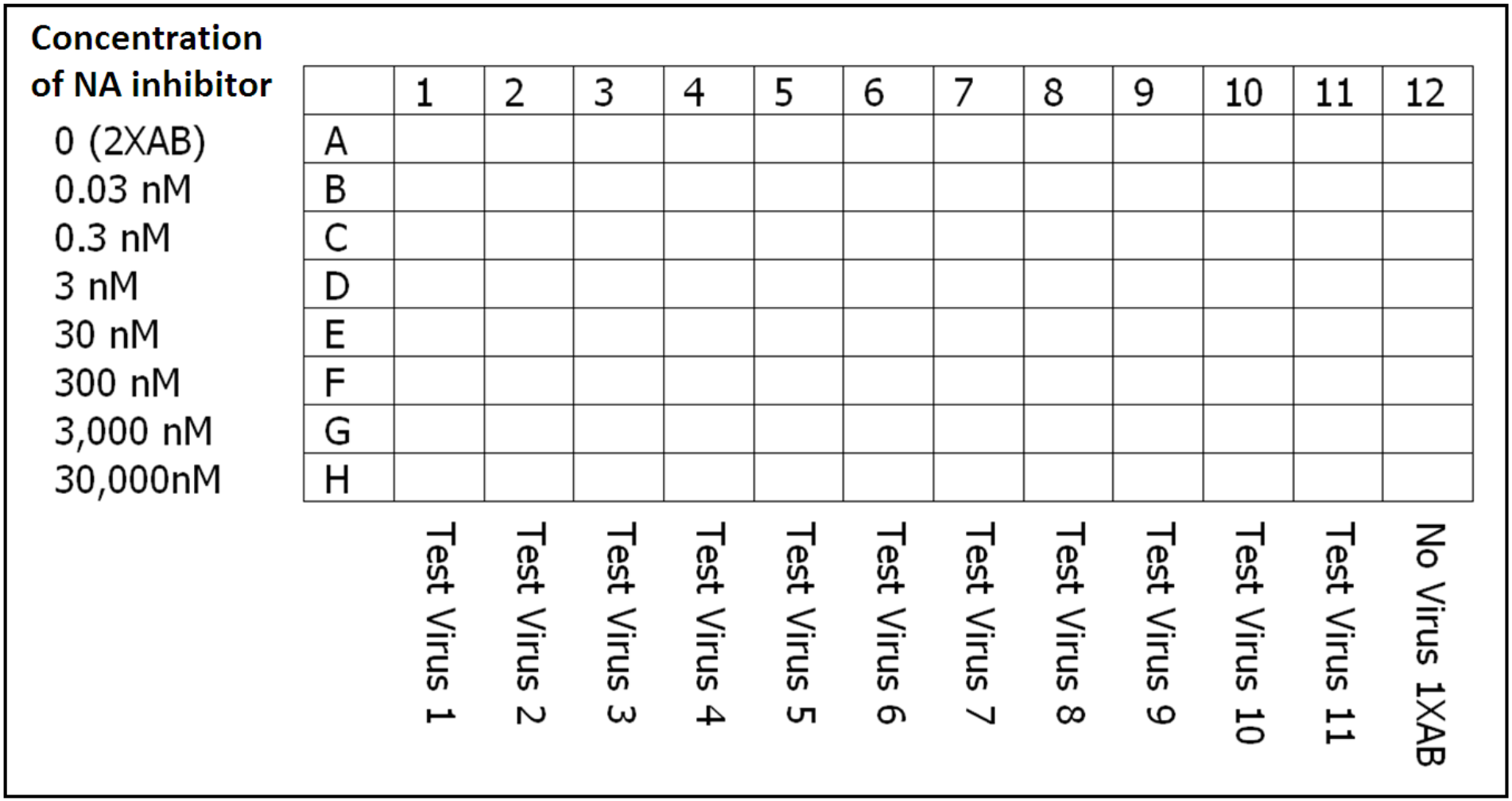

Figure 4

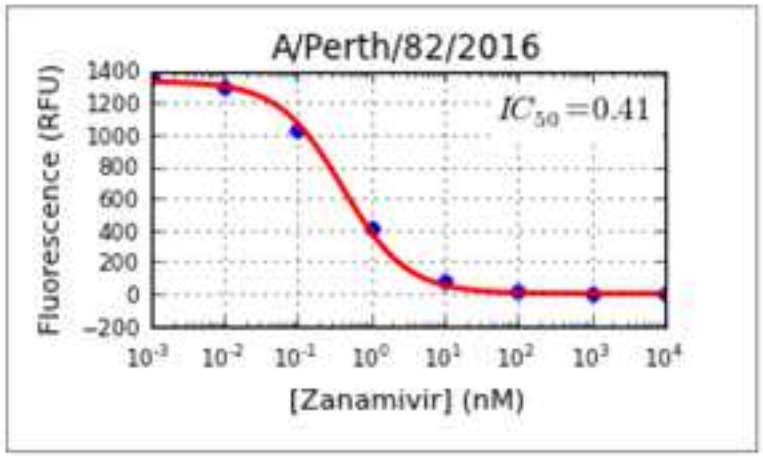

Table 1: Preparation of virus dilutions for VIRUS 1, 2, 3 in the NA inhibition as

Virus	Virus dilution required	1x assay buffer volume (µL)	Surfactant-Amps-NP-40 (10%) (µl)
1	1/20	940	10
2	1/400	965	10
3	1/100	890	10

say

Virus volume (μL)
50
25
10

Table 2: The WHO Antiviral Working Group recommended guidelines for the classification of influenza

Virus type/subtype/lineage	Normal inhibition (NI)	Reduced inhibition (RI)	Highly reduced inhibition (HRI)
A(H1N1)pdm09	< 10 fold	10 - 100 fold	> 100 fold
A(H3N2)	< 10 fold	10 - 100 fold	> 100 fold
B Yamagata and B Victoria	< 5 fold	5 - 50 fold	> 50 fold

virus susceptibility to NA inhibitors.

Table 3

Table 3: Median IC_{50} and IC_{50} range of normal inhibition (NI) viruses from 2015 derived in the WHO CCRRI, Melbourne

Virus type/subtype/lineage	N	Zanamivir	Oseltamivir	Peramivir	Laninamivir
		Median (range) IC_{50} nM	Median (range) IC_{50} nM	Median (range) IC_{50} nM	Median (range) IC_{50} nM
A(H1N1)pdm09	1326	0.42 (0.1 - 3.43)	0.36 (0.01 - 3.75)	0.19 (0.07 - 1.60)	0.55 (0.05 - 2.29)
A(H3N2)	1654	0.9 (0.11 - 4.0)	0.38 (0.01 - 3.65)	0.33 (0.12 - 3.06)	1.38 (0.01 - 9.38)
B Yamagata and B Victoria	1115	2.2 (1.24 - 10.72)	15.12 (2.39 - 70.75)	1.36 (0.57 - 6.67)	2.89 (1.62 - 9.15)

Table 4: Representative list of amino acid substitution linked to reduced inhibition (RI)

Amino acid substitution	Type/subtype/lineage	IC_{50} fold-change compared to refere	
		Zanamivir	Oseltamivir
H275Y	A(H1N1)pdm09	1	557 (HRI)
E119V	A(H3N2)	1	63 (RI)
H134Y	B Victoria	1	4
N151T	B Victoria	4	4
G104E	B Victoria	1220 (HRI)	87 (HRI)
E105K	B Victoria	3	5 (RI)
I222T	B Victoria	2	7 (RI)
H273Y	B Yamagata	1	230 (HRI)
D197N	B Yamagata	4	7 (RI)

or highly reduced inhibition (HRI) to NA inhibitors.

nce median IC_{50} values.

Peramivir	Laninamivir
123 (HRI)	2
1	1
76 (HRI)	2
42 (HRI)	1
17724 (HRI)	701 (HRI)
59 (HRI)	2
8 (RI)	3
377 (HRI)	2
32 (RI)	3

Table 5: Troubleshooting for potential problems in NA inhibition assay.

Problem	Possible reason(s)	Solution(s)
No or low NA activity	No virus was present or low virus yield.	Clinical specimen must be cultured in cell lines (*i.e.* Madin-Darby Canine Kidney cells) or in embryonated chicken eggs to a higher virus load for use in the NA inhibition assay.
	Some mutant viruses have extremely low NA activity despite at high virus load.	Use neat virus concentration for testing. Lower pH assay buffer (eg. pH 5.3) may be used. However, caution must be taken when comparing data.
No or low NA activity in NA inhibition assay	No virus was added.	Re-dilute the virus. Ensure the virus is directly added into the 1x assay buffer.
	Wrong virus dilution was used.	Repeat the NA activity assay.
	Insufficient incubation time.	Ensure the incubation time is followed.
Data points fall outside the IC_{50} curve	Cross contamination of NA inhibitor of higher concentration.	Ensure that the tips are not in contact with the NA inhibitor when dispensing diluted viruses into the 96 well plate.
		If an 8 deep well reservoir was used, discard and re-dispense the NA inhibitor concentrations into a fresh 8 deep well reservoir.
	The volume of the NA inhibitor or MUNANA or diluted virus was not added equally into each well.	Repeat the assay with a calibrated multi-channel pipette. Ensure equal volume of each reagent is dispensed into each well.
Unusually high IC_{50} values	Too high concentration of virus was added.	Repeat the NA activity assay and NA inhibition assay.
	Test sample contained mixtures of influenza A and influenza B.	Perform real-time PCR to identify the presence of virus mixtures.
	Bacterial contamination in the sample	Culture virus in the sterile condition with the presence of antibiotic.

High background fluorescence signal	MUNANA substrate may degrade over time.	Use a new batch of MUNANA subtrate.
	Detection of fluorescence from neighboring wells.	Use black 96-well flat-bottom plates

Excel Spreadsheet- Table of Materials/Equipment

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Tableofspecificmaterialsandequipments.xls

Name of Reagent/ Equipment	Company	Catalog Number
Influenza A and B viruses		Cultured in MDCK cells or 9 day old embryonated specific pathogen free (SPF) eggs
Madin-Darby Canine Kidney (MDCK) cells	ATCC	PTA-6500
2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA)	Biosynth AG	M-5507
2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA)	Sigma	M8639
4-Methylumbelliferone (4-MU)	Sigma	M1381-25G
2-[N-morpholino]ethanesulphonic acid (MES hydrate) (free acid)	Sigma	M8250-250G
Calcium Chloride ($Ca\ Cl_2$)	APS AJAX Finechem	127-500G
Surfactant-Amps-NP-40 (10% solution)	Thermo Fisher Scientific	PIE28324
Sodium Hydroxide (NaOH)	APS AJAX Finechem	482-2.5KG
Absolute Ethanol	APS AJAX Finechem	214-2.5L GL
96-well clear flat-bottom plates	NUNC	456537
96-well U-bottom plates	Greiner Bio-one	4650101
8 channel deep well block	Pacific Laboratory Products	RES-MW8-HP
96-well deep plates, 2.0mL square wells	Pacific Laboratory Products	P-2ML-SQ-C
Plate sealers	Thermo Fisher Scientific	236366
Bottle-top vacuum filter system (cellulose membrane (nitrate), pore size 0.2 µm, membrane area 33.2 cm2, filter capacity 500 mL)	Sigma-Aldrich	CLS430758-12EA
Single-channel pipettes (1 µL - 1000 µL)	Variety of suppliers (eg. Eppendorf, Sartorius)	
Multi-channel pipettes	Variety of suppliers (eg. Eppendorf, Sartorius)	8 or 12 channel electronic and manual pipette (5 - 1250 µL volume)
Pipette tips (1 µL - 1250 µL)	Variety of suppliers (eg. Eppendorf, Sartorius)	
Disposable pipettes (10 mL and 25 mL)	Greiner Bio-one	P7740-200EA and P7865-200EA

Pipette controller	Eppendorf	4430000018
Centrifuge tubes 50 mL	BD Bioscience	352070
Racked tubes	Scientific Specialties, Inc.	1750-00
Fluorometer with excitation wavelength setting of 355 nm and an emission wavelength setting of 460 nm	TermoFisher Scientific	ASCENT FL 374
Ascent software	TermoFisher Scientific	5185410CD
Incubator set at 37°C	Lab Supply	Biocell 1000
Zanamivir	GlaxoSmithKline	
Oseltamivir carboxylate	Roche	Request directly from
Peramivir (BCX-1812)	BioCryst	the company
Laninamivir (R-125489)	Daiichi-Sankyo	
		freely available upon request
JASPR v1.2	Influenza Division at the CDC	(fluantiviral@cdc.gov)

Comments/Description

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Author(s): SOOK-KWAN LEANG, AERON C. HURT

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• Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as…
Additional reagents and plastics supplies have been added to the Materials table.

• Please adjust the highlighting to identify 2.75 pages or less of text which are most important to include in the video. While highlighting text for filming, please keep the following in mind:
a) The highlighting must include all relevant details that are required to perform the step. For example, step 3.1 is highlighted for filming and the details of how to perform the step are given in steps 3.1.1 and 3.1.2, then the sub-steps where the details are provided must be included in the highlighting.

Author has high-lighted all the steps involved in the preparation of the NA inhibitor concentrations.

b) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
c) Notes will not be filmed, and should be excluded from highlighting.

• 2.1 – How are viruses cultured? Please provide a citation.

Author has included a citation for culturing viruses in MDCK cells and embryonated chicken eggs.

• Section 3 – Why are working stocks of the inhibitors in 2x assay buffer? This is combined with virus in 1x assay buffer, so the final concentration of the assay buffer will be greater than 1x in the reaction.

Editor has made a valid point. However, the use of 2x assay buffer in the NA inhibitor was to account for the use of neat virus (when the virus was not diluted in the 1x assay buffer). From our experience, the use of 1x or 2x assay buffer did not appear to affect the end IC_{50} values.

• Steps 1.9: Since you have highlighted this step for filming, it is important that the following is addressed:

o Mathematical calculations cannot be filmed. Please adjust the highlighting if this is a calculation lacking a graphical user interface.

Author has removed the high-lighting of the mathematical calculation as suggested by the editor.

o The above applies for graph plotting as well.

Author has removed the high-lighting of the graph plotting as suggested by the editor.

o Please consider removing the highlight here and adding a step that says “View the plot of RFU against 4-MU concentration and determine the linear region”, which will be highlighted.

Author has high-lighted and added the sentence Visualize the plot of RFU against 4-MU concentration (µM) to determine the linear range and the optimal target signal for Step 1.10 as suggested by the editor.

• Steps 1.8 and 2.6: This steps needs details for proper scripting. Please add menu item selections to adjust settings acquire the data.

Author has removed the high-lights for the fluorometer set up. Instead only the “Read the plate using a fluorometer” is high-lighted.

• Step 2.7: As this step is highlighted it is important to ensure sufficient filmable content. Please adjust this as suggested for step 1.9.

As the calculation is not easy for filming, the author has removed the high-light for Step 2.7.

• Step 4.8: The step was modified into a note and the note following the step was made into an action step, and highlighted. Please verify that this is appropriate.

Author verified that the modification is acceptable.

• Please expand the note following 4.8 and move it into the discussion.

Author has removed the note and added critical steps, modification and troubleshooting in the Discussion.

• Please move the in-text http weblinks into the reference list, and use superscripted citations.

Author has removed the weblink from the result and cited the website in the reference.

• Please ensure that the discussion covers discussion of the critical steps within the protocol, modifications and troubleshooting.

Please include additional citations from independent groups. The authors’ own work makes

up the majority of citations.
Additional citations have been added.

•Grammar: Discussion – “chemilunescent” and “chemilunescence” should be “chemiluminescent” and “chemiluminescence”

Author has made the corrections.

•Branding: Please remove trademark symbols from the materials table.

Author has made the corrections.

•Results:

-Please include titles for the Tables in the Figure Legends.

Author has made the changes.

-Please discuss Figure 4 in the results section. What do the data mean?

Author has added “The JASPR software presents the inhibition curve as Fluorescence (RFU) against the increasing concentrations (nM) of a NA inhibitor, with every point fit within the curve. Based on the curve, the IC_{50} value is determined as the concentration of NA inhibitor to reduce 50% of the virus NA activity.” in the figure legend of Figure 4.

•Discussion: Please discuss the critical steps of the protocol, the limitations, and the future applications. Please also discuss any modifications/troubleshooting that can be performed.

Author has included a paragraph in the Discussion, describing the critical steps of the protocol and the limitations

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript provides a method to conduct a fluorescence-based NA inhibition assay to assess susceptibility of influenza viruses to NA inhibitors.

Minor Concerns:
1. In my opinion, the determination of linear range using 4-MU should not be included in the main body of the method as it is not an essential component of the assay and will confuse those who choose to watch the video; I would recommend it be inserted in Table 5 as a means to verify that all reagents are performing well should there be low or no signal with virus.

Alternatively, include a section on Quality Control - routine inclusion of a 4-MU titration would provide a useful way to demonstrate that the assay is performing consistently. *[Editor: You can leave this as is and choose to not highlight it]*

Author has removed the high-lights for the entire section 1.

2. If the 4-MU titration is included, please explain in the method why you're including 300 µM MUNANA in each well (I assume it is to account for the background fluorescence observed in the 'actual' assay).

Author has included a sentence "This step is to account for the background fluorescence in the NA inhibition assay" to Step 1.5.

3. The signal at WHOCCRRI is very different to the signals we observe in our laboratory - our max is always >>>1,000,000 RFU; background values with 100 µM MUNANA are about 30,000. One criterion that we use is that the signal should be >10-fold the background. It may be helpful to give the readers an idea of the background values in your lab and whether you require a minimal signal:noise ratio.

Author has added the text "Additionally, JASPR also presents the IC_{50} values and signal to background ratio in excel format. The background values differ from lab to lab. At the Melbourne WHOCCRRI, the background values for 100 µM MUNANA ranges from 50 – 120 RFU. For a reliable IC_{50} value, the signal to background ratio of ≥10 is preferred, although ratio of less than 10 is still acceptable, particularly in mutant viruses that have very low NA activity…."in the note below Step 4.7.

4. Step 2.2. re-phrase to stipulate transfer of 60 µl from one well to the next.

Author has re-phrased the sentence.

5. Step 2.3. For fluorescence we always use black plates. This may be particularly important when using an instrument that is very sensitive. It may be helpful to include a line in Table 5 regarding detection of fluorescence from neighbouring wells and the potential need to use black plates.

Comparison of using black plates and clear Maxisorp plates had been performed in the past that showed no differences in detection of fluorescence. Author has added the suggestion in Table 5.

6. Step 2.4. How did you decide on 100 µM MUNANA (final concentration)? Is this critical? In my experience we get the same results when we use 100 µM and 20 µM MUNANA, but the advantage is that background is lower with the lower MUNANA concentration. May be another point to add to Table 5 for individuals who want to resolve background issues.

The protocol at the Melbourne WHOCCRRI has always been 100 µM MUNANA. Author has no data to support reviewer's comment. Because of concerns of how reducing MUNANA concentration may later IC_{50} values, author has chosen to not include the suggestion by the reviewer.

7. Section 3.1. I don't understand why the inhibitors are made in 2x assay buffer; this gets mixed with virus that is in 1x assay buffer and the MUNANA is also in 1x assay buffer. Correct if needed, or please explain.

The use of 2x assay buffer in the NA inhibitor was to account for the use of neat virus (where the virus was not diluted in the 1x assay buffer). From our experience, the use of 1x or 2x assay buffer did not appear to affect the IC_{50} values.

8. We haven't been adding detergent to our virus diluent. Sounds like a good idea but does it impact the signal at all? If addition of detergent either increases or decreases the signal, you may want to mention this in Table 5.

The addition of detergent NP-40 was to lyse the virus. In our experience, the presence of NP-40 does not affect signal.

9. Step 3.5 and figure 3 indicate that a single well is used per antiviral dilution. The WHO CCs are probably the only labs that have the need for such high throughput that replicates cannot be included. I would add a note somewhere that use of replicate wells or replicate assays is recommended to support documentation of IC50 values.

Author has included a sentence in Step 4.8 "For viruses that show unusually high IC_{50} values, the assay should be repeated to confirm the result."

10. The trouble shooting table (Table 5) is referenced in the text before Table 2. The table numbers probably need to be changed.

Author has moved the reference of Table 5 to the Discussion.

11. Figure 2 legend. State whether the background values were subtracted, and if so, state the RFU value of that background.

Author has included the sentence "The average background value of 50.61 RFU has been subtracted from every dilution point on the curves" in the Figure legend 2.

12. Table 3 lists the median IC50 for very large numbers of viruses. It would be helpful to include the IC50 range of each inhibitor for each virus type/subtype.

Author has listed the ranges of IC_{50} values (in brackets) in Table 3

13. Suggestion for Table 5. Bacterial contamination can also contribute to unusually high IC50 values. The solution is to use sterile or clean reagents.

Author has included the suggestion on Table 5

Reviewer #2:

Manuscript Summary:

The authors present an assay to determine the susceptibility of circulating viruses to neuraminidase inhibitors. This and similar assays are currently being used by several surveillance laboratories around the world for this purpose. This is of clear public health importance.

Overall, the paper reads well and the protocol is clear, but I do have a few suggestions listed below that I think could help improve the clarity of the paper.

Minor Concerns:

-In steps 1.10) and 2.8) it would be helpful to have a little more clarification on how the linear range, target signal, and mid-point of the linear section of the curve are determined. Are these visually assessed from the plots or are they calculated with specific software? Either way, a more complete description of this process would improve clarity.

Author has described how linear range and target signal are determined in Step 1.10. Author has also described in Step 2.8 the use of optimal target signal as a reference for the determination of virus dilution to use.

-Figure 3: Would be helpful to label the rows as concentration of NA inhibitor. Should also define the abbreviation AB in the legend.

Author has included the concentration of NA inhibitor in Figure 3 and defined AB as assay buffer in the figure legend.

-A few of the tables could use footnotes defining abbreviations so that they are able to stand alone.

Author did not include footnotes, however, author has defined all possible abbreviations.

-In the REPRESENTATIVE RESULTS section, annual reference median IC50 values are discussed. Are these values published anywhere that the authors could reference?

The median IC_{50} values at the Melbourne WHOCCRRI are updated annually, the most recent median IC_{50} values have not been published.

-The first paragraph in the discussion mentions an annual publication of influenza antiviral susceptibility. Could an example be referenced?

Author has included the three publications on global annual influenza antiviral susceptibility (Reference 2,3,4).

-If there is space, a comparison to the ELLA assay (Couzens et al, J Virol Methods 2014; 210:7-14) would be of interest. A question readers may have is whether the presented assay could also be extended to examine serum antibody titers against the NA. Discussion of the appropriateness of this in comparison to the ELLA assay would be of interest.

The assay presented cannot be extended to examine serum antibody titers against NA. The MUNANA substrates that are used in the assay are very small in size in comparison to the serum antibody. Therefore the NA enzyme will be able to react with the MUNANA despite being bound to the serum antibody.

Additional Comments to Authors:
N/A