Editorial comments:  
  
The manuscript has been modified by the Science Editor to comply with the JoVE standard. Please maintain the current formatting throughout the manuscript. The updated manuscript **55570\_R1\_101716** is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink to download the .docx file. **Please download the .docx file and use this updated version for future revisions.** The file is also attached.  
  
• 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 IC50 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 IC50 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 IC50 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 IC50 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 IC50 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 IC50 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 IC50 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 IC50 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 IC50 values at the Melbourne WHOCCRRI are updated annually, the most recent median IC50 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