# SensoLyte® 440 West Nile Virus Protease Assay Kit *Fluorimetric*

| Revision Number: 1.1 | *Last updated: October 2014* |
|---|---|
| **Catalog #** | **AS-72079** |
| **Kit Size** | 500 Assays (96-well) |

- ***Optimized Performance:*** This kit is optimized to detect West Nile Virus protease activity.
- ***Enhanced Value:*** It provides enough reagents to perform 500 assays in 96-well format.
- ***High Speed:*** The entire process only takes about half an hour.
- ***Assured Reliability:*** Detailed protocol and references are provided.

## Kit Components, Storage and Handling

| Component | Description | Quantity |
|---|---|---|
| Component A | Pyr-RTKR-AMC, WNV protease substrate, Ex/Em=354 nm/442 nm | 5mM DMSO solution, 250 µL |
| Component B | AMC, fluorescence reference standard, Ex/Em=354 nm/442 nm | 5 mM DMSO solution, 10 µL |
| Component C | Assay Buffer | 2 x 50 mL |
| Component D | WNV Protease Inhibitor undeca-D-Arg-$NH_2$ | 1 mM DMSO solution, 10 µL |

### Other Materials Required (but not provided)

- WNV protease: WNV protease can be produced in an *E. coli* expression system.[1,2] AnaSpec provides active recombinant WNV protease (Cat#72081).
- 96-Well microplate: Black, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 442 nm with excitation at 354 nm.

### Storage and Handling

- Store all kit components at -20°C.
- Protect Components A and B from light and from moisture.
- Component C can be stored at room temperature for convenience.

## Introduction

West Nile virus (WNV) is from the family Flaviviridae[3] found in both tropical and temperate regions. The main route of human infection is through the bite of an infected mosquito. Infection causes severe neurological disease and fatalities in both human and animal hosts. There is currently no effective vaccine or antiviral drug to protect against WNV infection.[4]

WNV contains a single-stranded, positive-sense RNA genome, which encodes three structural proteins (capsid (C), membrane (M), envelope (E)), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).[5,6] The West Nile viral NS3 protease is absolutely essential (along with viral-encoded cofactor NS2B) for post-translational processing of a viral polypeptide precursor in infected host cells. This polypeptide provides the structural and functional viral proteins, and inhibition of its processing could represent a potential treatment for viral infections. Thus, this protease has become one of the key targets for developing anti-WNV drugs.[1,7]

The SensoLyte® 440 West Nile Virus Protease Assay Kit provides a convenient, homogeneous assay for high throughput screening of West Nile Virus protease NS3 inhibitors. It allows for continuous quantification of protease activity using a fluorogenic peptide described in the literature[1]. This peptide, Pyr-RTKR-AMC generates the AMC (7-amino-4-methylcoumarin) fluorophore upon NS3 protease cleavage. AMC has bright blue fluorescence that can be detected with excitation at 354 nm and emission at 442 nm.

## Protocol

Note: For fluorometer calibration, please refer to Appendix II - recommended for first-time users.

### Screening WNV protease inhibitors or detection of WNV protease activity

Note: Allow all kit components to warm to room temperature before beginning.

**1. Prepare working solutions.**

- WNV protease substrate solution: Dilute protease substrate (Component A) 1:100 in assay buffer (Component C). For each experiment prepare fresh substrate solution.

  Table 1. WNV protease substrate solution for one 96-well plate (100 assays).

| Components | Volume |
|---|---|
| WNV protease substrate (100X, Component A) | 50 µL |
| Assay buffer (Component C) | 4.95 mL |
| Total volume | 5 mL |

- WNV protease diluent: Dilute WNV protease to an appropriate concentration in assay buffer (Component C). The recommended volume for WNV protease diluent is 85 µL/well for 96-well format

  Note: Prepare enzyme diluents immediately before use. Do not vortex the enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

- WNV protease inhibitor: [1] Dilute the 1 mM inhibitor solution (Component D) to 10 µM in assay buffer (Component C). Add 15 µl of the 10 µM inhibitor solution into each of the inhibitor control wells (DMSO concentration should not exceed 1%).

**2. Set up enzymatic reaction.**

- Add test compounds and protease to the microplate wells. For one well of a 96-well plate, the suggested volume of protease solution is 85 µL and of test compound is 15 µL.

- Establish the following control wells at the same time, as deemed necessary:
  - Positive control contains protease without test compound.
  - Inhibitor control contains protease and protease inhibitor.
  - Vehicle control contains protease and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
  - Test compound control contains assay buffer (Component C) and test compound. Some test compounds may themselves be fluorescent and may give false results.
  - Substrate control contains assay buffer (Component C).
- Using the assay buffer (Component C), bring the total volume of all controls to 100 µL.
- Pre-incubate the plate for 10 minutes at 37°C. This is important because the diluted enzyme stock had been kept on ice.

**3. Initiate the enzymatic reaction.**

- Add 50 µL of WNV protease substrate solution into each well except the test compound control wells. For best accuracy, it may be advisable to have the protease substrate solution preset to 37°C also. Mix the reagents completely by shaking the plate gently for no more than 30 seconds.
- Measure fluorescence signal:

  For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=354 nm/442 nm continuously and record data every 5 min. for 30 to 60 min (37°C recommended).

  For end-point reading: Incubate the reaction at 37°C for 30 to 60 min. Do not allow direct light to strike the plate. Measure the fluorescence intensity at Ex/Em=354 nm/442 nm.
- Data analysis: Refer to Appendix I.

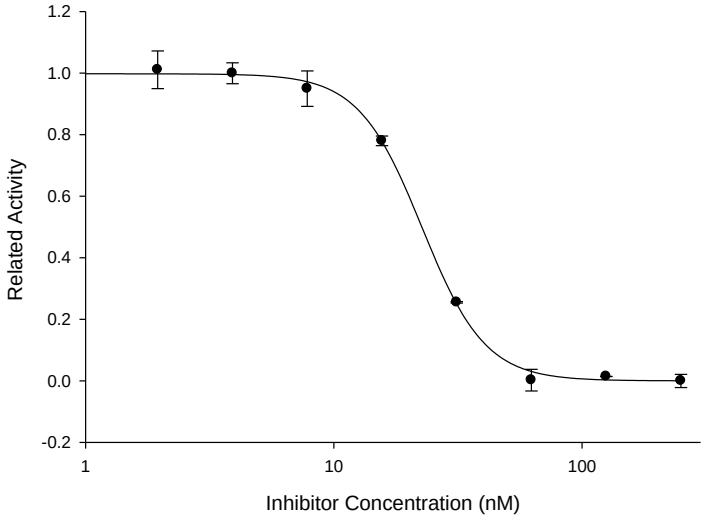


Figure 1. Inhibition of WNV by protease inhibitor, undeca-D-Arg-$NH_2$.

## Appendix I. Data Analysis

- The fluorescence reading from the substrate control well is the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. The fluorescence readings are expressed in relative fluorescence units (RFU).
- For kinetic reading:
  - Plot the data as RFU versus time for each sample. If you want to convert RFUs to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. Typically, the first 10-15% of the reaction will be the optimal range.
  - Obtain the initial reaction velocity ($V_o$) in RFU/min. Determine the slope of the linear portion of the data plot.
  - A variety of data analyses can be done, e.g., determining inhibition %, $EC_{50,}$ $IC_{50}$, $K_m$, $K_i$, etc.
- For endpoint reading:
  - Plot data as RFU versus concentration of test compounds.
  - A variety of data analyses can be done, e.g., determining inhibition %, $EC_{50}$, $IC_{50}$, etc.

## Appendix II. Instrument Calibration

- AMC fluorescence reference standard: Dilute 5 mM AMC (Component B) to 10 μM in the assay buffer (Component C). Perform 2-fold serial dilutions to obtain 5, 2.5, 1.2, 0.6, 0.3, and 0.15 μM AMC solutions, including an assay buffer blank. Add 100 μL/well of these serially diluted AMC solutions into the plate.
- Add 50 μL of WNV substrate solution (refer to Step 1 for preparation) to each well. Mix the reagents by shaking the plate gently for 3 to 5 sec.

  Note: WNV substrate solution is added to the reference standard to normalize for the intrinsic substrate fluorescence. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Measure the fluorescence intensity of the reference standard and substrate control wells at Ex/Em=354 nm/442 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the AMC fluorescence reference standard curve as RFU (relative fluorescent unit) versus concentration as shown in Figure 2.

  Note: The final concentration of AMC reference standard solutions are 7, 3.5, 1.7, 0.85, 0.4, 0.2, 0.1 and 0 μM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of enzymatic reaction final product.

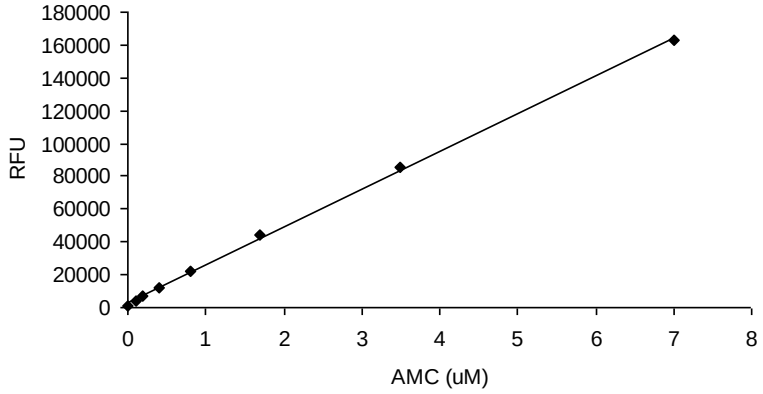


Figure 2. AMC reference standard.
AMC was serially diluted in assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=354 nm/442 nm. (Flexstation 384II, Molecular Devices)

## References

1. Shiryaev, SA. et al., *Biochem J.* **393**, 503 (2006).
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6. Lanciotti, RS. et al., *Science* **286,** 2333 (1999).
7. Mueller, NH. et al., *Int. J. Biochem. Cell Biol.* **39**, 606 (2007).