SensoLyte® 490 HCV Protease Assay Kit *Fluorimetric*

Revision Number: 1.2	*Last updated: October 2014*
Catalog #	**AS-72087**
Kit Size	200 Assays (96-well) or 500 Assays (384-well)

- ***Optimized Performance:*** Optimal conditions for the detection of HCV NS3/4A protease activity.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	HCV NS3/4A protease substrate EDANS/DABCYL FRET peptide, Ex/Em=340 nm/490 nm upon cleavage	250 µL
Component B	EDANS, fluorescence reference standard Ex/Em=340 nm/490 nm	100 µM DMSO solution, 10 µL
Component C	2X Assay buffer	25 mL
Component D	Stop solution	15 mL
Component E	DTT	1 M, 1 mL
Component F	Pep4AK HCV NS3 protease cofactor	150 µL, 600 µM

Other Materials Required (but not provided)

- 96-well or 384-well microplate: Black, flat bottom plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 490±30 nm with excitation 340±30 nm.
- HCV NS3/4A protease: HCV NS3/4A protease can be produced from *E. coli*[1-3]. AnaSpec provides highly active recombinant HCV NS3/4A protease (Cat#61017).

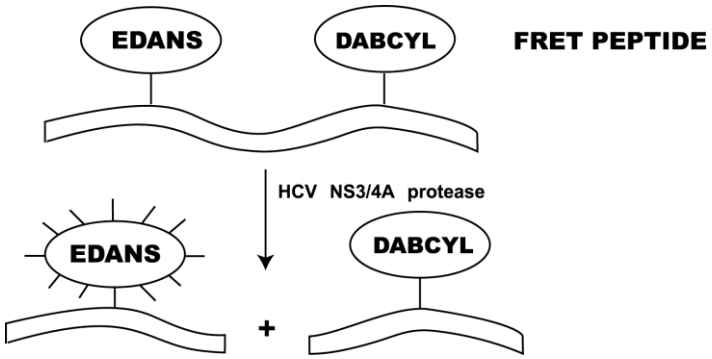
Storage and Handling

- Store all kit components at -20°C.
- Keep Components A and B away from light.
- If used frequently, Components C and D can be stored at room temperature for convenience.

Introduction

The NS3/4A protease of Hepatitis C Virus (HCV) is required for the cleavage of viral nonstructural polyprotein at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B sites. These cleavages are essential for the maturation of the viral proteins. Thus, this protease has become one of the key targets for developing anti-HCV drugs.

The SensoLyte® 490 HCV Protease Assay Kit provides a convenient assay for high throughput screening of HCV NS3/4A protease inhibitors and for continuous quantification of HCV NS3/4A protease activity using fluorescence resonance energy transfer (FRET) peptide. The sequence of this FRET peptide is derived from the cleavage site of NS4A/NS4B. The cysteine on the natural cleavage site is replaced with aminobutyric acid (Abu) and the scissile amide bond with an ester bond. These modifications improved k_{cat}/K_m values by more than 100 fold and enabled the detection of the activity of NS3/4A protease at subnanomolar concentrations. In the FRET peptide, the fluorescence of EDANS is quenched by DABCYL. Upon cleavage into two separate fragments by HCV NS3/4A protease at the Abu-Ala bond (see Scheme 1), the fluorescence of EDANS is recovered, and can be monitored at excitation/emission = 340 nm/490 nm. The assays are performed in a convenient 96-well or 384-well microplate format.



Scheme 1. Proteolytic cleavage of EDANS/DABCYL FRET peptide by HCV NS3/4A protease.

Protocol

Note 1: For fluorescence instrument calibration, please refer to Appendix II (recommended for first-time users).
Note 2: Please use protocol A or B based on your needs.

Protocol A. Screening protease inhibitors using purified HCV NS3/4A protease

Note: Warm all kit components until thawed to room temperature before starting the experiments.

1. Prepare working solutions

1.1 Assay buffer: Prepare fresh assay buffer for each experiment according to Table 1. **Use this DTT-containing 1X assay buffer in all the following steps.**

Table 1. Assay buffer for one 96-well plate (100 assays).

Components	Volume
2X assay buffer (Component C)	5 mL
1 M DTT (Component E)	300 µL
Deionized water	5 mL

Total volume	10 mL

1.2 HCV NS3/4A protease substrate solution: Dilute HCV protease substrate (Component A) 1:50 in assay buffer. For each experiment prepare fresh substrate solution.

Table 2. HCV protease substrate solution for one 96-well plate (100 assays).

Components	**Volume**
HCV protease substrate (50X, Component A)	100 µL
Assay buffer	4.9 mL
Total volume	5 mL

1.3 HCV NS3 protease diluent: Dilute HCV NS3 protease to an appropriate concentration in the assay buffer.

Note: Prepare enzyme diluent immediately before use. Do not vortex enzyme. Prolonged storage of diluent or vigorously vortexing will denature the enzyme. Keep the enzyme on ice.

2. Active HCV NS3 protease

Note: The following step is to activate HCV NS3 protease. If your HCV protease contains both NS3 and 4A domains, for example, the HCV NS3/4A protease (Cat#61017), step 2 can be skipped.

2.1 Pep4AK diluent: Dilute Pep4AK (Component F) 1:100 in assay buffer.

2.2 Mix an equal volume of the HCV NS3 protease diluent and Pep4AK diluent. Incubate the mixture at 23-25°C for 15 min.

3. Set up enzymatic reaction

3.1 Add test compounds and HCV NS3/4A protease diluent into a microplate. The suggested total volume of HCV NS3/4A protease diluent and test compound for a 96-well plate is 50 µL. The suggested total volume of HCV NS3/4A protease and test compound for a 384-well plate is 20 µL.

3.2 Simultaneously set up the following controls:

- Positive control contains HCV NS3/4A protease diluent without test compound.
- Inhibitor control contains HCV NS3/4A protease diluent and known HCV NS3/4A protease inhibitor (e.g. Ac-DE-Dif-E-Cha-C, AnaSpec Cat#25346).
- Vehicle control contains HCV NS3/4A protease diluent and vehicle used to deliver test compound (e.g. DMSO).
- Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains assay buffer.

3.2 Bring the total volume of all the controls to 50 µL/well for a 96-well plate or 20 µL/well for a 384-well plate with assay buffer.

4. Pre-incubation

4.1 Incubate the plate at the desired temperature for enzymatic reaction (e.g. 25°C or 37°C) for 10-15 min. In the meantime, also incubate the HCV NS3/4A protease substrate solution at the same temperature.

5. Initiate the enzymatic reaction

5.1 Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of HCV NS3/4A protease substrate solution. Mix the reagents completely by shaking the plate gently for 30-60 second.

5.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=340 nm/490 nm continuously and record data every 5 minutes for 30 to 60 minutes.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 minutes. Keep the plate away from direct light. Optional: Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of stop solution (Component D). Mix the reagents, then measure fluorescence intensity at Ex/Em=340 nm/490 nm.

Note: If stop solution looks cloudy, warm up in 37°C water bath to dissolve the precipitate.

5.3 Data analysis: Refer to Appendix I.

A sample data for HCV protease inhibitor is shown on Figure 1.

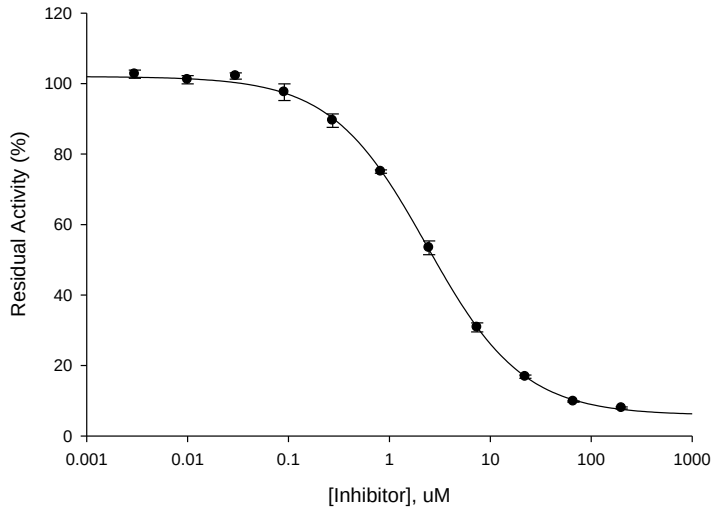


Figure 1. The inhibitory curve of HCV protease inhibitor, Ac-DE-Dif-E-Cha-C. Ac-DE-Dif-E-Cha-C (AnaSpec Cat#25346) was serially diluted in assay buffer and then pre-incubated with 20 ng/well HCV protease (Cat#61017) for 15 min at RT. The HCV NS3/4A protease substrate solution was prepared according to the protocol and 50 µL of substrate solution was added to each well to initiate the reaction. The fluorescence signal was continuously monitored for 30 min. The initial velocity of reactions and the percentage of residual activity were calculated. The IC_{50} of Ac-DE-Dif-E-Cha-C is 2.35 µM. (n=3, mean±S.D.)

Protocol B. Measuring HCV NS3/4A protease activity in biological samples

Note: Please check Appendix III for a sample protocol of preparing cell lysate containing HCV NS3 protease.

1. Prepare working solutions

1.1 2X Assay buffer: Add 60 µL of 1 M DTT (Component E) per mL of 2X assay buffer (Component C). **Use this DTT-containing 2X assay buffer in *all* the following steps.**

Note: Prepare the DTT -containing assay buffer fresh for each experiment.

1.2 HCV NS3/4A protease substrate solution: For each experiment, prepare fresh substrate solution by diluting the stock solution (Component A) 1:50 in 2X assay buffer.

2. Set up enzymatic reaction

2.1 Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of HCV NS3/4A protease containing biological sample.

2.2 Simultaneously set up the following controls:

- Positive control contains HCV NS/4A protease standard.
- Negative control contains biological sample without HCV NS/4A protease.
- Substrate control contains deionized water.

Note: Bring the total volume of all the controls to 50 µL/well for a 96-well plate or 20 µL/well for a 384-well plate.

3. Initiate the enzymatic reaction

3.1 Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of HCV NS3/4A protease substrate solution. Mix the reagents completely by shaking the plate gently for 30-60 seconds.

3.2 Measure fluorescence signal:

- For kinetics reading: Immediately start measuring fluorescence intensity at Ex/Em=340 nm/490 nm continuously and record data every 5 minutes for 30 to 60 minutes.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 minutes. Keep the plate away from direct light. Optional: Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of stop solution (Component D), then measure fluorescence intensity at Ex/Em=340 nm/490 nm.

4. Data analysis: Refer to Appendix I.

Appendix I: Data Analysis

- The fluorescence reading from the substrate control well is the background fluorescence. Subtract this background reading from the readings of the other wells to get the relative fluorescence unit (RFU).
- For kinetics reading:
  - Plot data as RFU versus time for each sample (Figure 2). To convert RFU to concentration of the product of enzymatic reaction, please refer to Appendix II for setting up the fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. 10-15% conversion appears to be the optimal range.
  - Obtain the initial reaction velocity (Vo) 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.

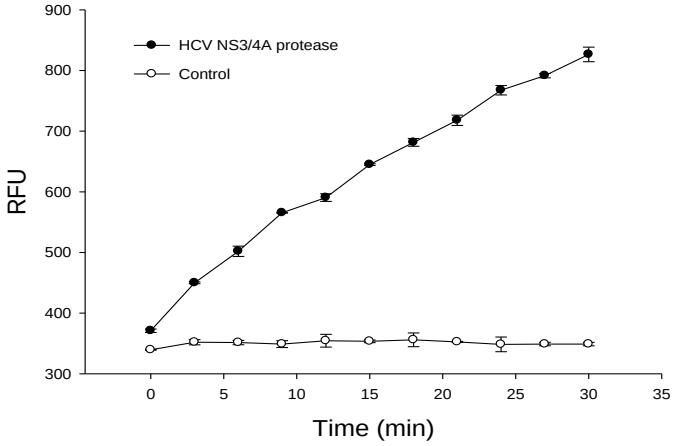


Figure 2. Proteolytic cleavage of EDANS/DABCYL FRET peptide by HCV NS3/4A protease. The FRET peptide was cleaved by HCV NS3/4A protease and the fluorescent signal was continuously monitored at Ex/Em=360 nm/ 460 nm on a microplate reader (FLx800, Bio-Tek Instruments) for 30 min. The control well contains FRET substrate but no enzyme. (n=2, Mean+S.D.)

Appendix II: Instrument Calibration

- EDANS fluorescence reference standard: Dilute 100 μM EDANS (Component B) to 1 μM in deionized water. Perform 2-fold serial dilutions to get concentrations of 500, 250, 125, 62.5, 31.25, 15.63, and include blank sample. Add 50 μL/well of these serially diluted EDANS from 1 μM to 0 nM into the 96-well plate or 20 μL/well into the 384-well plate.
- Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of HCV NS3/4A protease substrate solution (refer to protocol B step 1 for preparation).
  Note: The HCV NS3/4A protease substrate solution should be added to the EDANS reference standard to correct for the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Optional: If the stop solution (Component D) was added into the enzymatic reaction before taking the end-point reading, the same volume of stop solution should be added to the reference standard wells to obtain a better comparison.
- Measure the fluorescence intensity of the reference standard and substrate control wells at Ex/Em=340 nm/490 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot EDANS fluorescence reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 3.

  Note: The final concentrations of the EDANS reference standard are 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 0 nM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

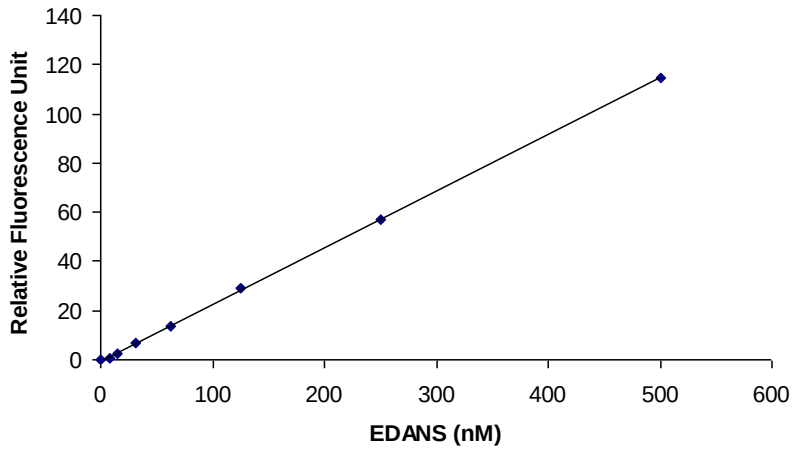


Figure 3. EDANS reference standard calibration curve.

EDANS was diluted in assay buffer containing HCV NS3/4A protease substrate. 100 μL of EDANS diluent at each concentration was added into a black 96-well microplate. The fluorescence signal was measured on a microplate reader (FLx800, Bio-Tek Instruments) at Ex/Em=360 nm/460 nm. (Samples were done in duplicates).

Appendix III: A sample protocol for preparing NS3-containing cellular membrane fractions[1,2]

- Grow the HCV replicon-containing cells (e.g. 1 x 10^7-10^8 Huh7 cells) to 90% confluence. Wash the cells with 1x phosphate-buffered saline once. Detach the cells by scraping. Harvest the cell pellet by centrifuging at 900x g for 10 min at 4°C.
- Resuspend the cell pellets with 1 mL of hypotonic buffer (10 mM Tris-HCl, pH 7.8, 10 mM NaCl). Incubate the cell pellets on ice for 15-20 min. Disrupt the cell pellets with 50 strokes of a tight fitting pestle in a Dounce homogenizer.
- Centrifuge the homogenate at 900x g for 5 min at 4°C to remove the nuclei, which is in the pellet.
- Collect the supernatant, which contains membrane fractions, and centrifuge it at 15,000x g for 20 min at 4°C to pellet the cellular membrane.
- Discard the supernatant and resuspend the pellet in 100-500 μL of storage buffer (hypotonic buffer plus 15% glycerol).
- Continue to **Step 2 in protocol B** for HCV NS3 protease assay. Typically, membrane from 1x 10^6 cells is used for one assay.
- The membrane fractions can be stored at -80°C for later use up to 3 months.

References:

1. Hardy, RW. et al. *J Virol* **77**, 2029 (2003).
2. Hamil, P. et al. *Biochem* **44**, 6586 (2005).