SensoLyte® 520 HCV Protease Assay Kit
Fluorimetric

Revision Number: 1.2 *Last updated: October 2014*

Catalog #	AS-71145
Kit Size	100 Assays (96-well plate) or 300 assays (384-well)

- ***Convenient Format:*** Complete kit including all the assay components.
- ***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 Ex/Em=490 nm/520 nm upon cleavage	120 µL
Component B	5-FAM, fluorescence reference standard Ex/Em=490 nm/520 nm upon cleavage	100 µM, 5 µL
Component C	2X Assay buffer	10 mL
Component D	Stop solution	10 mL
Component E	DTT	1 M, 0.5 mL
Component F	Pep4AK	50 µL, 600 µM

Other Materials Required (but not provided)

- 96-well or 384-well microplate: Black, flat-bottom 96-well or 384-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.
- HCV NS3 protease: HCV NS3 protease can be produced from *E. coli*.[1-3] AnaSpec provides highly active recombinant HCV NS3/4A protease (Cat#61017).

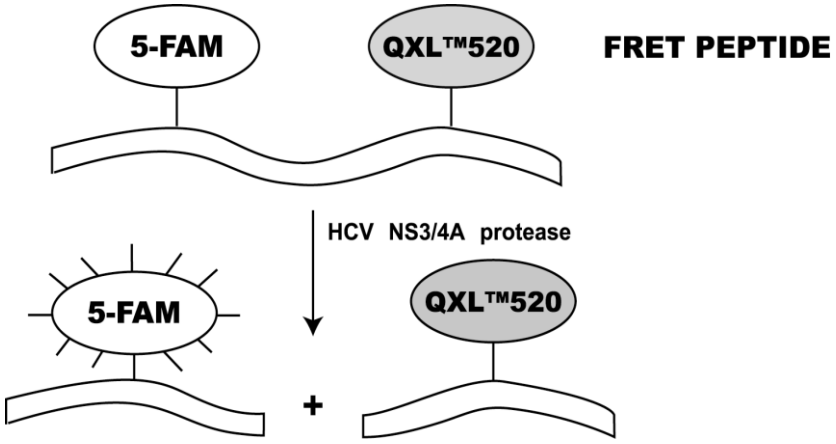
Storage and Handling

- Store all kit components at -20°C
- Protect Components A and B from light
- 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® 520 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 a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET) peptide. The sequence of this FRET peptide is derived from the cleavage site of NS4A/NS4B. In the FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon cleavage into two separate fragments by HCV NS3/4A protease (**Scheme 1**), the fluorescence of 5-FAM is recovered, and can be monitored at excitation/emission = 490 nm/520 nm. With superior fluorescence quantum yield and longer wavelength, the signal of 5-FAM is less interfered by the auto fluorescence of cell components and test compounds. The assay can detect as low as 1.56 ng/mL active HCV NS3/4A protease.



Scheme 1. Proteolytic cleavage of 5-FAM/QXL™520 FRET peptide by HCV NS3/4A protease

Protocol

Note 1: Please use protocol A or B based on your needs.
Note 2: For standard curve, please refer to Appendix II (optional).

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

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

1. Prepare working solutions.

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

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

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

2. Activate HCV NS3 protease.

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

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^{o}C for 15 min.

3. Set up enzymatic reaction.

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

3.2 Set up the following control wells at the same time, as deemed necessary:

- Positive control contains protease diluent without test compound.
- Inhibitor control contains protease diluent and known HCV NS3/4A protease inhibitor (e.g. Ac-DE-Dif-E-Cha-C, AnaSpec Cat# 25346).
- Vehicle control contains 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.

Note: Bring up the total volume of all the controls to 50 µL (96-well plate) or 20 µL (384-well plate) using assay buffer.

4. Pre-incubation.

4.1 Incubate the plate at the desired temperature for enzymatic reaction for 10-15 min. Also incubate the 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 substrate solution (Step 1.2). Mix the reagents completely by shaking the plate gently for 30-60 sec.

5.2 Measure the fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490/520 nm continuously and record data every 5 min for 30-60 min.

- For end-point reading: Incubate the reaction at room temperature for 30-60 min. Keep plate 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. Measure fluorescence intensity at Ex/Em=490/520 nm.

Note: If stop solution looks cloudy, warm it up in a water bath to dissolve the precipitate.

5.3 Data analysis: Refer to Appendix I. A sample data of HCV protease inhibitors is shown in Figure 1.

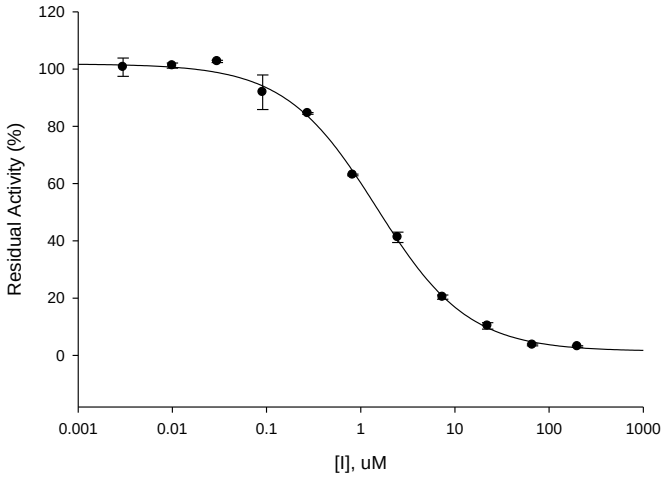


Figure 1. The inhibitory curve of HCV protease inhibitor, Ac-DE-Dif-E-Cha-C.
HCV protease inhibitor (AnaSpec Cat#25346) was serially diluted in assay buffer containing 5 ng/well HCV protease (Cat#61017) and HCV NS3/4A protease substrate and fluorescence signal was continuously monitored for 30 min at ex/em=490nm/520nm (Flex Station II384, Molecular Devices). The initial velocity of reactions and the percentage of residual activity were calculated. The IC_{50} of Ac-DE-Dif-E-Cha-C is 1.5 µM. (n=3, mean±S.D.)

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

Note: Please refer to Appendix III for a sample protocol on 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 freshly 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 Set up the following control wells at the same time, as deemed necessary:

- 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 up the total volume of all the controls to 50 µL (96-well plate) or 20 µL (384-well plate).

3. Initiate the enzymatic reaction.

3.1 Add 50 µL (96-well plate) or 20 µL (384-well plate) of substrate solution (Step 1.2). Mix the reagents completely by shaking the plate gently for 30-60 sec.

3.2 Measure fluorescence signal:

- For kinetics reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 nm continuously and record data every 5 min for 30 to 60 min.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 minutes. Keep the plate 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=490 nm/520 nm.

3.3 Data analysis: Refer to Appendix I.

Appendix I: Data analysis

- The fluorescence reading from the substrate control well is the background fluorescence. This background reading has to be subtracted from the readings of the other wells. This reading is 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 (setting up 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 the concentration of test compounds.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

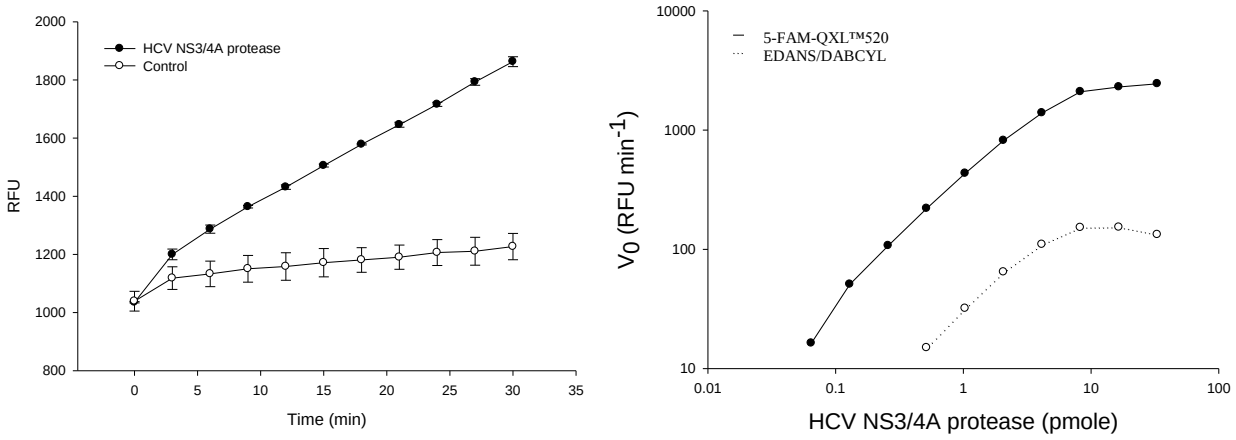


Figure 2. Proteolytic cleavage of 5-FAM/QXL™520 FRET substrate by HCV NS3/4A protease

Left panel: HCV NS3/4A substrate was cleaved by HCV NS3/4A protease and the fluorescent signal continuously monitored at Ex/Em=485±20 nm/ 528±20 nm for 30 min. Control well contains substrate without HCV NS3/4A protease. (n=2, mean±S.D.)
Right panel: 5-FAM/QXL™520 FRET substrate is 10 fold more sensitive than EDANS/DABCYL FRET substrate. The assay can detect 0.1 pmole of HCV NS3/4A protease. (n=2, mean±S.D.)

Appendix II: Instrument calibration

- 5-FAM fluorescence reference standard: Dilute 100 μM 5-FAM (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 0 nM. Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of serially diluted 5-FAM from 1 μM to 0 nM.
- Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of substrate solution (refer to protocol B step 1 for preparation).

  Note: Substrate solution should be added to the 5-FAM reference standard to correct the fluorescence inner filter effect.
- 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 reference standard wells for better comparison.
- Measure the fluorescence intensity of the reference standard wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot 5-FAM fluorescent reference standard as RFU (relative fluorescent unit) versus concentration as **Figure 3**.

Note: The final concentration of 5-FAM reference standard is 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 batch of experiments. It is also an indicator of the amount of final product of the HCV NS3/4A protease enzymatic reaction.

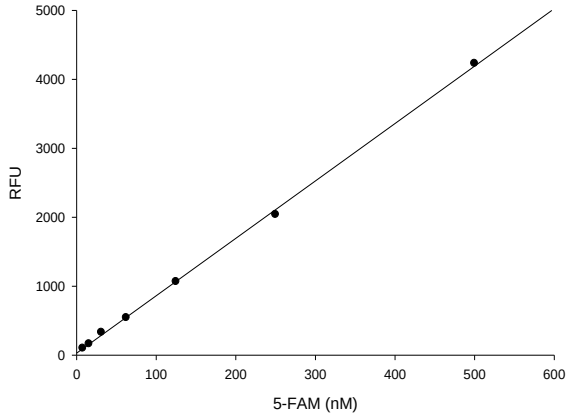


Figure 3. 5-FAM reference standard 5-FAM was serially diluted in assay buffer containing substrate, and fluorescence recorded at Ex/Em=485±20 nm/ 528±20 nm. (n=2, mean±S.D.) (Flex Station II384, Molecular Devices)

Appendix III: A sample protocol for preparing NS3-containing cellular membrane fractions

- Grow the HCV replicon-containing cells (e.g. 1 x 10^7-10^8 Huh7 cells) to 90% confluence. Wash cells with 1x phosphate-buffered saline once. Detach cells by scraping. Harvest the cell pellet by centrifuging at 900 x 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 900 x 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 15,000 x 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.
- Membrane fractions can be stored at -80°C for later use, good for up to 3 months.

References

1. Sali, DL. et al. *Biochemistry* **37**, 3392 (1998).
2. Steinkuhler, C. et al. *Biochemistry* **37**, 8899 (1998).
3. Gallinari, P. et al. *J. Virol.***72**, 6758 (1998).
4. Hardy, RW. et al. *J.Virol.* **77**, 2029 (2003).
5. Hamill, P. and Jean, F., *Biochemistry* **44**, 6586 (2005).