EnzoLyte™ 520 HCV Protease Assay Kit
Fluorimetric

Catalog #	71145
Unit Size	1 Kit
Kit Size	100 Assays (96-well) 300 assays (384-well)

This kit is optimized to detect the activity of hepatitis C virus NS3/4A protease using a 5-FAM/QXL™520 FRET peptide substrate, which can be monitored at Ex/Em=490 nm/520 nm upon proteolytic cleavage. It provides sufficient materials to perform 100 assays in 96-well format and 300 assays in 384-well plate. The kit has the following features:

- ***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

USA and Canada Ordering Information

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International Ordering Information

A list of international distributors is available at www.anaspec.com.

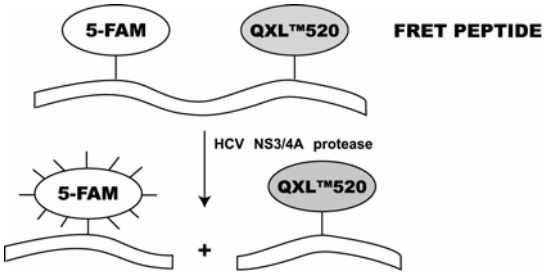
Version 1.2

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 EnzoLyte™ 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 autofluorescence of cell components and test compounds. Compared to the EDANS/DABCYL FRET substrate, the 5-FAM/QXL™520 FRET substrate is 10 folds more sensitive and can detect the activity of 0.1 pmole of HCV NS3/4A protease.

The assays are performed in a convenient 96-well or 384-well microplate format.



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

KIT COMPONENTS, STORAGE AND HANDLING

Note: Store all kit components at -20℃, and keep components A and B from light. If use frequently, components D and E can be stored at room temperature for convenience.

Component A: HCV NS3/4A protease substrate (1 vial)
5-FAM/QXL™520 FRET peptide
Ex/Em=490 nm/520 nm upon cleavage

Component B: 5-FAM, fluorescence reference standard (100 μM, 5 μL)
Ex/Em=490 nm/520 nm

Component C: DMSO (200 μL)

Component D: 2X Assay buffer (10 mL)

Component E: Stop solution (10 mL)

Component F: DTT (1 M, 0.5 mL)

Component G: Pep4AK (60 μL)
HCV NS3 protease cofactor

OTHER MATERIALS REQUIRED (BUT NOT PROVIDED)

96-well or 384-well microplate: Black microplate can provide better signal:noise value.
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).

STANDARD OPERATION PROTOCOL

Note 1: For fluorescence instrument calibration, please refer to Appendix II. This is recommended for the first-time users.

Note 2: Please use protocol A or B based on your needs.

Protocol A. Screen 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.**
   - Assay buffer: Dilute 2X assay buffer (component D) to 1X by deionized water. Add 30 µL of 1 M DTT (component F) per mL of 1X assay buffer. **Use this DTT-containing 1X assay buffer in all the following steps.**

     Note: Prepare fresh the DTT -containing 1X assay buffer for each experiment.
   - HCV NS3/4A protease substrate solution: At the first use, add 120 µL of DMSO (component C) into the vial of HCV NS3/4A protease substrate (component A) to reconstitute the substrate. Store the stock solution at -20°C. For each experiment, prepare fresh substrate solution by diluting the stock solution 1:50 in assay buffer.
   - HCV NS3 protease diluent: Dilute HCV NS3 protease to appropriate concentration in assay buffer.

     Note: Prepare enzyme diluent right before use. Don't vortex enzyme. Prolonged storage of dilutent or vigorously vortex will denature the enzyme. Preserve 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, step 2 can be skipped.
   - Pep4AK diluent: Dilute Pep4AK (component G) 1:100 in assay buffer.
   - 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.**
   - Add test compounds and HCV NS3/4A protease diluent into 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.
   - Set up the following controls at the same time:
     - 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.

   Note: Match the total volume of all the controls to 50 µL for 96-well plate or 20 µL for 384-well plate by assay buffer.
4. **Pre-incubation.**
   - Incubate the plate at the desired temperature for enzymatic reaction (e.g. 25°C or 37°C) for 10-15 min. In the mean time, also incubate the HCV NS3/4A protease substrate solution at the same temperature.
5. **Initiate the enzymatic reaction.**

- Add 50 μL of HCV NS3/4A protease substrate solution into the wells of a 96-well plate. Or add 20 μL into the wells of a 384-well plate. Mix the reagents completely by shaking the plate gently for 30-60 second.
- Measure fluorescence signal:

  For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 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 from direct light. (Optional) add 50 μL/well stop solution (component E) to 96-well plate or 20 μL/well to 384-well plate. Mix the reagents. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.
- Data analysis: Refer to Appendix I. A sample data of HCV protease inhibitor was showed in Figure 1.

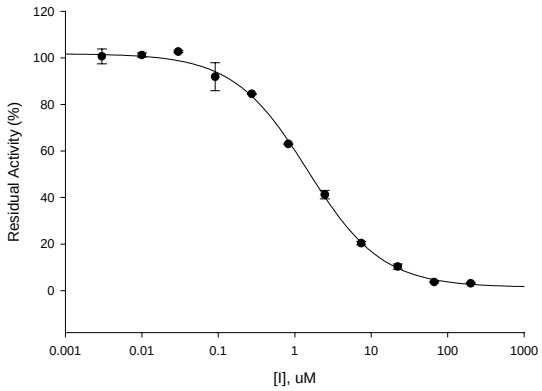


Figure 1. The inhibitory curve of HCV protease inhibitor, Ac-DE-Dif-E-Cha-C. Ac-DE-Dif-E-Cha-C (AnaSpec Cat#25346) was serially diluted in assay buffer and then pre-incubated with 5 ng/well HCV protease (Cat#61017) for 15 min at RT. The HCV NS3/4A protease substrate solution was prepared according to 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 IC50 of Ac-DE-Dif-E-Cha-C is 1.5 μM. (n=2, mean±S.D.)

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

1. **Prepare working solutions.**
   - 2X Assay buffer: Add 60 µL of 1 M DTT (component F) per mL of 2X assay buffer (component D). Use this DTT-containing 2X assay buffer in ***all*** the following steps.
     Note: Prepare the DTT -containing assay buffer freshly for each experiment.
   - HCV NS3/4A protease substrate solution: At the first use, add 120 µL of DMSO (component C) into the vial of HCV NS3/4A protease substrate (component A) to reconstitute the substrate. Store the stock solution at -20°C. For each experiment, prepare fresh substrate solution by diluting the stock solution 1:50 in 2X assay buffer.
2. **Set up enzymatic reaction.**
   - Add 50 µL/well HCV NS3/4A protease containing biological sample to a 96-well microplate. Or 20 µL/well to a 384-well plate.
   - Set up the following controls at the same time:
     - Positive control contains HCV NS/4A protease standard.
     - Negative control contains biological sample without HCV NS/4A protease.
     - Substrate control contains deionized water.

   Note: Match the total volume of all the controls to 50 µL for 96-well plate or 20 µL for 384-well plate.
3. **Initiate the enzymatic reaction.**
   - Add 50 µL of HCV NS3/4A protease substrate solution into the wells of a 96-well plate. Or add 20 µL into the wells of a 384-well plate. Mix the reagents completely by shaking the plate gently for 30-60 seconds.
   - Measure fluorescence signal:

     For kinetics reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 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 from direct light. (Optional) add 50 µL/well stop solution (component E) to 96-well plate or 20 µL/well to 384-well plate. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm
   - 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**). If you want to convert the RFU to the concentration of the product of enzymatic reaction, please refer to Appendix II for 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.

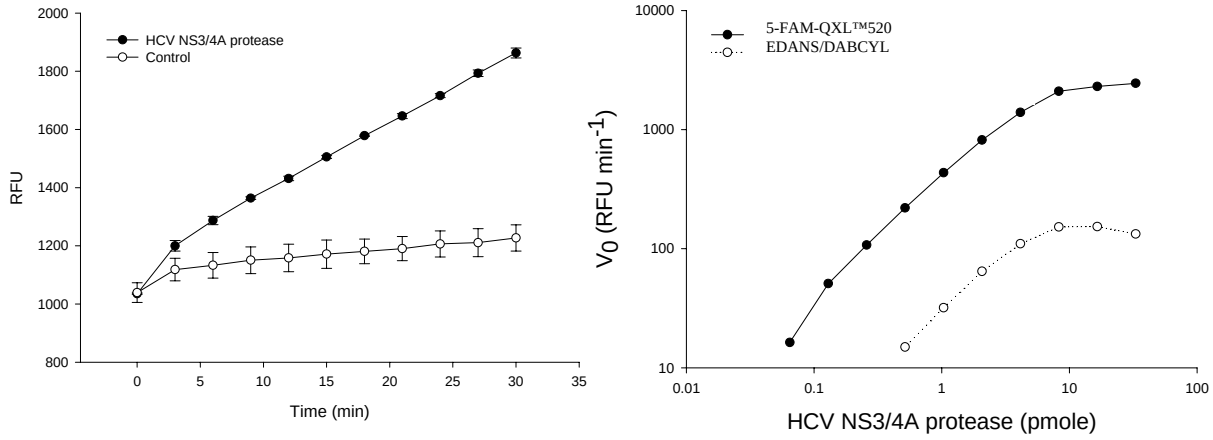


Figure 2. The proteolytic cleavage of 5-FAM/QXL™520 FRET substrate by HCV NS3/4A protease.
Left panel: The HCV NS3/4A substrate was cleaved by HCV NS3/4A protease and the fluorescent signal was continuously monitored at Ex/Em=485±20 nm/ 528±20 nm for 30 min. The control well contains substrate without 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. Do 1:2 serial dilutions to get concentrations of 500, 250, 125, 62.5, 31.25, 15.63 and 0 nM. Add 50 μL/well of serially diluted 5-FAM from 1 μM to 0 nM into the 96-well plate or 20 μL/well into the 384-well plate.
- Add 50 μL/well HCV NS3/4A protease substrate solution (refer to protocol B step 1 for preparation) to the 96-well plate or 20 μL/well into the 384-well plate.

  Note: The HCV NS3/4A protease substrate solution should be added to the 5-FAM reference standard to correct the fluorescence inner filter effect.
- (Optional) If the stop solution (component E) 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.
- 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.

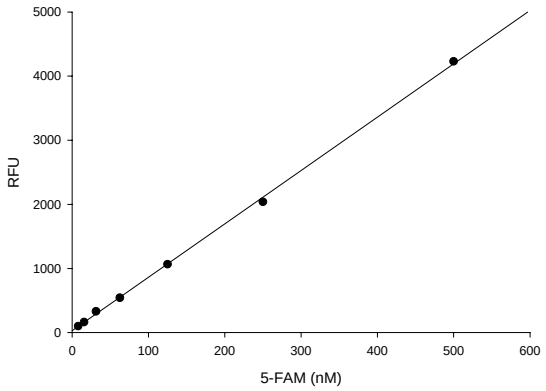


Figure 3. 5-FAM reference standard.
5-FAM was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=485±20 nm/ 528±20 nm. (n=2, mean±S.D.)

REFERENCES

1. D. L. Sali et al., *Biochemistry* 37, 3392-3401 (1998).
2. C. Steinkuhler et al., *Biochemistry* 37, 8899-8905 (1998).
3. P. Gallinari et al., *J.Virol.* 72, 6758-6769 (1998).