SensoLyte® 620 HCV Protease Assay Kit

*Fluorimetric*

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>71146</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit Size</td>
<td>1 Kit</td>
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<tr>
<td>Kit Size</td>
<td>100 Assays (96-well) or 300 Assays (384-well)</td>
</tr>
</tbody>
</table>

This kit is optimized to detect the activity of hepatitis C virus NS3/4A protease using a HiLyte Fluor™ TR/QXL™610 FRET peptide substrate which can be monitored at Ex/Em=591 nm/622 nm upon proteolytic cleavage. It provides ample materials to perform 100 assays in a 96-well format or 300 assays in a 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

AnaSpec Corporate Headquarter
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Website: [www.anaspec.com](http://www.anaspec.com)

### International Ordering Information

A list of international distributors is available at [www.anaspec.com](http://www.anaspec.com).

Version 2.1
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. Consequently, this protease has become one of the key targets for developing anti-HCV drugs.

The SensoLyte® 620 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 HiLyte Fluor™TR/QXL™610 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 HiLyte Fluor™TR is quenched by QXL™610. Upon cleavage into two separate fragments by the HCV NS3/4A protease (Scheme 1), the fluorescence of HiLyte Fluor™TR is recovered, and can be monitored at excitation/emission = 591 nm/622 nm. With near red wavelength, the signal of HiLyte Fluor™TR avoids the interference from most test compounds and is therefore ideal for high throughput screening of HCV NS3/4A protease inhibitors.

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

Scheme 1. Proteolytic cleavage of HiLyte Fluor™TR/QXL™610 FRET peptide by HCV NS3/4A protease.

KIT COMPONENTS, STORAGE AND HANDLING

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

Component A: HCV NS3/4A protease substrate (120 μL)
HiLyte Fluor™TR/QXL™610 FRET peptide
Ex/Em=591 nm/622 nm upon cleavage

Component B: HiLyte Fluor™TR, fluorescence reference standard (100 μM, 5 μL)
Ex/Em=591 nm/622 nm

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)
HCV NS3 protease cofactor

OTHER MATERIALS REQUIRED (BUT NOT PROVIDED)

96-well or 384-well microplate: Black microplate provides better signal to noise ratio.
Fluorescence microplate reader: Capable of detecting emission at 622 nm with excitation at 591 nm.
HCV NS3 protease: HCV NS3 protease can be produced from E. coli. AnaSpec provides highly active recombinant HCV NS3/4A protease (Cat#61017).
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**  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: Prepare fresh assay buffer for each experiment according to table 1. Use this DTT-containing 1X assay buffer in all the following steps.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X assay buffer (Component C)</td>
<td>5 mL</td>
</tr>
<tr>
<td>1 M DTT (Component D)</td>
<td>300 μL</td>
</tr>
<tr>
<td>Deionized water</td>
<td>5 mL</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

   **Note:** Use freshly prepared DTT-containing 1X assay buffer for each experiment.

   - 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>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV protease substrate (50X, Component A)</td>
<td>100 μL</td>
</tr>
<tr>
<td>Assay buffer</td>
<td>4.9 mL</td>
</tr>
<tr>
<td>Total volume</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

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

   **Note:** Prepare enzyme immediately right before use. Do not vortex enzyme. Prolonged storage of diluent or vigorous vortexing will denature the enzyme. Keep the 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, for example, the HCV NS3/4A protease (Cat#61017), step 2 can be skipped.

   - Pep4AK diluent: Dilute Pep4AK (Component F) 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 a microplate. The suggested total volume of HCV NS3/4A protease diluent and test compound for a 96-well plate is 50 μL or 20 μL for a 384-well plate.

   - 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-DEDif-EchaC, IC₅₀=5 μM, 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: Bring the total volume of all the controls to 50 μL for a 96-well plate or 20 μL for a 384-well plate with assay buffer.

4. Pre-incubation
   - Incubate the plate at the desired temperature for the 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
   - 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 sec.
   - Measure fluorescence signal:
     For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=591 nm/622 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 min. 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. Measure fluorescence intensity at Ex/Em=591 nm/622 nm.
     Note: If stop solution looks cloudy, warm it up in 37°C water bath to dissolve the precipitate.
   - Data analysis: Refer to Appendix I.

![Graph](image)

**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 100 ng/well HCV protease (Cat#61017) for 15 min at RT. The 620 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. (n=3, mean±S.D.)
Protocol B  Measure 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
   - 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: Use freshly prepared DTT-containing assay buffer for each experiment.
   - 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
   - Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of HCV NS3/4A protease containing biological sample.
   - 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: Bring the total volume of all the controls to 50 μL for a 96-well plate or 20 μL for a 384-well plate.

3. Initiate the enzymatic reaction
   - 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 sec.
   - Measure fluorescence signal:
     
     For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=591 nm/622 nm continuously and record data every 5 minutes for 30 to 60 min.
     
     For end-point reading: Incubate the reaction at room temperature for 30 to 60 min. 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). Measure fluorescence intensity at Ex/Em=591 nm/622 nm
   - Data analysis: Refer to Appendix I.
Appendix I: Data analysis

- The fluorescence reading from the substrate control well is the background fluorescence. To get the relative fluorescence unit (RFU), subtract this background reading from the readings of the other wells.

- For kinetic reading:
  - Plot data as RFU versus time for each sample (Figure 1). To convert RFU to concentration of enzymatic reaction product, 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<sub>50</sub>, IC<sub>50</sub>, K<sub>m</sub>, K<sub>i</sub>, 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<sub>50</sub>, IC<sub>50</sub>, etc.

Figure 1. Proteolytic cleavage of HiLyte Fluor™TR/ QXL™610 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=530±25 nm/ 590 ±35 nm for 15 min. The control well contains FRET substrate but no enzyme. (n=2, mean±S.D.)
Appendix II: Instrument calibration

- HiLyte Fluor™TR fluorescence reference standard: Dilute 100 μM HiLyte Fluor™TR (Component B) to 1 μM in deionized water. Perform 1:2 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 the serially diluted HiLyte Fluor™TR from 1 μM to 0 nM.

- 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 HiLyte Fluor™TR reference standard to correct for 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 the reference standard wells to obtain a better comparison.

- Plot HiLyte Fluor™TR fluorescent reference standard as RFU (relative fluorescent unit) versus concentration as Figure 2.

Note: The final concentrations of the HiLyte Fluor™TR reference standard solutions are 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 0 nM. This reference standard curve is used to calibrate the variation of different instruments and the different batches of experiments. It is also an indicator of the amount of HCV NS3/4A protease enzymatic reaction final product.

Figure 2. HiLyte Fluor™TR reference standard. HiLyte Fluor™TR was serially diluted in assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=530±25 nm/ 590 ±35 nm. (n=2, mean±S.D.)
Appendix III: A sample protocol for preparing NS3-containing cellular membrane fractions

- Grow the HCV replicon-containing cells (e.g. 1 x 10⁷-10⁸ 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 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 10⁶ cells is used for one assay.
- The membrane fractions can be stored at -80°C for later use up to 3 months.

REFERENCES