ProAssay™

HCV Protease Assay Kit

Catalog # P9001, P9002

User Manual

Store at –80 °C

For Research Use Only

PROTEINONE

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ProAssay™

HCV Protease Assay Kit

Features

- **Simple and rapid procedure**
- **Complete and comprehensive kit**
- **Extremely sensitive**
- **Reproducible results**
- **Useful system for drug screening against HCV protease**

Table of Contents

References

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Safety Precautions

Products from ProteinOne are for **Research Use Only** and are not to be used for any other purposes including, but not limited to, unauthorized commercial purposes, investigational use in foods, drugs, devices or cosmetics of any kind, or use in connection with humans or animals.

ProteinOne recommends that the kit components be used in accordance with the principles of good laboratory practice. Though the kit contains no preservatives and are non-hazardous, wearing appropriate protective attire during handling is recommended. Care should be taken to avoid contact with eyes and skin. In case of contact with eyes or skin, wash immediately with water.

Related Product

	Cat.#
ProAssay HCV protease assay kit (100 assays / 96-well format)	P9001-01
ProAssay HCV protease assay kit (200 assays / 96-well format)	P9001-02
ProAssay HCV protease assay kit (400 assays / 384-well format)	P9002-01
ProAssay HCV protease assay kit (800 assays / 384-well format)	P9002-02
HCV NS 4A/3-1b protease (Accession #: AF054247)	P5101
HCV NS 4A/3-1a protease (Accession #: AF009606)	P5103
HCV NS 4A/3-2a protease (Accession #: D00944)	P5104
HCV NS 4A/3-3 protease (Accession #: D17763)	P5105
ProAssay HIV-1 protease assay kit (100 assays / 96-well format)	P9003-01
ProAssay HIV-1 protease assay kit (200 assays / 96-well format)	P9003-02
ProAssay HIV-1 protease assay kit (400 assays / 384-well format)	P9004-01
ProAssay HIV-1 protease assay kit (800 assays / 384-well format)	P9004-02
HIV-1 protease (Accession #: AF459156)	P5102

Introduction

Persistent infection with hepatitis C virus (HCV) is a common cause of chronic liver disease, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1). HCV is an enveloped, single-stranded RNA virus with a 9.6-kb positive-polarity genome, which encodes a polyprotein precursor of about 3,000 amino acids. The HCV polyprotein is proteolytically processed by cellular and HCV proteases into at least 10 distinct products (reviewed in 2). The HCV NS3/4A protease is responsible for cleavage at four sites within the HCV polyprotein to generate the N termini of the NS4A, NS4B, NS5A, and NS5B proteins (3-6). The order and kinetics of cleavage as well as the extent of precursor processing appear to be critical steps in the generation of fully infectious, appropriately assembled viral particles. Therefore, inhibition of HCV NS3/4A protease represents an important avenue for antiviral therapy (4).

Description

Fluorescence resonance energy transfer (FRET) assays have become a popular and effective means for drug screening. ProAssay HCV Protease Assay Kit provides a convenient method for high throughput screening of HCV NS3/4A protease inhibitors and continuous quantification of HCV protease activity using FRET peptide substrate. The sequence of this FRET peptide is derived from NS3-dependent cleavage site:Asp/GluXaa$_4$Cys/Thr-Ser/Ala (7). In the FRET peptide, the green fluorescence is quenched by appropriate fluorescence quencher until this peptide is cleaved into two separate fragments by HCV NS3/4A protease at the cleavage site (see Figure 1). Upon cleavage, the green fluorescence is recovered and can be monitored at excitation/emission = 490 nm/530 nm. The assays can be performed in a black 96-well or 384-well plate format.

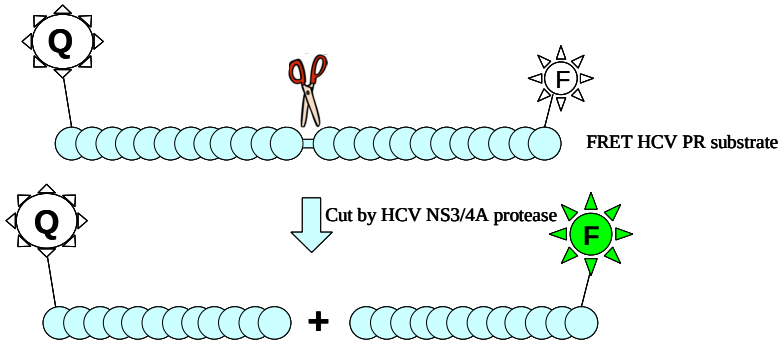

Fig.1 Green fluorescence (530 ± 10 nm) recovered from the proteolytic cleavage of FRET HCV PR substrate (component B) by HCV NS3/4A protease (component A). (Q: quencher, F: fluorophore).

Storage Conditions: All components may be stored at –80°C. If unable to do so, store liquid HCV NS 4A/3-1b Protease (Component A), HCV PR substrate (Component B) and Control inhibitor (Component F) at –80°C and all other components at –20°C until use. Properly stored products will be active for at least 6-12 months.

Kit Components:

Catalog#	P9001-01	P9001-02	P9002-01	P9002-02
Microplate format	96-well	96-well	384-well	384-well
Number of assays/kit	100 assays	200 assays	400 assays	800 assays
A: HCV NS3/4A protease	25 ul	50 ul	25 ul	50 ul
B: HCV PR substrate	180 ul	360 ul	180 ul	360 ul
C: 2X HCV PR assay buffer	30ml	30 ml	30 ml	30 ml
D. 1M DTT	300 ul	←	←	←
E. Fluoreascence STD (HCV)	20 ul	←	←	←
F. Control inhibitor (HCV)	50 ul	←	←	←
G. Stop Solution	1 ml	←	←	←
H. Deionized Water	30 ml	←	←	←
Black Microplate	1	2	1	2
User Manual	1	←	←	←

* For more information regarding Component A (HCV NS 4A/3-1b protease) please refer to the product sheet for HCV NS 4A/3-1b protease (Accession #: AF054247), catalog number P5101.

Additional reagents and equipment required:

- Fluorescent plate reader: capable of excitation at 490 ±10 nm and emission at 530 ±10 nm
- Multichannel pipettor or liquid handling device
- Microcentrifuge

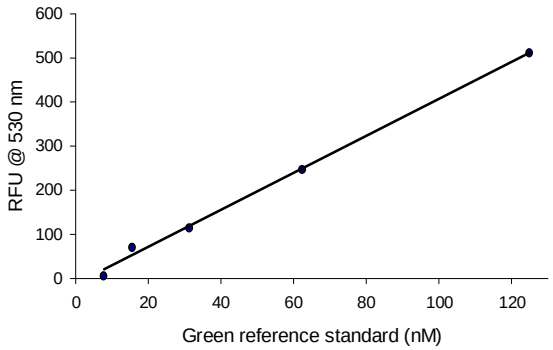

Fig.5. RFU values of green fluorescence reference standard using 125, 62.5, 32.25,15.6 and 7.8 nM were recorded at Ex/Em=490nm / 530 nm (n=2, mean ± SD). x=(y+13.389)/4.2 (R^2=0.99)

Troubleshooting Guide

If difficult to distinguish RFU signal from background noise:

1. Make sure instrument is set at proper level of sensitivity. Intermediate level of sensitivity usually is enough to detect signal over background.

2. Use **freshly** prepared HCV NS3/4A protease (component A). Repeated freeze-thaw of HCV protease will severely reduce its activity. Keep undiluted HCV protease at –80°C. Dilute the protease just before use and keep on ice.

3. **Keep the FRET HCV PR substrate (component B) or substrate containing solutions from light**. Wrap or cover these solutions with aluminum foil or proper way. Short-term exposure of substrate is acceptable.

4. Microplates with low background noise are provided for your convenience. The dimensions of the microplates are as follows:

unit (mm)	left edge to left well center	top edge to top left well center	well center to well center	well diameter
96-well plate	14.382	11	9	6.69
384-well plate	12.13	9	4.5	3.7

Appendix II: Instrument calibration.

1. Prepare 0.25μM FRET HCV PR substrate solution as shown in the following table for serial dilutions of green fluorescence reference standard for duplicated wells in a 96-well format.

	96-well format	384-well format	
HCV PR substrate (Component B)	13	3.50	**ul**
1X assay buffer	1.3	0.35	ml
0.25uM HCV PR substrate solution	**1.3**	**0.35**	ml

2. Prepare green fluorescence reference standard (component E) in the above 0.25μM FRET HCV PR substrate solution to eliminate any possible inner filter effect of the fluorescence substrate. Dilute 10 μM reference standard (component E) to make 1000 nM reference standard in FRET HCV PR substrate solution as shown in the following table:

	96-well format	384-well format
fluorescence standard (Component E)	5	1.3
0.25uM HCV PR substrate solution	45	11.7
1000nM standard solution	**50**	**13**

3. Prepare serial dilutions of 1000nM standard solution with 0.25μM FRET substrate solution as shown for the following 96-well plate format:

Serial dilutions (1 : 2.5) of standard solution	final conc. (nM)
45 ul of 1000 nM standard solution + 315 ul 0.25uM substrate solution	125
144 ul of above solution + 216 ul 0.25uM substrate solution	50
144 ul of above solution + 216 ul 0.25uM substrate solution	20
144 ul of above solution + 216 ul 0.25uM substrate solution	8
0.25uM substrate solution only	0

4. Add 100 μl/well of serially diluted standard and blank solutions into the duplicated wells of a 96-well plate, or 25 μl/well to a 384-well plate.

5. Read fluorescence signal (RFU) at excitation/emission = 490 ± 10 nm / 530 ± 10 nm. Plot data to RFU vs. concentration of standard to calibrate your machine as shown in Figure 5.

Example #1: HTS assay of HCV NS3/4A Protease inhibitors

1. Turn on plate reader about 30 min before use. Set up plate reader at proper level of sensitivity with excitation/emission = 490 ± 10 nm / 530 ± 10 nm and incubation temperature at 25°C.

2. Set up microplate format as Greiner bio-one's 96- or 384-well plate by choosing microplate format in your plate reader program or manually. (refer to Troubleshooting section for dimensions of plate).

3. Prepare solutions, set up reactions and data collections. ***Before opening tubes, briefly spin in order for solution to collect at the bottom. To test your machine you can prepare reduced amount of solutions. For example, prepare solutions for 7 assays and run 6 assays (3 assays without protease, 3 assays with HCV protease in master mix table as shown example in the following page) in a 96-well plate.***

A. 1X assay buffer: Freshly prepare 1X assay buffer containing DTT. Preparation of 20 ml 1X assay buffer for 100 assays in a 96-well plate or 400 assay in a 384-well plate is as follows:

2 X assay buffer (Component C)	10	ml
1M DTT (Component D)	200	ul
Deionized water (Component H)	9.8	ml
1 X assay buffer	20	ml

B. Preparation of test compound and control inhibitor (Component F): Prepare test compound in 1X aqueous assay buffer or proper vehicle. Deliver test compound, control inhibitor or 1X assay buffer in duplicated or triplicated wells up to 4 ul/well in a 96-well, 1ul/well in a 384-well plate.

C. Preparation of Master Mix and initiation of reaction: Prepare Master Mix just before use as Table in the following page. Keep them on ice or cool conditions are preferred before transferring into each wells containing test compound or control inhibitor. Add Master Mix 100 ul/well to a 96-well or 25 ul/well to a 384-well plate. **Protect Master Mix from light**. For kinetic mode (step D), immediately begin to collect fluorescence signal (RFU=relative fluorescence unit). Or, for end-point assay mode (step E) measure RFU at certain time points (ex, 30, 60 or 90 min) following incubation at 25°C.

Master Mix	96-well plate format (100ul/well/assay)	384-well plate format (25ul/well/assay)
1X assay buffer	100ul X 100 assays =10ml	25ul X 400 assays =10ml
HCV PR substrate (B)	1.5ul X 100 assays = 150ul	0.375ul X 400 assays =150ul
HCV protease (A)	0.2ul X 100 assays = 20ul	0.05ul X 400 assays =20ul
Total	10.17ml	10.17ml

D. Data collection at kinetic mode: Immediately begin to collect RFU signal continuously at excitation/emission = 490 ± 10 nm / 530 ± 10 nm as shown in Figure 2.

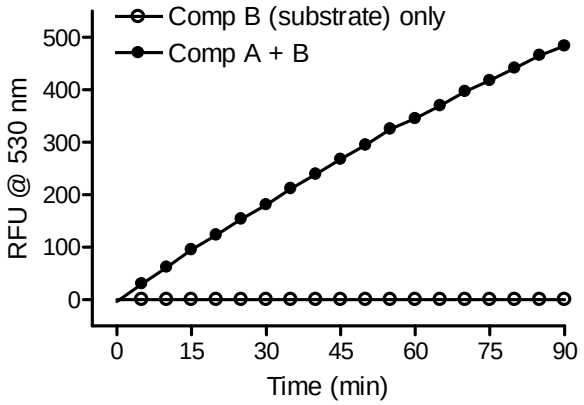

Fig. 2. Kinetic cleavage of FRET HCV PR substrate (component B) by HCV NS3/4A protease (component A).

E. Data collection at end-point mode: After initiation of reaction with test compound or control inhibitor at 25°C, add stop solution at certain time points (ex, 30, 60 or 90 min) (component G, 5ul/well of 96-well or 1ul/well of 384-well plate) and measure RFU values. After addition of stop solution, further cleavage reaction will be stopped and the RFU values are maintained as shown in Figure 3. This method is useful for the high throughput screening of test compounds to obtain potential drug candidates.

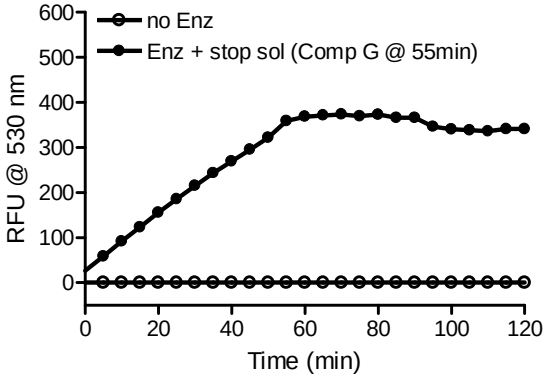

Fig. 3. Stop solution (component G) effectively maintained RFU value when added at any desired time points.

Appendix I: Effect of control inhibitor (Component F)

As shown in Figure 4, Control inhibitor (Comp F) blocked most of the proteolytic cleavage of FRET HCV PR substrate (Com B) by HCV NS3/4A protease (Comp A) over 2.5 mM (f.c.). This inhibitor can be used as a positive inhibitor control for the drug screening of small molecules. Add control inhibitor 4ul/well of 96-well plate or 1ul/well of 384-well plate.

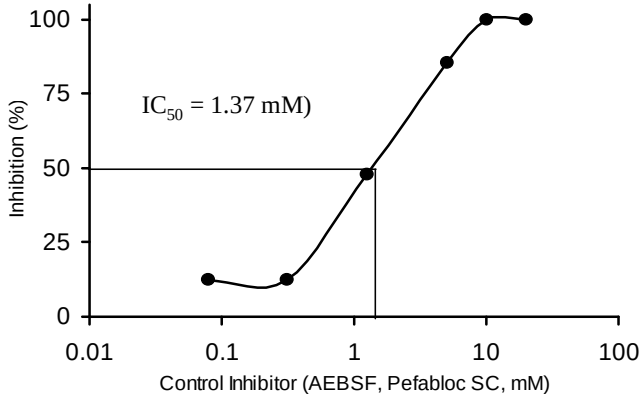

Fig. 4. Inhibitory effect of control inhibitor (component F) on the proteolytic cleavage of FRET PR substrate (component B) by HCV NS3/4A protease (component A).