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Fluorescence assay for Enterovirus D68 3C protease activity measurement

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We use this protocol and it's working

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Abstract

This protocol details the fluorescence assay for Enterovirus D68 (EV-D68) 3C protease activity measurement. This method is intended to measure the activity of viral proteases by using a specific labelled-peptide that allows the detection of the cleaved product. The substrate contains the cleavage-sequence specific to the tested protease and is labeled in C-terminal by the fluorophore Edans (ex 336 nm; em: 455 nm) and in N-ternimal by the quencher Dabcyl (ex 472 nm). In the case of a non-cleaved substrate, the proximity of Dabcyl to Edans prevents the emission and the detection of the fluorescence at 455 nm. The cleavage of the peptide by the protease allows Edans' fluorescence emission and detection.

Attachments

DOCX

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Materials

Reagents

- **Assay buffer**:

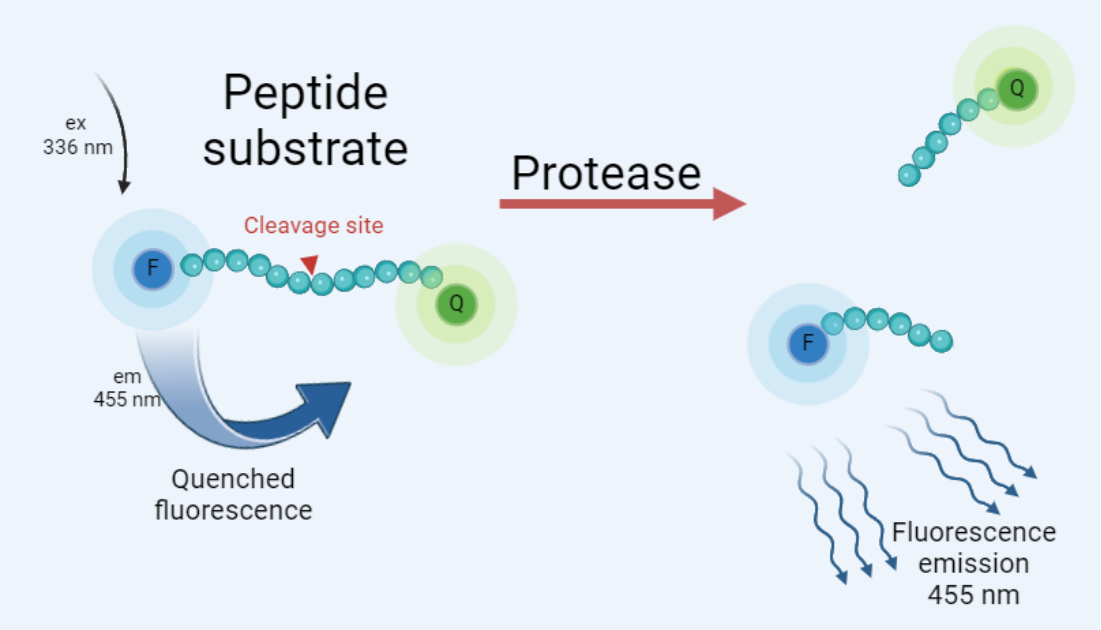
	A	B
	Tris pH 7.0	50 mM
	NaCl	150 mM
	Glycerol	10%
	DTT (optional)	0.5 mM

- **Incubation**: 01:00:00 at Room temperature .
- **EV-D68 3C**: Protein stocks were stored at -80 °C and used as 2x solution ([M] 1 micromolar (µM) , [M] 0.5 micromolar (µM) final assay concentration) in assay buffer.
- **Substrate**: Dabcyl-KEALFQGPPQFE-Edans (LifeTein, USA) prepared as a stock solution at [M] 5 millimolar (mM) in DMSO and used at 2x solution ([M] 40 micromolar (µM) , [M] 20 micromolar (µM) final concentration assay concentration) in assay buffer.
- **Positive control**: GC376 (Pubchem CID 71481119), [M] 50 micromolar (µM) top final assay concentration.
- **Plates**: ProxiPlate-384 Plus, white, Greiner cat# 6008280.
- **Liquid handler**: Echo® acoustic liquid handler (Beckman Coulter, USA).
- **Plate reade**r: Pherastar FS, BMG Labtech (Germany), 350-460 FI optic module, the plate is read every 30 s for 2 hours and shacked during 5 s before the first reading.

EV-D68 3C Pro Assay

3h

1 This method is intended to measure the activity of viral proteases by using a specific labelled-peptide that allows the detection of the cleaved product. The substrate contains the cleavage-sequence specific to the tested protease and is labeled in C-terminal by the fluorophore Edans (ex 336 nm; em: 455 nm) and in N-ternimal by the quencher Dabcyl (ex 472 nm). In the case of a non-cleaved substrate, the proximity of Dabcyl to Edans prevents the emission and the detection of the fluorescence at 455 nm. The cleavage of the peptide by the protease allows Edans' fluorescence emission and detection.



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Reagents and equipment

2 **Assay buffer:** [M] 50 millimolar (mM) Tris pH 7.0, [M] 150 millimolar (mM) NaCl, [M] 10 % (v/v) glycerol and [M] 1 millimolar (mM) TCEP (optional).

1h

Incubation: 01:00:00 at Room temperature .

EV-D68 3C: protein stocks were stored at -80C and used as 2x solution ([M] 1 micromolar (µM) , [M] 0.5 micromolar (µM) final assay concentration) in assay buffer.

Substrate: Dabcyl-KEALFQGPPQFE-Edans (LifeTein, USA) prepared as a stock solution at [M] 10 millimolar (mM) in DMSO and used at 2 [M] 2 x solution ([M] 40 micromolar (µM) , [M] 20 micromolar (µM) final concentration assay concentration) in assay buffer.

Plates: Non-binding, black 384-plate, small volume Greiner for the assay. Non-binding, 96-well polypropylene plates for the serial dilutions of the protease and substrate.
Plate reader: Pherastar FS, BMG Labtech (Germany), 350-460 FI optic module, the plate is read every 30 seconds for 2 hours and shacked 5 s before each reading.

EV-D68 3C Pro assay development and optimization

3h

3 This step aims to minimize the amount of costly reagents used for the assay and to find the best protease/substrate ratio allowing a high robustness determined by Z' calculation. A serial dilution of the protease is added to a substrate serial dilution and monitored for fluorescence intensity.

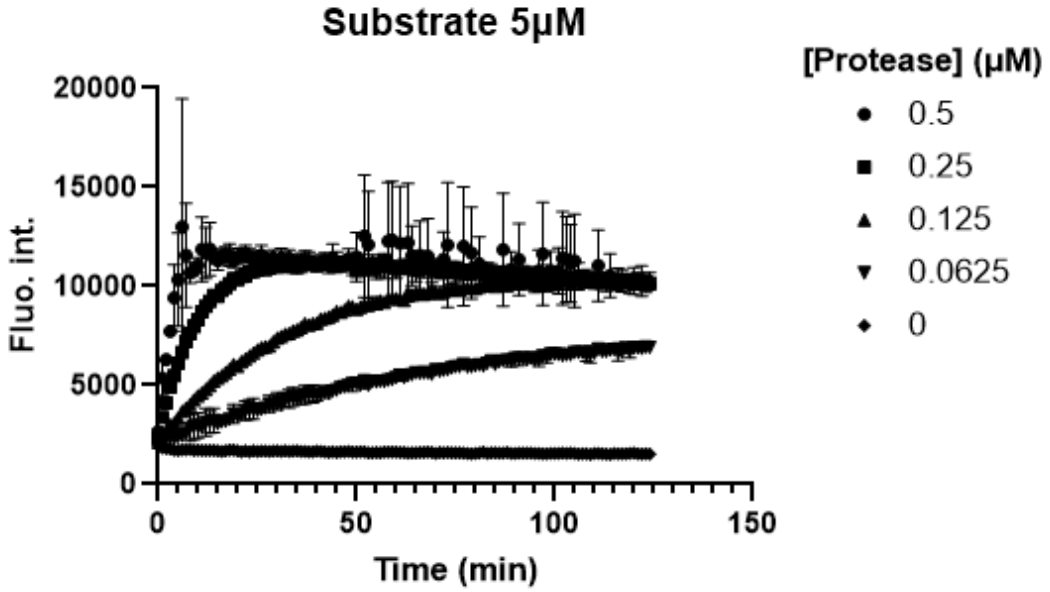
4 Prepare a serial dilution of the protease in assay buffer starting from [M] 1 micromolar (µM) [M] 2 x , at least 1000 µL , and perform a cascade dilution by transferring 500 µL of the first 2x solution to the next well which contains 500 µL of buffer. Transfer 10uL of 2x solution onto 384 well plate by repeat-pipetting, from the left to the right to cover several columns of the plate.

5 Prepare a serial dilution of the substrate starting from a solution of [M] 40 micromolar (µM) [M] 2 x , 1000 µL , by transferring in cascade 500 µL to the next well containing the same amount of buffer. Transfer 10uL of the 2x solution onto the protease solution by repeat-pipetting from the bottom to the top of the plate.

6 Read the fluorescence intensity at 350/460 nm every 00:00:30 for 02:00:00 in kinetic mode a with shaking step of the plate before each measurement. 2h 0m 30s

7 Plot the fluorescent intensity as a function of time for each substrate concentration and determine a reaction time for the analysis which shows a complete (or almost complete) reaction. In the example below, the reaction time for the analysis is set to 120 min.

8



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9 Calculate the mean (µ) and the standard deviation (σ) of fluorescence intensity and then calculate the signal-to-background ratio and the Z' or Zfactor. (s: signal; c: control).

$$\text{Estimated Z-factor} = 1 - \frac{3(\hat{\sigma}_s + \hat{\sigma}_c)}{|\hat{\mu}_s - \hat{\mu}_c|}$$

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11

		Substrate (µM)															
		20				10				5				2.5			
		average fluo	stdev fluo	S/BG	Z'	average fluo	stdev fluo	S/BG	Z'	average fluo	stdev fluo	S/BG	Z'	average fluo	stdev fluo	S/BG	Z'
[EV-D68 3C] (µM)	0.5	29967	3247	10.340	0.633	16832	605	8.491	0.861	10259	688	6.537	0.751	5350	287	4.271	0.744
	0.25	29931	2641	10.327	0.700	16922	842	8.537	0.814	10147	95	6.466	0.955	5883	328	4.696	0.746
	0.125	28805	2736	9.939	0.676	15629	1136	7.884	0.732	10234	356	6.521	0.866	5415	323	4.323	0.721
	0.0625	17527	1026	6.048	0.776	10653	453	5.374	0.814	6877	127	4.382	0.910	4163	371	3.323	0.552
	0	2898	67	ND	ND	1982	84	ND	ND	1569	32	ND	ND	1253	63	ND	ND

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Choose the substrate and protease concentrations that allow the best signal-to-background and the highest Z', in this example: 5µM substrate and 0.25µM protease.

EV-D68 3C Pro IC50 Measurement

3h

12 Add 10 µL of 2x protein [M] 0.5 micromolar (µM) solution to each well containing the compounds to be tested previously dispensed onto the plate.

13 Incubate the mix for 01:00:00 at Room temperature and initiate the enzymatic reaction by the addition of 10 µL of 2x ([M] 10 micromolar (µM)) substrate solution using the plate reader injector.

1h

14 Read the fluorescence intensity at 350/460 nm every 30 seconds for 02:00:00 in kinetic mode, which includes a shaking step of the plate before each measurement.

2h

15 Calculate the IC50 by plotting the initial velocity against various concentrations of tested inhibitor by using a four-parameter dose–response curve in Prism (v8.0) software.