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Enteroviruses (EV-D68/EV-A71) 3C and EVA71-2A proteases fluorescence single and dose response V.1

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

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Abstract

This is a **functional, biochemical assay** used to identify treatments for viral infectious diseases related to **Enteroviruses infection,** specifically **EV-D68 3C, EV-A71 3C and EV-A71 2A** proteases**.** In this current version we have added the Addgene id.

Utilizing a direct enzyme activity measurement method, the experiment was performed in a 384-well plate reading the fluorescence intensity. This assay tested the mode of action of inhibition.
Initial screening was conducted with 2 compound concentrations to determine %inhibition, followed by dose response for IC_{50} measurement (as specified below). In each run a reference compound was added to verify the assay reliability and robustness.
Assay protocol was developed at the Weizmann Institute of Science, as a part of the ASAP Discovery Consortium.
In this curre

Compounds Plate Design for 2-Point Assay:

Total Assay Volume: 20 µL
Compounds Assay Concentration: 100 µM and 50 µM
Dilution Factor: 2
Dose Response Points: 2
Number of Replicates: 2
Backfill with DMSO: Yes

Compound Plate Design for Dose Response:

Total assay volume: 20 µL
Compounds top assay concentration: 100 µM
Dilution factor: 2
Dose response points: 12
Number of replicates: 2
Backfill with DMSO: yes

Materials

Assay Buffer Reagents (Concentration listed are Stock Solution Concentrations)

1. [M] 50 Mass / % volume
   Glycerol - for molecular biology, ≥99% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G5516
2. [M] 1000 millimolar (mM)
   Tris(2-carboxyethyl)phosphine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**75259
3. [M] 100 millimolar (mM) Sodium Chloride **Fisher Scientific Catalog #**S271 (or similar)
4. [M] 10 mg/mL BSA-Molecular Biology Grade - 12 mg **New England Biolabs Catalog #**B9000S (or similar)
5. [M] 1000 millimolar (mM) TCEP HCl **P212121 Catalog #**SV-TCEP (or similar)
6. [M] 10 % volume Tween 20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P1379 (or similar)

*Note: all components are added fresh to the assay buffer before each experiment

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Additional Reagents:

Proteases and substrate:

- **3C Protease** stock concentration was [M] 1340 micromolar (µM) for **EV-D68** and [M] 1400 micromolar (µM) for **EV-A71 3C**. Stocks were diluted to their assay concentrations with freshly made **Assay Buffer** before each experiment
- The substrate for **EV-D68/EV-A71 3C** proteases was **5FAM- KEALFQGPPQFE-DABCYL** (custom made by GenScript Biotech (Singapore) PTE. LTD).
- The substrate for **EV-A71 2A** protease was **Dabcyl-KITTLGKFGQDE-Edans** (custom made by Peptide 2.0 Inc.).
- **Substrate stock** was created by dissolving the substrate powder in **DMSO** to create [M] 10 millimolar (mM) Substrate Stock aliquots and stored at -80 °C. Before each experiment, the Substrate Stock was diluted to its assay concentration with freshly made **Assay Buffer.**

Protocol materials

- Glycerol - for molecular biology, ≥99% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G5516
- Sodium Chloride **Fisher Scientific Catalog #**S271
- BSA-Molecular Biology Grade - 12 mg **New England Biolabs Catalog #**B9000S
- Tween 20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P1379
- Tris(2-carboxyethyl)phosphine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**75259
- TCEP HCl **P212121 Catalog #**SV-TCEP
- EV-A71 3C protease; aliases: Enterovirus A71 3C protease, non cleavable C-term 6xHis tag **addgene Catalog #**204816
- EV-D68 3C protease; aliases: Enterovirus A71 3C protease, non cleavable C-term 6xHis tag **addgene Catalog #**(Plasmid #204817)
- Enterovirus Coxsackievirus A16 2A protease **addgene Catalog #**228632
- Enterovirus A71 3C protease **addgene Catalog #**204816
- EV-D68 3C protease; aliases: Enterovirus A71 3C protease, non cleavable C-term 6xHis tag **addgene Catalog #**204817
- Human Coxsackievirus A16 strain G10 2A protease **addgene Catalog #**228632

Safety warnings

Please be sure to wear proper Personal Protective Equipment (PPE) while performing this experiment.

Before start

Dummy plate: In each step of the protocol, dispense the reagents into an **empty** plate before dispensing them into the assay compounds plate (this will detect and prevent dispensing issues).

Note: Inhibitor compounds stock concentration is 20 mM. Compounds are pre-dispensed into 384 plates and stored at -20˚C until use.

Protein expression and purification

1 Expression and purification protocol and the plasmid used for EV-A71 3C protease

Enterovirus A71 3C protease **addgene Catalog #**204816

Protocol

NAME

Enterovirus A71 3C protease small scale expression and purification protocol

CREATED BY

korvus.wang

Preview

2 Expression and purification protocol for the plasmid used EV-D68 3C protease

EV-D68 3C protease; aliases: Enterovirus A71 3C protease, non cleavable C-term 6xHis tag **addgene Catalog #**204817

Protocol

NAME

Enterovirus D68 3C protease small scale expression and purification protocol

CREATED BY

korvus.wang

Preview

3 Expression and purification for the plasmid used EV-A71 2A protease

Human Coxsackievirus A16 strain G10 2A protease **addgene Catalog #**228632

Protocol

NAME

Enterovirus coxsackievirus A16 2A protease small scale expression and purification protocol

CREATED BY
korvus.wang

Preview

Prepare Reagents

4 **PREPARE** all of the reagents/buffers required for this experiment.

	A	B	C	D	E
	Reagent	**Stock Concentration**	**Concentration Loaded into dispenser**	**Final Concentration in plate**	**Units**
	EV-D68 3C Enzyme stock	1340	0.04	0.02	uM
	EV-A71 3C Enzyme stock	1400	0.3	0.15	uM
	EV-A71 2A Enzyme stock	1200	0.4	0.2	uM
	EV-D68 3C Substrate				
	EV-A71 3C Substrate	10000	9	4.5	uM
	EV-A71 2A Substrate	10000	12	6	uM
	Assay Buffer				
	Tris (pH 7.5)	250	50	50	mM
	Sodium Chloride	5000	150	150	mM
	BSA	10	0.05	0.05	mg/mL

	A	B	C	D	E
	Tween 20	10	0.1	0.1	% by volume
	TCEP	1000	0.5	0.5	mM
	Glycerol	50	10	10	% by volume

For more information, please see the Materials sec

We used the following plasmid in the protein expression and purification protocol.

EV-A71 3C protease; aliases: Enterovirus A71 3C protease, non cleavable C-term 6xHis tag **addgene Catalog #**204816

EV-D68 3C protease; aliases: Enterovirus A71 3C protease, non cleavable C-term 6xHis tag **addgene Catalog #**(Plasmid #204817)

Enterovirus Coxsackievirus A16 2A protease **addgene Catalog #**228632

Prepare 384 Well Plate

32m

5 **PRIME** the dispenser with **Assay Buffer** by selecting the **PRIME** button until the tubes are filled completely.

5.1 **DISPENSE** 10 µL Assay Buffer to Columns **1** and **23** of assay plate

Note: These will represent the ***inhibitor control columns*** (Contain: Substrate + Assay Buffer + DMSO, no enzyme, no compounds)

5.2 **EMPTY** the dispenser tubes.

- Discard Assay Buffer discharged from the tubes.

6 **PRIME** the dispenser with [M] 40 nanomolar (nM) EV-D68 Enzyme or [M] 300 nanomolar (nM) EV-A71 Enzyme by selecting the **PRIME** button until the tubes are filled completely.

- **Note:** Be sure to cycle dispensing on an empty plate first to detect and avoid dispensing issues.

6.1 **DISPENSE** 10 µL [M] 40 nanomolar (nM) EV-D68 Enzyme , [M] 300 nanomolar (nM) EV-A71 3C Enzyme or [M] 400 nanomolar (nM) EV-A71 2A Enzyme to Columns **2 - 22** and Column **24**

Note:

- Enzyme concentrations dispensed to the plate are two times the final concentration in the plate for the assay.
- Plate final concentrations are [M] 20 nanomolar (nM) D68 Enzyme , [M] 150 nanomolar (nM) EV-A71 Enzyme and [M] 400 nanomolar (nM) EV-A71 2A Enzyme .
- Column 2 and Column 24 are ***neutral control columns*** (Contain: Enzyme, Substrate, DMSO, no compounds)

6.2 **EMPTY** the dispenser tubes.
- Discard the liquid discharged from the tubes.

7 **CENTRIFUGE** plate 1500 rpm, Room temperature, 00:01:00 to remove bubbles 1m

8 **INCUBATE** plate for 00:30:00 at Room temperature 30m

⚠ Make sure the plate is protected from light!

During Incubation: wash and prepare the dispenser to the next step

9 **PRIME** the dispenser with [M] 9 micromolar (µM) EV-A71 or [M] 6 micromolar (µM) EV-D68 Substrate by selecting the **PRIME** button until the tubes are filled completely.
- **Note:** Be sure to cycle dispensing on an empty plate to detect and avoid dispensing issues.

10 **DISPENSE** 10 µL EV Substrate into Columns **1 - 24** (the full plate)

Note:
- Be sure to cycle dispensing on an empty plate first to detect and avoid dispensing issues.
- EV Substrate concentration is two times the final assay concentration and diluted x2 with the enzyme/buffer in the plate.

11 **CENTRIFUGE** plate 1500 rpm, Room temperature, 00:01:00 in plate centrifuge to remove bubbles 1m

12 **INCUBATE** plate at Room temperature for **1 hr** for **EV-A71 3C, 30 min** for **EV-D68** and **45 min** for **EVA71-2A** enzyme .

⚠ **Make sure the plate is protected from light!**

Recommended: Clean the dispenser during this incubation step

Read Plate Fluorescence

13 **READ** and **RECORD** the plate Relative fluorescence units (RFU) on the **PHERAstar FS Control Software.**

- Software is a standard Fluorescence Assay set for Optimal excitation wavelength 485 nm, emission wavelength 528 nm for 3C proteases and 360/470 for EVA71 2A protease.

Equipment

PHERAstar FS

Microplate reader

BMG LABTECH

0471B0001A

https://www.bmglabtech.com/en/pherastar-fsx/?utm_term=pherastar%20plate%20reader&utm_campaign=usa.roi.products&utm_source
FwOoFR_5EUHUaAlkREALw_wcB

Expected result

For EV-A71: Gain 50 should yield ~32,000 RFU in full reaction and ~15,000 RFU in Buffer Control
For EV-D68: Gain 50 should yield ~37,000 RFU in full reaction and ~10,000 RFU in Buffer Control

Protein expression and purification 2d

14 For the EV-D68 3C protease we used the following protocol 2d

Protocol

NAME

Enterovirus D68 3C protease small scale expression and purification protocol

CREATED BY

korvus.wang

Preview

Protocol references

Tan, Jinzhi et al. “3C protease of enterovirus 68: structure-based design of Michael acceptor inhibitors and their broad-spectrum antiviral effects against picornaviruses.” *Journal of virology* vol. 87,8 (2013): 4339-51. doi:10.1128/JVI.01123-12