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Assay for Screening of Compounds that Inhibit Enzymatic Activity of EV-A71 2Apro

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Abstract

This protocol describes a biochemical assay used in screening to identify compounds that inhibit the enzymatic activity of the EV-A71 2A protease.
Under standard conditions, the enzyme cleaves its substrate (DABCYL-RDKITTLGKFGQDE-EDANS), releasing the fluorogenic moiety EDANS, increasing fluorescence. Prior to cleavage, fluorescence is quenched by the DABCYL moiety. In the presence of compounds that inhibit the enzyme's activity, the substrate remains uncleaved, resulting in a low fluorescent signal.
This enables the determination of % inhibition at a given compound concentration, and allows for subsequent IC_{50} determination when a dose-response format is applied.

This protocol was established through the transfer and adaptation of an assay originally developed at the Weizmann Institute of Science, as a part of the ASAP Discovery Consortium.

Guidelines

Introduction

	A	B
	Target name	**EV-A71 2A protease**
	Project Aim	To identify compounds that inhibit enzymatic activity of the 2A protease of enterovirus A71.
	Assay Principle	Under standard conditions the enzyme cleaves its substrate (DABCYL-RDKITTLGKFGQDE-EDANS) with the release of a fluorogenic moiety EDANS. Before EDANS is released, it is quenched by DABCYL moiety on the substrate. In the presence of compounds that inhibit activity of the enzyme, substrate is not cleaved which results in a low fluorescent signal.
	Assay readout	Fluorescence (RFU)

Summary of Assay Conditions

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Ref. at 2xIC50		Cpd 1	Cpd 9	Cpd 17	Cpd 25	Cpd 33	Cpd 41	Cpd 49	Cpd 57	Cpd 65	Cpd 73	Cpd 1	Cpd 9	Cpd 17	Cpd 25	Cpd 33	Cpd 41	Cpd 49	Cpd 57	Cpd 65	Cpd 73	0% Inhibition Control	100% Inhibition Control
B	Ref. at IC50		Cpd 1	Cpd 9	Cpd 17	Cpd 25	Cpd 33	Cpd 41	Cpd 49	Cpd 57	Cpd 65	Cpd 73	Cpd 1	Cpd 9	Cpd 17	Cpd 25	Cpd 33	Cpd 41	Cpd 49	Cpd 57	Cpd 65	Cpd 73		
C	Ref. at 100 uM		Cpd 2	Cpd 10	Cpd 18	Cpd 26	Cpd 34	Cpd 42	Cpd 50	Cpd 58	Cpd 66	Cpd 74	Cpd 2	Cpd 10	Cpd 18	Cpd 26	Cpd 34	Cpd 42	Cpd 50	Cpd 58	Cpd 66	Cpd 74		
D	Ref. at 50 uM		Cpd 2	Cpd 10	Cpd 18	Cpd 26	Cpd 34	Cpd 42	Cpd 50	Cpd 58	Cpd 66	Cpd 74	Cpd 2	Cpd 10	Cpd 18	Cpd 26	Cpd 34	Cpd 42	Cpd 50	Cpd 58	Cpd 66	Cpd 74		
E	0% Inhibition Control	100% Inhibition Control	Cpd 3	Cpd 11	Cpd 19	Cpd 27	Cpd 35	Cpd 43	Cpd 51	Cpd 59	Cpd 67	Cpd 75	Cpd 3	Cpd 11	Cpd 19	Cpd 27	Cpd 35	Cpd 43	Cpd 51	Cpd 59	Cpd 67	Cpd 75		
F			Cpd 3	Cpd 11	Cpd 19	Cpd 27	Cpd 35	Cpd 43	Cpd 51	Cpd 59	Cpd 67	Cpd 75	Cpd 3	Cpd 11	Cpd 19	Cpd 27	Cpd 35	Cpd 43	Cpd 51	Cpd 59	Cpd 67	Cpd 75		
G			Cpd 4	Cpd 12	Cpd 20	Cpd 28	Cpd 36	Cpd 44	Cpd 52	Cpd 60	Cpd 68	Cpd 76	Cpd 4	Cpd 12	Cpd 20	Cpd 28	Cpd 36	Cpd 44	Cpd 52	Cpd 60	Cpd 68	Cpd 76		
H			Cpd 4	Cpd 12	Cpd 20	Cpd 28	Cpd 36	Cpd 44	Cpd 52	Cpd 60	Cpd 68	Cpd 76	Cpd 4	Cpd 12	Cpd 20	Cpd 28	Cpd 36	Cpd 44	Cpd 52	Cpd 60	Cpd 68	Cpd 76		
I			Cpd 5	Cpd 13	Cpd 21	Cpd 29	Cpd 37	Cpd 45	Cpd 53	Cpd 61	Cpd 69	Cpd 77	Cpd 5	Cpd 13	Cpd 21	Cpd 29	Cpd 37	Cpd 45	Cpd 53	Cpd 61	Cpd 69	Cpd 77		
J			Cpd 5	Cpd 13	Cpd 21	Cpd 29	Cpd 37	Cpd 45	Cpd 53	Cpd 61	Cpd 69	Cpd 77	Cpd 5	Cpd 13	Cpd 21	Cpd 29	Cpd 37	Cpd 45	Cpd 53	Cpd 61	Cpd 69	Cpd 77		
K			Cpd 6	Cpd 14	Cpd 22	Cpd 30	Cpd 38	Cpd 46	Cpd 54	Cpd 62	Cpd 70	Cpd 78	Cpd 6	Cpd 14	Cpd 22	Cpd 30	Cpd 38	Cpd 46	Cpd 54	Cpd 62	Cpd 70	Cpd 78		
L			Cpd 6	Cpd 14	Cpd 22	Cpd 30	Cpd 38	Cpd 46	Cpd 54	Cpd 62	Cpd 70	Cpd 78	Cpd 6	Cpd 14	Cpd 22	Cpd 30	Cpd 38	Cpd 46	Cpd 54	Cpd 62	Cpd 70	Cpd 78		
M			Cpd 7	Cpd 15	Cpd 23	Cpd 31	Cpd 39	Cpd 47	Cpd 55	Cpd 63	Cpd 71	Cpd 79	Cpd 7	Cpd 15	Cpd 23	Cpd 31	Cpd 39	Cpd 47	Cpd 55	Cpd 63	Cpd 71	Cpd 79	Ref. at 100 uM	
N			Cpd 7	Cpd 15	Cpd 23	Cpd 31	Cpd 39	Cpd 47	Cpd 55	Cpd 63	Cpd 71	Cpd 79	Cpd 7	Cpd 15	Cpd 23	Cpd 31	Cpd 39	Cpd 47	Cpd 55	Cpd 63	Cpd 71	Cpd 79	Ref. at 50 uM	
O			Cpd 8	Cpd 16	Cpd 24	Cpd 32	Cpd 40	Cpd 48	Cpd 56	Cpd 64	Cpd 72	Cpd 80	Cpd 8	Cpd 16	Cpd 24	Cpd 32	Cpd 40	Cpd 48	Cpd 56	Cpd 64	Cpd 72	Cpd 80	Ref. at 2xIC50	
P			Cpd 8	Cpd 16	Cpd 24	Cpd 32	Cpd 40	Cpd 48	Cpd 56	Cpd 64	Cpd 72	Cpd 80	Cpd 8	Cpd 16	Cpd 24	Cpd 32	Cpd 40	Cpd 48	Cpd 56	Cpd 64	Cpd 72	Cpd 80	Ref. at IC50	

Figure 7. Plate loading for the test of compounds at 2 concentrations.

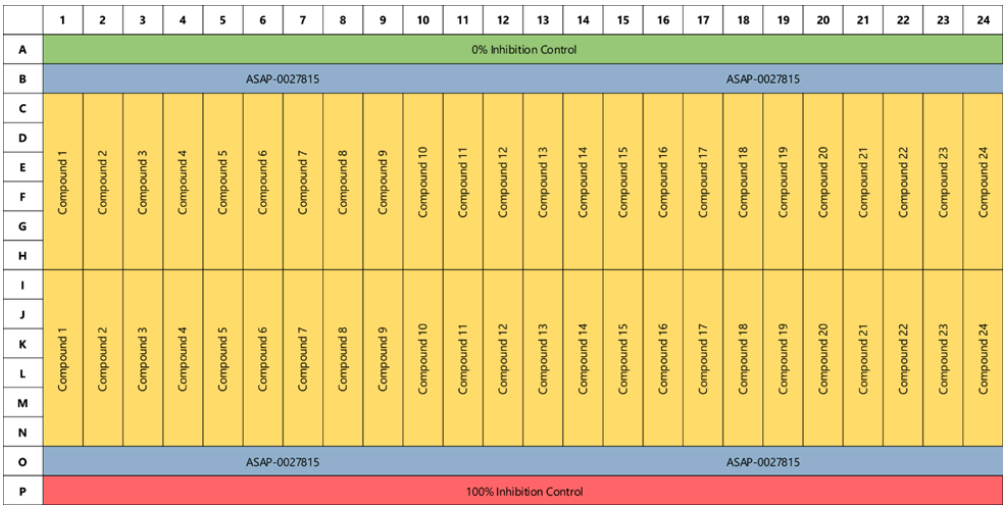


Figure 8. Plate loading for 6-point dose-response formats (options A and B).

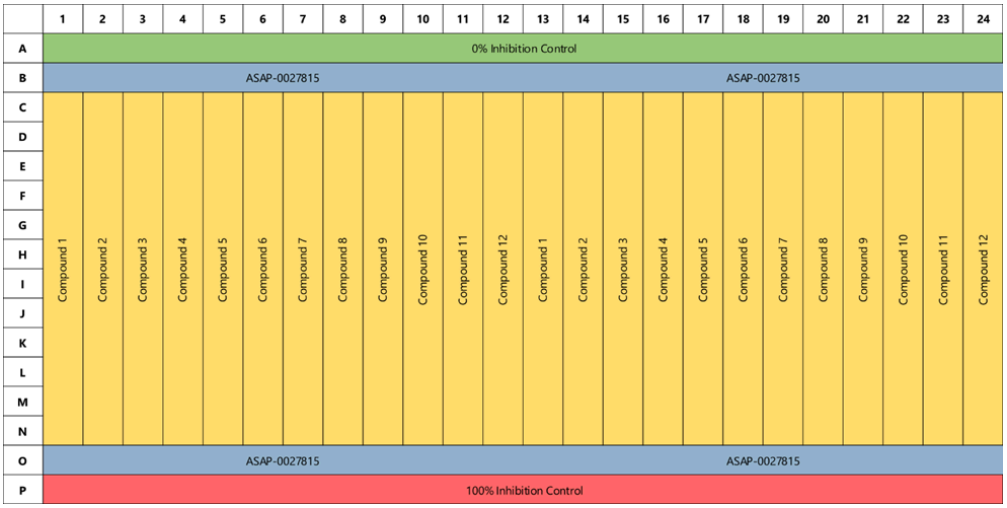


Figure 9. Plate loading for 12-point dose-response format.

	A	B
	Description	**Reaction volume composition, 10 µL**
	Negative control (100% of inhibition)	0.5% DMSO, 6 µM of Substrate
	Positive control (0% of inhibition)	0.5% DMSO, 2 µM of EVA71 2Apro, 6 µM of Substrate
	Reference compound	ASAP-0027815, at 4 concentrations, 100 µM, 50 µM, 2xIC50 [2 µM] and IC50 [1 µM] (n = 4), 2 µM of EVA71 2Apro, 6 µM of Substrate

	A	B
		ASAP- 0027815 in dose-response format, 2-fold dilution, 50 – 0.025 µM, 12 points (n = 4), 2 µM of EVA71 2Apro, 6 µM of Substrate
	Compounds	Compounds tested at 2 concentrations, 100 and 50µM, (n = 2), 2 µM of EVA71 2Apro, 6 µM of Substrate
		Compounds in 6-point dose-response format, option A: 2-fold dilution, 100 – 3 µM, (n=2), option B: 2-fold dilution, 5 – 0.16 µM, (n=2), 2 µM of EVA71 2Apro, 6 µM of Substrate
		Compounds in 12-point dose-response format, 2-fold dilution, 200 – 0.2 µM & point 150 µM, (n=2), 2 µM of EVA71 2Apro, 6 µM of Substrate

Materials

Tools/Equipment required

	A	B
	Tool Name	Tool Source
	Labcyte Echo 650	Beckman Coulter
	Biomek Span-8	Beckman Coulter
	Manual single channel pipettes 0.5-10 µL 10-100 µL 20-200 µL 20-200 µL 100-1000 µL	 Thermo Scientific Thermo Scientific Thermo Scientific Sartorius Thermo Scientific
	Multichannel electronic pipettes (E1-ClipTip) 1-30 µL 2-125 µL	Thermo Scientific
	Multidrop Combi nL	Thermo Scientific
	DLAB Levo plus (for stripettes)	DLAB
	SpectraMax Paradigm	Molecular Devices
	ASSAB incubator	Assab
	accuSpin 3 centrifuge	Fisher Scientific

Consumables

	A	B	C	D
	№	Disposable Name	Disposable Source	Catalogue Number

	A	B	C	D
	1	384 round bottom, small volume, non-binding, black plate	Corning	4514
	2	Multichannel Pipette tips (F1-ClipTip) 30 µL 125 µL	Thermo Scientific	94420103 94420153
	3	Stripettes 5 mL 10 mL 25 mL 50 mL	Thermo Scientific	170355N 170356N 170357N 170358N
	4	15 mL Polypropylene falcon tubes	Sente-Lab	SL50352/SG
	5	50 mL Polypropylene falcon tubes	Sente-Lab	SL65351/SG
	6	Manual Pipette tips 10 µL 200 µL 200 µL 1000 µL	Sente-Lab Sente-Lab Sartorius Sente-Lab	SL96102 SL96062 790202 SL96153
	7	Silverseal Sealer, Aluminium	Greiner	676090
	9	SealPlate film	Excel Scientific	Z369659-100EA
	9	Axygen Ultra-Clear, Pressure-Sensitive Sealing Film for Real-Time PCR	Corning	UC-500
	10	Minisart NY25 Syringe Filter, 0.2 µm Polyamide (Nylon)	Sartorius	17845----------Q
	11	Syringe 50,0ml, Luer Lock	Medicare	S-3S50,0

384-well Low Volume Black Round Bottom Polystyrene NBS Microplate, 10 per Bag, without Lid **Corning Catalog #**4514

ClipTip™ Filtered 384-Format Pipette Tips **Thermo Scientific Catalog #**94420103

ClipTip™ Filtered 384-Format Pipette Tips **Thermo Scientific Catalog #**94420153

Nunc™ Serological Pipettes **Thermo Scientific Catalog #**170355N

Nunc™ Serological Pipettes **Thermo Scientific Catalog #** 170356N

Nunc™ Serological Pipettes **Thermo Scientific Catalog #** 170357N

Nunc™ Serological Pipettes **Thermo Scientific Catalog #**170358N

SILVERSEAL SEALER, ALUMINIUM **greiner bio-one Catalog #**676090

Axygen® 70 µm Ultra Clear Pressure Sensitive Sealing Film for Real Time PCR, Nonsterile **Corning Catalog #**UC-500

Minisart® NY25 Syringe Filter, 0.2 µm Polyamide (Nylon) **Sartorius Catalog #**17845-Q

Reagents

	A	B	C	D	E	F
	№	Reagent Name	Stock Concentration	Reagent Source	Catalogue No.	Storage Conditions
	1	EVA71 2Apro	1.2 mM	Client Supplied	AddGene Catalog #228632	-80°C
	2	DABCYL-RDKITTLGKFGQDE-EDANS	-	GenScript	-	-20°C
	3	ASAP-0027815 Telaprevir	20 mM	Enamine	EN300-26212550	-80°C
	4	Tris hydrochloride	-	Bio Basic	TB0103	RT
	5	Glycerol	100%	ALLHIM	2905450000	RT
	6	TCEP, Hydrochloride	-	Santa Cruz	51805-45-9	+4°C
	7	DMSO	100%	Honeywell	34869-2.5L	RT

	A	B	C	D	E	F
	8	Tween 20	100%	Sigma-Aldrich	P7949-500ML	RT
	9	Bovine Serum Albumin (BSA)	-	Sigma-Aldrich	A3608-50G	+4°C
	10	SDS	-	Bio Basic	151-21-3	RT
	11	Sodium Chloride	-	Bio Basic	DB0483	RT

Tris hydrochloride **Bio Basic Inc.** **Catalog #**TB0103.SIZE.250G

TCEP, Hydrochloride **Santa Cruz**

Dimethyl sulfoxide **Honeywell Fluka** **Catalog #**34869

Sodium chloride **Bio Basic Inc.** **Catalog #**DB0483.SIZE.500G

TWEEN 20 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P7949

Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A3608

SDS **Bio Basic Inc.** **Catalog #**151-21-3

Standard Reagent Formulations

	A	B	C	D	E	F
	№	Prepared Reagent Name	Component Name	Prepared Reagent Volume	Component Quantity	Storage
	1	1 M Tris-HCl pH 7.5	Tris-HCl MQ H2O	10 mL	10 g 10 mL	4°C
	2	10 mM DABCYL-RDKITTLGKFGQDE-EDANS	DABCYL-RDKITTLGKFGQDE-EDANS DMSO	10×15 µL 9×10 µL	5 mg 237 µL	-20°C
	3	1 M Sodium chloride	Sodium chloride MQ H2O	500 mL	29.22 g 500 mL	RT

	A	B	C	D	E	F
	4	10% Tween 20	Tween 20 MQ H2O	50 mL	5 mL 45 mL	RT
	5	1% SDS	Sodium dodecyl sulfate MQ H2O	2 L	20 g 2 L	RT
	6	0.5 M TCEP	TCEP MQ H2O	20× 1.5 mL	4.30 g 30 mL	-20°C

Daily Reagent Formulations*

	A	B	C	D	E	F
	№	Prepared Reagent Name	Components	Component Quantity	Concentration in solution	Prepared Reagent Volume
	1	50 mg/mL BSA	Bovine Serum Albumin MQ H2O	5 mg 100 µL	50 mg/mL -	100 µL
	2	Assay Buffer	1 M Tris-HCl pH 7.5 Glycerol 0.5 M TCEP 1 M NaCl 10% Tween 20 50 mg/mL BSA Milli-Q H2O	2.5 mL 5 mL 50 µL 7.5 mL 500 µL 50µL 34.4 mL	50 mM 10% 1 mM 150 mM 0.1% 0.05 mg/mL -	50 mL
	4	4 µM EVA71 2Apro (2x solution)	1.2 mM EVA71 2Apro Assay Buffer	30 µL 8970 µL	4 µM -	9000 µL
	5	12 µM Substrate solution (2x solution)	10 mM DABCYL-RDKITTLGKFGQDE-EDANS Assay Buffer	12 µL 9988 µL	12 µM -	10000 µL
		*All calculations shown here are for a 3-plate HTS run. This consists of 3 assay plates with a dummy plate at the beginning of the dispense and dead volume of dispensing equipment.				

Protocol materials

- Enterovirus Coxsackievirus A16 2A protease **addgene** **Catalog #**228632
- SDS **Bio Basic Inc.** **Catalog #**151-21-3
- Sodium chloride **Bio Basic Inc.** **Catalog #**DB0483.SIZE.500G
- TWEEN 20 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P7949
- Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A3608
- ClipTip™ Filtered 384-Format Pipette Tips **Thermo Scientific** **Catalog #**94420103
- Nunc™ Serological Pipettes **Thermo Scientific** **Catalog #** 170357N
- Axygen® 70 µm Ultra Clear Pressure Sensitive Sealing Film for Real Time PCR, Nonsterile **Corning** **Catalog #**UC-500
- Dimethyl sulfoxide **Honeywell Fluka** **Catalog #**34869
- TCEP, Hydrochloride **Santa Cruz**
- 384-well Low Volume Black Round Bottom Polystyrene NBS Microplate, 10 per Bag, without Lid **Corning** **Catalog #**4514
- Nunc™ Serological Pipettes **Thermo Scientific** **Catalog #** 170356N
- Nunc™ Serological Pipettes **Thermo Scientific** **Catalog #**170358N
- Tris hydrochloride **Bio Basic Inc.** **Catalog #**TB0103.SIZE.250G
- SILVERSEAL SEALER, ALUMINIUM **greiner bio-one** **Catalog #**676090
- ClipTip™ Filtered 384-Format Pipette Tips **Thermo Scientific** **Catalog #**94420153
- Nunc™ Serological Pipettes **Thermo Scientific** **Catalog #**170355N
- Minisart® NY25 Syringe Filter, 0.2 µm Polyamide (Nylon) **Sartorius** **Catalog #**17845-Q

Assay Plate Preparation

1m

1 **Preparation of compounds for test at 2 concentrations:**
DMSO, tested compounds, and reference compound are prepared by Biomek Span-8 and printed onto assay-ready plates (assay plate - Corning #4514) using the Labcyte Echo 650.

Reference compound is formatted in the following order:

- **Reference compound 1st concentration** – C1-C2 and M23-M24 - 50 nL of ASAP-0027815 ([M] 20 millimolar (mM) , n=4)
- **Reference compound 2nd concentration** – D1-D2 and N23-N24 - 50 nL of ASAP-0027815 ([M] 10 millimolar (mM) , n=4)
- **Reference compound 3rd concentration** – A1-A2 and O23-O24 – 50 nL of ASAP-0027815 ([M] 400 micromolar (µM) , n = 4)
- **Reference compound 4th concentration** – B1-B2 and P23-P24 – 50 nL of ASAP-0027815 ([M] 200 micromolar (µM) , n = 4)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Ref. at 400 uM		Cpd 1 20 mM	9	17	25	33	41	49	57	65	73	1	9	17	25	33	41	49	57	65	73	DMSO	DMSO
B	Ref. at 200 uM		Cpd 1 10 mM	9	17	25	33	41	49	57	65	73	1	9	17	25	33	41	49	57	65	73	DMSO	DMSO
C	Ref. at 20 mM		2	10	18	26	34	42	50	58	66	74	2	10	18	26	34	42	50	58	66	74	DMSO	DMSO
D	Ref. at 10 mM		2	10	18	26	34	42	50	58	66	74	2	10	18	26	34	42	50	58	66	74	DMSO	DMSO
E	DMSO	DMSO	3	11	19	27	35	43	51	59	67	75	3	11	19	27	35	43	51	59	67	75	DMSO	DMSO
F	DMSO	DMSO	3	11	19	27	35	43	51	59	67	75	3	11	19	27	35	43	51	59	67	75	DMSO	DMSO
G	DMSO	DMSO	4	12	20	28	36	44	52	60	68	76	4	12	20	28	36	44	52	60	68	76	DMSO	DMSO
H	DMSO	DMSO	4	12	20	28	36	44	52	60	68	76	4	12	20	28	36	44	52	60	68	76	DMSO	DMSO
I	DMSO	DMSO	5	13	21	29	37	45	53	61	69	77	5	13	21	29	37	45	53	61	69	77	DMSO	DMSO
J	DMSO	DMSO	5	13	21	29	37	45	53	61	69	77	5	13	21	29	37	45	53	61	69	77	DMSO	DMSO
K	DMSO	DMSO	6	14	22	30	38	46	54	62	70	78	6	14	22	30	38	46	54	62	70	78	DMSO	DMSO
L	DMSO	DMSO	6	14	22	30	38	46	54	62	70	78	6	14	22	30	38	46	54	62	70	78	DMSO	DMSO
M	DMSO	DMSO	7	15	23	31	39	47	55	63	71	79	7	15	23	31	39	47	55	63	71	79	Ref. at 20 mM	
N	DMSO	DMSO	7	15	23	31	39	47	55	63	71	79	7	15	23	31	39	47	55	63	71	79	Ref. at 10 mM	
O	DMSO	DMSO	8	16	24	32	40	48	56	64	72	80	8	16	24	32	40	48	56	64	72	80	Ref. at 400 uM	
P	DMSO	DMSO	8	16	24	32	40	48	56	64	72	80	8	16	24	32	40	48	56	64	72	80	Ref. at 200 uM	

Figure 1. Plate Map of the test of compounds at 2 concentrations prepared by liquid handling department.

DMSO is formatted into wells E1-P2 and A23-L24.

Compounds are formatted in columns 3-22 in the following order:

- **Compounds 1st concentration** – rows A, C, E, G, I, K, M, O – 50 nL in each well at [M] 20 millimolar (mM) (n = 2).

- **Compounds 2nd concentration** – rows B, D, F, H, J, L, N, P – 50 nL in each well at [M] 10 millimolar (mM) (n = 2).

2 **Preparation of compounds for test at 6-point dose-response format, option A:**
DMSO, tested compounds and reference compound are prepared by Biomek Span-8 and printed onto assay-ready plates (assay plate – Corning #4514) using the Labcyte Echo 650.

Reference compound – rows B and O – 50 nL of ASAP-0027815 2-fold dilution, [M] 10 millimolar (mM) – [M] 5 micromolar (µM), 12 points (n = 4)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
B	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM
C	20 mM	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Compound 11	Compound 12	Compound 13	Compound 14	Compound 15	Compound 16	Compound 17	Compound 18	Compound 19	Compound 20	Compound 21	Compound 22	Compound 23	Compound 24
D	10 mM																							
E	5 mM																							
F	2.5 mM																							
G	1.2 mM																							
H	0.6 mM																							
I	20 mM	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Compound 11	Compound 12	Compound 13	Compound 14	Compound 15	Compound 16	Compound 17	Compound 18	Compound 19	Compound 20	Compound 21	Compound 22	Compound 23	Compound 24
J	10 mM																							
K	5 mM																							
L	2.5 mM																							
M	1.2 mM																							
N	0.6 mM																							
O	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM
P	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO

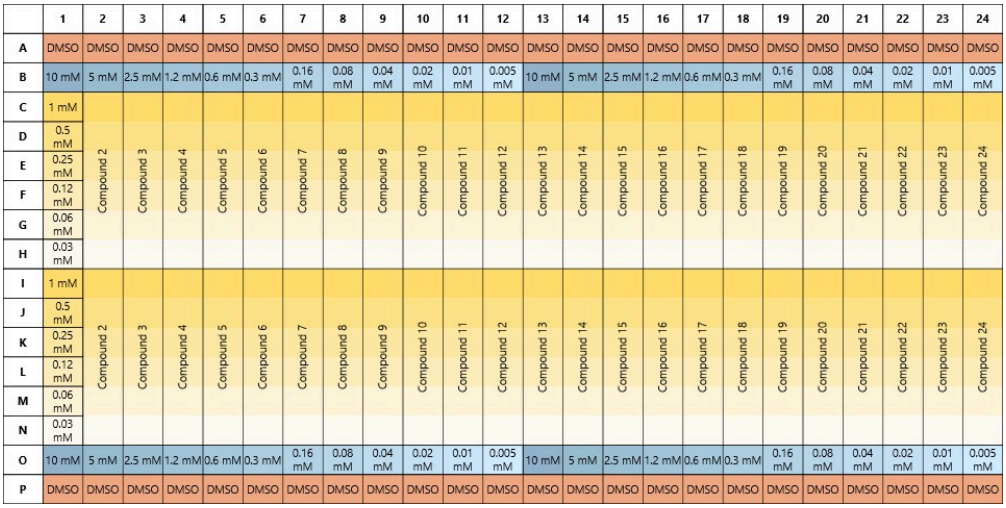
Figure 2A. Plate Map of 6-point dose-response scheme prepared by liquid handling department, option A.

DMSO is formatted into rows A and P.

Compounds are formatted into all columns, in rows C-N in dose-response format, 2-fold dilution, [M] 20 millimolar (mM) – [M] 0.6 millimolar (mM), 6 points (n=2), 50 nL in each well.

3 **Preparation of compounds for test at 6-point dose-response format, option B:**
DMSO, tested compounds and reference compound are prepared by Biomek Span-8 and printed onto assay-ready plates (assay plate – Corning #4514) using the Labcyte Echo 650.

Reference compound – rows B and O – 50 nL of ASAP-0027815 2-fold dilution, [M] 10 millimolar (mM) – [M] 5 micromolar (µM), 12 points (n = 4)



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
B	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM
C	1 mM	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Compound 11	Compound 12	Compound 13	Compound 14	Compound 15	Compound 16	Compound 17	Compound 18	Compound 19	Compound 20	Compound 21	Compound 22	Compound 23	Compound 24
D	0.5 mM																							
E	0.25 mM																							
F	0.12 mM																							
G	0.06 mM																							
H	0.03 mM																							
I	1 mM	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Compound 11	Compound 12	Compound 13	Compound 14	Compound 15	Compound 16	Compound 17	Compound 18	Compound 19	Compound 20	Compound 21	Compound 22	Compound 23	Compound 24
J	0.5 mM																							
K	0.25 mM																							
L	0.12 mM																							
M	0.06 mM																							
N	0.03 mM																							
O	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM
P	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO

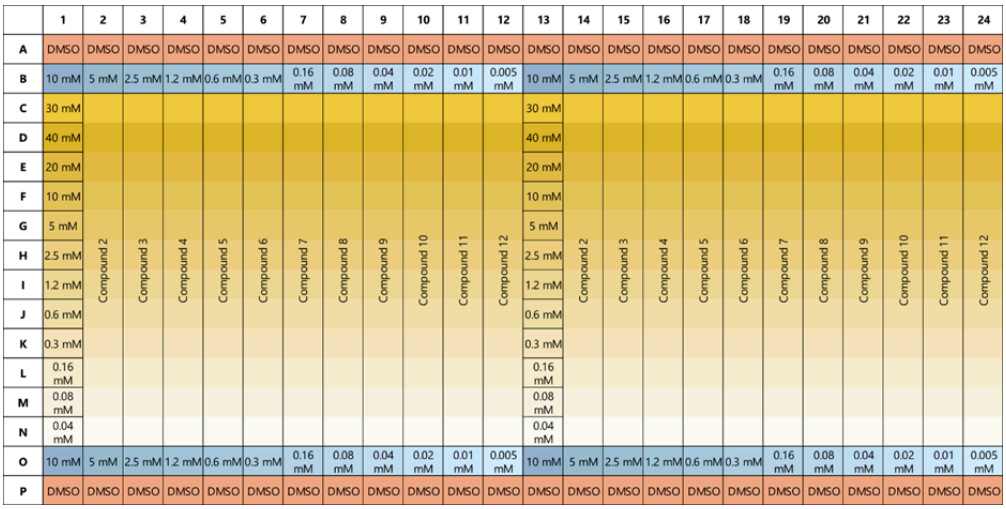
Figure 2B. Plate Map of 6-point dose-response scheme prepared by liquid handling department, option B.

DMSO is formatted into rows A and P.

Compounds are formatted into all columns, in rows C-N in dose-response format, 2-fold dilution, [M] 1 millimolar (mM) – [M] 0.03 millimolar (mM), 6 points (n=2), 50 nL in each well.

4 **Preparation of compounds for test at 12-point dose-response format:**
DMSO, tested compounds and reference compound are prepared by Biomek Span-8 and printed onto assay-ready plates (assay plate – Corning #4514) using the Labcyte Echo 650.

Reference compound – rows B and O – 50 nL of ASAP-0027815 2-fold dilution, [M] 10 millimolar (mM) – [M] 5 micromolar (µM), 12 points (n = 4)



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
B	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM
C	30 mM	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Compound 11	Compound 12	30 mM	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Compound 11	Compound 12
D	40 mM												40 mM											
E	20 mM												20 mM											
F	10 mM												10 mM											
G	5 mM												5 mM											
H	2.5 mM												2.5 mM											
I	1.2 mM												1.2 mM											
J	0.6 mM												0.6 mM											
K	0.3 mM												0.3 mM											
L	0.16 mM												0.16 mM											
M	0.08 mM												0.08 mM											
N	0.04 mM												0.04 mM											
O	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM	10 mM	5 mM	2.5 mM	1.2 mM	0.6 mM	0.3 mM	0.16 mM	0.08 mM	0.04 mM	0.02 mM	0.01 mM	0.005 mM
P	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO

Figure 3. Plate Map of 12-point dose-response scheme prepared by liquid handling department.

DMSO is formatted into A and P.

Compounds are formatted into all columns, in rows C-N in dose-response format, 2-fold dilution, [M] 40 millimolar (mM) – [M] 0.04 micromolar (µM) & point [M] 30 millimolar (mM), 12 points (n=2), 50 nL in each well.

5 The plates are sealed with Silverseal Sealer, Aluminium (no heating is applied) and stored at -20 °C. On the day of the assay, assay plates are removed from the -20°C freezer and left to warm up to Room temperature.

6 After liquid handling dispensing and before the start of assay procedures plates must be centrifuged at 1000 rpm, 00:01:00. 1m

7 Further assay procedures are similar for both screening formats: dose-response and compounds at 2 concentrations. Summarized in " Assay performance" section.

Assay Performance 1h 28m

8 **Multidrop Combi nL settings:**

- Plate type: 384 wells standard (16 mm)
- Predispense (per channel): 50 µL

- Dispense volume: 5 µL
- Correction factor: 1.031 (at RT)
- Dispense speed: medium (3)
- Dispense direction: by columns

Note

Multidrop Combi nL should be checked before screening procedures using gravimetric accuracy verification and photometric precision verification, in accordance to the Thermo Scientific Multidrop Combi nL User Manual. Multidrop Combi nL must have dispensing accuracy ± 2% & RCV ≤ 4% for each channel, and no obvious dispensing patterns.

9 **Multidrop Combi nL dispense #1.** Fill the tubes with freshly prepared filtered assay buffer, prime ~ 50 µL, preincubate for 00:05:00. 5 µL of assay buffer are then dispensed to half of the dummy plate, followed by the 3 assay plates. The plates are covered with plate lids and left at the table before next dispense. 5m

Select the following wells for dispense, based on a plate map format (fig. 4A, 4B):

2 concentrations format:

Dispensing of 5 µL into wells E2-P2 and A24-L24.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								5uL
B																								5uL
C																								5uL
D																								5uL
E		5uL																						5uL
F		5uL																						5uL
G		5uL																						5uL
H		5uL																						5uL
I		5uL																						5uL
J		5uL																						5uL
K		5uL																						5uL
L		5uL																						5uL
M		5uL																						
N		5uL																						
O		5uL																						
P		5uL																						

Figure 4A. Plate Map of dispensing 5 µL of **assay buffer** for 2 concentrations format.

Dose-response formats (all options):

Dispensing of 5 µL into row P.

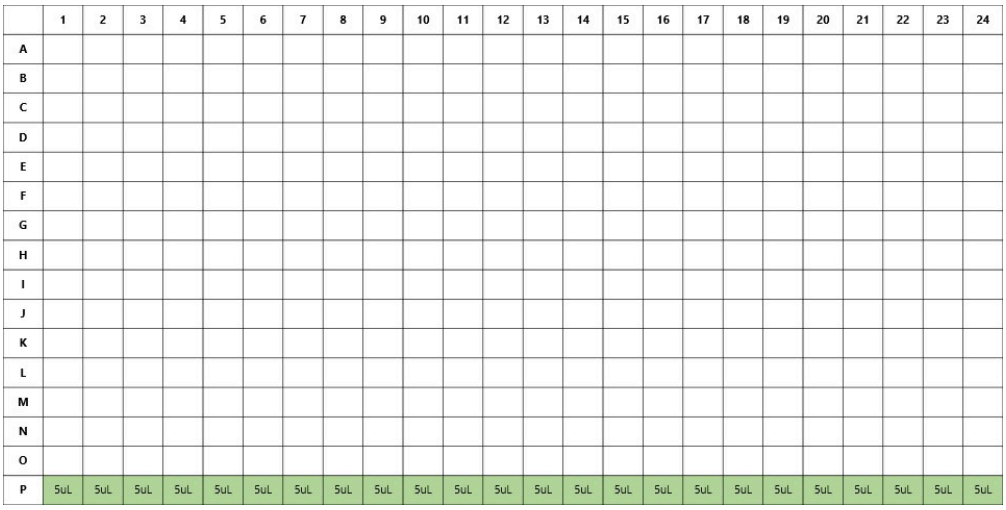


Figure 4B. Plate Map of dispensing 5 µL of **assay buffer** for dose-response format.

We used the following plasmid in the protein expression and purification protocol.
Enterovirus Coxsackievirus A16 2A protease **addgene Catalog #**228632

Protocol

NAME

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

CREATED BY

korvus.wang

Preview

10 **Multidrop Combi nL dispense #2.** Immediately after the first dispense the tubes are emptied, filled with 2x EVA71 2Apro enzyme solution and primed ~ 50 µL. Preincubate the solution in the tubes for 00:02:00. 5 µL are then dispensed to half of the dummy plate, followed by the 3 assay plates. The plates are then sealed with

33m

SealPlate film and centrifuged at 1000 rpm, 00:01:00 and then left to incubate for 00:30:00 at Room temperature in the incubator, while Multidrop Combi nL is flushed through with ~ 40 mL of 1% SDS and ~ 80 mL of MQ H_2O before the next dispense.

Select the following wells for dispense, based on a plate map format (fig. 5A, 5B):

2 concentrations format:

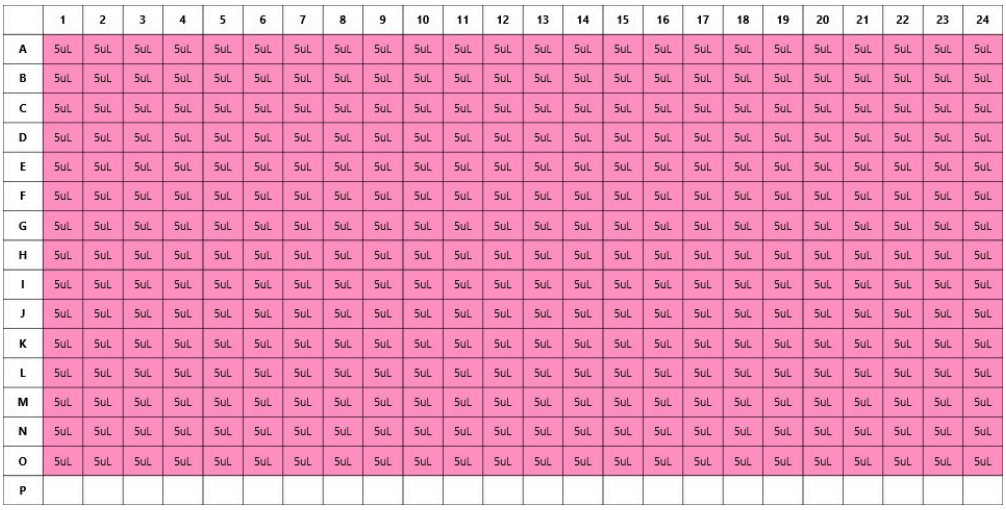
Dispensing of 5 µL into columns 1, 3-23 and wells A2-D2, M24-P24.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
B	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
C	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
D	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
E	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
F	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
G	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
H	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
I	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
J	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
K	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
L	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	
M	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
N	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
O	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
P	5uL		5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL

Figure 5A. Plate Map of dispensing 5 µL of **4 µM EVA71 2Apro** for 2 concentrations format.

Dose-response formats (all options):

Dispensing of 5 µL into rows A-O.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
B	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
C	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
D	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
E	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
F	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
G	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
H	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
I	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
J	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
K	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
L	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
M	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
N	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
O	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
P																								

Figure 5B. Plate Map of dispensing 5 µL of **4 µM EVA71 2Apro** for dose-response format.

11 **Multidrop Combi nL dispense #3.** Fill the tubes with assay buffer, prime ~ 50 µL, preincubate for 00:02:00, then empty the tubes. Fill the tubes with 2x Substrate solution, prime ~ 50 µL, preincubate the solution in the tubes for 00:02:00. 5 µL are then dispensed to half of the dummy plate, followed by the 3 assay plates (select dispensing of 5 µL of full plate). The plates are sealed with Axygen film and centrifuged at 1000 rpm, 00:01:00 and then left to incubate for 00:45:00 at Room temperature in the incubator, while Multidrop Combi nL is flushed through with ~ 40 mL of 1% SDS and ~ 80 mL of MQ H2O.

50m

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
B	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
C	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
D	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
E	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
F	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
G	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
H	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
I	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
J	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
K	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
L	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
M	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
N	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
O	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
P	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL

Figure 6. Plate Map of dispense #3 with Multidrop Combi. Full plate is dispensed with 5 µL of **12µM of Substrate**.

12 **Reader settings:**

- Mode: FL
- Ex./Em.: 360/510
- Integration Time: 140 ms
- Read Order: by rows
- Read Temperature: Room temperature

Data Analysis

13 Export the raw data in .xls files as RFU signals (in plate format).

14 The data is analyzed using specifically designed scripts in Python.

15 Parameters for QC:
Robust Signal to Background (RS/B) = median of high control (0% of inhibition) / median of low control (100% inhibition).
Plate Robust Z′-factor (RZ′) = 1 - (3*(RSD of high control (0% of inhibition) + RSD of low control (100% inhibition))/((median of high control (0% of inhibition) - median of low control (100% inhibition))).
Robust Coefficient of Variation (RCV) = RSD of data / median of data * 100%.

Note

NB: Plate must pass criteria:

- RZ' ≥ 0.5
- RS/B ≈ 2
- RCV < 20%

No obvious liquid handling-related patterns

16 Report the compound response as % inhibition based on 0% and 100% inhibition controls:

16.1 Compound response (% inhibition by median) = (median of high control (0% of inhibition) - data point median)/(median of high control (0% of inhibition) – median of negative control (100% of inhibition)) * 100%

17 The files with raw and analyzed data are uploaded to CDD Vault Protocols (EVA-71-2A_fluorescence-dose-response_bienta and EVA-71-2A_fluorescence-single-point_bienta) for further analysis.

Protocol references

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