

Supplemental information

Assessment of optimized FRET substrates as universal corona- and picornavirus main protease substrates for screening assays

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FIPV	SGLRKMAQPSGVVEPCIVRVAYGNNVLNGLWLGDEVICPRHVIASDTS-RVINYENELSS	59
PEDV	AGLRKMAQPSGVVEKCIVRVCYGNMALNGLWLGDTVMCPRHVIASSTT-STIDYDIALSV	59
SARS-CoV-2	SGFRKMAFPSPGKVEGCMVQVTCGTTTTLNGLWLDVVYCPRHVICTSEDMLNPNYEDLLIR	60
EqCoV	SGIVKMNPTSKVEPCIVSVTYGNMTLNGLWLDKVCPRHVICSASDMTNPDYTNLLCR	60
	:*: **: *:. ** *:* * * . *****.* * *****.: :* *	
FIPV	VRLHNFSIAKNNVFLGVVSAKYKGVNLVLKVNQVNPNTPEHKFKSVRPGESFNILACYEG	119
PEDV	LRLHNFSISSGNVFLGVVGTMRGALLQIKVNQNNVHTPKYTYRTVVRPGESFNILACYDG	119
SARS-CoV-2	KSNHNFLVQAGNVQLRVIGHSMQNCVCLKKVDTANPKTPKYKFVRIQPGQTFVSLACYNG	120
EqCoV	VTSSDFTVLFDRLSLTVMSYQMQCMLVLTVTLQNSRTPKYTFGVVKGGETFTVLAAYNG	120
	:* : ..: * *:. :. * :.* * .***:..: :***:*.***.*:*	
FIPV	CPGSVYGVNMRSQGTIKGSFIAGTCGSVGYVLENGTLYFVYMHHLGNGSHVGSNLEGE	179
PEDV	AAAGVYGVNMRSNYTIRGSFINGACGSPGYNINNGTVEFCYLHQLELGSGCHVGSDDLGV	179
SARS-CoV-2	SPSGVYQCAMRPNFTIKGSFLNGSCGSGVFNIDYDCVSFCYMHMELPTGVHAGTDLEGN	180
EqCoV	KPQGAFHVTMRSSYTIKGSFLCGSCGSGVGYVLMGDCVKFVYMHQLELSTGCHTGTDFNGD	180
	..: ** . ***:***: *:*:* * : . : * *:*:*:* * *.*:***:*	
FIPV	MYGGYEDQPSMQLEGTNVMSDDNVVAFLYAALINGERWVFTNTSMTLESYNSWAKTNSFT	239
PEDV	MYGGYEDQPTLQVEGASSLFTENVLAFLYAALINGSTWWLSSSRIAVDRFNEWAVHNGMT	239
SARS-CoV-2	FYGPVDRQTAQAAGTDTTITVNVLAFLYAALINGDRWFLNRFTTTLNDFNLVAMKYNYE	240
EqCoV	FYGPYKDAQVVQLPVQDYIQSVNFVAWLYAAI LNNCNWFVQSDKCSVEDFNVWALSNGFS	240
	:** : * * . : *.:*:*:*:*:*.* *:. :..: :* * .	
FIPV	EIVST--DAFNMLAAKTGYSVEKLLCCI-VRLNKGGFGRTILSYGSLCDEFTPIEVIRQM	296
PEDV	TVVNT--DCFSILAAGTGVQVRLASI-QSLHKNFGGKQILGYTSLTDEFTTGEVIRQM	296
SARS-CoV-2	PLTQDHVDILGPLSAQTGIAVLDMCASLKELLQNGMNGRTILGSALLEDEFTPFDDVVRQC	300
EqCoV	QVKSDLV--IDALASMTGVSLETLLAAI-KRLKNGFQGRQIMGSCSFEDELTPSDVYQQL	297
	: . :. *:. ** : : :. *:.:. *:* *:. : **:* :* :*	
FIPV	YGVNLQ	302
PEDV	YGVNLQ	302
SARS-CoV-2	SGVTFQ	306
EqCoV	AGIKLQ	303
	:..:	

Figure S1. Sequence alignment of coronavirus main proteases under investigation. The catalytic dyad (His41, Cys145) is highlighted in green, conserved sub-pocket residues (S1', S1 and S2) important for substrate recognition are highlighted in light grey.

NV	APPTLWSRVTKFGSGWGFWS-----PTVFITTTTHVVPTGVKE	38
HRV	-----GPNTEFALSLLRKNIMTITTSKGEFTGLGIHDRVCVIPTHAQPGDDVL	48
PV	-----GPGFDYAVAMAKRNIVTATTSKGEFTMLGVHDNVAILPTHASPGESIV	48
EV71	-----GPSLDFALSLLRRNIRQAQTDQGHFTMLGVRDRLAILPRHSQPGKTIW	48
	* . : :: : : : * *	
NV	FFGE-----PLSSIAIHQAGEFTQFRFSKKMRP-DLTGMVLEEGCPE	79
HRV	VNGQKIRVKDKYKLVDPENINLELTVLTLDRNEK---FRDIRGFISEDLE-----	95
PV	IDGKEVEILDAKALEDQAGTNLEITIIITLKRNEK---FRDIRPHIPTQIT-----	95
EV71	VEHKLINVLDARELVDEQGVNLELTVLTLDTNEK---FRDITKFIPEVIT-----	95
	. : : : : : : ** :	
NV	GTVCSVLIKRDSEGELLPLAVRMGAIASMRIQGRLVHGQSGMLLTGA---NAKGMDLGTIP	136
HRV	G-VDATLVVH-SNNFTNTILEVGPVTM---AGL-----INLSSTPTNRMIRYDYATKT	143
PV	ETNDGVLIVN-TSKYPNMYVPVGAVTE---QGY-----LNLGGRQTARTLMYNFPTRA	144
EV71	GASDATLVIN-TEHMPSMFVPGDVVQ---YGF-----LNLGKPTHRTMMYNFPTKA	144
	..* : . : . : : * : * . . : *	
NV	GDCGAPYVHKRGNDWVVCVHAAATKSGNTVVCAVQAGEGETALE-----	181
HRV	GQCGGVLCATG----KIFGIHV-----GGNGRQGFSAQLKKQYFVEKQ	182
PV	GQCGGVITCTG----KVIGMHV-----GGNGSHGFAAALKRSYFTQSQ	183
EV71	GQCGGVVTSVG----KIIGIHI-----GGNGRQGFCAGLKRSYFASEQ	183
	: : : * : * . * : *	

Figure S2. Sequence alignment of picornavirus and norovirus main proteases under investigation. The catalytic triad (His41, Cys147) is highlighted in green, conserved sub-pocket residues (S1', S1 and S2) important for substrate recognition are highlighted in light grey.

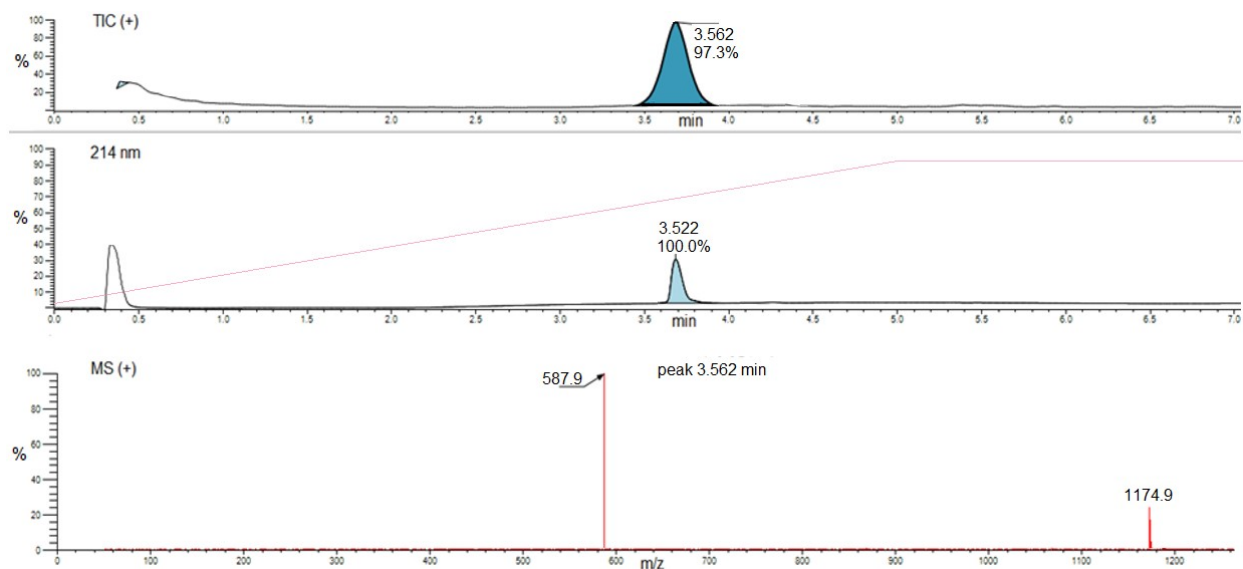


Figure S3. Total ion count chromatogram (TIC+) and mass spectral data for compound **Blanchard** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

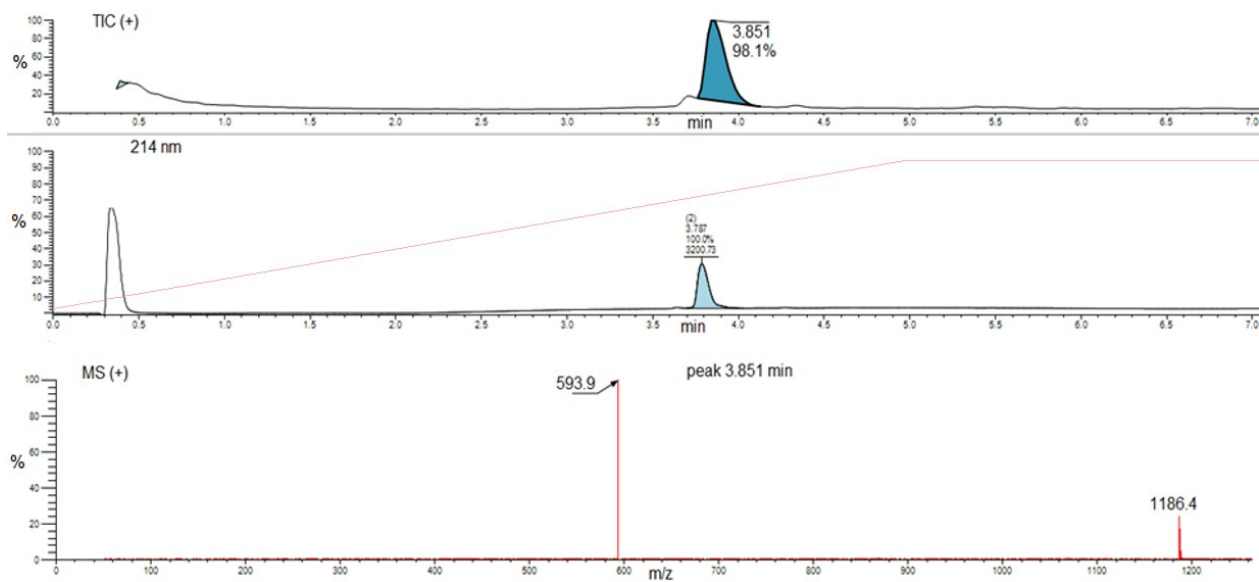


Figure S4. Total ion count chromatogram (TIC+) and mass spectral data for compound **Blanchard-VV** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

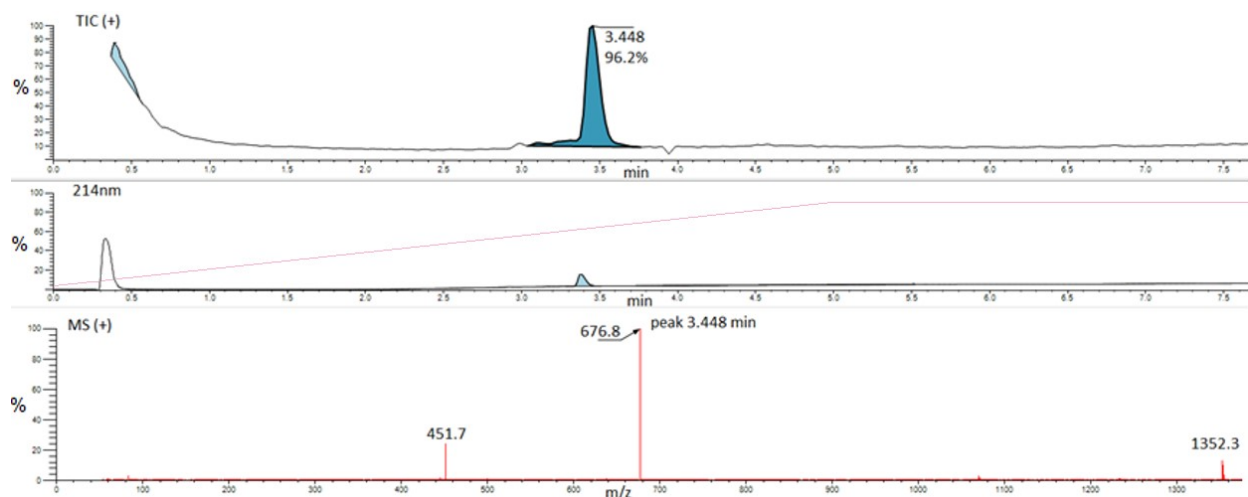


Figure S5. Total ion count chromatogram (TIC+) and mass spectral data for compound **PEDV 1** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

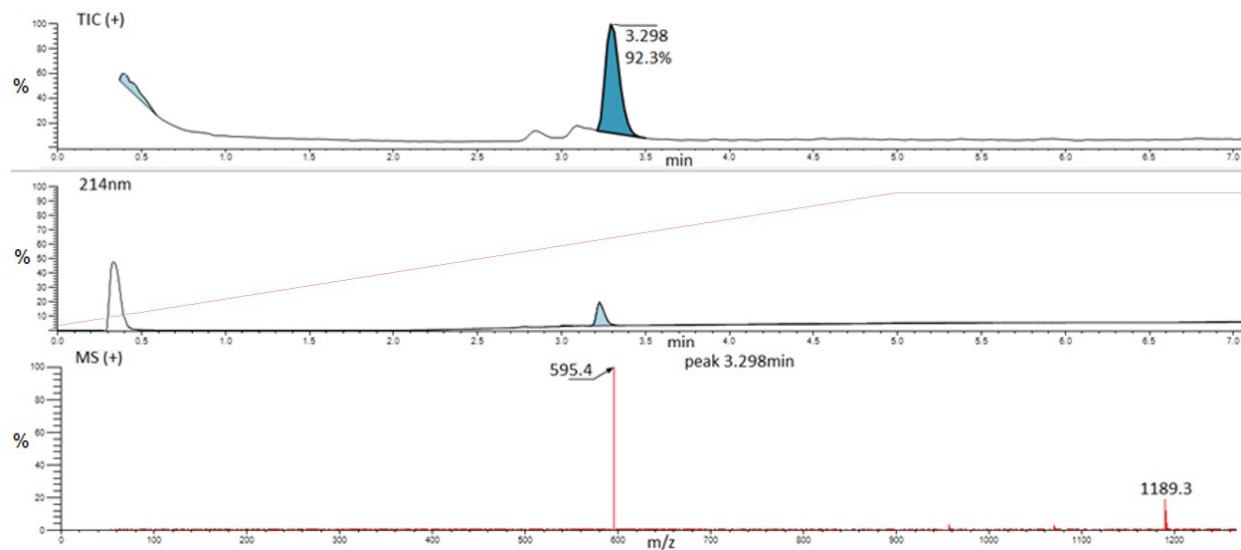


Figure S6. Total ion count chromatogram (TIC+) and mass spectral data for compound **PEDV 2** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

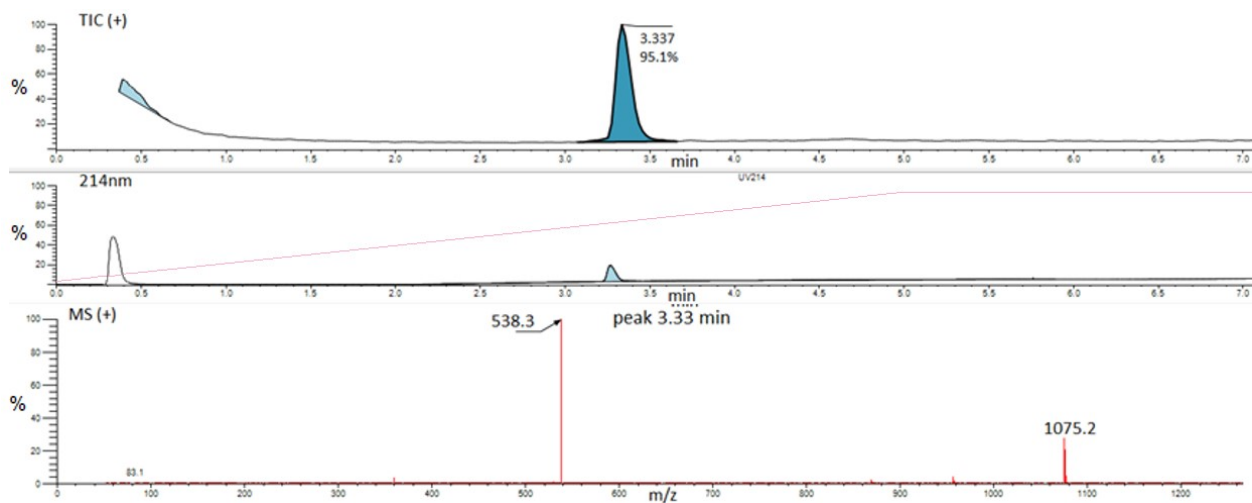


Figure S7. Total ion count chromatogram (TIC+) and mass spectral data for compound **PEDV 3** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

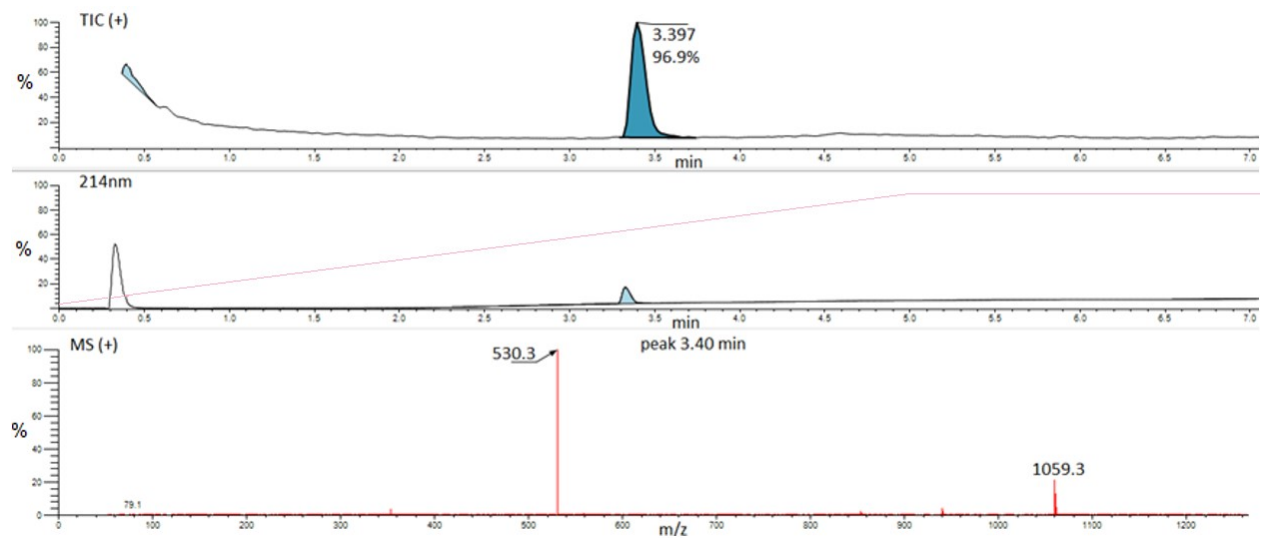


Figure S8. Total ion count chromatogram (TIC+) and mass spectral data for compound **PEDV 4** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

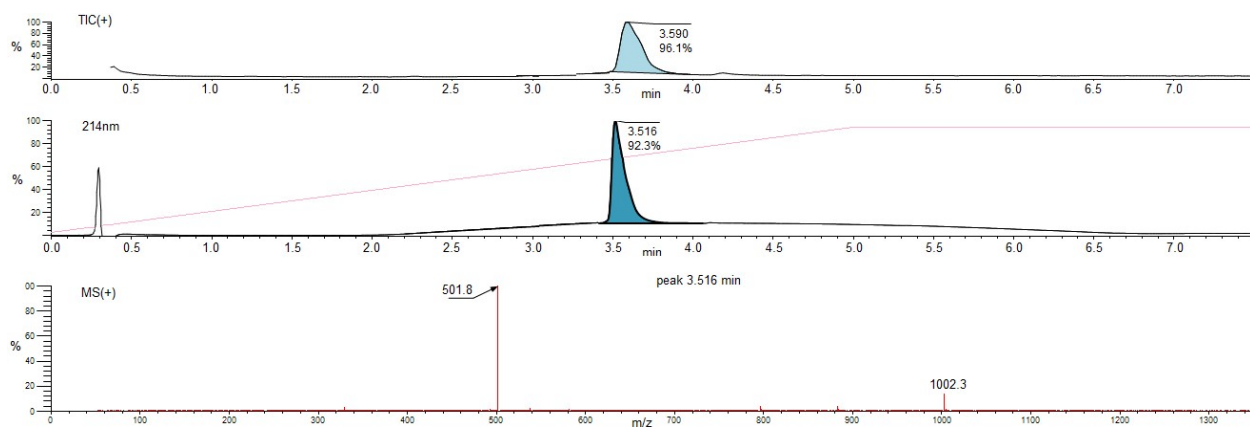


Figure S9. Total ion count chromatogram (TIC+) and mass spectral data for compound **(-)FRET** (negative control) after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

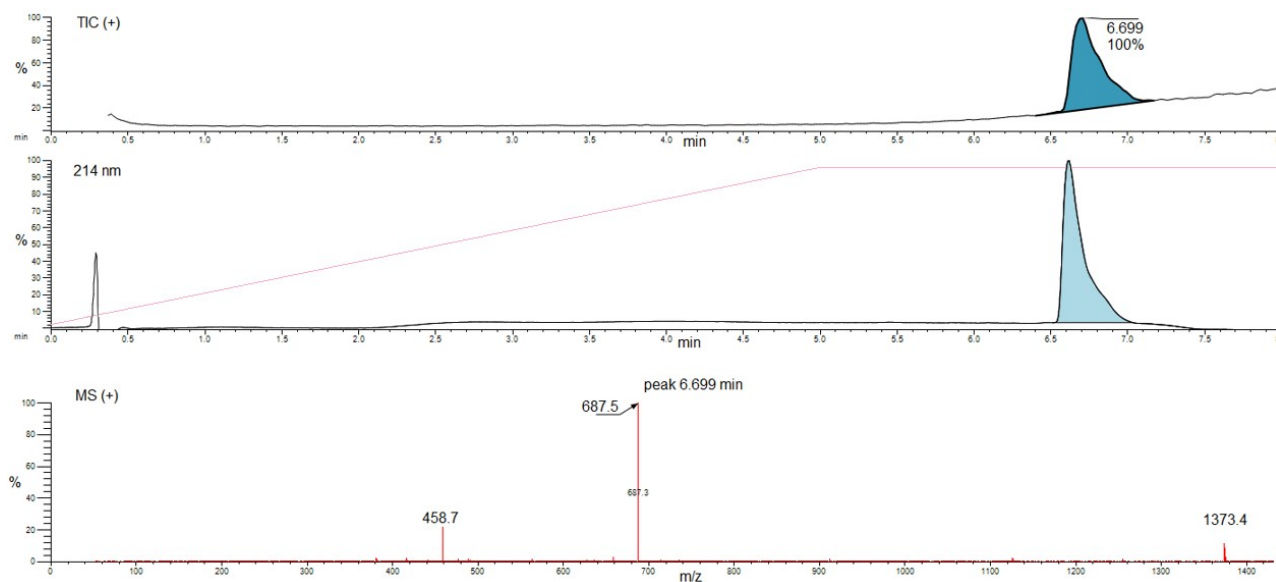


Figure S10. Total ion count chromatogram (TIC+) and mass spectral data for compound **HRVFRET** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

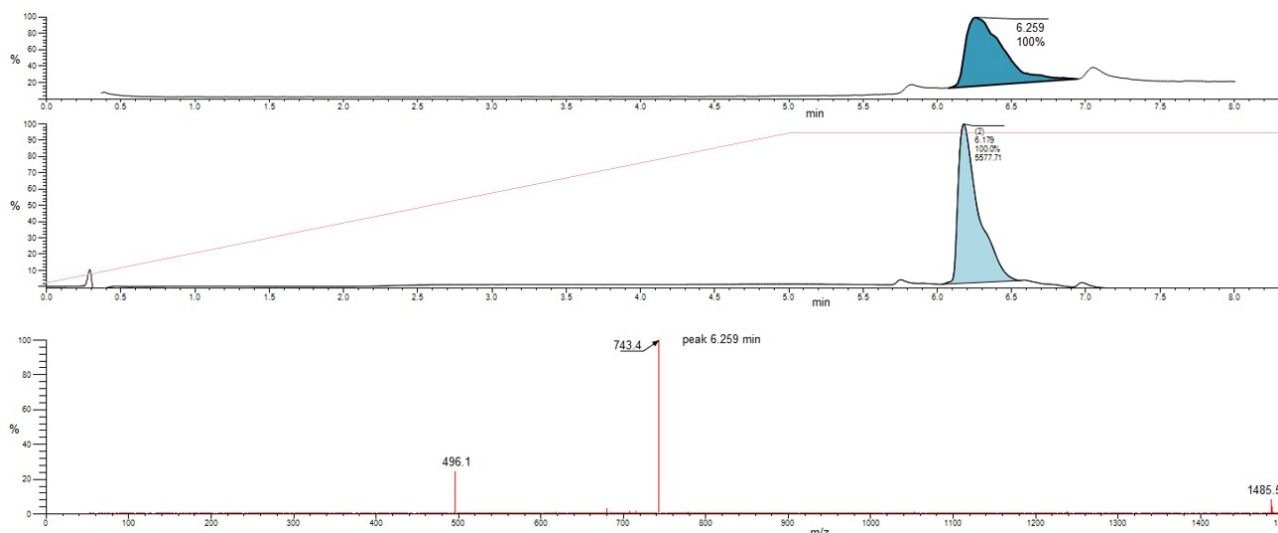


Figure S11. Total ion count chromatogram (TIC+) and mass spectral data for compound **PVFRET** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

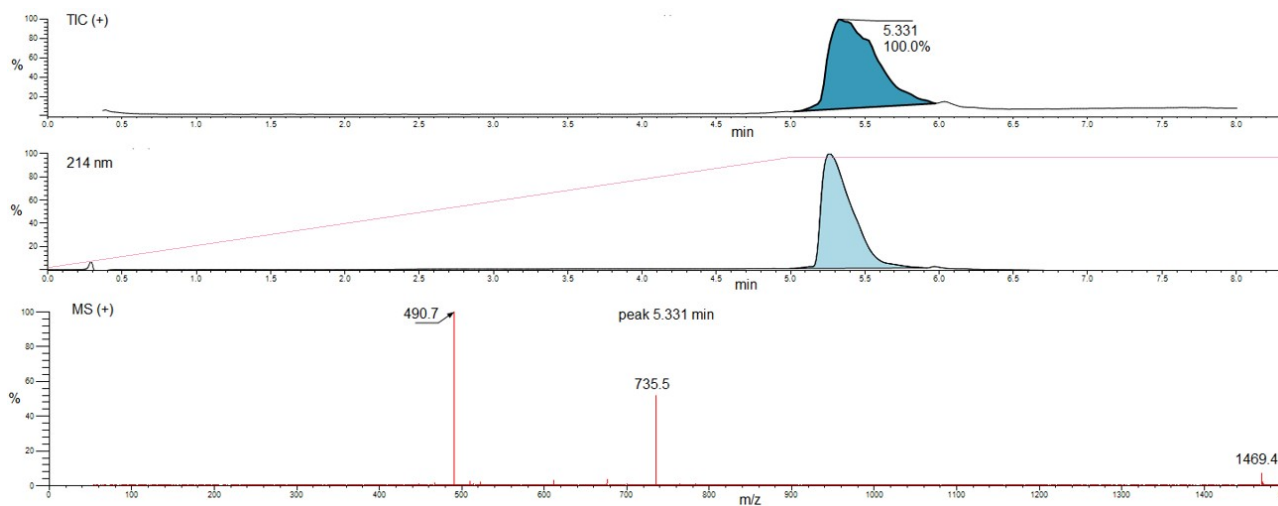


Figure S12. Total ion count chromatogram (TIC+) and mass spectral data for compound **EVFRET** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

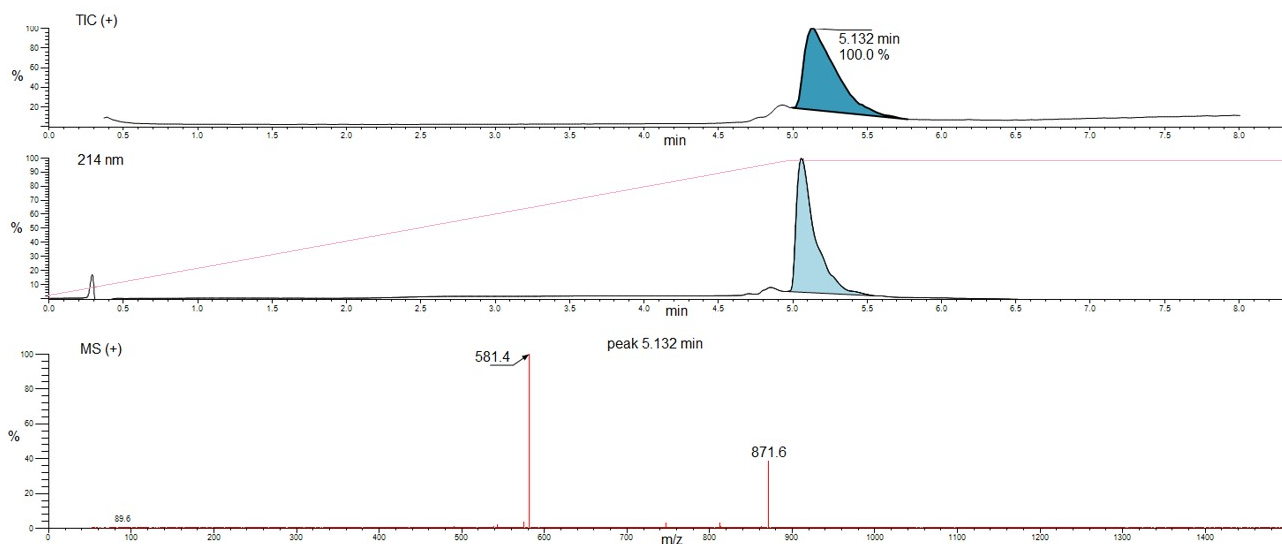


Figure S13. Total ion count chromatogram (TIC+) and mass spectral data for compound **NVFRET** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.

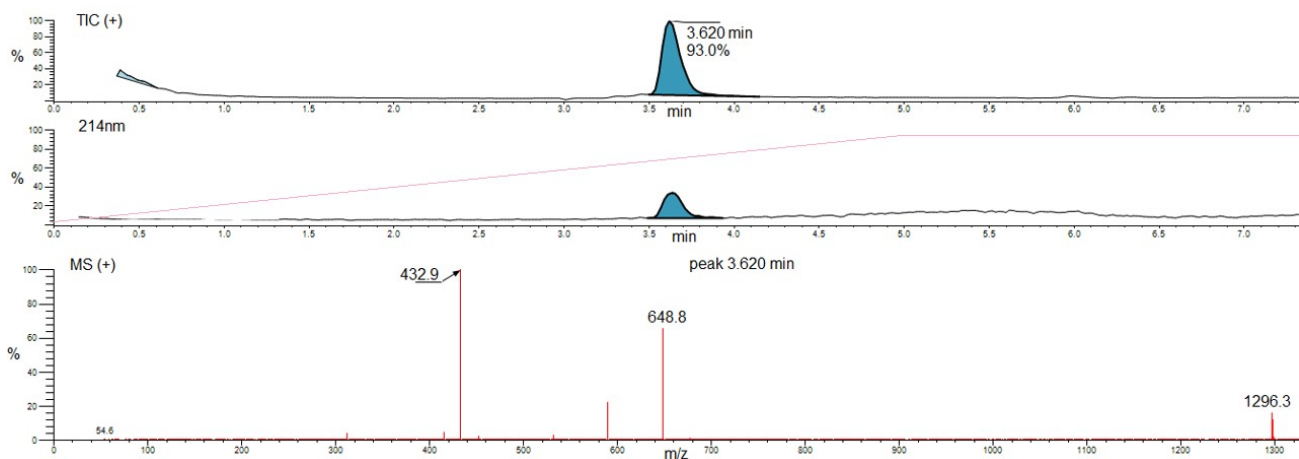
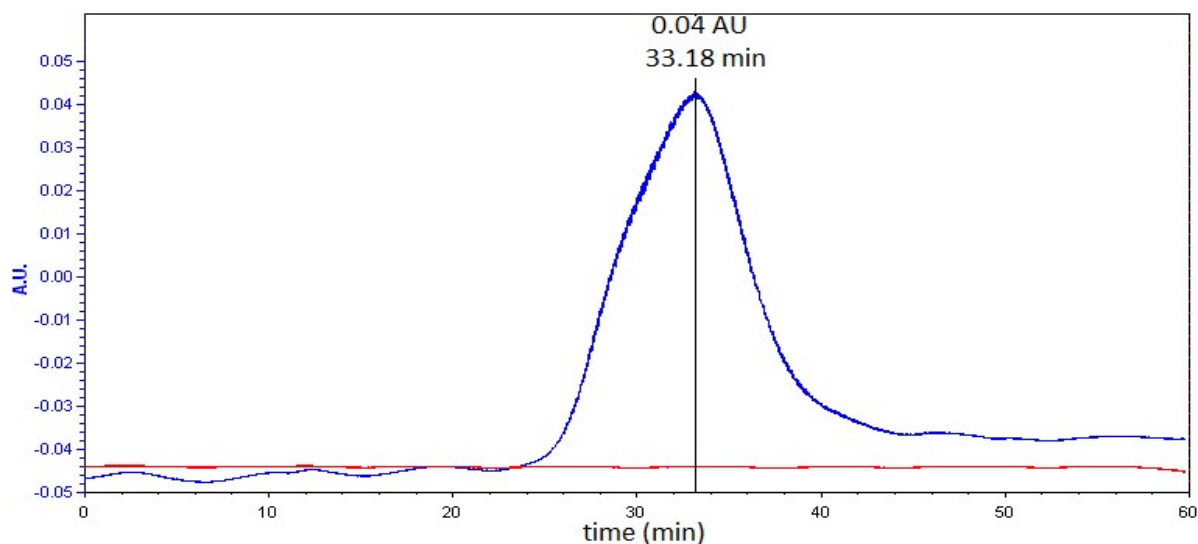
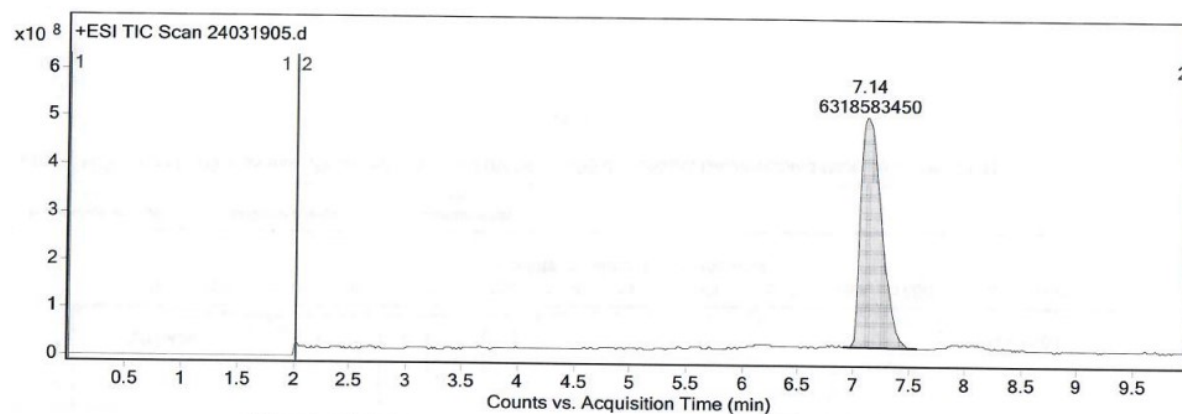


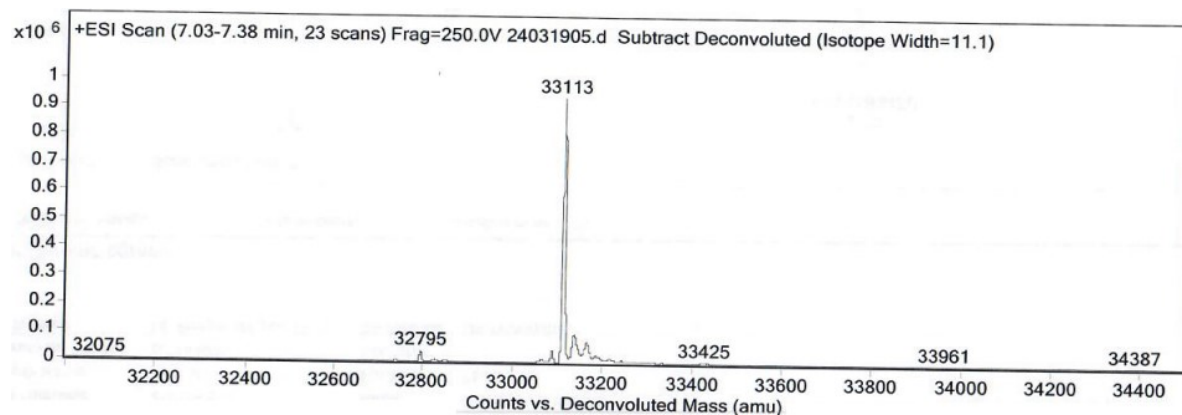
Figure S14. Total ion count chromatogram (TIC+) and mass spectral data for compound **NPFRET** after cleavage of the assembled peptide from SPPS resin and HPLC purification. The solvent gradient (%B = acetonitrile) is highlighted in pink in the DAD chromatogram at 214nm.



Gel filtration chromatogram (Sephadex G-15, 1 mL/min, 20 mM Bis-Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.8, 0.02% Tween) of FIPV-M^{Pro}

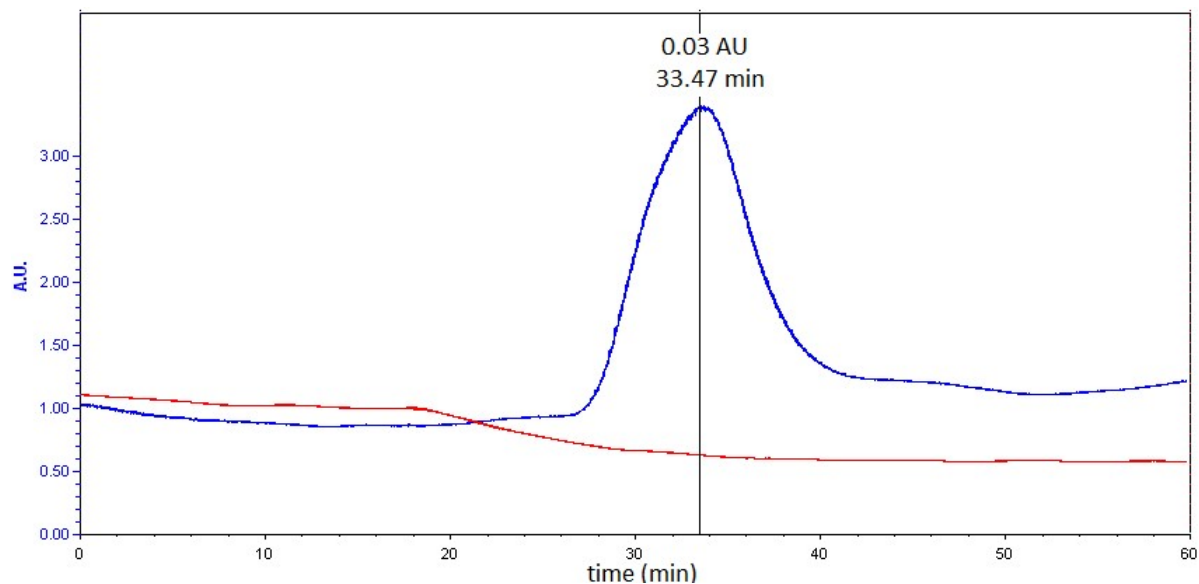


Integration Peak List							
Peak	Start	RT	End	Height	Area	Area %	
1	6.91	7.14	7.58	479186990	6318583450	100	

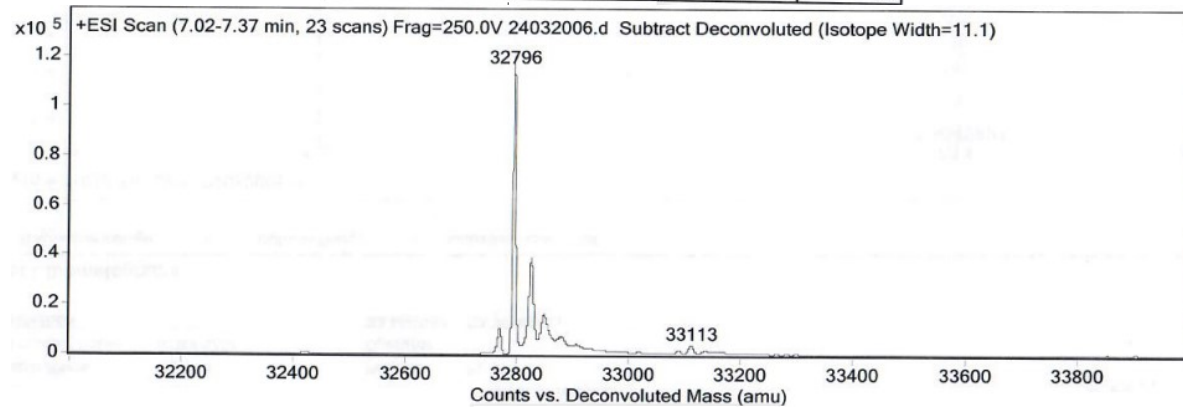
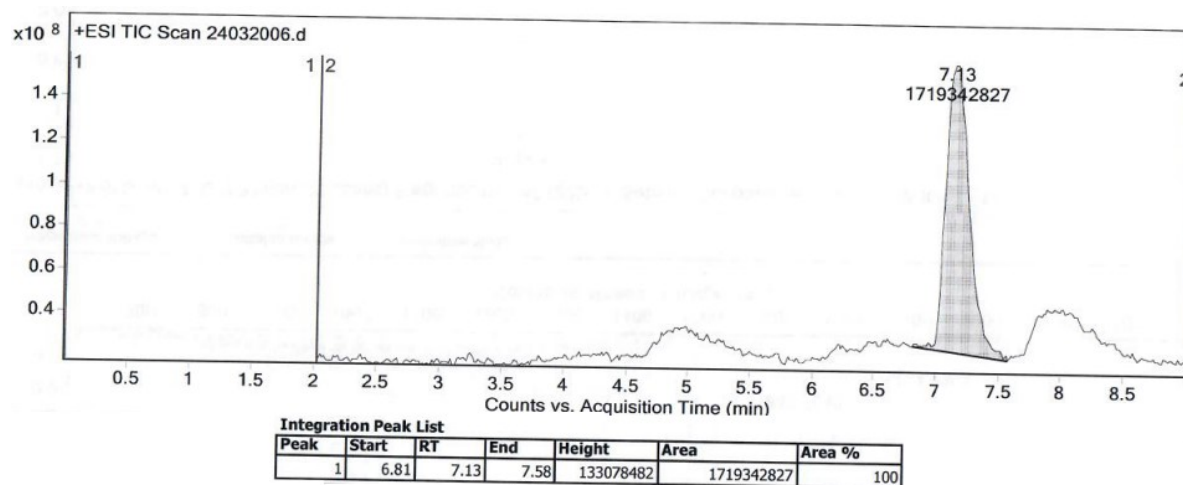


Chromatogram and deconvoluted mass spectrum of FIPV-M^{Pro}

Figure S15. Chromatographic and ESI mass-spectrometric characterization of FIPV-M^{Pro}.

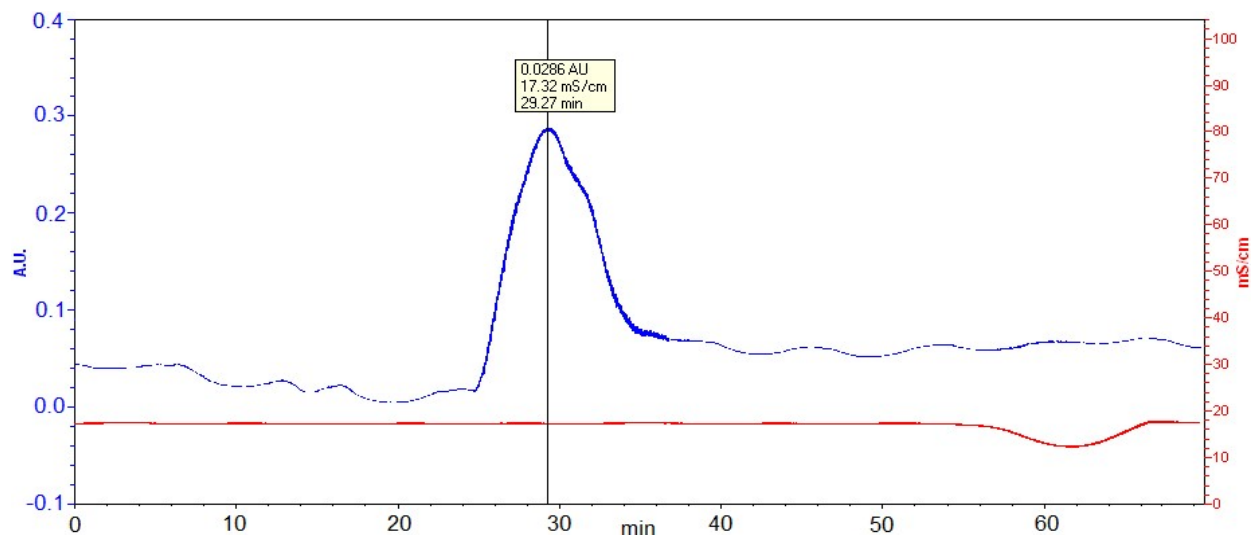


Gel filtration chromatogram (Sephadex G-15, 1 mL/min, 20 mM Bis-Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.8, 0.02% Tween) of PEDV-M^{pro}.

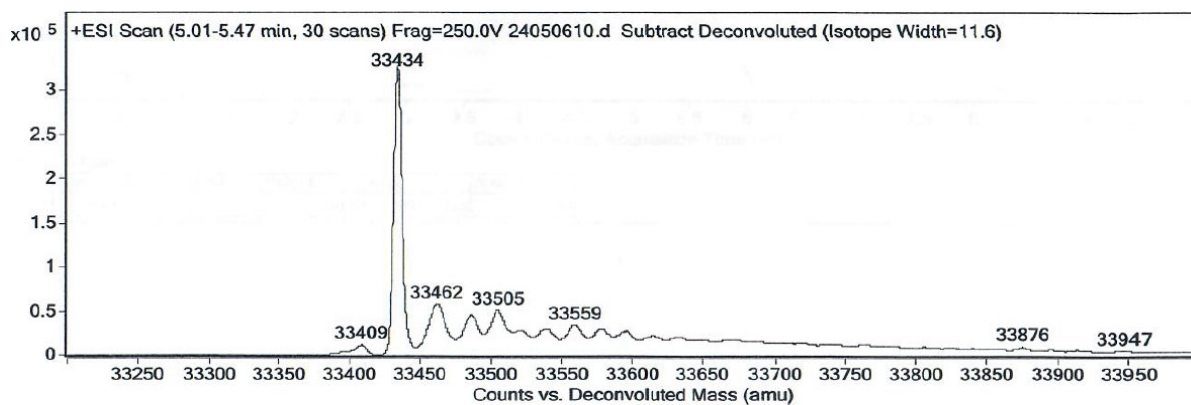
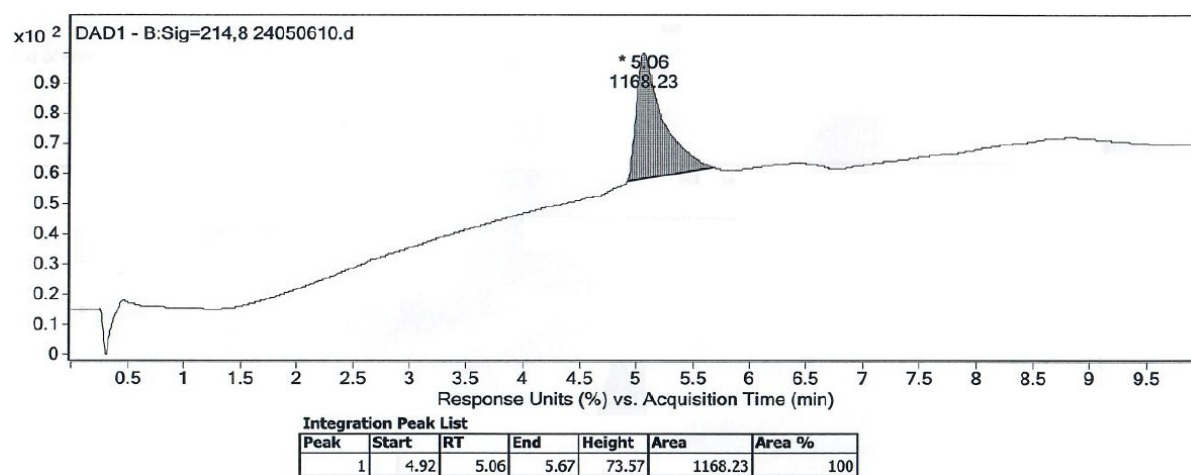


Chromatogram and deconvoluted mass spectrum of PEDV-M^{pro}.

Figure S16. Chromatographic and ESI mass-spectrometric characterization of PEDV-M^{pro}.

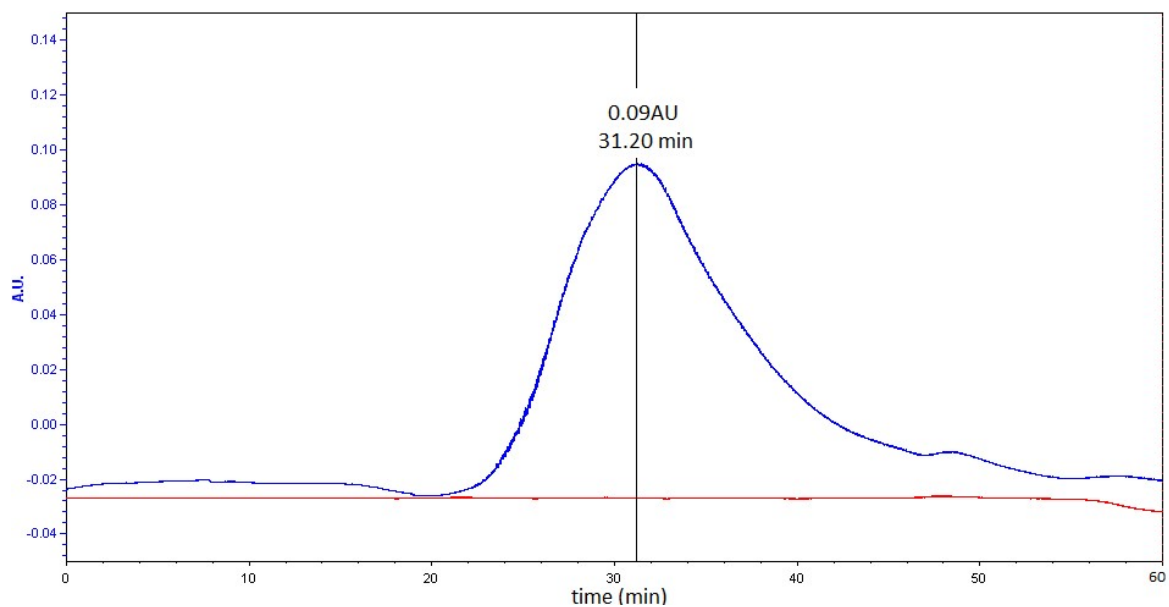


Gel filtration chromatogram (Sephadex G-15, 1 mL/min, 20 mM Bis-Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.8, 0.02% Tween) of EqCoV-M^{pro}.

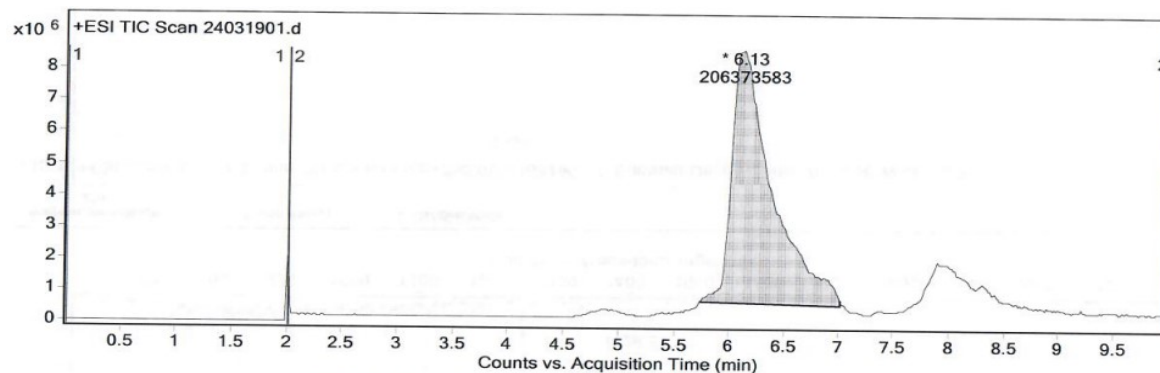


Chromatogram and deconvoluted mass spectrum of EqCoV-M^{pro}.

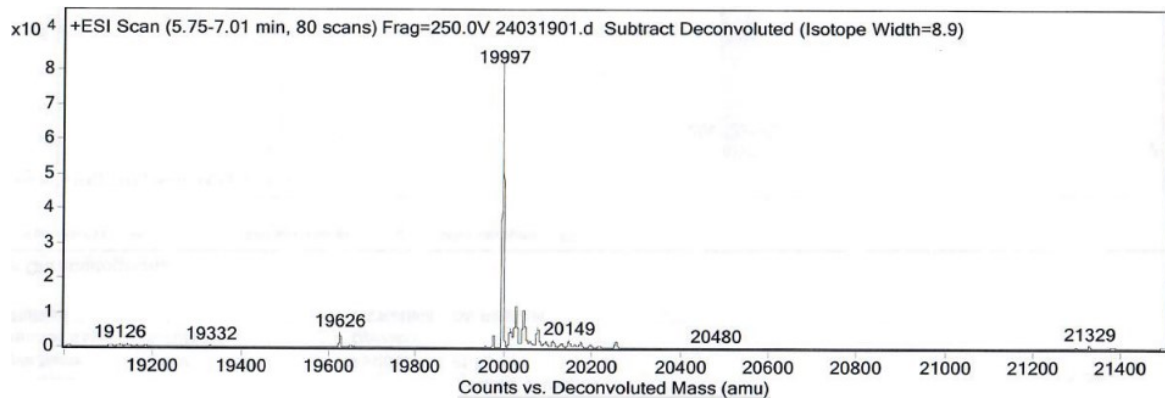
Figure S17. Chromatographic and ESI mass-spectrometric characterization of EqCoV-M^{pro}.



Gel filtration chromatogram (Sephadex G-15, 1 mL/min, 20 mM Bis-Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.8, 0.02% Tween) of HRV-M^{pro}

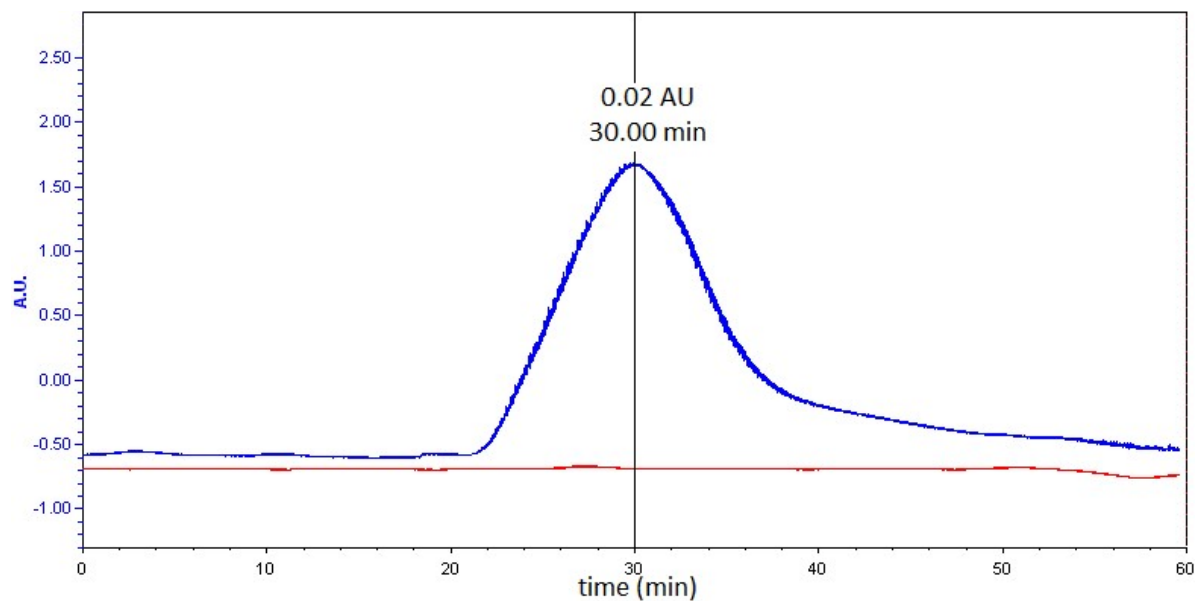


Peak	Start	RT	End	Height	Area	Area %
1	5.73	6.13	7.03	7992060	206373583	100

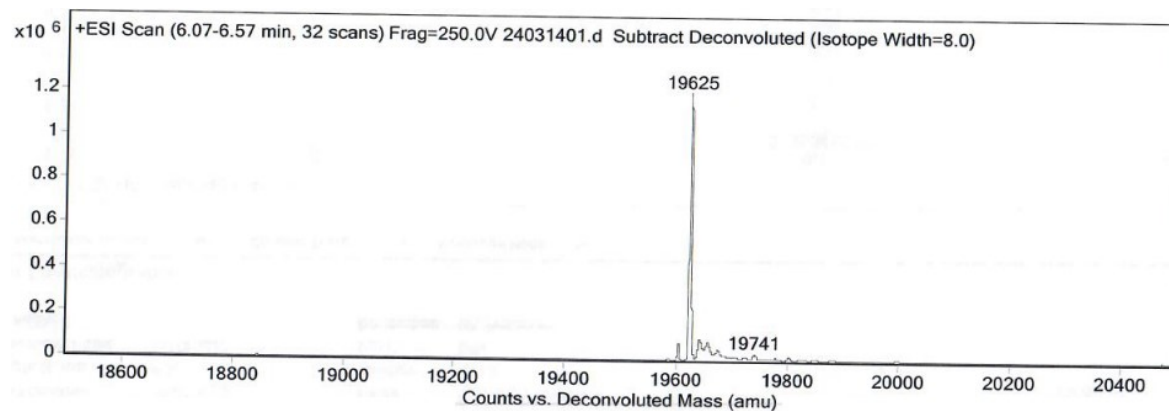
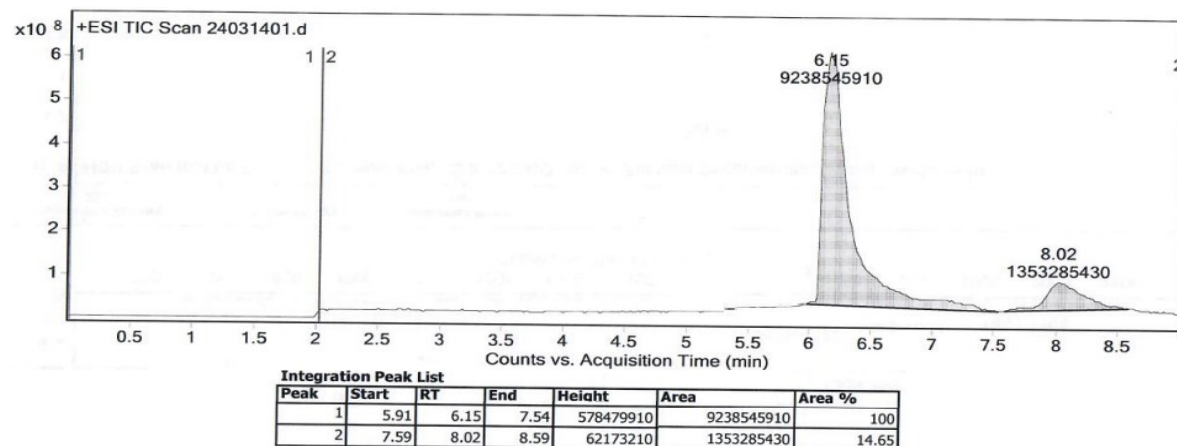


Chromatogram and deconvoluted mass spectrum of HRV-M^{pro}

Figure S18. Chromatographic and ESI mass-spectrometric characterization of HRV-M^{pro}.

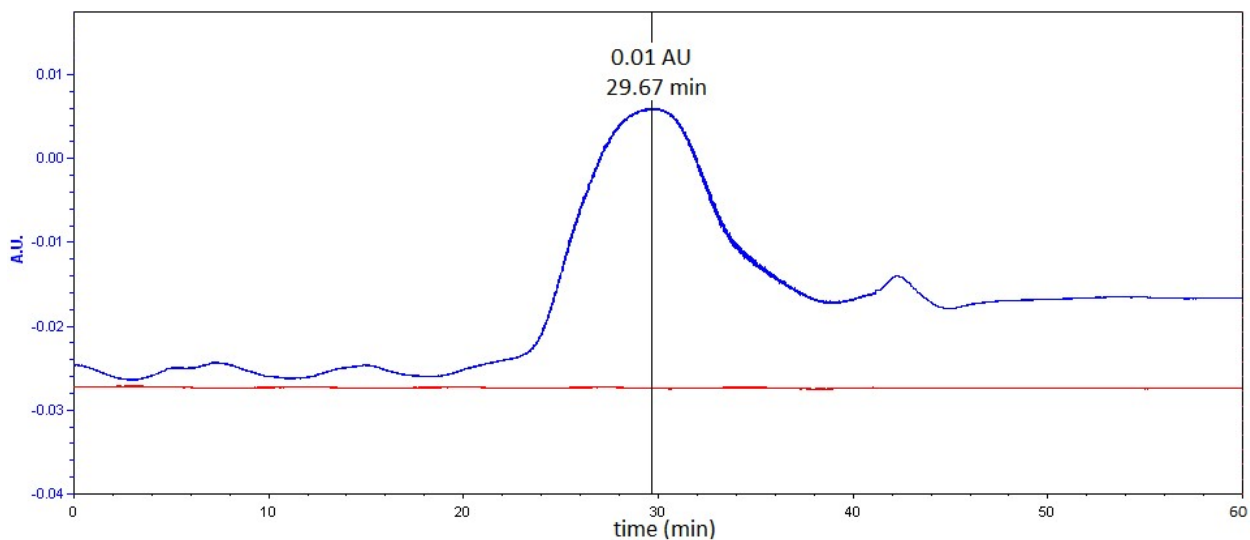


Gel filtration chromatogram (Sephadex G-15, 1 mL/min, 20 mM Bis-Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.8, 0.02% Tween) of PV-M^{pro}

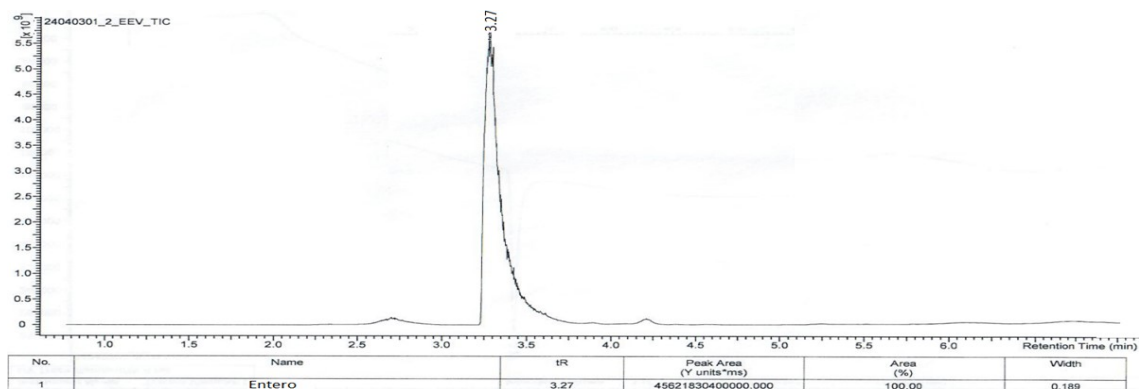


Chromatogram and deconvoluted mass spectrum of PV-M^{pro}

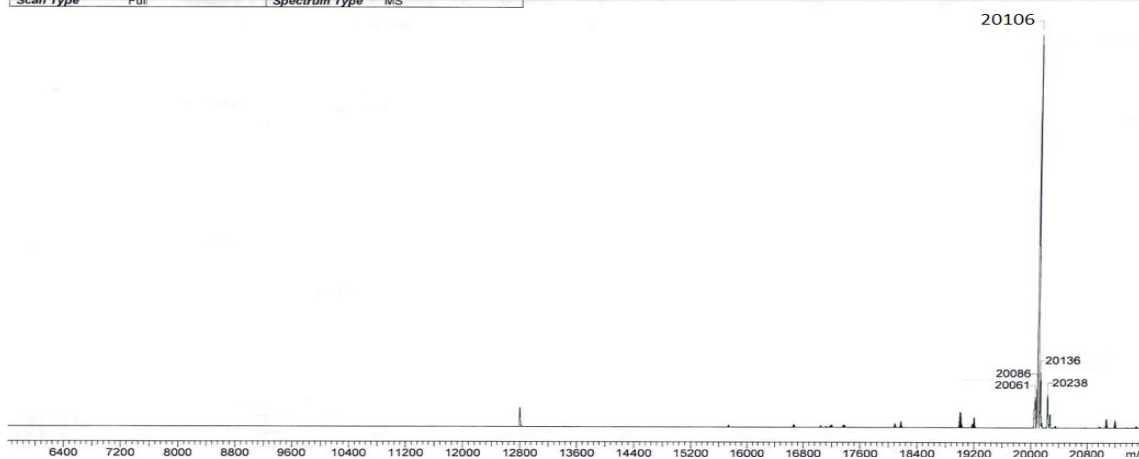
Figure S19. Chromatographic and ESI mass-spectrometric characterization of PV-M^{pro}.



Gel filtration chromatogram (Sephadex G-15, 1 mL/min, 20 mM Bis-Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.8, 0.02% Tween) of EV71-M^{pro}

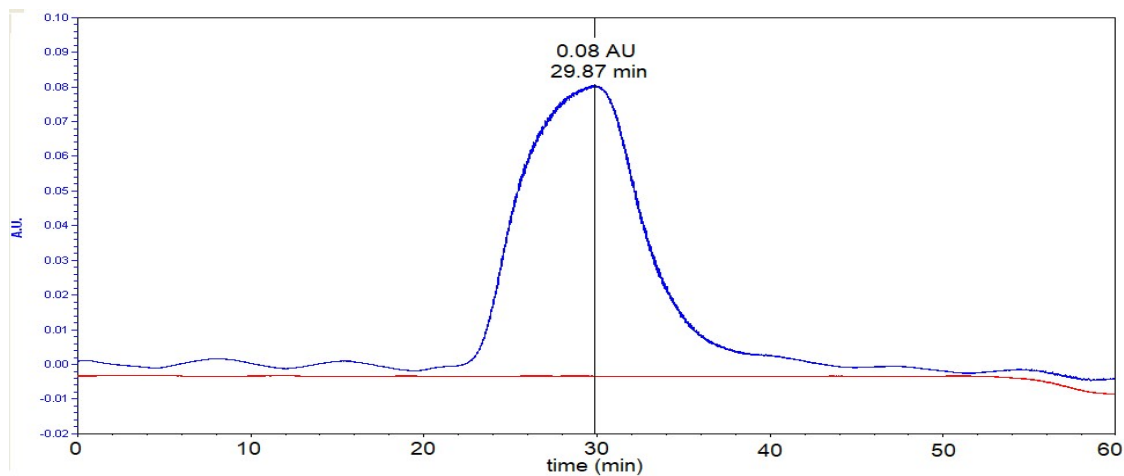


Sample	EEV	File Name	24040301_2_EEV_Combined_3-31_554_002_ChargeStateDeconvoluted		
Instrument Model	Orbitrap Exploris 240	Ion Mode	ESI+	Scan	554
Scan Type	Full	Spectrum Type	MS	Scan Filter	FTMS + p ESI Full ms [300.0000-3000.0000]

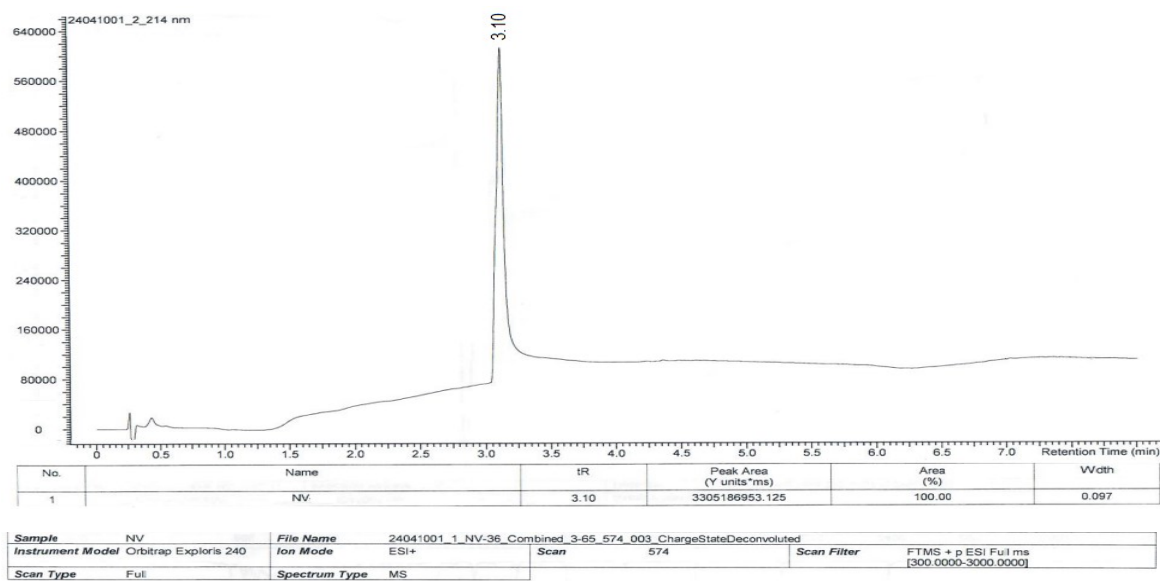


Chromatogram and deconvoluted mass spectrum of EV71-M^{pro}

Figure S20. Chromatographic and ESI mass-spectrometric characterization of EV71-M^{pro}.



Gel filtration chromatogram (Sephadex G-15, 1 mL/min, 20 mM Bis-Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.8, 0.02% Tween) of NV-M^{pro}



Chromatogram and deconvoluted mass spectrum of NV-M^{pro}

Figure S21. Chromatographic and ESI mass-spectrometric characterization of NV-M^{pro}.

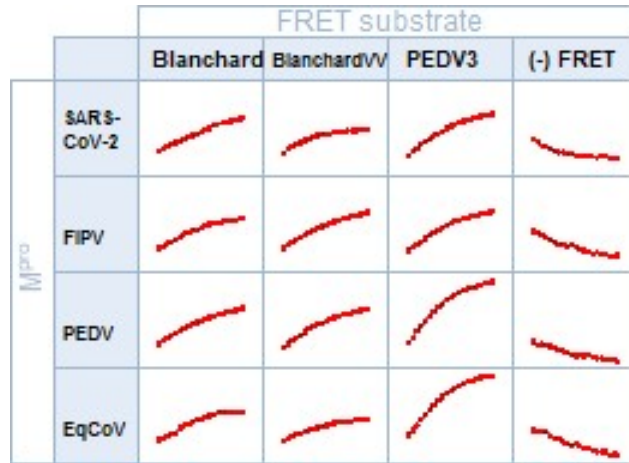
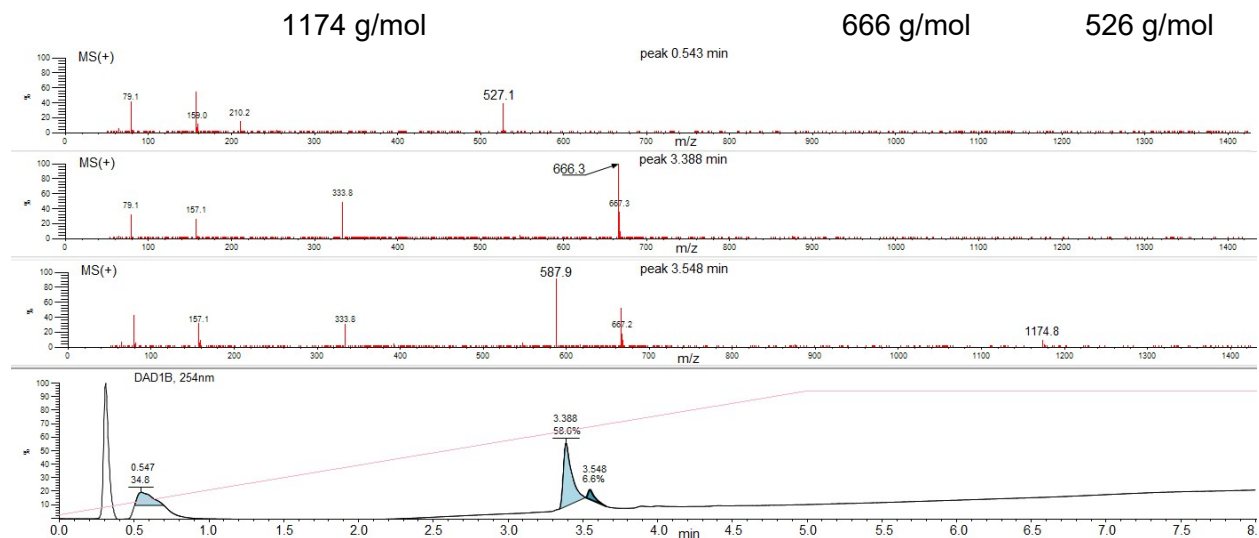
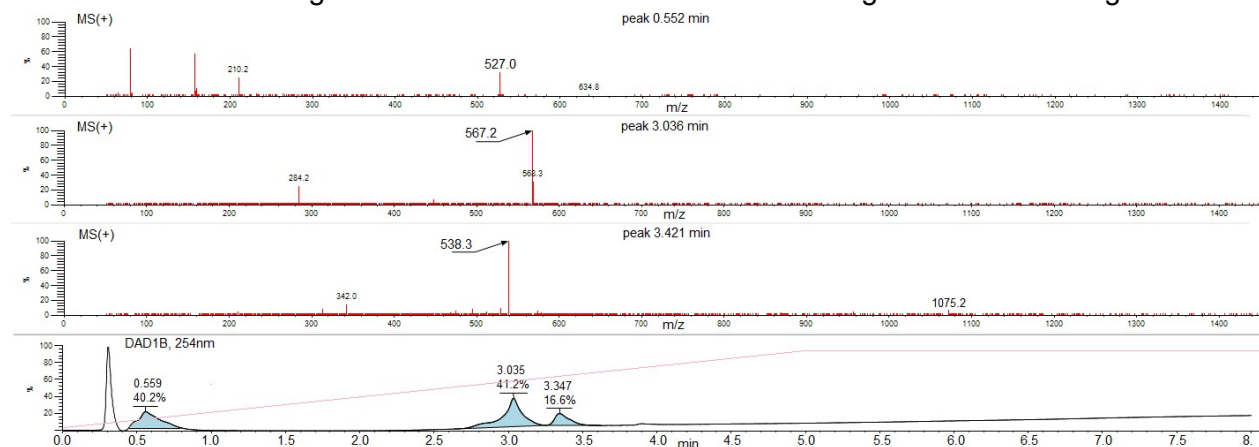


Figure S22. Reaction progression curves of various M^{pro}s with selected FRET substrates. The curves depict fluorescence released over the course of a 10 min assay.





PEDV3: 2-AbzSTLQ↓SGY^(NO2)R —SARS-CoV-2 M^{pro}→ **2-AbzSTLQ** + **SGY^(NO2)R**
 1075 g/mol 566 g/mol 526 g/mol



(-) FRET: 2-AbzSTLAAGY^(NO2)R —SARS-CoV-2 M^{pro}→ no cleavage
 1002 g/mol

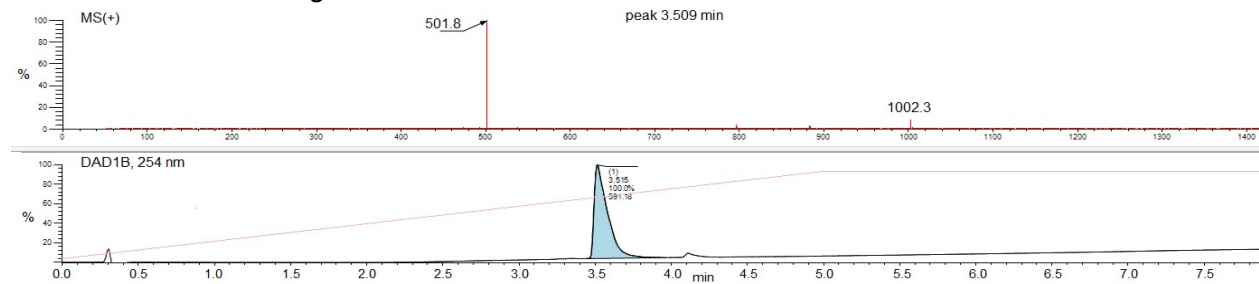
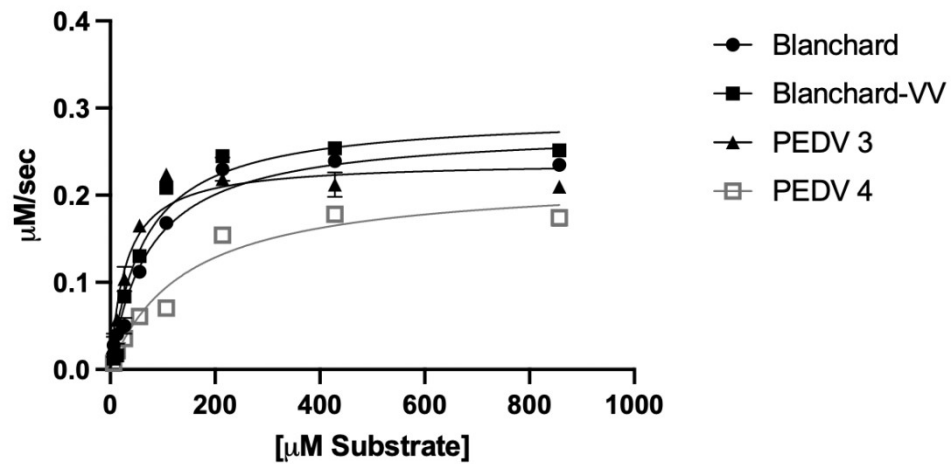


Figure S23. LCMS analysis of cleavage products formed by reaction of Blanchard FRET (top) and PEDV3 FRET (middle) and negative control (-)FRET, bottom) with SARS-CoV-2 M^{pro} after 10 min cleavage highlighting cleavage specificity of the main protease.

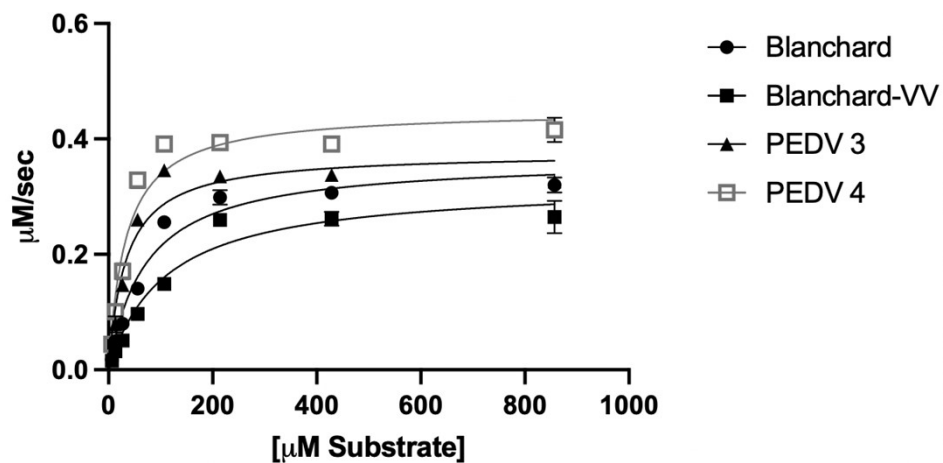
Michaelis-Menten plot SARS-CoV-2 M^{pro}



	Blanchard	Blanchard-VV	PEDV 3	PEDV 4
v_{max} , $\mu\text{M s}^{-1}$	0.2763 ± 0.02	0.2927 ± 0.02	0.2386 ± 0.04	0.2201 ± 0.03
K_M , μM	82 ± 14	64 ± 10	30 ± 8	142 ± 18
Goodness of fit				
R squared	0.9664	0.9619	0.9321	0.9545

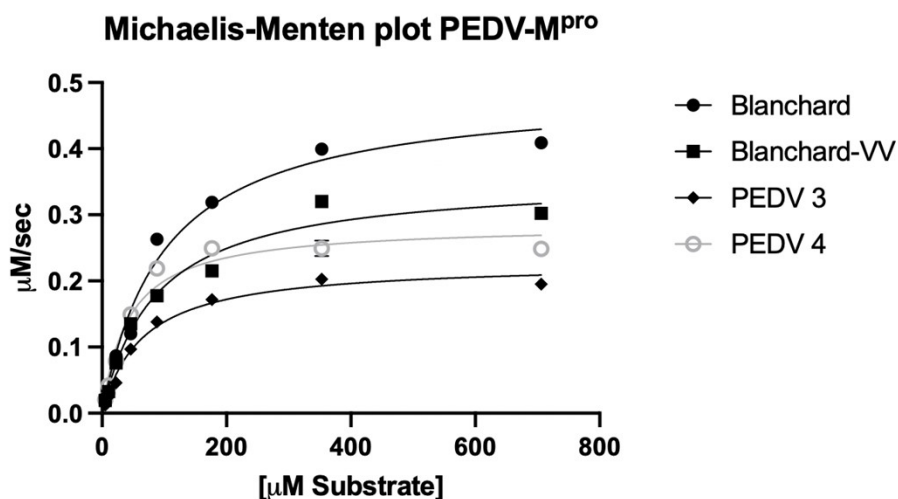
Figure S24. Michaelis-Menten plot of various coronavirus FRETs with SARS-CoV-2 M^{pro}.

Michaelis-Menten plot FIPV M^{pro}



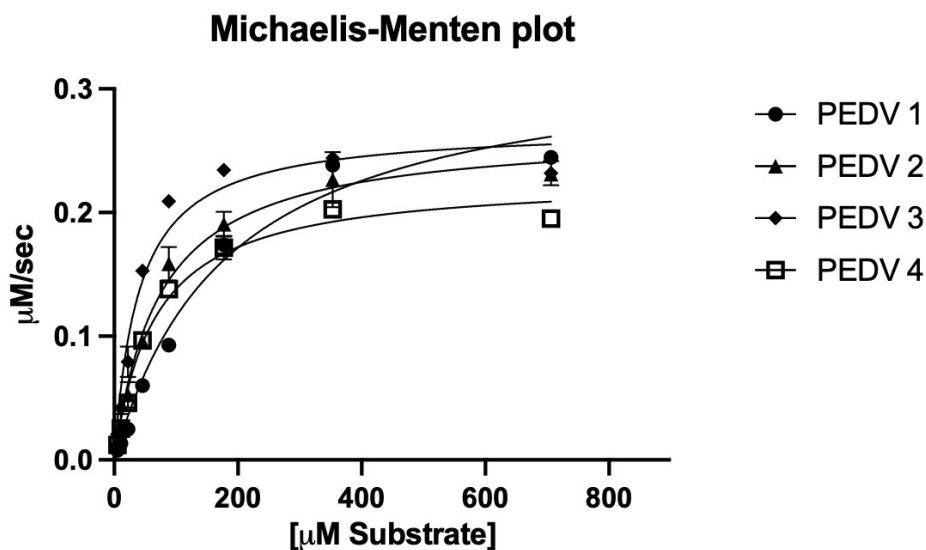
	Blanchard	Blanchard-VV	PEDV 3	PEDV 4
v_{max} , $\mu\text{M s}^{-1}$	0.3661 ± 0.03	0.3231 ± 0.04	0.3762 ± 0.04	0.4199 ± 0.04
K_M , μM	72 ± 12	85 ± 15	33.1 ± 12	32.9 ± 11
Goodness of fit				
R squared	0.9675	0.9556	0.9322	0.9471

Figure S25. Michaelis-Menten plot of various coronavirus FRETs with FIPV M^{pro}.



	Blanchard	Blanchard-VV	PEDV 3	PEDV 4
v_{max} , $\mu\text{M s}^{-1}$	0.489 ± 0.03	0.360 ± 0.04	0.269 ± 0.03	0.228 ± 0.01
K_M , μM	100 ± 18	85 ± 10	38 ± 11	65 ± 12
Goodness of fit				
R squared	0.9817	0.9801	0.9713	0.9892

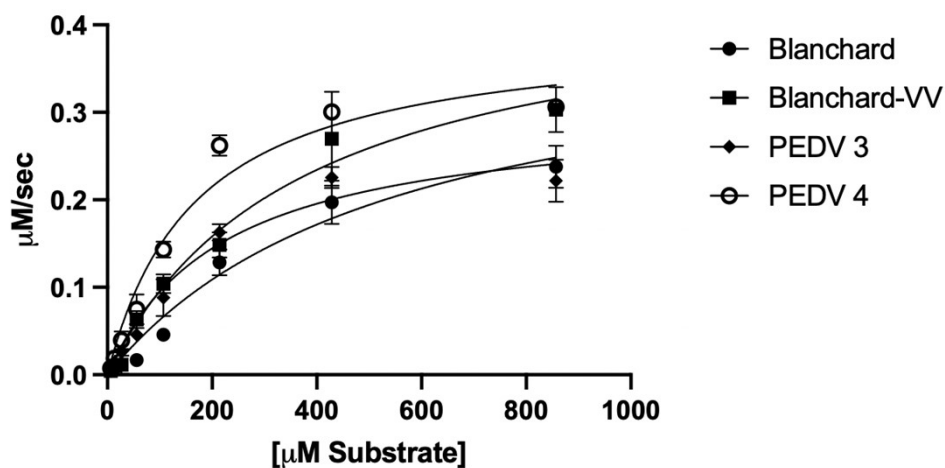
Figure S26. Michaelis-Menten plot of various coronavirus FRETs with PEDV M^{pro}.



	PEDV 1	PEDV 2	PEDV 3	PEDV 4
v_{max} , $\mu\text{M s}^{-1}$	0.330 ± 0.02	0.266 ± 0.02	0.269 ± 0.03	0.248 ± 0.01
K_M , μM	192 ± 26	63 ± 11	38 ± 11	65 ± 12
Goodness of fit				
R squared	0.9852	0.9819	0.9713	0.9892

Figure S27. Michaelis-Menten plot of various PEDV FRETs with PEDV M^{pro}.

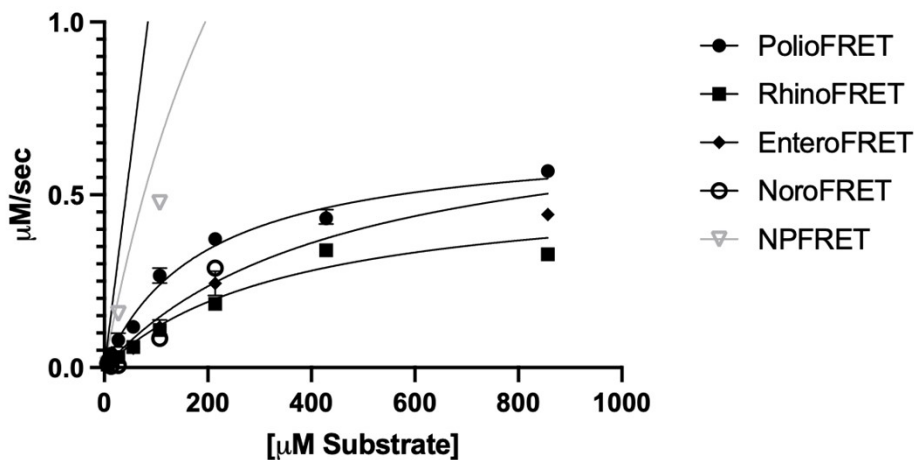
Michaelis-Menten plot EqCoV M^{pro}



	PEDV 1	PEDV 2	PEDV 3	PEDV 4
$v_{max}, \mu\text{M s}^{-1}$	0.4075	0.4516	0.3027	0.3963
$K_M, \mu\text{M}$	549.1	371.1	219.1	168.5
Goodness of fit				
R squared	0.9632	0.9630	0.9623	0.9664

Figure S28. Michaelis-Menten plot of various coronavirus FRETs with EqCoV M^{pro}.

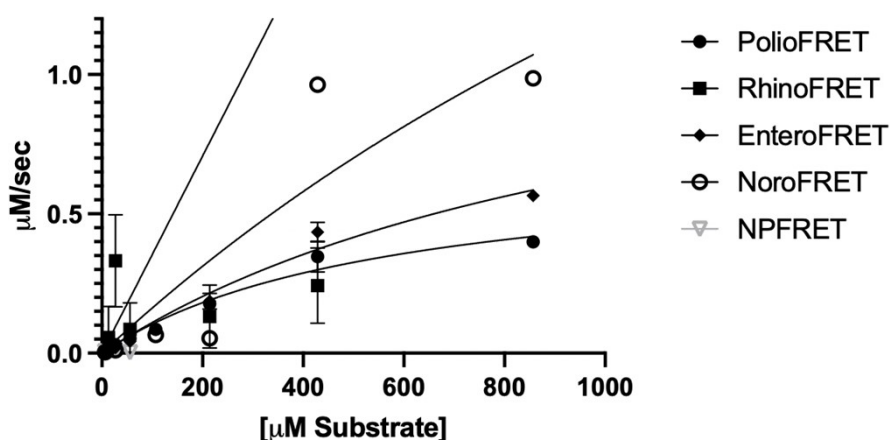
Michaelis-Menten plot HRV M^{pro}



	PVFRET	HRVFRET	EVFRET	NVFRET	NPFRET
$v_{max}, \mu\text{M s}^{-1}$	0.6694±0.06	0.5348±0.08	0.7709±0.08	n.d.*	n.d.*
$K_M, \mu\text{M}$	192.1±35	363.0±83	455.3±112	n.d.*	n.d.*
Goodness of fit					
R squared	0.9836	0.9593	0.9554	0.8414	0.07005

Figure S29. Michaelis-Menten plot of various picornavirus FRETs with HRV M^{pro}. No exact data for entries with (*) could be determined due lack of convergence.

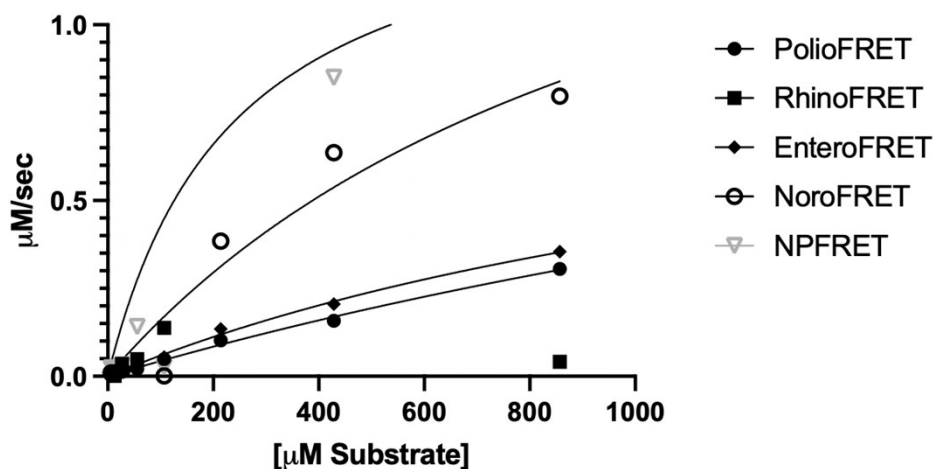
Michaelis-Menten plot Polio M^{pro}



	PVFRET	HRVFRET	EVFRET	NVFRET	NPFRET
$v_{max}, \mu\text{M s}^{-1}$	0.6969±0.06	n.d.*	1.366±0.30	4.136±1.36	n.d.*
$K_M, \mu\text{M}$	569.3±95	n.d.*	1143±205	2452±830	1386
Goodness of fit					
R squared	0.9693	0.7278	0.9756	0.8315	0.7265

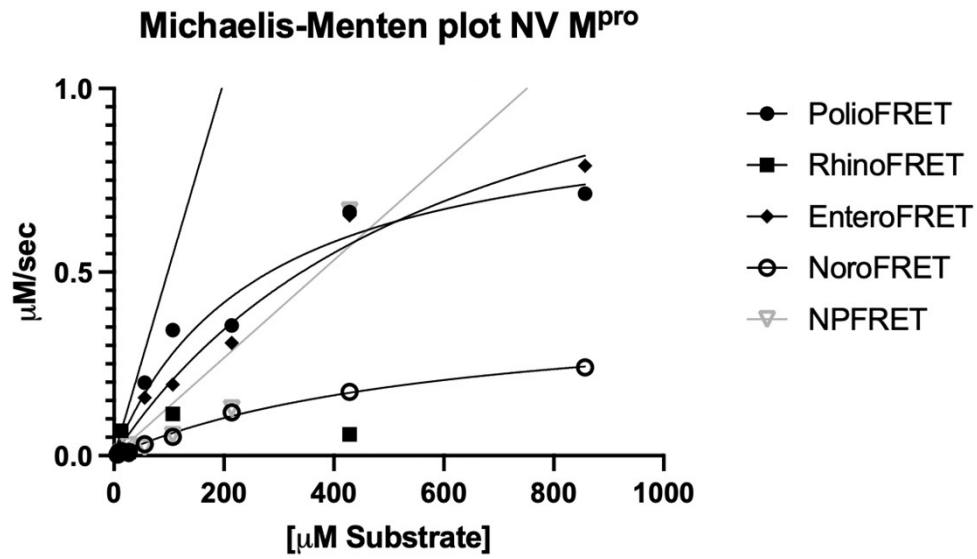
Figure S30. Michaelis-Menten plot of various picornavirus FRETs with PV M^{pro}. No exact data for entries with (*) could be determined due lack of convergence.

Michaelis-Menten plot Entero M^{pro}



	PVFRET	HRVFRET	EVFRET	NVFRET	NPFRET
$v_{max}, \mu\text{M s}^{-1}$	1.442±0.21	n.d.*	1.029±0.13	1.914±0.29	n.d.*
$K_M, \mu\text{M}$	3223	237.1	1641	1097	n.d.*
Goodness of fit					
R squared	0.9956	0.2027	0.9957	0.9246	0.04465

Figure S31. Michaelis-Menten plot of various picornavirus FRETs with EV71 M^{pro}. No exact data for entries with (*) could be determined due lack of convergence.



	PVFRET	HRVFRET	EVFRET	NVFRET	NPFRET
v_{max} , $\mu\text{M s}^{-1}$	0.9659 ± 0.10	n.d.*	1.410 ± 0.16	0.4163 ± 0.08	n.d.*
K_M , μM	263.5	n.d.*	621.5	613.0	n.d.*
Goodness of fit					
R squared	0.9565	0.7016	0.9735	0.9945	0.9643

Figure S32. Michaelis-Menten plot of various picornavirus FRETs with NV M^{pro}. No exact data for entries with (*) could be determined due lack of convergence.

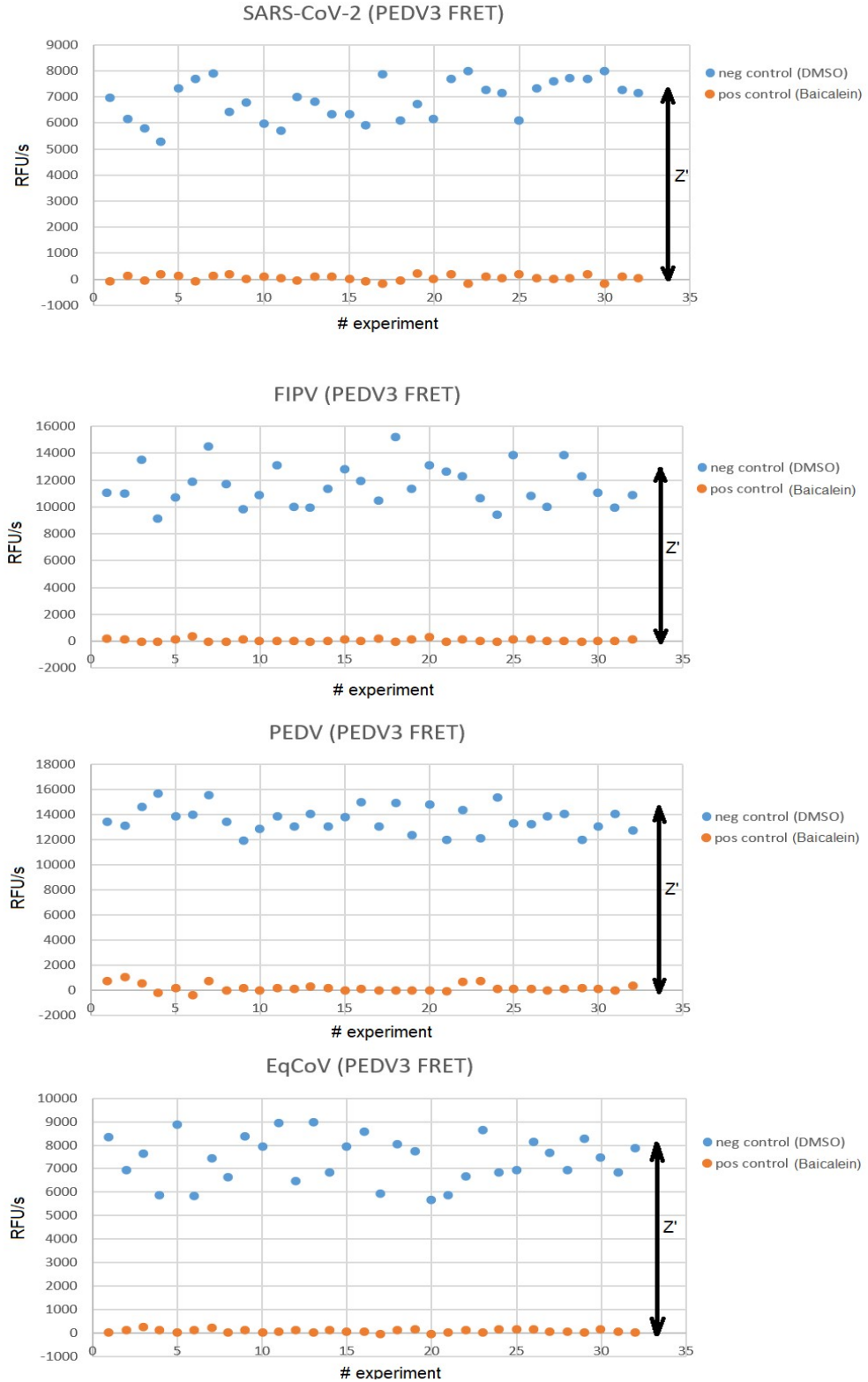


Figure S33. Z' assay quality statistics for different coronavirus M^{PRO}s (100 nM) with PEDV 3 FRET substrate (10 μ M). Samples were prepared with 50 μ M baicalein (positive control) or 50 μ M DMSO (negative control) and repeated in duplicate for $n=32$.