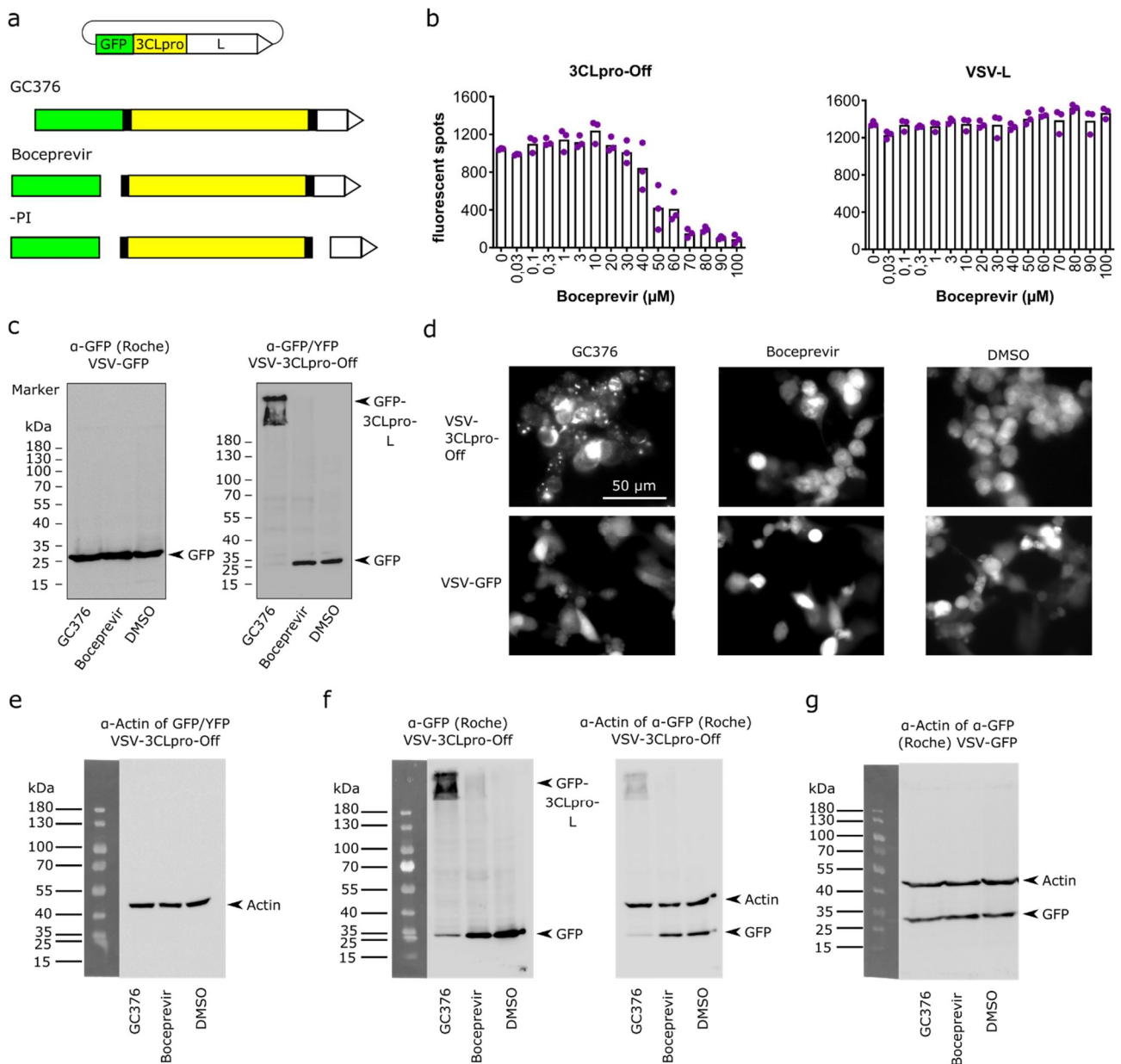


**Supplementary Figure 1:** Proof of principle of SARS-CoV-2 virus 3C-like protease incorporation into VSV genomes. **a:** Genome scheme of VSV vector with nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), reporter (rep) and polymerase / large protein (L). Replacement of the intergenic region (IGR) between the reporters GFP or luciferase (rep) and L with insertion of SARS-CoV-2 3C-like protease. **b:** Principle of 3CLpro-Off construct. The SARS-CoV-2 protease releases L in the absence of an inhibitor. The VSV replication and transcription complexes form, producing a reporter gene (rep). Adding a protease inhibitor

stalls L. **c:** Viral reporter genes GFP and *firefly* luciferase expression is blocked in the presence of more than 1  $\mu\text{M}$  of the bona fide protease inhibitor GC376. Control vectors cannot be regulated by addition of GC376. **d:** Genome scheme of wild-type VSV vector and VSV with intramolecular insertion of the SARS-CoV-2 protease into P. **e:** Principle of 3CLpro-On construct. The SARS-CoV-2 protease disintegrates P in the absence of a protease inhibitor. By adding a protease inhibitor, the construct is stabilized and the viral replication and transcription complexes can be formed. **f:** Viral reporter gene GFP expression is facilitated in the presence of more than 1  $\mu\text{M}$  of the bona fide protease inhibitor GC376. Control vectors cannot be regulated by addition of GC376. **g:** Fluorescent signal stability over continuous passaging. BHK21 3CLpro-On expressing cells and its mutated constructs red fluorescent signal in the presence of protease inhibitors (2 independent wells were imaged from each passage, images were enhanced linearly in contrast and brightness to visualize spot signal at low magnification). **h:** Spot count data for selected read-out days for all four 3CLpro-On expressing cell lines over the course of one month. Multiple data points were collected the same day (day 12 to 39) and separated to highlight them. Positive and negative controls are colored in green and red, respectively.



**Supplementary Figure 2: a:** Schemes of 3CLpro-Off in response to different inhibitors. **b:** 3CLpro-Off and control construct expressing VSV-L in response to different Boceprevir concentrations (n=3 biologically independent replicates per condition). **c:** Anti-GFP immunoblots of cells infected with VSV expressing GFP or VSV expressing 3CLpro-Off treated with either 50  $\mu\text{M}$  GC376, 100  $\mu\text{M}$  Boceprevir or DMSO. **d:** Fluorescence images of cells infected with VSV-3CLpro-Off with 50  $\mu\text{M}$  GC376 or 100  $\mu\text{M}$  Boceprevir. **e:** Mouse  $\alpha$ -Actin blot of unstripped rabbit GFP/YFP blot of cell lysates of cells treated with either 50  $\mu\text{M}$  GC376, 100  $\mu\text{M}$  Boceprevir or DMSO. **f:** Blot with mouse  $\alpha$ -GFP antibody from Roche and corresponding unstripped mouse  $\alpha$ -Actin blot loaded with cell lysates from cells treated with either 50  $\mu\text{M}$  GC376, 100  $\mu\text{M}$  Boceprevir or DMSO. **g:** Mouse  $\alpha$ -Actin blot of mouse  $\alpha$ -GFP antibody from Roche VSV-GFP blot loaded with cell lysates from cells treated with either 50  $\mu\text{M}$  GC376, 100  $\mu\text{M}$  Boceprevir or DMSO.

**Supplemental table 1:** Additional protease sequences tested in this project

**Rousettus bat coronavirus HKU9** (NCBI Reference Sequence: NC\_009021.1)

SVASAAALQAGLRMAHPSGLVEPCLVKVNYGSM TLNGLWLDNFVICPRHVMCSRDELANPDYPRLSMRAANYDFHVS  
QNGHNIRVIGHTMEGSLKLTVDVNNPKTPAYSFIRVSTGQAMSL LACYDGLPTGVYTCTLRNNGTMRASFLCGSCGSP  
GFVMNGKEVQFCYLHQLELPNGTHTGTDFSGVFYGFEDKQVPQLAAPDCTITVNVLAWLYAAVLSGENWFLTKSSISP  
AEFNCAVKYMCQSVTSESLQVLQPLAAKTGISVERMLSALKVLLSAGFCGRTIMGSCSLEDEHTPYDIGRQMLGVKLQ  
GKFQSMFR

**Human coronavirus 229E** (NCBI Reference Sequence: KF293666.1)

VSYGSTLQAGLRKMAQPSGFVEKCVVRVCYGN TVLNLGLWLDIVYCPRHVIASNTTSAIDYDHEYSIMRLHNFSIISGTA  
FLGVV GATMHGVTLKIKVSQTNMHTPRHSFRTLKSGEGFNILACYDGCAQGVFGVNMRTNWTIRGSFINGACGSPGY  
NLKNGEVEFVYMHQIELGSGSHVGSFDFGVMYGGFEDQPNLQVESANQMLTVNVVAFLYAAAILNGCTWWLKGEKLF  
VEHYNEWAQANGFTAMNGEDAFSILA AKTGVCVERLLHAIQVLNNGFGGKQILGYSSLNDEFSINEVVKQMFQVNLQS  
GKTTSMF

**Human coronavirus NL63** (NCBI Reference Sequence: YP\_010229075.1)

ISYNSTLQSGLKMAQPSGCVRCVVRVCYGSTV LNGLWLDVTCPRHVIAPSTTVLIDYDHAYSTMRLHNFSVSHNG  
VFLGVVGV TMHGSLVRIKVSQSNVHTPKHVFKTLKPGDSFNILACYEGIASGVFGVNLRTNFTIKGSFINGACGSPGYNV  
RNDGTVEFCYLHQIELGSGAHVGSDFGTGSVYGNFDDQPSLQVESANLMLSDNVVAFLYAALLNGCRWWLCSTRVNV  
GFNEWAMANGYTSVSSVECYSILA AKTGVSVEQLLASIQHLHEGFGGKNILGYSSLCDEFTLAEVVKQMYGVNLQSGKVI  
FGL

**Middle East respiratory syndrome-related coronavirus (MERS)** (NCBI Reference Sequence: KF600615.1)

SITSGVLQSGLVKMSHPSGDVEACMVQVTCGSM TLNGLWLDNTVWCPRHVMCPADQLSDPNYDALLISMTNHSFSV  
QKHIGAPANLRVVGHAMQGTLLKLTVDVANPSTPAYTFTTVKPGA AFVSLACYNGRPTGTFTVVMRPNYTIKGSFLCGS  
CGSVGYTKEGVSINFCYMHQMELANGTHTGSAFDGTMYGAFMDKQVHQVQLTDKYCSVNVVAVWLYAAAILNGCAWF  
VKPNRTSVVFNWALANQFTEFVGTQSV DMLAVKTGVAIEQLLYAIQQLYTGFGKQILGSTMLEDEFTPEDVNMQI  
MGVVMQSGVRKVTY

**Mouse Hepatitis Virus (MHV)** (NCBI Reference Sequence: NP\_045298.1)

SVTTSFLQSGIVKMSVPTSKEVPCIVSVTYGNM TLNGLWLDKDYCPRHVICSSADMTDPDYPNLLCRVTSSDFCVMSG  
RMSLTVMSYQM QGCQLVLTVTLQNPNTPKYSFGVVKPGETFTVLAAYNGRPQGAFHVTLRSSHTIKGSFLCGSCG SVG  
YVLTGDSVRFVYMHQLELSTGCHTGTDFSGNFYGPYRDAQV VQLPVQDYTQTVNVVAVWLYAAIFNRCNWFVQSDSCS  
LEEFNVWAMTNGFSSIKADLVLDALASMTGVTVEQVLA AIKRLHSGFQGKQILGSCVLEDETPSDVYQQLAGVKLQSKR  
TRVIK

**Poliovirus** (NCBI Reference Sequence: ACH87554.1)

TIRTAKVQGPFDYAVAMAKRNIVTATTSKGEFTMLGVHDNVA ILPTHASPGESIVIDGKEVEILDAKALEDQAGTNLEIT  
IITLKRNEKFRDIRPHIPTQITETNDGVLIVNTSKYPNM YVPVAVTEQGYLNLGGRQTARTLMYFNFPTRAGQCGGVITCT  
GKVIGMHVGGNGSHGFAAALKRSYFTQSQGEIQWMPR

**SARS-CoV-1** (NCBI Reference Sequence: AAP82976.1)

SITSAVLQSGFRKMAFPSGKVEGCMVQVTCGTTTLNGLWLD DTVYCPRHVIC TAEDMLNPNYEDLLIRKSNHSFLVQAG  
NVQLRVIGHSMQNCLLRLKVDTSNPKTPKYK FVRIQPGQTFSVLACYNGSPSGVYQCAMRPNHTIKGSFLNGSCG SVGF  
NIDYDCVSFCYMHMELPTGVHAGTDLEGGFYGPFVDRQTAQAAGTDTTITLNVLAWLYAAVINGDRWFLNRFTTTLN  
DFNLVAMKYNYEPLTQDHVDILGPLSAQTGIAVLDMCAAL KELLQNGMNGRTILGSTILEDEFTPFDVVRQCSGVTFQG  
KFKKIVK

**SARS-CoV-2** (NCBI Reference Sequence: NC\_045512.2)

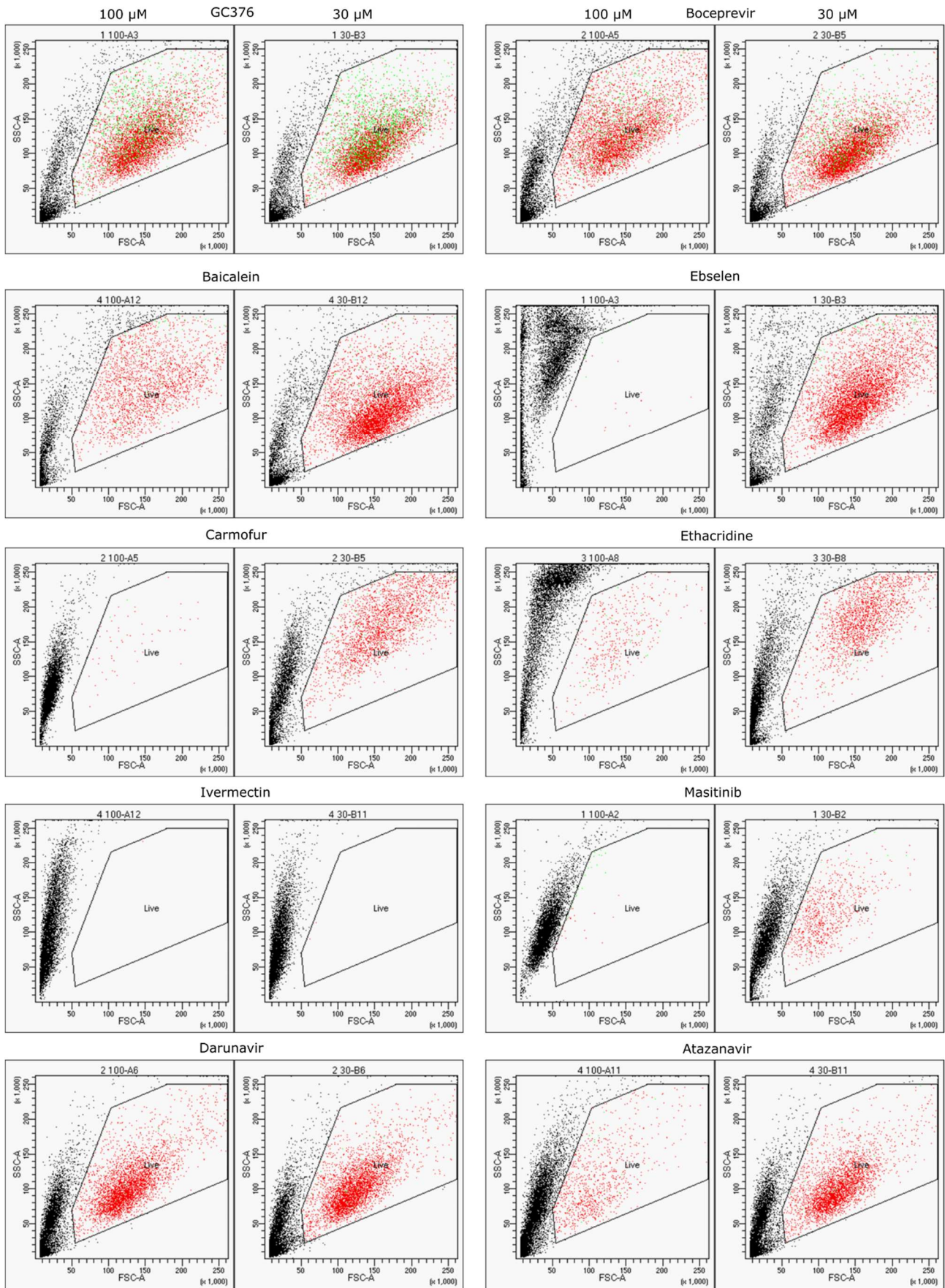
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NVQLRVIGHSMQNCV LKLVDTANPKTPKYK FVRIQPGQTFSVLACYNGSPSGVYQCAMRPNHTIKGSFLNGSCG SVGF  
NIDYDCVSFCYMHMELPTGVHAGTDLEGNFYGPFVDRQTAQAAGTDTTITVNVLAWLYAAVINGDRWFLNRFTTTL  
NDFNLVAMKYNYEPLTQDHVDILGPLSAQTGIAVLDMCASL KELLQNGMNGRTILGSALLEDEFTPFDVVRQCSGVTFQ  
SAVKRTI

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1          10          20          30          40
Consensus      S I T S A V L Q S G L R K M A X P S G K V E X C M V Q V T Y G S T T L N G L W L D D T V Y C P R H
Poliovirus     T I R T A K V Q Q P G F D Y A V A M A K R N I V T A T T S K G E F T M L G V H - D N V A I L P T H
Human coronavirus 229E V S Y G S T L Q A G L R K M A P S G F V E C V R V C Y G N T V L N G L W L G D V Y C P R H
Human coronavirus NL63 I S Y N S T L Q S G L K M A P S G V E C V R V C Y G S T V L N G W L G D T V T C P R H
Rousettus bat coronavirus HKU9 S V A S A L Q A G L T F M A P S G L V E C V K V N Y G S M T L N G W L D N F V I C P R H
SARS-CoV-1      S I T S A V L Q S G R K M A P S G K V E C M V Q V T G T T L N G L W L D D T V Y C P R H
SARS-CoV-2     I T S A V L Q S G F R K M A P S G K V E C M V Q V T G T T L N G L W L D D V Y C P R H
Mouse hepatitis virus S V T T S F L Q S G I V K M V S P T S K V E C I V S V T Y G N M T L N G L W L D D V Y C P R H
MERS           S I T S G V L Q S G L K M S H P S G D V E C M V Q V T G S M T L N G L W L D N T V W C P R H
50          60          70          80          90
Consensus      V I C S S X D M L X P D Y X X L L I R M X N H N F S V - - - X X G X X X L R V X G H T M Q G C L L
Poliovirus     A - - - - - S P G E S I V I D G K E V E I - - - L D A K A L E D Q A G T N L E I T I I
Human coronavirus 229E V I A S N T T S A - I D Y D H E Y S I M R L H N F S I - - - I S G T A F L G V V G A T M H G V T L
Human coronavirus NL63 V I A P S T T V L - I D Y D H A Y S T M R L H N F S V - - - S H N G V F L G V V G T M H G S V L
Rousettus bat coronavirus HKU9 V M C S R D E L A N P D Y P R L S M R A A N Y D F H V - - - S O N G H N R V I G H T M E G S L L
SARS-CoV-1      V I C T A E D M L P N Y E D L L I R K S N H F L V - - - Q A G N V Q L R V I G H M Q N C L L
SARS-CoV-2     V I C T S D M L P N Y E D L L I R K S N H N F L V - - - Q A G N V Q L R V I G H M Q N C V L
Mouse hepatitis virus V I C S S A D M T P D Y P N L L R V T S D F C V - - - M S G R M S L T V M S Y M Q G C Q L
MERS           V M C P A D Q L S D P N Y D A L L I S M T N H S F S V Q K H I G A P A N L R V V G H M Q G T L L
100         110         120         130         140
Consensus      K L K V D X X N P K T P K Y X F X X X K P G Q T F S V L A C Y N G X P S G V F X V X M R P N X T I
Poliovirus     T L K R N E - - - - - K F R D I R P H I P T Q I T E T N D G V L - - I V N T S K Y P N M Y V
Human coronavirus 229E K I K V S Q T N M H T P R H S F R T L K S G E G F N I L A C Y D G C A C G V F G V M R T N W T I
Human coronavirus NL63 R I K V S Q S N V H T P K H V F K T L K P G D S F N I L A C Y G I A S G V F G V L R T N F T I
Rousettus bat coronavirus HKU9 K L T V D V N N P K T P Y F I R V S T G Q A M S L L A C Y G L P G V Y T C T L R S N G T M
SARS-CoV-1      R L K V D T S N P K T P K Y K F V R I Q P G Q T F S V L A C Y N G P S G V Y Q C A M R P N H T I
SARS-CoV-2     K L K V D T A N P K T P K Y K F V R I Q P G Q T F S V L A C Y N G P S G V Y Q C A M R P N T I
Mouse hepatitis virus V L T V T L Q N P T P K Y F G V V K P G T F T V L A A Y N G R P G A F H V T L R S S H T I
MERS           K L T V D V A N P S T P Y T F T T V K P G A A F S V L A C Y N G R P T G T F T V M R P N Y T I
150         160         170         180         190
Consensus      K G S F L N G S C G S V G Y N J D G - D X V X F C Y M H Q M E L P T G X H T G T D F X G X F Y G P
Poliovirus     P - - - - V G A V T E Q G Y L N L G G R Q T A R T L M Y N F P T R A G Q C G G V - - - - -
Human coronavirus 229E R G S F I N G A C G S G Y N L K N - G E V F Y M H Q I E L G S G H V G S S F D G V M Y G G
Human coronavirus NL63 K G S F I N G A C G S G Y N V R N D G T V F C Y L H Q E L G S G A H V G S D F T G S V Y G N
Rousettus bat coronavirus HKU9 R A S F L G S C G S G F Y M N G - K E V F C Y L H Q E L P N G H T G T D F G V F Y G P
SARS-CoV-1      K G S F L N G S C G S V G N I D Y - D C V F C Y M H M E L P T G V H A G T D L E G K F Y G P
SARS-CoV-2     K G S F L N G S C G S V G N I D Y - D C V F C Y M H M E L P T G V H A G T D L E G N F Y G P
Mouse hepatitis virus K G S F L G S C G S V G Y V L T G - D S V R F Y M H Q E L S T G H T G T D F S G N F Y G P
MERS           K G S F L G S C G S V G Y T K E G - S V I N F C Y M H Q M E L A N G H T G S A F D G T M Y G A
200         210         220         230         240
Consensus      F X D X Q V X Q V A X T D T T I T V N V V A W L Y A A X L N G C R W F L X F X T S X X X F N E W
Poliovirus     - - - - - I T C T G K V I G - - - - -
Human coronavirus 229E F E D Q P N L Q V E S A N Q M L T V N V V A F L Y A A I L N G C T W L K G E K L F V E H Y N E W
Human coronavirus NL63 F D D Q P S L Q V E S A N L M L S D N V V A F L Y A A I L N G C R W L C S T R V N V D G F N E W
Rousettus bat coronavirus HKU9 F E D K Q V Q L A A P D C T I T V N V L A W L Y A A V L G E N W F L T K S S I S P A E F N N C
SARS-CoV-1      F V D R Q T A Q A A G T D T T I T N V L A W L Y A A V I N G R W F L N R F T T L N D F N L V
SARS-CoV-2     F V D R Q T A Q A A G T D T T I T V N V L A W L Y A A V I N G R W F L N R F T T L N D F N L V
Mouse hepatitis virus Y R D A Q V Q L P V C D Y T T V N V V A W L Y A A I F N C N W F V Q S D S C S L E E F N V W
MERS           F M D K Q V H Q V Q L T D K Y C S V N V V A W L Y A A I L N G C A W F V K P N R T S V V S F N E W
250         260         270         280         290
Consensus      A M X N G X T S X T X D H V - - L X P L A A K T G V A V E Q X L A A I K X - L H N G F N G K X I L
Poliovirus     - - - - - M H V G N G S H G F
Human coronavirus 229E A Q A N G F T A M N G E D A - - - F S I L A A K T G V C V E R L L H A I Q V - L N N G F G K Q I L
Human coronavirus NL63 A M A N G T S V S V E C - - - Y S I L A A K T G V V E Q L A I O H - L H E G F G K N I L
Rousettus bat coronavirus HKU9 A V K Y M C Q S V T S E S L Q V L G P L A A K T G I S V E R M L S A L K V L L S A G F C G R T I M
SARS-CoV-1      A M K Y N Y E P L T C D H V D I L G P L S A Q T G I A V L D M C A A L K E L L Q N G N G R T I L
SARS-CoV-2     A M K Y N Y E P L T C D H V D I L G P L S A Q T G I A V L D M C A S L K E L L Q N G N G R T I L
Mouse hepatitis virus A M T N G F S S I K A D L V - - L D A L A S M T G V T V E Q V L A A I K R - L H S G F Q G K Q I L
MERS           A L A N Q F T E F V G T Q S - - V D M L A V K T G V A I E Q L Y A I Q Q - L Y T G F Q G K Q I L
300         310         320         326
Consensus      G S X S L E D E F T P X D V V R Q M S G V X L Q S X X X X X I K
Poliovirus     A A A L K R S Y F T Q S - - - - - Q G E I Q W M R P
Human coronavirus 229E G Y S S L N D E F S I N E V V K Q M G V N L Q S G K T T S M F
Human coronavirus NL63 G Y S S L C D E F T L A E V V K Q M G V N L Q S G K V I F G L
Rousettus bat coronavirus HKU9 G S C S L E D E H T P Y D I G R Q M G V K L Q G K F Q S M F R
SARS-CoV-1      G S T I L E D E F T P D V V R Q S G V T F Q G K F K K I V K
SARS-CoV-2     G S A L L E D E F T P D V V R Q S G V T F Q S A V K R T I
Mouse hepatitis virus G S C V L E D E - T P S D V Y Q Q L A G V K L Q S K R T R V I K
MERS           G S T M L E D E F T P E D V N M Q I M G V V M Q S G V R K V T Y

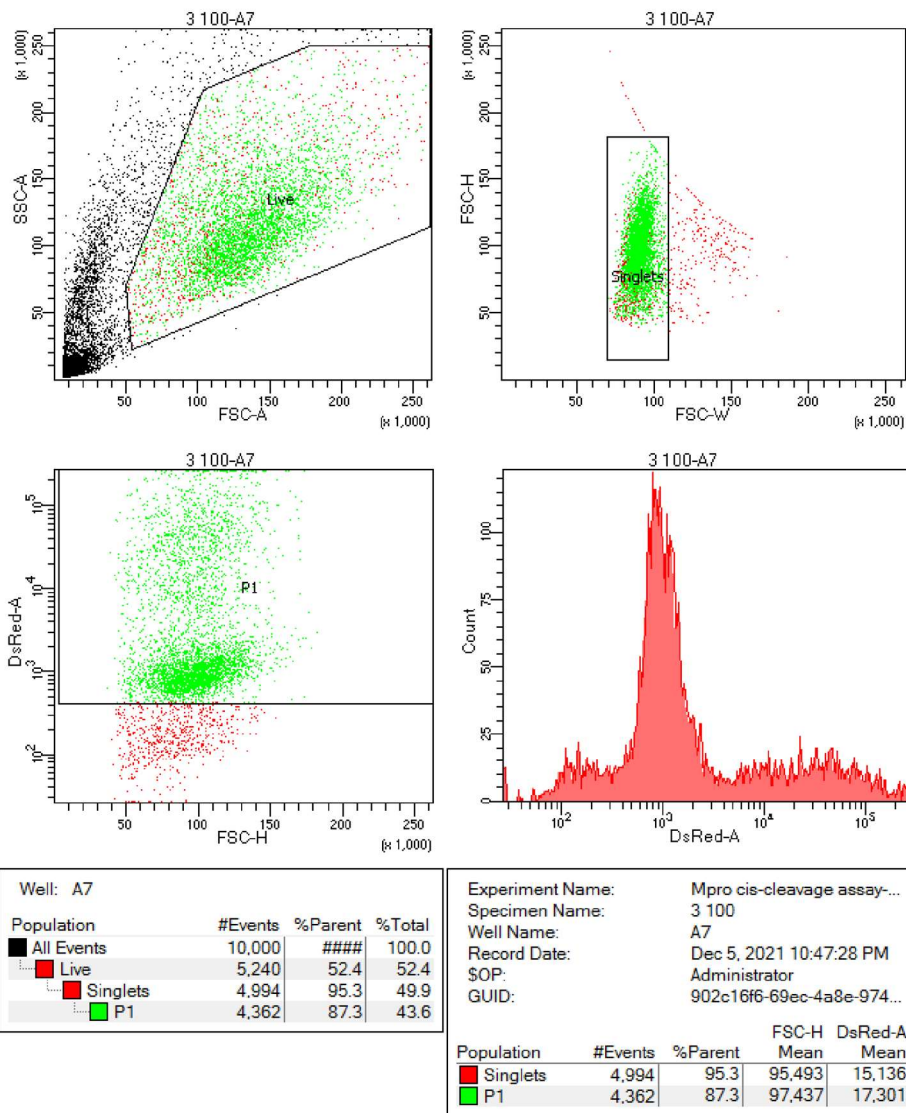
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**Supplementary Figure 3:** Amino acid sequence alignment of main proteases of Poliovirus, Human coronaviruses 229E and NL63, Rousettus bat coronavirus HKU9, SARS-Cov-1 and -2, mouse hepatitis virus and MERS.

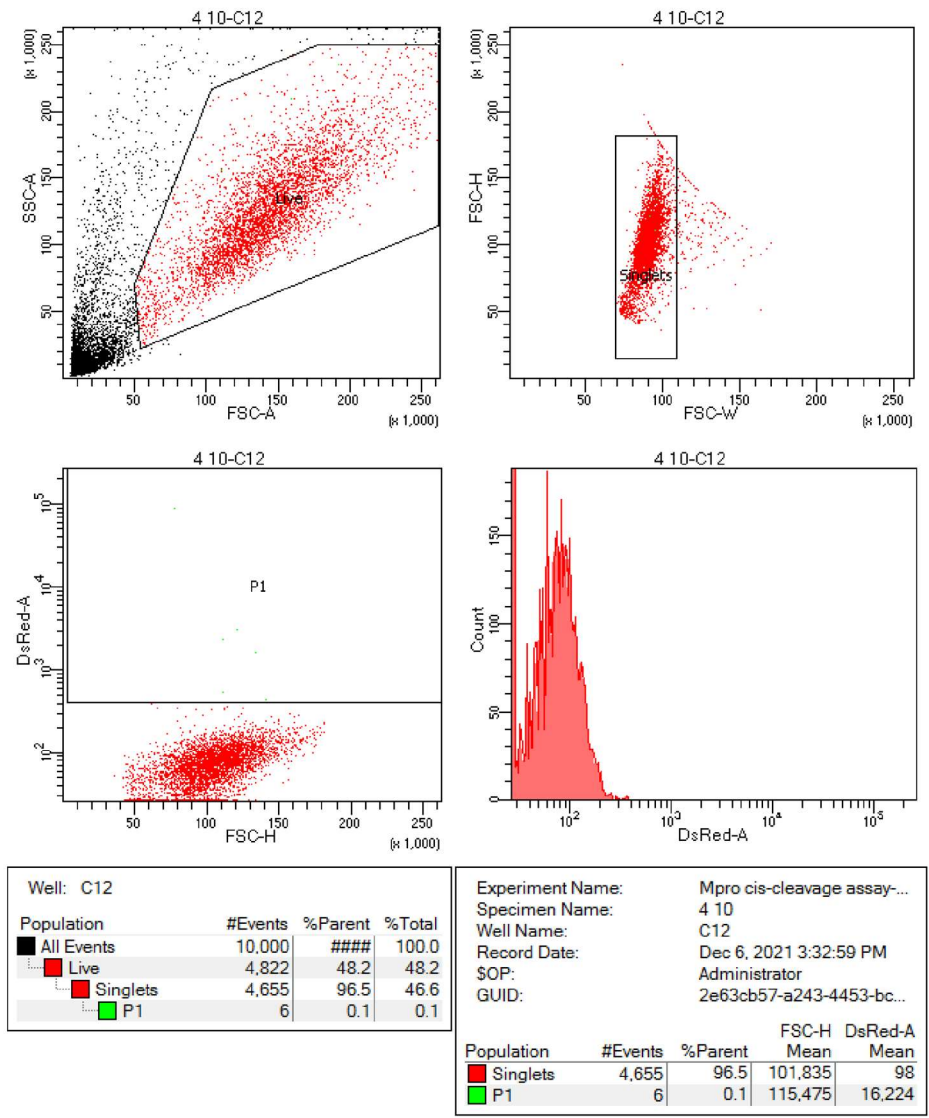


**Supplementary Figure 4:** Gating on live cells treated with different compounds and infected with VSV- $\Delta$ P-dsRed. One representative replicate of 100 and 30  $\mu$ M samples are shown. Red dots indicate below cut-off for following dsRed gate. Green dots indicate dsRed positivity in following gate (see also **Figure S5 and 6**).

BD FACSDiva 8.0.1

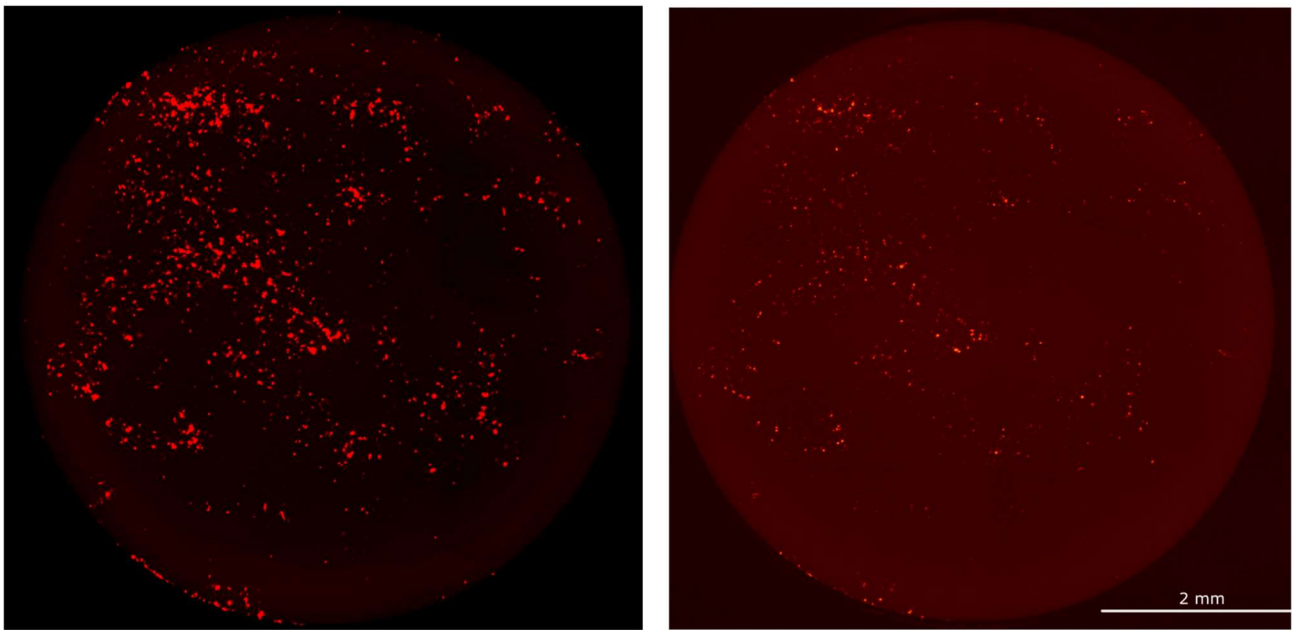
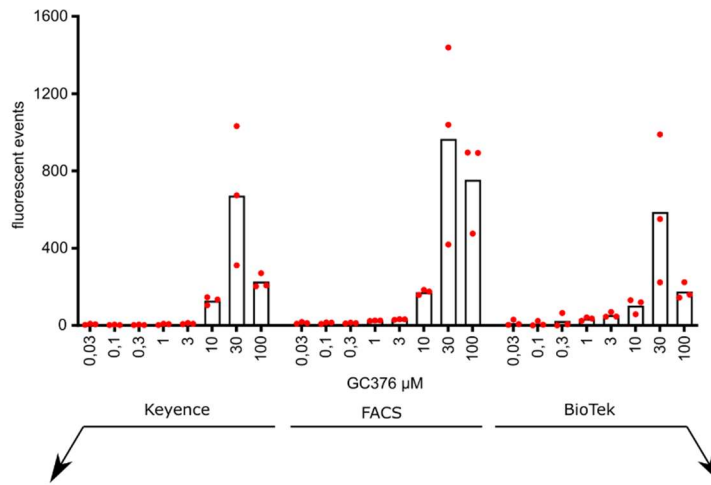


**Supplementary Figure 5:** Exemplary FACS read-out and gating strategy of VSV-ΔP-dsRed infected BHK-21 cells treated with 100 μM PF-07321332. DsRed-A Mean and P1 Events were used for quantitative read-outs. Red dots indicate below cut-off in dsRed gate. Green dots indicate dsRed positive cells.

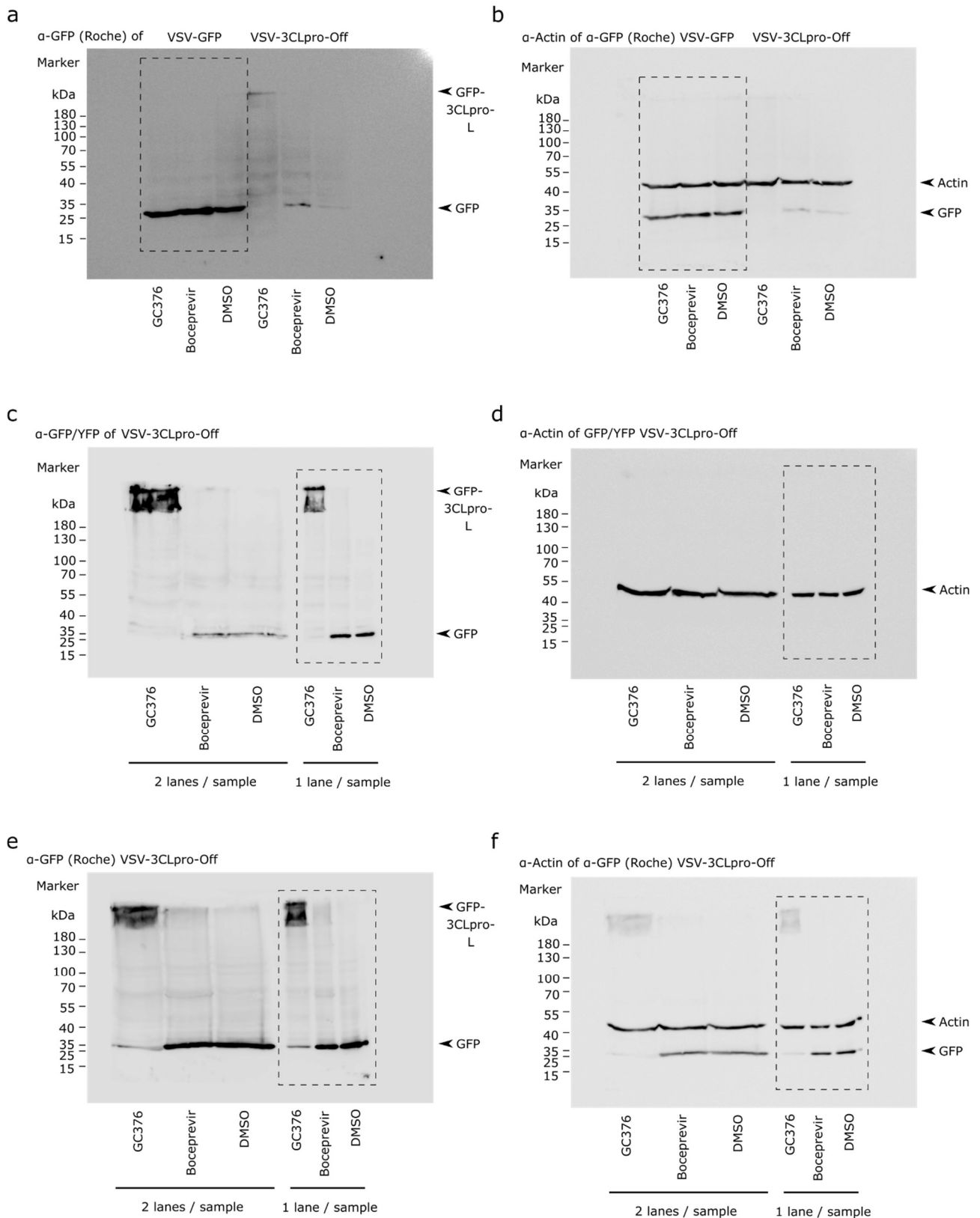


**Supplementary Figure 6:** Exemplary FACS read-out and gating strategy of VSV-ΔP-dsRed infected, but untreated BHK-21 cells. DsRed-A Mean and P1 Events were used for quantitative read-outs. Red dots indicate below cut-off in dsRed gate. Green dots indicate dsRed positive cells.





**Supplementary Figure 7:** Comparison of different fluorescent-based read-out methods with Rousettus bat coronavirus HKU9 main protease gain-of-signal BHK-21 cells (n=3 biologically independent replicates per condition). Representative images of the same 30  $\mu\text{M}$  GC376 well was chosen for comparison of camera sensitivity between Keyence and BioTek readers.



**Supplementary Figure 8:** **a:** Mouse anti-GFP (antibody from Roche) immunoblot of cells infected with VSV expressing GFP or VSV expressing 3CLpro-Off treated with either 50  $\mu$ M GC376, 100  $\mu$ M Boceprevir or DMSO. **b:** Unstripped mouse anti-actin blot of mouse anti-GFP immunoblot in panel a. **c:** Rabbit anti-GFP/YFP immunoblot of cells infected with VSV expressing 3CLpro-Off treated with either 50  $\mu$ M GC376, 100  $\mu$ M Boceprevir or DMSO. In the left side of the blot, 2 lanes were joined for each sample to load a larger sample volume. **d:** Unstripped mouse anti-actin blot of rabbit anti-GFP/YFP immunoblot in panel c. **e:** Mouse anti-GFP (antibody from Roche) immunoblot of cells infected with VSV expressing 3CLpro-Off treated with either

50  $\mu$ M GC376, 100  $\mu$ M Boceprevir or DMSO. In the left side of the blot, 2 lanes were joined for each sample to load a larger sample volume. **f**: Unstripped mouse anti-actin blot of mouse anti-GFP (Roche) immunoblot in panel e. Dashed rectangles indicate which part of the blots was used for **Supplementary Figure 2**.