

# Genetic Screen for Monitoring Severe Acute Respiratory Syndrome Coronavirus 3C-Like Protease

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**A novel coronavirus (SCoV) is the etiological agent of severe acute respiratory syndrome. Site-specific proteolysis plays a critical role in regulating a number of cellular and viral processes. Since the main protease of SCoV, also termed 3C-like protease, is an attractive target for drug therapy, we have developed a safe, simple, and rapid genetic screen assay to monitor the activity of the SCoV 3C-like protease. This genetic system is based on the bacteriophage lambda regulatory circuit, in which the viral repressor cI is specifically cleaved to initiate the lysogenic-to-lytic switch. A specific target for the SCoV 3C-like protease, P1/P2 (SAVLQ/SGFRK), was inserted into the lambda phage cI repressor. The target specificity of the SCoV P1/P2 repressor was evaluated by coexpression of this repressor with a chemically synthesized SCoV 3C-like protease gene construct. Upon infection of *Escherichia coli* cells containing the two plasmids encoding the cI, SCoV P1/P2-cro and the  $\beta$ -galactosidase–SCoV 3C-like protease constructs, lambda phage replicated up to 2,000-fold more efficiently than in cells that did not express the SCoV 3C-like protease. This simple and highly specific assay can be used to monitor the activity of the SCoV 3C-like protease, and it has the potential to be used for screening specific inhibitors.**

The recently identified severe acute respiratory syndrome (SARS) coronavirus (CoV) (SCoV) (5, 9, 12, 19) causes a life-threatening highly contagious pneumonia and is the most pathogenic human CoV identified so far. This disease was first recognized in southern China in November 2002. By August 2003, 8,422 cases had occurred in 29 countries and 908 individuals had died from the disease ([http://www.who.int/csr/sars/country/en/country2003\\_08\\_15.pdf](http://www.who.int/csr/sars/country/en/country2003_08_15.pdf)). Its rapid transmission and the high mortality (10%) make SARS a potential global threat. Recent reports of several SARS cases show that new SARS outbreaks are possible in the near future (<http://www.who.int/csr/don/en/>). To date, neither a vaccine nor an effective therapy is available.

The activity of specific proteases is essential in many fundamental cellular and viral processes. Viral polyprotein processing is indispensable in the replication and maturation of many viruses (6). Consequently, site-specific proteolysis has been an attractive target for the development of antiviral therapies based on potent and selective viral inhibitors. The generation of such therapies based on the inhibition of site-specific proteolysis has been clearly illustrated in the development of effective inhibitors of human immunodeficiency virus type 1 (HIV-1) (10, 30) and hepatitis C virus (HCV) (13).

CoVs are large, enveloped, plus-strand RNA viruses, which have the largest genomes of all RNA viruses (11). The SCoV genomic RNA is nearly 30 kb and is capped and polyadenylated (14, 21, 22). The primary translation product of the viral RNA is largely processed into multiple proteins by the viral main protease, also called 3C-like protease (Fig. 1) to indicate the similarity of its cleavage site specificity to that observed for

picornavirus 3C protease (1). The SCoV 3C-like protease has a molecular mass of nearly 35 kDa (7, 24, 31) and, like other CoV 3C-like proteases, has specificity for Gln at the P1 position (2). Recently, the crystal structure of the SCoV 3C-like protease has revealed that the protein fold can be described as a serine protease, but with a Cys-His at the active site (31).

It has been demonstrated that a bacteriophage lambda-based genetic screen can be used to isolate and characterize site-specific proteases (25). We have previously adapted this system, illustrated in Fig. 2, to study the HIV-1 and HCV proteases (3, 15, 16). This genetic screen system is based on the bacteriophage lambda cI-cro regulatory circuit, where the  $\lambda$ -



FIG. 1. Amino acid sequence of the SCoV 3C-like protease engineered in the present study. The autocleavage sites of the protease are marked with vertical arrows above the sequences. The cleavage site used as a target site in the genetic screen described here is shaded. Underlined are the catalytic-site residues Cys145 and His41.

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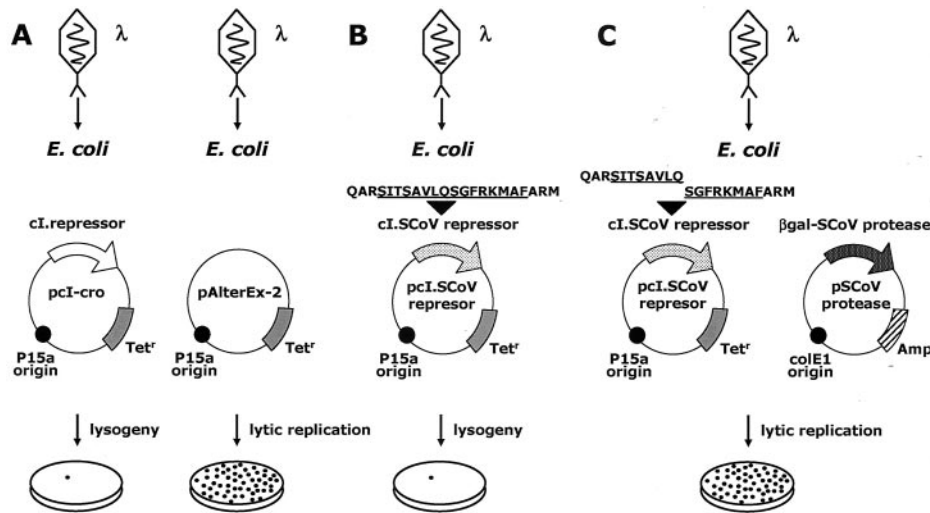


FIG. 2. Lambda-based genetic screen to monitor the activity of SCoV 3C-like protease. This genetic screen system is based on the bacteriophage lambda *cI*-cro regulatory circuit, where the viral repressor *cI* is specifically cleaved to initiate the lysogenic-to-lytic switch. (A) Expression of the phage-encoded repressor (*cI*) results in repression of the bacteriophage's lytic functions (lysogeny). (B) SCoV target repressor containing the P1/P2 cleavage site; as illustrated in Fig. 3 and 4, this repressor efficiently represses the infecting phage (lysogeny). (C) When phages infect *E. coli* cells that express recombinant *cI*.SCoV repressor and  $\beta$ -Gal-SCoV 3C-like protease, infection results in lytic replication.

encoded repressor *cI* is specifically cleaved to initiate the lysogenic-to-lytic switch (20). The inherent difficulties and safety requirements for the ex vivo propagation of SCoV prompted us to explore this genetic system as a simple alternative approach for the characterization of SCoV 3C-like protease activity. In this report, we demonstrate that the lambda-based genetic screen system can be used to monitor the activity of the SCoV 3C-like protease.

We chemically synthesized the SCoV 3C-like protease gene (Fig. 1) from synthetic oligonucleotides as chemical building blocks without employing any viral component formed in vivo

or ex vivo (4, 27). The strategy of synthesizing the SCoV 3C-like protease was as follows. Three overlapping DNA fragments of 340, 340, and 268 bp were combined by PCR, using the Overlap Extension protocol (23), to obtain the full-length SCoV 3C-like protease. Each of the former DNA fragments was synthesized by assembling eight purified oligonucleotides (average length, 60 nucleotides [nt]) of plus and minus polarities with an overlapping complementary sequence of 20 nt at their termini (Table 1). Synthetic oligonucleotides were assembled in an asymmetric PCR assay previously described (18) and were designed to synthesize the SCoV 3C-like protease gene

TABLE 1. Synthetic oligonucleotides for engineering the full-length SCoV 3C-like protease used in this work

Name	Sequence (5'-3')	nt <sup>a</sup>	Orientation
S1	TCTGCTGTCTGCAGAGTGGTTTTAGGAAAATGGCATTCCCGTCAGGCCAAAGTTGAAGGG	-15-45	Sense
S2	CATTAAGAGTTGTAGTTCACAGGTTACTTGTACCATGCACCCCTCAACTTTGGCTGACG	26-85	Antisense
S3	TGGAACACAACTCTTAATGGATTGTGGTTGGATGACACAGTATACTGTCCAAGACATGT	66-125	Sense
S4	TTCATAGTTAGGATTAAGCATGTCTTCTGCTGTGCAAATGACATGTCTTGGACAGTATAC	106-165	Antisense
S5	TGCTTAATCCTAACTATGAAGATCTGCTCATTGCGCAAATCCAACCATAGCTTTCTTGTT	146-205	Sense
S6	ATAGAATGGCCAATAACACGAAGTTGAACATTGCCAGCCTGAACAAGAAAAGCTATGGTTG	186-245	Antisense
S7	CGTGTATTGGCCATTCTATGCAAAAATTGTCTGCTTAGGCTTAAAGTTGATACTTCTAAC	226-285	Sense
S8	CAGGTTGGATACGGACAAATTTATACTTGGGTGTCTTAGGGTTAGAAGTATCAACTTTAA	266-325	Antisense
S9	ATTTGTCCGTATCCAACCTGGTCAAACATTTTCAGTTCTAGCATGCTACAATGGTTTACC	306-365	Sense
S10	GGTATGATTAGGTTCATGGCACACTGATAAACACAGATGGTGAAACATTGTAGCATGC	346-405	Antisense
S11	CCATGAGACCTAATCATACCATTAAAGGTTCTTTCTTAAATGGATCATGTGGTAGTGTG	386-445	Sense
S12	ATATAGCAGAAAAGACGCAATCATAATCAATGTTAAAACCAACTACCACATGATCCA	426-485	Antisense
S13	TGCGTGTCTTTCTGCTATATGCATCATATGGAGCTTCCAACAGGAGTACACGCTGGTACT	466-525	Sense
S14	GTCTGTCAACAAATGGACCATAGAATTTACCTTCTAAAGTCAGTACCAGCGTGTACTCTG	506-565	Antisense
S15	TGGTCCATTTGTTGACAGACAAACTGCACAGGCTGCAGGTACAGACACAACCATAACATT	546-605	Sense
S16	ACCATTGATAACAGCAGCATAACAGCCATGCCAAAACATTTAATGTTATGGTTGTGTCTGT	586-645	Antisense
S17	ATGCTGTGTATCAATGGTGATAGGTGGTTTCTTAAATGATGATGACACTACTTTGAATG	626-685	Sense
S18	AAAGGTTTCATAGTTGTACTTCATTGCCACAAGGTTAAAGTCATTCAAAGTAGTGGTGAAT	666-725	Antisense
S19	AAGTACAACATGAACCTTTGACACAAGATCATGTTGACATATTGGGACCTTTTCTGCT	706-766	Sense
S20	TCAAAGCAGCACACATATCTAAGACGGCAATTCCTGTTTGAGCAGAAAAGAGGTTCCCAATA	746-805	Antisense
S21	AGATATGTGTGCTGCTTTGAAAGAGCTGCTGCAGAATGGATGATGACACTACTATCCT	786-845	Sense
S22	ATCAAATGGTGTAAACTCATCTTCTAAAATAGTGCTACCAAGGATAGTACGACCATTTCAT	826-885	Antisense
S23	ATGAGTTTACACATTTGATGTTGTTAGACAATGCTCTGGTGTACCTTCCAAGGTAAGTTCAAGAAA	866-948	Sense
SProR	GGGAGGGGGCTCGAGTCAATTTCTTGAACCTTACCTTG <sup>b</sup>	916-948	Antisense

<sup>a</sup> Numerical position on the SCoV 3C-like protease.

<sup>b</sup> Underlining indicates an XhoI restriction site.

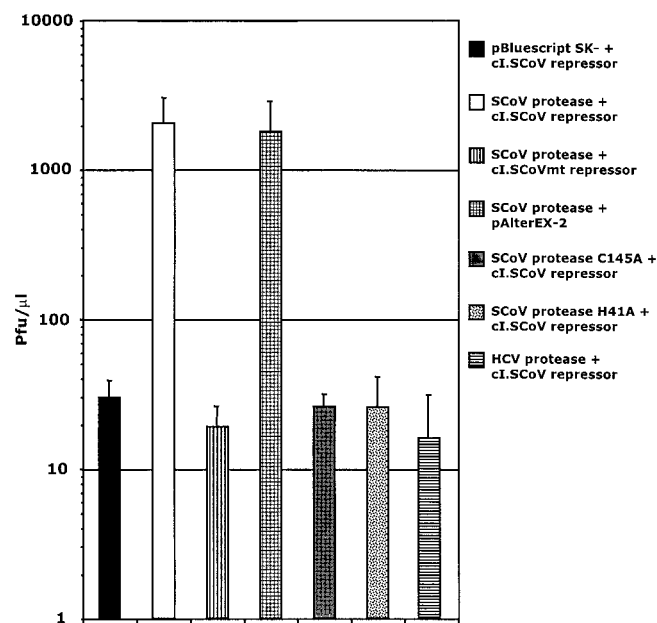


FIG. 3. Selective growth of  $\lambda$  in *E. coli* cells coexpressing the  $\beta$ -Gal-SCoV 3C-like protease construct and the cI.SCoV repressor. Expression of the protease was induced with IPTG for 1 h, and the cells were infected with  $\lambda$  for three additional hours. The graph illustrates the resulting phage titer per microliter. Plasmids pBSK- and pAlterEX-2 were used as controls for the  $\beta$ -Gal-SCoV 3C-like protease construct and the cI.SCoV repressor, respectively. cI.SCoVmt was also used as a negative control for the cI.SCoV repressor. As shown, selection in cells coexpressing the  $\beta$ -Gal-SCoV 3C-like protease construct and the cI.SCoV3 repressor resulted in  $\lambda$  replication, whereas the replication of  $\lambda$  was severely compromised in cells expressing the mutant cI.SCoVmt repressor. Lack of phage replication was also observed in cells expressing mutated forms of  $\beta$ -Gal-SCoV 3C-like protease that included catalytic-site residue substitutions C145A and H41A. Similarly, expression of another protease (HCV serine protease) also prevented phage replication. Values are the means  $\pm$  standard deviations (error bars) of at least four experiments.

reported by Anand et al. (2). Next, the full-length SCoV 3C-like protease gene was reamplified by PCR with oligonucleotides SProL (sense, 5'GGGTTGAATTCGCTGTTCTGCA GAGT 3'; underlining indicates an EcoRI restriction site) and SProR (antisense; Table 1), digested with EcoRI and XhoI, and cloned into pBluescript SK(-) (pBSK-; Stratagene) to generate the  $\beta$ -galactosidase ( $\beta$ -Gal)-SCoV 3C-like protease fusion (Fig. 2). Sequence analysis of several full-length  $\beta$ -Gal-SCoV 3C-like protease clones verified the accuracy of these synthetic genes. One of the former clones had exactly the intended sequence (Fig. 1).

By using a unique restriction site (BssH2 site) located in the coding sequence of the linker domain of cI (25), the SCoV 3C-like protease P1/P2 (SAVLO/SGFRK) cleavage site was inserted into the  $\lambda$  cI repressor (cI.SCoV) (Fig. 2B). The oligonucleotides encoding the SCoV proteolytic P1/P2 cleavage site were 5'GTTCAGGCGCGCTTCAATCACTTCTGCTGT TCTGCAGAGTGGTTTTAGGAAAATGGCATTTCGCGC GCATGTTTC3' (sense) and 5'GAACATGCGCGCAATGC CATTTCCTAAAACCACTCTGCAGAACAGCAGAAGT GATTGAAGCGCGCCTGAAC3' (antisense). A control mutant site (SAVLA/SGFRK) was also inserted in the  $\lambda$  cI repressor (cI.SCoVmt). As illustrated (Fig. 3), *Escherichia coli*

JM109 cells expressing these two repressors efficiently repressed the infecting phage.

We next tested the target specificity of the SCoV repressors by coexpressing these repressors with a  $\beta$ -Gal-SCoV 3C-like protease fusion construct. *E. coli* JM109 cells were then co-transformed with plasmids encoding the cI.SCoV repressor and the  $\beta$ -Gal-SCoV 3C-like protease constructs (Fig. 2C). The resulting cells were grown overnight at 30°C in the presence of 0.2% maltose, harvested by centrifugation, and resuspended to an optical density at 600 nm ( $OD_{600}$ ) of 2.0 per ml in 10 mM  $MgSO_4$ . To induce the expression of SCoV 3C-like protease, cells (200  $\mu$ l) were incubated in 1 ml of Luria-Bertani (LB) medium containing 12.5  $\mu$ g of tetracycline, 20  $\mu$ g of ampicillin, 0.2% maltose, 10 mM  $MgSO_4$ , and 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) for 1 h. Thereafter, cell cultures were infected with  $10^7$  PFU of  $\lambda$  phage. After 3 h at 37°C, the titer of the resulting phage was determined by co-plating the cultures with 200  $\mu$ l of *E. coli* XL-1 Blue cells ( $OD_{600} = 2.0$ /ml in 10 mM  $MgSO_4$ ) on LB plates using 3 ml of top agar containing 12.5  $\mu$ g of tetracycline per ml, 0.2% maltose, and 0.1 mM IPTG. After incubation at 37°C for 6 h, the resulting phage plaques were counted for growth scores. As shown in Fig. 3,  $\lambda$  phage replicated up to 2,000-fold more efficiently in cells expressing the cI.SCoV repressor and the  $\beta$ -Gal-SCoV 3C-like protease than in cells that did not express the  $\beta$ -Gal-SCoV 3C-like protease (Fig. 3).

The specificity of this *trans*-cleavage reaction was further demonstrated by the lack of phage replication in cells expressing mutated forms of the SCoV 3C-like protease that included catalytic-site residue substitutions C145A and H41A (Fig. 3). To demonstrate that the engineered cI.SCoV repressor is highly specific for the SCoV 3C-like protease, cells expressing the cI.SCoV repressor were transformed with an HCV serine protease construct. As shown in Fig. 3, the expression of the HCV serine protease did not allow phage replication. Likewise, phage replication was also abolished in cells expressing the control cI.SCoVmt repressor, arguing that cI.SCoV degradation was specifically mediated by the SCoV 3C-like protease.

Finally, a Western blot also demonstrated (Fig. 4) that the expression of the SCoV 3C-like protease resulted in nearly complete cleavage of the cI.SCoV repressor (Fig. 4, lane 1) but had no effect on the control cI.SCoVmt (Fig. 4, lane 4). Expression of proteases that included catalytic-site residue substitutions C145A and H41A (Fig. 4, lanes 6 and 5, respectively) completely abolished the cleavage of cI.SCoV. Furthermore, expression of another protease as the HCV serine protease also abolished wild-type cI.SCoV repressor cleavage (Fig. 4, lane 8).

The genetic screen system used here to monitor the activity of the SCoV 3C-like protease is based on the well-characterized bacteriophage  $\lambda$  lytic-lysogenic cycle (20). When the cI repressor is functional, lytic gene products are silenced and the phage enters a lysogenic phase. Endogenous bacterial protease RecA cleaves the cI repressor at a specific region. cI repressor cleavage allows the expression of *cro* and progression into the lytic replication cycle. This lysogenic-to-lytic switch was previously adapted to develop a genetic screen system for the characterization of the HIV-1 and HCV proteases (3, 15, 16, 25, 26). The simplicity and specificity of this system prompted us to explore this genetic system as a new approach for the charac-

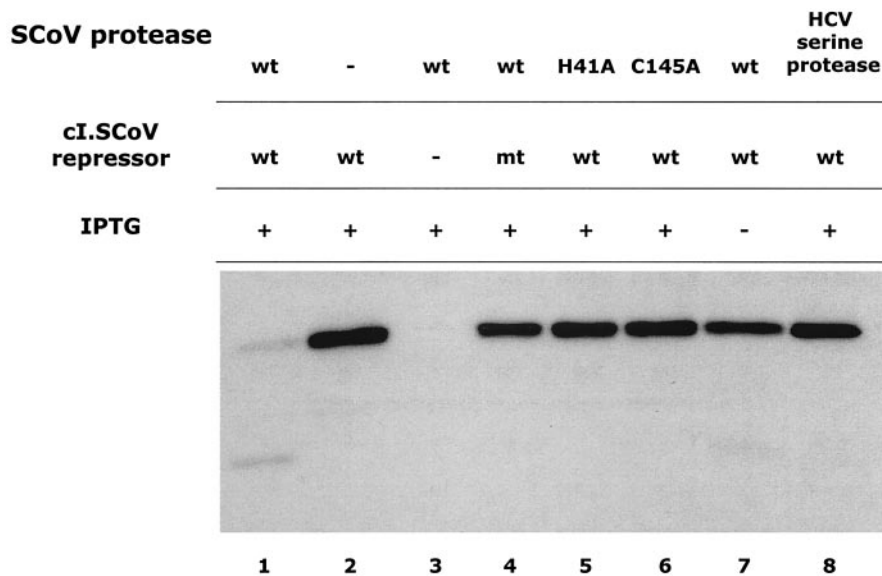


FIG. 4. The SCoV 3C-like protease reduces the expression levels of cI.CoV. The cI.SCoV and cI.SCoVmt (lane 4) repressors were coexpressed with the SCoV 3C-like protease. Expression of the protease was induced with IPTG for 3 h. The ODs of the cultures after 3 h (in the presence of IPTG) were measured to assure that equivalent amounts of total cell protein were blotted. No significant differences were observed when the ODs of the different cultures were compared, suggesting that the expression of the SCoV 3C-like protease did not affect the growth of the bacteria. Control proteases with catalytic residue substitutions C145A and H41A and another protease (HCV serine protease) were also included in this experiment (lanes 5, 6, and 8, respectively). Lane 7 cells were grown in the absence of IPTG. Reduced signal and cleavage products were observed only when the wild-type (wt) SCoV 3C-like protease was expressed (lane 1); cleavage products were also observed in the absence of IPTG, suggesting residual expression of the wild-type SCoV 3C-like protease (lane 7). *E. coli* JM109 cells were cotransformed with pAlterEx-2 repressor plasmids and the pBSK- plasmid containing wild-type or mutated SCoV 3C-like proteases. Cultures were lysed in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer, resolved in 18% gradient SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blocked in phosphate-buffered saline–0.1% Tween 20–10% nonfat dry milk. For immunochemical detection of the cI.SCoV repressor, membranes were subsequently incubated with rabbit serum containing polyclonal anti-cI antibodies (anti-cI sera; Invitrogen). Bound antibodies were visualized with peroxidase-linked anti-rabbit immunoglobulin G (Pierce) and the ECL Plus kit (Amersham Biosciences).

terization of SCoV 3C-like protease activity. Moreover, the different biological properties of the HIV-1, HCV, and SCoV proteases offered us the opportunity to explore whether this system can be used to characterize proteases with different mechanisms of action. Thus, the cI repressor was modified to replace the normal site of RecA-mediated cleavage with a SCoV 3C-like protease target cleavage site (Fig. 2B). The co-expression of the cI.SCoV repressor and the SCoV 3C-like protease resulted in  $\lambda$  phage replication (Fig. 2C, 3, and 4). In contrast, phage replication was efficiently repressed in control cells that did not express the SCoV 3C-like protease (Fig. 3 and 4). Therefore, we demonstrate here that this lambda-based system can be used to monitor the catalytic activity of the SCoV 3C-like protease. This simple assay can augment biochemical approaches to the analysis of this protease.

As mentioned in the beginning of this report, proteases have been an attractive target for developing effective HIV-1 and HCV therapeutics, and this seems to be the case for SCoV. SCoV can be detected at 14 days postexposure in 97% of patients (19); with influenza virus or rhinoviruses, nearly every patient will test negative for the virus at day 5 (29). This protracted period of SCoV replication means that there is a window of opportunity for intervention in SARS with an antiviral. Another reason for the development of in vitro cell-free enzymatic methods for the characterization of the different SCoV proteins is the safety procedures (biosafety level 3 [BSL-3]) required for SCoV ex vivo propagation. Even BSL-3 equip-

ment did not prevent the infection of laboratory researchers with this virus (17). It is important to emphasize that last winter the reported cases of SARS, after the outbreak was contained in July 2003, were due to laboratory contamination of researchers working with SCoV (17). The simplicity of our system can be seen as a complement to the classical biochemical approach for monitoring SCoV 3C-like proteolytic activity. As we and others have previously demonstrated for the HIV-1 protease (3, 15, 26), this system allows the characterization of enzymes with different proteolytic activities. Coupling mutant sequence libraries with this positive genetic selection system will allow the study of a huge number of functional mutants. Mutant proteases may be of interest for characterizing the catalytic properties of the enzyme in the absence or presence of specific inhibitors as well as for predicting the protease inhibitor resistance profile. To perform these experiments using classical biochemical approaches would be difficult and time-consuming.

Recently, a previously undescribed CoV associated with respiratory disease of unknown etiology in humans has been identified (8, 28). Easily, the system developed in this report can be extended to other CoV 3C-like proteases. Here we developed a safe, simple, and rapid genetic screen assay to monitor the activity of the CoV 3C-like protease. This system should be also useful for the development of a screening method to identify SCoV 3C-like protease inhibitors.

**Nucleotide sequence accession number.** The SCoV 3C-like protease nucleotide sequence constructed and used in the present work has been submitted to GenBank database under accession number AY609081.

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