Supplementary Information

Hybrid CRISPR/Cas protein for one-pot detection of DNA and RNA

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2. Materials and methods

2.1. Reagents and materials

MEGAshortscript™ T7 Transcription Kit, SuperScript™ II Reverse Transcriptase (200 U/μL), and nuclease-free water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ribonucleotide Solution Mix (25 mM of each), murine RNase inhibitor (40,000 U/mL), and T7 RNA polymerase (50,000 U/mL) were purchased from New England BioLabs (Ipswich, MA, USA). The QIAamp Viral RNA Mini Kit was purchased from Qiagen (Frederick, MD, USA). The TwistAmp™ Basic Kit was purchased from TwistDx Limited (Maidenhead, UK). All primers, clustered regularly interspaced short palindromic repeats (CRISPR) RNA (crRNA) templates, ssDNA reporter, and ssRNA reporter were synthesized by Bioneer Inc. (Daejeon, Korea). Nucleic acids were quantified individually using Thermo Fisher Nanodrop 2000c spectrophotometers. Fluorescence signals were measured by a Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, USA).

2.2. Viruses and clinical nasopharyngeal samples

The wild-type severe acute respiratory syndrome coronavirus 2 (WT SARS-CoV-2) (NCCP 43326 and NCCP 43330), B.1.617. 2 lineage (delta variant, NCCP 43390), AY.69 lineage (delta variant, NCCP 43409), B.1.351 lineage (beta variant, NCCP 43382), P. 1 lineage (gamma variant, NCCP 43388), and B.1.1.529 lineage (omicron variant, NCCP 43408) were provided by the Korea Disease Control and Prevention Agency and handled in the Biosafety Level 3 facility of the Korea Research Institute of Bioscience and Biotechnology in accordance with the biosafety guidelines. Human coronavirus, influenza A (pH1N1) virus, influenza B virus, parainfluenza virus, and respiratory syncytial virus (RSV) were obtained from the BioNano Health Guard Research Center (H-GUARD) of Korea.

To prepare viral lysates, the virus was mixed with tris(2-carboxyethyl) phosphine

(TCEP) (100 mM final)/ethylene-diamine-tetraacetic acid (EDTA) (1 mM final) and heated at 50 °C for 5 min and 64 °C for 5 min.

Nasopharyngeal samples of flu patients were taken with a swab of the crowded nasopharynx and placed in virus transport media (UTM, Copan Diagnostics Inc., Murrieta, CA, USA). Clinical samples of coronavirus disease 2019 (COVID-19) patients were also taken with a swab and placed in UTM. The samples from WT SARS-CoV-2-infected patients and the negatively diagnosed people were collected from April to June 2020 and the samples from omicron SARS-CoV-2-infected patients were collected during February 2022. PCR results of clinical samples were provided from Gyeongsang National University College of Medicine. This study was reviewed and approved by the Institutional Review Board (IRB) of Gyeongsang National University College of Medicine, Jinju, Korea (IRB approval number: 2020-10-002) and the IRB of Yonsei University Health Service, Severance Hospital, Seoul, Korea (IRB approval number: 4-2020-0465).

2.3. Expression and purification of LbCas12a-SpyCatcher protein

SpyCatcher and Spytag were fused with the C-terminus of LbCas12a (Addgene #113431) and the N-terminus of SUMO-LwCas13a (Addgene #90097), respectively. They were inserted into the pET21a expression vector using standard cloning procedures. The Cas12a-SpyCatcher and SpyTag-SUMO-LwCas13a constructs were verified by Sanger sequencing. LbCas12a-SpyCatcher was expressed in *Escherichia coli* BL21 (DE3) (Invitrogen, Waltham, MA, USA). Briefly, 100 mL of an overnight starting culture in Terrific Broth (TB) medium (12 g/L tryptone, 24 g/L yeast extract, 9.4 g/L K_2HPO, 2.2 g/L KH_2PO_4, Sigma, St. Louis, MO, USA) was inoculated into 8 L of TB and cultured at 37 °C until an OD_{600} of 0.6, followed by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) with a final concentration of 375 μM at 16 °C for 16 h to induce protein expression. The cell pellet was resuspended in a buffer

containing 500 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM TCEP, 5% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.25 mg/ml lysozyme and lysed by sonication (Sonifier 450, Branson, Danbury, CT, USA). Then, the protein was purified using Ni-NTA resin. After cleavage of tobacco etch virus (TEV) cleavage at 4 °C, protein purification was performed by cation exchange chromatography using MBPTrap HP and HiTrap Heparin HP columns (GE Healthcare, Chicago, IL, USA). Finally, the protein was subjected to a gel filtration (Superdex 200, GE Healthcare) in buffer consisting of 200 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM TCEP and 5% (v/v) glycerol.

2.4. Expression and purification of LwCas13a-SpyTag protein

LwCas13a-SpyTag expression in BL21 (DE3) was inoculated with overnight TB starter cultures and induced with 500 μM IPTG for 16 h at 18 °C. The cell pellet was resuspended in a buffer containing 500 mM NaCl, 5% glycerol, 20 mM Tris-HCl, 1 mM PMSF, pH to 8.0, protease inhibitors (Complete Ultra EDTA-free tablets) and lysozyme, lysed by sonication, and centrifuged at 4 °C for 1 h at 10,000 ×g. The LwCas13a-SpyTag was purified with a HiTrap SP HP cation exchange column (GE Healthcare) via fast protein liquid chromatography (FPLC) (AKTA PURE, GE Healthcare) and eluted with a salt gradient from 130 mM to 2 M NaCl in buffer containing 5 mM β-mercaptoethanol, 20 mM Tris-HCl, 5% glycerol, pH to 8.0. The elution fraction was confirmed by SDS-PAGE, concentrated to 5 mL using a centrifugal filter, and buffer changed to dialysis buffer (20 mM Tris-HCl, 150 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol). Using FPLC, the concentrated protein was applied to a gel filtration column (Superdex® 200 Increase 10/300 GL, GE Healthcare) and the resulting protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Finally, the LwCas13a protein was dialyzed against storage buffer containing 50 mM Tris-HCl (pH 7.5), 600 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol and all aliquots were stored at -

80 °C.

2.5. Construction of hybrid Cas protein

Cas12a-SpyCatcher and Cas13a-SpyTag were complexed in a molar ratio of 1:1.5 to confirm binding over time and concentrated via a centrifugal filter unit (100 kDa) to 5 mL. The resulting protein was loaded onto a gel filtration column (Superdex® 200 Increase 10/300 GL) and eluted fraction was verified by SDS-PAGE, and the hybrid Cas protein was buffer-exchanged with a storage buffer.

2.6. crRNA preparation

The crRNA transcription templates for in vitro transcription consisting of the T7 promoter sequence, repeat-derived sequences, and target-specific sequences were synthesized and purchased from Bioneer. In vitro transcription of crRNAs was attempted using the MEGAshortscript^{TM} T7 Transcription Kit. Briefly, the transcription reaction was performed by mixing T7 Reaction Buffer, template DNA, T7 Enzyme Mix, and 7.5 mM each of ATP, UTP, CTP, and GTP and incubated at 37 °C for 4 h. After incubation, the reaction was terminated with DNase and ammonium acetate stop solution. RNA was obtained by phenol/chloroform extraction and alcohol precipitation. Aliquots of RNA were frozen at -80 °C.

2.7. Validation of CRISPR/hybrid Cas system for simultaneous DNA and RNA detection

The CRISPR/hybrid Cas-mediated target cleavage assays were verified using ssDNA and ssRNA reporters. All fluorescence reactions were performed in 96-well microplates. Briefly, the CRISPR/hybrid Cas reaction was performed in a 100 μL reaction volume with 100 nM CRISPR/hybrid Cas, 200 nM Cas12a-crRNA, 300 nM Cas13a-crRNA, 20 nM ssDNA reporter, 400 nM ssRNA reporter, 1 μL murine RNase inhibitor, and varying amounts of input nucleic

acid target fragments in 1× assay buffer (10 mM Tris, 50 mM KCl, 10 mM $MgCl_2$; pH 7.5). The reaction mixtures were quickly transferred to a 96-well plate and then placed in a fluorescence reader and incubated at 37 °C. FAM and Cy3 fluorescence values were simultaneously collected every 5 min for 2 h using a multimode fluorescent plate reader (BioTek). For fluorescence resonance energy transfer (FRET) assays, the excitation wavelength was 470 nm and the emission detection wavelengths were 522 nm (FAM donor fluorescence emission wavelength) and 568 nm (Cy3 acceptor fluorescence emission wavelength). Slits for both excitation and emission were set at 20 nm. Fluorescence images were acquired in multichannel modality (Fluorescence and Cy3 channels) using the ChemiDoc™ MP imaging system (Bio-Rad, Hercules, CA, USA).

2.8. Isothermal amplification of viral RNA

Viral RNA was extracted from various SARS-CoV-2 strains, influenza A virus, influenza B virus, parainfluenza virus, RSV, and human coronavirus using the QIAamp Viral RNA Mini Kit. Reverse transcription-recombinase polymerase amplification (RT-RPA) was performed using a commercial TwistAmp Basic Kit. Briefly, one RPA pellet was hydrated with 29.5 μL of the rehydration buffer, 2.5 μL RPA forward primer (10 μM), 2.5 μL RPA reverse primer (10 μM), 2.5 μL SuperScript™ II Reverse Transcriptase (200 U/μL), 2.5 μL Murine RNase inhibitor (40 U/μL), and 8 μL experimental sample. The amplification reaction was started by adding 2.5 μL of 280 mM magnesium acetate. After incubation at 42 °C for 25 min, 5 μL of RT-RPA products were used for the CRISPR/hybrid Cas reaction. The viral lysates and clinical samples were directly used for RT-RPA without RNA extraction.

2.9. CRISPR/hybrid Cas-mediated viral RNA detection

The CRISPR/hybrid Cas-mediated viral RNA detection was performed in 200 nM

CRISPR/hybrid Cas in storage buffer, 200 nM Cas12a-crRNA, 300 nM Cas13a-crRNA, 20 nM ssDNA reporter, 400 nM ssRNA reporter, 8 μL NTP mix, and 2 μL T7 RNA polymerase (5 U/μL). Next, 5 μL of RT-RPA products was transferred to the CRISPR/hybrid Cas reaction tube. The fluorescence analysis was performed as described above.

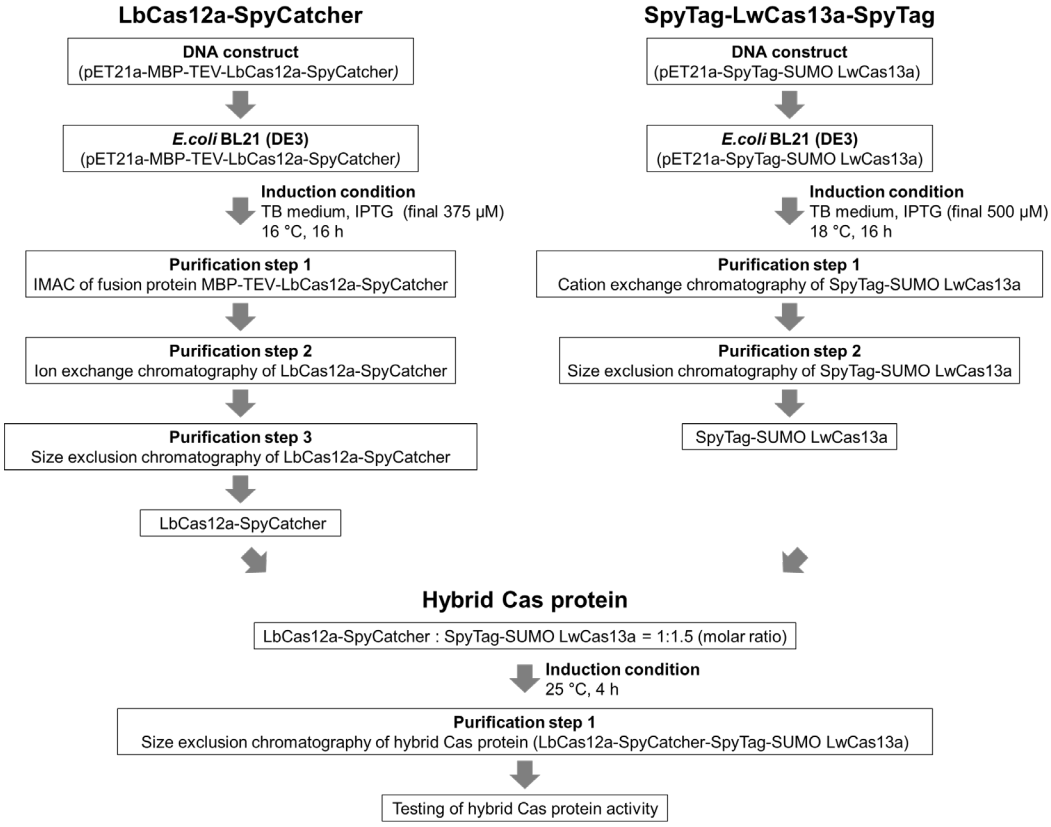

Figure S1. Experimental protocol for hybrid Cas protein

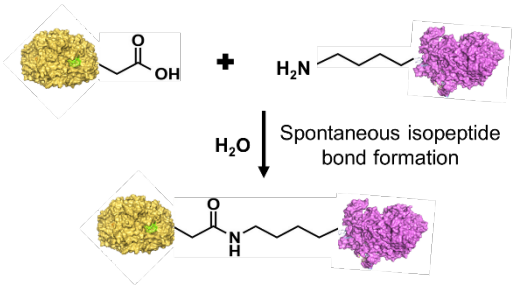

Figure S2. Schematic illustration of hybrid Cas protein formation.

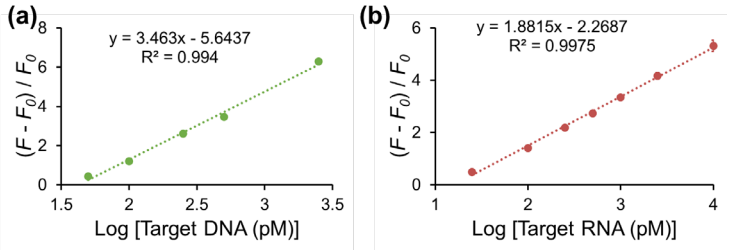

Figure S3. (a,b) Sensitivity of the CRISPR/hybrid Cas system for the detection of target (a) DNA and (b) RNA (n = 3, error bar = standard deviation). *F* and F_0 are fluorescence intensities obtained from the sample and negative control, respectively.

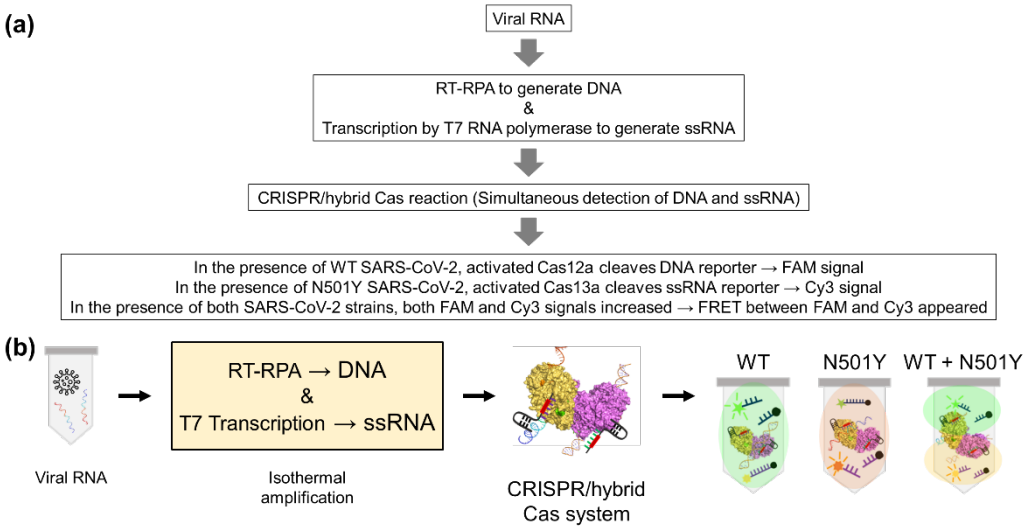

Figure S4. (a) Experimental protocol and (b) detailed schematic illustration of WT and N501Y SARS-CoV-2 identification using the CRISPR/hybrid Cas system.

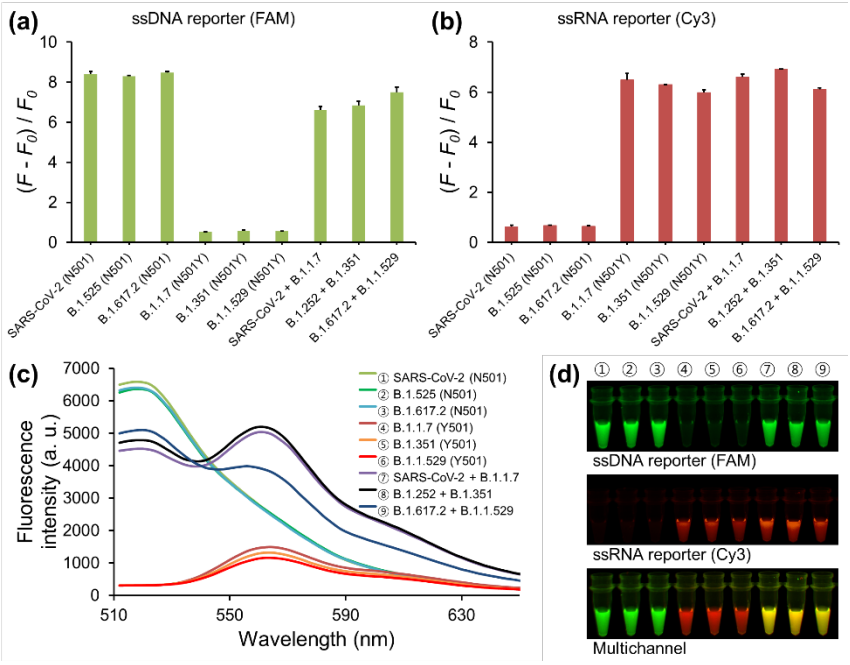

Figure S5. (a, b) $(F - F_0) / F_0$ values of (a) FAM (λ_{ex} = 470 nm and λ_{em} = 522 nm) and (b) Cy3 (λ_{ex} = 522 nm and λ_{em} = 568 nm) after the detection of viral RNA-spiked influenza-positive clinical samples using the CRISPR/hybrid Cas system (n = 3, error bar = standard deviation). F and F_0 are fluorescence intensities obtained from the sample and negative control, respectively. (c) Fluorescence emission spectra (λ_{ex} = 470 nm) after the detection of viral RNA-spiked influenza-positive clinical samples using the CRISPR/hybrid Cas system. (d) Fluorescence images of reaction tubes after the detection of viral RNA-spiked influenza-positive clinical samples using the CRISPR/hybrid Cas system.

(a)

	ssDNA reporter (FAM signal)									
	11	12	13	14	15	16	17	18	19	20
WT	4808	4295	4295	4885	4139	4912	4716	5159	5179	5340
Omicron	221	230	229	195	272	247	257	237	254	232
Mix	2930	2629	2487	2661	2760	2897	2593	2912	2662	2486
Negative	201	221	230	229	195	272	237	232	221	189

	ssRNA reporter (Cy3 signal)									
	11	12	13	14	15	16	17	18	19	20
WT	922	1372	1171	1274	1772	1208	1159	1408	1044	1738
Omicron	7771	8392	8536	7491	8686	7817	7348	7028	8214	7051
Mix	3474	3704	3763	3505	4188	3529	3406	3390	3530	3327
Negative	816	1013	1005	1056	1069	1082	1018	1028	1064	1129

(b)

	Ct Value (E gene)									
	1	2	3	4	5	6	7	8	9	10
WT	31.45	30.73	26.98	24.1	27.29	21.78	29.95	24.34	20.97	28.38
Omicron	21.49	28.99	18.85	19.7	23.47	18.06	19.8	20.1	26.97	26.21

	Ct Value (E gene)									
	11	12	13	14	15	16	17	18	19	20
WT	30.74	30.61	29.94	30.22	29.69	31.23	30.48	29.12	25.4	19.41
Omicron	21.15	20.45	20.27	22.04	18.77	20.99	26.47	27.3	20.64	26.91

Figure S6. (a) Fluorescence intensities of FAM (λ_{ex} = 470 nm and λ_{em} = 522 nm) and Cy3 (λ_{ex} = 522 nm and λ_{em} = 568 nm) after the diagnosis of clinical samples using the CRISPR/hybrid Cas system. (c) PCR results of clinical samples.

Table S1. Oligonucleotide sequences used in this study.

Name	Sequence (5' to 3')
Target DNA (N501)	tatagattgtttaggaagtctaatctcaaacctttgagagagatatttcaactgaaatctat caggccggtagcacaccttgtaatggtgttgaaggttttaattgttactttcctttacaatcat atggtttccaacccactaatggtgttggttaccaaccatacagagtagtagtactttctttg aacttctacatgcacca
Non-target DNA	tatagattgtttaggaagtctaatctcaaacctttgagagagatatttcaactgaaatctat caggccggtagcacaccttgtaatggtgttgaaggttttaattgttactttcctttacaatcat atggtttccaacccacttatggtgttggttaccaaccatacagagtagtagtactttctttg aacttctacatgcacca
Target RNA (Y501)	uauagauuguuuaggaagucuaaucucaaaccuuuugagagagauauuuca acugaaaucuaucaggccgguagcacaccuuguaaugguguugaaggguuua auuguuacuuuccuuuacaaucauaugguuuccaacccacuu[(a)]augguguu gguuaccaaccauacagaguaguaguacuuucuuuugaacuucuacaugcac ca
Non-target RNA	uauagauuguuuaggaagucuaaucucaaaccuuuugagagagauauuuca acugaaaucuaucaggccgguagcacaccuuguaaugguguugaaggguuua auuguuacuuuccuuuacaaucauaugguuuccaacccacuaaugguguugg uuaccaaccauacagaguaguaguacuuucuuuugaacuucuacaugcacca
RPA forward primer	GGTGTTGAAGGTTTTAATTGTTACTTTCCTTTACAATC
RPA reverse primer	TTTAGGTCCACAAACAGTTGCTGGTGCATGTAGAAGTT
Cas12a-crRNA	UAAUUUCUACUAAGUGUAGAU**caacccacuaaugguguugguua**[(b)]
Cas13a-crRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAAC**accaa caccauaaguggguuggaaacca**[(b)]
ssDNA reporter	FAM-TTATT-BHQ1
ssRNA reporter	Cy3-UUUUU-BHQ2

[(a)]Red character represents the mutation sequence.

[(b)]Bold characters represent the target binding sequences