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Coronavirus protease panel anti viral assay using 293T cells

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We use this protocol and it's working

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Abstract

Cell-based fluorescent assay with high sensitivity and low background that reports on the activity of viral proteases, which are key drug targets. Assay is compatible with not only the **SARS**-CoV-2 M^{Pro} protein but also orthologues from a range of human and nonhuman CoVs as well as clinically reported **SARS**-CoV-2 drug-resistant M^{Pro} variants. The assay is run using Cell-based (293T) Panel of transfected CoV proteases. 2 μ M of a P-gp inhibitor is also used.

Candidate perturbagens are screened as part of the assay

The assay is run as a panel to define the breadth of activity against of viral proteases and results reported against this set

Guidelines

Please be sure to wear proper Personal Protective Equipment (PPE) while performing this experiment.

Safety warnings

 Assay is performed under biosafety level 3 conditions.

Plasmids

- 1 The original coronavirus Mpro FlipGFP reporter was described previously (see [ref](#)). The improved coronavirus Mpro FKBP-FlipGFP reporter and pLex-FlipGFP β 1–9 template DNA sequences were generated via gBlock gene synthesis (IDT). Compared to the original reporter, three major changes were made:

This paper provides [more details](#)

- 1.1 An FKBP L106P fusion ORF was added to the N-terminus.
 - 1.2 A second viral protease cleavage site to allow removal of the FKBP protein was added.
 - 1.3 A GSG linker was added before the original 2A site (Supplemental Data).
- 2 Reporters for CoV PLpro, ZIKV NS2B-NS3, and EBV protease BVRF2 were generated containing their respective cleavage sequences but were otherwise identical to the CoV Mpro FKBP-FlipGFP reporter (see Supplemental Data in the paper).

All protease sequences were obtained through NCBI and cloned into pLex expression plasmids using codon-optimized gBlocks (IDT) (Accession numbers: SARS-CoV-2 Mpro, MT020880.1; SARS-CoV-2 PLpro, MN 985325.1; ZIKV NS2B3, KX087101.3; EBV BVRF2, NC_007605.1; SARS-CoV-1, KY417145.1; NL63, DQ445912; MERS-CoV, KU740200.1; OC43, NC_006213, 229E, AGW80947.1; HKU1, ON128612; CDPHE15, NC_022103; HKU8, NC_010438; HKU9, NC_009021; HKU11, NC_011547; HKU19, NC_016994; HKU33, MK720944; Lucheng-19, NC_032730; CHB25, MN611525; SW1, NC_010646; PDCOV, KX022605; IBV, NP_066134.1).

- SARS-CoV-2 (Severe acute respiratory syndrome coronavirus-2)
- SARS-CoV-1 (Severe acute respiratory syndrome coronavirus 1)
- MERS-CoV (Middle East respiratory syndrome coronavirus)
- NL63 (Human coronavirus NL63)
- OC43 (Human coronavirus OC43)
- 229E (Human coronavirus 229E)
- HKU1 (Human coronavirus HKU1)
- CDPHE15 (Bat coronavirus CDPHE15)
- HKU8 (Bat coronavirus HKU8)
- HKU9 (Bat coronavirus HKU9)

- HKU11 (Bulbul coronavirus HKU11)
- HKU19 (Night heron coronavirus HKU19)
- HKU33 (Bat coronavirus HKU33)
- Lucheng-19 (Rat coronavirus Lucheng-19)
- CHB25 (Bat coronavirus CHB25)
- SW1 (Beluga whale coronavirus SW1)
- PDCoV (Porcine deltacoronavirus)
- IBV (Avian infectious bronchitis virus)
- ZIKV (Zika virus)
- EBV (Epstein–Barr virus)

- 3 The CoV Mpro ORFs were cloned as the matured polypeptide sequence with an artificial initiator, methionine. The genes for (or the active protease domains of) the SARS-CoV-2 PLpro, EBV BVRF2, and soluble ZIKV NS2B3 were based on previous expression construct designs (see refs in the paper 49, 53, 82, 83).
- 4 SARS-CoV-2 nirmatrelvir-resistant mutants were all constructed on the USA/WA-1/2020 background. For all clones, PCR products were inserted into the pLex-expression vector using the BamHI and either XhoI or NotI restriction sites and HiFi DNA assembly (New England BioLabs [NEB]). DNA was transformed into chemically competent NEB 5-alpha high-efficiency cells (NEB, Cat. C2987H), and purified plasmids were confirmed using Sanger sequencing.

Cell Culture

- 5 293T cells were originally obtained from American Type Culture Collection (ATCC) and grown at 37°C in 5% CO₂. The 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, GlutaMAX, and penicillin-streptomycin, and kept at a low passage number (<15).

Transfections

- 6 For transfection, plates were poly-L-lysine-treated and seeded with 293Ts. After 24 h, plasmid DNA was combined with Opti-MEM and Lipofectamine 3000 (Invitrogen, Cat. L3000001) and incubated for 10 min at room temperature, before being added dropwise to cells.
- 7 For transfections including drug, a P-glycoprotein inhibitor, CP-100356, was included at the final concentration of 2 μ M to limit efflux of drug from the cells. CP-100356 was resuspended in DMSO, then diluted in medium, and added dropwise to the cells before the transfection mix.
- 8 Then, compounds to be tested were initially resuspended in DMSO to 10 mM, then diluted in medium. This compound/medium or DMSO-vehicle/medium mix was then

added to cells after the addition of the transfection mix to bring the total medium to the intended drug concentration.

Compounds

- 9 P-glycoprotein inhibitor CP-100356 monohydrochloride was purchased from Sigma-Aldrich (Cat. PZ0171). All compounds were resuspended in DMSO, aliquoted, and kept at -20°C .

Imaging and Quantification

- 10 At the intended timepoint, cells were fixed with 2% paraformaldehyde (PFA) at room temperature for 10 min. Cells were then incubated in 1:10,000 Hoechst 33342 (Life Technologies, Cat. H3570) in 1× phosphate-buffered saline at 4°C overnight.
- 11 Images were then obtained using the ZOE fluorescent cell imager (Bio-Rad), and quantification was performed using the Cell Insight CX5 platform (Thermo Fisher Scientific). A compartmental analysis protocol was used on the Cell Insight CX5 to capture the total live cell count stained by Hoechst 33342, as well as the percentage of the well that was GFP positive and the mean intensity of GFP signal.
- 12 The latter parameters were then multiplied and used to calculate the reporter signal. This calculation of reporter signal importantly takes into account both the number of cells that are GFP+ as well as their overall brightness, allowing for increased dynamic range.

Sequence and Structural Analyses

- 13 For phylogenetic analysis, coronavirus Mpro amino acid sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE). A maximum likelihood phylogenetic tree was constructed with IQ-Tree (v1.6.12) using automatic model selection and 10,000 ultra-fast bootstraps.
- 14 Sequence similarity was calculated using the Sequence Manipulation Suite. Multisequence alignments for consensus sequence site alignments were performed using CLUSTALW with shading at 50% identity or similarity on the Boxshade v3.3 software.
- 15 Sequence logo plots were made using WebLogo 3 software. All protein visualization and figure image generation were performed with UCSF Chimera protein modeling software (PDBs: SARS-CoV-2 Mpro in complex with nirmatrelvir, 8DZ2; SARS-CoV-2 PLpro, 7NFV; ZIKV NSB2-NS3, 7VXX; EBV viral protease, 1O6E).

Statistical Analysis and Graphing



- 16 All data were analyzed using GraphPad Prism 8 software, and the statistical analysis used to compare groups is indicated in the figure legends. All experiments were performed in biological replicates of four independent experiments, and significance values were determined using Mann–Whitney U tests.

Protocol references

<https://journals.asm.org/doi/10.1128/jvi.00597-23> A low-background, fluorescent assay to evaluate inhibitors of diverse viral proteases

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