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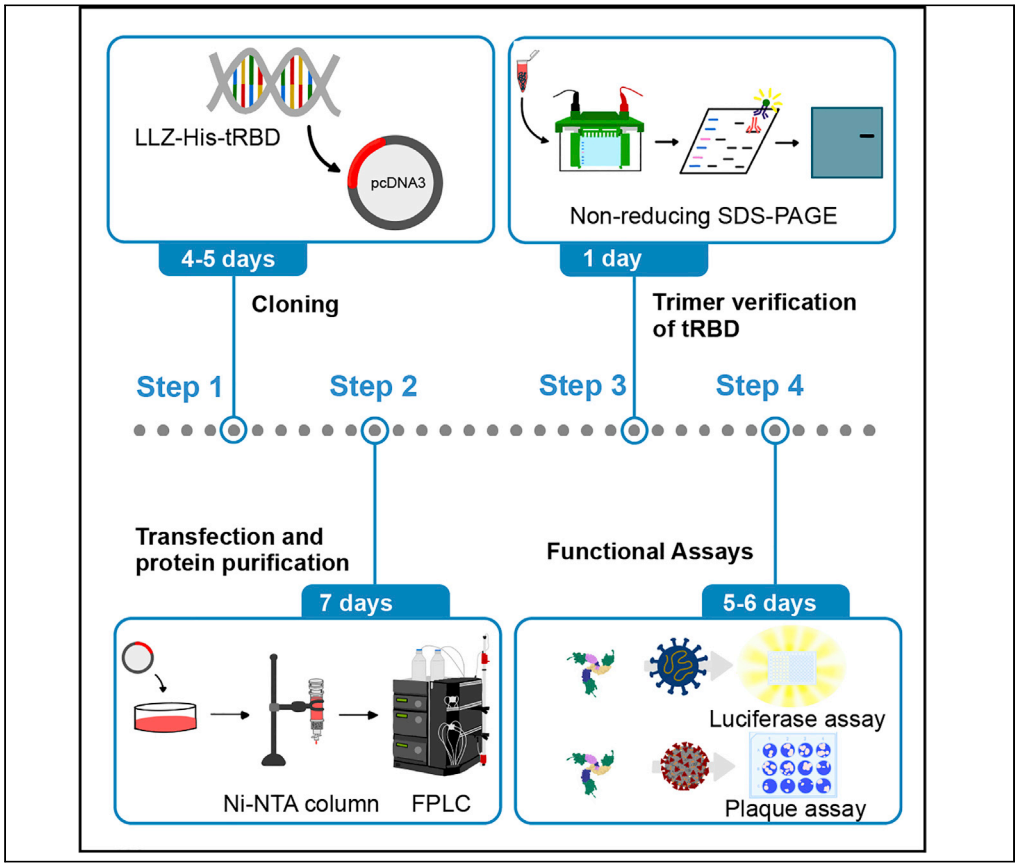
Protocol

Inhibition of SARS-CoV-2 infection in cellular systems using engineered trimeric receptor-binding domain of spike protein



Here, we provide a protocol for the design, expression, purification, and functional studies of an engineered trimeric version of the receptor-binding domain (tRBD) of SARS-CoV-2 spike protein. We describe the use of tRBD to block SARS-CoV-2 spike pseudovirus and true virus binding to cellular angiotensin converting enzyme-2 (ACE2), thereby blocking viral infection. This protocol is applicable to generate a trimeric version of any protein of interest.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Angela Rose Liu, Shrikanth C. Basavarajappa, Nandini Sarkar, Anna Bruchez, Parameswaran Ramakrishnan

pxr150@case.edu

Highlights

Cloning and expression of SARS-CoV-2 RBD as a secreted trimeric protein (tRBD)

Protocol to use tRBD in SARS-CoV-2 pseudovirus neutralization assay

Protocol to use tRBD to block true SARS-CoV-2 infection in plaque assay

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Inhibition of SARS-CoV-2 infection in cellular systems using engineered trimeric receptor-binding domain of spike protein

Angela Rose Liu,[1] Shrikanth C. Basavarajappa,[1] Nandini Sarkar,[1] Anna Bruchez,[1] and Parameswaran Ramakrishnan[1,2,3,4,5,*]

[1]Department of Pathology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

[2]The Case Comprehensive Cancer Center, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

[3]Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

[4]Technical contact: pxr150@case.edu

[5]Lead contact

*Correspondence: pxr150@case.edu
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SUMMARY

Here, we provide a protocol for the design, expression, purification, and functional studies of an engineered trimeric version of the receptor-binding domain (tRBD) of SARS-CoV-2 spike protein. We describe the use of tRBD to block SARS-CoV-2 spike pseudovirus and true virus binding to cellular angiotensin converting enzyme-2 (ACE2), thereby blocking viral infection. This protocol is applicable to generate a trimeric version of any protein of interest.

For complete details on the use and execution of this protocol, please refer to Basavarajappa et al. (2022).[1]

BEFORE YOU BEGIN

This protocol integrates molecular biology, biochemistry and cell biology approaches to engineer a trimeric version of the RBD of SARS-CoV-2 spike protein, and its use to inhibit the cellular entry of the virus. The RBD is the contact region between the SARS-CoV-2 and the host cell ACE2 receptor, an interaction that ultimately allows for infection of the host cell with SARS-CoV-2 virus. The engineered trimeric RBD protein binds with high affinity to host cell ACE2 receptors, thereby competitively inhibiting viral infection.[1] Protocols for plasmid construction, protein expression and purification, as well as functional assays are described in this manuscript.

Institutional permissions

Case Western Reserve University Institutional Biosafety Committee (IBC) approved all procedures involving recombinant DNA and viral infections. University Hospitals IRB approved procedures involving true virus infections.

Prepare gels and buffers for polyacrylamide gel electrophoresis

Prepare to run a 10% polyacrylamide gel as described in the Bio-Rad protocol.

https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf.

For manual preparation of 10% polyacrylamide gels, please refer to -

http://cshprotocols.cshlp.org/content/2015/7/pdb.rec087908.full?rss=1.

Prepare buffers for transfection

2.5 M $CaCl_2$		
Reagent	Final concentration	Amount
$CaCl_2$	2.5 M	27.75 g
MilliQ H_2O	N/A	Up to 100 mL
Total		100 mL
Can be stored at 4°C for up to 6 months.		

2× HEBS buffer (pH between 7.05–7.15)	
Reagent	Amount
NaCl	1.64 g
HEPES	1 g
Na_2HPO_4 $2H_2O$	0.027 g
MilliQ H_2O	Up to 100 mL
Total	100 mL
Store at 4°C for 3 months.	

Prepare glutaraldehyde for protein crosslinking

2.5% glutaraldehyde: dilute 25% glutaraldehyde in MilliQ H_2O.

Preparation SARS-CoV-2 pseudotyped lentivirus

Timing: 3 days

This section accomplishes preparation of pseudotyped virus for functional studies.

In this section, calcium phosphate transfection protocol was used. Detailed instructions are found in 'step by step instructions' section.

Note: The procedure for pseudovirus preparation is shown in Figure 3.

Seed 1.25 × 10^6 HEK-293T cells in 10 mL growth media (DMEM media with 2% v/v heat inactivated FBS) in a 10 cm culture dish the night before.

The next morning, transfect cells with the following plasmids:

1 μg of Luciferase-IRES-ZsGreen lentiviral backbone.

0.22 μg of plasmids HDM-Hgpm2 (NR-52517), pRC-CMV-Rev1b (NR-52519), and HDM-tat1b (NR-52518).

0.34 μg of either SARS-CoV-2 Spike protein, or VSV-G (positive control).

Change to fresh, prewarmed media after 4–6 h following transfection.

Collect virus 48 h following transfection by harvesting supernatant from cells and passing through a 45 μM filter. Store virus at −80°C until use.

Preparation SARS-CoV-2 isolate USA-WA1/2020 true virus

Timing: 1 day

This section accomplishes propagation and preparation of true virus at desired multiplicity of infection (MOI) for functional studies.

⚠ CRITICAL: SARS-CoV-2 is a biosafety level 3 (BSL-3) pathogen. This described protocol must be performed in a biosafety cabinet in a BSL-3 appropriate facility. Required safety training for researchers is needed and institutional guidelines are strictly followed. Sterile, filter barrier tips are used for all procedures. All liquid waste generated is disinfected with fresh bleach solution for at least 20 min and all items removed from the biosafety cabinet must be sprayed with 10% bleach prior to removal.

Note: The procedure for true virus preparation is shown in Figure 4.

One day prior to virus propagation, plate 1.8×10^7 Vero-E6 cells in a T175 flask.

Inoculate T175 of ~80% confluent VERO-E6 cells with SARS-CoV-2 virus isolate one day after seeding cells. Dilute the virus in 6 mL of VERO-E6 media per flask of VERO-E6 cells.

Note: The volume of SARS-CoV-2 virus added is calculated to a MOI of 0.01. SARS-CoV-2 isolate USA-WA1/2020 was used for all experiments (BEI Resources NR-52281).

Note: Virus tubes are placed in a sealable secondary containment for transport to the biosafety cabinet. Virus tubes are thawed at 21°C–25°C in the biosafety cabinet and decontaminated before opening.

⚠ CRITICAL: Following virus inoculation, the outside of each flask is wiped with bleach for decontamination. Flasks are placed into a sealable Tupperware container for secondary containment. Secondary containment is sprayed with 10% bleach solution.

Incubate flasks in their secondary container at 37°C. Gently rock every 15 min. Add 20 mL of true virus propagation media (DMEM media with 2% v/v FBS and L-glutamine) after 1 h absorption time. Allow flasks in their secondary container to incubate at 37°C for 24 h.

To harvest virus, collect all media into a 50 mL conical tube.

Note: Inspect cultures under microscope to monitor virus infection. Harvest virus once cells are all rounded and barely touching in a chain (looking like a string of pearls). Cells should not be lifted or dead. To inspect flasks under microscope, carefully remove flasks from secondary container in biosafety cabinet. Make sure the cap is tightly sealed prior to removing flask from biosafety cabinet to observe cells under the microscope. Keep microscope on a cart as close to biosafety cabinet as possible to minimize transport time out of the biosafety cabinet.

Centrifuge at 1000 *g* for 10 min to pellet cell debris. Decant supernatant into a fresh 50 mL conical tube.

Pause point: If virus is to be frozen down for stocks, aliquot 1 mL of virus into sterile 2 mL screw top tubes and freeze at −80°C. If virus is to be used fresh, virus can be stored at 4°C until ready to be used for 1–2 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal anti-HIS tag antibody produced in mouse	Sigma-Aldrich	SAB1305538; RRID: AB_2687993
Anti-DYKDDDDK (FLAG) antibody	BioLegend	637303: RRID: AB_1134265
Bacterial and virus strains		
SARS CoV-2 isolate USA-WA1/2020	BEI Resources	NR-52281
E. coli DH5alpha competent cells	Zymo Research	T3001
Chemicals, peptides, and recombinant proteins		
Recombinant SARS COV-2 spike protein (RBD HIS tag)	Sino biological Inc	40592-V08B
Ni-NTA agarose	QIAGEN	30210
Polybrene	Millipore sigma	TR-1003-G
NaCl	Sigma	S-7653
Sodium phosphate	Sigma	S-0751
Imidazole	ICN Biomedicals	102033
Tris	ACROS Organics	140500025
30% Acrylamide/Bis (37.5:1)	Bio-Rad	1610158
SDS	Bio-Rad	1610301
APS	MP Biomedicals	802829
TEMED	Bio-Rad	161-0801
25% Glutaraldehyde	Sigma	354400
Xho1	NEB	R0146L
Xba1	NEB	R0145L
HindIII	NEB	R0104L
Agarose	Fisher	BP160-500
Ethidium bromide	Sigma	E8751
Ligafast Rapid DNA Ligation System	Promega	M8221
2× Rapid DNA ligation buffer	Promega	M8221
Ampicillin	Fisher	BP176025
$CaCl_2$	Sigma	C1016
Na_2HPO_4	Sigma	S3264
HEPES	Santa Cruz Biotechnology	BP310-500
100% glycerol	Fisher	G33-1
0.5 M EDTA	Sigma	E9884
1 M Tris-HCl	Fisher	BP153-1
SDS	Bio-Rad	1610301
Bromophenol blue	Bio-Rad	161-0404
Tween-20	Fisher	BP337-500
Nitrocellulose membranes	Bio-Rad	1620115
Non-fat dry milk	Dot Scientific	DSM17200-1000
West Q Pico ECL solution	GenDepot	W3652-100
DMEM (high glucose)	Sigma	D6429
Fetal bovine serum (FBS)	Sigma	F2442
Penicillin-streptomycin	Corning	30-002-CI
L-glutamine	Corning	25-005-CI
Critical commercial assays		
Dual-Luciferase reporter assay system	Promega	E1960
Miniprep kit	Bio Basic	BS88136
Maxiprep kit	Omega Scientific	D6922-02
Experimental models: Cell lines		
HEK293T cells	ATCC	CRL-3216
HEK293T-ACE2 cells	Gift (Dr. Jesse D Bloom)	Crawford et al.[2]
VERO-E6 cells	ATCC	CRL-1586

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
SARS-CoV-2 Spike-ALAYT, pHAGE-CMV-Luc2-IRES-Zsgreen, HDM-HgPM2, HDM-tat1b, PRC-CMV-Rev1b plasmids	Gift (Dr. Jesse D Bloom)	Crawford et al.[2]
pcDNA3	Invitrogen	A-150228
Software and algorithms		
Prism software	GraphPad	V9.1.0
ImageJ	NIH, version 1.53	
Other		
15Q column with SOURCE 15Q beads	GE Healthcare	17-0947-20
10× premixed electrophoresis Tris-glycine-SDS buffer	Bio Basic	A0031
10× premixed transfer Tris-Glycine buffer	Bio Basic	A0029
45 µm syringe filter	Dot Scientific	CS-4040
Dialysis tubing clips	Thermo Scientific	68011
Dialysis tubing	VWR	470163-418

MATERIALS AND EQUIPMENT

Growth media for HEK293T cells

Reagent	Final concentration	Amount
DMEM (high glucose)	N/A	500 mL
Fetal Bovine Serum	2% (v/v)	10 mL
Penicillin-Streptomycin (100 IU/mL)	1% (v/v)	5 mL

Store at 4°C for up to 1 month.

Growth media for culturing VERO-E6 cells

Reagent	Final concentration	Amount
DMEM (high glucose)	N/A	500 mL
Fetal Bovine Serum	10% (v/v)	50 mL
Penicillin and streptomycin (100 IU/mL)	1% (v/v)	5 mL
L-glutamine	1%	5 mL

Store at 4°C for up to 1 month.

Growth media for true virus propagation

Reagent	Final concentration	Amount
DMEM (high glucose)	N/A	500 mL
Fetal Bovine Serum	2% (v/v)	10 mL
Penicillin and streptomycin (100IU/mL)	1% (v/v)	5 mL
L-glutamine	1%	5 mL

Store at 4°C for up to 1 month.

Equilibration buffer (pH 7.4)

Reagent	Final concentration	Amount
Sodium phosphate	20 mM	1.20 g
NaCl	0.5 M	14.61 g
MilliQ H_2O	N/A	Up to 500 mL
Total		500 mL

Store at 4°C for 3 months.

Wash Buffer A (pH 7.4)		
Reagent	Final concentration	Amount
Sodium phosphate	20 mM	1.2 g
NaCl	0.5 M	14.61 g
Imidazole	10 mM	0.34 g
MilliQ H_2O	N/A	Up to 500 mL
Total		500 mL
Store at 4°C for 3 months.		

Wash Buffer B (pH 7.4)		
Reagent	Final concentration	Amount
Sodium phosphate	20 mM	1.2 g
NaCl	0.5 M	14.61 g
Imidazole	20 mM	0.68 g
MilliQ H_2O	N/A	Up to 500 mL
Total		500 mL
Store at 4°C for 3 months.		

Wash Buffer C (pH 7.4)		
Reagent	Final concentration	Amount
Sodium phosphate	20 mM	1.2 g
NaCl	0.5 M	14.61 g
Imidazole	30 mM	1.02 g
MilliQ H_2O	N/A	Up to 500 mL
Total		500 mL
Store at 4°C for 3 months.		

Elution Buffer (pH 7.4)		
Reagent	Final concentration	Amount
Sodium phosphate	20 mM	1.2 g
NaCl	0.5 M	14.61 g
Imidazole	250 mM	8.51 g
MilliQ H_2O	N/A	Up to 500 mL
Total		500 mL
Store at 4°C for 3 months.		

Tris-NaCl Buffer A (pH 7.4)		
Reagent	Final concentration	Amount
Tris	25 mM	1.51 g
NaCl	25 mM	0.73 g
MilliQ H_2O	N/A	Up to 500 mL
Total		500 mL
Store at 21°C–25°C for 1 year.		

Note: Precool Tris-NaCl buffer A at 4°C prior to use.

Tris-NaCl Buffer B (pH 7.4)		
Reagent	Final concentration	Amount
Tris	25 mM	1.51 g
NaCl	1 M	29.2 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
MilliQ H_2O	N/A	Up to 500 mL
Total		500 mL
Store at 21°C–25°C for 1 year.		

Note: precool Tris-NaCl buffer B at 4°C prior to use.

STEP-BY-STEP METHOD DETAILS

Generation of SARS-CoV-2 RBD as a trimeric fusion protein construct

Timing: 4–5 days

This section accomplishes cloning of RBD in fusion with a trimerization domain, leader sequence and a protein tag. The cDNA encoding the leader sequence and leucine zipper was previously cloned.[3]

1. Design and order primers for the RBD region of the spike protein of SARS-CoV-2. Add cut sites to the 5′ and 3′ end to prepare insert for cloning into the vector, Table 1.
   a. To generate primers for the SARS-CoV-2 RBD insert, copy the DNA sequence encoding SARS-CoV-2 Spike protein RBD (YP_009724390.1) and add desired restriction enzymes on 5′ and 3′ ends.

   Note: The purchased recombinant RBD spike protein already has a His-tag. The protein tag can be changed as desired (i.e., The sequence: 5′ - GACTACAAGGACGACGATGACAAG-3′ can be used to add a FLAG tag). The amplified PCR product will have added XhoI and XbaI restriction enzyme site at the 5′ and 3′ end, respectively.

   b. Amplify the designed insert using PCR (insert 1) Table 2, Table 3.

   Note: The expected size of a single band is ~0.9 bp.

   c. Purify the PCR product of insert 1 using a commercially available PCR purification kit.
2. Prepare DNA fragment containing a leader sequence and modified leucine zipper (insert 2). Add desired restriction enzymes to the 5′ and 3′ ends, Table 1.
   a. Amplify the region of interest using PCR, Table 4, Table 5.

   Note: The amplified PCR product will have HindIII and XhoI at the 5′ and 3′ ends, respectively.

   Note: DNA sequence of the leader sequence and modified leucine zipper is provided (Figure 1A). This can be commercially ordered as a synthetic DNA fragment of about 0.2kB or used as the template DNA for PCR amplification of insert 2.

Table 1. Primer sequences

Primer	Sequence
SARS-CoV-2 RBD insert - forward primer	5′-CGC**CTCGAG**CACCATCACCACCATCATCACCACCAT-3′
SARS-CoV-2 RBD insert - reverse primer	5′-CTAG**TCTAGA**TTCAGAAGTTCACACACTTGTTCTTCAC-3′
Leader sequence and modified leucine zipper – forward primer	5′ -CCC**AAGCTT**GACATGCCGCTGCTGCTACTGCTGCCC-3′
Leader sequence and modified leucine zipper – reverse primer	5′- CCG**CTCGAG**AGACGTTCACCAATCAGCTTTTTGA-3′

Table 2. PCR reaction mix for RBD insert (insert 1)

Reagent	Final concentration	Amount
Forward primer	10 μM	1 μL
Reverse primer	10 μM	1 μL
Template: SARS-CoV-2 Spike Protein RBD	20 ng	1 μL
10× PfuUltra HF reaction buffer	1×	5 μL
PfuUltra HF DNA polymerase	2.5U	1 μL
dNTPs (10 mM)	200 μM	1 μL
DMSO	2%	1 μL
MilliQ H_2O		Up to 50 μL
Total		50 μL

b. Purify the PCR product using a commercially available PCR purification kit.

Note: The expected size of the leucine zipper is ~200 bp.

3. Digest the PCR product containing the leader sequence and leucine zipper (insert 2) using HindIII and Xho1. Digest the PCR product containing the RBD protein (insert 1) using Xho1 and Xba1. Digest a mammalian expression vector (e.g., pcDNA3) using HindIII and XbaI.

Note: Use 1 μg of the pcDNA3 vector and 500 ng of the PCR product inserts for digestion.

4. Purify the vector and inserts by running the DNA on an agarose gel and excising the appropriate bands.

Note: Prepare the final volume of the vector and inserts to 30 μL.

Note: For gel electrophoresis, use 1% agarose gel for PCR products and vectors.

5. Ligate the inserts into the vector with T4 DNA ligase (Figure 1B), Table 6.
   a. Incubate the reaction at 21°C–25°C for 30 min.
6. Transform competent bacteria with recombinant DNA.
   a. Thaw and aliquot of competent bacteria. Mix 2 μL of the ligation mix with 25 μL of competent cells. Incubate the mixture sequentially on ice for 10 min, a heating block 42°C for 30 s, and on ice for 10 min.

Note: E. coli DH5alpha competent cells are used for efficient transformation.

   b. Spread mixture onto a 37°C prewarmed 10 cm agar culture plate containing 15 mL of LB medium and ampicillin (100 μg/mL). Incubate 16 h at 37°C.

7. Confirm the presence of the desired insert into the vector.

Table 3. PCR cycling conditions for the RBD insert (insert 1)

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	1 min	30 cycles
Annealing	55°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

Table 4. PCR process for leader sequence and modified leucine zipper (insert 2)

Reagent	Final concentration	Amount
Forward primer	10 μM	1 μL
Reverse primer	10 μM	1 μL
Template: pcDNA3 vector with leucine zipper	20 ng	1 μL
10× PfuUltra HF reaction buffer	1×	5 μL
PfuUltra HF DNA polymerase	2.5U	1 μL
dNTPs (10 mM)	200 μM	1 μL
DMSO	2%	1 μL
MilliQ H_2O		Up to 50 μL
Total		50 μL

a. Select and inoculate individual colonies in a miniculture of 2 mL of liquid LB with ampicillin (100 μg/mL) at 37°C for 8–12 h in a shaking incubator at 220 rpm.
b. Isolate plasmid DNA using a commercially available miniprep DNA isolation kit.
c. Digest the plasmid with HindIII and XbaI and run on agarose gel.

Note: If insert 1 and insert 2 are correctly inserted into the vector, bands at 0.9 kb and 5.4 kb will be present, respectively (Figure 1C).

d. Verify presence of RBD protein in the vector by sequencing using the recommended primer: 5′- CGCAAATGGGCGGTAGGCGTG.

8. Purify verified DNA construct.
   a. Transfer 250 μL of miniculture into a maxiculture of 200 mL of autoclaved LB medium supplemented with 100 μg/mL of ampicillin. Incubate at 37°C with shaking at 220 rpm for 12–16 h.
   b. Isolate plasmid DNA using commercially available maxiprep DNA isolation Kit.

In vitro production of engineered trimeric RBD

Timing: 7 days

This section describes the *in vitro* production and purification of the tRBD.

Calcium phosphate transfection

9. Plate 2 × 10^6 HEK293T cells in a 15 cm culture dish with 20 mL of growth media (DMEM media with 2% v/v FBS) the night before.

Optional: If HEK293T cells are found to adhere loosely to the plate, pre-coat culture dish with polylysine to facilitate cell adherence.

10. Prepare DNA/$Ca_3(PO_4)_2$ mixture for transfection:

Table 5. PCR cycling conditions of the leader sequence and modified leucine zipper (insert 2)

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 s	30 cycles
Annealing	50°C	1 min	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

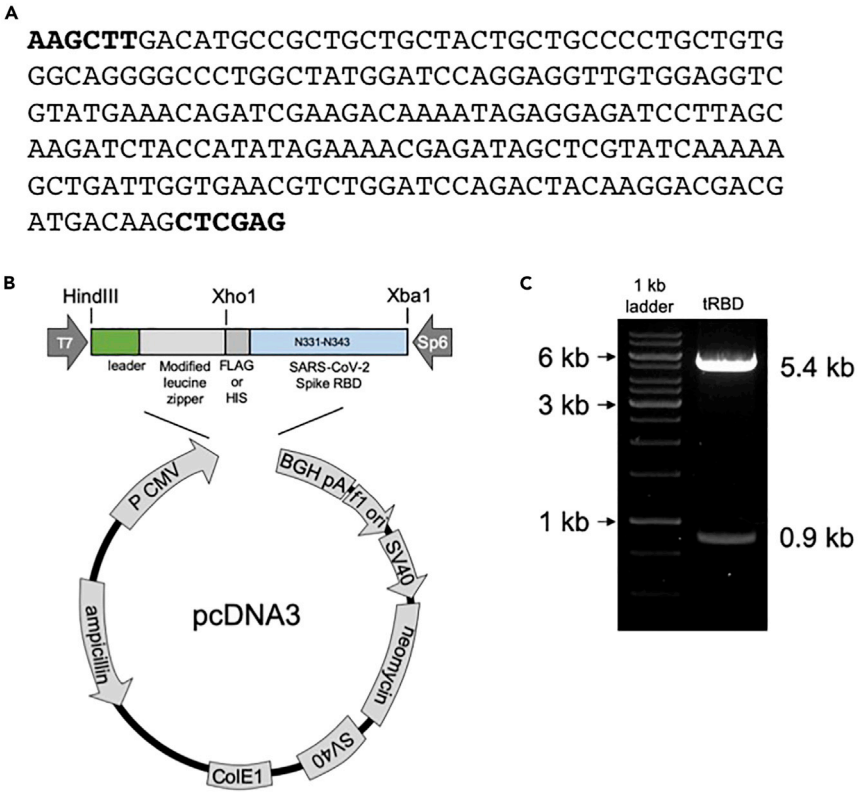


Figure 1. Schematics of tRBD cloning

(A) Sequence of the leader peptide and leucine zipper with HindIII and XhoI restriction sites added to its 5′ and 3′ end, respectively.

(B) Schematic of tRBD cloned into pcDNA3 vector.

(C) Digestion of vector with tRBD using HindIII and XbaI results in bands ~5.4 kb and ~0.9 kb.

a. Combine in a sterile tube (per plate).
   i. 24.0 μg tRBD plasmid DNA.
   ii. Up to 900 μL of MilliQ H_2O.
   iii. 100 μL of 2.5 M $CaCl_2$.
b. Add 1,000 μL of 2× HEBS buffer drop wise while slowly vortexing.
c. Incubate DNA at 21°C–25°C for 10–15 min.

11. Add DNA/Ca3$(PO_4)_2$ mixture to cells drop wise. Incubate at 37°C for 6 h.
12. Wash plates 2 times with 10 mL sterile PBS each time. Perform the washes very gently to prevent the cells dislodging from the plate. Add 20 mL of fresh, prewarmed growth media for HEK293T cells. Incubate at 37°C for 72 h.
13. Collect cell supernatant in conical tubes and centrifuge to remove cell debris.

Table 6. Ligation reaction

Reagent	Amount	Volume
T4 DNA ligase		1 μL
2× rapid DNA ligation buffer		5 μL
Vector	50 ng	
Insert 1: digested fragment containing RBD	100 ng	
Insert 2: digested fragment containing leader sequence and leucine zipper	50 ng	
H_2O		Up to 10 μL

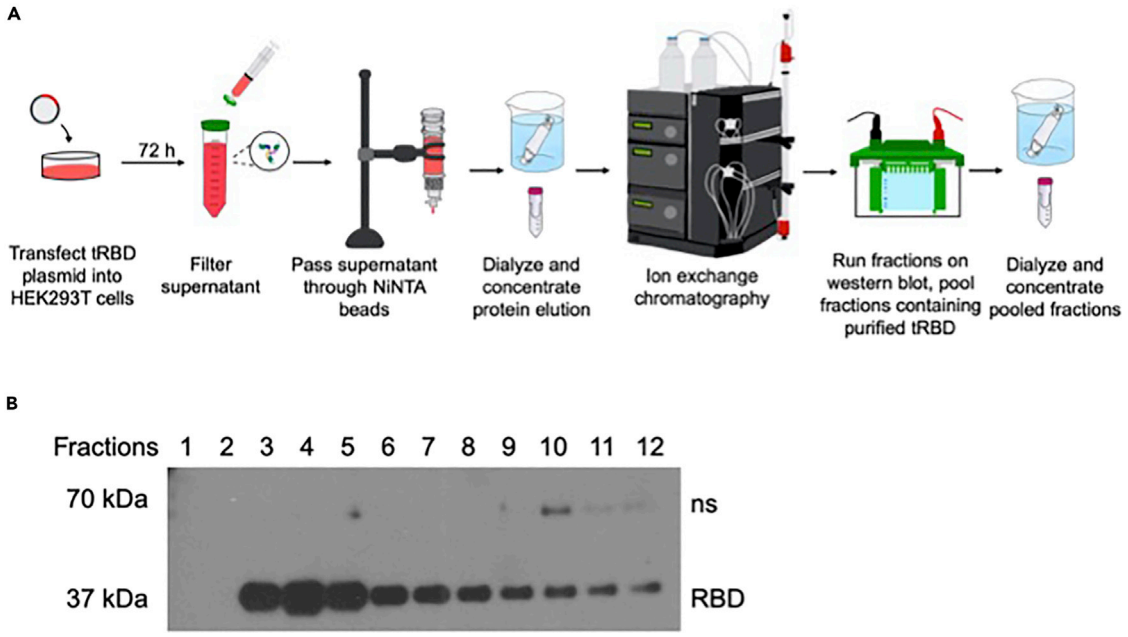


Figure 2. Schematics of tRBD expression and purification

(A) tRBD plasmid transfection and purification of tRBD protein from cell culture supernatant.

(B) Western blot analysis of purified tRBD fractions collected following ion-exchange FPLC. Samples were probed using anti-HIS antibody. ns, non specific band.

14. Pass cell supernatant through a 0.45 μm syringe filter.

Note: For difficulties with the producing tRBD via calcium phosphate transfection, see troubleshooting 1.

Pause point: If purified protein is not needed, conditioned media can be stored at this step at −80°C until usage. If protein purification is needed, do not freeze samples and store conditioned media at 4°C up to 16 h.

Prepare Ni-NTA column for protein purification

Note: These following steps are performed at 4°C.

Note: Schematic representing the purification of tRBD is shown in Figure 2A.

15. Clip a gravity column onto a stand. Close the column flow. Slowly add 10 mL of Ni-NTA beads into gravity flow column using a serological pipette. Allow beads to settle to the bottom of the column for 10 min. Open the column flow to allow ethanol to drain.

Note: Ni-NTA beads are 50% ethanol from the manufacturer. The final bead volume after this step will be 5 mL.

16. Change column flow rate to 1 mL/min. Slowly add 100 mL of Equilibration Buffer through the column.
17. Keep column flow rate at 1 mL/min. Pass RBD conditioned media through the column with equilibrated NiNTA beads. Collect flow-through media in a clean 1 L glass bottle.

18. Change column flow rate to 2 mL/min. Pass the flow-through through column to increase the amount of RBD bound to NiNTA beads.
19. Keep flow rate at 2 mL/min. Wash beads to remove nonspecific protein binding with sequentially increasing concentrations of imidazole.
    a. Flow 20 mL of Equilibration Buffer through the column.
    b. Flow 20 mL of Wash Buffer A through the column.
    c. Flow 20 mL of Wash Buffer B through the column.
    d. Flow 20 mL of Wash Buffer C through the column.

Note: Elution conditions may differ depending on the protein being purified. Refer to troubleshooting 4.

20. Change column flow rate to 1 mL/min. Elute bound RBD with 20 mL of Elution Buffer into a clean 50 mL conical tube.

Dialyze and concentrate protein elution

21. Transfer 20 mL of eluted RBD fraction to dialysis tube. Secure both ends with a dialysis bag clamp. Place bag in a 5 L glass beaker. Fill the beaker with 4 L of Tris-NaCl buffer until it covers the dialysis bag. Use a magnetic stirrer on the lowest setting.

Note: If the elution volume is less than 20 mL, use 200:1 of Tris-NaCl buffer volume:protein elution volume.

22. Allow dialysis to occur for 12 h at 4°C.
23. Add sample to the top compartment of a Millipore concentration tube with a molecular weight cutoff of 50 kDa. Centrifuge at 4000 *g* for 5 min at 4°C and discard the flow through.

Note: The Millipore concentration tube can hold up to 12 mL. Repeat this process in the same Millipore concentrator tube until all of the dialyzed RBD has been concentrated.

Ion exchange chromatography

24. Centrifuge concentrated RBD fraction at 10,000 *g* in a tabletop mini centrifuge for 10 min at 4°C to remove any precipitates that may clog the column.
25. Equilibrate Mono Q column in a AKTA FPLC (GE) system with Tris-NaCl buffer A. Inject samples into a Mono Q column and collect the flow through. Add more Tris-NaCl buffer A in batches depending on the capacity of the column and collect 15 more fractions.

Note: In our experiment, each fraction had roughly 1.5 mL.

Note: tRBD is generated as a secreted protein from HEK-293T cells into the growth media, which contains several proteins and a large amount of BSA. tRBD has a predicted PI of 8.7–8.8, with a positive charge. However, our attempt to purify tRBD using a Mono S cation exchange column proved unsuccessful as other unknown proteins as well as large amounts of BSA in the culture medium co-eluted with tRBD. Our major contaminant was BSA, which has a PI of 4.5–5.0, which, in theory, is not expected to bind to Mono S column. It is possible that the large amount of BSA in the FBS added to the culture medium was non-specifically sticking to the column. On the other hand, we found that tRBD does not bind to the Mono Q anion exchange column, which bound well to BSA and other proteins. Based on this, we performed a negative selection approach for tRBD using Mono Q column, which trapped most of the BSA and other proteins. tRBD was present mainly in early flowthrough fractions 3–8, collected in the equilibration buffer A (Figure 2B). This is a customized purification protocol

for tRBD. For other options to optimize elution of the protein of interest please see troubleshooting 6.

26. Elute proteins bound to Mono Q column using a salt gradient generated using Tris-NaCl buffer B, which contains 1 M NaCl. Carefully adjust the flow rate of buffer A and buffer B to obtain a salt gradient over time to elute proteins binding to the column with varying affinity. Depending on the protein content in the eluate, determine the number of fractions to be collected.

Note: In this protocol, we collected 25 more fractions in which no significant target protein content was observed in the eluates.

Note: Proteins eluted from the Mono Q column with Tris-NaCl buffer B contained no tRBD. The eluate can be discarded or kept as a negative or background control. This step will clean the column and keep it in good condition. Following this step, wash the column with 4 column volumes of distilled water, and then equilibrate with 4 column volumes of degassed 20% ethanol for its future use.

Sample preparation and gel running

27. Take 30 μL from each fraction. Dilute with 10 μL 4× Laemmli buffer. Boil at 95°C for 5 min. Vortex and quickly spin down samples in a tabletop centrifuge for 5 min at 10,000 *g*.

Note: Do not place samples on ice after this step, as samples have been denatured.

28. Clip the previously prepared 10% polyacrylamide gel into a gel runner tank.
29. Pour the 1× Running Buffer into the gel running tank chamber, ensuring that the buffer covers the top of the gel. Load the samples from step 27 into the gel.
30. Place the safety lid with electrodes over the entire apparatus. Plug the electrodes into a power pack and run gel for 100V for 10 min, then 200V for 55 min at 21°C–25°C.
31. Submerge an open Transfer Cassette plate into a tray with 1× transfer buffer.
32. On top of the cathode plate, place the following the materials in this specific order:
    a. Sponge support pad.
    b. Filter paper.
    c. Acrylamide gel.
    d. Nitrocellulose membrane.
    e. Filter paper.
    f. Sponge support pad.

Note: Be sure to submerge and saturate each layer with premade 1× transfer buffer prior to use.

Note: Gently use a roller to remove any air bubbles after adding each layer.

33. Gently close the transfer plate with the anode plate and place the assembled cassette into the transfer tank. Adjust the buffer level until 1× transfer buffer submerges the necessary level within the tank.
34. Move the power pack and transfer tank to 4°C or place it in an ice tray to prevent over heating. Connect the electrodes into the power pack. Run for 1 h at a constant voltage of 100V.

Note: We used constant voltage for transfer because it ensures constant field strength offering most efficient tank blotting method. This method was ideal for the proteins studied here. If suboptimal transfer efficiency is observed, please refer to other options provided under troubleshooting 5.

35. Immunoblot nitrocellulose membrane with an antibody detecting the HIS or FLAG tag or RBD itself.

Pool and aliquot purified tRBD

36. Note the fractions from ion exchange chromatography that contain tRBD. Pool fractions containing purified tRBD (Figure 2B).

Note: We pooled fractions ~3–8. More nonspecific proteins were often present in later fractions following FPLC, which resulted in poor tRBD protein purification (Figure 2B). See troubleshooting 3.

37. Dialyze and concentrate protein elution as described above.

Pause point: Aliquot purified tRBD. Store in −80°C until use.

Purified tRBD concentration quantification

Timing: 1 day

This section describes the method used to quantify the amount of protein purified using western blot analysis and the generation of a standard curve.

Protein sample preparation and gel running

38. Dilute 30 µL of tRBD conditioned media or purified tRBD and known concentrations of commercially available recombinant RBD (Sino Biological) with 4× Laemmli Sample buffer.
39. Boil samples at 95°C for 5 min. Vortex and quickly spin down samples in a tabletop mini-centrifuge for at least 5 s.
40. Resolve on SDS-PAGE and transfer to nitrocellulose membrane.

Densitometry analysis

41. Analyze western blot bands using the Gel Analyzer tool of ImageJ (NIH).
42. Prepare standard curve using densitometry values of known concentrations of commercially available recombinant RBD (Sino Biological).

Note: In this protocol we used Western blotting and densitometry to quantify RBD concentration. Therefore, we used commercial monomeric HIS-RBD as a standard and performed relative quantification of the trimeric HIS-RBD using anti-HIS antibody to simultaneously detect both proteins. Standard curve may also be prepared with known concentrations of bovine serum albumin (BSA) as an economical alternative option. However, if Western blotting and densitometry is used for quantification, using BSA will require the use of two different antibodies, i.e., one for BSA and one for the protein of interest, which may not give accurate signal intensity because of differences in the efficiency of antibodies used, thus skewing quantification of concentrations.

   a. Plot densitometry values against known RBD concentrations.
   b. Calculate regression equation.
43. Calculate concentrations of tRBD conditioned media and purified tRBD by inputting the obtained densitometry value into the obtained regression equation.

Confirm trimeric nature of purified tRBD

Timing: 1 day

This section describes validation of the trimeric nature of the purified RBD.

Usage of the modified leucine zipper allows for the trimerization of protein constructs. This section describes how to verify the trimeric nature of the purified protein.

44. Adjust concentration of 50 μL of purified tRBD to 20 μg/mL with PBS.
45. Preheat the 2.5% glutaraldehyde solution at 80°C. Add 3 μL of 2.5% glutaraldehyde to 20 μL of 20 μg/mL purified tRBD.
46. Incubate at 80°C for 5 min.
47. Stop the crosslinking reaction by adding 3 μL of 1 M Tris-HCl.
48. Add 9 μL of 4× Laemmli Sample buffer without beta mercaptoethanol (BME). Heat sample at 95°C for 5 min.

Note: As a control, prepare a sample of purified tRBD as stated above and add 9 μL of 4× Laemmli Sample buffer with BME as a reducing agent. Addition of BME to the Laemmli sample buffer will reduce the disulfide bonds and unfold the native conformation of the proteins in the sample facilitating its migration in the gel.

49. Resolve samples on 10% SDS-PAGE gel and transfer onto nitrocellulose membrane. Probe with antibody detecting HIS or FLAG tag or RBD itself.

Note: In its trimeric form, RBD will have a molecular weight of around 95 kDa while monomeric RBD will be around 37 kDa.

Note: Monomeric and trimeric proteins may also be comparatively analyzed without glutaraldehyde crosslinking using non-reducing native polyacrylamide gel electrophoresis.

Assess ability of engineered tRBD to inhibit SARS-CoV-2 viral entry

Timing: 5–6 days

This section accomplishes validation of the potential of tRBD to block SARS-CoV-2 infection in a pseudovirus assay and in a true virus infection assay.

tRBD inhibition of pseudotyped lentivral particles with SARS-CoV-2 spike protein

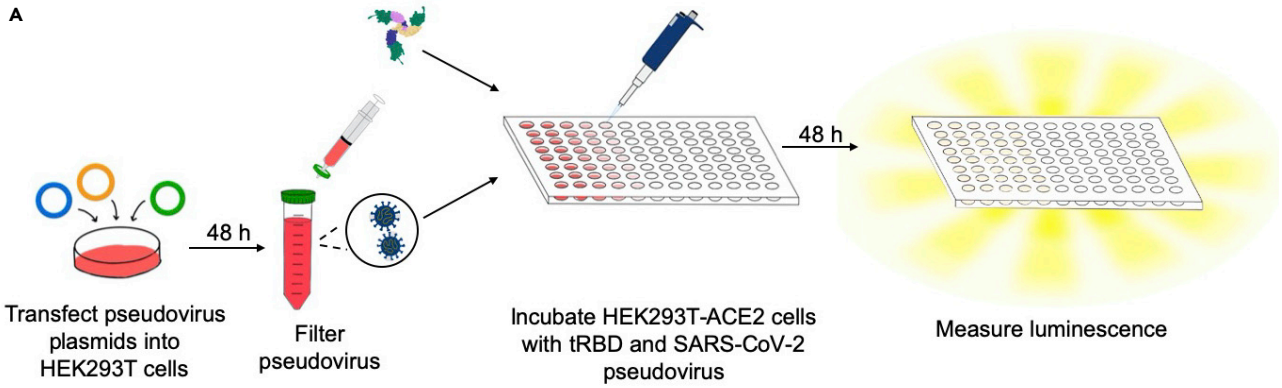
This section of the protocol is modified from a previous publication.[2] It assesses the ability of the engineered tRBD to inhibit entry of a SARS-CoV-2 psuedovirus into ACE2-expressing cells (Figure 3). See Figure 3A for the protocol overview.

50. Plate 1.25 × 10^4 HEK293T-ACE2 cells/well in a flat bottom 96 well plate. Incubate cells 16–24 h at 37°C, 5% CO_2. See Figure 3B for the layout of experimental and control samples in the plate.

Optional: If HEK293T-ACE2 cells used in the lab adhere only loosely to the plate, pre-coat culture dish with polylysine to facilitate cell adherence.

51. Incubate cells with 100 μL of SARS-CoV-2 or VSV-G pseudovirus and/or 50 ng (calculated from previous step) of tRBD (Figure 3B). Other dilution of tRBD can be used to calculate an IC50. 5 μg/mL polybrene was added to each well to facilitate lentiviral infection. Final volume of each well is adjusted to 200 μL. Incubate for 48 h at 37°C, 5% CO_2

A



B

	media only	HEK-ACE2 cells only	pseudovirus only	VSV-G only	HEK-ACE2 cells+tRBD	pseudovirus+ HEK-ACE2 cells	pseudovirus+ HEK-ACE2 cells+tRBD	VSVG+HEK-ACE2 cells	VSVG+HEK-ACE2 cells +tRBD			
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 3. Schematics of pseudovirus functional assay

(A) Luciferase assay to assess the ability of tRBD to inhibit SARS-CoV-2 psuedovirus entry.

(B) Example of plate layout for the assay run in triplicate.

52. Gently wash cells with 100 μL of PBS to remove cell media. Directly lyse cells on plate by adding 20 μL of 1× Passive Lysis Buffer from manufacturer's kit.
53. Allow samples to shake in lysis buffer at room 21°C–25°C for 15 min.

Note: During this time, prepare LARII solution, if needed, according to manufacturer's instructions. Allow LARII solution to adjust to 21°C–25°C.

54. Prepare plate reader to read luminescence with a 2 s delay and 10 s read time.
55. Centrifuge the lysate for 10,000 *g* for 5 min at 4°C and transfer 10 μL of the cell lysate supernatant/well of a white clear bottom 96 well plate. Dispense 50 μL of pre-thawed LARII solution and quickly pipette up and down 4–5 times to obtain a homogenous mix of lysate and LARII. Place plate in microplate reader with luminescence reading capacity Plot the graph using relative luminescence unit (RLU) readings obtained as described.[1,2] For difficulties with obtaining luciferase readings, see troubleshooting 2.

⚠ CRITICAL: The LARII reagent is extremely light sensitive. Keep in dark until ready to use. Read luminescence immediately, as signal will decrease with time.

Note: In the protocol described we used singe luciferase reading to detect firefly luciferase only. If high variability is expected in the assay, an alternative protocol to consider is the

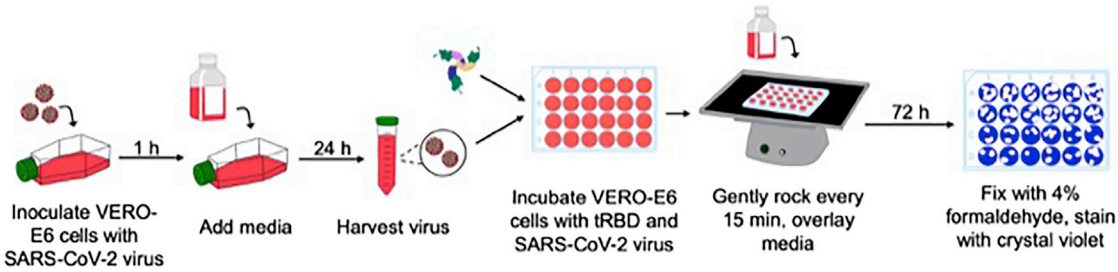


Figure 4. Schematics of true virus functional assay
Plaque reduction assay to assess the ability of tRBD to inhibit SARS-CoV-2 true virus infectivity.

use of dual luciferase system (Promega #1910), with firefly luciferase and Renilla luciferase as an internal control.

tRBD inhibition of SARS-CoV-2 true virus infection of Vero E6 cells

SARS-CoV-2 plaque reduction assay

This section describes a quantitative assay to assess the ability of SARS-CoV-2 to propagate and form plaques and the ability of tRBD to block the viral infection (Figure 4).

56. Plate 1 × 10^5 Vero E6 cells/well in a 24 well plate 24 h prior to infection.
57. Prepare tRBD and mock conditioned media to the desired concentration in virus media.
58. Incubate cells with 250 μL of tRBD or mock dilution for 30 min at 37°C, with gentle rocking after 15 min.
59. Infect each well with 50 μL of previously prepared SARS-CoV-2 true virus at a dilution of 10^{-4}. Incubate plate at 37°C for 1 h. Gently rock plate every 15 min.
60. Overlay 500 μL of media containing DMEM with 0.05% agarose and 2% FBS. Incubate at 37°C for 72 h.
61. Fix cells with 4% formaldehyde solution diluted in PBS for 30 min.
62. Stain with crystal violet for 5 min.
63. Calculate viral titers of SARS-CoV-2 true virus.
    a. Count plaque forming units per well for each dilution.
    b. Normalize each dilution to well with Mock media.

EXPECTED OUTCOMES

SARS-CoV-2 is the viral cause of the 2019 global pandemic. Successfully engineered tRBD will inhibit the viral entry of both pseudoviral and true virus of SARS-CoV-2. Following protein purification of tRBD, we obtained approximately 115 μg of tRBD per liter of transfected HEK293T cell culture supernatant. Undiluted tRBD conditioned media was able to inhibit ~75% of SARS-CoV-2 psuedovirus entry. The dosage of tRBD at which 50% of infectious true virus was infected (IC50) was 1,602 ng/mL[1].

This protocol can be adapted to produce any trimeric protein of interest. This protocol uses a modified leucine zipper of the protein GCN4, with isoleucine substitutions to allow for trimer formation of the fusion protein. We coupled a leader sequence to the isoleucine zipper to facilitate the secretion of the modified trimeric protein. Trimeric proteins may also be generated without the leader sequence, which will allow the fusion protein to be expressed within the cell. In principle, this approach is expected to work for the majority of the proteins, once primers of the specific fusion protein are designed. However, it is possible that some proteins with inherent complex structures may interfere with trimerization. It is also possible that certain proteins when forced to express as trimers may show proximity-induced activities such as cleavage, selected posttranslational modifications, enhanced stability and accumulation in the

cell, as well as uneven localization in cells. Preliminary experiments should be performed to examine such possibilities and ensure the appropriate expression of the desired protein in its trimeric form.

LIMITATIONS

Steps involving the preparation and usage of the true SARS-CoV-2 virus must be performed in a BSL-3 facility. This also requires preapproval from institutional biosafety committee, use of proper personal protective equipment, and proper disposal of wastes. Only those who have received adequate safety training should handle this. Viral infection of various cell types may also vary depending on the quality of the prepared virus as well as the cells used. This may require pilot screening to identify suitable target cell types and ideal viral MOI.

TROUBLESHOOTING

Problem 1

Poor transfection efficiency is observed following when generating tRBD from HEK293T cells.

Our protocol describes commonly used calcium phosphate transfection method, which is easily scalable and significantly economic for large-scale virus production. However, it may require some expertise to obtain consistent high transfection efficiency.

Potential solution

Collection of tRBD from the supernatant after 72 h is based on the number of cells used and transfection efficiency established in this protocol. If these parameters are changed, adjustments must be made accordingly. Transfection may also be performed with other methods such as liposome-mediated transfection or electroporation depending on the cell type.

Problem 2

Usage of SARS-CoV-2 spike pseudovirus did not yield high luciferase activity when compared to the VSV-G pseudotyped virus positive control.

Potential solution

The SARS-CoV-2 spike protein requires ACE-2 for its entry while VSV-G has a broad cell tropism, which enables it to infect most mammalian cell types. Ensure that the cell type you are using for the luciferase assay has high levels of endogenous or overexpressed ACE-2.

Problem 3

Purified tRBD protein has nonspecific (ns) background bands on Western blot (Figure 2B).

Potential solution

Later fractions of tRBD often have a higher amount of nonspecific proteins that are co-eluted in the purification steps (Figure 2B, nonspecific bands). Avoid adding later FPLC fractions to the tRBD pool during protein purification.

Problem 4

In step 20, we used imidazole 250 mM in the elution buffer to recover HIS-RBD from NiNTA beads. It is possible that elution of some proteins may not be efficient with this elution buffer.

Potential solution

Use elution buffer with higher imidazole concentrations such as 300 mM, 400 mM or 500 mM and examine the eluted fractions via Western blotting. Perform dialysis for additional 12 h if protein was eluted with higher concentrations of imidazole such as 400 mM or 500 mM.

Problem 5

Transfer of polyacrylamide gel onto a nitrocellulose membrane by applying a constant voltage of 100V for 1 h on ice or at 4°C was not efficient.

Potential solution

Some options that may be considered to improve transfer efficiency are the following: A constant current allows for a more stable directional movement of charged particles in the buffer, keeps the buffer relatively cooler than using constant voltage and may help to improve the transfer efficiency. Using a constant power may also be considered, which is an alternative to constant current to generate less heat. The transfer timings of these protocols would need to be adjusted based on the protein size and the actual transfer efficiency observed. Usage of PVDF membranes that have higher protein binding capacity than nitrocellulose membranes may be considered for detection of proteins expressed at low levels.

Problem 6

Difficulties with purification of the protein of interest from ion exchange column.

Potential solution

In step 25, we obtained purified tRBD in the flowthrough fractions collected from the Mono Q column. This approach is customized for tRBD and may not work for other proteins.

In general, use a cation exchange column (Mono S column) for proteins of interest that are positively charged and an anion exchange column (Mono Q column) for proteins of interest that are negatively charged. If the protein of interest shows tight binding to the ion exchange column, a gradual salt gradient elution may be considered with increasing NaCl concentrations. An alternate purification option is separation of the protein of interest using a size exclusion chromatography. Optimization of flow rate should be performed to limit elution of proteins with close molecular weights in the same fraction. A slower flow rate is expected to separate proteins into different fractions based on their molecular weight or charge.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Parameswaran Ramakrishnan (pxr150@case.edu).

Materials availability

Plasmids generated specifically for this study are available upon request.

Data and code availability

This study did not generate/analyze datasets/code.

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Cleveland, OH) for protein purification and Vinicius Suzart (Case Western Reserve University, Cleveland, OH) for plaque reduction assays.

AUTHOR CONTRIBUTIONS

Conceptualization, P.R.; methodology and investigation, S.B., A.R.L., A.B., P.R.; writing – original draft, A.R.L., P.R.; writing – review & editing, A.R.L., N.S., S.B., A.B., P.R.; visualization, A.R.L., N.S.; supervision, P.R.; project administration, P.R.; funding acquisition, P.R.

DECLARATION OF INTERESTS

P.R. has submitted an invention disclosure 2020–3814, on the use of trimeric RBD as a multipotential therapeutic target to Case Western Reserve University.

REFERENCES

1. Basavarajappa, S.C., Liu, A.R., Bruchez, A., Li, Z., Suzart, V.G., Liu, Z., Chen, Y., Xiao, T.S., Buck, M., and Ramakrishnan, P. (2022). Trimeric receptor-binding domain of SARS-CoV-2 acts as a potent inhibitor of ACE2 receptor-mediated viral entry. iScience *25*, 104716. https://doi.org/10.1016/j.isci.2022.104716.

2. Crawford, K.H.D., Eguia, R., Dingens, A.S., Loes, A.N., Malone, K.D., Wolf, C.R., Chu, H.Y., Tortorici, M.A., Veesler, D., Murphy, M., et al. (2020). Protocol and reagents for pseudotyping lentiviral particles with SARS-CoV-2 spike protein for neutralization assays. Viruses *12*, 513. https://doi.org/10.3390/v12050513.

3. Ramakrishnan, P., Wang, W., and Wallach, D. (2004). Receptor-specific signaling for both the alternative and the canonical NF-κB activation pathways by NF-κB-inducing kinase. Immunity *21*, 477–489. https://doi.org/10.1016/j.immuni.2004.08.009.