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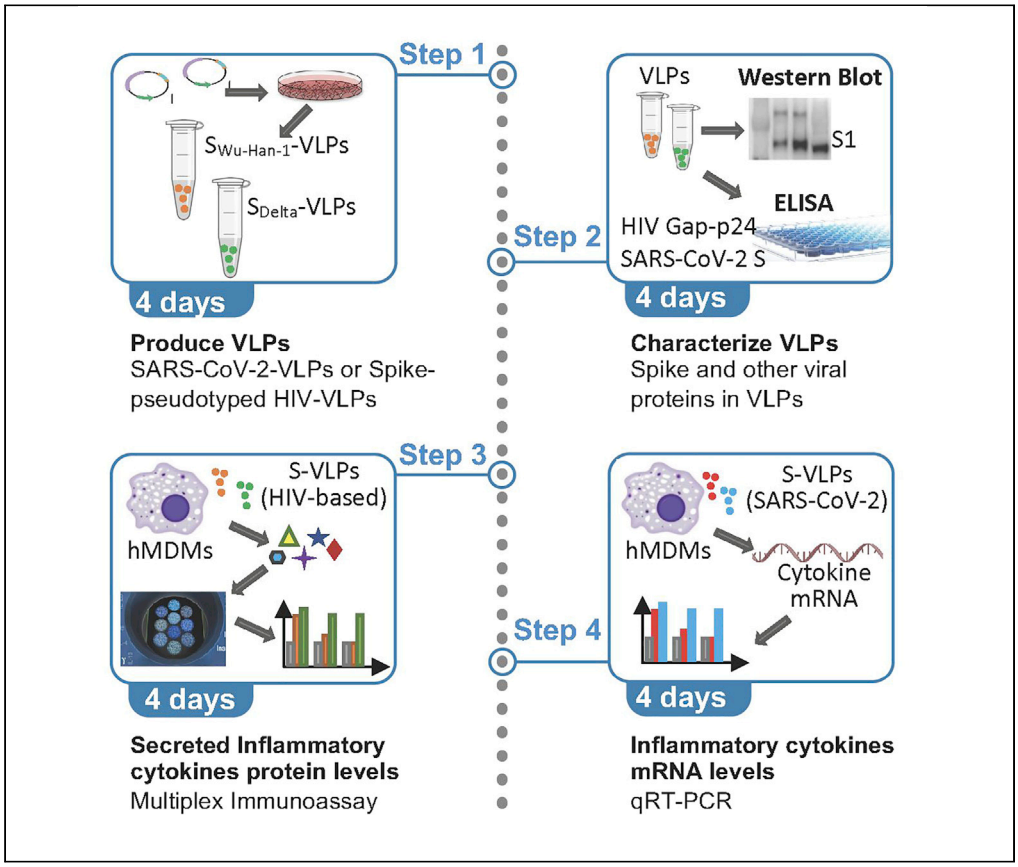
Protocol to evaluate the inflammatory response in human macrophages induced by SARS-CoV-2 spike-pseudotyped VLPs



The excessive release of pro-inflammatory cytokines in COVID-19 patients is deleterious to organs. The contribution of SARS-CoV-2 spike protein (S) to the inflammatory response is essential to understand its pathogenesis and virulence. Here, we present a protocol to produce and characterize HIV- and SARS-CoV-2-based virus-like particles and then evaluate the inflammatory cytokines' protein and mRNA levels produced in human macrophages by S of SARS-CoV-2 original strain and Delta variant. This protocol is applicable in evaluating S from different emerging variants.

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

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Highlights

Production and characterization of HIV- and SARS-CoV-2-based virus-like particles

Evaluation of the protein levels of inflammatory cytokines induced by SARS-CoV-2 S

Detection of the mRNA levels of inflammatory cytokines induced by SARS-CoV-2 S

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Protocol to evaluate the inflammatory response in human macrophages induced by SARS-CoV-2 spike-pseudotyped VLPs

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SUMMARY

The excessive release of pro-inflammatory cytokines in COVID-19 patients is deleterious to organs. The contribution of SARS-CoV-2 spike protein (S) to the inflammatory response is essential to understand its pathogenesis and virulence. Here, we present a protocol to produce and characterize HIV- and SARS-CoV-2-based virus-like particles and then evaluate the inflammatory cytokines' protein and mRNA levels produced in human macrophages by S of SARS-CoV-2 original strain and Delta variant. This protocol is applicable in evaluating S from different emerging variants.

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

BEFORE YOU BEGIN

Cytokine storm-associated severe lung inflammation and respiratory failure significantly contribute to the mortality of COVID-19.[2,3] The protocol below describes the evaluation and comparison of pro-inflammatory cytokine production induced by the SARS-CoV-2 Spike proteins (S) from the original strain and Delta variant. However, this protocol can be used for S from other emerging variants to evaluate the virus virulence of the new variant(s). The human monocyte-derived macrophages are used to investigate the inflammatory response in this protocol, but THP1-derived macrophages and human monocyte-derived dendritic cells also can be used. To safely handle SARS-CoV-2, the Containment Level-3 (CL3) lab is needed, and researchers are required to have a corresponding biosafety certificate. This protocol takes advantage of the S-pseudotyped Virus Like Particles (VLPs) to evaluate inflammatory responses. All the experiments in this protocol avoid using authentic SARS-CoV-2, therefore can be performed in a common biosafety level-2 (BSL2) lab.

Institutional permissions

The ethics statement and approval from the ethics committee for research involving human cells in the institute are required before the experiment. The blood of healthy donors is collected with their consent (e.g., by signing a consent form).

Preparation of plasmids

Timing: 4 days

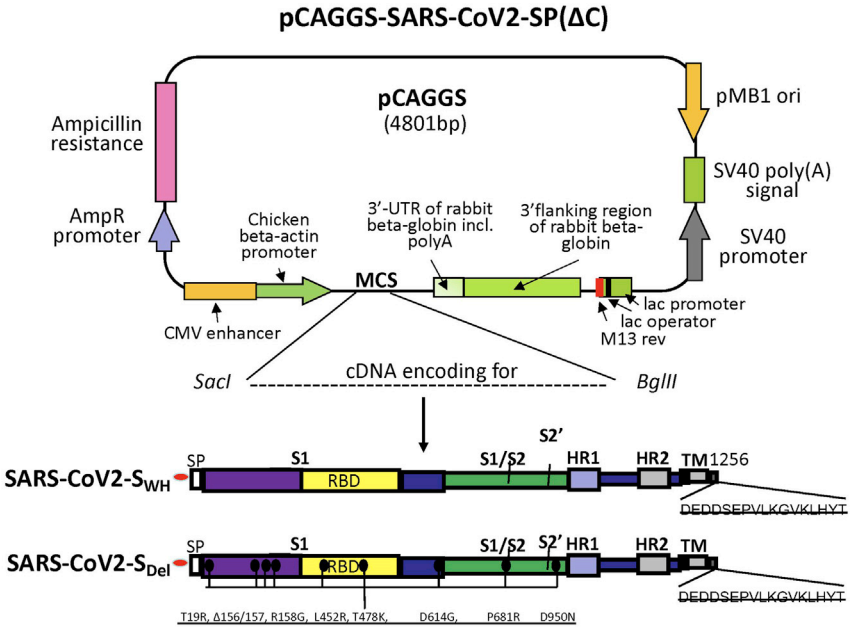

Figure 1. Schematic plasmid map of the pCAGGS plasmid expressing SARS-CoV2-S_{WH} or SARS-CoV2-S_{Del}

The cDNA of a C-terminal 17aa-deleted S from SARS-CoV-2 original strain (Wu-Han-1)(S_{WH}) or Delta variant (S_{Del}) was cloned into the plasmid though *SacI* and *BglII* restriction cleavage sites. MCS, multiple cloning sites.

The pCAGGS plasmids encoding for the optimized cDNA of the S from SARS-CoV-2 original strain (Wu-Han-1)(S_{WH}) or Delta variant (S_{Del}) were constructed as described previously[1] (Figure 1). Each SARS-CoV-2 S contains the C terminal 17 aa deletion (Figure 1). Other plasmids were purchased and listed in the key resources table.

1. Midi-Prepare the plasmids expressing SARS-CoV-2 S proteins, S_{WH}, and S_{Del}. The amount of plasmid needed is shown in the VLP production part (step 1 and step 4).
   a. Transform the plasmids into DH5α competent *E. coli* cells on Day 1.
      i. Thaw the DH5α competent cells on ice and take 50 μL of DH5α in a sterile pre-cold 1.5 mL tube. Refreeze the unused cells in a dry ice/ethanol bath for 5 min and return to the −80°C freezer.
      ii. Add 0.5 μL of the pCAGGS-S plasmid to the DH5 α cells and gently mix with the tip (not pipetting). Incubate the tube on ice for 30 min.
      iii. Heat shock cells at 42°C for 1 min and then put them on ice for 2 min. Add 950 μL of pre-warmed LB media and incubate the tube at 37°C for 1 h.
      iv. Spread 100 μL transformation on an Ampicillin (100 μg/mL) selective LB plate and incubate at 37°C overnight (8–18 h).

   Note: The time range for overnight in this protocol is 8–18 h.

   b. Day 2, pick up one colony from the transformation plate, and culture in 2 mL LB medium with shaking (250 rpm) overnight at 37°C.
   c. Day 3, take 0.5 mL of the above bacteria overnight culture to 50 mL LB medium and shake overnight at 37°C until the optical density at 600 nm (OD 600) read reaches 0.7–0.9.
   d. Day 4, midi-prep the plasmid from the culture by using a PureLink HiPure Plasmid Midiprep kit (https://www.thermofisher.com/order/catalog/product/K210004).

Note: The user guide of the PureLink HiPure plasmid DNA purification kit can be found here or use this link: https://www.thermofisher.com/document-connect/document-connect.html?

url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fpurelink_hipure_plasmid_dna_purification_man.pdf.

i. Centrifuge the DH5α culture (25–50 mL/tube) at 4000 *g* for 10 min and discard the medium. Add 4 mL R3 Buffer (with RNase A) to the cell pellet and resuspend the cells by vortex or pipetting until homogeneous.
ii. Add 4 mL L7 Buffer to lyse cells, invert the capped tube gently and mix thoroughly until homogeneous (less than 5 min).
iii. Add 4 mL N3 Buffer to precipitate the cellular debris. Invert the capped tube immediately until homogeneous. Centrifuge the mixture at 12,000 *g* for 10 min at room temperature.
iv. To equilibrate the column, place it in a flask with the Column Holder. Apply 10 mL EQ1 Buffer. Allow the solution to drain by gravity flow.
v. To bind DNA, load the cell lysate supernatant onto the equilibrated column and drain the column by gravity flow.
vi. Wash the column twice with 10 mL W8 Buffer. Drain the column by gravity flow after each wash. Discard the flow-through.
vii. To elute DNA, place a 15-mL tube under the column. Add 5 mL E4 Buffer to the column and drain by gravity flow. Purified DNA is in the elution tube.
viii. To precipitate the DNA, add 3.5 mL isopropanol to the tube, and mix well.

Pause point: The precipitation tube can be stored in a −20°C freezer overnight (8–18 h) or for days.

ix. Centrifuge the tube at 12,000 *g* for 30 min at 4°C, and carefully remove the supernatant.
x. Wash the DNA pellet with 3 mL 70% ethanol, and centrifuge at 12,000 *g* for 5 min at 4°C. Remove the supernatant and air-dry the DNA pellet for 10 min.
xi. Resuspend the DNA pellet in 200 μL TE Buffer or Nuclease-free ddH_2O.

e. Measure the plasmid DNA concentration by using the Nanodrop One spectrophotometer (Thermo Scientific). The machine calculates the DNA concentration based on the reads of OD280, OD260, and OD230 of plasmid DNA.
f. Adjust the plasmid concentration to 1 μg/μL. Store the plasmid at −20°C until use. Generally, about 500–800 μg of plasmid can be obtained from 50 mL of cell culture.

2. Midi-prepare the plasmids for VLP packaging. For HIV-based VLP, prepare the pCMVΔ8.2 that expresses the Gag-Pol. For SARS-CoV-2 VLP, prepare the pUNO1-SARS2-E (InvivoGen), pUNO1-SARS2-M and pCMV3-SARS2-NP-myc (Sino Biological). Procedures are similar to those above.

Preparation of 293T producer cells and human macrophages

Timing: 1 week

3. Prepare the cell culture complete media (500 mL/each), including Dulbecco's modified Eagle's (DMEM) and Roswell Park Memorial Institute (RPMI) 1640, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Store at 4°C for up to 1 month.
4. Prepare 293T producer cells.
   a. Defrost and culture the 293T cells (10^6/cryotube) in DMEM complete media.
   b. Passage the cells two or more times to obtain enough cells and adjust the cells to an actively growing status.
   c. Plate the cells in 10 cm dishes (1.8–2.5 ×10^6/dish) on the day before transfection.
5. Prepare human monocyte-derived macrophages (hMDMs) from peripheral blood mononuclear cells (PBMCs).
   a. To isolate PBMCs, collect whole blood from healthy donors in EDTA collection tubes, dilute the blood with PBS (1:1 v/v), and gently layer over an equal volume of Ficoll (Lymphoprep; Axis-Shield) in a Falcon tube.

b. Perform the density gradient centrifugation for 30–40 min at 400–500 *g* without brake. Collect PBMCs in the white and cloudy "blanket" layer, wash with PBS, and resuspend in DMEM (no FBS, with P/S). Pool PBMCs from different donors.
c. Because monocytes can attach to the plate but lymphocytes not, to purify the monocytes, incubate the PBMCs in the above medium in a 24-well flat-bottom plate (2.5×10^6 cells, 1 mL/well) at 37°C and 5% CO_2 for 2 h. Remove the supernatant and the floating lymphocytes.
d. To let the purified monocytes attached to the 24-well plate differentiate into macrophages, treat them with macrophage colony-stimulating factor (M-CSF, 10–50 ng/mL) in complete DMEM (1 mL/well) for 6–7 days. Replenish each well with 0.5 mL of new M-CSF medium supplemented every 2 days.

⚠ CRITICAL: To increase the efficiency of transfection and VLP production, the plasmids used in VLP packaging should be freshly prepared or not more than three months old.

⚠ CRITICAL: The macrophages do not proliferate in culture. After differentiation, hMDMs can be maintained for 2–3 weeks but can only be active for a short time (3–7 days). Therefore, the hMDMs preparation should be performed after the VLPs are well produced and characterized, and one week before the VLP treatment experiment (step 18 and step 24).

Alternatives: For hMDMs differentiation, the DMEM can be replaced by RPMI-1640.[4] However, for treatment, DMEM is preferable,[5] because nutrient factors in distinct media can affect macrophage activation.

Alternatives: This protocol tests the cytokine production from human Monocyte-Derived macrophages (hMDMs). It also can be used to test human Monocyte-Derived Dendritic Cells (MDDCs or moDCs) that are differentiated by the granulocyte-macrophage colony-stimulating factor (GM-CSF, 50 ng/mL) and IL-4 (20 ng/mL, R&D systems), or to test THP1-derived macrophages that can be differentiated by treating THP1 cells with phorbol 12-myristate 13-acetate (PMA, 100 nM) for 3 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal antibody against SARS-CoV-2 SP/RBD (1:2000)	Sino Biological	40592-T62
Recombinant human against SARS-CoV-2 S-NTD antibody (1:4000)	Elabscience	E-AB-V1030
Rabbit polyclonal anti-HIV-1 SF2 p24 antiserum (1:10000)	NIH HIV Reagent Program	ARP-4250
Donkey anti-rabbit IgG-HRP secondary antibody (1:6000)	GE Healthcare	NA934
Sheep anti-mouse IgG-HRP secondary antibody (1:6000)	GE Healthcare	NA931
Sheep anti-human IgG-HRP secondary antibody (1:4000)	GE Healthcare	NA933
Mouse monoclonal antibody against SARS-CoV-2 Spike RBD (clone#1034515) (1:2000)	R&D Systems	MAB105401
Rabbit monoclonal antibody against SARS-CoV-2 Nucleoprotein (NP) (1:2000)	Sino Biological	40143-R019
Bacterial and virus strains		
E. coli DH5α competent cells	Invitrogen	18265017
SARS-CoV-2 SPΔC pseudotyped HIV-1-pseudoviruses (PVLPs)(Wu-Han-1, Delta)	This study	N/A
SARS-CoV-2 SPΔC pseudotyped virus-like particles (VLPs)(Wu-Han-1, Delta)	This study	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Healthy human peripheral blood mononuclear cells (hPBMCs)	This study	N/A
Chemicals, peptides, and recombinant proteins		
Lymphoprep (Ficoll)	Axis-Shield Poc As	1114547
macrophage colony-stimulating factor (M-CSF)	R&D System	416-ML-010
Polyethylenimine (PEI)	Sigma-Aldrich	408727
Triton X-100	Sigma-Aldrich	T8787
Lipofectamine 3000 transfection reagent	Invitrogen	L3000001
Trypsin-EDTA (0.05%)	Gibco	25300062
DMEM	Gibco	11995065
RPMI 1640	Gibco	11875093
Fetal bovine serum (FBS)	Gibco	26140079
Penicillin-Streptomycin (10000 U/mL)	Gibco	15140122
Opti-MEM reduced serum	Gibco	31985062
TGX FastCast Acrylamide Kit, 10%	Bio-Rad	1610173
4× Laemmli Sample Buffer	Bio-Rad	1610747
TMB Chromogen solution for ELISA	Invitrogen	002023
ECL Western Blotting Substrate	Promega	W1001
Protease inhibitor cocktail	Promega	G6521
PureLink DNase I	Invitrogen	12185-010
Critical commercial assays		
Pierce bicinchoninic acid (BCA) Protein assay kit	Thermo Scientific	23227
Human SARS-CoV-2 RBD ELISA Kit	Thermo Scientific	EH492RB
HIV p24 Antigen Capture Assay kit	AIDS and Cancer virus Program (NCI)	N/A
V-PLEX proinflammatory Panel 1 (human) Kit	Meso Scale Discovery	K15049D-1
PureLink RNA mini kit	Invitrogen	12183020
SuperScript VILO MasterMix	Invitrogen	11755050
PowerUp SYBR Green Master Mix	Applied Biosystems	A25742
PureLink HiPure Plasmid Midiprep Kit	Invitrogen	K210004
Experimental models: Cell lines		
293T	ATCC	CRL-3216
293TN	System Biosciences	LV900A-1
Oligonucleotides		
hIFNγ-F/R (CAGGTCATTCAGATGTAGCGGAT, ACTCTCCTCTTTCCAATTCTTCAAAA), 71 bp	Invitrogen	N/A
hTNFα-F/R (CAGGTCCTCTTCAAGGGCCAA, GGGGCTCTTGATGGCAGAGA), 120 bp	Invitrogen	N/A
hIL-1β-F/R (ACAGATGAAGTGCTCCTTCCA, GTCGGAGATTCGTAGCTGGAT), 73 bp	Invitrogen	N/A
hIL-6-F/R (ACTCACCTCTTCAGAACGAATTG, CCATCTTTGGAAGGTTCAGGTTG), 149 bp	Invitrogen	N/A
hIL-10-F/ R (AAGGCGCATGTGAACTCCCT, CCACGGCCTTGCTCTTGTTTT), 103 bp	Invitrogen	N/A
hGAPDH-F/R (ACAAC TTTGGTATCGTGGAAGG, GCCATCACGCCACAGTTTC, 101 bp	Invitrogen	N/A
Recombinant DNA		
HIV helper packaging plasmid pCMVΔ8.2 encoding the HIV Gag-Pol (pCMV Delta R8.2)	Addgene	12263
SARS-CoV-2 (Wu-Han-1) spike protein expression plasmid (pCAGGS-SARS2-S_{WH} or pCAGGS-nCoVSPΔC)	Ao et al., 2021[6]	N/A
gene encoding $SP\Delta C_{Delta}$	Genscript	N/A
pCAGGS-$SP\Delta C_{Delta}$	Ao et al., 2022[1]	N/A
SARS-CoV-2-M expression plasmid (pUNO1-SARS2-M)	Sino Biological Inc.	puno1-cov2-m
SARS-CoV-2-E expression plasmid (pUNO1-SARS2-E)	Sino Biological Inc.	puno1-cov2-e
SARS-CoV-2 (2019-nCoV) Nucleoprotein expression plasmid, C-Myc tag (Codon Optimized), pCMV3-N	Sino Biological Inc.	VG40588-CM

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
ImageJ	Schneider et al., 2012[7]	https://imagej.nih.gov/ij/
Prism version 9	GraphPad	http://www.graphpad.com/scientific-software/prism/
Other		
Beckman Coulter ultracentrifuge XPN and type 70Ti rotor	Beckman Coulter	A94468, 337922
Western Blot Imager MicroChemi (with GelCapture)	DNR Bio Imaging Systems	MicroChemi
ELISA microplate reader SpectraMax (with SoftMax Pro)	Molecular Devices	M3
Meso QuickPlex SQ 120MM (with Discovery workbench 4.0)	Meso Scale Discovery	AI1AA-0
Real-time PCR thermocycler Stratagene MX3000P (with software MxPro)	Agilent	MX3000P

MATERIALS AND EQUIPMENT

Stock solutions and aliquots preparation		
Reagents	Stock concentration	Preparation procedures
Polyethylenimine (PEI)	1 mg/mL	Dissolve 50 mg in 50 mL sterile ddH_2O, and slowly add HCl to adjust to pH 7.0. Sterilize with a filter (0.22 μm). Aliquot 1 mL/each. Store at -80°C or -20°C for up to 1 year, or 4°C for up to 3 months Yang et al.[8]
Phosphate-Buffered Saline with Tween 20 (PBST)	10× (1.37 M NaCl, 27 mM KCl, 119 mM phosphates, and 0.5% Tween 20)	Dissolve the PBS 10× pH7.4 powder (Fisher BioReagents, #FLBP6651) and 5 mL Tween 20 in 1 L ddH_2O. Store at 4°C for 1 year.
Tris-Buffered Saline with Tween 20 (TBST)	10× (0.2 M Tris-HCl, 1.5 M NaCl, and 0.5% Tween 20)	Dissolve 24 *g* Tris-base, 88 *g* NaCl and 5 mL Tween 20 in 1 L ddH_2O. Adjust pH to 7.4. Store at 4°C for 1 year.
Tris-Glycine buffer	10× (250 mM Tris, 1.924 M glycine)	Dissolve 30.3 *g* Tris base and 144.4 *g* glycine in 1 L ddH_2O. Check pH 8.3. Store at room temperature for up to 1 year.
Tris-Glycine-SDS buffer	10× (250 mM Tris, 1.924 M glycine and 35 mM SDS)	Dissolve 30.3 *g* Tris base, 144.4 *g* glycine, and 10 *g* SDS in 1 L ddH_2O. Check pH 8.3. Store at room temperature for up to 1 year.

Note: The room temperature range is 15°C–28°C.

RIPA lysis buffer		
Reagent	Final concentration	Amount
Sodium chloride (5 M)	150 mM	3 mL
Tris-HCl (1 M, pH 8.0)	25 mM	2.5 mL
Tween 20	1%	1 mL
Sodium deoxycholate (10%)	0.5%	5 mL
SDS (10%)	0.1%	1 mL
ddH_2O	N/A	87.5 mL
Total	**N/A**	**100 mL**

Note: Sterilize with a 0.45-μm filter. Store at 4°C for up to 1 year. Add protease inhibitor cocktail immediately before use.

Coupling buffer (pH 9.6, carbonate-bicarbonate buffer)		
Reagent	Final concentration	Amount
Sodium Bicarbonate ($NaHCO_3$)	69 mM	5.76 *g*
Sodium Carbonate (Na_2CO_3)	31 mM	3.33 *g*
ddH_2O	N/A	1 L
Total	**N/A**	**1 L**

Note: Sterilize with a 0.45-μm filter. Store at 4°C for up to 1 month.

Sample diluent buffer		
Reagent	Final concentration	Amount
FBS	1%	1 mL
Tween 20 (10%)	0.2%	2 mL
RPMI 1640	N/A	97 mL
Total	N/A	100 mL

Note: Sterilize with a 0.45-μm filter. Store at 4°C for up to 1 month.

Primary antibody diluent buffer		
Reagent	Final concentration	Amount
FBS	10%	10 mL
BSA (5%)	0.2%	4 mL
Tween 20 (10%)	0.01%	0.1 mL
RPMI 1640	N/A	85.5 mL
Total	N/A	100 mL

Note: Sterilize with a 0.45-μm filter. Store at 4°C for up to 1 month.

Secondary antibody diluent buffer		
Reagent	Final concentration	Amount
BSA (5%)	0.5%	10 mL
Tween 20 (10%)	0.01%	0.1 mL
RPMI 1640	N/A	89.9 mL
Total	N/A	100 mL

Note: Sterilize with a 0.45-μm filter. Store at 4°C for up to 1 month.

Other solutions and media		
Name	Reagent	Storage
DMEM complete	10% Fetal Bovine Serum (FBS, v/v), 1% Penicillin (10000 I.U./mL), and Streptomycin (10 mg/mL) solution (v/v) in DMEM	4°C
RPMI 1640 complete	10% Fetal Bovine Serum (FBS, v/v), 1% Penicillin (10000 I.U./mL), and Streptomycin (10 mg/mL) solution (v/v) in RPMI 1640	4°C
Sucrose cushion	20% sucrose (w/v) in PBS (1×), sterilized with a 0.45 μm filter	4°C
Triton X-100	10% Triton X-100 in PBS (1×), sterilized with a 0.45 μm filter	4°C
5× sample buffer*	5% SDS (w/v), 200 mM Tris pH 6.8, 25% glycerol (v/v), 1.43 M β-mercaptoethanol, 0.032% bromophenol blue (w/v)	4°C or −20°C
SDS-PAGE running buffer	Tris-Glycine-SDS buffer (1×)	Room Temp.
Transfer buffer	Tris-Glycine buffer (1×), 20% methanol (v/v)	4°C
Blocking buffer 1 (Western Blot)	5% skimmed-milk (w/v) in PBST (1×)	Freshly prepared
Blocking buffer 2 (ELISA)	1% BSA (w/v) in TBS (1×); aliquoted 10 mL for each use	−20°C, thaw before use
ELISA stop buffer	1N HCl	4°C

Note: Store the media at 4°C for up to 1 month. For other buffers or solutions, store at 4°C or room temperature for up to 1 year, or store at −20°C for up to 2 years.

Alternatives: 4× Laemmli Sample Buffer (Bio-Rad, #1610747).

⚠ CRITICAL: Triton X-100 is a nonionic surfactant. It can cause acute toxicity if swallowed, skin irritation, and eye damage. When handling, wear personal protective equipment (PPE) (gloves and goggles) for protection.

⚠ CRITICAL: Sodium dodecyl sulfate (SDS) is an anionic detergent. It is a flammable solid and can irritate the skin, eyes, and respiratory system. When handling, keep away from heat, avoid inhaling dust, and wear PPE (gloves, goggles, and mask) for protection.

⚠ CRITICAL: Acrylamide in the TGX SDS-PAGE solution before polymerization can cause acute toxicity and carcinogenicity. When handling, wear PPE (gloves and lab coat) for protection.

⚠ CRITICAL: β-Mercaptoethanol in the SDS-PAGE sample buffer can cause acute toxicity to many organs and irritation to the skin and eyes. It is also flammable. When handling, keep away from heat, wear PPE (glove and lab coat) and manipulate it in the chemical fume hood.

Critical equipment

Name	Perform conditions
Cell culture incubator	37°C, 5% CO_2
Biological Safety Cabinet	BSL-2
Ultracentrifuge	e.g., Beckman 70Ti rotor, 100,000–120,000 × *g*, 4°C, 1–2 h
Electrophoresis Power Supply and cassettes	SDS-PAGE (120 V, 90 min), transfer (100V, 90 min, ice-bath)
Western Blot Imager	e.g., MicroChemi (DNR Bio-imaging Systems), software GelCapture
ELISA microplate reader	e.g., SpectraMax (Molecular Devices) and software SoftMax Pro
Meso QuickPlex SQ 120MM	Software Discovery workbench 4.0 (Meso Scale Discovery)
Real-time PCR thermocycler	e.g., Stratagene MX3000P (Agilent) and software MxPro

STEP-BY-STEP METHOD DETAILS

Produce SARS-CoV-2 VLPs

Timing: 4 days

The SARS-CoV-2 (SARS2) Virus-Like Particles (VLPs) incorporated with S protein from the S_{WH} or S_{Del} are produced in 293TN or 293T cells by using co-transfection methods, as previously described.[9] After the purification of SARS2-VLP-S_{WH} and SARS2-VLP-S_{Del}, these VLPs need to be characterized by western blotting (WB) and quantified by ELISA before using them to treat macrophages.

1. 293T cells plated in a 10 cm dish are co-transfected with plasmids expressing SARS-CoV-2 N (6.7 μg), M (3.3 μg), E (3.3 μg), and S (2.5 μg) by Polyethylenimine (PEI) on Day 1.
   a. Seed 293T cells in a 10 cm dish (1.8–2.5 ×10^6/dish) on Day 0 to reach 80% confluency on Day 1.
   b. Before transfection, change the cell culture medium with 5 mL of Opti-MEM or serum-free plain DMEM.
   c. Prepare two 1.5 mL Eppendorf tubes, and label them as "DNA" and "PEI". Thaw the plasmids prepared in the "before you begin" section (step 1 and step 2).
   d. In the DNA tube, add plasmids expressing SARS-CoV-2 N, M, E, and S (S_{WH} or S_{Del}) of the following amounts:
   pCMV3-SARS2-NP-myc 6.7 μg.
   pUNO1-SARS2-M 3.3 μg.

pUNO1-SARS2-E 3.3 μg.
pCAGGS-SARS2-S (S_{WH} or S_{Del}) 2.5 μg.
The total DNA amount is 15.8 μg. Dilute plasmid DNA with 158 μL H_2O (final concentration: 0.1 μg/μL). Vortex for 5 s and spin briefly.

e. Prepare 0.1 μg/μL PEI solution in the PEI tube by diluting 10 μL PEI stock (1 mg/mL) in 90 μL of PBS or medium. Add 17.38 μL PEI solution into the DNA tube (1.1 μL of PEI/1 μg of DNA). Vortex for 5 s and spin briefly.
f. Incubate the mixture for 15 min at room temperature to allow the DNA/PEI complex formation.
g. Add the PEI/DNA complex dropwise into the 293T cell dish and gently swirl the dish. Culture the co-transfected cells at 5% CO_2, 37°C for 2–3 h.
h. Remove the PEI/DNA-complex containing medium, add 10 mL of complete DMEM and continue culture for a total of 72 h.
    i. Collect the supernatant at 48 h in 50 mL tubes, and store at −80°C. Add 10 mL of new complete DMEM to cells in each dish and continue to culture.
    ii. Collect the supernatant again at 72 h. Store at −80°C or directly use for following purification steps.

Note: The PEI working solution (0.1 μg/μL) is freshly prepared for the same experiment. The PEI stock solution (1 mg/mL) store at −80°C or −20°C for up to 1 year, or at 4°C for up to 3 months.

Alternatives: Lipofectamine 3000 can be used instead of PEI. In this case, add the DNA plasmids, 30 μL P3000, and 250 μL of Opti-MEM in the "DNA" tube. Add 15 μL Lipofectamine 3000 and 250 μL Opti-MEM in the "Lipo" tube. Vortex, spin briefly and sit for 5 min at room temperature. Add the DNA solution to the Lipo tube. Vortex, spin briefly and incubate for 15 min at room temperature. Add the Lipo/DNA mixture dropwise into the cells, incubate for 4–6 h at 5% CO_2, 37°C, and change medium and culture as above described (step 1-g, 1h).

Alternatives: 293TN cells can be used to produce VLPs with higher production.

Alternatives: The supernatant collected at 48 h generally contains the major part of the VLPs that can be produced from the transfected cells, so it is optional to collect the supernatant at 72 h.

Pause point: The supernatants can be stored at −80°C for 4–8 weeks.

Note: The number of 10 cm dishes with cells used for VLP production depends on the yield and the amount of VLPs needed. Generally, the startup is 2 dishes of cells.

2. Purify the SARS2-VLPs by ultra-centrifugation in a Beckman 70Ti rotor.
    a. Thaw the SARS2-VLPs containing supernatants collected at 48 h and 72 h post-co-transfection, and combine them in 50 mL tubes.
    b. Clarify the supernatant with light centrifugation at 1000 × *g* for 15 min at room temperature, filter through a 0.45 μm membrane.
    c. Load the supernatant (20 mL) on the top of a 20% sucrose cushion (2–4 mL), ultracentrifuge at 100,000–120,000 × *g*, at 4°C for 3 h using a Beckman Type 70Ti rotor.
    d. Resuspend SARS2-VLPs containing pellet carefully in 100 μL of RPMI-1640 plain medium for each ultracentrifuge tube. Mix all the VLPs solutions, aliquot (30μL /tube), and store at −80°C.

Note: If the VLPs solution is turbid after ultracentrifugation, see troubleshooting 1.

⚠ CRITICAL: The tube balance before ultracentrifugation is very important. The removal of supernatant after ultracentrifugation should be done carefully and as soon as possible.

Because the pellet may dislodge over time, it is recommended to remove the supernatant immediately and finish all the tubes within 5–10 min.

3. Collect producer cells in cold PBS and spin down (1000 × *g*, 5 min at 4°C). See step 7.

Produce HIV-based SARS-CoV-2 S-pseudotyped VLPs

Timing: 4 days

HIV-based Pseudotyped Virus Like Particles (PVLPs) incorporated with SARS-CoV-2 S protein from the S_{WH} or S_{Del} is produced in 293T cells by using co-transfection methods, as previously described.[1] After the purification of PVLP-S_{WH} and PVLP-S_{Del}, these PVLPs needed to be characterized by WB and quantified by ELISA before the treatment of macrophages.

4. The 293T cells plated in a 10 cm dish are co-transfected with plasmids expressing HIV-1 Gag-Pol (5.0 μg) and SARS-CoV-2 S (2.5 μg) by Polyethylenimine (PEI) on Day 1.
   a. Seed the 293T cells in a 10 cm dish (1.8–2.5 ×10^6/dish) on Day 0 to reach 80% confluency on Day 1.
   b. Before transfection, change the cell culture medium with 5 mL of Opti-MEM or serum-free plain DMEM.
   c. Prepare two 1.5 mL Eppendorf tubes, and label them as "DNA" and "PEI".
   d. In the DNA tube, add plasmids expressing HIV-1 Gag-Pol and SARS-CoV-2 S (S_{WH} or S_{Del}) in the following amounts:
      pCMV-Δ8.2 5.0 μg.
      pCAGGS-SARS2-S (S_{WH} or S_{Del}) 2.5 μg.
      The total DNA amount is 7.5 μg. Dilute plasmid DNA with double distilled H_2O into 75 μL (0.1 μg/μL). Vortex for 5 s and spin briefly.
   e. Prepare 0.1 μg/ μL PEI solution by diluting the 10 μL of PEI stock (1 mg/mL) in 90 μL of PBS or medium in the PEI tube. Take 8.25 μL PEI solution into the DNA tube (1.1 μL of PEI/1 μg of DNA). Vortex for 5 s and spin briefly.
   f. Incubate together for 15 min at room temperature to allow the DNA/PEI complex to form.
   g. Add the PEI/DNA complex dropwise into the 293T cell dish and gently swirl the dish. Culture the co-transfected cells at 5% CO_2, 37°C for 2–3 h.
   h. Remove the PEI/DNA-containing medium, add 10 mL of complete DMEM and continue culture for a total of 72 h.
      i. Collect the supernatant at 48 h in 50 mL tubes, and store at −80°C. Add 10 mL of new complete DMEM to cells in each dish and continue to culture.
      ii. Collect the supernatant again at 72 h. Store at −80°C or directly use for following purification steps.

Note: The negative control HIV-VLP-Gag is produced at the same time via transfection without the SARS2-S plasmid. This empty HIV-VLP does not have the HIV envelope protein and SARS2-S.

Alternatives: Lipofectamine 3000 can be used instead of PEI. In this case, the DNA plasmids are diluted in 250 μL of Opti-MEM and mixed with 15 μL P3000 in the "DNA" tube. Add 7.5 μL Lipofectamine 3000 and 250 μL Opti-MEM in the "Lipo" tube. Vortex, spin briefly and sit both tubes for 5 min at room temperature. Add DNA solution into the Lipo tube. Vortex, spin briefly and incubate for 15 min at room temperature. Add the Lipo/DNA mixture dropwise into the cells, incubate for 4–6 h at 5% CO_2, 37°C, and change medium and culture as above described.

Alternatives: The supernatant collected at 48 h generally contains the major part of the VLPs that can be produced from the transfected cells, so it is optional to collect the supernatant at 72 h.

Pause point: The supernatants can be stored at −80°C for 4–8 weeks.

5. Purify the HIV-PVLPs by ultra-centrifugation in a Beckman 70Ti rotor same as in step 2.
   a. Thaw the PVLPs containing supernatant collected at 48 h and 72 h post-co-transfection, and combine them in 50 mL tubes.
   b. Clarify the supernatant with light centrifugation at 1000 × *g* for 10 min at room temperature, filter through a 0.45 μm membrane.
   c. Load the supernatant (20 mL) on the top of a 20% sucrose cushion (2–4 mL), ultracentrifuge at 90,000 × *g* (or 35000 × rpm), 4°C for 1.5 h using a Beckman Type 70Ti rotor.
   d. Resuspend PVLPs-containing pellet carefully in 100 μL of plain RPMI-1640. Mix all the PVLPs solution, an aliquot (30 μL/tube), and store at −80°C.

Note: If the PVLPs solution is turbid after ultracentrifugation, see troubleshooting 1.

⚠ CRITICAL: The removal of supernatant after ultracentrifugation should be done carefully and as soon as possible.

6. Collect producer cells in cold PBS and spin down (1000 × *g*, 5 min). See step 7.

Verify viral protein expressions in producer cells and VLPs by western blotting

Timing: 2 days

WB is performed to determine the S (S_{WH} and S_{Del}) expression/incorporation and the VLPs (SARS2-VLP and HIV-PVLP) formation. The expression of S, the SARS-CoV-2 VLP's core protein N (Nucleocapsid), and HIV Gag-p24 capsid in producer cells and VLPs/PVLPs samples are verified. The incorporation efficiency of different S proteins in VLPs is semi-quantified by comparing the ratio of S/N or S/p24 through the software ImageJ.

7. Prepare samples for Western Blot.
   a. Prepare the VLP samples.
      i. Defrost the SARS2-VLP and HIV-PVLP samples.
      ii. Mix 30 μL (2–10 μg total protein) of SARS2-VLP or HIV-PVLP with 10 μL of sample loading buffer (4×).
      iii. Boil the samples in capped tubes for 10 min in a boiling water bath.
   b. Prepare the producer cell samples.
      i. Wash the producer 293TN or 293T cells (5×10^6) with cold PBS.
      ii. Lyse cells in chilled 500 μL of RIPA lysis buffer containing 1× protease inhibitor (Promega) for 30 min on ice. Vortex every 15 min.
      iii. Centrifugate at 10,000 × *g* for 10 min at 4°C. The supernatant is transferred to a new tube.
      iv. Mix 30 μL of cell lysate with 10 μL of sample loading buffer (4×) and boil for 10 min.

Note: In the tubes of boiled samples, the evaporated water will condense on the top. To homogenize the samples, spin the tubes for 10 s, vortex for 10 s, and spin for 10 s again.

Note: The producer cell samples are used to determine if the transfection is successful and if plasmids can express proteins. One positive control for S, N, and p24 protein is needed in the same gel to confirm that the antibodies work well. Generally, the positive control is the commercial protein or peptide, or the cell lysate of cells transfected with the commercial expression plasmid.

Note: Store RIPA lysis buffer at 4°C for up to 1 year. Store sample loading buffer at 4°C for 1 year or −20°C for up to 2 year.

8. Run SDS-PAGE and transfer:
   a. Load the SARS2-VLP or HIV-PVLP of 20–40 μL (boiled sample) and producer cell lysates of 20–40 μL (boiled sample) to wells of SDS-PAGE gel (Bio-Rad, 10% TGX FastCast gel).
   b. Run the SDS-PAGE in a running buffer (1×) (120 V, ~90 min).

   Alternatives: The total protein concentration of VLP/PVLP samples or cell lysates can be quantified by using the Pierce bicinchoninic acid (BCA) protein assay kit to normalize the loading amount for SDS-PAGE. Load VLP/PVLP samples of 1–5 μg (total protein) or cell lysates of 10–30 μg (total protein) in each well of the gel.

   Note: Store the stock (10× Tris-Glycine-SDS buffer) and working running buffer at room temperature for up to 1 year.

   c. Wet transfer the proteins in gel to a nitrocellulose membrane (Bio-Rad, 0.45 μm pore size; 100 V, ~90 min) in an ice-cold transfer buffer.
   d. Block the membrane in 5% skimmed milk (in PBST) with gentle shaking for 1–2 h at room temperature or at 4°C overnight (8–18 h) with shaking (waving shaker, 20–30 rpm).

   Pause point: The membrane blocking can be performed at 4°C overnight with shaking.

   Note: Store the transfer buffer at 4°C for up to 1 year.

9. Antibody incubation and protein detection:

   Detect the S proteins (S_{WH} and S_{Del}) by incubating with human anti-S-NTD antibody (Elabscience; diluted in PBST, 1:4000) at room temperature for 2 h or at 4°C overnight (8–18 h) with shaking.

   a. Detect the N protein in SARS2-VLP by incubating with rabbit anti-SARS-CoV-2 N antibody (Sino Biological; diluted in PBST, 1:2000).
   b. Detect the HIV p24 in HIV-PVLP by incubating with rabbit anti-HIV Gag p24 antibody (NIH HIV Reagent Program; diluted in PBST, 1:10000).

   Alternatives: The S protein also can be detected with rabbit anti-RBD polyclonal antibody or mouse anti-RBD monoclonal antibody. The SARS2-VLP N protein also can be detected with rabbit anti-Myc antibodies.

   Note: The membrane can be cut into horizontal strips according to the appropriate protein size. For example, the S protein (full length is 180 kDa and S1 is 100 kDa) strip is from 65 kDa to the top edge of the membrane. The N protein (46 kDa) strip is from 30 kDa to 65 kDa. The HIV p24 protein (mature form 24 kDa; immature p55, 55 kDa) strip is from the bottom edge of the membrane to 60 kDa.

   Pause point: The membrane can be incubated in the primary antibody at 4°C overnight (8–18 h).

   Note: Store the stock PBST (10×) buffer at 4°C for up to 1 year. Store the working PBST (1×) buffer at room temperature for up to 3 months.

   c. Wash the transferred membrane 3 times at room temperature with shaking, in 20 mL PBST (1×) for 10 min per wash.
   d. Incubate the membrane with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG-HRP is used at 1:6000 dilution, anti-human IgG-HRP is used at 1:4000 dilution) for 1 h at room temperature with shaking. Wash 3 times with PBST.

e. The membrane is developed with ECL Substrate solution (Promega, #W1001) and exposed by using an Imager or film. Save the images for further data analysis.

Note: If the S protein is not or is weakly detected, see troubleshooting 2.

Note: If the N protein or p24 protein is not or is weakly detected, see troubleshooting 3.

10. Analyze the S incorporation efficiency with the software ImageJ.
    a. WB detects S protein in forms of both full-length (S) and cleaved (S1), so the sum of S and S1 represents the total S expression. When circling the bands of S, both two bands (S-180kDa and S1-100kDa) must be included.
    b. The density of S bands and the density of VLP capsid proteins (N of SARS2-VLP and p24 of HIV-PVLP) in the same sample (lane) are used to calculate the density ratio (S/N or S/p24). This ratio indicates the S incorporation efficiency in VLPs or PVLPs.
    c. Compare the ratio of S_{WH} and S_{Del} and use this to monitor the quality of VLPs used for further treatment experiments.

Note: The difference in antibody dilution, incubation/wash condition, and exposure time may affect the ratio of S/N or S/p24. This ratio is a relative parameter to monitor the S incorporation in the VLPs or PVLPs.

Quantify the S protein amount in SARS2-VLPs and HIV-PVLPs by ELISA

Timing: 2 days

The sandwich ELISA (enzyme-linked immunosorbent assay) is performed to quantify the S protein amount in SARS2-VLPs and HIV-PVLPs by using the human SARS-CoV-2 RBD ELISA Kit (Invitrogen, # EH492RB). The protein amount of S (RBD) is calculated by the curve-fitting software (SoftMax Pro) based on the standard curve. The S concentration is used to normalize the VLPs or PVLPs amount for the macrophage treatment experiment.

11. Dilute SARS2-VLP and HIV-PVLP samples.
    a. Dilute the SARS2-VLP and HIV-PVLP samples of 10 μL serially in Sample Diluent buffer from 10× to 100× dilution, and then dilute 2× serially from 100× to 200×, 400× and 800× dilution (see Figure 2).
    b. Use the four dilutions from 100× to 800× for ELISA detection. Add Triton X-100 (10%, v/v) into these samples to a final 1% concentration to lyse the VLP or PVLP samples. Incubate at 37°C for 0.5–1 h.
    c. The negative VLP control (HIV-VLP-Gag) is also prepared as described above.

12. Perform ELISA according to the manufacturer's instructions for the kit.

Note: The following description is brief. For more details, please refer to the manufacturer's instructions. (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FEH492RB.pdf).

    a. Standards are prepared from stock (25 ng/mL) by serial dilution and the standard concentrations are 1.5 ng/mL, 0.6 ng/mL, 0.24 ng/mL, 96 pg/mL, 38.4 pg/mL, 15.36 pg/mL, 6.14 pg/mL, and 0 pg/mL.
    b. Add 100 μL diluted and lysed samples (100×, 200×, 400× and 800×) to each well in the coated ELISA plate. Each dilution has two duplicates.
    c. Add standards in wells beside the samples (100 μL/well) and take one well as blank control.

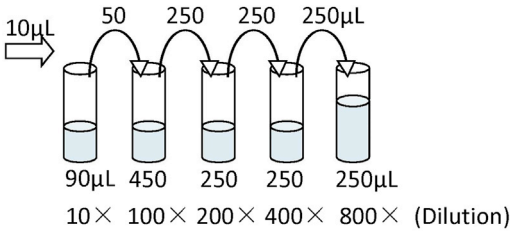

Figure 2. Serial dilution of VLP/PVLP samples for ELISA detecting SARS2-S

10 μL of purified VLPs or PVLPs are first diluted in 90 μL of sample diluent buffer to obtain the 10× dilution. 50 μL of this 10× dilution samples are transferred to the next tube with 450 μL of sample diluent buffer to obtain the 100× dilution. Next, 2 × serial dilutions are performed to obtain 200×, 400×, and 800× dilutions.

d. Cover wells with adhesive microplate-sealing film and incubate for 2.5 h at room temperature or overnight (8–18 h) at 4°C.

Pause point: The ELISA plate-loaded samples and standards can be incubated at 4°C overnight.

e. Wash the plate with wash buffer (300 μL/well) four times. Blot on paper towels.
f. Add primary antibody-biotin conjugate (100 μL/well; 1:80 diluted in assay diluent buffer) and incubate at room temperature for 1 h with gentle shaking.
g. Wash the plate four times. Add Streptavidin-HRP solution (100 μL/well; 1:200 diluted in assay diluent buffer within 15 min of usage) and incubate at room temperature for 45 min.
h. Wash the plate four times. Add TMB Substrate (100 μL/well) and incubate in the dark for 5–30 min.
i. Investigate the color of the substrate that turns blue. When the color is deep enough, add the stop solution (50 μL/well) to terminate the reaction. The color changes to yellow.

13. Read the absorbance at 450 nm (OD450) of each well through the ELISA microplate reader (SpectraMax, Molecular Devices) within 1 h after adding the stop solution.

Note: If the reads of standards are fine but the reads of samples are too low or higher than the highest standard concentration, see troubleshooting 4.

Note: If the reads of standards and samples all are weak or strong, see troubleshooting 5.

14. The software (SoftMax Pro) of the microplate reader is used to plot the standard curve and calculate the S protein concentration in SARS2-VLP and HIV-PVLP samples.

Quantification of Gag-p24 protein in HIV-PVLPs by ELISA

Timing: 2 days

HIV-Gag-p24 capsid protein in HIV-PVLPs is quantified by the HIV-1 p24 antigen capture assay ELISA kit (NCI-Frederick, AIDS, and Cancer Virus Program). The p24 concentration is used to normalize the HIV-PVLPs amount for the macrophage treatment experiment.

15. Dilute samples and standards.
    a. Dilute HIV-PVLPs samples of 10 μL serially in Sample Diluent buffer (1×) to 250× dilution, and then 2× serially dilute from 250× to 500×, 1000× and 2000× dilution (see Figure 3).
    b. Use the four dilutions from 250× to 2000× for detection. Add Triton X-100 (10%, v/v) into these samples to a final 1% concentration to lyse the PVLP samples and vortex well. Incubate at 37°C for 1 h.
    c. The negative VLP control HIV-VLP-Gag is also prepared as described above.
    d. Standard p24 protein (detergent-lysed HIV-1, with known p24 concentration) is prepared by 2× serial dilution and the concentrations used for ELISA start from 10000 pg/mL to 5000 pg/mL, 2500 pg/mL, 1250 pg/mL, 625 pg/mL, 312.5 pg/mL, 156.3 pg/mL, and 0 pg/mL.

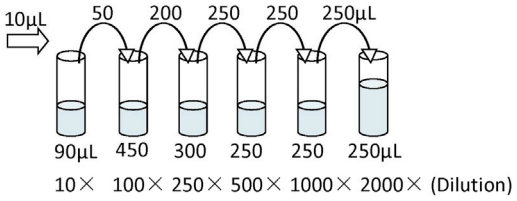

Figure 3. Serial dilution of PVLP samples for ELISA detecting HIV-Gag-p24

10 μL of purified PVLPs are first diluted in 90 μL of sample diluent buffer to obtain the 10× dilution. 50 μL of this 10× dilution samples are transferred to the next tube with 450 μL of sample diluent buffer to obtain the 100× dilution, and then the 250× dilution is obtained in the third tube. After that, 2 × serial dilutions are performed to obtain 500×, 1000×, and 2000× dilutions.

16. Perform ELISA according to the manufacturer's instruction for the kit.

Note: The following description is brief. For more details please refer to the manufacturer's instruction (to get the instruction, the contact information is in the link https://frederick.cancer.gov/research/aids-and-cancer-virus-program/cores/biological-products-core).

a. Pre-wash the ELISA plate with TBST (1×) two times (150 μL/well).
b. Add 100 μL diluted and lysed samples (250×, 500×, 1000× and 2000×) to each well in the coated ELISA plate. Each dilution has two duplicates.
c. Add standards in wells beside the samples (100 μL/well) and take one well as blank control and one as background control.
d. Seal the plate with adhesive film and incubate for 2 h at 37°C or overnight (8–18 h) at 4°C with gentle shaking.

Pause point: The ELISA plate loaded with samples and standards can be incubated at 4°C overnight.

Note: Store the stock TBST (10×) buffer at 4°C for up to 1 year. Store the working TBST (1×) buffer at 4°C for up to 3 months.

e. Wash the plate with TBST (1×) five times (150 μL/well). Add primary antibody (rabbit anti-HIV-1 p24 provided in the kit, 1:100 diluted in primary antibody diluent buffer) 100 μL/well and incubate at 37°C for 1 h.
f. Wash the plate five times.
g. Add secondary antibody (goat anti-rabbit IgG-HRP provided in the kit, 1:50 diluted in secondary antibody diluent buffer) 100 μL/well and incubate at 37°C for 1 h.
h. Wash the plate five times. Add TMB Substrate (100 μL/well) and incubate in the dark for 5–30 min. When the color is deep enough, add the stop solution (1N HCL; 100 μL/well) to stop the reaction.
i. Read the absorbance at 450 nm (OD450) of each well through the ELISA microplate reader (SpectraMax, Molecular Devices) within 1 h after adding the stop solution.

Note: If the reads of standards are fine but the reads of samples are too low or higher than the highest standard concentration, see troubleshooting 4.

Note: If the reads of standards and samples all are weak or strong, see troubleshooting 5.

17. The software (SoftMax Pro) of the microplate reader is used to plot the standard curve and calculate the p24 protein concentration in HIV-PVLP samples.

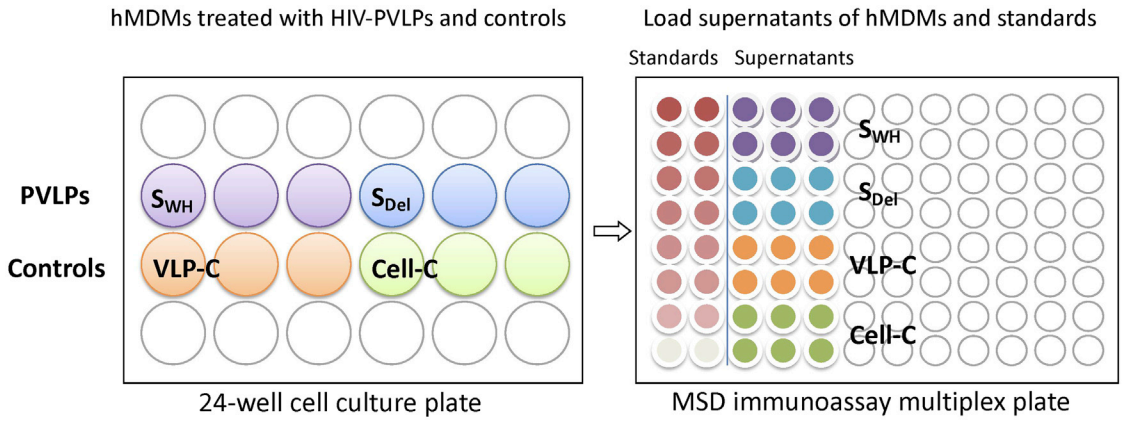

Figure 4. Human MDMs treatment with S-pseudotyped PVLPs and the plate arrangement of MSD immunoassay

In a 24-well plate, the hMDMs are treated with S_{WH}-pseudotyped and S_{Del}-pseudotyped HIV-PVLPs, negative VLP-control HIV-VLP-Gag, and non-treated cell-control. In the MSD 96-well plate, the first two columns (red to pink) are serially diluted standards and zero control, and each has two replicates. The next three columns are supernatant samples, and each has two replicates.

Determine the cytokine production (protein) from human MDMs treated with HIV-PVLPs

Timing: 4 days

Human monocyte-derived macrophages (hMDMs) are treated with HIV-PVLPs that are pseudotyped with SARS-CoV-2 S_{WH} or S_{Del}. The pro-inflammatory cytokines secreted from macrophages are quantified by using a multiplex immunoassay kit (Meso Scale Discovery, V-PLEX Proinflammatory Panel 1 Human Kit, #K15049D). This kit can detect ten proinflammatory cytokines (proteins) in one well, including IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α.

18. After 7 days of differentiation in a 24-well plate, the human MDMs reach the terminal state of macrophages and adhere to the bottom. There are about 2×10^5 hMDMs in each well. On Day 1, the supernatants are removed and replaced with 0.5 mL of 5% FBS DMEM fresh medium.
19. Treat MDMs with HIV-PVLPs.
    a. Add the same amount of each S_{WH}-pseudotyped and S_{Del}-pseudotyped HIV-PVLPs (20 ng, as adjusted by the p24 levels) into the wells containing MDMs.
    b. Add the same amount of HIV-VLP-Gag in a well as the negative VLP-control.
    c. The non-treated well is used as the negative cell control.
    d. For each PVLP or control group, treat two or three wells of MDMs as biological replicates (see Figure 4).

Alternatives: Here we used HIV-PVLPs to treat hMDMs and tested the cytokine production (protein). This protocol also can be used for the SARS2-VLPs. The amount of SARS2-VLPs can be adjusted by the S levels.

Note: The purpose of this protocol is to investigate the inflammatory immune response induced by S from different SARS-CoV-2 variants. MDMs are one of the contributors to this response. The contribution from other cells (Dendritic cells or lymphocytes) should also be considered. Here we used MDMs as a representative for the antigen-presentation cells (APCs). The MDMs purity at 80% or 100% will not affect the results.

20. Culture the MDMs with PVLPs for 24 h at 37°C, 5% CO_2.
21. On Day 2, collect the supernatants and aliquot (75 μL/well) in a 96-well plate.
    a. If the next step is performed immediately or the next day (Day 3), store the supernatants at 4°C.

b. If the MSD assay is performed later, store the supernatants at −80°C.

Note: The supernatant samples should be aliquoted in arranged wells as planned for the MSD immunoassay (Figure 4). One day before the assay, take the samples out of the −80°C freezer and put them in a 4°C fridge overnight (8–18 h) to let them thaw.

Alternatives: The supernatant can be collected at different time points (12–48 h) to investigate the production variation along with time.

Pause point: The supernatants can be frozen at −80°C for weeks (recommended less than 4 weeks) before the MSD immunoassay.

22. Measure the cytokine (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α) levels in the supernatants using the MSD V-PLEX proinflammatory Panel 1 (human) Kit (Meso Scale Discovery, USA, Cat# K15049D) following the manufacturer's procedure. For each sample, two or three technical replicates are needed. Standards are placed on the same plate.

Note: Here is a brief description of the MSD immunoassay. For more details, please refer to the manufacturer's instructions (https://www.mesoscale.com/~/media/files/product%20inserts/proinflammatory%20panel%201%20human%20insert.pdf).

a. Dilute supernatant samples (75 μL/well) 2-fold in Diluent 2 buffer (75 μL/well).
b. Prepare 7 standards (calibrators) by 4× serial dilution with Diluent 2 buffer.
c. Wash the plate three times with wash buffer (150 μL/well).

Optional: Washing before the sample loading is optional, but this can increase the uniformity of results.

d. Add the samples and standards to the coated 96-well plate (50 μL/well). Take two technical replicates for each sample. Seal the plate and incubate at room temperature with shaking for 2 h.
e. Wash the plate three times and add the detection antibodies in wells (25 μL/well). Seal the plate and incubate at room temperature with shaking for 2 h.
f. Wash the plate three times and add the 2× read buffer T in wells (150 μL/well).

23. Read the plate with the Meso QUICKPLEX SQ 120MM. The cytokine concentrations are calculated by the analysis software (Discovery workbench 4.0) based on the standard curve.

Note: If the MSD reads of samples are too low or higher than the highest standard concentration, see troubleshooting 6.

Determine the cytokine production (mRNA) from human MDMs treated with SARS2-VLPs

Timing: 4 days

Human MDMs are treated with SARS2-VLPs harboring S_{WH} or S_{Del}. The mRNA levels of pro-inflammatory cytokines in macrophages are measured by RT-qPCR, including IFN-γ, TNF-α, IL-1β, IL-6, and IL-10.

24. Similar to step 18. After 7 days of differentiation, the human MDMs reach the terminal state of macrophages and about 2×10^5 hMDMs in each well of the 24-well plate. Remove the supernatants and replace them with 0.5 mL of 5% FBS DMEM fresh medium.
25. Treat the MDMs with SARS-VLPs.
    a. Add an equal amount of each S_{WH}-SARS2-VLPs and S_{Del}-SARS2-VLPs (10 ng, as adjusted by the S levels) into the wells containing MDMs (similar to Figure 4 left).

b. The non-treated well is used as a negative cell control.
c. For each VLP and control group, treat two or three wells of MDMs as biological replicates.
d. Culture the hMDMs for 20 h at 37°C, 5% CO_2.

Alternatives: Here, we described how to measure the cytokine mRNA levels in the SARS2-VLPs treated hMDMs. This protocol can also be used for the HIV-PVLPs treated hMDMs.

26. Extraction of total RNA by using the PureLink RNA mini kit (Invitrogen). The following steps are brief descriptions. For more details, please refer to the manufacturer's instructions.

Note: The manufacturer's user guide is here or use the link (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fpurelink_rna_mini_kit_man.pdf).

Note: From step 26 to step 28, RNase-free techniques are required.

Note: Because the β-mercaptoethanol (β-ME) is used in steps 26 a–26e, perform these steps in a fume hood when the tube with β-ME is opened.

a. Prepare the fresh cell lysis buffer (Invitrogen, PureLink RNA mini kit) by adding 10 μL β-mercaptoethanol (β-ME) to each 1 mL lysis buffer. The final β-ME concentration is 1%.
b. To directly lyse the MDMs in wells, remove the media, add 300 μL of cell lysis buffer (1% β-ME), and pipet up and down over 10 times.
c. Transfer the cell lysates to a new RNase-free Eppendorf tube, vortexed for 1 min, and stored at −80°C according to the manufacturer's instruction.

Pause point: The cell lysates in the lysis buffer can be frozen at −80°C for days or weeks.

d. Extract the total RNA of macrophages. Mix the cell lysates with 300 μL of 70% ethanol with vortex.
e. Load the mixture in the column and wash with 350 μL of wash buffer I.
f. Remove genome DNA with 80 μL of PureLink DNase (Invitrogen, #12185-010) mixture at room temperature for 15 min according to the On-column DNase Treatment Protocol.
g. Wash with 350 μL of wash buffer I and 500 μL of wash buffer II. Elute the RNA with 50 μL Nuclease-free water.

27. Measure the RNA concentration with NanoDrop One Spectrophotometer (ThermoFisher Scientific, #ND-One-W).
28. Take 2 μg total RNA of each sample to an Eppendorf tube for reverse transcription (RT).
    a. Add 4 μL SuperScript VILO MasterMix (Invitrogen, #11755-050) and nuclease-free ddH_2O to a total volume of 20 μL for each reaction.
    b. After the gentle mix, incubate at 25°C for 10 min and then at 42°C for 1 h.
    c. Terminate the reverse transcription by heating it at 85°C for 5 min. Store the cDNAs at −20°C before qPCR.

Pause point: The RT products (cDNA) can be stored at −20°C for days or weeks (recommend less than 8 weeks).

29. The qPCR is performed using PowerUp SYBR Green Master Mix (Applied Biosystems, # A25741) in a qPCR thermocycler (e.g., Agilent Stratagene MX3000P) according to the manufacturer's instruction. For each cDNA sample, five cytokine genes and one housekeeping gene are detected. For each reaction, two or three technical replicates are needed.
    a. For each cDNA sample, use a nuclease-free tube to prepare the qPCR reaction cDNA-Mix, including cDNA, Master Mix (2×), and ddH_2O (without primers) for 20 reactions (10% extra).

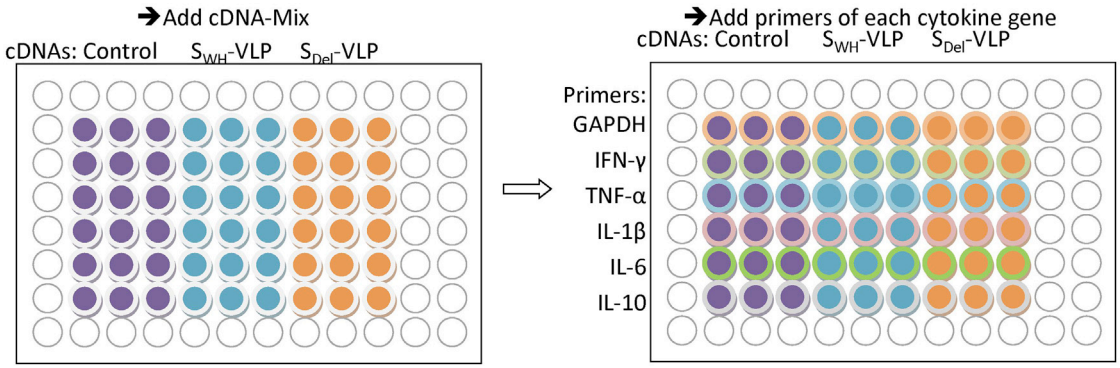

Figure 5. qPCR plate arrangement of hMDMs cDNAs and primers to detect cytokines induced by SARS2-VLPs

In the 96-well plate, the first three columns (purple) are the cDNA of non-treated cell control, and each sample for each gene has three replicates. The next three columns (blue) are the cDNA of S_{WH}-SARS2-VLP treated MDMs. The last three columns (orange) are the cDNA of S_{Del}-SARS2-VLP treated MDMs. The primers of each gene are added to wells in the same row.

b. Dispense 16 μL of the cDNA-Mix to each well in a PCR 96-well plate or 8-well strips (200 μL) as arranged in Figure 5.
c. Add primer pairs (F and R, 4 μL) of one specific gene to each well.
d. Cover the plate with film or caps. Gently vortex and spin shortly.
e. The PCR reaction system with a total volume of 20 μL is listed below. The primers used for qPCR are shown in the key resources table.

qPCR reaction

Reagent	Amount
cDNA template	10 ng, 0.1 μL
PowerUp SYBR Green Master Mix (2×)	10 μL
Primer-F (5 μM)	2 μL
Primer-R (5 μM)	2 μL
ddH_2O	5.9 μL
Total	**20 μL**

f. Load the 96-well plate or 8-well strips in the real-time PCR thermocycler. Set the qPCR cycling conditions (as below) and run the reaction. The fluorescence reads of each well for each cycle are recorded and obtained after the stop.

qPCR cycling conditions

Steps	Temperature	Time	Cycles
Initial UDG activation	50°C	2 min	1
Denaturation	95°C	2 min	
Denaturation	95°C	15 s	40
Annealing/ Extension	60°C	1 min	
Hold	4°C	Forever	

30. Perform a default dissociation curve after the qPCR.

Note: If no signal or low signal (the Ct is higher than 38) of qPCR is detected, see troubleshooting 7.

Note: If the amplification curve is not sigmoid, see troubleshooting 8.

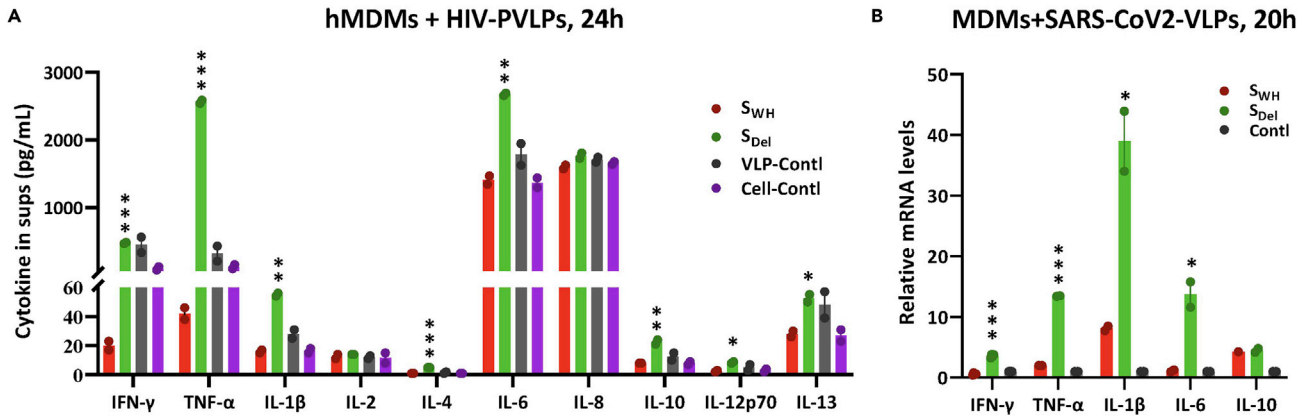

Figure 6. The production of pro-inflammatory cytokines from hMDMs

(A) The hMDMs are stimulated with S-pseudotyped HIV-VLPs for 24 h. The protein levels of cytokines in supernatants are measured by a Meso Scale immunoassay.

(B) The hMDMs are stimulated with S-pseudotyped SARS2-VLPs for 20 h. The relative mRNA levels of cytokines in cells are determined by RT-qPCR compared with non-treated cell control. Data are represented as mean ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001. This figure is reproduced from previous data in Ao et al. (2022).[1]

31. Calculate the relative RNA levels through the $2^{-\Delta\Delta Ct}$ method after normalization against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. This calculation can be done by using the software (MxPro) of the qPCR thermocycler. See quantification and statistical analysis section for more details.

EXPECTED OUTCOMES

The cytokines produced by human MDMs are shown in Figure 6. The protein levels of secreted cytokines in supernatants can be directly used in the Figure. The cytokine mRNAs in cells can be shown as relative levels after normalization against the housekeeping gene GAPDH. The secreted protein levels and cellular mRNA levels are similar and can be used to confirm the accuracy of data.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. The results in the Figures are shown as mean ± SEM of biological replicates.
2. The relative mRNA levels are calculated by the $2^{-\Delta\Delta Ct}$ method. This calculation can be done by the software (MxPro) of the qPCR thermocycler.
   a. Normalize the mRNA level of each interested gene against the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level by calculating the $\Delta Ct = (Ct_{gene} - Ct_{GAPDH})$ for each gene in each treated samples and untreated control.
   b. Set the mRNA level of each gene in the cell control as 1. Calculate the relative mRNA levels (fold change) of each gene in the treated samples (S_{WH}-SARS2-VLP and S_{Del}-SARS2-VLP) as compared with that in the cell control.
      i. Calculate $\Delta\Delta Ct_{gene\ A} = (Ct_{gene\ A} - Ct_{GAPDH})_{sample} - (Ct_{gene\ A} - Ct_{GAPDH})_{control}$ for gene A in the sample against the cell control.
      ii. Calculate the relative mRNA level (fold change) for gene A in the sample against the cell control: $2^{-\Delta\Delta Ct\ gene\ A}$.
3. To compare the cytokine production (protein levels and relative mRNA levels) induced by S proteins (S_{WH} and S_{Del}), statistical significance between the two groups is determined using the unpaired Student T-test. The significant p values are represented with asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Not significant (ns) is not shown.

LIMITATIONS

The S protein is located on the surface of HIV-PVLPs and SARS2-VLPs. The macrophages may be triggered not only by the engagement with surface protein on the particles but may also be triggered by internal proteins after the phagocytosis of the whole PVLPs or VLPs. Although we used a control HIV-Gag-VLP that contains all the HIV components in the S-pseudotyped VLPs, we could not exclude the possibility that the cytokine production contributed by S or other proteins is accumulated synergistically. In this situation, the results only exhibit a qualification relationship but not a quantification relationship.

However, for SARS2-VLPs, because the results may mimic the influence from a whole SARS-CoV-2 particle, it may be closer to the natural situation than the influence from the single S protein (HIV-PVLPs).

TROUBLESHOOTING

Problem 1

The purified SARS2-VLPs or HIV-PVLPs solution after ultracentrifugation may be turbid, and not clear.

Potential solution

There may be some cell debris, and it is also possible that VLPs or PVLPs aggregate in a high concentration after ultracentrifugation. Increase the RPMI-1640 volume or pipet up and down gently more times to increase the dissolution. Then spin at 1000 × *g* for 10 min to clear the solution.

Problem 2

A negative result in WB for the detection of S protein in HIV-PVLPs and SARS2-VLPs is observed.

Potential solution

The success of PVLP and VLP packaging requires both plasmid quality and cells to be in good condition. Check the GFP expression in GFP-plasmid transfected cells to make sure that the cells and PEI/Lipofectamine reagents work well. Simultaneously, check if S can be detected in the producer cell samples. If S is also not shown in producer cell samples, it indicates the S-expressing plasmid did not work. It is important to prepare the fresh plasmid and test if it works before the packaging experiment.

Problem 3

A negative result in WB that detect N protein in SARS2-VLPs or p24 in HIV-PVLPs is observed.

Potential solution

Similar to problem 2, first make sure cells and PEI/Lipofectamine reagents are good, and then test if the N-expressing plasmid or pCMV-Δ8.2 works by checking producer cell samples. Prepare fresh plasmids and test their functions before the packaging experiment.

Problem 4

The ELISA reads of standards are fine but those of VLP or PVLP samples are too low or higher than the highest standard concentration.

Potential solution

The ELISA reads of proteins (S or p24) in samples depend on the number of samples loaded in the wells. If the read is too low to detect, increase the amount of the sample by using a less diluted sample. Similarly, if the read is higher than the standards, an appropriate further dilution of the sample is needed.

Problem 5
The ELISA reads of standards and samples all are weak or strong.

Potential solution
If the ELISA reads all are weak, it suggests some reagents may lose their function. Check the standard protein, antibodies, and TMB substrate solution, and make sure they are stored properly, such as at a low temperature or in the dark. Test the functions of the reagents before the next use.

If the ELISA reads all are strong, the possible reasons include insufficient wash, too long incubation time, and contamination of reagents. Test the reagents before the next use. Wash five times and sit the plate for 1 min per wash. Shorten the incubation time in the range of 1–3 h at 37°C or room temperature.

Problem 6
The MSD reads of samples are too low or higher than the highest standard concentration.

Potential solution
If standard curves and controls are fine, this indicates the measuring system works well. The reason causing the reads out of the range of standards is that the concentration of the diluted sample is too low or too high. Adjust the sample dilution factor to let the expected read be in the range of the standard curve. If the read is higher than the highest standard concentration, the other option is to adjust the standard dilution/reconstitution, thus, increasing the highest standard concentration (to extend the standard curve). Multiple freeze-thaw cycles of the samples may cause signal loss. It is recommended to aliquot samples for future measurement.

Problem 7
No signal or low signal (the Ct is higher than 38) of qPCR.

Potential solution
There are two possible reasons: 1. the enzyme or other components in the master mix or primers do not work, or 2. the cDNA template amount is too low. The first reason can be confirmed by using a positive control cDNA to test . The second reason can be confirmed by checking the GAPDH Ct which should be around 15–25 for a normal reaction. But if the GAPDH Ct is higher than 30, this indicates that the cDNA template amount in the qPCR is not enough and needs to be increased.

Problem 8
The amplification curve is not sigmoid.

Potential solution
Check the dissociation curve of each gene. The single and symmetrical peak in the dissociation curve indicates good specificity of the primer pair and the accuracy and reliability of the qPCR data. If there are two or more peaks in the dissociation curve, change to another primer pair of this gene. The good specificity of primer pairs listed in the key resources table has been verified.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiao-Jian Yao (xiao-jian.yao@umanitoba.ca).

Materials availability
This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization, X.Y., M.J.O., and Z.A.; methodology, M.J.O., Z.A., and T.O.; analysis, M.J.O. and Z.A.; investigation, M.J.O., Z.A., and T.O.; writing – original draft preparation, M.J.O.; review and revision, M.J.O., Z.A., T.O., and X.Y.; supervision, X.Y.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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