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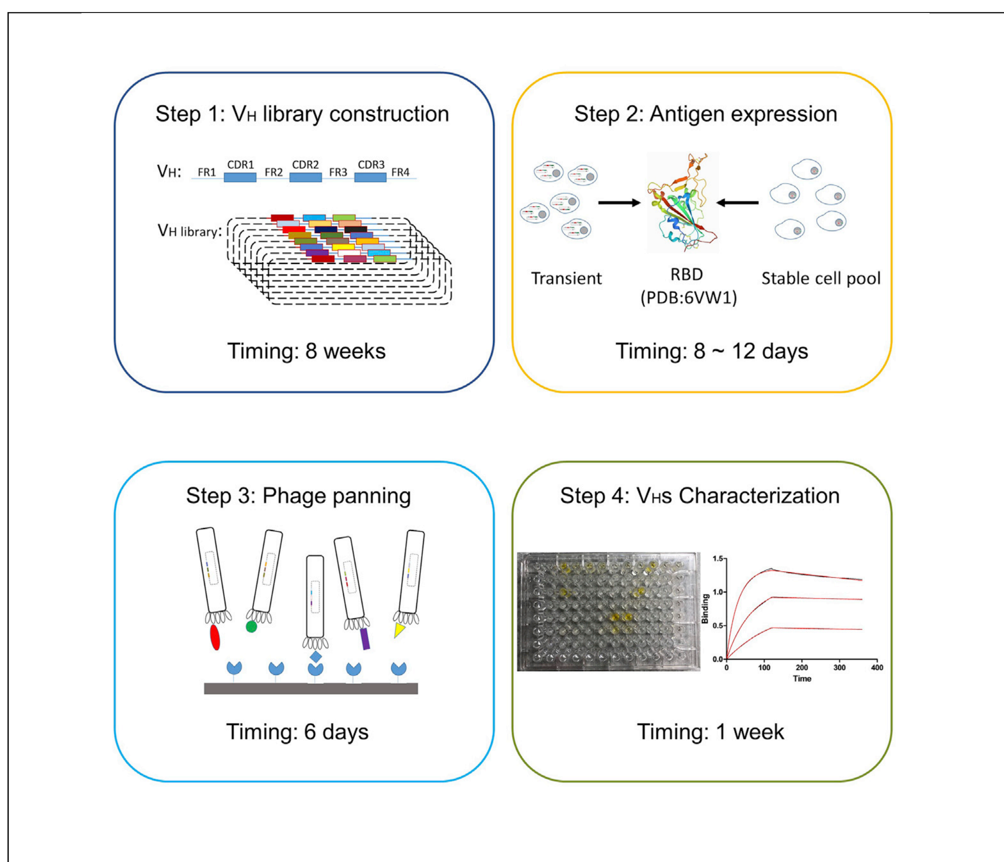
Protocol

Protocol for constructing large size human antibody heavy chain variable domain (V_H) library and selection of SARS-CoV-2 neutralizing antibody domains



This protocol is a comprehensive guide to phage display-based selection of virus neutralizing V_H antibody domains. It details three optimized parts including (1) construction of a large-sized (theoretically > 10^{11}) naïve human antibody heavy chain domain library, (2) SARS-CoV-2 antigen expression and stable cell line construction, and (3) library panning for selection of SARS-CoV-2-specific antibody domains. Using this protocol, we identified a high-affinity neutralizing human V_H antibody domain, V_H ab8, which exhibits high prophylactic and therapeutic efficacy.

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Highlights

Large-sized (> 10^{11}) naïve human antibody heavy chain domain library construction

Quick antigen expression and stable cell pool selection with Expi293^{TM} cells

Quick phage-display library panning (6 days)

High-affinity aggregation-resistant human antibody domain binder selection

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Protocol

Protocol for constructing large size human antibody heavy chain variable domain (V_H) library and selection of SARS-CoV-2 neutralizing antibody domains

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SUMMARY

This protocol is a comprehensive guide to phage display-based selection of virus neutralizing V_H antibody domains. It details three optimized parts including (1) construction of a large-sized (theoretically > 10^{11}) naïve human antibody heavy chain domain library, (2) SARS-CoV-2 antigen expression and stable cell line construction, and (3) library panning for selection of SARS-CoV-2-specific antibody domains. Using this protocol, we identified a high-affinity neutralizing human V_H antibody domain, V_H ab8, which exhibits high prophylactic and therapeutic efficacy.

For complete details on the use and execution of this protocol, please refer to Li et al. (2020)

BEFORE YOU BEGIN

Total RNA isolation and CDNA synthesis

⏲ Timing: 3–5 days

1. Collect peripheral blood mononuclear cells (PBMCs) from 12 healthy donors' blood samples before SARS-CoV-2 pandemic using Ficoll-Paque PLUS gradient (Sigma, Cat#GE17-1440-02) according to the manufacturer's protocol (https://www.sigmaaldrich.com/technical-documents/protocols/biology/isolation-of-mononuclear-cells/recommended-standard-method.html). 1.5×10^9 PBMCs were collected for RNA isolation.
2. Dissolving the cell pellet immediately with 60 mL RLT buffer (2.5×10^7 Cells/mL RLT buffer with 1% volume of 2-mercaptoethanol) and isolation of total RNA following the protocol of RNeasy Mini Kit.

Pause point: Total RNA in DEPC water can be kept at −80°C for short-term storage.

3. Preparation of cDNA using both random hexamer and Oligo dT as primers. Total RNA was prepared, and first strand cDNA was synthesized by Superscript™ III first-strand Synthesis System

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Table 1. Sequences of the primers for V_H assemble and RBD-AviTag cloning

Name	Sequence	Usage
ALL-F	GTTTCGCTACCGTGGCCCAGGCGGCCGAGGT GCAGCTGGTGGA	FR1 and CDR1
FR1R	ACAGGAGAGTCTCAG	
H1F-157	CTGAGACTCTCCTGTAAGGSTTCT	
H1F-2	CTGAGACTCTCCTGTACCKTCTCT	
H1F-3	CTGAGACTCTCCTGTGYAGCCTCT	
H1F-4	CTGAGACTCTCCTGTRCTGTCTCT	
H1F-6	CTGAGACTCTCCTGTGCCATCTCC	
H1R-13	CCTGGAGCCTGGCGGACCCAGTKCAT	
H1R-2	CCTGGAGCCTGGCGGACCCAGCYCAC	
H1R-3	CCTGGAGCCTGGCGGACCCAGCTCAT	
H1R-4	CCTGGAGCCTGGCGGACCCAGCYCCA	
H1R-5	CCTGGAGCCTGGCGGACCCAGCYGAT	
H1R-6	CCTGGAGCCTGGCGGACCCAGTTCCA	
H1R-7	CCTGGAGCCTGGCGGACCCAATTCAT	
H1R-ALL	CCTGGAGCCTGGCGGACCCA	
H2F167	TGGGTCCGCCAGGCTCCAGGACAASGSCTTGAGTGG	CDR2
H2F2	TGGGTCCGCCAGGCTCCAGGGAAGGCCCTGGAGTGG	
H2F1345	TGGGTCCGCCAGGCTCCAGGGAAGGGNCTRGAGTGG	
H2R1	ATTGTCTCTGGAGATGGTGACCCTKYCCTGRAACTY	
H2R36	ATTGTCTCTGGAGATGGTGAATCGGCCCTTCACNGA	
H2R24	ATTGTCTCTGGAGATGGTGACTMGACTCTTGAGGGA	
H2R5	ATTGTCTCTGGAGATGGTGACSTGGCCTTGGAAGGA	
H2R7	ATTGTCTCTGGAGATGGTAAACCGTCCTGTGAAGCC	
FR3F	ACCATCTCCAGAGACAATTCC	FR3
FR3R	GTCCTCGGCTCTCAGGCTG	
H3F3p	AGCCTGAGAGCCGAGGACACRGCYTTRTATTACTGT	CDR3
H3F1p257p	AGCCTGAGAGCCGAGGACACAGCCAYRTATTACTGT	
H3Fother	AGCCTGAGAGCCGAGGACACRGCYGTRTATTACTGT	
H3R	GTGGCCGGCCTGGCCACTTGAGGAGACGGTGACC	
ALL-R	GTCGCCGTGGTGGTGGTGGTGGTGGCCGGCCTGGCCACTTG	
EcoRV-RBD-P1	TCAGTGATATCCCAAATATAACCAATCTCTGCCCATTCG	RBD-AviTag-His
EcoRI-His-AviTag-P2	TCAGTGAATTCCTATTAGTGATGGTGGTGGTGATG GCTACCCTCGTGCCACTCG	

Degenerated bases: R = A,G; Y = C,T; M = A,C; K = G,T; S = C,G; N = A,C,G,T.
The restriction sites were marked yellow, complementary sequences were marked underline, overlapping sequences were marked red (Figures 1 and 4).

(Thermo, Cat#18080044) according to the manufacturer's protocol (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FsuperscriptIII_man.pdf&title=U3VwZXJTY3JpcHQgSUlJIFJldmVyc2UgVHJhbnNjcmlwdGFzZQ==).

Synthetic primers and genes

Timing: 3–5 days

4. PCR primers used for gene amplification and V_H assemble are listed in Table 1. The primers were synthesized by IDT with high quality (Purified by HPLC).
5. Codon Optimize the RBD-AviTag gene (GenBank: QHD43416.1, Figure 1) by "Codon Optimization Tool" in IDT (https://www.idtdna.com/pages/tools/codon-optimization-tool?returnurl=%2FCodonOpt). Optimized RBD-AviTag DNA was synthesized by IDT gBlock service. The gBlock DNA was diluted with ddH_2O into 10 ng/µL for PCR.

EcoRV-RBD-P1: TCAGTGATATCCCAAATATAACCAATCTCTGCCCATTCG

GCTCACCGTGCTCCCATCGGTAGTGGTGGTGGTAGTGATTATCCTTAAGTGACT:EcoRI-His-AviTag-P2

Figure 1. DNA sequence of RBD-AviTag

Codon optimized receptor-binding domain (RBD) of the SARS-CoV-2 spike (S) glycoprotein with AviTag™ and His-tag.

Sequences highlighted plum (RBD): Codon optimized receptor-binding domain of the SARS-CoV-2 spike (S) glycoprotein.

Sequences highlighted lavender (AviTag™): The AviTag™ sequences (GLNDIFEAQKIEWHE) for site-specific biotinylation of purified RBD-AviTag antigen with BirA Biotin-Protein Ligase Kit.

Sequences highlighted pink (His-tag): 6×His tag sequences for RBD-AviTag purification with Ni-NTA column.

Plasmids and other materials

Timing: 3–5 days

6. Generation of an empty plasmid named pIW-Zeo (Figure 2A) that contains a CMV promotor, Intron, a secret signal peptide (SPE) for extracellular expression, multiple cloning site (MCS), internal ribosome entry site (IRES), zeocin resistant gene, woodchuck posttranscriptional regulatory elements, BGH poly A, origin of replication and ampicillin resistant gene (This plasmid was generated before starting the protocol).
7. Prepare the phagemid for library construction with Qiagen plasmids Maxi-Prep kit (Qiagen Maxiprep, Cat#12663). The phagemid we used is a modified pComb3X vector (Cat#VPT4012, Creative Biogene), in which the HA-tag was replaced by Flag-tag (Figures 2C and 2D).
8. Maintain Expi293F™ cells (Thermo, Cat#A14527) with Expi293™ Expression Medium in a CO_2 resistant incubator at 135 rpm, 8% CO_2, 95% humidity according to the manufacturer's protocol (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0006283_Expi293_Cells_UG.pdf&title=VXNlciBHdWlkZTogRXhwaTI5M0YgQ2VsbHM=).

Note: Determine viability and cell clumping using the trypan blue dye exclusion method and make sure cell viability is higher than 97%. The Expi293F™ cells can maintain in Expi293™ Expression Medium for 2 months (20–30 passages) with no significant expression decrease. So, thawing a new vial of Expi293F™ cells every two months is needed.

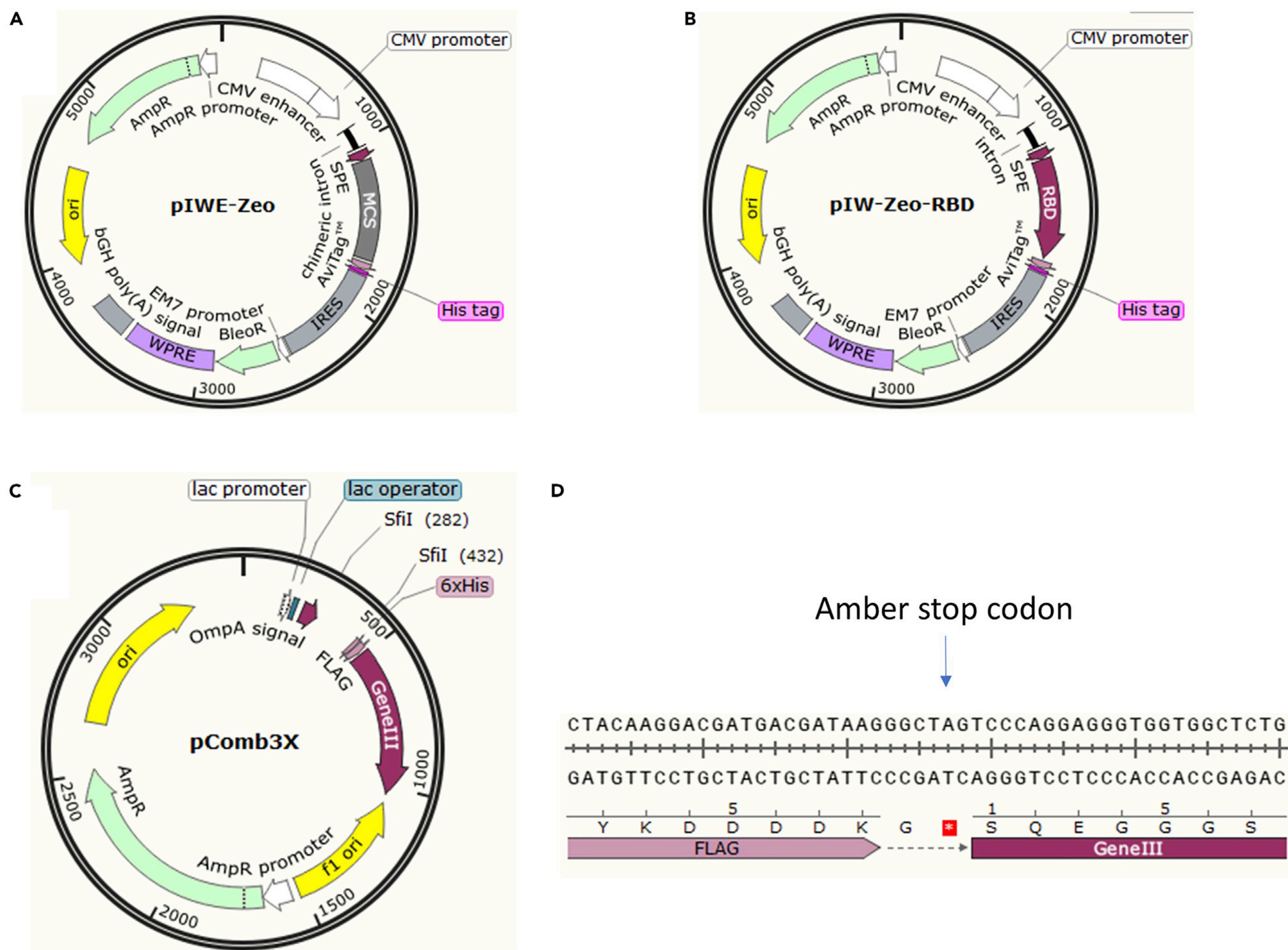

Figure 2. The schematic view of maps of Vectors pIW-Zeo, pIW-Zeo-RBD and pComb3X

(A) Maps of pIW-Zeo: A secret signal peptide (SPE) was inserted into the empty vector for extracellular expression.

(B) Maps of pIW-Zeo-RBD: RBD secret expression plasmid.

(C) Maps of pComb3x with an amber stop codon. TG1 is an amber codon (TAG) suppressor strain, allowing translation to read through the codon to produce a full-length V_H-gene III fusion protein. HB2151 is an amber codon non-suppressor strain, Only V_H gene can be translation to produce V_H in this strain.

(D) Amber stop codon in pComb3x

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
TG1 Electrocompetent Cells	Lucigen	60502-1
E. cloni 5-alpha Chemically Competent Cells	Lucigen	60602-2
HB2151 bacteria	CAT Lab, University of Pittsburgh	N/A
M13KO7 Helper Phage	Thermo Fisher Scientific	18311019
Antibodies		
Monoclonal ANTI-FLAG® M2-Peroxidase (HRP)	Sigma	A8592
Anti-M13 Antibody (HRP)	Sino Biological Inc.	11973-MM05T-H

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
T4 DNA Polymerase	NEB	M0203L
T4 DNA Ligase	NEB	M0202L
ACE2 Protein, Human, Recombinant (mFc Tag)	Sino Biological Inc.	10108-H05H
Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	15250061
UltraPure™ Agarose	Thermo Fisher Scientific	16500500
Agar for bacteriology	VWR	97064-332
Ampicillin Sodium Salt	Fisher Scientific	BP1760-25
Kanamycin Sulfate	Fisher Scientific	BP906-5
Dextrose (D-Glucose), Anhydrous	Fisher Scientific	D16-1
SfiI	NEB	R0123L
EcoRV-HF	NEB	R3195S
EcoRI-HF	NEB	R3101S
Polyethylene Glycol 8000	Fisher Scientific	BP233-1
Sodium Chloride	Fisher Scientific	S271-3
PEI MAX™	Polysciences	24765-1
Zeocin™ Selection Reagent	Thermo Fisher Scientific	R25001
Bovine Serum Albumin (BSA)	VWR	97063-626
Blotting-Grade Blocker (nonfat dry milk)	Bio-Rad	1706404
TWEEN® 20	Sigma-Aldrich	P1379-500ML
Glycerol	Sigma-Aldrich	G5516-1L
Isopropanol	Fisher Scientific	AC327272500
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Fisher Scientific	BP1755-10
SimplyBlue™ SafeStain	Thermo fisher scientific	LC6065
Polymyxin B sulfate salt	Sigma-Aldrich	P1004-50MU
2-Mercaptoethanol	Thermo fisher scientific	21985023
Imidazole	Sigma-Aldrich	I2399
50× TAE Buffer (Tris-acetate-EDTA)	Fisher Scientific	FERB49
Buffer RLT	QIAGEN	79216
ACK Lysing Buffer	Thermo Fisher scientific	A1049201
NuPAGE™ 4–12%, Bis-Tris, 1.0 mm, Mini Protein Gel	Thermo Fisher Scientific	NP0321BOX
Ficoll-Paque PLUS	GE Healthcare	17-1440-03
HisPur™ Ni-NTA Resin	Thermo Fisher Scientific	88222
Expi293™ Expression Medium	Thermo Fisher Scientific	A1435101
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190136
DPBS (10×), no calcium, no magnesium	Thermo Fisher Scientific	14200-075
Nuclease-Free Water	Thermo Fisher Scientific	AM9937
Teknova2-YT BROTH	Fisher Scientific	50-843-444
Teknova 2-YT AGAR	Fisher Scientific	50-843-447
Experimental models: cell line		
Expi293F™	Thermo Fisher Scientific	A14527
Oligonucleotides		
Primers (see Table 1)	IDT	N/A
Recombinant DNA		
pComb3X	CAT Lab, University of Pittsburgh	N/A
pIW-Zeo	CAT Lab, University of Pittsburgh	N/A
Critical commercial assays		
QIAquick Gel Extraction Kit	QIAGEN	28706
QIAprep Spin Miniprep Kit	QIAGEN	27106
HiSpeed Plasmid Maxi Kit	QIAGEN	12663
BirA Biotin-Protein Ligase Kit	Avidity	BirA-500
High Fidelity PCR Master	Roche	12140314001
Phusion Flash High-Fidelity PCR Master Mix	Thermo Fisher Scientific	F548L
Dynabeads™ MyOne™ Streptavidin T1	Thermo Fisher Scientific	65602

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
DNA Clean & Concentrator-5	Fisher Scientific	50-197-7310
Pierce™ Protein Concentrator PES, 10K MWCO, 5–20 mL	Thermo Fisher Scientific	88528
Pierce™ Protein Concentrator PES, 10K MWCO, 2–6 mL	Thermo Fisher Scientific	88517
RNeasy Midi Kit	QIAGEN	75144
SuperScript™ III First-Strand Synthesis System	Thermo Fisher Scientific	18080044
Other		
Gene Pulser/MicroPulser Electroporation Cuvettes, 0.1 cm gap	Bio-Rad	1652089
T100™ Thermal Cycler	Bio-Rad	1861096EDU
125 mL Fisherbrand™ Shaker Flasks	Fisher Scientific	PBV12-5
250 mL Fisherbrand™ Shaker Flasks	Fisher Scientific	PBV250
Pierce™ Disposable Columns, 5 mL	Thermo Fisher Scientific	29922
6 Tube Magnetic Stand	Thermo Fisher Scientific	AM10055
Corning® 96-well Half Area Clear Flat Bottom Polystyrene High Bind Microplate	Fisher Scientific	07-200-37
Amicon® Ultra-4 Centrifugal Filters	Sigma-Aldrich	UFC8030
Amicon® Ultra-15 Centrifugal Filters	Sigma-Aldrich	UFC9030

MATERIALS AND EQUIPMENT

Primers combinations for PCR amplification of CDRs

CDRs	Human Ig VH family	Primers combinations
CDR1	IGVH1	H1F-157 and H1R-13
	IGVH2	H1F-2 and H1R-2
	IGVH3	H1F-3 and H1R-13; H1F-3 and H1R-3
	IGVH4	H1F-4 and H1R-4
	IGVH5	H1F-157 and H1R-5
	IGVH6	H1F-6 and H1R-6
	IGVH7	H1F-157 and H1R-7
CDR2	IGVH1	H2F167 and H2R1; H2F1345 and H2R1
	IGVH2	H2F2 and H2R24
	IGVH3	H2F1345 and H2R36
	IGVH4	H2F1345 and H2R24
	IGVH5	H2F1345 and H2R5
	IGVH6	H2F167 and H2R36
	IGVH7	H2F167 and H2R7
CDR3	IGVH1	H3F1p257p and H3R; H3Fother and H3R
	IGVH2	H3F1p257p and H3R
	IGVH3	H3Fother and H3R
	IGVH4	H3Fother and H3R
	IGVH5	H3F1p257p and H3R
	IGVH6	H3Fother and H3R
	IGVH7	H3F1p257p and H3R; H3Fother and H3R

Binding buffer:

Reagent	Final concentration	Amount
MilliQ water	n/a	900 mL
DPBS (10 ×)	1 ×	100 mL
Imidazole	5 mM	0.34 g
NaCl	300 mM	17.53 g
Total	n/a	**1 L**

Note: Adjust the pH to 8.0, filter with 0.45 μm bottle top filter and store at 4°C. This buffer is guaranteed for one year when stored properly.

Wash buffer:

Reagent	Final concentration	Amount
MilliQ water	n/a	900 mL
DPBS (10×)	1 X	100 mL
Imidazole	20 mM	1.36 g
NaCl	300 mM	17.53 g
Total	**n/a**	**1 L**

Note: Adjust the pH to 8.0, filter with 0.45 μm bottle top filter and Store at 4°C. This buffer is guaranteed for one year when stored properly.

Elution buffer:

Reagent	Final concentration	Amount
MilliQ water	n/a	900 mL
DPBS (10×)	1 X	100 mL
Imidazole	250 mM	17.02 g
NaCl	300 mM	17.53 g
Total	**n/a**	**1 L**

Note: Adjust the pH to 8.0, filter with 0.45 μm bottle top filter and store at 4°C. This buffer is guaranteed for one year when stored properly.

PEG/NaCl solution

Reagent	Final concentration	Amount
MilliQ water	n/a	≈ 900 mL
Polyethylene Glycol 8000	25 mM	200 g
NaCl	2.5 M	150 g
Total	**n/a**	**1 L**

Note: Autoclave and mix after cooling down. Keep it at RT (20°C–25°C). This buffer is guaranteed for 6 months when stored properly.

Critical Reagents: TG1 Electrocompetent Cells (Lucigen, Cat#60502-1). DNA Clean & Concentrator-5 (Fisher Scientific, Cat#50-197-7310). These two reagents are critical for library construction.

Alternatives: Throughout this protocol, we refer to several specific kit for many standard biology techniques. Investigators may substitute other commercially available kit as needed.

STEP-BY-STEP METHOD DETAILS

V_H library construction

Timing: 8 weeks

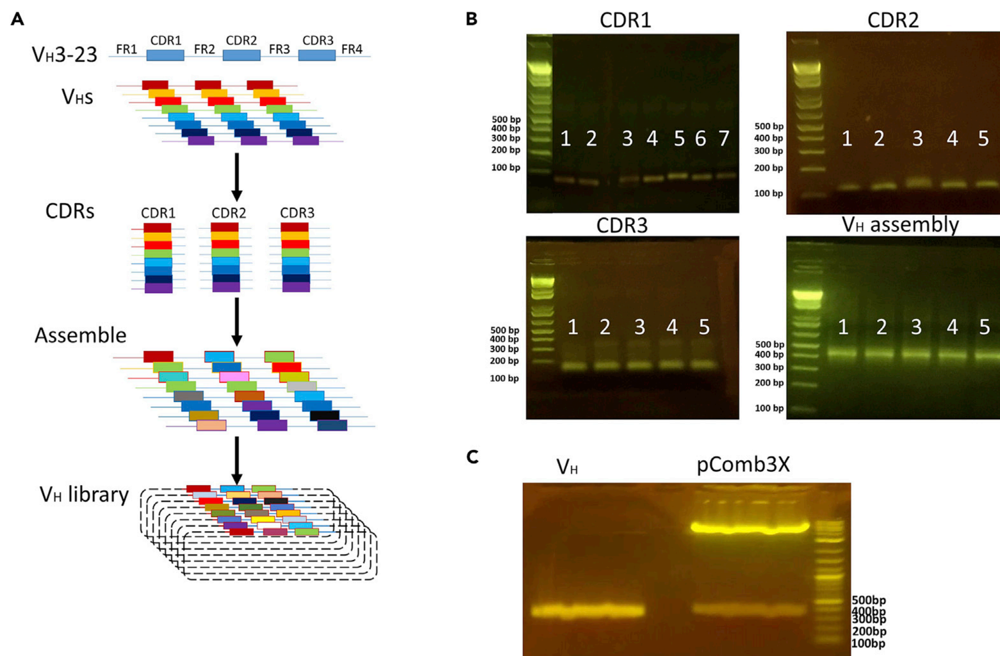

Figure 3. V_H antibody domain library construction and agarose gel results

(A) An overview of V_Hs library construction. The CDR1, CDR2 and CDR3 are amplified from human PBMC cDNA and grafted into a well-defined stable scaffold IGVH3-23.

(B) CDR PCR results of different immunoglobulin heavy-chain variable region gene (IGVH) subgroups (IGHV1 to IGHV7) and overlap-PCR assembled V_H. Below are illustrations of the results:

The CDR1 PCR products subgroups (~74 bp) in each row (Row 1: IGHV1. Row 2: IGHV2. Row 3: IGHV3. Row 4: IGHV4. Row 5: IGHV5. Row 6: IGHV6. Row 7: IGHV7).

The CDR2 PCR products subgroups (~114 bp) in each row (Row 1: IGHV1. Row 2: IGHV2, 4. Row 3: IGHV3, 6. Row 4: IGHV5. Row 5: IGHV7).

The CDR3 PCR products subgroups (~132 bp) in each row (Row 1–3: IGHV1, 2, 5, 7. Row 4: IGHV3. Row 5: other subgroups).

Overlap-PCR assembled V_H results (Row 1–5: IGHV1~7 mixture, ~430 bp).

(C) Digest results of V_H and pComb3x. The size of digested V_H~370 bp. The size of digested pComb3x~3300 bp

High diversity and large size are the characteristics of a good library and the basic requirements for successful library panning leading to selection of high affinity binders. Due to the limited diversity of PBMCs B cell antibody gene, it is unlikely to generate a large size V_H library by direct PCR amplification of V_H region from the cDNA of PBMCs. Therefore, overlap-PCR was used to combine different antibody heavy chain complementarity-determining regions (CDRs) to increase the size and diversity of V_H genes. In this protocol, a well-defined stable scaffold IGVH3-23 (Figure 3A) was chosen as basic scaffold for framework Region (FR): FR1, FR2, FR3 and FR4. The primer combinations used to amplify CDRs and overlap CDRs-FRs are listed in Table 1 and Figure 4.

1. Dissolve the primers with ddH_2O and adjust the concentration to 10 μM.

2. Assemble the V_H with PCR (Figure 3A).
   a. PCR amplify FR1, CDR1, CDR2, FR3 and CDR3 separately with High Fidelity PCR Master kit. Different primer combinations as shown in Table 1 (Figure 4) using the following cycling conditions:

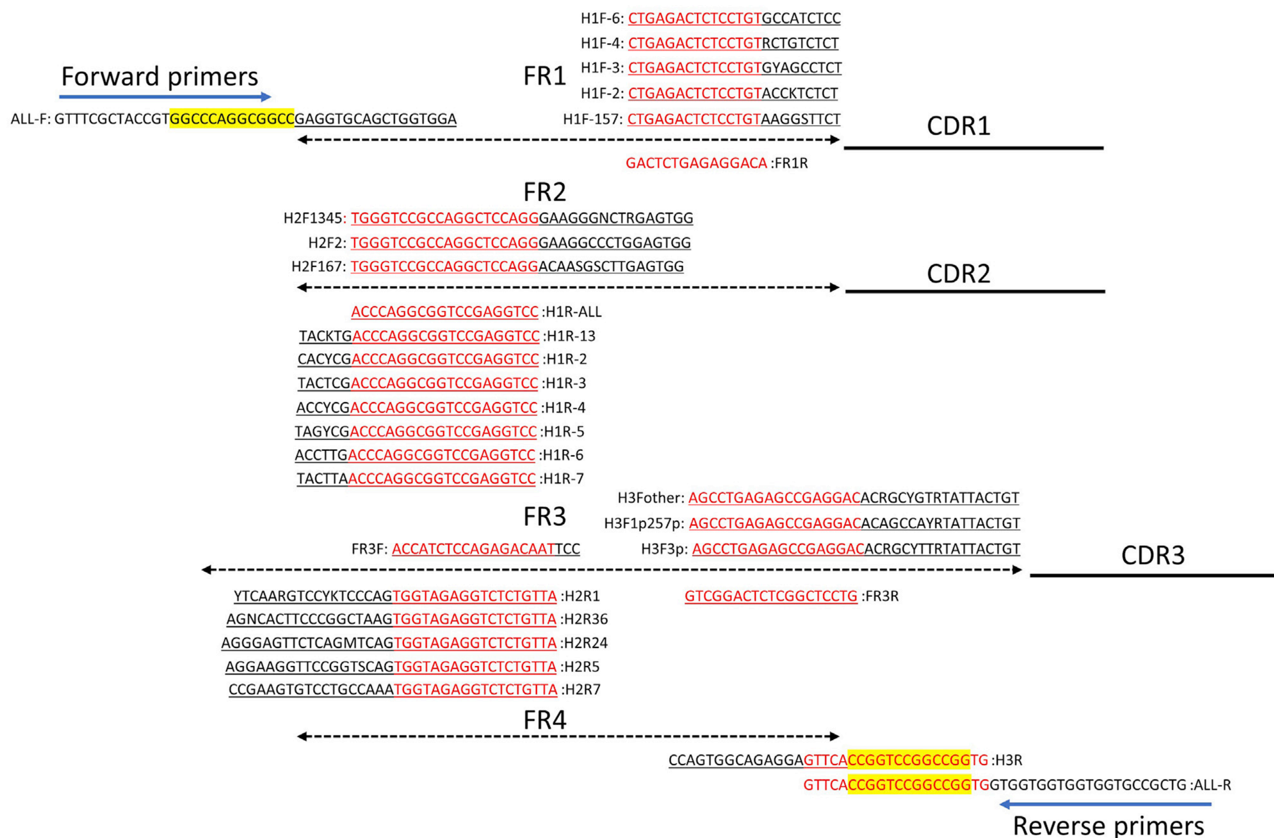

Figure 4. Schematic diagram of the assembled V_H and the primers for PCR

Degenerated bases: R = A,G; Y = C,T; M = A,C; K = G,T; S =C,G; N = A,C,G,T. The restriction sites were marked in yellow, complementary sequences were highlighted by underline, overlapping sequences were marked in red.

i. 50 μL PCR system:

Component	Volume/Weight	Final concentration
PCR master mix 1	25 μL	1 ×
10 μM forward primer	1 μL	0.2 μM
10 μM reverse primer	1 μL	0.2 μM
cDNA template	100 ng	2 ng/μL
ddH_2O	to 50 μL	n/a

Thermocycling conditions for PCR:

Steps	Temperature	Time
Initial denaturation	94°C	4 min
25 cycles	94°C	45 s
	55°C	45 s
	72°C	1 min
Final extension	72°C	5 min
Hold	4°C	

ii. Run all the PCR samples with 2% agarose gel to verify the size and purify the right size PCR products (Figure 3B) from the agarose gel with QIAquick Gel Extraction Kit.

⚠ CRITICAL: The template cDNA is a mixture; the primers may have non-specific binding with the cDNA to PCR out some non-specific DNA bands. In the experiment, only the target bands will be purified for further overlapping PCR process to assembly full-length V_H.

b. Over-lapping PCR to assemble full-length V_H with FR1, CDR1, CDR2, FR3 and CDR3 DNA purified from step 2a as template, ALL-F and ALL-R as primes using the following cycling conditions:
   i. 50 μL PCR system:

Component	Volume/Weight	Final concentration
PCR master mix 1	25 μL	1 ×
10 μM ALL-F primer	1 μL	0.2 μM
10 μM ALL-R primer	1 μL	0.2 μM
FR1, CDR1, CDR2, FR3 and CDR3 DNA template mixture	100 ng	2 ng/μL
ddH_2O	to 50 μL	n/a

Thermocycling conditions for PCR:

Steps	Temperature	Time
Initial denaturation	94°C	4 min
28 cycles	94°C	45 s
	55°C	45 s
	72°C	1 min
Final extension	72°C	5 min
Hold	4°C	

   ii. Run all the PCR samples with 2% agarose gel to verify the size and purify the right size PCR products (Figure 3B) from the agarose gel with QIAquick Gel Extraction Kit.

Note: If the over-lapping PCR productivity is low. Assemble the fragments with two steps (troubleshooting 1).

3. Digest the pComb3X plasmid and V_H gene repertoires with *SfiI* restriction enzyme in PCR tubes at 50°C for 12 h with the following conditions:
   a. 100 μL digest system:

Component	Volume/Weight	Final concentration
CutSmart buffer	10 μL	1 ×
plasmid or V_H DNA	2 μg	0.02 μg/μL
SfiI	1 μL for pComb-3X DNA 4 μL for V_H DNA	0.2 units/μL (pComb-3X) 0.8 units/μL (V_H DNA)
ddH_2O	to 100 μL	n/a

Total 1.5 mg pComb3X and 500 μg V_H gene needed.

⚠ CRITICAL: The size of pComb3X plasmid is ~9 fold larger than V_H DNA. With the same amount of DNA, V_H has higher molarity. So, more *SfiI* is needed to digest the V_H DNA completely.

b. Run all the digested DNA with 1% agarose gel to verify the size and purify the digested products (Figure 3C) from the agarose gel with QIAquick Gel Extraction Kit.

4. Ligation of purified V_H fragments to pCornb3X vector using a molar ratio of 3:1 V_H fragments to pComb3X at 16°C for 60 h with the following conditions:

100 μL ligation system:

Component	100 μL reaction
T4 DNA Ligase Buffer (10 ×)	10 μL
pComb3X	750 ng
V_H	250 ng
T4 DNA ligase	5 μL
ddH_2O	To 100 μL

Total 50 mL ligation is needed for a 10^{11} size library construction.

⚠ CRITICAL: In case of degradation, a 100 μL small-scale pilot experiments should be performed to check the degradation at 24, 48 and 60 h with 1% agarose gel before large scale experiment. Choose the time with no significant degradation detected for ligation. In this library construction, 60 h is a good choice.

5. Recover the ligation DNA with DNA Clean & Concentrator-5 according to the manufacturer's protocol (https://files.zymoresearch.com/protocols/_d4003t_d4003_d4004_d4013_d4014_dna_clean_concentrator_-5.pdf).

⚠ CRITICAL: Each column is load with 5 μg ligation DNA and eluted with 6 μL ddH_2O. The efficiency of ligation DNA recovery is ~30%. ~150 μg ligation DNA can be recovered with the DNA Clean & Concentrator-5 kit.

6. Pool all the eluted DNA and determine the DNA concentration by Nanodrop Lite.
7. Electroporate TG1 Electrocompetent Cells with the following conditions:
   a. Add 1 μg ligation DNA into each vial of TG1 cells (1 μg DNA in 50 μL competent bacteria per vial). Stir with tips 20 times and keep on ice 5–10 min.
   b. Transfer the bacteria into ice pre-chilled 0.1 cm cuvette.
   c. Electroporate using the pre-set program with setting at 1.8 kV/ 200 ohms/25 μF.
   d. Wash the cuvette with 1 mL pre-warm 2-YT medium three times and transfer the bacteria into 50 mL pre-warm 2-YT medium.

Note: During large scale electroporation, 10 electroporated/transformed TG1 vials are resuspended with 500 mL pre-warm 2-YT medium in a 2 L shake flask. Around 150 electroporation vials are needed for > 10^{11} size library construction, thus 15 × 2 L shake flasks with 7.5 L pre-warm 2-YT medium is needed to resuspend all the electroporated/transformed TG1 cells.

8. Recover the bacteria at 37°C, 200 rpm for 30 min, Aliquot $1/10^5$ bacteria (5 μL culture medium) from each bottle into a 1.5 mL centrifuge tube which contain 995 μL fresh 2-YT medium for titration. 10-fold serial dilute the bacteria and take $1/10^7$, $1/10^8$ and $1/10^9$ of total bacteria (100 μL of 10-, 10^2- and 10^3-fold diluted samples) from the dilutions, plate onto 2-YT-Agar plates with 100 μg/mL ampicillin. Select the transformants by adding 100 μg/mL ampicillin and 2% glucose, shaking at 37°C, 200 rpm for 2–3 h till the $OD6_{00}$ reach to ~0.6–0.8.
9. Add M13KO7 helper phage into the cells with multiplicity of infection (MOI) = 10:1, incubate at 37°C, 45 min. Mix every 15 min during incubation.

Note: MOI means the ratio of phages added to bacteria. OD_{600} of 1 corresponds to approximately 5×10^8 TG1 cells per ml. If the $OD_{600} \approx 0.6$, total TG1 cells equals $0.6 \times 5 \times 10^8$/mL ×

7500 mL ≈ 2.25×10^{12}, and ≈ 2.25×10^{13} M13KO7 helper phage are needed for the infection.

10. Centrifuge the bacteria at 5,000 *g* for 5 min at 4°C. Resuspend the bacterial pellet with fresh 2-YT contain 100 μg/mL ampicillin, 50 μg/mL kanamycin.
11. Shaking at 30°C, 200 rpm overnight (12–15 h).
12. Spin down the bacteria at 8,000 *g* for 15 min at 4°C, transfer the supernatant into new bottles, add 25% volume of PEG/NaCl solution into the supernatant and incubate on ice for 1 h.
13. Centrifugation of the mixture at 11,000 *g* for 20 min at 4°C. Discard supernatant and resuspend the pellet with 50 mL ice cold DPBS per liter of culture.
14. Centrifugation again at 10,000 *g* for 10 min at 4°C to eliminate the bacterial contamination.

⚠ CRITICAL: This bacteria elimination step is critical before storage, it will eliminate most of the bacteria in the phage library. The remaining bacteria will be eliminated by multiple rounds of wash during the phage panning process.

15. Transfer all the supernatant into a new bottle, add 20% glycerol and aliquot into 1 mL/vial, stock at −80°C for long-term storage. Determine the phage titer by detecting OD_{268} (1 OD ≈ 5×10^{12} phage)(Durr, Nothaft et al. 2010). The phage titer should above 5×10^{12}/mL

⚠ CRITICAL: 50 mL ligation with 375 μg digested pComb3X and 125 μg V_H is needed for a > 10^{11} size library. ~150 μg ligation DNA can be recovered with DNA Clean & Concentrator-5 kit (efficiency is ~30%). 1 μg ligation is used for one electroporation. Each electroporation will result in > 10^9 colonies, 150 electroporation will produce > 1.5×10^{11} colonies in total. According to the Sanger sequencing results, ~30% of the plasmid are empty vector, junk DNA or V_H with stop codon. So, 50 mL ligation should get a > 1.05×10^{11} size library.

16. Quality checks of the library:
    a. Randomly pick > 100 colonies and scale up in 4 mL 2-YT medium with 100 μg/mL ampicillin, shaking at 200 rpm overnight (12–15 h).
    b. Purify the plasmid with QIAprep Spin Miniprep Kit and elute with 30 μL water.
    c. Send all the plasmid for sequencing and analyze all the sequences.

⚠ CRITICAL: All the V_H sequences should be different from each other. ~30% of the plasmid are empty vector, junk DNA or V_H with stop codon.

Note: If repeat V_Hs are detected, the quality of the library is not good and library size might be smaller than 10^{11}, so re-construction of the library to make the size >10^{11} is needed.

Note: We are using two asymmetric SfiI sites for library construction. Sequencing results showed there is no issue of orientation of V_H insert in our pComb3X system.

Note: Beside randomly picked > 100 colonies for sequencing, we also evaluated the library quality and performance by panning with more than 5 antigens. For a high-quality library, several dozens of different V_H binders binding to each antigen were enriched. Multiple binders and the diversity of the selected V_H binders revealed good quality of library.

Molecular cloning, antigen expression, and protein purification

Compare with the microbial expression system, mammalian cell expression has the advantage of protein expression, more advanced peptide folding and post-translational modifications (Gray 2001, Khan 2013). In general, antigens expressed from mammalian cells which have proper folding and post-translational modifications are essential for full biological activity and successful selection

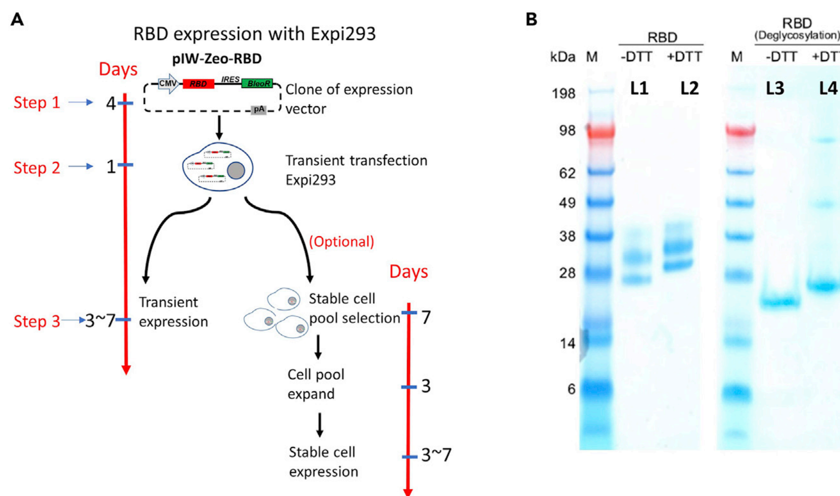

Figure 5. RBD-AviTag antigen expression

(A) An overview of RBD-AviTag transient expression and stable cell pool selection. There are three steps in RBD-AviTag expression: Step 1: Molecular cloning of the pIW-Zeo-RBD plasmid (4 days were needed). Step 2: Overnight transfection of the Expi293™ cell with pIW-Zeo-RBD plasmid (1 day was needed). Step 3: After transfection, cells were maintained at 37°C in incubator shaker rotating at 135 rpm with 8% CO_2 and 85% humidity for 3–7 days for RBD-AviTag transient expression. Or select stable cell pool with Zeocin antibiotic (step 18e, optional).

(B) SDS-PAGE results of RBD-AviTag with or W/O DTT. Line 1 (L1) and Line 2 (L2): Both RBD-AviTag samples with or W/O DDT shown three bands. Line 3 (L3) and line 4 (L4): After deglycosylation, only one band was detected in both L3 and L4. The results show multiple bands due to glycosylation of the RBD-AviTag protein. Figure reprinted with permission from Liu, X. et al., 2020.

of high affinity specific binders. Here we used the SARS-CoV-2 receptor-binding domain (RBD) as an example. This section includes molecular cloning, expression, and purification (Figures 1, 2, and 5).

Molecular cloning

Timing: 3 days

17. Clone the RBD into expression plasmid pIW-Zeo (Figure 2A):
    a. We have previously generated an expression plasmid named pIW-Zeo
    b. Amplify the SARS-CoV-2 RBD gene by the EcoRV-RBD-P1/EcoRI-His-AviTag-P2 primers (which contain the *EcoRV* and *EcoRI* restriction sites) with the Phusion Flash High-Fidelity PCR Master Mix according to the manufacturer's protocol (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0012774_Phusion_Flash_HiFi_PCR_MasterMix_100rxn_UG.pdf&title=VXNlciBHdWlkZTogUGh1c2lvbiBGbGFzaCBIaWdoLUZpZGVsaXR5IFBDUiBNYXN0ZXIgTWl4), using the following cycling conditions:
        i. 50 μL PCR system:

Component	Volume/Weight	Final concentration
2 × Phusion Flash PCR Master Mix	25 μL	1 ×
10 μM EcoRV-RBD-P1 primer	2 μL	0.4 μM
10 μM EcoRI-His-AviTag-P2 primer	2 μL	0.4 μM
gBlock RBD-AviTag	20 ng	0.4 ng/μL
ddH_2O	to 50 μL	n/a

Thermocycling conditions for PCR:

Steps	Temperature	Time
Initial denaturation	98°C	20 s
34 cycles	98°C	5 s
	55°C	10 s
	72°C	15 s
Final extension	72°C	5 min
Hold	4°C	

   ii. Run all the PCR product on a 1% agarose gel to verify the size and purify the PCR RBD-AviTag product from the agarose gel with QIAquick Gel Extraction Kit.

c. Digest the pIW-Zeo plasmid and the PCR amplified RBD-AviTag gene in separate reactions. Incubate each mixture at 37°C for 2 h with the following conditions:
50μL digest system:

Component	Volume/Weight	Final concentration
CutSmart buffer	5 μL	1 ×
pIW-Zeo or RBD-AviTag	1 μg	0.02 μg/μL
EcoRV-HF	2 μL	0.8 units/μL
EcoRI-HF	2 μL	0.8 units/μL
ddH_2O	to 50 μL	n/a

d. Run a 1% agarose gel, the digested PCR product and plasmid bands should be ~700 bp and ~5,000 bp, respectively.
e. Recovery the digested PCR product and plasmid DNA from the agarose gel. Elute with 10 μL water.
f. Ligate the digested RBD-AviTag gene into the pIW-Zeo plasmid. Leave the ligation reaction at 37°C for 15 min:
10 μL ligation system:

Component	10 μL reaction
T4 DNA Ligase Buffer (10 ×)	1 μL
pIW-Zeo	1 μL
RBD-AviTag	2 μL
T4 DNA ligase	1 μL
ddH_2O	to 10 μL

g. Transform 2 μL ligation reaction with 20 μL of E. coli DH5α competent cells. After recovering for 45–60 min at 37°C (shaking at 200 rpm), spread cells onto a 2-YT agar plate supplemented with 100 μg/mL ampicillin. Incubate the plate in a 37°C incubator overnight (12–15 h).
h. Randomly pick several colonies and scale up in 4 mL 2-YT medium with 100 μg/mL ampicillin, shaking at 200 rpm overnight (12–15 h).
i. Purify the plasmid with QIAprep Spin Miniprep Kit and elute with 50 μL water. Verification of the plasmid by DNA sequencing and keep the right clones (pIW-Zeo-RBD, Figure 2B).

RBD-AviTag antigen expression

Timing: 7 days

18. Transfection and Protein Expression (30 mL expression, Figure 5A):
   a. The day before transfection, seed the cells at a density of 2.0×10^6 viable cells/mL and incubate at 37°C in incubator shaker rotating at 135 rpm with 8% CO_2 and 85% humidity.

b. On the day of transfection, determine number and viability of the cells using an automated cell counter. Dilute the cells to 3 × 10^6 viable cells/mL with Expi293™ Expression Medium.
c. Add 27 mL cell suspension into a 125 mL Erlenmeyer shaker flask. Return the cells to the incubator.
d. prepare DNA-PEI complexes as follows:
   i. Dilute 30 μg of plasmid DNA into 1.5 mL Expi293™ Expression Medium, mix gently and incubate for 5 min at RT (20°C–25°C).
   ii. Dilute 120 μg of PEI into 1.5 mL Expi293™ Expression Medium, mix gently and incubate for 5 min at RT (20°C–25°C).
   iii. After 5 min incubation, mix the plasmid DNA with the PEI. Incubate at RT (20°C–25°C) for 10–20 min.
   iv. After the DNA-PEI complex incubation is complete, add the complex into shaker flask from step 18c. Gently swirl the flask.
   v. Return and incubate the cells in the incubator. Maintain 7 days at 37°C.

Note: Some expressed proteins might have degradation, denaturation, or aggregation during several days culturing at 37°C. Short time culture can protect protein from degradation, denaturation or aggregation to get proteins of higher quality, while long time culturing has higher yield. For the RBD-AviTag expression, the yield of 3 days culture is ≈ 5mg/L, and of 7 days is higher than 10 mg/L. We also found the activity of RBD-AviTag purified at day 3 is better than that of day 7. Thus, according to our experience, 3 days of culture is sufficient for RBD-AviTag expression.

e. (optional) 24 h after transfection, the transfected cells can be used for stable cell pool selection with the following steps (Figure 5A):
   i. Take 5 mL transfected cells into 50 mL centrifugation tube. Centrifugation at 300 *g*, 3 min. Return the supernatant into the expression bottle.
   ii. Resuspend the cells with 5 mL Expi293™ Expression Medium contain 250 μg/mL Zeocin. Return and incubate the cells in the incubator.
   iii. Change medium at day 1, day 2, day 3 and day 4 with 5 mL fresh Expi293™ Expression Medium containing 250 μg/mL Zeocin.

Note: Because of the transfection efficiency variation, the viability of the cells at day 4 might be different. If the viability is < 30% at day 4, reduce the Zeocin concentration to 50 μg/mL for the following three days selection.
In our RBD-AviTag stable cell pool selection process, the viability is ∼64% at day 4. So, 250 μg/mL Zeocin was used for 7 days selection.

   iv. Change medium at day 7 and resuspend the cells with 20 mL Expi293™ Expression Medium with 50 μg/mL Zeocin. Add the suspension cell into a 125 mL Erlenmeyer shaker flask, Return and incubate the cells in the incubator maintain 3–6 days (Stable cell pool is ready for expression).

RBD-AviTag-His purification with Ni-NTA column

Timing: 1 day

19. Protein purification
   a. Prepare the Ni-NTA gravity column:
      i. Transfer 1 mL His Pur™ Ni-NTA Resin (10 mg protein/ 1 mL beads) into a gravity column.
      ii. Allow the resin to settle, then let the excess buffer drain through the column by gravity flow.
      iii. Wash the resin with 20 mL Milli Q water.
      iv. Wash the resin with 20 mL binding buffer.

b. Cell culture media preparation:
   i. Transfer the expression medium at step 18 into a 50 mL conical tubes, spin down at 300 *g* for 5 min at 4°C.
   ii. Pour supernatant into new conical tubes. Spin down at 12,000 *g* for 10 min at 4°C.
c. Protein loading and elution:
   i. Load all the media into the column at step 19a.
   ii. Once the medium has completely entered the column, wash the column with 40 mL wash buffer.
   iii. Elution the RBD-AviTag with 4 mL elution buffer.
   iv. Change buffer with DPBS with 10 kD ultra-filter. Follow instructions in the manufacturer's protocol (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0015695_2162596_PierceProteinConcentrator_2_6mLPI.pdf&title=VXNlciBHdWlkZTogUGllcmNlIFByb3RlaW4gQ29uY2VudHJhdG9yLCBQRVMgLSAzSywgMTBLLCAzMEsgYW5kIDEwMEsgTVdDTzsgMi02bUw=).
   v. Measure the protein concentration on NanoDrop Lite. Run SDS-PAGE gel to characterize the purified RBD-AviTag (Figure 5B. Adapted from (Liu, Drelich et al. 2020)).

Note: The productivity of RBD-AviTag is ~10 mg/L. 1 μL purified RBD-AviTag sample is needed for concentration detection with NanoDrop Lite.

Phage-displayed library panning

Timing: 6 days

After obtaining the RBD-AviTag antigen, its quality was checked by human angiotensin-converting enzyme 2 (ACE2) binding ELISA. Then it is ready for bio-panning. This section describes streptavidin-magnetic beads-based library panning strategy.

20. Biotin-label the RBD-AviTag (RBD-Bio) antigen with BirA Biotin-Protein Ligase Kit according to the manufacture's protocol (https://www.avidity.com/showpdf.asp?N=B9B6C28E-1976-4CFE-89DE-E37D1D8DB2CA).

21. Library panning with streptavidin magnetic Beads:
   a. Thaw a phage library aliquot (1 mL/aliquot, $\approx 5 \times 10^{12}$ phage/mL). Add 250 μL ($V_{PEG/NaCl}$:V_{phage} = 1:4) PEG/NaCl solution into the phage, incubate on ice for 20 min.
   b. Centrifugation at 12,000 *g* for 10 min at 4°C. Discard supernatant and resuspend the pellet with 200 μL DPBS.
   c. Take 2×10^{12} library phage diluted into 1 mL 3% BSA-DPBS. Add 10 μg RBD-Bio antigen, rotation 1.5 h at RT (20°C–25°C).

   ⚠ CRITICAL: The amount of antigen used for panning are different in each round while the incubation time did not change. In our protocol we used 10 μg for 1st round, 2nd round 5 μg, 3rd round 1 μg, 4th round 0.2 μg. (Use high concentration antigen at 1st round can help to enrich more binders. Lower concentration of antigens in the following panning process will help to enrich high affinity V_H domains).

   d. At the same time, pick single colony from TG1 bacterial plate (LB-agar plate) or directly take 1 μL TG1 from commercial stock and scale up in 20 mL 2-YT medium at 37°C, 200 rpm until $OD_{600} \approx 0.5$.
   e. Take 25 μL streptavidin beads into a 1.5 mL centrifugation tube, wash the beads with 1 mL DPBS twice with magnets stand.

Note: Blocking the beads with 3% BSA-DPBS for 1 hour at RT (20°C–25°C) before use is good for panning. Because we have an extra step of depletion with streptavidin beads before panning (start from 2nd round), Streptavidin beads blocking is optional.

f. Transfer the phage at step 21c into washed streptavidin beads, rotate the tubes at 10 RPM/min for 1 h at RT (20°C–25°C).
g. Wash the beads with 1 mL 0.05% PBST (0.05% Tween-20 in DPBS) for 5 times, then wash with DPBS twice.

Note: Wash the beads with 0.05% PBST as follows: 1st round 5 times, 2nd round 8 times, 3rd round 15 times and 4th wash 20 times. (Increasing the washing numbers throughout the phage display selection can help to decrease low affinity binders. So that high affinity antibodies can be enriched efficiently).

h. Resuspend the beads with 5 mL TG1 cells (OD_{600} ≈ 0.5) in 15 mL culture tube, incubate the mixture at 37°C, 45 min. Mix every 15 min during incubation.

⚠ CRITICAL: The phage on the beads can infect TG1 cells effectively, so the elution step is not required.

i. Take $1/10^3$ infected TG1 cells (5 μL culture medium) into a 1.5 mL centrifuge tube which contain 995 μL fresh 2-YT medium. 10-fold serial dilute the bacteria with 2-YT medium and take $1/10^4$, $1/10^5$ and $1/10^6$ of total bacteria plate onto 2-YT-Agar plates with 100 μg/mL ampicillin. The rest add 100 μg/mL ampicillin and 2% glucose, shaking at 37°C, 200 rpm for 2 h.
j. Add 10 μL M13KO7 helper phage (Cat#18311019, Thermo) into the cells (10 μL/5 mL, phage titer: 10^{11}/mL), incubate at 37°C, 45 min. Mix every 15 min during incubation.
k. Centrifuge at 4,000 *g* for 10 min at RT (20°C–25°C). Discard supernatant and resuspend the pellet with 50 mL 2-YT contain 100 μg/mL ampicillin, 50 μg/mL kanamycin. shaking at 30°C, 200 rpm 12–15 h.

22. Phage purification and start of the next round:
   a. Check the colonies the next day.
   b. Transfer the cell culture into a 50 mL centrifuge tube, Spin down the bacteria at 8,000 *g* for 10 min at 4°C and collect the supernatant with a new 50 mL centrifugation tube.
   c. Add 25% volume of PEG/NaCl solution into the supernatant, mix and incubate on ice for 1 h.
   d. Centrifuge at 11,000 *g* for 20 min. Discard supernatant and resuspend the pellet with 2 mL cold DPBS.
   e. Separate the phage into two 1.5 mL tubes and centrifugation at 15,000 *g* for 1 min at 4°C to eliminate the bacteria.

Note: This bacterial eliminate step is critical before storage, it will eliminate most of the bacteria in the phage. The rest bacteria will be eliminated by multiple rounds of wash during the next round of phage panning.

   f. Transfer the phage to two new tubes and determined the titer by detecting OD_{268} (1 OD ≈ 5×10^{12} phage).

⚠ CRITICAL: If the phage titer is lower than 10^{11}/mL, do not start next round of panning. Check the quality of TG1 cell and M13KO7 helper phage to confirm both are good for experiment. Repeat the first round of panning and culture 15 h before purifying the phage.

   g. Add 20% glycerol into one tube and stock in −80°C. Take 10^{12} phage from the other tube for next round of panning.

Note: If the phage purified from previous round is lower than 10^{12}, use > 10^{11} phage for panning. The phage input can be less in the later rounds of panning due to the decreased diversity.

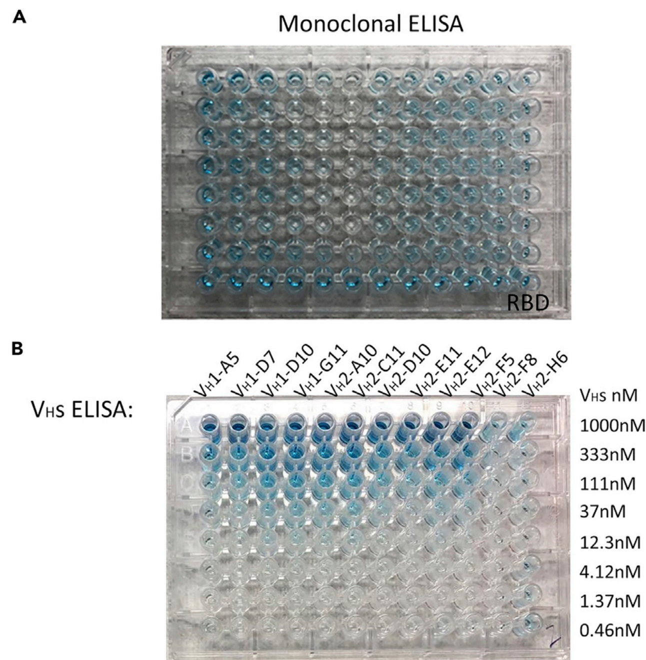

Figure 6. ELISA results of selected clones

(A) Plate based monoclonal ELISA Screening results. Clear wells mean no V_Hs or very low concentration (undetectable with ELISA) V_Hs binding with the coated plate. Blue color wells shown high concentration V_Hs binding with the RBD-AviTag coated plate. The colonies which get blue color are selected and expanded for sequencing.

(B) ELISA results of different purified V_H binders. The plate was coated with RBD-AviTag. 3-fold serial diluted V_H binders (start concentration=1 μM) were added into each well to detect the binding of V_Hs to RBD-AviTag. The results shown all the V_Hs have good binding with RBD-AviTag.

Pause point: add 20% glycerol into both tubes and stock in −80°C for long term storage till next round of panning.

23. Complete round 2, round 3 and round 4 (optional) phage panning.

CRITICAL: Before starting next round of panning, take 10^{11} to 10^{12} previous round purified phage, dilute the phage into 1 mL 5% Milk-DPBS (second and fourth rounds panning blocking the phage with 5% Milk-DPBS, third round panning blocking the phage with 3% BSA-DPBS) and incubate with 50 μL streptavidin-magnetic beads at 10 rpm for 1 hour at RT (20°C–25°C). Clean the beads with magnets. The beads depleted phage is used for next round of panning.

Note: Depending on the enrichment, in some cases round 4 is necessary to enrich high-affinity binders and narrow down the whole hits.

Optional: polyclonal phage ELISA to detect the enrichment of binders:

a. Coat the ELISA plates with RBD-AviTag (5 μg/mL in DPBS, 50 μL/well) overnight (12–15 h) at 4°C.
b. Wash plates 3 times with 0.05% PBST, block with 100 μL 5% Milk-DPBS 2 h.
c. Wash plates 3 times with 0.05% PBST.
d. Add 100 μL 5% Milk-DPBS diluted phage (~10^{11} phage from each round), shake 1 h with 200 rpm/min at RT (20°C–25°C).
e. Wash plates with 0.05% PBST, 4 times.

f. Add 100 μL Anti-M13 Antibody (HRP) (Sino Biological Inc, Cat#11973-MM05T-H, 1:2000 diluted in 5% Milk-DPBS), shake 45 min with 200 rpm/min at RT (20°C–25°C).
g. Wash plates with 0.05% PBST, 5 times.
h. Add 50 μL TMB into each well and reaction 5–10 min.
i. Stop the reaction with 50 μL 2 M H_2SO_4, read the optical density (OD) with a microplate reader at 450 nm.

24. Monoclonal ELISA Screening

After three rounds of panning, colonies will be picked for expression of heavy chain antibody domains which will be further screened by supernatant ELISA (Figure 6A. (Sun, Chen et al. 2020)).

a. Add 180 μL 2-YT medium with 100 μg/mL ampicillin to each well of 96-well plate.
b. Pick single colony into each well, Incubate the 96-well plates at 37°C, 200 rpm until $OD_{600} \approx 0.5$.

Note: Take 200 μL OD_{600} = 0.2, 0.4, 0.6, 0.8 TG1 cells into a 96 well plates, detect the OD_{600} with Synergy HTX Multi-Mode Reader to get a standard curve. Compare the absorbance with the standard curve. Once most wells reach to $OD_{600} \approx 0.5$, add IPTG to induce V_H expression.

c. Add 20 μL IPTG stock (10 mM) into each well (final IPTG concentration = 1 mM) to induce V_H expression. Incubate the plates at 30°C, 200 rpm 12–15 h.
d. Coat the ELISA plates with RBD-AviTag (2 μg/mL in DPBS, 50 μL/well) overnight (12–15 h) at 4°C.
e. Wash plates 3 times with 0.05% PBST, and block with 100 μL 3% BSA-DPBS 2 h.
f. Wash plates 3 times with 0.05% PBST.
g. Add 50 μL 6% BSA-DPBS into each well of the RBD-AviTag coated plates. Spin the bacteria expression plates (step 24c) at 4,000 *g* for 5 min at 4°C, transfer 50 μL supernatant into each well, shake 2 h with 200 rpm/min at RT (20°C–25°C).

Note: Keep the bacterial pellet for scale up and plasmid purification.

h. Wash plates with 0.05% PBST, 4 times.
i. Add 100 μL Monoclonal ANTI-FLAG® M2-Peroxidase (1:2500 diluted in 3% BSA-DPBS), shake 45 min with 200 rpm/min at RT (20°C–25°C).
j. Wash plates with 0.05% PBST, 5 times.
k. Add 50 μL TMB into each well and reaction 5–10 min.
l. Stop the reaction with 50 μL 2 M H_2SO_4, read the optical density (OD) with a microplate reader at 450 nm.
m. Take 10 μL bacterial from the positive wells, scale up with 2 mL 2-YT medium with 100 μg/mL ampicillin and incubate at 37°C, 200 rpm overnight (12–15 h). Purify the plasmid of selected positive clones and send for sequencing. Keep clones with unique sequences for V_Hs preparation and characterization.

25. V_H antibody domain expression and purification

a. Transform HB2151 competent cells with selected plasmid DNA by heat-shock 1 min at 42°C. Recovering 45–60 min at 37°C, 200 rpm, plate the transformed cells onto 2-YT-Agar plates with 100 μg/mL ampicillin and 1% glucose. Incubate at 37°C overnight (12–15 h).

⚠ CRITICAL: The pComb3X has an amber stop codon (TAG) between flag tag and gene III (Figure 2D). TG1 is an amber codon (TAG) suppressor strain, allowing translation to read through the codon and to produce a full-length V_H-gene III fusion protein. While the HB2151 is an amber codon non-suppressor strain, Only V_H gene can be translation to produce V_H in this strain. So, HB2151 is chosen for V_H expression without need to re-clone the V_H gene into another expression plasmid.

b. Pick single colony from the fresh transformed plate into 50 mL centrifugation tube which has 20 mL 2-YT medium containing 100 μg/mL of ampicillin, Incubate at 37°C, 200 rpm until $OD_{600} \approx 0.5$.
Add 20 μL IPTG stock (1 M) into each tube (final IPTG concentration = 1 mM). Incubate at 30°C, 200 rpm 12–15 h.
c. Centrifuge the bacteria at 5,000 *g* for 5 min at 4°C. Resuspend the bacterial pellet in 10 mL of DPBS, add 0.1 million units polymyxin B (5 MU polymyxin B for 1 L culture) and incubate at 37°C, 200 rpm for 30 min.

Note: Polymyxin B works by interacting with lipopolysaccharide (LPS), alters membrane permeability of gram-negative bacteria, ultimately leading to cell lysis. It is a simple and robust way to lysis outer membrane of gram-negative bacteria for periplasmic protein purification.

d. Centrifugation at 16,000 *g* for 30 min at 4°C. Transfer the supernatant into a new tube.
e. Purify the V_Hs with HisPur™ Ni-NTA Resin.
f. ELISA to confirm the binding of selected V_H antibody domains and detect their EC_{50} (Figure 6B).
g. Dynamic light scattering (DLS) and size exclusion chromatography (SEC) analysis to detect the aggregates of V_Hs candidates. Non-aggregates and low aggregates V_Hs are chosen for further study.
h. ELISA to detect the V_H antibody domains competition with ACE2 to RBD binding.
i. The V_H antibody domains competition with ACE2 are chosen for SPR to determine affinity and virus neutralization.

EXPECTED OUTCOMES

For V_H antibody domain library construction, each electroporation will result in > 10^9 colonies and 150 electroporation will make > 10^{11} size V_H library. Transient expression productivity of RBD-AviTag with Expi293 system should yield > 10 mg/L and the RBD-AviTag expression stable cell pool will be generated within 10 days. High affinity RBD V_H binders which compete with human ACE2 for binding to RBD will be selected after three rounds of panning. We have got 16 unique V_H binders with this protocol and the equilibrium dissociation constant of these binders is from 300 nM to 4 nM.

LIMITATIONS

Compared with single human B cell isolation, phage display is based on bacterial- expression system. In general, it has limitations on protein expression, folding and post-translational modification. Our V_H antibody domain library is generated from healthy human donors with the CDRs naturally grafted from human PBMC cDNA. It may lower the possibility of non-specific binding to human cells compare with synthetic library. However, non-specific binding was found in some of the selected V_H antibody domains.

Compare with scFv, Fab and VHH libraries, due to large size of our V_H library, we have selected out many high affinity V_H binders (nM range affinity). There are no significant affinity limitations compare with other libraries. However, the V_H antibody domains are much easier to aggregation and aggregations are detected in most of the selected antibody domains. So, characterization of the selected domains one by one to figure out the best functional candidates for further therapeutic development is needed.

TROUBLESHOOTING

Problem 1

The productivity of full-length V_H assemble by over-lapping PCR is low (step 2).

Potential solution

Assemble the full-length V_H with two steps:

1. Over-lapping PCR to assemble FR1, CDR1 and CDR2 with ALL-F/H2R1, H2R24, H2R36, H2R57 primers to get FR1-CDR1-CDR2-FR3. Assemble FR3 and CDR3 with FR3F/All-R primers to get FR3-CDR3-FR4 using the following cycling conditions:

50 μL PCR system:

Component	Volume/Weight	Final concentration
PCR master mix 1	25 μL	1 ×
10 μM forward primer	1 μL	0.2 μM
10 μM reverse primer	1 μL	0.2 μM
FR1, CDR1, CDR2 or FR3 and CDR3 DNA template mixture	100 ng	2 ng/μL
ddH_2O	to 50 μL	n/a

Thermocycling conditions for PCR:

Steps	Temperature	Time
Initial denaturation	94°C	4 min
25 cycles	94°C	45 s
	55°C	45 s
	72°C	1 min
Final extension	72°C	5 min
Hold	4°C	

Run all the PCR samples with 2% agarose gel to verify the size and purify the right size PCR products from the agarose gel with QIAquick Gel Extraction Kit.

2. Over-lapping PCR to assemble full-length V_H with FR1-CDR1-CDR2-FR3 and FR3-CDR3-FR4 DNA as template, ALL-F and ALL-R as primes using the following cycling conditions:

50 μL PCR system:

Component	Volume/Weight	Final concentration
PCR master mix 1	25 μL	1 ×
10 μM forward primer	1 μL	0.2 μM
10 μM reverse primer	1 μL	0.2 μM
FR1-CDR1-CDR2-FR3 and FR3-CDR3 DNA template mixture	100 ng	2 ng/μL
ddH_2O	to 50 μL	n/a

Thermocycling conditions for PCR:

Steps	Temperature	Time
Initial denaturation	94°C	4 min
28 cycles	94°C	45 s
	55°C	45 s
	72°C	1 min
Final extension	72°C	5 min
Hold	4°C	

Run all the PCR samples with 2% agarose gel to verify the size and purify the right size PCR products from the agarose gel with QIAquick Gel Extraction Kit.

Problem 2

Low efficiency of electroporation, hard to generate large size phage library (step 7).

Potential solution

The most common reason of low efficiency is the poor quality of digested V_H antibody domains (step 3) or DNA degradation by over- digestion and ligation (step 4). Long extra protection bases (such as 15 bp) in primers can be added in front of the *SfiI* restriction site. This will help improve the digestion of *SfiI* to get high quality digested V_H.

Run agarose gel to check the ligation DNA quality. If degradation is detected, shorten the ligation time to 48 h.

Problem 3

Low affinity of the selected V_H antibody domains (step 25f).

Potential solution

Use lower concentration of antigens for panning and increase the washing times to enrich high affinity domains.

Problem 4

Selected V_H domain candidates aggregate (step 25g).

Potential solution

Human variable domains rapidly aggregate when heated to 80°C–85°C (this condition well above their melting temperatures). The aggregated phage can be eliminated by centrifugation while the non-aggregation phage remains in the supernatant. Use the heat-treated phage supernatant for panning can help to get non-aggregating V_H domains (Dudgeon, Rouet et al. 2012).

Before panning (start from second round), heat the phage at 80°C for 10min, then keep on ice for 10 min and centrifugation for 10 min at 15,000 *g* (white pellet can be found at the bottom of the centrifuge tube after centrifugation). Collect the supernatant into a new tube to eliminate the aggregated V_Hs for panning.

Problem 5

During Expi293F™ cell maintain, cell clumping was detected, and cell viability is lower than 97% (In "before you begin, step 8").

Potential solution

Thaw a new Expi293F™ should always be the first choice. If clumping still detected, check the shake speed of the CO_2 Resistant incubator, and subculture the cells every two days with fresh medium.

Check the cell viability with trypan blue. If viability is lower than 97%, do not let the cell density over 5×10^6/mL and do not maintain the cells more than 5 days without subculture.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Chuan Chen (CHC316@pitt.edu), Wei Li (liwei171@pitt.edu), and Dimiter S. Dimitrov (mit666666@pitt.edu).

Materials availability

For cell lines and plasmid please contact Dimiter S. Dimitrov (mit666666@pitt.edu). All other materials are available commercially.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

C.C. drafted the manuscript, and the manuscript was edited by W.L., Z.S., X.L., and D.S.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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