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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™ 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 (VH) 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 VH antibody domains. It details three optimized parts including (1) construction of a large-sized (theoretically > 10^{11}) naive 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 VH antibody domain, VH 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 x 10^7 PBMCs were collected for RNA isolation.
2. Dissolving the cell pellet immediately with 60 mL RLT buffer (2.5 x 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
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?retursionurl=%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.

| Name | Sequence | Usage |
|------|----------|-------|
| ALL-F | GTTTGCTACCAGTGGCCAGGCGCCGAGGTGCAGTGTTGGA | FR1 and CDR1 |
| FR1R | ACAGGAAGAGTCTCAC | |
| H1F-157 | CTTAGACCTCTCGTAGGAAGSTTCCTCT | |
| H1F-2 | CTTAGACCTCTCGTACCKTCTCT | |
| H1F-3 | CTTAGACCTCTCGTAGGGCTCTCT | |
| H1F-4 | CTTAGACCTCTCGTGCCCCTCTCC | |
| H1F-6 | CTTAGACCTCTCGGCGCCAGGGAGGTCAT | |
| H1R-2 | CTTAGACCTCGGCGGAACCAGCAGC | |
| H1R-3 | CTTAGACCTCGGCGGAACCAGCTCAT | |
| H1R-4 | CTTAGACCTCGGCGGAACCAGCYCCA | |
| H1R-5 | CTTAGACCTCGGCGGAACCAGCYGAT | |
| H1R-6 | CTTAGACCTCGGCGGAACCAGCTTC | |
| H1R-7 | CTTAGACCTCGGCGGAACCAATTCAT | |
| H1R-ALL | CTTAGACCTCGGCGGAACCA | |
| H2F167 | TGGGTCCGCCAGGCGGCCGAGGTGCAGTGTTGGA | CDR2 |
| H2F2 | TGGGTCCGCCAGGCGGCCGAGGTGCAGTGTTGGA | |
| H2F345 | TGGGTCCGCCAGGCGGCCGAGGTGCAGTGTTGGA | |
| H2R1 | ATGTTCCTTGAGATGTGAAGCTTCTCTTGAGGGA | |
| H2R36 | ATGTTCCTTGAGATGTGAAGCTTCTCTTGAGGGA | |
| H2R24 | ATGTTCCTTGAGATGTGAAGCTTCTCTTGAGGGA | |
| H2R5 | ATGTTCCTTGAGATGTGAAGCTTCTCTTGAGGGA | |
| H2R7 | ATGTTCCTTGAGATGTGAAGCTTCTCTTGAGGGA | |
| FR3F | ACCATCTCCAGGAGAGATCC | FR3 |
| FR3R | GTCCTCGGCTCTCGGCTG | |
| H3F3p | AGGCGTGAAGGCGGGAACCAGCRGCGYTTTATTACTGT | CDR3 |
| H3F1p257p | AGGCGTGAAGGCGGGAACCAGCRGCGYTTTATTACTGT | |
| H3Father | AGGCGTGAAGGCGGGAACCAGCRGCGYTTTATTACTGT | |
| H3R | GTGGCGGCTCGCGCCACTTGAGGAGAGGCGGTGACC | |
| ALL-R | GTGCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG}
Plasmids and other materials

6. Generation of an empty plasmid named pIW-Zeo (Figure 2A) that contains a CMV promoter, 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 Maxi-prep, 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/C228 cells (Thermo, Cat#A14527) with Expi293/C228 Expression Medium in a CO₂ resistant incubator at 135 rpm, 8% CO₂, 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_UK.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 VH-gene III fusion protein. HB2151 is an amber codon non-suppressor strain, Only VH gene can be translation to produce VH 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 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| 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 | 152500500 |
| 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 | R31955 |
| EcoRI-HF | NEB | R3101S |
| Polyethylene Glycol 8000 | Fisher Scientific | BP233-1 |
| Sodium Chloride | Fisher Scientific | S271-3 |
| PEI MAX™ | Polyscience | 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 | Sigma-Aldrich | L2399 |
| 50 x TAE Buffer (Tris-acetate-EDTA) | Fisher Scientific | FER849 |
| 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 |
| Exp293™ Expression Medium | Thermo Fisher Scientific | A1435101 |
| DPBS, no calcium, no magnesium | Thermo Fisher Scientific | 14190136 |
| DPBS (10X), no calcium, no magnesium | Thermo Fisher Scientific | 14200-075 |
| Nuclease-Free Water | Thermo Fisher Scientific | AM9937 |
| Teknova2-YT BROTH | Fisher Scientific | SD-843-444 |
| Teknova 2-YT AGAR | Fisher Scientific | SD-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 |
| pW-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 |
| BiA Biotin-Protein Ligase Kit | Avidity | BiA-500 |
| High Fidelity PCR Master | Roche | 12140314001 |
| Phusion Flash High-Fidelity PCR Master Mix | Thermo Fisher Scientific | FS48L |
| Dynabeads™ MyOne™ Streptavidin T1 | Thermo Fisher Scientific | 65602 |
Continued

**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 x)   | 1 x                 | 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℃. This buffer is guaranteed for one year when stored properly.

**Wash buffer:**

| Reagent      | Final concentration | Amount |
|--------------|---------------------|--------|
| MilliQ water | n/a                 | 900 mL |
| DPBS (10X)   | 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℃. This buffer is guaranteed for one year when stored properly.

**Elution buffer:**

| Reagent      | Final concentration | Amount |
|--------------|---------------------|--------|
| MilliQ water | n/a                 | 900 mL |
| DPBS (10X)   | 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℃. 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℃–25℃). 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**

**VH library construction**

© Timing: 8 weeks
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<sub>H</sub> library by direct PCR amplification of V<sub>H</sub> 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<sub>H</sub> 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<sub>2</sub>O and adjust the concentration to 10 μM.

2. Assemble the V<sub>H</sub> 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 3. V<sub>H</sub> antibody domain library construction and agarose gel results](image)

(A) An overview of V<sub>H</sub> 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<sub>H</sub>. 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. Row 3: IGHV3. Row 4: IGHV4. Row 5: IGHV5. Row 6: IGHV6. Row 7: IGHV7).
- The CDR3 PCR products subgroups (~132 bp) in each row (Row 1–3: IGHV1, 2, 5. Row 4: IGHV3. Row 5: other subgroups).
- Overlap-PCR assembled V<sub>H</sub> results (Row 1–5: IGHV1~7 mixture, ~430 bp).

(C) Digest results of V<sub>H</sub> and pComb3x. The size of digested V<sub>H</sub>~370 bp. The size of digested pComb3x~3300 bp.
Forward primers

All-F: GTTTGCTACCCGTGCCCAGTGCCAGGCTAGCAGCTTGA

HFR-1: CTGAGACTCTCTGCTGGCACCTCC
HFR-1: CTGAGACTCTCTGCTGGCACCTCC
HFR-1: CTGAGACTCTCTGCTGGCACCTCC
HFR-1: CTGAGACTCTCTGCTGGCACCTCC

CDR1

GACTCTGAGAGGACA

CR2

H2F1345: TGAGCTCCGCGAGCGCTGAGGGAAGGGNNCTRNAAGTG
H2F12: TGAGCTCCGCGAGCGCTGAGGGAAGGGNNCTRNAAGTG
H2F167: TGAGCTCCGCGAGCGCTGAGGGAAGGGNNCTRNAAGTG

ACCCAGGCGTCCGAGGCTC
H1R-ALL
TACTGACCCAGGCGTCCGAGGCTC
CAGCGACCCAGGCGTCCGAGGCTC
TACCGACCCAGGCGTCCGAGGCTC
ACCTGACCCAGGCGTCCGAGGCTC
ACCTGACCCAGGCGTCCGAGGCTC
TACTTACCCAGGCGTCCGAGGCTC

CDR3

H3Rother: AGGCTGAAAGGGCGAGGCGAGGACACGCGCRAATTACTG
H3R1p257p: AGGCTGAAAGGGCGAGGCGAGGACACGCGCRAATTACTG
H3R3p: AGGCTGAAAGGGCGAGGCGAGGACACGCGCRAATTACTG

GTCGGACTCTCGGTCTCAG

Reverse primers

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 x                 |
| 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             |
| ddH₂O                     | 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 QIAGen 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 VH.

b. Over-lapping PCR to assemble full-length VH 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 x                 |
   | 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             |
   | ddH₂O                                          | 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 VH 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 x                 |
   | plasmid or VH DNA | 2 μg  | 0.02 μg/μL          |
   | SfiI       | 1 μL for pComb-3X DNA | 0.2 units/μL (pComb-3X) |
   |           | 4 μL for VH DNA   | 0.8 units/μL (VH DNA) |
   | ddH₂O     | to 100 μL       | n/a                 |

Total 1.5 mg pComb3X and 500 μg VH gene needed.

CRITICAL: The size of pComb3X plasmid is ~9 fold larger than VH DNA. With the same amount of DNA, VH has higher molarity. So, more SfiI is needed to digest the VH 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 pComb3X 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 X) | 10 μL |
| pComb3X             | 750 ng          |
| V_H                 | 250 ng          |
| T4 DNA ligase       | 5 μL            |
| ddH2O               | 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 ddH2O. 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 x 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 OD600 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 x 10^8 TG1 cells per ml. If the OD_{600} = 0.6, total TG1 cells equals 0.6 x 5 x 10^8/mL x
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 2YT 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^{5} 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_{H}s 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.
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=VXNlciBHdWlkZTogUGh1c2lvbiBGBgFzaCBiaWdoLUZpZGVsXRFsFBUiBNYXN0ZXJgTWl4), using the following cycling conditions:
   i. 50 μL PCR system:

| Component                          | Volume/Weight | Final concentration |
|------------------------------------|---------------|---------------------|
| 2 x Phusion Flash PCR Master Mix   | 25 μL         | 1 x                 |
| 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           |
| ddH₂O                              | to 50 μL      | n/a                 |

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 Exp293™ 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% CO₂ 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.
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-Avi-Tag 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:

| Component | Volume/Weight | Final concentration |
|-----------|--------------|---------------------|
| CutSmart buffer | 5 μL | 1 x |
| 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 |
| ddH2O | 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 x) | 1 μL |   |
| pIW-Zeo | 1 μL |   |
| RBD-AviTag | 2 μL |   |
| T4 DNA ligase | 1 μL |   |
| ddH2O | to 10 μL |   |

| Component | 10 μL reaction |   |
|-----------|----------------|---|
| T4 DNA Ligase Buffer (10 x) | 1 μL |   |
| pIW-Zeo | 1 μL |   |
| RBD-AviTag | 2 μL |   |
| T4 DNA ligase | 1 μL |   |
| ddH2O | 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 × 10⁶ viable cells/mL and incubate at 37°C in incubator shaker rotating at 135 rpm with 8% CO₂ 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 $3 \times 10^6$ 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 mg of plasmid DNA into 1.5 mL Expi293™ Expression Medium, mix gently and incubate for 5 min at RT ($20^\circ C$–$25^\circ C$).

   ii. Dilute 120 mg of PEI into 1.5 mL Expi293™ Expression Medium, mix gently and incubate for 5 min at RT ($20^\circ C$–$25^\circ C$).

   iii. After 5 min incubation, mix the plasmid DNA with the PEI. Incubate at RT ($20^\circ C$–$25^\circ C$) 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 $37^\circ C$.

   Note: Some expressed proteins might have degradation, denaturation, or aggregation during several days culturing at $37^\circ C$. 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 $\approx 5$ mg/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 $\approx 64\%$ 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%2FFTS-Assets%2FLSG%2Fmanuals%2FMAN0015695_2162596_PierceProteinConcentrator_2_6mLPI.pdf&title=VXNlciBHdWlkZTogUGllcmNlIFByb3RlaW4gQ29uY2VudHJvdG9yLCBQRVMgLSAzSywgMTBLLCAzMEsgYW5kIDEwMDMgMTBkMi4yMTQyMTMgMy0wMC0xMCwwZS0xMC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS0yMC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS0zMC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS00MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS01MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS02MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS03MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS04MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS05MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS06MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS07MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS08MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS09MC0wMCNjMTBiMzQxNjcgMC4yMTQyMTMgMy0xMC0wMCwwZS0AICzNjM0MjA5MTQxNjQyOQ==').
   v. Measure the protein concentration on Nano Drop 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, ≈ 5 x 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 x 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} = 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 (OD600 = 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 1/10^6, 1/10^5 and 1/10^4 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 4°C and collect the supernatant with a new 50 mL centrifugation tube.

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.

l. 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.
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.
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 OD₆₀₀ = 0.5.

Note: Take 200 μL OD₆₀₀ = 0.2, 0.4, 0.6, 0.8 TG1 cells into a 96 well plates, detect the OD₆₀₀ with Synergy HTX Multi-Mode Reader to get a standard curve. Compare the absorbance with the standard curve. Once most wells reach to OD₆₀₀ = 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 H₂SO₄, 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_H 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 OD₆₀₀ = 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 V₄ using HisPur™ Ni-NTA Resin.

f. ELISA to confirm the binding of selected V₄ antibody domains and detect their EC₅₀ (Figure 6B).

g. Dynamic light scattering (DLS) and size exclusion chromatography (SEC) analysis to detect the aggregates of V₄ candidates. Non-aggregates and low aggregates V₄ are chosen for further study.

h. ELISA to detect the V₄ antibody domains competition with ACE2 to RBD binding.

i. The V₄ antibody domains competition with ACE2 are chosen for SPR to determine affinity and virus neutralization.

EXPECTED OUTCOMES

For V₄ antibody domain library construction, each electroporation will result in > 10⁹ colonies and 150 electroporation will make > 10¹¹ size V₄ 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 V₄ binders which compete with human ACE2 for binding to RBD will be selected after three rounds of panning. We have got 16 unique V₄ 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 V₄ 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 compared with synthetic library. However, non-specific binding was found in some of the selected V₄ antibody domains.

Compare with scFv, Fab and VHH libraries, due to large size of our V₄ library, we have selected out many high affinity V₄ binders (nM range affinity). There are no significant affinity limitations compared with other libraries. However, the V₄ 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 V₄ assembled by over-lapping PCR is low (step 2).

Potential solution
Assemble the full-length V₄ 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 x                 |
| 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             |
| ddH₂O                                          | 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 Vₜ 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 x                 |
| 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             |
| ddH₂O                                          | 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 10 min, 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_{H}s for panning.

Problem 5
During Exp293F™ cell maintain, cell clumping was detected, and cell viability is lower than 97% (In “before you begin, step 8”).

Potential solution
Thaw a new Exp293F™ 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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