**Supplementary Methods**

This full protocol is similar to the protocol presented in our previous paper1. It incorporates several altered or updated procedures in addition to the modifications introduced in the methods section.

***Sample collection and B cell enrichment (Step 1)***

For best results, begin processing blood immediately after collection. If necessary, samples may be stored overnight at 4°C, but processing should begin within 18 hr of collection. Depending on the B cell subset, PBMCs frozen in appropriate tissue culture freeze-down media can also be used, albeit with reduced efficiency.

If ASCs are the cells of interest: ASCs become unstable and begin to die after removal from whole blood, so cells should be used immediately in ELISPOT and flow cytometry.

Materials:

RosetteSep (STEMCELL, 15064)

Lymphocyte Separation Media (Mediatech, 25072CV)

**1.1.** Collect 40 ml blood in citric acid dextrose tubes 7 days post-vaccination. Typically, one tube is for ELISPOT and three tubes are for sorting.

**1.2.** Pool the whole blood in a 50 ml conical tube and add 100 µl RosetteSep. Mix well by inverting. Incubate at room temperature for 20 min.

**1.3.** Aliquot the blood into three separate conical tubes, then dilute each with an equal volume of 0.02% BSA/PBS.

**1.4.** Set up three new 50 ml conical tubes with 15 ml lymphocyte separation media. To each tube, carefully overlay the diluted blood (no more than 30 ml blood per tube). Centrifuge at room temperature and 800g for 30 min with no brake.

**1.5.** Collect the PBMC layer, which is sandwiched between the serum and the lymphocyte separation media, and transfer to a new conical tube. Rinse the PBMCs by adding 0.02% BSA/PBS to 50 ml. Centrifuge at room temperature and 800g for 5-10 min with no brake. Remove the supernatant.

**1.6.** Combine cells from different tubes by filtering through a 40 µm cell strainer into a new conical tube. Add 0.02% BSA/PBS to 50 ml. Centrifuge at room temperature and 360 g for 5-10 min (brake can be used).

***ELISPOT (Step 2)***

Materials:

 Filter plate with hydrophilic MCE membrane (Millipore, MSHAN4B50)

 Goat anti-human IgA, IgG, IgM (KPL, 01-10-07)

 Goat anti-human IgA-biotin (Southern Biotech, 2050-08)

 Goat anti-human IgG-biotin (Southern Biotech, 2040-08)

 Goat anti-human IgM-biotin (Southern Biotech, 2020-08)

 Goat anti-mouse IgA, IgG, IgM (KPL, 01-18-07)

 Goat anti-mouse IgA-biotin (Southern Biotech, 1040-08)

 Goat anti-mouse IgG-biotin (Southern Biotech, 1030-08)

 Goat anti-mouse IgM-biotin (Southern Biotech, 1021-08)

 Streptavidin-AP (Southern Biotech, 7100-04)

 Tween 20 (Sigma, P9416)

 RPMI (Invitrogen, 11875135)

Penicillin-Streptomycin (Gibco, 15140)

L-Glutamine (Gibco, 25030)

FBS (Gibco, 16000)

 Beta-mercaptoethanol (Pierce, 35602)

 HEPES (Invitrogen, 15630)

 Sodium pyruvate (Invitrogen, 11360)

 1-Step NBT/BCIP (Thermo, 34042)

Reagents:

 Cell media:

 RPMI

 1% P/S

 1% HEPES

 1% sodium pyruvate

 1% L-glutamine

 20% FBS

 50 mM beta-mercaptoethanol

 Complete media:

 RPMI

 1% P/S

 1% HEPES

 1% L-glutamine

 10% FBS

 Blocking media:

 RPMI

 10% FBS

**2.1.** Coat individual columns of filter plates with 100 µl/well of coating antibody (10 µg/ml goat species-specific IgA, IgG, and IgM in PBS) and virus, vaccine (dilute approximately 1:20 in PBS), or other desired antigen. Determine exact coating protein concentrations experimentally to ensure adequate spot size. Spots should be large enough to be counted clearly. Incubate plates at 4°C for at least 18 hr and up to 7 days.

**2.2.** Wash 3x with 200 µl/well PBS/0.05% Tween (vol/vol) using a multichannel pipette.

Important: when washing, do not touch the membrane with pipette tips, and pipette with the plate at an angle. To remove liquid between washes, pour quickly. Refrain from tapping plates on paper towels to dry them unless necessary.

**2.3.** Wash 4x with 200 µl PBS using a multichannel pipette.

**2.4.** Block with 200 µl/well blocking media. Incubate at 37°C for 2 hr.

**2.5.** Aliquot 0.5-1 million PBMCs per condition (each coated column on the filter plate). Wash cells with cell media and centrifuge at room temperature and 360g for 5-10 min. Repeat twice. Resuspend PBMCs in complete media (200 µl per condition).

**2.6.** In a U-bottom plate, add 200 µl cells to row 1. Add 100 µl complete media to rows 2-7.

**2.7.** Make 1:2 serial dilutions of cells in rows 1-7 by progressively transferring and mixing 100 µl from each row to the next. Add 100 µl cell media to row 8.

**2.8.** Remove blocking media from ELISPOT plates by pouring.

**2.9.** Transfer 100 µl/well cell dilutions to the ELISPOT plate. Incubate overnight at 37°C and 5% CO2.

**2.10.** Wash 4x with 200 µl/well PBS/0.05% Tween (vol/vol) using a multichannel pipette.

**2.11.** Wash 4x with 200 µl/well PBS using a multichannel pipette.

**2.12.** Make the species-specific, isotype-specific, biotin-conjugated goat antibody mixture for cell detection. Add each at 1:1000 to PBS/0.05% Tween/1% FBS.

**2.13.** Add 100 µl/well revealing solution and incubate at room temperature for 2 hr.

**2.14.** Wash 4x with 200 µl/well PBS/0.05% Tween (vol/vol) using a multichannel pipette.

**2.15.** Prepare the secondary antibody by adding 1:500 streptavidin-AP to PBS/0.05% Tween/1% FBS.

**2.16.** Add 100 µl/well secondary antibody and incubate at room temperature for 2 hr.

**2.17.** Wash 3x with 200 µl/well PBS/0.05% Tween (vol/vol) using a multichannel pipette.

**2.18.** Wash 2x with 200 µl/well PBS using a multichannel pipette. Let the PBS sit for 5 min during each wash cycle.

**2.19.** Add 100 µl/well NBT/BCIP to reveal the substrate. Incubate under a paper towel for 1-5 min at room temperature, or until dark spots appear.

**2.20.** Stop the revealing reaction by washing the plate under a faucet. Pat dry the inverted plate on a paper towel, then air dry in a drawer before reading spots with an ELISPOT analyzer. Spots are stable for several years at room temperature.

***Flow cytometry (Step 3)***

Begin with 4-8 million enriched PBMCs. This flow strategy is designed to isolate human plasmablasts, but can be modified to suit different requirements.

Materials:

CD3 FITC (Invitrogen, MHCD0301)

CD19 Pacific Blue (Biolegend, 302224)

CD27 PE (Biolegend, 302808)

CD38 AlexaFluor 647 (Biolegend, 303514)

DMEM (Gibco, 11965)

FBS (Gibco, 16000)

Penicillin-Streptomycin (Gibco, 15140)

L-Glutamine (Gibco, 25030)

 RNasin (Promega, N2515)

TCL buffer (Qiagen, 1031576)

Beta-mercaptoethanol (Pierce, 35602)

Reagents:

Catch buffer (for ten half-plates; made fresh)

 5 ml RNase-free water

 50 µl 1M Tris pH 8.0

 125 µl RNasin

TCL buffer

 TCL buffer

 1% beta-mercaptoethanol (vol/vol)

Staining buffer

 PBS

 2% FCS

**3.1.** As controls, for each fluorophore, prepare an aliquot of 0.5 x 106 cells in 100 µl staining buffer. Prepare an additional aliquot of 0.5 x 106 cells in 100 µl staining buffer that will remain unstained. Prepare the remaining cells for sorting in 500 µl staining buffer.

**3.2.** Add fluorophores (CD3 FITC, anti-CD27 PE, anti-CD38 APC-Cy5.5, anti-CD19 Pacific Blue) to the sorting aliquot and also individually to the aliquots designated as compensation controls. Incubate cells at 4°C for 30 min.

Note: Each fluorophore should be titrated to distinguish single color populations before beginning the experiment.

**3.3.** Wash twice with 200 µl 0.2% BSA/PBS (vol/vol) and filter through a 40 µm cell strainer to prevent clogging.

**3.4.** Gate CD19+/CD3-/CD27high/CD38high cells. Bulk sort the cells into tubes that contain complete DMEM (DMEM with 10% FBS, 1% P/S, 1% L-Glutamine).

**3.5.** Sort plasmablasts on forward vs side scatter using a live cell gate with doublet discrimination into 96-well PCR plates containing the desired buffer (catch buffer or TCL buffer, depending on whether RNA purification will be performed).

Sort cells from each sample into half-plates (columns 1-6 or 7-12). Reserve row H for buffer-only controls, which will be used to detect PCR contamination.

Use RNase-free precautions. Immediately seal plates and put on dry ice, then store at -80°C (for several years if necessary).

***cDNA preparation (Step 4)***

Refer to the technical methods section in the main paper.

Materials:

 RNasin (Promega, N2515)

 TCL buffer (Qiagen, 1031576)

 SPRI beads (Beckman, A63987)

 Ring Super Magnet Plate (Beckman, A32782)

 RNase away (Thermo, 7003)

 DNA off (Takara, 9036)

 Maxima cDNA Synthesis Kit (Thermo, K1642)

 IGEPAL CA-630 (Sigma, I8896)

 SuperScript IV Synthesis System (Thermo, 18091200)

 Hard-Shell Low-Profile Thin-Wall 96-Well Skirted PCR Plates (Bio-Rad, HSP9601)

 Microseal “F” foil seals (Bio-Rad, MSF1001)

***PCR (Step 5A /5B)***

Important: to avoid contamination, avoid contact with the interior of Eppendorf tubes and limit airflow over open racks. Wipe pipettes and surfaces with DNA off before use.

***Human 1st and 2nd PCR (Step 5A)***

Three rounds of PCR are performed: 1st PCR, 2nd PCR, and Cloning PCR.

The template for the 1st PCR is the reverse transcription product from step 4A or 4B. The product of the 1st PCR is used as template DNA for both the 2nd PCR and the Cloning PCR. The 2nd PCR’s product is sent for sequencing and the Cloning PCR’s product is used in Gibson assembly and further cloning.

Materials:

DreamTaq Green PCR 2X MasterMix (Thermo, K1082)

 NEBuffer 3 (NEB, B7003S)

 CIP (NEB, M0290L)

 ExoI (NEB, M0293L)

Human primers:

 1st PCR heavy

|  |  |
| --- | --- |
| 5' L-VH 1  | ACAGGTGCCCACTCCCAGGTGCAG  |
| 5' L-VH 3 | AAGGTGTCCAGTGTGARGTGCAG  |
| 5' L-VH 4/6 | CCCAGATGGGTCCTGTCCCAGGTGCAG  |
| 5' L-VH 5 | CAAGGAGTCTGTTCCGAGGTGCAG  |
| 3' HuIgG-const-anti | TCTTGTCCACCTTGGTGTTGCT  |
| 3' Cm CH1 (IgM) | GGGAATTCTCACAGGAGACGA |
| 3' IgA1-RT | CCTGGCTGGGTGGGAAGTTT |

 1st PCR kappa

|  |  |
| --- | --- |
| 5' L Vk 1/2  | ATGAGGSTCCCYGCTCAGCTGCTGG  |
| 5' L Vk 3  | CTCTTCCTCCTGCTACTCTGGCTCCCAG  |
| 5' L Vk 4  | ATTTCTCTGTTGCTCTGGATCTCTG  |
| 3' Ck 543–566 | GTTTCTCGTAGTCTGCTTTGCTCA  |

 1st PCR lambda

|  |  |
| --- | --- |
| 5' L Vl 1  | GGTCCTGGGCCCAGTCTGTGCTG  |
| 5' L Vl 2  | GGTCCTGGGCCCAGTCTGCCCTG  |
| 5' L Vl 3  | GCTCTGTGACCTCCTATGAGCTG  |
| 5' L Vl 4/5/9 | GGTCTCTCTCSCAGCYTGTGCTG  |
| 5' L Vl 6  | GTTCTTGGGCCAATTTTATGCTG  |
| 5' L Vl 7  | GGTCCAATTCYCAGGCTGTGGTG  |
| 5' L Vl 8  | GAGTGGATTCTCAGACTGTGGTG  |
| 3' Cl  | CACCAGTGTGGCCTTGTTGGCTTG  |

 2nd PCR heavy

|  |  |
| --- | --- |
| 5' VH3a-sense | SARGTGCAGCTGGTGGAG  |
| 5' VH3b-sense | GAGGTGCAGCTGTTGGAG  |
| 5' VH1/5/7-sense | CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG |
| 5' VH4-sense | CTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG |
| 3' Cgamma (IgG) | AGTAGTCCTTGACCAGGCAGCCCAG  |
| 3' MuD (IgM) | GGAATTCTCACAGGAGACGA  |
| 3' IgA1 | CAGAGGCTCAGCGGGAAGACC |

 2nd PCR kappa

|  |  |
| --- | --- |
| 5' Pan Vk | ATGACCCAGWCTCCABYCWCCCTG |
| 3' Ck 494–516 | GTGCTGTCCTTGCTGTCCTGCT |

 2nd PCR lambda

|  |  |
| --- | --- |
| 5' AgeI Vl1 | CTGCTACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG |
| 5' AgeI Vl2 | CTGCTACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG |
| 5' AgeI Vl3 | CTGCTACCGGTTCTGTGACCTCCTATGAGCTGACWCAG |
| 5' AgeI Vl4/5 | CTGCTACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA |
| 5' AgeI Vl6 | CTGCTACCGGTTCTTGGGCCAATTTTATGCTGACTCAG |
| 5' AgeI Vl7/8 | CTGCTACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG |
| 5' XhoI Cl | CTCCTCACTCGAGGGYGGGAACAGAGTG |

 Vector sequencing

|  |  |
| --- | --- |
| Ab-vec-sense  | GCTTCGTTAGAACGCGGCTAC  |

**5A.1.** Prepare heavy, kappa, and lambda 1st PCR master mixes. Each half-plate of cDNA requires a total of three half-plate PCR setups (one for each chain).

|  |  |  |  |
| --- | --- | --- | --- |
| **1st PCR (for 1 half-plate)** | **VH** | **VK** | **VL** |
| 2X Green DreamTaq MasterMix | 500 µl | 500 µl | 500 µl |
| 5' primers (60 µM) | 8 µl (x4) | 8 µl (x3) | 8 µl (x7) |
| 3' primers (60 µM) | 8 µl (x3) | 8 µl (x1) | 8 µl (x1) |
| Nuclease-free H2O | to 900 µl | to 900 µl | to 900 µl |

|  |  |  |  |
| --- | --- | --- | --- |
| cDNA template | 2 µl | 2 µl | 2 µl |

Aliquot 18 µl master mix to each half-plate, then use a multichannel pipette to add 2 µl cDNA template for a total reaction volume of 20 µl.

**5A.2.** Thermocycler instructions, 1st PCR:

94°C for 4 min

 Repeat 15x:

 94°C for 30 sec

 51°C for 30 sec

 72°C for 55 sec

 Repeat 30x:

 94°C for 30 sec

 56°C for 30 sec

 72°C for 55 sec

 72°C for 8 min

 4°C forever

**5A.3.** Prepare heavy, kappa, and lambda 2nd PCR master mixes. Plate layouts remain the same from the 1st PCR.

Typically, VH3 primers (VH3a and VH3b) are sufficient to amplify all heavy chain gene segments. However, for repertoire studies, separate VH1/5 and VH4 reactions should be run to ensure complete coverage.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **2nd PCR (for 1 half-plate)** | **VH3** | **VH1/5, 4** | **VK** | **VL** |
| 2X Green DreamTaq MM | 625 µl | 625 µl | 625 µl | 625 µl |
| 5' primers, 60 µM | 8 µl (x2) | 8 µl (x1) | 8 µl (x1) | 8 µl (x6) |
| 3' primers, 60 µM | 8 µl (x3) | 8 µl (x3) | 8 µl (x1) | 8 µl (x1) |
| Nuclease-free H2O | to 1125 µl | to 1125 µl | to 1125 µl | to 1125 µl |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 1st PCR product | 2.5 µl | 2.5 µl | 2.5 µl | 2.5 µl |

Aliquot 22.5 µl master mix to each half-plate, then use a multichannel pipette to add 2.5 µl 1st PCR product for a total reaction volume of 25 µl.

**5A.4.** Thermocycler instructions, 2nd PCR:

94°C for 4 min

 Repeat 50x:

 94°C for 30 sec

 57°C for 30 sec

 72°C for 45 sec

 72°C for 10 min

 4°C forever

**5A.5.** Run 2 µl of product directly on a 1.2% Agarose gel (130V, 25 min). Successful reactions are identified by a band at approximately 400 bp. Ensure there are no bands of this size in the negative control wells (row H). Generate a list of wells that have a heavy chain amplicon and at least one light chain amplicon (infrequently, the kappa and lambda PCRs will both produce a positive result).

**5A.6.** Run a PCR clean-up with 5 µl of these desirable PCR products to prepare them for sequencing.

|  |  |  |
| --- | --- | --- |
| **PCR clean-up** | **1 well (7 µl)** | **200 wells** |
| dH2O | 0.7 µl | 140 µl |
| NEBuffer | 0.7 µl | 140 µl |
| CIP (10 units/ul) | 0.1 µl | 20 µl |
| ExoI | 0.5 µl | 100 µl |

|  |  |  |
| --- | --- | --- |
| 2nd PCR product | 5 µl |  |

 Run at 37°C for 30 min then 80°C for 20 min.

**5A.7.** Sequence with the 5' primers used in the 2nd PCR (VH3a/b-sense, VH1/5-sense, VH4-sense, Pan Vk, and XhoI Cl primers).

***Mouse 1st and 2nd PCR (Step 5B)***

For heavy chain DNA, perform two PCR steps (nested PCR and cloning PCR). For kappa chain DNA, perform three PCR steps (1st PCR, 2nd PCR, and cloning PCR).

Materials:

 Q5 Hot Start High-Fidelity Polymerase (NEB, M0493L)

 dNTPs, PCR Grade (Qiagen, 201901)

 One-Taq Hot Start Quick-Load 2X Master Mix (NEB, M0488L)

 ExoSAP-IT (Affymetrix, 78202 4X1 ML)

Mouse primers:

Heavy PCR

|  |  |
| --- | --- |
| 5' MsVHE | GGGAATTCGAGGTGCAGCTGCAGGAGTCTGG |
| 3' Cy1 outer (IgG1) | GGAAGGTGTGCACACCGCTGGAC |
| 3' Cy2b outer (IgG2b) | GGAAGGTGTGCACACTGGAC |
| 3' Cy2c outer (IgG2c) | GGAAGGTGTGCACACTGCTGGAC |
| 3' Ca outer (IgA) | GAGGCGAGGGCAGGTGGAAAGTTCACGG |
| 3' Cm outer (IgM) | AGGGGGCTCTCGCAGGAGACGAGG |

 Kappa 1st PCR

|  |  |
| --- | --- |
| 5' VK3 | TGCTGCTGCTCTGGGTTCCAG |
| 5' VK4 | ATTWTCAGCTTCCTGCTAATC |
| 5' VK5 | TTTTGCTTTTCTGGATTYCAG |
| 5' VK6 | TCGTGTTKCTSTGGTTGTCTG |
| 5' VK689 | ATGGAATCACAGRCYCWGGT |
| 5' VK14 | TCTTGTTGCTCTGGTTYCCAG |
| 5' VK19 | CAGTTCCTGGGGCTCTTGTTGTTC |
| 5' VK20 | CTCACTAGCTCTTCTCCTC |
| 3' MCK | GATGGTGGGAAGATGGATACAGTT |

 Kappa 2nd PCR

|  |  |
| --- | --- |
| 5' mVkappa | GAYATTGTGMTSACMCARWCTMCA |
| 3' BsiWI P-mJK01 | GCCACCGTACGTTTGATTTCCAGCTTGGTG |
| 3' BsiWI P-mJK02 | GCCACCGTACGTTTTATTTCCAGCTTGGTC |
| 3' BsiWI P-mJK03 | GCCACCGTACGTTTTATTTCCAACTTTGTC |
| 3' BsiWI P-mJK04 | GCCACCGTACGTTTCAGCTCCAGCTTGGTC |

**5B.1.** Set up wells for heavy chain amplification:

|  |  |  |  |
| --- | --- | --- | --- |
| **Heavy PCR (1 well)** | **IgG (40 µl)** | **IgA (40 µl)** | **IgM (40 µl)** |
| Q5 polymerase | 0.4 µl | 0.4 µl | 0.4 µl |
| 5X Q5 buffer | 8 µl | 8 µl | 8 µl |
| 5X Q5 high GC enhancer | 8 µl | 8 µl | 8 µl |
| dNTPs (10 mM) | 0.8 µl | 0.8 µl | 0.8 µl |
| 5' MsVHE (100 µM) | 0.2 µl | 0.2 µl | 0.2 µl |
| 3' primers (100 µM) | 0.2 µl (x3) | 0.2 µl (x1) | 0.2 µl (x1) |
| Nuclease-free H2O | 17 µl | 17.4 µl | 17.4 µl |

|  |  |  |  |
| --- | --- | --- | --- |
| cDNA template | 5 µl | 5 µl | 5 µl |

Transfer 35 µl master mix to each well then add 5 µl of cDNA template for a total reaction volume of 40 µl.

**5B.2.** Thermocycler instructions:

98°C for 30 sec

 Repeat 10x:

 98°C for 10 sec

 60°C for 20 sec

 72°C for 45 sec

 Repeat 50x:

 98°C for 10 sec

 72°C for 60 sec

 72°C for 10 min

 4°C forever

**5B.3.** Set up wells for the 1st PCR for kappa chain amplification:

|  |  |
| --- | --- |
| **Kappa 1st PCR** | **1 well (40 µl)** |
| Q5 polymerase | 0.4 µl |
| 5X Q5 buffer | 8 µl |
| dNTPs (10 mM) | 0.8 µl |
| 5' primers (100 µM) | 0.2 µl (x8) |
| 3' mCK (100 µM) | 0.2 µl |
| Nuclease-free H2O | 23.9 µl |

|  |  |
| --- | --- |
| cDNA template | 5 µl |

Transfer 35 µl master mix to each well then add 5 µl of cDNA template for a total reaction volume of 40 µl.

**5B.4.** Thermocycler instructions, 1st PCR:

98°C for 30 sec

 Repeat 50x:

 98°C for 10 sec

 62°C for 30 sec

 72°C for 45 sec

 72°C for 10 min

 4°C forever

**5B.5.** Set up wells for the 2nd PCR for kappa chain amplification:

|  |  |
| --- | --- |
| **Kappa 2nd PCR** | **1 well (40 µl)** |
| One-Taq 2X Master Mix | 20 µl |
| 5' mVkappa (10 µM) | 0.8 µl |
| 3' primers (10 µM) | 0.8 µl (x3) |
| Nuclease-free H2O | 14.8 µl |

|  |  |
| --- | --- |
| 1st PCR product | 2 µl |

Transfer 38 µl master mix to each well then add 2 µl of 1st PCR product for a total reaction volume of 40 µl.

**5B.6.** Thermocycler instructions, 2nd PCR:

94°C for 3 min

 Repeat 50x:

 94°C for 30 sec

 45°C for 30 sec

 72°C for 45 sec

 72°C for 10 min

 4°C forever

**5B.7.** Run 2 µl of product from the heavy PCR and the 2nd kappa PCR on a 1.2% Agarose gel (130V, 25 min). Generate a list of wells that have amplicons for both chains.

**5B.8.** Run a PCR clean-up with ExoSAP-IT, following the manufacturer’s protocol.

**5B.9.** Sequence the heavy chain sequences with 5' MsVHE and the kappa chain sequences with 3' mCK.

***Cloning PCR (Step 6)***

Prior to setting up the cloning PCR, use NCBI’s IgBLAST or the IMGT database to determine the exact V and J gene composition for each antibody’s heavy and light chains. V gene identity determines the forward primer for the cloning PCR and J gene identity determines the reverse primer. Many primers target conserved sequences so they can be used for more than one gene segment. For example, the human VH1/5/7 primer is used for any gene from the VH1, VH5, or VH7 families. The human VH3 primer is used for any gene from the VH3 family, except VH3-23, which instead uses the VH3-23 primer.

Typically, for a cloning PCR setup for all the antibodies derived from a half-plate of single cells, up to a few dozen master mixes (pairs of V/J primers) are required.

For details on running the human cloning PCR, refer to the technical methods section in the main paper.

Materials:

DreamTaq Green PCR 2X MasterMix (Thermo, K1082)

 NEBuilder DNA Assembly 2X MasterMix (New England Biolabs, E2621)

Human primers:

|  |  |
| --- | --- |
| VH1/5/7 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG |
| VH3 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG |
| VH3–23 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG |
| VH4 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG |
| VH4–34 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG |
| VH3–9/30/33 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG |
| VH6–1 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG |
| Vk1 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC |
| Vk1–9/1–13 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGACATCCAGTTGACCCAGTCT |
| Vk1D–43/1–8 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC |
| Vk2 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC |
| Vk2–28/2–30 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC |
| Vk3–11/3D-11 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATTGTGTTGACACAGTC |
| Vk3–15/3D-15 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGTC |
| Vk3–20/3D-20 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATTGTGTTGACGCAGTCT |
| Vk4–1 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCGGACATCGTGATGACCCAGTC |
| Vl1 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG |
| Vl2 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG |
| Vl3 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTTCTGTGACCTCCTATGAGCTGACWCAG |
| Vl4/5 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA |
| Vl6 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTTCTTGGGCCAATTTTATGCTGACTCAG |
| Vl7/8 | ATCCTTTTTCTAGTAGCAACTGCAACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG |
| JH1/2 | GGAAGACCGATGGGCCCTTGGTCGACGCCTGAGGAGACGGTGACCAG |
| JH4/5 | GGAAGACCGATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACCAG |
| JH3 | GGAAGACCGATGGGCCCTTGGTCGACGCTGAAGAGACGGTGACCATTG |
| JH6 | GGAAGACCGATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACCGTG |
| Jk1/2/4 | AAGACAGATGGTGCAGCCACCGTACGTTTGATYTCCACCTTGGTC |
| Jk3 | AAGACAGATGGTGCAGCCACCGTACGTTTGATATCCACTTTGGTC |
| Jk5 | AAGACAGATGGTGCAGCCACCGTACGTTTAATCTCCAGTCGTGTC |
| Cl | TGTTGGCTTGAAGCTCCTCACTCGAGGGYGGGAACAGAGTG |

Use the following instructions for mouse cloning PCR.

Materials:

 Q5 Hot Start High-Fidelity Polymerase (NEB, M0493L)

 dNTPs, PCR Grade (Qiagen, 201901)

Mouse primers:

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| mVH01 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGGCAGCCTGG |
| mVH02 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGGCAGTCTGG |
| mVH03 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGCTGAAGCAGTCTGG |
| mVH04 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGCTGAAGGAGTCTGG |
| mVH05 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGAAGCTGGAGGAGTCTGG |
| mVH06 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGGAGTCTGG |
| mVH07 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAAGTGCAGCTGTTGGAGACTGG |
| mVH08 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGCAGGCAGTCTGG |
| mVH09 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGCAGGGAGTCTGG |
| mVH10 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGCAGGCAGTCTGTG |
| mVH11 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGAAGCTGGTGGAGTCTGG |
| mVH12 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGATCCAGGCAGTCTGG |
| mVH13 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTTCAGACAGTCTGA |
| mVH14 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGTTCCAGGCAGTCTGG |
| mVH15 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGATGTACAGCTTCAGGAGTCAGG |
| mVH16 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGCAGCTTGTTGAGTCTGGTGGAGG |
| mVH17 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGCGTGAGGCAGTCTGG |
| mVH18 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGACGTGAAGCTGGTGGAGTCTGG |
| mVH19 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAAGTGATGCTGGTGGAGTCTGG |
| mVH20 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGCTTGTAGAGACCGG |
| mVH21 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGATGCAGCTTCAGGAGTCAGG |
| mVH22 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGCTTATCTACAGCAGTCTGG |
| mVH23 | CCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGTTCCAGGCAGTCTGG |
| mJH01 | GAAGACCGATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACCGTGG |
| mJH02 | GAAGACCGATGGGCCCTTGGTCGACGCTGAGGAGACTGTGAGAGTGG |
| fmJH03 | GAAGACCGATGGGCCCTTGGTCGACGGAGACAGTGACCAGAG |
| mJH04 | GAAGACCGATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACTGAGG |

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| **Mouse cloning PCR** | **1 well (50 µl)** |
| 5' primer (10 µM) | 2.5 µl |
| 3' primer (10 µM) | 2.5 µl |
| Q5 polymerase | 0.5 µl |
| 5X Q5 buffer | 10 µl |
| 5X Q5 high GC enhancer | 10 µl |
| dNTPs (10 mM) | 1 µl |
| Nuclease-free H2O | 21.5 µl |

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| 1st PCR product | 2 µl |

Thermocycle:

94°C for 3 min

Repeat 10x:

 94°C for 30 sec

 45°C for 30 sec

 72°C for 60 sec

Repeat 30x:

 94°C for 30 sec

 72°C for 90 sec

72°C for 10 min

4°C forever

***DNA assembly (Step 7)***

Refer to the technical methods section in the main paper.

***Transformation (Step 8)***

Materials:

 5-alpha competent E. coli (NEB; C2988 for individual samples, C2987P for 96-well format)

 SOC media

 LB agar (+ ampicillin)

**8.1**. Follow the transformation protocol given for the desired comptent cell line (the protocol can be performed with indivdual samples or in a 96-well format). Use 2 ul of assembled vector.

**8.2.** Plate on selection plates pre-warmed to 37°C (LB agar, 100 µg/ml ampicillin) and incubate overnight at 37°C. Successful transformations typically have 50-200 colonies per plate, but may have as few as 20.

***Plasmid DNA preparation (Step 9)***

Materials:

 QIAprep 96 Plus Kit (Qiagen, 27291)

 Genepure Plasmid Maxi Kit (Roche, 03143422001)

 LB broth (+ ampicillin)

 Glycerol (autoclaved)

**9.1.** Prepare a 96-well flat-bottom block for cell cultivation by adding 1.3 ml LB broth with 100 µg/ml ampicillin to each well. Pick four colonies from each transformation plate and individually inoculate wells in the flat-bottom block. Incubate cultures at 37°C for 20-24 hr with vigorous shaking.

**9.2.** Make glycerol stocks for each sample by transferring 700 µl from each well to 300 µl of a 1:1 mixture of LB:glycerol (without ampicillin). Store at -80°C; these stocks are viable for several years.

**9.3.** Follow instructions for preparing minipreps with the QIAprep 96 Plus Kit using the QIAvac vacuum manifold.

**9.4.** Sequence the plasmid DNA with the AbVec primer.

**9.5.** Compare the four miniprep sequences with sequence alignment to determine the consensus sequence. PCR error may introduce random errors in some sequences, but typically one or more of the sequences represents the consensus. Use this sequence for the following maxiprep steps.

In the rare cases where there is no consensus (even when adding the 1st PCR sequences and germline sequences to the alignment), pick four new colonies and repeat the miniprep process.

**9.6.** Prepare 14 ml round-bottom tubes with 5 ml of LB broth/ampicillin. Inoculate with the desired minipreps by scraping a small amount of bacteria from frozen glycerol stocks. Incubate at 37°C and 225 rpm for 4-5 hours.

**9.7.** Transfer the cultures to 500 ml flasks containing 250 ml LB broth/ampicillin. Incubate at 37°C and 225 rpm overnight.

**9.8.** Prepare plasmid DNA: follow instructions for the Genepure Plasmid Maxi Kit.

Note: for projects without the need for large amounts of DNA, a midiprep can be substituted for the maxiprep.

***Transfection and cell culture (Step 10)***

Materials:

 293A cells (Invitrogen, R70507)

 Advanced DMEM (Gibco, 12491)

L-glutamine (Gibco, 25030)

 FBS ultra-low IgG (Gibco, 16250)

 Antibiotic/Antimycotic (Gibco, 15240)

 PEI (Polysciences, 23966)

DMEM (Gibco, 11965)

 PFHM-II (Gibco, 12040)

 150 mm x 25 mm tissue culture plates (Falcon, 087726)

 Protein A agarose beads (Pierce, 20334)

Reagents:

 293A culture media

 Advanced DMEM

 1% L-glutamine

 1% Antibiotic/Antimycotic

 2% ultra-low IgG FBS

**10.1.** Maintain 293A cells in 150 mm x 25 mm tissue culture plates, following the instructions on the product sheet. Transfection-ready plates are 80-90% confluent. Keep track of the passage number of the cells; cells with more than 30 passages may exhibit lowered transfection efficiency (and simultaneously, lowered day-to-day growth rate).

**10.2.** Warm DMEM to room temperature. Set up transfections in 5 ml conical tubes.

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| **Transfection** | **1 plate** |
| DMEM | 2.4 ml |
| Heavy chain DNA | 9 µg |
| Light chain DNA | 9 µg |
| Polyethylenimine (1 mg/ml) | 100 µl |

Vortex immediately after addition of polyethylenimine (PEI). Incubate at room temperature for 15 min.

**10.3.** Remove all but 18 ml of media from each plate to be transfected. Add the transfection mixture, gently tilting plates to mix. Incubate at 37°C and 5% CO2.

**10.4.** After 12-18 hr, carefully remove the cell culture media, then gently add 25 ml PFHM-II media. Avoid physically disturbing adherent cells.

Warning: PEI is cytotoxic. Avoid longer incubation periods.

**10.5.** At day 3, collect the culture supernatants in a 50 ml conical tube and re-add 25 ml PFHM-II media.

**10.6.** At day 6, collect the culture supernatants again for a total of 50 ml from each plate.

**10.7.** Spin supernatants at 3000 rpm for 15 min to pellet cells, then transfer the supernatants to new 50 ml conical tubes.

**10.8.** Prepare protein A agarose beads by rinsing twice with PBS. Centrifuge in 50 ml conical tubes at 3000 rpm and 4°C for 10 min with no brake.

Important: Do not use a centrifuge brake on any steps involving agarose beads; braking can damage the beads.

**10.9.** Add 75 µl protein A agarose beads to each tube containing supernatants, then incubate overnight at 4°C on a slowly-agitating rocker. Place tubes horizontally to maximize bead binding surface area.

***Protein purification (Step 11)***

Materials:

 Mini Bio-Spin Chromatography Column (Biorad, 732-6207)

Reagents:

 10x Preservative

 dH2O

 5% BSA

 1.5M NaCl

 0.5% NaN3

**11.1.** Move the beads to a room temperature rocker for 1-2 hr. Centrifuge at 3000 rpm and 4°C for 10 min with no brake.

**11.2.** Aspirate the supernatant, then resuspend beads in 400 µl 1M NaCl.

**11.3.** Transfer to a chromatography column. Place columns on a vacuum manifold (such as Qiagen’s QIAvac 24) and apply vacuum, releasing upon completion.

**11.4.** Wash twice with 200 µl PBS, applying vacuum after each wash.

**11.5.** Prepare clean 1.5 ml Eppendorf tubes with 40 µl Tris pH 9.0 and transfer columns.

**11.6.** Add 200 µl glycine, then centrifuge at 500g for 1 min. Repeat once. Ensure that the pH of the solution is roughly 7.

**11.7.** Antibodies can be preserved by adding 50 µl of a 10x preservative (5% BSA, 1.5M NaCl, 0.5% sodium azide). Alternatively, buffer exchange to PBS and preserve with 0.05% sodium azide. Note that *in vivo* assays are sensitive to the addition of azide.

Smith, K. et al. Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. Nat. Protoc. 4, 372–384 (2009).