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Using the VRC01-class of anti-HIV-1 broadly neutralizing antibodies (bnAbs) elicited in sequentially immunized Ig-humanized mice as an example, we describe a protocol to identify key mutations for bnAb function by point mutagenesis and antibody binding and neutralization assays. We also describe steps to monitor how the key mutations arise in response to specific immunogens, which is critical for vaccine evaluation and design, via longitudinal antibody mutation profiling. This protocol can be customized for other V-gene-specific bnAbs and animal models.

Highlights
- Create germline revertants for every amino acid mutation of a target bnAb
- Identify key mutations for antibody function by binding and neutralization assays
- A procedure for fast antibody key mutation analysis and mutation profile visualization
- Longitudinal mutation profiling identifies immunogens inducing key antibody mutations

Chen et al., STAR Protocols 3, 101180
March 18, 2022 © 2022
https://doi.org/10.1016/j.xpro.2022.101180
Protocol to identify and monitor key mutations of broadly neutralizing antibody lineages following sequential immunization of Ig-humanized mice

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https://doi.org/10.1016/j.xpro.2022.101180

SUMMARY
Using the VRC01-class of anti-HIV-1 broadly neutralizing antibodies (bnAbs) elicited in sequentially immunized Ig-humanized mice as an example, we describe a protocol to identify key mutations for bnAb function by point mutagenesis and antibody binding and neutralization assays. We also describe steps to monitor how the key mutations arise in response to specific immunogens, which is critical for vaccine evaluation and design, via longitudinal antibody mutation profiling. This protocol can be customized for other V-gene-specific bnAbs and animal models.

For complete details on the use and execution of this profile, please refer to Chen et al. (2021).

BEFORE YOU BEGIN
The protocol below describes the specific steps for identifying and monitoring key mutations of VRC01-class anti-HIV-1 bnAbs from sequentially immunized Ig-humanized mice and determining which immunization step elicits each key mutation. However, this protocol can be customized to analyze the key mutation development of other bnAbs against other pathogens in different animal models.

Sequential immunization of Ig-humanized mice

© Timing: 4–20 months

The VH1-2/LC Ig-humanized mouse model (Chen et al., 2021; Tian et al., 2016) expresses diverse VRC01-class precursors composed of unmutated human VH1-2 heavy chains with diverse complementarity-determining regions (CDR) H3 and an unmutated human VK3-20 light chain with a fixed mature VRC01 CDR L3. The relatively high VRC01-class precursor frequency (∼25% among IgG+ B cells) made this mouse an ideal model for studying affinity maturation of VRC01-class antibodies during sequential immunization. All animal experiments were reviewed and approved by the Animal Care and Use Committee of the Vaccine Research Center, NIAID, NIH. The animal work was covered under protocol VRC 14-467 for breeding, and VRC 14-480 and 17-719 for immunization.

1. Ig-humanized mice expressing VRC01-class precursors of both genders at age of 4–6 months are sequentially immunized with a series of prime and boost immunogens at 3- or 4-week interval.
2. Sera are collected from each immunized animal before immunization and 2 weeks after each immunization step for serological analysis for serum binding and neutralization titers.
3. 1–2 mice are sacrificed 2 weeks after each step of sequential immunization for both sera and spleen collection.

4. Sera are heat-inactivated by incubation at 56 °C for 50 min and stored at −20 °C, and the spleens are processed to obtain splenocytes, which are resuspended in cell freezing medium: 10% DMSO/90% Fetal Bovine Serum (FBS), aliquoted and stored in a liquid nitrogen tank.

△ CRITICAL: Sufficient number of animals should be immunized in the beginning so that there are enough mice left at the terminal time point for statistical analysis, if needed, after the sequential sacrifice of 1–2 mice following each step of the sequential immunization.

**Design and ordering of primers for amplifying VRC01-class antibody heavy and light chains from a single transgenic mouse B cell by nested RT-PCRs**

○ Timing: 3 days

5. Design heavy and light chain V-gene-specific forward primers for 1st and 2nd round PCRs:
   a. Typically, design the 1st round forward primers against the 5' untranslated region (UTR) or the 5' half of the signal peptide coding region of the target VH and VK (or VL) genes, respectively, of the bnAb lineage you are studying. In this case, the VRC01-class bnAbs in this mouse model use human VH1-2 and VK3-20 for heavy and light chains, respectively.
   b. The 2nd round VH1-2 or VK3-20 gene-specific forward primers should start upstream of the beginning of V-gene framework 1, e.g., the 3' half of the signal peptide coding region, but downstream of the corresponding 1st round forward primer (Figures 1A and 1B).

△ CRITICAL: These forward primers should be as specific as possible to the target VH or VK-genes. A short sequence Blast search with the designed primers against the experimental animal genome should be performed to make sure there is no off-target binding site.

6. Design nested reverse primers for 1st and 2nd round PCR:
   a. The reverse primers are to be designed against the heavy and light chain constant regions, respectively, of the bnAb lineage expressed in the studied animal model. In this case, the constant regions are encoded by mouse gamma and kappa constant region genes.
   b. Align different isotypes or alleles of gamma constant genes in the animal model, and design primers to the most conserved regions so that the back primers can cover all isotypes/alleles using the least number of degenerative nucleotides in the primers (Figure 1C). Do the same for the light chain reverse primers if multiple light chain alleles exist.

△ CRITICAL: Try to reduce the number of degenerative nucleotides used in the primer. If there is too much difference between alleles, consider designing a specific primer to each allele and use the mixture as the reverse primers. The 2nd PCR primers must be of the 1st PCR primers inside (downstream of the 1st PCR 5' primers and upstream of the 1st PCR 3' primers) and should be close to the start of the constant region because they will be also used as the sequencing primers to sequence the PCR amplified V(D)J cassettes, which will range about 300–500 bp. Also try and match the melting temperatures for each PCR primer pair.

7. Order the designed primers from oligo-synthesis vendors like Sigma-DNA and IDTDNA. The primer sequences for amplifying VRC01-class antibody heavy and light chains in the VH1-2/LC mouse model are listed in the key resources table.

**Identification of a target bnAb or nAb from terminal mice**

○ Timing: 8 weeks
8. Serum neutralization assay to identify mice with cross-strain serum neutralization activity.
   a. Pick a panel of VRC01-sensitive viruses covering different clades of HIV-1 viruses and containing both glycan276 bearing- and deficient-viruses, such as the panel described in our previous publications (Chen et al., 2021; Tian et al., 2016).
   b. Perform TZM-bl neutralization assays of post-immune sera against the above sentinel panel of viruses and identify the mice with the highest serum neutralization breadth from the terminal immunization step, at which the most affinity-matured antibodies are expected to develop.

9. Single B cell sorting with multiple antigenic probes (i.e., BG505.SOSIP trimer probe in addition to eODGT6 and its CD4bs-disrupting mutant probes) to sort for B cells expressing cross-reactive VRC01-class antibodies from the splenocytes of terminal mice with broad neutralizing activity (Figure 5, step 8).

10. RT-PCR to amplify the heavy (VH1-2, VDJ region) and light (VK3-20, VJ region) chains of VRC01-class antibodies from each single-sorted B cells.
11. Gene synthesis and subcloning of the amplified VRC01-class antibody heavy and light chain V(D) J region into mouse IgG2a expression plasmids, VRC2742 and VRC3353.
12. Transient expression of the cloned VRC01-class antibodies (with mouse IgG2a constant region) by co-transfection of heavy and light chain expression plasmids in Expi293 cells and purifying them with Protein A Sepharose FastFlow.
13. Neutralization assays of the purified VRC01-class antibodies against a large panel of HIV-1 pseudoviruses to determine their breadth and potency.
14. Choose the most broad and potent VRC01-class antibody as the target bnAb for characterization in the following key mutation analysis.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| 2411a               | Chen et al., 2021 | GenBank: MT551696, MT551740 |
| VRC01               | Wu et al., 2010   | RRID: AB_2491019 |
| anti-mouse CD3 Cy5 SoldCP(1:160) | BD Pharmingen | Cat# 551163; RRID: AB_394082 |
| anti-mouse CD4 Cy5 SoldCP(1:400) | BioLegend | Cat# 100540; RRID: AB_893326 |
| anti-mouse CD8 Cy5 SoldCP(1:320) | BD Pharmingen | Cat# 551162; RRID: AB_394081 |
| anti-mouse F4/80 Cy5 SoldCP(1:400) | BioLegend | Cat# 123128; RRID: AB_893484 |
| anti-mouse B220 TrPE(1:200) | BD Pharmingen | Cat# 551489; RRID: AB_394219 |
| anti-mouse IgD BV711(1:133) | BioLegend | Cat# 405731; RRID: AB_2563342 |
| anti-mouse IgM Cy7PE(1:800) | eBioscience | Cat# 25-5790-82; RRID: AB_469655 |
| anti-mouse IgG1 FITC(1:100) | BD Pharmingen | Cat# 553443; RRID: AB_394862 |
| anti-mouse IgG2a FITC(1:100) | BD Pharmingen | Cat# 553399; RRID: AB_394833 |
| anti-mouse IgG3 FITC(1:100) | BD Pharmingen | Cat# 553403; RRID: AB_394840 |
| Chemicals, peptides, and recombinant proteins | | |
| C13.G3-AviHis       | Tian et al., 2016 | GenBank: KX462845 |
| ΔC13-AviHis         | Tian et al., 2016 | N/A |
| 426c-degly3 core-AviHis | Tian et al., 2016 | GenBank: KXS18319 |
| 426c-WT.DS SOSIP     | Tian et al., 2016 | GenBank: KX462847 |
| eOD-GT8 60mer       | Tian et al., 2016 | GenBank: KXS27857 |
| eOD-GT8-AviHis      | Tian et al., 2016 | GenBank: KXS27855 |
| ΔeOD-GT8-AviHis     | Tian et al., 2016 | GenBank: KXS27856 |
| eOD-GT6 60mer       | Tian et al., 2016 | GenBank: KXS27854 |
| eOD-GT6-AviHis      | Tian et al., 2016 | GenBank: KXS27852 |
| ΔeOD-GT6-AviHis     | Tian et al., 2016 | GenBank: KXS27853 |
| BG505.SOSIP.T332N   | Tian et al., 2016 | N/A |
| BG505.DS.SOSIP      | Kwon et al., 2015 | N/A |
| RSC3                | NIH AIDS Reagent Program; Wu et al., 2010 | Cat# 12042 |
| Bal.01 gp120        | VRC/NIH; Chen et al., 2021 | N/A |
| JRFL gp120          | VRC/NIH; Chen et al., 2021 | N/A |
| 6101 core           | VRC/NIH; Chen et al., 2021 | N/A |
| ZM53 core           | VRC/NIH; Chen et al., 2021 | N/A |
| ZM197 core          | VRC/NIH; Chen et al., 2021 | N/A |
| ZM215 core          | VRC/NIH; Chen et al., 2021 | N/A |
| KER2018.11 core     | VRC/NIH; Chen et al., 2021 | N/A |
| UG037.8 core        | VRC/NIH; Chen et al., 2021 | N/A |
| 45-01dG5 gp120      | Wu et al., 2012 | GenBank: AFE02253 |
| 45-01dH5 gp120      | Wu et al., 2012 | GenBank: AFE02270 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HxBc2 core          | Chen et al., 2009 | PDB: 3IDY_G |
| CH505.DS.SOSIP      | Immune Technology | Cat# IT-001-CONA1p |
| ConA1 gp120         | Immune Technology | Cat# IT-001-CONA2p |
| ConA2 gp120         | Immune Technology | Cat# IT-001-RC3p |
| Clade B gp140       | VRC/NIH, Chen et al., 2021 | N/A |
| Clade C (Du422.1) gp120 | Immune Technology | Cat# IT-001-RC12p |
| Cap210 gp120        | Immune Technology | Cat# IT-001-RC3p |
| Blocking/Diluent Solution | Thermo Fisher Scientific | Cat# 18064014 |
| SuperScript® II Reverse Transcriptase | Thermo Fisher Scientific | Cat# 18080044 |
| HotStarTag™ Plus DNA Polymerase | Thermo Fisher Scientific | Cat# 203607 |
| Protein G Sepharose™ 4 Fast Flow | Cytiva (GE Healthcare) | Cat# 17-0618-05 |
| rProtein A Sepharose™ 4 Fast Flow | Cytiva (GE Healthcare) | Cat# 17-1279-01 |
| SureBlue™ TMB Microwell Peroxidase Substrate | Kirkegaard & Perry Laboratories (KPL) | Cat# 52-00-00 |
| Extravidin-PE       | Sigma-Aldrich | Cat# E4011 |
| Streptavidin-APC     | Thermo Fisher Scientific | Cat# SA1005 |
| VIVID (LIVE/DEAD® fixable violet dead cell stain) | Thermo Fisher Scientific | Cat# L34964 |
| BD™ CompBead Anti-Mouse Ig, κ/Negative Control Particles Set | BD Biosciences | Cat# 552845 |
| Gibco® DMEM, high glucose, HEPES, 10 x 500 mL | Thermo Fisher Scientific | Cat# 12430062 |
| Gibco® Penicillin-Streptomycin (10,000 U/mL) | Thermo Fisher Scientific | Cat# 15140122 |
| 2% Agarose TAE w/ EtBr Long Gel, 4(24+1) well | Embiotech | Cat# GE-3642 |
| Experimental models: Cell lines | | |
| Human: Expi293™ cells | Thermo Fisher Scientific | Cat# A14528; RRID: CVCL_D615 |
| Human: TZM-bl cells | NIH AIDS Reagent Program | Cat# 8129; RRID: CVCL_B478 |
| Experimental models: Organisms/strains | | |
| Mus musculus: Vμ1-2/LC mouse model; mixed 129/Sv and C57BL/6 strains | Tian et al., 2016 | N/A |
| Oligonucleotides | | |
| Primers for single cell RT-PCR in the Vμ1-2/LC mouse model (see below) | | |
| IgH (VH1-2) 1st PCR forward primer VH1-LEADER-A: ATGGACT GACCTTGAGGAT | Tian et al., 2016 | N/A |
| Alternative IgH (VH1-2) 1st PCR forward primer VH1-2 S'UTR: GAGAGCTCC GTTCCTCACCATGGACTGG | This paper | N/A |
| IgH 1st PCR reverse primer 3'musCg-1st: CCGCTGACGGCGATCCYAG | Tian et al., 2016 | N/A |
| IgH (VH1-2) 2nd PCR forward primer x9-VH1-1st: ACAAGGCCAGGCACCTGG | Tian et al., 2016 | N/A |
| IgH 2nd PCR reverse primer 3'musCg-2nd: CCGGGGCGCCAGTGGATGATGCTTTGG | Tian et al., 2016 | N/A |
| IgK (VK3-20) 1st PCR forward primer S1VK3-20: CGCAGCTTCTCCTCCTCTCTCT | Tian et al., 2016 | N/A |

(Continued on next page)
### MATERIALS AND EQUIPMENT

**REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER**
--- | --- | ---
IgK 1st PCR reverse primer: 3’musCk-1st ACTGGATGGTGGGAAGATGGA | Tian et al., 2016 | N/A
IgK (VK3-20) 2nd PCR forward primer: 5’hVK3-20.4: ACTCTGGTCCCCAGATTACCA | Tian et al., 2016 | N/A
IgK 2nd PCR reverse primer: 3’musCk-2nd: GGAAGATGGATACAGTTGGTG | Tian et al., 2016 | N/A
Recombinant DNA
VRC2742: mouse IgG2a heavy chain expression vector | Chen et al., 2021 | N/A
VRC3353: mouse Kappa chain expression vector | Chen et al., 2021 | N/A
Software and algorithms
GraphPad Prism 8.01 Software | GraphPad Prism Software, (Ye et al., 2013) | RRID: SCR_002798
IgBlast | http://www.ncbi.nlm.nih.gov/igblast/ | 
IMGT/V-Quest | (Brochet et al., 2008; Giudicelli et al., 2011) | http://www.imgt.org/IMGT_vquest/vquest; RRID: SCR_010749
WebLogo | (Crooks et al., 2004) | http://weblogo.berkeley.edu/logo.cgi; RRID: SCR_010236
FlowJo v 9.9.1 and v.10.6.2 | FlowJo | https://www.flowjo.com; RRID: SCR_008520
R v3.5.2 | The Comprehensive R Archive Network | https://cran.r-project.org/
Other
MilliporeSigma™ Steriflip™ Sterile Disposable Vacuum Filter Units | Thermo Fisher Scientific | Cat# SCGP00525
ExpíFectamine™ 293 Transfection Kit (for 10 L culture) | Thermo Fisher Scientific | Cat# A14525
Corning® Costar 96-well Half Area Clear Microplates | Corning | Cat# 3690
Nalgene™ Rapid-Flow™ Sterile Single Use Vacuum Filter Units (250 mL, 0.2 μM PES filter) | Thermo Fisher Scientific | Cat# 568-0020
Slide-A-Lyzer™ G2 Dialysis Cassettes, 20K MWCO, 3 mL | Thermo Fisher Scientific | Cat# 87735
CulturPlate-96 Black, Sterile | PerkinElmer | Cat# 6005660

**Complete DMEM (cDMEM)**

| Reagent | Final concentration | Amount |
| --- | --- | --- |
| Gibco™ DMEM, high glucose, HEPES | 90% | 445 mL |
| Heat-inactivated FBS | 10% | 50 mL |
| Gibco™ Penicillin-Streptomycin (10,000 U/mL) | 100 U/mL | 5 mL |
| **Total** | **n/a** | **500 mL** |

Store at 4°C in dark, up to the earliest expiration date of the ingredients.

**Cell freezing medium**

| Reagent | Final concentration | Amount |
| --- | --- | --- |
| Heat-inactivated FBS | 90% | 90 mL |
| DMSO | 10% | 10 mL |
| **Total** | **n/a** | **100 mL** |

Store at 4°C for a month or at −20°C for a year.
## STEP-BY-STEP METHOD DETAILS

### Making germline revertant antibody expression constructs

- **Timing:** 3 weeks

To evaluate the importance of each amino acid mutation or indel in the target bnAb, we revert each of the changed residues in the heavy chain or light chain of the target bnAb back to its corresponding germline encoded sidechain one residue at a time.

1. Use the coding sequence of the bnAb heavy or light chain as bait to perform IgBlast against human database.

### RT/Lysis buffer

| Component                  | Final concentration | 1X (µL/well) | 100X (µL) |
|----------------------------|---------------------|-------------|----------|
| RNaseOut (40 U/µL)         | 1 U/µL              | 0.25        | 25       |
| 5X Superscript Buffer      | 1 x                 | 2.5         | 250      |
| DTT (0.5 M)                | 25 mM               | 0.63        | 62.5     |
| 10% IGEPAL                 | 0.25%               | 0.31        | 31.25    |
| H₂O                        | n/a                 | 6.31        | 631.25   |
| Subtotal                   | n/a                 | 10          | 1000     |

The RT/Lysis Buffer can be stored at –80°C for a month and the RT/SuperScript Mix should be made freshly and used immediately.

### 1st PCR mix

| Component                  | Final concentration | 1X (µL/well) | 100X (µL) |
|----------------------------|---------------------|-------------|----------|
| H₂O                        | n/a                 | 9.75        | 975      |
| 10X Qiagen PCR Buffer      | 1 x                 | 1.25        | 125      |
| 25 mM MgCl₂                | 0.5 mM              | 0.25        | 25       |
| 10 mM dNTP                 | 0.2 mM              | 0.25        | 25       |
| 20 µM 5’ primer for 1st PCR| 0.32 µM             | 0.2         | 20       |
| 20 µM 3’ primer for 1st PCR| 0.32 µM             | 0.2         | 20       |
| [cDNA template]            | 4%                  | 0.5         | 50       |
| HotStarTaq Plus (5 U/µL)   | 0.04 U/µL           | 0.1         | 10       |
| Total                      | n/a                 | 12.5        | 1250     |

Make freshly and use immediately.

### 2nd PCR mix

| Component                  | Final concentration | 1X (µL/well) | 100X (µL) |
|----------------------------|---------------------|-------------|----------|
| H₂O                        | n/a                 | 15          | 1500     |
| 10X CoralLoad PCR Buffer   | 1 x                 | 2.5         | 250      |
| 5X Q                       | 1 x                 | 5           | 500      |
| 10 mM dNTP                 | 0.2 mM              | 0.5         | 50       |
| 20 µM 5’ primer for 2nd PCR| 0.32 µM             | 0.4         | 40       |
| 20 µM 3’ primer for 2nd PCR| 0.32 µM             | 0.4         | 40       |
| [1st PCR]                  | 4%                  | 1           | 100      |
| HotStar Plus Enzyme        | 0.04 U/µL           | 0.2         | 20       |
| Total                      | n/a                 | 25          | 2500     |

Make freshly and use immediately.
a. Heavy chain IgBlast generates an alignment of the bnAb heavy chain with its inferred germline VH (VH1-2) gene
b. Light chain IgBlast generates an alignment of the bnAb light chain with its inferred germline VK (VK3-20) gene

2. Based on the search results, design a point mutagenesis mutant for each of the SHM-induced residue change or indel to revert the change back to corresponding germline residue. One revertant corresponds to one amino acid mutation in the heavy or light chain. An example of the germline revertants of a VRC01-class bnAb 2411a (Chen et al., 2021) is shown in Figure 2.

3. Order the designed germline revertants from GenScript with their point mutagenesis service, in which they will need the original heavy and light chain expression vectors of the target bnAb as templates to make a series of mutated heavy and light chain constructs, each containing only one germline reverted residue.

4. The expression plasmids for the revertants are usually ready in two-three weeks.

Note: The revertant constructs can also be generated using a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) or Q5 Site-Directed mutagenesis kit with NEBaseChanger web tool (New England Biolabs). While waiting for the germline revertant heavy and light chains to be synthesized, you could perform the single B cell sorting and RT-PCR of VRC01-class IgG sequences from splenocytes of mice sacrificed at different time points of the immunization course, as described in steps 31–32.

Expression and purification of germline revertant mutants of the target bnAb

© Timing: 8 days

This part describes how to produce and purify the germline revertant mutants of the target bnAb. We need to make germline revertant antibodies each containing only one germline-reverted residue, so we need to pair each mutant heavy chain with the parental bnAb light chain and pair each mutant light chain with the parental bnAb heavy chain for antibody production.

5. Transient transfection of Expi293 cells to produce germline revertants (7 days)
   a. Grow 2 L Expi293F™ cells in Expi293™ Expression Medium to 3–5 × 10^6/mL in a cell incubator at 37°C, 8% CO₂, 60% humidity with 125 rpm shaking. These cells should be sufficient to prepare 24–40 × 100 mL-transfections to express 24–40 mutant antibodies.
   b. On the day of transfection, transfect 100 mL Expi293 (2.5 × 10^6/mL) cells for expression of each germline revertant antibody. All procedures at this step are operated in a biosafety hood.
      i. Dilute the 2 L Expi293F™ cells to 2.5 × 10^6/mL with fresh warm Expi293™ Expression Medium and split into 100mL- aliquots in 250mL-baffled-flasks.
      ii. Warm 1× Opti-MEM medium in 37°C water bath.
      iii. For each transfection, mix 50 µg heavy chain plasmid and 50 µg light chain plasmid in 5 mL 1× Opti-MEM medium, and filter the mix through a MilliporeSigma™ Sterilip™ Sterile Disposable Vacuum Filter Unit (0.22 µm PES filter on a 50 mL conical tube).
      iv. For each transfection, add 0.27 mL ExpiFectamine™ 293 Reagent to 5 mL 1× Opti-MEM medium, mix gently and incubate the mixture at room temperature for 5 min.
      v. Add the ExpiFectamine™ mix from iv to the plasmid mix from iii in the 50 mL conical tube, mix gently by swirling and incubate at room temperature for 20 min.
      vi. Pour the transfection mix (~10 mL) to one 250 mL-flask containing 100 mL 2.5 × 10^6/mL Expi293™ cells.
   c. Incubate the transfected cells in a cell culture incubator at 37°C, 8% CO₂, 60% humidity with 125 rpm shaking.
   d. On day 2, add 0.5 mL ExpiFectamine™ 293 Transfection Enhancer 1 and 5 mL ExpiFectamine™ 293 Transfection Enhancer 2 to each flask of cells, and continue incubation under the same condition for 5–6 more days.
Note: To improve protein yield, the cells can also be incubated at 32°C during day 3–7 with the other conditions unchanged. It is better to produce the parental bnAb as a reference control together with the germline revertant antibodies.

6. Purification of the parental and germline revertant antibodies (1 day).
   a. On day 7 post transfection, collect the ~115 mL of cell culture from each flask and centrifuge the cells down in one-time use centrifugal flasks at 2000 \( \times \) g for 15 min.
   b. Sterile filter each supernatant with a 0.2 \( \mu \)m Nalgene Rapid-Flow 250 mL-filter unit and check the pH of the supernatant to make sure that it is between 7.0 and 8.0, for optimal rProtein A binding of mouse IgG2a at next step.
   c. Apply the filtered supernatant onto 0.8 mL rProtein A Sepharose FF (GE health) pre-equilibrated in PBS in a disposable 10 mL poly-prep column with a 250 mL funnel attached on top.
   d. Let the medium run through the column by gravity, remove the funnel and wash column 2 \( \times \), each with 10 mL PBS (pH7.4).
   e. Elute antibody with 5 mL GE IgG elution buffer and collect the eluate with constant shaking in a 15 mL conical tube containing 0.5 mL 1 M Tris, pH8.
   f. Concentrate the eluted antibodies to above 1 mg/mL, as needed, in Amicon Ultra-4 centrifugal filter units.
   g. Dialyze the antibodies in Slide-A-Lyzer G2 cassettes (3 mL size, 20 kD molecular weight cutoff) against 4 L PBS three times, with 1.5 h stirring at room temperature or 4°C overnight each time.

\( \triangle \) CRITICAL: Some antibodies tend to precipitate during pH neutralization. The drop-by-drop elution into 1M Tris, pH8 with constant shaking can effectively reduce or eliminate
precipitation. Constant shaking during elution is critical for quick and sufficient pH neutralization and prevention of antibody precipitation.

**Binding assay of germline revertants to identify key mutations**

- **Timing:** 2 days

ELISA of the parental bnAb and its germline revertant mutants against a panel of antigens to assess the impact of each SHM-resulted residue change of the bnAb on its binding activity.

7. Choose a panel of HIV-1 envelope protein antigens as the ELISA binding substrates for the tested bnAb and its germline revertants.

**Note:** The panel should include envelopes from different strains of HIV-1 viruses to examine the binding breadth of the tested antibodies, and some CD4bs-disrupting mutants of the envelope as negative control to check the CD4bs-specificity of the antibodies.

8. Coat 96-well costar half-area microplates with the panel of purified antigens:
   a. Prepare sufficient antigen coating solution in PBS at 2 μg/mL for each antigen: total volume = number of antibodies to test × 3 (3 antibody concentrations) × 50 μL × 1.05 (5% extra)
   b. For each antigen, coat a 96-well costar half-area plate with the 2 μg/mL antigen coating solution at 50 μL per well. Each coated plate is sufficient to test 32 antibodies (target bnAb and its germline revertants) at three different concentrations.
   c. Put all coated plates in stacks and cover the top plates with either a plate cover or plate sealing film and incubate the plates at 4°C overnight.

9. On the 2nd day, remove the coating solutions in all plates and wash them 3 times with 200 μL PBS/T (PBS with 0.05% Tween) per well in a BioTek plate washer.

10. Block all plates with 1:10 diluted (with PBS) Blocking/Diluent Solution (Immune Technology) at 50 μL per well and incubate them at room temperature for 1 h.

11. During the blocking period, prepare solutions of the parental or germline revertant antibodies each at three different concentrations (10, 1 and 0.1 μg/mL) in PBS/T containing 2% Immune-Tech Blocking Reagent.

12. Distribute 2 mL of the antibody solutions from step 11 into each well of a 96-well deep-well plate according to Figure 3A.

13. Wash blocked ELISA plates 5× with PBS/T (200 μL/well per wash) in a BioTek plate washer.

14. Transfer 50 μL/well from the deep-well plate to each well of the antigen-coated ELISA plate.

15. Incubate the plates at room temperature for 1 h.

16. Repeat step 13.

17. Add 50 μL/well SureBlue™ TMB Microwell Peroxidase Substrate (KPL) to all plates with a 96-well liquidator in order, wait for 10 min for color development at room temperature.

18. Add 50 μL/well 1 N H₂SO₄ to all plates in the same order, to quench the color development.

19. Read all plates for OD450 using a microplate reader.

20. Data analysis:
   a. Visualization of ELISA data in a heat map like Figure 3B.
   b. For each antigen, determine the antibody concentration at which the OD450 display the best dynamic range among the tested antibodies. For example, data obtained at 0.1 μg/mL for UG037.8 and 10 μg/mL for ZM53 core should be chosen for further analysis of binding titer changes of germline revertant antibodies.
   c. For each germline revertant, determine the OD450 ratio of the parental antibody to the germline revertant at the best antibody concentration for each antigen (as determine in 20b), and take the average of all the ratios for every antigen as the binding fold change index, Fb, for that germline revertant antibody.
Figure 3. Identify key mutations by ELISA

(A) ELISA plate/well setting for 1st antibody binding step. The graph shows the plate/well setting for testing the parent antibody and up to 31 germline revertant (Gr) antibodies in a 96-well ELISA plate coated with one antigen. Each antibody is tested in three different concentrations, 10, 1, and 0.1 μg/mL, to avoid overdosing or underdosing and to best detect the binding difference between a revertant and the parent antibody.

(B) Example of a heat map showing the binding (OD450) of a VRC01-class bnAb (Parental) and its 31 germline revertants (Gr) to a panel of 23 HIV envelope-based antigens at three different concentrations: 10, 1 and 0.1 μg/mL. * marks the optimal concentration for detecting changes between Gr and Parent.

\[
F_b = \frac{1}{n} \sum_{i=1}^{n} \left( \frac{OD_{450}^i}{OD_{450}^0} \right), n = \text{the number of antigens}
\]

d. We artificially define any fold change larger than 1.5 as significant, demonstrating that the SHM in the corresponding germline revertant has a significant impact on the binding of the target bnAb to its antigens.

21. Identify all key mutations that cause >= 1.5-fold change in binding index.
TZM-bl neutralization assay of germline revertants to identify key mutations

Timing: 1 week

22. Virus panel selection: based on the large-panel neutralization data of the target bnAb, select a panel of 10 bnAb-sensitive viruses from different clades with at least moderate neutralization titers by the bnAb. An exemplar panel for VRC01-class bnAb 2411a is shown in Figure 4A.

Figure 4. Identify key mutations by neutralization assays

(A) Exemplar neutralization results showing IC50s of a parental VRC01-class bnAb (2411a) and its germline revertants against a panel of 10 sensitive viruses from different clades. The color-code for IC50 goes from strong neutralization (red) to weak (green). No neutralization observed at greater than 50 μg/mL is shown in white.

(B) Exemplar neutralization curves of a parental bnAb 2411a and its germline revertants of non-key mutations (gray curves) vs. key mutations (red curves) against virus strain RHPA.7.

TZM-bl neutralization assay of germline revertants to identify key mutations

© Timing: 1 week
neutralizing titers to the bnAb: viruses with too high titers are often also sensitive to the germline precursor, whereas those with too low titers do not have the range to show significant titer change in response to SHM revertant.

23. Pseudovirus preparation: Pseudoviruses for use in TZM-bl neutralization assays were produced in 293T cells by cotransfection of a pSG3ΔEnv backbone plasmid and a full HIV-1 Env gp160-encoding plasmid (Li et al., 2005).
   a. 2 x 10⁶ cells in 20 mL cDMEM (see materials and equipment) were seeded in T75 flasks the day prior to cotransfection.
   b. For transfection, 40 μL of FuGene 6 reagent (Promega) was diluted into 800 μL of room-temperature Opti-MEM I reduced serum medium (Thermo Fisher), followed by addition of 10 μg of pSG3ΔEnv backbone plasmid.
   c. 3.3 μg of HIV Env plasmid was then added to the mixture, mixed, and incubated for 30 min at room temperature.
   d. Transfection mixture was then added to media of previously seeded 293T cells in the T75 flask and then distributed evenly on cells.
   e. The following day, media was replaced with 20 mL fresh cDMEM.
   f. Virus was harvested the following day by filtering cell supernatants with 0.45 μm Steriflip units (EMD Millipore) and aliquoted.

24. To measure neutralization of purified antibodies,
   a. 10 μL of five-fold serially diluted mAbs in cDMEM was incubated with 40ul of diluted HIV-1 Env-pseudotyped virus and incubated for 30 min at 37°C in a 96-well CulturPlate (Perkin Elmer).
   b. 20 μL of TZM-bl cells (10,000 cells/well) with or without 70 μg/mL DEAE-Dextran was then added and incubated overnight at 37°C.
   c. Each experiment plate also had a column of cells only (no Ab or virus) and a column of virus only (no Ab) as controls for background TZM-bl luciferase activity and maximal viral entry, respectively.

△ CRITICAL: Serial dilutions were performed with a change of tips at each dilution step to prevent carryover.

25. The following day, all wells received 100 μL of fresh cDMEM and were incubated overnight at 37°C.
26. The following day, 50 μL of Steadylite Plus Reporter Gene Assay System (PerkinElmer) was added to all wells, and plates were shaken at 600 rpm for 15 min.
27. Luminometry was then performed on a SpectraMax L (Molecular Devices) luminometer. Percent neutralization is determined by calculating the difference in average Relative Light Unit (RLU) between virus only wells (cells + virus column) and test wells (cells + plasma/Ab sample + virus), dividing this result by the average RLU of virus only wells (cell + virus column) and multiplying by 100. Background is subtracted from all test wells using the average RLU from the uninfected control wells (cells only column) before calculating the percent neutralization.
28. Neutralizing antibody titers are expressed as the antibody concentration required to achieve 50% or 80% neutralization (IC₅₀ or IC₈₀) and calculated using a dose-response curve fit with a 5-parameter nonlinear function.
29. For each germline revertant, determine the IC₅₀ ratio of the germline revertant to the parental antibody against each virus, and take the average of all the ratios for every tested virus as the neutralization fold change index, Fₙᵢ, for that germline revertant antibody.

\[
F_n = \frac{1}{m} \sum_{j=1}^{m} \left( \frac{IC_{50}^{G_r}}{IC_{50}^{P}} \right), \quad m = \text{the number of viruses}
\]

30. Identify all key mutations that cause >= 2-fold change in Fₙᵢ, and especially focus on those that also cause >=1.5-fold change in binding (Fₙ₀).
**Optional:** Structural analysis of identified key mutations.

If the structure of the parental bnAb in complex with its antigen is available or can be modeled based on known structures of similar antibodies, one can examine structurally why the identified key mutations contribute to improved antibody binding and neutralization activity, such as creating new antigen contact sites or reducing potential clash with antigens.

**Longitudinal analysis of antibody sequences to determine immunogens that elicit the key mutations**

© Timing: 3 weeks

This part describes steps for obtaining VRC01-class antibody heavy and light chain sequences from different stages of sequential immunization and how to perform the longitudinal analysis of the SHM and key mutations in these antibodies.

31. Single B cell sorting with VRC01-class specific probes
   a. Thaw frozen splenocytes (5–10 million cells) in 2-mL cryovials collected from different immunization stages in 37°C water bath.
   b. Spin down the cells at 500 x g for 3 min and remove the supernatants.
   c. Wash the cells with 1 mL PBS by vertexing to resuspend the cells and re-spinning them down at 500 x g for 3 min.
   d. Remove the PBS and resuspend the cells in 100 μl of 1:1,000 diluted (in PBS) ViViD dye (40-μL test vial) that was reconstituted in 50 μL DMSO. Incubate in dark for 15 min.
   e. In the meantime, prepare cell staining mix based on Table 1, and store on ice in dark.
   f. Wash off extra ViViD by adding 1 mL cold PBS and spinning the cells down at 500 g for 3 min.
   g. Resuspend cells in 100–200 μL of staining mix based on the number of cells (10⁷ cells per 100 μL staining mix), incubate on ice in darkness for 30–40 min.
   h. During the cell staining period, prepare compensation beads for each color used in the staining panel with an unstained bead control, following the recipe of 250 μL PBS + 1 drop of BD anti-mouse IgK CompBeads + specified amount of anti-mouse mAb as described in the last column of Table 1. Use 50 μL ViViD-conjugated Amide beads (BD Pharmingen) diluted in 250 μL PBS as compensation control for V450 channel.
   i. Wash the stained cells twice with 1 mL ice-cold PBS as f.
   j. Resuspend the cells in 0.5–0.7 mL of ice-cold PBS/1% BSA and filter through a 40 μm cell strainer.
   k. Set up the compensation in a BD FACSsurf Fusion cell sorter with the prepared CompBeads, and sort ViViD/CD3+CD4+CD8+ F4/80+ /IgD-IgM+IgG+/eOD-GT6+ /eOD-GT6 KO+ B cells (Figure 5) into 96 well plates containing 10 μL of freshly made reverse transcription (RT)/Lysis Buffer per well (see materials and equipment).

**Note:** Sorting strategy rationale is as follows: ViViD⁺ for live cells, CD3⁺ CD4⁺ and CD8⁺ to gate out T cells, F4/80⁺ to gate out macrophages, B220⁺ for B cells, IgD⁺ IgM⁺ and IgG⁺ for IgG⁺ memory B cells, eOD-GT6⁺ and its CD4bs-disrupting mutant eOD-GT6-KO⁺ for eOD-GT6-binding CD4bs-specific B cells (see Figure 5). We chose eOD-GT6 over eOD-GT8 because the former is more similar to gp120, has less engineered mutations and lower affinity to the unmutated VRC01-class precursors in the VH1-2/LC mice (Chen et al., 2021; Jardine et al., 2013, 2015), and can thus better select for and enrich VRC01-class B cell receptors with vaccine-elicited SHMs. However, do not use the additional BG505.SOSIP probe (Figure 5, step 8) for acquiring antibody sequences used for longitudinal mutation analysis, because the added selection by the BG505 probe artificially increases the frequency of more affinity matured antibodies among all acquired antibodies and miss out those with lower SHMs, which interferes with the evaluation of immunization impact on antibody SHM and key mutations.
1. Seal the 96 well plate with a sealing film, vortex and centrifuge the plate to make sure the sorted cells are in the lysis buffer.

m. Freeze the plate on dry ice or in a \(-80^\circ C\) freezer and store it frozen at \(-80^\circ C\) until next step.

32. Single B cell RT-PCR

a. Reverse Transcription (RT): For each 96 well plate, make a master mix of RT/Superscript mix (see materials and equipment) and add 2.5 µL to each well with a multichannel pipet; perform RT reaction as described in Table 2.

b. 1st PCR: Set up the 1st PCR reactions based on recipe in materials and equipment (make 100× master mix for each 96 well PCR plate) and run PCR under the conditions as described in Table 3. Choose corresponding 1st PCR 5’ (forward) and 3’ (reverse) primers to amplify the heavy and light chains of VRC01-class BCRs (see key resources table).

c. 2nd PCR: Set up the 2nd PCR reactions (see materials and equipment, make 100× master mix for each 96 well PCR plate) and run PCR under the conditions as described in Table 3. Choose corresponding 2nd PCR 5’ (forward) and 3’ (reverse) primers to amplify the heavy and light chains of VRC01-class BCRs (see key resources table).

33. Gel electrophoresis: load 5 µL of the 2nd PCR products to 4× (24+1)-well premade 2% agarose gel with EtBr, and run in 1× TAE buffer at 150V for 20 min. Take gel images on UV transilluminator in a Bio-Rad ChemiDoc imaging system.

△ CRITICAL: Ethidium Bromide (EtBr) is a carcinogen. Wear gloves when handling EtBr-

Agarose gels.

Figure 5. B cell sort gating steps for isolating CD4bs-specific B cells and VRC01-class antibodies
Sorting steps 1–7 are used to sort for eODGT6-reactive CD4bs-specific B cells which most likely encode VRC01-class antibodies in the VH1-2/LC mice. These antibodies should cover a wide range of SHM levels. Step 8 is added to select for B cells expressing more affinity matured and more cross-reactive VRC01-class antibodies, for example the target bnAb, that can bind the glycan276-containing BG505 SOSIP trimer.
34. Identify wells with positive PCR band of immunoglobulin heavy or light chains and send the remaining 2nd PCR products in those wells for Sanger sequencing (by Genewiz) with the corresponding 2nd PCR primers.

**Optional:** Can also obtain VRC01-class antibody sequences by H/L-paired deep sequencing or 10x genomics.

35. Sequence analyses
   a. Submit raw sequencing results to IgBlast and display mismatches with the germline (VH1-2 or VK3-20) sequences and check the sequencing chromatograms to correct any misread of nucleotides (including indels) and ensure that the mismatches from the germline sequences are not from sequencing errors.
   b. Submit all corrected sequences in FASTA format to IMGT/V-QUEST (≤ 50 sequences) or IMGT/HighV-QUEST (> 50 sequences) to define V-genes, D-genes, J-genes and CDRs of the queries; IMGT will also export the translated VH and VK sequences of the queries and align all sequences derived from the same V-gene.
   c. Align all heavy and light chains against their respective germline V-genes, VH1-2 or VK3-20, only displaying mismatched residues while showing all identical residues as a dot (.) or hyphen (-) in alignment. This can be done in public sequence alignment servers such as MUL-TALIN. In fact, the IMGT/V-QUEST protein sequence alignment is displayed this way and can be copied and pasted into cells [A3:A102] of a Microsoft Excel file template that we created specifically for analyzing VRC01-class Ab sequences and key mutations (Table S1). This file contains two sheets, each for analyzing VH1-2 HCs and VK3-20 LCs, respectively. It will

| Table 1. B cell staining panel (for up to 2 × 10^7 cells) |
|-------------------------------------------------------------|
| Detection channel | α-mouse mAb or probe | Dye | Probe vol (μl) | Note | mAb clone | Vol for making comp beads (μl) |
|-------------------|---------------------|-----|---------------|------|------------|-----------------------------|
| V710              | mlgD                | BV711 | 1.50          | IgD  | 11-26c.2a  | 0.5                         |
| B710              | CD3                 | PerCP-Cy5.5 | 1.25 | T cell marker | 145-2C11 | 0.5                         |
|                   | CD4                 | PerCP-Cy5.5 | 0.50 | T4 cell marker | RM4-5  |                             |
|                   | CD8                 | PerCP-Cy5.5 | 0.63 | T8 cell marker | S3-6.7 |                             |
|                   | F4/80               | PerCP-Cy5.5 | 0.50 | Macrophage marker | BMB  |                             |
| B515              | mlgG1               | FITC | 2.00 | IgG1         | A85-1  | 1                           |
|                   | mlgG2a              | FITC | 2.00 | IgG2a        | R19-15 |                             |
|                   | mlgG2b              | FITC | 2.00 | IgG2b        | R12-3  |                             |
|                   | mlgG3               | FITC | 2.00 | IgG3         | R40-82 |                             |
| G780              | mlgM                | PE-Cy7 | 0.25 | IgM          | II/41  | 0.5 (1/10)                 |
| G610              | B220                | PE-TR | 1.00 | B cell marker | RA3-682 | 0.5                         |
| G570              | BG505.SOSIP*        | PE | (3.00*) | Only used for bnAb isolation | 1.0 (1/10) |
| R670              | eODGT6-KO           | APC | 1.00 | Negative probe: CD4bs-KO | (1D3)  | 0.5 (1/10)                 |
| R710              | eODGT6             | AF700 | 1.00 | Positive probe |                  |                             |
|                   | PBS, 1% BSA         |       | 184.47 (181.47*) |                  |                  |                             |
|                   | Total               |       | 200.00 |                  |                  |                             |

*Only use this probe when sorting for bnAb.

| Table 2. RT reaction conditions |
|---------------------------------|
| Steps | Temperature | Time | Cycles |
|-------|-------------|------|--------|
| 1     | 42°C        | 10 min | 1      |
| 2     | 25°C        | 10 min | 1      |
| 3     | 50°C        | 60 min | 1      |
| 4     | 94°C        | 5 min  | 1      |
| 5     | 4°C         | ∞     |        |
output trimmed sequence alignment for making logo graphs and calculate frequencies of VRC01-class key mutations.

d. The aligned antibody sequences in Cells [A3:A102] of our Excel template file are automatically trimmed to remove the space and extra dot (.) in the IMGT/V-QUEST alignment and to keep the same length as the corresponding germline V-gene. The trimmed sequences are outputted in corresponding rows in column D. This trimming step is necessary for the following key mutation frequency analysis and logograph display of mutations induced by immunizations.

e. The frequency of each VRC01-class key mutation, as defined in our previous study (Chen et al., 2021), among all antibody sequences obtained from a certain immunization stage will be automatically calculated and output in cells [E103:M103] of “sheet1_VH1-2 HCs” and cells [E103:K103] of “sheet2_VK3-20 LCs” (Table S1). The total number of key mutations in each antibody heavy and light chains is also calculated and displayed in sheet1 [N3:N102] and sheet2 [L3:L102] VK3-20, respectively. The average number of key mutations in a HC or LC of the analyzed sequences is shown in Sheet1 [N103] or Sheet2 [L103], respectively.

f. Perform the above key mutation frequency analysis for every immunization stage and make a table to show how key mutation frequency changes with different immunization stages and last injected immunogens (Figure 6A).

36. Make logo graphs showing all the amino acid mutations (vs. the germline V-gene) and their frequencies in all the antibody sequences from a certain immunization stage.

a. Generate FASTA format of the aligned and trimmed antibody sequences:
   i. Copy the sequence numbers and the corresponding aligned and trimmed antibody sequences in cells [C3:D100] of the Excel template (Table S1) and paste them into a TextEdit file in Mac or a Word file in Windows.
   ii. Use letter “O” to replace any deleted residues (shown as “.”) compared to the germline V-gene in the aligned sequences in Table S1 the TextEdit or Word file with the “Replace” function. In the logo graph, “O” would represent a residue deletion.
   iii. In the Mac TextEdit file, select and copy all content to the designated sequence input field in WebLogo server, the sequences will be in FASTA format.
   iv. In Windows, save the Word file as a plain text file, and in the popup window, check the box under “options” for “insert line breaks”, and click OK. Reopen the saved plain text file with NotePad, select and copy all content to WebLogo server, and the pasted sequences will also be in FASTA format.

b. Use the default setting, including checked “Small Sample Correction”, to submit the pasted sequences.

c. A mutation logo graph will be displayed for the pasted sequences and can be copied.

37. Make a logo graph for VRC01-class Ab sequences obtained from every immunization stage, including naïve or preimmune stage. Align the graphs together as in Figure 6B, and it will be visually obvious at which stage or following injection of which immunogen each amino acid mutation, including the key mutations, initially appear or significantly rise in frequency.
CRITICAL: Only when aligned sequences all have the same number of characters and are in FASTA format, can the WebLogo output the correct logograph. To display only mutated residues, it is important to exclude the germline V-gene sequence in the alignment and display all germline residues in the aligned antibody sequences as a (.) or (-).

EXPECTED OUTCOMES

With this protocol, we should be able to identify which amino acid mutations in a bnAb are required for its binding and/or neutralizing function, and thus are key mutations. Since any one bnAb may not include all the mutations observed in the bnAb lineage, we may choose two or more different bnAbs of the same class to perform mutagenesis analysis and thus identify more key mutations for that class of bnAbs. Moreover, by longitudinal mutation profile analysis, we can infer which immunogens or immunization steps are important and likely induce certain key mutations, and therefore direct our vaccine design to elicit and better affinity mature the target bnAb lineages.
LIMITATIONS
This protocol is suited for analyzing key mutations of bnAb lineages that require and rely on specific V-genes for its functions, such as VRC01-class anti-HIV Env Abs and some anti-influenza HA stem antibodies that require usage of VH1-69, VH1-18, or VH6-1 genes. Because these V-gene dependent bnAb lineages have diverse CDRH3s but share the same V-gene, we only identify and monitor key mutations in the specific V-gene. Therefore, this protocol will not work on bnAbs that rely heavily on CDR3 regions for their functions, unless one can obtain enough antibody sequences covering the CDR3 regions, e.g., by deep sequencing, to analyze the development of key mutations in the CDR3s in response to immunizations.

As long as there are immunogens available to elicit a lineage of V-gene based bnAb against any pathogen, we can easily adapt this protocol to analyze the vaccine elicitation of key mutations in that bnAb lineage using any desired animal model with known immunoglobulin locus sequences, by designing PCR primers specific to the antibody V-genes and the animal model. Moreover, our VRC01-class key mutation analysis template (Table S1) can also be readily modified to analyze other antibodies once the germline antibody sequences and the key mutations are identified. This protocol works best in the humanized transgenic mouse system for VRC01-class antibodies due to the high precursor frequency but could also work in non-transgenic mouse system with properly designed 5’ primers. Without high bnAb precursor frequency, it may be more difficult to obtain sufficient bnAb-lineage sequences for longitudinal antibody mutation profiling analyses with the single cell sorting and RT-PCR method described here, but the application of next-generation-sequencing (NGS) could be an alternative for collecting sufficient antibody sequences.

TROUBLESHOOTING
Problem 1
Target bnAb does not cover all VRC01-class mutations that are found frequently in other bnAbs (before you begin: step 14).

Potential solution
Select additional bnAbs that contain mutations not covered by the first bnAb and perform functional analysis on the germline revertants of these mutations.

Problem 2
A germline revertant antibody does not express or expresses at a very low yield (step 5–6).

Potential solution
In the case of no expression at all, first double check the insert sequences of the antibody expression plasmids to make sure there is no unexpected mutation or reading frameshift in the antibody coding regions; if the coding sequences are correct, try to subclone them to a new batch of parental expression vector because the mutagenesis PCR sometimes generate unwanted mutation in the backbone of the expression vector and cause problem in expression of the insert. If the antibody expresses at a low yield, we can increase the yield by either increasing the transfection scale or by incubating transfected cells at 28°C–32°C, rather than 37°C, for a longer time (i.e., 7 days).

Problem 3
IMGT/V-Quest returns an error message when the input antibody sequences number more than 50 (step 35b).

Potential solution
Split the antibody sequences into multiple sets of 50 or less and perform the IMGT/V-Quest analysis for each set separately.

Problem 4
The current VRC01-class key mutation analysis template (Table S1) can only analyze 100 antibody sequences and might not be enough to cover all sequences obtained from a timepoint (step 35 c-35d).
Potential solution
The template can be easily modified to analyze more sequences by inserting more rows between row 3 and row 102.

Problem 5
The WebLogo outputs a logo graph different from Figure 6B (step 36).

Potential solution
Make sure 1) your input sequence alignments are in FASTA format, 2) the alignment shows residues identical to germlines as “-”, 3) “Small Sample Correction” box is checked in the submission page, 4) do not check the “Frequency Plot” box.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, John R. Mascola (jmascola@nih.gov).

Materials availability
All unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and code availability
The datasets generated during this study have been published (Chen et al., 2021).

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101180.

ACKNOWLEDGMENTS
We thank F.W. Alt and M. Tian for providing the mouse model for our immunization study that generated the VRC01-class antibodies. We thank D. Ambrozak and R. Nguyen for assistance in single-cell sorting and C. Chiedi, M. Dillon, and S. Gloria for animal care and maintenance. Support for this work was provided by the Intramural Research Program of the Vaccine Research Center, NIAID, NIH.

AUTHOR CONTRIBUTIONS
Conceptualization, X.C.; methodology, X.C., S.D.S., H.D., and N.A.D.; investigation, X.C., S.D.S., H.D., and N.A.D.; writing – original draft, X.C.; writing – review & editing, X.C., S.D.S., H.D., N.A.D., and J.R.M.; funding acquisition, J.R.M.; supervision, J.R.M.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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