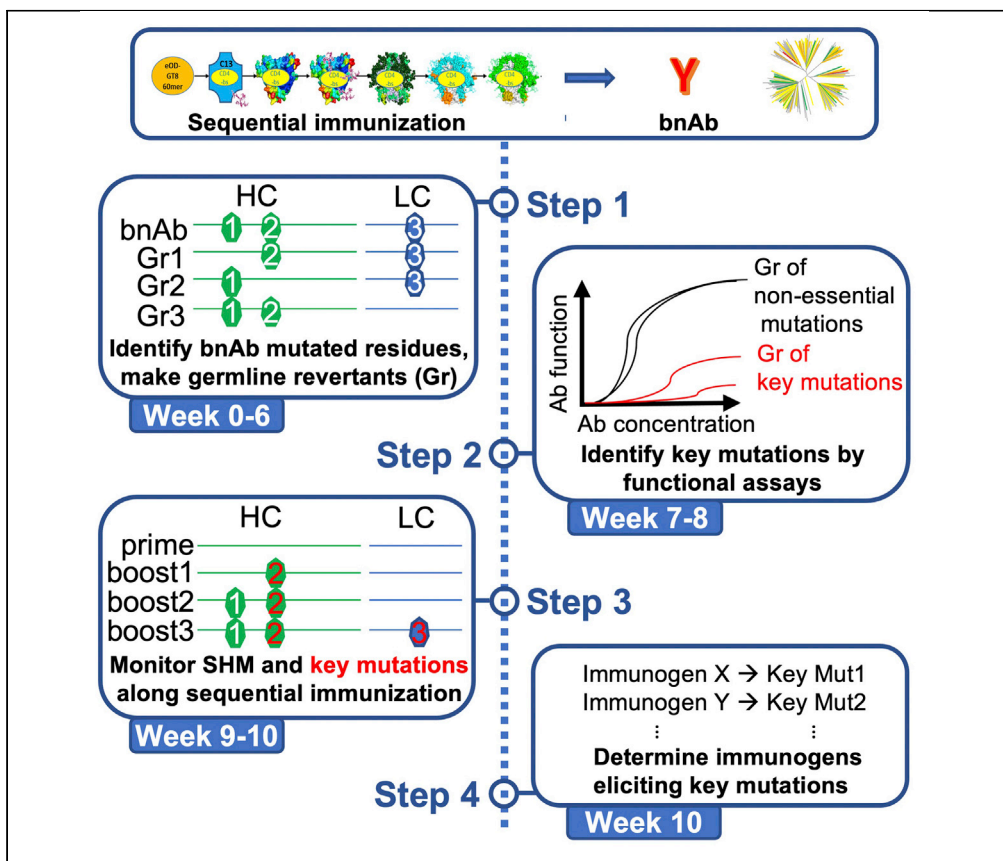


## Protocol

Protocol to identify and monitor key mutations of broadly neutralizing antibody lineages following sequential immunization of Ig-humanized mice



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### 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

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.

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## Protocol

## Protocol to identify and monitor key mutations of broadly neutralizing antibody lineages following sequential immunization of Ig-humanized mice

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## 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 1<sup>st</sup> and 2<sup>nd</sup> round PCRs:
  - a. Typically, design the 1<sup>st</sup> 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 2<sup>nd</sup> 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 1<sup>st</sup> 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.

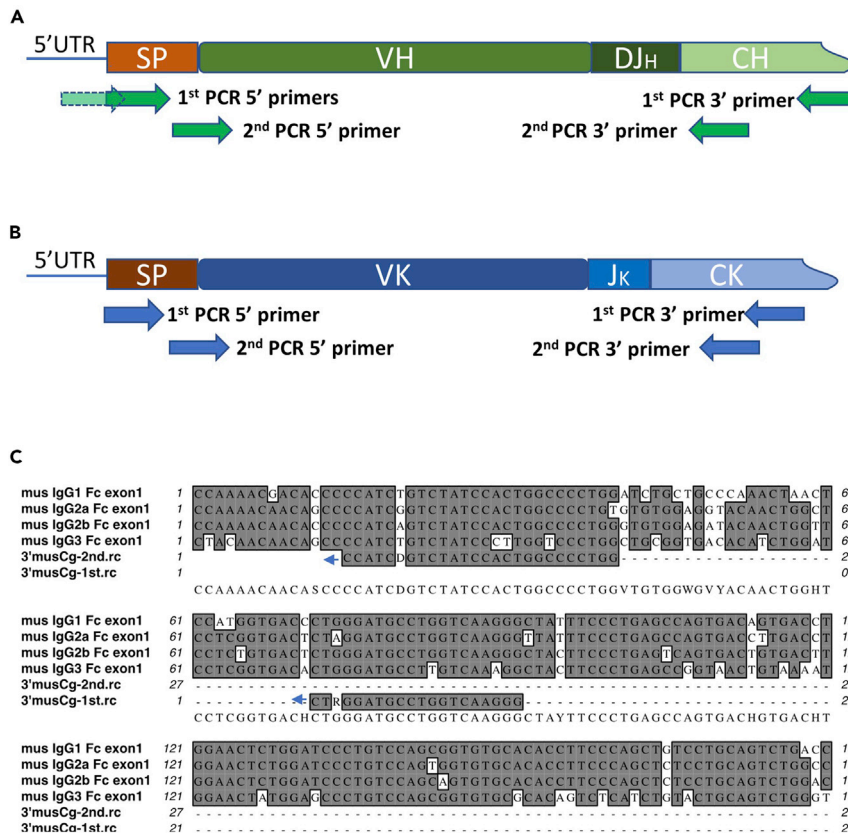
6. Design nested reverse primers for 1<sup>st</sup> and 2<sup>nd</sup> round PCR:
  - a. The reverse primers are to be designed against the heavy and light chain constant regions, respectively, of the bnAb lineage expressed in the studied animal model. In this case, the constant regions are encoded by 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 2<sup>nd</sup> PCR primers must be of the 1<sup>st</sup> PCR primers inside (downstream of the 1<sup>st</sup> PCR 5' primers and upstream of the 1<sup>st</sup> 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



**Figure 1. Locations of antibody PCR primers with respect to the antibody heavy and light chain coding regions**

(A) A diagram showing the locations of the 1<sup>st</sup> and 2<sup>nd</sup> round PCR 5' and 3' primers for a typical antibody heavy chain with respect to its different coding regions, including the 5' untranslated region (5' UTR) and the coding regions for the signal peptide (SP), the VH, the D and J (DJ<sub>H</sub>) and the 5' portion of the constant (CH) regions.

(B) A diagram showing the locations of the 1<sup>st</sup> and 2<sup>nd</sup> round PCR 5' and 3' primers for a typical antibody light (e.g., Kappa) chain with respect to its different coding regions.

(C) The alignment of two 3' gamma chain amplification primers (showing the reversed and complemented sequences) with the 5' coding regions of four different mouse gamma constant gene alleles (IgG1, IgG2a, IgG2b and IgG3). Real primer direction (5' → 3') is marked with an arrow at the left end of each primer. Primers are designed against the most conserved regions among the four alleles and to be proximal to the V-D-J junction for better allele and sequence coverage, especially if next generation sequencing is used. Use of degenerate nucleotides is necessary to cover allele differences in the 3' portion of the primers, but is not as critical for differences in the 5' end of the primers.

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 rProtein 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
<b>Antibodies</b>		
2411a	<a href="#">Chen et al., 2021</a>	GenBank: MT551696, MT551740
VRC01	<a href="#">Wu et al., 2010</a>	RRID: AB_2491019
anti-mouse CD3 Cy5.5PerCP (1:160)	BD Pharmingen	Cat# 551163; RRID: AB_394082
anti-mouse CD4 Cy5.5PerCP (1:400)	BioLegend	Cat# 100540; RRID: AB_893326
anti-mouse CD8 Cy5.5PerCP (1:320)	BD Pharmingen	Cat# 551162; RRID: AB_394081
anti-mouse F4/80 Cy5.5PerCP (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# 553390; RRID: AB_394828
anti-mouse IgG2b FITC (1:100)	BD Pharmingen	Cat# 553395; RRID: AB_394833
anti-mouse IgG3 FITC (1:100)	BD Pharmingen	Cat# 553403; RRID: AB_394840
<b>Chemicals, peptides, and recombinant proteins</b>		
C13.G3-AviHis	<a href="#">Tian et al., 2016</a>	GenBank: KX462845
ΔC13-AviHis	<a href="#">Tian et al., 2016</a>	N/A
426c-degly3 core-AviHis	<a href="#">Tian et al., 2016</a>	GenBank: KX518319
426c-WT.DS.SOSIP	<a href="#">Tian et al., 2016</a>	GenBank: KX462847
eOD-GT8 60mer	<a href="#">Tian et al., 2016</a>	GenBank: KX527857
eOD-GT8-AviHis	<a href="#">Tian et al., 2016</a>	GenBank: KX527855
ΔeOD-GT8-AviHis	<a href="#">Tian et al., 2016</a>	GenBank: KX527856
eOD-GT6 60mer	<a href="#">Tian et al., 2016</a>	GenBank: KX527854
eOD-GT6-AviHis	<a href="#">Tian et al., 2016</a>	GenBank: KX527852
ΔeOD-GT6-AviHis	<a href="#">Tian et al., 2016</a>	GenBank: KX527853
BG505.SOSIP.T332N	<a href="#">Tian et al., 2016</a>	N/A
BG505.DS.SOSIP	<a href="#">(Kwon et al., 2015)</a>	N/A
RSC3	NIH AIDS Reagent Program; <a href="#">Wu et al., 2010</a>	Cat# 12042
Bal.01 gp120	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
JRFL gp120	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
6101 core	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
ZM53 core	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
ZM197 core	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
ZM215 core	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
KER2018.11 core	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
UG037.8 core	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
45-01dG5 gp120	<a href="#">Wu et al., 2012</a>	GenBank: AFE02253
45-01dH5 gp120	<a href="#">Wu et al., 2012</a>	GenBank: AFE02270

(Continued on next page)

<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
HxBc2 core	<a href="#">Chen et al., 2009</a>	PDB: 3IDY_G
CH505.DS.SOSIP	<a href="#">Cheng et al., 2019</a>	N/A
ConA1 gp120	Immune Technology	Cat# IT-001-CONA1p
ConA2 gp120	Immune Technology	Cat# IT-001-CONA2p
Clade B gp140	VRC/NIH; <a href="#">Chen et al., 2021</a>	N/A
Clade C (Du422.1) gp120	Immune Technology	Cat# IT-001-RC3p
Cap210 gp120	Immune Technology	Cat# IT-001-RC12p
Blocking/Diluent Solution (concentrated) SuperScript® II Reverse Transcriptase	Thermo Fisher Scientific	Cat# 18064014
SuperScript® III Reverse Transcriptase	Thermo Fisher Scientific	Cat# 18080044
HotStarTaq Plus DNA Polymerase	QIAGEN	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 × 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	Embiotec	Cat# GE-3642
<b>Critical commercial assays</b>		
BD FACSAria™ III Cell Sorter	BD Biosciences	N/A
BioTek 405 TS Microplate Washer	BioTek (Now Agilent)	N/A
BioStack Microplate Stacker	BioTek (Now Agilent)	N/A
SpectraMax Plus 384 Microplate Reader	Molecular Devices	N/A
SpectraMax L luminometer	Molecular Devices	N/A
Bio-Rad ChemiDoc Imaging System	Bio-Rad	N/A
<b>Experimental models: Cell lines</b>		
Human: Expi293F™ cells	Thermo Fisher Scientific	Cat# A14528; RRID: CVCL_D615
Human: TZM-bl cells	NIH AIDS Reagent Program	Cat# 8129; RRID: CVCL_B478
<b>Experimental models: Organisms/strains</b>		
Mus musculus: V <sub>H</sub> 1-2/LC mouse model; mixed 129/Sv and C57BL/6 strains	<a href="#">Tian et al., 2016</a>	N/A
<b>Oligonucleotides</b>		
Primers for single cell RT-PCR in the V <sub>H</sub> 1-2/LC mouse model (see below)		
IgH (VH1-2) 1 <sup>st</sup> PCR forward primer VH1-LEADER-A: ATGGACT GGACCTGGAGGAT	<a href="#">Tian et al., 2016</a>	N/A
Alternative IgH (VH1-2) 1 <sup>st</sup> PCR forward primer VH1-2 5'UTR: GAGAGCTCC GTTCTCACCATGGACTGG	This paper	N/A
IgH 1 <sup>st</sup> PCR reverse primer 3'musCg-1 <sup>st</sup> : CCCTTGACCAGGCATCCYAG	<a href="#">Tian et al., 2016</a>	N/A
IgH (VH1-2) 2 <sup>nd</sup> PCR forward primer xj-VH1- 1 <sup>st</sup> : ACAGGAGCCCACTCCCAGGTGCG	<a href="#">Tian et al., 2016</a>	N/A
IgH 2 <sup>nd</sup> PCR reverse primer 3'musCg-2 <sup>nd</sup> : CCAGGGCCAGTGATAGACHGATGG	<a href="#">Tian et al., 2016</a>	N/A
IgK (VK3-20) 1 <sup>st</sup> PCR forward primer 5'hVK3-20.6: CGCAGCTTCTCTCTCTCTG	<a href="#">Tian et al., 2016</a>	N/A

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<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
IgK 1 <sup>st</sup> PCR reverse primer: 3'musCk-1st ACTGGATGGTGGGAAGATGGA	Tian et al., 2016	N/A
IgK (VK3-20) 2 <sup>nd</sup> PCR forward primer: 5'hVK3- 20.4: ACTCTGGCTCCCAGATACCA	Tian et al., 2016	N/A
IgK 2 <sup>nd</sup> PCR reverse primer: 3'musCk-2 <sup>nd</sup> : GGAAGATGGATACAGTTGGTG	Tian et al., 2016	N/A
<b>Recombinant DNA</b>		
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
<b>Software and algorithms</b>		
GraphPad Prism 8.01 Software	GraphPad Prism Software, (Ye et al., 2013)	RRID: SCR_002798
IgBlast		<a href="https://www.ncbi.nlm.nih.gov/igblast/">https://www.ncbi.nlm.nih.gov/igblast/</a>
IMGT/V-Quest	(Brochet et al., 2008; Giudicelli et al., 2011)	<a href="http://www.imgt.org/IMGT_vquest/vquest/">http://www.imgt.org/IMGT_vquest/vquest/</a> ; RRID: SCR_010749
WebLogo	(Crooks et al., 2004)	<a href="http://weblogo.berkeley.edu/logo.cgi">http://weblogo.berkeley.edu/logo.cgi</a> ; RRID: SCR_010236
FlowJo v.9.9.1 and v.10.6.2	FlowJo	<a href="https://www.flowjo.com/">https://www.flowjo.com/</a> ; RRID: SCR_008520
R v3.5.2	The Comprehensive R Archive Network	<a href="https://cran.r-project.org/">https://cran.r-project.org/</a>
<b>Other</b>		
MilliporeSigma™ Steriflip™ Sterile Disposable Vacuum Filter Units	Thermo Fisher Scientific	Cat# SCGP00525
ExpiFectamine™ 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

## MATERIALS AND EQUIPMENT

Complete DMEM (cDMEM)	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
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

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

Cell freezing medium	Final concentration	Amount
Heat-inactivated FBS	90%	90 mL
DMSO	10%	10 mL
<b>Total</b>	<b>n/a</b>	<b>100 mL</b>

Store at 4°C for a month or at -20°C for a year.

RT/Lysis buffer	Final concentration	1 × (μL/well)	100 × (μL)
RNaseOut (40 U/μL)	1 U/μL	0.25	25
5 × Superscript Buffer	1 ×	2.5	250
DTT (0.5 M)	25 mM	0.63	62.5
10% IGE PAL	0.25%	0.31	31.25
H <sub>2</sub> O	n/a	6.31	631.25
<b>Subtotal</b>	<b>n/a</b>	<b>10</b>	<b>1000</b>
RT/SuperScript Mix			
dNTP (10 mM)	0.8 mM	1	100
Random Hexamer (150 ng/ μL)	12 ng/μL	1	100
H <sub>2</sub> O	n/a	0.25	25
SuperScript III (200 U/μL)	4 U/μL	0.25	25
<b>Total</b>	<b>n/a</b>	<b>12.5</b>	<b>1250</b>

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.

1 <sup>st</sup> PCR mix	Final concentration	1 × (μL/well)	100 × (μL)
H <sub>2</sub> O	n/a	9.75	975
10 × Qiagen PCR Buffer	1 ×	1.25	125
25 mM MgCl <sub>2</sub>	0.5 mM	0.25	25
10 mM dNTP	0.2 mM	0.25	25
20 μM 5' primer for 1 <sup>st</sup> PCR	0.32 μM	0.2	20
20 μM 3' primer for 1 <sup>st</sup> 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
<b>Total</b>	<b>n/a</b>	<b>12.5</b>	<b>1250</b>

Make freshly and use immediately.

2 <sup>nd</sup> PCR mix	Final concentration	1 × (μL/well)	100 × (μL)
H <sub>2</sub> O	n/a	15	1500
10 × CoralLoad PCR Buffer	1 ×	2.5	250
5 × Q	1 ×	5	500
10 mM dNTP	0.2 mM	0.5	50
20 μM 5' primer for 2 <sup>nd</sup> PCR	0.32 μM	0.4	40
20 μM 3' primer for 2 <sup>nd</sup> PCR	0.32 μM	0.4	40
[1 <sup>st</sup> PCR]	4%	1	100
HotStar Plus Enzyme	0.04 U/μL	0.2	20
<b>Total</b>	<b>n/a</b>	<b>25</b>	<b>2500</b>

Make freshly and use immediately.

## 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.



- 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 NE-BaseChanger 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 Expi293<sup>FM</sup> cells in Expi293<sup>TM</sup> Expression Medium to  $3\text{--}5 \times 10^6/\text{mL}$  in a cell incubator at 37°C, 8% CO<sub>2</sub>, 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 ( $2.5 \times 10^6/\text{mL}$ ) cells for expression of each germline revertant antibody. All procedures at this step are operated in a biosafety hood.
    - i. Dilute the 2 L Expi293<sup>FM</sup> cells to  $2.5 \times 10^6/\text{mL}$  with fresh warm Expi293<sup>TM</sup> 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<sup>TM</sup> Steriflip<sup>TM</sup> 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<sup>TM</sup> 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<sup>TM</sup> 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  $2.5 \times 10^6/\text{mL}$  Expi293<sup>FM</sup> cells.
  - c. Incubate the transfected cells in a cell culture incubator at 37°C, 8% CO<sub>2</sub>, 60% humidity with 125 rpm shaking.
  - d. On day 2, add 0.5 mL ExpiFectamine<sup>TM</sup> 293 Transfection Enhancer 1 and 5 mL ExpiFectamine<sup>TM</sup> 293 Transfection Enhancer 2 to each flask of cells, and continue incubation under the same condition for 5–6 more days.

IGHV1-2*02	QVQLVQSGAEVKKPGASVKVSCKASGYFTFGYYMHVVRQAPGGLEWGMWINPNSGGTNYAQKFKQGRVTMTTRDTSISTAYMELSRLLSDDTAVYYCAR
2411a-H	M R E D-VL-M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr1	V R E D-VL-M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr2	M K E D-VL-M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr3	M R K D-VL-M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr4	M R E G-VL-M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr5	M R E D-YL-M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr6	M R E D-VM-M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr7	M R E D-VL-V I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr8	M R E D-VL-M L I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr9	M R E D-VL-M M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr10	M R E D-VL-M N I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr11	M R E D-VL-M K NY I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr12	M R E D-VL-M I-L-K-RS-AV-H S D-L-IN I SFDSYEWFFTYW
Gr13	M R E D-VL-M I-L-K-RY-GT I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr14	M R E D-VL-M I-L-K-RY-AT I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr15	M R E D-VL-M I-L-K-RY-AV-Q I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr16	M R E D-VL-M I-L-K-RY-AV-T I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr17	M R E D-VL-M I-L-K-RY-AV-S I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr18	M R E D-VL-M I-L-K-RY-AV-M I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr19	M R E D-VL-M I-L-K-RY-AV-SR I-L-K-RY-AV-H S D-L-IN I SFDSYEWFFTYW
Gr20	M R E D-VL-M I-L-K-RY-AV-S D-L-IN V SFDSYEWFFTYW
IGKV3-20*01	EIVLTQSPGTLSPGERATLSCRASQVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSP
2411a-K	YFI-RD R-K-TTT-S EFFLGLTKVQV
Gr21	QFI-RD R-K-TTT-S EFFLGLTKVQV
Gr22	YSI-RD R-K-TTT-S EFFLGLTKVQV
Gr23	YFV-RD R-K-TTT-S EFFLGLTKVQV
Gr24	YFI-SD R-K-TTT-S EFFLGLTKVQV
Gr25	YFI-RS R-K-TTT-S EFFLGLTKVQV
Gr26	YFI-RD K-K-TTT-S EFFLGLTKVQV
Gr27	YFI-RD R-R-TTT-S EFFLGLTKVQV
Gr28	YFI-RD R-K-ATT-S EFFLGLTKVQV
Gr29	YFI-RD R-K-TST-S EFFLGLTKVQV
Gr30	YFI-RD R-K-TTS-S EFFLGLTKVQV
Gr31	YFI-RD R-K-TTT-T EFFLGLTKVQV

**Figure 2. Amino acid sequence alignment of the HCs and LCs of a parental VRC01-class bnAb 2411a and its germline revertants (Gr1-Gr31) against the germline VH1-2\*02 and VK3-20\*01 respectively**

“-” indicates the same residue as the germline; mutated V-region residues in the bnAb and the revertants are shown as black single letter codes, whereas the germline reverted residues are marked in blue or red color. Mutation of the red-labeled residues are key mutations (Chen et al., 2021).

**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 × g for 15 min.
  - b. Sterile filter each supernatant with a 0.2 μm 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×, 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.

△ **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

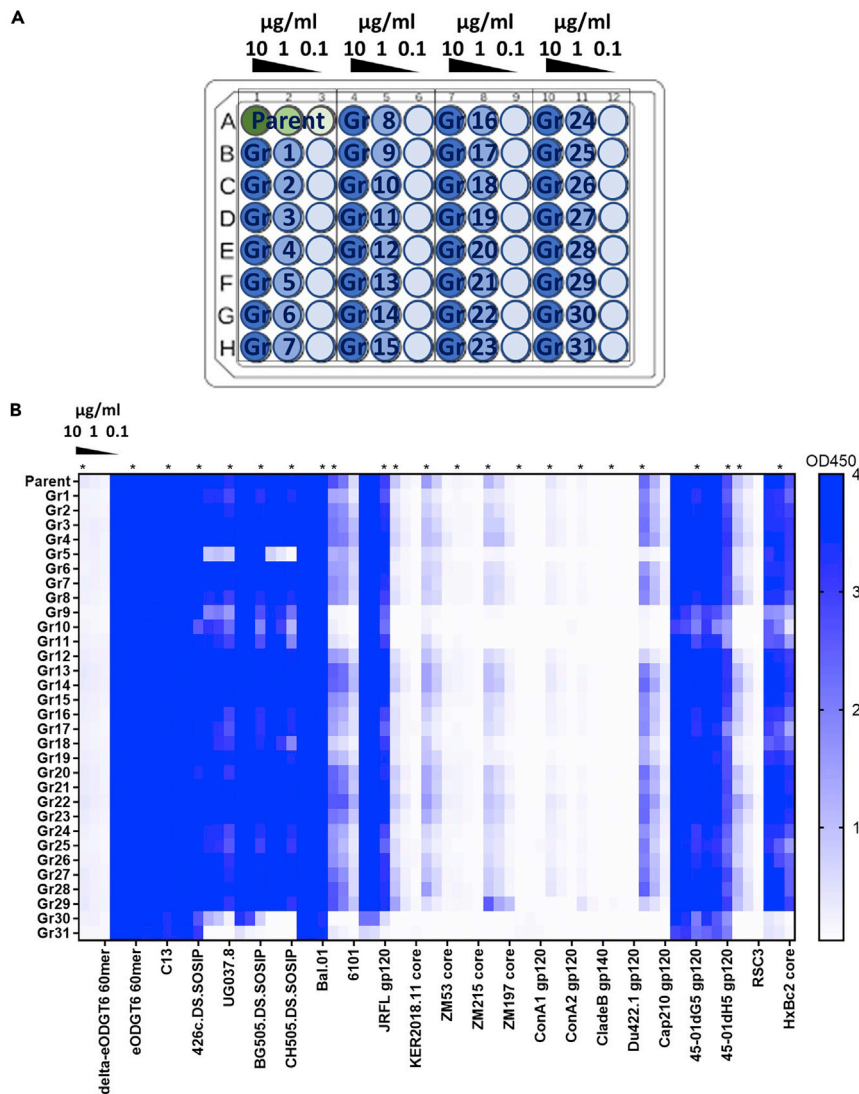
⌚ Timing: 2 days

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  $\mu\text{g}/\text{mL}$  for each antigen: total volume = number of antibodies to test  $\times$  3 (3 antibody concentrations)  $\times$  50  $\mu\text{L}$   $\times$  1.05 (5% extra)
  - b. For each antigen, coat a 96-well costar half-area plate with the 2  $\mu\text{g}/\text{mL}$  antigen coating solution at 50  $\mu\text{L}$  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 2<sup>nd</sup> day, remove the coating solutions in all plates and wash them 1  $\times$  with 200  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{g}/\text{mL}$ ) 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 $\times$  with PBS/T (200  $\mu\text{L}/\text{well}$  per wash) in a BioTek plate washer.
14. Transfer 50  $\mu\text{L}/\text{well}$  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  $\mu\text{L}/\text{well}$  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  $\mu\text{L}/\text{well}$  1 N  $\text{H}_2\text{SO}_4$  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  $\mu\text{g}/\text{mL}$  for UG037.8 and 10  $\mu\text{g}/\text{mL}$  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,  $F_b$ , for that germline revertant antibody.



**Figure 3. Identify key mutations by ELISA**

(A) ELISA plate/well setting for 1<sup>st</sup> 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  $\mu\text{g/mL}$ , 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  $\mu\text{g/mL}$ . \* marks the optimal concentration for detecting changes between Gr and Parent.

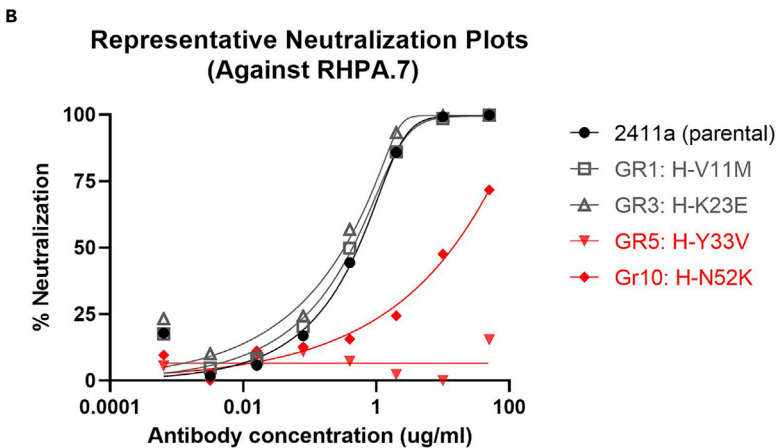
$$F_b = \frac{1}{n} * \sum_{i=1}^n \left( \frac{OD450_i^P}{OD450_i^{Gr}} \right), \quad n = \text{the number of antigens}$$

- 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  $\geq 1.5$ -fold change in binding index.

**A**

mAb ID	3365.v2.c 20	BG505, W5M.C2	BI369.9A	45_01dG 5	JRFLJB	RHPA.7	426c	001428- 2.42	16055-2.3	UG021.16	IC50 (µg/ml)
Clade	A	A	A	B	B	B	C	C	C	D	
	G/-G	G/-G	G/-G	-/-G	G/-G	G/-	G/GG	G/-G	G/-G	G/-G	
276/460462(3) glycan											
2411a parental (duplicate)	1.59	0.600	2.19	0.146	0.190	0.479	2.16	0.463	1.66	0.147	.001-.01
Gr1: H-V11M	1.27	0.630	1.84	0.138	0.185	0.529	2.16	0.485	1.88	0.120	.01-.100
Gr2: H-K19R	1.71	0.568	2.78	0.130	0.166	0.394	2.28	0.513	2.21	0.124	100-1000
Gr3: H-K23E	0.706	0.419	1.71	0.062	0.135	0.488	2.41	0.577	3.36	0.111	1.00-10.0
Gr4: H-G31D	1.14	0.516	2.41	0.134	0.279	0.312	2.21	0.410	1.39	0.072	10.0-.50
Gr5: H-Y33V	1.61	0.447	3.00	0.053	0.089	0.320	1.21	0.144	1.20	0.105	
Gr5&6: H-Y33V/M34L	>50	0.793	>50	0.161	0.222	>50	4.32	>50	>50	0.269	
Gr7: H-V37M	>50	0.577	>50	0.109	0.201	>50	2.93	>50	>50	0.174	
Gr8: H-L45I	3.40	0.579	7.83	0.206	0.201	0.453	2.44	1.03	7.51	0.127	
Gr9: H-M48L	1.52	0.650	2.90	0.116	0.190	0.438	2.00	0.439	2.28	0.120	
Gr10: H-N52K	0.728	0.394	1.35	0.123	0.133	0.333	1.43	0.315	1.72	0.103	
Gr11&12: H-N53R/S54Y	>50	0.913	>50	0.227	0.955	14.4	2.95	>50	24.0	0.149	
Gr12: H-S54Y	>50	1.130	>50	3.10	2.88	15.5	>50	>50	>50	1.51	
Gr13&14: H-G56A/T57V	>50	1.33	>50	0.342	1.20	2.60	47.6	>50	>50	1.29	
Gr14: H-T57V	>50	0.810	>50	0.258	0.908	12.5	>50	>50	>50	0.879	
Gr15: H-Q61H	>50	0.955	45.1	0.285	1.09	3.02	17.4	16.1	>50	0.515	
Gr16: H-T68S	1.89	0.552	7.88	0.141	0.574	1.27	2.79	3.54	>50	0.150	
Gr17: H-S76D	0.867	0.426	2.11	0.161	0.153	0.418	2.51	0.530	3.59	0.151	
Gr18: H-M80L	1.13	0.572	4.10	0.095	0.135	0.355	2.27	0.371	2.32	0.126	
Gr19: H-S83/R84N	1.09	0.359	3.07	0.088	0.129	0.533	2.48	0.717	4.11	0.110	
Gr20: H-V89I	0.754	0.336	1.60	0.097	0.099	0.254	1.30	0.297	1.63	0.119	
Gr21: K-Q27Y	1.25	0.606	3.04	0.129	0.232	0.385	2.28	0.354	1.89	0.132	
Gr22: K-S27A	36.5	0.782	>50	0.124	0.172	10.8	9.66	23.1	>50	0.348	
Gr23: K-V28I	4.31	0.467	24.4	0.100	0.134	1.03	3.09	5.85	29.7	0.216	
Gr24: K-S30R	1.52	0.381	3.20	0.078	0.139	0.551	2.66	1.11	7.50	0.154	
Gr25: K-S31D	3.63	0.541	3.57	0.117	0.182	0.510	2.71	1.38	8.06	0.196	
Gr26: K-K39R	0.540	0.342	1.90	0.106	0.131	0.361	2.63	0.266	1.76	0.115	
Gr27: K-R45K	1.04	0.440	2.11	0.103	0.179	0.397	2.19	0.537	2.57	0.136	
Gr28: K-A51T	1.25	0.442	2.07	0.093	0.122	0.393	1.94	0.445	2.39	0.149	
Gr29: K-S52T	1.10	0.492	1.90	0.100	0.165	0.441	2.50	0.360	2.05	0.149	
Gr30: K-S53T	1.16	0.382	1.86	0.094	0.112	0.371	1.93	0.435	2.31	0.149	
Gr31: K-T56S	0.968	0.532	1.75	0.101	0.157	0.476	2.47	0.387	1.77	0.162	
	0.313	0.220	0.594	0.061	0.083	0.356	1.63	0.369	2.00	0.099	



**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 IC<sub>50</sub> 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

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. The criteria for the virus panel selection include: 1) select from the bnAb-sensitive viruses, since a non-sensitive virus is not helpful for the antibody loss-of-function analysis; 2) select from different viral clades and be as representative as possible to the full panel; 3) avoid virus strains already sensitive to unmutated bnAb precursors; 4) choose viruses with moderate-high



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.  $2 \times 10^6$  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 40 μL 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 (IC<sub>50</sub> or IC<sub>80</sub>) and calculated using a dose-response curve fit with a 5-parameter nonlinear function.
29. For each germline revertant, determine the IC<sub>50</sub> 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, F<sub>n</sub>, for that germline revertant antibody.

$$F_n = \frac{1}{m} * \sum_{j=1}^m \left( \frac{IC50_j^{Gr}}{IC50_j^P} \right), \text{ m = the number of viruses}$$

30. Identify all key mutations that cause  $\geq 2$ -fold change in F<sub>n</sub>, and especially focus on those that also cause  $\geq 1.5$ -fold change in binding (F<sub>b</sub>).

**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.

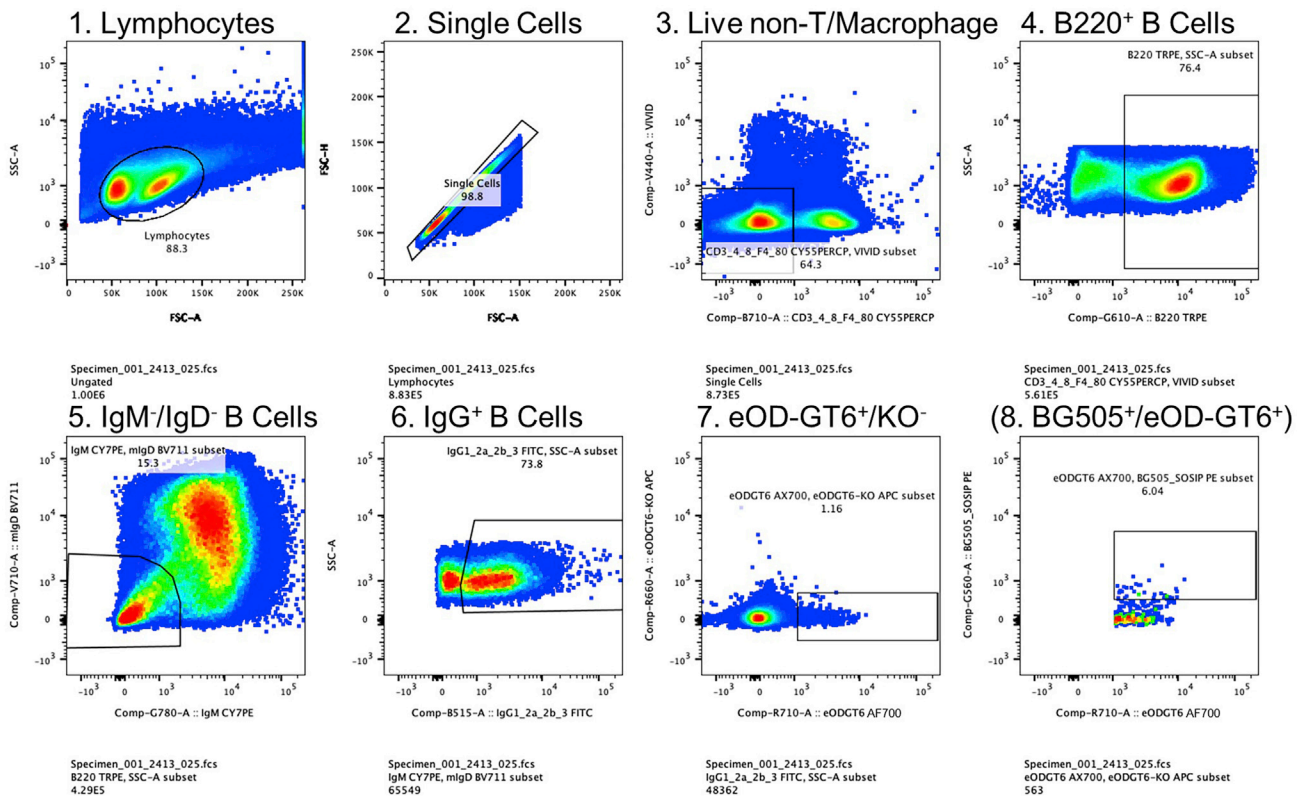
### Longitudinal analysis of antibody sequences to determine immunogens that elicit the key mutations

⌚ 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 × g for 3 min and remove the supernatants.
  - c. Wash the cells with 1 mL PBS by vortexing to resuspend the cells and re-spinning them down at 500 × 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-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 (10<sup>7</sup> 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 FACSAria Fusion cell sorter with the prepared CompBeads, and sort ViViD<sup>-</sup>/CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>F4/80<sup>-</sup>B220<sup>+</sup>/IgD<sup>-</sup>IgM<sup>-</sup>/IgG<sup>+</sup>/eOD-GT6<sup>+</sup>/eOD-GT6 KO<sup>-</sup> 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<sup>-</sup> for live cells, CD3<sup>-</sup> CD4<sup>-</sup> and CD8<sup>-</sup> to gate out T cells, F4/80<sup>-</sup> to gate out macrophages, B220<sup>+</sup> for B cells, IgD<sup>-</sup> IgM<sup>-</sup> and IgG<sup>+</sup> for IgG<sup>+</sup> memory B cells, eOD-GT6<sup>+</sup> and its CD4bs-disrupting mutant eOD-GT6-KO<sup>-</sup> 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.



**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.

- l. 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  $-80^{\circ}\text{C}$  freezer and store it frozen at  $-80^{\circ}\text{C}$  until next step.
32. Single B cell RT-PCR
- a. Reverse Transcription (RT): For each 96 well plate, makes a master mix of RT/Superscript mix (see [materials and equipment](#)) and add 2.5  $\mu\text{L}$  to each well with a multichannel pipet; perform RT reaction as described in [Table 2](#).
  - b. 1<sup>st</sup> PCR: Set up the 1<sup>st</sup> PCR reactions based on recipe in [materials and equipment](#) (make 100 $\times$  master mix for each 96 well PCR plate) and run PCR under the conditions as described in [Table 3](#). Choose corresponding 1<sup>st</sup> PCR 5' (forward) and 3' (reverse) primers to amplify the heavy and light chains of VRC01-class BCRs (see [key resources table](#)).
  - c. 2<sup>nd</sup> PCR: Set up the 2<sup>nd</sup> PCR reactions (see [materials and equipment](#), make 100 $\times$  master mix for each 96 well PCR plate) and run PCR under the conditions as described in [Table 3](#). Choose corresponding 2<sup>nd</sup> 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  $\mu\text{L}$  of the 2<sup>nd</sup> PCR products to 4 $\times$  (24+1)-well premade 2% agarose gel with EtBr, and run in 1 $\times$  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.



**Table 1. B cell staining panel (for up to  $2 \times 10^7$  cells)**

Detection channel	$\alpha$ -mouse mAb or probe	Dye	Probe vol ( $\mu$ l)	Note	mAb clone	Vol for making comp beads ( $\mu$ l)
V710	mIgD	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	53-6.7	
	F4/80	PerCP-Cy5.5	0.50	Macrophage marker	BM8	
B515	mIgG1	FITC	2.00	IgG1	A85-1	1
	mIgG2a	FITC	2.00	IgG2a	R19-15	
	mIgG2b	FITC	2.00	IgG2b	R12-3	
	mIgG3	FITC	2.00	IgG3	R40-82	
G780	mIgM	PE-Cy7	0.25	IgM	II/41	0.5 (1/10)
G610	B220	PE-TR	1.00	B cell marker	RA3-6B2	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.

34. Identify wells with positive PCR band of immunoglobulin heavy or light chains and send the remaining 2<sup>nd</sup> PCR products in those wells for Sanger sequencing (by Genewiz) with the corresponding 2<sup>nd</sup> PCR 3'primers.

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

35. Sequence analyses

- 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.
- Submit all corrected sequences in FASTA format to IMGT/V-QUEST ( $\leq 50$  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.
- 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 MULTALIN. 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 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	$\infty$	

**Table 3. PCR conditions**

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 s	50
Annealing	55°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	7 min	1
Hold	4°C	∞	

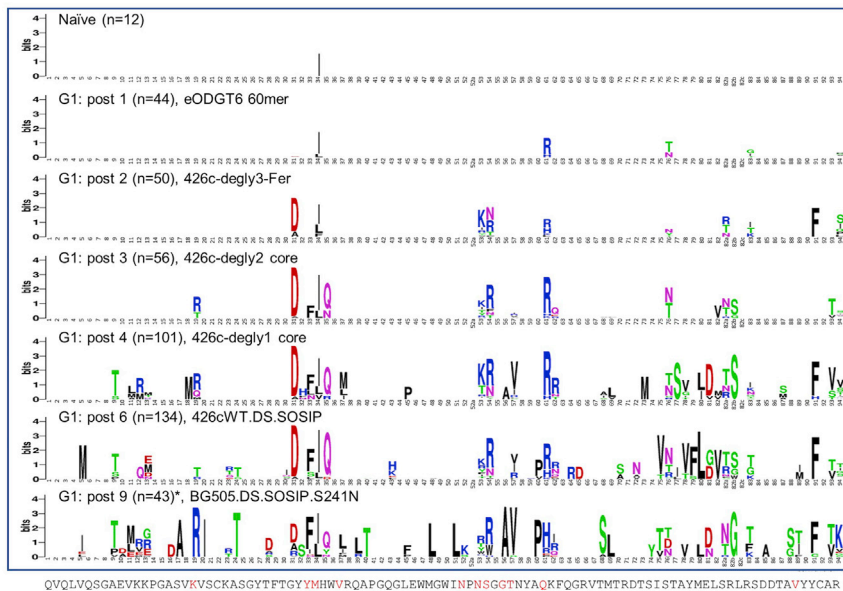
output trimmed sequence alignment for making logo graphs and calculate frequencies of VRC01-class key mutations.

- 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.

A

Groups	Naive	G1					
		1st	2nd	3rd	4th	6th	9th*
Immunizations	None	eOD-GT6 60mer	426c-degly3- Ferr	426c-degly2 core	426c-degly1 core	426cWT.DS. SOSIP	BG505.DS. SOSIP.S241
Immunogens	None						
<b>Key SHMs</b>							
H-Y33V/F/L/I	2.63%	2.27%	4.00%	7.14%	8.91%	7.46%	55.81%
H-V37M/I/L	0.00%	2.27%	0.00%	1.79%	12.87%	3.73%	23.26%
H-N52K/R	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	9.30%
H-N53K/R/N/Y/F/I/L/Y	7.89%	2.27%	34.00%	26.79%	57.43%	30.60%	55.81%
H-S54G/R/N/Y/F/I/L/T/H	2.63%	0.00%	46.00%	37.50%	65.35%	41.04%	65.12%
H-G56A	0.00%	0.00%	0.00%	0.00%	3.96%	1.49%	39.53%
H-T37V/I/P/R	0.00%	0.00%	2.00%	10.71%	31.68%	13.43%	67.44%
H-Q61R/N/K/W	0.00%	13.64%	18.00%	30.36%	23.76%	13.43%	39.53%
H-V89I/L/M/T	2.63%	2.27%	2.00%	0.00%	0.99%	4.48%	25.58%
K-CDR L1 del	0.00%	0.00%	0.00%	4.76%	2.54%	2.61%	21.05%
K-CDR L1 G/S	7.89%	0.00%	4.17%	9.52%	6.78%	11.76%	47.37%
K-Q27V/I/S	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	5.26%
K-S27aF/Y	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	18.42%
<b>Average # of Key SHMs per HC</b>	<b>0.16</b>	<b>0.24</b>	<b>1.11</b>	<b>1.14</b>	<b>2.43</b>	<b>1.55</b>	<b>3.81</b>

B



**Figure 6. Exemplar key mutation frequencies and VH1-2 mutation logo graphs of VRC01-class Abs at different immunization stages in response to different immunogens**

(A) Exemplar key mutation analysis results at different immunization stages. For the Key SHMs "H-" denotes heavy chain and "K-" denotes light chain mutations.

(B) Exemplar VH1-2 mutation logo graphs. Germline VH1-2\*02 sequence is shown at the bottom and the key mutation residues identified in Chen et al. (2021) are highlighted in red. Immunization stage (Post X), number of antibody sequences (n) and last injected immunogen are listed above each logo graph.

△ **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](mailto: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>.

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## 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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