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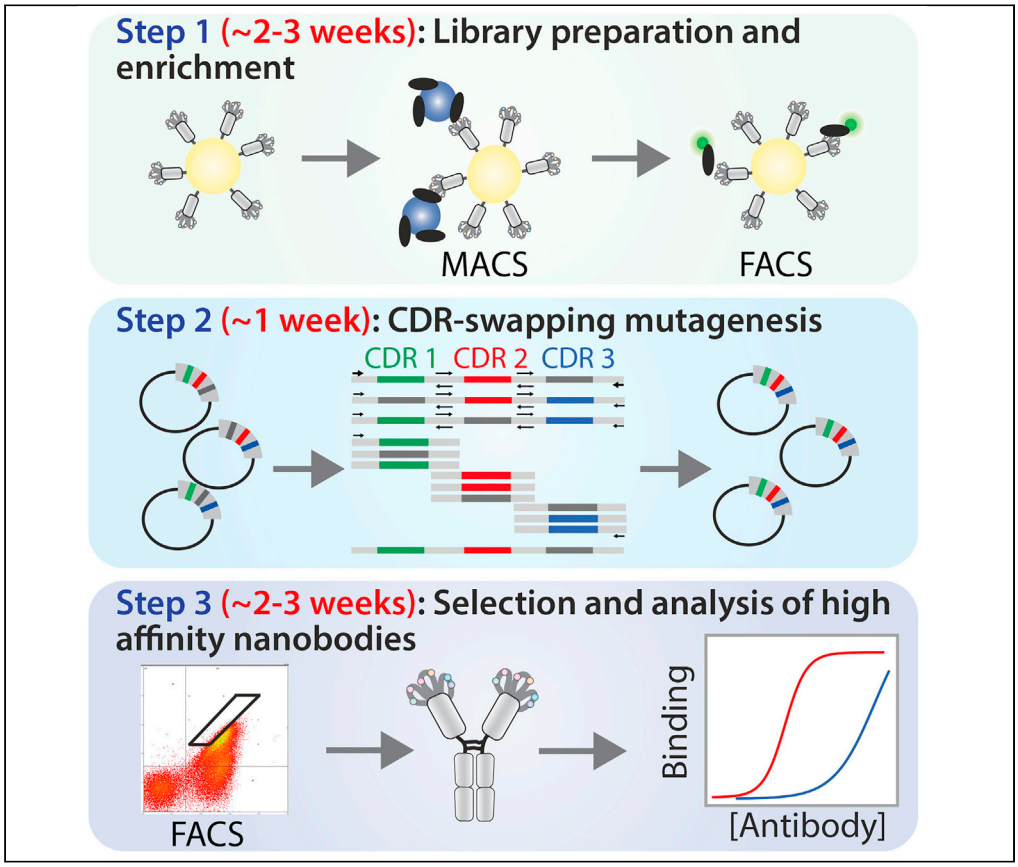
Facile isolation of high-affinity nanobodies from synthetic libraries using CDR-swapping mutagenesis



The generation of high-affinity nanobodies for diverse biomedical applications typically requires immunization or affinity maturation. Here, we report a simple protocol using complementarity-determining region (CDR)-swapping mutagenesis to isolate high-affinity nanobodies from common framework libraries. This approach involves shuffling the CDRs of low-affinity variants during the sorting of yeast-displayed libraries to directly isolate high-affinity nanobodies without the need for lead isolation and optimization. We expect this approach, which we demonstrate for SARS-CoV-2 neutralizing nanobodies, will simplify the generation of high-affinity nanobodies.

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Highlights

Protocol enables direct isolation of high-affinity nanobodies from synthetic libraries

Individual CDRs are amplified and recombined to obtain nanobodies with shuffled CDRs

Libraries of CDR-shuffled nanobodies are rapidly sorted to obtain high-affinity clones

Monovalent and bivalent nanobody affinities are determined using flow cytometry

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Facile isolation of high-affinity nanobodies from synthetic libraries using CDR-swapping mutagenesis

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SUMMARY

The generation of high-affinity nanobodies for diverse biomedical applications typically requires immunization or affinity maturation. Here, we report a simple protocol using complementarity-determining region (CDR)-swapping mutagenesis to isolate high-affinity nanobodies from common framework libraries. This approach involves shuffling the CDRs of low-affinity variants during the sorting of yeast-displayed libraries to directly isolate high-affinity nanobodies without the need for lead isolation and optimization. We expect this approach, which we demonstrate for SARS-CoV-2 neutralizing nanobodies, will simplify the generation of high-affinity nanobodies.

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

BEFORE YOU BEGIN

This protocol describes the steps for sorting nanobody libraries using CDR-swapping mutagenesis against target antigens (Figure 1). This process involves enriching nanobody libraries for binding to target antigens via yeast surface display and cell sorting methods [magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS)]. After several rounds of enrichment, the DNA of the enriched libraries is isolated and the CDRs of the enriched nanobodies are shuffled by PCR. Finally, the enriched and shuffled libraries are further sorted via yeast surface display and FACS to isolate high-affinity clones.

The protocol also describes the expression and purification of promising nanobodies as Fc fusion proteins for their characterization. The current protocol is an adaptation of methods previously described (Zupancic et al., 2021). Some steps need to be planned prior to starting nanobody sorting campaigns. The preparation of primers for CDR-swapping mutagenesis is recommended prior to starting library sorting.

Design of primers for CDR-swapping mutagenesis

Timing: 1 h

Note: Primers and plasmid sequences are given for one common framework nanobody library (Figure 2) (McMahon et al., 2018). Primers for the amplification of CDRs with overlapping DNA

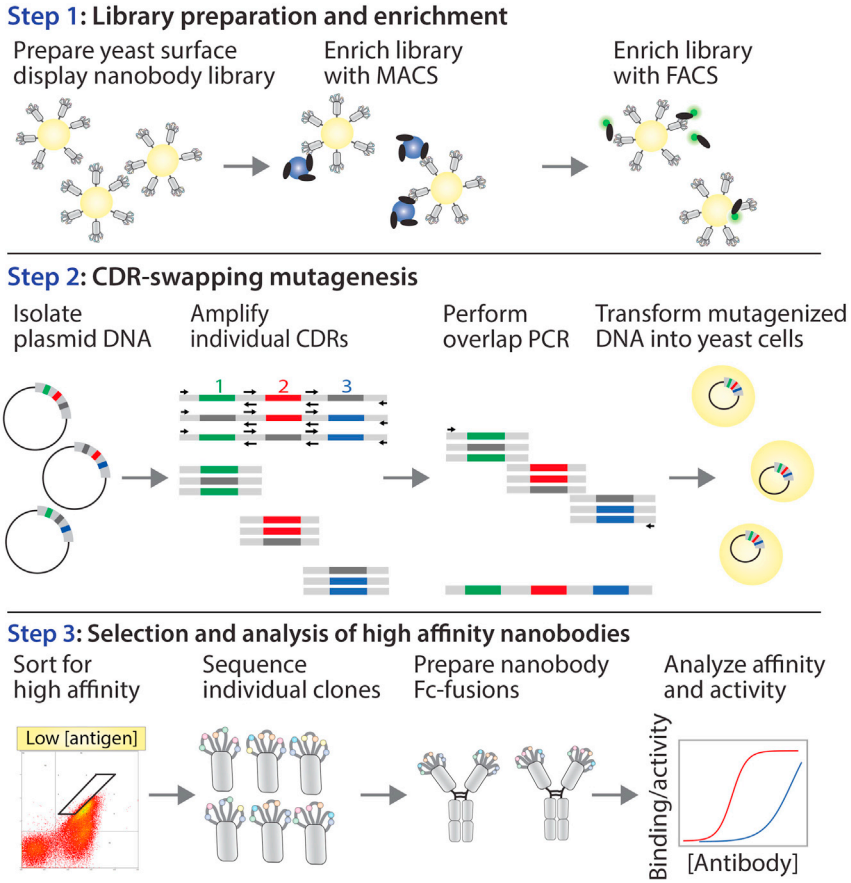

Figure 1. Schematic illustration of the nanobody selection process using yeast surface display and CDR-swapping mutagenesis

Step 1: A common framework nanobody library is prepared in a plasmid which enables nanobody display on yeast through a linker to the Aga2 protein. Magnetic-activated cell sorting (MACS) is first performed to enrich the naïve library for variants that bind the antigen. The enriched library is then sorted using fluorescence-activated cell sorting (FACS) to obtain a diverse population of cells that demonstrate antigen binding (see Figure 3 for details). Step 2: Nanobody plasmid DNA is isolated from yeast cells collected in the final sort performed in Step 1. PCRs are performed to amplify individual CDRs and overlapping DNA sequences from the nanobody framework and surrounding plasmid (see Figure 2 for details). DNA is then reassembled using overlap PCR to produce DNA sequences encoding entire nanobody genes composed of CDR sequences from one or more parental nanobodies. CDR-swapped nanobody library DNA is transformed into yeast cells to produce the CDR-swapped sub-library via homologous recombination in yeast. Step 3: The CDR-swapped library is displayed on the yeast surface and further sorted by FACS to select high-affinity variants (see Figure 3 for details). Plasmid DNA from yeast cells collected in the terminal sort is isolated, and the sequences of individual clones are determined. Individual nanobodies are cloned as Fc-fusion proteins and expressed via transient transfection in mammalian cells. The affinity and activity of nanobody-Fc fusion proteins is then analyzed.

sequences within common framework regions should be designed based on the DNA sequence of the nanobody plasmid.

1. Obtain the DNA sequence of a yeast surface display plasmid containing a representative nanobody.
2. Design forward (Forward primer #1) and reverse (Reverse primer #3) primers for amplifying the plasmid region encompassing the entire nanobody and additional plasmid DNA beyond the restriction sites that will be used for preparing the CDR-swapped library.

Note: Amplified DNA should extend at least ~30–50 base pairs beyond the restriction sites (i.e., N- and C-termini of the nanobody gene) that will be used for vector digestion. Overlap

Figure 2. CDR-swapping mutagenesis randomly combines CDRs from different nanobodies by individually amplifying each CDR using common framework sites and then recombining the DNA segments using overlap extension PCR

(A) Protein sequence of a representative nanobody from a common framework library (McMahon et al., 2018). Framework regions are shown in black. Variable sequences of the complementarity-determining regions (CDRs), namely CDR1 (red), CDR2 (blue), and CDR3 (green), are shown as Xs. The nanobody library used in this work incorporates variation in length of CDR3. A representative length of CDR3 is shown. CDR swapping can be used to shuffle CDR3 segments of different lengths between nanobodies.

(B) The DNA sequence of the nanobody and surrounding plasmid that are used for CDR-swapping mutagenesis. Gray sequences encode homologous sequences to the plasmid at the 5′ and 3′ ends. Nanobody framework regions are shown in black. Variable region DNA sequence corresponding to CDR1 (red), CDR2 (blue), and CDR3 (green) are shown as Xs. Individual PCRs amplify CDR1 (Forward and Reverse primer #1), CDR2 (Forward and Reverse primer #2), and CDR3 (Forward and Reverse primer #3). Regions recognized by primers that amplify individual CDRs are highlighted in yellow. Finally, the DNA segments are recombined using overlap extension PCR. Plasmid DNA at the 5′ and 3′ ends is amplified, including ~50–60 base pairs that flank the restriction sites used for vector digest, in order to prepare plasmid DNA for homologous recombination and transformation into yeast. Restriction sites (NheI and XhoI) are highlighted in purple.

between this sequence and the digested vector will allow for the preparation of a CDR-swapped library via homologous recombination in yeast.

Note: For the PCR conditions given below, the design of primers with melting temperatures of ~58°C–62°C is recommended.

3. Design primers to amplify individual CDRs and overlapping DNA sequences
   a. Amplify CDR1 using Forward primer #1 and a reverse primer that binds in framework 2 of the nanobody library (Reverse primer #1).
   b. Amplify CDR2 using a forward primer that binds in framework 2 (Forward primer #2) and a reverse primer that binds in framework 3 (Reverse primer #2).
   c. Amplify CDR3 using a forward primer that binds in framework 3 (Forward primer #3) and Reverse primer #3.

⚠ CRITICAL: The forward and reverse primers which bind in framework 2 and framework 3 should amplify enough overlapping DNA sequences (e.g., typically >15 base pairs) to perform overlap PCR using the forward and reverse primers designed in Step 3. It is recommended that the melting temperature of the primers designed in Step 3 and the overlapping regions of DNA amplified in framework 2 and framework 3 should not differ by >5°C.

Preparation of antigen-coated magnetic beads

Timing: 2 days

4. Add volume corresponding to 1 × 10^7 streptavidin Dynabeads to a 1.5 mL tube, and place tube on DynaMag™-2 Magnet.

Note: For antigen that is not biotinylated, immobilization on the surface of magnetic beads with alternative surface chemistries like primary amine reactive tosyl group (tosyl activated Dynabeads, Invitrogen, Cat# 14–203) may be performed.

Note: The volume of beads prepared may be scaled up or down depending upon the number of beads needed for analysis.

5. Allow sufficient time for beads to move to the side of the tube by the magnet and discard the liquid.
6. Wash the beads by resuspending in 1 mL of 1× PBS.

Note: Another buffer may also be used for washing and storing the beads. Buffer should not contain any components that will interact with the surface chemistry of the beads (e.g., a buffer with a primary amine should not be used for washing tosyl beads).

7. Place beads in 1× PBS on magnet, allow beads to settle on the side of the tube by the magnet, and discard the liquid.
8. Repeat Steps 6 and 7.
9. Add 0.1 μg antigen to the beads and resuspend the bead solution to a total volume of 400 μL in same buffer (1× PBS) as used for washing the beads.
10. Mix the beads by end-over-end mixing for ~12–48 h at room temperature (20°C–25°C).
11. Store beads at 4°C until ready to use.

Note: Beads can be stored at 4°C for several months. Antigen immobilization time is variable depending on the type of beads used. For example, for streptavidin Dynabeads, 12–24 h at 4°C is sufficient whereas for tosyl activated Dynabeads, longer time is preferred (recommended by the manufacturer).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Myc-Tag antibody (9B11) Working dilution: 1:1000	Cell Signaling Technology	Cat# 2276S; RRID: AB_331783
Goat anti-mouse IgG AlexaFluor 488 Working dilution: 1:200 (~10 μg/mL)	Invitrogen	Cat# A11001; RRID: AB_2534069
Goat anti-human IgG AlexaFluor 647 Working dilution: 1:300 (~5 μg/mL)	Jackson ImmunoResearch	Cat#109-605-098; RRID: AB_2337889
Bacterial and virus strains		
DH5α	Julian et al., 2019	N/A
Chemicals, peptides, and recombinant proteins		
Q5 High-Fidelity DNA Polymerase	New England BioLabs	Cat# M0491L
NheI-HF	New England BioLabs	Cat# R3131L
XhoI	New England BioLabs	Cat# R0146S
HindIII-HF	New England BioLabs	Cat# R3104L
Quick CIP calf intestinal alkaline phosphatase	New England BioLabs	Cat# M0525L
T4 DNA ligase	New England BioLabs	Cat# M0202L
F17 media	Thermo Fisher Scientific	Cat# A1383502
Glutamine	Invitrogen	Cat# 25030081
Kolliphor	Fisher Scientific	Cat# NC0917244
Geneticin (G418 Sulfate)	Thermo Fisher Scientific	Cat# 10131035
Yeastolate	Fisher Scientific	Cat# 292804
Streptavidin AlexaFluor 647	Invitrogen	Cat# S32357
Ampicillin Sodium Salt	Fisher Scientific	Cat# BP1760-25
Kanamycin Sulfate	Fisher Scientific	Cat# BP906-5
Penicillin Streptomycin (Pen Strep)	Gibco	Cat# 15140-122
Pellet Paint NF Co-Precipitant	Sigma-Aldrich	Cat# 70748-3
Protein A Agarose	Thermo Fisher Scientific	Cat# 20333
Bovine Serum Albumin (BSA)	Fisher Scientific	Cat# BP9706100
10x Phosphate Buffered Saline (PBS)	Fisher Scientific	Cat# BP39920
Sodium Citrate Dihydrate	Fisher Scientific	Cat# S279-500
Citric acid (anhydrous)	Fisher Scientific	Cat# A940-500
Yeast nitrogen base (without amino acids)	Fisher Scientific	Cat# DF0919-15-3
Acid casein peptone (casamino acids)	Fisher Scientific	Cat# BP1424-500
Dextrose (D-Glucose)	Fisher Scientific	Cat# D16-10
Sodium phosphate dibasic dihydrate	Fisher Scientific	Cat# S472-500
Sodium phosphate monobasic monohydrate	Fisher Scientific	Cat# S369-500
Galactose	Fisher Scientific	Cat# AC150610051
Tryptone	Fisher Scientific	Cat# BP1421-2
Sodium chloride	Fisher Scientific	Cat# BP358-10
Yeast extract	Fisher Scientific	Cat# BP1422-2
Bacto peptone	Fisher Scientific	Cat# DF0118-07-2
Agar	Fisher Scientific	Cat# BP1423-2
Tryptophan dropout media	Fisher Scientific	Cat# NC0931986
Sorbitol	Sigma-Aldrich	Cat# S1876
Lithium acetate	Fisher Scientific	Cat# AC268640010
1,4-Dithiothreitol (DTT)	Sigma-Aldrich	Cat# 10197777001
Glycine	Fisher Scientific	Cat# 60-090-287
Non-fat dry milk	Kroger	Cat# 0001111008733
Polyethylenimine Hydrochloride (PEI)	Fisher Scientific	Cat# NC1038561
Critical commercial assays		
Zymoprep Yeast Plasmid Miniprep II	Zymo Research	Cat# D2004
QIAquick Gel Extraction Kit	QIAGEN	Cat# 28704
QIAquick PCR Purification Kit	QIAGEN	Cat# 28104

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
QIAprep Spin Miniprep Kit	QIAGEN	Cat# 27106
Experimental models: Cell lines		
Human: HEK293-6E	National Research Council (NRC) of Canada	N/A
Yeast: EBY100	Julian et al., 2019	N/A
Oligonucleotides		
Forward primer #1: GCGCCTGCGGGAGGC	This work	n/a
Reverse primer #1: CTTTCCCAGGCGCCTGTC	This work	n/a
Forward primer #2: AGACAGGCGCCTGGGAAAG	This work	n/a
Reverse primer #2: GACCGCGGTATCTTCCGGTTTC	This work	n/a
Forward primer #3: GAAACCGGAAGATACCGCGGTC	This work	n/a
Reverse primer #3: TAAAAGTACAGTGGGAAC AAAGTCGATTTTG	This work	n/a
Recombinant DNA		
pCTCON2	Addgene	Cat# 41843
pTT5	National Research Council (NRC) of Canada	N/A
Other		
Synthetic nanobody library	McMahon et al., 2018 Provided by the laboratory of Andrew Kruse (Harvard Medical School)	N/A
Streptavidin MicroBeads	Miltenyi Biotec	Cat# 130-048-101
MACS MultiStand	Miltenyi Biotec	Cat# 130-042-303
MidiMACS Separator	Miltenyi Biotec	Cat# 130-042-302
MACS SmartStrainers (70 µm)	Miltenyi Biotec	Cat# 130-098-462
LS Columns	Miltenyi Biotec	Cat# 130-042-401
2 mm electroporation cuvettes	Fisher Scientific	Cat# FB102
DynaMag™-2 Magnet	Invitrogen	Cat# 12321D
Streptavidin Dynabeads	Invitrogen	Cat# 11047
Centrifuge columns	Thermo Fisher Scientific	Cat# 89898
Zeba Spin Desalting Columns	Thermo Fisher Scientific	Cat# 89894; Cat# 89892; Cat# 89890
96-well plates	VWR	Cat# 650261
Beckman Coulter MoFlo Astrios	Beckman Coulter	Cat# B52102

MATERIALS AND EQUIPMENT

PBSB

Reagent	Final concentration	Amount
BSA	1 g/L	1 g
10× PBS	1×	100 mL
DI H_2O	n/a	900 mL
Total	**n/a**	**1 L**

Sterile filter through a 0.22 µm filter before use. Store at 4°C until ready to use. PBSB is stable at 4°C for ~1 month. It is recommended to check for contamination before use.

SDCAA

Reagent	Final concentration	Amount
Sodium citrate	16.75 g/L (57 mM)	16.75 g
Citric acid (anhydrous)	4 g/L (20.8 mM)	4 g

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Reagent	Final concentration	Amount
Yeast nitrogen base (without amino acids)	6.7 g/L	6.7 g
Acid casein peptone (casamino acids)	5 g/L	5 g
Dextrose (D-Glucose)	20 g/L (111 mM)	20 g
DI H_2O	n/a	1 L
Total	**n/a**	**1 L**

Sterile filter through a 0.22 μm filter before use. Store at room temperature. SDCAA is stable at room temperature for ~2–3 weeks. It is recommended to check for contamination before use.

SDGCAA

Reagent	Final concentration	Amount
Sodium phosphate dibasic dihydrate	6.76 g/L (38 mM)	6.76 g
Sodium phosphate monobasic monohydrate	8.56 g/L (62 mM)	8.56 g
Yeast nitrogen base (without amino acids)	6.7 g/L	6.7 g
Acid casein peptone (casamino acids)	5 g/L	5 g
Galactose	20 g/L (111 mM)	20 g
Dextrose (D-Glucose)	11.1 mM	2 g
DI H_2O	n/a	1 L
Total	**n/a**	**1 L**

Sterile filter through a 0.22 μm filter before use. Store at room temperature. SDGCAA is stable at room temperature for ~2–3 weeks. It is recommended to check for contamination before use.

Alternatives: SGCAA may be prepared through the omission of dextrose from the above recipe for SDGCAA.

Note: Yeast induction media with small amounts of glucose (0.2% w/v) enhances cell growth but does not affect the surface display of nanobodies.

LB

Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
Sodium chloride	10 g/L (171 mM)	10 g
Yeast extract	5 g/L	5 g
DI H_2O	n/a	1 L
Total	**n/a**	**1 L**

Autoclave before use. Store at room temperature. LB is stable at room temperature for ~2–3 weeks. It is recommended to check for contamination before use.

YPD

Reagent	Final concentration	Amount
Yeast extract	10 g/L	10 g
Bacto peptone	20 g/L	20 g
Dextrose (D-Glucose)	20 g/L (111 mM)	20 g
DI H_2O	n/a	1 L
Total	**n/a**	**1 L**

Sterile filter through a 0.22 μm filter before use. Store at room temperature. YPD is stable at room temperature for ~2–3 weeks. It is recommended to check for contamination before use.

Tryptophan dropout plates

Reagent	Final concentration	Amount
Agar	15 g/L	15 g
Dextrose (D-Glucose)	20 g/L (111 mM)	20 g
Yeast nitrogen base (without amino acids)	6.7 g/L	6.7 g

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Reagent	Final concentration	Amount
Tryptophan dropout media	3.8 g/L	3.8 g
DI H_2O	n/a	1 L
Total	**n/a**	**1 L**

Store plates at 4°C and warm to room temperature prior to use. Tryptophan dropout plates are stable at 4°C for ~ 1 month. It is recommended to check for contamination before use.

Note: Autoclave agar in 800 mL DI H_2O to dissolve. Prepare supplement by dissolving dextrose, yeast nitrogen base (without amino acids), and the tryptophan dropout media in 200 mL DI H_2O. Sterile filter supplement into agar solution once agar solution has cooled sufficiently to pour plates.

LB ampicillin plates		
Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
Sodium chloride	10 g/L (171 mM)	10 g
Yeast extract	5 g/L	5 g
Agar	20 g/L	20 g
DI H_2O	n/a	1 L
Ampicillin (100 mg/mL)	100 µg/mL	1 mL
Total	**n/a**	**1 L**

Store plates at 4°C for long-term storage and warm to room temperature prior to use. LB plates are stable at 4°C for ~ 1 month. It is recommended to check for contamination before use.

Note: Autoclave tryptone, sodium chloride, yeast extract, and agar in 1 L DI H_2O to dissolve. Add ampicillin once agar solution has cooled sufficiently to pour plates.

Note: Ampicillin is used to select transformed bacteria containing a plasmid with an ampicillin resistance gene. Related antibiotics, including more stable ones (carbenicllin), are also appropriate. A suitable antibiotic and corresponding concentration should be chosen based on the plasmid being transformed.

Supplemented F17 media		
Reagent	Final concentration	Amount
F17 media	n/a	1 L
Glutamine	30 mL/L (5.8 mM)	30 mL
10% Kolliphor	0.1% (w/v)	10 mL
Geneticin	25 mg/L	0.5 mL
Total	**n/a**	**1.0405 L**

Store media at 4°C and warm in bead or water bath at 37 °C prior to use. Supplemented F17 media is stable for ~1 month. It is recommended to check for contamination before use.

Electroporation buffer		
Reagent	Final concentration	Amount
Sorbitol	1 M	182 g
Calcium chloride	1 mM	111 mg
DI H_2O	n/a	1 L
Total	**n/a**	**1 L**

Store at 4°C until use. Prepare fresh the day of use.

Conditioning buffer		
Reagent	Final concentration	Amount
Lithium acetate	0.1 M	1.98 g
1,4-Dithiothreitol (DTT)	10 mM	462 mg
DI H_2O	n/a	300 mL
Total	**n/a**	300 mL
Prepare immediately before use.		

Elution buffer (0.1 M glycine)		
Reagent	Final concentration	Amount
Glycine	0.1 M	7.5 g
DI H_2O	n/a	1 L
Total	**n/a**	**1 L**
Sterile filter through 0.22 μm filter before use. Store at room temperature. Elution buffer is stable at room temperature for ~2–3 months. It is recommended to check for contamination before use.		

Note: Adjust to pH 3 with hydrochloric acid and make up the solution to 1 L.

STEP-BY-STEP METHOD DETAILS

Recovering and inducing yeast libraries

Timing: 3 days

Yeast libraries are refreshed in growth media (SDCAA) prior to use in sorting. This growth step can be used to allow the library to recover after previous storage of the frozen library. Transferring the nanobody library to induction media (SDGCAA) then promotes nanobody expression on the surface of yeast cells for sorting.

1. Thaw a frozen aliquot of the nanobody yeast library on the benchtop at room temperature.
   a. The frozen library aliquot has >10-fold coverage of the diversity with $>10^9$ cells.
2. Add thawed yeast cells to 500 mL of SDCAA media in baffled flask.
   a. Supplement SDCAA media with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
   b. Incubate the flask at 30°C at 225 RPM for 18–24 h.
3. Calculate the cell density by measuring the optical density at 600 nm (OD_{600}). One OD_{600} unit is equivalent to approximately 3×10^7 cells/mL.
4. Calculate the volume of cells required to seed 500 mL SDGCAA culture at a final OD_{600} of 1.
5. Centrifuge the required number of cells at 2500×*g* for 5 min.
6. Discard the supernatant, resuspend the cells in SDGCAA, and move the cells to a 500 mL SDGCAA culture in shake baffled flask.
   a. Supplement SDGCAA media with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
   b. Incubate the flask at 20°C at 225 RPM for 36–48 h.

Note: Yeast induction could be performed at 30°C for 18–22 h or at 20°C for 36–48 h. In certain cases, longer induction times help with higher surface expression.

Magnetic-activated cell sorting, MACS (sort 1)

Timing: 5 h

Initial MACS selections allow for the processing of a large number of cells (10^8–10^9) in early rounds of sorting. In order to ensure full processing of the entire library diversity, approximately 10-fold

greater number of cells are processed than the expected library diversity. Performing a MACS selection enriches the library for clones that bind antigen and reduces library diversity to a level that can be processed via FACS.

7. Calculate the OD_{600} of the SDGCAA culture.
8. Transfer volume corresponding to 10^9 cells to a 50 mL conical tube.
9. Centrifuge the cells at 2500×*g* for 5 min at room temperature.
10. Discard the supernatant and wash the cells by resuspending in 25 mL PBSB.
11. Repeat Steps 9 and 10.
12. Centrifuge the cells at 2500×*g* for 5 min (room temperature) and discard the supernatant. Prepare 10% w/v milk solution in PBSB by dissolving 500 mg non-fat dry milk in 4.5 mL PBSB followed by end-over-end mixing at room temperature for 10–15 min.
    a. Spin down milk solution at 2500×*g* for 5 min (room temperature). Move the supernatant to a new tube.

Note: Centrifuging the milk solution prior to use removes insoluble aggregates and produces more consistent results.

13. Resuspend the cells in a final volume of 5 mL with 1% milk (dilute 10% milk solution in PBSB from Step 12) and 300 nM of biotinylated monovalent antigen (e.g., SARS-CoV-2 receptor-binding domain).
    a. Incubate cells with antigen for 3 h with end-over-end mixing at room temperature.
14. Post antigen incubation, centrifuge the cells at 2500xg for 5 min and wash the cells once by resuspending in 25 mL ice-cold PBSB.
15. Centrifuge the cells at 2500×*g* for 5 min (room temperature).
16. Discard the supernatant.
17. Re-suspend the cells in 40 mL ice-cold PBSB.
    a. Add 750 μL Miltenyi MACS Streptavidin MicroBeads to the resuspended cells.
    b. Incubate the cells and beads with end-over-end mixing at 4°C for 30–45 min.
18. Centrifuge the cells at 2500xg for 5 min and wash the cells once by resuspending in 25 mL ice-cold PBSB.
19. Centrifuge the cells at 2500×*g* for 5 min (room temperature).
20. Discard the supernatant.
21. Pass the cells through a LS column under a magnetic field.

Note: LS columns are gravity columns. Before passing cells through them, they need to be placed on magnetic adapter (MidiMACS Separator and MACS MultiStand). Refer to manufacturer's instructions (https://www.miltenyibiotec.com/upload/assets/IM0001298.PDF) for more details.

Optional: Pass cells through a 70 μm filter immediately prior to running cells through the LS columns. Filtering cells removes aggregates and allows cells to pass through the column without clogging.

22. Wash the captured beads once by passing 5 mL ice-cold PBSB through the LS column under the magnetic field.
23. Remove the LS column from the magnet and elute beads by passing 5 mL SDCAA through the column.
24. Transfer eluted beads and cells to a 50 mL SDCAA media.
    a. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
25. Plate dilutions (10–1000× from the 50 mL cultures) of the cell culture on yeast dropout plates to evaluate the numbers of cells retained during the selection.

26. Grow cells in SDCAA culture and on dropout plates at 30°C at 225 RPM for 2 d. The culture OD_{600} is typically >8–10 after 2 d of growth.

Pause point: Cells in SDCAA culture can be stored at 4°C for several weeks. After storing at 4°C, cells should be refreshed in fresh SDCAA for 18–24 h prior to induction in SDGCAA.

Fluorescence-activated cell sorting, FACS (sorts prior to CDR shuffling)

Timing: 5 h

FACS selections performed prior to CDR shuffling enrich the sorted library to obtain a population of yeast cells from which binding signal can be easily distinguished from the background. This stage of sorting maintains or sequentially decreases the antigen concentration in order to selection for a binding population that displays modest affinity for the target antigen.

27. Induce the yeast cells collected in sort 1 in SDGCAA media as described above in Steps 3–6. Culture volume at this stage may be decreased to 5–8 mL.
28. Calculate the OD_{600} of the SDGCAA culture.
29. Transfer volume corresponding to 10^7–10^8 yeast cells (adjusted to sample 10-fold greater number of cells compared to the isolated library diversity in the previous sort) to a sterile 1.5 mL tube. Typically, at least 10^7 cells are prepared to enable collection of at least 3,000–5,000 cells.
30. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
31. Resuspend cells in 1 mL of PBSB.
32. Repeat Steps 30 and 31.
33. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
34. Incubate the cells with antigen.
    a. Resuspend ~100–1000 nM antigen (see Note on determining concentrations below) and cell solution in a final volume of 200 μL in a sterile 1.5 mL tube along with a 1:1000 dilution of mouse anti-Myc tag antibody.
    b. Incubate with antigen at room temperature for 3 h with end-over-end mixing.
    c. A control sample containing the cells and all reagents except for the antigen should also be included.

⚠ CRITICAL: It is recommended to sample at least 10-fold coverage of nanobody diversity in the early FACS selections (e.g., rounds 2–3) to sample full diversity obtained from the MACS selections (e.g., round 1).

Note: Reducing the antigen concentration in progressive rounds of sorting allows for the separation and selection of nanobodies with higher affinities.

Note: It is recommended to prepare samples at two different concentrations for sorting. In cases in which enrichment for clones that bind to the antigen is modest, it is recommended that the samples are prepared with the same antigen concentration and a three-fold dilution compared to the previous round. In cases in which enrichment for clones progresses rapidly between rounds, it recommended that the samples are prepared with three-fold and 10-fold reductions in antigen concentration compared to the previous round. Collection of cells from the lower of the two examined antigen concentrations that shows a distinct binding population typically allows for more efficient isolation of nanobodies with increased affinity.

Optional: Add milk to achieve a final concentration of 1% milk. The presence of milk may help in reducing the collection of nanobodies with poor specificity.

35. Post primary incubation, centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
36. Wash the cells once by resuspending them in 1 mL ice-cold PBSB.
37. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
38. Resuspend cells in ice-cold secondary labeling reagents and incubate on ice for 4 min.
    a. Prepare secondary labeling solution in ice-cold PBSB
    b. Add a 1:200 dilution (final concentration of ~10 μg/mL) of goat anti-mouse IgG AlexaFluor 488 for detection of nanobody expression on yeast surface.
    c. For a bivalent antigen (antigen-Fc fusion protein), add a 1:300 dilution (final concentration of ~5 μg/mL) of goat anti-human Fc IgG AlexaFluor 647 for detection of antigen binding.
    d. For a monovalent biotinylated antigen, add a 1:1000 dilution (final concentration of ~2 μg/mL) of streptavidin AlexaFluor 647 for detection of antigen binding.

Note: Nanobody expression and antigen binding can be detected using different fluorophores. For optimal sorting results, it is recommended to choose fluorophores with minimal overlap in their emission spectra.

39. Post-secondary incubation, centrifuge the cells at 16,000xg for 1 min (room temperature) and wash the cells once by resuspending them in 1 mL ice-cold PBSB.
40. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
41. Place cells on ice.
42. Immediately before loading cells into a cell sorter, resuspend the cell pellet in 1 mL ice-cold PBSB.

⚠ CRITICAL: Cells should be resuspended immediately prior to sorting in order to prevent dissociation of nanobody-antigen binding. Keep cells and PBSB on ice for use in sorting.

43. Sort the cells on a cell sorter, such as a Beckman Coulter MoFlo Astrios, under sterile conditions.
44. Examine a plot of the forward scatter (FSC) and side scatter (SSC) of the yeast cells using the 488 nm laser.
45. Adjust the FSC and SSC voltages such that the population of cells spread out in a diagonal line (See Figure 3).
46. Draw a gate surrounding the main population of cells in the plot of FSC versus SSC such that events in the bottom left-hand corner (dead cells and debris) are not included.
47. Filter the cells from within the gate on the FSC versus SSC plot into a subplot. This subplot should be used to examine the log area of signal from the fluorophore used to detect nanobody expression on the x-axis and the log area of the signal for the fluorophore used to detect antigen binding on the y-axis.
48. Analyze a control sample to assess nanobody expression and secondary reagent binding. This sample should be labeled with primary and secondary antibodies for detecting nanobody expression as well as secondary reagent for detecting antigen binding.
49. Adjust voltages for lasers exciting the fluorophores for detecting nanobody expression and antigen binding.
    a. Adjust the voltage for the laser used for detecting nanobody expression such that two distinct cell populations are observed (See Figure 3).
        i. One population (left) is cells that do not express nanobodies on their surface.
        ii. The other population (right) is cells that express nanobodies.
        iii. Quadrants may be drawn to guide distinguishing the nanobody non-expressing and expressing cells.
    b. Adjust the voltage for the laser used for detecting antigen binding such that signal from this laser is minimal in the control sample.
        i. Voltage should be adjusted such that the signal is spaced away from the cytogram axis.

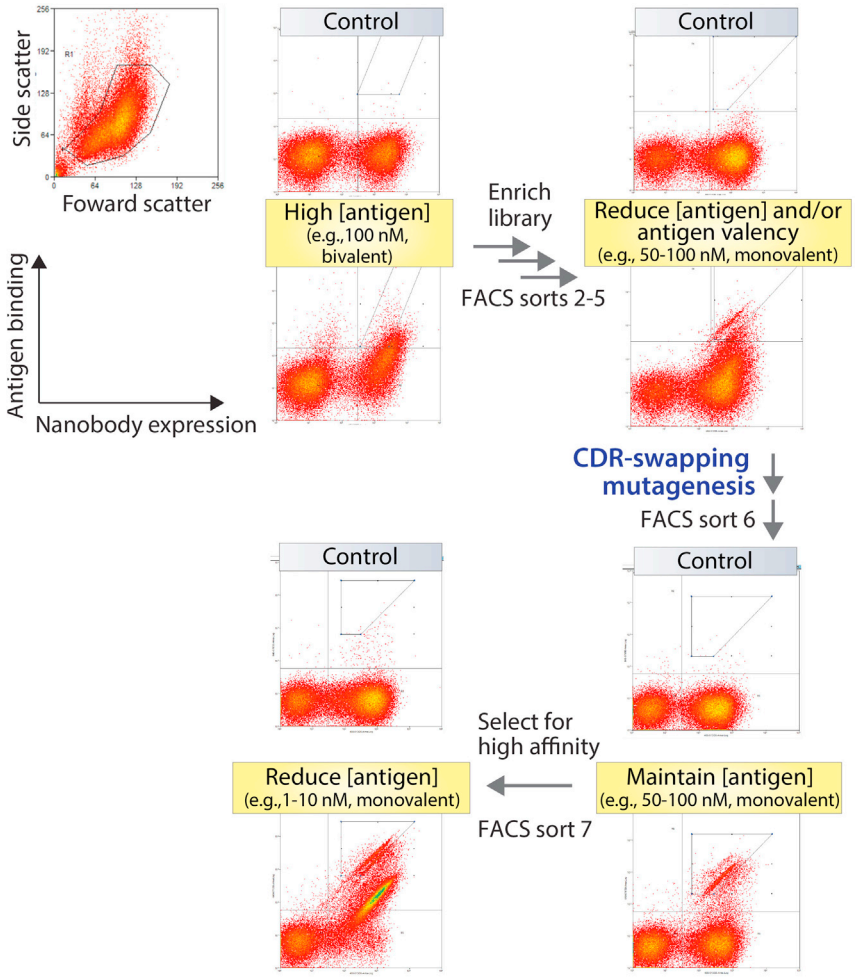

Figure 3. FACS selections incorporating CDR-swapping mutagenesis enable rapid selection of high-affinity nanobodies

The nanobody library is sorted first using MACS (e.g., one MACS sort against monovalent SARS-CoV-2 receptor binding domain, RBD). Next, the enriched library is sorted by FACS against bivalent antigen (e.g., RBD-Fc) at relatively high antigen concentrations, and then it is further sorted by FACS against monovalent antigens (e.g., monovalent RBD) and/or at reduced antigen concentrations. A diagonal gate is drawn during FACS selections to collect yeast cells that bind antigen in a manner that is proportional to nanobody expression to enrich for high-affinity clones. The gates are drawn to minimize the percentage of cells appearing within the gates in the control sample to avoid enrichment of nanobodies that bind the secondary reagents. After several rounds of enriching the library via FACS, yeast plasmid DNA is isolated and CDR-swapping mutagenesis is performed. Finally, additional FACS sorts are performed following CDR-swapping mutagenesis to isolate high-affinity nanobodies.

ii. Voltage and quadrants should be adjusted such that minimal signal is seen in the upper two quadrants.

50. Analyze positive samples to assess both nanobody expression and antigen binding. Positive samples should include samples in which cells have been incubated with antigen at desired concentrations and labeled with the secondary reagent for detecting bound antigen. These samples should also be labeled with primary and secondary reagents for detecting antibody expression.
    a. Draw a gate for cell collection in the upper right-hand quadrant of the cytogram.
        i. Gate should be drawn sufficiently above the horizontal line dividing the quadrants such that a minimal number of cells fall within this gate in the control sample.
        ii. Gate should be drawn to have a diagonal slope on the right side.

⚠ CRITICAL: Drawing a collection gate which minimizes the number of cells present in the control sample is critical to avoid enrichment of nanobodies that bind to secondary labeling reagents.

51. Sort cells and transfer into an 8 mL SDCAA culture.
    a. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
52. Grow cells in SDCAA culture at 30°C at 225 RPM for 2 d.

Pause point: Cells in SDCAA culture can be stored at 4°C for several weeks. After storing at 4°C, cells should be refreshed in fresh SDCAA for 18–24 h prior to induction in SDGCAA.

Recovering nanobody library plasmids

Timing: 1.5 h

This step isolates the plasmid DNA from the enriched library that will be used as a template for CDR shuffling.

53. Obtain 1 mL of the 8 mL SDCAA culture collected in final FACS sort prior to CDR-swapping mutagenesis.

Note: Remaining cell culture can be stored at 4°C for several weeks. For long-term storage, cells can be frozen at −80°C in a solution of 30% glycerol and 0.67% yeast nitrogen base (without amino acids).

54. Recover nanobody library plasmids from 1 mL of sorted cells using a Zymo Yeast Miniprep Kit according to the manufacturer's protocol (https://files.zymoresearch.com/protocols/_D2004_Zymoprep_Yeast_Plasmid_Miniprep_II.pdf).

PCR amplification and shuffling of nanobody CDR genes

Timing: 2 days

DNA segments encoding individual CDRs and overlapping constant regions of the nanobodies are amplified. DNA-encoding nanobodies, which consist of combinations of the individual CDRs, are then reassembled via overlap PCR.

55. Amplify individual CDR genes (Figure 2) by mixing DNA isolated in Step 54 with Q5 DNA polymerase and associated reagents (Table 1).
    a. Amplify DNA segment including CDR1 (N-terminal homology-framework 1 to framework 2) using Forward primer #1 and Reverse primer #1.

Table 1. Q5 PCR mix for amplification of individual CDRs

Reagent	Final concentration	Amount
5× Q5 buffer	1×	10 μL
dNTPs (10 mM each)	200 μM	1 μL
Forward primer (10 μM)	0.5 μM	2.5 μL
Reverse primer (10 μM)	0.5 μM	2.5 μL
DMSO	3% (v/v)	1.5 μL
DNA isolated from yeast miniprep	Variable	2 μL
DI H_2O	n/a	30 μL
Q5 DNA polymerase (2000 U/mL)	0.02 U/μL	0.5 μL
Total	**n/a**	**50 μL**

b. Amplify DNA segment including CDR2 (framework 2 to framework 3) using Forward primer #2 and Reverse primer #2.
c. Amplify DNA segment including CDR3 (framework 3 to framework 4-C-terminal homology) using Forward primer #3 and Reverse primer #3.

56. Place PCR tubes in PCR block with a heated lid and run PCR at the following conditions.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	30
Annealing	55°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	10 min	1
Hold	4°C	Infinite	

Pause point: PCR products can be stored at 4°C overnight (~12–24 h).

57. Purify the PCR product by separation using a 1% agarose gel followed by cutting the DNA bands at the expected size.
a. Cut band corresponding to ~190–200 bp for PCR amplifying CDR1 for given primers.
b. Cut band corresponding to ~160–170 bp for PCR amplifying CDR2 for given primers.
c. Cut band corresponding to ~200–210 bp for PCR amplifying CDR3 for given primers.

Pause point: Agarose gel pieces containing amplified DNA can be stored at 4°C overnight (~12–24 h).

58. Isolate DNA segments using a Qiagen Gel Extraction kit according to the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-gel-extraction-kit/?catno=28706).

Pause point: Isolated DNA can be stored at −20°C.

59. Mix amplified PCR products corresponding to each CDR gene in equal mass ratio along with terminal primers, Q5 polymerase, and reagents described below (Table 2) in a PCR tube to perform overlap PCR.

Table 2. Q5 PCR mix for overlap PCR

Reagent	Final concentration	Amount
5× Q5 buffer	1×	10 μL
dNTPs (10 mM each)	200 μM	1 μL
Forward primer #1(10 μM)	0.5 μM	2.5 μL
Reverse primer #3 (10 μM)	0.5 μM	2.5 μL
DMSO	3% (v/v)	1.5 μL
CDR1 PCR product (10 ng/ μL)	0.2 ng/ μL	1 μL
CDR2 PCR product (10 ng/ μL)	0.2 ng/ μL	1 μL
CDR3 PCR product (10 ng/ μL)	0.2 ng/ μL	1 μL
DI H_2O	n/a	29 μL
Q5 DNA polymerase (2000 U/mL)	0.02 U/μL	0.5 μL
Total	**n/a**	**50 μL**

60. Place PCR tube in a PCR block with a heated lid and run PCR at the same conditions as Step 56.

Note: Sufficient replicates (16–32 of the above described 50 μL PCR reactions is usually sufficient) of this overlap PCR reaction should be performed in order to generate 12 μg of insert DNA.

Pause point: PCRs can be stored at 4°C overnight (~12–24 h).

61. Purify the PCR product by separation using a 1% agarose gel followed by cutting the DNA band at the expected size (~520 base pairs for the given primers).
62. Isolate insert DNA using a Qiagen Gel Extraction kit according to the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-gel-extraction-kit/?catno=28706).
63. Incubate vector with *NheI-HF* and *XhoI* restriction enzymes at 37°C overnight (~12–18 h) according to the manufacturer's protocol (https://www.neb.com/protocols/2012/12/07/optimizing-restriction-endonuclease-reactions).

Note: Sufficient DNA should be digested in order to generate 4 μg of digested vector DNA (pCTCON2).

64. Add Quick CIP (calf intestinal alkaline phosphatase) to restriction digest reaction and incubate at 37°C for at least 15 min.
65. Purify the digested vector by separation using a 1% agarose.
66. Isolate digested vector DNA using a Qiagen Gel Extraction kit according to the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-gel-extraction-kit/?catno=28706).

Note: Digested vector DNA should be stored frozen at −20°C until use.

CDR-shuffled nanobody library generation

Timing: 5 days

Plasmid DNA encoding the CDR-shuffled nanobodies is transformed into yeast cells to generate a library for subsequent sorting.

67. Mix 12 μg of insert DNA (from Step 62) with 4 μg linearized plasmid (from Step 66).
68. Ethanol precipitate DNA with Pellet Paint NF Co-precipitant according to the manufacturer's protocol (https://www.emdmillipore.com/US/en/product/Pellet-Paint-NF-Co-Precipitant,EMD_BIO-70748#anchor_USP).
69. Cover ethanol precipitated DNA with aluminum foil and allow to dry completely overnight in a vacuum or chemical fume hood at room temperature.
70. Transform ethanol precipitated DNA into EBY100 yeast cells.

Note: The protocol below is adapted from previously reported yeast library transformation protocols (Benatuil et al., 2010; Desai et al., 2021; Julian et al., 2019; Zupancic et al., 2021).

71. Two days before transforming the library, start a 5 mL culture of EBY100 yeast cells and grow cells overnight (~12–18 h) at 30°C with agitation at 225 RPM.
    a. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
72. One day before transforming the library, seed a 50 mL culture of EBY100 yeast cells from the 5 mL culture started the previous day and grow cells overnight (~12–24 h) at 30°C with agitation at 225 RPM.
    a. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
73. The day that the library will be transformed, inoculate a culture of EBY100 yeast cells from the 50 mL culture started the previous day to achieve a final OD_{600} of 0.3.
    a. Start 50 mL of culture per library to be transformed.
    b. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
    c. Grow cells at 30°C with agitation at 225 RPM.

d. Grow cells until the OD_{600} reaches a value of ~1.6.

Note: The culture should reach the proper OD_{600} value in ~5–6 h.

74. Transfer 50 mL of the EBY100 cells to a 50 mL centrifuge tube.
75. Centrifuge the cells at 2500×*g* for 5 min (4°C) and discard the supernatant.
76. Resuspend the cells in 25 mL sterile ice-cold DI water.
77. Centrifuge the cells at 2500×*g* for 5 min (4°C) and discard the supernatant.
78. Resuspend the cells in 25 mL sterile ice-cold electroporation buffer.
79. Centrifuge the cells at 2500×*g* for 5 min (4°C) and discard the supernatant.
80. Resuspend the cells in 25 mL sterile ice-cold conditioning buffer.
81. Incubate the cells at 30°C with agitation at 225 RPMfor 15 min in a culture flask (baffled or non-baffled).
82. Transfer 25 mL of cells in conditioning buffer to 50 mL centrifuge tubes.
83. Centrifuge the cells at 2500×*g* for 5 min (4°C) and discard the supernatant.
84. Resuspend the cells in 25 mL sterile ice-cold electroporation buffer.
85. Centrifuge the cells at 2500×*g* for 5 min (4°C) and discard the supernatant.
86. Resuspend the cells in ice-cold electroporation buffer to a final volume of 400 μL.

Note: Approximately 100–200 μL of electroporation buffer should be added to achieve this final volume.

87. Resuspend the ethanol precipitated DNA in the prepared cells and electroporation buffer.
88. Transfer the cells to a 2 mm electroporation cuvette and place on ice for 5 min.
89. Electroporate cells using an appropriate electroporator, such as Bio-Rad Gene Pulser Xcell.
    a. Set the voltage to 2500 V.
    b. Set the resistance to 200 Ω.
    c. Set the capacitance to 25 μF.
90. Immediately add 1 mL of a 1:1 mixture of YPD and 1 M sorbitol mixture to the cells.
91. Transfer the cells to a 14 mL culture tube and add 1:1 YPD:sorbitol to achieve a final volume of 8 mL.
92. Incubate the cells at 30°C with agitation at 225 RPMfor 1 h.
93. Centrifuge the cells at 2500×*g* for 5 min (room temperature) and discard the supernatant.
94. Transfer the cells to 200 mL of SDCAA.
    a. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
95. Plate dilutions of the cell culture on yeast dropout plates to evaluate the number of transformants.
96. Grow the cells in SDCAA and dropout plates at 30°C with agitation at 225 RPMfor 2 days.

Pause point: A library in SDCAA culture can be stored at 4°C for several weeks. After storing at 4°C, the library should be refreshed in SDCAA for 18–24 h prior to induction in SDGCAA. For long-term storage, the library may be frozen at −80°C in solution of 30% glycerol and 0.67% yeast nitrogen base (without amino acids).

Fluorescence-activated cell sorting (sorting after CDR shuffling)

Timing: 5 h

FACS selections performed after CDR shuffling enrich the shuffled library to obtain nanobodies with high affinity. Sorts in this stage of the process may substantially decrease the antigen concentration in order to select for high-affinity nanobodies.

97. Induce the CDR-swapped library in 200 mL SDGCAA media.

a. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.

98. Calculate the OD_{600} of the SDGCAA culture.
99. Transfer volume corresponding to 10^7 yeast cells to a sterile 1.5 mL tube.
100. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
101. Resuspend cells in 1 mL PBSB.
102. Repeat Steps 100 and 101.
103. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
104. Incubate the cells with the desired concentration of antigen (see note below).
    a. Resuspend antigen and cell solution in a final volume of 200 μL in a sterile 1.5 mL tube with a 1:1000 dilution of mouse anti-Myc tag antibody.
    b. Incubate with antigen at room temperature for 3 h with end-over-end mixing.
    c. Antigen concentration should be maintained or reduced in progressive rounds of sorting.

Note: For the sort immediately following generation of the CDR-swapped library, antigen concentration should be maintained at the value used in the last selection before library generation.

Note: Subsequent rounds of sorting should further decrease the antigen concentration in order to select for high-affinity nanobodies. Preparation of samples with antigen at two distinct concentrations is recommended (as described above) because round-to-round enrichment is difficult to predict. Collection of cells from the lower of the two examined antigen concentrations typically allows for more efficient collection nanobodies with increased affinity.

105. Post primary incubation, centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min and discard supernatant.
106. Wash the cells once by resuspending them in 1 mL ice-cold PBSB.
107. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
108. Resuspend cells in ice-cold secondary labeling reagents and incubate on ice for 4 min.
    a. Prepare secondary labeling solution in ice-cold PBSB
    b. Add a 1:200 dilution (final concentration of ~10 μg/mL) of goat anti-mouse IgG AlexaFluor 488 for detection of nanobody expression on yeast surface.
    c. For a monovalent biotinylated antigen, add a 1:1000 dilution (final concentration of ~2 μg/mL) of streptavidin AlexaFluor 647 for detection of antigen binding.
109. Post-secondary incubation, centrifuge the cells at 16,000xg for 1 min and wash the cells once by resuspending in 1 mL ice-cold PBSB.
110. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
111. Place cells on ice.
112. Immediately before loading cells into a cell sorter, resuspend the cell pellet in 1 mL ice-cold PBSB.
113. Sort the cells as described in Steps 43–52.

Recovering nanobody library plasmids

Timing: 1.5 h

Plasmids encoding nanobodies selected in the final round of sorting are isolated, and the sequences of selected nanobodies are determined.

114. Obtain 1 mL of the 8 mL SDCAA culture collected in the final FACS sort (after CDR shuffling).

115. Recover nanobody library plasmids from 1 mL of sorted cells using a Zymo Yeast Miniprep Kit according to the manufacturer's protocol (https://files.zymoresearch.com/protocols/_D2004_Zymoprep_Yeast_Plasmid_Miniprep_II.pdf).

Monovalent nanobody affinity analysis on yeast

Timing: 5 h

Monovalent nanobody affinities are determined for individual nanobodies displayed on the surface of yeast. Yeast cells displaying nanobodies selected from the final round of sorting are incubated with a range of antigen concentrations, and binding signals are measured using flow cytometry. Binding curves can then be fit to the data in order to calculate dissociation constants for monovalent nanobodies.

116. Start a 5 mL culture of EBY100 cells containing individual nanobody plasmids isolated from the terminal selection using CDR-swapping mutagenesis in SDCAA.
    a. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
    b. Incubate the culture at 30°C at 225 RPM for 18–24 h
117. Measure the OD_{600} of the SDCAA culture and calculate the volume of cells from the above culture required to seed a 5 mL SDGCAA culture at a final OD_{600} of 1.
118. Centrifuge the corresponding volume of cells at 2500×*g* for 5 min (4°C) and discard the supernatant.
119. Resuspend the cells in SDGCAA, and move the cells to a 5 mL SDGCAA culture.
    a. Supplement culture with 100 μg/mL ampicillin, 100 μg/mL kanamycin, and 0.01× Pen-strep.
    b. Incubate the culture at 20°C at 225 RPM for 36–48 h
120. Measure the OD_{600} of the SDCAA culture and calculate the volume of cells required to obtain 1×10^5 yeast cells per antigen concentration to be examined.
121. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
122. Resuspend cells in 1 mL PBSB.
123. Repeat Steps 121 and 122 once.
124. Centrifuge the cells in a tabletop centrifuge at 16,000×*g* for 1 min (room temperature) and discard supernatant.
125. Prepare antigen dilutions in PBSB with a final concentration of 1% milk in individual wells of a 96-well plate.
    a. Add 1:1000 mouse anti-Myc antibody.
126. Add 1×10^5 cells to each well containing antigen and incubate at 350 RPM at room temperature for 3 h.
127. Centrifuge the plate containing the cells at 2500×*g* for 5 min (4°C) and discard supernatant.
128. Wash the cells once by resuspending them in 200 μL ice-cold PBSB.
129. Centrifuge the plate containing the cells at 2500×*g* for 5 min (4°C) and discard supernatant.
130. Resuspend cells in ice-cold secondary labeling reagents and incubate on ice for 4 min.
    a. Prepare secondary labeling solution in ice-cold PBSB
    b. Add 1:200 dilution (final concentration of ~10 μg/mL) of goat anti-mouse IgG AlexaFluor 488 for detection of nanobody expression on yeast surface.
    c. For a monovalent biotinylated antigen, add 1:1000 dilution (final concentration of ~2 μg/mL) of streptavidin AlexaFluor 647 for detection of antigen binding.
131. Centrifuge the plate containing the cells at 2500×*g* for 5 min (4°C) and discard supernatant.
132. Wash the cells once by resuspending them in 200 μL ice-cold PBSB.
133. Centrifuge the plate containing the cells at 2500×*g* for 5 min (4°C) and discard supernatant.
134. Resuspend the cells in ice-cold PBSB and immediately analyze by flow cytometry.

135. Analyze the antigen binding signal of yeast cells expressing nanobodies on the surface.

Cloning nanobodies as Fc-fusion proteins

Timing: 3 days

Plasmids are generated for expressing selected nanobodies in a bivalent Fc-fusion format.

136. Prepare nanobody Fc-fusion plasmids in pTT5 vector or other equivalent vectors (e.g., pBIO-CAM5). Amplify nanobody gene by PCR using terminal primers that have NheI and HindIII restriction sites.
137. Purify the insert by running on a 1% agarose gel
    a. Cut bands corresponding to nanobody coding sequences.
    b. Purify DNA using a Qiagen Gel Extraction kit according to the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-gel-extraction-kit/?catno=28706).
138. Digest purified insert and pTT5 vector with NheI-HF and HindIII-HF according to the manufacturer's protocol (https://www.neb.com/protocols/2012/12/07/optimizing-restriction-endonuclease-reactions). For the vector, add Quick CIP calf alkaline phosphatase to the reaction according to the manufacturer's protocol (https://www.neb.com/protocols/2019/06/04/protocol-for-dephosphorylation-of-5-ends-of-dna-using-quick-cip-neb-m0525).
139. Purify the digested insert using a Qiagen PCR Purification kit according to the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-pcr-purification-kit/?catno=28104).
140. Purify the digested vector by running on a 1% agarose gel. Purify DNA using a Qiagen Gel Extraction kit according to the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-gel-extraction-kit/?catno=28706).
141. Ligate digested insert and linearized backbone with T4 ligase according to the manufacturer's protocol (https://www.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202).
142. Transform the ligated plasmids into competent DH5α cells.
143. Plate transformed cells on LB ampicillin plates and grow cells at 37°C for 18–20 h.
144. Pick individual colonies and grow in LB media supplemented with 100 μg/mL ampicillin overnight (~18–24 h) at 37°C.
145. Isolate the plasmids from DH5α using a Qiagen Miniprep kit according to the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiaprep-spin-miniprep-kit/?catno=27106).
146. Confirm plasmid sequence via Sanger sequencing.

Nanobody-Fc fusion protein expression

Timing: 1 week

Nanobody-Fc fusion proteins are expressed via transient transfection in a mammalian cell line.

147. Grow HEK293-6E cells in supplemented F17 media at 37°C with 5% CO_2 and mild agitation (L'Abbé et al., 2018). Maintain and passage at a density of ~1.8–2 million cells/mL.

Note: It is recommended to passage cells no more than 40 times.

148. Transfect cells with 15 μg of sequence confirmed nanobody Fc-fusion
    a. Mix 15 μg plasmid, 45 μg PEI, and 3 mL un-supplemented F17 media and incubate at room temperature for 15–20 min.

b. Add prepared plasmid mixture to cells at a density of ~1.8–2 million cells/mL in culture tubes containing ~25 mL cell culture.

149. Add 750 μL of 20% w/v yeastolate to culture 24–48 h post transfection.
150. Grow cells for another 3–5 d at 37°C with 5% CO_2 and mild agitation.
151. Centrifuge the cell cultures at 3500×*g* for 40 min at 4°C.
152. Transfer the supernatant to a new tube and add ~0.5 mL Protein A agarose beads.
153. Incubate the supernatant and beads at 4°C overnight (~12–24 h) with mild agitation.
154. Capture beads in filter columns under vacuum and wash with ~50 mL of PBS.
155. Incubate beads with 0.1 M glycine (pH 3.0) for 15 min to elute the protein solution and collect it by centrifugation.
156. Buffer exchange the proteins into 20 mM acetate (pH 5.0) using Zeba desalting columns as per manufacturer's protocol (https://assets.fishersci.com/TFS-Assets/LSG/manuals/MAN0015751_2162579_89877-8_89882-3_89889-94_89807-8_Zeba_Desalting_UG.pdf).
157. Filter nanobody Fc-fusions through a 0.22 μm filter, aliquot, and store at −80°C until use.
158. Determine the protein concentrations by measuring the absorbance at 280 nm using a Nanodrop spectrophotometer and the purity using SDS-PAGE.

Nanobody-Fc affinity analysis

Timing: 5 h

Apparent affinities of bivalent nanobodies are determined for individual nanobodies formatted as Fc-fusion proteins. A range of soluble nanobody-Fc fusions are incubated with antigen immobilized on the surface of magnetic beads, and binding signals are measured using flow cytometry. Binding curves can then be fit to the data.

159. Prepare 1 × 10^5 antigen coated beads per sample to be examined.
    a. Transfer beads to a 1.5 mL tube and place tube on DynaMag™-2 Magnet.
    b. Discard the liquid.
    c. Wash the beads by resuspending in 1 mL of PBSB.
    d. Place tube containing beads on the magnet for 2–3 min and discard the liquid.
    e. Repeat sub steps c and d.
    f. Resuspend the beads in 1 mL of 10% milk and mix for 1 h with end-over-end mixing to block the beads.
    g. Place tube containing beads on the magnet and discard the liquid.
    h. Wash the beads by resuspending in 1 mL of PBSB.
    i. Place tube containing beads on the magnet for 2–3 min and discard the liquid.
    j. Resuspend beads in PBSB (typically 0.1–1 mL of PBSB).
160. Thaw an aliquot of nanobody-Fc fusion protein.
161. Centrifuge nanobody-Fc fusion in a tabletop centrifuge at max speed for 5 min. Transfer supernatant to a fresh tube.

Note: Centrifuging nanobody-Fc fusion proteins helps to remove any aggregates generated during each freeze/thaw cycle and produces more consistent results.

162. Measure the A_{280} signal for each nanobody using a Nanodrop to determine the protein concentration.
163. Prepare dilutions of nanobody-Fc fusion proteins in PBSB supplemented with 1% milk at concentrations (e.g., 0.1–1000 nM) desired for analysis.
164. Add 1 × 10^5 antigen-coated beads to antibody dilutions in each well of a 96-well plate.
165. Incubate soluble nanobody-Fc fusions and beads at 350 RPM and room temperature for 3 h in a final volume of 200 μL.
166. Centrifuge the plate containing the beads at 2500×*g* for 5 min (4°C) and discard supernatant.

167. Wash the beads once by resuspending them in 200 μL ice-cold PBSB.
168. Centrifuge the plate containing the beads at 2500×*g* for 5 min (4°C) and discard supernatant.
169. Resuspend the beads in 200 μL ice-cold secondary labeling reagents and incubate on ice for 4 min.
    a. Prepare secondary labeling solution in ice-cold PBSB
    b. Add 1:300 dilution (final concentration of ~5 μg/mL) of goat anti-human IgG AlexaFluor 647 for detection of antigen binding.
170. Centrifuge the plate containing the beads at 2500×*g* for 5 min (4°C) and discard supernatant.
171. Wash the beads once by resuspending them in 200 μL ice-cold PBSB.
172. Centrifuge the plate containing the beads at 2500×*g* for 5 min (4°C) and discard supernatant.
173. Resuspend the beads in ice-cold PBSB and immediately analyze by flow cytometry.
174. Analyze the antigen-binding signal of the singlet population of beads.

EXPECTED OUTCOMES

This protocol for CDR shuffling is expected to lead to the isolation of nanobody variants with increased affinity relative to those generated without the incorporation of CDR-swapping mutagenesis into the selection process. Isolation of high-affinity lead nanobodies from a synthetic library using CDR shuffling is expected to reduce the need for further affinity maturation of selected lead nanobodies using methods such as error-prone PCR to generate sub-libraries for further screening. This mutagenesis strategy has the potential to generate high-quality lead nanobodies that may be directly used in biological studies, such as potently neutralizing SARS-CoV-2 nanobodies (Zupancic et al., 2021).

LIMITATIONS

A limitation of this protocol is that it requires a common framework library, and it has yet to be evaluated if this method may be generalized to synthetic libraries that contain framework diversity or more complex libraries such as non-immune and immune libraries. The translation of this method to libraries with framework diversity would require more complex primer design, such as mixtures of primers or degenerate primers, to account for the diversity in the framework regions used for primer annealing in CDR-swapping mutagenesis.

TROUBLESHOOTING

Problem 1

Library does not show a clear binding population by FACS after one round of MACS (Step 50).

Potential solution

The library may not have been sufficiently enriched for clones that bind the antigen. Further enrichment of the library using a second round of MACS (as described in Steps 7–26) may lead to a clear binding population by FACS. Alternatively, if a bivalent antigen (e.g., antigen-Fc fusion protein) is available, FACS sorting may be performed with this antigen to detect a binding population through higher avidity.

Problem 2

Library shows enrichment of nanobodies binding to secondary labeling reagents by FACS (Step 48).

Potential solution

Selections have likely resulted in the enrichment of nanobodies that bind to secondary labeling reagents. For an antigen that is available with various detection tags (e.g., biotin, epitope tag, Fc region), the use of antigen with different tags in alternating rounds of sorting is encouraged. If antigen with multiple tags is not available or enrichment for nanobodies that bind to secondary reagents can otherwise not be avoided by gating on FACS, a negative selection to remove secondary binders should be performed using control samples without antigen, as described in Step 48. This selection

should collect cells that express nanobodies but do not show interaction with the secondary reagent used to detect antigen binding.

Problem 3

PCR product is not visible on agarose gel used to analyze the overlap PCR product (Step 61).

Potential solution

Ensure that PCR conditions for overlap PCR reactions (set up in Steps 59–60) agree with those described. An equal mass of each CDR product [e.g., 1–10 ng of each CDR (CDR1, CDR2, and CDR3)] should be used. The melting temperature of the primers and the overlapping DNA segments in frameworks 2 and 3 should be within 5°C of each other and greater than the PCR annealing temperature.

Problem 4

Expression of nanobody-Fc fusion proteins results in low yield (Step 158).

Potential solution

The expression conditions may be altered to increase nanobody yield. Nanobody-Fc fusion plasmids should be prepared using codon frequencies for the species of cell which will be utilized for protein expression (e.g., human codon frequencies should be used for HEK293-6E cells). Nanobody-Fc fusion protein expression may also be performed using gene dosing (10% nanobody-Fc plasmid and 90% salmon sperm DNA) for clones which do not express well under standard conditions. Gene dosing may improve the expression of some nanobody-Fc fusion proteins.

Problem 5

Binding curves for analyzing affinity of isolated nanobody-Fc fusion proteins do not saturate (Step 174).

Potential solution

Ensure that appropriate concentrations of both nanobody-Fc fusion protein and antigen are used. A range of nanobody-Fc fusion concentrations should be examined to obtain a sigmoidal binding curve from the signals measured in Step 174. If a sigmoidal binding curve cannot be obtained by adjusting the concentrations of the evaluated nanobody-Fc fusion protein, then antigen-coated beads with either higher or lower loading of antigen may be examined.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peter Tessier (ptessier@umich.edu).

Materials availability

This study generated a new Aga2 display format of the common framework nanobody library reported previously (McMahon et al., 2018). The original library was obtained from Andrew Kruse's lab and requires a Material Transfer Agreement (MTA).

Data and code availability

This study did not generate or analyze any unique datasets or codes.

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AUTHOR CONTRIBUTIONS

J.M.Z., A.A.D., and P.M.T. designed the method and protocols. J.M.Z. and A.A.D. performed the experiments. J.M.Z., A.A.D., and P.M.T. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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