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Protocol

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

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https://doi.org/10.1016/j.xpro.2021.101101

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

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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

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**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 naive 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.
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 \( \sim 58^\circ \text{C} \sim 62^\circ \text{C} \) 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 $1 \times 10^7$ 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 $1 \times$ 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 $1 \times$ 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 ($1 \times$ 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) | Cell Signaling Technology | Cat# 2276S; RRID: AB_331783 |
| Working dilution: 1:1000 | | |
| Goat anti-mouse IgG AlexaFluor 488 | Invitrogen | Cat# A11001; RRID: AB_2534069 |
| Working dilution: 1:200 (~10 µg/mL) | | |
| Goat anti-human IgG AlexaFluor 647 | Jackson ImmunoResearch | Cat#109-605-098; RRID: AB_2337889 |
| Working dilution: 1:300 (~5 µg/mL) | | |
| **Bacterial and virus strains** | | |
| DH5a | Julian et al., 2019 | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Q5 High-Fidelity DNA Polymerase | New England BioLabs | Cat# M0491L |
| Nhel-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 |
| Genetinic (G418 Sulfate) | Thermo Fisher Scientific | Cat# 10131035 |
| Yeastolate | Fisher Scientific | Cat# 292804 |
| Streptavidin AlexaFluor 647 | Invitrogen | Cat# 532357 |
| 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 |
| Tryptophan | 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# 51876 |
| 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# 00002711008733 |
| Polyethyleneimine 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 |
**Materials and Equipment**

### PBSB

| Reagent                    | Final concentration | Amount  |
|----------------------------|---------------------|---------|
| BSA                        | 1 g/L               | 1 g     |
| 10x PBS                    | 1 x                 | 100 mL  |
| DI H2O                     | n/a                 | 900 mL  |
| Total                      | n/a                 | 1 L     |

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     |

(Continued on next page)
**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.

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**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 H2O                                       | 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.

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**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 H2O           | 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.

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**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 H2O              | 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.

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**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 |

(Continued on next page)
Note: Autoclave agar in 800 mL DI H₂O to dissolve. Prepare supplement by dissolving dextrose, yeast nitrogen base (without amino acids), and the tryptophan dropout media in 200 mL DI H₂O. Sterile filter supplement into agar solution once agar solution has cooled sufficiently to pour plates.

Note: Autoclave tryptone, sodium chloride, yeast extract, and agar in 1 L DI H₂O 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 (carbenicillin), are also appropriate. A suitable antibiotic and corresponding concentration should be chosen based on the plasmid being transformed.

**Continued**

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| Tryptophan dropout media | 3.8 g/L             | 3.8 g  |
| DI H₂O                   | 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.

**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 H₂O               | 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.

**Supplemented F17 media**

| Reagent            | Final concentration | Amount |
|--------------------|---------------------|--------|
| F17 media          | n/a                 | 1 L    |
| Glutamine          | 30 mM/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 H₂O         | n/a                 | 1 L    |
| **Total**      | n/a                 | 1 L    |

Store at 4°C until use. Prepare fresh the day of 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 >109 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 (OD600). One OD600 unit is equivalent to approximately 3 × 107 cells/mL.
4. Calculate the volume of cells required to seed 500 mL SDGCAA culture at a final OD600 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 (10⁸–10⁹) in early rounds of sorting. In order to ensure full processing of the entire library diversity, approximately 10-fold

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### Conditioning buffer

| Reagent               | Final concentration | Amount |
|-----------------------|---------------------|--------|
| Lithium acetate       | 0.1 M               | 1.98 g |
| 1,4-Dithiothreitol (DTT) | 10 mM               | 462 mg |
| DI H₂O                | 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 H₂O  | 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.
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 2500×g 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 2500×g 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 \times 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 \times g for 1 min (room temperature) and discard supernatant.

34. Incubate the cells with antigen.
   a. Resuspend \( \sim 100–1000 \text{nM} \) 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.

\( \Delta \) 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 is 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 x 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 x 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 16,000 x g 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 x 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.
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.

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.
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   |
|----------------------------------------|---------------------|----------|
| 5x Q5 buffer                           | 1x                  | 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 H2O                                 | n/a                 | 30 μL    |
| Q5 DNA polymerase (2000 U/μL)          | 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-ma-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 |
| 5X Q5 buffer                        | 1 x                  | 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 H2O                              | n/a                  | 29 μL  |
| Q5 DNA polymerase (2000 U/μL)       | 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.
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<sub>600</sub> 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 RPM for 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 RPM for 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 RPM for 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.
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 16,000×g 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 SDGCAA 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 0.01x Pen-strep.
   b. Incubate the culture at 30°C at 225 RPM for 18–24 h

117. Measure the OD<sub>600</sub> 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<sub>600</sub> of 1.

118. Centrifuge the corresponding volume of cells at 2500 x 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 0.01x Pen-strep.
   b. Incubate the culture at 20°C at 225 RPM for 36–48 h

120. Measure the OD<sub>600</sub> of the SDCAA culture and calculate the volume of cells required to obtain 1 x 10<sup>5</sup> yeast cells per antigen concentration to be examined.

121. Centrifuge the cells in a tabletop centrifuge at 16,000 x 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 x 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.
   b. Prepare secondary labeling solution in ice-cold PBSB.
   c. 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.

126. Add 1 x 10<sup>5</sup> 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 x 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 x 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 x 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 x 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% CO₂ 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% CO₂ and mild agitation.

151. Centrifuge the cell cultures at 3500 x 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 x 10⁵ 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 A₂₈₀ 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 x 10⁵ 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 x 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.

**ACKNOWLEDGMENTS**
We thank Andrew Kruse for providing the nanobody library used in this work. We also thank members of the Tessier lab for their helpful suggestions. This work was supported by the National Institutes of Health (RF1AG059723 and R35GM136300 to P.M.T.), National Science Foundation (CBET...
1159943, 1605266, 1813963 and 1804313 to P.M.T.), Biointerfaces Institute (to P.M.T.), and Albert M. Mattocks Chair (to P.M.T.).

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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