Nanobody Library Selection by MACS

Introduction

We have written the protocol below with the goal of making steps of *in vitro* nanobody selection as clear and broadly applicable as possible, but it is important to recognize that any nanobody discovery effort can have unique requirements. Please view this protocol as a starting point for designing your experiment, rather than the final word on how to do the selection. Variations of this approach have been successful for isolating nanobodies for almost every antigen tested, with the notable exception of unstructured peptides.

The key challenge in any selection experiment is not to avoid "losing" active clones, but rather to efficiently clear out the millions of nanobodies without the desired activity. It is absolutely essential to diligently remove nanobodies that bind to secondary antibodies, microbeads, or protein tags. If you allow the selection process to find a shortcut, it will. Taking these considerations into account, selection with most antigens will yield nanobodies with affinities ranging from 10 nM to 1 µM.

Recipes and catalog numbers listed in appendix for items in 'single quotes'

Library recovery and expansion

1. Place frozen aliquots of yeast nanobody library (NbLib) at 30 °C to thaw, such that recoverable yeast exceed library diversity at least five-fold. For the full naïve library, a total of 2.5×10^9 viable cells are required.
2. Recover yeast in 1 L of 'Yglc4.5 –Trp', shaking at 230 RPM, 30 °C, overnight. You may want to take a small sample of the library cells immediately after resuspension and plate on YPD agar to measure the diversity.
3. Expand to 3 L of medium and allow yeast to grow to stationary phase. Approximately 48 hours, OD is usually between 10 and 30.
4. Measure OD_{600} of yeast to calculate density (OD_{600} of $1 \approx 1.5 \times 10^7$ yeast)
5. Spin down cultures at 3500×g for 5 minutes and resuspend in 'Yglc4.5 –Trp' supplemented with 10% DMSO, such that final density is 10^{10} cells per mL. Aliquot to 2 mL cryovials and freeze in a cell freezing chamber at -80°C (Thermofisher Scientific cat# 5100-0001). Each vial should contain enough viable cells upon recovery to reconstitute a full library.

Induction of nanobody expression

Nanobody expression is under the control of the GAL1 promoter, so nanobodies are only produced on the cell surface when the yeast are grown in galactose-containing medium.

1. Induce nanobody expression of NbLib by dilution of ≥10 x library diversity into –Trp +galactose medium followed by shaking for 48 hours, 25 °C, 220 rpm. Use at least 5×10^9 yeast cells for the inoculum to ensure no clones are lost in passaging.

(We find best expression levels to be between 48 and 72 hours.)

Note about sterility: It is essential to maintain a sterile environment throughout your selection. Unlike standard yeast work, your yeast cells will be passaged multiple times over the course of weeks, increasing the possibility of contamination. We recommend sterile filter tips and freshly sterilized media for every selection. Wipe down your hands, bench, and pipettes with ethanol often during the selection process. A sterile hood could be used as well, but usually a regular lab bench is fine so long as reasonable care is taken.

Nanobody expression test

Before any selection it's a good idea to confirm that nanobody expression was effectively induced. We check this with a quick analytical flow cytometry assay as described below.

1. Measure cell density of induced NbLib yeast (1 $OD_{600} \approx 1.5 \times 10^7$ cells/mL)
2. Add 500 µl of 'selection buffer' to two microcentrifuge tubes
3. Pipette approximately 1×10^6 induced yeast into each microcentrifuge tube and centrifuge for 1 min at 3500 × g, 4 °C
4. Aspirate supernatant and resuspend pellet in 100 µl selection buffer
5. To one tube add ~0.5 µg of anti-HA antibody labeled with AlexaFluor647 or AlexaFluor488 (to check for the percentage of cells expressing nanobody) and 1 µM final concentration of your protein labeled with a spectroscopically distinct dye (to check if your antigen binds nonspecifically to yeast). Leave the other tube unstained as a control
6. Rock both tubes at 4 °C for 15 minutes
7. Spin down cells for 1 minute at 3500 × g, 4 °C
8. Aspirate supernatant and resuspend cells in 500 µL of selection buffer
9. Spin down cells as before and aspirate supernatant
10. Resuspend cells in 100 µl of selection buffer and assess nanobody expression level on flow cytometer using unstained sample to set gates

Note: The maximum number of expressing cells from the naïve library is ~25%. The typical number of expressing cells is ~15-20%. If expression is on the low end (~8-12%) consider increasing the number of cells used for nanobody selection to compensate.

Nanobody Selection

The selection process below is at the heart of identifying clones that bind to your target protein. This protocol describes how to separate antigen binding nanobody clones from non-binders using magnetic cell sorting in two major steps: a "pre-clear" or negative selection step, followed by a positive selection step. These are usually done in series on the same day. The pre-clear step serves to deplete the library of any nanobodies that bind to things like secondary antibodies, magnetic beads, etc. The positive selection step isolates clones that bind to your antigen. After selection, you can expand your enriched cells and repeat the process until binders are highly enriched.

11. Spin down a suitable number of cells to have at least 10-fold over library diversity. For the naïve library (NbLib) you will need 5×10^9 cells. After one or more rounds of selection a smaller number of cells may be used based on estimated diversity. We recommend always using at least 1×10^7 cells, since smaller numbers are difficult to manipulate. Perform centrifugation for 3 minutes at 3500 × g, 4 °C
12. Aspirate media and resuspend yeast via pipetting in 10 mL of chilled selection buffer
13. Spin down yeast for 3 minutes at 3500 × g, 4 °C and aspirate supernatant
14. Resuspend yeast in 4.5 mL of selection buffer
15. Begin preclear to remove off-target binders from the naïve library by adding 500 µl of the 'Miltenyi anti-fluorophore beads' to the resuspended yeast (If using a secondary for selection, add that to the preclearing reaction as well. ~200 nM is typically sufficient to remove secondary binders)
16. Rock/Rotate slowly at 4 °C for 40 minutes
17. Approximately 20 minutes prior to the conclusion of incubation, place an 'LD column' on the Miltenyi MACS magnet. Put a 15 mL sterile falcon under the column and place a 50mL falcon cap over the top of the column to maintain sterility. Add 5 mL of selection buffer to the LD column to allow it to equilibrate before the next step. It will take ~20 minutes to flow through completely
18. Spin down yeast for 3 minutes at 3500 × g at 4 °C and aspirate buffer and unbound beads
19. Resuspend by pipetting in 5 mL of selection buffer.
20. Flow yeast over equilibrated LD column, collect flowthrough in new sterile 15mL centrifuge tube
21. Flush out remaining cells by washing column with an additional 2 mL of selection buffer
22. Remove LD column from magnet and discard
23. Spin down flow through at 3500 × g at 4 °C and aspirate supernatant

24. To resuspended yeast, add 5mL selection buffer containing fluorophore labeled antigen at a 1-1.5 µM concentration (if using a secondary to stain – preincubate the secondary with antigen before staining yeast and use a molar concentration of 2:3 secondary to antigen)
25. Rock or rotate slowly at 4 °C for 1 hour
26. Spin down yeast for 3 minutes at 3500 × g at 4 °C and aspirate supernatant
27. Resuspend yeast in 4.5 mL of selection buffer and 500 µl of anti-fluorophore beads
28. Rock or rotate slowly at 4 °C for 20 minutes
29. Approximately 5 minutes before end of incubation, place an 'LS column' on the Miltenyi MACS magnet. Put a 15 mL sterile falcon tube under the column and place a 50mL cap over the top of the column to maintain sterility. Keep plunger wrapped in package for later use. Add 5 mL of selection buffer to the LS column and allow it to equilibrate before the next step. It will take ~5 minutes to flow through completely
30. Spin down yeast for 3 minutes at 3500 × g at 4 °C and aspirate buffer and unbound beads
31. Resuspend yeast by pipetting in 3 mL of selection buffer and spin down as above
32. Aspirate supernatant and resuspend yeast in 3 mL of selection buffer
33. Remove 50 µl "pre-LS" aliquot of resuspended yeast and set aside for later analysis. Keep aliquot on ice.
34. Flow yeast over the equilibrated LS column and collect flow through in a new sterile 15 mL centrifuge tube
35. Wash column with 8 mL of selection buffer
36. Record approx. volume of flow through and remove 50 µl "flow through" aliquot for later analysis.
37. Remove LS column from magnet and place over fresh sterile 15 mL centrifuge tube
38. Add 5 mL of selection buffer to column and immediately use plunger to elute cells
39. Remove 50 µl "elution" aliquot from eluted cells and set aside for later analysis. Keep aliquot on ice.
40. Spin down elution at 3500 × g for 3 min at 4 °C and aspirate buffer taking care not to disturb the pellet
41. Resuspend yeast in 3 mL of –TRP +glucose in a 14 mL Falcon culture tube
42. Shake at 30 °C for about 24 hours to recover.
43. Use flow cytometry to compare the number of yeast from pre-LS and elution aliquots and use this ratio to get an estimate of the reduction in diversity of the library.

Next steps: After recovering yeast, you can reinduce in galactose medium for another round of selection, and iterate until you isolate binders. It's a good idea to use a different fluorophore for each selection round. We typically perform two MACS selections followed by a FACS sort to isolate binders.

For a FACS sort, you can prepare your yeast by staining with antigen exactly as described above. The details of how to sort will depend on the specific project and sorting equipment being used. Once your selected library has a high proportion of active clones (usually 20% or more of the total cells), you can move on to screening.

For screening, plate sorted yeast on –TRP plates for single clones. Grow single clones in a sterile deep-well 96-well plate, with one clone per well. Then induce expression and stain clones to identify those with the highest activity. Finally, a yeast miniprep or colony PCR can be used to isolate nanobody DNA for analysis and recombinant expression. Note that not every clone with activity on yeast retains activity in recombinant form. A large majority of clones will show activity in both formats, but there are some exceptions.

Appendix

Yglc4.5 –Trp (1 liter)
3.8 g of –Trp drop-out media supplement (US Biological)
6.7 g Yeast Nitrogen Base
10.4 g Sodium Citrate
7.4 g Citric Acid Monohydrate
10 mL Pen-Strep (10,000 units/mL stock)
20 g glucose

Adjust pH to 4.5; Sterifilter (or sterifilter a stock of 20% glucose and add with antibiotic after autoclaving).

–Trp (+glucose or +galactose - 1 liter)
3.8 g –Trp drop-out media supplement (US Biological)
6.7 g Yeast Nitrogen Base
10 mL Pen-Strep (10,000 units/mL stock)
20 g glucose or galactose (glucose for normal growth and galactose for induction of nanobodies)

Adjust pH to 6; Sterifilter (or sterifilter a stock of 20% glucose or galactose and add with antibiotic after autoclaving).

YPAD (1 liter)
20 g Bacto Peptone
20 g Glucose
10 g Yeast Extract
18 mg Adenine
10 mL Pen-Strep (10,000 units/mL stock)

Selection buffer*
20 mM HEPES pH 7.5
150 mM sodium chloride
0.1% (w/v) bovine serum albumin
5 mM maltose

Sterile filter before use.

*Many other buffers will work equally well, depending on your protein you may need to modify this. For GPCRs for instance, we usually add 0.1% (w/v) lauryl maltose neopentyl glycol detergent.
No pH adjustment needed. Sterifilter or autoclave as appropriate.

MACS accessories catalog numbers

Anti-Cy5/Anti-Alexa Fluor 647 MicroBeads	Miltenyi Biotec	Cat# 130-091-395
Anti-FITC MicroBeads	Miltenyi Biotec	Cat# 130-048-701
LD Columns	Miltenyi Biotec	Cat# 130-042-901
LS Columns	Miltenyi Biotec	Cat# 130-042-401