

Chapter 2

Yeast Surface Display of Lamprey Variable Lymphocyte Receptors

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Abstract

The variable lymphocyte receptors (VLRs) of lamprey and hagfish comprise leucine-rich repeat modules, instead of the immunoglobulin-like domain building blocks of antibodies and T-cell receptors in jawed vertebrates. Both types of vertebrate-rearranging antigen receptors are similarly diverse, with repertoires that can potentially exceed 10^{14} unique receptors. In order to characterize antigen-binding properties of the VLRs, we developed a high-throughput yeast surface display platform for the isolation of monoclonal VLRs. We have isolated VLRs that specifically bind hen egg lysozyme, β -galactosidase, cholera toxin subunit B, R-phycoerythrin, and the blood group trisaccharides A and B, with binding affinities in the mid-nanomolar to mid-picomolar range. VLRs may, thus, be excellent single-chain alternatives to Ig-based antibodies for biotechnology applications.

Key words: Lamprey, Variable lymphocyte receptors, Recombinant antibodies, Yeast surface display

1. Introduction

The variable lymphocyte receptors (VLRs) of jawless fish, such as lamprey and hagfish, are the only known rearranging antigen receptors that are built from leucine-rich repeats (LRRs) instead of the immunoglobulin (Ig) superfamily domains that are building blocks of the B- and T-cell receptors of jawed vertebrates from shark to man (1–3). Members of the LRR-containing protein superfamily serve as cardinal microbial recognition molecules in the innate immune systems of plants and animals, for instance the LRR-containing plant Disease Resistance genes, Toll and Toll-like receptors, and the cytoplasmic nucleotide-binding site (NBS)-LRR proteins (4). These innate microbial recognition

molecules have diverged to serve specific functions over very long evolutionary periods, like nearly all other genes in plant and animal genomes. In sharp contrast, in vertebrate lymphocytes, the rearranging antigen receptors are combinatorially assembled from hundreds of gene fragments, resulting in repertoires that can potentially exceed 10^{14} unique receptors (5, 6).

Experimental data indicates that Ig-based antibodies can specifically bind virtually all types of antigens with high affinity. Little is known, however, about the antigen-binding properties of VLRs. Antigen recognition by VLRs from immunized lamprey has been shown for spore coats of anthrax (*Bacillus anthracis*) and their BclA glycoprotein component, for the human blood group trisaccharide antigens, and for hen egg lysozyme (HEL) (5, 7). In order to isolate and characterize VLR binders of specific antigens, we developed a yeast surface display (YSD) platform for the VLRs. Thus far, we have isolated clones that bind HEL, *Escherichia coli* β -galactosidase, cholera toxin subunit B, R-phycoerythrin (RPE), and the blood group trisaccharides A and B, with binding affinities in the mid-nanomolar to mid-picomolar range comparable to high-affinity IgG antibodies with K_D s in the low-nanomolar range (8). These monoclonal VLRs were isolated from libraries originating from immunized lamprey, as well as from nonimmunized animals, indicating that for most antigens there is no need for immunization in order to isolate specific ligand-binding clones (7). VLRs may, thus, be excellent single-chain alternatives to Ig-based antibodies for biotechnology applications, since both of these antigen receptors were optimized over hundreds of millions of years of evolution (9).

The VLR diversity regions consist of sets of LRR modules, each with a highly variable sequence, as shown in Fig. 1. At both ends of the diversity region, there are capping modules, the N-terminal LRR (LRRNT), and the C-terminal LRR (LRRCT),

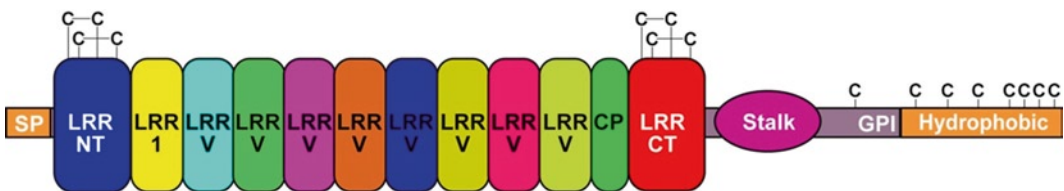


Fig. 1. A stick model of a lamprey mature VLRB. The VLR comprises a set of highly diverse LRR modules capped by disulfide-bonded N-terminal LRR (LRRNT, 24–32 amino acids) and C-terminal LRR (LRRCT, 45–62 amino acids). The 25-residue LRR1 is followed by one to ten 24-residue LRRVs and then a 16-residue truncated LRR, the connecting peptide (CP). The invariant portions of VLRBs include an N-terminal secretory signal peptide (SP) and an 81-residue C terminus that contains a threonine/proline-rich stalk (33 amino acids) and a glycosyl phosphatidylinositol (GPI) membrane anchor motif, which tethers the VLR to the lymphocyte surface. Seven cysteines in the 22-residue hydrophobic C-terminal domain may participate in VLR oligomerization.

which are stabilized by two sets of intramodular disulfide bonds (10, 11). These disulfide bonds are essential for proper folding and stability of the VLR structure. Expression of recombinant VLRs, therefore, requires a eukaryotic host, such as the yeast *Saccharomyces cerevisiae* that possesses an efficient oxidative protein-folding machinery and secretory pathway, and is amenable to high-throughput screens (12, 13). We also noted that for optimal antigen binding, the VLRs require free N-termini. We, therefore, developed a YSD vector based on C-terminal fusion of the VLRs to the yeast surface-anchored flocculation protein Flo1p, as shown in Fig. 2a, b.

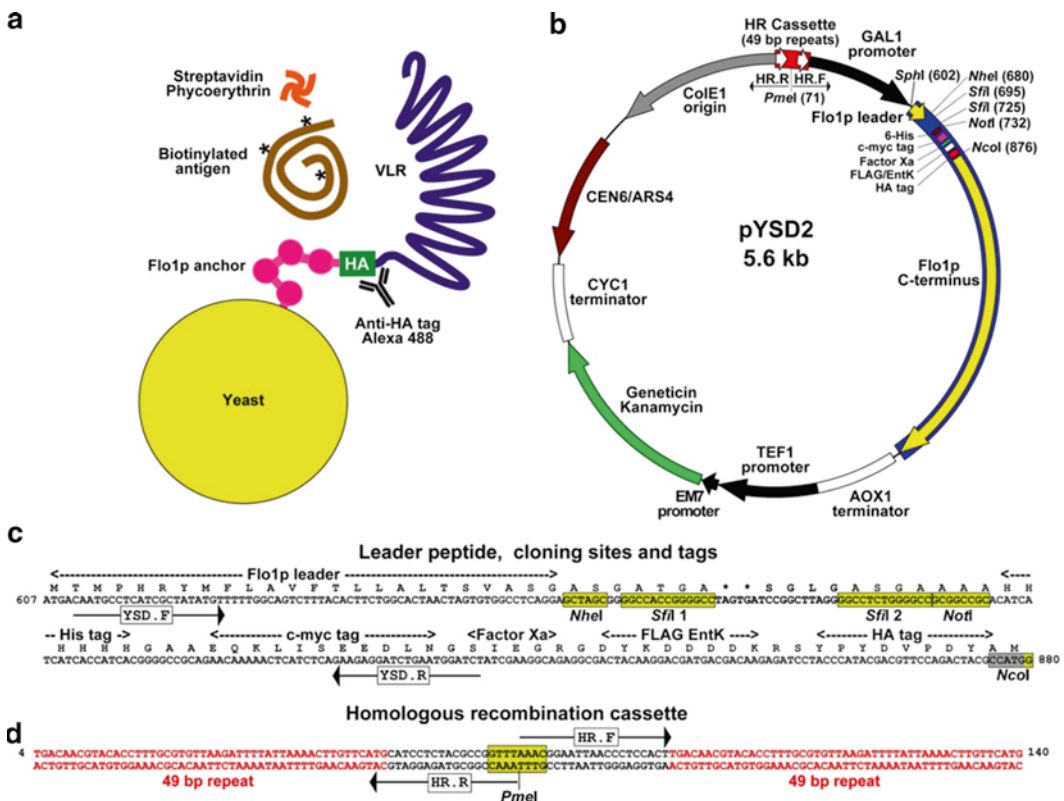


Fig. 2. (a) Yeast surface display of VLRs fused to the C-terminus of the Flo1p anchor. The hemagglutinin (HA)-tag serves for VLR detection via Alexa 488 labeled antibodies. Biotinylated ligands are detected via R-phycoerythrin conjugated to streptavidin (SA-PE). (b) The pYSD2 vector for VLR yeast surface display. The VLRs are expressed under the tightly regulated GAL1 promoter, fused between the authentic Flo1p leader and the yeast Flo1p C-terminus, which includes the surface-anchoring domain. The vector replicates in bacteria and yeast (ColE1, CEN6/ARS4) selected by kanamycin/geneticin resistance. (c) VLRs are cloned directionally between two unique *SfiI* sites. Protein detection and purification tags and the annealing sites for primers are indicated (YSD.F, YSD.R). (d) The homologous recombination cassette consists of two 49-bp direct repeats separated by a linker with a *PmeI* restriction site for plasmid linearization. HR.F, HR.R are primers for rolling-circle amplification across the plasmid.

2. Materials

2.1. Construction of VLR YSD Library

1. The pYSD2 vector is available upon request following MTA.
2. Primers for PCR amplification of VLRA and VLRB (see Note 1). The primers carry overhangs with two unique *Sfi*I sites (underlined). VLRA.F 5-aaaaaaggccaccggggccAAAACGTGTGAAACGGTC; VLRA.R 5-aaaaaaggccccagaggcccccCTCCACGAATGGGCACT; VLRB.F aaaaaaggccaccggggccGCATGTCCTTCGCAGTGT; VLRB.R aaaaaaggccccagaggcccccTGGGCATTTCGAGGGGCT.
3. QIAquick PCR purification kit (QIAGEN).
4. TempliPhi 100 amplification kit (GE Healthcare).
5. Primers for the homologous recombination cassette: HR.F 5-AAACGGAATTAACCCTCCACT, HR.R 5-AAACCGGCGTAGAGGATGCA.
6. dNTPs, 25 mM each (Roche).
7. Yeast inorganic pyrophosphatase, phi29 DNA polymerase, Bovine serum albumin (BSA), and *Pme*I restriction enzyme (all from New England Biolabs).

2.2. Yeast Transformation

1. Yeast strain BJ5464 (ATCC 208288).
2. Bacto Peptone, Bacto Yeast Extract, and Bacto Agar (BD Biosciences).
3. Salmon sperm carrier DNA, MB-grade (Roche).
4. YPD Plus (Zymo Research).
5. Geneticin (G-418 Sulfate, American Bioanalytical).
6. 1 L YPD medium: 20 g Bacto Peptone and 10 g Bacto Yeast Extract. For YPD plates, add 18 g Bacto Agar. Add water to 950 mL and autoclave. Allow to cool to 55°C and add 50 mL of filter-sterilized 40% glucose. When needed, add in YPD medium, G-418 to 100 µg/mL and for plates, add G-418 to 300 µg/mL. Store at room temperature for up to 1 month or at 4°C for up to 6 months.
7. 1 L YPD medium (pH 4.5): YPD, including 10.4 g sodium citrate and 7.4 g citric acid monohydrate.
8. 1 L 2× YPD medium: 40 g Bacto Peptone and 20 g Bacto Yeast Extract. Add water to 900 mL and autoclave. Allow to cool to 55°C and add 100 mL of filter-sterilized 40% glucose.
9. Transformation buffer 1: 0.1 M lithium acetate (LiAc), 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Prepare fresh from the stock of 1 M LiAc (10.2 g of lithium acetate dihydrate in 100 mL water. Autoclave and store at room temperature).

10. Transformation buffer 2: 40% PEG, 0.1 M LiAc, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Prepare fresh from the stocks of 1 M LiAc and 50% PEG (50 g of PEG 3350 in water in 100 mL volume. Stir to dissolve at 70°C on a heating plate. Autoclave and store at room temperature securely capped to prevent evaporation. See Note 2).

2.3. Isolation of Antigen-Specific Monoclonal VLRs

1. 1 L YPG medium: 20 g Bacto Peptone and 10 g Bacto Yeast Extract. Add water to 933 mL and autoclave. Allow to cool to 55°C and add 67 mL of filter-sterilized 30% galactose.
2. Penicillin, 5,000 units per mL and Streptomycin, 5,000 µg per mL (Invitrogen).
3. MidiMACS with LS column (Miltenyi). Can separate 1×10^9 labeled yeast cells from a total of 1×10^{10} cells.
4. MiniMACS with MS column (Miltenyi). Can separate 5×10^7 labeled yeast cells from a total of 5×10^8 cells.
5. MACS buffer: PBS with 0.5% BSA, 2 mM EDTA, 0.1% Tween 20. Filter-sterilize and store at 4°C for up to 6 months.
6. FACS buffer: PBS with 0.5% BSA, 2 mM EDTA. Filter sterilize and store at 4°C for up to 6 months.
7. Anti-biotin microbeads (Miltenyi).
8. Rat anti-HA (high-affinity clone 3F10, Roche) or rat anti-FLAG (Stratagene).
9. Streptavidin R-phycoerythrin (SA-PE) (Invitrogen).
10. Alexa Fluor 488 labeled donkey anti-rat IgG (Invitrogen).

3. Methods

The pYSD2 vector consists of the Flo1p leader peptide connected via a short linker to the cloning sites region, which consists of two unique *Sfi*I sites for directional cloning, followed by a set of tags for protein detection and purification, and then the Flo1p stalk and C-terminal anchor, as shown in Fig. 2b, c. The shuttle vector can propagate both in *E. coli* and yeast, selected for kanamycin or geneticin resistance.

To characterize the natural VLR repertoire, we developed a procedure for efficient library construction that circumvents recombination among the VLR inserts during yeast transformation. In *S. cerevisiae*, linear fragments of double-stranded DNA can efficiently recombine based on homology regions spanning 30–50 bp, and even several bases of identity suffice to initiate recombination events. Thus, co-transformation of yeast with a gapped vector and

molar excess of PCR amplicons of library inserts, which include overhangs homologous to the gap-flanking regions in the vector, result in highly efficient recombination between the vector and amplicons, as well as between related amplicons, reshuffling the genes in the library (14). Library construction by means of gap repair in vivo is about 100-fold more efficient than transformation with an equivalent aliquot of a plasmid library. However, this method produces low-quality VLR libraries perhaps due to the presence of multiple potential recombination sites in VLRs, which result in disrupted open reading frames.

To take advantage of the high efficiency of yeast transformation with linear DNA, we created a cassette for intra-plasmid homologous recombination in the pYSD2 vector, consisting of two 49-bp direct repeats separated by an 8-bp *PmeI* restriction enzyme site, as shown in Fig. 2d. After ligation of inserts into the vector *SfiI* sites, the library is amplified via rolling-circle amplification. The amplified circular library is then linearized by *PmeI* digest and used to transform yeast. The vector is then recircularized in vivo by homologous recombination between the two direct repeats in the pYSD2 plasmid.

3.1. Construction of VLR YSD Library

1. Amplify the diversity regions of lamprey VLRs from lymphocyte cDNA or genomic DNA (see Notes 1 and 3).
2. Digest 500 ng of the pYSD2 vector with *SfiI* restriction enzyme and gel purify the digested plasmid. Digest also 300 ng of the amplicon of VLR diversity regions with *SfiI* and column purify with QIAquick PCR. Set a ligation in 10 μ L volume using 50 ng of the digested vector and 30 ng of the VLRA insert or 25 ng of the VLRB insert (molar ratio of about 5:1 insert to vector). Ligate overnight at 16°C.
3. Use 2 μ L of the ligated library for rolling-circle amplification in 10 μ L reaction of TempliPhi. Incubate for 4 h at 30°C.
4. Add 100 pmol of each of the primers HR.F and HR.R, and then in a PCR cycler, heat for 2 min at 95°C and chill to 4°C. Increase the volume of the reaction to 400 μ L (can be split into two tubes of 200 μ L) adding dNTPs to 1 mM (16 μ L of 25 mM stock), 8 μ L BSA (10 mg/mL), 4 μ L pyrophosphatase (100 units/mL), 40 μ L of the 10 \times buffer, and 8 μ L of phi29 DNA polymerase (10 units/ μ L). Incubate 16 h at 30°C, and then add 100 pmol of the primers HR.F and HR.R and 6 μ L of *PmeI* restriction enzyme (10 units/ μ L). Incubate at 30°C for 3 h, and then at 37°C for 1 h. Finally, heat inactivate the enzymes at 65°C for 20 min. Purify the amplified DNA using two columns of QIAquick PCR. Reapply the flow through to increase the yield, and elute each sample using 100 μ L of the kit elution buffer heated to 70°C. Typical yields are 10–15 μ g of the linearized library ready for transformation.

3.2. Yeast Transformation

1. Inoculate a yeast colony from a freshly streaked plate (see Note 4) into 20 mL YPD medium and grow overnight shaking at 250–300 RPM at 30°C (or longer at room temperature, 20–22°C).
2. Determine the culture cell density using a spectrophotometer. Dilute a sample 1:10 in water (10 μ L culture in 90 μ L water) and prepare a blank similarly (10 μ L YPD medium in 90 μ L water). For optimal results, only use a culture that has reached an OD₅₄₆ between 2 and 4.
3. Dilute 30 OD units of the yeast culture into 200 mL of 2 \times YPD prewarmed to 30°C (OD₅₄₆ of 0.15).
4. Grow the cells at 30°C to an OD₅₄₆ of 0.6 (3–5 h; see Note 5).
5. Prepare Salmon sperm carrier DNA (10 mg/mL stock): Thaw the DNA on ice just before transformation. Aliquot 100 μ L DNA in a tube and boil for 5 min at 100°C. Immediately place the DNA tube in an ice/water bath for 5 min. Repeat the boiling and quenching once more and keep the DNA on ice.
6. Spin the 200 mL culture at 700 $\times g$ for 5 min.
7. Resuspend the pellet in 120 mL of sterile water by vortexing. Pipet up and down if necessary.
8. Spin again at 700 $\times g$ for 5 min.
9. Decant supernatant and resuspend the pellet in 4 mL of Transformation buffer 1.
10. Spin at 700 $\times g$ for 5 min.
11. Decant supernatant and spin briefly again to remove all residual fluid. Resuspend the pellet in 2.4 mL of Transformation buffer 1.
12. Set a 50-mL tube on ice and add 10–15 μ g of the linearized library DNA.
13. Add 80 μ L of the denatured carrier DNA.
14. Add the yeast cells from step 11 and vortex to mix.
15. Add 10 mL of Transformation buffer 2 and vortex for 1 min to thoroughly mix the components.
16. Incubate in a heat block at 30°C for 15 min, and then for 30 min at 30°C shaking at 100 RPM.
17. Remove the tube from the shaker and add 640 μ L DMSO. Immediately mix by gently swirling the tube (at this stage, the cells are becoming fragile).
18. Heat-shock in a heat block at 42°C for 5 min, and then for 20 min in an incubator shaking gently at 50 RPM.
19. Pellet the cells at 700 $\times g$ for 5 min, decant supernatant, and spin briefly again. Remove all residual fluid.

20. Resuspend the pellet in 10 mL YPD Plus by gently pipetting up and down a 10-mL pipette (takes about 5 min to reach a single-cell suspension).
21. Allow the cells to recover for 2 h at 30°C shaking at 150–200 RPM.
22. Pellet the cells at $700 \times g$ for 5 min.
23. Resuspend the cells in 10 mL of YPD (pH 4.5) supplemented with 100 $\mu\text{g}/\text{mL}$ G-418 (citrate buffer at pH 4.5 inhibits the growth of contaminating bacteria, which may be resistant to G-418).
24. Check the titer of the library by plating aliquots on YPD agar plates supplemented with 300 $\mu\text{g}/\text{mL}$ G-418. Typical yields are $5\text{--}50 \times 10^6$ individual clones.
25. Transfer all the transformed cells to a 2-L baffled flask containing 400 mL YPD (pH 4.5) supplemented with 100 $\mu\text{g}/\text{mL}$ G-418. Measure the OD_{546} at the start of culture.
26. Culture for 2 days at 30°C shaking at 250–300 RPM. After that, measure again the OD_{546} to calculate the actual growth of the library. Then, based on the original titer of the library, passage an aliquot representing at least tenfold of the calculated library size in a 2-L baffled flask containing 400 mL YPD (pH 4.5) supplemented with 100 $\mu\text{g}/\text{mL}$ G-418. For strain BJ5464, 1 unit of OD_{546} represents 3×10^7 cells. Repeat the passage once more (during the second and third passages, culture saturation should take less than a day).
27. The library can be stored for up to 1 month at 4°C. After that, passage an aliquot representing at least tenfold of the library size.
28. For long-term storage of the library, prepare frozen aliquots. Culture an aliquot representing at least tenfold of the library size in 100 mL YPD (pH 4.5) supplemented with 100 $\mu\text{g}/\text{mL}$ G-418 at 30°C for 3 days (freezing the cells in stationary phase enhances their survival).
29. Measure the OD_{546} of the culture to estimate cell number.
30. Spin the culture for 10 min at $3,000 \times g$ and decant supernatant.
31. Resuspend the pellet in YPD, 100 $\mu\text{g}/\text{mL}$ G-418, at a final volume of 2.6 mL.
32. Prepare three 2-mL cryogenic tubes. To each tube, add 150 μL of sterile glycerol and 850 μL of the cell suspension (each tube should contain about 10^9 cells).
33. Chill the cells gradually to -80°C . First, place the tubes in a Styrofoam box at -20°C . After 24 h, transfer the box with the tubes to a -80°C freezer.

34. To initiate culture from a frozen aliquot, thaw the cells at room temperature, transfer into 100 mL YPD, 100 µg/mL G-418, and culture at 30°C. Passage the library two to three times in order to dilute the dead cells.

3.3. Isolation of Antigen-Specific Monoclonal VLRs

1. In the morning, start a culture with an aliquot representing at least tenfold of the library size. Inoculate the culture at an OD₅₄₆ between 0.05 and 0.1 in YPD (pH 4.5), 100 µg/mL G-418. Incubate at 30°C with shaking at 250 RPM. The best results are obtained with cultures expanded to an OD₅₄₆ between 1 and 3. If the culture grew beyond OD₅₄₆ of 3, dilute with fresh medium to an OD₅₄₆ of 0.5 and culture at 30°C for about 2 h (doubling time is about 1.5 h at 30°C) to reach OD₅₄₆ of 1, and then proceed to induction of the library.
2. Inoculate the starter cells into prewarmed YPG supplemented with 100 µg/mL G-418 at an OD₅₄₆ of 0.05. Culture overnight at 30°C shaking at 250 RPM. The best results are obtained for induced cultures that reached an OD₅₄₆ between 1 and 2. Library passages and induction can also be done at room temperature (20–22°C), with culture periods of the starter and induction extended to 16–24 h (in YPD, yeast grows nearly twice as fast as in YPG).
3. Spin 1×10^{10} induced cells in a centrifuge at $2,500 \times g$ for 5 min and decant supernatant.
4. At this point, cells are prepared for magnetic separation (see Note 6). Wash the cell pellet with 50 mL of MACS buffer at room temperature, vortex to resuspend.
5. Repeat for a total of three washes. Resuspend the cell pellet in 5-mL MACS buffer.
6. Add biotinylated antigen to a final concentration of 0.5–1 µM (up to ten antigens may be used simultaneously). Rotate for 60 min at room temperature, followed by 10 min incubation on ice.
7. Pellet the cells in a refrigerated centrifuge at 4°C for 5 min at $2,500 \times g$ and decant supernatant.
8. Wash the cell pellet with 50 mL of ice-cold MACS buffer. Repeat for a total of three washes.
9. Resuspend the cell pellet in 5 mL of ice-cold MACS buffer and add 100 µl anti-biotin Microbeads (up to 200 µl anti-biotin Microbeads may be used for maximal enrichment). Rotate the tube for 30 min at 4°C.
10. Pretreat an LS column, loaded onto the magnet, by flowing 3 mL of ice-cold MACS buffer.
11. Pellet the cells at 4°C for 5 min at $2,500 \times g$ and decant supernatant.

12. Resuspend the cell pellet in 50 mL of ice-cold MACS buffer. Vortex to break any cell aggregates.
13. Immediately load 7 mL of the cell suspension onto the column on magnet. After the flow has stopped, briefly remove the column from magnet in order to release captured unlabeled cells, and immediately place it back on the magnet. Add 1 mL of ice-cold MACS buffer and let flow through.
14. Repeat until all cells have been loaded.
15. Wash the column with 3 mL of ice-cold MACS buffer. Make sure the upper loading chamber is washed of all the cells. To elute, remove the column from the magnet and place in a culture tube. Add 7 mL of YPD (pH 4.5) supplemented with 100 $\mu\text{g}/\text{mL}$ G-418 and 1:100 dilution of Pen-Strep (to inhibit contaminating bacterial growth), and use the plunger to push the eluted cell suspension into the tube. Check the titer of eluted cells by plating aliquots.
16. Expand the eluted cell population, and passage an aliquot representing at least tenfold the size of the enriched cell population. Set a culture for induction of the enriched cell population for further enrichment of antigen binders via fluorescence-activated cell sorting (FACS, see Note 7).
17. Pellet the induced cells in a microfuge at full speed (16,000–21,000 $\times g$) for 1 min and carefully aspirate the supernatant.
18. Wash the cells with 1-mL MACS buffer at room temperature. Vortex to resuspend the pellet.
19. Repeat for a total of three washes and resuspend the cells in MACS buffer (see Note 8).
20. Label the cells with 1:1,000 dilution in MACS buffer of rat anti-HA (100 $\mu\text{g}/\text{mL}$ stock) and with the biotinylated antigen at the desired concentration.
21. Rotate the cells at room temperature for 25 min, and then incubate on ice for 5 min.
22. Pellet the cells at full speed for 30 s at 4°C and wash with 1-mL ice-cold MACS buffer. Repeat for a total of three washes.
23. Label the yeast cells with a 1:200 dilution in ice-cold MACS buffer, of Alexa Fluor 488 labeled donkey anti-rat IgG and of SA-PE.
24. Incubate the cells on ice for 15–20 min shielded from light. Mix the tubes once or twice during the incubation.
25. Spin the cells at full speed at 4°C and aspirate supernatant, and then wash with 1 mL ice-cold FACS buffer for a total of three washes (FACS buffer used here, instead of the Tween-20 containing MACS buffer, to prevent distortion in fluid dynamics in the flow cytometer). Spin the cells for the last time, and decant

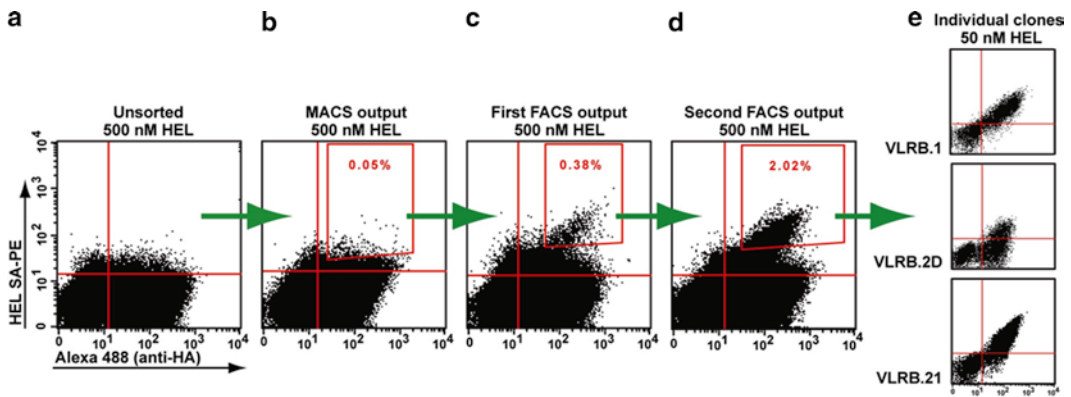


Fig. 3. Isolation of HEL-binding VLRB clones from a YSD library of 1×10^7 clones. Each dot-plot represents 10,000 events. (a) The unsorted library is stained with 500 nM HEL, with no visible antigen binders. (b) After one round of enrichment with anti-biotin magnetic microbeads (MACS), the small population of double-positive cells in the gate was sorted. (c) The output of the first fluorescence-activating cell sorting (FACS) separation shows an enriched population of true binders, (d) which were further enriched during the subsequent sort. (e) Representative individual clones resulting from this screen.

and keep the pellets on ice. Immediately prior to sorting, resuspend the cells in ice-cold FACS buffer at a concentration that is appropriate for your cytometer. Set a gate to sort and collect the population of double-positive cells (see Note 9).

26. Successful enrichment of antigen-binding clones will result in at least three- to fivefold increase in the population of double-positive cells after the second round of MACS, and after each subsequent round of FACS. An example of the process is shown in Fig. 3.

4. Notes

1. A set of primers devoid of overhangs may perform better for the first round of amplification of VLRA from cDNA or genomic DNA. Use these primers first, and then switch to the set listed in Subheading 2.1, item 2: VLRANT.F 5-AAAACGTGTGAAACGGTCACAG; VLRANT.R 5-CTCCACGAATGGGCACTCATA; VLRBNT.F 5-GCATGTCCTCGCAGTGTTTC; and VLRBNT.R 5-TGGGCATTTCGAGGGGCTAG.
2. It is essential to store the 50% PEG solution securely capped to prevent evaporation, which over time will increase the PEG concentration and affect the efficiency of transformation.
3. PCR amplification of VLRA yields substantial amounts of amplicons also from the nonassembled germline VLRA gene, which are invariant and include stop codons in all three

frames. The germline amplicons are shorter than the amplicons of mature VLRA and, in the intervening sequence, include a unique *AvrII* restriction site (TGCGCA). To eliminate the germline amplicons, amplify VLRA amplicons for the minimal number of PCR cycles required to see a product in an agarose gel, digest the PCR product with *AvrII*, and then gel purify the remaining longer band, which corresponds to the mature VLRA amplicons. These can be amplified again to obtain sufficient amount of DNA.

4. A colony from a freshly streaked plate yields the highest transformation efficiency.
5. Optimal transformation efficiencies are achieved only if the majority of cells have undergone two cell divisions. Adjust the inoculum in 2× YPD accurately to an OD₅₄₆ of 0.15 and proceed to the next step only once the culture has reached an OD₅₄₆ of 0.6. This may take 3–5 h or longer.
6. Using a flow cytometer (FACS), it is impractical in most cases to enrich antigen-binding clones from a primary library of 1×10^6 clones or larger, since the fraction of positive clones is usually below 0.01%. One or two rounds of enrichment with magnetic beads (MACS) should increase this fraction to a size that is practical for FACS. We describe here one round of MidiMACS enrichment. To use the MiniMACS, adjust the volumes proportionally.
7. Several cell samples are required every time the FACS is turned on to set the parameters for the cytometer and for color compensation: (1) sample of unstained cells; (2) sample of VLR surface display level (rat anti-HA followed by Alexa Fluor 488 labeled donkey anti-rat IgG); and (3) sample of antigen-binding level (a known control clone can be used, stained with biotinylated antigen followed by SA-PE). For any new antigen, it is recommended to stain uninduced cells with the biotinylated antigen followed by SA-PE to detect nonspecific binding on the surface of yeast. Since binders of the secondary reagents can also be enriched, it is important to frequently stain induced cell population with Alexa Fluor 488-labeled donkey anti-rat IgG, and separately with SA-PE, which should stain <0.01% of the cells. In the case of high background staining, the secondary reagents may be replaced with alternatives.
8. Typical labeling volume is 1 mL for up to 1×10^9 cells and 50–100 μL for $1\text{--}5 \times 10^6$ cells. It is recommended to maintain at least tenfold molar excess of antigen over the yeast-displayed receptors to prevent depletion of the antigen. Assuming $2.5\text{--}10 \times 10^3$ receptors per yeast cell, the receptor concentration for 10^6 cells in 100 μL is about 0.17 nM ($10^4 \times 10^6$), and the lowest antigen concentration is, therefore, 1.7 nM. For lower antigen

concentrations, increase the labeling volume proportionally. For the stage of labeling with secondary reagents, use 0.5 mL volume for 1×10^9 cells and 50–100 μ L for $1\text{--}5 \times 10^6$ cells.

9. Yeast cells grow poorly in liquid culture at concentrations of less than 10^4 cells per mL. When sorting small numbers of cells, use plates instead of liquid culture to recover the cells.

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References

1. Pancer, Z., Amemiya, C. T., Ehrhardt, G. R., Ceitlin, J., Gartland, G. L., and Cooper, M. D. (2004) Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey *Nature* **430**, 174–80.
2. Pancer, Z., Saha, N. R., Kasamatsu, J., Suzuki, T., Amemiya, C. T., Kasahara, M., and Cooper, M. D. (2005) Variable lymphocyte receptors in hagfish *Proc Natl Acad Sci USA* **102**, 9224–9.
3. Litman, G. W., Dishaw, L. J., Cannon, J. P., Haire, R. N., and Rast, J. P. (2007) Alternative mechanisms of immune receptor diversity *Curr Opin Immunol* **19**, 526–34.
4. Pancer, Z., and Cooper, M. D. (2006) The evolution of adaptive immunity *Annu Rev Immunol* **24**, 497–518.
5. Alder, M. N., Rogozin, I. B., Iyer, L. M., Glazko, G. V., Cooper, M. D., and Pancer, Z. (2005) Diversity and function of adaptive immune receptors in a jawless vertebrate *Science* **310**, 1970–3.
6. Rogozin, I. B., Iyer, L. M., Liang, L., Glazko, G. V., Liston, V. G., Pavlov, Y. I., Aravind, L., and Pancer, Z. (2007) Evolution and diversification of lamprey antigen receptors: evidence for involvement of an AID-APOBEC family cytosine deaminase *Nat Immunol* **8**, 647–56.
7. Tasumi, S., Velikovsky, C. A., Xu, G., Gai, S. A., Wittrup, K. D., Flajnik, M. F., Mariuzza, R. A., and Pancer, Z. (2009) High-affinity lamprey VLRA and VLRB monoclonal antibodies *Proc Natl Acad Sci USA* **106**, 12891–96.
8. Marks, J. D., and Bradbury, A. (2004) Selection of human antibodies from phage display libraries *Methods Mol Biol* **248**, 161–76.
9. Binz, H. K., Amstutz, P., and Plückthun, A. (2005) Engineering novel binding proteins from nonimmunoglobulin domains *Nat Biotechnol* **23**, 1257–68.
10. Kim, H. M., Oh, S. C., Lim, K. J., Kasamatsu, J., Heo, J. Y., Park, B. S., Lee, H., Yoo, O. J., Kasahara, M., and Lee, J. O. (2007) Structural diversity of the hagfish variable lymphocyte receptors *J Biol Chem* **282**, 6726–32.
11. Velikovsky, C. A., Deng, L., Tasumi, S., Iyer, M. L., Kerzic, M. C., Aravind, L., Pancer, Z., and Mariuzza, R. A. (2009) Structure of a lamprey variable lymphocyte receptor in complex with a protein antigen *Nat Struct Mol Biol* **16**, 725–30.
12. Chao, G., Lau, W. L., Hackel, B. J., Sazinsky, S. L., Lippow, S. M., and Wittrup, K. D. (2006) Isolating and engineering human antibodies using yeast surface display *Nat Protoc* **1**, 755–68.
13. Gai, S. A., and Wittrup, K. D. (2007) Yeast surface display for protein engineering and characterization *Curr Opin Struct Biol* **17**, 467–473.
14. Swers, J. S., Kellogg, B. A., and Wittrup, K. D. (2004) Shuffled antibody libraries created by *in vivo* homologous recombination and yeast surface display *Nucleic Acids Res* **32**, e36.

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