Alpaca Naïve Single Domain Antibody Library

Introduction

Phage display antibody library technology is one of the most prominent research advances in the field of antibody engineering in recent years. This technology mainly displays antibody molecules on the surface of bacteriophages, uses target antigen molecules to screen bacteriophages expressing specific antibody molecules, and uses genetic engineering methods to express and subsequently identify the functions of antibodies, thereby obtaining functional antibody molecules. According to whether the B cells used for constructing phage display antibody libraries have been immunized, the constructed phage antibody libraries can be divided into immune libraries and non-immune libraries. The B cells used to construct the immune bank are mainly derived from memory B cells that have been actively immunized or infected by pathogens in vivo; If the phage antibody library is constructed from non-sensitized B cells, it is called a non-immune library. Since the diversity of non-sensitized B cells depends on thediversity of B cell clones used to construct the antibody library, the larger the capacity of the non-immune library, the more high affinity antibodies can be screened.

The natural nanoantibody phage library sold by our company was constructed by collecting lymphocytes from 65 non-immunized Alpaca camels. 48 clones were randomly selected for PCR verification, and the positive rate was 100%. The sequencing results showed no sequence duplication, and NGS sequencing showed good diversity. The library capacity was 3 × 10^11, and the abundance exceeded 99.99%.

Reagent kit components

Phage display library 1mL (PBS containing 50% glycerol)

Auxiliary phage: M13K07 1mL (AlpVHHs, P006)

Host bacterium: E. coli TG1 (AlpVHHs, P008)

Antibody detection: Anti M13 Bacteriophage, AlpHcAbs Rabbit antibody(HRP) (AlpVHHs, 052-201-005)

Note: Please read the instructions carefully before use and configure the reagents used for screening

Phage display antibody library vector

This kit is constructed by pComb3XSS, a bacteriophage vector designed to display Fab fragments, ScFV, Polypeptides. pComb3XSS are particularly suitable for displaying nanobodies. By using SfiI single enzyme cleavage, pComb3XSS can be cleaved into two large fragments, 1672bp (SS stuffer) and 3301bp (target fragment of the vector). The preparation of the vector is particularly convenient, and uncut vectors will not be recovered. The screened positive clones can be directly transferred into non inhibitory strains of UAG (Top10F', HB2151, WK6, etc.) for direct expression. The vector contains His tag and HA tag for easy purification and detection.

Vector type: Phagemid vector

Vector length: 4973 bp

Tag: His tag; HA tag

Promoter: LacZ

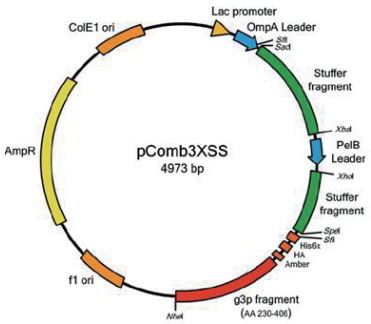
Resistance: Amp+

Vector backbone: pComb3H

Backbone size w/o insert (bp) 3300

OmpA: 5′ -AAG ACA GCT ATC GCG ATT GCA G-3′

gback: 5′ -GCC CCC TTA TTA CGG TTT GCC ATC-3′



Preparation of experimental materials

----1× PBS (0.01 M, pH 7.4): NaCl 8 g, KH2PO4 0.2 g, Na2HPO4·12H2O 2.9 g, KCl 0.2 g, Dilute deionized water to 1000 mL.

----PBST (0.05% Tween-20, v/v): Add 0.5 mL Tween-20 to 1000 mL of 1 × PBS.

----PEG/NaCl: 200 g PEG 8000, 146.1 g NaCl, Dissolve in 800 mL of deionized water, add water to make up to 1 L, sterilize at 121 ℃ for 20 min, and store at room temperature.

----2 × YT liquid culture medium: 16 g of peptone, 10 g of yeast extract, 5 g of sodium chloride, 800 mL of water, adjusted to pH 7.0 with sodium hydroxide, diluted to 1000 mL with water, sterilized at 121 ℃ for 20 min.

----Add 2% glucose to 2 × YT medium.

----Add 2% glucose and 100 μ g/mL ampicillin to 2 × YT medium.

----Add 100 μ g/mL ampicillin and 50 μ g/mL kanamycin to 2 × YT medium.

----2 × YT-A solid culture medium: LB solid culture medium was added with a final concentration of 100 μ g/mL ampicillin, mixed well, and spread on sterilized empty plates at a rate of 20 mL/block

----2 × YT-K solid culture medium: LB solid culture medium was added with a final concentration of 50 μ g/mL kanamycin, mixed well, and spread on sterilized empty plates at a rate of 20 mL/block

Host bacteria description and preparation

1.TG1 is derived from the E. coli K-12 strain and is currently the fastest growing cloning strain of E. coli. Clones can be seen on a plate at 37 ℃ for 7 hours. The main phage display strains can also be used for the construction of common plasmids. The presence of lacIqZ Δ M15 makes it suitable for experiments such as blue and white spot screening; But it does not contain the end A1 mutation of nuclease, and the content of nuclease in the body is high. When extracting plasmids, it is recommended to use the deproteinized solution in the plasmid extraction kit to remove a large amount of nuclease in the body.

2. Activation: Take out TG1 glycerol bacteria and place them on ice, dip a small amount of TG1 glycerol bacteria, and continuously make S-shaped or Z-shaped movements on M9 basic culture medium plate. Invert and culture overnight at 37 ℃. The next day, when inoculated into 2 × YT medium and grown to an OD600 of about 0.6, glycerol was added to a final concentration of 15% and stored at -80 ℃ in a refrigerator.

Note: TG1 is prone to losing flagella during long-term growth in 2YT medium. Activation on M9 basic medium allows TG1 to grow flagella.

Preparation of helper bacteriophage M13K07

Helper phage titer determination

1) Select a single colony TG1 and inoculate it into 5 mL of 2 × YT medium. Shake it on a shaker at 37 ℃ until the logarithmic phase is reached for later use. After ice bath, it can be stored at 4 ℃ for one week;

2) Dilute the Helper bacteriophages to be tested with PBS at a 10 fold gradient. Take 10 μ L of bacteriophages diluted at different gradients and add them to a 1.5 mL centrifuge tube containing 100 μ L TG1 culture. Shake quickly and mix well. Incubate at 37 ℃ for 15 minutes;

3) Coat the incubated mixture onto LB-K plates and invert at 37 ℃ overnight for cultivation; 4) Calculate the number of colonies on a plate with 30-300 plaques, and multiply by the corresponding dilution factor to obtain the titer of the Helper bacteriophage.

Helper phage amplification

1) Select a single colony from the phage titer assay plate and inoculate it into 5 mL of 2 × YT-K, shake and culture at 37 ℃ for 12 hours;

2) Inoculate 2mL of culture into 200 mL of 2 × YT-K and shake overnight (12h-16h) at 30 ℃ and 250 r/min; Collect overnight culture, centrifuge at 12000rpm for 10 minutes, collect supernatant, add 1/5 volume of PEG/NaCl, mix well (at this point, cloudy precipitate can be seen), precipitate at 4 ℃ for at least 60 minutes, preferably overnight;

3) Centrifuge at 12000rpm for 10 minutes, discard the supernatant, resuspend the bacteriophage precipitate in 2 mL PBS, measure the titer of M13K07, and add an equal volume of sterilized glycerol and freeze at -20 ℃ for future use;

4) If you want to expand the amplification system, it is recommended to concentrate it by 200 times.

Affinity screening

1) Dilute the target molecule with PBS to a final concentration of 10 μ g/mL, add it to the enzyme labeled well at a rate of 100 μ L/well, and coat at 4 ℃ for 12 hours (note that each target molecule should be coated in at least 4 wells);

2) Discard the package and wash it three times with PBS. Add 300 μ L of 3% BSA-PBS blocking solution to each well and seal at 37 ℃ for 2 hours;

3) Wash with PBS three times, take 10 μ L of phage library and 400 μ L PBS, then add them to the wells of the enzyme-linked immunosorbent assay plate and incubate at 37 ℃ for 2 hours;

4) Suck out unbound bacteriophages and wash them 5 times with PBST;

5) Add 100 μ L of 0.1M Gly HCl (pH 2.2-2.5, 1mg/mL BSA) eluent to each well, incubate at 37 ℃ for 8-10 minutes, and elute specific bound bacteriophages; Transfer the eluent to a sterile centrifuge tube and quickly neutralize it with 50 μ L of 1M Tris HCl (pH 8.0) buffer;

Note: Before neutralization, test how much neutralization buffer is added first

6) Take 10 μ L for gradient dilution, measure the titer, calculate the recovery rate of elution, and mix the remaining elution products for amplification and purification for the next round of affinity elution.

7) Perform the next round of screening on the library amplification results, changing the screening conditions. The screening conditions for each round are shown in the table below.

Table 1 Affinity selection conditions

Round	Antigencoatingconcentration(ug/ml)	Blocking buffer	Libraryinvestmentamount	Combiningtime(h)	CombinedPBSTwashingtimes
1	10	BSA-PBS	2.0×10^{11}	2	5
2	5	OVA-PBS	2.0×10^{11}	1	10
3	2.5	BSA-PBS	2.0×10^{11}	0.5	5

Amplification of Library after Selection

1) Mix the eluted material with 5 mL of TG1 culture in the early logarithmic growth stage at 37 ℃,

After shaking at 220 r/min for 45 minutes, transfer to 20 mL of 2 × YT-GA liquid medium and shake at 37 ℃ and 220 r/min for 2 hours. Add M13K07 bacteriophage in a cell: phage ratio of 1:20 at 37 ℃, let it stand for 15 minutes, and shake at 220 r/min for 30-45 minutes;

2) Divide the culture into centrifuge tubes, 4 ℃, 1000g, 10 min, resuspend the cell pellet in 25 mL of 2 × YT-AK liquid medium, and shake overnight at 30 ℃ and 250 r/min

3) Centrifuge the overnight culture at 4 ℃ and 12000 r/min for 10 minutes, transfer the supernatant to a new centrifuge tube, add 1/5 volume of PEG NaCl, mix well, and place at 4 ℃ for at least 2 hours;

4) Centrifuge at 4 ℃, 12000 r/min, for 10 minutes, remove the supernatant, resuspend the precipitate in 1 mL PBS, centrifuge at 12000 rpm for 1 minute, take the supernatant and add 1/5 volume of PEG/NaCl, mix well, and place at 4 ℃ for more than 1 hour;

5) Centrifuge at 4 ℃, 12000 r/min, 10 min, Remove the supernatant and suspend the precipitate in 200 μ L PBS to obtain the amplification product. Measure the titer for the next round of selection or analysis.

Titer determination

1) Select a single colony TG1 and inoculate it into 5 mL of 2 × YT medium. Shake the medium at 37 °C until the logarithmic phase is reached for future use. After ice bath, it can be stored for one week at 4 °C

2) Dilute the test bacteriophage with PBS at a 10 fold gradient, take 10 μ L of bacteriophages diluted at different gradients and add them to a microcentrifuge tube containing 100 μ L TG1 culture. Shake quickly and mix well, then incubate at 37 ℃ for 15 minutes

3) Evenly coat the infected bacterial cells onto a 2YT-A plate and culture overnight at 37 ℃;

4) Calculate the number of monoclonal antibodies on 30-300 monoclonal plates, and multiply by the corresponding dilution factor to obtain the

screening titer.

Monoclonal amplification

1) From the plate used to determine the titer of the elution material in the final round (with a colony count of 30-300), 96 single colonies were randomly selected using a sterilized toothpick and inoculated into 1 mL of 2 × YT-GA. The culture was shaken at 37 ℃ and 220 r/min for 12 hours.
2) Inoculate 1% of the vaccine into 2 × YT-GA at 37 ℃ and 220 r/min, and culture until the early stage of logarithmic growth.
3) Add M13K07 bacteriophage in a 1:1 ratio of cell: phage, incubate at 37 ℃ for 15 minutes, and shake at 220 r/min for 30-45 minutes;
4) At 4 ℃, 10000 r/min, centrifuge for 1 minute, resuspend the precipitate in an equal volume of 2 × YT-AK, and vigorously shake at 30 ℃ for 12 hours;
5) Centrifuge the above culture at 10000 rpm at 4 ℃ for 10 minutes, collect the supernatant, and use it for ELISA identification

Identification of positive phage clones

1) Dilute the target molecule to 2 μ g/mL with PBS, add it to the enzyme labeled well at a rate of 100 μ L/well, and coat overnight at 4 ℃;
2) Discard the package and wash it three times with PBST. Add 300-L of 3% skim milk to each well and seal at 37 ℃ for 2 hours;
3) Wash PBST three times, add 50 μ L of phage supernatant and 50 μ L of 3% skim milk powder, incubate at 37 ℃ for 1 hour;
4) Wash PBST 5 times, add Anti-M13 Bacteriophage, AlpHcAbs Rabbit Antibody (HRP) (AlpVHHs, 052-201-005) (diluted with 3% skim milk at a ratio of 1:5000), 100 μ L/well, and incubate at 37 ℃ for 1 hour;
5) Wash the PBST board 6 times. Add the reaction substrate TMB chromogenic solution for color development at 100 μ L/well, 37 ℃ for 10 minutes. Stop the reaction by adding termination solution at 50 μ L/well and measure the optical density at 450 nm. Package M13K07 (1 * 109/well) was used as a positive control, antigen package was added with M13K07 as a negative control, and antigen package was added with PBS as a blank control. Positive cloning criteria: The ratio (S/N) of the OD value (S) of the test sample to the OD value (N) of the negative control is ≥ 2.1.

Sequence analysis of positive phage clones

Sequencing of positive clones using primers gback 5 '- GCC CCC TTA TTA GCG TTT GCC ATC