CaV_HHL^{TM}

Phage Display

Camel naïve V_HH

Antibody Library Kit

User Manual

Cat. No. CaV_HH^{TM}

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I. List of Components

CaV_HHL™: Phage Display Camel Single Domain Antibody Library. ~1.0×10^{12} recombinant phages supplied in 100 µL PBS with 15% glycerol. Complexity=1.5×10^{9} transformants; ampicillin resistant.

E. coli TG1 **host strain:** K12 D(lac-pro), supE, thi, hsdD5/F'[traD36, proAB, lacIq, lacZDM15].

E. coli HB2151 **host strain:** K12 D(lac-pro), ara, nalr, thi/F'[proAB, lacIq, lacZDM15].

M13K07 helper phage: ~1.0×10^{11}pfu phages (~1.0×10^{12} pfu/mL) supplied in 100µL TBS with 15% glycerol. Kanamycin resistant.

The sequencing primers for CaV_HHL™ Camel Single domain Antibody Library:

L1: 5'-TGGAATTGTGAGCGGATAACAATT-3'

S6: 5'-GTAAATGAATTTTCTGTATGAGG-3'

II. Introduction & Library Overview

Principle of phage display

In a phage display library, a variety of peptides, small antibodies [e.g. scFv and Fab] or proteins are displayed on the surface of filamentous phage [M13, fd, and f1 strains] or lambda phage as fusion proteins with one of the coat proteins of the phage virions, while the genetic materials encoding the peptides/proteins are housed within the virion. Using a binding-based process called bio-panning [Figure 1], a small number of phages that display proteins specifically binding to a target of interest can be rescued from a phage library that usually displays a repertoire of many billions of unique peptides/proteins. Finally, the peptides/proteins displayed by the selected phages can be identified by phage amplification and DNA sequencing.

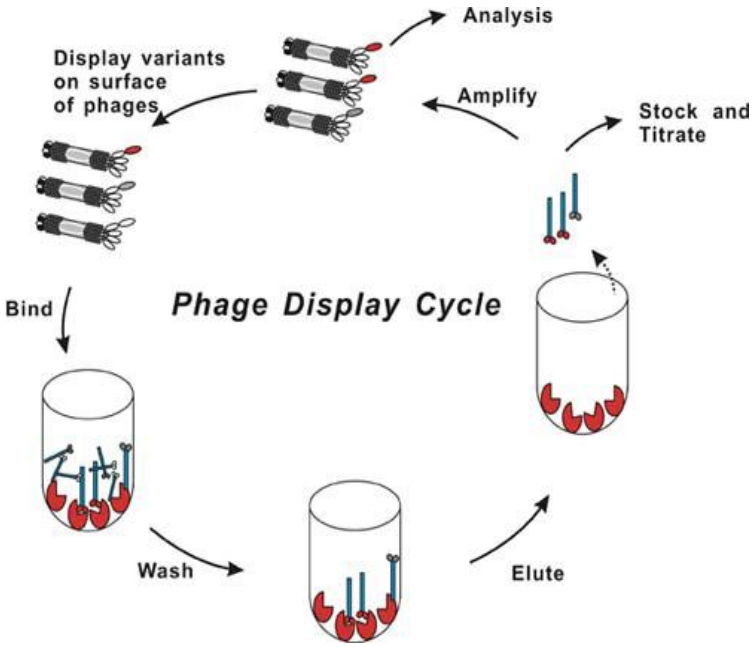


Figure 1. Illustration of phage display cycle

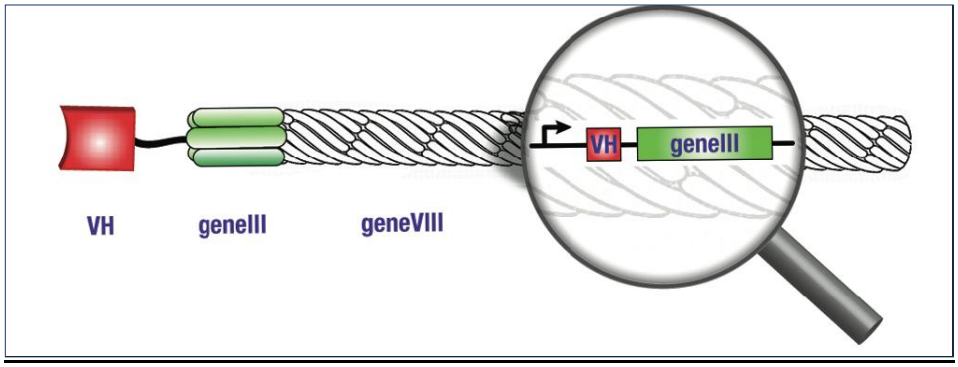


Figure 2. **Phage display of antibody fragments.**
The antibody fragment (such as variable heavy chain, VH, in red) is displayed as a fusion with the terminal phage geneIII protein (green). Both proteins are encoded by the phagmid DNA (magnified) and expressed from a common promoter. The major gene VIII coat protein is shown in white (other phage proteins not shown).

CaV_HHL^{TM} Phage Display Camel Single Domain Antibody Library

CaV_HHL^{TM} Phage Display Camel Single Domain Antibody Library was constructed by collecting antibody gene pools from camel lymphocytes derived from blood samples. In brief, the V_HH encoding genes were inserted into pCDisplay-2^{TM} vector, by which V_HH (single domain variable fragment) antibodies were fused with phage coat protein III and displayed on the surface of phage virions. Using the protocol provided in this manual, specific V_HH binders can be selected very rapidly (generally within two months).

Using the protocol provided in this manual, specific single domain antibody binders can be selected very rapidly (generally within two months). This library is an invaluable antibody resource for isolating camel single domain antibody binders for research, diagnostic and therapeutic applications.

III. Materials and Methods for Using the Library

Materials

CaV_HHL^{TM}: Phage Display Camel Single Domain Antibody Library, product of Creative Biolabs.

***E. coli* TG1 host strain:** From Stratagene TG1 is a suppressor strain, in which the amber stop codon would not be recognized and the single domain antibodies would be fused with phage coat protein III and displayed on the surface of phage virions.

***E. coli* HB2151 host strain:** From GE heathcare. HB2151 is a non-suppressor strain, in which the amber stop codon would be recognized and the soluble single domain antibodies would be produced.

M13K07 helper phage: GE Healthcare.

Media and solution

LB Medium: Per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl. Autoclave 20 minutes at 121°C

LB Plates: LB medium + 15 g/L agarose

Top agar: as for LB medium, but use 7 g/L Bacto-agar. Before use, melt and cool to 50°C

2TY medium: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, made up to 1 L in deionized H_2O, and adjusted to pH 7.0 with 1 *M* NaOH. Autoclave

2TY-G: 2TY containing 1%(w/v) glucose; **2TY-A:** 2TY containing 100ug/mL ampicillin (AMP)

2TY-AG: 2TY containing 100ug/mL ampicillin (AMP) and 1%(w/v) glucose

2TY-AK: 2TY containing 100 ug/mL ampicillin, 50 ug/mL kanamycin

TYE agar plates: Add 15 g agar to 1 L 2TY medium, autoclave, when cool, add glucose to 1% (w/v) and AMP

PBS: Per liter: 8 g NaCl, 0.2 g KCl, 1.7 g Na_2HPO_4, 0.163 g KH_2PO_4, pH to 7.4 with HCl

Blocking buffer: 0.1M $NaHCO_3$ (pH 8.6), 5 mg/mL BSA, 0.02% NaN_3. Filter sterilize, store at 4°C

Coating Buffer: 0.1M $NaHCO_3$ (pH 8.6).

Conjugated second antibody:

HRP-conjugated anti-M13 monoclonal antibody from mouse: GE Healthcare

HRP conjugate of anti-c-Myc Tag: Pierce

EIA/RIA strip well 96-well plates: Corning, USA

Substrate: OPD and H_2O_2 from sigma

5*PEG/NaCl: Dissolve in water: 200 g of **polyethylene glycol-8000,** 150 g of NaCl(2.5M). Bring volume to 1 liter; stir until dissolved. Filter through a 0.8 μm filter

Library Screening

1. Maintenance of Bacterial Strains and Viruses

***E. coli* TG1 host strain:** may be stored at 4 °C for up to two weeks on minimal medium; Prepare glycerol stocks (add sterile 80% glycerol to stationary phase cells, mix well) store at -70°C for long term storage.

***E. coli* HB2151 host strain:** may be stored at 4 °C for up to two weeks on minimal medium; Prepare glycerol stocks (add sterile 80% glycerol to stationary phase cells, mix well) store at -70°C for long-term storage.

M13K07 helper phage: This helper phage carries the kanamycin resistance gene for antibiotic selection. Stocks of the phage can be stored at –80°C in 7% (v/v) DMSO indefinitely, or for up to 6 months at 4°C.

HuSdL^{TM}: can be stored indefinitely at 4°C (in 1% BSA in PBS–0.01% [w/v] NaN3). Libraries should be freshly amplified before panning. Keep plasmid DNA stocks of the primary library and/or glycerol stocks of bacteria containing the phagemid library.

2. Amplification of M13KO7 helper phage

1) Prepare helper phage by infecting log-phase TG1 bacterial cells with M13K07 phage at different dilutions for 30 min at 37°C and plating in top agar onto 2TY plates.

2) Inoculate a small plaque in 3 mL liquid 2TY medium. Add 30 µL overnight culture of TG1 and grow for 2 h at 37°C.

3) Dilute the culture in 1 L 2TY medium and grow for 1 h. Add kanamycin to 50µg/mL and grow for 16 h at 37°C.

4) Remove cells by centrifugation (10 min at 5,000*g*) and precipitate phage from the supernatant by addition of 0.25 vol of phage precipitant. After 30 min incubation on ice, collect the phage particles by centrifugation during 10 min at 5,000*g*. Re-suspend the pellet in 5 mL PBS and sterilize through a 0.22-um filter.

5) Titrate the helper phage by determining the number of plaque-forming units (pfu) on 2TY plates with top-agar layers containing 100 µL TG1 (saturated culture) and various dilutions of phage. Dilute the phage stock solution to 1×10^{13} pfu/mL and store in small aliquots at –20°C.

3. Panning

1) Coat the target antigen (1-10µg/ml) in the panning plates in the coating buffer. Incubate at 37°C for two hours.

2) Block panning plates (NUNC) with the blocking buffer at 4 °C overnight.

3) Wash blocked wells 6 times with 0.1%PBST (PBS with 0.1% Tween 20(V/V))

4) Mix equal volumes of the phage library and 4% PBSM (PBS containing 4% milk) in a total volume of

0.5 mL.

5) During the first round of screening, the number of phage particles should be around 100× greater than the library size (e.g., 10^{12} pfu for a library of 10^{10} clones). Diversity drops to 10^6 after the first round and thus there is no such a requirement in the subsequent rounds of screening. Incubate 30 min at room temperature to block the binding sites.

6) Add input phage mix into panning wells and incubate at room temperature for 60 min.

7) Wash 10-20 times with PBSMT (PBS containing 2% milk and a certain percent of Tween-20).

8) Elute the phage by adding 200 µL either acidic eluting buffer or competitive eluting buffer and incubate 5 min at room temperature. Transfer the supernatant containing the phages to a new tube.

9) Infect a fresh exponentially growing culture of *Escherichia coli* TG1 with the eluted phages and amplify half of them for further rounds of selection.

   a) Inoculate 500 mL 2TY-G with the library glycerol stock and incubate at 37°C with shaking at 250 rpm until the optical density at 600 nm reaches 0.8–0.9.

   b) Add M13KO7 helper phage to a final concentration of 5 ×10^9 pfu/mL, and incubate for 30 min at 37°C without shaking, then for 30 min with gentle shaking (200 rpm), to allow phage infection.

   c) Recover the cells by centrifugation at 2,200*g* for 15 min and re-suspend the pellet in the same volume of 2TY-AK. Incubate overnight at 30°C with rapid shaking (300 rpm).

   d) Pellet the cells by centrifugation at 7,000*g* for 15 min at 4°C and recover the supernatant containing the phage into pre-chilled 1-L bottles.

   e) Add 0.3 vol of phage precipitant. Mix gently and allow the phage to precipitate for 1 h on ice.

   f) Pellet the phage by centrifuging twice at 7,000*g* for 15 min in the same bottle at 4°C. Remove as much of the supernatant as possible and re-suspend the pellet in 8 mL PBS.

   g) Re-centrifuge the phage in smaller tubes at 12,000*g* for 10 min and recover the phage via the supernatant. Ensure that any bacterial pellet that appears is left undisturbed.

   h) Finally, titer phage stocks by infecting TG1 cells with dilutions of phage stock, plating to 2TY-AG, incubation, and enumeration of the numbers of ampicillin resistant colonies that appear. The phages can then be stored in aliquots at 4°C for long periods, ready for screening.

4. Phage ELISA

1) Prepare single clones of antibody-displaying phages

a) Inoculate single clones of the eulate from the 4th round into 5 mL of 2YT-AG medium and incubate at 37°C overnight.

b) Prepare the glycerol stock for each clone with the overnight culture. Inoculate 100µL of overnight culture into 20mL of 2YT-AG medium. Grow for a few hours at 37°C until the optical density at

OD600 reach 0.4-0.5.

c) Add M13KO7 helper phage at a multiplicity of infection of 20 (i.e., the number of phage particles/host cell). Infect the cells by incubating 30 min at 37°C without shaking and another 30 min with shaking.

d) Collect infected cells by centrifugation (10 min at 5,000*g*). Re-suspend in 2YT-AK and grow the culture for 16 h at 30°C.

e) Precipitate phage particles from the supernatant as described above. Re-suspend the phage pellet in 1mL PBS and remove cellular debris by centrifugation (10 min at 5,000*g*).

f) To remove Ab fragments not associated to phage particles, carry out a second precipitation. Re-suspend the phage pellet in 250 µL PBS, clarify again by centrifugation.

2) Phage ELISA by coating the target directly

a) Coat 100 µL of non-biotinylated target (10µg/mL) in coating buffer by incubating at 4°C overnight

b) Shake out the coating solution and wash once with the washing buffer. Block all wells with 250 µL of blocking buffer. In order to test the binding of each selected sequence to BSA-coated plastic surface, enough uncoated wells should also be blocked. Incubate the blocked plates 1-2 hours at 4°C.

c) Shake out the blocking buffer and wash the plate 6 times with the washing buffer.

d) Add 100 µL of phage solution in washing buffer per well. Incubate at room temperature for 1–2 hours.

e) Wash 6 times with the washing buffer. Dilute HRP-conjugated anti-M13 antibody (GE healthcare) 1:5,000 in blocking buffer. Add 100 µL of diluted conjugate per well and incubate at room temperature for 1 hour.

f) Wash 6 times with the washing buffer. Prepare the HRP substrate solution as follows: a stock solution of OPD can be prepared in advance by dissolving 22 mg OPD (Sigma) in 100 mL of 50 mM sodium citrate, pH 4.0. Filter sterilize and store at 4°C. Immediately prior to the detection step, add 36 µL 30% H_2O_2 to 21 mL of OPD stock solution.

g) Add 100 µL of substrate solution per well and incubate at room temperature for 30 minutes.

h) Read plates using a microplate reader set at 490nm.

5. Soluble domain antibody ELISA

Since HB2151 is a non-suppressor strain, in which the amber stop codon between domain antibody gene and gene III can be recognized and the soluble domain antibody can be secreted into the periplasm.

1) Soluble expression in periplasm of HB2151 cells

a) Pick a single colony of HB2151 from a LB plate and grow overnight at 37°C in 2TY.

b) Add 100 µL HB2151 culture to 50 mL 2TY and grow at 37°C (with shaking) to an OD600 of 0.6–0.8.

c) Infect HB2151 with phages of positive clones identified by phage ELISA. Inoculate 1 µL of phages into 200-µL aliquots of the log-phase HB2151 culture. Incubate for 30 min at 37°C (no shaking). Plate onto TYE plates and incubate overnight at 37°C.

d) Inoculate a single colony into 5mL aliquot of 2TY-AG growth medium and grow overnight at 37°C (with gentle shaking).

e) Take a 250 µL aliquot of overnight culture from each well and transfer to 25 mL 2TY growth medium. Grow the cultures at 37°C until OD600 is approx 0.6.

f) Add IPTG to 1mM and continue to grow 20-24h at 30°C.

g) Collect cells via centrifugation at 3,500*g* for 10 min. Release soluble domain antibody by ultra-sonication of cell suspensions in 1/10 volume of PBS

h) Centrifuge at 9,000g for 10 min and collect the supernatant containing soluble domain antibody.

2) Soluble domain antibody ELISA

a) Coat the wells of an ELISA plate with the desired Ag (100 µL, 1-10µg/ mL) in coating buffer.

b) Wash the wells 3× with 200 µL PBS.

c) Block the wells with 200 µL PBSM for 2 h at room temperature.

d) Discard the block solution and add 100 µL of supernatant containing soluble domain antibody. Incubate for 2 h at room temperature.

e) Wash the wells 3× with 200 µL PBST.

f) Add 100 µL of HRP conjugate of anti-c-Myc Tag Ab diluted in PBSM to each well and incubate for 1 h at room temperature

g) Wash the wells 3× with 200 µL PBST.

h) Develop the HRP reaction using OPD and read optical density at 490nM

6. DNA Sequencing

1) Inoculate 2µL glycerol stock TG1 bacterial cells harboring the plasmid for each positive clone (determined by phage ELISA and/or soluble ELISA) into 5mL LB-A medium (LB medium added with 100µg/mL ampicillin). Grow overnight at 37°C with shaking.

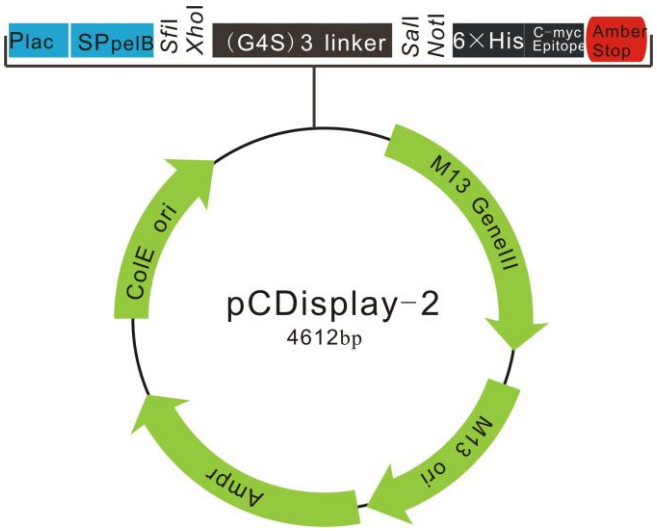
2) Isolate plasmids for each positive clone from bacteria using the commercialized Plasmid Isolation Kit (Such as Qiagen Miniprep kit).

3) Conduct DNA sequencing using “L1” and “S6” as the primers.

IV. Appendix:

1. Schematic map of pCDisplay-2^{TM}

(HuSdL^{TM} was constructed by inserting V$_H$H into pCDisplay-2^{TM})



2. One typical sequence of a single domain antibody:

DNA:

5'-gatgtgcagctggtggagtctgggggaggattagtgcagactggagggtctctgagactctcctgtgccgcctctggatacacacactgtaggtacg
acatggcttggtaccggcagactccagggaaggagcgcgagttcgtctcaagtattggtagggatggtagtacaacctacgcagcctccgtgaagggc
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ggtcggggatggaatgtactggtggtcactggggccaggggacccaggtcactgtctcctca-3'

AA:

CDR1 **CDR2**

DVQLVESGGGLVQTGGSLRLSCAASGYTHCRYDMAWYRQTPGKEREFVSSIGRDGSTTYAASVK

CDR3

GRFTISQDNTNNTVYLLMSSLRPEDTAMYYCKTLWSGMECTGGHWGQGTQVTVSS

References

1. C. F. Barbas III, D. R. Burton, J. K. Scott, and G. J. Silverman, (2001) *Phage Display: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. O'Brien P.M. Aitken R. Totowa, (2001) Antibody phage display: methods and protocols. In**:** Methods in Molecular Biology, Vol 178. Humana Press, NJ.
3. Sachdev S. Sidhu, (2005) Phage Display in Biotechnology and Drug Discovery. CRC Press/Taylor &

Francis Group, Boca Raton.

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