Target Background

Protein Type	Tight junction, tetraspan membrane protein. Key epitope: ECL2.
Family	Claudin family. The CLDN18gene encodes two major splice variants: CLDN18.1 (lung alveolar epithelium-specific) and CLDN18.2 (gastric epithelium-specific).
Expression	Normal: Restricted to gastric mucosa tight junctions. Cancer: Overexpressed & exposed in GC/GEJ (~50%), PDAC (~80%), others.
Function	Maintains paracellular barrier. In cancer: exposed, may support survival.
Clinical Role	A predictive companion diagnostic biomarker for 1L GC/GEJ therapy (HER2-negative).
Therapeutic Modalities	mAbs (ADCC/CDC), ADCs, Bispecifics, CAR-T.

Project Objective & Achievement

Objective	Develop CLDN18.2 -specific VHHs via alpaca immunization
Immunization & Library	Robust immune response after 4 injections; an immune VHH phage display library with a capacity exceeding 10_9 was constructed.
Panning & Screening	Three rounds of biopanning achieved good enrichment. From the enriched pool, 240 clones were picked, resulting in the identification of 37 unique VHH sequences.
Validation	Cell-based assay confirmed that 13 clones specifically recognized CLDN18.2.
Deliverable	A total of 13 unique CLDN18.2-specific nanobodies were discovered.

Milestone Overview

Animal Immunization

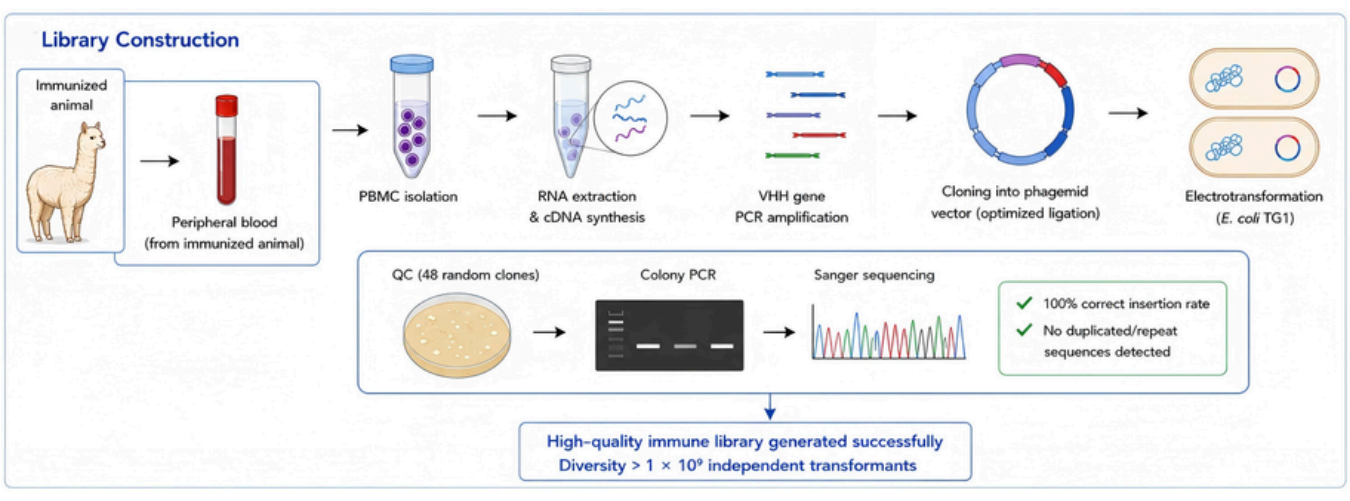
One naïve alpaca was immunized over 49 days with four multi-site subcutaneous injections at two-week intervals using CLDN18.2 OE cell line as immunogen. Serum antibody titers reached 1:1,000 after the third and fourth immunizations, with FACS MFI signals >3-fold higher than those of the negative control serum.

Table 1. Custom-designed immunization strategy

Date	Steps
Day 0	Pre-bleed
Day 0	Primary Injection 2E7 CHOK1-Human CLDN18.2 OE cells with equal volume of Adjuvant Camelid
Day 14	2nd Injection
Day 21	Bleeding and Titration
Day 28	3rd Injection 2E7 CHOK1-Human CLDN18.2 OE cells with equal volume of Adjuvant Camelid
Day 35	Bleeding, Titration and PBMC collection
Day 42	4th Injection 2E7 CHOK1-Human CLDN18.2 OE cells with equal volume of Adjuvant Camelid
Day 49	Bleeding, Titration and PBMC collection

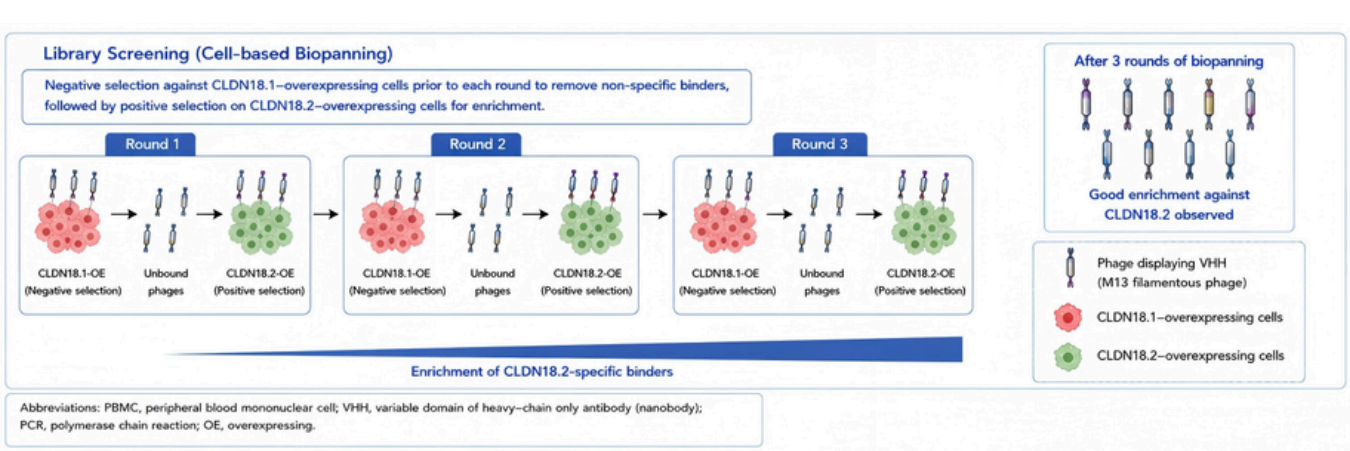
Library Construction

Peripheral blood samples collected after the third and fourth immunizations were used for PBMC isolation, RNA extraction, and cDNA synthesis. VHH repertoires were amplified using camelid-specific primers and cloned into a phagemid vector. Following electroporation into E. coli TG1, a high-quality immune phage display library with a diversity exceeding 1×10^9 independent transformants was generated. QC analysis of 48 random clones confirmed a 100% insert-positive rate with no duplicate sequences were identified.



Library Screening

A cell-based liquid-phase biopanning strategy was employed to isolate CLDN18.2-specific binders. Prior to each selection round, phages were depleted against CLDN18.1-overexpressing cells to remove non-specific and cross-reactive clones, followed by positive selection on CLDN18.2-overexpressing cells. After three rounds of biopanning, robust enrichment of CLDN18.2-specific phage clones was observed.



Binder validation

A total of 180 clones were randomly selected from the third-round output and screened by monoclonal phage ELISA. Positive clones were sequenced, resulting in the identification of 37 unique VHH sequences. Twenty representative clones were reformatted as recombinant antibodies for further characterization, of which 13 clones demonstrated specific binding to CLDN18.2 and exhibited favorable sequence developability profiles.

Table 2. Summary of Anti-CLDN18.2 VHH Evaluation (Representative Clones Shown)

Sample NO.	Length	Molecular weight (Da)	PI(VHH)	Purity		Cell binding		HIC	
				SDS-PAGE%	SEC-HPLC%	EC50-293T 18.2(nM)	293T 18.1(50ug/mL)	RetTime	Area%
BS059-7	123	13.4	7.96	>95	>95	5.39	No Binding	23.518	66.9291
BS059-4	121	13.3	8.6	>95	>95	7.369	No Binding	17.518	25.8483
BS059-6	133	14.34	8.02	>95	>95	8.879	No Binding	26.518	94.5839
BS059-8	123	13.32	6.42	>95	>95	15.88	No Binding	20.053	49.8326
SYSA-1801	N/A	N/A	N.A	>95	>95	5.519	No Binding	14.979	17.1718

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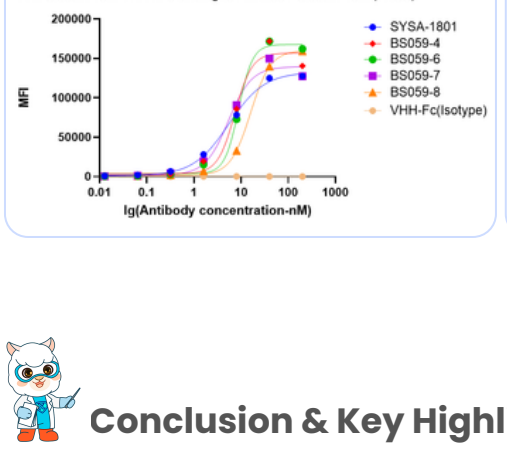

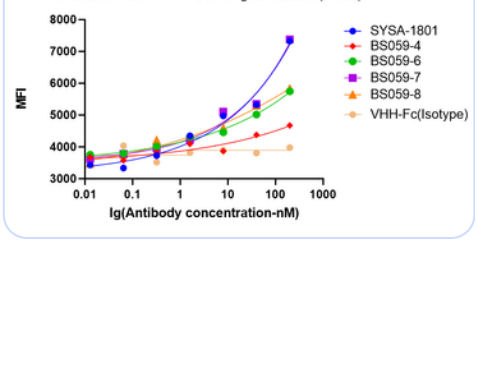

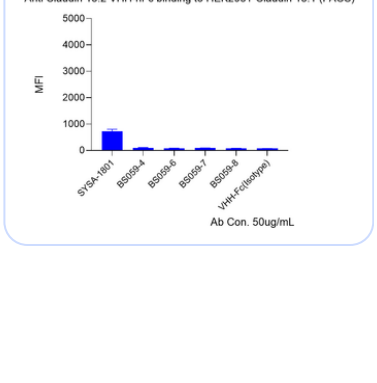

Conclusion & Key Highlights

Cell-Based Immunization

Effective for generating immune libraries against challenging multi-pass membrane proteins.

High-Quality Immune Library

10^9 diversity, 100% insert-positive rate, and no sequence redundancy detected.

High-Fidelity Screening

Cell-based biopanning with counter-selection enabled efficient enrichment of CLDN18.2-specific binders.

Two-Step Validation

Monoclonal phage ELISA and recombinant antibody validation ensured high-confidence hit identification.