

A FACS-microfluidics pipeline for rapid discovery of high-affinity rabbit monoclonal antibodies against hapten molecules

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1 Supplementary Methods

1.1 Sample pretreatment

The detailed preparation of the samples is presented as follows: Milk is without preparation; 3.0 g of chicken was homogenized and mixed with 6 mL of ethyl acetate and hexane in a 50 mL tube. After vortexing for 10 min, the mixture was centrifuged at 5000 rpm for 10 min, 4 mL of supernatant was separated and dried by nitrogen at 60 °C. The residue was dissolved in 3 mL of saturated salt solution, and after filtering through a filter membrane (0.22 µm), the sample solution could be used for analysis.

2 Supplementary Figures

2.1 Schematic structures of the immunogen

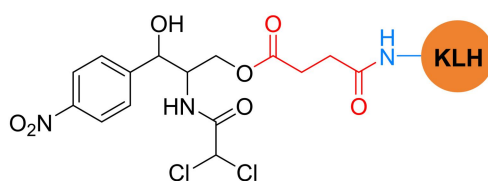


Figure S1. Schematic structures of CAP-HS-KLH. Black denotes the CAP-derived scaffold, while the colored segments highlight the modified hapten and protein-linking regions. CAP, chloramphenicol; KLH, keyhole limpet hemocyanin (orange).

2.2 Characterization of the prepared hapten-protein conjugates

All five prepared hapten-protein conjugates exhibited strong and dilution-dependent binding to the CAP-specific MmAb 5D11 in the iELISA (Figure S2). The recognition of CAP-HS-KLH, CAP-HS-BSA, CAP-OPA-OVA, CAP-N-6AS-OVA, and CAP-NH₂-PTSC-OVA by this antibody confirmed the successful conjugation of the haptens to their respective carrier proteins. These results supported the subsequent use of CAP-HS-KLH as the immunogen, CAP-HS-BSA as the coating antigen for antibody evaluation and as the precursor for fluorescent antigen probe preparation, and the OVA-based conjugates as candidate coating antigens.

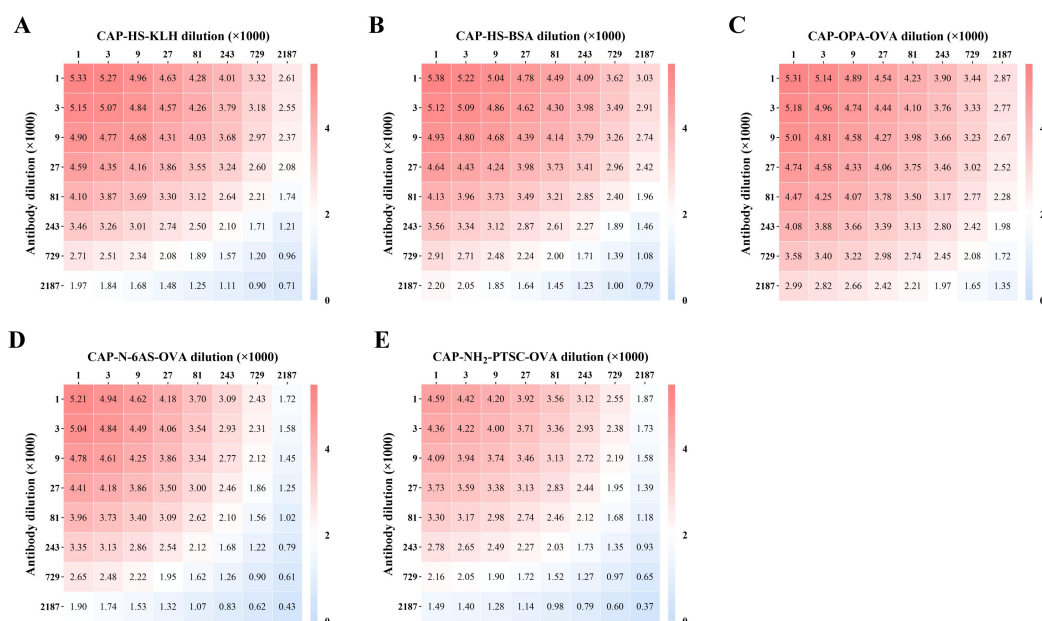


Figure S2. iELISA characterization of the prepared hapten-protein conjugates. The successful conjugation of (A) CAP-HS-KLH, (B) CAP-HS-BSA, (C) CAP-OPA-OVA, (D) CAP-N-6AS-OVA, and (E) CAP-NH₂-PTSC-OVA was confirmed using serial dilutions of each conjugate and a CAP-specific MmAb 5D11 previously generated in our laboratory. Columns and rows indicate the conjugate and antibody dilution factors, respectively. Dilution values are expressed in units of 1000, for example, 1 represents a dilution of 1:1000. The numbers within the heatmaps represent OD₄₅₀ values, with red and blue indicating higher and lower absorbance, respectively.

2.3 Characterization of CAP-HS-BSA and the fluorescent antigen probe

MALDI-TOF MS analysis of CAP-HS-BSA revealed a major molecular-ion peak at m/z 77923 (Figure S3A). The increase in molecular weight relative to native BSA (66430 Da)¹ corresponded to an approximate hapten-to-BSA coupling ratio of 28:1, confirming the successful conjugation of CAP-HS to BSA. FITC-CAP-HS-BSA exhibited a characteristic absorption peak at approximately 497 nm, whereas this peak was absent from unlabeled CAP-HS-BSA, confirming successful FITC labeling (Figure S3B). The fitted competitive inhibition curves obtained with CAP-HS-BSA and FITC-CAP-HS-BSA using the CAP-specific MmAb 5D11 did not differ significantly ($P > 0.05$), with IC_{50} values of 4.79 and 5.43 ng/mL, respectively (Figure S3C). These results indicated that FITC labeling did not substantially affect the immunoreactivity of CAP-HS-BSA toward the CAP-specific MmAb 5D11 and supported the use of FITC-CAP-HS-BSA as a fluorescent antigen probe for subsequent cell staining and FACS-based sorting.

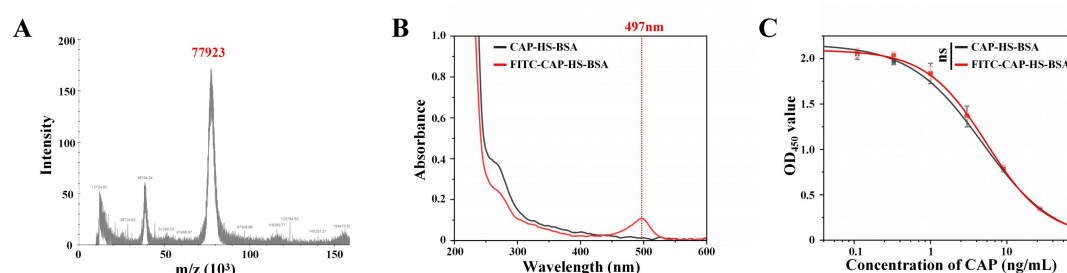


Figure S3. Characterization of CAP-HS-BSA and FITC-CAP-HS-BSA. (A) MALDI-TOF MS spectrum of CAP-HS-BSA, showing a major molecular-ion peak at m/z 77,923. The molecular-weight increase relative to native BSA (66430 Da) corresponded to an approximate hapten-to-BSA coupling ratio of 28:1. (B) UV-visible absorption spectra of CAP-HS-BSA and FITC-CAP-HS-BSA. The characteristic absorption peak at approximately 497 nm confirmed the successful labeling of CAP-HS-BSA with FITC. (C) Competitive inhibition curves for CAP obtained with CAP-HS-BSA and FITC-CAP-HS-BSA using the CAP-specific MmAb 5D11 previously generated in our laboratory. The corresponding IC_{50} values were 4.79 and 5.43 ng/mL, respectively, with no significant difference between the fitted curves ($P > 0.05$). ns, not significant.

2.4 FACS-based sorting antigen-specific memory B cells (MBCs).

Lymphocytes, singlets, and DAPI-negative viable cells were sequentially selected before analysis of IgG and CAP-probe binding (P1, P2, and P3, respectively; Figure S4A-C). IgG⁺ cells accounted for 29.17% of the gated live singlet population. Within the IgG⁺ population (P4, Figure S4D), 1.10% of cells bound FITC-CAP-HS-BSA and were therefore collected as antigen-specific MBCs (P5, Figure S4E).

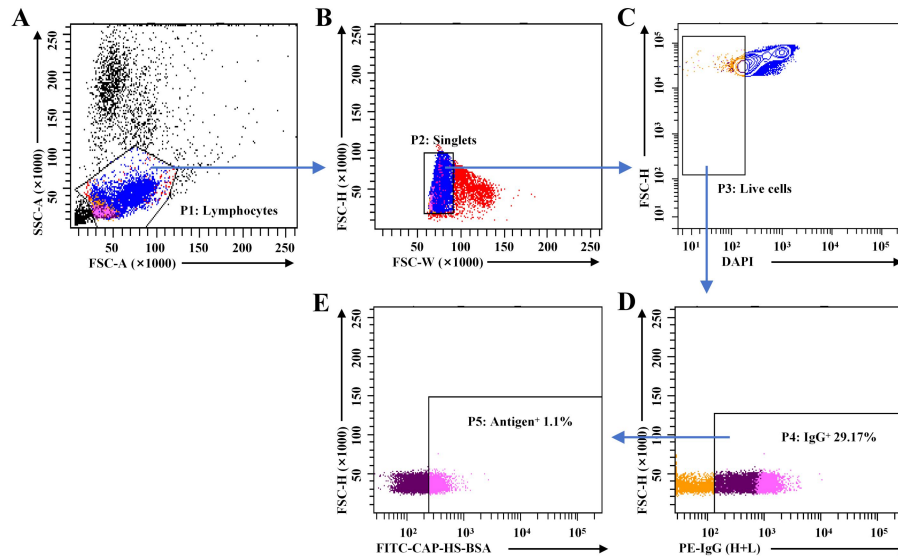


Figure S4. FACS gating strategy for the isolation of antigen-specific rabbit MBCs. (A) Lymphocytes were gated on the basis of forward- and side-scatter characteristics (FSC-A versus SSC-A; P1). (B) Singlets were selected from P1 using FSC-W versus FSC-H (P2). (C) DAPI-negative live cells were selected from P2 (P3). (D) IgG⁺ cells were identified within P3 using PE-conjugated goat anti-rabbit IgG (H+L) antibody (P4; 29.17%). (E) Antigen⁺ cells were identified within P4 using FITC-CAP-HS-BSA (P5; 1.10%) and collected as antigen-specific MBCs. Gates were defined using unstained and single-stained controls. Percentages indicate the proportions of events within the immediately preceding parent gate.

2.5 Expression and purification of full-length RabmAb-4E

Full-length RabmAb-4E was expressed in HEK293 cells and purified from the culture supernatant by Protein A affinity chromatography. Under non-reducing conditions, SDS-PAGE analysis showed a predominant band at approximately 150 kDa, consistent with the expected molecular mass of intact rabbit IgG (Figure S5, lane 2). Under reducing conditions in the presence of DTT, two bands were observed at approximately 50 and 25 kDa, corresponding to the antibody heavy and light chains, respectively (Figure S5, lane 3). Together, these banding patterns confirmed the expected assembly and subunit composition of purified full-length RabmAb-4E.

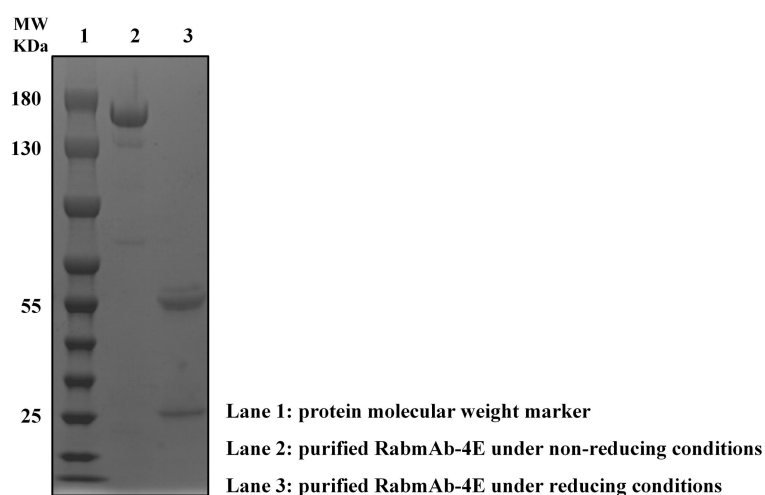


Figure S5. SDS-PAGE analysis of purified full-length RabmAb-4E. Lane 1, protein molecular weight marker; lane 2, purified RabmAb-4E under non-reducing conditions, showing a predominant band at approximately 150 kDa corresponding to intact rabbit IgG; lane 3, purified RabmAb-4E under reducing conditions in the presence of DTT, showing bands at approximately 50 and 25 kDa corresponding to the antibody heavy and light chains, respectively.

2.6 Analytical performance of the RabmAb-4E-based icELISA

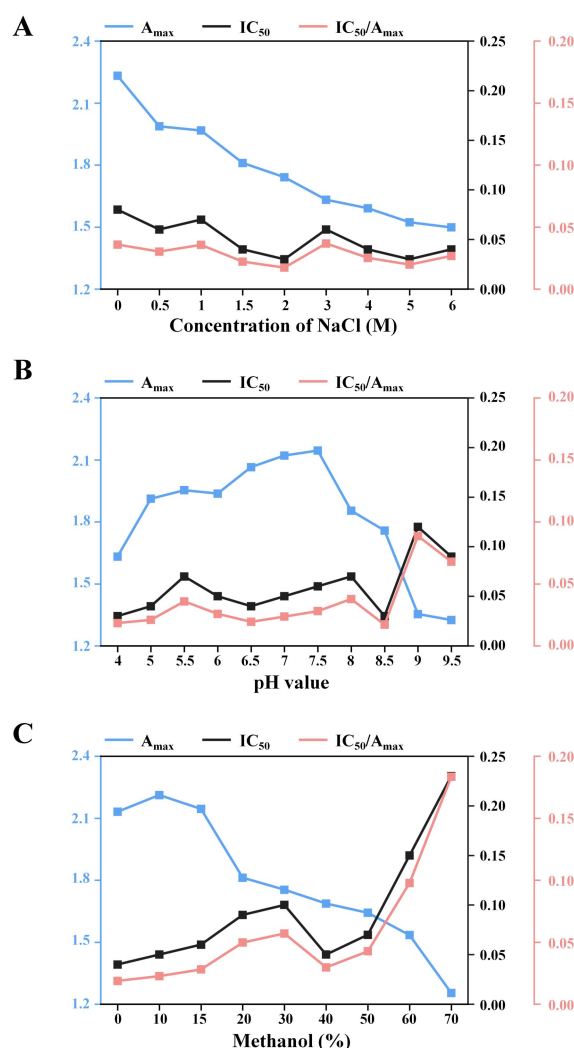


Figure S6. Effects of NaCl concentration, pH, and methanol content on the performance of the RabmAb-4E-based icELISA. (A) Salt tolerance analysis of RabmAb-4E evaluated over a NaCl concentration range of 0-6.0 M. RabmAb-4E maintained stable CAP recognition across 0-6.0 M NaCl, with IC_{50} values ranging from 0.03 to 0.08 ng/mL and IC_{50}/A_{max} ratios from 0.017 to 0.040, indicating exceptionally high ionic strength tolerance. (B) pH tolerance of the assay assessed from pH 4.0 to 9.5. RabmAb-4E retained stable recognition performance between pH 4.0 and 8.5, with IC_{50} values of 0.03-0.07 ng/mL and IC_{50}/A_{max} ratios of 0.017-0.037. At pH 9.0 and above, assay performance declined, as evidenced by decreased A_{max} and increased IC_{50} values. (C) Methanol tolerance evaluated over 0-70% methanol (v/v). The icELISA maintained stable performance in the presence of 0-60% methanol, with IC_{50} values of 0.04-0.15 ng/mL and IC_{50}/A_{max} ratios of 0.019-0.098. At 70% methanol, A_{max} decreased and IC_{50} increased to 0.23 ng/mL, indicating compromised assay performance. Error bars represent the standard deviation of

triplicate measurements. $A_{\max}$, maximum absorbance at 450 nm in the absence of competitor; IC_{50} , half-maximal inhibitory concentration; icELISA, indirect competitive enzyme-linked immunosorbent assay. OD, optical density; RabmAb, rabbit monoclonal antibody.

2.7 Validation of the RabmAb-4E Fv homology model

The predicted Fv structure of RabmAb-4E was evaluated before molecular docking. Ramachandran plot analysis showed that more than 90% of the residues were located in favored regions (Figure S7A), indicating acceptable backbone stereochemistry. Profile-3D analysis showed predominantly positive residue-level Verify scores across the modeled sequence (Figure S7B), supporting the compatibility between the amino acid sequence and its local three-dimensional environments. Together, these results supported the suitability of the predicted Fv model for subsequent molecular docking.

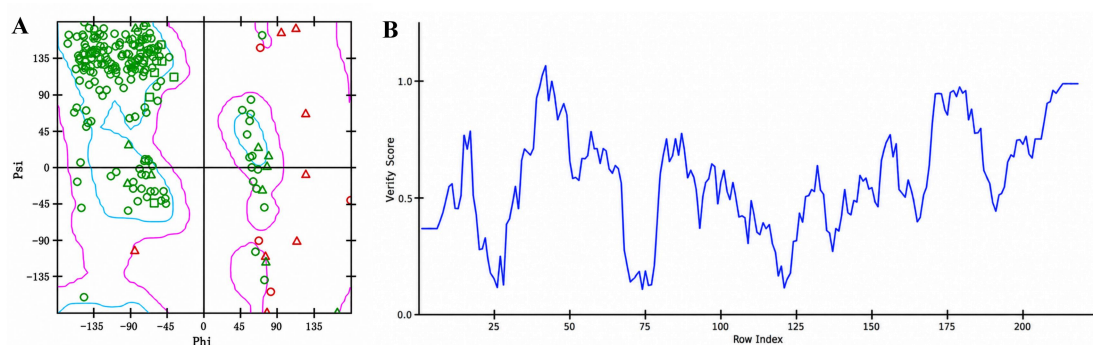


Figure S7. Structural validation of the RabmAb-4E Fv homology model. (A) Ramachandran plot showing the distribution of backbone dihedral angles (ϕ and ψ), with more than 90% of the residues located in favored regions. (B) Profile-3D analysis showing the residue-level Verify scores across the modeled Fv sequence. The predominantly positive Verify scores supported the sequence-structure compatibility of the predicted model.

3 Supplementary Tables

Table S1. IC₅₀ values of antigen-binding scFv clones determined by icELISA.

scFv clone	IC ₅₀ (ng/mL)
scFv-2A	30.92
scFv-2B	79.63
scFv-12B	>1000
scFv-11C	25.04
scFv-12C	31.28
scFv-4E*	4.16
scFv-9E	ND
scFv-4G	>1000
scFv-1H	ND
scFv-6H	29.03

Note: IC₅₀ values were calculated from the competitive inhibition curves obtained by icELISA. “>1000” indicates that 50% inhibition was not reached at the highest CAP concentration tested (1,000 ng/mL). ND, not determined because a reliable concentration-dependent inhibition curve could not be obtained. *Clone selected for full-length RabmAb construction.

Table S2. Optimized concentrations of candidate coating antigens and RabmAb-4E determined by checkerboard titration.

Coating antigen	Coating antigen concentration (ng/mL)	RabmAb-4E concentration (ng/mL)	B ₀	B/B ₀ (%)	IC ₅₀ (ng/mL)
CAP-HS-BSA	8.23	2.74	1.8436	84.67	1.04
CAP-OPA-OVA	8.23	24.69	1.9533	73.84	0.54
CAP-N-6AS-OVA	24.69	8.23	2.1098	13.41	0.03
CAP-NH ₂ -PTSC-OVA	24.69	2.74	1.8746	69.45	0.37

Note: B₀ and B represent the OD₄₅₀ values measured in the absence and presence of 0.1 ng/mL CAP, respectively. B/B₀ (%) was calculated as $(B/B_0) \times 100$. A lower B/B₀ value indicates stronger competitive inhibition.

Table S3. Specificity analysis of RabmAb-4E against CAP and structurally related analogues.

Compound	IC ₅₀ (ng/mL)	CR (%)
CAP	0.01	100
Thiamphenicol	>1000	<0.001
Florfenicol	>1000	<0.001
Florfenicol amine	>1000	<0.001

Note: IC₅₀ values were determined by icELISA. CR (%) = [(IC₅₀ of CAP)/(IC₅₀ of analogues)]×100. An IC₅₀ value of >1000 ng/mL indicates that 50% inhibition was not achieved at the highest concentration tested (1000 ng/mL). CAP, chloramphenicol; CR, cross-reactivity; IC₅₀, half-maximal inhibitory concentration; icELISA, indirect competitive enzyme-linked immunosorbent assay; RabmAb, rabbit monoclonal antibody.

References

- (1) Hirayama, K.; Akashi, S.; Furuya, M.; Fukuhara, K. Rapid Confirmation and Revision of the Primary Structure of Bovine Serum-Albumin by Esims and Frit-Fab Lc Ms. *Biochem. Biophys. Res. Commun.* **1990**, *173* (2), 639–646.