

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

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Abstract: Single B-cell technology has become increasingly important for discovering of rabbit monoclonal antibody (RabmAb) with naturally paired heavy- and light-chain variable-region genes (VH/VL), which offers many advantages for detecting hapten contaminants in food safety testing, clinical diagnostics, and environmental monitoring. However, the efficient recovery of naturally paired VH/VL genes from single B cells remains challenging. To address this challenge, we developed an integrated pipeline that couples fluorescence-activated cell sorting (FACS) with a closed-nanoliter microfluidic system for automated single-cell cDNA preparation, thereby bridging hapten-specific B-cell enrichment and antibody gene recovery. Using chloramphenicol (CAP) as a model hapten, FACS identified antigen-specific memory B cells (MBCs) representing 1.10% of the IgG⁺ population, and the pipeline recovered 22 paired VH/VL sequences, of which 10 were confirmed as antigen-specific, representing a high positive hit rate of 45.5%. The entire process from cell sorting to RabmAb validation was completed within one week, eliminating conventional manual steps of cell lysis, reverse transcription, and sample transfer that are prone to RNA loss and contamination. The lead candidate, RabmAb-4E, demonstrated exceptional analytical performance in an indirect competitive ELISA, with an IC₅₀ of 0.01 ng/mL, negligible cross-reactivity (<0.001%) against structurally related analogues, and robust tolerance to high ionic strength (0-6.0 M NaCl), broad pH range (4.0-8.5), and organic solvent (0-60% methanol). Recoveries from spiked skim milk and chicken samples ranged from 90.28% to 117.53%, confirming its practical utility in food matrix analysis. Molecular docking further suggested that a shape and chemical complementary within the paratope pocket in which multiple noncovalent interactions including hydrogen bonds, π - π stacking, and hydrophobic contacts, cooperatively stabilize the antibody-CAP complex. This work establishes a FACS-microfluidics pipeline that not only overcomes the technical barriers to hapten-specific RabmAb discovery but also provides a rapid, efficient, and scalable platform for generating high-performance antibodies against hapten molecules.

Keywords: Rabbit monoclonal antibody, Single B-cell antibody discovery, Hapten-specific antibody, Fluorescence-activated cell sorting, Microfluidics-based single-cell cDNA preparation, Chloramphenicol

1 Introduction

Monoclonal antibodies (mAbs) are among the most powerful biorecognition elements in immunoassays for food safety, clinical diagnostics, and environmental monitoring. Their utility derives from their ability to bind defined antigenic determinants with high specificity and affinity and to provide standardized reagents for reproducible assay development^{1,2}. In the case of hapten molecules, which typically possess a molecular weight below 1000 Da and only a single antigenic determinant, the quality of the antibody reagent becomes the single most critical determinant of assay sensitivity, specificity, and robustness. Hapten-specific mAbs are therefore not merely useful but indispensable for achieving regulatory-level detection limits in complex food and environmental matrices. Among various antibody formats, rabbit monoclonal antibodies (RabmAbs) have gained increasing attention as superior recognition reagents for small-molecule immunoassays. Compared with murine monoclonal antibodies (MmAbs), RabmAbs often offer higher affinity, broader epitope coverage, and greater conformational stability, because of a larger B-cell repertoire, distinct mechanisms of affinity maturation, and a unique antibody structure characterized by exceptionally long CDR-H3 and diverse CDR-L3 loops³. These features make RabmAbs particularly well suited for recognizing the constrained, often planar architectures of haptenic compounds, where the antibody-hapten interface relies on a limited number of intermolecular contacts and demands a high degree of shape complementarity^{4,5}.

Despite these advantages, the generation of RabmAbs against haptens remains technically challenging. The conventional hybridoma approach, while well-established for MmAbs, has met with limited success in rabbits exemplified by platforms such as RabMab® due to intrinsically low fusion efficiency, progressive genetic instability of rabbit-mouse heterohybridomas, loss of antibody secretion during prolonged culture, and the laborious nature of repeated subcloning^{6,7}. Beyond these technical hurdles, the widespread adoption of hybridoma-based RabmAb generation is further constrained by intellectual property and commercial availability of myeloma cell lines for rabbit. To overcome these limitations, display library-based technologies, including phage, yeast, and mammalian cell display, have been applied to RabmAb discovery in combination with recombinant expression systems^{7,8}. These in vitro selection platforms avoid the need for cell fusion and enable high-throughput

screening. However, in most display libraries, antibodies are generated by artificial pairing of heavy-chain variable region (VH) and light-chain variable region (VL) genes, resulting in the loss of naturally VH and VL pairing information from the B-cell repertoire^{9,10}. Moreover, artificially constructed libraries cannot fully recapitulate the *in vivo* processes of antigen-driven B-cell selection and affinity maturation^{11,12}. For hapten-specific antibodies, where the combining site relies on exquisitely coordinated VH/VL pairing to form a complementary pocket for the small molecule, the loss of native pairing information imposes a particularly severe constraint on the recovery of high-quality antibodies¹³. Therefore, there is a pressing need for alternative strategies that preserve naturally VH/VL pairing while enabling efficient recovery of hapten-specific RabmAb genes.

Single B-cell antibody technologies have emerged as an important solution to this challenge, offering a direct route from individual antigen-experienced B cells to mAbs with naturally paired VH and VL genes¹⁴. A critical prerequisite for this approach is the selective isolation of antigen-specific B cells. Fluorescence-activated cell sorting (FACS) enables the identification and sorting of antigen-specific B cells by combining antibodies against cell-surface markers with fluorescently labeled antigens that bind to surface B-cell receptors (BCRs), provides a powerful means to achieve this selection. Indeed, FACS-based enrichment of IgG⁺ antigen-specific memory B cells (MBCs) has been successfully applied to generate mAbs from both mice and rabbits¹⁵. However, the downstream processing of FACS-sorted single cells remains a significant source of technical variability. Conventional workflows generally rely on sorting single cells into microplate wells, followed by manual or semi-automated cell lysis, reverse transcription, and PCR amplification performed at microliter-scale volumes. Because each primary B cell contains only picogram quantities of messenger RNA, these open-system, manually operated workflows are susceptible to RNA loss, enzymatic degradation, and exogenous contamination, all of which can compromise the recovery and fidelity of antibody variable-region transcripts. Moreover, the multi-step, manually intensive nature of these procedures inherently extends processing timelines and introduces batch-to-batch variability that complicates reproducibility across experiments. This vulnerability is particularly acute for rabbit B cells, for which the limited availability of validated primer sets and the relatively low abundance of immunoglobulin transcripts add

further layers of technical difficulty.

Microfluidic platforms provides a powerful technical basis to these limitations by enabling the confinement, manipulation, and molecular processing of single cells within miniaturized, closed-volume reaction chambers^{16,17}. In these miniaturized compartments, molecular processing steps such as cell lysis, reverse transcription, and single-cell cDNA preparation can be integrated into a controlled pipeline^{18,19}. In addition, the reduction of reaction volumes from the microliter scale to the nanoliter or picoliter scale substantially increase the effective template concentration, reduce reagent consumption, and minimizes sample loss and the risk of exogenous nucleic acid contamination²⁰. The Fluidigm C1 Single-Cell Auto Prep System (hereafter, the Fluidigm C1 system) is a microfluidics-based integrated fluidic circuit (IFC) platform for automated single-cell processing. This system performs single-cell capture, lysis, reverse transcription, and cDNA preparation on a single IFC chip, making it suitable for preparing single-cell cDNA for downstream amplification of antibody variable-region fragments^{21,22}. Titcombe et al. used this system to recover paired heavy- and light-chain sequences from a subset of FACS-sorted citrullinated peptide-specific human MBCs for subsequent antibody generation²³. This parallelized, chamber-based workflow reduced manual handling during single-cell RNA processing and provide a technical route for improving antibody gene recovery from sorted antigen-specific B cells. However, despite these advantages, the application of the Fluidigm C1 system integrated with FACS to antibody discovery, particularly for the recovery of hapten-specific antibodies from rabbit memory B cells, remains limited. In this study, we developed a streamlined single B-cell antibody discovery pipeline that integrates FACS-based antigen-specific MBCs sorting with microfluidics-based automated single-cell cDNA preparation, specifically tailored for the generation of hapten-specific RabmAbs.

2 Materials and Methods

2.1 Reagents and chemicals

Bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), and

4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS), isopropyl β -D-1-thiogalactopyranoside (IPTG), and anhydrous dimethyl sulfoxide (DMSO) were sourced from Thermo Fisher Scientific Inc. (Massachusetts, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) and phycoerythrin (PE)-conjugated goat anti-rabbit IgG (H+L) were purchased from Beyotime (Beijing, China). Lymphocyte separation medium was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). All other reagents were obtained from Beijing Chemical Reagent Co., Ltd. (Beijing, China). The CAP-specific MmAb 5D11 was previously prepared in our laboratory and used in this study²⁴.

The following buffers and solutions were used in this study. Phosphate-buffered saline (PBS, 0.01 M, pH 7.4) was used unless otherwise specified. Carbonate-bicarbonate buffer (CB; 0.05 M, pH 9.0) was used as the coating buffer. The blocking buffer consisted of 0.25% casein (w/v) and 5% calf serum (v/v) in PBS. The washing buffer (PBST) consisted of 0.05% Tween-20 (v/v) in PBS (0.01 M, pH 7.4). The FACS buffer consisted of PBS containing 2% fetal bovine serum (FBS). The substrate solution was freshly prepared by mixing equal volumes of solutions A and B. Solution A contained 0.5 g of urea hydrogen peroxide dissolved in 500 mL of citrate-phosphate buffer (0.1 M, pH 5.0). Solution B contained 0.25 g of 3,3',5,5'-tetramethylbenzidine and 5.2 g of citric acid dissolved in 20 mL of DMSO and 480 mL of distilled water.

2.2 Preparation of hapten-protein conjugates and fluorescent antigen probe

Hapten-protein conjugates, including the immunogen (CAP-HS-KLH) and coating antigens (CAP-HS-BSA, CAP-OPA-OVA, and CAP-NH₂-PTSC-OVA), were prepared using the active ester method²⁵. Briefly, the carboxyl groups of the haptens were activated with EDC and NHS at a molar ratio of 1:1.5:1.5 (hapten:EDC:NHS) by gentle shaking at room temperature for 3 h. The activated hapten solution was then slowly added dropwise to KLH, BSA or OVA dissolved in PBS (0.01 M, pH 7.2) at a hapten-to-protein molar ratio of approximately 60:1 for BSA/OVA conjugates and 50:1 for KLH conjugates. The reaction mixture was incubated with gentle agitation in the dark at room temperature for 12 h and then dialyzed against PBS (0.01 M, pH 7.2) at 4 °C for 72 h, with buffer replacement every 8 h.

CAP-N-6AS-OVA was prepared by glutaraldehyde-mediated coupling²⁶. Briefly, CAP-N-6AS and OVA were dissolved in PBS (0.01 M, pH 7.4) at a molar ratio of approximately 60:1. Subsequently, a 25% (v/v) glutaraldehyde solution was added dropwise to the mixture under stirring. The reaction proceeded at room temperature for 3 h in the dark. Sodium borohydride was then added, and the mixture was stirred for a further 1 h to reduce and stabilize the Schiff-base linkages. The resulting CAP-N-6AS-OVA conjugate was dialyzed against PBS (0.01 M, pH 7.2) at 4 °C for 72 h, with buffer replacement every 8 h. All conjugates were collected and stored at -20 °C. The hapten-protein conjugates were characterized by indirect enzyme-linked immunosorbent assay (iELISA), using the previously prepared CAP-specific MmAb 5D11, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to verify successful conjugation and determine the hapten-to-protein coupling ratios. The structure of the immunogen is shown in Figure S1. The coating-antigen structures are shown in Figure 2C and Figure 3A-C.

FITC-CAP-HS-BSA was prepared by conjugating CAP-HS-BSA with FITC-NHS. Briefly, CAP-HS-BSA was dissolved in CB (0.05 M, pH 9.0), and FITC-NHS dissolved in DMSO was added at a FITC-to-protein molar ratio of 5:1. The mixture was protected from light and incubated in the dark at room temperature for 2 h with gentle agitation, after which unreacted FITC-NHS was removed by three rounds of ultrafiltration using a 30 kDa molecular weight cut-off centrifugal filter. The purified FITC-CAP-HS-BSA was aliquoted and stored at -20 °C. It was characterized by UV-visible absorption spectroscopy and indirect competitive enzyme-linked immunosorbent assay (icELISA), performed using the previously prepared CAP-specific MmAb 5D11, to confirm that FITC labeling did not compromise the antigen-recognition properties of CAP-HS-BSA.

2.3 Rabbit immunization, antisera monitoring, and PBMC preparation

Male New Zealand White rabbits (n = 3) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were approximately 6 months old and weighed approximately 3 kg. All animal experiments were performed in accordance with protocols approved by the Experimental Animal Ethics Committee of China Agricultural University (approval number: AW81905202-2-03). The immunogen CAP-HS-KLH was diluted in PBS to a final concentration of 1 mg/mL and emulsified with an equal volume of Freund's

adjuvant by vigorous vortexing until a stable water-in-oil emulsion was formed. For primary immunization, CAP-HS-KLH was emulsified with complete Freund's adjuvant, whereas incomplete Freund's adjuvant was used for all booster immunizations. Rabbits were immunized by multiple subcutaneous injection at six to eight multiple sites in the dorsal neck region to maximize antigen drainage to regional lymph nodes. Each rabbit received six immunizations at 30-day intervals, with 1 mg of CAP-HS-KLH administered per immunization.

Antisera was collected from the marginal ear vein 7 days after each booster immunization and monitored by icELISA. Briefly, the coating antigen CAP-HS-BSA (as a heterologous coating antigen to minimize carrier-protein interference) was diluted in CB (0.05 M, pH 9.0) to an optimized concentration of 1 µg/mL and added to 96-well microplates at 100 µL per well. The plates were incubated at 37 °C for 2 h, two additional washed three times with PBST, and blocked at 37 °C for 1 h. After two washes, 50 µL of PBS or CAP standard solution at serial concentrations was added to each well, followed by 50 µL of diluted antisera at a pre-determined optimal dilution. The plates were incubated at 37 °C for 30 min. After two washes, 100 µL of HRP-conjugated secondary antibody diluted 1:5000 in PBS was added to each well and incubated at 37 °C for 30 min. After two further washes, 50 µL of substrate solution was added to each well. Following incubation at room temperature for 15 min in the dark, the reaction was stopped by adding 50 µL of 2 M H₂SO₄. Absorbance was measured at 450 nm using a microplate reader, and the optical density was recorded as OD₄₅₀. The inhibition rate (IR) was calculated using Eq. (1):

$$IR (\%) = [(B_0 - B) / B_0] \times 100 \quad (1)$$

where B₀ represents the OD₄₅₀ value in the absence of CAP, and B represents the OD₄₅₀ value in the presence of CAP.

After the sixth immunization, the rabbit with the highest antisera sensitivity (defined as the lowest IC₅₀ value in the icELISA) was selected for PBMC preparation. Approximately 30 mL of blood was collected from the central auricular artery and processed for PBMC isolation. The blood sample was diluted with an equal volume of PBS (0.01 M, pH 7.2) and layered onto lymphocyte separation medium in a 1:1 volume ratio while maintaining a clear interface between the two liquid phases. The sample was then centrifuged at 1500× g for 30 min at

room temperature. After centrifugation, the PBMC layer at the plasma-separation medium interface was carefully collected using a Pasteur pipette. The cells were washed three times with PBS (0.01 M, pH 7.2) and centrifuged at $250\times g$ for 10 min after each wash. After the final wash, the supernatant was discarded, and the PBMCs were resuspended for subsequent FACS-based sorting of antigen-specific MBCs.

2.4 FACS-based sorting of antigen-specific MBCs from PBMCs

PBMCs prepared as described above were resuspended in ice-cold FACS buffer and adjusted to a final concentration of 1×10^6 cells/mL. For cell staining, 10 μg of PE-conjugated goat anti-rabbit IgG (H+L) antibody and 20 μg of FITC-CAP-HS-BSA were sequentially added to the cell suspension. The cells were incubated on ice for 30 min in the dark to prevent capping and internalization of surface BCRs. After staining, the cells were centrifuged at $400\times g$ for 10 min and washed three times with ice-cold FACS buffer to remove unbound antibody and fluorescently labeled antigen. The stained cells were resuspended in 500 μL of FACS buffer and transferred to sterile flow cytometry tubes with cell-strainer caps to remove aggregates. A 10 μL aliquot of the stained cell suspension was used for confocal laser scanning microscopy to examine the dual-positive fluorescence signals on the cell surface. Before FACS-based sorting, DAPI was added at a final concentration of 1 $\mu\text{g/mL}$, after which the flow cytometer settings, sorting gates, and flow rate were optimized using unstained and single-stained controls as fluorescence reference standards. During sorting, lymphocytes were first identified based on their FSC/SSC characteristics to exclude debris (P1), followed by singlet discrimination using FSC-W versus FSC-H (P2). Live cells were then selected by excluding DAPI⁺ events (P3). IgG⁺ events were subsequently selected in P4, and antigen⁺ events within the IgG⁺ population were selected in P5. The resulting IgG⁺antigen⁺ double-positive live cells were sorted as antigen-specific MBCs and collected in ice-cold RPMI 1640 medium supplemented with 5% FBS in a 1.5 mL microcentrifuge tube pre-coated with 5% FBS to minimize cell adhesion for subsequent microfluidics-based automated single-cell cDNA preparation.

2.5 Microfluidics-based single-cell cDNA preparation

The overall workflow for single-cell cDNA preparation on the Fluidigm C1 system comprised four sequential stages, including chip priming, cell loading and single-cell capture,

on-chip lysis/reverse transcription/cDNA preparation, and harvest of single-cell cDNA products.

Chip priming. The IFC chip (designed for cell diameters of 5-10 μm) was primed according to the manufacturer's instructions with minor modifications to ensure complete hydration and removal of air bubbles from the microfluidic channels. A 200 μL aliquot of C1 Harvest Reagent was added to the red-circled inlet, and 20 μL of C1 Harvest Reagent was added to each of the 40 red-marked inlets. C1 Preloading Reagent (20 μL), C1 Blocking Reagent (15 μL), and Cell Wash Buffer (20 μL) were loaded into the purple-, white-, and gray-marked inlets, respectively. The IFC chip was then placed into the Fluidigm C1 system and primed under vacuum for 10 min at room temperature.

Cell loading and single-cell capture. FACS-sorted antigen-specific MBCs were counted using a hemocytometer, adjusted to a concentration of 66000-333000 cells/mL, and mixed with C1 Suspension Reagent at a ratio of 3:2 (v/v). A 6 μL aliquot of the cell mix was loaded into the green-marked inlet. After removal of the C1 Blocking Reagent from the white-marked inlet to open the microfluidic capture valves, the IFC chip was placed in the Fluidigm C1 system for single-cell capture in individual microfluidic chambers. The cell loading process was performed under programmed pressure control to ensure Poisson-distributed single-cell occupancy.

On-chip lysis, reverse transcription, and cDNA preparation. After microscopic inspection of single-cell capture using an inverted phase-contrast microscope to confirm single-cell occupancy and record chamber coordinates, reagents for single-cell cDNA preparation were loaded into the IFC chip. Briefly, 180 μL of C1 Harvest Reagent was added to the red-marked harvest reservoir, 7 μL of Lysis Mix A containing cell lysis buffer and RNase inhibitor was added to the orange-marked inlet, 7 μL of RT Mix B containing reverse transcriptase, buffer, dNTPs, and oligo(dT) primers was added to the yellow-marked inlet, and 24 μL of PCR Mix C containing pre-amplification reagents was added to inlets 7 and 8. The IFC chip was then processed in the Fluidigm C1 system to perform cell lysis, reverse transcription, and cDNA preparation in nanoliter-scale reaction chambers for 5.5 h for a total of 5.5 h following the manufacturer's standard protocol.

Harvest of single-cell cDNA products. After completion of the on-chip reaction, the

single-cell cDNA products were harvested from the IFC chip into a 96-well PCR plate according to the chamber order of the chip by applying C1 Harvest Reagent to flush the cDNA from each chamber and stored at -20 °C for downstream amplification of antibody variable-region fragments.

2.6 Amplification and sequence analysis of paired VH and VL fragments

The concentration of the single-cell cDNA products was measured using a Qubit 3.0 fluorometer with the Qubit dsDNA HS Assay Kit. Briefly, 2 µL of each cDNA product was mixed with 198 µL of Qubit working solution and incubated at room temperature for 2 min in the dark before measurement. The integrity of the single-cell cDNA products was analyzed using an Agilent 2100 Bioanalyzer with the High Sensitivity DNA Kit according to the manufacturer's instructions to assess the fragment size distribution and confirm the presence of high-molecular-weight cDNA. VH and VL fragments were amplified from single-cell cDNA templates using previously validated rabbit variable-region-specific primers that were designed to target the leader peptide and constant-region sequences, enabling amplification of variable regions while preserving natural VH/VL pairing information²⁷. PCR was performed under the following conditions: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. A no-template control was included in each PCR run to exclude contamination. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and target bands corresponding to the VH and VL fragments at approximately 400 bp were excised and purified. The purified VH and VL fragments from each single-cell cDNA reaction were subjected to TA cloning into the pMD19-T vector and Sanger sequencing using M13 forward and reverse primers to determine the paired VH and VL sequences. The productive paired VH/VL recovery rate was calculated using Eq. (2):

$$\text{Productive paired VH/VL recovery rate (\%)} = (\text{number of productive VH/VL pairs} / \text{number of single-cell-occupied IFC capture chambers}) \times 100 \quad (2)$$

where the number of single-cell-occupied IFC capture chambers was determined by microscopic counting of chambers containing exactly one viable cell.

2.7 Construction and expression of recombinant antibodies

The VH and VL fragments were used to construct scFvs expression plasmids using

sequence- and ligation-independent cloning²⁸ with a flexible (G₄S)₃ linker connecting the VH and VL domains. Each recombinant scFv plasmid was transformed into *E. coli* RV308 electrocompetent cells by electroporation at 2.5 kV, 25 μ F, and 200 Ω . Positive transformants were identified by colony PCR using vector-specific primers, and scFv expression was induced with 0.5 mM IPTG at 25 °C for 12 h to enhance soluble protein yield. After induction, the bacterial cells were harvested by centrifugation at 8,000 $\times$ g for 10 min and disrupted by sonication on ice (20 min, 30% amplitude, 5 s on/5 s off). The supernatant obtained after centrifugation at 12,000 $\times$ g for 20 min sonication was collected as the crude scFv extract for initial functional screening. For RabmAb expression, the codon-optimized VH and VL sequences corresponding to the lead scFv clone 4E were synthesized commercially and cloned into the pFUSE expression vectors containing the rabbit IgG heavy-chain (CH1-CH3) and light-chain (C _{κ}) constant-region sequences, respectively. HEK-293 cells in the exponential growth phase at 70-80% confluence were transfected with the recombinant plasmids at a 1:1 molar ratio (heavy chain:light chain) using Lipofectamine 2000. The culture supernatant was harvested on day 4 after transfection, and RabmAb was purified by HiTrap Protein A affinity chromatography. The molecular integrity of the purified RabmAb was evaluated by SDS-PAGE under both reducing (with 100 mM DTT) and non-reducing conditions.

2.8 Development of RabmAb-4E-based icELISA and sample evaluation

To improve the sensitivity of the RabmAb-4E-based icELISA, the coating antigen-antibody combinations and their working concentrations were optimized using a checkerboard titration in which each combination of coating antigen (CAP-HS-BSA, CAP-OPA-OVA, CAP-N-6AS-OVA, and CAP-NH₂-PTSC-OVA) and antibody concentration was tested to identify the pair with the lowest B/B₀ ratio at a given CAP concentration. The effects of assay buffer conditions, including ionic strength and pH, were also evaluated by preparing standard curves in PBS containing varying concentrations of NaCl (0-6.0 M) or at different pH values (4.0-9.5). In addition, the tolerance of RabmAb-4E to methanol was assessed in the icELISA system by incorporating 0-70% methanol into the assay buffer. The maximum absorbance ($A_{\max}$), defined as the OD₄₅₀ value in the absence of CAP under each tested condition, together with IC₅₀ and IC₅₀/ $A_{\max}$ ratio were used to evaluate these parameters

as indicators of signal intensity, sensitivity, and overall assay robustness, respectively. The specificity of RabmAb-4E was evaluated by determining the cross-reactivity (CR) with structurally related CAP analogues including thiamphenicol, florfenicol, and florfenicol amine using icELISA. CR was calculated by using Eq. (3):

$$\text{CR (\%)} = [(\text{IC}_{50} \text{ of CAP})/(\text{IC}_{50} \text{ of analogues})] \times 100 \quad (3)$$

The analytical performance of the optimized icELISA was evaluated based on the IC_{50} , limit of detection (LOD), and linear range defined as IC_{20} - IC_{80} from the standard curve. The standard inhibition curve was fitted using a four-parameter logistic model according to Eq. (4).

$$Y = (A-D)/[1+(X/C)^B] + D \quad (4)$$

where A represents the responses at high asymptotes of the curve, B acts as the slope factor, C is the IC_{50} of the curve, D is the responses at low asymptotes of the curve, and X is the calibration concentration.

The LOD was determined by analyzing 20 blank skim milk and chicken samples that had been confirmed negative for CAP by LC-MS/MS and was defined as the CAP concentration corresponding to the mean blank signal minus three times the standard deviation of the blank measurements on the standard curve. Recovery experiments using spiked samples were performed to evaluate the accuracy and precision of the developed icELISA. CAP-negative skim milk and chicken samples were obtained from the Beijing Key Laboratory of Diagnostic and Traceability Technologies for Food poisoning (Beijing, China). The samples were pretreated according to a previously reported method to suit the matrix characteristics²⁷, with detailed procedures provided in Section 1.1 of the Supplementary Information.

Recovery and coefficient of variation (CV) values were calculated using Eq. (5) and Eq. (6), respectively. All measurements were performed in triplicate.

$$\text{Recovery (\%)} = (\text{measured concentration}/\text{spiked concentration}) \times 100 \quad (5)$$

$$\text{CV (\%)} = (\text{standard deviation}/\text{mean value}) \times 100 \quad (6)$$

2.9 Structure prediction and molecular docking of RabmAb-4E

The three-dimensional (3D) structure of the Fv region of RabmAb-4E was predicted from the VH and VL amino acid sequences using the homology modeling module of Discovery Studio 2019 with a suitable rabbit antibody template selected based on sequence

identity. The quality of the predicted model was evaluated using Ramachandran plot analysis to assess stereochemical quality and Profile-3D scoring to verify compatibility of the model with its amino acid sequence. The 3D structure of CAP was obtained from the PubChem database (CID: 5959) and subjected to energy minimization using the CHARMM force field with 1,000 steps of steepest descent before docking. Molecular docking between the RabmAb-4E Fv model and CAP was performed using the CDOCKER module in Discovery Studio 2019 with the CHARMM force field and a grid-based binding-site definition centered at the predicted antigen-binding pocket. Docking poses were ranked by CDOCKER interaction energy, and the optimal binding conformation was selected for further analysis based on the lowest interaction energy and visual inspection. The docking results were visualized using ChimeraX 1.11, and the amino acid residues, hydrogen bonds, hydrophobic interactions, and electrostatic surface properties potentially associated with CAP recognition were analyzed to generate a structural hypothesis for the observed binding specificity and affinity.

3 Results and discussions

The discovery of mAbs from single B cells depends on two critical, sequentially linked steps, the precise selective enrichment of antigen-specific B cells from a complex polyclonal repertoire, and the efficient recovery of their cognate antibody variable-region genes with minimal transcript loss. Since surface BCRs on antigen-experienced B cells can specifically recognize their cognate antigens, fluorescent antigen probes combined with FACS have been widely used to sort antigen-specific B cells for downstream antibody discovery^{15,29,30}. However, conventional workflows of antibody discovery generally separate antigen-specific B-cell sorting from subsequent cell lysis, reverse transcription, and antibody variable-region gene amplification, resulting in multiple manual processing steps in multiwell plates that are inherently susceptible to RNA loss, transcript degradation, and cross-contamination. Therefore, there is an highly demand for integrated pipeline that link single-cell isolation with downstream molecular processing³¹. In recent years, microfluidic technologies have provided powerful tools for single-cell compartmentalization, manipulation, and analysis, enabling miniaturized and integrated workflows for single-cell studies^{32–34}. Microfluidic platforms

offer three distinctive advantages for single B-cell antibody discovery, i.e., (i) closed reaction chambers that minimize exogenous contamination; (ii) nanoliter-scale reaction volumes that increase effective template concentrations; and (iii) automated, parallelized processing that reduces batch-to-batch variability and hands-on time. However, the combination of FACS-based antigen-specific B-cell sorting with microfluidic-based single-cell cDNA preparation has rarely been applied to antibody discovery, particularly in the rabbit system for hapten, where the limited abundance of immunoglobulin transcripts in single cells poses additional technical difficulties. To establish a more direct connection between antigen-specific B-cell enrichment and antibody gene recovery, we developed a single B-cell antibody discovery pipeline that bridges the gap between phenotypic B-cell selection at the single-cell level and genotype recovery within a closed, automated nanoliter environment, thereby preserving the natural VH/VL pairing information that is lost in conventional display-based approaches.

3.1 FACS-microfluidics pipeline for the discovery of hapten-specific RabmAbs

As shown in Scheme 1, the pipeline comprises three integrated modules. In the left module, we immunized New Zealand White rabbits with CAP-HS-KLH as immuogen and antisera were monitoring using CAP-HS-BSA as antigen, and isolated PBMCs from peripheral blood of the rabbit with the highest antisera affinity against CAP. In the middle module, antigen-specific MBCs were sorted by FACS based on IgG⁺antigen⁺ dual positivity using PE-conjugated goat anti-rabbit IgG (H+L) and FITC-CAP-HS-BSA as staining reagents (Step 1). The sorted antigen-specific MBCs were then automatically processed on a microfluidics platform based on the Fluidigm C1 system for single-cell capture, cell lysis, reverse transcription, and cDNA harvest in a single-cell capture chamber within an IFC chip (Step 2). In the right module, the harvested cDNA products from the microfluidics platform were used for recovery paired VH/VL genes, which were then sequenced, assembled into scFv constructs for functional screening, and the lead clone was subsequently reformatted into full-length RabmAb for functional characterization in term of affinity, specificity, stability and adaptation of real samples. By integrating FACS-based enrichment of antigen-specific MBCs with automated microfluidic cDNA processing, this pipeline substantially reduces manual intervention and accelerates the path from antigen-specific rabbit MBCs to functional

RabmAbs relative to hybridoma or display-based methods.

3.2 FACS-based sorting of CAP-specific MBCs from PBMCs

The antisera responses of these immunized rabbits were reported previously by our group²⁷, and the rabbit with the highest antisera affinity with IC₅₀ values of 0.27 ng/mL was selected for PBMC isolation and subsequent processing within the FACS-microfluidics pipeline. The efficiency of single B-cell antibody discovery is critically dependent on the purity of the input cell population³⁵. Before proceeding to cell sorting, we first characterized the hapten-protein conjugates including CAP-HS-KLH, CAP-HS-BSA, CAP-OPA-OVA, CAP-N-6AS-OVA, CAP-NH₂-PTSC-OVA, and the fluorescent antigen probe to confirm their suitability for immunization, plate coating, and cell sorting. ELISA results showed positive binding signals of CAP for all hapten-protein conjugates, indicating successful conjugation (Figure S2). The BSA-based conjugate was further verified by MALDI-TOF MS, as evidenced by the increased molecular weights relative to native BSA, with a hapten-to-BSA coupling ratio of approximately 28:1 (Figure S3A). Since the immunogen was CAP-HS-KLH, BSA was selected as the heterologous carrier protein for both antisera evaluation and fluorescent antigen probe preparation. This heterologous strategy was intended to minimize carrier-specific interference during sorting and to preferentially enrich MBCs recognizing CAP-associated epitopes rather than those specific to the KLH carrier²⁸. Fluorescent antigen FITC-CAP-HS-BSA was subsequently prepared as a probe for FACS-based sorting of CAP-specific MBCs. UV-visible absorption spectroscopy revealed a characteristic FITC absorption peak at approximately 497 nm, primary showing successful FITC labeling (Figure S3B). To address whether FITC conjugation would compromise the antibody-recognition properties of CAP-HS-BSA, we compared the competitive inhibition profiles of CAP-HS-BSA before and after FITC labeling by icELISA. As shown in Figure S3C, the competitive inhibition curves of icELISA based on CAP-HS-BSA before and after FITC labeling did not differ significantly ($p > 0.05$), and the corresponding IC₅₀ values of icELISA were comparable (4.79 vs. 5.43 ng/mL, respectively), indicated that FITC labeling retained the CAP-recognition performance of CAP-HS-BSA. For cell staining, we adopted a multiparameter staining strategy. DAPI was used to exclude dead cells, PE-conjugated goat anti-rabbit IgG (H+L) was used to identify the IgG⁺ MBC population, and

FITC-CAP-HS-BSA was used to label antigen-specific MBCs. Among live cells, those positive for both markers (IgG⁺antigen⁺) were operationally defined as antigen-specific MBCs. As shown in Figure 1A, PE and FITC fluorescence signals were detected in the red and green channels, respectively. Comparison of the corresponding fields showed that the arrow-marked cells were detected in both channels, confirming the presence of double-positive cells. The presence of these double-positive cells indicated that FITC-CAP-HS-BSA could specifically bind to a subset of MBCs, supporting the technical feasibility of using these probes for FACS-based enrichment of IgG⁺antigen⁺ cells.

Following optimization of the FACS parameters, gating strategy, and sorting flow rate, IgG⁺antigen⁺ double-positive cells from rabbit PBMCs were isolated from rabbit PBMCs by FACS. After lymphocyte gating, singlet discrimination, and live-cell selection during FACS (P1, P2, and P3, respectively; Figures S4A-C), the downstream gates shown in Figure 1B identified that IgG⁺ cells accounted for 29.17% of the gated live population (P4). This value exceeded the previously reported mean IgG⁺ cell frequency of 16.62% for rabbits immunized with the same antigen, including the rabbit analyzed here ⁵. The results may reflect interindividual variation in IgG⁺ cell frequencies within the immunized cohort. Within the IgG⁺ population, antigen⁺ cells represented 1.10% (P5), indicating that antigen-specific MBCs constituted a rare subset of the gated lymphocyte singlets. This low frequency, approximately one in every 100 IgG⁺ cells, is broadly consistent with the antigen⁺ fractions reported for the same CAP-HS-KLH-immunized rabbits (2.8-7.2%)⁵ and for MON-HS-KLH-immunized rabbits (3.21%)³⁶. Notably, both studies likewise employed a heterologous strategy, used hapten-KLH as immunogens and fluorescently labeled hapten-BSA as probes for FACS-based sorting. The results highlight the need for high-resolution FACS to recover sufficient numbers of antigen-specific cells for subsequent single-cell processing. The sorted IgG⁺antigen⁺ cells were subsequently subjected to microfluidics-based single-cell cDNA preparation without intermediate culture or expansion, thereby preserving the transcriptional state of the cells at the time of sorting.

3.3 Microfluidics-based single-cell cDNA preparation

A critical step following FACS-based sorting is the conversion of the limited transcript content of each primary B cell into amplifiable cDNA templates while preserving the natural

VH/VL pairing information. Following FACS-based sorting, the sorted IgG⁺antigen⁺ cells were immediately subjected to microfluidics-based single-cell cDNA preparation using the Fluidigm C1 system, without intermediate culture, for antibody gene recovery. A 5-10 μ m IFC chip was selected according to the diameter of the sorted MBCs (approximately 6-9 μ m), as determined by microscopic measurement (Figure 1A). Microscopic inspection of the IFC chip confirmed the presence of single cells distributed across the capture chambers (Figure 1C). After optimization of the cell-loading conditions, single cells were observed in 41.7% of the capture chambers (40 of 96), defined as the percentage of chambers containing a single viable cell. The subsequent microfluidics-based single-cell cDNA preparation process was performed entirely on-chip and included cell lysis, reverse transcription, cDNA preparation, and final cDNA harvest within nanoliter-scale reaction chambers³⁴. This closed-system approach eliminated multiple manual transfer steps that are otherwise required in conventional microplate-based protocols. The resulting single-cell cDNA was evaluated by both concentration measurement and fragment-size analysis to assess yield and quality prior to variable-region amplification. Qubit quantification showed that the cDNA concentrations ranged from 0.212 to 0.341 ng/ μ L across the harvested cDNA products. Fragment-size analysis using the Agilent 2100 Bioanalyzer detected high-molecular-weight cDNA products at approximately 5,000 bp, indicating that the prepared cDNA contained sufficiently long fragments and was of adequate quality to support downstream amplification of antibody variable-region genes (Figure 1D). Importantly, the presence of full-length cDNA rather than degraded fragments suggested that the on-chip reverse transcription proceeded efficiently despite the low RNA input from individual primary B cells. Then, all harvested single-cell cDNA products were subjected to amplification of antibody variable-region fragments regardless of their quantified concentration using rabbit VH- and VL-specific primer sets. As shown in Figure 1E, of the 40 chambers in which single-cell capture was confirmed, 23 VH/VL pairs were successfully amplified, with both VH and VL amplicons showing the expected size of approximately 400 bp on agarose gel electrophoresis. Since each VH/VL pair originated from an individual single-cell cDNA product within a distinct capture chamber, the natural VH/VL pairing information of the corresponding B-cell clone was preserved. All 23 amplicon-positive reactions yielded paired VH/VL products, indicating that the

microfluidics-generated cDNA supported co-amplification of both chains without preferential loss of either variable-region transcript. After cloning and Sanger sequencing of the purified PCR products, 22 of the 23 VH/VL pairs were identified as intact based on open reading frame analysis and sequence integrity, and selected for scFv construction and functional screening.

Recovery of paired antibody variable-region genes from individual rabbit B cells remains technically challenging because of the low abundance of immunoglobulin transcripts in each cell. To contextualize the performance of our FACS-microfluidics pipeline, we compared our recovery metrics with those previously reported studies using manual or alternative single-cell approaches. In a recent study targeting haptenic florfenicol, paired VH/VL sequences were recovered from 10 of 30 individually isolated rabbit antibody-secreting cells (ASCs) following manual cDNA synthesis⁴. Ozawa et al. employed the immunospot array assay to isolate 189 hen egg lysozyme-specific ASCs from the peripheral blood lymphocytes of immunized rabbits, from which 56 paired VH/VL sequences were successfully recovered³⁷. Similarly, in a study targeting WNT1-inducible signaling pathway protein 1, Starkie et al. failed to recover paired VH/VL products from wells containing a single rabbit MBC and obtained variable-region genes from only 18% of wells containing three cells¹⁵. In the present study, single cells were observed in 41.7% (40/96) of the IFC capture chambers, and from these, 22 VH/VL pairs were ultimately recovered from the corresponding cDNA products, yielding an estimated productive paired VH/VL recovery rate of 55.0% (calculated as 22 pairs divided by the 40 single-cell-occupied chambers). This recovery rate was substantially higher than the 0% recovery from single MBCs reported by Starkie et al. and comparable to the recovery rates achieved from ASCs in other studies, despite our starting material being an MBC population with a lower abundance of immunoglobulin transcripts. These results indicated that the microfluidics-generated cDNA supported downstream recovery of rabbit antibody variable-region genes, and that the closed, nanoliter-scale microfluidic format substantially reduced hands-on steps and minimized the risk of transcript loss and cross-contamination while achieving recovery rates comparable to manual workflows.

3.4 Generation and functional validation of recombinant antibodies

A central question after recovering paired VH/VL sequences is whether the preserved

natural pairing translates into functional antigen-binding activity. To address this, we adopted a two-stage validation strategy. The first stage involved rapid functional screening of recombinant antibodies in the scFv format using crude bacterial extracts, whereas the second involved reformatting the lead candidate as full-length RabmAb for detailed characterization. The 22 VH/VL pairs obtained above were assembled into scFv constructs and expressed in *E. coli* RV308 for functional screening. After IPTG induction and cell disruption by sonication, the soluble fractions were collected as crude scFv extracts without further purification, enabling parallel screening of all 22 clones in a single batch. As shown in Figure 2A, initial screening by direct binding ELISA against the CAP-HS-BSA coating antigen identified 10 antigen-binding scFv clones, yielding a positive hit rate of 45.5% (10/22). This positive rate was substantially higher than typical scFv phage-display outputs³⁸, suggesting that the pipeline effectively enriched for antigen-specific B cells and preserved functional VH/VL pairing during cDNA preparation and amplification. These 10 clones were subsequently evaluated by icELISA using free CAP as the competitor to assess their hapten-binding specificity and sensitivity, and the corresponding IC₅₀ values are summarized in Table S1. Among them, clone 4E showed the highest affinity against free CAP with an IC₅₀ value of 4.16 ng/mL in the scFv format, and was therefore selected for full-length RabmAb construction. The amino acid sequences of the 4E-derived VH and VL regions, with the framework regions (FRs) and complementarity-determining regions (CDRs) annotated according to the IMGT numbering system, are shown in Figure 2B with the CDRs highlighted by colored arrows. Although scFvs provide a convenient format for initial functional screening, their monovalent binding format and reduced conformational stability relative to full-length RabmAbs can affect antigen-recognition properties³⁹. Therefore, we reformatted clone 4E as a full-length RabmAb for more comprehensive characterization. RabmAb-4E was expressed in HEK293 cells and purified from the culture supernatant by Protein A affinity chromatography. SDS-PAGE analysis confirmed the successful purification of RabmAb-4E (Figure S5). Under non-reducing conditions, RabmAb-4E migrated as a single band at approximately 150 kDa, consistent with the expected molecular mass of intact rabbit IgG with two heavy chains and two light chains assembled via disulfide bonds. Under reducing conditions in the presence of DTT, two distinct bands were observed at approximately 50 and

25 kDa, corresponding to the antibody heavy and light chains, respectively, confirming the expected subunit composition. In the icELISA, RabmAb-4E retained CAP-recognition activity, with an IC_{50} value of 1.04 ng/mL, which was approximately fourfold lower than that of scFv-4E under the tested conditions (Figure 2C). This affinity enhancement upon reformatting is consistent with the avidity effect conferred by the bivalent antigen binding of full-length RabmAb compared with the monovalent scFv format^{40,41}, and it indicates the value of evaluating lead candidates in their final antibody format. In addition, the greater structural stability reported for full-length antibody relative to scFv may help preserve antigen-binding activity and contribute to the lower IC_{50} observed here⁴². Similar advantages of full-length antibodies over corresponding scFvs have been reported for the haptens ofloxacin and dihydroartemisinin^{43,44}. These results confirmed that the 4E-derived VH/VL pair remained functional after reformatting as a full-length RabmAb, supporting the selection of RabmAb-4E for subsequent icELISA optimization and analytical evaluation. This two-stage validation strategy enabled rapid identification of functional leads while ensuring that the final antibody format retained the desired antigen-recognition properties.

3.5 Analytical performance of the RabmAb-4E-based icELISA for CAP detection

To improve the analytical performance of the RabmAb-4E-based icELISA, a checkerboard titration was performed to systematically evaluate four coating antigens across different coating antigen and antibody concentrations including CAP-HS-BSA, CAP-OPA-OVA, CAP-N-6AS-OVA, and CAP-NH₂-PTSC-OVA. Previous studies have shown that an appropriate heterologous coating antigen can significantly improve the analytical sensitivity of competitive immunoassays for hapten molecules^{45,46}. At the selected working concentrations, the four antigen-antibody pairs yielded B_0 values of 1.84-2.11. At 0.1 ng/mL CAP, the corresponding B/ B_0 values were 84.67%, 73.84%, 13.41%, and 69.45%, respectively (Table S2). The icELISA using CAP-N-6AS-OVA as the coating antigen therefore showed the greatest competitive inhibition in this preliminary screen. Competitive inhibition curves for CAP were generated using each of the four coating antigens with IC_{50} values of 1.04, 0.54, 0.03, and 0.37 ng/mL, respectively (Figure 2C and Figures 3A-C). The icELISA using CAP-N-6AS-OVA as the coating antigen showed the lowest IC_{50} , approximately 35-fold lower than that obtained using CAP-HS-BSA, suggesting that the

effect of coating-hapten heterology on assay sensitivity may depend on the specific structural modification. Such differences facilitate competition by free CAP for RabmAb-4E binding. The significant improvement of sensitivity for hapten in competitive immunoassay has often observed. In the case of enrofloxacin, modification of the coating hapten reduced the IC_{50} from 1.3 to 0.07 $\mu\text{g/L}$ ⁴⁷. Likewise, a heterologous coating hapten reduced the IC_{50} for tetracycline from 40.7 to 1.95 ng/mL, and further assay optimization reduced it to 0.7 ng/mL⁴⁸. Accordingly, CAP-N-6AS-OVA and RabmAb-4E were selected at working concentrations of 24.69 and 8.23 ng/mL, respectively, for subsequent analytical evaluation.

Complex sample matrices and extraction solvents can affect icELISA performance. Therefore, the effects of ionic strength, pH, and methanol concentration were evaluated using A_{max} , IC_{50} , and the IC_{50}/A_{max} ratio as performance indices. A lower IC_{50}/A_{max} ratio indicates a more favorable combination of signal intensity and assay sensitivity. RabmAb-4E maintained stable CAP recognition over a NaCl concentration range of 0-6.0 M. Although A_{max} gradually decreased from 2.232 to 1.498 with increasing NaCl concentration, IC_{50} values remained within 0.03-0.08 ng/mL, and the corresponding IC_{50}/A_{max} ratios ranged from 0.017 to 0.040, indicating strong tolerance to high ionic strength (Figure S6A). This extreme salt tolerance extending to 6.0 M NaCl, which is well beyond physiological conditions, suggests that the RabmAb-4E-CAP interaction is likely driven predominantly by hydrophobic and aromatic contacts rather than electrostatic interactions^{49,50}. The pH tolerance of the assay was further assessed (Figure S6B). RabmAb-4E maintained relatively stable recognition performance from pH 4.0 to 8.5, with A_{max} values of 1.632-2.145, IC_{50} values of 0.03-0.07 ng/mL, and IC_{50}/A_{max} ratios of 0.017-0.037. In contrast, at pH 9.0 and 9.5, A_{max} decreased to 1.325 and IC_{50} values increased to 0.12 ng/mL, indicating that the assay was less suitable under more alkaline conditions, possibly due to conformational changes in the antibody paratope or altered ionization states of key antigen-contact residues⁵¹. Because organic solvents are often introduced during sample extraction, particularly for veterinary drug residues in animal-derived foods, the methanol tolerance of RabmAb-4E was evaluated. The icELISA retained stable performance in the presence of 0%-60% methanol, with A_{max} values ranging from 1.534 to 2.212 and IC_{50} values from 0.04 to 0.15 ng/mL. The IC_{50}/A_{max} ratios ranged from 0.019 to 0.098 over this concentration range. At 70% methanol, the A_{max} decreased to

1.254 and the IC_{50} increased to 0.23 ng/mL, indicating that excessive methanol compromised assay performance, possibly through disruption of the antibody's conformational integrity or altered hapten solubility (Figure S6C). Overall, these results demonstrate that the RabmAb-4E-based icELISA exhibited broad tolerance to variations in ionic strength, pH, and methanol concentration.

Under these optimized conditions, the standard inhibition curve of icELISA for CAP showed an IC_{50} value of 0.01 ng/mL (Figure 3D), which was approximately 27-fold lower than that obtained with rabbit polyclonal antibodies (0.27 ng/mL) and 8-fold lower than that of the RmAb3-based icELISA (0.08 ng/mL)²⁷. RabmAb-4E and RmAb3 were isolated from MBCs and ASCs of the same immunized rabbit, indicating that both antigen-specific MBCs and ASCs can serve as sources of RabmAbs with high affinity. The specificity of RabmAb-4E was evaluated by determining its CR against structurally related CAP analogues, including thiamphenicol, florfenicol, and florfenicol amine. Similar to RmAb3 and rabbit polyclonal antibodies, RabmAb-4E showed negligible CR values (<0.001%) toward all tested analogues (Table S3). The high sensitivity and specificity of icELISA with RabmAb-4E support the utility of the FACS-microfluidics pipeline for discovering hapten-specific RabmAbs, with performance characteristics that meet or exceed those of previously reported antibodies generated by conventional hybridoma- or display-based approaches from both rabbit and mouse (Table 1).

3.6 Analysis of samples

To evaluate the practical applicability of the RabmAb-4E-based icELISA, skim milk and chicken samples were selected as representative food matrices. Matrix effects were assessed by comparing calibration curves prepared in assay buffer with those prepared in untreated blank skim milk and pretreated blank chicken extract. As shown in Figures 3E and 3F, the calibration curves prepared in both matrices were nearly superimposable on those obtained in buffer, with similar slopes and comparable IC_{50} values. These results suggest that matrix interference were negligible in skim milk and effectively minimized in chicken through sample pretreatment. The LODs of the RabmAb-4E-based icELISA were calculated to be 0.0012 µg/L for skim milk and 0.0025 µg/kg for chicken, and the corresponding linear ranges were 0.003-0.039 µg/L and 0.004-0.053 µg/kg as shown in Table2, respectively. To assess

accuracy and precision, recovery experiments were conducted using spiked samples at three concentrations spanning the linear range of the assay. As summarized in Table 2, recoveries ranged from 95.12% to 117.53% in skim milk, with CVs below 8.56%. In chicken samples, the recoveries ranged from 90.28% to 112.15%, with CVs below 10.75%. These results indicate that the RabmAb-4E-based icELISA was applicable to CAP determination in skim milk and chicken, with acceptable accuracy and precision that meet the requirements for routine monitoring applications.

3.7 Recognition mechanism of RabmAb-4E

To gain structural insights into the molecular basis of CAP recognition by RabmAb-4E and to explain the high sensitivity and specificity observed in the icELISA, we performed homology modeling of the Fv region followed by molecular docking of CAP. The quality of the predicted Fv model was first evaluated by Ramachandran plot analysis and Profile-3D scoring. As shown in Figure S7, the Ramachandran plot indicated that over 90% of the residues were located in the favored regions, and the Profile-3D score was well above the threshold for a reliable model, confirming the stereochemical quality and overall fold compatibility of the predicted structure. As is typical for most hapten-antibody interactions, CAP was predicted to be deeply buried within a cavity formed by the antibody paratope, with favorable shape complementarity between the binding site and the CAP epitope (Figure 4A). Surface analysis of the binding pocket revealed a complementary distribution of hydrogen-bond donor and acceptor regions around CAP (Figure 4B), and the pocket also contained spatially organized hydrophobic regions surrounding the aromatic and nonpolar portions of CAP (Figure 4C). These surface features are consistent with the extreme salt tolerance observed in the icELISA (Figure S6A), where the RabmAb-4E-CAP interaction remained stable at NaCl concentrations up to 6.0 M, indicating that hydrophobic and aromatic interactions may predominate over electrostatic forces in antigen binding. Furthermore, the precise hydrogen-bonding network involving the nitro group of CAP and the shape-complementary pocket may explain the negligible cross-reactivity with thiamphenicol, florfenicol, and florfenicol amine (Table S3), as these analogues differ at the C-1 position bearing a methylsulfonyl group (thiamphenicol), a fluorine and hydroxyl group (florfenicol), or a hydroxyl group (florfenicol amine) instead of the nitro group and lack the exact

hydrogen-bonding geometry required for optimal recognition.

The selected docking pose ranked by CDOCKER interaction energy placed CAP within a pocket formed primarily by all six CDRs (CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3) of the heavy and light chains (Figure 4D). Among the six CDRs, CDR-H3 and CDR-L3 contributed the majority of the contact residues, consistent with the prominent roles of these two hypervariable loops in antigen recognition by rabbit antibodies^{52,53}. Within this six-CDR pocket, four residues, TrpH52 (FR-H2), GlyH110 (CDR-H3), AlaH113 (CDR-H3), and AspL116 (CDR-L3), were predicted to form the core of the CAP-binding interface and to stabilize CAP through a combination of polar, electrostatic, and aromatic interactions (Figures 4E, F). The aromatic side chain of TrpH52 was predicted to interact with the phenyl ring of CAP through a π - π T-shaped interaction and to form a carbon-hydrogen bond with an oxygen atom of the nitro group, whereas GlyH110 was predicted to form a carbon-hydrogen bond with CAP. AlaH113 was predicted to provide an additional π -alkyl contact, whereas AspL116 was predicted to contribute attractive charge and π -anion interactions with the nitro group and phenyl ring of CAP, respectively. The interaction network involves both heavy-chain (GlyH110, AlaH113, TrpH52) and light-chain (AspL116) residues, indicating that the CAP-binding pocket is formed by the cooperative contribution of both chains. This cooperative VH/VL engagement shows the importance of preserving natural heavy-light chain pairing, a key advantage of our pipeline over display libraries that randomize chain combinations.

In contrast to the predominantly VL-mediated CAP recognition reported for RmAb3²⁷, RabmAb-4E was predicted to interact with CAP through three VH residues and one VL residue. This difference in the spatial distribution of binding contacts between RabmAb-4E (MBC-derived) and RmAb3 (ASC-derived) from the same immunized rabbit suggests that the MBC and ASC compartments may harbor antibodies with distinct antigen-recognition modes, potentially reflecting differences in the stage of affinity maturation or the selection pressures acting on these two B-cell populations. Despite this difference, both models suggested that polar interactions help position the CAP nitro group, hydrophobic contacts help stabilize CAP within the binding pocket, and CDR-L3 contributes to CAP recognition. In summary, the docking analysis suggested that shape complementarity and coordinated hydrogen-bonding,

electrostatic, aromatic, and hydrophobic interactions may stabilize the RabmAb-4E-CAP complex. These predictions provide a testable structural hypothesis for the sensitive and specific recognition of CAP by RabmAb-4E observed in the icELISA, and they provide a rational basis for future engineering efforts aimed at further enhancing affinity or altering specificity profiles.

4 Conclusions

In this study, we developed an integrated FACS-microfluidics pipeline for generating hapten-specific RabmAbs from individual antigen-specific MBCs while preserving natural VH/VL pairing. Using CAP as a model hapten, the pipeline recovered 22 paired VH/VL sequences and identified 10 antigen-binding scFvs, corresponding to a positive hit rate of 45.5%, which is substantially higher than typical scFv phage-display outputs (typically <5%) and hybridoma-derived antibody recovery rates (<10%). This high success rate likely reflects the efficient enrichment of antigen-specific MBCs by FACS and the preservation of functional VH/VL pairing during automated microfluidic cDNA preparation. The lead clone, 4E, was reformatted as a full-length RabmAb, and the resulting RabmAb-4E not only retained CAP-recognition activity but also exhibited superior analytical performance in an icELISA based on a heterogenous coating antigen. Under optimized conditions, the RabmAb-4E-based icELISA achieved an IC_{50} of 0.01 ng/mL, representing a 27-fold improvement over rabbit polyclonal antibodies and an 8-fold improvement over the previously reported ASC-derived RmAb3 isolated from the same immunized rabbit. The results suggest that the MBC compartment may harbor antibodies with more advanced affinity maturation than the ASC compartment at the time of sampling. Moreover, RabmAb-4E showed CR values below 0.001% toward structurally related analogues thiamphenicol, florfenicol, and florfenicol amine, and retained stable detection performance across broad ranges of ionic strength (0-6.0 M NaCl), pH (4.0-8.5), and methanol concentration (0-60%). Recoveries of 90.28%-117.53% in spiked skim milk and chicken samples, further supported its practical applicability for CAP residue analysis in food matrices. Molecular docking suggested that CAP recognition may be supported by shape-complementary pocket formed by the CDRs and multiple noncovalent interactions. The results demonstrate the practical utility of combining FACS-based sorting of

antigen-specific MBCs with microfluidics-based automated single-cell cDNA preparation for the rapid discovery of hapten-specific RabmAbs. This pipeline may facilitate the development of antibody against other small-molecule contaminants relevant to food safety monitoring.

CRediT authorship contribution statement

Chen Xing: Conceptualization, Investigation, Formal analysis, Writing-original draft.
Yuan Li: Investigation, Formal analysis, Writing-review & editing. **Yantong Pan:** Investigation, Methodology. **Bochen Lv:** Validation, Visualization. **Congming Wu:** Conceptualization, Investigation, Resources, Writing-review & editing. **Zhanhui Wang:** Conceptualization, Writing-review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article is available.

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Figure captions

Scheme 1. Integrated FACS-microfluidics pipeline for hapten-specific rabbit monoclonal antibody discovery. The pipeline comprises three sequential modules. **Left (Immunization and PBMC isolation):** New Zealand White rabbits are immunized with CAP-HS-KLH, and PBMCs are isolated from the peripheral blood of animal exhibiting the highest antisera affinity against CAP. **Middle (FACS-based sorting and microfluidics-based single-cell cDNA preparation):** **Step 1**, antigen-specific MBCs are sorted by FACS based on dual positivity ($\text{IgG}^+\text{antigen}^+$) using PE-conjugated goat anti-rabbit IgG (H+L) and FITC-CAP-HS-BSA as staining reagents. **Step 2**, the sorted antigen-specific MBCs are automatically processed on the Fluidigm C1 system for single-cell capture, cell lysis, reverse transcription, and cDNA harvest in the nanoliter-scale closed single-cell capture chambers within an IFC chip. The dashed inset shows a magnified view of a single-cell capture chamber containing an individual antigen-specific MBC. **Right (Paired VH/VL recovery and functional RabmAb generation):** The harvested cDNA products are used for paired VH/VL genes amplification, sequence analysis, scFv construction and functional screening, and subsequent reformatting of the lead clone into full-length RabmAb for functional characterization. The entire pipeline from cell sorting to antibody confirmation could be completed within one week, with automated microfluidic processing minimizing manual handling and preserving natural VH/VL pairing. BSA, bovine serum albumin; CAP, chloramphenicol; FACS, fluorescence-activated cell sorting; IFC, integrated fluidic circuit; MBC, memory B cell; PBMC, peripheral blood mononuclear cell; RabmAb, rabbit monoclonal antibody; scFv, single-chain variable fragment; VH, heavy-chain variable region; VL, light-chain variable region.

Figure 1. FACS-based sorting and microfluidics-based single-cell cDNA preparation of antigen-specific rabbit MBCs. (A) Confocal microscopy images of rabbit PBMCs stained with PE-conjugated goat anti-rabbit IgG (H+L) (red channel) and FITC-CAP-HS-BSA (green channel). Arrows indicate cells positive for both markers (double-positive, $\text{IgG}^+\text{antigen}^+$). Scale bars, 25 μm . (B) FACS gating strategy for isolating $\text{IgG}^+\text{antigen}^+$ cells from rabbit PBMCs. IgG^+ cells were selected in P4 (29.17% of live singlet lymphocytes), and antigen^+ cells within the IgG^+ population were further gated in P5 (1.10% of IgG^+ cells). The complete P1-P5 gating strategy is shown in Figure S4. (C) Bright-field microscopy image showing single-cell capture in an IFC capture chamber using the Fluidigm C1 system. (D) Fragment-size distribution of single-cell cDNA analyzed by Agilent 2100 Bioanalyzer, showing high-molecular-weight

cDNA products at approximately 5,000 bp. (E) Agarose gel electrophoresis of paired VH and VL fragments amplified from single-cell cDNA templates. Both VH and VL amplicons show the expected size of approximately 400 bp. M, DNA marker. FACS, fluorescence-activated cell sorting; PBMC, peripheral blood mononuclear cell; IFC, integrated fluidic circuit; PE, phycoerythrin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; CAP, chloramphenicol; VH, heavy-chain variable region; VL, light-chain variable region.

Figure 2. Generation and functional validation of CAP-specific antibodies. (A) The initial screening of 22 crude scFv extracts for binding to CAP-HS-BSA by direct binding ELISA. Numbers indicate the OD₄₅₀ values measured in the absence of free CAP (0 ng/mL). Light-green squares denote the 10 binding-positive clones (10/22, 45.5%), whereas gray squares labeled 0 denote the non-binding clones. (B) Amino acid sequences of the VH and VL regions of clone 4E, annotated according to the IMGT numbering system. The complementarity-determining regions (CDRs; CDR1-CDR3 for both heavy and light chains) are indicated by colored arrows above the sequences. Framework regions (FRs, FR1-FR4) are not highlighted for clarity. (C) Competitive inhibition curve of RabmAb-4E (full-length rabbit IgG) against free CAP. The inset shows the schematic structure of CAP-HS-BSA, the coating antigen used in the assay. CAP-HS-BSA and RabmAb-4E were used at their respective optimal concentrations, as determined by checkerboard titration (Table S2). RabmAb-4E showing an IC₅₀ value of 1.04 ng/mL, approximately fourfold lower than that of scFv-4E under the same assay conditions (4.16 ng/mL), confirming that the 4E-derived VH/VL pair retained functional antigen-binding activity after reformatting. Error bars represent the standard deviation of triplicate measurements. CAP, chloramphenicol; CDR, complementarity-determining region; ELISA, enzyme-linked immunosorbent assay; FR, framework region; BSA, bovine serum albumin; IC₅₀, half-maximal inhibitory concentration; icELISA, indirect competitive enzyme-linked immunosorbent assay; IgG, immunoglobulin G; OD, optical density; RabmAb, rabbit monoclonal antibody; scFv, single-chain variable fragment; VH, heavy-chain variable region; VL, light-chain variable region.

Figure 3. Analytical performance and matrix evaluation of the RabmAb-4E-based icELISA. (A-C) Competitive inhibition curves for CAP obtained using the RabmAb-4E-based icELISA with CAP-OPA-OVA (A), CAP-N-6AS-OVA (B), and CAP-NH₂-PTSC-OVA (C) as coating antigens. For each assay, the coating antigen and RabmAb-4E were used at their respective optimized concentrations, as

determined by checkerboard titration (Table S2). The corresponding IC_{50} values were 0.54, 0.03, and 0.37 ng/mL, respectively. The insets show the schematic structures of the corresponding coating antigens. (D) Standard inhibition curve for CAP detection under optimized conditions, fitted using a four-parameter logistic model. The assay achieved an IC_{50} of 0.01 ng/mL with a linear range (IC_{20} - IC_{80}) of 0.003-0.043 ng/mL. The inset shows the schematic structure of CAP-N-6AS-OVA, the coating antigen used in the optimized assay. (E) Comparison of CAP standard curves prepared in assay buffer and skim milk matrix. The curves were nearly superimposable, indicating negligible matrix interference. (F) Comparison of CAP standard curves prepared in assay buffer and chicken extract. The curves showed good agreement, confirming effective matrix effect minimization. Error bars represent the standard deviation of triplicate measurements. CAP, chloramphenicol; IC_{50} , half-maximal inhibitory concentration; IC_{20} and IC_{80} , concentrations giving 20% and 80% inhibition, respectively; icELISA, indirect competitive enzyme-linked immunosorbent assay; OD, optical density; OVA, ovalbumin; RabmAb, rabbit monoclonal antibody.

Figure 4. Molecular docking analysis of RabmAb-4E-CAP interaction. (A) Surface representation of the CAP-binding pocket, showing close surround of the ligand, suggesting favorable shape complementarity between the antibody paratope and the small-molecule hapten. The pocket surface is shown in light purple. (B) Molecular surface of the binding pocket colored by hydrogen-bonding potential, showing a complementary distribution of hydrogen-bond donor (magenta) and acceptor (green) regions around CAP. (C) Molecular surface colored by hydrophobicity (brown, hydrophobic; blue, hydrophilic), revealing spatially organized hydrophobic regions surrounding the aromatic and nonpolar portions of CAP. The predominance of hydrophobic and aromatic contacts is consistent with the extreme salt tolerance observed experimentally (Figure S6A), whereas the precise hydrogen-bonding geometry surrounding the nitro group of CAP provides a structural rationale for the negligible cross-reactivity with structurally related analogues (Table S3). (D) Overall docking pose of the Fv region of RabmAb-4E (ribbon representation) docked with CAP (stick representation, colored by atom type). The CDR loops (CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3) are labeled. The selected pose, ranked by CDOCKER interaction energy, places CAP within a pocket formed primarily by the CDRs of both heavy and light chains. (E) Close-up view of the ligand-binding interface showing the four key residues, TrpH52 (FR-H2), GlyH110 (CDR-H3), AlaH113 (CDR-H3), and AspL116 (CDR-L3), predicted to contribute to CAP recognition. (F) Two-dimensional interaction diagram depicting the predicted intermolecular contacts between RabmAb-4E

1043 and CAP. TrpH52 engages in a π - π T-shaped interaction with the phenyl ring of CAP and form a
 1044 carbon-hydrogen bond with an oxygen atom of the nitro group; GlyH110 forms a carbon-hydrogen bond;
 1045 AlaH113 provides a π -alkyl contact; and AspL116 contributes attractive charge and π -anion interactions.
 1046 CAP, chloramphenicol; CDR, complementarity-determining region; FR, framework region; Fv, fragment
 1047 variable; RabmAb, rabbit monoclonal antibody; VH, heavy-chain variable region; VL, light-chain variable
 1048 region.

Table 1. Comparison of RabmAb-4E with previously reported CAP-specific antibodies.

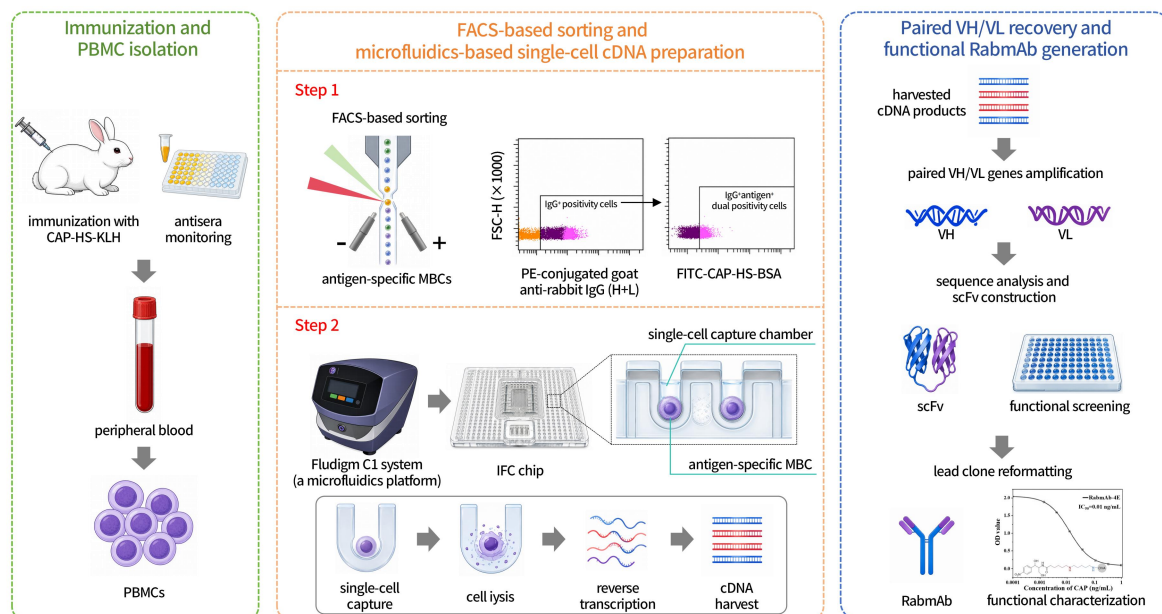
Antibody	Host/format	Discovery method	IC ₅₀ (ng/mL)	Reference
RabmAb-4E	Rabbit IgG	FACS-microfluidics pipeline ^a	0.01	This study
RmAb3	Rabbit IgG	CSMN ^b	0.08	²⁷
CAP-9-3	Rabbit IgG	Rabbit hybridoma	1.06	⁵⁴
6F11	Mouse IgG	Mouse hybridoma	0.01	⁵⁵
2D2	Mouse IgG	Mouse hybridoma	0.46	⁵⁶
4B5	Mouse IgG	Mouse hybridoma	0.23	⁵⁷
Chl-D1	Llama VHH	Phage display	1034.7*	⁵⁸

Note: ^aThe FACS-microfluidics pipeline developed in this study integrates FACS-based sorting of antigen-specific MBCs with microfluidics-based automated single-cell cDNA preparation. ^bCSMN denotes an indirect competitive screening method in nanowells used to select individual CAP-specific rabbit ASCs. *The IC₅₀ of Chl-D1 was converted from 3.202 μM using the molecular weight of CAP (323.13 g/mol). ASC, antibody-secreting cell; CSMN, competitive screening method in nanowells; FACS, fluorescence-activated cell sorting; MBC, memory B cell; VHH, variable domain of a heavy-chain-only antibody.

Table 2. Recovery, CV, LOD and linear range for CAP in spiked samples using the RabmAb-4E-based icELISA.

Sample	Spiking level ^a (µg/L or µg/kg)	Recovery (%)	CV (%)	LOD ^a (µg/L or µg/kg)	Linear range ^a (µg/L or µg/kg)
Skim milk	0.005	95.12	8.56	0.0012	0.003-0.039
	0.01	112.84	3.94		
	0.02	117.53	6.69		
Chicken	0.01	90.28	10.75	0.0025	0.004-0.053
	0.02	105.02	6.12		
	0.04	112.15	8.73		

Note: Each spiking level was analyzed in triplicate ($n = 3$), and recovery values are reported as the mean. Recovery (%) = (measured concentration/spiked concentration) $\times$ 100, and CV (%) = (standard deviation/mean value) $\times$ 100. The LOD was defined as the CAP concentration corresponding to the mean signal of 20 CAP-negative blank samples minus three times the standard deviation. The linear range was defined as IC₂₀-IC₈₀. ^aConcentrations are expressed in µg/L for skim milk and µg/kg for chicken samples. CAP, chloramphenicol; CV, coefficient of variation; IC₂₀ and IC₈₀, concentrations producing 20% and 80% inhibition, respectively; icELISA, indirect competitive enzyme-linked immunosorbent assay; LOD, limit of detection; RabmAb, rabbit monoclonal antibody.



Scheme 1.

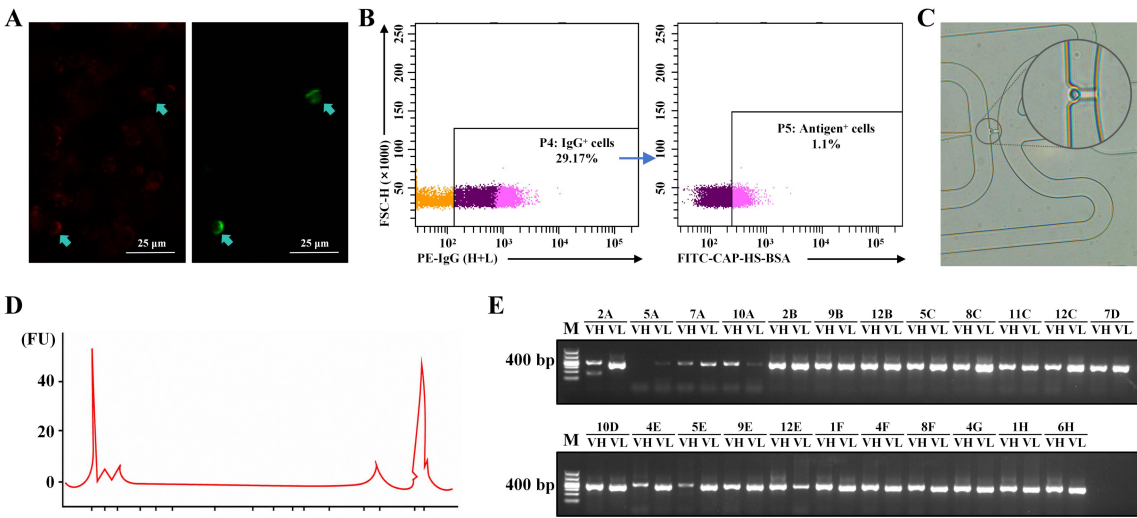


Figure 1.

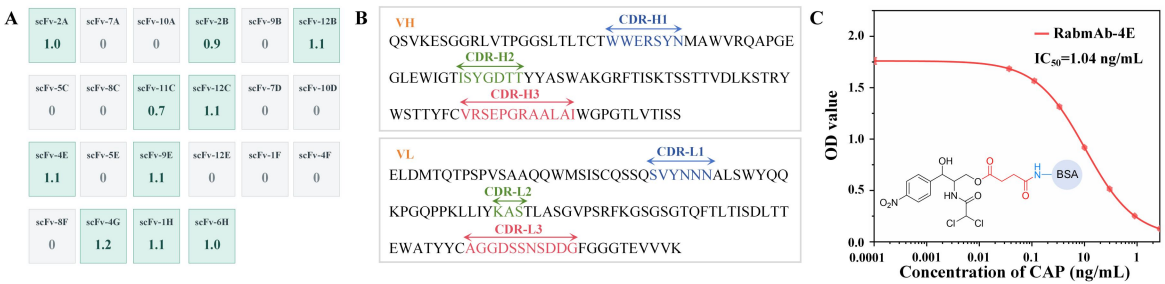


Figure 2.

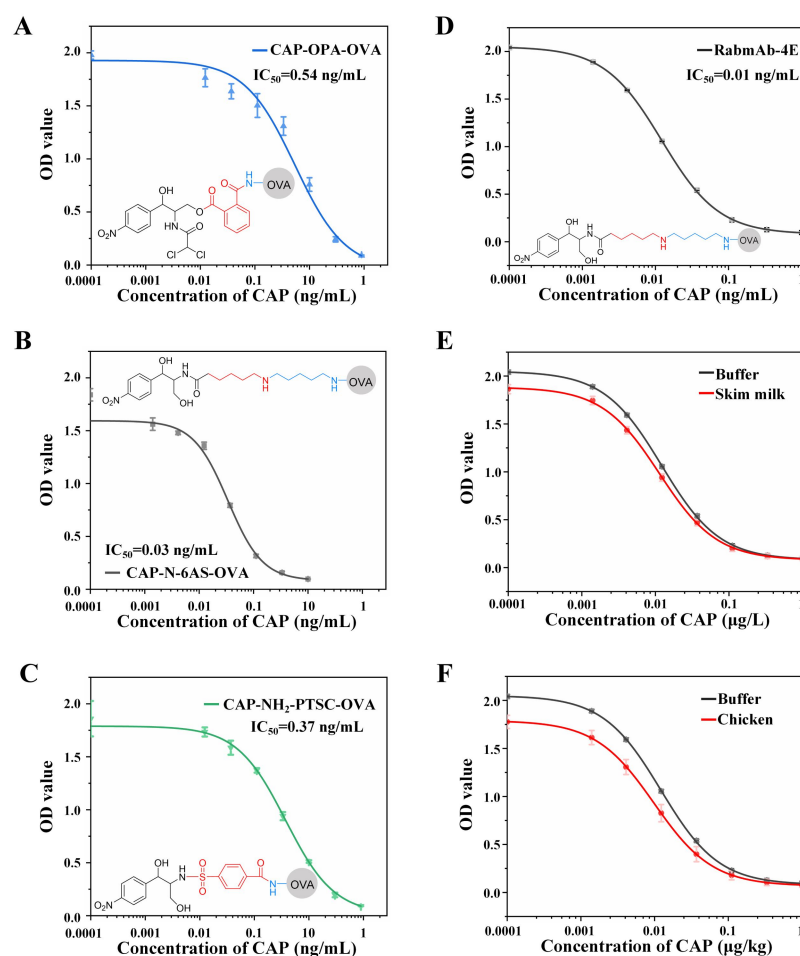


Figure 3.

