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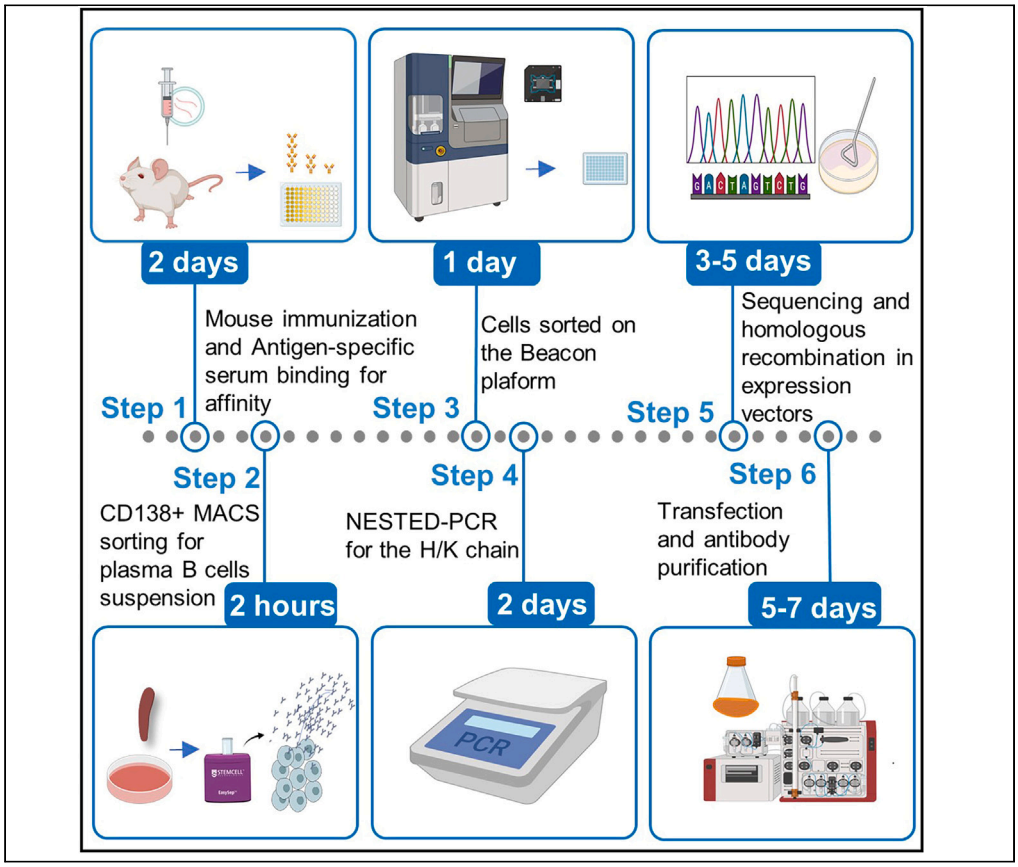
Protocol for isolating antigen-specific monoclonal antibodies from immunized mice utilizing the Beacon platform



Isolation of antigen-specific plasma B cells had been challenging until the recent arrival of the Beacon platform. Leveraging light-sorting technology, Beacon can perform high-throughput screening of plasma B cells on a chip to sort single cells with the desired antigen specificity. Here, we present a protocol for isolating antigen-specific plasma B cells from immunized mice using Beacon, sequencing the encoded B cell receptors (BCRs), and cloning and expressing the resulting antibodies. This protocol can easily be extended to human samples.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for enriching plasma B cells from mouse spleens

Isolates single antigen-specific antibody-secreting cells using the Beacon platform

Performs PCR on RNA purified from sorted single cells to retrieve antibody sequences

Procedures for cloning, expressing, and purifying the paired antibody chains

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Protocol for isolating antigen-specific monoclonal antibodies from immunized mice utilizing the Beacon platform

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SUMMARY

Isolation of antigen-specific plasma B cells had been challenging until the recent arrival of the Beacon platform. Leveraging light-sorting technology, Beacon can perform high-throughput screening of plasma B cells on a chip to sort single cells with the desired antigen specificity. Here, we present a protocol for isolating antigen-specific plasma B cells from immunized mice using Beacon, sequencing the encoded B cell receptors (BCRs), and cloning and expressing the resulting antibodies. This protocol can easily be extended to human samples.

BEFORE YOU BEGIN

Antibody response is a critical arm of adaptive immunity and antibody-mediated therapeutics have been explored for treating human diseases ranging from cancers to viral infections. The recent development of the ability to interrogate antibody response at single B cell level not only advances our understanding of humoral immune response but also facilitate the discovery of antibody with desired potency and specificity from the natural repertoires. Most of these advancements have been on memory B cells, which express BCR receptors on their surface, making them isolatable using flow cytometry after being labeled with cognate antigens.[1,2] With antibodies releasing from the producing cells, single-cell analysis of the antibody-secreting B cell subpopulation, the plasma cells, is more challenging. A conventional screening method often entails time- and material-consuming cell expansion to reach antibody levels in the cell culture supernatants sufficient for antigen-based assessments such as an enzyme-linked immunosorbant assay (ELISA) and, to a greater extent, functional characterizations. This scenario is radically changes with the advent of microfluid technology, which enable maneuvering single B cells in a micro volume to attain highly concentrated antibody production. The Berkeley Beacon and Lightning system (abbreviated as Beacon) is a recently developed platform that combines microfluidic technology with OptoElectro Positioning (OEP).[3,4] Based on the principle of light-induced dielectrophoresis, OEP utilizes a light beam to nondestructively manipulate in a high-throughput manner on a chip. The chip contains thousands of nanoliter-size

microchambers, known as nanopen chambers, designed to hold single cells that can be individually moved in and out. Currently, Berkeley Lighting produces four types of chips, 3600/11K/14K/20K, with the corresponding numbers of nanopens being 3600, 11000, 14000, and 20000, respectively. In the case of plasma cells, the secreted antibodies are analyzed per nanopen for their binding to the circulating fluorescence-based detection reagent in the chip, with positive hits being identified by fluorescent "blooms" above the chambers. The positive cells are then exported from the chip and subjected to single-cell sequencing of the antibody's heavy chain variable domain (VH) and the light chain variable domain (VL). Subsequently, antibody cloning and expression are performed to purify antibodies for experimental validation and further characterization.

This protocol is divided into four parts as follows: Enriching CD138+ plasma B cells from the spleens of vaccinated mice; Using the Beacon platform for high-throughput screening and sorting of plasma B cells producing antigen-specific antibodies; Performing single B-cell sequencing on sorted plasma cells to obtain paired antibody VH and VL sequences; Cloning, expressing, and purifying antibodies for subsequent analysis of antigen binding affinity and functional properties.

Experimental design considerations

In this study, the plasma cells used in Beacon screening were derived from mice immunized with an mRNA vaccine encoding the RABV-G protein of the rabies virus,[5] aiming to identify RABV-G-specific antibodies, particularly those with neutralizing activities. When using immunized animals as the source of plasma cells, the vaccine type(s) and immunization regimen should be optimized to ensure the production of an antibody repertoire with the desired breadth and target specificity. Additionally, the timing of collecting plasma cells for maximum yield should be determined. Considering individual variation in immune response, we recommend screening the immunized animals for the highest serum antigen-specific antibody titers using the ELISA assay, as was performed in this study.

Depending on the number of positive hits that need to be unpenned from the chip, the operations on Beacon could be time-consuming, and scheduling in advance may be necessary.

Sequence analyses of VH and VL segments, including their V (D) J composition and the location of mutations differing from their germline counterpart, can be conducted using the IgBlast or IMGT website (IgBLAST, http://www.ncbi.nlm.nih.gov/igblast; IMGT, http://www.imgt.org).

Given that mouse antibodies strongly favor kappa light chains over lambda light chains with a ratio of 10:1 to 20:1,[1] we only recovered the kappa light chains in this study.

Institutional permissions

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Public Health Clinical Center and conducted in strict adherence to animal ethics guidelines.

Preparation 1: Mouse immunization and selection

Timing: Mouse immunization: 1–2 h; ELISA-based serum sample analysis: 2 days

In this study, the plasma cells used in Beacon screening were derived from mice immunized with an mRNA vaccine encoding the RABV-G protein of the rabies virus, namely the RABV-G mRNA-LNP vaccine.[5] The vaccination was administered using a prime-boost scheme, with one-and-a-half-year interval between the two doses. Choosing such a long interval between primary and booster immunization was based on preliminary studies indicating that it is advantageous compared to a short interval in promoting germinal center B cell response and increasing the number of antigen-specific antibody-producing cells. Animals that showed the highest antigen-specific serum titers were selected based on enzyme-linked immunosorbent assays (ELISA) of their blood samples and

subsequently used to collect spleens for plasma cell isolation. The ELISA EC50 value for the serum of the mice used in the study, calculated as the serum dilution at which the optical density was half of the maximum absorbance after subtracting the background, was approximately 78709. Spleen cell separation was conducted 7 days after the booster immunization.

⚠ CRITICAL: Animal manipulation should be conducted in accordance with relevant institutional guidelines and regulations, adhering to protocols approved by local animal ethics committees. As such, in this study, all animal experiments received approval from the Shanghai Public Health Center Local Ethical Committee.

Preparation 2: Biotinylating detection antigens

Timing: 1 h

In this study, we utilized biotin-labeled RABV-G proteins coated on assay beads for screening plasma cells on the Beacon platform. The biotinylation procedures were conducted using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, A39257), following the manufacturer's instructions (Protocol for EZ-Link Sulfo-NHS-LC-Biotin). The biotinylated protein could be kept at 4°C for up to one week or at −80°C for long-term storage.

Preparation 3: Preparation of buffers for use on the beacon platform

Timing: 1 h

The Opto Plasma B Discovery Workflows require four separate media, which are all prepared using the Opto Plasma B Discovery Sample Prep Kit, Mouse (750-02050) as listed in the key resources table.

Note: We regularly prepare the media freshly. In case where advance preparation is necessary, we recommend storing the prepared media in a 4-degree refrigerator for up to 1 month without losing activity. If storage exceeds 7 days, filtration through a 0.22 μm-pore-size membrane is required to remove potential precipitate that could block the channels or paths of the Beacon machine.

Contents of Opto Plasma B Discovery Sample Prep Kit (Mouse)

Reagent	Storage
1 × DNA Clean Up	−20°C
1 × Loading Reagent	−20°C
1 × Basal Media	4°C
1 × Media Additive A1	−20°C
1 × Media Additive A2	4°C
1 × Media Additive P1 (M)	4°C
1 × Media Additive P2 (M)	−20°C
2 × Wetting Additive	4°C

1. Prepare Wash Media.
   (Wash Media is used to wash the collected mouse cells).
   a. Transfer 18 mL of RPMI + 2 mL of FBS to a 50-mL conical tube.
   b. Add 20 μL of thawed DNA Clean Up and pipette to ensure thorough mixing.
   c. Label the bottle with the preparation date tore it at 4°C until use.

Note: The resulting medium should contain 10% FBS and DNA Clean Up at a 1:1,000 dilution.

2. Prepare Base Media.

(Base Media is used to prepare Loading Media and Plasma B Cell Media).

a. Thaw Media Additives A1, P1, and P2 and Loading Reagent at room temperature (20°C–25°C in our lab).

b. Prepare Base Media by mixing the reagents in a 250-mL Nalgene bottle in a biosafety cabinet.

c. Label the bottle with the preparation date tore it at 4°C until use.

Base Media Preparation

	Volume per workflow			
Reagent	1 chip	2 chips	3 chips	4 chips
Basal Media	44.7 mL	73.1 mL	100.6 mL	129 mL
Media Additive A1	7.3 mL	11.9 mL	16.4 mL	21 mL
Media Additive A2	52 µL	85 µL	117 µL	150 µL
Media Additive P1	52 µL	85 µL	117 µL	150 µL
Media Additive P2	52 µL	85 µL	117 µL	150 µL
Final Volume	52.2 mL	85.3 mL	117.4 mL	150.5 mL

Note: Given the larger volume of the bottle of Media Additive A1, begin thawing it at room Temperature (20°C–25°C in our lab), 15–20 min earlier than other reagents.

3. Prepare Loading Media.

(Loading Media is used to resuspend cells for loading on the beacon).

a. Prepare Loading Media by mixing the following components in a 125-mL Nalgene bottle in a biosafety cabinet.

Loading Media Preparation

	Volume per workflow			
Reagent	1 chip	2 chips	3 chips	4 chips
Basal Media	12.5 mL	22 mL	31 mL	40 mL
Loading Reagent	250 µL	440 µL	620 µL	800 µL
Wetting Additive (stock solution)	250 µL	220 µL	310 µL	400 µL
Final Volume	12.9 mL	22.7 mL	31.9 mL	41.2 mL

b. Label the bottle with the preparation date and store it at 4°C until use.

4. Prepare Plasma B Cell Media.

(Plasma B Cell Media is used during the workflow steps).

a. Prepare Plasma B Cell Media using the following recipe in a 125-mL Nalgene bottle in a biosafety cabinet.

Plasma B Cell Media Preparation

	Volume per workflow			
Reagent	1 chip	2 chips	3 chips	4 chips
Base Media	37 mL	58 mL	79 mL	100 mL
Wetting Additive (stock solution)	370 µL	580 µL	790 µL	1000 µL
Final Volume	37.4 mL	58.6 mL	79.8 mL	101 mL

b. Label the bottle with the preparation date.

c. Set aside 1.5 mL of the media in a 1.5-mL microcentrifuge tube at 4°C for later preparation of assay reagents.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 568 (1:250)	Invitrogen	Cat#A-11031; RRID: AB_144696
Goat anti-mouse IgG Fc secondary antibody, FITC 2 mg (1:250)	Invitrogen	Cat#31547; RRID: AB_228294
HRP-conjugated goat anti-human IgG (H + L) (1:5,000)	ZSGB-BIO	Cat#ZB-2304; RRID: AB_3608281
HRP-conjugated anti-mouse IgG (1:5,000)	ABclonal	Cat#AS003; RRID: AB_2769851
Bacterial and virus strains		
DH5α chemically competent cell (alternative referred)	Tsingke Biotech	TSC-C14
RABV-G mRNA-LNP vaccine	Bai et al.[5]	N/A
Biological samples		
Spleens from BALB/cJGpt[GF] mice	GemPharmatech	RRID:IMSR_GPT:N000296
Chemicals, peptides, and recombinant proteins		
RABV-G protein	AtaGenix	Cat#ATAP10593
Mineral oil	Sigma	Cat#M5904
Fetal bovine serum	EallBio	Cat#03.U16001DC
Buffer TCL, 2×	QIAGEN	Cat#1070498
Superscript III Reverse Transcriptase (50 reactions)	Invitrogen	Cat#18080044
NP40S-100 mL, TERGITOL solution	Sigma	Cat#NP40S-100ML
USB dithiothreitol (DTT), 0.1 M solution	Thermo Scientific	Cat#707265ML
HotStarTaq DNA polymerase (250 U)	QIAGEN	Cat#203203
dNTP Set, 100 mM solutions	Thermo Scientific	Cat#R0182
RNaseOUT Recombinant ribonuclease inhibitor	Thermo Scientific	Cat#10777019
IMDM	Gibco	Cat#31980030
DMEM	Corning	Cat#10-013-CV
PBS	Absin	Cat#abs962
SMM 293TII Expression medium	Sino Biological	Cat#M293TII
Opti-MEM	Gibco	Cat#31985070
Tris pH 8.0	Sangon Biotech	Cat#B548127-0500
Oxoid Tryptone	Thermo Scientific	Cat#LP0042B
Oxoid yeast extract	Thermo Scientific	Cat#LP0021B
NaCl	Yeasen	Cat#60372ES76
DPBS (-Mg^{2+}, -Ca^{2+})	Thermo Scientific	Cat#14190250
EDTA (0.5 M), pH 8.0, RNase-free	Thermo Scientific	Cat#AM9260G
10× loading buffer (alternative referred)	Takara Bio	Cat#9157
Trypan blue solution, 0.4%	Thermo Scientific	Cat#15250061
Ampicillin	Sigma	Cat#A9518-100G
Penicillin-streptomycin (alternative referred)	NCM Biotech	Cat#C100C5
FastDigest SalI	Thermo Scientific	Cat#FD0644
Pfl23II (BsiWI)	Thermo Scientific	Cat#ER0851
FastDigest AgeI (BshTI)	Thermo Scientific	Cat#FD1464
PEI MAX 40K	Polysciences	Cat#24765
RIPA lysis buffer	Thermo Scientific	Cat#89901
4× SDS PAGE loading buffer (alternative referred)	Takara Bio	Cat#9173
180 kDa prestained protein marker	Vazyme	Cat#MP201
1 M Tris-HCl solution, pH 8.5, sterile (or self-made)	Sangon Biotech	Cat#B548141-0500
QuickBlue	Biodragon	Cat#BF06152
Agarose	Yeasen	Cat#10208ES60
$Na_2HPO_4 \cdot 12H_2O$	Sinopharm Chemical	Cat#100203008
Glycine (alternative referred)	Yeasen	Cat#60112ES76
Skim milk (alternative referred)	Yeasen	Cat#36120ES76

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NCM ECL ultra	NCM Biotech	Cat#P10300B
H_2SO_4 (1 M)	Sinopharm Chemical	Cat#10021618
EZ-Link Sulfo-NHS-LC-Biotin	Thermo Scientific	Cat#A39257
SuperSignal West Pico Plus chemiluminescent substrate	Thermo Scientific	Cat#34580
Substrate OPD	Thermo Scientific	Cat#34006
Critical commercial assays		
EasySep Mouse CD138 Pos Selection Kit	STEMCELL Technologies	Cat#18957
ClonExpress One Step Cloning Kit	Vazyme	Cat#C112
Wizard SV Gel and PCR Clean-Up system	Promega	Cat#A9282
Agencourt RNAClean XP Kit	Beckman Coulter	Cat#A63987
Plasmid Mini Kit I	Omega Bio-tek	Cat#D6943-02
Endo-free Plasmid Mini Kit II	Omega Bio-tek	Cat#D6950-02
NucleoBond Xtra Midi Kit EF	MACHEREY-NAGEL	Cat#740420.50
Opto Plasma B Discovery Sample Prep Kit, mouse	Berkeley Lights	Cat#750-02050
OptoSelect Chip 11k	Berkeley Lights	Cat#750-08090
Eppendorf twin.tec 96-well PCR plates	Eppendorf	Cat#951020401
Adhesive PCR plate seals	Thermo Scientific	Cat#AB0558
Flush Chip	Berkeley Lights	Cat#500-00030
Assay Beads (Clear, 4°C)	Berkeley Lights	Cat#520-00053
BLI cleaning solution	Berkeley Lights	Cat#520-08000
Import Wells (100 pack)	Berkeley Lights	Cat#750-08096
MagDTR 96-well magnetic separator.	EdgeBio	Cat#57624
AbcapA/G 4FF (alternative referred)	Smart-Lifesciences	Cat#SA032C55
Experimental models: Cell lines		
HEK293T cell	ATCC	Cat#CRL-3216; RRID:CVCL_0063
Gibco FreeStyle 293-F	Thermo Scientific	Cat#R79007; RRID:CVCL_D603
Experimental models: Organisms/strains		
Mice: BALB/cJGpt[GF] (Female, 6–8 weeks old)	GemPharmatech	RRID:IMSR_GPT:N000296
Oligonucleotides		
Random Primers (0.3 mg/mL) (100 μL)(200 reactions)	Invitrogen	Cat#48190011
Primers for NEST-PCR	von Boehmer et al.[1]	See Table S1
Primers for cloning PCR	von Boehmer et al.[1]	See Table S1
Recombinant DNA		
IGg1 expression vector: AbVec2.0-IGHG1	Addgene	RRID:Addgene_80795
IGk expression vector: AbVec1.1-IGKC	Addgene	RRID:Addgene_80796
Software and algorithms		
IgBlast	NCBI	RRID:SCR_002873: http://www.ncbi.nlm.nih.gov/igblast/
GraphPad Prism version 9	GraphPad Software	RRID:SCR_002798: https://www.graphpad.com/features
BioRender	BioRender online	RRID:SCR_018361: https://www.biorender.com/
Opto Plasma B Discovery 2.0 Workflow	Berkeley Lights	https://techsupport.berkeleylights.com/
Other		
Beacon	Berkeley Lights	https://techsupport.berkeleylights.com/
PCR thermocycler (compatible with full-skirted plates)	Eppendorf	Cat#6311000070
ChemiDOC MP imaging system	Bio-Rad	Cat#12003154
Biosafety cabinet	Hair	Cat#HR-40IIA2
Incubator shakers	SH Scientific	Cat#IS-RDS3
Automated cell counter TC-20	Bio-Rad	Cat#14501002-0G
Synergy microplate reader	Bio-Tek	Cat#CYTATION imaging reader 5
NanoDrop One	Thermo Scientific	Cat#701-058112
15-mL High-Clarity PP centrifuge tube, conical bottom, with dome seal screw cap, sterile	yueyiBio	Cat#YB0019-15

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
50-mL High-Clarity PP centrifuge tube, conical bottom, with dome seal screw cap, sterile	yueyiBio	Cat#YB0019-50
Nunc EasYDish -6 cm	Thermo Scientific	Cat#150462
Nunc EasYFlask 25 cm^2	Thermo Scientific	Cat#156367
Nunc EasYFlask 75 cm^2	Thermo Scientific	Cat#156499
PCR plates	SAVIFICO	Cat#20296-201
Nalgene Square PETG media bottles with closure, 125 mL	Thermo Scientific	Cat#2019-0125
Nalgene Square PETG media bottles with closure, 250 mL	Thermo Scientific	Cat#2019-0250
Nalgene Square PETG media bottles with closure, 500 mL	Thermo Scientific	Cat#2019-0500
Eppendorf 12 multichannel pipette: 10 μL, 100 μL, 300 μL	Eppendorf	Cat#3122000027
Eppendorf pipette	Eppendorf	Cat#3122000043
Falcon 70 μm cell strainer, white, sterile, individually packaged, 50/case	Corning	Cat#352350
Amicon Ultra-15 centrifugal filter, 10 kDa MWCO	Millipore	Cat#UFC901096
1.5-mL microcentrifuge tube	Thermo Scientific	Cat#509-GRD-Q
Tubes, 0.2-mL, flat cap	Thermo Scientific	Cat#AB0620
3 mL more longer transfer pipets	WHB	Cat#WHB-S4
Nalgene single-use PETG Erlenmeyer flasks with plain bottom: sterile	Thermo Scientific	Cat#4115-0500

MATERIALS AND EQUIPMENT

- Solutions for antibody column purification using the AKTA system.

Solution 1: Equilibration and wash buffer, pH = 7.4

Reagent	Final concentration	Amount
Na_2HPO_4	20 mM	7.16 g
Nacl	150 mM	8.8 g
ddH_2O	N/A	1 L
Total	N/A	1 L

Filtration and removal of bacteria by 0.22 μm filter membrane vacuum pump. Store at 4°C for up to 2 weeks.

Solution 2: Elution buffer, pH = 3.0

Reagent	Final concentration	Amount
glycine	100 mM	7.5 g
ddH_2O	N/A	1 L
Total	N/A	1 L

Filtration and removal of bacteria by 0.22 μm filter membrane vacuum pump. Store at 4°C for up to 2 weeks.

- Bacterial growth mediums

LB liquid medium, pH = 7.0

Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
Nacl	10 g/L	10 g
Yeast extract	5 g/L	5 g
ddH_2O	N/A	1 L
Total	N/A	1 L

Autoclave at 121°C for 20 min. After cooling down, add ampicillin (50 mg/mL) to the medium at a ratio of 1:1,000. Use immediately or stored at 4°C for up to 1 month.

Note: To prepare LB + Ampicillin (LBA) plates, use the same recipe and autoclaving conditions but include 20 g/L agar in the mixture. Then, pour the medium into 6 cm plates rapidly after cooling down and adding ampicillin. Use immediately or stored at 4°C for up to 1 month.

- Buffer used in plasma B cell isolation.

MACS buffer		
Reagent	Final concentration	Amount
1× DPBS	N/A	97.8 mL
FBS	2%	2 mL
EDTA (0.5 M)	1 mM	200 μL
Total	N/A	100 mL
Store at 4°C for up to 2 weeks.		

Alternative reagents for items with limited availability		
Reagent or resource	Source	Identifier
DH5α Chemically Competent Cells	Thermo Scientific	CAT#EC0112
DNA Gel Loading Dye (6×)	Thermo Scientific,	CAT#R0611
Penicillin-Streptomycin	Gibco	CAT#15070063
4× SDS sample buffer	Merck Millipore;	CAT#70607
Skim milk powder	Serva Electrophoresis, Germany	CAT#42590.01,
HiTrap Protein A HP antibody purification column, 1 mL	Cytiva	CAT#17040201

STEP-BY-STEP METHOD DETAILS

Plasma cell sample preparation

Timing: 2 h

This step involves preparation of single B-cell suspension from mouse spleen tissue and the enrichment of plasma B cells that are ready to be loaded onto the Beacon optofluidic system.

1. Preparation of single B-cell suspension.
   a. Euthanize mice according to the institutional guidelines.
   b. Remove the mouse spleen tissue and place it in a petri dish containing 5 mL of RPMI+10% FBS culture medium.
   c. Place a 40 μm cell strainer on a sterile 50 mL conical tube. Pass 2 mL of RPMI+10% FBS to prime it.
   d. Wrap the spleen in double layers of gauze and clamp the gauze with tweezers.
      i. Gently rub it repeatedly.
      ii. Use a 1 mL pipettor to draw the cell suspension through the gauze, and transfer it to the primed cell strainer.
      iii. Rinse the tissue with the medium in the dish and transfer the medium to the strainer.
   e. Centrifuge the filtrate at 800 × *g* for 5 min.
   f. Carefully remove the supernatant without disturbing the pellet.
   g. Resuspend the pellet in 1 mL of pre-cooled MACS buffer.
   h. Repeat gentle pipetting to make a single-cell suspension.
   i. After counting the cells by using automated cell counters or a hemocytometer, adjust the volume to achieve a cell concentration of 1 × 10^8 cells/mL.

2. Enrichment of plasma B cells from the cell suspension by using mouse CD138 MicroBeads, following the manufacturer's protocol. Protocol for EasySep Mouse CD138 Positive.

Note: Ensure that pre-cooled MACS buffer is used for all incubation and wash steps.

3. Preparation of cell sample for loading onto the Beacon platform
   a. Take an aliquot of the isolated plasma B cells and stain them with trypan blue. Count the cells and check their viability.
   b. Centrifuge the tube at 300 × *g* at 4°C for 5 min.
   c. Resuspend the cell pellets in an appropriate amount of Loading Media attain a target density of 7.0 × 10^6 cells/mL.
   d. Depending on the number of chips to be used, transfer the desired amount of plasma B cells into a 0.2-mL microcentrifuge tube.

Note: Keep the tube on ice until ready to load the cells onto the Beacon platform.

   e. The information regarding target density and sample volumes (number of cells) required for 1–4 Chips is provided in the table below.

Targeted cell loading

OptoSelect Chip type	On-chip cell density (target)	1 Chip	2 chips	3 chips	4 chips
11k	7.0 × 10^6 cells/mL	5.5 μL (38,500 cells)	9 μL (63,000 cells)	12.5 μL (87,500 cells)	16 μL (112,000 cells)

Note: All procedures should be processed aseptically inside a biosafety cabinet.

Assay beads preparation

Timing: 4 h

The Beacon system detects antibodies secreted by the chambered plasma B cells using antigen-specific bead-based assays. The following protocol describes a method for coating assay beads with biotinylated soluble antigen, which will be utilized for antibody detection.

4. Preparation of assay beads.
   a. Transfer 100 μL of assay beads to a 1.5-mL microcentrifuge tube.
   b. Centrifuge the beads at 3,000 × *g* for 5 min, 4°C, then remove the supernatant.
   c. Resuspend the beads in 1 mL of DPBS.
   d. Centrifuge the beads at 3,000 × *g* for 5 min, 4°C, then remove the supernatant.
   e. Repeat steps c & d two more times.
   f. Resuspend the beads in 1 mL of DPBS in 1.5-mL microcentrifuge tube for a final bead concentration of 0.5% w/v.
5. Coating assay beads with antigen.
   a. Add 5 μg of biotinylated soluble antigen (RABV-G protein in this protocol) to 1 mL of the assay bead suspension in step 4f. The final concentration of biotinylated antigen protein in the mixture should be 5 μg/mL.
   b. Rotate the mixture on a rocker at 4°C for a minimum of 3 h.
   c. Centrifuge the tube at 3,000 × *g* at 4°C for 5 min. Remove the supernatant, which contains the unbound biotinylated protein.
   d. Resuspend the beads in 1 mL of DPBS.
   e. Centrifuge the tube at 3,000 × *g* for 5 min. Then, remove the supernatant.

f. Repeat steps d & e two more times.
g. Resuspend the beads in DPBS to achieve a final volume of 50 μL (half volume of the initial volume) and transfer them to a 1.5-mL microcentrifuge tube (Typically 25 μL for one assay at a time, depending on desired bead density).

Note: Bead loss may occur during preparation and may require adjustment of the final suspension volume. The recommended density is 5%–6.25% w/v.

h. Store the bead suspension at 4°C until use.

Loading cells onto the beacon platform

Timing: 3 h

This step involves loading the isolated plasma B cells onto the Beacon platform to distribute single cells into individual nanopens.

6. Create an Opto Plasma B Discovery 2.0 Workflow on the Beacon system by combining all or part of the following core operations in sequence:
   a. Chip prep.
   b. Load.
   c. Culture.
   d. Image and Count.
   e. Assay.
   f. Unload.

Note: The Berkeley Lights Beacon Instrument (BLI) provides detailed instructions for creating and running an Opto Plasma B Discovery 2.0 Workflow. These instructions can be found on the website under the posted protocols (https://techsupport.berkeleylights.com/).

On-chip screening of antigen-specific antibody-secreting cells (ASCs)

Timing: 1 h

The screening assay is initiated by running the assay script on the Cell Analysis Suite (CAS). Once the channel priority is established, schedule the image capture to occur every 5 min for a total of 10 iterations.

7. Mix the antigen-coated Assay Beads prepared above with the fluorophore (Alexa Fluor 568 or FITC)-conjugated Goat anti-mouse IgG (H + L) secondary antibody. Typically, dilute the antibody by a factor of 10 and then add 2 μL–50 μL of assay beads.
8. To identify ASCs producing antigen-specific antibodies, image each of the 22 fields of view (FOVs) that cover the entire chip per fluorophore channel every 5 min over a total duration of 45 min.
9. Analyze the collected images using onboard image detection software algorithms. Afterward, manually verify the identified positive nanopens by observing the characteristic fluorescent "bloom" at their mouth. See problem 1:
10. Prepare a 96-well cell-receiving plate by sequentially loading 5 μL of 2× TCL buffer supplemented with 5 μM dithiothreitol (DTT) and 10 μL of mineral oil into each well before centrifugation for 1 min at 400 × *g*.
11. Use OEP to unpen the antigen-specific nanopens and export the enclosed content in a total volume of 5 μL directly into the receipt plate.

12. Store the plate at −80°C until proceeding to the next step.

Single-cell RNA purification and reverse transcription

Timing: 2.75 h

This step involves purifying RNA from the isolated individual B cells, and then performing reverse transcription to obtain cDNA.

13. Bring Agencourt RNAClean beads out of the refrigerator and keep them at room temperature (20°C–25°C in our lab) for at least 30 min.

Note: Thoroughly mix the beads by vortexing to ensure complete resuspension of any settled particles.

14. Take the 96-well plate containing the sorted B cells from −80°C and place it on ice for 5–10 min to thaw.
15. Centrifuge the plate at 400 × *g* for 30 s at 4°C.
16. Add 22 μL of 1: 2.2 diluted Agencourt RNAClean beads per well.
17. Mix the contents by pipetting, and then incubate the plate at room temperature (20°C–25°C in our lab) for 10 min.
18. During the incubation time of step 17, prepare an 80% ethanol wash buffer using nuclease-free water.
19. Place the plate on a MagDTR 96-Well Magnetic Separator at room temperature (20°C–25°C in our lab) 5 min, or until beads are clearly attached to the side of the tube that is against the magnet.
20. Remove the supernatants from the wells using a multichannel pipettor, being careful not to disturb the bead pellets.
21. Add 125 μL of 80% ethanol to each well using a multichannel pipettor.
    a. Wash the beads by moving the plate to an adjacent magnet column, whereby the beads are allowed to shift from one side of the tube to the other side for effective washing.
    b. Remove the wash buffer, and repeat this washing procedure three more times.
22. Carefully and completely remove the wash buffer. Then, air dry the bead pellets for 8–10 min at room temperature (20°C–25°C in our lab).
23. Add 11 μL of Reverse Transcription Mix-1 to each well.
24. Pipette up and down several times to resuspend the bead pellets.
25. Seal the plate by wrapping the sides with paraffin films and then centrifuge the plate at 400 × *g* for 30 s at 4°C.

Reverse Transcription Mix-1

Reagent	Final concentration	Volume 1 (μL)	Volume 2 (μL)
Random Primers (0.3 mg/mL)	~18.3 ng/mL	6.7	67
NP40 (10%)	~0.61%	6.7	67
RNaseOUT Recombinant Ribonuclease (40 U/μL)	~0.78 U/μL	2. 1	21
Nuclease-Free Water	N/A	94.5	945
total	11 μL/well	110 μL (10 samples)	1100 μL (96-well plate)

26. Incubate the plate at 65°C for 5 min in a thermocycler.
27. Take out the plate and chill it on ice for 5 min.
28. Add 7 μL of Reverse Transcription Mix-2 to each well, and pipette up and down several times to ensure thorough mixing.

Reverse Transcription Mix-2			
Reagent	Final concentration	Volume 1 (μL)	Volume 2 (μL)
SuperScript III Reverse Transcriptase (200 U/μL)	~7.14 ng/mL	2.75	27.5
RNaseOUT Recombinant Ribonuclease (40 U/μL)	~0.57 U/μL	1.1	11
0.1 M DTT	~14.29 mM	11	110
dNTP (25 mM)	~1.79 mM	5.5	55
5* First-Strand Buffer	N/A	33	330
Nuclease-Free Water	N/A	23.65	236.5
total	7 μL/well	77 μL (10 samples)	770 μL (96-well plate)

Note: The Reverse Transcription Mix-1 and Mix-2 can be prepared in advance and stored at 4°C for several hours before use.

29. Seal the plate by wrapping the sides with paraffin films, and then briefly centrifuge at 4°C.
30. Place the plate in a PCR thermocycler and initiate a Reverse Transcription program with the following settings.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Denaturing	42°C	10 min	1
Annealing	25°C	10 min	
Extension	50°C	60 min	
Inactivation	94°C	5 min	
Hold	4°C Forever		

31. After the program is over, remove the plate from the PCR thermocycler and briefly centrifuge it at 4°C to precipitate the evaporated solution.

Pause point: Seal the plate by wrapping the sides with paraffin films before storing it at −80°C. The transcribed cDNA should remain stable at −80°C for at least several months.

⚠ CRITICAL: Avoiding RNase contamination is crucial for the RNA isolation step. Thus, we recommend performing this step in an RNase-free environment, such as a flow hood that is cleaned using RNAse Away Surface Decontaminant or other products with a similar function. Additionally, all the pipettes to be used should also be cleaned with RNase Away. Microcentrifuge tubes and pipette tips should be DNase/RNase free as well.

Nested PCR amplification of antibody sequences-1st PCR

Timing: 3 h

This step represents the first PCR for amplifying antibody sequences using cDNA from the isolated single B cells as templates. We have adopted a protocol originally described by Nussenzweig's laboratory[6] and Qiao Wang's laboratory.[7]

32. Prepare primers for the 1st PCR reactions.
    a. Dilute all the primers to a concentration of 50 μM using nuclease-free water.
    b. Prepare the forward primer mixture for the heavy and Kappa light chains separately by equally mixing all the forward primers.
    c. Use the forward primer mixture and the corresponding individual reverse primer designed for the first round of nested PCR reactions as a primer pair in the PCR reaction.

33. Thaw the cDNA plate from the previous step on ice.
    a. Use a multichannel pipette to add 10 μL of pre-chilled nuclease-free water to each well, and then pipette up and down 10 times.
    b. Centrifuge the plate at 300 × *g* for 1 min at 4°C.
34. Prepare new 96-well PCR plates for performing the 1st PCR amplification
    a. Use the recipe table below to prepare the 1st PCR MIX: Volumes 1 and 2 represent preparations sufficient for 10 wells and a whole 96-well plate, respectively.
    b. Use a multichannel pipette to dispense 38 μL of 1st PCR MIX into each well of the new 96-well PCR plates.

Note: Be careful during the addition to avoid bubble formation.

1st PCR MIX

Reagent	Final concentration	Volume 1 (μL)	Volume 2 (μL)
HotStarTaq DNA Polymerase (5 U/μL)	~0.05 U/μL	4.2	42
Forward Primer Mixture (50 μM)	~0.3 μM	2.3	23
Reverse Primer (50 μM)	0.2 μM	1.5	15
dNTP (25 mM)	0.3 mM	4.8	48
10*PCR Buffer	N/A	42	420
Nuclease-Free Water	N/A	363.2	3632
Total	N/A	418 μL (10 samples)	4180 μL (96-well plate)

Note: Use two different PCR plates to separately amplify the variable heavy and light chain genes.

35. Use a multichannel pipette to transfer 4 μL of diluted cDNA from each well of the cDNA plate to the corresponding well in the new 96-well plate containing 1st PCR MIX. Mix the contents by pipetting 10 times.

Note: After use, store the cDNA plate at 4°C for short-term storage or return it to −80°C for long-term storage.

36. Seal the plate by wrapping the sides with paraffin films before centrifuging at 300 × *g* for 1 min at 4°C.
37. Place the plate in a thermocycler and perform the first round of PCR amplification using a PCR program with the following settings:

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	94°C	30 s	50
Annealing	46°C	30 s	
Extension	72°C	55 s	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

38. After completing the program, remove the PCR plates from the thermocyclers and then subject them to rapid centrifugation at 4°C.

Pause point: The plates can be wrapped with paraffin film and stored at −20°C for several weeks before proceeding to the next round of PCR amplification.

Nested PCR amplification of antibody sequences-2nd PCR

Timing: 3 h

This step represents the second nested PCR for amplifying the antibody sequence. Subsequently perform sequencing on the obtained PCR products after gel electrophoresis to validate successful recovery of heavy and light chains.

39. Dilute and mix the primers designed for the 2nd PCR reactions in the same way as for the 1st PCR reaction as step 32 described.
40. Place the 1st PCR plates on ice and allow them to thaw for 5 min.
41. Prepare new 96-well PCR plates to perform 2nd PCR amplification
    a. Use the recipe table below to prepare the 2nd PCR MIX: Volumes 1 and 2 represent preparations sufficient for 10 wells and a whole 96-well plate, respectively.

2nd PCR MIX

Reagent	Final concentration	Volume 1 (μL)	Volume 2 (μL)
HotStar Taq DNA Polymerase (5 U/μL)	~0.05 U/μL	4.2	42
Forward Primer Mixture (50 μM)	~0.2 μM	1.5	15
Reverse Primer (50 μM)	~0.2 μM	1.5	15
dNTP (25 mM)	~0.3 mM	4.8	48
10*PCR Buffer	N/A	42	420
Nuclease-Free Water	N/A	364	3640
Total	38 μL/well	418 μL (10 samples)	4180 μL (96-well plate)

    b. Use a multichannel pipette to dispense 38 μL of the 2nd PCR MIX into each well of the new 96-well PCR plates.

Note: Be careful during the addition to avoid bubble formation.

42. Use a multichannel pipette to thoroughly mix the contents in each well of the 1st PCR plates by pipetting.
43. Then transfer 4 μL from each well to the corresponding well in the 2nd PCR plates, followed by mixing the contents by pipetting 10 times.

Note: After use, return the 1st PCR plates to −80°C for storage.

44. Place the 2nd PCR plate in a thermocycler and perform the second round of PCR amplification using a PCR program with the following settings:

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	94°C	30 s	50
Annealing	57°C for Heavy, 46°C for kappa	30 s	
Extension	72°C	50 s	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

45. Load 5 μL of the 2nd PCR product from each well into individual lanes of a 2% agarose gel.
    a. Run the gel at 140 V for 20 min.

b. Visualize the bands of linearized vectors under UV lights, then excise them with razor blades. See problem 3.
c. Isolate DNA from the bands using the Wizard SV Gel and PCR Clean-Up System (Promega, A9282) following the manufacturer's instructions (Protocol for Wizard SV Gel and PCR Clean-Up System).

Note: The expected size of the PCR product is approximately 400 bp for the heavy chain and about 350 bp for the light chain.

Pause point: The plates can be stored at 4°C for several days before analysis.

46. Sequence the 2nd PCR products in step 45c verified by gel analysis using Sanger sequencing with the respective reverse primers listed in the primer Table S1.

Note: These primers are located at the constant region.

Pause point: The 2nd PCR Plates can be stored at 4°C for several days before proceeding to sequencing.

PCR amplification of antibody sequences for cloning-2nd PCR

Timing: 2.5 h

This step involves cloning PCR, which also uses the product of the first nested PCR as the template. However, it differs from the second nested PCR in that the primers contain restriction enzyme sites and homology arms that match the antibody expression vector. This allows for cloning the products into the antibody expression vector.

47. Prepare primers for the cloning-2nd PCR.
    a. Dilute all the primers to 50 μM.
    b. Mix equal amounts of all the forward primers to obtain forward primer mixture for heavy and Kappa light chains, respectively.
    c. Use the forward primer mixtures and their corresponding individual reverse primers to amplify the heavy and Kappa light chains in separate PCR plates.
48. Place the 1st PCR plates on ice for 5 min or longer to thaw. Centrifuge the plates at 400 × *g* for 1 min at 4°C.
49. Prepare the Cloning-2nd PCR plates.
    a. Prepare the Cloning-2nd PCR MIX separately for reactions to amplify the heavy and kappa light chains, following the recipe below.

Cloning-2nd PCR MIX

Reagent	Final concentration	Volume 1 (μL)	Volume 2 (μL)
HotStar Taq DNA Polymerase (5 U/μL)	~0.05 U/μL	4.2	42
clone-Forward Primer Mixture (50 μM)	~0.3 μM	2.3	23
clone-Reverse Primer (50 μM)	~0.2 μM	1.5	15
dNTP (25 mM)	~0.3 mM	4.8	48
10*PCR Buffer	N/A	42	420
Nuclease-Free Water	N/A	363.2	3632
–	38 μL/well	418 μL (10 samples)	4180 μL (96-well plate)

b. Use a multichannel pipette to dispense 38 μL of the Cloning-PCR MIX into each reaction well of new 96-well PCR plates.

Note: Keep heavy and light chain amplification reactions separate by using different plates. Be careful during the addition to avoid bubble formation.

50. Add 4 μL of PCR products from each well of the 1st PCR plates to the corresponding wells in the Cloning-2nd PCR plates.
51. Place the Cloning-2nd PCR plates in thermocyclers and perform the PCR amplification using a program with the following settings:

PCR cycling conditions

Cloning–2nd PCR steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	94°C	30 s	35
Annealing	46°C for Heavy, 52°C for kappa	30 s	
Extension	72°C	50 s	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

⚠ CRITICAL: It is not appropriate to use the product of the sequences-2nd PCR as the template for the cloning PCR, as it involves 50 more PCR amplification cycles than starting with the product from the 1st PCR, thus increasing the risk of introducing mutations in the PCR product. Indeed, the cloning-2nd PCR uses 35 cycles instead of the 50 cycles used in the sequences-2nd PCR, also due to the consideration of limiting mutation accumulation associated with increased PCR amplification cycles.

52. Subject 5 μL of the reaction from each well to electrophoresis with a 2% agarose gel.
    a. Run the gel at 140 V for 20 min.
    b. Visualize the bands of linearized vectors under UV lights, then excise them with razor blades. See problem 2.
    c. Isolate DNA from the bands using the Wizard SV Gel and PCR Clean-Up System (Promega, A9282) following the manufacturer's instructions (Protocol for Wizard SV Gel and PCR Clean-Up System).

Note: The expected size of the PCR product is approximately 400 bp for the heavy chain and about 350 bp for the light chain.

Pause point: The plates can be stored at 4°C for several days before analysis.

Cloning of heavy and light chain genes into expression vectors

Timing: 2 days

This step involves cloning the antibody sequence obtained into the cloning PCR into the antibody expression vector through homologous recombination.

53. Vector preparation:
    a. Plasmid digestion:
        i. Set up two 50-μL reactions in two 1.5-mL microcentrifuge tubes to digest the two cloning vectors with appropriate restriction enzymes.

Note: In this protocol, we use FastDigest AgeI (BshTI) and FastDigest SalI for the heavy chain-cloning vector, FastDigest AgeI (BshTI) and Pfl23II (BsiWI) for the light chain-expressing vector.

ii. Incubate the tubes in a 37°C water bath for 5–6 h.

Restriction enzyme digestion (1 reaction)	
Reagent	Volume (μL)
Nuclease-free water	To final 50 μL
Vector (10 μg)	Depending on the vector concentration
Enzyme 1 (20,000 U/mL)	2.5
Enzyme 2 (20,000 U/mL)	2.5
10× Fast digest buffer	5

b. Purification of the linearized vector:
   i. Electrophorese the digestion reactions on a 1% agarose gel.
   ii. Visualize the bands of linearized vectors under UV lights, then excise them with razor blades.
   iii. Isolate DNA from bands using the Wizard SV Gel and PCR Clean-Up System (Promega, A9282) following the manufacturer's instructions (Protocol for Wizard SV Gel and PCR Clean-Up System).

54. Insert purification:
   a. Electrophorese the remaining Cloning PCR reactions that have been confirmed to successfully amplify both heavy and light chains on a 2% agarose gel.
   b. Visualize and excise the bands of interest, and extract DNA from the excised bands, as described in Step 53 (Protocol for Wizard SV Gel and PCR Clean-Up System).

Pause point: The purified linearized vector and PCR products can be stored at −20°C for months. For temporary storage (several days), storing at 4°C is also suitable.

55. *In vitro* vector-insert ligation using homologous recombination:
   a. Prepare the ligation mix on ice using the following recipe.

Homologous recombination-based ligation (1 reaction)	
Reagent	Volume (μL)
Nuclease-free water	To final 8 μL
Linearized vector(~20 ng)	Depending on the concentration
5× CE II buffer	2
Exnase II	1

   b. Mix 8 μL of the ligation mix with 2 μL of the purified PCR insert (diluted to 10 ng/μL with water) in a new 96-well plate on ice.
   c. Incubate the plate at 37°C for 30 min in a thermocycler.
   d. After completing the incubation, immediately place the plate on ice.

Note: Keep the place on ice for at least 10 min to stop the reaction.

56. Bacterial transformation:
   a. Thaw chemically competent DH5α cells on ice, usually 100 μL per tube. Pipette the thawed cells.
   b. While on ice, pipette 10 μL of the ligation reaction into the competent cell suspension without mixing.
   c. Incubate the tube on ice for 30 min.
   d. Transfer the tube to a thermocycler pre-warmed to 42°C and perform heat shocking of the bacteria-DNA mix for 90 s.

e. Immediately put the tube back on ice for approximately 2 min.
f. Add 500 μL of pre-warmed LB medium to each well.
g. Incubate it in a 37°C shaker at a 45° angle. Shake at 210 rpm for 60 min.
h. Spread bacterial cells onto pre-warmed LBA plates (LB agar plates with 100 μg/mL ampicillin).
i. Incubate the plates 12–16 h in a 37°C incubator.

Pause point: The LBA plates can be sealed and stored at 4°C for several days before conducting colony screening.

Colony screening and sequencing

Timing: 2–3 days

This step involves screening the recombinants using colony PCR and then performing transfection with the identified successful constructs to express antibodies in mammalian cells for further validation. In our protocol, the primers used in colony-screening PCR are located on the vector sequence near the cloning sites of the insert. The forward primer (Colony-F) is common to both the heavy chain- and light chain-expressing vectors, while the reverse primer is specific to each vector (Colony-Heavy-R for the heavy chain-expressing vector and Colony-Kappa-R for the light chain-expressing vector).

Primers used in Colony-PCR screening

Primer name	Sequences (5′–3′)
Colony-F	CCACCATGGGATGGTCATGTATC
Colony-Heavy-R	GCTCTTGGAGGAGGGTGCCAGG
Colony-Kappa-R	GGCACACAACAGAGGCAGTTCCAG

57. Colony screening with PCR:
    a. Prepare the Colony-PCR MIX following the recipe below.

Colony-PCR MIX (one reaction)

Reagent	Final concentration	Volume (μL)/
2× Taq Plus Master Mix (Dye Plus)	N/A	10
Colony-F (10 μM)	0.25 μM	0.5
Colony-Heavy/ Kappa -R (10 μM)	0.25 μM	0.5
Nuclease-Free Water	N/A	8
Total	N/A	20 μL

    b. Prepare a Colony PCR plate by adding 20 μL of Colony-PCR MIX to individual well of a new 96-well plate pre-cooled on ice.
    c. Pick at least three individual bacterial colonies per ligation reaction using sterile pipette tips.
        i. Dip the tips a couple of times into individual wells of the Colony PCR plate containing the Colony-PCR MIX.
        ii. Then streak each tip onto an LBA plate to prepare a bacterial stock.
        iii. Incubate the stock plate at 37°C for 12–18 h until visible bacterial streaks form.

Note: We recommend dividing the LBA plate into grids to facilitate the organization of the bacterial stocks. It is also important to label the stocks clearly for easy tracking.

d. Place the Colony-PCR plate in a thermocycler and perform PCR amplification using a program with the following settings:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	94°C	30 s	30
Annealing	62°C	50 s	
Extension	72°C	50 s	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

e. After completing the program, electrophorese the Colony-PCR reactions.
   i. Take 5 μL from each reaction, mix with loading dye.
   ii. Load onto a 2% agarose gel. Run the gel at 120 V for approximately 30 min.
   iii. Visualize the DNA bands under UV lights. See problem 4:

Note: The expected size of the amplified product is ~400 bp for the heavy chain and ~350 bp for the light chain.

f. Using sterile pipette tips, transfer a small patch of each bacterial stock with a positive colony PCR into 1 mL of LB medium with 100 μg/mL ampicillin (LBA medium).
g. Grow the cultures at 37°C for ~7–8 h or until they become turbid.
h. Purify DNA from the bacterial cultures using the preferred commercial DNA miniprep kit following the manufacturer's protocol (Protocol for Endo-free Plasmid Mini Kit II).
i. Subject the purified plasmids to Sanger sequencing using the universal SP6 primer (5′-ATT TAGGTGACACTATAG-3′), which is located immediately upstream of the antibody sequence insertion site in both cloning vectors.
j. Utilize online immunoinformatic tools, such as IgBLAST (http://www.ncbi.nlm.nih.gov/igblast) and IMGT (https://www.imgt.org/), to analyze the sequence results and identify antibody clones with paired, functional heavy and light chains.

Pause point: The bacterial stock plate(s) and the Colony PCR plate(s) can be stored at 4°C for several days.

Antibody-expressing plasmid purification and transfection

Timing: 5–7 days

This step involves performing a medium-scale purification of individual heavy chain and light chain expression plasmids. And then by Plasmid transfection of Gibco FreeStyle 293-F cells to get the expression of the antibodies.

58. Plasmid preparation:

This process involves using an transfection-grade DNA isolation kit (MACHEREY-NAGEL, 740420.50) following the manufacturer's protocol to purify plasmid DNA from 100 mL of 12–16 h bacterial culture (220 rpm, 37°C) grown in LBA medium (Protocol for MACHEREY-NAGEL, 740420.50).

59. Plasmid transfection of Gibco FreeStyle 293-F cells: See problem 5:
   a. Prepare the plasmid mixture by adding 50 μg of heavy chain plasmid and 75 μg of light chain plasmid to Gibco-Opti-MEM I (1×) in a total volume of 2 mL.

b. Immediately agitate the mixture on a vortex mixer for 15 s.
c. Dilute 375 μL of PEI transfection reagent with 1625 μL of Gibco-Opti-MEM I (1×).
d. Immediately agitate the mixture on a vortex mixture for 15 s.
e. Add the plasmid mixture to the PEI mixture and immediately vortex for at least 15 s before letting it sit at room temperature (20°C–25°C in our lab) for 15–20 min.
f. In a dropwise manner, add the plasmid-PEI mixture to the Gibco FreeStyle 293-F cells cultures.
g. Gently rock the culture vessel back and forth and from side to side to evenly distribute the PEI: plasmid complex.
h. Incubate the transfected cells for 5–7 days in a shaker incubator at 130 rpm, 37°C, 95% humidity and 5.0% CO_2.

Note: (1) If time allows, it is best to conduct a small-scale expression of the selected antibodies in 293T cells to analyze their expression by detecting the presence of the antibody in the supernatants of transfected cells through Western blotting. Only the antibodies that show high yields of both heavy and light chains are chosen for upscaled plasmid preparation and transfection using HEK293-6E cells as outlined above.

Note: (2) Gibco FreeStyle 293-F suspension cells are recommended for the preparative expression and purification of antibodies. A log-phase culture with 1 × 10^6 cells per mL is suitable for transfection. For a single antibody expression, 125 mL of cell culture in a 500 mL Erlenmeyer flask is used. Transfection must be performed in a laminar air flow cabinet under sterile conditions.

Antibody purification by affinity chromatography

Timing: 1–2 days

This step involves purifying antibodies from the supernatants of cells transfected with antibody expression vectors using affinity chromatography.

60. Collect the supernatants of cell cultures from step 59.
61. Centrifuge the supernatants at 3600 × *g* for 30 min at 16°C to remove any cell debris.

⚠ CRITICAL: The supernatants used for chromatography need to be filtered through 0.22 μm pore size filters to remove any particles that could potentially block the tubing and column.

62. Load the cleared supernatants onto an ÄKTA Pure Protein Purification System equipped with a Protein A/G column (AbcapA/G 4FF from Smart-lifesciences Inc, China(Cat# SA032C55) for affinity antibody purification.

Buffers used in protein chromatography

Solutions	Composition	PH
Solution 1: Equilibration and wash buffer	20 mM Na_2HPO_4, 150 mM NaCl	PH = 7.4
Solution 2: Elution buffer	0.1 M glycine	PH = 3.0
Solution 3: Neutralizing Buffer	1 M Tris-HCl Solution	pH = 8.5

⚠ CRITICAL: The buffers used for chromatography need to be filtered through 0.22 μm pore size filters to remove any particles that could potentially block the tubing and column.

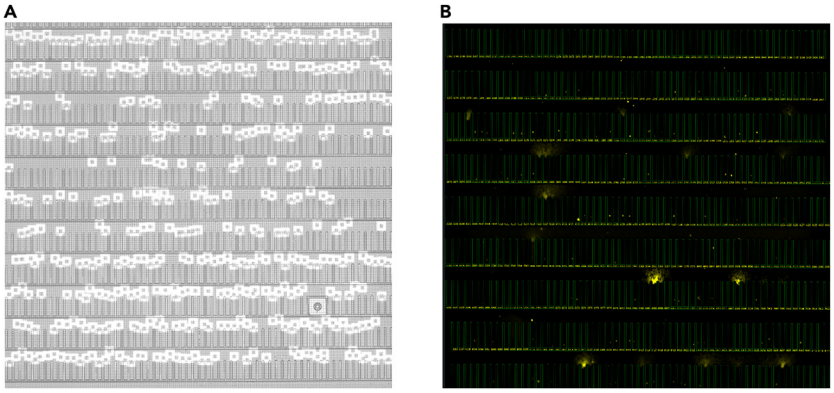

Figure 1. Representative on-chip screening of antibody-secreting cells (ASCs)

(A) Bright-field image of the chip showing the distribution of ASCs in nanopens after loading.

(B) 45-min images of the chip after stitching all the fields of vision (FOV) together, depicting the fluorescent blooms formed on the mouth of the nanopens containing the desired secreted IgG, as captured in the filter cube.

a. After loading the sample, wash the column with 10 column volumes (CV) of Solution 1.
b. Use 10 CV of Solution 2 to elute the bound proteins, and collect the peak fraction using a Frac30 fraction collector.
c. Immediately neutralize the eluates by adding one tenth of 1 M Tris-HCl Solution, pH = 8.5.
d. Use 10 column volumes (CV) of Solution 1 to neutralize the column, wash with 20% ethanol, and store at 4°C after sealing both ends.
e. Subject the collected antibody samples to a Centrifugal Filter with a 10 kDa MWCO for buffer exchange into PBS, following the manufacturer's instructions (Protocol for Amicon Ultra-15 Centrifugal Filter, 10 kDa MWCO).
f. Use NanoDrop One to measure the concentrations of the purified antibodies.
g. Submit the samples to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reduced and reduced conditions.
h. Coomassie blue staining of the gels to determine the productivity and purity.

Pause point: After adding an equal volume of PBS containing 20% glycerol, the antibody preparations can be stored −20°C for several years without losing activity.

EXPECTED OUTCOMES

It would be expected that the vast majority of B cells loaded on the chip should be single cells. In this study, after imaging each of the 22 FOVs on the 2 chips, we recorded a total of 8434 nanopens receiving cells on the first chip. Among these, 6686 (~79%) contained a single cell. On the second chip, there were 6544 nanopens with cells, among which 5313 (~81%) held a single cell (Figure 1A).

The antigen-positive clones on the chip are identified by the presence of a fluorescent bloom resulting from the binding of secreted antibodies to antigens coated on the assay beads and the fluorophore-conjugated secondary antibody for detection (Figure 1B). The percentage of these clones is expected to vary from case to case, depending on the ASC-induction efficiency of vaccination and sampling time. In this study, we identified 220 individual cells (nanopens) that were positive for secreting antibodies against the RABV-G protein on the first chip, and 178 on the second chip. These accounted for 3.2% and 3.3% of the total single cells analyzed, respectively.

The single B cell antibody cloning represents the most challenging step in the procedure. We conducted an optimization by extensively comparing different published protocols and the protocol provided by Berkeley Light. Following the optimized protocol, as shown in the representative gel

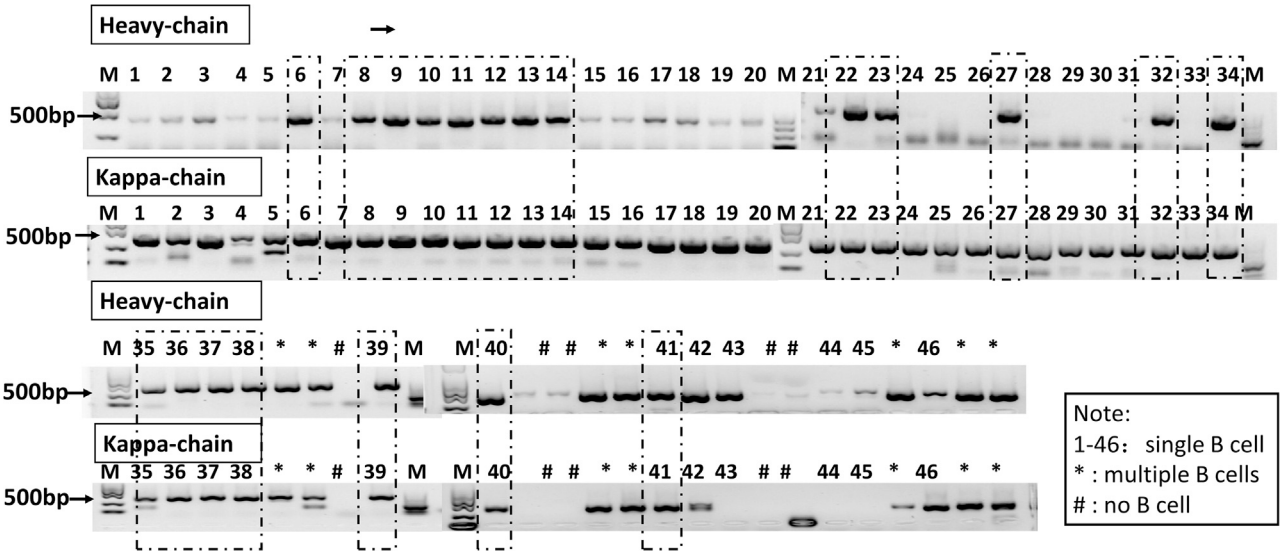

Figure 2. Representative gel electrophoresis results of antibody fragments amplified from individual ASCs imported from Beacon

The amplification was achieved through two rounds of nested PCR reactions. Clones with both Heavy and Kappa chains successfully amplified are indicated with a dotted frame.

electrophoresis results of the products of the two-step PCR amplification (Figure 2), we were able to achieve nearly 100% amplification efficiency for the Kappa light chain. The amplification of the heavy chain is less effective, with approximately 80% of the clones showing amplification products with varying band intensities. When only considering clear and strong bands of amplification products, paired heavy and light chains were successfully recovered from 20 out of 46 single B cell clones analyzed, resulting in an acquisition ratio of approximately 40%. It should be noted that we did not subject the clones to Lambda light chain analysis, considering the significantly higher abundance (10:1 to 20:1) of mouse B cells expressing kappa light chains compared to those expressing lambda light chains. A total of 20 paired amplification products of heavy and kappa chains were sequenced and subsequently analyzed using IgBlast or IMG online tool. The analysis revealed that 19 out of the 20 pairs were naturally paired antibody sequences.

To express and purify the cloned antibodies, a preliminary experiment can be conducted using HEK293T cells. This involves small-scale co-transfection of the corresponding paired heavy chain and light chain plasmids, followed by Western blotting analyses of the supernatants from transfected cells to detect antibody expression. Only antibodies that have been confirmed to be effectively expressed are then subjected to preparative expression using Gibco FreeStyle 293-F cells and subsequent purification using Protein A/G affinity chromatography. In our study, out of the nineteen antibodies we attempted to express, eight showed high expression, while the remaining were expressed at significantly lower levels. The eight highly expressed antibodies were purified, and the purity of the isolated antibodies was demonstrated by Coomassie blue staining (Figure 3).

Although the protocol described here is used to identify antigen-specific antibodies from mouse ASCs, it can be easily extended to samples from other species, including ASCs harvested from the peripheral blood of human subjects, by adjusting the primer sets for amplifying antibody sequences.

LIMITATIONS

The protocol described here is optimized for screening IgG-secreting plasma cells on the Beacon platform. Theoretically, the Beacon platform can also be used to screen other subtypes of secretory antibodies, such as IgM. The real challenge lies in the lower abundance of cells secreting these subtypes compared to IgG-secreting cells and the lack of specific surface marker(s) for cell enrichment.

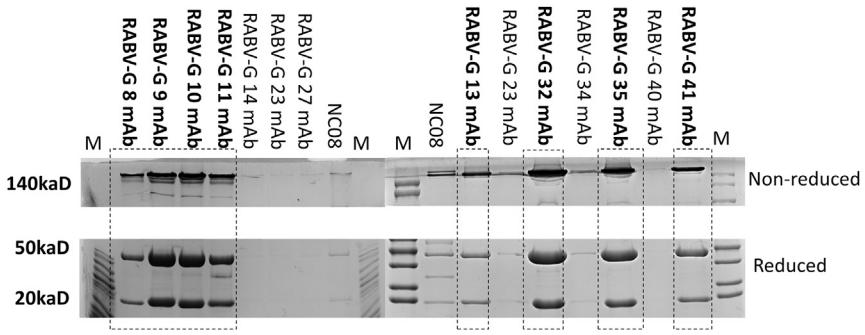

Figure 3. Representative Coomassie blue staining analysis result of purified antibodies

Gel electrophoresis was performed under both non-reduced conditions (above) and reduced conditions (below). Antibodies with good expression are indicated with a dotted frame.

Quantification of these cells in the total population of plasma cells, for example using a Plasma cell ELISPOT assay,[8] is necessary to assess the feasibility of sorting them through the Beacon platform. While the Beacon platform is designed to screen secretory antibodies, memory B cells, in which antibodies are expressed in the form of BCRs on the cell surface, can also serve as the initial antibody reservoir. However, they need to be converted into antibody-secreting cells through *in vitro* activation before implementing this protocol.[9]

We adopted a two-step nested PCR method, along with the primer sets used, from a protocol published by Nussenzweig's laboratory.[1] As noted in the original protocol, the forward primer mixture used in the first PCR, particularly for immunoglobulin heavy chains, cannot cover the V genes entirely. This limitation could explain why the success rate for amplifying heavy chains was approximately 80%, compared to the almost full recovery of kappa light chains.

It is important to note that antigen-specific ASCs induced by immunization in mice make up only a small proportion of the total ASC pool. Therefore, researchers need to optimize the immunization regimen and the sampling time to maximize the presence of the antigen-specific subpopulation in the ASC preparation that is submitted for Beacon screening.

TROUBLESHOOTING

Described below are some potential problems and recommendations for troubleshooting.

Problem 1

No positive clones, or very few, are identified on the Beacon platform (step 9).

Potential solution

This likely indicates a very low presence of antigen-specific ASCs in the sample. To address this issue, the immunization regimen and collection time should be optimized to increase the yield of antigen-specific ASCs available for isolation. We also recommend performing a live/dead staining to confirm that the sample has high cell viability, as low viability can lead to a decreased hit rate and lower antibody sequence recovery efficacy.

Problem 2

Low rate of amplification of heavy chain and/or light chain: no band or weaker bands observed in gel electrophoresis (step 52).

Potential solution

This problem could arise from the steps of mRNA isolation, PCR amplification, or both. It can be solved by paying attention to the following.

- Ensure that mRNA isolation is performed in an RNase-free environment to prevent mRNA degradation. Be careful not to overdry the RNAClean beads. Additionally, consider including an RNase inhibitor in the 2× TCL buffer, into which single B cells are sorted.
- PCR primers should be stored separately and only mixed right before each use. If there is insufficient amplification (a weak product band of the correct size), consider increasing the PCR cycles by 2–3.

Problem 3

The Sanger sequencing of the 2nd sequences PCR product either fails or yields poor quality results (step 45).

Potential solution

It may indicate that the PCR product is a mixture resulting from amplifying antibody sequences from multiple B cells, which could have occurred accidentally during loading on the Beacon. This can be verified by cloning the recovered antibody sequences into the expression vector and sequencing multiple resulting clones.

Problem 4

Colony screening with PCR does not yield any colonies containing the antibody sequence (step 57).

Potential solution

This problem is likely a result of inadequate linearization of the vector. Repeat the vector digestion with a longer incubation period and include a treatment with calf intestinal alkaline phosphatase (CIP) after digestion to prevent re-ligation.

Problem 5

No expression of antibody in plasmid-transfected 293T cells (step 59).

Potential solution

This most likely indicates that the heavy and light chains are mis-paired. To be cautious, sequence the plasmid preparations to verify their identity, thus excluding the loss or alteration of the cloned antibody sequence due to improper plasmid amplification. If no expression or low expression is observed for all the cloned antibodies, check the status of expressing cells (i.e., low passage and no mycoplasma contamination) and make sure that the transfection reagent is effective using positive controls such as a GFP-expressing plasmid.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jianqing Xu (xujianqing@fudan.edu.cn).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Meilan Fu (fumeilan_987@126.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets or codes.

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AUTHOR CONTRIBUTIONS

M.F. and X.W. conducted the experiments and developed the protocol. S.B. provided the immunized mice. S.B., L.D., and Y.H. assisted with protocol development. C.Z. and S.T. provided advice for improving the protocol. M.F. wrote the original draft. C.Z. and S.T. revised the draft. J.X. and X.Z. supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103389.

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