Materials required:

spleen from an immunized animal
spleen from a naive animal

msCD40L-3T3 irradiated with 5000 rads (referred to as feeder cells)

Heat-inactivated FBS
RPMI medium
IMDM-c medium (IMDM supplemented with 10% FBS, penicillin/streptomycin, L-glutamine)
PBS
ELISA wash buffer (0.02% Tween-20 in PBS)
BSA block solution (3% BSA in PBS)
NFM block solution (10% Non-Fat dry Milk, 0.3% Tween-20 in PBS)
BSA Dilution Buffer (0.2% BSA in PBS)
Secondary antibody (Goat anti-mouse Ig-HRP, BD Biosciences, cat. 554002)
SureBlue Reserve TMB Substrate
ELISA stop solution (1N H_2SO_4)

IL-4 (BioLegend, cat. # 574302)
LPS (Sigma-Aldrich, cat. # L4391)

70-µm filter screens (Falcon/Corning)
Nitex filter paper (Sefar)
Ficoll (optional)
β-mercaptoethanol (βME)

EasySep negative selection mouse B cell enrichment kit (StemCell cat. # 19754)

Biotinylated target antigen
Biotinylated decoy antigen

Fc block (BD Biosciences, cat. # 553141)
IgD-AF700 (BioLegend cat# 405730)
CD38-APC (BioLegend cat# 102712)
IgM-FITC (BioLegend cat# 406506)
strep-BV785 (Streptavidin-Brilliant Violet 785™, Biolegend, cat. # 405249)
strep-BV510 (Streptavidin-Brilliant Violet 510™, Biolegend, cat. # 405233)

anti-B220-BV786 (BD Biosciences, cat. # 563894)
anti-CD4-AF700 (BD Biosciences, cat. # 561025)

anti-CD4-APC (BD Biosciences, cat. # 561091)
anti-IgD-FITC (BD Biosciences, cat. # 562022)
anti-CD4-BV510 (BD Biosciences, cat. # 563106)

RLT lysis buffer (Qiagen) with freshly-added 1% βME (VWR)

SMARTer RACE 5'/3' Kit (Clontech, cat. # 634858)
NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs, cat. # M0543S)
NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, cat. # E5520S)

Protocol:

Splenocyte preparation:

From fresh spleen

- ❏ In a 10-cm tissue culture plate, measure out 5 mL of FACS buffer (2% v/v heat-inactivated FBS in PBS) and pre-wet a 70-μm filter screen
- ❏ Add the whole spleen to the filter screen and gently mash with the rubber end of a 3-mL syringe plunger
- ❏ Filter the splenocyte suspension through a nitex filter
- ❏ Wash the plate, 70-μm filter and the nitex filter with another 5-mL volume of FACS buffer, and add to the main suspension
- ❏ Count a 1:10 dilution
- ❏ Pellet cells by centrifugation at 300×g for 7 minutes and resuspend (gently, by flicking the pellet) in FACS buffer to produce a 100 million cells/mL stock suspension for the B-cell enrichment protocol (below)

(Optional) From frozen preparation

- ❏ Use a 37°C water bath to rapidly thaw the required number of previously frozen splenocytes (see the "From fresh spleen" section above)
  Note: we frequently recover only 25-50% of previously frozen cells, so the number of cells thawed should be compensated, accordingly
- ❏ For each cryovial: dilute the thawed cells by adding 1 mL of prewarmed RPMI medium
- ❏ For each cryovial: transfer the thawed diluted cells into a fresh tube containing 8 mL of prewarmed RPMI medium
- ❏ Pellet cells by centrifugation at 300×g for 7 minutes and resuspend (gently, by flicking the pellet) in enough FACS buffer to produce ~10 million cells/mL suspension

Note: if cell clumping is an issue, the cell suspension may be filtered through nitex

- ❏ (Optional step) if the viability is lower than 70%, live cells may be purified using a Ficoll gradient (e.g., resuspend cells in 3 mL of RPMI and stack over 2 mL of Ficoll, centrifuge at 860×g for 15 min without brakes)
- ❏ Count cells
- ❏ Pellet cells by centrifugation at 300×g for 7 minutes and resuspend (gently, by flicking the pellet) in enough FACS buffer to produce 100 million cells/mL stock suspension for the B-cell enrichment protocol (below)

(Optional) Freezing excess splenocytes

- ❏ Pellet cells by centrifugation at 300×g for 7 minutes and resuspend in ice-cold Freezing medium (90% FBS + 10% DMSO) to produce a 10 million cells/mL suspension
  Note: Best results are obtained by resuspending cells in cold FBS, with DMSO added subsequently to reach final concentrations

B-cell enrichment (EasySep negative selection mouse B cell enrichment kit [StemCell cat #19754])

- ❏ Place a maximum of 2 mL of stock splenocyte suspension (100 million cells/mL) into a polystyrene FACS tube (e.g., Corning™ 352008)
- ❏ Add Normal Rat Serum at 50 µL for each mL of cell suspension
- ❏ Add EasySep Mouse B Cell Enrichment Cocktail at 50 µL for each mL of cell suspension
- ❏ Mix well and incubate at 4°C for 15 minutes
- ❏ Add EasySep Biotin Selection Cocktail at 100 µL for each mL of cell suspension
- ❏ Mix well and incubate at 4°C for 15 minutes
- ❏ Vortex the EasySep D Magnetic Particles for 30 seconds to ensure uniform suspension
- ❏ Add EasySep D Magnetic Particles at 100 µL for each mL of cell suspension
- ❏ Mix well and incubate at 4°C for 5 minutes
- ❏ Bring the cell suspension up to a total volume of 2.5 mL with FACS buffer
- ❏ Mix cells gently. Insert the tube containing cell suspension (without cap) into the magnet and set aside for 5 minutes at room temperature.
- ❏ Pick up the magnet with the tube, and invert in one continuous motion to pour the resulting cell suspension into a fresh tube
- ❏ Add 10 mL FACS buffer to the cells to wash; count cells
- ❏ Pellet cells by centrifugation at 300×g for 7 min, resuspend in 100 µL FACS buffer, and proceed to Staining (below)

Preparation of fluorescent antigen stain (complexation with streptavidin-fluorophore)

- ❏ Add ~4 molar-equivalents of biotinylated target protein to 1 molar-equivalent of strep-BV785.
  Note: we typically complex ~5 µg of biotinylated protein (with molecular weight of ~50 kDa) with ~1 µg conjugated streptavidin in a total volume of ~10 µL.
  Note: addition is done slowly to allow all 4 streptavidin sites to become occupied with biotinylated protein.
- ❏ Add ~4 molar-equivalents of biotinylated decoy protein to 1 molar-equivalent of strep-BV510.
- ❏ Incubate at room temperature for 30 min

Staining

- ❏ Transfer the 100 µL of cell suspension from B cell enrichment procedure (above) into a polystyrene FACS tube (e.g., Corning™ 352008)
- ❏ Add Fc block to each tube at 1:100 (1 µL per 100 µL)
- ❏ Incubate 5 minutes at room temperature
- ❏ Add decoy:strep-BV510 complex to each tube at 1:25 (4 µL per 100 µL)
- ❏ Incubate 10 minutes at room temperature, in the dark
- ❏ Add decoy:strep-BV785 complex to each tube at 1:25 (4 µL per 100 µL)
- ❏ Add anti-IgD-AF700 to each tube at 1:200 (0.5 µL per 100 µL)
- ❏ Add anti-CD38-APC to each tube at 1:80 (1.25 µL per 100 µL)
- ❏ Add anti-IgM-FITC to each tube at 1:25 (4 µL per 100 µL)
- ❏ Add anti-B220-PacBlue to each tube at 1:50 (2 µL per 100 µL)
- ❏ Incubate at 4°C for 25 min, in the dark
- ❏ Wash target cells by diluting them in 5 mL FACS buffer and pelleting at 300×g for 7 min, resuspend to a final density of ~8 million cells/mL

- ❏ Single Stain Compensation - Use naive cells (~350,000 cells in 100-µL aliquots) to set up single-stain controls for each color plus an unstained control (7 tubes total)
  - ❏ anti-B220-BV786: use at 1:250 (0.4 µL per 100 µL)
  - ❏ anti-CD4-AF700: use at 1:200 (0.5 µL per 100 µL)
  - ❏ anti-CD4-APC: use at 1:200 (0.5 µL per 100 µL)
  - ❏ anti-IgD-FITC: use at 1:100 (1 µL per 100 µL)
  - ❏ anti-CD4-BV510: use at 1:200 (0.5 µL per 100 µL)
  - ❏ anti-B220-PacBlue: use at 1:50 (2 µL per 100 µL)
  - ❏ Incubate at 4°C for 25 min in the dark
- ❏ Wash single-stain control cells by diluting them in 2 mL FACS buffer for each sample, and pelleting at 300×g for 7 min; resuspend in 250 µL FACS buffer

Sorting

- ❏ Filter final target cell suspension through nitex
- ❏ Prepare receiving test tubes with 500 µL of IMDM-c
  Note: we found the following cytometer settings useful
  - *100-µm nozzle*
  - *Pressure at 20 psi*
  - *Sort chamber set to 20°C.*
  - *Sort speed is between 3000-4000 events/second.*

Sorted B-cell culture

Note: in order to avoid the plate-edge effect, we only use the 60 central wells; the edge wells (top/bottom rows, and left/right columns) are filled with sterile water, as a temperature and humidity buffer
Note: in order to generate a set of control wells, reserve a column for feeder cells alone

- ❏ Dilute sorted target cells with IMDM-c to generate a suspension of 270 cells (for 5 cells per well, final seeding density) or 540 cells (for 10 cells per well, final seeding density) in a final volume of 10.8 mL of IMDM-c
- ❏ Aliquot 200 µL sorted target cell suspension per well
- ❏ Aliquot 200 µL IMDM-c in the control “feeder-alone” wells
- ❏ Prepare a 1 million cells/mL suspension of feeder cells for each 96-well plate
- ❏ Prepare the 5× concentrate for B-cell plating, in 2.2 mL IMDM-c (final volume)
  - ❏ 11 ng IL-4 (for a final concentration of 1 ng/mL)
  - ❏ 0.22 mg LPS (for a final concentration of 20 µg/mL)
  - ❏ 55 µL 10 mM βME (for a final concentration of 50 µM)
  - ❏ 0.55×10^6 feeder cells (for a final seeding density of 10,000 cells per well)
- ❏ Aliquot 50 µL of 5× concentrate into each of the wells
- ❏ Incubate undisturbed at 37°C, 5% CO_2 for 12–14 days

Streptavidin Capture-ELISA assay

- ❏ Aliquot 100 µL per well (of a 96-well plate) of 0.5 µg/mL streptavidin in 0.1 M $NaHCO_3$ (pH 9.4)
- ❏ Seal and incubate at room temperature overnight
- ❏ Wash the plate(s) with ELISA wash buffer
- ❏ Block with 100 µL per well BSA block solution for 1 hour at 37°C
- ❏ Wash the plate(s)
- ❏ Incubate with 2 µg/mL biotinylated target protein in 100 µL/well BSA dilution buffer for 1 hr at 37°C

- ❏ Wash the plate(s) with ELISA wash buffer
- ❏ Incubate with 100 µL/well NFM block solution for 1 hour at 37°C
- ❏ Wash the plate(s) with ELISA wash buffer
- ❏ Add 90 µL BSA dilution buffer per well
- ❏ Add 10 µL Sorted B-cell culture supernatant per well
- ❏ Incubate for 1 hour at 37°C
- ❏ Wash the plate(s) with ELISA wash buffer
- ❏ Incubate with 100 µL/well BSA dilution buffer with the secondary antibody diluted 1:2000 for 1 hour at 37°C
- ❏ Wash the plate(s) with ELISA wash buffer
- ❏ Incubate with 50 µL/well of SureBlue Reserve TMB Substrate for 3 min at room temperature
- ❏ Add 50 µL/well of ELISA stop solution
- ❏ Measure absorbance at 450 nm

RNA harvesting

- ❏ Gently remove and save cell-culture supernatant from the well(s)
- ❏ Add 200 µL RLT buffer

Immunoglobulin sequence isolation and generation of expression constructs

- ❏ Extract RNA with All-Prep DNA/RNA Mini Kit
- ❏ Use 50–250 ng RNA to generate first strand of cDNA with SMARTer RACE 5’/3’ Kit
    - ❏ Gene-specific primers added to the reaction: Mm IgG, Mm IgK, Mm IgL.
- ❏ Purify race-ready cDNA using Qiaquick PCR Purification Kit
- ❏ Use 1.0 µL of purified race-ready cDNA to amplify heavy- and light-chain fragments
    - ❏ IgG reaction: “SMARTer step-out” and “Mm IgG CH1” primers
    - ❏ IgK reaction: “SMARTer step-out” and “Mm IgK CH1” primers
    - ❏ Cycling conditions: 98°C – 30s; 30 cycles (98°C – 10s, 63°C – 30s, 72°C – 30s); 72°C – 5 min
- ❏ Gel-purify PCR products
- ❏ Use 1.0 µL of purified PCR products to adaptor the products for Gibson cloning
    - ❏ IgG reaction: “Gibson SMARTer fwd” and “Mm IgG rev” primers
    - ❏ IgK reaction: “Gibson SMARTer fwd” and “Mm IgK rev” primers
    - ❏ Cycling conditions: 98°C – 30s; 15 cycles (98°C – 10s, 63°C – 30s, 72°C – 30s); 72°C – 5 min
- ❏ Set up Gibson cloning reactions using the heavy- and light-chain adaptored PCR products with the appropriate acceptor construct (predigested with NotI to expose the insertion site) using the NEBuilder DNA Assembly Cloning Kit

- ❏ Transform competent cells with the Gibson cloning products and sequence plasmid purified from multiple colonies from each reaction to ensure completeness of the heavy- and light-chain ORFs
- ❏ At this point paired sequence-verified expression constructs may be tested for expression and antigen recognition