Product Manual

DiSH™ Kit

A Revolutionary mAb Technology for Hybridoma Selection and Cloning

Catalog #: ENZ-71001

Manufactured by Abeome

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NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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DESCRIPTION

Components

- 1 vial of 2x10^6 Sp2ab myeloma fusion partner (ENZ-70008-0001)
- 200 mL PCM (Prefusion) (ENZ-70001-0200)
- 200 mL WCM x 2 (Wash) (ENZ-70002-0200)
- 200 mL FCM (Fusion) (ENZ-70003-0200)
- 100 mL RCM x 2 (Recovery) (ENZ-70005-0100)
- 200 mL ECM (Expansion) (ENZ-70006-0200)
- 1.5 mL Polyethylene Glycol (PEG) (ENZ-70007-0001)

Size

For fusion of 1x10^8 total lymphocytes

Product format

Kit

Storage

- **Cells:** Cells should be stored in liquid nitrogen or seeded immediately upon arrival.
- **Media (PCM, WCM, FCM, RCM, ECM) and PEG:** Store at 2-8°C. Expiration date is indicated on the bottle label.

PRINCIPLE OF DiSH™

The isolation of antigen-specific hybridomas is performed in a four-step procedure. First, lymphocytes are harvested from a mouse that has been immunized with an antigen of interest. The B cells are prepared as a single-cell suspension and fused with the Igα and Igß-expressing myeloma, Sp2ab, resulting in hybridomas that secrete and surface express their specific immunoglobulin. At this point, antigen-specific hybridomas can be isolated using magnetic-labeled antigen or fluorescent-labeled antigen. The antigen-specific hybridomas are subsequently single cell sorted by fluorescence-activated cell sorting (FACS), and supernatant analyzed for secreted antibody after 10 days.

The total elapsed time from harvest to the first screening is typically 16 days, and ranges from 14 to 18 days.

BACKGROUND AND PRODUCT APPLICATIONS

The DiSH™ (Direct Selection of Hybridomas) technology offers a much-improved method for selecting desired hybridomas that produce valuable antibodies. Through genetic engineering of the myeloma fusion partner, Enzo Life Sciences has developed a process that allows one to label only the desired hybridomas with identifying markers. Once labeled, these hybridomas can be isolated from among a pool of hundreds of thousands of cells in a matter of hours using Fluorescence activated cell sorting (FACS) or magnetic separation.

Abeome products and product applications are covered by US Patent No. 7,148,040.

ADDITIONAL REAGENT AND INSTRUMENT REQUIREMENTS

Equipment

- Biohazard safety cabinet certified for level II handling of biological material
- Low speed benchtop centrifuge
- 37°C incubator with humidity and gas control to maintain 95% humidity and 5% CO2 atmosphere.
- Pipette-aid
- Hemocytometer
- Inverted microscope
- 37°C water bath
- Liquid nitrogen tank

Reagents

- Sterile PBS
- 0.4% Trypan blue
- Biotinylated antigen
- 95% ethanol

The following for use with fluorescence-activated cell sorting

- Streptavidin-fluorochrome (e.g., Streptavidin conjugated PE (phycoerythrin) BD Pharmingen Cat # 554061)
- Goat anti-mouse IgG (human & mouse Ig adsorbed), (ATTO 647N conjugate) (Enzo Life Sciences, cat# ALX-211-205TS-C100)
- Flow cytometer capable of single cell sorting (e.g., BD FACS Aria II)

The following for use with magnetic separation

- Miltenyi reagents/supplies required:
- MidiMACS starting kit (LS) Order# 130-042-301 (request Anti-Biotin Microbeads Order# 130-090-485 with this kit)
- MACS BSA stock solution Order# 130-091-376
- AutoMACS Rinsing Solution Order# 130-091-222

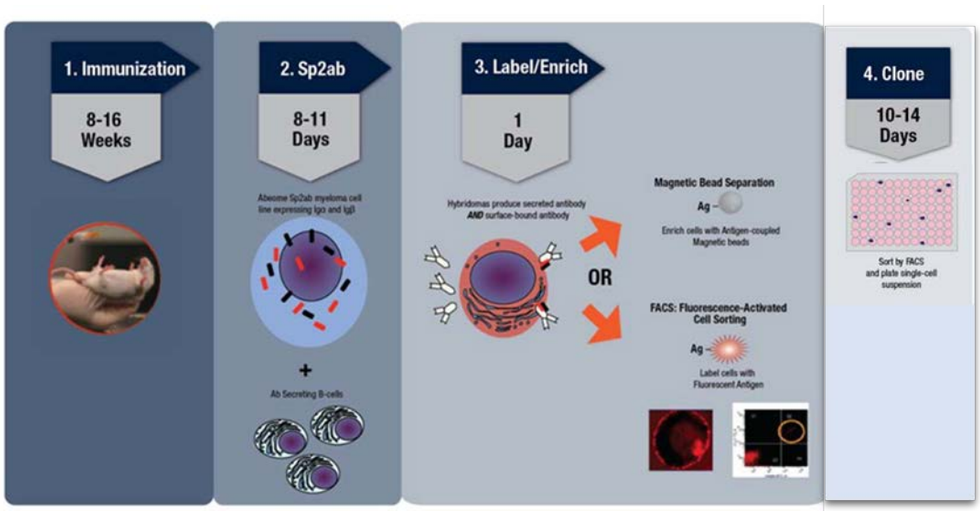
Supplies

- Sterile serological pipettes
- Tissue culture flasks
- ELISA plates (e.g., Nunc Immulon 2HB, Costar Maxisorp)
- 12 mL syringe
- Forceps
- Scissors
- Disposable cell strainer
- Multi-channel pipettor

Biologicals

Primed mouse 1-4 days after antigen boost

DiSH Flowchart



PROTOCOL

All procedures should be carried out using sterile technique in a certified biosafety cabinet.

Mouse (BALB/c) immunization

Ideally, mice should be immunized 8-10 weeks before performing fusion with purified antigen to generate hybridomas (intact cells or complex antigenic mixtures may also be used if desired). This time period allows for the production and development of antibodies specific to the antigen of interest. The most optimal immunization schedule is dependent on the type and dose of antigens and desired affinity of the specific antibodies. Generally, longer periods between booster injections stimulate the production of higher affinity antibodies.

This is an example of a typical injection schedule for immunizing BALB/c mice prior to fusion.

1. Collect a sample of serum or plasma prior to immunization to use as a baseline control for antibody screening. Mice can be bled by a variety of methods including saphenous or tail vein which do not require anesthesia or orbital sinus which does require anesthesia. Use a method that is approved by your Institution's IACUC.
2. Inject 3-6 adult BALB/c mice with antigen. Typically 20-100µg of purified antigen or 100-200µg of antigen mixture is injected intraperitoneally in a total volume of 200µL (i.e., 200µL of a 1:1 stable emulsion of antigen in adjuvant).
3. Repeat the injection 14-28 days later.
4. 10-14 days after the second injection, collect 100-200µL of blood. Prepare plasma from the blood sample and measure antibody levels in an ELISA.
5. Compare plasma collected after antigen boost with the pre-immune serum from the same animal.
6. Continue to give injections at 2-4 week intervals until a good titer (for protein, peptide, or viral lysate antigens >1:30,000; for whole cell antigen > 1:3000) of antibody is obtained.
7. 1-4 days before the day of the fusion, boost the selected mouse with antigen only (no adjuvant) intravenously or subcutaneously in maximum volume of 200µL. Prepare to fuse spleen cells to Sp2ab myelomas 1-4 days later.

Preparation of myeloma cells

Thaw the parental myeloma cells (refer to Appendix, “Thawing and recovering myelomas from frozen stock”) and culture in the Prefusion Medium (PCM) at least one week prior to fusion. Seed cells at a density of 5 x10^4 cells/mL and passage every 2 days. The suggested maximum density is 8 x 10^5 cells/mL.

1. Collect myeloma cells from culture, and transfer to 50 mL centrifuge tube(s).
2. Pellet the cells at 290 × *g*, 4°C, 5 min, aspirate supernatant.
3. Wash cells 1x with an excess of WCM (290×g, 4°C, 5 min.) and aspirate the supernatant.
4. Resuspend the cells with WCM, and determine cell density.

Lymphocyte harvest

The procedures used to harvest spleens, lymph nodes, and bone marrow from immunized animals, for the purpose of preparing lymphocytes for fusion are reviewed elsewhere. Typically ~50% of splenic lymphocytes are B cells.

1. Harvest tissues, and prepare “single-cell” suspensions. Transfer cells to a 50 mL tube, wash cells (290 × *g* (~1200 rpm), 4°C, 5 min.) with an excess of WCM. (It is important to remove serum in the culture before fusing the cells with polyethylene glycol).
2. Optional: Lyse red blood cells using ammonium chloride.
    - 2.1. Add 10 mL 0.8% ammonium chloride that has been warmed to 37°C to cell pellet.
    - 2.2. Incubate for 5 min. at 37°C.
        - 2.2.1. Add PBS to 50 mL.
        - 2.2.2. Centrifuge the cells (290 × *g* (~1200 rpm), 4°C) for 10 min.
        - 2.2.3. Aspirate the supernatant and proceed to Step 3.
3. Resuspend the pellet in 10 mL WCM. Prepare a dilution of cells in 0.4% trypan blue and count live cells using a hemocytometer. (Count white blood cells only if not lysing red blood cells as described in Step 2). Keep cells at 37°C, 95% humidity and 5% CO2 atmosphere until fusion.

Fusion

Herein, we describe a “standard” fusion protocol. Other fusion methods (e.g., electrofusion) should be evaluated empirically for suitability. Since the following steps should be performed in a biological safety cabinet, we recommend preparing a water bath with sterilized water in a wide-mouthed bottle or flask, and warming it to 37°C. The external surface of the bottle can be wiped with 70% ethanol prior to bringing it into the cabinet.

1. Add myeloma cells to the lymphocyte suspension at a ratio of one myeloma cell per five lymphocytes, and add WCM to 50 mL. (Enzo Life Sciences has investigated myeloma to murine B cell ratios between 1:3 and 1:10, and found that the optimal ratio for Sp2ab fusions is 1:5).

2. Centrifuge the combined cell suspension at 290 x *g* (~1200 rpm), 4°C for 5 min.

3. Carefully remove the supernatant as completely as possible. Do not disturb the pellet.

4. Warm the cells to 37°C, about 1-2 min in water bath.

5. While holding the tube in a water bath, add PEG 1000 warmed to 37°C drop-wise over the course of 1 min (10 µL per 1 x 10^6 total cells to a maximum of 1 mL), gently swirling the tube to allow disruption of the pellet.

6. Incubate the cells for 60 sec in 37°C water bath with gentle agitation.

7. Keeping the cells in the water bath, add 4 mL sterile WCM (37°C) to the cells over the course of 2 min while gently swirling the tube. Add an additional 5.0 mL over the next minute with continued swirling. Finally, add an additional 6 mL over one minute. (The purpose of this step-wise addition is to dilute out the PEG slowly allowing adequate time for cell membranes to fuse).

8. Incubate the newly fused cells for 10 minutes in a 37°C water bath. This can be done outside of the biological safety cabinet as long as the vial containing the cells is sprayed down with 70 % ethanol prior to placing it back in the cabinet.

9. Slowly add 30 mL of FCM and centrifuge the cells at 150 x *g* (~1000 rpm), 5 min at room temperature, aspirate supernatant. (Our fusion medium FCS is specifically formulated to enhance the fusion process).

10. Gently resuspend the pellet in 10 mL of FCM. Transfer the suspension to a T-75 tissue culture flask containing 20 mL FCM and incubate for 16-24 hours at 37°C in 5% CO_2 atmosphere.

Selection and Cloning

After allowing the newly fused cells to incubate for 16-24 hours at 37°C in 5% CO_2, the antigen specific hybridomas can be labeled and specifically selected using magnetic separation. Alternatively, hybridomas can be aliquoted into multiple flasks for flow-activated cell sorting at a later date. Biotinylated antigen must be used with both procedures (see Appendix for biotin conjugation of antigen for cell selection).

If selecting for antigen specific clones by FACS (after 16-24 hr fusion recovery incubation) aliquot the cell suspension into multiple T25 tissue culture flasks containing sufficient 37°C FCM to achieve a density of 5-7×105 cells per mL (aliquotting into multiple flasks and sorting before the flasks reach too high of a density will help reduce sibling rate). Add HAT (100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine) to each flask and incubate at 37°C in 5% CO2 atmosphere for 3 days. After 3 days in HAT, collect and centrifuge (290 x g (~1200 rpm), 4°C for 5 min) the contents of each flask into separate 15 mL conical tubes (do not throw away flasks). Aspirate the media and resuspend cells in RCM pre-warmed to 37°C. Return cells suspended in RCM to respective flasks and culture for 4-7 days until the cells have recovered from HAT treatment and begun to divide. Proceed to “Antigen-specific sorting by flow.”

If selecting for antigen specific clones by MACS separation, proceed to “Antigen-specific selection by MACS separation.”

Antigen-specific sorting by flow

Reagents/supplies required:

- Streptavidin-fluorochrome (eg. Streptavidin conjugated PE BD Pharmingen Cat # 554061)
- Goat anti-mouse IgG (human & mouse Ig adsorbed), (ATTO 647N conjugate) (Enzo Life Sciences, cat# ALX-211-205TS-C100)
- Flow cytometer capable of single cell sorting (ex. BD FACS Aria II)

Fluorescent labeling, sorting and screening of hybridomas

Conditioned medium provides important cell growth factors. Sorting media RCM is formulated with conditioned media and additional factors for optimal survival and proliferation of single cell sorted hybridomas. Cells should be kept on ice and protected from light throughout this procedure.

1. Transfer newly fused hybridomas into one centrifuge tube per respective culture flask, pellet the cells at 290 × *g* (~1200 rpm), 4°C, 5 min, and aspirate the supernatant.

    1.1. Transfer control cells (myeloma fusion partner-negative control; an established hybridoma-positive control) into respective centrifuge tubes pellet the cells at 290 × *g* (~1200 rpm), 4°C, 5 min, and aspirate the supernatant.

2. Wash control and experimental cells once with 10 mL WCM, centrifuging at 290 × *g*, 4°C, 5 min, and aspirate the supernatant.

    2.1. Determine density of control cells, and transfer approximately 1×10^6 cells into respective tubes, generating staining and compensation controls:

    Negative (eg. myeloma fusion partner)– a) unstained, b) anti-Ig only, c) antigen only, d) antigen and Ig

    Positive (eg. established hybridoma) – a) unstained, b) anti-Ig only, c) antigen only, d) antigen and Ig

    2.2. Pellet control cells at 290 × *g*, 4°C, 5 min, and aspirate supernatants.

3. Disrupt the pellets by gently tapping the tubes and add 5 ul of purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™-BD Pharmingen Cat# 553141) per10^6 cells. Incubate on ice for 5 min.

4. Add 200µl RCM for every 10^6 cells, and incubate 30 min on ice.

5. Label cells with biotin conjugated antigen.

    5.1. To each tube of fused cells add 10µl biotin conjugated antigen per 10^6 cells.

    5.2. To each tube of control cells marked “antigen only” and “Ig and antigen” add 10µl biotin conjugated control

specific antigen.

🗁 Each 10µl of biotin conjugated antigen should contain approximately 1-10µg of target for proteins and peptides, 10 to 50µg for viral lysate, or, for lysed cells, approximately 5×10^5 whole cell equivalents.

5.3. Incubate 30 min. on ice, protected from light.

6. Label with antibody specific to surface immunoglobulin.

6.1. To each vial of fused cells add 5µl fluorochrome-conjugated antibody specific to surface immunoglobulin.

6.2. To each vial of cells marked “Ig only” and “Ig and antigen” add 5µl fluorochrome-conjugated antibody specific to surface immunoglobulin.

🗁 Since each lot of antibody can differ, it is recommended that the final concentration be determined empirically using a control hybridoma.

6.3. Incubate 30 min. on ice, protected from light.

7. Wash all cells with 10 mL RCM, centrifuging at 290 × *g*, 4°C, 5 min. Aspirate supernatants.

8. Resuspend pellets with 200µl RCM for every 10^6 cells.

9. To newly fused cells and controls marked “antigen only” and “Ig and antigen” add 5µg fluorochrome-conjugated streptavidin, and incubate 10 min on ice, protected from light.

10. Wash all cells with 10 mL RCM, centrifuging at 290 × *g*, 4°C, 5 min. Aspirate supernatants.

11. Resuspend pellets with 500µl WCM.

11.1. A high density of cells may be diluted further to achieve an optimal cytometer flow rate of about 3,000 to 5,000 events per second at 20 psi and nominal flow rate setting of “2.”

12. Sort double positive cells (those postitive for antigen-reactivity and surface Ig), into wells of 96-well culture plate (1 cell/well) containing 130µl RCM.

13. Incubate at 37°C, 5% CO_2, in humidified incubator.

14. After 7–10 days, or when identified colonies become confluent, screen cells for secretion of specific antigen-reactive antibody.

15. Analyze supernatant from each hybridoma for antibody secretion by ELISA or comparable assay and add 100µl fresh

16. Resuspend hybridomas that show secretion, and expand by transferring 100µl of cells to a 24-well plate containing 1 mL of RCM.

17. When cells have grown to a suitable density (~4 x 10^5 cells/mL), expand the cells into 2 wells of a 6 well tissue culture plate containing 5 mL ECM.

18. When cells have grown to a suitable density (~4 x 10^5 cells/mL), freeze the cells (Appendix, “Freezing hybridomas”) from one well and expand the remaining positive clones into a T75 tissue culture flask containing 30 mL ECM.

19. Continue expanding positive hybridoma cultures as desired for downstream procedures, seeding cells at 1 x 10^4 - 5x10^4 cells/mL. At this point, additional vials may also be frozen.

Antigen-specific selection by magnetic separation

Miltenyi reagents/supplies required:

- MidiMACS starting kit (LS) Order# 130-042-301 (request Anti-Biotin Microbeads Order# 130-090-485 with this kit)
- MACS BSA Stock Solution Order# 130-091-376
- autoMACS Rinsing Solution Order# 130-091-222

Magnetic labeling of hybridomas

1. Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA) and 2mM EDTA by diluting MACS BSA Stock Solution 1:20 with autoMACS Rinsing Solution. Keep buffer cold (2-8°C). Degas buffer before use, as air bubbles could block the column.

2. Collect newly fused cells from culture into a 50 mL conical tube. Pellet the cells at 290 × *g*, 4°C, 5 min, and aspirate the supernatant.

3. Wash cells by resuspending in 10 mL of WCM. Determine cell density. Pellet the cells at 290 × *g*, 4°C, 5 min, and aspirate the supernatant.

4. Add purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™-BD Pharmingen Cat# 553141). Incubate on ice for 5 min.

5. Label with biotin-conjugated antigen prepared in Section 2.4.

> 🗁 Each 10µl of biotin conjugated antigen should contain approximately 1-10µg of target for proteins and peptides, 10 to 50µg for viral lysate, or, for lysed cells, approximately 5×10^5 whole cell equivalents.

5.1. Add 10 µl biotin-conjugated antigen per 10^6 cells.

5.2. Incubate 30 min. on ice, protected from light.

6. Wash cells with 1-2 mL buffer per 10^7 cells and centrifuge at 290 × *g* (~1200 rpm), 4°C, 5 min.

7. Repeat step 6.

8. Resuspend cell pellet in 70 µl of buffer.

9. Add 20µl of Miltenyi Anti-Biotin MicroBeads per 10^7 total cells.

10. Mix well and incubate for 15 minutes in the refrigerator (2-8°C).

11. Wash cells by adding 1-2 mL of buffer per 10^7 cells and centrifuge at 290 × *g* (~1200 rpm), 4°C, 5 min.

12. Resuspend up to 10^8 cells in 500µl of buffer.

13. Proceed to magnetic separation.

Magnetic separation of hybridomas

1. Place column in the magnetic field of the MidiMACS separator. For details refer to the MACS Column data sheet.

2. Prepare column by rinsing with 3 mL buffer.

3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.

4. Wash column with the 3x3 mL buffer. Collect unlabeled cells that pass through and combine with the effluent from step 3.

> 🗁 Perform wash steps by adding buffer aliquots only when the column reservoir is empty.

5. Remove the column from the separator and place it on a suitable collection tube.

6. Pipette 5 mL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

7. To increase the purity of magnetically labeled cells the eluted fraction can be enriched over a second LS Column. Repeat the magnetic separation procedure as described in steps 1-6 by using a new column.

8. Pellet the cells at 290 × *g*, 4°C, 5 min, and aspirate the supernatant.

Please refer to data sheets for Miltenyi product warnings and warranties. All data sheets for Miltenyi reagents are available at www.miltenyibiotec.com.

Cloning and harvest

1. Plate cells into a semisolid medium following manufacturer's instructions
2. Alternatively, distribute 1 cell per well in RCM into 96 well culture plates.
   - 2.1. Incubate at 37°C, 5% CO_2, in humidified incubator.
   - 2.2. After 7–10 days, or when identified colonies become confluent, screen cells for secretion of specific antigen-reactive antibody.
   - 2.3. Analyze supernatant from each hybridoma for antibody secretion by ELISA or comparable assay and add 100µl fresh
   - 2.4. Resuspend hybridomas that show secretion, and expand by transferring 100µl of cells to a 24-well plate containing 1 mL of RCM.
   - 2.5. When cells have grown to a suitable density (~4 x 10^5 cells/mL), expand the cells into 2 wells of a 6 well tissue culture plate containing 5 mL ECM.
   - 2.6. When cells have grown to a suitable density (~4 x 10^5 cells/mL), freeze the cells (Appendix, "Freezing hybridomas") from one well and expand the remaining positive clones into a T75 tissue culture flask containing 30 mL ECM.
   - 2.7. Continue expanding positive hybridoma cultures as desired for downstream procedures, seeding cells at 1 x 10^4 - 5x10^4 cells/mL. At this point, additional vials may also be frozen.

APPENDIX

Preparation of antigen for cell selection: biotin conjugation

Conjugation of peptides, proteins, and viral lysates

1. **FluoReporter Mini-biotin-XX kit (Molecular Probes Cat# F-6347) or Sulfo-NHS-LC-Biotin (Pierce Cat# 21335, Cat# 21430 for kit) (follow manufacturer's directions) both are amine-reactive.** Follow either of the manufacturers' protocol to label these antigens. Coupling reactions use a 100mM sodium bicarbonate, pH 8.4 buffer which should be compatible with most antigens.

    1.1. After the reaction is completed, dialyze the biotinylated antigen for 24 hours against 100 volumes of PBS. Alternately, we recommend using Millipore's Amicon Ultra centrifugal filter units (10,000 MWCO) to buffer exchange the protein solution against PBS or spin columns provided if using kit.

    1.2. Transfer the biotinylated material to a sterile tube, and filter sterilize through a 0.22µm syringe filter, store at 4°C.

    1.3. Determine efficiency of biotin conjugation (below).

2. **Method using EZ-Link-biotin-PEO-LC-amine (Pierce Cat. #21347) carboxyl-reactive. Follow the manufacturers' protocol to label these antigen types.** This coupling reaction is buffered to a pH to 4.5 to 5.0 which should be compatible with many antigens.

Determining efficiency of biotin conjugation (example protocol)

1. To confirm the biotinylation of the antigen, coat 96-well ELISA plates (eg. Nunc Immulon 2HB or Costar Maxisorp) by diluting avidin or streptavidin to 1µg/mL in borate buffered saline (BBS) at a volume appropriate for the number of wells to be coated.
2. Add 50µl avidin or streptavidin suspension to each well of ELISA plate ([final] = 50ng/well). Incubate overnight at 4°C, protected from light.
3. Wash plates three times with 350µl per well PBS-T.
4. Block by adding 200µl ELISA blocking solution to each well and incubate 2 hr at room temp to overnight at 4°C. Prepare serial dilutions of biotinylated protein/polypeptide in 1% BSA PBS-T, and add 50µl to respective wells.
5. Incubate at room temperature for 1 hour.
6. Wash plate three times with PBS-T (350µl per well if using plate washer).
7. Dilute alkaline phosphatase conjugated streptavidin (SA-alkPhos Sigma Catalog# S2890) to an appropriate concentration in 1% BSA PBS-T. Typically, SA-alkPhos is diluted 1:500, but this should be verified for each new lot. Add 50µl SA-alkPhos to each well of ELISA plate.
8. Incubate at room temperature for 1 hour.
9. Wash plate three times with 350µl PBS-T per well using plate washer.
10. Colorimetric reaction (Phosphatase Substrate (Sigma #S0942, 5mg/tablet). Calculate amount of substrate solution needed at 50µl per well. Dilute one Phosphatase Substrate tablet per 8.33 mL substrate buffer.

    11.1 Dispense 50µl per well and incubate 35 min at room temp.
11. Quantitate results using plate reader at dual wavelengths of 405nm and 492nm.

Confirming antigenicity

It is possible that biotin conjugation can result in loss of epitopes. Thus, Enzo Life Sciences recommends verifying that biotin conjugated proteins and peptides continue to react with immune serum. Test by ELISA, coating plates with biotin conjugated antigen

and unbiotinylated antigen, and assay using serum obtained from immunized mice during test bleed.

Thawing and recovering myelomas from frozen stock

1. Remove vial of frozen cells (containing 2 x 10^6 cells) from liquid nitrogen freezer and immediately thaw culture in a 37°C water bath (thaws in 60-90 seconds).

2. Spray vial with 70% ethanol before opening in a biosafety hood.

3. Gently transfer thawed cell suspension into a sterile 15 mL conical tube containing 9 mL warm PCM. Centrifuge for 10 minutes at 150 x *g* (~1000 rpm).

4. Gently resuspend cell pellet in 1 mL of pre-warmed PCM and transfer to 1 T-25 cm^2 flask containing 9 mL prewarmed PCM.

5. Passage cells as needed to maintain early-mid log phase growth.

Freezing hybridomas

1. Prepare FBS (HyClone Cat# SH30080.03) containing 20% DMSO (dimethylsulfoxide Fisher Scientific Cat #BP231-100).

    1.1. Cool FBS on ice for 1 hr.

    1.2. Add DMSO slowly, a fraction of the total quantity at a time.

        1.2.1. Adding DMSO to FBS produces an exothermic reaction. Adding the DMSO too quickly will result in the generation of heat sufficient to denature some serum proteins.

        1.2.2. After all DMSO has been added, allow to cool to room temperature.

        1.2.3. Sterilize by filtration.

2. Suspend cells to be frozen in incomplete culture medium to a concentration of not less than 2×10^6 cells/mL.

3. Add FBS 20% DMSO to the cell suspension to a final volume ratio of 1:1.

4. Aliquot 1mL of cell suspension per cryovial.

5. Place vials into insulated container and incubate overnight to no more than 7 days at <-70°C.

6. Transfer cells to liquid nitrogen.

7. It is recommended that one vial be tested after two weeks by thawing and culturing.

FORMULATIONS/ABBREVIATIONS (unless otherwise noted, dry chemicals can be purchased from Fisher Scientific)

- 0.8% Ammonium chloride

  1000 mL ddH_2O

  8g Ammonium chloride (NH_4Cl)

  Filter Sterilize

  Store at 4°C, 6-month expiration.

- Borate Buffered Saline (BBS)

  900 mL distilled, deionized

  H_2O **6.19g** Boric Acid (H_3BO)

  9.5g Sodium Borate ($Na_2B_4O_7$)

  4.39g Sodium Chloride (NaCl)

  Adjust pH to 7.2±0.05.

  Bring volume to 1000 mL with distilled, deionized H_2O.

- 1% BSA in PBS-T

  1000 mL Phosphate Buffered Saline (PBS) **10g** Bovine Serum Albumin, fraction V **10 mL** Sodium Azide, 2% (NaN_3)

  0.5 mL Tween 20 (polyoxyethylene sorbitan monolaurate)

- ELISA blocking solution (3% BSA in PBS-T)

  1000 mL Phosphate Buffered Saline (PBS)

  10 mL Sodium Azide, 2% (NaN_3)

  0.5 mL Tween 20 (polyoxyethylene sorbitan monolaurate)

  30g Bovine Serum Albumin, fraction V (BSA)

- Phosphate Buffered Saline (PBS)

  800 mL distilled, deionized H_2O

  8.0g NaCl

  0.2g KCl

  1.44g $Na_2HPO_4 \bullet 7H_2O$

  0.24g KH_2PO_4

  Adjust pH to 7.2±0.05.

  Bring volume to 1000mL with distilled, deionized H_2O.

  0.22µm filter sterilize

- PBS-T (PBS, 0.05% Tween 20)

  1000 mL Phosphate Buffered Saline (PBS)

  10 mL Sodium Azide, 2% (NaN_3)

  0.5 mL Tween 20 (polyoxyethylene sorbitan monolaurate)

- 100mM sodium bicarbonate, pH 8.4

  950 mL distilled, deionized H_2O

  1.36g sodium carbonate

  7.35g sodium bicarbonate

  Adjust pH, if necessary.

  Bring volume to 1000 mL with distilled, deionized H_2O.

- Substrate buffer (10mM diethanolamine)

  800 mL ddH_2O

  97 mL diethanolamine (Sigma #D8885)

  100mg magnesium chloride ($MgCl_2$)

  Adjust pH to 9.8 with 10 M HCl then adjust volume to 1000 mL with ddH_2O

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