

Chapter 6

Production and Purification of Monoclonal Antibodies

Masami Nakazawa, Mari Mukumoto, and Kazutaka Miyatake

Abstract

Monoclonal antibodies (mAb's) have been used extensively in biochemical and biomedical studies, including immunoelectron microscopy. Production of mAb's consists of four steps: immunizing the animal usually a mouse, obtaining immune cells from the spleen of the immunized mouse, fusing the spleen cells with myeloma cells to obtain hybridomas, and selecting the hybridoma cell line producing the desired mAb. In this chapter, we present a general method for monoclonal antibody production.

Key words: Monoclonal antibody, myeloma cell, hybridoma, cell fusion, mouse ascites method, protein A.

1. Introduction

Monoclonal antibodies (mAb's) are used extensively in basic biomedical and biochemical research, including immunoelectron microscopy. In general, mAb shows lower nonspecific binding than do polyclonal antibodies because they are a homogeneous antibody recognizing a single epitope.

Construction of cell lines producing a desired mAb requires the following four steps: immunizing an animal, usually a mouse; obtaining immune cells from its spleen, fusing the spleen cells with myeloma cells to make them immortal, and selecting the hybridoma cell lines producing the desired mAb. The hybrid antibody-producing cell resulting from the fusion of an antibody-secreting cell and an immortal cell (i.e., myeloma) is called a hybridoma. The development of the immortal hybridoma requires the use of animals.

Producing excellent mAb's needs antigens which are suitable for the experiment and a good selection system. In this chapter, we also describe the indirect ELISA antibody assay method, which is one of the most popular and convenient assay systems used in the selection of mAb's.

2. Materials

2.1. Immunization of Mice

1. Institutional Animal Care and Use Committee approval.
2. Sterile phosphate-buffered saline (PBS): prepare 10× PBS by mixing 87.7 g NaCl, 30 g Na₂HPO₄·12H₂O, 2.9 g NaH₂PO₄·2H₂O, and distilled water for a final volume of 1 L. Dilute the 10× PBS tenfold with distilled water to prepare PBS as needed. Sterilize by autoclaving at 121°C for 20 min.
3. Antigen solution: resuspend antigen in sterile PBS for a final concentration of 500 µg/mL. A minimum of 2 mg antigen is required to immunize five mice.
4. Balb/c female mice, 7 weeks old (*see Note 1*).
5. Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO; *see Note 2*).
6. Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO).
7. Luer-lock, all-plastic type syringes for preparing emulsion (*see Note 3*).
8. 22-Gauge 1.5-in. syringe needles.
9. 26-Gauge 0.5-in. syringe needles.
10. 18-Gauge double hub micro-emulsifying needle with reinforcing bar (Popper & Sons, New Hyde Park, NY).

2.2. Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA)

1. PBS: prepare 10× PBS by mixing 87.7 g NaCl, 30 g Na₂HPO₄·12H₂O, 2.9 g NaH₂PO₄·2H₂O, and distilled water for a final volume of 1 L. Dilute the 10× PBS tenfold with distilled water to prepare PBS as needed.
2. Antigen solution: dilute antigen with PBS for a final concentration of 5 µg/mL.
3. PBS-T: PBS containing 0.05% Tween 20.
4. Blocking buffer: 1% BlockAce (DS Pharma Biomedical, Osaka, Japan) in water.
5. Microtiter plate washer (Bio-Rad Laboratories, Hercules, CA).

6. Microtiter plate reader with 490-nm filter (Bio-Rad Laboratories, Hercules, CA).
7. Test antisera or test antibodies.
8. Horseradish peroxidase-conjugated goat anti-mouse IgG- (γ chain) (SouthernBiotech, Birmingham, AL).
9. 10 mg *o*-Phenylenediamine dihydrochloride tablets (Sigma-Aldrich, St. Louis, MO).
10. 30% Hydrogen peroxide.
11. 0.5 M H₂SO₄.
12. Multichannel pipette and disposable pipette tips.
13. 96-Well ELISA plate (Asahi Techno Glass, Chiba, Japan).
14. Citrate phosphate buffer: prepare 10 $\times$ citrate phosphate buffer stock solution by mixing 36.8 g Na₂HPO₄•12H₂O, 10.2 g citric acid, and distilled water for a final volume of 100 mL. Store stock solution at 4°C. Dilute 10 $\times$ citrate phosphate buffer stock solution tenfold as needed.

2.3. Preparation of Myeloma Cells

1. Frozen stock of P3X63-Ag8.653 murine myeloma cell line (Japanese Collection of Research Bioresources, Tokyo, Japan).
2. DMEM: sterile serum-free Dulbecco's Modified Eagle's Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate (supplied as sterile liquid medium; Life Technologies, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin (use Penicillin-Streptomycin, liquid, Life Technologies, Carlsbad, CA).
3. 10% FBS DMEM: DMEM supplemented with 10% fetal bovine serum (FBS; supplied as sterile liquid; Thermo Trace, Sydney, Australia; *see Note 4*).
4. 8-Azaguanine myeloma medium: 10% FBS DMEM supplemented with 130 μ M 8-azaguanine (Sigma-Aldrich, St. Louis, MO; *see Note 5*).
5. Sterile 100-mm tissue culture dishes (Asahi Techno Glass, Chiba, Japan).
6. Sterile 15-mL plastic centrifuge tubes with conical bottom.
7. Humidified 37°C, 5% CO₂ tissue culture incubator (HIRASAWA WORKS, Tokyo, Japan).
8. Inverted phase contrast microscope.
9. Alcohol swabs.
10. 0.4% Trypan blue solution (Life Technologies, Carlsbad, CA).
11. Hemocytometer.

2.4. Fusion of Murine Myeloma Cells with Murine Spleen Cells

1. DMEM: sterile serum-free Dulbecco's Modified Eagle's Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate (supplied as sterile liquid medium; Life Technologies, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (use penicillin–streptomycin, liquid; Life Technologies, Carlsbad, CA).
2. 15% FBS DMEM: DMEM supplemented with 15% fetal bovine serum (FBS; supplied as sterile liquid; Thermo Trace, Sydney, Australia; *see Note 4*).
3. HAT (hypoxanthine/aminopterin/thymidine) medium: prepare 500 mL 15% FBS DMEM containing 5% BriClone (Archport, Dublin, Ireland; *see Note 6*). Take 10 mL of this medium and reconstitute one vial of HAT Media Supplement (50×) Hybri-Max (Sigma-Aldrich, St. Louis, MO). Add the contents of the vial to the 490 mL of 15% FBS DMEM containing 5% BriClone for a final concentration of 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine
4. HT (hypoxanthine/thymidine) medium: prepare 500 mL 15% FBS DMEM containing 5% BriClone (Archport, Dublin, Ireland; *see Note 6*). Take 10 mL of this medium and reconstitute one vial of HT Media Supplement (50×) Hybri-Max (Sigma-Aldrich, St. Louis, MO). Add the contents of the vial to the 490 mL of 15% FBS DMEM containing 5% BriClone for a final concentration of 100 µM hypoxanthine, 16 µM thymidine.
5. Red blood cell lysis buffer: mix 180 mL of 0.16 M NH₄Cl (8.3 g/L) and 20 mL of 0.17 M Tris–HCl, pH 7.2. Autoclave at 121°C for 15 min and store at 4°C.
6. DMSO: sterile-filtered, hybridoma tested dimethyl sulfoxide (Hybri-Max; Sigma-Aldrich, St. Louis, MO).
7. PEG solution: autoclave 1.5 g of polyethyleneglycol 4000 (gas chromatography grade; Merck Chemicals, Nottingham, UK) at 121°C for 20 min in a glass bottle. Add 1.5 mL of DMEM and 0.3 mL of DMSO (hybridoma tested grade, Sigma-Aldrich, St. Louis, MO) before cooling in a sterile hood (*see Note 7*).
8. 50-mL Sterile plastic centrifuge tubes with conical bottom.
9. Dissecting board or plastic tray.
10. Sterile scissors: two general operating scissors with sharp/blunt points (14.5 cm) and one general operating scissors with sharp/sharp points (11.5 cm).
11. Sterile forceps: one forceps with teeth to hold the skin of the mouse tightly (15 cm), two forceps without teeth (15 cm), and one forceps without teeth (11 cm).

12. Sterile 60-mm plastic culture dishes (Asahi Techno Glass, Chiba, Japan).
13. Sterile 100-mm plastic culture dishes (Asahi Techno Glass, Chiba, Japan).
14. Sterile 70 μm mesh cell strainer (BD Bioscience, San Jose, CA).
15. Sterile 10-mL plastic syringe with 22-gauge needle.
16. Sterile polystyrene 96-well microtiter plates (Asahi Techno Glass, Chiba, Japan).
17. Stopwatch
18. Humidified 37°C, 5% CO₂ tissue culture incubator (HIRASAWA WORKS, Tokyo, Japan).

2.5. Cloning of Hybridoma Cell Lines

1. HT medium: prepare 500 mL 15% FBS DMEM containing 5% BriClone (Archport, Dublin, Ireland; *see Note 6*). Take 10 mL of this medium and reconstitute one vial of HT Media Supplement (50 $\times$) Hybri-Max (Sigma-Aldrich, St. Louis, MO). Add the contents of the vial to the 490 mL of 15% FBS DMEM containing 5% BriClone for a final concentration of 100 μM hypoxanthine, 16 μM thymidine.
2. Sterile polystyrene 96-well microtiter plates (Asahi Techno Glass, Chiba, Japan).
3. Sterile polystyrene 24-well microtiter plates (Asahi Techno Glass, Chiba, Japan).
4. Sterile 15-mL plastic centrifuge tubes with conical bottom.
5. Humidified 37°C, 5% CO₂ tissue culture incubator (HIRASAWA WORKS, Tokyo, Japan).
6. Inverted phase contrast microscope.
7. 0.4% Trypan blue solution (Life Technologies, Carlsbad, CA).
8. Hemocytometer.
9. Additional reagents and equipment for indirect ELISA (*see Section 2.2*).

2.6. Freezing and Recovery of Hybridoma Cell Lines

1. DMEM: sterile serum-free Dulbecco's Modified Eagle's Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate (supplied as sterile liquid medium; Life Technologies, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin (use Penicillin-Streptomycin, liquid, Life Technologies, Carlsbad, CA).
2. Sterile 15-mL plastic centrifuge tubes with conical bottom.
3. Sterile 50-mL plastic centrifuge tubes with conical bottom.
4. CELLBANKER 1 (Nippon zenyaku kogyo, Fukushima, Japan).

5. Sterile 2-mL cryotubes with internal thread (Asahi Techno Glass, Chiba, Japan).
6. Inverted phase contrast microscope.
7. 0.4% Trypan blue solution (Life Technologies, Carlsbad, CA).
8. Hemocytometer.
9. 15% FBS DMEM: DMEM supplemented with 15% fetal bovine serum (FBS; supplied as sterile liquid; Thermo Trace, Sydney, Australia; *see* **Note 4**).
10. Alcohol swabs.
11. Pasteur pipettes.
12. 37°C water bath.
13. Humidified 37°C, 5% CO₂ tissue culture incubator (HIRASAWA WORKS, Tokyo, Japan).
14. -80°C freezer (SANYO Electric, Tokyo, Japan).
15. Liquid nitrogen freezer (TAIYO NIPPON SANSO CORPORATION, Tokyo, Japan).

2.7. Production of mAb in the Murine Ascites Fluid

1. Cloned hybridoma cell line producing desired antibody.
2. Male Balb/c mice (syngenic host) older than 8 weeks.
3. Pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, St. Louis, MO; *see* **Note 8**).
4. DMEM: sterile serum-free Dulbecco's Modified Eagle's Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate (supplied as sterile liquid medium; Life Technologies, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (use penicillin-streptomycin, liquid; Life Technologies, Carlsbad, CA).
5. 15% FBS DMEM: DMEM supplemented with 15% fetal bovine serum (FBS; Thermo Trace, Sydney, Australia; *see* **Note 4**).
6. Pasteur pipettes.
7. Scissors with sharp/blunt points (14.5 cm).
8. Forceps, 15 cm, with teeth.
9. Sterile normal saline solution: 0.9% NaCl.
10. Sterile 5-mL luer-lock all-plastic type syringes for pristane injections (*see* **Note 3**).
11. Sterile 5-mL plastic syringes.
12. 22-Gauge needle.
13. 26-Gauge needle.

14. 0.4% Trypan blue solution (Life Technologies, Carlsbad, CA).
15. Hemocytometer.
16. Inverted phase contrast microscope.
17. No. 2 filter paper.
18. Sterile 25 mm mixed cellulose acetate 0.45- μ m syringe filter (Toyo roshi kaisya, Tokyo, Japan).

2.8. Purification of mAb Using Affinity Chromatography

1. IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche diagnostics, Mannheim, Germany).
2. HiTrap Protein A HP (1 mL) column (GE Healthcare UK, Buckinghamshire, England).
3. 25 mm Mixed cellulose acetate 0.45- μ m syringe filter, not sterile (Toyo roshi kaisya, Tokyo, Japan).
4. 47-mm 0.45- μ m Mixed cellulose acetate membrane filter.
5. Binding buffer:
 - a. For IgG₁ subclass (1.5 M glycine-NaOH, pH 8.9, 3 M NaCl): dissolve 56.3 g of glycine and 87.7 g of NaCl in 350 mL of Milli-Q water. Adjust the pH to 8.9 with NaOH and add Milli-Q water for a final volume of 500 mL. Filter with a 47-mm 0.45- μ m mixed cellulose acetate membrane filter and degas under vacuum supplied by an aspirator.
 - b. For IgG_{2a}, IgG_{2b}, and IgG₃ subclasses (20 mM sodium phosphate buffer, pH 7.0): Dissolve 1.56 g of NaH₂PO₄•2H₂O in 350 mL of Milli-Q water. Adjust the pH to 7.0 with NaOH and add Milli-Q water for a final volume of 500 mL. Filter with a 47-mm 0.45- μ m mixed cellulose acetate membrane filter and degas under vacuum supplied by an aspirator.
6. Elution buffer:
 - a. For IgG₁, IgG_{2a}, and IgG₃ subclasses (100 mM sodium citrate buffer, pH 4.5): dissolve 10.5 g of citric acid monohydrate in 350 mL of Milli-Q water. Adjust the pH to 4.5 with NaOH and add Milli-Q water for a final volume of 500 mL. Filter with a 47-mm 0.45- μ m mixed cellulose acetate membrane filter and degas under vacuum supplied by an aspirator.
 - b. For IgG_{2b} subclass (100 mM sodium citrate buffer, pH 3.5): dissolve 10.5 g of citric acid monohydrate in 350 mL of Milli-Q water. Adjust the pH to 3.5 with NaOH and add Milli-Q water for a final volume of 500 mL. Filter with a 47-mm 0.45- μ m mixed cellulose acetate membrane filter and degas under vacuum supplied by an aspirator.

7. Ascites fluid containing desired mAb.
8. 1 M Tris-HCl, pH 9.0.
9. 2.5-mL Plastic syringes.
10. 10-mL Plastic syringes.

3. Methods

3.1. Immunization of Mice

1. Prepare an emulsion (200 μ L/mouse) using equal volumes of sterile PBS containing 50 μ g antigen/mouse and Freund's complete adjuvant, by linking two locking syringes, one loaded with antigen solution (500 μ g/mL of antigen in sterile PBS) and the other loaded with adjuvant (using 22-gauge needle), using a double ended Luar-Lock connector. Press the syringe barrels back and forth until a stable emulsion is produced (for 5–10 min till thickened). Let a drop of the emulsion fall into water to check the endpoint of emulsification. A stable emulsion will not disperse.
2. Inject a mouse intraperitoneally with 200 μ L of the antigen emulsion using a 26-gauge needle. Five mice are immunized for each antigen.
3. Boost mice 2 weeks later by intraperitoneally injecting an emulsion (200 μ L/mouse) of equal volumes of sterile PBS containing 50 μ g antigen/mouse and Freund's incomplete adjuvant. The emulsion is prepared and injected as in Steps 1 and 2.
4. Collect blood 1 week after the booster injection to determine the antibody titer. Place mice in a retaining device and use a 26-gauge needle to make a small incision in the mouse's tail vein. Immediately collect the blood (<20 μ L) in a microcentrifuge tube using a 200- μ L pipette or glass capillary. Keep the sample blood at 37°C for 1 h and centrifuge at 5,000 $\times g$ for 10 min to separate the serum sample (supernatant fraction).
5. Determine the antibody titer in the serum by indirect ELISA (*see Section 3.2, Note 9*).
6. If the antibody titer is too low for cell fusion, boost mice as in Step 3 every 2 weeks until a sufficient titer is achieved (e.g., a significant signal at <1/10,000 dilution).
7. When the antibody titer is sufficient, boost mice by injecting 50 μ g antigen in sterile PBS without adjuvant intraperitoneally (100 μ L).

8. Four days after the final immunization (Step 7), fuse murine myeloma cells with spleen cells from the immunized mice (*see Section 3.4*).

3.2. Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA)

1. Coat plate(s) with antigen by dispensing 100 μL of antigen solution (5 $\mu\text{g}/\text{mL}$ in PBS) into each well of an ELISA plate using a multichannel pipette and tips. Close the lid and incubate at 37°C for 1 h or 4°C overnight.
2. Rinse the coated plate once with PBS-T using microplate washer apparatus.
3. Add 200 μL of 1% BlockAce solution to each well and incubate at 37°C for 1 h or 4°C overnight.
4. Rinse plate once with PBS-T using microplate washer apparatus.
5. Add 100 μL of antibody samples diluted in PBS to each of the coated wells, close the lid, and incubate at 37°C for 1 h.
6. Wash plate five times with PBS-T using microplate washer and remove residual liquid completely by vigorously patting on a paper towel.
7. Add 100 μL of diluted antibody-enzyme conjugate (Goat anti-mouse IgG (γ chain)-horseradish peroxidase conjugated; 1:3,000 dilution in PBS-T) to each well (*see Note 10*), close the lid, and incubate at 37°C for 1 h.
8. Wash plate five times with PBS-T using microplate washer and remove residual liquid completely by vigorously patting on a paper towel.
9. Prepare the substrate solution by dissolving one tablet of *o*-phenylenediamine dichloride in 25 mL of citrate phosphate buffer in the dark and then add 5 μL of 30% H_2O_2 .
10. Add 200 μL of substrate solution to each well and incubate at 37°C for 30 min in the dark.
11. Add 50 μL of 0.5 M H_2SO_4 to each well to stop the peroxidase reaction.
12. Read the absorbance at 490 nm with a microtiter plate reader (*see Note 9*).

3.3. Preparation of Myeloma Cells

3.3.1. Culture of Myeloma Cells

1. At least 7 days before the cell fusion experiment, start culturing myeloma cells. Place a sterile 15-mL centrifuge tube containing 5 mL of DMEM prewarmed in a 37°C water bath and a sterile 100-mm culture dish in the sterile hood.

2. Recover a cryotube containing frozen myeloma cells from the liquid nitrogen storage tank.
3. Put the cryotube in 37°C water bath for 2–5 s to thaw the surface of frozen cells and move it to a sterile hood.
4. Wipe the surface and top of the cryotube with an alcohol swab and transfer the cells to a 15-mL centrifuge tube. Steps 1–4 should be completed within 1 min in order to maintain maximum cellular viability.
5. Centrifuge at $200\times g$ for 5 min. The cells will thaw while centrifuging.
6. Aspirate the supernatant.
7. Resuspend the cell pellet in 10 mL of 10% FBS DMEM.
8. Transfer the resuspended cells to a 100-mm culture dish and spread the cells.
9. Incubate the culture overnight in a CO₂ incubator at 37°C.
10. Aspirate the culture medium and add 10 mL of new culture medium (10% FBS DMEM).
11. Incubate the culture in a CO₂ incubator at 37°C until the culture becomes confluent. Check the condition of the cells with an inverted microscope.
12. Prepare four new culture dishes containing 8 mL of 10% FBS DMEM.
13. Completely resuspend the cell culture with a Pasteur pipette and subculture 2 mL of cell suspension in the new dishes.
14. Continue to subculture as in Steps 12–13 when the cells become almost confluent (*see Note 11*).

3.3.2. Cell Viability Test by Trypan Blue Exclusion

1. Suspend cell culture with a Pasteur pipette.
2. Mix 10 μ L of trypan blue solution and 10 μ L of cell suspension.
3. Place a clean coverslip on the hemocytometer. Introduce the trypan blue cell mixture (Step 2) into the counting chamber from the edge of the coverslip. Allow the suspension to be drawn into the chamber by capillary action. Fill the chamber on the opposite side in the same manner.
4. Count the unstained (viable) and stained (dead) cells separately in the hemocytometer. Each counting chamber consists of nine 1-mm squares divided into smaller squares. One of the 1-mm squares represents a volume of 10^{-4} mL. Count all the cells within each of the four corner squares. In your counting, count the cells that lie on the top and the left-hand lines of each square but not those on the bottom

or the right-hand lines. Count the chamber on the opposite side in the same manner.

5. Calculate the mean number of cells in each of the 1-mm squares. Multiply by 10^4 and the dilution factor ($2\times$ in this case) to obtain the cell concentration in cells/mL culture.
6. Calculate the percentage of viable cells from the number of total cells (viable + dead) and unstained cells (viable).

3.4. Fusion of Murine Myeloma Cells with Murine Spleen Cells

3.4.1. Isolation of Splenocytes

1. Euthanize a mouse by cervical dislocation and fully immerse the mouse in a beaker containing 70% ethanol (*see Note 12*).
2. Place the mouse with its right side down on a dissecting board or plastic tray (head-side left).
3. Lift the skin over the thorax using sterile forceps and cut the skin away with sterile scissors to expose the left side of the rib cage.
4. Place the mouse in a sterile hood.
5. Using another set of small sterile forceps and scissors, open the muscle layer. Remove the spleen from the left upper abdomen of the mouse. The spleen is a small dark red organ.
6. Place the spleen in a sterile 60-mm culture dish containing 5 mL of DMEM and dissect away surface fat and other adhering tissue using a sterile forceps and scissors.
7. Transfer the cleaned spleen to a sterile 100-mm culture dish.
8. Set a cell strainer on a sterile 50-mL centrifuge tube.
9. Fill a 10-mL sterile syringe with a 22-gauge needle with 10 mL of DMEM. Inject DMEM into the spleen at several sites to push immune cells out of the spleen. Transfer the cell suspension to the cell strainer with a serological pipette. Repeat three times each time transferring the cell suspension to the cell strainer (*see Note 13*).
10. Centrifuge the combined cell suspension at $200\times g$ for 5 min at room temperature.
11. Remove the supernatant and resuspend the splenocytes in 10 mL of ice-cold red blood cell lysis buffer and let the suspension stand for 10 min on ice to ensure erythrocyte hemolysis.
12. Centrifuge the tube containing the spleen cells at $200\times g$ for 5 min at room temperature.

13. Remove the supernatant. Wash the spleen cells once with 10 mL of DMEM followed by centrifugation at $200\times g$ for 5 min and discard the supernatant.

3.4.2. Fusion of Myeloma Cells and Spleen Cells

1. While the spleen cells are incubating on ice in the Red blood cell lysis buffer (**Section 3.4.1**, Step 11), transfer the myeloma cells (almost confluent) to a 50-mL centrifuge tube. Count the myeloma cells and check their viability using the trypan blue exclusion method (*see* **Section 3.3.2**). Incubate the centrifuge tube at 37°C in a CO_2 incubator until cell fusion.
2. Resuspend the washed spleen cells in 10 mL of DMEM in a sterile 50-mL centrifuge tube (*see* **Section 3.4.1**, Step 13) and add 4×10^7 viable myeloma cells. Recover the mixed cells by centrifugation at $200\times g$ for 5 min (*see* **Note 13**).
3. Wash the mixed cells twice by resuspension in 10 mL of DMEM and centrifuge at $200\times g$ for 5 min at room temperature.
4. After the second wash, discard the supernatant and loosen the mixed cell pellet by gently flicking the tip of the tube.
5. Initiate fusion of the spleen and myeloma cells in the 50-mL centrifuge tube by adding 1 mL of PEG solution at a rate of 0.1 mL/6 s by micropipette with gentle mixing (*see* **Note 14**).
6. Mix the PEG cell solution gently for 1 min.
7. Add 5 mL of DMEM solution at a rate of 0.1 mL/6 s with gentle mixing.
8. Add 4 mL of DMEM and 2 mL of FBS and mix gently.
9. Centrifuge the cells at $100\times g$ for 7 min.
10. Pour off the supernatant and wash the cells twice with 15% FBS DMEM by centrifugation for 10 min at $150\times g$.
11. Resuspend the cell pellet in 15 mL of 15% FBS DMEM. Transfer the cell suspension to a sterile 100 mm culture dish and incubate for 3–15 h at 37°C in a CO_2 incubator.
12. Resuspend the cells from the 100-mm culture dish in 160 mL of HAT medium placing 40 mL of cell suspension in each of four 50-mL centrifuge tubes.
13. Add 200 μL of cell suspension to each of the wells of eight 96-well culture plates using a multichannel pipette.
14. To select for hybrid cells, incubate the plates at 37°C in a CO_2 incubator (*see* **Note 15**).
15. After 24 h, remove 100 μL of the culture medium from each well and add 100 μL fresh HAT medium.

16. Grow the hybrids in HAT medium for 2 weeks by removing 100 μ L of the culture medium from each well and adding 100 μ L fresh HAT medium every 3 days.
17. Check for antibody production by indirect ELISA assay using 100 μ L of culture supernatant.

3.5. Cloning of Hybridoma Cell Lines

1. From each well containing antigen-specific antibody in the hybridoma supernatant (positive signal in the indirect ELISA), transfer all of the cells into a separate well of a 24-well culture plate that has been preincubated with 0.5 mL of HT medium and culture 2 or 3 days in a CO₂ incubator (*see Note 16*).
2. Check the antibody titer by indirect ELISA. Select the higher titer and/or faster growing hybridoma cell lines.
3. Perform a trypan blue cell viability test (*see Section 3.3.2*) of the ELISA-positive hybridoma cell lines.
4. Transfer 200 viable hybridoma cells of a selected hybridoma cell line to a sterile 15-mL centrifuge tube containing 5 mL of HT medium producing a 40 cells/mL hybridoma cell suspension. Mix well.
5. Using sterile 15-mL centrifuge tubes containing 2.5 mL of HT medium, perform three serial dilutions of the 40 cells/mL hybridoma suspension by mixing 2.5 mL of hybridoma suspension with 2.5 mL HT medium producing cell suspensions containing 20, 10, and 5 cells/mL.
6. Fill 24 wells of a 96-well plate with 100 μ L of each dilution for 4, 2, 1, and 0.5 cells/well.
7. Incubate the plate(s) at 37°C in a CO₂ incubator for 5 days. Monitor cell growth with an inverted microscope and mark the wells containing monoclonal cells. Monoclonal cells grew from a single cell in each well.
8. Feed the culture by adding 100 μ L of HT medium to every well and continue incubation in a CO₂ incubator until cell growth in the bottom of the wells is 10–50% confluent.
9. Check the antibody titer by indirect ELISA. Cells in the positive wells are transferred to a new 24-well culture plate.
10. Repeat the cloning procedure (Steps 4–9) to establish a stable and monoclonal hybridoma cell line.
11. Established hybridomas are maintained in 15% FBS DMEM.

3.6. Freezing and Recovery of Hybridoma Cell Lines

1. For each cryotube, grow two cultures in 100-mm dishes to 80–100% confluency.
2. Resuspend the cell culture in the culture media with a Pasteur pipette and transfer to a sterile 50-mL centrifuge tube.

3. Perform a trypan blue cell viability test (*see* **Section 3.3.2**). Cells should be nearly 100% viable.
4. Centrifuge at $200\times g$ for 5 min at room temperature.
5. Aspirate the supernatant and resuspend the cell pellet in 10 mL of DMEM.
6. Centrifuge at $200\times g$ for 5 min.
7. Resuspend the cell pellet in CELLBANKER 1 to give a cell density of 1×10^7 cells/mL.
8. Aliquot 1 mL/cryotube.
9. Label the cryotubes with the date and information concerning the hybridoma line.
10. Freeze in a -80°C freezer overnight.
11. Transfer the cryotubes to a liquid nitrogen freezer.
12. To start a hybridoma culture from frozen cells, remove a cryotube from the freezer, and initiate a culture as was done for the frozen myeloma cells but use 15% FBS DMEM (*see* **Section 3.3.1**).
13. Propagate the cells in 15% FBS DMEM by incubating at 37°C in a CO_2 incubator. Subculture the cells when the cells are almost confluent. Check the condition of the cells with an inverted microscope.

3.7. Production of mAb in the Murine Ascites Fluid

1. Ten days and 3 days prior to inoculation with hybridomas, inject mice intraperitoneally with 0.5 mL pristane/mouse using a 26-gauge needle and an all-plastic syringe.
2. Grow hybridoma cells in 100-mm culture dishes in 15% FBS DMEM until almost confluent to obtain at least 5×10^6 cells/mouse.
3. Transfer the cultures to 50-mL centrifuge tubes. Centrifuge at $200\times g$ for 5 min and resuspend the cells in 3 mL of DMEM.
4. Count the cells and determine viability by the trypan blue exclusion method (*see* **Section 3.3.2**). Cells should be nearly 100% viable.
5. Centrifuge the cells at $200\times g$ for 5 min. Aspirate the supernatant to remove serum completely. Adjust the cell concentration to 1×10^7 cells/mL with DMEM.
6. Inject mice intraperitoneally with 0.5 mL of cell suspension using a 26-gauge needle. The number of mice depends on the amount of the monoclonal antibody needed. Typically, five mice are injected at one time. Wait 1–2 weeks for ascites to form (5–10 mL/mouse).
7. Euthanize mice by cervical dislocation. Lift the abdominal skin using a forceps wiped with an alcohol swab and cut

the skin away with a sterile scissors. Make a small cut in the peritoneum and insert a Pasteur pipette to harvest the ascites fluid.

8. Inject a volume of ice-cold normal saline equal in volume to the ascites fluid removed from the mouse into the mouse's peritoneal cavity washing the inside of the mouse. Combine the wash with the ascites fluid.
9. Centrifuge $3,000 \times g$ for 20 min at 4°C. Harvest the ascites fluid layer in the middle layer between the pristane and cell pellet (*see Note 17*).
10. Filter the ascites fluid using No.2 filter paper to remove residual pristane and large cell debris.
11. Filter-sterilize the ascites fluid using a 0.45- μ m filter. Freeze at -80°C. Keep a part of the ascites fluid (<50 μ l) before freezing at 4°C to determine the antibody subclass (*see Section 3.8*, Step 1).

3.8. Purification of mAb Using Affinity Chromatography

1. Determine the monoclonal antibody subclass using the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche).
2. Equilibrate the HiTrap Protein A column (1 mL) with 10 mL of binding buffer appropriate to the IgG subclass. The column should be used for ascites fluid from a single mouse (5–15 mg of immunoglobulin/single mouse ascites fluid).
3. Dilute the ascites fluid with an equal volume of binding buffer and apply to the column at a flow rate of 0.5 mL/min. Keep the flowthrough fraction to avoid losing the unbound antibody.
4. Wash the column with 10 mL of binding buffer at a flow rate of 1 mL/min.
5. Elute the bound protein with 2–5 column volumes of elution buffer appropriate to the IgG subclass and collect 0.5 mL fractions using a fraction collector. Measure A_{280} to locate the eluted protein. Combine the protein fractions and neutralize with 1 M Tris-HCl buffer, pH 9.0 (check with pH test paper).
6. Dialyze eluted protein overnight at 4°C against 2 L of PBS.
7. Freeze 1 mL aliquots at -80°C

4. Notes

1. Other mouse strains can be used as fusion partners with the P3X63-Ag8.653 murine myeloma cell line (derived from

Balb/c). If other mice are used there is a risk that ascites fluid may not accumulate following injection of mAb producing hybridoma

2. Freund's complete adjuvant (FCA) elicits a strong response to most antigens. It, however, may cause inflammation, induration, pain, and necrosis at the injection site, and its use is therefore ethically controversial. It has also the risk to workers, as it can cause localized soft tissue damage following accidental needlestick injuries. Due to the harmful responses produced by this adjuvant, the ability of other adjuvants such as Titermax Gold (Titermax USA, Norcross, GA) to elicit the desired immune response should be considered.
3. Syringes should be all-plastic and Luer-lock type to prepare adjuvant and pristane. Plastic syringes with rubber pistons contain a lubricant that fails in the presence of them and causes syringes to stick.
4. All FBS used in this chapter are heat inactivated (56°C for 30 min) and aliquots are prepared in a sterile hood and stored at -80°C.
5. The myeloma cells used for cell fusion should lack hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity due to mutation. Myeloma cells are cultured with 8-azaguanine to prevent growth of HPRT-positive revertants when the cells are subcultured. Use of HPRT-negative cells ensures that only splenocyte-myeloma fusion cells will grow during HAT selection. In detail, aminopterin blocks the synthesis of DNA by inhibiting dihydrofolate reductase. Cells that lack the ability to utilize the salvage pathway for nucleotide synthesis are eliminated in the HAT selection. Only cells that possess both HPRT and thymidine kinase can utilize the salvage pathway if supplied with hypoxanthine and thymidine. One week before cell fusion experiment, myeloma cells are grown in medium without 8-azaguanine (*see Section 3.3.1*).
6. BrlClone is an IL-6-enriched hybridoma cloning medium. It eliminates the need for feeder cells for the generation of hybridomas.
7. The PEG solution needs to be freshly prepared.
8. Freund's incomplete adjuvant can be used instead of pristane. Inject 0.5 mL Freund's incomplete adjuvant/mouse once 1-2 weeks before injecting mice with hybridoma.
9. The ELISA assay is a rapid, sensitive, and quantitative assay making it the most popular immunoassay for hybridoma selection. However, ELISA-positive mAb's may not be suitable for immunoelectron microscopy. Monoclonal

antibodies to be used for immunoelectron microscopy should be identified using both ELISA and a method such as Western blotting which identifies antibodies that have a high affinity for denatured proteins. Western blotting could also detect if the nonspecific binding for non-desired protein exists or not.

10. The antibody dilution should be according to the manufacturer's instructions.
11. A total of 4×10^7 myeloma cells with viability greater than 95% are used for cell fusion. Healthy myeloma cells have a doubling time of about 20 h.
12. If you need to obtain antisera from the mouse, anesthetize the mouse and collect blood prior to killing the mouse and removing the spleen.
13. 8×10^7 – 4×10^8 immune cells will be obtained from one mouse spleen. Ratios for mixing myeloma and splenocytes should be around 1:5.
14. The timing and the rate of liquid addition are critical and should be measured with a stopwatch. They ensure higher cell viability and higher fusion efficiency.
15. To ensure that selection of hybridomas will be successful, confirm that the myeloma cells that have not undergone the fusion process cannot grow in the HAT medium. In detail, prepare 2 mL of HAT medium in a sterile 35-mm culture dish in the sterile hood and add several drops of myeloma culture. After 1 week, myeloma cells should die and float on the culture medium.
16. Cloning the hybridoma is a critical step required to determine whether a hybridoma producing the desired mAb has been obtained. A positive signal in the indirect ELISA prior to cloning the hybridoma may be a false positive due to the presence of antibody-producing immune cells that have not fused with myeloma cells.
17. The pristane was injected at the start of ascites production and it stays in the peritoneal cavity during the ascites production.