STANDARD OPERATING PROCEDURE

Title: Antibody Production at Strategic Diagnostics Inc.

SOP#: M-119

Version #: 1 **Author: Strategic Diagnostic Inc.**

Date Approved: August 6, 2009 **Date Modified:**

1. PURPOSE

This procedure is to describe the methods used by Strategic Diagnostic Inc. (SDI) to produce and grow up monoclonal antibodies for the Clinical Proteomic Technologies for Cancer (CPTC) program.

2. SCOPE

This document describes the procedure for production and growth of monoclonal antibodies to specific cancer related target proteins.

3. RESPONSIBILITIES

It is the responsibility of the person(s) preparing these reagents to be familiar with lab safety procedures and to have basic laboratory skills.

4. EQUIPMENT

- Motic Microscope, Model AE30
- Reichert Bright Line Hemacytometer (0.1mm deep)
- Thermo Scientific Centrifuge, Model FL40R
- Thermo Scientific Shaker, Model 2314
- Thermo Fisher Incubator. Model Hera Cell 240
- SpectraMax M5 Plate Reader
- CryoMed Liquid Nitrogen Tank, Model 1520HE
- Puffer Hubbard -70°C Freezer, Model 10F8517

5. MATERIALS

- Swiss Webster Mice (Taconic)
- Complete Freunds Adjuvant (Sigma, Cat. #F5881)

- Incomplete Freunds Adjuvant (Sigma, Cat. #F5506)
- Sterile instruments (forceps, scissors and/or tweezers)
- Strainer (Fisher Scientific, Cat. #22-363-547)
- Iscoves Modified Dulbeccos Medium (Media Tech, Cat. #10-016-CM)
- Hypoxanthine Aminopterin Thymidine (Invitrogen, Cat. #31062)
- Hypoxanthine Thymidine (Invitrogen, Cat. #11067030)
- Polyethylene Glycol (Roche, Cat. #0783641001)
- P3x63Ag8.653 cells
- ELISA plates (Fisher Scientific, Cat. #21-377-270)
- 96 well TC Plates (Fisher Scientific, Cat. #07-200-91)
- Cryovials (Fisher Cat. #03-337-7W)
- TMB (Strategic Diagnostics, Cat. #1026218)
- HRP- conjugated Goat anti-mouse Gamma secondary antibody (Jackson Immuno, Cat. #115-035-008)
- PBS (DiaMedix, Cat. #1000-3)
- Casein (Sigma, Cat. #C5890-500G)
- Tween 20 (Fisher Scientific, Cat. #BP337-500)

6. REAGENTS

- Antigen for desired cancer related protein

7. PROCEDURE

7.1. Immunization Strategy

7.1.1. Swiss Webster mice (8 – 10 weeks of age) were immunized with the antigen of interest at either 50 or 100 μg dose.

7.1.2. The initial dose contains Complete Freunds adjuvant, applied Intraperitoneal (IP). Boost every 14 days with Incomplete Freunds, IP

7.1.3. A Tail bleeds are performed after the fourth injection and is performed only once to determine titer with Direct ELISA (7.5)

7.2. Cellular Fusion

7.2.1. Euthanize the selected mouse

7.2.2. Using forceps grasp the midsection of the mouse in two positions, close together and pull open the skin. Spray the opening with 70% ethanol. Using tweezers lift the peritoneum away from the internal organs. Using scissors carefully cut open the peritoneum from undercarriage to spine. Spray internal organs with 70% ethanol. Gently lift the spleen out of the peritoneum, and cut out of mouse. Transfer the spleen to a sterile reservoir containing ~ 10ml of Iscoves Modified Dulbeccos Medium (IMDM).

7.2.3. Collect splenocytes by using two 1.5 inch 18 gauge needles to remove the cells from the spleen. Use the protective cap to bend the needles at right angles. Hold the spleen to the reservoir with one needle. Using the second needle to puncture the outer capsule and to push the cells out of the spleen. Transfer the cells to a sterile 50mL conical tube by passing through a cell strainer containing ~25mL IMDM.

7.2.4. Centrifuge the collected cells at 1200 rpm for 10 minutes at 21-23°C. Decant the supernatant into a waste container. Gently resuspend the pellet, and wash with ~25mL of IMDM. Perform this step twice. After the second wash resuspend the pellet in 25 mL IMDM.

7.2.5. Concurrently the mouse myeloma cells p3x63Ag8.653 (P3) are harvested by tapping the flask gently and pouring the cell suspension into a 50 mL centrifuge tube(s). Centrifuge the collected cells at 1200 rpm for 10 minutes at 21-23°C, resuspend in ~25mL of IMDM. Perform this step twice.

7.2.6. Count splenocytes and P3 cells using hemacytometer to determine density

7.2.7. Determine the amount of P3 cells needed for a 1:5 splenocyte:P3 ratio

7.2.8. Centrifuge the cells at 1200 rpm for 10 minutes at 21-23°C.

7.2.9. Fuse the cells with the addition of 1 mL Polyethylene Glycol (PEG) followed by the addition of 2 ml of IMDM.

7.2.10. Following a four minute incubation at room temperature, centrifuge the cells at 1200 rpm for 10 minutes at 21-23°C.

7.2.11. Resuspend the cells in the selection medium, Hypoxanthine Aminopterin Thymidine (HAT), and transfer to 225 cm^2 flask.

7.2.12. Incubate the cells for 72 hours in 37°C incubator

7.2.13. After 72 hours, distribute the cells at 0.2 ml/well into ten 96 well plates.

7.2.14. Approximately seven days post fusion the HAT medium was aspirated and refeed with Hypoxanthine Thymidine medium

7.2.15. At 10 – 14 days post fusion, growing hybridomas are screened for antibody activity using ELISA (7.5)

7.3. Limited Dilution Cloning

7.3.1. Visual assessment of cells under microscope to ensure no bacterial or fungal contaminants

7.3.2. Count the cells (hemacytometer) and determine the percent viability of cells/ml

7.3.3. Prepare cells in exponential growth phase for limited dilution cloning

7.3.3.1. Take 1 ml sample and serial dilute to 1:1000 in media to ensure cell concentration will be ~4.5 cells/ml

7.3.3.2. Plate the diluted cells into three 96 well plates

7.3.3.3. Monitor plates for single colonies using a microscope on days 3, 7 and 14

7.3.3.4. Eliminate wells with more than a single colony

7.3.3.5. On day 14 wells with visible single colonies will be screened by ELISA for antibody production (7.5)

7.4. Freezing Suspension Cell Lines

7.4.1. Visual assessment of cells under microscope to ensure no bacterial or fungal contaminants

7.4.2. Count the cells (hemacytometer) to determine the concentration

7.4.3. Cells should be frozen at 3×10^5 – 8×10^5 with ≥ 85% viability

7.4.4. The necessary amount of cell culture containing appropriate number of cells (7.3.3.) was centrifuged at 1200 rpm for 10 minutes at 21-24°C and resuspended with cold freezing media (90% Fetal Bovine Serum and 10% DMSO). Cell are frozen at a concentration of 5×10^6 per ml in 1 ml aliquots.

7.4.5. Immediately transfer 1 ml of cell suspension to each appropriately labeled cryovial.

7.4.6. Transfer the vials to -70°C freezer for a minimum of 12 hours and no longer than 72 hours.

7.4.7. After 12 hours at -70°C transfer the vials to liquid nitrogen for long term storage.

7.5. Direct ELISA

7.5.1. Prepare a coating solution with 1 – 2 µg/ml of appropriate antigen in 0.1M Carbonate Buffer pH 9.6.

7.5.2. Coat flat bottom 96 well plate (Maxisorb) with 100 µl per well of coating solution (7.5.1)

7.5.3. Incubate the plate(s) on a shaker for 1 hour at room temperature

7.5.4. After the incubation, wash 3 times with distilled water, 300 µL per well.

7.5.5. Block the plate(s) with 200 µl per well with PCT (PBS containing 1.0% Casein, 0.5% Tween 20)

7.5.6. Incubate the plate(s) on a shaker for 1 hour at room temperature

7.5.7. Wash 3 times with distilled water, 300 µL per well.

7.5.8. Dilute sample containing antibody with PCT

7.5.8.1. Sera samples should be evaluated in serial dilutions using PCT buffer

7.5.8.2. Supernatants should be diluted 1:20 in PCT

7.5.8.3. Final diluted sample volume should be 100 µl per well

7.5.9. Incubate plate(s) on a shaker for 1 hour at room temperature

7.5.10. Wash 3 times with distilled water, 300 µL per well.

7.5.11. Add 100 µl of diluted HRP conjugated Goat anti-mouse Gamma secondary antibody at a dilution of 1:5000.

7.5.12. Incubate plate(s) on a shaker for 1 hour at room temperature

7.5.13. Wash 3 times with distilled water, 300 µL per well.

7.5.14. Develop with 100 µl of Tetramethylbenzidine (TMB) color solution for 15 minutes

7.5.15. Read plate(s) using a plate reader set to 650 nm wavelength

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8. REFERENCED DOCUMENTS

8.1. Operation Manual, Centrifuge

8.2. Operation Manual, Shaker

8.3. Operation Manual, Incubator

8.4. Operation Manual, Plate Reader

8.5. Operation Manual, Microscope