PROTOCOL FOR MONOCLONAL ANTIBODY PRODUCTION

MATERIALS AND EQUIPMENT

1. Sp2 cells
2. Immunized mice
3. 5mL Serological Pipettes VWR-89130-896 or VWR-14672-920

   10mL Serological Pipettes VWR-89130-898 or VWR-14672-922

   25mL Serological Pipettes VWR-89130-900 or VWR-89089-888
4. 200uL Pipette Tips VWR-30128-376 or VWR-89368-950
5. Micropipette (20uL-200uL)
6. 1.5µL microtubes VWR-20901-551 or VWR-10170-038
7. 50mL centrifuge Tubes VWR-89039-658 or VWR 21008-690
8. Dissecting board
9. 1L Filter Unit Receiver VWR-28199-246 or VWR-28199-762
10. Inverted Microscope
11. Washing Solution
12. 1X PBS
13. Plating Medium
14. Dissecting kits
15. 150mm and 60mm petri dishes VWR-25384-326 or VWR-82050-596, VWR-25384-092 or VWR-82050-548
16. 5mL syringes VWR-BD309646 or VWR-53548-019
17. VWR® Autoclave Bags VWR-14220-082 or VWR-11215-822
18. VWR® Light Duty Tissue Wipers VWR-21908-697 or VWR-89174-728
19. Isoflurane

6. PROCEDURE

Preceeding the Fusion Day

Four days before the fusion prepare the following materials:

A. Methyl cellulose –

1. Weigh 20 grams of methylcellulose and add to 810 mL of distilled water in a bottle placed on a hot/stir-plate. Heat the solution to 90 C with stirring and allow the methylcellulose to suspend until only small lumps remain.

2. When the methylcellulose solution is at least 80% homogeneous prepare another bottle (1L capacity) with 210 mL of distilled water. Sterilize the MC and water bottles by autoclaving.

3. Allow bottles to cool to room temperature. The bottle containing methylcellulose is returned to a stir-plate (without heat) to completely disperse the methyl cellulose.

4. Weigh 13.48 g of DMEM and 3.7 g of $NaHCO_3$ and add them to bottle containing 210 mL of distilled water sterile inside of laminar flow cabinet. Add first the DMEM and then add $NaHCO_3$. Mix vigorously and let stand for approximately 1 minute. Use 1.5ml of 3M HCl to adjust the pH to 7.0-7.4. Again mix vigorously and let stand for approximately 1 minute, and verify the pH value.

5. Inside of laminar flow cabinet place the filter unit at the opening of the bottle containing methylcellulose solution and filter the concentrated DMEM into the MC solution. Stir the DMEM concentrate into the methylcellulose solution at room temperature with constant agitation. Once the homogenization appears complete, place the bottle at 4°C overnight stirring to complete the mixing.

6. After 24 hours, prepare 30mL aliquots in sterile 50mL tubes and store them at 4 C.

B. Autoclave water, dissecting kits and tips as outlined in Material Preparation 10-014-SOP.

C. Prepare and 0.2 um filter Washing Medium- DMEM, 297 mL plus P/S, 3 mL.

D. Prepare the myeloma line (Sp2/Ag0), which is maintained in high glucose (4.5 g/L) DMEM/ 10% FBS/1 % P/S, as follows:

1. At least two days before the fusion, expand the cells in a sufficient number of T600 flasks to have 5 or 6 Sp2 T600 flasks the day of the fusions. Each Sp2 T600 flask must have a total volume of 100mL of cell culture. You must prepare at least 4 T600 flasks with different dilutions of cell culture into new medium, ex: 30:70, 35:65, 40:60 & 45:55 This way you can select the flask with the best percent viability at 3-7 x 10^5 cells/mL density the day of the fusion process.

2. On Mondays, prepare an Sp2 control to verify aminopterin sensitivity. Add 9mL of 10% Regular Medium to a T25 flask and 200uL of HAT 50x solution. Before

splitting the Sp2 cells, take 1mL of one T600 flask and transfer it to the T25. Label it as Sp2 control and the date. On Tuesday, scan the cells. At least >95% of the cells should be dead. This control is valid for a week only and therefore must be done weekly. If the control is not satisfactory then a new vial of Sp2 cells must be thawed from the liquid nitrogen tanks.

FUSION DAY

A. Prepare the following before dissecting the mice.

1. Place the vial of PEG vial in a dry 37C bath.

2. Warm the washing medium to room temperature.

3. Thoroughly clean the safety cabinet with distilled water and then with a solution of 70%EtOH.

4. Prepare at least 70 150mm petri dishes with 35mm and 60mm plates inside as stated in “Material Preparation SOP”.

5. Spray with 70% EtOH and place inside the laminar flow hood the following materials:

One dissecting kit.

One box of autoclaved mesh screens.

Three 50mL tubes.

Two microtubes; one microtube must be empty and the other one filled with 90uL of 1XPBS.

One 100mm petri dish per mouse with 11mL of Washing Media.

One dissecting board and several pins.

One 5mL syringe.

6. Scan the myeloma line (Sp2/Ag0) in the microscope to verify morphology, lack of contamination and confluence of the cells.

7. Count the number of cells in each flask using the Cell Counter 10-025-SOP. Document the number of live cells, viability and split # per flask.

B. Euthanize the mice with isoflurane.

6.4.1 In one of the selected plastic boxes used as chambers for containing the mouse, put several cottons soaked in isoflurane and close the box cover.

6.4.3 Put the mouse inside the box with isoflurane and close with the box cover. Wait until the mouse has stopped breathing.

6.4.3.1 Once the mouse has stopped breathing, wait another minute before taking the mouse out of the chamber.

6.4.3.3 You have only 90 minutes after dissection to complete the fusion process to minimize the loss of B cell viability due to time and temperature exposure.

6.5 Take note of the time. Proceed to dissect the left lymph node. Put both lymph nodes in a 100mm petri dish with Washing Medium and dispose of the mice.

6.6 Macerate the lymph nodes using a mesh and the plunger head of a syringe to generate a cell suspension.

6.7 Transfer the suspension from the 100mm petri dish to a 50mL tube using a serological pipette. Wash the plate to remove remaining cells with 5mL of Washing Medium and transfer it to the same tube.

6.8 Resuspend the cells gently and take a 1mL sample and place it in a microtube. Put the 50mL tube with the solution of cells in the incubator at 37C/5%CO_2 (see figure 6).

6.9 To a microtube with 90uL of 1xPBS previously prepared add a 10uL aliquot of the 1mL sample and resuspend.

6.10 Count the cells using an hemacytometer.

6.11 When dissecting spleens, before taking the 1mL sample to be diluted, the cells must be centrifuged at 5min/500rcf/10C. Discard the supernatant and add a solution of 0.8% ammonium chloride to lyse the red blood cells. Incubate for 2 minutes at room temperature while moving the tube in a circular motion. Then add slowly, in a period of 1 min, 6mL of Washing Media using a serological pipette. Centrifuge again using the same parameters, discard supernatant, resuspend in 11mL of Washing Medium and then continue in step 6.3.8

6.12 To determine number of fusions do the following:

Calculate the number of B cells in the pool by multiplying the counting with the dilution factor. The number of B cells obtained after counting are cells/mL from the 10uL in 90uL dilution in the microtube, a dilution of 1/10. Also when recovering the B cells from the lymph nodes the cells were resuspended in 16mL of Washing Medium and 1 mL sample was used for dilution. The cells are then in 15mL, another 1/15 dilution. Use the template located in Dropbox>CDI Labs 2.1 named ‘Fusion Calculation Worksheet 10-003-FORM”. Here is an example of how the fusions are calculated.

Pool	Mice ID Tags	Cell Counting ($x10^6$)	# of Bcells ($x10^6$) = (Cell Counting)(10)(15)	# of Sp2/Ag0 ($x10^6$) = # of Bcells/4	Total cells ($x10^6$) = # of Bcells + # Sp2/Ag0
A1	1512/1513	0.54	81	20.25	101.25
A2	1514/1515	0.30	45	11.25	56.25

Fusions should contain a range of 50 to 100 million total cells. If the total number of cells is more than 100 million cells, the group must be divided in two fusions. As shown

above, two fusions are suggested for A1: split into two fusions called A1.1 & A1.2, each of which will have 50.62 million total cells. For A2 a single fusion of 56.25 million total cells is adequate.

Note: The name of the fusion depends on the name of the group (# of mice immunized with X antigen). For example, if group A1 is composed of 2 mice immunized with X and Y antigens, the fusions resulting from that pool will be named A1.1, A1.2, A1.3 … and so on.

Fusion name	Mice ID Tags	Total Bcells ($x10^6$)	Total Sp2/Ag0 ($x10^6$)	Total cells ($x10^6$)= Total Bcells + Total Sp2/Ag0
A1.1	1512/1513	40.5	10.12	50.62
A1.2	1512/1513	40.5	10.12	50.62
A2.1	1514/1515	45	11.25	56.25

To determine the volume of Sp2s needed use the cell counts determined for each flask and the viability.

Flask ID	Cell Counting (cells $x10^6$)	Viability
1	0.65	97%
2	0.60	100%
3	0.21	100%

NOTE: Use the flask of Sp2/Ag0 with the best viability, a cell density near 0.7 x 10^6 cells/mL and with no contamination. For example, the best flask in the above example is number 2.

Fusion name	Mice ID Tags	Total cells ($x10^6$)	Total Sp2/Ag0 ($x10^6$)	Flask ID	mL of Sp2/Ag0 ($x10^6$) = [Total Sp2/Ag0] / [Same day counting]
A1.1	1512/1513	50.62	10.12	2	10.12/0.60 = 17mL
A1.2	1512/1513	50.62	10.12	2	10.12/0.60 = 17mL
A2.1	1514/1515	56.25	11.25	2	11.25/0.60 = 19mL

6.13 After determining the number of fusions and the quantity of Sp2 cells needed, transfer the components of the fusion to one 50mL tube. Mix the 15mL solution of B cells with the calculated volume of Sp2/Ag0. For pools with two fusions mix the total amount of B cells with the total amount of Sp2 cells and divide in two tubes. Centrifuge each fusion at 600rcf/6min/21°C. Using a serological pipette, gently remove all of the supernatant without disrupting the pellet.

NOTE: The following part must be done without any interruption. Read the protocol and be sure to understand every detail of it to avoid losing cell viability and fusion efficiency. You can fuse two fusions at the same time for divided pools.

6.14 Over a total time of 2 minutes, slowly add PEG dropwise at a rate of 500uL per minute. Move the tube in circular motions during the entire 2 min.

6.15 Incubate for 2 minutes at room temperature. Continue moving the cells in a circular motion.

6.16 To stop the fusion the PEG has to be diluted. Gently and with continued circular motion add 4mL of Washing Medium over a total time of two minutes. First add 1mL in 1 minute and then 3mL in 1 minute. Then add 16mL of Washing Medium over 2 minutes. Centrifuge the cells at 600rcf/6min/21C. Incubate 10 minutes at 37°C. **NOTE:** The first drops of Washing Medium to dilute the PEG must be added very slowly.

6.17 To perform two fusions at the same time follow the above instructions but stagger the start of each fusion by two minutes. Each step is then delayed by two minutes for the fusions. For example, while you are adding media to dilute the first fusion, the second fusion will be in the 2 minute incubation.

6.18 After the 37 C incubation, discard the supernatant. Resuspend the pellet in the corresponding quantity of Plating Medium, depending on the quantity of tubes with MC needed. Mix well in a 250mL receiver and aliquot into the calculated number of tubes of MC. Aliquot 18mL of the solution for every 30mL of MC. Replace cap and mix end over end for 10 minutes in a rotator at room temp. Make sure medium is homogeneous before plating.

NOTE: the total number of cells per fusion determines the number of plates, number of MC tubes and the volume of plating medium needed.

Fusion name	Total cells ($x10^6$)	60mm plates needed = Total cells ($x10^6$) / $0.5x10^6$ cells per plate	150mm plates needed = # of 60mm plates / 3	MC tubes needed = (#150mm plates needed)*(3/2)*(2/10)
A1.1	50.62	102	34	10
A1.2	50.62	102	34	10
A2.1	56.25	113	38	11

6.19 Prepare the designated number of plates per fusion. Place three 60mm petri dishes inside one 150mm petri dish and a 35mm petri dish. Using the Zebra Designer Pro labeler, prepare a label to identify each fusion with the name and plating date. With a serological pipette aliquot approximately 5 mL of each fusion mixture into the 60mm plates per 150mm petri dish. The 35mm petri dish must be filled with 2.5mL of autoclaved water to avoid dehydration.

6.20 Incubate at 37C, 5% CO_2 for 8-10 days before picking colonies.