SOP: AB104.6
Modified: 7/27/22 KE

Screening and Subcloning of Monoclonal Hybridomas

Materials and Reagents:

1. Complete IMDM media (SOP: M028.3) or DMEM/F12 media (SOP:M027.2)
    - ***Use same media that was used for SP2/0 cells and during fusion***
    - ***Different % reagents needed for protocol (can make smaller volumes of each type of media, check to see how much of each you will need prior to making media – for entire protocol):***
        - ***20% FBS with 1X HAT***
        - ***20% FBS with 0.5X HAT***
        - ***20% FBS with 1X HT***
        - ***10% FBS with 1X HT***
2. HAT supplement, 50X (Fisher Scientific, Cat#21060017)
3. HT supplement, 50X (Fisher Scientific, Cat#11067-030)
4. Sterile reservoirs (VWR, Cat# 89094-680)
5. 200 µL wide orifice ***filter*** tips (Fisher Scientific, Cat# 2069GPK) (Note 12)
6. ***Filter*** tips (normal), sterile (200 µL and 1000 µL) (Note 12)
7. P200 and P1000 piptmen
8. P200 ***multichannel*** pipetman
9. Automated pipet-aid
10. Sterile, round U-bottom 96-well tissue culture microplates (Fisher Scientific, Cat# 08-772-17)
    - *Individually wrapped is best*
11. Sterile, flat-bottom, 48, 24, and 12-well tissue culture treated microplates (Thomas Scientific (Nest brand), Cat # 1194Y81, 1194Y82, 1194Y81, respectively)
    - *Individually wrapped is best*
12. Tissue culture flasks (VWR Corning, T25 (29185-302) and T75 (BD353136))
13. ELISA reagents (SOP: SP039.2)
14. Western blotting reagents (SOP: SP011.3 or SP011a)
15. 1.2 mL sterile, external thread Cryovials (Fisher Scientific, Cat# 09-761-74)
16. Cryovial storage boxes
17. Mr. Frosty freezing container
    - ***See SOP: AB105.5 materials section for Mr. Frosty maintenance directions***
    - Isopropyl alcohol
18. 0.22 µm Millipore Steriflip vacuum filter unit, PES membrane (Fisher Scientific, Cat# SCGP00525)
19. 0.22 µm 250 mL or 500 mL filter units, PES membrane
20. 0.65mL Eppendorf tubes
21. 50 mL Falcon conical tubes
22. 15 mL Falcon conical tubes
23. Hemocytometer (SOP: SP067.2) or EVE™ Automatic Cell Counter (See SOP: SP078.1)
24. Serological pipets (assorted volumes)
25. Water Bath 37˚C
26. 2.5 % Vesphene (or other disinfectant of choice)
27. 70% Ethanol
28. Inverted light microscope
29. 5% CO_2 humidified, 37°C tissue culture incubator
30. Biosafety cabinet

Establishing Parental Cell Lines Protocol:

1._____ 7-14 days after the fusion process, the hybridomas will begin to make the media slightly acidic and turn a bright yellow color. *These colonies are polyclonal and will be referred to as parentals*.

2._____ Coat an ELISA plate (SOP: SP039.2) in anticipation of screening the next day (so as to not have to freeze/thaw supernatants). Coat wells at 1 µg/well with ***target*** antigen.

3._____ Remove 110 µL of culture supernatant from wells with hybridoma cell growth and transfer them to a 96-well microplate (tissue culture treated microplates not necessary for sampling) (Note 1).

4._____ Re-feed original well with 110 µL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HAT***.

5._____ Continue screening over several days, circling the selected clones on the original 96-well microplate with a different colored marker every day to track screening.

6._____ Develop ELISA with 100 µL of the culture supernatant ("primary antibody") collected in step 3 (see SOP: SP039.2) (Note 2).

7._____ For the wells that are positive via ELISA, upscale cells from the original 96-well plate to a 24-well microplate. Re-suspend cells gently and use wide orifice filter tips to transfer as to not damage the hybridomas. Add 2 mL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 0.5X HAT*** to transferred cell suspension in the 24-well microplate (Notes 3-5).

- **Sufficient reactivity is (generally) regarded as an OD405 of ≥ 1 at a supernatant dilution/titer of 1:1,000 for ELISA.**

8._____ Strongly positive wells by ELISA should also be tested by Western blot (SOP: SP011.3 or SP011a) to determine if the epitope is conformational or linear. After cells have been allowed to grow in the 24-well microplate for at least 24-48 hr, and have a moderately confluent layer, they can be tested by Western blot. Remove 1 mL of cell culture supernatant from each well that will be tested and replace (re-feed) with 1 mL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 0.5X HAT***. *Be sure to not touch the bottom of the well or to mix the supernatant so the cells are not lost during this screening process* (Notes 6 & 7).

- **Sufficient reactivity is (generally) regarded as a dark band (corresponding to appropriate molecular weight of antigen being tested) at a supernatant dilution/titer of 1:1,000 for Western blotting.**

9._____ If positive via Western blotting, upscale the parental hybridoma cells from a 24-well microplate to a 12-well microplate. Re-suspend cells gently using a 5 mL serological pipet and transfer to the larger volume well microplate. Add an additional 2 mL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HT*** to the transfer cell suspension (~ 2 mL from 24-well microplate) (Note 8).

Subcloning Cell Lines Protocol:

10._____ Hybridomas that test positive via Western blotting (Note 5) are subcloned directly from the 12-well microplate stage. These hybridomas are the ***parental lines*** (***one positive well = one parental line***). *It is crucial to not lose any of the positive parental lines before subcloning or freezing down at least one line from each parental.*

11._____ To subclone, count the cells in the 12 well plate using a hemocytometer (SOP:SP067.2) or EVE™ Automatic Cell Counter (See SOP: SP078.1). The cells should be highly viable and have a density between 2 x 10^5 and 8 x 10^5 cells/ml. If the cell density is too low, it is better to leave the clones to grow for 1-2 days more. ***See Note 9 for an alternative subcloning method.***

- _____ Seed cells at 0.5 cells/mL in round bottom 96-well microplates. Total volume should be 10mL of complete IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HT***. This will be enough to cover approximately 1 full plate.

12._____ Place the subcloned plates in a 5% CO_2 humidified, 37°C tissue culture incubator. Make sure to appropriately label subcloned plates with *parental name, date, initials, media.*

13._____ After approximately 4 hours, check each individual well for a single hybridoma cell. ***Use the 4X objective lens on the light microscope to help visualize single cells***. This step is important to make sure these subclones are truly monoclonal. *Truly monoclonal hybridoma cells are referred to as subclones.*

14._____ Circle the wells with a single cell. These cells are monoclonal. Cross out the wells with multiple cells as these are still polyclonal.

15._____ Place the plates back in a 5% CO_2 humidified, 37°C tissue culture incubator.

16._____ Let the monoclonal hybridoma subclones grow up (takes approximately 5 days to see a colony form under the light microscope and 9-10 days before the media starts changing color and a clear colony is seen by the naked eye). ***Visually inspect plates every day (naked eye and under microscope).***

Upscaling and Freezing of Parental Cell Lines:

17._____ Upscale the rest of the parental cells in the 12-well plate to a T25 flask containing ~3 mL of cell suspension and 5mL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HT***. Re-suspend the cells gently before transferring to flask.

18._____ Once the parental cells in the T25 flask reach confluency (80-90% of well surface is covered with cells), they are ready for freezing. Freeze down 1-2 vials of at least 10-15 parental lines (select the lines that give the highest signal for ELISA and Western blot) (See SOP: AB105.5).

Upscaling and Freezing of Subcloned (Monoclonal) Cell Lines:

19._____ 6-7 days after subcloning, add 100 µL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HT*** to each subclone that was circled in step 14 (if there is a visible colony growing).

20._____ By days 9 or 10, individual monoclonal hybridoma cell wells should be growing well. Repeat ELISA (steps 2-6) as before. However, re-feed cells with **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS +1X HT*** (Note 10).

21._____ From the positive ELISA wells (96-well microplate), pick *15-20 subclones* with the strongest signal and transfer these to a 24-well microplate. Re-suspend cells gently and use wide orifice filter tips to transfer as to not damage the hybridomas. Add 2 mL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HT*** to the transferred cell suspension.

- **Sufficient reactivity is (generally) regarded as an OD405 of ≥ 1 at a supernatant dilution/titer of 1:1,000 for ELISA.**

22._____ Repeat steps 8 and 9 for Western blotting analysis. However, re-feed cells with **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS +1X HT***.

- **Sufficient reactivity is (generally) regarded as a dark band (corresponding to appropriate molecular weight of antigen being tested) at a supernatant dilution/titer of 1:1,000 for Western blotting.**

23._____ Positive subclones (Note 6), at least 10-12, should be grown up to 1 x T75 flask. At least 2-3 vials of each subcloned line should be frozen down (SOP: AB105.5). ***The two best subclones selected for BEI should be upscaled into multiple T75 flasks and at least 5-10 x 1mL vials of cells with 5x10^6 cells/mL – 1x10^{7}cells/mL should be frozen down***.

24._____ **For antibody production (BEI) using the monoclonal (subcloned) hybridoma cell lines, see SOP: AB100.6, *section* "Antibody Production Protocol (Hybridoma Cells Only)" (Note 11).**

- ***ALL BEI antibody supernatant products should be 0.22 µm sterile filtered for qualification and final products.***

Notes:

1. Be careful to not touch the bottom of the well or to mix the supernatant so the cells are not lost during this screening process. If necessary, the culture supernatants may be stored at 4°C overnight an ELISA the next day. Culture supernatants can be stored at -20°C if ELISA cannot be performed immediately.
2. Be sure to include a positive control of polyclonal antibody serum from the corresponding mouse (immunized mice). Also include negative controls, like pre-immune serum (naïve bleeds) from the corresponding mouse. See SOP: SP039.2 for more negative control ideas.
3. If cells are struggling to grow, upscaling to a 48-well microplate may help before upscaling to a 24-well microplate. Add 750 µL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 0.5X HAT*** to transferred cell suspension. When transferring to a 48 or 24-well microplate be sure to gently re-suspend the cells to free the hybridomas from the bottom of the 96-well microplate (use wide orifice tips so as to not damage the hybridoma cells).
4. Wells will turn a bright yellow color and/or cells reach confluency when ready to upscale. It is a good idea to transfer a little bit of the spent medium to the next size well with each transfer, since it will contain growth factors the cells need.
5. Weaning parental cell lines off HAT and into HT completely is recommended. However, as demonstrated, this should be done gradually as to not lose any polyclonal hybridoma cells. Each "step down" of HAT (1X → 0.5X → NO HAT, only HT) *should* be tested via ELISA and Western blotting to make sure reactivity is still high in these parental lines before freezing them down.
6. Last version of protocol (AB104.4) stated: "***only linear epitopes (positive for Western blot) are selected for subcloning unless otherwise directed***". **This should be discussed with supervisor as antibodies reactive to both ELISA and Western blotting are beneficial.**
7. Run 1 µg of the ***target*** antigen (against purified antigen) and test for multiple dilutions for Western blotting. ***Generally, an ideal OD405 value of ≥1 should be reached (for dilutions) to be considered strongly positive.*** Crude fractions (like culture filtrate protein and whole cell lysate or other subcellular fractions) are strongly encouraged to be run alongside the purified antigen. This is due to the fact that some of the purified target antigens are recombinant proteins. Using the crude samples allows for identification of reactivity to the native version of the protein as well.
8. When positive parentals are upscaled (24-well to 12-well microplate), SWITCH FROM **complete** IMDM media ***OR*** DMEM/F12 media with ***20% FBS + 1X HAT*** TO **complete** IMDM media ***OR*** DMEM/F12 media ***with 20%FBS + 1X HT.*** *This ensures the subclones will be completely weaned off HAT supplement*.
9. ***An alternative method for subcloning was used in an older version (AB104.5):***

   To subclone, gently re-suspend the cells in the 12-well microplate and count cells using a hemocytometer or EVE™ Automatic Cell Counter (See SOP: SP067.2 or SOP: SP078.1, respectively). The cells should be highly viable and have a density between 2 x 10^5 and 1 x 10^6 cells/mL. ***Round U-bottom 96 well plates should be used for subcloning (this allows for visualization of a single cell in one well). Use wide orifice filter tips with subcloning.***

   a. _____ Calculate the number of cells that need to be added to 5 mL of **fresh, warm, complete** IMDM media ***OR*** DMEM/F12 media ***with 20% FBS + 1X HT*** to achieve a density of 1 x 10^3 cells/mL. Never add less than 25 µL or more than 200 µL of cells to be subcloned. If the cell density is too low, it is better to leave the clones to grow for 1-2 more days. If the density is too high, the volume of media can be increased accordingly.

   b. _____ Transfer cell suspension to a sterile reagent reservoir. Rock to mix.

   c. _____ From this stock, add 100 µL/well to row A, Plate 1 (100 cells/well).

   d. _____ Into ***another*** sterile reagent reservoir, add 1.8 mL of complete IMDM media ***OR*** DMEM/F12 media ***with 20% FBS +1X HT*** and 0.2 mL of the previous cell suspension. *This will make a 2 mL stock of a 1:10 dilution.*

   e. _____ Rock to mix the cell suspension and plate 100 µL/well of this stock to row B, Plate 1 (10 cells/well).

f. _____ To the remaining 0.8mL in the reservoir (from Step D), add 7.2 mL of complete IMDM media ***OR*** DMEM/F12 media ***with 20% FBS +1X HT*** to make a second 1:10 dilution.

g. _____ From this stock, plate 100 µL/well in rows C and D, Plate 1 (1 cell/well).

h. _____ To the remaining 5.6 mL in the reservoir (from Step D), add 9.5 mL of complete IMDM media ***OR*** DMEM/F12 media ***with 20% FBS +1X HT***.

i. _____ Using a P200 multichannel pipet and 200 µL wide orifice filter tips, pipet 100 µL/well on the rest of Plate 1 and all of Plate 2. ***This represents 0.3 cell/well***. The goal behind subcloning is to get a ***single hybridoma in a single well***. *So, if everything goes as planned, 1/3 of the 0.3 cells/well wells will grow up and each of these hybridomas will have been derived from a single B-cell.*

10. Some wells will not be positive via ELISA, but still have growth. These can be tested further via Western blotting. However, if these subclones are ALSO negative via Western blotting, these subclones should not be kept as they are not reactive against the antigen. This happens sometimes with subclones that are actually polyclonal – a cell that does not produce a reactive antibody outcompetes a cell that does produce a reactive antibody.
11. Subcloned cell lines can be kept going (from original T75 cultures) or a frozen subclone vial can be thawed to start a fresh culture. IF the subcloned cell lines are kept going (from original T75 cultures), media (IMDM ***OR*** DMEM/F12) can be switched to include a reduction of FBS (10% FBS) instead of 20% FBS, still with 1X HT. This helps the cells grow a little slower and aids in better cultures/more robust antibody production.
12. ALL work with hybridomas should utilize filter tips. Normal filter tips can be used to collect supernatant for testing. It is recommended to use wide orifice filter tips when manipulating the hybridoma cells directly as to not sheer/harm the cells.

References:

1. Antibodies Production Manual (Dobos). Version 1. August 26, 2005.
2. Harlow, Ed, and Lane, David. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, New York. 1988. pp.139- 281.
3. Kohler, G., and Milstein, C. *Nature* 256, 495-497. August 7, 1975. Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity.
4. Wayne M. Yokoyama, Michelle Christensen, Gary Dos Santos, and Diane Miller. *Current Protocols in Immunology*. Unit 2.5 - Production of Monoclonal Antibodies. Published Online: September 1, 2006. Retrieved February 5, 2012.