SOP: AB100

Cell Line Maintenance

Materials and Reagents:

1. Complete RPMI (note 1)
2. Fetal Calf Serum (FCS)
3. Water Bath 37˚C
4. CO_2(5%) Humidified Tissue Culture Incubator 37˚C
5. 0.2 um, 25mm Acrodisc syringe filters
6. 50 ml falcon centrifuge tubes
7. 15 ml falcon centrifuge tubes
8. 225 ml falcon tubes
9. Nalgene 250ml filter unit, 0.2um
10. Hemocytometer
11. Pasteur pipettes
12. Serological pipettes
13. Tissue culture/biosafety hood
14. Tabletop centrifuge
15. Tissue culture flasks (T25,T75, T125)
16. Rollerbottle
17. Rollerbottle apparatus
18. Inverted microscope

Protocol:

1. _____ Remove cell line that is to be used from liquid Nitrogen tank and note in LN2 log book.

2. _____ Immediately transfer cell line to 37°C waterbath. It takes about 2-4 minutes to defrost.

3. _____ While cells are thawing, in a tissue culture hood, place 6 ml of complete RPMI media (note 1) in a 15 ml falcon tube and underlay with 1 ml of FCS (note 2).

4. _____ When the cells are thawed, aseptically transfer the cells to the RPMI layer in the 15 ml conical. Reserve 10µl of cell suspension (note 3).

5. _____ Centrifuge cells at 1000-1200 rpm at room temperature for 6-10 minutes.

6. _____ While cells are spinning, transfer the reserved cells (from step 4) to a hemocytometer and look at cells using an inverted microscope (note 4).

7. _____ If viability is good, then prepare a T25 flask containing 10ml of complete RPMI and label flask with all the information that is on the cryovial (note 5).

8. _____ Retrieve cells from centrifuge, decant supernatant and discard.

9. _____ Carefully resuspend cells in 1 ml of RPMI complete media and transfer to T25 (note 6).

10. _____ Transfer cells to 37°C incubator with 5% CO_2.

11. _____ Check cells daily, to see when media is beginning to turn acidic. For hybridomas, the cells usually need to be fed every 2-3 days.

12. _____ To check if the cells need to be fed, counts cells under a hemocytometer (note 7).

13. _____ Cells that are being refed are usually split 1:10 or 1:20 (note 8).

14. _____ Make note on flask that it has been fed and return cells to 37°C incubator with 5% CO_2.

15. _____ When media begins to turn acidic, upscale from a T25 to two T75 containing a total volume of 50 ml RPMI complete media plus cells (note 9).

16. _____ Refeed the original T25 and make note on flask that it has been fed.

17. _____ Return cells to 37°C incubator with 5% CO_2.

18. _____ When the media begins to turn acidic again, upscale to two T125 each containing a total volume of 150-200 ml of media plus cells (note 10).

19. _____ Return cells to 37°C incubator with 5% CO_2.

20. _____ In two days, the cells should be upscaled to rollerbottles. One T125 in each rollerbottle and bring the total volume up to 1L-1.2L.

21. _____ Gas the rollerbottles for at least 4 hours in the CO_2 incubator with the caps loosened (note 11).

22. _____ Close the caps on the rollerbottles very tight and seal with several layers of parafilm.

23. _____ Place on the rollerbottle apparatus at 37°C for 5-6 days (note 12).

24. _____ When the media is very acidic (yellow), harvest the supernatant by centrifuging the supernatant at 3200 rpm for 10 minutes. Repeat as needed and discard the pellets.

25. _____ Transfer the new supernatant to a new rollerbottle.

26. _____ Adjust pH to 7.4 (note 13).

27. _____ Store at -20°C until needed.

Notes:

1. See SOP:M012 for Complete RPMI.
2. The FCS acts as a barrier to separate the cells from the DMSO that is in the Freezing Media.
3. The reserved amount of cell suspension is to check for viability using a hemocytometer.
4. If cell viability looks low it is best to start cells in a 24 well tissue culture plate or the amount of FCS can be increased to 20%.
5. Check the vial in which the cells were frozen to see which media they were frozen in. If the vials says 'HAT' the cells must be grown in HAT media. HAT media is 600 ml RPMI complete media with 1 vial of HAT supplement added. Otherwise, RPMI complete should be used.
6. If using vented cap flasks: screw the lid on firmly, if not using vented cap, make sure the cap is loose to allow CO_2 exchange in the incubator.
7. Add 10µl of cell suspension to hemocytometer and count the number of cells in 16 squares. Multiple this number by 1×10^4 to obtain how many cells are in each ml of media. The cells will grow best when they are between 4×10^5 and 7×10^5. Cell greater than this density should be expanded to new flasks.
8. This means that the cell density must be adjusted to be between 5×10^3 and 2×10^4 cells per ml. The fewer the cells the longer they will take to recover, but usually the density will be back up in 2-4 days.
9. Do this by adding 5 ml of slightly acidic cells to each T75 with 45 ml of media.
10. Transfer the entire contents of one T75 into one T125 and add 100-150 ml media depending on the density of the T75. Do this for the other T75 flask.
11. The rollerbottles can be gassed overnight if necessary.
12. Check for leaks. If not tightly sealed, the bottles will leak or ambient air will replace the CO2 inside the flask and this will make the media alkaline (pink).
13. It is not necessary to use a pH meter. The media is a pH indicator and the color should be red at this pH (not pink or yellow).