Care of Lymphoblast Cell Lines From Human Peripheral Blood

Note: This protocol assumes the investigator is using proper sterile tissue culture techniques.

1) Place complete RPMI-1640 in 37° C water bath.
2) After the lymphoblast cell line initiation (See ***GMB004 Lymphoblast Protocol***), place the T25 culture flask into CO_2 incubator #3 (5% CO_2, 37° C, ~85% humidity) for seven days.
3) After seven days, add 3mL of complete RPMI to the flask. Place culture back into the incubator, on the second shelf down.
4) After an additional seven days, carefully remove 5mL of media, without disturbing the cells on the bottom of the flask. If the lymphoblast cells have excessive red blood cell contamination, an attempt to remove some red blood cells can be done at this time to "clean up" the sample. Use caution not to remove any of the lymphoblast cells. Replace the old media with 5 mL of fresh complete RPMI. Place back into the incubator on the third shelf down.

   Media is discarded by placing into a waste container containing bleach, which will serve to decontaminate the media. The pipette is then disposed of by placing in a biohazard was container.

5) After seven more days, if the culture has yellowing media and visible clusters / balls of lymphoblast cells, continue onto the next step (6). If the culture is struggling after the seven days, with little/no visible cell clumps, then skip to step number 7.
6) Add 5mL of fresh complete RMPI to the culture and move to incubator #5 Follow the steps listed below to bring samples to maturity in preparation for the freeze process.
   a. Lymphoblasts prefer to live and grow in clusters. Maintain a robust culture and bring the total volume in the T25 flask to about 10-15mL.
   b. Once the T25 flask has reached 10-15ml and it growing reasonably well, carefully remove ~7mL of the old media without removing any of the cell clusters.
   c. Transfer the remaining cells to a T75 flask (along with the flask label) and bring the volume to 20mL with fresh complete media and move to incubator #7.
   d. Maintain the culture until ready to freeze.
7) For struggling samples, carefully remove 5mL of media and place culture back into the incubator for another seven days, placing on the bottom shelf.
8) If the cultures begin to have visible cells and yellowing media, then follow step 6a-d to bring samples to maturity. If the samples continue to struggle after the seven days then the culture will undergo re-virusing as follows:
   a. Remove as much media as possible and reintroduce 3mL EBV conditioned media and placed in incubator #2.
   b. After seven days, add 1mL complete RPMI.
   c. Continue to remove media and reintroduce EBV every two weeks until successful initiation or confirmed failure.
      i. If cells are visible after a reintroduction of EBV, add 1-2mL complete RPMI and begin to follow step 6 a-d to bring samples to maturity.

ii. Typically allow 2 attempts for re-initiation before recording the cell line as unsuccessful in the failed cell line log.

Decontaminate of any pipets used to with the virus containing media by placing them in the pipet chimney that contains 10% Clorox solution. Once the pipets have been decontaminated, they can be removed from the chimney, drained of liquid and disposed of in a biohazard box.

RPMI-1640 (complete)
500mL of RPMI-1640
100mL heat-inactivated FBS (20%)
6mL antibiotic-antimycotic (1x) – *Gibco/Invitrogen: Cat. No. 15240-062*
1mL Tylosin solution – *Sigma-Aldrich: Cat. No. T3397*

EBV Media
This is complete RPMI-1640 media that has been conditioned by B95.8 cells. The B95.8 cell line is a lymphoblastoid cell line from the marmoset monkey that expresses the human EBV virus. Please see protocol ***GMB008*** for preparation details.