Chapter 5

Establishment of Lymphoblastoid Cell Lines

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1. Introduction

The ability to establish long-term B lymphocyte cultures from patients carrying particular genetic characteristics or with the ability to secrete specific antibodies (*1*) is an extremely valuable technique. However, there are several basic principles to follow in the approach to this technology. It is the purpose of this chapter to provide all the information necessary to run an efficient and safe Epstein-Barr Virus (EBV) transformation system. More detailed information regarding the use of this technique for the preparation of human monoclonal antibodies is given elsewhere (2).

2. Materials

1. B95–8 marmoset cell line.
2. RPMI 1640 culture medium containing 10%, 5%, and 2% FBS.
3. Culture medium (without FBS) RPMI 1640 containing 10 U/mL heparin (preservative-free), plus penicillin, streptomycin, and Polymixin B sulfate.
4. 1 mL B95.8 cell culture supernatant.

5. 10% FBS RPMI 1640 culture medium, plus 0.5% PHA Wellcome.
6. Ficoll-hypaque or lymphroprep.

3. Methods

3.1. Production of Epstein-Barr Virus Stock (3)

1. The B95.8 cell line (Note 1) is cultured routinely in 5% FBS RPMI 1640. Routine passage consists of diluting the cells between 1 in 2 and 1 in 5 at weekly intervals. A culture in the logarithmic growth phase would usually yield 10^6 cells/mL at 37°C.
2. For preparation of virus stocks, dilute from 10^6/mL to 0.2×10^6/mL in 2% FBS RPMI 1640.
3. Incubate at 33°C for 2 w without any medium additions or changes.
4. Allow the cells to settle out at 4°C.
5. Centrifuge the supernatant at low speed (150*g* for 5 min) in order to clarify.
6. Pass the supernatant through a 0.2 µm filter.
7. Aliquot the filtered supernatant in 2 mL sterile plastic ampules, and store either short-term (1 mo) at –20°C, medium-term (6mo) at –70°C, or preferably in a gaseous phase liquid nitrogen container at –130°C.
8. A sample of each batch should be examined for microbiological purity (bacteria, fungi, mycoplasma) and also tested for transforming ability.

3.2. EB-Virus Transformation: Blood Sample Preparation

1. The heparinized blood is mixed 1:1 with RPMI 1640/heparin (*see* Note 2).
2. Layer the diluted blood sample onto Ficoll-hypaque at a ratio of 2:1, i.e., 8 mL Ficoll-hypaque/4 mL blood, in a 15-mL conical centrifuge tube. Care must be taken to prevent the two solutions from mixing.
3. Centrifuge at room temperature at 300*g* for 20 min.
4. Carefully remove the buffy coat interphase with a Pasteur pipet. This contains the mononuclear cell fraction.
5. Add RPMI 1640/heparin to the cells to make the volume up to 15 mL.
6. Centrifuge at 150*g* for 4 min.
7. Pour off the supernatant, and resuspend the lymphocyte pellet in RPMI 1640/heparin.
8. Repeat this procedure twice more. IMPORTANT: It is essential to ensure that the lymphocyte pellet forms after each centrifugation step. If not, dilute the sample further (step 5 or recentrifuge). Avoid centrifuging too hard; otherwise platelets will sediment.

9. Resuspend the mononuclear cells in 5 mL of RPMI 1640/heparin.
10. Mix 0.2 mL of cell suspension with 0.2 mL Trypan blue (0.4% in PBS), and count the viable cells on an improved Neubauer counting chamber.
11. At this point, cells can be set up immediately for transformation or stored in liquid nitrogen (*see* Note 3).

3.3. Cell-Freezing Procedure

1. Centrifuge the cell suspension at 150*g* for 4 min.
2. Resuspend the pellet in the correct vol of 91% newborn calf serum (NBCS) + 9% DMSO to achieve 5 x 10^6 cells mL.
3. Freeze in a programmable freezer at –3°C/min. Alternatively, freeze overnight in a polystyrene container in a –80°C refrigerator and then remove ampules to –196°C.

3.4. Virus Transformation

Either:

1. Carefully thaw the ampule in a 37°C water bath, and transfer contents to 10 mL of 10% FBS RPMI 1640 to remove DMSO. Centrifuge at 100*g* for 5 min, or
2. Centrifuge cells in RPMI 1640/heparin at 150*g* for 4 min.

Then:

3. Resuspend cell pellet in 1 mL EBV (B95.8) supernatant per 5 x 10^6 cells.
4. Incubate at 37°C for 1–1.5h. Agitate the suspension at least once during the incubation period.
5. Centrifuge at 100*g* for 5 min.
6. Discard supernatant.
7. Resuspend lymphocytes in an appropriate vol of 10% FBS RPMI 1640 + penicillin/streptomycin + 0.5% PHA (Wellcome) to give 10^6 cells/mL (*see* Note 4).
8. Transfer 1 mL of cell suspension to each well of a 24-well tissue culture tray (*see* Note 5). Incubate at 37°C in 95% air/5% CO_2 for a minimum of 5 d before changing the medium.
9. With smaller cell numbers, 0.2 mL of cell suspension can be transferred to each well in a 96-well plate (*see* Note 6).
10. Cells can be transferred sequentially from a 96-well tray to a 24-well tray to a 25-cm^2 flask over the next 14–21 d. This depends upon the rate of appearance of cell growth in the wells. The technique can be amended if particular problems are anticipated with cells. *See* Notes 4–7.

4. Notes

1. A major consideration is the source of EBV. Most laboratories currently use the persistently infected Marmoset cell line B95.8 (4) as a source of virus, although in some cases cocultivation with the cell line QIMR–WIL has been employed. Unfortunately, many samples of the B95.8 cell lines distributed informally between research laboratories are mycoplasma contaminated. It is therefore of the utmost importance that the basic starting material for the technique, the virus source, is obtained from a reliable source, such as an established culture collection.
2. Blood samples (5 mL min.) should be transferred from the syringe immediately into preservative-free heparin (final concentration, 10 U/mL). If the samples are to be stored prior to preparation, this must be done at room temperature. Storage at 4°C leads to rapid cell death. The maximum period of storage is 4 d.
3. There are two approaches to EBV-transformation: either cells can be transformed immediately or, if this is not convenient, the cells, once separated, can be stored in liquid nitrogen until transformation. It is recommended that all procedures should be carried out in a Class II containment cabinet.
4. The mononuclear cell suspension prepared by Ficoll-hypaque separation contains both B + T cells. There are several approaches to the removal of T cells necessary to prevent specific cytotoxic T cell killing of B cells carrying EBV antigens (*see* Chapter 4, this volume for details). The alternatives are:
   a. Rosetting with sheep red blood cells to deplete the T cells from the suspension. This has the disadvantage of reducing the B cell yield and contaminating T cells may remain.
   b. Cyclosporin-A can be added to kill T cells. This has the disadvantages of requiring assay before use, has carcinogenic properties, and needs to be dissolved in ethanol for use. It may be difficult for some laboratories to obtain supplies. It can have certain advantages in microtechniques.
   c. PHA can be added. This results in the proliferation of T cells and has a mitogenic effect on B cells. There is no evidence of specific T cell killing following PHA stimulation. In our laboratory, this has proved to be the most efficient technique available: hence, it is reproduced here. (One possible drawback is that mitogenesis leads to differentiation of committed B cells to

plasma cells and then the loss of the C3 receptor, and thus, the cells are not transformed.)

5. Mouse peritoneal macrophages can be prepared as feeder-layers (at 2 x 10^4 cells/well) in a 24-well plate 24 h prior to cell transformation.
6. FCS concentration can be increased to 20% with small cell vol.
7. It is important to test the cell line for mycoplasma following establishment in culture. It has been noted in our laboratory that, even with precautions taken, i.e., pretested virus and media components, mycoplasma can be introduced with the original blood sample. Finally, it is advisable to test staff for antibody to EBV (VCA test) prior to commencement of work with the virus. If immunity is not present or is at a very low level, additional caution should be exercised in the handling of this agent. However, over 98% of adults aged 23+ have antibody to VCA.

References

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