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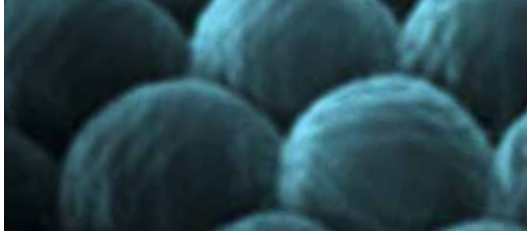

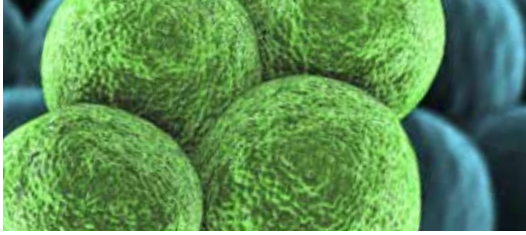

ATCC®

LYMPHOCYTE TRANSFORMATION USING ATCC® VR-1492™

Tech Bulletin
No. 10

Epstein-Barr virus (EBV), also referred to as Human herpesvirus 4, is a gamma herpesvirus of the genus *Lymphocryptovirus*. It is the cause of infectious mononucleosis and has been associated with several human neoplastic diseases, including Burkitt's and Hodgkin's lymphomas. Most adults are seropositive for EBV, demonstrating its ubiquitous presence in the human population. Infection is usually latent. However, explanted lymphoma cells sometimes produce infectious virus. Several decades ago, supernatants from cell lines derived from lymphoma explants were used to confirm the neoplastic potential of EBV infection by virus-mediated immortalization of B lymphocytes from human blood in cell culture. Since then, EBV transformation of B lymphocytes *in vitro* has become a routine procedure for obtaining permanent human cell lines for use in diagnostics and research[1,2].

In 1972, G. Miller and colleagues found that EBV transformed marmoset lymphocytes produce more virus than infected human lymphocytes, providing a ready source of the virus in the B95-8 cell line they established[3,4]. EBV-containing supernatants from the B95-8 cell line are distributed by ATCC as VR-1492™. Prior to distribution, each preparation is tested to verify that the content of one vial (1 mL) is capable of inducing transformation in a sample of ~106 normal human lymphocytes isolated from frozen normal human blood (~0.5-1 mL).

This technical bulletin describes the procedure recommended for transformation with this product as described by Caputo *et al.* in 1995, which is reported to have a success rate of 95%[5]. This high success rate is attributed to the use of a feeder layer to support the culture during the transformation process. Other laboratories have reported other modifications that may improve transformation success rate[6-9]. These are generally aimed at ensuring reagent quality or preventing the development of cytotoxic T lymphocytes reactive with EBV antigens that might kill virally infected B cells before a transformed cell line can be established. Because blood samples vary in quality (and quantity), each laboratory must verify the efficacy of any EBV transformation methods it adopts, and modify them as needed for specific applications.

MATERIALS

- Defibrinated or anticoagulant-treated whole human blood
- DMSO (ATCC® 4-X™ or equivalent)
- Cryovials to hold 0.5-1 mL
- Histopaque®-1077 Ficoll® gradient solution (Sigma-Aldrich or equivalent)
- Iscove's modified DME (IMDM; ATCC® 30-2005™ or equivalent)
- RPMI-1640 (ATCC® 30-2001™ or equivalent)
- FBS (ATCC® 30-2020™ or equivalent)
- Penicillin/streptomycin solution, 10,000 I.U./mL penicillin, 10,000 µg/mL streptomycin (ATCC® 30-2300™)
- PBS, calcium- and magnesium-free (ATCC® 30-2200™ or equivalent)
- Filtered supernatant from EBV-expressing cells, ATCC® VR-1492™
- Irradiated MRC-5 feeder layer cells (ATCC® 55-X™). NOTE: Irradiated MRC-5 cells are packaged as frozen suspensions, 1-mL per vial. Each vial is sufficient for seeding up to 225 cm^2 of surface area (9 - T-25 flasks)
- 25 cm^2 (T-25) flasks, cell culture treated
- 75 cm^2 (T-75) flasks, cell culture treated
- 15 mL sterile polystyrene centrifuge tubes, capped

PRECAUTIONS

- All work should be completed using aseptic technique under BSL-2 conditions using universal precautions for handling blood products[10].
- B95-8 cells may also carry and secrete a transmissible type D retrovirus, SMRV-B95-8 (squirrel monkey retrovirus – B95-8), which can infect human B and T cell lines. This retrovirus does not interfere with EBV's transforming capabilities[11].

PROCEDURE

Freeze blood sample for later use (optional)

1. Dispense 0.1 mL DMSO into labeled cryovials for blood storage.
2. Pool defibrinated or anticoagulant-treated blood from containers.
3. Dispense 0.9 mL of blood per cryovial, mixing periodically to ensure an even cell suspension.
4. Cap the vials and mix gently.
5. Freeze the vials in a cryopreservation container in ethyl alcohol at -80°C for 3-18 hours or in a controlled rate freezer at 1°C per minute.
6. Store vials of cryopreserved blood in liquid nitrogen.

Prepare feeder layers 1-5 days prior to use for transformation

1. Prepare complete culture medium by addition of FBS to either EMEM or IMDM to 10% v/v and penicillin/streptomycin solution to 1% v/v (1X).
2. Thaw a vial of ATCC® 55-X™ and mix with 27 mL of complete culture medium in a sterile 50 mL conical tube.
3. Dispense 3 mL of cell suspension per T-25 flask (~4 X 10^5 cells).
4. Examine flasks microscopically next day. Cultures should be about 30% confluent.
5. Use for transformation from 1-5 days after planting.

Prepare lymphocytes for transformation

1. Mix 3 mL of whole blood (fresh or thawed) with an equal volume of PBS or RPMI-1640.
2. Separate lymphocytes from red blood cells and granulocytes using Ficoll®.
   a. Carefully layer the diluted whole blood over 4.5 mL of room temperature Histopaque®-1077 in a 15 mL polystyrene centrifuge tube.
   b. Centrifuge at 400 x g for 40 minutes at room temperature.
   c. Remove and discard the upper, clear plasma layer to within 0.3 cm of intermediate layer.
   d. Transfer the opaque lymphocyte band at the intermediate layer and the Histopaque®-1077 layer down to within 0.3 cm of the pellet at the bottom of the tube to a new 15 mL centrifuge tube.
   e. Fill the tube with IMDM with 20% FBS and penicillin/streptomycin; mix the tube well and centrifuge at 260 x g for 15 minutes. Remove and discard the supernatant.
   f. Resuspend the cell pellet in 10 mL IMDM with 10% FBS and penicillin/streptomycin; mix and collect cells again by centrifugation at 260 x g for 15 minutes. Remove and discard the supernatant.
   g. Resuspend the cell pellet in 3 mL IMDM with 10% FBS and 1X penicillin/streptomycin.
3. Perform a viable count on the resulting lymphocyte preparation using a dye exclusion method.
4. Use cells immediately for transformation or freeze them for future use as for whole blood.

Set up transformation cultures (2 flasks per sample are recommended)

1. Thaw 1 vial of ATCC® VR-1492™ (1 mL) per transformation flask.
2. Remove medium from feeder layer(s).
3. Per flask add:
   a. 2 mL IMDM with 20% FBS
   b. 1 mL lymphocyte suspension containing 1-6 x 106 lymphocytes.
   c. 1 mL (1 vial) ATCC® VR-1492™
4. Mix gently and incubate flasks at 37°C under a 5% CO_2 atmosphere.

Maintain and observe cultures for transformed cells

1. 7 days after initiation, observe each flask for signs of transformation and add 4 mL of fresh medium (IMDM with 20% FBS and 1X penicillin/streptomycin).
   a. Early signs of lymphocyte transformation include cone shaped cells, cells with spike-like projections and clusters of refractile, healthy-looking cells.
   b. Later signs of transformation include increased turbidity of the culture and acidification of the culture medium, indicated by yellowing.
2. Every 3-4 days thereafter, examine cultures for signs of transformation and add fresh medium.
   a. Carefully withdraw 3-4 mL of medium without removing cells.
   b. Add back the same volume of complete culture medium (IMDM with 20% FBS and 1X penicillin/streptomycin).

Expand transformed cultures to establish lypmhoblast cell lines

1. Transfer cells from a densely populated T-25 flask to a T-75 flask and add sufficient IMDM with 10% FBS and penicillin/streptomycin to obtain a cell density of 1-3 x 10^5 cells per mL.
2. Maintain cell density in the T-75 flask at 1-3 x 10^5 cells per mL by addition of culture medium A maximum density of 1-2 x 10^6 is achievable and maintainable.
3. Expand to additional flasks to maintain cell density at 1-3 x 10^5cells per mL.

Freeze transformed cells at 3-5 X 10^6 cells per mL for preservation and later use

1. Pool cultures to be frozen and complete a viable cell count to determine the cell density and viability in the suspension, and the total number of cells to be frozen.
2. Collect cells from culture fluids by centrifugation at 260 x g for 15 minutes.
3. Discard the supernatant and resuspend the cell pellet in a volume of chilled freezing medium (IMDM with 10% FBS, penicillin/streptomycin, and 10% DMSO) that will give a cell density of 3-5 X 10^6 cells per mL.
4. Dispense the resulting cell suspension into labeled cryovials.
5. Freeze vials in a cryopreservation container in ethyl alcohol at -80°C for 3-18 hours or in a controlled rate freezer at 1°C per minute, then store them in liquid nitrogen.

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TB-0117-02