SOP: D03.01.23 GENERATION OF EBV-TRANSFORMED CELL LINES (LYMPHOBLASTOID CELL LINES - LCL)

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1. **Purpose:**

1.1. Epstein-Barr Virus-transformed B cell lines for use as stimulators for cytotoxic T-lymphocytes may be derived from peripheral blood of the stem cell donor (ETNA protocol), or the patient (ANGEL, ANGELA or SCAEBV protocols). B cells are infected by a well-characterized laboratory strain of EBV, to generate a lymphoblastoid cell line (LCL).

2. Scope

2.1. This procedure is to be followed by GMP staff to prepare lymphoblastoid cells as intermediates during the preparation of cytotoxic T cells.

3. Definitions and Abbreviations:

3.1.	B95-8	A laboratory strain of EBV
3.2.	EBV	Epstein Barr Virus
3.3.	Complete Medium	RPM1 1640, 10% Serum, 2mM L-Glutamine
3.4.	CSA	Cyclosporin A
2.4.	CTL	Cytotoxic T-lymphocytes
2.2.	LCL	Lymphoblastoid Cell Line
3.5.	PBMC	Peripheral Blood Mononuclear Cells
3.6.	SOP	Standard Operating Procedure

4. Materials and Equipment:

NOTE: All materials in contact with cells must be sterile, pyrogen-free and used according to the manufacturer's directions unless stated otherwise. Equivalent materials and equipment may be used but all changes must be recorded in the appropriate worksheets.

4.1. Specimen:

4.1.1. Fresh or frozen PBMC prepared according to the appropriate SOP

NOTE: Infectious disease testing must be performed on the donor within 30 days of collection.

4.2. Materials

4.2.1.	RPMI 1640	Invitrogen
4.2.2.	Advanced RPMI 1640	Invitrogen

4.2.3.	Fetal Calf Serum (Heat inactivated)	HyClone
4.2.4.	L-glutamine (200 Mm)	Invitrogen
4.2.5.	EBV-containing Viral Supernatant	Produced by cell line B95-8
4.2.6.	Cyclosporin A	Sandoz
4.2.7.	Acyclovir	Wellcome
4.2.8.	Freezing medium	Origen (Fisher Cat.#1G-50-0715)
4.2.8.1.	(10%DMSO, 10% HI FBS,80% Iscove's Dulbecco's Medium)	
4.2.9.	Centrifuge tubes	Corning
4.2.10.	Tissue culture plates	Costar
4.2.11.	Disposable pipets	Costar
4.2.12.	Tissue culture flasks - vented	Costar
4.2.13.	Ficoll	Pharmacia

4.3. Equipment

4.3.1. Biological safety cabinet, certified

4.3.2. Centrifuge, calibrated

4.3.3. Humidified incubator with atmosphere of 5% CO_2 in air

4.3.4. Hemacytometer

5. Procedure:

NOTE: Perform all steps in a certified biological safety cabinet, using aseptic technique and following Universal Precautions for handling blood and blood products.

5.1. Generation of EBV-transformed B cell lines from patients or donors

5.1.1. (Use worksheet DW3.1.xA)

5.1.2. Prepare 5 ml of culture medium containing 1ug/ml Cyclosporin A for later use

5.1.3. Separate mononuclear cells from a minimum of 5 ml of peripheral blood as described in the SOP "Density Gradient Separation of Lymphocytes Using Ficoll ®"

5.1.4. Save 1 ml of plasma in a cryovial and store at –80°C if required.

5.1.5. Centrifuge 5 x 10^6 PBMC for 5' @ 400 g. Remove supernatant and resuspend pellet.

5.1.6. Add 200ul of concentrated B95-8 virus supernatant to PBMC. Resuspend the cells by gently flicking the base of the tube with the fingers.

5.1.7. Add 1.8 ml of complete culture medium containing 1ug/ml Cyclosporin A.

5.1.8. Place 200ul of cell mixture in each of 5 wells of a 96-well flat-bottomed plate. Place 100ul of cell mixture into another 10 wells of the same 96 well flat bottom plate. Add an additional 100ul of CSA-containing medium to the 10 wells containing 100ul of cell suspension. The final volume per well is 200ul.

5.1.9. Fill empty wells with sterile water.

5.1.10. Place plate(s) in 37°C, 5% CO_2 incubator.

5.1.11. Remaining PBMC should be frozen in aliquots of ~5 x 10^6/ml as backup as described in section 5.3

NOTE: For patients with allogeneic donors, peripheral blood should be requested at this time to initiate PHA blasts according to SOP D.3.6

5.1.12. Feed plate weekly as follows:

5.1.12.1. Remove plate(s) from incubator and aspirate ~0.1 ml of medium from each well of the 96-well flat-bottom plate.

5.1.12.2. Add ~0.1ml of fresh medium to each well and return to incubator.

5.1.13. After 2-3 weeks of incubation, if transformed cell clumps are expanding, combine cells from three wells of the 96-well plate into one well of a 24-well flat-bottomed plate. Retain remaining cells in 96-well plate as reserves and feed harvested wells with medium, as these usually grow back. (Worksheet DW03.01.xb)

5.1.14. Continue with weekly feeds and expansion of the 96 well plate. Begin twice weekly feeds of 24 well plates.

5.1.15. Once cells are clearly proliferating, according to microscopic examination, remove cells from 24-well plate and place them into a 25 cm^2 vented flask with 5ml

complete medium plus 100μM of Acyclovir. Acyclovir is added to assure that there will be no infectious virus present in the culture. At this time, also add acyclovir to 2 mL wells to keep all cells synchronized for acyclovir

5.1.16. Feed cells twice weekly with complete medium, adding 100μM Acyclovir each time. After approximately two weeks, freeze 3 vials with at least 5 x 10^6 LCLs as described below. (Form DW3.1.1d) The residual cells are for cytotoxic T lymphocyte (CTL) generation.

5.1.17. LCLs should be cultured to appropriate cell numbers for CTL stimulation and maintained in culture as stimulator cells for CTLs. (Form DW3.1.1c)

5.2. *Expansion of LCLs in bioreactors (G40 and G500)*

NOTE: If large numbers of LCLs are required or if LCLs are growing poorly, they may be expanded in bioreactors, in which cells grow to higher cell densities and are less susceptible to oxygen deprivation.

5.2.1. Once LCLs are established, transfer 1 x 10^6 into a GP40 bioreactor. Add LCL medium to 30 mLs. Culture for ~ 7 days in 37^o C/5% CO2 incubator**.**

5.2.2. On day 7, count. Expect ~ 5 x 10^7 LCLs.

5.2.2.1. If fewer than 5 x 10^7, cells may be cultured for 2-3 more days

5.2.3. Cells may be removed for irradiation and use as APCs, transduction with adenovirus vectors or cryopreservation.

5.2.4. Cells may be maintained in GP40 or expanded in GP500

5.2.4.1. To maintain in GP40,

5.2.4.1.1.Reseed GP40 flask at ~1 x 10^6 cells per bioreactor in 30 mLs medium

5.2.4.2. To expand in GP500

5.2.4.2.1. Transfer 10 x 10^6 cells to GP500 bioreactor(s)

5.2.4.2.2.Add medium to 100 mL and culture for ~7 days

5.2.4.2.3.On ~ day 7, count, remove cells if required as in section 5.2.3

5.2.4.2.4. Add 200 mL fresh medium. Return to incubator and culture for 7

days.

5.2.4.2.5.On day 14, count. Expect ~ 800 x 10^6 cells. Remove cells as required, as in section 5.2.3

5.2.4.2.6.Reseed into flasks or bioreactors as required.

NOTE: Cell numbers used to reseed bioreactors can be increased depending on the timing of the cells required for use as APCs.

5.3. Freezing of LCLs or PBMCs (See Worksheet DW03.01.XB)

5.3.1. Centrifuge cells to be frozen at 400xg for 5 minutes.

5.3.2. Transfer 5 ml of supernatant from centrifuged cells to a 15ml centrifuge tube and store at –80ºC for future QC testing. This supernatant can be discarded when all release criteria have been met for this CTL population.

5.3.3. Place cells on ice for 10 minutes to cool.

5.3.4. Resuspend cells in 1 ml freezing medium for each 5-10 x 10^6 cells

5.3.5. Distribute 1ml aliquots of cells/freezing medium mixture into labeled cryotubes.

5.3.6. Place tubes in Nalgene freezing containers and transfer to -80^{o}C freezer and freeze overnight.

5.3.7. Transfer to the designated liquid nitrogen freezer for storage.

6. Expected Results

6.1. This procedure should produce a minimum of 2x10^7 LCL for both a back-up freeze and subsequent CTL initiation. Viability should be >75%. The cultures should be capable of growth indefinitely.

7. Notes

7.1. Since all cells prepared are intended for infusion to patients, it is essential to adhere to proper procedures to prevent misidentification or contamination of patient samples.

7.2. **ALL** flasks and centrifuge tubes must be labeled with a patient name and number, donor number. **UNLABELED MATERIAL WILL BE DISCARDED.**

7.3. **NEVER** work with more than one patient cell line at any one time.

7.4. **ALWAYS** use medium prepared and labeled specifically for each patient's cells. **NEVER** use medium prepared for one patient's cells to feed or process cells from a different patient.

8. References:

8.1. Rooney CM, Smith CA, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation, *The Lancet* 1995; 345: 9-13.

9. Attachments:

9.1. Appendix 1: Flow diagram

9.2. Worksheets DW3.6.Xa and DW3.6.Xb (blank and completed versions)

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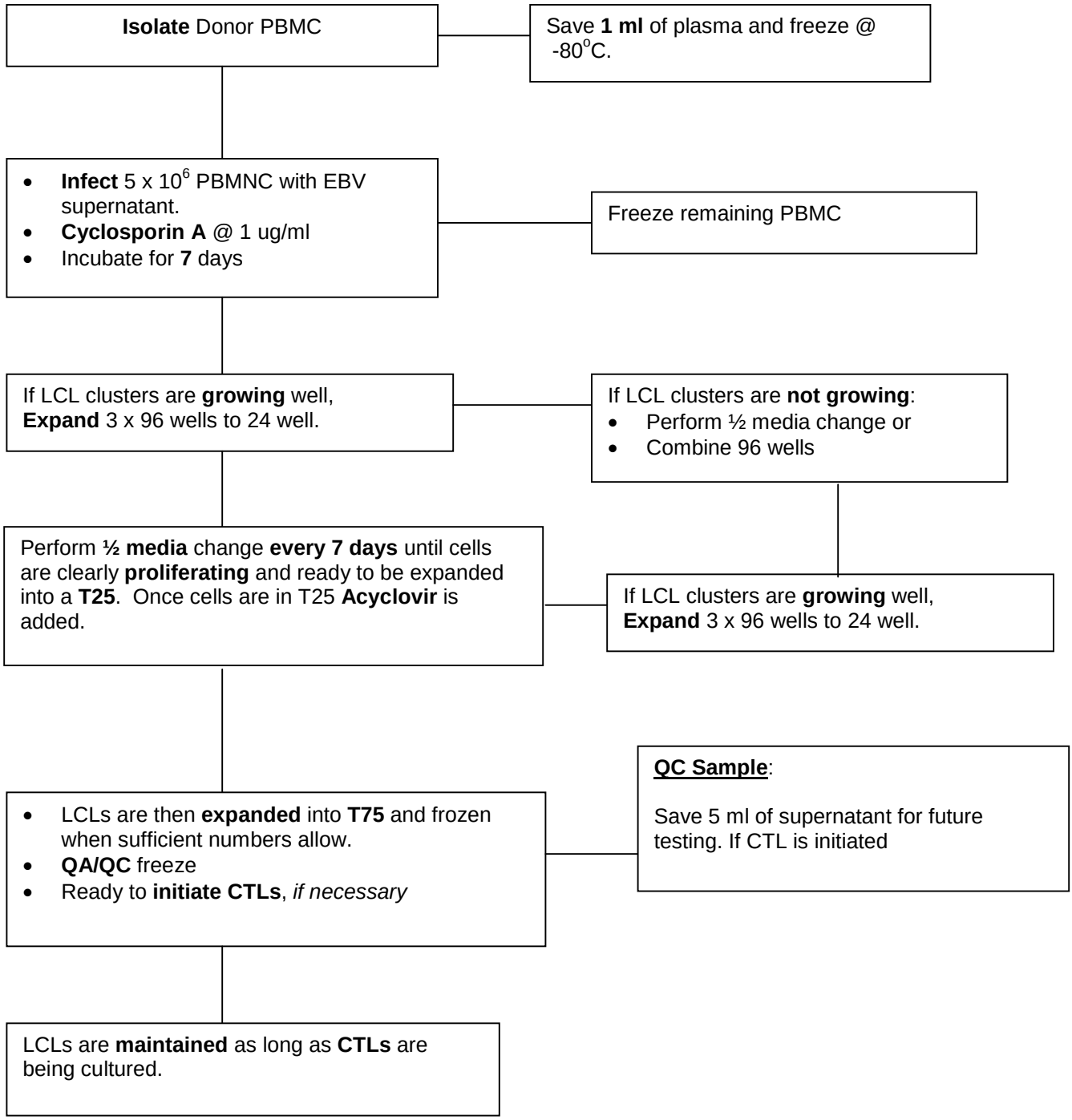
APPENDIX 1: FLOW CHART FOR LCL INITIATION/EXPANSION AND MAINTENANCE



10. Review and Revisions

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