Generation of cell lines using Epstein-Barr Virus (EBV) transformation of small volumes of cryo-preserved whole blood and the use of bench-top flow cytometry to achieve high and reproducible success rates.

Abstract.

ECACC has developed a procedure for the generation of lymphoblastoid cell lines (LCLs) by the direct EBV transformation of small amounts (800 μl) of cryo-preserved whole blood eliminating any requirement for prior separation of peripheral blood lymphocytes (PBLs). The ability to directly transform frozen whole blood avoids the cost of separating and storing PBLs while retaining the option to make a cell line at any time from all or a subset of the collection. Consistent transformation success rates of over 90% have been achieved using bench-top flow cytometry to track lymphoblastoid proliferation from source blood that has been cryo-preserved for more than four years.

Introduction.

The generation of an LCL from a human blood sample can be regarded as a long term means of protecting the genomes of valuable study subjects. Cell line DNA generated from these cell lines can be widely distributed, representing a potentially limitless source of the subject's genome that will be available many years after venesection allowing follow-on studies that may not have been originally anticipated or which are only possible with the development of new technologies.

Despite the advantages and benefits of LCLs, conventional methods for their generation rely on processing freshly venesected blood samples by density gradient centrifugation to produce enriched, erythrocyte – free PBL preparations. This process of PBL preparation is both labour and consumables intensive, and most protocols rely on 5 ml or more of blood to provide an adequate yield of PBLs to assure successful LCL generation. Often PBLs are cryo-preserved and held as intermediates in liquid nitrogen storage before EBV transformation is carried out. Thus the cost of preparing and storing PBLs for every subject in a large population study may be considered prohibitive to many researchers, particularly where cell lines may not be required from every participant.

Here we present a protocol for the direct EBV transformation and cell line generation from 800 μl of cryo-preserved whole blood. This procedure does not require pre-isolation of PBLs or standardisation of lymphocyte densities and transformation success rates in three separate studies were consistently above 90%.

Methods overview.

Whole blood was cryo-preserved by addition of 10% (v/v) DMSO. Samples were mixed thoroughly, before being frozen to -180°C in a rate controlled freezer and stored in vapour phase liquid nitrogen (-196°C). Frozen bloods were thawed and 100 μl removed for analysis using a bench-top flow cytometer. The remaining sample (1.5 ml or 800 μl) was washed with warmed RPMI 1640 (Sigma) supplemented with 10% Fetal Bovine serum and the cell pellet re-suspended in 1 ml of a transformation media containing 80% cyclosporin media (foetal calf serum (FCS-96% v/v), 1000u/ml

penicillin/streptomycin (2% v/v),1000u/ml neomycin (1% v/v), cyclosporin A (a T-lymphocyte inhibitor – 1% v/v)and 20% EBV supernatant. The cell suspension was transferred to a 5 ml, round bottom transformation tube using a sterile pipette and placed in an incubator at 37^0C at 5% CO_2 for five days. After five days samples were media changed. This was repeated every four - five days until up to six media changes had occurred. Samples were then expanded into T25 cm flasks containing 5 mls of warmed RPMI 1640 20% FCS. 100 μl of sample was removed for analysis using a bench-top flow cytometer. Samples were checked microscopically for signs of transformation. Over the course of the next two – three weeks, cultures were expanded to a volume of 50 mls. Aliquots of the cell pellet were then frozen in medium supplemented with 10% DMSO in a rate controlled freezer.

The use of bench-top flow cytometery to measure lymphocyte proliferation in transformed whole blood samples.

During ECACCs standard transformation procedure of PBLs, transformed cells can easily be visualised by inverted phase contrast microscopy because of the clarified nature of the starting material (erythrocytes and cell debris are removed by density gradient centrifugation). Cell proliferation, an indication of successful transformation, can easily be monitored by the appearance of characteristic clusters of dividing cells. In this standard process the developing cultures are fed by the replenishment of medium without increasing the culture volume (media changing). After about three media changes the culture is ready to be expanded into 25 cm^2 culture flasks. Once in culture flasks the cells rarely senesce or die. This point is therefore recognised as a key stage in the transformation process and subsequent generation of a cell bank.

However, during the development of this whole blood transformation procedure it was evident that for blood samples visual or microscopic analysis to monitor successful transformation was difficult due to the presence of un-clarified, glutinous blood debris. Thus identifying the optimum point to expand the samples into culture flasks, which usually results in the successful generation of a transformed cell line, was difficult resulting in poor reproducibility and transformation success rates for the small volume blood samples in early studies.

This problem was resolved with the use of bench-top flow cytometry. Bench-top flow cytometry is a key tool in achieving high transformation success rates from frozen whole blood.The basis of the assay relies on the differential permeability of viable and non-viable cells to DNA-binding dyes in a proprietary reagent.

Once incubated in the dye the cells are taken up into the flow cell and the patterns of fluorescence detected by a number of lasers. Each cell produces an ‘event’ which is displayed graphically. Live cell events are shown on the left hand side of the viability discriminator (diagonal red line in Figure 1) and dead cell events on the right hand side of the line. Figure 1 shows a comparison of the cytometric analyses of a PBL and frozen blood sample immediately after recovery from cryogenic storage.

(A) (B)

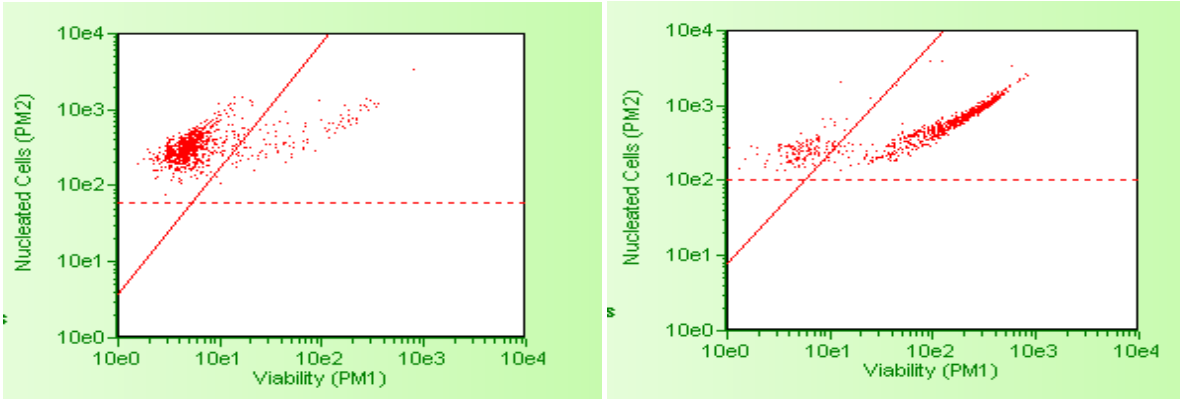


Figure 1. Comparison of the cytometric analyses of a PBL and a frozen blood sample immediately after recovery from cryogenic storage. The PBL sample (A) is showing 90% viable lymphocytes and the frozen blood sample (B) about 30% viable lymphocytes.

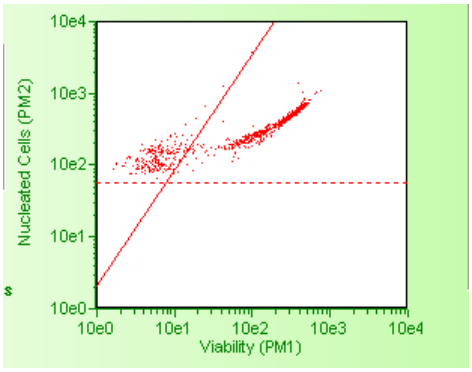
In the second graph (B) representing the blood sample, there are many more events to the right of the viability discriminator showing there are more dead cells in this sample which displays only about 30% viable lymphocytes. This is probably a result of the non-optimised blood cryo-preservation procedure.

Bench-top flow cytometery is a key tool in achieving high transformation success rates from frozen whole blood.

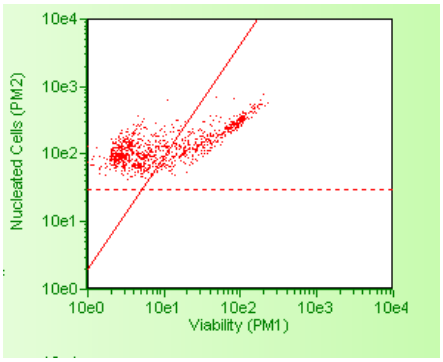
Bench-top flow cytometry gives a method of assessing the progress of transformation by measuring lymphocyte proliferation (Figure 2, graphs A – D). This analysis gives the operator the information they require to expand the culture from its initial transformation vessel into culture flasks. This results in reproducible transformation success rates of over 90%. Without this analysis, this decision is often arbitrary and in our experience resulted in transformation success rates ranging from 30% – 90%.

Figure 2 shows a sequence of cytometric analyses at four time points throughout the transformation of a frozen blood sample. This represents a period of roughly seven weeks from resuscitation of the blood (graph A) to freezing the final transformed cell line (graph D). During this period the percentage of events can be seen to move from the right of the viability discriminator (dead events) to the left (live events) representing successful lymphocyte proliferation, despite the poor viability of the starting material. Evidence for successful transformation would be backed up by microscopic analysis after expansion into culture flasks.

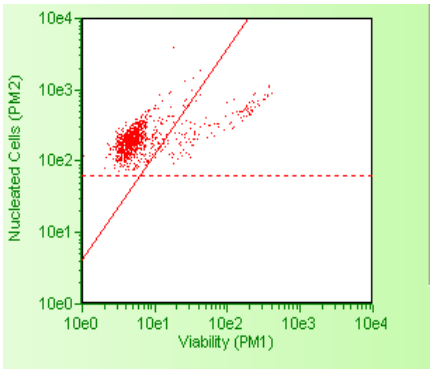
(A)



(B)



(C)



(D)

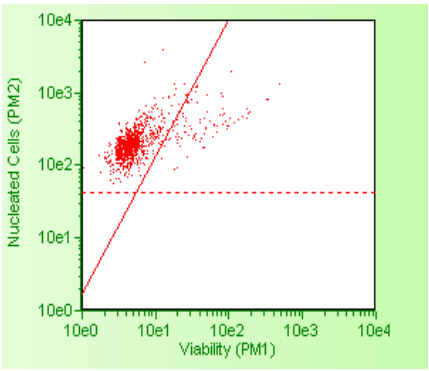


Figure 2. Cytometric analyses of the transformation of a cryo-preserved whole blood sample. Graph (A) immediately after resuscitation. Graph (B) after three weeks in the initial transformation tube. Graph (C) after five weeks just prior to expansion to culture flasks. Graph (D) transformed cell line prior to freezing (week seven).

Using this strategy, three studies were carried out on separate sets of four year old cryo-preserved blood samples. The control PBLs were generated from the same source as the bloods used in study 1. Study 2 was a repeat of study 1 to assess how reproducible the technique was. Figure 3 shows high transformation success rates in both cases. Finally, the objective of study 3 was to establish if the volume of blood could be reduced to 800 μl. The results show a 100% transformation success rate for this volume.

Study	Number of samples	Blood volume	Transformation success rate
Control PBLs	20	n/a	100%
1	20	1.5 ml	100%
2	20	1.5 ml	95%
3	20	800 μl	100%

Figure 3. Summary of results of three separate studies to establish the transformation success rate of the whole blood transformation procedure.

Conclusions.

(1) Small quantities of cryo-preserved blood (800 μl) can be directly transformed, without the need to separate PBLs, with transformation success rates comparable to those achieved with separated PBLs (over 90% success at first attempt even with starting material with low lymphocyte viability).

(2) High transformation success rates are achievable through the use of bench-top flow cytometry in the early stages of transformation to detect lymphocyte proliferation where visual analysis is not possible.

(3) Since the process mimics the standard PBL transformation process it is suitable for high throughput (50 transformations per day).

(4) Cryopreservation of aliquots of whole blood offers a viable and cost effective alternative to the preparation and storage of PBLs, particularly where cell lines may not be required form a complete collection, but only a selected sub-Cohort.

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