An Efficient, Cost Effective Method for the Generation of Immortalized Cell Lines from Small Quantities of Cryopreserved Whole Blood.

Deborah Blick, James Cooper, Natalie Baker, Pippa Bracegirdle, James Biggins, Edward Burnett

Health Protection Agency Culture Collections, Health Protection Agency Centre for Emergency Preparedness & Response, Salisbury, Wiltshire SP4 0JG, UK

Introduction

The conversion of a human blood sample to a lymphoblastoid cell line can be regarded as a means of immortalising a very valuable study subject and is the soundest means of underpinning a biobank. Cell line DNA can be widely distributed, representing a potentially limitless source of genomic DNA that will be available many years after collection, for follow-on studies that may not have been originally anticipated, or which may not be possible with existing technologies.

However, the cost of preparing and storing peripheral blood lymphocytes (PBLs) for every subject in a study cohort may be considered prohibitive, particularly where cell lines may not be required from every participant.

ECACC has developed a procedure for direct EBV transformation of small amounts of cryopreserved whole blood without the requirement for prior separation of 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. High transformation success rates have been achieved using blood that has been cryopreserved for more than 4 years.

Requirements

- Transformation success rates must be reproducible and comparable to those achievable from separated PBLs, i.e. 95% at first attempt.
- The procedure must be suitable for operation at high throughput, (50 samples per day) and amenable to miniaturisation.
- Costs for EBV transformation must be comparable to those achievable through high efficiency, high throughput transformation from frozen PBLs.

Source Material

- Ethical approval to use rejected blood samples (insufficient volume for transfusion) as a source for all whole blood transformation (WBT) experiments was obtained from the National Blood Service. Samples were obtained from 80 individuals.
- As a control, separated PBLs from each sample were transformed using ECACC's standard procedure.

Methods

Whole blood was cryopreserved 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 specimens were thawed and 100µl removed for analysis using a benchtop flow cytometer (see below). The remaining sample (1.5ml or 800µl) was washed with warmed RPMI 1640 (Sigma) supplemented with 10% foetal bovine serum and the cell pellet resuspended in 1ml of a transformation medium containing Cyclosporin A (a T-lymphocyte inhibitor) foetal calf serum (FCS), Penicillin, Streptomycin and Neomycin and EBV reagent. The Cell suspension was transferred to a round bottom transformation tube or 24 well plate using a sterile pipette and placed in an incubator at 37ºC at 5% CO_2 for 5 days.

After 5 days samples were media changed. This was repeated every 4-5 days until up to six media changes had occurred. Samples were then expanded into T25cm flasks containing 5mls of warmed RPMI 1640 20% FCS. 100µl of sample was removed for analysis using the benchtop flow cytometer. Over the course of the next 2-3weeks, cultures were expanded to a volume of 50mls. Aliquots of the cell pellet were then frozen in media and 10% DMSO in a rate controlled freezer.

Benchtop flow cytometry is a key tool in achieving high transformation success rates from frozen whole blood

During ECACC's standard transformation procedure of PBLs cells can be visualised in the bottom of the transformation vessel because of the clean nature of the starting material. Cell proliferation, an indication of successful transformation, can easily be monitored. After about 3 media changes the culture is ready to be expanded into culture flasks. Once in culture flasks the transformation rarely fails so it is recognised as a key stage in the process.

However, during development of the whole blood transformation procedure it was evident with blood samples that visual or microscopic analysis to monitor successful transformation was difficult due to the presence of glutinous blood debris. This meant that making the important decision 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 with the small volume blood samples in early studies.

This problem was resolved with the use of benchtop flow cytometry. 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 on a graph. Live cell events are shown on the left hand side of the viability discriminator (diagonal red line on the graph below) and dead cell events on the right hand side of the line. Figure 1 below, shows a typical cytometric analysis of the kind described for a resuscitated PBL.

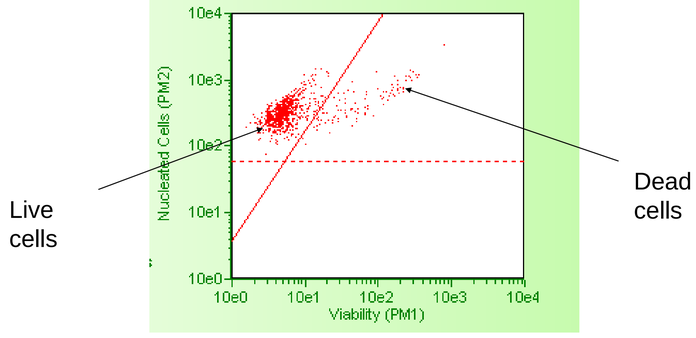

Figure 1. Cytometric analysis of a PBL sample after resuscitation. This sample is showing 90% viable lymphocytes, that is 90% of the events are to the left of the viability discriminator. The small smear of events in between the live and dead cell populations represent dying or apoptotic cells.

When a resuscitated blood sample was analysed using this method the resulting graph was very different to that for a resuscitated PBL. This is shown in Figure 2, below.

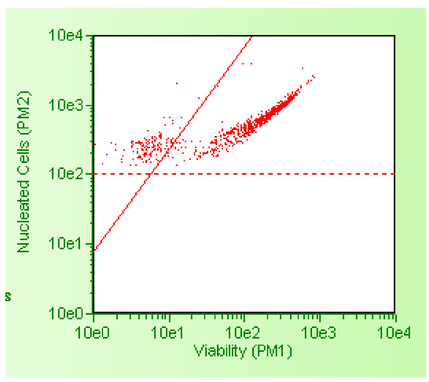

Figure 2. Cytometric analysis of a frozen blood sample after resuscitation.

In the second graph representing resuscitated blood there are many more events to the right of the viability discriminator showing there are lots more dead cells in this sample which displays only about 30% viable lymphocytes. This is probably a result of the un-optimised cryopreservation procedure.

Figure 3, below, 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. Successful transformation would be backed up by microscopic analysis after expansion into culture flasks .

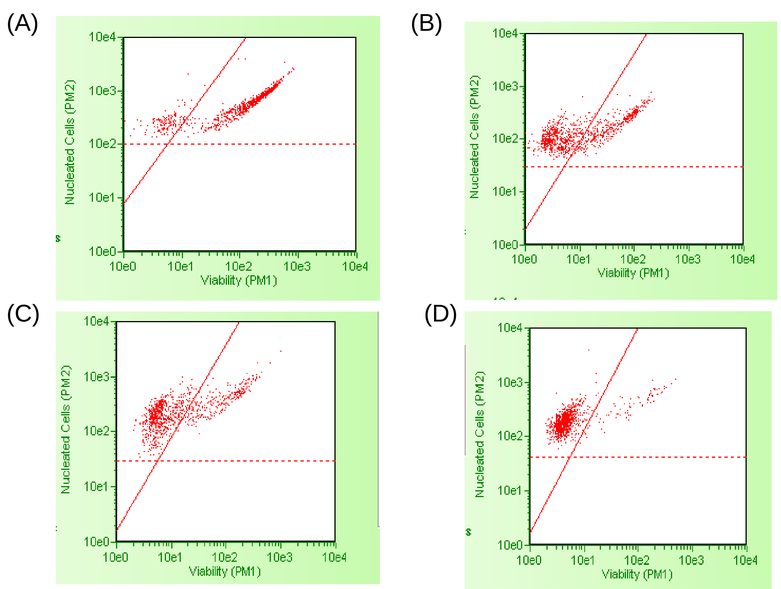

Figure 3. Cytometric analyses of the transformation of a frozen whole blood sample. Graph (A) immediately after resuscitation. Graph (B) after three weeks in the initial transformation vessel. Graph (C) after five weeks just prior to expansion to culture flasks. Graph (D) transformed cell line prior to freezing.

It is important to note that for the first five weeks of the transformation process (graphs A to C) microscopic analysis to determine successful transformation, and therefore to determine when to expand the cultures to culture flasks , was very difficult and thus the only indicator of successful transformation and lymphocyte proliferation was this cytometric analysis. In the absence of this data, the operator would be expanding the cultures on a 'best guess' approach resulting in lower transformation success rates

It is also important to note that not all samples would behave the same as the sample represented by the analyses in Figure 3. In fact about 30% of samples would show little signs of lymphocyte proliferation three weeks after the initiation of transformation represented by graph (B) in Figure 3. Cytometric analyses of a typical example of this kind is shown below in Figure 4. Experience in developing this protocol has shown that if a sample of this type is expanded into culture flasks then the culture usually fails to transform. Samples exhibiting this kind of result are therefore not expanded but are continued on the media change phase of the process and rechecked by cytometry . On the majority of occasions such samples subsequently transform resulting in high transformation success.

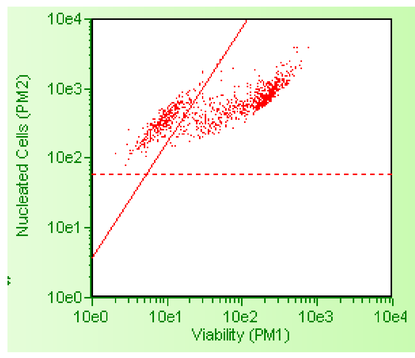

Figure 4. Cytometric analysis of a blood sample three weeks after the initiation of transformation. This graph shows less evidence of lymphocyte proliferation than graph (B) in Figure 3 and many more dead cells still present. Experience shows this sample would not be successful if expanded into flasks at this stage.

Results

The method described above, utilising benchtop flow cytometry to provide visibility to parts of the blood transformation process where microscopic analysis is difficult, allows the operator to make the important decisions of when to expand the cell cultures to culture flasks resulting in high transformation success rates. Without this analysis the process would result in lower, less reproducible transformation success rates.

Using this strategy, four studies were carried out on separate sets of four year old cryopreserved blood samples. The objective of studies 1 and 2 was to compare the success rates of transformations initiated in 24 well plates compared to transformation tubes. The results in Table 1 show a higher success in tubes principally because it was much easier to clear the blood debris and media change the samples in this format. Study 3 was a repeat of study 2 to assess how reproducible the technique was. Table 1 shows high transformation success rates in both cases. Finally, the objective of study 4 was to establish if the volume of blood could be reduced to 800µl. The results in Table 1 show a 100% transformation success rate for this volume.

study	Number of samples	Blood volume	Transformation vessel	Transformation success rate
Control PBL's	20	n/a	Tube	100%
1	20	1.5ml	Plate	70%
2	20	1.5ml	Tube	100%
3	20	1.5ml	Tube	95%
4	20	800µl	Tube	100%

Table 1. Summary of results of four separate studies to establish the transformation success rate of the whole blood transformation procedure. Parameters established in these studies were the best transformation vessel, the reproducibility of the technique and the fact that blood volumes could be reduced to 800µl.

Conclusions

(1) Small quantities of cryopreserved blood (800µl) can be directly transformed, without the need to separate PBLs, with transformation success rates comparable to those achieved with PBLs (over 95% 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 ECACC's standard PBL transformation process it is amenable to 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.