Capacity of robot handling for Epstein-Barr virus transformation

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Abstract

Objectives: Epstein-Barr virus (EBV) transformation has been described as a routine method to establish human B lymphoblastoid cell lines. Each established lymphoblastoid cell line represents one unique genetic information carrier and can produce unlimited quantities of DNA materials available for downstream applications and research. Undoubtedly, it is of great value to human clinical and experimental genetic studies. However, the current process of EBV transformation requires much manpower in the routine renewal of medium, which is time-consuming. This situation can become a serious problem especially when establishing a human B lymphoblastoid cell bank. A modified and cost-effective protocol for EBV transformation should be considered. **Materials and methods:** In the present study, process in EBV transformation was modified to fit the requirements of robot handling.

Results: 1 mL of whole blood was demonstrated to be sufficient to perform EBV transformation. Additionally, EBV transformation can be performed in 96-deep-well plates that are directly and widely used with automatic work platforms.

Conclusions: Based on these facts, a process of EBV transformation can be modified to fit the requirements of robot handling.

Introduction

It is well accepted that establishment of B lymphocyte cell lines can produce unlimited quantities of DNA materials available for downstream applications and research, which would be of great value to human clinical and experimental genetic studies (1). Because of this, several international cell banks, such as the Bioresources Collection and Research Center, European Collection of Animal Cell Cultures, American Type Culture Collection, and Coriell Institute for Medical Research, have employed Epstein-Barr virus

(EBV) transformation to establish human B lymphoblast cultures. For the purpose of rapidly establishing human B lymphoblast banks, the quantities of samples which need to be processed are great.

In this situation, avoiding unnecessary costs of materials, labour, and processing times is important. The scale of EBV transformation currently employed in the laboratory is so small that it cannot meet the requirements of human B lymphoblast banks. Without modifications to the current process of EBV transformation, much manpower will be needed to perform the routine renewal of media to establish human B lymphoblast banks, which is time-consuming. This serious problem has not been addressed in previous studies (2–5). In addition, in previous studies (2–5), at least 5 mL of collected blood was required to perform EBV transformation. It should be noted that the physiological state of collected blood, which is determined by biochemical assays or a haematology test, might be important reference data needed to establish human B lymphoblastoid cell banks. This means that the use of collected blood should be more economical and efficient. Taking all of these situations mentioned above together, a modified and more-efficient process is necessary for EBV transformation when it is employed to deal with a great number of samples such as required to establish a human B lymphoblastoid cell bank. The minimum required blood volume and development of robot handling compatibility for EBV transformation are two important topics for modifying EBV transformation. In the present study, it was found that numbers of mononuclear cells isolated from 1 mL of whole blood were sufficient to use as starter material for EBV transformation. In addition to this, the present study shows that EBV transformation can be carried out using microplates. This implies that an automatic system can be applied to the process of EBV transformation, especially in the routine renewal of medium. Based on this, a more-efficient process of EBV transformation with robot handling compatibility is presented.

Materials and methods

Isolation of lymphocytes

Blood was diluted with an equal volume of phosphate-buffered saline. Two millilitres of phosphate-buffered saline-diluted

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blood was carefully layered onto 1.5 mL of Ficoll plaque for density gradient centrifugation at 620 *g* for 30 min. After centrifugation, the interface band (buffy coat layer), enriched with lymphocytes was transferred to a new clean tube. The enriched lymphocytes were then re-suspended in 10 mL wash medium (RPMI 1640 with 2% (v/v) foetal bovine serum). After centrifugation (270 *g*, 10 min), discard supernatant. Repeat wash step one more time. Then, the resultant cell pellet immediately underwent EBV transformation.

Preparation of EBV

The B95-8 EBV-transformed marmoset cell line (the Bioresources Collection and Research Center 60199) was the source of EBV. B95-8 cells were maintained in Iscove's modified Dulbecco's media with 10% foetal bovine serum and incubated at 37 °C in a 5% CO₂ atmosphere. Before harvesting the EBV, the density of the B95-8 cell culture had to reach $(1-2) \times 10^6$ viable cells/mL and also had to be incubated for 10–12 days without the medium being renewed. Then, the yellowish medium, enriched with EBV, was collected and centrifuged at 270 *g* for 10 min to remove cell or debris. Then, the supernatant was sterilized by 0.2- μ m filtration. The filtered supernatant was split into aliquots and stored at –80 °C.

Cell number counting and cell viability determination

In the present study, haemocytometer was employed to count cell number and cell viability was determined by trypan blue dye exclusion.

Epstein-Barr virus transformation

Epstein-Barr virus transformation was performed based on our previous study (6). Briefly, the cell pellet containing lymphocytes (cell number is in the range of 10^6 – 10^7 cells) was infected with 1 mL EBV at 37 °C for 1 h. Then, the sample was centrifuged at 270 *g* for 10 min to remove the EBV. The resultant lymphocyte pellet was re-suspension and added to each well of a microplate, and incubated at 37 °C in a 5% CO₂ atmosphere. The medium was renewed twice weekly with fresh culture medium. During the period of incubation, signs of an exponential increase in cell numbers, clumping of cells, and production of a yellowish (acidic) medium were noted to determine whether immortalization had occurred.

Amplification of EBV-infected B lymphocytes in the microplates

In previous study (7), microplates were employed to perform B lymphocyte infection with the EBV. However,

application of microplate in EBV transformation was not mentioned in studies of lymphoblastoid cell lines establishment (4–6,8–10). Thus, it is worthy to examine whether microplates could be employed or not in EBV transformation to establish lymphoblastoid cell lines (LCLs). In this experiment, the infected lymphocytes were re-suspended in 1 mL of culture medium (RPMI 1640 with 20% (v/v) foetal bovine serum) in the presence of 1% phytohemagglutinin (PHA). The resultant lymphocytes were seeded (0.5×10^6 cells/mL) in 96-well (0.2 mL/well) and 96-deep-well microplates (1 mL/well). The cell number and viability of EBV-infected B lymphocytes were determined after 3-week cultivation. In this way, application of microplate in EBV transformation could be illustrated.

Effects of PHA on growth of EBV-infected B lymphocytes in deep-well microplates

It has well accepted that growth of LCLs could be enhanced by mitogen such as PHA (4,11,12) and utilization of PHA in EBV transformation has been as standard EBV transformation protocol (12). Dosages of PHA that had been employed in EBV transformation were varied (10% PHA in Beck *et al.* (4); 1% PHA in Chang *et al.* (6)). In purpose of material saving, EBV transformation in this experiment was performed in the presence or absence of 1% PHA. Thus, infected lymphocytes were re-suspended in 1 mL of culture medium (RPMI 1640 with 20% (v/v) foetal bovine serum) in the presence or absence of 1% PHA. The former and latter ones were, respectively, denoted the PHA and non-PHA groups, and both were seeded in 96-deep-well microplates. The cell number and viability of EBV-infected B lymphocytes were determined weekly during the 3-week cultivation.

Amplification of EBV-infected B lymphocytes from deep-well microplates to flasks

Amplification of EBV-infected B lymphocytes is an important topic in EBV transformation. Only EBV-infected B lymphocytes with well amplification capacity could be as sources of genetic material which is the ultimate end of EBV transformation. The aim of this experiment is try to explore the effects of different transformed periods on amplification capacity of EBV-infected B lymphocytes. Transformed periods were defined as 1–3 weeks of cultivation after EBV infection based on our experiences (6). Cell number was as a parameter of amplification capacity. Therefore, EBV-infected B lymphocytes which had been, respectively, cultured in 96-deep-well microplates for 1, 2 and 3 weeks, were transferred to 25-cm² flasks, and cultivated for another 3 weeks. These three groups were denoted groups D-1,

Collected blood volume (mL)	Isolated lymphocyte		
	Total numbers of viable cell ($\times 10^6$)	Viability (%)	Recovery rate (%)
9	11.8 ± 0.7 (11)*	96.3 ± 0.5 (11)	20.5 ± 1.2 (11)
1	1.4 ± 0.1 (11)	97.7 ± 0.1 (11)	23.3 ± 2.4 (11)

Values are presented as the mean $\pm$ SE (n). An asterisk (*) indicates a significant difference between two isolations (Student's t -test, $P < 0.05$).

The recovery rate was calculated from the ratio of total numbers of viable isolated lymphocyte cells to total numbers of white blood cells. Total numbers of white blood cell in 9 and 1 mL of collected blood were 59.3 ± 4.5 (11) and 6.4 ± 0.5 (11), respectively.

D-2, and D-3, respectively. Cell numbers of EBV-infected B lymphocytes in these groups were determined weekly.

Statistical analysis

All values are presented as the mean $\pm$ standard error (n). Significant differences of major factors among groups with different culture times were assessed by Student's t -test and one-way ANOVA ($P < 0.05$).

Results

Numbers of lymphocytes isolated from whole blood

Isolation of lymphocyte cells was initiated with 9 and 1 mL of collected blood (Table 1). Except for total numbers of isolated cells, there were no significant differences in the viability or recovery rate between the two preparations.

Amplification of EBV-infected B lymphocytes in the microplates

In the present experiment, 96-well microplates (0.2 mL/well) and 96-deep-well microplates (1 mL/well) were both applied for EBV transformation, and the optimal microplate for EBV transformation was evaluated. After 3 weeks of culture, total cell of each well in the 96-deep-well microplate group and 96-well microplate group were $(1.6 \pm 0.1) \times 10^6$ ($n = 14$) and $(1.2 \pm 0.0) \times 10^5$ cells ($n = 11$), respectively. The multiple of increase in total cell numbers in the 96-deep-well microplate group was 2.6 ± 0.4 -fold ($n = 14$) which was significantly greater ($P = 0.00608$) than that in the 96-well microplate group (1.2 ± 0.1 , $n = 11$). Viabilities of the two manipulations were $95.3 \pm 0.7\%$ (96-deep-well microplate group) and $99.0 \pm 0.0\%$ (96-well microplate group). The efficiencies of EBV transformation in the two manipulations were 92.3% for the 96-deep-well microplate group and 54.8% for the 96-well microplate group.

Table 1. Isolation of lymphocyte cells from different volumes of collected whole blood

Figure 1. Effects of phytohemagglutinin (PHA) on the growth of Epstein-Barr virus (EBV)-infected B lymphocytes in deep-well microplates.

Based on the above data, it was suggested that the 96-deep-well microplate was more suitable than the 96-well microplate for EBV transformation.

Effects of PHA on the growth of EBV-infected B lymphocytes in deep-well microplates

Initially, a 1-mL cell suspension [$(0.47 \pm 0.00) \times 10^6$ cells] was seeded in each well of the 96-deep-well microplates. The cell number and viability were determined weekly. The level of cell numbers of the PHA group dramatically increased about 3-fold over the initial level after 7 days of cultivation. It remained at a constant level during subsequent periods of cultivation (Fig. 1). On the contrary, the cell number of the non-PHA group did not increase with cultivation time. With regard to cell viabilities, the PHA group maintained a level of about 97% without a significant difference (7-day cultivation: $97.57 \pm 0.11\%$; 14-day cultivation: $97.47 \pm 0.07\%$; 21-day cultivation: $97.47 \pm 0.07\%$). Cell viabilities of the non-PHA group showed levels of 76–87%.

Table 2. Comparison of initial total number and cell viability of seeded lymphocyte cells in groups with different deep-well cultivation times for expansion in flasks

Groups with different deep-well cultivation times	Seeded lymphocytes	
	Total numbers of viable cells ($\times 10^6$)	Viability (%)
D-1	1.1 ± 0.1 (25) ^a	87.3 ± 3.0 (25) ^a
D-2	1.4 ± 0.1 (11) ^a	91.1 ± 3.0 (11) ^a
D-3	1.5 ± 0.1 (23) ^a	91.6 ± 1.0 (23) ^a

Values are presented as the mean $\pm$ SE (*n*). The superscript letters indicate that there was significant difference in total numbers of viable cells or viability among groups with different cultivation times in deep-well plates (one-way ANOVA, $P < 0.05$).

Figure 2. Growth of Epstein-Barr virus (EBV)-infected lymphocytes with different cultivation periods.

Amplification of EBV-infected B lymphocytes from deep-well microplates to flasks

At the beginning of cultivation in flasks, there were no significant differences in total seeded cells or viabilities (Table 2). The total number of EBV-infected B lymphocytes in group D-1 increased 2-fold over the initial level after the 21-day cultivation in the flask. In the meantime, the increase in group D-2 reached 7-fold above the initial level (Fig. 2). After the 14-day cultivation in flasks, the total cell number in group D-3 had dramatically increased, to about 6-fold higher than that of the initial level. At the end of cultivation in the flasks, it was found that the increase in the total cell number was about 10-fold that at the beginning of cultivation.

Discussion

The present study shows that the blood volume required for EBV transformation can be decreased to 1 mL, which is less than that required in the current protocol of EBV

transformation. This indicates that more collected blood can be saved, and other necessary biochemical assays or haematology tests for establishing human B lymphoblastoid cell banks can be performed or the blood can be applied to other interesting research projects. In addition, it confirms that EBV transformation can be performed in 96-deep-well microplates that are directly and widely used with automatic work platforms. This means that the process of EBV transformation can be modified to fit the requirements of robot handling. Based on these two lines of data, a modified process of EBV transformation, which is more efficient and has robot handling compatibility, is proposed.

In the current protocol of EBV transformation (2–6), the volume of whole blood required for EBV transformation is reported to be at least about 5 mL. It can be expected that there will be a significant difference in total isolated lymphocytes when making comparisons between 1- and 5-mL collected blood samples (Table 1). However, it should be emphasized that the amount of isolated lymphocytes not the total volume of collected blood is critical for EBV transformation. In our preliminary study, isolated lymphocytes (at a cell density of 10^5 cells/mL) were used as the starting materials to perform EBV transformation, which displayed signs of immortalization such as clumping of cells and production of a yellowish (acidic) medium after 96 h of culture. It was suggested that 10^5 isolated lymphocyte cells could be used as starting materials for EBV transformation. In the present study, it was found that 1 mL of collected blood contains about 1.4×10^6 lymphocyte cells (Table 1) which is greater than that what we used in the preliminary study. In addition, it was demonstrated that there were no significant differences in the viability or recovery rate between the two preparations that were started with different blood volumes (Table 1). This implies the possibility that a 1-mL blood sample of lymphocyte cells is sufficient for the initial EBV transformation. In a previous study (7), 96-well microplates were employed to perform B lymphocyte infection with the EBV. Although application of microplates in EBV transformation was not the topic of Maruo *et al.*'s study, it revealed that microplates could possibly be applied for EBV transformation. Thus, 96-well microplates and 96-deep-well microplates were selected to perform EBV transformation which was initiated with 1-mL blood sample of isolated lymphocytes. Interestingly, in the 96-deep-well microplate group, increased multiples of total cell number and efficiency of EBV transformation were higher than those in the 96-well microplate group. The reason for this phenomenon was not addressed. We suggest that the greater amount of space in the deep-well microplate might be one factor contributing to this phenomenon. This indicates that cultivation in 96-deep-well

microplates might be the way to expand EBV-infected B lymphocytes. Because microplates are widely applied in lots of automated equipment, it can be expected that the present EBV transformation process would have the capacity for robot handling.

In our previous study (6) and several other studies (4,11,12), it was accepted that mitogen stimulation such as PHA can enhance the growth of LCLs during EBV transformation. Therefore, it was necessary to determine effects of the presence and absence of PHA in the culture medium in deep-well microplate cultivation of EBV transformation. Based on our data, it was found that PHA was necessary for growth and cell viabilities of EBV-infected B lymphocytes (Fig. 1).

The advantage of a human B lymphoblastoid cell bank is unlimited expansion of each established LCL, which can be a source of DNA materials for downstream applications and research. However, in practice, it is impossible to expand all established LCLs at one time and deposit them, as that would require a lot of manpower and deposit facilities. To conserve manpower and materials, two factors should be considered: selection standards with high efficiency of EBV transformation and the expansion capacity of established LCLs. The former was shown in our previous study (6). In the present study, we found that EBV-infected B lymphocytes with different cultivation times displayed distinctive expansion capacities (Fig. 2), even though the initial total cell numbers were at the same level (Table 2). It is worth exploring the molecular mechanisms behind this phenomenon.

The data presented here first show that lymphocytes isolated from 1 mL of collected blood can be used as starting materials for EBV transformation. In this way, it can be expected that more collected blood can be saved and applied to other interesting research. We not only developed a process of EBV transformation which meets the requirements of robot handling by employing microplates, but also found that the expansion capacity of EBV-infected B lymphocytes is dependent on the cultivation period.

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References

- 1 Heidemarie N (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum. Genet* **73**, 320–326.
- 2 Doyle A, Griffiths JB, Newell DG (1996) *Cell & Tissue Culture: Laboratory Procedures*. New York: John Wiley & Sons Inc.
- 3 Biddison WE (1998) Preparation and culture of human lymphocytes. *Curr. Protoc. Cell Biol.* **Chapter 2**, Unit 2.1–2.13.
- 4 Beck JC, Beiswanger CM, Esther MJ, Satariano E, West D (2001) Successful transformation of cryopreserved lymphocytes: a resource for epidemiological studies. *Cancer Epidemiol. Biomarkers Prev.* **10**, 551–554.
- 5 Oh HM, Oh JM, Choi SC, Kim SW, Han WC, Kim TH *et al.* (2003) An efficient method for the rapid establishment of Epstein-Barr virus immortalization of human B lymphocytes. *Cell Prolif.* **36**, 191–197.
- 6 Chang IC, Wu JY, Lu HI, Ko HW, Kuo JL, Wang CY *et al.* (2006) High potentiality-preliminary selection criteria and transformation time dependent factors analysis for establishing Epstein-Barr virus transformed human lymphoblastoid cell lines. *Cell Prolif.* **39**, 457–469.
- 7 Maruo S, Johannsen E, Illanes D, Cooper A, Kieff E (2003) Epstein-Barr virus nuclear protein EBNA3A is critical for maintaining lymphoblastoid cell line growth. *J. Virol.* **77**, 10437–10447.
- 8 Tremblay S, Khandjian EW (1998) Successful use of long-term frozen lymphocytes for the establishment of lymphoblastoid cell lines. *Clin. Biochem* **31**, 555–556.
- 9 Kilger E, Kieser A, Baumann M, Hammerschmidt W (1998) Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. *EMBO J.* **17**, 1700–1709.
- 10 Hayes RB, Smith CO, Huang WY, Read Y, Kopp WC (2002) Whole blood cryopreservation in epidemiological studies. *Cancer Epidemiol. Biomarkers Prev.* **11**, 1496–1498.
- 11 Penno MB, Pedrotti-Krueger M, Ray T (1993) Cryopreservation of whole blood and isolated lymphocytes for B-cell transformation. *J. Tissue Cult. Methods* **15**, 43–48.
- 12 Borrebaeck CAK, Ohlin M (1996) *In vitro* immunization of peripheral lymphocytes. In: Doyle A, Griffiths JB, Newell DG, eds. *Cell and Tissue Culture: Laboratory Procedures*, p. 25E:1. New York: John Wiley & Sons Inc.