ORIGINAL ARTICLE

Optimising Transfection Strategies in PBMCs: A Comparative Study of Lipofection and Electroporation for Single and Multi-plasmids

Nahil Hassan Mahmoud Ahmed[1], Ling King Hwa[2], Lai Mei I[1]

[1] Haematology Unit, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2] Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

ABSTRACT

Introduction: Transfection involves the introduction of exogenous nucleic acids into cells to modify their genomic content. Each blood cell type has specific requirements for effective transfection, making uniform transfection across all cell types potentially challenging. This study investigated lipofection-mediated transfection and electroporation techniques for plasmid delivery to identify an effective and cost-efficient method for transfecting peripheral blood mononuclear cells (PBMCs). **Methods:** This study utilised three plasmids: EGFP, ABE8, and sgRNA. PBMCs were isolated from human blood and cultured. Transfection of PBMCs was performed using the two above-mentioned methods. Following transfection, the cells were cultured and analysed to assess the efficiency of gene delivery and protein expression. **Results and Conclusion**: Electroporation demonstrated a higher transfection efficiency compared to lipofection reagents, achieving 17.81±7.20% versus 5.18±5.93%, respectively. Furthermore, the co-transfection of PBMCs with three plasmids yielded lower but statistically insignificant efficiency to transfection with a single plasmid. Notably, $CD34^+$ HSPCs exhibited a higher transfection efficiency compared to T cells, with rates of 26.39±3.47% versus 4.73±2.28%, respectively. Although electroporation is a viable method for introducing plasmid DNA into primary blood cells, a major drawback is its potential for causing substantial cell cytotoxicity. This study provides a practical and cost-effective approach for co-transfection PBMCs, $CD34^+$ HSPCs, and T cells with multiple plasmids. However, future research with larger cohorts is necessary to further optimise the protocol and overcome its limitations.
Malaysian Journal of Medicine and Health Sciences (2025) 21(SUPP12): 11-20.doi:10.47836/mjmhs.21.s12.3

Keywords: Transfection, PBMCs, $CD34^+$HSPCs, Electroporation, Lipofectamine

Corresponding Author:
Lai Mei I, PhD
Email: lmi@upm.edu.my
Tel: +60 3-9769 2494

INTRODUCTION

Transfection is the process of inserting exogenous nucleic acids into a eukaryotic cell in order to change its genome. Transfection can be classified into two categories: stable and transient transfection. Stable transfection refers to the establishment of persistent expression of a transgene, achieved either through the integration of exogenous DNA into the host cell's chromosomal genome or by retaining an episomal vector as an extra-chromosomal element within the host nucleus. Stable transfection allows for continuous transgene expression, even during cellular replication. Meanwhile, transient transfection involves temporarily introducing nucleic acids into the recipient cell without integration into the genome. Consequently, transgene expression diminishes over time as host cells undergo replication. A vector construct designed to deliver specific nucleic acids for transfection can be categorised into viral or plasmid vectors. Both types of vectors enable the expression of a foreign transgene by incorporating an appropriate eukaryotic promoter. Viral vectors, however, have the potential to induce an immunogenic response in the host cells, whereas plasmid vectors generally exhibit a lower immunogenic profile (1).

Various chemical and physical techniques have been employed to deliver DNA expression vectors into mammalian cells. Delivery vehicles, such as lipid-based

methods, are employed to facilitate the interaction between the vector-vehicle complex and the host cell membrane. This interaction enhances the uptake of the complex into cells (2). Lipofection employs liposomes, which are small vesicular structures that share the same composition as the cell membrane. DNA sequences can be encapsulated within liposomes. Depending on the specific properties of the liposome and the type of cell involved, the liposome can either undergo endocytosis or fuse directly with the cell membrane, facilitating the delivery of the DNA construct into the cells (3). The transfer of genes into cells by cationic liposome/DNA complexes has gained a lot of traction because of its versatility, low toxicity, and relative efficacy *in vitro* (2). Electroporation is a physical technique that utilises an electric pulse to facilitate the transfection of cells with DNA. The application of an electric field to cells causes transient pores in the cell membrane, thereby facilitating the taking up of DNA molecules. After applying the electric field, the cell membrane undergoes resealing, thereby retaining some of the DNA introduced during electroporation within the cell. Electroporation offers several advantages, including its versatility in transfecting different cell types, including primary cells, across various *in vivo* and *in vitro* environments (3).

Peripheral blood mononuclear cells (PBMCs) are nucleated blood cells including lymphocytes (T, B, and NK cells), monocytes, dendritic cells, and haematopoietic stem and progenitor cells (HSPC) (4). The majority of these cells play a crucial role in the immune system's defence against pathogens and adaptation to foreign agents. Cultivation of PBMCs *in vitro* has emerged as indispensable for studying immunological responses, including cytokine production upon exposure to specific antigens (5).

As PBMCs do not divide or divide slowly in normal conditions, transfection can be extremely difficult Since different cell types have distinct needs for transfection, achieving consistent transfection efficiency across all cell types can be a complex task. Various approaches have been used to overcome this problem. Majumdar et al. demonstrated successful transfection of PBMCs using the BLOCK-iT™ RNAi expression system. They found that incorporating low-speed centrifugation significantly enhanced transfection efficiency (6). Polyethylenimine (PEI) was shown to be an effective transfection agent for delivering genes to human PBMC when conjugated with an anti-CD3 antibody (7). Using an RNA electroporation technique has demonstrated increased transfection efficacy while reducing or eliminating associated toxicity. The study highlighted mRNA electroporation as a proficient approach to introducing genes into PBMCs (8).

An alternative approach for transfecting PBMCs involved pre-activating T-lymphocytes with human anti-CD3 and anti-CD28 antibodies. Activated T cells enhanced transfection efficiency when co-transfected with mRNA encoding ABE (Adenine Base Editor) and single guide RNA (sgRNA) using electroporation. Following transfection, activated T cells could be cultured for several days with human interleukin2 (IL2), allowing for subsequent evaluation of gene editing efficacy (9, 10, 11).

Plasmid co-transfection refers to the simultaneous introduction of multiple plasmids into a cell. This technique is particularly valuable for the co-expression of multiple genes and is widely employed in gene editing and repair applications, such as CRISPR/Cas9 and ABE, where coordinated expression of the Cas9 enzyme and sgRNA is required (9, 10).

This study employed lipofection-mediated transfection and electroporation techniques for plasmid delivery to identify an effective and cost-efficient method for transfecting PBMCs. Additionally, this study aimed to develop an optimised protocol for the concurrent transfection of $CD34^{+}$HSPCs and T cells with ABE, sgRNA, and EGFP (Enhanced Green Fluorescent Protein) plasmids.

MATERIALS AND METHODS

Plasmid DNA preparation and extraction

This study utilised the following plasmids: an EGFP plasmid pRP[Exp]-Neo-CMV>EGFP was made by VectorBuilder (vector ID: VB210516-1024ddy), ABE8 plasmid, NG-ABE8.20-d, which was a gift from Nicole Gaudelli (Addgene plasmid # 136301; http://n2t.net/addgene:136301; RRID: Addgene_136301), and a sgRNA plasmid, pU6-sp-sgRNA-RNF2_+41nick mammalian expression vector (was a gift from David Liu (Addgene plasmid #135958; http://n2t.net/addgene:135958; RRID: Addgene_135958)). According to the manufacturer's instructions, Plasmids DNA was isolated using the ZymoPURE Plasmid Maxiprep (Zymo Research, USA) from 150-300 mL of LB broth cell culture (Lennox, Sigma-Aldrich, USA). The concentration of plasmid DNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), then visualised post-agarose gel electrophoresis.

PBMC isolation

This study was approved by Medical Research and Ethics Committee, Ministry of Health Malaysia (NMRR-19-4169-51907). This pilot study represents a preliminary exploratory laboratory investigation; therefore, the availability of resources and donors influenced the sample size. Following the acquisition of written informed consent, 20 mL of heparinised venous blood was collected from ten donors. The blood was diluted 1:1 with 1X phosphate-buffered saline (PBS, GibcoTM, USA) and then carefully layered on top of a Ficoll-Paque Plus (Sigma-Aldrich, USA) in a ratio of 2:1. With the

brake-off, the tubes were centrifuged in a swing bucket centrifuge (Eppendorf, Centrifuge 5702) for 30 minutes at 1100 x g. The PBMC layer was gently removed from the plasma-Ficoll interface and transferred to a 15mL tube before being washed twice with 1X PBS at 500 x g for ten minutes and 300 x g for five minutes. The supernatant was removed, and the cells were suspended in 1mL 1X PBS. To determine the total number of PBMCs, a small aliquot was taken out, and PBMC viability and number were assessed employing a haemocytometer (Neubauer chamber) and Trypan blue dye (Sigma-Aldrich) under a phase contrast microscope (Olympus CX31, Germany), wherein the nucleus of dead cells is stained blue.

CD34⁺ HSPC cells isolation and expansion

According to manufacturer instructions, CD34⁺HSPC cells were isolated from freshly isolated PBMC using EasySepTM Human CD34 Positive Selection Kit II (STEMCELLTM technologies, Canada). Subsequently, CD34⁺HSPC cells were cultured in StemSpan™ Serum-Free Expansion Medium II (SFEM II) media supplemented with StemSpan™ CD34+ Expansion Supplement (10X) (STEMCELLTM technologies) for five to ten days. Supplemented StemSpan™ SFEM II media was added every other date to keep the cell concentration < 1 x 10^5 cell/mL.

T cells activation

A subset of PBMCs was cultured with supplemented 1X RPMI 1640+GlutaMAX ((Roswell Park Memorial Institute 1640 Medium, Gibco™, USA) supplemented with 10% Foetal bovine serum (FBS, TICO EUROPE, Netherlands), and 1% Penicillin-Streptomycin (Pen-strep, Elabscience, China), and CD28 Monoclonal Antibody (eBioscience, Clone CD28.2) in a 6-well plate coated with CD3 Monoclonal Antibody (eBioscience, Clone OKT3). Cultures were maintained at 37°C in a 5% CO2 incubator for three days to induce activation of the T cells.

PBMC culture

PBMC were cultured in supplemented 1X RPMI 1640 media, and Phytohemagglutinin-L (PHA-L) 500X (eBioscienceTM, USA) at 2 µL/mL at a concentration of 1 x 10^6 cell/mL for 24-hours. After 24-hours the cells were centrifuged at 500 x g for five minutes before proceeding with transfection.

HEK293T cell culture

HEK293T cells were cultured in DMEM (1X) (Dulbecco's Modified Eagle Medium, Gibco™) supplemented with 10% FBS and 1% pen-strep, 1% MEM Non-Essential Amino Acids Solution (100X) (Gibco™), and 1% Sodium Pyruvate (100 mM) (Gibco™).

Transfection using Lipofectamine reagents

PBMC cells at a density of 2 × 10^5 cells/500 µL were seeded onto a 24-well tissue culture plate in Opti MEM® (1X) (Gibco™) reduced serum medium and without antibiotics. Various amounts of Lipofectamine® 2000 (Thermo Fisher Scientific) were diluted in Opti MEM® (1X) medium (2,3,4,5µL/50µL). Finally, 5 µg of DNA (EGFP) was diluted in 250 µL of Opti MEM® (1X) medium and mixed gently at a 1:1 ratio with the diluted Lipofectamine® 2000 Reagent. The DNA-lipofectamine mix was incubated at room temperature (RT) for 20 minutes before being added dropwise to the cells. The cells were incubated at 37°C, 5% CO2, overnight. After incubation, the cells were harvested and centrifuged at 500 x g for five minutes and resuspended and cultured in supplemented 1X RPMI 1640 media. The cells were maintained for three days before the transfection efficiency was assessed.

For Lipofectamine® 3000, 2 × 10^5 cells were plated similarly. Two amounts of Lipofectamine™ 3000 were diluted in Opti MEM® (1X) Medium (0.75 and 1.5 µL/25 µL) and mixed. Then 1 µg of EGFP DNA was diluted in 50 µL Opti MEM® (1X) Medium, and 2 µL of P3000™ Reagent was added and mixed well. The diluted DNA was added to each tube of diluted Lipofectamine™ 3000 reagent (1:1 ratio). The DNA-lipofectamine mix was Incubated for 20 minutes at RT and was added dropwise to cells. The cells were maintained similarly to the Lipofectamine 2000 protocol, and transfection efficiency was assessed three days after transfection.

HEK293T cell transfection

HEK293T cells were seeded onto a 48-well polystyrene tissue culture plate at a density of 4 × 104 cells per well and transfected 24 hours after plating. Cells were transfected with Lipofectamine 2000 and Lipofectamine 3000, similarly to PBMC. 6 hours after transfection; the transfection media was replaced with supplemented DMEM. The transfection efficiency was assessed three days after transfection.

PBMC, CD34⁺ HSPC, and T cells electroporation

PBMC, and CD34⁺HSPC, and T cells were suspended in 1 mL of Opti-MEM medium (EP buffer) and centrifuged at 500 × g for five minutes. Cell numbers and viability were assessed using trypan blue and a haemocytometer. Meanwhile, supplemented culture media were added to 12 or 24-well plates (1mL/well) and incubated at 37°C for post-electroporation. For electroporation, 1 × 10^6 cells (5 × 10^6 for T cells) diluted in EP buffer were combined with 10 µg of plasmid DNA in a sterile 1.5 mL tube, resulting in a final volume of 100 µL. The cells and DNA were gently mixed without foaming and then transferred to a 2mm gap NEPA Electroporation cuvette (NepGene, Japan). The cuvette was gently tapped to briefly mix the cells without inducing foam. Per manufacturer instruction, the electroporation parameters were set, and the electroporation was carried out using the NEPA21 super electroporator (NepaGene) (Table I). Following electroporation, 300 µL of prewarmed culture media was added to the cells. The cells and media were thoroughly mixed within the cuvette before the sample

was transferred into prewarmed culture plates and placed in an incubator for 24-72 hours.

Table I: Settings for the NEPA21 Super Electroporator

The electroporation parameters employed for PBMCs, CD34+HSPCs, and T cells using the NEPA21 Super Electroporator were as follows:

PBMC electroporation parameters		
Parameter	Poring pulse	Transfer Pulse
Voltage (V)	275	20
Pulse length (ms)	2.5	50
Pulse interval (ms)	50	50
Number of pulses	2	5
Decay rate (%)	10	40
Polarity switching	+	+/-
CD34+ HSPCs& T cells electroporation parameters		
Parameter	Poring pulse	Transfer Pulse
Voltage (V)	250	20
Pulse length (ms)	2.5	50
Pulse interval (ms)	50	50
Number of pulses	2	5
Decay rate (%)	10	40
Polarity switching	+	+/-

Abbreviation; ms: milliseconds.

Determination of transfection efficiency using flow cytometry

Cells were assessed for transfection efficiency using flow cytometry. The cells were stained with 7-Amino-Actinomycin D (7-AAD) according to the manufacturer's instructions (BD Biosciences, USA) to exclude non-viable cells. Flow cytometric analysis was conducted using a BD FACSCanto™ II Flow Cytometer (BD Biosciences). Data acquisition involved recording ≥10,000 events, and analysis was performed using BD FACSDiva Software V6.1.2 (BD Biosciences). The evaluation included monitoring EGFP expression and 7-AAD staining on the dot plot of cells. Following data acquisition, a gating strategy was applied to ensure the selection of a homogeneous cell population. Initially, cells were gated from all detected events (including cells and debris) based on forward scatter (FSC) and side scatter (SSC) parameters, allowing for the exclusion of debris. Subsequently, single cells were identified within the "Cells" gate, with larger events indicative of cell aggregates excluded to define the "Single Cell" gate. From this population, viable cells were selected for transfection analysis using 7-AAD staining in an SSC versus 7-AAD dot plot. Transfection efficiency was then assessed by determining the proportion of EGFP-positive cells within the viable cell population (7-AAD versus EGFP dot plot).

Effect and toxicity of electroporation

Electroporation can cause transient or permanent damage to the cell membrane, possibly resulting in cell death. T cells were employed to assess the impact and toxicity of electroporation on primary blood cells when transfected simultaneously with three plasmids. Cell viability was evaluated at various time points (zero, three, six, and nine days) following electroporation using trypan blue exclusion dye to count the number of viable cells.

Statistical analysis

Pairwise comparisons were conducted using Student's t-test and ANOVA (two-way). $p \leq 0.05$ value was considered statistically significant for all tests. Data analysis was executed using GraphPad Prism (10.2.3). Measurement values were expressed as mean ± standard deviation (SD).

RESULTS

Lipofection-mediated transfection of PBMC and HEK293T cells

The transfection process utilised Lipofectamine 2000 reagent at four distinct concentrations (2 µL, 3 µL, 4 µL, and 5 µL) for transfecting PBMC and HEK293T cells. Three days post-transfection, cells were examined using an inverted fluorescent microscope (Olympus 1X51, Japan). HEK293T cells exhibited a high transfection rate (data not shown). However, subsequent flow cytometric analysis revealed a decreased transfection efficiency, particularly in cells transfected with Lipofectamine 3000. This reduction in observed transfection efficiency may be attributed to cell death occurring during the flow cytometry preparation process, leading to the exclusion of these dead cells in the flow cytometry analysis. In contrast, for PBMCs, the results obtained from the inverted fluorescent microscope and flow cytometer analysis were comparable (data not shown).

Following visualisation, cells were prepared and analysed with the flow cytometer. The percentage of EGFP-positive cells in PBMCs was 1.68±1.86%, 2.03±1.89%, 3.03±2.93%, and 5.18±5.93% for the 2 µL, 3 µL, 4 µL, and 5 µL concentrations, respectively. In contrast, the percentage of EGFP-positive cells in HEK293T cells was 34.15±12.69 %, 51.5±12.23 %, 43.68±4.41 %, and 42.23±11.52% for the corresponding concentrations. In both cells, lipofectamine 3000 was also evaluated; for 0.75µL and 1.5µL, respectively, the transfection efficiency in PBMC was 1.78±0.28% and 4.73±1.58%. Meanwhile, the percentages for HEK293T cells were 12.93±2.97 % and 11.9±1.84 % (mean ± SD, n=4), respectively (Fig. 1). HEK293T cells demonstrated significantly higher transfection efficiency compared to PBMCs when transfected using Lipofectamine 2000 reagent. Although transfection with Lipofectamine

3000 also increased efficiency in HEK293T cells, the difference was statistically insignificant compared to the transfection efficiency observed in PBMCs.

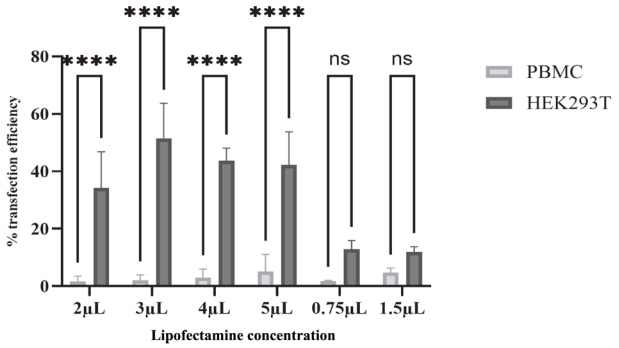


Fig. 1. PBMCs and HEK293T cells transfection using Lipofectamine reagents. The figure compares cell transfection efficiencies vs lipofectamine concentration. For PBMCs and HEK293T cells, Lipofectamine 2000 was used at concentrations of 2, 3, 4, and 5 µL, while Lipofectamine 3000 was used at concentrations of 0.75 and 1.5 µL. Transfection efficiency was evaluated using flow cytometry. HEK293T cells exhibited significantly higher transfection efficiency than PBMCs when transfected with Lipofectamine 2000. While using Lipofectamine 3000 increased transfection efficiency in HEK293T cells, the difference was not statistically significant compared to the efficiency observed in PBMCs. Values and error bars represent the mean ± SD (n=4, P<0.0001, two-way ANOVA with Šídák's multiple comparisons test).

PBMC co-transfection

PBMCs were subjected to electroporation with 10 µg of either EGFP plasmid alone or a combination of two plasmids (EGFP and ABE8) in a 1:1 molar ratio (MR). Alternatively, cells were co-transfected with ABE8, sgRNA, and EGFP plasmids in a 2:2:1 MR, respectively. Compared to un-transfected cells, which had a transfection percentage of 1.45±1.44%, the percentage of transfected cells was 17.81±7.20% for cells transfected with EGFP alone, 11.71±7.41% for cells transfected with both EGFP and ABE8 plasmids, and 8.5±7.59% for cells transfected with EGFP, ABE8, and sgRNA plasmids (mean ± SD, n=7) (Fig. 2). Interestingly, cells transfected with the EGFP plasmid exhibited higher transfection efficiency than those transfected with EGFP+ABE8 or EGFP+ABE8+sgRNA. However, this difference in efficiency was not statistically significant.

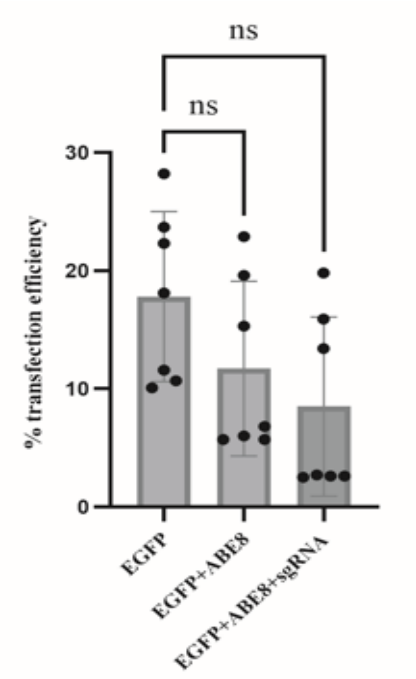


Fig. 2. PBMCs co-transfection using electroporation. The figure compares PBMC transfection efficiencies vs plasmid used for transfection. Cells were transfected with either the EGFP plasmid alone, a combination of two plasmids (EGFP and ABE8), or a combination of three plasmids (EGFP, ABE8, and sgRNA) simultaneously. Cells transfected with the EGFP plasmid exhibited higher transfection efficiency than those transfected with EGFP+ABE8 or EGFP+ABE8+sgRNA. However, this difference was not statistically significant. Values and error bars represent the mean ± SD (n=7, P value 0.0835,Kruskal-Wallis's with Dunn's).

ABE8, sgRNA, EGFP plasmids co-transfection in CD34⁺HSPC and T cells

CD34⁺HSPC and T cells were co-transfected with ABE8, sgRNA, and EGFP plasmids using electroporation in 2:2:1 MR, respectively. Notably, CD34⁺HSPCs exhibited a higher transfection efficiency, with a mean of 26.39±3.47%, compared to T cells, which had a mean transfection efficiency of 4.73±2.28% (mean ± SD, n=4) (Fig. 3).

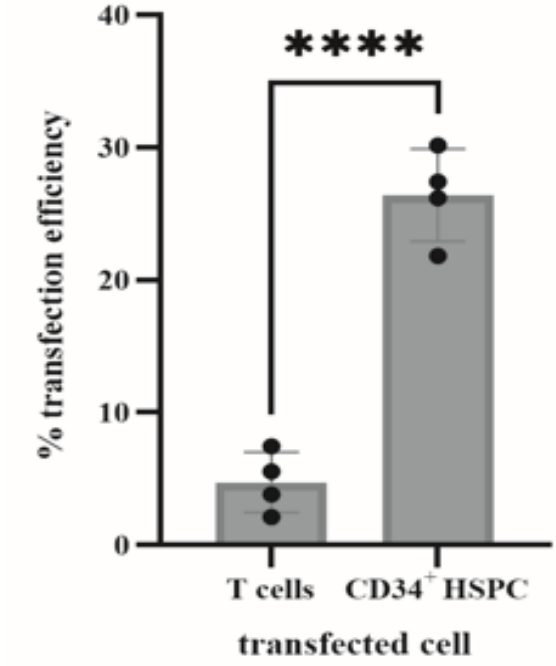
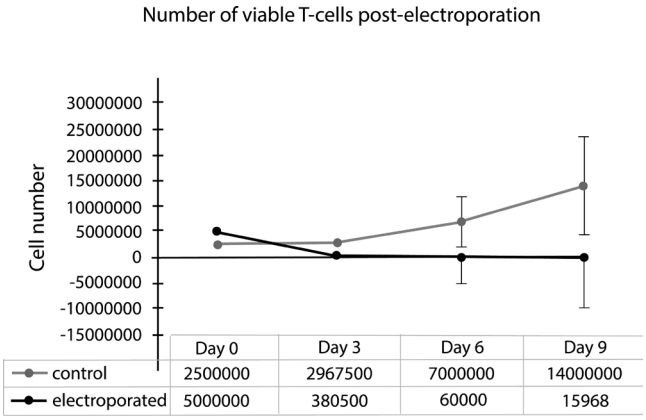


Fig. 3. CD34+ HSPC and T cells co-transfection. The figure compares transfection efficiencies vs the cell transfected. The cells were co-transfected with three plasmids (EGFP+ABE8+sgRNA). CD34+HSPCs demonstrated significantly higher transfection efficiency than T cells. Values and error bars represent mean ± SD (n=4, p<0.0001, unpaired t-test).

Effect and toxicity of electroporation

The number of viable T cells significantly declined following electroporation at various intervals. The manufacturer recommends using 5×10^6 cells per electroporation, and due to primary cell availability constraints, the un-transfected control (un-treated) was performed with 2.5×10^6 cells. At day 0, the initial cell count for un-transfected control samples was 2.5 × 106, whereas 5×10^6 T cells underwent electroporation. By day three post-electroporation, the un-transfected control cell count increased to $2.9675 \times 10^6 \pm 1.06 \times 10^4$, while electroporated cells were reduced to $3.8 \times 10^5 \pm 6.13 \times 10^4$. On day six, the un-transfected control cells further increased to $7 \times 10^6 \pm 4.94 \times 10^6$, whereas electroporated cells decreased to $6 \times 10^4 \pm 1.4 \times 10^4$. Finally, on day nine, the un-transfected control cell count rose to $14 \times 10^6 \pm 9.8 \times 10^6$, while the number of electroporated cells dropped to $1.59 \times 104 \pm 4.1 \times 10^3$ (mean ± SD n=4) (Fig. 4).



	Day 0	Day 3	Day 6	Day 9
control	2500000	2967500	7000000	14000000
electroporated	5000000	380500	60000	15968

Fig. 4. The number of viable electroporated T cells (black line) compared to un-transfected T cells (grey line). The number of viable T cells significantly decreased following electroporation at various time points (0, 3, 6, and 9 days). In contrast, the number of un-transfected T cells increased over the same intervals. Data are presented as the mean ± SD, n = 4.

DISCUSSION

The discovery of transfection has allowed for researchers to modify cellular functions, express specific genes, and study gene expression and regulation. Transfection has numerous applications, including gene therapy, vaccine development, protein production, and gene editing. By precisely controlling the introduction of genetic material, scientists can investigate cellular processes, develop novel therapeutics, and engineer cells for various purposes. Despite tremendous advancements, no strategy works equally well for every cell type or even for every cell within a specific type. Certain cell types present particular challenges; for instance, neuron cells due to their low rate of cell division, resting $CD4^+$ T-lymphocyte cells owing to their low metabolic activity, and $CD34^+$HSPCs due to a combination of defined and undefined cellular characteristics (12). Furthermore, transfecting suspension cells presents significant challenges, primarily attributed to reduced binding of the transfection complex — typically composed of the plasmid DNA and liposome-based reagents—to the cell surface, resulting in decreased uptake of the target DNA (13). These factors collectively contribute to the challenges of transfecting primary blood cells.

In this study, we investigated the transfection of PBMCs using Lipofectamine reagents, specifically Lipofectamine 2000 and 3000. Our results demonstrated that transfection with these lipofection reagents yielded low efficiency comparable to that observed in un-transfected controls. This finding is congruent with that of O'Neill et al., who reported that transfection of

human PBMCs with EGFP using Lipofectamine or DEAE-dextran was ineffective. Conversely, consistent gene expression was achieved through receptor-mediated gene delivery using anti-CD3 PEI conjugates, resulting in 56 ± 43% of cells expressing GFP with significant fluorescence intensity detectable for up to 72 hours (7). Okano et al. employed an mRNA lipofection technique on human PHA-stimulated lymphocytes, resulting in a transfection efficiency of up to 64.4 ± 30.2% EGFP-positive cells (14). In contrast, Majumdar et al. applied low-speed centrifugation to enhance the transfection of unstimulated PBMCs with scrambled siRNA using Lipofectamine 2000 reagent. This approach markedly improved transfection efficiency compared to conventional methods, achieving 99.8% versus 28.3% transfection (6).

The type of nucleic acids used for transfection (such as DNA or RNA) and the delivery method significantly influence the transfection outcome. While plasmid DNA is relatively easy to handle and cost-effective for transfecting eukaryotic cells compared to RNA, it generally exhibits lower transfection efficiency and reduced protein production. By contrast, RNA transfection can offer higher efficiency since RNA does not require traversal of the nuclear membrane. Additionally, RNA transfection bypasses the need for genomic integration, transcription, and post-transcriptional modifications, potentially leading to accelerated protein expression. However, RNA-based expression is typically transient, and RNA molecules are less stable than DNA, rendering them more susceptible to degradation during intracellular transport (1).

Ribonucleoproteins (RNPs), including BEs and CRISPR/Cas9 complexes, provide high precision in genome editing and circumvent reliance on the host cell's transcription or translation machinery, thereby reducing off-target effects. Like mRNA, RNPs do not integrate into the host genome, thus mitigating the risks associated with plasmid integration. Additionally, RNPs, similar to mRNA, facilitate transient effects, which is advantageous for applications requiring temporary modifications or short-term experiments. However, the delivery of RNPs into cells poses significant challenges, as they can be difficult to introduce efficiently and are prone to rapid degradation. Specialised delivery techniques, such as electroporation, are often necessary for effective transfection. Moreover, preparing RNPs can be complex and costly, involving precise synthesis and rigorous purification processes (15).

On average, adherent cells such as HEK 293T exhibit higher transfection efficiencies compared to suspension cells like T cells. This increased efficiency is attributed to the enhanced stability and contact area between the transfection reagent and the cells, facilitated by cell adhesion to a rigid surface. Adhesion is partially mediated by membrane-bound heparan sulfate proteoglycans (HSPGs), with adherent cells expressing far more significant amounts of HSPGs than suspension cells. For instance, researchers have demonstrated that T cell lines, such as Jurkat cells, exhibit approximately 100-fold lower levels of HSPGs compared to adherent cells (16). Besides aiding cell adhesion, the negative charge of HSPGs facilitates the initial electrostatic interaction with certain gene delivery vehicles, such as cationic polymers and lipids, and promotes viral transduction through specific interactions with viral proteins. Consequently, the relatively low expression of HSPGs in T cells may impair the cellular uptake of therapeutic transgenes, whether delivered by viral or non-viral methods (17). All the above may explain the low transfection results with lipofectamine 2000 and 3000 presented in this study. Additionally, Ebert et al. demonstrated that non-viral transfection of lymphocytes induces apoptosis in a significant proportion of cells. This apoptosis appears to be mediated through the TNF/TNF receptor pathway; they found that lipofection resulted in high TNF levels, whereas electroporation produced lower TNF amounts (18).

Although electroporation is highly efficient and often preferred for transfecting primary blood suspension cells, it can compromise cell membrane integrity. This disruption can result in cell trauma and subsequent cell death. In this study, DNA electroporation was found to be highly toxic for blood cells, particularly in T cells. However, more recent methods involving the electroporation of RNP complexes of recombinant Cas9 and *in vitro*–transcribed or synthetic sgRNA have demonstrated transfection efficiencies ranging from 50% to 90% across various targets in activated human T and $CD34^+$HSCP cells (19) (20). Seki et al. recently optimised RNP transfection for efficient CRISPR/Cas9-mediated gene knockout in primary T cells using the Lonza 4D Nucleofector system, achieving up to 80% knockout efficiency (21).

This study used electroporation with the NEPA electroporation system to co-transfect primary blood cells with plasmids encoding an ABE8, sgRNA, and EGFP. Co-transfection, which involves introducing multiple plasmid DNA types into host cells, generally aims to incorporate several foreign genes simultaneously. Co-transfection is typically more complex than the transfection of a single nucleic acid type, as the efficiency of transferring several nucleic acids into a particular cell can vary. This variability often depends on the transfection technique and the transfected cell type.

T cells generally present more significant transfection challenges than other cell types due to their robust and complex structure. While electroporation can be an effective method for transfection, its efficiency can vary significantly. Optimisation of electroporation parameters is essential to enhance transfection efficiency

and reduce cellular damage. Studies have demonstrated improved results in activated and resting T cells using Neon and nucleofector electroporation systems and RNP complexes (19) (20) (21).

Zhang et al. investigated the factors influencing electro-transfection outcomes and developed an optimised DNA electroporation strategy suitable for T-cell engineering. Their findings indicated that T-cell stimulation had the most significant impact on transfection efficiency, with stimulation for up to three days markedly enhancing transfection efficiency (~40%). Additionally, they reported that the voltage applied during electroporation influenced plasmid permeation and exhibited a negative correlation with cell viability post-electroporation. Furthermore, increasing plasmid concentration resulted in a higher proportion of successfully transfected cells but reduced overall cell viability (22).

Notably, Zhang et al. conducted their study using CTSTM OpTmizerTM T-Cell Expansion SFM, a medium specifically designed for the growth and expansion of primary human T lymphocytes, supplemented with either CTSTM Immune Cell Serum Replacement (SR) or FBS. Their results demonstrated that transfection efficiency was significantly higher in the SR-supplemented group compared to the FBS group, while no significant differences in cell viability were observed between the two conditions. This distinction in culture medium composition may have contributed to our study's relatively low transfection efficiency, as RPMI supplemented with FBS was used (22).

$CD34^+$HSPCs exhibit lower transfection efficiency compared to more differentiated cell types. While electroporation can be an effective method for gene delivery, it often necessitates precise optimisation of experimental conditions. Transfection of $CD34^+$HSPCs with multiple plasmids poses additional challenges due to their inherently low proliferation rate and delicate nature. Providing optimal recovery conditions following electroporation to alleviate any adverse effects is crucial. Specifically, using media formulations and growth factors tailored to support $CD34^+$HSPCs can be beneficial. For instance, in this study, culturing $CD34^+$HSPCs in Supplemented StemSpan™ SFEM II with StemSpan™ $CD34^+$ Expansion Supplement (10X) proved advantageous. This specialised media is designed to enhance the growth and maintenance of $CD34^+$HSPCs, thereby improving outcomes in gene editing applications. STEMCELL Technologies has recently published a case study evaluating optimal culture methods for high-efficiency genome editing in $CD34^+$ HSPCs on their website (Genome Editing of Human $CD34^+$ Hematopoietic Stem and Progenitor Cells Using the ArciTect™ CRISPR-Cas9 System and StemSpan™ Media (stemcell.com). The researchers assessed various pre- and post-editing media to determine optimal conditions for efficient genome editing while preserving the HSPC phenotype. They reported that among all the media and supplements tested, SFEM II supplemented with StemSpan™ $CD34^+$ Expansion Supplement (10X) was found to most effectively support the maintenance of CD34 expression and the expansion of primitive HSPC subsets following genome editing.

Limitations and Future Recommendation

Lipofectamine has shown to produce lower transfection efficiency when compared to electroporation in our study. However, electroporation was associated with significant cytotoxicity, particularly noted in T lymphocytes. Such adverse effects may elucidate the suboptimal transfection outcomes observed, particularly when multiple plasmids are co-transfected.

Transfecting blood cells with three plasmids presents significant challenges in achieving an optimal balance between transfection efficiency and cytotoxicity. Several strategies can be employed to enhance transfection outcomes while minimising cellular stress: 1) Optimisation of the electroporation protocol: Fine-tuning electroporation parameters, such as voltage and pulse duration, based on the specific cell type can improve transfection efficiency while preserving cell viability. 2) Post-transfection culture conditions: The recovery and expansion of transfected cells are highly dependent on the culture environment. Using xeno-free or serum-free media, such as SFEM II supplemented for $CD34^+$ HSPCs and CTSTM OpTmizerTM T-Cell Expansion SFM supplemented for T cells and appropriate cytokine supplementation, can enhance cell survival and proliferation. 3) Plasmid concentration and quality: Reducing the overall plasmid concentration while maintaining the appropriate molar ratio can mitigate cytotoxic effects. Additionally, using high-purity, endotoxin-free plasmids minimises immune activation and enhances transfection efficiency. 4) Plasmid design considerations: Reducing the number of plasmids required for transfection and utilising smaller plasmids can improve transfection efficiency and reduce cellular burden, thereby enhancing overall cell viability. 5) Finally, utilising RNP complexes or mRNA of the recombinant Cas9/ABE and *in vitro*-transcribed or synthetic sgRNA presents a promising approach for transfecting-sensitive primary blood cells. This strategy offers several advantages, including enhanced transfection efficiency, reduced cellular toxicity, and transient expression, thereby minimising potential off-target effects and immune activation.

While this pilot study offers valuable insights into the co-transfection of plasmids in primary suspension cells, it is limited by a small sample size. Future research involving a larger cohort could yield more comprehensive data. Additionally, employing alternative electroporation devices for comparative performance analysis could enhance the robustness of the findings.

In summary, DNA co-transfection in primary suspension cells presents significant challenges; however, electroporation remains a robust method for introducing plasmids into primary blood cells. The effectiveness and toxicity of this approach are highly dependent on the cell type and the specific conditions employed. Therefore, meticulous optimisation is essential to balance transfection efficiency, cell viability, and functionality.

ACKNOWLEDGEMENT

This work was financially supported by the Fundamental Research Grant Scheme (FRGS/1/2019/SKK08/UPM/02/8) from the Ministry of Higher Education, Malaysia, awarded to LMI. The authors certify that there is no actual or potential conflict of interest in relation to this article.

REFERENCES

1. Chong ZX, Yeap SK, Ho WY. Transfection types, methods and strategies: A technical review. PeerJ [Internet]. 2021 Apr 21 [cited 2024 Jul 5];9. Available from: /pmc/articles/PMC8067914/ doi: 10.7717/peerj.11165
2. Maurisse R, De Semir D, Emamekhoo H, Bedayat B, Abdolmohammadi A, Parsi H, et al. Comparative transfection of DNA into primary and transformed mammalian cells from different lineages. BMC Biotechnol [Internet]. 2010 Feb 8 [cited 2024 Jul 7];10. Available from: https://pubmed.ncbi.nlm.nih.gov/20144189/ DOI: 10.1186/1472-6750-10-9
3. Carter M, Shieh J. Gene Delivery Strategies. Guid to Res Tech Neurosci. 2015 Jan 1;239–52. DOI:10.1016/B978-0-12-818646-6.00017-8
4. Kleiveland C, Kleiveland C. Peripheral Blood Mononuclear Cells. Impact Food Bioact Heal Vitr Ex Vivo Model [Internet]. 2015 Jan 1 [cited 2024 Jul 27];161–7. Available from: https://www.ncbi.nlm.nih.gov/books/NBK500157/ DOI:10.1016/B978-0-12-818646-6.00017-8
5. de Mello VDF, Kolehmanien M, Schwab U, Pulkkinen L, Uusitupa M. Gene expression of peripheral blood mononuclear cells as a tool in dietary intervention studies: What do we know so far? Mol Nutr Food Res. 2012 Jul;56(7):1160–72. DOI: 10.1002/mnfr.201100685
6. Majumdar M, Ratho R, Chawla Y, Singh MP. Evaluating the role of low-speed centrifugation towards transfecting human peripheral blood mononuclear cell culture. Indian J Med Microbiol [Internet]. 2014 [cited 2024 Jul 8];32(2):164–8. Available from: https://pubmed.ncbi.nlm.nih.gov/24713904/ DOI: 10.4103/0255-0857.129806
7. O'Neill MM, Kennedy CA, Barton RW, Tatake RJ. Receptor-mediated gene delivery to human peripheral blood mononuclear cells using anti-CD3 antibody coupled to polyethylenimine. Gene Ther [Internet]. 2001 [cited 2024 Jul 8];8(5):362–8. Available from: https://pubmed.ncbi.nlm.nih.gov/11313812/ DOI: 10.1038/sj.gt.3301407
8. Zhao Y, Zheng Z, Cohen CJ, Gattinoni L, Palmer DC, Restifo NP, et al. High-efficiency transfection of primary human and mouse T lymphocytes using RNA electroporation. Mol Ther [Internet]. 2006 Jan [cited 2024 Jul 8];13(1):151–9. Available from: https://pubmed.ncbi.nlm.nih.gov/16140584/ DOI: 10.1016/j.ymthe.2005.07.688
9. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, et al. Programmable base editing of A T to G C in genomic DNA without DNA cleavage. Nat 2017 5517681 [Internet]. 2017 Nov 1 [cited 2021 Nov 17];551(7681):464–71. Available from: https://www.nature.com/articles/nature24644 DOI: 10.1038/nature24644
10. Gaudelli NM, Lam DK, Rees HA, Sol6-Esteves NM, Barrera LA, Born DA, et al. Directed evolution of adenine base editors with increased activity and therapeutic application. Nat Biotechnol 2020 387 [Internet]. 2020 Apr 13 [cited 2023 May 7];38(7):892–900. Available from: https://www.nature.com/articles/s41587-020-0491-6 DOI: 10.1038/s41587-020-0491-6
11. Richter MF, Zhao KT, Eton E, Lapinaite A, Newby GA, Thuronyi BW, et al. Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. Nat Biotechnol [Internet]. 2020 Jul 1 [cited 2023 Oct 30];38(7):883–91. Available from: https://pubmed.ncbi.nlm.nih.gov/32433547/ DOI: 10.1038/s41587-020-0453-z
12. Sebrow J, Goff SP, Griffin DO. Successfully transfected primary peripherally mobilized human CD34+ hematopoietic stem and progenitor cells (HSPCs) demonstrate increased susceptibility to retroviral infection. Virol J [Internet]. 2020 Feb 10 [cited 2024 Feb 29];17(1):1–5. Available from: https://virologyj.biomedcentral.com/articles/10.1186/s12985-020-1297-3 doi 10.1186/s12985-020-1297-3
13. Basiouni S, Fuhrmann H, Schumann J. High-Efficiency Transfection of Suspension Cell Lines. Biotechniques [Internet]. 2012 Aug 1 [cited 2024 Jul 17];53(2):1–4. Available from: https://www.tandfonline.com/doi/abs/10.2144/000113914 DOI: 10.2144/000113914
14. Okano K, Fukui M, Suehiro Y, Hamanaka Y, Imai K, Hinoda Y. Evaluation of an mRNA lipofection procedure for human dendritic cells and induction of cytotoxic T lymphocytes against enhanced green fluorescence protein. Tumour Biol [Internet]. 2003 [cited 2024 Jul 27];24(6):317–24. Available from: https://pubmed.ncbi.nlm.nih.gov/15004492/ DOI: 10.1159/000076464
15. Zhang S, Shen J, Li D, Cheng Y. Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. Theranostics [Internet]. 2021 [cited 2024 Aug 18];11(2):614. Available

from: /pmc/articles/PMC7738854/ DOI: 10.7150/thno.47007
16. Bobardt MD, Armand-Ugyn M, Clotet I, Zhang Z, David G, Este JA, et al. Effect of polyanion-resistance on HIV-1 infection. Virology [Internet]. 2004 Aug 1 [cited 2024 Jul 28];325(2):389–98. Available from: https://pubmed.ncbi.nlm.nih.gov/15246277/ DOI: 10.1016/j.virol.2004.05.011
17. Harris E, Elmer JJ. Optimization of electroporation and other non-viral gene delivery strategies for T cells. Biotechnol Prog [Internet]. 2021 Jan 1 [cited 2024 Jul 28];37(1). Available from: https://pubmed.ncbi.nlm.nih.gov/32808434/ DOI: 10.1002/btpr.3066
18. Ebert O, Finke S, Salahi A, Herrmann M, Trojaneck B, Lefterova P, et al. Lymphocyte apoptosis: induction by gene transfer techniques. Gene Ther [Internet]. 1997 [cited 2024 Jul 28];4(4):296–302. Available from: https://pubmed.ncbi.nlm.nih.gov/9176514/ DOI: 10.1038/sj.gt.3300394
19. Hendel A, Bak RO, Clark JT, Kennedy AB, Ryan DE, Roy S, et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat Biotechnol [Internet]. 2015 Sep 10 [cited 2024 Aug 18];33(9):985–9. Available from: https://pubmed.ncbi.nlm.nih.gov/26121415/ DOI: 10.1038/nbt.3290
20. Gomes-Silva D, Srinivasan M, Sharma S, Lee CM, Wagner DL, Davis TH, et al. CD7-edited T cells expressing a CD7-specific CAR for the therapy of T-cell malignancies. Blood [Internet]. 2017 Jul 20 [cited 2024 Aug 18];130(3):285–96. Available from: https://pubmed.ncbi.nlm.nih.gov/28539325/ DOI: 10.1182/blood-2017-01-761320
21. Seki A, Rutz S. Optimized RNP transfection for highly efficient CRISPR/Cas9-mediated gene knockout in primary T cells. J Exp Med [Internet]. 2018 Mar 3 [cited 2024 Aug 18];215(3):985. Available from: /pmc/articles/PMC5839763/ DOI: 10.1084/jem.20171626
22. Zhang Z, Qiu S, Zhang X, Chen W. Optimized DNA electroporation for primary human T cell engineering. BMC Biotechnol [Internet]. 2018 Jan 30 [cited 2025 Mar 13];18(1). Available from: https://pubmed.ncbi.nlm.nih.gov/29378552/ DOI: 10.1186/s12896-018-0419-0