An Innovative, Highly Efficient Method for Transfecting Large Quantities of Primary Human T-Cells

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Introduction

With 18.1 million new cases and 9.6 million related deaths observed worldwide in 2018, cancer continues to pose a significant threat to health and well-being. Therefore, a strong need exists for improved cancer treatment options. One promising approach relies on genetically modified, patient-derived cells like T-cells or natural killer cells which express chimeric antigen receptors (CAR) targeting cancer cells.

Primary human T-cells are difficult to genetically modify with chemical transfection reagents, as virtually all non-dividing primary cells. Current viral transduction methods require expensive, cumbersome viral vector production. Classical electroporation methods are often limited in throughput as well as volume and can result in cells with impaired viability and functionality. The study objective was to optimize transfection and culture protocols for large quantities of primary human T-cells using the 4D-Nucleofector® System

Methods

Cell preparation: Cryopreserved human peripheral blood mononuclear cells (PBMC, Lonza, CC-2702) were thawed according to manufacturer's instructions and incubated for 1-2 hours (h) at 37°C/5% CO_2 in X-Vivo® 15 (Lonza, 04-744Q) supplemented with 5% Human Serum (HS, Sigma, H4522). Fresh PBMC were isolated from 48 hours-old buffy coats using Ficoll (GE Healthcare, 17-1440-03) and SepMate™-50 Tubes (Stemcell Technologies, 85450).

Transfection: 3.0 x 10^7 – 10.0 x 10^7 PBMC were resuspended in 1 mL P3 Nucleofector® Solution containing 40 µg/mL or the indicated concentrations of pmaxGFP™ Vector or Chimeric Antigen Receptor Vector[1] (anti-CEA CAR, kindly provided by Prof Dr. Abken, University Hospital Regensburg, Germany). Cells were transferred into the indicated Nucleocuvette® Vessels and transfected in the 4D-Nucleofector® X Unit or LV Unit with program EO-115.

Cell culture: After transfection, cells were diluted in X-Vivo® 15/5% HS to a cell concentration of 2.5 x 10^6 cells/mL and transferred into standard T25 or T75 tissue culture flasks. 6 hours post transfection, interleukin 2 (IL-2, final concentration 20 IU/mL, Peprotech, 200-02) and T Cell TransAct™ (human, Miltenyi 130-111-160) were added.

Analysis: On the indicated time points, cells were stained for CD3, CAR (Southern Biotech, 2043-09) and DAPI and the number Green Fluorescent Protein (GFP)- or CAR-positive, DAPI-negative, CD3-positive T-cells determined using flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher). If not stated otherwise, cell viability is expressed as the number of viable cells in comparison to an untreated culture control.

Conclusion

- Transfection efficiencies of up to 70% were obtained with the 4D-Nucleofector® System in the 20 µL Nucleocuvette® Strip (data not shown), 100 µL Nucleocuvette® Vessel, 1 mL and LV Nucleocuvette® Cartridge. This corresponds to 1 million PBMC in 20 µL and up to 1 billion PBMC in 20 mL transfection volume.
- The presented protocol is suitable for cryopreserved and freshly isolated PBMC.
- The CD3+ T-cell population expanded five-fold during 10 days in standard tissue culture flasks. The number of GFP-positive T-cells peaked around day 6 with a decline thereafter, most likely caused by the loss of the transient pmaxGFP™ Vector.
- Transfection efficiencies between 39% and 55% were obtained for a CAR-expression vector.
- In summary, we present a reliable method for the efficient transfection and subsequent expansion of large quantities

Figure 1.
Nucleofection® Principle

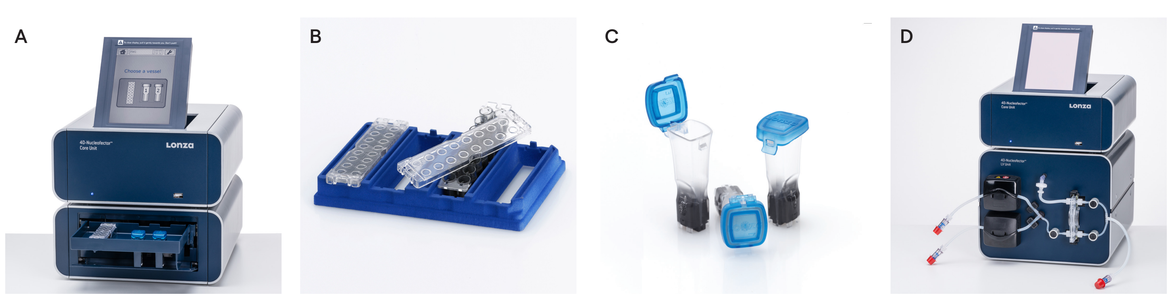

Figure 2. 4D-Nucleofector® System
(A) 4D-Nucleofector® Core Unit and X Unit for transfection in 20 µL and 100 µL volume.
(B) 20 µL Nucleocuvette® Strip for transfection of 10^4 – 10^6 cells
(C) 100 µL Nucleocuvette® Vessel for transfection of 10^5 – 10^7 cells
(D) 4D-Nucleofector® LV Unit for transfection in up to 20 mL volume.
(E) 1 mL Nucleocuvette® Cartridge for up to 1×10^8 cells
(F) LV Nucleocuvette® Cartridge for up to 1×10^9 cells in a closed system

Figure 3. 4D-Nucleofector® LV Unit Process
1. Cartridge inserted in slot
2. Short venting tubing with filter attached to the male Luer connector on the front plate
3. Tubings inserted in pinch valves
4. T connector inserted into T connector holder. Two larger diameter tubings completely inserted into liquid sensors
5. Upper tubing (for cell suspension) inserted into the upper pump
6. Optional - when feeding substrate separately - lower tubing inserted into lower pump
7. Holder for cell suspension reservoir placed on a magnetic stirring platform
8. Connection to input reservoir for cell suspension
9. Connection to input reservoir for substrate
10. Connection to output reservoir

Results

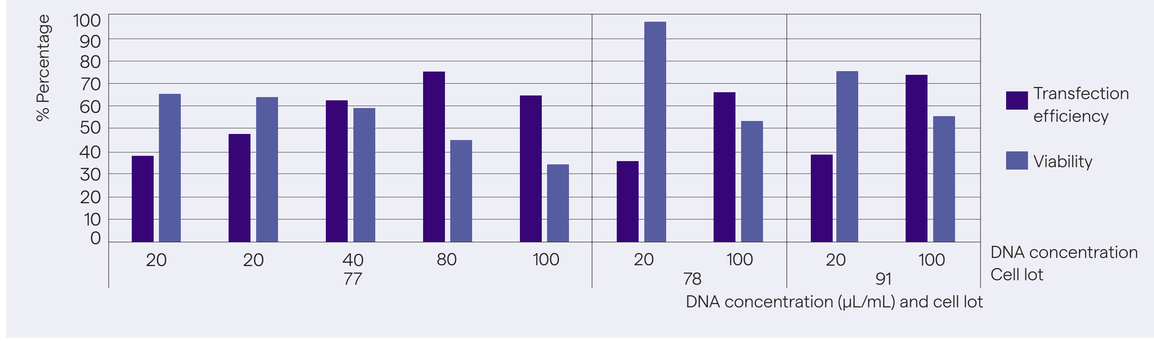

Figure 4.
Three independent lots of cryopreserved PBMC were transfected in 100 µL Nucleocuvette® Vessels with the indicated amount of pmaxGFP™ Vector. 24 hours after the Nucleofection® Process, transfection efficiencies of up to 70% were achieved. Cell viability is expressed as the number of viable cells in comparison to an untreated culture control. Transfection efficiency increased and cell viability decreased with increased DNA concentration. Therefore, 40 µg/mL pmaxGFP™ Vector were chosen as the optimal DNA concentration for further experiments.

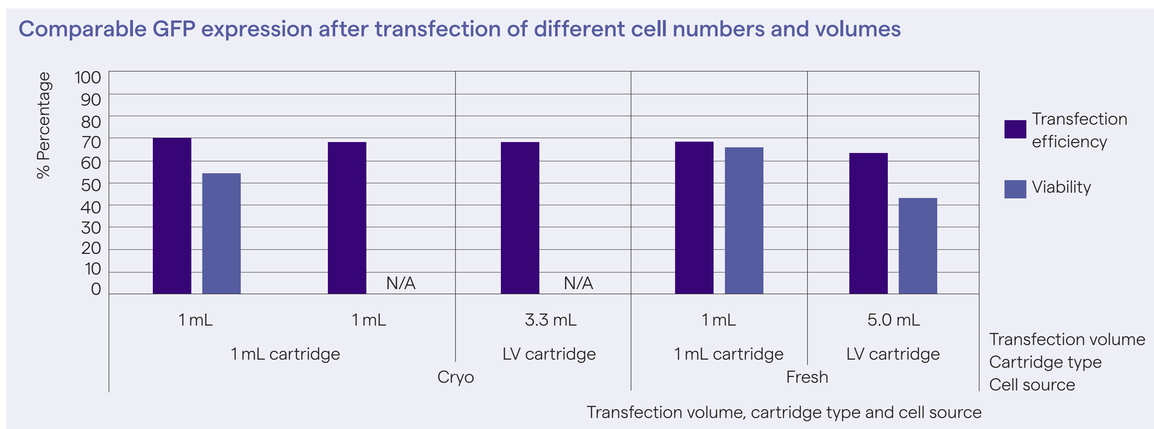

Figure 5.
The optimized transfection conditions (see Figure 4) were applied to larger cell numbers using the 4D-Nucleofector® LV Unit. 1.0 – 5.0 mL of PBMC suspension were transfected. 24 hours after the Nucleofection® Process, transfection efficiencies of up to 70% were observed in 3 independent experiments for different transfection volumes and cartridge types as well as cryopreserved and fresh PBMC isolated from 48 hours-old buffy coats. The number of viable cells was between 43% and 66% of culture controls. (n/a = No culture control available to allow calculation of % cell viability.)

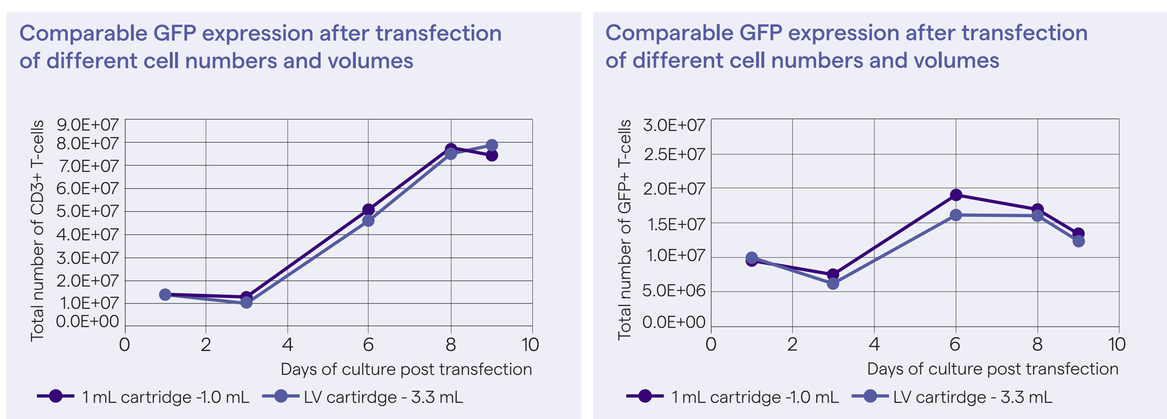

Figure 6.
1.0 mL or 3.3 mL cryopreserved PBMC were transfected in the 4D-Nucleofector® LV Unit, stimulated via anti-CD3/CD28 and cultured for up to 10 days during which the CD3+ T-cell population expanded five-fold. The number of GFP-positive T-cells peaked around day 6 with a decline thereafter, most likely caused by the loss of the transient pmaxGFP™ Vector. Stable integration approaches (*i.e.* CRISPR) would presumably support stable gene of interest expression.

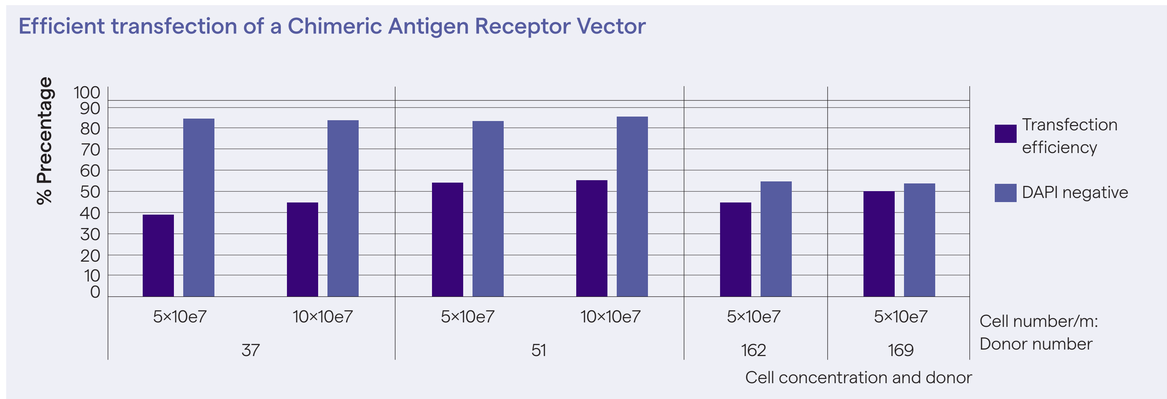

Figure 7.
In two independent experiments, PBMC were isolated from four independent, 48 hours-old buffy coats. The indicated amount of cells was resuspended in 1 mL P3 Nucleofector® Solution containing 50 µg/mL CAR vector and transfected in the 4D-Nucleofector® LV Unit. Cells were analyzed 24 hours post transfection. Between 39% and 55% of T-cells stained positive for CAR. 54% - 86% of CD3+ T-cells were DAPI-negative and considered to be viable.

References

[1] Chmielewski, M. & Abken, H. (2017) CAR T cells releasing IL-18 convert to T-bethigh FoxO1low effectors which exhibit augmented activity against advanced solid tumors. Cell Reports 12, 3205 – 3219, doi: 10.1016/j.celrep.2017.11.063