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Cell & Gene

The Cocoon® Platform Combined With the 4D-Nucleofector® LV Unit

A Non-viral Workflow for Modifying Primary T Cells

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Introducing a functionally closed and automated non-viral method for gene-modified cell therapy manufacturing

Cell Immunotherapy Market Need

The market need for gene-modified T cellular immunotherapies has increased significantly since two CAR (chimeric antigen receptor) T cell products (1, 2) were commercialized in 2017. Kymriah® (Tisagenlecleucel; Novartis) and Yescarta® (axicabtagene ciloleucel; Kite Gilead) demonstrated marked clinical benefits in patients with B-cell haematological malignancies. These products illustrated a critical patient need for curative cellimmunotherapies in oncology. Consequently, more than 500 clinical trials worldwide are testing CAR-T cells in oncology applications with the number of programs entering the clinic climbing annually (3).

Autologous T cell immunotherapy research and development has quickly evolved but a key bottleneck is scaling manufacturing to meet commercial demand (4). Lonza has provided a new tool to address this pain-point, the Cocoon® Platform, which enables automation of cell isolation, activation, transduction, expansion, cell washing and harvest while providing real-time biofeedback (temperature, CO_2, pH, and DO) throughout the process.

Although manufacturing is a critical pain-point requiring solutions, others issues exist, including the expense (hundreds of thousands to millions USD), time (9-12 months), and availability (up to 24 months for a manufacturing slot) of GMP viral vectors (lentiviral & γ- retroviral). One solution to address this pain point is utilizing non-viral transfection methods to gene modify cells. Lonza has such a system, the 4D-Nucleofector® LV Unit. The Nucleofection® Unit utilizes electroporation technology which enables automated, scalable transfection capabilities for multiple cell types and applications.

To address multiple pain-points, these complimentary technologies (the Cocoon® Platform and 4D-Nucleofector® LV Unit) were combined to enable non-viral gene delivery for cell immunotherapy manufacturing in a functionally-closed, automated workflow. In doing this, Lonza provides a platform which can reduce costs, improve process efficiency, and increase final product quality. The study objective was to demonstrate the ability of the combined platforms to modify primary T cells in a functionally-closed, automated fashion.

Methods

Experimental design

9×10^8 fresh or frozen healthy donor peripheral blood mononuclear cells (PBMCs) were transfected with the pmaxGFP™ Vector (DNA plasmid encoding for GFP) using the 4D-Nucleofector® LV Unit. Frozen PBMCs were prepared by thawing PBMCs in X-VIVO® 15 Media containing 5% human AB serum (X-VIVO® Complete Media) with DNase I. The PBMCs were seeded in T225 flasks with X-VIVO® Complete Media at 2×10^6 cells/mL. Flasks were placed upright in a 37°C, 5% CO_2 incubator for 2 hours prior to transfection. Following transfection, the cells were transferred through a functionally-closed fluid path connection to the Cocoon® Platform for a 10 day expansion step which included automated media exchanges. PBMCs were activated with TransAct™ and expanded in X-VIVO® Complete Media and 20 IU/mL IL-2. The cell washing and harvesting process steps were automated and performed within the Cocoon® Platform. In-process cell samples were collected (in a functionally-closed manner) and analyzed for CD3, CD4, CD8, and GFP expression via flow cytometry. Cell counts and viability were assessed using a NucleoCounter® NC-200™ (n=3 per condition).

Cocoon® Platform integration with the 4D-Nucleofector® LV Unit via functionally-closed tubing connection

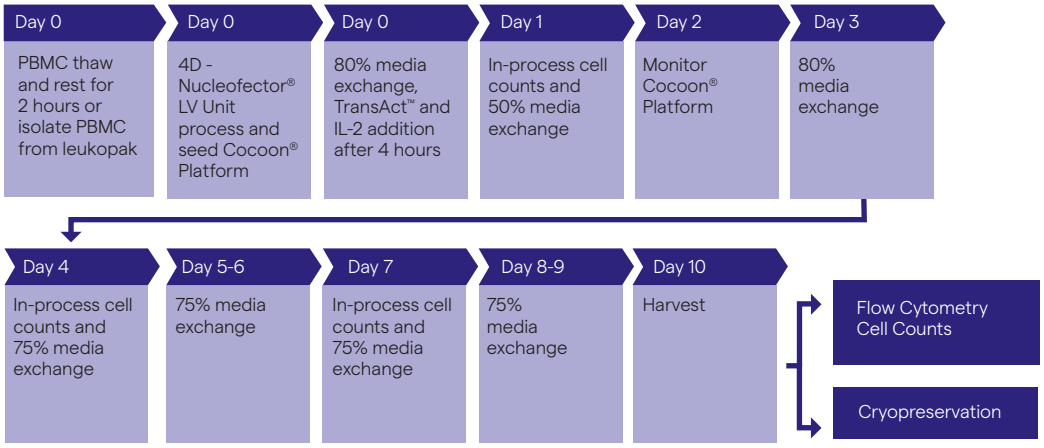
The PBMCs were mixed with the P3 Nucleofector® Solution containing 40 µg/mL pmaxGFP™ Vector. PBMCs were manually loaded into the input 4D-Nucleofector® LV Reservoir prior to initiating transfection. PBMCs were pumped into the LV Nucleocuvette® Cartridge and electroporated using the EO115 program at 5×10^7 PBMCs/mL per cycle. After transfection, the PBMCs were transferred to the output 4D-Nucleofector® LV reservoir pre-loaded with 45 mL of X-VIVO® 15 Media and 5% human AB serum. Once all PBMCs had undergone the Nucleofection® Process, the pooled PBMCs were transferred to the Cocoon® Platform proliferation chamber in an automated step via integrated, functionally-closed fluid paths.

Figure 1: Cocoon® Platform integration to the 4D-Nucleofector® LV Unit via functionally-closed tubing connections. Standard Nucleofection® Process tubing set with the 4D-Nucleofector® LV Output Reservoir connected to the Cocoon® Platform

Key process steps

As illustrated in Figure 2, following electroporation;
Day 0 – The PBMCs recovered in the Cocoon® Cassette proliferation chamber for 4 hrs in 180 mL of X-VIVO® Complete Media. An 80% media exchange was performed and fresh activation media containing IL-2 and TransAct™ beads were added to the PBMCs increasing the total volume to 276 mL. The activation substrate ratio was 4 mL TransAct™ to 2×10^8 PBMCs.
Day 1 – 50% media exchange (276 mL volume) was performed.
Day 3 – 80% media exchange (450 mL volume) was performed to remove and dilute the TransAct™ beads.
Day 4 thru 9 – 75% daily media exchange (450 mL volume) was performed.
Day 10 - Expanded T cells were harvested in 90 mL of Plasma-Lyte A containing 3% HSA, counted, and cryopreserved in CryoStor® CS10 at 2.5×10^7 cells per cryovial at 1 mL. FACS and cell counts were analyzed on Day 0, 1, 4, 7 and 10.

Key Process Steps



Final product characteristics

The transfection efficiency, cell yield, cell viability and percent CD3⁺ cell purity were assessed in the final cell therapy product. Acceptance criteria for the processes using the combined platforms were established based on prior knowledge from other cell therapy processes. The 50% transfection efficiency threshold was set based on the expected total viable cell number from a 10-day process (1.5×10^9 total viable cells; Table 1) which, if met, would produce clinically-relevant cell numbers. Utilizing these thresholds, predicted gene-modified cell numbers can be extrapolated using Day 1 transfection efficiency, assuming a stable-expression system. Cell viability and purity were established based on past cell therapy process experience.

Parameter	Acceptance criteria
Transfection efficiency, day 1	≥ 50%
Cell yield, day 10	≥1.5×10 total viable cells
Cell viability, day 10	70% viability
Purity, day 10	CD3⁺ >90%

Table 1: Final product acceptance criteria.
The table lists the defined acceptance criteria to determine a successful manufacturing process for the combined platforms (Cocoon® Platform and 4D-Nucleofector® LV Unit)

Results

Assessing transfection efficiency in fresh and frozen PBMCs

Comparable GFP⁺ transfection efficiency was observed in frozen and fresh PBMCs over the course of a 10 day expansion (Figure 3A-C). Day 1 CD3⁺ GFP⁺ transfection efficiency in the frozen and fresh PBMCs was 61% and 58% respectively (Figure 3A). Similar transfection efficiencies in the CD4⁺ and CD8⁺ T cell populations were observed (Figure 3B & C). Because the pmaxGFP™ Vector does not integrate into the cell's genome, GFP expression was transient and decreased over time as the cells proliferated. The data illustrated this with GFP expression decreasing after Day 1 (Figure 3A-C). Cell recoveries immediately after the Nucleofection® Process on Day 0 averaged 73% for Frozen and 56% for Fresh PBMCs (Figure 4). Twenty-hours later, on Day 1, cell recovery dropped further to 30% on average, indicating additional cell death (Figure 4). Cells subsequently recovered and viabilities exceeded 90% at all time points (Figure 5).

In-process and final product cell characteristics

All Cocoon® Process Runs, whether using frozen or fresh PBMCs as starting material, exhibited comparable expansion profiles (Figure 4). A lag period was observed between Day 1 and Day 4 where cell expansion was limited. Cells began to rapidly proliferate after Day 4 and averaged 10.6 fold expansion by process end. Total viable cell numbers averaged 2.0×10^9 on Day 7 and 2.8×10^9 on Day 10 (Figure 4). Doubling time averaged 29 hours between frozen and fresh PBMCs from day 4 to 7. Cell viabilities remained high, greater than 95%, during expansion and harvest (Figure 5).

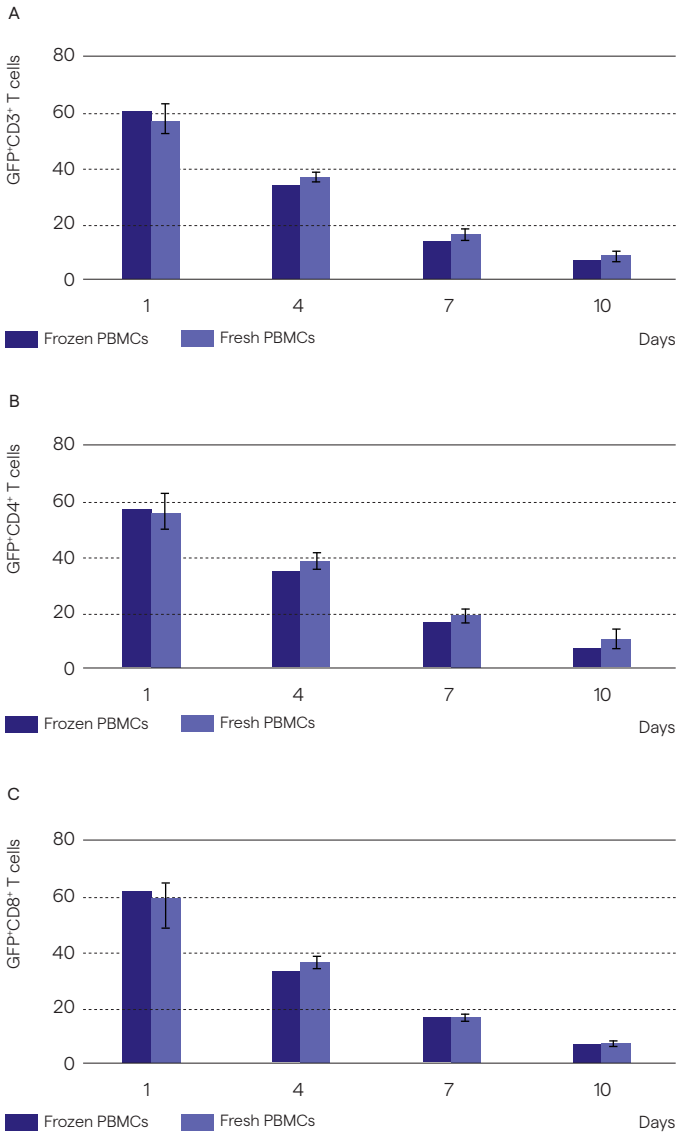


Figure 3A-C: Transient GFP expression degrades over time in frozen and fresh transfected PBMCs. A: % GFP⁺CD3⁺ T cell population; **B:** % GFP⁺CD4⁺ T cell population; **C:** % GFP⁺CD8⁺ T cell population. (n=2 Frozen samples from 1 donor; n=3 Fresh samples from 2 donors)

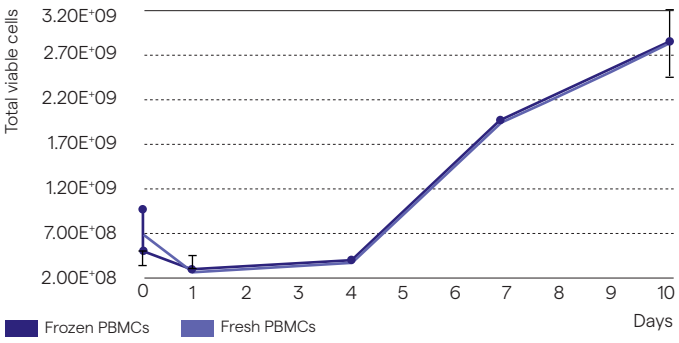


Figure 4: Frozen and fresh PBMC expansion profile in the Cocoon® Platformduring a 10 day non-viral transfection process. Expansion profiles werecomparable pre- and post- Nucleofection® Process steps whether using frozen or fresh PBMCs as starting material. (n=2 Frozen samples from 1 donor; n=3 Fresh samples from 2 donors)

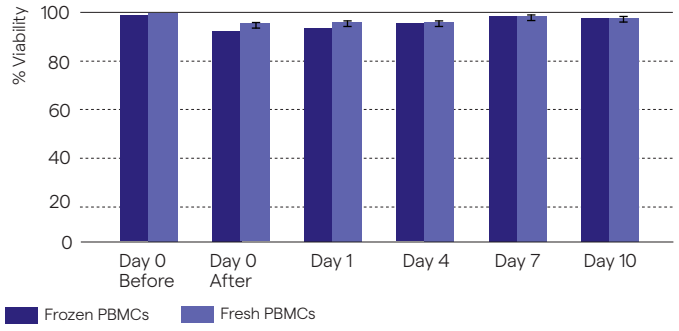


Figure 5: In-process and final product cellular viability remained high in cells expanded in the Cocoon® Platform. Cell viabilities above 90% were observed in both frozen and fresh PBMCs pre- and post- Nucleofection® Process steps. High cell viabilities continued to Day 10. Data of Day 0 represented viabilities measured before and after transfection. (n=2 Frozen samples from 1 donor; n=3 Fresh samples from 2 donors)

CD3⁺ T cell purity and CD4⁺:CD8⁺ ratio in frozen and fresh PBMCs

In-process sampling showed high CD3⁺ (T cell) purity by Day 4 and greater than 90% by Day 7 when using frozen or fresh PBMCs as starting material (Figure 6). A similar CD4⁺:CD8⁺ ratio, marginally favoring CD4⁺ T cells, was observed on Day 1 (Figure 7 A-B). By Day 10, the CD4⁺:CD8⁺ ratio trended closerto parity (Figure 7A-B).

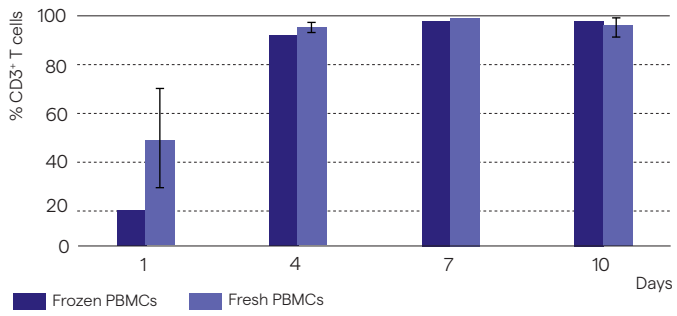


Figure 6: CD3⁺ T cell purity over a 10-day process. Greater than 90% CD3⁺ T cells was observed by Day 7 and purities were comparable between frozen and fresh PBMCs. (n=2 Frozen samples from 1 donor; n=3 Fresh samples from 2 donors)

Final product criteria

In the 5 process runs performed using the combined platform, the final product critical quality attributes met the acceptance criteria defined in Table 2. In an integrated process utilizing a stable-expression system, the extrapolated cell yield is 1.7×10^9 transfected cells by Day 10 (assuming 60% transfection efficiency on Day 1).

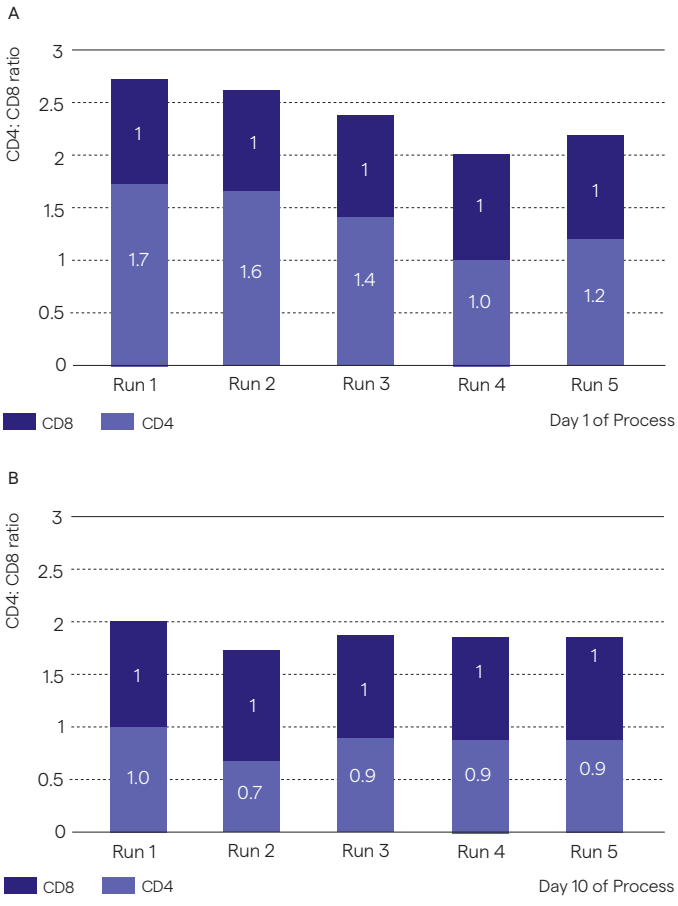


Figure 7A-B: The CD4⁺:CD8⁺ ratio Day 1 versus Day 10 comparison. A: CD4⁺:CD8⁺ population ratio on Day 1; B: CD4⁺:CD8⁺ population ratio on Day 10. (n=2 frozen samples from 1 donor; n=3 fresh samples from 2 donors)

Parameter	Acceptance criteria	Frozen PBMC	Fresh PBMC	Combined frozen & fresh PBMC
Number of runs	N/A	2	3	5
Transfection efficiency, day 1	≥ 50%	61%	58% ± 5%	61% ± 6%
Cell yeild day 10	≥ 1.5×10^9 Total viable cells	2.80×10^9	2.82×10^9 ± 4.35×10^8	2.81×10^9 ± 3.42×10^8
Cell viability,- day 10	> 70% viablity	97.2%	97.2% ± 0.6%	97.2% ± 0.004%
Purity, day 10	$CD3^+$ >90%	99%	96% ± 4.4%	97% ± 3.5%

Table 2: Final product critical quality attributes compared to acceptance criteria. The transfection efficiency, cell yield, cell viability and % $CD3^+$ cellpurity met the established acceptance criteria.

Conclusions

These study results illustrate the utility of combining the 4D-Nucleofector® LV Unit with the Cocoon® Platform to enable automation of non-viral cell therapy manufacturing processes. By transfecting PBMCs prior to activation and expansion, the combined platforms produced a gene-modified T cell product with clinically-relevant characteristics (high cell yield, transfection efficiency, and purity).

The combination of the Cocoon® Platform and the 4D-Nucleofector® LV Unit provides an alternative, non-viral gene delivery system which removes the need for costly, time-consuming viral vector preps. The integrated platform provides transfection and operation scalability, high cell yield, manufacturing COGS reduction, reduced operator error, and better process control to generate high quality cell immunotherapies.

Follow-on studies will focus on transfecting a CAR-coding plasmid using a stable expression system.

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