Scalable CAR-NK Cell Expansion Using Vertical-Wheel Bioreactors with XCell ATF Perfusion

App Note | NK Cell

Joyce Hee Jin Kim[1], Sanket Dahotre[1], Kenny Cruz[2], Isobelle Espiritu[2], Muhammad Shamim[3], Yifan Effie Huang[3], Yas Hashimura[2], Rachel Legmann[3], Sharon Harvey[2], Wesley Gorman[1]

[1]GeneFab, Alameda, California, USA

[2]PBS Biotech, Camarillo, California, USA

[3]Repligen Corporation, Waltham, Massachusetts, USA

Introduction

Engineered immune cells are emerging as powerful modalities in modern immunotherapy, but their expansion requires highly controlled, scalable bioprocesses. Conventional flask-based methods lack the sterility, automation, and environmental control needed for reliable performance. PBS Biotech's Vertical-Wheel® Bioreactors (VWBs), combined with Repligen's XCell® Alternating Tangential Flow (ATF) filtration, deliver a closed and scalable low-shear environment optimized for high-quality immune-cell production. This application note investigates nutrient-delivery and perfusion-feeding approaches for high-potency expansion of chimeric antigen receptor natural killer (CAR-NK) cells at a 3 L working scale.

For additional details regarding small-scale NK cell expansion and process optimization in PBS-Mini® Vertical-Wheel bioreactors, please consult the full application note: Achieving Clinically Relevant Yields of High-Potency CAR-NK Cell Expansion in PBS Vertical-Wheel Bioreactors through Integrated Perfusion Via XCell ATF from GeneFab and Repligen

Materials and Methods

Cell Source and Activation

Human peripheral blood NK cells were thawed, activated with irradiated K562 feeder cells, and transduced with CAR retrovirus.

Cell Expansion

PBS-3 VWB (3L) used an agitation rate, seeding cell density, and medium exchange strategy optimized in the PBS-Mini scale-down model. Batch, fed batch, and perfusion feeding strategies were then evaluated in the PBS-3.

Perfusion-Based Medium Exchange Strategy Using XCell ATF 2

The XCell ATF 2 device was connected to PBS-3 VWBs to enable continuous medium exchange. Perfusion initiation was triggered by cell density (~2 × 10^6 cells/mL) or glucose depletion (~2 g/L).

The XCell ATF 2 System includes several components: a controller (with air and vacuum accessories), software, sensors and XCell ATF Device.

XCell Lab Controller and Accessories (Figure 1)

1. XCell Lab Controller
2. XCell Lab Software and HMI
3. Flow sensor
4. Supply air protection assembly
5. Vacuum manifold
6. Vacuum pump

Figure 1. XCell Lab Controller and accessories

Consumables: XCell ATF 2 Single-Use Device (Figure 2)

1. CPC AseptiQuik® S Connector
2. Top permeate
3. XCell ATF filter housing
4. Bottom permeate
5. Diaphragm
6. XCell ATF to controller tubing (A2C)
7. A2B tubing (sterile connectors optional)
8. Permeate pressure sensor
9. Air filter

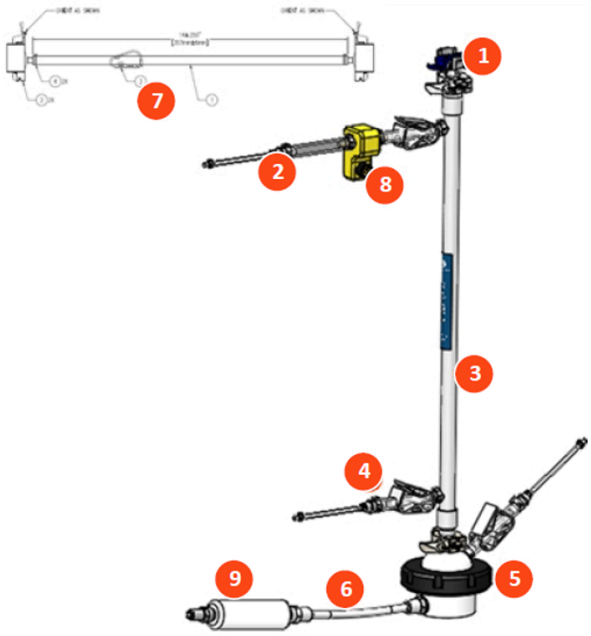


Figure 2. XCell ATF 2 Device components

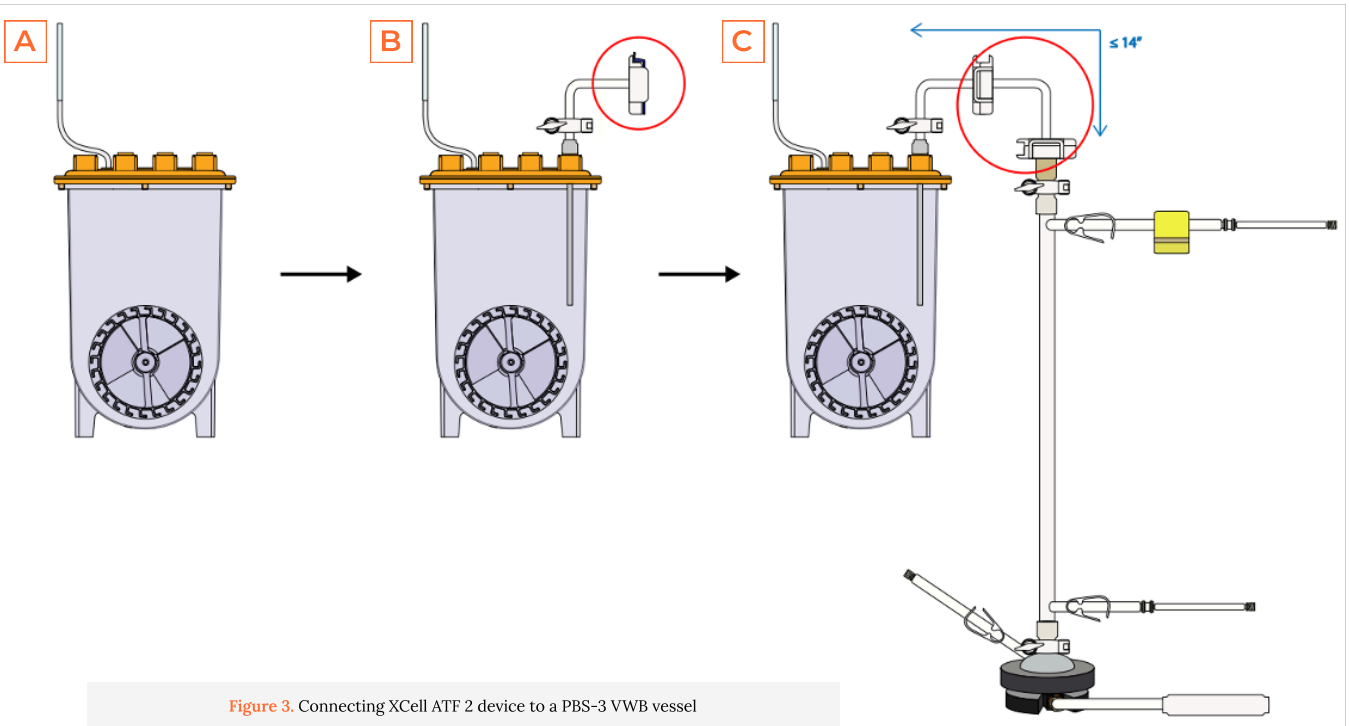


Figure 3. Connecting XCell ATF 2 device to a PBS-3 VWB vessel

Most single-use, bench-scale bioreactors are equipped with a dip tube and associated tubing. Use the following steps to connect the XCell ATF 2 Device to a single-use bioreactor as illustrated above (Figure 3):

1. Insert a sterilized dip tube and A2B tubing (with or without aseptic connector) into the single use bioreactor using head plate fittings and aseptic practices (Figure 3B).
2. Connect the XCell ATF filter device to the bioreactor using the CPC AseptiQuik S Sterile Connector (circled in red) or by directly welding the A2B tubing (Figure 3C).

Note: the total length of the A2B tube from the top of the XCell ATF Device to the bioreactor headplate must not exceed 14". If unable to meet this requirement, please consult with your local Field Applications Specialist (FAS).

The PBS-3 bioreactor vessel lid contains six ports with universal Pg-13.5 connections to support flexible integration of probes and accessories, along with a 1/8" addition port for liquid input. Three PG-13.5 ports are equipped with pre-installed accessories, including a single-use pH probe, a DO probe sleeve, and a thermal well, while the remaining three ports are left unoccupied and capped for optional use (Figure 4A). A sterile dip tube was inserted aseptically into one of the three unoccupied lid ports of the PBS-3 single-use vessel and attached to the ATF 2 device to enable perfusion-based medium exchange for expansion of CAR-NK cells (Figure 3, Figure 4B). Media was equilibrated overnight under the same culture parameters as a batch culture, and the cells were inoculated at 1.0 x cells/mL the following day. Before cell inoculation, the ATF hollow fiber filter was primed by recirculating pre-equilibrated media through both the lumens and shell side before integration. Daily cell counts and metabolite measurements, particularly glucose levels, were taken to inform the timing of perfusion initiation. To initiate perfusion, the waste media was pulled across the ATF hollow fiber filter using a permeate pump at a fixed pump rate of 1 mL/min and an equivalent amount of fresh formulated media was added to the bioreactor via PBS-3 level control. A bag of fresh NK cell culture medium was welded to the addition line then installed through one of the native peristaltic pumps on the PBS bioreactor unit (Figure 4B). The culture perfusion rate was set to 0.5VVD, corresponding to an exchange rate of 1.5 L/day. The ATF flow rate was maintained at a set rate of 0.4 L/min, intended to minimize shear until harvest.

Figure 4. (A) PBS-3 vessel lid with capped Pg-13.5 threaded ports. The rightmost capped port was utilized for sterile ATF dip tube insertion and fresh medium was added through the addition port on the vessel lid. (B) PBS-3/ATF setup including feed and waste bags.

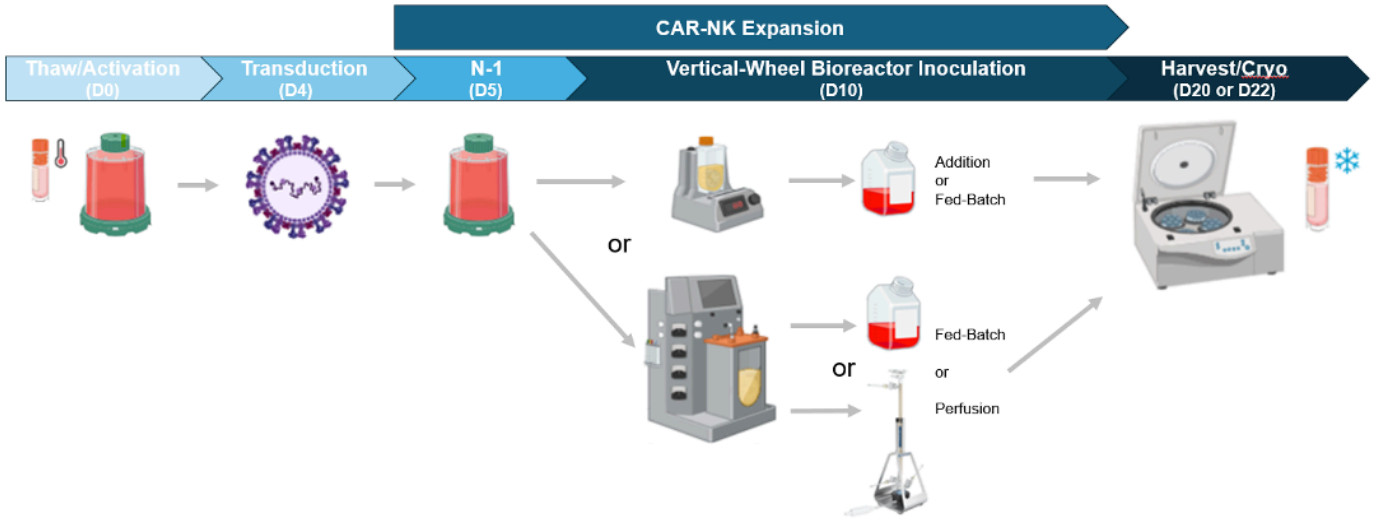


Figure 5. Process workflow. A diagram of the processing steps of NK activation and expansion until cryopreservation for a fed-batch or perfusion system.

Analytics

Counting and Metabolite Measurements

From activation day through harvest, cell counts were obtained using the NucleoCounter® NC-3000 (ChemoMetec), which utilizes fluorescence-based imaging to assess viability and total cell number. Metabolite measurements, including glucose and lactate, were performed using the BioProfile® FLEX2 Analyzer (Nova Biomedical). These samples were collected and analyzed to monitor nutrient consumption and byproduct accumulation over time.

Cell Health Assessment

Samples were collected at multiple timepoints throughout the culture period to assess apoptosis. Apoptosis was evaluated using the Annexin V Apoptosis Assay on the NucleoCounter NC-3000 (ChemoMetec), following the manufacturer's protocol. Each sample was tested in duplicate to ensure reproducibility. Proper population gating was applied by utilizing FMO's (fluorescence minus one) samples for both reagents (Annexin 5 and propidium iodide (PI)). A positive control, HT1080 cells treated with camptothecin to induce apoptosis, was included in each run to validate assay performance.

Potency

Cell potency was evaluated by comparing CAR-NK cells expanded in static G-Rex® culture against those expanded in dynamic PBS-3 VWB culture for 7 and 8 days, using a cell-based lactate dehydrogenase (LDH) assay. At designated time-points, CAR-NK cells were co-cultured at serial dilutions against a target cell line HT1080 then supernatants were collected and LDH levels were quantified using this colorimetric assay in which absorbance was measured at 490nm. A 4PL curve was generated to estimate the EC50, and relative potency was calculated by comparing the EC50 values of the samples. This potency assessment enabled a comparison of functional cell activity and potency between the static G-Rex and dynamic PBS-3 VWB cell expansion platforms.

CAR Expression

CAR expressions were evaluated using flow cytometry to quantify transduction efficiency and assess cell viability. Cells were stained with a fluorophore-conjugated protein, which specifically binds to the CAR construct expressed on the cell surface, allowing detection of transduced cells. To assess viability, 7-AAD was included in the panel to exclude dead cells from analysis. Samples were incubated with the staining reagents according to the manufacturer's protocols, washed, and analyzed on a flow cytometer.

Harvest and Cryopreservation

NK cells expanded in VWBs were harvested under aseptic conditions. For the PBS-0.1 VWB, CAR-NK cells were manually collected using a sterile serological pipette. In the PBS-3 VWB, a sterile weld was performed to attach a collection bag to the harvest tubing, and cells were collected by gravity flow. The harvested cells were centrifuged, supernatants discarded, and the cell pellets were resuspended in CryoStor® CS10 cryopreservation medium (BioLife Solutions). The cells were placed in a controlled-rate freezing container and stored at -80°C for 24 hours prior to transfer into vapor-phase liquid nitrogen for long-term storage.

Results and Discussion

Large-Scale CAR-NK Cell Expansion

Batch/Fed-Batch Feeding Strategy

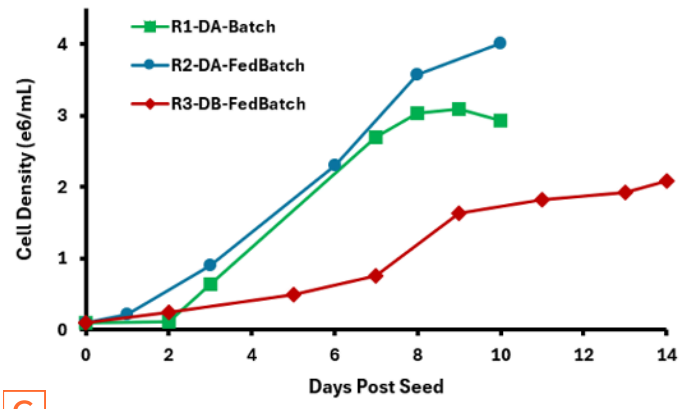
To assess donor variability and the effects of different feeding strategies, NK cells from two donors were selected, transduced, and expanded using the described protocol. Three runs were performed: two with Donor A and one with Donor B. Each run began on seed day with a starting seeding density of 1.0 x 105 cells/mL in the PBS-3 VWB.

- Run 1 (R1): The batch culture was initiated and maintained at maximum working volume (3L) and did not receive any media top-off throughout the expansion period.
- Run 2 (R2): Process modifications were introduced to improve expansion outcomes. A fed-batch approach was employed, where the culture started at a 2L working volume, followed by a 1L media top-off three days post-seeding to reach the full 3L capacity.
- Run 3 (R3): Similar to Run 2, this was a fed-batch study with a 1L top-off three days post-seeding. Additionally, both runs 2 and 3 were maintained under 50% DO and pH control throughout the process.

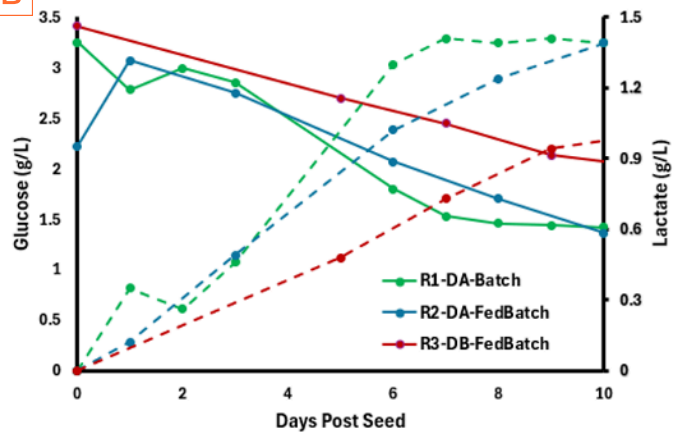
The results of all three runs including starting and final cell counts as well as total fold expansion are presented in Figure 6. The differences in expansion trends between the R1/R2 and R3 were attributed to donor variability. For Donor A runs, the fed-batch model in Run 2, where the media was topped off to maximum working volume, likely explains the improved expansion from Run 1. Run 3, which extended for four additional days compared to Runs 1 and 2, confirmed the growth plateau.

These controlled variables, media feeding strategy, initial working volume, pH and DO control, were assessed for their impact on cell expansion kinetics and culture health. Growth kinetics as well as metabolite consumption and accumulation from these batch runs were used to inform perfusion initiation.

A



B



C

Run	Donor	Feeding Strategy	ID	Culture Duration (days)	Seeding Density (cells/mL)	Total Viable Cells (D12)	Fold Expansion (D12)
R1	DA	Batch		10	1.0×10^5	8.8×10^9	29
R2	DA	Fed-Batch		10	1.0×10^5	1.2×10^{10}	40
R3	DA	Fed-Batch		14	1.0×10^5	5.2×10^9	17

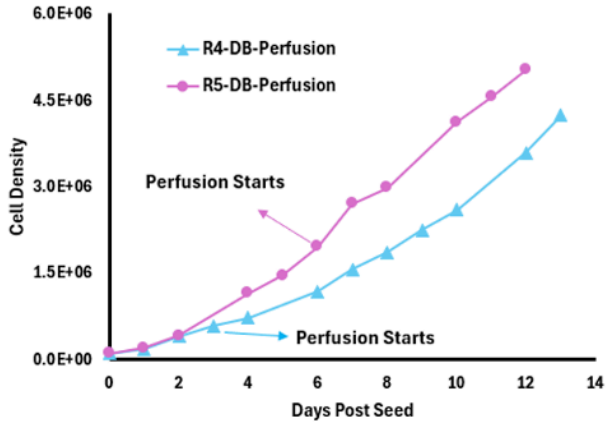
Figure 6. Expansion graphs displaying (A) cell density of the non-perfused runs, (B) glucose and lactate FLEX2 metabolite measurements (solid line = glucose, dashed line = lactate), and (C) summary of culture conditions and NK expansion yields, including fold expansion of the three runs showing improved cell yield using a fed-batch medium exchange strategy over batch culture.

Glucose and lactate were sampled throughout the processing days using the FLEX2 metabolite analyzer. Glucose consumption and lactate accumulation correlated with cell expansion kinetics, where rapid glucose depletion indicated high metabolic activity. Lactate buildup reflected increased acidity, potentially leading to reduced culture health and a plateau in cell growth due to nutrient limitations. Plateaus often began when glucose approached 2.0 g/L and lactate exceeded 1.0 g/L, consistent with nutrient-limited growth kinetics (Figure 6B).

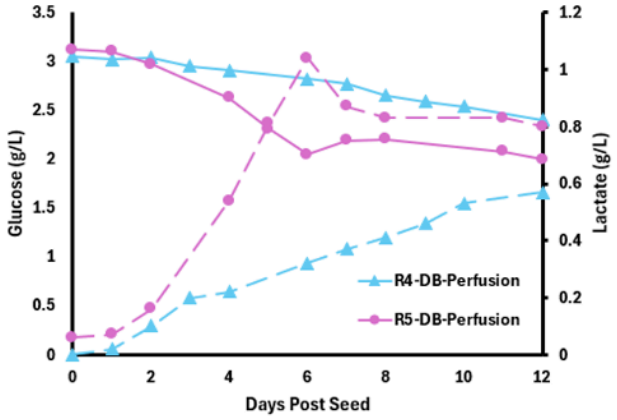
Perfusion Feeding Strategy

Two studies were conducted to evaluate the feasibility and impact of perfusion medium exchange on CAR-NK cell expansion in VWBs using the same NK donor in both studies to ensure consistency. The two studies employed different perfusion initiation strategies, fixed and responsive. In the first study, perfusion was initiated three days after cells were seeded into the vessel and in the second study, initiation began once the culture reached a cell density of 2×10^6 cells/mL and/or glucose levels dropped to 2.0 g/L. In both studies, the perfusion rate was maintained consistently at 0.5 VVD from initiation through culture harvest.

A



B



C

Run	Donor	Feeding Strategy	Perfusion Strategy	ID	Culture Duration (days)	Seeding Density (cells/mL)	Total Viable Cells (D12)	Fold Expansion (D12)
R4	DB	Perfusion	Fixed	▲	13	1.0×10^5	1.1×10^{10}	36
R5	DB	Perfusion	Responsive	●	12	1.0×10^5	1.5×10^{10}	46

D

Run	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13
R4	PBS-3 Seed	Expansion		Perfusion Starts D3 (0.5 VVD - 1.5L/day)										Harvest
R5	PBS-3 Seed	Expansion					Perfusion Starts D6 (0.5 VVD - 1.5L/day)						Harvest	

Figure 7. Perfusion operation strategy. (A) Cell density of Run 4 (R4-DB-Perfusion) and Run 5 (R5-DB-Perfusion) with indicated perfusion start, (B) glucose and lactate trends (solid line = glucose, dashed line = lactate), (C) summary of culture conditions and NK expansion yields, and (D) perfusion schedule (VVD = Vessel Volume per Day).

Optimization of the perfusion schedule between Run 4 and 5, using cell density and metabolite levels as better indicators for initiating perfusion medium exchange rather than relying solely on culture day, led to improved cell expansion. This approach allowed perfusion to be initiated only when needed, enhancing media utilization efficiency (Figure 7A). The initial increase and subsequent decrease of lactate levels observed in Run 5 align with the start of perfusion (Figure 7B). Importantly, no significant changes in cell health were observed before and after perfusion initiation, suggesting that the ATF flow rate of 0.4 L/min is gentle on NK cells (Figure 8).

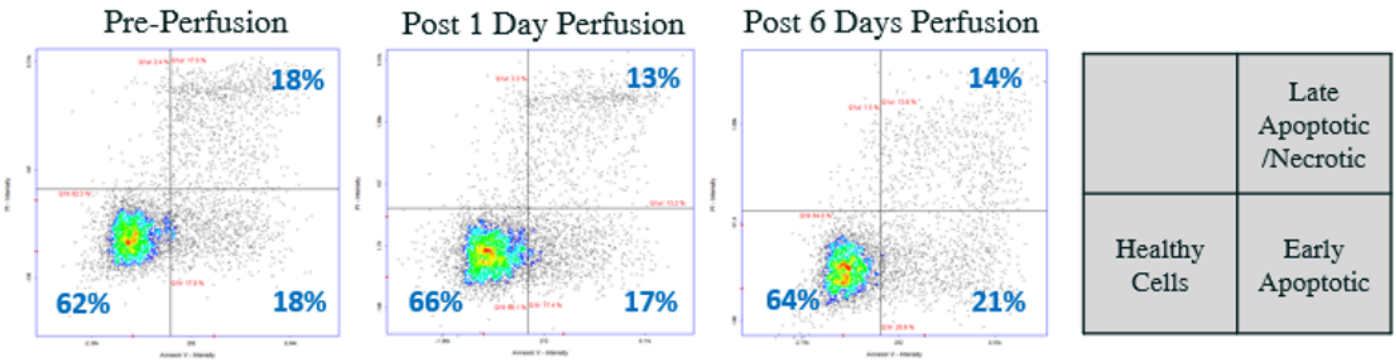


Figure 8. R5-DB-Perfusion (Run 5). Cell health assessment of pre- and post-perfusion initiation demonstrates similar cell health before and after perfusion.

In this study, cells harvested from the PBS-3 VWB demonstrate greater CAR-NK cell potency compared to a static G-Rex control condition (Figure 9), with G-Rex samples collected at their peak expansion. Cells expanded in the G-Rex vessel were used throughout the study as a batch static large-scale comparator. These findings suggest that the PBS-3 may support various medium exchange strategies and improved functional outcomes.

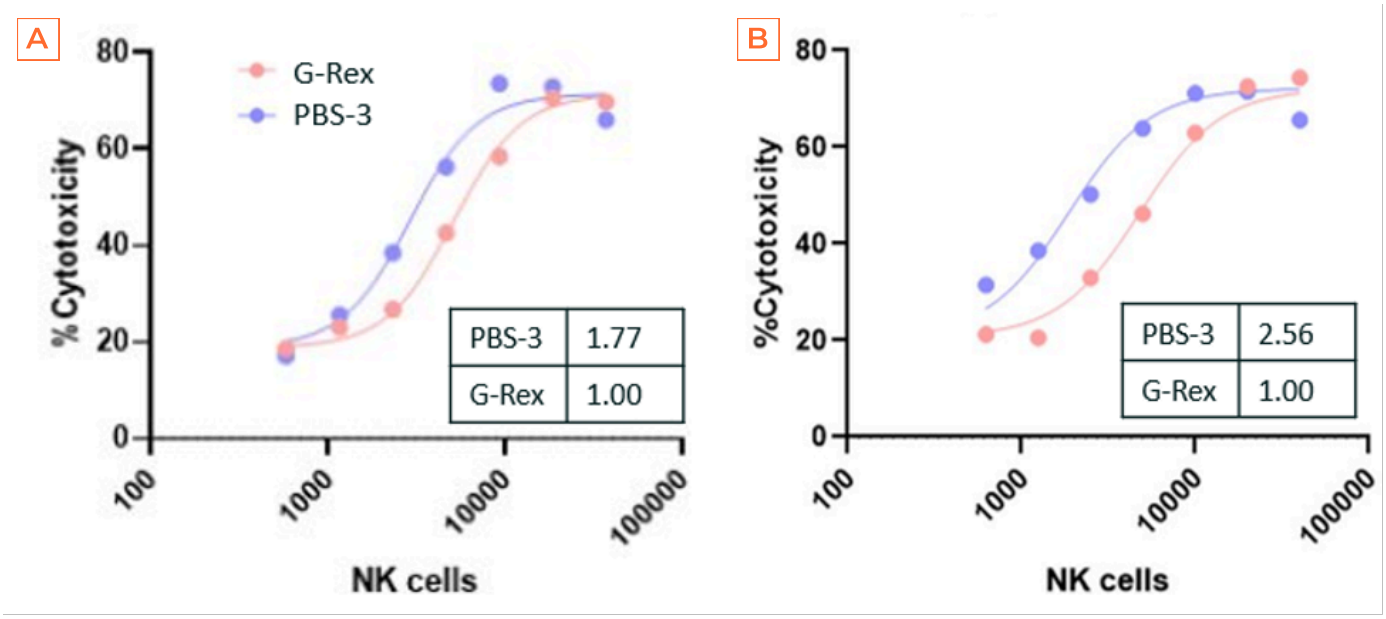


Figure 9. Relative potency (RP) of cells from static G-Rex and R5-DB-Perfusion. (A) RP of PBS-3 and G-Rex condition seven days post seed and (B) RP of PBS-3 and G-Rex condition eight days post seed.

This case study highlights the scalability of CAR-NK cell expansion in PBS VWBs. The use of the PBS-0.1 VWBs provides a robust small-scale model for early process development, facilitating rapid optimization with minimal resource consumption. Due to the linear scalability between the PBS-0.1 and PBS-3 systems, growth kinetics and culture behavior observed at small scales can be reliably translated to mid-scale runs, ensuring a more streamlined and efficient scale-up process (Figure 10A).

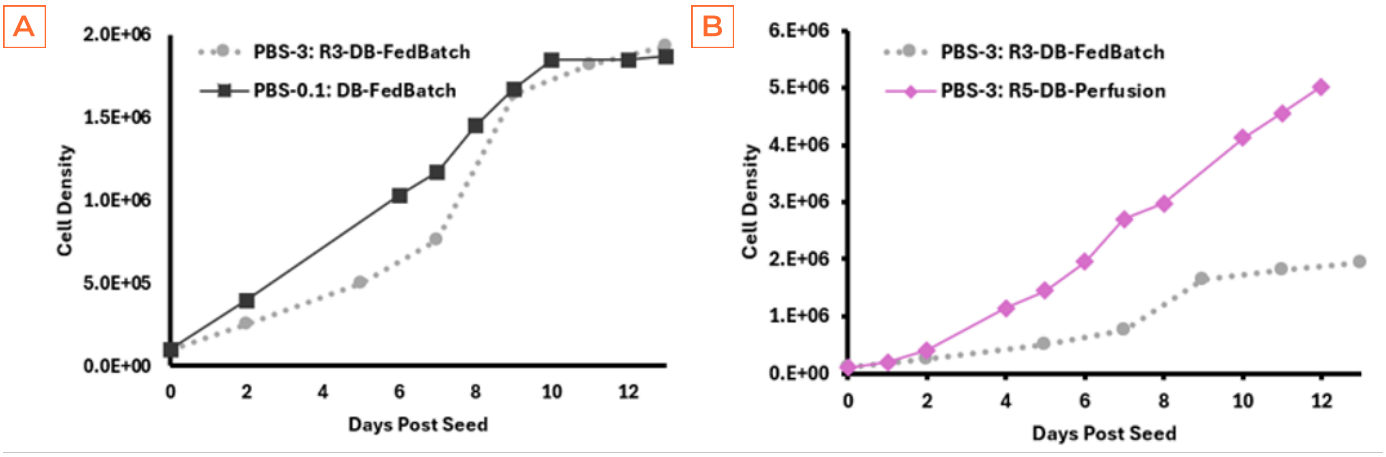


Figure 10. CAR-NKcell density using VWBs using single donor (Donor B). (A) Fed-batch runs with similar growth kinetics in both PBS-3 and PBS-0.1. (B) Fed-batch and perfusion run in PBS-3 demonstrates improved cell yield using perfusion medium exchange strategy.

Perfusion culture yielded a 2.6-fold higher expansion than the fed-batch run from the same donor, highlighting its advantage in achieving substantially greater cell yields (Figure 10B). These findings highlight the benefits of perfusion in supporting robust cell growth and promoting a more productive culture environment. While not measured in this study, ammonia is another metabolite known to accumulate during high-density cell culture and can negatively impact cell viability and proliferation. Future users of the PBS system may benefit from monitoring a broader range of metabolites, such as ammonia, to better inform perfusion timing and rate adjustments. In addition, greater expansion outcomes may be achievable with further optimization of both culture conditions and perfusion parameters.

Conclusion

PBS Vertical-Wheel Bioreactors integrated with XCell Alternating Tangential Flow perfusion enabled scalable, high-yield, and potent expansion of CAR-NK cells and demonstrates strong potential to accelerate the clinical translation of a wide range of engineered immune-cell therapies. The VWB platform provides a low-shear, uniform mixing environment with the flexibility to integrate auxiliary systems, while the ATF enables gentle, continuous perfusion to maintain glucose levels and remove lactate. Together, these complementary technologies outperform batch and fed-batch modes and establish a robust, scalable foundation for advanced immune-cell manufacturing.