# Large-Scale Non-Viral Genetic Modification and Feeder-Free Production of CAR-NK Cells

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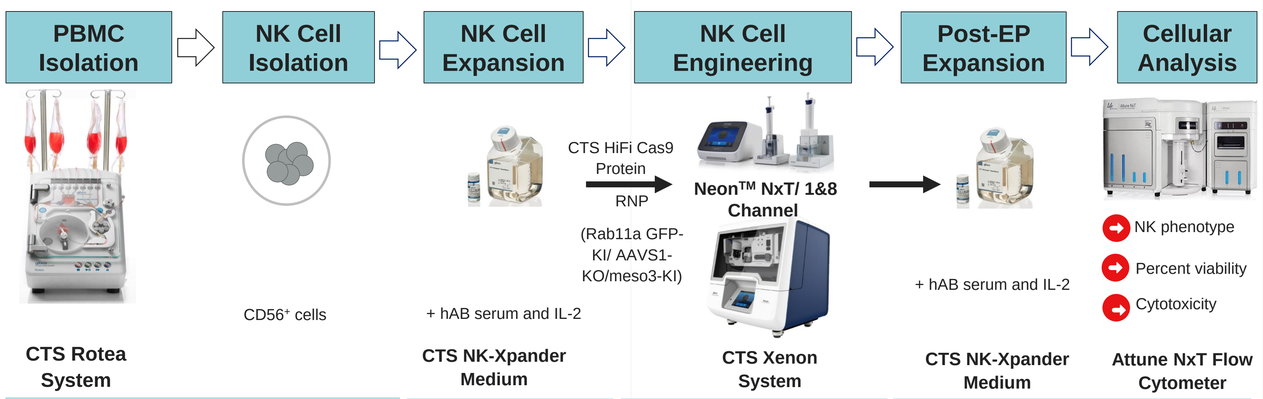
## Introduction

Genetic engineering is a major driving force in advancing adoptive immunotherapy, a promising approach for treating cancers. In particular, engineering primary human natural killer (NK) cells holds significant potential owing to their potent antitumor properties and demonstrated safety in an allogeneic applications. NK cells function independently of human leukocyte antigen (HLA) matching, eliminating the risk of graft-versus-host disease in allogeneic settings. As a result, they offer the potential to be safer, more cost-effective, and possibly more efficient than current autologous T-cell therapies. One of the key challenges faced by the cell and gene therapy industry is the ability to efficiently expand, gene edit and process NK cells at clinically relevant scales. Additionally, there is a need for regulatory compliant reagents and automated instruments. Here we have addressed these pain points and developed optimized protocols for non-viral based NK cell engineering and feeder free production of CAR-NK cells.

CTS™ NK-Xpander™ Medium used in this work is capable of producing high yield of hNK cells without the need for feeder cells and it also enables robust expansion of enriched NK cells from qualified donor derived PBMC's. The Gibco™ CTS™ Rotea™ system was used for PBMC isolation and, for wash, concentration and buffer exchange milestones. The Neon™ NxT and Gibco™ CTS™ Xenon™ Electroporation Systems along with CTS™ HiFi™ Cas9 protein and TrueGuide™ synthetic gRNA were used for gene editing milestones. The workflow and protocols developed through this work addresses research use to clinical scale needs for engineered NK cell manufacturing while maintaining high cell viability and/or recovery pre and post *ex vivo* genetic modification steps. Furthermore, combining Rotea and Xenon systems can help overcome some of the challenges faced with manual cell processing and viral based cell engineering protocols.

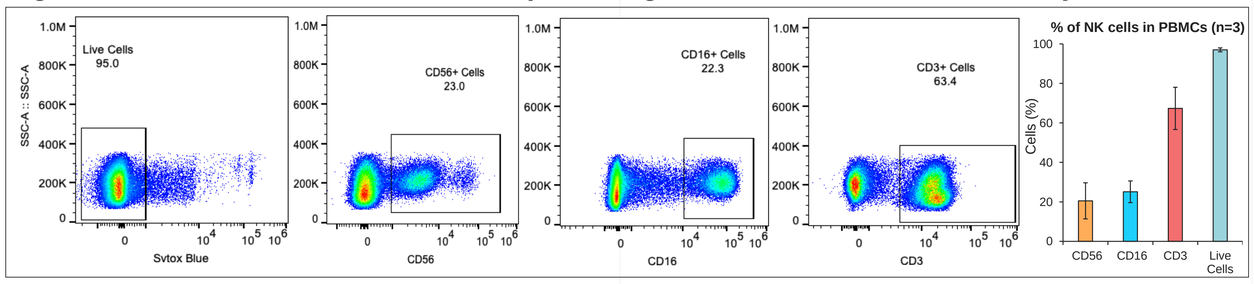
## Materials and Methods

Human PBMCs were isolated from Leukopak using Rotea. Human NK cells were enriched from PBMCs using negative isolation kit. Isolated NK cells were cultured with NK Xpander media supplemented with human serum and IL-2. On day 6, cells were electroporated with Neon NxT/Xenon to deliver CRISPR-Cas9 protein, gRNA and donor DNA. For gene knock in applications either a GFP or CAR construct was used to target Rab11a or AAVS1 locus and CRISPR/Cas9 RNP system was used for all editing experiments. Post-EP cells were cultured for 20 days and analyzed by Attune™ flow cytometry over multiple time intervals.

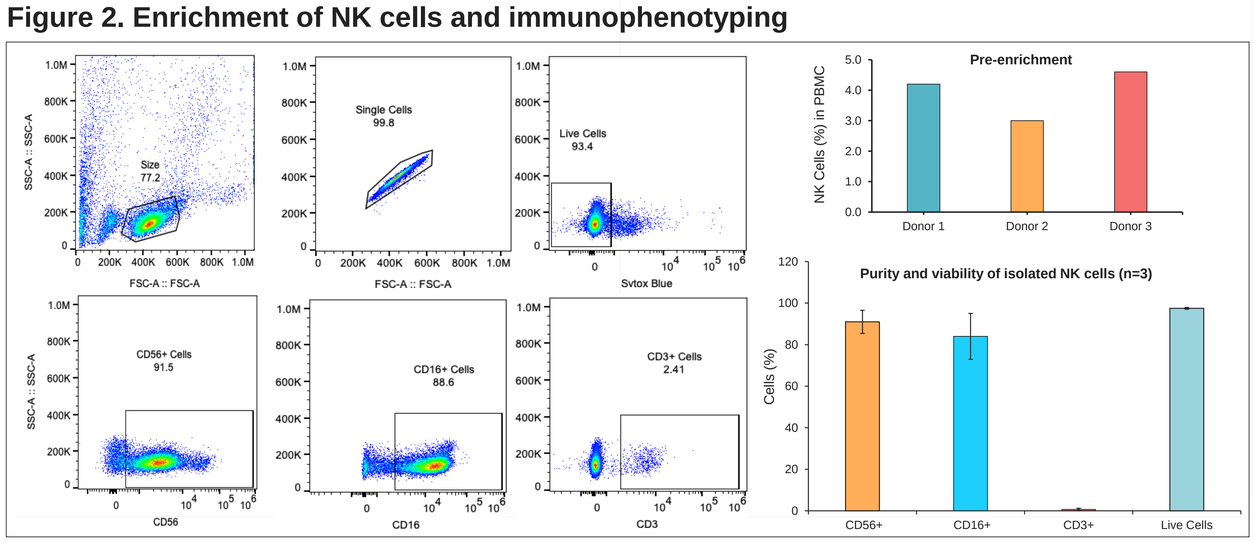



## Results

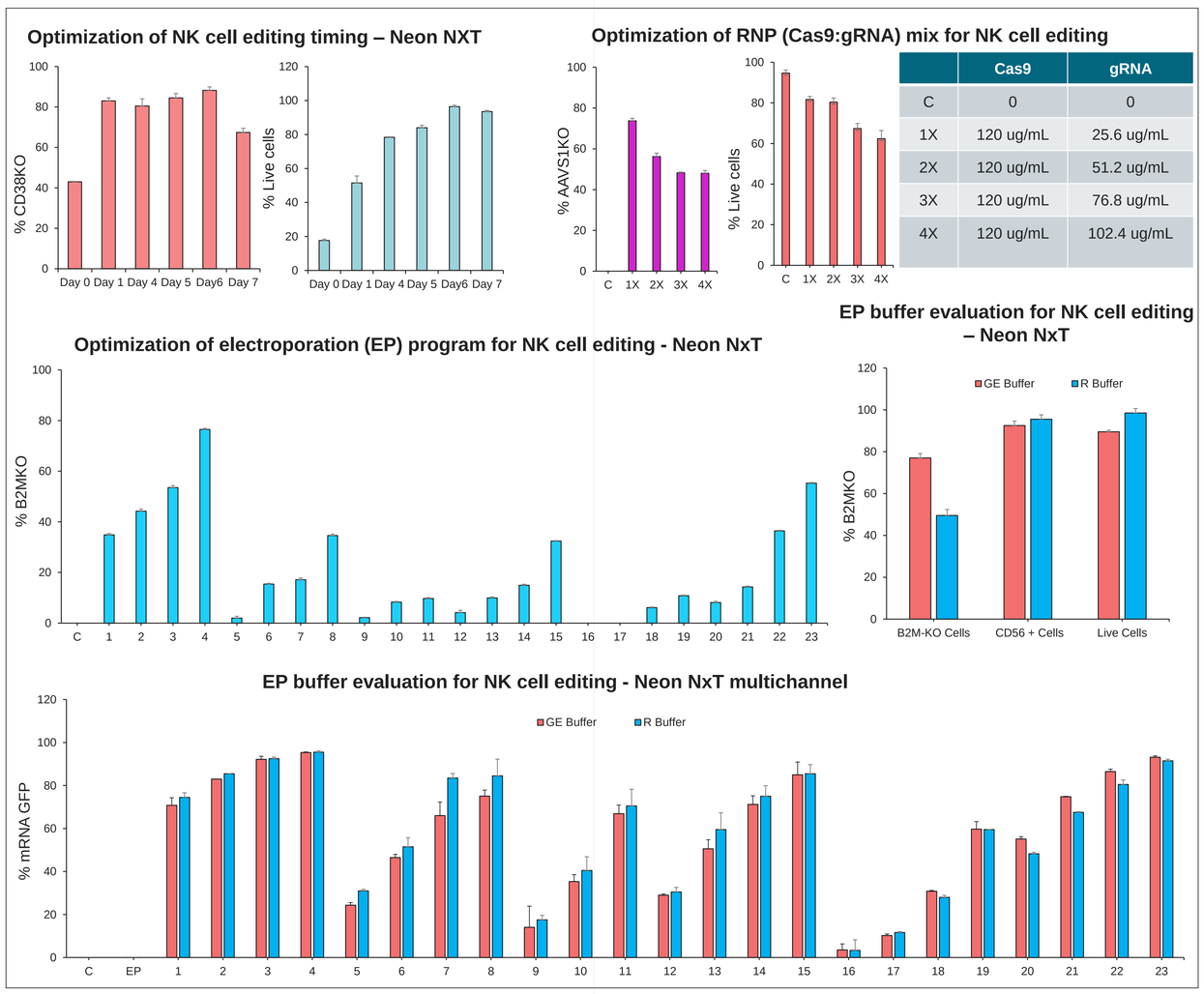
### Figure 1. Isolation of PBMC from leukopak using CTS™ Rotea™ counterflow system




### Figure 2. Enrichment of NK cells and immunophenotyping

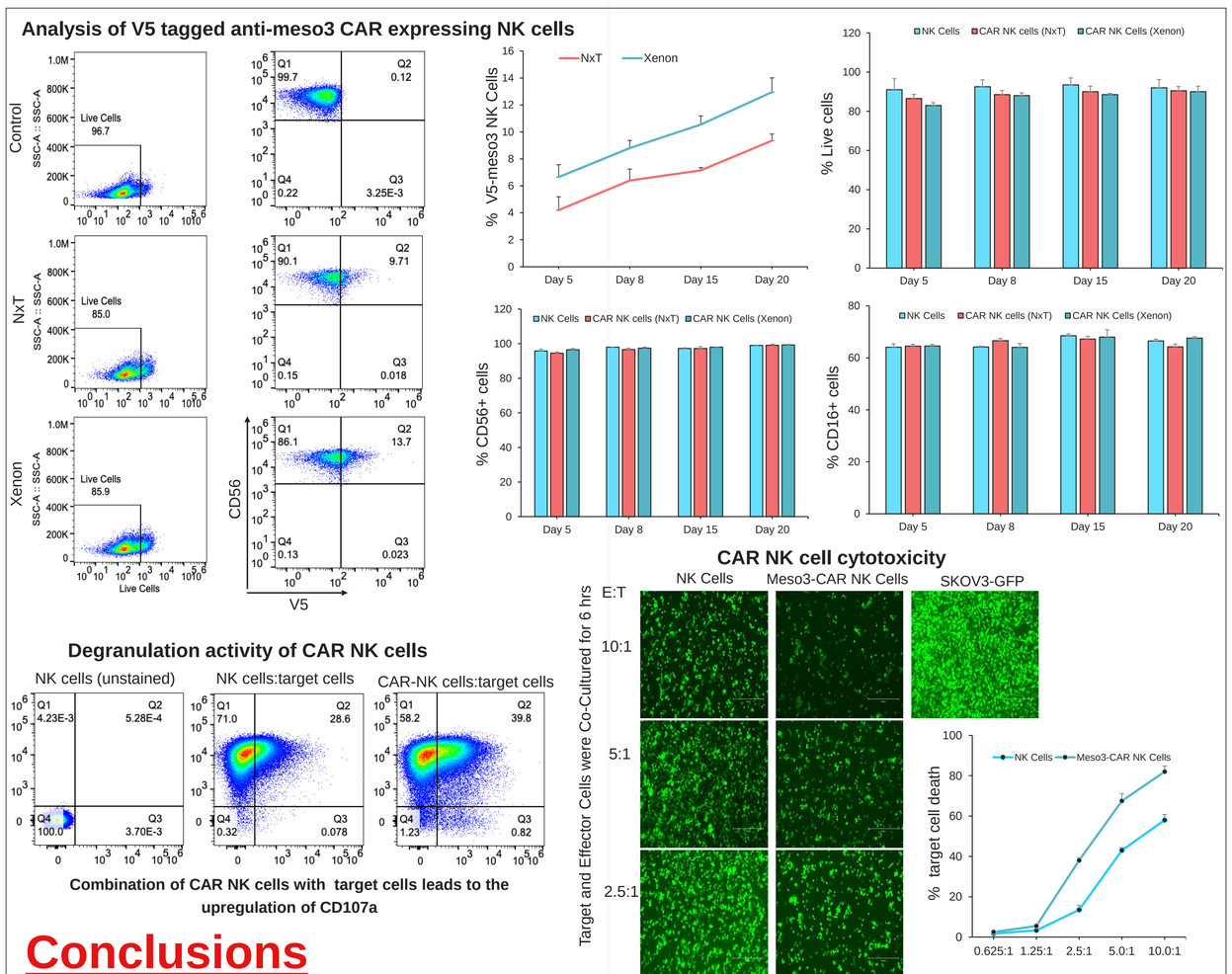



### Figure 3. Optimizing gene delivery & editing using Neon NxT single & multichannel




| | Cas9 | gRNA |
|---|---|---|
| C | 0 | 0 |
| 1X | 120 ug/mL | 25.6 ug/mL |
| 2X | 120 ug/mL | 51.2 ug/mL |
| 3X | 120 ug/mL | 76.8 ug/mL |
| 4X | 120 ug/mL | 102.4 ug/mL |

### Figure 4. Generation of V5 tagged anti-mesothelin CAR NK cells – Scaling from research use platform (Neon NxT) to GMP compatible CTS Xenon Electroporation System




## Conclusions

- PBMCs were successfully isolated using the CTS™ Rotea system.
- Neon™ NxT 8 channel system delivered GFP mRNA into NK cells with over 90% expression.
- The CTS™ Xenon and Neon™ NxT Electroporation Systems along with CTS™ HiFi Cas9 showed around 15-25% KI efficiency with fully non viral protocol using either ss-DNA Rab11a GFP (data not shown) or ss-DNA meso3 payloads
- The edited NK cells/CAR NK cells expanded well in CTS™ NK-Xpander Medium and maintained relevant NK cell phenotype, viability and functionality.
- CAR NK cells were able to kill around 80% of target cells (SKOV3) within 6 hours of co-incubation.
- Data demonstrated here used modular approaches incorporating Rotea and Xenon instruments to manufacture clinically relevant CAR-NK cells

**CAR NK cells expanded in CTS NK-Xpander Medium can efficiently kill SKOV3 ovarian cancer cell line.**



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