Lonza
Specialized Modalities

Large-volume non-viral genetic modification using the 4D-Nucleofector® LV Unit in cell and gene therapy

Selected publications

Our customers' work teaches us that large-volume transfection is a relevant tool throughout the whole cell and gene therapy pipeline, be it during earlier research, pre-clinical phases or for the manufacture of an ATMP.

The ability to scale up undoubtedly becomes a requirement though when moving closer to the clinical translation of a cell and gene therapy. Furthermore, it gets increasingly important to document that the raw materials used in a process are compatible with manufacturing a therapeutic to meet GMP guidelines and ensure the patient's safety.

The 4D-Nucleofector® LV Unit can help you address these challenges, no matter if you are still in research or advancing towards the clinic or commercialization. The system allows for closed and scalable transfection of up to 1 billion cells and comes with consumables for research use and GMP manufacturing as well as a 21CFR part11 compliant software.

Researchers from around the globe have trusted the 4D-Nucleofector® LV Unit to efficiently modify large volumes of cells in their non-viral, *ex vivo* cell and gene therapy processes. The use of the technology spans from research to clinical phases and covers approaches including mRNA, CRISPR knock-out, base or prime editing and more.

With this overview on selected publications, we aim to provide insights into these researchers' work and achievements. Discover the short summaries of these publications below and find out more about the genetic modification approaches that are used by your peers.

Are you using the 4D-Nucleofector® LV Unit and are happy to share your data with the Nucleofector® Community?

Get in touch with us, we would highly appreciate hearing back from you. How are you using the technology? What challenges do you face and what successes have you achieved?

Contact our Scientific Support Team.

4D-Nucleofector® LV Unit and base-editing

Chiesa *et al*. (2023) Base-edited CAR7 T cells for relapsed T-Cell acute lymphoblastic leukemia. *N Engl J Med* 389(10):899-910

Phase:	Clinical trial phase I
Disease:	Acute lymphoblastic leukemia (ALL)
Treatment:	CAR-T cells (allogeneic)

Genetic modification approach:
The authors' genetic modification approach combines inactivation of CD52, CD7 and TCR beta chain via base editing (cytidine deamination) to prevent graft-versus-host disease, followed by lentiviral integration of a CD7-specific CAR. For base-editing, 2-day activated T cells from healthy donor PBMCs were transfected with coBE mRNA (encoding the base-editor protein: a catalytically impaired Cas9 nickase fused to a rat-derived single-stranded DNA deaminase and a uracil glycosylase) and sgRNA targeting both TRBC1 and TRBC2 using the 4D-Nucleofector® LV Unit. On the next day, cells were transduced for CAR insertion and cultured for another 10 days until cryopreservation. Knock-out of CD52, CD7 and TCR was between 92 and 99%.

In a phase I trial, the safety of these allogeneic CAR-T cells was investigated in 3 children with relapsed ALL. The study shows proof-of-concept for use of base-editing as therapeutic approach but also points to complications expected for immunotherapy.

Woodruff *et. al.,* (2023) Large-scale manufacturing of base-edited chimeric antigen receptor T cells. *Mol Ther Methods Clin Dev* 31:101123

Phase:	Preclinical
Disease:	N/A
Treatment:	CAR-T cells (allogeneic)

Genetic modification approach:
The researchers knocked-out PD-1 in healthy donor T cells using base-editing and successive lentiviral transduction with an anti-CD19 CAR. For base editing, they compared the use of linear mRNA with circular mRNA. After proof-of-concept in small scale, they transferred the application to larger scale to manufacture a clinical dose of 1×10^8 CAR-T cells. In brief, after a 2-day activation stimulated PBMCs or $CD4^+/CD8^+$ T cells at a concentration of 5×10^7 mL were transfected with different amounts of BE4max circular RNA and sgRNA targeting PD-1. One day post Nucleofection®, cells were transduced with anti-CD19 CAR and then further cultured for 9 days. Depending on the circular mRNA amount used base conversion was between 86% and near complete conversion.

The authors showed at clinical scale that when using circular mRNA higher editing efficiencies can be achieved with 8x less RNA, thus leading to significant cost reduction.

Georgiadis *et. al.,* (2021) Base-edited CAR T cells for combinational therapy against T cell malignancies. *Leukemia* 35(12):3466-3481

Phase:	Preclinical
Disease:	T-cell acute lymphoblastic leukemia (T-ALL)
Treatment:	CAR-T cells (allogeneic)

Genetic modification approach:
In this publication the group from UCL knocked out TCR/CD3 and CD7 via base editing (or CRISPR NHEJ) prior to lentiviral introduction of a CAR. The knock-out of CD3 and CD7 was aimed to avoid so called "fractricide" when treating T cell malignancies. In brief, PBMCs were isolated from healthy donors and activated. Activated T cells were then transfected with sgRNA targeting the TCR constant β chain (TRBC) and CD7 together with either SpCas9 mRNA or coBE3 dCas9 mRNA using the 4D-Nucleofector® LV Unit. The cells were transduced, 24 hours later, with lentiviral vectors either expressing a CAR targeting CD3 (3CAR) and CD7 (7CAR). Results showed robust CAR expression and efficient reduction of surface expression for TCRαβ/CD3 and CD7 in the range of 50 to >95% for single knock-out and 37% to 60% for double knockout. Co-culture of 3CAR and 7CAR cultures led to 'self-enrichment', yielding to populations with 99.6% of TCR−/CD3−/CD7− cells. It was shown that those cells revealed specific cytotoxicity, no problematic off-target activity or base conversion. The 3CAR and 7CAR T cells were also analyzed in humanized mouse xenograft models for anti-leukemic potency.

4D-Nucleofector® LV Unit and CRISPR-based knock-out

Ottoviano *et. al.,* (2022) Phase 1 clinical trial of CRISPR-engineered CAR19 universal T cells for treatment of children with refractory B cell leukemia. *Sci. Transl. Med.* 14, eabq3010

Phase:	Clinical trial phase I
Disease:	B cell acute lymphoblastic leukemia (B-ALL)
Treatment:	CAR-T cells (allogeneic)

Genetic modification approach:
The authors used a combination of lentiviral introduction of both, a CAR targeting CD19 and CRISPR gRNA targeting TRAC and CD52, respectively, with non-viral transfer of Cas9 mRNA. In brief, PBMCs from a healthy donor were activated for 1 day and transduced with a lentiviral vector

encoding the anti-CD19 CAR gene and sgRNAs targeting TRAC and CD52 for multiplex editing. Three days later, the cells were transfected with Cas9 mRNA using the 4D-Nucleofector® LV Unit. Post transfection, cells were further expanded and after another 7 days residual TCRαβ expressing T cells were depleted via magnetic beads. With this approach a CAR19 expression of 86 to 98% was achieved with a residual 0.1 to 1 % TCR expression.

Using this approach, six children with refractory/relapsed CD19-positive B-ALL were treated in a phase I clinical trial. The study showed feasibility, safety and therapeutic potential of CRISPR-based immunotherapy. Two of these six are in remission.

Vazquez-Lombardi *et. al.,* (2022) High-throughput T cell receptor engineering by functional screening identifies candidates with enhanced potency and specificity. *Immunity* 55:1953–1966

Phase: Research
Disease: N/A
Treatment: TCR

Genetic modification approach:
The aim of this study was to develop a method to identify engineered T cell receptors (TCRs) with high potency and specificity. The established high-throughput method, called "TCR engine", is based on a combination of CRISPR genome editing, computational TCR design and deep sequencing. In brief, a TCR-accepting T cell line (TnT) was generated by modifying Jurkat cells via CRISPR-Cas using the small scale 4D-Nucleofector® X Unit. In five successive steps Cas9, human CD8, NFAT-GFP and mRuby were integrated via HDR and endogenous CD4, TCRα and Fas were deleted via NHEJ. After each step, cells were sorted by FACS and clones were expanded and validated by flow cytometry and sequencing. Then, a combinatorial HDR template library was designed comprising of >30 mutation variants of a TCR targeting MAGE-A3 (TCR-A3). Together with a gRNA targeting the endogenous TCRβ region, this library was transfected into 100 million TnT cells using the 4D-Nucleofector® LV Unit. Those cells carrying reconstituted TCRs did undergo a comprehensive selection procedure to identify TCR variants with enhanced target recognition. Selected synthetic TCR-A3 variants were further characterized in primary T cells by transfecting those in small scale for CRISPR-based knock-out of endogenous TCR and knock-in of TCR-A3 variants into the TRAC locus. TCR variants were assessed for safety and efficacy via multiple *in vitro* and *in vivo* assays.

Lattanzi *et. al.,* (2021) Development of β-globin gene correction in human hematopoietic stem cells as a potential durable treatment for sickle cell disease. *Science Translational Med* 13(598):eabf2444

Phase: Preclinical (IND-enabling study)
Disease: Sickle cell disease (SCD)
Treatment: Gene correction

Genetic modification approach:
The researchers used a combination of non-viral, electroporation-based delivery of CRISPR RNP and recombinant adeno-associated virus mediated delivery of the HDR donor template to correct point-mutated β-globin (HBB) gene causing sickle cell disease (SCD). In brief, healthy and SCD patient CD34⁺ hematopoietic stem cells were transfected at small scale and medium scale with an RNP targeting HBB for knocking out the mutated sequence.

Immediately after Nucleofection®, cells were plated and transduced with rAAV6 to knock-in the corrected sequence. At clinical scale, up to 60% HBB allelic correction was achieved. Upon transplantation into NSG mice a 20% gene correction was observed. Besides high frequency gene correction, long-term engraftment of HBB gene-corrected cells in mice and absence of genotoxicity and tumorigenic potential was shown.

The focus of this study was to demonstrate efficacy, safety, and clinical-scale manufacturing feasibility.

4D-Nucleofector® LV and virus production

Nawandar *et. al.*, (2022) Human anelloviruses produced by recombinant expression of synthetic genomes (bioRxiv preprint)

Phase: Research

Disease: N/A

Genetic modification approach:
Anelloviruses seem to have high prevalence in humans with low immunogenic effect and do not cause disease. Therefore, the development of gene therapies based on these viruses could provide a promising alternative to other commonly used viruses, also allowing for re-dosing. So far, their biology hasn't been studied extensively due to the lack of an *in vitro* system to produce such viruses. In this study, an effective recombinant production of two types of Betatorquevirus, one of the anellovirus genera, was achieved by transfection of the T-cell derived MOLT-4 cell line with a plasmid encoding the LY2 genome or nrVL4619 genome of this genus. In brief, after initial tests in small scale transfecting 10 million cells, larger scale production was performed via transfection of 2 billion cells with 2 mg of LY2 or nrVL4619 encoding plasmid in 20 mL flow-through process using the 4D-Nucleofector® LV Unit. Analyses involved viral gene expression, replication and imaging via electron microscopy as well as tissue-specific *in vivo* infectivity. This work enables further studies of this promising virus family.

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