mRNA-Lipid Nanoparticles Circumvent Limitations Associated With Both Viral Vectors and Electroporation for Cell and Gene Therapy Development

Driven by the blockbuster clinical successes of chimeric antigen receptor (CAR) T cell cancer immunotherapies, scientific and capital investment for these technologies is at an all-time high. It is estimated that the global cell and gene therapy market will reach US$ 45.4 billion by 2028 with many therapies progressing rapidly through a robust clinical pipeline; regulatory approval milestones for ten product candidates are expected in 2022 alone with additional therapies poised for market approval in the coming years [1]. With the addition of Legend Biotech and Janssen's CARVYKTI™ for multiple myeloma, there are now six FDA-approved CAR-T therapies since the landmark approvals in 2017, and these "living drugs" have revolutionized the standard of care for hematological cancers such as leukemia, lymphoma and multiple myeloma. In addition to increased investment in this highly transformative field, regulatory agencies have created expedited approval pathways (i.e., FDA's RMAT designation) to keep pace with the demand, which has concomitantly put pressure on therapy developers to drive down development timelines to bring therapies to market faster than ever before. The complex logistics of autologous therapies including long vein-to-vein times and high cost, has led to a desire to move towards allogeneic "off the shelf" therapies, where economies of scale can drive down production cost and timelines.

With promising results in many clinical trials using CAR-T, researchers are actively seeking to expand the current scope of this technology to address other indications in hopes of achieving the same therapeutic efficacy. However, current CAR constructs appear to have limited efficacy against solid tumor cancers as well as exhibiting undesirable adverse toxicities that require further development work, and novel approaches are needed to overcome them. More complex genetic modifications are being evaluated where T cells transduced with the CAR construct to create CAR-T cells, which are then further engineered to improve *in vivo* efficacy and toxicity (i.e., inclusion of suicide genes or molecular switches). In addition, other cell populations such as natural killer (NK) cells, γδ T cells, dendritic cells and macrophages armed with CAR constructs have shown promising animal and preclinical study results that may eventually expand the repertoire of engineered cell therapies against cancer.

The genetic modification of cells using gene delivery vehicles called vectors, subdivided into viral and non-viral classifications, play an integral role in the development of many immunotherapies (Figure 1). Predominantly, viral vectors based on modified retroviruses, lentiviruses, adenoviruses (Ads) or adeno-associated viruses (AAVs) are used as gene delivery systems for gene therapy and gene-

modified cell therapies like CAR-T[2-4]. Here, a patient's T cells are isolated via leukapheresis and genetically engineered *ex vivo* with viral vectors encoding the chimeric antigen receptor (CAR) transgenes designed to recognize tumor-specific antigens. These CAR-modified T cells are then re-infused back to the patient to target and kill antigen-expressing cancer cells *in vivo*.

Gene Delivery Systems
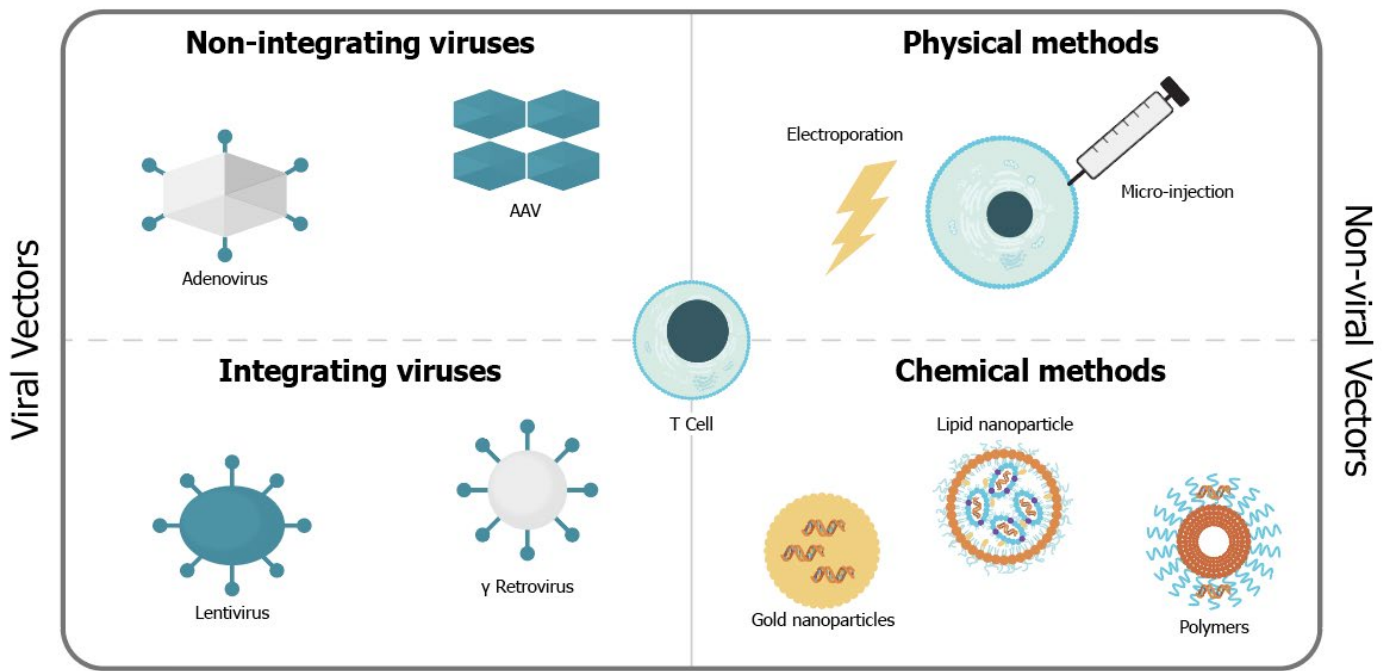
Figure 1. Gene delivery systems for gene editing of T cells are divided into viral and non-viral vectors. For viral transduction, integrating and non-integrating viral vectors are available. Non-viral delivery systems span physical methods like electroporation and microinjection while chemical methods include nanoparticles composed of gold, polymers, or lipids.

While viral vectors are a popular mode of gene delivery, because of their high transduction levels, there are multiple core challenges such as limitations in the gene cargo capacity (posing barriers to the co-delivery of multiple genes), the risk of patient immunogenicity and carcinogenicity, as well as the complexity and expense of large-scale production[2-5]. Additionally, certain cell types, like NK cells are not amenable to transduction by viral vectors. VSV-G lentivirus, classically used to engineer CAR-T cells, do not efficiently transduce NK-cells, which has hampered NK cell-based immunotherapy development efforts[6]. The increasing number and diversity of therapies in the pipeline combined with condensed timelines has greatly increased the demand for viral vectors that far exceeds the current production capacity and poses a roadblock for developing therapies[7]. Because of these limitations, alternative non-viral gene delivery systems such as electroporation and lipid nanoparticles offer advantages that can overcome these issues.

NON-VIRAL GENE DELIVERY SYSTEMS

Both physical and chemical non-viral gene delivery systems for *ex vivo* genetic modification offer advantages over viral vectors, including smaller scale production and the low risk of immunogenicity being just a few. Previously, low transfection efficiencies held non-viral methods at a disadvantage; however, recent advances in non-viral technologies have greatly improved transfection efficiencies, similar to that observed with viral vector-based mechanisms[8,9].

ELECTROPORATION

Electroporation is a widely used physical cell transfection method, used to move genes across the cell membrane where an applied electrical pulse temporarily disrupts the phospholipid bilayer of the cell membrane resulting in the formation of pores that allows a diverse range of cargo molecules to pass into the cell. Once optimal electroporation conditions are established, it can be easy to execute with versatility in payload size and utility across all cell types.

However, a major drawback of electroporation is the cellular cytotoxicity caused by the high voltage pulses that can induce changes to the lipid membranes, proteins, and DNA, and cause oxidative damage through the generation of reactive oxygen species[9]. Other harmful side effects of electroporation result from local pH changes, the release of toxic electrode products and temperature increase from Joule heating[10]. For autologous CAR-T therapies, the starting quantity of patient cells (owing to prior lymphodepleting treatments) is already limited and that coupled with low cell viability post-electroporation can pose significant challenges downstream[11]. In fact, these patient T cells may be less tolerant to the cytotoxic effects of electroporation *ex vivo* because of their pre-existing condition. Moreover, the heterogeneous cell permeabilization observed with electroporation can make precise dose control difficult to achieve, leading to non-uniform transgene expression[12]. As the industry shifts to more complex genetic modifications where T cells are required to undergo multiple rounds of genetic engineering, the challenges associated with post-electroporation mortality, loss of proliferative potential, and decreased potency reported for primary cell types will be difficult to reconcile[12,13]. Moreover, the consequences of electroporation-induced disruptions on global gene expression, cytokine production, lineage markers, and *in vivo* function requires further characterization, particularly in the context of primary cells for cell therapy[13]. From a commercial perspective, market-ready electroporation platforms are designed for small-scale R&D operation and have not demonstrated the scalability needed for industrialized manufacturing[11]. This has driven many in the field to investigate chemical methods like lipid nanoparticles which have proven to be a more consistent, scalable approach with high transfection efficiency and reduced perturbations of cellular function and viability for nonviral gene delivery into T cells for cell therapy applications.

LIPID NANOPARTICLES (LNPs)

Lipid nanoparticles (LNPs) have been extensively characterized and are the most clinically advanced non-viral vector system having several key advantages over electroporation and viral vectors. Owing to the recent successes of Moderna and BioNTech SARS-CoV-2 mRNA vaccines, which relies on lipid nanoparticles (LNPs) for mRNA delivery, and their utilization in the first FDA-approved RNAi therapeutic, ONPATTRO® (patisiran), the first small interfering RNA-based drug, LNPs have a proven history of *in vivo* clinical efficacy and safety. Combined, LNP technology is in a strong position for clinical

implementation in *ex vivo* gene-modification strategies as evidenced by emerging investigational candidates[8,14]. Intellia Therapeutics has several *ex vivo* candidates under early-stage clinical evaluation (Phase I/II) developed using an LNP-based CRISPR/Cas9 genome editing platform. Notably, OTQ923 and HIX763 (NCT04443907) are two *ex vivo* CRISPR-edited autologous hematopoietic stem cell therapies, in adults and children, developed in collaboration with Novartis, and NTLA-5001 (NCT05066165) is an *ex vivo* T cell receptor (TCR)-T cell therapy to treat acute myeloid leukemia (AML). Moreover, production platforms suited for clinical and commercial scale manufacturing of LNPs overcome scalability limitations of electroporation and are lower cost compared with viral vectors, make LNP's an attractive alternative to meet rising industry demand.
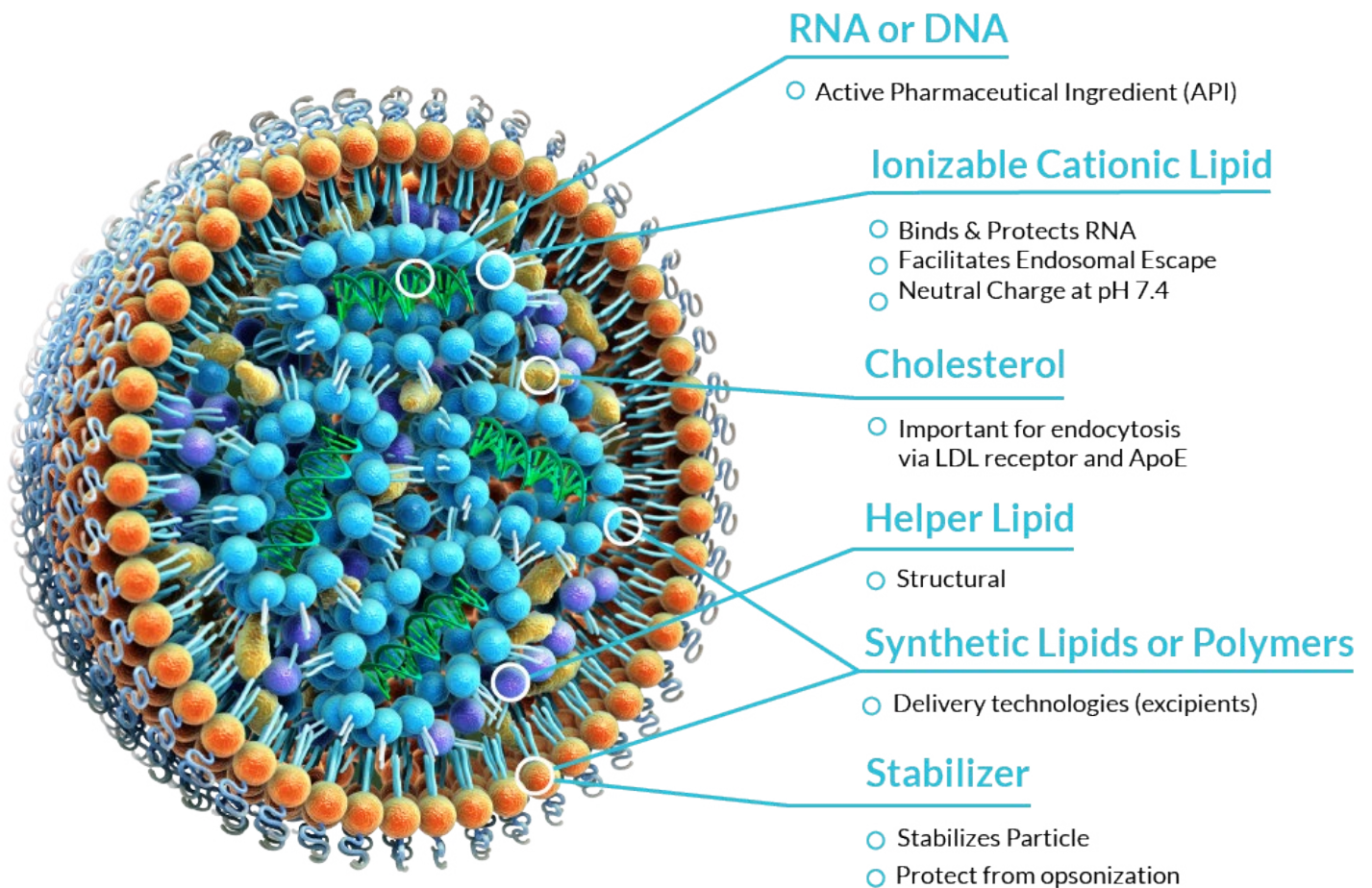

RNA or DNA
O Active Pharmaceutical Ingredient (API)

Ionizable Cationic Lipid
O Binds & Protects RNA
O Facilitates Endosomal Escape
O Neutral Charge at pH 7.4

Cholesterol
O Important for endocytosis via LDL receptor and ApoE

Helper Lipid
O Structural

Synthetic Lipids or Polymers
O Delivery technologies (excipients)

Stabilizer
O Stabilizes Particle
O Protect from opsonization

Figure 2. Detailed structure and the components that make up a lipid nanoparticle and their importance

LNPs are complex nanostructures that are composed of a mixture of lipid species, including cationic/ionizable lipids, helper lipids, cholesterol, and stabilizers (Figure 2). The ionizable lipids exhibit a positive charge at low pH, which facilitates nucleic acid encapsulation through electrostatic forces and enables endosomal escape and release of the genetic payload in the cytoplasm after they are internalized via receptor-mediated endocytosis[16]. The near neutral charge of LNPs at physiological pH makes them less toxic and immunogenic compared to cationic particles like polyplexes and lipoplexes and other delivery mechanisms[14-17]. The helper lipids, PEG-lipids and cholesterol are responsible for nanoparticle properties, such as particle stability, delivery efficacy, tolerability and biodistribution and it is their specific ratios along with the ionizable lipids that dictate the efficacy for a given cell type and application[14]. Cholesterol, in particular, plays an important role in cellular uptake via apoprotein E (apoE)-mediated association to membrane LDL receptors. Researchers have demonstrated the potential of LNPs for co-delivery of nucleic acids, which can overcome the payload size limitations associated with viral vectors and streamline workflows to accelerate the advancement of gene-modification strategies[18].

Technological solutions to support LNP production resulting in a highly reproducible and scalable system for large-scale gene delivery and gene editing, are key to meeting clinical demand now and in the future[19]. A comprehensive approach where specialized knowledge around the technology and an understanding of cell biology are needed to design and optimize multiple parameters to produce biocompatible LNP formulations to support *in vivo* and *ex vivo* gene modification strategies across many cell types for a diversity of payloads.

LNP REAGENTS OPTIMIZED FOR THE DELIVERY OF MRNA INTO ACTIVATED PRIMARY HUMAN T CELLS

With a recognized need in the cell therapy arena for an optimized, scalable platform for *ex vivo* T cell engineering to support CAR-T and other novel immunotherapies, Precision NanoSystems' GenVoy-ILM™ T Cell Kits for mRNA are LNP reagents optimized for the delivery of mRNA into activated primary human T cells, which are known to be difficult to transfect. The reagents are designed to easily integrate into any standard T cell culture workflow, to enable the rapid generation of mRNA encapsulated LNPs with minimal T cell manipulation using the NanoAssemblr® Spark™ and Ignite™ instruments and cartridges (Figure 3). Automated generation of mRNA-LNP's in this manner, provide valuable time savings over electroporation protocols, thereby expediting workflow execution and easy scale up of mRNA-LNP production from early-stage development to preclinical studies.
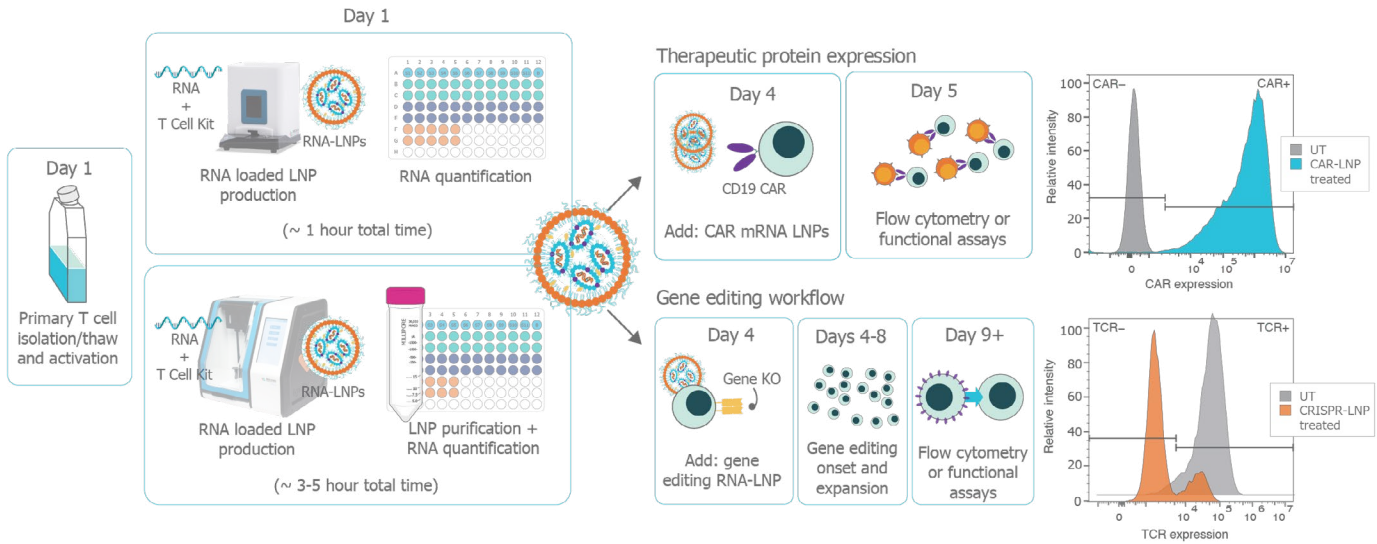
Figure 3. Schematic overview of a typical T cell workflow where the GenVoy-ILM T Cell Kits for mRNA on the NanoAssemblr Spark and Ignite can be utilized to produce CAR-T cells in a consistent manner.

LNP technology leverages endogenous receptor-mediated endocytosis for the delivery of genetic material into the cell. This method is gentle and well-tolerated by cells, resulting in a higher transfection efficiency and viable yield of engineered T cells with uniform gene expression compared to standard, highly manual electroporation processes (Figure 4).
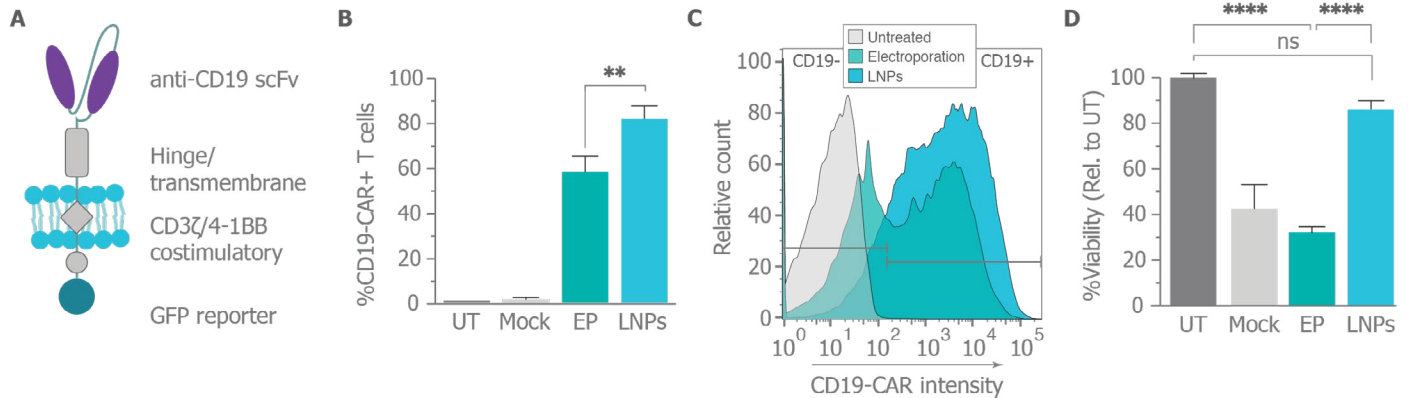
Figure 4. Lipid nanoparticles show higher transfection efficiency and high cell viability than electroporation in human primary T cells. A) Schematic illustration of the custom CAR mRNA construct. B) Percentage of CD19 CAR+ T cells at 24-h post RNA-LNP addition in human primary T cells. C) Surface expression of CD19 CAR as detected by flow cytometry. D) Percent cell viability normalized to untreated (UT) cells. One-way ANOVA analysis was conducted with ns p>0.05,**p<0.01, and **** p ≤ 0.0001.

Electroporation methods pose a significant barrier to support the increasing need for complex genetic manipulations, due to cytotoxicity challenges and resulting poor cell viability coupled with the lack of scalability (i.e., multi-step or multiplex gene transfection) to address new therapeutic targets more effectively (Figure 4)[20].
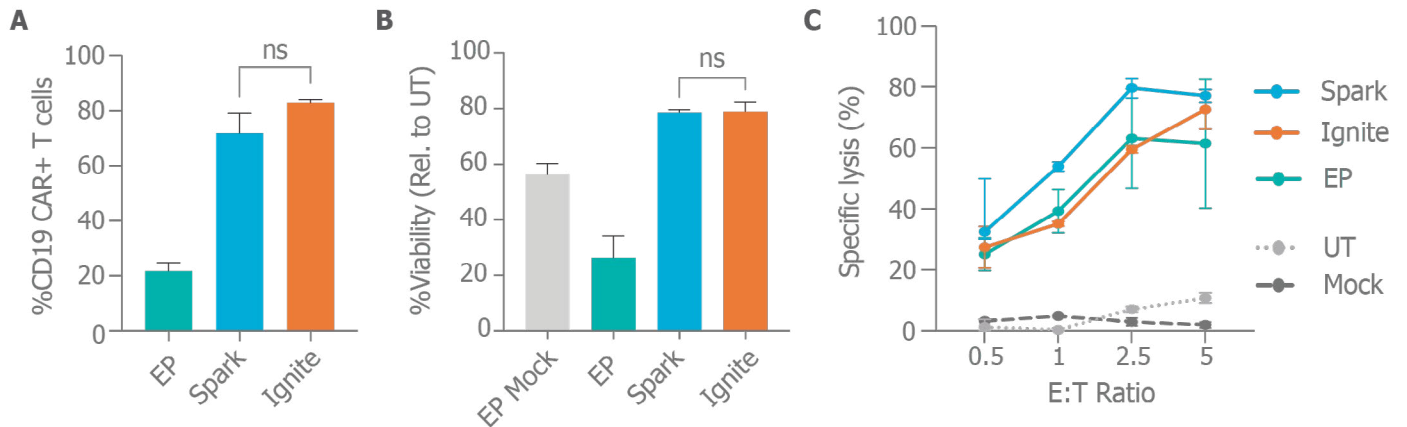
Figure 5. *Seamless scale-up of LNP manufacturing from discovery to pre-clinical stages of drug development from the discovery scale (NanoAssemblr Spark) to the preclinical scale (NanoAssemblr Ignite) while maintaining key performance metrics. A) Percentage of CD19 CAR+ T cells at 24 hours post RNA-LNP addition in human primary T cells. B) Percentage cell viability relative to untreated cells (UT). C) Tumor-specific T cell cytotoxicity was assessed. Anti-CD19 CAR-T cells (effector, E) were co-cultured with SUP-B15 cells (target, T) at indicated E:T ratios. The specific lysis (%) was determined by normalizing target cell viability to untreated SUP-B15 controls. One-way ANOVA analysis was conducted with ns p>0.05.*

mRNA-LNP production can be easily scaled for clinical and commercial manufacturing using advanced microfluidics technology such as the NxGen™, enabling easy tech transfer across instrument and reagent platforms. The produced CAR-T cells demonstrate excellent transfection efficiency, viability and leukemia targeted cytotoxicity (Figure 5).
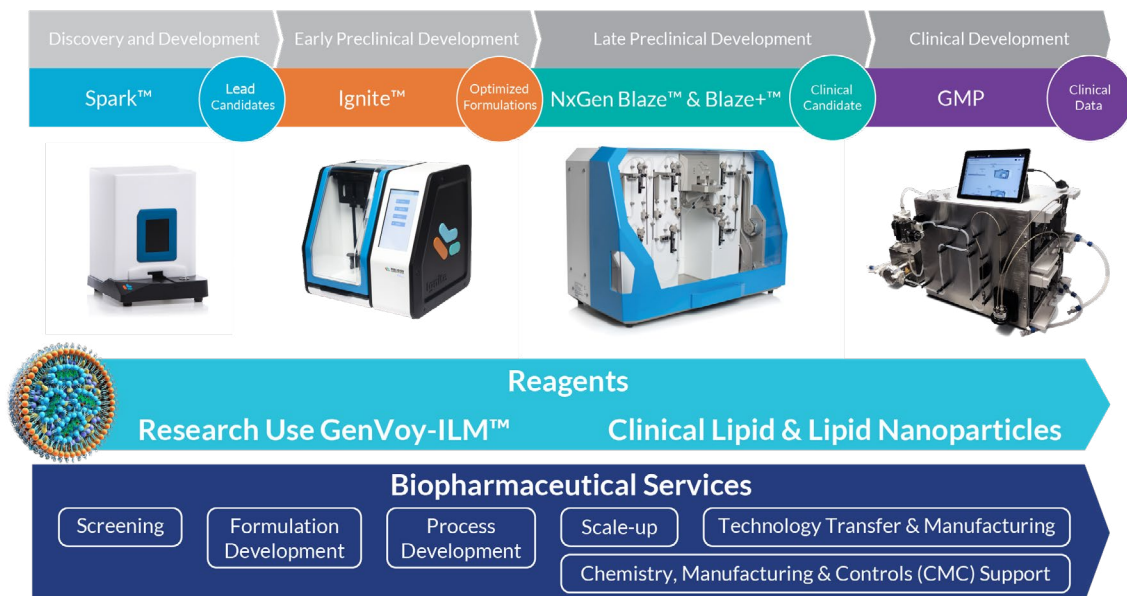
CONCLUDING REMARKS

The interest and demand for novel cell and gene therapies continues to grow as the field turns its focus to addressing new diseases and therapeutic targets. The complexity and scope of genetic engineering will only increase, yet the pressure to bring these novel therapies to market quickly and reduce costs remains high. As a clinically validated technology, LNPs are well-positioned to circumvent the limitations outlined here with regards to electroporation and viral vectors to successfully address a rapidly evolving clinical landscape where condensed timelines and increased market competition are a reality. LNPs can bring scalable, cost-effective gene delivery technology to support and expedite downstream development and optimization that can ultimately help bring CAR-T and other novel therapies to market faster to meet the growing clinical demand.

ABOUT PRECISION NANOSYSTEMS

Precision NanoSystems has years of expertise in LNP formulation, platform development and industry know-how accumulated through their active involvement in the novel COVID-19 mRNA vaccines that goes beyond just the LNP technology. This specialized knowledge and expertise paired with highly scalable instrument platforms and reagents can be leveraged by researchers and therapy developers looking to adopt LNP technology in the cell and gene therapy workflows at any stage from discovery to the clinic and beyond.

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