

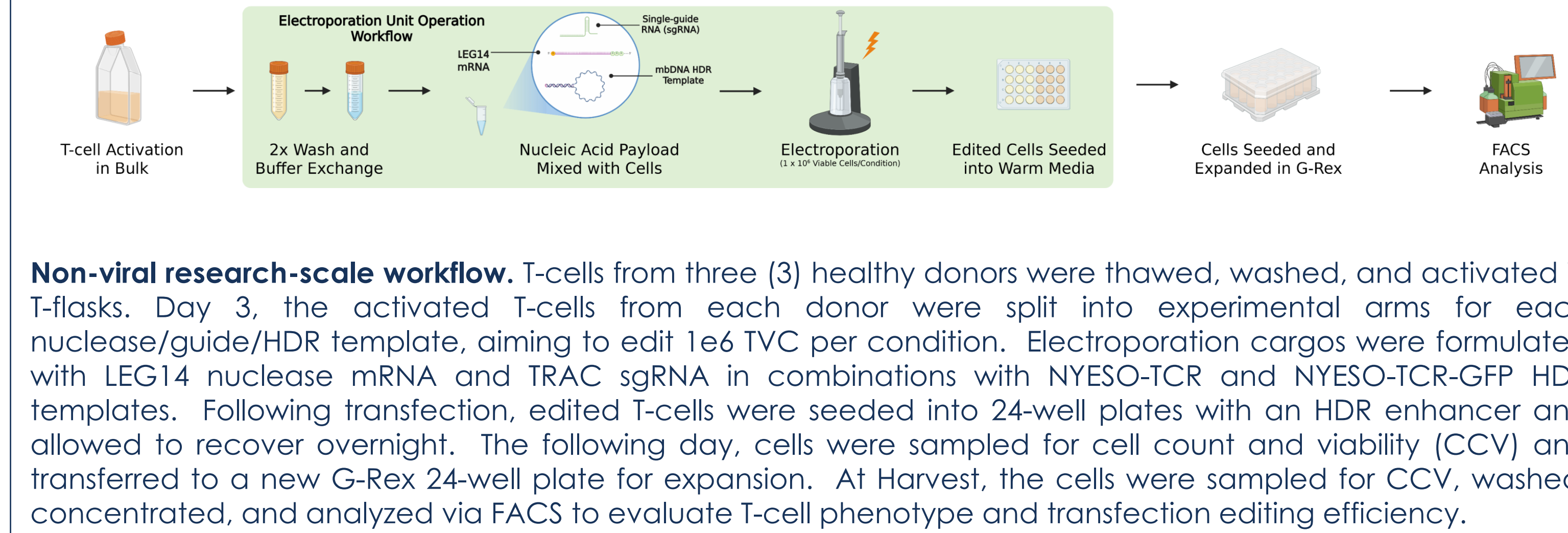
# Non-Viral TCR Knock-In Using LEG14 Nuclease and HDR Templates for T-Cell Engineering

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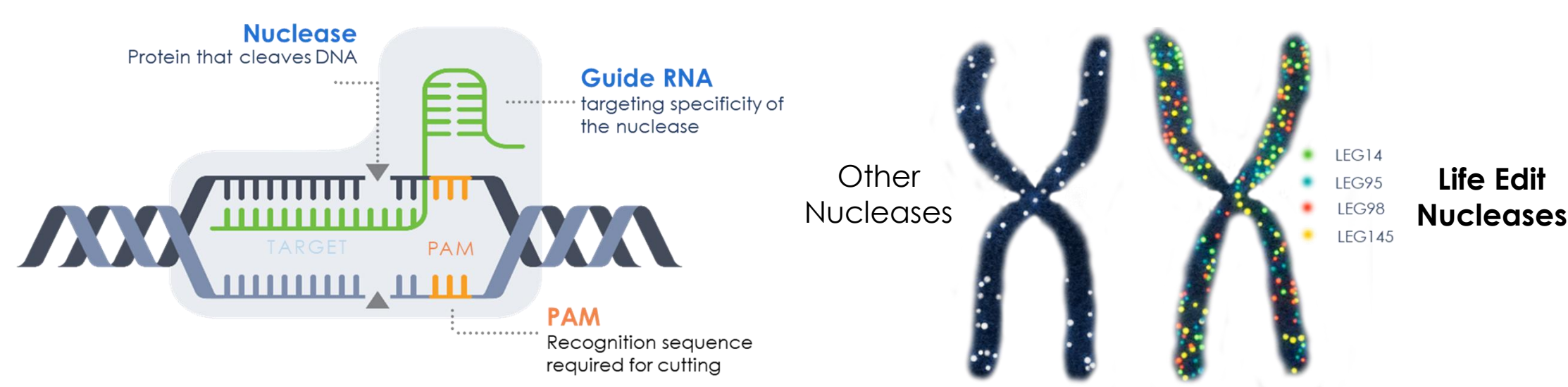
## Introduction

CRISPR based technologies offer some clear advantages to traditional viral based transduction methods. Most importantly the capability to much more precisely and efficiently engineer cells. This has led to a surge in the development of novel and engineered nucleases, each designed to overcome existing limitations and broaden the applicability of CRISPR-based therapeutics. We introduce an ElevateBio CRISPR nuclease, LEG14, a member of Life Edit's proprietary collection of Type II CRISPR nuclease system, offering versatile targeting capabilities for diverse gene editing applications. In this study, we used LEG14 nuclease to modify autologous T-cells. Specifically, we knocked out the endogenous TCR and inserted an exogenous TCR targeting the NYESO-1 antigen. The transgenic T-cell receptor was engineered using HDR-mediated target knock-in, with two circular donor DNA templates: Touchlight's mbDNA™ (MegabulbDNA), single-stranded DNA, and next generation plasmid (NG pDNA), double-stranded DNA, each evaluated independently for genome integration efficiency. Here, we demonstrated that both DNA templates facilitated stable genome integration, however delivery of mbDNA template, featuring a customizable stem region and fully user-defined sequence, resulted in 2-fold more anti-NYESO-1 TCR T-cells compared to the plasmid approach. These data show that novel nucleases, combined with DNA donors, represent an effective approach for creating engineered T-cells ex-vivo.

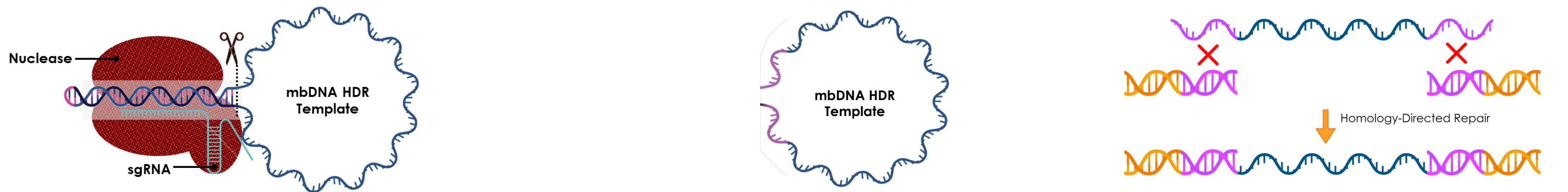
## Materials and Methods



## Life Edit Type II CRISPR nuclease system



## Touchlight mbDNA™ (MegabulbDNA) HDR Template

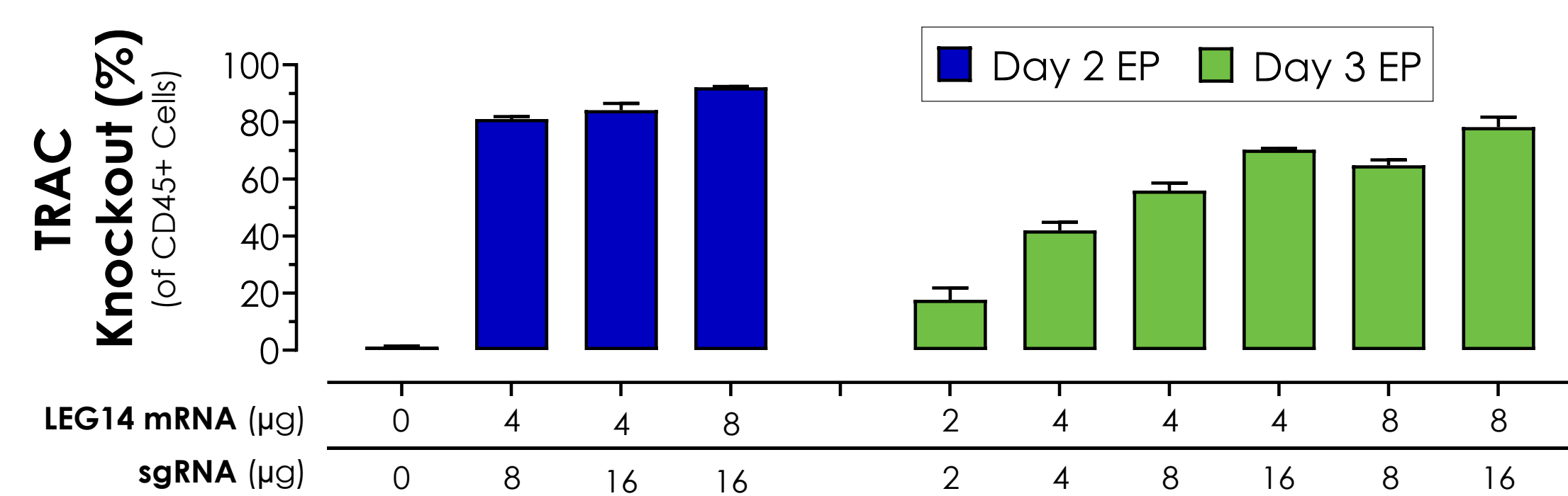


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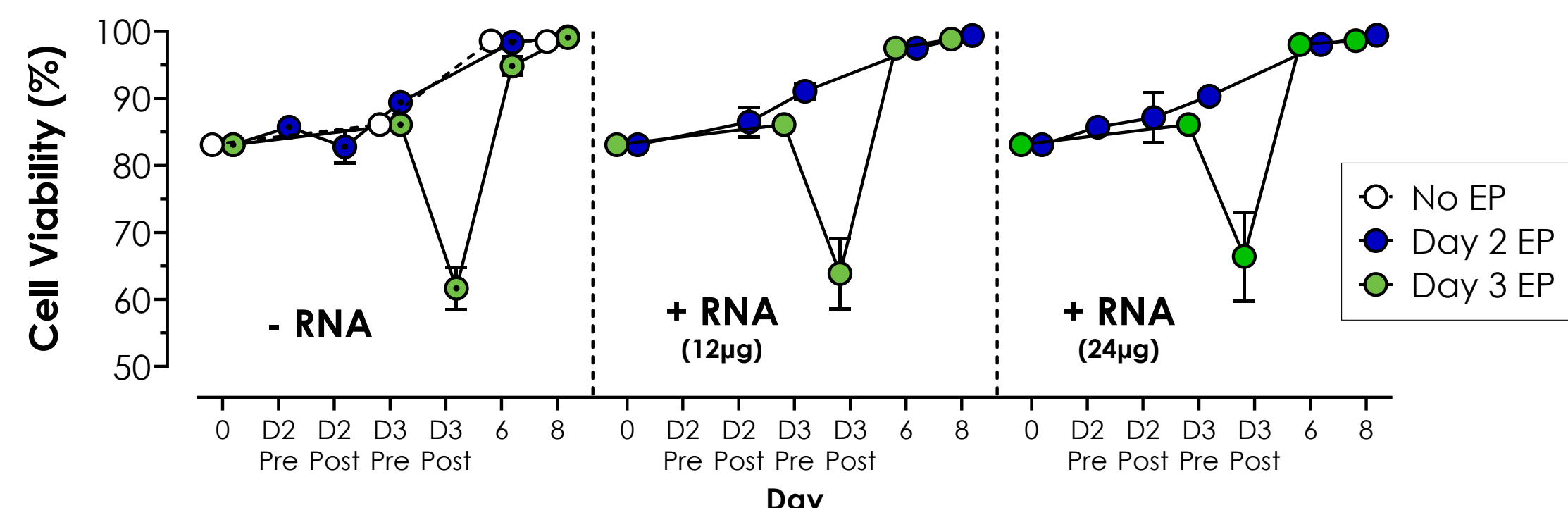
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## Improved knockout efficiency achieved through early EP, regardless of LEG14 mRNA/sgRNA balance



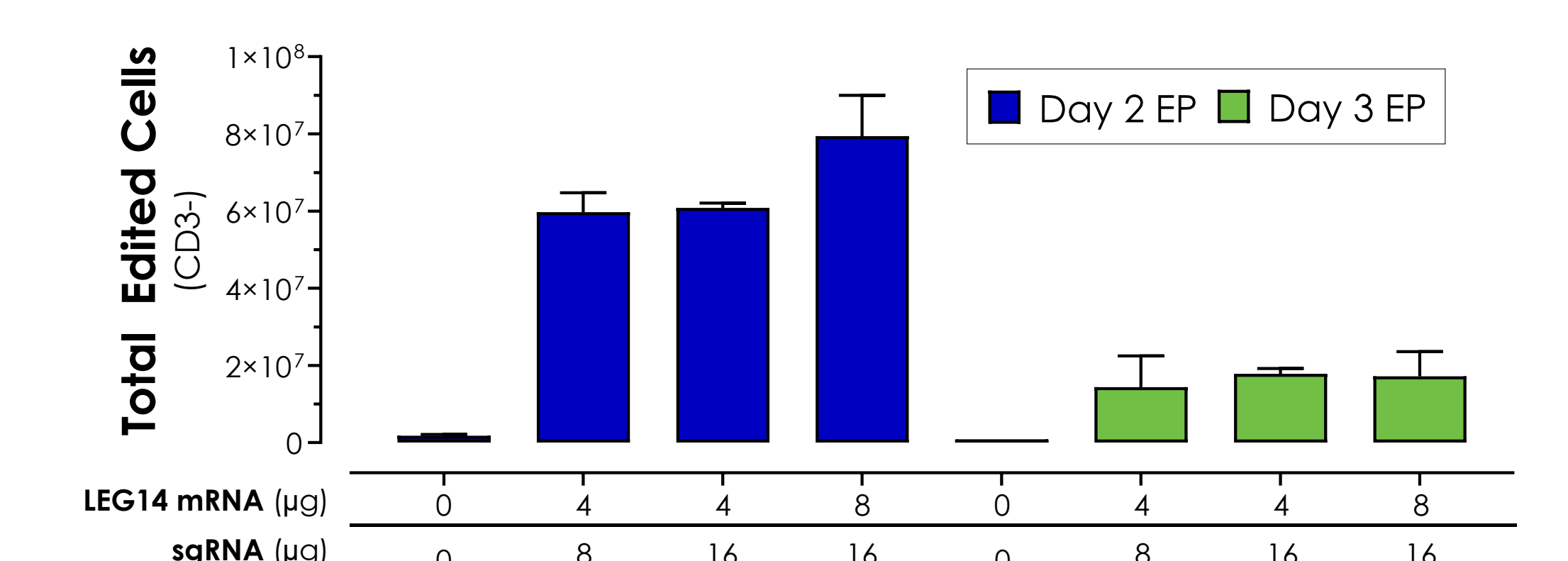
**Figure 1. RNA ratio, concentration and transfection timing impacts TRAC knockout efficiency.** To optimize gene editing efficiency, we evaluated 3 key factors, RNA ratio, concentration, and day of delivery. An optimal TRAC KO efficiency was achieved at 8µg LEG14 mRNA/16µg sgRNA delivered on day 2.

## Absence of T-cell toxicity from early electroporation (EP) despite elevated RNA cargo



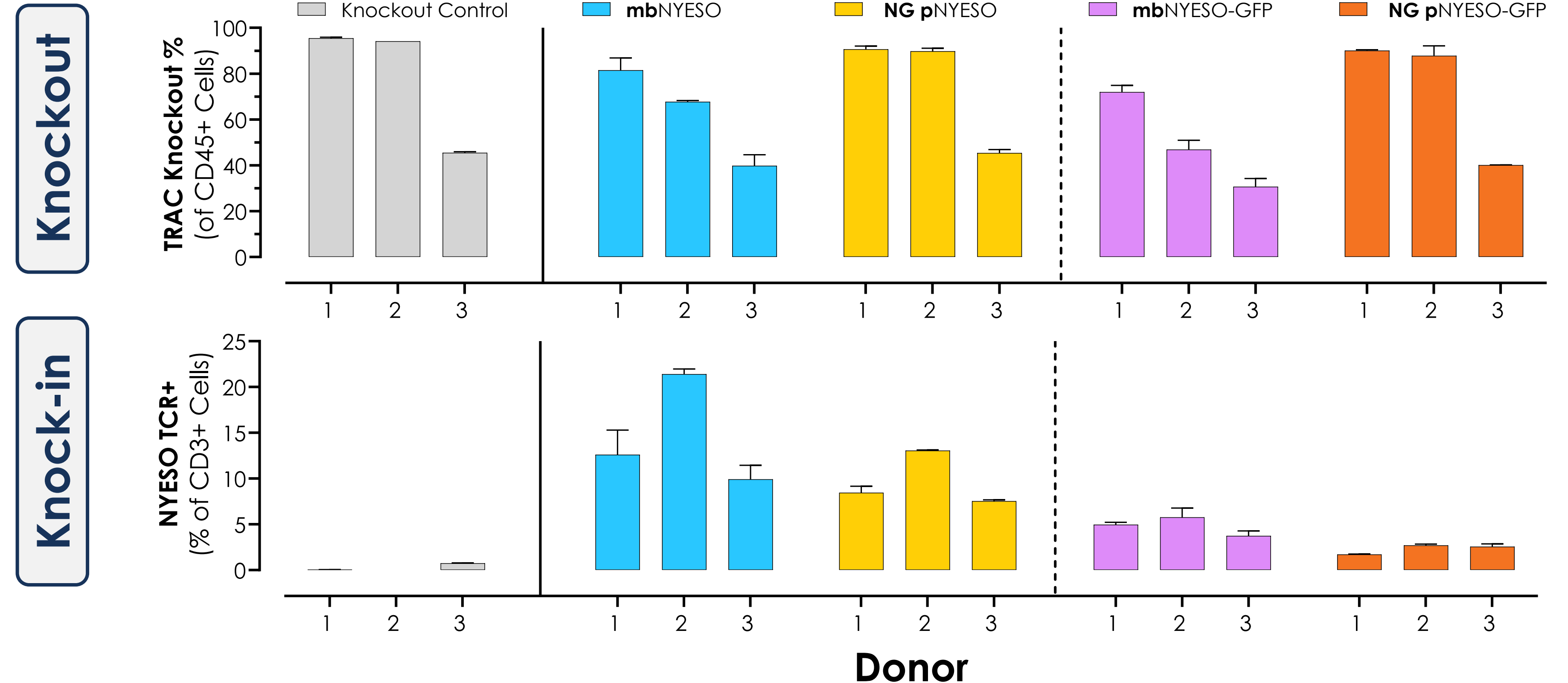
**Figure 2. Early transfection protects T-cells from EP-induced cytotoxicity.** Cell viability is extremely sensitive to EP parameters including pulse strength and duration, EP buffer composition, and the cell characteristics at time of delivery. T-cells activated for 2 days maintained high cell viability after EP, whereas T-cells activated for 3 days showed a marked drop in cell health. Importantly, differences in total RNA did not account for these variations in cell viability.

## Facilitating increased gene-edited cells and T-cell growth with early EP



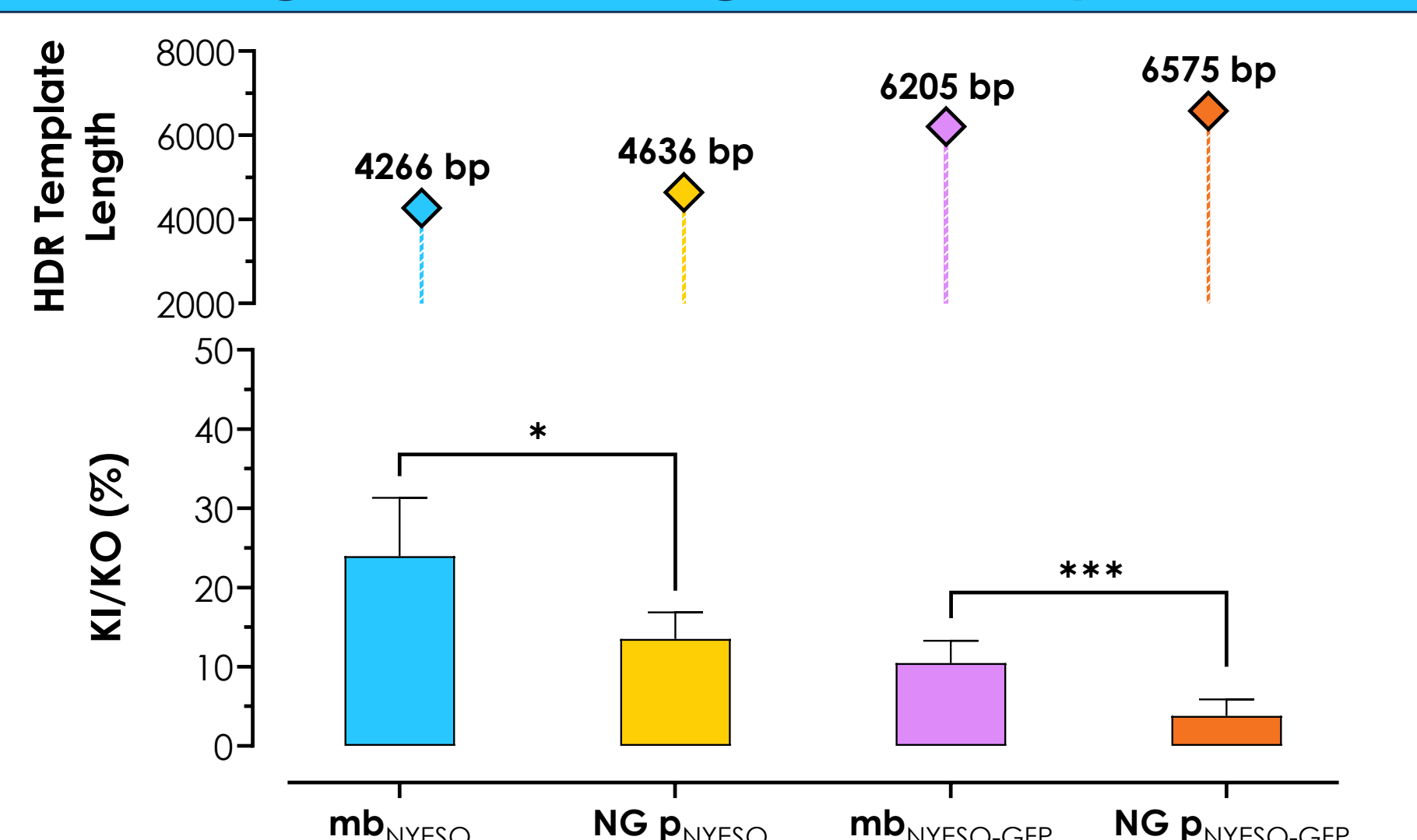
**Figure 3. Early electroporation promotes T-cell expansion.** Early transfection and increased RNA had minimal negative impact on T-cell growth kinetics, leading to a higher proportion of gene-edited viable cells.

## Non-viral transgenic T-cells engineered with mbDNA HDR template, resulted in improved anti-NYESO-1 TCR integration



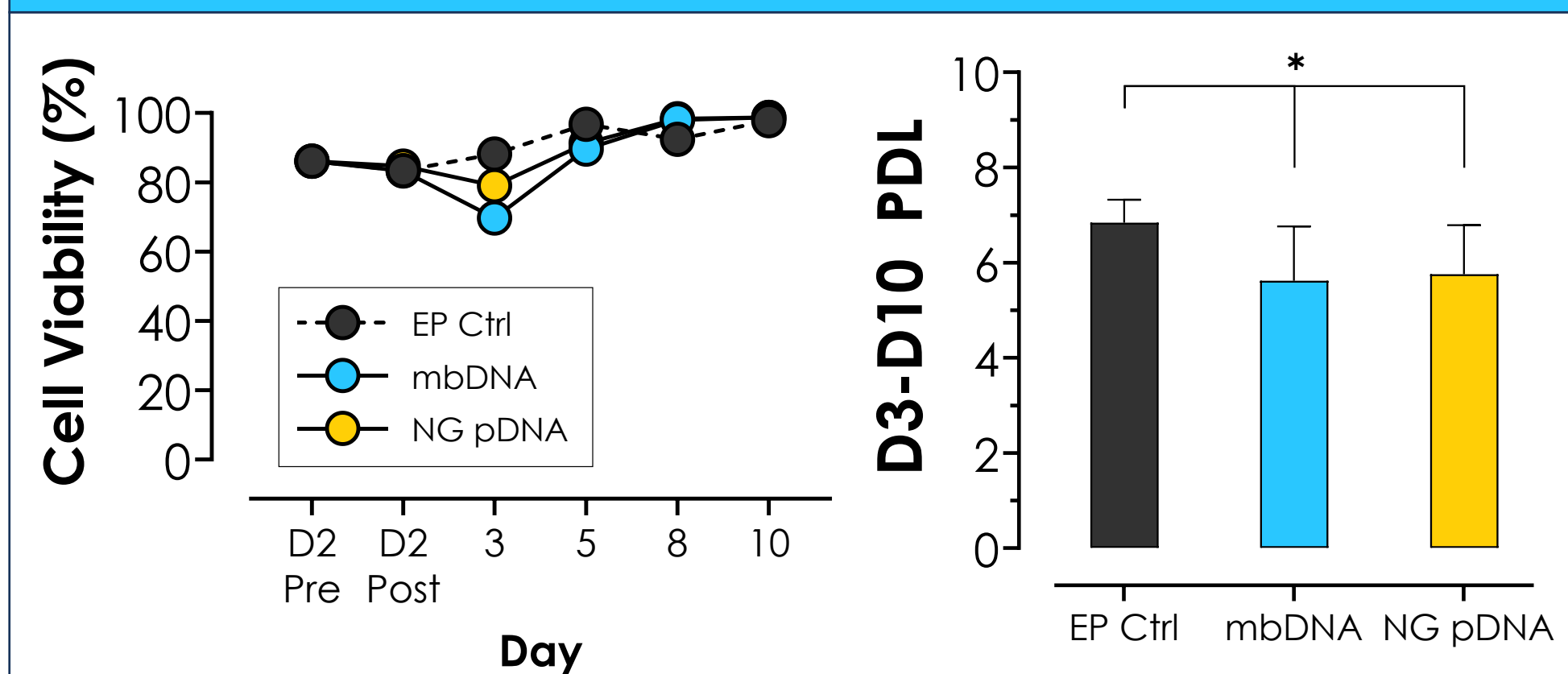
**Figure 4. Non-viral precision engineered transgenic T-cells.** Having optimized LEG14-guided nuclease for efficient TRAC deletion, we next used cell-free DNA to engineer a non-viral transgenic T-cell. Activated T-cells from three (3) healthy donors were transfected via EP. We used an optimized LEG14 mRNA/sgRNA ratio and amount. This was combined with four (4) different HDR templates: mbDNA encoding NYESO-TCR (mbNYESO), NYESO-TCR with an added GFP construct (mbNYESO-GFP), as well as next generation plasmid DNA encoding the same constructs (NG pNYESO and NG pNYESO-GFP). TRAC knockout efficiencies were similar to the control group in conditions transfected with NG pDNA, but showed more variability in conditions transfected with mbDNA. This variability suggests that further optimization of mRNA/sgRNA ratio and amount is required when used with the mbDNA template. This may be due to the mbDNA sequestering mRNA/sgRNA in the T-cell cytoplasm, to protect it from degradation and support its translocation into the nucleus. Overall knock-in efficiency for mbDNA templates, resulted in 2-fold more anti-NYESO-1 TCR T-cells compared to the plasmid approach.

## mbDNA facilitated more efficient delivery and integration of a larger DNA sequence



**Figure 5. Single-stranded mbDNA design facilitated better TCR insertion.** Donor DNA template significantly impacted endogenous TCR knockout and HDR knock-in efficiency. Single-stranded mbDNA outperformed NG plasmid DNA, exhibiting both improved TCR insertion and enhanced compatibility with larger DNA sequences. Results represent the mean KI/KO expression. Unpaired t test, \*, p > 0.05; \*\*\*, p > 0.001.

## T-cells experienced a rapid recovery post DNA delivery



**Figure 6. Transient Impact of Donor DNA on Growth Kinetics.** Introducing large DNA constructs during T-cell genetic engineering causes toxicity and cell death. Our non-viral delivery approach for gene editing tools, including large DNA sequence templates, resulted in an initial 10-20% reduction in cell viability. While the engineered T-cells recovered, reaching >95% cell viability at harvest, the transient cellular stress negatively affected their subsequent population doubling capacity and overall yield. Process optimization is needed to enhance long-term growth and reduce initial stress. Results represent the mean. Unpaired t test, \*, p > 0.05.

## Summary/Conclusion

ElevateBio's suite of gene-editing technologies at Life Edit combined with industry-leading expertise to design, develop, and manufacture, advanced next-generation therapies at BaseCamp enables us and our partners to bring novel treatments to patients. Here, we presented LEG14, a member of Life Edit's proprietary collection of Type II CRISPR nucleases and demonstrated its functionality through targeted deletion of the TRAC locus. Non-viral delivery of the LEG14 guided nuclease, in combination with HDR templates, successfully knocked in an NYESO-1 transgene to engineer transgenic T cells. When paired with LEG14-mediated precision knockout, Touchlight's mbDNA—a single-stranded gene-editing template—facilitated production of 2-fold more anti-NYESO-1 TCR T cells compared to the plasmid approach. Additionally, the cell-free mbDNA template enhanced insertion of larger DNA sequences. While challenges remain in non-viral gene editing—including donor material variation, low efficiencies with large insertions, and reduced cell expansion—our results demonstrated clear progress toward solving these limitations. Through systematic optimization of process parameters, nuclease properties, and HDR template design, we are advancing non-viral gene editing to achieve efficiencies that will match or exceed viral vectors, bringing safer and more accessible cell therapies to patients.

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