Improved CRISPR gene editing with doggybone DNA (dbDNA™) as a knock-in template

Elena Stoyanova, Linda Meggiolaro, Lisa Caproni, Tom Adie

Touchlight Genetics Ltd. Morelands & Riverdale Buildings, Lower Sunbury Road, Hampton, TW12 2ER

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1 Abstract

Recent advancements in non-viral gene editing technologies offer precise genome engineering at significantly reduced costs and biosafety risks compared to viruses. CRISPR is a programmable genome targeting system, which relies on a donor DNA as a template for homology-directed repair (HDR) to introduce exogenous sequences for cell and gene therapy. Therefore, as viruses are increasingly being replaced by safer and cheaper technologies, there is a growing need for reliable and effective vectors for targeted gene insertions. Plasmid DNA (pDNA), which has traditionally been used as the incumbent template for gene-length knock-ins, however, is associated with high toxicities, poor immunogenicity profiles and low HDR efficiencies, all of which has served to limit the success of non-viral gene therapy.

Touchlight's doggybone DNA (dbDNA™) is a fully synthetic, GMP-grade, covalently closed, linear vector, which directly addresses these challenges. The enzymatic manufacture eliminates bacterial sequences and produces a minimal vector of higher purity and lower immunogenicity compared to fermentation-derived DNA. Furthermore, the platform offers unparalleled scalability with reduced timelines overcoming pDNA manufacture bottlenecks.

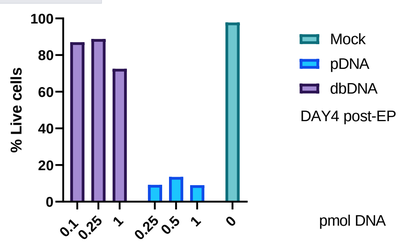
dbDNA™ has been robustly evaluated as an HDR template in ex vivo CRISPR gene editing platforms internally and by gene therapy industry leaders and is shown to maintain significantly better cell viability compared to pDNA. Improved cell survival leads to accelerated recovery and expansion in culture to produce higher edited primary immune cell counts. With its reduced size, dbDNA™ has a copy-number advantage over pDNA, allowing improved CRIPSR knock-in rates at a lower template concentration, further minimising the DNA bioburden. Importantly, the improved toxicity and immunogenicity profiles lead to better and more reliable knock-in efficiencies across primary T cells isolated from different blood donors, reducing undesired donor-to-donor variability. This altogether makes dbDNA™ highly suitable for autologous ex vivo gene and cell therapies, where cell numbers are typically limited and genome editing outcomes inconsistent.

dbDNA™'s improved performance in cells, alongside Touchlight's robust and rapidly scalable manufacturing technology, offers a reliable and effective alternative to pDNA as a knock-in template for CRISPR and, thus, promises to advance non-viral gene therapy.

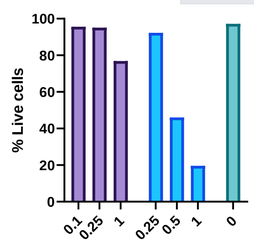
3 Results

dbDNA's improved viability compared to plasmid allows for increased HDR template concentrations to improve knock-in in primary T cells

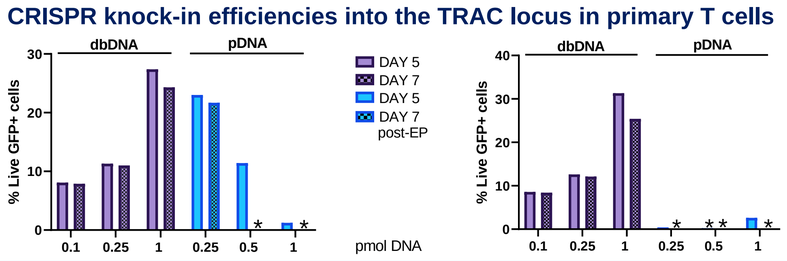
Donor 1



Donor 2



CRISPR knock-in efficiencies into the TRAC locus in primary T cells



dbDNA	
pmol	µg
1.00	2.00
0.25	0.5
0.1	0.2

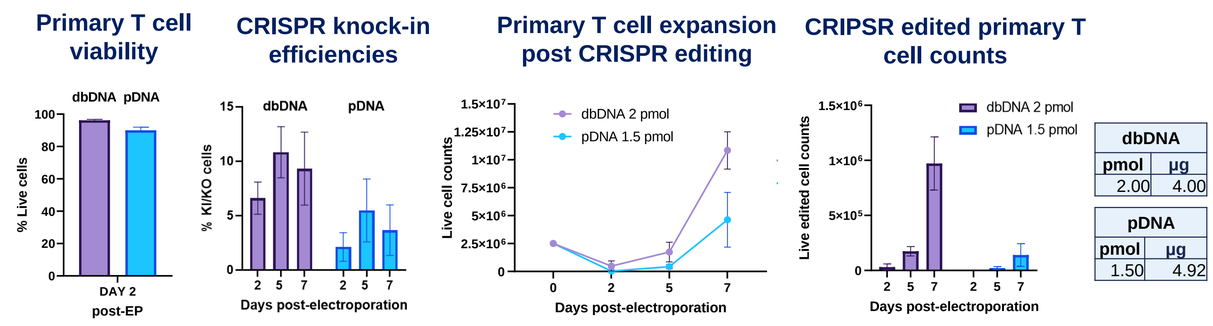
pDNA	
pmol	µg
1.00	3.28
0.5	1.65
0.25	0.82

- dbDNA is less toxic than plasmid (pDNA) to primary T cells from two different blood donors.
- Lower toxicity from dbDNA allows for introduction of higher template concentrations.
- dbDNA mediates higher CRIPSR knock-in rates than pDNA, especially at high template concentrations
- dbDNA has a copy-number advantage over pDNA, further reducing toxicity per mole of DNA and minimising template DNA costs

Methods: EGFP dbDNA or pDNA with 800 bp homology arms to the TRAC locus were co-electroporated with 50 pmol Cas9 RNPs into 1x10^6 primary T cells in 20 µl EP (Lonza 4D)

*Viability too low to produce usable data

dbDNA's improved viability compared to plasmid allows for faster cell recovery and expansion



dbDNA	
pmol	µg
2.00	4.00

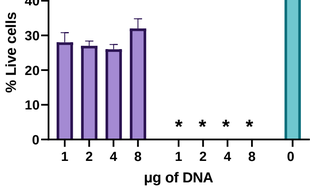
pDNA	
pmol	µg
1.50	4.92

- dbDNA is less toxic than plasmid (pDNA) to primary T cells (dbDNA and pDNA are roughly mass-matched)
- Primary T cells that have been CRISPR edited with dbDNA recover faster and expand better than those edited with pDNA.
- Lower toxicity, higher knock-in rates and better expansion of cells edited with dbDNA produce higher edited cell counts.
- All the above makes dbDNA a highly suitable CRIPSR HDR template for ex vivo cell editing.

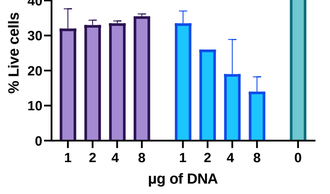
Methods: EGFP dbDNA or pDNA with 800 bp homology arms to the TRAC locus were co-electroporated with 50 pmol Cas9 RNPs into 2.5x10^6 primary T cells in 25 µl EP (MaxCyte ATX). Post-EP cells were expanded in 24-well G-Rex in up to 4.5 ml media.

dbDNA has improved consistency across blood donors

Donor A

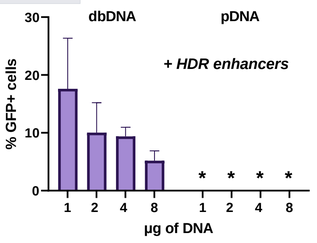


Donor B

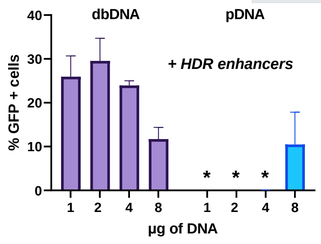


CRISPR knock-in efficiencies into the TRAC locus in primary T cells

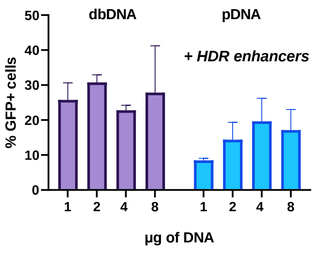
Donor A



Donor B



Donor C



dbDNA	
µg	pmol
1.00	0.50
2.00	1.00
4.00	2.00
8.00	4.00

pDNA	
µg	pmol
1.00	0.31
2.00	0.61
4.00	1.22
8.00	2.44

*Viability too low to produce usable data

- dbDNA has more consistent toxicity profile than pDNA in primary T cells isolated from different blood donors
- dbDNA produces more reliable knock-in efficiencies across blood donors
- Therefore, dbDNA reduces undesired donor-to-donor variability, making it highly suitable for autologous applications

Methods: EGFP dbDNA or pDNA with 800 bp homology arms to the TRAC locus were electroporated into 2x10^6 primary T-cells (Lonza 4D) in 20 ul EP. Viability was measured 24 h and GFP 8 days post-EP.

2 Introduction

Doggybone DNA (as a template for gene editing)

Touchlight's doggybone DNA (dbDNA™)

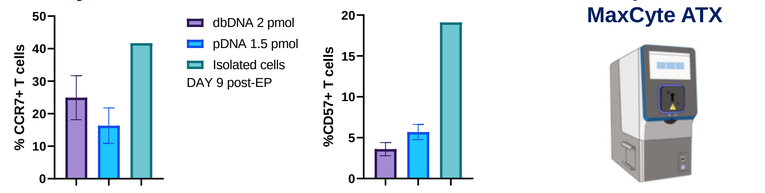
- Linear, covalently closed DNA vector
- GMP production process in a week
- No bacterial sequences
- Lower toxicity & immunogenicity

Scan for poster #360 on Touchlight's dbDNA™ technology

- **Minimal vector: copy number advantage over plasmid DNA for further reduced toxicity**
- **Supporting larger cargo capacity > 20kb – more complex and multiple genes**
- **High purity, low endotoxin, closed ended dsDNA – better editing efficiency**
- **Avoiding viral vector supply chain – cost, complexity & timeline**
- **Excellent template for (CRIPSR) targeted genome insertions**
- **A bulk of CRISPR editing data in primary T cells demonstrating dbDNA is superior to other non-viral vectors**

dbDNA preserves the physiological T cell profile better than plasmid

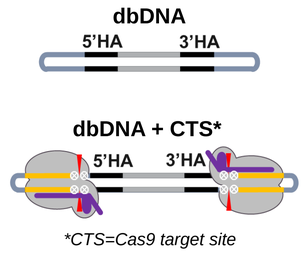
CRISPR edited T cell characterisation



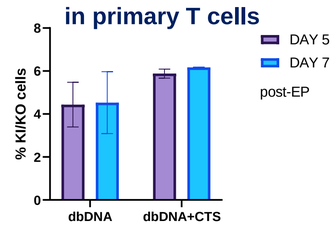
- T cells edited with dbDNA retain their central and effector memory identity better than those edited with pDNA.
- dbDNA-based editing allows for the generation of a less differentiated/exhausted T cell population than pDNA.

Methods: EGFP dbDNA or pDNA with 800 bp homology arms to the TRAC locus were co-electroporated with 50 pmol Cas9 RNPs into 2.5x10^6 primary T cells in 25 µl EP (MaxCyte ATX). Post-EP cells were expanded in 24-well G-Rex in up to 4.5 ml media and stained for CCR7 and CD57 at day 9 post-EP.

Addition of nuclease target sites to dbDNA may improve HDR yields



*CTS=Cas9 target site

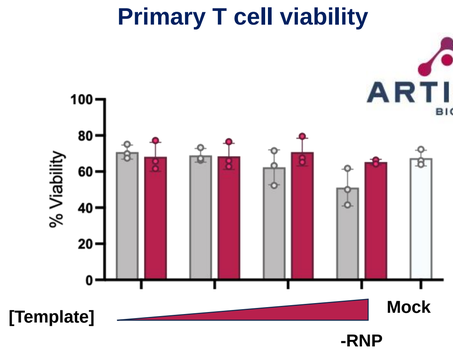


- Preliminary data suggests that addition of fully cleavable Cas9 target sites (CTSs) on either end of the repair template sequence on the dbDNA HDR template may improve knock-in efficiencies
- This would be in line with published data, where CTSs have been shown to improve HDR (*Nguyen et al., 2020; Shy et al., 2022*)

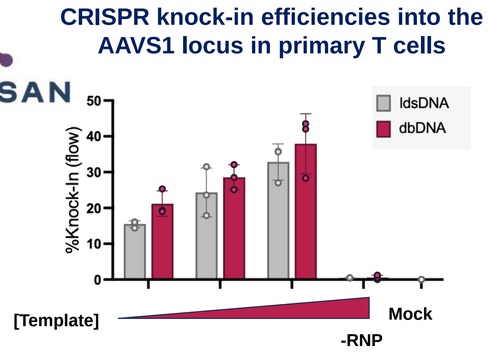
Methods: EGFP dbDNA, dbDNA+CTS or pDNA with 800 bp homology arms to the TRAC locus were co-electroporated with 50 pmol Cas9 RNPs into 2.5x10^6 primary T cells in 25 µl EP (MaxCyte ATX).

dbDNA outperforms linear dsDNA in primary T cells

Primary T cell viability



CRISPR knock-in efficiencies into the AAVS1 locus in primary T cells

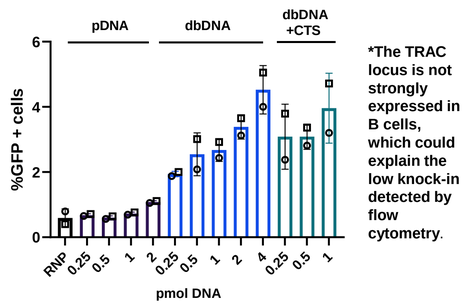


Methods: Primary human T cells were edited using Artisan's optimized protocol for STAR-CRISPR nuclease + split gRNA targeting AAVS1 Safe Harbor Site. Linear double stranded DNA (ldsDNA) and Touchlight Doggybone DNA (dbDNA) were used as HDR templates at increasing concentrations.

- Artisan Bio has optimized the STAR-CRISPR system to deliver Touchlight's dbDNA, producing insertion efficiencies greater than linear dsDNA (ldsDNA) at a range of template concentrations (Day 11 flow)
- The STAR-dbDNA system maintained high cell viability & recovery (Day 1 post-editing)
- dbDNA allows for improved T cell viability at high template concentrations
- The combination of Touchlight's dbDNA and Artisan's STAR-CRISPR system with optimized safe harbor site guides provides an attractive platform for cell engineering.

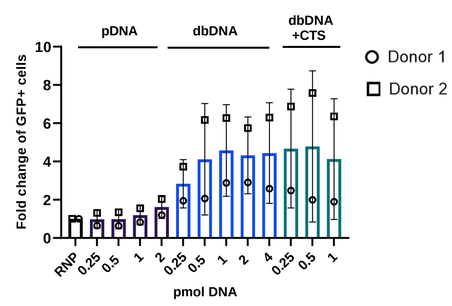
dbDNA & dbDNA+CTS outperform plasmid in primary B cells

CRISPR knock-in efficiencies into the TRAC locus* in primary B cells



***The TRAC locus is not strongly expressed in B cells, which could explain the low knock-in detected by flow cytometry.**

Fold expansion of edited primary B cells (normalised to RNP only control)



dbDNA	
pmol	µg
4.00	8.00
2.00	4.00
1.00	2.00
0.25	0.5

pDNA	
pmol	µg
2.00	6.56
1.00	3.28
0.5	1.65
0.25	0.82

Methods: EGFP dbDNA, dbDNA+CTS or pDNA with 800 bp homology arms to the TRAC locus with Cas9 RNP were electroporated into primary B cells.

- dbDNA and dbDNA+CTS produce higher CRIPSR knock-in rates than pDNA in primary B cells from two different blood donors
- dbDNA+CTS shows slightly increased HDR efficiencies than dbDNA
- The higher editing efficiencies with dbDNA and dbDNA+CTS produce higher CRISPR edited primary B cell counts than pDNA, which expand better to allow for higher fold expansion over control (RNP only sample)

4 FAQs & More Touchlight technology

- dbDNA works well for stem cell editing:
  - CRISPR editing data
  - dbDNA has improved viability over pDNA in stem cells
- dbDNA works with a variety of nuclease targeting systems:
  - Cas9 (T cells, B cells, iPSCs)
  - Cas12a (T cells, iPSCs)
  - STAR-CRISPR (T cells)
- dbDNA has shown lower immunogenicity
- dbDNA formulates well in LNP compared to pDNA:
  - Smaller size
  - Improved DNA cargo
- Lower level of immunogenicity markers (TLR9 pathway) in iPSC generated with dbDNA compared to those generated using pDNA

dbDNA as a vector for Lentivirus Production – scan for poster #233

mbDNA: Touchlight's circular ssDNA HDR template