# Development of a Clinical-Grade Meganuclease for Allogeneic CAR T Cell Production

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PRECISION BIOSCIENCES

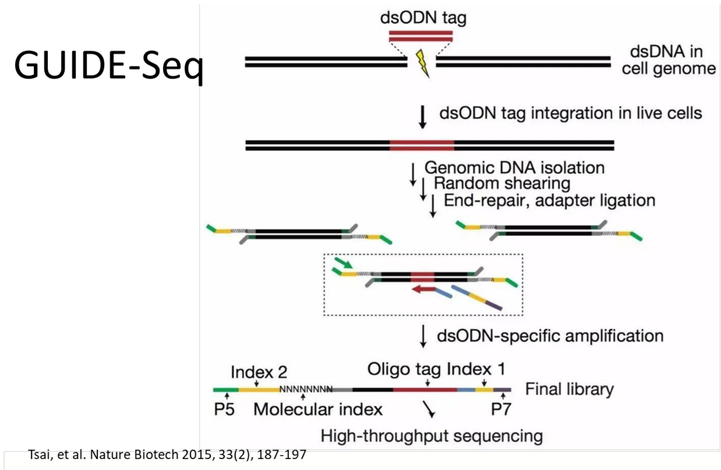
## ABSTRACT

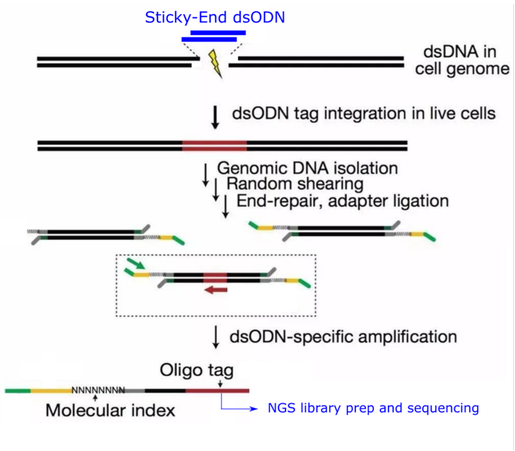
Chimeric Antigen Receptor T cells (CAR Ts) are revolutionizing the treatment of hematologic malignancies. Autologous (patient-derived) therapies, though effective, are challenging to produce. Allogeneic (donor-derived) CAR T cells may be an attractive alternative if they can be modified to prevent graft-vs-host disease (GvHD). We previously reported an efficient genome editing strategy to produce allogeneic CAR T cells by targeting the insertion of a CAR transgene directly into the native TRAC locus using an engineered meganuclease and an AAV donor template (MacLeod, et. al., 2017). The resulting cells are CAR+ and do not elicit GvHD by virtue of having the native T cell receptor gene knocked-out by the CAR transgene.

To better understand the extent and impact of nuclease activity on the CAR T cell product, we developed a high-sensitivity assay for off-target cutting that takes advantage of the 3' overhangs generated by meganucleases. Based on this analysis, we were able to engineer a second-generation nuclease with greatly reduced off-target activity. By comparing the performance of the optimized nuclease to the parent, we found a surprisingly robust relationship between nuclease specificity and CAR T cell performance. These results suggest that monitoring and control of off-target nuclease activity is critical to optimizing the overall fitness and function of gene edited CAR T cells.

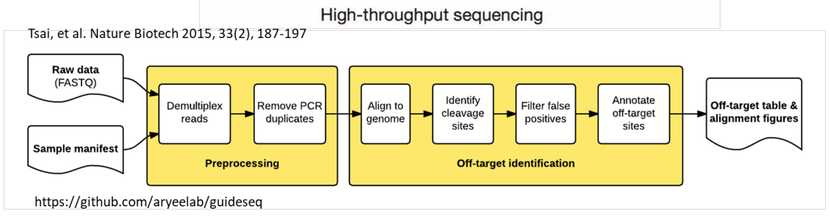
## METHODS

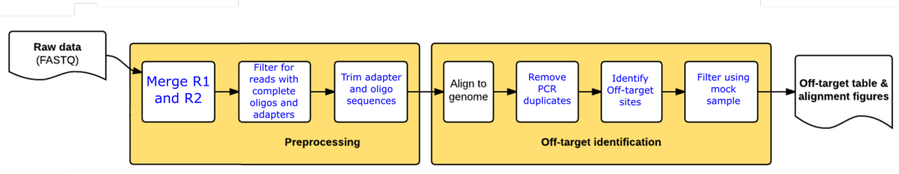
Oligo capture parallels GUIDE-Seq in wet lab procedure with changes at a few key points. Instead of using a blunt-ended double stranded oligo for insertion, we use a double stranded oligo with randomized, protected, 4bp overhangs at the 3' ends. A new data processing pipeline has been customized for meganucleases, including changes in target site identification based on the single chain structure.

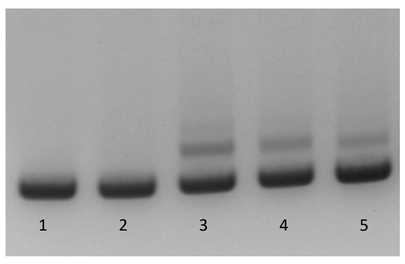



Tsai, et al. Nature Biotech 2015, 33(2), 187-197

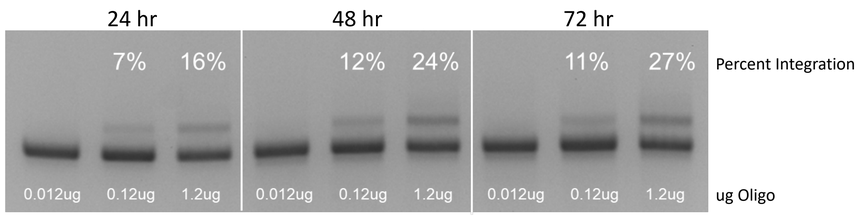



| | | Integration |
|---|---|---|
| 1 | Mock | 0% |
| 2 | Nuclease only | 0% |
| 3 | Nuclease + oligo with site specific overhangs | 19% |
| 4 | Nuclease + oligo with random overhangs | 14% |
| 5 | Nuclease + oligo with blunt ends | 8% |

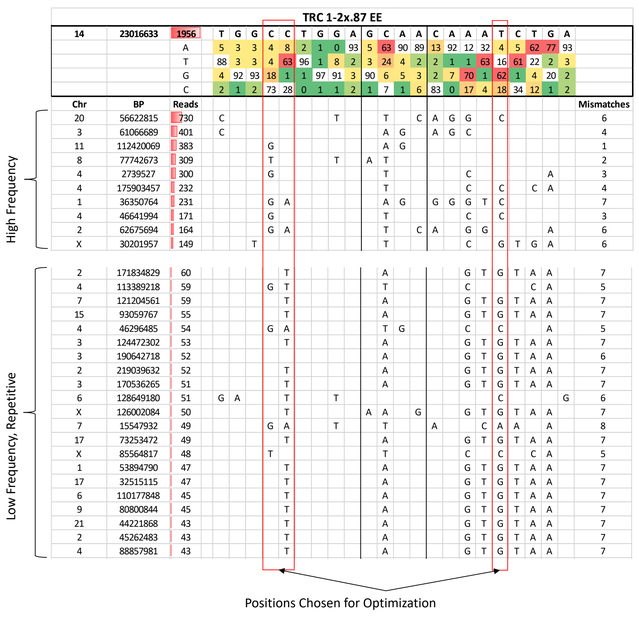
Adding a randomized 4bp 3' overhang increases the integration of the oligo at the intended target site from 8% to 14%.



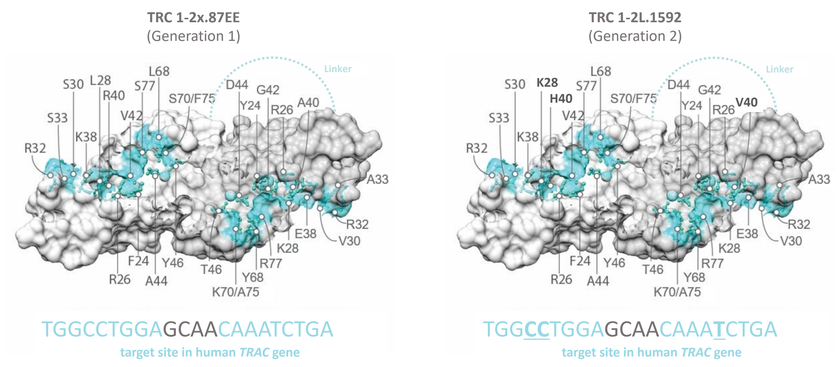

Integration scales with amount of oligo electroporated into the cells and peaks around 48 hours. No significant increase in integration occurs between 48 and 72 hours post-electroporation.

## RESULTS

Oligo capture performed on the TRC 1-2x.87 EE nuclease identified two types of off-target sites. The first type were high frequency hits: sites that were cut many times resulting in a large number of reads. The second type were low frequency repeats: sites that were cut a few times and had lower numbers of reads but with a target site sequence that occurred multiple times in the genome.




Three positions in the target sequences were chosen for optimization and a second generation enzyme was produced (TRC 1-2L.1592). The second generation nuclease differs from the original at only 3 amino acid positions (**designated in bold**).




Analysis of TRC 1-2L.1592 by Guide-seq produced only two off-target sites, both of which were shown to be false positives by targeted indel analysis. Oligo capture identified a further 47 targets, 5 of which produced indels above the mock background.

Guide-seq

| site # | Chr | Pos | Sequence | Indel Frequency (%) |
|---|---|---|---|---|
| site 0 | 14 | 23016633 | TGGCCTGGAGCAACAAATCTGA | 61.35 |
| site 200 | 3 | 45249461 | G AGA TC C | N.S |
| site 201 | 4 | 149232383 | TT TT TT T | N.S |

Oligo Capture

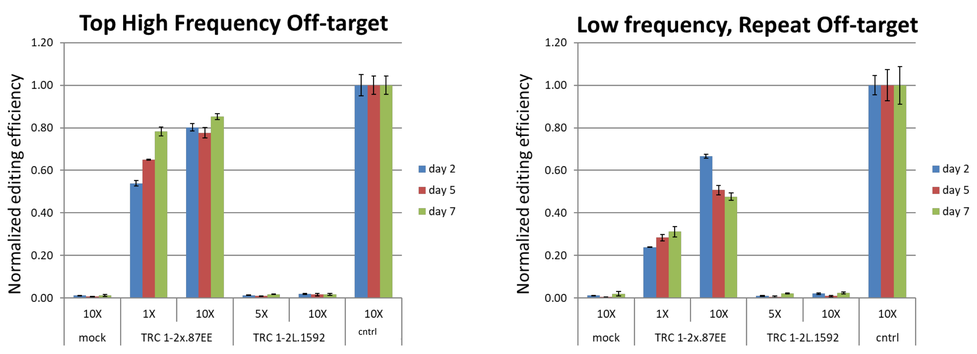
| site # | Chr | Pos | Sequence | Indel Frequency (%) |
|---|---|---|---|---|
| site 0 | 14 | 23016633 | TGGCCTGGAGCAACAAATCTGA | 61.35 |
| site 8 | 11 | 112420069 | G AG | N.S |
| site 14 | 17 | 70963906 | T C C | 0.23 |
| site 43 | 7 | 45886383 | G G A T A C | 0.44 |
| site 46 | 11 | 117707291 | T A C CT | 0.85 |
| site 79 | 8 | 70953326 | G T A C C | 2.83 |
| site 80 | 15 | 91730805 | T T G T A C T | 0.45 |
| site 81 | 6 | 134362399 | G T AGG GA A T | N.S |
| site 83 | 20 | 51802795 | T A A T TG A C T | N.S |
| site 84 | 2 | 85864762 | A A CACTT C A C | N.S |
| site 85 | 5 | 75148730 | AA T T T TAA | N.S |
| site 86 | 14 | 96013249 | A TG T T GTG C | N.S |
| site 89 | 8 | 68898594 | AA GG C C AAT | N.S |
| site 92 | 22 | 48584419 | AA A AG TA C CT | N.S |
| site 93 | 2 | 9176023 | AAGA TTTC T C T | N.S |
| site 94 | 4 | 183627898 | T T T A TC TG C A | N.S |
| site 95 | 6 | 86300063 | A TTTTGGA AA T | N.S |
| site 97 | 11 | 67957795 | AAG T TGTC GC T | N.S |
| site 98 | 10 | 94357492 | T A C C GGGG G | N.S |
| site 100 | 2 | 200426071 | T T A TTTGT GA C | N.S |
| site 101 | 7 | 102266445 | A TG A TGA C G T | N.S |
| site 102 | 12 | 133336771 | A A ACTC C C AAT | N.S |
| site 103 | 1 | 91577468 | A T A C TA G C A | N.S |
| site 105 | 2 | 207017087 | A T A TTTTA A A | N.S |

N.S.: not significant (<1 STDEV over mock)

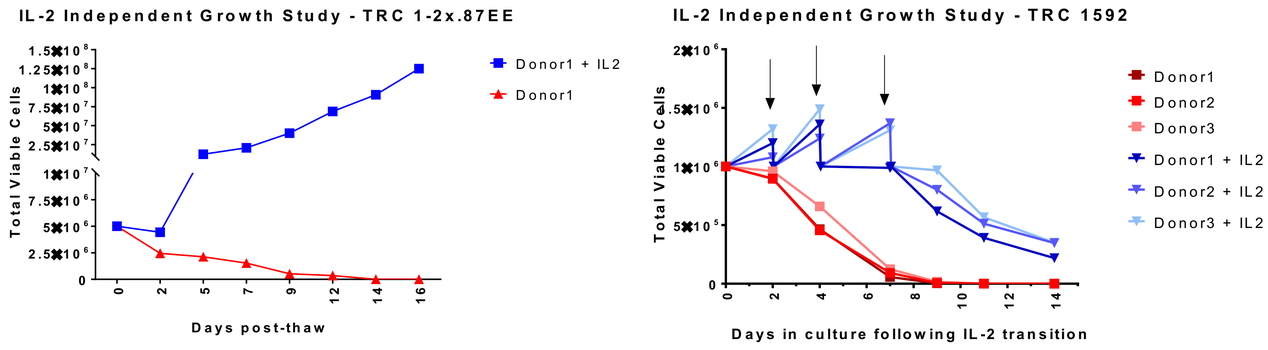
- No sites in exons
- Three sites in non-coding regions of genes
- No known oncogenes
- No known association with relevant pathology

| Site # | % indel | Location | |
|---|---|---|---|
| site 14 | 0.23 | Intron 4 of ***SLC39A11*** , >10 kb from the nearest exon | Zinc transporter |
| site 43 | 0.44 | >10 kb from closest known gene transcript | |
| site 46 | 0.85 | 3' UTR of ***FXYD6,*** 3225 bp from the nearest exon | Na,K-ATPase regulation |
| site 79 | 2.83 | >10 kb from closest known gene transcript | |
| site 80 | 0.45 | Intron 1 of ***SV2B*** , >10kb from the nearest exon | Uncharacterized secretory role |

Reporter assays showed that the second generation nuclease did not cut either the top high frequency off-target site or the low frequency, repetitive off-target site.

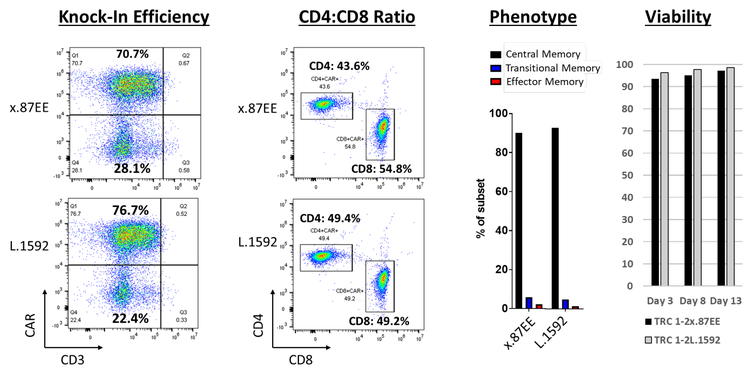



CAR T cells produced with either the first or second generation nuclease fail to survive in the absence of exogenous IL-2 indicating that no neoplastic transformation has occurred.

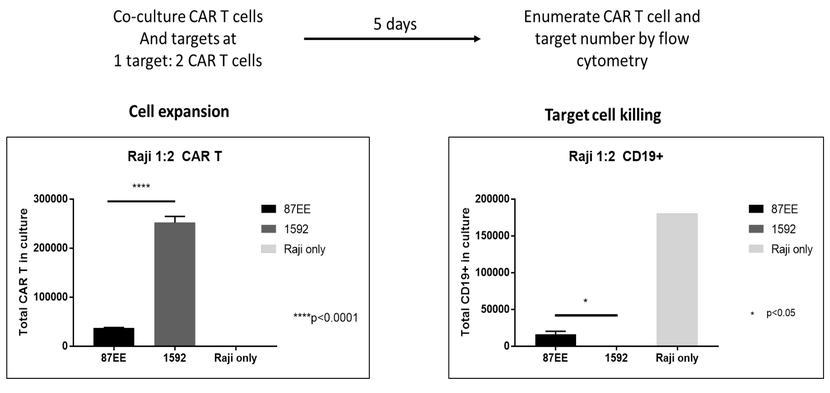



*Arrows indicate that samples with IL-2 supplementation were reduced to 1e6 cells after counting at days 2, 4, and 7.*

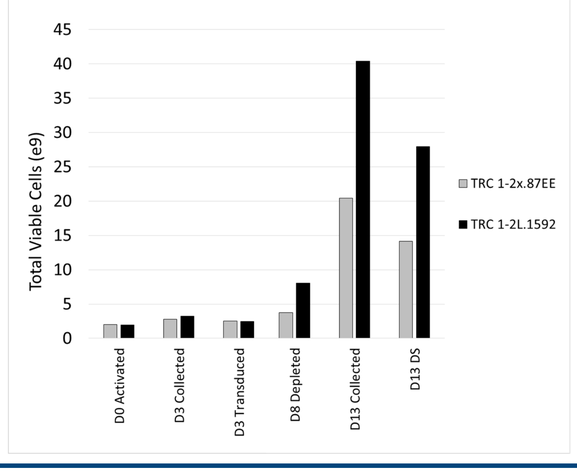
The optimized TRC 1-2L.1592 showed a better knock-in efficiency, CD4:CD8 ratio, and phenotype. Viability was unaffected.




CAR T cells produced using TRC 1-2L.1592 expanded 5x better than those made with TRC 1-2x.87EE and killed target cells significantly better.




Significant increases were seen in the number of total viable cells produced in a standard production run.




## Conclusions

Guide-seq has become the gold standard for identification of off-target genome editing using CRISPR, but several changes are needed to allow discovery of possible off-targets generated by a meganuclease. Using our customized method, we were able to identify and eliminate multiple off-target sites by creating an optimized generation of our TRC 1-2 nuclease. While the first version of the nuclease produced modified CAR T cells that performed to specifications and were phenotypically normal, the optimized nuclease allowed for a large increase in the amount of cells produced during the manufacturing process and increased the effectiveness of the CAR T cells in killing the target cells. In this case, it appears that off-target genome breaks that occur during the production of cell therapy products may have a greater effect on the efficiency and cost of manufacturing than the safety of the final product.

## REFERENCES

1. Tsai, S. Q., Zheng, Z., Nguyen, N. T., Liebers, M., Topkar, V. V., Thapar, V., … Joung, J. K. (2015). GUIDE-Seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology*, *33*(2), 187–197.
2. MacLeod, D. T., Antony, J., Martin, A. J., Moser, R. J., Hekele, A., Wetzel, K. J., … & Bartsevich, V. V. (2017). Integration of a CD19 CAR into the TCR alpha chain locus streamlines production of allogeneic gene-edited CAR T cells. *Molecular Therapy*, *25*(4), 949-961.