# MAD7-based gene editing platform for rapid and efficient iPSC engineering

Jasdeep Mann[1a], Laura Prochazka[1b], Justin Yoo[1a], Nabil Zeidan[1b], Zahra Sahaf[1b], Dhaarini Murugan[1a], Sam Lam[1a], Julia Bershadsky[1b], Avisek Deyati[1c], Chris Bond[1a]

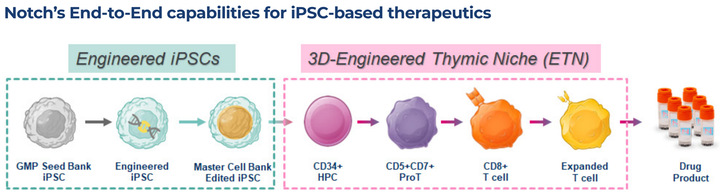
[1]Notch Therapeutics, [a]Seattle, WA, US; [b]Toronto, OT, CA; [c]Vancouver, BC, CA | Keystone

## ABSTRACT

Induced pluripotent stem cells (iPSCs) have enormous potential in the field of adoptive cellular therapy as they can provide homogeneous sources of diverse immune cell subtypes. Their ability to self-replicate and generate identical clones and/or master cell lines with increasingly complex genetic modifications makes them an attractive source for off-the-shelf cellular therapies. We have built a gene-editing platform to alter several clinically relevant targets utilizing MAD7. Our platform enables the identification of gRNA sequences with more than 80% editing efficiency. Using orthogonal biased and unbiased methods, these gRNAs have been shown to have a high level of specificity. We have further validated these gRNA sites to demonstrate a strong genotype-phenotype correlation in relevant cell types. In addition, we have established a gene-editing method for simultaneous editing of several sites in iPSCs and subsequent clonal selection. Using this method, we were able to create multi-edited clonal lines with more than 70% of the clones harboring bi-allelic knockouts at desired targets at research scale in less than 2 months. Finally, our process has been successfully translated to a GMP compliant suite for production of clinically grade iPSC-derived drug products. Our gene editing platform allows production of multi-target edited iPSC lines at unprecedented efficiency, speed, and compliance with GMP with opportunities to generate increasingly complex genetically engineered iPSC-derived therapeutics.

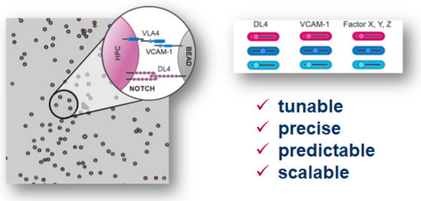
## OVERVIEW

### Notch's End-to-End capabilities for iPSC-based therapeutics




- Developing T cell products from renewable iPSCs with expertise in control of Notch signaling during cell differentiation
- Improving iPSC-derived T cell function by producing uniform and potent phenotype
- Implementing commercially compatible stem cell materials and manufacturing
- Aiming to produce sophisticated product design by multiplexed genome engineering

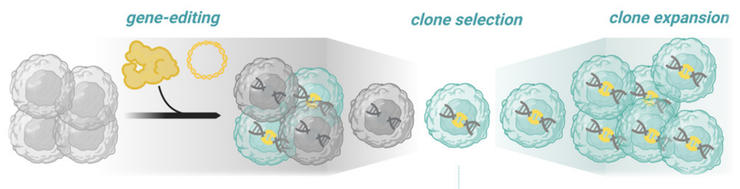
**proprietary ETN bead-based manufacturing**




- ✓ **tunable**
- ✓ **precise**
- ✓ **predictable**
- ✓ **scalable**

- Notch designed the 3D ETN platform for scalable T-cell manufacturing, built on our discovery that immobilized DLL4/VCAM supports T-cell differentiation *in vitro*[1]
- ETN technology provides a foundation for time and dose-dependent Notch signaling in a manner that enables repeatable manufacturing of iPSC-derived T cells

**iPSC engineering platform and analytics to generate clonal cells**




| Identity | Consistency | Genomic Stability | *Safety (GMP only)* |
|---|---|---|---|
| Knock-Out [TIDE, ICE, NGS] | Cell Viability | Karyotyping | Residual editing reagents |
| Frameshift mutation [NGS] | Cell Count | Array CGH | Sterility (USP<71>) |
| Knock-In sequence [Sanger seq] | Cell Morphology | FISH | Mycoplasma (USP<63>) |
| Vector Copy Number [ddPCR] | Transgene Expression | Off-target detection | Endotoxin (USP<85>) |
| Off-targets [in-silico, in-situ] | Pluripotency markers | Translocations | |

- Notch has streamlined the iPSC engineering process, which entails genome editing with MAD7, single-cell cloning using high-efficiency dispenser (VIPS™), screening, and expansion
- This workflow yields genetically stable homogeneous iPSCs with validated characteristics and modifications in less than five weeks
- Implemented and developed various complementary analytical techniques to analyze edits and ensure genomic stability to expedite decision making and banking of clonal lines

## RESULTS

### Section I: MAD7 enabled high editing efficiencies in iPSCs

**Enhanced editing efficiencies by 3-fold through multi-parameter optimization**

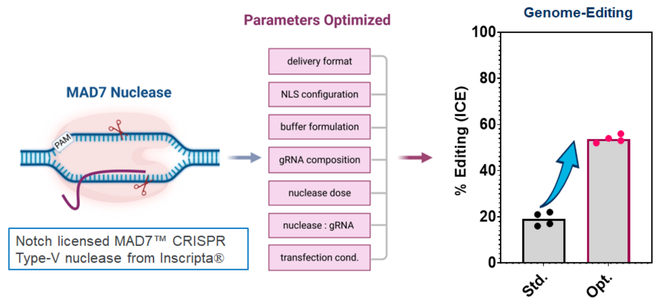



**Fig 1:** MAD7 nuclease belongs to the Class 2 type V-A CRISPR-Cas family, which recognizes thymidine-rich PAM 'YTTV' and creates double stranded staggered breaks. We optimized and combined multiple parameters such as MAD7 delivery format, dose, gRNA composition and ratio, to establish a robust gene-editing protocol in iPSCs.

**Demonstrated >80% editing efficiencies in GMP compliant iPSC line**

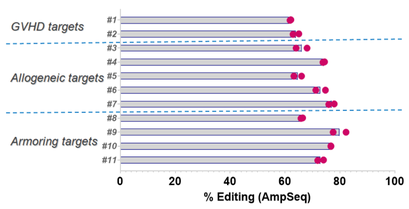



**Fig 2:** RNP complexed MAD7 was nucleofected in Notch's GMP compliant iPSC line using proprietary editing protocol to target clinically relevant genes. We developed a high-throughput gRNA screening workflow to incorporate viability assessment using high-titer glo and indel detection through ICE followed by deep analysis via targeted amplicon sequencing.

### Section II: Characterization of MAD7 gRNAs and edits

**InDel fingerprints profile informed frequency of frame-shift mutations**

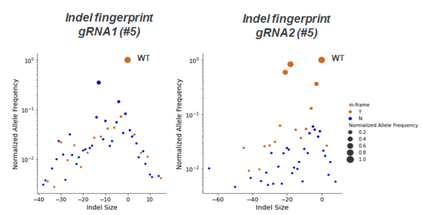



**Fig 3**: InDel profiling of targeted site using amplicon sequencing revealed the frequencies of frame-shift perturbations. Indel fingerprints plots facilitated the characterization and comparison of gRNA sequences for the same site. These probabilities dictated nominating the gRNA sequences for GMP process.

**Confirmed genotype-phenotype association by loss-of-expression**

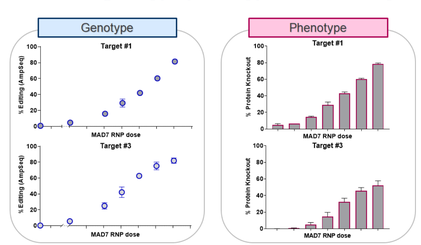
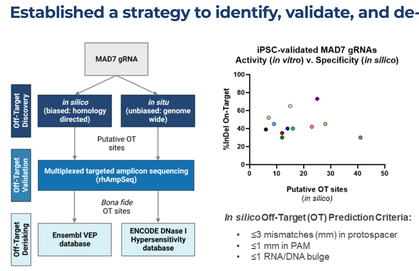



**Fig 4**: Following MAD7-mediated editing of target sites, the extent of InDel formation and disruption of phenotype was evaluated for a range of RNP doses by amp-seq and flow cytometry, respectively in primary donor derived T-cells. A dose-dependent increase in the frequency of functional knockout was observed at both the genomic and phenotypic levels.

**Established a strategy to identify, validate, and de-risk off-target sites**



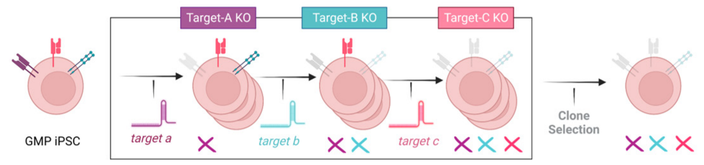

*In silico* Off-Target (OT) Prediction Criteria:

- ≤3 mismatches (mm) in protospacer
- ≤1 mm in PAM
- ≤1 RNA/DNA bulge

**Fig 5**: An overview of Notch's three-part strategy to identify and validate off-target (OT) editing associated with MAD7 gRNAs. We have developed in silico pipeline and in-situ method to predict OT sites and validate using multiplex and single-plex amplicon sequencing. The validated *bona fide* OT sites will be de-risked through prediction of functional consequences. MAD7 gRNAs, in pipeline, screened for high-editing on-target activity in iPSCs are predicted to exhibit high specificity using in-silico off-target analysis

### Section III: Generation of multiplexed edited iPSC clones

**Notch's multiplex gene-editing platform**




**Generated triple knock-out homozygous clones in less than 5-weeks**

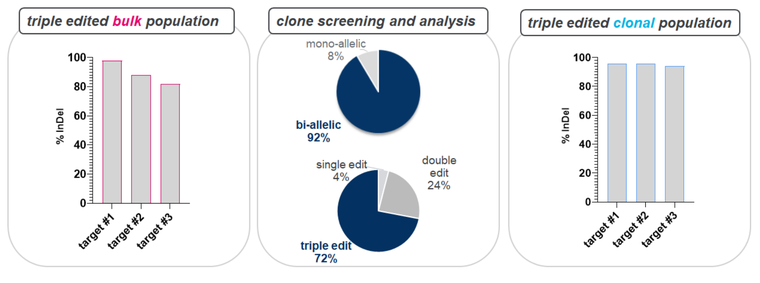



**Fig 6:** We have developed a modular multiplex method to produce clonal iPSC lines with multiple edits at unprecedented efficiency and speed.

**Fig 7:** The editing methodology can be repeated up to at least 3 times as shown by the case study, without compromising the cell viability and editing efficiency in a single target. Bulk edited population was single cell seeded using VIPS™ with cloning efficiencies and outgrowth > 80%. Most of the clones (>70%) exhibited bi-allelic triple knockout, confirmed using multiplex PCR, ddPCR and sanger sequencing. The combination of high editing rates and cloning efficiency drastically reduced the number of clones assessed for the desired genotype.

### Section IV: Site-specific transgene integration using MAD7 in iPSCs

**Attained >20% CAR knock-in using nonviral templates**

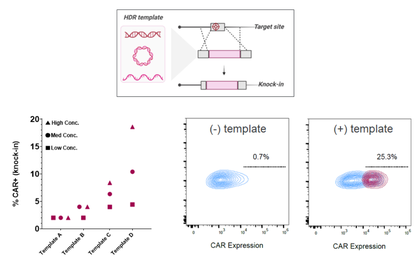
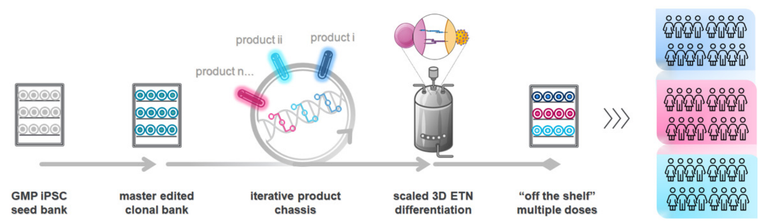



**Fig 8:** Transgene knock-in is attained by cointroduction of a nonviral (DNA) template for homology-directed repair at the target site. We screened several template formats and concentrations to achieve efficient transgene integration and cell recovery. Our current template design can accomplish up to 20% gene integration measured as CAR expression on iPSC surface, which would expedite clone screening process.

## SUMMARY

**Notch's vision for clinical development of drug product**




- **Notch's efficient and multiplexed gene editing along with 3D-ETN platform would enable rapid product iteration for "off-the-shelf" cellular therapy**
- Optimized CRISPR-MAD7 editing process achieved >80% editing efficiency consistently across several clinically relevant target genes in GMP compliant iPSCs
- Nominated, characterized, and validated gRNA sequences for GMP manufacturing
- Developed modularized multiplex editing process to simultaneously target multiple sites while preserving high editing efficiency
- Generated triple edited validated iPSC clones in 5-week period using MAD7
- Improved knock-in efficiency by applying modified non-viral templates, which would expedite the manufacturing of GMP compliant reagents and process

1. Shukla et al (2017) Progenitor T-cell differentiation from hematopoietic stem cells using Delta-like-4 and VCAM-1. Nature methods 14 531-538.