Using iPSC and gene editing technologies to generate immune-cloaked “off-the-shelf” allogeneic cell therapies.

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1 Introduction

Human induced pluripotent stem cells (iPSCs) possess the remarkable ability to be regenerated, genetically modified, scaled, and differentiated into a wide array of cell types. These characteristics make iPSCs an excellent tool for cell therapies. Simultaneously, development of the clustered regularly interspaced short palindromic repeats (CRISPR) technology has revolutionized genome engineering, enabling the creation of genetically modified cell products with enhanced efficacy, persistence and safety. The combination of these scientific findings are paving the way for a bright future for “off-the-shelf” allogeneic cell therapies. However, the application of gene editing technologies, particularly CRISPR, poses challenges when attempting to introduce multiple genetic alterations in iPSCs, as these cells can be sensitive to such procedures. Overcoming these challenges in achieving efficient and precise gene edits in iPSCs will be crucial for the successful development and implementation of immune-cloaked cell therapies (**Figure 1**).
Here we present a streamlined pipeline to produce gene-edited iPSCs with high efficiency.

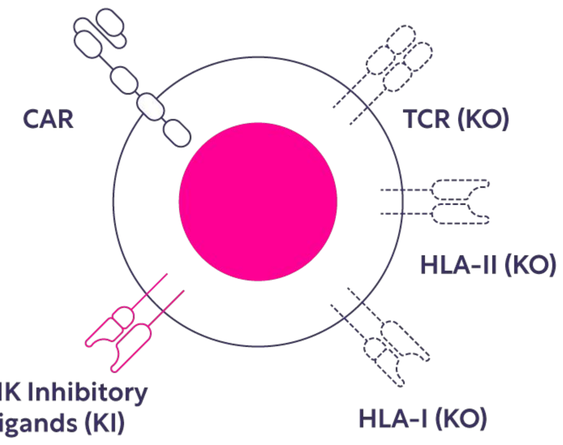

Fig. 1. A) Persistence of gene-edited CAR cells can be increased by disruption of major histocompatibility complex class I molecules, possibly major histocompatibility complex class II molecules and addition of immune modulatory proteins.

2 Editing Strategy Design

- Design gRNAs and validate editing efficiencies by introducing CRISPR components as ribonucleotides complexes (RNPs) via electroporation.
- Forty-eight hours post-transfection, iPSCs are harvested and DNA is extracted.
- The targeted loci is amplified using PCR, followed by Sanger sequencing.
- Editing efficiencies are calculated by deconvolution of the Sanger traces (**Figure 2**).

Fig. 2A

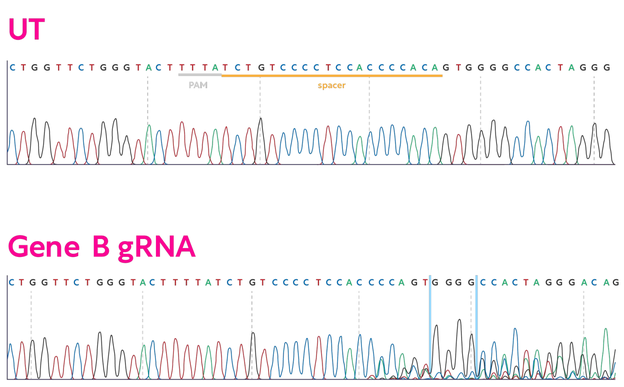

Fig. 2B

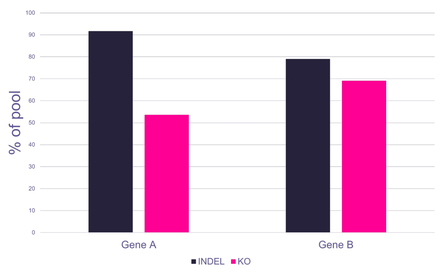

***Fig. 2. Validating gRNA. A)** Sanger traces following PCR of targeted loci. Yellow line indicates the targeting (spacer) sequence, blue line is the PAM sequence, required for the nuclease to bind and cut the target loci **B)** Editing efficiencies for Gene A and Gene B obtained 48 hours post delivery of CRISPR components via electroporation.*

- Design HDR donor templates taking the following considerations into account:
  - Disruption of target (spacer) site, with/without PAM blocking mutation
  - Use 2A self-cleaving peptides or IRES between transgenes in the same donor template
  - Inclusion “insulators” to prevent transgene silencing
  - If needed, selection markers can be included, allowing enrichment of the edited cells
- Validate knock-in rates using gRNA selected in the previous step.
- Forty-eight hours post-transfection, iPSCs are harvested and DNA is extracted.
- Knock-in of transgenes is validated by junction (‘In-Out’ PCR) (**Figure 3**).

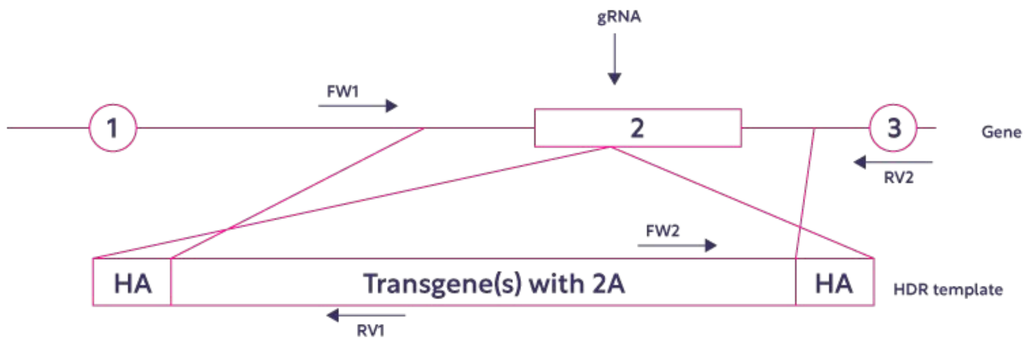

***Fig. 3. Validating HDR donor template.** Junction PCR to evaluate correct integration of the transgene(s), using primers binding outside of the homology arms (HA) in the genomic locus and within the transgene.*

3 Generation of Single Cell Clones

To produce monoclonally derived iPSC lines, the edited (enriched) pool undergoes single cell sorting into 384 well plates using the UP.SIGHT, as single cell seeder with built-in imaging (**Figure 4A**). Cells are plated in complete pluripotent cell media with a supplement that increases the cloning efficiency and single-cell survival. Upon plating, the cells will be imaged (nozzle and 3D imaging). All plates will be imaged at the different timepoints to evaluate growth of the clones.

Fig. 4A

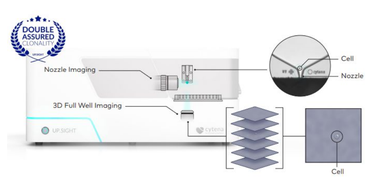

Fig. 4B

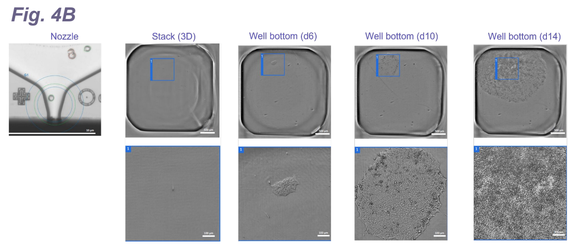

***Fig. 4. Single cell sorting. A)** The UP.SIGHT, a single cell seeder with built-in imaging; **B)** Tracking of the clones to confirm monoclonality (nozzle, 3D stack and d0 image) and evaluate growth.*

4 Clone Picking and Screening

Upon monoclonality confirmation, confluent colonies will be dissociated and transferred into two 96 well plates (known as ‘masterplates’). One plate will be used for expansion and one for screening **(Figure 5**)**.**
Following DNA extraction and PCR, clones are Sanger sequences to identify the clones with the desired genotype. Next Generation Sequencing (NGS) allows further confirmation of accurate gene editing.

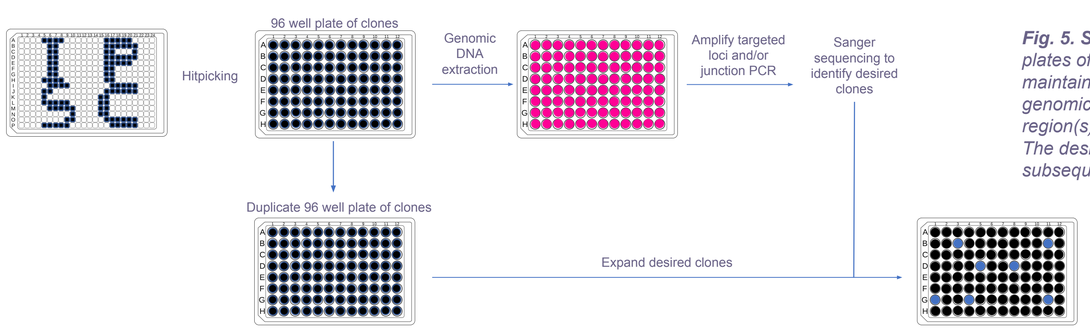

***Fig. 5. Screening workflow.** Duplicate 96 well plates of cell clones are generated. One plate is maintained for later use. From the other plate, genomic DNA is extracted, and the targeted region(s) is/are amplified and Sanger sequenced. The desired clones are then expanded for subsequent QC and pre-banking.*

5 QC

To qualify the correct iPSC clone for further Master Cell Bank (MCB) production, multiple clones will be characterized. The purpose of testing is to confirm the identity (identical to donor), purity (pluripotent and absence CLD generation components), quantity (cell count and viability), genetic (e.g., transgene copy number, genomic stability) and microbiological safety of the cell substrate for manufacturing (**Figure 6**).

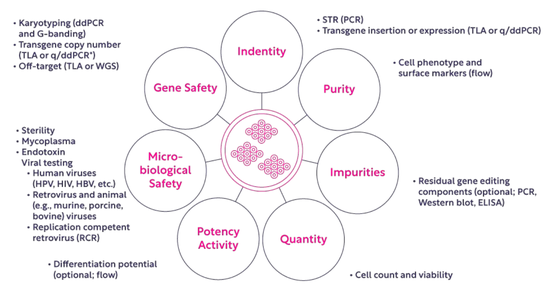

***Fig. 6. QC assessment.** Selected clones will undergo a full characterization to select the desired clone to use for further manufacturing.*

6 Conclusion

Using our optimized pipeline, we achieve up to 90% editing efficiencies and >30% recovery from single iPSCs, enabling the generation of monoclonally-derived, gene-edited iPSCs.

The generation of these immune-cloaked “off-the-shelf” iPSC lines will greatly reduce time and expenses of allogeneic cell therapies, with huge benefits for the patients.

Our bespoke gene-editing platform offers state-of-the-art facilities and fits in seamlessly with our good manufacturing practice (GMP)-compliant master cell banking and proprietary cell-specific differentiation pipelines.