

Site-specific integration of a 50 kb multi-gene payload into human iPSCs enables advanced engineering of next-generation cell therapy platforms

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BACKGROUND

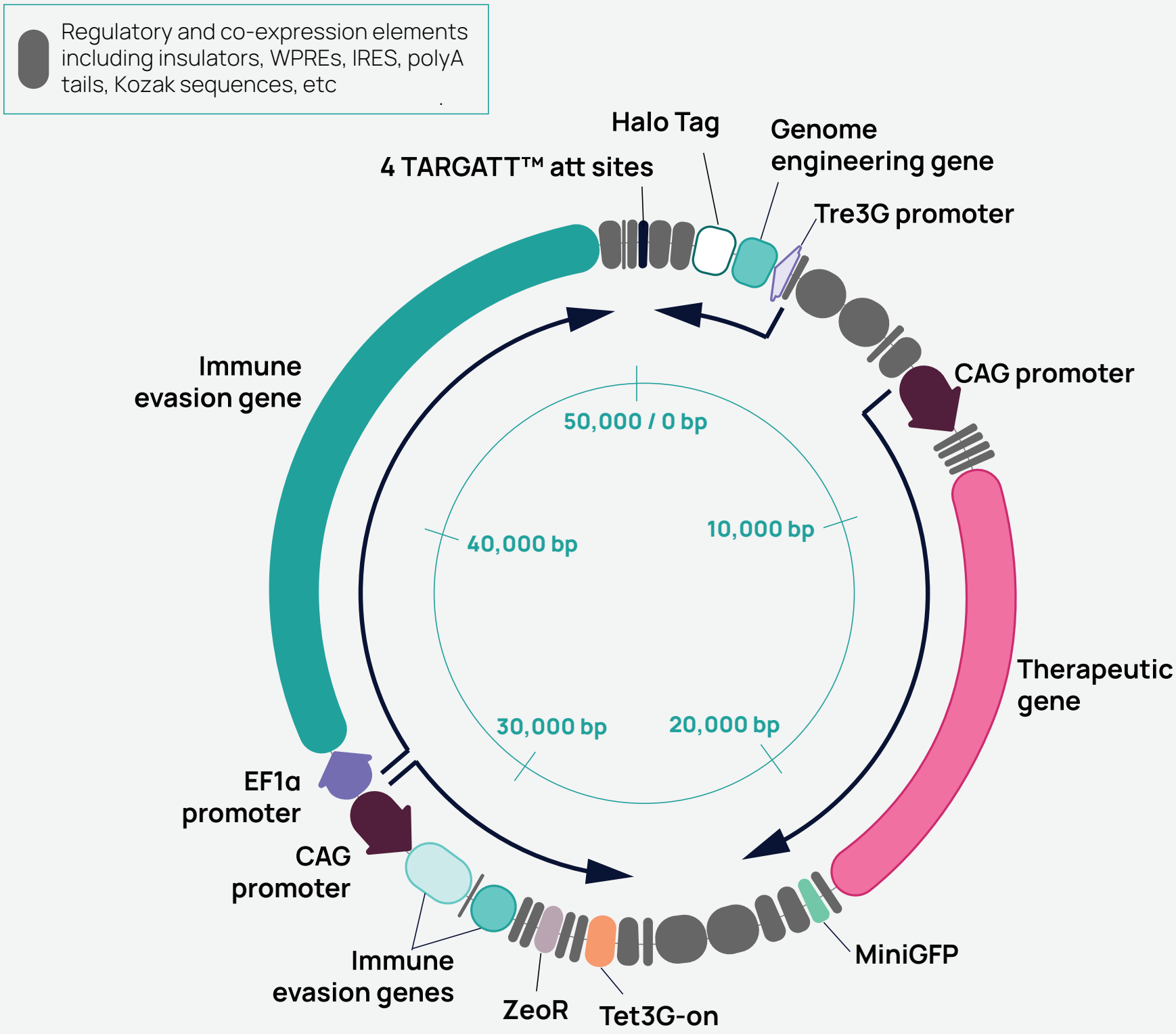
Next-generation cell therapies require stable integration of complex, multi-gene constructs that include immune evasion factors, therapeutic payloads, and logic gates. Human iPSCs are a scalable starting material for off-the-shelf cellular medicines, but safe integration of large DNA constructs has been challenging.

Here, we demonstrate that our serine integrase-based TARGATT™ technology enables efficient knock-in of very large multi-cassette payloads in hiPSCs. We also demonstrate the utility and speed of using a synthetic 50 kb donor plasmid for efficient creation of large donor plasmids.

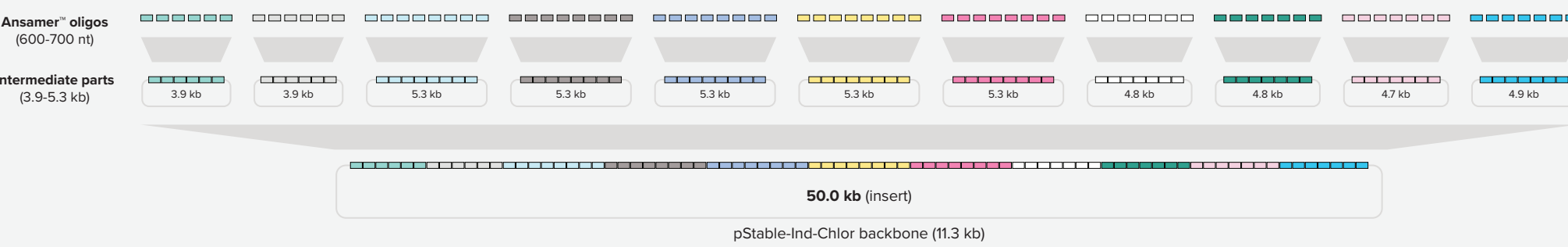
METHODS

We designed a 50 kb donor construct containing four expression cassettes relevant to engineered cell therapies, including constitutive immune evasion modules, selectable markers, fluorescent reporters, and an inducible element.

Expression cassettes are indicated by the curved arrows, with key elements in color, as indicated, and elements to support robust gene and protein expression in gray.



The donor construct was synthesized as a 60 kb plasmid—10 kb backbone and 50 kb donor—using Ansa Biotechnologies proprietary enzymatic DNA synthesis platform. The sequence was divided into 80 segments of 600 bp - 700 bp that were synthesized as Ansamer™ contiguous oligonucleotides and amplified using PCR. The amplicons were cloned using *E. coli* and hierarchically assembled through two subsequent stages into the final full-length construct with the Ansa pStable-Ind-Chlor inducible-copy BAC backbone.

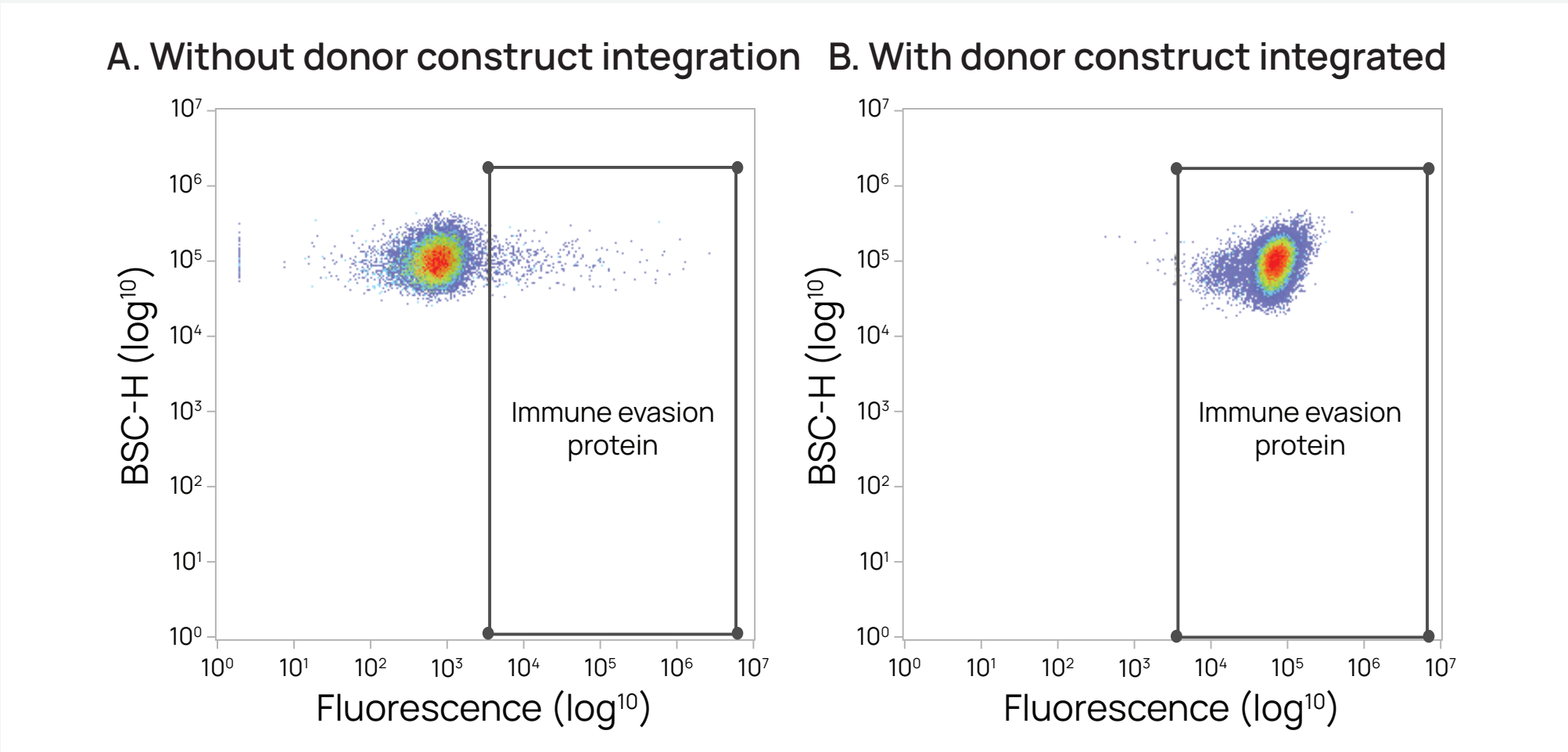


We integrated the donor plasmid into hiPSCs at the H11 safe harbor locus using TARGATT™ site-specific recombinase technology. Following antibiotic selection, we screened clones by junction PCR and assessed functional expression by flow cytometry under constitutive and doxycycline-induced conditions.

RESULTS

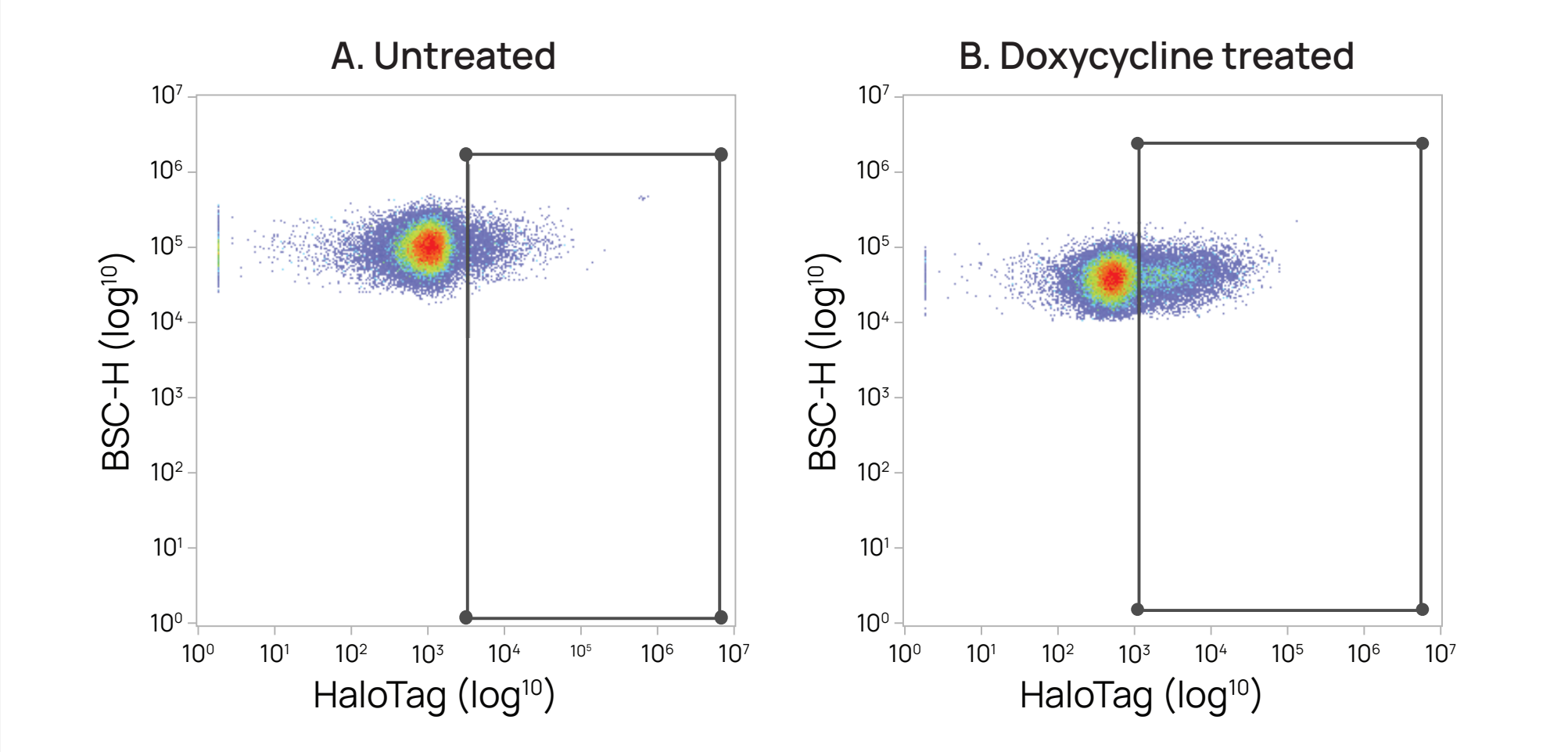
We achieved targeted knock-in of the full construct with ~88% integration efficiency across screened clones. Junction PCR confirmed precise insertion at the H11 locus (data not shown). Flow cytometry showed robust constitutive expression of an immune evasion protein in nearly all donor-integrated cells. We also validated inducible control, with doxycycline treatment activating the reporter module in 23% of treated cells compared with negligible background in untreated controls.

Virtually 100% of donor-integrated cells show constitutive expression.



(A) The isotype control without the donor construct integrated into the H11 safe harbor site shows no surface expression of the assessed immune evasion protein. (B) With the donor construct integrated into the H11 site, almost 100% of donor-integrated cells express the immune evasion protein, demonstrating the robust and consistent expression delivered by the TARGATT™ platform.

Donor-integrated cells show inducible HaloTag expression.



(A) Uninduced cells show negligible HaloTag expression. (B) After induction with doxycycline for 72 hrs, 23% of cells show HaloTag expression. The number of cells expressing the HaloTag increased over time (data not shown), consistent with robust inducibility.

CONCLUSION

We demonstrate efficient safe-harbor integration and functional expression of a 50 kb multi-gene payload in human iPSCs. This expands the genetic complexity achievable in a single targeted event and enables uniformly engineered iPSC master lines for next-generation hypoimmunogenic and regulated cell therapy platforms.

The ability to install complex genetic programs in a single targeted event has broad implications for developing robust, next-generation engineered cell therapies with enhanced functionality and control.