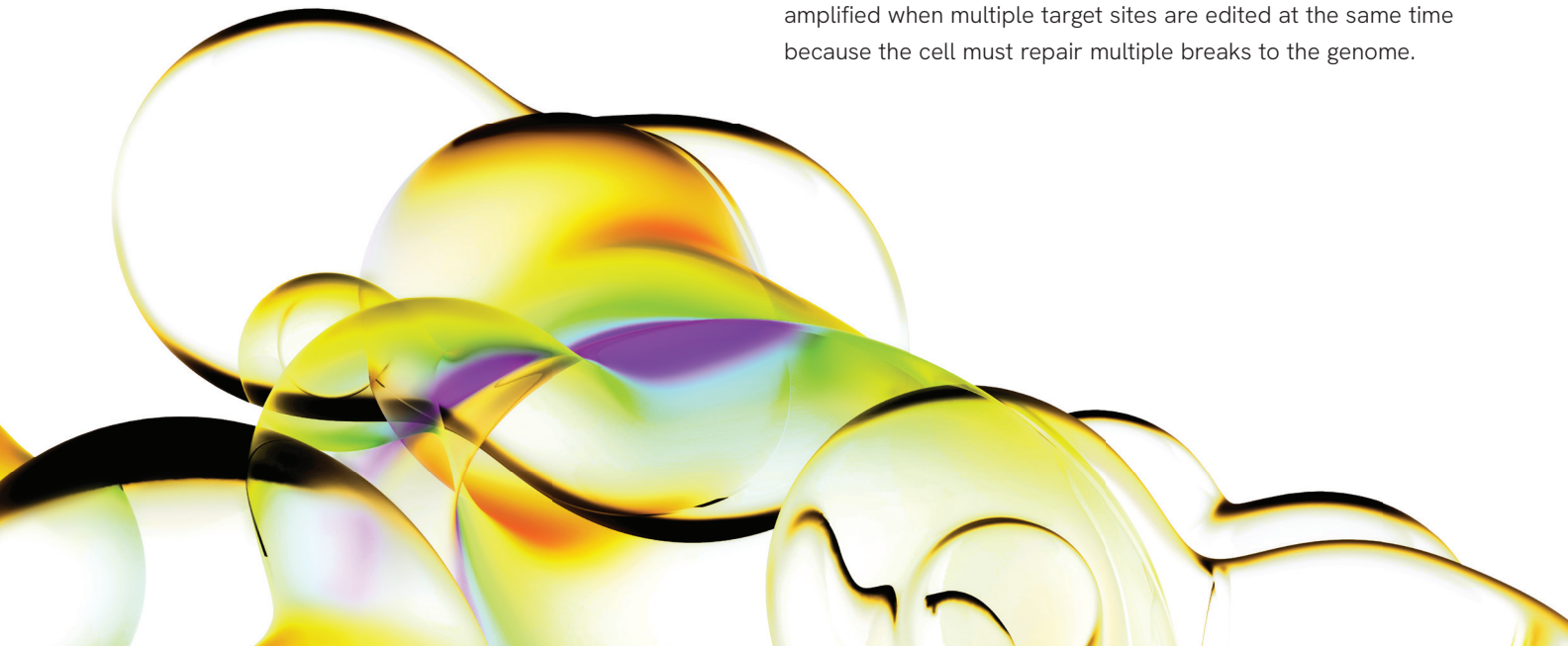
Complex genome engineering with the Pin-point base editing system, even in sensitive cell types.

Introduction

Genome editing technologies are rapidly developing to be more specific, less detrimental to cells, and capable of performing increasingly complex engineering tasks. One of these next generation genome editing technologies is base editing, originally developed by David Liu and colleagues at Harvard University in 2016[1]. Thanks to its versatility and enhanced safety profile, base editing has quickly progressed to clinical use with the technology first appearing in clinical trials in 2023[2].

As the name implies, base editing changes single bases in the genome (either C:G to T:A with cytidine base editors, or A:T to G:C with adenine base editors), but that doesn't mean that the applications of the technology are limited. In fact, it's quite the opposite. Base editing can be used to introduce specific point mutations for disease modeling or correction[3], but it can also be used to knockout proteins through introduction of premature stop codons[4] or splice site disruptions[5]. In some cases, base editing can also activate silenced genes[6], and could be used for rendering proteins semi-functional through single or multiple amino acid changes in specific protein domains[7]. The Pin-point™ base editing platform employed here can also be used for simultaneous multiplex knockout and knock-in applications[8].

Base editing is different from first generation CRISPR-Cas technologies that rely on the generation of double strand DNA breaks (DSBs) to edit DNA sequences. Base editing does not rely on DSBs because the RNA-guided nuclease is either deactivated or modified to only nick a single strand of DNA. The DSBs introduced by CRISPR-Cas systems can be cytotoxic, particularly in sensitive cell types. These cytotoxic effects are amplified when multiple target sites are edited at the same time because the cell must repair multiple breaks to the genome.



The occurrence of DSBs can also lead to undesirable genomic rearrangements and translocations[9]. Base editing is therefore a unique solution for editing the genomes of cell types that are sensitive to DSBs, as well as being a next generation tool for complex cell engineering projects.

The Pin-point base editing platform is comprised of three different components (Fig. 1). An RNA-guided nuclease is directed to the genomic target through a single guide RNA (sgRNA) that includes an RNA aptamer sequence. The RNA aptamer sequence recruits a deaminase-aptamer binding protein fusion to perform base editing on the target DNA. All three components of the Pin-point base editing platform can be configured and optimized for specific applications. Data shown here was generated with a nickase Cas9 and rat APOBEC configuration of the Pin-point base editing platform.

Here, we demonstrate improved viability and reduced translocations in clinically relevant T cells and human induced pluripotent stem cells (iPSCs) when performing complex genome engineering to knockout multiple proteins in a single transfection with the Pin-point base editing platform compared to first generation CRISPR-Cas9 gene editing.

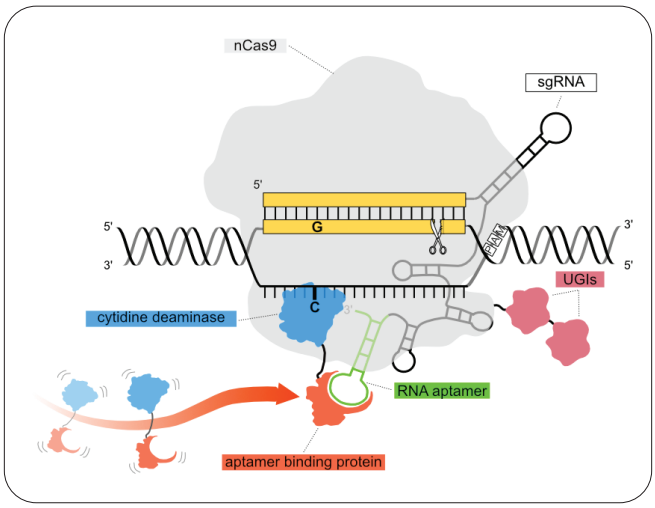


Figure 1: Illustration of the Pin-point™ base editing platform, shown with a nickase Cas9 and cytidine deaminase configuration.

Results

Multiplex editing without compromising efficiency

For applications where multiple single base changes need to be introduced, or multiple proteins need to be knocked out at the same time, the ability to do complex engineering in a single step can save significant time and costs in the laboratory and can shorten manufacturing time for cell therapy production. Maintaining high editing efficiencies while multiplexing is also critical to result in a population of cells where most individual cells have all intended edits so that the population can be used without further enrichment, or clones can easily be generated from the population.

To demonstrate the ability of the Pin-point™ platform to edit multiple loci simultaneously in primary T cells without loss of efficiency compared to editing one gene at a time, synthetic sgRNAs were designed to cause protein knockout through C to T conversion in four target genes: beta-2 macroglobulin (B2M), cluster of differentiation 52 (CD52), T cell receptor alpha constant (TRAC), and programmed cell death protein 1 (PCDC1). When looking at both genotypic (Fig. 2A) and phenotypic (Fig. 2B) data, we observed no difference in C to T conversion and target protein loss, respectively, between editing one locus or multiple loci simultaneously.

The Pin-point platform was also used to evaluate single and multiplex editing efficiencies in human induced pluripotent stem cells (iPSCs), which are known to be highly sensitive to DNA damage (Fig. 2C, D). Of the four proteins targeted, iPSCs only expressed B2M in their undifferentiated state, therefore B2M was used as a representative target for genotypic and phenotypic data post-editing. In a population of iPSCs electroporated with either a single sgRNA or four sgRNAs, the efficiency of target base editing was minimally impacted by the increased number of sgRNAs (Fig. 2C). Additionally, cell populations edited with the Pin-point system showed less than 10% difference in functional knockout between single or multiplex (Fig. 2D).

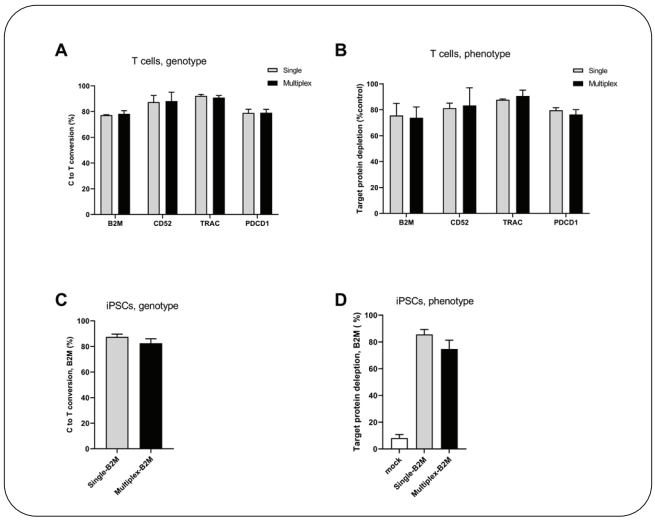


Figure 2: A, B) Primary human T cells were electroporated with 1 (single) or 4 (multiplex) sgRNAs targeting different genes using the Pin-point base editing platform. Data is representative from two T cell donors, spanning two independent experiments. A) Editing of the Pin-point platform treated cells was measured by % C to T conversion, detected by Sanger sequencing. B) Cell populations were also subjected to flow cytometric analysis for protein loss. C, D) Two human iPSCs lines were electroporated with 1 (single) or 4 (multiplex) sgRNAs targeting different genes. C) Editing of the Pin-point platform treated cells was measured by % C to T conversion, detected by Sanger sequencing. D) B2M is shown as an example of % protein loss induced by the Pin-point platform under mock, single, or multiplex conditions.

Multiplex editing with the Pin-point™ platform has improved cell health outcomes compared to traditional CRISPR-Cas editing

When choosing a technology to perform multiple edits simultaneously in a sensitive cell type, it is important to ensure not only that editing efficiency is maintained, but also that viability and cell health is not compromised.

First, viability and rate of cell expansion of T cells edited by a single sgRNA or in a multiplex of four sgRNAs by CRISPR-Cas9 (WT Cas9) or the Pin-point platform were compared. First, viability and rate of cell expansion of T cells edited by a single sgRNA or in a multiplex of four sgRNAs by WT Cas9 or the Pin-point platform were compared. Over the course of three days, the T cell populations edited with a single sgRNA and either WT Cas9 or the Pin-point platform both maintained nearly 100% viability (Fig. 3A).

However, differences emerged when multiple genes were targeted in the same electroporation. The population of cells multiplex edited by WT Cas9 suffered nearly a 40% decrease in viability, while the population multiplex edited with the Pin-point platform maintained high viability with a less than 10% decrease in viability (Fig. 3A). Additionally, simultaneous editing at three or four loci with WT Cas9 adversely impacts T cell expansion measured over three days compared to the Pin-point platform (Fig. 3B).

Human iPSCs are very sensitive to DNA damage[10]. When editing such cell types, additional introduction of DSBs by way of targeting multiple genes simultaneously may decrease the cell viability exponentially. When introducing multiple edits at one time, the viability of edited iPSCs was unaffected as the number of sgRNAs increased from one to four when using the Pin-point platform, while the number of live cells significantly decreased as the number of sgRNAs increased in WT Cas9 edited iPSCs (Fig. 3C).

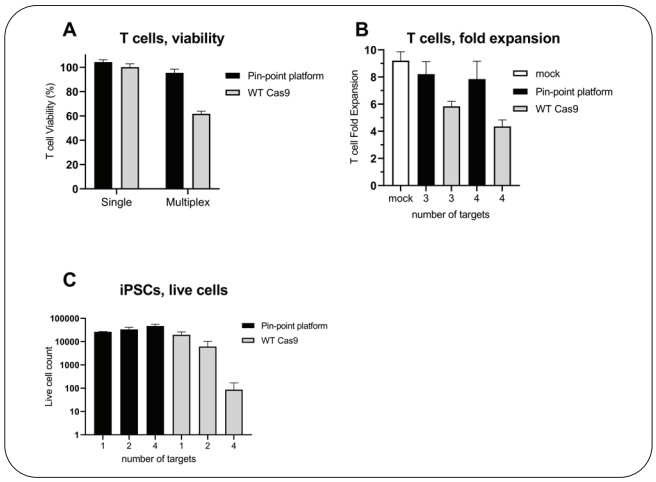


Figure 3: Primary T cells (A, B) or iPSCs (C) were electroporated either with single or multiple sgRNAs and WT Cas9 or the Pin-point platform. A) T cell viability was measured for each condition by flow cytometric analysis, using DAPI DNA stain to differentiate the live and dead populations. B) Fold expansion of T cell populations, determined by dividing the day 3 live count by the day 0 live count, was also assessed to determine T cell proliferative capacity. C) iPSC viability was assessed 7 days after electroporation by live cell count.

Multiplex editing with the Pin-point™ platform reduces translocations compared to traditional CRISPR-Cas editing

Genomic rearrangements or translocations happen when one part of a cell's genome breaks and gets fused to another part in an unnatural way. This could occur even when only one sgRNA is introduced in a single transfection with traditional CRISPR-Cas9 editing because a DSB occurring on-target could translocate with a DSB occurring at an off-target site. The probability of translocations increases as the number of DSBs in the genome increases, such as when multiple edits are simultaneously introduced with traditional CRISPR-Cas9 systems.

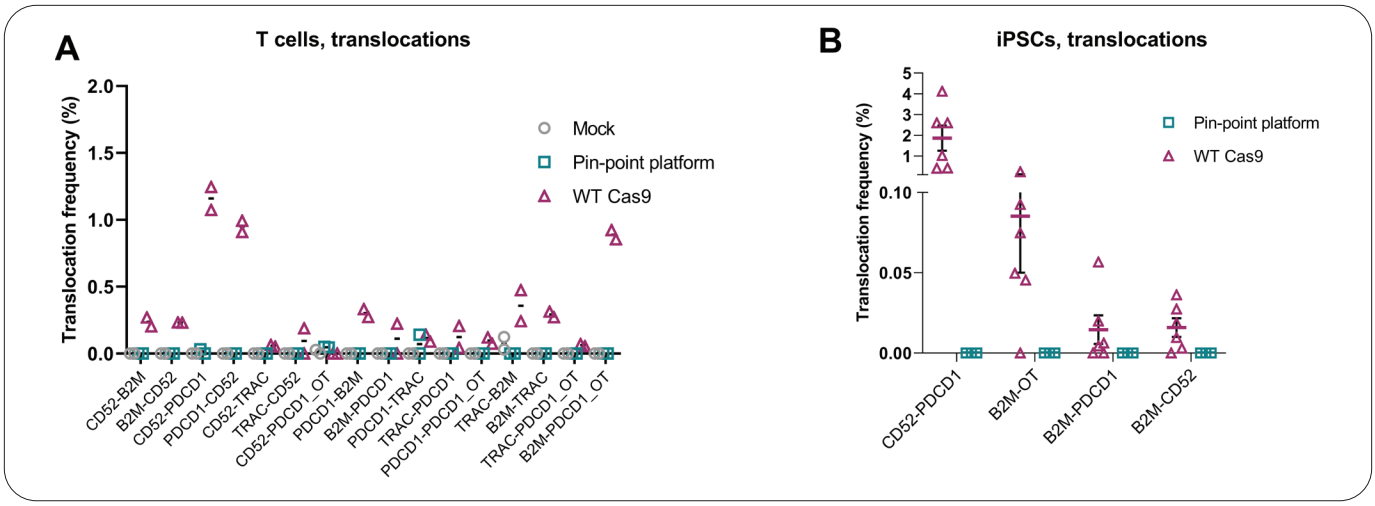


Figure 4: Primary T cells (A) or human iPSCs (B) were electroporated simultaneously with four sgRNAs targeting B2M, CD52, PDCD1, and TRAC genes, and evidence of translocations between on-target and predicted off-target sites were measured by ddPCR.

To assay for evidence of translocations, primary T cells edited with a multiplex of sgRNAs targeting four different genes simultaneously by either WT Cas9 or the Pin-point platform were assayed by digital droplet PCR (ddPCR). As expected, translocations were observed between the targeted sites, but also between target and off-target sites when the cells were edited with WT Cas9 (Fig. 4A). However, translocations were not detected over baseline when the Pin-point platform was used to introduce the same multiple edits.

Using four of the frequent translocations validated in T cells, we then looked for similar evidence of translocations in iPSCs after editing at the same four sites edited in T cells using either WT Cas9 or the Pin-point platform. While translocations were undetectable in cells edited with the Pin-point platform, they were detected in WT Cas9 edited cells (Fig. 4B).

Conclusion

Base editing can be used as a genome engineering tool to generate cell models, explore functional genomics, and is also emerging as a clinically relevant method for developing cell and gene therapies[6,8,11]. As understanding of monogenic disorders and individual protein function expands, the need for increasingly complex models and more granular interrogation of the genome is growing. Genome engineering technologies like base editing can contribute to better comprehension of complex diseases, pathways, and networks.

Introducing edits in multiple genes at one time can save cost and time in the laboratory so long as editing efficiencies, cell health, and genomic integrity are maintained. Editing technologies such as traditional CRISPR-Cas9 that rely on DSBs can have negative impacts on cell health and genomic integrity, particularly in sensitive, clinically relevant cell types such as human primary T cells and iPSCs.

To achieve complex genome engineering projects, base editing offers an attractive solution. When editing multiple genes simultaneously with the Pin-point™ base editing platform, it is possible to achieve high editing efficiencies without reducing cell viability or impacting the rate of cell expansion. Technologies like the Pin-point base editing platform that do not rely on DSBs also reduce the frequency of editing-induced translocations, which could otherwise potentially compromise the downstream use of cell models and products.

Materials and methods

Primary human T cell isolation and culture and electroporation

Primary human T cells were cultured and electroporated as reported in Porreca et al[8]. Briefly, CD3+ T cells were isolated from fresh whole peripheral blood (CPD Blood bags, Cambridge Bioscience). T cells were isolated by immunomagnetic negative selection using the EasySep™ Human T Cell Isolation Kit (STEMCELL Technologies). T cells were cultured in Immunocult™-XF T cell expansion medium (STEMCELL Technologies), supplemented with Penicillin-

Streptomycin (Gibco) and IL-2 (100 IU/mL; STEMCELL Technologies), and activated with DynaBeads Human T-Activator CD3/28 (Gibco) at a 1:1 bead to cell ratio for 48 hours prior to electroporation.

After removing DynaBeads from culture medium, the activated T cells were counted, pelleted by centrifugation, and resuspended in the total volume of R buffer needed for all conditions. Activated T cells were electroporated with sgRNA at 2 µM and 1.6 µg of of Pin-point nCas9-UGI-UGI and 0.2 µg of Pin-point rApobec1 or 1 µg WT Cas9 mRNA (Horizon Discovery™ now part of the Revvity™ group and TriLink Biotechnologies®) using the Neon Electroporation System (Invitrogen). Post-electroporation, T cells were cultured in antibiotic free ImmunoCult™-XV T cell expansion medium (STEMCELL Technologies) supplemented with IL-2 (100 IU/ml; STEMCELL Technologies), IL-7 (100 IU/ml; Peprotech, New Jersey, USA) and IL-15 (100 IU/ml; Peprotech, New Jersey, USA) and incubated at 37 °C, 5% CO_2 for 3-7 days.

iPSC culture and electroporation

WTC-11 (Corriell Institute, www.AJSC.us/ISSN:2160-4150/AJSC1304002) and NH50191 (NINDS) lines were cultured on Geltrex® (ThermoFisher). The Gibco iPSC line (A18945, doi:10.1371/journal.pone.0018293) was cultured on Vitronectin XF (STEMCELL Technologies). All lines were maintained in mTeSR™-PLUS medium (STEMCELL Technologies) Y-27632 (STEMCELL Technologies).

Electroporation was performed with the Amaxa 4D-Nucleofector (Lonza) and in either 20 µL or 100 µL Amaxa NucleoCuvette® Cartridges (Lonza) at a density of 2e5 or 1e6 cells per cuvette, respectively. Pelleted iPSCs were resuspended in P3 Primary Cell Nucleofector® Solution (Lonza), and electroporated with 40 pmol sgRNA and either 2.56 µg Pin-point nCas9-UGI-UGI and 0.74 µg of Pin-point rApobec1 mRNAs or 2 µg WT Cas9 mRNA (Agilent, Horizon Discovery™ now part of the Revvity™ group, and TriLink Biotechnologies®) using the Amaxa program CM138. After electroporation, cells were recovered in mTeSR™-PLUS medium (STEMCELL Technologies) and Y-27632 (STEMCELL Technologies).

Cell lysis, PCR, and base editing analysis

For genomic DNA preparations, cells were resuspended (T cells) or lysed (iPSCs) in 40 µL of lysis buffer (direct PCR lysis reagent; Viagen #732-3260) and incubated at 55 °C for 30 minutes, followed by 95°C for an additional 30 minutes. Lysates were used to generate PCR amplicons spanning the region containing the base editing site(s). PCR amplicons between 400-1000 bp in length were generated and sequenced by Sanger sequencing. Base editing efficiencies were calculated from Sanger sequencing reads and displayed as % C to T editing, using the Chimera™ analysis tool, an adaptation of the open-source tool BEAT. Chimera first determines the background noise to define the expected variability in a sample, using a geometric mean with outliers capped to the median value. Following this, Chimera subtracts the background noise to determine the editing efficiency of the base editor over the span of the input guide sequence.

T cell flow cytometry

Prior to flow cytometric analysis, T cells were stimulated to induce expression of PD-1 (PDCD1). T cells were split into two groups, stimulated and unstimulated. T cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma-Aldrich) and ionomycin (250 ng/mL; Millipore) in the presence of IL-2 (100 IU/mL; STEMCELL Technologies) for 48 h prior flow cytometry analysis to induce the expression of PD-1, while unstimulated cells were treated with an equal volume of non-stimulating media containing IL-2 (100 IU/mL; STEMCELL Technologies). After 48 h, T cells were stained with the following fluorophore conjugated anti-human antibodies: TCR alpha/beta-BV785 (BioLegend #306742), B2M-FITC (BioLegend # 316304), CD52-PE (BD Biosciences #562945), and PD1-APC (BioLegend #329908). Fluorescence minus one (FMO) controls were included for accurate gating strategy. DAPI was used to stain for live cells. Cells were acquired on an IntelliCyte IQue PLUS or Sartorius iQue3 flow cytometer using iQue ForeCyt® Enterprise Client Edition 9.0 (R3) Software. Single, live, cells negative for each specific marker were gated. The level of PD-1 knockout was evaluated in cells stimulated with PMA/ionomycin while levels of TRAC, B2M and CD52 in non-stimulated cells.

T cell viability and fold expansion explanation

At 2 h, 24 h, 48 h, 72 h and 7 d post electroporation, T cells were stained with DAPI to measure percent cell viability and live cell counts as measured by flow cytometric analysis. Fold expansion was calculated 72 h post-electroporation by dividing the day 3 live count by the day 0 live count. For fold expansion calculation, CountBright™ Absolute Counting Beads (Invitrogen) were added to flow cytometry samples to allow counting of the absolute number of live (DAPI negative). A flow cytometry count was performed 2 h after editing

(baseline), and 3 days after editing. Fold expansion was calculated by dividing the live cell count for each sample by its own baseline count.

iPSC flow cytometry and viability

iPSC lines were washed with PBS and dissociated with Accutase (STEMCELL Technologies) prior to being transferred to v-bottom plates for centrifugation. iPSCs were then stained for 30 minutes at 4 °C with B2M-FITC (BioLegend # 316304) and DAPI for cell viability. Cells were acquired on an IntelliCyte IQue PLUS or Sartorius iQue3 flow cytometer using iQue ForeCyt® Enterprise Client Edition 9.0 (R3) Software.

Digital droplet polymerase chain reaction (ddPCR) quantification of translocations

ddPCR was performed as reported in Porreca et al[8]. Briefly, genomic DNA from electroporated iPSCs or CD3+ T cells was isolated using DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions and genomic DNA was subsequently digested with EcoR1-HF (NEB). ddPCR Supermix (No dUTP, BioRad) was used for PCR reactions consisting of a PPIA primer, a translocation primer, and the EcoRI-HF digested genomic DNA. Droplets were generated using the QX200 Droplet-digital PCR system (Bio-Rad) according to manufacturer's instructions.

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