Precision CRISPR Editing of Induced Pluripotent Stem (iPS) Cells

Key Takeaways

- Synthego offers three types of edits in iPS cells: knockouts, SNVs, and tags.
- Edited iPS cells are generated using a streamlined process that retains pluripotency, viability, and genome stability.
- Optimized protocols deliver knockout efficiencies of ~90% in iPS cell pools.
- All projects are 100% guaranteed to contain your desired edit.

Abbreviations:

iPS cell: induced pluripotent stem cell
ESC: embryonic stem cell
CRISPR: clustered regularly interspaced short palindromic repeats
Cas9: CRISPR-associated protein 9
gRNA: guide RNA
NHEJ: non-homologous end joining
HDR: homology-directed repair
SNV: single nucleotide variant
sgRNA: single guide sgRNA
ICE: Inference of CRISPR Edits
ssODN: single-stranded oligodeoxynucleotide

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Introduction

A significant amount of biomedical research relies on the culture of immortalized cell lines, which are easy to use and manipulate. However, these cell lines are often genetically abnormal and may not faithfully recapitulate the characteristics of the tissue they are intended to represent. Takahashi and Yamanaka's 2006 work on **induced pluripotent stem (iPS) cells**[1] was a seminal innovation in the biomedical field. By ectopically expressing key transcription factors in mouse cells, these researchers found that fully differentiated cells could be reverted to a pluripotent state characteristic of embryonic stem cells (ESCs). Once reprogrammed, an iPS cell could then be directed to differentiate into any cell of the three germ layers (endoderm, mesoderm, and ectoderm). A year later, these researchers achieved the same feat in human cells[2], ending the reliance on human embryos for a source of stem cells. The advent of iPS cells has provided researchers with unprecedented access to virtually any cell type and has enabled the creation of diploid, genetically stable models that resemble primary tissues. The vastly improved validity and reliability of data generated from iPS cell-based models continues to accelerate research timelines from discovery to the clinic.

In the age of personalized medicine, we have characterized thousands of genomes. However, identifying genetic changes that modulate biology and affect disease outcomes requires the ability to edit the genome with high specificity. The relatively new gene-editing tool **CRISPR (clustered regularly interspaced short palindromic repeats)** has provided an unparalleled level of precision and efficacy to genome engineering.[3] The CRISPR system comprises a programmable guide RNA (gRNA) that binds to a target genomic sequence, allowing a Cas9 endonuclease to cleave both strands of DNA. The innate DNA repair that follows can then be co-opted to generate edits at the cut site. For instance, non-homologous end joining (NHEJ) can be utilized to induce frameshift mutations that disrupt protein function (knockout). Alternatively, homology-directed repair (HDR) can be used to knock in a sequence of interest or generate single-nucleotide variants (SNVs).

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CRISPR-edited iPS cells are making incredibly valuable contributions to the clinical space. For instance, CRISPR editing is used to produce disease models in relevant cell types, generate isogenic cell lines, and correct mutations in patient-derived cells. Edited pluripotent or differentiated iPS cell lines are also used in high-throughput screens to search for new therapeutic compounds. Together, these two revolutionary technologies are greatly advancing our understanding of fundamental molecular mechanisms, disease pathways, and are accelerating the establishment of the next generation of therapies (Fig 1).

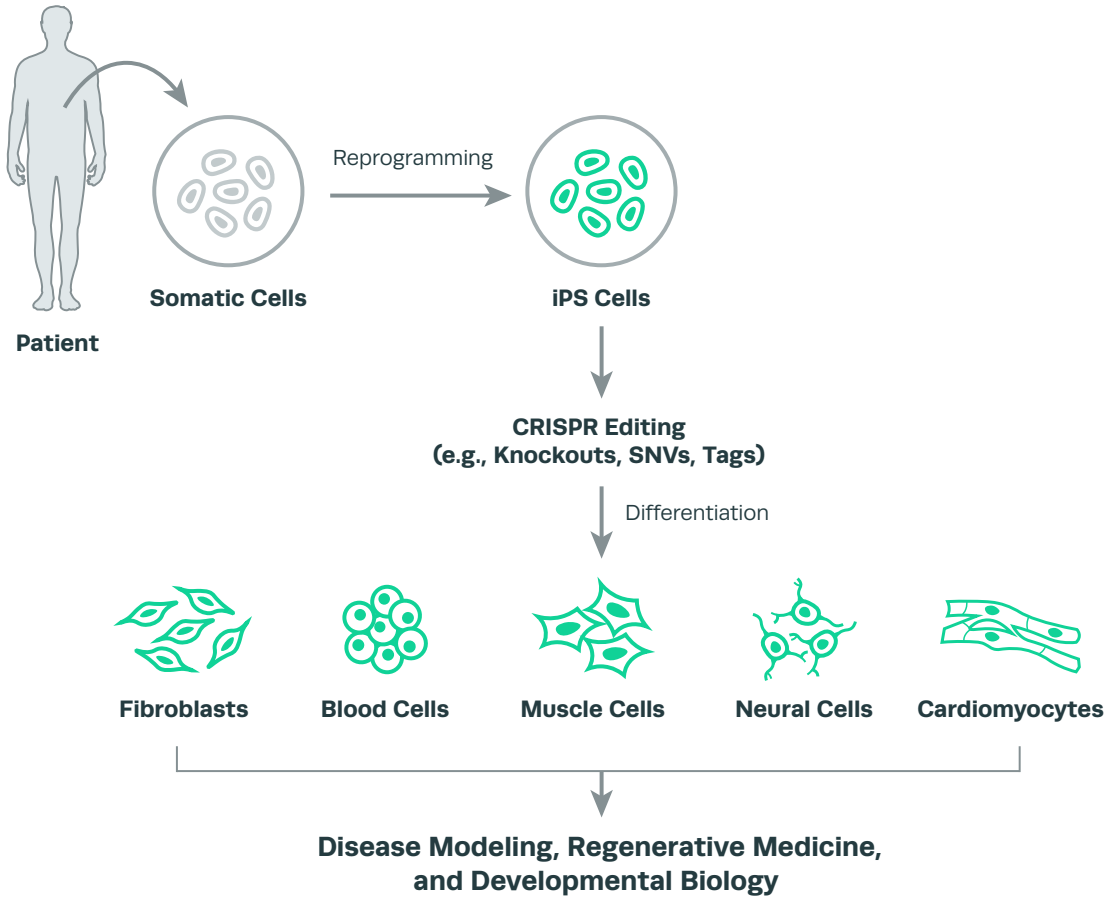

Figure 1. Genetic modifications in iPS cells accelerate research in a variety of fields.
Somatic cells derived from a patient or donor are re-programmed into an embryonic-like pluripotent state by integrating a set of transcription factors into the genome. CRISPR is then used to generate the desired edit, such as knockout, SNV, or tag insertion. Edited iPS cells can then be differentiated into any cell type. Human iPS cells are now used in a variety of scientific fields, including disease research, regenerative medicine, and developmental biology.

Despite the rising enthusiasm for editing iPS cells, unique challenges still remain. First, iPS cells are notoriously difficult to work with and require constant monitoring to maintain in a pluripotent state. Second, CRISPR editing requires a considerable amount of optimization to ensure high on-target editing efficiencies while minimizing off-target effects. Further, generating and screening clones requires weeks or even months of tedious work. Mastering the CRISPR workflow while maintaining pluripotency and viability requires a substantial amount of skill, time, and financial investment. All of these factors can significantly stymie one's research or serve as a roadblock to benefiting from iPS cell-based approaches altogether.

The Synthego Solution

Leveraging our expertise in automated high-throughput genome engineering, Synthego has implemented a robust process for editing human iPS cells using CRISPR. We are currently offering three types of edits in Synthego-supplied or customer-supplied iPS cell lines:

Knockouts

Single Nucleotide Variants

Tags

Each of these genomic modifications is available in pool (i.e., an edited cell culture that contains mostly edited sequences) and clone (100% edited genetically identical cells) formats. All iPS cell projects undergo rigid quality control checks and are **guaranteed** to have your desired editing outcome. Here, we present our manufacturing process for iPS cell gene editing as well as example data depicting our three iPS cell offerings: knockouts, SNVs, and tags.

Synthego's Process for Engineering iPS cells

Synthego has developed a highly streamlined workflow for generating edited iPS cell pools and clones (Fig 2). Each project begins with the submission and assessment of your desired edit in your supplied or Synthego-supplied iPS cell line. Once accepted, the steps for engineering the iPS cell project are delineated below.

- ***Guide RNA Design and Transfection***

  Synthetic single guide RNAs (sgRNAs) and Cas9 (*Streptococcus pyogenes*) are used for all iPS cell editing projects. The sgRNAs are designed using our bioinformatics-driven design parameters and synthesized with our high-throughput Halo™ Platform. All guides contain chemical modifications that increase stability and resist triggering an innate immune response within the cell. For SNV and tag projects, single-stranded oligodeoxynucleotides (ssODN) are also designed and manufactured to serve as a template for HDR.

  Each iPS cell project is subject to several quality checks prior to transfection, including adaptation to our growth conditions and karyotyping to ensure cells are genomically normal. Guides and Cas9 are transfected as ribonucleoproteins (RNPs) using an optimized electroporation protocol. Because RNPs persist only briefly inside the cells, the chance of off-target editing is reduced.[4,5]

  After transfection, the resulting iPS cell pool contains a heterogeneous mix of edited and unedited cells. To evaluate the editing efficiency of the pool, the edited and control regions of the target site are PCR-amplified, Sanger-sequenced, and analyzed using Synthego's Inference of CRISPR Edits (ICE) software.* After the analysis of the pool, the remaining steps of the workflow differ depending on whether a pool or clone is ordered.

- ***Pools***

  For pool projects, the edited iPS cell population is subjected to QC genotyping and mycoplasma testing. Pluripotency testing is available as an add-on service. The final shipment includes two vials of edited pools and two vials of mock-transfected pools. Additionally, a retain vial will be stored at Synthego for twelve months

- ***Clones***

  For clone projects, single cells are isolated from the pool using an automated workcell. Colonies originating from the single cells are monitored for morphology using automated imaging, and any differentiating cells are removed. Clonal colonies are hit picked, then analyzed by PCR, Sanger sequencing, and ICE to select those that contain the edit of interest. Once validated, the clones are expanded over several weeks, and final clonal populations undergo QC genotyping and mycoplasma testing before shipment. Pluripotency and karyotyping analyses are available as add-on services. Orders include two vials per clone (2 clones), each containing an independent monoclonal population, and two vials of mock-transfected pools.

* Synthego's ICE tool is available at ice.synthego.com. For an explanation of how ICE works, please see https://www.synthego.com/guide/how-to-use-crispr/ice-analysis-guide

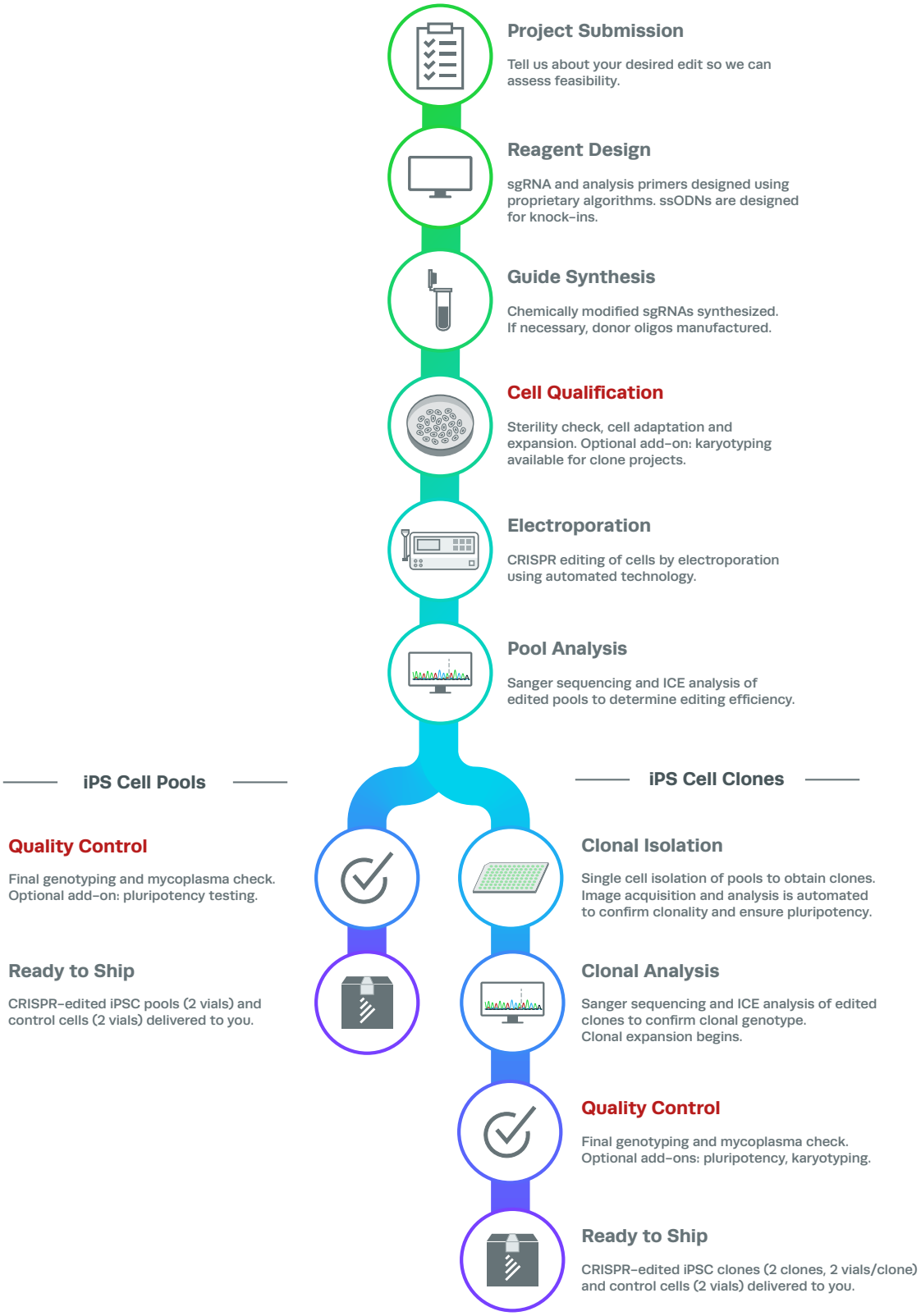

Figure 2. Synthego's streamlined process for editing iPS cell pools and clones.
Synthego's process for iPS cell editing utilizes an automated workflow and stringent quality checks (red text) to ensure our edited pool and clone projects contain the desired edit and are free of contaminants.

Reliable Knockouts

Knockout iPS cells are incredibly valuable for a variety of applications, including gene function studies, validation of drug targets, disease pathway analysis, and screening applications. By knocking out a gene of interest in an iPS cell line, one can assess causal gene-phenotype relationships in a biologically relevant context. Synthego offers both knockout iPS cell pools and clones.

Here we illustrate a successful knockout in the pool format via the hypoxanthine (guanine) phosphoribosyl transferase (HPRT) assay,[6,7] which uses the toxic nucleotide analog 6-thioguanine (6-TG) to indicate functional knockout at the HPRT locus. Given that functional HPRT protein is required for 6-TG cytotoxicity, treatment with the agent is expected to kill unedited cells. In HPRT knockout pools, however, cells with a functional knockout are expected to survive 6-TG treatment. Negative control knockout and parental controls (receiving no 6-TG) are also expected to maintain viability (see Fig 3 for predicted results).

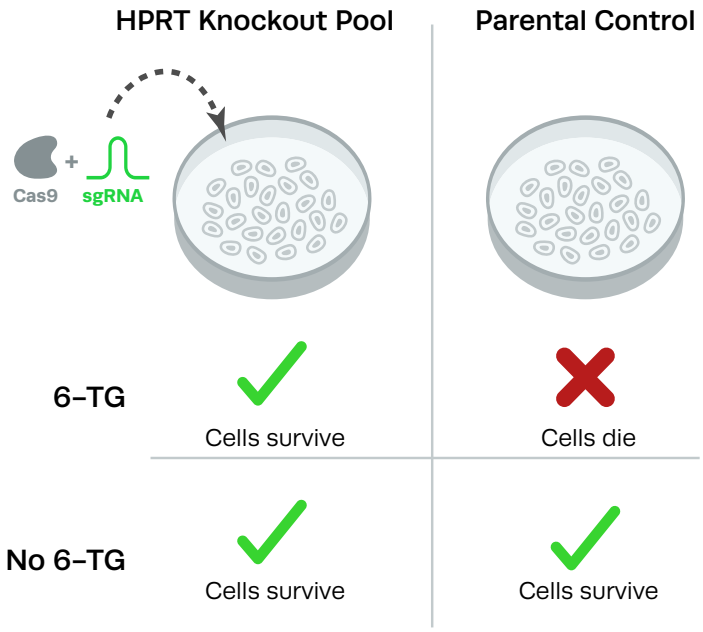

Figure 3. Experimental design and expected results of the HPRT assay.
Knockout of the HPRT gene desensitizes cells from the toxic nucleotide analog, 6-TG. Upon treatment with 6-TG, parental cells are expected to die (upper right, red X), whereas HPRT knockout cells are expected to survive (upper left, green check). HPRT knockout and parental controls not treated with 6-TG serve as negative controls and are expected to retain viability (lower left and right, respectively).

For three iPS cell lines (CR0003, CR0005, and NN5200), duplicate HPRT knockout cell pools were generated and compared with parental controls. In all three lines, 6-TG treatment resulted in the expected loss of cell viability in the parental control (Fig 4, upper right panels marked by a red X). Cell death was characteristically followed by the propagation of isolated colonies, which appear as dark spots.

By contrast, cell viability was maintained in the HPRT knockout pools for all three cell lines (upper left panels marked by a green check), similar to the negative control pools that did not receive 6-TG (Fig 4, 2nd row of panels marked by a green check). These functional results, along with accompanying high Knockout Scores in the HPRT knockout treatments (96%, 88%, 94% respectively) indicate successful ablation of the HPRT gene.

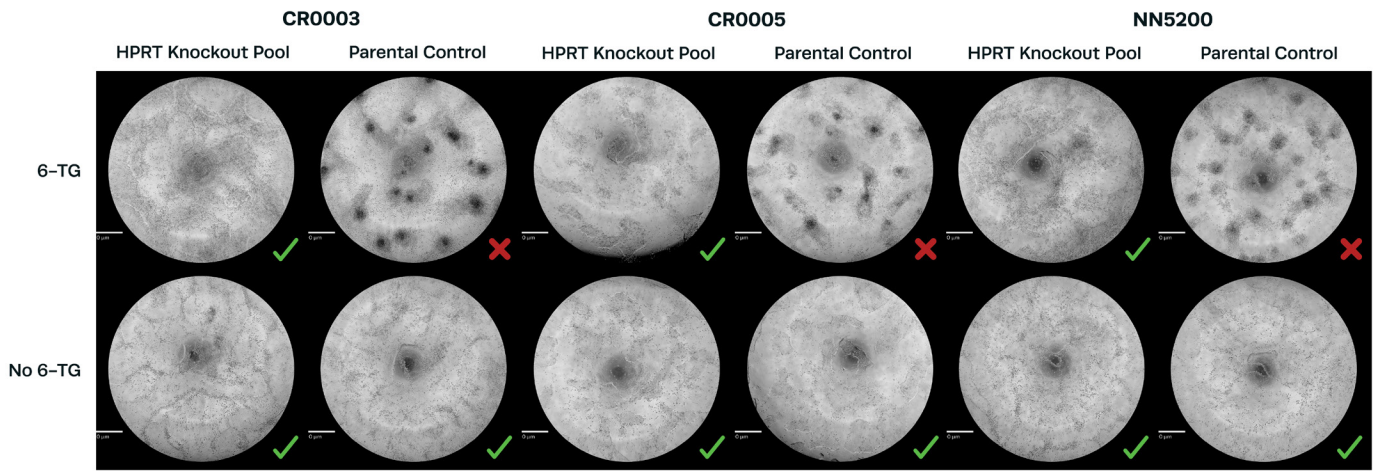

Figure 4. Functional Knockout of HPRT confirmed by resistance to 6-TG toxicity.
Three iPS cell lines (CR0003, CR0005, and NN5200) were treated with 6-TG to confirm HPRT functional knockout. For each cell line, reduced cell viability was apparent in the parental controls following treatment with 6-TG (upper right panels, red X). The HPRT knockout pools retained cell viability upon 6-TG treatment (upper left panels, green check), appearing similar to the negative control pools not treated with 6-TG (second row). All iPS cell lines were cultured in StemFlex medium on hESC-qualified Matrigel-coated culture plates. HPRT knockout treatments were transfected with RNPs via electroporation and analyzed using ICE. All pools were visualized using a compound microscope (4x magnification).

Precise Single Nucleotide Variants (SNVs)

A substantial amount of variability in the human genome is characterized as SNVs. Such variability poses a challenge to disentangling disease-associated mutations from diverse genetic backgrounds. Using CRISPR, it is now possible to make precise single-nucleotide changes in iPS cells to facilitate a variety of capabilities in disease research and functional genetics. For instance, an isogenic control can be produced from a patient by reverting a disease-causing SNV to the unedited state. Alternatively, different SNVs can be generated in normal cells from a healthy donor in order to identify which variants recapitulate a disease.

Using optimized workflows for HDR-based editing, Synthego will generate your SNV of interest in a pool and/or clone format. For clone projects, both homozygous and heterozygous SNVs can be generated. Figure 5 depicts an ICE readout of a homozygous single nucleotide change from cytosine in the parental cells (bottom trace) to thymine in the edited sample (top trace).

ICE results for this SNV edit are available at: Synthego.com/ice3

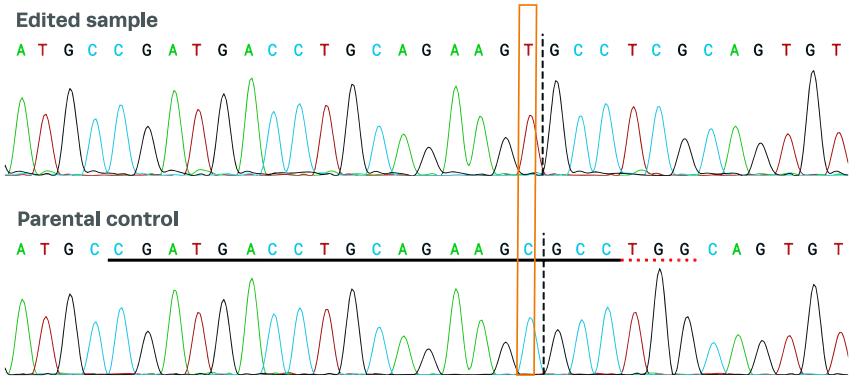

Figure 5. 100% SNV editing in iPS cell clones.
A CRISPR-edited homozygous clone containing a single nucleotide change from cytosine (C, bottom) to thymine (T, top). The SNV change is enclosed by an orange box across both traces. The target sequence (underlined in black) and the PAM site (underlined with a red dashed line) are indicated in the control trace. Human iPS cells were electroporated with sgRNA and Cas9 (as RNPs) along with a ssODN containing the single nucleotide change. To produce clonal populations, single cells were isolated using limiting dilution and expanded.

High–Efficiency Tags

Protein tags are exogenous sequences introduced to genomic loci via HDR and are expressed endogenously with the target protein. These molecular tools are used for many applications, including protein purification, detection, localization, and solubilization.

Tags have a variety of functionalities in iPS cells, including tracking the expression of target genes during cell differentiation and validating protein-level knockouts. Maximizing HDR using CRISPR is a challenging process, and typically yields only low knock-in rates of desired insertions. Synthego has developed an optimized methodology for enhancing HDR over NHEJ in order to drive maximal knock-in efficiency.

As demonstrated in Figure 6 below, our iPS cell pools have a high success rate of tag integration (ranging from 54 to 97%). Our tagged iPS cell pools often have such high insertion percentages that they resemble clones and can be assayed directly. In addition, we are able to maintain high levels of pluripotency, as demonstrated by the HA-tag insertion in Figure 7.

Synthego offers several small protein tags (Table 1) in iPS cell pool and clone formats.

Table 1. Protein tags offered by Synthego and their uses.

Tags	Size (nt)	Common Uses
HA	27	Purification, Detection, Protein:Protein Interactions
Flag	24	Purification, Biochemical Assays, Detection, Protein:Protein Interactions
3Flag	66	Purification, Detection, Protein:Protein Interactions
S Tag	45	Solubility
CBP	78	Purification, Solubility
V5	42	Purification, Activity Assays
Myc	30	Detection, Activity Assays, Biochemical Assays
HiBit	33	Detection, Activity Assays, Protein:Protein Interactions, Reporter Assays

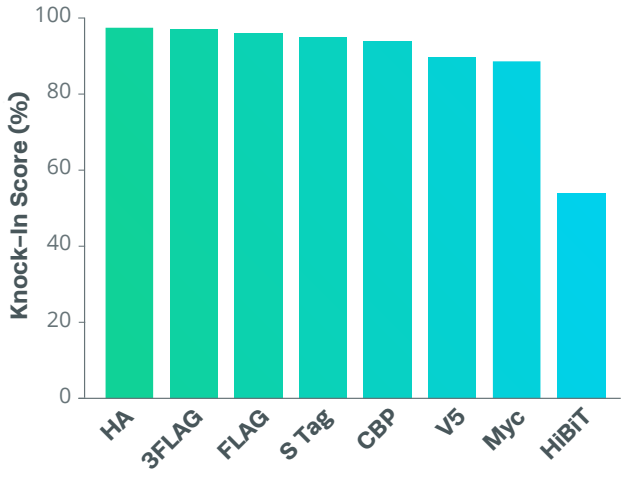

Figure 6. High knock-in efficiency of gene tags in iPS cells pools. Eight small tags were inserted via electroporation of ribonucleoproteins (RNP) targeting the N-terminus of the GAPDH locus. Knock-in efficiency was determined following DNA isolation, PCR amplification, and Sanger sequencing. ICE analysis of traces generated a Knock-In Score, a measure of knock-in efficiency defined as the percentage of sequences containing the desired edit.

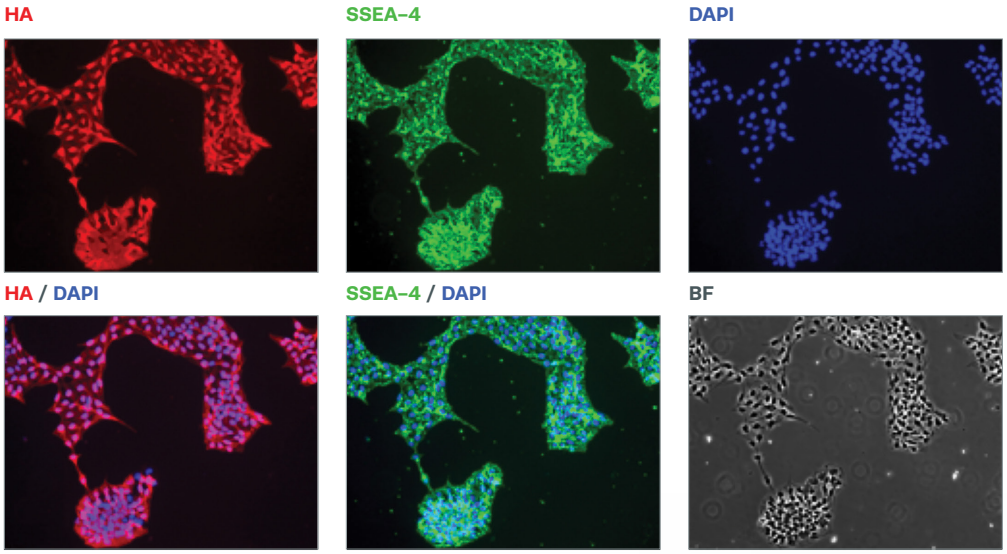

Figure 7. Maintained pluripotency following knock-in of HA tag in iPS cells.
Immunofluorescence images of iPS cells transfected with a ribonucleoprotein (RNP) and ssODN facilitating HA-tag knock-in (a, red) demonstrate maintained pluripotency as measured by SSEA-4 protein expression (b, green). DAPI (c, blue) was used to mark nuclei. Overlays of DAPI and the HA-tag (d) and DAPI and SSEA-4 (e), and bright field, BF (f) are shown.

Conclusion

Two monumental breakthroughs—iPS cells and CRISPR editing— have opened the door to an exciting new era of biological discovery. These technologies have facilitated novel translational research that is shaping the therapies of tomorrow. Already, great strides have been made in treating neurodegenerative diseases, β-hemoglobinopathies, cardiovascular diseases, and ocular disorders. Some exciting *ex vivo* therapies are already in clinical trials. With each passing year, both academic and commercial institutions are marching steadily toward making more personalized medicine a reality.

At Synthego, we strive to enable researchers to make groundbreaking advances in fundamental biomedical research and therapy development. Working with iPS cells is hard enough, and editing them while maintaining their pluripotent state is even more difficult. Furthermore, CRISPR optimization and clonal expansion can take months of valuable time. Relying on Synthego to provide edited iPS cells can free researchers' hands so that they can concentrate on more salient aspects of their research. With our expertise in genome engineering, we are committed to providing you with high-quality iPS cells containing your desired knockout, SNV, or tag—100% guaranteed.

Interested in Synthego's edited iPS cells? Please visit Synthego.com/iPS.

References

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Additional Information

For an up-to-date list of all Synthego Protocols and other resources, please visit Synthego.com/Resources

For technical assistance, contact our Scientific Support Team:

Ph: 888.611.6883 Email: support@synthego.com

About Synthego

Synthego is the leading genome engineering innovation company. The company's automated, full stack genome engineering platform enables broader access to CRISPR to accelerate basic scientific discovery, uncover cures for diseases, and develop novel synthetic biology applications. Headquartered in Silicon Valley, Synthego is used by scientists from the largest global biotechnology companies and global biology universities to unlock the potential of gene editing.