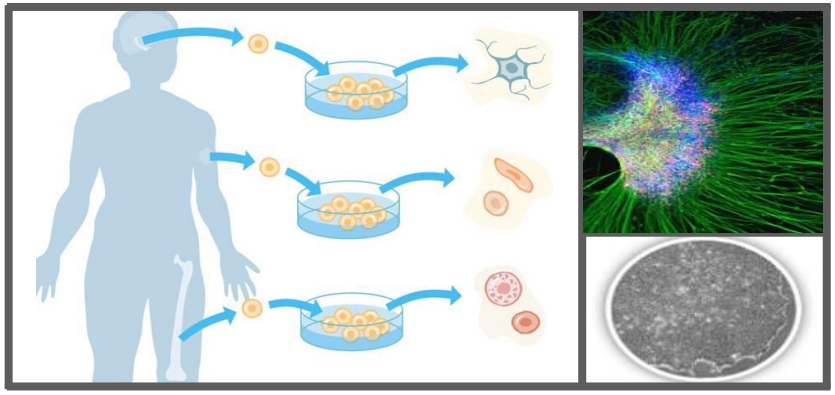
TaKaRa

Clontech TaKaRa cellartis

Efficient, Footprint-Free Gene Editing and Single-Cell Cloning of iPS Cells Using CRISPR/Cas9



Liz Quinn, PhD

June 15, 2017

that's GOOD science!®

Takara Bio USA, Inc.
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Stem Cell Research Expertise

TaKaRa

GMP Manufacturing
Cell & Gene Therapy

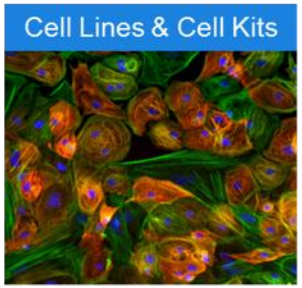
cellartis

Clinical grade iPS & ES
Cell Lines & Media

Clontech

Gene Editing
Reporter lines (FPs)

Product & Service Solution Portfolio



We focus on innovative research & clinical grade stem cell products & services

Comprehensive Products & Services

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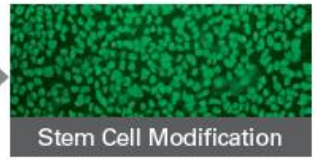
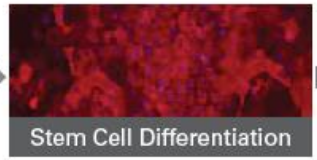
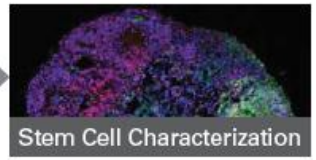
Cellartis® Products

Reprogramming Kits	Media & Supplements	Genome Editing Kits	Media & iPS Derived Cells	Antibodies & Reagents
				
Stem Cell Generation	Stem Cell Culture	Stem Cell Modification	Stem Cell Differentiation	Stem Cell Characterization
Cell Sourcing & iPS Cell Generation	hPS Cell Line Banking	Genetic Modification of hPS Cells	Directed Differentiation into Cell Types	Assay Development

Cellartis Services

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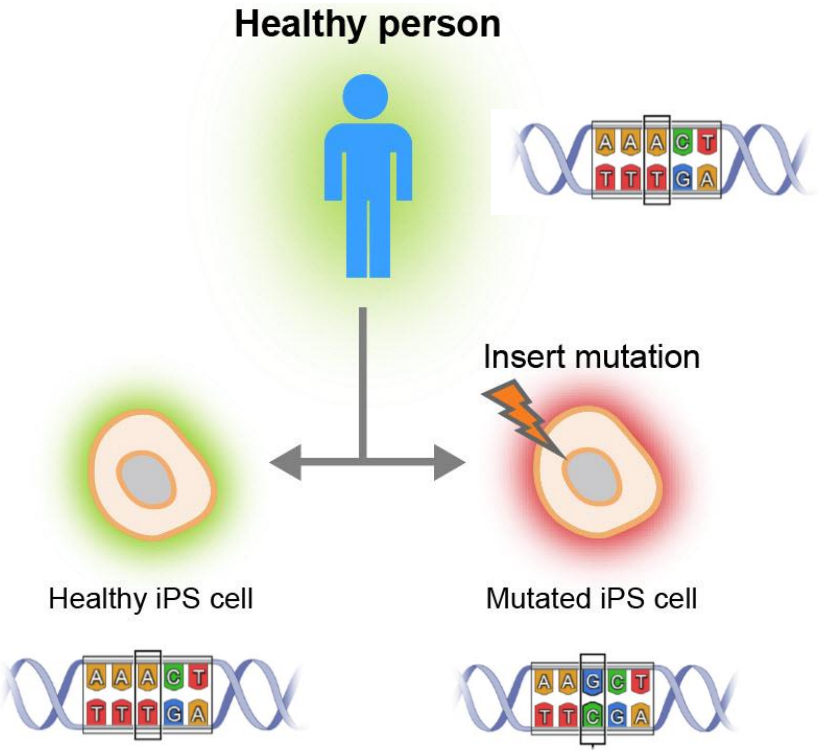
Utility of Stem Cells and Gene Editing

- Stem cells offer a renewable, expandable source of edited cells that can become multiple cell types



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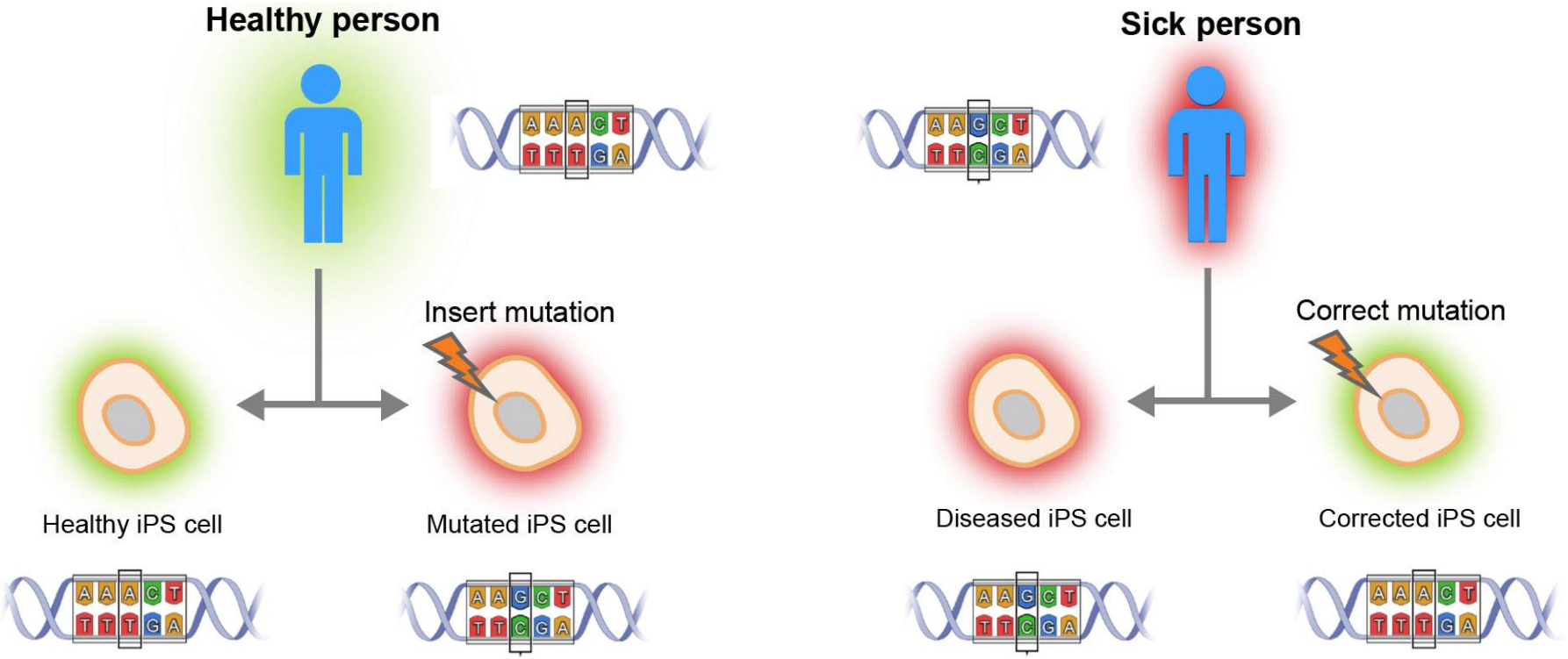

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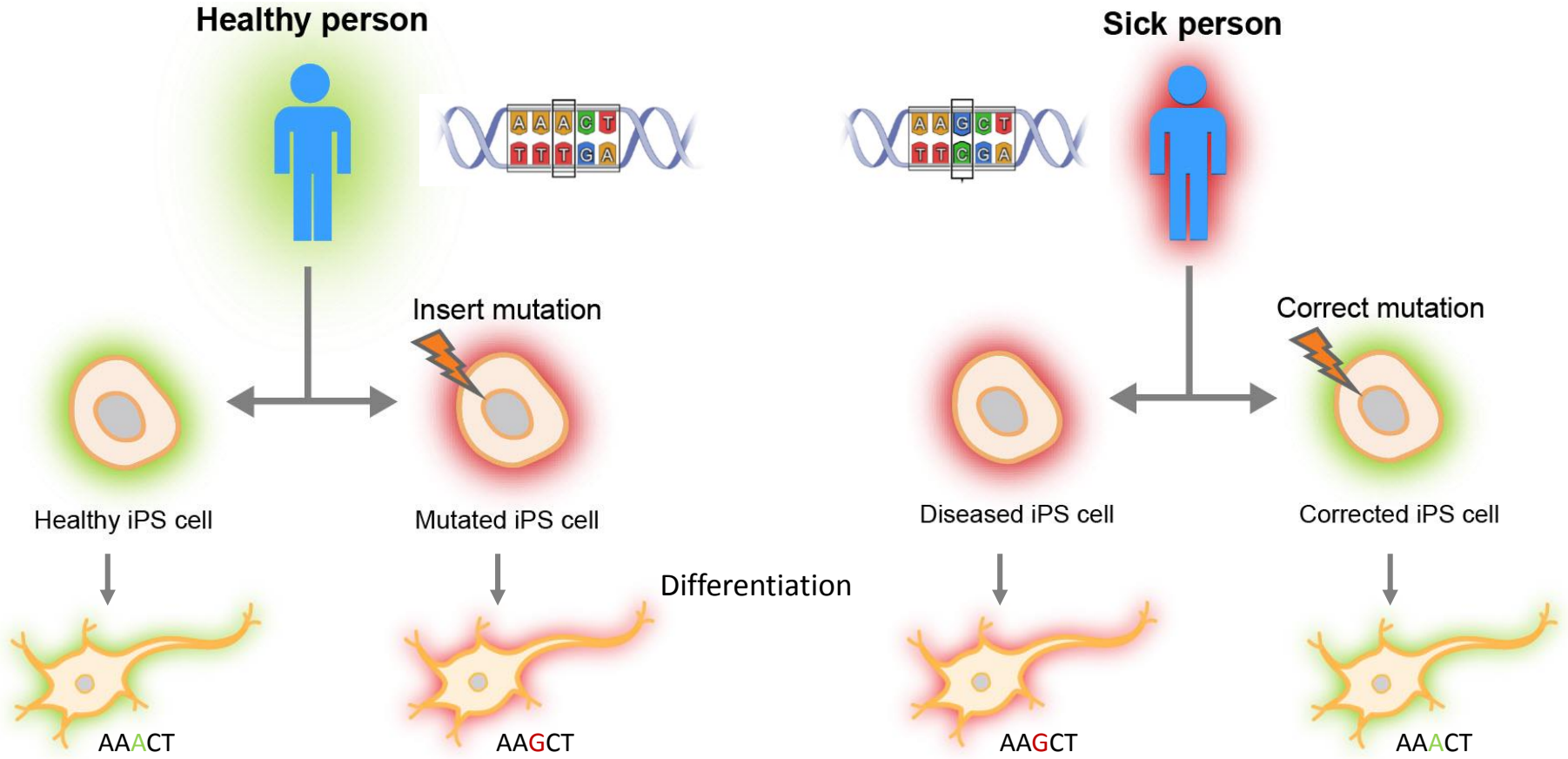


Challenges in Editing Stem Cells

Editing machinery design (ZFN, TALEN, sgRNA)

- Accuracy
- Specificity

Cas9/sgRNA delivery into cells (gene editing)

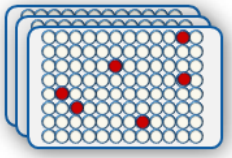
- Minimize toxicity
- Maximize pluripotency
- Efficient editing
- No off-target effects

Single-cell cloning

- Maximize survival
- Maximize pluripotency

Screening

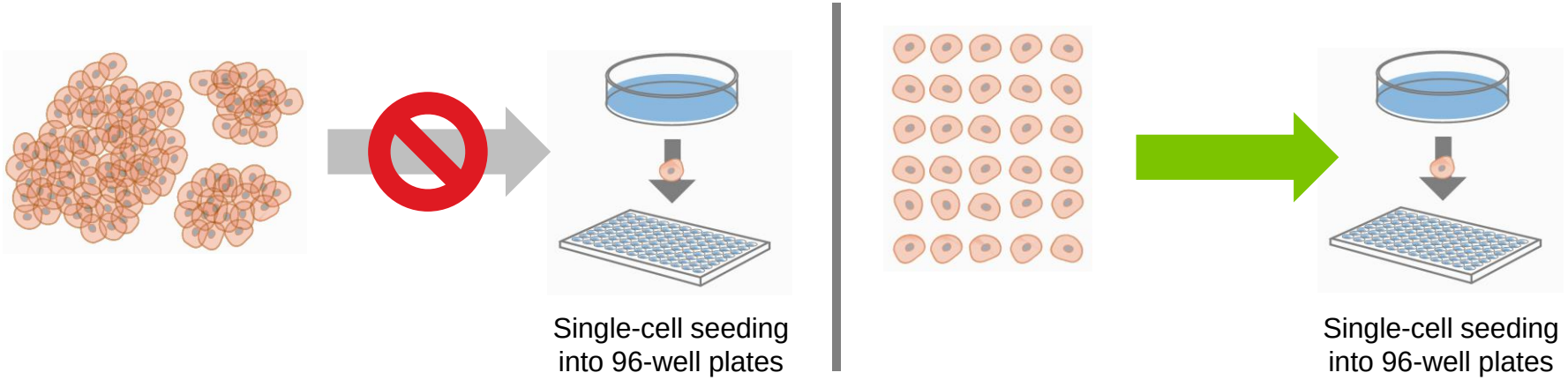
- Identification
- Specificity

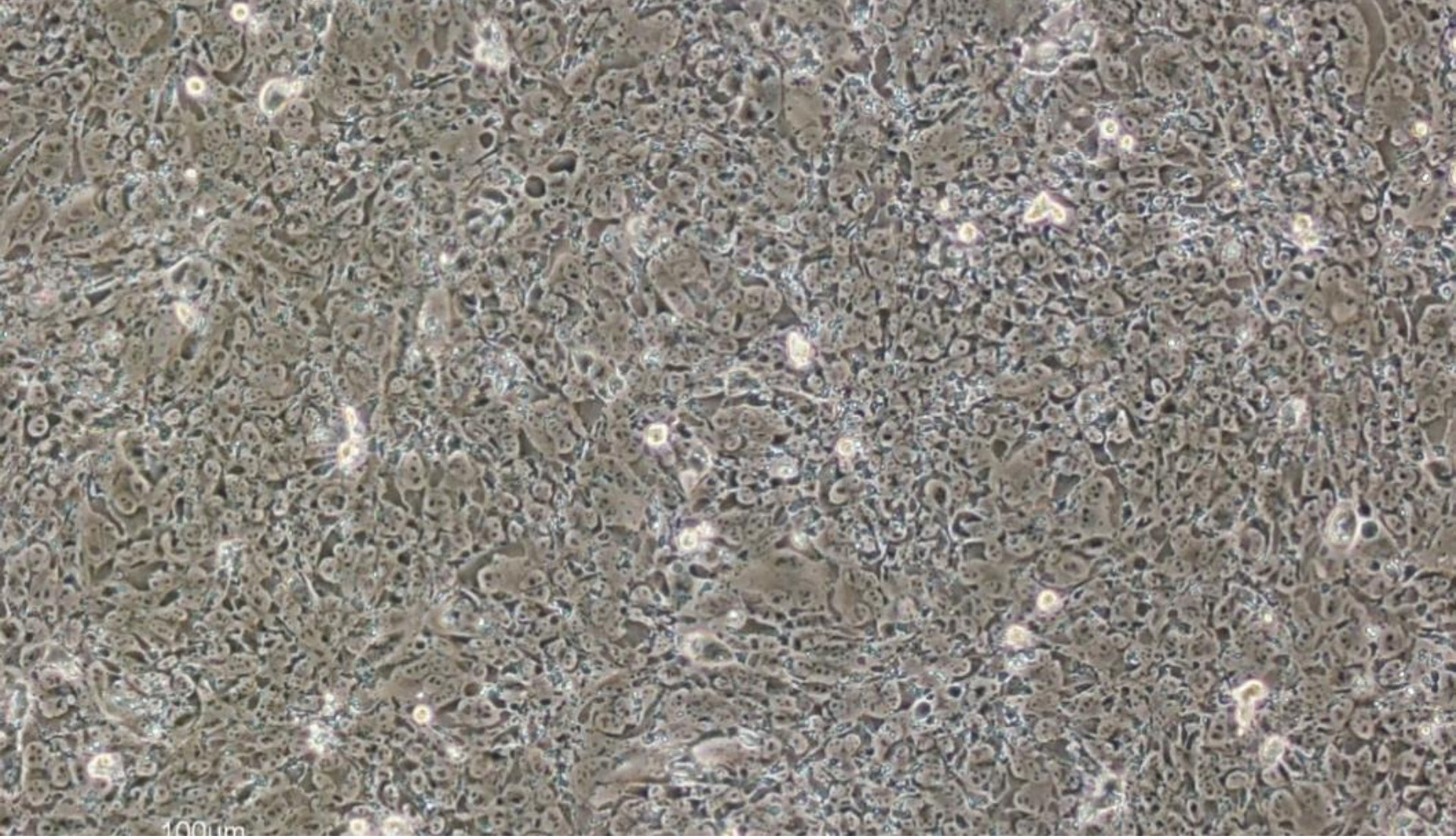


Goal: Isolate & expand a single, edited cell

Clonal Expansion of Single Pluripotent Stem Cells is a Bottleneck

- Isolating and clonally expanding edited cells
  - Pluripotent stem cells traditionally grow in colonies
  - Screening a colony is time-consuming and challenging
  - Single pluripotent cells die or differentiate
  - Need for single-cell culture of pluripotent stem cells





Human iPSC Culture System

Cellartis DEF-CS™ System & Single-Cell Cloning of iPSC

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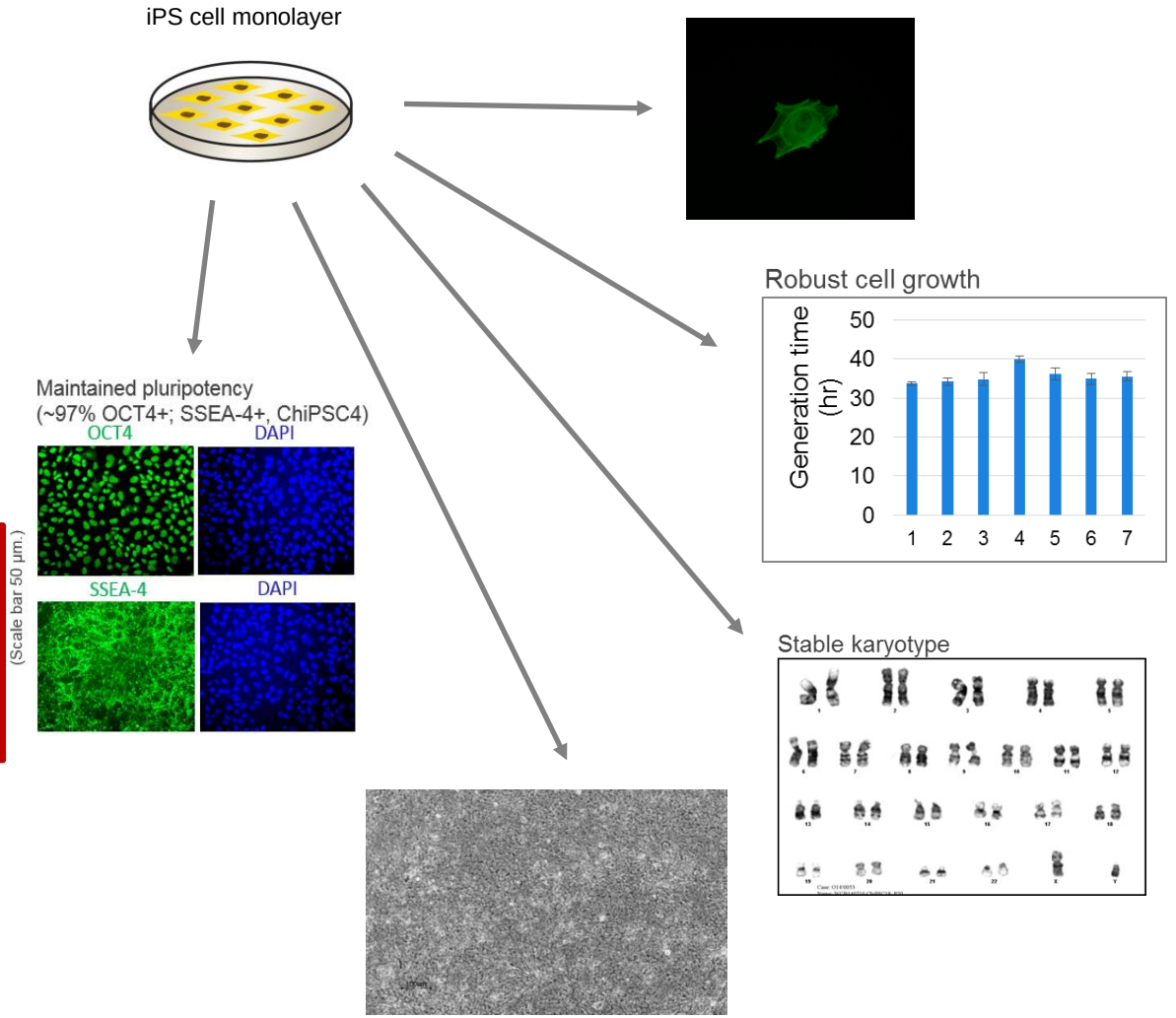
- Maintains cells in a highly undifferentiated state
- Allows for culturing iPS cells in a monolayer
- Feeder-free — no contamination, less time consuming, increased consistency
- Enables survival and expansion of single cells
- Maintains normal karyotype
- Allows rapid expansion for further downstream applications and analysis



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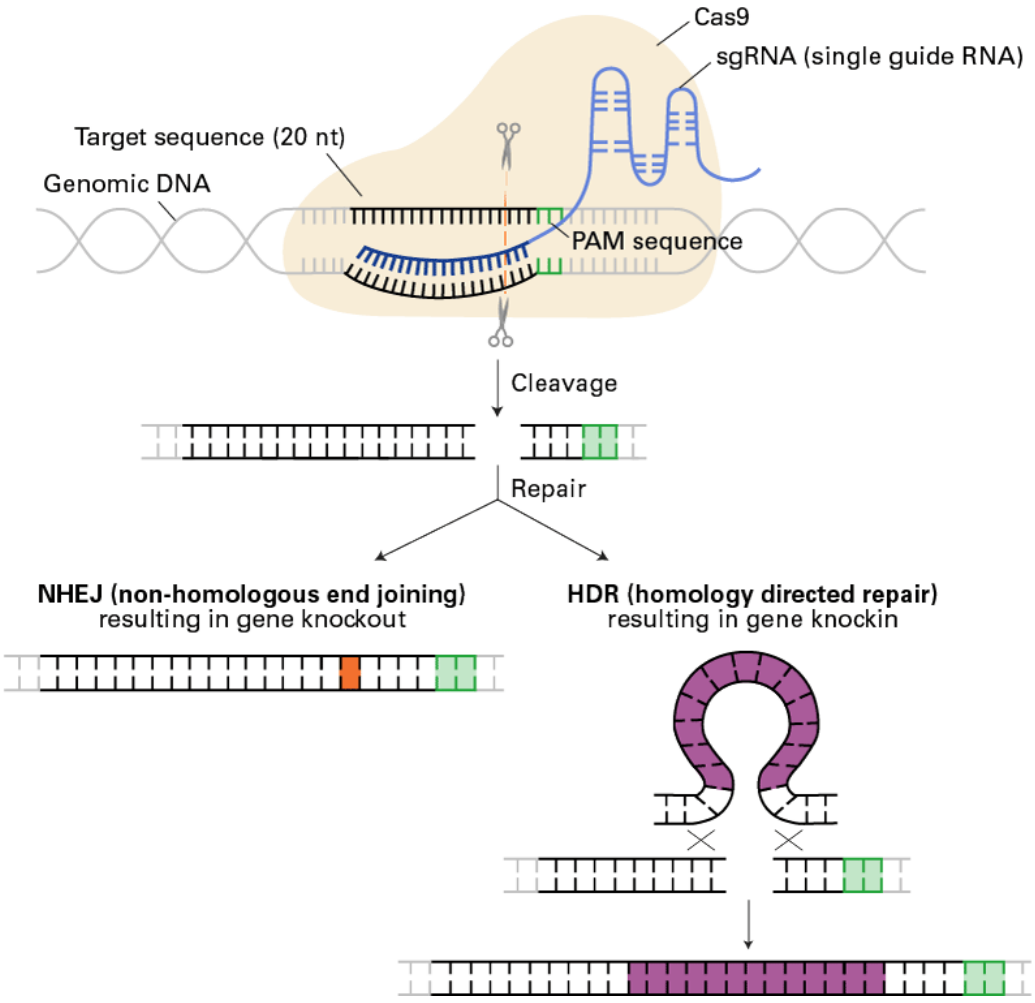
Gene Editing Tools

Gene Editing with Cas9



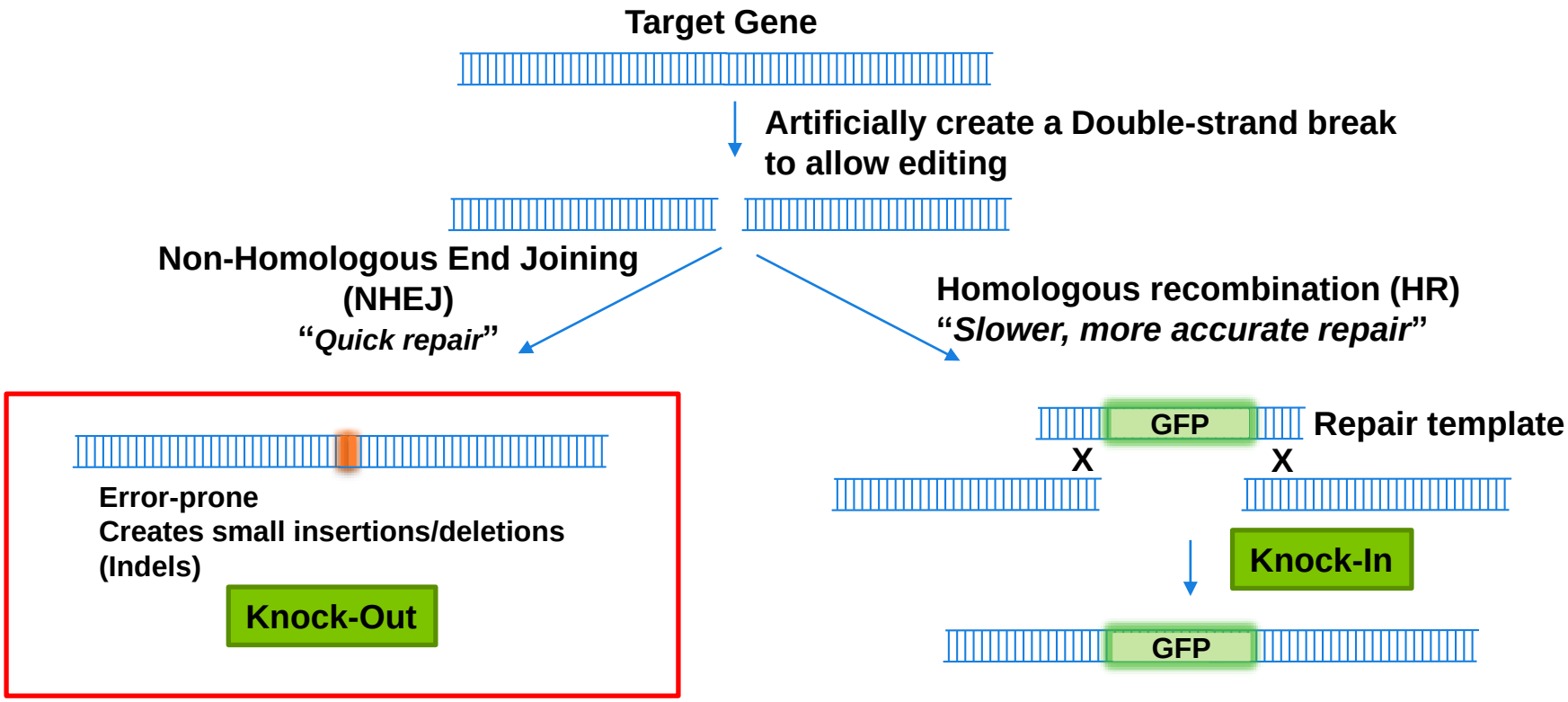
Repair template (HDR template) production methods

- AAV
- PCR
- Plasmid
- Synthetic oligos
- Takara Long ssDNA Kit

3 Components required:

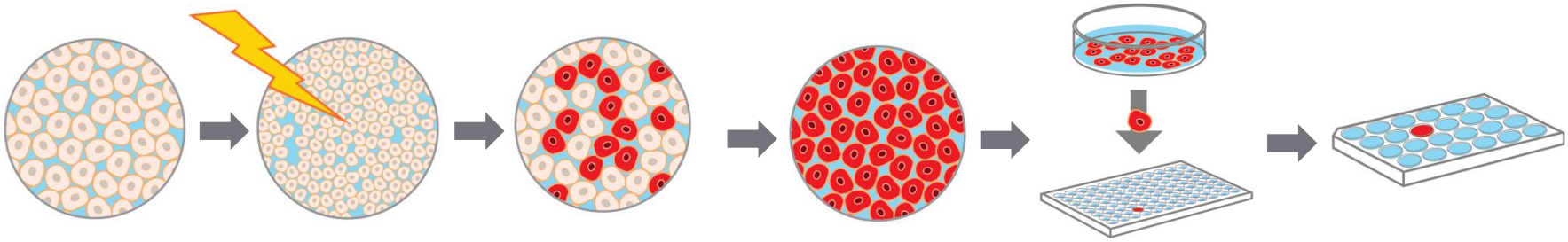
- Cas9 nuclease
- Guide RNA
- Repair template

CRISPR/Cas9 Genome Editing



- Delivery of two components
  - ❑ Cas9/sgRNA (RNP)
- Knockout efficiency via RNP acceptable/high even in primary cells or hiPS cells

CRISPR/Cas9 Gene Editing Workflow



ChiPSC22 or ChiPSC18

Editing of hiPS cells to KO *CD81* using CRISPR/Cas9

Analysis of the population to determine pluripotency and % KO

Sorted population (Isolate edited population)

Single cell seeded into each well of a 96-well plate (by FACS sorting or limiting dilution)

Expansion into edited clonal lines and characterization

Two options for Cas9 Delivery

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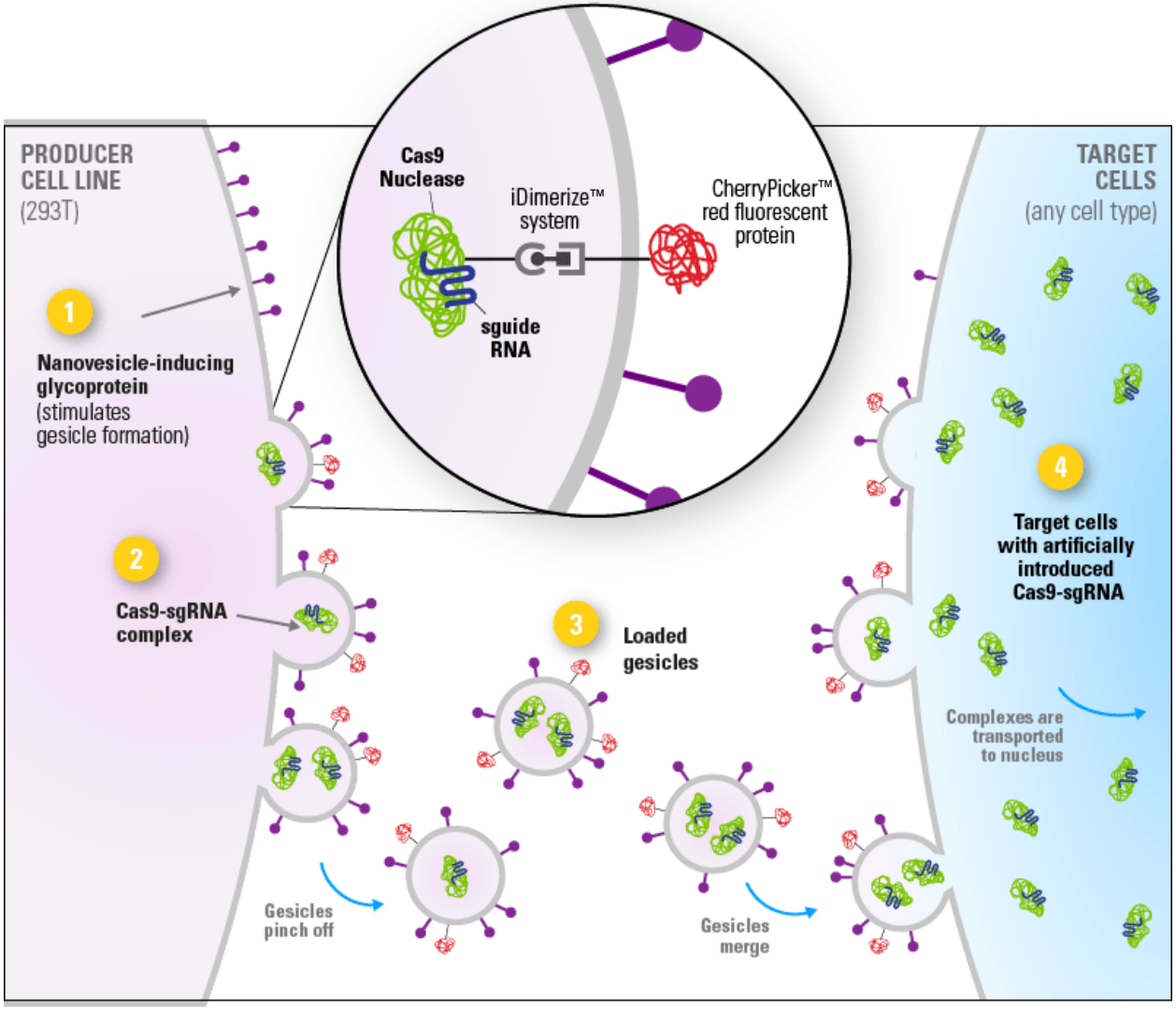
Guide-it™ rCas9 for electroporation:

- ~2 µg rCas9
- ~0.5 µg sgRNA (*in vitro transcribed*)
- ~0.5–1µg of donor DNA
- Using a Neon Electroporator

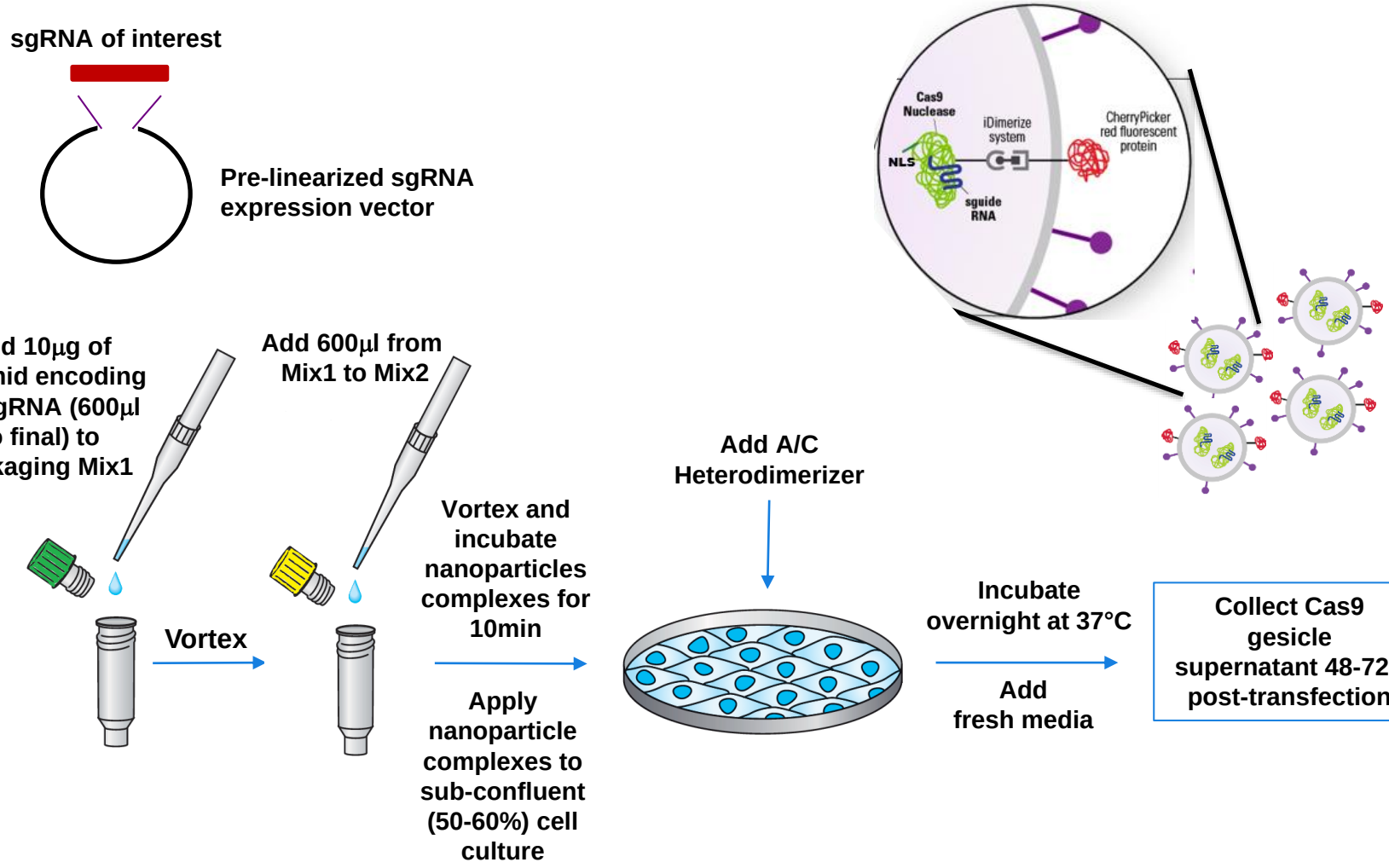
Guide-it CRISPR/Cas9 gesicle production kit:

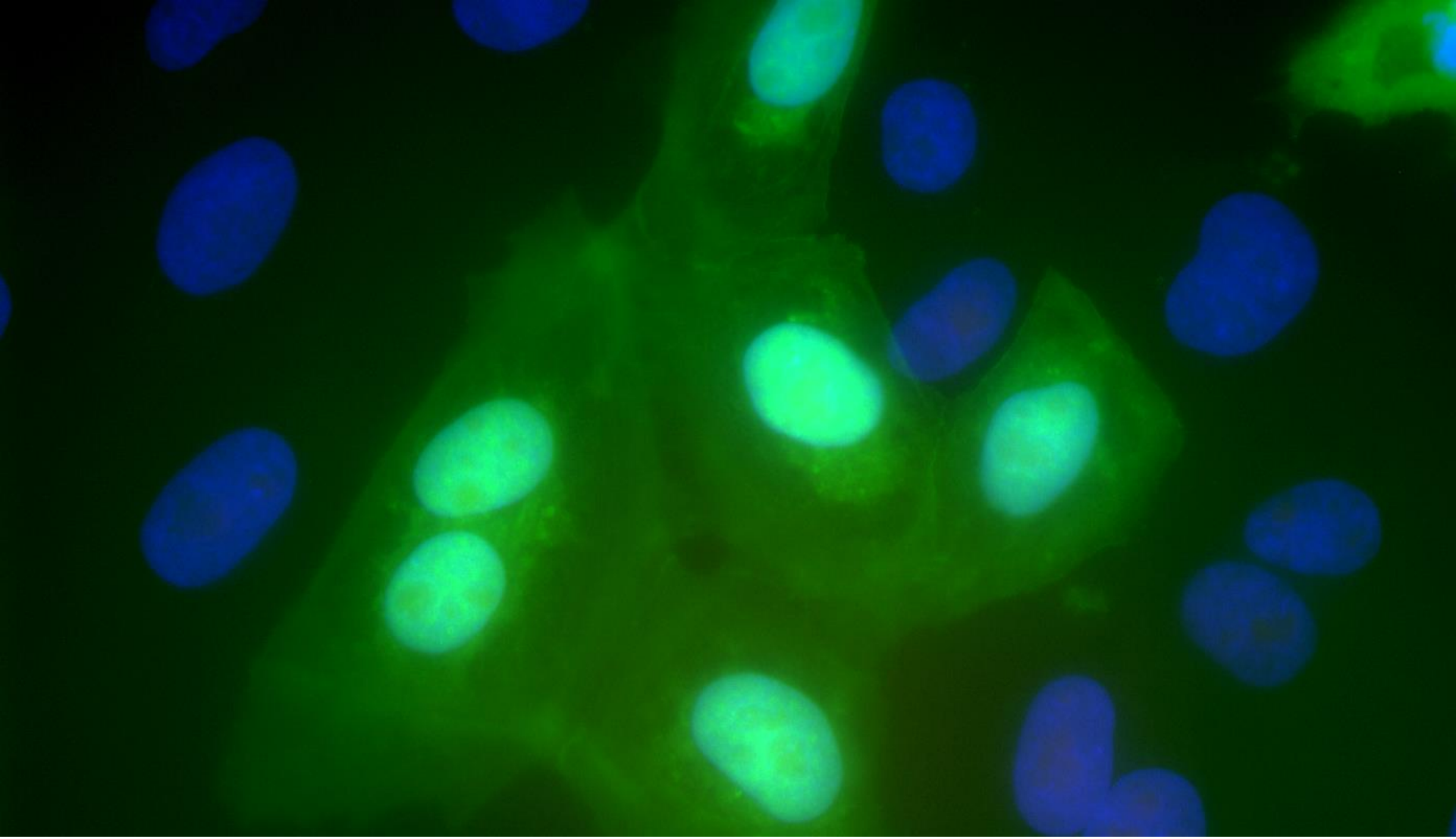
- Delivery of a Cas9/sgRNA RNP complex via cell derived nanovesicles

Guide-it CRISPR/Cas9 Gesicle Production Kit



Guide-it CRISPR/Cas9 Gesicle Production Kit

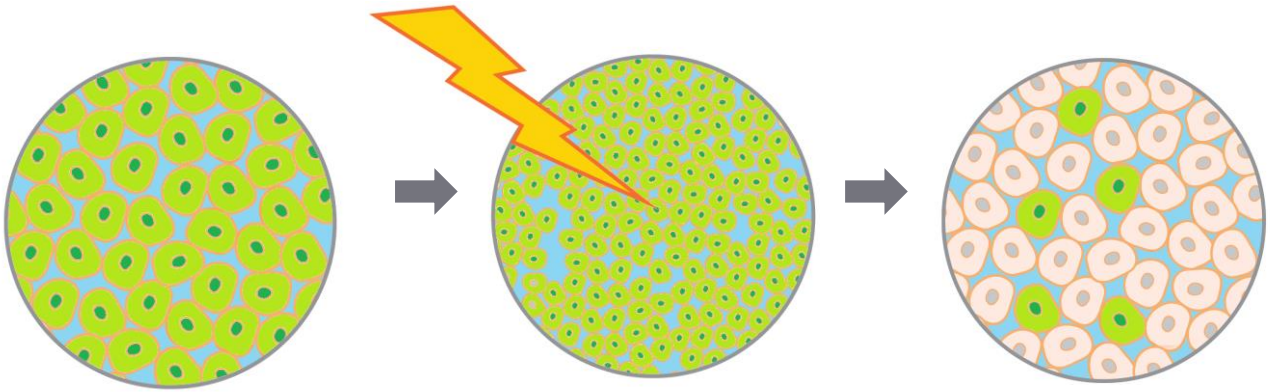




Case Studies - Knock Out

Maintenance of Pluripotency after Gene Editing: *AcGFP1* KO Test Case

Cellartis Human iPS Cell Line 22 (ChiPSC22) stably expressing AcGFP1 → CRISPR/Cas9 gene editing targeting *AcGFP1* → Cells grown for 9 days → FACS / Immunocytochemistry



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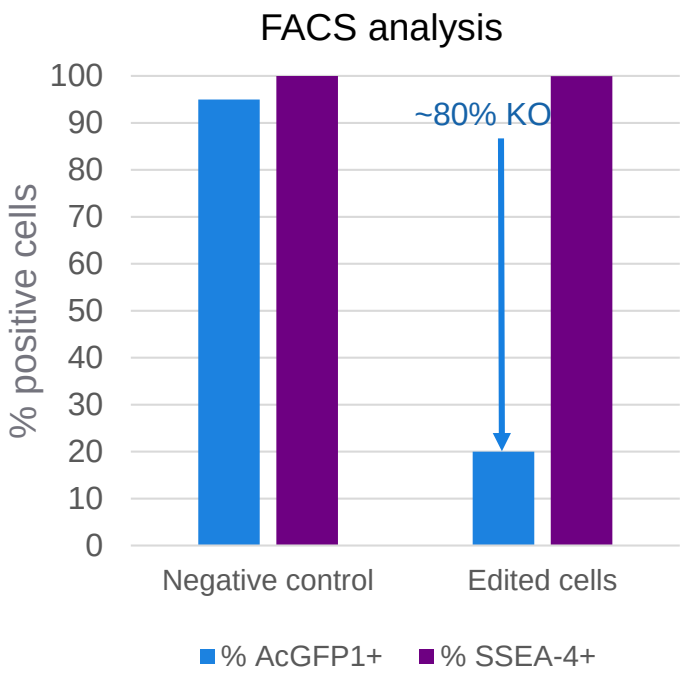

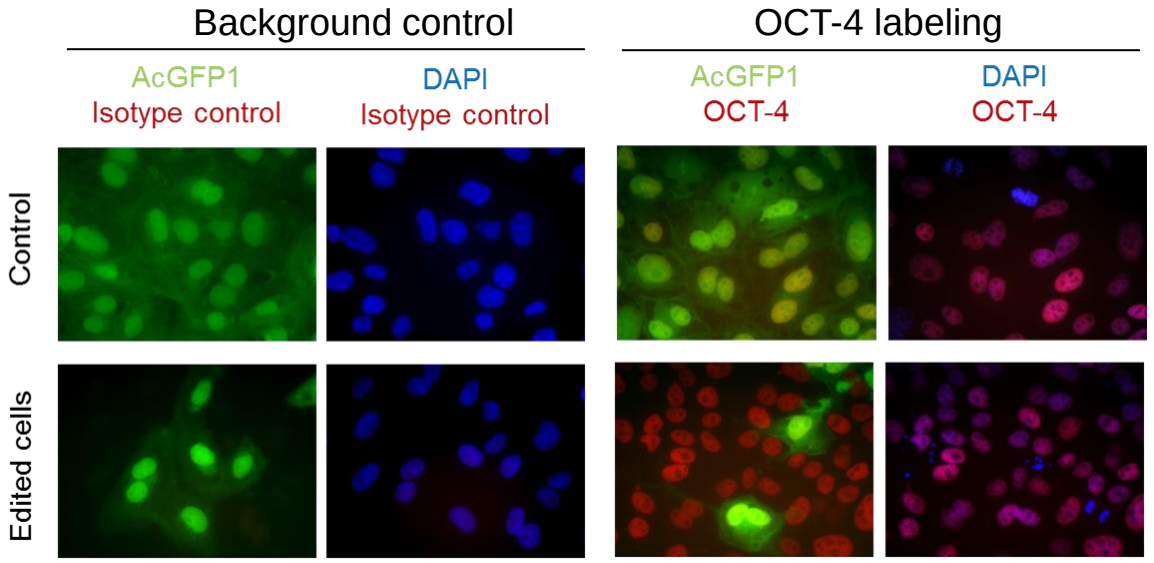

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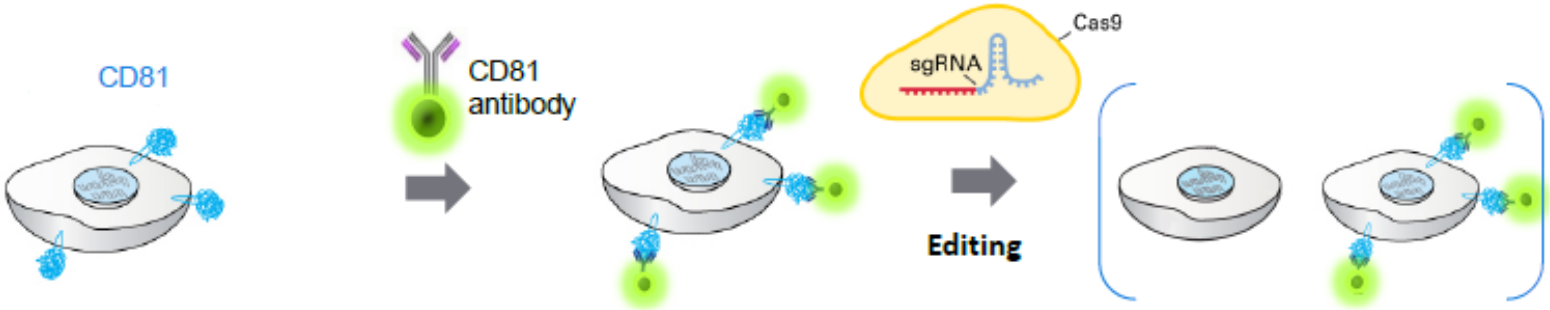
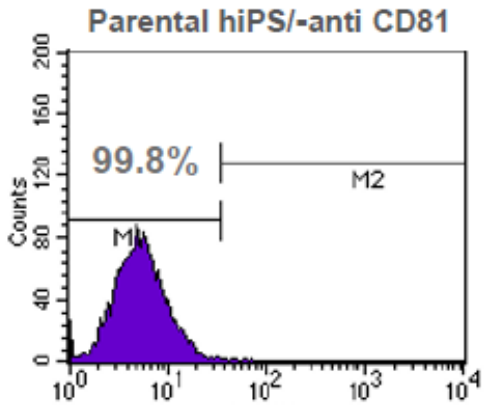
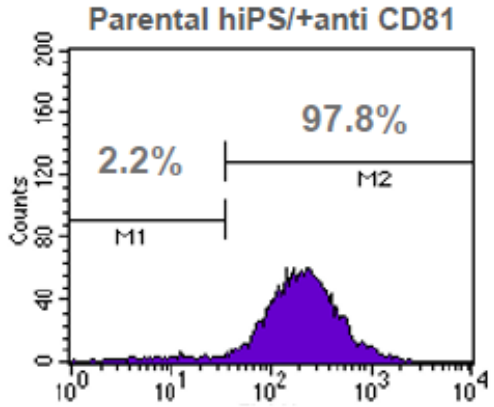
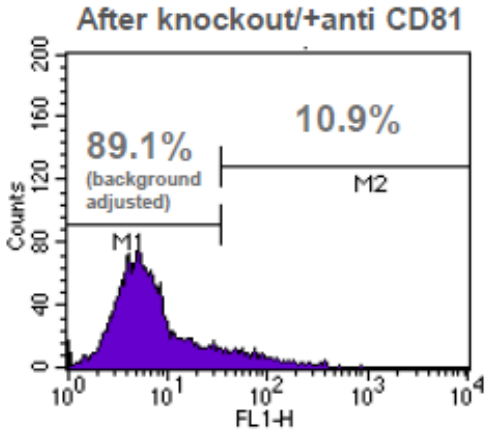
Model System - *CD81* KO in hiPSC

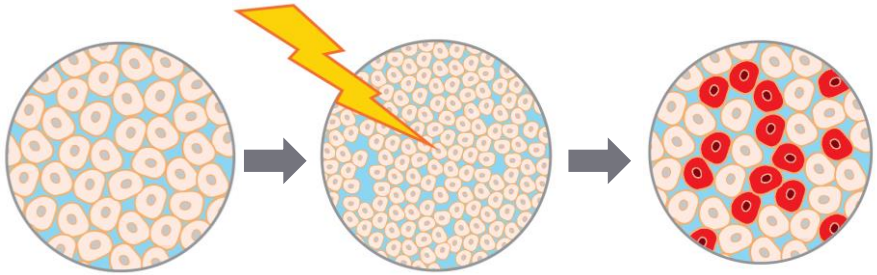








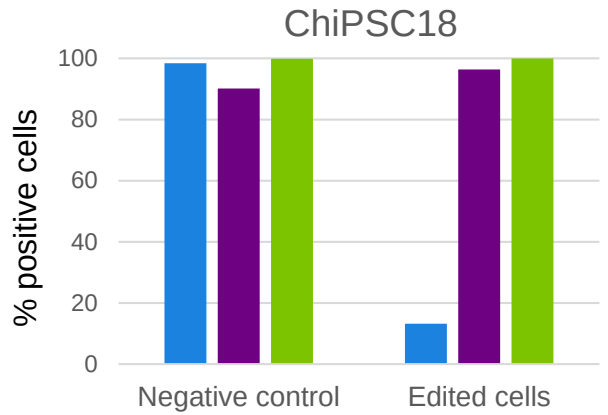
Pluripotency Maintained after *CD81* Knockout

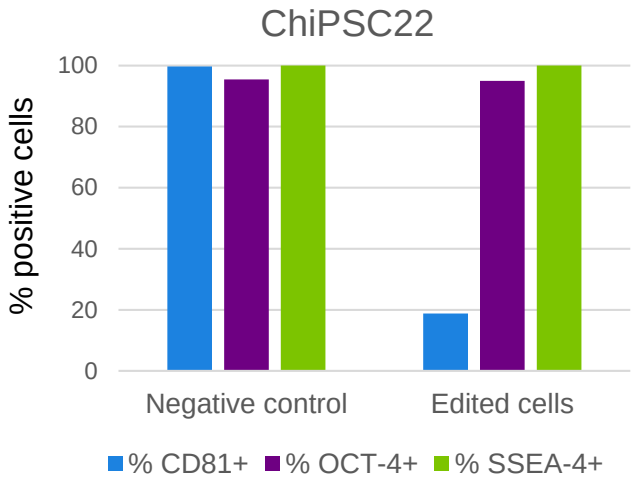


ChiPSC22 or ChiPSC18

Editing of hiPS cells to KO *CD81*

FACS analysis of the population to determine pluripotency and % KO





Sorting the *CD81* Negative Cell Population

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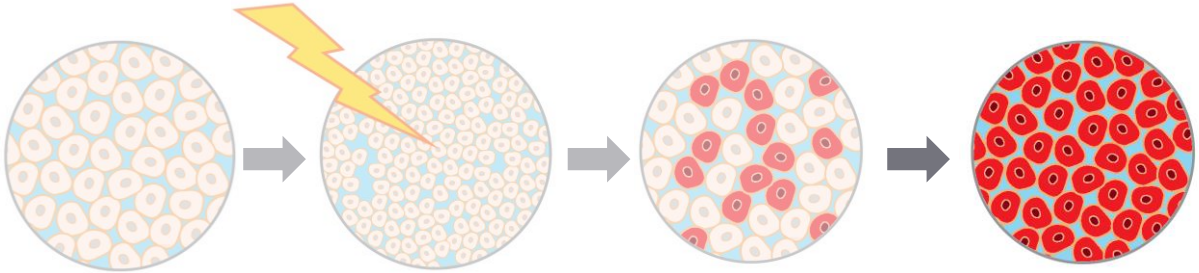
CD81/SSEA-4 double labeling



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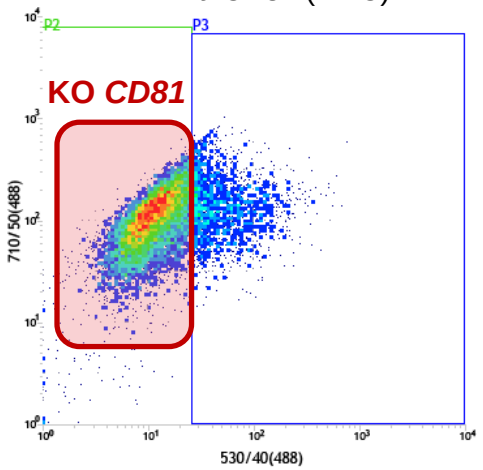
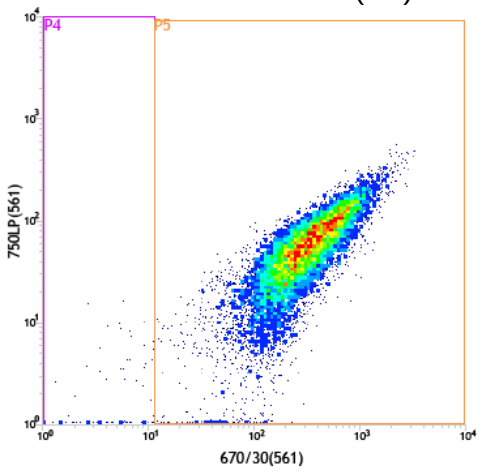
Anti-CD81 (FITC)



Anti-SSEA-4 (PE)



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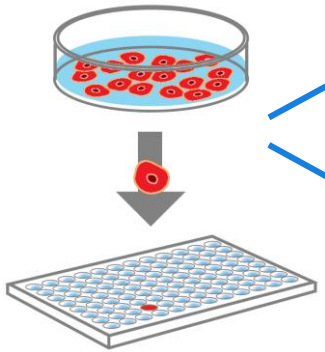
Cloning of Edited hiPSCs via FACS or Limiting Dilution

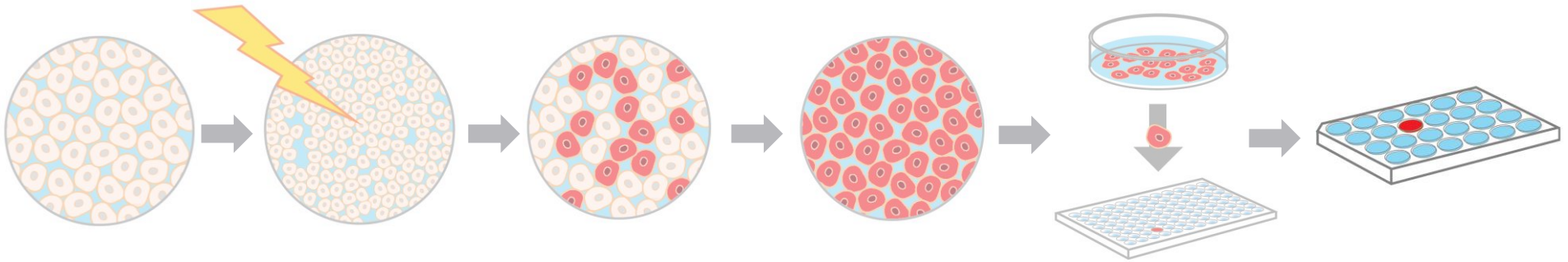


FACS into 96-well plate

Serial dilution into 96-well plate (~0.5 cells/well)

Cell line	Isolation method	Single clones	Double clones	Total clones (proportion)	Total clones (%)
ChiPSC22	FACS	8	0	8/96	8.5%
ChiPSC22	Limiting dilution	39	15	54/55	98%
ChiPSC18	FACS	52	0	52/96	54%
ChiPSC18	Limiting dilution	46	12	58/55	105%

Expansion of Edited Clonal Lines



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Expansion into edited clonal lines and characterization

Robust Expansion of Edited Clones

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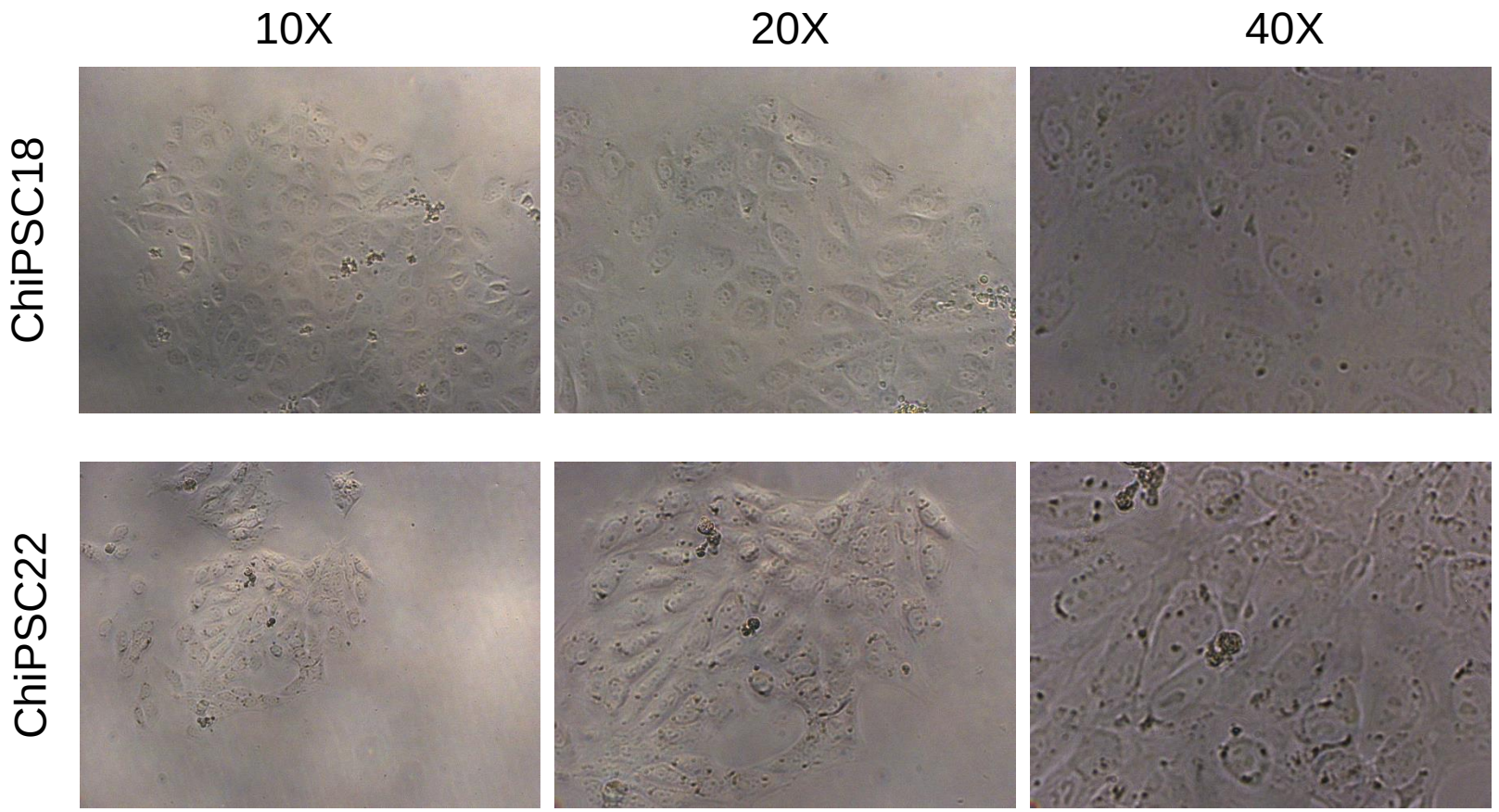
Images of colonies that originated from a single cell



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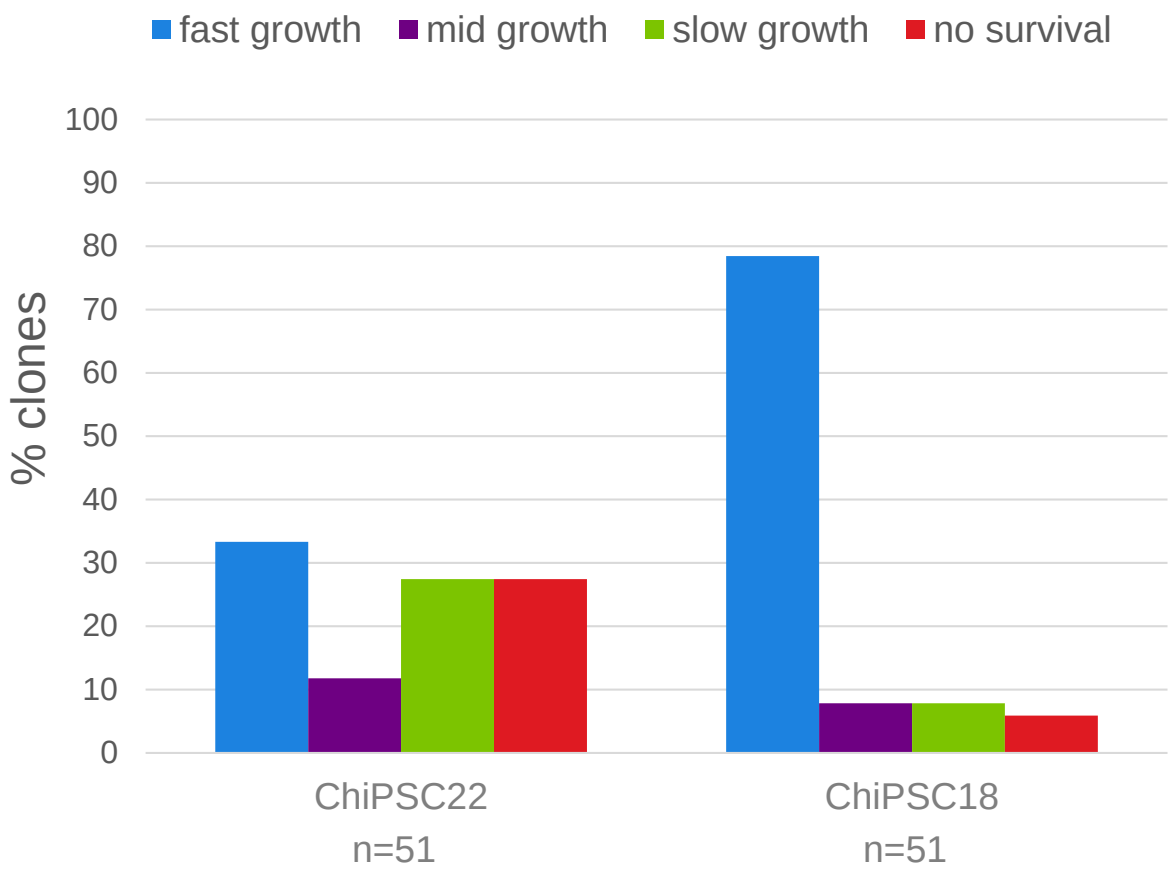
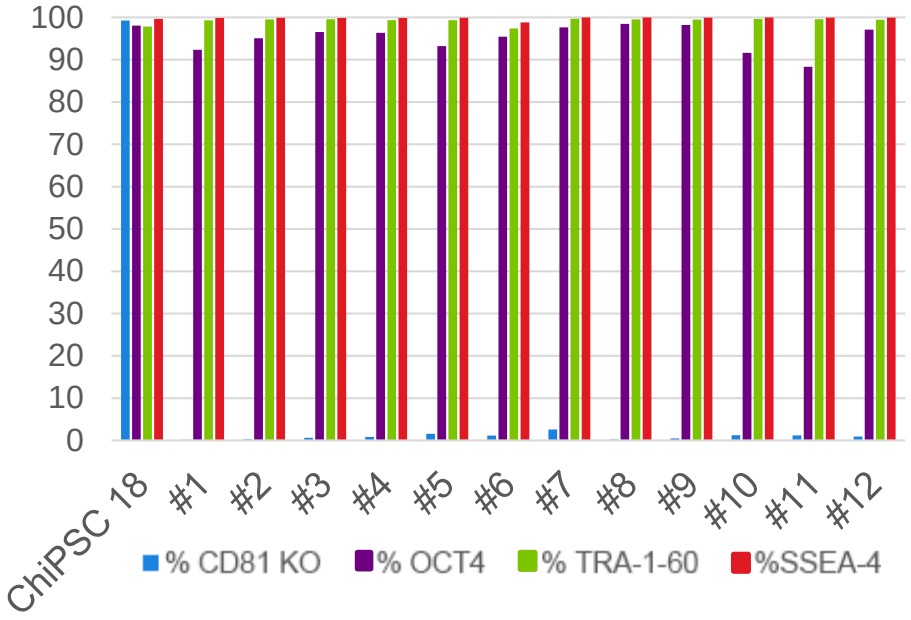
Robust Expansion of Edited Clones

Growth characteristics of clonal lines

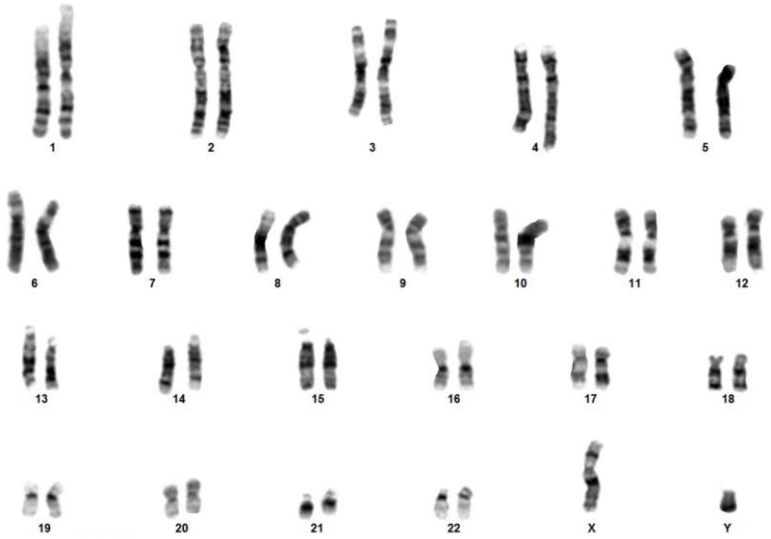


Edited Single-Cell Clones in DEF-CS Maintain Pluripotency & Stable Karyotype

Clone Analysis



Karyotype Analysis



ChiPSC18, c.8, Passage 14

Pluripotency maintained following Limiting Dilution

Stable karyotype observed in all edited clones tested after 14 to 15 passages (21 doublings)

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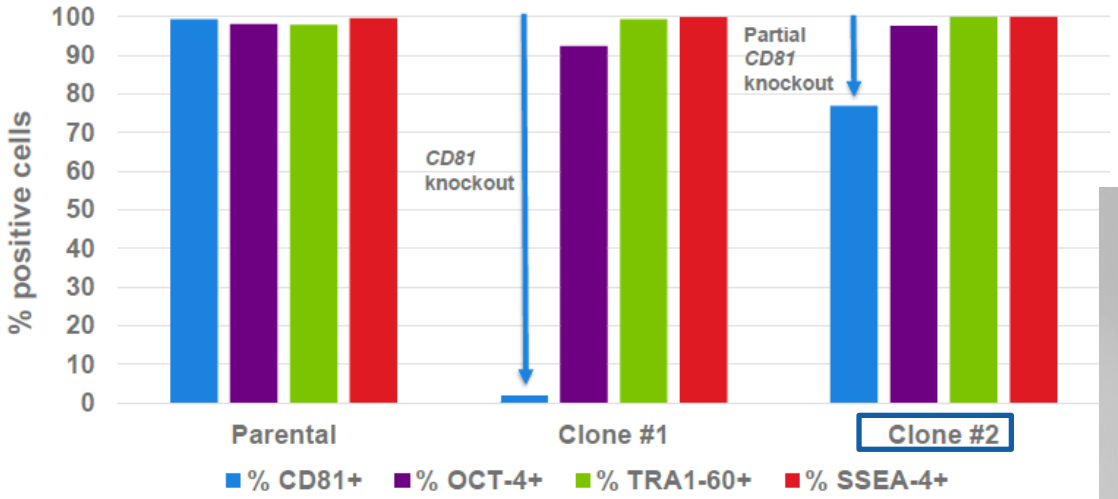
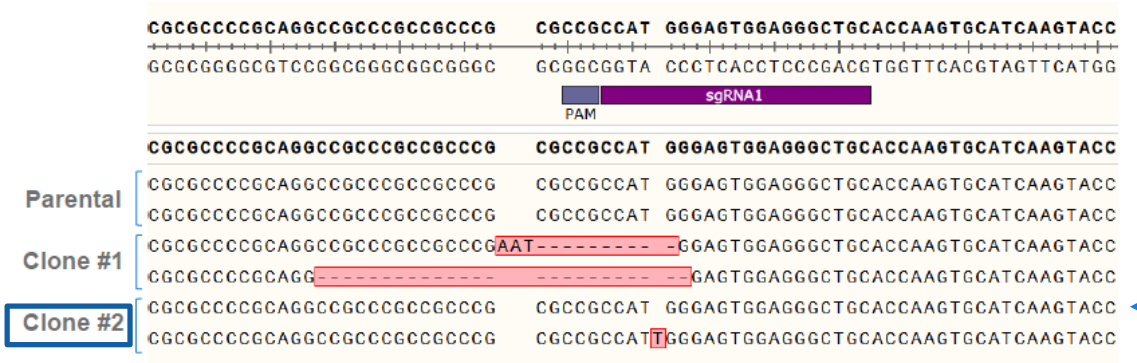
Genomic Characterization of Edited Clones in ChiPSC18

CD81

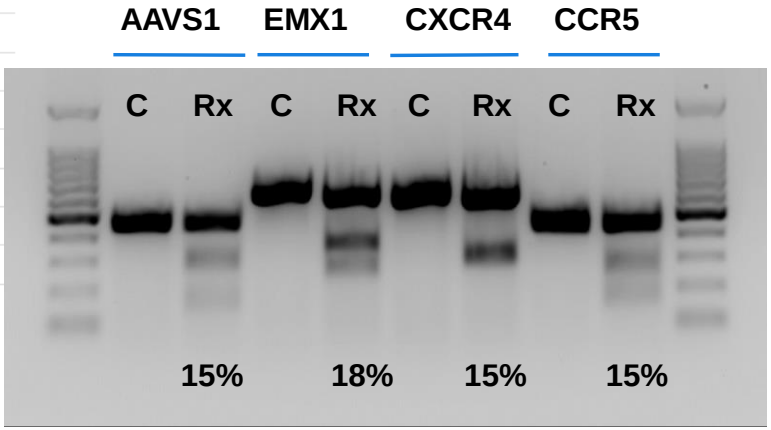




← CD81 WT allele

Other Genes

"*Mutation detection assay*"



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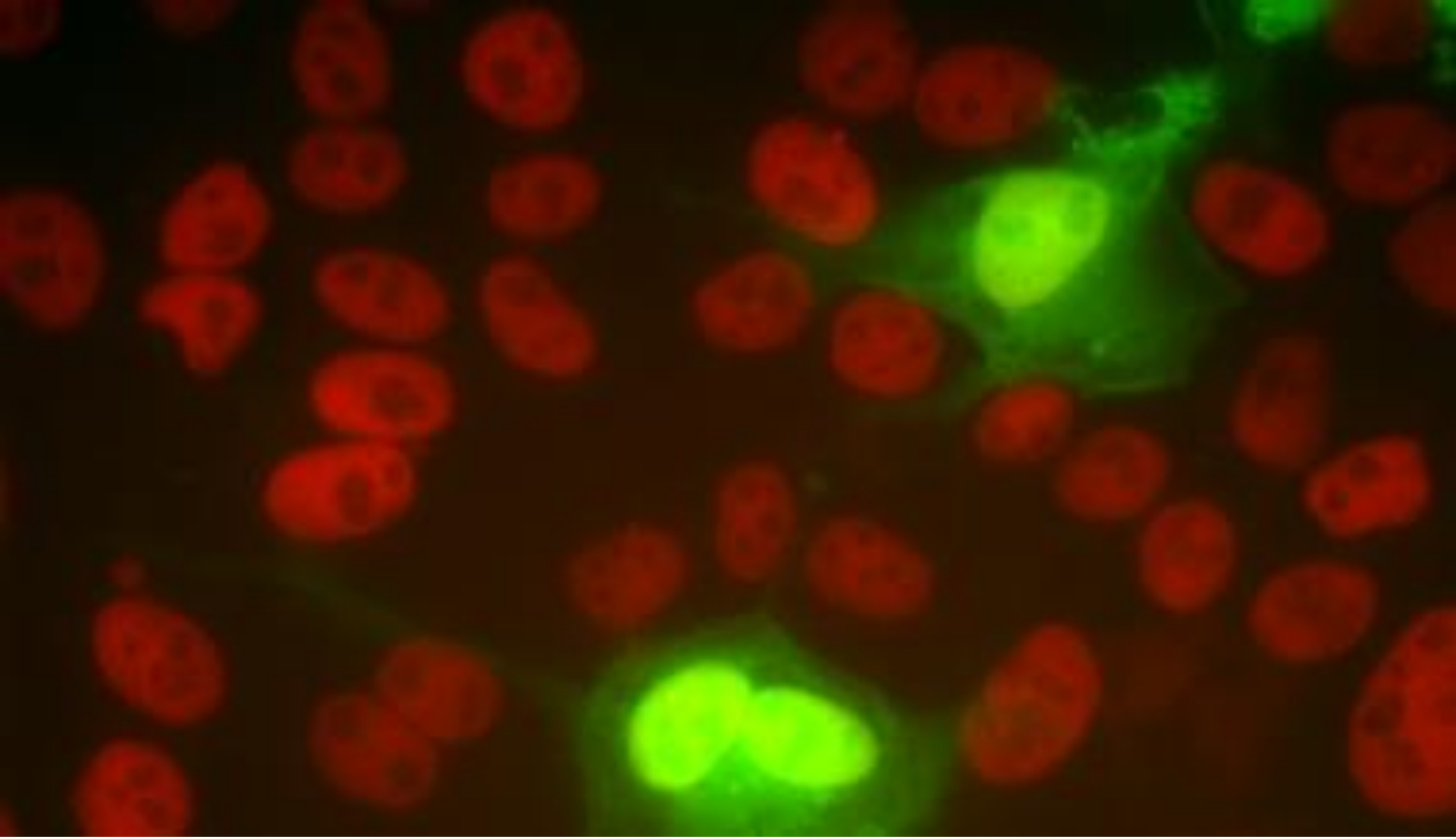
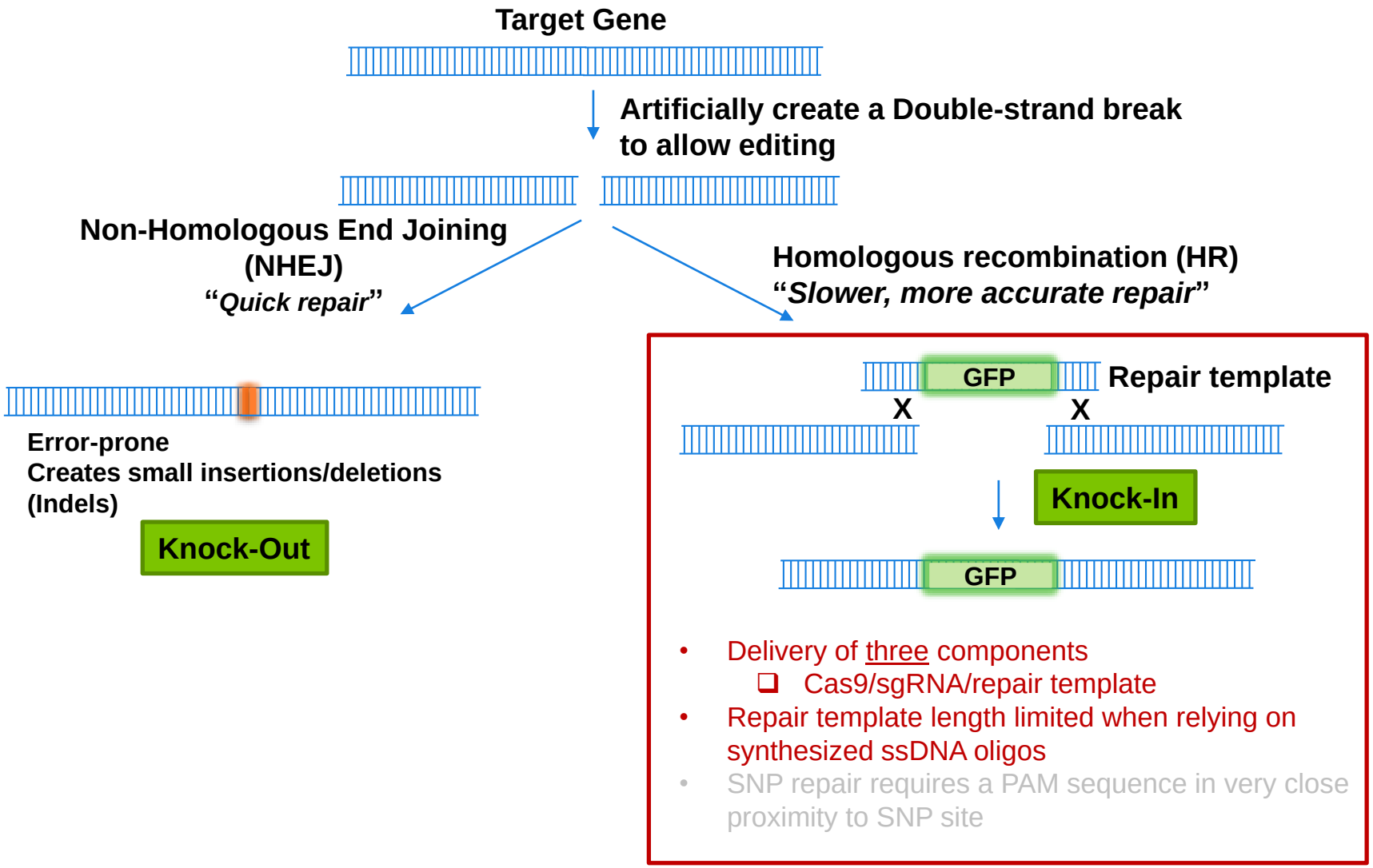
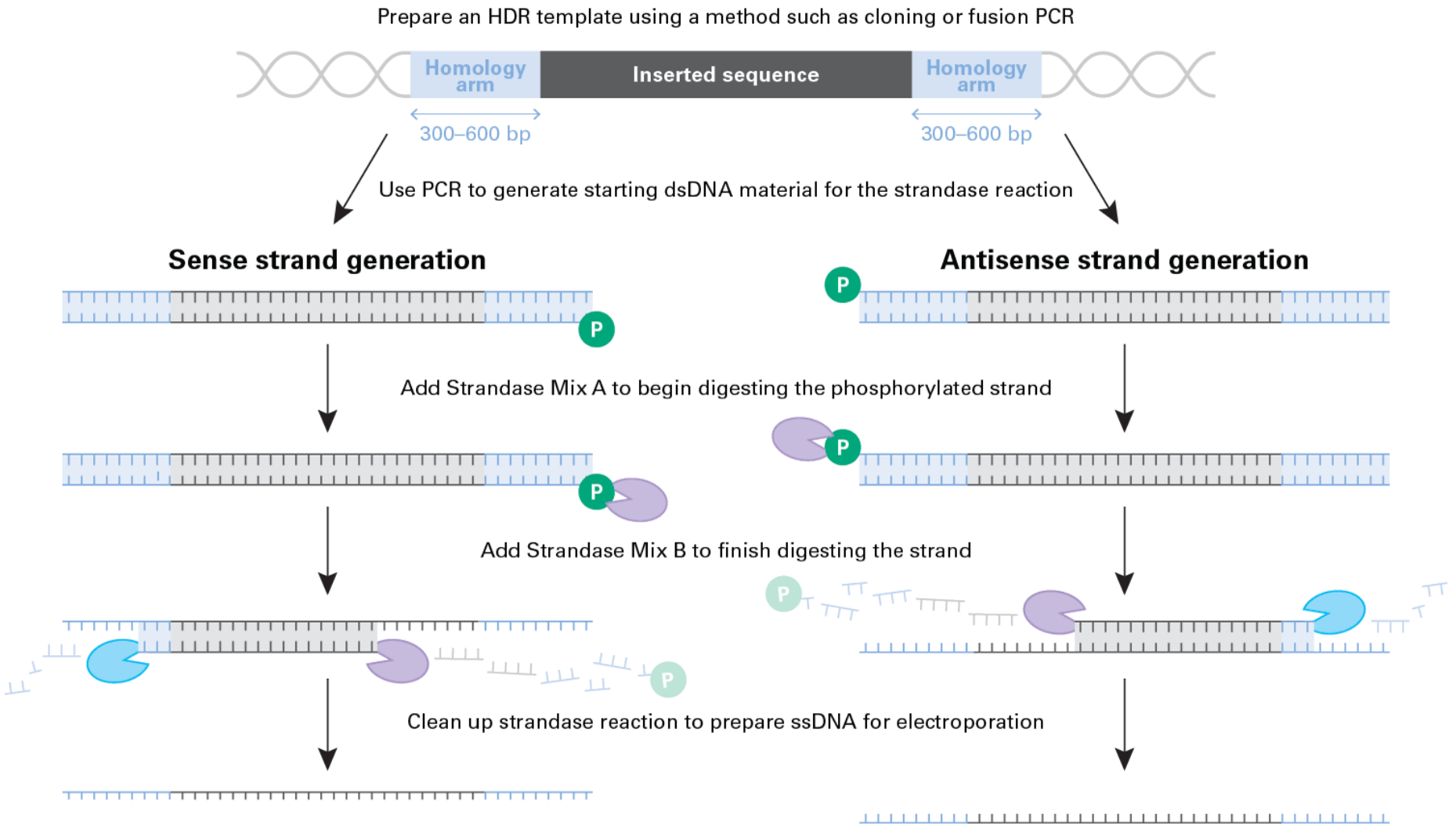


Case Study - Knockin

Bottleneck: Homologous Recombination



Guide-it Long ssDNA Production System – How it Works



Long ssDNA Prep:
Agarose gel

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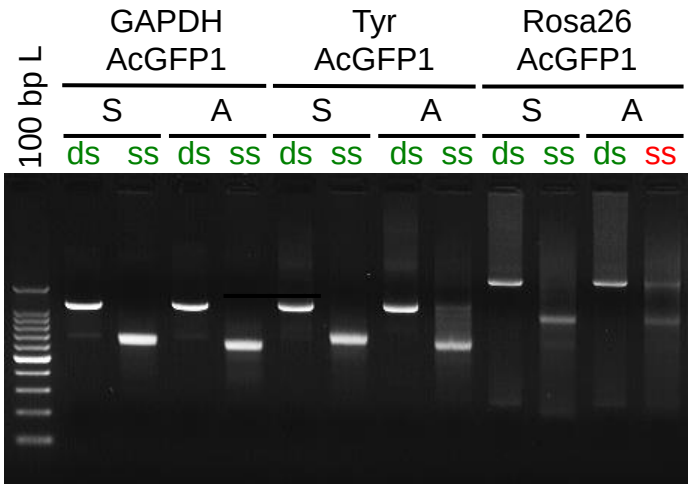
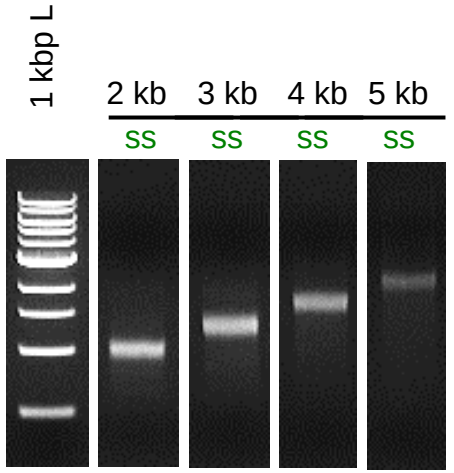
* ssDNA is much less sensitive to Et-Br





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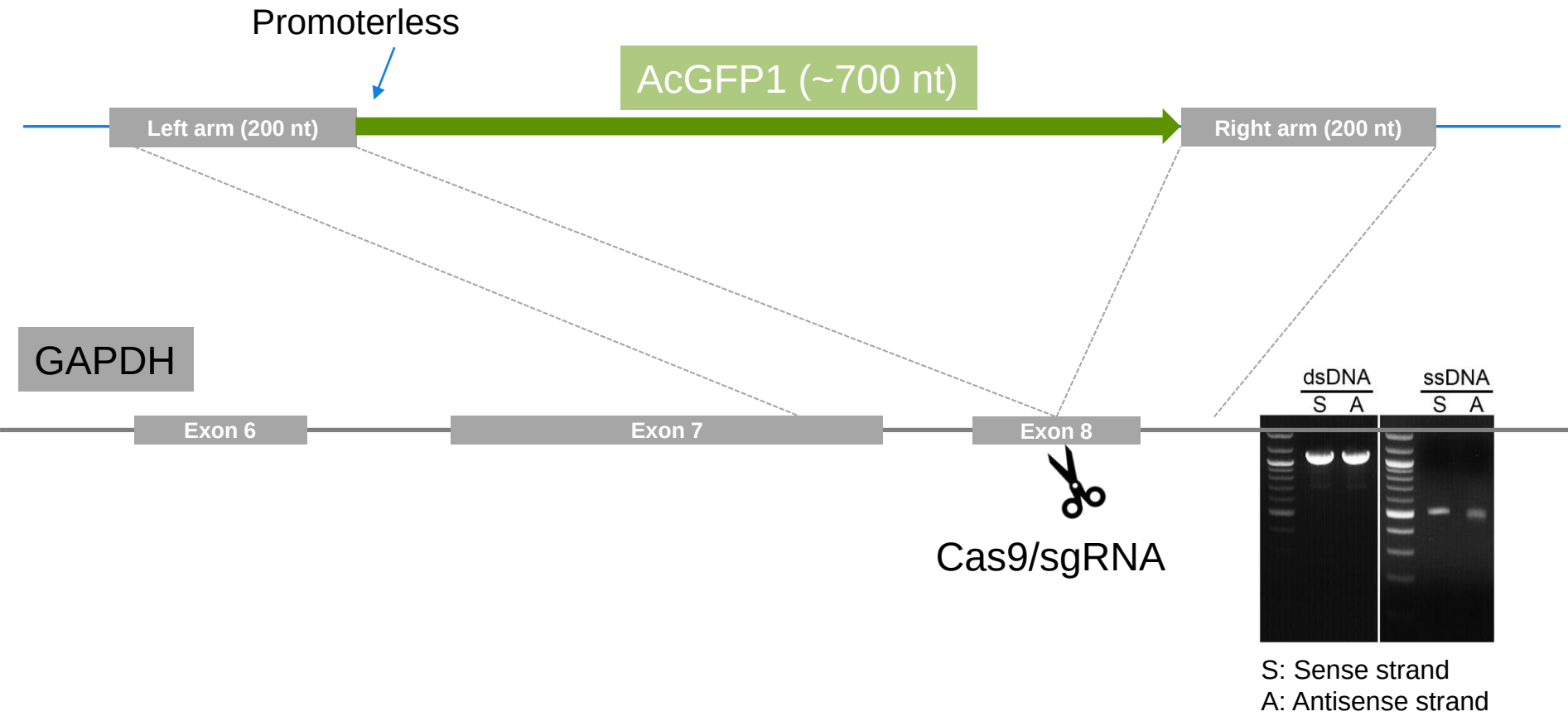
Gene Editing Data

Key things to note:

- dsDNA can result in random integration in the absence of Cas9
- ssDNA integrates seamlessly with a very low error rate at the junctions
- dsDNA will have significant background expression when delivering expression cassettes (e.g. CMV→GFP), making selection for edited cells difficult
- dsDNA can cause significant cellular toxicity

Proof of Concept in HEK293 cells

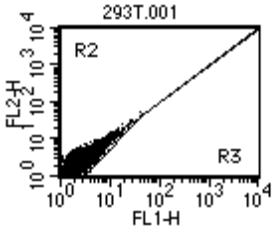
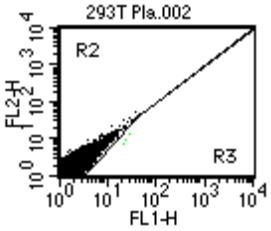
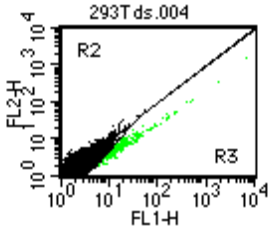
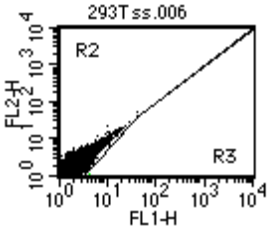
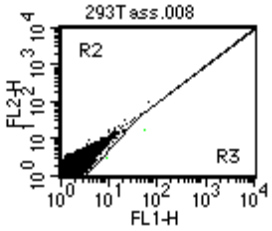
Knock-in of promoterless AcGFP1 at the C-terminus of GAPDH



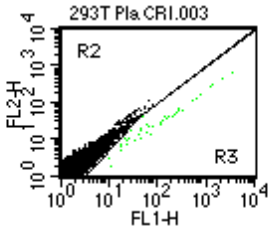
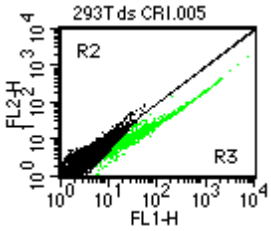
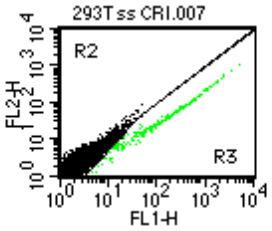
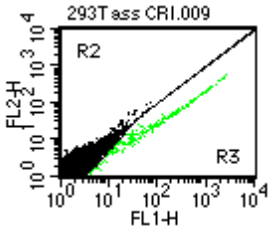
dsDNA Integrates Randomly

HEK293 (GAPDH/AcGFP1 donor)

Without Cas9/sgRNA

Non	Plasmid	dsDNA	ssDNA(S)	ssDNA(A)	
					
0% / ---	**0.04% / 35**	**2.68% / 60**	**0.01% / 4**	**0.02% / 32**	**%AcGFP+ / MFI**

With Cas9/sgRNA

Plasmid	dsDNA	ssDNA(S)	ssDNA(A)	
				
0.35% / 317	**7.91% / 176**	**2.34% / 334**	**2.43% / 268**	**%AcGFP+ / MFI**

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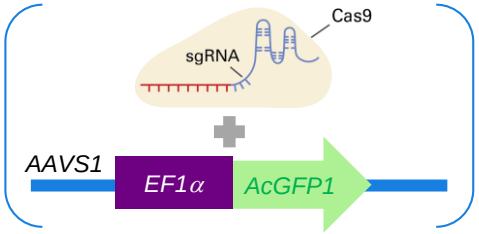
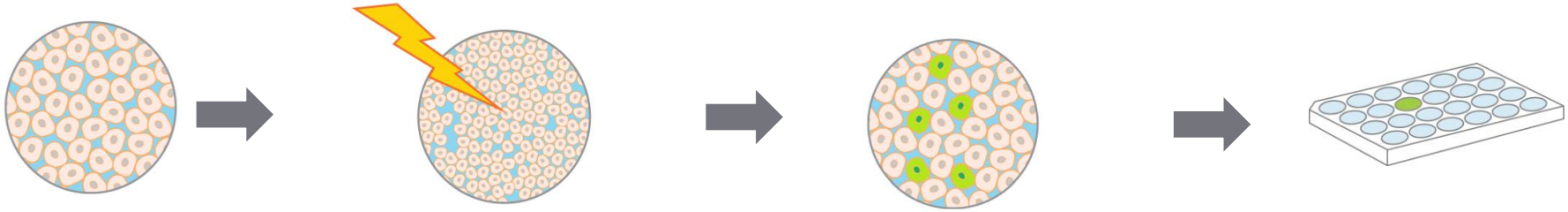
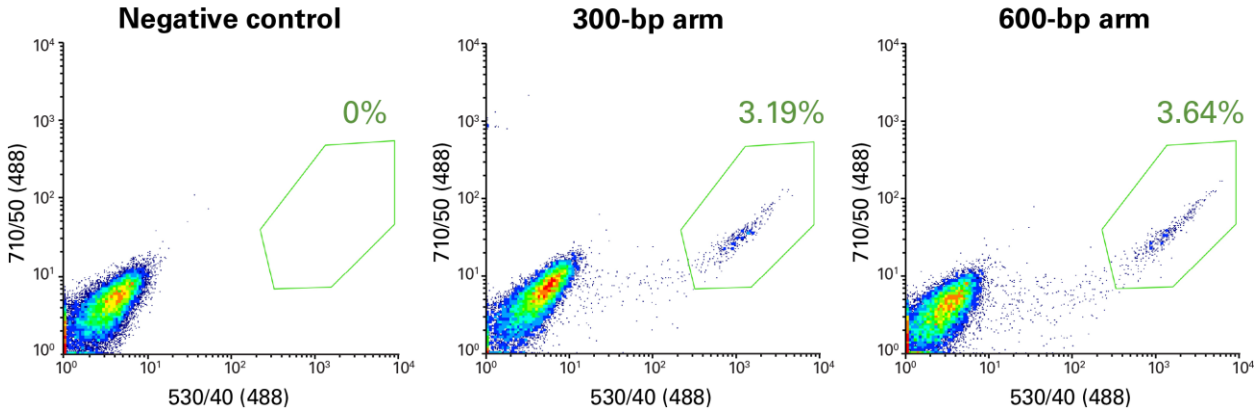
Knock-in of EF1α-AcGFP1 at AAVS1 Site of hiPS cells (Clone 18)

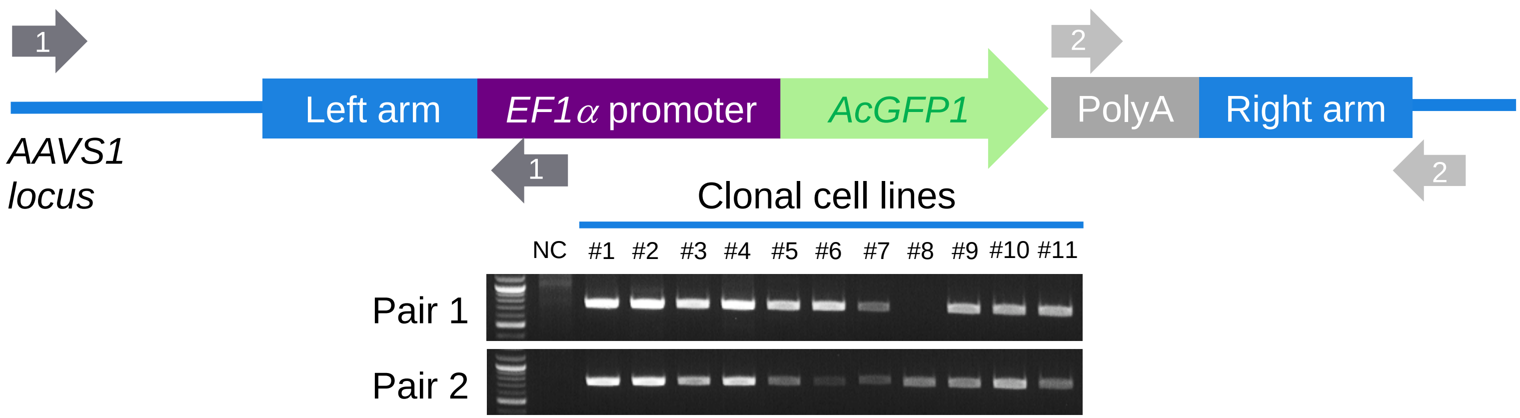






Isolated AcGFP1 positive single cells

Isolated Clonal Lines Do Not Contain Mutations



Left arm	Right arm
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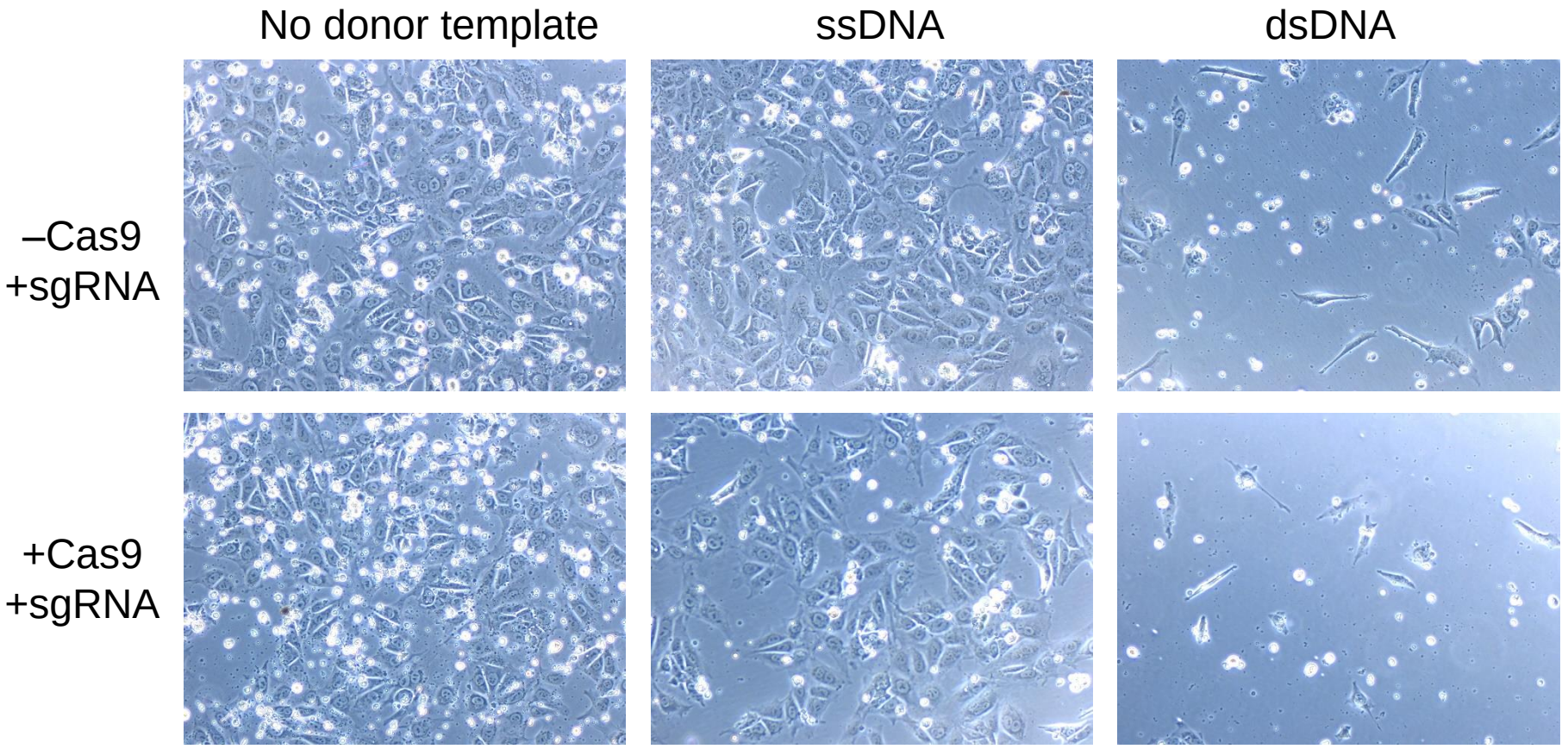
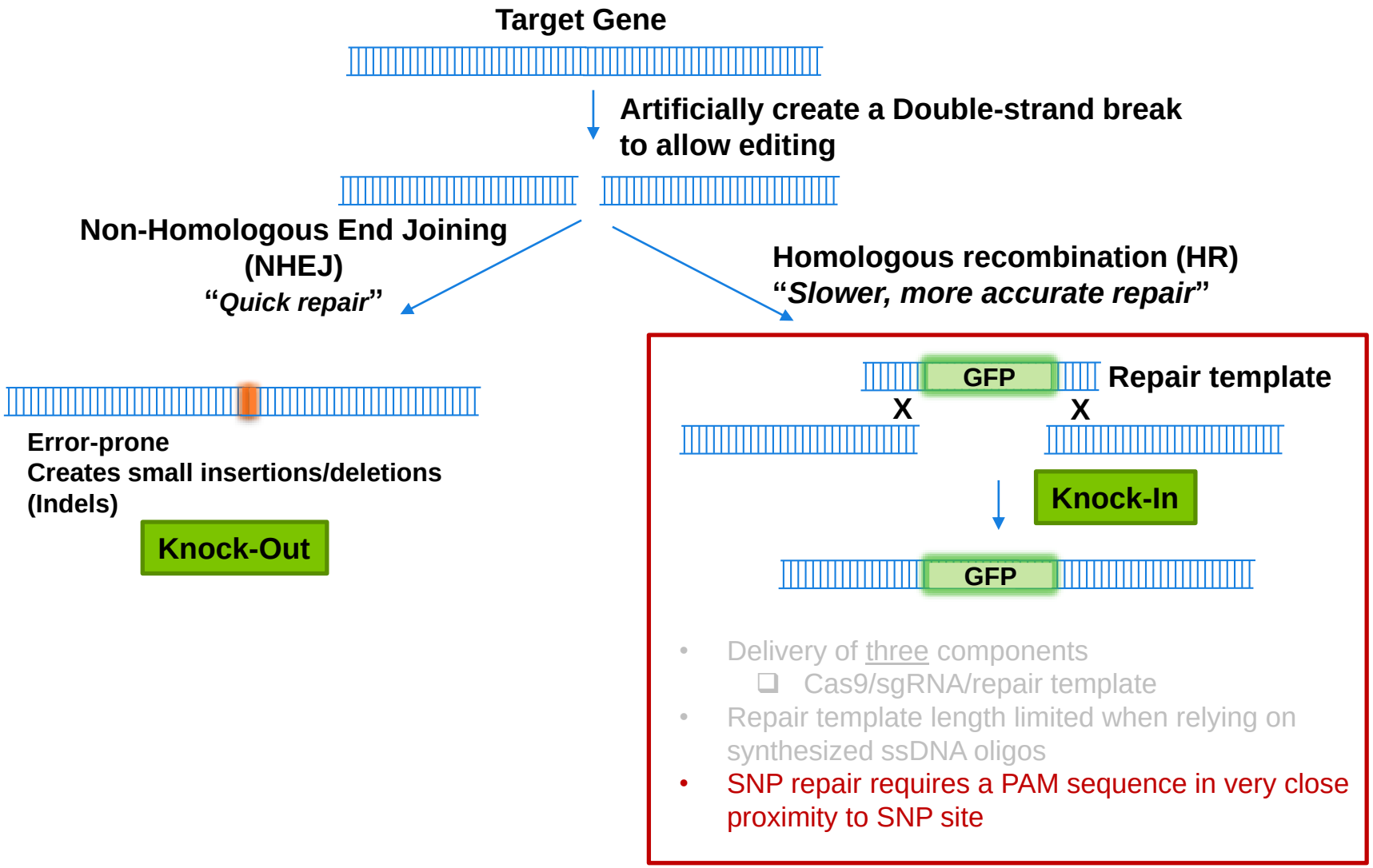
Clonal cell lines

dsDNA-induced toxicity in ChiPSC18



Bottleneck: Homologous Recombination



Why is SNP Editing/Screening Important?

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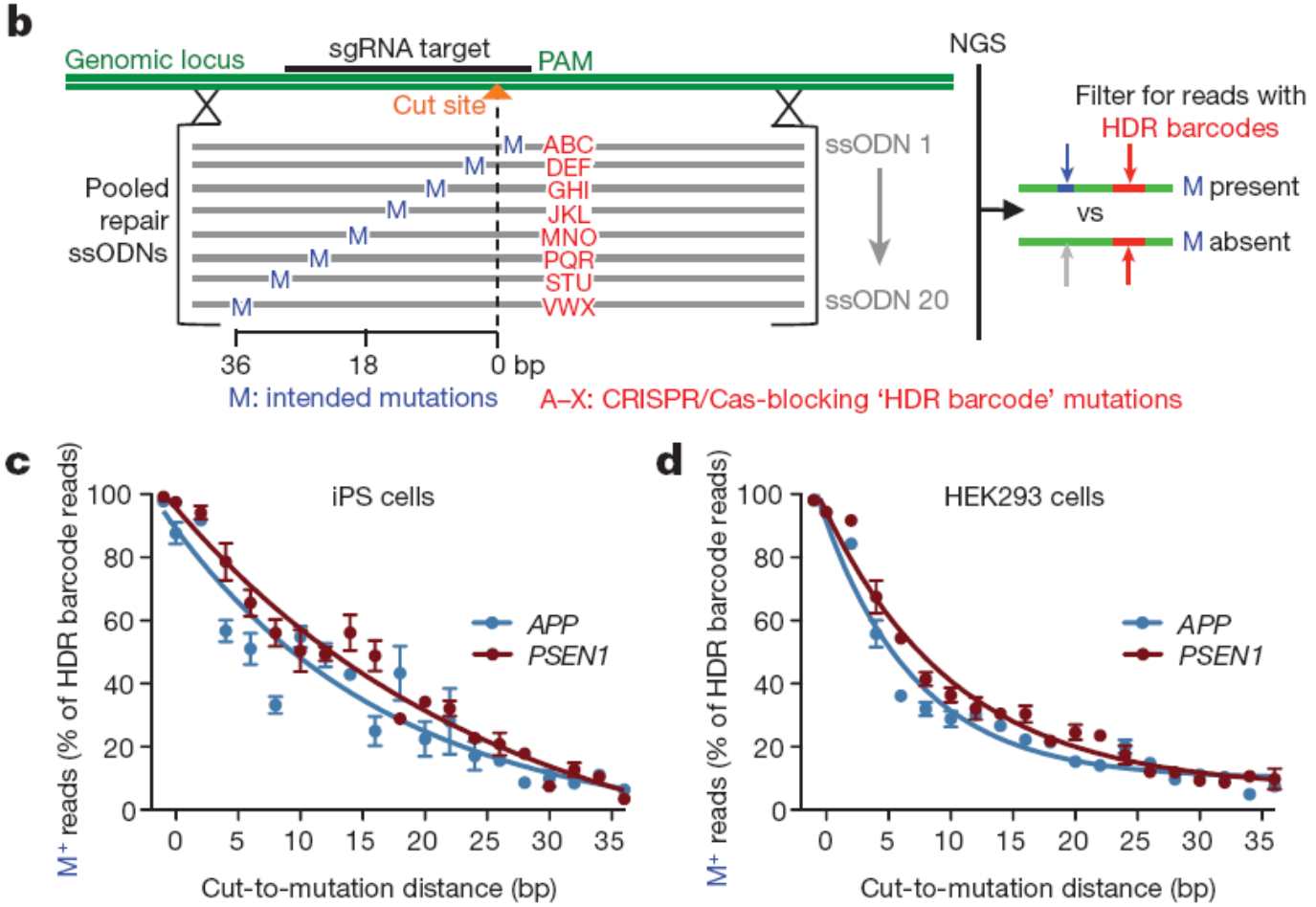
- Majority of genetic diseases are “caused” by (or can be correlated to) SNPs
- 46,589 SNPs and 6,356 diseases and phenotypes (source: http://www.disgenet.org/)
- Essential for disease model development
- “Personalized medicine” by predicting efficacy of a new drug dependent on a SNP “fingerprint”
- Identifying how a complex set of different SNPs can cause similar disease phenotypes

Efficiency of SNP Repair Relies on Close Proximity to PAM Site



Nature 533, 125–129 (05 May 2016)

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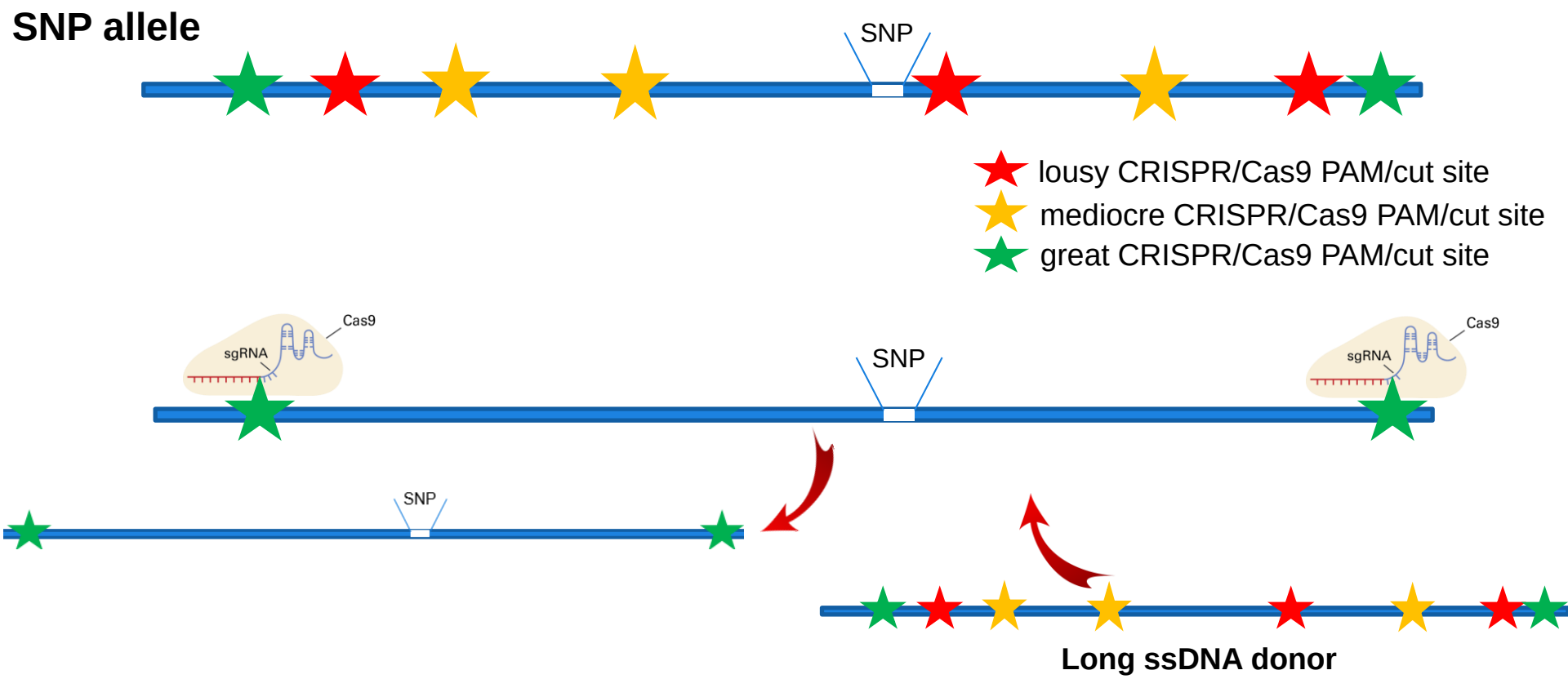
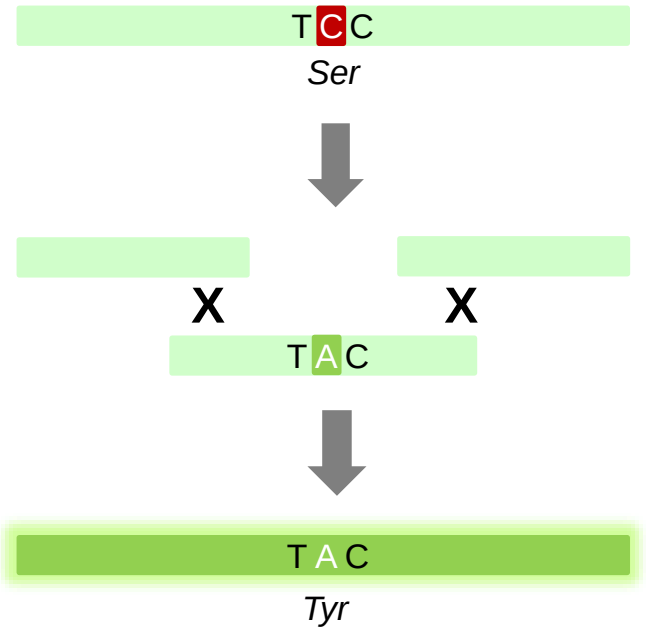
Advantage of Using Long ssDNA for SNP Repair via Homologous Recombination

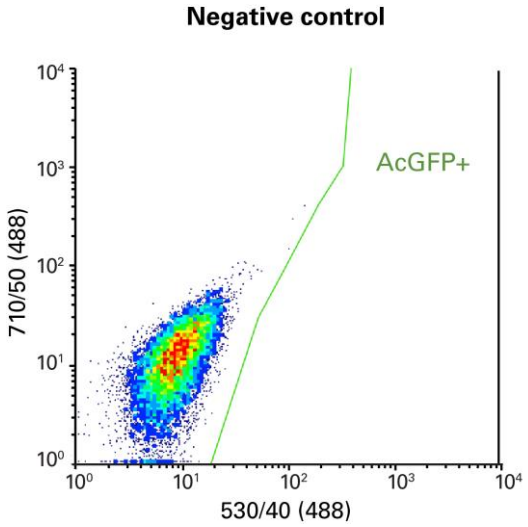


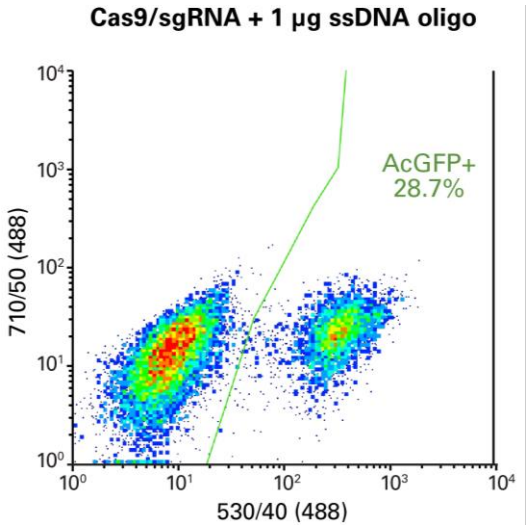
Homology-Directed Knockin of Point Mutations in hiPSCs



Non-fluorescent mutant AcGFP1

Fluorescent AcGFP1





Cell Biology Portfolio Enabling Gene Function Analysis

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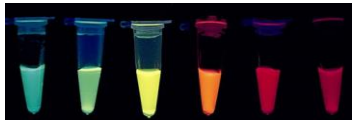
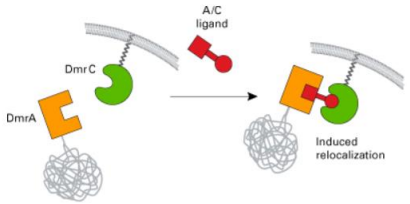
Cellartis:
-Products
-Service business

Genome editing

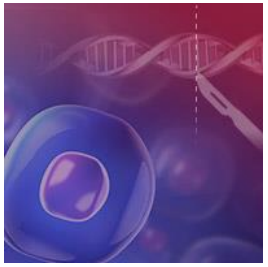
Fluorescent proteins and reporters



Ligand induced protein interaction

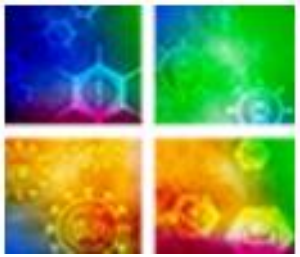


hiPS

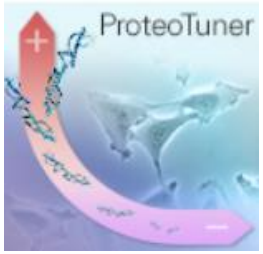


Gene Function

Viral delivery



Ligand induced protein stabilization



Inducible gene expression systems

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Stem Cell Research

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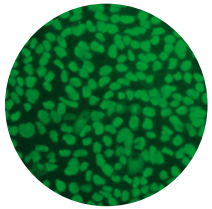
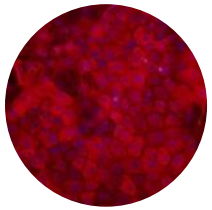
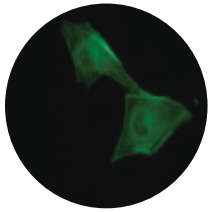
Stem cell innovations for today and the future



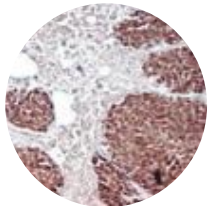
Cell Culture
- Expansion & maintenance
- Research-grade, Xeno-Free & GMP
- Differentiation



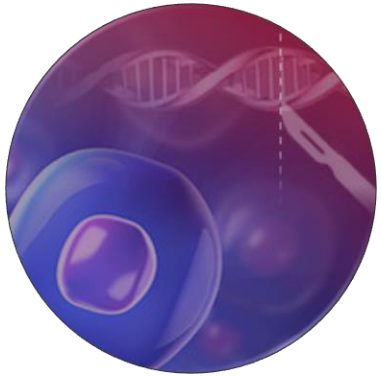
ES/iPS-derived Cells
- Hepatocytes & cardiomyocytes
- DE cells
- Beta cells
- Neural progenitors



Stem Cell Services
- Sourcing & reprogramming
- Clinical-grade cell line generation & banking
- Genome modification



Characterization and Detection
- Antibodies to verify pluripotency, differentiation, etc.
- qPCR primer sets



New products: Human iPS Gene Editing Systems
- Optimized delivery for clonal expansion
- Flexible gene methods

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TaKaRa

that's GOOD science!®

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