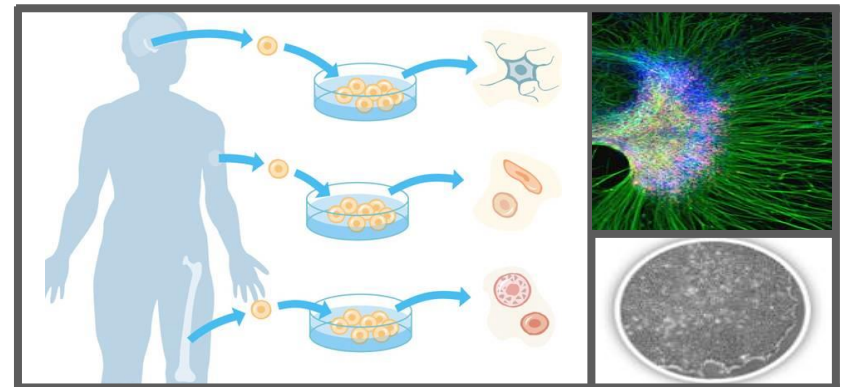


Clontech **Takara** cellartis

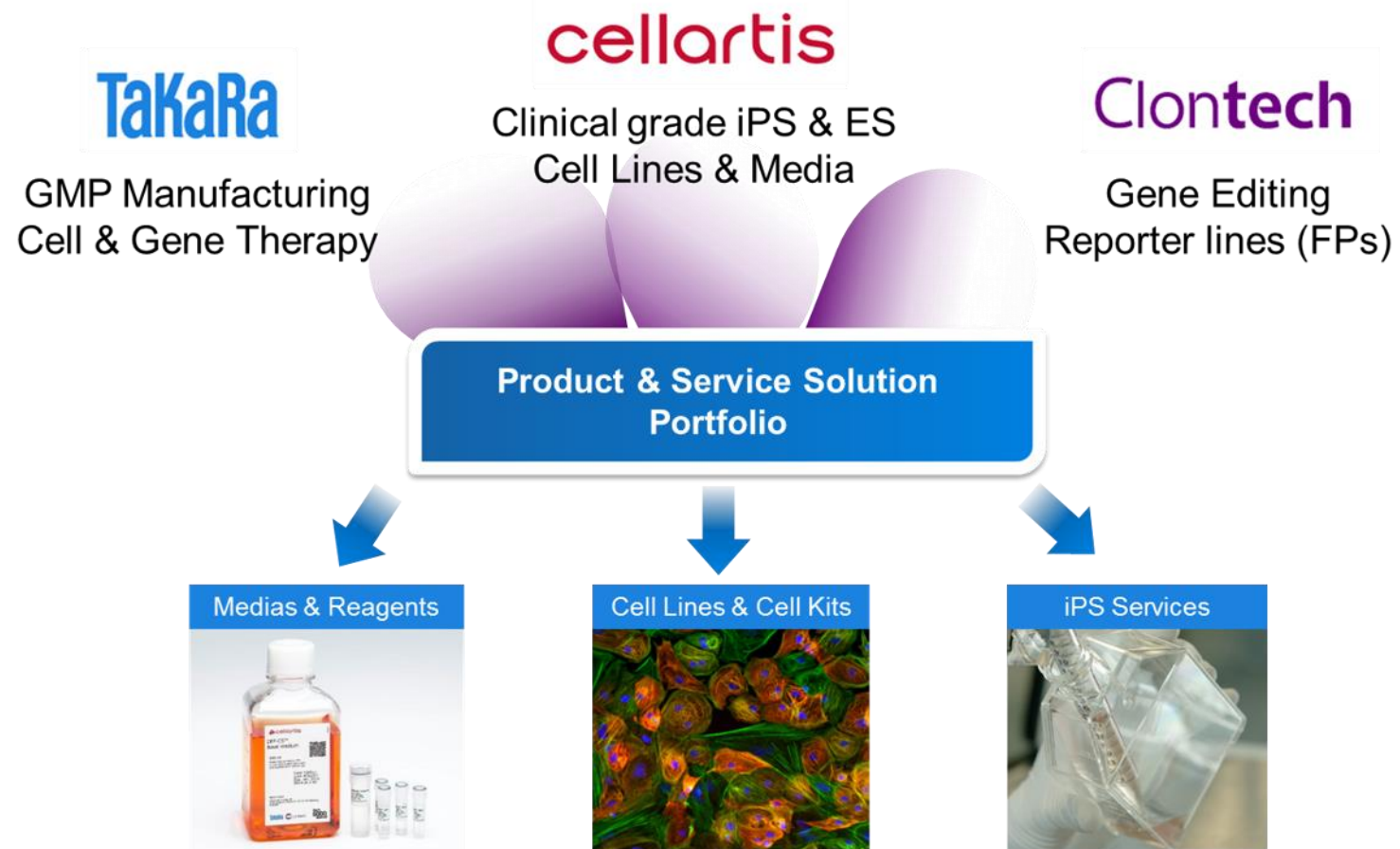
# Efficient, Footprint-Free Gene Editing and Single-Cell Cloning of iPS Cells Using CRISPR/Cas9

Liz Quinn, PhD



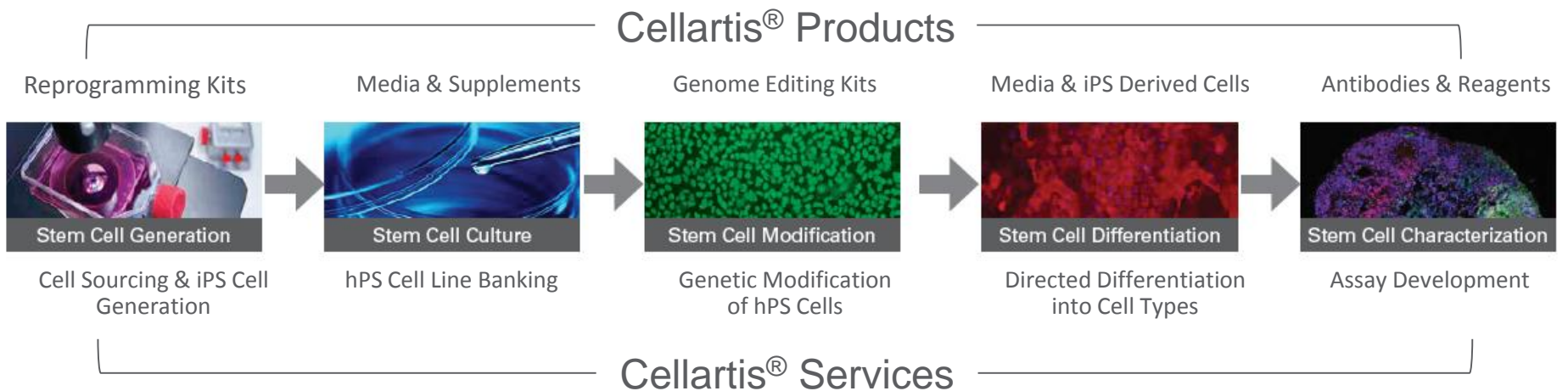
that's  
**GOOD**  
science!®

# Stem Cell Research Expertise



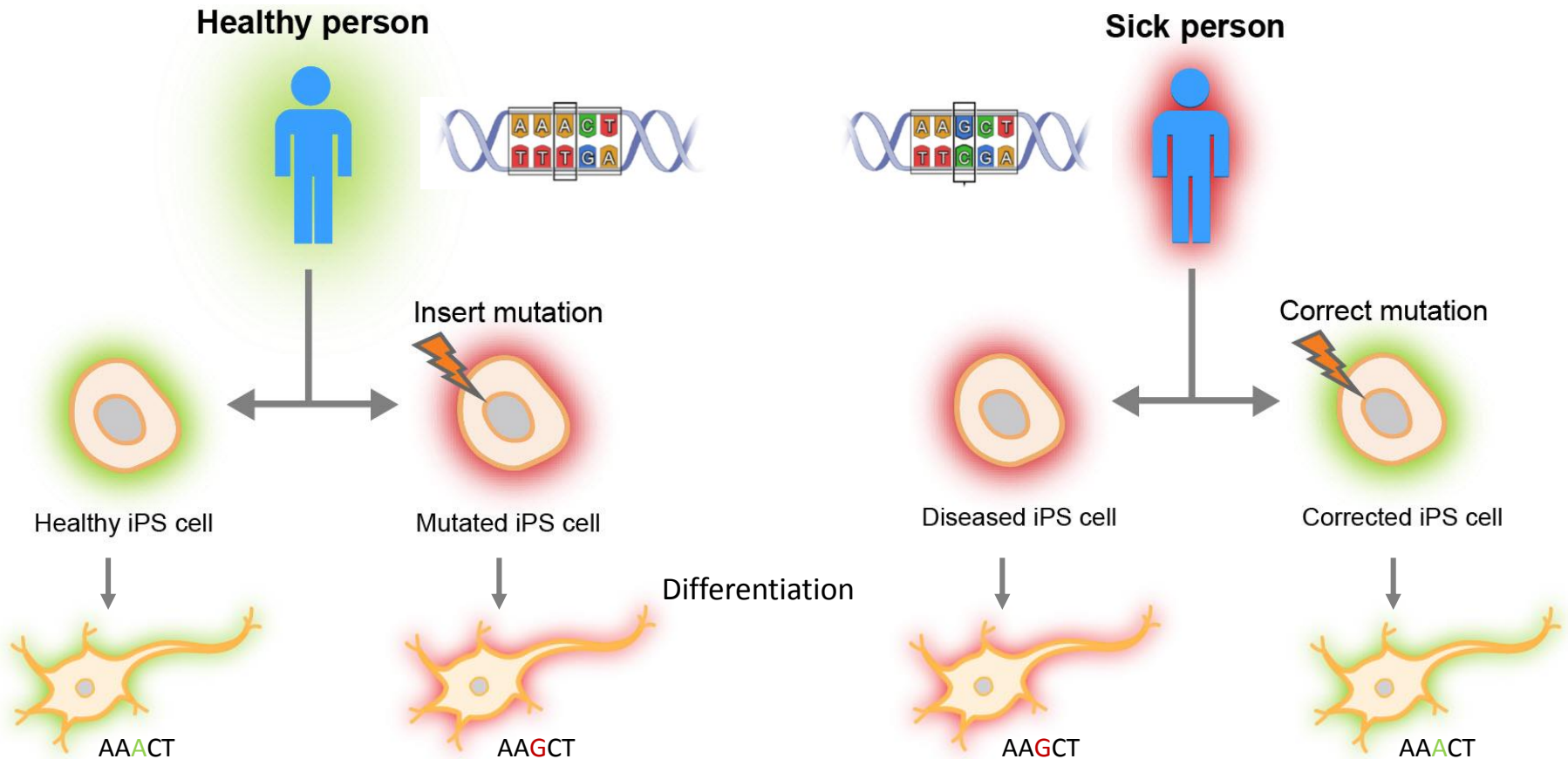
We focus on innovative research & clinical grade stem cell products & services

# Comprehensive Products & Services

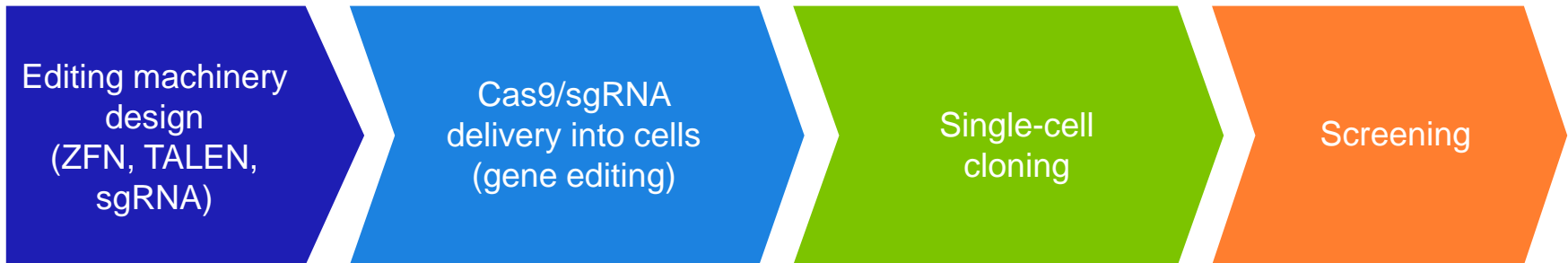


# Utility of Stem Cells and Gene Editing

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



# Challenges in Editing Stem Cells

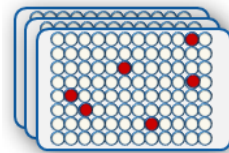


- Accuracy
- Specificity

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

- Maximize survival
- Maximize pluripotency

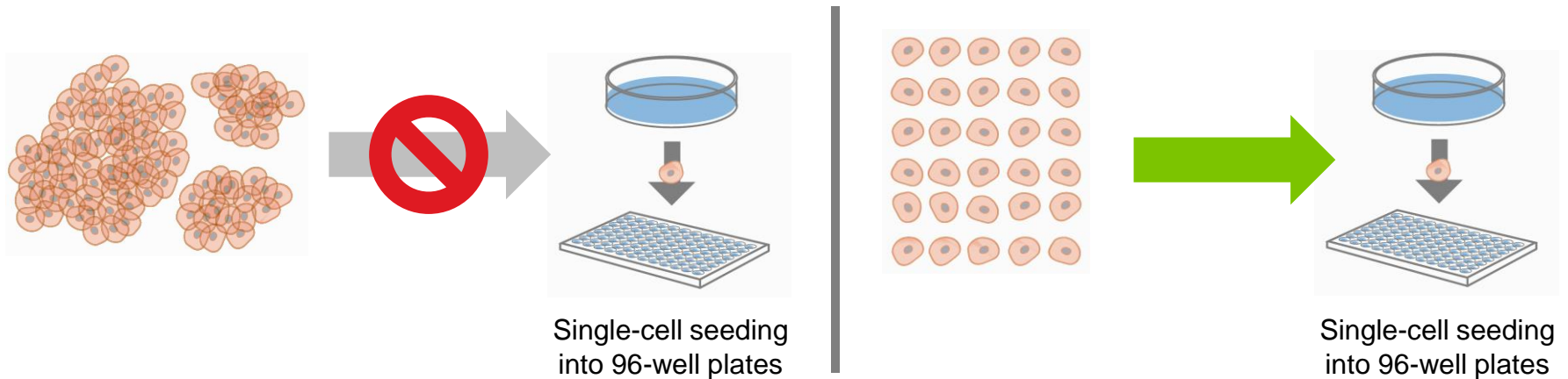
- Identification
- Specificity

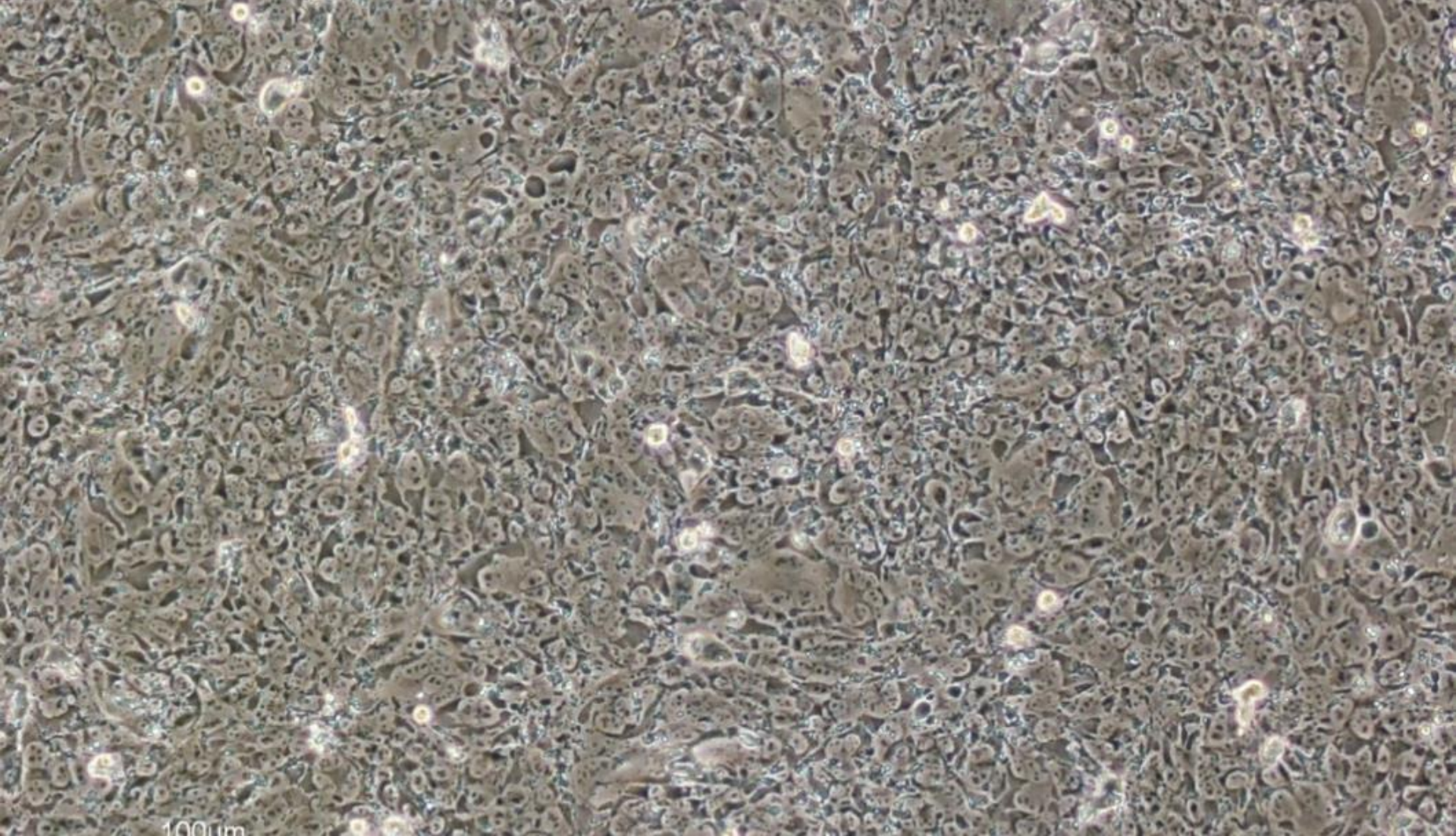


**Goal:** Isolate & expand a single, edited cell

# 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

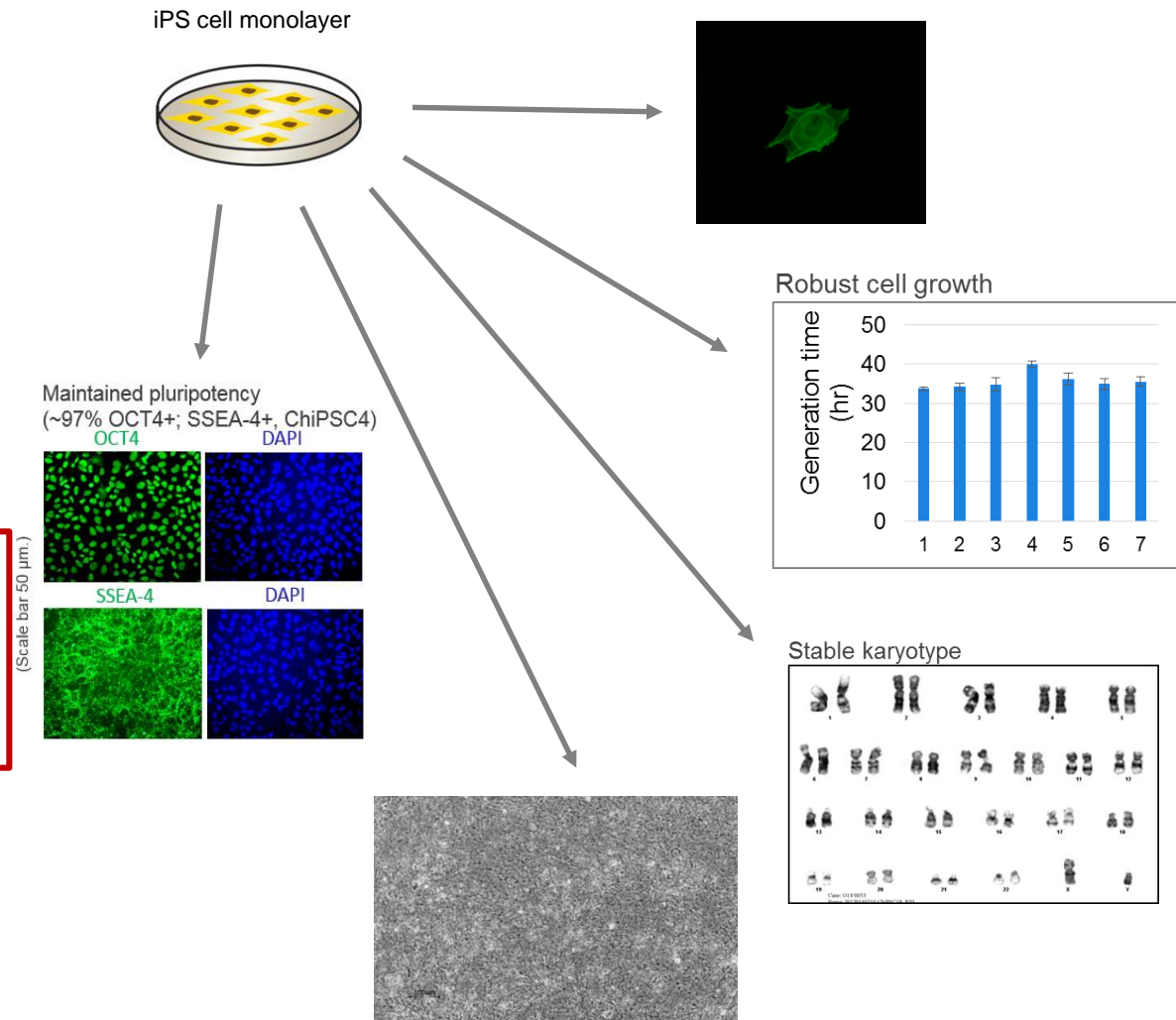


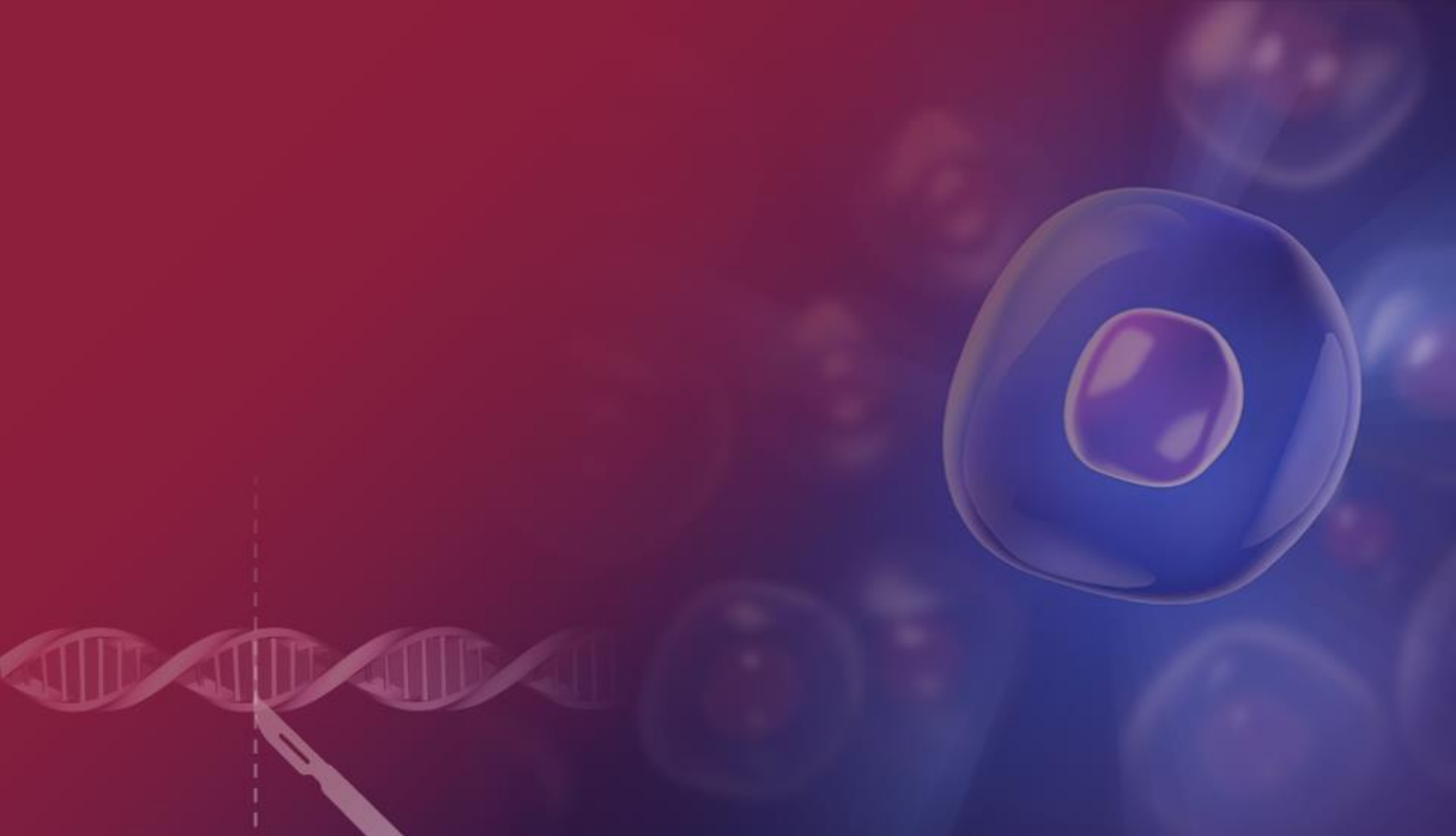


# Human iPSC Culture System

# DEF-CS™ & Single-Cell Cloning of iPSC

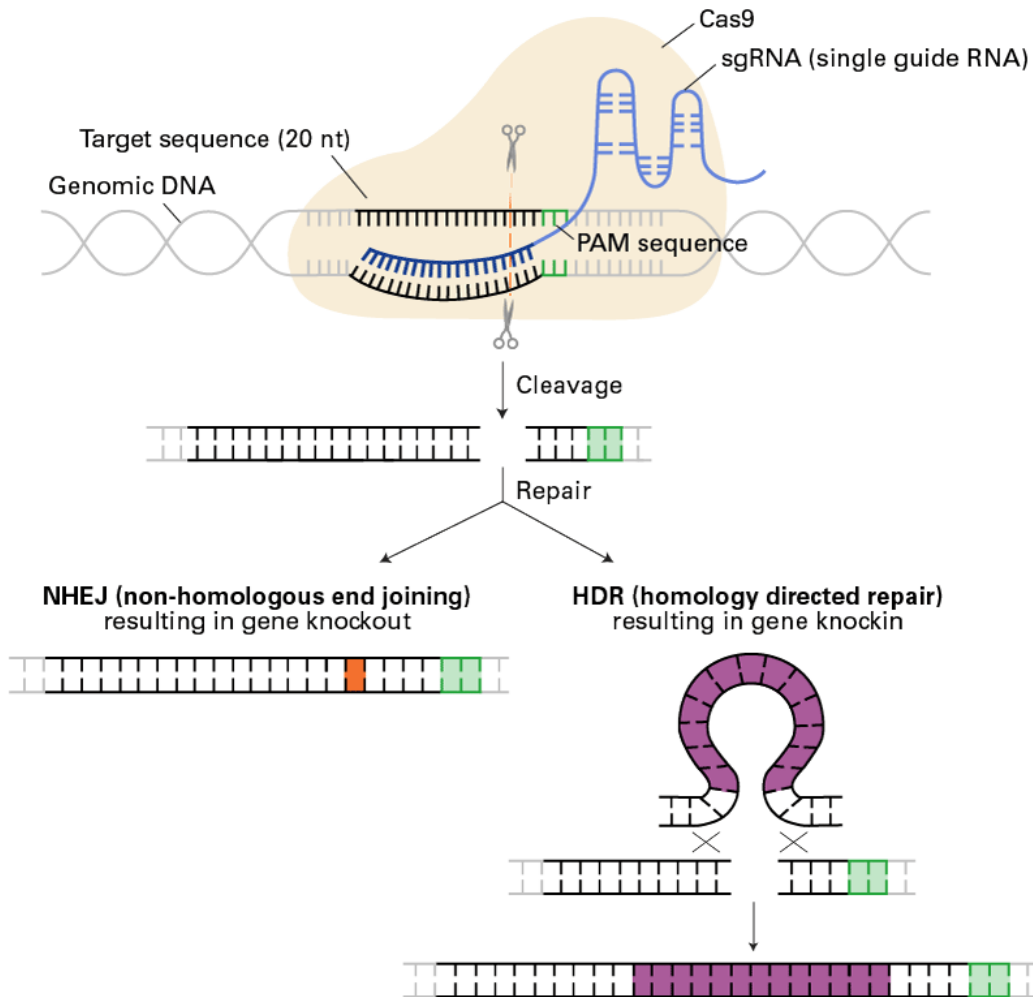
- Maintains cells in a highly undifferentiated state
- Allows for culturing iPSC cells in a monolayer
- Feeder-free — no contamination, less time consuming, increased consistency
- Enables survival and expansion of single cells
- Maintains normal karyotype
- Allows rapid expansion for further downstream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





# Gene Editing Tools

# Gene Editing with Cas9



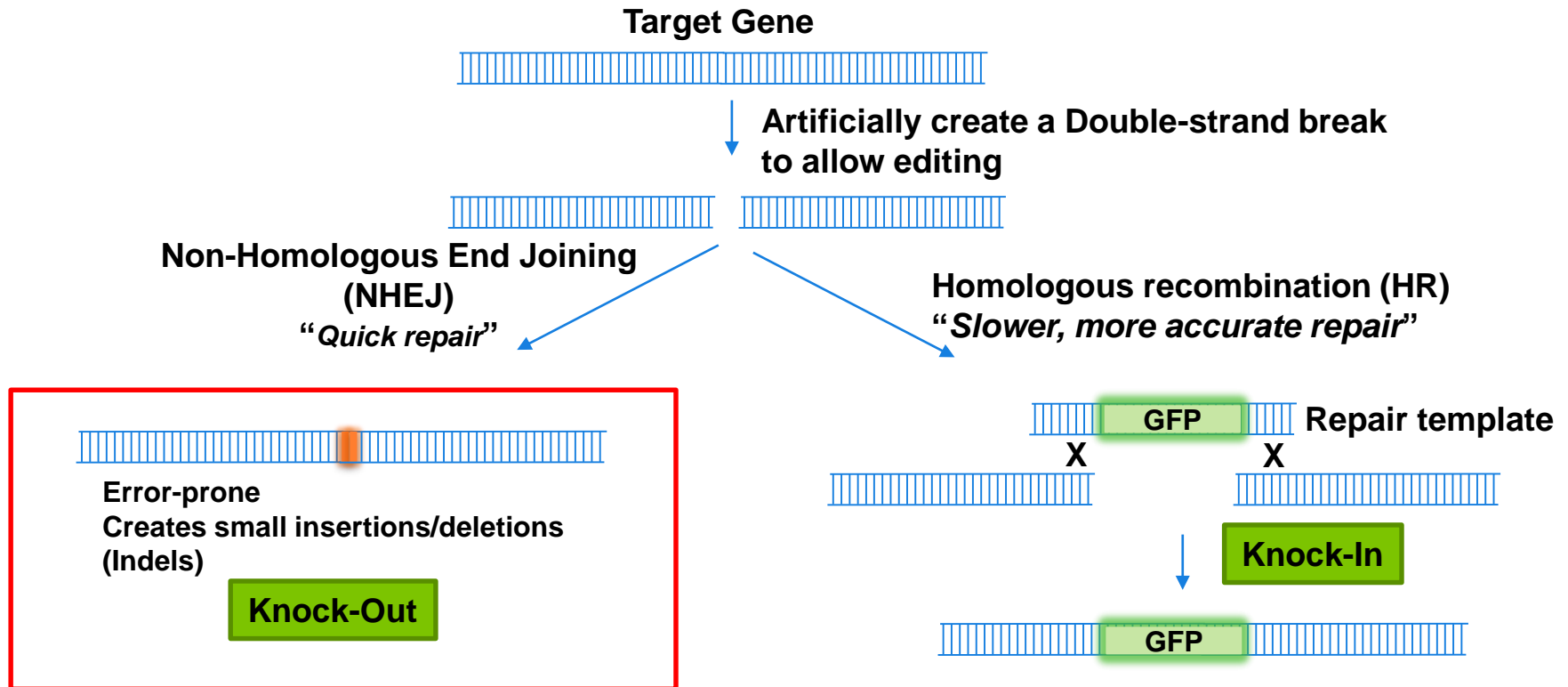
## Repair template (HDR template) production methods

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

## 3 Components required:

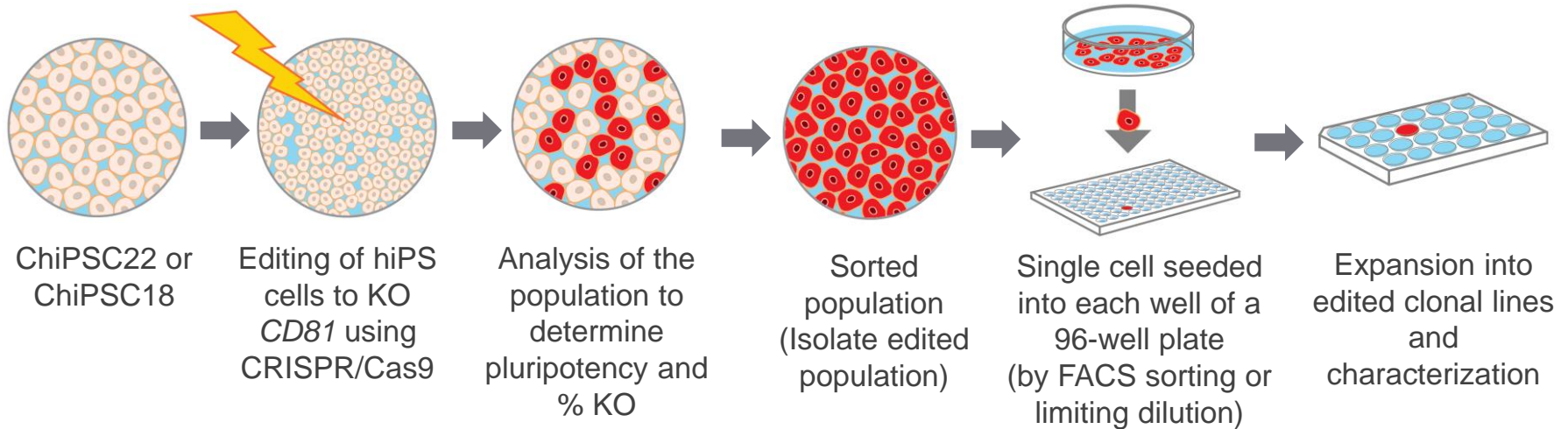
- Cas9 nuclease
- Guide RNA
- Repair template

# 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



# Two options for Cas9 Delivery

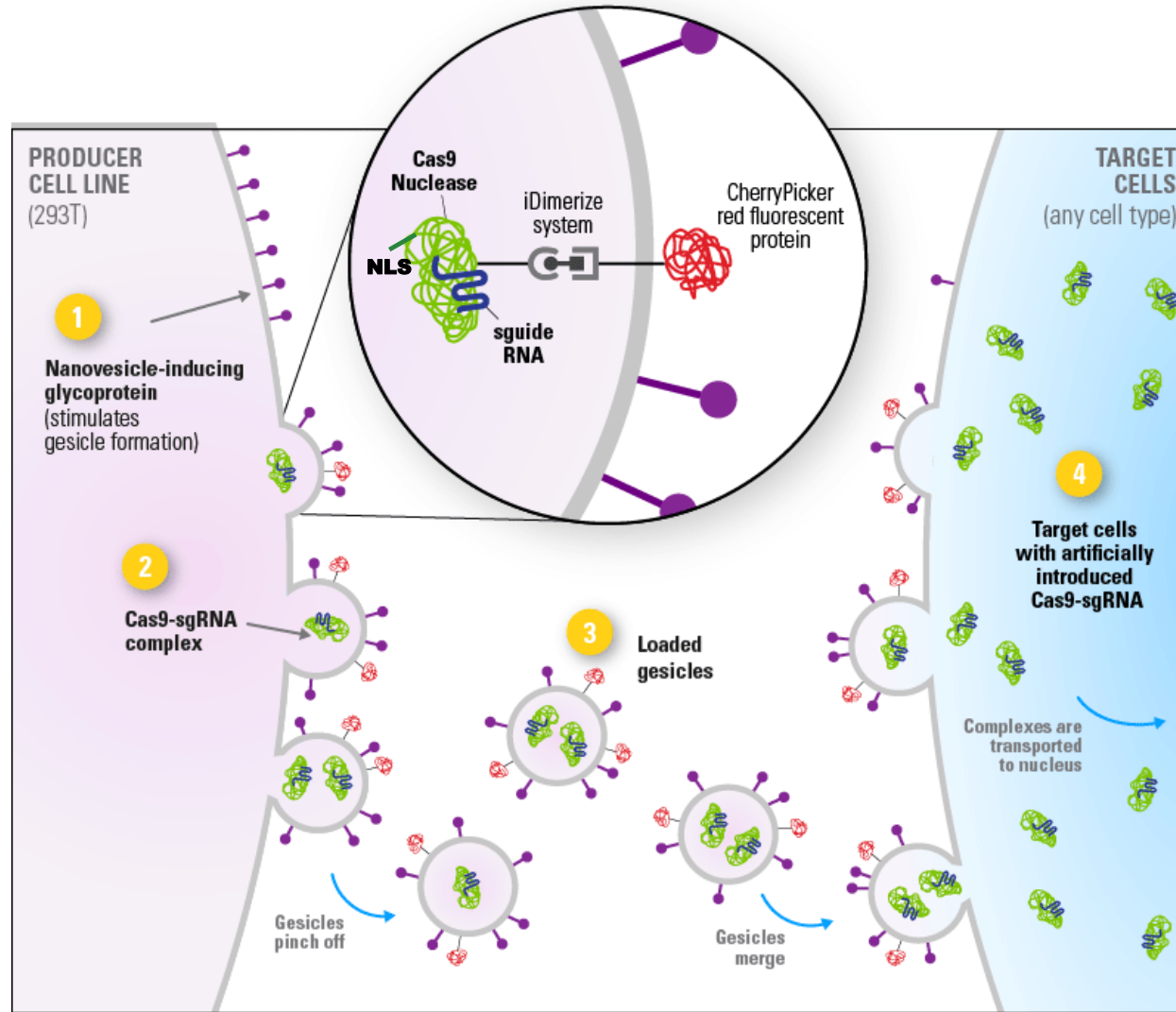
## **Guide-it™ rCas9 for electroporation:**

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

## **Guide-it CRISPR/Cas9 vesicle production kit:**

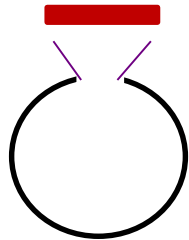
- Delivery of a Cas9/sgRNA RNP complex via cell derived nanovesicles

# Guide-it CRISPR/Cas9 Gesicle Production Kit

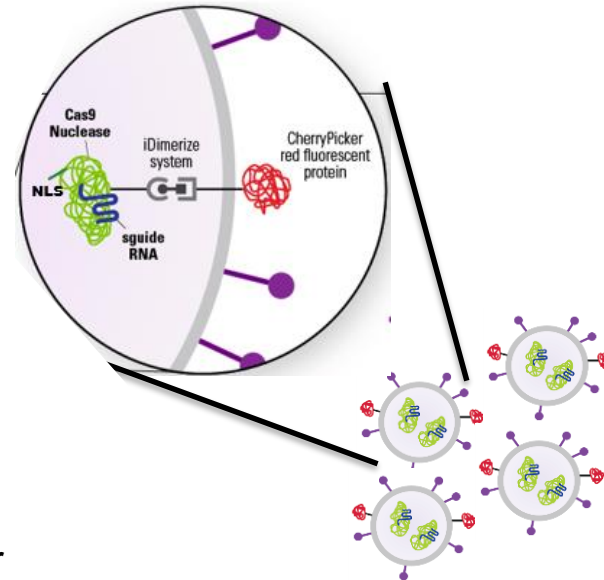


# Guide-it CRISPR/Cas9 Gesicle Production Kit

sgRNA of interest



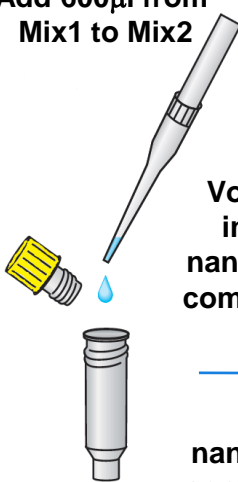
Pre-linearized sgRNA expression vector



Add 10µg of Plasmid encoding for sgRNA (600µl to final) to Packaging Mix1



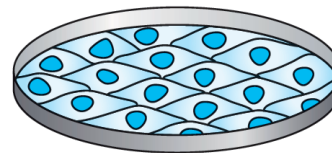
Add 600µl from Mix1 to Mix2



Vortex and incubate nanoparticles complexes for 10min

Apply nanoparticle complexes to sub-confluent (50-60%) cell culture

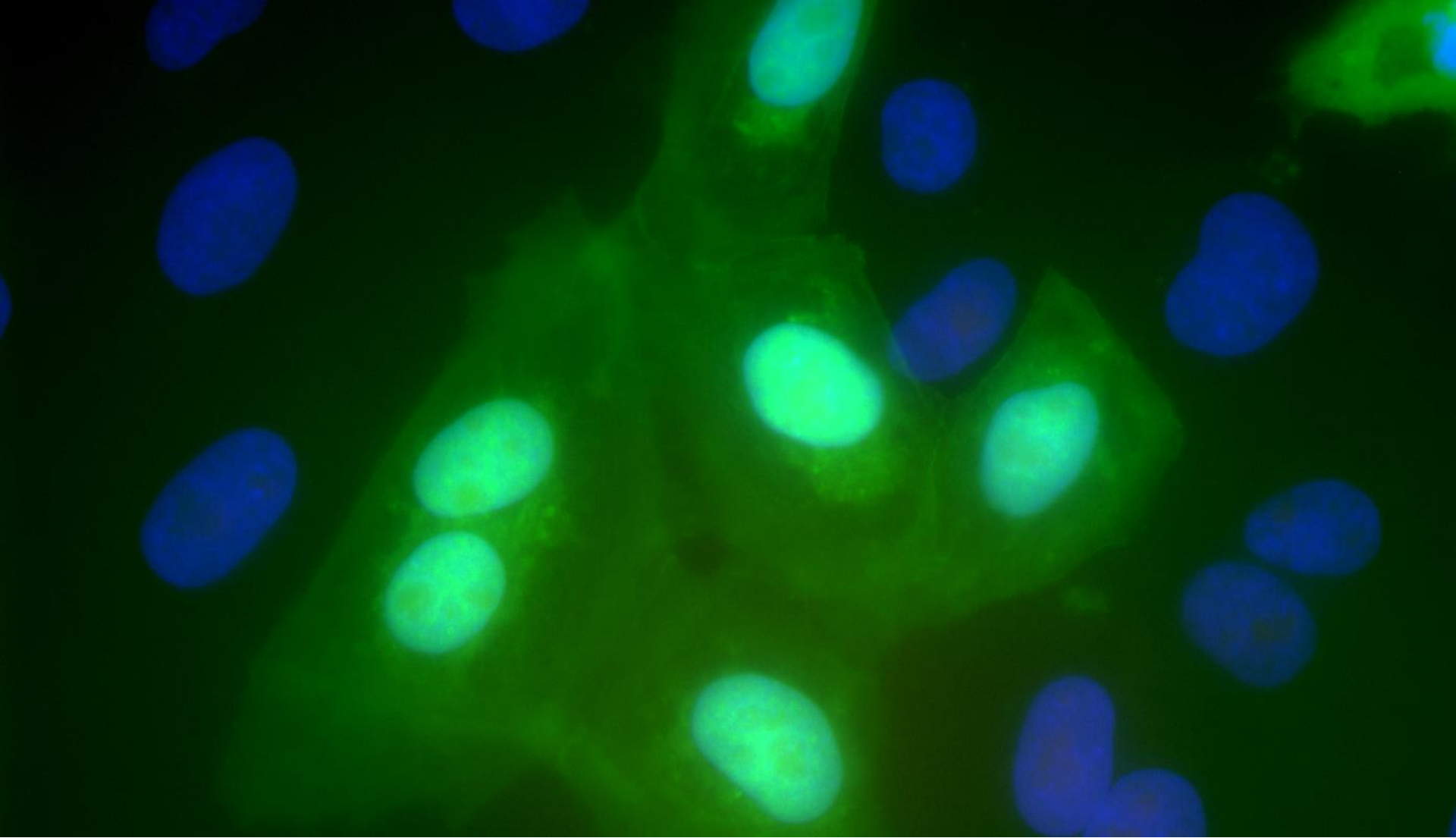
Add A/C Heterodimerizer



Incubate overnight at 37°C

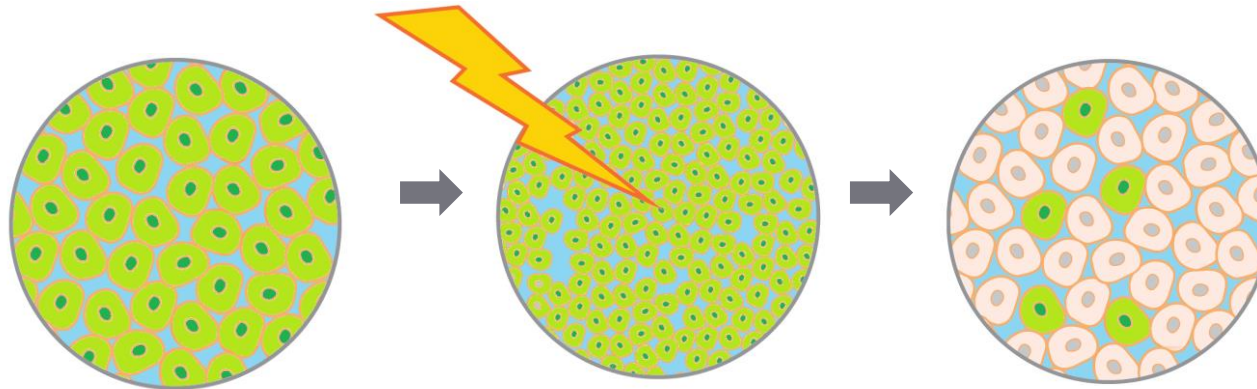
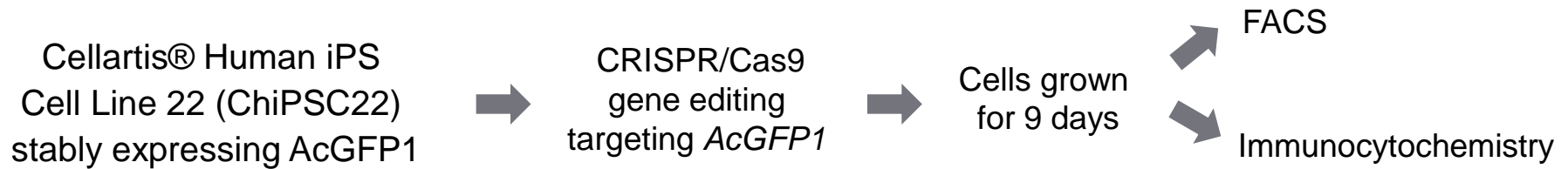
Add fresh media

Collect Cas9 gesicle supernatant 48-72h post-transfection

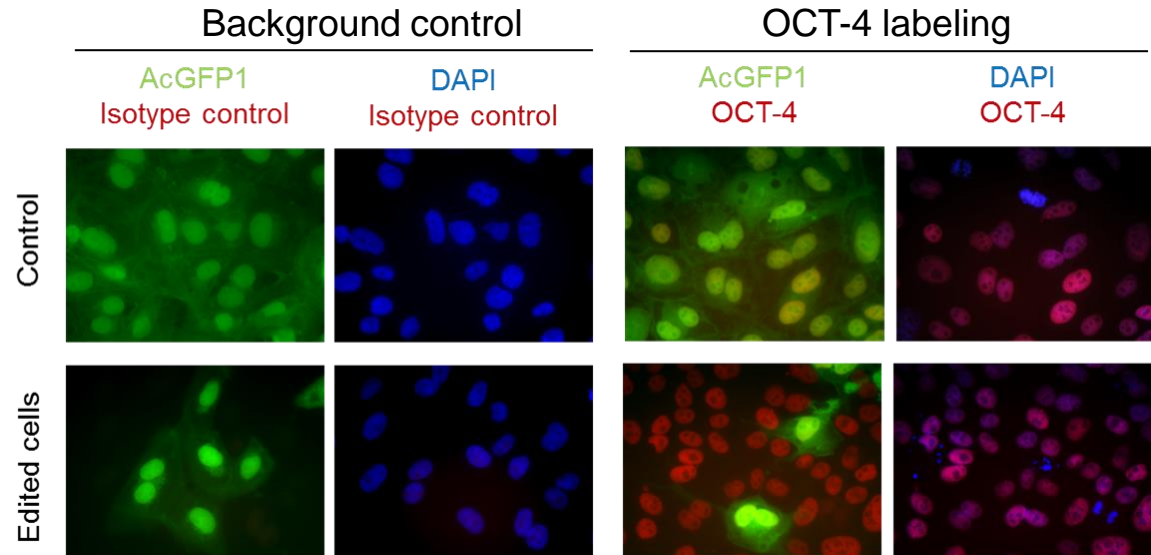
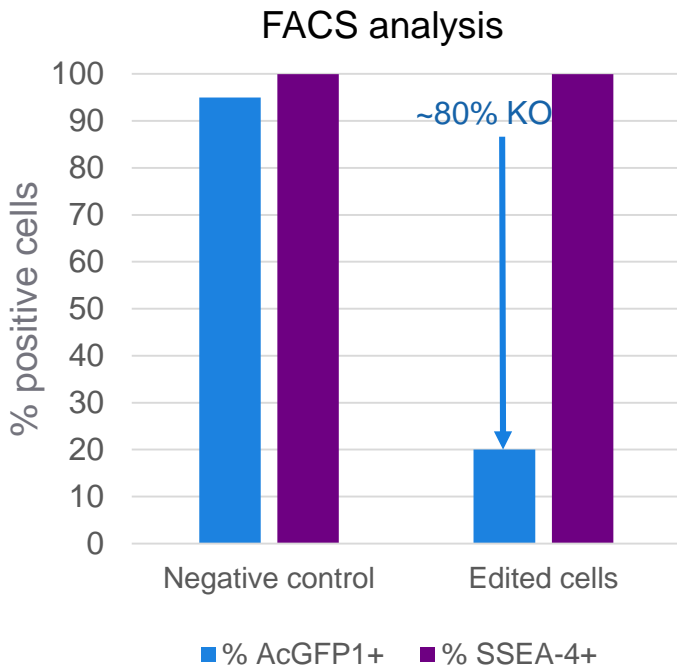
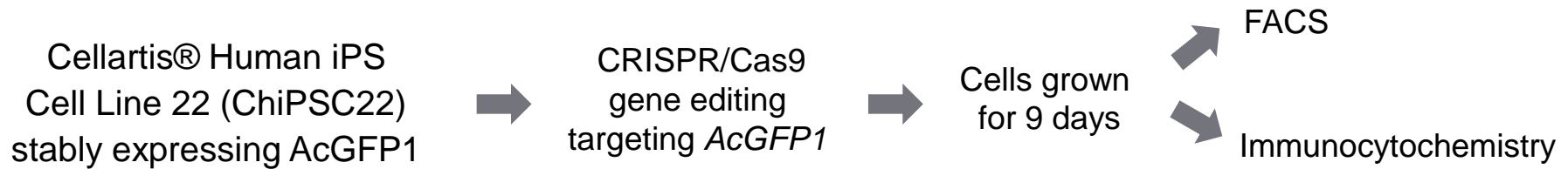


# Case Studies - Knock Out

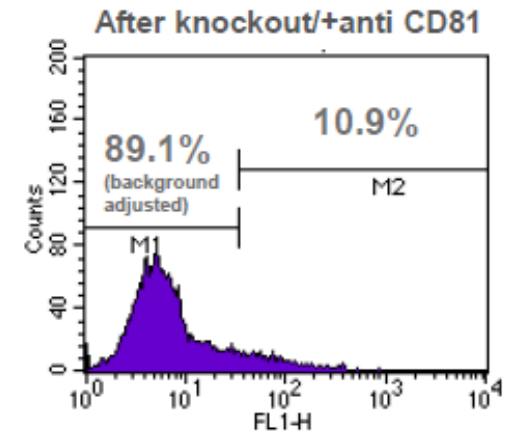
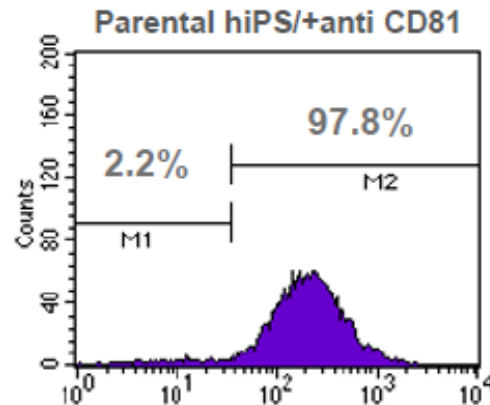
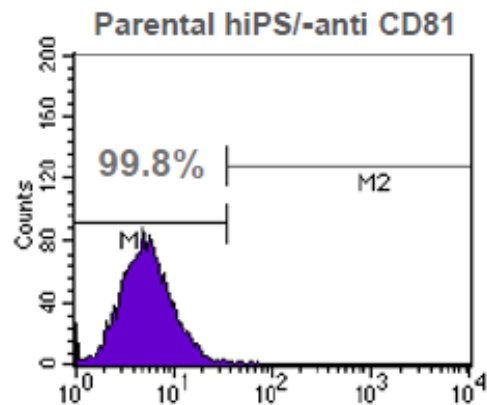
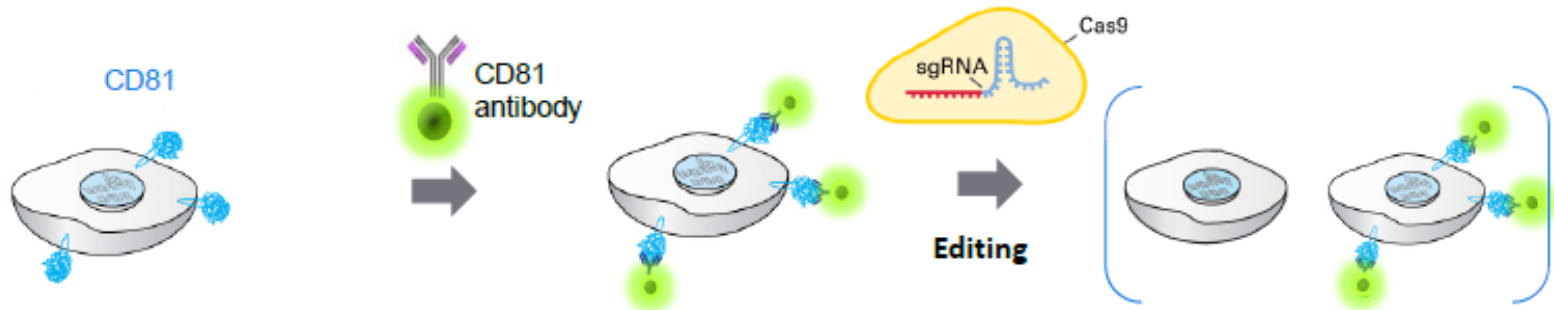
# Maintenance of Pluripotency after Gene Editing: *AcGFP1* KO Test Case



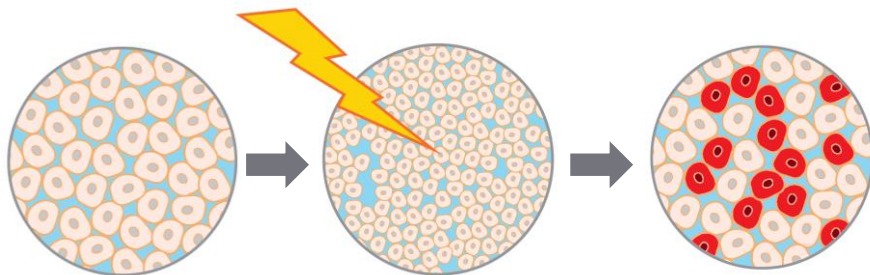
# Maintenance of Pluripotency after Gene Editing: *AcGFP1* KO Test Case



# Model System - *CD81* KO in hiPSC



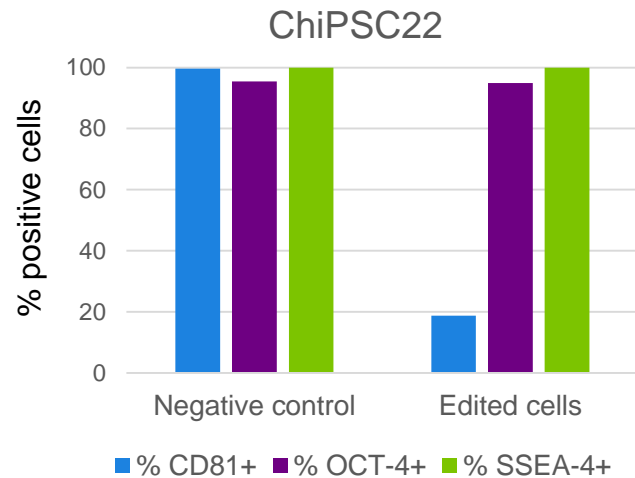
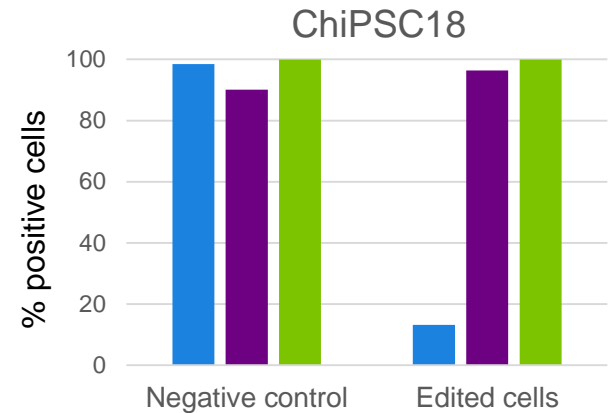
# Pluripotency Maintained after *CD81* Knock-Out



ChiPSC22 or  
ChiPSC18

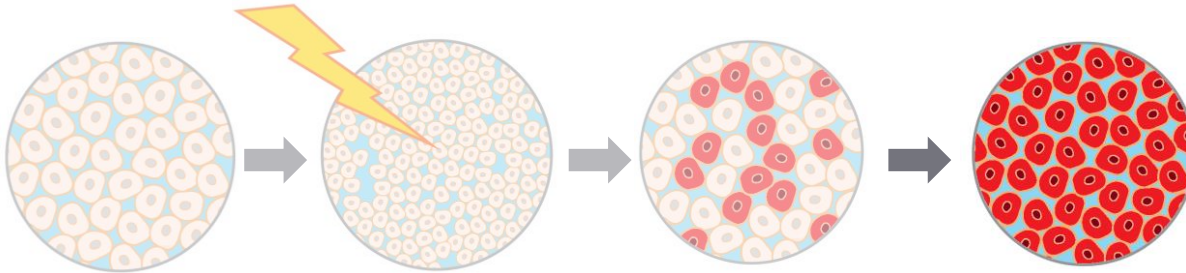
Editing of  
hiPS cells to  
KO *CD81*

FACS analysis  
of the population  
to determine  
pluripotency and  
% KO



# Sorting the *CD81* Negative Cell Population

## CD81/SSEA-4 double labeling

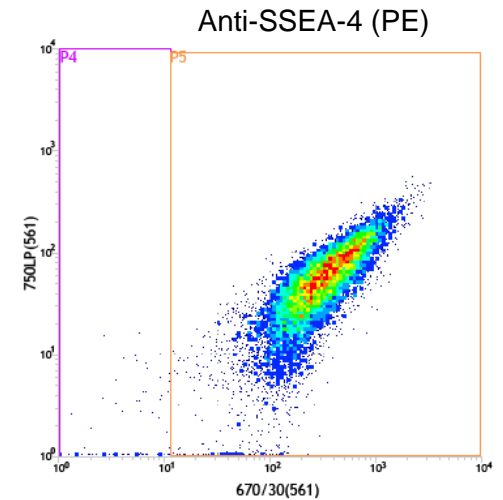
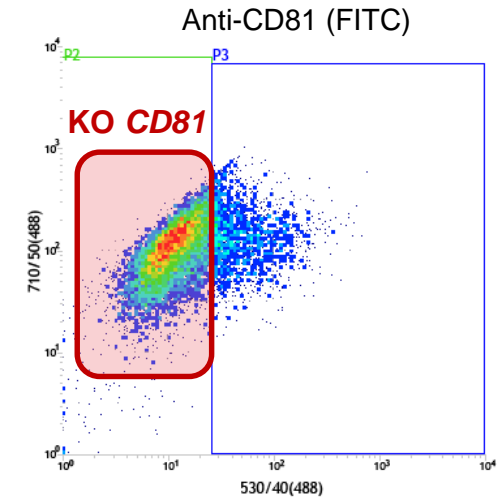


ChiPSC22 or  
ChiPSC18

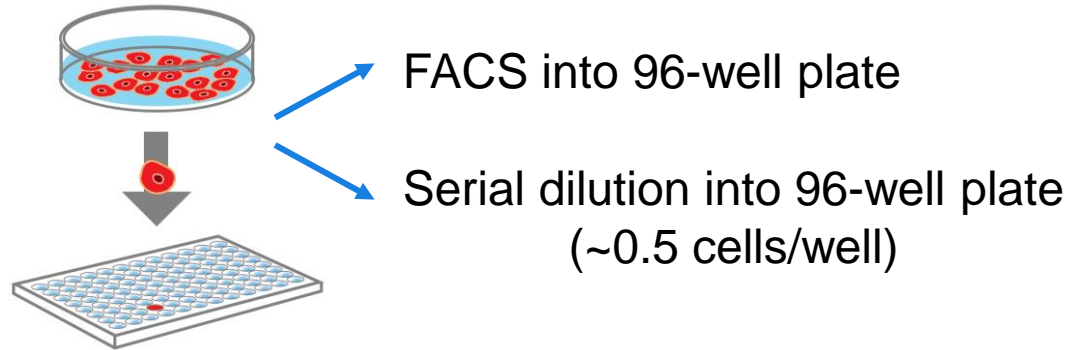
Editing of  
hiPS cells to  
KO *CD81*

FACS analysis  
of the population  
to determine  
pluripotency and  
% KO

Sorted  
population

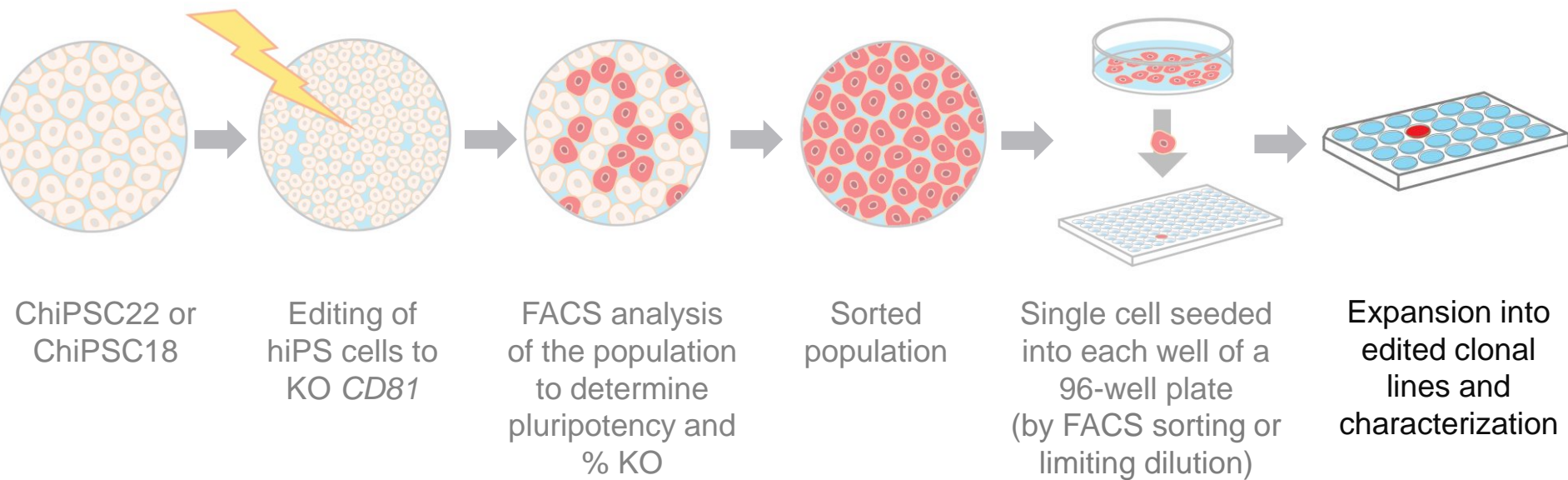


# Cloning of Edited hiPSCs via FACS or Limiting Dilution



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



# Robust Expansion of Edited Clones

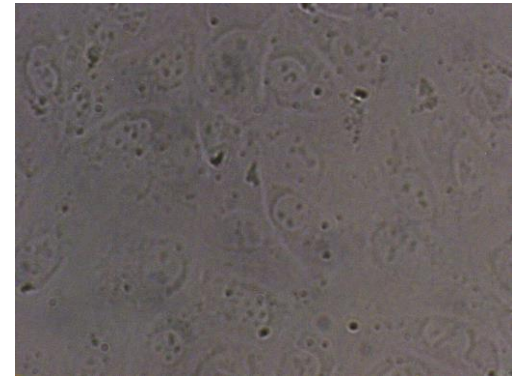
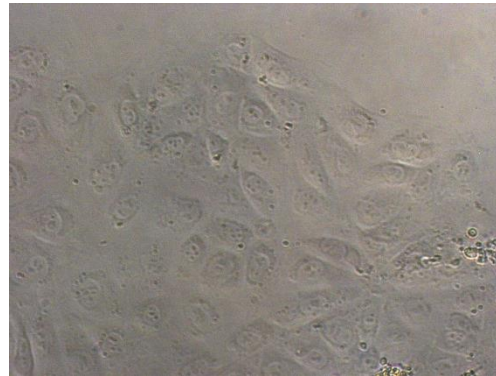
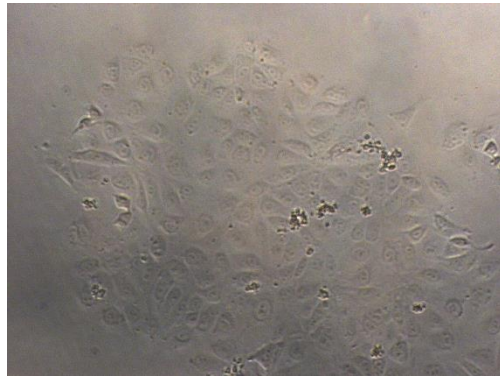
Images of colonies that originated from a single cell

10X

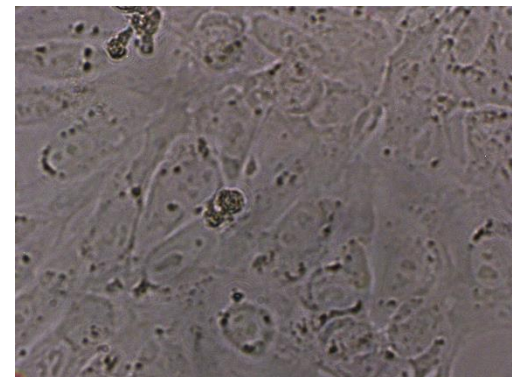
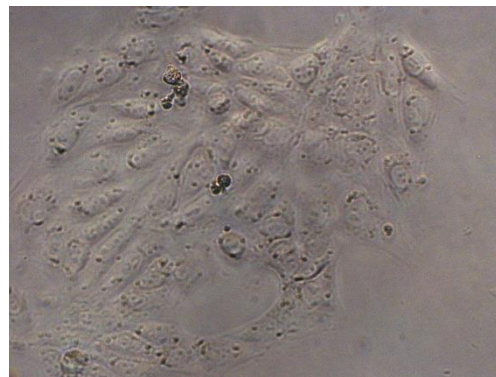
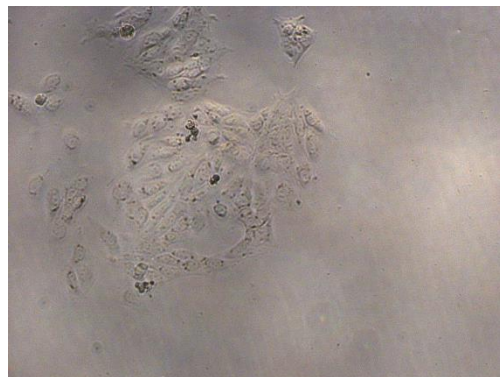
20X

40X

ChiPSC18

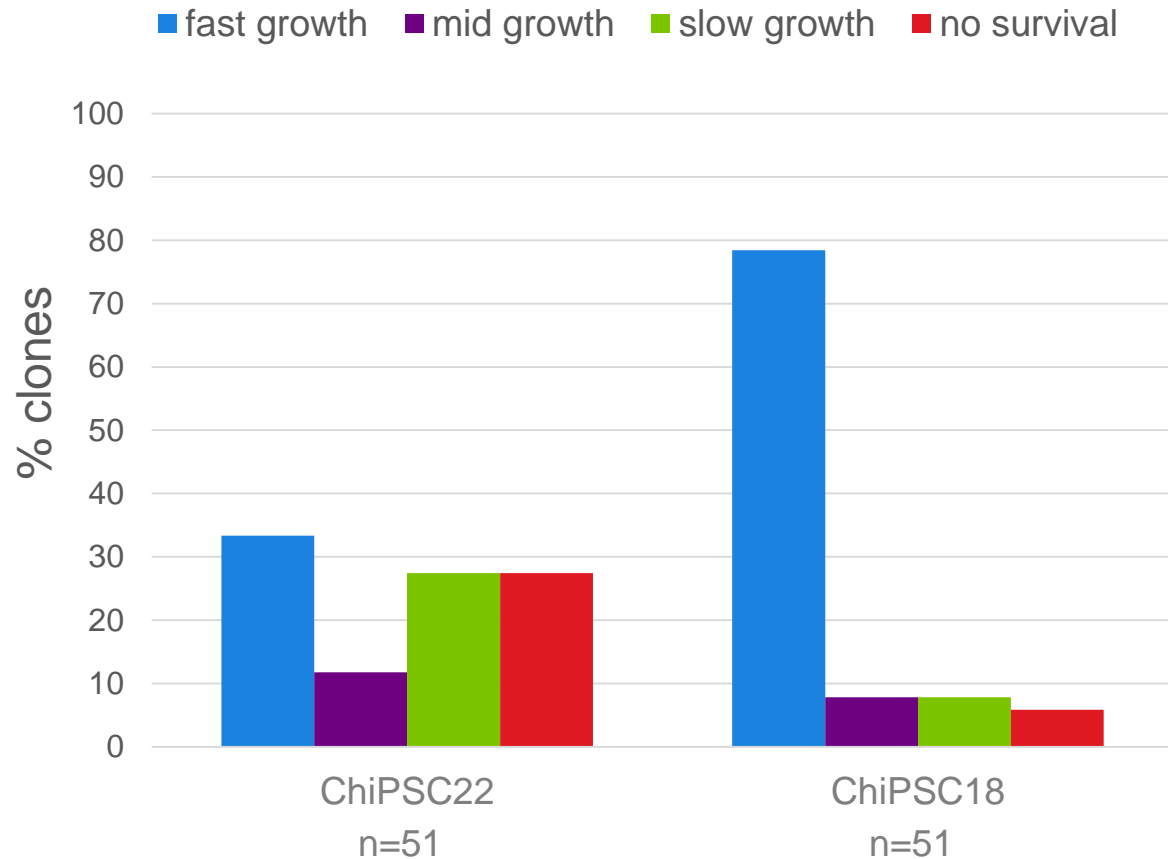


ChiPSC22



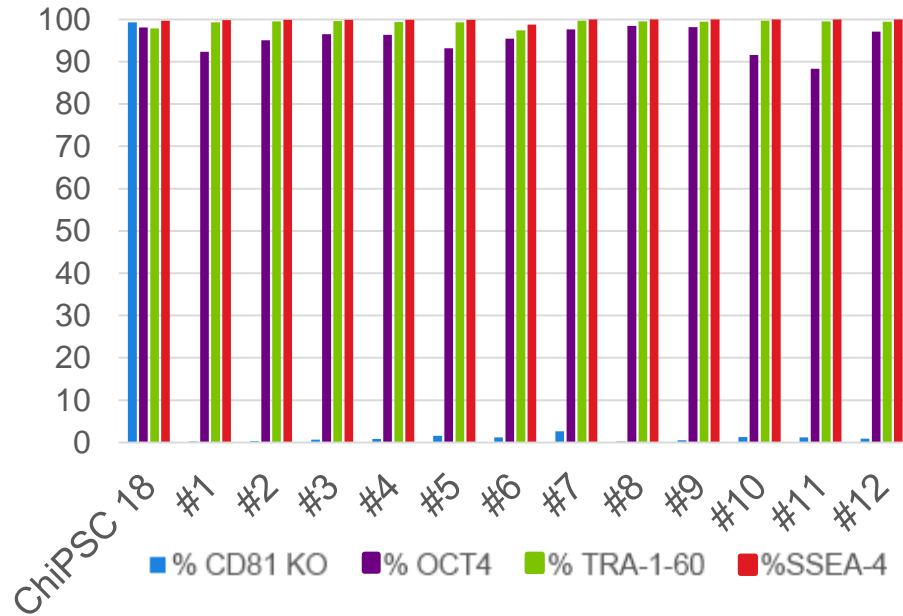
# Robust Expansion of Edited Clones

## Growth characteristics of clonal lines



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

## Clone Analysis



Pluripotency maintained following Limiting Dilution

## Karyotype Analysis

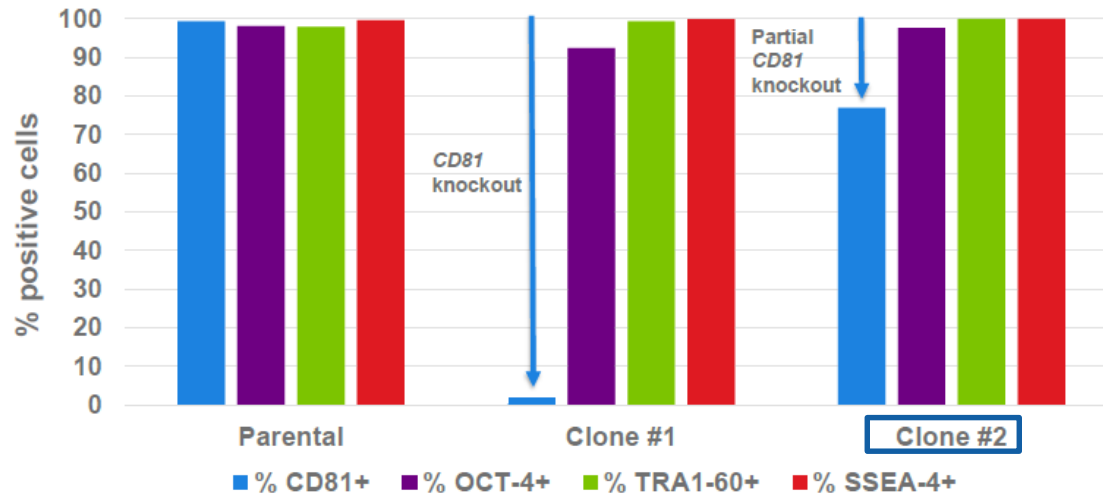


ChiPSC18, c.8, Passage 14

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

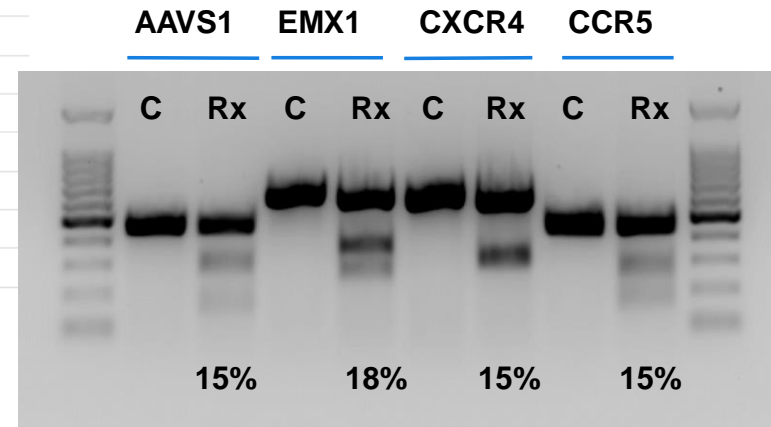
# Genomic Characterization of Edited Clones in ChiPSC18

## CD81



## Other Genes

“Mutation detection assay”

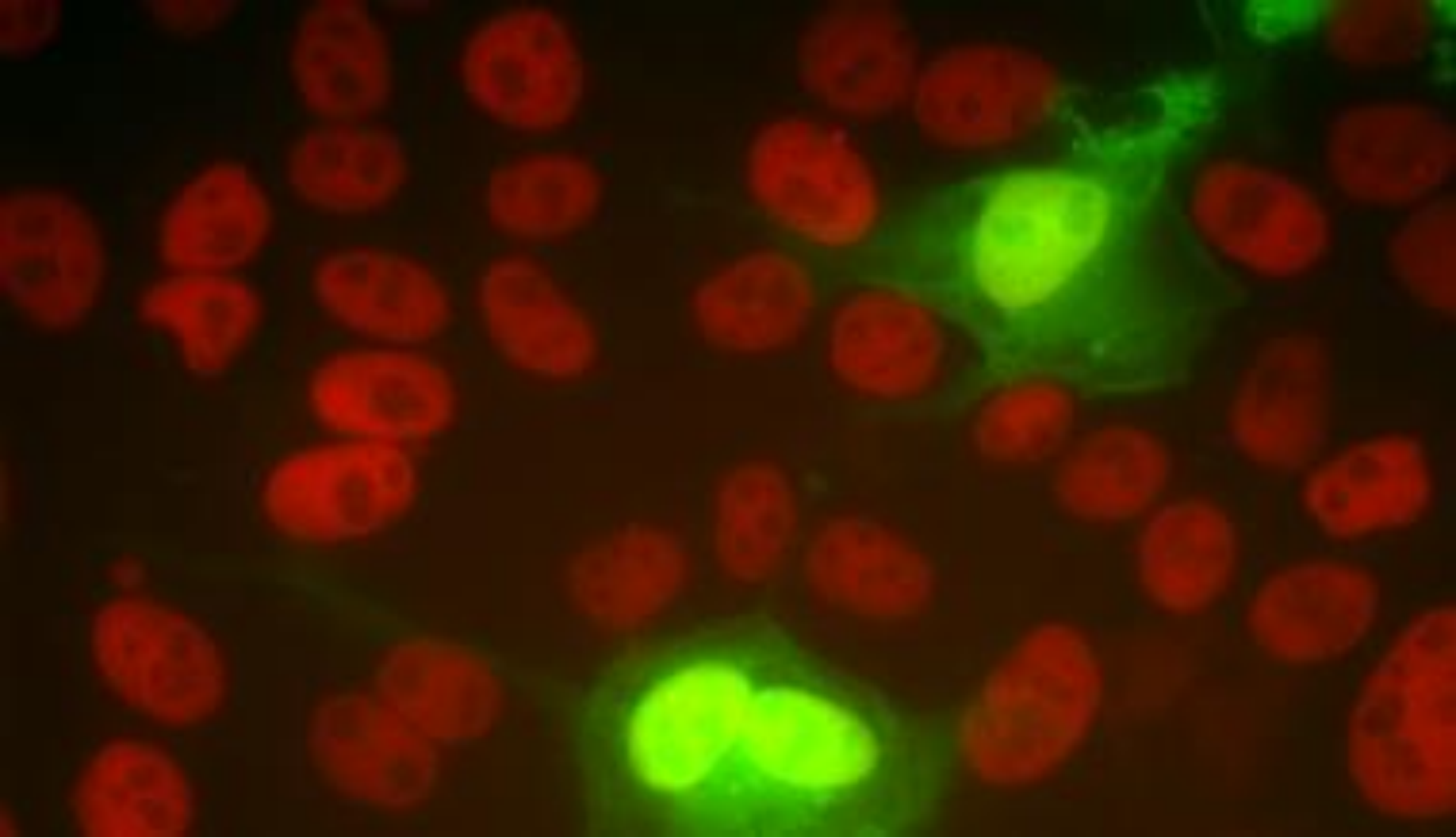


Genomic sequence alignment showing the CD81 gene structure and sgRNA1 binding site. The parental sequence is shown in black, and the edited sequences for Clone #1 and Clone #2 are shown in blue. The sgRNA1 binding site is highlighted in purple, and the PAM sequence is highlighted in grey. The CD81 WT allele is indicated by a blue arrow pointing to the sequence in Clone #2.

```

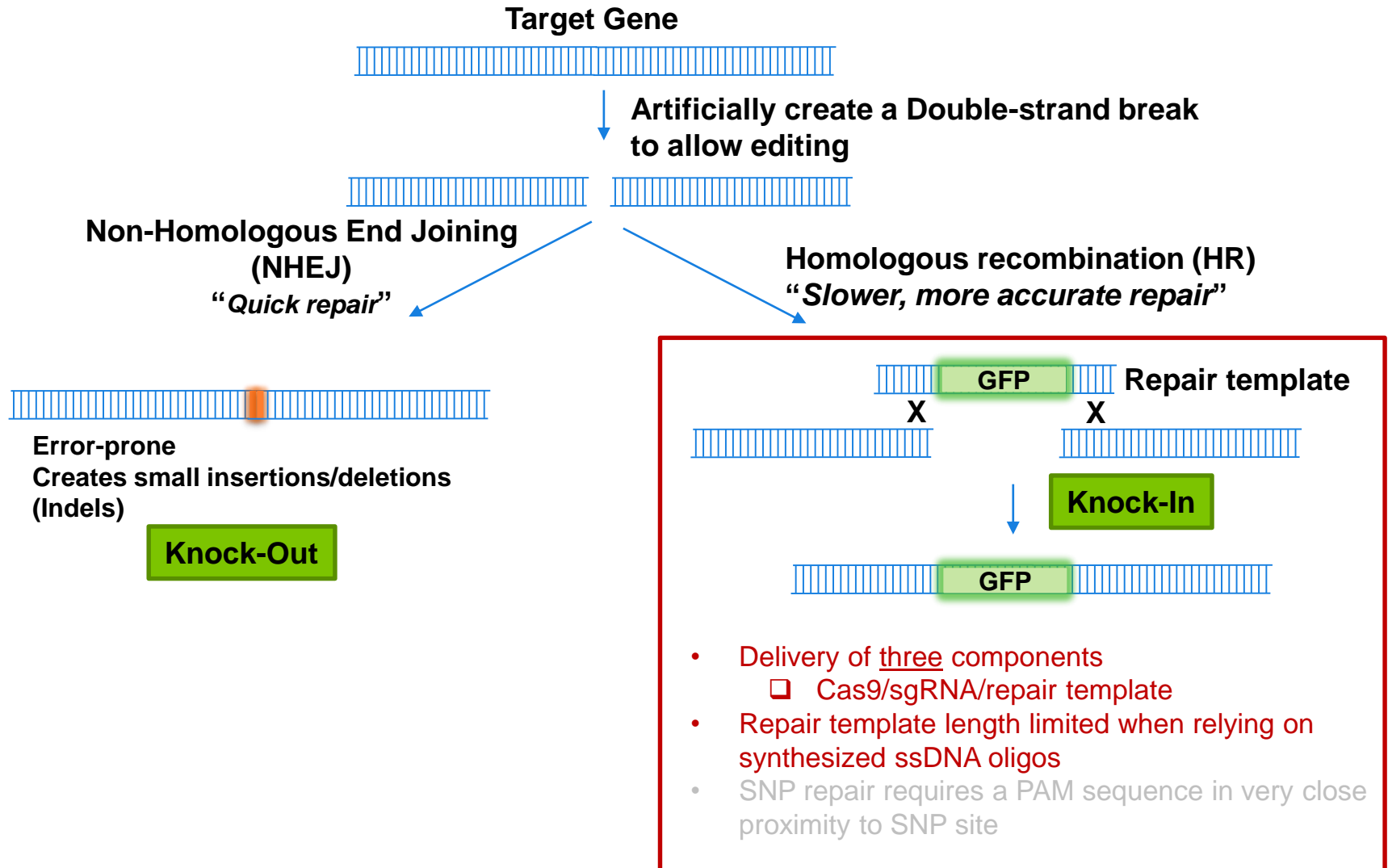
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GGCGGGGGCGTCCGGCGGGCGGGCGGGC  GCGCGGTA  CCTCACCTCCCGACGTGGTTACGTTAGTTCATGG
                                     PAM  sgRNA1
CGCGCCCCGCAAGCCGCCCGCCCGCCCG  CGCCGCCAT  GGGAGTGGAGGGCTGCACCAAAGTCATCAAAGTACC
CGCGCCCCGCAAGCCGCCCGCCCGCCCG  CGCCGCCAT  GGGAGTGGAGGGCTGCACCAAAGTCATCAAAGTACC
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```

← CD81 WT allele



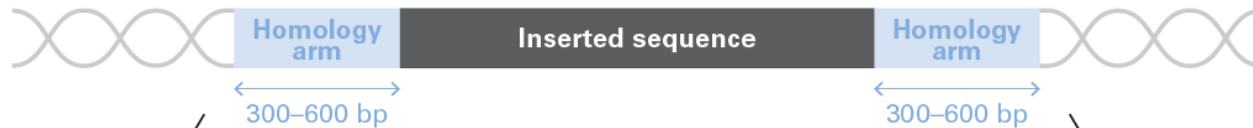
# Case Study - Knockin

# Bottleneck: Homologous Recombination



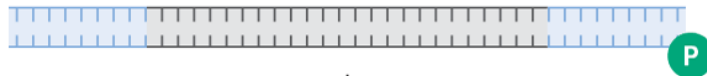
# Guide-it™ Long ssDNA Production System – How it Works

Prepare an HDR template using a method such as cloning or fusion PCR

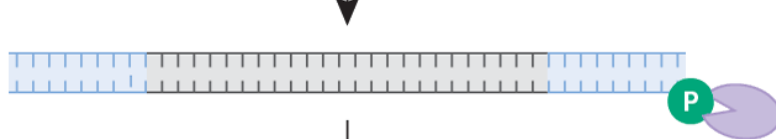


Use PCR to generate starting dsDNA material for the strandase reaction

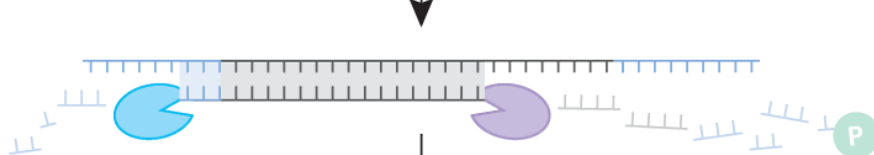
## Sense strand generation



Add Strandase Mix A to begin digesting the phosphorylated strand



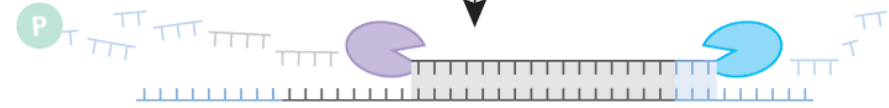
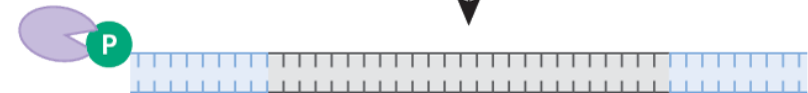
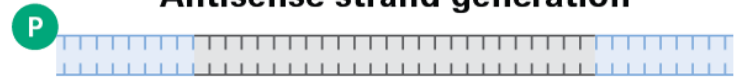
Add Strandase Mix B to finish digesting the strand



Clean up strandase reaction to prepare ssDNA for electroporation

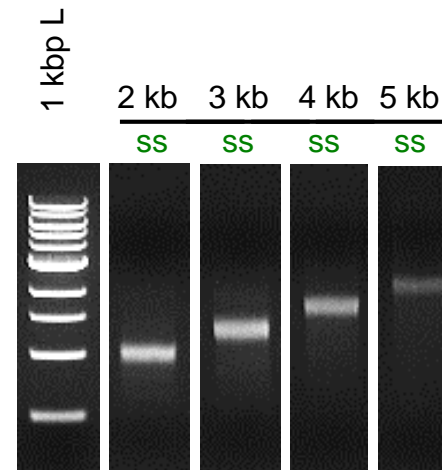
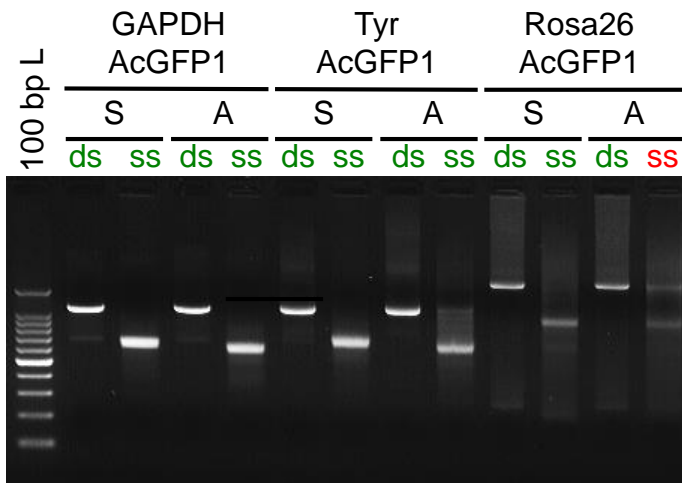


## Antisense strand generation



# Long ssDNA Prep: *Agarose gel*

\* ssDNA is much less sensitive to Et-Br



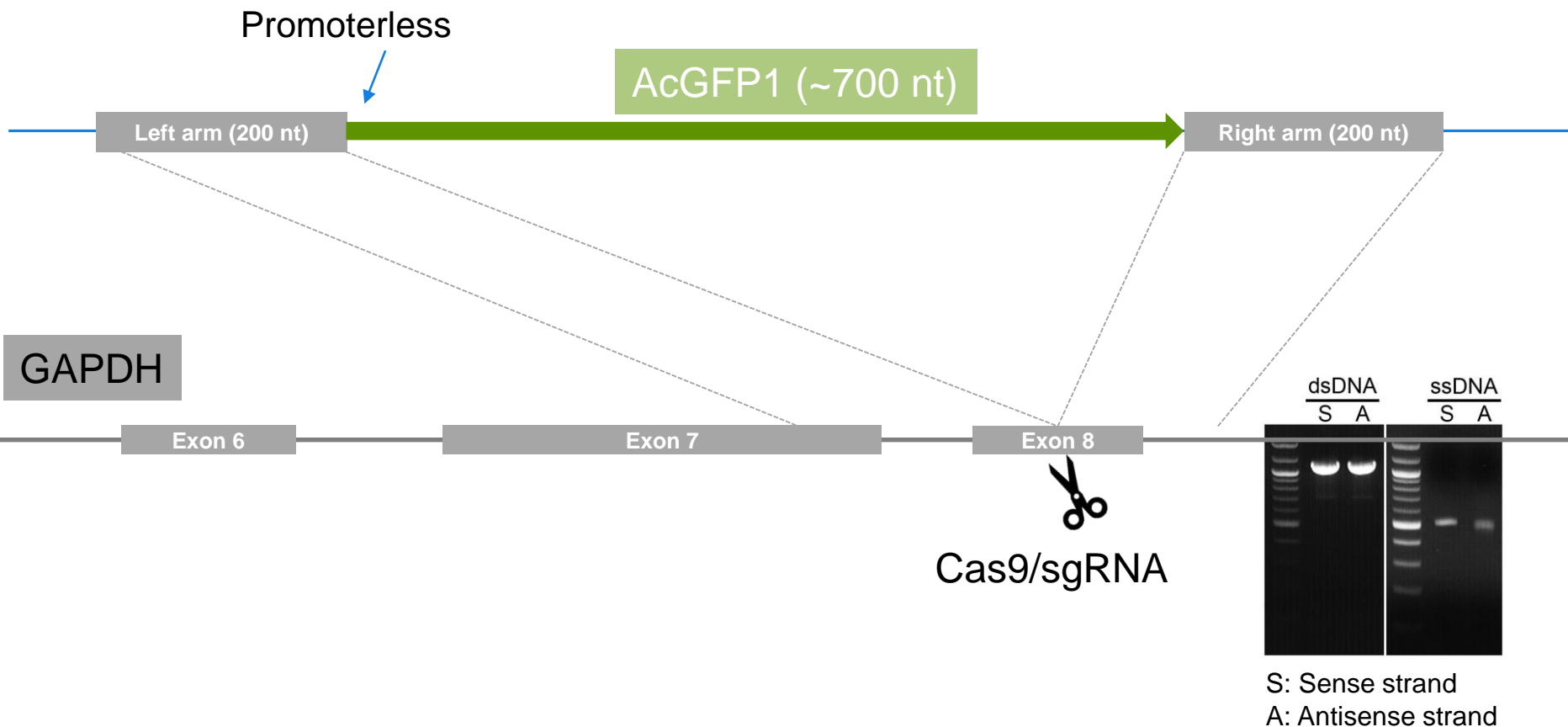
# Gene Editing Data

## 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

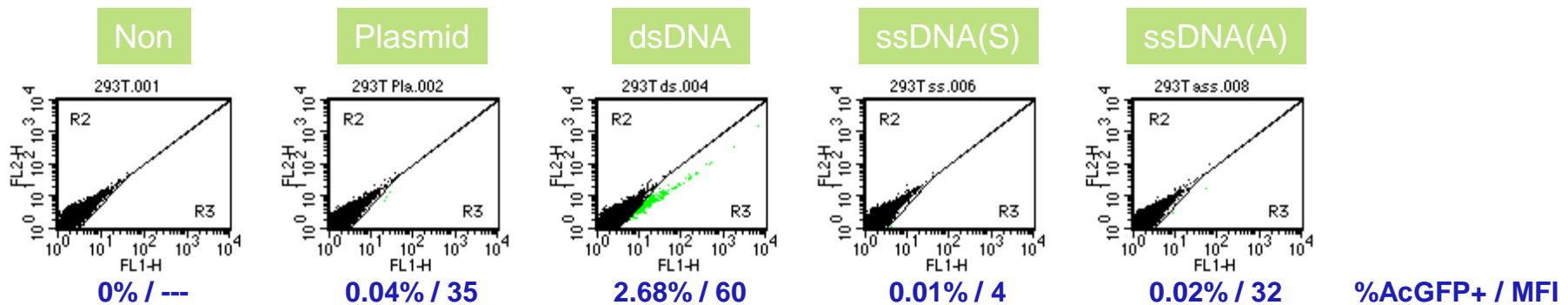
Knock-in of promoterless AcGFP1 at the C-terminus of GAPDH



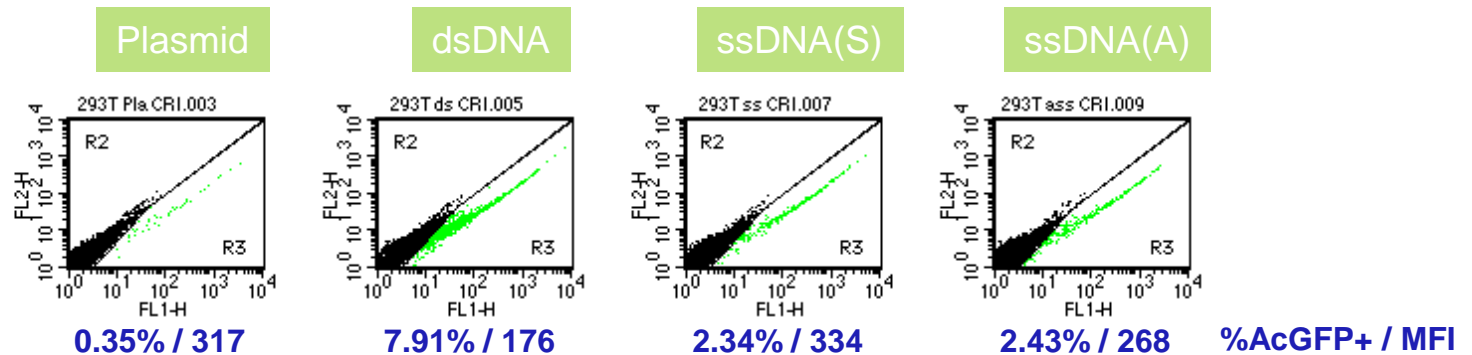
# dsDNA Integrates Randomly

## HEK293 (GAPDH/AcGFP1 donor)

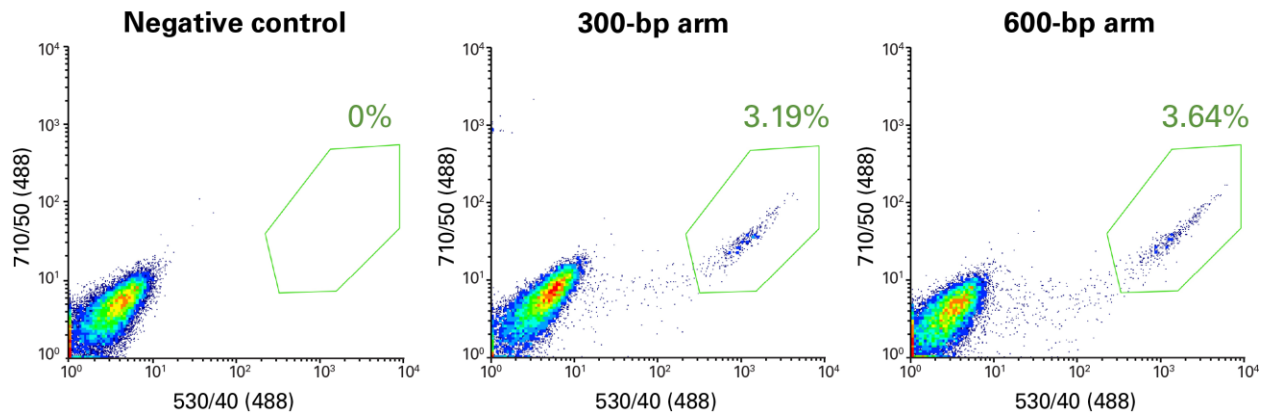
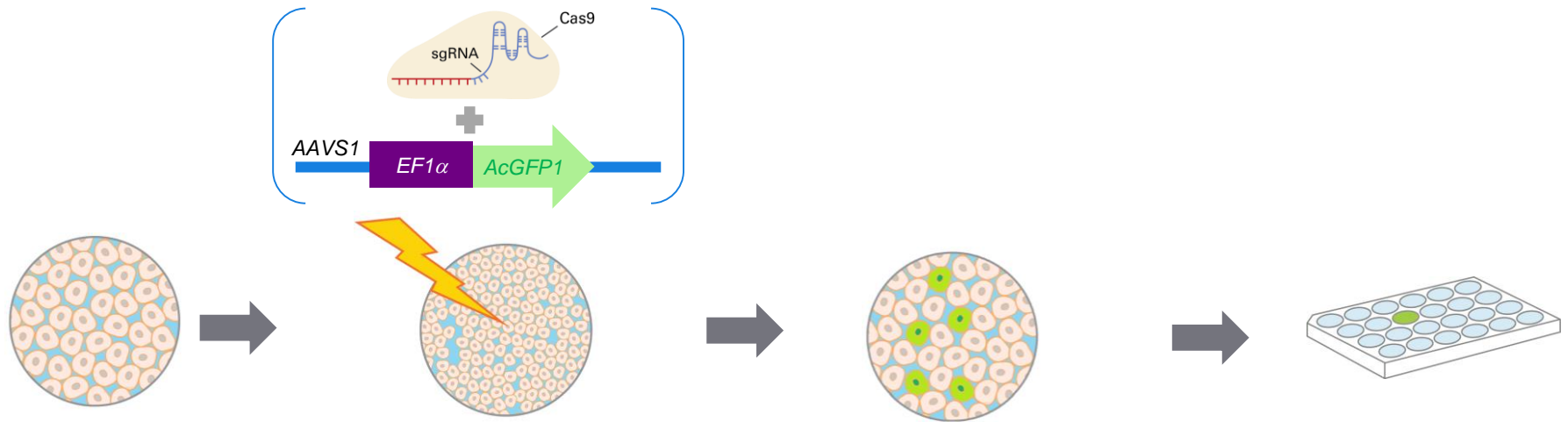
### Without Cas9/sgrNA



### With Cas9/sgrNA

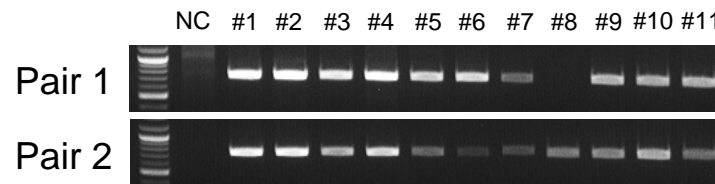
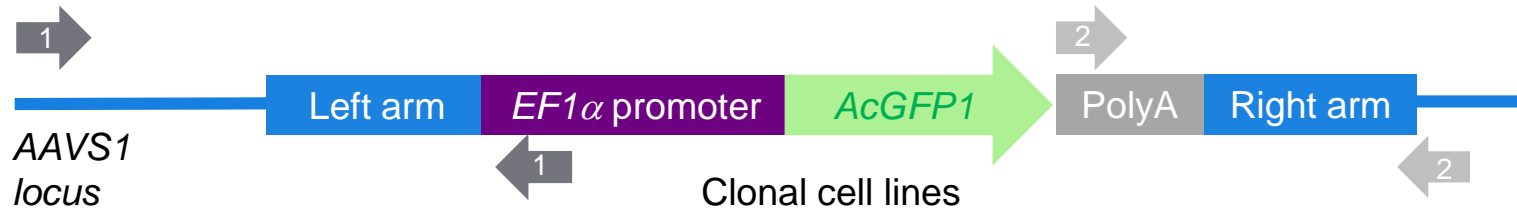


# Knock-in of EF1 $\alpha$ -AcGFP1 at AAVS1 Site of hiPS cells (Clone 18)



Isolated AcGFP1 positive single cells

# Isolated Clonal Lines Do Not Contain Mutations



Left arm

Right arm

ttccggagcacttccttctcggcgctgaccacgtagtgctctgagcggatcctcccgtgctctgggtcctctcgg  
 TTCCGGAGCAC TTCCTCTCGGCCTGCACCACGTGATGTCCTCTGAGCGGATCCTCCCCTGTCCTGGTCTCTCCGG  
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taggtgttcaccaggctggtggccgctctactcccttctcttctccatccttcttctcctaaagagtccccagtgcta  
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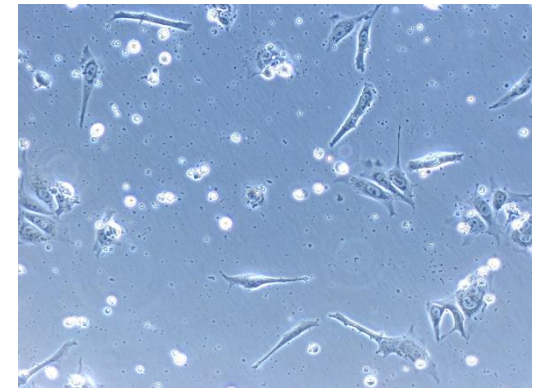
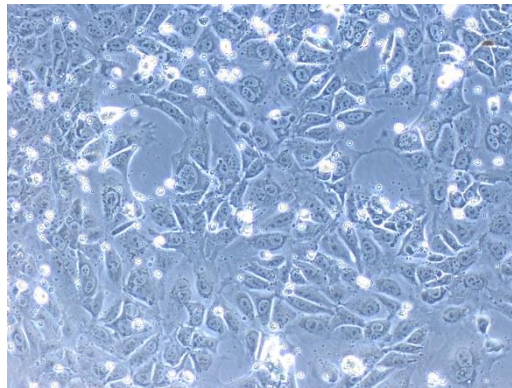
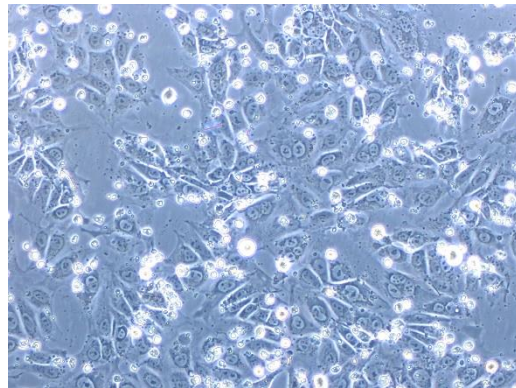
Clonal  
cell  
lines

# dsDNA-induced toxicity in hiPSC-18

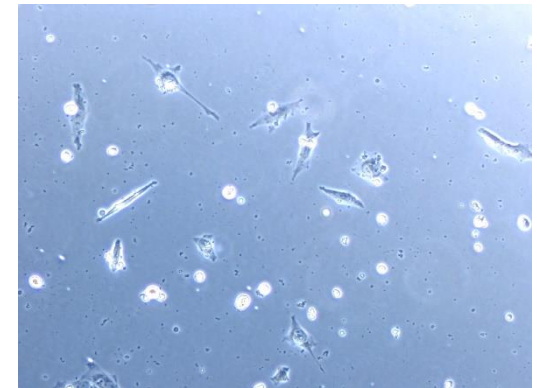
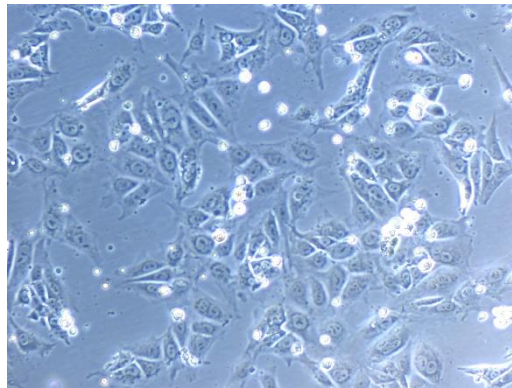
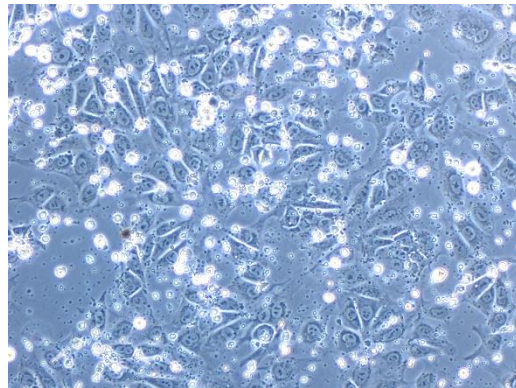
No donor template

ssDNA

dsDNA

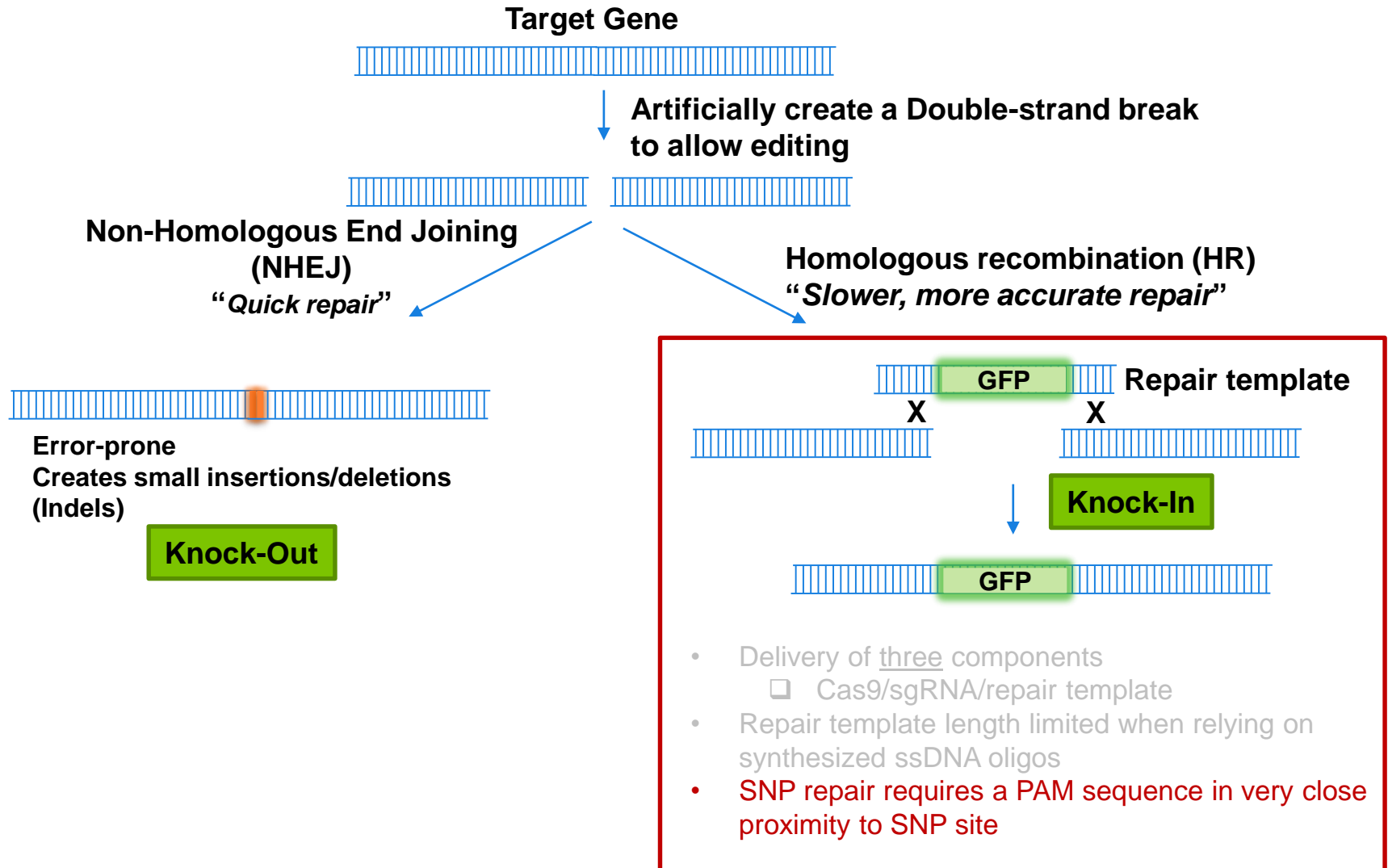


-Cas9  
+sgRNA



+Cas9  
+sgRNA

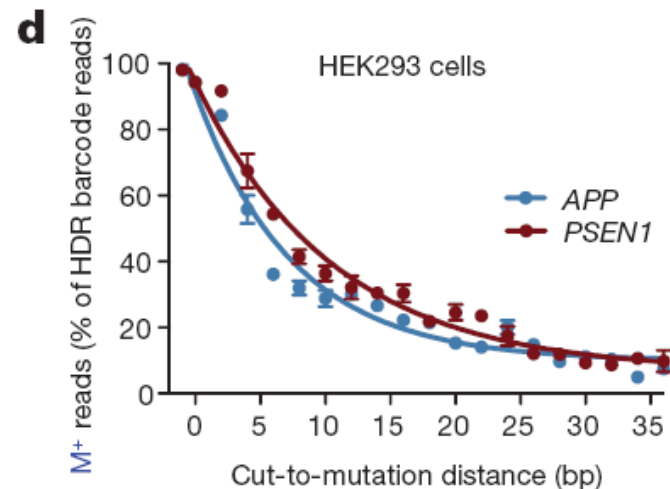
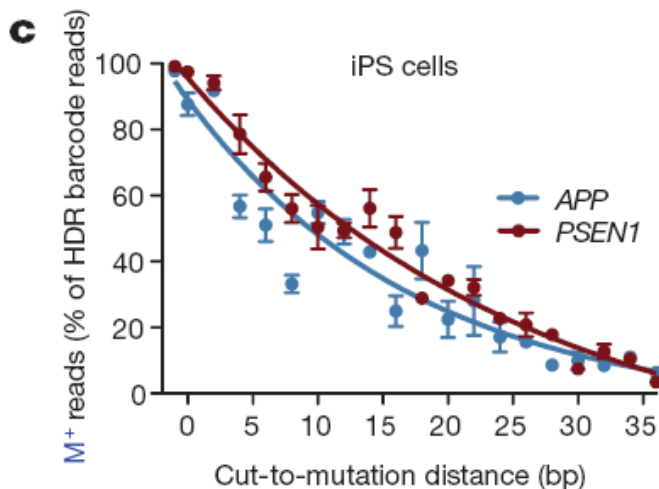
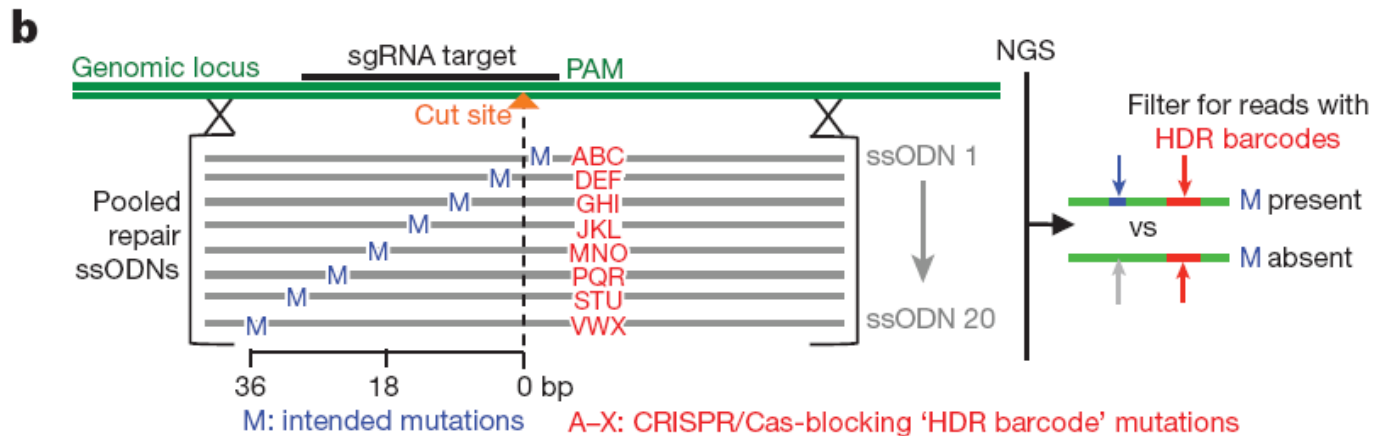
# Bottleneck: Homologous Recombination



# Why is SNP Editing/Screening Important?

- 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






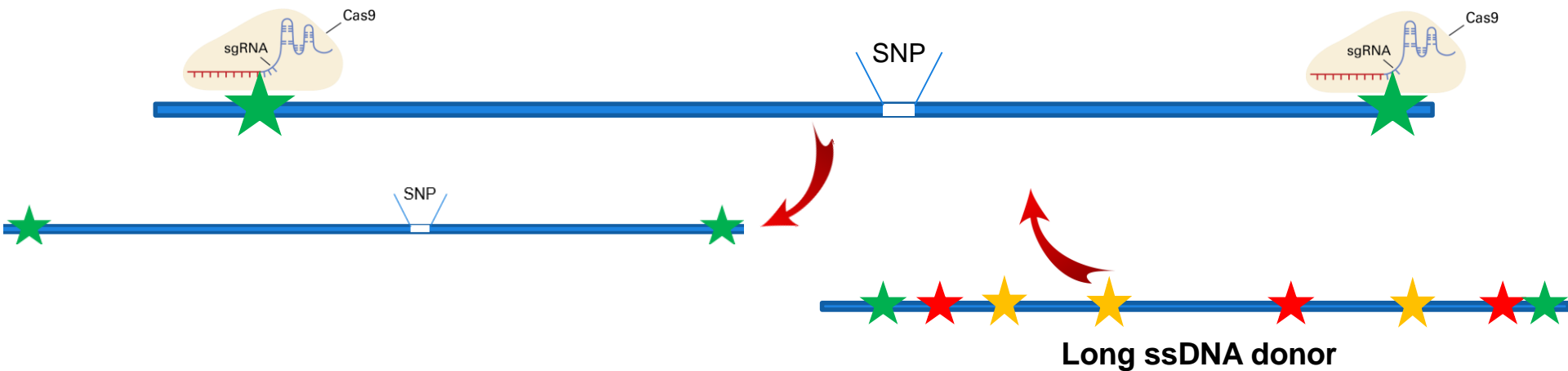
*Nature* 533, 125–129 (05 May 2016)

# Advantage of Using Long ssDNA for SNP Repair via Homologous Recombination

SNP allele



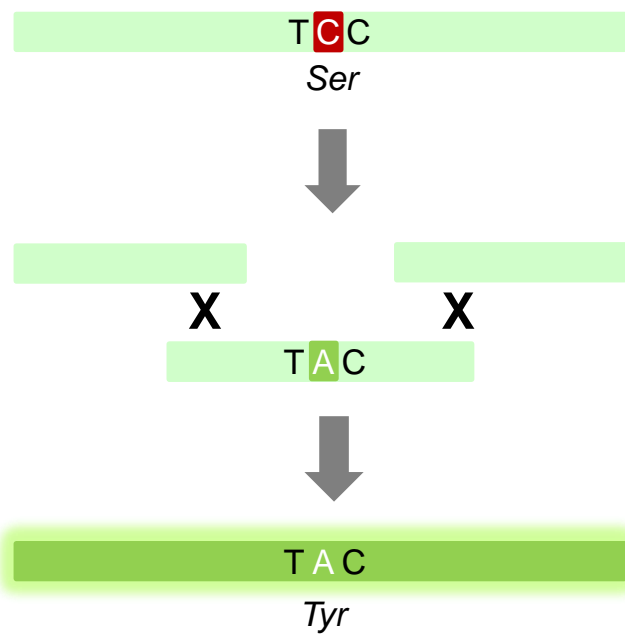
-  lousy CRISPR/Cas9 PAM/cut site
-  mediocre CRISPR/Cas9 PAM/cut site
-  great CRISPR/Cas9 PAM/cut site



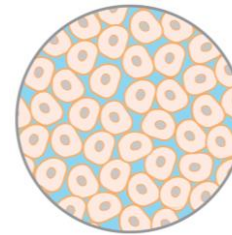
Repaired allele



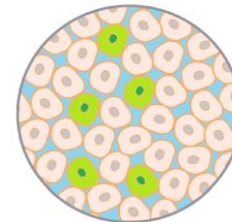
# Homology-Directed Knockin of Point Mutations in hiPSCs



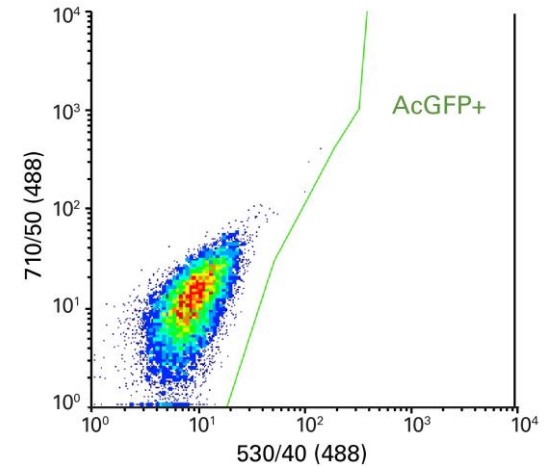
Non-fluorescent mutant AcGFP1



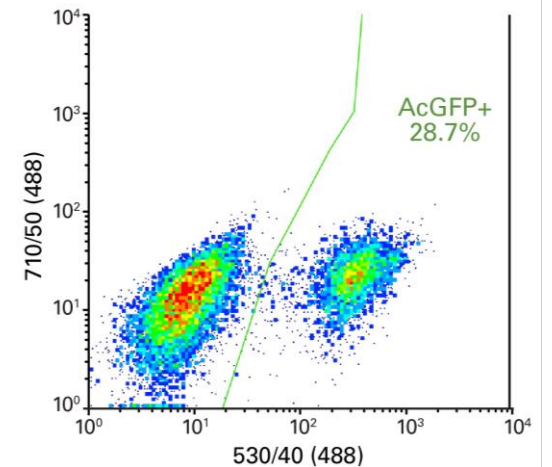
Fluorescent AcGFP1



Negative control

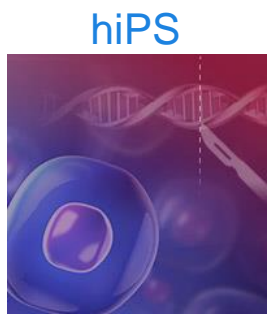


Cas9/sgRNA + 1 μg ssDNA oligo



# Cell Biology Portfolio Enabling Gene Function Analysis

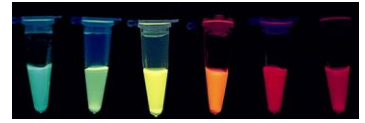
Cellartis:  
-Products  
-Service business



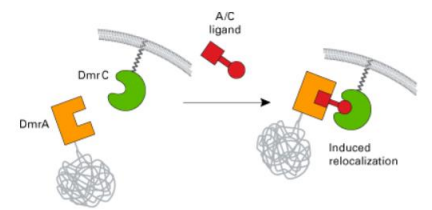
Genome editing



Fluorescent proteins and reporters

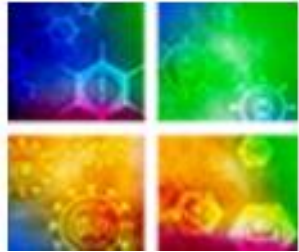


Ligand induced protein interaction

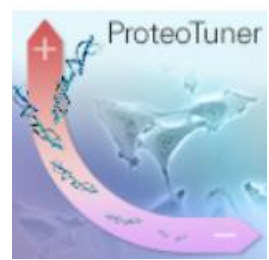


**Gene Function**

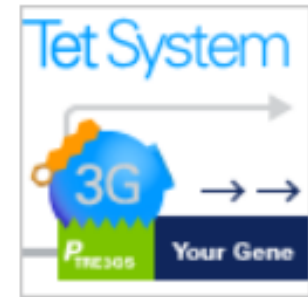
Viral delivery



Ligand induced protein stabilization

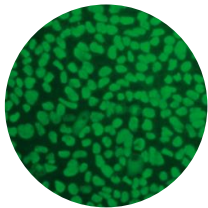


Inducible gene expression systems



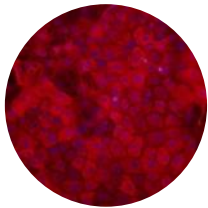
# Stem Cell Research

## Stem cell innovations for today and the future



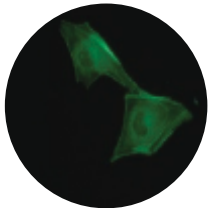
### Cell Culture

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



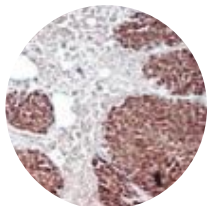
### ES/iPS-derived Cells

- Hepatocytes & cardiomyocytes
- DE cells
- Beta cells
- Neural progenitors



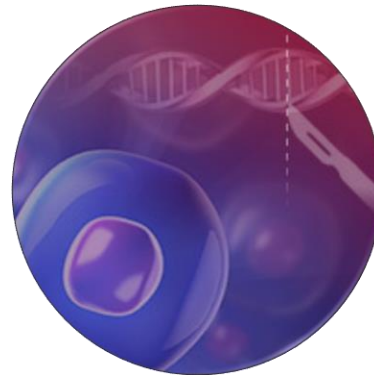
### Stem Cell Services

- 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



that's  
**GOOD**  
science!®

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Clontech **TAKARA** cellartis