TaKaRa

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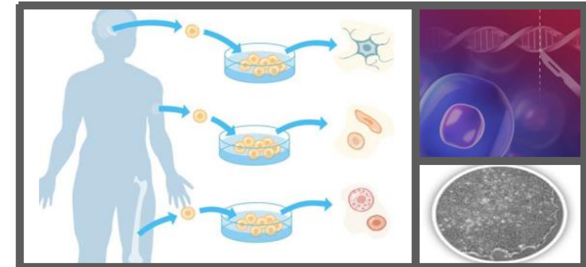
Innovative CRISPR/Cas9 gene knockin and SNP-detection tools:

Establishing human iPS-derived disease model lines for drug screening



Elizabeth Quinn, PhD

Society for Laboratory Automation and Screening (SLAS) 2018 conference

that's GOOD science!®

Outline

- Introduction to genome editing and hiPSCs
- Knocking out genes in hiPSCs
  - Key considerations
  - Test case: generation of clonal cell lines with KO in *CD81*
- Knocking in genes in hiPSCs
  - Key considerations
  - Knockin of point mutations using ssDNA oligos (<200 bp)
  - Knockin of longer sequences (>200 bp) with ssDNA repair template

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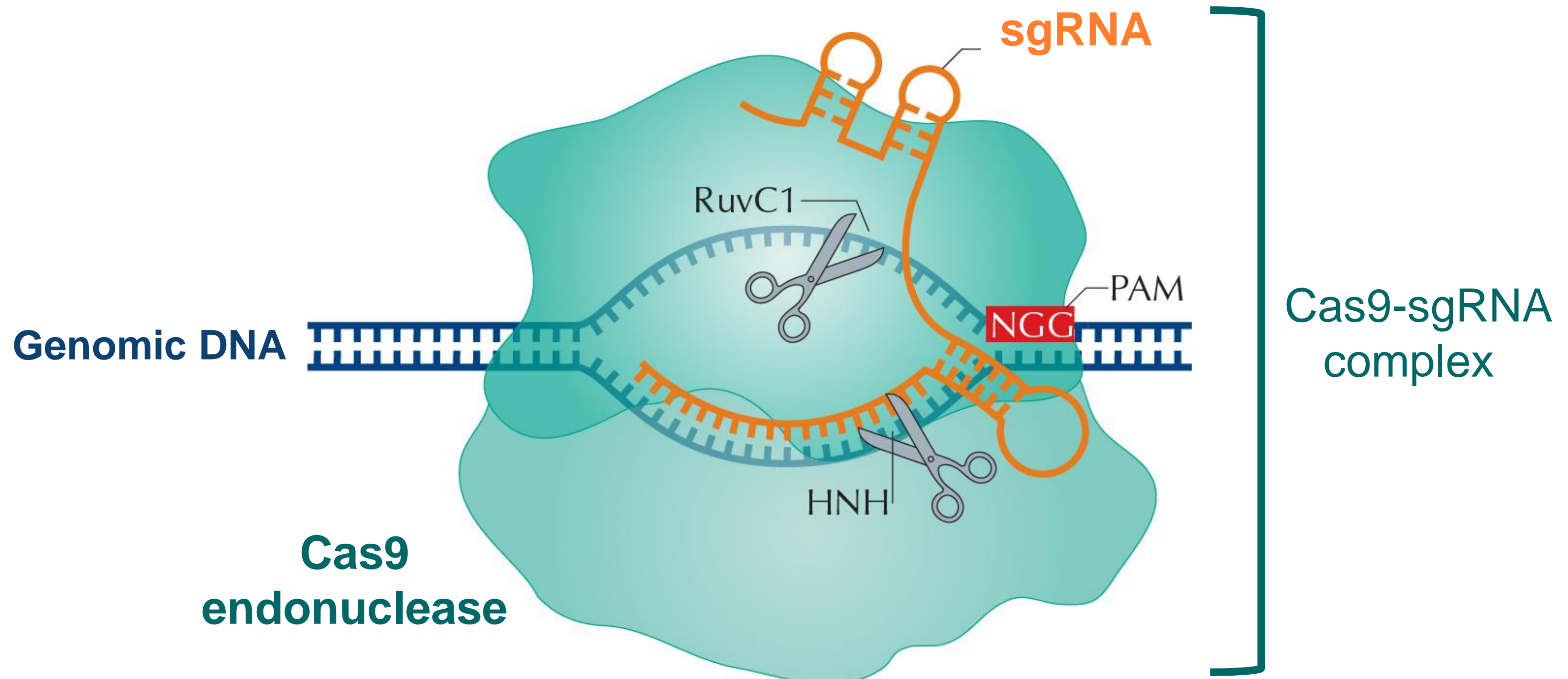
Genome editing technologies

CRISPR/Cas9: bacterial mechanism of self-defense repurposed as an editing tool



DNA target site recognition + DNA cleavage → Genome editing tool

sgRNA — Cas9 protein

Dominguez *et al. Nat Rev Mol Cell Biol*. **17**, 5–15 (2016).

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Human iPSCs meet genome editing

Application of CRISPR/Cas9 in hiPSC-based disease modeling

Reprogramming factors

Human iPSCs

Reprogramming somatic cells

Creating isogenic iPSC controls

Patient

Differentiating into desired cell types

Personalized medicine

Neural cells

Cardiomyocytes

Islet cells

Haematopoietic cells

Identifying molecular targets

Characterizing disease phenotypes

Shi *et al. Nat Reviews Drug Discovery* **16**, 115–130 (2017).

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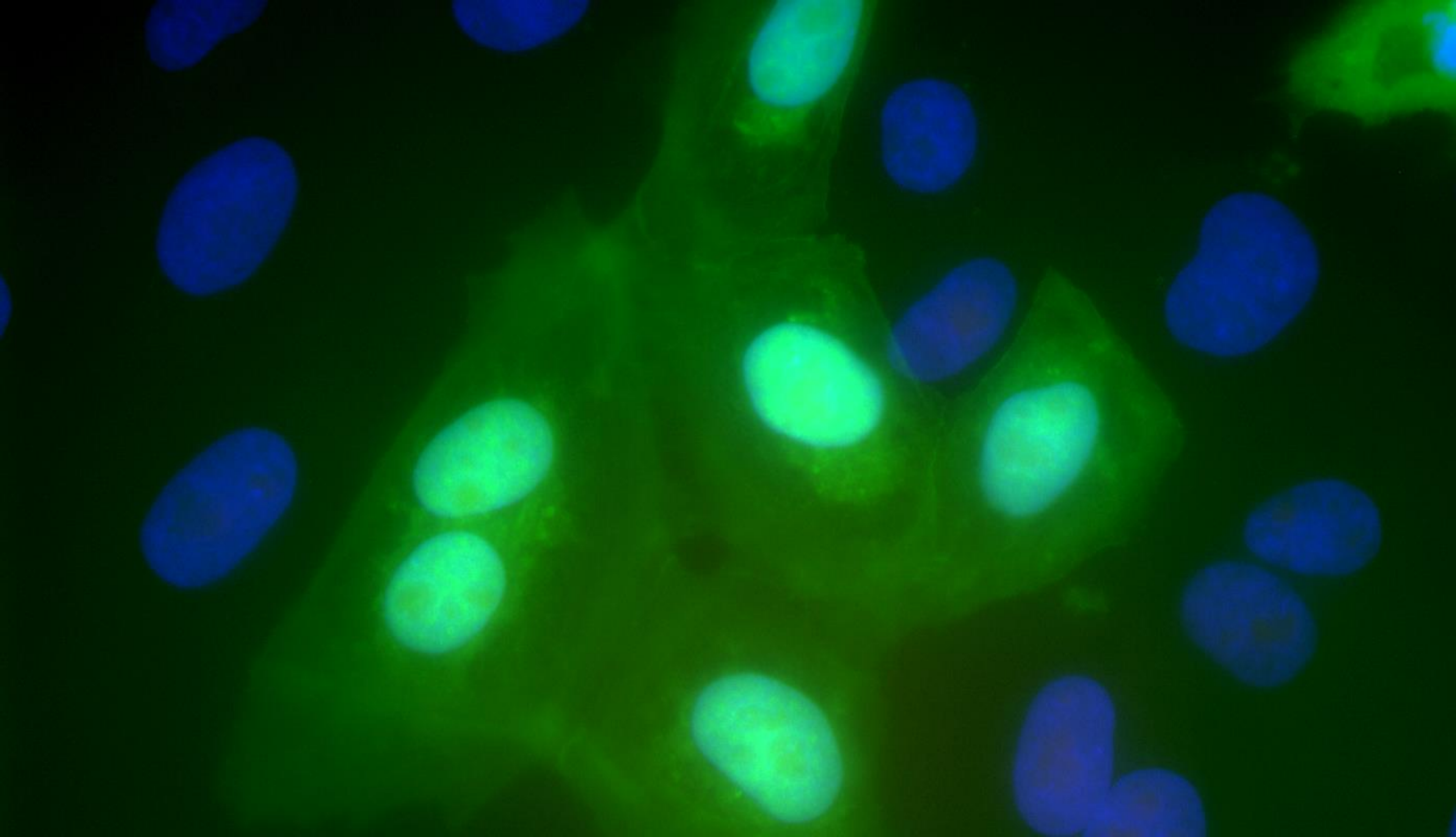

Knockout

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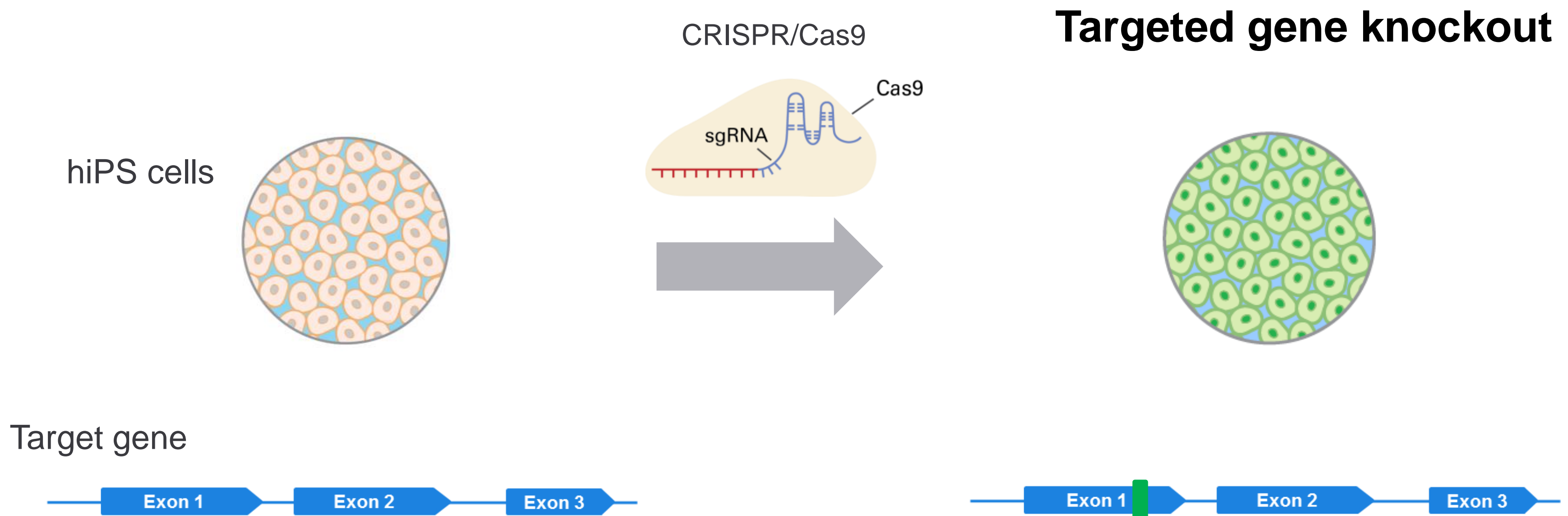
Knocking out genes in hiPSCs



Experimental design is essential in order to maximize success.

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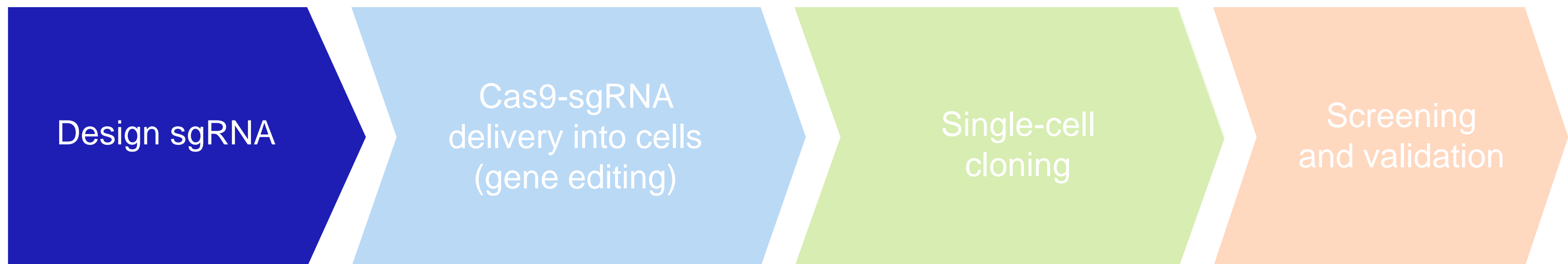
Knocking out genes in hiPSCs

Key considerations



- Take into account different protein isoforms and alternative start codons
- Design sgRNAs targeting key exons or essential functional domains
- Use online tools to choose sgRNAs with predicted low off-target effects (http://chopchop.cbu.uib.no/index.php, https://www.deskgen.com/landing/)
- Check sgRNAs' activity *in vitro* (Guide-it™ sgRNA Screening Kit)
- Use optimized sgRNA scaffold

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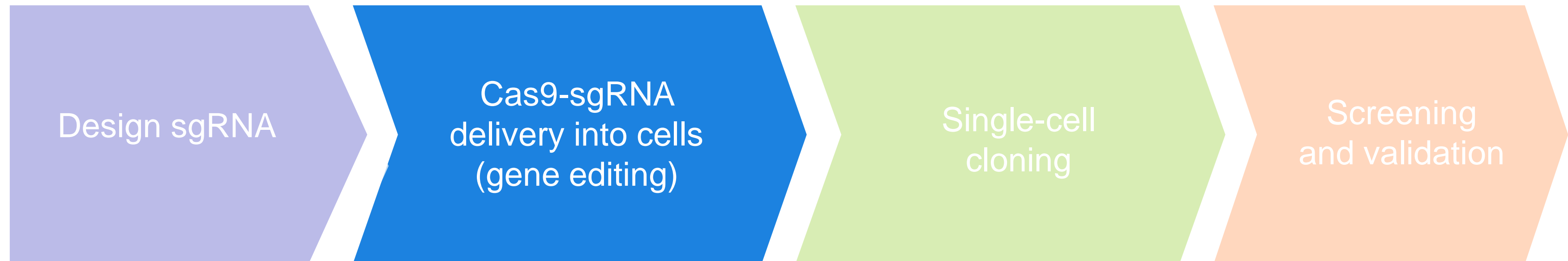
Knocking out genes in hiPSCs
Key considerations



- Minimize cell toxicity due to the delivery of the Cas9-sgRNA
- Delivery in the form of CRISPR/Cas9 ribonucleoprotein complex (RNP)
  - Lowest toxicity in cells
  - No need for cellular transcription/translation machinery
  - Relative transience: lower off-target effects
  - No integration events: footprint-free genome editing

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Guide-it rCas9 Electroporation Ready

Recombinant Cas9 purified from *E. coli* and ready for gene editing experiments using electroporation

- Sterile
- Contains one C-terminus Nuclear Localization Signal (NLS) protein
- Low glycerol content for higher electroporation efficiency/reduced toxicity
- Consistently effective when combined with Guide-it *In Vitro* sgRNA Transcription Kit
- Mix rCas9 and sgRNA, incubate for 5 minutes, and then use!

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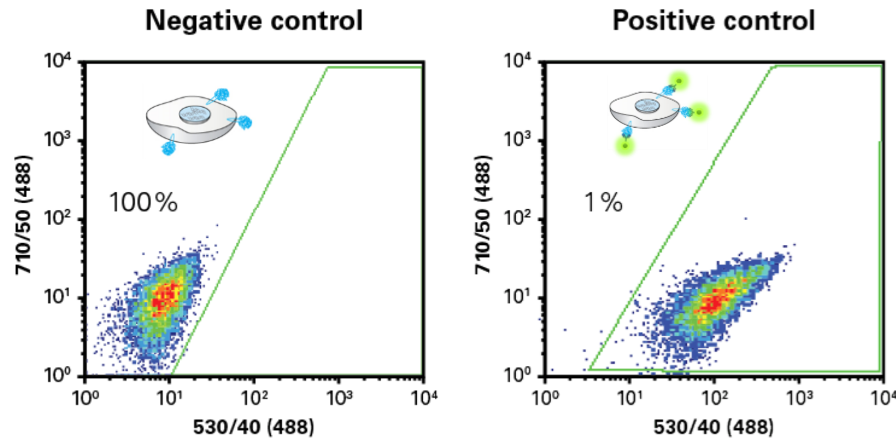
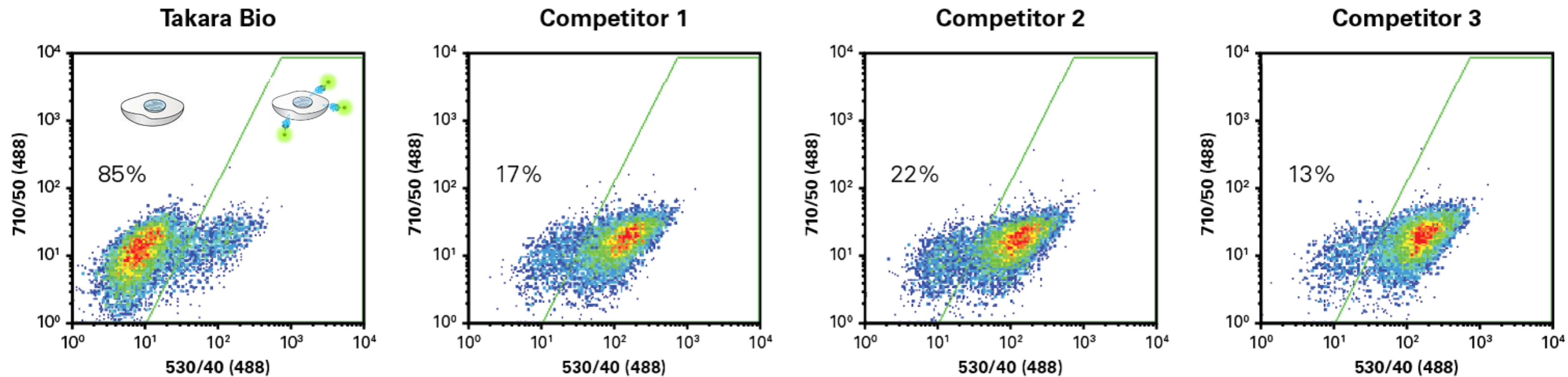
Guide-it rCas9 Electroporation Ready

KO of CD81 protein in hiPSCs





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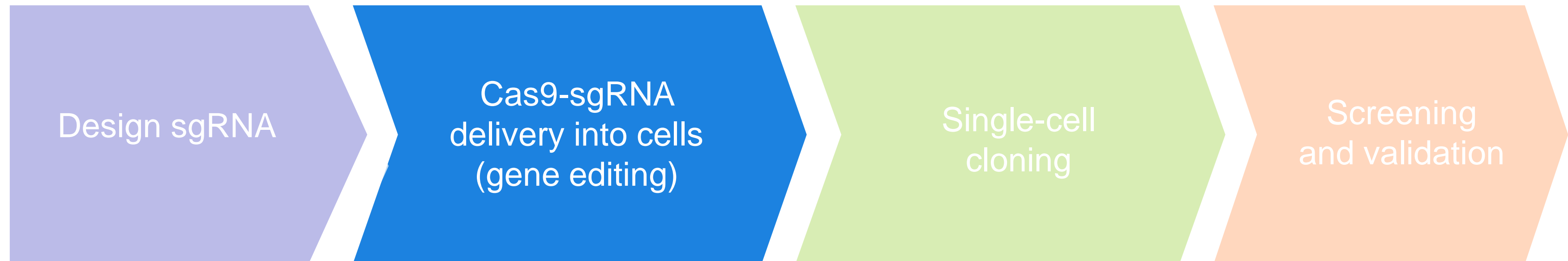
Knocking out genes in hiPSCs

Key considerations



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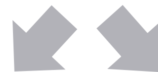

Electroporation   Gesicles

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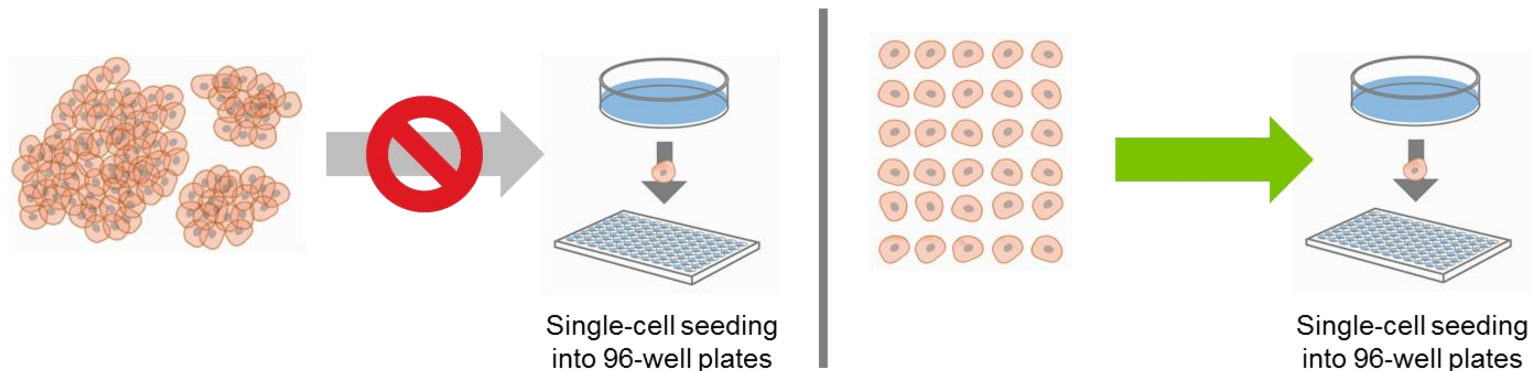
Knocking out genes in hiPSCs

Key considerations

- Isolating and clonally expanding edited cells
  - Single pluripotent cells die or differentiate when seeded alone
  - Need for single-cell culture of pluripotent stem cells



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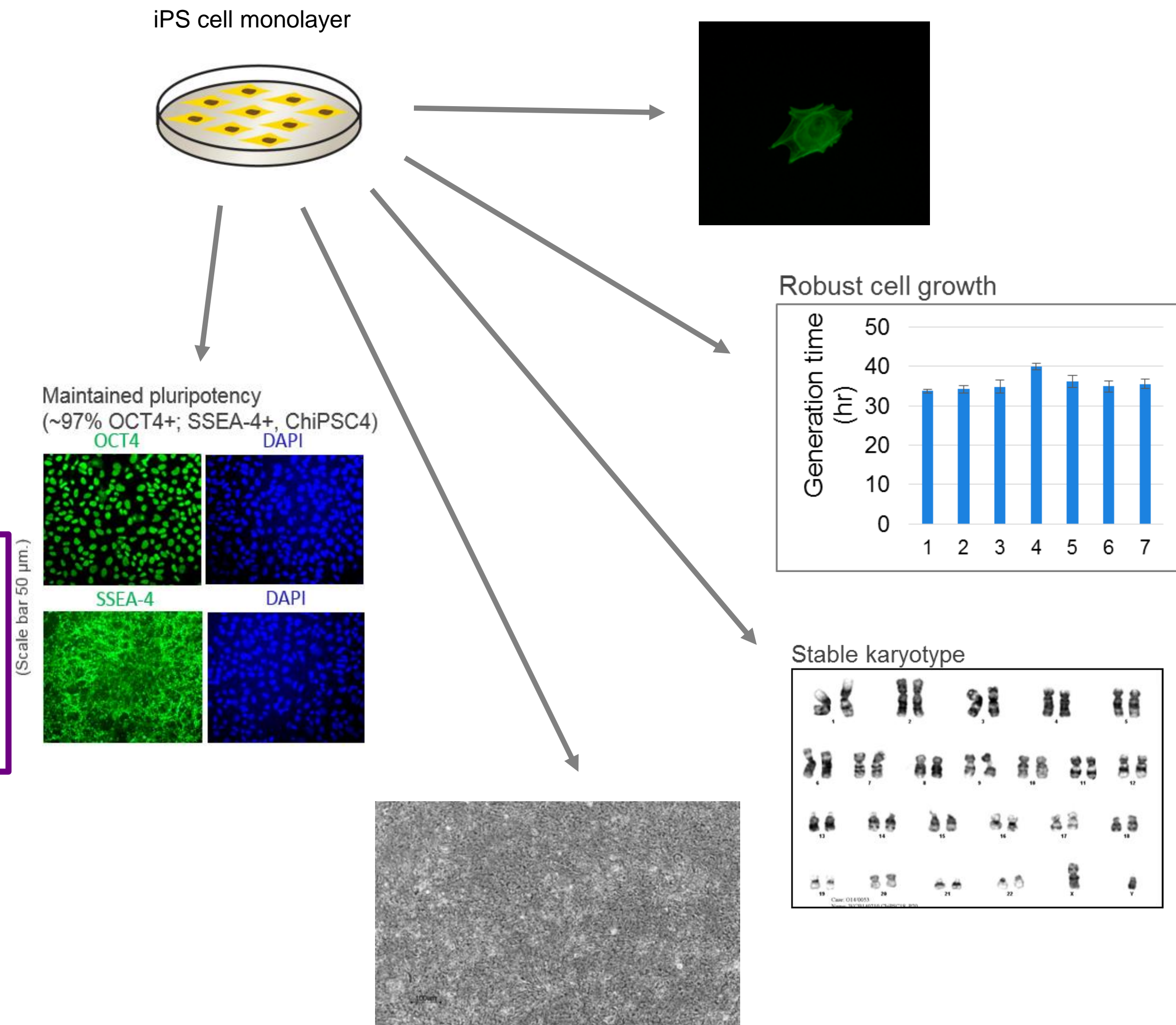
Clontech TaKaRa cellartis

Single-cell cloning of hiPSCs with the Cellartis® DEF-CS™ culture system

- 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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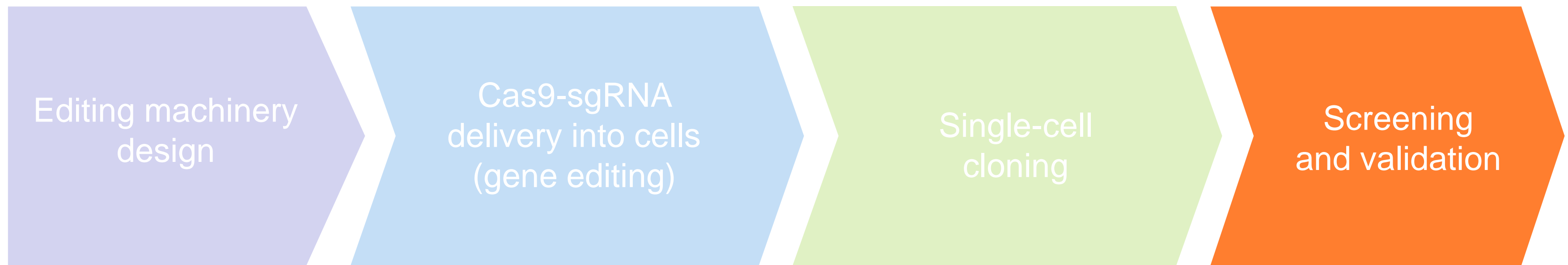
Knocking out genes in hiPSCs
Key considerations



- Check pluripotency
- Check karyotype
- Characterization of the indels (Guide-it Indel Identification Kit)
- Check for nonexpression of your transcript by RT-PCR

Sharpe *et al. Genome Biology* **18,** 109–113 (2017).

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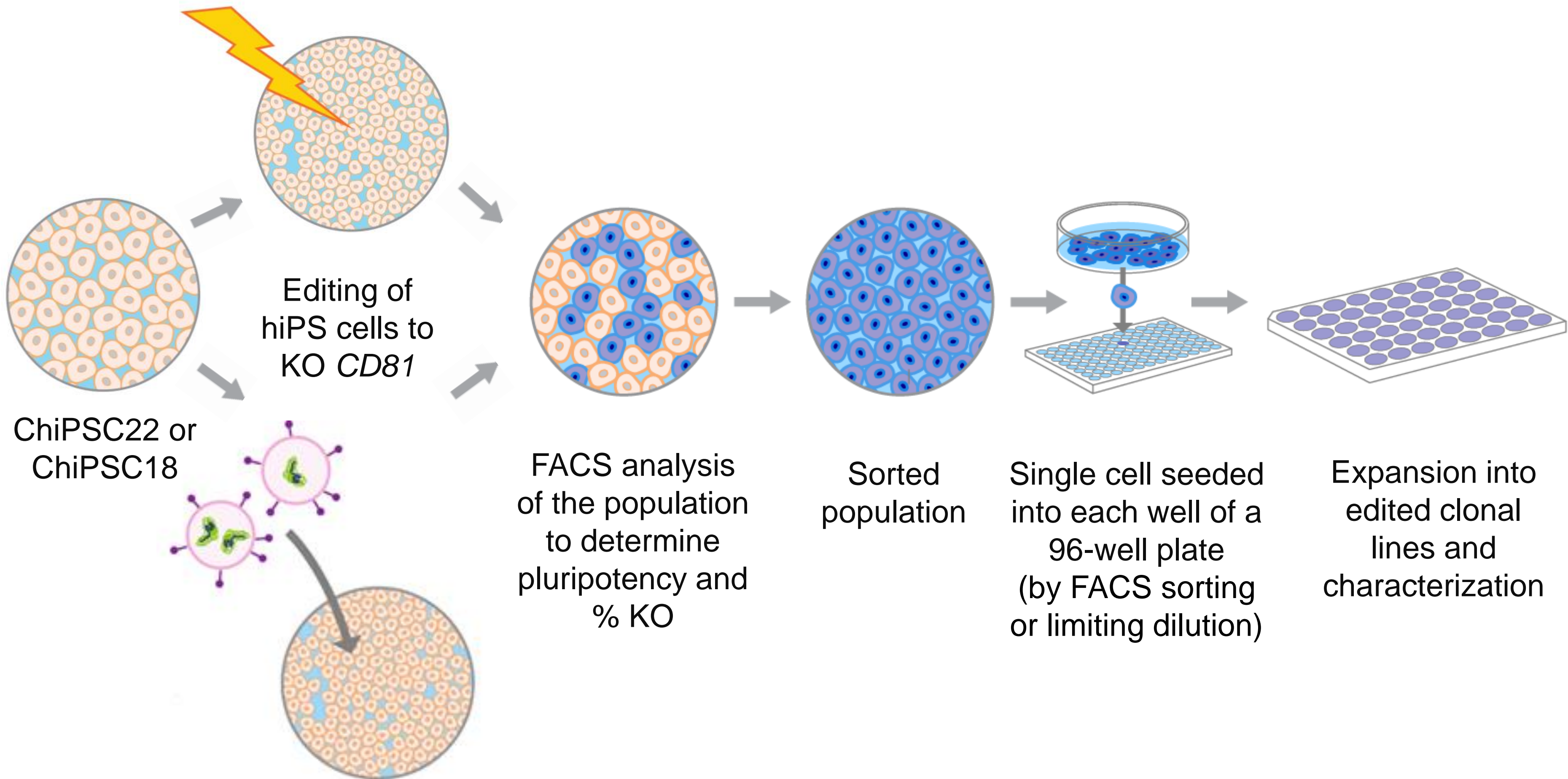
Test case: knocking out *CD81*

Workflow



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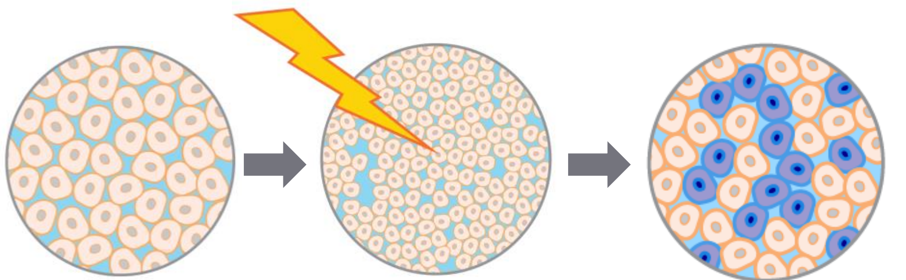
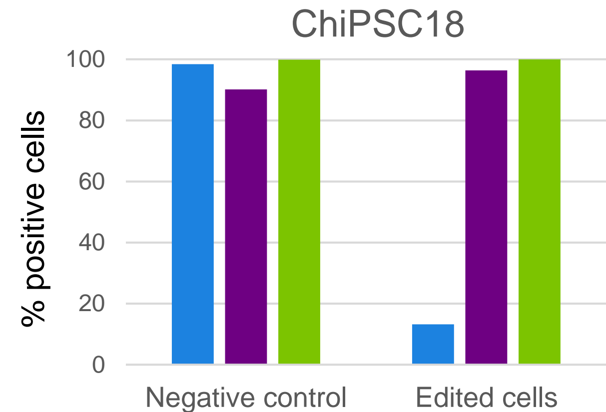
Pluripotency maintained after *CD81* KO

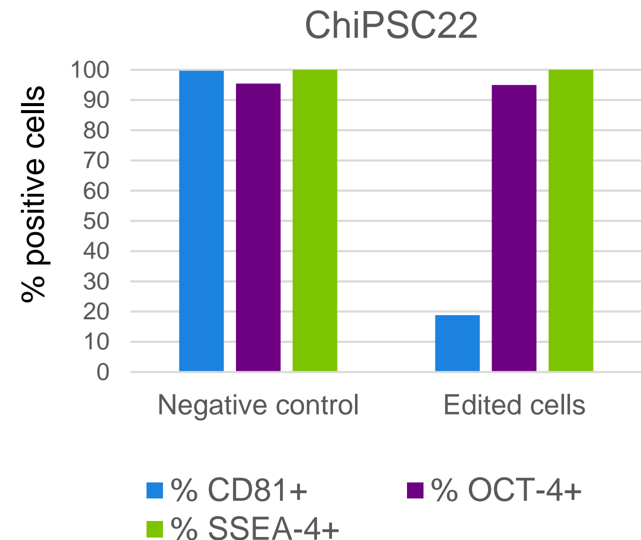


ChiPSC22 or ChiPSC18

Editing of hiPS cells to KO *CD81*

FACS analysis of the population to determine pluripotency and % KO





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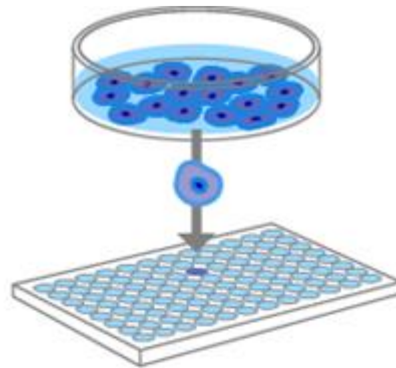
Cloning of edited hiPSCs

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 (%)
ChiPSC18	FACS	52	0	52/96	54%
ChiPSC18	Limiting dilution	46	12	58/55	105%*

*Percent expected versus total theoretical clones

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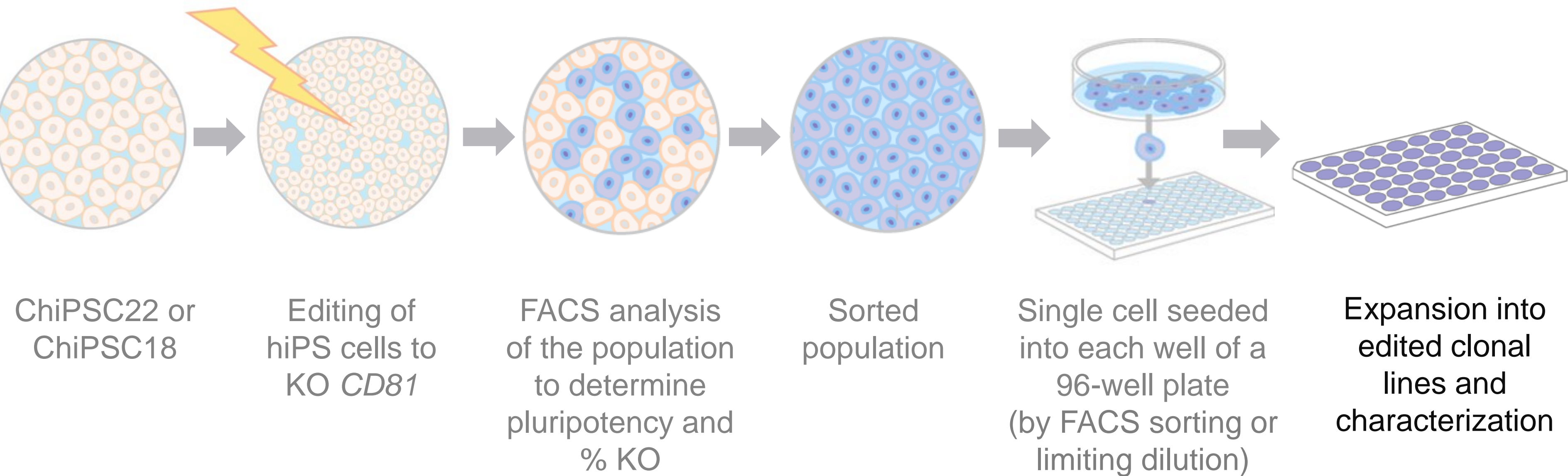
Expansion of edited clonal lines



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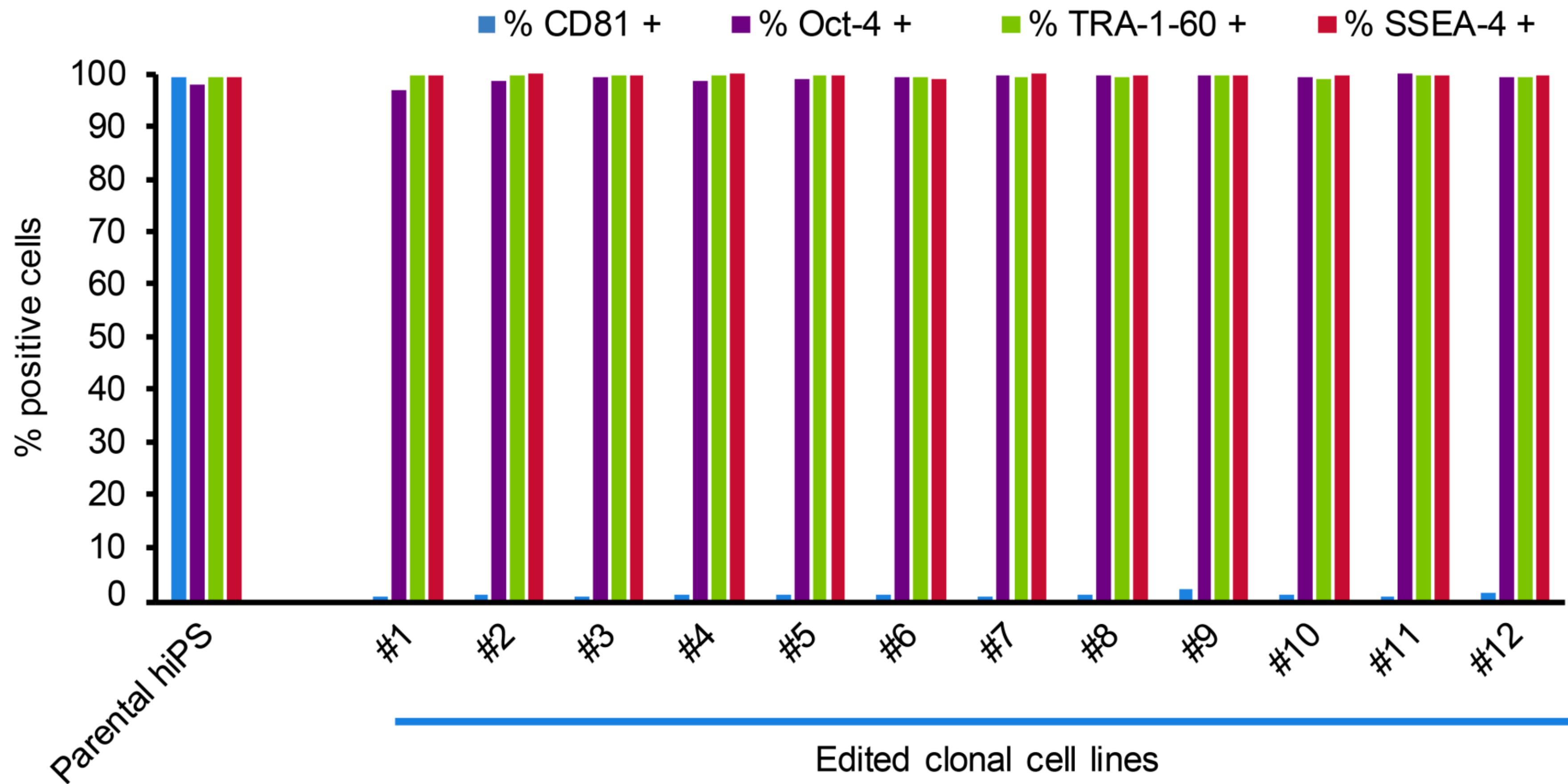
Edited, pluripotent single-cell clones

The DEF-CS culture system maintains stemness



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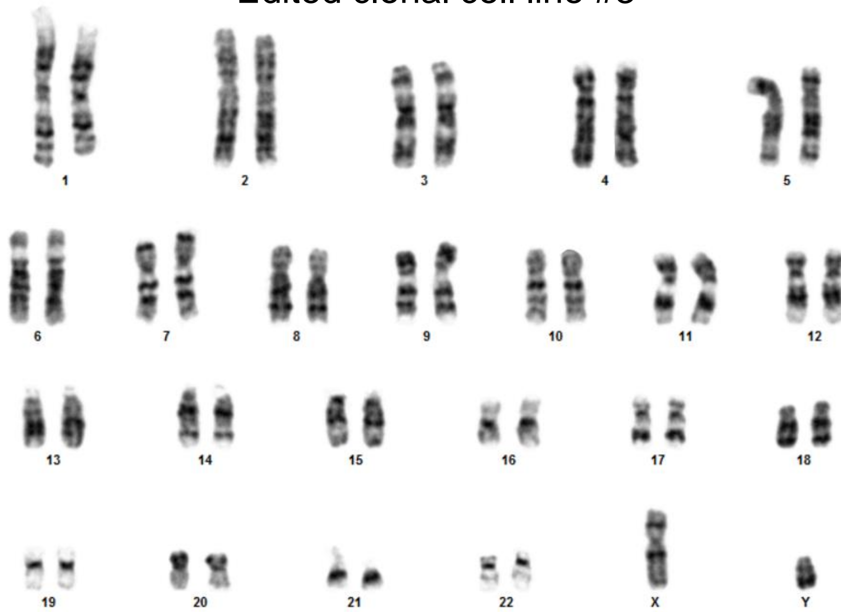
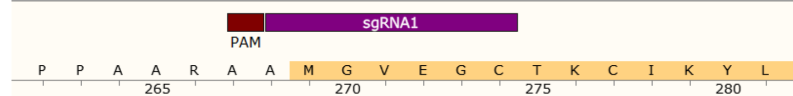
Edited, pluripotent single-cell clones

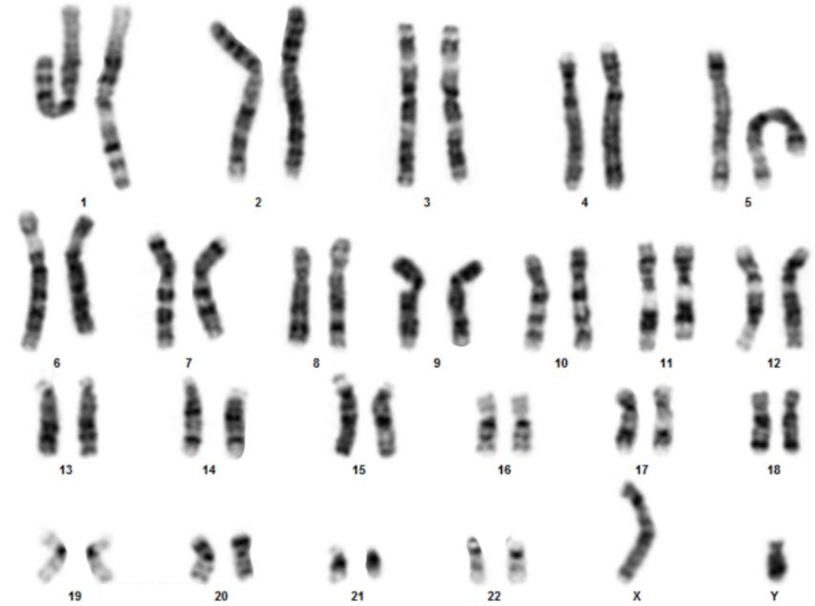
Karyotype analysis

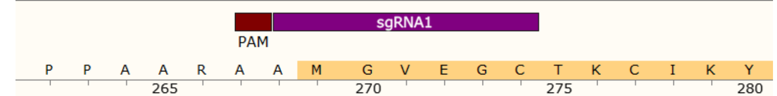
Edited clonal cell line #3





Edited clonal cell line #5





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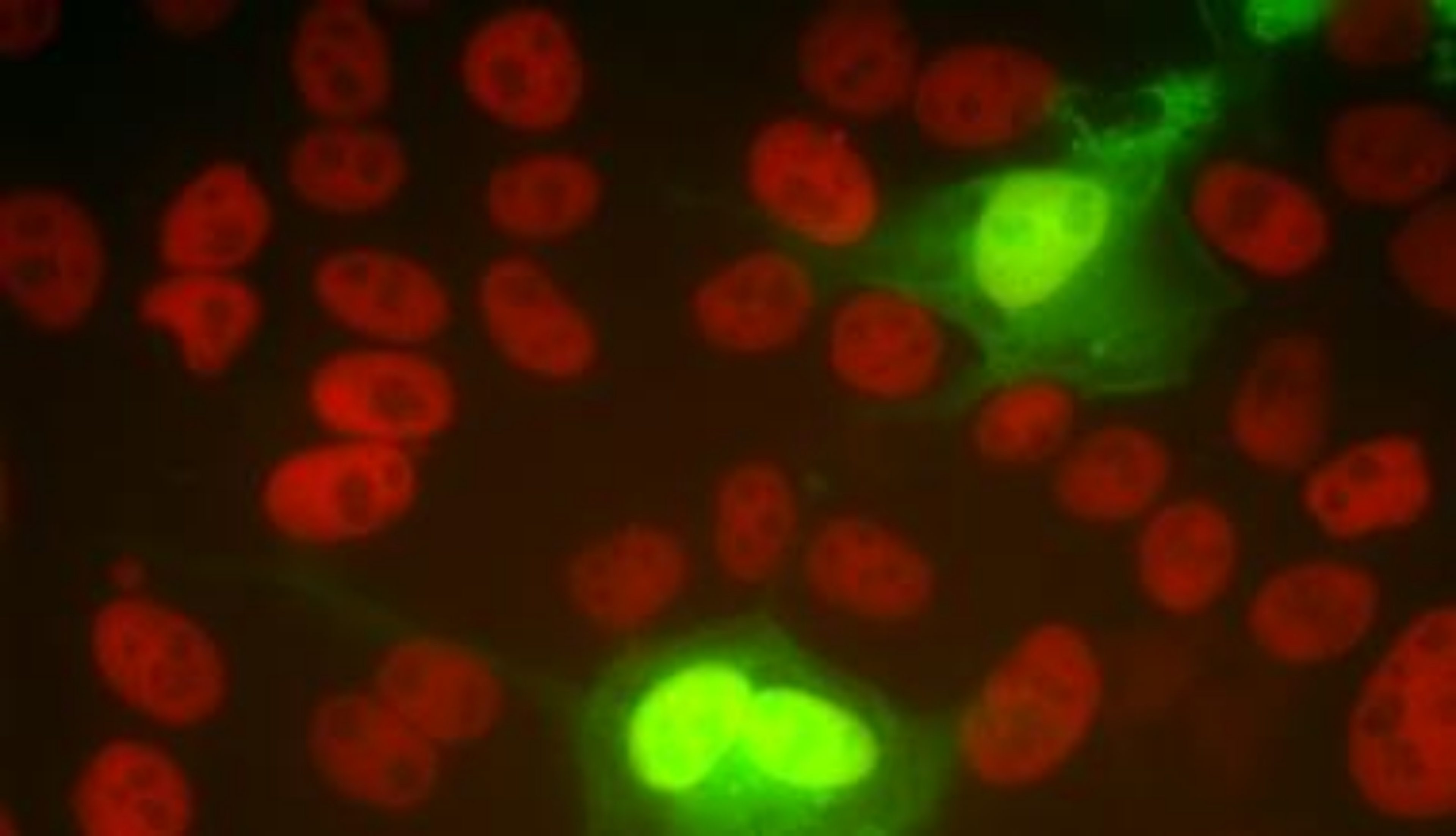
Clontech TaKaRa cellartis



Knockin

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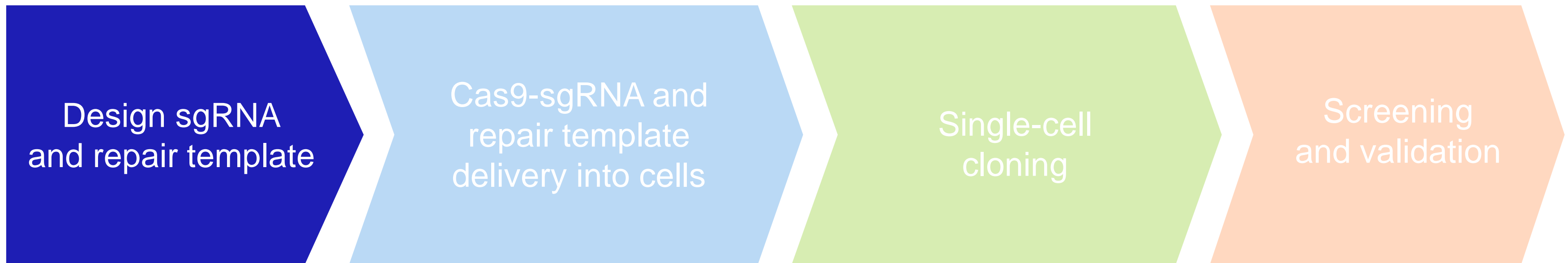
Knocking in genes in hiPSCs

Key considerations



- Check the sequence of the homologous recombination arms
- Pick sgRNAs as close as possible to the modification

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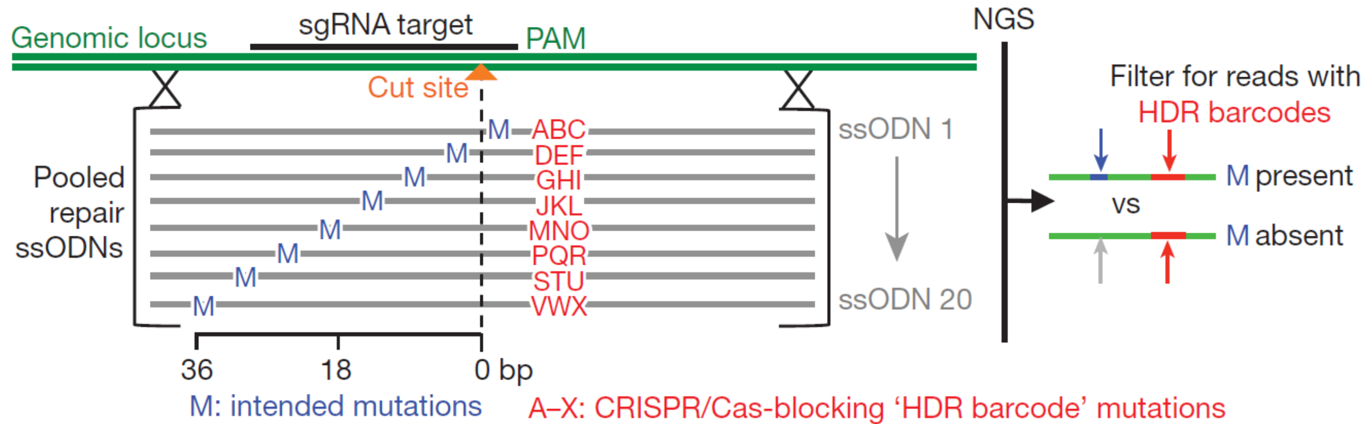
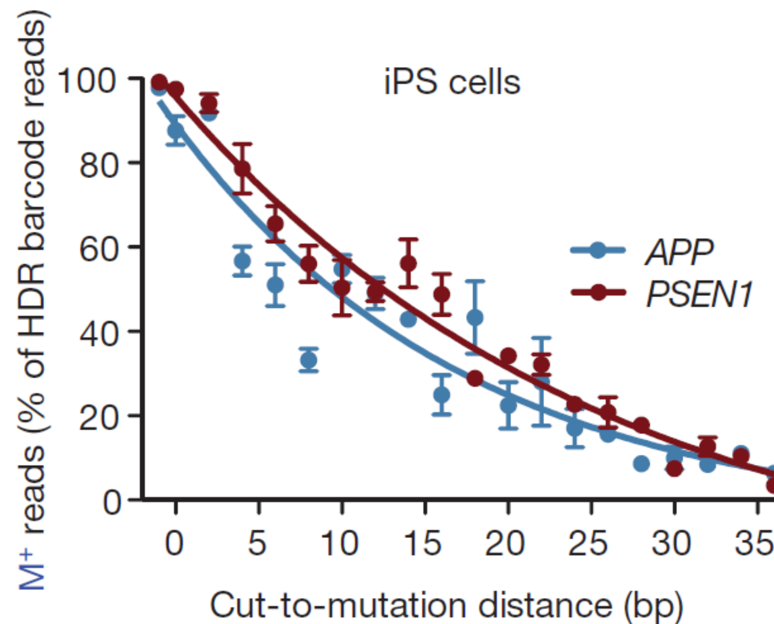
Knocking in genes in hiPSCs

Efficiency of SNP repair relies on close proximity to PAM site





Paquet *et al.* *Nature* **533**, 125–129 (2016).

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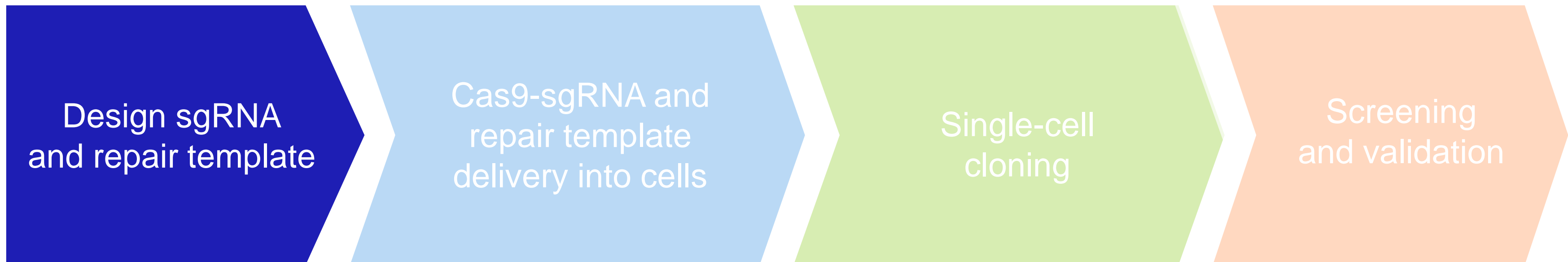
Knocking in genes in hiPSCs

Key considerations



- Check the sequence of the homologous recombination arms
- Pick sgRNAs as close as possible to the DSB
- Use single-stranded donor templates
  - No background expression when delivering expression cassettes (e.g. CMV→GFP)
  - Lower rate of random integration than dsDNA

Chen *et al. Nature Methods* ***8***, 753-755 (2011)

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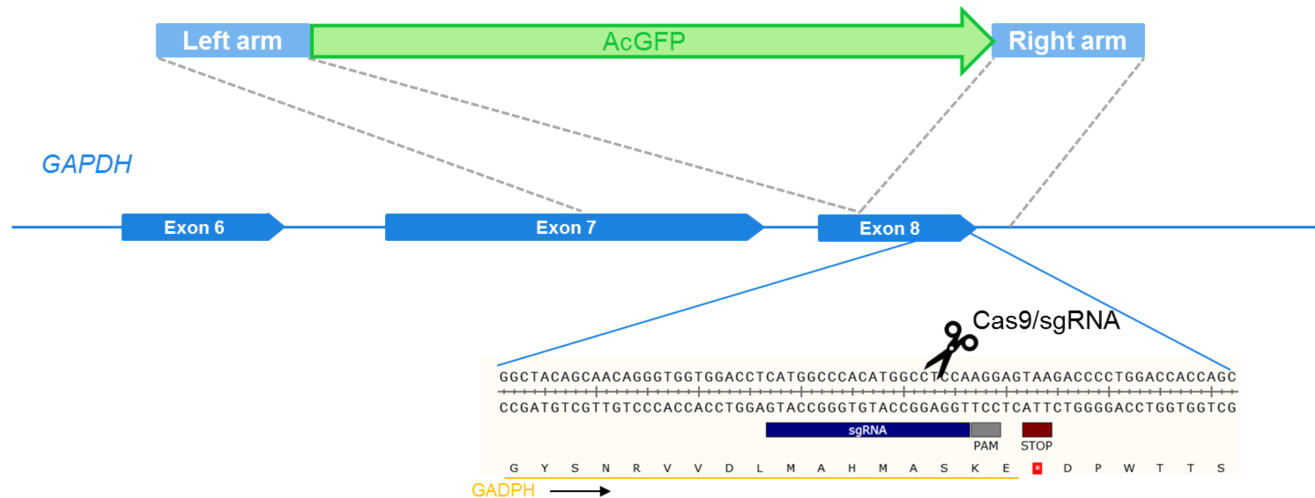
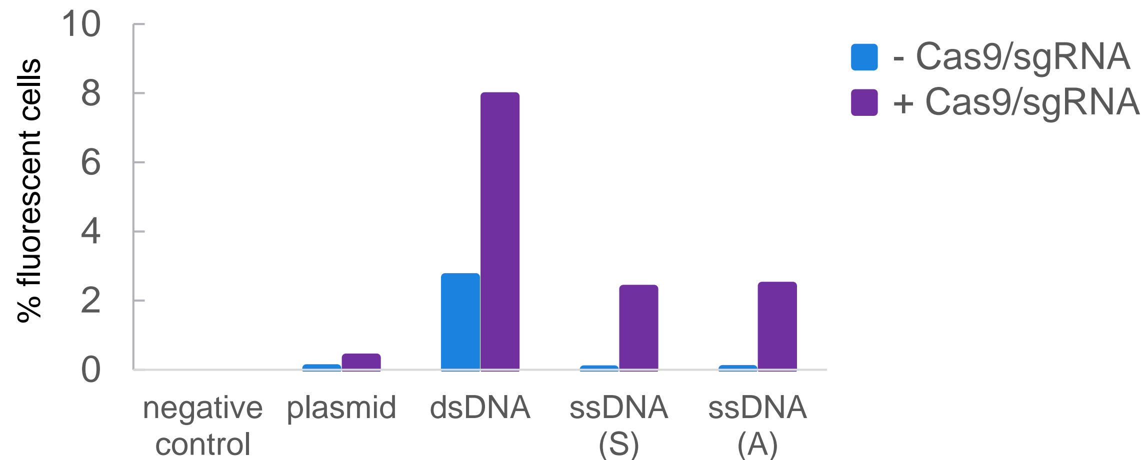
Knocking in genes in hiPSCs

Random integration of dsDNA donors: GAPDH-AcGFP fusion in HEK293





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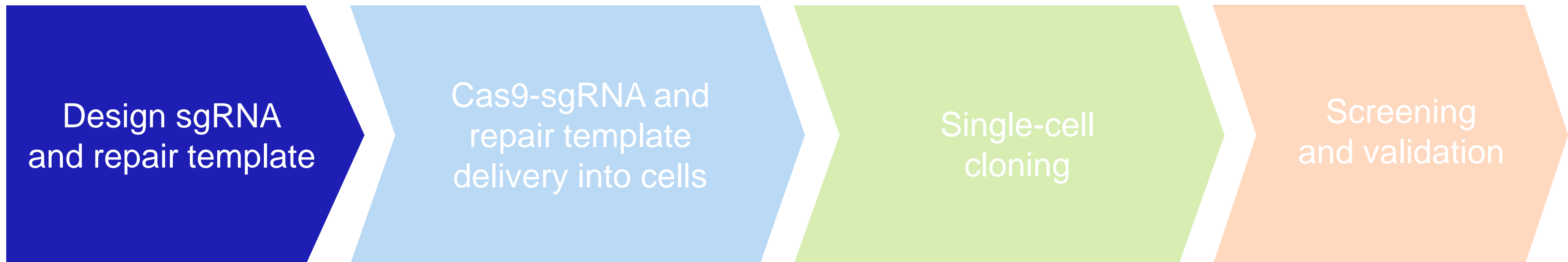
Knocking in genes in hiPSCs
Key considerations



- Check the sequence of the homologous recombination arms
- Pick sgRNAs as close as possible to the DSB
- Use single-stranded donor templates
  - No background expression when delivering expression cassettes (e.g. CMV→GFP)
  - Lower rate of random integration than dsDNA
  - Lower toxicity than dsDNA donors

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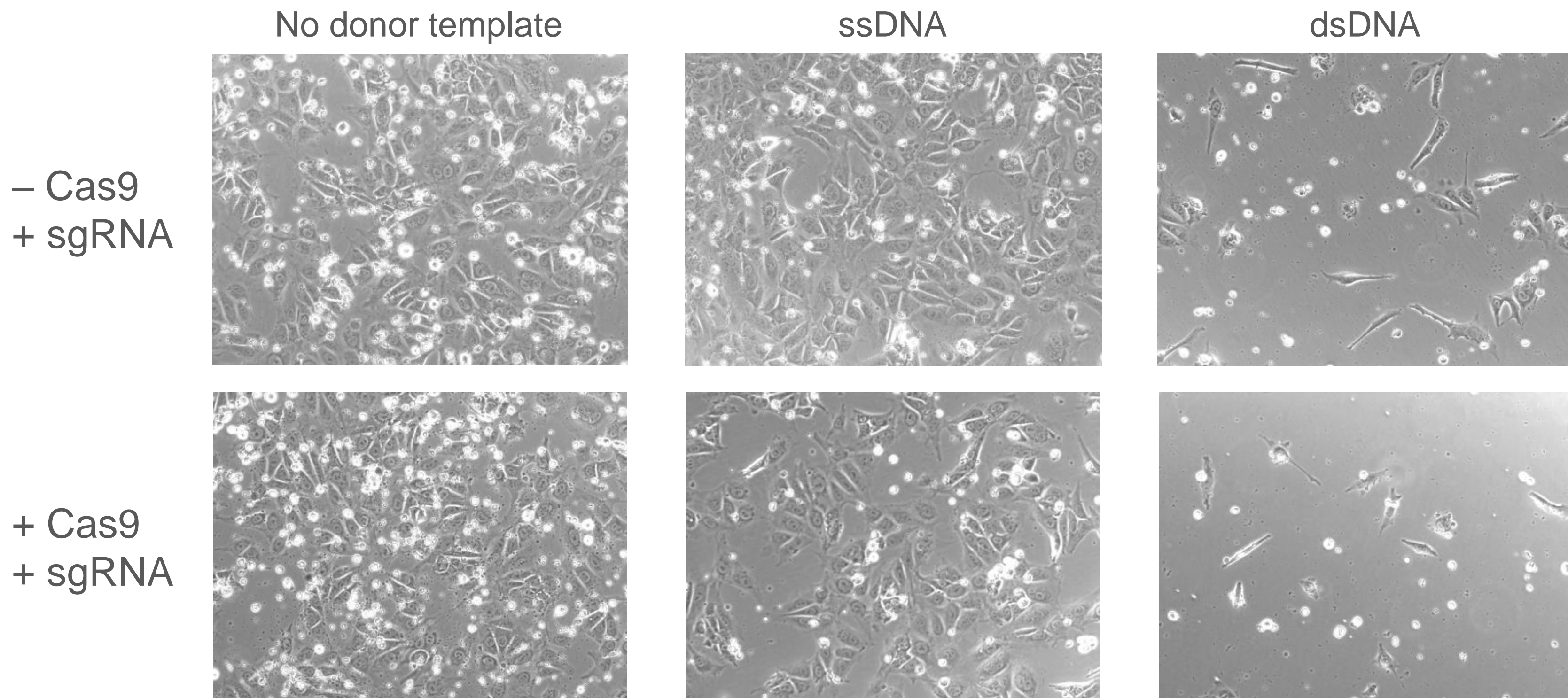
Knocking in genes in hiPSCs

ssDNA is less toxic than dsDNA

Cellular toxicity induced by dsDNA and ssDNA in hiPSCs



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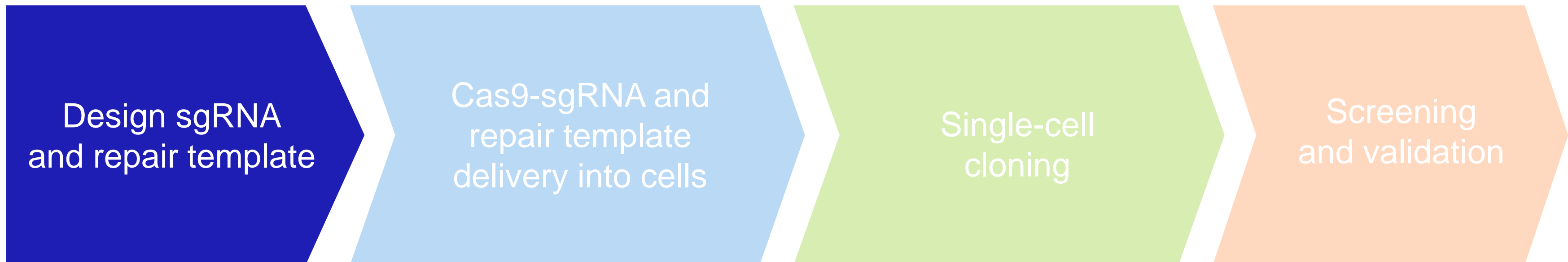
Knocking in genes in hiPSCs

Key considerations



- Check the sequence of the homologous recombination arms
- Pick sgRNAs as close as possible to the DSB
- Use single-stranded donor templates
  - No background expression when delivering expression cassettes (e.g. CMV→GFP)
  - Lower rate of random integration than dsDNA
  - Lower toxicity than dsDNA donors
  - Repair mechanism more efficient than using dsDNA donors

Richardson *et al. Biorxiv* (2017).
Yan *et al. Genome Res* **27,** 1099–1111 (2017).

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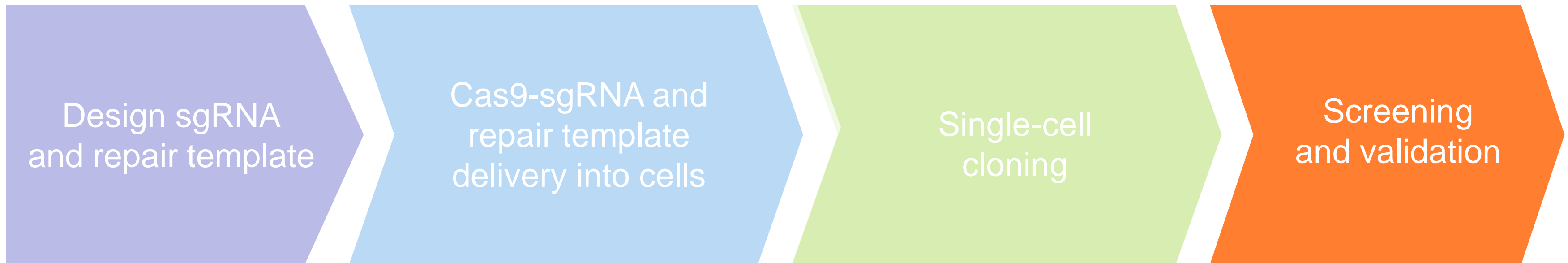
Knocking in genes in hiPSCs
Key considerations



- Bottleneck in homologous recombination experiments
- Homozygous vs. heterozygous

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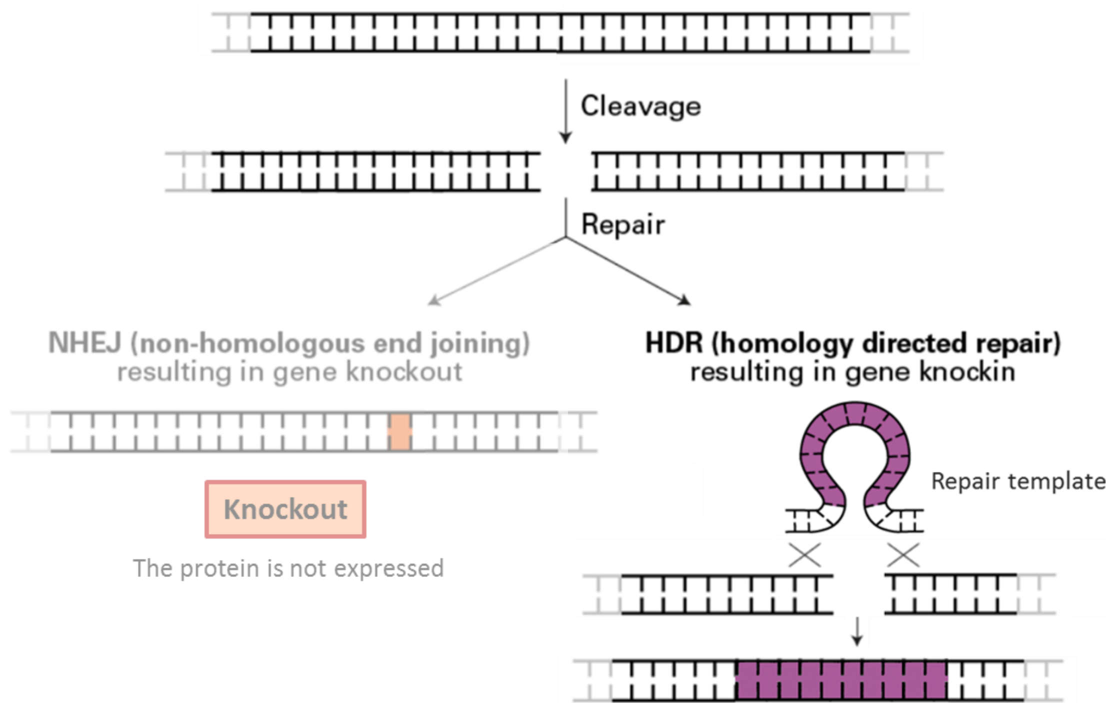
Knocking in genes in hiPSCs



Knockin

- Introducing a mutation
- Tag a protein
- Introduce a expression cassette in a safe harbor (like *AAVS1*)

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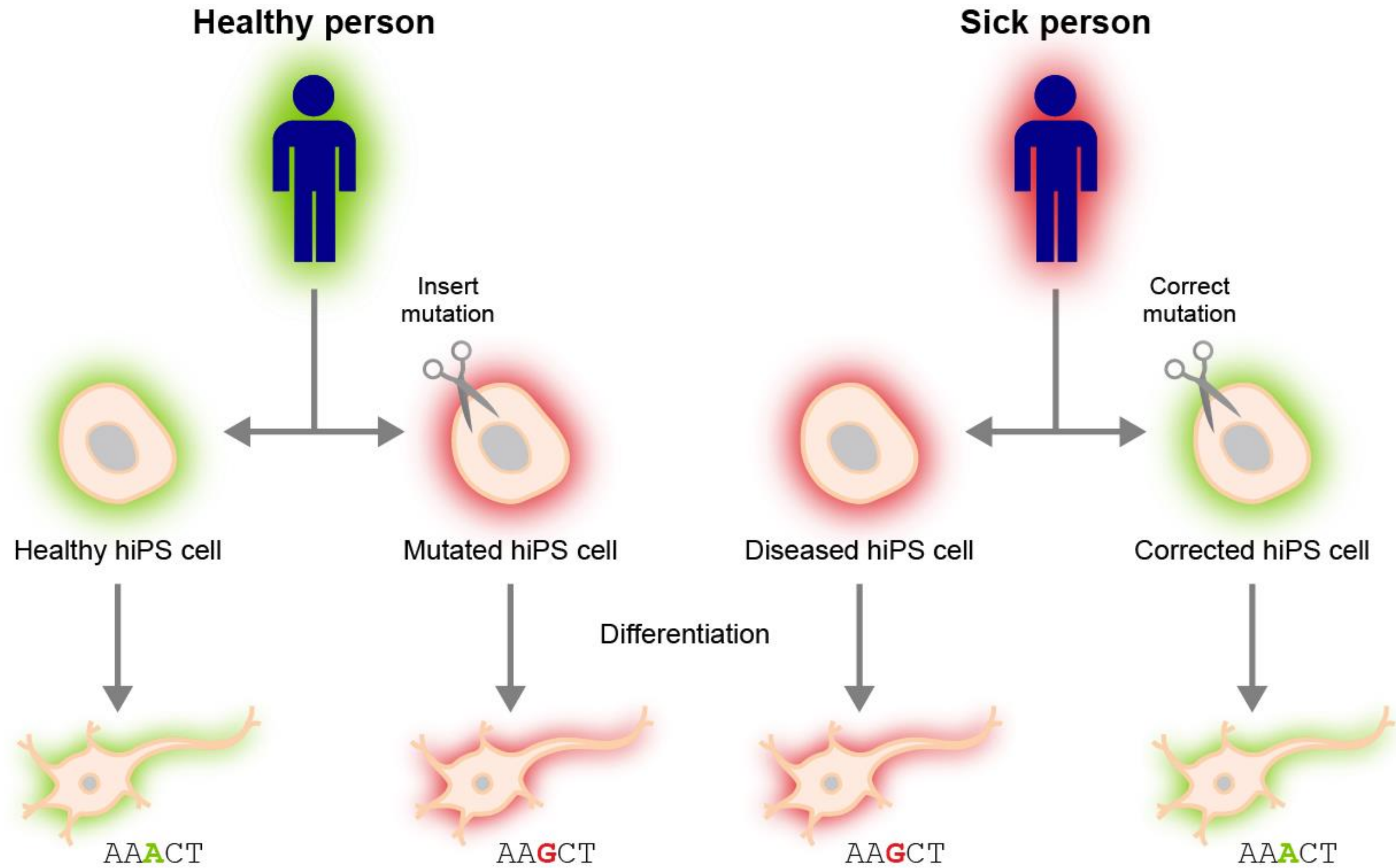
Knocking in point mutations in hiPSCs

Creation of isogenic cell lines



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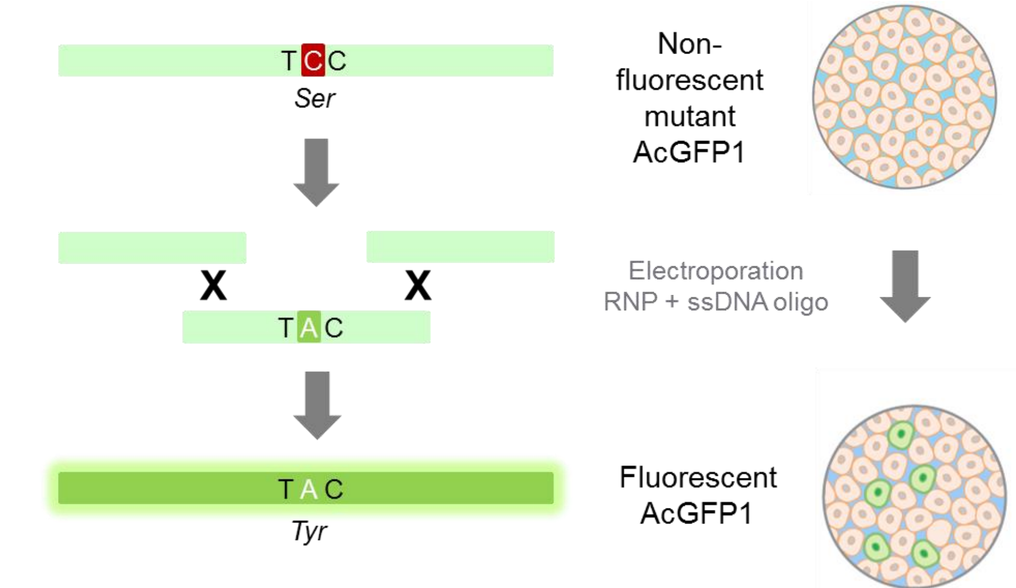
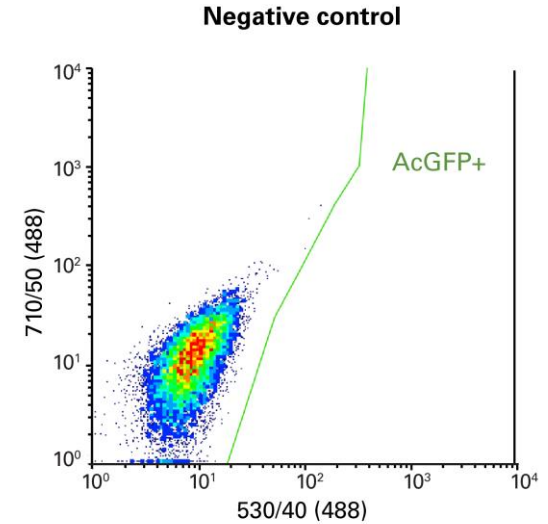
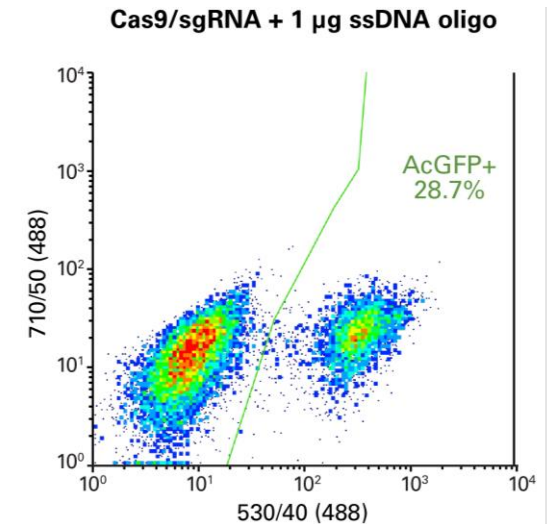
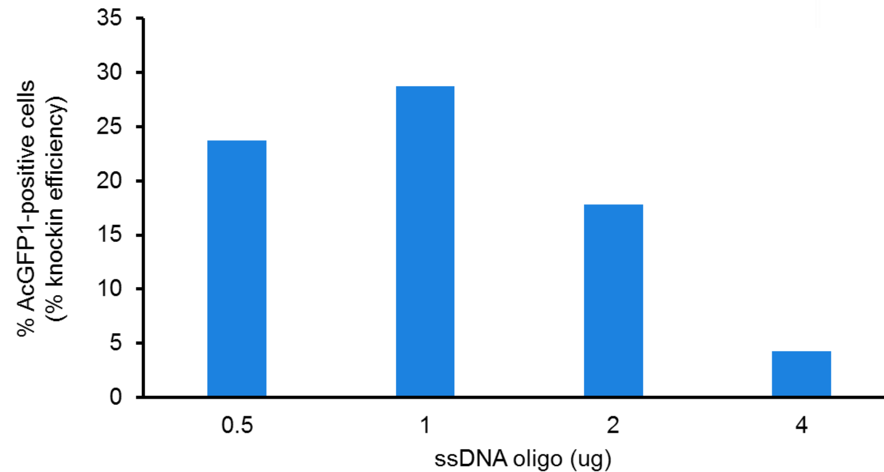
Homology-directed knockin of point mutations

Use of synthetic ssDNA oligos (<200 bp)









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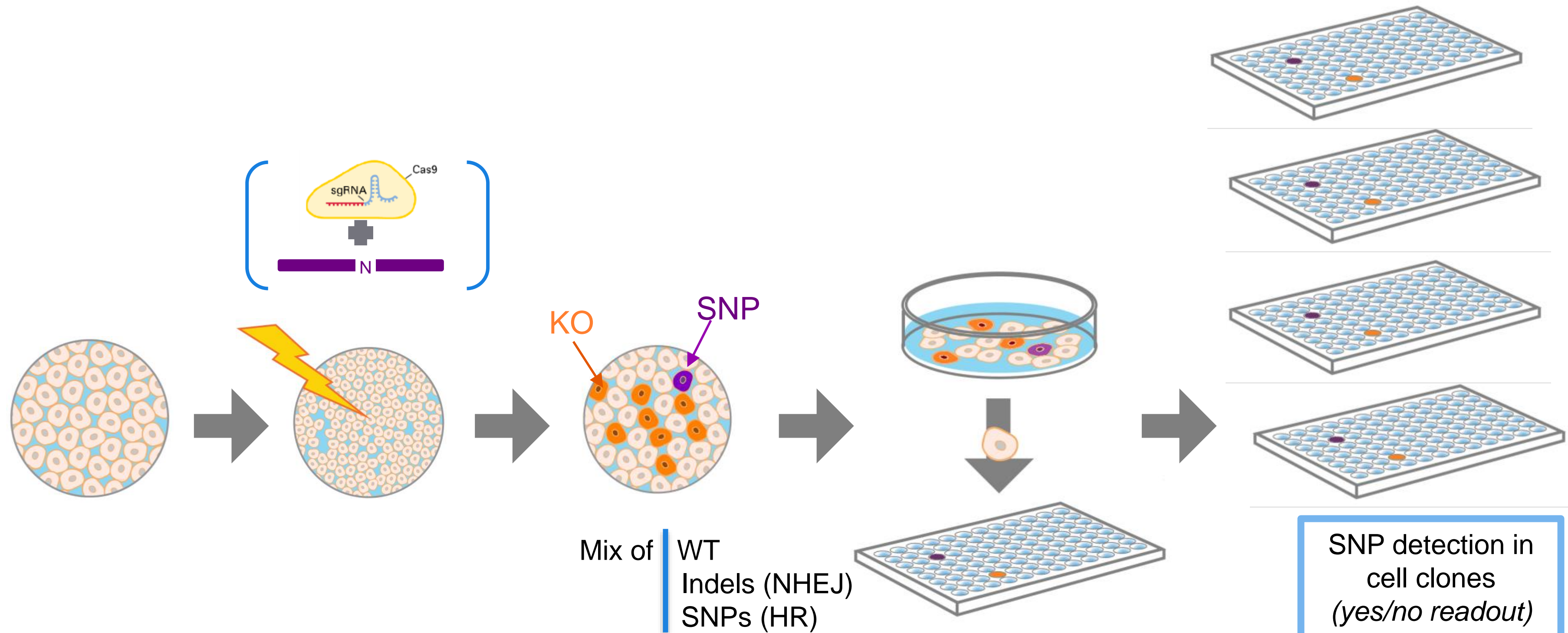
Bottleneck in HR experiments: clone screening



CONFIDENTIAL

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Screening for SNP-containing clones using the SNP detection kit

Wt>SNP
G>A

Single-cell cloning

Non-edited (G)

Edited (A)

DNA extraction

PCR amplification

5′ G 3′ C

5′ A 3′ T

Displacement oligo

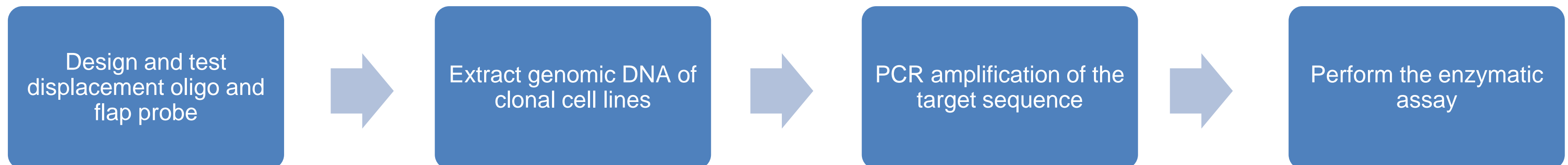
Flap probe

No signal

Guide-it Flapase

Guide-it Flap detector

Workflow of SNP detection kit



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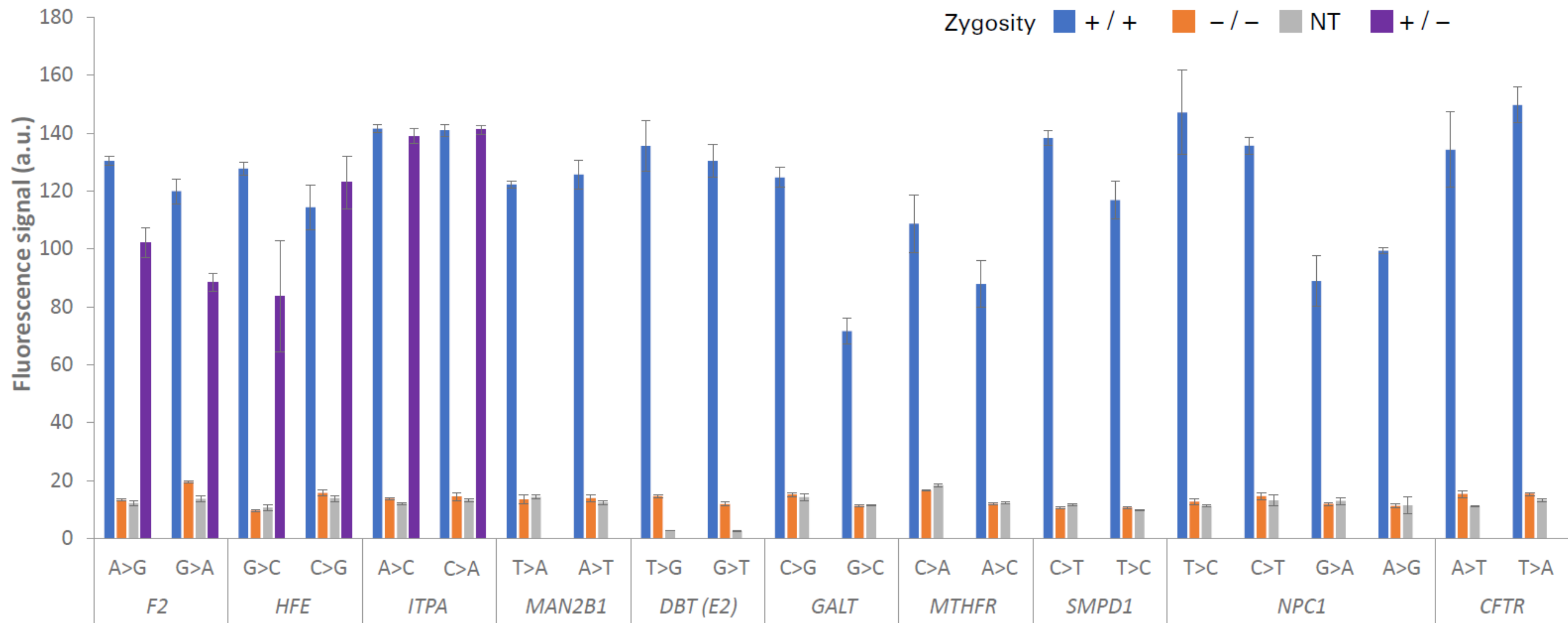
Detection of all possible base mutations



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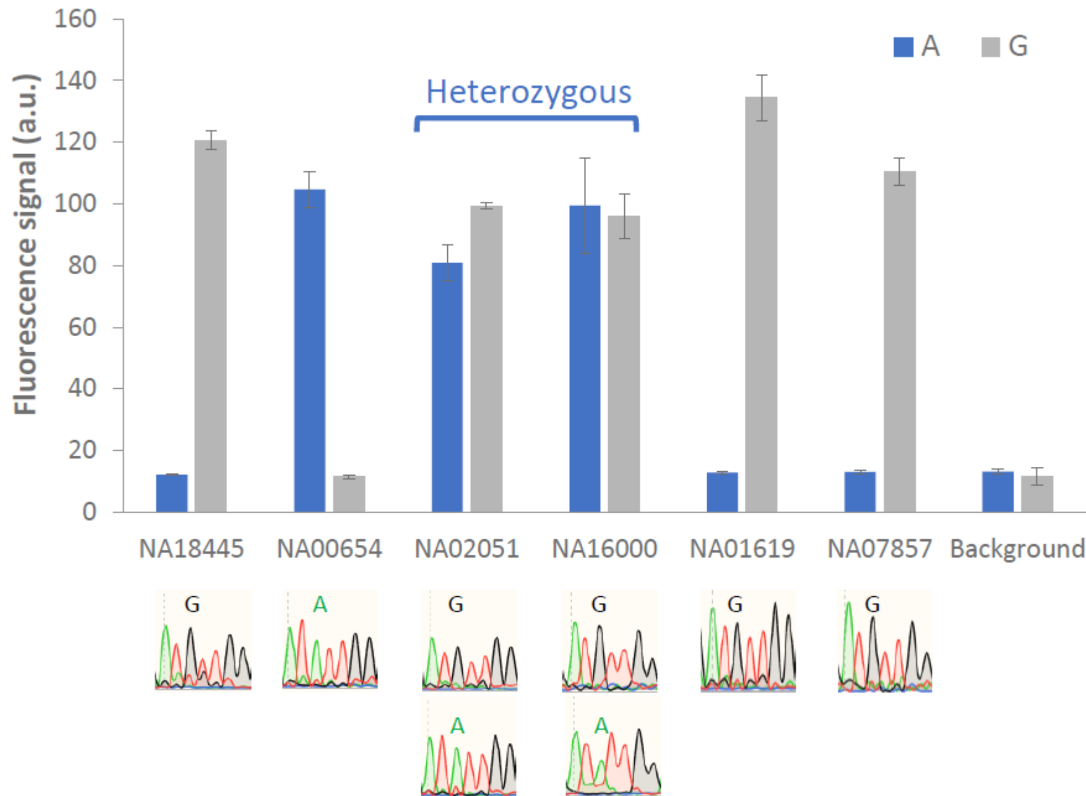
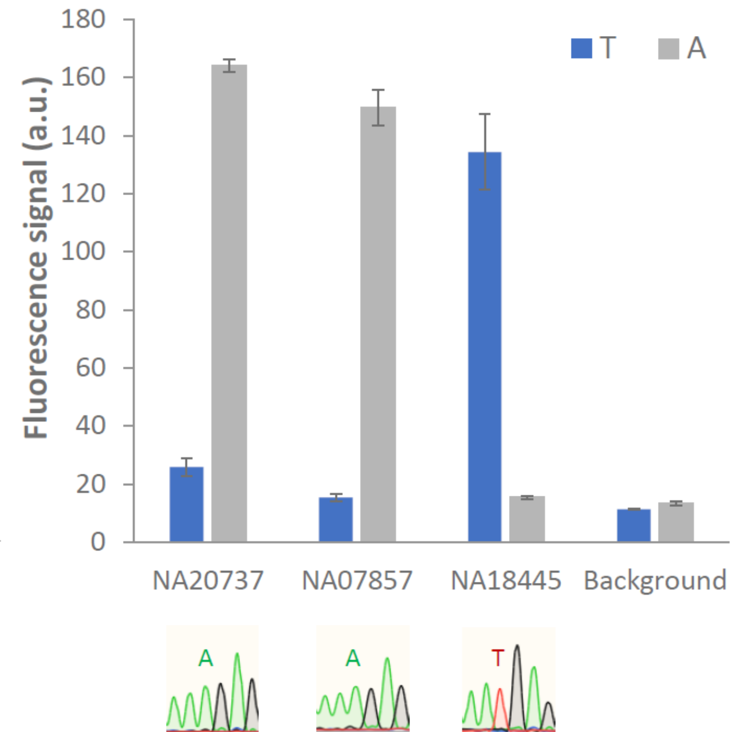
Application in sample genotyping





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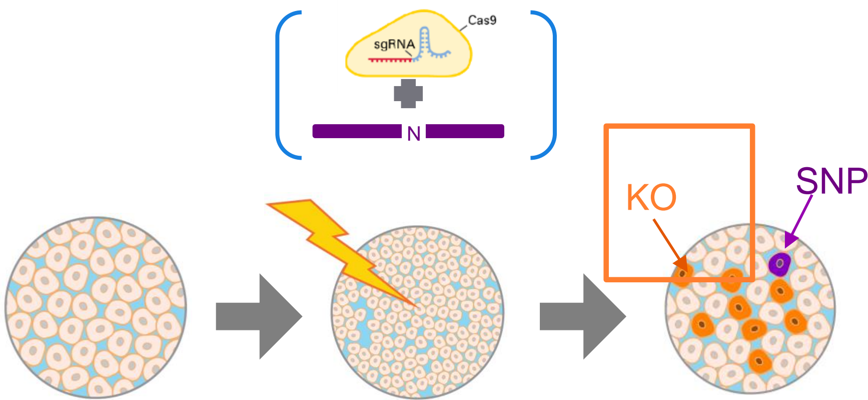
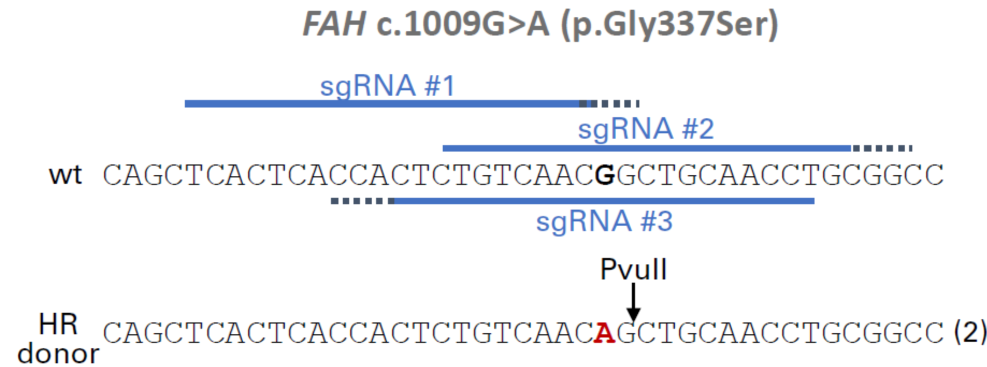
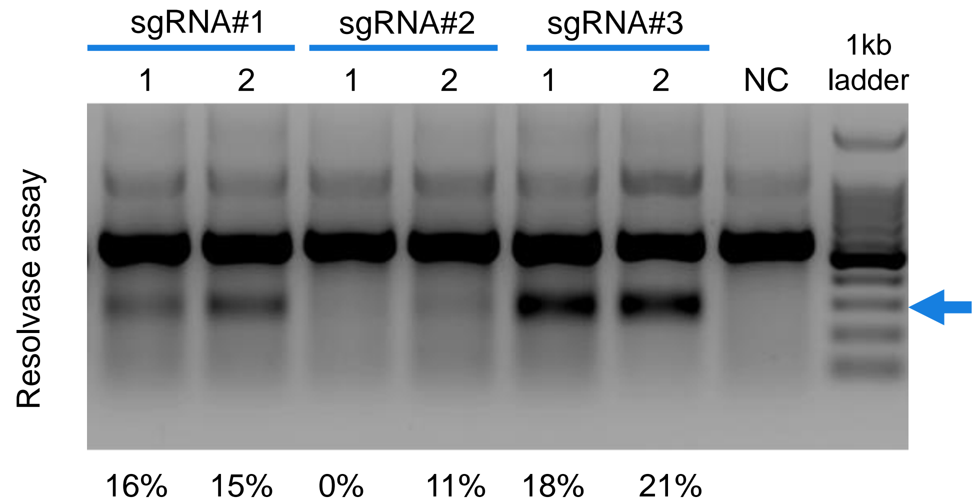
Screening for SNPs related to tyrosinemia







1: RNP
2: RNP + HR donor

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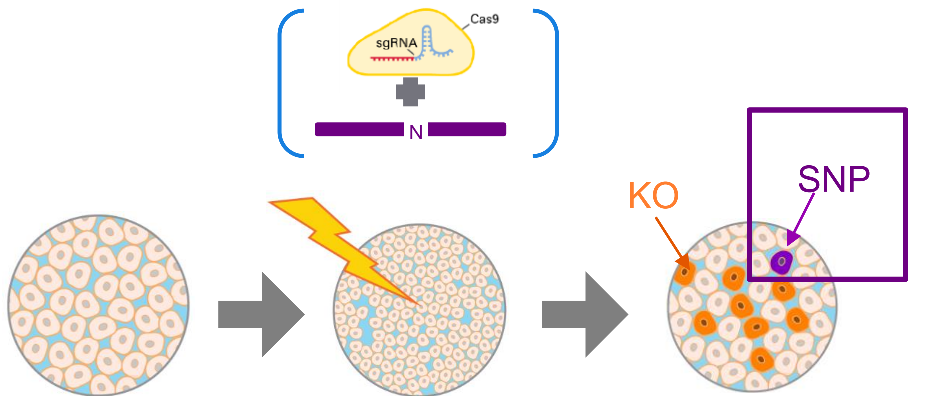
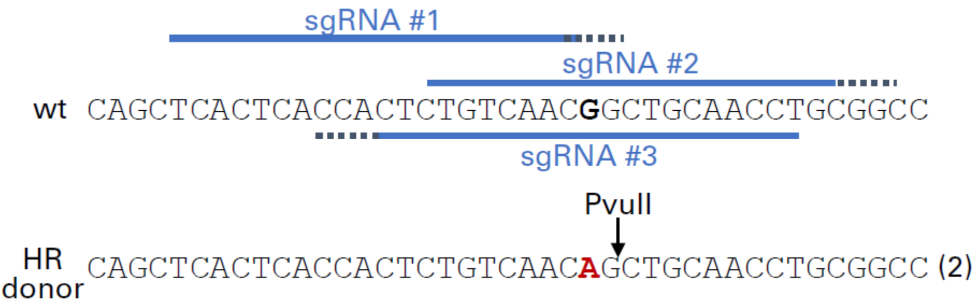
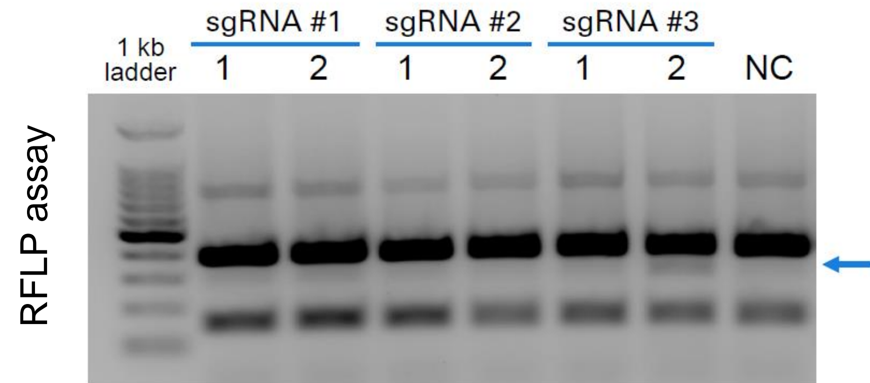
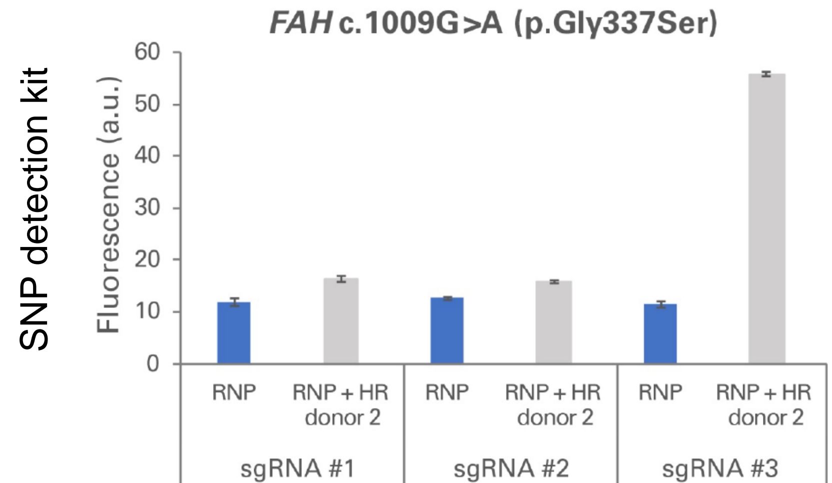
Screening for SNPs related to tyrosinemia









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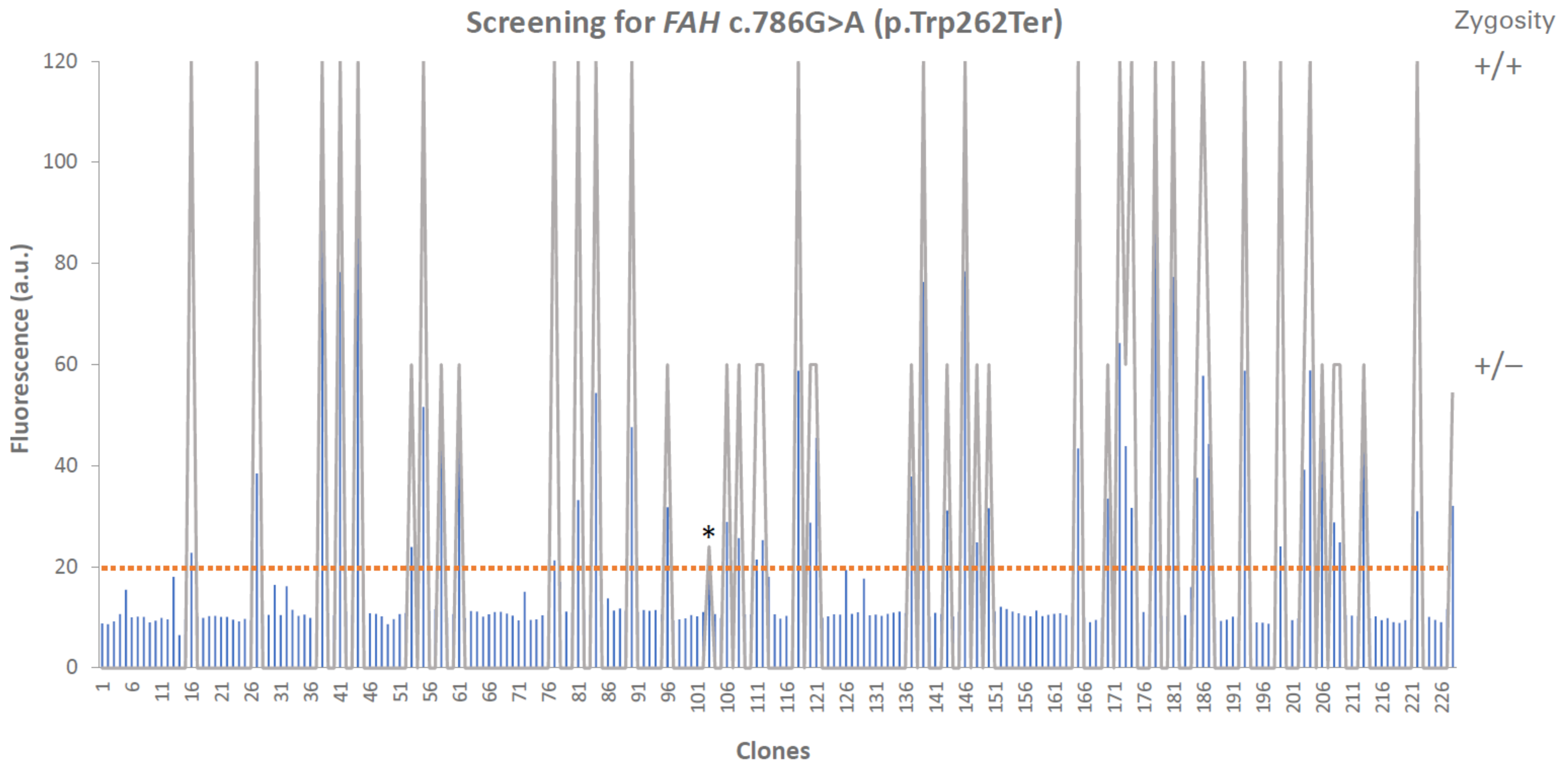
Screening for SNPs related to tyrosinemia



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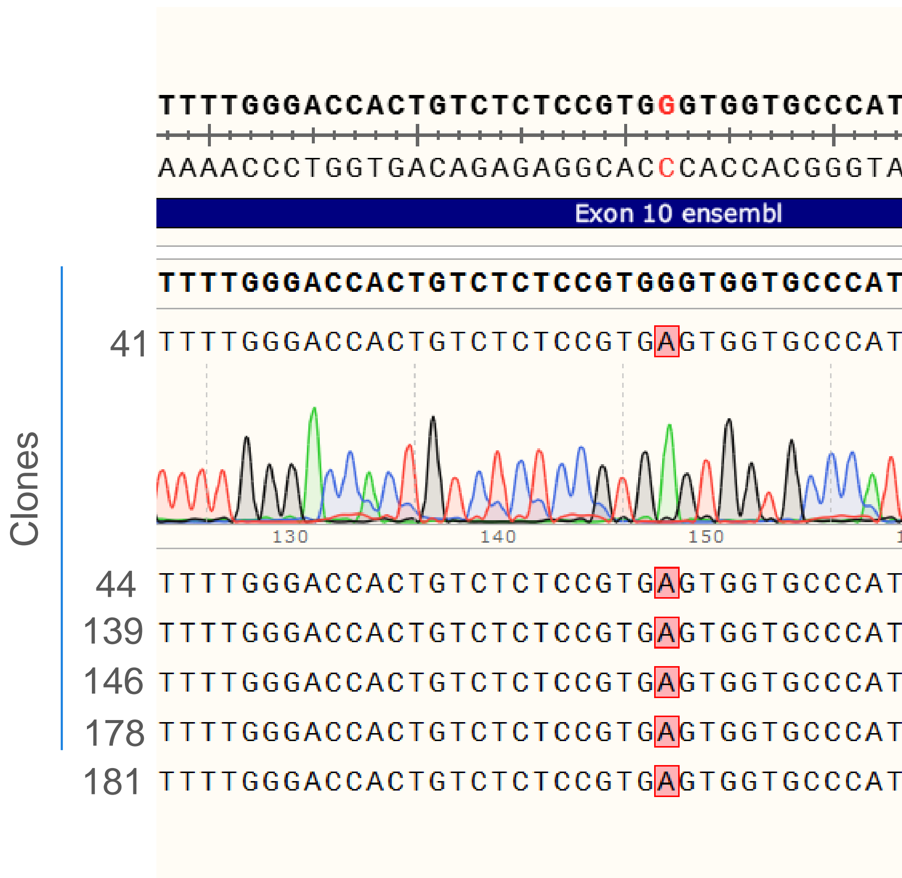
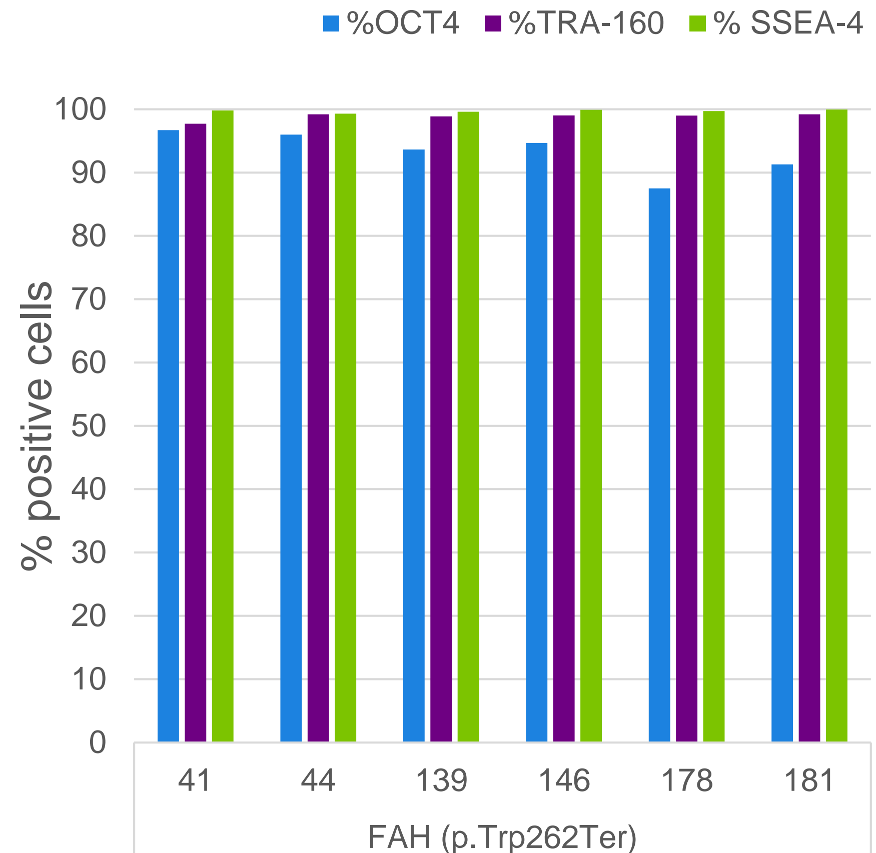
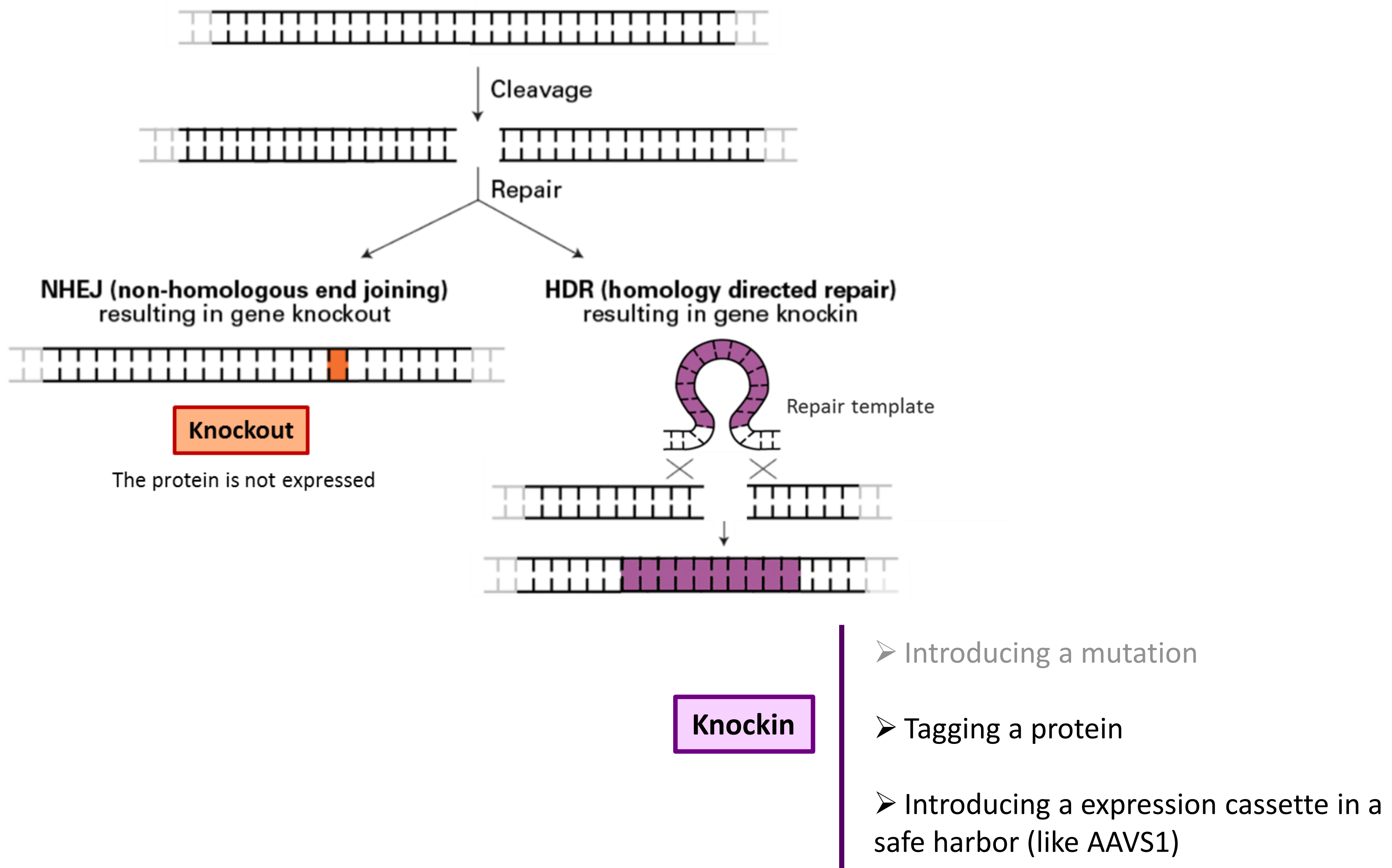
Screening for SNPs related to tyrosinemia





Knocking in genes in hiPSCs



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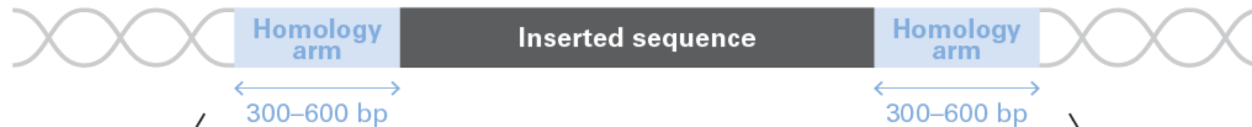
Guide-it Long ssDNA Production System

PCR-based method to create long ssDNA donors (>200 bp)

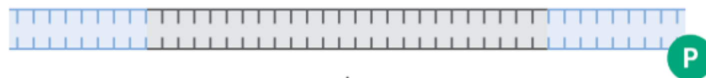
Prepare an HDR template using a method such as cloning or fusion PCR

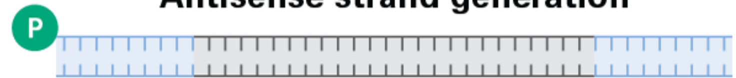


Use PCR to generate starting dsDNA material for the strandase reaction

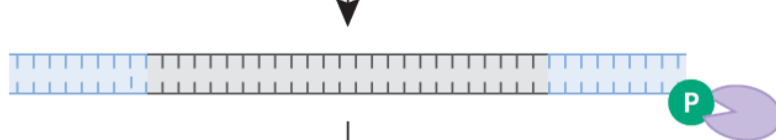
Sense strand generation

Antisense strand generation

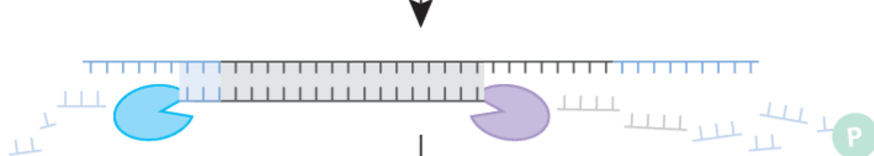


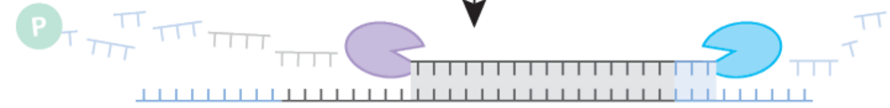


Add Strandase Mix A to begin digesting the phosphorylated strand



Add Strandase Mix B to finish digesting the strand





Clean up strandase reaction to prepare ssDNA for electroporation

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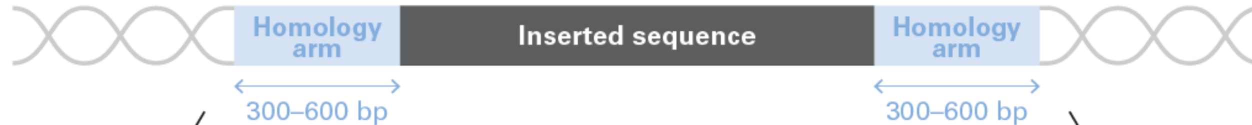
Guide-it Long ssDNA Production System

PCR-based method to create long ssDNA donors (>200 bp)

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 ... nd generation

PCR and "Strandase"-based preparation

- No cloning or gel purification
- So far, successful up to 5 kb
- Yield: 2–4 µg from 10 µg dsDNA
- Creating an ssDNA from a dsDNA PCR product takes 30–40 minutes

Clean up strandase reaction to prepare ssDNA for electroporation

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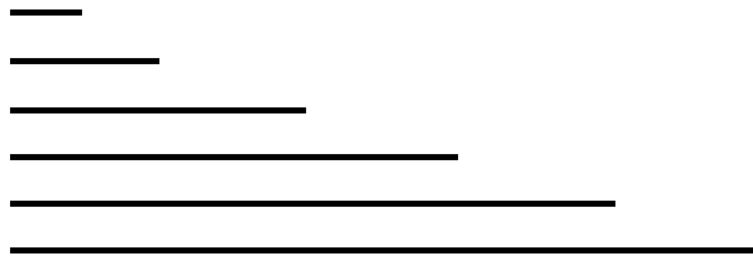
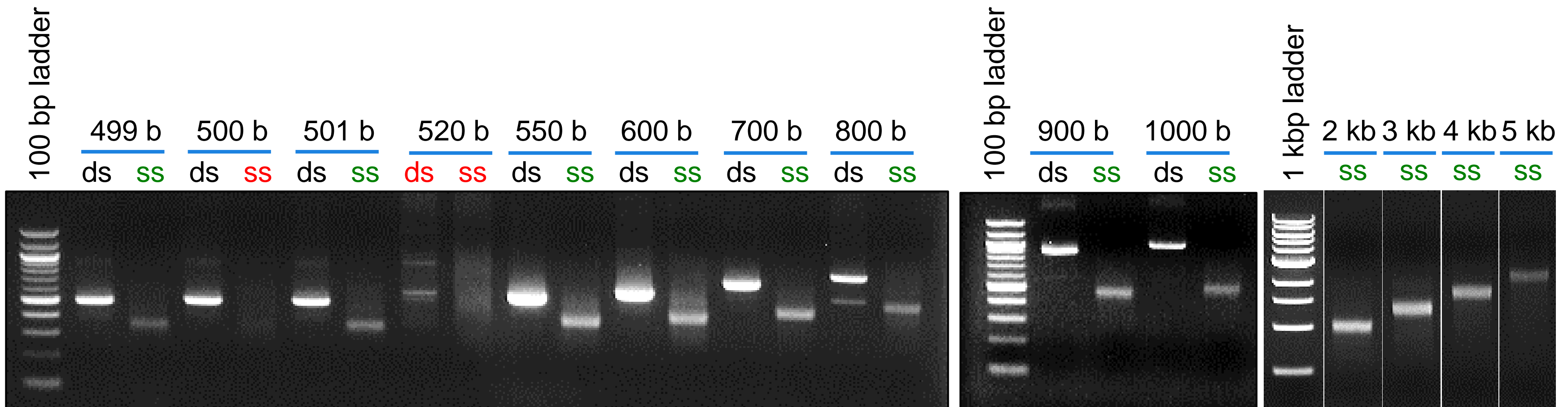
Production of ssDNA ranging from 0.5–5 kb in length

CCR5 region
(0.5~5 kb)





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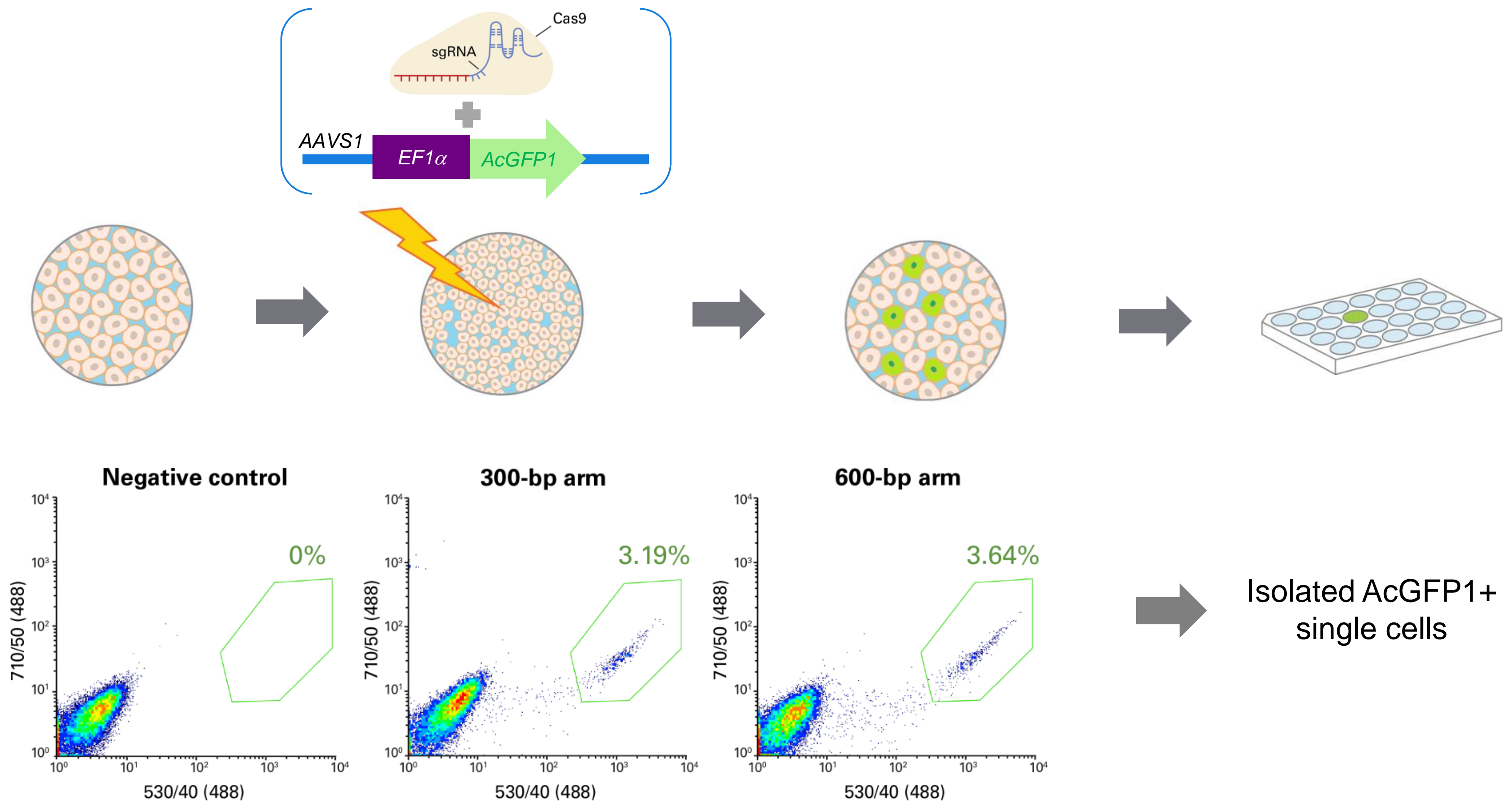
Knockin of *EF1α-AcGFP1* at *AAVS1* site



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Isolated clonal lines without mutations

L1
R1
Left arm
EF1α promoter
AcGFP1
PolyA
Right arm
AAVS1
locus
L2
R2

Clonal cell lines

NC #1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11

L1/L2

R1/R2

EF1α
promoter
SV40 PolyA
signal
L1
R1
Left arm
AcGFP1
Right arm
L2
R2

ctgagcggatcctccccgtgtctgggtcctctc
CTGAGCGGATCCTCCCCGTGTCTGGGTCCTCTC
CTGAGCGGATCCTCCCCGTGTCTGGGTCCTCTC
CTGAGCGGATCCTCCCCGTGTCTGGGTCCTCTC
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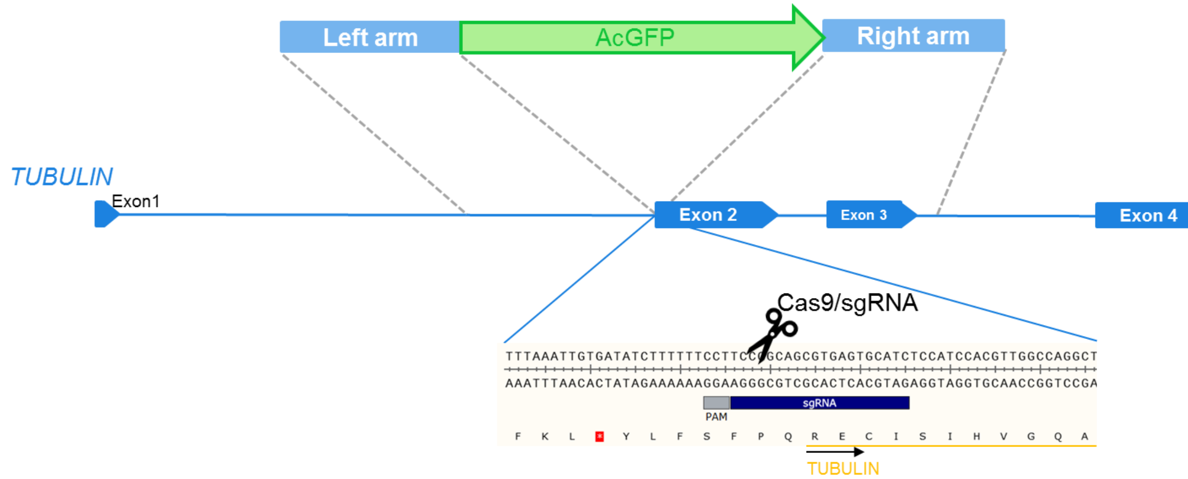
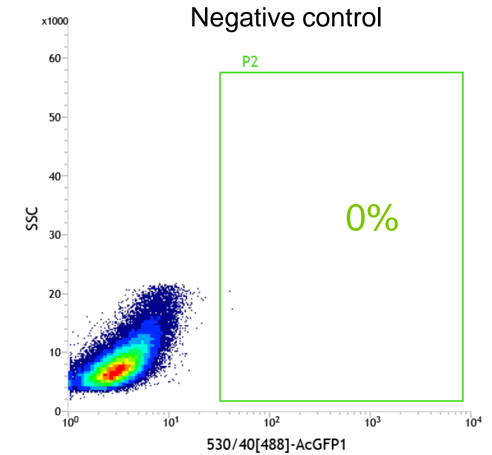
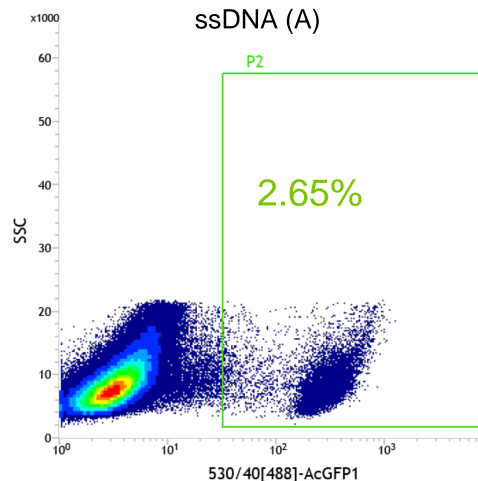
Tagging of endogenous genes in hiPSCs

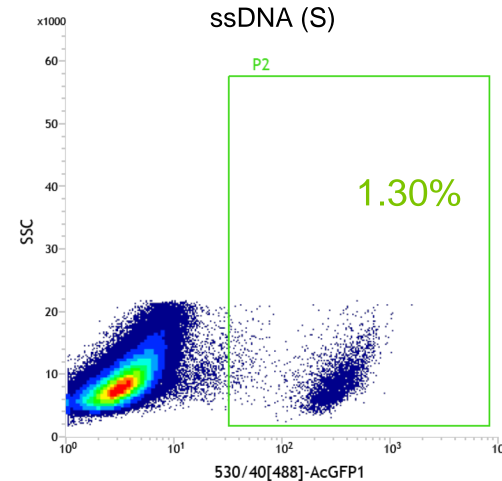
N-terminal fusion of AcGFP1 to Tubulin





ssDNA (*AcGFP1-Tubulin*)





Roberts *et al. Biorxiv* (2017).

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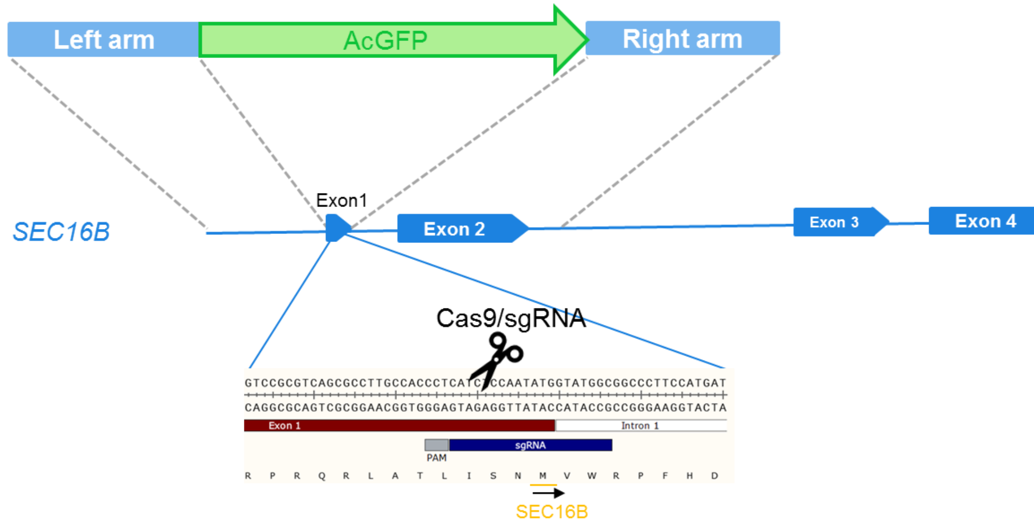
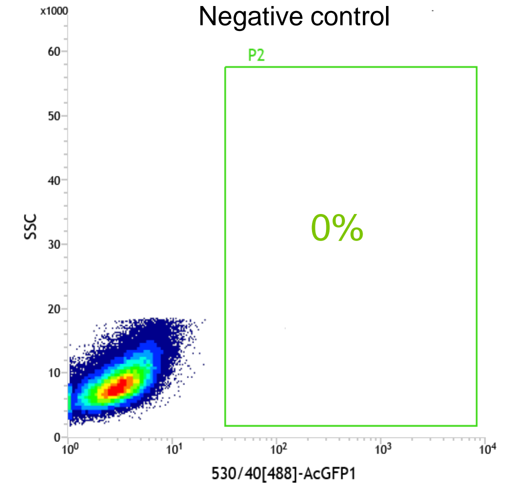
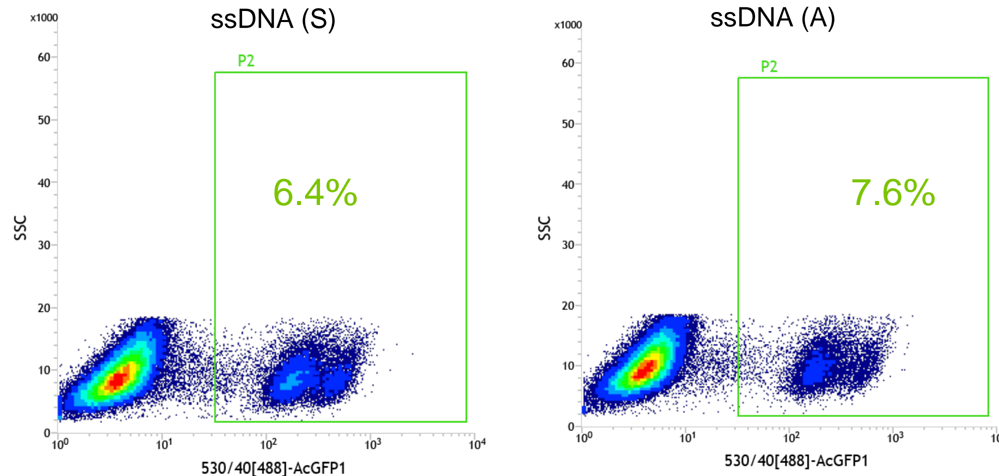
Tagging of endogenous genes in hiPSCs

N-terminal fusion of AcGFP to SEC16B





ssDNA (*AcGFP1-SEC16B*)



Roberts *et al. Biorxiv* (2017).

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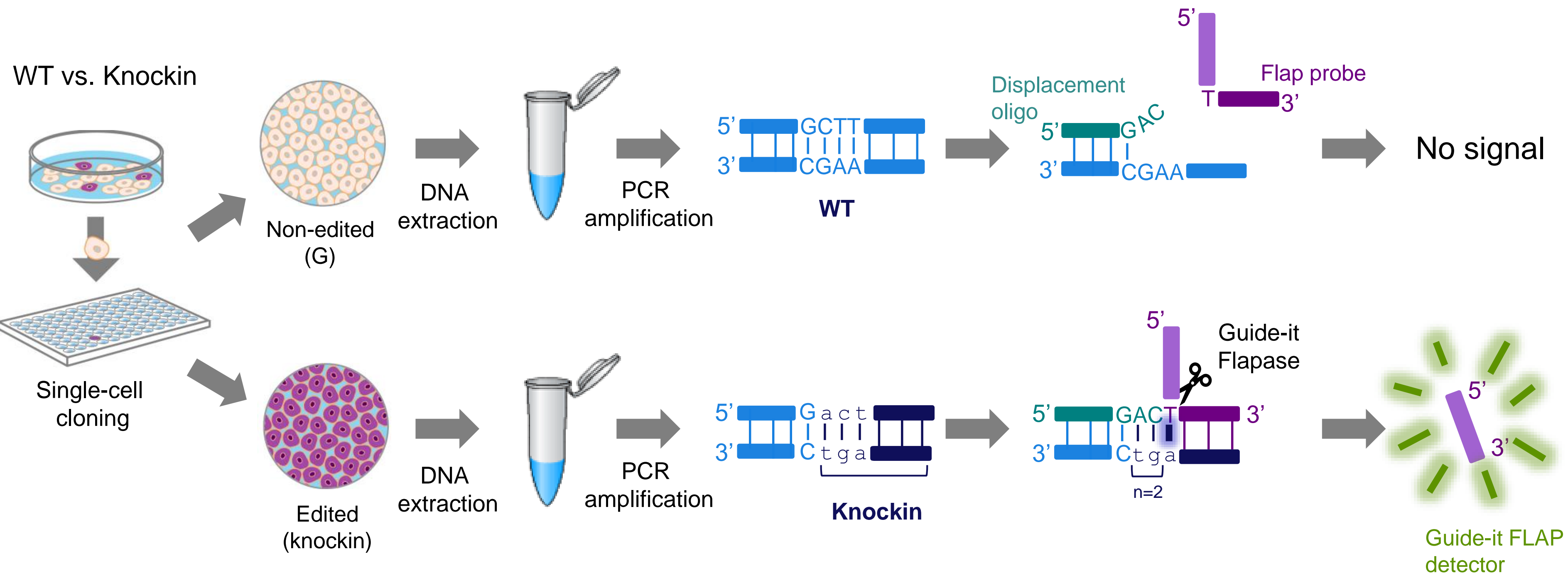
Knockin detection strategy



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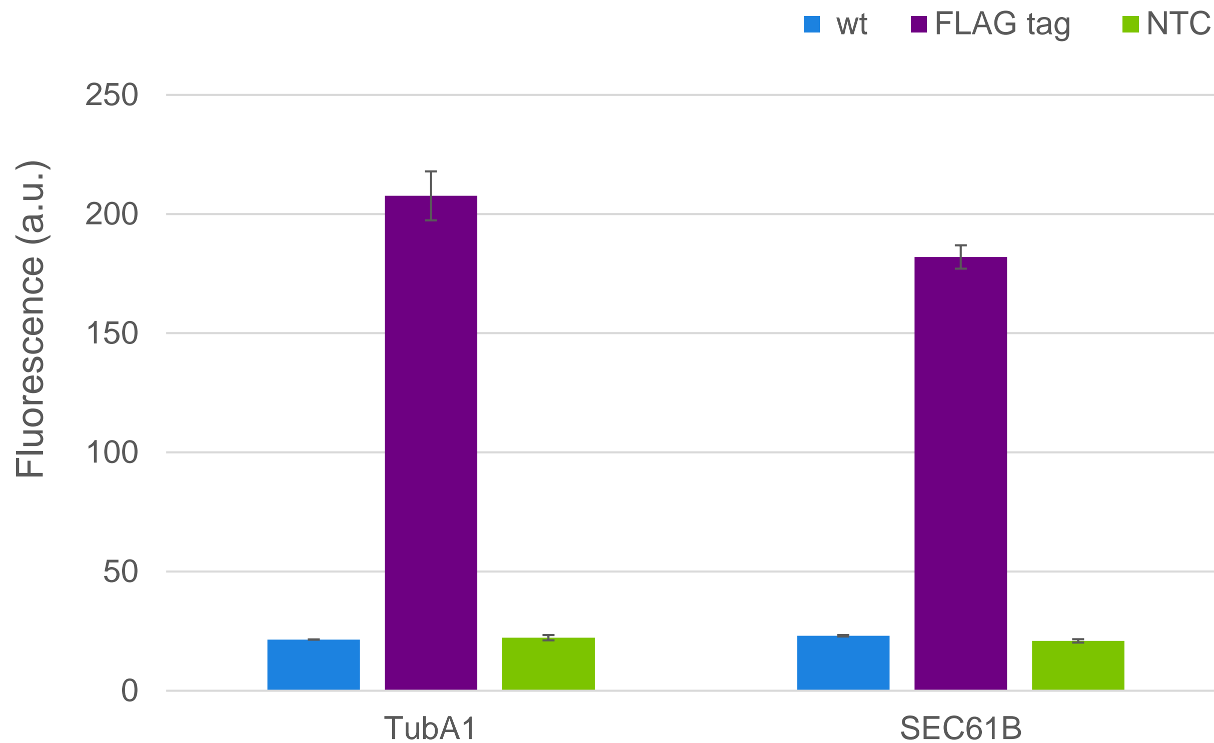
Knockin detection strategy

Proof of concept



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Takara Bio and stem cell expertise

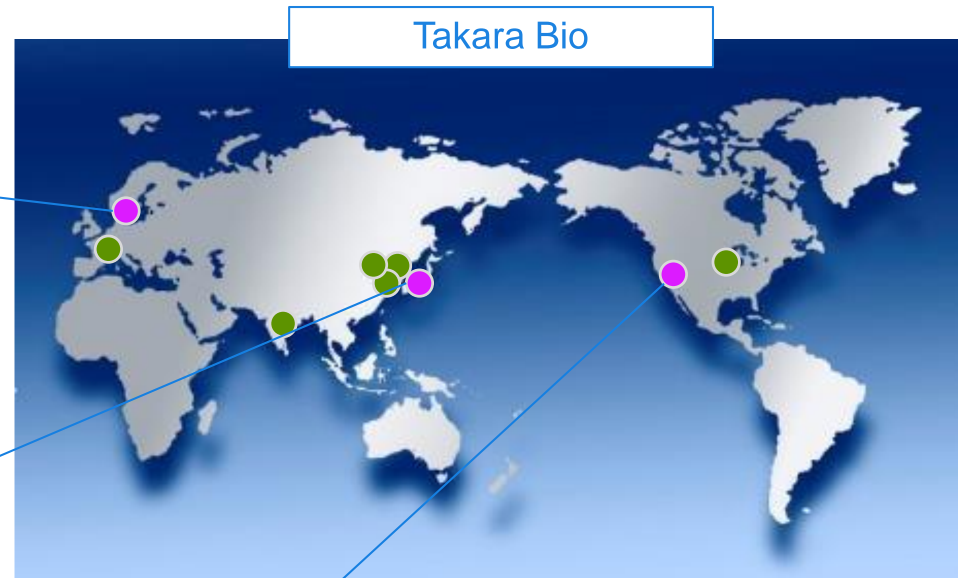
● **Production & Development**

Takara Bio Europe AB
Gothenburg, Sweden

Takara Bio Inc.
Kusatsu, Shiga, Japan

Takara Bio USA, Inc.
Mountain View, CA

Takara Bio



● **Sales & Distribution**

- Takara Bio Europe S.A.S.
- Takara Bio USA, Inc.
- Takara Biomedical Technology Co. Ltd.
- Takara Korea Biomedical Inc. DSS
- Takara Bio India Pvt. Ltd.

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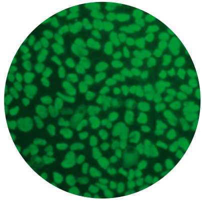
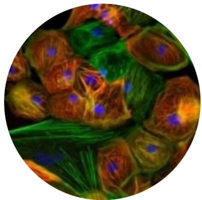
Cellartis products and services



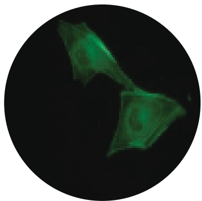
Undifferentiated human pluripotent stem (hPS) cells

- hPSC lines
- DEF-CS and DEF-CS Xeno-Free (2D, 3D, and GMP) for expansion and maintenance
- Media kits for gene editing and single-cell cloning
- Differentiation kits



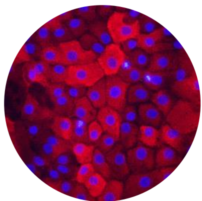
Specialized cells, differentiated from hPS cells

- Definitive endoderm cells
- Hepatocytes
- Beta cells
- Cardiomyocytes



Human pluripotent stem cell services

- Sourcing, reprogramming, and banking
- Directed differentiation into multiple cell types
- Clinical-grade human embryonic stem cell line generation and banking
- Gene editing



Miscellaneous

- Power™ Primary HEP Medium for primary hepatocytes
- Adult neural stem cells
- Medium for expansion, maintenance, and differentiation of neural stem cells
- Antibodies and qPCR primers for characterization and detection

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THANKS!

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GOOD
science!®

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