

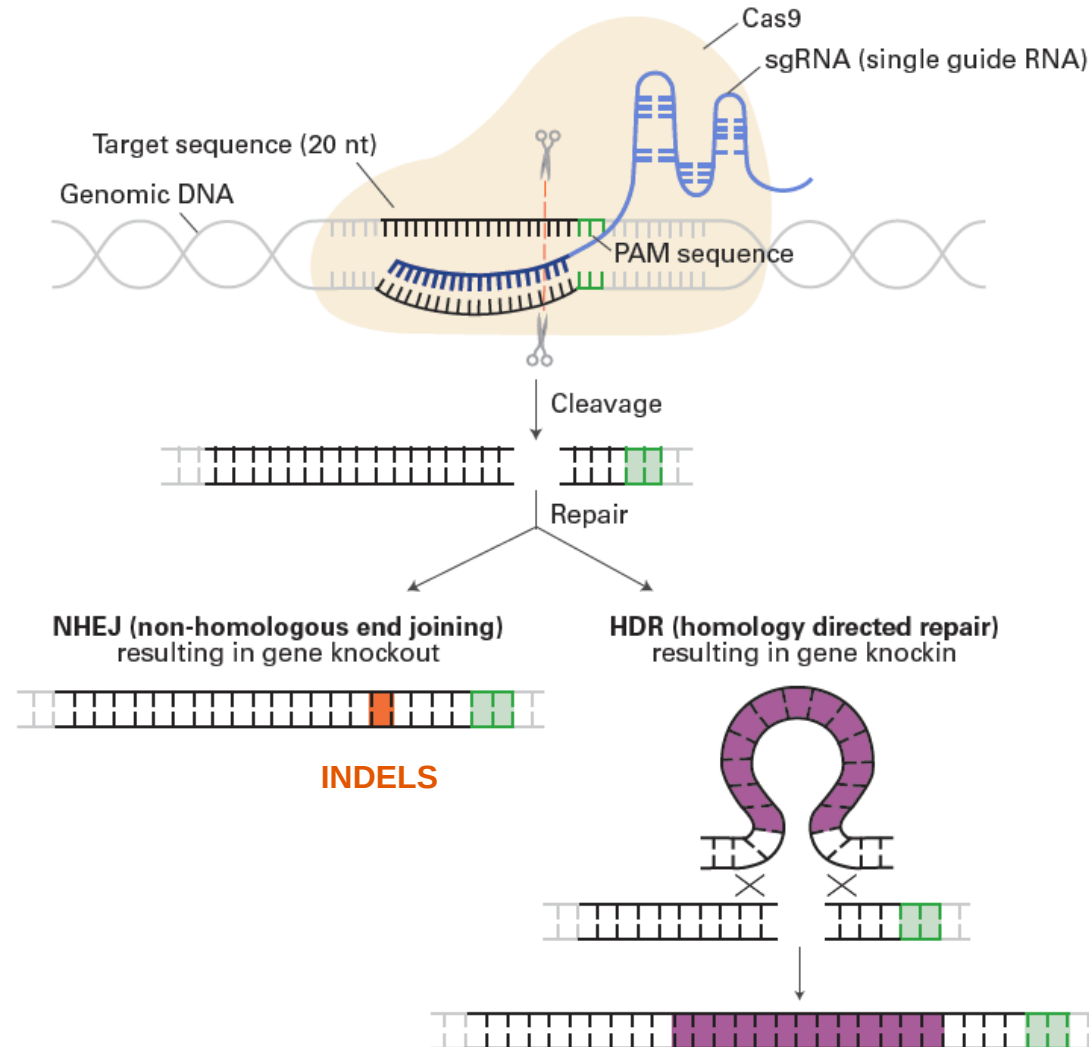
Cas9/sgRNA ribonucleoprotein (RNP) delivery to improve your KO and KI gene editing experiments

Cornelia Hampe, PhD
Product Manager Gene Function & Delivery
Takara Bio Europe

This presentation

- CRISPR/Cas9 principle and workflow
- Benefits of Cas9/sgRNA RNP delivery
 - Guide-it™ CRISPR/Cas9 Gesicles
 - Electroporation
- Knockin of short versus long sequences
 - Limitations of knockin using long sequences
 - Benefits of single-stranded DNA HDR templates
 - Guide-it™ SNP Screening Kit
 - Guide-it™ Long ssDNA Production System

CRISPR/Cas9 Gene Editing Principle



CRISPR/Cas9 Gene Editing Workflow

sgRNA design & screening

- Efficacy
- Specificity

Cas9/sgRNA delivery into cells (gene editing)

- Efficient delivery
- Efficient editing
- Minimize toxicity
- No off-target effects

Check editing efficiency

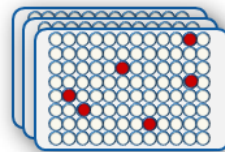
- Quick screening of cell population

Single-cell cloning

- Maximize survival

Screening

- Identification
- Specificity



Isolate & expand an edited single cell

CRISPR/Cas9 Gene Editing Workflow

sgRNA
design

Cas9/sgRNA
delivery into cells
(gene editing)

Check editing
efficiency

Single-cell
cloning

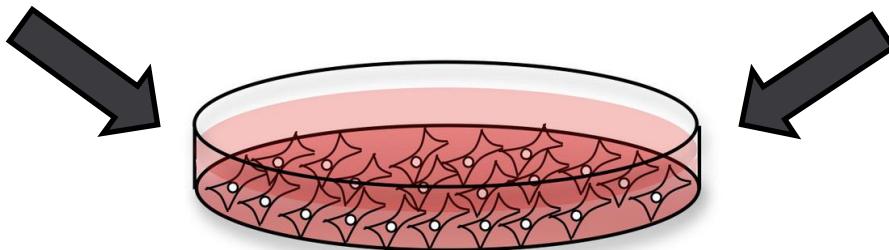
Screening

Source of Cas9

- Plasmid
- AAV
- Lentiviral
- mRNA
- Protein

Source of sgRNA

- Plasmid
- AAV
- Lentiviral
- Synthetic or IVT sgRNA



CRISPR/Cas9 Gene Editing Workflow

sgRNA
design &
production

Cas9/sgRNA
delivery into cells
(gene editing)

Check editing
efficiency

Single-cell
cloning

Screening

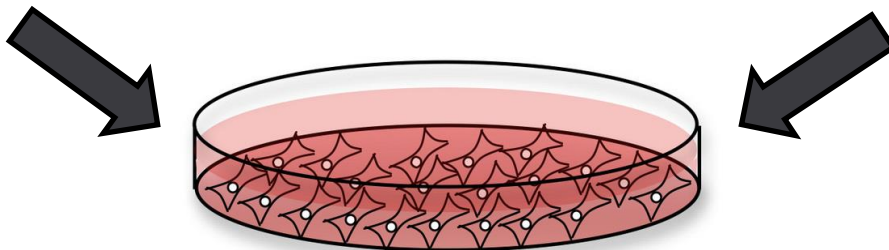
Source of Cas9

- Plasmid
- AAV
- Lentiviral
- mRNA
- **Protein**

Source of sgRNA

- Plasmid
- AAV
- Lentiviral
- **Synthetic or IVT sgRNA**

Ribonucleoprotein
(RNP) delivery



The benefits of RNP delivery

Method

Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins

Sojung Kim,^{1,2} Daesik Kim,^{1,2} Seung Woo Cho,^{1,2} Jungeun Kim,^{1,2} and Jin-Soo Kim^{1,2,3}
¹Department of Chemistry, Seoul National University, Seoul 151-747, South Korea; ²Center for Genome Engineering, Institute for Basic Science, Seoul 151-747, South Korea

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journal homepage: www.elsevier.com/locate/jbiotec

Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection

Xiquan Liang,¹ Jason Potter^{*,1}, Shantanu Kumar, Yanfei Zou, Rene Quintanilla, Mahalakshmi Sridharan, Jason Carte, Wen Chen, Natasha Roark, Sridhar Ranganathan, Namritha Ravinder, Jonathan D. Chesnut
Thermo Fisher Scientific, 5781 Van Allen Way, Carlsbad, CA 92008, USA



Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing *in vitro* and *in vivo*

John A Zuris, David B Thompson, Yilai Shu, John P Guilinger, Jeffrey L Bessen, Johnny H Hu, Morgan L Maeder, J Keith Joung, Zheng-Yi Chen & David R Liu
Affiliations | Contributions | Corresponding author

Nature Biotechnology 33, 73–80 (2015) | doi:10.1038/nbt.3081
Received 22 July 2014 | Accepted 23 October 2014 | Published online 30 October 2014

Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery

Steven Lin, Brett T Staahl, Ravi K Alla, Jennifer A Doudna
University of California, Berkeley, United States; Howard Hughes Medical Institute, University of California, Berkeley, United States; Lawrence Berkeley National Laboratory, United States

DOI: <http://dx.doi.org/10.7554/eLife.04766>
Published December 15, 2014
Cite as eLife 2014;3:e04766

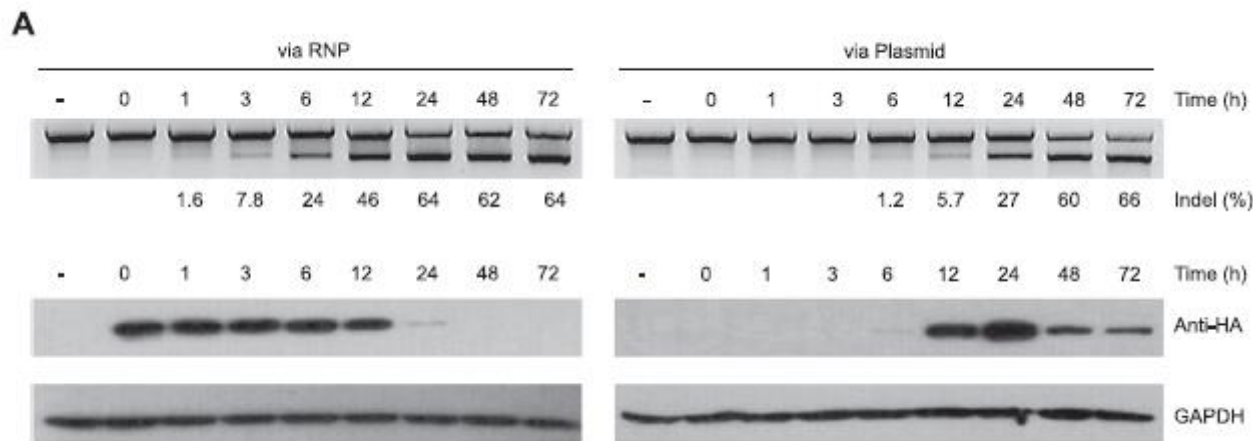
The benefits of RNP delivery

Method

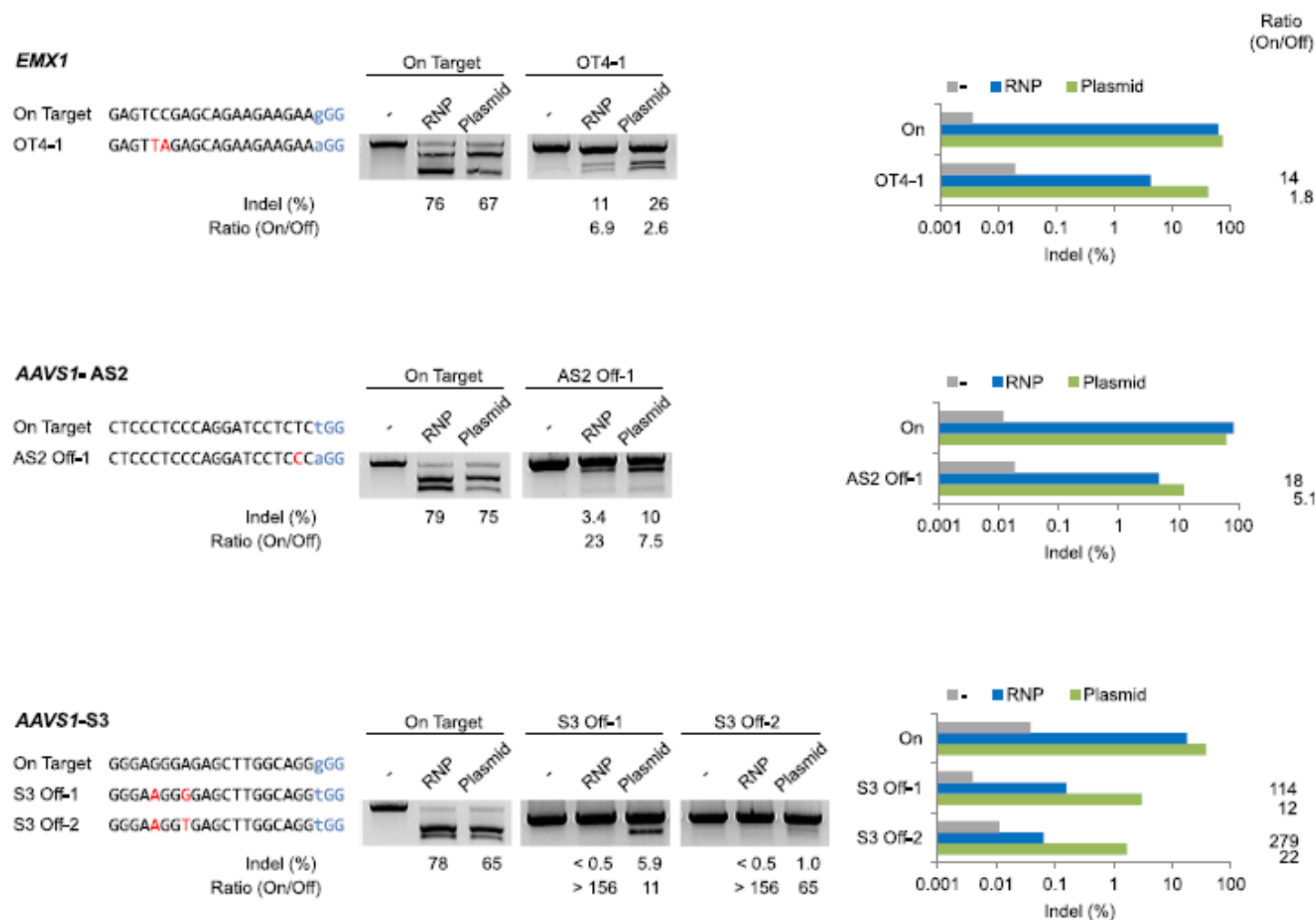
Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins

Sojung Kim,^{1,2} Daesik Kim,^{1,2} Seung Woo Cho,^{1,2} Jungeun Kim,^{1,2} and Jin-Soo Kim^{1,2,3}

¹Department of Chemistry, Seoul National University, Seoul 151-747, South Korea; ²Center for Genome Engineering, Institute for Basic Science, Seoul 151-747, South Korea



The benefits of RNP delivery



Kim et al., 2014

RNP delivery via Gesicles

sgRNA
design &
production

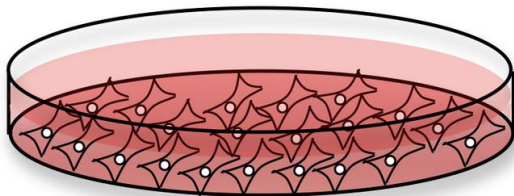
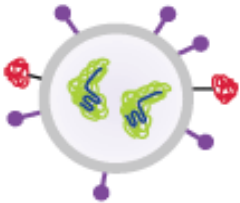
Cas9/sgRNA
delivery into cells
(gene editing)

Check editing
efficiency

Single-cell
cloning

Screening

Gesicles deliver Cas9
Ribonucleoprotein
(RNP) complexes



Guide-it CRISPR/Cas9 Gesicle Production System

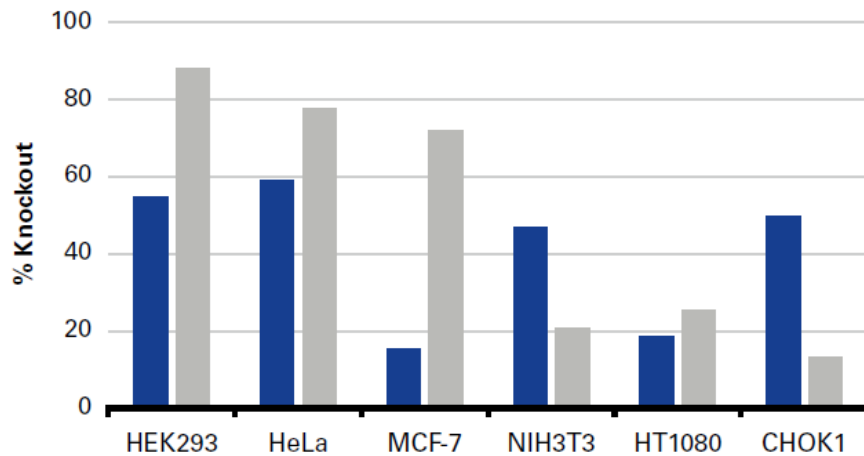
- Cell-derived **nanovesicles**, made by a producer cell line after overexpression of a specific glycoprotein. Broad mammalian tropism
- **Complete system** to produce gesicles to deliver Cas9 protein together with a target specific sgRNA
- Suitable for **knockouts**
- **Reduced off-target effects** compared to DNA delivery (plasmid, lentiviral)



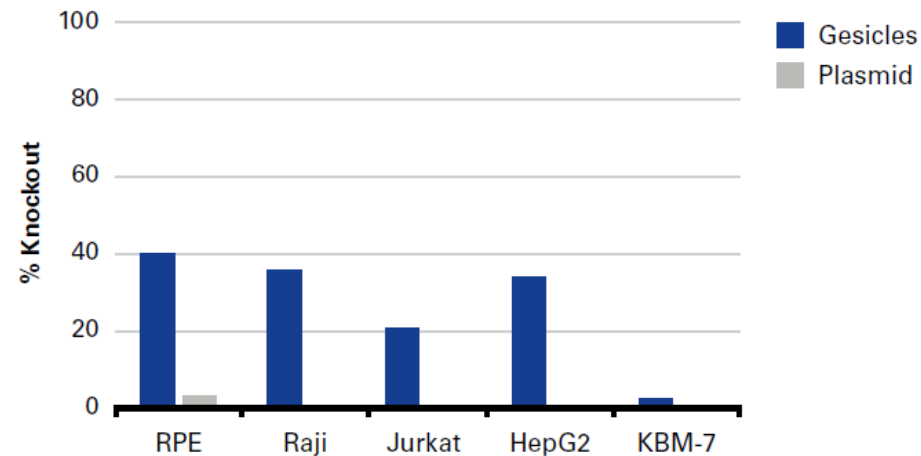
Gene editing in a wide range of cell types

Knockout of ZsGreen1 in Multiple Cell Types

Easier-to-transfect cell types



Harder-to-transfect cell types



- Suitable for adherent and suspension cells
- Suitable for proliferating and non-proliferating cells (i.e. primary cells)
- Suitable for human iPS cells
- Based on customer feedback, low efficiency with primary T-Cells, B-Cells, or CD34+ hematopoietic stem cells

Gesicle-mediated KO

ARTICLES

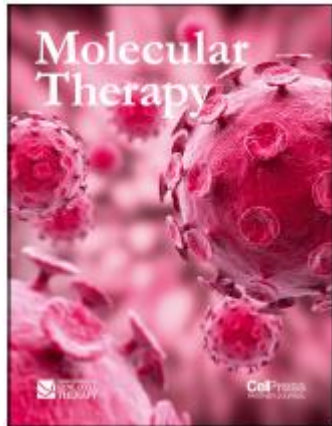
<https://doi.org/10.1038/s41591-018-0095-6>

nature
medicine

Suppression of antitumor T cell immunity by the oncometabolite (R)-2-hydroxyglutarate

Lukas Bunse^{1,2,3,4,33}, Stefan Pusch^{5,6,33}, Theresa Bunse^{1,3,7,33}, Felix Sahm^{5,6}, Khwab Sanghvi^{1,4},

The authors used CRISPR/Cas9 gesicles to knockout SLC13A3 in Jurkat cells.



Gesicle-Mediated delivery of CRISPR/Cas9 Ribonucleoprotein Complex for Inactivating the HIV Provirus.

Campbell LA, Coke LM, Richie CT, Fortuno LV, Park AY, Harvey BK
Molecular Therapy, Oct 11, 2018.

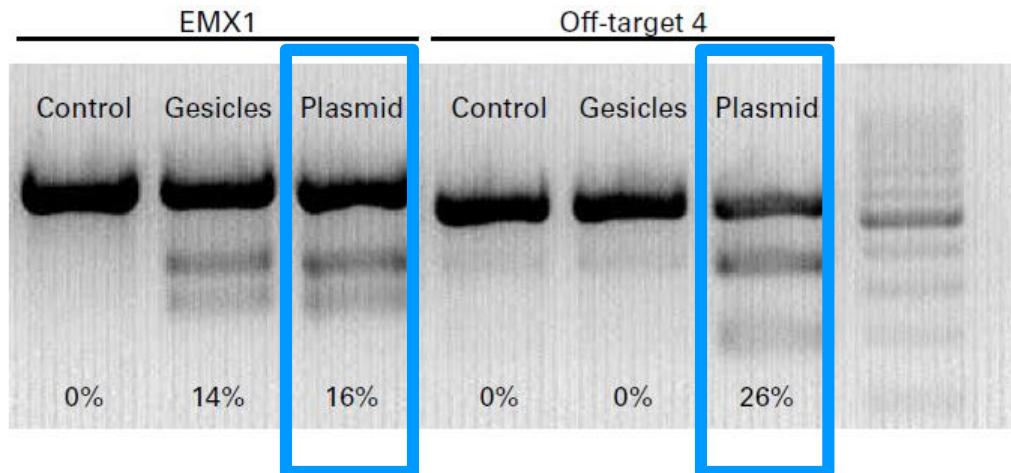
Gesicle-mediated delivery of Cas9 RNP successfully inactivated an HIV proviral genome in CHME-5 microglial cell line.

Reduced off-target effects

Target sequence *EMX1*:
GAGTCCGAGCAGAAGAAGAAGGG

Potential off-target sequence:
GAGTC**TA**AGCAGAAGAAGAAG**AG**

PAM
site



Cleavage indicates
Cas9-mediated
editing

Reduced off-target effects

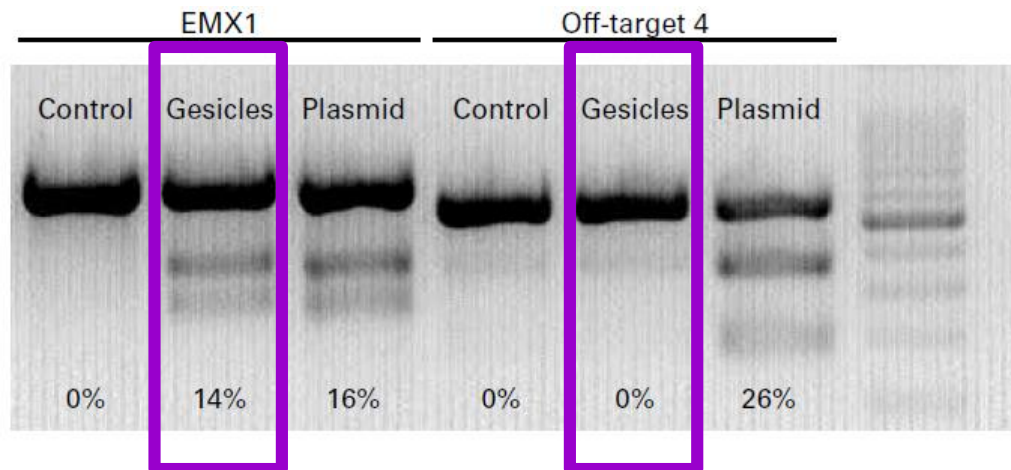
Target sequence *EMX1*:

GAGTCCGAGCAGAAGAAGAAGGGG

Potential off-target sequence:

GAGTCTAAGCAGAAGAAGAAGAG

PAM
site



← Cleavage indicates
Cas9-mediated
editing

RNP delivery via Electroporation

sgRNA
design &
production

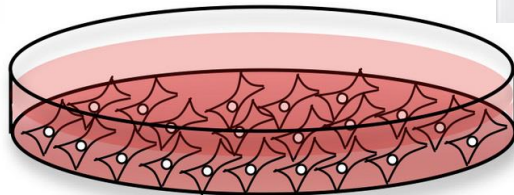
**Cas9/sgRNA
delivery into cells
(gene editing)**

Check editing
efficiency

Single-cell
cloning

Screening

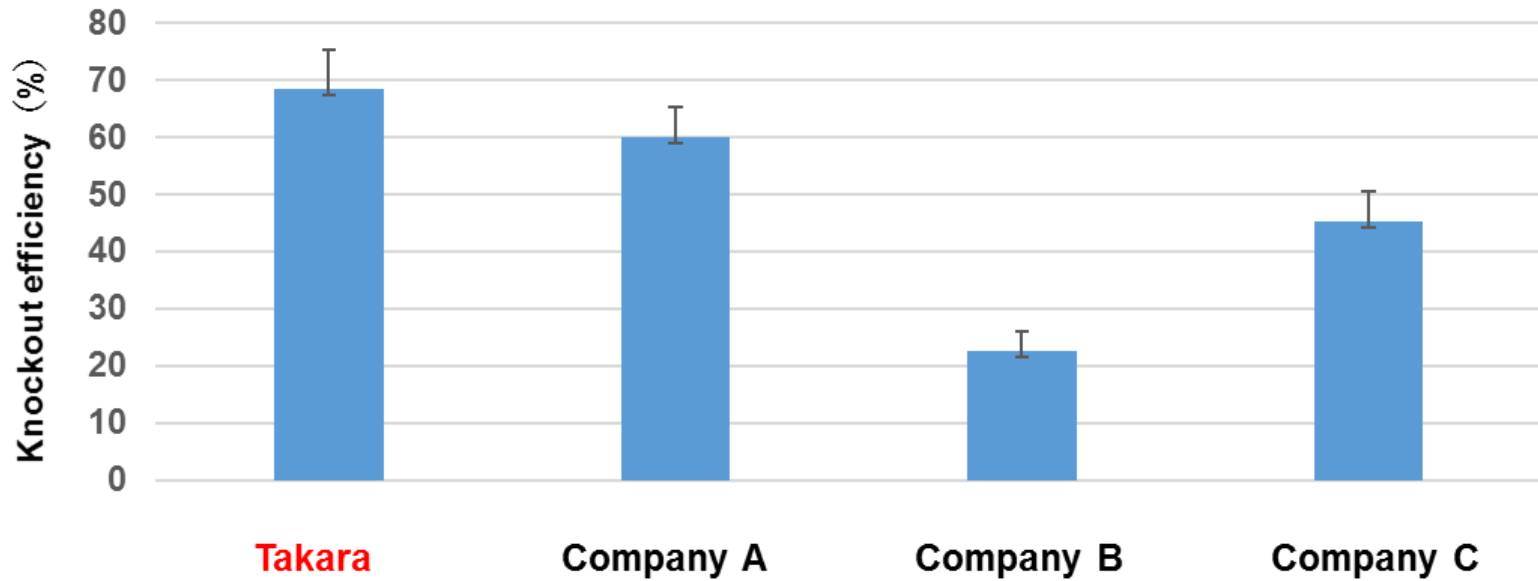
Electroporate Cas9
Ribonucleoprotein
(RNP) complexes



Guide-it Recombinant Cas9

- **Reduced off-target effects** compared to DNA delivery (plasmid, lentiviral)
- Suitable for **knockouts & knockins**
- Target hard-to-edit cells, including primary hematopoietic cells
- High concentration (3 ug/ul) & **lower glycerol (10%)** compared to other vendors enzymes, ensuring highly efficient delivery via electroporation

rCas9 – low glycerol ensures high efficiency



Knockout of CD81 in iPSCs

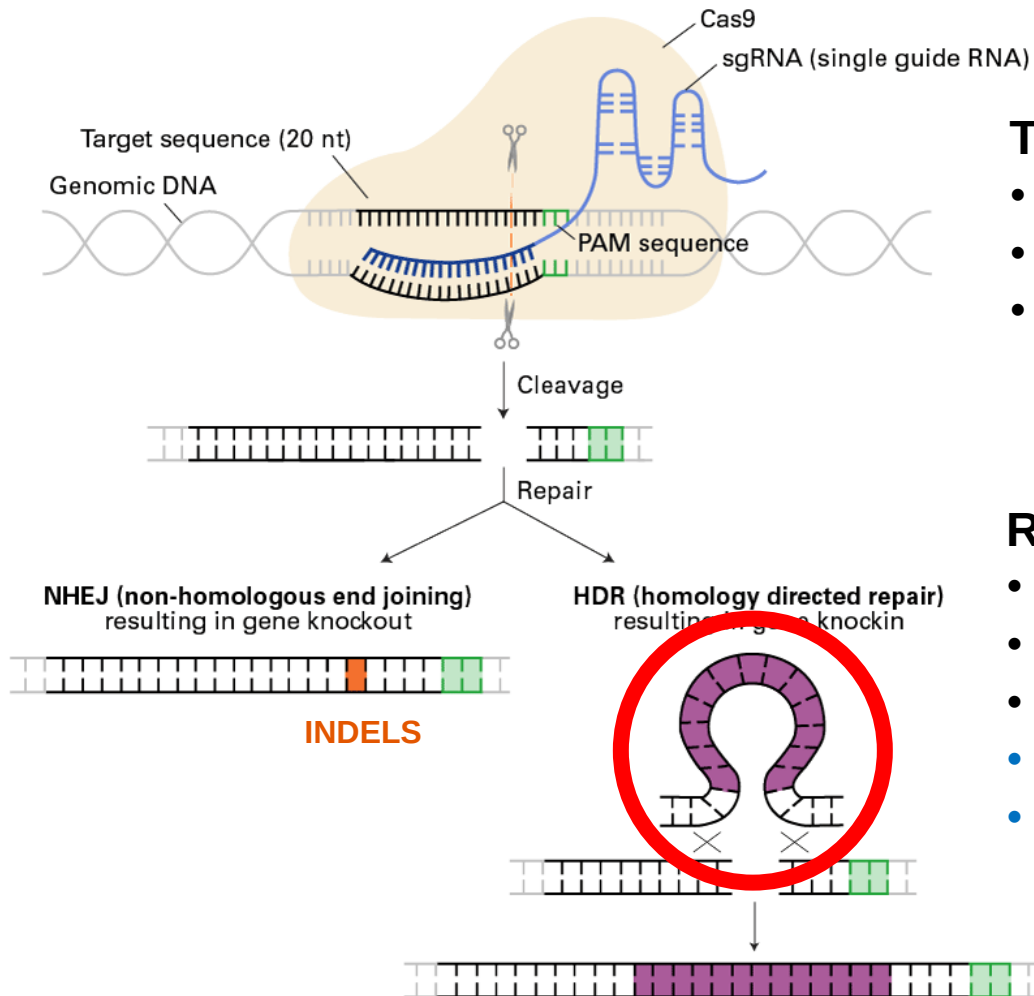
Electroporation using Neon Transfection System (Thermo Fisher Scientific)

For all experiments, the same sgRNA (produced with Guide-it IVT kit) has been used
N=2 × 3 times

This presentation

- CRISPR/Cas9 principle and workflow
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 - Electroporation
- Knockin of short versus long sequences
 - Limitations of knockin using long sequences
 - Benefits of single-stranded DNA HDR templates
 - Guide-it™ SNP Screening Kit
 - Guide-it™ Long ssDNA Production System

Knockin with CRISPR/Cas9



Three Components required:

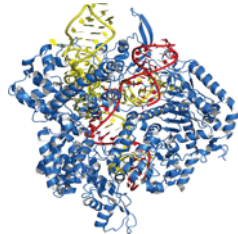
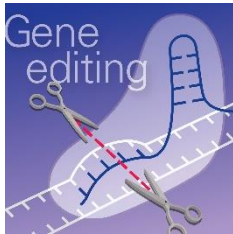
- Cas9 nuclease
- Guide RNA
- Repair template

Repair template production methods:

- PCR
- Plasmid
- AAV
- Synthetic oligos (ssODN)
- Guide-it Long ssDNA Production System

Knockin with CRISPR/Cas9 - Limitations

- Gene knockout using CRISPR/Cas9 is fairly routine
 - Delivery of Cas9/gRNA RNP complexes reduces off-target edits
 - Design of guide RNA is important, use optimized scaffold
 - Typical %KO = 50-90%
- Knockin of SNPs using ssODNs <200nt is also straightforward
 - Typical %KI = 5-40%
- Knockin of long sequences is not so easy
 - Typical %KI = <10%
 - dsDNA HDR templates can be toxic and often integrate randomly
 - Lack of easy method to produce long ssDNA donors



CCCGC	GCCGCCAT	GGGAG
CCCGC	GCCGCCAT	GGGAG
CC	-----T	GGGAG
CCCGC	GCCGCCAT	GGGAG
CC	TGCC-----GGAG	
CCCGC	GCCGCCA	---AG

Knockin of small sequences using ssODNs and electroporation

Use of ssODN donors

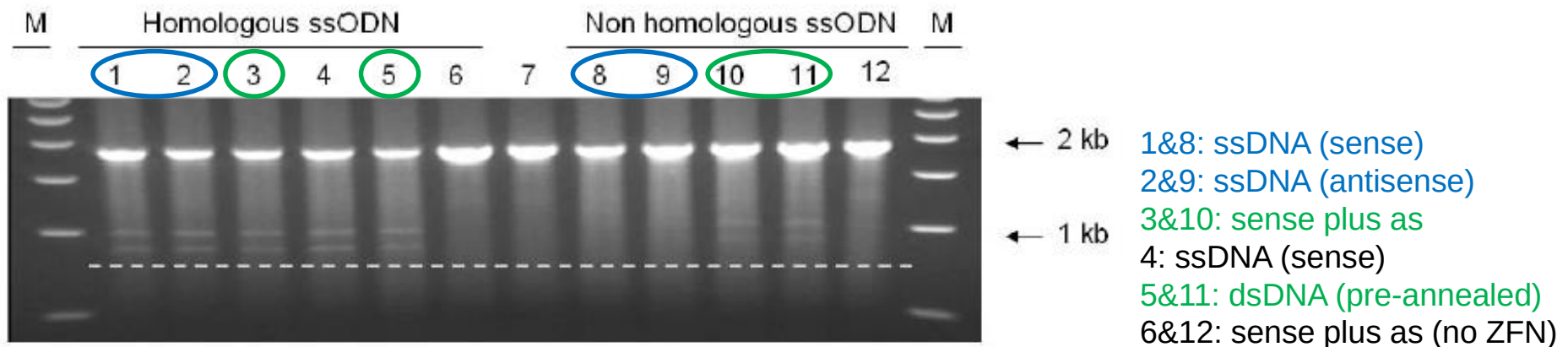
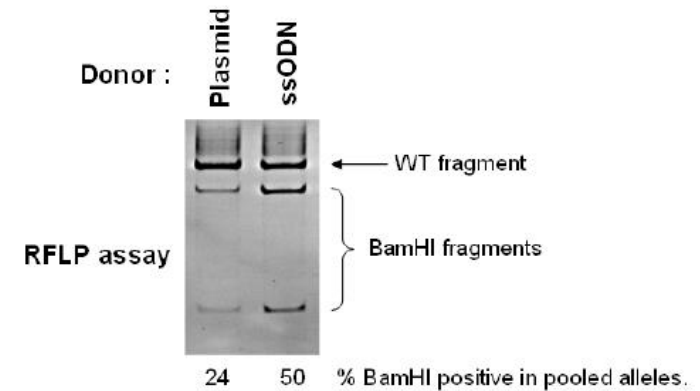
High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases

Fuqiang Chen, Shondra M Pruett-Miller, Yuping Huang, Monika Gjoka, Katarzyna Duda, Jack Taunton, Trevor N Collingwood, Morten Frodin & Gregory D Davis

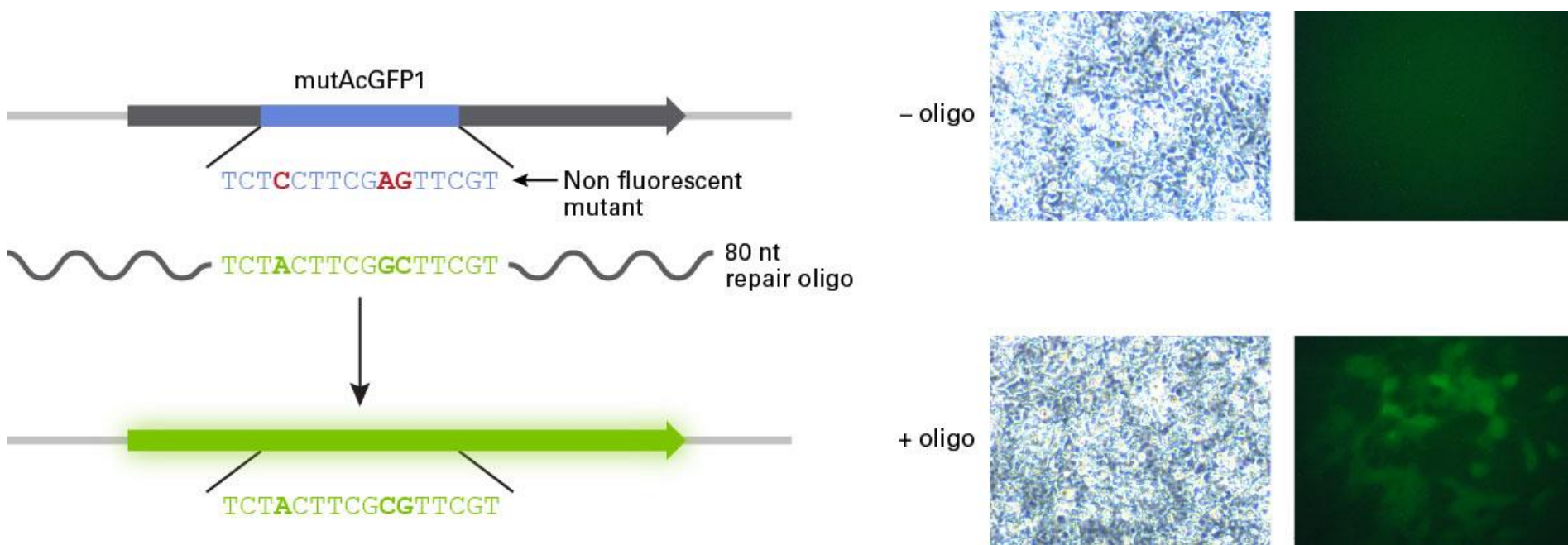
Affiliations | Contributions | Corresponding author

Nature Methods **8**, 753–755 (2011) | doi:10.1038/nmeth.1653

Received 04 April 2011 | Accepted 27 June 2011 | Published online 17 July 2011

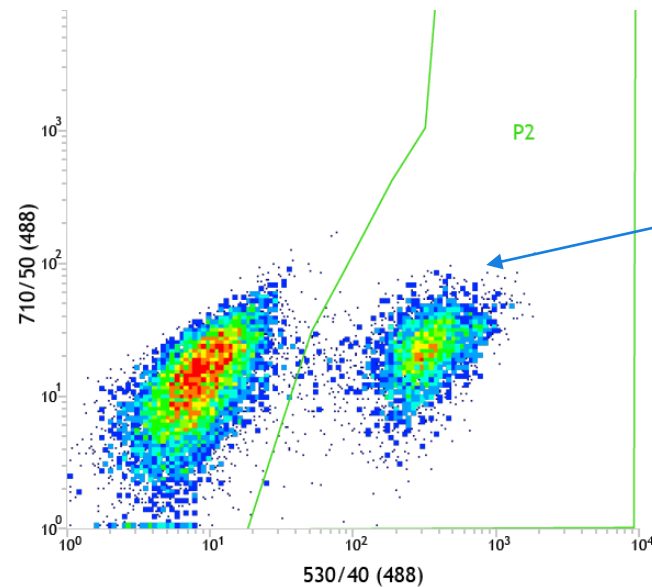
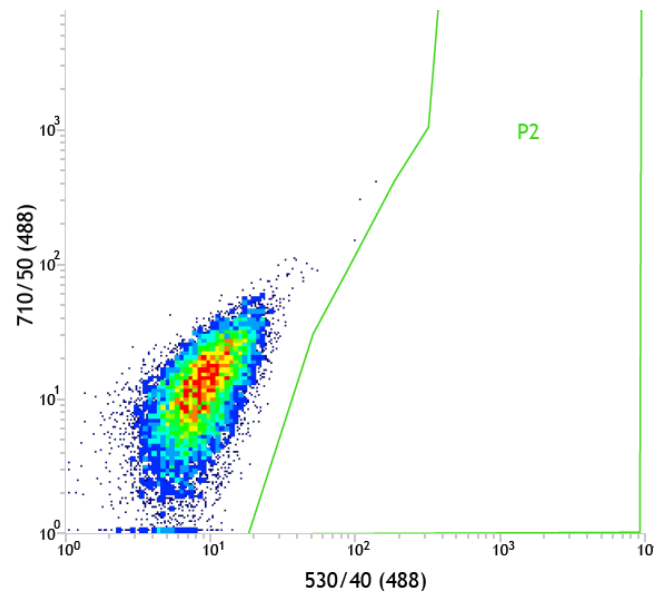


SNP repair in iPS cells using an 80nt ssODN



Repair of point mutation in AcGFP1

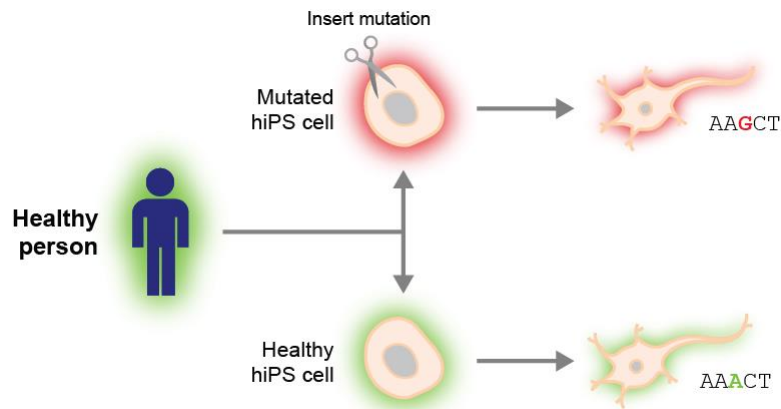
- 1.5 µg Guide-it Recombinant Cas9
- 0.5 µg sgRNA (Guide-it sgRNA IVT Kit)
- 1 µg repair oligo (ssODN)
- 1.5×10^5 iPS cells
- Electroporation using Neon Transfection System (ThermoFisher)



29% of cells repaired

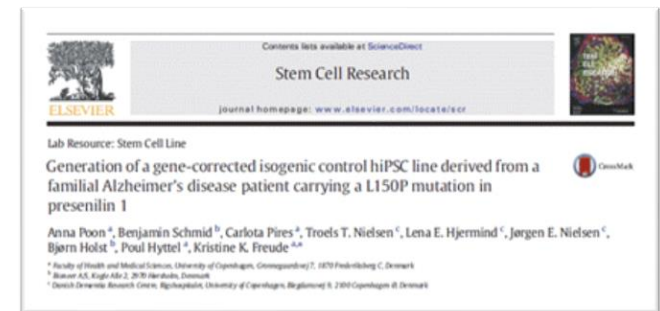
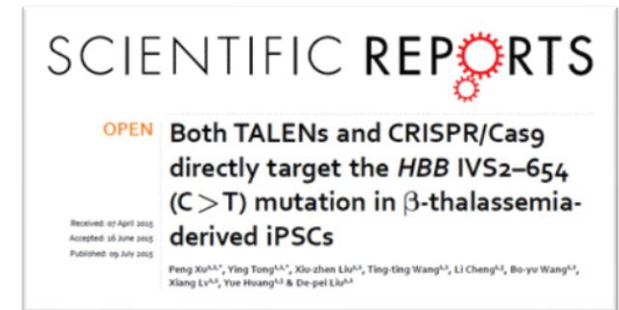
Engineering single nucleotide substitutions

Creation of isogenic cell lines to model SNP-related diseases

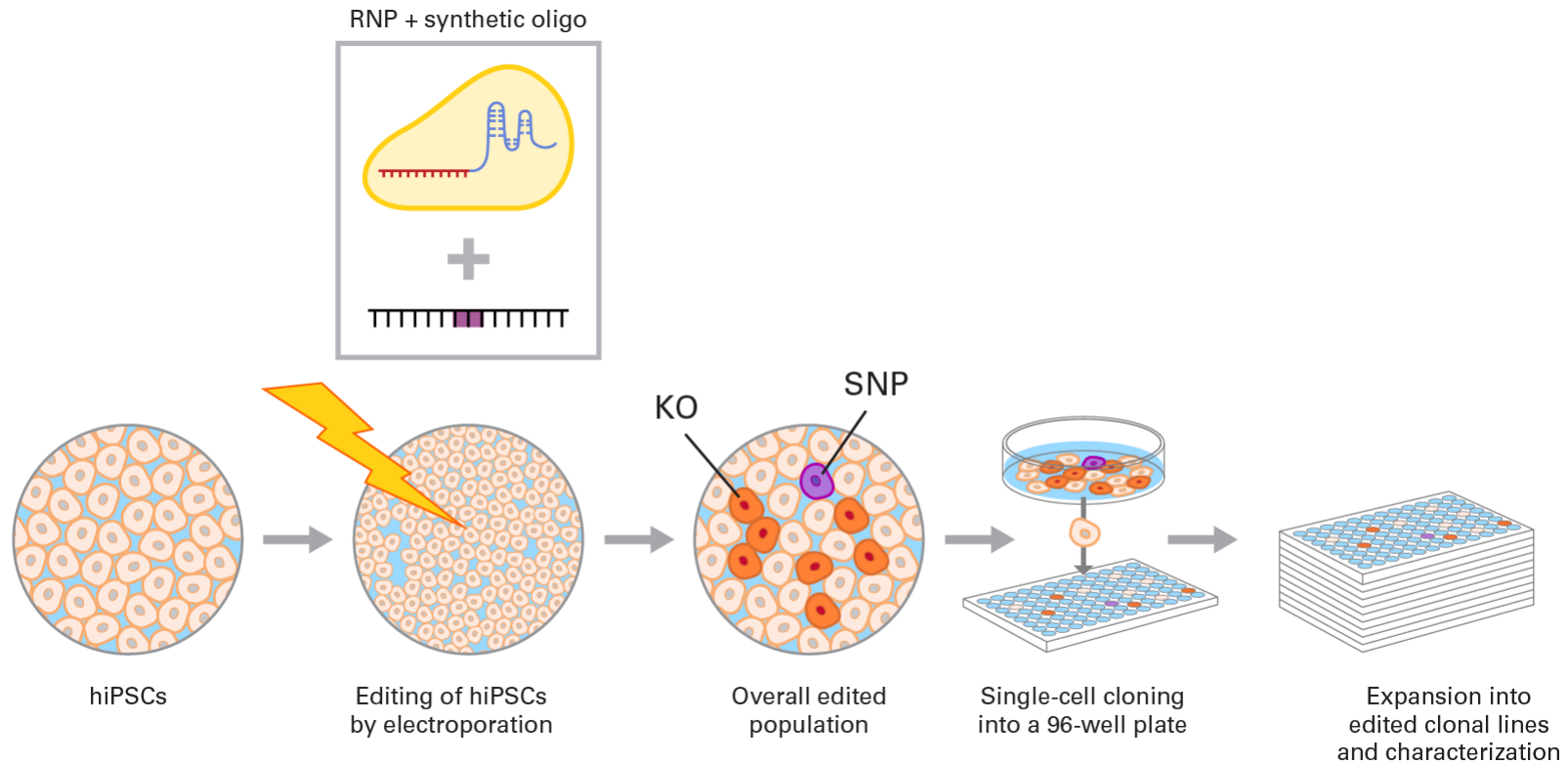


Pathogenic human SNPs
(approx. 32,000)

Disease	Gene	SNP
Retinitis pigmentosa	DHDDS	c.124A>G (p.Lys42Glu)
Spinocerebellar ataxia 21	TMEM240	c.509C>T (p.Pro170Leu)
Cardiomyopathy 1A	LMNA	c.673C>T (p.Arg225Ter)
Usher syndrome	USH2A	c.14803C>T (p.Arg4935Ter)



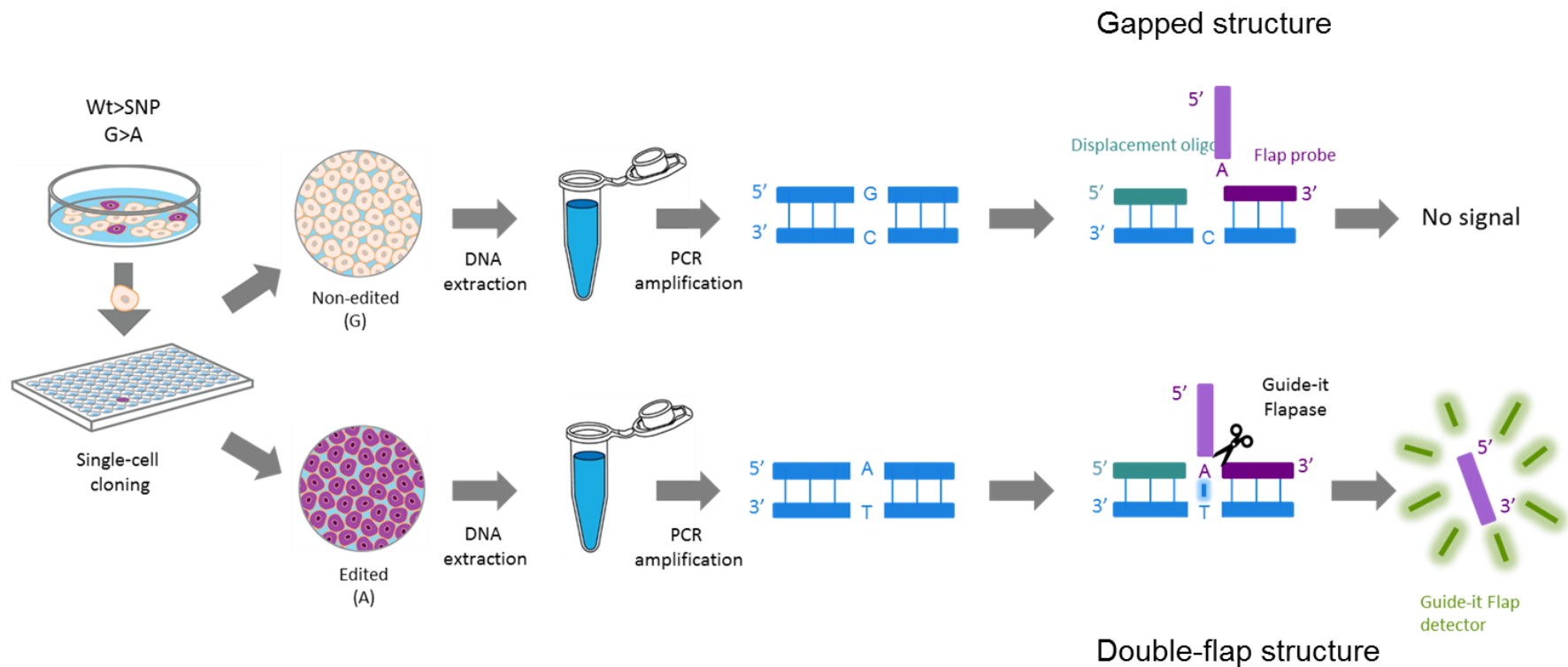
Workflow of knockin experiments



**Detection of
successful HDR in
edited population**

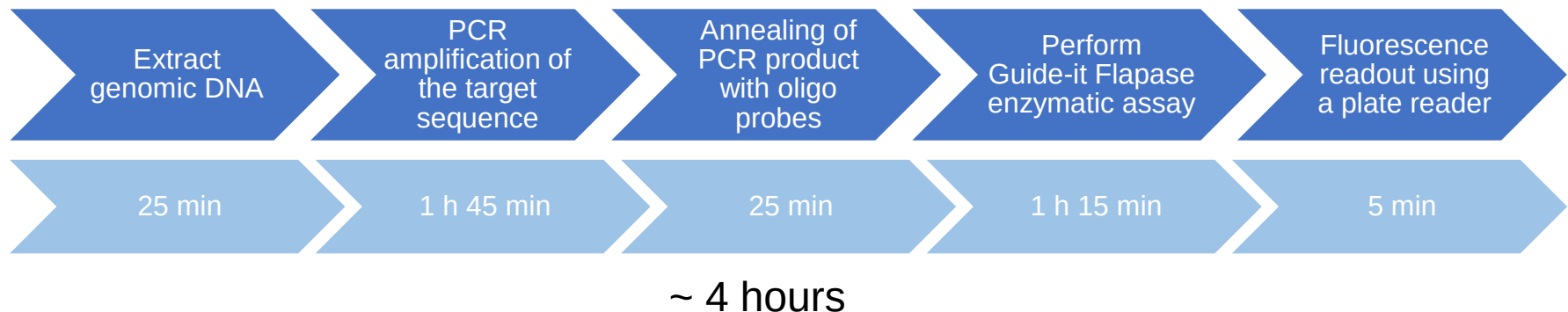
**SNP detection
in single
clones**

Guide-it SNP Screening Kit



Guide-it SNP Screening Kit

- Fast (4 hrs) and high-throughput SNP detection in any genomic region
- Qualitative (yes/no) output (fluorescence readout)
- Detection of HDR in edited cell population and in single clones
- Reliable results without PCR optimization



Screening for SNPs related to tyrosinemia

FAH c.1009G>A (p.Gly337Ser)

sgRNA #1

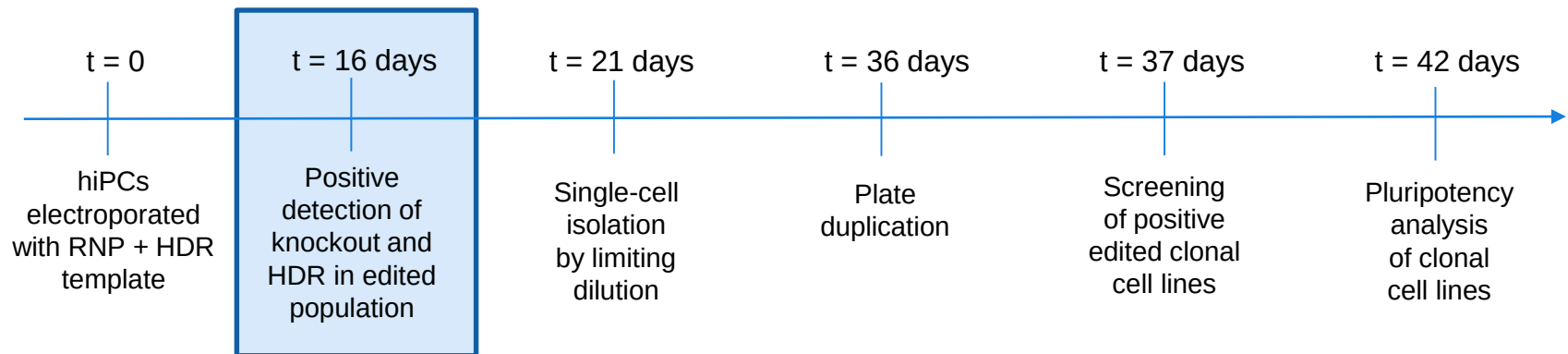
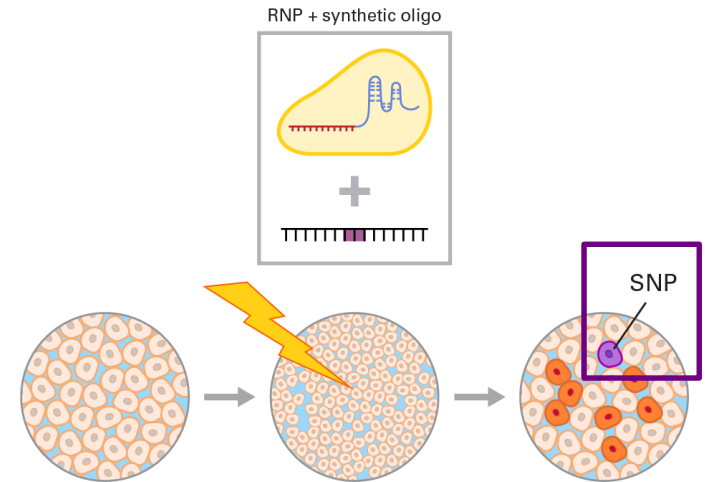
sgRNA #2

wt CAGCTCACTCACCACCTCTGTCAAC**G**GCTGCAACCTGCGGCC

sgRNA #3

PvuII

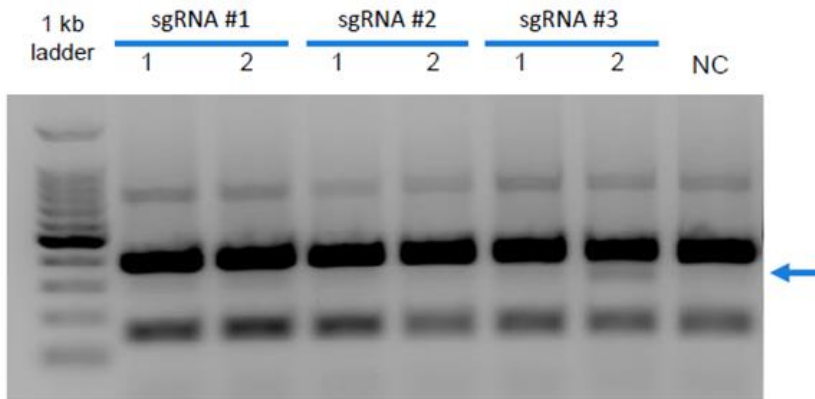
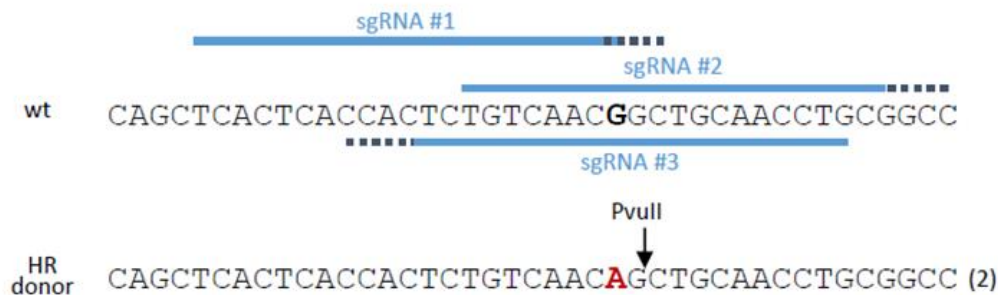
HR donor CAGCTCACTCACCACCTCTGTCAAC**A**GCTGCAACCTGCGGCC



SNP detection in edited cell population

RFLP assay

FAH c.1009G>A (p.Gly337Ser)

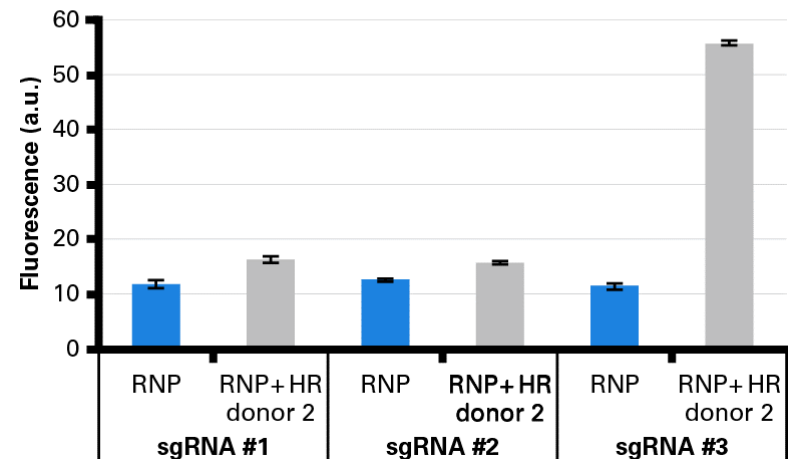


1: RNP only

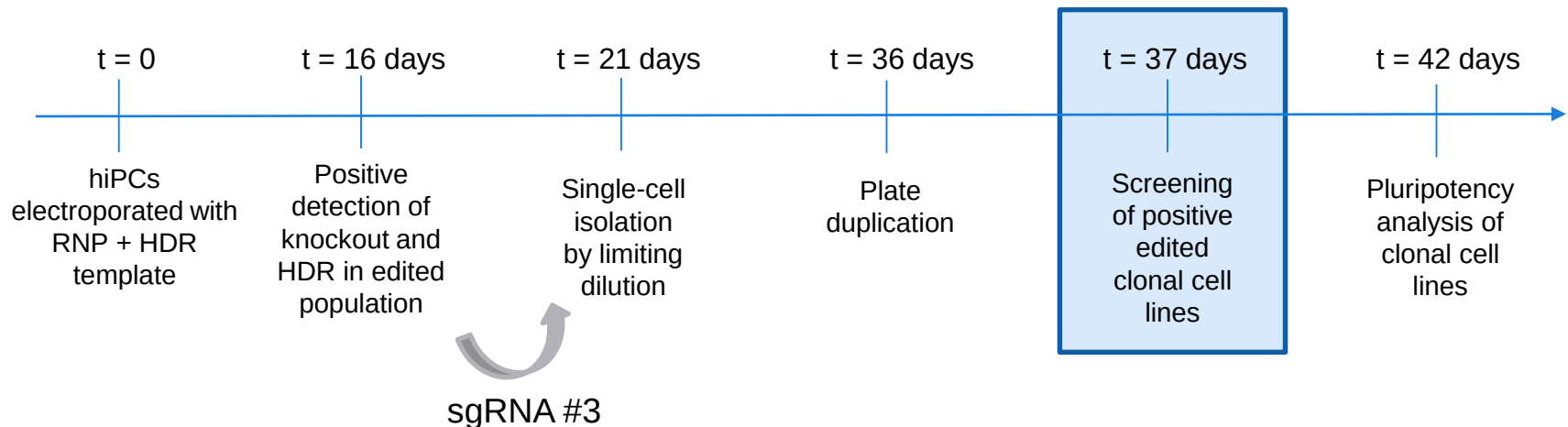
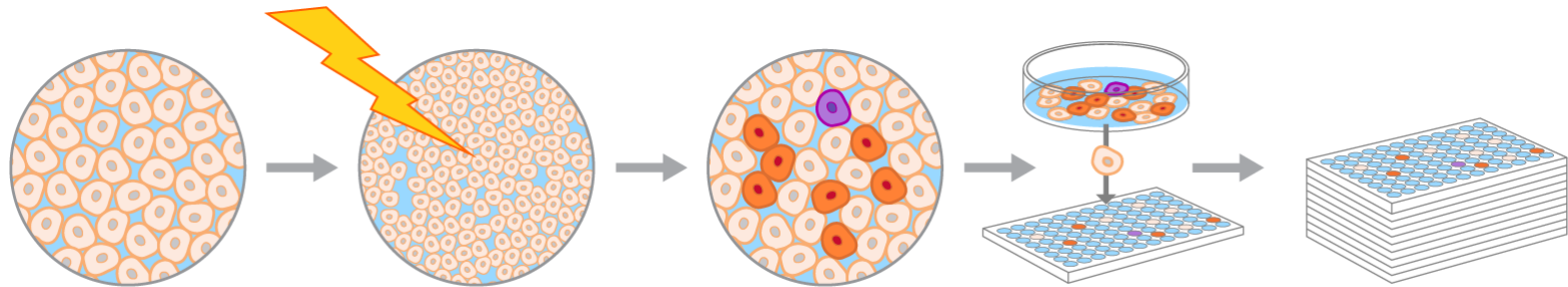
2: RNP + HR donor 2

SNP Screening Kit

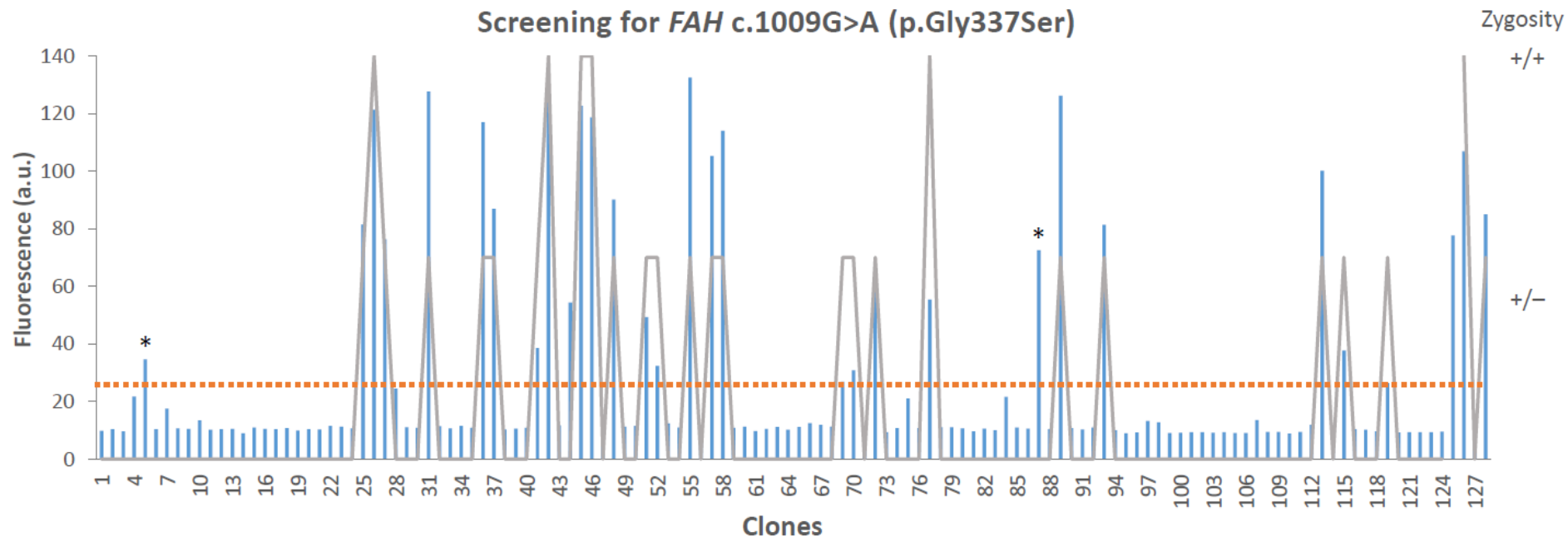
FAH c.1009G>A (p.Gly337Ser)



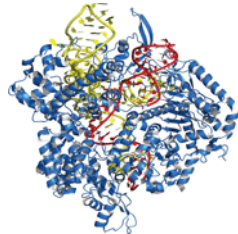
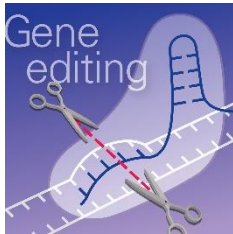
Screening for SNPs related to tyrosinemia



SNP Screening in single clones



- 19–24% of the clonal cell lines generated a positive fluorescent signal
- The existence of the SNPs was confirmed by Sanger sequencing



CCCGC	GCCGCCAT	GGGAG
CCCGC	GCCGCCAT	GGGAG
CC	-----T	GGGAG
CCCGC	GCCGCCAT	GGGAG
CC	TGCC-----	GGGAG
CCCGC	GCCGCCA	---AG

Knockin of large >200nt
sequences by electroporation

Donor templates for long sequences

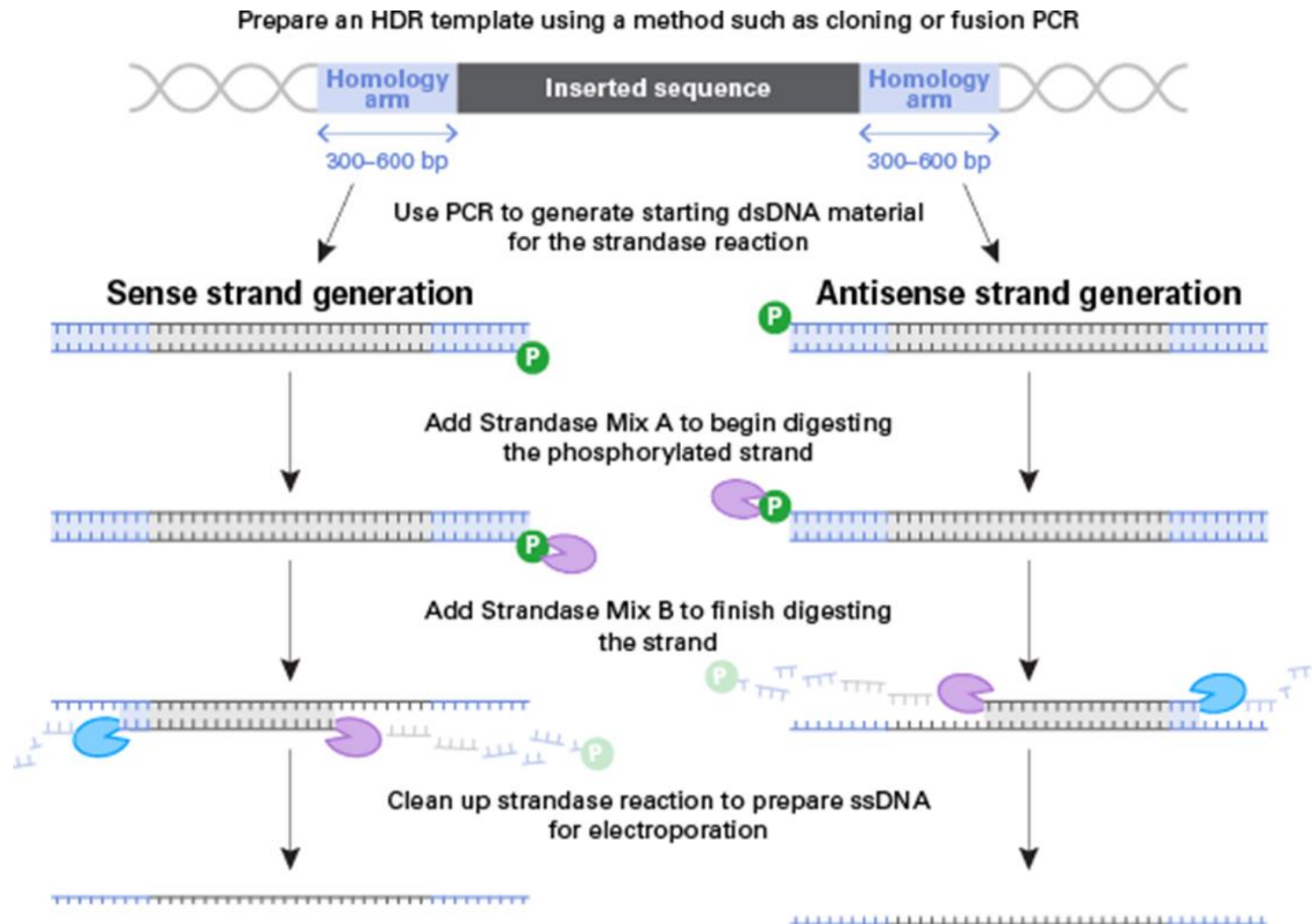
- PCR products and plasmids
 - Easy and inexpensive to create
 - Often toxic when delivered
 - Can integrate randomly
- Single stranded DNA oligos (ssODN)
 - Far less toxic
 - Far more specific
 - SNP repair - very efficient
 - Long sequences - size limitation (2 kb) & cost

Guide-it™ Long ssDNA Production System

Kit for producing **ssDNA** for knockin of **long sequences**, starting from a PCR product

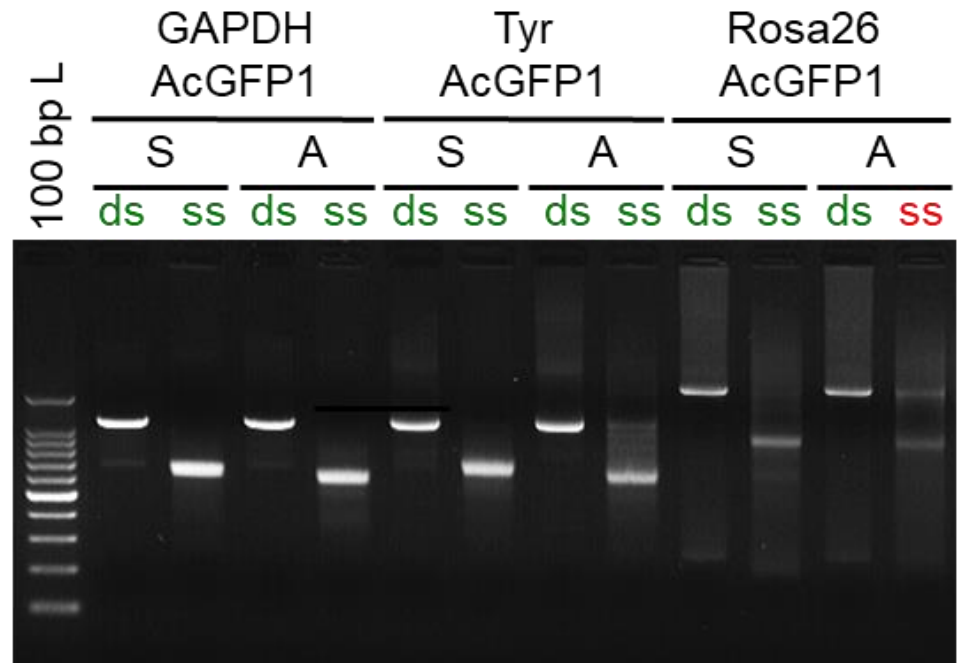
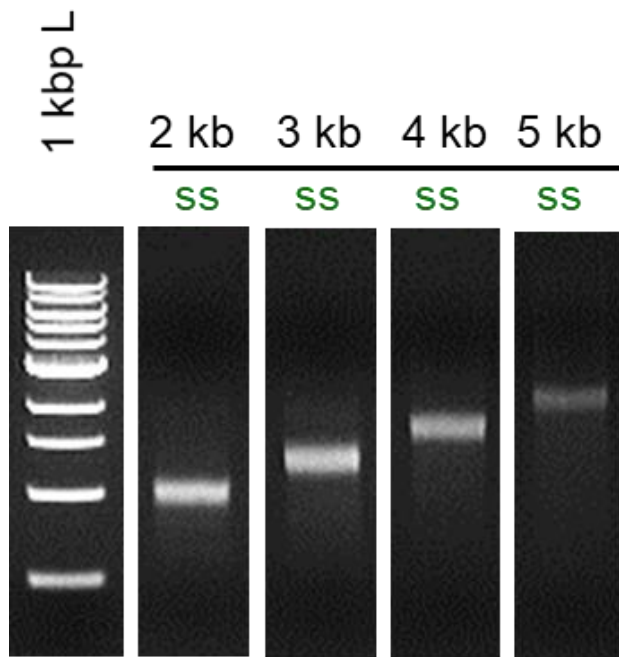
- Works for sequences from **0.5–5 kb**
- The resulting ssDNA can be combined with recombinant Cas9 protein and target specific sgRNA for **electroporation** into target cells
- ssDNA produced in this way is superior to dsDNA repair templates for gene editing:
 - **lower toxicity**
 - **no random integration**
 - **no background expression**

Principle of long ssDNA Production

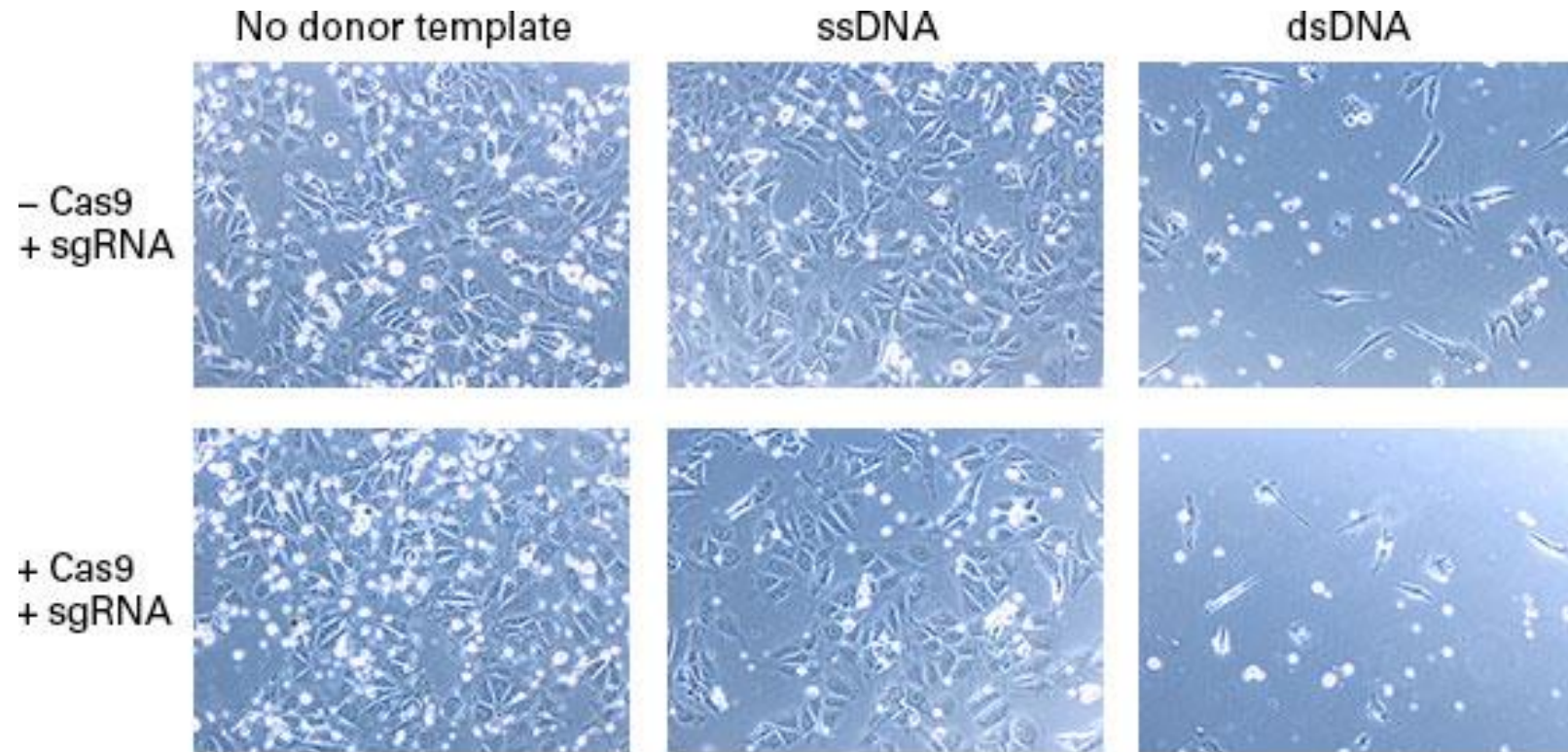


Long ssDNA Production

- ssDNA production **up to 5 kb**.
- Each reaction produces **~2-4 µg of each strand** (sense and antisense).
- **Single band** should be visible on Agarose gel. Difficult targets – shift primer position by 1 nt.

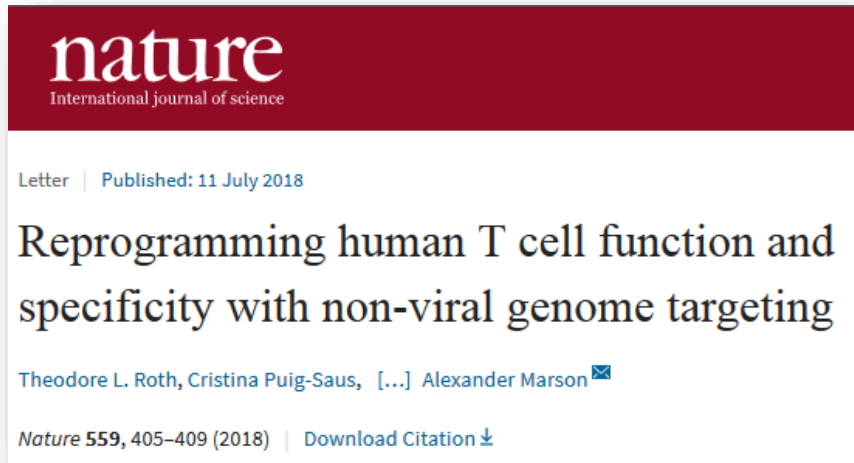


dsDNA vs ssDNA – Toxicity

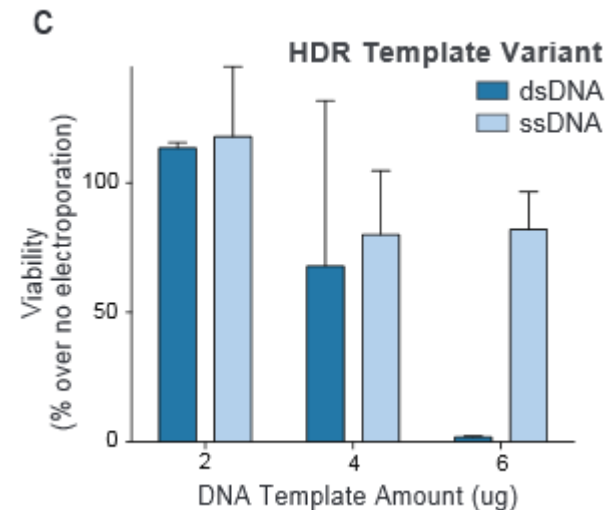


Electroporation of Cellartis hiPSC18 cells to tag tubulin with AcGFP1 (1.5 kb donor templates with 350 bp homology arms).

Comparison with dsDNA HDR templates



Long ssDNA donors are less toxic



At 4 days post-electroporation, long ssDNA HDR templates (~1.3 kb) did not show the decreasing viability in CD3+ T cells electroporated with a linear dsDNA HDR template of the same length.

Comparison with dsDNA HDR templates

nature
International journal of science

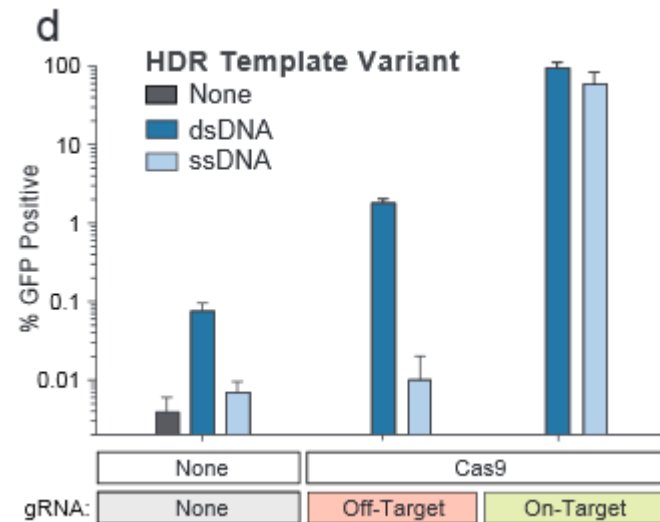
Letter | Published: 11 July 2018

Reprogramming human T cell function and specificity with non-viral genome targeting

Theodore L. Roth, Cristina Puig-Saus, [...] Alexander Marson [✉](#)

Nature 559, 405–409 (2018) | [Download Citation](#) [↓](#)

Long ssDNA donors are more specific



ssDNA HDR templates reduced functional off-target integrations approximately 100-fold, while maintaining efficient on-target integration.

Summary

- Vesicles and electroporation are tools to effectively deliver **Cas9/sgRNA RNP complexes** to a broad range of cell types.
 - No Cas9 coding sequence present: no chance for genomic integration, no persistent nuclease overexpression → **Reduced off-target effects**
- dsDNA HDR templates can result in **random integration and background expression**, ssDNA HDR templates integrate with very high **specificity**
- dsDNA HDR templates can cause **cellular toxicity**, ssDNA templates do not
- The Guide-it SNP Screening Kit is a fast method for SNP detection in **edited populations** as well as **clonal cell lines**
- The Guide-it Long ssDNA Production System is a simple method to **produce ssDNA donor templates up to 5 kb**

that's
GOOD
science!®