



Hematopoietic Innovative Therapies



*Example. Gene Therapy of primary cells with CRISPR:*  
**Gene Editing to correct Pyruvate Kinase Deficiency**

**Oscar Quintana Bustamante, PhD.**

*Differentiation and Cytometry Unit*

*Hematopoietic Innovative Therapies Division.*

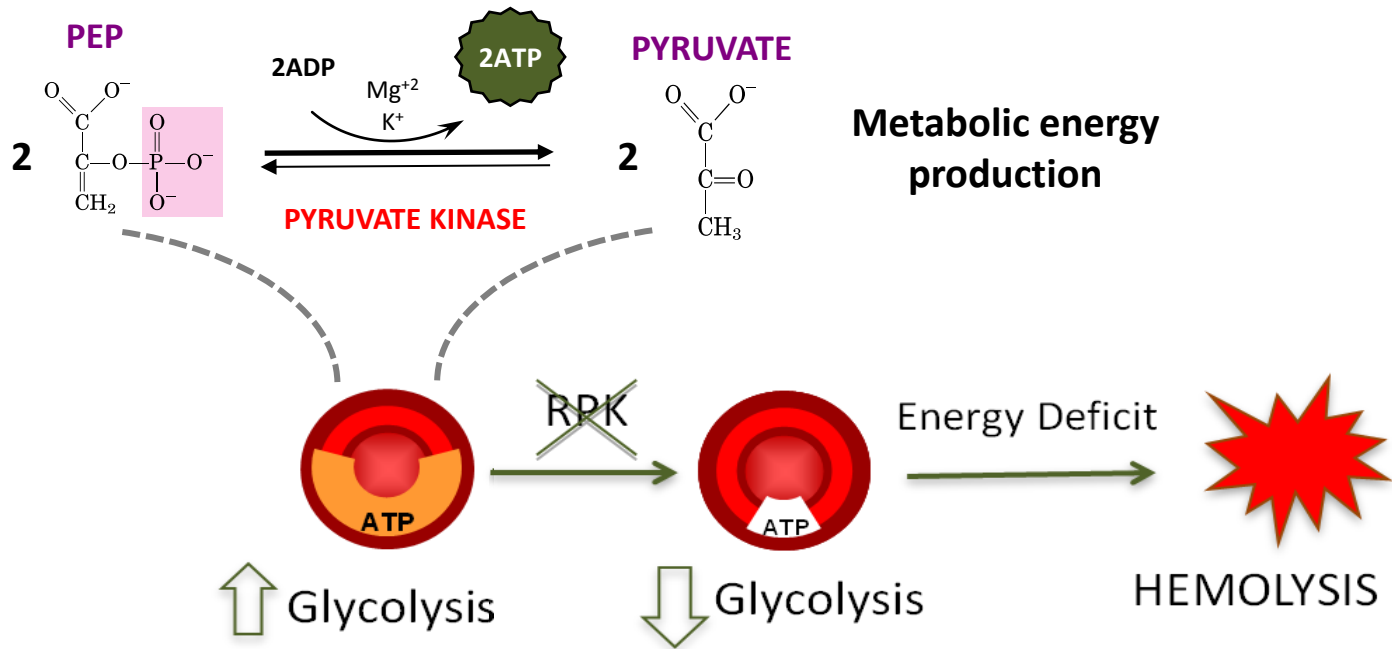
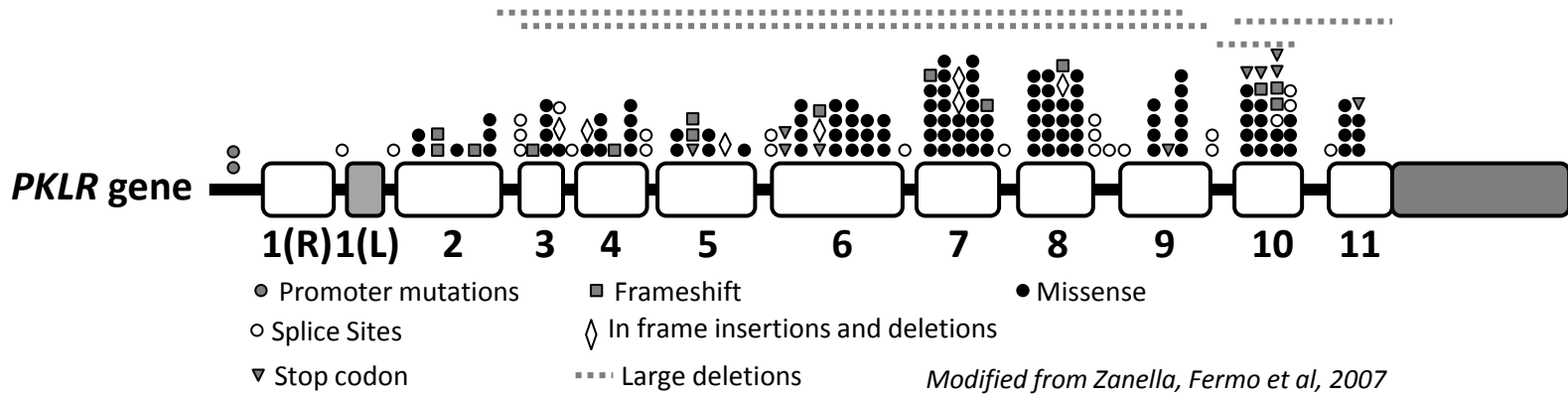
CIEMAT/CIBERER/IIS-FJD

[oscar.quintana@ciemat.es](mailto:oscar.quintana@ciemat.es)

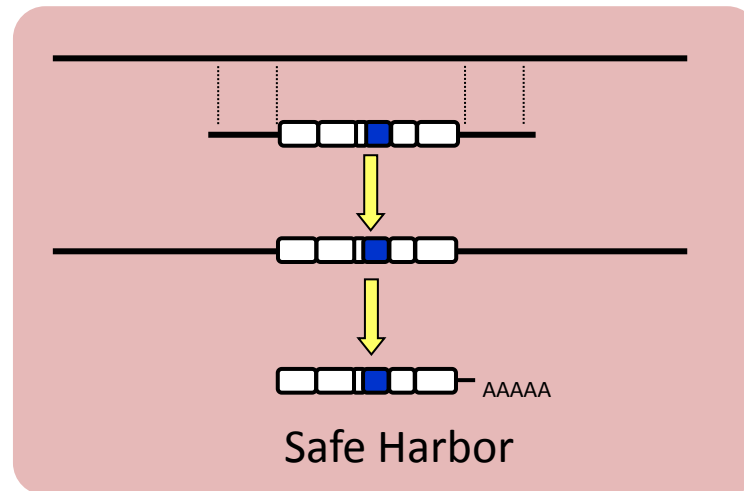
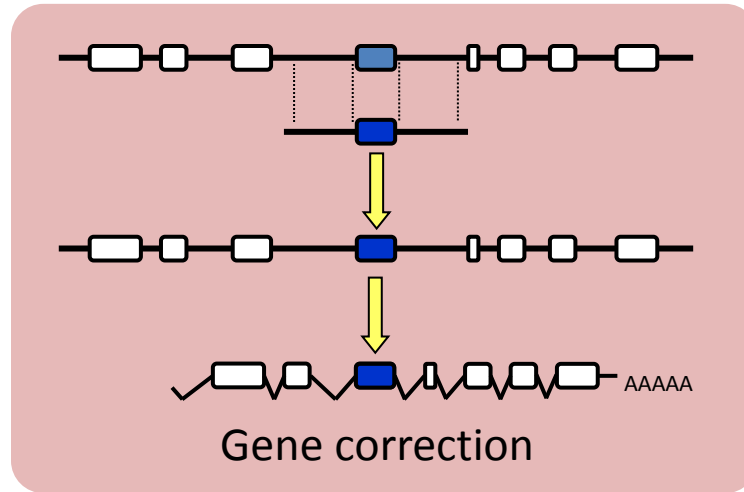
## Summary

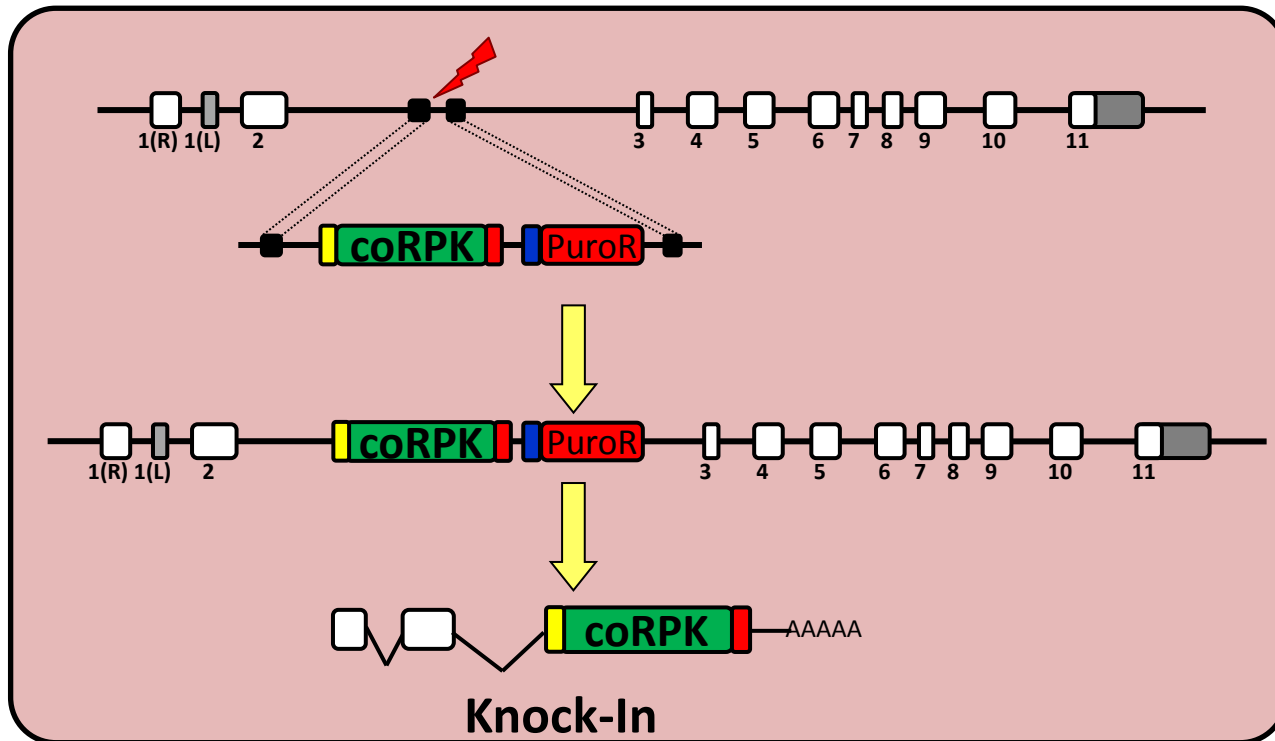
1. Pyruvate Kinase Deficiency
2. Gene Editing Strategies
3. “Proof of concept”: PKDiPSC
4. Gene editing in Hematopoietic progenitors
  - a. “Old tools”
  - b. Patient-specific gene editing tool
  - c. Gene editing to correct all PKD patients

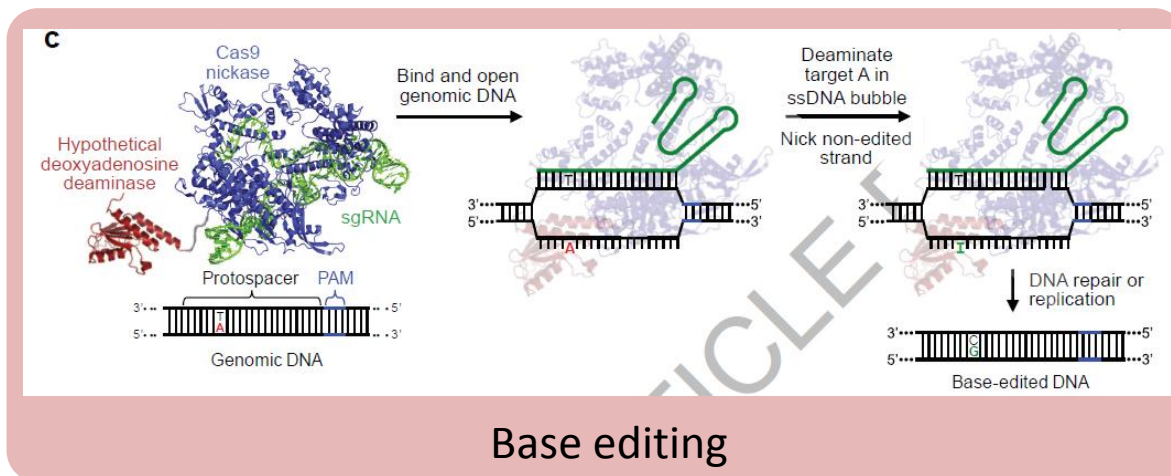
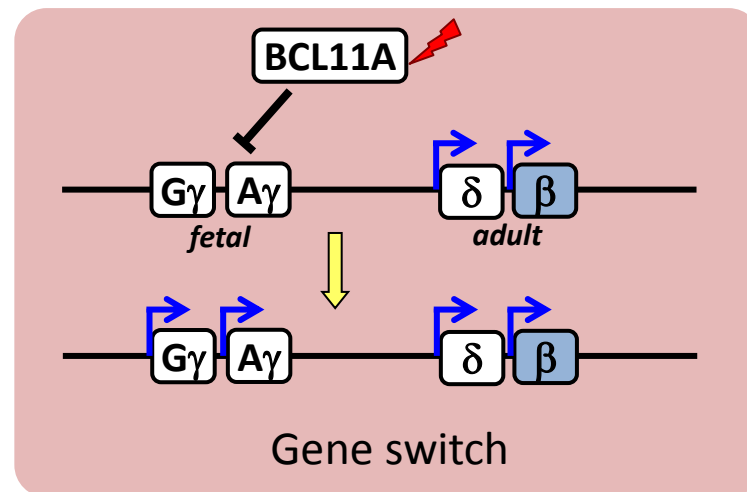
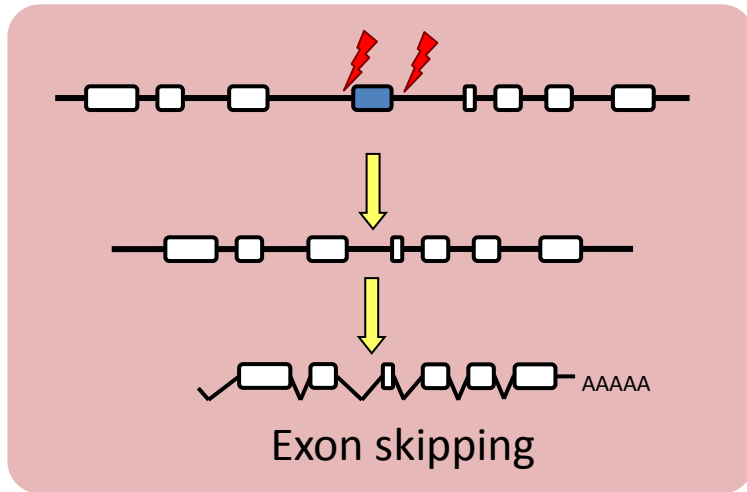
# Pyruvate Kinase Deficiency



**Definitive cure: Allogenic Hematopoietic Progenitor Transplantation**

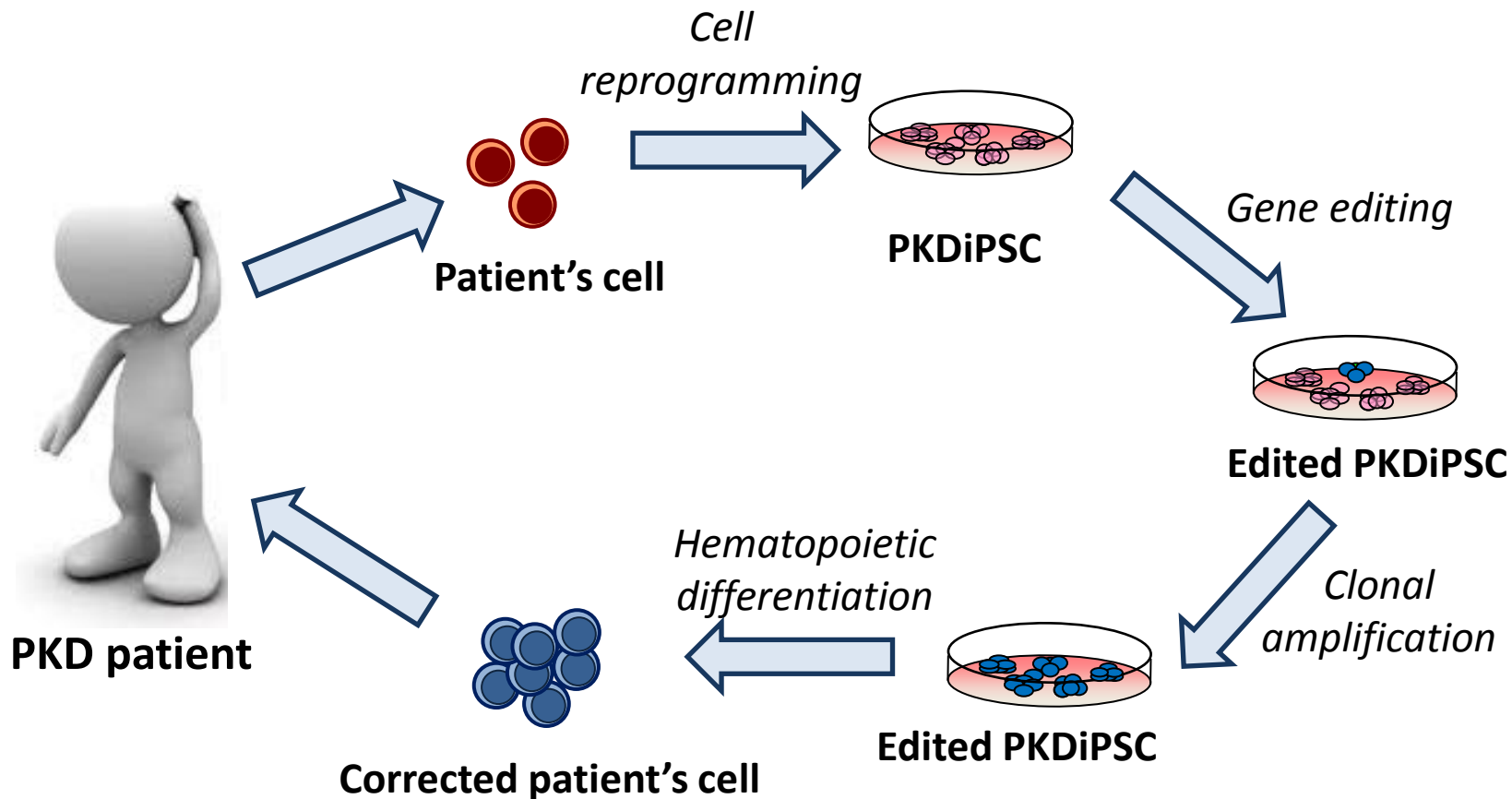




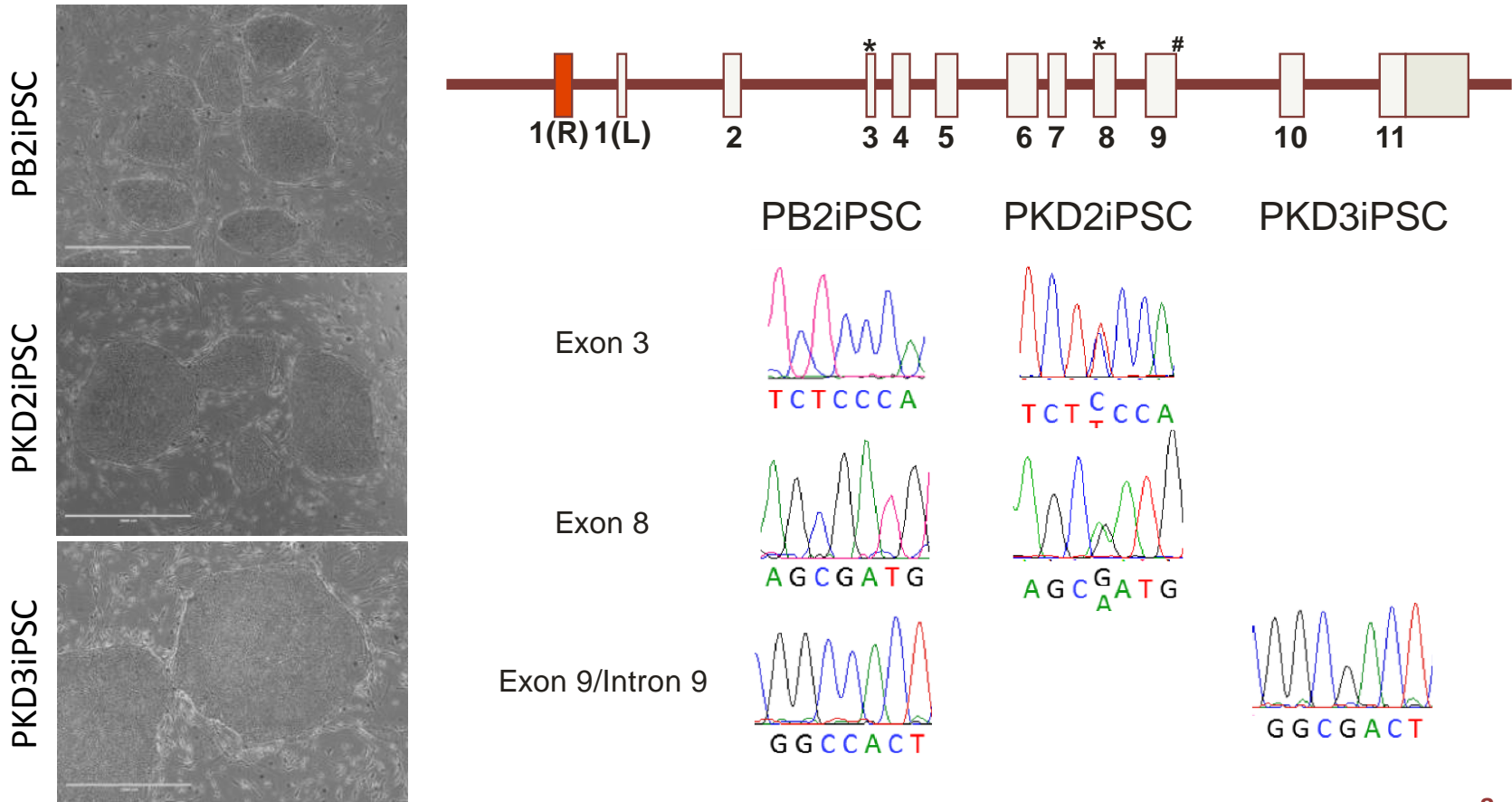
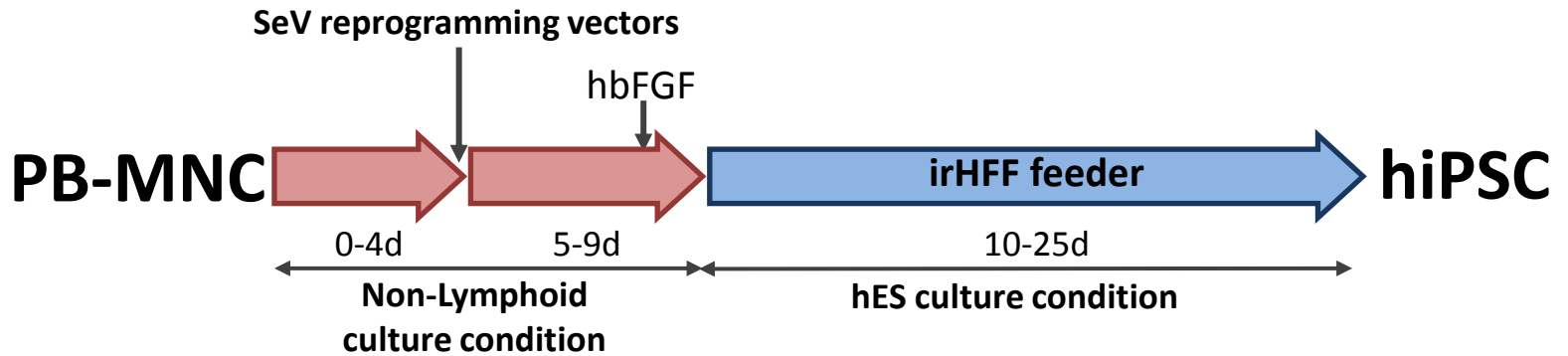


## Induced Pluripotent Stem Cells

*Long period of in vitro culturing to select “rare” gene edited Stem Cells*

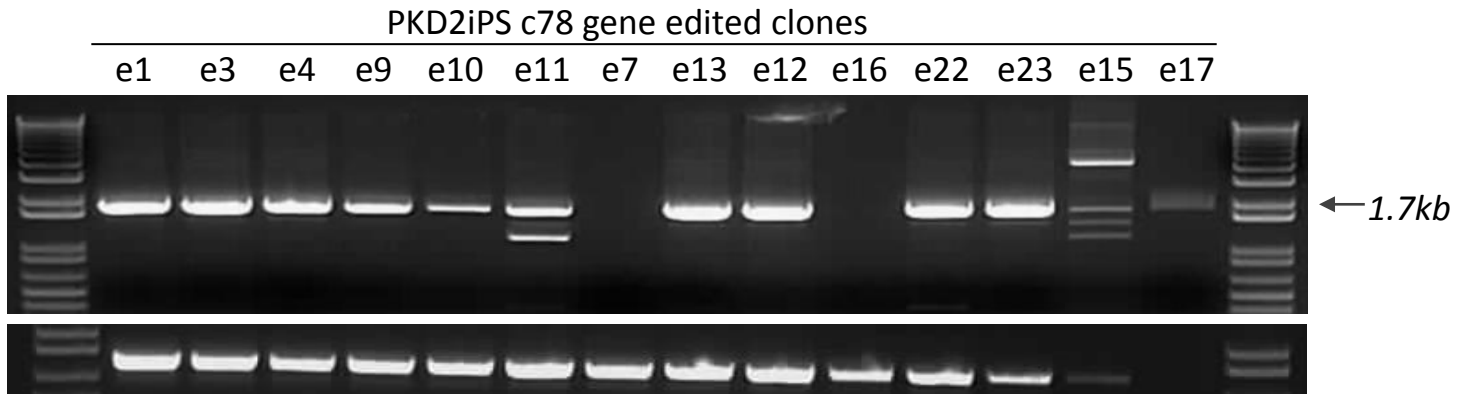
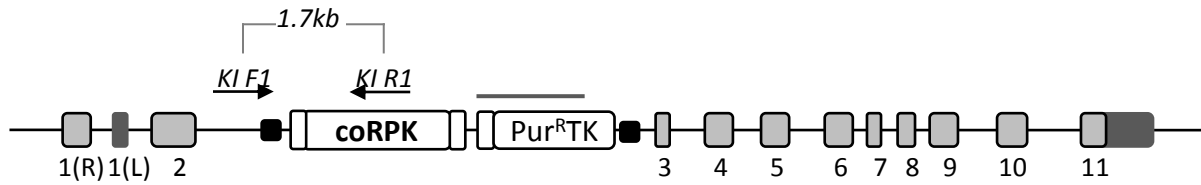
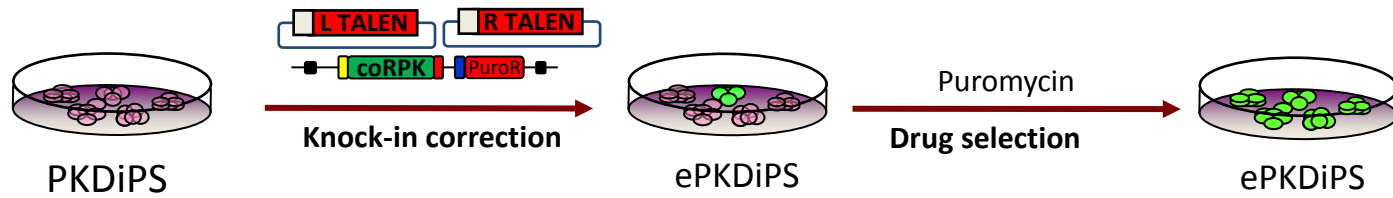








# “Proof of concept”: PKDiPSC



	PuroR clones	Edited clones*	Edited in both alleles	NHEJ in unedited allele
PKD2iPSC	<b>13</b>	<b>77%</b>	<b>0%</b>	<b>40%</b>
PKD3iPSC	<b>40</b>	<b>76%</b>	<b>11%</b>	<b>31%</b>

\*2.85 edited PKDiPSC per  $1 \times 10^5$  nucleofected cells

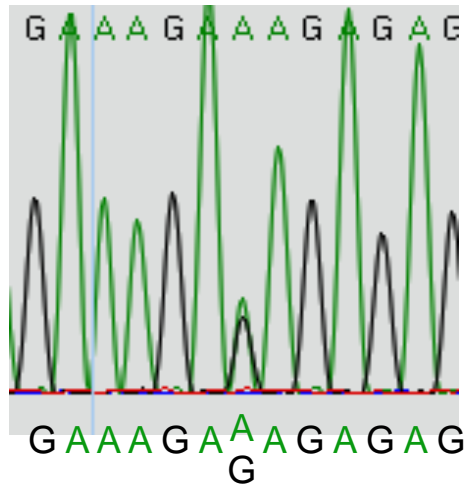
**Patient  
specificity**



**Nuclease  
activity**



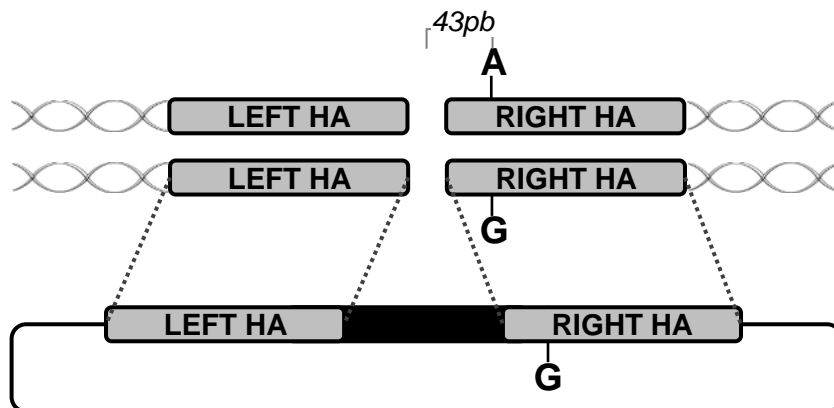
## SNP in PKD2 patient



## Sequencing of unedited allele

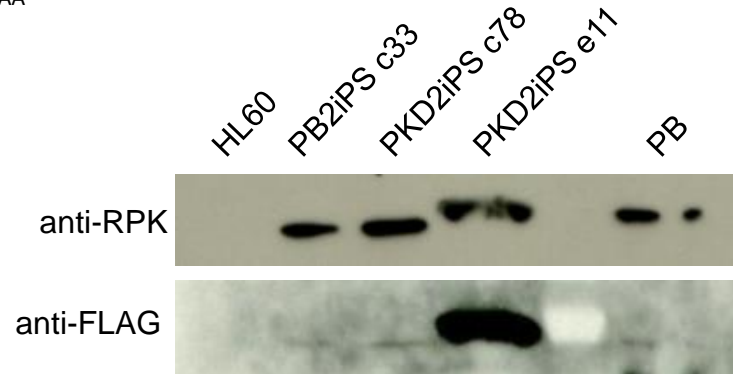
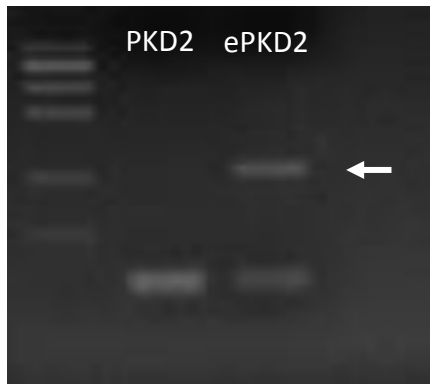
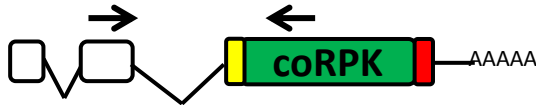
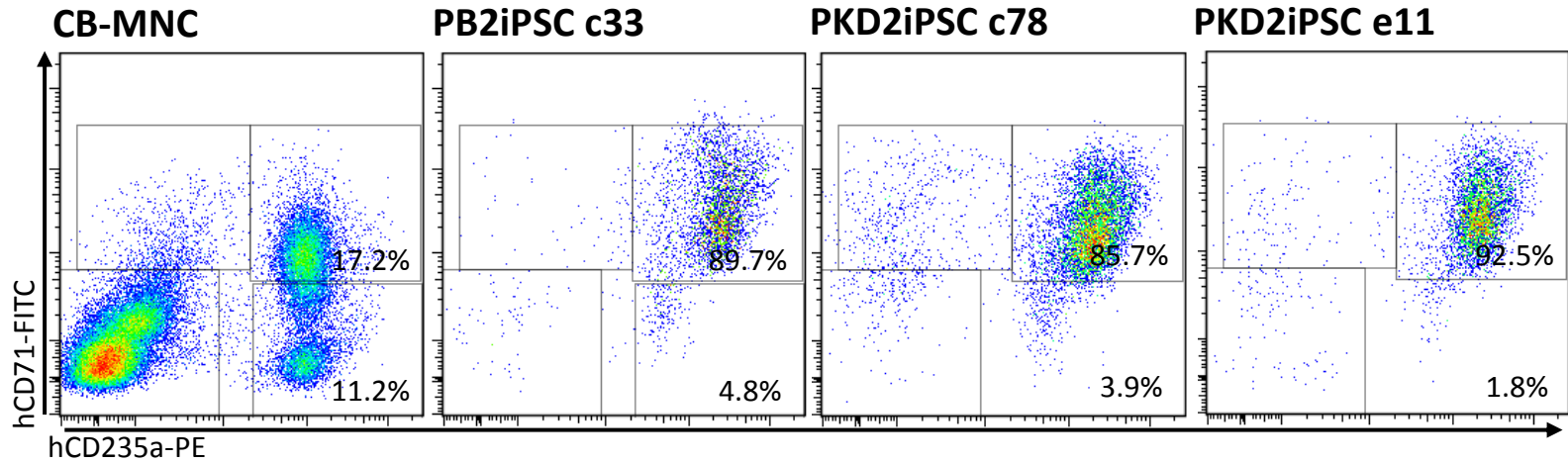
PKLR seq .aagaaaagaaaagaaaagagagagagagaaaagaagc

PKD2iPS e3	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
PKD2iPS e4	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
PKD2iPS e1	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
PKD2iPS e13	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
PKD2iPS e11	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
PKD2iPS e12	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
PKD2iPS e22	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
PKD2iPS e23	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
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PKD2iPS e9	PKLR On R	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC
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	PKLR On F	AAGAAAAGAAAAGAAAAGAAAGAGAGAGAAAAGAAGC



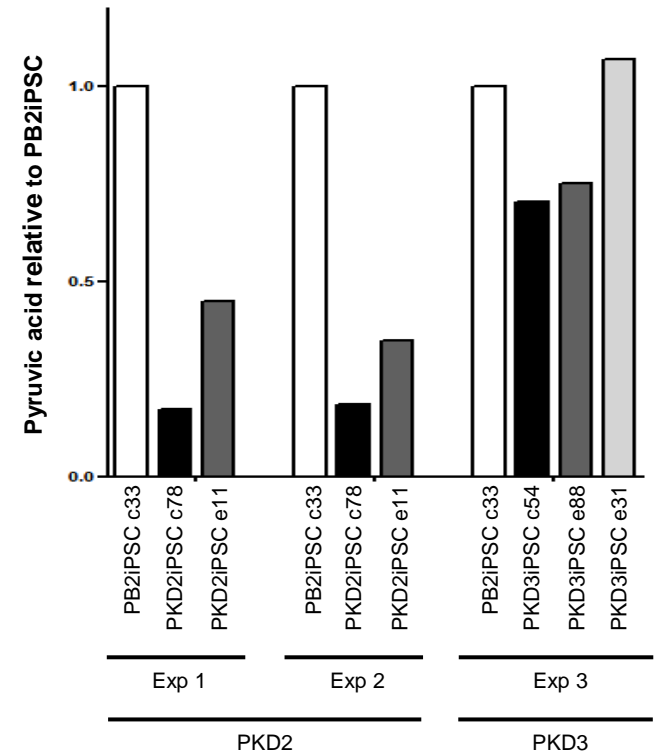
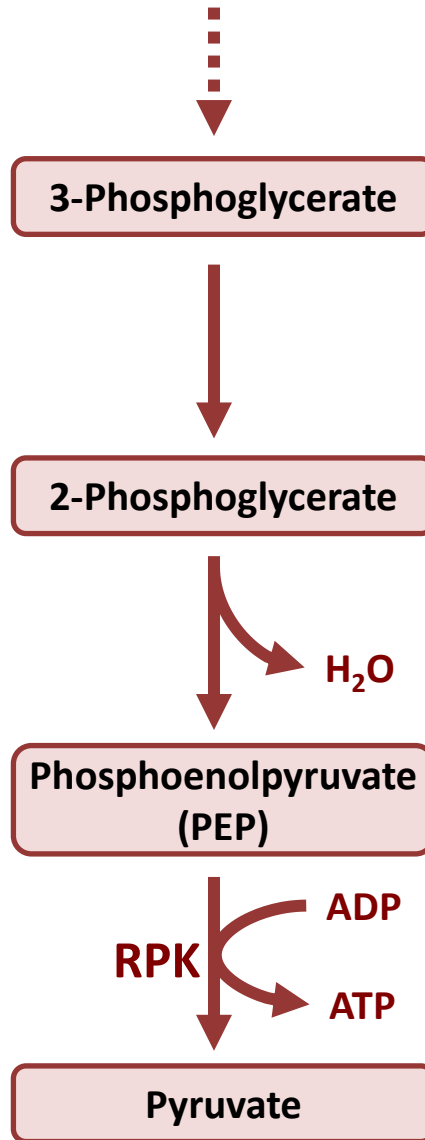
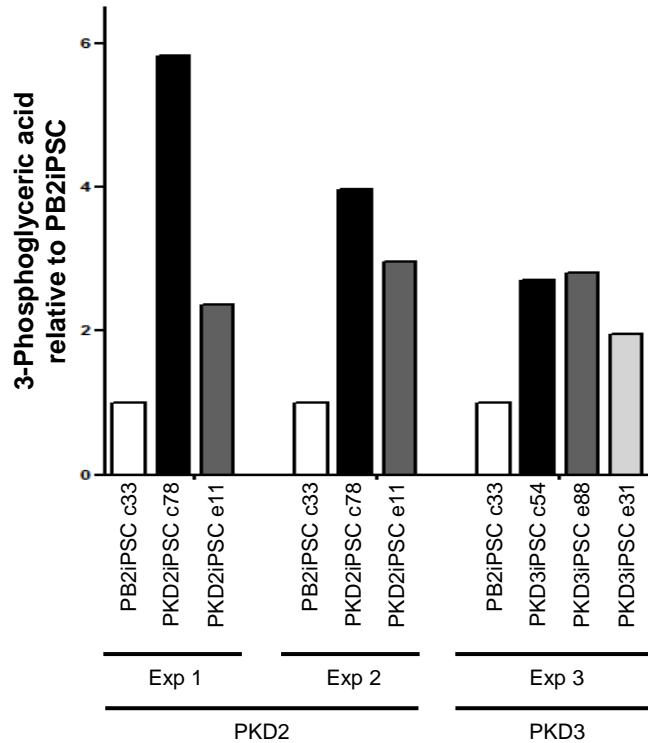
## Patient specific gene therapy

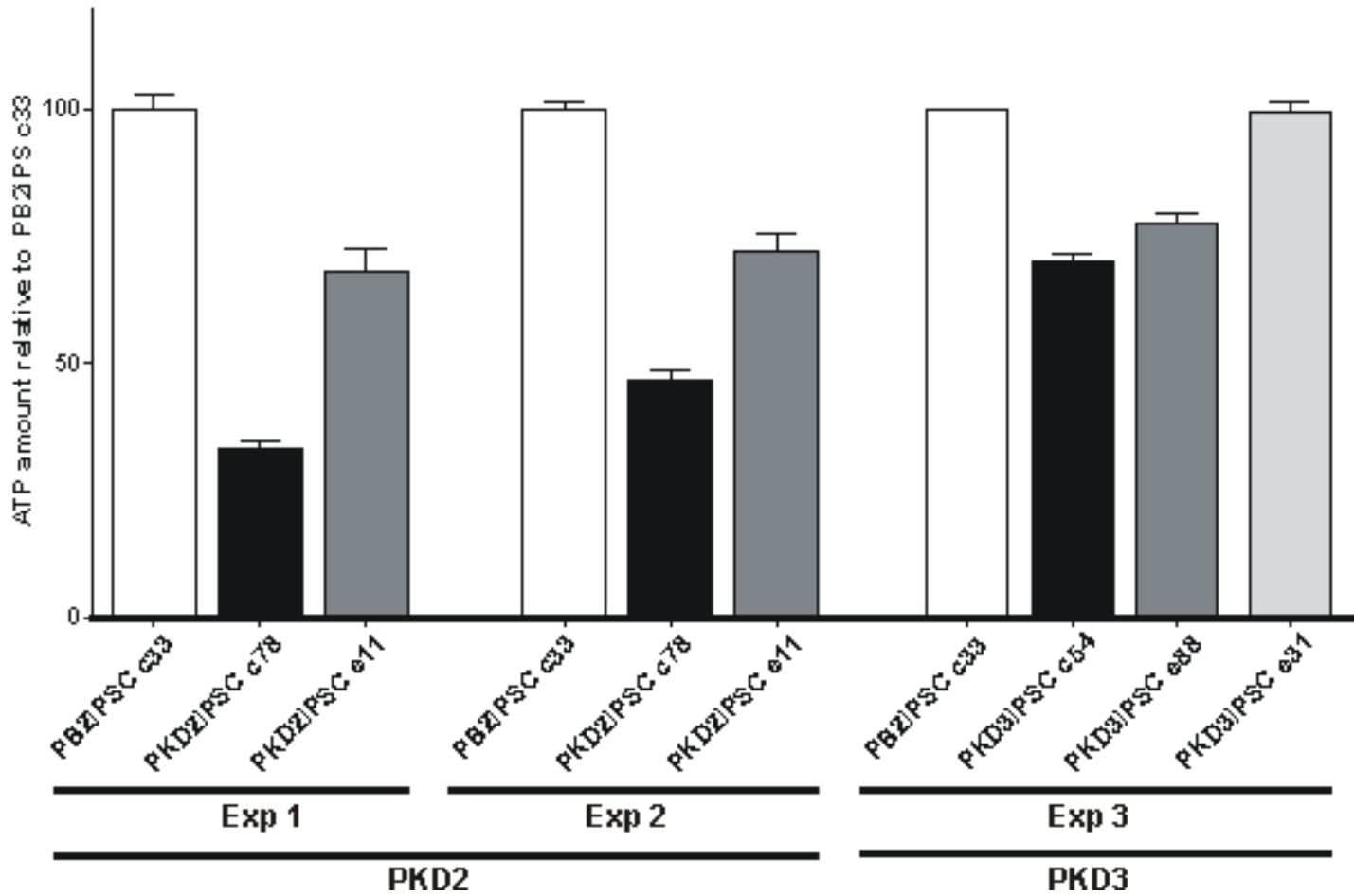
# “Proof of concept”: PKDiPSC



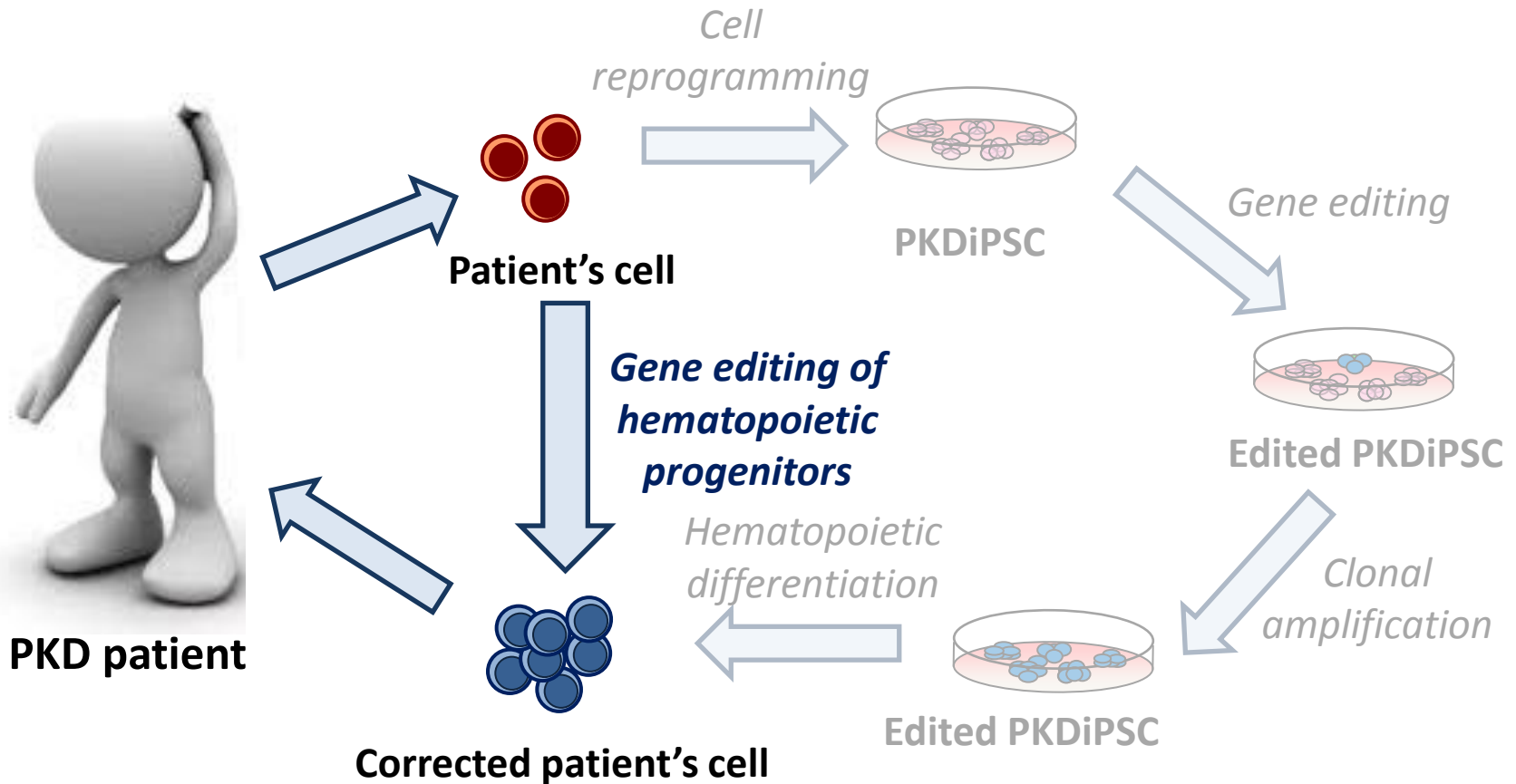
# “Proof of concept”: PKDiPSC

## Erythroid differentiation





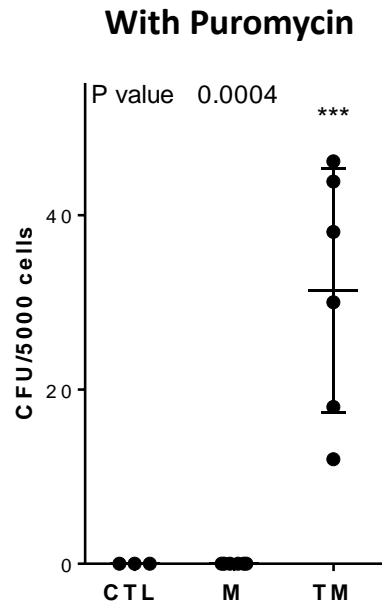
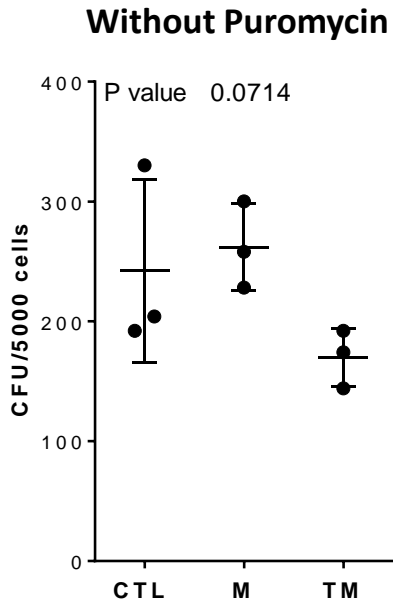
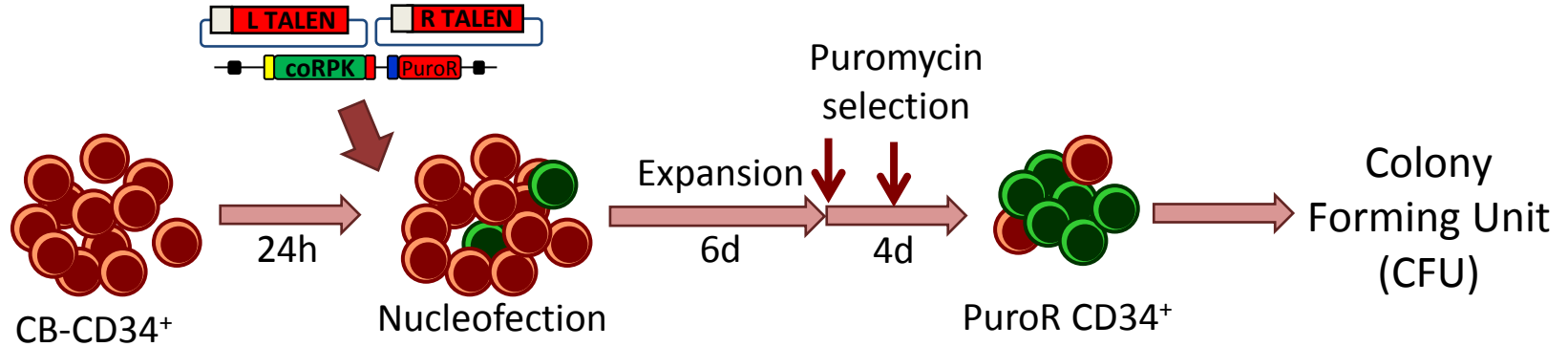
***ATP level: improved in Erythroid cell derived from Edited PKDiPSC***



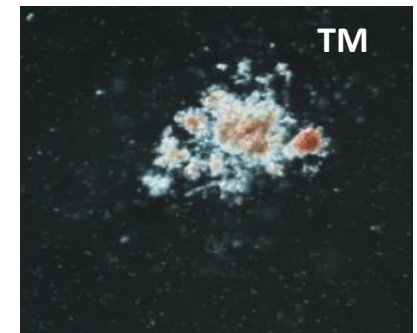
***Ready to use cell source***

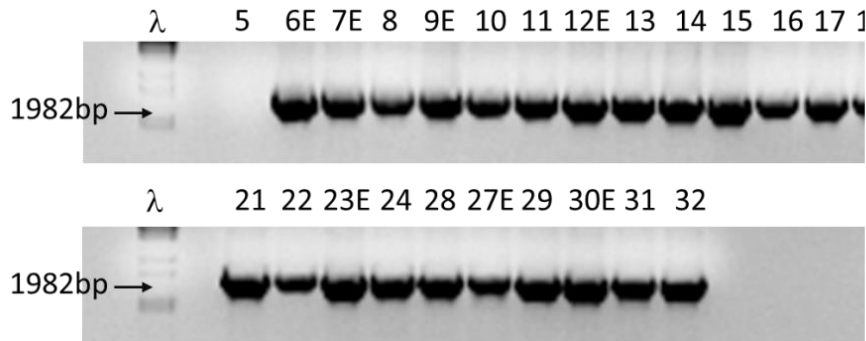
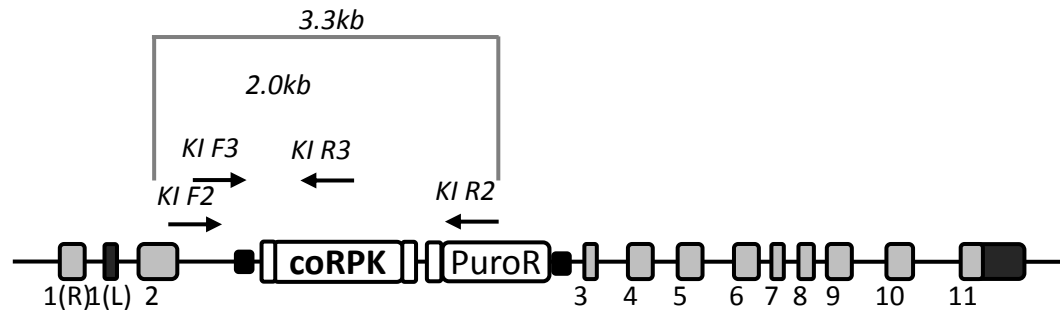
***Gene editing of hematopoietic progenitors is technically challenging***



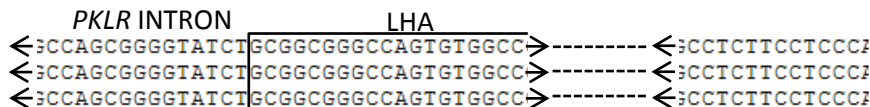


With Puro





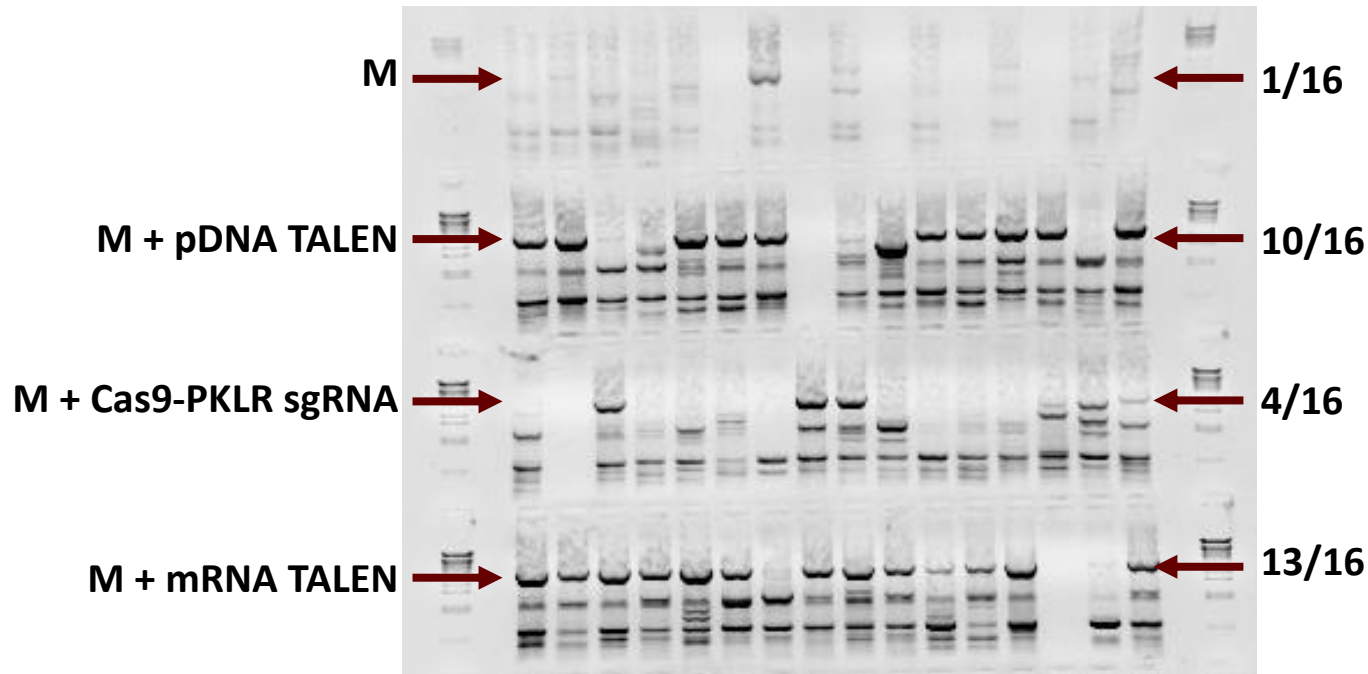
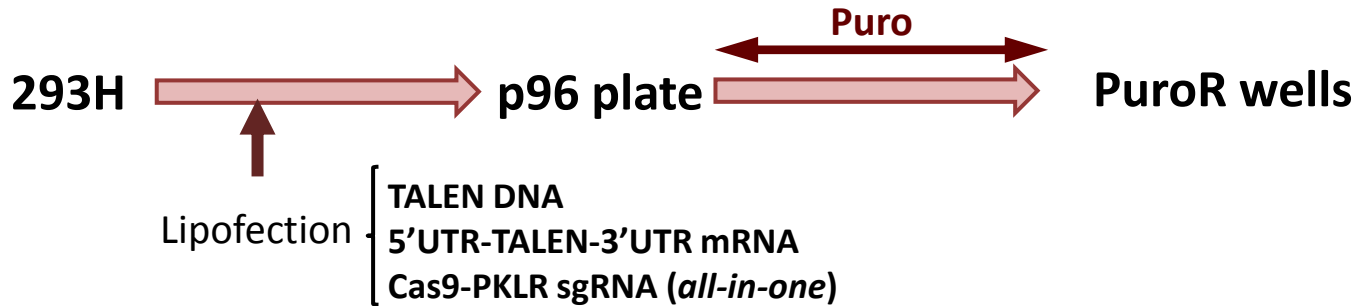
	HR CFU	%HR
CD34-HR1	29	96.7
CD34-HR2	2	40.0
CD34-HR3	18	85.7
<b>Mean</b>		<b>74.1</b>
<b>SEM</b>		<b>17.4</b>



**\*5.8 edited CFU per 1x10<sup>4</sup> nucleofected CFUs**

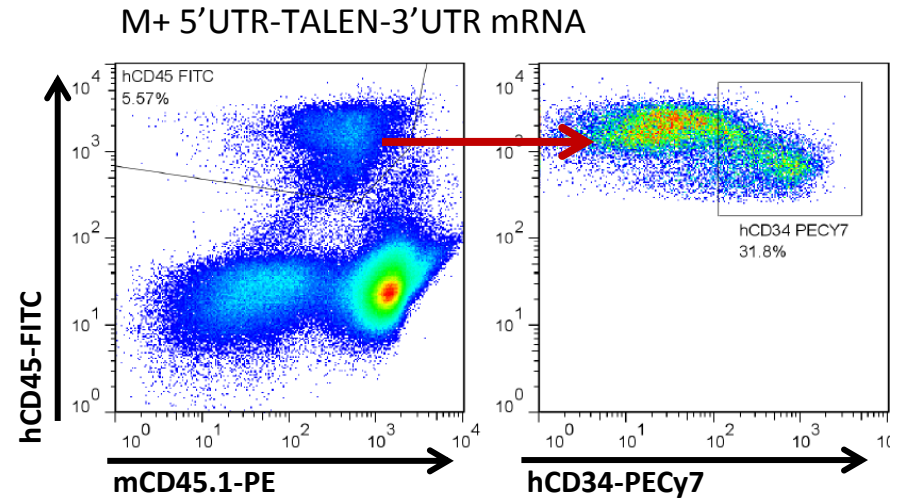
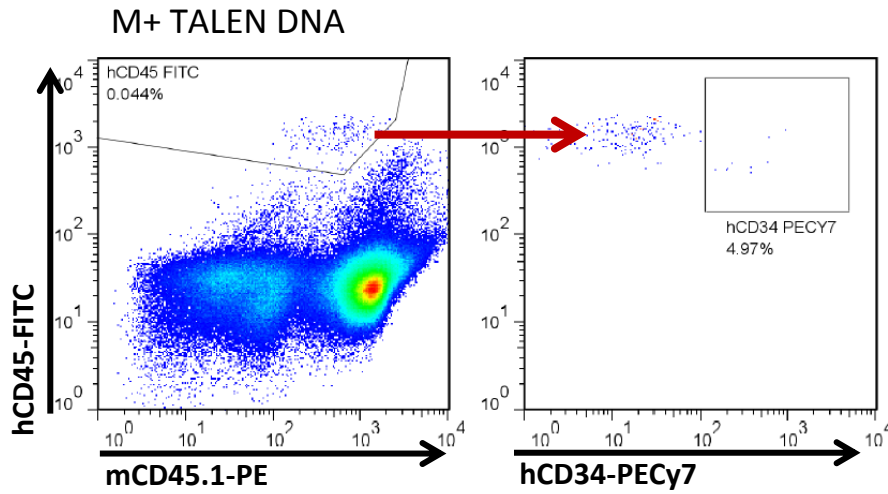
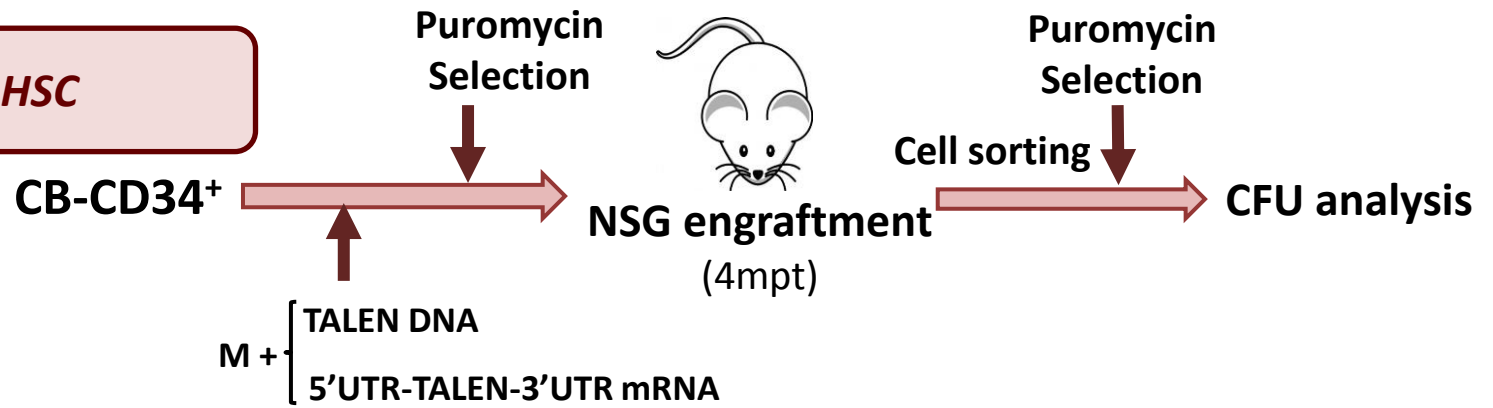
*Knock-in gene editing: feasible in HPCs*

## CRISPR-Cas9: PKLR TALEN vs PKLR sgRNA

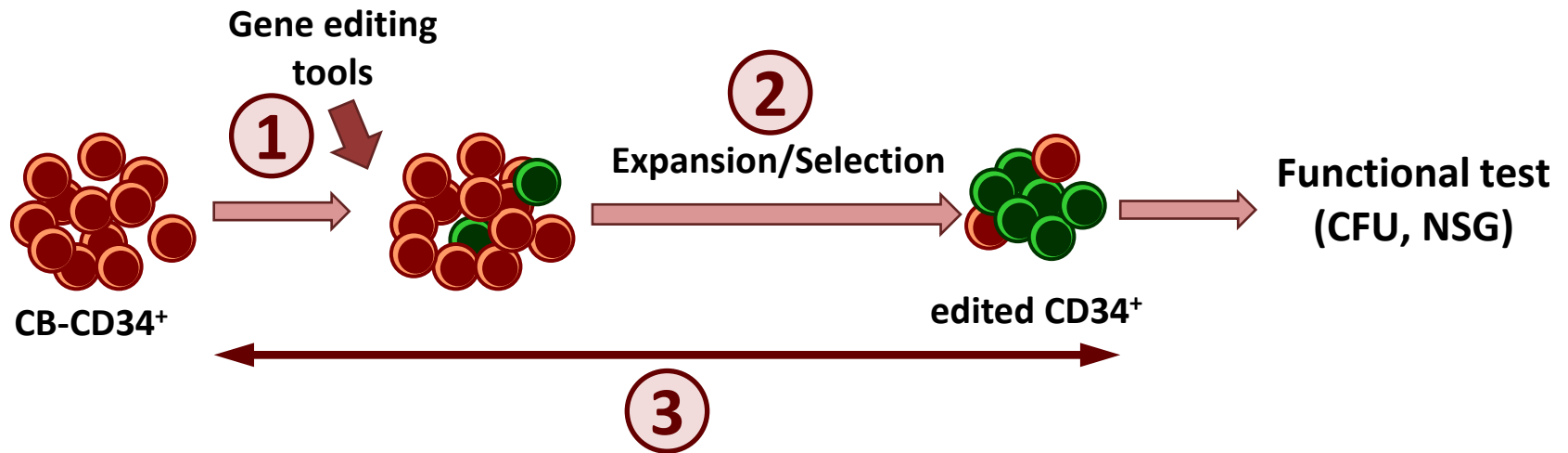


*Cas9-PKLR sgRNA (all-in-one): less efficient*

**Gene editing of HSC**



**TALEN mRNA: gene editing of engraftable hematopoietic progenitors**

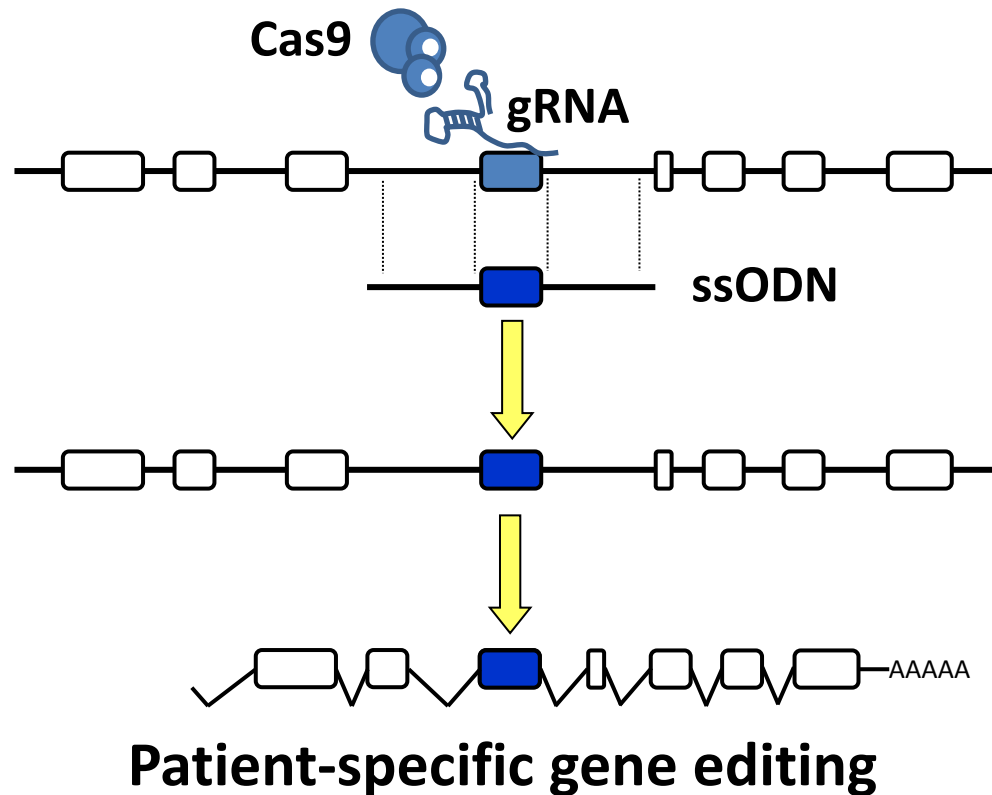


- Patient-specific gene editing tool
- Gene editing to correct all PKD patients

### 3. New strategies of gene editing

- *Most suitable for Hematopoietic Progenitors*

## Patient-specific gene correction by CRISPR-Cas9/ssODN



**Tool desing:** *mutation specific sgRNA and therapeutic ssODN*

## sgRNA

### CRISPOR

### Benchling

### Breaking-Cas

### IDT

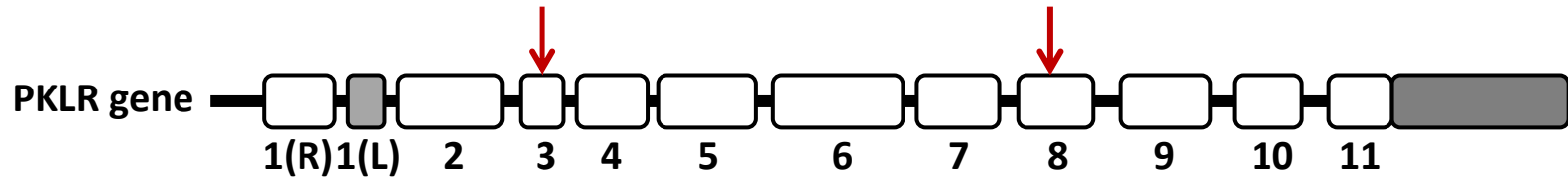
- High On-target score
- Low Off-target score
- Specific for the mutation:  
*mutated allele specificity*

## ssODN

- Correct the mutation: *restore aminoacid sequence*
- Eliminate PAM or near nucleotides of the target sequence sequence without modifying de amino acids sequence: *Prevent cutting of gene edited allele*
- Introduction of a restriction site without modifying de amino acids sequence: *Restriction Fragment Length Polymorphisms (optional)*
- Symmetric: *40nt around cutting site*



## *PKD2-specific gene correction:*



**Mutation**

**SNP**

```
ctcaaggagatgatcaaggccgggatgaacattgcgcgactcaacttctTccacggctcccacgaggtgcgggacTggccgcccgggcagtggggtgg
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
gagttcctctactagttccggccctacttgtaacgcgctgagttgaagaAgggtgccgaggggtgctccacgccctgAccggcgggcccgtcaccacc
```

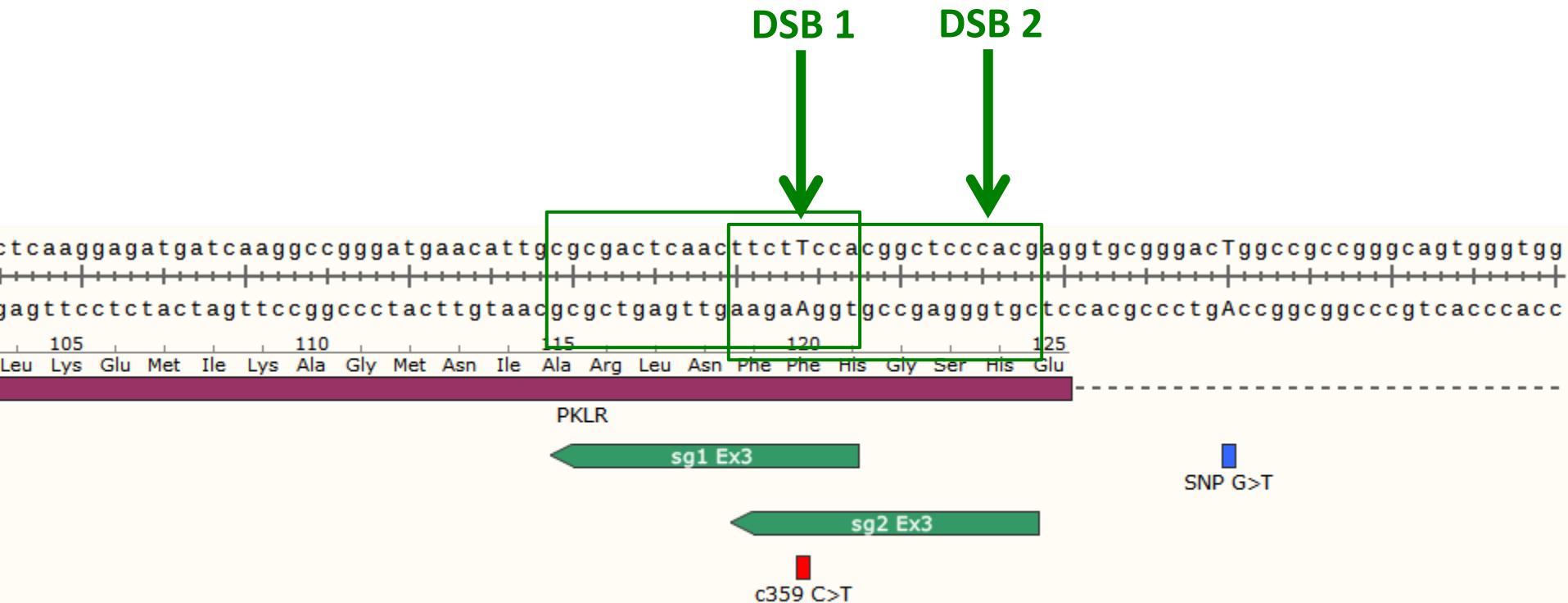
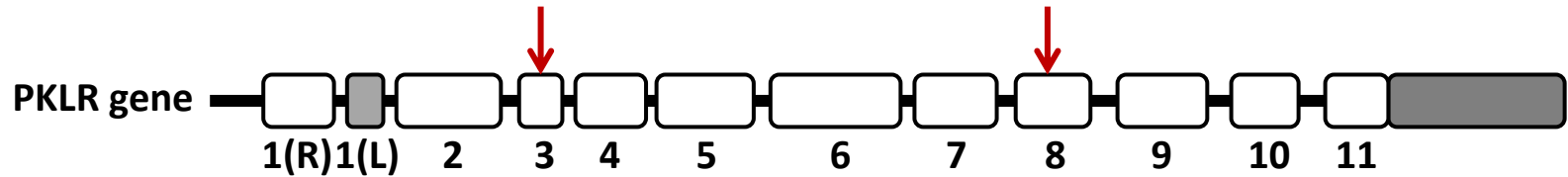
105                      110                      115                      120                      125  
Leu Lys Glu Met Ile Lys Ala Gly Met Asn Ile Ala Arg Leu Asn Phe Phe His Gly Ser His Glu

PKLR

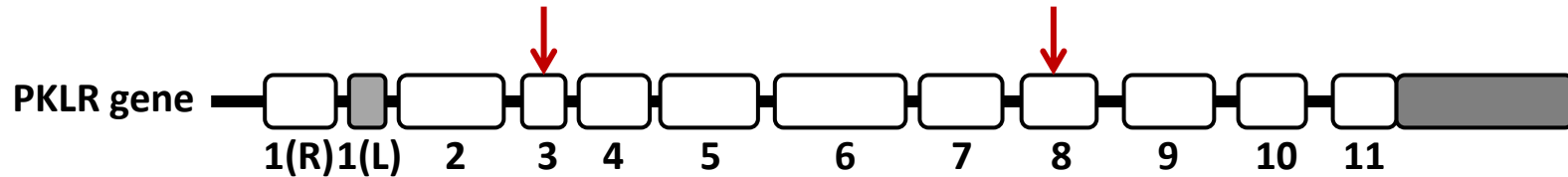
**c359 C>T**

**SNP G>T**

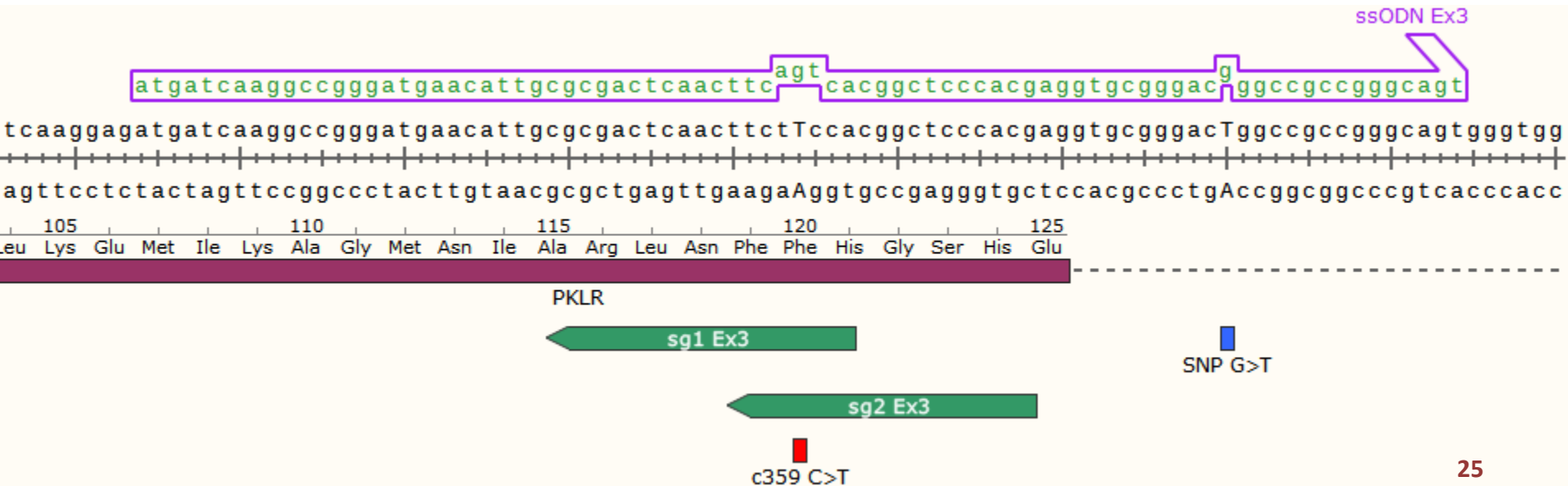
## PKD2-specific gene correction: patient specific sgRNA



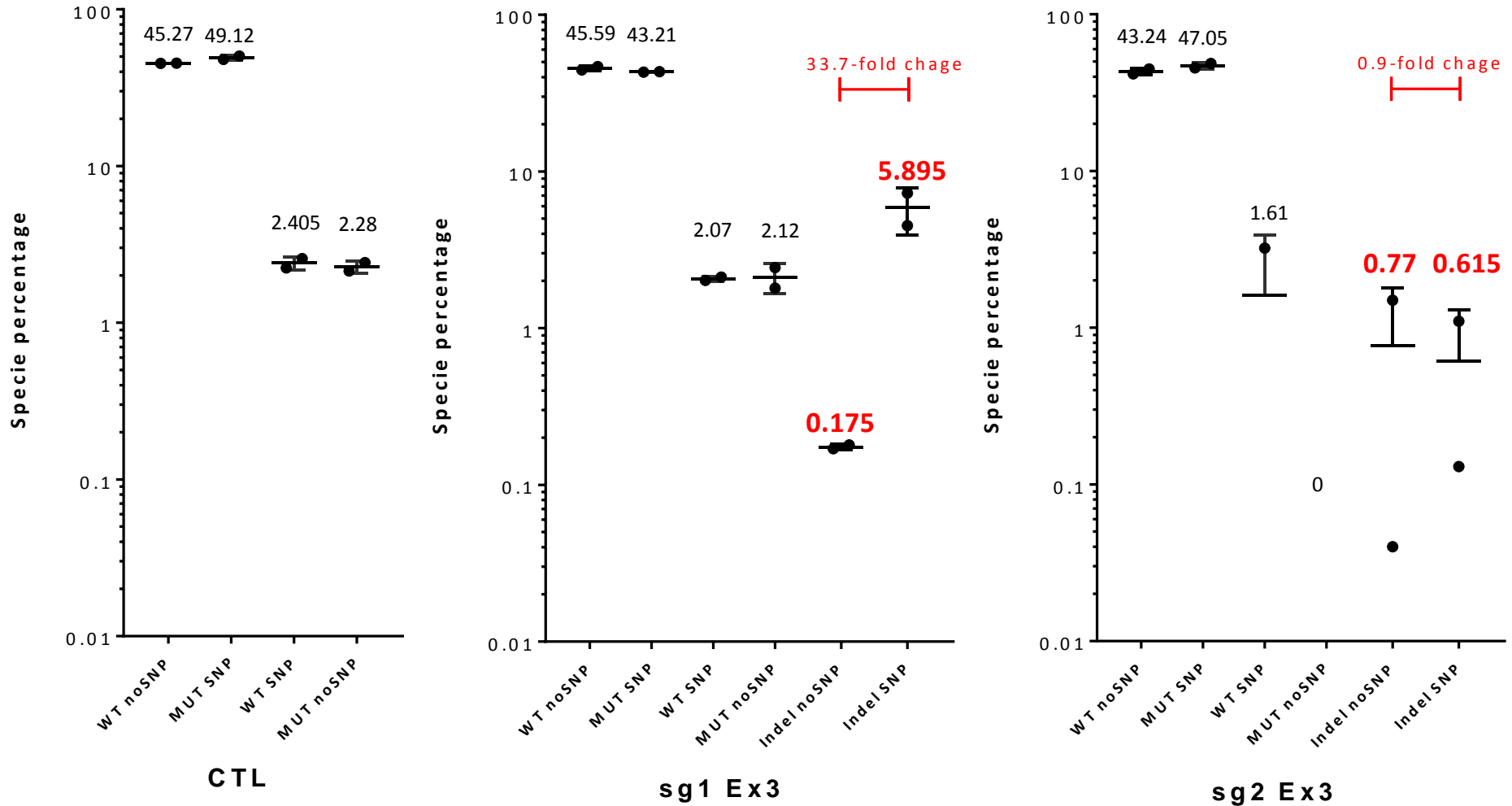
*PKD2-specific gene correction: patient specific ssODN*



*Gene editing?*

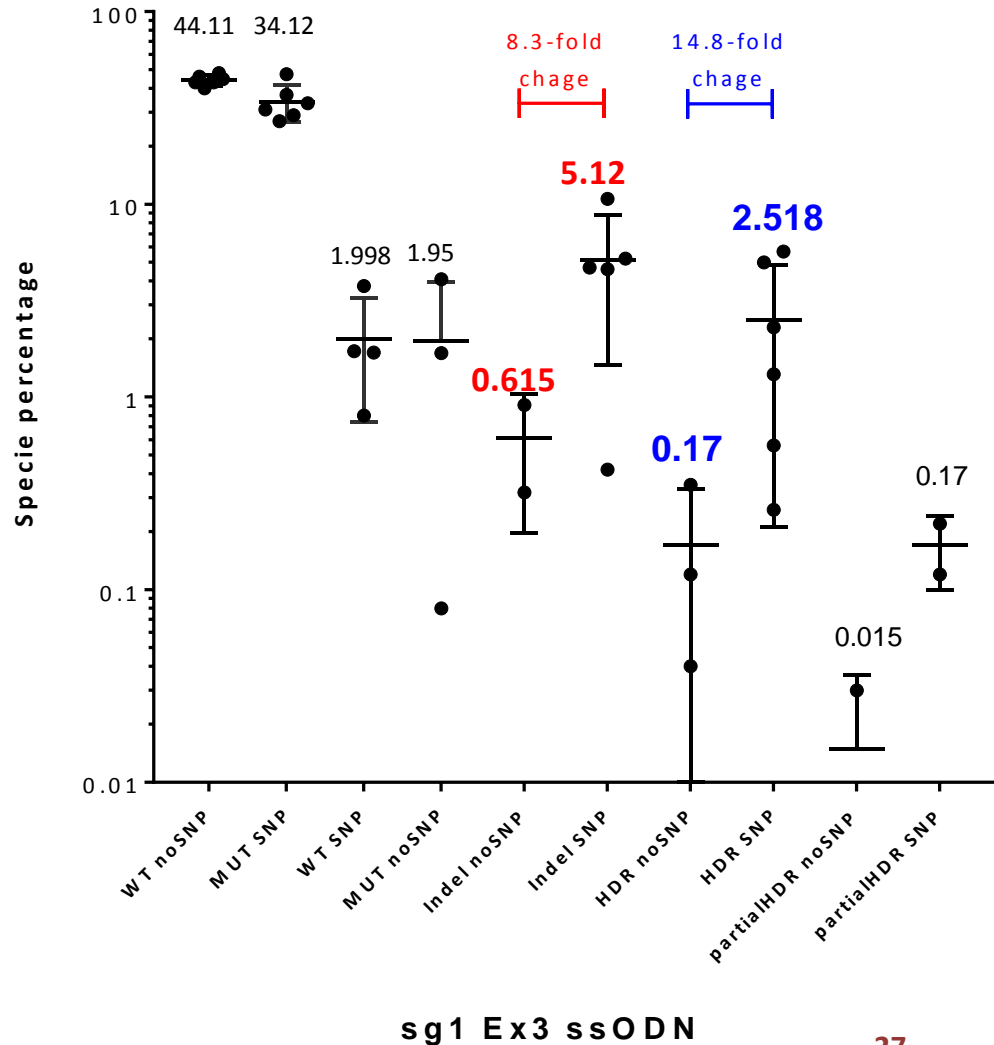
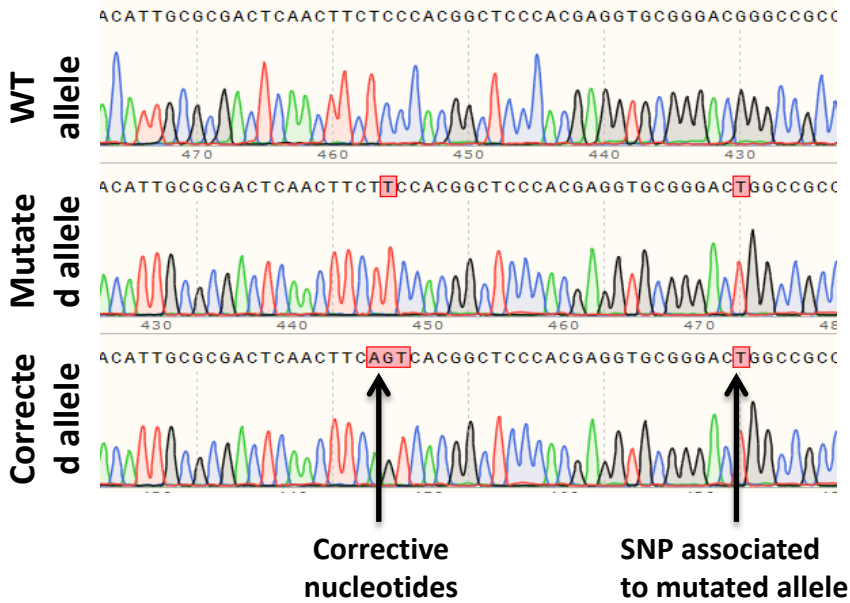


## *PKD2-specific gene correction: mutation-specific guide RNA. NGS (Matías Morín)*



*PKD2-specific gene correction: mutation-specific HDR. NGS (Matías Morín)*

## Topo Cloning Analysis



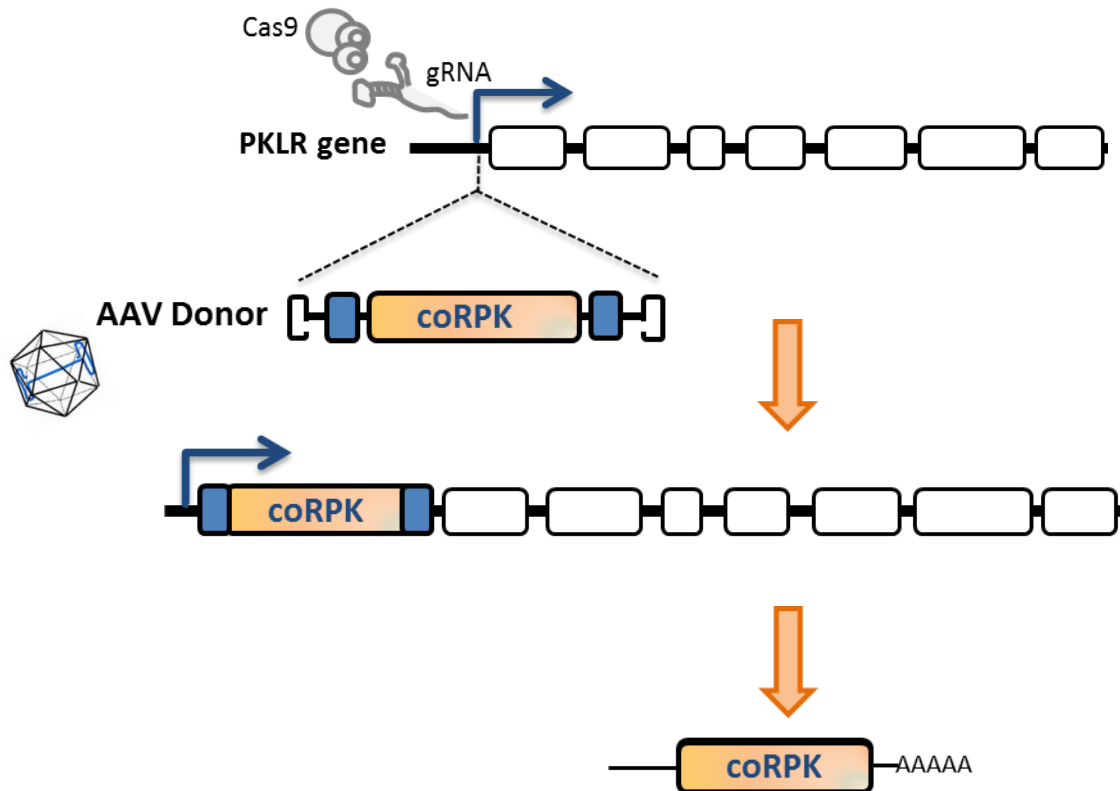
*Patient-specific gene editing tools*

## CRISPR/Cas9 RNP and AAV6-delivery of donor templates: (Matthew Porteus)

 **Stanford University**  
Dr. Porteus lab

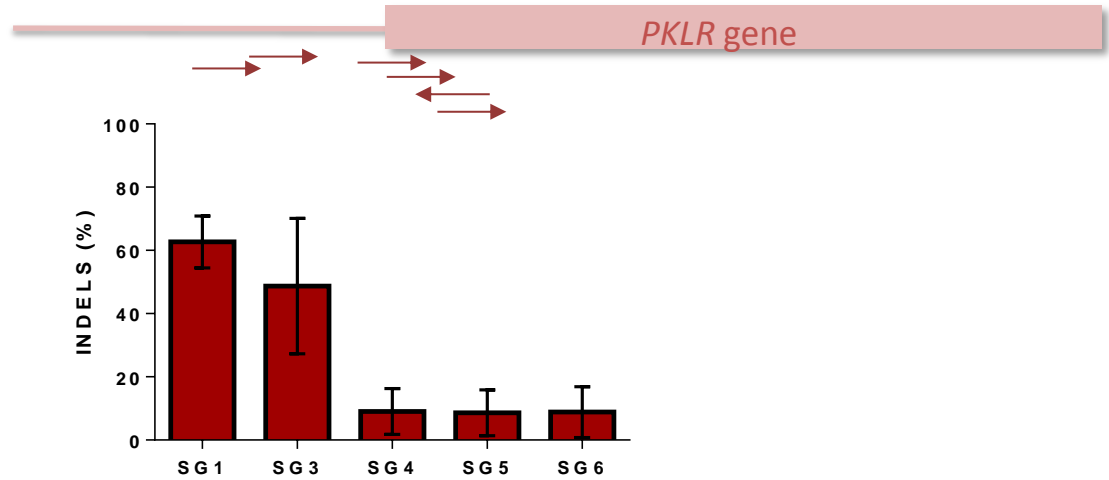
Electroporation of  
CRISPR/Cas9 as RNP

AAVs as donor  
delivery system



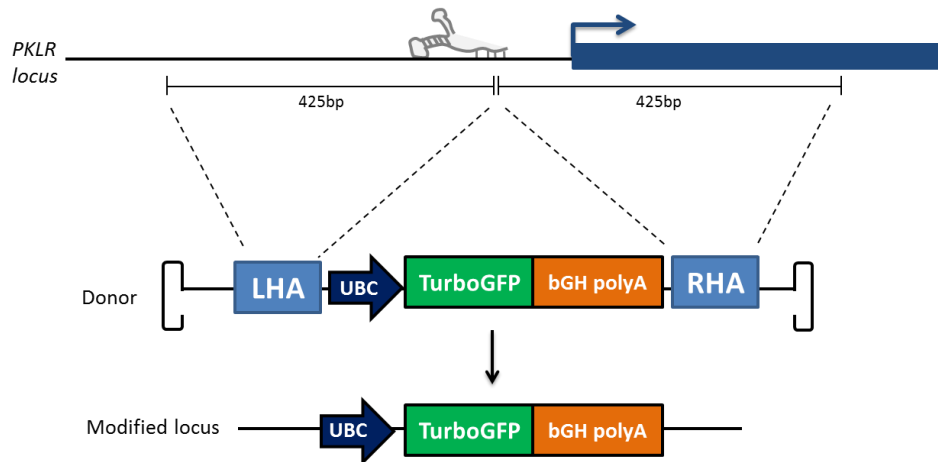
## CRISPR/Cas9 RNP and AAV6-delivery of donor templates: (Matthew Porteus)

PKLR-RNP

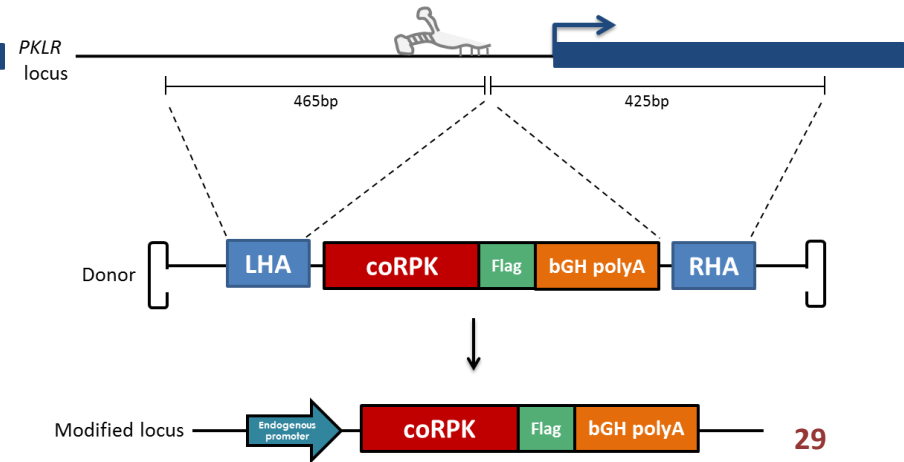


rAAV-6 Donors

Reporter Donor

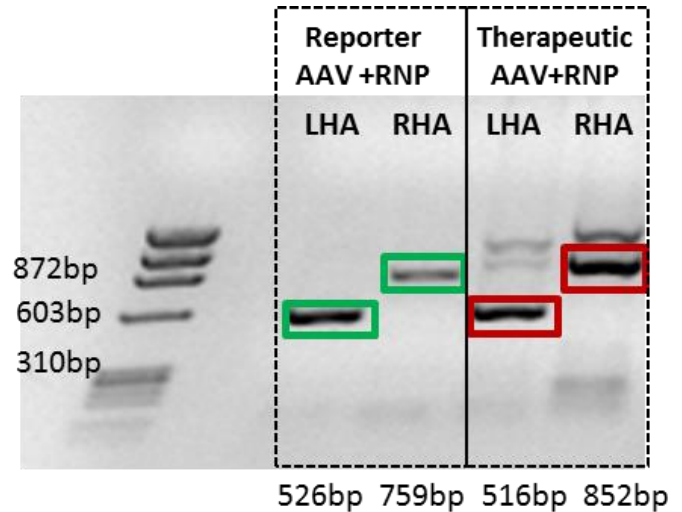
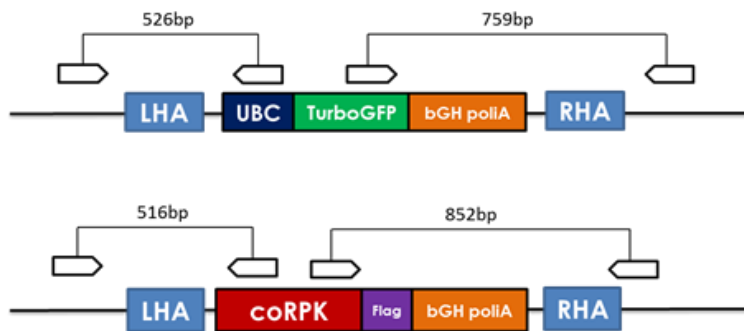


Therapeutic Donor

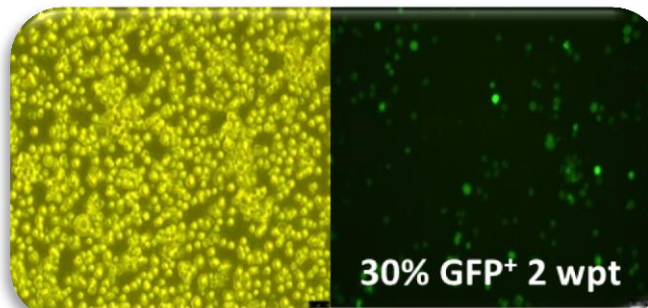




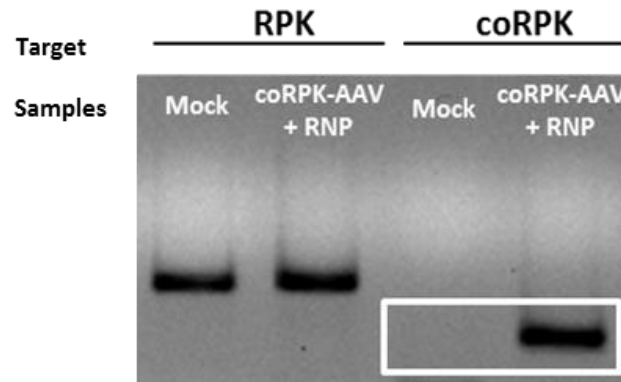
## Gene editing tools testing: human erythroleukemia cell line (K562)



Reporter donor+RNP

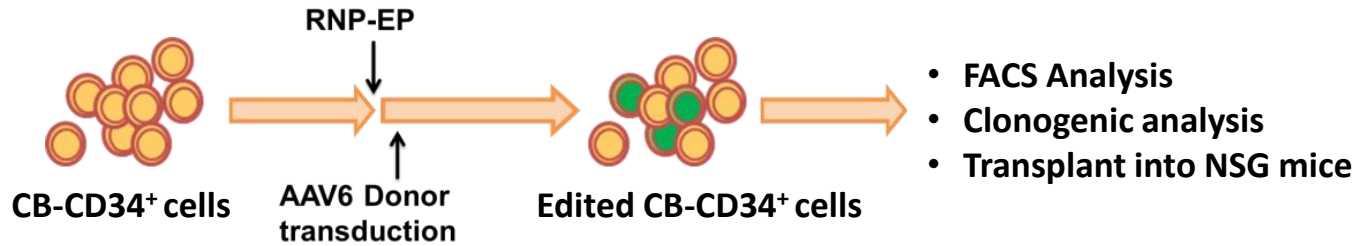


Therapeutic donor+RNP



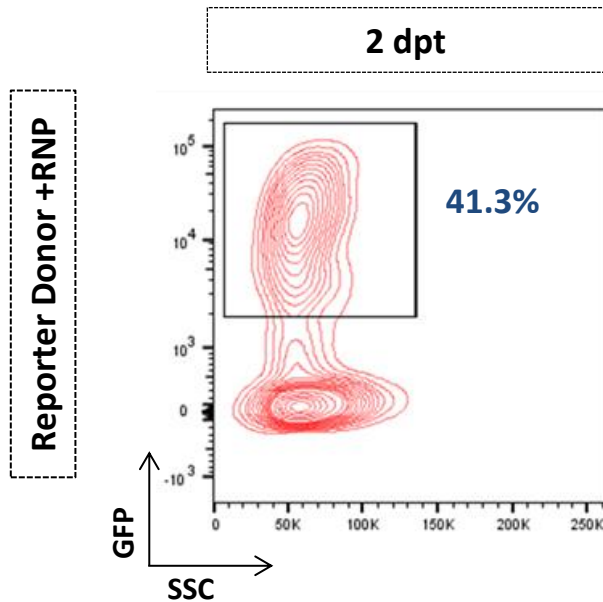
Stable expression of coRPK driven by endogenous promoter

## Gene editing of human HSPCs:



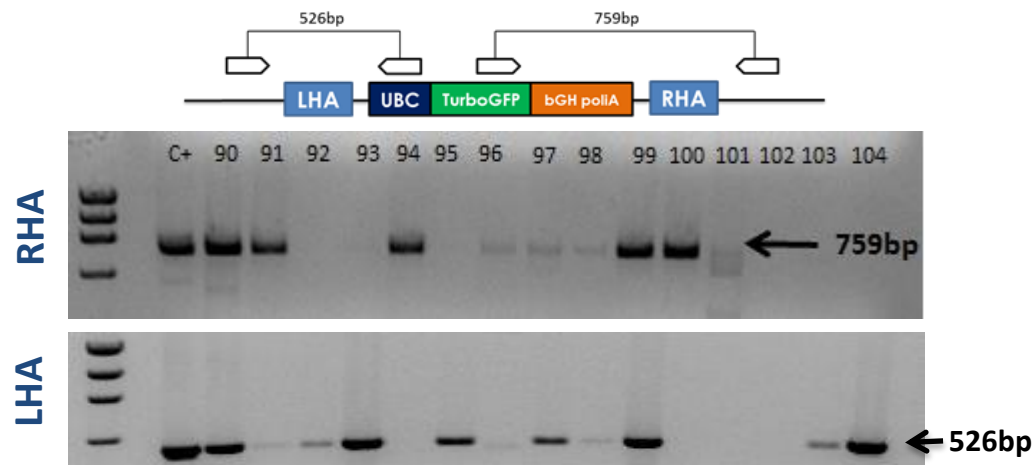
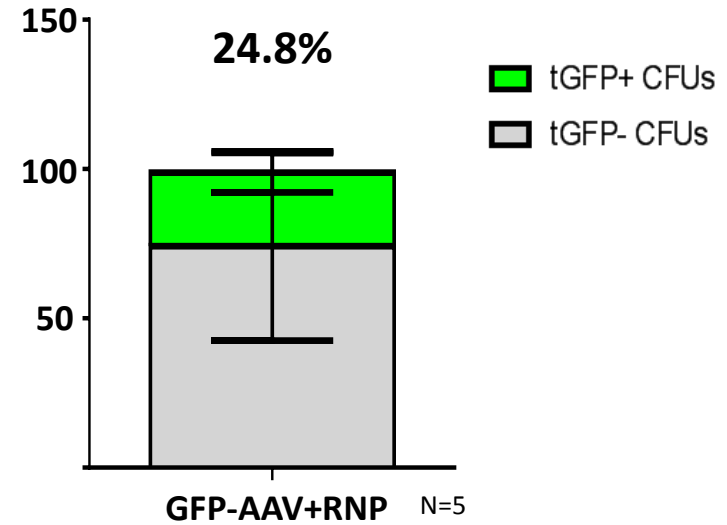
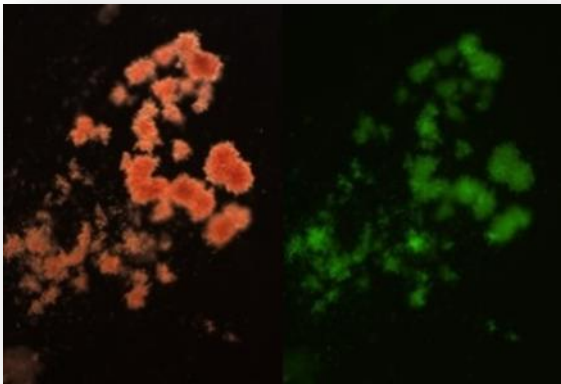
## FACS Analysis

*CD34<sup>+</sup> cells*



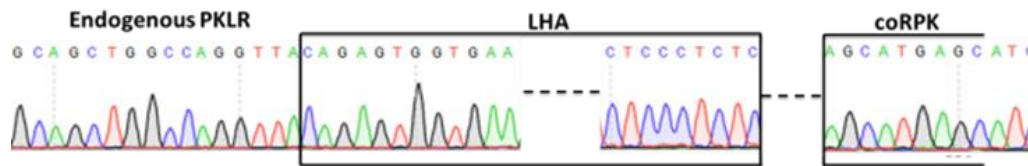
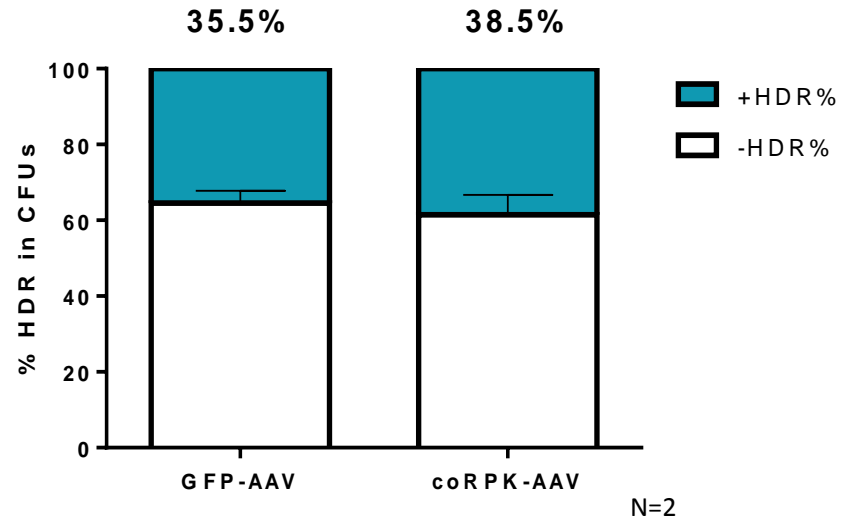
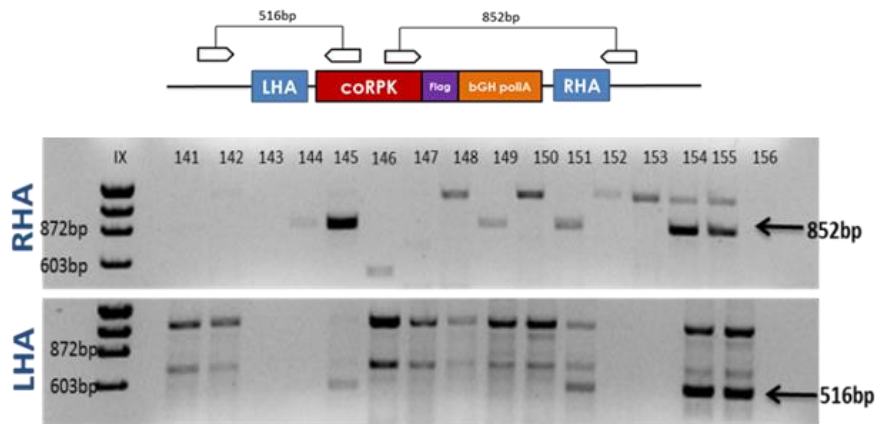
## Gene editing of human HSPCs:

Reporter donor+RNP



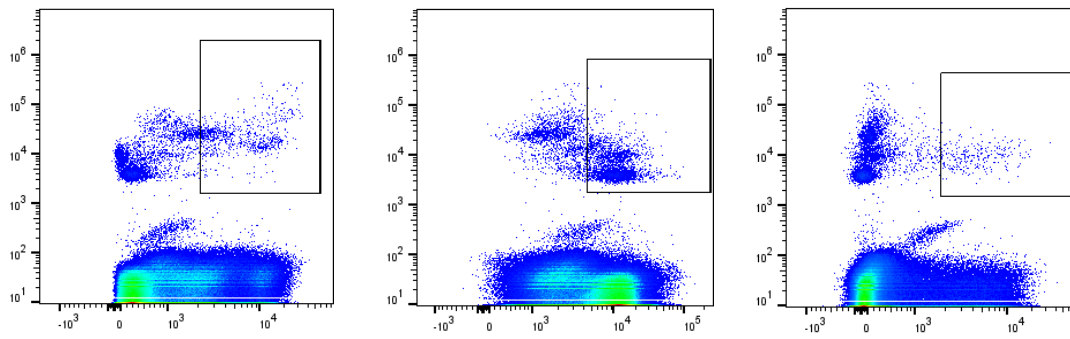
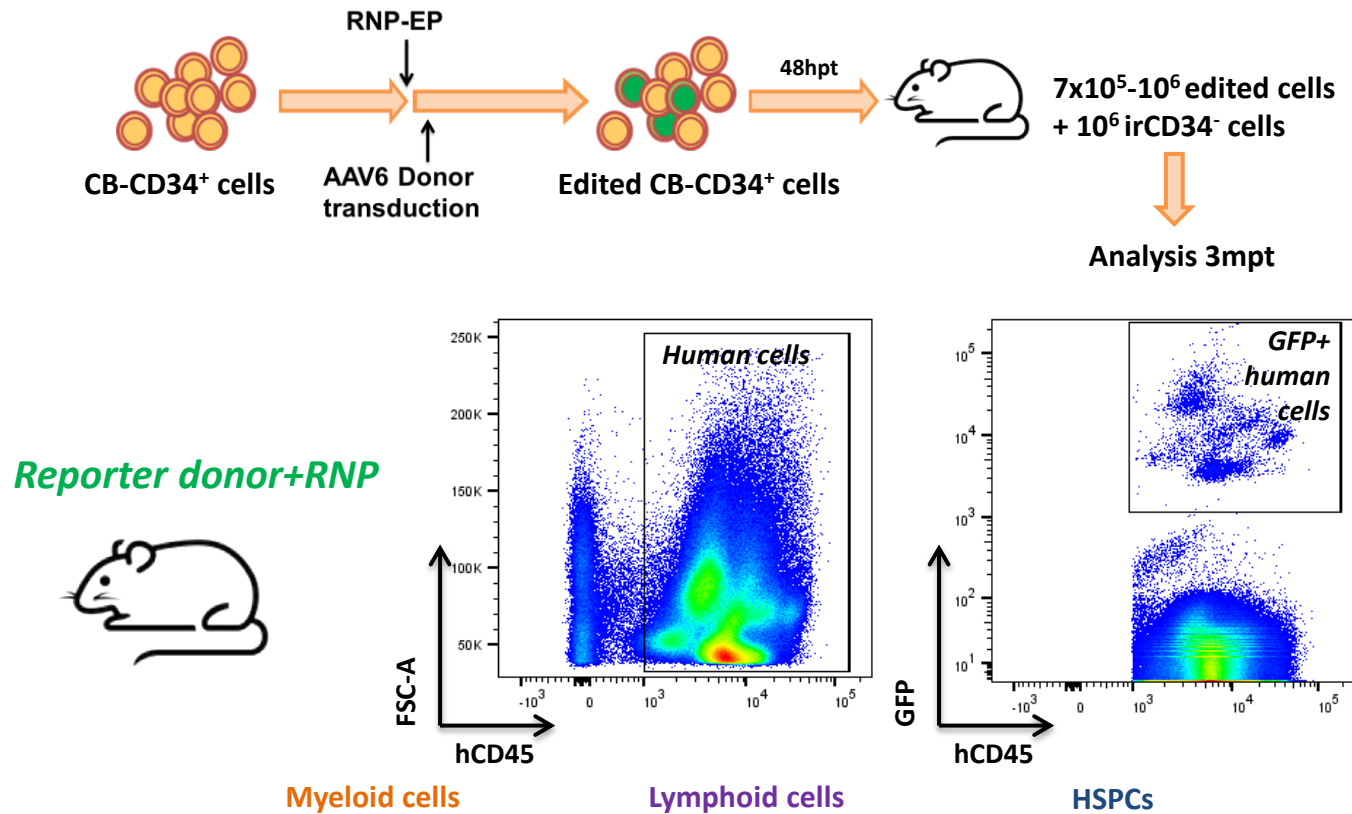
## Gene editing of human HSPCs:

### Therapeutic donor+RNP

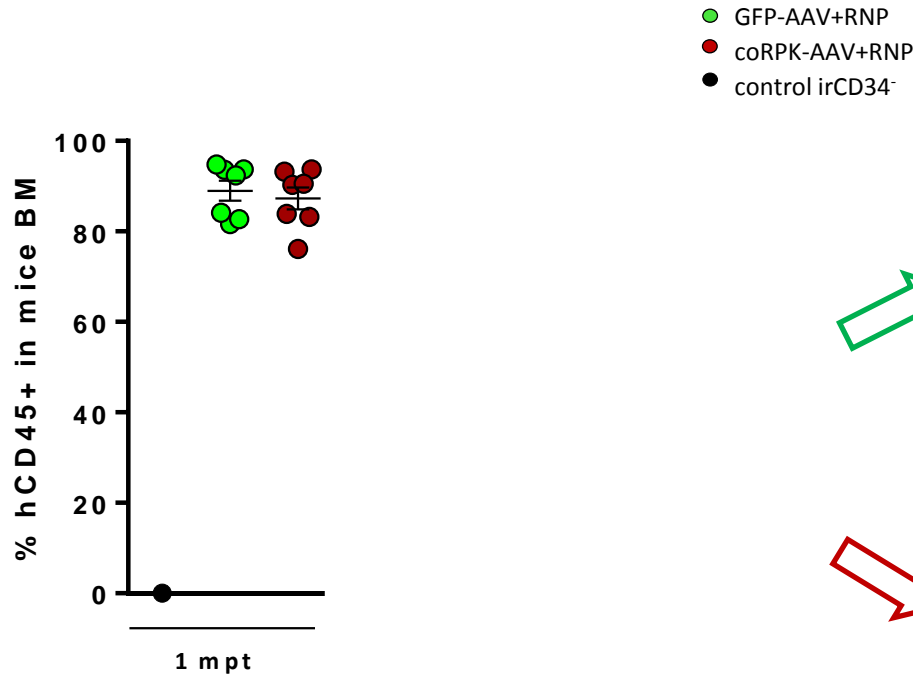


> 20-40% of CFUs were positive for specific HR

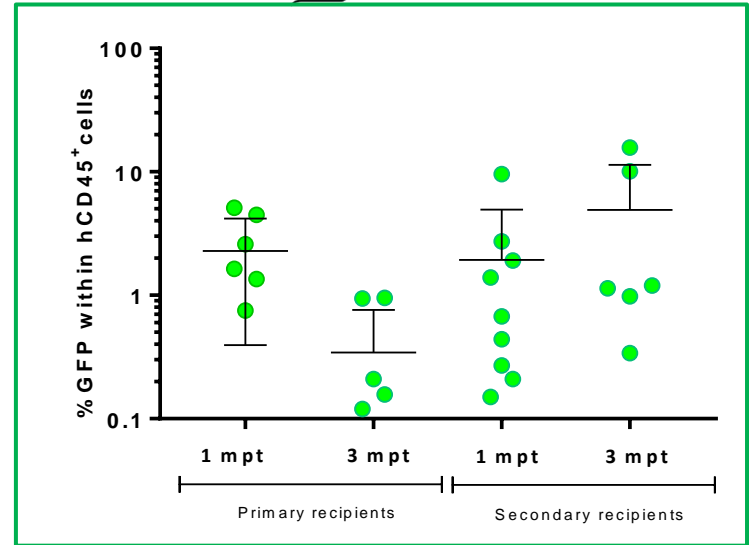
## Gene editing of human HSPCs: In vivo assay



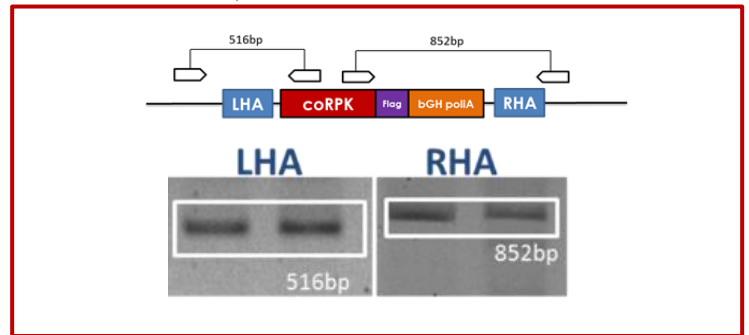
## Gene editing of human HSPCs: In vivo assay



### Reporter donor+RNP



### Therapeutic donor+RNP



## *Gene Editing to correct Pyruvate Kinase Deficiency*

Knock-in gene editing correct PKD phenotype

Knock-in strategy is feasible in hematopoietic progenitors

Patient-specific gene correction is feasible with RNP/ssODN

*Increase efficacy?*

***Clinically applicable?***





**Hematopoietic Innovative Therapies**

**Advance Therapies Unit**

**CIEMAT/IIS Fundación Jiménez Díaz**

**Sara Fañanas  
Zita Garate  
Israel Ormán  
Mercedes Dessy  
Omaira Alberquilla  
Rebeca Sánchez**

**Fátima Rodríguez  
Francisco J. Román  
Paula Rio  
Maria Garcia Bravo**

**Juan Bueren  
Jose Carlos Segovia**

**Collectis**

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Laurent Poirot  
Frédéric Paques**

**IMM-UT**

**Brian Davis**

**Agios Pharmaceutical**

**Penelope Kosinski  
Collin Hill  
Charles Kung  
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**Hospital Univ.**

**Ramón y Cajal  
Matías Morín  
Miguel Ángel Moreno–Pelayo**

**Glasgow University**

**Joanne Mountford  
Emmanuel Olivier**

**DNAvec**

**Noemi Fusaki**

**Stanford University**

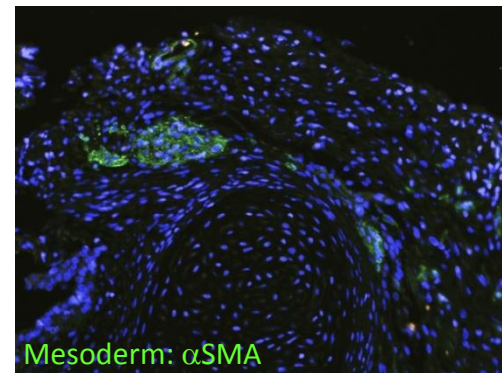
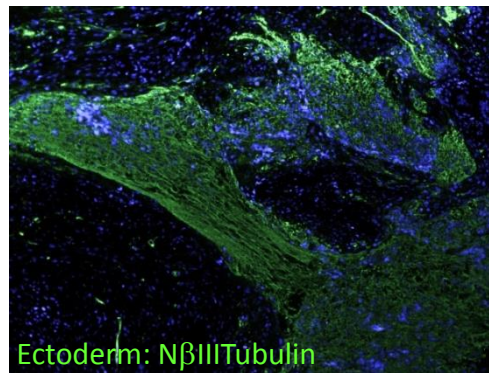
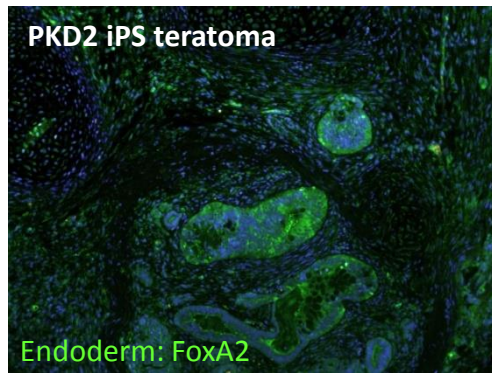
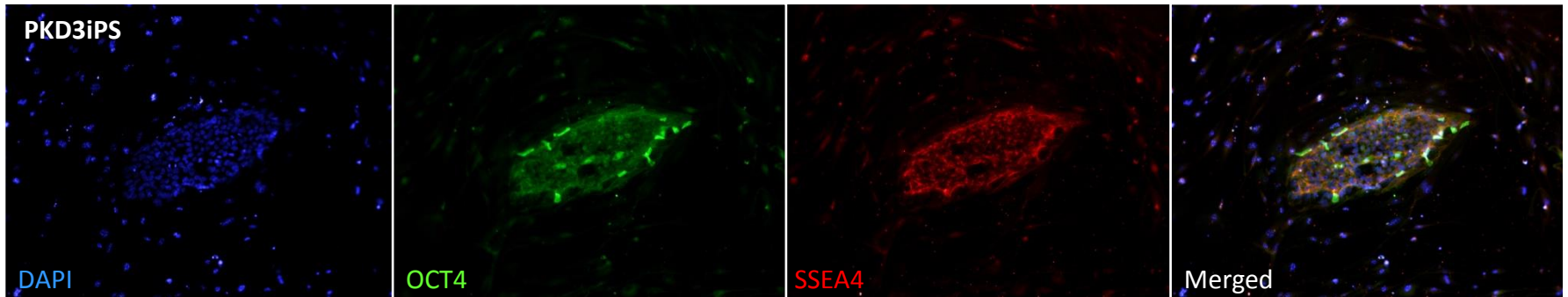
**Daniel P. Dever  
Joab Camarena  
Matthew Porteus**

*Example. Gene Therapy of primary cells with CRISPR:*

# Gene Editing to correct Pyruvate Kinase Deficiency

[oscar.quintana@ciemat.es](mailto:oscar.quintana@ciemat.es)





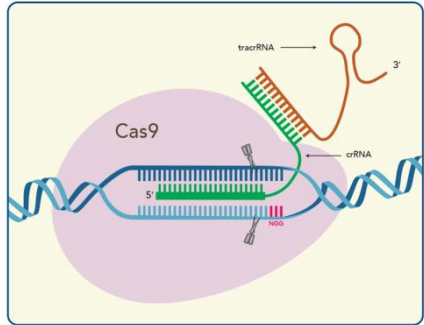
# 1. Gene editing tool: Nuclease.

**Nuclease delivery: format Cas9/sgRNA**

**CB-CD34<sup>+</sup>** → **FACS**

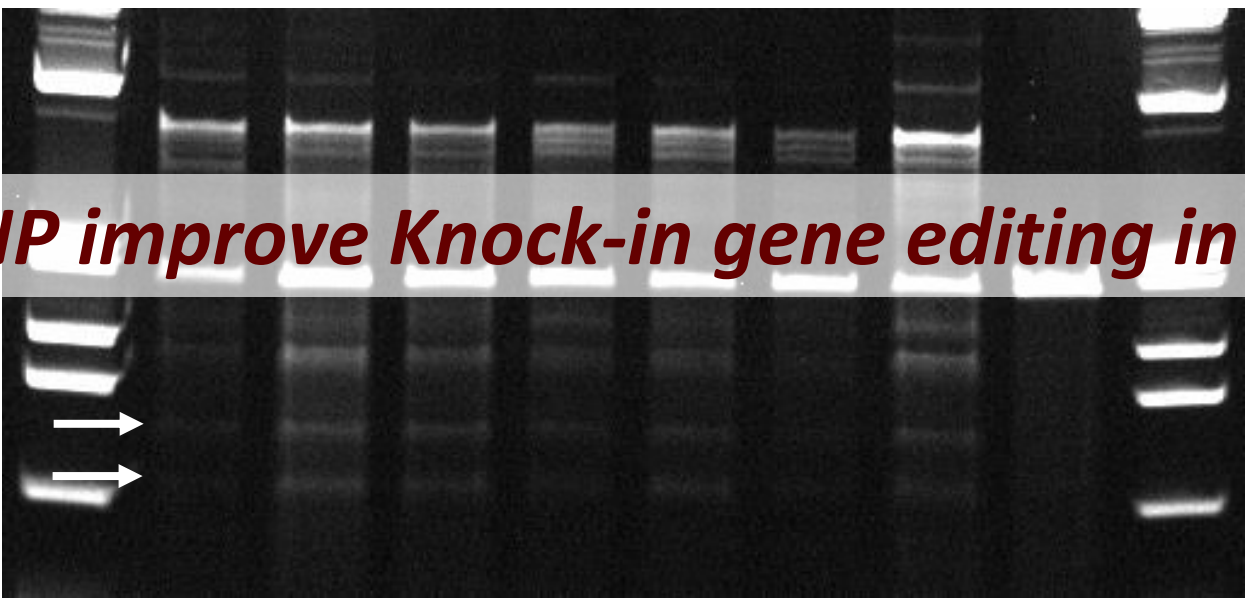
Nucleofection  
(strips Amaxa4D)

- IX
- Control+
- RNP IDT (Cas9 6.6µg)
- RNP IDT (Cas9 3.3µg)
- RNP (Cas9 9µg+12µg SG)
- RNP (Cas9 4.5µg+6µg SG)
- SG + C9G mRNA
- SG + Cas9 mRNA
- Cas9-PKLR sgRNA (all-in-one):
- IX



<https://eu.idtdna.com>

**Can RNP improve Knock-in gene editing in HSC?**



**RNP (IDT): easy, efficient and non-toxic**

*ssODN delivery: delivery method*

CB-CD34<sup>+</sup> → FACS

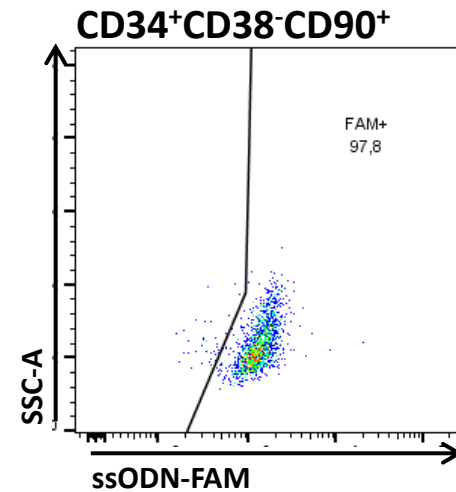
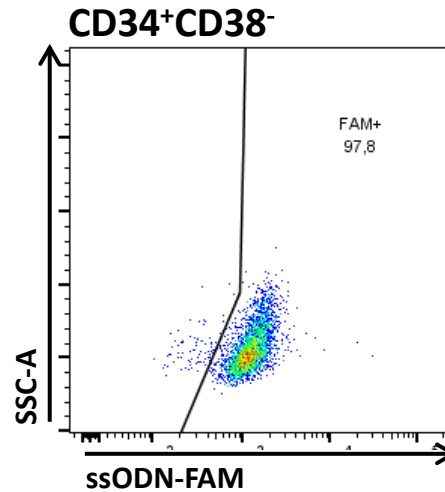
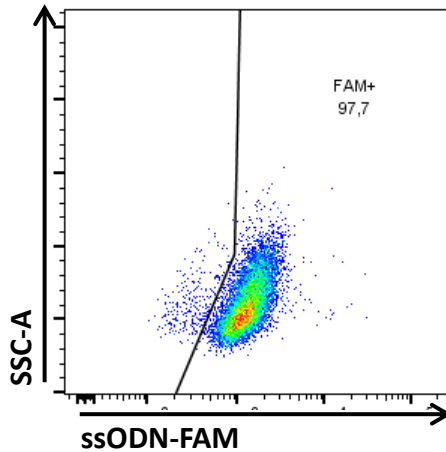
Nucleofection

(Amaxa II vs cuv Amaxa4D vs strips Amaxa4D)

ssODN-FAM (0, 3μM, 10μM)

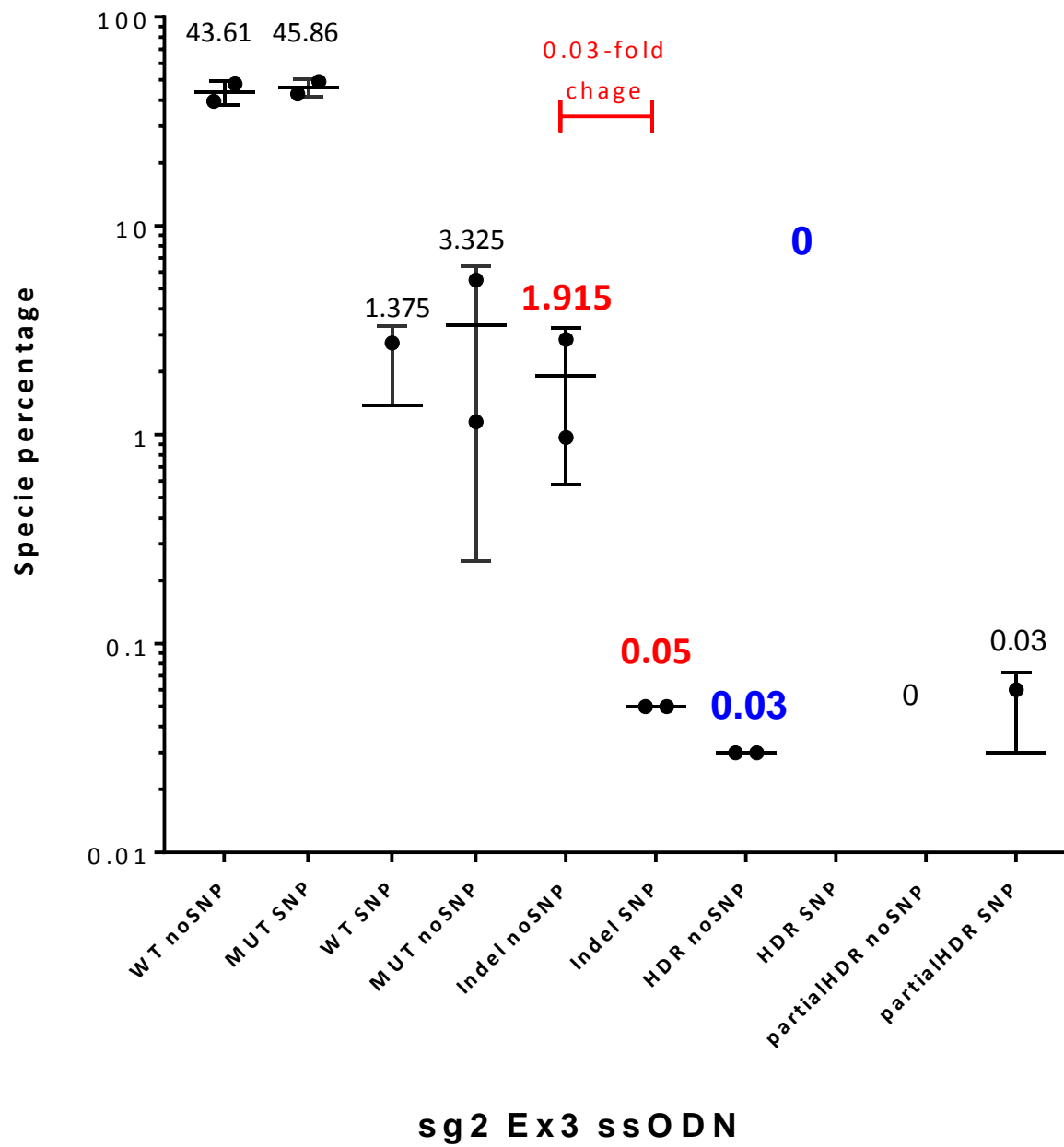
Nucleofection (strip Amaxa4D)

ssODN-FAM (3μM)



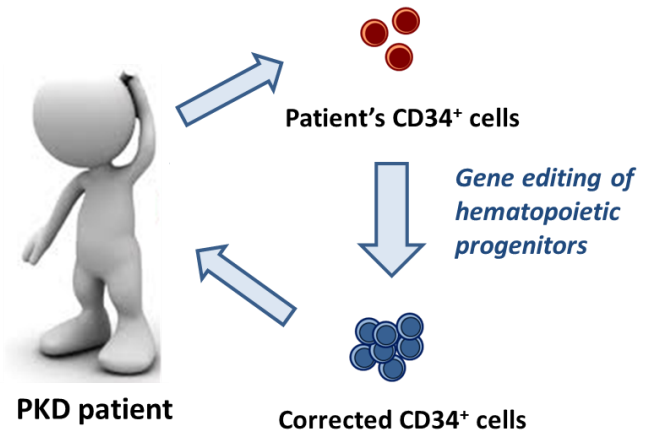
Amaxa	[ssODN] (μM)	%ssODN-FAM 2hpe
Amaxa II	3	98.8
	10	98.8
Cuv Amaxa 4D	3	98.5
	10	97.2
Strips Amaxa 4D	3	97.7
	10	93.8

*ssODN: very efficient and non-toxic*



- › **Specific integration** and **stable expression** of coRPK cDNA driven by *PKLR* endogenous promoter
- › Up to **40% specific integration** of the donor was detected in hematopoietic progenitors generated from gene edited CB-CD34<sup>+</sup> cells
- › **No evidence of toxicity** associated with the procedure
- › Gene edited HSCs were able to **engraft in primary and secondary immunodeficient mice**

**RNP+rAAV6** donor is an **efficient tool** to mediate HDR in the *PKLR* locus





# Acknowledgement



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José C. Segovia



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Joab Camarena  
Matthew Porteus

**PKDefin**



*Patients and healthy donors*

